รายงานวิจัยฉบับสมบูรณ์

**ความหลากหลายและนิเวศวิทยาของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์
จากใบของพืชเศรษฐกิจในประเทศไทยและการผลิตสารส่งเสริมการเจริญพืช
ออกซินโดยสายพันธุ์ที่คัดเลือกว่ามีประสิทธิภาพสูง
พร้อมกับการศึกษาชีววิถีการสังเคราะห์**

Biodiversity and ecology of endophytic and epiphytic yeasts from leaves of agronomic crops in Thailand and production of plant growth promoting auxins by the selected promising strain with the elucidation of it biosynthetic pathway

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รายงานวิจัยฉบับสมบูรณ์

ความหลากหลายและนิเวศวิทยาของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์จากใบของพืชเศรษฐกิจในประเทศไทย และการผลิตสารส่งเสริมการเจริญพืชออกซินโดยสายพันธุ์ที่คัดเลือกว่ามีประสิทธิภาพสูงพร้อมกับการศึกษาชีววิถีการสังเคราะห์

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บทคัดย่อ

โครงการวิจัยนี้มีวัตถุประสงค์ 3 ข้อ คือ เพื่อตรวจวิเคราะห์ความหลากหลายและนิเวศวิทยาของยีสต์เอนโดไฟต์ (endophytic yeast) และยีสต์อีพิไฟต์ (epiphytic yeast) จากใบพืชเศรษฐกิจ คือข้าว อ้อยและข้าวโพดทั้งโดยวิธีที่อาศัยการเพาะเลี้ยงเชื้อ (culture-dependent method) และวิธีที่ไม่อาศัยการเพาะเลี้ยงเชื้อ (culture-independent method) เพื่อผลิตกรดอินโดล-3-อะซิติก (indole-3-acetic acid, IAA) โดยคัดเลือกจากยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์ที่แยกได้และศึกษาชีววิถีการสังเคราะห์ IAA ของยีสต์ที่คัดเลือกได้ และเพื่อประเมินและคัดเลือกยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์ที่แยกได้มีศักยภาพในการควบคุมเชื้อราที่ทำให้เกิดโรคกับพืชพืชเศรษฐกิจทั้ง 3 ชนิด

สำหรับตัวอย่างใบพืชที่ใช้ในการวิจัยความหลากหลายและนิเวศวิทยาของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์ด้วยวิธีที่อาศัยการเพาะเลี้ยงเชื้อและวิธีที่ไม่อาศัยการเพาะเลี้ยงเชื้อนั้น เก็บจากพื้นที่เพาะปลูกในภาคกลางและตะวันออกเฉียงเหนือระหว่างตุลาคม 2554 ถึงเมษายน 2555 ประกอบด้วยใบข้าว 100 ตัวอย่าง จาก 11 จังหวัด ใบอ้อย 102 ตัวอย่าง จาก 13 จังหวัด และใบข้าวโพด 109 จาก 12 จังหวัด จากตรวจวิเคราะห์จุลินทรีย์จากใบข้าว อ้อย และข้าวโพดนั้น พบว่าในเนื้อเยื่อและผิวด้านนอกของใบพืชทั้ง 3 ชนิดมีทั้งยีสต์ แบคทีเรีย และรา โดยมีจำนวนผันแปรค่อนข้างสูง

การศึกษาความหลากของยีสต์เอนโดไฟต์ด้วยการแยกยีสต์จากเนื้อเยื่อที่กำจัดเชื้อที่ผิวด้านนอกของใบข้าว อ้อย และข้าวโพด จำนวน 100, 102 และ 109 ตัวอย่าง ได้ยีสต์ 125, 149 และ 143 สายพันธุ์ ตามลำดับ จากการจัดจำแนกโดยอาศัยอนุกรมวิธานระดับโมเลกุลและไฟโลจีนีพบว่ายีสต์เอนโดไฟต์จากใบข้าว 117 สายพันธุ์เป็นสปีชีส์ที่รู้จักแล้ว (known species) 23 สปีชีส์ ใน 12 สกุล ประกอบด้วย 90 สายพันธุ์ (76.9%) ในไฟลัมแบสิดิโอมัยโคตา 7 สกุล และ 27 สายพันธุ์ (23.1%) ในไฟลัมแอสโคมัยโคตา 5 สกุล มี 1 สายพันธุ์เหมือนกับยีสต์สปีชีส์ที่ยังไม่มีการอธิบาย 1 สายพันธุ์เป็นยีสต์สปีชีส์ใหม่ 3 สายพันธุ์เป็นยีสต์สกุลและสปีชีส์ใหม่ และสายพันธุ์ที่เหลือเป็นรา ส่วนเอนโดไฟต์จากใบอ้อยพบว่า 100 สายพันธุ์เป็น 29 สปีชีส์ที่รู้จักแล้ว โดย 65 สายพันธุ์ (65.0%) อยู่ในแบสิดิโอมัยโคตา (10 สกุล) มี 35 สายพันธุ์ (35.0%) อยู่ในแอสโคมัยโคตา (4 สกุล) ส่วน 21 สายพันธุ์เหมือนกับ 10 สปีชีส์ที่ยังไม่มีการอธิบาย และสายพันธุ์ที่เหลือเป็นรา สำหรับยีสต์เอนโดไฟต์จากใบข้าวโพดนั้น 120 สายพันธุ์เป็นสปีชีส์ที่รู้จักแล้ว 37 สปีชีส์ โดย 90 สายพันธุ์ (75.0%) อยู่ในแบสิดิโอมัยโคตา (7 สกุล) และ 30 สายพันธุ์ (25.0%) อยู่ในแอสโคมัยโคตา (5 สกุล) ส่วน 1 สายพันธุ์เป็นยีสต์สปีชีส์ใหม่ มี 3 สายพันธุ์เหมือนกับ 3 สปีชีส์ที่ยังไม่มีการอธิบาย และที่เหลือเป็นรา ยีตส์เอนโดไฟต์ที่พบบ่อยที่สุดในเนื้อเยื่อใบข้าว อ้อย และข้าวโพด คือ *Pseudozyma aphidis*, *Meyerozyma caribbica* และ *Pseudozyma churashimaensis* โดยมีความถี่ของการปรากฏ (frequency of occurrence) 25.0, 17.6 และ 20.0% ตามลำดับ

สำหรับการศึกษาความหลากหลายของยีสต์อีพิไฟต์จากผิวใบด้วยการเพาะเชื้อจากน้ำล้างใบในจานเพาะเชื้อ พบว่าในกรณีของผิวใบข้าว 112 ตัวอย่าง แยกยีสต์ได้ 379 สายพันธุ์ เมื่อนำ 354 สายพันธุ์มาจัดจำแนก พบว่า 302 สายพันธุ์เป็นยีสต์ที่รู้จักแล้วถึง 50 สปีชีส์ ใน 18 สกุล โดย 246 สายพันธุ์ (81.5%) อยู่ในแบสิดิโอมัยโคตา 10 สกุล ส่วนอีก 56 สายพันธุ์ (18.5%) อยู่ในแอสโคมัยโคตา 9 สกุล มี 21 สายพันธุ์เหมือนกับสปีชีส์ที่ยังไม่มีการอธิบาย 14 สปีชีส์ ใน 4 สกุล พบ 31 สายพันธุ์ที่คาดว่าจะเป็นยีสต์สปีชีส์ใหม่ 20 สปีชีส์ ใน 8 สกุล สำหรับยีสต์อีพิไฟต์จากผิวใบอ้อย แยกยีสต์ 173 สายพันธุ์จากใบอ้อย 84 ตัวอย่าง พบว่า 87 สายพันธุ์ เป็นยีสต์สปีชีส์ที่รู้จักแล้ว โดย 61 สายพันธุ์ (35.3%) เป็นยีสต์ 24 สปีชีส์ 10 สกุล ในแบสิดิโอมัยตา มี 26 สายพันธุ์ (15%) เป็นยีสต์ 12 สปีชีส์ 3 สกุล ในแอสโคมัยโคตา ส่วนอีก 67 สายพันธุ์อาจเป็นยีสต์สปีชีส์ใหม่หรือสปีชีส์ที่รู้จักแล้วซึ่งต้องตรวจสอบเพิ่มเติม มี 20 สายพันธุ์ที่เป็นยีสต์สปีชีส์ใหม่ 12 สปีชีส์ สำหรับยีสต์อีพิไฟต์จากผิวใบข้าวโพดแยกยีสต์ได้ 284 สายพันธุ์จาก 109 ตัวอย่าง เมื่อจัดจำแนก 244 สายพันธุ์ พบว่า 151 สายพันธุ์เป็นยีสต์สปีชีส์ที่

รู้จักแล้ว 43 สปีชีส์ ใน 15 สกุล โดย 130 สายพันธุ์ (86.1%) อยู่ในแบสิดิโอมัยตา 10 สกุล ส่วน 21 สายพันธุ์ (13.9%) อยู่ในแอสโคมัยโคตา5 สกุล อีก 18 สายพันธุ์เหมือนกับยีสต์สปีชีส์ที่ยังไม่มีการอธิบาย 3 สกุล นอกจากนั้นมี 21 สายพันธุ์ที่คาดว่าเป็นยีสต์สปีชีส์ใหม่ 16 สปีชีส์ และสายพันธุ์ที่เหลือพบว่าเป็นรา ในขณะที่การศึกษาความหลากหลายของยีสต์อีพิไฟต์ในผิวใบข้าว ใบข้าวโพด และใบอ้อย ด้วยการแยกยีสต์โดย enrichment technique จากตัวอย่างใบพืชรวม 244 ตัวอย่าง ได้ยีสต์จากใบข้าว อ้อย และข้าวโพด 156, 126 และ 159 สายพันธุ์ตามลำดับ เมื่อจัดจำแนก พบว่ายีสต์จากใบข้าว 155 สายพันธุ์เป็นยีสต์สปีชีส์ที่รู้จักแล้ว โดย 99 สายพันธุ์ (63.9%) เป็น 23 สปีชีส์ใน 13 สกุล ในแอสโคมัยโคตา ส่วน 56 สายพันธุ์ (36.1%) เป็น 8 สปีชีส์ 5 สกุล ในแบสิดิโอมัยโคตา ยีสต์จากใบอ้อย 146 สายพันธุ์ เป็น 23 สปีชีส์ 13 สกุล ในแอสโคมัยโคตา ในขณะที่ 46 สายพันธุ์ (31.7%) อยู่ในแบสิดิโอ-มัยโคตา 12 สปีชีส์ใน 6 สกุล ส่วนอีก 8 สายพันธุ์เหมือนกับยีสต์สปีชีส์ที่ยังไม่มีการอธิบายและ 9 สายพันธุ์เป็นยีสต์สปีชีส์ใหม่ 6 สปีชีส์ และยีสต์จากใบข้าวโพด 123 สายพันธุ์เป็นยีสต์สปีชีส์ที่รู้จักแล้ว โดย 81 สายพันธุ์ (65.9%) เป็น 28 สปีชีส์ 12 สกุลแอสโคมัยโคตา มี 42 สายพันธุ์ (34.1%) เป็น 10 สปีชีส์ ใน 5 สกุล ในแบสิดิโอมัยโคตา สำหรับยีสต์อีพิไฟต์ที่พบบ่อยที่สุดบนผิวใบข้าว อ้อย และข้าวโพด เมื่อแยกยีสต์ด้วยการเพาะเชื้อจากน้ำล้างใบในจานเพาะเชื้อ คือ *Pseudozyma antarctica*, *Meyerozyma caribbica* และ *Sporidiobolus pararoseus* โดยมีความถี่ที่ปรากฏ 21.8, 20.2 และ 11.3% ตามลำดับ โดย ***M. caribbica*** ที่พบบ่อยที่สุดในผิวใบอ้อยนี้เป็นสปีชีส์เดียวกับที่พบเมื่อแยกเชื้อโดย enrichment technique ซึ่งมีความถี่ที่ปรากฏ 12.0% ในขณะที่เมื่อแยกเชื้อโดย enrichment technique ที่สปีชีส์ที่พบบ่อยที่สุดผิวใบข้าว คือ *Rhodotorula taiwanensis* (14.3%) ส่วนผิวใบข้าวโพดพบ *Kodamaea ohmeri, M. caribbica* และ *R. taiwanensis* โดยมีความถี่ที่ปรากฏ 7.9%

จากผลการวิจัยความหลากหลายของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์ที่ใบข้าว อ้อย และข้าวโพด โดยใช้วิธีที่อาศัยการเพาะเลี้ยงเชื้อโดยตรงจากน้ำล้างใบว่าพบแบสิดิโอมัยซีตัสยีสต์มากกว่าแอสโคมัยซีตัสยีสต์ ซึ่งเหมือนกับที่รายงานในประเทศในเขตอื่นของโลก อย่างไรก็ตามถ้าการแยกยีสต์ทำโดยใช้ enrichment technique ยีสต์ที่พบมากกว่ากลับเป็นแอสโคมัยซีตัสยีสต์ ดังนั้นวิธีการที่ใช้แยกยีสต์มีผลต่อชนนิดของยีสต์ที่ตรวจพบ นอกจากนั้นในการวิจัยครั้งนี้ได้พบและรายงานยีสต์สกุลใหม่ 1 สกุล คือ *Diutina* และสปีชีส์ใหม่ 15 สปีชีส์ประกอบด้วยสปีชีส์ในไฟลัมแบสิดิโอมัยโคตา คือ *Hannaella phyllophyla*, *Hannaella phetchabunensis*, *Hannaealla siamensis*, *Occultifur tropicalis*, *Papiliotrema siamensis* และ *Pseudozyma vetiver* และสปีชีส์ในไฟลัมแอสโคมัยโคตา คือ *Diutina siamensis*, *Metschnikowia saccharicola*, *Metschnikowia lopburiensis*, *Nakazawaea siamensis*, *Wicherhamomyces siamensis*, *Wickerhamiella siamensis*, *Yamadazyma epiphylla*, *Yamadazyma phyllophila* และ *Yamadazyma siamensis* โดยยังอยู่ในระหว่างการศึกษาเพื่อเสนอเป็นสปีชีส์ใหม่อีกหลายสปีชีส์

การศึกษาความหลากหลายของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์ในใบพืชด้วยวิธีที่ไม่อาศัยการเพาะเลี้ยงเชื้อ ทำโดยเพิ่มปริมาณดีเอ็นเอบริเวณ D1/D2 ของ large subunit rDNA ด้วยปฏิกิริยา PCR และสร้าง D1/D2 library คัดเลือกโคลน (clone) ที่มีลำดับนิวคลีโอไทด์ในบริเวณ D1/D2 ที่แตกต่างกันในแต่ละ library ด้วยวิธี PCR-RFLP โดยใช้เอนไซม์ *Hae*III, *Hin*fI และ *Cfo*I และวิเคราะห์หาลำดับนิวคลีโอไทด์และเปรียบเทียบกับลำดับนิวคลีโอไทด์ในฐานข้อมูล GenBank เพื่อระบุชนิดและหาความสัมพันธ์ทางพันธุกรรม สำหรับยีสต์เอนโดไฟต์ในเนื้อเยื่อใบข้าว 46 ตัวอย่าง พบโคลนของยีสต์ 101 โคลน (5.5%) จากทั้งหมด 1,826 โคลน และจัดจำแนก phylotype ที่เป็นของยีสต์ที่รู้จักแล้ว 5 สปีชีส์ใน 4 สกุล (43.6%) คือ *Cryptococcus victoriae*, *Debaryomyces hansenii*, *Debaryomyces vindobonensis*, *Meyerozyma guilliermondii* และ *Pseudozyma antarctica* และพบ phylotype ของยีสต์ที่อาจเป็นยีสต์สปีชีส์ใหม่ 17 phylotype ที่มีความใกล้เคียงกับ *Candida metapsilosis*, *Cryptococcus foliicola*, *Cryptococcus laurentii*, *Pseudozyma abaconensis*, *Pseudozyma aphidis* และ *Trichosporon asahii* ส่วนในใบอ้อย 29 ตัวอย่าง พบโคลนของยีสต์ 224 โคลน (19.8%) จาก 1,144 โคลน และจัดจำแนก phylotype ที่เป็นของยีสต์ที่รู้จักแล้ว 3 สปีชีส์ใน 3 สกุล (76.3%) คือ *Candida palmioleophila, D.*

hansenii และ *Kodamaea ohmeri* และพบ phylotype ของยีสต์ที่อาจเป็นสปีชีส์ใหม่ 10 phylotype ที่ใกล้เคียงกับ 7 สปีชีส์ สำหรับเนื้อเยื่อใบข้าวโพด 35 ตัวอย่าง พบยีสต์ 285 โคลน (24.4%) จาก 1,166 โคลน และจัดจำแนก phylotype ที่เป็นของยีสต์ที่รู้จักแล้ว 6 สปีชีส์ใน 6 สกุล (33.0%) คือ *Hannaella sinensis*, *Mal. restricta*, *Pseudozyma parantarctica*, *Rhodotorula taiwanensis*, *T. asahii* และ *Yarrowia deformans* และพบ phylotype ของยีสต์ที่อาจเป็นยีสต์สปีชีส์ใหม่ 26 phylotype ที่ใกล้เคียงกับ 5 สปีชีส์ โดย phylotype ที่พบบ่อยที่สุดในเนื้อเยื่อใบข้าว อ้อย และข้าวโพด ได้แก่ phylotype ที่มีความใกล้เคียงกับ *Cryp. foliicola* (47.5%), phylotype ของ *K. ohmeri* (43.8%) และ phylotype ที่มีความใกล้เคียงกับ *T. faecale* (30.1%) ตามลำดับ และยังพบ phylotype ของยีสต์ที่อาจเป็นยีสต์สปีชีส์ใหม่มากถึง 56.4%, 32.6% และ 69.2% ของ phylotype ของยีสต์ทั้งหมดในตัวอย่างใบข้าว อ้อย และข้าวโพด ตามลำดับ ส่วนของยีสต์อีพิไฟต์จากใบข้าว (44 ตัวอย่าง) พบโคลนของยีสต์ 451 โคลน (40.2%) จาก 1,121 โคลน โดย 329 โคลน (72.9%) จัดจำแนกได้เป็นยีสต์ที่รู้จักแล้ว 9 สปีชีส์ ใน 6 สกุล คือ *Bullera japonica*, *Metschnikowia koreensis*, *Mey. guilliermondii*, *P. antarctica*, *P. aphidis*, *Sporobolomyces blumeae*, *Sporobolomyces carnicolor*, *Sporobolomyces oryzicola* และ *Wickerhamomyces anomalus* สำหรับผิวด้านนอกของใบอ้อย (45 ตัวอย่าง) พบยีสต์ 426 โคลน (31.7%) จาก 1,342 โคลน โดย phylotype ส่วนใหญ่ไม่สามารถระบุสปีชีส์ได้ (410 โคลน) โดยใกล้เคียงกับยีสต์ 11 สปีชีส์ ใน 7 สกุล ได้แก่ *Cryp. flavus*, *Hannaella coprosmaensis*, *R. taiwanensis*, *Jaminaea angkorensis*, *Mal. restricta*, *P. antarctica*, *P. aphidis*, *Pseudozyma hubeiensis*, *Pseudozyma prolifica*, *Pseudozyma shanxiensis* และ *Sp. vermiculatus* โดยยีสต์ที่พบส่วนใหญ่อยู่ในสกุล *Pseudozyma* สำหรับยีสต์บนผิวใบข้าวโพด (37 ตัวอย่าง) พบว่ายีสต์ 357 โคลน (34.0%) จาก 1,049 โคลน ซึ่งเป็นสปีชีส์ที่รู้จักแล้ว 12 สปีชีส์ ใน 7 สกุล จาก 98 โคลน (27.5%) ได้แก่ *Bullera derxii, Bullera oryzae, Cryptococcus flavescens, H. sinensis, J. angkorensis, P. antarctica, P. aphidis, P. hubeiensis, P. prolifica, Sporidiobolus pararoseus, Sp. carnicolor* และ *Sporobolomyces odoratus* ที่เหลือส่วนใหญ่ใกล้เคียงกับยีสต์ในสกุล *Pseudozyma* เป็นที่น่าสนใจว่า phylotype ที่พบบนผิวใบพืชทั้ง 3 ชนิดส่วนใหญ่มีความใกล้เคียงกับยีสต์ในไฟลัมแบสิดิโอมัยโคตา และไม่สามารถระบุได้ว่าเป็นยีสต์สปีชีส์ใด ดังนั้นจึงอาจเป็นลำดับนิวคลีโอไทด์ของยีสต์สปีชีส์ใหม่ ยีสต์อีพิไฟต์สปีชีส์ที่ศึกษาโดยใช้วิธีที่ไม่อาศัยการเพาะเลี้ยงเชื้อที่พบบ่อยที่สุดในผิวใบข้าว คือ *P. antarctica* ด้วยความถี่ที่ปรากฏ 63.6% ส่วนในผิวใบอ้อยยีสต์ที่พบบ่อยอยู่ในสกุล *Pseudozyma* และในผิวใบข้าวโพดสปีชีส์ที่พบบ่อยใกล้เคียงกัน คือ *P. hubeiensis* และ *P. antarctica* ด้วยความถี่ที่ปรากฏ 24.3และ 21.6% ตามลำดับ

การคัดเลือกยีสต์ที่มีประสิทธิภาพเพื่อการผลิตกรดอินโดล-3-แอซีติก (IAA) จากยีสต์อีพิไฟต์และยีสต์เอนโดไฟต์ที่แยกจากใบข้าว อ้อย และข้าวโพด และการศึกษาสภาวะที่เหมาะสมของการผลิต IAA โดยวิธีศึกษาทีละปัจจัยและพื้นผิวตอบสนอง รวมกับการศึกษาชีววิถีสังเคราะห์ IAA จากยีสต์จำนวนทั้งสิ้น 1,388 สายพันธุ์ ที่แยกจากใบข้าวจำนวน 430 สายพันธุ์ จากใบอ้อยจำนวน 605 สายพันธุ์ และจากใบข้าวโพดจำนวน 353 สายพันธุ์ โดยเลี้ยงในอาหาร yeast extract peptone dextrose (YPD) broth ที่เติมเอล-ทริปโตเฟน 0.1% วิเคราะห์ปริมาณ IAA โดย colorimetric method และ HPLC พบยีสต์ 2 สายพันธุ์ สกุล *Rhodosporidium* ที่สามารถผลิต IAA ได้สูง คือ *Rh. fluviale* DMKU-CP293 ที่แยกจากผิวใบข้าวโพด และ *Rh. paludigenum* DMKU-RP301 ที่แยกจากผิวใบข้าว โดยผลิต IAA ได้เท่ากับ 141.5 และ 321.7 mg/L ตามลำดับ โดยคิดเป็น 31.9 และ 29.3 mg/ g dry cell weight ตามลำดับ หลังจากนั้นศึกษาสภาวะที่เหมาะสมของการผลิต IAA ในฟลาส์กโดยวิธีศึกษาทีละปัจจัย พบว่า *R. fluviale* DMKU-CP293 และ *Rh. paludigenum* DMKU-RP301 ผลิต IAA ได้สูงขึ้นเป็น 1,559.4 และ 1,464.7 mg/L ตามลำดับ ขณะที่เมื่อใช้วิธีพื้นผิวตอบสนองโดยใช้แผนการทดลอง Box-Benhken สำหรับออกแบบการทดลอง พบว่า *Rh. fluviale* DMKU-CP293 และ *Rh. paludigenum* DMKU-RP301 ผลิต IAA ได้สูงขึ้นเป็น 2,420.2 และ 1,623.9 mg/L ตามลำดับ จากการปรับปรุงการผลิต IAA ในปริมาตรที่ใหญ่ขึ้นโดยใช้ถังหมัก พบว่า *Rh. fluviale* DMKU-CP293 สามารถผลิต IAA ได้สูงสุดเท่ากับ 8,930.0 mg/L โดยการหมักแบบแบตช์ในถังหมัก

ขนาด 5 ลิตร และ *Rh. paludigenum* DMKU-RP301 ผลิต IAA ได้สูงสุดเท่ากับ 2,707.9 mg/L โดยการหมักแบบเฟด-แบตช์ในถังหมักขนาด 2 ลิตร เมื่อนำส่วนใสหลังจากการผลิต IAA ของ *R. paludigenum* DMKU-RP301 มาวิเคราะห์หาสารสื่อกลางของชีววิถีสังเคราะห์โดย gas chromatography–mass spectrometry (GC-MS) และ HPLC พบ indole-3-pyruvic acid, indole-3-lactic acid และ indole-3-ethanol ในอาหารเลี้ยงเชื้อ และพบกิจกรรมเอนไซม์ tryptophan aminotransferase ดังนั้นแสดงให้เห็นว่ายีสต์ *R. paludigenum* DMKU-RP301 ผลิต IAA ผ่านทางวิถีกรดอินโดล-3-ไพรูวิก นอกจากการผลิตกรดIAA พบว่ายีสต์ *R. paludigenum* DMKU-RP301 ยังผลิตแอมโมเนีย, คาตาเลส (catalase) และไซเดอโรฟอร์ (siderophore) ซึ่งเป็นลักษณะที่สนับสนุนการเจริญของพืชทั้งทางตรงและทางอ้อม

จากการประเมินและคัดเลือกยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์ที่แยกได้มีศักยภาพในการควบคุมเชื้อราที่ทำให้เกิดโรคกับพืชเศรษฐกิจทั้ง 3 ชนิด รวม 10 สายพันธุ์ ประกอบด้วยราโรคข้าว (*Fusarium moniliforme*, *Helminthosporium oryzae* และ *Rhizoctonia solani*) ราโรคข้าวโพด (*Bipolaris maydis, Bipolaris zeicola, Exserohilum turcicum, F. moniliforme* และ *Macrophomina phaseolina*) และราโรคอ้อย (*Colletotrichum falcatum* และ *F. moniliforme*) พบว่าเมื่อตรวจการยับยั้งราโรคพืชของยีสต์เอนโดไฟต์ 417 สายพันธุ์ พบว่ามียีสต์เพียง 11 สายพันธุ์ที่ยับยั้งราโรคพืชได้ ประกอบด้วย *Wickerhamomyces anomalus* 7 สายพันธุ์ และ *Kodamaea ohmeri* 4 สายพันธุ์ จากการศึกษาการยับยั้งราโรคพืชของยีสต์อีพิไฟต์ พบว่ายีสต์จากผิวใบอ้อย 173 สายพันธุ์ มีเพียง 4 สายพันธุ์ที่ยับยั้งการเจริญของราโรคพืชได้ คือ *Pap. siamense* DMKU-SP85^{T}, *Candida* sp. DMKU-SP81, *P. alboarmeniaca* DMKU-SP290 และ *K. ohmeri* DMKU-SP45 ในขณะที่ยีสต์อีพิไฟต์จากผิวใบข้าวและข้าวโพด 221 สายพันธุ์ พบว่ามี 37 สายพันธุ์ ใน 17 สปีชีส์ 12 สกุลที่ยับยั้งราโรคพืชได้ในจานเพาะเชื้อ โดย *Torulaspora globosa* DMKU-RP31 และ *T. globosa* DMKU-RP35 ยับยั้งราสาเหตุโรคพืชได้ 8 ชนิด *Wickerhamomyces anomalus* DMKU-RP04 ยับยั้งราสาเหตุโรคพืชได้ 7 ชนิด *K. ohmeri* DMKU-RP06 และ *W. anomalus* DMKU-RP25 ยับยั้งราสาเหตุโรคพืชได้ 5 ชนิด ยีสต์ 3 สายพันธุ์ ได้แก่ *W. anomalus* DMKU-CP122*W. anomalus* DMKU-CP127 และ *K. ohmeri* DMKU-RP34 ยับยั้งราสาเหตุโรคพืชได้ 4 ชนิด แต่ละสายพันธุ์ ของยีสต์ที่เหลือยับยั้งราสาเหตุโรคพืชได้น้อยกว่า 4 ชนิด จากการทดลองนี้ยีสต์ปฏิปักษ์ทั้ง 37 สายพันธุ์พบว่า *Candida floricola* DMKU-CP149, *Hanseniaspora guilliermondii* DMKU-CP295, *C. floricola* DMKU-CP367, *Bullera sinensis* DMKU-CP437, *W. anomalus* DMKU-RP04, *W. anomalus* DMKU-RP25, *T. globosa* DMKU-RP31, *K. ohmeri* DMKU-RP34 และ *T. globosa* DMKU-RP35 ยับยั้งราโรคพืชสูงกว่า 50% จากการศึกษากลไกควบคุมราโรคพืชของยีสต์ทั้ง 37 สายพันธุ์ที่คัดเลือกได้ พบมียีสต์ที่ผลิตไซเดอโรฟอร์ได้ 8 สายพันธุ์ มียีสต์ที่ผลิตสารระเหยเพื่อยับยั้ง *R. solani* 7 สายพันธุ์ โดยยับยั้งได้ 57.0-73.9% มียีสต์ 33 สายพันธุ์ ที่ผลิตเอนไซม์เบต้า-กลูคาเนสได้ในช่วง 0.5-42 mU/ และ 21 สายพันธุ์ที่ผลิตไคติเนสในช่วง 2-249 mU/ml ความสามารถในการแข่งขันใช้สารอาหารและพื้นที่พบว่ายีสต์ 12 สายพันธุ์ยับยั้งราสาเหตุโรคพืชสูงเมื่อมีธาตุอาหารความเข้มข้นต่ำลง จากการทดสอบการควบคุมโรคกาบใบแห้ง (*R. solani*) ในเรือนทดลองโดยยีสต์ปฏิปักษ์ 7 สายพันธุ์ (*B. sinensis* DMKU-CP437, *C. floricola* DMKU-CP149, *C. floricola* DMKU-CP367, *H. guilliermondii* DMKU-CP295, *W. anomalus* DMKU-RP25, *T. globosa* DMKU-RP31 และ *T. globosa* DMKU-RP35) พบว่าการพ่นเซลล์แขวนลอยของยีสต์ *H. guilliermondii* DMKU-CP295 กดการเกิดโรคกาบใบแห้งในข้าวได้อย่างมีนัยสำคัญ โดยกดการเกิดโรคสูงกว่า 65%

Abstract

This research project aims to investigate and analyze the diversity and ecology of endophytic and epiphytic yeasts from leaves of rice, sugarcane and corn by culture-dependent and culture-independent methods, to produce indole-3-acetic acid (IAA) by the selected promising yeast strain obtain from the screening of endophytic and epiphytic yeasts, and elucidate the biosynthetic pathway of IAA and to evaluate the efficiency to be biological control agents for rice, sugarcane and corn pathogenic fungi of endophytic and epiphytic yeasts.

To understand the diversity and ecology of endophytic and epiphytic yeasts leaves of healthy agronomic crops (rice, sugarcane and corn) were collected from their cultivation fields in the central and north-eastern parts during October 2011 to April 2012. The sample consisted of 100 rice leaf samples collected from 11 provinces, 102 sugarcane leaf samples collected from 13 provinces and 109 sugarcane leaf samples collected from 12 provinces. The number of yeasts, bacteria and fungi from leaf samples was found to vary among the samples.

Study on the endophytic yeast diversity based on culture-dependent method yeasts was carried out by isolation of yeast from the surface-sterilized leaves tissue. From 100, 102 and 109 samples of rice, sugarcane and corn leaf we obtained 125, 149 and 143 yeast strains, respectively. Identification of yeast was based on molecular taxonomy and genetic analysis revealed that 117 yeast strains from rice leaf samples were 23 known species in 12 genera. Ninety 90 strains (76.9%) were in phylum Basidiomycota (7 genera) and 27 strains (23.1%) in the phylum Ascomycota
(5 genera). One strain was identical with one undescribed species, three strains represented a novel genus and species, whereas the other strains were fungi. Of endophytic yeasts from sugarcane leaf, 100 strains were known yeast species. A total of 65 strains (65.0%) were in Basidiomycota (10 genera). Thirty-five strains were in Ascomycota (4 genera). Six strains were found to represent two novel yeast species while 21 strains were fungi. A total of 120 strains obtained from corn leaves were 37 known yeast species. Among these 90 strains (75.0%) were in Basidiomycota (7 genera) and 30 strains (25.0%) were in Ascomycota (5 genera). One strain was a new species, three strains were identical with three undescribed species and the other were fungi. The most prevalent species of endophytic yeasts from rice, sugarcane and corn leaves were *Pseudozyma aphidis*, *Meyerozyma caribbica* and *Pseudozyma churashimaensis* with 25.0, 17.6 and 20.0% frequency of occurrence, respectively.

To investigate the diversity of epiphytic yeasts in the surface of rice, sugarcane and corn leaved by culture-dependent method, yeasts were isolated from leaf surface by plating of leaf washing. A total of 379 strains were isolated from 112 rice leaf samples and 354 strains were identified. The result revealed that 302 strains were 50 known species in 18 genera. Two hundred and forty six strains (81.5%) were in Basidiomycota (10 genera) and 55 strains were in Ascomycota (9 genera). Twenty one strains were identical with 14 undescribed species in four genera. As many as 31 strains were expected to be 20 new yeast species in 8 genera. A total of 173 strains were obtained from the surface of 84 sugarcane leaf samples. Eighty seven strains were known yeast

species. Sixty one strains (35.3%) were 24 species in Basidiomycota (10 genera) and 26 strains (15%) were 12 species in genera in Ascomycota. The other 67 strains may be new or known yeast species that need more analysis. As many as 20 strains were found to represent 12 new yeast species. From the surface of corn leaves (109 samples) 284 strains were obtained and 244 strains were identified. One hundred and fifty one strains were 43 known species in 15 genera. A total of 130 strains (86.1%) were in Basidiomycota (10 genera) and 21 strains (13.9%) were in Ascomycota (5 genera). Eighteen strains were identical with undescribed yeast species in 3 genera and 21 strains were expected to be 16 new yeast species, while the other strains were fungi. Investigation of epiphytic yeasts in the surface of 244 leaf samples by using an enrichment isolation technique revealed that 156, 126 and 159 strains were obtained from rice, sugarcane and corn leaf samples, respectively.

Identification of epiphytic yeasts from rice leaf samples showed that 155 strains were known yeast species. Among the known species 99 strains (63.9%) were 23 species of 13 genera in Ascomycota. Fifty six strains (36.1%) were 8 species of 5 genera in Basidiomycota. One hundred and forty six strains of epiphytic yeast from the surface of sugarcane leaves were 23 species of 13 genera in Ascomycota and 46 strains (31.7%) were 12 species of 6 genera in Basidiomycota. The other eight strains were identical with undescribed species and nine strains were six new species. Epiphytic strains (123 strains) from the surface of corn leaves were known yeast species. Eighty one 81 strains (65.9%) were 28 species of 12 genera in Ascomycota and 42 strains (34.1%) were 10 species of 5 genera in Basidiomycota. The most prevalent species in the surface of rice, sugarcane and corn leaves, which were isolated by plating of leaf washing, were *Pseudozyma antarctica*, *Meyerozyma caribbica* and *Sporidiobolus pararoseus* at 21.8, 20.2 and 11.3% frequency of occurrence, respectively. ***M. caribbica***, which was the most prevalent species in the surface of sugarcane leaf when plating of leaf washing was used, was also be the most prevalent species (12.0%) in the surface of sugarcane leaf when enrichment isolation technique was used. By using the enrichment isolation technique, the most prevalent species in the surface of rice leaf was *Rhodotorula taiwanensis* (14.3%) while *Kodamaea ohmeri* and *Meyerozyma caribbica* and *Rhodotorula taiwanensis* occurred in the surface of corn leaf at the same frequency of 7.9%.

The result of investigation of endophytic and epiphytic yeasts diversity by using culture-dependent method revealed higher number of basidiomycetous yeasts than ascomycetous yeasts were obtained same as that were reported in the other countries and areas. However, when enrichment isolation technique was used higher number of ascomycetous yeasts than basidiomycetous yeasts were detected. Therefore, our results indicated the influence of isolation methods on the obtained yeast species. Besides, one new genus *viz. Diutina* and 15 new yeast species were proposed *viz. Hannaella phyllophyla*, *Hannaella phetchabunensis*, *Hannaealla siamensis*, *Occultifur tropicalis*, *Papiliotrema siamensis* and *Pseudozyma vetiver* in the phylum Basidiomycota, and *Diutina siamensis*, *Metschnikowia saccharicola*, *Metschnikowia lopburiensis*, *Nakazawaea siamensis*, *Wicherhamomyces siamensis*, *Wickerhamiella siamensis*, *Yamadazyma epiphylla*, *Yamadazyma phyllophila* and *Yamadazyma siamensis* in the phylum Ascomycota.

Moreover, various new yeast species are under characterization and will be proposed in the near future.

A culture-independent method was used to examine endophytic and epiphytic yeasts associated with rice, sugarcane and corn leaves based on PCR amplification of the D1/D2 domain of the large subunit rRNA gene, construction of D1/D2 libraries, PCR-RFLP using a single digestion with *Hae*III, *Hin*fI and *Cfo*I, sequencing and phylogenetic analysis. For the investigation of endophytic yeasts in rice, sugarcane and corn leaves, the results showed that 101 (5.5%) out of 1,826 clones from 46 rice leaf samples were yeasts. Of total yeast related phylotypes, 44 phylotypes (43.6%) were five known species in four genera *viz. Cryptococcus victoriae, Debaryomyces hansenii, Debaryomyces vindobonensis, Meyerozyma guilliermondii* and *Pseudozyma antarctica*, together with 17 new phylotypes closest to six species. For sugarcane, 224 (19.8%) out of 1,144 clones from 29 samples were yeasts. Of total yeast related phylotypes, 171 phylotypes (76.3%) were three known species in three genera *viz. Candida palmioleophila, D. hansenii* and *K. ohmeri*, together with 10 new phylotypes closest seven species. In case of corn leaves, 285 (24.4%) out of 1,166 clones from 29 samples were yeasts. Of total yeast related phylotypes, 94 phylotypes (33.0%) were identified as six known species in six genera including *Hannaella sinensis, Mal. restricta, Pseudozyma parantarctica*, *Rhodotorula taiwanensis*, *T. asahii* and *Yarrowia lipolytica*), together with 26 new phylotypes closest five species. The most prevalent phylotypes in tissue of rice, sugarcane and corn leaves were new phylotypes closest to *Cryp. foliicola*, phylotypes identified as *K. ohmeri* and new phylotypes closest to *T. faecale, respectively*. Moreover, the new phylotypes were found at high frequency as 56.4%, 32.6% and 69.2% in rice, sugarcane and corn leaves, respectively.

The epiphytic yeast diversity in the leaf surface of three economic crop were also investigated by culture-independent method. For rice leaf samples (n=44), of total 1,121 clones, 451 clones (40.2%) were yeasts. Of total yeast related clones, 329 clones (72.9%) were 9 known yeast species in 6 genera *viz. Bullera japonica*, *Metschnikowia koreensis*, *Mey. guilliermondii*, *P. antarctica*, *P. aphidis*, *Sporobolomyces blumeae*, *Sporobolomyces carnicolor*, *Sporobolomyces oryzicola* and *Wickerhamomyces anomalus*. For sugarcane leaf samples (n=45), of total 1,342 clones, 426 clones (31.7%) were yeasts. Nevertheless, the majority of yeast related sequences (410 clones) were unable to be identified as known yeasts species. These were closest to 11 yeast species in seven genera *viz*. *Cryp. flavus*, *Hannaella coprosmaensis*, *R. taiwanensis*, *Jaminaea angkorensis*, *Mal. restricta*, *P. antarctica*, *P. aphidis*, *P. hubeiensis*, *P. prolifica*, *P. shanxiensis* and *Sp. vermiculatus*. In case of corn phyllosphere samples (n=37), of total 1,049 clones, 357 clones (34.0%) were yeasts. Of total yeast related clones, 98 clones (27.5%) were 12 known yeast species in seven genera including *B. derxii, B. oryzae, Cryp. flavescens, H. sinensis, J. angkorensis, P. antarctica, P. aphidis, P. hubeiensis, P. prolifica, Sporidiobolus pararoseus, Sp. carnicolor* and *Sporobolomyces odoratus*. The majority of yeast related sequences were closest to yeasts in genus *Pseudozyma.* Interestingly, most of the phylotypes detected in all three leaf samples were identified as yeasts in phylum Basidiomycota and were unable to identify as known yeast species, implying the representative phylotypes of the new yeast taxa. The most prevalent

yeast species in the surface of rice leaf was *P. antarctica* with 63.6% frequency of occurrence. The most prevalent genus in the surface of sugarcane leaf was *Pseudozyma*. *P. hubeiensis* and *P. antarctica* were most prevalent species in the surface corn leaf samples with 24.3% and 21.6% frequency of occurrence.

Selection of potential yeast for indole-3-acetic acid (IAA) production was carried out in epiphytic yeasts and endophytic yeasts isolated from rice leaves, sugar cane leaves and corn leaves. Optimization of IAA production by the selected yeast strains was performed using one factor at a time (OFAT) as well as response surface methodology (RSM) experimental designs. IAA biosynthetic pathway of the selected yeast isolate was also investigated. A total of 1,388 yeasts isolated from rice leaves (430 strains), sugar cane leaves (605 strains) and corn leaves (353 strains) were subjected to screening for high IAA producing yeast in yeast extract peptone dextrose (YPD) broth supplemented with 0.1% tryptophan. IAA assay was performed by colorimetric method and high-performance liquid chromatography (HPLC). As a result, two yeast strains i.e. corn phylloplane yeast *Rhodosporidium fluviale* DMKU-CP293 and rice phylloplane yeast *R. paludigenum* DMKU-RP301 were found as high IAA producers by producing 141.5 and 321.7 mg IAA/L which corresponding to 31.9 and 29.3 mg IAA/g dry cell weight respectively. Optimization of IAA production in flasks by the selected yeasts using one factor at a time (OFAT) revealed that *R. fluviale* DMKU-CP293 and *R. paludigenum* DMKU-RP301 produced 1,559.4 and 1,464.7 mg IAA/L, respectively. Respond Surface Methodology (RSM) was used to improve IAA production and Box-Benhken experimental design was employed. Results indicated that *R. fluviale* DMKU-CP293 and *R. paludigenum* DMKU-RP301 improved their IAA production to 2,420.2 and 1,623.9 mg/L, respectively. The production of IAA in batch cultivation was then scaled up in a 5-l fermenter for *R. fluviale* DMKU-CP293 and the maximum IAA of 8,930.0 mg/L was obtained whereas 2-l stirred tank fermenter was used for fed-batch cultivation of *R. paludigenum* DMKU-RP301 and the maximum IAA of 2,707.9 mg/l was obtained. To investigate on IAA biosynthetic pathway, culture broth of *R. paludigenum* DMKU-RP301 was analysed by both gas chromatography–mass spectrometry (GC-MS) and HPLC. Indole-3-pyruvic acid, indole-3-lactic acid and indole-3-ethanol were detected in the culture broth as well as tryptophan aminotransferase activity. This therefore indicates that the yeast *R. paludigenum* DMKU-RP301 produced IAA via Indole-3-pyruvic acid pathway. In addition to IAA production, *R. paludigenum* DMKU-RP301 also showed the production of ammonia, siderophore and catalase which are both direct and indirect plant growth promoting characters.

To evaluate the efficiency of endophytic and epiphytic yeasts to be biological control agent rice, sugarcane and corn pathogenic fungi. Ten plant pathogenic fungi (PPF) including rice pathogenic fungi (*Fusarium moniliforme*, *Helminthosporium oryzae* and *Rhizoctonia solani*), corn pathogenic fungi (*Bipolaris maydis*, *Bipolaris zeicola*, *Exserohilum turcicum*, *Fusarium moniliforme* and *Macrophomina phaseolina*) and sugarcane pathogenic fungi (*Colletotrichum falcatum* and *Fusarium moniliforme*) were used. By dual cultivation of 417 endophytic yeast strains and 10 PPF only 11 strains in two species (*Wickerhamomyces anomalus* and *Kodamaea ohmeri*) showed inhibition on photogenic fungi. Determination of antagonistic activity of 173 epiphytic yeast strains against 10 PPF revealed that only four yeast strains *viz. Pap. siamense* DMKU-SP85^{T}, *K. ohmeri*

DMKU-SP45, *Candida* sp. DMKU-SP81 and *P. alboarmeniaca* DMKU-SP290 inhibited growth of some PPF with various percentages of inhibition. The four antagonistic yeast strains produced siderophore and two enzymes (ß-glucanase and chitinase). *P. alboarmeniaca* DMKU-SP290 produced volatile compounds and inhibited growth of pathogenic fungi by competition of nutrients. *Candida* sp. DMKU-SP81 and *K. ohmeri* DMKU-SP45 produced the highest ß-glucanase and chitinase, respectively. A total of 221 epiphytic yeast strains from the surface of rice and corn leaves were determined for their antagonistic activity and 37 strains of 17 species in 12 genera inhibited growth of PPF. Among these *Torulaspora globosa* DMKU-RP31 and *T. globosa* DMKU-RP35 inhibited 8 PPF, *W. anomalus* DMKU-RP04 inhibited 7 PPF, *K. ohmeri* DMKU-RP06 and *W. anomalus* DMKU-RP25 inhibited 5 PPF, *W. anomalus* DMKU-CP122, *W. anomalus* DMKU-CP127 and *K. ohmeri* DMKU-RP34 inhibited 4 PPF and the others inhibited less than 4 PPF. Considering of inhibition, *Candida floricola* DMKU-CP149, *Hanseniaspora guilliermondii* DMKU-CP295, *C. floricola* DMKU-CP367, *Bullera sinensis* DMKU-CP437, *W. anomalus* DMKU-RP04, *W. anomalus* DMKU-RP25, *T. globosa* DMKU-RP31, *K. ohmeri* DMKU-RP34 and *T. globosa* DMKU-RP35 inhibited growth of PPF greater than 50%. Inhibitory mechanisms study revealed that eight antagonistic strains produced siderophore, seven strains produced volatile compounds to inhibit growth of *R. solani* by 57.0-73.9% inhibition, 34 strains produced β-glucanase in the range of 0.5-42 mU/ml and 21 strains produced chitinase in the range of 2-249 mU/ml. The result of investigation on competition of nutrient and space revealed that 12 yeast strains inhibited growth of PPF when nutrient concentration was low. Rice sheath blight disease (*R. solani*) control was tested in greenhouse by the seven antagonistic yeast strains (*Bullera sinensis* DMKU-CP437, *C. floricola* DMKU-CP149, *C. floricola* DMKU-CP367, *H. guilliermondii* DMKU-CP295, *T. globosa* DMKU-RP31, *T. globosa* DMKU-RP35 and *W. anomalus* DMKU-RP25). The result showed that cell suspension of *H. guilliermondii* DMKU-CP295 applied by spraying significantly suppressed the rice sheath blight disease by up to 65 %.

บทสรุปผู้บริหาร

บทนำ

ใบพืชถือเป็นแหล่งที่อยู่ (habitat) ที่สำคัญของจุลินทรีย์ทั้งจุลินทรีย์เอนโดไฟต์ที่อาศัยอยู่ในเนื้อเยื่อของพืชและจุลินทรีย์อีพิไฟต์ที่อาศัยอยู่ที่ผิวด้านนอกของพืช สำหรับยีสต์เอนโดไฟต์ (endophytic yeast) เป็นยีสต์ที่อาศัยอยู่ในเนื้อเยื่อของพืชโดยไม่ทำให้เกิดรอยแผลหรือผลเสียใดๆ ต่อพืชที่อาศัยอยู่ ปัจจุบันเป็นที่ยอมรับโดยทั่วไปว่าพืชทุกชนิดมีราอาศัยอยู่ในเนื้อเยื่อแต่มีการศึกษาไม่มากที่รายงานการเข้าไปอาศัย จนถึงปัจจุบันมีรายงานการแยกยีสต์เอนโดไฟต์ในพืชไม่กี่ชนิดในบางประเทศ แต่ไม่มีรายงานการวิจัยที่ตรวจหายีสต์เอนโดไฟต์ในพืชชนิดใด ๆ ในประเทศไทย ในขณะที่มีการวิจัยและรายงานเกี่ยวกับราเอนโดไฟต์จำนวนไม่มากนักในประเทศไทยโดยนักวิจัยบางกลุ่ม ดังนั้นคณะนักวิจัยกลุ่มนี้จึงสนใจทำการศึกษาความหลากหลายและนิเวศวิทยาของยีสต์เอนโดไฟต์ในประเทศไทย สำหรับยีสต์อีพิไฟต์อาศัยที่ผิวด้านนอกของพืช โดยอาจมีความสัมพันธ์กับพืชเป็นแบบแซโพรไฟต์ (saprophyte) ปรสิต (parasite) และแบบอยู่ร่วมกัน (symbiosis) ในบรรดาผิวด้านนอกของพืชนั้น ผิวด้านนอกของใบพืชซึ่งมักเรียกว่า ฟิลโลเพลน (phylloplane) หรือ ฟิลโลสเฟียร์ (phyllosphere) จัดว่าเป็นแหล่งที่อยู่ (habitat) ที่สำคัญของจุลินทรีย์อีพิไฟต์รวมทั้งยีสต์ แม้ว่ามีการศึกษาความหลากหลายและการแยกยีสต์จากฟิลโลเพลนของพืชหลายชนิดในหลายประเทศแต่จนถึงปัจจุบันความรู้ทางด้านความหลากหลายของยีสต์ในผิวใบพืช (ฟิลโลเพลน) และบทบาททางนิเวศวิทยาของยีสต์ยังมีไม่มากนัก ในประเทศไทยมีการวิจัยและรายงานเกี่ยวกับยีสต์อีพิไฟต์น้อย จนมีผลให้ไม่สามารถสรุปว่าความหลากหลายและนิเวศวิทยาของยีสต์ที่ฟิลโลเพลนได้ว่าเหมือนหรือแตกต่างจากที่พบในประเทศอื่นและภูมิภาคอื่นหรือไม่ การศึกษาความหลากหลายของจุลินทรีย์ด้วยวิธีที่อาศัยการเพาะเลี้ยงเชื้อ (culture-dependent method) ปัญหาหลักก็คือการที่ไม่สามารถหาสภาวะที่เหมาะสมสำหรับการเพาะเลี้ยงและแยกจุลินทรีย์แต่ละชนิดทุกสปีชีส์ได้ และนอกจากนั้นจุลินทรีย์บางชนิดอาจไม่สามารถเพาะเลี้ยงได้ ปัจจุบันเทคนิคด้านชีวโมเลกุลช่วยให้การศึกษาด้านความหลากหลายของจุลินทรีย์ในระบบนิเวศต่างๆ ทำได้รวดเร็วขึ้นและแก้ไขปัญหาในส่วนของจุลินทรีย์ที่ไม่สามารถเพาะเลี้ยงได้ในห้องปฏิบัติการด้วยอาหารเลี้ยงเชื้อ ดังนั้นการใช้วิธีที่ไม่อาศัยการเพาะเลี้ยงเชื้อจะทำให้ทราบถึงข้อมูลประชากรของจุลินทรีย์ทั้งชนิดที่เพาะเลี้ยงได้และชนิดที่เพาะเลี้ยงไม่ได้ อย่างไรก็ตามจุลินทรีย์ที่ได้จากวิธีที่อาศัยการเพาะเลี้ยงเชื้อสามารถนำไปใช้ประโยชน์ในด้านต่าง ๆ ได้ทันที ซึ่งถือว่าเป็นข้อดีของการศึกษาความหลากหลายด้วยวิธีนี้ที่เหนือกว่าวิธีที่ไม่อาศัยการเพาะเลี้ยงเชื้อ

ข้าว อ้อย และข้าวโพด เป็นพืชที่มีความสำคัญทางเศรษฐกิจอันดับต้น ๆ ของประเทศไทย ดังนั้นคณะนักวิจัยกลุ่มนี้จึงเลือกใบของพืชเศรษฐกิจซึ่งเป็นพืชใบเลี้ยงเดี่ยวทั้ง 3 ชนิด เพื่อใช้เป็นต้นแบบในการตรวจวิเคราะห์ความหลากหลายและนิเวศวิทยาของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์ในใบพืชประเทศไทย

ยีสต์แซโพรไฟต์หลายสปีชีส์สามารถเข้าไปเจริญอยู่ในหรืออยู่ที่ผิวใบพืชและทำหน้าที่เป็นแนวกั้นตามธรรมชาติต่อราโรคพืช ดังนั้นยีสต์เหล่านี้จึงได้รับความสนใจที่จะใช้เป็นตัวควบคุมทางชีวภาพ (biocontrol agent) ยิ่งไปกว่านั้นจุลินทรีย์ที่มีความสัมพันธ์กับพืชอาจสามารถส่งเสริมการเจริญและการพัฒนาของพืชด้วยหลายกลไก บางชนิดสร้างฮอร์โมนพืช (phytohormone) ซึ่งทำหน้าที่เป็นสารสำคัญในการสื่อสารระหว่างจุลินทรีย์กับพืชให้อาศัย บทบาทของจุลินทรีย์ทั้งจุลินทรีย์เอนโดไฟต์และจุลินทรีย์อีพิไฟต์ที่สัมพันธ์กับพืชได้รับความสนใจมากขึ้น เนื่องจากจุลินทรีย์เหล่านั้นสามารถนำไปใช้ประโยชน์เป็นสารส่งเสริมการเจริญพืช (plant growth promoter) เพื่อเพิ่มการผลิตพืชผล และใช้เป็นตัวควบคุมทางชีวภาพสำหรับราโรคพืช ดังนั้นคณะนักวิจัยกลุ่มนี้จึงสนใจนำยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์ที่ได้จากการวิจัยในครั้งนี้มาคัดเลือกเพื่อหาสายพันธุ์ที่มีศักยภาพในการผลิตสารส่งเสริมการเจริญพืช และสายพันธุ์ที่มีศักยภาพในการควบคุมราโรคพืช อย่างไรก็ตามคาดการณ์ว่าการใช้เซลล์ยีสต์ที่มีชีวิตอาจจะต้องเผชิญกับปัญหาหลายอย่างทำให้คณะนักวิจัยสนใจที่จะผลิตเป็นสารส่งเสริมการเจริญพืชบริสุทธิ์ ซึ่งจากผลการวิจัยในเบื้องต้นของคณะนักวิจัยกลุ่มนี้พบยีสต์ที่แยกจากผิวใบพืชหลายสายพันธุ์ผลิตกรดอินโดล-3-แอซีติก (indole-3-acetic acid, IAA) ได้

กรดอินโดล-3-แอซีติกเป็นฮอร์โมนพืชหลักที่จัดอยู่ในกลุ่มของออกซิน (auxin) ที่เป็นอนุพันธ์ของสารประกอบอินโดล มีบทบาทที่สำคัญในการควบคุมและการพัฒนา ตลอดจนกระบวนการทางสรีรวิทยาของพืชหลายประการ เช่น กระตุ้นการแบ่งเซลล์ราก การยืดขยายตัวของเซลล์ราก กระตุ้นให้เซลล์เกิดความแตกต่าง ควบคุมการร่วงของใบและการสุกของผลไม้ และยังช่วยการตอบสนองของแสงและแรงโน้มถ่วง นอกจากนั้นยังมีผู้พบว่าไม่เพียงแต่ IAA ที่ผลิตภายในเซลล์ของพืชเท่านั้นที่ส่งเสริมการเจริญพืช IAA ที่สร้างและขับจากจุลินทรีย์ที่สัมพันธ์กับพืชมีส่วนในการส่งเสริมการเจริญพืชด้วยเช่นกัน จุลินทรีย์ที่สร้างสารส่งเสริมการเจริญพืชที่มีการวิจัยและรายงานว่ามีบทบาทกับการเจริญพืชมีทั้งแบคทีเรีย รา และยีสต์ สำหรับชีววิถีการสังเคราะห์ IAA ในพืชมีการศึกษากันมาก ซึ่งข้อมูลความรู้ที่ได้จากการศึกษาวิจัยในพืชได้ขยายไปยังนักวิจัยจุลินทรีย์ ทำให้รู้ถึงกิจกรรมและคุณสมบัติของ IAA จากจุลินทรีย์ และปฏิสัมพันธ์ระหว่างพืชกับจุลินทรีย์ อย่างไรก็ตามการวิจัยส่วนใหญ่มุ่งที่แบคทีเรียและรา โดยการวิจัยการสร้าง IAA ในยีสต์มีมีน้อย ดังนั้นคณะผู้วิจัยกลุ่มนี้จึงสนใจทำวิจัยเพื่อศึกษารายละเอียดชีววิถีการสังเคราะห์ IAA ของยีสต์ โดยคัดเลือกสายพันธุ์ที่สร้าง IAA ได้สูงจากยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์ที่ได้จากการวิจัยของโครงการนี้ นอกจากนั้นยังศึกษาถึงอิทธิพลของปัจจัยต่าง ๆ ต่อการผลิต IAA จากยีสต์สายพันธุ์ที่คัดเลือกว่ามีประสิทธิภาพสูง เพื่อให้รู้ถึงศักยภาพสูงสุดของยีสต์ในการผลิต IAA เพื่อใช้เป็นสารส่งเสริมการเจริญพืช การวิจัยเน้นการศึกษาอิทธิพลของปัจจัยต่าง ๆ ต่อการผลิต IAA และเนื่องจากเทคโนโลยีในการผลิตเซลล์ยีสต์และผลิตภัณฑ์จากยีสต์มีการพัฒนามาแล้วอย่างดีดังนั้นจึงเชื่อว่าไม่ใช่เป็นเรื่องยากในการผลิต IAA จากยีสต์เพื่อใช้เป็นสารส่งเสริมการเจริญพืช

ยีสต์หลายสปีชีส์โดยเฉพาะยีสต์ที่มาจากพืชหรือจากแหล่งที่อยู่ที่สัมพันธ์กับพืชพบว่ามีประสิทธิภาพในการควบคุมโรคพืชและโรคพืชหลังเก็บเกี่ยว โดยยีสต์ที่นำมาศึกษาประสิทธิภาพการควบคุมโรคพืชและโรคพืชหลังเก็บเกี่ยวมาจากหลายแหล่งที่อยู่ โดยเฉพาะจากพืชหรือแหล่งที่อยู่ที่สัมพันธ์กับพืช อย่างไรก็ตามยีสต์ที่ใช้เป็นตัวควบคุมทางชีวภาพอาจได้มาจากแหล่งอื่น เช่น น้ำทะเล และดิน ในประเทศไทยมีการวิจัยและรายงานการใช้ยีสต์เป็นตัวควบคุมทางชีวภาพสำหรับราที่ทำให้เกิดโรคพืชหลังเก็บเกี่ยว อย่างไรก็ตามยีสต์ส่วนใหญ่ได้จากหน่วยเก็บรักษาจุลินทรีย์ มีงานวิจัยไม่มากนักที่แยกและคัดเลือกยีสต์เพื่อนำมาใช้เป็นตัวควบคุมทางชีวภาพจากยีสต์ที่ได้จากแหล่งธรรมชาติในประเทศไทย ดังนั้นคณะผู้วิจัยนี้จึงนำยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์ที่แยกได้จากใบพืชทั้ง 3 ชนิด ในการศึกษาความหลากหลายโดยวิธีที่อาศัยการเพาะเลี้ยงเชื้อมาศึกษาการยับยั้งหรือต่อต้านการเจริญของราที่ทำโรคกับพืชเศรษฐกิจทั้ง 3 ชนิด คือ ข้าว อ้อย และข้าวโพด ตลอดจนตรวจหากลไกลการต่อต้านราโรคพืช

วัตถุประสงค์

1. เพื่อตรวจและวิเคราะห์ความหลากหลายและนิเวศวิทยาของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์ในใบของข้าว อ้อย และข้าวโพด โดยวิธีที่อาศัยการเพาะเลี้ยงเชื้อและวิธีที่ไม่อาศัยการเพาะเลี้ยงเชื้อ
2. เพื่อการผลิตกรดกรดอินโดล-3-แอซีติก (IAA) โดยสายพันธุ์ที่คัดเลือกจากยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์ และศึกษาชีววิถีการสังเคราะห์ IAA ของยีสต์สายพันธุ์ที่คัดเลือกได้
3. เพื่อประเมินประสิทธิภาพในการใช้เป็นตัวควบคุมทางชีวภาพของราที่ทำให้เกิดโรคกับข้าว อ้อย และข้าวโพด ของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์ที่แยกได้

ผลการวิจัย

Investigation and analysis biodiversity and ecology of endophytic and epiphytic yeasts from leaves of agronomic plants by culture-dependent technique

เก็บตัวอย่างใบพืชทั้งหมด 311 ตัวอย่าง จากพื้นที่เพาะปลูกในภาคกลางและภาคตะวันออกเฉียงเหนือของประเทศไทยระหว่างเดือนตุลาคม 2011 ถึงเมษายน 2012 ประกอบด้วย ใบข้าว (n=100) จาก 11 จังหวัด ใบอ้อย (n=102) จาก 13 จังหวัด และใบข้าวโพด (n=109) จาก 12 จังหวัด จากตรวจวิเคราะห์จุลินทรีย์เอนโดไฟต์จากใบข้าว อ้อย และข้าวโพดนั้น พบว่าในเนื้อเยื่อของใบพืชทั้ง 3 ชนิดมีทั้งยีสต์ แบคทีเรีย และรา โดยมีจำนวนผันแปรค่อนข้างสูง คือ 0 - $1.2x10^3$, 0 - $1.9x10^4$ และ $1.1x10^4$ colony forming unit (CFU)/กรัมของใบพืช ตามลำดับ สำหรับจุลินทรีย์อีพิไฟต์ที่พบในผิวใบพืชมีทั้ง ยีสต์ แบคทีเรีย และรา และมีจำนวนผันแปรค่อนข้างสูงเช่นกัน คือ 0 - $5.26x10^7$, $1.1x10^3$ - $4.58x10^9$ และ 0 - $86.0x10^6$ CFU/กรัมของใบพืช ตามลำดับ

การศึกษาความหลากของยีสต์เอนโดไฟต์ด้วยการแยกยีสต์จากเนื้อเยื่อที่กำจัดเชื้อที่ผิวด้านนอกของใบข้าว อ้อย และข้าวโพด จำนวน 100, 102 และ 109 ตัวอย่าง ได้ยีสต์ 125, 149 และ 143 สายพันธุ์ ตามลำดับ เมื่อจัดจำแนกโดยอาศัยอนุกรมวิธานระดับโมเลกุลและไฟโลจีนีด้วยการวิเคราะห์ลำดับนิวคลีโอไทด์บริเวณ D1/D2 ของ large subunit (LSU) rRNA gene พบว่ายีสต์เอนโดไฟต์จาก ใบข้าว 117 สายพันธุ์เป็นสปีชีส์ที่รู้จักแล้ว (known species) 23 สปีชีส์ ใน 12 สกุล ประกอบด้วย 90 สายพันธุ์ (76.9%) ในไฟลัมแบสิดิโอมัยโคตา 7 สกุล ได้แก่ *Bullera* (1 สปีชีส์), *Cryptococcus* (1 สปีชีส์), *Pseudozyma* (5 สปีชีส์), *Rhodotorula* (4 สปีชีส์), *Rhodosporidium* (2 สปีชีส์), *Sporobolomyces* (2 สปีชีส์) และ *Sporidiobolus* (1 สปีชีส์) และ 27 สายพันธุ์ (23.1%) ในไฟลัมแอสโคมัยโคตา 5 สกุล ได้แก่ *Candida* (3 สปีชีส์), *Cyberlindnera* (1 สปีชีส์)ม *Kodamaea* (1 สปีชีส์)ม *Meyerozyma* (1 สปีชีส์) และ *Wickerhamomyces* (1 สปีชีส์) และอีก 90 สายพันธุ์ อยู่ในเบสิดิโอมัย-โคตา (7 สกุล) ได้แก่ *Bullera* (1 สปีชีส์), *Cryptococcus* (1 สปีชีส์), *Pseudozyma* (5 สปีชีส์), *Rhodotorula* (4 สปีชีส์), *Rhodosporidium* (2 สปีชีส์), *Sporobolomyces* (2 สปีชีส์) และ *Sporidiobolus* (1 สปีชีส์) นอกจากนั้น 1 สายพันธุ์เหมือนกับยีสต์สปีชีส์ที่ยังไม่มีการอธิบาย 1 สายพันธุ์เป็นยีสต์สปีชีส์ใหม่ และ 3 สายพันธุ์เป็นยีสต์สกุลและสปีชีส์ใหม่ สายพันธุ์ที่เหลือเป็นรา ส่วนเอนโดไฟต์จากใบอ้อยพบว่า 100 สายพันธุ์เป็นสปีชีส์ที่รู้จักแล้ว 29 สปีชีส์ โดยพบ 65 สายพันธุ์ (65.0%) ในแบสิดิโอมัยโคตา 10 สกุล คือ *Bullera* (4 สปีชีส์), *Cryptococcus* (5 สปีชีส์), *Dioszegia* (1 สปีชีส์), *Jaminaea* (1 สปีชีส์), *Pseudozyma* (5 สปีชีส์), *Rhodotorula* (3 สปีชีส์), *Rhodosporidium* (1 สปีชีส์), *Sporobolomyces* (1 สปีชีส์), *Sporidiobolus* (1 สปีชีส์) และ *Trichosporon* (1 สปีชีส์) และ *Trichosporon* ส่วนอีก 35 สายพันธุ์ (35.0%) อยู่ในแอสโคมัยโคตา ได้แก่ *Candida* (1 สปีชีส์) *Cyberlindnera* (1 สปีชีส์) *Meyerozyma* (2 สปีชีส์) และ *Pichia* (2 สปีชีส์) นอกจากนี้ 6 สายพันธุ์เป็นยีสต์สปีชีส์ใหม่ ส่วน 21 สายพันธุ์เหมือนกับ 10 สปีชีส์ที่ยังไม่มีการอธิบาย และสายพันธุ์ที่เหลือเป็นรา สำหรับยีสต์เอนโดไฟต์จากใบข้าวโพดนั้น 120 สายพันธุ์เป็นสปีชีส์ที่รู้จักแล้ว 37 สปีชีส์ โดยมี 90 สายพันธุ์ (75.0%) อยู่ในแบสิดิโอมัยโคตา (7 สกุล) ได้แก่ *Bullera* (1 สปีชีส์), *Cryptococcus* (9 สปีชีส์) *Rhodotorula* (2

สปีชีส์) *Pseudozyma* (3 สปีชีส์) *Rhodosporidium* (2 สปีชีส์) *Sporobolomyces* (2 สปีชีส์) และ *Sporidiobolus* (2 สปีชีส์) และ 30 สายพันธุ์ (25.0%) อยู่ในแอสโคมัยโคตา (5 สกุล) ได้แก่ *Candida* (5 สปีชีส์) *Kluyveromyces* (1 สปีชีส์) *Meyerozyma* (1 สปีชีส์) *Pichia* (1 สปีชีส์) และ *Wickerhamomyces* (1 สปีชีส์) นอกจากนี้ 1 สายพันธุ์เป็นยีสต์สปีชีส์ใหม่ มี 3 สายพันธุ์เหมือนกับ 3 สปีชีส์ที่ยังไม่มีการอธิบาย และที่เหลือเป็นรา ยีตส์เอนโดไฟต์ที่มีความถี่ในการพบมากที่สุดในเนื้อเยื่อใบของพืชทั้ง 3 ชนิดไม่ได้เป็นยีสต์ชนิดเดียวกัน โดยยีตส์ในเนื้อเยื่อใบข้าว อ้อย และข้าวโพด คือ *Pseudozyma aphidis*, *Meyerozyma caribbica* และ *Pseudozyma churashimaensis* โดยมีความถี่ที่ปรากฏเท่ากับ 25.0 17.6 และ 20.0% ตามลำดับ

สำหรับการศึกษาความหลากหลายของยีสต์อีพิไฟต์จากผิวใบด้วยการเพาะเชื้อจากน้ำล้างใบในจานเพาะเชื้อ (plating of leaf washing) พบว่าในกรณีของผิวใบข้าว 112 ตัวอย่าง แยกยีสต์ได้ 379 สายพันธุ์ เมื่อนำ 354 สายพันธุ์มาจัดจำแนก พบว่า 302 สายพันธุ์เป็นยีสต์ที่รู้จักแล้วถึง 50 สปีชีส์ ใน 18 สกุล โดย 246 สายพันธุ์ (81.5%) อยู่ในแบสิดิโอมัยโคตา สกุล *Blastobotrys*, *Bullera*, *Cryptococcus*, *Jaminaea*, *Pseudozyma* (พบถึง 7 สปีชีส์ จาก128 สายพันธุ์), *Rhodosporidium*, *Rhodotorula*, *Sporidiobolus*, *Sporobolomyces* และ *Trichosporon* ส่วนอีก 56 สายพันธุ์ (18.5%) อยู่ในแอสโคมัยโคตา สกุล *Candida*, *Debaryomyces*, *Hyphopichia*, *Kodamaea*, *Lodderomyces*, *Meyerozyma*, *Pichia*, *Torulaspora* และ *Wickerhamomyces* ส่วน 21 สายพันธุ์เหมือนกับสปีชีส์ที่ยังไม่มีการอธิบาย 14 สปีชีส์ ใน 4 สกุล คือ *Candida, Cryptococcus, Pseudozyma* และ *Rhodotorula* นอกจากนี้พบ 31 สายพันธุ์ที่คาดว่าจะเป็นยีสต์สปีชีส์ใหม่ 20 สปีชีส์ ใน 8 สกุล คือ *Bullera, Cryptococcus, Occultifur, Pseudozyma, Rhodosporidium, Rhodotorula, Sakaguchia* และ *Candida*

ส่วนยีสต์อีพิไฟต์จากผิวใบอ้อย โดยแยกยีสต์ 173 สายพันธุ์ จากใบอ้อย 84 ตัวอย่าง พบว่า 87 สายพันธุ์เป็นยีสต์สปีชีส์ที่รู้จักแล้ว โดย 61 สายพันธุ์ (35.3%) เป็นยีสต์ 24 สปีชีส์ ในแบสิดิโอมัยตา 10 สกุล คือ *Cryptococcus*, *Dioszegia*, *Hannaella*, *Jaminaea*, *Occultifur*, *Pseudozyma*, *Rhodotorula*, *Rhodosporidium*, *Sporobolomyces* และ *Tremella* และ 26 สายพันธุ์ (15%) เป็นยีสต์ 12 สปีชีส์ ในแอสโคมัยโคตา สกุล *Candida*, *Kodamaea* และ *Meyerozyma* ส่วนอีก 67 สายพันธุ์อาจเป็นยีสต์สปีชีส์ใหม่หรือสปีชีส์ที่รู้จักแล้วซึ่งต้องตรวจสอบเพิ่มเติม นอกจากนั้นมี 20 สายพันธุ์ที่เป็นยีสต์สปีชีส์ใหม่ 12 สปีชีส์ เมื่อวิเคราะห์ดัชนีความหลากหลายของแชนนอน-เวียเนอร์และดัชนีของซิมสันพบว่าจังหวัดลพบุรีมีจำนวนสปีชีส์ของยีสต์มากที่สุดคือ 24 สปีชีส์ 39 สายพันธุ์และมีความสม่ำเสมอของแต่ละสปีชีส์ในพื้นที่ (Evenness index; EH = 1) นอกจากนี้เป็นบริเวณที่มีความหลากหลายของยีสต์มากที่สุด (Shanon index (H') = 2.97 และ Simson index (D') = 0.93) รองมาคือ จังหวัดสระบุรี ซึ่งมีจำนวนยีสต์สปีชีส์ 21 สปีชีส์ 38 สายพันธุ์ และมีค่าดัชนีความหลากหลาย H' = 2.87 และ D' = 0.93

สำหรับยีสต์อีพิไฟต์ในผิวใบข้าวโพด แยกยีสต์ได้ 284 สายพันธุ์จาก 109 ตัวอย่าง เมื่อจัดจำแนก 244สายพันธุ์ พบว่า 151 สายพันธุ์เป็นยีสต์สปีชีส์ที่รู้จักแล้ว 43 สปีชีส์ ใน 15 สกุล โดย 130 สายพันธุ์ (86.1%) อยู่ในแบสิดิโอมัยตา คือ *Bullera*, *Cryptococcus*, *Dioszegia, Jaminaea*, *Occultifur*, *Pseudozyma*, *Rhodosporidium, Rhodotorula*, *Sporidiobolus* และ *Sporobolomyces* ส่วน 21 สายพันธุ์ (13.9%) อยู่ในแอสโคมัยโคตา สกุล *Candida, Hanseniaspora*, *Kodamaea*, *Meyerozyma* และ *Wickerhamomyces* ส่วน 18 สายพันธุ์เหมือนกับยีสต์สปีชีส์ที่ยังไม่มีการอธิบายสกุล *Cryptococcus*, *Pseudozyma* และ *Rhodotorula* นอกจากนั้นมี 21 สายพันธุ์ที่คาดว่าเป็นยีสต์สปีชีส์ใหม่ 16 สปีชีส์ และสายพันธุ์ที่เหลือเป็นรา

ในขณะที่การศึกษาความหลากหลายของยีสต์อีพิไฟต์ในผิวใบข้าว ใบข้าวโพด และใบอ้อย ด้วยการแยกยีสต์โดย enrichment technique จากตัวอย่างใบพืชรวม 244 ตัวอย่าง ได้ยีสต์จากใบข้าว อ้อย และข้าวโพด 156, 126 และ 159 สายพันธุ์ ตามลำดับ เมื่อจัดจำแนก พบว่ายีสต์จากใบข้าว 155 สายพันธุ์เป็นยีสต์สปีชีส์ที่รู้จักแล้ว โดย 99 สายพันธุ์ (63.9%) อยู่ในแอสโคมัยโคตา 23 สปีชีส์ใน 13 สกุล คือ *Candida*, *Clavispora, Cyberlindnera*, *Debaryomyces*, *Kodamaea*, *Lachancea*, *Metschnikowia*, *Meyerozyma*, *Millerozyma*, *Pichia*, *Wickerhamomyces, Yamadazyma* และ *Yarrowia* ส่วน 56 สายพันธุ์ (36.1%) อยู่ในแบสิดิโอมัยโคตา 8 สปีชีส์ ใน 5 สกุล ได้แก่ *Cryptococcus*, *Pseudozyma*, *Rhodotorula*, *Sporobolomyces* และ *Trichosporon* ยีสต์จากใบอ้อย 146 สายพันธุ์ จัดจำแนกเป็นแอสโคมัยซีตัสยีสต์ 23 สปีชีส์ใน 13 สกุล คือ *Candida, Cyberlindnera, Debaryomyces, Hanseniaspora, Kluyveromyces, Lachancea, Lodderomyces, Metschnikowia, Meyerozyma, Millerozyma, Pichia, Torulaspora* และ *Wickerhamomyces* ในขณะที่ 46 สายพันธุ์ (31.7%) อยู่ในแบสิดิโอมัยโคตา 12 สปีชีส์ใน 6 สกุล คือ *Hannaella, Cryptococcus, Rhodosporidium, Rhodotorula, Sporidiobolus* และ *Sporobolomyces* ส่วนอีก 8 สายพันธุ์

เหมือนกับยีสต์สปีชีส์ที่ยังไม่มีการอธิบายและ 9 สายพันธุ์เป็นยีสต์สปีชีส์ใหม่ 6 สปีชีส์ และยีสต์จากใบข้าวโพด 123 สายพันธุ์เป็นยีสต์สปีชีส์ที่รู้จักแล้ว โดย 81 สายพันธุ์ (65.9%) จำแนกเป็นแอสโคมัยซีตัสยีสต์ถึง 28 สปีชีส์ใน 12 สกุล คือ *Candida*, *Cyberlindnera*, *Geotrichum*, *Hanseniaspora, Kluyveromyces*, *Kodamaea, Meyerozyma, Millerozyma, Pichia, Torulaspora, Wickerhamomyces* และ*Yamadazyma* มี 42 สายพันธุ์ (34.1%) ในแบสิดิโอมัยโคตา 10 สปีชีส์ ใน 5 สกุล คือ *Cryptococcus, Rhodosporidium, Rhodotorula, Sporobolomyces* และ *Sporidiobolus* สำหรับยีสต์อีพิไฟต์ที่พบบ่อยที่สุดบนผิวใบข้าว อ้อย และข้าวโพด เมื่อแยกยีสต์ด้วยการเพาะเชื้อจากน้ำล้างใบในจานเพาะเชื้อ คือ *Pseudozyma antarctica*, *Meyerozyma caribbica* และ *Sporidiobolus pararoseus* โดยมีความถี่ที่ปรากฏ 21.8, 20.2 และ 11.3% ตามลำดับ โดย ***M. caribbica*** ที่พบบ่อยที่สุดในผิวใบอ้อยนี้เป็นสปีชีส์เดียวกับที่พบเมื่อแยกเชื้อโดย enrichment technique โดยจากการแยกเชื้อโดยวิธีนี้พบโดยมีความถี่ที่ปรากฏ 12.0% ในขณะที่เมื่อแยกเชื้อโดย enrichment technique ที่สปีชีส์สุดในผิวใบข้าว คือ *Rhodotorula taiwanensis* (14.3%) ส่วนผิวใบข้าวโพดยีสต์สปีชีส์ที่พบบ่อยเท่าๆ กัน คือ *Kodamaea ohmeri, Meyerozyma caribbica* และ *Rhodotorula taiwanensis* โดยมีความถี่ที่ปรากฏ 7.9% เท่าๆ กัน

จากผลการวิจัยความหลากหลายของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์ที่ใบข้าว อ้อย และข้าวโพด โดยใช้วิธีที่อาศัยการเพาะเลี้ยงเชื้อแสดงว่าพบแบสิดิโอมัยซีตัสยีสต์มากกว่าแอสโคมัยซีตัสยีสต์ ซึ่งเหมือนกับที่รายงานในประเทศอื่นๆ ในเขตอื่นของโลก อย่างไรก็ตามถ้าการแยกยีสต์ทำโดยใช้ enrichment technique ยีสต์ที่พบมากกว่ากลับเป็นแอสโคมัยซีตัสยีสต์ ดังนั้นวิธีการที่ใช้แยกยีสต์มีอิทธิพลต่อผลของการวิจัยที่ได้

จากการวิจัยความหลากหลายของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์โดยวิธีที่อาศัยการเพาะเลี้ยงเชื้อครั้งนี้ได้พบและรายงานยีสต์สกุลใหม่ 1 สกุล คือ *Diutina* gen. nov. และสปีชีส์ใหม่ 15 สปีชีส์ ประกอบด้วยสปีชีส์ในไฟลัมแบสิดิโอมัยโคตา คือ *Hannaella phyllophyla* sp. nov., *Hannaella phetchabunensis* sp. nov., *Hannaealla siamensis* sp. nov., *Occultifur tropicalis* sp. nov., *Papiliotrema siamensis* f.a., sp. nov. และ *Pseudozyma vetiver* sp. nov. และสปีชีส์ในไฟลัมแอสโคมัยโคตา คือ *Diutina siamensis* f.a. sp. nov., *Metschnikowia saccharicola* sp. nov., *Metschnikowia lopburiensis* sp. nov., *Nakazawaea siamensis* sp. nov., *Wicherhamomyces siamensis* sp. nov., *Wickerhamiella siamensis* sp. nov., *Yamadazyma epiphylla f.a.* sp. nov., *Yamadazyma phyllophila* sp. nov. และ *Yamadazyma siamensis* sp. nov. โดยยังอยู่ในระหว่างการศึกษาเพื่อเสนอเป็นสปีชีส์ใหม่อีกหลายสปีชีส์

Investigation and analysis biodiversity and ecology of endophytic and epiphytic yeasts from leaves of agronomic plants by culture-independent technique

ศึกษาความหลากหลายของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์บนผิวใบพืชเศรษฐกิจในประเทศไทยด้วยวิธีการไม่เพาะเลี้ยงเชื้อ โดยสกัดดีเอ็นเอของยีสต์เอนโดไฟต์จากตัวอย่างใบพืชที่ผ่านการการทำความสะอาดผิวใบและสกัดดีเอ็นเอของยีสต์อีพิไฟต์จากน้ำล้างใบพืช จากเพิ่มปริมาณดีเอ็นเอบริเวณ D1/D2 ของ LSU rDNA ของยีสต์เอนโดไฟต์ด้วยปฏิกิริยา semi-nested PCR โดยใช้คู่ไพร์เมอร์ ITS1F_KYO1 (Toju et al., 2012)/NL4 และ NL1/NL4 (Kurztman and Robnett, 1998) และปฏิกิริยา PCR โดยใช้คู่ไพร์เมอร์ NL1/NL4 สำหรับยีสต์อีพิไฟต์ จากนั้นสร้าง D1/D2 library ของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์บนข้าว อ้อย และข้าวโพด และคัดเลือก clone ที่มีลำดับนิวคลีโอไทด์ในบริเวณ D1/D2 ที่แตกต่างกันในแต่ละ library ด้วยวิธี PCR-RFLP โดยใช้เอนไซม์ตัดจำเพาะ *Hae*III, *Hin*fI และ *Cfo*I และจัดกลุ่มโคลนที่มีรูปแบบ RFLP เหมือนกันอยู่ในกลุ่มเดียวกัน (Operational taxonomic unit, OTU) สุ่มเลือกโคลนในแต่ละ OTU ไปวิเคราะห์หาลำดับนิวคลีโอไทด์และเปรียบเทียบกับลำดับนิวคลีโอไทด์ในฐานข้อมูล GenBank และจัดจำแนกการวิเคราะห์ลำดับนิวคลีโอไทด์บริเวณ D1/D2

สำหรับการศึกษาความหลากหลายของยีสต์เอนโดไฟต์จากตัวอย่างใบพืชทั้ง 3 ชนิดนั้น จำเป็นต้องทดสอบสภาพปลอดเชื้อและดีเอ็นเอบนผิวใบพืชเพื่อป้องกันการปนเปื้อนของยีสต์อิพิไฟต์ในกลุ่มประชากรของยีสต์

เอนโดไฟต์ ซึ่งผลการทดสอบพบว่าขั้นตอนการทำความสะอาดผิวใบพืชที่ใช้สามารถชะล้างจุลินทรีย์และดีเอ็นเอบนผิวใบพืชได้ดี เนื่องจากผลการวิจัยในระยะแรกชี้ให้เห็นว่า คู่ไพร์เมอร์ NL1 และ NL4 ซึ่งเป็นคู่ไพรเมอร์ที่นิยมใช้ในการเพิ่มปริมาณดีเอ็นเอบริเวณ D1/D2 ของ LSU rDNA เพื่อการระบุชนิดของยีสต์บริสุทธิ์ สามารถเพิ่มปริมาณของ LSU rDNA ของข้าว อ้อย และข้าวโพดได้ ดังนั้นจึงได้ปรับเปลี่ยนมาใช้ปฏิกิริยา semi-nested PCR โดยใช้คู่ไพร์เมอร์ ITS1F_KYO1/NL4 และ NL1/NL4 เพื่อเพิ่มความจำเพาะต่อการเพิ่มปริมาณดีเอ็นเอบริเวณ D1/D2 ของ LSU rDNA ของราให้สูงขึ้นและลดความจำเพาะต่อการเพิ่มปริมาณดีเอ็นเอ บริเวณ D1/D2 ของ LSU rDNA ของพืชให้ต่ำลง พบว่าปฏิกิริยา semi-nested PCR ที่ใช้มีความจำเพาะต่อดีเอ็นเอบริเวณ D1/D2 ของ LSU rDNA ของรา แต่อย่างไรก็ตามพบว่า PCR product ที่ได้ส่วนใหญ่เป็นของราเส้นใย ซึ่งแสดงให้เห็นว่ายีสต์เป็นประชากรส่วนน้อยของประชากรเอนโดไฟต์ โดยในใบข้าว 46 ตัวอย่าง พบโคลนของยีสต์ 101 โคลน (5.5%) จากจำนวนทั้งหมด 1,826 โคลน และสามารถจัดจำแนก phylotype ที่เป็นของยีสต์ที่สามารถระบุสปีชีส์ได้ 5 สปีชีส์ใน 4 สกุล (43.6%) คือ *Cryptococcus victoriae*, *Debaryomyces hansenii*, *Debaryomyces vindobonensis*, *Meyerozyma guilliermondii* และ *P. antarctica* และพบ phylotype ของยีสต์ที่อาจเป็นยีสต์สปีชีส์ใหม่ 17 phylotype ที่มีความใกล้เคียงกับ *Candida metapsilosis*, *Cryptococcus foliicola*, *Cryptococcus laurentii*, *P. abaconensis*, *P. aphidis* และ *Trichosporon asahii* ส่วนในใบอ้อย 29 ตัวอย่าง พบโคลนของยีสต์ 224 โคลน (19.8%) จากจำนวนทั้งหมด 1,144 โคลน และสามารถจัดจำแนก phylotype ที่เป็นของยีสต์ที่สามารถระบุสปีชีส์ได้ 3 สปีชีส์ใน 3 สกุล (76.3%) คือ *Candida palmioleophila, D. hansenii* และ *Kodamaea ohmeri* และพบ phylotype ของยีสต์ที่อาจเป็นยีสต์สปีชีส์ใหม่ 10 phylotype ที่มีความใกล้เคียงกับ *D. hansenii*, *Cryptococcus flavus*, *Malassezia restricta*, *P. aphidis*, *Rhodotorula cassiicola*, *Rhodotorula marina* และ *Sporobolomyces vermiculatus* และจากใบข้าวโพด 35 ตัวอย่าง พบโคลนของยีสต์ 285 โคลน (24.4%) จากจำนวนทั้งหมด 1,166 โคลน และจัดจำแนกเป็น phylotype ของยีสต์ที่สามารถระบุสปีชีส์ได้ 6 สปีชีส์ใน 6 สกุล (33.0%) คือ *Hannaella sinensis*, *Mal. restricta*, *Pseudozyma parantarctica*, *R. taiwanensis*, *T. asahii* และ *Yarrowia deoformans* และพบ phylotype ของยีสต์ที่อาจเป็นยีสต์สปีชีส์ใหม่ 26 phylotype ที่มีความใกล้เคียงกับ *Mal. restricta*, *P. antarctica*, *P. parantarctica*, *Rhodotorula colostri* และ *Trichosporon faecale* โดย phylotype ที่พบมากที่สุดในใบข้าว อ้อย และข้าวโพด ได้แก่ phylotype ที่มีความใกล้เคียงกับ *Cryp. foliicola* (47.5%), phylotype ของ *K. ohmeri* (43.8%) และ phylotype ที่มีความใกล้เคียงกับ *T. faecale* (30.1%) ตามลำดับ จากผลการเปรียบเทียบความหลากหลายของ phylotype ที่พบในใบพืชทั้ง 3 ชนิด พบว่าในใบข้าวโพดมีความหลากหลายของยีสต์สูงสุด รองลงมาคือใบข้าว และใบอ้อย ตามลำดับ นอกจากนี้พบว่า phylotype ของยีสต์เอนโดไฟต์ในใบพืชทั้งสามชนิดส่วนใหญ่เป็นของยีสต์ในไฟลัมแบสิดิโอมัยโคตา และยังพบ phylotype ของยีสต์ที่อาจเป็นยีสต์สปีชีส์ใหม่มากถึง 56.4%, 32.6% และ 69.2% ของ phylotype ของยีสต์ทั้งหมดในตัวอย่างใบข้าว อ้อย และข้าวโพด ตามลำดับ

ผลการศึกษาความหลากหลายของยีสต์อีพิไฟต์จากตัวอย่างใบพืชทั้ง 3 ชนิด โดยจากตัวอย่างใบข้าว (44 ตัวอย่าง) พบโคลนของยีสต์ 451 โคลน (40.2%) จาก 1,121 โคลน โดยจำนวน 329 โคลน (72.9%) สามารถจัดจำแนกได้เป็นยีสต์ที่สามารถระบุสปีชีส์ได้ 9 สปีชีส์ ใน 6 สกุล ได้แก่ *Bullera japonica*, *Metschnikowia koreensis*, *Mey. guilliermondii*, *P. antarctica*, *P. aphidis*, *Sporobolomyces blumeae*, *Sporobolomyces carnicolor*, *Sporobolomyces oryzicola* และ *Wickerhamomyces anomalus* โคลนอื่นๆ อีก 122 โคลน (27.0%) ไม่สามารถระบุสปีชีส์ได้ ซึ่งมีความใกล้เคียงกับยีสต์ 17 สปีชีส์ ได้แก่ *B. japonica*, *Cryptococcus cellulolyticus*, *Cryptococcus curvatus*, *Cryptococcus flavescens*, *Cryp. flavus*, *Cryptococcus heimaeyensis*, *Cryp. laurentii*, *H. sinensis*, *Hannaella zeae*, *Met. koreensis*, *Mey. guilliermondii*, *P. antarctica*, *P. aphidis*, *Pseudozyma hubeiensis*, *Sporidiobolus pararoseus*, *Sp. carnicolor* และ *W. anomalus* ยีสต์สปีชีส์ที่พบโดดเด่นบนผิวใบข้าว ได้แก่ *P. antarctica* และ *B. japonica* ด้วยความถี่ที่พบเป็น 63.6% และ 22.7% ตามลำดับ ค่าดัชนีความหลากหลายและความสม่ำเสมอของ phylotype

ที่ตรวจพบบนผิวใบข้าวเป็น 2.19 และ 0.54 ตามลำดับ สำหรับตัวอย่างใบอ้อย (45 ตัวอย่าง) พบ โคลนของยีสต์ 426 โคลน (31.7%) จากทั้งสิ้น 1,342 โคลน มีเพียง 4 phylotypes (16 โคลน) ที่สามารถระบุสปีชีส์ได้ ได้แก่ *Jaminaea angkorensis*, *P. hubeiensis*, *R. taiwanensis* และ *Sp. vermiculatus* โดย phylotype ส่วนใหญ่ไม่สามารถระบุสปีชีส์ได้ (410 โคลน) ซึ่งมีความใกล้เคียงกับยีสต์ 11 สปีชีส์ ใน 7 สกุล ได้แก่ *Cryp. flavus*, *H. coprosmaensis*, *R. taiwanensis*, *J. angkoreiensis*, *Mal. restricta*, *P. antarctica*, *P. aphidis*, *P. hubeiensis*, *P. prolifica*, *P. shanxiensis* และ *Sp. vermiculatus* โดยยีสต์ที่พบส่วนใหญ่จัดอยู่ในลำดับ Ustilaginales (89.4%) และอยู่ในสกุล *Pseudozyma* ค่าดัชนีความหลากหลายและความสม่ำเสมอของ phylotype ที่ตรวจพบบนผิวใบอ้อยเป็น 2.33 และ 0.64 ตามลำดับ สำหรับตัวอย่างใบข้าวโพด (37 ตัวอย่าง) พบว่าโคลนของยีสต์ 357 โคลน (34.0%) จากทั้งสิ้น 1,049 โคลน สามารถระบุสปีชีส์ได้ 12 สปีชีส์ ใน 7 สกุล จาก 98 โคลน (27.5%) ได้แก่ *Bullera derxii*, *Bullera oryzae*, *Cryp. flavescens*, *H. sinensis*, *J. angkorensis*, *P. antarctica*, *P. aphidis*, *P. hubeiensis*, *P. prolifica*, *Spori. pararoseus*, *Sp. carnicolor* และ *Sporobolomyces odoratus* ส่วนโคลนที่เหลือจำนวน 259 โคลนนั้นไม่สามารถระบุสปีชีส์ได้ ซึ่งมีความใกล้เคียงกับยีสต์ในไฟลัมแอสโคมัยโคตา 1 สปีชีส์ และแบสิดิโอมัยโคตา 26 สปีชีส์ ได้แก่ *Bullera coprosmaensis*, *B. derxii*, *B. oryzae*, *Bullera pseudovariabilis*, *Cryp. flavescens*, *Cryptococcus luteolus*, *Cryp. zeae*, *H. sinensis*, *J. angkorensis*, *Mey. guilliermondii*, *P. abaconensis*, *P. antarctica*, *P. aphidis*, *Pseudozyma fusiformata*, *Pseudozyma graminicola*, *P. hubeiensis*, *Pseudozyma prolifica*, *Pseudozyma rugulosa*, *Pseudozyma shanxiensis*, *Rhodotorula bacarum*, *Rhodotorula crocea*, *R. marina*, *Spori. pararoseus*, *Sp. oryzicola*, *Sp. odoratus*, *Sympodiomycopsis kandeliae* และ *Sympodiomycopsis paphiopedili* ยีสต์ส่วนใหญ่ที่ตรวจสอบพบจัดอยู่ในซับไฟลัม Ustilaginomycotina (72.3%) และมีความใกล้เคียงกับยีสต์ในสกุล *Pseudozyma* เป็นส่วนใหญ่ โดยยีสต์ที่สามารถระบุสปีชีส์ได้ที่พบบนตัวอย่างผิวใบข้าวโพดส่วนใหญ่คือ *P. hubeiensis* และ *P. antarctica* ด้วยความถี่ที่พบบนผิวใบข้าวโพด 24.3% และ 21.6% ตามลำดับ ค่าดัชนีความหลากหลายและความสม่ำเสมอของ phylotype ที่ตรวจพบบนผิวใบข้าวโพดเป็น 4.03 และ 0.83 ตามลำดับ และจากผลการเปรียบเทียบความหลากหลายของยีสต์บนผิวใบพืชทั้ง 3 ชนิด พบว่าใบข้าวโพดมีความหลากหลายของยีสต์สูงสุด รองลงมาคือใบอ้อย และใบข้าว ตามลำดับ

Production of plant growth promoting auxins by the selected promising strain with the elucidation of it biosynthetic pathway

กรดอินโดล-3-แอซีติก (Indole-3-acetic acid, IAA) เป็นฮอร์โมนพืชที่จัดอยู่ในกลุ่มของออกซินที่เป็นอนุพันธ์ของสารประกอบอินโดล มีบทบาทหน้าที่ที่สำคัญต่อพืชหลายประการ เช่น กระตุ้นการแบ่งเซลล์ราก การยืดขยายตัวของเซลล์ราก กระตุ้นให้เซลล์เกิดความแตกต่าง ควบคุมการร่วงของใบและการสุกของผลไม้ และยังช่วยการตอบสนองของแสงและแรงโน้มถ่วง เป็นต้น การสังเคราะห์ IAA มี 2 ชีววิถีสังเคราะห์หลัก คือ วิถีที่ใช้ tryptophan และวิถีที่ไม่ใช้ tryptophan การสังเคราะห์ IAA ที่ใช้ tryptophan มีหลายวิถี ได้แก่ indole-3-acetamide pathway, indole-3-pyruvic acid pathway, indole-3-acetonitrile pathway, tryptamine pathway และ tryptophan side-chain oxidase pathway มีการเสนอว่าการสังเคราะห์ IAA ที่ไม่ใช้ tryptophan นั้นมาจาก indole หรือ indole-3-glycerol phosphate นอกจากพืชที่สามารถผลิต IAA ได้แล้ว ยังพบว่าจุลินทรีย์ทั้งแบคทีเรีย รา สาหร่าย และยีสต์สามารถผลิต IAA ได้เช่นกัน ยีสต์เป็นหนึ่งในจุลินทรีย์ที่น่าสนใจสำหรับการผลิต IAA แต่ยังมีการศึกษาอยู่ไม่มากนักและยังไม่พบรายงานเกี่ยวกับสภาวะที่เหมาะสมต่อการผลิต IAA ในยีสต์ ดังนั้นในงานวิจัยนี้จึงมุ่งคัดเลือกยีสต์ที่มีศักยภาพสูงในการผลิต IAA จากยีสต์อีพิไฟต์และยีสต์เอนโดไฟต์ที่แยกจากใบข้าวอ้อย และข้าวโพด และการศึกษาสภาวะที่เหมาะสมของการผลิต IAA โดยวิธีศึกษาทีละปัจจัยและพื้นผิวตอบสนอง รวมทั้งการศึกษาชีววิถีสังเคราะห์ IAA ของยีสต์

คัดเลือกยีสต์จำนวนทั้งสิ้น 1,388 สายพันธุ์ที่แยกจากใบข้าวจำนวน 430 สายพันธุ์(เป็นยีสต์เอนโดไฟต์ 125 สายพันธุ์ และยีสต์อีพิไฟต์ 307 สายพันธุ์) จากใบอ้อยจำนวน 605 สายพันธุ์ (เป็นยีสต์เอนโดไฟต์ 149 สายพันธุ์ และยีสต์อีพิไฟต์ 456 สายพันธุ์) และจากใบข้าวโพดจำนวน 353 สายพันธุ์ (เป็นยีสต์อีพิไฟต์ทั้งหมด) โดยเปรียบเทียบการผลิต IAA ในอาหารเหลว YPD ที่เติม tryptophan 0.1 เปอร์เซ็นต์ วิเคราะห์หาปริมาณ IAA โดย colorimetric method และ HPLC พบยีสต์ 2 สายพันธุ์ สกุล *Rhodosporidium* สามารถผลิต IAA ได้สูง คือ *Rhodosporidium fluviale* DMKU-CP293 ที่แยกจากผิวใบข้าวโพด และ *R. paludigenum* DMKU-RP301 ที่แยกจากผิวใบข้าว โดยผลิต IAA ได้เท่ากับ 141.5 และ 321.7 มิลลิกรัมต่อลิตร ตามลำดับ โดยคิดเป็น 31.9 และ 29.3 มิลลิกรัมต่อกรัมน้ำหนักเซลล์แห้ง ตามลำดับ หลังจากนั้นศึกษาสภาวะที่เหมาะสมของการผลิต IAA ในฟลาส์ก โดยวิธีศึกษาทีละปัจจัย ซึ่งปัจจัยที่ศึกษา ได้แก่ ระยะเวลาการเพาะเลี้ยง แหล่งและความเข้มข้นของคาร์บอน แหล่งและความเข้มข้นของไนโตรเจน แหล่งและความเข้มข้นของ growth factor ความเข้มข้นของ tryptophan อุณหภูมิการเพาะเลี้ยง ความเร็วรอบการเขย่า และพีเอชเริ่มต้นของอาหารเลี้ยงเชื้อ พบว่าทั้ง 2 สายพันธุ์สามารถผลิต IAA ได้สูงขึ้นเป็น 1,559.4 มิลลิกรัมต่อลิตร สำหรับ *R. fluviale* DMKU-CP293 โดยเมื่อเพาะเลี้ยงในอาหารที่เติมกาแลคโตสความเข้มข้น 3.0 เปอร์เซ็นต์ ยีสต์เอ็กซ์แทรคความเข้มข้น 1.0 เปอร์เซ็นต์ เปปโตนความเข้มข้น 2.0 เปอร์เซ็นต์ และ ทริปโตเฟนความเข้มข้น 0.5 เปอร์เซ็นต์ ปรับพีเอชเป็น 6.0 เป็นเวลา 10 วัน และ 1,464.7 มิลลิกรัมต่อลิตร สำหรับ *R. paludigenum* DMKU-RP301 โดยเมื่อเพาะเลี้ยงในอาหารที่เติมซูโครสความเข้มข้น 1.0 เปอร์เซ็นต์ corn steep liquor ความเข้มข้น 0.05 เปอร์เซ็นต์ ยีสต์เอ็กซ์แทรคความเข้มข้น 1.0 เปอร์เซ็นต์ และทริปโตเฟนความเข้มข้น 0.4 เปอร์เซ็นต์ ปรับพีเอชเป็น 7.0 บ่มที่ 30 องศาเซลเซียส บนเครื่องเขย่าแบบหมุน ความเร็ว 200 รอบต่อนาที เป็นเวลา 7 วัน ขณะที่เมื่อใช้วิธีพื้นผิวตอบสนองโดยใช้แผนการทดลอง Box-Benhken สำหรับออกแบบการทดลอง พบว่าทั้ง 2 สายพันธุ์สามารถผลิต IAA ได้สูงขึ้นเป็น 2,420.2 และ 1,623.9 มิลลิกรัมต่อลิตร โดยสภาวะที่เหมาะสมของการผลิตกรดอินโดล-3-แอซีติกของ *R. fluviale* DMKU-CP293 คือ เมื่อเพาะเลี้ยงในอาหารที่เติมกาแลคโตสความเข้มข้น 2.41 เปอร์เซ็นต์ ยีสต์เอ็กซ์แทรคความเข้มข้น 1.52 เปอร์เซ็นต์ และทริปโตเฟนความเข้มข้น 0.41 เปอร์เซ็นต์ บ่มที่ 30 องศาเซลเซียส บนเครื่องเขย่าแบบหมุน ความเร็ว 200 รอบต่อนาที เป็นเวลา 8 วัน ขณะที่สภาวะที่เหมาะสมของการผลิต IAA ของ *R. paludigenum* DMKU-RP301 คือ เมื่อเพาะเลี้ยงในอาหารที่เติมซูโครสความเข้มข้น 0.9 เปอร์เซ็นต์ ยีสต์เอ็กซ์แทรคความเข้มข้น 0.9 เปอร์เซ็นต์ และทริปโตเฟนความเข้มข้น 0.45 เปอร์เซ็นต์ ปรับพีเอชเป็น 7.2 บ่มที่ 30 องศาเซลเซียส บนเครื่องเขย่าแบบหมุน ความเร็ว 100 รอบต่อนาที เป็นเวลา 7 วัน และเพื่อปรับปรุงการผลิตกรดอินโดล-3-แอซีติกให้สูงขึ้น ได้ศึกษาการผลิต IAA ในปริมาตรที่ใหญ่ขึ้นโดยใช้สภาวะที่เหมาะสมที่ได้จากวิธีพื้นผิวตอบสนอง โดยใช้ถังหมักขนาด 5 ลิตร สำหรับ *R. fluviale* DMKU-CP293 และถังหมักขนาด 2 ลิตร สำหรับ *R. paludigenum* DMKU-RP301 โดยปัจจัยที่ศึกษาเพิ่มเติม ได้แก่ อัตราการกวน (100, 200, 300, 400 และ 500 รอบต่อนาที) และอัตราการให้อากาศ (0.67, 1, 1.33 และ 2 ลิตรอากาศต่อลิตรอาหารต่อนาที) รวมถึงอัตราการเจือจาง (0.01 และ 0.05 ต่อชั่วโมง) จากการศึกษาพบว่า *R. fluviale* DMKU-CP293 สามารถผลิต IAA ได้สูงสุดเท่ากับ 8,930.0 มิลลิกรัมต่อลิตร (คิดเป็นความสามารถในการผลิตเท่ากับ 37.2 มิลลิกรัมต่อลิตรต่อชั่วโมง และค่า yield เท่ากับ 380.1 มิลลิกรัม IAA ต่อกรัมกาแลคโตส) เมื่อเพาะเลี้ยงในสภาวะอัตราการให้อากาศ 1 ลิตรอากาศต่อลิตรอาหารต่อนาที อัตราการกวน 500 รอบต่อนาที โดยการหมักแบบแบตช์เป็นเวลา 240 ชั่วโมง และ *R. paludigenum* DMKU-RP301 สามารถผลิต IAA ได้สูงสุดเท่ากับ 2,707.9 มิลลิกรัมต่อลิตร (คิดเป็นความสามารถในการผลิตเท่ากับ 25.1 มิลลิกรัมต่อลิตรต่อชั่วโมง และค่าผลได้ของ IAA เท่ากับ 0.23 มิลลิกรัม IAA ต่อมิลลิกรัมทริปโตเฟน) เมื่อเพาะเลี้ยงในสภาวะอัตราการให้อากาศ 2 ลิตรอากาศต่อลิตรอาหารต่อนาที อัตราการกวน 400 รอบต่อนาที และอัตราการเจือจาง 0.01 ต่อชั่วโมง โดยการหมักแบบเฟด-แบตช์เป็นเวลา 108 ชั่วโมง โดยการศึกษาครั้งนี้เป็นรายงานครั้งแรกเกี่ยวกับการศึกษาสภาวะที่เหมาะสมต่อการผลิตกรดอินโดล-3-แอซีติกโดยยีสต์ *Rhodosporidium* ทั้งในฟลาส์กและถังหมัก

การศึกษาชีววิถีสังเคราะห์ IAA ศึกษาโดยนำส่วนใสที่ได้จากการผลิตกรดอินโดล-3-แอซีติกของ *R. paludigenum* DMKU-RP301 มาวิเคราะห์หาสารสื่อกลางของชีววิถีสังเคราะห์โดย GC-MS พบกรดอินโดล-3-แล

คติก และอินโดล-3-เอทานอลในอาหารเลี้ยงเชื้อซึ่งเป็นผลของปฏิกิริยารีดักชันของกรดอินโดล-3-ไพรูวิกและอินโดล-3-แอซีทัลดีไฮด์ในวิถีกรดอินโดล-3-ไพรูวิก รวมกับ HPLC ในการระบุกรดอินโดล-3-ไพรูวิกซึ่งเป็นสารสื่องกลางหลักของวิถีกรดอินโดล-3-ไพรูวิก และพบกิจกรรมเอนไซม์ tryptophan aminotransferase ซึ่งเป็นเอนไซม์ที่สำคัญในวิถีกรดอินโดล-3-ไพรูวิก แสดงให้เห็นว่ายีสต์ *R. paludigenum* DMKU-RP301 ผลิต IAA ผ่านทางวิถีกรดอินโดล-3-ไพรูวิก นอกจากนั้นยังพบสารประกอบอินโดล ซึ่งเป็นผลจากการย่อยสลายของ tryptophanและพบ 3-เมทิลอินโดล และกรดอินโดล-3-แอซีติกเมทิลเอสเทอร์ ซึ่งเป็นผลจากการย่อยสลายของ IAA โดยการศึกษาครั้งนี้เป็นรายงานครั้งแรกเกี่ยวกับการศึกษาชีววิถีสังเคราะห์ IAA ใน basidiomycetous yeast

นอกจากการผลิต IAA แล้ว ยังได้นำ *R. paludigenum* DMKU-RP301 มาศึกษาคุณลักษณะอื่นที่ส่งเสริมการเจริญของพืช ซึ่งพบว่ายีสต์ *R. paludigenum* DMKU-RP301 สามารถผลิตแอมโมเนีย สร้างเอนไซม์คาตาเลส และสร้างสารไซเดอโรฟอร์นอกเหนือไปจากการผลิต IAA ที่ส่งผลสนับสนุนการเจริญของพืชอีกด้วย

Evaluation of biological control activity of endophytic and epiphytic yeast strains against rice, sugarcane and corn pathogenic fungi

การควบคุมทางชีวภาพด้วยการใช้จุลินทรีย์ชนิดต่างๆ รวมทั้งการใช้ยีสต์เป็นตัวควบคุมเป็นทางเลือกหนึ่งสำหรับเกษตรกร กลไกของยีสต์ในการควบคุมราโรคพืชมีหลายกลไก เช่น การเจริญแก่งแย่งอาหารและพื้นที่ (competition for nutrient and space) กับราก่อโรคพืช โดยยีสต์มีการเจริญรวดเร็วกว่ารา ดังนั้นสารอาหารบนพื้นผิวของพืชจะถูกใช้โดยทำให้ไม่เพียงพอต่อการเจริญของรา และเมื่อยีสต์เจริญปกคลุมรอยแผลบนพืช ราโรคพืชจะไม่สามารถเข้าทำลายพืชต่อไปได้ การเกิดภาวะปฏิปักษ์ (antagonism) กับรา ได้แก่ การผลิตสารอินทรีย์เพื่อยับยั้งราโรคพืช เช่น การผลิตเอนไซม์เบต้า-กลูคาเนส (β-glucanase) และไคติเนส (chitinase) เพื่อทำลายผนังเซลล์ของรา และการชักนำให้เนื้อเยื่อพืชเกิดความต้านทาน โดยยีสต์สร้างสารต่างๆที่ทำให้พืชสามารถเจริญได้ดีและการสร้างสารที่ต้านทานการเจริญของราโรคพืช เช่น สารไซเดอโรฟอร์ (siderophore)

งานวิจัยส่วนนี้มีวัตถุประสงค์เพื่อคัดเลือกยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์ที่มีศักยภาพในการควบคุมเชื้อราที่ทำให้เกิดโรคกับพืชเศรษฐกิจทั้ง 3 ชนิด สำหรับใช้เป็นตัวควบคุมทางชีวภาพของโรคพืชที่เกิดจากรา รวม 10 สายพันธุ์ ประกอบด้วยราโรคข้าว (*Fusarium moniliforme*, *Helminthosporium oryzae* และ *Rhizoctonia solani*) ราโรคข้าวโพด (*Bipolaris maydis, Bipolaris zeicola, Exserohilum turcicum, Fusarium moniliforme* และ *Macrophomina phaseolina*) และราโรคอ้อย (*Colletotrichum falcatum* และ *Fusarium moniliforme*) โดยการเพาะเลี้ยงเชื้อร่วมกันในจานเพาะเชื้อ พบว่าเมื่อตรวจการยับยั้งราโรคพืชของยีสต์เอนโดไฟต์ 417 สายพันธุ์ พบว่ามียีสต์เพียง 11 สายพันธุ์ ที่ยับยั้งราโรคพืชได้ โดยเป็นยีสต์ที่แยกได้จากใบข้าว 9 สายพันธุ์ คือ *Wickerhamomyces anomalus* (DMKU-RE13, DMKU-RE38, DMKU-RE41, DMKU-RE62, DMKU-RE103) and *Kodamaea ohmeri* (DMKU-RE04, DMKU-RE27, DMKU-RE65 และ DMKU-RE114) และแยกได้จากใบข้าวโพด 2 สายพันธุ์ คือ *W. anomalus* (DMKU-CE52 และ DMKU-CE55) โดย *W. anomalus* ทั้ง 7 สายพันธุ์ สามารถยับยั้งราโรคข้าว *C. Lunata*, *F. moniliforme* และ *R. solani* ได้ 36.6-56.6, 36.7-54.4 และ 68.3-80.0% ตามลำดับ ในขณะที่ยับยั้งราโรคข้าวโพด *F. moniliforme* และ *M. phaseolina* ได้ 24.2-38.3 และ 27.0-34.1% ตามลำดับ และสามารถยับยั้งราโรคอ้อย *C. falcatum* และ *F. moniliforme* ได้ 35.4-45.8 และ 42.5-65.0% ตามลำดับ ส่วน *K. ohmeri* 4 สายพันธุ์ (DMKU-RE04, DMKU-RE27, DMKU-RE65 และ DMKU-RE114) ยับยั้ง *F. moniliforme* (ราโรคข้าว), *F. moniliforme* (ราโรคข้าวโพด) และ *C. falcatum* (ราโรคอ้อย) ได้ 33.0-38.4, 25.0-35.0 และ 34.6-36.6% ตามลำดับ จากการศึกษาตรวจสอบการสร้างสาระเหยของยีสต์เอนโดไฟต์ทั้ง 11 สายพันธุ์ที่พบว่าทุกสายพันธุ์สร้างสารระเหยที่ยับยั้งการเจริญของราโรคพืช และสารไซเดอโรฟอร์ได้ แต่ไม่สามารถยับยั้งราโรคพืชเมื่อเลี้ยงบนอาหารที่มีสารอาหารความเข้มข้นต่ำ ดังนั้นการสร้างสารระเหยและสารไซเดอโรฟอร์อาจเป็นกลไกของยีสต์ในการยับยั้งการเจริญของเส้นใยราโรคพืชได้ ทั้งนี้จะมีการศึกษากลไลอื่น ๆ ต่อไป

จากการศึกษาการยับยั้งราโรคพืชของยีสต์อีพิไฟต์พบว่า ยีสต์จากผิวใบอ้อย 173 สายพันธุ์ มีเพียง 4 สายพันธุ์ที่ยับยั้งการเจริญของราโรคพืชได้ คือ *Pap. siamense* DMKU-SP85^{T} ยับยั้งการเจริญของ *B. maydis* ได้ 50.9% ส่วน *K. ohmeri* DMKU-SP45 ยับยั้ง *H. oryzae* และ *F. moniliforme* (ราโรคข้าว) 28.6 และ 20.3% ตามลำดับ และยับยั้ง *C. falcatum* 39.8% สำหรับ *Candida* sp. DMKU-SP81 ยับยั้ง *F. moniliforme* (ราโรคข้าวโพด) และ *C. falcatum* 12.9 และ 29.3% ตามลำดับ ในขณะที่ *P. alboarmeniaca* DMKU-SP290 ยับยั้ง *R. solani*, *B. maydis* และ *E. turcica* 50, 40.2 และ 41.1% ตามลำดับ เมื่อตรวจกลไกในการยับยั้งราโรคพืชในจานเพาะเชื้อ พบว่า ยีสต์ทั้ง 4 สายพันธุ์สร้างสารประกอบไซเดอโรฟอร์ เอนไซม์บีตา-กลูคาเนสและเอนไซม์ไคติเนส ในขณะที่ *P. alboarmeniaca* DMKU-SP290 สร้างสารระเหยและแก่งแย่งสารอาหารได้ *Candida* sp. DMKU-SP81 สร้างบีตา-กลูคาเนสได้สูงที่สุด 16.4 mU/ml ในขณะที่ *P. alboarmeniaca* DMKU-SP290, *K. ohmeri* DMKU-SP45, *Pa. siamense* DMKU-SP85 สร้างได้ 12.7, 10.4 และ สร้างได้ 3.9 mU/ml ตามลำดับ สำหรับเอนไซม์ไคติเนสพบว่า *K. ohmeri* DMKU-SP45 สร้างได้สูงสุด 147.7 mU/ml ส่วน *Pa. siamense* DMKU-SP85, *Candida* sp. DMKU-SP81 และ *P. alboarmeniaca* DMKU-SP290 สร้างได้ 90.4, 56.7 และ 51.2 mU/ml ตามลำดับ

ส่วนยีสต์อีพิไฟต์จากผิวใบข้าวและข้าวโพด 221 สายพันธุ์ พบว่ามี 37 สายพันธุ์ ใน 17 สปีชีส์ 12 สกุลที่ยับยั้งราโรคพืชได้ในจานเพาะเชื้อ โดย *Torulaspora globosa* DMKU-RP31 และ *T. globosa* DMKU-RP35 ยับยั้งราสาเหตุโรคพืชได้ 8 ชนิด *Wickerhamomyces anomalus* DMKU-RP04 ยับยั้งราสาเหตุโรคพืชได้ 7 ชนิด *K. ohmeri* DMKU-RP06 และ *W. anomalus* DMKU-RP25 ยับยั้งราสาเหตุโรคพืชได้ 5 ชนิด ยีสต์ 3 สายพันธุ์ ได้แก่ *W. anomalus* DMKU-CP122*W. anomalus* DMKU-CP127 และ *K. ohmeri* DMKU-RP34 ยับยั้งราสาเหตุโรคพืชได้ 4 ชนิด แต่ละสายพันธุ์ ของยีสต์ที่เหลือยับยั้งราสาเหตุโรคพืชได้น้อยกว่า 4 ชนิด จากการทดลองนี้ยีสต์ปฏิปักษ์ทั้ง 37 สายพันธุ์พบว่า *Candida floricola* DMKU-CP149, *Hanseniaspora guilliermondii* DMKU-CP295, *C. floricola* DMKU-CP367, *Bullera sinensis* DMKU-CP437, *W. anomalus* DMKU-RP04, *W. anomalus* DMKU-RP25, *T. globosa* DMKU-RP31, *K. ohmeri* DMKU-RP34 และ *T. globosa* DMKU-RP35 ยับยั้งราโรคพืชสูงกว่า 50% จากการศึกษากลไกควบคุมราโรคพืชของยีสต์ทั้ง 37 สายพันธุ์ที่คัดเลือกได้ พบมียีสต์ที่ผลิตไซเดอโรฟอร์ได้ 8 สายพันธุ์ มียีสต์ 7 สายพันธุ์ที่ผลิตสารระเหยที่ยับยั้ง *R. solani* ได้ 57.0-73.9% มียีสต์ 33 สายพันธุ์ที่ผลิตเอนไซม์เบต้า-กลูคาเนส 0.5-42 mU/ml โดย 5 สายพันธุ์มีกิจกรรมของเบต้า-กลูคาเนสสูงกว่า 25 mU/ml และมี ยีสต์ 21 สายพันธุ์ผลิตไคติเนสในช่วง 2-249 mU/ml โดยมี 3 สายพันธุ์ มีกิจกรรมของไคติเนส 166.7, 166.8 และ 249.0 mU/ml ความสามารถในการแข่งขันใช้สารอาหารและพื้นที่โดยวิธีเพาะเลี้ยงร่วมกันบน potato dextrose agar (PDA) ที่มีธาตุอาหารความเข้มข้นต่างๆ พบว่ายีสต์ 12 สายพันธุ์ ยับยั้งราสาเหตุโรคพืชสูงเมื่อมีธาตุอาหารความเข้มข้น โรคกาบใบแห้งของข้าวที่เกิดจาก *Rhizoctonia solani* เป็นโรคที่ก่อให้เกิดความรุนแรงในทุกพื้นที่เพาะปลูกของโลกรวมถึงประเทศไทยด้วย จากการทดสอบการควบคุมโรคกาบใบแห้งของข้าว ในสภาวะเรือนทดลองโดยยีสต์ปฏิปักษ์ที่คัดเลือกได้ 7 สายพันธุ์ (*B. sinensis* DMKU-CP437, *C. floricola* DMKU-CP149, *C. floricola* DMKU-CP367, *H. guilliermondii* DMKU-CP295, *W. anomalus* DMKU-RP25, *T. globosa* DMKU-RP31 และ *T. globosa* DMKU-RP35) ทดสอบโดยฉีดพ่นเซลล์แขวนลอยของยีสต์ปฏิปักษ์เข้มข้น 10^{8} cells/ml ปริมาตร 20 ml บริเวณกาบใบของต้นข้าวที่เป็นโรค ตรวจผลการเกิดโรคโดยวัดจากความสูงของรอยแผล และความสูงของต้นข้าว การควบคุมโรคกาบใบแห้งคำนวณเป็นเปอร์เซ็นต์การกดการเกิดโรค พบว่าการพ่นเซลล์แขวนลอยของยีสต์ *H. guilliermondii* DMKU-CP295 กดการเกิดโรคกาบใบแห้งในข้าวได้อย่างมีนัยสำคัญ สูงกว่า 65 เปอร์เซ็นต์ ในขณะที่ยีสต์สายพันธุ์อื่นแสดงการกดการเกิดโรคต่ำในช่วง 12.0-53.9 เปอร์เซ็นต์

Executive Summary

Introduction

Plant leaf is one of the prominent habitats for microorganisms both endophytes and epiphytes. Endophytic yeasts inhabit the internal plant tissues without causing any symptoms or negative effects to the host plant. At present it is commonly accepted that almost every plant species are inhibited by endophytic fungi but only a limit number of reports described the isolation, localization or diversity of endophytic yeasts. To date endophytic yeasts have been isolated from a few varieties of plant in some countries. So far, no study have been undertaken to access the incidence of endophytic yeasts in any plant varieties in Thailand, though researches on endophytic filamentous fungi have been carried out by a few researchers. Therefore, we interested to investigate the biodiversity and ecology of endophytic yeasts in Thailand. The endophytic yeasts inhabit the internal plant tissue epiphytic yeasts colonize plant surface and their association on the plant surface ranges from saprophytic, commensal symbiotic to parasite. Among surface of various plant parts, surface of leaf which usually refers to the phylloplane or phyllosphere has been recognized as an important habitat for epiphytic microorganisms. Phylloplane has been reported to harbor epiphytic yeasts as a substantial component. Although epiphytic yeasts have been isolated from phylloplane of various plant varieties in many countries in the world, until present little is known about the biodiversity and ecological role of these yeasts. Few investigations of diversity and utilization of epiphytic yeasts especially from phylloplane were undertaken in Thailand. Consequently, the diversity and ecology of phylloplane yeasts in Thailand cannot be concluded so far. To access the biodiversity of any microorganisms the cultured dependent techniques is a major obstacle in isolation due to unknown cultivation conditions of all species and some species might be unculturable. However, the cultures which are obtained from culture-dependent technique can be directly and immediately utilized for any purposes. Since the molecular approach permit the direct evaluation of microbial population in the communities, the information of biodiversity of both culturable and unculturable microorganisms may be obtained by characterization of the rRNA gene using culture-independent technique. However, the cultures which are obtained from culture-dependent technique can be directly and immediately utilized for any purposes. This may be an advantage of using culture-dependent technique over culture-independent technique.

Rice, corn and sugarcane are the major agronomic crops in Thailand. Area for cultivation of rice covers the major portion of the area of Thailand followed by field crop such as corn and sugarcane. Therefore, leaves from these agronomic important crops are selected to be used as monocotyledon model plants to investigate and analyze the biodiversity and ecology of endophytic and epiphytic yeasts in Thailand.

Several genera of saprophytic yeasts are colonized on leaf surfaces and provide natural barrier against plant pathogens. Therefore, these yeasts are being interested to be used as biocontrol agents. Moreover, many plant associate microorganisms are capable of promoting plant growth and development through different mechanisms. Some of them synthesize plant

growth promoters similar to phytohormones produce in plant which is necessary as mediator in communication between microorganisms and host plant. The role of plant associate microorganisms including epiphytic and endophytic microorganisms has receiving increasing attention due to the interest to exploit them as plant growth promoters for increasing crop productivity and biocontrol agents for plant pathogens. Therefore, the endophytic and epiphytic yeast strains which will be obtained from this study can be screened for their potential to produce plant growth promoters, as well as to control plant pathogens and further employed in agricultural fields. However, it is foreseen that using living yeast cells as plant growth promoters in the fields may encounter several difficulties, this make us interested in producing high purity plant growth promoting substance. From our own preliminary investigation we found that various phylloplane yeasts show the ability to produce auxins mainly indole-3-acetic acid (IAA).

Indole 3-acetic acid (IAA) is the principle hormone which regulates various developmental and physiological processes in plants. It has been found that not only endogenouse IAA produced by plant cell that stimulate plant growth but exogenous IAA secreted by plant-associated microorganisms also share contribution. Plant growth promoting microorganisms including bacteria, actinomycetes, fungi and yeasts have been isolated and identified to elucidate the crucial role of such microbes to plant growth. As a result of stimulating activity to plant growth observed, biosynthetic pathway of IAA and pathway intermediates are most interest for biological researchers. In plant, biosynthetic pathway of IAA has been widely investigated to characterize pathway intermediates and intermediate steps. Using information obtained from plant researches, knowledge on IAA biosynthetic pathway has then been provided and expanded to microbial researches. There have been various report published on microbial IAA activity and plant-microbe interaction. However, many of those have been geared to bacteria and fungi. IAA production in yeast has also been exploited but little information has yet been made compared to bacteria. Endophytic and epiphytic yeasts associated with plant leaves obtained in this project have therefore been of most interest to study in more details on IAA biosynthetic pathway. Characterization of pathway as well as pathway intermediates will be elucidated on either views of endophytic or epiphytic plant-yeast relationship. Finally, to reach utmost potential application of the selected yeast isolate collected in Thailand, production of IAA by the selected promising yeast isolate will be focused in terms of biotechnological aspect. Production parameters influencing IAA yield will be investigated and optimized using response surface methodology, a mathematical and statistical analysis which found to be useful for the modeling and analysis of problems that the response of interest is influenced by several variables. Due to the technology for production of yeast cells and yeast products are well developed, and yeast can grow rapidly on simple and inexpensive substrates, therefore, we believe that it is quite easy to produce auxins in large quality with low cost of production.

Various yeast species have been found to be the effective biocontrol agents of plant and postharvest pathogenic fungi. Yeasts which have been studied for their efficiency as biocontrol agents were derived from various habitats, especially from plants and habitats related to plants, for example: fruits, inner tissue of medicinal plant, phylloplane and rhizosphere. However, some other biocontrol yeast species could be obtained from the other habitats such as,

marine habitat, seawater and soil. In Thailand there were some reports on using yeasts as biocontrol agents of postharvest pathogenic fungi. However, most yeasts used in the studies were obtained from culture collections. Only a few works on isolation and selection of biocontrol yeasts from natural habitats in Thailand was carried out in Thailand. Therefore endophytic and epiphytic yeasts obtained from the study on diversity by culture-dependent methods were determined for their antagonistic activity agent rice, and sugarcane corn pathogenic fungi. The antagonistic mechanisms of the antagonistic yeast strains were also determined.

Objectives

1. To investigate and analyze the diversity and ecology of endophytic and epiphytic yeasts from leaves of rice, sugarcane and corn by culture-dependent and culture-independent methods

2. To produce indole-3-acetic acid (IAA) by the selected promising yeast strain obtained from the screening of endophytic and epiphytic yeasts, and elucidate the biosynthetic pathway of IAA

3. To evaluate the efficiency to be biological control agents for rice, sugarcane and corn pathogenic fungi of endophytic and epiphytic yeasts

Results

There are four main parts in this research project; therefore, research methodology will be explained according to each part.

Investigation and analysis biodiversity and ecology of endophytic and epiphytic yeasts from leaves of agronomic plants by culture-dependent technique

To understand the diversity and ecology of endophytic and epiphytic yeasts leaves of healthy agronomic crops (rice, sugarcane and corn) were collected from their cultivation fields in the central and north-eastern parts during October 2011 to April 2012. The sample consisted of 100 rice leaf samples collected from 11 provinces, 102 sugarcane leaf samples collected from 13 provinces and 109 sugarcane leaf samples collected from 12 provinces. The number of yeasts, bacteria and fungi in leaf tissue was found to vary among the samples i.e. 0 - $1.2x10^3$, 0 - $1.9x10^4$, $1.1x10^4$ colony forming unit (CFU)/g of leaf sample, respectively. Likewise the number of epiphytic yeasts, bacteria and fungi in the surface of leaf samples were 0 - $5.26x10^7$, $1.1x10^3$ - $4.58x10^9$ and 0 - $86.0x10^6$ CFU/g of leaf sample, respectively.

Study of the endophytic yeast diversity based on culture-dependent method yeasts was by isolation of yeast from the surface-sterilized leaves tissue. From 100, 102 and 109 samples of rice, sugarcane and corn leaf we obtained 125, 149 and 143 yeast strains, respectively. Identification of yeast was based on molecular taxonomy and phylogenetic analysis of the D1/D2 region of the large subunit (LSU) rRNA gene revealed that 117 yeast strains from rice leaf samples were 23 known species in 12 genera. Ninety 90 strains (76.9%) were in the phylum Basidiomycota *viz.* *Bullera* (1 species) *Cryptococcus* (1 species) *Pseudozyma* (5 species) *Rhodotorula* (4 species) *Rhodosporidium* (2 species) *Sporobolomyces* (2 species) and *Sporobolomyces* (2 species), and 27

strains (23.1%) in the phylum Ascomycota *viz. Candida* (3 species) *Cyberlindnera* (1 species) *Kodamaea* (1 species) *Meyerozyma* (1 species) and *Wickerhamomyces* (1 species). One strain was identical with one undescribed species and three strains were found to represent a novel genus and species which was proposed as *Diutina siamensis* f.a. sp. nov., and the other strains were fungi. Of endophytic yeasts from sugarcane leaf, 100 strains were 29 known yeast species. A total of 65 strains (65.0%) were in Basidiomycota *viz. Bullera* (4 species) *Cryptococcus* (5 species) *Dioszegia* (1 species) *Jaminaea* (1 species) *Pseudozyma* (5 species) *Rhodotorula* (3 species) *Rhodosporidium* (1 species) *Sporobolomyces* (1 species) *Sporidiobolus* (1 species) and *Trichosporon* (1 species). Thirty-five strains were in Ascomycota *viz. Candida* (1 species) *Cyberlindnera* (1 species) *Meyerozyma* (2 species) and *Pichia* (2 species). Six strains were found to represent two novel yeast species while 21 strains were fungi. A total of 120 epiphytic yeast strains obtained from corn leaves were identified to be 37 known species. Among these 90 strains (75.0%) were in Basidiomycota *viz. viz. Bullera* (1 species), *Cryptococcus* (9 species), *Rhodotorula* (2 species), *Pseudozyma* (3 species), *Rhodosporidium* (2 species), *Sporobolomyces* (2 species) and *Sporidiobolus* (2 species), while 30 strains (25.0%) were in Ascomycota *viz. Candida* (5 species), *Kluyveromyces* (1 species), *Meyerozyma* (1 species), *Pichia* (1 species) and *Wickerhamomyces* (1 species). One strain was new species, three strains were identical with three undescribed species and the other were fungi. The most prevalent species of endophytic yeasts from rice, sugarcane and corn leaves were *Pseudozyma aphidis*, *Meyerozyma caribbica* and *Pseudozyma churashimaensis* with 25.0, 17.6 and 20.0% frequency of occurrence, respectively.

To investigate the diversity of epiphytic yeasts in the surface of rice, sugarcane and corn leaved by culture-dependent method, yeasts were isolated from leaf surface by plating of leaf washing. A total of 379 strains were isolated from 112 rice leaf samples and 354 strains were identified. The result revealed that 302 strains were 50 known species in 18 genera. Two hundred and forty six strains (81.5%) were in Basidiomycota *viz.* genus *Blastobotrys*, *Bullera*, *Cryptococcus*, *Jaminaea*, *Pseudozyma* (as many as 128 strains in 7 species), *Rhodosporidium*, *Rhodotorula*, *Sporidiobolus*, *Sporobolomyces* and *Trichosporon*. Fifty-five strains were in Ascomycota *viz.* genus *Candida*, *Debaryomyces*, *Hyphopichia*, *Kodamaea*, *Lodderomyces*, *Meyerozyma*, *Pichia*, *Torulaspora* and *Wickerhamomyces*. Twenty one strains were identical with 14 undescribed species in genus *Candida, Cryptococcus, Pseudozyma* and *Rhodotorula*. As many as 31 strains were expected to be 20 new yeast species in 8 genera *viz. Bullera, Cryptococcus, Occultifur, Pseudozyma, Rhodosporidium, Rhodotorula, Sakaguchia* and *Candida*.

One hundred and forty six strains of epiphytic yeast from the surface of sugarcane leaves were in Ascomycota consisted of 23 species in 13 genera *viz. Candida, Cyberlindnera, Debaryomyces, Hanseniaspora, Kluyveromyces, Lachancea, Lodderomyces, Metschnikowia, Meyerozyma, Millerozyma, Pichia, Torulaspora* and *Wickerhamomyces*. While 46 strains (31.7%) were in Basidiomycota consisted of 12 species in 6 genera *viz. Hannaella, Cryptococcus, Rhodosporidium, Rhodotorula, Sporidiobolus* and *Sporobolomyces*. The other 8 strains were identical with undescribed species and 9 strains were 6 new species. Epiphytic strains (123 strains) from the surface of corn leaves were known yeast species. Eighty one 81 strains (65.9%) were in Ascomycota consisted of 28 species in 12 genera *viz. Candida*, *Cyberlindnera*, *Geotrichum*,

Hanseniaspora, Kluyveromyces, *Kodamaea, Meyerozyma, Millerozyma, Pichia, Torulaspora, Wickerhamomyces* and *Yamadazyma* and 42 strains (34.1%) were in Basidiomycota (10 species in 5 genera) *viz. Cryptococcus, Rhodosporidium, Rhodotorula, Sporobolomyces* and *Sporidiobolus*. The most prevalent species in the surface of rice, sugarcane and corn leaves, which were isolated by plating of leaf washing, were *Pseudozyma antarctica*, *Meyerozyma caribbica* and *Sporidiobolus pararoseus* at 21.8, 20.2 and 11.3% frequency of occurrence, respectively. ***M. caribbica***, which was the most prevalent species in the surface of sugarcane leaf when plating of leaf washing was used, was also the prevalent species (12.0%) in the surface of sugarcane leaf when enrichment isolation technique was used. By using the enrichment isolation technique, the most prevalent species in the surface of rice leaf was *Rhodotorula taiwanensis* (14.3%) while *Kodamaea ohmeri* and *Meyerozyma caribbica* and *Rh. taiwanensis* occurred in the surface of corn leaf at the same frequency of 7.9%.

The result of investigation of endophytic and epiphytic yeasts diversity by using culture-dependent method revealed that higher number of basidiomycetous yeasts than ascomycetous yeasts were obtained same as reports of studies in the other countries in the other areas. However, if enrichment isolation technic was used higher number of ascomycetous yeasts than basidiomycetous yeasts were detected. Therefore, the method use for yeast isolation may affect the result of investigation.

In the investigation of endophytic and epiphytic yeasts diversity by culture-dependent method one new genus *viz. Diutina* gen. nov was proposed. Fifteen new yeast species were also proposed including *Hannaella phyllophyla* sp. nov., *Hannaella phetchabunensis* sp. nov., *Hannaealla siamensis* sp. nov., *Occultifur tropicalis* sp. nov., *Papiliotrema siamensis* f.a., sp. nov. and *Pseudozyma vetiver* sp. nov. in the phylum Basidiomycota, and *Diutina siamensis* f.a. sp. nov., *Metschnikowia saccharicola* sp. nov., *Metschnikowia lopburiensis* sp. nov., *Nakazawaea siamensis* sp. nov., *Wicherhamomyces siamensis* sp. nov., *Wickerhamiella siamensis* sp. nov., *Yamadazyma epiphylla f.a.* sp. nov., *Yamadazyma phyllophila* sp. nov. and *Yamadazyma siamensis* sp. nov. in the phylum Ascomycota. Moreover, various new yeast species are being studied and will propose in the near future.

Investigation and analysis biodiversity and ecology of endophytic and epiphytic yeasts from leaves of agronomic plants by culture-independent technique

Recent studies have employed molecular biology techniques to rapidly investigate microbial diversity including the unculturable microbes within and between ecosystems. Therefore, in this study a culture-independent method was used to examine endophytic and epiphytic yeasts associated with rice, sugarcane and corn leaves based on PCR amplification of the D1/D2 domain of the large subunit (LSU) rDNA. The endophytic DNA was extracted from surface-sterilized leaf samples and the amplification of D1/D2 domain via the semi-nested PCR was carried out using ITS1-F_KYO1/NL4 and NL1/NL4 primer sets, whereas the epiphytic DNA was extracted from leaf washing samples and the D1/D2 domain was amplified by NL1/NL4 primers. Then, D1/D2 libraries were constructed and screened by PCR-RFLP using a single digestion with *Hae*III, *Hin*fI and *Cfo*I. Clones were grouped into operational taxonomic units (OTUs) according to

the RFLP pattern and the representative OTUs were sequenced, submitted to BLASTn search and identified according to the guidelines described by Kurztman and Robnett (1998) and Fell et al (2000) for ascomycete and basidiomycete, respectively. Phylogenetic tree was constructed using the MEGA version 6.0.

For the investigation of endophytic yeasts in rice, sugarcane and corn leaves, the surface sterilization and the removal of DNA from leaf surfaces were verified prior to DNA extraction and the results showed the effectiveness of the decontamination of the epiphytic DNA from the endophytic population. Since the LSU rDNA of rice, sugarcane and corn were remarkably amplified by NL1/NL4 primer set commonly used for yeast culture identification, semi-nested PCR was carried out using ITS1-F_KYO1, having high percentage of fungal taxa and low percentage of plant taxa amplified *in silico*, and NL4 as the outer primer set in order to increase the specificity to fungal DNA and NL1/NL4 as the inner primer set. The fungal D1/D2 domain of the LSU rRNA gene was successfully amplified by semi-nested PCR, however, most of PCR products were D1/D2 domain of filamentous fungi, implying that yeast is a minority among the endophytic population. In cease of rice leaves, 101 (5.5%) out of 1,826 clones from 46 rice leaf samples were classified as yeasts. Of total yeast related phylotypes, 44 phylotypes (43.6%) were designated as 5 known species in 4 genera *viz. Cryptococcus victoriae, Debaryomyces hansenii, Debaryomyces vindobonensis, Meyerozyma guilliermondii* and *Pseudozyma antarctica*, together with 17 novel phylotypes closest to *Candida metapsilosis, Cryptococcus foliicola, Cryptococcus laurentii, Cryptococcus abaconensis, Cryptococcus aphidis* and *Trichosporon asahii.* For sugarcane, 224 (19.8%) out of 1,144 clones from 29 samples were classified as yeasts. Of total yeast related phylotypes, 171 phylotypes (76.3%) were designated as 3 known species in 3 genera *viz. Candida palmioleophila, D. hansenii* and *Kodamaea ohmeri*, together with 10 novel phylotypes closest to *D. hansenii*, *Cryptococcus flavus*, *Malassezia restricta*, *P. aphidis, Rhodotorula cassiicola, Rhodotorula marina* and *Sporobolomyces vermiculatus.* In case of corn leaves, 285 (24.4%) out of 1,166 clones from 29 samples were classified as yeasts. Of total yeast related phylotypes, 94 phylotypes (33.0%) were designated as 6 known species in 6 genera *viz. Hannaella sinensis, Mal. restricta, Pseudozyma parantarctica, Rh. taiwanensis, T. asahii* and *Yarrowia deoformans*, together with 26 novel phylotypes closest to *Mal. restricta, P. antarctica, P. parantarctica, Rhodotorula colostri* and *Trichosporon faecale*. The most prevalent phylotypes in rice, sugarcane and corn leaves were novel phylotypes closest to *Cryp. foliicola*, phylotypes identified as *K. ohmeri* and novel phylotypes closest to *T. faecale,* respectively. Among the 3 agronomic crops, corn leaves showed the highest species richness of the endophytic yeasts, followed by rice and sugarcane leaves. Moreover, the majority of endophytic yeast phylotypes found in rice, sugarcane and corn leaves belonged to Basidiomycota. Remarkably, the novel phylotypes were found at high frequency as 56.4%, 32.6% and 69.2% in rice, sugarcane and corn leaves, respectively.

In case of epiphytic yeast diversity in 3 agronomic crop phyllospheres, the results showed that 451 clone s (40.2%) of total 1,121 clones from rice phyllosphere samples (n=44), were classified as yeasts. Of total yeast related clones, 329 clones (72.9%) were identified as 9 known yeast species in 6 genera *viz. Bullera japonica*, *Metschnikowia koreensis*, *Mey. guilliermondii*, *P. antarctica*, *P. aphidis*, *Sporobolomyces blumeae*, *Sporobolomyces carnicolor*, *Sporobolomyces*

oryzicola and *Wickerhamomyces anomalus*. Other 122 clones (27.0%) were unable to identify as yeast species but closest to 17 yeast species. These consisted of *B. japonica*, *Cryptococcus cellulolyticus*, *Cryptococcus curvatus*, *Cryptococcus flavescens*, *Cryp. flavus*, *heimaeyensis heimaeyensis*, *Cryp. laurentii*, *H. sinensis*, *H. zeae*, *Met. koreensis*, *Mey. guilliermondii*, *P. antarctica*, *P. aphidis*, *P. hubeiensis*, *Spori. pararoseus*, *Sp. carnicolor* and *Wick. anomalus*. The dominant yeasts in rice phyllospheres were *P. antarctica* and *B. japonica* with 63.6% and 22.7% frequency of occurrence, respectively. The diversity index and community evenness of yeast phylotypes in rice phyllospheres were 2.19 and 0.54, respectively. For sugarcane phyllosphere samples (n=45), of total 1,342 clones, 426 clones (31.7%) were classified as yeasts. Only 4 phylotypes (16 clones) were identified as known yeast species. These included *Jaminaea angkoriensis*, *P. hubeiensis*, *Rh. taiwanensis* and *Sp. vermiculatus*. Nevertheless, the majority of yeast related sequences (410 clones) were unable to be identified as known yeasts species. These were closest to 11 yeast species in 7 genera *viz. Cryp. flavus*, *H. coprosmaensis*, *Rh. taiwanensis*, *J. angkoreiensis*, *Mal. restricta*, *P. antarctica*, *P. aphidis*, *P. hubeiensis*, *P. prolifica*, *Pseudozyma shanxiensis* and *Sp. vermiculatus*. The most predominant yeasts detected be longed to Ustinaginales (89.4%) and the prevalent genus was *Pseudozyma*. The diversity index and community evenness of yeast phylotypes in sugarcane phyllospheres were 2.33 and 0.64, respectively. In case of corn phyllosphere samples (n=37), of total 1,049 clones, 357 clones (34.0%) were classified as yeasts. Of total yeast related clones, 98 clones (27.5%) were identified as 12 known yeast species in 7 genera *viz*. *Bullera derxii*, *Bullera oryzae*, *Cryp. flavescens*, *H. sinensis*, *J. angkoriensis*, *P. antarctica*, *P. aphidis*, *P. hubeiensis*, *P. prolifica*, *Spori. pararoseus*, *Sp. carnicolor* and *Sporobolomyces odoratus*. Other 259 clones could not be identified as known species, however, closest to 1 and 26 species in Ascomycota and Basidiomycota, respectively, *viz. Bullera coprosmaensis*, *B. derxii*, *B. oryzae*, *B. pseudovariabilis*, *Cryp. flavescens*, *Cryp. luteolus*, *Cryp. zeae*, *H. sinensis*, *J. angkoriensis*, *Mey. guilliermondii*, *P. abaconensis*, *P. antarctica*, *P. aphidis*, *Pseudozyma fusiformata*, *Pseudozyma graminicola*, *P. hubeiensis*, *P. prolifica*, *Pseudozyma rugulosa*, *Pseudozyma shanxiensis*, *Rhodotorula bacarum*, *Rhodotorula crocea*, *Rh. marina*, *Spori. pararoseus*, *Sp. oryzicola*, *Sp. odoratus*, *Sympodiomycopsis kandeliae* and *Sympodiomycopsis paphiopedili.* The majority of yeast related sequences were closest to yeasts in subphylum Ustilaginomycotina (72.3%) especially in genus *Pseudozyma*. *P. hubeiensis* and *P. antarctica* were predominant species in corn phyllosphere samples with 24.3% and 21.6% frequency of occurrence. The diversity index and community evenness of yeast phylotypes in corn phyllospheres were 4.03 and 0.83, respectively. Among the 3 agronomic crops, corn leaves showed the highest species richness of the epiphytic yeasts, followed by sugarcane and rice leaves.

Production of plant growth promoting auxins by the selected promising strain with the elucidation of it biosynthetic pathway

Indole-3-acetic acid (IAA) is a plant hormone classified into indole derivative of auxin family. IAA has a number of significant roles in plants such as stimulation of cell division, cell elongation, cell differentiation, regulating leaf fall and fruit ripening, light and gravitational

response. Two major pathways for IAA biosynthesis have been proposed: tryptophan-dependent and tryptophan-independent pathways. Several pathways using tryptophan as a precursor for IAA biosynthesis have been proposed, *viz.* an indole-3-acetamide pathway, an indole-3-pyruvic acid pathway, an indole-3-acetonitrile pathway, a tryptamine pathway and a tryptophan side-chain oxidase pathway. The tryptophan-independent IAA biosynthetic pathway has been proposed as a branch from either indole or indole-3-glycerol phosphate. Apart from plant, microorganisms *viz.* bacteria, fungi, algae and yeast produce IAA. Yeast is one of the interesting IAA producers but only few reports on the optimization of yeast IAA production have been published. This study therefore aimed to screen for the potent high IAA producing yeasts from epiphytic yeasts and endophytic yeasts isolated from rice leaves, sugar cane leaves and corn leaves. Optimization of IAA production by the selected yeast isolates was performed using One Factor at A Time (OFAT) as well as Response Surface Methodology (RSM) experimental designs. IAA biosynthetic pathway of the selected yeast isolate was also investigated.

A total of 1,388 yeasts isolated from rice leaves (430 isolates which are 125 endophyte and 307 epiphyte), sugar cane leaves (605 isolates which are 149 endophyte and 456 epiphyte) and corn leaves (353 isolates which are all epiphyte) were subjected to screening for high IAA producing yeast in YPD broth supplemented with 0.1% tryptophan. IAA was analysed by colorimetric method and high-performance liquid chromatography (HPLC). As a result, two yeast isolates i.e. corn phylloplane yeast *Rhodosporidium fluviale* DMKU-CP293 and rice phylloplane yeast *R. paludigenum* DMKU-RP301 were found as high IAA producers by producing 141.5 and 321.7 mg IAA/L which corresponding to 31.9 and 29.3 mg IAA/g DCW respectively.

Optimization of IAA production in flasks was investigated using OFAT. The study looked at the effects of incubation time, carbon and nitrogen sources, growth factor, tryptophan, temperature, shaking speed and pH on the production of IAA. The results showed that the two isolates showed improved IAA production. *R. fluviale* DMKU-CP293 produced 1,559.4 mg IAA/L after 10 days cultivation in the medium supplemented with 3% galactose, 1% yeast extract 2% peptone and 0.5% tryptophan, pH was adjusted to 6.0 whereas *R. paludigenum* DMKU-RP301 produced 1,464.7 mg IAA/L after 7 days cultivation at 30 °C on an orbital shaker (200 rpm) in the medium supplemented with 1% sucrose, 0.05% corn steep liquor, 1% yeast extract and 0.4% tryptophan, pH was adjusted to 7.0. RSM was used to improve IAA production and Box-Benhken experimental design was employed. Results indicated that *R. fluviale* DMKU-CP293 and *R. paludigenum* DMKU-RP301 improved their IAA production to 2,420.2 and 1,623.9 mg IAA/L, respectively. Optimized conditions for IAA production by *R. fluviale* DMKU-CP293 was shown after 8 days cultivation at 30°C on an orbital shaker (200 rpm) in the medium supplemented with 2.41% galactose, 1.52% yeast extract and 0.41% tryptophan. *R. paludigenum* DMKU-RP301 showed optimized conditions for IAA production after 7 days cultivation at 30°C on an orbital shaker (100 rpm) in the medium supplemented with 0.9% sucrose, 0.9% yeast extract and 0.45% tryptophan, pH was adjusted to 7.2.

The production of IAA in batch cultivation of *R. fluviale* DMKU-CP293 and *R. paludigenum* DMKU-RP301 was scaled up in a 5-l fermenter and a 2-l fermenter, respectively, using optimized

conditions obtained by RSM with additional parameters studied i.e. agitation speed (100, 200, 300, 400 and 500 rpm), aeration rate (0.67, 1, 1.33 and 2 vvm) and dilution rate (0.01 and 0.05 h^{-1}). Results indicated that *R. fluviale* DMKU-CP293 produced the maximum IAA of 8,930.0 mg/L (corresponding to 37.2 mg/l/h productivity and 380.1 mg IAA/g galactose used) when cultivated under batch cultivation for 240 h with aeration rate of 1 vvm and agitation speed of 500 rpm whereas *R. paludigenum* DMKU-RP301 produced the maximum IAA of 2,707.9 mg/L (corresponding to 25.1 mg/l/h productivity and 0.23 mg IAA/mg tryptophan used) when cultivated under fed-batch cultivation for 108 h with aeration rate of 2 vvm, agitation speed of 400 rpm and dilution rate of 0.01 h^{-1}. This is the first report on optimization of IAA production in flask and fermenter by the yeast *Rhodosporidium*.

To investigate on IAA biosynthetic pathway, culture broth of *R. paludigenum* DMKU-RP301 was analysed by gas chromatography–mass spectrometry (GC-MS) and HPLC. Results obtained from HPLC revealed indole-3-pyruvic acid (IPA) which is an intermediate of IPA pathway for IAA biosynthesis whereas those from GC-MS showed the detection of indole-3-lactic acid and indole-3-ethanol in the culture broth as a result of the enzymatic reduction of indole-3-pyruvic acid and indole-3-acetaldehyde in IPA pathway. Activity of tryptophan aminotransferase, a key enzyme in the IPA pathway, was also observed. This therefore indicates that the yeast *R. paludigenum* DMKU-RP301 synthesizes IAA via IPA pathway. This is also the first report on IAA biosynthetic pathway in basidiomycetous yeast.

In addition to IAA production, *R. paludigenum* DMKU-RP301 also showed other plant growth promoting characters viz. the production of ammonia, siderophore and catalase.

Evaluation of biological control activity of endophytic and epiphytic yeast strains against rice, sugarcane and corn pathogenic fungi

Biological control, which is an environmental friendly approach, is an alternative method for plant disease control by using microorganisms including yeasts as biological control agents. Therefore, biological control by using the antagonistic yeast could be one alternative way to for farmers. Various mechanisms of yeasts in controlling of plant pathogenic fungi have been reported such as competition for nutrient and space; competition for nutrient and space better than the plant pathogenic fungi so the nutrients on plant surface used so its not be enough for plant pathogenic fungi, and when yeast grows cover the plant scar so the pathogens cannot infect, antagonism status; producing β-glucanase and chitinase to damage mold cell to destroy some plant pathogenic fungi and induced resistance in the host tissue; produce plant promoting substance and make plants be healthy such as siderophore.

This part aimed to evaluate biological control activity of endophytic and epiphytic yeast strains against rice, sugarcane and corn pathogenic fungi. Ten plant pathogenic fungi (PPF) including rice pathogenic fungi (*Fusarium moniliforme*, *Helminthosporium oryzae* and *Rhizoctonia solani*), corn pathogenic fungi (*Bipolaris maydis*, *Bipolaris zeicola*, *Exserohilum turcicum*, *Fusarium moniliforme* and *Macrophomina phaseolina*) and sugarcane pathogenic fungi (*Colletotrichum falcatum* and *Fusarium moniliforme*) were used. By dual cultivation of 417 endophytic yeast strains and 10 PPF only 11 strains in two species (*Wickerhamomyces anomalus* and *Kodamaea*

ohmeri) showed inhibition on photogenic fungi. These consisted of nine strains from rice *viz. W. anomalus* (DMKU-RE13, DMKU-RE38, DMKU-RE41, DMKU-RE62 and DMKU-RE103) and *K. ohmeri* (DMKU-RE04, DMKU-RE27, DMKU-RE65 and DMKU-RE114), and two *W. anomalus* strains from corn (DMKU-CE52 and DMKU-CE55). Seven *W. anomalus* strains (DMKU-RE13, DMKU-RE38, DMKU-RE41, DMKU-RE62, DMKU-RE103, DMKU-CE52 and DMKU-CE55) inhibited mycelium growth of three rice pathogenic fungi *viz. C. lunata, F. moniliforme* and *R. solani* by 56.6, 36.7-54.4 and 68.3-80.0%, respectively, two corn pathogenic fungi *viz. F. moniliforme* and *M. phaseolina* by 24.2-38.3 and 27.0-34.1%, respectively, and two sugarcane pathogenic fungi *viz. Col. falcatum* and *F. moniliforme* by 35.4-45.8 and 42.5-65.0%, respectively. Four *K. ohmeri* strains (DMKU-RE04, DMKU-RE27, DMKU-RE65 and DMKU-RE114) inhibited growth of three PPF *viz. F. moniliforme* (rice pathogenic fungus), *F. moniliforme* (corn pathogenic fungus) and *Col. falcatum* (sugarcane pathogenic fungus) by 33.0-38.4, 25.0-35.0 and 34.6-36.6%, respectively. All 11 antagonistic yeast strains produce volatile compounds and siderophore. Therefore, the volatile production and siderophore production may be mechanism of antagonistic yeast strains against plant pathogenic fungi. Determination of antagonistic activity of 173 epiphytic yeast strains from sugarcane leaves against 10 PPF revealed that only four yeast strains inhibited growth of some PPF with various degree of inhibition. *Pap. siamense* DMKU-SP85^{T} revealed the highest inhibition (50.9%) on growth of *B. maydis. K. ohmeri* DMKU-SP45 inhibited growth of *H. oryzae* (28.6%), *F. moniliforme* (20.3%) and *Col. falcatum* (39.8%). *Candida* sp. DMKU-SP81 inhibited *F. moniliforme* and *Col. falcatum* by 12.9% and 29.3%, respectively. *P. alboarmeniaca* DMKU-SP290 inhibited growth of *R. solani* (50%), *B. maydis* (40.2%) and *E. turcica* (41.1%). The four antagonistic yeast strains produced siderophore and two enzymes (ß-glucanase and chitinase). *P. alboarmeniaca* DMKU-SP290 produced volatile compounds and inhibited growth of pathogenic fungi by competition of nutrients. A total of 221 epiphytic yeast strains from the surface of rice and corn leaves were determined for their antagonistic activity and 37 strains of 17 species in 12 genera inhibited growth of PPF. Among these *Torulaspora globosa* DMKU-RP31 and *T. globosa* DMKU-RP35 inhibited 8 PPF, *W. anomalus* DMKU-RP04 inhibited 7 PPF, *K. ohmeri* DMKU-RP06 and *W. anomalus* DMKU-RP25 inhibited 5 PPF, *W. anomalus* DMKU-CP122, *W. anomalus* DMKU-CP127 and *K. ohmeri* DMKU-RP34 inhibited 4 PPF and the other strains inhibited less than 4 PPF. Considering of inhibition, *Candida floricola* DMKU-CP149, *Hansespora guilliermondii* DMKU-CP295, *C. floricola* DMKU-CP367, *H. sinensis* DMKU-CP437, *W. anomanialus* DMKU-RP04, *W. anomalus* DMKU-RP25, *T. globosa* DMKU-RP31, *K. ohmeri* DMKU-RP34 and *T. globosa* DMKU-RP35 inhibited growth of PPF greater than 50%. Inhibitory mechanisms study revealed that eight antagonistic strains produced siderophore, seven strains produced volatile compounds to inhibit growth of *R. solani* by 57.0-73.9% inhibition, 34 strains produced ß-glucanase in the range of 0.5-42 mU/ml among these five strains produced higher than 25 mU/ml, and 21 strains produced chitinase in the range of 2-249 mU/ml, where *H. guilliermondii* DMKU-CP295, *T. globosa* DMKU-RP35 and *K. ohmeri* DMKU-RP57 produced relatively high activities of 166.7, 166.8 and 249 mU/ml, respectively. The competition of nutrient and space determined by dual cultivation on PDA with various nutrient concentrations revealed that 12 yeast strains showed high inhibition when the nutrient concentrations were low. Rice sheath blight disease (*R. solani*) control was tested in greenhouse by the seven antagonistic yeast strains *B. sinensis* DMKU-CP437, *C. floricola* DMKU-CP149, *C. floricola* DMKU-CP367, *H. guilliermondii* DMKU-CP295, *T. globosa* DMKU-RP31, *T.*

globosa DMKU-RP35 and *W. anomalus* DMKU-RP25). The result showed that cell suspension of *H. guilliermondii* DMKU-CP295 applied by spraying (20 ml of 10^8 cells/ml of the antagonistic yeast cell suspension) significantly suppressed the rice sheath blight disease by up to 65 %, whereas the other yeast strains expressed the low disease suppression of 12.0-53.9 %.

ผลงาน

ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ รวม 22 เรื่อง ดังนี้

1. ผลงานที่ตีพิมพ์แล้ว	19	เรื่อง
2. ผลงานที่กำลังอยู่ในระหว่างการเสนอให้ตีพิมพ์	2	เรื่อง
3. ผลงานที่อยู่ในระหว่างการเตรียมต้นฉบับ	1	เรื่อง

ผลงานตีพิมพ์ที่ไม่ใช่งานของโครงการโดยตรงแต่ใช้ยีสต์ที่แยกได้จากโครงการที่ยอมรับให้ตีพิมพ์ 1 เรื่อง

(สำเนาผลงานตีพิมพ์ แนบในภาคผนวก 1)

ผลงานที่ตีพิมพ์แล้ว 19 เรื่อง ดังนี้

(Names in bold are researchers and students of the project)

1. **Kaewwichian R.**, W. Yongmanitchai, H. Kawasaki and **S. Limtong***. 2012. *Metschnikowia saccharicola* sp. nov. and *Metschnikowia lopburiensis* sp. nov., two novel yeast species isolated from phylloplane in Thailand. *Antonie van Leeuwenhoek*. 102: 743–751. (IF 2012: 2.072) (*Corr)
2. **Kaewwichian, R.**, W. Yongmanitchai, H. Kawasaki, P.-H. Wang, S.-H. Yang and **S. Limtong***. 2013. *Yamadazyma siamensis* sp. nov., *Yamadazyma phyllophila* sp. nov. and *Yamadazyma paraphyllophila* sp. nov., three novel yeast species isolated from phylloplane in Thailand and Taiwan. *Antonie van Leeuwenhoek.* 103: 777-788. (IF 2013: 2.137) (*Corr)
3. **Kaewwichian, R.**, H. Kawasaki and **S. Limtong*.** 2013. *Wickerhamomyces siamensis* sp. nov., a novel yeast species isolated from phylloplane in Thailand. *International Journal of Systematic and Evolutionary Microbiology.* 63: 1568-1573. (IF 2014: 2.511) (*Corr)
4. Chamnanpa T., P. Limtong, **N. Srisuk** and **S. Limtong***. 2013. *Pseudozyma vetiver* sp. nov., a novel anamorphic ustilaginomycetous yeast species isolated from the phylloplane in Thailand. *Antonie van Leeuwenhoek*. 104: 637-644. (IF 2014: 2.137) (*Corr)
5. **Nutaratat, P., N. Srisuk*, P. Arunrattiyakorn** and **S. Limtong**. 2014. Plant growth promoting traits of epiphytic and endophytic yeasts isolated from rice and sugar cane leaves in Thailand. *Fungal Biology.* 118: 683-694. (IF 2014: 2.342) (*Corr)

6. **Kaewwichian, R.** and **S. Limtong***. 2014. *Nakazawaea siamensis* f.a., sp. nov., a novel yeast species isolated from phylloplane in Thailand. *International Journal of Systematic and Evolutionary Microbiology.* 64: 266–270. (IF 2014: 2.511) (*Corr)

7. **Limtong, S.*, R. Kaewwichian**, W. Yongmanitchai and H. Kawasaki. 2014. Diversity of cultivable yeasts in phylloplanes of sugarcane in Thailand and their capability to produce indole-3-acetic Acid. *World Journal of Microbiology* and *Biotechnology*. 30: 1785–1796. (IF2014: 1.779) (*Corr)

8. **Surussawadee, J.**, **P. Khunnamwong**, **N. Srisuk** and **S. Limtong***. 2014. *Papiliotrema siamensis* f.a., sp. nov., a novel yeast species isolated from plant leaf in Thailand. 2014. *International Journal of Systematic and Evolutionary Microbiology.* 64: 3058-3062. (IF 2014: 2.511) (*Corr)

9. **Khunnamwong P., J. Surussawadee, S. Jindamorakot** and **S. Limtong*** 2014. *Wickerhamiella siamensis* f.a., sp. nov., a novel endophytic and epiphytic yeast species isolated from sugarcane leaf in Thailand. *International Journal of Systematic and Evolutionary Microbiology*. 64: 3849-3855. (IF 2014: 2.511) (*Corr)

10. **Nutaratat, P.**, W. Amsri, **N. Srisuk***, **P. Arunrattiyakorn** and **S. Limtong***. 2015. Indole-3-acetic acid production by newly isolated red yeast *Rhodosporidium paludigenum. Journal of General and Applied Microbiology.* 61: 1–9. (IF 2014: 0.943) (*Corr)

11. **Limtong, S***. and **R. Kaewwichian**. 2015. The diversity of culturable yeasts in the phylloplane of rice in Thailand. *Annals of Microbiology*. 65: 667–675. (IF 2014: 0.990) (*Corr)

12. **Jindamorakot S., S. Am-In, R. Kaewwichian** and **S. Limtong***. 2015. *Yamadazyma insecticola* f.a., sp. nov. and *Yamadazyma epiphylla* f.a., sp. nov., two novel yeast species isolated in Thailand". *International Journal of Systematic and Evolutionary Microbiology.* 65: 1290–1296. (IF 2014: 2.511) (*Corr)

13. **Kaewwichian R., S. Jindamorakot, S. Am-In**, M. Sipiczki and **S. Limtong***. 2015. *Hannaella siamensis* sp. nov. and *Hannaella phetchabunensis* sp. nov., two new anamorphic basidiomycetous yeast species isolated from plants. *International Journal of Systematic and Evolutionary Microbiology.* 65: 1297–1303. (IF 2014: 2.511) (*Corr)

14. **Nasanit R., K. Krataithong, M. Tantirungkij** and **S. Limtong*.** 2015. Assessment of epiphytic yeast diversity in rice (*Oryza sativa*) phyllosphere in Thailand by a culture-independent approach. *Antonie van Leeuwenhoek*. 107:1475–1490. (IF 2014: 1.806) (*Corr)

15. **Surussawadee, J., S. Jindamorakot**, T. Nakase, C.-F. Lee and **S. Limtong***. 2015. *Hannaella phyllophila* sp. nov., a novel basidiomycetous yeast species associated with plants in Thailand and Taiwan. *International Journal of Systematic and Evolutionary Microbiology*.65 (7): 2135-2140. (IF 2014: 2.511) (*Corr)

16. **Khunnamwong, P., J. Surussawadee, S. Jindamorakot,** J. R. A. Ribeiro, A. N. Hagler and **S. Limtong*.** 2015. *Occultifur tropicalis* f.a., sp. nov., a novel Cystobasidiomycetous yeast

species isolated from tropical regions. *International Journal of Systematic and Evolutionary Microbiology*. 65 (5): 1578–1582. (IF 2014: 2.511) (*Corr)

17. **Tantirungkij M., R. Nasanit** and **S. Limtong***. 2015. Assessment of endophytic yeast diversity in rice leaves by a culture-independent approach. *Antonie van Leeuwenhoek*. 108 (3): 633–647. (IF 2014: 1.806) (*Corr)

18. **Nasanit, R., A. Tangwong-o-thai, M. Tantirungkij** and **S. Limtong***. 2015. The assessment of epiphytic yeast diversity in sugarcane phyllosphere in Thailand by culture-independent method. *Fungal Biology.* http://dx.doi.org/10.1016/j.funbio.2015. 08.021 (*Corr) (online)

19. **Khunnamwong, P.***, N. Lertwattanasakul, S. Jindamorakot, **S. Limtong** and M-A. Lachance. 2015. The genus *Diutina*, description of *Diutina siamensis*, f.a. sp. nov., and reassignment *of Candida catenulata*, C. mesorugosa, *C. neorugosa*, *C. pseudorugosa*, *C. ranongensis*, *C. rugosa* and *C. scorzettiae* to the genus *Diutina*. *Int J Syst Evol Microbiol*. doi: (Digital Object Identifier) doi: 10.1099/ijsem.0.000634 (*Corr) (on line)

ผลงานที่กำลังอยู่ในระหว่างการเสนอตีพิมพ์ 2 เรื่อง

1. **Nutaratat, P., N. Srisuk*, P. Arunrattiyakorn** and **S. Limtong**. Indole-3-acetic acid biosynthetic pathways in the basidiomycetous yeast *Rhodosporidium paludigenum.* Archives of Microbiology. (*Corr) (revision 10 October 2015)

2. **Nutaratat, P., N. Srisuk*, P. Arunrattiyakorn** and **S. Limtong**. Fed-batch fermentation of indole-3-acetic acid production in stirred tank fermenter by red yeast *Rhodosporidium paludigenum*. Annals of Microbiology. (*Corr) (revision 7 September 2015)

ผลงานที่อยู่ในระหว่างการเตรียมต้นฉบับ 1 เรื่อง

1. **Limtong, S.*** and **R. Kaewwichian.** Yeasts in the corn phylloplane in Thailand and their antagonistic activities against plant pathogenic Fungi. (*Corr) (in preparation)

ผลงานตีพิมพ์ที่ไม่ใช่งานของโครงการโดยตรงแต่ใช้ยีสต์ที่แยกได้จากโครงการ ที่ยอมรับให้ตีพิมพ์ 1 เรื่อง

1. Polbureea, P., W. Yongmanitchaia, N. Lertwattanasakula, T. Ohashic, K. Fujiyama and **S. Limtong***. Characterization of oleaginous yeasts accumulating high levels of lipid when cultivated in glycerol and their potential for lipid production from biodiesel-derived crude glycerol. Fungal Biology. (*Corr) (in press)

ผลงานหนังสือ

ต้นฉบับหนังสือ เรื่อง อนุกรมวิธานและความหลากหลายของยีสต์ จำนวน 204 หน้า เขียนโดย สาวิตรี ลิ่มทอง และ มณี ตันติรุ่งกิจ (แนบสำเนาต้นฉบับหนังสือมาพร้อมกับรายงานนี้)

การเสนอผลงานในฐานะผู้บรรยายรับเชิญ (invited speaker)

ศ.ดร. สาวิตรี ลิ่มทอง ได้รับเชิญไปบรรยายพิเศษในเรื่องที่เกี่ยวข้องกับโครงการ ดังนี้

1. Diversity and indole-3-acetic acid production of phylloplane yeasts in Thailand with description of five novel ascomycetous yeast species บรรยายใน IUMS International Congress of Bacteriology and Applied Microbiology (BAM), and the International Congress of Mycology (IUMS 2011), 6-10 September 2011, Sapporo, Japan
2. Yeasts on agronomic crops phylloplanes: Diversity and potential in agricultural utilization บรรยายใน International Conference on Microbial Taxonomy, Basic and Applied Microbiology 4-6 October 2012 ณ โรงแรมโฆษะ จังหวัดขอนแก่น ประเทศไทย
3. Yeasts on agronomic crops phylloplanes: Diversity and potential in agricultural utilization บรรยายใน The 90th Anniversary Meeting of the Society for Biotechnology, Japan (SBJ) 23-26 October 2012 ณ KICC, Kobe Port Island, Japan
4. Polyphasic taxonomy of yeasts บรรยายใน Seminar on Curation and Management of Microbial Resources ซึ่งจัดที่ Thailand Science Park, Pathum Thani, Thailand ในระหว่าง 14–17 มกราคม 2556
5. ความหลากหลายของยีสต์ในแหล่งที่อยู่ธรรมชาติและการค้นพบยีสต์สปีชีส์ใหม่ในประเทศไทย บรรยายในการประชุมชีสเทมาติคส์ในประเทศไทยครั้งที่ 5 ซึ่งจัดที่ คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ในระหว่าง 25-27 พฤษภาคม 2558

(สำเนาโปรแกรม บทคัดย่อ และ power point แนบในภาคผนวก 2)

การเสนอผลงานในการประชุมวิชานานาชาติในต่างประเทศ

(Names in bold are researchers and students of the project)

1. **Limtong, S., P. Khunnamwong.** Diversity of endophytic yeasts in sugarcane leaves in Thailand. เสนอใน 14th International Congress of Bacteriology and Applied Microbiology, the 14thInternational Congress of Mycology and Eukaryotic Microbiology and the 16th International Congress of Virology (IUMS 2014) ระหว่าง 27 กรกฎาคม - 1 สิงหาคม 2557 ณ เมือง มอนทรีออน ประเทศแคนาดา
2. **Jindamorakot, S., S. Am-In, S. Limtong.** Diversity of epiphytic yeasts on rice leaves in Thailand. เสนอใน 14th International Congress of Bacteriology and Applied Microbiology, the 14thInternational Congress of Mycology and Eukaryotic Microbiology and the 16th International Congress of Virology (IUMS 2014) ระหว่าง 27 กรกฎาคม - 1 สิงหาคม 2557 ณ เมือง มอนทรีออน ประเทศแคนาดา
3. **Kaewwichian, R.**, W. Yongmanitchai, **S. Limtong.** Diversity of yeasts in corn phylloplanes and their antagonistic activities against pathogenic fungi. เสนอใน14th International Congress of Bacteriology and Applied Microbiology, the 14thInternational Congress of Mycology and

Eukaryotic Microbiology and the 16th International Congress of Virology (IUMS 2014) ระหว่าง 27 กรกฎาคม - 1 สิงหาคม 2557 ณ เมือง มอนทรีออน ประเทศแคนาดา

4. **Limtong, S., J. Surussawadee** and **N. Srisuk.** Diversity of cultivable yeast in sugarcane phyllosphere in Thailand, a tropical country. เสนอ ใน ISSY 32 International Specialized Symposium on Yeasts biodiversity and Biotechnology in the Twenty-first Century ระหว่าง 13 - 17 กันยายน 2558 ณ เมือง Perugia ประเทศอิตาลี
5. **Tantirungkij, M., R. Nasanit** and **S. Limtong.** Diversity of culture-independent endophytic yeasts from sugarcane leaves in Thailand (ดร.มณี ตันติรุ่งกิจ นำเสนอ) เสนอ ใน ISSY 32 เสนอ ใน ISSY 32 International Specialized Symposium on Yeasts Biodiversity and Biotechnology in the Twenty-first Century ระหว่าง 13 - 17 กันยายน 2558 ณ เมือง Perugia ประเทศอิตาลี
6. **Nasanit, R., S. Jaibangyang, M. Tantirungkij** and **S. Limtong.** Diversity of epiphytic yeasts in phyllosphere of corn in Thailand by culture-independent approach (ดร. รุจิกาญจน์ นาสนิท นำเสนอ) เสนอ ใน ISSY 32 เสนอ ใน ISSY 32 International Specialized Symposium on Yeasts Biodiversity and Biotechnology in the Twenty-first Century ระหว่าง 13 - 17 กันยายน 2558 ณ เมือง Perugia ประเทศอิตาลี

(สำเนาโปรกรม บทคัดย่อ และโปสเตอร์ หรือ power point แนบในภาคผนวก 3)

การเสนอผลงานในการประชุมวิชาการในประเทศไทย

(Names in bold are researchers and students of the project)

1. **Kaewwichian, R.**, W. Yongmanitchai and **S. Limtong**. Description of six novel yeast species isolated from phylloplane of rice, corn and sugarcane เสนอใน RGJ Seminar Series ครั้งที่ 88 หัวข้อ Microbial Resources: Their Biodiversity and Utilization จัดโดยโครงการปริญญาเอกกาญจนาภิเษก ร่วมกับภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ วันที่ 24 สิงหาคม 2555 ณ คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์
2. **Nutaratat, P., N. Srisuk** and **S. Limtong.** Screening for a promising yeast capable on plant growth promotion เสนอใน RGJ Seminar Series ครั้งที่ 88 หัวข้อ Microbial Resources: Their Biodiversity and Utilization จัดโดยโครงการปริญญาเอกกาญจนาภิเษก ร่วมกับภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ วันที่ 24 สิงหาคม 2555 ณ คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์
3. **Kaewwichian, R.**, W. Yongmanitchai and **S. Limtong**. Diversity of yeasts on phylloplane of rice, corn and sugarcane in Thailand and description of seven novel ascomycetous yeast species เสนอในการประชุมวิชาการ The 6th Thai Mycological Conference and Annual Meeting of Thai Mycological Association (TMA) 2012, 6 March 2012 at Maruay Garden Hotel, Bangkok, Thailand
4. **Krataithong, K., R. Nasanit, M. Tantirungkij** and **S. Limtong.** Identification of epiphytic yeast on rice population in Thailand based on molecular biological techniques. TSB 2013 Conference Proceedings. The 25th Annual Meeting of the Thai Society for Biotechnology

and International Conference. 16-19 Ocotober 2013, The Emerald Hotel, Bangkok, Thailand.

5. **Tangwong, A., R. Nasanit, M. Tantirungkij** and **S. Limtong.** Identification of epiphytic yeast from sugarcane phyllospheres using PCR and RFLP techniques. TSB 2013 Conference Proceedings. The 25th Annual Meeting of the Thai Society for Biotechnology and International Conference. 16-19 Ocotober 2013, The Emerald Hotel, Bangkok, Thailand.
6. **Jindamorakot, S., S. Am-In** and **S. Limtong.** Investigation on diversity of epiphytic yeasts on rice leaves in Thailand. เสนอใน The 1st ASEAN Microbial Biotechnology Conference. 19-21 Febuary 2014. BITEC, Bangkok, Thailand**.**
7. **Am-In S, S Jindamorakot and S Limtong.** Biodiversity of epiphytic yeasts from corn leaves by culture dependent technique. เสนอใน The 1st ASEAN Microbial Biotechnology Conference. BITEC, Bangkok Thailand**.** 19-21 February 2014.
8. **Jaibangyang, S., R. Nasanit, M. Tantrirungkij.** and **S. Limtong**. Study on epiphytic yeasts in corn phylloplane from Phetchabun province, Thailand by culture-independent approach. เสนอใน The 26th Annual Meeting of the Thai Society for Biotechnology and International Conference is organized jointly by the Thai Society for Biotechnology (TSB) and Mae Fah Lunag University (School of Science), Chiang Rai, Thailand, 26th and 29th November, 2014.
9. **Thaicharoena, P., R. Chanklana** and **S. Limtong**. **Selection of Indole-3-acetic Acid Producing-epiphytic Yeasts Isolated from Corn Leave**. เสนอใน The 4th International Biochemistry and Molecular Biology Conference. April 2-3, 2014, Bangkok, Thailand**.**
10. **จันทร์จีรา สุรัสวดี, นันทนา สีสุข และสาวิตรี ลิ่มทอง** ความหลากหลายของยีสต์อีพิไฟต์บนใบอ้อยและการอธิบายยีสต์สปีชีส์ใหม่ เสนอใน การประชุมชีสเทมาติคส์ในประเทศไทยครั้งที่ 5 ที่ คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ ระหว่าง 25-27 พฤษภาคม 2558
11. **ศศิธร จินดามรกฎ, สมจิต อ่ำอินทร์และสาวิตรี ลิ่มทอง** ความหลากหลายของยีสต์อีพิไฟต์บนใบข้าว เสนอใน การประชุมชีสเทมาติคส์ในประเทศไทยครั้งที่ 5 ที่ คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ ระหว่าง 25-27 พฤษภาคม 2558
12. **สมจิต อ่ำอินทร์, ศศิธร จินดามรกฎ และสาวิตรี ลิ่มทอง** การวิจัยความหลากหลายของยีสต์อีพิไฟต์บนใบข้าวโพดในประเทศไทย เสนอใน การประชุมชีสเทมาติคส์ในประเทศไทยครั้งที่ 5 ที่ คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ในระหว่าง 25-27 พฤษภาคม 2558

(สำเนาโปรแกรม บทคัดย่อ และเรื่องเต็ม แนบในภาคผนวก 4)

การจัดประชุมเมธีวิจัยอาวุโส สกว

1. จัดการประชุมเมธีวิจัยอาวุโส สกว ครั้งที่ 1 วันที่ 23 สิงหาคม 2556 คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ จตุจักร กรุงเทพมหานคร มีผู้เข้าประชุม 72 คน
2. จัดการประชุมเมธีวิจัยอาวุโส สกว ครั้งที่ 2 วันที่ 13 มิถุนายน 2557 คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ จตุจักร กรุงเทพมหานคร มีผู้เข้าประชุม 60 คน
3. จัดการประชุมเมธีวิจัยอาวุโส สกว ครั้งที่ 2 วันที่ 3 กันยายน 2558 คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ จตุจักร กรุงเทพมหานคร มีผู้เข้าประชุม 81 คน

   (สำเนาโปรแกรม และบทคัดย่อ แนบในภาคผนวก 5)

ผลิตมหาบัณฑิตและดุษฎีบัณฑิต

1. ผลิตมหาบัณฑิต 5 คน ดังนี้
   1) น. ส.กุลธรา กระต่ายทอง สำเร็จการศึกษา ภาควิชาเทคโนโลยีชีวภาพ คณะวิศวกรรมศาสตร์และเทคโนโลยีอุตสาหกรรม มหาวิทยาลัยศิลปากร
   2) นายอภิรัฐ ตั้งวงษ์อุทัย สำเร็จการศึกษา ภาควิชาเทคโนโลยีชีวภาพ คณะวิศวกรรมศาสตร์และเทคโนโลยีอุตสาหกรรม มหาวิทยาลัยศิลปากร
   3) นางสาวพรพรรณ ไทยเจริญ สำเร็จการศึกษา จากภาควิชาเทคโนโลยีชีวภาพ คณะวิทยาศาสตร์และเทคโนโลยี มหาวิทยาลัยธรรมศาสตร์
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3. นิสิตปริญญาเอกที่กำลังศึกษา 3 คน ดังนี้
   1) นายภูมิน นุตรทัต กำลังศึกษาที่ ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์
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   3) นางสาวปาริฉัตร อินโต ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ (รับทุน คปก)

การเชื่อมโยงกับต่างประเทศ

สำหรับความเชื่อมโยงนักวิจัยของโครงการนี้กับวิชาการอื่นๆ ในต่างประเทศที่เกี่ยวข้องกับโครงการนี้ หลายท่านดังนี้

1) มีร่วมมือทางวิชาการและการวิจัย กับ Dr. Hiroko Kawasaki, Senior Chief & Leader of Yeast Section, Resource collection Division & International Affairs Division, NITE Biological Resource Center (NBRC), National Institute of Technology and Evaluation, Japan ซึ่งมีความเชี่ยวชาญทางด้าน Molecular taxonomy และ Phylogenetic taxonomy ของยีสต์ มาเป็นระยะเวลานาน Dr. Kawasaki รับนางสาวรุ่งลักษณ์ แก้ววิเชียร ในขณะที่เป็นนักศึกษาทุน คปก ไปทำวิจัยที่ NITE Biological Resource Center เป็นเวลา 6 เดือน และได้ตีพิมพ์ผลงานของโครงการนี้ร่วมกัน 4 ฉบับ

2) มีร่วมมือทางวิชาการและการวิจัยกับ Prof. Ching-Fu Lee ผู้เชี่ยวชาญทางด้าน Yeast Molecular Taxonomy จาก Department of Applied Science, National Hsinchu University of Education, Hsinchu, Taiwan ได้ร่วมศึกษาและตีพิมพ์ผลงานการพบยีสต์สปีชีส์ใหม่จากสายพันธุ์ที่แยกจากใบพืชในโครงการนี้กับสายพันธุ์ของ Prof. Lee

3) มีร่วมมือทางวิชาการและการวิจัยกับ Prof. Allen N. Hagler และ Jose Roberto ผู้เชี่ยวชาญทางด้าน Yeast Molecular Taxonomy จาก Program de Pos-Graduacao em Biotechnologia Vegetal, CC, Bloco K, Rio de Janeiro, Brazil โดยได้ร่วมกันศึกษาและรายงานยีสต์สปีชีส์ใหม่จากสายพันธุ์ที่แยกจากใบพืชในโครงการนี้ กับยีสต์ที่ทั้งสองท่านแยกและศึกษาไว้โดยการตีพิมพ์ในวารสารนานาชาติแล้ว 1 สปีชีส์

4) มีร่วมมือทางวิชาการและการวิจัยกับ Prof. Dr. Marc-André Lachance, Department of Biology, University of Western Ontario, London, Ontario, Canada ซึ่งเป็นผู้เชี่ยวชาญทางด้าน Yeast Diversity และ Yeast Molecular Taxonomy Prof. Lachance ได้รับ นางสาวพัณณิดา ขุนนามวงษ์ นักศึกษาทุนจาก คปก ไปวิจัยโดยทุน คปก เป็นเวลา 6 เดือน โดยได้วิจัยยีสต์สกุลใหม่และสปีชีส์ใหม่จากยีสต์เอนโดไฟต์ที่แยกได้จากเนื้อเยื่อของใบพืชที่ได้จากโครงการ ได้ตีพิมพ์ผลงานร่วมกันแล้ว 1 เรื่อง และอยู่ในระหว่างการเตรียมผลงานเกี่ยวกับความหลากหลายของยีสต์เอนโดไฟต์ในใบพืชเพื่อการตีพิมพ์

5) มีความร่วมมือทางวิชาการและการวิจัยกับ Prof. Shao-Lan Li ผู้เชี่ยวชาญทางด้าน Yeast Molecular Taxonomy ที่ Yunnan Institute of Microbiology, Yunnan University, Kunming, Yunnan, PR China โดยได้ร่วมกับศึกษายีสต์สปีชีส์ใหม่จากสายพันธุ์ที่แยกจากใบพืชในโครงการนี้ กับยีสต์ที่ Prof. Li แยกและศึกษาไว้ เนื่องจากพบว่ามีลำดับนิวคลีโอไทด์บริเวณ D1-D2 ของ large subunit เหมือนกัน

6) มีความร่วมมือทางวิชาการและการวิจัยกับ Prof. Dr. Kazuhito Fujiyama และ Assist.Prof.Dr. Takao Ohashi, International Center for Biotechnology (ICBiotech), Osaka University, Japan โดยร่วมมือทำวิจัยพื้นฐานและการใช้ประโยชน์ยีสต์ โดยขณะนี้ได้นักวิชาการคณะนี้ได้รับอาจารย์รุ่นใหม่ จากภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ ที่ทำงานวิจัยด้านยีสต์ จากกลุ่มวิจัยของ ศ.ดร. สาวิตรี ลิ่มทองรวมทั้งรับนิสิตปริญญาเอกและปริญญาโทภายใต้การดูแลของ ศ.ดร. สาวิตรี ลิ่มทอง ไปทำวิจัยทั้งระยะยาว (6 เดือน) และระยะสั้น โดยมีการนำยีสต์จากผิวใบพืชที่ได้จากโครงการไปวิจัยต่อเรื่องการผลิตลิพิด และตีพิมพ์ผลงานการใช้ยีสต์จากผิวใบพืชสำหรับการผลิตลิพิดร่วมกัน

7) มีความร่วมมือทางวิชาการและการวิจัยกับ Assoc. Prof. Hiroya Yurimoto, Laboratory of Microbial Biotechnology, Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Japanโดยร่วมมือทำวิจัยเกี่ยวกับการยีสต์จากใบพืชเพื่อการผลิตออกซิน

8) มีความร่วมมือทางวิชาการและการวิจัยกับ Dr. Marizeth Groenewald, CBS-KNAW Fungal Biodiversity Center, Uppsalalaan, Utrecht, The Netherlands ซึ่งทำงานวิจัยทางด้านอนุกรมวิธานและการ

เก็บรักษายีสต์ โดยได้วิจัยร่วมกันเพื่อเสนอยีสต์ชนิดใหม่ที่ค้นพบในประเทศไทย ตลอดจนให้การสนับสนุนยีสต์สายพันธุ์ wild type เพื่อใช้ในการเปรียบเทียบ

รางวัลที่ได้รับ

1. วิทยานิพนธ์ปรัชญาดุษฎีบัณฑิต (จุลชีววิทยา) โดย ดร.รุ่งลักษณ์ แก้ววิเชียร เรื่อง Diversity of phylloplane yeasts and elucidation of their biocontrol mechanisms on pathogenic fungi (ความหลากหลายของยีสต์บนผิวใบพืชและการอธิบายกลไกการควบคุมทางชีวภาพของยีสต์ต่อราก่อโรคพืช) ได้รับรางวัลวิทยานิพนธ์ดีเด่น ระดับปริญญาเอก จาก บัณฑิตวิทยาลัย มหาวิทยาลัยเกษตรศาสตร์ 2555ประจำปี และได้รับวิทยานิพนธ์ระดับดี จากสำนักงานคณะกรรมการวิจัยแห่งชาติ เมื่อ 2 กุมภาพันธ์ 2558 (ศ.ดร. สาวิตรี ลิ่มทอง ได้รับโล่ ในฐานะอาจารย์ที่ปรึกษาหลักที่ให้คำแนะนำและการควบคุมการจัดทำวิทยานิพนธ์)
2. วิทยานิพนธ์วิทยาศาสตรมหาบัณฑิต (จุลชีววิทยา) โดยนางสาวจันทร์จีรา สุรัสวดี เรื่อง ความหลากหลายของยีสต์ที่ผิวใบอ้อยและความสามารถในการยับยั้งการเจริญของราโรคพืช ได้รับรางวัลวิทยานิพนธ์ดีเด่น ระดับปริญญาโท จากบัณฑิตวิทยาลัย มหาวิทยาลัยเกษตรศาสตร์ ประจำปี 2557

ภาคผนวก 1

ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ รวม 22 เรื่อง ดังนี้

1. ผลงานที่ตีพิมพ์แล้ว	19	เรื่อง
2. ผลงานที่กำลังอยู่ในระหว่างการเสนอให้ตีพิมพ์	2	เรื่อง
3. ผลงานที่อยู่ในระหว่างการเตรียมต้นฉบับ	1	เรื่อง

ผลงานตีพิมพ์ที่ไม่ใช่งานของโครงการโดยตรงแต่ใช้ยีสต์ที่แยกได้จากโครงการที่ยอมรับให้ตีพิมพ์ 1 เรื่อง

ผลงานที่ตีพิมพ์แล้ว 19 เรื่อง ดังนี้

1. **Kaewwichian R.**, W. Yongmanitchai, H. Kawasaki and **S. Limtong***. 2012. *Metschnikowia saccharicola* sp. nov. and *Metschnikowia lopburiensis* sp. nov., two novel yeast species isolated from phylloplane in Thailand. *Antonie van Leeuwenhoek*. 102: 743–751. (IF 2012: 2.072) (*Corr)

2. **Kaewwichian, R.**, W. Yongmanitchai, H. Kawasaki, P.-H. Wang, S.-H. Yang and **S. Limtong***. 2013. *Yamadazyma siamensis* sp. nov., *Yamadazyma phyllophila* sp. nov. and *Yamadazyma paraphyllophila* sp. nov., three novel yeast species isolated from phylloplane in Thailand and Taiwan. *Antonie van Leeuwenhoek.* 103: 777-788. (IF 2013: 2.137) (*Corr)

3. **Kaewwichian, R.**, H. Kawasaki and **S. Limtong*.** 2013. *Wickerhamomyces siamensis* sp. nov., a novel yeast species isolated from phylloplane in Thailand. *International Journal of Systematic and Evolutionary Microbiology.* 63: 1568-1573. (IF 2014: 2.511) (*Corr)

4. Chamnanpa T., P. Limtong, **N. Srisuk** and **S. Limtong***. 2013. *Pseudozyma vetiver* sp. nov., a novel anamorphic ustilaginomycetous yeast species isolated from the phylloplane in Thailand. *Antonie van Leeuwenhoek*. 104: 637-644. (IF 2014: 2.137) (*Corr)

5. **Nutaratat, P., N. Srisuk*, P. Arunrattiyakorn** and **S. Limtong**. 2014. Plant growth promoting traits of epiphytic and endophytic yeasts isolated from rice and sugar cane leaves in Thailand. *Fungal Biology.* 118: 683-694. (IF 2014: 2.342) (*Corr)

6. **Kaewwichian, R.** and **S. Limtong***. 2014. *Nakazawaea siamensis* f.a., sp. nov., a novel yeast species isolated from phylloplane in Thailand. *International Journal of Systematic and Evolutionary Microbiology.* 64: 266–270. (IF 2014: 2.511) (*Corr)

7. **Limtong, S.*, R. Kaewwichian**, W. Yongmanitchai and H. Kawasaki. 2014. Diversity of cultivable yeasts in phylloplanes of sugarcane in Thailand and their capability to produce indole-3-acetic Acid. *World Journal of Microbiology* and *Biotechnology*. 30: 1785–1796. (IF2014: 1.779) (*Corr)

8. **Surussawadee, J.**, **P. Khunnamwong**, **N. Srisuk** and **S. Limtong***. 2014. *Papiliotrema siamensis* f.a., sp. nov., a novel yeast species isolated from plant leaf in Thailand. 2014. *International Journal of Systematic and Evolutionary Microbiology.* 64: 3058-3062. (IF 2014: 2.511) (*Corr)

9. **Khunnamwong P., J. Surussawadee, S. Jindamorakot** and **S. Limtong*** 2014. *Wickerhamiella siamensis* f.a., sp. nov., a novel endophytic and epiphytic yeast species isolated from sugarcane leaf in Thailand. *International Journal of Systematic and Evolutionary Microbiology*. 64: 3849-3855. (IF 2014: 2.511) (*Corr)

10. **Nutaratat, P.**, W. Amsri, **N. Srisuk***, **P. Arunrattiyakorn** and **S. Limtong***. 2015. Indole-3-acetic acid production by newly isolated red yeast *Rhodosporidium paludigenum. Journal of General and Applied Microbiology.* 61: 1–9. (IF 2014: 0.943) (*Corr)

11. **Limtong, S***. and **R. Kaewwichian**. 2015. The diversity of culturable yeasts in the phylloplane of rice in Thailand. *Annals of Microbiology*. 65: 667–675. (IF 2014: 0.990) (*Corr)

12. **Jindamorakot S., S. Am-In, R. Kaewwichian** and **S. Limtong***. 2015. *Yamadazyma insecticola* f.a., sp. nov. and *Yamadazyma epiphylla* f.a., sp. nov., two novel yeast species isolated in Thailand". *International Journal of Systematic and Evolutionary Microbiology.* 65: 1290–1296. (IF 2014: 2.511) (*Corr)

13. **Kaewwichian R., S. Jindamorakot, S. Am-In**, M. Sipiczki and **S. Limtong***. 2015. *Hannaella siamensis* sp. nov. and *Hannaella phetchabunensis* sp. nov., two new anamorphic basidiomycetous yeast species isolated from plants. *International Journal of Systematic and Evolutionary Microbiology.* 65: 1297–1303. (IF 2014: 2.511) (*Corr)

14. **Nasanit R., K. Krataithong, M. Tantirungkij** and **S. Limtong*.** 2015. Assessment of epiphytic yeast diversity in rice (*Oryza sativa*) phyllosphere in Thailand by a culture-independent approach. *Antonie van Leeuwenhoek*. 107:1475–1490. (IF 2014: 1.806) (*Corr)

15. **Surussawadee, J., S. Jindamorakot**, T. Nakase, C.-F. Lee and **S. Limtong***. 2015. *Hannaella phyllophila* sp. nov., a novel basidiomycetous yeast species associated with plants in Thailand and Taiwan. *International Journal of Systematic and Evolutionary Microbiology*.65 (7): 2135-2140. (IF 2014: 2.511) (*Corr)

16. **Khunnamwong, P., J. Surussawadee, S. Jindamorakot,** J. R. A. Ribeiro, A. N. Hagler and **S. Limtong*.** 2015. *Occultifur tropicalis* f.a., sp. nov., a novel Cystobasidiomycetous yeast species isolated from tropical regions. *International Journal of Systematic and Evolutionary Microbiology*. 65 (5): 1578–1582. (IF 2014: 2.511) (*Corr)

17. **Tantirungkij M., R. Nasanit** and **S. Limtong***. 2015. Assessment of endophytic yeast diversity in rice leaves by a culture-independent approach. *Antonie van Leeuwenhoek*. 108 (3): 633–647. (IF 2014: 1.806) (*Corr)

18. **Nasanit, R., A. Tangwong-o-thai, M. Tantirungkij** and **S. Limtong***. 2015. The assessment of epiphytic yeast diversity in sugarcane phyllosphere in Thailand by culture-independent method. *Fungal Biology.* http://dx.doi.org/10.1016/j.funbio.2015. 08.021 (*Corr) (online)

19. **Khunnamwong, P.***, N. Lertwattanasakul, S. Jindamorakot, **S. Limtong** and M-A. Lachance. 2015. The genus *Diutina*, description of *Diutina siamensis*, f.a. sp. nov., and reassignment *of Candida catenulata*, C. mesorugosa, *C. neorugosa*, *C. pseudorugosa*, *C. ranongensis*, *C. rugosa* and *C. scorzettiae* to the genus *Diutina*. *Int J Syst Evol Microbiol*. doi: (Digital Object Identifier) doi: 10.1099/ijsem.0.000634 (*Corr) (on line)

ผลงานที่กำลังอยู่ในระหว่างการเสนอตีพิมพ์ 2 เรื่อง

1. **Nutaratat, P., N. Srisuk*, P. Arunrattiyakorn** and **S. Limtong**. Indole-3-acetic acid biosynthetic pathways in the basidiomycetous yeast *Rhodosporidium paludigenum.* Archives of Microbiology. (*Corr) (submitted 8 September 2015)

2. **Nutaratat, P., N. Srisuk*, P. Arunrattiyakorn** and **S. Limtong**. Fed-batch fermentation of indole-3-acetic acid production in stirred tank fermenter by red yeast *Rhodosporidium paludigenum*. Annals of Microbiology. (*Corr) (revision 7 September 2015)

ผลงานที่อยู่ในระหว่างการเตรียมต้นฉบับ 1 เรื่อง

1. **Limtong, S.*** and **R. Kaewwichian.** Yeasts in the corn phylloplane in Thailand and their antagonistic activities against plant pathogenic Fungi. (*Corr) (in preparation)

ผลงานตีพิมพ์ที่ไม่ใช่งานของโครงการโดยตรงแต่ใช้ยีสต์ที่แยกได้จากโครงการ ที่ยอมรับให้ตีพิมพ์ 1 เรื่อง

1. Polbureea, P., W. Yongmanitchaia, N. Lertwattanasakula, T. Ohashic, K. Fujiyama and **S. Limtong***. Characterization of oleaginous yeasts accumulating high levels of lipid when cultivated in glycerol and their potential for lipid production from biodiesel-derived crude glycerol. Fungal Biology. (*Corr) (in press)

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ORIGINAL PAPER

Metschnikowia saccharicola sp. nov. and *Metschnikowia lopburiensis* sp. nov., two novel yeast species isolated from phylloplane in Thailand

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Abstract Five strains representing two novel anamorphic yeast species were isolated from the external surface of sugarcane leaves (DMKU-RK16, DMKU-RK24, DMKU-RK198 and DMKU-RK500^T) and rice leaf (DMKU-RK277^T) by an enrichment technique. On the basis of morphological, biochemical, physiological and chemotaxonomic characteristics, the sequence analysis of the D1/D2 domain of the large subunit (LSU) rRNA gene and the internal transcribed spacer region, the five strains were assigned to be two novel species of the *Metschnikowia,* although the formation of ascospores was not observed. Four strains (DMKU-RK16, DMKU-RK24, DMKU-RK198 and DMKU-RK500^T) where the sequences of the D1/D2 domain of the LSU rRNA gene were identical and differed by 4.1 % nucleotide substitutions (20 nucleotide substitutions and four gaps out of 490 nt) from the type strain of *Candida succicola*, their closest known species in terms of pairwise sequence similarity, represent a single novel species, for which the name *Metschnikowia saccharicola* sp. nov. is proposed. The type strain is DMKU-RK500^T (= BCC 50735^T = NBRC 108904^T = CBS 12575^T). Strain DMKU-RK277^T was closest to *Candida hainanensis* but with 4.0 % nucleotide substitutions (18 nucleotide substitutions and three gaps out of 449 nt) in the D1/D2 domain of the LSU rRNA gene was assigned to be *Metschnikowia lopburiensis* sp. nov. (type strain DMKU-RK277^T = BCC 50731^T = NBRC 108902^T = CBS 12574^T)

Keywords *Metschnikowia saccharicola* sp. nov. · *Metschnikowia lopburiensis* sp. nov. · Phylloplane yeast · Ascomycetous yeast · New yeast species · Thailand

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Introduction

Many yeast species of the genus *Metschnikowia* and a few related *Candida* species are often found associated with a variety of substrates including insects and plants, especially beetles and flowers (Lachance et al. 2011, 2005; Manson et al. 2007; Nguyen et al. 2006). More recently, yeast species of the genus *Candida* in *Metschnikowia* clade were reported to have been collected from flowers, tree sap, gum produced by trees and exudates of *Ficus racemosa* Linn.; these species included *Candida hainanensis*, *Candida heveicola*, *Candida pseudoflosculorum*, *Candida robnettiae*, *Candida eppingiae, Candida danieliae* and *Candida succicola* (Groenewald et al. 2011; Nakase et al. 2011; Wang et al. 2008).

The external surface of plant leaves is usually referred to as the phylloplane or phyllosphere (Fonseca and Inacio 2006; Phaff and Starmer 1987). Phylloplane from various regions of the world are found to be colonized by members of both basidiomycetous and ascomycetous yeasts (Fonseca and Inacio 2006; Glushakova et al. 2007; Landell et al. 2010; Nakase et al. 2001; Slavikova et al. 2009). Although most common phylloplane yeasts are reported to be basidiomycete species (Fonseca and Inacio 2006; Nakase et al. 2001) such as the genera *Cryptococcus*, *Rhodotorula*, *Sporobolomyces* and *Trichosporon* (de Azeredo et al. 1998; Glushakova and Chernov 2010; Slavikova et al. 2009), various ascomycetous yeast species have also been detected on the phylloplane i.e. *Debaryomyces hansenii*, *Hanseniaspora uvarum, Kazachstania barnetii*, *Metschnikowia pulcherrima*, *Pichia membranifaciens*, *Saccharomyces cerevisiae* and various species of *Candida* including *Candida aechmeae*, *Candida olephila*, *Candida chumphonensis, Candida mattranensis* and *Candida vrieseae* (Glushakova et al. 2007; Glushakova and Chernov 2010; Koowadjanakul et al. 2011; Landell et al. 2010; Slavikova et al. 2009).

During an investigation of yeasts on the external surface of sugarcane leaves and rice leaves in Thailand, various known yeast species were found and five strains, including four strains from phylloplane of sugarcane and one strain from phylloplane of rice, were found to represent two novel *Metschnikowia* species. In this paper, the four strains (DMKU-RK16, DMKU-RK24, DMKU-RK198 and DMKU-RK500^T) are described as *Metschnikowia saccharicola* sp. nov. and strain DMKU-RK277^T is described as *Metschnikowia lopburiensis* sp. nov.

Materials and methods

Sample collection and yeast isolation

Green and undamaged leaves of rice (85 samples) and sugarcane (95 samples) were respectively collected from rice and sugarcane fields in central and northeastern Thailand. Yeasts were isolated from surface of plant leaves by an enrichment technique using acidified yeast extract malt extract (YM) broth (0.3 % yeast extract, 0.3 % malt extract, 0.5 % peptone and 1 % glucose) supplemented with 0.025 % sodium propionate and 0.02 % chloramphenicol (Limtong et al. 2007). Three grams of cut leaves were aseptically placed in 250 ml Erlenmeyer flask, containing 50 ml of acidified YM broth and incubated on a rotary shaker at room temperature (27 ± 3 °C) for 3 days. The enrichment culture was then spread on YM agar supplemented with 0.025 % sodium propionate and 0.02 % chloramphenicol and incubated at room temperature until yeast colonies appeared. Yeast colonies of different morphologies were picked and purified by cross streaking on YM agar. Purified yeast strains were suspended in YM broth supplemented with 10 % glycerol and maintained at −80 °C.

A total of 154 strains were isolated from rice leaf samples and 166 strains were from sugarcane leaf samples. Four strains (DMKU-RK16, DMKU-RK24, DMKU-RK198 and DMKU-RK500^T) were obtained from four leaf samples and strain DMKU-RK277^T was from a rice leaf sample (Table 1).

DNA sequencing and phylogenetic analysis

The sequences of the D1/D2 domain of the LSU rRNA gene and the ITS region were determined from PCR products amplified from genomic DNA. Methods for DNA extraction and amplification of the D1/D2 domain of the LSU rRNA gene were described previously (Limtong et al. 2007). The ITS region was amplified with primers, ITS1 and ITS4, following the method of White et al. (1990). The PCR products were checked by agarose gel electrophoresis and purified by using the QIAquick purification kit (Qiagen, Germany). The purified products were submitted to Macrogen Inc. (Korea) for sequencing with primers, NL1 (5′ GCATATCAATAAGCGGAGGA AAAG 3′) and NL4 (5′ GGTCCGTGTTTCAAGACG G 3′), for the D1/D2 domain of the LSU rRNA gene, and with primers, ITS1 (5′ TCCGTAGGTGAACCT GCGG 3′) and ITS4 (5′ TCCTCCGCTTATTGAT ATGC 3′), for the ITS region. The sequences were compared pairwise using a BLAST search (Altschul et al. 1997) and were aligned with the sequences of related species retrieved from GenBank using the multiple alignment program CLUSTAL_X version 1.81 (Thompson et al. 1997). A phylogenetic tree was constructed from the evolutionary distance data with Kimura's two-parameter correction (Kimura 1980), using the neighbor joining method (Saitou and Nei 1987) by MEGA software version 4.0 (Tamura et al.

Table 1 Strains of *M. saccharicola* sp. nov. and *M. lopburiensis* sp. nov. isolated from surface of plant leaves used in this study

Strain	Accession number			GenBank accession number		Sample for isolation		
	BCC	NBRC	CBS	D1/D2	ITS	Leaves	Locality	Date
M. saccharicola sp. nov.								
DMKU-RK16	50727	–	–	AB697752	AB697757	Sugarcane 1 (*S. officinarum*)	Sikhio district, Nakhon Ratchasima Province, Thailand	28 Sep. 2009
DMKU-RK24	50728	–	–	AB697753	AB697758	Sugarcane 2 (*S. officinarum*)	Sikhio district, Nakhon Ratchasima Province, Thailand	28 Sep. 2009
DMKU-RK198	5072	–	–	AB697754	AB697759	Sugarcane 3 (*S. officinarum*)	Dan Chang district, Suphan Buri Province, Thailand	22 Mar. 2010
DMKU-RK500^{T}	50735^{T}	108904^{T}	12575^{T}	AB697755	AB697760	Sugarcane 4 (*Saccharum officinarum*)	Khon Buri district, Nakhon Ratchasima Province, Thailand	6 May 2010
M. lopburiensis sp. nov.								
DMKU-RK277^{T}	50731^{T}	108902^{T}	12574^{T}	AB697756	AB697761	Rice (*Oryza sativa*)	Ban Mi district, Lop Buri Province, Thailand	5 May 2010

BCC BIOTEC Culture Collection, *NBRC* NITE Biological Resources Center, *CBS* Centraalbureau voor Schimmelcultures

2007). Confidence levels of the clades were estimated from bootstrap analysis (1,000 replicates) (Felsenstein 1985).

Examination of taxonomic characteristics

The strains were characterized morphologically, biochemically, and physiologically according to the standard methods described by Yarrow (1998). Mycelium formation was investigated on potato dextrose agar (PDA) in slide culture at 25 °C for up to 7 days. Ascospores formation was investigated individually or in pair on 5 % malt extract agar, McClary's acetate agar, Gorodkowa agar, corn meal agar, V8 agar and yeast carbon base supplemented with 0.01 % ammonium sulfate (YCBAS) agar at 15 and 25 °C for up to 4 weeks. Carbon assimilation tests were conducted in liquid medium according to the method described by Yarrow (1998). Assimilation of nitrogen compounds was examined on solid media with starved inocula following the method of Nakase and Suzuki (1986). Growth at various temperatures was determined by cultivation in YM broth. Ubiquinones were extracted from cells cultivated in a 500 ml Erlenmeyer flask containing 250 ml of yeast extract peptone dextrose (YPD) broth (1 % yeast extract, 2 % peptone and 2 % dextrose) on a rotary shaker at 28 °C for 24–48 h and purified according to the method described by Yamada and Kondo (1973) and Kuraishi et al. (1985). Isoprenologues were identified by HPLC as described previously (Limtong et al. 2007). The DNA base composition was determined by HPLC as described by Tamaoka and Komagata (1984) using the DNA-GC kit (Yamasa Shoyu Co., Japan).

Results and discussion

Novel species delineation and identification

Analysis of the D1/D2 domain of the LSU rRNA gene sequence revealed that the sequences of the four strains (DMKU-RK16, DMKU-RK24, DMKU-RK198 and DMKU-RK500^{T}) were identical and differed by only two nucleotide substitutions from the two strains of undescribed *Candida* species namely strains HN1-1 and HN1-2(a), however, the strains are not available for further comparison. The sequences of these strains showed 4.1 % nucleotide substitutions

(20 nucleotide substitutions and four gaps out of 490 nt) from the type strain of *C. succicola*, their closest known species, in terms of pairwise sequence similarity. The nucleotide sequences of the ITS regions of the four strains were identical and differed by 12.6 % nucleotide substitutions (37 nucleotide substitutions and one gap out of 294 nt) from *C. succicola.* Strain DMKU-RK277^T was closest to *C. hainanensis* but with 4.0 % nucleotide substitutions (18 nucleotide substitutions and three gaps out of 449 nt) in the D1/D2 domain of the LSU rRNA gene. The sequences of ITS region of strain DMKU-RK277^T and *C. hainanensis* differed by 31.9 % nucleotide substitutions (145 nucleotide substitutions and 51 gaps out of 455 nt).

The phylogenetic tree base on the sequences of the D1/D2 domains of the LSU rRNA gene further demonstrated that all strains were in *Metschnikowia* clade. Four strains (DMKU-RK16, DMKU-RK24, DMKU-RK198 and DMKU-RK500^T) were in the same position and connected to the two undescribed *Candida* species (strains HN1-1 and HN1-2(a)) that closest to them. They connected to *C. succicola*, the closest known species, and form a subclade with *Metschnikowia lunata* though the bootstrap was low (Fig. 1). Strain DMKU-RK277^T connected to *C. hainanensis*, it closest relative in term of pairwise sequence similarity, with 100 % bootstrap support and was near the subclade containing *Metschnikowia pulcherrima.*

Cells of the four strains (DMKU-RK16, DMKU-RK24, DMKU-RK198 and DMKU-RK500^T) were globose, subglobose and ovoid (Fig. 2a) and the strain DMKU-RK277^T was ovoid to ellipsoid (Fig. 3). Cells of the five strains proliferated by multilateral budding. Pseudohyphae but not true hyphae were formed by the four strains (DMKU-RK16, DMKU-RK24, DMKU-RK198 and DMKU-RK500^T) (Fig. 2b). Neither pseudohyphae and true hyphae were formed by the strain DMKU-RK277^T. Ascospores were not produced by any of the strains either individually or when paired on YM agar, YPD agar, 5 % malt extract agar, McClary's acetate agar, corn meal agar, Gorodkowa agar, V8 agar and YCBAS agar after 4 weeks at 15 and 25 °C.

On the basis of morphological, biochemical, physiological, chemotaxonomic characteristics, and the sequence analysis of the D1/D2 domain of the LSU rRNA gene and the ITS region, we concluded that the five strains represent two novel *Metschnikowia* species. The name *M. saccharicola* sp. nov. (MB564869) is proposed for the four strains (DMKU-RK16, DMKU-RK24, DMKU-RK198 and DMKU-RK500^T) and the name *M. lopburiensis* sp. nov. (MB564870) is assigned for the strain DMKU-RK277^T.

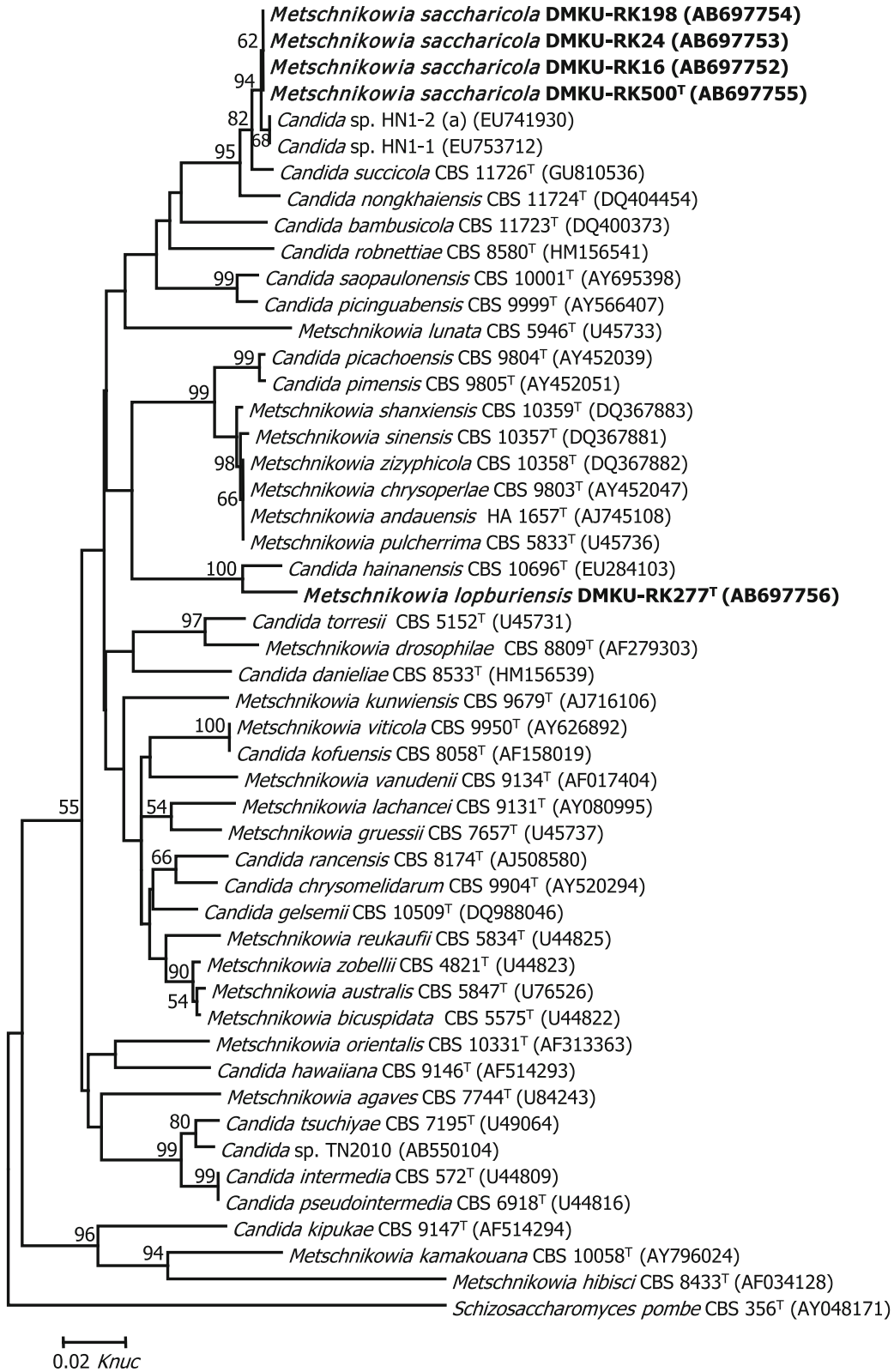
Fig. 1 Phylogenetic tree based on the sequences of the D1/D2 domain of the LSU rRNA gene, showing positions of *M. saccharicola* sp. nov. (DMKU-RK500^T) and *M. lopburiensis* sp. nov. (DMKU-RK277^T), with respect to closely related species. The phylogenetic tree was constructed from evolutionary distance data with Kimura's two-parameter correction, using the neighbor joining method by MEGA software version 4.0. *Numbers* indicate percentages of bootstrap sampling, derived from 1,000 samples. The numbers in *parentheses* are GenBank accession numbers. *Schizosaccharomyces pombe* was the outgroup in the analysis

In practice, *M. saccharicola* sp. nov. and *M. lopburiensis* sp. nov. can be distinguished from their closest species *C. succicola* and *C. hainanensis*, respectively, not only on the basis of the sequences of the D1/D2 domain of the LSU rRNA gene and the ITS region but also some phenotypic characteristics as shown in Table 2.

Most of the members of the *Metschnikowia* clade such as *C. nongkhaiensis, C. bambusicola, C. saopaulonensis, C. picinguabensis, C. tsuchiyae, C. intermedia* and *C. pseudointermedia* exhibit an association with insect frass, flowers and mosses (Lachance et al. 2011, Nakase et al. 2011, Ruivo et al. 2006). In the most recent report, the novel *Candida* species in *Metschnikowia* clade, namely *C. succicola,* the species closest to *M. saccharicola* sp. nov., was isolated from exudate of a tree in Thailand (Nakase et al. 2011). *C. hainanensis,* the species closest to *M. lopburiensis* sp. nov., and *C. heveicola* were isolated from flowers and tree sap collected, respectively from tropical forest in Yunnan and Hainan, China (Wang et al. 2008). *C. danieliae*, *C. pseudoflosculorum*, *C. robnettiae* and *C. eppingiae* were reported to isolate from gum produced by trees, and flowers in Thailand and Guyana (Groenewald et al. 2011). Therefore, plants seem to be the habitat of numerous yeast species in the *Metschnikowia* clade.

Description of *M. saccharicola* Kaewwichian, Yongmanitchai, Kawasaki & Limtong sp. nov.

Growth in yeast extract malt extract (YM) broth: After 3 days at 25 °C, cells are globose, subglobose to ovoid (4–7 × 5–8 µm) and occur singly, in pairs or in groups (Fig. 2a). Budding is multilateral. Growth on YM


Metschnikowia saccharicola DMKU-RK198 (AB697754)
62
Metschnikowia saccharicola DMKU-RK24 (AB697753)
Metschnikowia saccharicola DMKU-RK16 (AB697752)
94
Metschnikowia saccharicola DMKU-RK500T (AB697755)
82
Candida sp. HN1-2 (a) (EU741930)
68
Candida sp. HN1-1 (EU753712)
95
Candida succicola CBS 11726T (GU810536)
Candida nongkhaiensis CBS 11724T (DQ404454)
Candida bambusicola CBS 11723T (DQ400373)
Candida robnettiae CBS 8580T (HM156541)
99
Candida saopaulonensis CBS 10001T (AY695398)
Candida picinguabensis CBS 9999T (AY566407)
Metschnikowia lunata CBS 5946T (U45733)
99
Candida picachoensis CBS 9804T (AY452039)
Candida pimensis CBS 9805T (AY452051)
99
Metschnikowia shanxiensis CBS 10359T (DQ367883)
Metschnikowia sinensis CBS 10357T (DQ367881)
98
Metschnikowia zizyphicola CBS 10358T (DQ367882)
Metschnikowia chrysoperlae CBS 9803T (AY452047)
66
Metschnikowia andauensis HA 1657T (AJ745108)
Metschnikowia pulcherrima CBS 5833T (U45736)
100
Candida hainanensis CBS 10696T (EU284103)
Metschnikowia lopburiensis DMKU-RK277T (AB697756)
97
Candida torresii CBS 5152T (U45731)
Metschnikowia drosophilae CBS 8809T (AF279303)
Candida danieliae CBS 8533T (HM156539)
Metschnikowia kunwiensis CBS 9679T (AJ716106)
100
Metschnikowia viticola CBS 9950T (AY626892)
Candida kofuensis CBS 8058T (AF158019)
Metschnikowia vanudenii CBS 9134T (AF017404)
55
54
Metschnikowia lachancei CBS 9131T (AY080995)
Metschnikowia gruessii CBS 7657T (U45737)
66
Candida rancensis CBS 8174T (AJ508580)
Candida chrysomelidarum CBS 9904T (AY520294)
Candida gelsemii CBS 10509T (DQ988046)
Metschnikowia reukaufii CBS 5834T (U44825)
90
Metschnikowia zobellii CBS 4821T (U44823)
54
Metschnikowia australis CBS 5847T (U76526)
Metschnikowia bicuspidata CBS 5575T (U44822)
Metschnikowia orientalis CBS 10331T (AF313363)
Candida hawaiiana CBS 9146T (AF514293)
Metschnikowia agaves CBS 7744T (U84243)
80
Candida tsuchiyae CBS 7195T (U49064)
99
Candida sp. TN2010 (AB550104)
99
Candida intermedia CBS 572T (U44809)
Candida pseudointermedia CBS 6918T (U44816)
96
Candida kipukae CBS 9147T (AF514294)
94
Metschnikowia kamakouana CBS 10058T (AY796024)
Metschnikowia hibisci CBS 8433T (AF034128)
Schizosaccharomyces pombe CBS 356T (AY048171)
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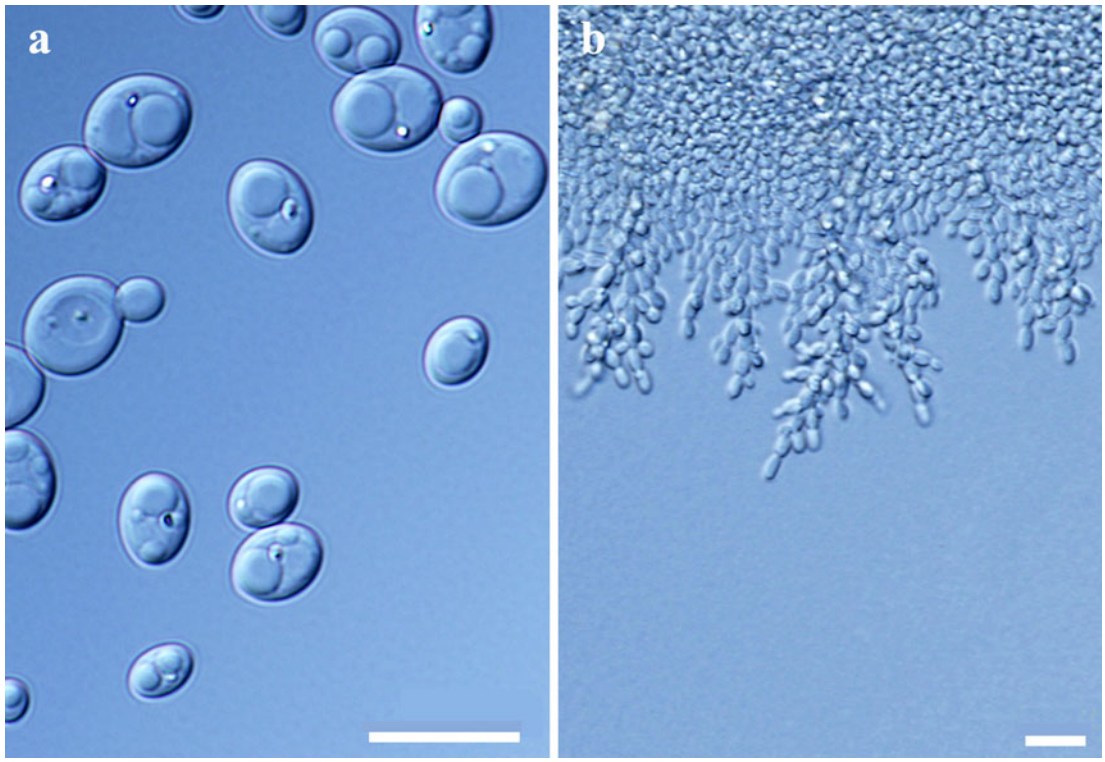


Fig. 2 *Metschnikowia saccharicola* sp. nov. (DMKU-RK500^T) **a** budding cells in YM broth after 3 days at 25 °C and **b** pseudohyphae formed on PDA after 3 days at 25 °C. *Scale bar* 10 μm

agar: After 3 days at 25 °C, the streak culture is grayish white, smooth, soft to butyrous and has an entire margin. Pseudohyphae are formed but true hyphae are not formed in slide culture on potato dextrose agar (PDA) after 3 days at 25 °C (Fig. 2b). Ascospores were not produced by any of the strains either individually or when paired on YM agar, YPD agar, 5 % malt extract agar, McClary's acetate agar, corn meal agar, Gorodkowa agar, V8 agar and YCBAS agar after 4 weeks at 15 and 25 °C. Fermentation of D-glucose and trehalose are positive but negative for D-galactose, sucrose, maltose, lactose, raffinose, xylose and cellobiose. D-glucose, L-sorbose, sucrose, maltose, trehalose, melezitose, D-xylose, ethanol, ribitol, D-mannitol, D-glucitol, α-methyl-D-glucoside, 2-keto-D-gluconic acid, D-glucono-δ-lactone, succinic acid, citric acid, xylitol, ethylamine, L-lysine and cadaverine are assimilated, but D-galactose, cellobiose, lactose, melibiose, raffinose, inulin, soluble starch, L-arabinose, D-arabinose, L-rhamnose, D-ribose, N-acetyl-D-glucosamine, methanol, glycerol, erythritol, galactitol, salicin, D-gluconic acid, D-glucuronic acid, D-galacturonic acid, 5-keto-D-gluconic acid, DL-lactic acid, inositol, D-glucosamine, arbutin, hexeadecane, propane-1,2-diol, butane-2,3-diol, arabinitol, nitrate and nitrite are non-assimilated. Growth in vitamin free medium is positive (weak). Growth on medium containing 50 % (w/v) glucose and 60 % (w/v) glucose are positive, but 10 % (w/v) sodium chloride/5 % (w/v) glucose and 16 % (w/v) sodium chloride/5 % (w/v) glucose are negative. Growth with 0.01 % cylcloheximide is positive but 0.1 % cylcloheximide is negative. Growth at 25, 30 and 35 °C are positive, but at 37, 40, 42 and 45 °C are negative. Acid formation is present. Starch like compounds are not produced. Diazonium blue B color and urease reactions are negative. DNA G+C content is 41.7 mol %. The major ubiquinone is Q-9.

Holotype

DMKU-RK500^T is the holotype of *M. saccharicola* (MB564869). The strain was isolated from phylloplane of sugarcane (*Saccharum officinarum*) collected from Nakhon Ratchasima province, Thailand. The living culture from type was deposited at the BIOTEC



Fig. 3 *Metschnikowia lopburiensis* sp. nov. (DMKU-RK277^T) budding cells in YM broth after 3 days at 25 °C. *Scale bar* 10 μm

Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathumthani, Thailand, as BCC 50735^T; NITE Biological Resources Center (NBRC), Department of Biotechnology, National Institute of Technology and Evaluation, Chiba, Japan, as NBRC 108904^T and Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands as CBS 12575^T.

Etymology

The species epithet "*saccharicola*" was chosen because this species was isolated from phylloplane of sugarcane, *S. officinarum* collected in Thailand.

Description of *M. lopburiensis* Kaewwichian, Yongmanitchai, Kawasaki & Limtong sp. nov

Growth in yeast extract malt extract (YM) broth: After 3 days at 25 °C, cells are ovoid to ellipsoid (2–3 × 3–5 μm) and occur singly, in pairs or in groups (Fig. 3). Budding is multilateral. Growth on YM agar after 3 days at 25 °C, the streak culture is cream, smooth soft to butyrous and has an entire margin. Pseudohyphae and true hyphae are not formed in slide culture on PDA. Ascospores were not produced on YM agar, YPD agar, 5 % malt extract agar, McClary's acetate agar, corn meal agar, Gorodkowa agar, V8 agar and YCBAS agar after 4 weeks at 15 and 25 °C. Fermentation of D-glucose and trehalose (latent) are positive but negative for D-galactose, sucrose, maltose, lactose, raffinose, xylose and cellobiose. D-glucose, L-sorbose, sucrose, maltose, cellobiose, trehalose, melezitose, D-xylose, D-arabinose (weak), *N*-acetyl-D-glucosamine, ethanol, glycerol (latent), ribitol, D-mannitol, D-glucitol, α-methyl-D-glucoside, salicin, D-gluconic acid, D-glucuronic acid, 2-keto-D-gluconic acid, D-glucono-δ-lactone, succinic acid, citric acid, D-glucosamine, arbutin, xylitol, ethylamine, L-lysine and cadaverine are assimilated, but D-galactose, lactose, melibiose, raffinose, inulin, soluble starch, L-arabinose, L-rhamnose, D-ribose, methanol, erythritol, galactitol, D-galacturonic acid, 5-keto-D-gluconic acid, DL-lactic acid, inositol, hexeadecane, propane-1,2-diol, butane-2,3-diol, arabinitol, nitrate and nitrite are non-assimilated. Growth in vitamin free medium is positive (weak). Growth on medium containing 50 % (w/v) glucose, 60 % (w/v) glucose and 10 % (w/v) sodium chloride/5 % (w/v) glucose (weak) are positive, but 16 % (w/v) sodium chloride/5 % (w/v) glucose is negative. Growth with 0.01 % cylcloheximide is positive but 0.1 % cylcloheximide is negative. Growth at 25, 30, 35 and 37 °C are positive, but at 40, 42 and 45 °C are negative. Acid formation is absent. Starch like compounds are not produced. Diazonium blue B color and urease reactions are negative. DNA G+C content is 51.0 mol %. The major ubiquinone is Q-9.

Holotype

DMKU-RK277^T is the holotype of *M. lopburiensis* (MB564870). The strain was isolated from phylloplane of rice (*Oryza sativa*) collected from Lop Buri province, Thailand. The living culture from type was deposited at the BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathumthani, Thailand, as BCC 50731^T; NITE Biological Resources Center (NBRC), Department of Biotechnology, National Institute of

Table 2 Phenotypic characteristics that differentiate *M. saccharicola* sp. nov. and *M. lopburiensis* sp. nov. from their closest species, *C. succicola* and *C. hainanensis*, respectively

Characteristics	Species			
	M. saccharicola	*C. succicola*[a]	*M. lopburiensis*	*C. hainanensis*[b]
Fermentation				
Sucrose	−	+	−	−
Maltose	−	+	−	−
Assimilation of carbon compounds				
D-Galactose	−	l	−	+
D-Arabinose	−	−	w	−
Glycerol	−	v	l	+
Ribitol	+	l	+	+
D-Glucono-δ-lactone	+	l	+	n
Succinic acid	+	+	+	l
Hexeadecane	−	+	−	−
Arabinitol	−	l	−	n
Other growth characteristics				
Vitamin-free medium	w	w	w	+
Growth at 37 °C	−	+	+	+
Acid formation from glucose	w	−	−	n

+ positive, − negative, *l* delayed positive, *w* weak, *v* variable, *n* no data

[a] Data from Nakase et al. (2011)

[b] Data from Wang et al. (2008)

Technology and Evaluation, Chiba, Japan, as NBRC 108902^T and Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands as CBS 12574^T.

Etymology

The species epithet *lopburiensis* refers to Lop Buri province, Thailand, where the type strain was isolated.

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ORIGINAL PAPER

Yamadazyma siamensis sp. nov., *Yamadazyma phyllophila* sp. nov. and *Yamadazyma paraphyllophila* sp. nov., three novel yeast species isolated from phylloplane in Thailand and Taiwan

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Abstract Four strains representing three novel anamorphic yeast species were isolated from the external surface of sugarcane leaves (DMKU-RK254^T), corn leaves (DMKU-RK548^T), bean leaves (K129) in Thailand and hengchun pencilwood leaves (TrB1-1^T) in Taiwan. On the basis of morphological, biochemical, physiological and chemotaxonomic characteristics, the sequence analysis of the D1/D2 region of the large subunit (LSU) rRNA gene, the internal transcribed spacer (ITS) region, the actin gene (ACT1) and the elongation factor 2 gene (EF2), the four strains were determined to represent novel *Yamadazyma* species although formation of ascospores was not observed. Strain DMKU-RK254^T was determined to be related to *Candida diddensiae*, *Candida naeodendra* and *Candida kanchanaburiensis* but with 1.8, 1.8 and 2.0 % nucleotide substitutions in the D1/D2 region of the LSU rRNA gene, respectively. It was assigned to *Yamadazyma siamensis* sp. nov. (type strain DMKU-RK254^T = BCC 50730^T = NBRC 108901^T = CBS 12573^T). The sequences of the D1/D2 region of the LSU rRNA gene, the ITS region, ACT1 gene and EF2 gene of two strains (DMKU-RK548^T and K129) were identical but differed from that of strain TrB1-1^T by 0.6, 1.0, 3.3 and 5.9 % nucleotide substitutions, respectively. Therefore, the two strains (DMKU-RK548^T and K129) and strain TrB1-1^T were assigned to be two separate species. The closest species in terms of pairwise sequences similarity of the D1/D2 region to the two novel species was *Yamadazyma philogaea* but with 1.1–1.7 % nucleotide substitutions. The two strains (DMKU-RK548^T and K129) were assigned to *Yamadazyma phyllophila* sp. nov. (type strain DMKU-RK548^T = BCC 50736^T = NBRC 108906^T = CBS 12572^T) and the strain TrB1-1^T was named *Yamadazyma paraphyllophila* sp. nov. (type strain TrB1-1^T = BCRC 23030^T = CCTCC AY 204005^T = CBS 9928^T).

Keywords *Yamadazyma siamensis* sp. nov. · *Yamadazyma phyllophila* sp. nov. · *Yamadazyma paraphyllophila* sp. nov. · Phylloplane yeast · Ascomycete yeast · New yeast species · Thailand · Taiwan

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Introduction

The genus *Yamadazyma* was described by Billon-Grand (1989) to accommodate species that had previously been assigned to the genus *Pichia* and that formed CoQ-9 as their major ubiquinone and formed hat-shaped ascospores (Kurtzman and Robnett 1998). Analysis of the D1/D2 region of the large subunit (LSU) rRNA gene by Kurtzman and Robnett (1998) showed that species assigned to the genus *Yamadazyma* are placed in several clade. Later some of the species that had been assigned to this genus by Billon-Grand (1989) were transferred to the newly described genera *Babjeviella*, *Meyerozyma*, *Millerozyma* and *Priceomyces* based on phylogenetic analysis of the D1/D2 region of the LSU rRNA gene and the nearly complete small subunit rRNA gene (Kurtzman and Suzuki 2010). Then after the genus *Yamadazyma* became a well-support clade (Kurtzman and Suzuki 2010) and a generally accepted genus in the Family Debaryomycetaceae of Order Saccharomycetales (Kurtzman 2011) in The Yeast, a taxonomic study 5th edition *Y. philogaea*, the type species, as well as *Y. akitaensis*, *Y. mexicana*, *Y. nakazawae*, *Y. scolyti*, *Y. triangularis* and 23 *Candida* species are placed in the *Yamadazyma* clade (Kurtzman 2011; Lachance et al. 2011). More recently, a few novel *Candida* species in this clade have been described i.e. *Candida kanchanaburiensis*, which was isolated from the fruit body of a mushroom and rotted fruit of guava collected in the western Thailand (Nakase et al. 2008), *C. khaothaluensis*, which was isolated from unidentified tree in Thailand, *C. vaughaniae* and *C. tallmaniae*, which were isolated from flowers of unidentified plants in French Guiana (Groenewald et al. 2011), and *C. oceani*, which was obtained from an unidentified deep-sea coral, water samples and the stomach of a marine fish on the Mid-Atlantic Ridge (Burgaud et al. 2011).

The external surface of plant leaves is usually referred to as the phylloplane, or phyllosphere (Fonseca and Inacio 2006; Phaff and Starmer 1987). The phylloplane has been found to be colonized by members of both basidiomycete and ascomycete yeasts (Fonseca and Inacio 2006; Glushakova et al. 2007; Landell et al. 2010; Nakase et al. 2001; Slavikova et al. 2009). Although most common phylloplane yeasts are yeast species in basidiomycete genera such as *Cryptococcus*, *Rhodotorula*, *Sporobolomyces* and *Trichosporon* (de Azeredo et al. 1998; Fonseca and Inacio 2006; Glushakova and Chernov 2010; Nakase et al. 2001; Slavikova et al. 2009), various ascomycete yeast species have also been detected on the phylloplane i.e. *Debaryomyces hansenii*, *Hanseniaspora uvarum, Kazachstania barnetii*, *Metschnikowia lopburiensis, Metschnikowia pulcherrima*, *Metschnikowia saccharicola, Pichia membranifaciens*, *Saccharomyces cerevisiae* and various *Candida* species including *Candida aechmeae*, *Candida olephila*, *Candida chumphonensis, Candida mattranensis* and *Candida vrieseae* (Glushakova et al. 2007; Glushakova and Chernov 2010; Kaewwichian et al. 2012; Koowadjanakul et al. 2011; Landell et al. 2010; Slavikova et al. 2009).

During an investigation of yeasts on the external leaf surfaces of plants in Thailand and Taiwan, four strains representing three novel species of *Yamadazyma* were isolated from the phylloplane of sugarcane, corn, bean and hengchun pencilwood. In this paper, one strain (DMKU-RK254^T) is described as *Yamadazyma siamensis* sp. nov., two strains (DMKU-RK548^{T}and K129) are described as *Yamadazyma phyllophila* sp. nov. and one strain (TrB1-1^T) is described as *Yamadazyma paraphyllophila* sp. nov.

Materials and methods

Yeast isolation

Strain DMKU-RK254^T, DMKU-RK548^T and K129 were isolated from leaf surfaces of sugarcane, corn and bean collected in Thailand (Table 1) by an enrichment technique using acidified yeast extract malt extract (YM) broth (0.3 % yeast extract, 0.3 % malt extract, 0.5 % peptone and 1 % glucose) supplemented with 0.025 % sodium propionate and 0.02 % chloramphenicol (Limtong et al. 2007). 3 g of cut leaves were aseptically placed in 250 ml Erlenmeyer flask, containing 50 ml of acidified YM broth and incubated on a rotary shaker at room temperature (27 ± 3 °C) for 3 days. The enrichment culture was then spread on YM agar supplemented with 0.025 % sodium propionate and 0.02 % chloramphenicol and incubated at room temperature until yeast colonies appeared. Yeast colonies of different morphologies were picked and purified by cross streaking on YM agar. Strain TrB1-1^T

Table 1 Strains of *Yamadazyma siamensis* sp. nov., *Yamadazyma phyllophila* sp. nov. and *Yamadazyma paraphyllophila* sp. nov. isolated from surface of plant leaves used in this study

Strain	Accession number	GenBank accession number				Sample for isolation		
		D1/D2	ITS	ACT1	EF2	Leaves	ocality	Date
Y. siamensis sp. nov.								
DMKU-RK254^T	BCC 50730^T NBRC 108901^T CBS 12573^T	AB734046	AB734049	–	–	Sugarcane (*Saccharum officinarum*)	Bueng Sam Phan district, Phetchabun province, Thailand	23 Mar 2010
Y. phyllophila sp. nov.								
DMKU-RK548^T	BCC 50736^T NBRC 108906^T CBS 12572^T	AB734047	AB734050	AB761313	AB761316	Corn (*Zea mays*)	Pak Chong district, Nakhon Ratchasima province, Thailand	6 May 2010
K129	–	AB734048	AB734051	AB761312	AB761315	Bean (*Phaseolus vulgaris*)	Kanjanaburi province, Thailand	15 Nov 2009
Y. paraphyllophila sp. nov.								
TrB1-1^T	BCRC 23030^T CCTCC AY 204005^T CBS 9928^T	AY562397	AY559447	AB761314	AB761317	Hengchun pencilwood (*Dysoxylum hongkongense*)	NanJen-Shan Natural Reserve on Pindung, Taiwan	2004

BCC BIOTEC Culture Collection, *NBRC* NITE Biological Resources Center, *CBS* Centraalbureau voor Schimmelcultures, *BCRC* Bioresource Collection and Research Center, *CCTCC* China Center for Type Culture Collection

was isolated from hengchun pencilwood leaves collected in Taiwan (Table 1) according to the procedures described by Rosa et al. (1995) with slight modification. Leaves were collected in clean polyethylene plastic bags, transported to the laboratory and washed with 30–40 ml of sterile distilled water, vortexed for 30 min, an aliquot of 0.1 ml was spread on acidified YM agar supplemented with 0.034 % chloramphenicol adjusted to pH 3.7 by 1 N HCl and incubated at 26 °C. After 2 days, yeast colonies were picked and purified. Purified yeast strains were suspended in YM broth supplemented with 10 % glycerol and maintained at −80 °C.

DNA sequencing and phylogenetic analysis

The sequences of the D1/D2 region of the LSU rRNA gene, the internal transcribed spacer (ITS) region, the actin gene (ACT1) and the elongation factor 2 gene (EF2) were determined from PCR products amplified from genomic DNA. The D1/D2 region of the LSU rRNA gene, the ITS region, the ACT1 gene and EF2 gene were amplified and sequenced with primers, NL1 and NL4, ITS1 and ITS4 or ITS5 and ITS4, ACT1 and ACT2, and EFIIF1 and EFIIR2 (Table 2). Methods for DNA extraction and amplification of the D1/D2 region of the LSU rRNA gene, ITS region, and ACT1 gene and EF2 gene of strain DMKU-RK548^T, K129 and TrB1-1^T were as described previously (Limtong et al. 2007). The PCR products were checked by agarose gel electrophoresis and purified by using the QIAquick purification kit (Qiagen, Germany). The purified products were submitted to Macrogen Inc. (Korea) for sequencing. The DNA was extracted for the amplification of the D1/D2 region of the LSU rRNA gene and the ITS region of strain TrB1-1^T by using a variation of a CTAB method (Doyle and Doyle 1990). Dichloromethane was substituted for chloroform in the extraction procedures. Yeast cells were suspended in 500 µl of CTAB extraction buffer [1 ± 4 M NaCl, 100 mM Tris–HCl, pH 8 ± 0, 20 mM EDTA, 2 % w/v PVP-40, 20 mM cetyl trimethyl ammonium bromide (CTAB), and 0 ± 5 % v/v 2-mercaptoethanol] in an eppendorf and placed in a dry bath at 95 °C for 30 min and then in a water bath at 60 °C for 30 min with occasional gentle mixing. An equal volume of dichloromethane:isoamyl alcohol (24:1) was added, mixed by gentle inversion and centrifuged at 1,500×*g* for 4 min at 20 °C. The upper aqueous phase was recovered and DNA was precipitated by the addition of 0.6 volume of isopropanol. The tube was inverted gently and then centrifuged at 460×*g* for 2 min. The DNA pellet was rinsed with 500 µl wash buffer (76 % ethanol and 10 mM ammonium acetate) for 2 min, and rinsed again with wash buffer. The tube was spun at 1,500×*g* for 10 min and the supernatant was decanted. The tube was inverted and air-dried for 10–30 min and then the pellet was resuspended in 20 µl SDW at 37 °C for 30 min. DNA was quantified with a fluorimeter, according to the manufacturer's instructions, and stored at −20 °C. The PCR product was sequenced according to the manufacturer's instructions (ABI prism 3100 Genetic Analyzer, PE Applied Biosystems, USA). The sequences were compared pairwise using a BLAST search (Altschul et al. 1997) and were aligned with the sequences of related species retrieved from GenBank using the multiple alignment program CLUSTAL_X version 1.81 (Thompson et al. 1997).

Table 2 Primers used for PCR amplification and sequencing

Region/gene	Primer	Sequence	Reference
D1/D2	NL1 (forward)	5′ GCATATCAATAAGCGGAGGAAAAG 3′	Kurtzman and Robnett (1998)
	NL4 (reverse)	5′ GGTCCGTGTTTCAAGACGG 3′	
ITS	ITS1 (forward)	5′ TCCGTAGGTGAACCTGCGG 3′	White et al. (1990)
	ITS5 (forward)	5′ GGAAGTAAAAGTCGTAACAACG 3′	
	ITS4 (reverse)	5′ TCCTCCGCTTATTGATATGC 3′	
ACT1	ACT1 (forward)	5′ TACCCAATTGAACACGGTAT 3′	Diezmann et al. (2004)
	ACT2 (reverse)	5′ TCTGAATCTTTCGTTACCAAT 3′	
EF2	EFIIF1 (forward)	5′ AAGTCTCCAAACAAAGCATAAC 3′	Diezmann et al. (2004)
	EFIIR2 (reverse)	5′ GGGAAAGCTTGACCACCAGTAGC 3′	

ITS5 (forward primer) was used for strain TrB1-1

A phylogenetic tree was constructed from the evolutionary distance data with Kimura's two-parameter correction (Kimura 1980), using the neighbor joining method (Saitou and Nei 1987) by MEGA software version 4.0 (Tamura et al. 2007). Confidence levels of the clades were estimated from bootstrap analysis (1,000 replicates) (Felsenstein 1985).

Examination of taxonomic characteristics

The strains were characterized morphologically, biochemically, and physiologically according to the standard methods described by Yarrow (1998). Mycelium formation was investigated on potato dextrose agar (PDA) in slide culture at 25 °C for up to 7 days. Ascospores formation was investigated individually or in pairs on 5 % malt extract agar, McClary's acetate agar, Gorodkowa agar, corn meal agar, V8 agar and yeast carbon base supplemented with 0.01 % ammonium sulfate (YCBAS) agar at 15 and 25 °C for up to 4 weeks. Carbon assimilation tests were conducted in liquid medium according to the method described by Yarrow (1998). Assimilation of nitrogen compounds was examined on solid media with starved inocula following the method of Nakase and Suzuki (1986). Growth at various temperatures was determined by cultivation in YM broth. Ubiquinones were extracted from cells cultivated in a 500 ml Erlenmeyer flask containing 250 ml of yeast extract peptone dextrose (YPD) broth (1 % yeast extract, 2 % peptone and 2 % dextrose) on a rotary shaker at 28 °C for 24–48 h and purified according to the method described by Yamada and Kondo (1973) and Kuraishi et al. (1985). Isoprenologues were identified by HPLC as described previously (Limtong et al. 2007). The DNA base composition was determined by HPLC as described by Tamaoka and Komagata (1984) using the DNA-GC kit (Yamasa Shoyu Co., Japan).

Results and discussion

Novel species delineation and identification

Analysis of the D1/D2 region of the LSU rRNA gene sequence revealed that the sequence of strain DMKU-RK254^T was closely related to *Candida diddensiae* and *Candida naeodendra* with 1.8 % nucleotide substitutions (9 nucleotide substitutions out of 493 nt) and to *Candida kanchanaburiensis* with 2.0 % nucleotide substitutions (10 nucleotide substitutions out of 493 nt). The sequences of the ITS region of strain DMKU-RK254^T differed by 6.6 % nucleotide substitutions (36 nucleotide substitutions and 13 gaps out of 545 nt) from *C. diddensiae* by 6.6 % nucleotide substitutions (39 nucleotide substitutions and 30 gaps out of 589 nt) from *C. naeodendra* and by 5.8 % nucleotide substitutions (34 nucleotide substitutions and 11 gaps out of 585 nt) from *C. kanchanaburiensis*.

The D1/D2 region of the LSU rRNA gene sequences of the two strains (DMKU-RK548^T and K129) were identical and differed by three nucleotide substitutions from strain TrB1-1^T. The closest known species to the three strains was *Yamadazyma philogaea*; the two strains (DMKU-RK548^T and K129) differed by 1.1 % nucleotide substitutions (6 nucleotide substitutions out of 530 nt) while strain TrB1-1^T differed by 1.7 % nucleotide substitutions (9 nucleotide substitutions out of 530 nt). The sequences of ITS region of the two strains (DMKU-RK548^T and K129) were identical and differed from strain TrB1-1^T by six nucleotide substitutions and one gap out of 582 nt. The two strains (DMKU-RK548^{T}and K129) differed by 5.2 % nucleotide substitutions (30 nucleotide substitutions and five gaps out of 582 nt) while strain TrB1-1^T differed by 5.5 % nucleotide substitutions (32 nucleotide substitutions and 6 gaps out of 582 nt) from *Y. philogaea*. Moreover, the sequences of the ACT1 gene and the EF2 gene of the two strains (DMKU-RK548^T and K129) were identical and differed from strain TrB1-1^T by 17 nucleotide substitutions out of 523 nt (3.3 % nucleotide substitutions) and 36 nucleotide substitutions out of 606 nt (5.9 % nucleotide substitutions), respectively. The difference in the ACT1 and EF2 genes of the two strains (DMKU-RK548^T and K129) from strain TrB1-1^T is high enough to justify them as two separate species as report for the ACT1 gene in *Clavispora* spp. by Lachance et al. (2003) and for EF2 gene in *Wickerhamomyces* spp. by Imanishi et al. (2009).

The phylogenetic tree based on the sequences of the D1/D2 region of the LSU rRNA gene further demonstrated that the four strains of the three novel species are in *Yamadazyma* clade. Strain DMKU-RK254^T connects to *Candida kanchanaburiensis* and is placed in the same subclade that contains *C. naeodendra*, *C. diddensiae* and the three strains of another two novel species (Fig. 1). Strains DMKU-RK548^T and

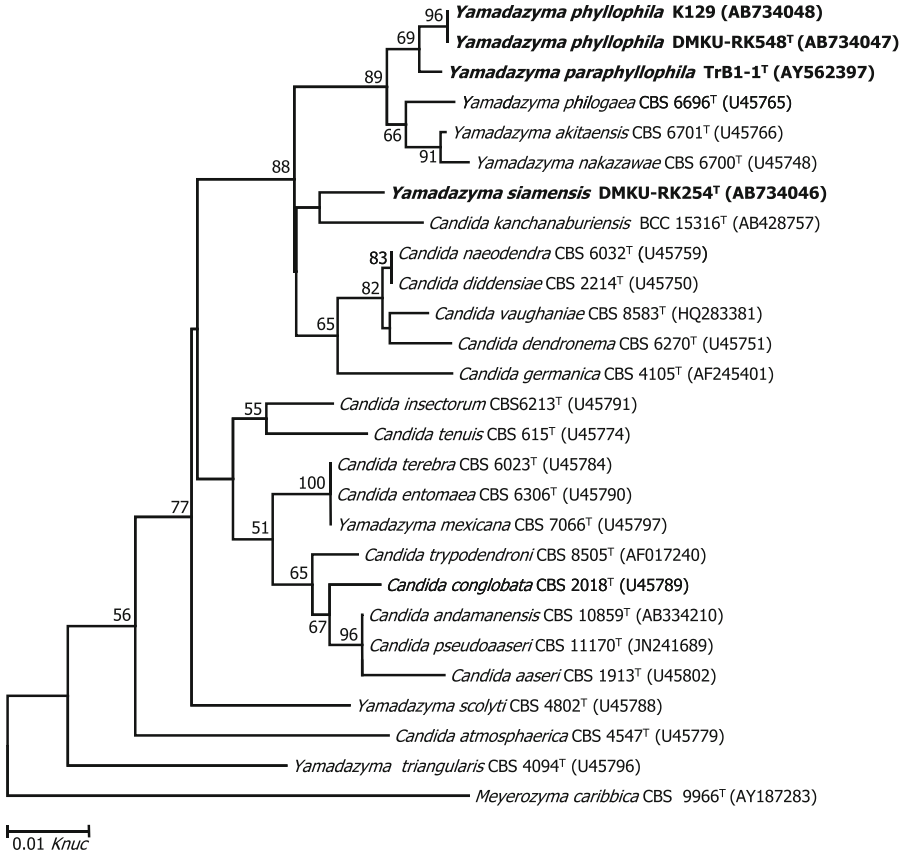


Fig. 1 Phylogenetic tree based on the sequences of the D1/D2 region of the LSU rRNA gene, showing positions of *Yamadazyma siamensis* sp. nov. (DMKU-RK254^T), *Yamadazyma phyllophila* sp. nov. (DMKU-RK548^T and K129) and *Yamadazyma paraphyllophila* sp. nov. (TrB1-1^T), with respect to closely related species. The phylogenetic tree was constructed from evolutionary distance data with Kimura's two-parameter correction, using the neighbor joining method by MEGA software version 4.0. Numbers indicate percentages of bootstrap sampling, derived from 1,000 samples. The *numbers* in *parentheses* are GenBank accession numbers. *Meyerozyma caribbica* was the outgroup in the analysis

K129 are located in the same position and connect to strain TrB1-1^T. They cluster with *Yamadazyma philogaea*, the closest known species, and *Yamadazyma nakazawae* and *Yamadazyma akitaensis* with high bootstrap support (Fig. 1).

On the basis of morphological, biochemical, physiological, chemotaxonomic characteristics, and the sequence analysis of the D1/D2 region of the LSU rRNA gene, the ITS region, the ACT1 gene and the EF2 gene, we concluded that the four strains represent three novel *Yamadazyma* species although formation of ascospores was not observed. The name *Yamadazyma siamensis* sp. nov. (MB801056) is proposed for the strain DMKU-RK254^T, the name *Yamadazyma phyllophila* sp. nov. (MB801057) is assigned for the two strains (DMKU-RK548^{T}and K129) and the name *Yamadazyma paraphyllophila* sp. nov. (MB802274) is proposed for the strain TrB1-1^T.

In practice, *Yamadazyma siamensis* sp. nov., *Y. phyllophila* sp. nov. and *Y. paraphyllophila* sp. nov. can be distinguished from their close related species *C. diddensiae*, *C. naeodendra* and *C. kanchanaburiensis*, and *Y. philogaea*, respectively, not only on the basis of the sequences of the D1/D2 region of the LSU rRNA gene and the ITS region but also several phenotypic characteristics as shown in Table 3. *Y. phyllophila* sp. nov. can be distinguished from *Y. paraphyllophila* sp. nov. only by the different in genotypic characteristics while the phenotypic characteristics were the same.

Most of the members of the *Yamadazyma* clade such as *C. michaelii*, *C. gorgasii*, *C. lessepsii*, *C. cerambycidarum*, C. *endomychidarum*, *C. amphixiae*, *C. diddensiae*, *C. naeodendra* and *C. dendronema* exhibit an association with the gut of beetles and other insects (Lachance et al. 2011). However, some strains of the recognized species were reported to have been isolated from plants i.e. a strain of *Yamadazyma akitaensis* (CBS 6701^T) that was isolated from exudate of a willow, two strains of *Yamadazyma mexicana* (CBS 7066^T and CBS 7067) that were isolated from cactus, *Candida diospyri* (CBS 9769^T) that was isolated from a kaki fruit and *Candida buinensis*

Table 3 Phenotypic characteristics that differentiate *Yamadazyma siamensis* sp. nov., *Yamadazyma phyllophila* sp. nov. and *Yamadazyma paraphyllophila* sp. nov. from their closest species, *Candida diddensiae*, *C. naeodendra*, *C. kanchanaburiensis* and *Yamadazyma philogaea*, respectively

Characteristics	Species						
	1	2[a]	3[b]	4[c]	5	6	7[d]
Fermentation							
Glucose	+	+	+	w	+	+	+
Galactose	w	+	s	w	+	+	w
Sucrose	−	v	−	−	−	−	−
Maltose	−	v	−	−	−	−	v
Lactose	−	−	−	n	−	−	−
Raffinose	−	−	−	n	−	−	−
Trehalose	−	+	+	−	−	−	+
Cellobiose	+	n	n	−	−	−	n
Xylose	−	n	n	−	−	−	n
Assimilation of carbon/nitrogen compounds							
D-Glucose	+	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+	+
L-Sorbose	+	v	s	l	−	−	+
Sucrose	+	+	−	+	+	+	+
Maltose	+	+	−	+	+	+	+
Cellobiose	+	+	+	+	+	+	+
Trehalose	+	v	+	+	+	+	+
Lactose	−	−	−	−	+	+	−
Melibiose	−	−	−	−	−	−	−
Raffinose	−	−	−	−	−	−	−
Melezitose	+	+	+	+	+	+	+
Inulin	−	−	−	−	−	−	−
Soluble starch	−	−	+	−	−	−	−
D-Xylose	+	+	+	+	+	+	+
L-Arabinose	+	v	+	+	w	w	+
D-Arabinose	s	v	s	v	−	−	+
D-Ribose	+	v	+	v	−	−	+
L-Rhamnose	+	−	s	l	+	+	−
Ethanol	+	+	+	+	+	+	+
Glycerol	+	+	+	+	+	+	+
Erythritol	+	+	+	+	+	+	+
Ribitol	+	+	+	+	+	+	+
Galactitol	−	−	−	−	−	−	−
D-Mannitol	+	+	+	+	+	+	+
D-Glucitol	+	+	+	+	+	+	+
α-Methyl-D-glucoside	+	v	+	+	+	+	+
Salicin	+	v	+	+	+	+	−
DL-Lactic acid	w	−	−	−	−	−	−
Succinic acid	+	v	+	+	+	+	+
Citric acid	+	v	+	+	−	−	+
Inositol	−	−	−	−	−	−	−
Arbutin	+	n	n	+	−	−	n
D-Gluconic acid	+	+	+	+	+	+	+
D-Glucosamine	+	v	+	s	−	−	+
N-Acetyl-D-glucosamine	+	+	+	+	+	+	+
2-Keto-D-gluconic acid	−	v	−	−	−	−	+
5-Keto-D-gluconic acid	−	n	n	−	−	−	−
Hexeadecane	−	w	+	−	−	−	+
Methanol	−	−	−	−	−	−	−
Propane-1,2-diol	−	n	n	l	−	−	n
Butane-2,3-diol	−	n	n	−	−	−	n
D-Glucono-δ-lactone	−	+	−	−	w	w	n
Xylitol	+	v	+	+	+	+	n
D-Glucuronic acid	−	−	−	−	−	−	n
D-Galacturonic acid	−	n	n	−	−	−	n
Arabinitol	+	n	n	+	−	−	n
Nitrate	−	−	−	−	−	−	−
Nitrite	−	−	−	−	−	−	n
Ethylamine	+	+	+	+	+	+	n
L-Lysine	+	+	+	w	+	+	+
Cadaverine	+	+	+	+	+	+	n
Other growth characteristics							
Vitamin free medium	w	−	−	n	−	−	−
50 % Glucose	+	v	+	n	+	+	n
60 % Glucose	+	n	n	n	+	+	n
10 % NaCl + 5 % glucose	+	+	−	v	w	w	+
16 % NaCl + 5 % glucose	−	n	n	n	−	−	n
0.01 % Cycloheximide	w	−	−	n	−	−	−
0.1 % Cycloheximide	−	−	−	−	−	−	n
Growth at 25 °C	+	+	+	+	+	+	+
Growth at 30 °C	+	+	+	+	+	+	+
Growth at 35 °C	+	+	+	+	+	+	+
Growth at 37 °C	+	+	+	+	+	+	w
Growth at 40 °C	+	n	n	n	+	+	n
Growth at 42 °C	−	n	n	n	−	−	n
Growth at 45 °C	−	n	n	n	−	−	n
Starch production	−	−	−	−	−	−	−

Table 3 continued

Characteristics	Species						
	1	2[a]	3[b]	4[c]	5	6	7[d]
Diazonium blue B	–	–	–	n	–	–	–
Urease	–	n	n	n	–	–	n
Major ubiquinone	Q9	Q9	Q9	Q9	Q9	Q9	Q9

+, positive; −, negative; l, latent positive; s, slow positive; w, weakly positive; v, variable; n, no data

1 Yamadazyma siamensis sp. nov., *2 Candida diddensiae*, *3 C. naeodendra*, *4 C. kanchanaburiensis*, *5 Y. phyllophila* sp. nov., *6 Y. paraphyllophila* sp. nov., *7 Y. philogaea*

[a,b] Data from Lachance et al. (2011)

[c] Data from Nakase et al. (2008)

[d] Data from Kurtzman (2011)

(CBS 6796^T) that was isolated from the gelatinous exudate of a tree fern (Kurtzman 2011; Lachance et al. 2011). In Thailand few *Candida* species in the *Yamadazyma* clade have been isolated i.e. *Candida khaothaluensis*, which was isolated from an unidentified tree (Groenewald et al. 2011) and *Candida kanchanaburiensis*, which was isolated from a fruit body of a unidentified mushroom (*Coprinus* sp.) and a rotted guava fruit (Nakase et al. 2008). Until the present, no members of this clade have been reported to have been isolated from the phylloplane. In this study, four strains of the two novel *Yamadazyma* species are proposed, but from limited number of strains derived in this study, we could not conclude that the phylloplane is one of the habitats of yeast in this clade. However, the phylloplane is interesting as the subject for further investigation of yeast in this clade.

Description of *Yamadazyma siamensis* Kaewwichian, Yongmanitchai, Kawasaki, Wang, Yang & Limtong sp. nov.

Growth in yeast extract malt extract (YM) broth: After 3 days at 25 °C, cells are globose, subglobose to ovoid (2–7 × 3–8 µm) and occur singly, in pairs or in groups (Fig. 2a). Budding is multilateral. Growth on YM agar: after 3 days at 25 °C, the streak culture is grayish white, smooth, soft to butyrous and has an entire margin. Pseudohyphae are formed but true hyphae are not formed in slide culture on PDA after 3 days at 25 °C (Fig. 2b). Ascospores were not produced on YM agar, YPD agar, 5 % malt extract agar, McClary's acetate agar, corn meal agar, Gorodkowa agar, V8 agar and YCBAS agar after 4 weeks

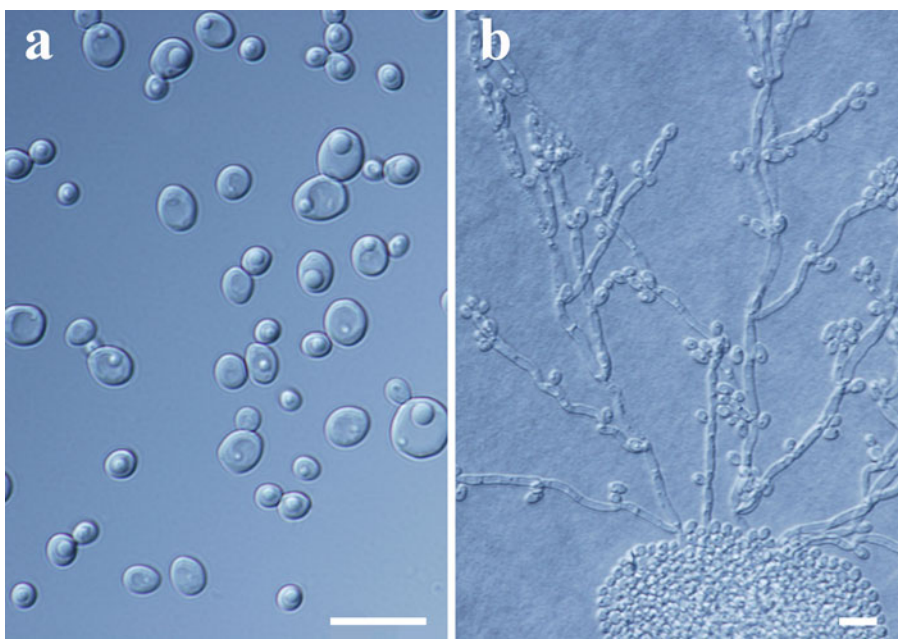


Fig. 2 *Yamadazyma siamensis* sp. nov. (DMKU-RK254^T) **a** budding cells in YM broth after 3 days at 25 °C **b** pseudohyphae formed on PDA after 3 days at 25 °C. *Scale bar*, 10 µm

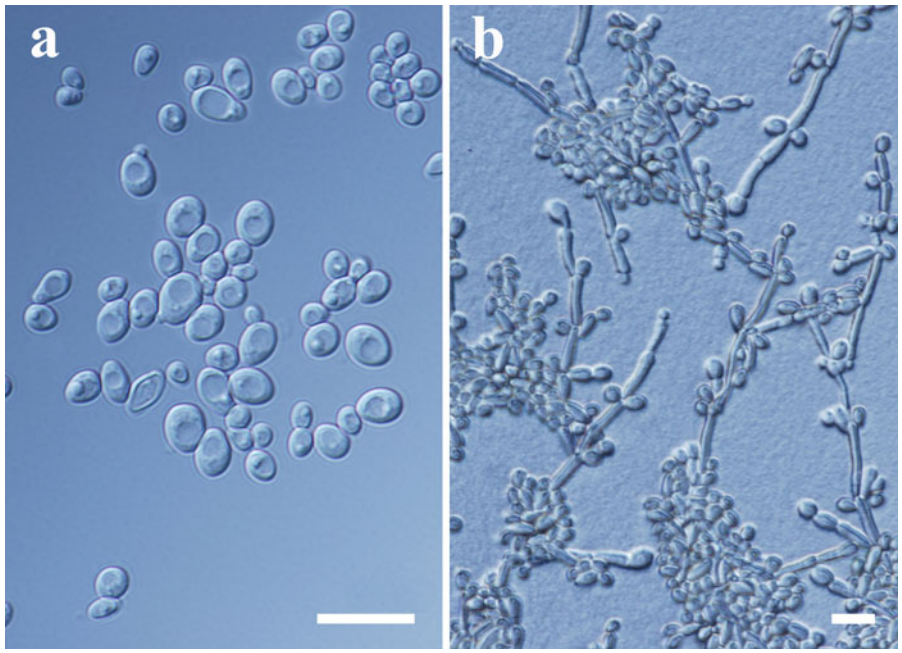


Fig. 3 *Yamadazyma phyllophila* sp. nov. (DMKU-RK548^T) **a** budding cells in YM broth after 3 days at 25 °C **b** pseudohyphae formed on PDA after 3 days at 25 °C. *Scale bar*, 10 μm

at 15 and 25 °C. DNA G+C content is 46.2 mol %. The other phenotypic characteristics of the species are shown in Table 3.

Holotype

DMKU-RK254^T is the holotype of *Yamadazyma siamensis* (MB801056). The strain was isolated from phylloplane of sugarcane (*Saccharum officinarum*) collected from Phetchabun province, Thailand. The living culture from type was deposited at the BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathumthani, Thailand, as BCC 50730^T; NITE Biological Resources Center (NBRC), Department of Biotechnology, National Institute of Technology and Evaluation, Chiba, Japan, as NBRC 108901^T and CBS-KNAW Fungal Biodiversity Center (CBS), Utrecht, The Netherlands as CBS 12573^T.

Etymology

The species epithet *siamensis* refers to Siam the old name of Thailand, where the strain was isolated.

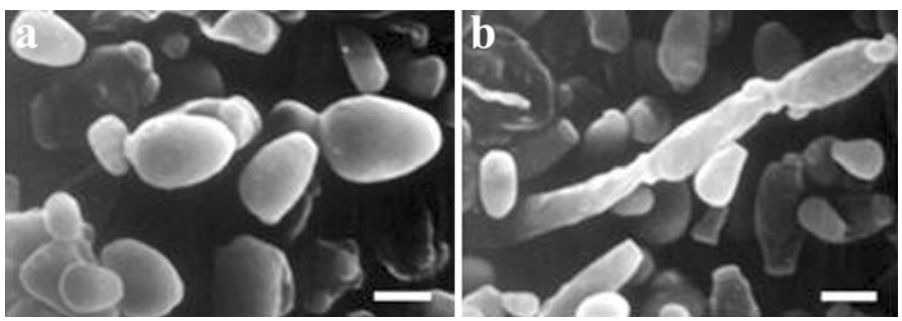


Fig. 4 *Yamadazyma paraphyllophila* sp. nov. (TrB1-1^T) **a** budding cells in YM broth after 3 days at 25 °C **b** pseudohyphae formed on PDA after 3 days at 25 °C. *Scale bar*, 1 μm. Scanning electron microscopy was prepared according to the procedures described by Lee et al. (1994)

Description of *Yamadazyma phyllophila* Kaewwichian, Yongmanitchai, Kawasaki, Wang, Yang & Limtong sp. nov.

Growth in yeast extract malt extract (YM) broth: after 3 days at 25 °C, cells are ovoid to ellipsoid (2–4 × 3–6 μm) and occur singly, in pairs or in groups (Fig. 3a). Budding is multilateral. Growth on YM agar after 3 days at 25 °C, the streak culture is white, smooth and has an entire margin. Pseudohyphae are formed but true hyphae are not formed in slide culture on PDA after 3 days at 25 °C (Fig. 3b). Ascospores were not produced by any of the strains either individually or when paired on YM agar, YPD agar, 5 % malt extract agar, McClary's acetate agar, corn meal agar, Gorodkowa agar, V8 agar and YCBAS agar after 4 weeks at 15 and 25 °C. DNA G+C content is 38.7 mol %. The other phenotypic characteristics of the species are shown in Table 3.

Holotype

DMKU-RK548^T is the holotype of *Yamadazyma phyllophila* sp. nov. (MB801057). The strain was isolated from phylloplane of corn (*Zea mays*) collected from Nakhon Ratchasima province, Thailand. The living culture from type was deposited at the BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathumthani, Thailand, as BCC 50736^T; NITE Biological Resources Center (NBRC), Department of Biotechnology, National Institute of Technology and Evaluation, Chiba, Japan, as NBRC 108906^T and CBS-KNAW Fungal Biodiversity Center (CBS), Utrecht, The Netherlands as CBS 12572^T.

Etymology

The species epithet *phyllophila* refers to phylloplane where the three strains of this species were isolated.

Description of *Yamadazyma paraphyllophila* Wang, Yang, Kaewwichian, Yongmanitchai, Kawasaki & Limtong sp. nov.

Growth in yeast extract malt extract (YM) broth: after 3 days at 25 °C, cells are ovoid to ellipsoid (2–3 × 3–5 μm) and occur singly, in pairs or in groups (Fig. 4a). Budding is multilateral. Growth on YM agar after 3 days at 25 °C, the streak culture is white, smooth and has an entire margin. Pseudohyphae are formed but true hyphae are not formed in slide culture on PDA after 3 days at 25 °C (Fig. 4b). Ascospores were not produced on YM agar, YPD agar, 5 % malt extract agar, McClary's acetate agar, corn meal agar, Gorodkowa agar, V8 agar and YCBAS agar after 4 weeks at 15 and 25 °C. The other phenotypic characteristics of the species are shown in Table 3.

Holotype

TrB1-1^T is the holotype of *Yamadazyma paraphyllophila* sp. nov. (MB802274). The strain was isolated from phylloplane of hengchun pencilwood (*Dysoxylum hongkongense*) collected from NanJen-Shan Natural Reserve on Pindung, Taiwan. The living culture from type was deposited at the Bioresource Collection and Research Center (BCRC), Hsinchu, Taiwan R.O.C., as BCRC 23030^T; China Center for Type Culture Collection, Wuhan, Hubei, China, as CCTCC AY 204005^T and CBS-KNAW Fungal Biodiversity Center (CBS), Utrecht, The Netherlands as CBS 9928^T.

Etymology

The species epithet *paraphyllophila* indicating affinity with *phyllophila* species.

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Wickerhamomyces siamensis sp. nov., a novel yeast species isolated from the phylloplane in Thailand

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Strain DMKU-RK359^T, representing a novel yeast species, was isolated from the external surface of a sugar-cane leaf collected in Thailand. On the basis of morphological, biochemical, physiological and chemotaxonomic characteristics, sequence analysis of the D1/D2 region of the large-subunit (LSU) rRNA gene and the internal transcribed spacer (ITS) region, strain DMKU-RK359^T was assigned to a novel *Wickerhamomyces* species. The novel species was closest to *Wickerhamomyces ciferrii*, but differed from it by 0.7 % nucleotide substitutions in the D1/D2 region of the LSU rRNA gene and 6 % nucleotide substitutions in the ITS region. The name *Wickerhamomyces siamensis* sp. nov. is proposed (type strain DMKU-RK359^T =BCC 50732^T =NBRC 108900^T =CBS 12570^T).

The genus *Wickerhamomyces* was proposed to accommodate 17 species that were transferred from the genera *Pichia*, *Williopsis* and *Hansenula* as a result of phylogenetic analysis of nucleotide divergence in the genes coding for the large-subunit (LSU) and small-subunit rRNAs and for elongation factor 1α (Kurtzman *et al.*, 2008; Kurtzman, 2011). The number of *Wickerhamomyces* species was subsequently increased by the discovery of *Wickerhamomyces edaphicus* (Limtong *et al.*, 2009), *W. queroliae* (Rosa *et al.*, 2009), *W. patagonicus* (de García *et al.*, 2010), *W. chaumierensis* (Groenewald *et al.*, 2011), *W. ochangensis* (Shin *et al.*, 2011), *W. tratensis* (Nakase *et al.*, 2012) and *W. xylosica* (Limtong *et al.*, 2012).

The phylloplane, the external surface of the plant leaf, has been recognized as an important habitat for epiphytic micro-organisms (Phaff & Starmer, 1987; Fonseca & Inácio, 2006). Both basidiomycete and ascomycete yeasts have been found to be phylloplane colonizers (Nakase *et al.*, 2001; Fonseca & Inácio, 2006; Glushakova *et al.*, 2007; Slavikova *et al.*, 2009; Landell *et al.* 2010). Although most common phylloplane yeasts are members of basidiomycete genera such as *Cryptococcus*, *Rhodotorula*, *Sporobolomyces* and *Trichosporon* (de Azeredo *et al.*, 1998; Nakase *et al.*, 2001; Fonseca & Inácio, 2006; Slavikova *et al.*, 2009; Glushakova & Chernov, 2010), various ascomycete yeast species have also been reported, e.g. *Debaryomyces hansenii*, *Hanseniaspora uvarum*, *Kazachstania barnetii*, *Metschnikowia lopburiensis*, *M. pulcherrima*, *M. saccharicola*, *Pichia membranifaciens*, *Saccharomyces cerevisiae* and various *Candida* species including *Candida aechmeae*, *C. olephila*, *C. chumphonensis*, *C. mattranensis* and *C. vrieseae* (Glushakova *et al.*, 2007; Slavikova *et al.*, 2009; Glushakova & Chernov, 2010; Landell *et al.*, 2010; Kaewwichian *et al.*, 2012; Koowadjanakul *et al.*, 2011).

During an investigation of yeasts on the external leaf surfaces of sugar cane in Thailand, strain DMKU-RK359^T, representing a novel species of *Wickerhamomyces*, was obtained. In this paper, it is described as the type strain of *Wickerhamomyces siamensis* sp. nov.

One hundred and sixty-six yeast strains were isolated from the external surfaces of 95 samples of sugar cane (*Saccharum officinarum* L.) leaf collected in Thailand. Strain DMKU-RK359^T was isolated from a sample collected from Nong Muang district, Lop Buri Province, on 5 May 2010, by an enrichment technique using acidified yeast extract-malt extract (YM) broth (0.3 % yeast extract, 0.3 % malt extract, 0.5 % peptone and 1 % glucose) supplemented with 0.025 % sodium propionate and 0.02 % chloramphenicol (Limtong *et al.*, 2007). Cut leaves (3 g) were placed aseptically in a 250-ml Erlenmeyer flask containing 50 ml acidified YM broth and incubated on a rotary shaker at room temperature (27 ± 3 °C) for 3 days. The enrichment culture was then spread on YM agar supplemented with 0.025 % sodium propionate and 0.02 % chloramphenicol and incubated at room temperature (27 ± 3 °C) until yeast colonies appeared. Yeast colonies of different morphologies were picked and

Abbreviations: ITS, internal transcribed spacer; LSU, large subunit.

The GenBank/EMBL/DDBJ accession numbers for the sequences of the D1/D2 region of the LSU rRNA gene and the ITS region of strain DMKU-RK359^T are AB714248 and AB714257, respectively.

The Mycobank number for *Wickerhamomyces siamensis* is MB802886.

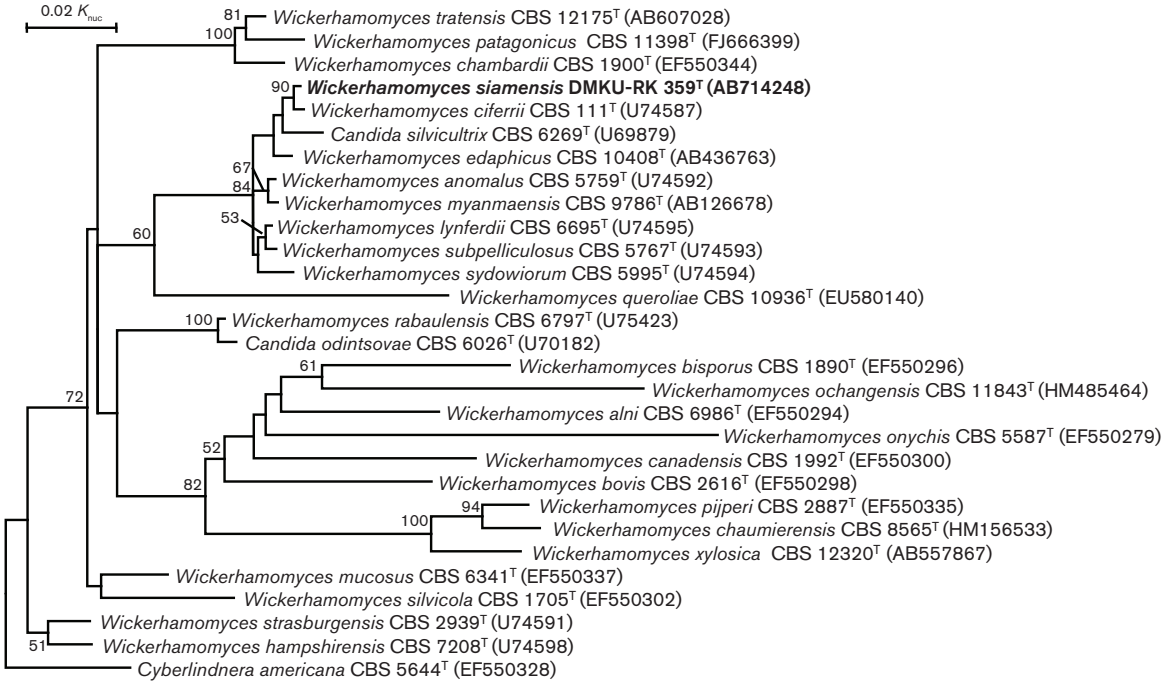


Fig. 1. Phylogenetic tree based on sequences of the D1/D2 region of the LSU rRNA gene, showing the position of *W. siamensis* sp. nov. DMKU-RK359^T with respect to closely related species. The phylogenetic tree was reconstructed from evolutionary distance data with Kimura's two-parameter correction, using the neighbour-joining method with MEGA version 5.0. Numbers at nodes indicate percentages of bootstrap sampling, derived from 1000 samples. *Cyberlindnera americana* CBS 5644^T was the outgroup. Bar, 0.02 K_{nuc}.

purified by cross-streaking on YM agar. Purified yeast strains were suspended in YM broth supplemented with 10 % glycerol and maintained at −80 °C.

The sequences of the D1/D2 region of the LSU rRNA gene and the internal transcribed spacer (ITS) region were determined from PCR products amplified from genomic DNA, using primers NL1 and NL4 (Kurtzman & Robnett, 1998) and ITS1 and ITS4 (White *et al.*, 1990), respectively, for sequencing and amplification. Methods of DNA extraction and amplification were as described previously (Limtong *et al.*, 2007). The PCR products were checked by agarose gel electrophoresis and purified by using the QIAquick purification kit (Qiagen) and the purified products were submitted to Macrogen Inc. (Seoul, Republic of Korea) for sequencing. The sequences were compared pairwise using a BLAST search (Altschul *et al.*, 1997) and were aligned with the sequences of related strains retrieved from GenBank using the multiple alignment program CLUSTAL_X version 1.81 (Thompson *et al.*, 1997). A phylogenetic tree was reconstructed from the evolutionary distance data with Kimura's two-parameter correction (Kimura, 1980) using the neighbour-joining method (Saitou & Nei, 1987) and MEGA software version 5.0 (Tamura *et al.*, 2011). Confidence levels of the clades were estimated from bootstrap analysis (1000 replicates) (Felsenstein, 1985).

Strain DMKU-RK359^T was characterized morphologically, biochemically and physiologically according to standard methods described by Yarrow (1998). Mycelium formation was investigated on potato dextrose agar (PDA; 20 % potato infusion, 2 % glucose and 1.5 % agar) in slide culture at 25 °C for up to 7 days. Ascospore formation was investigated on YPD agar (1 % yeast extract, 2 % peptone, 2 % glucose and 1.5 % agar) and 5 % malt extract agar (5 % malt extract and 1.5 % agar) at 25 °C. Carbon assimilation tests were conducted in liquid medium according to the method described by Yarrow (1998). Assimilation of nitrogen compounds was examined on solid medium with starved inocula following the method of Nakase & Suzuki (1986). Growth at various temperatures was determined by cultivation in YM broth. Ubiquinones were extracted from cells cultivated in a 500-ml Erlenmeyer flask containing 250 ml YPD broth on a rotary shaker at 28 °C for 24–48 h and purified according to the method described by Yamada & Kondo (1973) and Kuraishi *et al.* (1985). Isoprenologues were identified by HPLC as described previously (Limtong *et al.*, 2007). The DNA base composition was determined by HPLC as described by Tamaoka & Komagata (1984) using the DNA-GC kit (Yamasa Shoyu).

Morphological examination of ascospores under the scanning electron microscope was performed as follows. Strain DMKU-RK359^T was grown on YPD agar at 25 °C

Table 1. Phenotypic characteristics that differentiate *Wickerhamomyces siamensis* sp. nov. DMKU-RK359^T from closely related species

Species/strains: 1, *W. siamensis* sp. nov. DMKU-RK359^T; 2, *W. ciferrii* (data from Kurtzman, 2011); 3, *W. edaphicus* (Limtong *et al.*, 2009); 4, *C. silvicultrix* (Lachance *et al.*, 2011). +, Positive; W, weakly positive; −, negative; ND, no data available.

Characteristic	1	2	3	4
Assimilation of:				
Melibiose	−	−	+	+
L-Arabinose	−	+/W	W/−	+
D-Arabinose	−	−	−	+
D-Ribose	−	+	ND	+
L-Rhamnose	−	+/W	+	−
Ribitol	−	+	+	+
Citric acid	−	+	+	+
Hexadecane	−	+	ND	−
D-Glucono-δ-lactone	−	ND	ND	+
Nitrate	−	+	+	−
Nitrite	−	ND	+	−
Ethylamine	+	ND	+	−
Growth in/at:				
Vitamin-free medium	−	+	+	+
10 % NaCl/5 % glucose	−	W	+	+
37 °C	+	W/−	W	+
40 °C	+	ND	−	ND

for 3 days or until ascospores were produced. An ascospore suspension was prepared in 1 ml normal saline and then centrifuged. An aliquot (0.5 ml) of 0.1 M phosphate buffer, pH 7.0, was added to the cell suspension and the cells were harvested by centrifugation. Ascospores were fixed with 0.5 ml of 3.5 % glutaraldehyde in 0.1 M phosphate buffer, pH 7.0, at room temperature (27 ± 3 °C) for 1 h and then centrifuged and washed three times with phosphate buffer. An aliquot (0.5 ml) of 2 % osmium tetroxide in 0.1 M phosphate buffer was added and left at room temperature for 1 h and then the suspension was washed three times with phosphate buffer. Fixed ascospores were washed stepwise with 0.5 ml aliquots of increasing ethanol concentrations (30, 50, 70, 80, 90 and 95 %) for 30 min and two 30-min washes in 100 % ethanol. Fixed ascospores were dried with 0.5 ml ethanol/isoamyl alcohol (2 : 1) for 30 min, centrifuged and then repeatedly dried with ethanol/isoamyl alcohol (1 : 1 and 1 : 2). After centrifugation, isoamyl alcohol was added, left for 30 min and then the suspension was centrifuged; this step was repeated. Ascospores were moved to a critical-point dryer and then coated with platinum. Finally, ascospores were observed by scanning electron microscopy (Hitachi S-5200).

Determination of whether strain DMKU-RK359^T was homothallic or heterothallic was performed by the heat-treatment method described by Wickerham & Burton (1954). A loopful of cells from a YM agar plate (24–48 h, 25 °C) was suspended in 2 ml YM broth in a 16-mm test tube and the test tube was placed in a water bath at 62 °C. After 1, 2, 3, 4 and 5 min, loopfuls of the cell suspension were taken and spread on YM agar. The plates were examined after incubation to determine the time taken to kill the vegetative cells. A suspension of a sporulated culture of strain DMKU-RK359^T (3 days, 25 °C on YPD agar) was then prepared in YM broth. The suspension was heated at 62 °C for 7 min and spread on YM agar. Vegetative cells were killed, so that the colonies arising on plates were derived only from surviving ascospores. Twelve to twenty colonies were checked for sporulation. If all or nearly all colonies show good sporulation, the strain is homothallic; otherwise, the culture is heterothallic.

Novel species delineation and identification

Analysis of the D1/D2 region of the LSU rRNA gene sequence revealed that the sequence of strain DMKU-RK359^T was closest to that of *W. ciferrii*, with 0.7 % nucleotide substitutions (four nucleotide substitutions and four gaps out of 571 nt). The sequence of the ITS region of strain DMKU-RK359^T was also analysed; it differed by 6 % nucleotide substitutions (33 substitutions and eight gaps out of 550 nt) from that of *W. ciferrii*.

The phylogenetic tree based on sequences of the D1/D2 domain of the LSU rRNA gene shows that strain DMKU-RK359^T is closely related to *W. ciferrii*, *W. edaphicus* and *Candida silvicultrix* with high bootstrap support and belongs to the *Wickerhamomyces* clade at a position distinct from the other members of the clade (Fig. 1).

Ascospores were produced by strain DMKU-RK359^T on YPD agar and 5 % malt extract agar after 7 days at 25 °C. Asci were persistent and unconjugated and formed four hat-shaped ascospores. Strain DMKU-RK359^T was found to be homothallic.

On the basis of morphological, biochemical, physiological and chemotaxonomic characteristics and sequence analysis of the D1/D2 domain of the LSU rRNA gene and ITS region, we conclude that strain DMKU-RK359^T represents a novel *Wickerhamomyces* species, for which the name *Wickerhamomyces siamensis* sp. nov. is proposed. *W. siamensis* sp. nov. is distinguished from *W. ciferrii*, *W. edaphicus* and *C. silvicultrix*, the most closely related described species, by the phenotypic characteristics shown in Table 1.

Member of *Wickerhamomyces* species are often found associated with plants; however, only a few species have been isolated from the phylloplane, such as *W. edaphicus*, which was isolated from *Terminalia catappa* L. (Limtong & Koowadjanakul, 2012). In this study, of 166 yeast strains obtained from the phylloplane of 95 samples of sugar-cane leaf, strain DMKU-RK359^T was the only strain of *W. siamensis* sp. nov. that was isolated; therefore, it is clear that the novel species is not common in the phylloplane. *Meyerozyma guilliermondii* was found to be the most

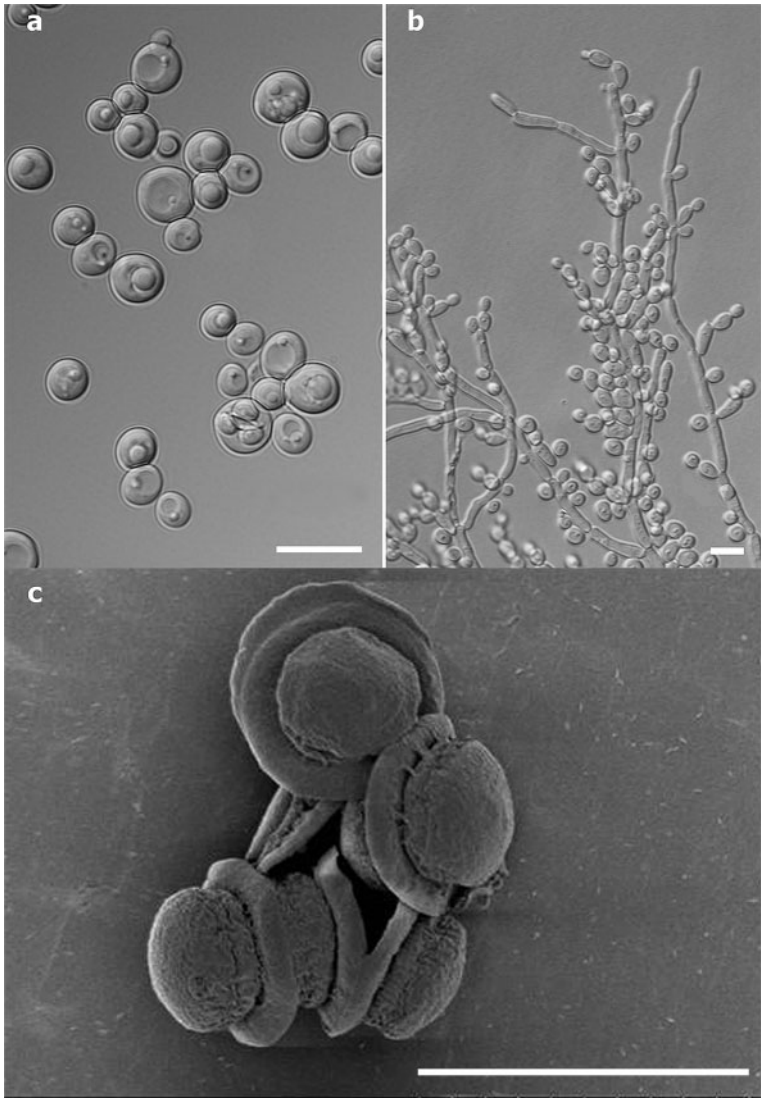


Fig. 2. Morphological analysis of *W. siamensis* sp. nov. DMKU-RK359^T. (a) Budding cells and an ascus with hat-shaped ascospores in YM broth after 3 days at 25 °C. (b) Pseudohyphae formed on PDA after 3 days at 25 °C. (c) Scanning electron micrograph of hat-shaped ascospores produced on 5 % malt extract agar after 7 days at 25 °C. Bars, 10 μm (a, b) and 5 μm (c).

common yeast species in the sugar-cane phylloplane; 25 strains of this species were obtained.

Description of *Wickerhamomyces siamensis* Kaewwichian, Kawasaki & Limtong sp. nov.

Wickerhamomyces siamensis (si.a.men′sis. N.L. fem. adj. *siamensis* of or belonging to Siam, the old name of Thailand, where the type strain was isolated).

Growth in YM broth: after 3 days at 25 °C, cells are globose, subglobose to ovoid (3–8 × 4–8 μm) and occur singly, in pairs or in groups (Fig. 2a). Budding is multilateral. Growth on YM agar: after 3 days at 25 °C, the streak culture is greyish white, smooth, soft to butyrous and has an entire margin. Pseudohyphae are formed but true hyphae are not formed in slide culture on PDA after 3 days at 25 °C (Fig. 2b). Ascospores are produced on YPD agar and 5 % malt extract agar after 7 days at 25 °C. Asci are persistent, unconjugated and formed four hat-shaped ascospores (Fig. 2c). D-Glucose, D-galactose, sucrose, maltose (weak) and raffinose (weak) are fermented but lactose, trehalose, xylose and cellobiose are not fermented. Compounds assimilated include D-glucose, D-galactose (slow), sucrose, maltose, cellobiose (weak), trehalose (weak), raffinose (weak), melezitose (slow), soluble starch (weak), D-xylose (slow), ethanol, glycerol, erythritol (weak), D-mannitol (weak), D-glucitol (weak), methyl α-D-glucoside (weak), salicin (weak), D-gluconic acid, DL-lactic acid, succinic acid, arbutin (weak), propane-1,2-diol (weak), xylitol (weak), ethylamine, L-lysine (weak) and cadaverine. Compounds that are not assimilated include L-sorbose, lactose, melibiose, inulin, L- and D-arabinose, L-rhamnose, D-ribose, *N*-acetyl-D-glucosamine, methanol, ribitol, galactitol, D-glucuronic acid, D-galacturonic acid, 2-keto-D-gluconic acid, 5-keto-D-gluconic acid, D-glucono-δ-lactone, citric acid, inositol, D-glucosamine, hexadecane, butane-2,3-diol, arabitol, nitrate and nitrite. No growth in vitamin-free medium. Growth occurs on medium containing 50 and 60 % glucose, but not on 10 % NaCl/5 %

glucose or 16 % NaCl/5 % glucose. No growth with 0.01 or 0.1 % cycloheximide. Growth occurs at 25, 30, 35, 37, 40 and 42 °C, but not at 45 °C. Starch-like compounds are not produced. Diazonium blue B colour and urease reactions are negative. The major ubiquinone is Q-7. The DNA G+C content of the type strain is 46.2 mol%.

The holotype is DMKU-RK359^T, isolated from the phylloplane of sugar cane (*Saccharum officinarum* L.) collected from Lop Buri province, Thailand. Living cultures from the type have been deposited at the BIOTEC Culture Collection, National Center for Genetic Engineering and Biotechnology, Pathumthani, Thailand, as BCC 50732^T, the NITE Biological Resources Center, Department of Biotechnology, National Institute of Technology and Evaluation, Chiba, Japan, as NBRC 108900^T, and the Centraalbureau voor Schimmelcultures, Utrecht, Netherlands, as CBS 12570^T. The Mycobank registration number is MB802886.

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ORIGINAL PAPER

Pseudozyma vetiver sp. nov., a novel anamorphic ustilaginomycetous yeast species isolated from the phylloplane in Thailand

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Abstract Three strains representing one novel yeast species were isolated from the phylloplanes of the vetiver grasses (DMKU-LV90 and DMKU-LV99^T) and sugarcane (DMKU-SP260) collected in Thailand by leaf washing followed by a plating technique. On the basis of morphological, biochemical, physiological and chemotaxonomic characteristics and the sequence analysis of the D1/D2 region of the large subunit (LSU) rRNA gene and the internal transcribed spacer region (ITS), the three strains were found to represent a single novel anamorphic ustilaginomycetous yeast species in the genus *Pseudozyma*. The name *Pseudozyma vetiver* sp. nov. is proposed for this novel species. The type strain is DMKU-LV99^T (BCC 61021 = CBS 12824). The novel species showed phylogenetic relationships to the other members of the genus *Pseudozyma* and to teleomorphic fungal genera, namely *Ustilago*, *Sporisorium* and *Anomalomyces* in Ustilaginaceae, Ustilaginales. The three strains showed identical sequences both in the D1/D2 and ITS regions. The *Pseudozyma* species closest to the novel species in terms of pairwise sequence similarity in the D1/D2 region was *Pseudozyma pruni* but with 2.3 % nucleotide substitutions (14 nucleotide substitutions and no gaps out of 606 nt). The novel species and *P. pruni* differed by 10.9 % nucleotide substitutions (75 nucleotide substitutions and 31 gaps out of 691 nt) in the ITS region. The phylogenetic analysis based on the combined sequences of the ITS region and the D1/D2 region of the LSU rRNA gene showed that the novel species was found to be most closely related to *Pseudozyma fusiformata* but with 2.9 % nucleotide substitutions in the D1/D2 region and 7.4 % nucleotide substitutions in the ITS region.

Keywords *Pseudozyma vetiver* sp. nov. · Anamorphic yeast · Ustilaginomycetous yeast · Phylloplane · Vetiver grass · Sugarcane · Thailand

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Introduction

The anamorphic genus *Pseudozyma* Bandoni emend. Boekhout (1995) is a member of the Ustilaginaceae, Ustilaginales, Ustilaginomycetes, Ustilaginomycotina, which are closely related to species of the teleomorphic genera *Ustilago* and *Sporisorium*, the causal agents of smut diseases of plants. At present the genus *Pseudozyma* contains 15 described species, namely *Pseudozyma abaconensis*, *P. antarctica*, *P. aphidis*,

P. flocculosa, *P. fusiformata*, *P. graminicola*, *P. hubeiensis*, *P. jejuensis*, *P. parantarctica*, *P. prolifica*, *P. pruni*, *P. rugulosa*, *P. shanxiensis*, *P. thailandica* and *P. tsukubaensis* (Wang et al. 2006; Golubev et al. 2007; Seo et al. 2007; Liou et al. 2009; Statzell-Tallman et al. 2010; Boekhout 2011). The genus *Pseudozyma* is polyphyletic based on the distribution of the species in this genus among various teleomorphic genera of Ustilaginales (Boekhout 2011; Boekhout et al. 2011) that are placed in different clades including *Ustilago* sensu lato, *Sporisorium* and *Ustilago–Sporisorium* clades previously defined by Stoll et al. (2005).

The phylloplane, which usually refers to the external surface of plant leaves, has been recognized as an important natural habitat for epiphytic microorganisms, including bacteria, actinomycetes, cyanobacteria, yeast and filamentous fungi (Phaff and Starmer 1987; Fonseca and Inacio 2006). Both basidiomycetous and ascomycetous yeasts have been found to be phylloplane colonizers (Nakase 2000; Nakase et al. 2001; Fonseca and Inacio 2006; Glushakova et al. 2007; Slavikova et al. 2009; Landell et al. 2010). Most common phylloplane yeasts are in basidiomycetous genera such as *Cryptococcus*, *Rhodotorula*, *Sporobolomyces* and *Trichosporon* (de Azeredo et al. 1998; Slavikova et al. 2009; Glushakova and Chernov 2010).

During the investigation of yeasts on the phylloplane of vetiver grasses and sugarcane in Thailand, three strains representing a novel species were isolated. Detailed analyses demonstrated that they belong to the genus *Pseudozyma*. In this report the three strains are described as *Pseudozyma vetiver* sp. nov.

Materials and methods

Yeast isolation

Green and undamaged leaves of vetiver grass (*Vetiveria zizanioides*) and sugarcane (*Saccharum officinarum*) in the Family Poaceae were collected from various provinces in Thailand in 2012. In total 15 samples of vetiver grass leaf and 102 samples of sugarcane leaf were collected.

Yeasts were isolated from the surfaces of the plant leaves by a technique including leaf washing followed by plating. Three grams of cut leaf were aseptically placed in 250 ml Erlenmeyer flask containing 50 ml of 0.85 % saline solution and shaken on a rotary shaker at room temperature (27 ± 3 °C) for 1 h. An aliquot (0.1 ml) of washing solution was then spread on yeast extract-malt extract (YM) agar (0.3 % yeast extract, 0.3 % malt extract, 0.5 % peptone, and 2.0 % agar) supplemented with 0.025 % sodium propionate and 0.02 % chloramphenicol and incubated at room temperature until yeast colonies appeared. Yeast colonies of different morphologies were selected and purified by cross streaking on YM agar. Purified yeast strains were suspended in YM broth supplemented with 10 % glycerol and maintained at −80 °C.

DNA sequencing and phylogenetic analysis

The sequences of the D1/D2 region of the large subunit (LSU) rRNA gene and the internal transcribed spacer (ITS) region were determined from PCR products amplified from genomic DNA using primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) (Kurtzman and Robnett 1998) and ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′), and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990), respectively. Methods for DNA extraction and amplification were described previously (Limtong et al. 2007). The PCR products were checked by agarose gel electrophoresis and purified by using the QIAquick purification kit (Qiagen, Germany). The purified products were submitted to Macrogen Inc. (Korea) for sequencing with primers, NL1 and NL4, for the D1/D2 region of the LSU rRNA gene, and with primers, ITS1 and ITS4, for the ITS region. The sequences were compared pairwise using a BLAST search (Altschul et al. 1997) and were aligned with the sequences of related species retrieved from GenBank using the multiple alignment program CLUSTAL_X version 1.81 (Thompson et al. 1997). A phylogenetic tree was constructed from the combined sequences of the ITS and the D1/D2 region of the LSU rRNA gene by the evolutionary distance data with Kimura's two-parameter correction (Kimura 1980) using the neighbor joining method (Saitou and Nei 1987) and MEGA software version 5.0 (Tamura et al. 2011). Confidence levels of the clades were estimated from bootstrap analysis (1,000 replicates) (Felsenstein 1985).

Examination of taxonomic characteristics

The strains were characterized morphologically, biochemically, and physiologically according to the

standard methods described by Kurtzman et al. (2011). Formation of hyphae was investigated by cultivation in YM broth and in slide culture on potato dextrose agar (PDA) at 20 and 23 °C for up to 28 days and the cultures was examined microscopically at intervals of a few days. Basidiospore formation was investigated for individual strains or strain pairs on 5 % malt extract agar, YM agar and corn meal agar at 20 and 25 °C for up to 3 weeks. Carbon assimilation tests were conducted in liquid medium as described by Kurtzman et al. (2011). Assimilation of nitrogen compounds was examined in liquid media with starved inocula following the method of Kurtzman et al. (2011). Growth at various temperatures was determined by cultivation in YM broth. Ubiquinones were extracted from cells cultivated in 500 ml Erlenmeyer flasks containing 250 ml of yeast extract peptone dextrose (YPD) broth (1 % yeast extract, 2 % peptone and 2 % dextrose) on a rotary shaker at 28 °C for 24–48 h and purified according to the methods described by Yamada and Kondo (1973) and Kuraishi et al. (1985). Isoprenologues were identified by HPLC as described previously (Limtong et al. 2007).

Results and discussion

Yeast isolation, identification and novel species delineation

In total 25 and 366 yeast strains were isolated from 15 samples of vetiver grass leaf and 102 samples of sugarcane leaf, respectively. Identification on the basis of similarities of the D1/D2 region of the LSU rRNA gene sequences revealed that three strains (DMKU-LV90, DMKU-LV99^T and DMKU-SP260) were found to represent one novel yeast species. Two strains, DMKU-LV90 and DMKU-LV99^T, had been isolated from the phylloplanes of two samples of vetiver grass leaf collected from Mueang district, Ratchaburi province and strain DMKU-SP260 had been obtained from the phylloplane of a sugarcane leaf sample collected from Mueang district, Saraburi province.

The three strains (DMKU-LV90, DMKU-LV99^T and DMKU-SP260) were the same in morphological, biochemical and physiological characteristics. Colonies of the three strains were dull, whitish to pale pinkish-cream, zonate towards the margin, and with fringed margins. Cells were ovoidal and ellipsoidal, and contained oil droplets (Fig. 1). Budding was polar on a short denticle. Ballistoconidia were absent. No mating reaction or sexual structure was observed. Fermentation was absent. Diazonium blue B (DBB) and urease reactions were positive. Myo-inositol (latent) and D-glucuronic acid assimilation were positive. Starch-like compounds were not produced. The major ubiquinone was CoQ-10. The phenotypic characteristics suggested that the three strains belong to the genus *Pseudozyma* as defined by Boekhout (2011).

The phylogenetic tree based on the combined sequences of the ITS region and the D1/D2 region of the LSU rRNA gene (Fig. 2) showed that the tree strains were placed in the same position, connected to *Pseudozyma fusiformata*, and clustered with other two *Pseudozyma* species (*P. pruni* and *P. graminicola*) and the species of the teleomorphic genus *Sporisorium* in the *Sporisorium* 2 subclade of *Sporisorium* clade defined by Stoll et al. (2005). This phylogenetic analysis further demonstrated that the three strains were a single species in the genus *Pseudozyma* but were not conspecific with any of the recognized *Pseudozyma* species. The novel species was found to be most closely phylogenetically related to *P. fusiformata* but with 2.9 % nucleotide substitutions (17 nucleotide substitutions and one gap out of 589 nt) in the D1/D2 region. In the ITS region the two species differed by 7.4 % nucleotide substitutions (50 nucleotide substitutions and 20 gaps out of 680 nt). The most closely related teleomorphic taxon was *Anomalomyces yakirrae* but with 2.5 % nucleotide substitutions in the D1/D2 region and 13.5 % nucleotide substitutions in the ITS region. However, in terms of pairwise sequence similarity of the D1/D2 region, the closest relative to the novel species was *P. pruni* but with 2.3 % nucleotide substitutions (14 nucleotide substitutions out of 606 nt). These two species differed by 10.9 % nucleotide substitutions (75 nucleotide substitutions and 31 gaps out of 691 nt) in the ITS region. The novel species differed from the other recognized *Pseudozyma* species by 2.9-4.3 % nucleotide substitutions in the D1/D2 region and 7.4–15.8 % nucleotide substitutions in the ITS region.

On the basis of morphological, biochemical, and physiological characteristics, and the sequence analysis of the D1/D2 region of the LSU rRNA gene and ITS region, we concluded that the three strains represent a

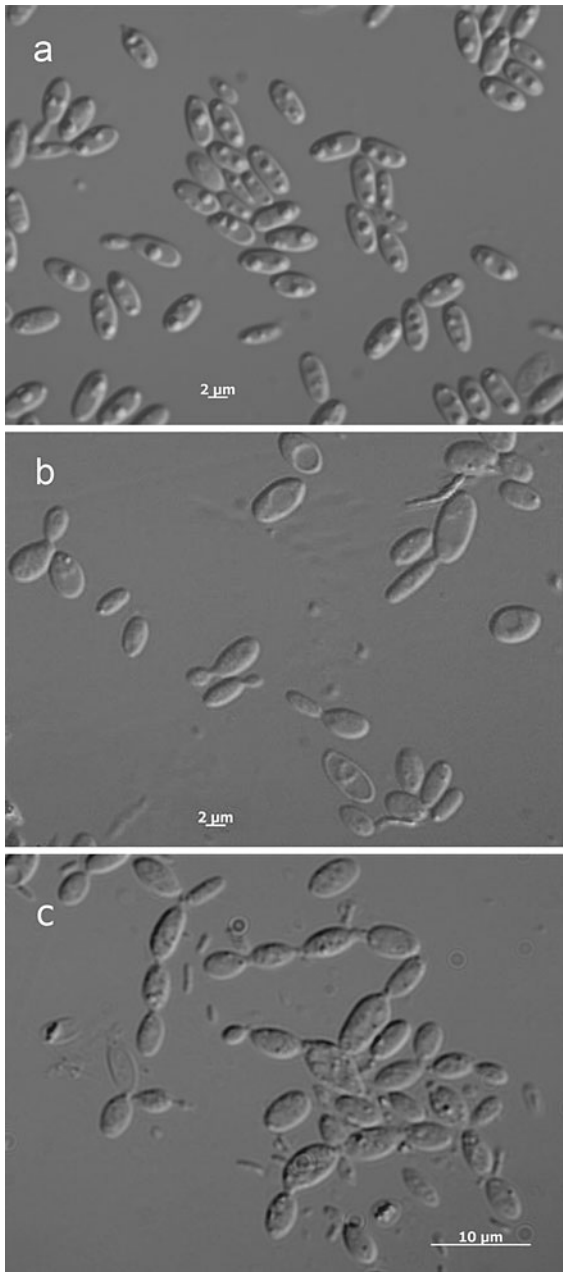


Fig. 1 *Pseudozyma vetiver* sp. nov. (DMKU-LV99^T) **a** cells showing oil droplets inside on 5 % malt extract agar after 4 days at 25 °C. *Bar* 2 µm. **b** Cells on YM agar after 4 days at 25 °C. *Bar* 2 µm. **c** Rudimentary pseudohyphae produced in YM broth after 23 days at 25 °C. *Bar* 10 µm

novel *Pseudozyma* species for which the name *P. vetiver* sp. nov. (MB804174) is proposed.

Pseudozyma vetiver can be is distinguished from the other 15 recognized *Pseudozyma* species not only on the basis of the sequences of the D1/D2 and the ITS regions but also by several phenotypic characteristics as shown in Table 1.

Pseudozyma species are mainly associated with plants (Boekhout and Fell 1998; Wang et al. 2006; Golubev et al. 2007; Seo et al. 2007; Liou et al. 2009) although two species, namely *P. parantarctica* and *P. thailandica*, were isolated from clinical specimens (Sugita et al. 2003) and one species, namely *P. abaconensis*, was collected from water (Statzell-Tallman et al. 2010). Among 15 *Pseudozyma* species, six species, namely *P. flocculosa*, *P. graminicola*, *P. hubeiensis*, *P. jejuensis*, *P. rugulosa* and *P. shanxiensis*, were isolated from plants leaves while the other six species, namely *P. aphidis*, *P. antarctica*, *P. fusiformata*, *P. prolifica*, *P. pruni* and *P. tsukubaensis*, were obtained from other plant materials or materials associated with plants (Boekhout and Fell 1998; Wang et al. 2006; Golubev et al. 2007; Seo et al. 2007; Liou et al. 2009). In this study the three strains were isolated from the phylloplane at the surface of plant leaves. Therefore, plant materials, especially leaves, seem to be natural habitats of yeasts in this genus. However, *P. vetiver* seems not common in the phylloplane because in this study, of 391 yeast strains obtained from the phylloplanes of 117 leaf samples, only three strains were isolated.

Description of *Pseudozyma vetiver* Chamnanpa and Limtong sp. nov

Growth on YM agar after 4 days at 25 °C, cells are ovoid and ellipsoidal (1.8–3.8 × 3.7–8.1 µm), occur singly or in pairs and contain oil droplets (Fig. 1). Budding is polar on a short denticle. Colonies are dull, whitish to pale pinkish-cream, zonate towards the margin, and with the margin fringed. After 3 days at 25 °C in YM broth, sediment and a pellicle are present. Rudimentary pseudohyphae are formed in YM broth after 10 days at 25 °C (Fig. 1), but are not formed on PDA and corn meal agar after 28 days at 20 °C. No mating reaction or sexual structure was observed in cultures of individual strains or strain pairs on 5 % malt extract agar, YM agar and corn meal agar at 20 and 25 °C for up to 3 weeks. Ballistoconidia are absent. Fermentation is absent. D-Glucose, sucrose, raffinose, trehalose, maltose, melezitose, methyl-α-D-glucoside (weak), soluble starch, cellobiose (weak),

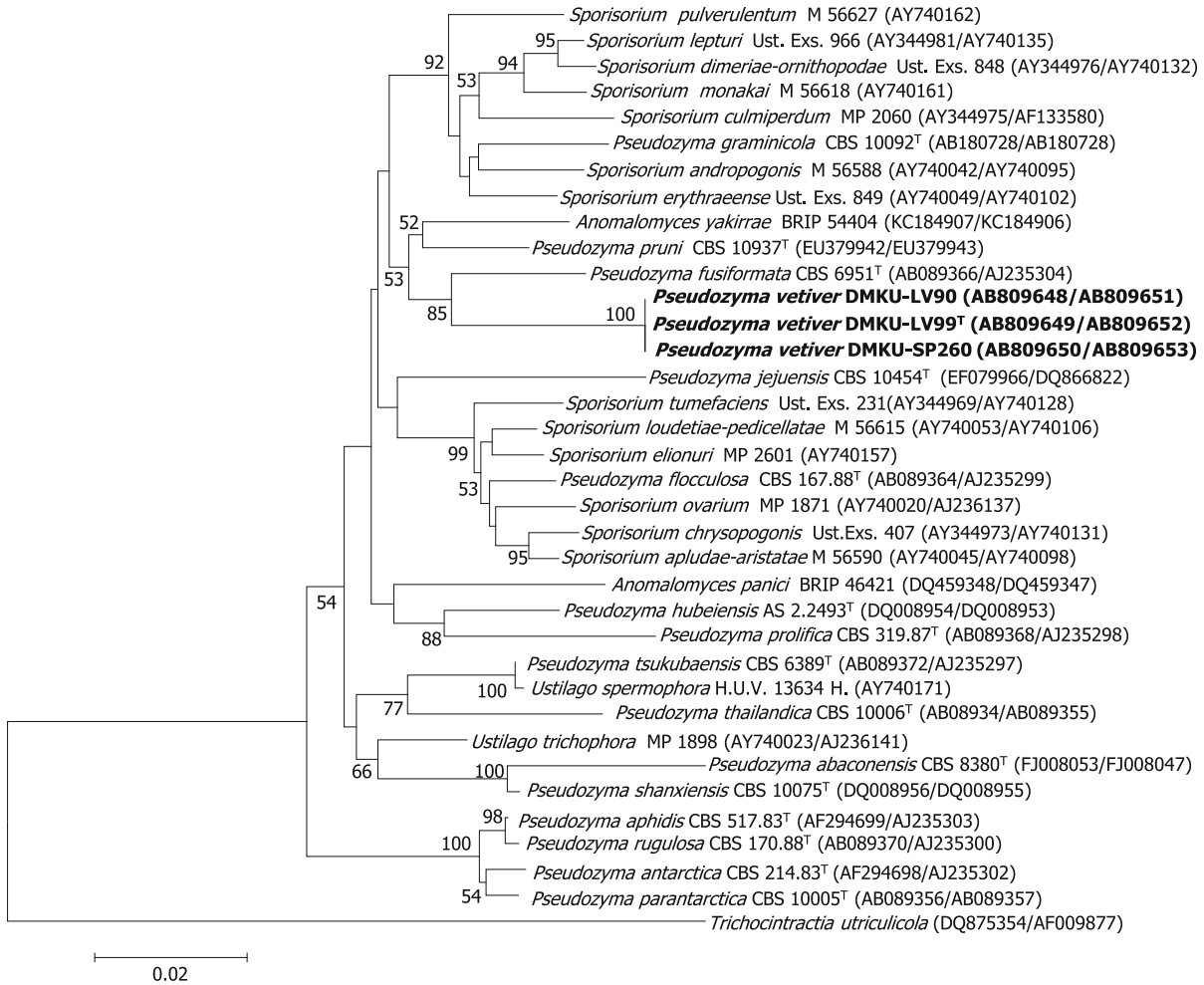


Fig. 2 Phylogenetic tree based on the combined sequences of the ITS region and the D1/D2 region of the LSU rRNA gene, showing positions of *Pseudozyma vetiver* sp. nov. (DMKU-LV99^T, DMKU-LV90 and DMKU-SP260) with respect to closely related species. The phylogenetic tree was constructed from evolutionary distance data with Kimura's two-parameter correction, using the neighbor joining method by MEGA software version 5.0. *Numbers* indicate percentages of bootstrap sampling, derived from 1,000 samples. The *numbers* in *parentheses* are GenBank accession numbers. *Trichocintractia utriculicola* was the outgroup in the analysis

salicin (weak), L-sorbose (slow), D-xylose, xylitol, D-mannitol (slow), D-glucitol, *myo*-inositol (latent), DL-lactic acid, succinic acid, citric acid, gluconolactone (slow), 2-keto-D-gluconic acid, D-glucuronic acid, D-galacturonic acid, ammonium sulfate, potassium nitrate, sodium nitrite, L-lysine (weak) and cadaverine (weak) are assimilated, but inulin, melibiose, D-galactose, lactose, L-rhamnose, L-arabinose, D-arabinose, D-ribose, methanol, ethanol, glycerol, erythritol, ribitol, galactitol, D-gluconic acid, 5-keto-D-gluconic acid, ethylamine are not assimilated. Growth is present in vitamin free medium. Growth occurs on medium containing 50 % (w/v) glucose and 60 % (w/v) glucose, growth on 10 % (w/v) sodium chloride/5 % (w/v) glucose is weakly present, but on 16 % (w/v) sodium chloride/5 % (w/v) glucose growth is absent. Growth with 0.01 % cylcloheximide and 0.1 % cylcloheximide are negative. Growth at 15, 25 and 30 is present, but is absent at 35, 37, 40, 42 and 45 °C. Acid formation is absent. Starch-like compounds are not produced. Diazonium blue B color and urease reactions are positive. The major ubiquinone is Q-10.

Table 1 Physiological characteristics that differentiate *Pseudozyma vetiver* sp. nov. from the other species of the genus *Pseudozyma*

Characteristic	1	2[a]	3[b]	4[c]	5[c]	6[c]	7[c]	8[d]	9[e]	10[f]	11[c]	12[c]	13[c]	14[e]	15[c]	16[c]
Assimilation of carbon compounds																
Inulin	−	w	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Melibiose	−	+	s	−	+	s	v	+	+	−	+	−	s	+	+	−
D-Galactose	−	+	+	+	+	s	−	s	+	s	+	−	+	+	+	s
Lactose	−	+	−	+	+	−	−	s	+	+	+	+	−	+	−	+
Trehalose	+	+	+	+	+	+	+	s	+	+	+	+	+	+	+	+
Maltose	+	+	w	+	+	+	+	+	+	+	+	+	+	+	+	+
Melezitose	+	+	s	+	+	+	+	+	+	+	+	+	+	+	+	+
Methyl-α-D-glucoside	w	+	+	+	+	+	+	−	+	−	+	+	+	+	+	+
Soluble starch	+	+	−	+	+	s	−	s	d/w	−	+	+	+	d/w	+	+
Cellobiose	w	+	−	+	s	s	s	w	+	d	+	+	s	−	+	+
Salicin	w	+	−	+	d	+	v	w	−	d	+	−	d	−	+	−
L-Sorbose	s	w	w	+	d	d	d	w	−	d	w	d	+	−	+	d
L-Rhamnose	−	w	–	v	+	−	−	−	−	−	+	+	+	–	–	–
D-Xylose	+	+	s	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Arabinose	−	+	w/s	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Arabinose	−	s	s	+	+	+	+	−	+	−	+	+	+	v	−	s
D-Ribose	−	+	s	+	+	+	−	w	+	+	+	+	+	+	+	s
Methanol	−	−	−	−	−	+	−	−	−	−	−	−	w	−	−	−
Ethanol	−	+	w	+	−	−	+	+	−	−	+	+	+	−	+	+
Glycerol	−	+	−	+	s	−	s	s	+	−	+	+	+	+	+	+
Erythritol	−	+	s	+	+	+	s	w	+	d	d	+	s	+	+	+
Ribitol	−	v	s	s	s	s	s	w	+	−	+	s	s	+	d	−
D-Mannitol	s	+	w	+	+	+	+	s	+	+	+	+	+	+	+	−
D-Glucitol	+	+	w	+	+	+	+	s	+	+	+	+	+	+	+	−
myo-Inositol	d	+	+	+	+	+	+	w	−	−	+	+	+	−	+	+
DL-Lactic acid	+	w	s	+	+	s	s	s	−	−	+	s	+	−	+	+
Succinic acid	+	+	+	+	+	+	+	s	+	−	+	+	+	+	+	+
Citric acid	+	w	+	+	+	+	s	s	−	+	+	−	+	−	+	+
D-Gluconic acid	−	w	+	+	s	s	+	s	n	d	+	+	+	n	w	−
D-Glucuronic acid	+	+	+	+	+	+	+	+	n	+	+	+	+	n	+	+
Gluconolactone	s	+	n	n	n	n	n	n	n	n	n	n	n	n	n	n
D-Galacturonic acid	+	w	n	n	n	n	n	n	n	n	+	n	n	n	−	n
2-Keto-D-gluconic acid	+	w	+	+	+	+	+	n	n	+	+	+	+	n	+	+
5-Keto-D-gluconic acid	−	n	−	n	n	n	n	w	n	n	d/w	n	n	n	−	n
Xylitol	+	s	n	n	n	n	n	−	n	−	d	n	n	n	−	n
Assimilation of nitrogen compounds																
Ammonium sulfate	+	d	n	n	n	n	n	n	+	n	n	n	n	+	n	n
L-Lysine	+	+	n	+	+	+	+	+	+	+	+	+	+	+	+	−
Cadaverine	w	w	n	+	+	+	+	+	+	+	+	+	+	+	−	+
Ethylamine	−	w	n	+	+	−	+	−	+	−	−	+	+	+	−	+

Table 1 continued

Characteristic	1	2[a]	3[b]	4[c]	5[c]	6[c]	7[c]	8[d]	9[e]	10[f]	11[c]	12[c]	13[c]	14[e]	15[c]	16[c]
Growth in/at																
Vitamin-free medium	+	+	+	−	−	+	+	+	+	+	−	+	w	+	−	+
50 % glucose	+	−	n	−	−	−	−	−	−	−	−	−	−	−	−	−
10 % sodium chloride + 5 % glucose medium	w	n	−	n	n	n	n	w	n	n	n	n	n	n	n	n
0.01 % cycloheximide	−	+	n	n	n	n	n	n	n	n	n	n	n	n	n	n
35 °C	−	n	n	n	n	n	n	+	n	n	n	n	n	n	n	n
37 °C	−	+	−	v	+	−	−	n	+	+	d/w	−	+	+	+	−
40 °C	−	n	n	−	−	−	−	n	−	−	−	−	w	+	−	−

Species 1 *P. vetiver* sp. nov., 2 *P. pruni*, 3 *P. abaconensis*, 4 *P. antarctica*, 5 *P. aphidis*, 6 *P. flocculosa*, 7 *P. fusiformata*, 8 *P. graminicola*, 9*P. hubeiensis*, 10 *P. jejuensis*, 11 *P. parantarctica*, 12 *P. prolific*, 13 *P. rugulosa*, 14 *P. shanxiensis*, 15 *P. thailandica*, 16 *P. tsukubaensis*

+ positive, − negative, *d* delayed positive, *s* slow positive, *w* weak positive, *v* variable, *n* no data

[a] Data from Liou et al. (2009)

[b] Data from Statzell-Tallman et al. (2010)

[c] Data from Boekhout (2011)

[d] Data from Golubev et al. (2007)

[e] Data from Wang et al. (2006)

[f] Data from Seo et al. (2007)

Holotype

DMKU-LV99^T is the holotype of *P. vetiver* (MB804174). The strain was isolated from phylloplane of vetiver grass (*Vetiveria zizanioides*) collected in 2012 from Ratchaburi province, Thailand. The holotype has been deposited at the BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathumthani, Thailand, as BCC 61021^T and CBS-KNAW Fungal Biodiversity Center (CBS), Utrecht, The Netherlands as CBS 12824^T.

Etymology

The species epithet "*vetiver*" refers to vetiver grass (*Vetiveria zizanioides*), where the type strain and strain DMKU-LV99^T were isolated.

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Plant growth-promoting traits of epiphytic and endophytic yeasts isolated from rice and sugar cane leaves in Thailand

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ABSTRACT

A total of 1035 yeast isolates, obtained from rice and sugar cane leaves, were screened primarily for indole-3-acetic acid (IAA) production. Thirteen isolates were selected, due to their IAA production ranging from 1.2 to 29.3 mg g^{-1} DCW. These isolates were investigated for their capabilities of calcium phosphate and ZnO_3 solubilisation, and also for production of NH_3, polyamine, and siderophore. Their 1-aminocyclopropane-1-carboxylate (ACC) deaminase, catalase and fungal cell wall-degrading enzyme activities were assessed. Their antagonism against rice fungal pathogens was also evaluated. Strain identification, based on molecular taxonomy, of the thirteen yeast isolates revealed that four yeast species – i.e. *Hannaella sinensis* (DMKU-RP45), *Cryptococcus flavus* (DMKU-RE12, DMKU-RE19, DMKU-RE67, and DMKU-RP128), *Rhodosporidium paludigenum* (DMKU-RP301) and *Torulaspora globosa* (DMKU-RP31) – were capable of high IAA production. Catalase activity was detected in all yeast strains tested. The yeast *R. paludigenum* DMKU-RP301 was the best IAA producer, yielding 29.3 mg g^{-1} DCW, and showed the ability to produce NH_3 and siderophore. Different levels of IAA production (7.2–9.7 mg g^{-1} DCW) were found in four strains of *C. flavus* DMKU-RE12, DMKU-RE19, and DMKU-RE67, which are rice leaf endophytes, and strain DMKU-RP128, which is a rice leaf epiphyte. NH_3 production and carboxymethyl cellulase (CMCase) activity was also detected in these four strains. Antagonism to fungal plant pathogens and production of antifungal volatile compounds were exhibited in *T. globosa* DMKU-RP31, as well as a moderate level of IAA production (4.9 mg g^{-1} DCW). The overall results indicated that *T. globosa* DMKU-RP31 might be used in two ways: enhancing plant growth and acting as a biocontrol agent. In addition, four *C. flavus* were also found to be strains of interest for optimal IAA production.

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Introduction

Microorganisms can promote plant growth by both direct and indirect means. Production of phytohormones, such as auxins or gibberellins, is a direct mechanism that is used by plant growth-promoting microorganisms. Auxins are a group of compounds having an indole ring, that have a positive effect on plants by stimulating seed germination, root initiation, cell elongation, and seedling growth (El-Tarabily 2008). Indole-3-acetic acid (IAA), a plant growth hormone classified as an indole derivative of the auxin family, has been widely studied and found to be a dominant type of auxin in plants. Several important roles that IAA has been shown to play in plants include stimulation of cell division, cell elongation, cell differentiation, light and gravitational responses, and regulation of leaf fall and fruit ripening (Trotsenko *et al.* 2001; Teale *et al.* 2006). Different levels of IAA production have been reported in several groups of bacteria and yeasts.

As well as phytohormone production, microorganisms directly enhance plant growth by making nutrients more available in various ways. Ammonia (NH_3) production by plant-associated microorganisms is advantageous to plants because NH_3 provides available nitrogen that supports plant growth. Microorganisms also assist in phosphorus uptake. Plants receive phosphorus in the form of phosphate, which is sometimes present in nature in an insoluble form that is unusable by plants. Microorganisms that possess phosphate solubilising capabilities provide phosphorus in a soluble form, as required by plants. Similarly, the micronutrient zinc needs to be converted from an insoluble to a soluble form, and this is done by microbial activity. In the case of iron, siderophores produced by microbes help in the accumulation of Fe in Fe-deficient environments, and in its uptake by plants (Vessey 2003). By producing siderophores that trap iron, plant growth-promoting microorganisms support plant growth directly. However, siderophores can also have a negative effect on fungal pathogens through iron depletion (Calvente *et al.* 1999; Wang & Chi 2009). In this way, the growth of phytopathogens is limited. Therefore, siderophore production by plant growth-promoting microorganisms may also be regarded as an indirect mechanism for plant growth enhancement.

In addition to siderophore production, microbes also possess other indirect mechanisms for the suppression of plant pathogens, *via* the production of antibiotics and fungal wall-degrading enzymes such as chitinase, cellulase and β-1,3-glucanase (Chaiharn *et al.* 2008). Polyamines – compounds involved in many cellular processes, including the maintenance of cell macromolecular biosynthesis, survival and growth (Amprayn *et al.* 2012) – also function in plant growth promotion. The production of polyamines (spermidine, spermine, putrescine) has been claimed to be a tool to stimulate root cell division and, hence, increase root growth (Tang & Newton 2005; Cloete *et al.* 2009). ACC deaminase is an enzyme that cleaves ACC to form α-ketobutyrate and ammonia, and decreases ethylene levels in host plants (Sessitsch *et al.* 2005; Sun *et al.* 2009). Plant growth-promoting bacteria with ACC deaminase activity can sequester ACC and potentially lower deleterious levels of ethylene in the plant host, which alters its physiology (Glick 2004, 2005; Glick *et al.* 2007a,b). The function of ACC deaminase in plant-microbe systems results in the stimulation of plant root elongation, a phenomenon which has been well-studied (Glick *et al.* 2007a; Husen *et al.* 2008; Yim *et al.* 2010). Microorganisms can also produce hydrogen cyanide (HCN), which inhibits the hyphal growth of fungi (Freeman *et al.* 1975; Fry & Evans 1977).

The direct and indirect plant growth-promoting characters at different levels of various yeast species have been reviewed. In terms of direct promotion, nutrient uptake and phytohormone production have been exhibited in a diverse range of yeast. IAA and indole-3-pyruvic acid (IPYA) were produced *in vitro* by the endophytic yeast *Lindera* (*Williopsis*) *saturnus*, isolated from maize roots (Nassar *et al.* 2005), whereas *Rhodotorula*, the first endophytic yeast isolated from the *Populus* plant, was also shown to produce IAA (Xin *et al.* 2009). In addition, the endophytic yeast *Cryptococcus* sp. CBSB78, isolated from rape (*Brassica chinensis*), although producing low concentrations of IAA (11.7 $\mu g\ ml^{-1}$), showed heavy metal resistance and hence promoted the growth of *Brassica* spp. in metal-contaminated soils (Deng *et al.* 2012).

IAA production was not only found in endophytic yeast but also in the epiphytic yeast *Candida maltosa*, isolated from the phylloplane; relatively high concentrations of IAA (121.4–234.1 $mg\ l^{-1}$) were produced when cultivated in YPD broth, supplemented with 0.1 % L-tryptophan (Limtong & Koowadjanakul 2012). In the case of the soil yeast *Candida tropicalis* HY, the production of small quantities of IAA was accompanied by tri-calcium phosphate solubilisation and the production of phytase, which enhances phosphate release from phytate-containing materials available in nature.

In addition to direct plant growth-promoting characters, *C. tropicalis* HY showed indirect characters such as polyamine production and high ACC deaminase activity, which enhanced plant growth by lowering ethylene levels (Amprayn *et al.* 2012). Recently, high ACC deaminase activity has been reported in two yeasts: *Hansenula saturnus* and *Issatchenkia occidentalis* (Palmer *et al.* 2007). The production of polyamines – i.e. putrescine, spermidine, and spermine – was exhibited in different strains of the yeast *Debaryomyces hansenii* (Reyes-Becerril *et al.* 2011). As in other plant growth-promoting microbes, siderophore production by yeast is another critical tool that bolsters plant growth. This may be considered a direct or an indirect role of yeast. *Rhodotorula glutinis* can be claimed to indirectly promote plant growth, through the antagonistic action of siderophores affecting the postharvest pathogen *Penicillium expansum* (Calvente *et al.* 1999).

Diverse yeast species can act as biocontrol agents against fungal plant pathogens. It has been shown that *Candida valida*, *Rhodotorula glutinis*, and *Trichosporon asahii*, isolated from sugar beet rhizospheres, possess biocontrol activity against *Rhizoctonia solani*, a soil-borne fungal plant pathogen that causes root diseases (El-Tarabily 2004). The yeast *Cryptococcus laurentii*, in combination with IAA, was shown to inhibit gray mold, *Botrytis cinerea*, in harvested apple fruit (Yu *et al.* 2008). The yeast *Rhodotorula glutinis* ySL 30 was shown to inhibit *Botrytis cinerea* (Sansone *et al.* 2005). The biological control by the yeast *Torulaspora globosa* against *Colletotrichum sublineolum* in sorghum was evaluated, and one strain of *T. globosa* exhibited killer activity against two sensitive strains of *C. sublineolum*, which is

a novel capacity for *T. globosa* resulting in hyphal deformities but not cell death (Rosa *et al.* 2010).

The capacity to produce enzymes which destroy the fungal cell wall, such as chitinase and β-1,3-glucanase, was exhibited in the yeasts *Pichia anomala*, *Candida guilliermondii*, and *C. oleophila* (Jijakli & Lepoivre 1998; Saligkarias *et al.* 2002). Another putative action mode is the production of toxic volatile compounds, as observed in different *Saccharomyces cerevisiae* species (Fialho 2004).

The combined effects of both direct plant growth-promoting characters, such as plant nutritional supplementation and IAA production, and indirect characters, such as antifungal activity or the scavenging of nutrients required by plant pathogens, are much more supportive of plant growth overall. Microbial activities working in this concerted fashion can augment both the growth of the plant and the economy of the farmer. Our literature review has so far indicated that Amprayn *et al.* (2012) have reported the widest study on plant growth-promoting characters in yeast. In this paper, we report on plant growth-promoting traits of Thai yeast species isolated from rice and sugar cane leaves, including some traits that have not yet been reported.

Materials and methods

Microorganisms

Epiphytic and endophytic yeasts were isolated from rice and sugar cane leaves collected in Thailand, and maintained in a private culture collection at the Department of Microbiology, Kasetsart University, Thailand. The yeasts used in this study consisted of 125 endophytic yeasts and 305 epiphytic yeasts from rice leaves, plus 149 endophytic yeasts and 456 epiphytic yeasts from sugar cane leaves. Yeast strains designated with the initial code of DMKU-RE refer to endophytic yeasts isolated from rice leaves, whereas DMKU-RP refers to epiphytic yeasts isolated from rice leaves. Yeast strains isolated from sugarcane leaves were designated with the initial code DMKU-SE and DMKU-SP for endophytic and epiphytic yeasts, respectively. Rice fungal pathogens – i.e. *Fusarium moniliforme*, *Helminthosporium oryzae*, and *Rhizoctonia solani* – were used in this study. The yeasts were maintained on YPD medium while the fungal strains were maintained on PDA medium.

Screening of indole 3-acetic acid (IAA)-producing yeast

Assessment of IAA production was carried out using a modification of the method described by Xin *et al.* (2009). Yeast inoculum was prepared on YPD agar (10 g l^{-1} yeast extract, 20 g l^{-1} peptone, 20 g l^{-1} dextrose, and 15 g l^{-1} agar) and incubated at 28 °C for 24 h. One loop of inoculum was transferred into 5 ml of YPD broth, amended with 0.1 % (w/v) L-tryptophan. All culture tubes were incubated in a reciprocal shaker at 180 strokes/min at 28 ± 2 °C for 7 d. Culture supernatant was collected by centrifugation for 5 min at 8000 rpm, prior to quantitative analysis of IAA. The amount of IAA was determined by a colorimetric method. The colorimetric assay of IAA was carried out by mixing 1 ml of culture supernatant with 1 ml of Salkowski reagent containing 12 g l^{-1} $FeCl_3$ and 7.9 M H_2SO_4 (Glickmann & Dessaux 1994) followed by 30 min incubation in the dark for complete pink–red colour development. Colour intensity was determined as A_{530} using a spectrophotometer (Thermo Electron Corporation, USA). The IAA concentration of the sample was compared with a linear calibration curve obtained from an authentic IAA standard. The high IAA-producing isolates were selected for use in further studies.

Yeast identification

Yeasts were identified by analysis of the D1/D2 region of the large subunit of rRNA (LSU rRNA) gene sequence similarities (Kurtzman & Robnett 1998). Methods for DNA extraction and amplification of the D1/D2 domain were described previously (Limtong *et al.* 2007). The D1/D2 domain of the LSU rRNA gene was PCR amplified from yeast genomic DNA. The D1/D2 region of the LSU rRNA gene was amplified and sequenced with primers, NL1 and NL4 (Kurtzman & Robnett 1998). The PCR products were checked by agarose gel electrophoresis and purified using HiYield™ Gel/PCR DNA Fragments Extraction Kit (RBC Bioscience, Chung Ho City, Taipei, Taiwan). The purified products were submitted to Macrogen (Korea) for sequencing. Sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler, using an ABI PRISM® BigDye™ Terminator Cycle Sequencing Kit with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems, USA), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using the primers NL1 and NL4 for the D1/D2 region of the LSU rRNA gene. The fluorescent-labelled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730XL sequencer (Applied Biosystems, USA). The sequences were compared pairwise using a BLAST search (Altschul *et al.* 1997).

Study on plant growth-promoting traits

IAA production

The culture broth from the high IAA-producing isolates was subjected to HPLC analysis to accurately quantify the amount of IAA produced. Supernatants of IAA were analysed by an HPLC (Agilent 1100, Agilent Technologies, USA), equipped with a Cosmosil SC18-MS-II column (Nacalai Tesque, Japan) and UV detector at 280 nm. A mixture of 40 % of solution A (methanol:acetic acid:water were 10:0.3:89.7 v/v/v) and 60 % of solution B (methanol:acetic acid:water were 90:0.3:9.7 v/v/v) was used as the mobile phase with a flow rate of 0.5 ml min^{-1}, as described by Kim *et al.* (2006), except that isocratic elution was used instead of gradient elution. Authentic IAA (Sigma, USA) was used as a standard.

Siderophore production

Yeast culture grown on YPD agar at 28 ± 2 °C for 24 h was used as inoculum, which was point inoculated onto chrome azurol S (CAS) agar (Schwyn & Neilands 1987). The cultures were incubated at 28 ± 2 °C for 5 d. Isolates showing colonies surrounded by orange zones were considered siderophore-producing isolates.

Phosphate solubilisation, zinc oxide solubilisation and polyamine production
Yeast inoculum was prepared by cultivation in a 5 ml YPD broth (10 g l^{-1} yeast extract, 20 g l^{-1} peptone and 20 g l^{-1} dextrose) and the culture was incubated on a reciprocal shaker at 180 strokes/min at 28 ± 2 °C for 24 h. The inoculum density was adjusted to an optical density at 600 nm (OD600) of 0.10 and 3 μl was spotted onto Pikovskaya's agar (Zaidi *et al.* 2006), zinc oxide agar (Rokhbakhsh-Zamin *et al.* 2011), and Long Ashton decarboxylase (LAD) agar (Cloete *et al.* 2009), and incubated at 28 ± 2 °C for 5 d. The phosphate and zinc oxide solubilisation efficiency (SE) was calculated as a ratio between the diameter of the halo zone and the diameter of the colonies.

1-aminocyclopropane-1-carboxylate (ACC) deaminase activity
The presence of ACC deaminase activity was determined as described by Dell'Amico et al., 2005. The ability to use ACC as an N source is a consequence of the enzymatic activity of ACC deaminase. Yeast inoculum was prepared by cultivation in 5 ml YPD broth and tube cultures were incubated on a reciprocal shaker at 180 strokes/min at 28 ± 2 °C for 24 h. Inoculum was transferred to 5 ml N-free YCB broth supplemented with 3.0 mM ACC (filter sterilised) as sole nitrogen source whereas N-free YCB broth without ACC was used as a control. Tube cultures were incubated at 28 ± 2 °C on a reciprocal shaker at 180 strokes/min for 14 d. Yeast growth, determined by culture turbidity, indicated the potential of microorganisms to use ACC as their N-source through deamination.

Ammonia (NH_3) production
Yeast culture grown in 5 ml YPD broth was used as inoculum, and tube cultures were incubated on a reciprocal shaker at 180 strokes/min at 28 ± 2 °C for 24 h. The inoculum density was adjusted to an OD600 of 0.10, and 100 μl was transferred to 5 ml peptone water in a tube and incubated on a reciprocal shaker at 180 strokes/min at 28–30 °C for 5 d. Nessler's reagent was mixed with each sample. The development of a yellow-to-brown colour indicated a positive result for ammonia production, as described by Cappuccino & Sherman (1992).

Cell wall-degrading enzyme production
Yeast inoculum was prepared by cultivation in 5 ml YPD broth, and tube cultures were incubated on a reciprocal shaker at 180 strokes/min at 28 ± 2 °C for 24 h. Inoculum was adjusted to an OD600 of 0.10 at 600 nm, and 3 μl was spotted onto different media and incubated at 28 ± 2 °C for 1, 3, 5, and 7 d. Protease activity was determined by the presence of a clear zone on skimmed milk agar (Cattelan *et al.* 1999). Chitinolytic activity was determined by a clear zone on YNB medium (Difco, USA), amended with colloidal chitin as described by Shanmugaiah *et al.* (2008), except that YNB agar and 0.5 % colloidal chitin were used instead of YNB broth and 0.3 % colloidal chitin, respectively. Two kinds of cellulase activity were determined by the method described previously (Mangunwardoyo *et al.* 2011), except that 1 % substrate – i.e. carboxymethyl cellulose (CMC) or avicel – was used.

Catalase test
Yeasts were grown on YPD agar at 28 ± 2 °C for 18–24 h. The cultures were mixed with appropriate amounts of H_2O_2 on a glass slide to observe the evolution of oxygen.

Production of hydrogen cyanide (HCN)
HCN production was detected by the modified method of Bakker & Schippers (1987). Yeast inoculum was prepared by cultivation in 5 ml YPD broth, and tube cultures were incubated on a reciprocal shaker at 180 strokes/min at 28 ± 2 °C for 24 h. Inoculum intensity was adjusted to an OD600 of 0.10, and 3 μl was transferred onto YPD agar, amended with 4.4 g l^{-1} glycine by streaking. A disk of Whatman filter paper No. 1 was dipped into 0.5 % picric acid, and 2 % Na_2CO_3 was placed on the lid of the Petri plates. The plates were sealed with parafilm. After 1–7 d incubation at 28 ± 2 °C, any colour change of the filter paper was noted and the HCN production potential of the antagonists was assessed.

Evaluation of the in vitro *antagonism*
Yeast inoculum was prepared in YPD broth and incubated on a reciprocal shaker at 180 strokes/min at 28 ± 2 °C for 24 h. A loop full of inoculum was transferred onto potato dextrose agar (PDA) (Difco, USA) by streaking and the plate culture was incubated at 25 °C for 48 h. To evaluate the control of fungal growth by yeast, an assay in which yeast and fungi were grown side by side was set up, as described by Rosa *et al.* (2010). A control plate was prepared with only fungal inoculation. The experiment was performed with three replicates, incubating the plates at 25 °C. Mycelial growth was measured at 3, 5, and 7 d of incubation.

Production of antifungal volatile compounds
Yeast inoculum was prepared by a method similar to that used to evaluate the *in vitro* antagonism described above. To evaluate the production of antifungal volatile compounds by yeast, a plate culture in which yeast and fungi were grown side by side was prepared, as described by Rosa *et al.* (2010). However, it should be emphasised that a division was made in the agar medium in the Petri dishes to prevent the non-volatile exudates produced by the yeast from reaching the fungus through the culture medium.

Results and discussion

Screening of indole-3-acetic acid (IAA) producing yeast

IAA production was determined for a total of 1035 yeast isolates in tube cultivation. Colorimetric assay of IAA revealed that 167 isolates were IAA producers, whereas 868 isolates were non-IAA producers. It is possible to classify as low (<12.4 mg g^{-1} DCW) and high (>12.5 mg g^{-1} DCW) IAA-producing yeast by the numbers of 154 and 13 isolates, respectively.

Yeast identification

Based on colorimetric assay of IAA production, thirteen yeast isolates possessing high levels of IAA production

(12.5–64.4 mg g^{-1} DCW) were identified using the D1/D2 domain of the large subunit rRNA gene sequence similarity. Results revealed nine yeast species, which were: *Hannaella sinensis* (DMKU-RP45) (AB920308) (Wang & Bai 2008), *Cryptococcus flavus* (DMKU-RE12 (AB863475), DMKU-RE19 (AB863482), DMKU-RE67 (AB863528), and DMKU-RP128 (AB920310)) (Fonseca et al., 2011), *Kodamaea ohmeri* (DMKU-RP233) (AB920313) (Lachance & Kurtzman 2011), *Meyerozyma guilliermondii* (DMKU-RP168) (AB920311) (Kurtzman 2011a), *Pseudozyma antarctica* (DMKU-RP163 (AB920315), DMKU-RP213 (AB920312)) (Boekhout 2011a), *P. rugulosa* (DMKU-RE60) (AB863521) (Boekhout 2011a), *Rhodosporidium paludigenum* (DMKU-RP301) (AB920314) (Sampaio 2011a), *Rhodotorula taiwanensis* (DMKU-RP105) (AB920309) (Sampaio 2011b), and *Torulaspora globosa* (DMKU-RP31) (AB920307) (Kurtzman 2011b) among the thirteen isolates selected.

Characterisation of plant growth-promoting traits in yeast strains

Determination of IAA production

According to results obtained from colorimetric assay, the cell-free culture broth of the yeast isolates that showed high IAA production were subjected to HPLC analysis to confirm the amount of IAA produced. Results indicated production in a range of 1.2–29.3 mg IAA/g DCW (Table 1). Four yeast species produced more than 4.8 mg IAA/g DCW. They were: *Hannaella sinensis* (DMKU-RP45), *Cryptococcus flavus* (DMKU-RE12, DMKU-RE19, DMKU-RE67, and DMKU-RP128), *Torulaspora globosa* (DMKU-RP31), and *Rhodosporidium paludigenum* (DMKU-RP301), which was the best IAA-producing strain obtained in this study. *R. paludigenum*, a carotenoid-producing yeast, was first described by Fell & Tallman (1980), and was reported to produce carotenoids when grown in glycerol as the sole carbon source (Yimyoo *et al.* 2011). In addition, this yeast has also been used in various applications: it proved effective as an antioxidant when applied to *Litopenaeus vannamei* aquaculture (Yang *et al.* 2010; Yang *et al.* 2013), and it helped in control of postharvest decay on cherry tomatoes (Wang *et al.* 2010) and in biocontrol of *Penicillium expansum* infection in pear fruit (Yu *et al.* 2013). Among all the yeast studied in the present work, *R. paludigenum* DMKU-RP301 was the best IAA producer at 29.3 mg g^{-1} DCW and also exhibited siderophore production (see below). The present work is therefore the first to report that *R. paludigenum* possesses plant growth-promoting characters (i.e. IAA- and siderophore-producing abilities). *Bullera sinensis*, previously described as *B. derxii*, is a basidiomycetous yeast that may form ballistoconidia (Nakase & Suzuki 1986). There has been a report of secretion of a fungicidal thermolabile and protease-sensitive toxin with a cureless killer phenotype of *B. sinensis* (Golubev & Nakase 1997); however, *H. sinensis* DMKU-RP45 found in this study may not possess killing activity and showed neither production of antifungal volatile compounds nor *in vitro* antagonism in a solid medium. However, *H. sinensis* DMKU-RP45 has been characterised here for its IAA production activity at 7.1 mg g^{-1} DCW.

IAA-producing microorganisms are receiving attention as good sources of biofertilizer (Sasikala & Ramana 1998). For almost a decade, there have been reports on IAA production by yeasts. El-Tarabily (2004) studied the plant growth-promoting yeasts *Candida valida*, *Rhodotorula glutinis*, and *Trichosporon asahii*, isolated from sugar beet rhizospheres. These three yeast species were found to support beet growth by production of IAA at 29.5, 24.1, and 31.7 μg ml^{-1}, respectively. Nassar *et al.* (2005) investigated IAA production of endophytic yeasts isolated from maize (*Zea mays* L.) root. Each isolate was cultured in glucose peptone broth (GPB), with or without 1.25 % L-tryptophan, at 25 °C for 7 d, under shaking at 250 rev/min. Only eight out of 24 isolates were shown to produce IAA in the medium with and without L-tryptophan. Xin *et al.* (2009) characterised three IAA-producing endophytic yeasts (WP1, PTD2 and PTD3) from the *Populus* tree and found the highest IAA production (39 mg g^{-1} dry cell) in the isolate WP1, whereas the isolates PTD2 and PTD3 produced 18 and 8 mg g^{-1} dry cell of IAA, respectively. Deng *et al.* (2012) reported a low IAA production of 11.7 μg ml^{-1} (quantified by a colorimetric

Table 1 – Direct plant growth-promoting traits of four endophytic and eleven epiphytic yeasts isolated from rice leaves (RE and RP, respectively); Solubilisation efficiency (SE) = Halo zone/Diameter of colonies.

Isolates	IAA production (mg g^{-1} DW)	Phosphate solubilisation (SE)	Zinc oxide solubilisation (SE)	Polyamine production[a]	NH_3 production[b]	Catalase test[a]
DMKU-RE12	7.2	0	0	–	++	+
DMKU-RE19	7.9	0	0	–	+	+
DMKU-RE60	2.7	0	0	+	++	+
DMKU-RE67	9.7	0	0	–	++	+
DMKU-RP31	4.9	1.2	1.2	–	–	+
DMKU-RP45	7.1	0	0	–	+	+
DMKU-RP105	2.8	0	0	–	+	+
DMKU-RP128	7.2	0	0	–	+	+
DMKU-RP163	1.5	0	0	–	++	+
DMKU-RP168	4.5	0	0	–	+	+
DMKU-RP213	1.2	0	0	+	+	+
DMKU-RP233	2.0	0	0	–	+	+
DMKU-RP301	29.3	0	0	–	+	+

a + positive, – not detected.
b + slight positive result, ++ strong positive result.

method using Salkowski reagent) by heavy metal resistant endophytic *Cryptococcus* sp. CBSB78 from rape (*Brassica chinensis*).

Siderophore production, phosphate solubilisation, and zinc oxide solubilisation

To determine as many of the plant growth-promoting characters as may possibly exist in yeast, a group of yeast isolates which were high IAA producers were studied. Thirteen isolates possessing 1.2–29.3 mg g^{-1} DCW of IAA (by HPLC) were then tested for phosphate solubilisation, siderophore production and some other important characters for plant growth promotion (Tables 1 and 2). We found that five isolates – i.e. *Pseudozyma rugulosa* DMKU-RE60, *Hannaella sinensis* DMKU-RP45, *Rhodotorula taiwanensis* DMKU-RP105, *P. antarctica* DMKU-RP213 and *Rhodosporidium paludigenum* DMKU-RP301 – showed positive results for siderophore production, as they altered the CAS-blue medium colour from blue to orange (Table 2; Fig 1). However, none of these isolates possessed antifungal activity either in the manner of antagonism on a solid medium or in production of antifungal volatile compounds (Table 2). Investigation of phosphate solubilising activity revealed that only *Torulaspora globosa* DMKU-RP31 indicated *in vitro* calcium phosphate solubilisation capability and showed an obvious solubilising activity, expressed in the solubilisation efficiency (SE) unit of 1.22 (Table 1; Fig 2A). In addition to phosphate solubilisation, *T. globosa* DMKU-RP31 also exhibited zinc oxide solubilisation, expressed in the SE unit of 1.17 (Table 1; Fig 2B). The yeast *T. globosa* was reported to be antagonistic towards the phytopathogenic mold *Colletotrichum sublineolum*, even though no siderophores, volatile compounds or fungal wall hydrolytic enzymes were detected (Rosa *et al.* 2010).

Yeasts have been reported to produce siderophores, chelating agents for the ferric ion (Fe^{3+}) which not only support plant growth *via* iron chelating but also indirectly inhibit plant pathogenic fungi by scavenging available iron from the environment (Calvente *et al.* 1999; Wang & Chi 2009). However, it is not necessarily the case that yeasts which show antifungal activity will definitely produce siderophores as a key element of such activity. Evidence of this was found in the yeast *Torulaspora globosa* 1S112, isolated from sugarcane rhizosphere, which reduced growth of the phytopathogenic mold *Colletotrichum sublineolum* but did not produce siderophores, volatile compounds or hydrolytic enzymes (Rosa *et al.* 2010).

Investigation of phosphate solubilising activity revealed that the yeast *Torulaspora globosa* DMKU-RP31 showed an obvious solubilising activity and also exhibited zinc oxide solubilisation. Results on phosphate solubilisation appeared to be on the same level as those reported in rhizospheric *Acinetobacter* strains of pearl millet, whereas the degree of zinc oxide solubilising activity found in *T. globosa* DMKU-RP31 was far less than that observed in the *Acinetobacter* strains (Rokhbakhsh-Zamin *et al.* 2011). To date, the literature related to yeast mineral solubilisation remains limited, although zinc solubilisation has been reported in fungi (White *et al.* 1997).

Polyamine, NH_3 and ACC deaminase production

Study of polyamine production indicated that two yeasts – *Pseudozyma rugulosa* DMKU-RE60 and *P. antarctica* DMKU-RP213 – produce polyamine in culture broth (Table 1; Fig 3). NH_3 production was detected at two different levels – i.e. a low or a high level – in nearly all the yeasts, except *T. globosa* DMKU-RP31. No relationship between mineral solubilisation and ammonia production in this yeast could be drawn. However, ACC deaminase activity was not detected in any of the yeast isolates studied here.

Polyamines are organic polycations having variable hydrocarbon chains and two or more primary amino groups. Production of diamine putrescine, triamine spermidine, and tetraamine has been claimed to stimulate root cell division

Table 2 – Indirect plant growth-promoting traits of four endophytic and eleven epiphytic yeasts isolated from rice leaves (RE and RP, respectively); Diameter of halo zone = halo zone (mm) – colony zone (mm); Diameter of clear zone = clear zone (mm) – colony zone (mm).

Isolates	Siderophore production; (diameter of halo zone, mm)	Cell wall-degrading enzyme production		Antagonism in solid medium[a]	Production of antifungal volatile compounds[a]
		Protease (diameter of clear zone, mm)	Cellulase		
			CMC (diameter of halo zone, mm)		
DMKU-RE12	–	–	36	–	–
DMKU-RE19	–	–	36	–	–
DMKU-RE60	6	15	–	–	–
DMKU-RE67	–	–	34	–	–
DMKU-RP31	–	–	–	+	+
DMKU-RP45	2	–	–	–	–
DMKU-RP105	2	–	–	–	–
DMKU-RP128	–	–	35	–	–
DMKU-RP163	–	1	–	–	–
DMKU-RP168	–	–	–	–	–
DMKU-RP213	6	6	–	–	–
DMKU-RP233	–	–	–	–	–
DMKU-RP301	5	–	–	–	–

a + positive, - not detected.

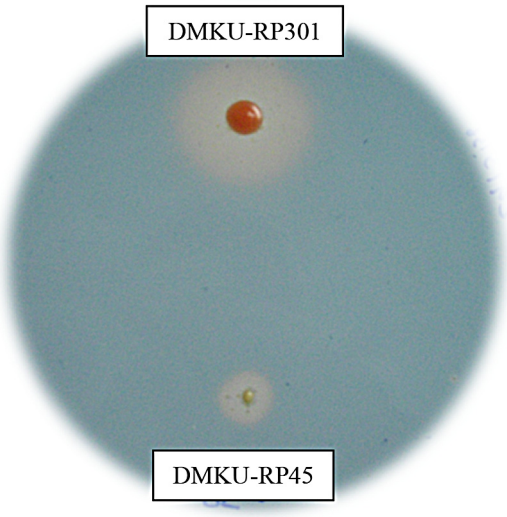


Fig 1 – Siderophore was produced by DMKU-RP301 and DMKU-RP45 on chrome azurol S (CAS) agar by point inoculation and incubation at 28 °C for 5 d.

and, hence, increase root growth (Tang & Newton 2005; Cloete *et al.* 2009). As one of the general characteristics studied in plant growth-promoting bacteria (Joseph *et al.* 2007; Chaiharn *et al.* 2008; Ahemad & Khan 2012), it is therefore of interest to investigate NH_3 production in the yeast isolates studied here. However, it should be noted that ammonia production is quite rare in yeast, an exception being the yeast *Debaryomyces hansenii*, which is able to produce NH_3 when grown on cheese agar (Gori *et al.* 2007). Samuel & Muthukkaruppan (2011) reported that NH_3 is commonly produced in both rhizobacteria and fungi associated with rice, mangrove and effluent-contaminated soil.

In our literature review, ACC deaminase activity was reported in only a few yeast strains, such as *Hansenula saturnus*, *Issatchenkia occidentalis*, *Candida tropicalis*, and *Cryptococcus* sp. (Minami *et al.* 1998; Palmer *et al.* 2007; Amprayn *et al.* 2012; Deng *et al.* 2012). Therefore, it is not surprising that we found none of our yeast isolates exhibited this enzyme activity.

Catalase test and HCN production

Catalase, a very important enzyme in protecting the cell from oxidative damage by reactive oxygen species (ROS), was investigated in all thirteen yeast isolates. Since catalase production is one of the plant growth-promoting character found in bacteria (Joseph *et al.* 2007), it was therefore of interest to determine catalase production in all selected yeast strains. Catalase activity was found in all yeasts studied here (Table 1).

Catalase activity can be detected in yeasts, especially those associated with plant leaves and utilising methanol as carbon and energy sources. This is due to phyllospheric methanol emission as a result of pectin degradation. The methanol utilisation pathway requires catalase activity to react with hydrogen peroxide in peroxisome. Leaf-associated yeast may be able to utilise methanol and hence produce catalase to detoxify hydrogen peroxide in peroxisome. Results indicated that all of the strains tested showed catalase activity whereas none of them exhibited HCN production (data not shown). These results agreed with those reported in plant growth-promoting rhizobacteria associated with chickpea (Joseph *et al.* 2007) and rice (Samuel & Muthukkaruppan 2011), indicating that the filamentous fungi *Penicillium* spp. and *Aspergillus niger*, associated with rice, mangrove and effluent-contaminated soil, showed the same contrasting results of catalase and HCN tests, respectively. However, HCN has long been reported as a secondary metabolite produced by many antagonistic *Pseudomonas* species (Freeman *et al.* 1975; Michelsen & Stougaard 2012).

Production of cell wall-degrading enzymes

It is well known that the fungal wall consists of rather complicated contents, such as chitin, β-1,3-glucan, mannoprotein and cellulose in some species. The ability of yeast to produce

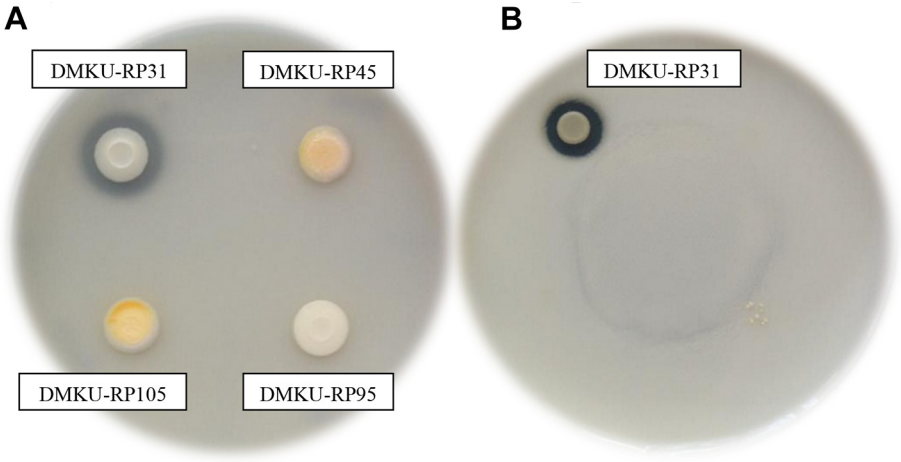


Fig 2 – Phosphate and zinc oxide solubilising halos produced by DMKU-RP31 on Pikovskaya's agar (A) and zinc oxide agar (B), respectively by spot inoculation and incubation at 28 °C for 5 d.

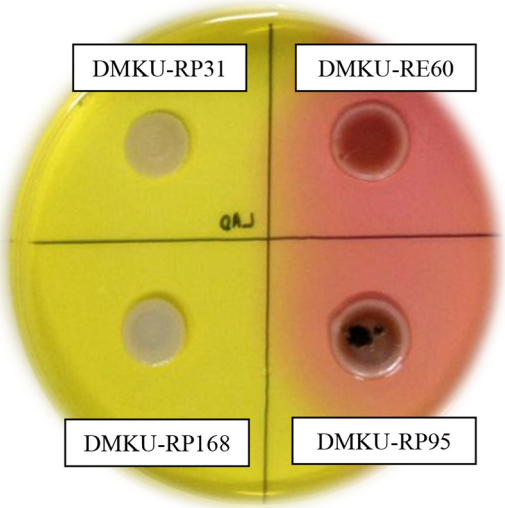


Fig 3 – Polyamine was produced by DMKU-RE60 and DMKU-RP95 on Long Ashton decarboxylase (LAD) agar by spot inoculation and incubation at 28 °C for 5 d.

some hydrolytic enzymes, such as glucanase, chitinase, protease, and cellulase, helps to promote antagonistic activity against plant pathogenic fungi. As a consequence, plant growth will therefore be indirectly promoted. Assay of fungal cell wall-degrading enzymes indicated that the yeasts *Pseudozyma rugulosa* DMKU-RE60 and two strains of *P. antarctica* (DMKU-RP163 and DMKU-RP213) produced protease (Fig 4A). None of the yeast isolates studied here showed avicelase or chitinase activity. However, all strains of *Cryptococcus flavus* identified in this study (DMKU-RE12, DMKU-RE19, DMKU-RE67 and DMKU-RP128) produced carboxy methyl cellulase (CMCase), as shown in Fig 4B. *C. flavus* is a basidiomycetous yeast that has been shown to possess amylase, CMCase and xylanase activities (Wanderley *et al.* 2004; Parachin *et al.* 2009). In our study, CMCase activity was detected in four strains of *C. flavus*, which additionally possessed plant growth hormone, IAA production activity. The levels of IAA production of 7.2, 7.9, 9.7 and 7.2 mg g^{-1} DCW were found in *C. flavus* DMKU-RE12, DMKU-RE19, DMKU-RE67, and DMKU-RP128, respectively.

Production of hydrolytic enzymes, such as exo-β-1,3-glucanase which destroys fungal cell wall, has been reported in the yeast *Pichia anomala* (Jijakli & Lepoivre 1998). Mangunwardoyo *et al.* (2011) screened yeasts isolated from soil, sediment and river water in Indonesia for CMCase, avicelase and cellobiase and found that a total of 16 yeast isolates possessed those wall-degrading enzymes in the form of either a single enzyme or two enzyme components.

Evaluation of in vitro *antagonism*

The thirteen yeast isolates that possessed high IAA production abilities were investigated for their antagonistic activity against rice fungal pathogens i.e. *Fusarium moniliforme*, *Helminthosporium oryzae*, and *Rhizoctonia solani*. Our results indicated that only the yeast *Torulaspora globosa* DMKU-RP31 showed significant antagonistic effect against all fungi tested on PDA (Fig 5A).

Production of antifungal volatile compounds

Similar to the antagonism studies, results on production of antifungal volatile compounds revealed that only the yeast *Torulaspora globosa* DMKU-RP31 produced volatile compounds that significantly inhibited fungal growth (Fig 5B). Rosa *et al.* (2010) also reported the antagonistic action of *T. globosa* 1S112 against *Colletotrichum sublineolum,* which is a sorghum pathogen. However, no siderophores, volatile compounds or fungal wall hydrolytic enzymes were detected in *T. globosa* 1S112. It should be emphasised that the report on *T. globosa* 1S112 indicated a lack of volatile compound production which

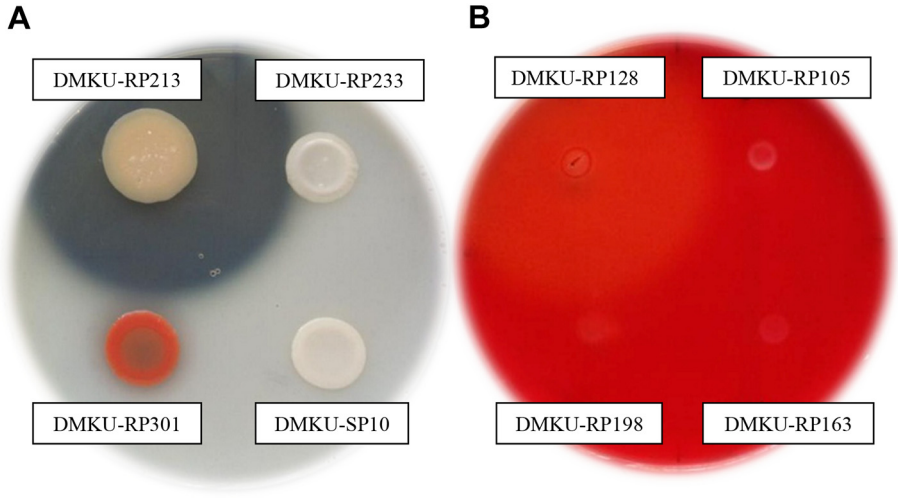


Fig 4 – Cell wall-degrading enzyme production: (A) Protease was produced by DMKU-RP213 on skimmed milk agar. (B) Cellulase was produced by DMKU-RP128 on YNB agar amended with 1 % carboxymethyl cellulose (CMC) agar.

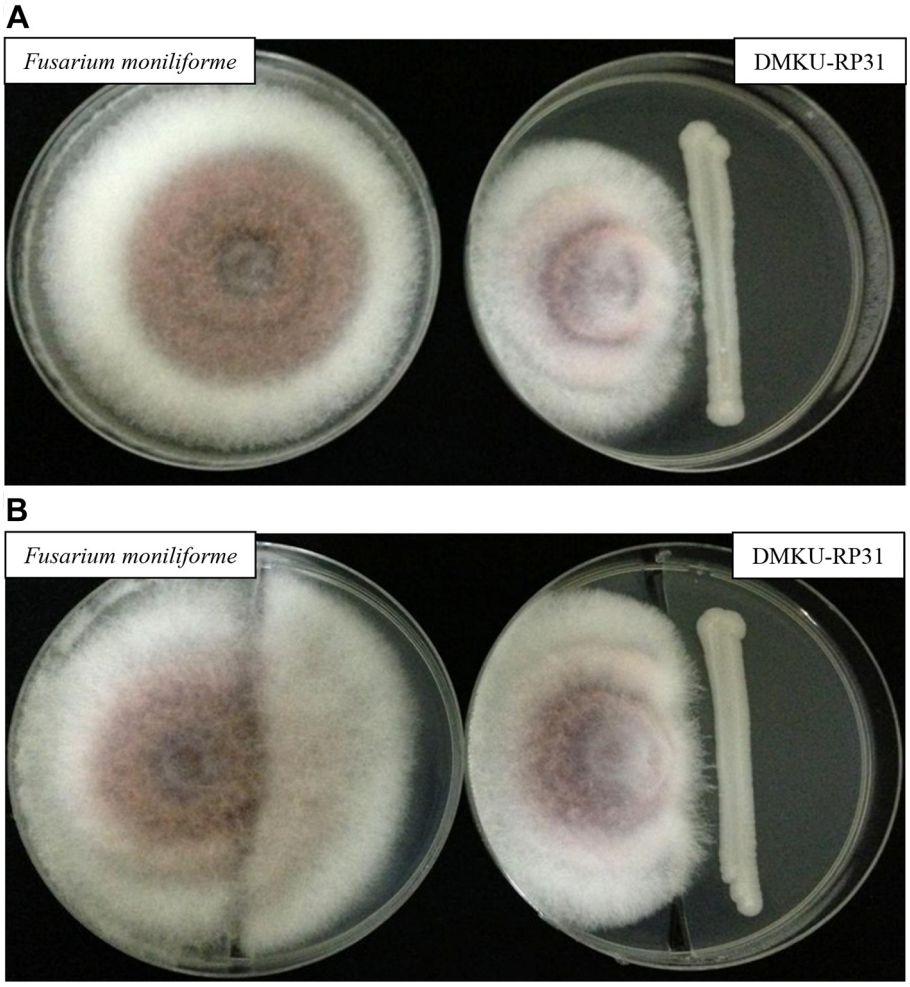


Fig 5 – Antagonism by DMKU-RP31 against *Fusarium moniliforme*, which causes disease in rice plants. (A) Evaluation of *in vitro* antagonism in a solid medium: *F. moniliforme* alone (left) and yeast antagonistic to *F. moniliforme* (right). (B) Test of the production of antifungal volatile compounds: *F. moniliforme* alone (left) and yeast antagonistic to *F. moniliforme* (right).

inhibited the mold growth, whereas *T. globosa* DMKU-RP31, isolated in our work, demonstrated this ability.

Previous studies of IAA-producing yeast were reported in *Lindera* (*Williopsis*) *saturnus* (Nassar *et al.* 2005), *Rhodotorula graminis* and *R. mucilaginosa* (Xin *et al.* 2009), *Candida tropicalis* (Amprayn *et al.* 2012), *Candida valida*, *Rhodotorula glutinis*, and *Trichosporon asahii* (El-Tarabily 2004), *Cryptococcus* sp. (Deng *et al.* 2012) and 20 yeast species isolated from the phylloplanes of various plant species collected from seven provinces in Thailand (Limtong & Koowadjanakul 2012). Although a higher IAA level was reported in the yeast *Rhodotorula* spp. by Xin *et al.* (2009), no other plant growth-promoting traits have yet been proposed by the authors. It must be noted that the lower NH_3 and siderophore production found in *R. paludigenum* DMKU-RP301 might not recommend this yeast as a good candidate for plant growth promotion. However, a higher level of IAA production was foreseen through optimisation of either medium composition or growth conditions. Experimental design has been set up and placed under investigation not only for *R. paludigenum* DMKU-RP301 but also for *Torulaspora globosa* DMKU-RP31. The latter has received more attention than the others because of its capability to produce antifungal volatile compounds, hence showing significant antagonistic effects against three molds on a solid medium. This is due to the fact that not only phytohormones such as IAA are necessary to support plant growth, but also other indirect plant growth-promoting traits are required to obtain good plant yield.

From overall results, we found three plant growth-promoting traits – i.e. antagonism to fungal plant pathogens, antifungal volatile compound production and a moderate level of IAA production (4.9 mg g^{-1} DCW) – in *Torulaspora globosa* DMKU-RP31, whereas *Rhodotorula paludigenum* DMKU-RP301 showed a much higher IAA production (29.3 mg g^{-1} DCW) with some production of siderophore as an additional character. We therefore propose that *T. globosa* DMKU-RP31 might be used in two ways: enhancing plant growth and acting as a biocontrol agent. However, we also found high levels of IAA production (7.2–9.7 mg g^{-1} DCW) in four *Cryptococcus flavus* strains, accompanied with NH_3 production as well as CMCase activity. These yeasts are also strains of interest for optimal IAA production.

Acknowledgements

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Plant growth-promoting traits of epiphytic and endophytic yeasts isolated from rice and sugar cane leaves in Thailand

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ABSTRACT

A total of 1035 yeast isolates, obtained from rice and sugar cane leaves, were screened primarily for indole-3-acetic acid (IAA) production. Thirteen isolates were selected, due to their IAA production ranging from 1.2 to 29.3 mg g^{-1} DCW. These isolates were investigated for their capabilities of calcium phosphate and ZnO_3 solubilisation, and also for production of NH_3, polyamine, and siderophore. Their 1-aminocyclopropane-1-carboxylate (ACC) deaminase, catalase and fungal cell wall-degrading enzyme activities were assessed. Their antagonism against rice fungal pathogens was also evaluated. Strain identification, based on molecular taxonomy, of the thirteen yeast isolates revealed that four yeast species – i.e. *Hannaella sinensis* (DMKU-RP45), *Cryptococcus flavus* (DMKU-RE12, DMKU-RE19, DMKU-RE67, and DMKU-RP128), *Rhodosporidium paludigenum* (DMKU-RP301) and *Torulaspora globosa* (DMKU-RP31) – were capable of high IAA production. Catalase activity was detected in all yeast strains tested. The yeast *R. paludigenum* DMKU-RP301 was the best IAA producer, yielding 29.3 mg g^{-1} DCW, and showed the ability to produce NH_3 and siderophore. Different levels of IAA production (7.2–9.7 mg g^{-1} DCW) were found in four strains of *C. flavus* DMKU-RE12, DMKU-RE19, and DMKU-RE67, which are rice leaf endophytes, and strain DMKU-RP128, which is a rice leaf epiphyte. NH_3 production and carboxymethyl cellulase (CMCase) activity was also detected in these four strains. Antagonism to fungal plant pathogens and production of antifungal volatile compounds were exhibited in *T. globosa* DMKU-RP31, as well as a moderate level of IAA production (4.9 mg g^{-1} DCW). The overall results indicated that *T. globosa* DMKU-RP31 might be used in two ways: enhancing plant growth and acting as a biocontrol agent. In addition, four *C. flavus* were also found to be strains of interest for optimal IAA production.

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Introduction

Microorganisms can promote plant growth by both direct and indirect means. Production of phytohormones, such as auxins or gibberellins, is a direct mechanism that is used by plant growth-promoting microorganisms. Auxins are a group of compounds having an indole ring, that have a positive effect on plants by stimulating seed germination, root initiation, cell elongation, and seedling growth (El-Tarabily 2008). Indole-3-acetic acid (IAA), a plant growth hormone classified as an indole derivative of the auxin family, has been widely studied and found to be a dominant type of auxin in plants. Several important roles that IAA has been shown to play in plants include stimulation of cell division, cell elongation, cell differentiation, light and gravitational responses, and regulation of leaf fall and fruit ripening (Trotsenko *et al.* 2001; Teale *et al.* 2006). Different levels of IAA production have been reported in several groups of bacteria and yeasts.

As well as phytohormone production, microorganisms directly enhance plant growth by making nutrients more available in various ways. Ammonia (NH_3) production by plant-associated microorganisms is advantageous to plants because NH_3 provides available nitrogen that supports plant growth. Microorganisms also assist in phosphorus uptake. Plants receive phosphorus in the form of phosphate, which is sometimes present in nature in an insoluble form that is unusable by plants. Microorganisms that possess phosphate solubilising capabilities provide phosphorus in a soluble form, as required by plants. Similarly, the micronutrient zinc needs to be converted from an insoluble to a soluble form, and this is done by microbial activity. In the case of iron, siderophores produced by microbes help in the accumulation of Fe in Fe-deficient environments, and in its uptake by plants (Vessey 2003). By producing siderophores that trap iron, plant growth-promoting microorganisms support plant growth directly. However, siderophores can also have a negative effect on fungal pathogens through iron depletion (Calvente *et al.* 1999; Wang & Chi 2009). In this way, the growth of phytopathogens is limited. Therefore, siderophore production by plant growth-promoting microorganisms may also be regarded as an indirect mechanism for plant growth enhancement.

In addition to siderophore production, microbes also possess other indirect mechanisms for the suppression of plant pathogens, *via* the production of antibiotics and fungal wall-degrading enzymes such as chitinase, cellulase and β-1,3-glucanase (Chaiharn *et al.* 2008). Polyamines – compounds involved in many cellular processes, including the maintenance of cell macromolecular biosynthesis, survival and growth (Amprayn *et al.* 2012) – also function in plant growth promotion. The production of polyamines (spermidine, spermine, putrescine) has been claimed to be a tool to stimulate root cell division and, hence, increase root growth (Tang & Newton 2005; Cloete *et al.* 2009). ACC deaminase is an enzyme that cleaves ACC to form α-ketobutyrate and ammonia, and decreases ethylene levels in host plants (Sessitsch *et al.* 2005; Sun *et al.* 2009). Plant growth-promoting bacteria with ACC deaminase activity can sequester ACC and potentially lower deleterious levels of ethylene in the plant host, which alters its physiology (Glick 2004, 2005; Glick *et al.* 2007a,b). The function of ACC deaminase in plant-microbe systems results in the stimulation of plant root elongation, a phenomenon which has been well-studied (Glick *et al.* 2007a; Husen *et al.* 2008; Yim *et al.* 2010). Microorganisms can also produce hydrogen cyanide (HCN), which inhibits the hyphal growth of fungi (Freeman *et al.* 1975; Fry & Evans 1977).

The direct and indirect plant growth-promoting characters at different levels of various yeast species have been reviewed. In terms of direct promotion, nutrient uptake and phytohormone production have been exhibited in a diverse range of yeast. IAA and indole-3-pyruvic acid (IPYA) were produced *in vitro* by the endophytic yeast *Lindera* (*Williopsis*) *saturnus*, isolated from maize roots (Nassar *et al.* 2005), whereas *Rhodotorula*, the first endophytic yeast isolated from the *Populus* plant, was also shown to produce IAA (Xin *et al.* 2009). In addition, the endophytic yeast *Cryptococcus* sp. CBSB78, isolated from rape (*Brassica chinensis*), although producing low concentrations of IAA (11.7 $\mu g\ ml^{-1}$), showed heavy metal resistance and hence promoted the growth of *Brassica* spp. in metal-contaminated soils (Deng *et al.* 2012).

IAA production was not only found in endophytic yeast but also in the epiphytic yeast *Candida maltosa*, isolated from the phylloplane; relatively high concentrations of IAA (121.4–234.1 $mg\ l^{-1}$) were produced when cultivated in YPD broth, supplemented with 0.1 % L-tryptophan (Limtong & Koowadjanakul 2012). In the case of the soil yeast *Candida tropicalis* HY, the production of small quantities of IAA was accompanied by tri-calcium phosphate solubilisation and the production of phytase, which enhances phosphate release from phytate-containing materials available in nature.

In addition to direct plant growth-promoting characters, *C. tropicalis* HY showed indirect characters such as polyamine production and high ACC deaminase activity, which enhanced plant growth by lowering ethylene levels (Amprayn *et al.* 2012). Recently, high ACC deaminase activity has been reported in two yeasts: *Hansenula saturnus* and *Issatchenkia occidentalis* (Palmer *et al.* 2007). The production of polyamines – i.e. putrescine, spermidine, and spermine – was exhibited in different strains of the yeast *Debaryomyces hansenii* (Reyes-Becerril *et al.* 2011). As in other plant growth-promoting microbes, siderophore production by yeast is another critical tool that bolsters plant growth. This may be considered a direct or an indirect role of yeast. *Rhodotorula glutinis* can be claimed to indirectly promote plant growth, through the antagonistic action of siderophores affecting the postharvest pathogen *Penicillium expansum* (Calvente *et al.* 1999).

Diverse yeast species can act as biocontrol agents against fungal plant pathogens. It has been shown that *Candida valida*, *Rhodotorula glutinis*, and *Trichosporon asahii*, isolated from sugar beet rhizospheres, possess biocontrol activity against *Rhizoctonia solani*, a soil-borne fungal plant pathogen that causes root diseases (El-Tarabily 2004). The yeast *Cryptococcus laurentii*, in combination with IAA, was shown to inhibit gray mold, *Botrytis cinerea*, in harvested apple fruit (Yu *et al.* 2008). The yeast *Rhodotorula glutinis* ySL 30 was shown to inhibit *Botrytis cinerea* (Sansone *et al.* 2005). The biological control by the yeast *Torulaspora globosa* against *Colletotrichum sublineolum* in sorghum was evaluated, and one strain of *T. globosa* exhibited killer activity against two sensitive strains of *C. sublineolum*, which is

a novel capacity for *T. globosa* resulting in hyphal deformities but not cell death (Rosa *et al.* 2010).

The capacity to produce enzymes which destroy the fungal cell wall, such as chitinase and β-1,3-glucanase, was exhibited in the yeasts *Pichia anomala*, *Candida guilliermondii*, and *C. oleophila* (Jijakli & Lepoivre 1998; Saligkarias *et al.* 2002). Another putative action mode is the production of toxic volatile compounds, as observed in different *Saccharomyces cerevisiae* species (Fialho 2004).

The combined effects of both direct plant growth-promoting characters, such as plant nutritional supplementation and IAA production, and indirect characters, such as antifungal activity or the scavenging of nutrients required by plant pathogens, are much more supportive of plant growth overall. Microbial activities working in this concerted fashion can augment both the growth of the plant and the economy of the farmer. Our literature review has so far indicated that Amprayn *et al.* (2012) have reported the widest study on plant growth-promoting characters in yeast. In this paper, we report on plant growth-promoting traits of Thai yeast species isolated from rice and sugar cane leaves, including some traits that have not yet been reported.

Materials and methods

Microorganisms

Epiphytic and endophytic yeasts were isolated from rice and sugar cane leaves collected in Thailand, and maintained in a private culture collection at the Department of Microbiology, Kasetsart University, Thailand. The yeasts used in this study consisted of 125 endophytic yeasts and 305 epiphytic yeasts from rice leaves, plus 149 endophytic yeasts and 456 epiphytic yeasts from sugar cane leaves. Yeast strains designated with the initial code of DMKU-RE refer to endophytic yeasts isolated from rice leaves, whereas DMKU-RP refers to epiphytic yeasts isolated from rice leaves. Yeast strains isolated from sugarcane leaves were designated with the initial code DMKU-SE and DMKU-SP for endophytic and epiphytic yeasts, respectively. Rice fungal pathogens – i.e. *Fusarium moniliforme*, *Helminthosporium oryzae*, and *Rhizoctonia solani* – were used in this study. The yeasts were maintained on YPD medium while the fungal strains were maintained on PDA medium.

Screening of indole 3-acetic acid (IAA)-producing yeast

Assessment of IAA production was carried out using a modification of the method described by Xin *et al.* (2009). Yeast inoculum was prepared on YPD agar (10 g l^{-1} yeast extract, 20 g l^{-1} peptone, 20 g l^{-1} dextrose, and 15 g l^{-1} agar) and incubated at 28 °C for 24 h. One loop of inoculum was transferred into 5 ml of YPD broth, amended with 0.1 % (w/v) L-tryptophan. All culture tubes were incubated in a reciprocal shaker at 180 strokes/min at 28 ± 2 °C for 7 d. Culture supernatant was collected by centrifugation for 5 min at 8000 rpm, prior to quantitative analysis of IAA. The amount of IAA was determined by a colorimetric method. The colorimetric assay of IAA was carried out by mixing 1 ml of culture supernatant with 1 ml of Salkowski reagent containing 12 g l^{-1} $FeCl_3$ and 7.9 M H_2SO_4 (Glickmann & Dessaux 1994) followed by 30 min incubation in the dark for complete pink–red colour development. Colour intensity was determined as A_{530} using a spectrophotometer (Thermo Electron Corporation, USA). The IAA concentration of the sample was compared with a linear calibration curve obtained from an authentic IAA standard. The high IAA-producing isolates were selected for use in further studies.

Yeast identification

Yeasts were identified by analysis of the D1/D2 region of the large subunit of rRNA (LSU rRNA) gene sequence similarities (Kurtzman & Robnett 1998). Methods for DNA extraction and amplification of the D1/D2 domain were described previously (Limtong *et al.* 2007). The D1/D2 domain of the LSU rRNA gene was PCR amplified from yeast genomic DNA. The D1/D2 region of the LSU rRNA gene was amplified and sequenced with primers, NL1 and NL4 (Kurtzman & Robnett 1998). The PCR products were checked by agarose gel electrophoresis and purified using HiYield™ Gel/PCR DNA Fragments Extraction Kit (RBC Bioscience, Chung Ho City, Taipei, Taiwan). The purified products were submitted to Macrogen (Korea) for sequencing. Sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler, using an ABI PRISM® BigDye™ Terminator Cycle Sequencing Kit with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems, USA), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using the primers NL1 and NL4 for the D1/D2 region of the LSU rRNA gene. The fluorescent-labelled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730XL sequencer (Applied Biosystems, USA). The sequences were compared pairwise using a BLAST search (Altschul *et al.* 1997).

Study on plant growth-promoting traits

IAA production

The culture broth from the high IAA-producing isolates was subjected to HPLC analysis to accurately quantify the amount of IAA produced. Supernatants of IAA were analysed by an HPLC (Agilent 1100, Agilent Technologies, USA), equipped with a Cosmosil SC18-MS-II column (Nacalai Tesque, Japan) and UV detector at 280 nm. A mixture of 40 % of solution A (methanol:acetic acid:water were 10:0.3:89.7 v/v/v) and 60 % of solution B (methanol:acetic acid:water were 90:0.3:9.7 v/v/v) was used as the mobile phase with a flow rate of 0.5 ml min^{-1}, as described by Kim *et al.* (2006), except that isocratic elution was used instead of gradient elution. Authentic IAA (Sigma, USA) was used as a standard.

Siderophore production

Yeast culture grown on YPD agar at 28 ± 2 °C for 24 h was used as inoculum, which was point inoculated onto chrome azurol S (CAS) agar (Schwyn & Neilands 1987). The cultures were incubated at 28 ± 2 °C for 5 d. Isolates showing colonies surrounded by orange zones were considered siderophore-producing isolates.

Phosphate solubilisation, zinc oxide solubilisation and polyamine production
Yeast inoculum was prepared by cultivation in a 5 ml YPD broth (10 g l^{-1} yeast extract, 20 g l^{-1} peptone and 20 g l^{-1} dextrose) and the culture was incubated on a reciprocal shaker at 180 strokes/min at 28 ± 2 °C for 24 h. The inoculum density was adjusted to an optical density at 600 nm (OD600) of 0.10 and 3 μl was spotted onto Pikovskaya's agar (Zaidi *et al.* 2006), zinc oxide agar (Rokhbakhsh-Zamin *et al.* 2011), and Long Ashton decarboxylase (LAD) agar (Cloete *et al.* 2009), and incubated at 28 ± 2 °C for 5 d. The phosphate and zinc oxide solubilisation efficiency (SE) was calculated as a ratio between the diameter of the halo zone and the diameter of the colonies.

1-aminocyclopropane-1-carboxylate (ACC) deaminase activity
The presence of ACC deaminase activity was determined as described by Dell'Amico et al., 2005. The ability to use ACC as an N source is a consequence of the enzymatic activity of ACC deaminase. Yeast inoculum was prepared by cultivation in 5 ml YPD broth and tube cultures were incubated on a reciprocal shaker at 180 strokes/min at 28 ± 2 °C for 24 h. Inoculum was transferred to 5 ml N-free YCB broth supplemented with 3.0 mM ACC (filter sterilised) as sole nitrogen source whereas N-free YCB broth without ACC was used as a control. Tube cultures were incubated at 28 ± 2 °C on a reciprocal shaker at 180 strokes/min for 14 d. Yeast growth, determined by culture turbidity, indicated the potential of microorganisms to use ACC as their N-source through deamination.

Ammonia (NH_3) production
Yeast culture grown in 5 ml YPD broth was used as inoculum, and tube cultures were incubated on a reciprocal shaker at 180 strokes/min at 28 ± 2 °C for 24 h. The inoculum density was adjusted to an OD600 of 0.10, and 100 μl was transferred to 5 ml peptone water in a tube and incubated on a reciprocal shaker at 180 strokes/min at 28–30 °C for 5 d. Nessler's reagent was mixed with each sample. The development of a yellow-to-brown colour indicated a positive result for ammonia production, as described by Cappuccino & Sherman (1992).

Cell wall-degrading enzyme production
Yeast inoculum was prepared by cultivation in 5 ml YPD broth, and tube cultures were incubated on a reciprocal shaker at 180 strokes/min at 28 ± 2 °C for 24 h. Inoculum was adjusted to an OD600 of 0.10 at 600 nm, and 3 μl was spotted onto different media and incubated at 28 ± 2 °C for 1, 3, 5, and 7 d. Protease activity was determined by the presence of a clear zone on skimmed milk agar (Cattelan *et al.* 1999). Chitinolytic activity was determined by a clear zone on YNB medium (Difco, USA), amended with colloidal chitin as described by Shanmugaiah *et al.* (2008), except that YNB agar and 0.5 % colloidal chitin were used instead of YNB broth and 0.3 % colloidal chitin, respectively. Two kinds of cellulase activity were determined by the method described previously (Mangunwardoyo *et al.* 2011), except that 1 % substrate – i.e. carboxymethyl cellulose (CMC) or avicel – was used.

Catalase test
Yeasts were grown on YPD agar at 28 ± 2 °C for 18–24 h. The cultures were mixed with appropriate amounts of H_2O_2 on a glass slide to observe the evolution of oxygen.

Production of hydrogen cyanide (HCN)
HCN production was detected by the modified method of Bakker & Schippers (1987). Yeast inoculum was prepared by cultivation in 5 ml YPD broth, and tube cultures were incubated on a reciprocal shaker at 180 strokes/min at 28 ± 2 °C for 24 h. Inoculum intensity was adjusted to an OD600 of 0.10, and 3 μl was transferred onto YPD agar, amended with 4.4 g l^{-1} glycine by streaking. A disk of Whatman filter paper No. 1 was dipped into 0.5 % picric acid, and 2 % Na_2CO_3 was placed on the lid of the Petri plates. The plates were sealed with parafilm. After 1–7 d incubation at 28 ± 2 °C, any colour change of the filter paper was noted and the HCN production potential of the antagonists was assessed.

Evaluation of the in vitro *antagonism*
Yeast inoculum was prepared in YPD broth and incubated on a reciprocal shaker at 180 strokes/min at 28 ± 2 °C for 24 h. A loop full of inoculum was transferred onto potato dextrose agar (PDA) (Difco, USA) by streaking and the plate culture was incubated at 25 °C for 48 h. To evaluate the control of fungal growth by yeast, an assay in which yeast and fungi were grown side by side was set up, as described by Rosa *et al.* (2010). A control plate was prepared with only fungal inoculation. The experiment was performed with three replicates, incubating the plates at 25 °C. Mycelial growth was measured at 3, 5, and 7 d of incubation.

Production of antifungal volatile compounds
Yeast inoculum was prepared by a method similar to that used to evaluate the *in vitro* antagonism described above. To evaluate the production of antifungal volatile compounds by yeast, a plate culture in which yeast and fungi were grown side by side was prepared, as described by Rosa *et al.* (2010). However, it should be emphasised that a division was made in the agar medium in the Petri dishes to prevent the non-volatile exudates produced by the yeast from reaching the fungus through the culture medium.

Results and discussion

Screening of indole-3-acetic acid (IAA) producing yeast

IAA production was determined for a total of 1035 yeast isolates in tube cultivation. Colorimetric assay of IAA revealed that 167 isolates were IAA producers, whereas 868 isolates were non-IAA producers. It is possible to classify as low (<12.4 mg g^{-1} DCW) and high (>12.5 mg g^{-1} DCW) IAA-producing yeast by the numbers of 154 and 13 isolates, respectively.

Yeast identification

Based on colorimetric assay of IAA production, thirteen yeast isolates possessing high levels of IAA production

(12.5–64.4 mg g^{-1} DCW) were identified using the D1/D2 domain of the large subunit rRNA gene sequence similarity. Results revealed nine yeast species, which were: *Hannaella sinensis* (DMKU-RP45) (AB920308) (Wang & Bai 2008), *Cryptococcus flavus* (DMKU-RE12 (AB863475), DMKU-RE19 (AB863482), DMKU-RE67 (AB863528), and DMKU-RP128 (AB920310)) (Fonseca et al., 2011), *Kodamaea ohmeri* (DMKU-RP233) (AB920313) (Lachance & Kurtzman 2011), *Meyerozyma guilliermondii* (DMKU-RP168) (AB920311) (Kurtzman 2011a), *Pseudozyma antarctica* (DMKU-RP163 (AB920315), DMKU-RP213 (AB920312)) (Boekhout 2011a), *P. rugulosa* (DMKU-RE60) (AB863521) (Boekhout 2011a), *Rhodosporidium paludigenum* (DMKU-RP301) (AB920314) (Sampaio 2011a), *Rhodotorula taiwanensis* (DMKU-RP105) (AB920309) (Sampaio 2011b), and *Torulaspora globosa* (DMKU-RP31) (AB920307) (Kurtzman 2011b) among the thirteen isolates selected.

Characterisation of plant growth-promoting traits in yeast strains

Determination of IAA production

According to results obtained from colorimetric assay, the cell-free culture broth of the yeast isolates that showed high IAA production were subjected to HPLC analysis to confirm the amount of IAA produced. Results indicated production in a range of 1.2–29.3 mg IAA/g DCW (Table 1). Four yeast species produced more than 4.8 mg IAA/g DCW. They were: *Hannaella sinensis* (DMKU-RP45), *Cryptococcus flavus* (DMKU-RE12, DMKU-RE19, DMKU-RE67, and DMKU-RP128), *Torulaspora globosa* (DMKU-RP31), and *Rhodosporidium paludigenum* (DMKU-RP301), which was the best IAA-producing strain obtained in this study. *R. paludigenum*, a carotenoid-producing yeast, was first described by Fell & Tallman (1980), and was reported to produce carotenoids when grown in glycerol as the sole carbon source (Yimyoo *et al.* 2011). In addition, this yeast has also been used in various applications: it proved effective as an antioxidant when applied to *Litopenaeus vannamei* aquaculture (Yang *et al.* 2010; Yang *et al.* 2013), and it helped in control of postharvest decay on cherry tomatoes (Wang *et al.* 2010) and in biocontrol of *Penicillium expansum* infection in pear fruit (Yu *et al.* 2013). Among all the yeast studied in the present work, *R. paludigenum* DMKU-RP301 was the best IAA producer at 29.3 mg g^{-1} DCW and also exhibited siderophore production (see below). The present work is therefore the first to report that *R. paludigenum* possesses plant growth-promoting characters (i.e. IAA- and siderophore-producing abilities). *Bullera sinensis*, previously described as *B. derxii*, is a basidiomycetous yeast that may form ballistoconidia (Nakase & Suzuki 1986). There has been a report of secretion of a fungicidal thermolabile and protease-sensitive toxin with a cureless killer phenotype of *B. sinensis* (Golubev & Nakase 1997); however, *H. sinensis* DMKU-RP45 found in this study may not possess killing activity and showed neither production of antifungal volatile compounds nor *in vitro* antagonism in a solid medium. However, *H. sinensis* DMKU-RP45 has been characterised here for its IAA production activity at 7.1 mg g^{-1} DCW.

IAA-producing microorganisms are receiving attention as good sources of biofertilizer (Sasikala & Ramana 1998). For almost a decade, there have been reports on IAA production by yeasts. El-Tarabily (2004) studied the plant growth-promoting yeasts *Candida valida*, *Rhodotorula glutinis*, and *Trichosporon asahii*, isolated from sugar beet rhizospheres. These three yeast species were found to support beet growth by production of IAA at 29.5, 24.1, and 31.7 μg ml^{-1}, respectively. Nassar *et al.* (2005) investigated IAA production of endophytic yeasts isolated from maize (*Zea mays* L.) root. Each isolate was cultured in glucose peptone broth (GPB), with or without 1.25 % L-tryptophan, at 25 °C for 7 d, under shaking at 250 rev/min. Only eight out of 24 isolates were shown to produce IAA in the medium with and without L-tryptophan. Xin *et al.* (2009) characterised three IAA-producing endophytic yeasts (WP1, PTD2 and PTD3) from the *Populus* tree and found the highest IAA production (39 mg g^{-1} dry cell) in the isolate WP1, whereas the isolates PTD2 and PTD3 produced 18 and 8 mg g^{-1} dry cell of IAA, respectively. Deng *et al.* (2012) reported a low IAA production of 11.7 μg ml^{-1} (quantified by a colorimetric

Table 1 – Direct plant growth-promoting traits of four endophytic and eleven epiphytic yeasts isolated from rice leaves (RE and RP, respectively); Solubilisation efficiency (SE) = Halo zone/Diameter of colonies.

Isolates	IAA production (mg g^{-1} DW)	Phosphate solubilisation (SE)	Zinc oxide solubilisation (SE)	Polyamine production[a]	NH_3 production[b]	Catalase test[a]
DMKU-RE12	7.2	0	0	–	++	+
DMKU-RE19	7.9	0	0	–	+	+
DMKU-RE60	2.7	0	0	+	++	+
DMKU-RE67	9.7	0	0	–	++	+
DMKU-RP31	4.9	1.2	1.2	–	–	+
DMKU-RP45	7.1	0	0	–	+	+
DMKU-RP105	2.8	0	0	–	+	+
DMKU-RP128	7.2	0	0	–	+	+
DMKU-RP163	1.5	0	0	–	++	+
DMKU-RP168	4.5	0	0	–	+	+
DMKU-RP213	1.2	0	0	+	+	+
DMKU-RP233	2.0	0	0	–	+	+
DMKU-RP301	29.3	0	0	–	+	+

a + positive, – not detected.
b + slight positive result, ++ strong positive result.

method using Salkowski reagent) by heavy metal resistant endophytic *Cryptococcus* sp. CBSB78 from rape (*Brassica chinensis*).

Siderophore production, phosphate solubilisation, and zinc oxide solubilisation

To determine as many of the plant growth-promoting characters as may possibly exist in yeast, a group of yeast isolates which were high IAA producers were studied. Thirteen isolates possessing 1.2–29.3 mg g^{-1} DCW of IAA (by HPLC) were then tested for phosphate solubilisation, siderophore production and some other important characters for plant growth promotion (Tables 1 and 2). We found that five isolates – i.e. *Pseudozyma rugulosa* DMKU-RE60, *Hannaella sinensis* DMKU-RP45, *Rhodotorula taiwanensis* DMKU-RP105, *P. antarctica* DMKU-RP213 and *Rhodosporidium paludigenum* DMKU-RP301 – showed positive results for siderophore production, as they altered the CAS-blue medium colour from blue to orange (Table 2; Fig 1). However, none of these isolates possessed antifungal activity either in the manner of antagonism on a solid medium or in production of antifungal volatile compounds (Table 2). Investigation of phosphate solubilising activity revealed that only *Torulaspora globosa* DMKU-RP31 indicated *in vitro* calcium phosphate solubilisation capability and showed an obvious solubilising activity, expressed in the solubilisation efficiency (SE) unit of 1.22 (Table 1; Fig 2A). In addition to phosphate solubilisation, *T. globosa* DMKU-RP31 also exhibited zinc oxide solubilisation, expressed in the SE unit of 1.17 (Table 1; Fig 2B). The yeast *T. globosa* was reported to be antagonistic towards the phytopathogenic mold *Colletotrichum sublineolum*, even though no siderophores, volatile compounds or fungal wall hydrolytic enzymes were detected (Rosa *et al.* 2010).

Yeasts have been reported to produce siderophores, chelating agents for the ferric ion (Fe^{3+}) which not only support plant growth *via* iron chelating but also indirectly inhibit plant pathogenic fungi by scavenging available iron from the environment (Calvente *et al.* 1999; Wang & Chi 2009). However, it is not necessarily the case that yeasts which show antifungal activity will definitely produce siderophores as a key element of such activity. Evidence of this was found in the yeast *Torulaspora globosa* 1S112, isolated from sugarcane rhizosphere, which reduced growth of the phytopathogenic mold *Colletotrichum sublineolum* but did not produce siderophores, volatile compounds or hydrolytic enzymes (Rosa *et al.* 2010).

Investigation of phosphate solubilising activity revealed that the yeast *Torulaspora globosa* DMKU-RP31 showed an obvious solubilising activity and also exhibited zinc oxide solubilisation. Results on phosphate solubilisation appeared to be on the same level as those reported in rhizospheric *Acinetobacter* strains of pearl millet, whereas the degree of zinc oxide solubilising activity found in *T. globosa* DMKU-RP31 was far less than that observed in the *Acinetobacter* strains (Rokhbakhsh-Zamin *et al.* 2011). To date, the literature related to yeast mineral solubilisation remains limited, although zinc solubilisation has been reported in fungi (White *et al.* 1997).

Polyamine, NH_3 and ACC deaminase production

Study of polyamine production indicated that two yeasts – *Pseudozyma rugulosa* DMKU-RE60 and *P. antarctica* DMKU-RP213 – produce polyamine in culture broth (Table 1; Fig 3). NH_3 production was detected at two different levels – i.e. a low or a high level – in nearly all the yeasts, except *T. globosa* DMKU-RP31. No relationship between mineral solubilisation and ammonia production in this yeast could be drawn. However, ACC deaminase activity was not detected in any of the yeast isolates studied here.

Polyamines are organic polycations having variable hydrocarbon chains and two or more primary amino groups. Production of diamine putrescine, triamine spermidine, and tetraamine has been claimed to stimulate root cell division

Table 2 – Indirect plant growth-promoting traits of four endophytic and eleven epiphytic yeasts isolated from rice leaves (RE and RP, respectively); Diameter of halo zone = halo zone (mm) – colony zone (mm); Diameter of clear zone = clear zone (mm) – colony zone (mm).

Isolates	Siderophore production; (diameter of halo zone, mm)	Cell wall-degrading enzyme production		Antagonism in solid medium[a]	Production of antifungal volatile compounds[a]
		Protease (diameter of clear zone, mm)	Cellulase		
			CMC (diameter of halo zone, mm)		
DMKU-RE12	–	–	36	–	–
DMKU-RE19	–	–	36	–	–
DMKU-RE60	6	15	–	–	–
DMKU-RE67	–	–	34	–	–
DMKU-RP31	–	–	–	+	+
DMKU-RP45	2	–	–	–	–
DMKU-RP105	2	–	–	–	–
DMKU-RP128	–	–	35	–	–
DMKU-RP163	–	1	–	–	–
DMKU-RP168	–	–	–	–	–
DMKU-RP213	6	6	–	–	–
DMKU-RP233	–	–	–	–	–
DMKU-RP301	5	–	–	–	–

a + positive, - not detected.

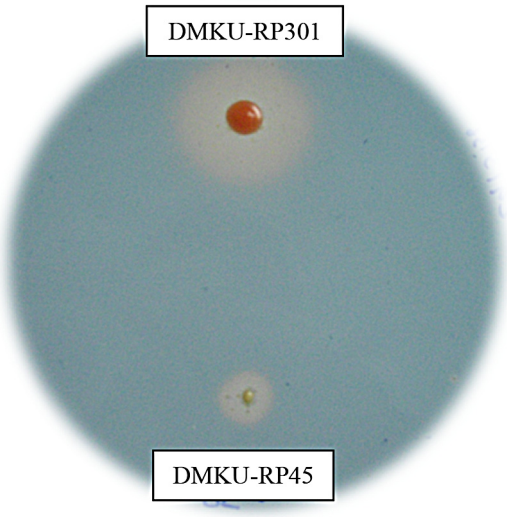


Fig 1 – Siderophore was produced by DMKU-RP301 and DMKU-RP45 on chrome azurol S (CAS) agar by point inoculation and incubation at 28 °C for 5 d.

and, hence, increase root growth (Tang & Newton 2005; Cloete *et al.* 2009). As one of the general characteristics studied in plant growth-promoting bacteria (Joseph *et al.* 2007; Chaiharn *et al.* 2008; Ahemad & Khan 2012), it is therefore of interest to investigate NH_3 production in the yeast isolates studied here. However, it should be noted that ammonia production is quite rare in yeast, an exception being the yeast *Debaryomyces hansenii*, which is able to produce NH_3 when grown on cheese agar (Gori *et al.* 2007). Samuel & Muthukkaruppan (2011) reported that NH_3 is commonly produced in both rhizobacteria and fungi associated with rice, mangrove and effluent-contaminated soil.

In our literature review, ACC deaminase activity was reported in only a few yeast strains, such as *Hansenula saturnus*, *Issatchenkia occidentalis*, *Candida tropicalis*, and *Cryptococcus* sp. (Minami *et al.* 1998; Palmer *et al.* 2007; Amprayn *et al.* 2012; Deng *et al.* 2012). Therefore, it is not surprising that we found none of our yeast isolates exhibited this enzyme activity.

Catalase test and HCN production

Catalase, a very important enzyme in protecting the cell from oxidative damage by reactive oxygen species (ROS), was investigated in all thirteen yeast isolates. Since catalase production is one of the plant growth-promoting character found in bacteria (Joseph *et al.* 2007), it was therefore of interest to determine catalase production in all selected yeast strains. Catalase activity was found in all yeasts studied here (Table 1).

Catalase activity can be detected in yeasts, especially those associated with plant leaves and utilising methanol as carbon and energy sources. This is due to phyllospheric methanol emission as a result of pectin degradation. The methanol utilisation pathway requires catalase activity to react with hydrogen peroxide in peroxisome. Leaf-associated yeast may be able to utilise methanol and hence produce catalase to detoxify hydrogen peroxide in peroxisome. Results indicated that all of the strains tested showed catalase activity whereas none of them exhibited HCN production (data not shown). These results agreed with those reported in plant growth-promoting rhizobacteria associated with chickpea (Joseph *et al.* 2007) and rice (Samuel & Muthukkaruppan 2011), indicating that the filamentous fungi *Penicillium* spp. and *Aspergillus niger*, associated with rice, mangrove and effluent-contaminated soil, showed the same contrasting results of catalase and HCN tests, respectively. However, HCN has long been reported as a secondary metabolite produced by many antagonistic *Pseudomonas* species (Freeman *et al.* 1975; Michelsen & Stougaard 2012).

Production of cell wall-degrading enzymes

It is well known that the fungal wall consists of rather complicated contents, such as chitin, β-1,3-glucan, mannoprotein and cellulose in some species. The ability of yeast to produce

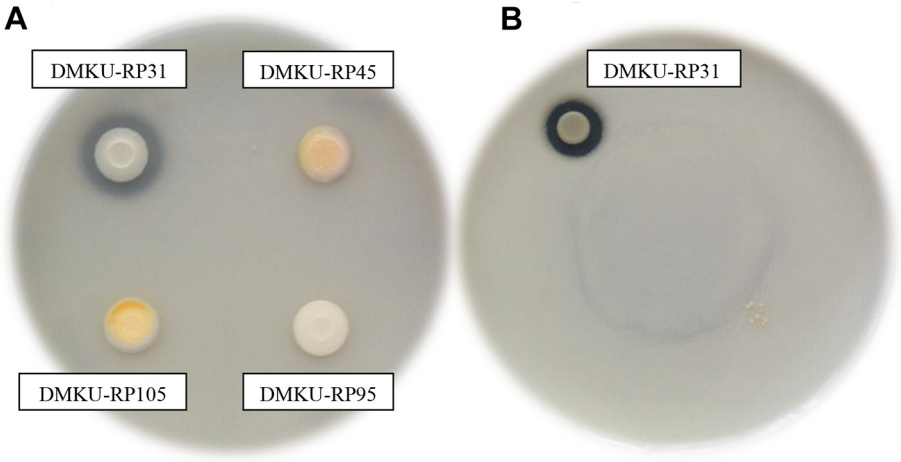


Fig 2 – Phosphate and zinc oxide solubilising halos produced by DMKU-RP31 on Pikovskaya's agar (A) and zinc oxide agar (B), respectively by spot inoculation and incubation at 28 °C for 5 d.

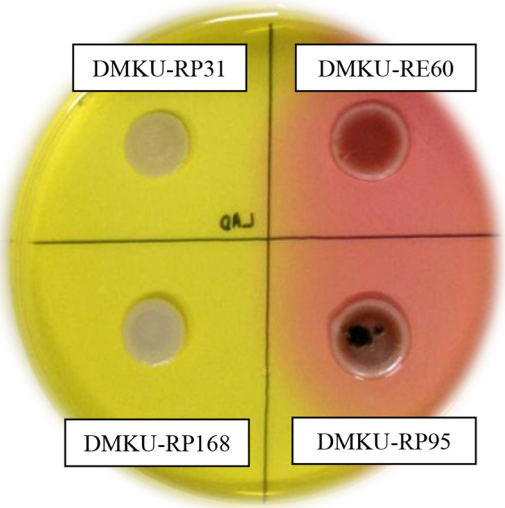


Fig 3 – Polyamine was produced by DMKU-RE60 and DMKU-RP95 on Long Ashton decarboxylase (LAD) agar by spot inoculation and incubation at 28 °C for 5 d.

some hydrolytic enzymes, such as glucanase, chitinase, protease, and cellulase, helps to promote antagonistic activity against plant pathogenic fungi. As a consequence, plant growth will therefore be indirectly promoted. Assay of fungal cell wall-degrading enzymes indicated that the yeasts *Pseudozyma rugulosa* DMKU-RE60 and two strains of *P. antarctica* (DMKU-RP163 and DMKU-RP213) produced protease (Fig 4A). None of the yeast isolates studied here showed avicelase or chitinase activity. However, all strains of *Cryptococcus flavus* identified in this study (DMKU-RE12, DMKU-RE19, DMKU-RE67 and DMKU-RP128) produced carboxy methyl cellulase (CMCase), as shown in Fig 4B. *C. flavus* is a basidiomycetous yeast that has been shown to possess amylase, CMCase and xylanase activities (Wanderley *et al.* 2004; Parachin *et al.* 2009). In our study, CMCase activity was detected in four strains of *C. flavus*, which additionally possessed plant growth hormone, IAA production activity. The levels of IAA production of 7.2, 7.9, 9.7 and 7.2 mg g^{-1} DCW were found in *C. flavus* DMKU-RE12, DMKU-RE19, DMKU-RE67, and DMKU-RP128, respectively.

Production of hydrolytic enzymes, such as exo-β-1,3-glucanase which destroys fungal cell wall, has been reported in the yeast *Pichia anomala* (Jijakli & Lepoivre 1998). Mangunwardoyo *et al.* (2011) screened yeasts isolated from soil, sediment and river water in Indonesia for CMCase, avicelase and cellobiase and found that a total of 16 yeast isolates possessed those wall-degrading enzymes in the form of either a single enzyme or two enzyme components.

Evaluation of in vitro *antagonism*

The thirteen yeast isolates that possessed high IAA production abilities were investigated for their antagonistic activity against rice fungal pathogens i.e. *Fusarium moniliforme*, *Helminthosporium oryzae*, and *Rhizoctonia solani*. Our results indicated that only the yeast *Torulaspora globosa* DMKU-RP31 showed significant antagonistic effect against all fungi tested on PDA (Fig 5A).

Production of antifungal volatile compounds

Similar to the antagonism studies, results on production of antifungal volatile compounds revealed that only the yeast *Torulaspora globosa* DMKU-RP31 produced volatile compounds that significantly inhibited fungal growth (Fig 5B). Rosa *et al.* (2010) also reported the antagonistic action of *T. globosa* 1S112 against *Colletotrichum sublineolum,* which is a sorghum pathogen. However, no siderophores, volatile compounds or fungal wall hydrolytic enzymes were detected in *T. globosa* 1S112. It should be emphasised that the report on *T. globosa* 1S112 indicated a lack of volatile compound production which

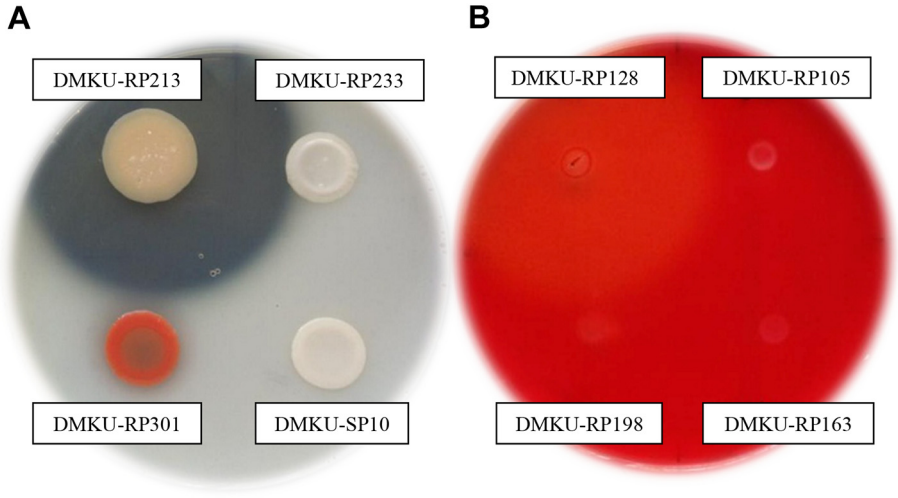


Fig 4 – Cell wall-degrading enzyme production: (A) Protease was produced by DMKU-RP213 on skimmed milk agar. (B) Cellulase was produced by DMKU-RP128 on YNB agar amended with 1 % carboxymethyl cellulose (CMC) agar.

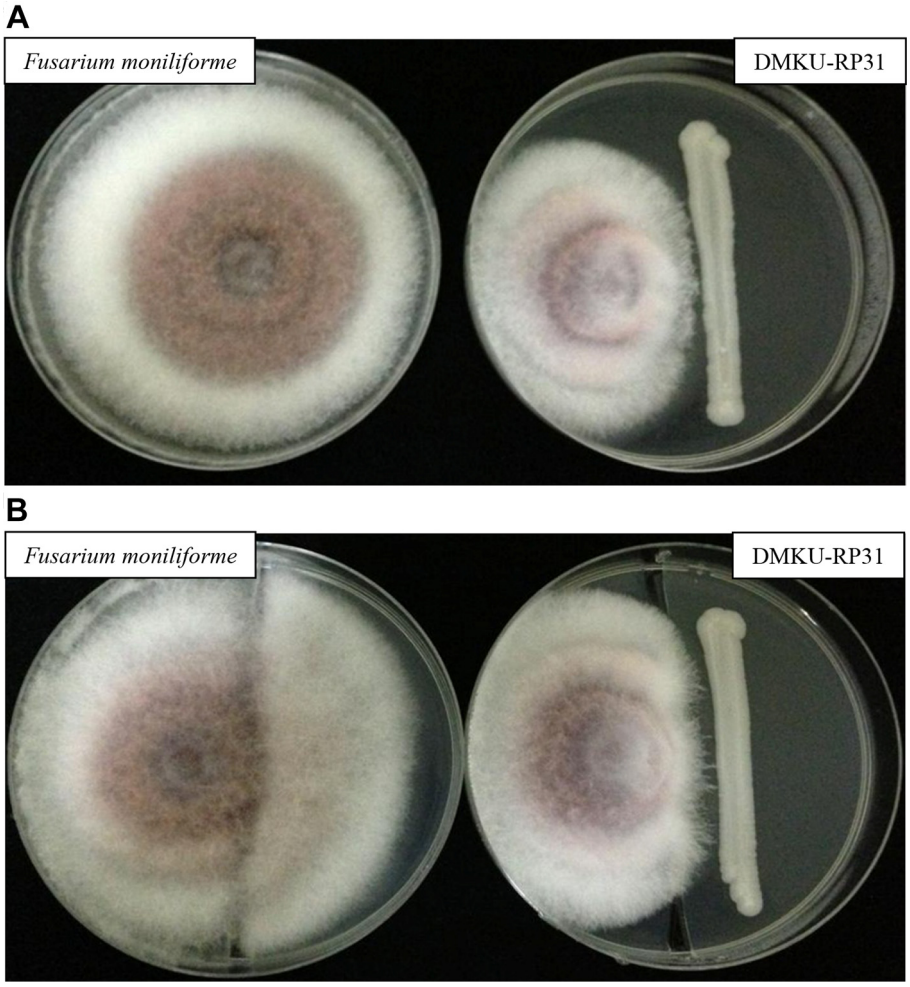


Fig 5 – Antagonism by DMKU-RP31 against *Fusarium moniliforme*, which causes disease in rice plants. (A) Evaluation of *in vitro* antagonism in a solid medium: *F. moniliforme* alone (left) and yeast antagonistic to *F. moniliforme* (right). (B) Test of the production of antifungal volatile compounds: *F. moniliforme* alone (left) and yeast antagonistic to *F. moniliforme* (right).

inhibited the mold growth, whereas *T. globosa* DMKU-RP31, isolated in our work, demonstrated this ability.

Previous studies of IAA-producing yeast were reported in *Lindera* (*Williopsis*) *saturnus* (Nassar *et al.* 2005), *Rhodotorula graminis* and *R. mucilaginosa* (Xin *et al.* 2009), *Candida tropicalis* (Amprayn *et al.* 2012), *Candida valida*, *Rhodotorula glutinis*, and *Trichosporon asahii* (El-Tarabily 2004), *Cryptococcus* sp. (Deng *et al.* 2012) and 20 yeast species isolated from the phylloplanes of various plant species collected from seven provinces in Thailand (Limtong & Koowadjanakul 2012). Although a higher IAA level was reported in the yeast *Rhodotorula* spp. by Xin *et al.* (2009), no other plant growth-promoting traits have yet been proposed by the authors. It must be noted that the lower NH_3 and siderophore production found in *R. paludigenum* DMKU-RP301 might not recommend this yeast as a good candidate for plant growth promotion. However, a higher level of IAA production was foreseen through optimisation of either medium composition or growth conditions. Experimental design has been set up and placed under investigation not only for *R. paludigenum* DMKU-RP301 but also for *Torulaspora globosa* DMKU-RP31. The latter has received more attention than the others because of its capability to produce antifungal volatile compounds, hence showing significant antagonistic effects against three molds on a solid medium. This is due to the fact that not only phytohormones such as IAA are necessary to support plant growth, but also other indirect plant growth-promoting traits are required to obtain good plant yield.

From overall results, we found three plant growth-promoting traits – i.e. antagonism to fungal plant pathogens, antifungal volatile compound production and a moderate level of IAA production (4.9 mg g^{-1} DCW) – in *Torulaspora globosa* DMKU-RP31, whereas *Rhodotorula paludigenum* DMKU-RP301 showed a much higher IAA production (29.3 mg g^{-1} DCW) with some production of siderophore as an additional character. We therefore propose that *T. globosa* DMKU-RP31 might be used in two ways: enhancing plant growth and acting as a biocontrol agent. However, we also found high levels of IAA production (7.2–9.7 mg g^{-1} DCW) in four *Cryptococcus flavus* strains, accompanied with NH_3 production as well as CMCase activity. These yeasts are also strains of interest for optimal IAA production.

Acknowledgements

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Nakazawaea siamensis f.a., sp. nov., a yeast species isolated from phylloplane

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Strain DMKU-RK467^T, representing a novel yeast species, was isolated from the external surface of sugar cane leaves collected in Thailand. On the basis of morphological, biochemical, physiological and chemotaxonomic characteristics, and sequence analysis of the D1/D2 region of the LSU rRNA gene and the internal transcribed spacer (ITS) region, strain DMKU-RK467^T was assigned to a novel species of the genus *Nakazawaea*. The novel species was related most closely to the type strain of *Candida wickerhamii* but they differed by 1.9 % nucleotide substitutions in the D1/D2 region of the LSU rRNA gene and by 5.2 % nucleotide substitutions in the ITS region. The name *Nakazawaea siamensis* f.a., sp. nov. is proposed (type strain DMKU-RK467^T=BCC 50734^T=NBRC 108903^T=CBS 12569^T).

The genus *Nakazawaea* was first proposed to accommodate *Pichia holstii* by Yamada *et al.* (1994) based on data obtained from partial sequences of the LSU and SSU rRNA genes that separate *Pichia holstii* from other hat-shaped ascospore-forming and nitrate-assimilating species of the genus *Pichia*, and *Nakazawaea holstii* was proposed as the type species. This reclassification was not initially accepted because only a few species were included in the rRNA gene sequence analysis (Kurtzman, 1998). Moreover, sequence analysis of the D1/D2 region of the LSU rRNA gene of all recognized species of the genus *Pichia* by Kurtzman & Robnett (1998) and of the SSU rRNA gene by Suzuki & Nakase (1999) did not support the reclassification. However, later analysis of multigene sequences including nuclear sequences of the largest subunit and second largest subunit of the RNA polymerase II gene, actin, the second subunit of the mitochondrial cytochrome oxidase gene, and D1/D2 LSU rRNA gene provided much stronger support for the proposal of the genus *Nakazawaea* (Tsui *et al.*, 2008).

On the basis of analysis of multigenes, including the D1/D2 region of the LSU rRNA gene, translation elongation factor-1α (EF-1α) gene and mitochondrial SSU rRNA gene, the *Nakazawaea* clade was found to be basal to the *Peterozyma* clade that accommodates two species transferred from the genus *Pichia*, namely *Peterozyma toletana* and *Peterozyma xylosa*, and close to *Pachysolen tannophilus* (Kurtzman & Robnett, 2010). The *Nakazawaea* clade as defined by Kurtzman (2011) comprises *N. holstii*, which is the only known ascospore-producing species in the genus *Nakazawaea*, and nine species of the genus *Candida*, namely *Candida anatomiae*, *Candida chilensis*, *Candida heliconiae*, *Candida ishiwadae*, *Candida peltata*, *Candida pomicola*, *Candida populi*, *Candida wickerhamii* and *Candida wyomingensis*. *N. holstii* is heterothallic and only asporogenous haploid strains have been isolated from nature.

The phylloplane, an external surface of plant leaves (Phaff & Starmer, 1987; Fonseca & Inácio, 2006), has been recognized as an important habitat for epiphytic microorganisms. Both basidiomycete and ascomycete yeasts have been found to be phylloplane colonizers (Nakase *et al.*, 2001; Fonseca & Inácio, 2006; Glushakova *et al.*, 2007; Slavikova *et al.*, 2009; Landell *et al.* 2010). Although most common phylloplane yeasts are members of the basidiomycete genera *viz. Cryptococcus*, *Rhodotorula*, *Sporobolomyces* and *Trichosporon* (de Azeredo *et al.*, 1998; Nakase *et al.*, 2001; Fonseca & Inácio, 2006; Slavikova *et al.*, 2009; Glushakova & Chernov, 2010), various ascomycete yeast species have also been reported, for example *Debaryomyces hansenii*, *Hanseniaspora uvarum*, *Kazachstania barnettii*, *Metschnikowia lopburiensis*, *Metschnikowia pulcherrima*, *Metschnikowia saccharicola*, *Pichia membranifaciens*, *Saccharomyces cerevisiae*, *Wickerhamomyces siamensis*, *Yamadazyma siamensis*, *Yamadazyma phyllophila*, *Yamadazyma paraphyllophila* and some species of the genus *Candida* including *Candida aechmeae*, *Candida oleophila*, *Candida chumphonensis*, *Candida mattranensis* and *Candida vrieseae* (Glushakova *et al.*, 2007; Slavikova *et al.*, 2009; Glushakova & Chernov, 2010; Landell *et al.*, 2010; Koowadjanakul *et al.*, 2011; Kaewwichian *et al.*, 2012; Kaewwichian *et al.*, 2013a, b).

Abbreviation: ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the D1/D2 region of the LSU rRNA gene and ITS region of strain DMKU-RK467^T are AB772177 and AB793784, respectively. The Mycobank number for *Nakazawaea siamensis* f.a., sp. nov. is MB 803008.

During an investigation of yeasts in the external leaf surfaces of sugar cane in Thailand, strain DMKU-RK467^T, representing a novel species of the genus *Nakazawaea*, was obtained. After isolation of yeasts using a technique including leaf washing followed by plating, we obtained 372 strains from 102 leaf samples but we were unable to derive a second strain of this novel species. Moreover, we have identified more than 900 yeast strains isolated from the phylloplane of various plants species both by an enrichment technique and by leaf washing followed by plating but we did not obtain another strain of the novel species. According to Kurtzman (2010), the description of species based on a single strain will add to an understanding of yeast phylogeny and species diversity, and we thus decided to describe the novel species based on this single strain. In this paper, strain DMKU-RK467^T is described as the type strain of *Nakazawaea siamensis* f.a., sp. nov.

In total, 166 yeast strains were isolated from the external surfaces of 95 samples of sugar cane (*Saccharum officinarum* L.) leaves collected in Thailand. Strain DMKU-RK467^T was isolated from a sample collected from Chok chai district, Nakhon Ratchasima province (14° 43′ 56″ N 102° 9′ 47″ E), on 6 May 2010, by an enrichment technique using acidified yeast extract-malt extract (YM) broth (0.3 % yeast extract, 0.3 % malt extract, 0.5 % peptone and 1 % glucose) supplemented with 0.025 % sodium propionate and 0.02 % chloramphenicol (Limtong *et al.*, 2007). Three grams of leaves, cut to a size appropriate for placement in a 250 ml Erlenmeyer flask, was inoculated to 50 ml of acidified YM broth in the flask and incubated on a rotary shaker at room temperature (27±3 °C) for 3 days. The enrichment culture was then spread on YM agar supplemented with 0.025 % sodium propionate and 0.02 % chloramphenicol and incubated at room temperature (27±3 °C) until yeast colonies appeared. Yeast colonies of different morphologies were picked and purified by cross streaking on YM agar. Purified yeast strains were suspended in YM broth supplemented with 10 % glycerol and maintained at −80 °C.

The sequences of the D1/D2 region of the LSU rRNA gene and the internal transcribed spacer (ITS) region were determined from PCR products amplified from genomic DNA, using primers NL1 and NL4 (Kurtzman & Robnett, 1998) and ITS1 and ITS4 (White *et al.*, 1990), respectively, for sequencing and amplification. Methods of DNA extraction and amplification were as described previously (Limtong *et al.*, 2007). The PCR products were checked by agarose gel electrophoresis and purified by using the QIAquick purification kit (Qiagen) and the purified products were submitted to Macrogen (Seoul, Republic of Korea) for sequencing with primers NL1 and NL4 for the D1/D2 region and primers ITS1 and ITS4 for the ITS region. The sequences were compared pairwise using a BLAST search (Altschul *et al.*, 1997) and were aligned with the sequences of related strains retrieved from GenBank using the multiple alignment program CLUSTAL X version 1.81 (Thompson *et al.*, 1997). A phylogenetic tree was reconstructed from the evolutionary distance data with Kimura's two-parameter correction (Kimura, 1980) using the neighbour-joining method (Saitou & Nei, 1987) and MEGA software version 5.0 (Tamura *et al.*, 2011). Confidence levels of the clades were estimated from bootstrap analysis (1000 replicates) (Felsenstein, 1985).

Strain DMKU-RK467^T was characterized morphologically, biochemically and physiologically according to standard methods described by Yarrow (1998). Hyphae formation was investigated in slide culture on potato dextrose agar (PDA; 20 % potato infusion, 2 % glucose and 1.5 % agar) at 25 °C for up to 7 days. Ascospore formation was investigated on YPD agar (1 % yeast extract, 2 % peptone, 2 % glucose and 1.5 % agar), 5 % malt extract agar (5 % malt extract and 1.5 % agar), McClary's acetate agar (0.1 % glucose, 0.18 % potassium chloride, 0.82 % sodium acetate trihydrate, 0.25 % yeast extract and 1.5 % agar), Gorodkowa agar (0.1 % glucose, 0.5 % sodium chloride, 1 % peptone and 2 % agar) and corn meal agar (1.7 % corn meal agar) at 15 and 25 °C for up to 4 weeks. Carbon assimilation tests were conducted in liquid medium according to the method described by Yarrow (1998). Assimilation of nitrogen compounds was examined on solid medium with starved inocula following the method of Nakase & Suzuki (1986). Growth at various temperatures was determined by cultivation in YM broth. Ubiquinones were extracted from cells cultivated in a 500 ml Erlenmeyer flask containing 250 ml YPD broth on a rotary shaker at 28 °C for 24–48 h and purified according to the methods described by Yamada & Kondo (1973) and Kuraishi *et al.* (1985). Isoprenologues were identified by HPLC as described previously (Limtong *et al.*, 2007).

Novel species delineation and identification

Analysis of the D1/D2 region of the LSU rRNA gene revealed that the sequence of strain DMKU-RK467^T was closest to that of *Candida wickerhamii* CBS 2928^T, with 1.9 % nucleotide substitutions (11 nt substitutions out of 569 nt). The sequence of the ITS region of strain DMKU-RK467^T differed by 5.2 % nucleotide substitutions (35 nt substitutions and 99 gaps out of 680 nt) from that of *Candida wickerhamii* CBS 2928^T.

The phylogenetic tree based on sequences of the D1/D2 region of the LSU rRNA gene further demonstrated that strain DMKU-RK467^T was in the subclade that contained *Candida wickerhamii*, *N. holstii*, *Candida anatomiae*, *Candida ishiwadae*, *Candida pomicola*, *Candida populi* and *Candida wyomingensis* at a position distinct from the other members of the clade (Fig. 1).

Cells of strain DMKU-RK467^T were globose to subglobose and proliferated by multilateral budding (Fig. 2). Pseudohyphae and true hyphae were not formed in slide culture on PDA. Ascospores were not produced on YPD agar, 5 % malt extract agar, McClary's acetate agar, Gorodkowa agar or corn meal agar after 4 weeks at 15 and 25 °C.

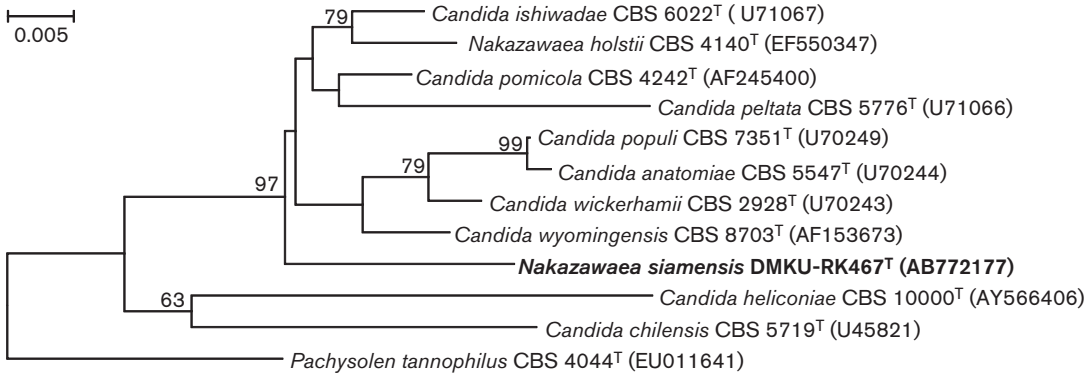


Fig. 1. Phylogenetic tree based on sequences of the D1/D2 region of the LSU rRNA gene, showing the position of *Nakazawaea siamensis* sp. nov. DMKU-RK467^T with respect to strains of closely related species. The phylogenetic tree was reconstructed from evolutionary distance data with Kimura's two-parameter correction, using the neighbour-joining method with MEGA version 5.0. Numbers at nodes indicate percentages of bootstrap sampling, derived from 1000 datasets. *Pachysolen tannophilus* CBS 4044^T was used as the outgroup. Bar, 0.005 K_{nuc} distance.

On the basis of morphological, biochemical, physiological and chemotaxonomic characteristics and sequence analysis of the D1/D2 region of the LSU rRNA gene and ITS region, we conclude that strain DMKU-RK467^T represents a novel species. Although formation of ascospores was not observed, according to the International Code of Nomenclatural for algae, fungi and plants concerning the nomenclatural rules for fungi that the most important is the adoption of 'one fungus, one name' (Miller *et al.*, 2011), the novel species is assigned to the genus *Nakazawaea*, and the designation *forma asexualis* (f.a.) is included following the recommendation of Lachance (2012). The name *Nakazawaea siamensis* f.a., sp. nov. (MB 803008) is proposed.

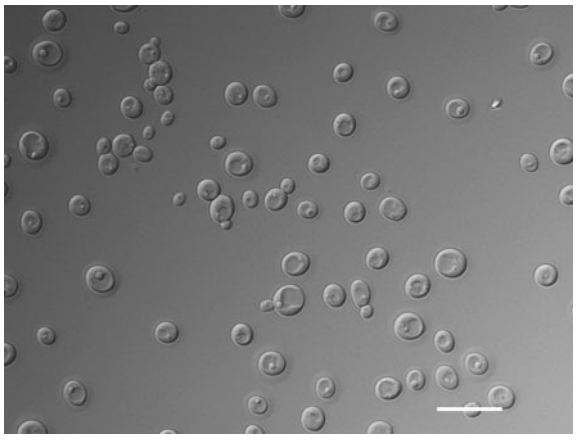


Fig. 2. Budding cells of *Nakazawaea siamensis* sp. nov. DMKU-RK467^T in YM broth after 3 days at 25 °C. Bar, 10 μm.

Description of *Nakazawaea siamensis* Kaewwichian & Limtong f.a., sp. nov.

Nakazawaea siamensis (si.am.en′sis. N.L. fem. adj. *siamensis* of or belonging to Siam, the old name of Thailand, where the type strain was isolated).

After 3 days at 25 °C in YM broth, cells are globose to subglobose (3–5 × 3–5 μm) and occur singly, in pairs or in groups (Fig. 2). Budding is multilateral. After 3 days at 25 °C in YM agar, the streak culture is white to cream, smooth, soft and has an entire margin. Pseudohyphae and true hyphae are not formed in slide culture on PDA after 7 days at 25 °C. Ascospores are not produced on YPD agar, 5 % malt extract agar, McClary's acetate agar, Gorodkowa agar or corn meal agar after 4 weeks at 15 and 25 °C. Positive for fermentation of D-glucose but negative for D-galactose, sucrose, maltose, raffinose, lactose, trehalose, xylose and cellobiose. D-Glucose, D-galactose (slow), L-sorbose (slow), sucrose, maltose, cellobiose, trehalose, melezitose, soluble starch, inulin, D-xylose, L-arabinose (latent), D-arabinose (slow), L-rhamnose, D-ribose, ethanol, glycerol, erythritol, ribitol, galactitol (latent), D-mannitol, D-glucitol, methyl α-D-glucoside, salicin, D-gluconic acid (slow), succinic acid, citric acid, arbutin, *N*-acetyl-D-glucosamine, 2-keto-D-gluconic acid (weak), 1,2-propanediol (weak), 2,3-butanediol (weak), D-glucono-δ-lactone, xylitol, D-galacturonic acid, arabinitol, ethylamine, L-lysine (weak) and cadaverine are assimilated but lactose, melibiose, raffinose, DL-lactic acid, methanol, D-glucuronic acid, 5-keto-D-gluconic acid, inositol, D-glucosamine, hexadecane, nitrate and nitrite are not. No growth in vitamin-free medium. Growth on medium containing 50 % glucose, 60 % glucose and 10 % sodium chloride/5 % glucose is positive, but on 16 % sodium chloride/5 % glucose is negative. Growth with 0.01 and 0.1 % cycloheximide is positive (weak). Grows at 25, 30, 35, 37 and

40 °C, but not at 42 or 45 °C. Starch-like compounds are not produced. Diazonium blue B colour and urease reactions are negative. The major ubiquinone is Q-7.

The holotype is DMKU-RK467^T, isolated from the phylloplane of sugar cane (*Saccharum officinarum* L.) collected from Nakhon Ratchasima province, Thailand. Living cultures from the type have been deposited at the BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathumthani, Thailand, as BCC 50734^T, the NITE Biological Resources Center (NBRC), Department of Biotechnology, National Institute of Technology and Evaluation, Chiba, Japan, as NBRC 108903^T and the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands, as CBS 12569^T. The Mycobank registration number is MB 803008.

In practice, *N. siamensis* can be distinguished from its closest relative, *Candida wickerhamii*, not only on the basis of the sequences of the D1/D2 region of the LSU rRNA gene and ITS region but also on some phenotypic characteristics. *N. siamensis* sp. nov. assimilates sucrose, maltose, melezitose, inulin, soluble starch, erythritol (weak), galactitol (latent), methyl α-D-glucoside and xylitol, in contrast to the type strain of *Candida wickerhamii*. The novel species does not assimilate nitrate or nitrite, in contrast to the type strain of *Candida wickerhamii*. Growth on medium containing 50 % glucose and 10 % NaCl plus 5 % glucose is positive for *N. siamensis* but negative for the type strain of *Candida wickerhamii*.

Members of the *Nakazawaea* clade are often found on plants and substrates associated with plants (Kurtzman, 2011). Many strains of *N. holstii* seem to be primarily associated with bark beetles infesting pine, spruce and fir trees (Kurtzman, 2011), but it has also been isolated from other substrates, such as cider and apples, insect frass, streams and lichens. *Candida chilensis* was collected from *Tritoma* sp., a basidiocarp-feeding beetle, *Candida heliconiae* was isolated from water accumulated in flower bracts of *Heliconia*, *Candia populi* from sap flux of trembling aspen, *Candida wickerhamii* from silage and *Candida wyomingensis* from sap fluxes of trees (Kurtzman, 2011; Lachance *et al.*, 2011). However, members of the *Nakazawaea* clade have not previously been isolated from the leaf surface. In this study of 166 yeast strains obtained from the phylloplane of 95 samples of sugar cane leaf, strain DMKU-RK467^T was the only strain of *Nakazawaea siamensis* isolated; therefore, it is clear that the novel species is not common in the phylloplane. *Meyerozyma caribbica* was found to be the most common yeast species in the sugar cane phylloplane in this study.

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ORIGINAL PAPER

Diversity of culturable yeasts in phylloplane of sugarcane in Thailand and their capability to produce indole-3-acetic acid

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Abstract Yeasts were isolated by the enrichment technique from the phylloplane of 94 samples of sugarcane leaf collected from seven provinces in Thailand. All sugarcane leaf samples contained yeasts and 158 yeast strains were obtained. On the basis of the D1/D2 domain of the large subunit rRNA gene sequence analysis, 144 strains were identified to 24 known species in 14 genera belonging to the Ascomycota *viz. Candida akabanensis*, *Candida dendronema*, *Candida mesorugosa*, *Candida michaelii*, *Candida nivariensis*, *Candida rugosa*, *Candida orthopsilosis*, *Candida quercitrusa*, *Candida tropicalis*, *Candida xylopsoci*, *Cyberlindnera fabianii*, *Cyberlindnera rhodanensis*, *Debaryomyces nepalensis*, *Hannaella* aff. *coprosmaensis*, *Hanseniaspora guilliermondii*, *Kluyveromyces marxianus*, *Lachancea thermotolerans*, *Lodderomyces elongisporus*, *Metschnikowia koreensis*, *Meyerozyma caribbica*, *Millerozyma koratensis*, *Pichia kudriavzevii*, *Torulaspora delbrueckii* and *Wickerhamomyces edaphicus,* and 12 species in six genera of the Basidiomycota *viz. Cryptococcus flavescens*, *Cryptococcus laurentii*, *Cryptococcus rajasthanensis*, *Kwoniella heveanensis*, *Rhodosporidium fluviale*, *Rhodosporidium paludigenum*, *Rhodotorula mucilaginosa*, *Rhodotorula sesimbrana*, *Rhodotorula taiwanensis*, *Sporidiobolus ruineniae*, *Sporobolomyces carnicolor* and *Sporobolomyces nylandii.* Seven strains were identical or similar to four undescribed species. Another seven strains represented four novels species in the genus *Metschnikowia*, *Nakazawaea*, *Wickerhamomyces* and *Yamadazyma*. The results revealed 69 % of the isolated strains were ascomycete yeasts and 31 % were basidiomycete yeast. The most prevalent species was *M. caribbica* with a 23 % frequency of occurrence followed by *Rh. taiwanensis* (11 %) and *C. tropicalis* (10 %). All strains were assessed for indole-3-acetic acid (IAA) producing capability showing that 69 strains had the capability of producing IAA when cultivated in yeast extract peptone dextrose broth supplemented with 1 g/L L-tryptophan. The highest IAA concentration of 565.1 mg/L was produced by *R. fluviale* DMKU-RK253.

Keywords Phylloplane · Sugarcane · Yeast · Indole-3-acetic acid · Thailand

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Introduction

Sugarcane is the second most important crop in Thailand after rice. The Office of the Cane and Sugar Board under the jurisdiction of the Thailand Ministry of Industry reported a sugarcane planted area of approximately 1,439,700 hectares and a production of 106,319,900 tons of sugar for the year 2012.

The phylloplane or phyllosphere, a term which usually refers to the external surface of plant leaves, has been recognized as an important habitat for epiphytic microorganisms that are capable of surviving, growing and reproducing

(Phaff and Starmer 1987; Fonseca and Inacio 2006). The growth of microorganisms on the phylloplane depend mainly on nutrients from plant metabolites that are secreted to the leaf surface or on materials from external sources that drop on. The plant metabolites are organic substances, mostly simple sugars e.g. glucose, fructose and sucrose, while amino acids, organic acids and sugar alcohols are also present (Fiala et al. 1900; Xin et al. 2009). On the other hand, the materials from external sources are inorganic nutrients (Xin et al. 2009). In addition to having relatively limited nutrient sources, the microbial inhabitants of the phylloplane encounter strong ultraviolet radiation, low or inconsistent water activity, fluctuating temperature, and probably antimicrobial compounds that are produced by plants or other microorganisms. As a consequence, the microorganisms on the phylloplane are often irregularly distributed (Fonseca and Inacio 2006; Inácio et al. 2005; Remus-Emsermann et al. 2012; Vorholt 2012). While bacteria are the most abundant phylloplane microorganisms, yeasts and yeast-like fungi are also active phylloplane colonizers (Andrews and Harris 2000; Lindow and Brandl 2003). The phylloplane of vascular plants are offenly colonized by both basidiomycete and ascomycete yeasts (Nakase et al. 2001; Inácio et al. 2005; Fonseca and Inacio 2006; Slavikova et al. 2009; Glushakova and Chernov 2010; Landell et al. 2010). There have been few studies on yeasts and other fungi associated with sugarcane. Yeast communities associated with sugarcane leaves, stems and rhizospheres during different phases of plant development in Brazil were reported by de Azeredo et al. (1998). They found that the prevalent yeast species isolated from sugarcane were *Cryptococcus laurentii, Cryptococcus albidus*, *Rhodotorula mucilaginosa* and *Debaryomyces hansenii*. *Candida pseudointermedia*, *Candida tropicalis*, *Hanseniaspora guilliermondii*, *Pichia guilliermondii*, *Saccharomyces cerevisiae* and *Trichosporon asahii* were found form sugarcane peels from dumping sites in Nigeria and *C. tropicalis* being the species most frequently isolated (Olasupo et al. 2003). From our previous study on yeasts in phylloplane of various plants species in Thailand *Hanseniaspora opuntiae* and *T. asahii* were isolated from phylloplane of sugarcane (Limtong and Koowadjanakul 2012). Various fungi and yeasts were isolated from decaying sugarcane plants in nature (Shrestha et al. 2011) and the ascomycete fungi were dominant. The two most frequently found classes were Dothideomycetes, the more common class, and Sordariomycete. Four novel yeast species *viz. Metschnikowia saccharicola*, *Nakazawaea siamensis*, *Wickerhamomyces siamensis* and *Yamadazyma siamensis* were proposed from the strains isolated from phylloplane of sugarcane in Thailand (Kaewwichian et al. 2012, 2013a, b; Kaewwichian and Limtong 2013).

Plant growth promoters produced by plants and microorganisms at low concentrations play an important role in growth and development of plants through induction of some important physiological responses at different stages of plant development such as regulation of seed germination, root formation, branching and tillering, and fruit ripening (Tsavkelova et al. 2006). Plant growth promoters have been classified into five groups, namely, auxins, gibberellins, cytokinins, ethylene and ascorbic acid (Tsavkelova et al. 2006). In practice, plant growth promoting agents can be applied directly, or inoculation of microorganisms capable of producing plant growth promoters may be used (Nassar et al. 2005; Tsavkelova et al. 2006). Indole-3-acetic acid (IAA) is the major member of the auxin plant growth promoter group and is known to stimulate both rapid and long-term responses in plants by regulation of various developmental and physiological processes (Cleland 1990). Microorganisms which are capable of IAA production include bacteria (Sasirekha et al. 2012), actinomycetes (Khamna et al. 2010), filamentous fungi (Ruanpanun et al. 2010) and yeasts. Some yeast species that have been reported to produce IAA include *Saccharomyces cerevisiae*, *Saccharomyces uvarum*, *Cyberlindnera* (*Williopsis*) *saturnu* and *Scheffersomyces (Pichia) spartinae* (Nakamura et al. 1991; Shin et al. 1991; Nassar et al. 2005; Rao et al. 2010). In both plants and microorganisms there are multiple IAA biosynthesis pathways and L-tryptophan has been identified as a main precursor (Delker et al. 2008; Spaepen et al. 2007). Apart from tryptophan-dependent IAA biosynthesis, the tryptophan-independent pathways are also found in both plants (Woodward and Bartel 2005) and microorganisms. A tryptophan-independent IAA biosynthetic pathway in a *S. cerevisiae* mutant defective in two *ALD* genes was recently elucidated (Rao et al. 2010). A number of recent reports indicated that IAA can be a signaling molecule in microorganisms, both IAA producing and non-IAA producing species (Remans et al. 2006; Spaepen et al. 2007). In *S. cerevisiae* and *Candida albicans* addition of IAA into the culture medium stimulated filamentation and differential gene expression which suggested that IAA was an important signal that triggers dimorphic transition (Prusty et al. 2004; Rao et al. 2010). Applications of IAA producing yeasts, such as *Candida valida, Cy. saturnus*, *Rhodotorula glutinis*, *Rhodotorula mucilaginosa*, *Sporobolomyces roseus* and *T. asahii* to promoting the growth of plants have been reported (Perondi et al. 1996; El-Tarabily 2004; Nassar et al. 2005; Xin et al. 2009).

Although yeasts inhabiting the phylloplane have been studied intensively elsewhere, the diversity of yeasts in the phylloplane in Thailand has received little attention. Until now, except our report (Limtong and Koowadjanakul 2012), no study have been undertaken of yeasts in the phylloplane of sugarcane. Moreover, there have been few studies on yeasts associated with sugarcane in the other

countries. Therefore, the objectives of this study were to investigate the diversity of yeasts in the phylloplane of sugarcane in Thailand and to assess the capacity of such yeasts to produce IAA in vitro.

Materials and methods

Sample collection

Green and healthy leaves of sugarcane (*Saccharum officinarum*) were collected from sugarcane plantations and placed in plastic bags, sealed and transferred in an ice-box to the laboratory. The samples were kept at 8 °C until subjected to yeast isolation.

Yeast isolation

Yeasts were isolated by an enrichment technique using yeast extract malt extract (YM) broth (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone and 10 g/L glucose) supplemented with 250 mg/L sodium propionate and 200 mg/L chloramphenicol (Limtong et al. 2007). Three grams of cut leaves were put into a 250 mL Erlenmeyer flask containing 50 mL enrichment broth. The inoculated flasks were incubated on a rotary shaker at room temperature (30 ± 2 °C) for 2 days. A loopful of the enriched culture was streaked on YM agar supplemented with 250 mg/L sodium propionate and 200 mg/L chloramphenicol. Yeast colonies of different morphologies were picked and purified by cross streaking on YM agar. Purified yeast strains were maintained by suspending in YM broth supplemented with 10 % v/v glycerol and kept at −80 °C.

Yeasts identification

Yeasts were identified by analysis of the D1/D2 region of the large subunit (LSU rRNA) gene sequence similarities (Kurtzman and Robnett 1998). The sequence of the D1/D2 region of the LSU rRNA gene was determined from PCR products amplified from genomic DNA. The D1/D2 region of the LSU rRNA gene was amplified and sequenced with primers, NL1 and NL4 (Kurtzman and Robnett 1998). Methods for DNA extraction and amplification of the D1/D2 region of the LSU rRNA gene and ITS region were as described previously (Limtong et al. 2007). The PCR products were checked by agarose gel electrophoresis and purified by using the Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taiwan). The purified products were submitted to Macrogen Inc. (Korea) for sequencing. Sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler using a ABI PRISM® BigDye TM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems, USA), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using the primers NL1 and NL4 for the D1/D2 region of the LSU rRNA gene. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730XL sequencer (Applied Biosystems, USA). The sequences were compared pairwise using a BLAST search (Altschul et al. 1997).

Determination of indole-3-acetic acid production

Production of indole-3-acetic acid (IAA) by the phylloplane yeasts was investigated by the method of Xin et al. (2009). A yeast culture grown for 1–2 days on YM agar at 25 °C was inoculated into 5 mL of yeast extract peptone dextrose (YPD) broth (10 g/L yeast extract, 20 g/L peptone and 20 g/L dextrose) supplemented with 1 g/L L-tryptophan in a test tube and incubated on a shaker at 30 ± 2 °C and 150 rpm for 7 days. An aliquot of 1.5 mL of the culture broth was centrifuged at 8,000 rpm for 5 min and the supernatant was collected for determination of IAA concentration. One mL of supernatant was mixed with 1 mL of Salkowski reagent (12 g/l $FeCl_3$ and 7.9 M H_2SO_4) (Glickmann and Dessaux 1994), and the intensity of pink color developed in the mixture after 30 min was quantified with a spectrophotometer (UV–Vis 1700, Shimizu, Japan) at a wavelength of 530 nm. A calibration curve using authentic IAA was established for calculation of IAA concentration. Cultivation for IAA production was performed in duplicate. Cell dry weight was determined by resuspending cell pellet in distilled water, washed twice with distilled water, dried at 90 °C for 48 h and then weighed.

IAA production by the high IAA producing strains was determined by cultivation in 50 mL of previous mentioned medium in a 250 mL Erlenmeyer flask and incubated on a rotary shaker (IS-917R Floor Model Incubated Shaker, Lab Companion, Korea) at 150 rpm and 25 °C for 7 days. IAA was quantified by HPLC (Agilent 1100, Agilent Technologies, USA) equipped with a Cosmosil SC18-MS-II column (NacalaiTesque, Japan) and UV detector at 280 nm, using 40 % of solution A (methanol:aceticacid:water = 10:0.3:89.7 v/v/v) and 60 % of solution B (methanol:aceticacid:water = 90:0.3:9.7 v/v/v) as mobile phase with the flow rate of 0.5 mL/min as described by Kim et al. (2006). Authentic IAA (Sigma, USA) was used as a standard.

Results

Sample collection and yeast isolation

Yeasts were isolated by the enrichment technique from the phylloplane of 94 sugarcane leaf samples which had been collected from 15 sampling sites in different districts of seven provinces of Thailand between September 2009 and May 2010 (Table 1). The sampling sites were in the north-eastern (A) and central parts (B–G) of Thailand, where the average maximum temperature (32–36 °C) and minimum temperature (22–25 °C) were not much different during the sampling period. Yeasts could be isolated from all 94 samples and in total 159 strains were obtained (Table 1).

Yeast identification and diversity of yeasts in sugarcane phylloplane

On the basis of the D1/D2 region of the large subunit rRNA gene sequence analysis, 144 strains were identified as shown in Tables 2 and Supplementary Table 1. A total of 24 known yeast species represented 14 genera of the Phylum Ascomycota and 12 species belonged to six genera of the Phylum Basidiomycota. The ascomycete yeasts consisted of two species of *Cyberlindnera*, one species each of *Debaryomyces*, *Hannaella*, *Hanseniaspora*, *Kluyveromyces*, *Lachancea*, *Lodderomyces*, *Metschnikowia*, *Meyerozyma*, *Millerozyma Pichia*, *Torulaspora* and *Wickerhamomyces* and 10 species of *Candida*. The *Candida* species were in seven clades including *Candida tanzawaensis* (one species), *Kurtzmaniella* (one species), *Lodderomyces-Spathospora* (two species), *Metschnikowia* (one species), *Nakaseomyces* (one species), *Yamadazyma* (two species) and unaffiliated clades (two species).The basidiomycete yeasts were belonging to six genera *viz*. *Cryptococcus* in *Bulleromyces* clade (three species), *Kwoniella* (one species), *Rhodosporidium* (two species), *Rhodotorula* in Sporidiobolales clade (three species), *Sporidiobolus* (one species) and *Sporobolomyces* (two species). One strain similar to *Candida* sp. BG01-8-20-001B-C1, two strains identical to *Candida* sp. 05-7-186^T and three strains similar to *Rhodosporidium* sp. APSS 849 were obtained. Seven of these strains have already been proposed as four novel species: *M. saccharicola*, *N. siamensis*, *W. siamensis* and *Y. siamensis* (Kaewwichian et al. 2012, 2013a, b; Kaewwichian and Limtong 2013).

The most prevalent yeast species on sugarcane leave samples were *Meyerozyma caribbica,* with a 23 % frequency of occurrence followed by *Rhodotorula taiwanensis* (11 %), *Candida tropicalis* (10 %), *Cryptococcus laurentii* (6 %), *Cyberlindnera rhodanensis* (5 %), *Candida nivariensis* (4 %), *Cyberlindnera fabianii* (3 %), *Rhodosporidium fluviale* (3 %), and *Millerozyma koratensis* (3 %). The other species were detected only 1–3 strains (1 %). It should be noted that *M. carribica* was widely distributes as it was isolated from sugarcane leaf samples collected from 13 sampling sites out of 15 sites (Table 2).

Iodole-3-acetic acid production

Among the 159 yeast strains, 71 assigned to 21 known species and two representatives of two undescribed species showed the capacity to produce IAA when cultivated

Table 1 Sugarcane leaves collection, sampling site and number of yeast strains isolated from phylloplane

Site	District, province	Location	Date of sampling	Sample no.	No. of yeast strains
A-1	Sikhio, Nakhon Ratchasima	14°53′30″N101°43′24″E	28/9/2009	1–5	7
A-2	Dan Khun Thot, Nakhon Ratchasima	15°12′35″N101°45′57″E	5/5/2010	72–74	5
A-3	Mueang, Nakhon Ratchasima	14°58′16″N102°5′59″E	6/5/2010	75–80	8
A-4	Chok Chai, Nakhon Ratchasima	14°43′56″N102°9′47″E	6/5/2010	81–88	14
A-5	Khon Buri, Nakhon Ratchasima	14°31′24″N102°14′54″E	6/5/2010	89–94	15
B	Phak Hai, Phra Nakhon Si Ayutthaya	14°27′30″N100°22′12″E	3/2/2010	6–16	16
C	Dan Chang, Suphan Buri	14°50′30″N99°41′50″E	22/3/2010	17–21	11
D-1	Ban Rai, Uthai Thani	15°5′2″N99°31′16″E	22/3/2010	22–24	7
D-2	Lan Sak, Uthai Thani	15°27′10″N99°34′34″E	22/3/2010	25–26	4
E	Takhli, Nakhon Sawan	15°15′47″N100°20′37″E	5/5/2010	35–46	18
F-1	Bueng Sam Phan, Phetchabun	15°47′48″N101°0′30″E	5/5/2010	27–31	8
F-2	Wichian Buri, Phetchabun	15°39′26″N101°6′24″E	5/5/2010	32–34	4
G-1	Nong Muang, Lop Buri	15°14′50″N100°39′15″E	5/5/2010	47–55	14
G-2	Khok Samrong, Lop Buri	15°4′11″N100°43′26″E	5/5/2010	56–60	8
G-3	Tha Luang, Lop Buri	15°4′6″N101°6′25″E	5/5/2010	61–71	20

Table 2 Identification of yeast strains isolated from phylloplane of sugarcane and their GenBank accession numbers of the D1/D2 region of LSU rRNA gene and their frequency of occurrence

Species	Genus or clade	No. of total strain	Frequency of occurrence (%)[c]	GenBank accession no. of the closest species	Nucleotide substitution to closest species	Strain example		Sampling site where this species was present
						Strain	D1/D2 GenBank accession no.	
Phylum Ascomycota								
Candida akabanensis	*Metschnikowia*	1	0.63	EU100744	2	DMKU-RK433	AB772160	A-3
Candida dendronema	*Yamadazyma*	1	0.63	U45751	1	DMKU-RK365	AB772126	G-2
Candida mesorugosa	Unaffiliated	1	0.63	FJ768919	2	DMKU-RK179	AB772048	E
Candida michaelii	*Yamadazyma*	2	1.27	AY520329	0	DMKU-RK343	AB772108	E
Candida nivariensis	*Nakaseomyces*	6	3.80	AY627305	0–1	DMKU-RK290	AB772094	A-3, A-4, A-5, E, G-3
						DMKU-RK393	AB772137	
Candida orthopsilosis	*Lodderomyces-Spathospora*	1	0.63	FJ746056	1	DMKU-RK28	AB772042	A-1
Candida quercitrusa	*Kurtzmaniella*	1	0.63	U45831	1	DMKU-RK14	AB772038	A-1
Candida rugosa	Unaffiliated	3	1.90	U45727	1–3	DMKU-RK299	AB772100	A-5, H, G-3
Candida tropicalis	*Lodderomyces-Spathospora*	15	9.49	U45749	0	DMKU-RK26	AB772040	A-1, A-2, A-4, A-5, C, D-2, E, F-1, F-2, G-3
						DMKU-RK196	AB772062	
						DMKU-RK210	AB772074	
						DMKU-RK256	AB772084	
Candida xylopsoci	*Candida tanzawaensis*	1	0.63	AY013719	1	DMKU-RK209	AB772073	D-1
Similar to *Candida* sp. BG01-8-20-001B-C1	Unidentified	1	0.63	AY242320	1	DMKU-RK15	AB772039	A-1
Identical to *Candida* sp. 05-7-186^T	Unidentified	2	1.27	FJ537069	0	DMKU-RK206	AB772070	D-1
						DMKU-RK470	AB772180	A-4
Nakazawaea siamensis[a]	*Nakazawaea*	1	0.63	U70243	11	DMKU-RK467	AB772177	E

Table 2 continued

Species	Genus or clade	No. of total strain	Frequency of occurrence (%)[e]	GenBank accession no. of the closest species	Nucleotide substitution to closest species	Strain example		Sampling site where this species was present
						Strain	D1/D2 GenBank accession no.	
Cyberlindnera fabianii	*Cyberlindnera*	5	3.16	U73573	0	DMKU-RK208	AB772072	A-2, A-4, A-5, D-1, G-3
				U73573		DMKU-RK413	AB772146	
Cyberlindnera rhodanensis	*Cyberlindnera*	8	5.06	U73571	0–3	DMKU-RK349	AB772114	A-3, A-4, A-5, G-1
						DMKU-RK492	AB772186	
Debaryomyces nepalensis	*Debaryomyces*	1	0.63	U45839	1	DMKU-RK176	AB772047	B
Hanseniaspora guilliermondii	*Hanseniaspora*	1	0.63	U84230	0	DMKU-RK497	AB772191	A-5
Hannaella aff. *coprosmaensis*	*Hannaella*	1	0.63	FN428908	1	DMKU-RK250	AB772081	F-1
Kluyveromyces marxianus	*Kluyveromyces*	1	0.63	U94924	0	DMKU-RK458	AB772174	A-4
Lachancea thermotolerans	*Lachancea*	1	0.63	U69581	0	DMKU-RK361	AB772123	G-1
Lodderomyces elongisporus	*Lodderomyces-Spathospora*	1	0.63	U45763	0	DMKU-RK440	AB772161	A-3
Metschnikowia koreensis	*Metschnikowia*	3	1.90	AF296438	0	DMKU-RK382	AB772129	A-3, G-2, B
Metschnikowia saccharicola[b]	*Metschnikowia*	4	2.53	GU810536	20	DMKU-RK16	AB697752	A-1, A-5, C
						DMKU-RK24	AB697753	
						DMKU-RK198	AB697754	
						DMKU-RK500	AB697755	
						Strain	D1/D2 GenBank	

Table 2 continued

Species	Genus or clade	No. of total strain	Frequency of occurrence (%)[e]	GenBank accession no. of the closest species	Nucleotide substitution to closest species	Strain example		Sampling site where this species was present
						Strain	D1/D2 GenBank accession no.	
Meyerozyma caribbica	*Meyerozyma*	36	22.78	EU348786	0–1	DMKU-RK27	AB772041	A-1, A-2, A-4, A-5, B, C, D-1, D-2, E, F-2, G-1, G-2, G-3
						DMKU-RK203	AB772068	
						DMKU-RK287	AB772091	
						DMKU-RK346	AB772111	
						DMKU-RK366	AB772127	
						DMKU-RK391	AB772135	
						DMKU-RK426	AB772156	
						DMKU-RK499	AB772193	
Millerozyma koratensis	*Millerozyma*	4	2.53	DQ404484	0	DMKU-RK297	AB772099	E, G-3,A-3
						DMKU-RK412	AB772145	
Pichia kudriavzevii	*Pichia*	3	1.90	U76347	0	DMKU-RK490	AB772184	A-5, D-1, G-3
Torulaspora delbrueckii	*Torulaspora*	1	0.63	U72156	0	DMKU-RK193	AB772061	C
Wickerhamomyces edaphicus	*Wickerhamomyces*	1	0.63	AB436763	2	DMKU-RK411	AB714243	G-3
Wickerhamomyces siamensis[c]	*Wickerhamomyces*	1	0.63	U74587	4	DMKU-RK359	AB714248	G-1
Yamadazyma siamensis[d]	*Yamadazyma*	1	0.63	U45750	9	DMKU-RK254	AB734046	F-1
Phylum Basidiomycota								
Cryptococcus flavescens	*Bulleromyces*	2	1.27	AB035042	0	DMKU-RK204	AB772069	C, D-1
Cryptococcus laurentii	*Bulleromyces*	9	5.70	AF075469	1–3	DMKU-RK248	AB772079	A-4, C, F-1,G-1
						DMKU-RK345	AB772110	

Table 2 continued

Species	Genus or clade	No. of total strain	Frequency of occurrence (%)[e]	GenBank accession no. of the closest species	Nucleotide substitution to closest species	Strain example		Sampling site where this species was present
						Strain	D1/D2 GenBank accession no.	
Cryptococcus rajasthanensis	*Bulleromyces*	2	1.27	AM262324	0	DMKU-RK175	AB772046	B, F-1
Kwoniella heveanensis	*Kwoniella*	2	1.27	AF075467^T	1	DMKU-RK302	AB772103	E
Rhodosporidium fluviale	Sporidiobolales	5	3.16	AF070422	1–2	DMKU-RK253	AB772082	A-2, A-5, G-2, G-3, F-1
						DMKU-RK416	AB772148	
Rhodosporidium paludigenum	Sporidiobolales	1	0.63	AF363640	0	DMKU-RK342	AB772107	E
Similar to *Rhodosporidium*sp. APSS 849	unidentified	3	1.90	FM178290	1	DMKU-RK354	AB772118	G-1
Rhodotorula mucilaginosa	Sporidiobolales	1	0.63	AF070432	0	DMKU-RK294	AB772097	E
Rhodotorula sesimbrana	Sporidiobolales	1	0.63	AF387144	0	DMKU-RK494	AB772188	A-5
Rhodotorula taiwanensis	Sporidiobolales	17	10.76	GU646863	0–1	DMKU-RK188	AB772057	A-3, A-4, B, C, D-2, E, F-1, F-2
						DMKU-RK427	AB772157	
						DMKU-RK443	AB772163	
						DMKU-RK454	AB772172	
Sporidiobolus ruineniae	Sporidiobolales	2	1.27	AF070434	0	DMKU-RK392	AB772136	G-2, G-3
Sporobolomyces carnicolor	Sporidiobolales	1	0.63	AY158641	0	DMKU-RK249	AB772080	F-1
Sporobolomyces nylandii	Sporidiobolales	3	1.90	AB279629	0	DMKU-RK207	AB772071	D-1, G-1, G-3

[a] Kaewwichian and Limtong 2013

[b] Kaewwichian et al. 2012

[c] Kaewwichian et al. 2013a

[d] Kaewwichian et al. 2013b

[e] Number of plant leaf sample from which that species was isolated/number of plant leaf sample examined × 100

Table 3 Indole-3-acetic acid productions by yeast strains isolated from sugarcane phylloplane cultivated in YPD broth supplemented with 1 g/L L-tryptophan in a test tube and incubated on a shaker at 30 ± 2 °C and 150 rpm for 7 days and IAA was analyzed by colorimetric method

Species	No. of strain	IAA (mg/L) (minimal/average/maximal)	IAA (mg/g dry cell weight) (minimal/average/maximal)
Phylum Ascomycota			
Candida mesorugosa	2	24.2/118.0/130.1	1.6/4.9/8.2
Candida michaelii	1	259	27.1
Candida orthopsilosis	1	129.2	10.6
Candida quercitrusa	1	37.5	4.0
Candida tropicalis	7	2.5/49.8/95.2	0.2/4.1/7.0
Cyberlindnera fabianii	1	6.9	0.8
Debaryomyces nepalensis	1	47.6	4.8
Lodderomyces elongisporus	1	105.7	8.4
Metschnikowia koreensis	1	58.5	6.1
Metschnikowia saccharicola	2	0.5/7.5/14.4	0.1/1.15/2.2
Meyerozyma caribbica	20	5.7/47.0/131.8	0.7/5.5/16.6
Pichia kudriavzevii	1	24.2	2.4
Torulaspora delbrueckii	1	69.6	6.1
Similar to *Candida* sp. BG01-8-20-001B-C1	1	106.7	11.8
Phylum Basidiomycota			
Cryptococcus flavescens	2	17.5/47.0/76.4	3.0/6.7/10.3
Cryptococcus laurentii	2	31.5/40.8/50.0	4.1/8.9/13.7
Cryptococcus rajasthanensis	1	57.7	6.2
Rhodosporidium fluviale	5	10.4/220.5/417.8	3.7/30.8/66.8
Rhodosporidium paludigenum	1	144.6	14.4
Similar to *Rhodosporidium* sp. APSS 849	1	8.7	1.0
Rhodotorula taiwanensis	10	14.4/57.3/137	2.0/7.4/19.0
Sporidiobolus ruineniae	2	11.0/16.6/21.5	1.4/2.5/3.6
Sporobolomyces nylandii	2	10.6/50.9/91.2	1.6/7.5/13.3

in YPD broth supplemented with 1 g/L L-tryptophanand IAA was analyzed by colorimetric method in the range of 0.5–418 mg/L (Table 3). The highest IAA concentration of 418 mg/L (67 mg/g dry cell weight) was produced by *R. fluviale* DMKU-RK253 followed by the other two strains of *R. fluviale*, DMKU-RK498 and DMKU-RK422, which produced 269 and 259 mg/L, respectively. The three high IAA producing strains were re-checked for IAA production in the same medium but by shake flask cultivation at 25 °C. The three strains, DMKU-RK253, DMKU-RK498 and DMKU-RK422 produced IAA determined by HPLC as 565.1, 456.0 and 487.4 mg/L, respectively (Table 4).

Discussion

The results indicated that 69 % of the strains isolated by the enrichment technique were ascomycete yeasts, and 31 % represented basidiomycete yeast strains. This is in contrast with other investigations, which have reported the dominance of basidiomycete yeasts on the phylloplane of plants in other regions (de Azeredo et al. 1998; Nakase et al. 2001; Fonseca and Inacio 2006; Slavikova et al. 2009; Glushakova and Chernov 2010). This difference may have resulted from differences in the climatic conditions and in the technique employed for isolation. While in most investigations by plating of leaf washing at 25 °C or lower was used (de Azeredo et al. 1998; Slavikova et al. 2009), Nakase et al. (2001) used the ballistoconidium fall method with YM agar without any antibacterial and antifungal agents and found more yeast species when the incubation temperature was at 23 °C than at 30 °C. However, the present study agreed well with our previous report, which revealed the dominance of ascomycete yeasts species on the phylloplane of various plant species in Thailand when enrichment isolation and a relatively high incubation temperature were used (Limtong and Koowadjanakul 2012).

Among yeast species found in the phylloplane of sugarcane in this study only two basidiomycete yeast species,

Table 4 Indole-3-acetic acid productions by the three high IAA producing strains cultivated in 50 mL YPD broth supplemented with 1 g/L L-tryptophan in 250 Erlenmeyer flask, incubated on a rotary shaker at 25 °C and 150 rpm for 7 days and IAA was analyzed by HPLC

Species	IAA (mg/L)[a]	IAA (mg/g dry cell weight)[a]
Rhodosporidium fluviale DMKU-RK253	565.1	74.4
Rhodosporidium fluviale DMKU-RK422	487.4	37.0
Rhodosporidium fluviale DMKU-RK498	456.0	42.1

[a] Values presented were averaged from three replication

namely *Cr. laurentii* and *Rh. mucilaginosa*, were previously reported to associate with sugarcane in Brazil (de Azeredo et al. 1998); however, they were identified based on growth response data, which might not coincide with sequence-based identification. In addition three yeast species, *viz. C. tropicalis, H. guilliermondii* and *Rh. mucilaginosa*, were previously isolated from sugarcane peels in Nigeria (Olasupo et al. 2003). But the species obtained from sugarcane phylloplane in our previous investigation *viz. H. opuntiae* and *T. asahii* could not detect in this study (Limtong and Koowadjanakul 2012). The differences between the yeasts associated with sugarcane in Thailand and those studies in Brazil and Nigeria could be the consequence of differences in geographic location because Thailand is in the Southeast Asia in Asia continent while Brazil is in South America of the American continent and Nigeria is in Western Africa of the African continent.

Many yeast species found in the phylloplane of sugarcane had also been detected previously in the phylloplane of diverse plants species in Thailand. These included *Candida nivariensis*, *C. rugosa*, *C. tropicalis*, *Cy. rhodanensis*, *Debaryomyces nepalensis, Kluyveromyces marxianus, Lachancea thermotolerans*, *Pichia kudriavzevii*, *Wickerhamomyces edaphicus, Sporidiobolus ruineniae* and *Sporobolomyces nylandii* (Takashima and Nakase 2000; Limtong and Koowadjanakul 2012). However, two common yeast species in sugarcane phylloplane in this study *viz. M. caribbica* and *Rh. taiwanensis* have never been detected in the phylloplane of other plants growing naturally in Thailand. The differences between the yeast communities on sugarcane leaf surface and those of other plants growing naturally in Thailand previously reported by our group could be the impact of differences in plant species. Also, the differences may be the consequence of the areas that located between 10°29′ 34″N90°10′45″E and 14° 12′44″N 101°12′06″E in which the samples were collected. *S. ruineniae*, which had a low frequency of occurrence in this study, was the only species for which the phylloplane has been stated to be a possible habitat and is the species that has been found only in Asia and Africa (Sampaio 2011).

Investigation of the IAA production capability of the sugarcane phylloplane yeasts in this study revealed that approximately 43 % of the investigated strains in both Ascomycota and Basidiomycota possessed this ability. It was found that within a single species of yeast it could be an IAA producing or non-IAA producing strain. It therefore seems that IAA production capability is strain-dependent in these phylloplane yeasts. Variation in IAA biosynthesis among yeast strains within the same species and the other microorganisms has also been reported by other investigators and our group (Tsavkelova et al. 2006; Ruanpanun et al. 2010; Limtong and Koowadjanakul 2012).

Quantitative analysis of IAA by Salkowski colorimetric technique could produce erroneous results because this technique appeared to be not only to IAA but also the other indole compounds such as indole pyruvic acid and indole acetamide (Glickmann and Dessaux 1994). Therefore, IAA production by the three high IAA producing strains was confirmed by using HPLC. The quantity of IAA production has been reported to depend on strain, culture conditions, growth state as well as the concentration of L-tryptophan in the medium (Chaihara and Lumyong 2011; Mujahid et al. 2011; Sasirekha et al. 2012). In this study although 1 g/L of L-tryptophan was added to the medium but the concentration of L-tryptophan could be higher because of releasing of this compound from peptone and yeast extract in the medium.

IAA production by *R. fluviale* DMKU-RK253 (565.1 mg/L) was higher than that reported by other yeasts such as *Cy. saturnus* (22.5 mg/L) (Nassar et al. 2005) and *Candida maltose* LM114 (314.3 mg/L) (Limtong and Koowadjanakul 2012); fungi such as *Aspergillusniger* (132.7 mg/L) (Bilkay et al. 2010); some actinomycetes such as *Streptomyces* sp. CMU-H009 (143.95 mg/L) (Khamna et al. 2010); and bacteria such *Klebsiellas* sp. SN1 (291 mg/L) (Xinxian et al. 2011) and *Pseudomonas aeruginosa* (132 mg/L) (Sasirekha et al. 2012).

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Papiliotrema siamense f.a., sp. nov., a yeast species isolated from plant leaves

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Two strains representing a novel species were isolated from the external surface of a sugar cane leaf (DMKU-SP85^T) and tissue of a rice leaf (DMKU-RE97) collected in Thailand. On the basis of morphological, biochemical, physiological and chemotaxonomic characteristics, and sequence analysis of the D1/D2 region of the large subunit (LSU) rRNA gene and the internal transcribed spacer (ITS) region, the two strains were determined to represent a novel species of the genus *Papiliotrema* although sexual reproduction was not observed. The sequences of the D1/D2 region of the LSU rRNA gene and ITS region of the two strains were identical, but differed from those of the type strain of *Cryptococcus nemorosus* by 0.6 % nucleotide substitutions (four nucleotide substitutions out of 597 nucleotides) in the D1/D2 region of the LSU rRNA gene and 1.8 % nucleotide substitutions (nine nucleotide substitutions out of 499 nucleotides) in the ITS region. The name *Papiliotrema siamense* f.a., sp. nov. is proposed. The type strain is DMKU-SP85^T(=BCC 69499^T=CBS 13330^T).

The genus *Papiliotrema* was proposed by Sampaio *et al.* (2002) based on an integrated analysis of morphological, ultrastuctural, physiological and molecular data as a dimorphic and teleomorphic yeast genus in the order Tremellales, class Tremellomycetes, subphylum Agaricomycotina and phylum Basidiomycota. In the same article *Papiliotrema bandonii* was proposed as a novel species (Sampaio *et al.*, 2002) and was the only species of this genus at the time of writing (Sampaio, 2011). Phylogenetic relationships among the order Tremellales show that the genus *Papiliotrema* is located in the clade of *Bulleromyces*/*Papiliotrema*/*Auriculibuller* which is referred to as the *Bulleromyces* clade. This clade consists not only of species of the teleomorphic genera, *Bulleromyces*, *Papiliotrema* and *Auriculibuller* but also of species of the two polyphyletic anamorphic genera *Bullera* and *Cryptococcus* (Boekhout, 2011). Sequence analysis of the D1/D2 region of the large subunit (LSU) rRNA gene placed *Papiliotrema bandonii* in a subclade with *Cryptococcus nemorosus* and *Cryptococcus perniciosus* (Fonseca *et al.*, 2011; Sampaio, 2011).

While epiphytic micro-organisms inhabit the surface of various parts of plants (Andrews & Harris, 2000; Lindow & Brandl, 2003), endophytes colonize intercellular and/or intracellular healthy plant tissue without causing disease symptoms or abnormal changes in the plant (Petrini, 1991; Isaeva *et al.*, 2010). Both ascomycete and basidiomycete yeasts have been found to be epiphytes and endophytes, and one yeast species can be both an epiphyte and an endophyte (Isaeva *et al.*, 2010). Some examples of epiphytic yeast species isolated from plant leaves and belong to the Ascomycota are *Candida tropicalis*, *Hanseniaspora opuntiae*, *Kodamaea ohmeri* and *Meyerozyma caribbica* and *Cryptococcus laurentii*, *Rhodosporidium fluviale*, *Rhodotorula taiwanensis* and *Sporidiobolus ruineniae* belong to the Basidiomycota (de Azeredo *et al.*, 1998; Fonseca & Inacio, 2006; Slávikova *et al.*, 2009; Glushakova & Chernov, 2010; Limtong *et al.*, 2014). Though there are reports of endophytic yeasts, they are fewer compared with those of epiphytic yeasts. Examples of endophytic yeast species which have been found in plant leaves are *Debaryomyces hansenii* and *Pichia guilliermondii*, belonging to the Ascomycota and *Cryptococcus flavescens*, *Cryptococcus laurentii*, *Rhodotorula mucilaginosa*, *Sporidiobolus pararoseus* and *Sporobolomyces roseus*, belonging to the Basidiomycota (Camatti-Sartori *et al.*, 2005; Gai *et al.*, 2009).

During the investigations of epiphytic yeasts on the external leaf surfaces of sugar cane and endophytic yeasts in leaf tissue of rice in Thailand, two strains (DMKU-SP85^T and DMKU-RE97) representing a novel species of the genus *Papiliotrema* were obtained.

Abbreviations: LSU, large subunit; ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the sequences of the D1/D2 region of the LSU rRNA gene and the ITS region of strain DMKU-SP85^T are AB909023 and AB915387, respectively; those for the corresponding sequences of strain DMKU-RE97 are AB863558 and AB921216, respectively. The MycoBank number for *Papilitrema siamense* is MB808372.

Yeast isolation and characterization

A total of 267 yeast strains were isolated from the external surface of 102 samples of sugar cane (*Saccharum officinarum* L.) leaves, and 125 yeast strains were obtained from the tissue of 100 samples of surface-sterilized rice (*Oryza sativa* L.) leaves. Strain DMKU-SP85^T was isolated from the external surface of a sugar cane leaf collected from Sankhaburi district, Chai Nat province (15° 11′ 14″ N 100° 07′ 42″ E), on 2 March 2012 and strain DMKU-RE97 was obtained from tissue of a rice leaf collected from Bang Pla Ma district, Suphanburi province (14° 28′ 03″ N 100° 07′ 01″ E), on 2 March 2012.

Strain DMKU-SP85^T was isolated by plating of leaf washings as described by Inácio *et al.* (2002). A 3 g sample of leaf was aseptically suspended in 50 ml of 0.85 % saline solution in a 250 ml Erlenmeyer flask and shaken on a rotary shaker at 25 °C for 1 h to detach yeast cells from the surface. An aliquot of 0.1 ml of the washing solution was then spread on yeast extract–malt extract (YM) agar (0.3 % yeast extract, 0.3 % malt extract, 0.5 % peptone and 2.0 % agar) supplemented with 0.025 % sodium propionate and 0.02 % chloramphenicol and incubated at 25 °C until yeast colonies appeared. Yeast colonies of different morphologies were selected and purified by cross-streaking on YM agar. Purified yeast strains were suspended in YM broth supplemented with 10 % v/v glycerol and maintained at −80 °C. Strain DMKU-RE 97 was isolated from a surface-sterilized leaf using the method of Abdel-Motaal *et al.* (2009) with slight modifications. The leaf was surface sterilized by immersion in 70 % ethanol for 3 min followed by 5 % sodium hypochlorite solution for 3 min and rinsed five times for 5 min with sterilized deionized water. The effectiveness of the surface sterilization procedure was verified by spreading the final rinse water onto YM agar and direct placing of sterilized leaf pieces onto YM agar; no growth of micro-organisms indicated effective sterilization. After surface sterilization, the leaf was cut into small pieces (50 × 50 mm), slightly ground in a sterile mortar to expose the inner tissue and a few pieces of the tissue were placed directly onto YM agar supplemented with 0.02 % chloramphenicol in a sterilized Petri dish. The Petri dish was incubated at 25 °C until yeast colonies appeared. Yeast colonies of different morphologies were picked up, purified and maintained by the same methods as for strain DMKU-SP85.

DNA sequencing and phylogenetic analysis

The sequences of the D1/D2 region of the LSU rRNA gene and the internal transcribed spacer (ITS) region were determined from PCR products amplified from genomic DNA extracted from yeast cells. Methods of DNA extraction and amplification were as described by Limtong *et al.* (2007). Amplification of the D1/D2 of the LSU rRNA gene was carried out by PCR with the forward primer NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and the reverse primer NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) (Kurtzman & Robnett, 1998). The ITS region was amplified with forward primer ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al.*, 1990). The PCR products were checked by agarose gel electrophoresis and purified by using the HiYield Gel/PCR Fragments Extraction kit (RBC Bioscience). The purified products were sequence commercially by Macrogen (Seoul, Korea) using primers, NL1 and NL4 for the D1/D2 and primers, ITS1 and ITS4 for the ITS region. The sequences were compared pairwise using a BLAST search (Altschul *et al.*, 1997) and were aligned with the sequences of related species retrieved from the GenBank database using the multiple alignment program CLUSTAL_X version 1.81 (Thompson *et al.* 1997). A phylogenetic tree was reconstructed from the evolutionary distance data with Kimura's two-parameter correction (Kimura, 1980), by the neighbour joining method (Saitou & Nei, 1987) using MEGA software version 5.0 (Tamura *et al.*, 2011). Confidence levels of the clades were estimated from bootstrap analysis (1000 replicates) (Felsenstein, 1985). *Cryptococcus bromeliarum* BI20^T was used as the outgroup species in the analysis.

Phenotypic characterization

The strains were characterized morphologically, biochemically and physiologically according to the standard methods described by Kurtzman *et al.* (2011b). Hyphae formation was investigated on potato dextrose agar (PDA) and corn meal agar in slide cultures at 25 °C for up to 14 days. Formation of ballistoconidia was determined by the method described by Kurtzman *et al.* (2011b) on inverted PDA plates incubated at 15 °C for up to 4 weeks. Sexual processes were investigated for individual strains or strain pairs on 5 % malt extract agar, corn meal agar, PDA agar and YM agar at 15 °C and 25 °C for up to 6 weeks. Carbon and nitrogen assimilation tests were conducted in liquid medium, and for the nitrogen assimilation test a starved inoculum was used (Kurtzman *et al.*, 2011b). Growth at various temperatures was determined by cultivation in YM broth. Ubiquinones were extracted from cells cultivated in 500 ml Erlenmeyer flasks containing 250 ml of yeast extract peptone glucose (YPD) broth (1 % yeast extract, 2 % peptone and 2 % glucose) on a rotary shaker at 25 °C for 24–48 h and purified according to the methods described by Yamada & Kondo (1973) and Kuraishi *et al.* (1985). Isoprenologues were identified by HPLC as described previously (Limtong *et al.*, 2007).

Species delineation and classification

The two strains, DMKU-SP85^T and DMKU-RE97, showed identical sequences of the D1/D2 region of the LSU rRNA gene. In terms of pairwise sequence similarity, the two strains were closest to *Cryptococcus nemorosus* with 0.6 % nucleotide substitutions (four nucleotide substitutions out of 597 nucleotides). The sequences of the ITS region of strains DMKU-SP85^T and DMKU-RE97 were also identical and differed by 1.8 % nucleotide substitutions (nine nucleotide substitutions out of 499 nucleotides) from the corresponding sequence of *Cryptococcus nemorosus*.

The phylogenetic tree based on sequences of the D1/D2 region of the LSU rRNA gene and ITS region demonstrate that the two strains of the novel species clustered with *Cryptococcus nemorosus* and *Cryptococcus periniciosus* and formed a subclade with *Cryptococcus* sp. CBS 8363 and *Papiliotrema bandonii* (Fig. 1).

On the basis of morphological, biochemical, physiological and chemotaxonomic characteristics and the sequence analysis of the D1/D2 region of the LSU rRNA gene and ITS region, we concluded that the two strains represent a novel species. Due to the position of the novel species in the phylogenetic tree based on the D1/D2 region of the LSU rRNA gene, which is closer to *Papiliotrema bandonii*, the only species of the genus *Papiliotrema*, than to the other species of the two teleomorphic genera of the *Bulleromyces/Papiliotrema/Auriculibullera* clade, *Bulleromyces* and *Auriculibullera*, the novel species was assigned to the same genus as *Papiliotrema bandonii*. According to the International Code of Nomenclature for algae, fungi and plants concerning the nomenclatural rules for fungi and the adoption of 'one fungus, one name' (Miller *et al.*, 2011), the novel species is assigned to the genus *Papiliotrema*, and the designation *forma asexualis* (f.a.) is included following the recommendation of Lachance (2012). The name *Papiliotrema siamense* f.a., sp. nov. is proposed. The other species of the genus *Cryptococcus* in the clade can be assigned regarding the 'one fungus, one name' adoption to the genera *Bulleromyces*, *Papiliotrema* or *Auriculibuller* depending on their phylogenetic positions.

Description of *Papiliotrema siamense* Surussawadee, Khunnamwong & Limtong sp. nov.

Papiliotrema siamense (si.am.en′se. N.L. fem. adj. *siamense* referring to Siam, the old name of Thailand, where the two strains were isolated).

Growth is observed on YM broth after 3 days at 25 °C, cells are globose to oval (2–6.5 × 2–8 μm) and occur singly or in pairs. (Fig. 2) Budding is polar. Colonies are butryous, white to cream, with a smooth surface and an entire margin. After 14 days in Dalmau plate cultures on corn meal agar and PDA agar at 25 °C, pseudohyphae and true hyphae are not formed. Ballistospores are not produced on PDA agar after culture at 15 °C for up to 4 weeks. Sexual reproduction is not observed on 5 % malt extract agar, corn meal agar, PDA agar or YM agar after incubation at 15 °C and 25 °C for up to 6 weeks. Fermentation of D-glucose is negative. D-Glucose, D-galactose, L-sorbose,

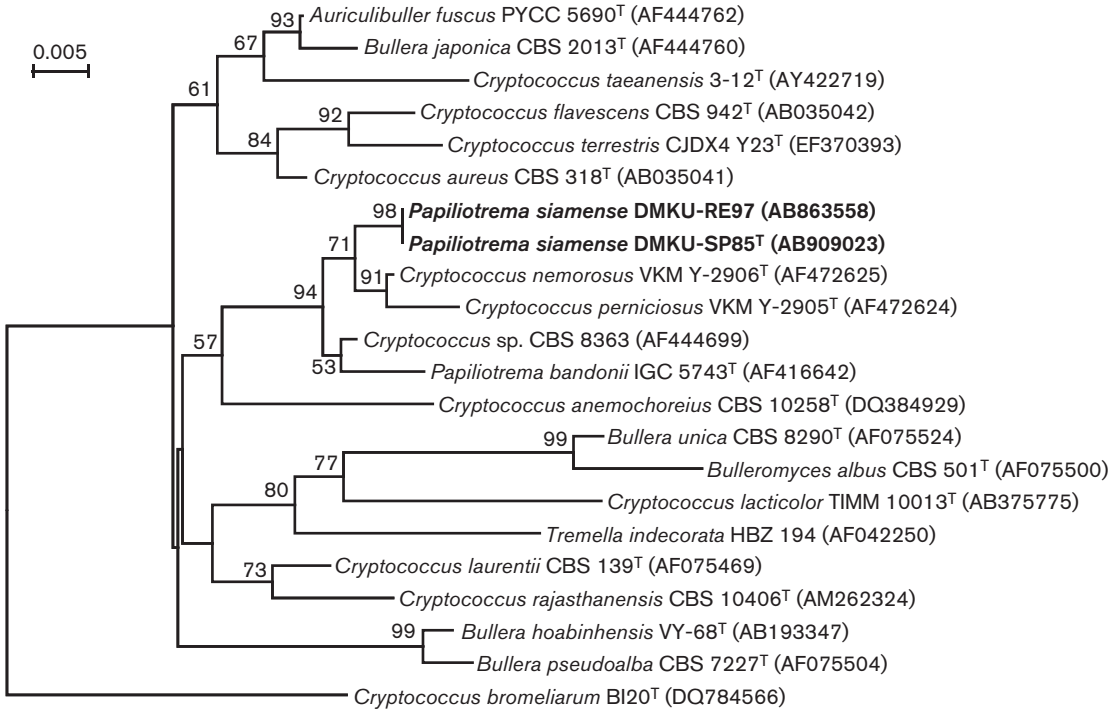


Fig. 1. Phylogenetic tree based on the sequences of the D1/D2 region of the LSU rRNA gene, showing positions of *Papiliotrema siamense* f.a., sp. nov. (DMKU-SP85^T and DMKU-RE97) with respect to closely related species. The phylogenetic tree was reconstructed from evolutionary distance data with Kimura's two-parameter correction (Kimura, 1980), using the neighbour-joining method with MEGA software version 5.0. Numbers at nodes indicate percentages of bootstrap sampling, derived from 1000 replicates; only values >50 % are shown. *Cryptococcus bromeliarum* BI20^T was the outgroup. Bar, 0.005 K_{nuc}.

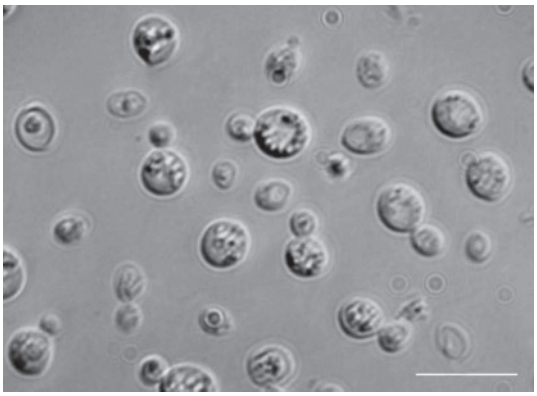


Fig. 2. Budding cells of *Papiliotrema siamense* f.a., sp. nov. DMKU-SP85^T grown in YM broth for 3 days at 25 °C. Bar, 10 μm.

N-acetylglucosamine, D-ribose (weakly), D-xylose, L-arabinose, D-arabinose (slowly), L-rhamnose (latent), sucrose, trehalose, methyl α-D-glucoside (slowly), melibiose, lactose, raffinose, melezitose, soluble starch (weakly), ribitol, D-mannitol, *myo*-inositol, D-glucono-1,5-lactone, 5-ketogluconic acid, D-gluconate, D-galacturonic acid, succinate, citrate, xylitol, ethylamine HCl, L-lysine and cadaverine are assimilated, but nitrate and nitrite are not assimilated. Growth at 25 and 30 °C is positive, but growth is negative at 35 °C. No growth is observed in vitamin-free medium. Growth on medium containing 50 % glucose, 60 % glucose (weakly) and 10 % sodium chloride/5 % glucose are positive but on 15 % sodium chloride/5 % glucose growth is negative. No growth is observed with 0.01 % cycloheximide. Starch-like compounds are produced. Diazonium blue B colour and urease reaction are positive. The major ubiquinone is Q-10.

Strain DMKU-SP85^T is the holotype of *Papiliotrema siamense*. The strain was isolated from the external surface of a sugar cane (*Saccharum officinarum* L.) leaf collected from Chai Nat province, Thailand. The living culture from the type has been deposited at BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology, Thailand (Pathumthani, Thailand) as BCC 69499^T and the Centraalbureau voor Schimmecultures (CBS), Utrecht, the Netherlands as CBS 13330^T. The Mycobank registration number is MB808372.

Concluding remarks

In practice, *Papiliotrema siamense* sp. nov. can be distinguished from *Cryptococcus nemorosus*, its closest relative, on the basis of not only the sequences of the D1/D2 region of the LSU rRNA gene and the ITS region but also some phenotypic characteristics, as shown in Table 1. Members of the *Bulleromyces/Papiliotrema/Auriculibuller* clade comprised ballistoconidia-producing and non-ballistoconidia-producing species. Plant leaves are the main habitat of ballistoconidia-producing species, *Auriculibuller fuscus*, *Bullera albus*, *Bullera hannae*, *Bullera hoabinhensis*, *Bullera japonica*, *Bullera penniseticola*, *Bullera pseudoalba* and *Bullera unica* (Boekhout, 2011). On the other hand, non-ballistoconidia-producing species, *Cryptococcus anemochorus*, *Cryptococcus aureus*, *Cryptococccus flavescens*, *Cryptococcus laurentii*, *Cryptococcus nemorosus*, *Cryptococcus perniciosus*, *Cryptococcus rajasthanensis* and *Cryptococcus taeanensis* have been identified from various sources such as plant parts, atmosphere, rhizophere, soil, fermenting soy beans sauce, caterpillar gut and wild rabbit faeces (Fonseca *et al.*, 2011), and a single strain of *Papiliotrema bandonii* was obtained from a fruiting body that was found to be associated with pyrenomycetous ascomycetes at the base of the inflorescences of the pampas grass (Sampaio, 2011). Among non-ballistoconidia-producing species, *Cryptococcus flavescens*, *Cryptococcus laurentii* and *Cryptococcus rajasthanensis* have been reported from the phylloplane of sugar cane in Thailand (Limtong *et al.*, 2014). In this study, one strain of the novel species was isolated from the phylloplane of sugar cane and the other from rice leaf tissue. Therefore, not only phylloplane but also leaf tissue is a habitat that warrants further investigation for non-ballistoconidia-producing species in this clade. It should be noted that strains of the novel species, *Cryptococcus nemorosus*, *Cryptococcus periniciosus* and *Papiliotrema bandonii*, which formed a subclade with high bootstrap value

Table 1. Phenotypic characteristics that differentiate *Papiliotrema siamense* f.a., sp. nov. from the closest species, *Cryptococcus nemorosus*

Species: 1, *Papiliotrema siamense* sp. nov.; 2, *Cryptococcus nemorosus* (data from Kurtzman *et al.*, 2011a). +, Positive; W, weakly positive; S, slow; L, latent; −, negative.

Characteristic	1	2
Assimilation of carbon compounds		
L-Sorbose	+	W
N-Acetylglucosamine	+	S
D-Ribose	W	S
L-Rhamnose	L	S
Maltose	−	+
Cellobiose	−	+
Salicin	−	+
Inulin	−	W
Soluble starch	W	S
Glycerol	−	S
D-Glucitol	−	+
Galactitol	−	+
2-Ketogluconic acid	−	+
D-Glucuronate	−	+
DL-Lactate	−	W
Ethanol	−	+
Assimilation of nitrogen compounds		
Potassium nitrate	−	+
Sodium nitrite	−	+
Growth in/at:		
60 % Glucose	W	S
30 °C	+	−

(94 %), were obtained from herbaceous plants (Fonseca *et al.*, 2011; Sampaio, 2011). Therefore, herbaceous plants and yeast species in this subclade seems to have ecological association.

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Wickerhamiella siamensis f.a., sp. nov., an endophytic and epiphytic yeast species isolated from sugar cane leaf

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Six strains representing a novel yeast species were isolated from tissue (DMKU-SE106^T, DMKU-SE110, DMKU-SE112 and DMKU-SE132) and the external surface (DMKU-SP335 and DMKU-SP406) of sugar cane leaves collected in Thailand. On the basis of morphological, biochemical, physiological and chemotaxonomic characteristics, and sequence analysis of the D1/D2 region of the LSU rRNA gene and the internal transcribed spacer (ITS) region, the six strains were found to represent a single novel species of the genus *Wickerhamiella* although the formation of ascospores was not observed. The sequences of the D1/D2 region of the LSU rRNA gene and ITS region of the six strains differed from each other by 0–2 and 2–3 nt substitutions, respectively. The novel species was related most closely to *Candida infanticola* but with 4.5–4.6 % nucleotide substitutions in the D1/D2 region of the LSU rRNA gene and 6.6–7.1 % nucleotide substitutions in the ITS region. The name *Wickerhamiella siamensis* f.a., sp. nov. is proposed. The type strain is DMKU-SE106^T (=BCC 61185^T=NBRC 109697^T=CBS 13331^T).

The *Wickerhamiella* clade appears to be monophyletic from the phylogenetic analysis of sequences from a nearly complete LSU rRNA gene, the mitochondrial small subunit (MtSM) rRNA gene and the cytochrome oxidase II (COXII) gene (Kurtzman & Robnett, 2007). At the time of writing, the *Wickerhamiella* clade consisted of 30 species and included six species with a sexual state viz. *Wickerhamiella australiensis*, *Wickerhamiella cacticola*, *Wickerhamiella domercqiae*, *Wickerhamiella lipophila*, *Wickerhamiella occidentalis* and *Wickerhamiella pagnoccae* and 24 anamorphic species. (Kurtzman *et al.*, 2011; Barbosa *et al.*, 2012; Badotti *et al.*, 2013; Hagler *et al.*, 2013; Dayo-Owoyemi *et al.*, 2014). Based on the phylogenetic analysis of the D1/D2 region of the LSU rRNA gene and the combined sequences of the nearly complete LSU rRNA gene, MtSM rRNA gene and COXII gene, the *Wickerhamiella* species with a sexual state are separated into two subclades. *Wickerhamiella domercqiae*, the type species of the genus *Wickerhamiella*, is placed in one subclade while the other five species are in another subclade (Kurtzman & Robnett, 2007; Kurtzman *et al.*, 2011; Hagler *et al.*, 2013).

Endophytic micro-organisms are micro-organisms including bacteria, yeast and filamentous fungi that can colonize healthy plant tissues during at least part of their life cycle without causing any damage to the host plant or developing external structures (Petrini, 1991; Azevedo *et al.*, 2000; Linnakoski *et al.*, 2012). On the other hand, epiphytic micro-organisms are bacteria, filamentous fungi, yeasts and algae that inhabit the external surface of various parts of plants (Andrews & Harris, 2000; Inácio *et al.*, 2002; Lindow & Brandl, 2003). Both ascomycete and basidiomycete yeasts have been found to be endophytes and epiphytes, and one yeast species can be both an endophyte and an epiphyte (Isaeva *et al.*, 2010). Endophytic yeasts which have been found in plant leaves include both basidiomycetous yeast viz. *Cryptococcus albidus*, *Cryptococcus flavescens*, *Cryptococcus laurentii*, *Cryptococcus magnus*, *Rhodotorula graminis*, *Rhodotorula mucilaginosa*, *Rhodotorula pinicola* and *Rhodotorula rubra* (Gai *et al.*, 2009; Abdel-Motaal *et al.*, 2009; Isaeva *et al.*, 2010; Khan *et al.*, 2012; Akhtyamova & Sattarova, 2013), and ascomycetous yeasts viz. *Candida guilliermondii*, *Candida oleophila*, *Candida*

Abbreviations: COXII, cytochrome oxidase II; ITS, internal transcribed spacer; MtSM, mitochondrial small subunit.

The GenBank/EMBL/DDBJ accession numbers for the D1/D2 region sequences of the LSU rRNA gene are AB915236–AB915239 and AB924020–AB924021, and for the ITS region are AB915240–AB915243 and AB924024–AB924025. The MycoBank number for *Wickerhamiella siamensis* is MB 808650.

railenensis, *Cyberlindnera saturnus*, *Metschnikowia pulcherrima* and *Wickerhamomyces anomalus* (Nassar *et al.*, 2005; Isaeva *et al.*, 2010; Rodríguez *et al.*, 2011; Oliveira *et al.*, 2012). Epiphytic yeasts which have been found associated with plant leaves include *Cryptococcus aerius*, *Pseudozyma vetiver*, *Pseudozyma graminicola*, *Rhodotorula glutinis*, *Rhodotorula colostri*, *Rhodosporidium fluviale* and *Sporobolomyces fushanensis*, which belong to Basidiomycota (Golubev *et al.*, 2007; Sláviková *et al.*, 2009; Pozo *et al.*, 2011; Chamnanpa *et al.*, 2013; Limtong *et al.*, 2014) and *Candida tropicalis*, *Metschnikowia saccharicola*, *Ogataea phyllophila* and *Yamadazyma siamensis*, which belong to Ascomycota (de Azeredo *et al.*, 1998; Limtong *et al.*, 2012; Kaewwichian *et al.*, 2012, 2013). Reports of endophytic yeasts, especially of those associated with plant leaves, are fewer compared with reports of epiphytic yeasts.

During the investigation of endophytic and epiphytic yeasts of sugar cane leaves in Thailand, four endophytic yeast strains (DMKU-SE106^T, DMKU-SE110, DMKU-SE112 and DMKU-SE132) and two epiphytic yeast strains (DMKU-SP335 and DMKU-SP406) representing a novel *Wickerhamiella* species were obtained. In this article, these six strains are described as representatives of *Wickerhamiella siamensis* f.a., sp. nov.

Endophytic yeasts were isolated from surface sterilized sugar cane (*Saccharum officinarum*) leaves by the method described by Abdel-Motaal *et al.* (2009) with slight modification. Three grams of leaf were cut into fragments (3 × 3 cm) and surface sterilization was conducted by immersion in 70 % ethanol for 3 min, then in 5 % sodium hypochlorite solution for 3 min and then rinsing five times for 5 min with sterile deionized water. The effectiveness of the surface sterilization procedure was verified by spreading the final rinse water onto yeast extract-malt extract (YM) agar (0.3 %, w/v, yeast extract; 0.3 %, w/v, malt extract; 0.5 %, w/v, peptone; 2.0 %, w/v, agar) and placing a few leaf fragments directly onto YM agar; no growth of micro-organisms indicated effective sterilization. After surface sterilization, leaves were cut into smaller fragments (0.5 × 0.5 cm), slightly ground in a sterile mortar to expose the inner tissue and then five fragments were placed onto YM agar supplemented with 0.02 % chloramphenicol in a Petri dish. A total of 15 leaf fragments per sample were used for yeast isolation. The Petri dishes were incubated at 25 °C until yeast colonies appeared. Yeast colonies of different morphologies were selected and purified by cross streaking on YM agar. Purified yeast strains were suspended in YM broth supplemented with 10 % (v/v) glycerol and maintained at −80 °C. Yeast cells were enumerated by mixing 1 g of slightly ground leaf fragments with 9 ml of 0.85 % normal saline solution in a test tube and an aliquot (100 µl) was spread on YM agar supplemented with 0.02 % chloramphenicol in a Petri dish and incubated at 25 °C for 3–7 days. Yeast colonies were counted and reported as c.f.u. per g of fresh leaf weight. Epiphytic yeasts were isolated by plating the leaf washings as described by Inácio *et al.* (2002). Three grams of leaf were suspended in 50 ml of 0.85 % saline solution in a 250 ml Erlenmeyer flask and shaken on a rotary shaker at 150 r.p.m. and 25 °C for 1 h to detach yeast cells from the surface. An aliquot of 100 µl of the washing solution was then spread on YM agar supplemented with 0.025 % sodium propionate and 0.02 % chloramphenicol and incubated at 25 °C until yeast colonies appeared. Yeast colonies were counted and colonies of different morphologies were purified and maintained as described above.

In total, 127 endophytic yeast strains and 267 epiphytic yeast strains were isolated from tissue and external surfaces, respectively, of 102 samples of sugar cane leaf collected in the central and north-eastern parts of Thailand between October 2011 and March 2012.

Endophytic yeast enumeration revealed low yeast numbers in sugar cane leaf tissue, 6.0×10^2–9.3×10^3 c.f.u. (g fresh leaf)$^{-1}$was found. The number of endophytic yeast in plant leaf was not different from the other parts of plant. One study reported the endophytic yeast number of 2.7×10^3 c.f.u. (g fresh maize root)$^{-1}$ (Nassar *et al.*, 2005), a second study reported that the number of yeast cells in storage tissues was 10^2–10^4 (Isaeva *et al.*, 2010); however, in some cases they reached up to 10^6–10^7 c.f.u. (g storage tissue)$^{-1}$. On the other hand, the number of epiphytic yeast of sugar cane leaf found in this study was in the range of 4.5×10^2–3.7×10^6 c.f.u. (g leaf)$^{-1}$.

The sequences of the D1/D2 region of the LSU rRNA gene and the ITS (ITS1-5.8S rRNA-ITS2) region were determined from PCR products amplified from genomic DNA, using the primers NL1 and NL4 (Kurtzman & Robnett, 1998), and ITS1 and ITS4 (White *et al.*, 1990), respectively. DNA extraction and amplification of the D1/D2 region of the LSU rRNA gene and the ITS region were performed as described previously (Limtong *et al.*, 2007).

The PCR product was checked by agarose gel electrophoresis and purified using the HiYield Gel/PCR DNA Fragments Extraction kit (RBC Bioscience), according to the manufacturer's protocol. The purified products were sequenced (Macrogen, Korea) with primers NL1 and NL4 for the D1/D2 region of the LSU rRNA gene, and with ITS1 and ITS4 for the ITS region. The sequences were compared pairwise using the BLAST search program (Altschul *et al.*, 1997) and were aligned with the sequences of related species retrieved from GenBank using the multiple alignment program CLUSTAL_X version 1.81 (Thompson *et al.*, 1997). The phylogenetic tree was reconstructed from the evolutionary distance data with Kimura's two-parameter correction (Kimura, 1980), using the neighbour-joining method (Saitou & Nei, 1987) and the MEGA software version 5.03 (Tamura *et al.*, 2011). Confidence levels of the clades were estimated from bootstrap analysis (1000 replicates) (Felsenstein, 1985).

The strains of the novel yeast species were characterized morphologically, biochemically and physiologically according to the standard method described by Kurtzman *et al.* (2011). Mycelium formation was investigated by cultivation on potato dextrose agar (PDA; 20 %, w/v, potato

infusion; 2 %, w/v, glucose; 1.5 %, w/v, agar) and corn meal agar (2 %, w/v, corn meal infusion; 1.5 %, w/v, agar) in slide culture at 25 °C for up to 14 days. Ascospores formation was investigated for individual strains or strain pairs on PDA, corn meal agar, 5 % malt extract agar (5 %, w/v, malt extract; 1.5 %, w/v, agar), YPD agar (1 %, w/v, yeast extract; 2 %, w/v, peptone; 2 %, w/v, glucose; 1.5 %, w/v, agar), Fowell's acetate agar (0.5 %, w/v, sodium acetate; 2 %, w/v, agar) and Gorodkowa agar (0.1 %, w/v, glucose; 0.5 %, w/v, sodium chloride; 1 %, w/v, peptone; 2 %, w/v, agar) at 15 °C and 25 °C for up to 4 weeks. Carbon source and nitrogen source assimilation tests were performed in liquid medium and starved inocula was used in nitrogen assimilation tests (Kurtzman *et al.*, 2011). Growth at various temperatures was determined by cultivation in YM broth. Ubiquinones were extracted from cells cultivated in 500 ml Erlenmeyer flasks containing 250 ml of yeast extract peptone glucose (YPD) broth (1 %, w/v, yeast extract; 2 %, w/v, peptone; 2 %, w/v, glucose) on a rotary shaker at 28 °C for 48 h and purified according to the methods described by Yamada & Kondo (1973) and Kuraishi *et al.* (1985). Isoprennologues were identified by HPLC as described previously (Limtong *et al.*, 2007).

Analysis of the D1/D2 region of the LSU rRNA gene sequence revealed that the six strains represented a single novel species. Four strains (DMKU-SE106^T, DMKU-SE110, DMKU-SE112 and DMKU-SE132) were isolated from tissue and two strains (DMKU-SP406 and DMKU-SP335) were isolated from the external surface of sugar cane leaves (Table 1). The sequence of the D1/D2 region of the LSU rRNA gene of the endophytic yeast strain DMKU-SE106^T differed by only one nucleotide substitution from the sequences of the other three endophytic strains (DMKU-SE110, DMKU-SE112 and DMKU-SE132) and by two nucleotide substitutions from the sequences of the two epiphytic strains (DMKU-SP406 and DMKU-SP335). The sequences of the D1/D2 region of the LSU rRNA gene of these six strains differed by 4.5–4.6 % nucleotide substitutions (25–26 nt substitutions and five gaps out of 562 nt) from *Candida infanticola* NRRL Y-17858^T (DQ438230), the closest species in terms of pairwise sequence similarity. The nucleotide sequence of the ITS region of strain DMKU-SE106^T differed by only two nucleotide substitutions from that of two strains (DMKU-SE110 and DMKU-SE132) and by three nucleotide substitutions from the sequences of the other three strains (DMKU-SE112, DMKU-SP335 and DMKU-SP406). The ITS sequences of the six strains differed by 6.6–7.1 % nucleotide substitutions (27–29 nt substitutions and 64–69 gaps out of 409 nt) from the sequences of *Candida infanticola* NRRL Y-17858^T (DQ911458).

A phylogenetic analysis based on sequences of the D1/D2 region of the LSU rRNA gene placed the six strains (DMKU-SE106^T, DMKU-SE110, DMKU-SE112, DMKU-SE132, DMKU-SP335 and DMKU-SP406) as a sister clade to *Candida infanticola* NRRL Y-17858^T (DQ911458) and *Candida sorbophila* NRRLY-17921^T (U45852) with strong bootstrap support (Fig. 1). Although the sequence is polymorphic for the six strains, strong bootstrap values also support their reciprocal monophyly with respect to the sister species. A phylogenetic species concept therefore applies.

The primary criterion of proposing the six strains as representatives of a single novel species was the sequence analysis of the D1/D2 region of the LSU rRNA gene and ITS region. Although formation of ascospores was not observed, according to the nomenclatural rules for fungi described within the International Code of Nomenclature for Algae, Fungi and Plants, the most important requirement is the adoption of 'one fungus, one name' (Miller *et al.*, 2011). Consequently, the novel species was assigned to the genus *Wickerhamiella*, and the designation *forma asexualis* (f.a.) was included following the recommendation as described by Lachance (2012). The name *Wickerhamiella siamensis* f.a., sp. nov. (MB 808650) is proposed.

Table 1. Strains isolated from tissue and the external surface of sugar cane leaves collected on 19 April 2012 used in this study

Strain	Endophyte/epiphyte	Location of sample collection
DMKU-SE106^T=BCC 61185^T=NBRC 109697^T=CBS 13331^T	Endophyte	Chaloem Phra Kiat district, Saraburi province, Thailand (14° 36′ 30 N 100° 58′ 6 E)
DMKU-SE110	Endophyte	Chaloem Phra Kiat district, Saraburi province, Thailand (14° 36′ 30 N 100° 58′ 6 E)
DMKU-SE112	Endophyte	Chaloem Phra Kiat district, Saraburi province, Thailand (14° 36′ 30 N 100° 58′ 6 E)
DMKU-SP335	Epiphyte	Kaeng Khoi district, Saraburi province, Thailand (14° 36′ 30 N 100° 58′ 6 E)
DMKU-SE132	Endophyte	Nong Muang district, Lopburi province, Thailand (15° 7′ 9 N 100° 53′ 10 E)
DMKU-SP406	Epiphyte	Nong Muang district, Lopburi province, Thailand (15° 7′ 19 N 100° 53′ 10 E)

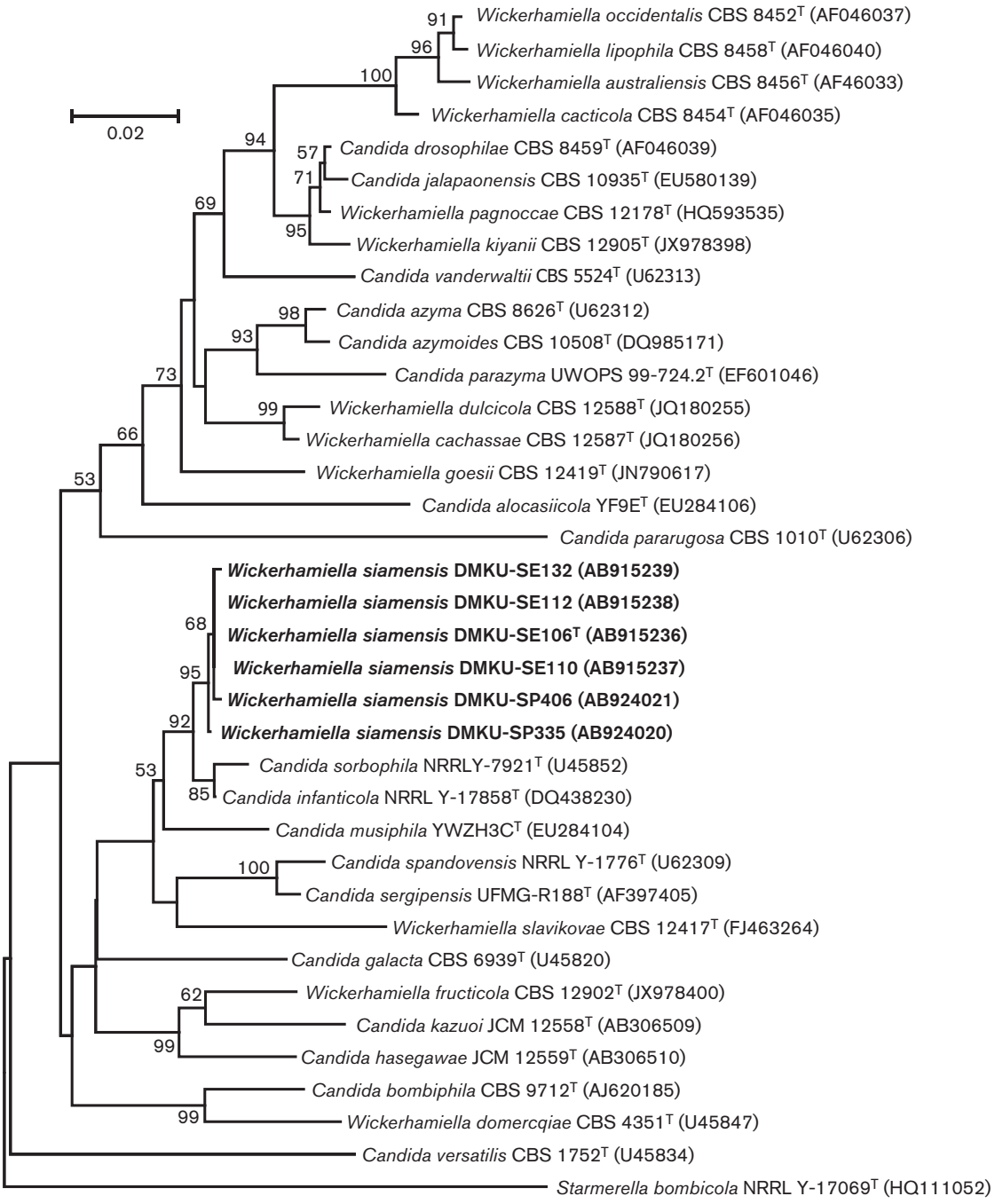


Fig. 1. Phylogenetic tree based on the sequence of the D1/D2 region of the LSU rRNA gene, showing positions of strains DMKU-SE106^T, DMKUSE110, DMKU-SE112, DMKU-SE132, DMKU-SP335 and DMKU-SP406 with respect to closely related species. The phylogenetic tree was reconstructed using the neighbour-joining method by MEGA software version 5.03, and the nucleotide substitution rate (K_{nuc} value) was computed from evolutionary distance data by Kimura's two-parameter model. Bar, evolutionary distance of 0.02 K_{nuc}. Numbers at nodes indicate percentages of bootstrap sampling, derived from 1000 samples. Numbers in parentheses are GenBank accession numbers. *Starmerella bombicola* NRRL Y-17069^{T}was the outgroup in the analysis.

Description of *Wickerhamiella siamensis* Khunnamwong, Surussawadee, Jindamorakot & Limtong sp. nov.

Wickerhamiella siamensis (si.am.en′sis. N.L. fem. adj. *siamensis* of or belonging to Siam, the old name of Thailand, where the type strain was isolated).

Growth occurs in YM broth. After 3 days at 25 °C, cells are subglobose to ovoid (1.6–3 × 2–4 μm) and occur singly or in pairs (Fig. 2a). Budding is multilateral. After 3 days of growth on YM agar at 25 °C, the streak culture is white to cream in colour, soft with a smooth surface and has an entire margin. Rudimentary pseudohyphae are formed in slide culture on PDA after 14 days at 25 °C (Fig. 2b), but true hyphae are not formed in slide culture on PDA or corn meal agar after 28 days at 25 °C. Ascospores are not produced for individual strains or strain pairs on PDA, corn meal agar, 5 % malt extract agar, YPD agar, Fowell's acetate agar or Gorodkowa agar at 15 °C or 25 °C after 4 weeks. Fermentation is absent. D-Glucose, D-galactose, L-sorbose, D-xylose (weak), soluble starch, glycerol, D-glucitol, D-mannitol, D-glucono-1,5-lactone, 5-ketogluconic acid, succinate, ethanol (weak), xylitol (latent) are assimilated, but *N*-acetylglucosamine, D-ribose, L-arabinose, D-arabinose, L-rhamnose, sucrose, maltose, α-α-trehalose, methyl α-D-glucoside, cellobiose, salicin, melibiose, lactose, raffinose, melezitose, inulin, ribitol, erytritol, galactitol, *myo*-inositol, 2-ketogluconic acid, D-gluconate, D-glucuronate, D-galacturonic acid, DL-lactate, citrate and methanol are not assimilated. Ammonium sulfate, ethylamine hydrochloride, L-lysine and cadaverine are assimilated, but potassium nitrate is not assimilated. No growth occurs in vitamin-free medium or with 16 % (w/v) sodium chloride/5 % (w/v) glucose, 0.01 % cycloheximide or 0.1 % cycloheximide. Growth on medium containing 50 % (w/v) glucose, 60 % (w/v) glucose and 10 % (w/v) sodium chloride/5 % (w/v) glucose is present. Growth is present at 15, 25, 30, 37 °C, but absent at 40, 42 and 45 °C. Acid formation is absent. Starch-like compounds are not produced. Diazonium blue B colour and urease reaction are negative. The major ubiquinone is Q-9.

The type strain, DMKU-SE106^T (=BCC 61185^T=NBRC 109697^T=CBS 13331^T), was isolated from the tissue of a sugar cane (*Saccharum officinarum*) leaf collected from Chaloem Phra Kiat district, Saraburi province, Thailand. The MycoBank accession number is MB 808650.

In practice, *Wickerhamiella siamensis* can be distinguished from the closest related species, *Candida infanticola*, not only by the analysis of the sequence of the D1/D2 region of the LSU rRNA gene and the ITS region but also by some phenotypic characteristics. Rudimentary pseudohyphae are formed by *Wickerhamiella siamensis* but are not formed by *Candida infanticola*. *Wickerhamiella siamensis* assimilates D-xylose (weakly), soluble starch, 5-ketogluconic acid, ethanol (weakly) and xylitol (latent) while *Candida infanticola* does not. Growth with 0.01 % cycloheximide is absent for *Wickerhamiella siamensis* but is present for *Candida infanticola*.

The members of the *Wickerhamiella* clade have been obtained from various parts of plants such as flowers (*Candida alocasiicola*, *Candida musiphila*, *Wickerhamiella cacticola*, *Wickerhamiella kiyanii* and *Wickerhamiella pagnoccae*) and fruits (*Candida sergipensis* and *Wickerhamiella fructicola*) (Lachance *et al.*, 1998; Trindade *et al.*, 2004; Wang *et al.*, 2008; Barbosa *et al.*, 2012; Dayo-Owoyemi *et al.*, 2014). Interestingly, among the plant-associated species, *Wickerhamiella slavikovae* and *Wickerhamiella goesii* were isolated from the surface of sugar cane leaves (Hagler *et al.*, 2013), the same source as the novel species in this study. Some of the members have been found associated with insects, e.g. *Wickerhamiella occidentalis* was reported to have been isolated from morning glories and their associated insects in Hawaii, and *Candida kazuoi* and *Candida hasegawae* were isolated from insect frass in Thailand (Nakase *et al.*, 2007; Kurtzman *et al.*, 2011). Therefore, plants and associated insects may be important habitats for yeasts in this clade. However, no member of the *Wickerhamiella* clade isolated from plant leaf tissue has ever been reported. In this study, among the 127 endophytic

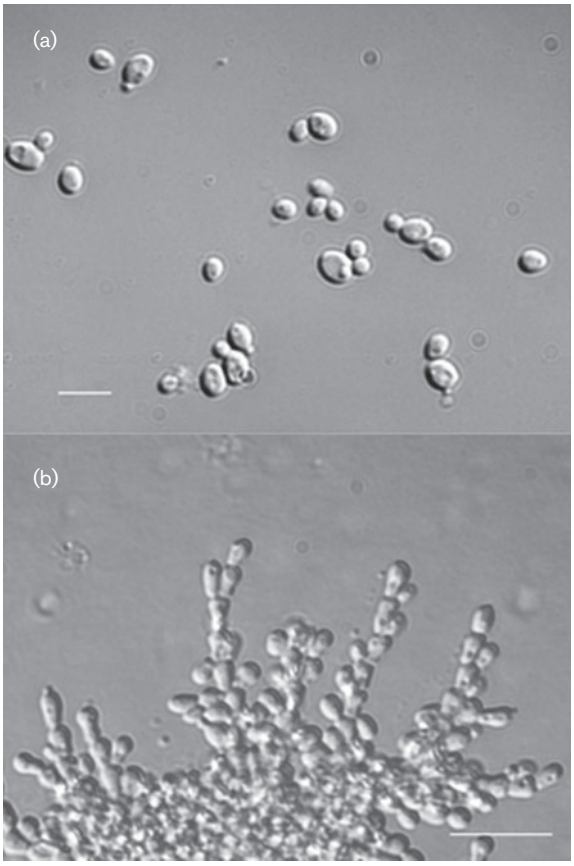


Fig. 2. Strain DMKU-SE106^T (a) budding cells in YM broth after 3 days at 25 °C (bar, 5 μm) and (b) rudimentary pseudohyphae formed on PDA after 14 days at 25 °C (bar, 10 μm).

yeast strains obtained from the tissue of 102 samples of sugar cane leaves, four strains (DMKU-SE106^T, DMKU-SE110, DMKU-SE112 and DMKU-SE132) represented a novel species, *Wickerhamiella siamensis*. The most frequently isolated yeast species from sugar cane leaf tissue was *Meyerozyma caribbica* (18 strains, equivalent to 14.2 % frequency of isolation). The novel species was found among 42 endophytic yeast species including 29 known species, eight undescribed species and five novel species, which was calculated to be 2.4 % of total species. At the same time, two yeast strains (DMKU-SP335 and DMKU-SP406) were isolated from the external surface of sugar cane leaves. Therefore, it is clear that the novel species was present not only in tissue but also on the external surface of plant leaves. In fact, in one sample, strain DMKU-SE132 was detected in the tissue and strain DMKU-SP406 was found on the external surface of sugar cane leaves.

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ORIGINAL ARTICLE

The diversity of culturable yeasts in the phylloplane of rice in Thailand

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Abstract One hundred and fifty-six yeast strains were obtained by the enrichment technique from the phylloplanes of 85 rice leaf samples collected from seven provinces in Thailand. On the basis of the D1/D2 domain of the large subunit rRNA gene sequence analysis, 156 strains were identified as 34 known species in 18 genera consisting of 25 species in 13 genera of the phylum Ascomycota and nine species in five genera of the phylum Basidiomycota. The species in the phylum Ascomycota comprised 24 species in 12 genera of the order Saccharomycetales and one species viz. *Yarrowia lipolytica* in Saccharomycetales *incertae sedis*. The 24 species viz. *Candida glabrata* in the *Nakaseomyces* clade of Saccharomycetaceae, *Candida jaroonii*, *Candida membranifaciens* and *Candida terebra* in the *Yamadazyma* clade of Debaryomycetaceae, *Candida pseudolambica* in the *Pichia* clade of Pichiaceae, *Candida ruelliae* in the *Metschnikowia* clade of Metschnikowiaceae, and three unaffiliated clade *Candida* species (*Candida catenulata*, *Candida rugosa* and *Candida tropicalis*); *Clavispora lusitaniae*, *Kodamaea ohmeri*, *Metschnikowia koreensis* and *Metschnikowia lopburiensis* in Metschnikowiaceae; *Cyberlindnera fabianii*, *Cyberlindnera rhodanensis* and *Wickerhamomyces ciferrii* in Wickerhamomycetaceae; *Debaryomyces nepalensis*, *Meyerozyma caribbica*, *Meyerozyma guilliermondii*, *Millerozyma koratensis*, and *Yamadazyma mexicanum* in Debaryomycetaceae; *Pichia kudriavzevii* in Pichiaceae; and *Lachancea thermotolerans* in Saccharomycetaceae. The species in Basidiomycota viz. *Cryptococcus flavescens*, *Cryptococcus laurentii*, *Cryptococcus* aff. *laurentii* and *Cryptococcus rajasthanensis* in the Tremellales lineage, Bulleromyces clade, Tremellales, Tremellomycetes, Agaricomycotina; *Pseudozyma antarctica* and *Pseudozyma aphidis* in Ustilaginales, Ustilaginomycetes, Ustilaginomycotina; *Rhodotorula taiwanensis* and *Sporobolomyces blumeae* in Sporidiobolales, Microbotryomycetes, Pucciniomycotina; and *Trichosporon asahii* in Trichosporonales, Tremellomycetes, Agaricomycotina. The most prevalent species was *R. taiwanensis* with a 23 % frequency of occurrence followed by *Candida tropicalis* (16 %) and *Cryptococcus fabianii* (12 %).

Keywords Phylloplane · Rice · Yeast · Thailand · D1/D2 domain

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Introduction

Rice is one of the world's most important food crops. About 90 % of the world's rice is cultivated in Asia. In Thailand, a tropical Asian country, the Office of Agricultural Economics, Ministry of Agriculture and Cooperatives reported the rice cultivation area to be approximately 13,192,400 hectares and the production of rice for the year 2012 to be 38,830,600 tons. Rice is a type of grass belong to the family Poaceae, a group of monocotyledonous plants. The rice species that is cultivated in Thailand is Asian rice, *Oryza sativa*.

The phylloplane, or phyllosphere, is a term for the above ground surface of plants and usually refers to the external surface of plant leaves, a dominant aerial part of plant (Phaff and Starmer 1987; Fonseca and Inacio 2006). The phylloplane is known to be colonized by a large number of microorganisms including bacteria, yeasts and fungi (Andrews and Harris 2000; Lindow and Brandl 2003). The growth of microorganisms in the phylloplane is dependent on nutrients from plant metabolites that are secreted to the phylloplane or materials from external sources that drop on the plant surface. The plant metabolites are organic substances, mostly simple sugars, e.g., glucose, fructose and sucrose, while amino acids, organic acids and sugar alcohols are also present (Fiala et al. 1990; Xin et al. 2009). On the other hand, nutrients in the materials from external sources are inorganic nutrients (Xin et al. 2009). In addition to having relatively limited nitrogen sources, the microbial inhabitants of the phylloplane encounter strong ultraviolet radiation, low or fluctuating water activity, fluctuating temperature, and antimicrobial compounds that are produced by plants or other microorganisms; as a consequence, the microorganisms on the phylloplane are often irregularly distributed (Inácio et al. 2005; Fonseca and Inacio 2006; Remus-Emsermann et al. 2012; Vorholt 2012).

Bacteria are the most abundant phylloplane microorganisms; however, yeasts and yeast-like fungi are also active phylloplane colonizers (Andrews and Harris 2000; Lindow and Brandl 2003). The phylloplanes of both dicotyledonous and monocotyledonous plants are colonized by both basidiomycete and ascomycete yeasts (Nakase and Suzuki 1985; de Azeredo et al. 1998; Nakase et al. 2001; Inácio et al. 2005; Fonseca and Inacio 2006; Glushakova et al. 2007; Slavikova et al. 2007, 2009; Glushakova and Chernov 2010; Landell et al. 2010). Fewer studies on yeasts that colonize the phylloplanes of monocotyledonous plants have been reported. The ballistoconidium-fall method was used to isolate ballistoconidium-forming yeasts from dead leaves and stems of rice in Japan and from rice and various plants in Thailand (Nakase and Suzuki 1985; Nakase et al. 2001). The prevalent yeast species associated with leaves, stems and rhizospheres of sugarcane in Brazil were found to be *Cryptococcus laurentii, Cryptococcus albidus*, *Rhodotorula mucilaginosa* and *Debaryomyces hansenii* (de Azeredo et al. 1998). In a recent investigation, *Pseudozyma graminicola* was proposed from two strains isolated from the leaves of pasture plants in Russia (Golubev et al. 2007). *Candida aechemae* and *Candida vrieseae* were proposed as two novel yeast species isolated from phyllophanes of bromolids in southern Brazil (Landell et al. 2010). *Bensingtonia rectispora*, a ballistoconidium-forming yeast, was a new species proposed from strains isolated from bamboo (Wang et al. 2012). *Metschnikowia lopburiensis*, was proposed as a new species from one strain isolated from the surface of green rice leaves in Thailand (Kaewwichian et al. 2012). Four novel yeast species viz. *Metschnikowia saccharicola*, *Yamadazyma siamensis*, *Wickerhamomyces siamensis* and *Nakazawaea siamensis* were proposed from strains isolated from the phylloplane of sugarcane in Thailand (Kaewwichian et al. 2012, 2013a, 2013b; Kaewwichian and Limtong 2014).

Although yeasts inhabiting the phylloplane have been studied intensively, the diversity of yeasts in the phylloplane of rice has received little attention, and only a few articles have been published so far. Among them, one article reported the use of the ballistoconidium-fall isolation method to study yeasts in rice phylloplane in Thailand (Nakase et al. 2001). Therefore, this study aimed to investigate the diversity of yeasts in the phylloplane of rice in Thailand by using the enrichment isolation technique.

Materials and methods

Sample collection

Green and healthy leaves of rice (*Oryza sativa*) were random collected from rice fields. Leaf samples were put in plastic bags, sealed and brought in an ice-box to the laboratory. The samples were kept at 8 °C until subjected to yeast isolation.

Yeast isolation

Yeasts were isolated by an enrichment technique using yeast extract malt extract (YM) broth (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone and 10 g/L glucose) supplemented with 250 mg/L sodium propionate and 200 mg/L chloramphenicol (Limtong et al. 2007). Three grams of cut leaves, derived from cutting a few leaves to the size that could be put into a 250 ml Erlenmeyer, were inoculated into 50 ml enrichment broth in the flask and incubated on a rotary shaker at room temperature (30±2 °C) for 2 days. A loopful of the enriched culture was directly streaked on YM agar supplemented with 250 mg/L sodium propionate and 200 mg/L chloramphenicol and incubated at room temperature (30±2 °C) for 2–7 days or until yeast colonies appeared. Yeast colonies of different morphologies were picked and purified by cross streaking on YM agar. Purified yeast strains were suspended in YM broth supplemented with 10 % v/v glycerol and maintained at −80 °C.

Yeasts identification

Yeasts were identified by analysis of the D1/D2 domain of the large subunit (LSU rRNA) gene sequence similarities (Kurtzman and Robnett 1998). The sequence of the D1/D2 region of the LSU rRNA gene was determined from PCR products amplified from genomic DNA. The D1/D2 region of the LSU rRNA gene was amplified and sequenced with primers, NL1 and NL4 (Kurtzman and Robnett 1998). Methods for DNA extraction and amplification of the D1/D2 region of the LSU rRNA gene and ITS region were as described previously (Limtong et al.

2007). The PCR products were checked by agarose gel electrophoresis and purified by using the Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taiwan). The purified products were submitted to Macrogen Inc. (Korea) for sequencing. Sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems, USA), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using the primers NL1 and NL4 for the D1/D2 region of the LSU rRNA gene. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730XL sequencer (Applied Biosystems, USA). The sequences were compared pairwise using a BLAST search (Altschul et al. 1997).

Determination of growth at high temperature

Active yeast culture was prepared by cultivation on YM agar at room temperature (30±2 °C) for 24 h and cells was suspended in sterilized 0.85 % normal saline solution. Cell suspension (200 μl) was inoculated in to YM broth and incubated at 35, 37 and 40 °C. Growth was observed after seven days of incubation.

Results and discussion

Sample collection and yeast isolation

Eighty-five samples of rice leaf were collected from 14 sampling sites in seven provinces in Thailand between February 2010 and May 2010 (Table 1). These seven provinces were in the central and north-eastern parts of Thailand, where the average maximum temperature was 32-36 °C and average minimum temperature was 22-25 °C throughout the year. Yeasts were isolated by the enrichment technique at a temperature which varied in the range of 28 to 32 °C, and 156 strains were obtained (Table 1).

Yeast Identification and diversity of yeasts in rice phylloplanes

On the basis of the D1/D2 domain of the large subunit rRNA gene sequence analysis, 156 strains were identified to be 34 known yeast species (Tables 2 and Supplementary Table 1). Among the 34 known species, 25 species were in 13 genera of the phylum Ascomycota and nine species belonged to five genera of the phylum Basidiomycota.

The genera belonging to Ascomycota consisted of *Clavispora* (one species), *Cyberlindnera* (two species), *Debaryomyces* (one species), *Lachancea* (one species), *Kodamaea* (one species), *Metschnikowia* (two species), *Meyerozyma* (two species), *Millerozyma* (one species), *Pichia* (one species), *Wickerhamomyces* (one species), *Yamadazyma* (one species), *Yarrowia* (one species), and *Candida* (nine species). The seven *Candida* species were in five clades, and two were in unaffiliated clades including *Lodderomyces-Spathospora* (one species), *Metschnikowia* (two species), *Nakaseomyces* (one species), *Pichia* (one species), *Yamadazyma* (three species) and unaffiliated (two species) clades. The basidiomycete yeast genera were *Cryptococcus* (four species in the *Bulleromyces* clade), *Pseudozyma* (two species), *Rhodotorula* (one species),

Table 1 Rice leaves collection, sampling site and number of the isolated yeast strains

Site	District, Province	Location	Date of sampling	Sample No.	No. of yeast strain
A-1	Bang Pa In, Ayutthaya	14°13′39″N 100°34′32″E	3/2/2010	1-7	14
A-2	Bang Ban, Ayutthaya	14°22′25″N 100°29′8″E	3/2/2010	8-9, 40-41	10
A-3	Sena, Ayutthaya	14°19′38″N 100°24′16″E	3/2/2010	10-15	14
A-4	Phak Hai, Ayutthaya	14°27′30″N 100°22′12″E	3/2/2010	16-24	19
B	Samko, Ang Thong	14°36′20″N 100°14′41″E	3/2/2010	34-39	12
C	Samkhok, Pathum Thani	14°3′55″N 100°31′21″E	3/2/2010	42-51	18
D-1	Si Prachan, Suphan Buri	14°37′11″N 100°8′40″E	3/2/2010	25-33	17
D-2	Doem Bang Nang Buat, Suphan Buri	14°51′13″N 100°5′52″E	22/3/2010	52-58	10
E-1	Krok Phra, Nakhon Sawan	15°33′24″N 100°4′24″E	23/3/2010	59-66	11
E-2	Chum Saeng, Nakhon Sawan	15°52′52″N 100°18′9″E	23/3/2010	67-69	5
F	Ban Mi, Lop Buri	15°2′41″N 100°32′13″E	5/5/2010	70-76	11
G-1	Mueang, Nakhon Ratchasima	14°58′16″N 102°5′59″E	6/5/2010	77-79	4
G-1	Chok Chai, Nakhon Ratchasima	14°43′56″N 102°9′47″E	6/5/2010	80-82	6
G-1	Khon Buri, Nakhon Ratchasima	14°31′24″N 102°14′54″E	6/5/2010	83-85	5

Table 2 Identification of yeast strains isolated from phylloplane of rice and their GenBank accession numbers of the D2 region of LSU rRNA gene

Species	Taxa		No. of total strain	Frequency of occurrence (%)[1]	GenBank accession no. of the closest species	Nucleotide substitutions to closest species	Strain example		Sampling site where this species was present
							Strain	D1/D2 GenBank accession no.	
Phylum Ascomycota									
Candida catenulata	Saccharomycetales	Unaffiliated clade	2	1.3	U45714	0-1	DMKU-RK53	AB772448	A-3, A-4
Candida glabrata		*Nakaseomyces* clade, Saccharomycetaceae	1	0.6	U45727	1	DMKU-RK165	AB772536	D-2
Candida jaroonii		*Yamadazyma* clade, Debaryomycetaceae	3	1.9	DQ404493	0	DMKU-RK94	AB772479	D-1, C
Candida membranifaciens		*Yamadazyma* clade, Debaryomycetaceae,	1	0.6	U45792	0	DMKU-RK57	AB772451	A-3
Candida pseudolambica		*Pichia* clade, Pichiaceae	1	0.6	U71063	1	DMKU-RK48	AB772444	A-2
Candida ruelliae		*Metschnikowia* clade, Metschnikowiaceae	1	0.6	AM262326	2	DMKU-RK434	AB772568	G-1
Candida rugosa		Unaffiliated clade	2	1.3	U45727	1-3	DMKU-RK164	AB772535	D-2
Candida terebra		*Yamadazyma* clade,	1	0.6	U45784	0	DMKU-RK436	AB772570	
		Debaryomycetaceae							G1
Candida tropicalis		*Lodderomyces-Spathospora* clade,	25	16.1	U45749	0-1	DMKU-RK34	AB772431	A-1, A-2,
							DMKU-RK60	AB772453	A-3, D-1,
		Debaryomycetaceae					DMKU-RK104	AB772486	D-2, C, E-1,
							DMKU-RK121	AB772497	E-2, F, G-2
							DMKU-RK226	AB772547	
							DMKU-RK284	AB772567	
							DMKU-RK462	AB772575	
							DMKU-RK485	AB772583	
Clavispora lusitaniae		Metschnikowiaceae	1	0.6	AJ539561	1	DMKU-RK484	AB772582	G-2
Cyberlindnera fabianii		Wickerhamomycetaceae	19	12.3	U73573	0-2	DMKU-RK32	AB772429	A-1, A-2,
							DMKU-RK59	AB772452	A-3, A-4, D-1
							DMKU-RK95	AB772480	
							DMKU-RK108	AB772490	
							DMKU-RK122	AB772498	
							DMKU-RK134	AB772510	
Cyberlindnera rhodanensis		Wickerhamomycetaceae	5	3.2	U73571	1	DMKU-RK144	AB772518	C, G-2
							DMKU-RK481	AB772579	
Debaryomyces nepalensis		Debaryomycetaceae	7	4.5	U45839	0-3	DMKU-RK71	AB772461	A-4, C, F
							DMKU-RK153	AB772526	C
							DMKU-RK280	AB772563	
Kodamaea ohmeri		Saccharomycetaceae	2	1.3	U45702	0	DMKU-RK67	AB772458	A-4
Lachancea thermotolerans		Metschnikowiaceae	1	0.6	U69581	1	DMKU-RK275	AB772561	F
Metschnikowia koreensis		Metschnikowiaceae	3	1.9	AF296438	0	DMKU-RK61	AB772454	A-3, A-4

Table 2 (continued)

Species	Taxa		No. of total strain	Frequency of occurrence (%)[1]	GenBank accession no. of the closest species	Nucleotide substitutions to closest species	Strain example		Sampling site where this species was present
							Strain	D1/D2 GenBank accession no.	
Metschnikowia lopburiensis		Metschnikowiaceae	1	0.6	EU284103	18	DMKU-RK277	AB697756	F
Meyerozyma caribbica		Debaryomycetaceae	7	4.5	EU348786	0	DMKU-RK237	AB772554	E-2, F, G-2
							DMKU-RK461	AB772574	
Meyerozyma guilliermondii		Debaryomycetaceae	4	2.6	U62311	0-1	DMKU-RK149	AB772522	C, E-1
Millerozyma koratensis		Debaryomycetaceae	3	1.9	DQ404484	0	DMKU-RK238	AB772555	E-2, F
Pichia kudriavzevii		Pichiaceae	6	3.9	U76347	0-1	DMKU-RK105	AB772487	A-2, C , D-1, F, G-2
							DMKU-RK460	AB772573	
Wickerhamomyces ciferrii		Wickerhamomycetaceae	2	1.3	U74587	2	DMKU-RK30	AB772427	A-1, A-2
Yamadazyma mexicana		Debaryomycetaceae	2	1.3	U45790	0	DMKU-RK465	AB772578	G-1, G-2
Yarrowia lipolytica	Saccharomycetales *incertae sedis*		1	0.6	U40080	1	DMKU-RK82	AB772472	A-4
Phylum Basidiomycota									
Cryptococcus flavescens	Agaricomycotina	Tremellomycetes	3	1.9	AB035042	0	DMKU-RK99	AB772482	C , D-1
Cryptococcus laurentii		Tremellales	1	0.6	AF075469	1	DMKU-RK76	AB772466	A-4
Cryptococcus aff. *laurentii*		Bulleromyces clade	3	1.9	AM931019	0	DMKU-RK169	AB772540	D-2, E-1
Cryptococcus rajasthanensis			3	1.9	AM262324	0	DMKU-RK54	AB772449	A-3, D-2, E-1
Pseudozyma antarctica	Ustilaginomycotina	Ustilaginomycetes	1	0.6	AJ235302	0	DMKU-RK112	AB772493	D-1
Pseudozyma aphidis		Ustilaginales	1	0.6	AJ235303	3/588	DMKU-RK86	AB772475	A-4
Rhodotorula taiwanensis	Pucciniomycotina	Microbotryomycetes Sporidiobolales	35	22.6	GU646863	0-3	DMKU-RK33	AB772430	A-1, A-2, A-3,
							DMKU-RK47	AB772443	A-4, D-1, D-2,
							DMKU-RK52	AB772447	C, E-1, F, G-1
							DMKU-RK73	AB772463	
							DMKU-RK90	AB772476	
							DMKU-RK103	AB772485	
							DMKU-RK128	AB772504	
							DMKU-RK146	AB772520	
							DMKU-RK157	AB772529	
							DMKU-RK168	AB772539	
							DMKU-RK227	AB772548	
							DMKU-RK274	AB772560	
Sporobolomyces blumeae			2	1.3	AY213010	0-1	DMKU-RK31	AB772428	A-1
Trichosporon asahii	Agaricomycotina	Tremellomycetes	6	3.9	AF105393	0	DMKU-RK51	AB772446	A-3, A-4, D-1
		Trichosporonales					DMKU-RK130	AB772506	
			156	100					

[1] Number of plant leaf samples from which that species was isolated/ number of plant leaf sample examined ×100

Sporobolomyces (one species) and *Trichosporon* (one species).

The most prevalent yeast species was *Rhodotorula taiwanensis*, with a 23 % frequency of occurrence followed by *Candida tropicalis* (16 %), *Cyberlindnera fabianii* (12 %), *Debaryomyces nepalensis* (5 %), *Meyerozyma caribbica* (5 %), *Pichia kudriavzevii* (4 %), and *Cyberlindnera rhodanensis* (4 %). Only 1–4 strains of the remaining species were detected. The result revealed that *R. taiwanensis* was isolated from rice leaf samples collected from ten out of the 14 sampling sites (Table 2), therefore, it could be interpreted to mean that it is a common yeast species in rice phylloplane.

The results indicated that 64 % of the isolates obtained by the enrichment isolation technique at 30±2 °C were ascomycete yeasts, and only 36 % represented basidiomycete yeasts. This is in contrast with other investigations, which have reported the dominance of basidiomycete yeasts in the phylloplanes of other regions (de Azeredo et al. 1998; Nakase et al. 2001; Fonseca and Inacio 2006; Slavikova et al. 2009; Glushakova and Chernov 2010). The difference in the results may derive from the difference in cultivation temperature and the technique employed for isolation. In most investigations, plating of leaf washings and incubating at 25 °C or lower was used (de Azeredo et al. 1998; Slavikova et al. 2009) while Nakase et al. (2001) used the ballistoconidium-fall method with YM agar without any antibacterial and antifungal agents and found more yeast species when the incubation temperature was at 23 °C than at 30 °C. However, the present results agreed well with the previous studies of ours, which also reported the dominance of ascomycete yeast species on the phylloplanes of various plants in Thailand when enrichment isolation and a relatively high incubation temperature were used (Limtong and Koowadjanakul 2012; Limtong et al. 2014). These results indicated that the species of the small population in Ascomycota are possible to be isolated by using an enrichment technique.

Rhodotorula taiwanensis, the most prevalent species in the rice phylloplane in this study, was proposed recently from the strain isolated from plant stem tissue in Taiwan (Huang et al. 2011). This species was also found with high frequency of occurrence in sugarcane phylloplane in Thailand (Limtong et al. 2014). These confirmed that *R. taiwanensis* is a plant associated yeast species. *Candida tropicalis* has been reported to be one of the dominant yeast species in the phylloplanes of various plants in Russia (Kvasnikov et al., 1975 cited in Fonseca and Inacio 2006). It was isolated from the phylloplanes of fruit trees in the Czech Republic (Slavikova et al. 2009). This species was previously reported to be present in the phylloplanes of the other plant species in Thailand with relatively high frequency of isolation (14 %) (Limtong and Koowadjanakul 2012; Limtong et al. 2014). I It is also present in other habitats in Thailand such as water, sediment, decaying plant materials submerged in water in mangrove forest (Limtong et al. 2008; Boonmak et al. 2009; Limtong and Yongmanitchai 2010) and forest soil (Kaewwichian et al. 2010). *Cyberlindnera* (*Lindnera*) *fabianii* (a teleomorph of *Candida fabianii*) has been reported to be a contaminant in industrial and food fermentations consisting of starch-based substrates and a common yeast of a Zimbabwean wild fruit (Kurtzman 2011a). Among the species found in rice phylloplanes, *Cryptococcus laurentii*, which was obtained in only one isolate, has been reported in many previous investigations (Slavikova et al. 2007, 2009; Glushakova and Chernov 2010; Limtong et al. 2014). *Candida catenulata* and *L. thermotolerans* were also detected in the phylloplanes of trees in the Czech Republic (Slavikova et al. 2007). On the other hand, *Candida catenulata*, *Candida membranifaciens*, *Candida pseudointermedia*, *Wickerhamomyces ciferrii* and *Yamadazyma mexicana* that never been reported to be isolated from phylloplane of any plant species were found in rice phylloplane in this study. The first three species have been reported to be isolated from insects (Lachance et al. 2011), which makes it possible to bring these yeasts to the phylloplane by their visiting the plants. *C. pseudointermedia*, *W. ciferrii* and *Y. mexicana* were reported to be isolated from plants (Kurtzman 2011b, 2011c), therefore, it is possible it is also present in phylloplane.

Comparison of the prevalent yeast species in the rice phylloplane in this study with the prevalent species in the sugarcane phylloplane investigated by us (Limtong et al. 2014) revealed that *R. taiwanensis, Candida tropicalis*, *Cyberlindnera fabianii*, *Cyberlindnera rhodanensis* and *Meyerozyma caribbica* were common species found in the phylloplanes of both plant species. Many yeast species found in the rice phylloplane had also been detected previously in the phylloplanes of diverse plant species in Thailand. These included *Candida glabrata*, *Candida jaroonii*, *Candida rugosa*, *Clavispora lusitaniae*, *Cryptococcus flavescens*, *Cryptococcus rajasthanensis*, *Debaryomyces nepalensis*, *Kodamaea ohmeri*, *Metschnikowia koreensis*, *Meyerozyma guilliermondii*, *Meyerozyma koratensis*, *Pichia kudriavzevii*, *Sporobolomyces blumeae* and *Trichosporon asahii* (Nakase et al. 2001; Limtong and Koowadjanakul 2012; Limtong et al. 2014). These results could be interpreted to mean that the species of a plant has no effect on the yeast species in its phylloplane; on the other hand, the climate may influence the yeasts in the phylloplane.

It should be noted that many rice phylloplane yeast species, such as *Candida glabrata*, *Candida tropicalis*, *Clavispora lusitaniae*, *Meyerozyma guilliermondii*, *Pichia kudriavzevii* and *Trichosporon asahii*, are Risk Group 2 microorganisms, which are defined by many organizations in many countries as pathogens that can cause human or animal disease but are unlikely to be a serious hazard to laboratory workers, the community, livestock or the environment, and, while laboratory exposure may cause serious infection, effective treatment

and preventative measures are available and the risk of the spread of infection is limited (Madsen and Tendal 2012). This may be due to the fact that human beings are involved in growing rice in Thailand and microorganisms considered to

Table 3 Growth at 35, 37 and 40 °C of yeast strains isolated from rice phylloplane

Species	Strain	Growth		
		35 °C	37 °C	40 °C
Phylum Ascomycota				
Candida glabrata	DMKU-RK165	+	+	+
Candida jaroonii	DMKU-RK94, DMKU-RK101, DMKU-RK138	+	+	−
Candida membranifaciens	DMKU-RK57	+	+	w
Candida ruelliae	DMKU-RK434	+	+	+
Candida rugosa	DMKU-RK164, DMKU-RK483	+	+	+
Candida terebra	DMKU-RK436	+	+	+
Candida tropicalis	DMKU-RK34, DMKU-RK36, DMKU-RK37, DMKU-RK41, DMKU-RK43, DMKU-RK45, DMKU-RK60, DMKU-RK62, DMKU-RK104, DMKU-RK121, DMKU-RK123, DMKU-RK131, DMKU-RK137, DMKU-RK145, DMKU-RK152, DMKU-RK161, DMKU-RK163, DMKU-RK166, DMKU-RK219, DMKU-RK226, DMKU-RK235, DMKU-RK284, DMKU-RK462, DMKU-RK464, DMKU-RK485	+	+	+
Clavispora lusitaniae	DMKU-RK484	+	+	+
Cyberlindnera fabianii	DMKU-RK35, DMKU-RK38, DMKU-RK40, DMKU-RK42, DMKU-RK56, DMKU-RK59, DMKU-RK63, DMKU-RK95, DMKU-RK108, DMKU-RK110, DMKU-RK114, DMKU-RK118, DMKU-RK122, DMKU-RK124, DMKU-RK127, DMKU-RK134	+	+	+
Cyberlindnera rhodanensis	DMKU-RK144, DMKU-RK151, DMKU-RK463, DMKU-RK481, DMKU-RK482	+	+	+
Debaryomyces nepalensis	DMKU-RK153	+	+	w
	DMKU-RK158	+	+	−
	DMKU-RK280	+	+	+
Kodamaea ohmeri	DMKU-RK67, DMKU-RK79	+	+	+
Lachancea thermotolerans	DMKU-RK275	+	+	−
Meyerozyma caribbica	DMKU-RK240, DMKU-RK273, DMKU-RK279, DMKU-RK281, DMKU-RK282, DMKU-RK461	+	+	+
Meyerozyma guilliermondii	DMKU-RK149, DMKU-RK216, DMKU-RK221, DMKU-RK228	+	+	+
Millerozyma koratensis	DMKU-RK238, DMKU-RK241, DMKU-RK272	+	+	+
Pichia kudriavzevii	DMKU-RK105, DMKU-RK135, DMKU-RK140, DMKU-RK283, DMKU-RK460	+	+	+
Wickerhamomyces ciferrii	DMKU-RK136	+	+	−
Yamadazyma mexicanum	DMKU-RK465	+	+	+
Yarrowia lipolytica	DMKU-RK82	+	w	−
Phylum Basidiomycota				
Cryptococcus aff. *laurentii*	DMKU-RK169	w	−	−
Cryptococcus flavescens	DMKU-RK99	w	−	−
	DMKU-RK129, DMKU-RK141	+	−	−
Cryptococcus laurentii	DMKU-RK76	+	+	−
Rhodotorula taiwanensis	DMKU-RK44, DMKU-RK49, DMKU-RK64, DMKU-RK73, DMKU-RK75, DMKU-RK85, DMKU-RK90, DMKU-RK98, DMKU-RK103, DMKU-RK107, DMKU-RK113, DMKU-RK128, DMKU-RK146, DMKU-RK150, DMKU-RK155, DMKU-RK274, DMKU-RK435, DMKU-RK437	w	−	−
Trichosporon asahii	DMKU-RK69, DMKU-RK72, DMKU-RK102	+	+	+
	DMKU-RK77,	+	+	w
	DMKU-RK130	+	+	−

Remark: +, growth; −, no growth; w, weak growth

be of Risk Group 2 may be brought to rice fields by these farmers. Not only that, many animals in the field can also be sources of Risk Group 2 microorganism contamination. Many Risk Group 2 yeast strains isolated from rice phylloplane were revealed to have high number of these strains in the field. Moreover, they are ascomycete yeast species so that our enrichment isolation technique at relative high temperature was favorabe for their isolation.

Growth at high temperature of rice phylloplane yeasts

The ability of growing at high temperatures, 35, 37 and 40 °C, of the strains isolated from rice phylloplane was determined and the results shown in Table 3. Because the isolation of yeast was carried out by the enrichment technique at relatively high temperature, in the range of 28 to 32 °C, therefore, these yeast strains should have the ability to grow in this range of temperature or higher, which is likely to be characteristic of yeast present in a tropical country like Thailand. This assumption seems correct because, when we determined the ability of growing at high temperatures, 35, 37 and 40 °C, we found that all strains could grow at 35 °C although all strains of *Rhodotorula taiwanensis*, *Cryptococcus* aff. *laurentii* (one strain) and *Cryptococcus flavescens* (one strain) revealed weak growth. At 37 °C, most strains grew but all strains of *R. taiwanensis*, *Cryp.* aff. *laurentii* and *Cryp. flavescens* could not. When increasing the temperature to 40 °C, more than half of the tested strains could grow, although many strains could not grow.

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Yamadazyma insecticola f.a., sp. nov. and *Yamadazyma epiphylla* f.a., sp. nov., two novel yeast species

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Two yeast strains representing two novel yeast species were isolated from frass of an unidentified insect (ST-78^T) and the external surfaces of rice leaves (YE170^T) collected in Thailand. The two strains were genetically, morphologically and phenotypically distinct from recognized species and were found to represent two novel species of the genus *Yamadazyma* although formation of ascospores was not observed. In terms of pairwise sequence similarity of the D1/D2 region of the large subunit rRNA gene, the closest relative of strain ST-78^T was *Candida lessepsii* CBS 9941^T but with 3.8 % nucleotide substitutions, while the closest relative of strain YE170^T was strain ST-78^T but with 4.3 % nucleotide substitutions. Analysis of the sequences of the internal transcribed spacer 1 and 2 (ITS1–2) regions revealed that strain ST-78 differed from *C. lessepsii* CBS 9941^T by 8.8 % nucleotide substitutions and from strain YE170^T by 9.4 % nucleotide substitutions. The result of pairwise sequence similarity of the D1/D2 and ITS1–2 regions together with phylogenetic analysis indicated that strains ST-78^T and YE170^T represented two novel species within the *Yamadazyma* clade. The names *Yamadazyma insecticola* f.a., sp. nov. (type strain ST-78^T=BCC 8314^T=NBRC 110421^T=CBS 13382^T; MycoBank no. MB810546) and *Yamadazyma epiphylla* f.a., sp. nov. (type strain YE170^T=BCC 63466^T=NBRC 110423^T=CBS 13384^T; MycoBank no. MB810547) are proposed for the two novel yeast species.

The genus *Yamadazyma* was described by Billon-Grand (1989) to accommodate species that were transferred from the genus *Pichia*, which formed hat-shaped ascospores that were released from the ascus at maturity and had coenzyme Q-9 as their major ubiquinone (Kurtzman & Robnett, 1998). Phylogenetic analysis of the D1/D2 region of the large subunit (LSU) rRNA gene placed the species assigned to the genus *Yamadazyma* in several clades and indicated that the genus is polyphyletic (Kurtzman & Robnett, 1998); it was therefore not generally accepted. Analysis of the D1/D2 region of the LSU rRNA gene and the nearly complete small subunit rRNA gene supported transferring some species that had been assigned to the genus *Yamadazyma* by Billon-Grand (1989) to the newly described genera *Babjeviella*, *Meyerozyma*, *Millerozyma* and *Priceomyces*. Thereafter, the genus *Yamadazyma* became a well-supported clade (Kurtzman & Suzuki, 2010) and a generally accepted genus in the family *Debaryomycetaceae*, order *Saccharomycetales* (Kurtzman, 2011). In Kurtzman (2011), *Yamadazyma philogaea*, the type species of the genus, as well as *Y. akitaensis*, *Y. mexicana*, *Y. nakazawae*, *Y. scolyti*, *Y. triangularis* and 28 *Candida* species are placed in the *Yamadazyma* clade. Since then, many novel species of the genus *Candida* in this clade have been described, for example *Candida kanchanaburiensis*, *C. khao-thaluensis*, *C. vaughaniae*, *C. tallmaniae*, *C. oceani*, *C. sirachaensis*, *C. sakaeoensis*, *C. phyllophilla* and *C. vitiphila* (Nakase *et al.*, 2008; Burgaud *et al.*, 2011; Groenewald *et al.*, 2011; Limtong *et al.*, 2012; Limtong & Kaewwichian, 2013). Recently, five species of the genus *Yamadazyma*, namely

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Abbreviations: ITS, internal transcribed spacer; LSU, large subunit.

The GenBank/EMBL/DDBJ accession numbers for the sequences of the D1/D2 region of the large subunit rRNA gene and the ITS1–2 regions of *Yamadazyma insecticola* ST-78^T are DQ400379 and LC006081, respectively, and of *Yamadazyma epiphylla* YE170^T are LC006026 and LC006082, respectively.

Y. terventina, *Y. siamensis*, *Y. phyllophila*, *Y. paraphyllophila* and *Y. ubonensis*, were proposed from strains isolated in Italy, Taiwan and Thailand (Ciafardini *et al.*, 2013; Kaewwichian *et al.*, 2013; Junyapate *et al.*, 2014). At the time of writing, the *Yamadazyma* clade consists of 11 species of the genus *Yamadazyma* and more than 30 species of the genus *Candida* (Lachance *et al.*, 2011; Kurtzman, 2011). The habitats of species belonging to the *Yamadazyma* clade are very diverse and include water, plants, animals, and the guts of termites and other insects (Suh *et al.*, 2005; Ganter, 2006).

In the present paper, two yeast strains isolated from frass of an unidentified insect (ST-78^T) and the external surfaces of rice leaves (YE170^T) collected in Thailand are described and considered to represent two novel species of the genus *Yamadazyma*, *Yamadazyma insecticola* f.a., sp. nov. and *Yamadazyma epiphylla* f.a., sp. nov.

Yeast isolation

Strain ST-78^T was isolated from frass of an unidentified insect by direct streaking on yeast extract-malt extract (YM) agar (0.3 % yeast extract, 0.3 % malt extract, 0.5 % peptone and 2.0 % agar) supplemented with 100 μg chloramphenicol ml^{-1} and 200 μg sodium propionate ml^{-1} in a Petri dish. Strain YE170^T was isolated by the plating of leaf washings as described by Inácio *et al.* (2002). A rice leaf sample (10 g) was aseptically suspended in 50 ml of 0.85 % saline solution in a 250 ml Erlenmeyer flask and shaken on a rotary shaker at 25 °C for 1 h to detach yeast cells from the surfaces. An aliquot (0.1 ml) of the washing solution was then spread on YM agar supplemented with 100 μg chloramphenicol ml^{-1} and 250 μg sodium propionate ml^{-1}, and incubated at 25 °C until yeast colonies appeared. Purification of yeast strains was performed by cross streaking on YM agar. Purified yeast strains were suspended in YM broth supplemented with 10 % (v/v) glycerol and maintained at −80 °C.

DNA sequencing and phylogenetic analysis

The nucleotide sequences of the D1/D2 region of the LSU rRNA gene and the internal transcribed spacer 1 and 2 (ITS1–2) regions were determined from PCR products amplified from genomic DNA extracted from cells. DNA extraction, PCR primers, PCR reaction conditions and the sequencing of amplicons were determined according to the methods outlined by Kurtzman & Robnett (1998) and White *et al.* (1990). Amplification of the D1/D2 region of the LSU rRNA gene was carried out by PCR with the forward primer NL1 (5′-GCATATCAATAAGCGGAGGA-AAAG-3′) and the reverse primer NL4 (5′-GGTCCGTGT-TTCAAGACGG-3′) (Kurtzman & Robnett, 1998). The ITS1–2 regions were amplified with forward primer ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al.*, 1990). Sequence divergence of the D1/D2 region of the LSU rRNA gene and the ITS1–2 regions of the yeast strains was compared with similar sequences in the GenBank database by the nucleotide BLAST analysis tool (Altschul *et al.*, 1997). The phylogenetic placement of the proposed novel species along with neighbouring taxa was analysed using the concatenated sequences of the ITS1–2 and the D1/D2 regions of the LSU rRNA gene. The sequences of the novel strains and related species retrieved from GenBank (accession numbers indicated in Fig. 1) were aligned using the program MUSCLE (Edgar, 2004) and phylogenetic trees were reconstructed by using the maximum-likelihood method based on the Kimura two-parameter model. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013).

Phenotypic characterization

The strains were characterized morphologically, biochemically and physiologically according to the standard methods given by Kurtzman (2011). Pseudohypha and hypha formation was investigated on YM agar, corn meal agar (CMA) and potato dextrose agar (PDA) and on slide culture after growth at 25 °C for 7 days. Ascospore formation was investigated on YM agar, acetate agar, 5 % malt extract agar, CMA, Gorodkowa agar and Fowell's acetate agar after growth at 15 and 25 °C for 4 weeks. Carbon assimilation tests were conducted in a liquid medium according to the method described by Kurtzman (2011). Nitrogen assimilation was examined on solid media with starved inocula (Nakase & Suzuki, 1986a). The ability to grow at various temperatures was determined in YM broth using a metal block bath. Ubiquinone was extracted from yeast cells grown in yeast peptone glucose broth (1.0 % yeast extract, 2.0 % peptone and 2.0 % glucose) at 25 °C on a rotary shaker at 150 r.p.m. for 3 days. The cells were washed with distilled water and then freeze-dried. Ubiquinone extraction and purification were performed as described by Nakase & Suzuki (1986b). Ubiquinone isoprenologues were identified by HPLC (Waters 2690) with a Cosmosil column (Waters 5C18) and eluted with a 2 : 1 (v/v) ratio of methanol/2-propanol as the mobile phase at a flow rate of 1 ml min^{-1} and the eluent was analysed using a UV spectrophotometer (Waters 996 photodiode array detector) at 275 nm.

Novel species delineation and phylogenetic placement

In terms of pairwise alignment, the sequence of strain ST-78^T was related most closely to *Candida lessepsii* CBS 9941^T, but with 3.8 % nucleotide substitutions (20 nt substitutions, two indels out of 535 nt) in the D1/D2 region of the LSU rRNA gene and 8.8 % nucleotide substitutions (49 nt substitutions, 25 indels out of 579 nt) in the ITS1–2 regions. Strain YE170^T was related most closely to strain ST-78^T but with 4.3 % nucleotide substitutions (22 nt substitutions, five indels out of 521 nt) and 9.4 % nucleotide substitutions (56 nt substitutions, 24 indels out of 623 nt) in the D1/D2 region of the LSU rRNA gene and the ITS1–2 regions, respectively. Its closest

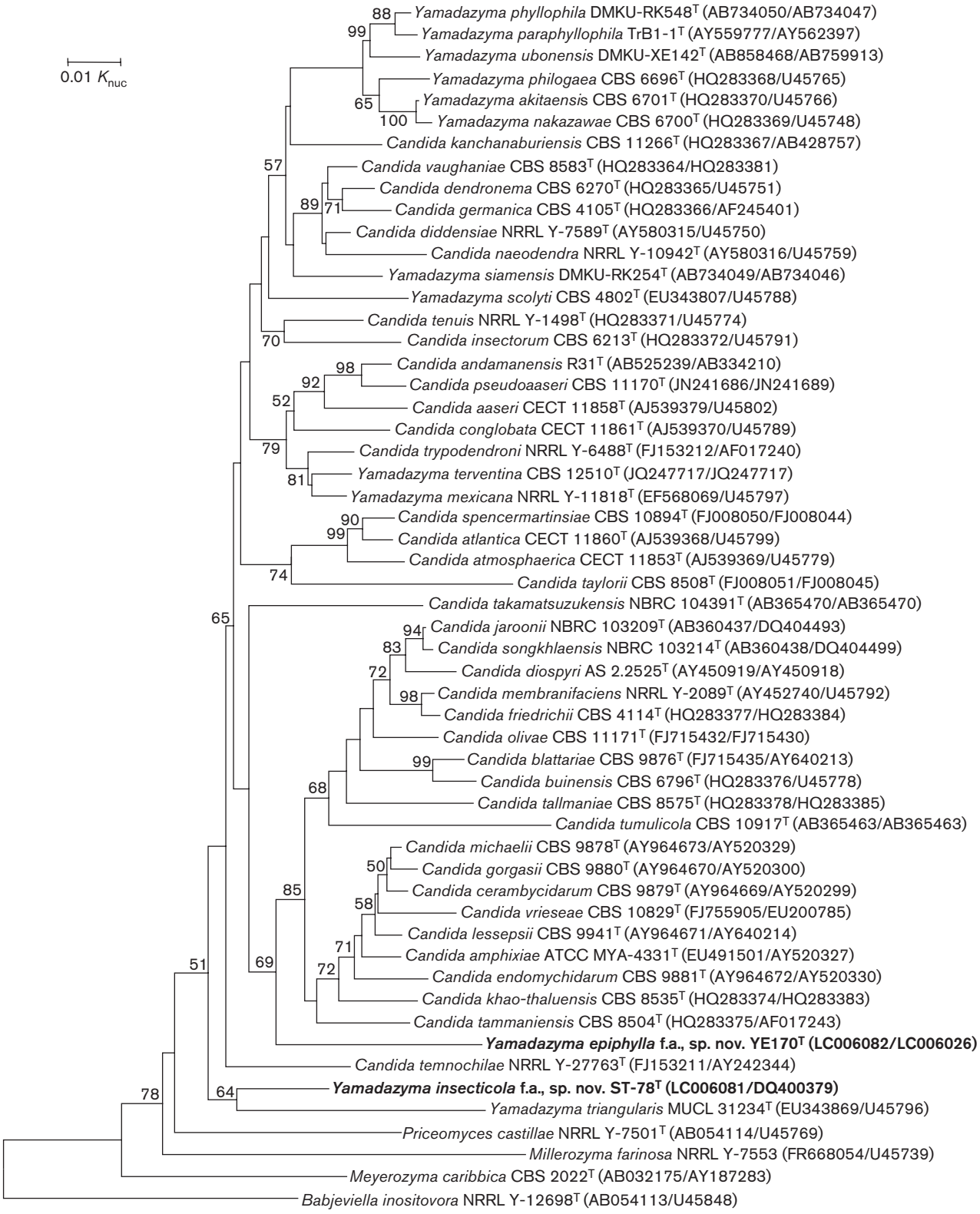


Fig. 1. Phylogenetic tree showing the positions of *Yamadazyma insecticola* f.a., sp. nov. (ST-78^T) and *Yamadazyma epiphylla* f.a., sp. nov. (YE170^T) with respect to closely related species based on the concatenated sequences of the ITS1–2 regions and the D1/D2 region of the LSU rRNA gene. The phylogenetic tree was reconstructed from evolutionary distance data with Kimura's two-parameter correction, using the maximum-likelihood method by MEGA software version 6.0. Numbers indicate percentages of bootstrap sampling, derived from 1000 samples. Numbers in parentheses are GenBank accession numbers. *Babjeviella inositovora* NRRL Y-12698^T was the outgroup in the analysis. Bar, 0.01 substitutions per site.

recognized relatives were *Candida amphixiae* CBS 9877^T and *Candida tammaniensis* CBS 8504^T, but with 4.5 % nucleotide substitutions (23 nt substitutions, eight indels out of 524 nt) and 6.2 % nucleotide substitutions (38 nt substitutions, 25 indels out of 640 nt) in the D1/D2 region of the LSU rRNA gene and the ITS1–2 regions, respectively. Therefore, BLAST similarity searches with the D1/D2 region of the LSU and ITS1–2 regions clearly indicated that strains ST-78^T and YE170^T were representatives of two novel species.

Phylogenetic analysis based on the concatenated sequences of the ITS1–2 regions and D1/D2 region of the LSU rRNA gene using the maximum-likelihood method indicated that strains ST-78^T and YE170^T represented two novel species placed in the *Yamadazyma* clade (Fig. 1). The phylogentically closest relative of strain ST-78^T was *Y. triangularis*. Strain YE170^T was distinct from strain ST-78^T and placed in a subclade that contained *C. lessepsii*, the closest relative of strain ST-78^T in terms of pairwise sequence similarity of the sequence of the D1/D2 region of the LSU rRNA gene. The affinity of the subclades containing the two novel species with the subclade containing the type species of the genus *Yamadazyma* was supported by bootstrap values of 51 and 65 %, respectively. The phylogenetic analysis indicated the reciprocal monophyly of the two novel species with respect to related species. Because the phylogenetic tree was reconstructed from the concatenated sequences of the ITS1–2 regions and the D1/D2 region of the LSU rRNA gene, the two novel species did not show the same relationship as the result of pairwise sequence similarity of the sequences of the D1/D2 region of the LSU rRNA gene. No differences were detected between the neighbour-joining and maximum-parsimony methods, particularly with regard to the position of strains ST-78^T and YE170^T. A phylogenetic species concept therefore applies.

According to the recent International Code of Nomenclature for algae, fungi and plants, the most important requirement is the adoption of 'one fungus, one name' (Miller *et al.*, 2011). Consequently, although the formation of ascospores was not observed, the two novel species are described based on a single strain each and sexual spore reproduction was not observed, the novel species are assigned to the genus *Yamadazyma*, and the designation *forma asexualis* (f.a.) is included following the recommendation of Lachance (2012). We propose the names *Yamadazyma insecticola* f.a., sp. nov. (type strain ST-78^T, MycoBank accession no. MB810546) and *Yamadazyma epiphylla* f.a., sp. nov. (type strain YE170^T, MycoBank accession no. MB810547) for these species.

Physiologically, *Y. insecticola* and *Y. epiphylla* can be differentiated from each other and from their closest known relatives not only based on analysis of the sequences of the D1/D2 region of the LSU rRNA gene and the ITS1–2 regions but also based on several phenotypic characteristics (Table 1). *Y. insecticola* does not assimilate melibiose, raffinose or DL-lactic acid and does not grow at 40 °C while *Y. epiphylla* assimilates these compounds and grows at 40 °C. *Y. insecticola* grows in 60 % glucose and with 0.1 % cycloheximide whereas *Y. epiphylla* does not. *Y. insecticola* can be distinguished from *C. lessepsii* based on several phenotypic characteristics; for example, *Y. insecticola* assimilates sucrose, L-sorbose, L-rhamnose and ethanol, grows in vitamin-free medium and 0.1 % cycloheximide and does not ferment trehalose while *C. lessepsii* does not assimilate sucrose, L-sorbose or L-rhamnose, does not grow in vitamin-free medium or 0.1 % cycloheximide and ferments trehalose. Phenotypic characteristics that distinguish *Y. epiphylla* from *C. amphixiae* are the inability to ferment D-galactose and trehalose of *Y. epiphylla* in contrast to *C. amphixiae*. *Y. epiphylla* assimilates melibiose and raffinose while *C. amphixiae* does not.

Members of the *Yamadazyma* clade exhibit an association with the gut of beetles and other insects, flowers, tree exudates, plants, mushroom, deep-sea coral, water samples, and marine and mangrove habitats (Nakase *et al.*, 2008; Burgaud *et al.*, 2011; Groenewald *et al.*, 2011; Kurtzman, 2011; Lachance *et al.*, 2011; Kaewwichian *et al.*, 2013; Junyapate *et al.*, 2014). According to these reports, 20

Table 1. Differential phenotypic characteristics between *Y. insecticola* sp. nov. and *Y. epiphylla* sp. nov. and closely related species of the genera *Candida* and *Yamadazyma*

Species: 1, *Y. insecticola*; 2, *Y. epiphylla*; 3, *C. lessepsii*; 4, *C. amphixiae*; 5, *C. tammaniensis*; 6, *Y. triangularis*. +, Positive; W, weak; V, variable; D, delayed; −, negative; N, no data. Data for *C. lessepsii*, *C. amphixiae* and *C. tammaniensis* were taken from Kurtzman *et al.* (2011) and for *Y. triangularis* from Kurtzman (2011).

Characteristic	1	2	3	4	5	6
Fermentation of:						
D-Galactose	W	−	D	+	+	−
Trehalose	−	−	+	+	+	−
Assimilation of:						
L-Sorbose	Latent	Slow	−	W	−	−
Sucrose	+	+	−	+	+	+
Melibiose	−	+	−	−	−	+
Raffinose	−	+	−	−	−	−
Soluble starch	W	W	−	−	−	−
D-Arabinose	+	+	+	+	+	−
L-Rhamnose	+	+	−	+	+	+
Ethanol	+	+	−	+	+	+
Salicin	+	+	+	+	+	−
2-Keto-D-gluconate	−	−	−	−	+	−
DL-Lactic acid	−	+	−	D	+	−
Growth in/at/with:						
Vitamin-free medium	+	W	−	−	−	N
50 % Glucose	+	+	+	+	−	N
60 % Glucose	+	−	N	−	N	N
0.01 % Cycloheximide	+	−	+	W	+	N
0.1 % Cycloheximide	+	−	−	−	+	N
37 °C	+	+	N	N	−	−
40 °C	−	+	N	N	N	−

species in the *Yamadazyma* clade have been isolated from the gut or frass of insects and 10 species have been obtained from plants. In the present study, two yeast strains representing two novel species of the genus *Yamadazyma*, *Y. insecticola* f.a. and *Y. epiphylla* f.a., were isolated from frass of an unidentified insect and the external surfaces of rice leaves. Since the first description of four yeasts species isolated from insect frass reported by Phaff and do Carmo-Sousa (1962), many species have been described belonging to several genera of ascomycetous yeasts, such as *Ascobotryozyma*, *Barnettozyma*, *Kuraishia*, *Lipomyces*, *Lindnera*, *Meyerozyma*, *Nakazawaea*, *Ogataea*, *Peterozyma*, *Pichia*, *Schefferomyces*, *Starmera*, *Sugiyamaella*, *Trichomonascus*, *Wickerhamomyces* and *Yamadazyma*, and some genera of basidiomycetous yeasts, such as *Bulleromyces*, *Curvibasidium*, *Cryptococcus*, *Sporobolomyces* and *Trichosporon*. The basidiomycetous yeasts often colonize the phylloplane at approximately $10–10^4$ cm^{-2} (Shivas & Brown, 1984) and they move from leaf to leaf by passive air dispersal. In general, the phylloplane yeast community includes species of the genera *Bullera*, *Bensingtonia*, *Cystofilobasidium*, *Cryptococcus*, *Leucosporidium*, *Pseudozyma*, *Rhodotorula* and *Sporobolomyces* (Starmer & Lachance, 2011). However, ascomycetous yeasts have also been obtained from the phylloplane, as reported by Limtong *et al.* (2014).

The strains isolated from insect frass and phylloplane have been isolated from many countries in all continents (Kurtzman *et al.*, 2011). This indicates that insect frass and phylloplane or plants are important habitats of yeasts worldwide in terms of species distribution.

This paper describes two novel yeast species of the genus *Yamadazyma* based each on a single strain, supporting by several phenotypic and genetic properties that differentiate them from any recognized species. This is despite the lack of phenotypic and genetic heterogeneity information regarding the proposed species, and understanding of their species diversity, ecology and phylogeny.

Description of *Yamadazyma insecticola* Jindamorakot, Am-in, Kaewwichian & Limtong sp. nov.

Yamadazyma insecticola (in.sec.ti′cola. N.L. fem adj. *insecticola* referring to an association between yeast and insect).

After growth in YM broth at 25 °C for 3 days, cells are globose, subglobose to ovoid (2–8 × 3–8 μm) and occur singly, in pairs or in groups (Fig. 2a). Budding is multilateral. After growth on YM agar at 25 °C for 3 days, the streak culture is greyish white, smooth, soft to butyrous with entire margin. Pseudohyphae are formed in slide culture on YM agar, CMA and PDA after 3 days at 25 °C (Fig. 2b) and true hyphae are not formed in slide culture on those agar media after 21 days at 25 °C. Ascospores are not produced on YM agar, acetate agar, 5 % malt extract agar, CMA, Gorodkowa agar and Fowell's acetate agar at 15 or 25 °C after 4 weeks. Positive for fermentation of D-glucose, weakly positive for fermentation of D-galactose but negative for fermentation of sucrose, maltose, lactose, trehalose and raffinose. Assimilates D-glucose, D-galactose, L-sorbose (latent), sucrose, maltose, cellobiose, trehalose, melezitose, soluble starch (weakly), D-xylose, L-arabinose, D-arabinose, D-ribose (weakly), L-rhamnose, ethanol, glycerol, erythritol, ribitol, D-mannitol, D-glucitol, methyl α-D-glucoside, salicin, succinic acid, citric acid, xylitol (weakly), L-arabitol and D-glucosamine, but not lactose, melibiose, raffinose, inulin, galactitol, D-glucono-δ-lactone, D-glucuronate, D-galacturonate, 2-keto-D-gluconate, 5-keto-D-gluconate, DL-lactic acid, inositol or hexadecane. Ethylamine-HCl, L-lysine-HCl and cadaverine are assimilated, but nitrate and nitrite are not. Growth in vitamin-free medium is positive. Growth occurs on media containing 50 % (w/w) glucose, 60 % glucose, 10 % NaCl/5 % glucose, 15 % NaCl/5 % glucose, 0.01 % cyclohexemide and 0.1 % cyclohexemide. Grows at 25, 30, 35 and 37 °C but not at 40 °C. Acid formation from glucose is positive. Starch-like compounds are not produced. Diazonium blue B and urease reactions are negative. The major ubiquinone is Q-9.

Strain ST-78^T (=BCC 8314^T=NBRC 110421^T=CBS 13382^T) is the holotype of *Y. insecticola* f.a., sp. nov. (MycoBank accession number MB810546). The strain was isolated from insect frass collected from Udon Thani province, Thailand. The culture is maintained by freezing and/or lyophilization.

Description of *Yamadazyma epiphylla* Jindamorakot, Am-in, Kaewwichian & Limtong sp. nov.

Yamadazyma epiphylla (e.pi.phyl′la. N.L. fem. adj. *epiphylla* referring to an association between yeast and plant surface).

After growth in YM broth at 25 °C for 3 days, cells are globose, subglobose to ovoid (2–7 × 2.5–9 μm) and occur

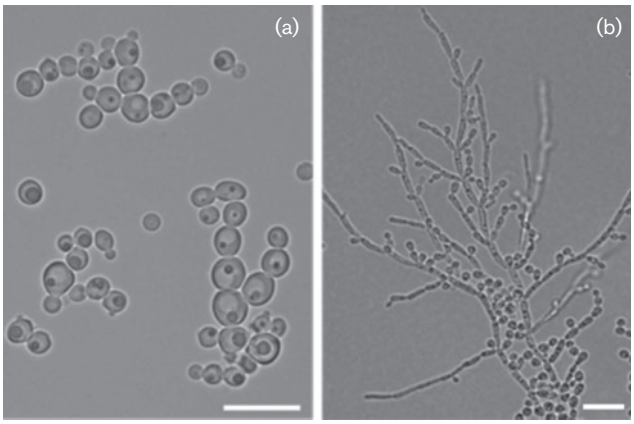


Fig. 2. *Yamadazyma insecticola* f.a., sp. nov. (ST-78^T): (a) budding cells in YM broth after 3 days at 25 °C; (b) pseudohyphae formed on CMA after 3 days at 25 °C. Bars, 10 μm.

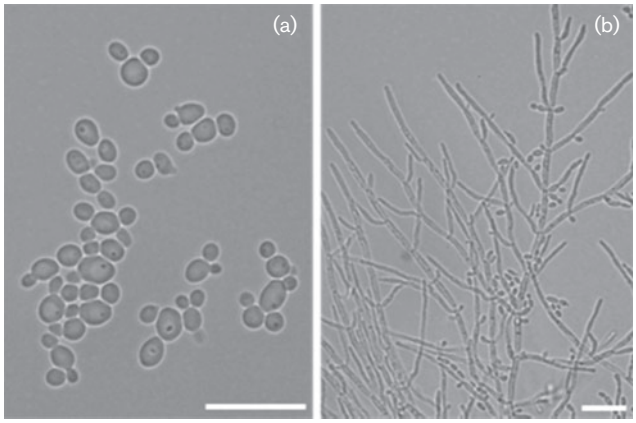


Fig. 3. *Yamadazyma epiphilla* f.a., sp. nov. (YE170^T): (a) budding cells in YM broth after 3 days at 25 °C; (b) pseudohyphae formed on YM agar after 3 days at 25 °C. Bars, 10 μm.

singly, in pairs or in groups (Fig. 3a). Budding is multilateral. After growth on YM agar at 25 °C for 3 days, the streak culture is greyish white, smooth, soft to butyrous with entire margin. Pseudohyphae are formed in slide culture on YM agar, CMA and PDA after 3 days at 25 °C (Fig. 3b) but true hyphae are not formed in slide culture on those agar media after 21 days at 25 °C. Ascospores are not produced on YM agar, acetate agar, 5 % malt extract agar, CMA, Gorodkowa agar or Fowell's acetate agar at 15 or 25 °C after 4 weeks. Fermentation of D-glucose is positive but negative for D-galactose, sucrose, maltose, lactose, trehalose and raffinose. Assimilates D-glucose, D-galactose, L-sorbose (slowly), sucrose, maltose, cellobiose, trehalose, melibiose, raffinose, melezitose, soluble starch (weakly), D-xylose, L-arabinose, D-arabinose, D-ribose, L-rhamnose, ethanol, glycerol, erythritol, ribitol, D-mannitol, D-glucitol (latent), methyl α-D-glucoside, salicin, DL-lactic acid, succinic acid, citric acid, xylitol, L-arabitol and D-glucosamine, but not lactose, inulin, galactitol, D-glucono-δ-lactone, D-glucuronate, D-galacturonate, 2-keto-D-gluconate, 5-keto-D-gluconate, inositol or hexadecane. Ethylamine-HCl, L-lysine-HCl and cadaverine are assimilated, but nitrate and nitrite are not. Growth in vitamin-free medium is weakly positive. Growth occurs on media containing 10 % NaCl/5 % glucose, 15 % NaCl/5 % glucose (weakly) and 50 % glucose but not on media containing 60 % glucose, 0.01 % cyclohexemide or 0.1 % cyclohexemide. Grows at 25, 30, 35, 37 and 40 °C but not at 42 °C. Acid formation from glucose is positive. Starch-like compounds are not produced. Diazonium blue B and urease reactions are negative. The major ubiquinone is Q-9.

Strain YE170^T (=BCC 63466^T=NBRC 110423^T=CBS 13384^T) is the holotype of *Y. epiphylla* f.a., sp. nov. (MycoBank accession number MB810547). The strain was isolated from the surfaces of rice leaves collected from Nonthaburi province, Thailand. The culture is maintained by freezing and/or lyophilization.

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Hannaella siamensis sp. nov. and *Hannaella phetchabunensis* sp. nov., two new anamorphic basidiomycetous yeast species isolated from plants

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Eight strains, representing two novel anamorphic yeast species, consisted of five strains isolated from the external surfaces of rice leaves (DMKU-RP72^T, DMKU-RP109, DMKU-RP119, YE-124 and YE-156) and one from a corn leaf (DMKU-CP430^T)4 collected in Thailand, and one strain isolated from each of a composite flower (11-1114) and a fallen dead leaf (12-301); the latter two were collected in Belize. On the basis of sequence analysis of the D1/D2 region of the large subunit rRNA gene and the internal transcribed spacer (ITS) region, they were suggested to be two novel species of the genus *Hannaella*. Seven strains (DMKU-RP72^T, DMKU-RP109, DMKU-RP119, YE-124, YE-156, 11-1114 and 12-301) differed from each other by 0–3 nt substitutions in the D1/D2 region and by 0–1 nt substitutions in the ITS region. In terms of pairwise sequence similarities of the D1/D2 region these seven strains were closest to *Hannaella zeae*, but with 1.2–1.7 % (7–9) nucleotide substitutions. The sequences of the ITS region of these seven strains differed from *H. zeae* by 3.7–3.9 % (16–17) nucleotide substitutions. Therefore, they were assigned to a single novel species and the name *Hannaella siamensis* sp. nov. has been proposed. The type strain is DMKU-RP72^T (=BCC 69493^T=NBRC 110425^T=CBS 13533^T). Strain DMKU-CP430^T represents the second novel species and was also most closely related to *H. zeae*, but with 1.0 % (6) nucleotide substitutions in the D1/D2 region and 3.2 % (14) nucleotide substitutions in the ITS region. It was assigned to the proposed novel species, *Hannaella phetchabunensis* sp. nov. (type strain DMKU-CP430^T=BCC 69492^T=NBRC 110424^T=CBS 13386^T).

The genus *Hannaella* is an anamorphic genus of basidiomycetous yeast in the Tremellales clade, Subphylum Agaricomycotina, Phylum Basidiomycota; it is closely related to the genera *Dioszegia* and *Derxomyces*. It was proposed on the basis of analysis of multigenes, including the small subunit (SSU) rRNA gene, the D1/D2 region of the large subunit (LSU) rRNA gene, the ITS region including the 5.8S rRNA gene and the mitochondrial cytochrome *b* gene to accommodate the

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Abbreviations: ITS, internal transcribed spacer; LSU, large subunit.

The GenBank/EMBL/DDBJ accession number for the sequences of the D1/D2 region of the LSU rRNA gene and the ITS region of the type strain of *Hannaella siamensis*, DMKU-RP72^T are AB922844 and AB922850, respectively, and of the type strain of *Hannaella phetchabunensis*, DMKU-CP430^T, are AB922849 and AB922855, respectively. The living culture of the type strain of *Hannaella siamensis* and *Hannaella phetchabunensis* were deposited at the BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathumthani, Thailand, as BCC 69493^T and BCC 69492^T, respectively; NITE Biological Resources Center (NBRC), Department of Biotechnology, National Institute of Technology and Evaluation, Chiba, Japan, as NBRC 110425^T and NBRC 110424^T and Centraalbureauvoor Schimmelcultures (CBS), Utrecht, the Netherlands as CBS 13533 ^{T} and CBS 13386^T, respectively. The MycoBank numbers of *Hannaella siamensis* and *Hannaella phetchabunensis* are MB809601 and MB809602, respectively.

species in the *Bullera sinensis* clade including species of *Bullera* and *Cryptococcus* (Wang & Bai, 2008). At present, the genus *Hannaella* is composed of eight described species, namely *Hannaella coprosmaensis, Hannaella kunmingensis, Hannaella luteola, Hannaella oryzae, Hannaella pagnoccae, Hannaella sinensis, Hannaella surugaensis* and *Hannaella zeae* (Wang & Bai, 2008; Boekhout *et al.*, 2011a; Landell *et al.*, 2014). Strains of all species of the genus *Hannaella*, except for those of *H. surugaensis*, have been reported to have been isolated from phylloplanes (Wang & Bai, 2008; Boekhout *et al.*, 2011b; Landell *et al.*, 2014), which refer to the external surface of plant leaves (Phaff & Starmer, 1987; Fonseca & Inacio, 2006).

Phylloplanes from various regions of the world have been found to be colonized by members of both the basidiomycetous and the ascomycetous yeasts (Nakase *et al.*, 2001; Fonseca & Inacio, 2006; Glushakova *et al.*, 2007; Slavikova *et al.*, 2009; Landell *et al.*, 2010). The most common phylloplane yeasts are members of the basidiomycetous genera, such as *Cryptococcus*, *Rhodotorula*, *Sporobolomyces* and *Trichosporon* (Slavikova *et al.*, 2009; de Azeredo *et al.*, 1998; Glushakova & Chernov, 2010).

During an investigation of yeasts on the external surfaces of rice and corn leaves in Thailand, six strains, including five strains from the phylloplane of rice and one strain from the phylloplane of corn, were found, which represent two novel species of the genus *Hannaella*. In this paper, the five strains (DMKU-RP72^T, DMKU-RP109, DMKU-RP119, YE-124 and YE-156) together with two strains (11-1114 and 12-301) isolated from plants in Belize are described and proposed to represent *Hannaella siamensis* sp. nov. Strain DMKU-CP430^T isolated from corn leaves is described and proposed as *Hannaella phetchabunensis* sp. nov.

Six yeasts were isolated from the surfaces of rice and corn leaves collected in Thailand by the plating of leaf washes. Leaves (3 g) were aseptically suspended in 50 ml of 0.85 % (w/v) saline solution in a 250 ml Erlenmeyer flask and shaken on a rotary shaker at 150 rpm and room temperature (27 ± 3 °C) for 1 h to detach yeast cells from surfaces. An aliquot of 0.1 ml of the washing solution was then spread on yeast extract malt extract (YM) agar (0.3 % (w/v) yeast extract, 0.3 % (w/v) malt extract, 0.5 % (w/v) peptone, and 2.0 % (w/v) agar) supplemented with 0.025 % (w/v) sodium propionate and 0.02 % (w/v) chloramphenicol and incubated at room temperature until yeast colonies appeared. Yeast colonies of different morphologies were selected and purified by cross streaking on YM agar plates. Purified yeast strains were suspended in YM broth supplemented with 10 % glycerol and maintained at −80 °C. Five strains (DMKU-RP72^T, DMKU-RP109, DMKU-RP119, YE-124 and YE-156^T) were obtained from five samples of rice leaves and strain DMKU-CP430^T from a corn leaf sample (Table 1). Strain 11-1114 was isolated from a composite flower and strain 12-301 from fallen dead leaves, both collected in Belize. The sample was macerated in sterile water and the macerated plant material was placed on yeast extract peptone glucose (YPG) agar (1.0 % yeast extract, 1.0 % peptone, 2.0 % glucose and 2.0 % agar). After incubation at 25 °C for 7 days, the yeast colonies were isolated and purified by streaking on YPG agar plates. The purified clones were maintained on YPG agar.

The sequences of the D1/D2 region of the LSU rRNA gene and the internal transcribed spacer (ITS) (ITS1-5.8S-ITS2) region were determined from PCR products, which had been amplified from genomic DNA. Genomic DNA was isolated from cultures grown overnight on YM agar or in YPG broth. The isolated DNA was used for the amplification of the D1/D2 region of the LSU rRNA gene and the ITS region. The primers used were NL-1 and NL-4 for the D1/D2 region (O'Donnell, 1993), and ITS1 and ITS4 for the ITS region (White *et al.*, 1990). The PCR products were checked by agarose gel electrophoresis, purified and sequenced with NL1 and NL4 primers for the D1/D2 region, and with the ITS1 and ITS4 primers for the ITS region.

The sequences were compared pairwise using a BLAST search (Altschul *et al.*, 1997) and were aligned with the sequences of related species retrieved from GenBank using the multiple alignment program CLUSTAL X version 2.0 (Larkin *et al.*, 2007). A phylogenetic tree was reconstructed from the evolutionary distance data with Kimura's two-parameter correction (Kimura, 1980), with the neighbour joining method (Saitou & Nei, 1987) using MEGA software version 5.0 (Tamura *et al.*, 2011). Confidence levels of the clades were estimated from bootstrap analysis (1000 replicates) (Felsenstein, 1985).

The strains from Thailand were characterized morphologically, biochemically, and physiologically according to the standard methods described by Kurtzman *et al.* (2011). Formation of hyphae was investigated on potato dextrose agar (PDA) and YPG agar in slide cultures at 25 °C for up to 28 days with cultures examined microscopically at intervals of a few days. Basidiospore formation was investigated individually or in pairs on YM agar, YPG agar, 5 % malt extract agar, acetate agar, corn meal agar and Gorodkowa agar at 25 °C for up to 4 weeks. Formation of ballistoconidia was investigated by the ballistoconidia-fall method using corn meal agar. Fermentation was tested with Durham tubes in 5 ml of medium at 25 °C. Carbon and nitrogen assimilation tests were conducted in liquid medium or solid medium. Growth at various temperatures was determined by cultivation in YM broth. Ubiquinones were extracted from cells cultivated in a 500 ml Erlenmeyer flask containing 250 ml of YPG broth on a rotary shaker at room temperature for 24–48 h and purified according to the method described by Yamada & Kondo (1973) and Kuraishi *et al.* (1985). Isoprenologues were identified by HPLC, as described previously (Limtong *et al.*, 2007).

Analysis of the D1/D2 region of the LSU rRNA gene sequence revealed that the eight strains (DMKU-RP72^T, DMKU-RP109, DMKU-RP119, DMKU-CP430^T, YE-124, YE-156, 11-1114 and 12-301) represented two novel yeast species. The first species consisted of seven strains, of which four strains (DMKU-RP72^T, DMKU-RP119, YE-124 and

Table 1. Strains of *Hannaella siamensis* sp. nov. and *Hannaella phetchabunensis* sp. nov.

Strain (accession number)	GenBank accession number		Source/locality	Date
	D1/D2	ITS		
Hannaella siamensis sp. nov.				
DMKU-RP72^T (BCC 69493^T, NBRC 110425^T, CBS 13533^T)	AB922844	AB922850	Rice leaves (*Oryza sativa*)/Nakhon Pathom province, Thailand	12 January 2012
DMKU-RP109 (BCC 69494)	AB922845	AB922851	Rice leaves (*Oryza sativa*)/Suphan Buri province, Thailand	12 January 2012
DMKU-RP119 (BCC 69495)	AB922846	AB922852	Rice leaves (*Oryza sativa*)/Suphan Buri province, Thailand	12 January 2012
YE124 (BCC 63420)	AB922847	AB922853	Rice leaves (*Oryza sativa*)/Nonthaburi province, Thailand	13 February 2012
YE156 (BCC 63452)	AB922848	AB922854	Rice leaves (*Oryza sativa*)/Suphan Buri province, Thailand	13 February 2012
11-1114	KM206722	KM206725	Unknown composite flower/Bird Island, Belize City, Belize	Unknown
12-301	–	–	Dead fallen leaf of unknown tree/Benque Viejo del Carmen, Belize City, Belize	Unknown
Hannaella phetchabunensis sp. nov.				
DMKU-CP430^T (BCC 69492^T, NBRC 110424^T, CBS 13386^T)	AB922849	AB922855	Corn leaves (*Zea mays*)/Phetchabun Province, Thailand	24 May 2012

YE-156) had identical D1/D2 region sequences of the LSU rRNA gene, and these differed by two nucleotide substitutions from strain DMKU-RP109 and by three nucleotide substitutions from the two strains (11-1114 and 12-301) that had identical sequences. The ITS region sequences of the six strains (DMKU-RP72^T, DMKU-RP119, YE-124, YE-156, 11-1114 and 12-301) were identical and differed from strain DMKU-RP109 by only one nucleotide substitution. In terms of pairwise sequence similarity of the D1/D2 region of the LSU rRNA gene the closest species was *H. zeae*. From the type strain of *H. zeae*, the four strains (DMKU-RP72^T, DMKU-RP119, YE-124 and YE-156) differed by 1.2 % nucleotide substitutions (7 nt substitutions out of 576 nt), strain DMKU-RP109 differed by 1.6 % nucleotide substitutions (9 nt substitutions out of 576 nt), and the two strains (11-1114 and 12-301) differed by 1.7 % nucleotide substitutions (10 nt substitutions out of 576 nt). The sequences of the ITS region of the six strains (DMKU-RP72^T, DMKU-RP119, YE-124, YE-156, 11-1114 and 12-301) were identical and differed to that of strain DMKU-RP109 by only one nucleotide substitution. The sequence difference in the ITS region between the seven strains and *H. zeae* was 3.7 % nucleotide substitutions (16 nt substitutions and three gaps out of 438 nt) for the six strains (DMKU-RP72^T, DMKU-RP119, YE-124, YE-156, 11-1114 and 12-301) and 3.9 % nucleotide substitutions (17 nt substitutions and two gaps out of 438 nt) for strain DMKU-RP109. The strain of the second novel species, strain DMKU-CP430^T, was closest to *H. zeae*, but with 1.0 % nucleotide substitution (6 nt substitutions out of 576 nt) in the D1/D2 region of the LSU rRNA gene. The ITS region sequences of strain DMKU-CP430^T and *H. zeae* differed by 3.2 % nucleotide substitutions (13 nt substitutions and four gaps out of 437 nt). The two novel species differed from each other by 1.2–1.7 % in their nucleotide substitutions (7–10 nt substitutions out of 597 nt) in the D1/D2 region of the LSU rRNA gene and by 5.0–5.3 % nucleotide substitutions (22–23 nt substitutions and three gaps out of 437 nt) in the ITS region.

The phylogenetic tree, based on the combined sequences of the ITS and D1/D2 regions of the LSU rRNA gene, demonstrated further that all eight strains were in the *Hannaella* clade. The four strains (DMKU-RP72^T, DMKU-RP119, YE-124 and YE-156) were located in the same position and connected to strain DMKU-RP109 and strain 11-1114 with high bootstrap support. They clustered with the type strain of *H. zeae*, their most closely related species, with high bootstrap values (Fig. 1). Strain DMKU-CP430^T was also connected to *H. zeae* (Fig. 1).

The novel species delineation was based primarily on the analysis of the combined sequences of the ITS region and D1/D2 region of the LSU rRNA gene, which indicated the reciprocal monophyletic nature of the species in this clade; therefore, a phylogenetic species concept applies. Consequencely, on this basis, we concluded that the eight strains represent two novel species of the genus *Hannaella*. The name *Hannaella siamensis* sp. nov. (MB809601) is proposed for seven of the strains (DMKU-RP72^T, DMKU-RP109, DMKU-119, YE-124, YE-156, 11-1114 and 12-301) and the name *Hannaella phetchabunensis* sp. nov. (MB809602) is assigned to the remaining single strain DMKU-CP430^T.

In practice, *H. siamensis* sp. nov.and *H. phetchabunensis* sp. nov. can be distinguished from their closest species, *H. zeae*, on the basis not only of the sequences of the D1/D2

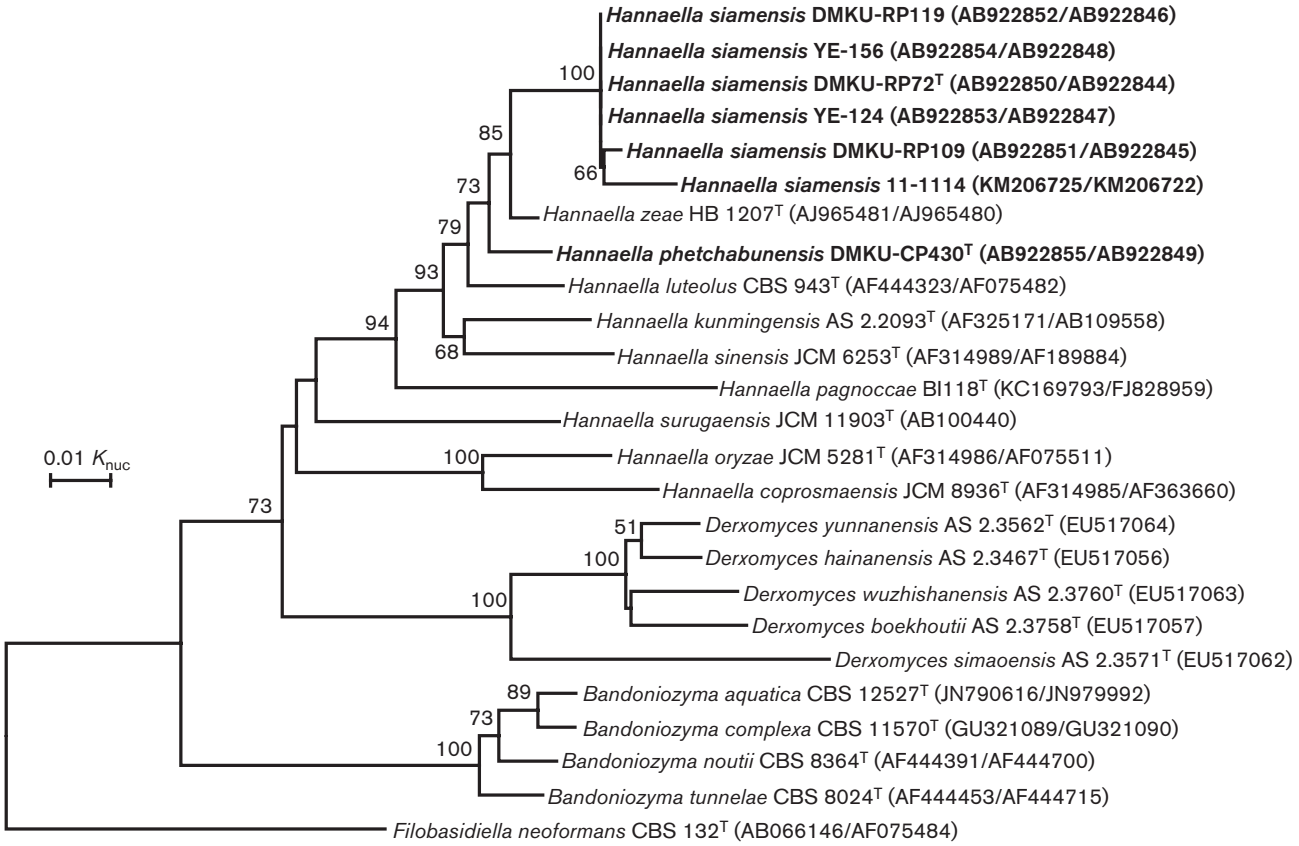


Fig. 1. Phylogenetic tree based on the concatenated sequences of the ITS region and the D1/D2 region of the LSU rRNA gene, showing the positions of *Hannaella siamensis* sp. nov. (DMKU-RP72^T) and *Hannaella phetchabunensis* sp. nov. (DMKU-CP430^T), with respect to closely related species. Numbers indicate percentages of bootstrap sampling, derived from 1000 samples. The numbers in parentheses are GenBank accession numbers. *Filobasidiella neoformans* CBS132^T was the outgroup in the analysis. Bar, 0.01 K_{nuc}.

region of the LSU rRNA gene and the ITS region, but also from some phenotypic characteristics, as shown in Table 2.

Members of the genus *Hannaella* are found in diverse habitats. *H. coprosmaensis*, for example, was isolated from a dead leaf and the fruit of *Coprosma tenuifolia* and *H. kunmingensis* was isolated from a dead leaf of a climbing plant, but some strains were isolated from a snail and a bee. *H. oryzae* was isolated from the surfaces of grasses, such as rice, bamboo and Chinese silver grass, *Hannaella sinensis* var. *sinensis* was isolated from the living and dead leaves of the grasses: rice, Chinese silver grass and wheat. *Hannaella sinensis* var. *lactis* was isolated from a ladybird beetle (Boekhout *et al.*, 2011b), while *H. luteola* was isolated from various habitats, such as air, plant leaves, seawater and acidic sludge; *H. surugaensis* was isolated from deep-sea sediment (Fonseca *et al.*, 2011) and *H. zeae* was isolated from corn and corn pests (Molnár & Prillinger, 2006). Strains of the latest species to be proposed, *H. pagnoccae*, were obtained from plants and soil (Landell *et al.*, 2014). In this study, the six strains were isolated in Thailand from rice and corn leaf surfaces, while two strains were isolated in Belize from a composite flower and a fallen dead leaf. Therefore, plant materials, especially leaf surfaces, appear to be the natural habitats of yeasts in this genus.

Description of *Hannaella siamensis* Kaewwichian, Jindamorakot, Am-In, Sipiczki & Limtong sp. nov.

Hannaella siamensis (si.am.en'sis. N.L. fem. adj. *siamensis* referring to Siam, the old name of Thailand, where the type strain was isolated.)

Growth in YM broth: after 3 days at 25 °C, cells are oval, subglobose, ellipsoidal (3–5 × 5–7 μm) and occur singly, in pairs or in groups (Fig. 2). Budding is polar budding. Growth on YM agar: after 3 days at 25 °C, the streak culture is yellowish-cream, semi-glistening, soft, low convex and has an entire margin. Pseudohyphae and true hyphae are not formed in slide cultures on PDA. Basidiospores are not produced by any of the strains either individually or when paired on YM agar, 5 % malt extract agar, corn meal agar, acetate agar and Gorodkowa agar after 4 weeks at 25 °C. Ballistoconidia are absent. Fermentation of D-glucose,

Table 2. Phenotypic characteristics that differentiate *Hannaella siamensis* sp. nov. and *Hannaella phetchabunensis* sp. nov. from the most closely related species, *H. zeae*

Taxa: 1, *Hannaella siamensis*; 2, *Hannaella phetchabunensis*; 3, *H. zeae*. +, Positive; D, delayed positive; S, slowly positive; W, weak; −, negative.

Characteristics	1	2	3*
Assimilation			
Lactose	S	−	−
Soluble starch	+	+	−
D-Ribose	+	+	D
Ethanol	+	S	+
Ribitol	+	S	+
α-Methyl-D-glucoside	+	S	+
Salicin	+	S	+
DL-Lactic acid	W	S	+
Citric acid	+	+	W
Inositol	+	S	+
Xylitol	+	S	+
D-Glucosamine	+	W	+
Propane-1,2-diol	−−	−	+
Growth in			
Vitamin-free medium	+	+	−
50 % (w/v) glucose	+	+	W
0.01 % (w/v) cycloheximide	W	S	W
10 % (w/v) NaCl + 5 % (w/v) glucose	+	+	−
Amyloid formation	−	−	+

*Data from Molnár & Prillinger (2006).

D-galactose, maltose, sucrose, trehalose, lactose, melezitose, raffinose and xylose are absent. D-Glucose, D-galactose, L-sorbose, sucrose, maltose, cellobiose, trehalose, lactose (slow), melibiose, raffinose, melezitose, soluble starch, D-xylose, L-arabinose, D-arabinose, L-rhamnose, D-ribose, ethanol (weak), glycerol, erythritol, ribitol, galactitol, D-mannitol, D-glucitol, α-methyl-D-glucoside, salicin, 2-keto-D-gluconic acid, 5-keto-D-gluconic acid, D-glucono-δ-lactone, DL-lactic acid (weak), succinic acid, citric acid, inositol, D-glucuronic acid, D-galacturonic acid, xylitol, L-arabinitol, D-gluconic acid, D-glucosamine, *N*-acetyl-D-glucosamine (delay) and propane-1, 2-diol (weak) are assimilated, but methanol, butane-2,3-diol and hexeadecane are not assimilated. Ethylamine, L-lysine and cadaverine are assimilated, but nitrate and nitrite are not assimilated. Growth in vitamin-free medium is positive. Growth on medium containing 50 % (w/v) glucose, 60 % (w/v) glucose, 10 % (w/v) sodium chloride/5 % (w/v) glucose, 15 % (w/v) sodium chloride/5 % (w/v) glucose is positive. Growth with 0.01 % cylclohex-imide is weakly positive, but with 0.1 % cylcloheximide is negative. Growth at 25 and 30 °C is positive, but at 35, 37, 40 and 42 °C is negative. Acid formation is absent. Starch-like compounds are not produced. The diazonium blue B colour and urease reactions are positive. The major ubiquinone is Q-10.

Strain DMKU-RP72^T is the holotype of *Hannaella siamensis* (MB809601). The strain was isolated from the phylloplane of rice (*Oryza sativa*) collected from Nakhon Pathom Province, Thailand.

Description of *Hannaella phetchabunensis* Kaewwichian, Jindamorakot, Am-In & Limtong sp. nov.

Hannaella phetchabunensis (phet.cha.bun.en'sis. N.L. fem. adj. *phetchabunensis* referring to Hetchabun Province, where the strain was isolated.

Growth in YM broth: after 3 days at 25 °C, cells are ovoid to ellipsoid (2–3 × 3–6 μm) and occur singly, in pairs or in groups (Fig. 3). Budding is polar. Growth on YM agar after 3 days at 25 °C: the streak culture is yellowish-cream, semi-glistening, soft, low convex and has an entire margin. Pseudohyphae and true hyphae are not formed in slide cultures on PDA. Basidiospores are not produced on YM

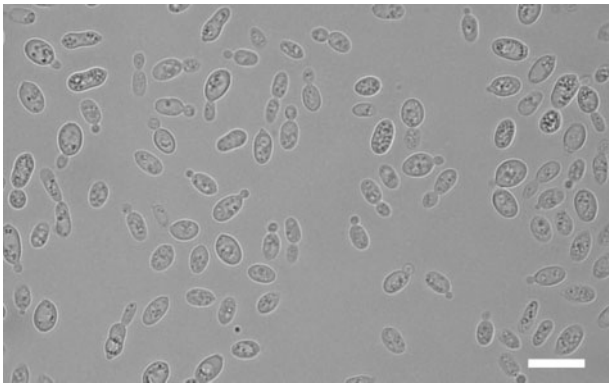


Fig. 2. Micrograph of budding cells of *Hannaella siamensis* sp. nov. (DMKU-RP72^T) in YM broth after 3 days at 25 °C. Bar, 10 μm.

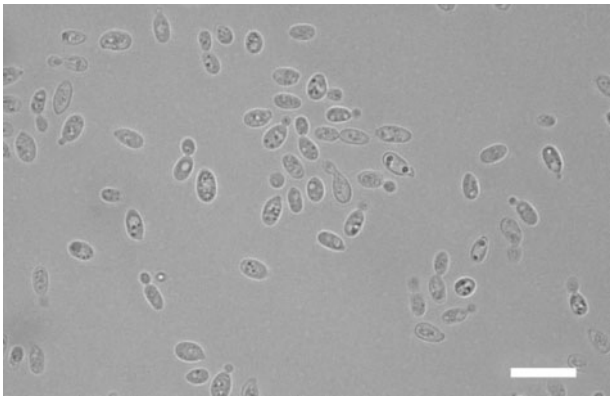


Fig. 3. Micrograph of budding cells of *Hannaella phetchabunensis* sp. nov. (DMKU-CP430^T) in YM broth after 3 days at 25 °C. Bar, 10 μm.

agar, 5 % malt extract agar, acetate agar, corn meal agar or Gorodkowa agar after 4 weeks at 25 °C. Ballistoconidia are absent. The fermentation of D-glucose, D-galactose, sucrose, maltose, lactose, melezitose and raffinose is absent. D-Glucose, D-galactose, L-sorbose, sucrose, maltose, cellobiose, trehalose, melibiose, raffinose, melezitose, soluble starch, D-xylose, L-arabinose, D-arabinose, L-rhamnose, D-ribose, ethanol (slow), glycerol, erythritol, ribitol (slow), galactitol, D-mannitol, D-glucitol, α-methyl-D-glucoside (slow), salicin (slow), 2-keto-D-gluconic acid, 5-keto-D-gluconic acid, D-glucono-δ-lactone, DL-lactic acid (slow), succinic acid, citric acid, inositol (slow), D-glucuronic acid, D-galacturonic acid, xylitol (slow), L-arabinitol, D-gluconic acid, D-glucosamine (weak), and *N*-acetyl-D-glucosamine are all assimilated, but lactose, inulin, methanol, propane-1,2-diol, butane-2,3-diol and hexeadecane are not assimilated. Nitrate is not assimilated. Growth in vitamin-free medium is positive. Growth on medium containing 50 % (w/v) glucose, 10 % (w/v) sodium chloride/5 % (w/v) glucose and 15 % (w/v) sodium chloride/5 % (w/v) glucose (weakly) is positive. Growth with 0.01 % cylcloheximide is slow, but positive; with 0.1 % cylcloheximide it is negative. Growth at 25 and 30 °C is positive, but at 35, 37, 40 and 42 °C is negative. Acid formation is absent. Starch-like compounds are not produced. The diazonium blue B colour and urease reactions are positive. The major ubiquinone is Q-10.

Strain DMKU-CP430^T is the holotype of *Hannaella phetchabunensis* (MB809602). The strain was isolated from the phylloplane of corn (*Zea mays*) collected from Phetchabun Province, Thailand.

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ORIGINAL PAPER

Assessment of epiphytic yeast diversity in rice (*Oryza sativa*) phyllosphere in Thailand by a culture-independent approach

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Abstract The epiphytic yeast diversity in rice phyllosphere in Thailand was investigated by a culture-independent technique based on the RFLP pattern and the sequence of the D1/D2 domain of the large subunit rRNA gene. Forty-four samples of rice leaf were collected randomly from six provinces. The DNA was extracted from leaf washing samples and the D1/D2 domain was amplified using PCR technique. The PCR products were cloned and then screened by colony PCR. Of total 1121 clones, 451 clones (40.2 %) revealed the D1/D2 domain sequences closely related to sequences of yeasts in GenBank, and they were clustered into 45 operational taxonomic units (OTUs) at 99 % homology. Of total yeast related clones, 329 clones (72.9 %) were identified as nine known yeast species, which consisted of 314 clones (8 OTUs) in the phylum Basidiomycota including *Bullera japonica*, *Pseudozyma antarctica*, *Pseudozyma aphidis*, *Sporobolomyces blumeae*, *Sporobolomyces carnicolor* and *Sporobolomyces oryzicola* and 15 clones (6 OTUs) in the phylum Ascomycota including *Metschnikowia koreensis*, *Meyerozyma guilliermondii* and *Wickerhamomyces anomalus.* The D1/D2 sequences (122 clones) that could not be identified as known yeast species were closest to 3 and 14 species in Ascomycota and Basidiomycota, respectively, some of which may be new yeast species. The most predominant species detected was *P. antarctica* (42.6 %) followed by *B. japonica* (25.9 %) with 63.6 and 22.7 % frequency of occurrence, respectively. The results of OTU richness of each sampling location revealed that climate condition and sampling location could affect epiphytic yeast diversity in rice phyllosphere.

Keywords Epiphyte · Yeast · Phyllosphere · Rice · rRNA gene

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Introduction

Rice is a monocotyledonous plant of the family Poaceae. Rice is one of the most widely produced and consumed foods in the world, making its

cultivation of vital global social and economic importance. In Thailand, rice (*Oryza sativa*) is the main economic crop which is mostly cultivated in north, northeast and central Thailand. During the period 2013–2014 the rice cultivation area was approximately 12.64 million hectares and grain production approximately 38.24 million tons as reported by the Department of Rice, Ministry of Agriculture and Cooperative, Thailand.

Phyllosphere or phylloplane describes the above ground surfaces of the plant as habitat for microorganisms; the phyllosphere is dominated by the leaves. The total surface area available for microbial colonization has been estimated to be about 2–6 $\times$ 10^8 km^2 (Morris 2001; Lindow and Brandl 2003). Microbial epiphytes including bacteria, yeasts and filamentous fungi can inhabit the phyllosphere by using leaf surface nutrients as carbon sources including carbohydrates, amino acids, organic acids, sugar alcohols and salts (Tukey 1970; Fiala et al. 1990; Weibull et al. 1990; Dik et al. 1991; Leveau and Lindow 2001). In addition, the epiphytes have been reported to produce plant hormones which promote cell wall loosening and release of saccharides from plant cells as substrates (Fry 1989; Brandl and Lindow 1998; Lindow and Brandl 2003). Furthermore, it has been reported that epiphytes may produce biosurfactants which facilitate wettability and further enhance the leaching of substrates (Bunster et al. 1989; Schreiber et al. 2005). The abundance of available nutrients is dependent on leaf age, plant species and growing conditions all of which shape microbial diversity in the phyllosphere. Moreover, environmental factors including radiation, pollution and nitrogen fertilization, plus biotic factors including leaf age and competing microorganisms all affect microbial diversity (Irvine et al. 1978; Jumpponen and Jones 2010; Zimmerman and Vitousek 2012).

The diversity of microorganisms in the environment has been evaluated by culture-dependent methods, however a major obstacle has been unculturable or slow growing strains which may lead to exclusion of important members of the composting microbial community (Amann et al. 1995; Takaku et al. 2006). Therefore recently culture-independent methods have been applied to more accurately elucidate microbial diversity (Su et al. 2012). Over the past 20 years, molecular ecological methods based on the direct amplification and analyses of ribosomal RNA genes have been developed for investigations of microbial communities in diverse environments such as denaturing gradient gel electrophoresis (DGGE) (Muyzer 1999; Liu et al. 2002), temperature gradient gel electrophoresis (TGGE) (Muyzer 1999), restriction fragment length polymorphisms (RFLP) (Esteve-Zarzoso et al. 1999), terminal restriction fragment length polymorphisms (T-RFLP) (Schutte et al. 2008), random amplified polymorphic DNA (RAPD) (Chiocchetti et al. 1999), amplified fragment length polymorphism (AFLP) (de Barros Lopes et al. 1999), quantitative PCR (qPCR) (Hierro et al. 2006) single-strand-conformation polymorphism (SSCP) (Peters et al. 2000) and capillary electrophoresis-single strand conformation polymorphism (CE-SSCP) (Martins et al. 2014). Meanwhile, alternative molecular techniques such as fluorescence in situ hybridization (FISH) (Moter and Gobel 2000) and micro arrays (Bodrossy and Sessitsch 2004) have successfully been used for the identification of environmental microorganisms. Moreover, the high-throughput sequencing in combination with DNA tagging were utilized efficiently in evaluation of fungal communities (Jumpponen and Jones 2009).

The most numerous microorganisms colonising the phyllosphere are bacteria. However yeast and filamentous fungi are often considered active inhabitants of leaf surfaces (Vorholt 2012). Most studies identifying microorganisms in the phyllosphere have focused on bacteria. In recent years the diversity of yeast communities in the phyllosphere has been studied intensively, however most of which were culture-dependent. Additionally, only a small number of articles have focused on yeasts that colonize the phyllosphere of monocotyledonous plants (Nakase et al. 2001; Limtong and Kaewwichian 2014) and very few of those investigating the diversity of epiphytic yeasts from the phyllosphere were undertaken with samples from Thailand. Therefore, this study is the first research aimed at investigating the diversity of epiphytic yeasts of rice phyllosphere in Thailand by using a culture-independent technique.

Materials and methods

Sample collection

Forty-four samples of rice (*Oryza sativa*) leaf were randomly collected from rice fields in six provinces in Thailand between October 2011 and March 2012

(Table 1). Leaf samples were put in plastic bags, sealed and kept in an ice-box before transfering to the laboratory. The samples were stored at 4 °C until used. DNA was extracted from the leaf washing of the samples within a maximum of 7 days post collection.

DNA extraction of epiphytes

Leaf samples (6 g) were cut and submerged in washing buffer (1X Phosphate buffered saline, Tween 20), sonicated for 7 min using ultrasonic cleaning bath (Bransonic, USA) to dislodge microbes from sample surfaces. The leaf washing was centrifuged at 5000×*g* for 5 min and the supernatant was discarded. For rapid extraction of DNA from cell pellets the method of Makimura et al. (Makimura et al. 1994) with slight modification was performed. Briefly, the cell pellet was suspended in 100 µl of lysis buffer (100 mM Tris–HCl, pH 7.5, 0.5 % w/v SDS, 30 mM EDTA) and incubated at 95 °C for 15 min. A 100 µl of 2.5 M potassium acetate was added and incubated on ice for 60 min, then centrifuged at 12,000×*g* for 5 min. The supernatant was transfered to new tube and DNA precipitated with an equal volume of isopropanol, then washed with 70 % ethanol. The precipitated DNA was air dried, resuspended in 30 µl of TE buffer (10 mM Tris–Cl, 1 mM EDTA, pH 8.0) and stored at −20 °C.

PCR amplification of D1/D2 domain of large subunit (LSU) rRNA gene

PCR amplification of D1/D2 domain of LSU rRNA gene was performed using the following primers: NL-1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and NL-4 (5′-GGT CCG TGT TTC AAG ACG G-3′) (Kurtzman and Robnett 1998). The amplification was carried out in 25 µl of the reaction mixture containing 10–100 ng of genomic DNA, 1X PCR buffer (Vivantis, USA), 1.5 mM $MgCl_2$ (Vivantis, USA), 100 µM of each dNTP (Vivantis, USA), 20 pmol of each primer, and 1 U of *Taq* DNA polymerase (Vivantis, USA). The PCR reactions were performed on a Geneamp PCR system 2400 (Perkin Elmer, USA). The reaction conditions were 94 °C for 5 min; 35 cycles of 45 s at 94 °C, 30 s at 52 °C, and 45 s at 72 °C; and a final extension at 72 °C for 10 min. PCR products with expected size of 600 bp were visualized on 1 % agarose gel (w/v) and purified by Nucleic Acid Extraction Kit (Vivantis, USA).

PCR cloning and screening

The purified PCR products were ligated into pTG19-T cloning vector (Vivantis, USA) performed using manufacturer's recommended protocol, and transformed into *E.coli* DH5α competent cells. The recombinant clones were screened by colony PCR using the primers described above. The reaction mixture was manipulated as described above except that cell suspension (2 µl) was used as template. The reaction conditions were 94 °C for 10 min; 35 cycles of 45 s at 94 °C, 30 s at 52 °C, and 45 s at 72 °C; and a final extension at 72 °C for 10 min. PCR products with expected size of 600 bp were visualized on 1 % agarose gel (w/v).

RFLP analyses

PCR products of the expected size were analyzed using RFLP after enzymatic digestion with the

Table 1 Rice leaf samples collected from six provinces in Thailand

Region	Province	Location	Sampling period	No. of samples	Average atmospheric temperature (°C)	Average rainfall (mm.)
East	Chachoengsao	13°41′15″N, 101°4′13″E	Dec 2011	6	28.1	3.4
Central	Nakhon Nayok	14°12′44″N, 101°12′6″E	Dec 2011	3	25.1	0.8
	Nakhon Pathom	13°49′14″N, 100°3′45″E	Jan 2012	13	27.3	19.9
	Suphanburi	14°28′3″N, 100°7′1″E	Mar 2012	9	30.0	29.9
North-East	Sisaket	15°7′12″N, 104°19′18″E	Oct 2011	10	26.4	182.5
	Surin	14°52′48″N, 103°29′24″E	Oct 2011	3	26.4	182.5

restriction enzymes, *Hae*III, *Hin*fI and *Cfo*I. The restriction fragment patterns were visualized using a 2 % agarose gel (w/v). The RFLP patterns were clustered and the representatives of the PCR products of each pattern from each clone libraries were further purified and sequenced.

Sequencing and phylogenetic analyses

The DNA sequencing was performed by Sanger dideoxy sequencing with four colour fluorescence using NL1 primer and an ABI Prism 3730XL DNA sequencer (Applied Bioscience, USA) at the DNA sequencing services, Bio Basic Inc. (Ontario, Canada). The sequences of the D1/D2 domain were sumitted to BLASTn homology search (Altschul et al. 1997). The collection of taxonomically related type sequences and related matches were obtained from GenBank for further analyses. The sequences were aligned with related species using CLUSTALW (Thompson et al. 1994). The GenBank accession numbers of related yeast type strain sequences are AF444760 (*Bullera japonica*), AF075525 (*Cryptococcus cellulolyticus*), AF189834 (*Cryptococcus curvatus*), AB035042 (*Cryptococcus flavescens*), AF075497 (*Cryptococcus flavus*), DQ00317 (*Cryptococcus heimaeyensis*), AF075525 (*Cryptococcus laurentii*), AF189884 (*Hannaella sinensis*), AJ965480 (*Hannaella zeae*), AF189977 (*Sporidiobolus pararoseus*), AB279628 (*Sporobolomyces carnicolor*), AF189990 (*Sporobolomyces oryzicola*), AJ235302 (*Pseudozyma antarctica*), AB089363 (*Pseudozyma aphidis*), DQ008953 (*Pseudozyma hubeiensis*), AF296438 (*Metschnikowia koreensis*), U45709 (*Meyerozyma guilliermondii*) and U74592 (*Wickerhamomyces anomalus*).

The recombinant clones of the D1/D2 domain sequences were clustered into operational taxonomic units (OTUs) at 99 % homology using the furthest neighbour approach of mothur v.1.10 (Schloss et al. 2009). The sequence analysis regarding to the number of the nucleotide substitution compared to the D1/D2 sequences of yeast type strains were also considered (Kurtzman and Robnett 1998; Fell et al. 2000).

A phylogenetic tree was constructed from the evolutionary distance data using Kimura 2-parameter model (Kimura 1980) and the neighbor-joining method (Saitou and Nei 1987) performed with MEGA 6 (Tamura et al. 2013). All positions containing gaps were eliminated. Confidence level of the clades was estimated using bootstrap analysis (1000 replicates).Yeast related sequence data have been submitted to the GenBank database under accession numbers KJ937682, KJ937684-KJ937698, KJ937700-KJ937728, KJ937730-KJ937750, KJ937752-KJ937781, KM078919-KM078921 and KM999142-KM999219.

Data analyses

Diversity indices were calculated using the Shannon–Wiener index (H') and Shannon equitability (E_H);

$$H' = -\sum_{i=0}^{S} P_i(\ln P_i)$$

where P_i is the ratio of the number of clones of the ith OTU to the total number of clones.

$$E_H = H'/\ln S$$

where S is the total number of OTUs in the total samples of rice phyllosphere.

Equitability assumes a value between 0 and 1. A value close to 1 means that the sample is diverse, while a value close to 0 means that the sample is not diverse.

Relative frequency (%) was calculated as the number of clones of a particular OTU as a propotion of the total number of clones.

Frequency of occurance (%) was calculated as the number of samples, where a particular OTU was observed, as a propotion of the total number of samples.

Statistical data analyses

Comparisons of multiple means with standard deviations (SDs) obtained from OTU richness, the number of OTUs of each sample, in association with different provinces were performed by StatPlus v2009 (AnalystSoft Inc., USA), with one-way analysis of variance (ANOVA) and Fisher LSD tests at appropriate significant level of $P = 0.05$ or lower.

Results and discussion

Yeast identification and phylogenetic analyses

Genomic DNA from the cell pellets of leaf washings of 44 samples of rice phyllospheres was successfully

isolated. The amplified PCR products of the D1/D2 domain of LSU rRNA gene were cloned. A total of 1121 clones were obtained from 44 clone libraries. The representative sequences (621 sequences) of each library as a result of restriction analysis were sequenced. Among 1121 clones, 53.3 % (405 sequences, 598 clones) were related to filamentous fungi, 40.2 % (176 sequences, 451 clones) were related to yeasts and 6.4 % (40 sequences, 72 clones) were of unknown origin, the sequences with no or low significant similarly found in database. For the purposes of this study we focused on yeasts present in the rice phyllosphere samples.

Ascomycetous yeasts were identified by analysis of areas of homology for the D1/D2 domain of LSU rRNA gene sequences and those of type strains. Sequences showing greater than 1 % nucleotide substitutions (6 nucleotides) in the ca. 600-nucleotide D1/D2 domain are likely to be of different species and that strains with 0-3 nucleotide differences are either conspecific or sister species (Kurtzman and Robnett 1998). According to the suggestion of Fell et al. (2000) for basidiomycetous yeasts, strains that differ by two or more nucleotides in the D1/D2 region, represent different taxa. Therefore to identify yeasts species present in this study the numbers of nucleotide substitutions within the D1/D2 domain were also considered according to ascomycetous and basidiomycetous yeast identification suggested above.

A total of 176 sequences of 451 clones related to yeasts were classified into 45 OTUs by 99 % similarity cut off. The sequences under the same OTUs that had different number of nucleotide substitutions were distinguished and identified (Table 2). The basidiomycetous yeasts (96 %) were the major components of the clone libraries, in contrast to only 4 % of the ascomycetous yeasts. The total clones were classified into 4 yeast subphylum, three were under phylum Basidiomycota (Agaricomycotina, Pucciniomycotina and Ustilaginomycotina) and one under phylum Ascomycota (Saccharomycotina). The majority of yeast related clones were in subphylum Ustilaginomycotica (60.0 %) followed by Agaricomycotina (33.7 %). The number of clones classified in Saccharomycotina and Pucciniomycotina were only 4.0 and 2.2 %, respectively. As can be seen, some sequences under the same OTUs had different identification results according to the number of nucleotide substitutions (see OTU1, OTU23, OTU31). The sequences that differ by more than 1 nucleotide substitution in the case of basidiomycetous yeasts and 6 nucleotide substitutions in the ascomycetous yeasts were treated as different species in this study. 329 out of 451 clones (72.9 %) were precisely identified into nine known yeast species in six genera. Of these total, 314 clones (under 8 OTUs) were identified as basidiomycetous yeasts including *B. japonica, P. antarctica, P. aphidis, Sporobolomyces blumeae*, *S. carnicolor* and *S. oryzicola*. Other 15 clones (under 6 OTUs) were identified as known ascomycetous yeast species including *M. koreensis*, *M. guilliermondii* and *W. anomalus*.

The sequences (119 clones) of basidiomycetous yeasts that could not have been identified to species level regarding to the substitution numbers were closest to 14 yeast species. These included *B. japonica*, *C. cellulolyticus*, *C. curvatus*, *C. flavescens*, *C. flavus*, *C. heimaeyensis*, *C. laurentii*, *H. sinensis*, *Hannaela zeae*, *P. antarctica*, *P. aphidis*, *P. hubeiensis*, *S.pararoseus* and *S. carnicolor*. For their identification to species level further study such as ITS sequence analysis is needed. Some of these sequences may represent the sequences of new yeast taxa. The sequences (3 clones) of ascomycetous yeasts which differed by more than 6 nucleotide substitutions represented new yeast species closest to *M. koreensis*, *M. guilliermondii* and *W. anomalus*.

The most frequently detected OTU from the clone libraries was OTU23 (47.2 %, 213 clones) closest to *P. antarctica* followed by OTU1 (27.5 %, 124 clones) closest to *B. japonica* and OTU31 (8.2 %, 37 clones) closest to *P. aphidis* (Table 2). Other OTUs were detected within the clone libraries but showed low relative frequencies (0.2–1.8 %). The relative frequencies of the total number of clones from all OTUs identified as *P. antarctica* and *B. japonica* were 42.6 % (192 clones) and 25.9 % (117 clones), respectively.

Phylogenetic trees based on analysis of the D1/D2 domain sequences of subphylum Agaricomycotina (Fig. 1), Pucciniomycotina (Fig. 2), Ustilaginomycotina (Fig. 3) and Saccharomycotina (Fig. 4) were constructed. The phylogenetic tree of OTUs in the subphylum Agaricomycotina illustrated that the sequences with high number in substitutions (differ by 6 nucleotides) such as OTU3, OTU4, OTU5, OTU6, OTU7, OTU8, OTU9, OTU11, OTU12, OTU13, OTU15, OTU16, OTU19 and OTU20 were confirmed of long distance relation to the closest strains. These

Table 2 Yeast identification for 45 OTUs based on BLAST analysis and their frequency of occurrence (%) from rice phyllospheres

OTU	No. of clones	Closest yeast species	Nucleotide substitution (gap)[a]	Identity (%)	Identification	Frequency of occurrence (%)
Phylum Basidiomycota						
Subphylum Agarinomycotina						
1	40	*Bullera japonica* CBS 2013^T (AF444760)	0 (0–2)	99.7–100	*Bullera japonica*	13.6
	77	*Bullera japonica* CBS 2013^T (AF444760)	1 (0–3)	99.5–99.8	*Bullera japonica*	18.2
	3	*Bullera japonica* CBS 2013^T (AF444760)	2 (0, 3)	99.1–99.6	Closest to *Bullera japonica*	6.8
	4	*Bullera japonica* CBS 2013^T (AF444760)	3 (1)	99.1–99.3	Closest to *Bullera japonica*	4.5
2	1	*Bullera japonica* CBS 2013^T (AF444760)	4 (0)	99.3	Closest *to Bullera japonica*	2.3
3	1	*Bullera japonica* CBS 2013^T (AF444760)	19 (4)	96.1	Closest to *Bullera japonica*	2.3
4	1	*Bullera japonica* CBS 2013^T (AF444760)	20 (1)	96.4	Closest to *Bullera japonica*	2.3
5	1	*Bullera japonica* CBS 2013^T (AF444760)	35 (10)	92.0	Closest to *Bullera japonica*	2.3
6	1	*Cryptococcus cellulolyticus* CBS 8294^T (AF075525)	52 (4)	90.1	Closest to *Cryptococcus cellulolyticus*	2.3
7	1	*Cryptococcus curvatus* CBS570^T (AF189834)	6 (2)	98.6	Closest to *Cryptococcus curvatus*	2.3
	1	*Cryptococcus curvatus* CBS570^T (AF189834)	7 (2)	98.5	Closest to C*ryptococcus curvatus*	2.3
	1	*Cryptococcus curvatus* CBS570^T (AF189834)	8 (1)	98.5	Closest to *Cryptococcus curvatus*	2.3
8	2	*Cryptococcus curvatus* CBS570^T (AF189834)	6 (1–2)	98.6	Closest to *Cryptococcus curvatus*	2.3
9	1	*Cryptococcus flavescens* CBS 942^T (AB035042)	3 (1)	99.3	Closest to *Cryptococcus flavescens*	2.3
10	8	*Cryptococcus flavus* CBS 331^T (AF075497)	3 (0–1)	99.1–99.5	Closest to *Cryptococcus flavus*	9.1
11	1	*Cryptococcus heimaeyensis* CBS 8933^T (DQ00317)	48 (11)	89.8	Closest to *Cryptococcus heimaeyensis*	2.3
12	2	*Cryptococcus laurentii* CBS139^T (AF075525)	23 (10)	94.4	Closest to *Cryptococcus laurentii*	4.5
13	1	*Hannaella sinensis* CBS7238^T(AF189884)	50 (15)	88.6	Closest to *Hannaella sinensis*	2.3
14	1	*Hannaella zeae* HB 1207^T (AJ965480)	7 (0)	98.8	Closest to *Hannaella zeae*	2.3
	1	*Hannaella zeae* HB 1207^T (AJ965480)	8 (0)	98.6	Closest to *Hannaella zeae*	2.3
	1	*Hannaella zeae* HB 1207^T (AJ965480)	9 (1)	98.2	Closest to *Hannaella zeae*	2.3
15	1	*Hannaella zeae* HB 1207^T (AJ965480)	53 (6)	88.7	Closest to *Hannaella zeae*	2.3
16	1	*Hannaella zeae* HB 1207^T (AJ965480)	54 (13)	87.7	Closest to *Hannaella zeae*	2.3

Table 2 continued

OTU	No. of clones	Closest yeast species	Nucleotide substitution (gap)[a]	Identity (%)	Identification	Frequency of occurrence (%)
Subphylum Pucciniomycotina						
17	1	*Sporidiobolus pararoseus* CBS491^T (AF189977)	3 (2)	99.1	Closest to *Sporidiobolus pararoseus*	2.3
18	3	*Sporidiobolus pararoseus* CBS491^T (AF189977)	5 (0–1)	98.9–99.1	Closest to *Sporidiobolus pararoseus*	2.3
19	1	*Sporobolomyces blumeae* CBS 9094^T (AB279628)	0 (0)	100	*Sporobolomyces blumeae*	2.3
20	2	*Sporobolomyces carnicolor* CBS 4215^T (AY070008)	1 (0)	99.8	*Sporobolomyces carnicolor*	2.3
	1	*Sporobolomyces carnicolor* CBS 4215^T (AY070008)	2 (0)	99.6	Closest to *Sporobolomyces carnicolor*	2.3
21	1	*Sporobolomyces carnicolor* CBS 4215^T (AY070008)	5 (1)	98.8	Closest to *Sporobolomyces carnicolor*	2.3
22	1	*Sporobolomyces oryzicola* CBS7228^T (AF189990)	0 (1)	99.7	*Sporobolomyces oryzicola*	2.3
Subphylum Ustilaginomycotina						
23	117	*Pseudozyma antarctica* CBS 214.83^T (AJ235302)	0 (0–2)	99.6–100	*Pseudozyma antarctica*	54.5
	70	*Pseudozyma antarctica* CBS 214.83^T (AJ235302)	1 (0–2)	99.5–99.8	*Pseudozyma antarctica*	34.1
	21	*Pseudozyma antarctica* CBS 214.83^T (AJ235302)	2 (0–2)	99.3–99.6	Closest to *Pseudozyma antarctica*	25.0
	5	*Pseudozyma antarctica* CBS 214.83^T (AJ235302)	3 (0–3)	98.9–99.3	Closest to *Pseudozyma antarctica*	6.8
24	1	*Pseudozyma antarctica* CBS 214.83^T (AJ235302)	1 (17)	96.7	*Pseudozyma antarctica*	2.3
25	4	*Pseudozyma antarctica* CBS 214.83^T (AJ235302)	1 (2)	99.5	*Pseudozyma antarctica*	2.3
26	1	*Pseudozyma antarctica* CBS 214.83^T (AJ235302)	2 (0)	99.6	Closest to *Pseudozyma antarctica*	2.3
27	1	*Pseudozyma antarctica* CBS 214.83^T (AJ235302)	2 (2)	99.3	Closest to *Pseudozyma antarctica*	2.3
28	1	*Pseudozyma antarctica* CBS 214.83^T (AJ235302)	5 (1)	99.0	Closest to *Pseudozyma antarctica*	2.3
29	1	*Pseudozyma antarctica* CBS 214.83^T (AJ235302)	7 (0)	98.9	Closest to *Pseudozyma antarctica*	2.3
30	2	*Pseudozyma antarctica* CBS 214.83^T (AJ235302)	7 (1)	98.6	Closest to *Pseudozyma antarctica*	2.3
31	1	*Pseudozyma aphidis* JCM 10318 (AB089363)	1 (0)	99.8	*Pseudozyma aphidis*	2.3
	21	*Pseudozyma aphidis* JCM 10318 (AB089363)	2 (1)	99.5	Closest to *Pseudozyma aphidis*	4.5
	5	*Pseudozyma aphidis* JCM 10318 (AB089363)	3 (0, 2)	99.1–99.4	Closest to *Pseudozyma aphidis*	6.8
	10	*Pseudozyma aphidis* JCM 10318 (AB089363)	4 (1)	99.1	Closest to *Pseudozyma aphidis*	4.5
32	1	*Pseudozyma aphidis* JCM 10318^T (AB089363)	6 (1)	98.8	Closest to *Pseudozyma aphidis*	2.3

Table 2 continued

OTU	No. of clones	Closest yeast species	Nucleotide substitution (gap)[a]	Identity (%)	Identification	Frequency of occurrence (%)
33	4	*Pseudozyma aphidis* JCM 10318^T (AB089363)	41 (5)	91.8	Closest to *Pseudozyma aphidis*	2.3
34	3	*Pseudozyma aphidis* JCM 10318^T (AB089363)	44 (7)	91.0	Closest to *Pseudozyma aphidis*	2.3
35	1	*Pseudozyma aphidis* JCM 10318^T (AB089363)	45 (3)	91.4	Closest to *Pseudozyma aphidis*	2.3
36	1	*Pseudozyma hubeiensis* CBS 10077^T (DQ008953)	4 (1)	99.2	Closest to *Pseudozyma hubeiensis*	2.3
Phylum Ascomycota						
Subphylum Saccharomycotina						
37	1	*Metschnikowia koreensis* KCTC 7998T (AF296438)	1 (2)	99.4	*Metschnikowia koreensis*	2.3
38	1	*Metschnikowia koreensis* KCTC 7998T (AF296438)	1 (1)	99.6	*Metschnikowia koreensis*	2.3
39	1	*Metschnikowia koreensis* KCTC 7998T (AF296438)	13 (2)	97.0	Closest to *Metschnikowia koreensis*	2.3
40	3	*Meyerozyma guilliermondii* NRRL Y-2075T (U45709)	2 (1)	99.5	*Meyerozyma guilliermondii*	2.3
	1	*Meyerozyma guilliermondii* NRRL Y-2075T (U45709)	3 (1)	99.3	*Meyerozyma guilliermondii*	2.3
41	1	*Meyerozyma guilliermondii* NRRL Y-2075T (U45709)	17 (4)	95.9	Closest to *Meyerozyma guilliermondii*	2.3
42	1	*Wickerhamomyces anomalus* NRRL Y-366T (U74592)	0 (1)	99.8	*Wickerhamomyces anomalus*	2.3
	1	*Wickerhamomyces anomalus* NRRL Y-366T (U74592)	1 (1)	99.6	*Wickerhamomyces anomalus*	2.3
	3	*Wickerhamomyces anomalus* NRRL Y-366T (U74592)	2 (1)	99.5	*Wickerhamomyces anomalus*	4.5
43	3	*Wickerhamomyces anomalus* NRRL Y-366T (U74592)	3 (1)	99.3	*Wickerhamomyces anomalus*	2.3
44	1	*Wickerhamomyces anomalus* NRRL Y-366T (U74592)	3 (2)	99.1	*Wickerhamomyces anomalus*	2.3
45	1	*Wickerhamomyces anomalus* NRRL Y-366T (U74592)	20 (6)	95.3	Closest to *Wickerhamomyces anomalus*	2.3

[a] Number of nucleotides that does not match with the closest yeast sequence subtract with number of gaps

sequences may represent the sequences of new yeast taxa. Other OTUs with lower numbers of substitutions such as OTU1, OTU2, OTU9 and OTU10 with 2-4 substitutions were closely related to the type strains seen in the phylogenetic tree however OTUs could not have been identified by only one region in DNA. The similar interpretation could be described for the phylogenetic results in subphylum Pucciniomycotina (Fig. 2), Ustilaginomycotina (Fig. 3) and Saccharomycotina (Fig. 4).

Frequency of occurrence and diversity of epiphytic yeasts

The frequency of occurrence of yeast OTUs within the 44 rice samples (Table 2) showed that OTU23, which was identified as *P. antarctica*, was commonly found in rice samples followed by OTU1 identified as *B. japonica*. Other OTUs occurred but at lower frequencies of occurrence. Of the yeast species detected *P. antarctica* and *B. japonica* were the most common

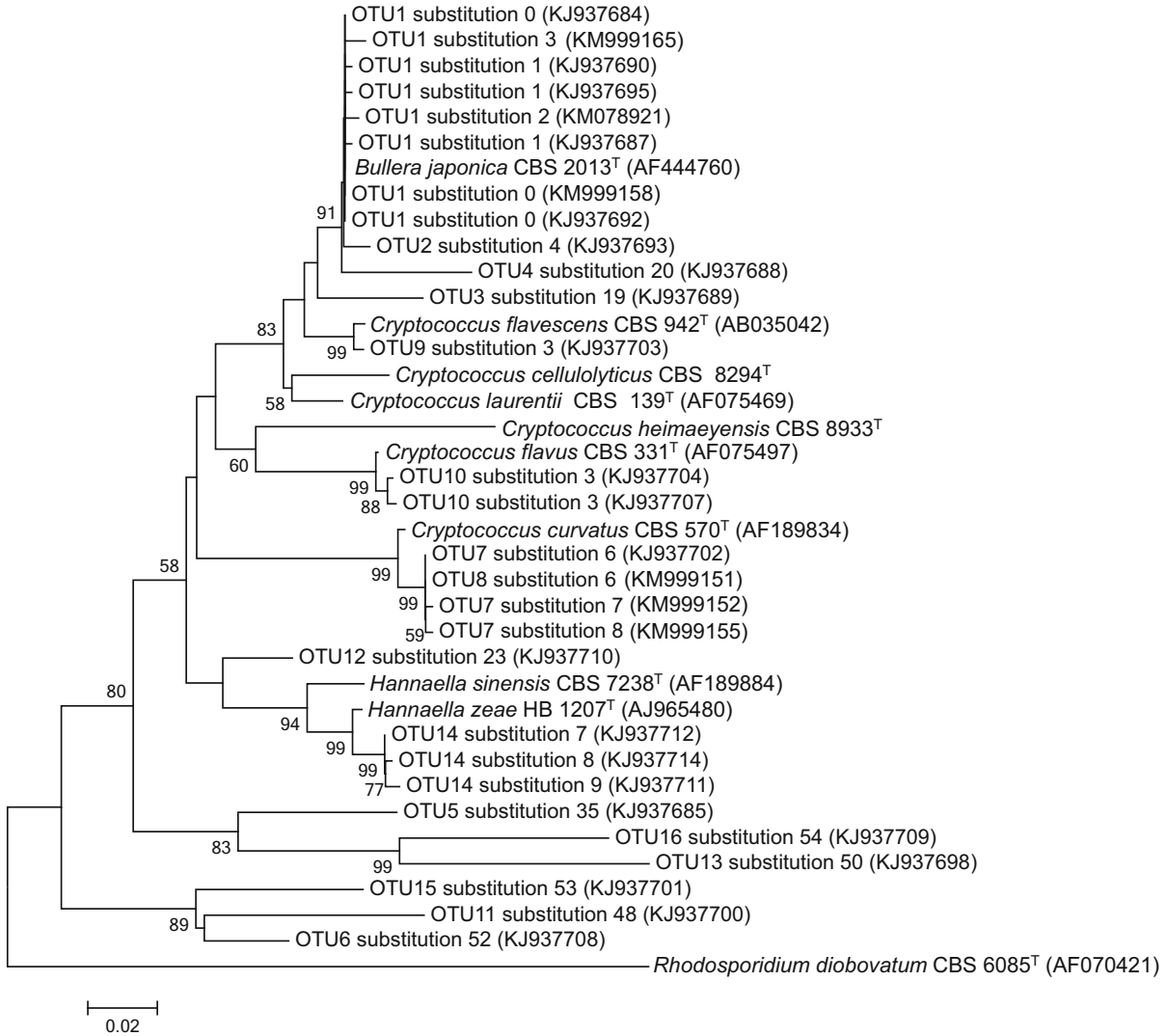


Fig. 1 Phylogenetic tree calculated from neighbor-joining of OTU sequences in subphylum Agarinomycotina (D1/D2 domain, LSU rRNA gene) and reference sequences retrieved from the GenBank database using Kimura 2-parameter model. Numbers on branches are the bootstrap percentage (>50 %) from 1000 random replications. The *scale bar* corresponds to a genetic distance of 0.02 substitutions per position

with 63.6 % (28 samples) and 22.7 % (10 samples) frequencies of occurrence, respectively. Other species were less frequently detected with less than 10 % frequency of occurrence.

The average OTU richness values ranged from 1 to 3 over 6 provinces (Fig. 5). This figure showed that there were significant differences between the values of OTU richness for samples collected in different provinces. The OTU richness of the samples collected in Chachoeangsao (CC, 1.00 ± 0.89) was lowest and was significantly different from those of Surin (SR, 3.67 ± 1.00, $P = 0.007$), Nakhon Nayok (NN, 3.00 ± 1.00, $P = 0.039$) and Nakhon Pathom (NP, 3.08 ± 1.71, $P = 0.003$) except Suphanburi (SP, 2.11 ± 1.05, $P = 0.89$) and Sisaket (SK, 1.5 ± 1.08, $P = 0.312$) which showed no significant difference. In addition, those of the samples collected in Sisaket was also significantly different from those of Surin ($P = 0.030$) and Nakhon Pathom ($P = 0.018$). These results revealed that the OTU richness of the rice samples collected in Chachoeangsao and Sisaket provinces were significantly lower than other provinces. These could

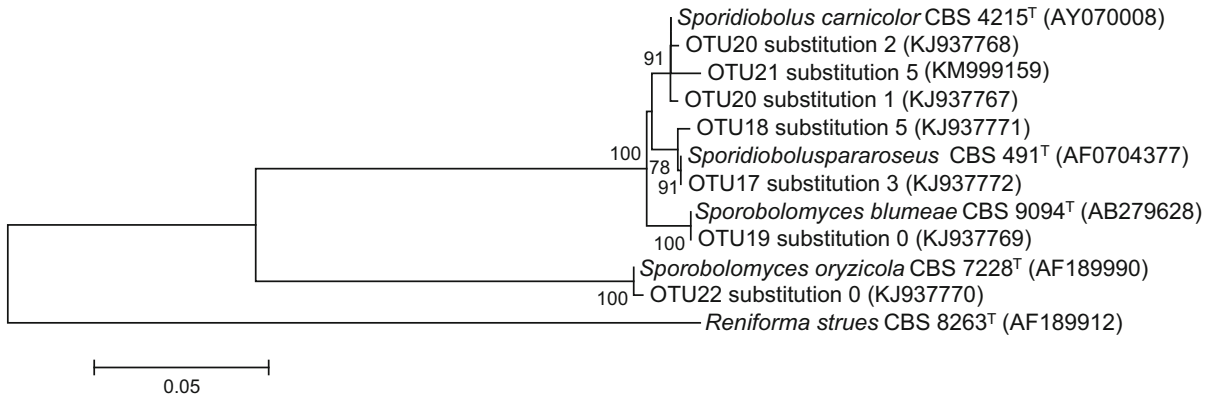


Fig. 2 Phylogenetic tree calculated from neighbor-joining of OTU sequences in subphylum Pucciniomycotina (D1/D2 domain, LSU rRNA gene) and reference sequences retrieved from the GenBank database using Kimura 2-parameter model. Numbers on branches are the bootstrap percentage (>50 %) from 1000 random replications. The *scale bar* corresponds to a genetic distance of 0.05 substitutions per position

suggest that the richness of yeast strains varied by sampling locations. However, the inter-provincial variation was not the focus of this study.

The diversity index and equitability of yeast evaluated in this study were calculated based on the identification results according to nucleotide substitution numbers. Therefore the sequences that could not be identified at the species level were distinguished as different genotypes in those particular OTUs. Thus, we could separate these sequences into 57 genotypes. The Shannon–Weiner index and equitability of yeast genotypes detected in rice samples were 2.191 and 0.542, respectively, as shown in Table 3.

Discussion

This is the first study that we are aware of that has used a culture-independent molecular approach to determine the diversity of epiphytic yeasts in rice (*Oryza sativa*) phyllosphere in Thailand. The basidiomycetous yeasts were the major components of the clone libraries in our study. These results for rice are in accordance with a previous study in Moscow by Glushakova and Chernov (2010), which reported that the number of basidiomycetous yeasts were greater than those of ascomycetous yeasts on the phyllosphere of plants. This study was performed using a culture-dependent technique and yeasts were identified based on morphological, physiological characteristics and the analysis of the rDNA ITS1-5.8S-ITS2 and D1/D2 nucleotide sequences. Additionally, the basidiomycetous yeasts have been reported as the dominant yeasts in previous studies of other plants and with alternative methodologies. For instance, the study of yeast communities associated with sugarcane in Rio de Janeiro, Brazil showed that the prevalent species were basidiomycetous yeasts including *C. laurentii*, *Cryptococcus albidus* and *Rhodotorula mucilaginosa* (de Azeredo et al. 1998). It has been postulated that the yeast species most commonly found on monocotyledonous plants are from the phylum Basidiomycota (Spencer and Spencer 1997). The ballistoconidium-fall isolation method was used to isolate ballistoconidium-forming yeasts from dead leaves and stems of rice (*Oryza sativa* L.) in Japan. The isolated yeasts belonged to the genera *Bullera*, *Sporobolomyces* and *Tilletopsis* (Nakase and Suzuki 1985). The common ballistosporous yeasts isolated from rice leaf and other plants collected in the Asia-Pacific region were the genera *Bensingtonia*, *Bullera*, *Kockovaella*, *Sporidiobolus Sporobolomyces*, *Tilletiopsis* and *Udeniomyces* (Nakase 2000). A year later Nakase et al. (2001) also reported that the basidiomycetous yeasts isolated from plant phyllosphere collected in Thailand by using the ballistoconidium-fall isolation method, were identified as 21 species in six genera including *Bensingtonia*, *Bullera*, *Kockovaella*, *Sporidiobolus*, *Sporobolomyces* and *Tilletiopsis*. In the present study we also detected the common ballistosporous yeasts in the genera *Bullera* and *Sporobolomyces* by using culture-independent method from rice phyllosphere in Thailand.

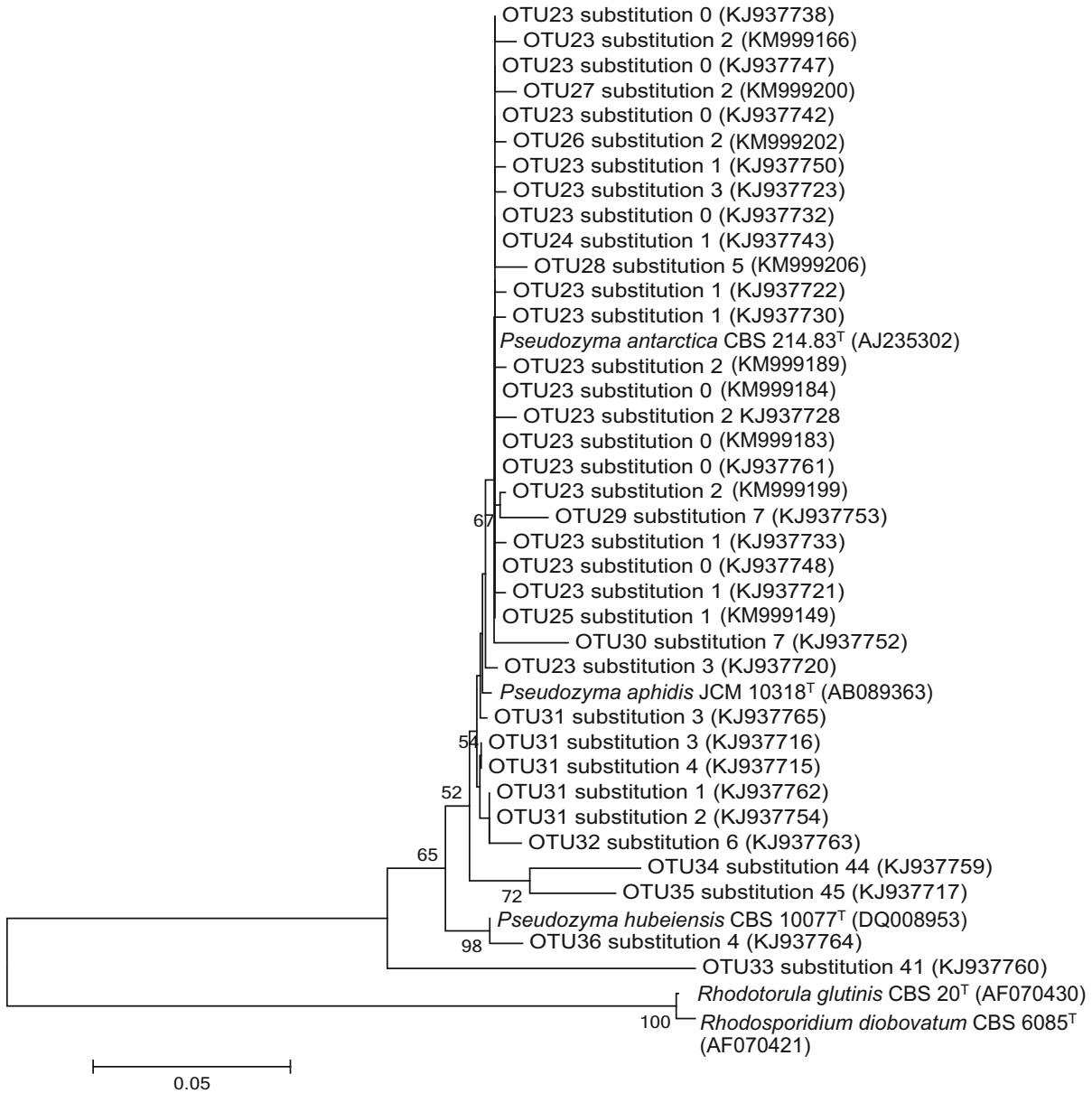


Fig. 3 Phylogenetic tree calculated from neighbor-joining of OTU sequences in subphylum Ustilaginomycotina (D1/D2 domain, LSU rRNA gene) and reference sequences retrieved from the GenBank database using Kimura 2-parameter model. Numbers on branches are the bootstrap percentage (>50 %) from 1000 random replications. The *scale bar* corresponds to a genetic distance of 0.05 substitutions per position

Although the most common phyllosphere yeasts are basidiomycete yeast species various ascomycete yeast species have also been isolated from the phyllophere such as *Debaryomyces hansenii*, *Hanseniaspora uvarum, Kazachstania barnetii*, *Metschnikowia lopburiensis, Metschnikowia pulcherrima*, *Metschnikowia saccharicola, Pichia membranifaciens*, *Saccharomyces cerevisiae* and various *Candida* species including *Candida aechmeae*, *C. olephila*, *C. chumphonensis, C. mattranensis* and *C. vrieseae* (Glushakova et al. 2007; Slavikova et al. 2007; Glushakova and Chernov 2010; Landell et al. 2010; Koowadjanakul et al. 2011; Kaewwichian et al. 2012). The ascomycetous yeasts were mostly detected in phyllosphere when using the

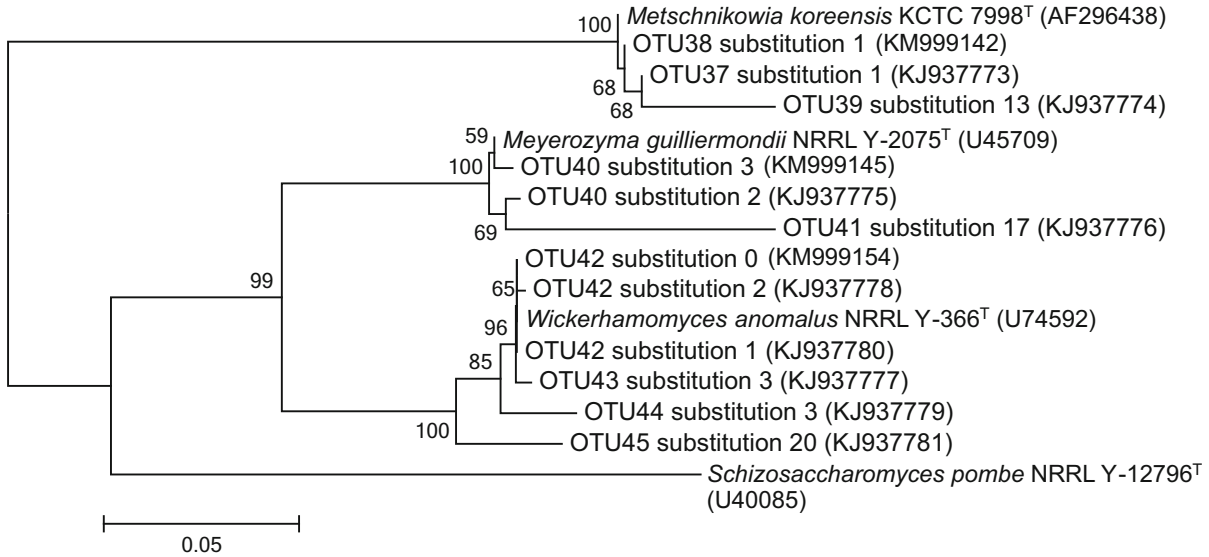


Fig. 4 Phylogenetic tree calculated from neighbor-joining of OTU sequences in subphylum Saccharomycotina (D1/D2 domain, LSU rRNA gene) and reference sequences retrieved from the GenBank database using Kimura 2-parameter model. Numbers on branches are the bootstrap percentage (>50 %) from 1000 random replications. The *scale bar* corresponds to a genetic distance of 0.05 substitutions per position

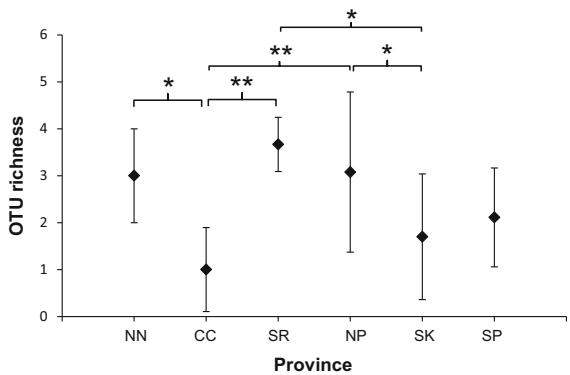


Fig. 5 OTU richness in the sub-samples depending on province; Nakhon Nayok (NN), Chachoengsao (CC), Surin (SR), Naphon Pathom (NP), Sisaket (SK) and Suphanburi (SP). *Bars* are confidence interval and middle points are the respective mean. Significant differences are indicated by $*P \leq 0.05$ and $**P \leq 0.01$

Table 3 Diversity indices of yeast genotypes presenting in 44 rice phyllosphere samples

Diversity indices	Values
Total number of clones	451
Total number of genotypes (S)	53
Average population size	8.51
Shannon–Weiner index (H')	1.996
Equitability index (E_H)	0.5028

culture-dependent approach based on the enrichment isolation technique and the D1/D2 domain sequence analysis (Limtong and Koowadjanakul 2012; Limtong and Kaewwichian 2014; Limtong et al. 2014). Only 7 related yeast species detected in this study were also found in rice phyllospheres when using the enrichment isolation technique (Limtong and Kaewwichian 2014). These consisted of *C. flavescens, C. laurentii, Metshnikowia koreensis*, *P. antarctica*, *P. aphidis*, *M. guilliermondii* and *S. blumeae*. Furthermore, the dominant species detected in our study were present in relatively low frequencies (*P. antarctica*) and was not found (*B. japonica*) when using enrichment culture. Also of note is that the yeast species showing a relatively high frequency of isolation using the enrichment isolation technique did not appear in this study. These included *Rhodotorula taiwanensis*, *Candida tropicalis* and *Cyberlindnera fabianii* (Limtong and Kaewwichian 2014). These results indicated that different yeasts were obtained from different techniques of investigations. The enrichment technique is likely to support the growth of small populations of

ascomycetous yeasts leading to a greater representation of these yeast species in the results. The species that were not found by enrichment technique but sequences of which were obtained in this study were related to that of *B. japonica*, *C. curvatus, C. flavus, H. zeae, P. hubeiensis, S. pararoseus, S. carnicolor*, *S. oryzicola* and *W. anomalus*. This likely indicates that the culture techniques used during enrichment do not support growth of these populations and these maybe under-represented in the results.

Some of the yeast species found in this study had also been detected previously in various plant species in Thailand, including *C. flavescens*, *M. koreensis*, *M. guilliermondii*, *S. blumeae* and *S. carnicolor* (Nakase 2000; Limtong and Kaewwichian 2014; Limtong et al. 2014). *Pseudozyma* was the most prevalent yeast genus in the rice phyllospheres of this study and this is not unexpected as the *Pseudozyma* genus is known generally inhabit numerous plant phyllospheres (Allen et al. 2004; Seo et al. 2007; Kitamoto et al. 2011; Limtong and Kaewwichian 2014; Yoshida et al. 2014). In the present study, *P. antarctica* was detected in relatively high frequency and was a common species in the rice phyllosphere samples. Some strains of this yeast species have been reported to provide beneficial effects to the host plant, such as microbiological control (Clement-Mathieu et al. 2008). There have been reported that *P. antarctica* secreted mannosylerythritol lipids (MELs) on plant surfaces that could be expected to play a significant role in fungal morphological development and propagation on plant surfaces (Morita et al. 2010; Yoshida et al. 2014). Therefore, these epiphytic yeast species detected in this study could be used as promising plant pathogenic biocontrol agents. In recent years, the enzyme production ability of this species which allows for the breakdown of biodegradable plastics has been reported (Kitamoto et al. 2011; Shinozaki et al. 2013a, 2013b). Other predominant yeasts in this study were in the genus *Bullera*, which have also been commonly found in other plant phyllopheres (Nakase 2000; Sampaio et al. 2004; Inácio et al. 2005; Fungsin et al. 2006). *B. japonica* another predominant species in this study has also been found to be so in other plant phyllospheres (Sampaio et al. 2004). Other epiphytic yeasts that have been reported of their capability to promote plant growth by producing plant growth hormone, indole-3-acetic acid (IAA) were also found in this study such as *P. aphidis* and *C. flavus* (Sun et al. 2014). Therefore these yeasts could be useful for further development in rice production.

It should be noted that a few yeasts species found in this study have been reported of their pathogenic risk in human i.e. *P. antarctica* has been isolated from the blood of a patient with a spontaneous pneumothorax in Thailand (Sugita et al. 2003). This should be aware of as this yeast species was predominantly found in rice phyllosphere in Thailand. *M. guilliermondii*, Risk Group 2 microorganism, was also detected in this study (San Millan et al. 1997; Pfaller et al. 2006). An appearance of pathogenic yeasts in rice fields may be due to the fact that human beings are involved in growing rice in Thailand and these microorganisms may be brought by farmers. In addition, many animals can also be sources of pathogenic microorganism contamination in the field.

Since the diversity of epiphytic yeasts in the rice phyllysphere has received little attention, only few novel yeast species isolated from the rice phyllosphere in Thailand have been proposed so far. These included *M. saccharicola and M. lopburiensis* (Kaewwichian et al. 2012). In our study, there were several OTUs that could not have been identified into a species level on the basis of the D1/D2 domain of the LSU rRNA gene sequence analysis with strongly supporting phylogenetic analysis, and some of these were suggested to be the sequences of new yeast taxa.

This study was not intended to investigate the relation between sampling locations and yeast communities. However, we primarily found significant differences in the OTU richness between some provinces. The significantly lowest OTU richness was shown in Chachoeansao province. This may be a result of the minimal level of rainfall (3.4 mm) during the sampling period in comparison with the rainfall level in other locations. This result is accordance with the report of Swinfield et al. (2012) which indicated that dry climates and when rainfall is less frequent could reduce fungal diversity. Nevertheless, the rainfall level was lowest (0.8 mm) in Nakhon Nayok province but this factor did not affect the OTU richness. There may be other factors that have an effect on yeast diversity. There have been reported that environmental factors such as seasonal and climatic conditions also geography could affect microbial biodiversity (Lachance et al. 2003; Glushakova and Chernov 2010; Jumpponen and Jones 2010; Gayevskiy and Goddard 2012; Voriskova et al. 2014).

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Hannaella phyllophila sp. nov., a basidiomycetous yeast species associated with plants in Thailand and Taiwan

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Five strains representing one novel anamorphic yeast species were isolated from plant leaves collected in Thailand (strains DMKU-SP186^T, ST-111 and ST-201) and Taiwan (strains FN20L02 and SM13L16). On the basis of morphological, biochemical, physiological and chemotaxonomic characteristics and sequence analysis of the D1/D2 region of the large subunit (LSU) rRNA gene and the internal transcribed spacer (ITS) region, they were assigned to a single novel species of the genus *Hannaella*. The sequences of the D1/D2 regions of the LSU rRNA genes of four of the strains (DMKU-SP186^T, ST-111, FN20L02 and SM13L16) were identical, while differing from strain ST-201 by 2 substitutions and 2 gaps. The nucleotide sequence of the ITS regions of the five strains differed from each other by between 0 and 3 nucleotide substitutions. The novel species was most closely related to *Hannaella luteola*, but showed 1.0–1.3 % nucleotide substitutions (between 6 substitutions out of 568–606 nt and 8 substitutions, and 2 gaps out of 597 nt) in the D1/D2 region of the LSU rRNA gene and 1.4–2.0 % nucleotide substitutions (6–9 substitutions out of 435 nt) in the ITS region. Ballistospores were produced by three of the strains on cornmeal agar at 15 and 20 °C after 4 weeks, while *H. luteola* did not produce ballistospores. The name *Hannaella phyllophila* sp. nov. is proposed. The type strain is DMKU-SP186^T (=BCC 69500^T=NBRC 110428^T=CBS 13921^T).

The genus *Hannaella* was proposed on the basis of phylogenetic relationships of the small-subunit (SSU) rRNA gene, the D1/D2 region of the large-subunit (LSU) rRNA gene, the internal transcribed spacer (ITS) region including the 5.8S rRNA gene and the mitochondrial cytochrome *b* gene, and colony morphology, to accommodate seven species that were transferred from the *Bullera sinensis* clade: *Hannaella coprosmaensis*, *H. kunmingensis*, *H. luteola*, *H. oryzae*, *H. sinensis*, *H. surugaensis* and *H. zeae* (Wang & Bai, 2008). The *B. sinensis* clade was in the *Cryptococcus luteolus* lineage (Takashima & Nakase, 1999) or the *luteolus* clade of Scorzetti *et al.* (2002), which also included two other distinct clades, namely *Dioszegia* and *Bullera mrakii* in the order *Tremellales* (Takashima & Nakase, 1999; Wang & Bai, 2008). On the same basis as for the proposal of *Hannaella*, the new genus *Derxomyces* was proposed to accommodate the species in the *B. mrakii* clade (Wang & Bai, 2008). At present, the genus *Hannaella* consists of eight species: the seven species listed above and *Hannaella pagnoccae* (Wang & Bai, 2008; Boekhout *et al.*, 2011; Landell *et al.*, 2014).

During independent investigations of yeasts in Thailand and Taiwan, five yeast strains (DMKU-SP186^T, ST-111, ST-201, FN20L02 and SM13L16) representing a novel

Abbreviations: ITS, internal transcribed spacer; LSU, large subunit

The GenBank/EMBL/DDBJ accession numbers for the sequences of the ITS and D1/D2 regions of the LSU rRNA gene of strains DMKU-SP186^T, ST-111, ST-201, FN20L02 and SM13L16 are AB934926/AB934929, AB934927/DQ404459, AB934928/DQ404475, HQ623591/EU523600 and HQ623592/HQ623511, respectively. The Mycobank number for *Hannaella phyllophila* is MB81068.

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species of *Hannaella* were obtained. In this paper, they are described as *Hannaella phyllophila* sp. nov.

Yeast isolation

Strains DMKU-SP186^T, ST-111 and ST-201 were obtained from plant leaves collected in Thailand. They were respectively isolated from a sample of sugar cane (*Saccharum officinarum* L.) leaf collected from Chai Badan, Lop Buri province, on 3 March 2012, a leaf of morning glory (*Merremia* sp.) collected from Than Tong waterfall, Sangkhom, Nong Khai province, on 2 February 2001, and a leaf of cassava (*Manihot esculenta*) collected from Ban Tai, Sakon Nakhon province, on 4 February 2001. Strains FN20L02 and SM13L16 were isolated from leaves of plants in Hsinchu, Taiwan, specifically from a leaf of Taiwan fir (*Cunninghamia lanceolata*) collected from Wufong in November 2005 and a leaf of *Villerbrunea pedunculata* collected from Jianshih in November 2006, respectively.

Strain DMKU-SP186^T was isolated by plating of leaf washings. Three grams of leaf were suspended aseptically in 50 ml 0.85 % saline solution in a 250 ml Erlenmeyer flask and shaken on a rotary shaker at 25 °C for 1 h to detach yeast cells from the surface. An aliquot of 0.1 ml of the washing solution was then spread on yeast extract-malt extract (YM) agar (0.3 % yeast extract, 0.3 % malt extract, 0.5 % peptone and 2.0 % agar) supplemented with 0.025 % sodium propionate and 0.02 % chloramphenicol and incubated at 25 °C until yeast colonies appeared. Yeast colonies of different morphologies were selected and purified by cross-streaking on YM agar. Purified yeast strains were suspended in YM broth supplemented with 10 % glycerol and maintained at −80 °C. Strains ST-111 and ST-201 were isolated by direct streaking of samples on YM agar supplemented with 0.02 % sodium propionate and 0.02 % chloramphenicol and incubated at 30 ± 2 °C until yeast colonies appeared. Strains FN20L02 and SM13L16 were isolated by the method described by Lee *et al.* (2008).

DNA sequencing and phylogenetic analysis

Sequences of the D1/D2 region of the LSU rRNA gene and the ITS region were determined from PCR products amplified from genomic DNA extracted from yeast cells. Amplification of the D1/D2 region of the LSU rRNA gene was carried out by PCR with the forward primer NL1 (5′-CATATCAATAAGCGGAGGAAAAG-3′) and reverse primer NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) (Kurtzman & Robnett, 1998). The ITS region was amplified with the forward primer ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al.*, 1990). The PCR products were checked by agarose gel electrophoresis and purified. The purified products were sequenced using an automated DNA sequencer (3730 DNA Analysis System; Applied Biosystems) or commercially by Macrogen Inc. (Seoul, Korea) using primers NL1 and NL4 for the D1/D2 region and primers ITS1 and ITS4 for the ITS region. The sequences were compared pairwise using a BLAST search (Altschul *et al.*, 1997) and were aligned with sequences of related species retrieved from GenBank using the multiple alignment program CLUSTAL_X version 1.81 (Thompson *et al.* 1997). A phylogenetic tree was reconstructed from the evolutionary distance data with Kimura's two-parameter correction (Kimura, 1980) by the neighbour-joining method (Saitou & Nei, 1987) using the MEGA software version 6.0 (Tamura *et al.*, 2013). The confidence levels of clades were estimated from bootstrap analysis (1000 replicates) (Felsenstein, 1985). *Bulleromyces albus* CBS 501^T was used as the outgroup in the analysis.

Phenotypic characterization

The five strains were characterized morphologically, biochemically and physiologically according to the standard methods described by Kurtzman *et al.* (2011). Formation of pseudohyphae and true hyphae was investigated on potato dextrose agar (PDA) and cornmeal agar in slide culture at 25 °C for up to 14 days. Ballistospore formation was investigated on inverted PDA and cornmeal agar plates and incubated at 15 °C and 25 °C for up to 4 weeks. The sexual process was investigated for individual strains or strain pairs on 5 % malt extract agar, cornmeal agar, PDA and YM agar at 15 and 25 °C for up to 6 weeks. Carbon and nitrogen assimilation tests were conducted in liquid medium and a starved inoculum was used for the nitrogen assimilation test (Kurtzman *et al.*, 2011). Growth at various temperatures was determined by cultivation in YM broth. Ubiquinones were extracted from cells cultivated in 500 ml Erlenmeyer flasks containing 250 ml yeast extract-peptone-dextrose (YPD) broth (1 % yeast extract, 2 % peptone and 2 % glucose) on a rotary shaker at 25 °C for 24–48 h and purified according to the methods described by Yamada & Kondo (1973) and Kuraishi *et al.* (1985). Isoprenologues were identified by HPLC as described previously (Limtong *et al.*, 2007).

Novel species delineation and identification

Analysis of the D1/D2 region of the LSU rRNA gene sequence revealed that the five strains represented a single novel species. Four of the strains (DMKU-SP186^T, ST-111, FN20L02 and SM13L16) had identical sequences, while differing from strain ST-201 by 2 substitutions and 2 gaps. In terms of pairwise sequence similarities of the D1/D2 region of the LSU rRNA gene, these five strains were closest to the type strain of *Hannaella luteola*, CBS 943^T. Four of the strains (DMKU-SP186^T, ST-111, FN20L02 and SM13L16) differed from *Hannaella luteola* CBS 943^T by 1.0–1.1 % nucleotide substitutions (6 substitutions out of 604–606 nt to 6 substitutions out of 568–574 nt), and strain ST-201 differed by 1.3 % nucleotide substitutions (8 substitutions and 2 gaps out of 597 nt). The nucleotide sequences of the ITS regions of the five strains differed from each other by 0–3

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substitutions, and from that of *H. luteola* CBS 943^T by 1.4–2.0 % nucleotide substitutions (6–9 substitutions out of 435 nt).

The phylogenetic tree based on the concatenated sequences of the ITS and D1/D2 regions of the LSU rRNA gene further demonstrated that all five strains were clearly placed in the *Hannaella* clade, and they clustered together with high bootstrap support (Fig. 1). They were also connected to *H. luteola* CBS 943^T, the type strain of the closest known species, with a high bootstrap value.

Among the species of the genus *Hannaella*, at least two, *H. kunmingensis* and *H. pagnoccae*, were reported to exhibit high intraspecific sequence variation; however, high intraspecific sequence variation was found only in the ITS region, not in the D1/D2 region of the LSU rRNA gene (Dayo-Owoyemi *et al.*, 2013; Landell *et al.*, 2014). Dayo-Owoyemi *et al.* (2013) reported that all 11 strains of *H. kunmingensis* had identical sequences of the D1/D2 region of the LSU rRNA gene, but showed variation of about 2.5 % (9 substitutions and 2 gaps) in the ITS region. In the case of *H. pagnoccae*, 32 strains proposed to belong to this species revealed more than 99 % similarity (0–3 substitutions and 0–2 gaps) in the D1/D2 region and between 98 and 100 % similarity (0–7 substitutions and 0–3 gaps) in the ITS region (Landell *et al.*, 2014). Because the differences between the sequences of the D1/D2 and ITS regions of the five strains in this study and the sequence of the type strain of the closest species, *H. luteola* CBS 943^T, were not so great, an analysis was carried out to confirm that the five strains did not belong to *H. luteola*. Sequences of the D1/D2 region of the LSU rRNA gene

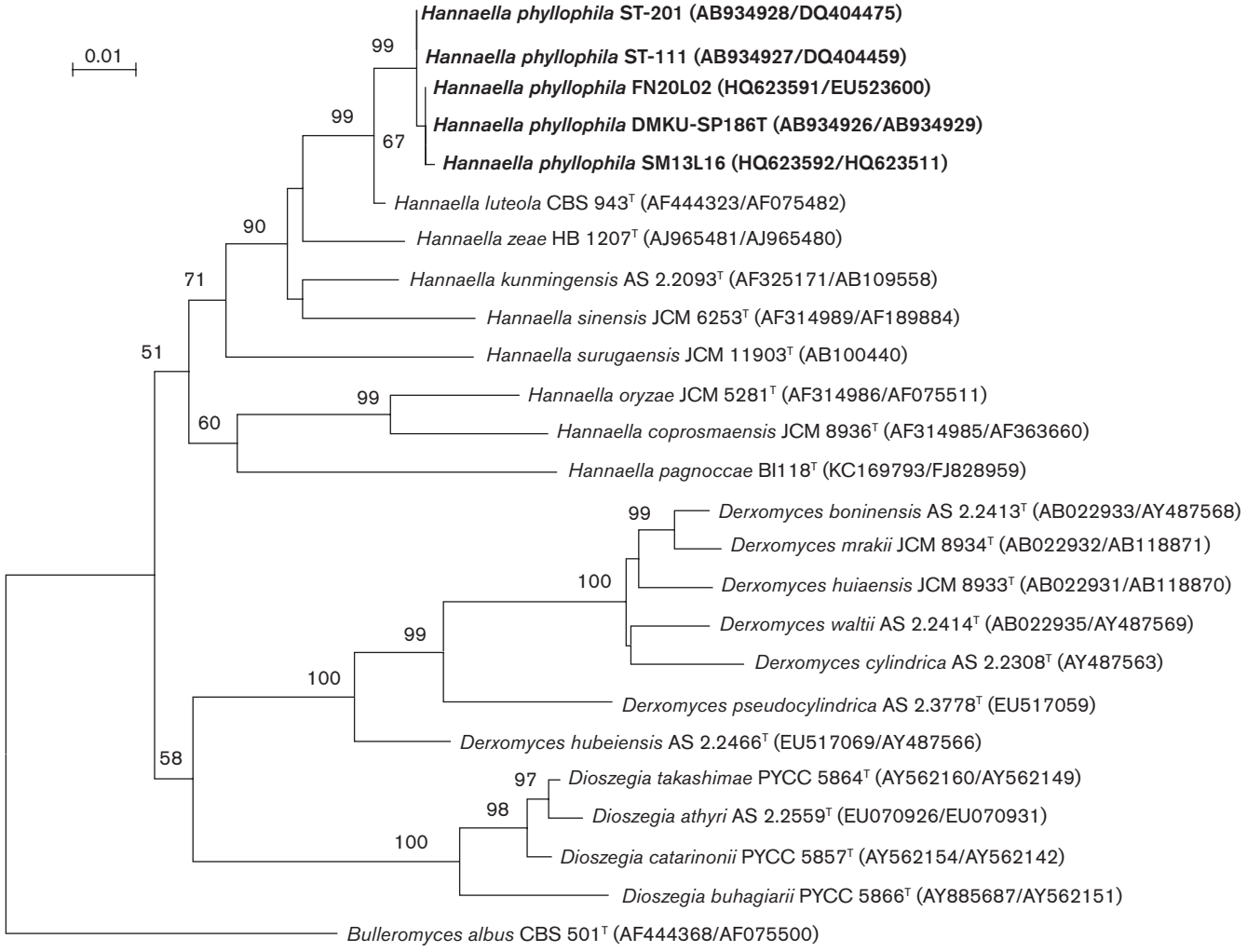


Fig. 1. Phylogenetic tree, based on sequences of the D1/D2 region of the LSU rRNA gene and the ITS region, showing the positions of strains of *H. phyllophila* sp. nov. with respect to closely related species. The phylogenetic tree was reconstructed from evolutionary distance data with Kimura's two-parameter correction (Kimura, 1980), using the neighbour-joining method and MEGA version 6.0.Numbers at nodes indicate percentages of bootstrap sampling derived from 1000 replicates; bootstrap percentages higher than 50 % are shown. *Bulleromyces albus* CBS 501^T was the outgroup. Bar, 0.01 K_{nuc}.

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of strains of *H. luteola* that have been deposited in GenBank were retrieved and analysed. They included KC798402, AM160633, GQ328023, GQ328025, FJ743611, EF599106, AM160632, GQ328024, KC494722, HQ111504, KJ159047, JQ964209, JQ968519 and FJ527155. The result showed that the sequences of the D1/D2 regions of the 13 strains were identical to the sequence of the type strain of *H. luteola* or differed by only 1 gap or 1 substitution (data not shown). However, there was one sequence, KC798402, which revealed high divergence (6 substitutions) from the type strain and the other strains of *H. luteola*. This may have been the result of an error in identification. Interestingly, the sequence KC79840 was identical to that of four of the strains (DMKU-SP186^T, ST-111, FN20L02 and SM13L16) of the novel species, and differed from that of strain ST-201 by 2 substitutions and 2 gaps. Therefore, it seems to be a member of the novel species. The sequences of the ITS region of the other 10 strains that have been deposited in GenBank as *H. luteola* were also retrieved and analysed in comparison with the sequence of the type strain of *H. luteola*; they were JX188158, AM176654, AM176651, AM160632, AM160633, KP132260, EU252551, FJ591129, AY301024 and AF360371. All strains of *H. luteola* showed identical ITS sequences (data not shown). The result of sequence similarity analysis of both the D1/D2 and ITS regions indicated that intraspecific sequences variation in *H. luteola* was very limited. Therefore, the differences between the five strains in this study and the type strain of *H. luteola* were great enough to separate them into two species, as suggested by Fell *et al.* (2000).

Cells of the five strains were globose, oval and ellipsoidal, and budding was polar. Colonies on YM agar were cream-coloured to yellowish cream, mucoid, shiny to dull, low convex with a smooth surface and an entire margin, which is a common colony morphology of the genus *Hannaella* (Wang & Bai, 2008). Three strains (SP186^T, FN20L02 and SM13L16) produced ballistospores, which were ellipsoidal or ellipsoidal with a constriction in the middle. Pseudohyphae and true hyphae were not formed. Fermentation was absent. Diazonium blue B colour and urease reaction were positive. The major ubiquinone was Q-10. These phenotypic characteristics confirmed that the five strains belong to the genus *Hannaella*.

On the basis of analysis of the concatenated sequences of the ITS region and the D1/D2 region of the LSU rRNA gene and morphological, biochemical, physiological and chemotaxonomic characteristics, we therefore concluded that the five strains represent a novel *Hannaella* species, *Hannaella phyllophila* sp. nov.

In practice, *H. phyllophila* sp. nov. can be distinguished from its closest relative *H. luteola* not only on the basis of sequences of the D1/D2 region of the LSU rRNA gene and the ITS region, but also by a few phenotypic characteristics. Some strains of *H. phyllophila* sp. nov. form ballistospores, but they are not formed by *H. luteola*. *H. phyllophila* sp. nov. assimilates soluble starch, grows at 30 °C and grows on media containing 50 % glucose and 10 % NaCl/5 % glucose (weakly), while *H. luteola* does not. *H. luteola* assimilates DL-lactate (weakly), but *H. phyllophila* sp. nov. does not. Strains of some members of the genus *Hannaella*, including *H. coprosmaensis*, *H. kunmingensis*, *H. luteola*, *H. oryzae*, *H. pagnoccae*, *H. sinensis* var. *sinensis*, *H. sinensis* var. *lactis* and *H. zeae*, but excluding *H. surugaensis*, were reported to have been isolated from plant leaves (Molnár & Prillinger, 2006; Wang & Bai, 2008; Boekhout *et al.*, 2011; Landell *et al.*, 2014), some from dead leaves and some from fresh and healthy leaves. In the current study, the five strains of the novel species were isolated from fresh leaves of different plants; this finding confirms that leaves are important habitats of yeasts in the genus *Hannaella*.

Description of *Hannaella phyllophila* Surussawadee, Jindamorakot, Am-In, Nakase, Lee & Limtong sp. nov

Hannaella phyllophila (phyl.lo′phi.la. N.L. fem. adj. *phyllophila* liking leaves, referring to the phylloplane, where the first strains were isolated).

When grown in YM broth for 3 days at 25 °C, cells are globose, oval and ellipsoidal (2–5.5 × 4–11 μm) and occur singly, in pairs or in short chains (Fig. 2a). Budding is polar. Colonies on YM agar are cream-coloured to yellowish cream, mucoid, shiny to dull, low convex with a smooth surface and an entire margin. After 14 days in Dalmau plate culture on cornmeal agar and PDA at 25 °C, pseudohyphae and true hyphae are not formed. Ballistospores are produced by strains SP186^T, FN20L02 and SM13L16 on PDA and cornmeal agar at 15 and 25 °C after 4 weeks. Ballistospores are ellipsoidal or ellipsoidal with a constriction in the middle (Fig. 2b). Sexual reproduction is not observed by any of the known strains or when strains are paired on 5 % malt extract agar, cornmeal agar, PDA or YM agar at 15 or 25 °C for up to 6 weeks. Fermentation of D-glucose is negative. Assimilates D-glucose, D-galactose, L-sorbose, *N*-acetylglucosamine, D-xylose, L- and D-arabinose, L-rhamnose, sucrose, maltose, trehalose, methyl α-D-glucoside, cellobiose, salicin, melibiose, raffinose, melezitose, soluble starch, glycerol (slow), erythritol, ribitol (weak), D-glucitol, D-mannitol, galactitol (slow), *myo*-inositol, D-glucono-1,5-lactone, 2-keto-D-gluconate, 5-keto-D-gluconate, D-gluconate, D-glucuronate, D-galacturonate, succinate, citrate, ethanol and xylitol, but not inulin, lactose, DL-lactate or methanol. Nitrite, ethylamine hydrochloride, L-lysine and cadaverine are assimilated, but nitrate is not. Grows at 30 °C, but not at 35 °C. No growth in vitamin-free medium. Grows on medium containing 50 % glucose, 60 % glucose (weakly) and 10 % NaCl/5 % glucose (weakly), but not on 15 % NaCl/5 % glucose. No growth with 0.01 % cycloheximide. Starch-like compounds are produced. Diazonium blue B colour and urease reaction are positive. The major ubiquinone is Q-10.

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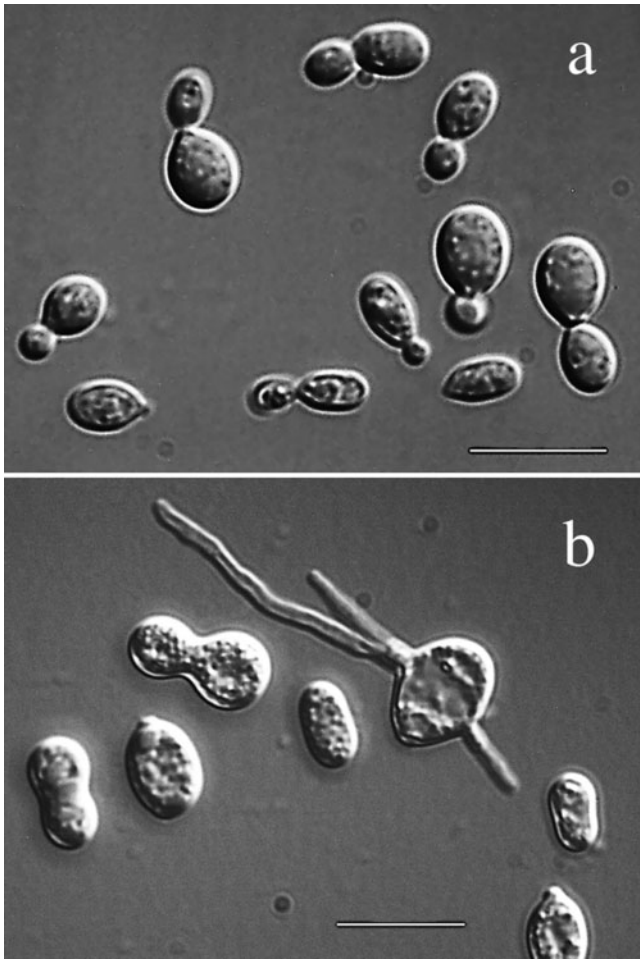


Fig. 2. Morphology of *H. phyllophila* sp. nov. DMKU-SP186^T. (a) Budding cells in YM broth after 3 days at 25 °C. (b) Ballistospores on cornmeal agar at 15 °C for 4 weeks. Bars, 10 μm.

Strain DMKU-SP186^T is the holotype of *Hannaella phyllophila*. The strain was isolated from the external surface of a sugar cane (*Saccharum officinarum* L.) leaf collected from Lop Buri province, Thailand. The living culture from the type has been deposited with the BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology, Thailand (Pathumthani, Thailand), as BCC 69500^T and with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands, as CBS 13921^T. The MycoBank registration number is MB81068.

Acknowledgements

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Occultifur tropicalis f.a., sp. nov., a novel cystobasidiomycetous yeast species isolated from tropical regions

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Five strains representing a single novel anamorphic yeast species were isolated from sugar cane. Two strains were from tissue (DMKU-SE38, DMKU-SE59^T) and two from the external surface (DMKU-SP385, DMKU-SP403) of leaves collected in Thailand and the fifth (IMUFRJ 52020) from the rhizoplane of sugar cane in an organically cultivated field in Brazil. On the basis of sequence analysis of the D1/D2 region of the large subunit (LSU) rRNA gene and the internal transcribed spacer (ITS) region, they were classified as representing a single species of the genus *Occultifur*. The sequences of the D1/D2 region of the LSU rRNA genes and the ITS regions of the five strains were either identical or differed from each other by only one nucleotide substitution. The novel species was related most closely to *Occultifur brasiliensis* f.a. CBS 12687^T but with 0.7–1.0 % nucleotide substitutions (4–6 nt) in the D1/D2 region of the LSU rRNA gene and 2.5–2.7 % nucleotide substitutions (14–15 nt) in the ITS region. The name *Occultifur tropicalis* f.a., sp. nov. is proposed. The type strain is DMKU-SE59^T (=BCC 61184^T=NBRC 109696^T=CBS 13389 ^{T}).

The genus *Occultifur* was proposed by Oberwinkler (1990) to accommodate *Occultifur internus*, a mycoparasitic, simple-pored, auricularioid and diomorphic basidiomycetous yeast (Sampaio & Oberwinkler, 2011). It is a teleomorphic genus and belongs to the subphylum Pucciniomycotina, the class Cystobasidiomycetes and the order Cystobasidiales. In the latest edition of The Yeasts: A Taxonomic Study there are only three species in this genus with validly published names: *Occultifur internus, Occultifur externus* and *Occultifur corticiorum* (Sampaio & Oberwinkler, 2011). However, living cultures and molecular sequence data are available only for *O. externus* (Sampaio & Oberwinkler, 2011). An additional anamorphic species (*O. brasiliensis* f.a.) that was isolated from tanks of the bromeliad *Vriesea minarum* has recently been proposed (Gomes *et al.*, 2015).

Plant leaves and soil are two important and interrelated habitats of yeasts, as microbes are expected to be washed off the leaves into the soil. Endophytic and epiphytic yeasts can colonize healthy plant leaf tissue and external leaf surfaces, respectively, and they belong to either the Ascomycota or the Basidiomycota although the most common species are members of the phylum Basidiomycota (Isaeva *et al.*, 2010; Oliveira *et al.*, 2012; Akhtyamova & Sattarova, 2013; Kaewwichian *et al.*, 2013; Khunnamwong *et al.*, 2014; Limtong *et al.*, 2014). The presence of yeast in soil depends on many factors, e.g. type of soil, rainfall and climate (Slávikóvá and Vadkertiová, 2003). Soil yeasts can also be either ascomycetous or basidiomycetous species. (Slávikóvá & Vadkertiová, 2003; Connell *et al.*, 2008; Yurkov *et al.*, 2012; Polburee *et al.*, 2014). In the course of the investigation of endophytic and epiphytic yeasts from sugar cane leaves in Thailand and yeast diversity associated with the soil, rhizoplane and phylloplane of sugar cane in Brazil, five strains representing a novel species of the genus *Occultifur* were

The GenBank/EMBL/DDBJ accession numbers for the sequences of the ITS region and the D1/D2 region of the large subunit rRNA genes of strains DMKU-SE38, DMKU-SE59^T, DMKU-SP385, DMKU-SP403 and IMUFRJ 52020 are AB921283 and AB921281, AB921282 and AB921280, AB924026 and AB924022, AB924027 and AB924023 and FN428928, respectively (the last accession incorporates both sequences for IMUFRJ52020). The MycoBank number for *Occultifur tropicalis* f.a., sp. nov. is MB 810993.

obtained. In this article, these five strains are described as *Occultifur tropicalis* f.a., sp. nov.

Yeast isolation

Strains DMKU-SE38, DMKU-SE59^T, DMKU-SP385 and DMKU-SP403 were isolated from surface-sterilized sugar cane (*Saccharum officinarum* L.) leaves collected in Thailand (Khunnamwong *et al.*, 2014). Strains DMKU-SE38 and DMKU-SE59^T were isolated by plating of leaf washings from two samples of sugar cane leaf collected on 2 March 2012 from Kao Liao district, Nakhon Sawan province (15° 51′ 1″ N 100° 4′ 43″ E) and Chai Badan district, Lopburi province (15° 11′ 22 N 101° 7′ 37″ E), respectively. Strains DMKU-SP385 and DMKU-SP403 were derived from two samples of sugar cane leaf collected on 19 April 2012 from Nong Muang district, Lopburi province (15° 14′ 50″ N 100° 39′ 15″ E). Strain IMUFRJ 52020 was isolated by spreading yeast cells from rhizoplane of sugar cane organically cultivated at an experimental farm [Sistema Integrado de Produção Agroecológica (Integrated System of Agroecological Production) (SIPA)], in Seropédica district, Rio de Janeiro, Brazil (22° 45′ 21″ S 43° 40′ 38″ W) in January 2008 (Ribeiro *et al.*, 2011). The purified yeast strains were suspended in YM broth supplemented with 10 % (v/v) glycerol and maintained at −80 °C.

DNA sequencing and phylogenetic analysis

The sequences of the D1/D2 region of the LSU rRNA gene and the ITS (ITS1–5.8S rRNA–ITS2) region were determined from PCR products amplified from genomic DNA, using primers NL1 and NL4 (Kurtzman & Robnett, 1998) and ITS1and ITS4 (White *et al.*, 1990), respectively. DNA extraction and amplification of the D1/D2 region of the LSU rRNA gene and the ITS region were performed as described previously (Limtong *et al.*, 2007). The PCR product was checked by agarose gel electrophoresis and purified using a HiYield Gel/PCR DNA Fragments Extraction kit (RBC Bioscience), according to the manufacturer's protocol. The purified product was submitted to Macrogen (Seoul, Republic of Korea) for sequencing with primers NL1 and NL4 for the D1/D2 region of the LSU rRNA gene and ITS1 and ITS4 for the ITS region. For strain IMUFRJ 52020, DNA was extracted and amplified as described previously (Ribeiro *et al.*, 2011). Genomic DNA was amplified using ITS1f (Gardes & Bruns, 1993) and NL4 (O'Donnell, 1993) primers for the ITS region and the D1/D2 region of the LSU rRNA gene. Automated sequencing was performed using the MegaBACE system (GE Healthcare). The sequences were compared pairwise using the BLASTN search program (Altschul *et al.*, 1997) and were aligned with the sequences of related species retrieved from GenBank using the multiple alignment program CLUSTAL_X version 2.1 (Thompson *et al.*, 1997). A phylogenetic tree was reconstructed from the evolutionary distance data with Kimura's two-parameter correction (Kimura, 1980), using the neighbour joining method (Saitou & Nei, 1987) and MEGA software version 6.06 (Tamura *et al.*, 2013). Confidence levels of the clades were estimated from bootstrap analysis (1000 replicates) (Felsenstein, 1985). *Erythrobasidium hasegawianum* CBS 8253^T was the outgroup in the analysis.

Phenotypic characterization

Strains DMKU-SE38, DMKU-SE59^T, DMKU-SP385, DMKU-SP403 and IMUFRJ 52020 were characterized morphologically, biochemically and physiologically according to the standard methods described by Kurtzman *et al.* (2011). Formation of pseudohyphae and true hyphae was investigated by cultivation on potato dextrose agar (PDA; 20 % potato infusion, 2 % glucose and 1.5 % agar) and corn meal agar(2 % corn meal infusion and 1.5 % agar) in slide culture at 25 °C for up to 4 weeks. Ballistospore formation was investigated by the method described by Kurtzman *et al.* (2011) on inverted PDA and corn meal agar plates and incubated at 15 °C and 25 °C for up to 4 weeks. Sexual processes were investigated for individual strains or strain pairs on PDA, corn meal agar, 5 % malt extract agar (5 % malt extract and 1.5 % agar) and YM agar at 15 °C and 25 °C for up to 6 weeks. Carbon source and nitrogen source assimilation tests were conducted in liquid medium and starved inocula was used in nitrogen assimilation tests (Kurtzman *et al.*, 2011). Growth at various temperatures was determined by cultivation in YM broth. Ubiquinones were extracted from cells cultivated in 500 ml Erlenmeyer flasks containing 250 ml of yeast extract peptone glucose (YPD) broth (1 % yeast extract, 2 % peptone and 2 % glucose) on a rotary shaker at 28 °C for 48 h and purified according to the methods described by Yamada & Kondo (1973) and Kuraishi *et al.* (1985). Isoprennologues were identified by HPLC as described previously (Limtong *et al.*, 2007).

Novel species delineation and identification

Analysis of the D1/D2 region of the LSU rRNA gene sequence and ITS region revealed that the five strains (DMKU-SE38, DMKU-SE59^T, DMKU-SP385, DMKU-SP403 and IMUFRJ 52020) represented a single novel species. The sequences of the D1/D2 region of the LSU rRNA genes of the four strains isolated in Thailand comprising the two endophytic strains (DMKU-SE59^T and DMKU-SE38) and the two epiphytic strains (DMKU-SP385 and DMKU-SP403) were either identical or differed by one gap or one nucleotide substitution with one gap from the sequence of strain IMUFRJ 52020 from Brazil. In terms of pairwise sequence similarity of the D1/D2 region of the LSU rRNA gene the most closely related species to the five strains was *O. brasiliensis* f.a. CBS 12687^T (KC698874). The sequence of the D1/D2 region of the most closely related species differed from those of the four strains from Thailand (DMKU-SE38, DMKU-SE59^T, DMKU-SP385 and DMKU-SP403) by 1.0 % nucleotide substitutions (six nucleotides) and from strain IMUFRJ 52020 by 0.7 % nucleotide substitutions (four nucleotides and three gaps). The sequence of the D1/D2 region of the LSU rRNA gene of the novel species differed from that of *O. externus* CBS 8732^T (AF189909) by 1.3–1.4 % nucleotide substitutions (seven to

eight nucleotides and zero to three gaps). The nucleotide sequences of the ITS region of three strains DMKU-SE59^T, DMKU-SE38 and IMUFRJ 52020 were either identical or differed by only one gap or one nucleotide substitution with one gap from those of the other two strains (DMKU-SP385 and DMKU-SP403), which were identical to each other. The ITS sequences of the five strains differed by 2.5–2.7 % nucleotide substitutions (14–15 nucleotides and 10–11 gaps) from the sequence of *O. brasiliensis* f.a. CBS 12687^T (KC698874) and 3.4–3.6 % nucleotide substitutions (19–20 nucleotides and seven to eight gaps) from that of *O. externus* CBS 8732^T (AF444567). A phylogenetic tree based on sequences of the ITS region and the D1/D2 region of the LSU rRNA gene indicated that the four strains from Thailand (DMKU-SE59^T, DMKU-SE38, DMKU-SP385 and DMKU-SP403) were located in the same position, clustered with IMUFRJ 52020 and connected to a cluster that contained *O. brasiliensis* f.a. CBS 12687^T and *O. externus* CBS 8732^T to form a clade with strong bootstrap support (Fig. 1.).

The novel species delineation was based primarily on the analysis of the combined sequences of the ITS region and D1/D2 region of the LSU rRNA gene, which indicated the reciprocal monophyletic nature of the species in this clade; therefore, a phylogenetic species concept applies. Therefore, on this basis, we concluded that the five strains represent a novel species. Although sexual reproduction was not observed, according to the nomenclatural rules for fungi of the International Code of Nomenclature for algae, fungi and plants, the most important requirement is the adoption of 'one fungus, one name' (Miller *et al.*, 2011). Consequently, the novel species was assigned to the genus *Occultifur*, and the designation *forma asexualis* (f.a.) was included following the recommendation of Lachance (2012). The name *Occultifur tropicalis* f.a., sp. nov. (MB 810993) is proposed.

Description of *Occultifur tropicalis* Khunnamwong, Surussawadee, Jindamorakot, Ribeiro, Hagler & Limtong sp. nov.

Occultifur tropicalis (tro.pi.ca′lis. N.L. masc. adj. *tropicalis* of or belonging to tropical area where the type strain was isolated). MycoBank accession number is MB 810993.

Growth occurs in YM broth. After 3 days at 25 °C, cells are subglobose to ovoid (3–5 × 3.5–9 μm) and occur singly or in pairs (Fig. 2). Budding is polar. After 3 days of growth on YM agar at 25 °C, the streak culture is pink to orange in colour, soft with a smooth surface and has an entire margin. Pseudohyphae and true hyphae are not formed in slide culture on PDA and corn meal agar after 4 weeks at 25 °C. Ballistospores are not produced when cultured on PDA agar at 15 °C for up to 4 weeks. Sexual reproduction is not observed in any of the strains or when strains are

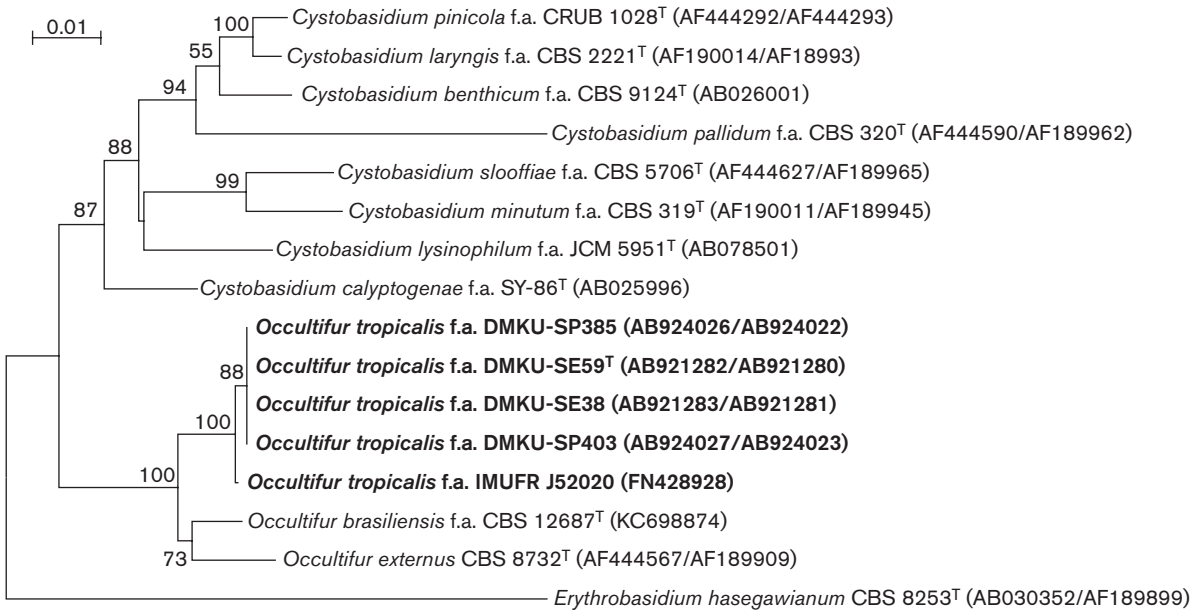


Fig. 1. Phylogenetic tree based on the sequence of the D1/D2 region of the LSU rRNA gene and ITS region showing positions of *Occultifur tropicalis* f.a. sp. nov. (DMKU-SE59^T, DMKU-SE38, DMKU-SP385, DMKU-SP403 and IMUFRJ 52020) with respect to closely related species. The phylogenetic tree was reconstructed using the neighbour joining method by MEGA software version 6.0, and the nucleotide substitution rates (K_{nuc} values) were computed from evolutionary distance data using Kimura's two-parameter model. The scale bar indicates an evolutionary distance of 0.01 K_{nuc}. Numbers at the nodes indicate percentages of bootstrap samples, derived from 1000 replicates; only values >50 % are shown. The numbers in parentheses are GenBank accession numbers. *Erythrobasidium hasegawianum* CBS 8253^T was the outgroup in the analysis.

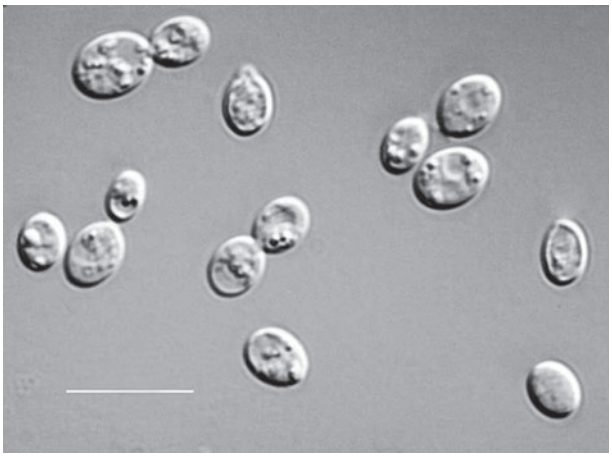


Fig. 2. *Occultifur tropicalis* f.a. sp. nov. (DMKU-SE59^T) grown in YM broth for 3 days at 25 °C. Bar, 10 μm.

paired on PDA agar, corn meal agar, 5 % malt extract agar and YM agar at 15 °C and 25 °C for up to 6 weeks. Fermentation is absent. D-glucose, D-galactose, L-sorbose, *N*-acetylglucosamine, D-xylose, L-arabinose, D-arabinose, sucrose, maltose, α-α-trehalose, met-α-D-glucoside (weakly), cellobiose, salicin (slowly), melibiose (weakly), soluble starch (weakly), lactose (slowly), melezitose, glycerol, ribitol (weakly), D-glucitol, D-mannitol, D-glucono-1,5-lactone, 2-ketogluconic acid, 5-ketogluconic acid, D-gluconate, D-glucuronate, D-galacturonic acid (slowly), DL-lactate, succinate, citrate (weakly) and ethanol are assimilated, but D-ribose, L-rhamnose, raffinose, inulin, erytritol, galactitol, *myo*-inositol and methanol are not assimilated. Ammonium sulfate, ethylamine HCl, L-lysine and cadaverine are assimilated, but potassium nitrate and sodium nitrite are not assimilated. No growth occurs in vitamin-free medium or with 0.01 % cycloheximide or 0.1 % cycloheximide or on medium containing 15 % (w/v) sodium chloride/5 % (w/v) glucose. Growth on medium containing 50 % (w/v) glucose, 60 % (w/v) glucose and 10 % (w/v) sodium chloride/5 % (w/v) glucose is present. Growth is present at 15, 25, 30 and 35 °C, but absent at 37, 40, 42 and 45 °C. Acid formation is absent. Starch-like compounds are not produced. Diazonium blue B colour and urease reaction are positive. The major ubiquinone is Q-10.

The type strain, DMKU-SE59^T, was isolated from the tissue of a sugar cane (*Saccharum officinarum*) leaf collected from Chai Badan district, Lopburi province, Thailand. This strain has been deposited at the BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC), Phathumthani, Thailand as BCC 61184^T, the NITE Biological Resource Center (NBRC), Department of Biotechnology, National Institute of Technology and Evaluation, Chiba, Japan as NBRC 109696^T and the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands as CBS 13389^T. The MycoBank registration number is MB 810993.

In practice, *O. tropicalis* f.a. can be distinguished from the most closely related species in terms of pairwise sequence similarity of the D1–D2 region, *O. brasiliensis* f.a., not only by the analysis of the sequence of the D1/D2 region of the LSU rRNA gene and the ITS region, but also by some phenotypic characteristics. *O. tropicalis* f.a. sp. nov. assimilates D-arabinose (weakly), sucrose (delayed), maltose, melibiose (weakly), soluble starch (weakly), ribitol (weakly) and DL-lactate (slowly) but *O. brasiliensis* f.a. does not. *O. tropicalis* f.a. does not assimilate D-ribose but *O. brasiliensis* f.a. does. *O. brasiliensis* f.a. grows (weak) at 37 °C but *O. tropicalis* f.a. does not. Growth on medium containing 50 % glucose and 10 % (w/v) sodium chloride/5 % (w/v) is present for *O. tropicalis* f.a. sp. nov. but absent for the type strain of *O. brasiliensis* f.a. *O. brasiliensis* f.a. grows on medium containing 0.01 % cycloheximide but *O. tropicalis* f.a. sp. nov. does not. The phenotypic characteristics of the novel species reveal few differences from *O. externus*. These differences include that *O. tropicalis* f.a. sp. nov. assimilates *N*-acetylglucosamine and creatine but *O. externus* does not, growth on medium containing 50 % glucose is present for *O. tropicalis* f.a. sp. nov. but absent for *O. externus* and that no growth occurs with 0.01 % cycloheximide and 0. 1 % cycloheximide for *O. tropicalis* f.a. sp. nov., but growth occurs for *O. externus*.

Two species of yeast of the genus *Occultifur* have been reported to be mycoparasites, i.e. *O. internus*, which is a parasite of dacrymycetaceous fungi, and *O. corticiorum*, which is a parasite of *Hyphoderma praetermissum*, whereas one species of the genus, namely *O. externus*, may be as saprophyte because it has been reported to have been isolated from plant litter (Sampaio & Oberwinkler, 2011). All 10 strains of *O. brasiliensis* f.a. were isolated from water tanks of *Vriesea minarum*, indicating that this is an important habitat for it. In this study, two strains (DMKU-SE38 and DMKU-SE59) of the novel species were isolated from sugar cane leaf tissue, two strains (DMKU-SP385 and DMKU-SE403) were isolated from the surfaces of sugar cane leaves collected in Thailand and one strain was isolated from rhizoplane of sugar cane collected in Brazil. However, it was difficult to conclude that the novel species is associated with sugar cane because sugar cane is a cultivated plant so the novel species might be an exotic microbe. It should be noted that both Thailand and Brazil are in the tropics, but they are on opposite sides of the planet.

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ORIGINAL PAPER

Assessment of endophytic yeast diversity in rice leaves by a culture-independent approach

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Abstract Endophytic microorganisms inhabit internal plant tissues in the host plant without causing any symptoms or negative effects. Although the diversity of endophytes has been evaluated by both culture-dependent and culture-independent methods, less information is available on yeast communities. Therefore, in this study a culture-independent method was used to examine endophytic yeasts associated with rice leaves based on the large subunit of ribosomal DNA using a semi-nested PCR technique. Sequence analysis indicated that the colonization frequency and the relative species frequency (RF) of endophytic yeast phylotypes were 0.41 and 0.06, respectively, and the majority of the yeast phylotypes were basidiomycetous yeasts. The phylotypes were designated as five known species (*Cryptococcus victoriae, Debaryomyces hansenii, Debaryomyces vindobonensis, Meyerozyma guilliermondii* and *Pseudozyma antarctica*), together with seventeen phylotypes closest to *Candida metapsilosis, Cryp. foliicola, Cryp. laurentii, Pseudozyma abaconensis, Pseudozyma aphidis* and *Trichosporon asahii*, among which some could be novel species. The most prevalent phylotypes were those closest to *Cryp. foliicola* (47.5 % RF) followed by *D. hansenii* (22.8 % RF) and *P. antarctica* (16.8 % RF). The presence of the phylotypes related to species known for their potential applications as biocontrol agents and plant growth promoting hormone producers suggests that they may have valuable applications. In addition, our findings revealed the occurrence of novel phylotypes at high frequency, which should encourage extensive studies to discover novel yeast species and to understand their roles in the rice leaves.

Keywords Endophyte · Yeast · Rice · Culture-independent diversity · PCR · *Cryptococcus*

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Introduction

Endophytic microorganisms comprising bacteria, filamentous fungi and yeasts inhabit internal tissues of

the host plant without causing any symptoms or negative effects (Petrini 1991). Endophytes have been receiving attention due to their ability to produce various beneficial bioactive compounds which are involved in plant health and adaptation to both abiotic and biotic stress and thus have potential agricultural, industrial and pharmaceutical applications (Trindade et al. 2002; Arnold et al. 2003; Tian et al. 2004; Higginbotham et al. 2013). At present it is commonly accepted that almost every plant species is inhabited by endophytic fungi and that more than one type of fungal endophyte may be found within a single plant (Petrini et al. 1992). Endophytes associated with crop plants, including rice have been reported. The reports have focused on bacterial and filamentous fungal community structures and their biological activities, but only a few reports have described the isolation, localization or diversity of endophytic yeasts. For instance, *Fusarium* and *Streptomyces* were respectively predominant endophytic fungi and actinomycetes in rice leaves and roots from Guangdong province in southern China while yeasts were detected only in leaves and only at low frequency (3.7 %); no yeasts were detected in roots (Tian et al. 2004). Yuan et al. (2010) characterized the fungal communities in the roots of rare wild rice (*Oryza granulate*) using both culture-dependent and culture-independent approaches. The results demonstrated that most of the fungal isolates belonged to Ascomycota with 34.5 % being of undescribed species, whereas the frequently detected clones were classified as Basidiomycota, 60.2 % being of unknown taxa. Among the detected clones, only one yeast phylotype was found; this was closely related to *Trichosporon mucoides*. To date endophytic yeasts have been isolated from a few plant species; for example *Cryptococcus* sp. and *Rhodotorula* sp. in leaves of tomato (Larran et al. 2001), *Cyberlindnera saturnus*, producing indole-3-acetic acid (IAA) and indole-3-pyruvic acid (IPYA), in roots of maize (Nassar et al. 2005), *Rhodotorula graminis* and *Rhodotorula mucilaginosa*, producing IAA, in stems of wild cottonwood and hybrid poplar (Xin et al. 2009).

Although microbial strains have been isolated by conventional methods, it has not been possible to isolate numerous microorganisms in the environment due to the fact that their cultivation conditions are unknown and that there is a possibility that some species might be unculturable. Culture-independent methods are increasingly employed to analyze the microbial diversity directly from tissue or environmental samples via DNA extraction, along with PCR amplification of nuclear ribosomal gene (rDNA) and separation techniques such as random cloning, amplified rDNA restriction analysis (ARDRA), amplified ribosomal intergenic spacer analysis (ARISA), denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), and terminal restriction fragment length polymorphism (T-RFLP) (Anderson and Cairney 2004; Nielsen et al. 2007; Yuan et al. 2010; Singh et al. 2011). These techniques are subject to biases and their own limitations because of the DNA quality, the primers used, copy numbers of the target gene and the length of amplified products (Jones and Richards 2011). To reduce biases, several primers have been developed as universal primers (Toju et al. 2012) and specific primers (White et al. 1990; Gardes and Bruns 1993; Harrison et al. 2011) together with the optimization of amplification programs such as nested PCR or semi-nested PCR which increases the sensitivity and/or specificity of PCR (Ibeas et al. 1996; Oros-Sichler et al. 2006; Chae et al. 2012; Díaz et al. 2013).

Rice is a major grain food crop and its production for both local consumption and export is the main agricultural activity in Thailand. Aware that endophytes possess beneficial biological properties and that attention has not yet been focused on endophytic yeasts, we aimed to evaluate the biodiversity of endophytic yeast in rice leaves using a culture-independent approach via semi-nested PCR for construction of a D1/D2 library followed by ARDRA and sequencing.

Materials and methods

Sample collection

Forty-six samples of healthy leaves of rice (*Oryza sativa*) were collected from rice fields in five provinces in Thailand between October 2011 and December 2011 (Table 1). Leaf samples were kept in ice boxes during collection and then at 4 °C in the laboratory. DNA extraction was conducted within 24 h after returning to the laboratory and not longer than 1 week after collection.

Table 1 Details of rice leaf samples used in this study

Region	Province	Location	Average atmospheric temperature (°C)	Annual rainfall (mm)	Sampling period	No. of samples	Sample code no.
North-East	Surin	14°52′48″N, 103°29′24E	26.4	1901.8	Oct, 2011	3	R1–R3
	Buriram	14°36′24″N 103°7′18″E	27.3	1655.5	Oct, 2011	2	R4–R5
	Sisaket	15°7′12″N, 104°19′18″E	26.4	1709.9	Oct, 2011	12	R6–R13, R15–R18
Central	Nakhon Pathom	14°1′16″N, 99°58′54″E	26.9	1295.5	Nov, 2011	24	R31–R40, R42–R55
East	Chachoengsao	13°41′15″N, 101°4′13″E	28.1	1706.5	Dec, 2011	5	R58–R59, R61–R62, R64

DNA extraction from leaf samples

The leaves were cut into fragments (3 cm) and surface-sterilized. The leaf fragments were submerged in 70 % (w/v) ethanol for 2.5 min, followed by 5 % (w/v) sodium hypochlorite for 2.5 min, and then washed 5 times by soaking in 50 ml sterile water and shaking for 2 min. The effectiveness of the surface sterilization procedure was verified by spreading 0.1 ml of the final rinse water onto yeast extract-malt extract (YM) agar (3 g l^{-1} yeast extract, 3 g l^{-1} malt extract, 5 g l^{-1} peptone, 10 g l^{-1} glucose and 20 g l^{-1} agar) and placing of a few leaf fragments directly onto YM agar. Furthermore, the efficiency of the removal of DNA from leaf surfaces was examined by the semi-nested PCR amplification (described below in PCR Amplification of D1/D2 domain of large subunit ribosomal DNA section) using DNA extracted from the pellets collected from the rinse water as well as washed leaves and unwashed leaves, that were submerged in washing buffer (1X phosphate buffered saline, Tween 20), and sonicated for 7 min using an ultrasonic bath (Yang et al. 2001), as DNA template. The quantity of the amplified D1/D2 fragment was calculated based on the approximate mass of 100 bp DNA ladder (NEB, USA).

Total genomic DNA from fresh surface-sterilized rice leaves was extracted according to the method of Kurtzman and Robnett (1998) with modification. The leaf sample was ground with mortar and pestle under liquid nitrogen and then treated with 2 × CTAB extraction buffer (2 % (w/v) CTAB, 100 mM Tris–HCl pH 8.4, 1.4 M NaCl, 25 mM EDTA) at 65 °C for 1 h with occasional gentle swirling. DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1), followed by chloroform:isoamyl alcohol (24:1). Then, DNA was precipitated by adding 0.54 volume of isopropanol and washed once with 70 % (w/v) ethanol and finally resuspended in 100 µl of sterile distilled water.

PCR Amplification of D1/D2 domain of the large subunit ribosomal DNA

The total genomic DNA extracted from rice leaves was used as a DNA template for the amplification of the D1/D2 domain of the large subunit ribosomal DNA (LSU rDNA) with primers, NL1 and NL4 (Kurtzman and Robnett 1998). However, considerable amplification of rice LSU rDNA was detected. To exclude this disturbance, semi-nested PCR was carried out. Forward primers for amplifying the ITS-D1/D2 domain as an outer fragment were sought out from fungal ITS primers previously published, NSI1 (Martin and Rygiewicz 2005), ITS1-F (Gardes and Bruns 1993), ITS1-F_KYO1 and ITS1-F_KYO2 (Toju et al. 2012). ITS1-F_KYO1 was selected due to its amplification performance in vitro as well as the high percentage of fungal taxa and low percentage of plant taxa amplified in silico (Toju et al. 2012). The primer pairs used for semi-nested PCR were ITS1-F_KYO1 and NL4 as the outer set to amplify the ITS-D1/D2 domain (~1300 bp) and NL1/NL4 as the inner set to amplify only the D1/D2 domain (~600 bp). Although the ITS-D1/D2 domain contained the D1/D2 domain, the amplified D1/D2 domain was used to construct the clone library due to its smaller size and higher yield. The

sequences of the primers used are shown in Table 2. Amplification of the ITS-D1/D2 domain was carried out in 25 µl of a reaction mixture containing 50 ng of genomic DNA, 1X PCR buffer, 1X Q-solution, 200 µM of each dNTP, 10 pmol of each primer of the outer set, and 2.5 U of Taq polymerase (Qiagen, Germany). The reaction was performed as follows: initial DNA denaturation at 94 °C for 5 min, followed by 45 cycles of denaturation at 94 °C for 1 min, annealing at 47 °C for 30 s and extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. For the first amplification, 45 cycles were used to enhance the yield of PCR product. Amplification of the D1/D2 domain was carried out in 25 µl of a reaction mixture containing 1/25 dilution of the ITS-D1/D2 PCR product, 1X PCR buffer, 1X Q-solution, 200 µM of each dNTP, 10 pmol of each primer of the inner set, and 2.5 U of Taq polymerase (Qiagen, Germany). The reaction was performed as follows: initial DNA denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 30 s and extension at 72 °C for 30 s and a final extension at 72 °C for 10 min. Visualization of the amplified DNA was performed by electrophoresis using 0.8 % agarose in 1X TBE buffer (0.09 M Tris–borate, 0.001 M EDTA; pH 8.0); it was then stained with ethidium bromide and observed under a UV illuminator. The expected amplified DNA fragment (~600 bp) was excised and purified with QIAquick® Gel Extraction Kit (Qiagen, Germany) according to the manufacturer's instructions.

Construction of clone libraries and phylogenetic analysis

The purified PCR products were ligated into pGEM-T$^{®}_{easy}$ (Promega, USA) and used to transform into *Escherichia coli* JM109. The randomly selected transformants containing the insert were confirmed by the colony PCR method with NL1 and NL4 primers and screened by amplified ribosomal DNA restriction analysis (ARDRA). PCR products having the correct size of insert were digested with the restriction enzymes, *Cfo*I, *Hae*III, and *Hin*fI (Guillamón et al. 1998) and then separated in 2 % agarose gel electrophoresis for 45 min. Clones were grouped into operational taxonomic units (OTUs) according to the RFLP pattern and the representative OTUs were sequenced by First Base Laboratories Sdn Bhd (Malaysia) with NL1 primer using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The sequences of the D1/D2 domain of the LSU rDNA were submitted to BLASTn search (Altschul et al. 1997) at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) to determine their similarity and phylogenetic affiliation. The sequences were aligned with related species by Multiple Alignment using Fast Fourier Transform (MAFFT) (Katoh and Standley 2013) at the European Bioinformatics Institute (EBI; http://www.ebi.ac.uk/) and the phylogenetic tree was constructed from the evolutionary distance data according to Kimura (1980) by the maximum likelihood method (Felsenstein 1981) using the MEGA

Table 2 Primers used in this study

Primer	Sequence 5′–3′	Position[a]	T_m (°C)	Reference
Forward				
NSI1	GATTGAATGGCTTAGTGAGG	1644–1663	59	Martin and Rygiewicz (2005)
ITS1-F	CTTGGTCATTTAGAGGAAGTAA	1723–1744	50	Gardes and Bruns (1993)
ITS1-F_KYO1	CTHGGTCATTTAGAGGAAGTAA	1723–1744	58	Toju et al. (2012)
ITS1-F_KYO2	TAGAGGAAGTAAAAGTCGTAA	1733–1753	56	Toju et al. (2012)
NL1	GCATATCAATAAGCGGAGGAAAAG	41–64	68	Kurtzman and Robnett (1998)
Reverse				
NL4	GGTCCGTGTTTCAAGACGG	655–637	60	Kurtzman and Robnett (1998)

[a] NSI1, ITS1-F, ITS1-F_KYO1 and ITS1-F_KYO2 primer positions are corresponding to a reference ribosomal RNA sequence of *Serpula himantioides* (AM946630) (Toju et al. 2012). NL1 and NL4 primer positions are corresponding to *Saccharomyces cerevisiae* NC_001144.5:455181-451786 (Porter and Golding 2012)

version 6.0 (Tamura et al. 2013) with 1000 bootstrap replicates.

Nucleotide sequence accession number

The nucleotide sequence data reported in this work were deposited in GenBank under the accession numbers KM872031 to KM872060.

Data analyses

The colonization frequency (CF) was calculated as number of leaf samples colonized by endophytic yeasts divided by the number of total leaf samples collected. The relative frequency (RF) was calculated as the number of clones in each yeast phylotype, species, genus or phylum divided by the total number of clones in the library, depended on the group involved. Diversity of the clone library was investigated by rarefaction analysis and the rarefaction curve was calculated using the RarefactWin program (Holland 2003).

Results

Diversity of endophytic yeasts by a culture-independent approach

Diversity of the endophytic yeast species in rice leaves was investigated by a culture-independent method in this study. Prior to DNA extraction, the sterility of the washed leaf surface was verified and the results showed that there was no growth of microorganisms. In case of the removal of DNA on the leaf surface, the quantity of PCR products of DNA obtained from the rinse water, washed leaves and unwashed leaves showed a significant reduction of the epiphytic yeast DNA since the quantity of amplified band at each washing step reduced gradually from 100 % in unwashed leaves (R0) to 0 % in the final rinse water (R5) and washed leaves (R6) (Fig. 1). The results indicated that most of the epiphytic yeast DNA was removed from the washed leaves. Due to the long distance from the sampling sites to the laboratory, total DNA from rice leaf samples collected in the Central Region (n = 24), the Eastern Region (n = 5) and the Northeastern Region (n = 17) was extracted within 1, 3 and 5 days of collection. The extracted DNA was used as template for the amplification of the D1/D2 domain of the LSU rDNA of endophytic yeasts. The D1/D2 domain of the LSU rDNA was successfully amplified by semi-nested PCR using ITS1-F_KYO1 and NL4 as the outer set and NL1/NL4 as the inner set. Therefore, 46 D1/D2 libraries were constructed and a total of 1826 randomly picked clones were grouped into 124 operational taxonomic units (OTUs), according to the ARDRA patterns obtained from the independent digestion by three restriction endonucleases: *Cfo*I, *Hae*III and *Hin*fI. Representative OTUs from each library were sequenced and analyzed. Through the BLAST analysis, 12 OTUs had sequences typical of yeasts (101clones), whereas 112 OTUs belonged to filamentous fungi (1725 clones) and no OTU matched the rice sequence. Moreover, the number of yeast phylotypes in rice leaves ranged from 0 to 3 phylotypes per sample. Among 46 leaf samples, yeast phylotypes were found in 19 leaf samples, whereas all leaf samples contained filamentous fungal phylotypes. The results revealed that the primer sets used in this study effectively amplified the fungal D1/D2 domain of the LSU rDNA and that yeasts were in a minority among endophytic fungi in rice leaves. The CF and the RF of endophytic yeast phylotypes in rice leaves were 0.41 and 0.06, respectively. The rarefaction curve did not reach a horizontal asymptote (Fig. 2), indicating that more species could be retrieved. Since the objective of this work is the assessment of the biodiversity of endophytic yeast in rice leaves, only yeast clones (101 clones) were further analyzed and are discussed.

Based on sequence analysis of the D1/D2 domain of the LSU rDNA, the D1/D2 clone sequences of endophytic yeasts derived from rice leaves were identified and affiliated by their similarity and phylogenetic relationships to the sequences of the closest related strains in NCBI and related type strains. The criteria described by Kurtzman and Robnett (1998), namely, that ascomycetous yeast strains with 0 to 3 nucleotide differences in the D1/D2 domain (~600 bp) are either conspecific or sister species and yeast strains showing nucleotide substitutions greater than six are usually different species and the criteria of Fell et al. (2000), namely, that the majority of basidiomycetous yeast can be identified using the D1/D2 region and strains that differed by two or more nucleotides represent different taxa, were used as the guidelines. A total of 101 sequences of yeast clones

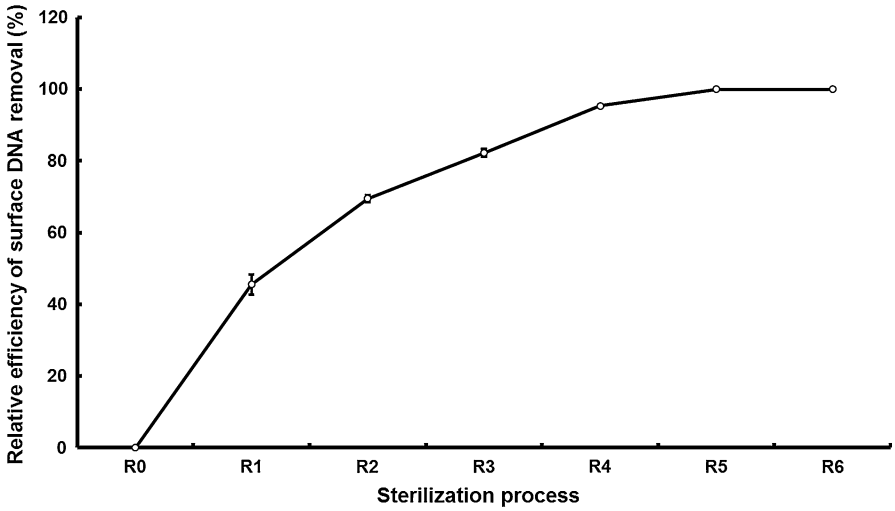


Fig. 1 Relative efficiency of the removal of DNA from rice leaf surface by the sterilization protocol. Values are mean ± standard deviation and N = 3 for each treatment; R0 is the unwashed leaves, R1–R5 are the 1st–5th rinse water and R6 is washed leaves. The relative efficiency of the removal of DNA was calculated as a percentage of the quantity of amplified D1/D2 fragment of each sample divided by the quantity of amplified D1/D2 fragment of unwashed leaf sample

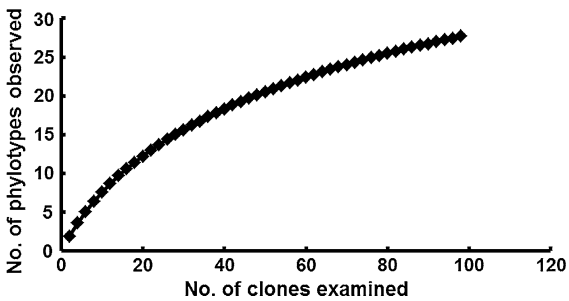


Fig. 2 Rarefaction curve of endophytic yeast clones derived from rice leaves

showed that 12 OTUs were composed of 28 different phylotypes (Table 3). According to the sequence analysis by BLAST search, three OTUs had diverse phytotypes; ten phylotypes closest to *Cryptococcus foliicola* in OTU1, one phylotype closest to *Pseudozyma antarctica* and one phylotype closest to *P. aphidis* in OTU8, and six phylotypes closest to *Debaryomyces hansenii* and one phylotype closest to *Debaryomyces vindobonensis* in OTU11. The closest related species of the clone sequences showed nine (26 clones, 25.7 % RF) and nineteen (75 clones, 74.3 % RF) phylotypes belonged to the Ascomycota and Basidiomycota, respectively. The genera belonging to the Ascomycota consisted of *Candida* (one phylotype in the *Lodderomyces–Spathaspora* clade), *Debaryomyces* (seven phylotypes) and *Meyerozyma* (one phylotype), whereas the genera belonging to the Basidiomycota were *Cryptococcus* (two phylotypes in the *Bulleromyces* clade and twelve phylotypes in the *victoriae* clade), *Pseudozyma* (two phylotypes) and *Trichosporon* (one phylotype). The phylotypes, most closely related to type strains of ascomycetous yeasts, which were either identical or had only two to three nucleotides differences (Kurtzman and Robnett 1998) and the phylotypes most closely related to basidiomycetous yeasts, having fewer than two nucleotides difference (Fell et al. 2000), were designated as five known species (*Cryp. victoriae, D. hansenii, D. vindobonensis, Meyerozyma guilliermondii* and *P. antarctica*), whereas the others may be novel species closest to *Candida metapsilosis, Cryp. foliicola, Cryp. laurentii, Pseudozyma abaconensis, Pseudozyma aphidis* and *Trichosporon asahii* (Table 4). The majority of the endophytic yeast phylotypes in rice leaves were basidiomycetous yeasts and the most prevalent phylotypes were the ones closest to *Cryp. foliicola* (47.5 % RF) followed by *D. hansenii* (22.8 % RF) and *P. antarctica* (16.8 % RF). The phylotypes close to *C. metapsilosis, Cryp. laurentii, D. vindobonensis, M. guilliermondii, P. aphidis, P. abaconensis* and *T. asahii* were found at the low frequency of 1.0–5.0 % (Table 4). The phylogenetic tree showed that the analyzed sequences were distributed in four taxonomic orders, namely,

Table 3 Number of phylotypes per OTU and their RFLP patterns

OTU	Identification[a]	Phylotype ID[b]	No. of clones	D1/D2 amplified product (bp)	RFLP pattern		
					*Cfo*I	*Hae*III	*Hin*fI
Basidiomycota							
1	Closest to *Cryptococcus foliicola*	3R32	12	607	507, 100	607	411, 192, 4
		5R2	3	606	507,99	606	411, 192, 5
		10R21	4	606	509, 97	606	413, 191, 2
		31R22	2	598	506, 92	598	410, 188
		33R2	3	604	506, 94	604	410, 194
		34R2	3	607	507, 100	607	411, 193, 3
		35R27	8	606	507, 99	606	411, 192, 3
		37R42	4	605	507, 98	605	411, 192, 2
		38R1	5	603	507, 96	603	411, 192
		40R4	2	606	508, 98	606	412, 191, 3
2	Closest to *Cryptococcus foliicola*	3R33	1	600	332, 268	348, 93, 50, 48,35, 26	412, 141, 47
3	Closest to *Cryptococcus foliicola*	15R19	1	606	606	606	413, 193
4	Closest to *Cryptococcus laurentii*	43R8	3	596	332, 211, 53	273, 155, 93, 75	412, 137, 47
5	Closest to *Cryptococcus laurentii*	43R21	2	605	605	529, 76	413, 192
6	*Cryptococcus victoriae*	64R2	2	606	507, 99	531, 75	411, 192, 3
7	Closest to *Pseudozyma abaconensis*	58R6	1	627	273, 178, 176	430, 123, 39, 35	239, 194, 184, 10
8	*Pseudozyma antarctica*	44R1	17	613	448, 165	371, 123, 48, 39, 32	236, 193, 184
	Closest to *Pseudozyma aphidis*	51R6	1	623	449, 174	380, 123, 48, 39, 33	237, 202, 184
9	Closest to *Trichosporon asahii*	3R41	1	607	509, 98	531, 72, 4	230, 183, 192, 2
Ascomycota							
10	Closest to *Candida metapsilosis*	33R1	1	579	579	398, 111, 52, 18	386, 145, 48
11	*Debaryomyces hansenii*	13R4	1	580	580	416, 164	389, 144, 47
		31R1	6	581	581	414, 167	392, 142, 47
		31R5	1	577	577	414, 163	388, 142, 47
		34R6	1	578	578	414, 164	389, 142, 47
		40R1	12	574	574	409, 165	390, 137, 47
		48R31	2	580	580	416, 164	389, 144,47
	Debaryomyces vindobonensis	47R6	1	581	581	416, 165	390, 144, 47
12	*Meyerozyma guilliermondii*	37R32	1	578	525, 53	415, 163	218, 170, 143, 47

[a] Identification of each phylotype was based on BLASTn analysis

[b] Phylotype ID was designated as sample No. followed by clone No.

Table 4 Distribution of D1/D2 clone sequences derived from endophytic yeasts in rice leaves

Phylotype ID[a]	Accession no.	Relative frequency (%)	Closest related type strain (accession no.)	Nucleotide substitution/total nucleotide	Identification
Basidiomycota					
37R42	KM872049	3.96	*Cryptococcus foliicola* CBS 9920^T (AY557599)	2/605	Closest to *Cryptococcus foliicola*
40R4	KM872052	1.98	*Cryptococcus foliicola* CBS 9920^T (AY557599)	2/606	Closest to *Cryptococcus foliicola*
35R27	KM872047	7.92	*Cryptococcus foliicola* CBS 9920^T (AY557599)	3/606	Closest to *Cryptococcus foliicola*
38R1	KM872050	4.95	*Cryptococcus foliicola* CBS 9920^T (AY557599)	3/603	Closest to *Cryptococcus foliicola*
31R22	KM872040	1.98	*Cryptococcus foliicola* CBS 9920^T (AY557599)	3/598	Closest to *Cryptococcus foliicola*
33R2	KM872042	2.97	*Cryptococcus foliicola* CBS 9920^T (AY557599)	3/604	Closest to *Cryptococcus foliicola*
34R2	KM872044	2.97	*Cryptococcus foliicola* CBS 9920^T (AY557599)	4/607	Closest to *Cryptococcus foliicola*
10R21	KM872035	3.96	*Cryptococcus foliicola* CBS 9920^T (AY557599)	5/606	Closest to *Cryptococcus foliicola*
5R2	KM872034	2.97	*Cryptococcus foliicola* CBS 9920^T (AY557599)	5/606	Closest to *Cryptococcus foliicola*
3R32	KM872031	11.88	*Cryptococcus foliicola* CBS 9920^T (AY557599)	11/607	Closest to *Cryptococcus foliicola*
15R19	KM872037	0.99	*Cryptococcus foliicola* CBS 9920^T (AY557599)	17/606	Closest to *Cryptococcus foliicola*
3R33	KM872032	0.99	*Cryptococcus foliicola* CBS 9920^T (AY557599)	63/600	Closest to *Cryptococcus foliicola*
43R21	KM872054	1.98	*Cryptococcus laurentii* CBS 139^T (AF075469)	5/605	Closest to *Cryptococcus laurentii*
43R8	KM872053	2.97	*Cryptococcus laurentii* CBS 139^T (AF075469)	51/596	Closest to *Cryptococcus laurentii*
64R2	KM872060	1.98	*Cryptococcus victoriae* CBS8685^T (AF363647)	1/606	*Cryptococcus victoriae*
58R6	KM872059	0.99	*Pseudozyma abaconensis* CBS8380^T (FJ008047)	6/627	Closest to *Pseudozyma abaconensis*
44R1	KM872055	16.83	*Pseudozyma antarctica* CBS 214.83^T (AJ235302)	0/613	*Pseudozyma antarctica*
51R6	KM872058	0.99	*Pseudozyma aphidis* CBS517.83^T (AB089363)	3/623	Closest to *Pseudozyma aphidis*
3R41	KM872033	0.99	*Trichosporon asahii* CBS 2479^T (AF105393)	19/607	Closest to *Trichosporon asahii*
Ascomycota					
33R1	KM872041	0.99	*Candida metapsilosis* ATCC 96144^T (FJ746055)	5/579	Closest to *Candida metapsilosis*
48R31	KM872057	1.98	*Debaryomyces hansenii* NRRL Y-7426^T (JQ689041)	0/580	*Debaryomyces hansenii*
13R4	KM872036	0.99	*Debaryomyces hansenii* NRRL Y-7426^T (JQ689041)	1/580	*Debaryomyces hansenii*
31R5	KM872039	0.99	*Debaryomyces hansenii* NRRL Y-7426^T (JQ689041)	1/577	*Debaryomyces hansenii*

Table 4 continued

Phylotype ID[a]	Accession no.	Relative frequency (%)	Closest related type strain (accession no.)	Nucleotide substitution/total nucleotide	Identification
34R6	KM872045	0.99	*Debaryomyces hansenii* NRRL Y-7426^T (JQ689041)	2/578	*Debaryomyces hansenii*
40R1	KM872051	11.88	*Debaryomyces hansenii* NRRL Y-7426^T (JQ689041)	2/574	*Debaryomyces hansenii*
31R1	KM872038	5.94	*Debaryomyces hansenii* NRRL Y-7426^T (JQ689041)	3/581	*Debaryomyces hansenii*
47R6	KM872056	0.99	*Debaryomyces vindobonensis* CBS:11666^T (FN598875)	3/581	*Debaryomyces vindobonensis*
37R32	KM872048	0.99	*Meyerozyma guilliermondii* NRRL Y-2075^T (JQ689407)	2/578	*Meyerozyma guilliermondii*

[a] Phylotype ID was designated as sample No. followed by clone No.

Ustilaginales, Trichosporonales and Tremellales in Basidiomycota (Fig. 3) and Saccharomycetales in Ascomycota (Fig. 4). The majority of phylotypes were affiliated in the order Tremellales and most of them were located in the *victoriae* clade. The tree also revealed the clusters of the novel sequences (15R19, 3R33 and 43R8), which did not include any reference sequences as their phylogenetic neighbors in the *victoriae* clade, Tremellales. Moreover, the other phylotypes were affiliated in the genera *Candida* in the *Lodderomyces-Spathaspora* clade, *Debaryomyces*, *Meyerozyma*, *Pseudozyma* and *Trichosporon.* Remarkably, the basidiomycetous phylotypes with two or more nucleotides differences were grouped in different clusters of the closest related type strains, implying that they may belong to novel yeast species.

Discussion

Investigation of endophytic yeast species in rice leaves was carried out by a culture-independent method. Therefore, the removal of viable cells and their DNA on the leaf surface should be performed to avoid the contamination of the endophytic population with epiphytes. After treating the leaves with 70 % (w/v) ethanol for microbial decontamination and 5 % (w/v) sodium hypochlorite for DNA decontamination together with several times washing with sterile water, no growth of microorganisms and no PCR products of the D1/D2 domain from the washed leaves were detected, indicating adequate sterilization and DNA removal of the leaf surface, respectively. The inoculation of *Saccharomyces cerevisiae* as a control organism on the leaf surface of wheat was proposed for the evaluation of the surface sterilization efficiency in culture-independent fungal endophyte studies (Burdorf et al. 2014); however, a known species used as control organism must not be an endophyte of the plant and must produce amplified products that are different in size from the native endophytes. Therefore, we used epiphytic microorganisms as control organisms to elucidate the reduction in the epiphytic DNA and the comparison of the PCR products with and without sterilization revealed that the sterilization process could significantly reduce the contamination of epiphytic yeast DNA and sufficiently prevent the amplification of the D1/D2 domain of the epiphytic yeast DNA. However, a small amount of residual DNA may remain on the leaf surface. Furthermore, it is possible that epiphytic yeasts colonized the inner tissues of the leaves prior to surface sterilization due to the long storage period of leaf samples collected in the Eastern and Northeastern regions.

Although internal transcribed spacer (ITS) has been proposed as the official fungal barcode, a culture-independent method based on LSU rDNA analyses was used because LSU rDNA is primarily used for standard identification of yeasts and has less variation in length than ITS. Also, there is an extensive reference database for taxonomic study. The primers: NL1 and NL4, have been commonly used for amplification of the D1/D2 domain of pure cultures, but have been applied to mixed DNA samples in only a few instances such as investigations of fruit-associated fungal communities (Taylor et al.

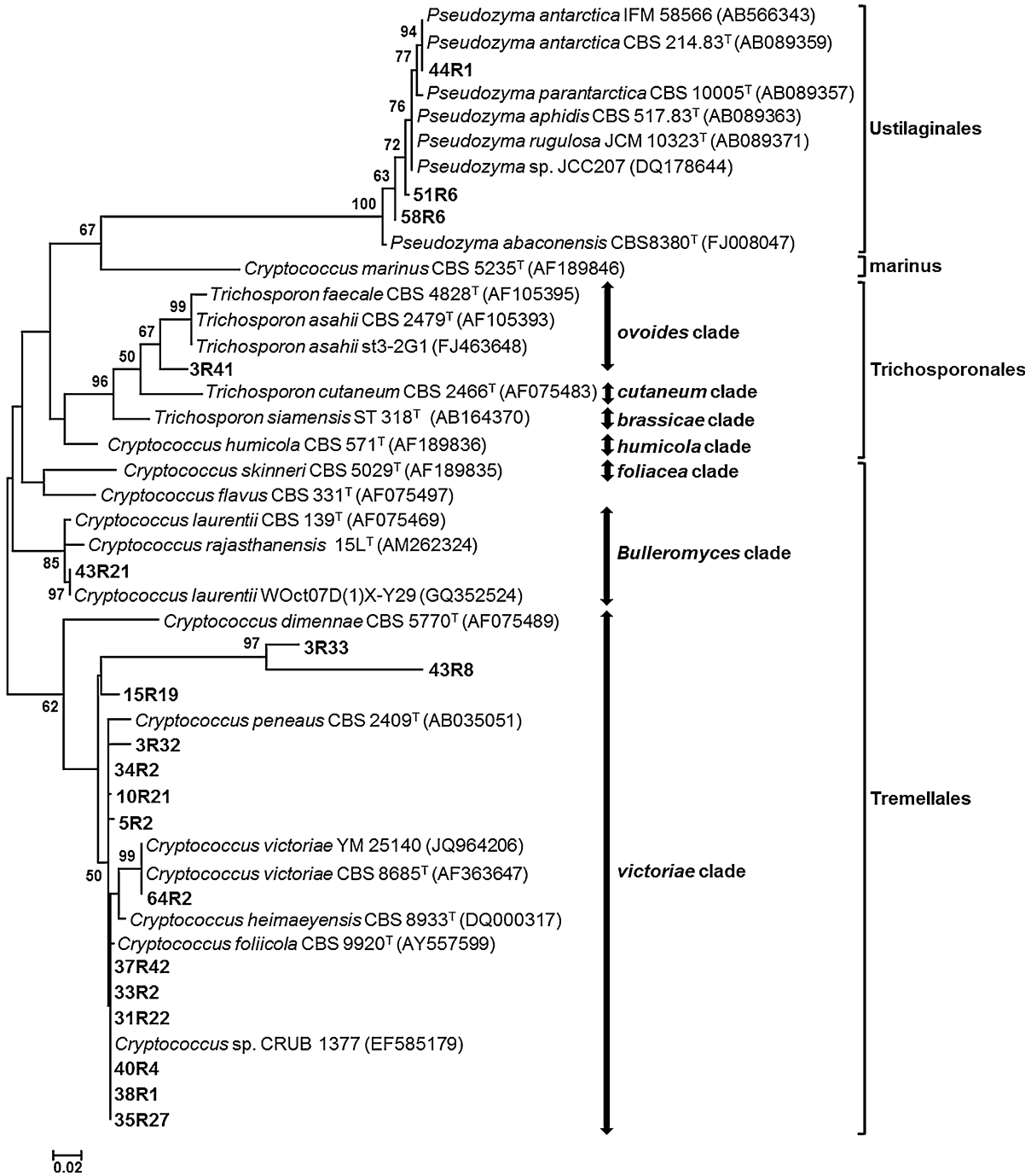


Fig. 3 Phylogenetic tree, based on the sequences of the D1/D2 region of the LSU rDNA, showing the placement of basidiomycetous phylotypes with respect to closely related species. The phylogenetic tree was constructed from evolutionary distance data with Kimara's two-parameter correction, using the maximum likelihood method and MEGA version 6.0. *Numbers* at nodes indicate percentages of bootstrap sampling derived from 1000 replicates and bootstrap percentages higher than 50 % are shown. *Bar* 0.02 Knuc. Sequences obtained in the present study are printed in *bold letters* and the sequences of type strains are indicated by *superscript T*

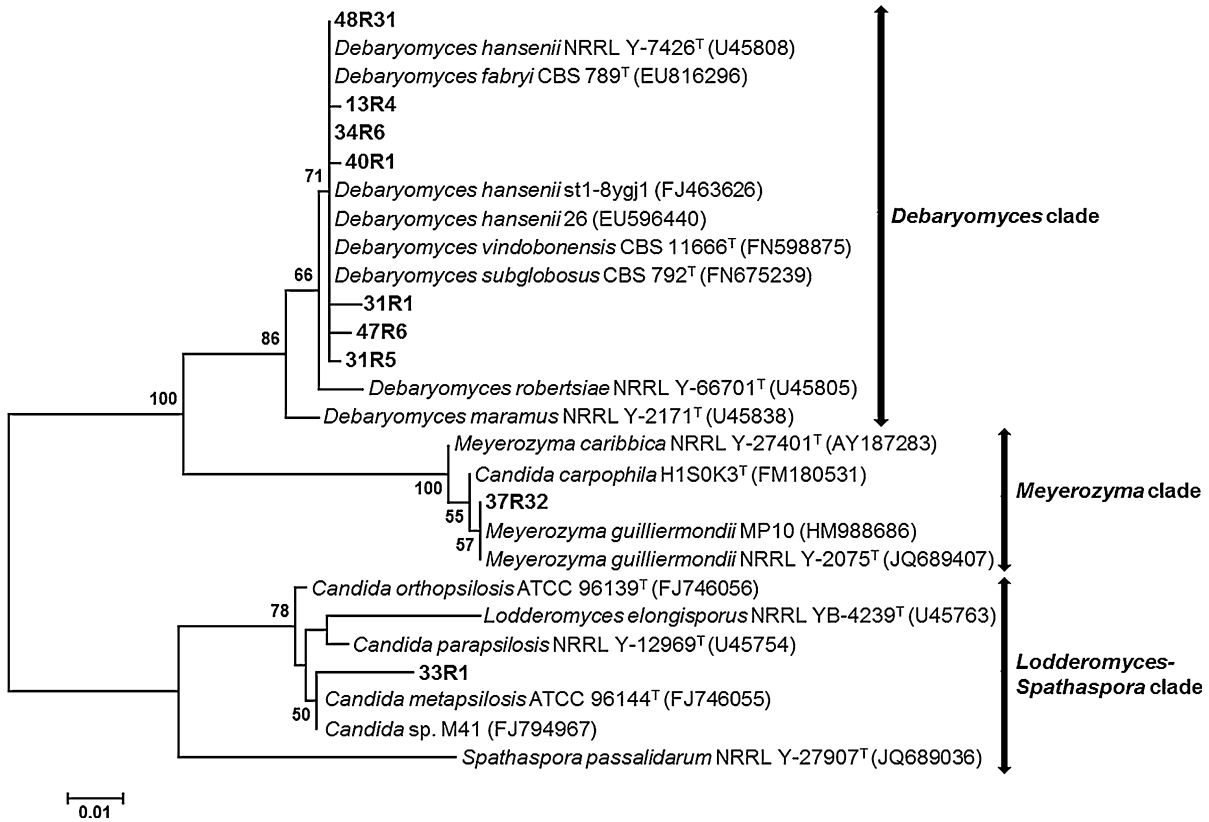


Fig. 4 Phylogenetic tree, based on the sequences of the D1/D2 region of the LSU rDNA, showing the placement of ascomycetous phylotypes with respect to closely related species. The phylogenetic tree was constructed from evolutionary distance data with Kimara's two-parameter correction, using the maximum likelihood method and MEGA version 6.0. Numbers at nodes indicate percentages of bootstrap sampling derived from 1000 replicates and bootstrap percentages higher than 50 % are shown. *Bar* 0.01 Knuc. Sequences obtained in the present study are printed in *bold letters* and the sequences of type strains are indicated by *superscript T*

2014), of microorganisms associated with mosquitoes (Gusmão et al. 2010) and of yeast diversity associated with invasive bark beetle (Lou et al. 2014). Although there was no report about biased amplification of other eukaryotic sequences, our results showed that these universal primers could also amplify the LSU rDNA of rice, and this interfered with the amplification of fungal LSU rDNA. Nested PCR or semi-nested PCR, involving two sets of primers for two successive runs of PCR reaction, the second set intended to amplify a secondary target within the first run product, is used to increase the sensitivity and/or specificity of PCR products. Furthermore, PCR primers used in the exploration of microbial diversity in environmental samples should have high specificity for the representative target genes. The ITS1-F_KYO1 primer designed by Toju et al. (2012) showed enhanced coverage across diverse fungal taxa and the in silico analysis revealed that the primer matched 99 % of ascomycete and basidiomycete ITS taxa without significant taxonomic biases whilst inhibiting the amplification of plant sequences. Therefore, semi-nested PCR combined with the highly specific primers (ITS1-F_KYO1) was carried out to enhance the specific amplification of small amounts of fungal DNA compared to rice DNA in the reaction. The primer sets of ITS1-F_KYO1 and NL4 as the outer set and NL1/NL4 as the inner set used for semi-nested PCR confirmed their specificity to fungal sequences in the presence of many copies of rice LSU rDNA and the products of the D1/D2 domain were successfully obtained. However, the competition of yeast DNA with the large amount of filamentous fungal DNA resulted in numerous filamentous fungal sequences in

the clone libraries. Accordingly, only 12 OTUs (101 clones) out of 124 OTUs (1826 clones) were identified to be yeast phylotypes by the ARDRA technique followed by sequencing, homology search and phylogenetic analyses. The OTUs were clustered by the restriction patterns with different restriction enzymes for rapid identification; however, this technique was unable to distinguish the difference among the phylotypes related to *Cryp. foliicola* (OTU1), *P. antarctica* and *P. aphidis* (OTU8), and *D. hansenii* and *D. vindobonensis* (OTU11). Therefore, the sequence analysis was needed for the identification of these phytotypes. The yeast phylotypes found in 19 rice leaf samples ranged from 0 to 3 phylotypes per sample and among these phylotypes belonging to the Basidiomycota were dominant, while all samples contained filamentous fungi phylotypes (86–99 % similarity to closely related species) ranging from 2 to 11 phylotypes per sample. Moreover, the leaf samples collected from the Northeastern region, except for the sample No. R3, contained fewer endophytic phylotypes than those from Central and Eastern regions and only the filamentous fungal phylotypes related to *Passalora janseana* were observed. On the other hand, various genera of the filamentous fungal phylotypes; for instance *Bipolaris, Cladosporium, Passalora, Ramularia, Setosphaeria* and *Xylaria,* were found in the leaf samples collected from the Central and Eastern regions (data not shown), suggested that the sampling site may affect the distribution of fungal endophytes in rice leaf. The endophytic distribution in the plant is affected by various ecological factors such as geographic location, sampling site, season and type of plant tissue (Collado et al. 1999; Gai et al. 2009; Naik et al. 2009; Solis et al. 2015). However, the rarefaction curve indicated that additional sampling is needed to retrieve the remaining yeast species. This finding confirmed that filamentous fungi were relatively much more abundant than yeasts in rice leaves, corresponding to the culture-dependent study reported by Tian et al. (2004) that endophytic yeasts could be isolated only from leaves but not roots of rice plants at the isolation frequency of 3.7 %. Likewise, endophytic fungi of wild rice (*Oryzae granulate*) analyzed by a culture-independent approach based on ITS sequences showed that the phylotype accumulation curve did not reach saturation level. The frequently detected clones were classified as Basidiomycota and only one yeast phylotype, closely related to *T. mucoides*, was found (Yuan et al. 2010). On the culture-independent basis, the biases of clone library construction, PCR amplification condition and/or ARDRA screening may limit the sampling saturation (Jones and Richards 2011).

The endophytic yeast community in rice leaves was dominated by phylotypes that belonged to the Basidiomycota. The prevalent phylotypes were *Cryptococcus* spp. located in the *victoriae* clade, Tremellales. Interestingly, the novel phylotypes were found at high frequency (56.4 %), suggesting much more exploration of endophytic yeasts in rice leaves is needed. Our findings demonstrated that the endophytic yeast phylotypes consisted of five known species and seventeen novel phylotypes, indicating high proportion of novel yeast species in rice leaves. Although the contamination by epiphytic yeast DNA in this study is possible, it could be noted that the phylotypes stated in this study are endophytic yeasts in rice leaves according to the following reasons. The phylotypes related to *Cryp. foliicola*, *Cryp. victoriae*, *C. metapsilosis*, *D. hansenii*, *D. vindobonensis*, *P. abaconensis*, and *T. asahii* are endophytic yeasts in rice leaves because the assessment of epiphytic yeasts from rice leaves in Thailand by the enrichment technique (Limtong and Kaewwichian 2015) and culture-independent approach (Nasanit et al. 2015) showed that none of these yeast species were detected. In relation to the species reported as rice epiphytic yeast, the phylotypes related to *Cryp. laurentii* and *P. aphidis* may be novel phylotypes due to the high number of nucleotide substitutions (3–51 nucleotides), whereas *P. antractica* and *M. guilliermondii*, are probably endophytic phylotypes since their DNA was present in the leaf samples (37R and 44R), from which DNA was extracted within 24 h of collection. Hence, the colonization in the inner tissues of rice leaves probably occurred in the paddy field. Nonetheless, it should be noted that a culture-independent approach may not depict the actual yeast diversity in rice leaves due to the presence of large quantity of fungal DNA and the limitation of primer sets for specific annealing with the yeast LSU rDNA. The direct amplification method could remarkably enhance detection of endophytic yeasts that may be novel species and/or unculturable. Furthermore, the development of specific primers designed for amplification of yeast rDNA and the use of a multi primer approach might make it possible to

recover diverse phylotypes from the environmental samples of interest (Nakano et al. 2010; Singh et al. 2011; Singh et al. 2012).

The yeast phylotypes discussed in this study were reported to be associated with plants such as grasses, rice, sugarcane, medicinal plants, fruits, flowers and nectar as both epiphytes and endophytes (Middelhoven 1997; Trindade et al. 2002; Wang et al. 2011; Glushakova et al. 2014, Limtong and Kaewwichian 2015, Limtong et al. 2014) and the ones related to *Cryptococcus,* especially *Cryp. foliicola,* were found at high frequency. Moreover, the high frequency of occurrence of novel phylotypes, mainly the basidiomycetous ones, revealed undiscovered yeast species by a culture-dependent approach. *Cryp. foliicola* inhabits substrates of plant origins, such as leaves (Bakys et al. 2009; Wang et al. 2011) and nectar (Mittelbach et al. 2015), as well as thrives in the extreme environment, such as acidic (Russo et al. 2008) and cold (Arenz et al. 2010; de Garcia et al. 2012) environments. In the present study, the phylotypes related to *Cryp. foliicola* were found in leaf samples collected from the Northeastern and Central regions, mainly from Jasmine rice (3R, 5R, 10R, 15R, 33R, 34R and 35R). Although it is not possible to specify the variety of rice with which the other phytotypes were associated, the results suggested the possibility that they were associated with Jasmine rice. *D. hansenii,* the second most common phylotypes in the present study, is widespread in nature, such as the phylloplane, soil, and many habitats with low water activity (Suzuki et al. 2011). Moreover, some phylotypes presented in this study such as *M. guilliermondii,* and *D. hansenii* were reported as yeast associated with the brown planthopper (*Nilaparvata* lugens), an insect pest of rice (Hou et al. 2013), and *Cryp. laurentii* was isolated from internal organs of beetles (Vega and Dowd 2005). Therefore, it seems likely that insects are a route for the transmission of yeasts into plant tissues. Furthermore, it can be assumed that *M. guilliermondii, and P. antractica* might inhabit both the leaf surface and internal leaf tissue due to their occurrence as epiphytes (Limtong and Kaewwichian 2015; Nasanit et al. 2015) and endophytes (the present study). The occurrence of yeast species being both epiphyte and endophyte of the same plant species was recently reported by Khunnamwong et al. (2014) that *Wickerhamiella siamensis*, was a novel endophytic and epiphytic yeast species isolated from sugarcane leaf in Thailand. Furthermore, when the plant growth-promoting traits of epiphytic and endophytic yeasts isolated from rice leaves in Thailand were investigated, 13 strains of nine yeast species, including *M. guilliermondii,* produced indole-3-acetic acid (IAA) (Nutaratat et al. 2014). IAA production of epiphytic yeasts on sugarcane leaves in Thailand was also determined and the results revealed that 69 strains of 23 yeast species, including *Cryp. laurentii,* had the capability of producing IAA (Limtong et al. 2014). *Cryp. victoriae* isolated from fruits had high antagonistic activity against *Penicillium expansum*, *Pen. digitatum* and *Botrytis cinera* (Lutz et al. 2013; Ghosh et al. 2013). Trindade et al. (2002) reported that *P. antarctica* isolated from pitanga ripe fruits produced mycotoxin, protease, pectinase or β-glucosidase. *D. hansenii* isolates obtained from citrus fruits were proved to be a potential biocontrol agent against green mold (*Pen. digitatum*), blue mold (*Pen. italicum*) and sour rot (*Galactomyces candidum*) of citrus fruits (Chalutz and Wilson 1990; Hernández-Montiel et al. 2010). Although it is not possible to specify the roles of these phylotypes, the reviewed information suggests, there are potentially beneficial endophytic yeast strains in rice leaves.

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Compliance with Ethical Standards

Conflict of interest The authors declare that there are no conflicts of interest.

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The assessment of epiphytic yeast diversity in sugarcane phyllosphere in Thailand by culture-independent method

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ABSTRACT

The diversity of epiphytic yeasts from sugarcane (*Saccharum officinarum* Linn.) phyllospheres in Thailand was investigated by culture-independent method based on the analysis of the D1/D2 domains of the large subunit rRNA gene sequences. Forty-five samples of sugarcane leaf were collected randomly from ten provinces in Thailand. A total of 1342 clones were obtained from 45 clone libraries. 426 clones (31.7 %) were closely related to yeast strains in the GenBank database, and they were clustered into 31 operational taxonomic units (OTUs) with a similarity threshold of 99 %. All OTU sequences were classified in phylum *Basidiomycota* which were closely related to 11 yeast species in seven genera including *Cryptococcus flavus*, *Hannaella coprosmaensis*, *Rhodotorula taiwanensis*, *Jaminaea angkoreiensis*, *Malassezia restricta*, *Pseudozyma antarctica*, *Pseudozyma aphidis*, *Pseudozyma hubeiensis*, *Pseudozyma prolifica*, *Pseudozyma shanxiensis*, and *Sporobolomyces vermiculatus*. The most predominant yeasts detected belonged to *Ustilaginales* with 89.4 % relative frequency and the prevalent yeast genus was *Pseudozyma*. However, the majority were unable to be identified as known yeast species and these sequences may represent the sequences of new yeast taxa. In addition, The OTU that closely related to *P. prolifica* was commonly detected in sugarcane phyllosphere.

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Introduction

Phyllosphere or phylloplane, the aboveground surfaces of the plant, has been recognized as an important habitat for microorganisms. Bacteria, yeasts, and filamentous fungi can inhabit the phyllosphere using nutrients on leaf surfaces as carbon sources such as carbohydrates, amino acids, organic acids, sugar alcohols, and salts (Tukey 1970; Fiala *et al.* 1990;

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Weibull et al. 1990; Dik et al. 1991; Leveau & Lindow 2001). In addition, some epiphytic microorganisms have been reported to produce plant hormones which promote cell wall loosening and release of saccharides from plant cells as their substrates (Fry 1989; Brandl & Lindow 1998; Lindow & Brandl 2003). Furthermore, they may produce biosurfactants which facilitate wettability and further enhance the leaching of substrates (Bunster et al. 1989; Schreiber et al. 2005). The microbial communities of leaf surfaces depend on many factors including the availability of nutrients which is dependent on leaf age, plant species and growing conditions as well as environmental factors such as radiation, pollution and nitrogen fertilization. Moreover, biotic factors including competing microorganisms affect microbial communities of the plant phyllosphere (Irvine et al. 1978; Jumpponen & Jones 2010; Zimmerman & Vitousek 2012).

Sugarcane is a monocotyledonous plant of the family *Poaceae*. It is hypothesized that sugarcane originated in New Guinea (Daniels et al. 1996). To date, sugarcane is grown in more than 70 countries, mainly in tropical areas. In Thailand, sugarcane (*Saccharum officinarum* Linn.) is the second most important economic crop. The Office of Cane and Sugar Board under the jurisdiction of the Thailand Ministry of Industry reported in 2012–2013 approximately 1.52 million hectares was used for sugarcane cultivation with a production yield of 107.44 million tons of sugar. In 2013 the Food and Agriculture Organization of the United Nations reported Thailand was the fourth largest sugarcane producer. However, destructive plant diseases caused by pathogenic fungi are a major problem in the sugarcane cultivation industry. It has been previously reported that leaf surfaces are colonized by members of several genera of saprophytic yeasts and that these provide a natural barrier to protect against plant pathogens (Avis & Bélanger 2002; De Costa et al. 2006; Pusey et al. 2009). Therefore, the study of yeast communities associated with this crop and their antagonistic effects on pathogens may facilitate the application of novel and effective biocontrol approaches. In addition, yeast diversity data is fundamental to the comprehensive study of microbiological functions within nature. In recent years the diversity of yeast and fungal communities within the phyllosphere has been studied intensively. However, only a small number of these studies have focused on yeast communities associated with sugarcane (de Azeredo et al. 1998; Nakase et al. 2001; Limtong et al. 2014). De Azeredo et al. (1998) studied the yeast communities associated with sugarcane in Campos, Rio de Janeiro, Brazil by culture dependent method and reported that basidiomycetous yeasts were prevalent on leaf (79.6 %). The prominent yeast species isolated from leaf washings were in phylum *Basidiomycota* including *Cryptococcus albidus*, *C. laurentii*, and *Rhodotorula mucilaginosa*. Whereas *Debaryomyces hansenii* was the prevalent ascomycetous yeast species isolated from sugarcane leaves in Brazil. However using enrichment isolation technique the ascomycetous yeasts (69 %) were found to be more prevalent than basidiomycetous yeasts (31 %) in sugarcane phyllospheres in Thailand (Limtong et al. 2014). The predominant species was *Meyerozyma caribbica* followed by *Rhodotorula taiwanensis* and *Candida tropicalis*, respectively. Nakase et al. (2001) reported that yeasts isolated from phyllospheres of various plant species including sugarcane collected in Thailand by using the ballistoconidium-fall isolation method, were identified as 21 species within the following six genera *Bensingtonia*, *Bullera*, *Kockovaella*, *Sporidiobolus*, *Sporobolomyces*, and *Tilletiopsis*. These differing results appear to be due to the use of different isolation techniques. However, other factors that may affect yeast diversity and cannot be ruled out. It has been reported that environmental factors such as seasonal fluctuations and climatic conditions are likely to have an effect on microbial biodiversity (Glushakova and Chernov, 2010; Jumpponen & Jones 2010; Kachalkin & Yurkov 2012; Voriskova et al. 2014) as well as geography (Maksimova et al. 2009; Yurkov et al. 2015). Additionally, Finkel et al. (2011) demonstrated that microbial phyllosphere communities between plant species show significant similarity within the same locale, whereas trees of the same species and growing in different climatic regions are host to distinct microbial communities.

Since the 1980s, molecular based methods based on the direct amplification and analyses of ribosomal RNA genes have been developed for investigation of microbial diversity in diverse environments. These methods overcome the problems of culture-dependent methods as it is estimated that 99 % of microorganisms presented in nature are not typically able to be cultivated (Amann et al. 1995; Takaku et al. 2006). For yeast identification the rRNA gene has been preferred for molecular taxonomy over other genes because of (1) its universality, which allows comparisons to virtually any organism; (2) the presence of multiple gene copy numbers which evolve in concert; and (3) the belief that it is ancestrally homologous, having originated from a single evolutionary event (Sogin et al. 1986), allowing systematic study of yeast relationships. The nucleotide sequence of the D1/D2 domain of the large subunit (LSU) rRNA gene has been widely used to identify the majority of yeast species in phylums *Ascomycota* and *Basidiomycota* by direct sequence comparison, since the D1/D2 domain nucleotide sequences of nearly all known yeast species are available within the GenBank database (Kurtzman & Robnett 1998; Fell et al. 2000). The aim of this study was therefore to investigate the diversity of epiphytic yeasts of sugarcane phyllosphere using a culture-independent method based on the D1/D2 domain of the large subunit rRNA gene using a PCR based molecular technique.

Materials and methods

Sample collection

Forty-five samples of sugarcane (*S. officinarum* Linn.) leaf were randomly collected from sugarcane plantations in ten provinces in Thailand between January 2012 and April 2012 (Table 1). Leaf samples were stored in plastic bags, sealed and kept refrigerated on ice before transfer to the laboratory. The samples were stored at 4 °C until analysis. DNA was extracted from leaf washings of the samples within 7 d of collection.

DNA extraction of microbial epiphytes

Leaf samples (6 g) were cut and submerged in washing buffer (1× Phosphate buffered saline, Tween 20), sonicated for 7 min

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Table 1 – Sugarcane leaf samples collected from ten provinces in Thailand.

Province	Location	Sampling period	No. of samples	Average atmospheric temperature (°C)	Average rainfall (mm)
Buriram (BR)	14°59′39″N,103°6′8″E	Mar 2012	1	28.1	37.8
Chainat (CN)	15°11′6″N, 100°7′29″E	Mar 2012	3	30.0	29.9
Lopburi (LB)	14°48′0″N,100°37′37″E	Apr 2012	1	30.8	51.9
Nakhon Pathom (NP)	13°49′14″N,100°3′45″E	Jan 2012	2	27.3	19.9
Nakhon Sawan (NK)	15°42′48″N, 100°8′2″E	Mar 2012	3	30.0	29.9
Phetchabun (PB)	16°25′1″N, 101°9′12″E	Mar 2012	14	30.0	29.9
Phichit (PC)	16°26′38″N,100°20′52″E	Mar 2012	1	30.0	29.9
Phitsanulok (PN)	16°48′57″N, 100°15′49″E	Mar 2012	4	30.0	29.9
Suphanburi (SP)	14°28′3″N, 100°7′1″E	Mar 2012	9	30.0	29.9
Saraburi (SB)	14°31′43″N, 100°54′41″E	Apr 2012	7	30.8	51.9

using an ultrasonic cleaning bath (Bransonic, USA) to dislodge microbes from sample surfaces. The leaf washing was centrifuged at 5000× *g* for 5 min and the supernatant discarded. For rapid extraction of DNA from cell pellets the method of (Makimura *et al.* 1994) was performed with slight modification. Briefly, the cell pellet was suspended in 100 µl of lysis buffer (100 mM Tris–HCl, pH 7.5, 0.5 % w/v SDS, 30 mM EDTA) and incubated at 95 °C for 15 min. 100 µl of 2.5 M potassium acetate was added and incubated on ice for 60 min, then centrifuged at 12 000× *g* for 5 min. The supernatant was transferred to a new tube and DNA precipitated with an equal volume of isopropanol, then washed with 70 % ethanol. The precipitated DNA was air dried, resuspended in 30 µl of TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) and stored at −20 °C.

Amplification of the D1/D2 domain of the large subunit (LSU) rRNA gene

The D1/D2 domain of the LSU rRNA gene was amplified by PCR prior to sequencing using the following universal primers: NL-1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and NL-4 (5′-GGT CCG TGT TTC AAG ACG G-3′) to identify ascomycetes and basiomycetes as reported by Kurtzman & Robnett (1998) and Fell *et al.* (2000). The PCR amplification step was carried out in 25 µl of reaction mixture consisting of 10–100 ng of genomic DNA, 1X PCR buffer (Vivantis, USA), 1.5 mM $MgCl_2$ (Vivantis, USA), 100 µM of each dNTP (Vivantis, USA), 20 pmol of each primer, and 1 U of *Taq* DNA polymerase (Vivantis, USA). The PCR reactions were performed using the Geneamp PCR system 2400 (Perkin Elmer, USA). The reaction conditions were as follows; 94 °C for 5 min; 35 cycles of 45 s at 94 °C, 30 s at 52 °C, and 45 s at 72 °C; and a final extension step at 72 °C for 10 min. PCR products with expected size of 600 bp were visualized on a 1 % agarose gel (w/v) and purified using an Nucleic Acid Extraction Kit (Vivantis, USA).

Clone library and screening

The purified PCR products were ligated into the pTG19-T cloning vector (Vivantis, US) using the manufacturer's recommended protocol, and transformed into *E. coli* DH5α competent cells. The recombinant clones were screened by colony PCR using the primers described above. A cell suspension volume of 2 µl was used in the reaction mix as template. The reaction conditions were 94 °C for 10 min; 35 cycles of 45 s at 94 °C, 30 s at 52 °C, and 45 s at 72 °C; and a final extension at 72 °C for 10 min. PCR products were visualized on a 1 % agarose gel (w/v).

Restriction analyses

PCR products of the expected size were analysed using restriction fragment length polymorphism (RFLP) by digestion with the restriction enzymes, *Hae*III, *Hin*fI, and *Cfo*I. The restriction fragment patterns were visualized using a 2 % agarose gel (w/v). The RFLP patterns were clustered and representatives of the PCR products from each pattern of each clone library were further purified and sequenced.

Sequencing and phylogenetic analyses

DNA sequencing was performed using Sanger dideoxy sequencing with four colour fluorescence and the ABI Prism 3730XL DNA analyser (Applied Bioscience, USA) and NL1 primers described above at the DNA sequencing services, Bio Basic Inc. (Ontario, Canada). The DNA sequences of the D1/D2 domains were submitted to BLASTn homology search (Altschul *et al.* 1997). The collection of taxonomically related type sequences and related matches were obtained from GenBank for further analyses. The sequences were aligned with related species using CLUSTALW (Thompson *et al.* 1994). The yeast related sequences were clustered into operational taxonomic units (OTUs) at 99 % homology using the furthest neighbour approach of Mothur v.1.10 (Schloss *et al.* 2009). A phylogenetic tree was constructed from the evolutionary distance data using the Kimura 2-parameter model (Kimura 1980) and the neighbour-joining method (Saitou & Nei 1987) performed with MEGA 6 (Tamura *et al.* 2013). All positions containing gaps were eliminated. Confidence level of the clades was estimated using bootstrap analysis (1000 replicates).

Yeast related sequence data has been submitted to the GenBank database under the following accession numbers: KM114629–KM114634, KM114636–KM114682, and KP058903–KP058927.

Data analyses

The community diversity was calculated using the Shannon–Wiener index (*H*) and community evenness with Shannon equitability index (E_H). Relative frequency (%) was

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Table 2 – Distribution of amplified clones, RFLP fragments of D1/D2 DNA sequences and closest yeast species from BLAST analysis of 45 sugarcane samples.

Samples	No. of positive clones[a]	No. of yeast OTUs/total OTUs	No. of clones	D1/D2 size (bp)	RFLP[b]			Closest species (Accession no.)	Identity (%)	GenBank accession no.
					*Hae*III	*Hinf*I	*Cfo*I			
Buriram										
S60BR	37	1/7	10	588	44,58,74,155,257	186,402	71,172,345	*Jaminaea angkoriensis* (EU587489)	99.7 (582/584)	KM114673
Chainat										
S24CN	19	2/2	15	580	26,39,123,392	166,184,230	136,444	*Pseudozyma prolifica* (AB089369)	99.3 (537/541)	KM114644
			4	567	20,39,44,75,389	163,404	133,187,247	*Pseudozyma hubeiensis* (DQ008953)	92.5 (532/575)	KM114645
S25CN	37	5/11	22	594	20,39,123,412	184,186,224	158,436	*Pseudozyma prolifica* (AB089369)	99.1 (557/562)	KM114646
			6	595	21,39,48,123,364	184,186,225	158,437	*Pseudozyma aphidis* (AB089363)	99.5 (559/562)	KM114647
			1	597	44,66,70,74,86,257	183,414	83,172,342	*Jaminaea angkoriensis* (EU587489)	99.0 (509/514)	KM114648
			1	588	44,59,74,154,257	185,403	46,72,172,298	*Jaminaea angkoriensis* (EU587489)	99.5 (587/590)	KM114649
			1	594	20,39,123,412	184,186,224	156,438	*Pseudozyma prolifica* (AB089369)	98.6 (554/562)	KP058905
S26CN	21	3/12	3	604	31,39,123,411	184,185,235	157,447	*Pseudozyma prolifica* (AB089369)	98.9 (556/562)	KM114650
			1	588	18,39,119,412	186,402	158,187,243	*Pseudozyma shanxiensis* (DQ008955)	92.9 (553/595)	KM114651
			1	590	20,39,123,408	182,184,224	152,438	*Pseudozyma prolifica* (AB089369)	99.3 (553/557)	KP058906
Lopburi										
S79LB	40	1/7	29	591	17,39,123,412	184,186,221	158,433	*Pseudozyma prolifica* (AB089369)	98.6 (555/563)	KP058927
Nakhon Pathom										
S01NP	25	0/6								
S09NP	24	1/9	3	596	22,39,123,412	184,186,226	158,438	*Pseudozyma prolifica* (AB089369)	99.1 (557/562)	KM114629
Nakhon Sawan										
S27NK	19	1/8	3	575	214,361	11,183,184,197	155,420	*Pseudozyma aphidis* (AB089363)	96.8 (425/439)	KM114652
S28NK	30	4/19	1	590	20,39,123,408	182,184,224	154,436	*Pseudozyma prolifica* (AB089369)	99.1 (552/557)	KM114653
			2	589	20,39,123,407	84,97,184,224	153,436	*Pseudozyma hubeiensis* (DQ008953)	99.5 (586/589)	KM114654
			2	588	20,39,123,406	180,184,224	152,436	*Pseudozyma prolifica* (AB089369)	98.9 (550/556)	KP058907
			1	577	18,39,123,397	171,184,222	141,436	*Pseudozyma prolifica* (AB089369)	99.1 (551/556)	KP058908
S29NK	18	0/5								
Phetchabun										
S36PB	33	0/6								
S37PB	40	2/11	2	586	53,125,408	82,100,183,221	2,71,86,427	*Sporobolomyces vermiculatus* (AB279731)	99.0 (583/589)	KP058914
			2	591	18,39,123,411	184,185,222	157,434	*Pseudozyma prolifica* (AB089369)	98.9 (556/562)	KP058915
S38PB	22	1/7	1	591	18,39,123,408	181,184,222	153,434	*Pseudozyma prolifica* (AB089369)	99.3 (552/556)	KM114662
S40PB	39	1/11	23	593	25,39,123,406	180,184,229	152,441	*Pseudozyma prolifica* (AB089369)	99.1 (551/556)	KP058916
S41PB	39	4/11	13	591	17,39,123,412	184,186,221	158,433	*Pseudozyma prolifica* (AB089369)	98.6 (555/563)	KM114663
			2	585	20,565	67,75,114,329	154,431	*Pseudozyma prolifica* (AB089369)	78.4 (406/518)	KP058917
			1	592	18,26,39,123,386	184,186,222	46,112,434	*Pseudozyma hubeiensis* (DQ008953)	99.3 (588/592)	KM114664
			1	586	53,125,408	82,100,183,221	2,71,86,427	*Sporobolomyces vermiculatus* (AB279731)	99.0 (583/589)	KM114665
S42PB	34	2/4	5	591	17,39,123,412	184,186,221	158,433	*Pseudozyma prolifica* (AB089369)	98.6 (555/563)	KP058918
			1	572	21,39,48,123,341	163,184,225	135,437	*Pseudozyma aphidis* (AB089363)	99.4 (535/538)	KM114666
S43PB	35	1/7	1	588	18,39,48,123,360	182,184,222	154,434	*Pseudozyma aphidis* (AB089363)	98.9 (551/557)	KM114667
S44PB	24	1/5	4	592	18,39,123,412	184,186,222	158,434	*Pseudozyma prolifica* (AB089369)	99.1 (557/562)	KM114668
S45PB	36	2/4	2	594	20,39,123,412	184,186,224	158,436	*Pseudozyma prolifica* (AB089369)	99.1 (557/562)	KM114669
			1	587	44,57,75,154,257	185,402	46,71,172,298	*Jaminaea angkoriensis* (EU587489)	98.8 (582/589)	KM114670
S46PB	28	2/8	5	591	18,39,123,411	184,185,222	157,434	*Pseudozyma prolifica* (AB089369)	98.8 (555/562)	KM114671
			6	587	13,39,123,412	184,186,217	158,429	*Pseudozyma prolifica* (AB089369)	98.8 (555/562)	KP058919
S48PB	34	1/2	10	591	18,39,123,411	184,185,222	157,434	*Pseudozyma prolifica* (AB089369)	98.9 (556/562)	KM114672

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S49PB	39	0/4								
S50PB	24	0/7								
S51PB	33	0/6								
Phichit										
S30PC	27	2/17	2	594	20,39,123,412	184,186,224	158,436	*Pseudozyma prolifica* (AB089369)	99.1 (557/562)	KM114655
			2	577	18,29,123,397	171,184,222	143,434	*Pseudozyma prolifica* (AB089369)	98.9 (540/546)	KP058909
Phitsanulok										
S31PN	21	0/11								
S32PN	40	1/12	15	598	20,38,123,408	182,184,223	154,435	*Pseudozyma prolifica* (AB089369)	98.4 (543/552)	KM114656
S33PN	35	4/7	1	593	20,39,48,123,363	184,185,224	157,436	*Pseudozyma aphidis* (AB089363)	99.3 (558/562)	KM114657
			19	593	20,39,48,123,363	184,185,224	155, 438	*Pseudozyma aphidis* (AB089363)	99.1 (557/562)	KP058911
			2	593	25,39,123,406	180,184,229	152,441	*Pseudozyma prolifica* (AB089369)	99.1 (551/556)	KP058910
			1	592	44,48,58,114,123,205	48,137,184,223	17,111,154,310	*Pseudozyma aphidis* (AB089363)	91.4 (427/467)	KM114658
S35PN	38	5/11	1	584	63,521	184,400	88,496	*Hannaella coprosmaensis* (AF363660)	93.8 (549/585)	KM114659
			1	593	20,39,123,411	185,408	157,436	*Pseudozyma prolifica* (AB089369)	98.8 (555/562)	KM114660
			2	583	53,125,405	82,97,183,221	2,30,41,83,427	*Sporobolomyces vermiculatus* (AB279731)	99.5 (581/584)	KM114661
			2	586	53,125,408	82,100,183,221	2,71,86,427	*Sporobolomyces vermiculatus* (AB279731)	99.0 (583/589)	KP058912
			24	562	20,39,123,380	154,184,224	126,436	*Pseudozyma prolifica* (AB089369)	99.1 (524/529)	KP058913
Suphanburi										
S06SP	16	0/7								
S07SP	18	0/6								
S08SP	13	0/4								
S15SP	34	1/3	32	608	34,39,123,412	184,186,238	158,450	*Pseudozyma prolifica* (AB089369)	99.1 (557/562)	KM114630
S16SP	40	1/7	4	593	25,39,123,406	180,184,229	152,441	*Pseudozyma prolifica* (AB089369)	99.1 (551/556)	KM114631
S17SP	40	3/11	15	591	18,39,123,411	184,185,222	157,434	*Pseudozyma prolifica* (AB089369)	98.9 (556/562)	KM114632
			1	625	15,37,39,123,411	184,185,256	157,468	*Pseudozyma prolifica* (AB089369)	98.9 (556/562)	KM114633
			1	588	12,19,40,159,358	67,74,114,150,183	153,435	*Pseudozyma antarctica* (AJ235302)	92.6 (512/553)	KM114634
S21SP	18	0/8								
S22SP	18	5/5	1	594	20,39,48,123,364	184,186,224	158,436	*Pseudozyma aphidis* (AB089363)	99.1 (557/562)	KM114637
			5	576	21,39,123,393	167,184,225	139,437	*Pseudozyma prolifica* (AB089369)	99.3 (538/542)	KM114638
			1	546	178,368	170,376	546	*Rhodotorula taiwanensis* (GU646863)	98.9 (534/540)	KM114639
			8	586	32,61,493	183,188,215	586	*Cryptococcus flavus* (AF075497)	99.1 (581/586)	KM114636
			2	569	20,39,48,123,339	161,184,224	131,438	*Pseudozyma aphidis* (AB089363)	99.6 (534/536)	KP058903
S23SP	26	5/8	9	593	20,39,123,411	184,185,224	593	*Pseudozyma prolifica* (AB089369)	93.6 (527/563)	KM114641
			1	594	20,39,123,412	184,186,224	156,436	*Pseudozyma prolifica* (AB089369)	99.1 (557/562)	KM114642
			11	586	8,578	40,143,185,218	586	*Malassezia restricta* (AF064026)	98.4 (562/571)	KM114640
			1	585	20,39,123,162,241	47,48,82,184,224	74,183,328	*Pseudozyma prolifica* (AB089369)	87.7 (490/559)	KM114643
			1	590	20,39,128,161,247	48,53,82,183,224	80,183,327	*Pseudozyma prolifica* (AB089369)	86.9 (491/565)	KP058904
Saraburi										
S52SB	25	0/8								
S63SB	34	2/9	2	606	25,33,39,48,123,338	184,185,237	157,449	*Pseudozyma aphidis* (AB089363)	98.8 (555/562)	KM114674
			4	598	53,125,408	82,100,183,221	2,71,86,427	*Sporobolomyces vermiculatus* (AB279731)	99.3 (414/417)	KM114675
S64SB	36	2/9	11	590	20,39,123,408	182,184,224	42,112,436	*Pseudozyma hubeiensis* (DQ008953)	99.5 (587/590)	KM114676
			5	601	31,39,48,123,360	182,184,235	154,447	*Pseudozyma aphidis* (AB089363)	98.4 (548/557)	KM114677
S66SB	33	0/13								
S68SB	35	3/12	22	593	25,39,123,406	180,184,229	152,441	*Pseudozyma prolifica* (AB089369)	99.1 (551/556)	KP058920
			1	593	20,39,123,173,238	184,185,224	157,436	*Pseudozyma prolifica* (AB089369)	98.8 (555/562)	KM114678
			1	579	21,39,123,138,258	169,184,226	143,436	*Pseudozyma prolifica* (AB089369)	94.1 (515/547)	KP058921

(*continued on next page*)

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Table 2 — (continued)

Samples	No. of positive clones[a]	No. of yeast OTUs/total OTUs	No. of clones	D1/D2 size (bp)	RFLP[b]			Closest species (Accession no.)	Identity (%)	GenBank accession no.
					HaeIII	*HinfI*	*CfoI*			
S69SB	31	5/11	14	595	21,39,123,412	184,186,225	158,437	*Pseudozyma hubeiensis* (DQ008953)	99.5 (593/596)	KM114679
			2	608	35,39,123,411	184,185,239	157,451	*Pseudozyma prolifica* (AB089369)	98.8 (555/562)	KM114680
			1	609	36,39,123,411	184,185,240	45,112,452	*Pseudozyma hubeiensis* (DQ008953)	99.7 (596/598)	KM114681
			1	593	20,39,123,411	184,185,224	45,112,436	*Pseudozyma hubeiensis* (DQ008953)	99.7 (593/595)	KP058922
			2	593	20,39,123,411	184,185,224	157,436	*Pseudozyma prolifica* (AB089369)	98.8 (555/562)	KP058923
S70SB	34	4/13	6	593	22,39,123,409	183,184,226	155,438	*Pseudozyma prolifica* (AB089369)	98.9 (552/558)	KM114682
			1	582	32,39,48,123,340	236,346	132,450	*Pseudozyma aphidis* (AB089363)	99.1 (533/538)	KP058924
			2	575	20,39,123,393	48,119,184,284	28,547	*Pseudozyma prolifica* (AB089369)	95.3 (523/549)	KP058925

a Number of positive clones analysed by colony PCR.
b Restriction fragments were obtained from in-silico analysis using NEBcutter V2.0 (http://nc2.neb.com/NEBcutter2/index.php).

calculated as the number of clones of a particular OTU as a proportion of the total number of clones. Frequency of occurrence (%) was calculated as the number of samples, where a particular OTU was observed, as a proportion of the total number of samples. The EstimateS software was used to calculate rarefaction curve and expected OTU richness on the sampling effort by Chao1 and Bootstrap estimators with sample-based abundance data (classic EstimateS input) (Colwell 2006).

Results

Yeast OTUs

A total of 1342 clones were obtained from 45 clone libraries of sugarcane phyllosphere. The restriction analysis was used to preliminarily cluster the recombinant clones in each library. Since the RFLP technique has certain limitations; (1) we cannot exclude the presence of false-positive and false negative results since there is no available database for RFLP profiles of LSU and (2) fragments differing in 20–50 bp cannot be sufficiently distinguished. Therefore the PCR products of each clone library showing distinct RFLP profiles were selected for sequencing and identified using BLAST analysis. Of the total number of analysed clones, 53.1 % (713 clones, 226 sequences) were related to filamentous fungi and 31.7 % (426 clones, 77 sequences) were related to yeasts. The remaining 15.1 % (203 clones, 68 sequences) were of unknown origin, as the sequences had none or low significant similarity using BLAST analysis of the GenBank database. Only the yeast related sequences were further analysed and are discussed in this study. The distribution of amplified clones is shown in Table 2. The sequence fragments from restriction analysis were obtained from in-silico analyses of yeast related sequences and the closest matching yeast species obtained from GenBank. The sequence fragments of these samples were compared to those of the closest yeast type strains and expected yeast species reported in previous studies of sugarcane phyllospheres in Thailand using the enrichment cultivation method (Limtong *et al.*,2014) (supplementary data). Only two sequences, KM114659 (S35PN) and KM114639 (S22SP), found in this study were comparable to RFLP patterns of yeast species obtained using the enrichment method, *Hannaella* aff. *coprosmaensis* (AB772081) and *Rhodoturula taiwanensis* (AB772163), respectively. These results were confirmed by phylogenetic analysis (Fig 1).

The yeast related sequences were clustered into a total of 31 OTUs at 99 % homology cut off (Table 3). All were classified as yeasts in the phylum *Basidiomycota*. Basidiomycetous yeasts were identified by analysis of areas of homology in the D1/D2 domains of LSU rRNA gene sequences and those of type strains. Sequences that differ by two or more nucleotides in the D1/D2 region represent different taxa according to the recommendations of Fell *et al.* (2000). Therefore sequences within the same OTUs that varied in the number of nucleotide substitutions by two or more nucleotides were distinguished as different phylotypes. This analysis showed there were 38 phylotypes within 31 OTUs. The rarefaction curve of epiphytic yeast clones reached a horizontal

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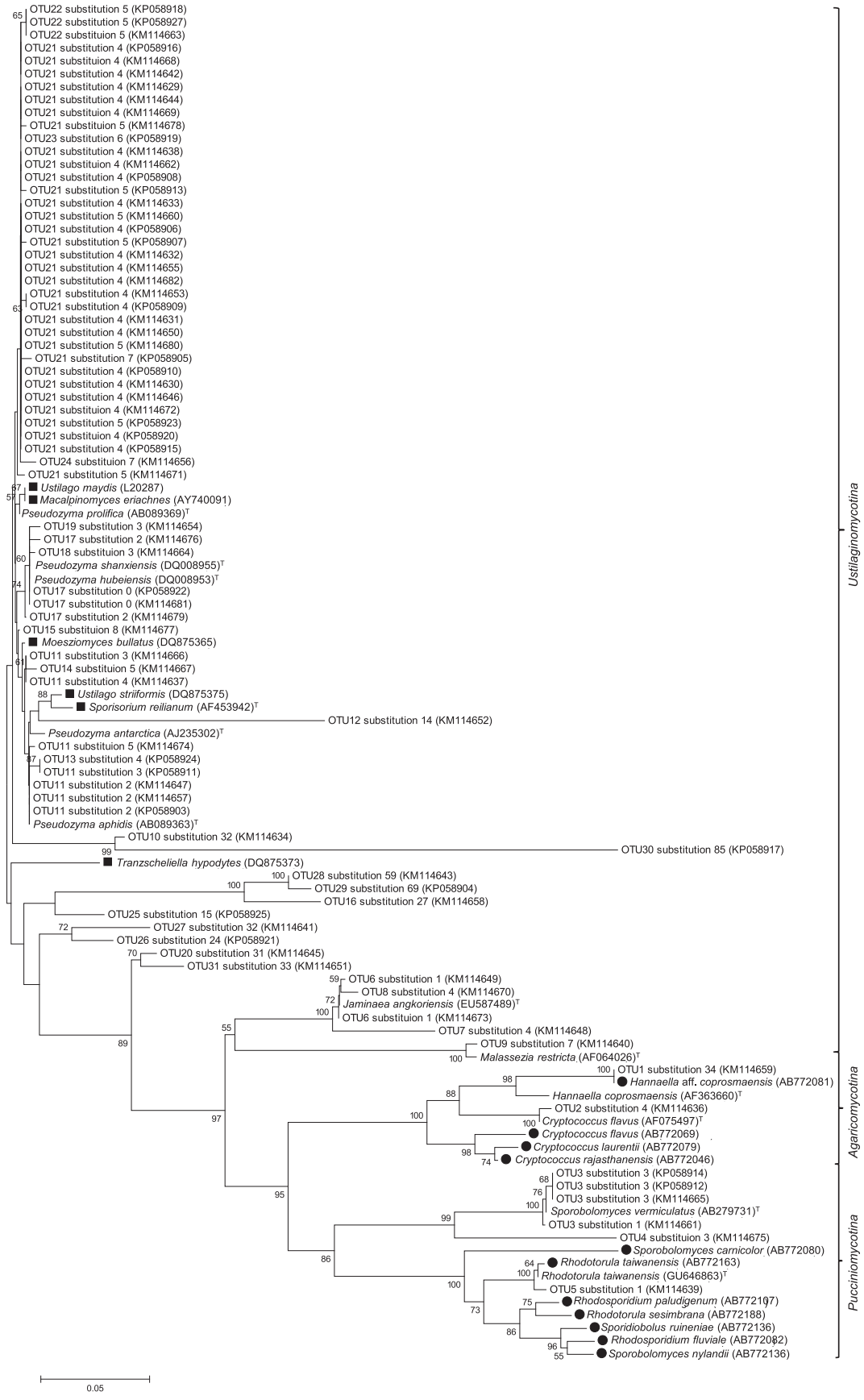


Fig 1 – Phylogenetic tree calculated from neighbour-joining of each OTU sequence (D1/D2 domains, LSU rRNA gene) from 31 OTUs and reference sequences of yeast type strains, expected yeast strains (●) and the representative strains of telemorphic fungi in *Ustilaginomycotina* (■) retrieved from the GenBank database using a Kimura 2-parameter model. Numbers on branches represent the bootstrap percentage (>50 %) from 1000 random replications. The scale bar corresponds to a genetic distance of 0.05 substitutions per position.

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Table 3 – BLAST results for 31 OTUs based on the D1/D2 domain sequences and their frequencies of occurrence in 45 samples of sugarcane phyllospheres.

OTU	No of sequences	No of clones	Closest species	GenBank accession no.	Identity (%)	Nucleotide substitution	Coverage (%)	Identification	Frequency of occurrence (%)	Province
Subphylum *Agaricomycotina*										
Order *Tremellales*										
1	1	1	*Hannaella coprosmaensis*	AF363660	93.8	34	100	Closest to *Hannaella coprosmaensis*	2.2	PN
2	1	8	*Cryptococcus flavus*	AF075497	99.1	4	100	Closest to *Cryptococcus flavus*	2.2	SP
Subphylum *Pucciniomycotina*										
Order *Sporidiobolales*										
3	1	2	*Sporobolomyces vermiculatus*	AB279731	99.5	1	99	*Sporobolomyces vermiculatus*	2.2	PN
	3	5	*Sporobolomyces vermiculatus*	AB279731	99.0	3	100	Closest to *Sporobolomyces vermiculatus*	6.7	PB,PN
4	1	4	*Sporobolomyces vermiculatus*	AB279731	99.3	3	69	Closest to *Sporobolomyces vermiculatus*	2.2	SB
Order *Sporidiales*										
5	1	1	*Rhodotorula taiwanensis*	GU646863	98.9	1	98	*Rhodotorula taiwanensis*	2.2	SP
Subphylum *Ustilaginomycotina*										
Order *Microstromatales*										
6	2	11	*Jaminaea angkoriensis*	EU587489	99.5–99.7	1	99–100	*Jaminaea angkoriensis*	4.4	BR, CN
7	1	1	*Jaminaea angkoriensis*	EU587489	99.0	4	85	Closest to *Jaminaea angkoriensis*	2.2	CN
8	1	1	*Jaminaea angkoriensis*	EU587489	98.8	4	100	Closest to *Jaminaea angkoriensis*	2.2	PB
Order *Malasseziales*										
9	1	11	*Malassezia restricta*	AF064026	98.4	7	97	Closest to *Malassezia restricta*	2.2	SP
Order *Ustilaginales*										
10	1	1	*Pseudozyma antarctica*	AJ235302	92.6	32	92	Closest to *Pseudozyma antarctica*	2.2	SP
11	3	9	*Pseudozyma aphidis*	AB089363	99.3–99.6	2	94	Closest to *Pseudozyma aphidis*	6.7	CN,PN,SP
	2	20	*Pseudozyma aphidis*	AB089363	99.1–99.4	3	94	Closest to *Pseudozyma aphidis*	4.4	PB,PN
	1	1	*Pseudozyma aphidis*	AB089363	99.1	4	94	Closest to *Pseudozyma aphidis*	2.2	SP
	1	2	*Pseudozyma aphidis*	AB089363	98.8	5	92	Closest to *Pseudozyma aphidis*	2.2	SB
12	1	3	*Pseudozyma aphidis*	AB089363	96.8	14	76	Closest to *Pseudozyma aphidis*	2.2	NK
13	1	1	*Pseudozyma aphidis*	AB089363	99.1	4	92	Closest to *Pseudozyma aphidis*	2.2	SB
14	1	1	*Pseudozyma aphidis*	AB089363	98.9	5	94	Closest to *Pseudozyma aphidis*	2.2	PB
15	1	5	*Pseudozyma aphidis*	AB089363	98.4	8	92	Closest to *Pseudozyma aphidis*	2.2	SB
16	1	1	*Pseudozyma aphidis*	AB089363	91.4	27	77	Closest to *Pseudozyma aphidis*	2.2	PN
17	2	2	*Pseudozyma hubeiensis*	DQ008953	99.7	0	99–100	*Pseudozyma hubeiensis*	2.2	SB
	2	25	*Pseudozyma hubeiensis*	DQ008953	99.5	2	99–100	Closest to *Pseudozyma hubeiensis*	4.4	SB
18	1	1	*Pseudozyma hubeiensis*	DQ008953	99.3	3	91	Closest to *Pseudozyma hubeiensis*	2.2	PB
19	1	2	*Pseudozyma hubeiensis*	DQ008953	99.5	3	91	Closest to *Pseudozyma hubeiensis*	2.2	NK
20	1	4	*Pseudozyma hubeiensis*	DQ008953	92.5	31	88	Closest to *Pseudozyma hubeiensis*	2.2	CN
21	24	180	*Pseudozyma prolifica*	AB089369	98.9–99.3	4	92–94	Closest to *Pseudozyma prolifica*	44.4	CN,NK,NP,PB, PC,PN,SB,SP
	7	37	*Pseudozyma prolifica*	AB089369	98.8–99.1	5	92–94	Closest to *Pseudozyma prolifica*	11.1	NK,PB,PN,SB
	1	1	*Pseudozyma prolifica*	AB089369	98.6	7	94	Closest to *Pseudozyma prolifica*	2.2	CN
22	3	47	*Pseudozyma prolifica*	AB089369	98.6	5	94	Closest to *Pseudozyma prolifica*	6.7	LB,PB
23	1	6	*Pseudozyma prolifica*	AB089369	98.8	6	95	Closest to *Pseudozyma prolifica*	2.2	PB
24	1	15	*Pseudozyma prolifica*	AB089369	98.4	7	93	Closest to *Pseudozyma prolifica*	2.2	PN
25	1	2	*Pseudozyma prolifica*	AB089369	95.3	15	94	Closest to *Pseudozyma prolifica*	2.2	SB

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26	1	1	*Pseudozyma prolifica*	AB089369	94.1	24	93	Closest to *Pseudozyma prolifica*	2.2	SB
27	1	9	*Pseudozyma prolifica*	AB089369	93.6	32	94	Closest to *Pseudozyma prolifica*	2.2	SP
28	1	1	*Pseudozyma prolifica*	AB089369	87.7	59	94	Closest to *Pseudozyma prolifica*	2.2	SP
29	1	1	*Pseudozyma prolifica*	AB089369	86.9	63	94	Closest to *Pseudozyma prolifica*	2.2	SP
30	1	2	*Pseudozyma prolifica*	AB089369	78.4	85	85	Closest to *Pseudozyma prolifica*	2.2	PB
31	1	1	*Pseudozyma shanxiensis*	DQ008955	92.9	33	100	Closest to *Pseudozyma shanxiensis*	2.2	CN
	77	426								

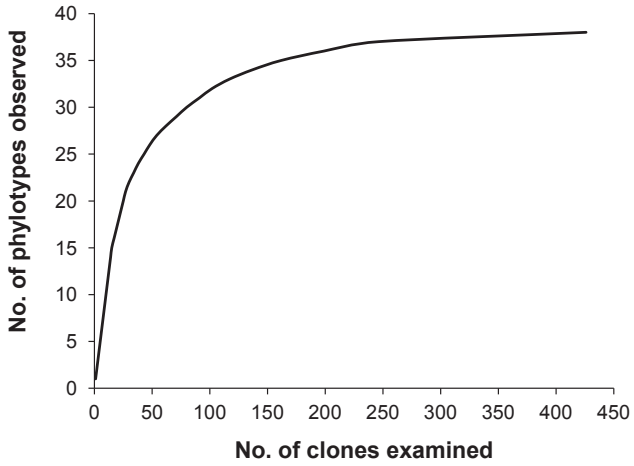


Fig 2 – Rarefaction curve of epiphytic yeast clones derived from sugarcane leaf samples.

asymptote indicating that the number of clones sampled covered the expected OTU (phylotype) richness (Fig 2). Yeast OTUs were classified into six taxonomic orders within three subphylums which were most closely related to 11 known yeast species in seven genera according to the BLAST search results (Table 3). Most of these represent sequences of putative new species. Only four phylotypes could be identified as known yeast species. OTU 21, closely related to *Pseudozyma prolifica*, was the most frequently detected and widely distributed within the sugarcane leaf samples. The results indicated that the common epiphytic yeasts of the sugarcane phyllosphere belonged to yeasts from the subphylum *Ustilaginomycotina* (95.1 % relative frequency) and the majority were in the order *Ustilaginales* (89.4 % relative frequency). These results were confirmed by the phylogenetic tree (Fig 1). The diversity indice and community evenness were 2.33 and 0.64, respectively. However, the estimation of the expected phylotype richness on the sampling effort by Chao1 and Bootstrap estimators revealed that the observed phylotype richness was lower than the expected phylotype richness, indicating that the sugarcane leaf was under-sampled and there were likely

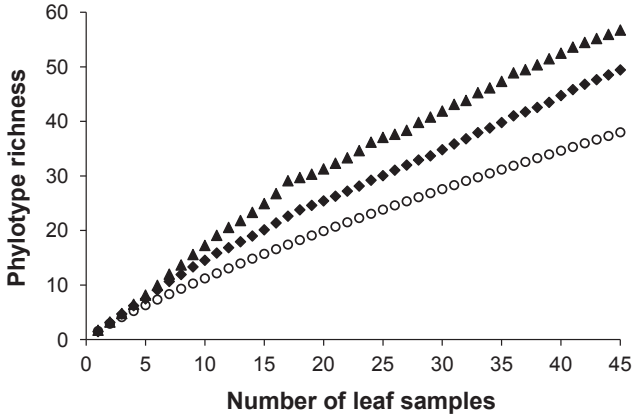


Fig 3 – Observed (○) and estimated phylotype richness of epiphytic yeasts in sugarcane phyllosphere *versus* sample size using Chao1 (▲) and Bootstrap (◆) estimators.

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yeast phylotypes that remained unobserved within this study (Fig 3).

Discussion

Though yeast communities of plant phyllospheres have been studied intensively; there have not been many studies to date of yeasts associated with sugarcane and all of these were studied using culture-dependent methods. In this study the diversity of epiphytic yeasts in sugarcane phyllosphere in Thailand was evaluated using a culture-independent method based on the nucleotide sequence of the D1/D2 domain of the large subunit rRNA gene. The phyllospheres of vascular plants are generally colonized by epiphytic yeasts, both basidiomycetes and ascomycetes (Nakase *et al.* 2001; Inácio *et al.* 2005; Fonseca & Inacio 2006; Slavikova *et al.* 2007; Glushakova and Chernov, 2010; Landell *et al.* 2010). However, in this study culture independent technique has led to the discovery of basidiomycetous yeasts only. It has been postulated that the yeast species most commonly found on monocotyledonous plants are from the phylum *Basidiomycota* (Spencer & Spencer 1997). In previous studies using culture-dependent method for investigation of yeast communities of the plant phyllosphere it has been reported that the basidiomycetous yeasts are the predominant yeasts (de Azeredo *et al.* 1998; Inácio *et al.* 2005; Slavikova *et al.* 2009; Glushakova and Chernov, 2010) Nakase *et al.* (2001) reported that the basidiomycetous yeasts are present on the phyllospheres of various plant species collected in Thailand using the ballistoconidium-fall isolation method, these were identified as 21 species in the genera *Bensingtonia*, *Bullera*, *Kockovaella*, *Sporidiobolus*, *Sporobolomyces*, and *Tilletiopsis*. Culture-independent method has been used to assess epiphytic yeasts of rice phyllospheres in Thailand and this has revealed that the predominant yeasts are from the phylum *Basidiomycota* (Nasanit *et al.* 2015). In contrast, the ascomycetous yeasts were most often isolated when using the enrichment technique (Limtong & Kaewwichian 2014; Limtong & Koowadjanakul 2012; Limtong *et al.* 2014). Previous to this study the yeast genera obtained from sugarcane phyllospheres using the enrichment method belonged to the *Ascomycota viz. Candida*, *Debaryomyces*, *Hannaella*, *Hanseniaspora*, *Kluyveromyces*, *Lachancea*, *Lodderomyces*, *Metschnikowia*, *Meyerozyma*, *Millerozyma*, *Pichia*, *Torulaspora*, and *Wickerhamomyces*, and to the *Basidiomycota viz. Cryptococcus*, *Kwoniella*, *Rhodotorula*, *Sporidiobolus*, *Sporomolomyces*, and *Tricosporon* (Limtong & Koowadjanakul 2012; Limtong *et al.* 2014). In this study, using uncultured technique in investigation of epiphytic yeasts, only a limited number of genera were found in common to those found using enrichment method. These common genera included *Hannaella*, *Rhodotorula*, and *Sporobolomyces*. The prevalent yeast genus detected in our study *Pseudozyma* could not be isolated using the enrichment technique but was detected using leaf washing followed by a plating technique (Chamnanpa *et al.* 2013). These results indicated that different yeasts are obtained when using different investigational techniques. Species not found on sugarcane using the enrichment method but were found in our study were *Cryptococcus flavus*, *Jaminaea angkoreiensis*, *Malassezia restricta*, *Pseudozyma antarctica*, *P. aphidis*, *P. hubeiensis*, *P. prolifica*, *P. shanxiensis*, and *Sporobolomyces vermiculatus*. This indicates that enrichment cultivation technique is unreliable for phylloplane yeasts as it does not reveal the predominant yeast communities, basidiomycete yeasts do not grow at the elevated temperatures used and can be suppressed in the stationary liquid cultures of enrichment cultivation technique. Though this technique can be useful for species isolation, e.g. fermentation of sugarcane (Rosimeire *et al.* 2011).

Yeasts in the order *Ustilaginales* were the most prevalently detected in sugarcane phyllospheres sampled within this study. Interestingly, ustilaginomycetes are economically important fungi well characterized for their parasitism of monocotyledonous plants. *Pseudozyma* was the most prevalent yeast genus found and this is not unexpected as the *Pseudozyma* genus is known to widely inhabit numerous plant phyllospheres (Allen *et al.* 2004; Wang *et al.* 2006; Seo *et al.* 2007; Kitamoto *et al.* 2011; Limtong & Kaewwichian 2014; Yoshida *et al.* 2014). This genus seems to represent the asexual counterpart of *Ustilaginales* that parasitize grasses (Begerow *et al.* 2006; Begerow *et al.* 2014). In the present study, DNA sequences closely related to *P. prolifica* was detected in relatively high frequency and was a common species of sugarcane phyllosphere followed by *P. aphidis* and *P. hubeiensis*. *Pseudozyma prolifica*, and *P. aphidis* have also been found in the gut of several pests of maize (Molnar *et al.* 2008). Therefore it can be hypothesized that pathogenic yeasts found in sugarcane fields may have been introduced or spread by the crop pests. *P. hubeiensis* and *P. shanxiensis* isolated from wilt leaves of various plants in China and which were first proposed as a novel yeast species by Wang *et al.* (2006). *Pseudozyma aphidis*, *P. flocculosa*, *P. fusiformata*, and *P. tsukubaensis* have all been previously reported for their potential as antagonists of phytopathogenic fungi infection (Avis & Bélanger 2002; Golubev *et al.* 2006; Clement-Mathieu *et al.* 2008; Buxdorf *et al.* 2013). In recent years, *P. antarctica* has been found to be a producer of a biodegradable plastic degrading enzyme (Kitamoto *et al.* 2011; Watanabe *et al.* 2014). *Pseudozyma jejuensis* isolated from orange leaves is capable of producing cutinase and degrading polycaprolactone which may be useful for the biological degradation of plastic waste (Seo *et al.* 2007).

Malassezia is difficult to culture under routine conditions since it has a rather narrow temperature growth range growing poorly at 25 °C and some species will not grow above 37 °C. Additionally, lipid supplement is also required for growth. However it is readily detected in environmental DNA samples (Amend 2014). DNA sequences with homology to *M. restricta* have been detected in habitats as diverse as deep-sea sediments (Lai *et al.* 2007), Antarctic soils (Arenz *et al.* 2006), and various plant roots such as orchids (Roy *et al.* 2009). In this study we also detected DNA samples closely related to *M. restricta* in the sugarcane phyllosphere by using a molecular biology approach. This highlights the advantages of using molecular techniques for the detection of poorly cultivable yeast species.

The following yeast species; *Bullera coprosmaensis*, *Cryptococcus flavus*, *Jaminae angkoriensis*, *Rhodotorula taiwanensis*, *Sporobolomyces vermiculatus*, and other *Sporobolomyces* spp., detected in the present survey have been isolated from the phyllosphere before (Hamamoto & Nakase 1996; Takashima & Nakase 2000; Allen *et al.* 2004; Glushakova and Chernov,

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2004; Wanderley *et al.* 2004; Sipiczki & Kajdacsi 2009; Huang *et al.* 2011; Limtong *et al.* 2014). Since the diversity of epiphytic yeasts of the sugarcane phyllosphere has received little attention in Thailand, limited novel yeast species have been newly described to date. These species include *Metschnikowia saccharicota*, *Nakazawaea siamensis*, *Papiliotrema siamensis*, *Wickerhamomyces siamensis*, and *Yamadazyma siamensis*. In our study, there were in total 34 novel phylotypes that are likely to represent sequences from new yeast taxa (Table 3).

In conclusion, using a culture-independent technique to study epiphytic yeast diversity of sugarcane leaf phyllosphere showed that basidiomycetous were the prevalent yeasts and the subphylum *Ustilaginomycotina* were most predominant. Detection of novel yeast sequences and the occurrence of multiple species within sugarcane leaf samples suggest that extensive studies of epiphytic yeasts from sugarcane phyllospheres would enable discovery of novel yeast species and therefore lead to a better understanding of yeast diversity. Further study of a greater number of sugarcane leaf samples will be required to describe the entire range of epiphytic yeasts found in Thailand. However this understanding could facilitate development of novel applications for the agricultural, industrial, and pharmaceutical industries.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funbio.2015.08.021.

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The genus Diutina, description of Diutina siamensis, f.a. sp. nov., and reassignment of Candida catenulata, C. mesorugosa, C. neorugosa, C. pseudorugosa, C. ranongensis, C. rugosa and C. scorzettiae to the genus Diutina (/content/journal/ijsem/10.1099/ijsem.0.000634)

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Archives of Microbiology

Indole-3-acetic acid biosynthetic pathways in the basidiomycetous yeast Rhodosporidium paludigenum

--Manuscript Draft--

Manuscript Number:	
Full Title:	Indole-3-acetic acid biosynthetic pathways in the basidiomycetous yeast Rhodosporidium paludigenum
Article Type:	Original Paper
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Abstract:	Microorganisms produce plant growth regulators, such as auxins, cytokinins and gibberellins, to promote plant growth. Auxins are a group of compounds with an indole ring that have a positive effect on plant growth. Indole-3-acetic acid (IAA) is a plant growth hormone classified as an indole derivative of the auxin family. IAA biosynthesis pathways have been reported and widely studied in several groups of bacteria. Only a few studies on IAA biosynthesis pathways have been conducted in yeast. This study aimed to investigate IAA biosynthesis pathways in a basidiomycetous yeast (Rhodosporidium paludigenum DMKU-RP301). Investigations were performed both with and without a tryptophan supplement. Indole compound intermediates were detected by gas chromatography-mass spectrometry. Indole-3-lactic acid and indole-3-ethanol were found as a result of the enzymatic reduction of indole-3-pyruvic acid and indole-3-acetaldehyde, in IAA biosynthesis via an indole-3-pyruvic acid pathway. In addition, we also found indole-3-pyruvic acid in culture supernatant determined by high-performance liquid chromatography. Identification of tryptophan aminotransferase activity supports indole-3-pyruvic acid routed IAA biosynthesis in R. paludigenum DMKU-RP301. We hence concluded that R. paludigenum DMKU-RP301 produces IAA through an indole-3-pyruvic acid pathway.
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Author Comments:	Dear Editors, We prevoiusly submitted our manuscript entitled "Indole-3-acetic acid biosynthetic pathways in the basidiomycetous yeast Rhodosporidium paludigenum" to Archives of

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Microbiology since May 15th 2015, and it was rejected with some valuable comments from reviewers. However, Associate Editor gave us a kind suggestion as "after an extensive revision and the inclusion of additional experiments you are encouraged to submit your work as a new manuscript to Archives of Microbiology (reject and resubmit)." After addition of experimental results that supports our findings, we would very much like to submit our work for evaluation for publication in your journal once again. We do confirm that our manuscript is an original study that has not been published or is currently under consideration for publication in any journal.

For this MS resubmission, N. Srisuk will be solely corresponding author by permission of S. Limtong (who previously stated as co-corresponding author. In addition, this manuscript has already been English proofed by Academic proof reading agency.

On behalf of all the authors;
Yours sincerely,

Nantana Srisuk

****In addition, we attach here responses to comments of the reviewers from our previous submission :

Manuscript AOMI-D-15-00181 "Indole-3-acetic acid biosynthetic pathways in the basidiomycetous yeast Rhodosporidium paludigenum"

Dear All Reviewers,
Thank you for all of your kind comments and corrections. May I make a response to your comments as indicate in red letters below each comment.

Comments for the Author:

Reviewer #1: This manuscript describes IAA production in the basidiomycetous yeast Rhodosporidium paludigenum. Being that there is little research done on IAA production by these types of microorganisms, this manuscript will further scientific understanding of the mechanisms by which yeast synthesize IAA. That being said, there are some gaps in the information provided. The novelty is not that yeast produce IAA but rather how they produce it. The basis of the paper is the comparison of IAA production in two different media compositions in an attempt to identify which pathways are operating. However, it is difficult to draw conclusions as to the factors affecting IAA production, if more than one variable in the media composition is being changed at the same time. Because IAA production is affected by many different aspects (pH, temperature, carbon/ nitrogen source, amount of trp, growth phase, stress etc…) these factors can be simultaneously affecting the IAA and IAA derivative profiles observed. Therefore, while this manuscript is useful in telling us that IAA is indeed produced by this yeast, and also identifying some of the intermediates present, it is not clear as to which conditions have the biggest affect on the production. Moreover, the conclusions made are preliminary at best and require further testing. One cannot state that the IPA pathway is solely operating in this bacterium if IPA and IAld intermediates are not directly observed. Although these intermediates are labile, it is possible to detect them under certain conditions. I strongly recommend repeating the experiments and attempting to identify these two intermediates, in order to concretely state that the IPA pathway is the main mode of IAA production. Moreover, change one variable of the media composition at a time, in order to isolate where the biggest difference in IAA profiles is coming from. A more extensive explanation of the observed indole derivatives listed in Table 1 and how they are associated with IAA production would also be helpful.
> Conclusion about the effect of different yeast growth conditions e.g. pH, yeast extract and tryptophan to IAA production and IAA intermediates was omitted
.
Please see below for specific comments:

Page 3, line 57-58 "However, there are only a few reports of IAA production in basidiomycetous yeast and therefore no reports on the biosynthesis of IAA have been established" - reword this sentence
> Already done

Page 4, line 84 " 0.1% w/v tryptophan" is better reported as ug/mL
> We would like very much to express tryptophan concentration as 0.1% w/v as usually found in many articles of IAA production.

Page 4, line 86 : what was the OD600 of the culture after 7 days?
> Already done as:
"The initial pH and initial OD600 of the culture medium were adjusted to 6 and 0.1, respectively, prior to incubation on an orbital shaker at 170 rpm, 30°C, for seven days." has been rewritten to "The initial pH and initial OD600 of the culture medium were adjusted to 6 and 0.1, respectively, prior to incubation on an orbital shaker at 170 rpm. The cultures were incubated at 30°C for 7 days."

For the two different media used (BB17, BB23) You need to change only one variable (i.e pH, yeast extract, tryptophan concentration) at a time between them in order to determine which variable has the effect on IAA production.
> We have decided not to compare factors affecting IAA production as in previous MS.

Page 5, line 96: what volume of sample was taken for analysis?
What was the OD 600 of the cultures at each time point that a sample was taken?
Which growth phase were the cells in? Growth phase affects IAA production significantly
> Already done
"Samples were taken" has been rewritten to "The whole culture broths were taken." However, growth was not assessed.

Page 5, line 98: What volume of methanol was used to dissolve the residue?
> 1 ml of methanol was used

Don't use acronyms and short forms (i.e IAM, IPA, mass spec etc…) write out the entire word.
> Full names have already been stated along with their abbreviations in the revised MS.

Page 6, line 134-140: "The results indicated that the main product of tryptophan metabolism, produced by R. paludigenum DMKU-RP301 cultivated under BB17 conditions, was not IAA but indole-3-ethanol (IEt) and indole-3-lactic acid (ILA) instead" - You should mention that this trend was observed at all the time points that a sample was taken at (1, 3, 5, 7 days).
> Already explained as "The result notices that the relative amounts of indole-3-lactic acid and indole-3-ethanol had a tendency to decrease over time, whereas, the relative amounts of IAA increased. This could be explained by the conversation of indole-3-lactic acid and indole-3-ethanol to be IAA. Furthermore, due to increase of IAA, it resulted in increase of IAA methyl ester and 3-methylindole as well because these two products were derived from IAA degradation."

Page 6, line 146- there is trace amounts of tryptophan present in yeast extract too. Generally when IAA is being assayed a minimal media is used instead of a nutrient rich media, and only exogenous tryptophan is added
> We would like to explain that we were not worry about the interference of tryptophan in the medium use because IAA was not detected even in the culture grown in YPD broth containing as high as 1% yeast extract. Therefore, using the medium BB17 and BB23 which contained 0.1% and 1% yeast extract should not interfere the GC-MS analysis result.

Page 6 line 147-148: There is also a pH difference between the 2 media used. What if the difference in IAA and derivative ratios was due to a pH effect?
> The low pH (4.0) of BB17 medium may affect IAA production or ratio of derivatives. However, under such environment, we still observed IAA since 24hr incubation and throughout the cultivation. As long as IAA was detectable, we then focused on the spectrum of IAA intermediates or derivatives rather than the ratio of those derivatives.

What is the optimal pH for your yeast to grow? Is it possible that the low pH 4 of the BB17 media stresses the yeast and this in turn will affect IAA production. Or perhaps

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	the intermediates ILA and IEt are biochemically forming more readily under a low pH Also, perhaps the different media composition and pH caused the cells to enter stationary phase at different times. > From our previous work on optimization of IAA production (using OFAT experimental design), we found an optimum pH for growth of R. paludigenum DMKU-RP301 was 5.0. Yeast growth was varied from high to low level at pH 5>pH 6>pH 7> pH 4. However, optimum IAA production was found at pH 7 and varied from high to low level at pH 7 > pH 6 > pH 5 > pH 4. From these results, it appeared that pH 4 of the BB17 media possibly stressed the yeast and this possibly in turn affected IAA production. However, as long as IAA was detectable, the intermediates and derivatives should be observed even in lesser amount. Page 7, line 176: a similar profile to what? > The paragraph has been rewritten. Page 7, line 177: you cannot conclude this, you can only infer or suggest > Already done Page 7, line 179: pH 4 and 7, not 4-7 > Already done Page 8, line 180-182: The % is not clear. Is this a % of the total volume of sample analyzed? It's better to report as a concentration in ug/mL instead of %. In Table 1 you need to explain your units (% total of what) > The % shown in the result text and table 1 indicated the relative amounts of the existing intermediates compare to all intermediates detected not the actual amount of such intermediates. Page 8, line 188: IAM pathway not IMA > Already changed Page 8 line 192-193: reword this sentence. It's not clear how conjugated forms help in IAA catabolism > Already changed Page 8, line 195: indicate that in your study IAA was converted to its methyl ester > Already changed (In addition In this study) Reviewer #2: The authors have investigated whether the marine yeast Rhodosporidium paludigenum is able to generate indole acetic acid (IAA) from tryptophan via GC-MS analysis of culture supernatants grown with or without tryptophan. They detected IAA in supernatants of cultures supplemented with but not in cultures lacking tryptophan. In addition, they did a time-course analysis of the occurrence of indole derivatives in culture supernatants and could detect presence of indole, 3-methyl indole, indole-3-ethanol, indole-3-acetic acid methyl ester, indole-3-acetic acid, indole-3-lactic acid methyl ester, and indole-3-lactic acid. They provide a Figure that links these molecules in a possible pathway proposing that R. paludigenum generates IAA via indole pyruvic acid from tryptophan. The manuscript is clearly written and the experiments well-described and discussed. Although the contrast and size of molecule peak descriptions can be improved for Fig. 1, the Figures are generally suitable for publication. One major problem of the paper is that it claims to offer insights into the auxin biosynthetic pathways of R. paludigenum, when all that is shown are existence of pathway intermediates. The suggestive explanations of how pathway intermediates could be linked, is not backed up by measurement of enzyme activities or by gene functional analyses. > Determination of tryptophan aminotransferase activity was carried out and IPA was found as indicated in Lines 113-123 of the revised MS. I have the following suggestions for improving the text. Lines 19, 67, 156, and 168 replace "which" by "that" > Already changed

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	L40 delete "Previous studies indicate that" > Already changed L42 replace "tryptophan-dependent (as a precursor) pathways" by "pathways using tryptophan as a precursor" > Already changed L83 "The inoculum was transferred" -> A part of the inoculum was transferred?? > Already changed L118-120 This paragraph discusses/presents data. Either cite reference or supply data including experimental description and microorganism analysis. > Reference has been already cited L121 What is GPB medium? > Already changed (GPB medium glucose peptone broth) L169/170 replace "tried mostly to convert tryptophan into IAA along with some other storage intermediate compounds as a minority" by "converted tryptophan to IAA and some minor storage intermediates" > Already replaced L176/177 replace "We could then conclude" by "By GC-MS analysis, we found" > Already replaced L179 replace "while" by "and" and delete "and all intermediates were analysed by GC-MS" > Already replaced L188 What is IMA? > Already changed (IMA IAM) L197 replace "which also showed that" by "that also converted" > Already replaced L198 delete "was converted" and replace "We are therefore the first to" by "Here we" > Already changed L202 include reference > Already included Associate Editor: Both reviewers are of the opinion that the results described in the manuscript are too preliminary and additonal experiments are needed (see reviewer's comments). For this reason the manuscript cannot be accepted for publication in its present form in Archives of Microbiology. However, after an extensive revision and the inclusion of additional experiments you are encouraged to submit your work as a new manuscript to Archives of Microbiology (reject and resubmit). > As indicates in underlined sentence, we would therefore like to resubmit our work as a new MS.
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Indole-3-acetic acid biosynthetic pathways in the basidiomycetous yeast *Rhodosporidium paludigenum*

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Abstract

Microorganisms produce plant growth regulators, such as auxins, cytokinins and gibberellins, to promote plant growth. Auxins are a group of compounds with an indole ring that have a positive effect on plant growth. Indole-3-acetic acid (IAA) is a plant growth hormone classified as an indole derivative of the auxin family. IAA biosynthesis pathways have been reported and widely studied in several groups of bacteria. Only a few studies on IAA biosynthesis pathways have been conducted in yeast. This study aimed to investigate IAA biosynthesis pathways in a basidiomycetous yeast (*Rhodosporidium paludigenum* DMKU-RP301). Investigations were performed both with and without a tryptophan supplement. Indole compound intermediates were detected by gas chromatography-mass spectrometry. Indole-3-lactic acid and indole-3-ethanol were found as a result of the enzymatic reduction of indole-3-pyruvic acid and indole-3-acetaldehyde, in IAA biosynthesis via an indole-3-pyruvic acid pathway. In addition, we also found indole-3-pyruvic acid in culture supernatant determined by high-performance liquid chromatography. Identification of tryptophan aminotransferase activity supports indole-3-pyruvic acid routed IAA biosynthesis in *R. paludigenum* DMKU-RP301. We hence concluded that *R. paludigenum* DMKU-RP301 produces IAA through an indole-3-pyruvic acid pathway.

Keywords Basidiomycetous yeast · Red yeast · *Rhodosporidium* · Indole-3-acetic acid · Biosynthetic pathway

Introduction

Indole-3-acetic acid (IAA) is the principle plant hormone classified into the family of indole derivatives of auxins. IAA has several important roles in plants; it has been shown to regulate various developmental and physiological processes (Teale et al. 2006), as well as increasing plants' protection from external stress (Bianco and Defez 2009). Moreover, IAA serves as a regulating agent for microbial cell differentiation (Rao et al. 2010).

Two major pathways for IAA biosynthesis have been proposed: tryptophan-dependent and tryptophan-independent pathways. The IAA biosynthetic pathways in bacteria and plants show a high degree of similarity, although some different intermediates can be found in each. Tryptophan-dependent IAA production has been extensively studied in bacteria (Carreno-Lopez et al. 2000; Kumavath et al. 2010; Mujahid et al. 2011; Szkop and Bielawski 2013). Several pathways using tryptophan as a precursor for IAA biosynthesis have been proposed, including an indole-3-acetamide pathway, an indole-3-pyruvic acid pathway, an indole-3-acetonitrile pathway, a tryptamine pathway and a tryptophan side-chain oxidase pathway. The indole-3-pyruvic acid, indole-3-acetamide and indole-3-acetonitrile pathways were considered to be the major IAA biosynthetic pathways in bacteria (Duca et al. 2014). However, a tryptophan-independent pathway for IAA biosynthesis was exhibited in *Azospirillum brasilense*, but no specific enzymes or genes involved with this pathway have yet been identified (Prinsen et al. 1993). The tryptophan-independent IAA biosynthetic pathway has been proposed as a branch from either indole or indole-3-glycerol phosphate (Normanly et al. 2004; Rao et al. 2010). Similarly to bacteria, many fungi (such as *Colletotrichum gloeosporioides* f. sp. *aeschynomene*, *Fusarium proliferatum*, *F. verticillioides*, *F. fujikuroi*, *F. oxysporum*, *Piriformospora indica*, *Ustilago esculenta* and *U. maydis*) mainly use indole-3-pyruvic acid and indole-3-acetamide pathways for IAA biosynthesis (Robinson et al. 1998; Chung and Tzeng 2004; Reineke et al. 2008; Hilbert et al. 2012; Tsavkelova et al. 2012). In the case of yeast, only a few studies on IAA biosynthetic pathways have been conducted. Rao et al. (2010) have studied IAA biosynthetic pathways in the ascomycetous yeast *Saccharomyces cerevisiae* and proposed that this yeast possesses a tryptophan-dependent pathway for IAA biosynthesis, through an indole-3-pyruvic acid pathway. In addition, they also found a tryptophan-independent pathway for IAA biosynthesis in *S. cerevisiae*. Up to date, there have

been only a few reports of IAA production in basidiomycetous yeast and no report on IAA biosynthetic pathway has been established.

The red yeast *Rhodosporidium paludigenum* CBS 6566^{T}, a basidiomycetous yeast, was originally isolated from the intertidal waters of South Florida (Fell and Statzell-Tallman 1980). The red yeasts in the genus *Rhodosporidium* are known as microbial lipid producers, or the so-called oleaginous yeasts (Wu et al. 2011; Wiebe et al. 2012), and are recognized as potential alternative natural sources of carotenoids (Yimyoo et al. 2011). In addition, a versatile *Rhodosporidium* strain has been reported to produce L-phenylalanine (Jia et al. 2008) and, moreover, acts as a biological control (Wang et al. 2009). Reviews of the literature suggest that the production of plant growth regulators like IAA in the basidiomycetous yeast *Rhodosporidium* is still poorly understood. Hence, the present study aims to investigate the IAA biosynthetic pathway in the red yeast *Rhodosporidium paludigenum* DMKU-RP301 that was isolated from a rice phyllosphere in Thailand (Nutaratat et al. 2014).

Material and methods

Microorganism

Rhodosporidium paludigenum DMKU-RP301 (DDBJ accession number: AB920314) was isolated from the rice phyllosphere using the plating of leaf washings technique, as described by Inácio et al. (2002). This yeast was isolated and identified by analysis of the D1/D2 region of the large subunit of rRNA gene sequence similarities, as described in Nutaratat et al. (2014). The yeasts were maintained on YPD agar medium (1% yeast extract, 2% peptone, 2% dextrose and 1.5% agar).

Tryptophan-dependent and tryptophan-independent pathway analysis

Yeast inoculum was prepared in a 250 ml Erlenmeyer flask containing 50 ml YPD, and incubated on an orbital shaker (Jeio Tech, South Korea) at 150 rpm at 30°C, for 16-18 hrs. A part of the inoculum was transferred into a 250 ml Erlenmeyer flask containing 50 ml YPD broth, with or without a 0.1% L-tryptophan supplement. Initial pH and initial OD_{600} of the culture medium were adjusted to 6 and 0.1, respectively, prior to incubation on an

orbital shaker at 170 rpm. The cultures were incubated at 30°C for 7 days. The culture supernatant was analysed for IAA using high-performance liquid chromatography (HPLC) (Agilent 1100, Agilent Technologies, USA), as described in Nutaratat et al. (2014).

Detection of indole compound intermediates

Rhodosporidium paludigenum DMKU-RP301 inoculum was prepared in YPD broth and incubated at 28°C for 24 hrs. The cells were washed, resuspended in sterile distilled water and transferred to two different culture media, differing in their ratio of medium components. The first medium, namely BB17, contained 2% sucrose, 0.1% yeast extract, 0.1% L-tryptophan, and the initial pH was adjusted to 4; the second medium, namely BB23, contained 2% sucrose, 1% yeast extract, 0.5% L-tryptophan, and the initial pH was adjusted to 7. All tests were performed on an orbital shaker at 150 rpm, at 28°C. The whole culture broth samples were taken at one, three, five and seven days of cultivation. After incubation, the supernatant was extracted twice with equal volumes of ethyl acetate. The ethyl acetate fraction was evaporated at 45°C. The residue was dissolved in 1 ml methanol prior to analysis (Mujahid et al. 2011). The indole compound intermediates were analysed using the gas chromatography–mass spectrometry (GC-MS) (GC-G1530N and MS-G2573A, Agilent Technologies Co. Ltd., USA) with a HP-5 column (30 m x 0.25 mm ID, 0.25 mm film thickness); the analysis was performed at the Faculty of Science, King Mongkut's Institute of Technology Ladkrabang (KMITL), Thailand. The oven temperature was maintained at 80°C, for 2 mins, then programmed to rise from 80 to 200°C at 10°C min^{-1}, and then finally programmed to shift from 200 to 280°C at 20°C min^{-1} for 7 mins. Full scan mass spectra were acquired from *m*/*z* 30 to 500. The mass spectra of indole compound intermediates were compared with databases.

Determination of tryptophan aminotransferase activity

Cell pellets *Rhodosporidium paludigenum* DMKU-RP301 were washed twice with 50 ml of 0.2 M sodium phosphate buffer (pH 7.2) and resuspended in the same buffer. Cells were lysed by ultrasonication at 4 °C (Sonics Vibra-Cell CV33, Sonics & Materials Inc., USA). After sonication, the lysate was centrifuged (10,000 rpm, 10 min, 4 °C) and the clear supernatant was used as a source of the enzymes for the determination. Determination of tryptophan aminotransferase activity was carried out in final volume of 2.5 ml sodium

phosphate buffer consist of 40 µmol of L-tryptophan, 20 µmol α-ketoglutarate, 0.1 µmol of pyridoxal-5-phosphate and 0.5 ml of enzyme extract. The reaction mixture was incubated for 5 min at 30°C. The reaction was stopped by 10% trichloroacetic acid. The sample was centrifuged and the formation of indole 3-pyruvic acid was analyzed by injecting 20 µl in HPLC.

Results and discussion

Analysis of the IAA biosynthetic pathways

When cultivated in YPD broth supplemented with 0.1% L-tryptophan, *Rhodosporidium paludigenum* DMKU-RP301 produced a high level of IAA (321.7 mg/l). However, this yeast did not produce IAA in YPD broth when the L-tryptophan was omitted. This result was consistent with the report on the characteristics of three IAA-producing endophytic yeasts (*Rhodotorula graminis* WP1, and *Rh. mucilaginosa* PTD2 and PTD3) that showed high IAA production in YPD broth supplemented with 0.1% L-tryptophan, while IAA was undetectable when the yeast strains were cultivated in YPD medium without the L-tryptophan supplement (Xin et al. 2009). The results we observed here therefore indicate that *R. paludigenum* DMKU-RP301 possesses a tryptophan-dependent pathway for IAA biosynthesis. The IAA production of endophytic yeast isolated from maize root (*Zea mays* L.) was investigated using colorimetric analysis, and it was found that only eight out of 24 isolates produced IAA in both the media supplemented with and without L-tryptophan. The results showed that a greater amount of indole-3-acetic acid was obtained in glucose peptone broth with L-tryptophan than in the medium without L-tryptophan (Nassar et al. 2005). Tryptophan is therefore necessary for IAA production in many yeast species.

Detection of the indole compound intermediates of IAA biosynthetic pathways

According to our previous study on the optimization of IAA production by *R. paludigenum* DMKU-RP301 (Nutaratat et al. 2015), it would be of interest to further investigate the intermediates or precursors existing under conditions of low and high IAA production. In order to avoid an undetectable level of intermediates, we used a culture supernatant obtained from the conditions that gave a considerably low amount of IAA, but not the lowest. Similarly, the optimized culture conditions that yielded the highest IAA production were not used, to

avoid a situation in which all of the precursors have shifted to IAA production and the amount of intermediates remains undetectable, as suggested by prior evidence. We therefore chose two culture conditions, namely BB23 and BB17, that showed relatively high and low levels of IAA production by *R. paludigenum* DMKU-RP301, yielding 1415.3 mg/l and 261.6 mg/l, respectively (Nutaratat et al. 2015).

The identification of indole derivatives from the culture supernatant of *Rhodosporidium paludigenum* DMKU-RP301, cultivated in BB17 and BB23 media (as representatives of culture conditions that gave low and high IAA, respectively), is shown in Table 1. The mass spectrometry fragmentation patterns for the individual compounds are shown in Fig. 1. The result indicated that the relative amount of indole-3-lactic acid and indole-3-ethanol had a tendency to decrease over time, whereas, the relative amount of IAA increased. This could be explained by the conversion of indole-3-lactic acid and indole-3-ethanol to IAA. Furthermore, an increase of IAA resulted in an increase of IAA methyl ester and 3-methylindole as well since these two products were derived from IAA degradation.

Regarding the relative amounts of the existing intermediates, we found 36.61%, 26.39% and 12.92% of indole-3-lactic acid, indole-3-ethanol and IAA, respectively, in the BB17 culture supernatant, whereas 17.75%, 0.07% and 52.73% of indole-3-lactic acid, indole-3-ethanol and IAA, respectively, were found in the BB23 culture supernatant (Table 1). The results indicated that the main product of tryptophan metabolism, produced by *R. paludigenum* DMKU-RP301 cultivated under BB17 conditions, was not IAA but indole-3-ethanol and indole-3-lactic acid throughout of cultivation. It was possible that the low pH 4 of the BB17 media stressed the yeast and this in turn affected IAA production. However, IAA appeared as the main product of tryptophan catabolism when this yeast was cultivated in the BB23 medium. This result indicates that *R. paludigenum* DMKU-RP301 favoured BB23 conditions for IAA production over BB17 conditions. This may be due to the higher amount of tryptophan – the IAA precursor – contained in the BB23 medium (0.5% tryptophan supplement) compared to the BB17 medium (0.1% tryptophan supplement), which therefore supported IAA production via a tryptophan-dependent pathway in the yeast *R. paludigenum* DMKU-RP301. Moreover, the BB23 medium also contained a higher amount of yeast extract: a vitamin and growth factor favourable for yeast growth. Both tryptophan and yeast extract promoted high levels of IAA production as well as good growth of the *R. paludigenum* DMKU-RP301.

It appeared that IAA might not be the main product of tryptophan catabolism of *R. paludigenum* DMKU-RP301 under high tryptophan supplement conditions (BB17 medium). After one day of cultivation, *R. paludigenum* DMKU-RP301 started to produce and gradually release IAA into the culture medium, as well as

increasing in cell mass. As a secondary metabolite, IAA production had increased rapidly after three days of incubation when a stationary growth phase was reached in the BB23 medium (Table 1). The highest relative amount of IAA detected by GC-MS was shown after seven days of incubation (Table 1). This result was similar to that for the yeasts *Rhodotorula graminis* WP1, *Rh. mucilaginosa* PTD2 and *Rh. mucilaginosa* PTD3 that obtained their maximum IAA levels after the seventh day of cultivation (Xin et al. 2009).

We also noticed that incubation time affected the detection of indole derivatives in the culture supernatant. In the case of BB17 conditions, we found IAA, indole-3-lactic acid and indole-3-ethanol in the culture supernatant after one day of incubation. However, we did not find IAA, indole-3-lactic acid and indole-3-ethanol in the culture supernatant of the BB23 medium before five days of incubation. This indicates that the longer the incubation, the more variety will be detected in the indole derivative profile.

Some intermediates of IAA biosynthesis can be reversibly converted to storage compounds, such as indole-3-lactic acid and indole-3-ethanol. In this study, indole-3-pyruvic acid and indole-3-acetaldehyde were not detected, but we did find indole-3-lactic acid and indole-3-ethanol, which are products of the enzymatic reduction of indole-3-pyruvic acid and indole-3-acetaldehyde, respectively, via the indole-3-pyruvic acid pathway of IAA biosynthesis. This result was consistent with that reported by Szkop and Bielawski (2013), as the indole-3-pyruvic acid and indole-3-acetaldehyde were unstable and were not intracellularly accumulated (Carreno-Lopez et al. 2000). In addition, it has been proposed that indole-3-ethanol serves as a reservoir for IAA, and maintains the level of indole-3-acetaldehyde in equilibrium in plants (Brown and Purves 1980). Our results suggested that, under the conditions that were less supportive of IAA production (BB17 conditions), tryptophan had been converted to other storage compounds, e.g. indole-3-pyruvic acid and indole-3-ethanol as the majority. On the other hand, under the conditions that promoted IAA production (BB23 conditions), almost all of the tryptophan had been used for IAA formation. However, it should be noted that the BB23 medium contained 0.5% tryptophan, a level that is almost toxic to *R. paludigenum* DMKU-RP301 growth (Nutaratat et al. 2015). Therefore, the yeast *R. paludigenum* DMKU-RP301 converted tryptophan to IAA and some minor storage intermediates.

As mentioned above, indole-3-pyruvic acid was unstable then small amount could be observed. Under this study, indole-3-pyruvic acid was not detected by using GC-MS because GC-MS required extraction procedure before detection and therefore caused the loss of indole-3-pyruvic acid during the extraction process. To avoid this, we then decided to immediately detect indole-3-pyruvic acid in the supernatant by using HPLC without extraction process. The results showed the low amount of indole-3-pyruvic acid (Fig. 3). The presence

of indole-3-pyruvic acid, indole-3-lactic acid and indole-3-ethanol thus suggested that *R. paludigenum* DMKU-RP301 used an indole-3-pyruvic acid pathway as the sole route to produce IAA under conditions in which sucrose and yeast extract were used as carbon and nitrogen sources respectively, and the medium pH was adjusted to 4 and 7 respectively (Fig. 2). This pathway is the major IAA biosynthesis pathway in microorganisms (Tsavkelova et al. 2006). The indole-3-pyruvic acid pathway has mainly been reported in plant growth promoting bacteria (PGPB), whereas the indole-3-acetamide pathway has mainly been reported in phytopathogenic bacteria (Spaepen and Vanderleyden 2011; Duca et al. 2014). However, indole-3-pyruvic acid and indole-3-acetamide pathways occur in both PGPB and pathogenic bacteria.

IAA biosynthetic pathway of *R. paludigenum* DMKU-RP301 was further confirmed by feeding yeast with indole-3-pyruvic acid as a precursor instead of L-tryptophan. Yeast was grown in medium containing 2% sucrose, 0.1% KNO_3, with or without 0.1% indole-3-pyruvic acid and adjusted an initial pH to 4. IAA from culture supernatant was analysed by HPLC. The results confirmed that IAA accumulation was found in the medium supplemented with indole-3-pyruvic acid (Fig. S1). Yeast did not produce IAA when grown in the medium without 0.1% indole-3-pyruvic acid supplement.

In plants, indole-3-acetic acid is mostly found in conjugated form (IAA linked to sugars, amino acids or peptides); only a small amount of free IAA is present (Woodward and Bartel, 2005). The role of IAA's conjugated form includes transport, storage and the protection of the IAA from enzymatic degradation. Regarding the regulation of IAA levels in plants by enabling the catabolism of IAA to its conjugated forms that therefore help to control IAA in plant cells (homeostatic mechanism) (Cohen and Bandurski 1982; Seidel et al. 2006). In bacteria, IAA conjugates have been found only in *Pseudomonas savastanoi* pv. *savastanoi*, as an IAA-lysine conjugate (Glass and Kosuge 1986, 1988). In this study, IAA was converted to its methyl ester. The present results are consistent with the studies of plants (Yang et al. 2008), the bacteria *Pseudomonas amygdali* NCPPB 2610 (Evidente et al. 1993) and the fungi *Fusarium poae* FUN1 (Nagia et al. 2012) that also converted IAA to its methyl ester. Here we report the discovery of IAA methyl ester in yeast. However, the *in vivo* functions of IAA methyl ester remain unclear.

We also found skatole (3-methylindole) in the BB17 culture medium of *R. paludigenum* DMKU-RP301. Skatole was found in the black mould *Aspergillus niger* but not in the yeast *Saccharomyces cerevisiae* (Bau 1981). However, previous studies indicate that skatole has mostly been found in bacteria (Deslandes et al. 2001; Leveau and Lindow 2005; Mujahid et al. 2011). Therefore, this is also the first report of the discovery of skatole in yeast. Skatole is an organic compound belonging to the indole family; it is a product of the microbial

degradation of indole-3-acetic acid. Yeast IAA degradation is probably similar to that of bacteria, in that IAA degradation provides both the carbon and nitrogen needed for survival. In addition, some bacteria degrade IAA in IAA-rich environments, due to its toxicity to bacterial cells. IAA is a weak acid that becomes deprotonated in the neutral cytoplasm and may inhibit growth of bacteria by acidifying the cytoplasm (Tromas and Perrot-Rechenmann 2010). Apart from stakole, bacteria can degrade IAA into catechol, indole-3-methanol, isatin, indole-3-carboxylic acid, methoxy-indole 3-acetic acid, 2-aminobenzoyl-coenzyme A, and benzoyl-coenzyme A (Ernstsen et al. 1987; Deslandes et al. 2001; Leveau and Lindow 2005; Kumavath et al. 2010; Mujahid et al. 2011; Ebenau-Jehle et al. 2012). However, none of these intermediates have yet been reported in yeast. It seems likely that microorganisms (including yeast) promote plant growth by providing IAA to the plant and simultaneously degrading the excess IAA to minimize IAA level, since high IAA concentration has a negative effect on cell survival.

Determination of tryptophan aminotransferase activity

Apart from indolic compound intermediates detection, the determination of enzyme activity that involved with IAA biosynthetic pathway was carried out. Tryptophan aminotransferase activity was determined by measuring the loss of L-tryptophan and the formation of indole 3-pyruvic acid using HPLC. HPLC analysis of enzyme reaction mixture indicated the formation of products having an R_t of 14.7 which corresponds to authentic indole 3-pyruvic acid standard. This result indicated that *R. paludigenum* DMKU-RP301 had trytophan aminotranferase activity which is the key enzyme of indole 3-pyruvic acid-routed IAA biosynthesis.

Conclusions

Rhodosporidium paludigenum DMKU-RP301 was shown to possess a tryptophan-dependent pathway for IAA biosynthesis, since the strain produced IAA in a culture medium supplemented with L-tryptophan. By using GC-MS analysis, we found that *R. paludigenum* DMKU-RP301 mainly used an indole-3-pyruvic acid pathway for IAA production from tryptophan, when sucrose and yeast extract were used as carbon and nitrogen sources respectively, regardless of whether the medium pH was 4 or 7.

Acknowledgments

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Table
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Table 1 Identification of indole derivatives from the culture supernatant (BB17 and BB23 medium) of *Rhodosporidium paludigenum* DMKU-RP301 using the GC-MS

Sample name	R_t (min)	% of total*	m/z	Indole derivatives	Sample name	R_t (min)	% of tota*l	m/z	Indole derivatives
BB17 (1 Day)	8.716	5.88	117	Indole	BB23 (1 Day)	8.711	4.08	117	Indole
	9.984	1.02	131	3-methylindole		9.990	1.11	131	3-methylindole
	14.479	32.70	161	Indole-3-ethanol		14.425	5.56	161	Indole-3-ethanol
	15.018	0.58	189	Indole-3-acetic acid methyl ester		15.547	4.16	175	Indole-3-acetic acid
	15.569	9.61	175	Indole-3-acetic acid			85.09		Other and unidentified
	16.502	1.67	219	Indole-3-lactic acid methyl ester					
	17.425	28.09	205	Indole-3-lactic acid					
		20.45		Other and unidentified					
BB17 (3 Days)	8.722	5.75	117	Indole	BB23 (3 Days)	8.711	5.22	117	Indole
	9.990	1.68	131	3-methylindole		9.984	1.97	131	3-methylindole
	14.517	26.45	161	Indole-3-ethanol		14.411	19.67	161	Indole-3-ethanol
	15.024	0.24	189	Indole-3-acetic acid methyl ester		15.574	21.17	175	Indole-3-acetic acid
	15.612	7.84	175	Indole-3-acetic acid			51.97		Other and unidentified
	16.508	1.30	219	Indole-3-lactic acid methyl ester					
	17.544	46.35	205	Indole-3-lactic acid					
		10.39		Other and unidentified					
BB17 (5 Days)	8.722	5.19	117	Indole	BB23 (5 Days)	8.722	2.36	117	Indole
	9.990	1.41	131	3-methylindole		10.017	5.97	131	3-methylindole
	14.527	26.72	161	Indole-3-ethanol		14.452	3.17	161	Indole-3-ethanol
	15.024	0.28	189	Indole-3-acetic acid methyl ester		15.018	0.62	189	Indole-3-acetic acid methyl ester
	15.628	12.02	175	Indole-3-acetic acid		15.806	44.50	175	Indole-3-acetic acid

Sample name	R_t (min)	% of total*	m/z	Indole derivatives	Sample name	R_t (min)	% of tota*l	m/z	Indole derivatives
	16.513	1.26	219	Indole-3-lactic acid methyl ester		17.538	25.56	205	Indole-3-lactic acid
	17.592	47.30	205	Indole-3-lactic acid			17.82		Other and unidentified
		5.82		Other and unidentified					
BB17 (7 Days)	8.997	5.47	117	Indole	BB23 (7 Days)	8.997	3.96	117	Indole
	10.281	2.81	131	3-methylindole		10.373	16.63	131	3-methylindole
	14.802	26.39	161	Indole-3-ethanol		14.711	0.07	161	Indole-3-ethanol
	15.277	2.75	189	Indole-3-acetic acid methyl ester		15.310	5.94	189	Indole-3-acetic acid methyl ester
	15.881	12.92	175	Indole-3-acetic acid		16.167	52.73	175	Indole-3-acetic acid
	16.761	7.41	219	Indole-3-lactic acid methyl ester		16.766	2.28	219	Indole-3-lactic acid methyl ester
	17.673	36.61	205	Indole-3-lactic acid		17.711	17.75	205	Indole-3-lactic acid
		5.64		Other and unidentified			0.64		Other and unidentified

* the relative amounts of the existing intermediates compared to all intermediates detected

Figure
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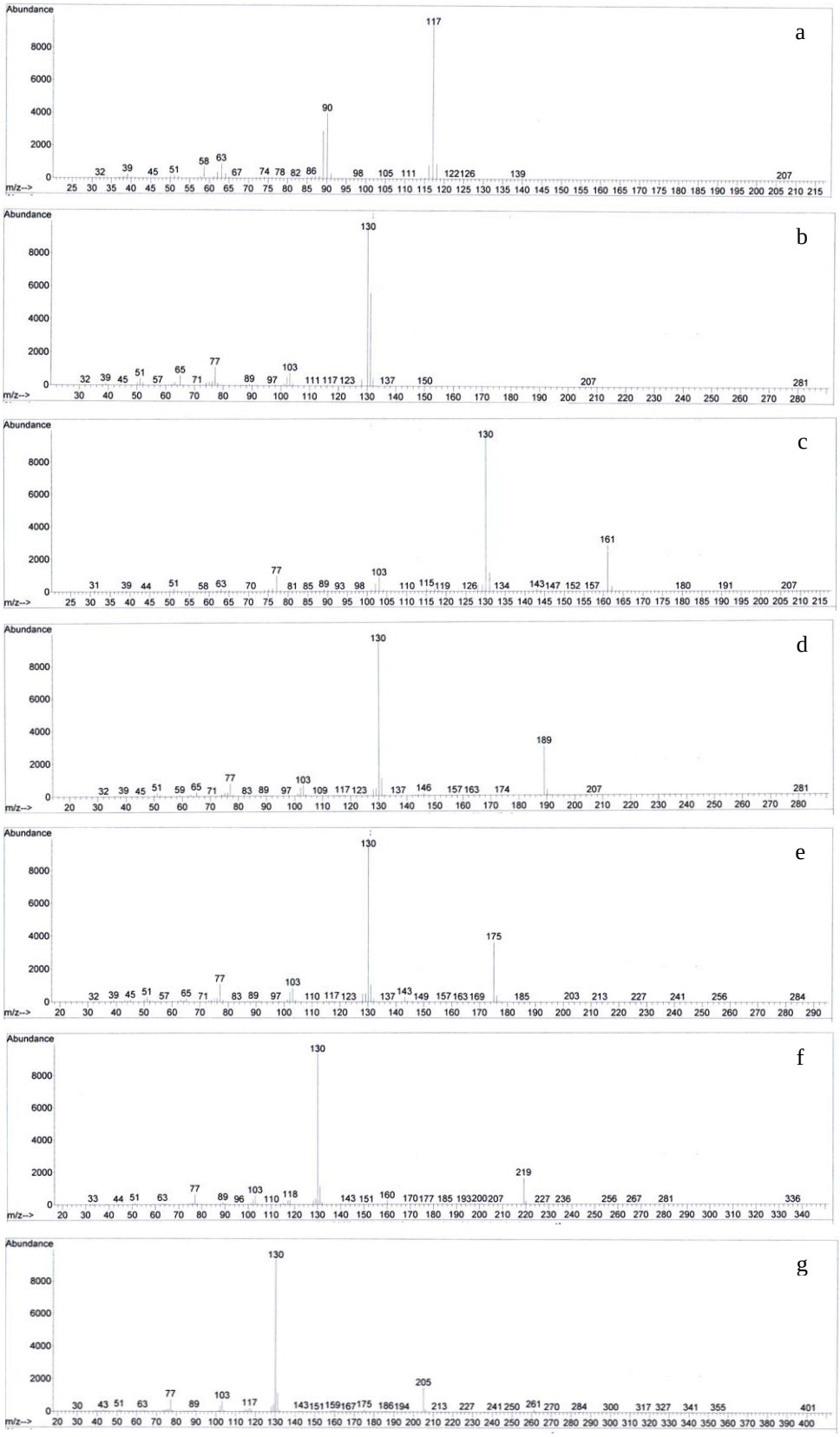


Fig. 1 GC–MS analysis of extracted broth showed specific mass spec patterns of indole (a), 3-methylindole (b), indole-3-ethanol (c), indole-3-acetic acid, methyl ester (d), indole-3-acetic acid (e), indole-3-lactic acid, methyl ester (f) and indole-3-lactic acid (g).

Figure
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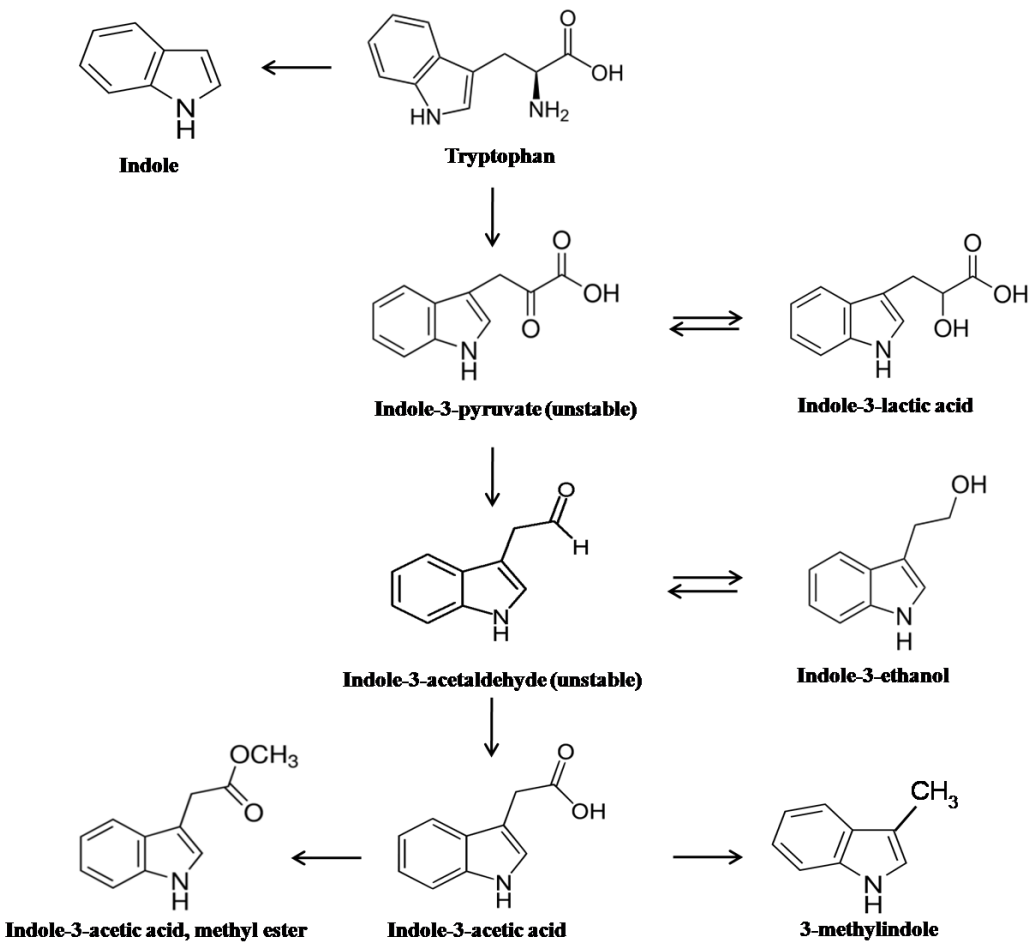


Fig. 2 Proposed IAA biosynthetic pathway of *Rhodosporidium paludigenum* DMKU-RP301 including IAA degradation. The proposed pathway for L-tryptophan catabolism is based on experimental evidence observed in the present study and the evidence of the intermediate was also obtained from the literature (Mujahid et al 2011; Spaepen and Vanderleyden 2011).

Figure
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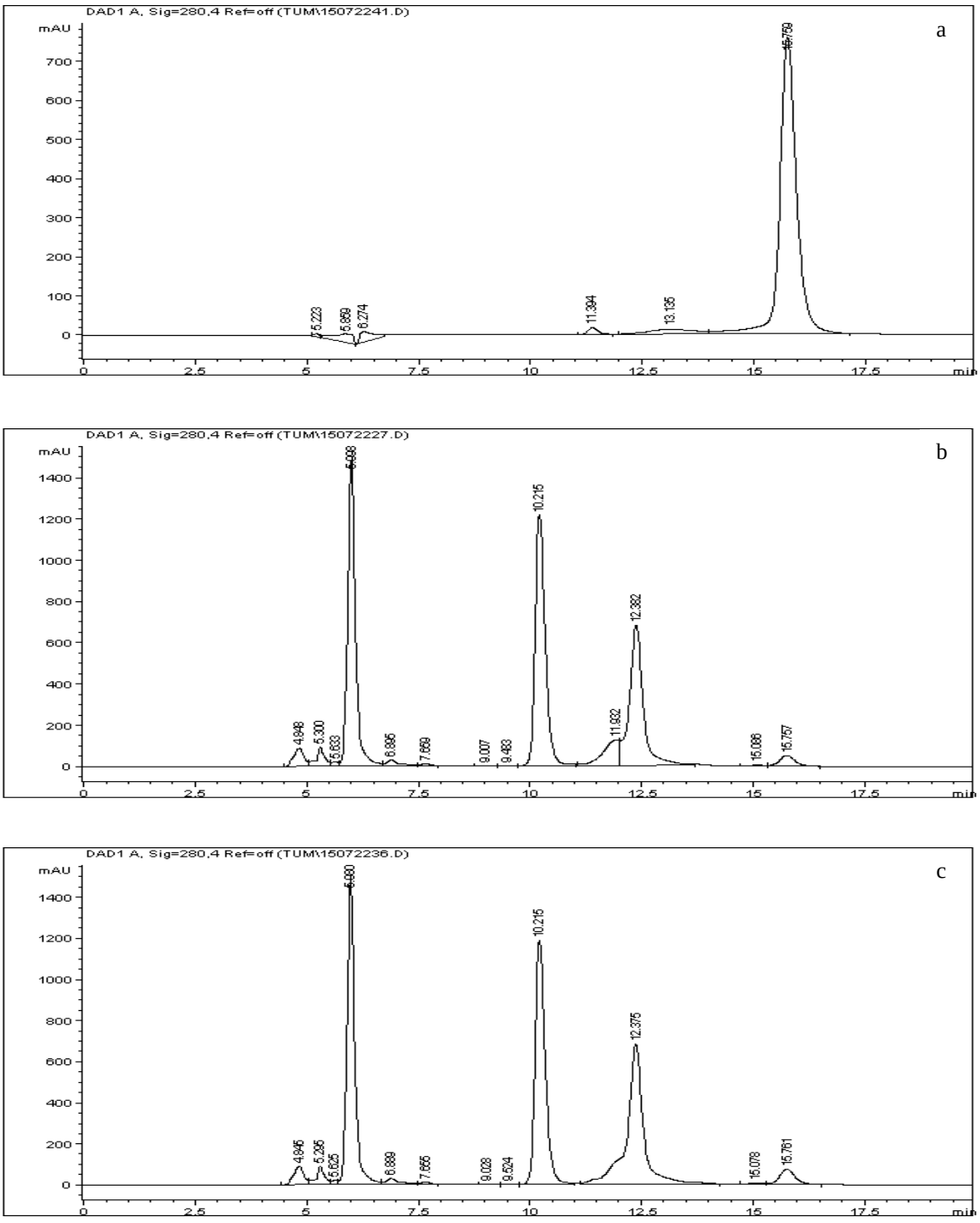


Fig. 3 Chromatogram of authentic indole-3-pyruvic acid (a), BB17 23 hrs culture supernatant of *Rhodosporidium paludigenum* DMKU-RP301 (b) and BB17 supernatant of *R. paludigenum* DMKU-RP301 mixed with authentic indole-3-pyruvic acid (c)**.**

Supplementary Material

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Supplementary Material

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Yeasts in Corn Phylloplane in Thailand and their Antagonistic Activities against Plant Pathogenic Fungi

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ABSTRACT

This study aimed to investigate the diversity of yeasts in corn phylloplane and evaluate antagonistic activity against pathogenic fungi causing corn and rice diseases of the isolated yeasts. One hundred and twenty-six yeast strains were isolated by the enrichment technique from the phylloplane of 65 corn leaf samples collected in Thailand. On the basis of the D1/D2 region of the large subunit rRNA gene sequence analysis, 121 strains were identified to 28 known species belonging to the Ascomycota *viz. Candida*, *Cyberlindnera*, *Geotrichum*, *Hanseniaspora*, *Kluyveromyces*, *Kodamaea*, *Meyerozyma*, *Millerozyma*, *Pichia*, *Torulaspora*, *Wickerhamomyces* and *Yamadazyma*, and 10 species of the Basidiomycota *viz. Cryptococcus*, *Rhodosporidium*, *Rhodotorula*, *Sporidiobolus* and *Sporobolomyces*. Four strains had identical sequences with two undescribed species. One strain represents the recently described species

Yamadazyma phyllophila. The prevalent yeast species was *Meyerozyma caribbica* with a frequency of occurrence of 9.5% followed by *Kodamaea ohmeri* and *Rhodotorula taiwanensis*, each of which having a 7.9% frequency of occurrence. In vitro evaluation of antagonistic activity against eight plant pathogenic fungi of all yeast strains revealed that none of them showed antagonistic activity against the five pathogenic fungi causing corn diseases. However, one strain, *Torulaspora globosa* DMKU-RK547, inhibited mycelium growth of the three rice pathogens *viz. Fusarium moniliforme, Rhizoctonia solani* and *Helminthosporium oryzae* with 31.4, 25.0 and 14.2% inhibition, respectively. The antagonistic mechanisms of *T. globosa* DMKU-RK547 were based on antifungal volatile compound and siderophore production.

Keywords: phylloplane yeast, enrichment technique, antagonistic activity, pathogenic fungi, corn

1. INTRODUCTION

The phylloplane, or phyllosphere, is a term for the above ground surface of plants and usually refers to the external surface of plant leaves, a dominant aerial part of plant [1]. The phylloplane is known to be colonized by a large number of microorganisms including bacteria, yeasts and fungi [2-3]. The phylloplanes of both dicotyledonous and monocotyledonous plants are colonized by both basidiomycete and ascomycete yeasts [1, 4-9]. Fewer studies on yeasts that colonize the phylloplanes of monocotyledonous plants have been reported. The ballistoconidium-fall method was used to isolate ballistoconidium-forming yeasts from dead leaves and stems of rice in Japan and from rice and various plants in Thailand [4, 6]. The prevalent yeast species associated with leaves, stems and rhizospheres of sugarcane in Brazil were found to be *Cryptococcus laurentii, Cryp. albidus, Rhodotorula mucilaginosa* and *Debaryomyces hansenii* [5]. In a recent study, our group investigated yeasts in sugarcane phylloplanes by using an enrichment technique and reported that 69% of the strains were ascomycete yeasts and 31 % of the strains were basidiomycete yeasts [10]. The most prevalent species in sugarcane phylloplanes in that study was *Meyerozyma caribbica* with a 23% frequency of occurrence followed by *Rhodotorula taiwanensis* (11%) and *Candida tropicalis* (10%).

Corn, a monocotyledonous plant, is one of the important crops in Thailand. The Office of Agricultural Economics, Ministry of Agriculture and Cooperatives reported the corncultivation area to be approximately 9,874,300 hectares and the production of corn for the year 2012 to be approximately 26,200,000 tons.

Although application of fungicides is still an effective method for controlling plant diseases caused by fungi, the widespread use of chemical fungicides has become a subject of concern due to their harmful effects on non-target organisms. Biological control, which is the use of living agents to control plant diseases, is increasingly considered to be a reliable alternative to chemical fungicides. Among the organisms being studied for their biological

control activities, yeasts are one group that shows promise as biological control agents. Antagonistic yeasts which have been studied for their biological control activities have been derived from various habitats. Many of them have been isolated from the same habitats as those in which the diseases occurred while some yeast strains showing antagonistic activity have been obtained from the other sources. However, yeasts obtained from the environment, especially those from agricultural areas, have shown better results in controlling plant diseases [11]. *Candida glabrata, C. maltose, C. slooffii, Rhodotorula rubura* and *Trichosporon cutanum*, which were obtained from the rhizosphere of maize, were used as biocontrol agents for kidney bean wilt disease caused by *Fusarium oxysporum* [12]. *Tolulaspora globosa* isolated from the rhizosphere of sugarcane was reported to have antagonistic activity against *Colletotrichum sublineolum*, the causative agent of sorghum anthracnose [13]. *Pichia albicans* and *Candida sake*, which were isolated from the surface of orange fruits, were used as biocontrol agents for controlling foliar diseases of sugar beet [14].

Yeasts inhabiting the phylloplane have been studied intensively, however, as far as we know there is no report on diversity of yeasts in the corn phylloplane. Therefore, this study aimed to investigate the diversity of yeasts in the phylloplane of corn in Thailand by culture-dependent approach using the enrichment isolation technique. The isolated corn phylloplane yeast strains were evaluated for their antagonistic activity against pathogenic fungi causing corn and rice diseases, and the antagonistic mechanisms of the antagonistic yeast were studied.

2. MATERIALS AND METHODS

2.1 Sample Collection and Yeast Isolation

Green and healthy leaves of corn (*Zea mays* Linn.) were random collected from corn fields. Leaf samples were put in plastic bags, sealed and brought in an ice-box to the

laboratory. The samples were kept at 8°C until subjected to yeast isolation that usually within five days after collection.

Yeasts were isolated by an enrichment technique using yeast extract malt extract (YM) broth (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone and 10 g/L glucose) supplemented with 250 mg/L sodium propionate and 200 mg/L chloramphenicol [15]. Three grams of cut leaves, derived from cutting a few leaves to the size that could be put into a 250 ml Erlenmeyer, was inoculated into 50 ml enrichment broth in the flask and incubated on a rotary shaker at 30±2°C for 2 days. A loopful of the enriched culture was streaked on YM agar supplemented with 250 mg/L sodium propionate and 200 mg/L chloramphenicol. Yeast colonies of different morphologies were picked and purified by cross streaking on YM agar. Purified yeast strains were suspended in YM broth supplemented with 10% v/v glycerol and maintained at -80°C.

2.2 Yeasts Identification

Yeasts were identified by analysis of the D1/D2 region of the large subunit (LSU rRNA) gene sequence similarities [16-17]. The sequence of the D1/D2 region of the LSU rRNA gene was determined from PCR products amplified from genomic DNA. The D1/D2 region of the LSU rRNA gene was amplified and sequenced with primers, NL1 and NL4 [16]. Methods for DNA extraction and amplification of the D1/D2 region of the LSU rRNA gene and ITS region were as described previously [15]. The PCR products were checked by agarose gel electrophoresis and purified by using the Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taiwan). The purified products were submitted to Macrogen Inc. (Korea) for sequencing. Sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler using a ABI PRISM® BigDye™ Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems, USA), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using the primers NL1 and NL4 for the D1/D2 region of the LSU rRNA gene. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation

protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730XL sequencer (Applied Biosystems, USA). The sequences were compared pairwise using a BLAST search [18].

2.3 Evaluation of the in Vitro Antagonistic Activity against Plant Pathogenic Fungi

To evaluate the antagonistic activity of the isolated phylloplane yeasts five corn pathogenic fungi *viz. Bipolaris maydis* (southern leaf blight disease), *Bipolaris zeicola* (northern leaf spot disease), *Exserohilum turcicum* (northern leaf blight disease), *Fusarium moniliforme* (stem rot disease) and *Macrophomina phaseolina* (stem rot disease), and three rice pathogenic fungi *viz. Fusarium moniliforme* (bakanae disease), *Helminthosporium oryzae* (brown spot disease) and *Rhizoctonia solani* (sheath blight disease) were used.

The inhibition of fungal pathogens by the phylloplane yeasts was determined by using the dual culture technique where the pathogenic fungus and yeast were grown side by side on potato dextrose agar (PDA) plates following Rosa et al. [13]. Each PDA plate was inoculated near its edge with a 5-mm diameter disk of fungal mycelium grown on PDA at 25°C for 5 days. At the opposite edge, one loop of yeast culture grown on yeast extract malt extract agar (YM) agar at 25°C for 1-2 days was inoculated at approximately 3 cm from the fungal disk. The dual culture PDA plate was incubated at 25°C for 7 days. Three replicates of each treatment were carried out. The percentage inhibition of the pathogenic fungal growth was calculated as % Inhibition = $\underline{D1 - D2}/D \times 100$ (where D1 is the diameter of control fungal colony and D2 is the diameter of fungal colony grown with yeast).

2.4 Evaluation of in Vitro Antagonistic Mechanisms

To evaluate the in vitro antagonistic mechanisms against the plant pathogenic fungi of the antagonistic yeast various characteristics were determined. Three replicates were carried out in each treatment.

To investigate the damage to mycelium of the pathogenic fungi by the antagonistic yeast, the dual culture technique on PDA, which was used to evaluate the antagonistic

activity, was performed as described by Rosa et al. [13]. Mycelium disks of 5-mm diameter were removed from different parts of the dual culture plate where the pathogenic fungal mycelium growth inhibition was observed after 5 days incubation at 25°C. The mycelia were studied microscopically at 400x magnification. Production of antifungal volatile compounds by the antagonistic yeast was investigated using the dual culture technique on a PDA plate, which was partitioned in the middle [13]. The pathogenic fungus and the antagonistic yeast were inoculated on different sides of the plate. The mycelium growth inhibition was determined after 7 days of incubation at 25°C. The production of siderophores by the antagonistic yeast was determined by cultivation on chrome azurol S (CAS) blue agar, which was prepared according to Schwyn and Neilands [19]. Yeast culture grown on YM agar at 25°C for 1-2 days was inoculated on CAS blue agar and incubated at 25°C for 10 days in the dark. A yellow halo around the colony was taken as indicative of siderophore production. Production of hydrolytic enzyme (β-glucanase and chitinase) by the antagonistic yeast was determined by the method of Saligkarias et al. [20]. Yeast was cultivated in YM broth in a test tube on a shaker at 150 rpm and 30±2°C for 3 days. Yeast cells were removed from the culture broth by centrifugation at 10,000g for 5 min, the supernatant was collected and analyzed for hydrolytic enzyme activity. The β-glucanase and chitinase activities were assayed by measuring the release of reducing sugar (glucose and N-acetylglucosamine, respectively) from their respective substrates, laminarin and colloidal chitin, using dinitrosalicylic (DNS) acid reagent [21]. β-1,3-glucanase activity was assayed by mixing 200 µl of the culture supernatant, 50 µl of sodium acetate buffer (0.1 M, pH 5.0) and 250 µl of laminarin (4 mg/ml) and incubating at 37°C for 1 h. Then, the reaction was stopped by adding 250 µl of DNS reagent to 250 µl of the reaction mixture and the concentration of reducing sugar was determined. Chitinase was assayed by mixing 100 µl of the culture supernatant with 200 µl of Mcllvaine buffer (50 mM citric acid; 100 mM sodium phosphate; pH 6.0) and

100 µl of 0.01% w/v colloidal chitin (prepared in the same buffer). After incubation at 37°C for 15 min, the reaction was stopped by adding 250 µl of DNS reagent to 250 µl of the reaction mixture and the reducing sugar concentration was determined.

3. RESULTS AND DISCUSSION

3.1 Sample Collection and Yeast Isolation

Sixty-five samples of corn leaf were collected from eight sampling sites in four provinces in Thailand between September 2009 and May 2010 (Table 1). These four provinces were in the central and north-eastern parts of Thailand, where the average maximum temperature was 32-36°C and average minimum temperature was 22-25°C throughout the year. Yeasts were isolated by the enrichment technique, and 126 isolates were obtained (Table 1).

3.2 Yeast Identification and Diversity of Yeasts in Corn Phylloplanes

On the basis of the D1/D2 region of the LSU rRNA gene sequence analysis of the 126 strains, 121 strains were identified to belong to 38 yeast species, one strain represents the recently described species *Yamadazyma phyllophila* [22] and four strains represented two undescribed species (Tables 2). Among the known species, 29 species were in 12 genera of the phylum Ascomycota and 10 species belonged to five genera of the phylum Basidiomycota. The known yeast species in 12 genera belonging to Ascomycota consisted of *Cyberlindnera* (one species), *Geotrichum* (one species), *Hanseniaspora* (three species), *Kluyveromyces* (one species), *Kodamaea* (one species), *Meyerozyma* (two species), *Millerozyma* (two species), *Pichia* (three species), *Torulaspora* (one species), *Wickerhamomyces* (one species), *Yamadazyma* (two species), and *Candida* (11 species). The 11 *Candida* species were in five clades including *Lodderomyces-Spathaspora* (three species), *Metschnikowia* (one species), *Kurtzmaniella* (one species), *Nakaseomyces* (one species), *Yamadazyma* (one species) and four unaffiliated clades (four species). The known yeast

species in five basidiomycete yeast genera *viz*. *Cryptococcus* (two species in the *Bulleromyces* clade and one species in *heveanensis* clade), *Rhodosporidium* (three species), *Rhodotorula* (one species), *Sporidiobolus* (two species) and *Sporobolomyces* (one species). Among four strains of undescribed species two strains had identical sequences of the D1/D2 region with an undescribed species which the sequence has been deposit in GenBank, *Candida* sp. 05-7-186T (FJ537069), and two strains were identical with *Cryptococcus* aff. *laurentii* APSS 823 (AM931019).

The prevalent yeast species was *M. caribbica* with a frequency of occurrence of 9.5% followed by *Kodamaea ohmeri* and *R. taiwanensis*, each of which having a 7.9% frequency of occurrence then *Candida tropicalis*, *Hanseniaspora opuntiae*, *Pichia kudriavzevii* and *Rhodosporidium fluvial*, each with a frequency of occurrence of 6.4% and *Crypt. laurentii*, with a frequency of occurrence of 5.6%. Only 1-5 strains (1-4%) of the remaining species were detected. The results revealed that *M. caribbica* and *R. fluvial* were isolated from corn leaf samples collected from 5 out of the 8 sampling sites while *K. ohmeri* and *Rh. taiwanensis* were found from 3 and 4 sampling sites, respectively (Table 2).

The results revealed that 64% of the strains obtained from corn phylloplanes by the enrichment isolation technique at 30$\pm$2°C were ascomycete yeasts, and only 36% represented basidiomycete yeasts. The present results agree well with the previous studies of ours, which also reported the dominance of ascomycete yeast species on the phylloplanes of sugarcane and various plants in Thailand when enrichment isolation and a relatively high incubation temperature were used [10, 23]. Also, in some reports ascomycetes were found to be important leaf colonists [5]. However, it is in contrast with other investigations, which have reported the dominance of basidiomycete yeasts in the phylloplanes of other regions [1, 5-8]. The difference in the results may derive from the difference in cultivation temperature and the technique employed for isolation. In most investigations, plating of leaf washings and incubating at 25 °C or lower was used [5, 7] while Nakase et al. [6] used the

ballistoconidium-fall method with YM agar without any antibacterial and antifungal agents and found more yeast with the incubation temperature of 23°C instead of 30°C.

Among the prevalent species in corn phylloplanes in this study, *M. caribbica*, *Rh. taiwanensis*, *C. tropicalis*, and *Crypt. laurentii* were the prevalent species in sugarcane phylloplanes, while *P. kudriavzevii* and *R. fluvial* have also been previously found in sugarcane phylloplanes in Thailand [10]. *K. ohmeri*, *H. opuntiae* and *P. kudriavzevii* have been reported to isolate from other plants species in Thailand. *Rh. taiwanensis*, the prevalent species in the corn phylloplane in this study, was proposed recently from the strain isolated from plant stem tissue in Taiwan [24]. *C. tropicalis* has been reported to be one of the dominant yeast species in the phylloplanes of various plants in Russia [1]. It was isolated from the phylloplanes of fruit trees in the Czech Republic [7]. This species was previously reported to be present in the phylloplanes of the other plant species in Thailand with a relatively high frequency of occurrence (14%) [23]. Among the species found in corn phylloplanes, *Crypt. laurentii* seems to be a common phylloplane yeast regardless of the plant species and geography because it presence in phylloplanes has been reported in many investigations [1, 5, 7-8, 10]. Many yeast species found in the corn phylloplane had also been detected previously in the phylloplanes of diverse plants species in Thailand although with low frequencies of occurrence. These included *Candida glabrata*, *C. mesorugosa*, *C. orthopsilosis*, *C. rugosa*, *C. sorboxylosa*, *Cyberlindnera rhodanensis*, *Hanseniaspora guilliermondii*, *H. opuntiae*, *H. thailandica*, *Kluyveromyces marxianus*, *Meyerozyma guilliermondii*, *P. kudriavzevii*, *Wickerhamomyces edaphicus* and *Sporidiobolus ruineniae* [10, 23].

These results could be interpreted to mean that the plant species has no effect on the yeast species in the phylloplane; on the other hand, the climate may influence the yeasts in the phylloplane. The species of the small population in Ascomycota are possible to be isolated by using enrichment technique. From the result of this work we could confirm that *M. caribbica*,

Rh. taiwanensis, C. tropicalis and *R. fluvial* are often found in phylloplane of plants in tropical area.

3.3 Evaluation of the in Vitro Antagonistic Activity against Plant Pathogenic Fungi

Evaluation of the in vitro antagonistic activity of all 126 yeast strains isolated from corn phylloplanes revealed that none of them inhibited mycelium growth of five corn pathogenic fungi. Only one strain, *Torulaspora globosa* DMKU-RK547, was able to inhibit mycelium growth of all three rice pathogenic fungi. Its inhibitions of *F. moniliforme, R. solani* and *H. oryzae* were 28.7%, 29.4% and 11.8%, respectively (Table 3).

The low number of antagonists isolated from the environment was not a surprise, since many other workers have reported similar results Chanchaichaovivat et al. [25] demonstrated that from 225 yeast strains isolated from fruits and vegetables, only four strains showed effective growth inhibition of *Colletotrichum capsici*, a pathogenic fungus causing chili anthracnose. The four strains were *Candida musae, C. quercitrusa, Pichia guilliermondii* and *P. kudriavzevii*. Wang et al. [26] showed that 24 out of 463 plant epiphytic yeast strains inhibited bacterial fruit blotch (BFB) pathogen, *Acidovorax avenae* subsp. *Citrulli*, the strain showing the highest reduction of BFB disease being *Pichia anomala* Rosa et al. [27] reported that eight out of the 317 yeast strains tested inhibited growth of *Colletotrichum graminicola* and *Colletotrichum sublineolum*, and of the eight, six strains were *Torulaspora globosa* while two strains were *Candida intermedia.*

3.4 Evaluation of in Vitro Antagonistic Mechanisms

The result of investigation on the three rice pathogenic fungi mycelium damage by *T. globosa* DMKU-RK547 revealed that mycelia obtained from two locations, close to and far away from the yeast culture, appeared normal. This result suggested that *T. globosa* DMKU-RK547 did not cause lethal hyphal deformity. This was different from the report of Rosa et al. [27] that another strain of *T. globosa* caused some changes in hyphal morphology, but that this was non-lethal to the mycelia. Investigation of antifungal volatile compound production

revealed that *T. globosa* DMKU-RK547 produced volatile compounds that inhibited the mycelial growth of all three rice pathogenic fungi. Production of antifungal volatile compounds has been reported in other antagonistic yeasts such as *Candida melibiosica* [28]. On the other hand, not all *T. globosa* strains exhibited this ability [27]. Study of siderophore production showed that *T. globosa* DMKU-RK547 grew and changed the color of the CAS-blue medium suggesting that it produced siderophores. However, siderophores are not always produced by *T. globosa* strains [27], and some other antagonistic yeast species such as *Rhodotorula glutinis* [29] produced siderophores. Siderophore production is a mechanism involving competition for nutrients because siderophore producing microorganisms are capable of taking up iron (ferric) ion from the substrate, making it unavailable to other microorganisms [29]. This mechanism may be involved in the pathogenic fungal growth inhibition of *T. globosa* DMKU-RK547. Investigation of the production of the hydrolytic enzymes β-glucanase and chitinase, which is a mechanism which inhibits fungal growth by digesting the fungal cell wall, revealed that *T. globosa* DMKU-RK547 could not produce either β-glucanase and chitinase. However, some strains of other antagonistic yeasts *Metschnikowia pulcherrima* and *P. guilliermondii* were reported to have the capacity to produce hydrolytic enzymes [30-31].

4. CONCLUSION

Our study has demonstrated that one hundred and twenty-six yeast strains were isolated from the phylloplane of 65 corn leaf samples collected in Thailand. On the basis of the D1/D2 region of the large subunit rRNA gene sequence analysis, 121 strains were identified to 28 known species belonging to the Ascomycota *viz. Candida*, *Cyberlindnera*, *Geotrichum*, *Hanseniaspora*, *Kluyveromyces*, *Kodamaea*, *Meyerozyma*, *Millerozyma*, *Pichia*, *Torulaspora*, *Wickerhamomyces* and *Yamadazyma*, and 10 species of the Basidiomycota *viz. Cryptococcus*, *Rhodosporidium*, *Rhodotorula*, *Sporidiobolus* and *Sporobolomyces*. Four strains had identical sequences with two undescribed species. One strain represents the

recently described species *Yamadazyma phyllophila*. The prevalent yeast species was *Meyerozyma caribbica* with a frequency of occurrence of 9.5% followed by *Kodamaea ohmeri* and *Rhodotorula taiwanensis*, each of which having a 7.9% frequency of occurrence. In vitro evaluation of antagonistic activity against seven plant pathogenic fungi of all yeast strains revealed that none of them showed antagonistic activity against the five pathogenic fungi causing corn diseases. However, one strain, *Torulaspora globosa* DMKU-RK547, inhibited mycelium growth of the two rice pathogens *viz. Fusarium moniliforme* and *Helminthosporium oryzae* with 31.4 and 14.2% inhibition, respectively. The antagonistic mechanisms of *T. globosa* DMKU-RK547 were based on antifungal volatile compound and siderophore production.

ACKNOWLEDGMENTS

This work was supported by the Thailand Research Fund through the TRF Research-Team Promotion Grant (RTA54800009), the Royal Golden Jubilee Ph.D. program grant no. PHD/0215/2551, the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission and NITE Biological Resource Center (NBRC), Japan. Special thank go to Dr. Hiroko Kawasaki and Dr. Wichien Yongmanitchai for their valuable comments.

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ภาคผนวก 2

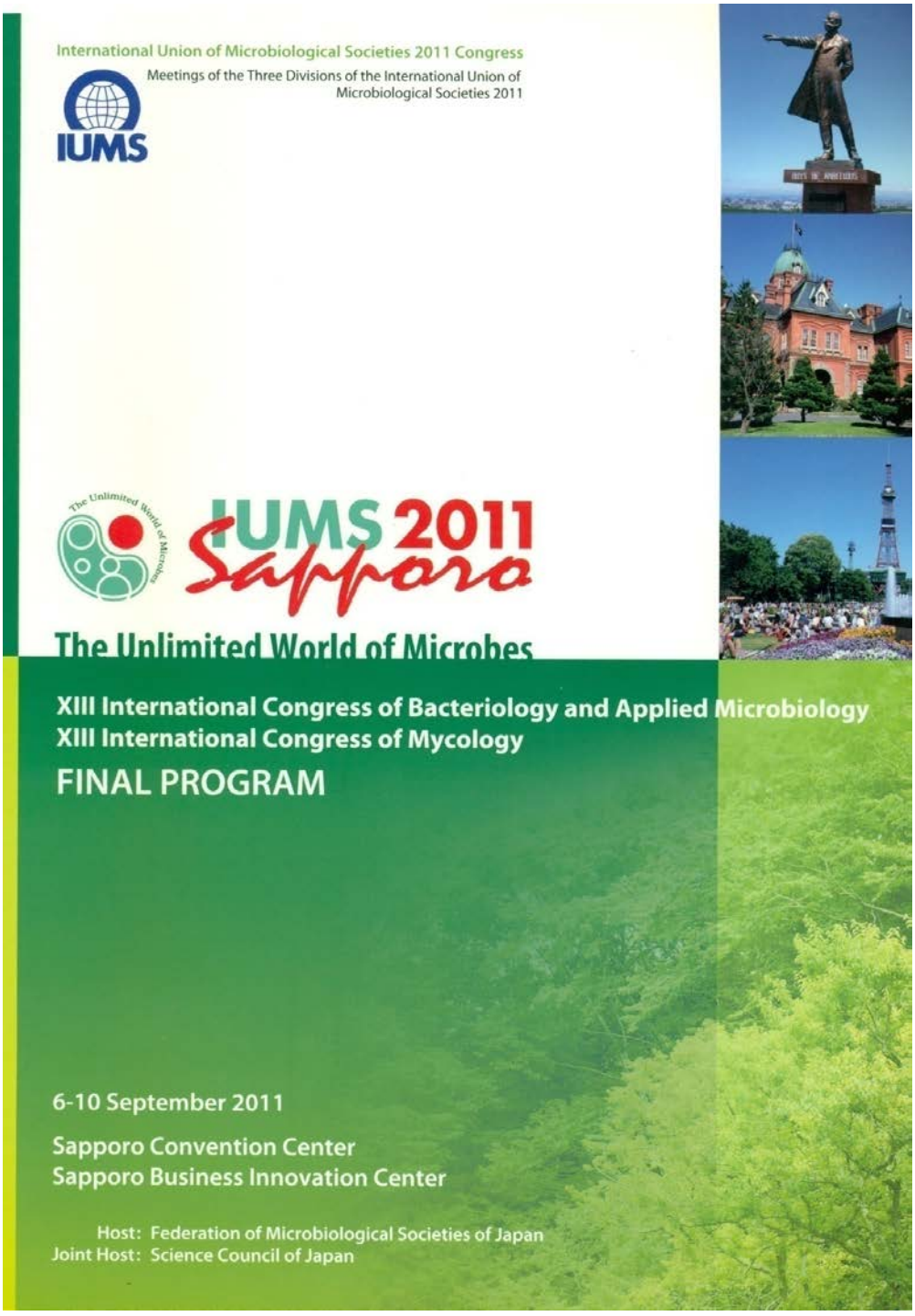
การเสนอผลงานในฐานะผู้บรรยายรับเชิญ (invited speaker)

ศ.ดร. สาวิตรี ลิ่มทอง ได้รับเชิญไปบรรยายพิเศษในเรื่องที่เกี่ยวข้องกับโครงการ ดังนี้

1. Diversity and indole-3-acetic acid production of phylloplane yeasts in Thailand with description of five novel ascomycetous yeast species บรรยายใน IUMS International Congress of Bacteriology and Applied Microbiology (BAM), and the International Congress of Mycology (IUMS 2011), 6-10 September 2011, Sapporo, Japan
2. Yeasts on agronomic crops phylloplanes: Diversity and potential in agricultural utilization บรรยายใน International Conference on Microbial Taxonomy, Basic and Applied Microbiology 4-6 October 2012 ณ โรงแรมโฆษะ จังหวัดขอนแก่น ประเทศไทย
3. Yeasts on agronomic crops phylloplanes: Diversity and potential in agricultural utilization บรรยายใน The 90th Anniversary Meeting of the Society for Biotechnology, Japan (SBJ) 23-26 October 2012 ณ (KICC), Kobe Port Island, Japan
4. Polyphasic taxonomy of yeasts บรรยายใน Seminar on Curation and Management of Microbial Resources ซึ่งจัดที่ Thailand Science Park, Pathum Thani, Thailand ในระหว่าง 14–17 มกราคม 2556
5. ความหลากหลายของยีสต์ในแหล่งที่อยู่ธรรมชาติและการค้นพบยีสต์สปีชีส์ใหม่ในประเทศไทย บรรยายในการประชุมชิสเทมาติคส์ในประเทศไทยครั้งที่ 5 ซึ่งจัดที่ คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ในระหว่าง 25-27 พฤษภาคม 2558

Diversity and indole-3-acetic acid production of phylloplane yeasts in Thailand with description of five novel ascomycetous yeast species

บรรยายใน IUMS International Congress of Bacteriology and Applied Microbiology (BAM), and the International Congress of Mycology (IUMS 2011), 6-10 September 2011, Sapporo, Japan



Wednesday, 7 September 14:00-16:00 Room C

BR9 Genomics and Resources in Microbiology (Symposium for the 60th Anniversary of JSCC)

Supported by Japan Society for Culture Collections (JSCC)

Conveners: Hans-Peter Klenk *Germany*
Kevin McCluskey *USA*

BR9-1 **CHARACTERIZATION OF CLASSICAL MUTANT STRAINS OF *NEUROSPORA* FROM THE FUNGAL GENETICS STOCK CENTER COLLECTION USING GENE AND WHOLE GENOME SEQUENCE ANALYSIS**

Kevin McCluskey[1], Aric Wiest[1], Scott Baker[2]
[1]*School of Biological Sciences, University of Missouri- Kansas City, USA,* [2]*Pacific Northwest National Laboratory*

BR9-2 **COMPARATIVE GENOMICS OF *ASPERGILLUS* AND GENE DIVERSITY**

Masayuki Machida[1,2], Hideaki Koike[1], Yoshinori Koyama[1], Hiroko Hagiwara[1], Itaru Takeda[2], Myco Umemura[1], Koichi Tamano[1]
[1]*Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Japan,* [2]*Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology*

BR9-3 **THE GENOMIC STANDARDS CONSORTIUM: AN OPEN INVITATION TO PARTICIPATE**

George M Garrity
Microbiology & Molecular Genetics, Michigan State University and Editorial Office, Standards in Genomic Sciences, USA

BR9-4 **THE ROLE OF CULTURE COLLECTIONS AND DNA BANKS FOR LARGE SCALE GENOMICS**

Hans-Peter Klenk
Microbiology, German Collection of Microorganisms and Cell Cultures, Germany

Wednesday, 7 September 14:00-18:30 Room I

BR10 Microbial Diversity in Asia and their Application

Supported by the National Institute of Technology and Evaluation (NITE)

Conveners: Katsuhiko Ando *Japan*
Wellyzar Sjamsuridzal *Indonesia*

BR10-1 **DISTRIBUTION OF *TRICHODERMA* SPECIES IN ASIA**

Yasuhisa Tsurumi[1], Tsetseg Balijinova[2], Nyunt Phay[3], Duong Van Hop[4], Haji Saidin Salleh[5], Yantyati Widyastuti[6], Ken-Ichiro Suzuki[1], Katsuhiko Ando[1]
[1]*Biological Resource Center, National Institute of Technology and Evaluation (NITE), Japan,* [2]*Institute of Biology, Mongolian Academy of Science (MAS),* [3]*Botany Department, Pathein University (PU),* [4]*Institute of Microbiology and Biotechnology, Vietnam National University, Hanoi (VNUH),* [5]*Department of Forestry, Ministry of Industry and Primary Resources (MIPR),* [6]*Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI)*

BR10-2 **HONEYBEE-ASSOCIATED YEASTS IN INDONESIA: DIVERSITY AND THEIR USE AS POLLEN SUBSTITUTES**

Wellyzar Sjamsuridzal, Adi Basukriadi, Ariyanti Oetari, Novia Rachmayanti, Bangga Beristama Putera, Retno Widowati, Estriningtyas Agus Rismawanti, Virgine Enfinali, Bregas Adi Luhur
Department of Biology, Faculty of Mathematics and Natural Sciences, University of Indonesia, Indonesia

BR10-3 **DIVERSITY AND INDOLE-3-ACETIC ACID PRODUCTION OF PHYLLOPLANE YEASTS IN THAILAND WITH DESCRIPTION OF FIVE NOVEL ASCOMYCETOUS YEAST SPECIES**

Savitree Limtong[1,2], Nampueng Koowadjanakul[1], Wichien Yongmanitchai[1,2], Sasitorn Jindamorakot[3]
[1]*Department of Microbiology, Faculty of Science, Kasetsart University, Thailand,* [2]*Center for Advanced Studies in Tropical Natural Resources, National Research University-Kasetsart University, Kasetsart University,* [3]*Bioresources Technology Unit, National Center for Genetic Engineering and Biotechnology (Biotec)*

BR10-4 **BACTERIAL AND ARCHAEAL DIVERSITY IN SOME SALINE ENVIRONMENTS OF MONGOLIA**

Tsetseg Baljinova[1], Tsuyoshi Yasuta[2], Marjangul Nyrimkhan[1], Katsuhiko Ando[2]
[1]*Laboratory of Microbiology, Institute of Biology, Mongolian Academy of Sciences, Ulaanbaatar-51, Mongolia,* [2]*Department of Biotechnology, National Institute of Technology and Evaluation (Nite), Chiba*

Diversity and Indole-3-Acetic Acid Production of Phylloplane Yeasts in Thailand with Description of Five Novel Ascomycetous Yeast Species

Savitree Limtong[1],
Nampueng Koowadjanakul[1],
Wichien Yongmanitchai[1]
and Sasitorn Jindamorakot[2]

[1]Department of Microbiology, Faculty of Science, Kasetsart University
and [2]National Center for Genetic Engineering and Biotechnology, Thailand

Phylloplane / phyllosphere

- External surface of plant leaves
- Important habitat for microorganisms
- Growth of microorganisms on phylloplane depend on nutrients derive by 2 ways:
  - endogenously from plant exudates that provide organic substances e.g. simple sugars (glucose, fructose and sucrose), organic acids, amino acids and methanol
  - exogenously from compounds found in materials that land on it, mostly are inorganic nutrients (Tukey, 1971)

Phylloplane Yeasts

Basidiomycota →
Sporobolomyces roseus
Rhodotorula glutinis
Rhodotorula mucilaginosa
Cryptococcus aurentii
***Cryptococcus magnus*, etc.**
(Inacio *et al.*, 2002; Glushakova *et al.*, 2006, etc.)

Ascomycota →
Torulaspora delbrueckii
Torulaspora pretoriensis
Kluyveromyces thermotolerans
(Spencer and Spencer, 1997)
Ogataea allantospora (methylotrophic yeast) (Peter *et al.*, 2007) **etc.**

Plant Growth Promoter

- Many plant-associate microorganisms are capable of promote plant growth and development through different mechanisms
- Some of them synthesize plant growth promoters similar to phytohormones produce in plant

Auxins, especially indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA), are the dominant plant growth promoters produced by yeasts

Candida valida
Rhodotorula glutinis
Rhodotorula mucilaginosa
Rhodotorula graminis
Saccharomyces roseus
Trichosporon asahii and *Lindnera (Williopsis) saturnus*
(Nassar *et al.*, 2005; Xin *et al.*, 2009)

Objectives

- To study biodiversity of phylloplane yeasts in Thailand
- To discover novel yeast species
- To investigate the ability of indole-3-acetic acid (IAA), a plant growth promoting auxin, production of the phylloplane yeasts

Research Outline

Collection of plant leaves
↓
Isolation
Yeasts | Methylothrophic yeasts
↓
Identification of yeast isolates
All isolates: Molecular Taxonomy and Phylogeny | Novel taxa: Polyphasic Taxonomy
↓
Determination of IAA production of all isolates

Plant leaves collection



- 97 leaf samples:
  - 79 samples of 47 plant species
  - 18 samples of unknown plants
- 20 locations in 7 provinces in Thailand

Provinces:
1. Buri Ran
2. Sakaeo
3. Prachin Buri
4. Nakhon Nayok
5. Chonburi
6. Prachuap Khiri Khan
7. Chumphon

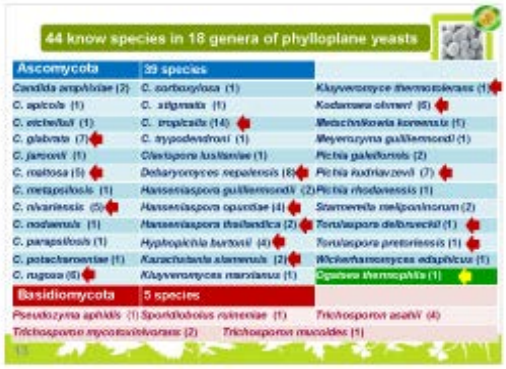
Yeast isolation: by enrichment in YM broth

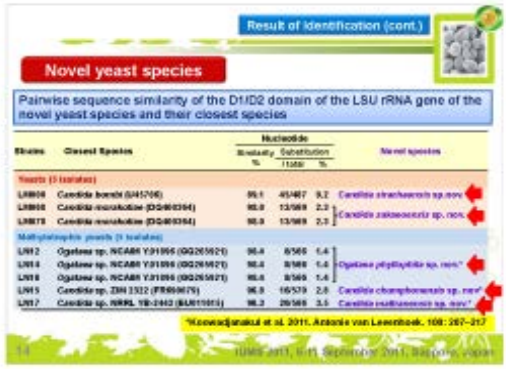


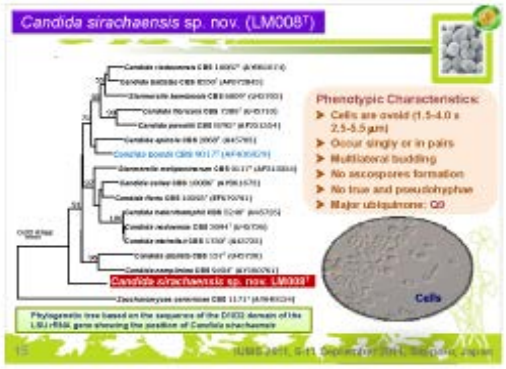
YM broth:
0.3% Yeast extract
0.3% Malt extract
0.5% Peptone
1.0% Glucose
250 mg/L sodium propionate
200 mg/L chloramphenicol

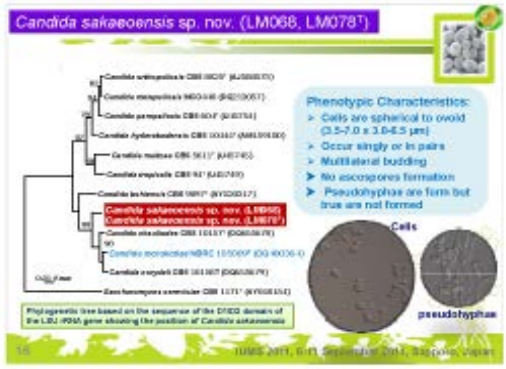
YM broth (50 ml)
Shaker at 150 rpm, 30±2°C, 2 days
↓
Streak on YM agar supplemented with sodium propionate and chloramphenicol
↓
30±2°C 1-2 day
↓
Pick yeast colony and purify by streaking on YM agar
↓
Pure culture



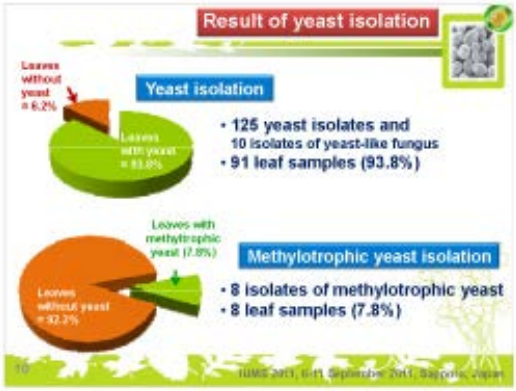

44 know species in 18 genera of phylloplane yeasts
Ascomycota
39 species
Candida amphixiae (2)
C. apicola (1)
C. etchellsii (1)
C. glabrata (7)
C. jamonii (1)
C. mattosa (5)
C. metapsilosis (1)
C. nivariensis (5)
C. nodaensis (1)
C. parapsilosis (1)
C. potaciarorantiae (1)
C. rugosa (6)
C. sorbosivora (1)
C. stigmata (1)
C. tropicalis (14)
C. oryzodesmonii (1)
Clavispora lusitaniae (1)
Debaryomyces nepalensis (8)
Hanseniaspora guilliermondii (2)
Hanseniaspora opuntiae (4)
Hanseniaspora thailandica (2)
Hyphopichia burtonii (4)
Kazachstania slooffiae (2)
Kluyveromyces marxianus (1)
Kluyveromyce thermotolerans (1)
Kodamaea ohmeri (6)
Metschnikowia koreensis (1)
Meyerozyma guilliermondii (1)
Pichia galeiformis (2)
Pichia kudriavzevii (7)
Pichia rhodanensis (1)
Starmerella meliponinorum (2)
Torulaspora delbrueckii (1)
Torulaspora prestoriensis (1)
Wickerhamomyces edaphicus (1)
Ogataea thermophila (1)
Basidiomycota
5 species
Pseudozyma aphidis (1)
Sporidiobolus ruinenae (1)
Trichosporon asahii (4)
Trichosporon mycotoxinivorans (2)
Trichosporon mucoides (1)

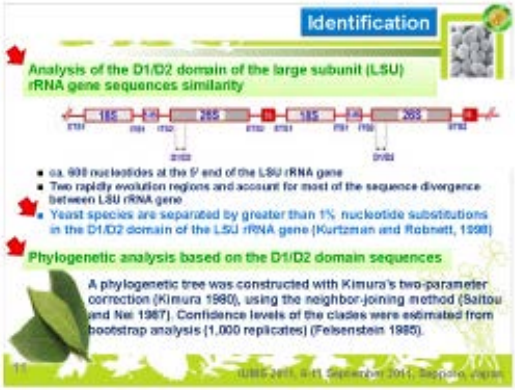

Result of Identification (cont.)
Novel yeast species
Pairwise sequence similarity of the D1/D2 domain of the LSU rRNA gene of the novel yeast species and their closest species
Nucleotide
Strains
Closest Species
Similarity %
Substitution
Novel species
Yeasts (9 isolates)
LM008 Candida boidinii (U45706) 99.1 4/467 0.2 Candida sirachaensis sp. nov.
LM068 Candida membranifaciens (DQ480364) 98.8 13/969 2.3
LM078 Candida membranifaciens (DQ480364) 98.8 13/969 2.3 Candida sakaeoensis sp. nov.
Methylotrophic yeasts (3 isolates)
LM12 Ogataea sp. NCAIM Y.01995 (GQ265621) 99.4 6/565 1.4
LM14 Ogataea sp. NCAIM Y.01995 (GQ265621) 99.4 6/565 1.4 Ogataea phyllophila sp. nov.*
LM16 Ogataea sp. NCAIM Y.01995 (GQ265621) 99.4 6/565 1.4
LM15 Candida sp. ZSN 2322 (FR686675) 96.8 16/529 2.8 Candida champhorensis sp. nov.*
LM17 Candida sp. NRRL YB-2462 (EU011051) 96.2 20/565 3.5 Candida nakhonsawanensis sp. nov.*
*Kaewwajanakul et al. 2011. Antonie van Leeuwenhoek. 108: 207–217

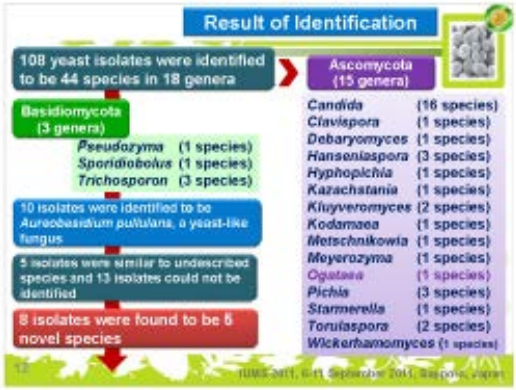

Candida sirachaensis sp. nov. (LM008T)
Phenotypic Characteristics:
Cells are ovoid (1.5–4.0 x 2.5–5.5 µm)
Occur singly or in pairs
Multilateral budding
No ascospores formation
No true and pseudohyphae
Major ubiquinone: Q9
Candida sirachaensis sp. nov. LM008T
Cells
Phylogenetic tree based on the sequence of the D1/D2 domain of the LSU rRNA gene showing the position of Candida sirachaensis

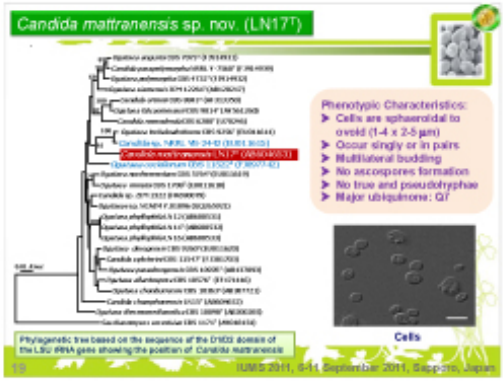

Candida sakaeoensis sp. nov. (LM068, LM078T)
Phenotypic Characteristics:
Cells are spherical to ovoid (3.5–7.0 x 3.0–6.5 µm)
Occur singly or in pairs
Multilateral budding
No ascospores formation
Pseudohyphae are form but true are not formed
Candida sakaeoensis sp. nov. (LM068)
Candida sakaeoensis sp. nov. (LM078T)
Cells
pseudohyphae
Phylogenetic tree based on the sequence of the D1/D2 domain of the LSU rRNA gene showing the position of Candida sakaeoensis

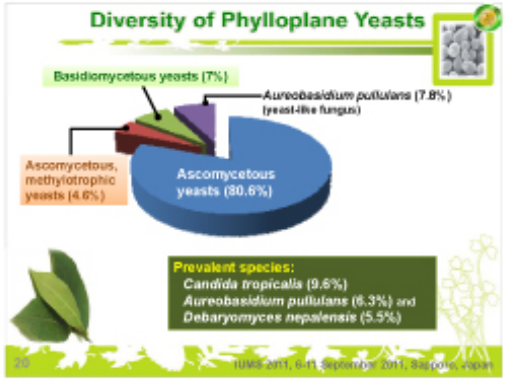

Methylotrophic yeast isolation:
by three consecutive enrichment in methanol broth
methanol-YNB broth
0.67% Yeast-Nitrogen Base
0.5% Methanol
MtOH-YNB broth (50 ml)
shaker at 180 rpm, 30±2°C, 5 days
5 ml
MtOH-YNB broth (50 ml)
shaker at 180 rpm, 30±2°C, 5 days
5 ml
MtOH-YNB broth (50 ml)
Shaker at 180 rpm, 30±2°C, 5 days
Streak on MtOH-YNB agar
Pure culture
Pick yeast colony and purify by streaking on YM agar
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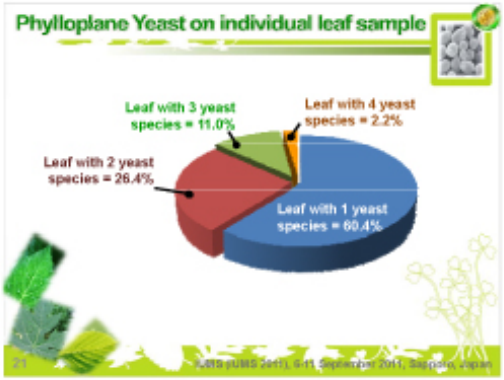

Result of yeast isolation
Yeast isolation
Leaves without yeast = 6.2%
Leaves with yeast = 93.8%
• 125 yeast isolates and 10 isolates of yeast-like fungus
• 91 leaf samples (93.8%)
Methylotrophic yeast isolation
Leaves with methylotrophic yeast (7.8%)
Leaves without yeast = 92.2%
• 8 isolates of methylotrophic yeast
• 8 leaf samples (7.8%)

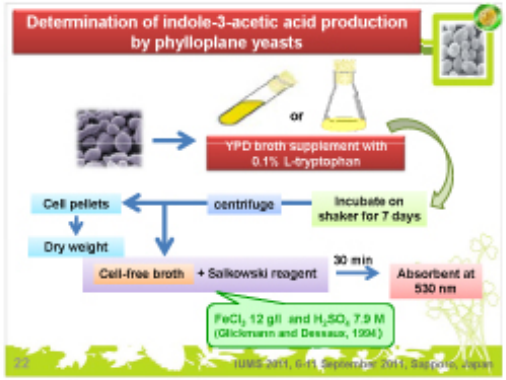

Identification
Analysis of the D1/D2 domain of the large subunit (LSU) rRNA gene sequences similarity
18S
26S
5S
5.8S
ca. 600 nucleotides at the 5' end of the LSU rRNA gene
Two rapidly evolution regions and account for most of the sequence divergence between LSU rRNA gene
Yeast species are separated by greater than 1% nucleotide substitutions in the D1/D2 domain of the LSU rRNA gene (Kurtzman and Robnett, 1998)
Phylogenetic analysis based on the D1/D2 domain sequences
A phylogenetic tree was constructed with Kimura's two-parameter correction (Kimura 1980), using the neighbor-joining method (Saitou and Nei 1987). Confidence levels of the clades were estimated from bootstrap analysis (1,000 replicates) (Felsenstein 1985).

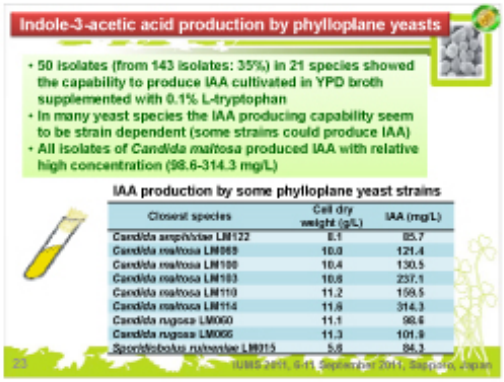

Result of Identification
108 yeast isolates were identified to be 44 species in 18 genera
Ascomycota (15 genera)
Candida (16 species)
Clavispora (1 species)
Debaryomyces (1 species)
Hanseniaspora (3 species)
Hyphopichia (1 species)
Kazachstania (1 species)
Kluyveromyces (2 species)
Kodamaea (1 species)
Metschnikowia (1 species)
Meyerozyma (1 species)
Ogataea (1 species)
Pichia (3 species)
Starmerella (1 species)
Torulaspora (2 species)
Wickerhamomyces (1 species)
Basidiomycota (3 genera)
Pseudozyma (1 species)
Sporidiobolus (1 species)
Trichosporon (3 species)
10 isolates were identified to be Aureobasidium pullulans, a yeast-like fungus
5 isolates were similar to undescribed species and 13 isolates could not be identified
8 isolates were found to be 8 novel species

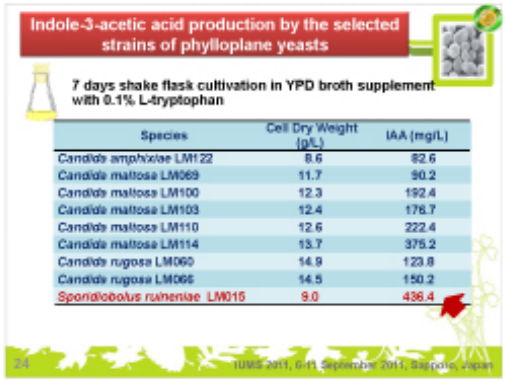

Candida mattranensis sp. nov. (LN17T)
Phenotypic Characteristics:
Cells are sphaeroidal to ovoid (1-4 x 2-5 μm)
Occur singly or in pairs
Multilateral budding
No ascospores formation
No true and pseudohyphae
Major ubiquinone: Q7
Cells
Phylogenetic tree based on the sequence of the D1/D2 domain of the LSU rRNA gene showing the position of Candida mattranensis
19
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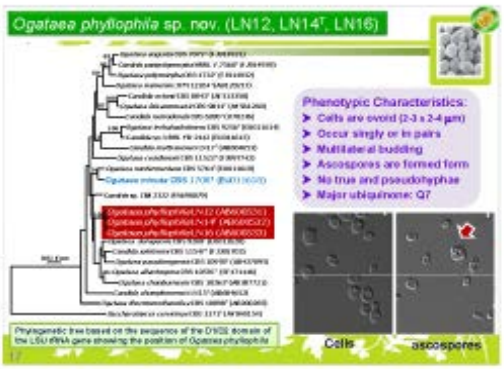

Diversity of Phylloplane Yeasts
Basidiomycetous yeasts (7%)
Aureobasidium pullulans (7.8%) (yeast-like fungus)
Ascomycetous, methylotrophic yeasts (4.6%)
Ascomycetous yeasts (80.6%)
Prevalent species:
Candida tropicalis (9.6%)
Aureobasidium pullulans (6.3%) and
Debaryomyces nepalensis (5.5%)
20
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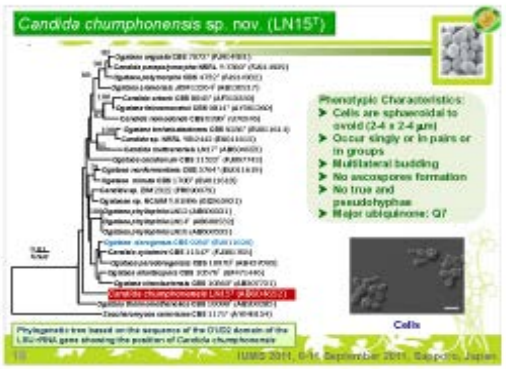

Phylloplane Yeast on individual leaf sample
Leaf with 3 yeast species = 11.0%
Leaf with 4 yeast species = 2.2%
Leaf with 2 yeast species = 26.4%
Leaf with 1 yeast species = 60.4%
21
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Determination of indole-3-acetic acid production by phylloplane yeasts
or
YPD broth supplement with 0.1% L-tryptophan
Cell pellets
centrifuge
Incubate on shaker for 7 days
Dry weight
Cell-free broth
+ Salkowski reagent
30 min
Absorbent at 530 nm
FeCl3 12 g/l and H2SO4 7.9 M (Gliclemann and Dessaux, 1994)
22
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Indole-3-acetic acid production by phylloplane yeasts
• 50 isolates (from 143 isolates: 35%) in 21 species showed the capability to produce IAA cultivated in YPD broth supplemented with 0.1% L-tryptophan
• In many yeast species the IAA producing capability seem to be strain dependent (some strains could produce IAA)
• All isolates of Candida maltosa produced IAA with relative high concentration (98.6-314.3 mg/L)
IAA production by some phylloplane yeast strains
Closest species | Cell dry weight (g/L) | IAA (mg/L)
Candida amphixiae LM122 | 8.1 | 85.7
Candida maltosa LM069 | 10.8 | 121.4
Candida maltosa LM100 | 10.4 | 130.5
Candida maltosa LM103 | 10.8 | 237.1
Candida maltosa LM110 | 11.2 | 159.5
Candida maltosa LM114 | 11.8 | 314.3
Candida rugosa LM060 | 11.1 | 98.6
Candida rugosa LM066 | 11.3 | 101.9
Sporidiobolus ruineniae LM015 | 5.8 | 84.3
23
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Indole-3-acetic acid production by the selected strains of phylloplane yeasts
7 days shake flask cultivation in YPD broth supplement with 0.1% L-tryptophan
Species | Cell Dry Weight (g/L) | IAA (mg/L)
Candida amphixiae LM122 | 8.6 | 82.6
Candida maltosa LM069 | 11.7 | 90.2
Candida maltosa LM100 | 12.3 | 192.4
Candida maltosa LM103 | 12.4 | 176.7
Candida maltosa LM110 | 12.6 | 222.4
Candida maltosa LM114 | 13.7 | 375.2
Candida rugosa LM060 | 14.9 | 123.8
Candida rugosa LM066 | 14.5 | 150.2
Sporidiobolus ruineniae LM015 | 9.0 | 436.4
24
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Ogataea phyllophila sp. nov. (LN12, LN14T, LN16)
Phenotypic Characteristics:
Cells are ovoid (2-3 x 2-4 μm)
Occur singly or in pairs
Multilateral budding
Ascospores are formed form
No true and pseudohyphae
Major ubiquinone: Q7
Cells
ascospores
Phylogenetic tree based on the sequence of the D1/D2 domain of the LSU rRNA gene showing the position of Ogataea phyllophila
17
IUMS 2011, 6-11 September 2011, Sapporo, Japan


Candida chumphonensis sp. nov. (LN15T)
Phenotypic Characteristics:
Cells are sphaeroidal to ovoid (2-4 x 2-4 μm)
Occur singly or in pairs or in groups
Multilateral budding
No ascospores formation
No true and pseudohyphae
Major ubiquinone: Q7
Cells
Phylogenetic tree based on the sequence of the D1/D2 domain of the LSU rRNA gene showing the position of Candida chumphonensis
18
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Conclusions

- 93.8% of leaf samples contained yeasts and 7.8% contained methylotropic yeasts
- Among the isolates obtained, 80.6% was ascomycetous yeasts, 4.6% was methylotrophic yeasts and 7% was basidiomycetous yeasts
- Five novel species, *Candida chumphonensis* sp. nov., *Candida mattranensis* sp. nov., *Candida sakaeoensis* sp. nov., *Candida sirachaensis* sp. nov. and *Ogataea phyllophila* sp. nov., were discovered and proposed
- The prevalent species on phylloplane were *Candida tropicalis* and *Debaryomyces nepalensis* but with low frequencies, 9.6 and 5.5% of the total isolates, respectively

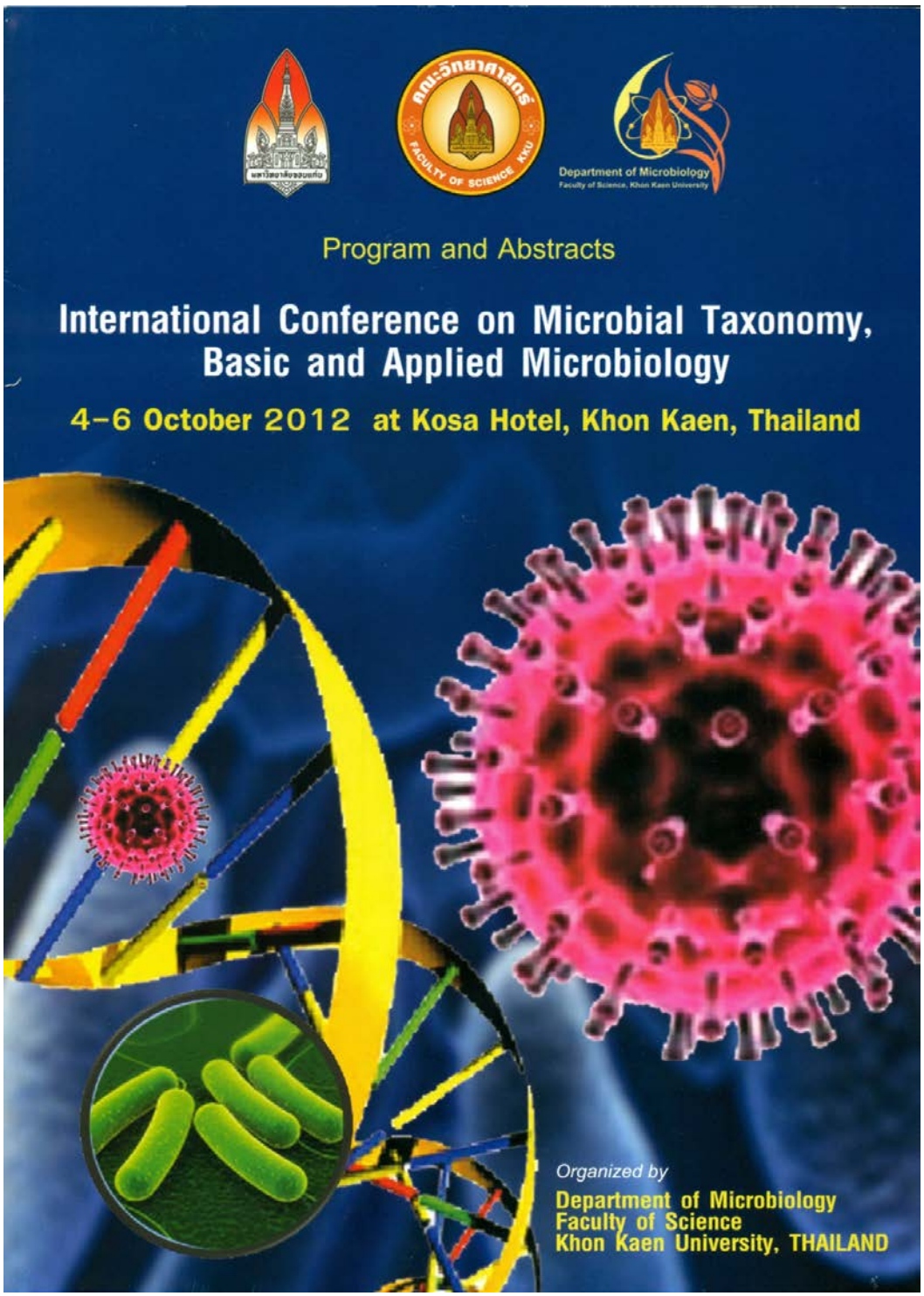
Conclusions (cont.)

- Individual leaf sample was generally accommodated by only one yeast species (60.4%) while two or three species were presented in 26.4 and 11.0% of the samples, respectively, and only 2.2% contained up to four species
- Fifty isolates in 21 species showed the capability to produce IAA cultivated in YPD broth supplemented with 0.1% L-tryptophan. All strains of *Candida maltosa* produced IAA with relatively high concentration (98.6-314.3 mg/L)
- By shake flask cultivation *Sporidiobolus ruineniae* LM015 produced as high as 436.4 mg/L of IAA

Yeasts on agronomic crops phylloplanes: Diversity and potential in agricultural utilization

บรรยายใน International Conference on Microbial Taxonomy, Basic and Applied Microbiology

4-6 October 2012 ณ โรงแรมโฆษะ จังหวัดขอนแก่น ประเทศไทย



Schedule and Program

4 October 2012

Venue : Mongkut Phet 1 (2nd floor)

Time	Topics
8.30 – 9.00	Registration
9.00 – 9.15	Opening Ceremony by President of Khon Kaen University
9.15 – 9.45	Keynote Speaker : Professor Dr. Takao Terashita "Role of protease and amylase on the fruit-body growth of mushroom fungi and edible mushroom production in Japan"
9.45 – 10.00	Coffee Break
10.00 – 10.30	Plenary speaker : Professor Dr. Kohei Oda "New family of peptidase : glutamic peptidase"
10.30 – 11.00	Plenary speaker : Associate Professor Dr. Napavarn Noparatnaraporn "Microbes and microbial technology - from research to business"
11.00 – 11.30	Plenary speaker : Professor Dr. Tsutomu Morinaga "The mechanism of fruiting body formation in mushroom"
11.45 – 13.00	Lunch

Oral Session 1 (S1)

Venue : Green A (1st floor)

Session Chair : Dr. Suriyanarayanan Sarvajayakesavalu

Assistant Chair : Wilailak Siripornadulsil

Time	Topics
13.00 – 13.30	Invited Speaker : Professor Dr. Savitree Limtong "Yeasts on agronomic crops phylloplanes: diversity and potential in agricultural utilization"
13.30 – 13.50	Screening of an anaerobic alkalithermophilic cellulolytic-xylanolytic bacterium Amornrat Watthanalamloet (S1-01)
13.50 – 14.10	The identification and classification of a novel Ascomycete fungus discovered on bamboo Wuttiwat Jitjak (S1-02)
14.10 – 14.30	Isolation and screening of lactic acid bacteria from gastrointestinal tracts of fish and fermented foods for potential probiotic use in aquaculture Ratchanu Meidong (S1-03)
14.30 – 14.50	Molecular identification of total plate count bacteria isolated from drinking water vending machines Thippayarat Chahomchuen (S1-04)
14.50 – 15.00	Coffee Break
15.00 – 15.20	Demineralization of shrimp shell by lactic acid bacteria Ekkalak Ploydee (S1-05)
15.20 – 15.40	*Annulatascus aquatorba*, a novel aquatic fungus from Sirindhorn peat swamp forest, Narathiwat, Thailand Nattawut Boonyuen (S1-06)
15.40 – 16.00	Isolation and characterization of lactic acid bacteria for potential use as probiotics in fermented tilapia belly flap meat Siriyanapat Tasaku (S1-07)
16.00 – 17.30	Poster presentation at Mongkut Ngen (2nd floor)
18.00	Banquet

INVITED SPEAKER



Professor Dr. Savitree Limtong

Yeasts on agronomic crops phylloplanes : Diversity and potential in agricultural Utilization

Savitree Limtong, Rungluk Kaewwichian and Wichien Yongmanitchai
Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand (fscistl@ku.ac.th)

Abstract

Phylloplane is an external surface of plant leaf and it has been recognized as an important habitat of microorganisms. This research attempted to study the diversity of yeasts on plylloplanes of important agronomic crops (rice, corn and sugarcane) in Thailand and determine their potential in agricultural utilization namely biocontrol and plant growth promoting activities. A total of 453 yeast strains were isolated from 261 leaf samples collected from nine provinces. On the basis of molecular taxonomy 432 strains were identified to be 50 species in 25 ascomycete yeast genera and 20 species in nine basidiomycete yeast genera. However, only seven species were found from all three crop phylloplanes including six ascomycete species i.e. *Candida tropicalis, Lindnera rhodanensis, Meyerozyma caribbica, Meyerozyma guilliermondii, Pichia koratensis* and *Pichia kudriavzevii*, and one basidiomycete species i.e. *Rhodotorula mucilaginosa*. Eight strains represented five novel species and 13 strains were found similar to undescribed species. Antagonistic activity against five corn and three rice pathogenic fungi of these 453 strains were tested and the result showed that only *Torulaspora globosa* RK547 isolated from corn phylloplane could inhibit growth of *Fusarium moniliforme*, *Rhizoctonia solani* and *Helminthosporium oryzae*. Thirty-five percentages of the strains showed the capability to produce indole-3-acetic acid (IAA) cultivated in yeast extract peptone dextrose broth supplemented with L-tryptone in the range of 0.2-66.3 mg/g DCW and *Rhodosporidium fluviale* RK253 yielded the highest IAA concentration of 66.3 mg/g DCW while the other two strains (RK 532 and RK498) of this species produced 40.6 and 37.4 mg/g DCW, respectively.

Keywords: yeasts, plylloplanes, biocontrol, antagonistic, plant pathogenic fungi, plant growth promoter, indole-3-acetic acid

YEASTS ON AGRONOMIC CROPS PHYLLOPLANES: DIVERSITY AND POTENTIAL IN AGRICULTURAL UTILIZATION

Savitree Limtong, Rungluk Kaewwichian and Wichien Yongmanitchai

Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok, Thailand

International Conference on Microbial Taxonomy, Basic and Applied Microbiology, 4-6 October 2012 at Kosa Hotel, Khon Kaen, Thailand

Kasetsart University

Phylloplane / phyllosphere

- External surface of plant leaves
- Important habitat for microorganisms
- Growth of microorganisms on phylloplane depend on nutrients derive:
  - endogenously from plant exudates (provide organic substances e.g. simple sugars organic acids, amino acids and methanol)
  - exogenously from compounds found in materials that drop on it, mostly are inorganic nutrients (Tukey, 1971)

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Phylloplane Yeasts

Yeasts in Basidiomycota →	*Sporobolomyces roseus* *Rhodotorula mucilaginosa* *Cryptococcus laurentii* etc. (Inacio *et al.*, 2002; Glushakova *et al.*, 2006)
Yeasts in Ascomycota →	*Torulaspora delbrueckii* *Kluyveromyces thermotolerans* (Spencer and Spencer, 1997) *Ogataea allantospora* (Peter *et al.*, 2007) etc.
Yeast-like fungi →	*Aureobasidium pullulans* (Andrews and Harris, 2000)

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Phyllophane yeast in Thailand

- *Ogataea phyllophila*, *Candida chumphonensis* *Candida mattranensis* (Koowadjanakul *et al.* 2011)
- *Candida sirachaensis* *Candida saksaoensis* (Limtong *et al.* 2012)
- *Metschnikowia siamensis* *Metschnikowia phyllophila* (Limtong *et al.* 2012)



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Plant Growth Promoter

Many plant-associate microorganisms are capable of promote plant growth and development through different mechanisms. Some of them synthesize plant growth promoters similar to phytohormones produce in plant

Auxins, especially indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA), are the dominant plant growth promoter produced by yeasts

Candida valida (A)
Candida maltosa (A)
Lindnera saturnus (A)
Saccharomyces roseus (A)
Rhodotorula glutinis (B)
Rh. mucilaginosa (B)
Sporidiobolus ruineniae (B)
Trichosporon asahii (B)
(Limtong *et al.* 2012; Nassar *et al.*, 2005; Xin *et al.*, 2009)

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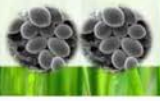
Yeast as Biological Control Agents

Pichia membranaefaciens *Candida tropicalis* *Hanseniula saturnus*	Controlled fruit rot of banana (Montatip *et al.*, 1997)
Candida guilliermondii *Endomycopsis fibuligera*	Controlled crown rot of banana cv. Hom Tong, caused by *Lasiodiplodia theobromae* (Sumitra *et al.*, 2004)
Candida utilis, *Candida tropicalis* *Debaryomyces hansenii*	Controlled green mold (*Penicillium digitatum*) of citrus fruit cv. Sai-Numphaung (Suangsan *et al.*, 2006)

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Yeast as Biological control Agents

Yeast isolated from environment, especially from the agricultural areas, may show better results in the biological control of plant pathogens (Ahansal et al. 2008; Rosa et al. 2010)



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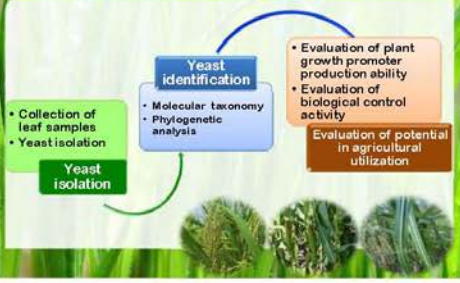
Objectives

- To study diversity of yeasts in phylloplane of important agronomic crops (rice, corn and sugarcane) in Thailand
- To evaluate the potential in agricultural utilization namely plant growth promoting and biological control activities in vitro



Kasetsart University

Research Outline



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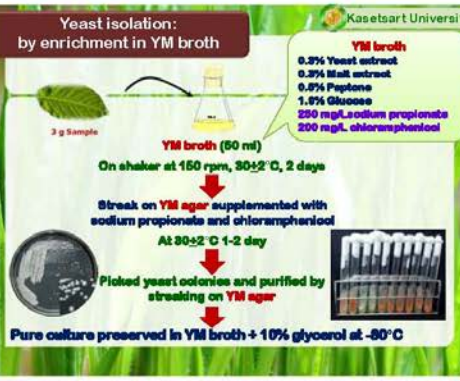
Collection of leaf samples



245 samples (9 provinces)
- rice leaf 85 samples
- corn leaf 65 samples
- sugarcane leaf 95 samples

Yeast isolation: by enrichment in YM broth

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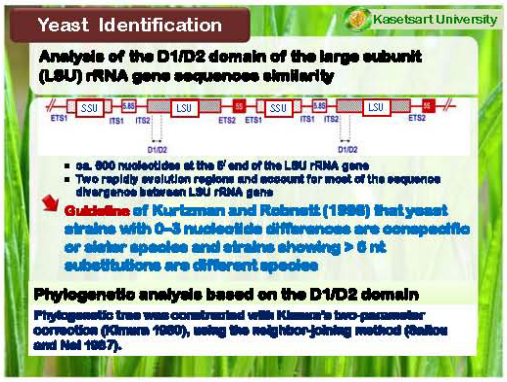


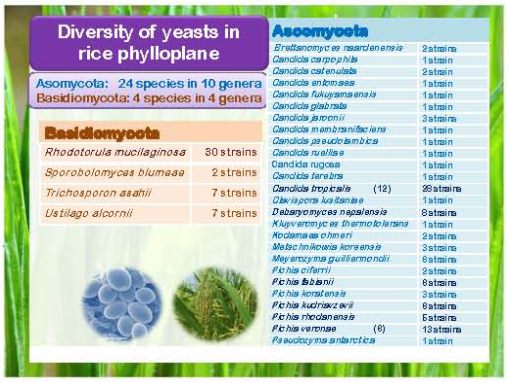
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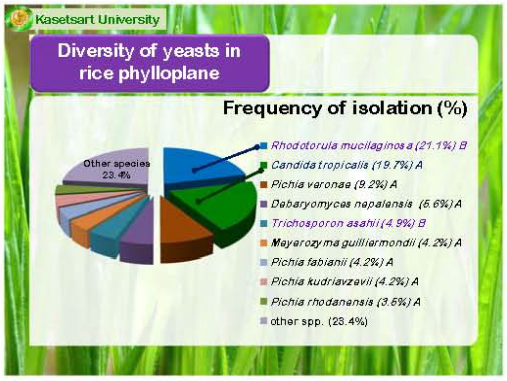
Result of yeast isolation by enrichment technique

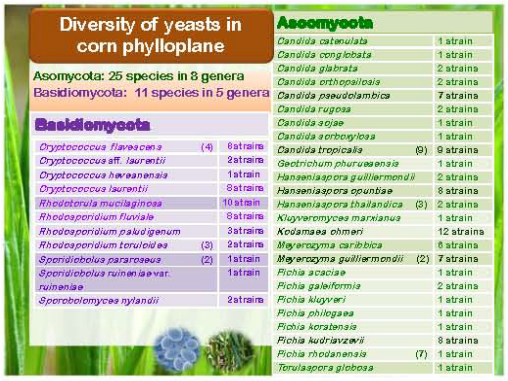
	No. of sample	No. of yeast isolates
Rice phylloplane	85	154
Corn phylloplane	65	134
Sugarcane phylloplane	95	165
Total	245	453

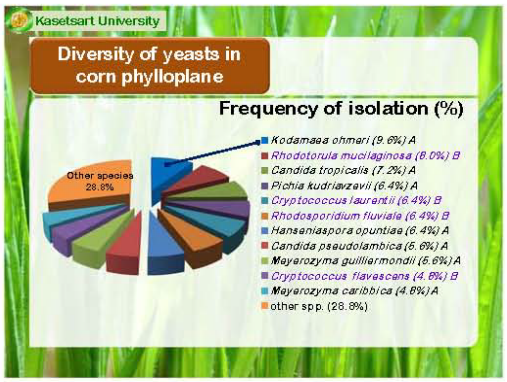


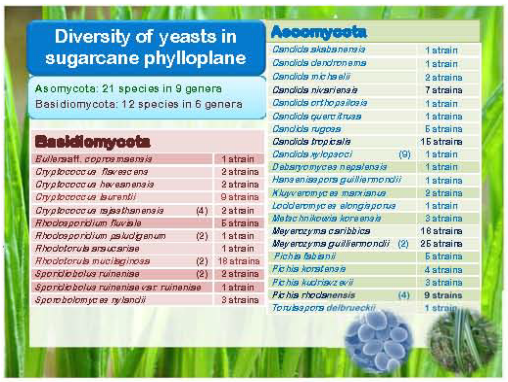

Yeast Identification
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Analysis of the D1/D2 domain of the large subunit (LSU) rRNA gene sequences similarity
SSU
LSU
ITS1
ITS2
D1/D2
ca. 600 nucleotides at the 5' end of the LSU rRNA gene
Two rapidly evolution regions and account for most of the sequence divergence between LSU rRNA gene
Guideline of Kurtzman and Robnett (1998) that yeast strains with 0–3 nucleotide differences are conspecific or sister species and strains showing > 6 nt substitutions are different species
Phylogenetic analysis based on the D1/D2 domain
Phylogenetic tree was constructed with Kimura's two-parameter correction (Kimura 1980), using the neighbor-joining method (Saitou and Nei 1987).


Diversity of yeasts in rice phylloplane
Asomycota: 24 species in 10 genera
Basidiomycota: 4 species in 4 genera
Basidiomycota
Rhodotorula mucilaginosa 30 strains
Sporobolomyces blumaea 2 strains
Trichosporon asahii 7 strains
Ustilago alcornii 7 strains
Ascomycota
Brettanomyces naardenensis 2 strains
Candida carpophila 1 strain
Candida catenulata 2 strain
Candida entomaea 1 strain
Candida fukuyamaensis 1 strain
Candida glabrata 1 strain
Candida jaroonii 3 strains
Candida membranifaciens 1 strain
Candida pseudolambica 1 strain
Candida ruellae 1 strain
Candida rugosa 1 strain
Candida tenuis 1 strain
Candida tropicalis (12) 28 strains
Clavispora lusitaniae 1 strain
Debaryomyces nepalensis 8 strains
Kluyveromyces thermotolerans 1 strain
Kodamaea ohmeri 2 strains
Metschnikowia koreensis 3 strains
Meyerozyma guilliermondii 6 strains
Pichia ciferrii 2 strains
Pichia fabianii 6 strains
Pichia kofaniensis 3 strains
Pichia kudriavzevii 6 strains
Pichia rhodanensis 5 strains
Pichia varonae (6) 12 strains
Pseudozyma antarctica 1 strain


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Diversity of yeasts in rice phylloplane
Frequency of isolation (%)
Other species 23.4%
Rhodotorula mucilaginosa (21.1%) B
Candida tropicalis (19.7%) A
Pichia varonae (9.2%) A
Debaryomyces nepalensis (5.6%) A
Trichosporon asahii (4.9%) B
Meyerozyma guilliermondii (4.2%) A
Pichia fabianii (4.2%) A
Pichia kudriavzevii (4.2%) A
Pichia rhodanensis (3.6%) A
other spp. (23.4%)


Diversity of yeasts in corn phylloplane
Asomycota: 25 species in 8 genera
Basidiomycota: 11 species in 5 genera
Basidiomycota
Cryptococcus flavescens (4) 8 strains
Cryptococcus aff. laurentii 2 strains
Cryptococcus hawaianensis 1 strain
Cryptococcus laurentii 8 strains
Rhodotorula mucilaginosa 10 strain
Rhodosporidium fluviale 8 strains
Rhodosporidium paludigenum 3 strains
Rhodosporidium toruloides (3) 2 strains
Sporidiobolus pararoseus (2) 1 strain
Sporidiobolus ruinenia e var. ruinenia e 1 strain
Sporobolomyces nylandii 2 strains
Ascomycota
Candida catenulata 1 strain
Candida conglobata 1 strain
Candida glabrata 2 strains
Candida orthopsilosis 2 strains
Candida pseudolambica 7 strains
Candida rugosa 2 strains
Candida sojae 1 strain
Candida sorbosivora 1 strain
Candida tropicalis (9) 9 strains
Geotrichum phurueaensis 1 strain
Hanseniaspora guilliermondii 2 strains
Hanseniaspora opuntiae 8 strains
Hanseniaspora thailandica (3) 2 strains
Kluyveromyces marxianus 1 strain
Kodamaea ohmeri 12 strains
Meyerozyma caribbica 6 strains
Meyerozyma guilliermondii (2) 7 strains
Pichia acaciae 1 strain
Pichia galeiformis 2 strains
Pichia kluyveri 1 strain
Pichia philogaea 1 strain
Pichia koratensis 1 strain
Pichia kudriavzevii 8 strains
Pichia rhodanensis (7) 1 strain
Torulaspora globosa 1 strain


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Diversity of yeasts in corn phylloplane
Frequency of isolation (%)
Other species 28.8%
Kodamaea ohmeri (9.6%) A
Rhodotorula mucilaginosa (8.0%) B
Candida tropicalis (7.2%) A
Pichia kudriavzevii (6.4%) A
Cryptococcus laurentii (6.4%) B
Rhodosporidium fluviale (6.4%) B
Hanseniaspora opuntiae (6.4%) A
Candida pseudolambica (5.6%) A
Meyerozyma guilliermondii (5.6%) A
Cryptococcus flavescens (4.8%) B
Meyerozyma caribbica (4.8%) A
other spp. (28.8%)

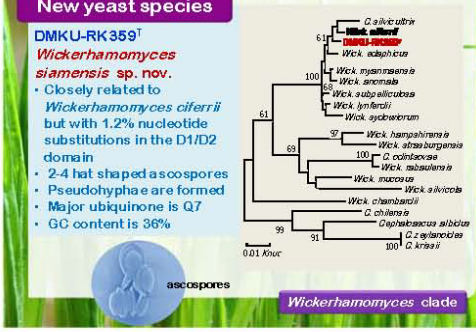

Diversity of yeasts in sugarcane phylloplane
Asomycota: 21 species in 9 genera
Basidiomycota: 12 species in 6 genera
Basidiomycota
Bullera aff. coprosmaensis 1 strain
Cryptococcus flavescens 2 strains
Cryptococcus hawaianensis 2 strains
Cryptococcus laurentii 9 strains
Cryptococcus rajasthanensis (4) 2 strain
Rhodosporidium fluviale 5 strains
Rhodosporidium paludigenum (2) 1 strain
Rhodotorula araucariae 1 strain
Rhodotorula mucilaginosa (2) 19 strains
Sporidiobolus ruinenias (2) 2 strains
Sporidiobolus ruinenias var. ruinenias 1 strain
Sporobolomyces nylandii 3 strains
Ascomycota
Candida akabanensis 1 strain
Candida dendronema 1 strain
Candida michaelii 2 strains
Candida nivariensis 7 strains
Candida orthopsilosis 1 strain
Candida quercitrusa 1 strain
Candida rugosa 5 strains
Candida tropicalis 15 strains
Candida wylopacci (9) 1 strain
Debaryomyces nepalensis 1 strain
Hanseniaspora guilliermondii 1 strains
Kluyveromyces marxianus 2 strains
Lodderomyces elongisporus 1 strain
Metschnikowia koreensis 3 strains
Meyerozyma caribbica 18 strains
Meyerozyma guilliermondii (2) 25 strains
Pichia fabianii 5 strains
Pichia kofaniensis 4 strains
Pichia kudriavzevii 3 strains
Pichia rhodanensis (4) 9 strains
Torulaspora delbrueckii 1 strain

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New yeast species

DMKU-RK359^T
Wickerhamomyces siamensis sp. nov.

- Closely related to *Wickerhamomyces ciferrii* but with 1.2% nucleotide substitutions in the D1/D2 domain
- 2-4 hat shaped ascospores
- Pseudohyphae are formed
- Major ubiquinone is Q7
- GC content is 36%



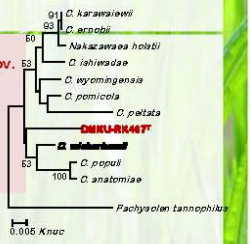
Wickerhamomyces clade

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New yeast species

DMKU-RK467^T
Nakazawaea siamensis sp. nov.

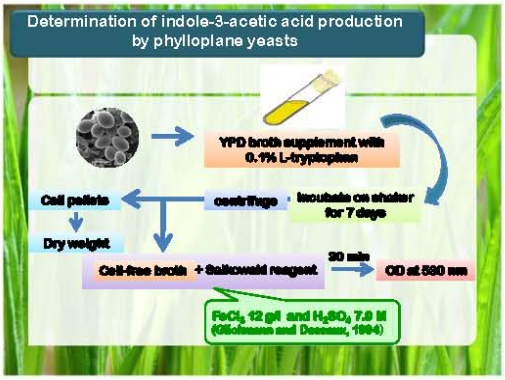
- Closely related to *Candida wickerhamii* but with 1.4% nucleotide substitutions in the D1/D2 domain
- Ascospores are not produced
- Pseudohyphae are not formed
- Major ubiquinone is Q7





Nakazawaea clade

Determination of indole-3-acetic acid production by phylloplane yeasts



Indole-3-acetic acid production by phylloplane yeasts

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Ascomycota



- 96 strains (292 strains) in 29 species produced IAA: 2.2-259 mg/L
- 10 strains produced >100 mg/L

Ascomycete yeast species	Cell Dry Weight (g/L)	IAA (mg/L)
Candida michaelii DMKU-RK301	10.0	259.0 25.9 mg/g CDW
Candida orthopsilosis DMKU-RK28	12.2	129.2
Candida ruelliae DMKU-RK434	9.0	131.9
Candida rugosa DMKU-RK179	14.5	118.0
Hanseniaspora opuntiae DMKU-RK551	4.4	106.9
Kodamaea ohmeri DMKU-RK552	8.4	110.2
Meyerozyma caribbica DMKU-RK220	8.0	131.8
Meyerozyma guilliermondii DMKU-RK331	5.6	130.2
Meyerozyma caribbica DMKU-RK6	7.5	104.1
Pichia philogaea DMKU-RK544	9.2	115.9

Indole-3-acetic acid production by phylloplane yeasts

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Basidiomycota



- 70 strains (from 128 strains) in 13 species produce IAA: 3.3-417 mg/L, 15 strains produced >100

Basidiomycete yeast species	Cell Dry Weight (g/L)	IAA (mg/L)
Rhodosporidium fluviale DMKU-RK253	6.3	417.8 (66.3 mg/g CW)
Rhodosporidium fluviale DMKU-RK498	7.2	269.1
Rhodosporidium fluviale DMKU-RK442	9.3	259.1
Rhodosporidium fluviale DMKU-RK539	8.4	217.3
Rhodosporidium fluviale DMKU-RK537	5.5	223.0
Rhodosporidium fluviale DMKU-RK545	7.2	198.3
Rhodosporidium fluviale DMKU-RK416	8.2	146.0
Rhodosporidium fluviale DMKU-RK472	7.2	115.5
Rhodosporidium fluviale DMKU-RK406	6.8	105.4
Rhodosporidium paludigenum DMKU-RK342	10.1	144.6
Rhodosporidium paludigenum DMKU-RK528	8.5	186.2
Rhodosporidium paludigenum DMKU-RK266	5.2	144.0
Rhodosporidium toruloides DMKU-RK334	7.0	153.0
Rhodosporidium toruloides DMKU-RK336	6.3	125.0
Rhodotorula mucilaginosa DMKU-RK247	7.2	137.1

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Evaluation of the biological control of plant pathogenic fungi by phylloplane yeasts in vitro

- **Rice pathogenic fungi**
  - *Fusarium moniliforme*: bakanae disease
  - *Helminthosporium oryzae*: brown spot
  - *Rhizoctonia solani*: sheath blight
- **Corn pathogenic fungi**
  - *Bipolaris maydis*: southern leaf spot
  - *Bipolaris zeicola*: northern leaf spot
  - *Exserohilium turcica*: northern leaf blight
  - *Fusarium moniliforme*: stalk rot
  - *Macrophomina phaseolina*: charcoal stalk rot

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Evaluation of the biological control of plant pathogenic fungi by phylloplane yeasts in vitro

Method: dual cultivation (Rosa et al., 2010) on PDA agar, incubated at 25 °C for 7 days

Yeast: 454 strains



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Result: Evaluation of the biological control of plant pathogenic fungi by phylloplane yeasts in vitro

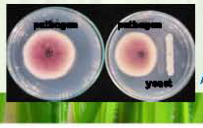
Torulaspora globosa DMKU-RK547 (isolated from corn phylloplane) inhibited growth of the 3 rice pathogenic fungi



Fusarium moniliforme



Rhizoctonia solani



Helminthosporium oryzae

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Growth inhibition (%) of rice pathogenic fungi by *Torulaspora globosa* DMKU-RK547

	fungal colony diameter (cm)	% Inhibition
Fusarium moniliforme (Control)	6.55	
DMKU-RK547 + *F. moniliforme*	4.65	29.0
Rhizoctonia solani (Control)	5.9	
DMKU-RK547+ *R. solani*	4.15	29.7
Helminthosporium oryzae (Control)	5.1	
DMKU-RK547+ *H. oryzae*	4.5	11.8

Inhibition (%) =

$$\frac{\text{fugal colony diameter (control)} - \text{diameter of fungal colony inoculate with yeast)} \times 100}{\text{Fungal colony diameter (control)}}$$

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Modes of action of *T. globosa* DMKU-RK547 in controlling rice pathogenic fungi in vitro (1)

Detection of antifungal volatile compounds production:

dual cultivation (Rosa et al., 2010) on two separate section of PDA agar in plate, incubated at 25 °C for 7 days



Fusarium moniliforme *Rhizoctonia solani* *Helminthosporium oryzae*

T. globosa DMKU-RK547 produced volatile compound(s) that inhibited growth of all 3 rice pathogenic fungi

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Modes of action of *T. globosa* DMKU-RK547 in controlling rice pathogenic fungi in vitro (2)

Detection of siderophore production:

cultivation of yeast on Chrome Azurol S (CAS) Blue Agar and incubated at 25 °C, 7 days (Milagres *et al.* 1999)



T. globosa DMKU-RK547 grew and changed medium color from blue to yellow, indicated siderophore producing capability

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Conclusion

- High diversity of yeast on phylloplane of rice, corn and sugarcane was observed
- Both ascomycete and basidiomycete yeast were detected, though higher frequency of isolation was ascomycete yeast, which may due to enrichment isolation technique was used
- Six new yeast species i.e. *Metschnikowia saccharicola* , *Metschnikowia lopburiensis*, *Yamadazyma siamensis*, *Yamadazyma phyllophila*, *Wickerhamomyces siamensis*, *Nakazawaea siamensis* were discovered and proposed

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Conclusion (cont.)

- Determination of IAA production by cultivation yeast in YPD broth supplemented with 0.1% L-tryptophan showed that
  - Among the ascomycete yeast strains tested 39% produced IAA, 10 strains produced IAA >100 mg/L and *Candida michaelii* DMKU-RK301 produced 259 mg/L (25.9 mg/g CDW)
  - Among the basidiomycete yeast strains tested 55% produced IAA, 14 strains produced in the rang of 100- 300 mg/L and *Rhodosporidium fluviale* DMKU-RK253 produced 417.8 mg/L (66.3 mg/g CDW)

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Conclusion (cont.)



- Evaluation of the biological control of plant pathogenic fungi by the phylloplane yeasts in vitro revealed that only *Torulaspora globosa* DMKU-RK547 inhibited *Fusarium moniliforme*, *Rhizoctonia solani* and *Helminthosporium oryzae*, which are the rice pathogenic fungi
- Preliminary evaluation of mechanisms of *T. globosa* DMKU-RK547 in controlling rice pathogenic fungi revealed that it produced antifungal volatile compound(s) which inhibited rice pathogen and produced siderophore

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Acknowledgements

- **Royal Golden Jubilee Ph.D. program, Thailand Research Fund**
- **TRF Research-Team Promotion Grant, Thailand Research Fund**
- **NITE Biological Resource Center (NBRC), Japan and Dr. Hiroko Kawasaki**



Thank you

Rungluk Kaewwichian

創立90周年記念

第64回 日本生物工学会大会
講演要旨集

2012年10月23・24・25・26日

創立90周年記念式典・授賞式・受賞講演・特別講演
神戸国際会議場　メインホール（神戸市中央区港島中町6-9-1）

国際シンポジウム・シンポジウム・一般講演
神戸国際会議場

記念祝賀会
神戸ポートピアホテル（神戸市中央区港島中町6-10-1）

2012（平成24年度）



公益社団法人
日本生物工学会

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90th Anniversary Meeting 2012

The Society for Biotechnology, Japan (SBJ)

October 23rd – 26th, 2012

Kobe International Conference Center
Kobe, Japan

64th SBJ Annual Meeting Program

Supported by
Japan Science Technology Agency (JST)
Institute for Fermentation, Osaka (IFO)
Kobe Covention & Visitors Association
Nakauchi Tsutomu Convention Shinko Zaidan Foundation
Suntory Institute for Bioorganic Research

Room D Afternoon (13:30~14:30)

Invited Lectures

13:30 2Dp01 Genetic and Phenotypic Diversity of *Saccharomyces cerevisiae*: Evidence for the Origin of Domestic Populations of the Yeast

.....○Feng-Yan Bai, Qi-Ming Wang, Pei-Jie Han, Wan-Qiu Liu
(State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, China)

13:45 2Dp02 Taxonomy of yeasts-associated with *Apis cerana* and their potential use for pollen substitutes

.....○Wellyzar Sjamsuridzal[1], Adi Basukriadi[1], Ariyanti Oetari[1], Effionora Anwar[2], Novia Rachmayanti[1], Bangga Beristama Putera[1], Irvan Maulana[1], Retno Widowati[1], Estriningtyas Agus Rismawanti[1], Virgine Enfinali[1], Bregas Adi Luhur[1], Dafina Ghossani Nurlaili[1]
([1]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Indonesia, [2]Faculty of Pharmacy, Universitas Indonesia, Indonesia)

14:00 2Dp03 Yeasts on Agronomic Crop Phylloplane: Description of Novel Species and Potential in Agricultural Application

.....○Savitree Limtong[1], Rungluk Kaewwichian[1], Hiroko Kawasaki[2], Wichien Yongmanitchai[1]
([1]Dept. Microbiol., Fac. Sci., Kasetsart Univ., Thailand, [2]NITE Biological Resource Center, Japan)

14:15 2Dp04 TISTR Algal Culture Collection and Its Utilization

.....○Aparat Mahakhant
(Bioscience Dept., Thailand Institute of Scientific and Technological Research TISTR, Thailand)

Room D Afternoon (14:30~18:54)

General Presentaion (Taxonomy, phylogenetics, Genetic engineering / Plant cell, tissue engineering)

14:30 2Dp06 Draft Genome Sequence of *Pediococcus lolii* NGRI 0510Q^T isolated from silage in Okinawa

.....○Katsumi Doi[1], Kazuki Mori[2], Kosuke Tashiro[2], Yasuhiro Fujino[3], Satoru Kuhara[2], Toshihisa Ohshima[1] ([1]Inst. Genet., Fac. Agr. Kyushu Univ., [2]Mole. Gene. Tech., Fac. Agric., Kyushu Univ., [3]Fac. Arts & Sci., Kyushu Univ.)

14:42 2Dp07 Presence of halophilic and alkaliphilic lactic acid bacteria in various cheeses

.....○Mioko Matsuyama, Morio Ishikawa, Akiko Okamoto-Kainuma, Yukimichi Koizumi
(Dept. Ferment. Sci., Tokyo Univ. Agric.)

14:54 2Dp08 Phosphorylation site regulating intracellular localization and transcriptional activity in *Saccharomyces cerevisiae* transcription activator Gln3

.....○Minori Numamoto[1], Yusuke Ueda[1], Yusuke Imabeppu[1], Minetaka Sugiyama[1], Hiromi Maekawa[2], Satoshi Harashima[1] ([1]Dept. Biotech., Grad. Sch. Eng., Osaka Univ., [2]Y. G. R. L., Osaka Univ.)

15:06 2Dp09 Search and diversity analysis of Mating type gene in *Penicillium purpurogenum*

.....○Kasumi Koganei[1], Teppei Arai[1], Jun Kato[2], Takahumi Kasumi[1], Jun Ogihara[1]
([1]Dept. Biores. Util. Sci., Grad. Sch. Biores. Sci., Nihon Univ., [2]Dept. Chem. Life. Sci., Nihon Univ.)

15:18 2Dp10 Molecular Evolution of [NiFeSe]H2ase based on Phylogenetic Tree and Modeling

.....○Takashi Tamura[1,2], Naoki Tsunekawa[3], Toshiyuki Hirano[3], Fumitoshi Sato[3], Kenji Inagaki[1]
([1]Grad. Sch. Environ. Life Sci., [2]JST Presto, [3]Univ. Tokyo)

15:30 2Dp11 Phylogenetic analysis of jatropha from Mexco, Asia, and Africa using SSR markers

.....○Tomohiro Sasai[1], Atefeh Alipour[1], Hideki Hirakawa[2], Shusei Sato[2], Hisashi Tsujimoto[3], Alfredo Zamarripa Colmenero[4], Hiroe Sakai[1], Suguru Tsuchimoto[1], Fukui Kiichi[1]
([1]Dept. Mat. Life Sci., Osaka Univ., [2]Kazusa DNA Res. Inst., [3]Tottori Univ., [4]INIFAP)

15:42 2Dp12 Characterization of *Alcaligenes faecalis* strain AD15 which indicates biocontrol activity against plant pathogens

.....○Shin-ichiro Yokoyama[1], Yoshitomi Adachi[1], Shuichi Asakura[1], Erina Kohyama[2]
([1]Indust. Tech. Center, Gifu Pref. Gov., [2]Gifu Pref. Res. Inst. Health Environ. Sci.)

9:35 3Ea02 Efficient production of shochu and bioethanol using a novel submerged culture of *Aspergillus kawachii* which has multiple enzyme activities
.....○Hikaru Tanaka[1], Toshikazu Sugimoto[2], Tomohiro Makita[3], Koutaro Watanakbe[1], Hiroshi Shoji[1]
([1]Asahi Breweries, Ltd., [2]The Nikka Whisky Distilling Co., Ltd, [3]Asahi Group Holdings, Ltd)

10:00 3Ea03 Molecular mechanism of acetaldehyde tolerance in *Saccharomyces cerevisiae*
.....○Tomoyuki Nakagawa (Dept. Appl. Life Sci., Gifu Univ.)

10:25 Break

Chairperson: **Hiroshi Kitagaki**

10:35 3Ea04 How has sake yeast acquired high alcohol fermentation ability?
.....○Daisuke Watanabe (Natl. Res. Inst. Brewing)

11:00 3Ea05 Redox responses and signaling by *Aspergillus nidulans*
.....○Naoki Takaya, Motoyuki Shimizu, Shunsuke Masuo (Life & Environ. Sci., Univ. Tsukuba)

11:25 3Ea06 NO-mediated antioxidative mechanism of yeast and its application to baker's yeast breeding
.....○Hiroshi Takagi (Grad. Sch. Biol. Sci., NAIST)

11:50 Closing remarks
..... Hiroshi Takagi

Room E Afternoon (14:00~16:20)

Symposium (The innovative treatment strategy for biofilm infection)

14:00 Openign remarks
..... Satoshi Tsuneda

Chairperson: **Satoshi Tsuneda**

14:05 3Ep01 Control and prevention of biofilm formation based on quorum sensing inhibition
.....○Tsukasa Ikeda[1,2], Tomohiro Morohoshi[1,2]
([1]Dept. Mat. Environ. Chem., Utsunomiya Univ., [2]CREST, JST)

14:35 3Ep02 Potential of Macrolide Antibiotics as Quorum-Sensing Inhibitor to *Pseudomonas aeruginosa*
.....○Kazuhiro Tateda (Dept. Microbiol. Infect. Dis., Toho Univ.)

15:05 Break

Chairperson: **Tsukasa Ikeda**

15:15 3Ep03 Cell density dependent RelE-mediated domancy of *Escherichia coli*
.....○Satoshi Okabe, Yosuke Tashiro, Asami Taniuchi, Thithiwat May, Kenji Kakinuma, Koji Kawata
(Dept. Environ. Eng., Hokkaido Univ.)

15:45 3Ep04 *c*-di-GMP with potentiality as a medicine for biofilm-infection diseases
.....○Yoshihiro Hayakawa (Dept. Appl. Chem., Fac. Eng., Aichi Inst. Tech.)

16:15 Closing remarks
..... Tsukasa Ikeda

Room E Afternoon (16:40~19:00)

Symposium (Thermotolerant microorganisms leading to high-temperature fermentation)

Chairperson: **Kazunobu Matsushita**

16:40 3Ep05 Introduction: Thermotolerant microorganisms leading to high-temperature fermentation
.....○Kazunobu Matsushita (Dept. Biol. Chem., Yamaguchi Univ.)

16:50 3Ep06 Isolation, Selection and Characterization of Thermotolerant Yeasts for Ethanol Production
.....○Savitree Limtong[1], Wichien Yongmanitchai[1], Napatchanok Yuangsaard[1], Mamoru Yamada[2]
([1]Dept. Microbiol., Kasetsart Univ., Thailand, [2]Fac. Agri. and Grad. Sch. Med., Yamaguchi Univ., Japan)

2Dp02　Taxonomy of yeasts-associated with *Apis cerana* and their potential use for pollen substitutes

○Wellyzar Sjamsuridzal[1], Adi Basukriadi[1], Ariyanti Oetari[1], Effionora Anwar[2], Novia Rachmayanti[1], Bangga Beristama Putera[1], Irvan Maulana[1], Retno Widowati[1], Estriningtyas Agus Rismawanti[1], Virgine Enfinali[1], Bregas Adi Luhur[1], Dafina Ghossani Nurlaili[1]
([1]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Indonesia, [2]Faculty of Pharmacy, Universitas Indonesia, Indonesia)
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The mutually beneficial interaction between yeasts and their associated honeybees has been reported. However, the yeasts associated with *Apis cerana* (the Asiatic honeybees) were least studied. *Apis cerana* is a native honeybee domesticated by many Indonesian villagers. The taxonomic positions of yeasts from larvae, pupae, workers, and honey of *A. cerana* and its visited flowers was analyzed based on sequence data of internal transcribed spacers regions of ribosomal DNA (ITS rDNA). A total of 123 yeast isolates were isolated from flowers, larvae, pupae, workers, and honey from an apiary in West Java, Indonesia. Our study revealed that *ascomycetous* yeasts from Order *Saccharomycetales* are the dominant yeasts in domesticated *A. cerana*. Our phylogenetic analysis showed that several yeast species from the honeybee were clustered with other known yeast species associated with bees or other insects and their visited flowers. Four yeast species found in *A. cerana* visited flowers (e.g., *Candida* cf. *azyma*, *C. hawaiiana* , and *Debaryomyces hansenii*) were commonly found in larvae, pupae, and digestive tracks of workers of *A. cerana*. All yeast species found in honeybee's products were also found either in the digestive tracts of workers, or larvae or pupae. We found that domesticated *A. cerana* harbors higher number of yeast species than wild honeybees. Yeasts have been reported to have the most important role on in honeybee's nutrition. Yeast isolated from *A. cerana* were used as a component of pollen substitutes. The pollen substitutes were consisted of dry yeasts, defatted soybean flour and dry skim milk. We made six pollen substitutes with variation of yeast species and sugar. The pollen substitutes were fed to colonies of *A. cerana* for 45 days. Our results showed that pollen substitutes containing yeast *Candida hawaiiana* were more preferred by *A. cerana* compared to other pollen substitutes. The pollen substitute without yeasts was not preferred by *A. cerana*. We found that the uses of *A. cerana*-associated yeast in pollen substitutes resulted in an increase number of honey combs and honey production.

Acknowledgments：This presentation was supported by a grant from the Institute for Fermentation, Osaka（IFO）.

2Dp03　Yeasts on Agronomic Crop Phylloplane: Description of Novel Species and Potential in Agricultural Application

○Savitree Limtong[1], Rungluk Kaewwichian[1], Hiroko Kawasaki[2], Wichien Yongmanitchai[1]
([1]Dept. Microbiol., Fac. Sci., Kasetsart Univ., Thailand, [2]NITE Biological Resource Center, Japan)
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Phylloplane is an external surface of plant leaf and it has been recognized as an important habitat of microorganisms. This research attempted to study the diversity of yeasts on plylloplane of important agronomic crops (rice, corn and sugarcane) in Thailand and determine their potential for agricultural application i.e. biocontrol of plant pathogenic fungi and plant growth promoting activity. A total of 453 yeast strains were isolated from 261 leaf samples collected from nine provinces. On the basis of molecular taxonomy 431 strains were identified to be 50 species in 20 ascomycete yeast genera including *Candida* (20 species), *Hanseniaspora* (3 species), *Lindnera* (3 species), *Meyerozyma* (2 species), *Pichia* (5 species), *Torulaspora* (2 species), *Yamadazyma* (2 species), and one species each of *Brettanomyces*, *Clavispora*, *Debaryomyces*, *Geotrichum*, *Kodamaea*, *Kluyveromyces*, *Lachancea*, *Lodderomyce*, *Metschnikowia*, *Millerozyma*, *Pseudozyma*, *Wickerhamomyces* and *Yarrowia*, and 17 species in nine basidiomycete yeast genera including *Bullera* (1 species), *Cryptococcus* (5 species), *Rhodosporidium* (3 species), *Rhodotorula* (2 species), *Sporobolomyces* (1 species), *Sporidiobolus* (2 species), *Sporobolomyces* (1 species), *Trichosporon* (1 species) and *Yamadazyma* (1 species). The result showed high diversity of yeast in phylloplane and the predominant species was *Rhodotorula mucilaginosa* (13.5% of the strains) followed by *Candida tropicalis* (12.1% of the strains) and *Meyerozyma guilliermondii* (8.8% of the strains). Seven species were found in phylloplane of all three crops including six ascomycete species i.e. *Candida tropicalis*, *Lindnera rhodanensis*, *Meyerozyma caribbica*, *Meyerozyma guilliermondii*, *Pichia koratensis* and *Pichia kudriavzevii* and one basidiomycete species i.e. *Rhodotorula mucilaginosa*. Nine strains represented five novel species and 13 strains were similar to undescribed species. Until present, two novel species were already proposed as *Metschnikowia saccharicola* and *Metschnikowia lopburiensis* on the basis of polyphasic taxonomy. Investigation on potential to be biocontrol yeast, antagonistic activities of all 453 yeast strains against plant pathogenic fungi including one rice and corn pathogen (*Fusarium moniliforme*), two rice pathogens (*Helminthosporium oryzae* and *Rhizoctonia solanic*) and four corn pathogens (*Bipolaris maydis*, *Bipolaris zeicola*, *Exserohilum turcicum* and *Macrophomina phaseolina*) were tested. The result showed that only *Torulaspora globosa* RK547 which was isolated from corn phylloplane could inhibit growth of three plant pathogenic fungi *viz. Fusarium moniliforme*, *Helminthosporium oryzae* and *Rhizoctonia solani*. To investigate capacity to produce plant growth promoter, all 453 yeast strains were cultivated in yeast extract peptone dextrose (YPD) broth supplemented with L-tryptophan and indole-3-acetic acid (IAA), a major member of plant growth promoter in the auxin class, as determined in culture broth. Thirty-five percentages of the strains showed capability to produce indole-3-acetic acid (IAA) and *Rhodosporidium fluviale* RK253 yielded the highest IAA concentration.

Acknowledgments：This presentation was supported by a grant from the Institute for Fermentation, Osaka（IFO）.

Taxonomy of yeasts-associated with *Apis cerana* and their potential use for pollen substitutes

○Wellyzar Sjamsuridzal[1], Adi Basukriadi[1], Ariyanti Oetari[1], Effionora Anwar[2], Novia Rachmayanti[1], Bangga Beristama Putera[1], Irvan Maulana[1], Retno Widowati[1], Estriningtyas Agus Rismawanti[1], Virgine Enfinali[1], Bregas Adi Luhur[1], Dafina Ghossani Nurlaili[1]
([1]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Indonesia, [2]Faculty of Pharmacy, Universitas Indonesia, Indonesia)

Key words　*Apis cerana*, honeybee, yeast taxonomy, pollen substitutes

Yeasts on Agronomic Crop Phylloplane: Description of Novel Species and Potential in Agricultural Application

○Savitree Limtong[1], Rungluk Kaewwichian[1], Hiroko Kawasaki[2], Wichien Yongmanitchai[1]
([1]Dept. Microbiol., Fac. Sci., Kasetsart Univ., Thailand, [2]NITE Biological Resource Center, Japan)

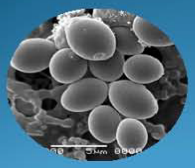
Key words　yeast, plylloplane, plant pathogenic fungi, indole-3-acetic acid

Polyphasic taxonomy of yeasts

บรรยายใน Seminar on Curation and Management of Microbial Resources

ณ Thailand Science Park, Pathum Thani, Thailand ระหว่าง 14–17 มกราคม 2556

Polyphasic Taxonomy of Yeasts



สาวิตรี ลิ่มทอง
ภาควิชาจุลชีววิทยา
คณะวิทยาศาสตร์
มหาวิทยาลัยเกษตรศาสตร์

Seminar on Curation and Management of Microbial Resources, 14 – 17 January 2013, Thailand Science Park, Pathum Thani, Thailand

Kasetsart University Kasetsart University Kasetsart University

อนุกรมวิธานของยีสต์ (Yeast Taxonomy)

ตามกฎของอนุกรมวิธาน ยีสต์และราชนิดอื่น อยู่ภายใต้ International Code of Nomenclature for algae, fungi, and plant (ICN) ซึ่งเปลี่ยนแปลงหลังสุดใน International Botanical Congress ที่ Melbourne ประเทศออสเตรเลีย ใน เดือนกรกฎาคม ค.ศ. 2011 โดยเป็นส่วนหนึ่งของ Melbourne Code แทนที่ Vienna Code ปี ค.ศ. 2005



อนุกรมวิธานของยีสต์ (Yeast Taxonomy) (ต่อ)

- International Code of Nomenclature for algae, fungi, and plant (Melbourne Code, 2011) มีการเปลี่ยนแปลงจากเดิม (Vienna Code, 2005) 4 ข้อ และที่เกี่ยวข้องกับราที่สำคัญ
  - "One fungus, one name", concepts anamorph and teleomorph has eliminate
  - เปลี่ยนข้อบังคับของการอธิบายลักษณะ (description/ diagnosis) ที่ต้องมีภาษา Latin เป็นจะอธิบายด้วยภาษาอังกฤษ หรือภาษา Latin ก็ได้
  - ต้องมี "registration of name" ปัจจุบันมี 2 repositories คือ Index Fungorum และ MycoBank

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อนุกรมวิธานของยีสต์ (Yeast Taxonomy) (ต่อ)

- อนุกรมวิธาน ของยีสต์เริ่มเมื่อ ค.ศ.1838
- Emil Christian Hansen (ค.ศ.1896): แยกเชื้อ และจำแนกประเภท (classification) โดยอาศัย
  - morphology of cells and ascospores
  - assimilation of C-compounds
  - fermentation of Carbohydrates
  - ความทนอุณหภูมิสูง



การจำแนกประเภทยีสต์ (Classification of Yeasts)

Classification: The Yeasts: A Taxonomic Study, 1st edition (1952)

- vegetative cell division
- morphology of ascospores
- biochemical characteristics
- physiological characteristics

} Phenotypic characteristics

Conventional Taxonomy

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การจำแนกประเภทยีสต์ (Classification of Yeasts) (ต่อ)

- ตั้งแต่ ค.ศ. 1960 ได้มีการนำ chemotaxonomy และ molecular taxonomy มาใช้ในการจำแนกประเภทยีสต์ (Nakase, 2001)
- ลักษณะที่ใช้ คือ
  - องค์ประกอบของเบส guanine และ cytosine (G+C content) ของ nuclear DNA (nDNA)
  - องค์ประกอบของน้ำตาลในเซลล์
  - ชนิดของ coenzyme Q หรือ ubiquinone
  - องค์ประกอบของกรดไขมันในเซลล์

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การจำแนกประเภทยีสต์ในปัจจุบัน

Polyphasic Taxonomy

- Conventional taxonomy
- Chemotaxonomy
- Molecular taxonomy
- Phylogenetic taxonomy

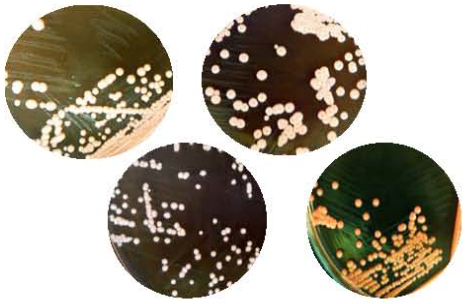


Phenotypic Characteristics for Conventional Taxonomy

1. ลักษณะสัณฐานวิทยา
   1) การเจริญบนอาหารแข็ง
   2) สัณฐานวิทยาของเซลล์ที่เจริญแบบไม่อาศัยเพศ (asexual cell)
   3) การสร้างเส้นใยแท้และเส้นใยเทียม
2. การสืบพันธุ์แบบอาศัยเพศใน ascomycetous yeast
3. การสืบพันธุ์แบบอาศัยเพศใน basidiomycetous yeast
4. การทดสอบทางสรีรวิทยาและชีวเคมี
   1) การหมักคาร์โบไฮเดรต
   2) การเจริญ (การแอสซิมิเลต) บนสารประกอบคาร์บอน
   3) การเจริญ (การแอสซิมิเลต) บนไนโตรเจน

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สัณฐานวิทยาของโคโลนี (Colony Morphology)



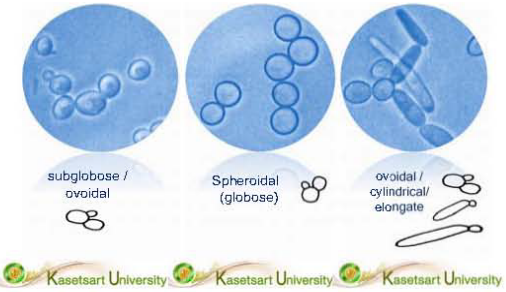
สัณฐานวิทยาของเซลล์ที่เจริญแบบไม่อาศัยเพศ (Morphology of Asexual Cell)

- กลม (spheroidal)
- ค่อนข้างกลม (subglobose)
- รี (ellipsoidal)
- ไข่ (ovoidal)

} พบมาก

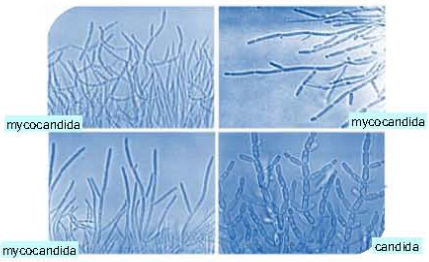
- ทรงกระบอก (cylindrical)
- ยาว (elongate)
- สาย (filamentous)
- แหลมหัวแหลมท้ายแบบมะนาว (apiculate)
- ปลายด้านหนึ่งแหลม (ogival)
- สามเหลี่ยม (triangular)
- คนโท (flask)

สัณฐานวิทยาของเซลล์ที่เจริญแบบไม่อาศัยเพศ (Morphology of Asexual Cell) (ต่อ)

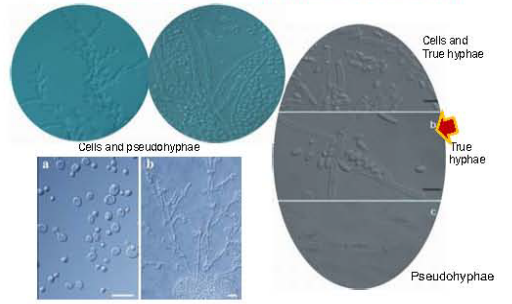


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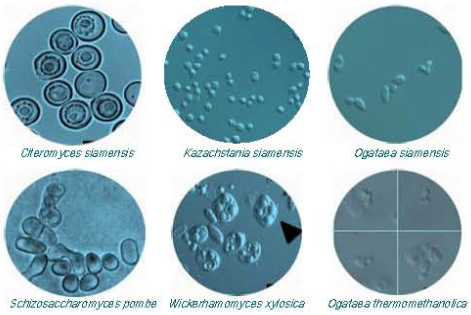
การสร้างเส้นใยเทียม (Pseudohyphae Formation)



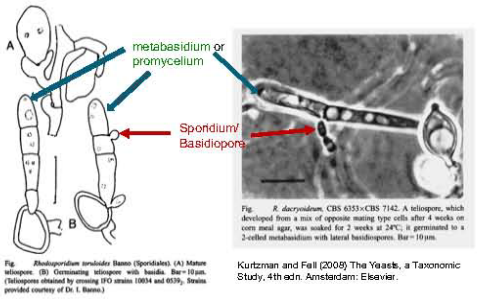
การสร้างเส้นใยแท้และเส้นใยเทียม (True and Pseudohyphae Formation)



รูปร่างของแอสโคสปอร์



การสร้างแบสิดิโอสปอร์



Kurtzman and Fell (2008) The Yeasts, a Taxonomic Study, 4th edn. Amsterdam: Elsevier.

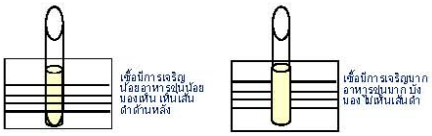
การหมักคาร์โบไฮเดรต

- น้ำตาลที่ใช้ประจำ คือ glucose, galactose, sucrose, maltose, lactose, raffinose, alpha-alpha trehalose
- ถ้ายีสต์ชนิดนั้นมีความสามารถในการหมัก จะหมักกลูโคสได้เสมอ
- ตรวจผลโดยดูการสร้างแก๊ส (CO_2) ใน Durham fermentation tube



การเจริญ (การแอสสิมิเลต) บนสารประกอบคาร์บอนในอาหารเหลว

- ใช้ yeast nitrogen base ที่มีสารประกอบคาร์บอนที่ต้องการทดสอบ
- เพาะเชื้อ และ บ่มโดยการวางเอียง หรือ บน rotary shaker หรือ roller drum ที่ 25°C 3-4 สัปดาห์
- ตรวจผล ที่ 1 และ 3 สัปดาห์ และรายงานการเจริญ จากความขุ่น โดยการหาบหลอดบนกระดาษขาวที่มีขีดเส้นดำ พิจารณาความชัดของเส้นดำที่หาย



การเจริญ (การแอสสิมิเลต) บนสารประกอบคาร์บอนโดยใช้ test kid



Phenotypic characteristics for conventional taxonomy (ต่อ)

5. การตรวจการเจริญอื่นๆ เช่น

1) การเจริญในอาหารที่ปราศจากวิตามิน
2) การเจริญในอาหารที่มีแรงดันออสโมซิสสูง
3) การเจริญที่ 37 องศาเซลเซียส และอุณหภูมิอื่น
4) การสร้างกรดจากคาร์โบไฮเดรต
5) การไฮโดรไลซ์ยูเรีย
6) การสร้างสารประกอบอะมีลอยด์ (amyloid compound)
7) การทำปฏิกิริยากับสีไดอะโซเนียมบลู บี (diazonium blue B)
8) ความต้านทานต่อสารปฏิชีวนะ cycloheximide
9) การละลายเจลาติน (gelatin liquefaction)
10) กิจกรรมของไลเพส (lipase activity)

อนุกรมวิธานระดับโมเลกุล (Molecular Taxonomy)

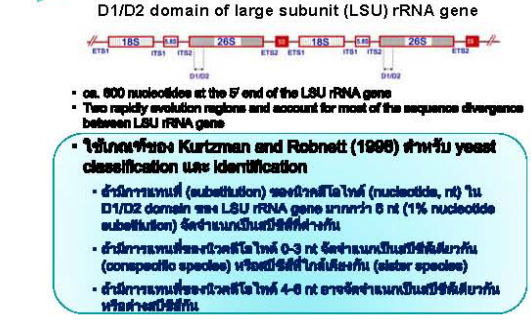
การศึกษาระดับ DNA และ RNA

- G+C content in nDNA (จัดเป็น chemotaxonomyด้วยเช่นกัน)
- การศึกษาความสัมพันธ์ระหว่าง nuclear DNA โดยการทำ DNA hybridization
- โครโมโซมโดยใช้ pulsed field gel electrophoresis (PFGE)
- restriction fragment length polymorphism (RFLP)
- random amplified polymorphic DNA (RAPD)
- amplified fragment length polymorphism (AFLP)
- nucleotide (base) sequence

การเปรียบเทียบลำดับนิวคลีโอไทด์

- การเปรียบเทียบลำดับนิวคลีโอไทด์ (เบส) ใน nucleic acid, genes, locus
- เป็นวิธีการที่เป็นให้ความน่าเชื่อถือมากกว่าวิธีอื่น เพราะใช้ได้กับชนิดที่มีความสัมพันธ์น้อยหรือมาก
- ใช้เพื่อบอกความสัมพันธ์ของยีสต์ โดยเฉพาะในระยะ 10 ปีที่ผ่านมา
- ลำดับนิวคลีโอไทด์ที่ใช้มาก คือ ลำดับของ rRNA gene (rDNA)

การจัดจำแนกยีสต์โดยการเปรียบเทียบลำดับนิวคลีโอไทด์



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การจัดจำแนกยีสต์โดยการเปรียบเทียบลำดับนิวคลีโอไทด์

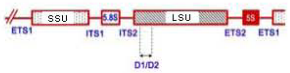
D1/D2 domain of large subunit (LSU) rRNA gene

- ca. 600 nucleotides at the 5' end of the LSU rRNA gene
- Two rapidly evolution regions and account for most of the sequence divergence between LSU rRNA gene

- ใช้เกณฑ์ของ Kurtzman and Robnett (1998) สำหรับ yeast classification และ identification
  - ลำดับการแทนที่ (substitution) ของนิวคลีโอไทด์ (nucleotide, nt) ใน D1/D2 domain ของ LSU rRNA gene มากกว่า 6 nt (1% nucleotide substitution) จัดจำแนกเป็นสปีชีส์ที่ต่างกัน
  - ลำดับการแทนที่ของนิวคลีโอไทด์ 0-3 nt จัดจำแนกเป็นสปีชีส์เดียวกัน (conspecific species) หรือสปีชีส์ที่ใกล้เคียงกัน (sister species)
  - ลำดับการแทนที่ของนิวคลีโอไทด์ 4-6 nt อาจจัดจำแนกเป็นสปีชีส์เดียวกัน หรือต่างสปีชีส์กัน

การจัดจำแนกยีสต์โดยการเปรียบเทียบลำดับนิวคลีโอไทด์ (ต่อ)

Internal Transcribe Spacer region (ITS1 and ITS2)



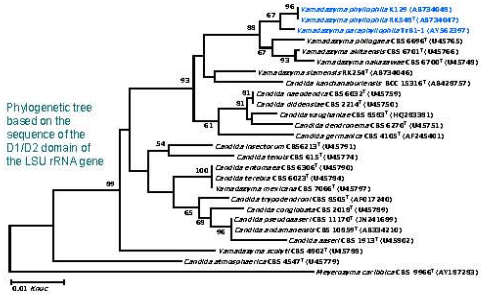
สำหรับยีสต์ขนาดของ ITS1 และ ITS2 มักต่ำกว่า 300 base pair (bp) (จากศึกษายีสต์ 373 strains 86 species) ยกเว้นบางชนิด เช่น

- ITS1: *Candida glabrata* = 402 bp
- ITS2: *Saccharomyces cerevisiae* = 366 bp

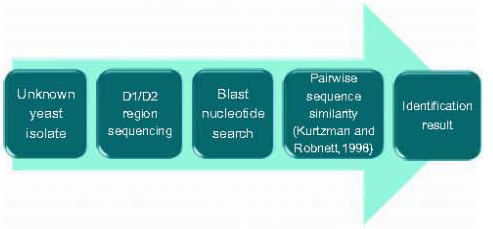
(Leaw et al. 2006. J Clinical Microbiol. 44:693-699)

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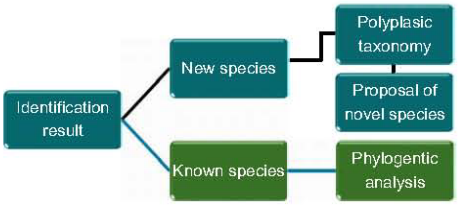
การวิเคราะห์ความสัมพันธ์ทางวิวัฒนาการ



Yeast Identification



Yeast Identification (ต่อ)



หนังสือที่ใช้สำหรับ Yeast classification และ Yeast Identification

The Yeasts: a Taxonomic Study

1st edition (1952) โดย Lodder and Kreger-van Rij
- มี 26 genera 164 species
- ascomycetous yeasts

2nd edition (1970) โดย Lodder
- มี 39 genera 349 species
- ascomycetous yeasts

3rd edition (1984) โดย Kreger-van Rij
- มี 60 genera 500 species
- ascomycetous yeasts และ basidiomycetous yeasts

The Yeasts: a Taxonomic Study (cont.)

4th Revised and enlarged edition (1998) โดย C.P. Kurtzman and J.W. Fell
- มี >100 genera มากกว่า 700 species
- ascomycetous yeasts และ basidiomycetous yeasts

5th edition (2011) โดย C.P. Kurtzman, J.W. Fell and T. Boekhout
- มี 3 volumes
- มี 149 genera เกือบ 1500 species
- ascomycetous yeasts และ basidiomycetous yeasts

หลักการจำแนกประเภทของยีสต์ในปัจจุบัน

Kurtzman, C.P. , Fell ,J.W. and Boekhout, T. (2011). The Yeasts: a Taxonomic Study, 5th edition

การ Classification ตามหลักการ ดังนี้
- **Mycota** ที่แนะโดย Hibbett et al. (2007) และ
- **Multigene analysis** ที่ทำตามโดย Kurtzman and Robnett (2003, 2007, 2010); Kurtzman and Suzuki (2010); Kurtzman et al. (2007, 2008)
  - Large subunit (LSU) rRNA gene
  - Small subunit (SSU) rRNA gene
  - Elongation factor-1α (EF-1α)
  - Mitochondrial SSU rRNA gene

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การจำแนกประเภทของยีสต์

Kingdon Fungi 2 phylums

1. Phylum Ascomycota
2. Phylum Basidiomycota

แต่ละ phylum ประกอบด้วย

- anamorphic genera: มีเฉพาะ anamorphic state
- teleomorphic genera: มี anamorphic state และ teleomorphic state

Phylum Ascomycota

Characteristics:

- ส่วนใหญ่เป็น multilateral budding อาจมี bipolar และ fission
- ascospores ใน ascus
- อาจมีการสร้าง pseudohyphae และ/หรือ true hyphae
- ปฏิกิริยากับ Diazonium blue B เป็นลบ (-) (มียกเว้น 2-3 สปีชีส์)
- Urease เป็น ลบ
- Mol% G+C ต่ำกว่า 50 (30-50%)

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Phylum Ascomycota

Characteristics:

- ส่วนใหญ่เป็น multilateral budding อาจมี bipolar และ fission
- ascospores ใน ascus
- อาจมีการสร้าง pseudohyphae และ/หรือ true hyphae
- ปฏิกิริยากับ Diazonium blue B เป็นลบ (-) (มียกเว้น 2-3 สปีชีส์)
- Urease เป็น ลบ
- Mol% G+C ต่ำกว่า 50 (30-50%)

Phylum Ascomycota (ต่อ)

Characteristics (ต่อ):

- Coenzyme Q system มี Co-Q5 ถึง Co-Q10
- วิธีการเกิดหน่อเป็นแบบ holoblastic budding
- Cell wall polysaccharides มี 3 patterns:
  1. Mannose - glucose
  2. Mannose - glucose – galactose
  3. Mannose - glucose – galactose –rhamnose

holoblastic budding: ระหว่างเกิดหน่อเซลล์ลูกจะมีขนาดเท่ากับเซลล์แม่ทุกขั้น



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Phylum Ascomycota

(Yeast and Yeast-Like Taxa)

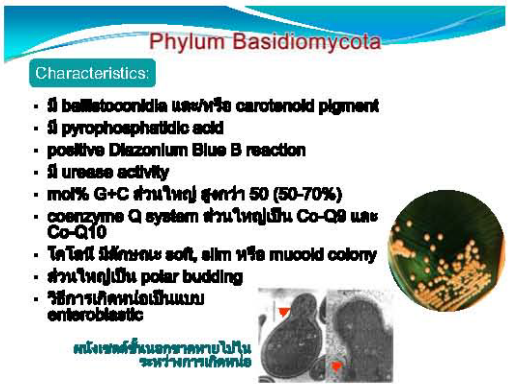
มี 2 subphylums:

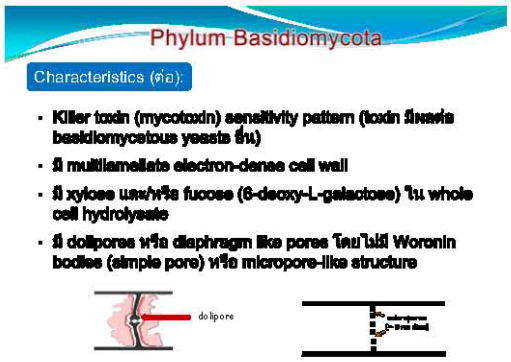
1. Subphylum Taphrinomycotina มี 4 classes
   - Class 1: Neolectomycetes
     - 1 order, 1 family, 1 genus (*Neolecta*)
   - Class 2: Pneumocystidomycetes
     - 1 order, 1 family, 1 genus (*Pneumocystidomycetes*)
   - Class 3: Schizosaccharomycetes
     - 1 order, 1 family, 1 genus (*Schizosaccharomyces*)
   - Class 4: Taphrinomycetes
     - 1 order, 2 families, 7 genera
2. Subphylum Saccharomycotina มี 1 class
   - Class Saccharomycetes
     - Order Saccharomycetales and Saccharomycetales *Incertae sedis* : 14 families; 82 genera

Phylum Basidiomycota

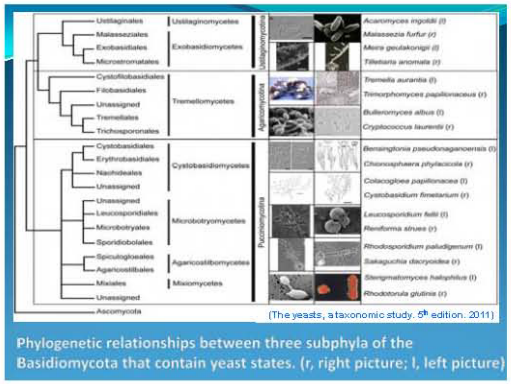
- ยีสต์ในไฟลัมนี้มีจำนวนมากที่เป็น dimorphic
  - มีทั้ง unicellular budding haploid (yeast) state และ dikaryotic hyphal state (บางสปีชีส์เป็น diploid/ หรือ aneuploid state)
  - Yeast / Yeast state
    - เซลล์ยีสต์ได้จากการงอก/ budding ของ basidiospore หรือ budding ของ basidia โดยตรง
    - ขั้นสุด yeast state เมื่อเริ่ม dikaryotic hyphal mycelia
  - Hyphal / dikaryotic hyphal state
    - ใน teleomorphic species

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Phylum Basidiomycota
Characteristics:
มี ballistoconidia และ/หรือ carotenoid pigment
มี pyrophosphatidic acid
positive Diazonium Blue B reaction
มี urease activity
mol% G+C ส่วนใหญ่ สูงกว่า 50 (50-70%)
coenzyme Q system ส่วนใหญ่เป็น Co-Q9 และ Co-Q10
โดโลไล มีลักษณะ soft, slim หรือ mucoid colony
ส่วนใหญ่เป็น polar budding
วิธีการเกิดหมอเป็นแบบ enteroblastic
สปอร์เซลล์ขึ้นนอกราดหายไปในระหว่างการเกิดหมอ


Phylum Basidiomycota
Characteristics (ต่อ):
Killer toxin (mycotoxin) sensitivity pattern (toxin มีผลต่อ basidiomycetous yeasts อื่น)
มี multilamellate electron-dense cell wall
มี xylose และ/หรือ fucose (6-deoxy-L-galactose) ใน whole cell hydrolysate
มี dolipores หรือ diaphragm like pores โดยไม่มี Woronin bodies (simple pore) หรือ micropore-like structure
dolipore


Phylum Basidiomycota
แบ่งเป็น 3 subphylum คือ
Subphylum 1 Agaricomycotina มี 1 classes:
1. Tremellomycetes
Subphylum 2 Pucciniomycotina มี 5 classes and unassigned:
1. Pucciniomycetes
2. Mixiomycetes
3. Agaricostilbomycetes
4. Cystobasidiomycetes
5. Microbotryomycetes
6. Unassigned
Subphylum 3 Ustilaginomycotina มี 2 classes:
1. Exobasidiomycetes
2. Ustilaginomycetes
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(The yeasts, a taxonomic study. 5th edition. 2011)
Phylogenetic relationships between three subphyla of the Basidiomycota that contain yeast states. (r, right picture; l, left picture)

The 5th TST
Conference on Taxonomy and Systematics in Thailand

การประชุมวิชาการอนุกรมวิธานและซิสเทมาติคส์ในประเทศไทยครั้งที่ 5

The 5th Conference on Taxonomy and Systematics in Thailand



คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์

25-27 พฤษภาคม 2558

กำหนดการประชุม
อนุกรมวิธานและซิสเทมาติคส์ในประเทศไทย ครั้งที่ 5

ณ อาคารปฏิบัติการวิทยาศาสตร์กายภาพ (อาคาร 45 ปี)
คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์
วันที่ 25-27 พฤษภาคม 2558

วันจันทร์ที่ 25 พฤษภาคม 2558 (อาคารวิทยาศาสตร์ 45 ปี)			
08:00 น. – 09:00 น.	ลงทะเบียน (หน้าห้อง 341)		
09:00 น. – 09:45 น.	พิธีเปิดการประชุม ณ คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ (ห้อง 341)		
09:45 น. – 10:30 น.	บรรยายพิเศษ (ห้อง 341) โดย: Professor Dr. Peter Ng Lee Kong Chain Natural History Museum, National University of Singapore		
10:30 น. – 11:00 น.	รับประทานอาหารว่าง		
11:00 น. – 11:40 น.	บรรยายพิเศษ (ห้อง 341) โดย: Assistant Professor Dr. Cameron D. Siler Department of Biology, University of Oklahoma, USA		
11:40 น. – 13:00 น.	รับประทานอาหารกลางวัน		
13:00 น. – 14:30 น.	กลุ่มพืช (I) (ห้อง 307)	กลุ่มพืช (II) (ห้อง 308)	กลุ่มสัตว์ (I) (ห้อง 341)
14:30 น. – 14:45 น.	รับประทานอาหารว่าง		
14:45 น. – 16:15 น.	กลุ่มพืช (I) (ห้อง 307)	กลุ่มสัตว์ (I) (ห้อง 341)	
16:15 – 18:00 น.	การนำเสนอผลงานโปสเตอร์โดยนักวิจัย และนิทรรศการ Thai Discovery		
18:00 น. เป็นต้นไป	งานเลี้ยงรับรอง ณ คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์		
วันอังคารที่ 26 พฤษภาคม 2558 (อาคารวิทยาศาสตร์ 45 ปี)			
08:30 น. – 09.15 น.	บรรยายพิเศษ (ห้อง 341) โดย: ศาสตราจารย์ ดร. สาวิตรี ลิ่มทอง คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์		

Diversity of Yeasts in Natural Habitats and Discovery of Novel Yeast Species in Thailand

Savitree Limtong

Department of Microbiology, Faculty of Science, Kasetsart University, Chatuchak, Bangkok 10900
E-mail: fscistl@.ku.ac.th

Thailand is a tropical country that is very rich in biodiversity and bioresources. Yeasts are unicellular fungi and many of them are important bioresources because they can be applied for industrial, agricultural and environmental utilization. Our group have carried out researches on diversity of yeasts in natural habitats *viz.* water, soil and decaying materials, plant leaf, and tree bark and exudate in various ecosystems *viz.* mangrove forest, terrestrial forest and agricultural ecosystems in Thailand by culture-dependent technique and yeast identification was based on molecular and phylogenetic taxonomies. The yeast diversity researches by our group during the past 10 years (2004-2014) resulted in discovery and reporting by publication in international journals of 58 novel yeast species in both phyla *viz.* Ascomycota and Basidiomycota. They were in genus *Candida, Barnettozyma*, *Geotrichum*, *Kazachstania, Kluyveromyces*, *Metschnikowia*, *Nakazawaea Ogataea*, *Pichia*, *Torulaspora*, *Wickerhamiella*, *Wickerhamomyces* and *Yamadazyma* belonging to the phylum Ascomycota such as *Barnettozyma siamensis*, *Candida chanthaburiensis, Candida phangngensis*, *Candida ranon-gensis*, *Candida siamensis*, *Candida thaimueangensis, Geotrichum siamensis*, *Kazachstania siamensis*, *Kluyveromyces siamensis*, *Metschnikowia lopburiensis*, *Nakazawaea siamensis*, *Ogataea chonburiensis*, *Ogataea kanchanaburiensis, Ogataea nakhonphanomensis*, *Ogataea siamensis*, *Ogataea thermomethanolica*, *Pichia jaroonii*, *Torulaspora maleeae*, *Wickerhamiella siamensis*, *Wickerhamomyces edaphicus*, *Wickerhamomyces xylosica*, *Yamadazyma epiphylla*, *Yamadazyma insecticola*, *Yamadazyma paraphyllophila*, *Yamadazyma phyllophila*, *Yamadazyma siamensis* and *Yamadazyma ubonensis*, and genus *Hannaella*, *Occultifur*, *Papiliotrema* and *Pseudozyma* belonging to the phylum Basidiomycota such as *Hannaella siamensis*, *Occultifur tropicalis*, *Pseudozyma vetiver* and *Papiliotrema siamense*. Moreover, the author joined the description of more than 30 novel yeast species that discovered in Thailand.

ความหลากหลายของยีสต์ในแหล่งที่อยู่ธรรมชาติและการค้นพบยีสต์สปีชีส์ใหม่ในประเทศไทย

สาวิตรี ลิ่มทอง
ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์
มหาวิทยาลัยเกษตรศาสตร์

อนุกรมวิธานและชีสเทมาติคส์ในประเทศไทย ครั้งที่ 5 คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ 25-27 พ.ค. 2558

ยีสต์

- ยีสต์เป็นราชนิดที่ส่วนใหญ่มีการดำรงชีวิตแบบเป็นเซลล์เดี่ยว (unicellular form)
- ยีสต์เป็น eukaryotic microorganism
- มีรูปร่างหลายแบบ
  - ที่พบบ่อย คือ กลม (round, spheroidal, spherical), รี (ellipsoidal) หรือ รูปไข่ (oval, ovoidal)
  - บางชนิดมีการสร้างเส้นใยเทียม (pseudomycelium) และบางชนิดสร้างเส้นใยแท้ (true mycelium)
- การเพิ่มจำนวน
  - การแตกหน่อ (budding)
  - การแบ่งแบบ fission
  - สร้างสปอร์ และวิธีอื่น

การจำแนกประเภทยีสต์

- อนุกรมวิธาน ยีสต์และราชนิดอื่นอยู่ภายใต้ International Code of Nomenclature for algae, fungi, and plant (ICN)
- ICN เปลี่ยนแปลงหลังสุดจาก International Botanical Congress ที่ Melbourne ประเทศออสเตรเรีย เมื่อกรกฎาคม พ.ศ. 2554 (ค.ศ. 2011): Melbourne Code
- การเปลี่ยนแปลงมี 4 ข้อ และที่เกี่ยวข้องกับราที่สำคัญ
  - "One fungus, one name", concepts anamorph and teleomorph has eliminate
  - การอธิบายลักษณะ (description/ diagnosis) ที่เดิมต้องมีภาษาลาตินด้วย เป็นจะอธิบายด้วยภาษาอังกฤษ หรือภาษาลาตินก็ได้
  - ต้องมี registration of name ปัจจุบันมี 2 repository คือ Index Fungorum และ MycoBank

หนังสือสำหรับการจำแนกประเภทยีสต์ (yeast classification) และการจัดจำแนกยีสต์ (yeast identification)

The Yeasts: A Taxonomic Study

- 5th edition (2011) โดยมี C.P. Kurtzman, J.W. Fell และ T. Boekhout เป็นบรรณาธิการ
  - มี 3 volume
  - อธิบายยีสต์ 149 สกุล เกือบ 1500 สปีชีส์

Kingdon Fungi : 2 phylum

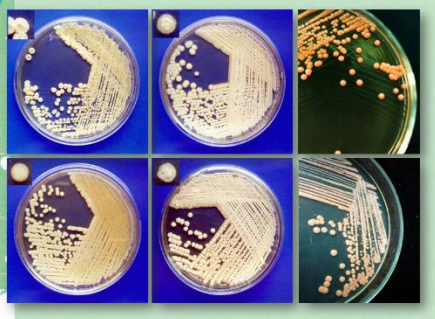
1. Phylum Ascomycota
2. Phylum Basidiomycota

การจำแนกประเภทยีสต์ (yeast classification)

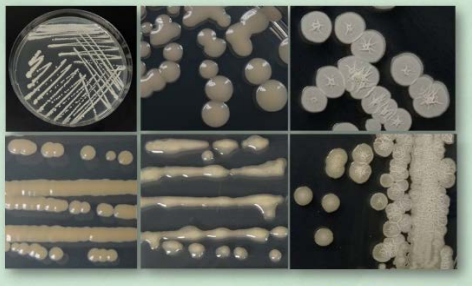
Polyphasic Taxonomy

- **Conventional taxonomy** (สัณฐานวิทยา ลักษณะทางชีวเคมี และสรีรวิทยา)
- **Chemotaxonomy** (องค์ประกอบทางเคมีต่าง ๆของเซลล์)
- **Molecular taxonomy** (การหาลำดับนิวคลีโอไทด์ของยีนต่าง ๆ และการเปรียบเทียบ DNA)
- **Phylogenetic taxonomy** (ความสัมพันธ์ทางวิวัฒนาการ)

สัณฐานวิทยาของโคโลนีของยีสต์



สัณฐานวิทยาของโคโลนีของยีสต์ (ต่อ)



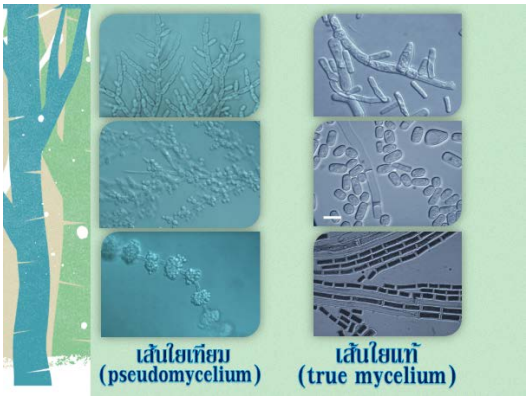
ถ่ายโดย ดร. ศศิธร จินดามรกฎ

สัณฐานวิทยาของโคโลนีของยีสต์ (ต่อ)



ถ่ายโดย ดร. ศศิธร จินดามรกฎ และคณะ


สัณฐานวิทยาของเซลล์ยีสต์
Budding yeast


เส้นใยเทียม
(pseudomycelium)
เส้นใยแท้
(true mycelium)

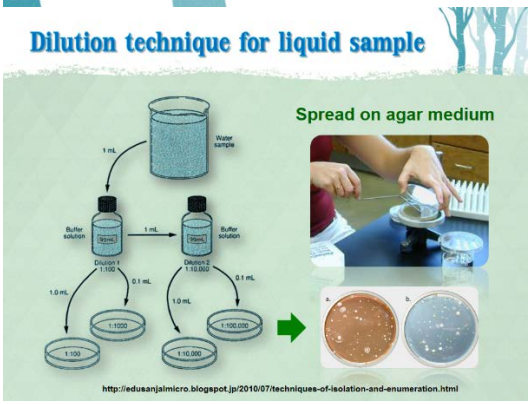

แหล่งที่อยู่ (habitat) ของยีสต์
• ทุกแหล่งที่อยู่ ทุกสภาวะแวดล้อม ทุกระบบนิเวศ
น้ำ
ต้นข้าว
ดอกไม้
เปลือกไม้
ใบไม้
ดิน
ตะกอนดิน


ระบบนิเวศที่ศึกษายีสต์ในประเทศไทย
ระบบนิเวศป่าชายเลน
ระบบนิเวศป่าบก
ระบบนิเวศการเกษตร

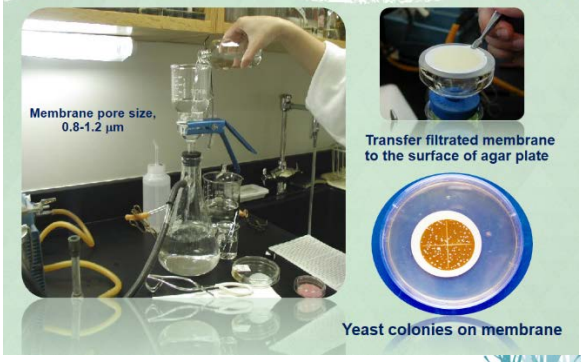

การวิจัยความหลากหลายของยีสต์ในแหล่งที่อยู่
• เทคนิคที่อาศัยการเพาะเลี้ยง
(culture-dependent technique)
• แยกเซลล์ยีสต์ออกมาเป็นยีสต์บริสุทธิ์ และ
จัดจำแนก
• เทคนิคที่ไม่อาศัยการเพาะเลี้ยง
(culture-independent technique)
• สกัด DNA ของยีสต์ออกมา และจัดจำแนกจาก DNA


เทคนิคที่อาศัยการเพาะเลี้ยง
(culture-dependent technique)
การเก็บตัวอย่าง
และการแยกยีสต์จากแหล่งที่อยู่

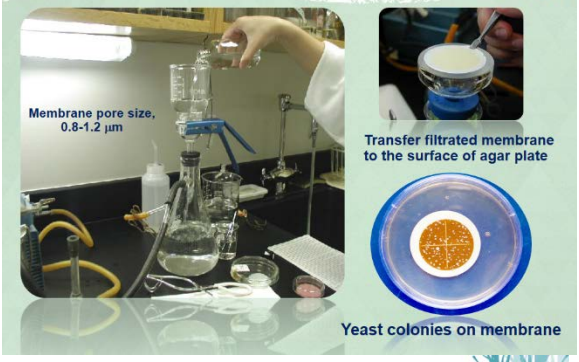

การเก็บตัวอย่างน้ำ และตะกอนดิน
Niskin biosampler
grab


Dilution technique for liquid sample
Spread on agar medium
Water sample
Buffer solution
1 mL
0.1 mL
1.0 mL
http://edusanjaimicro.blogspot.jp/2010/07/techniques-of-isolation-and-enumeration.html

Membrane filtration technique for liquid sample



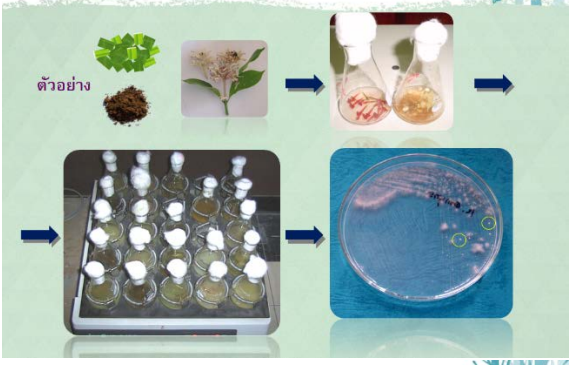
Membrane filtration technique for liquid sample



Dilution technique from solid sample



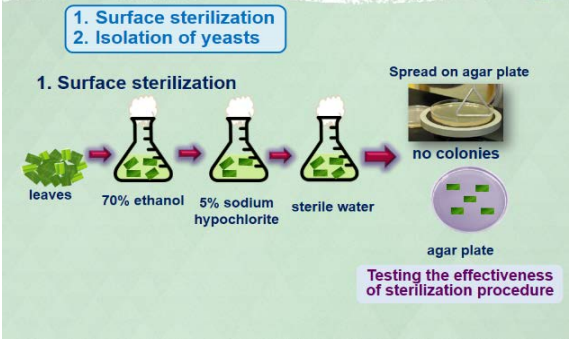
Enrichment technique



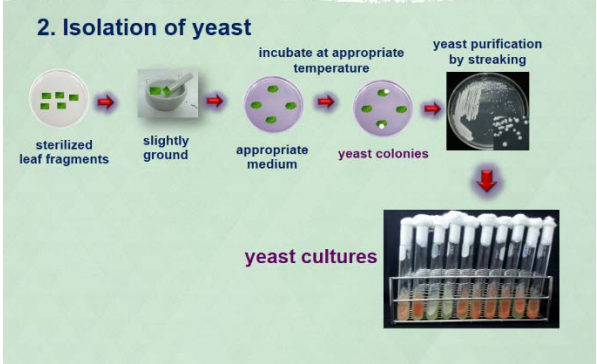
Isolation from surface of plant leaves by plating of leaf washing



Isolation from plant tissue

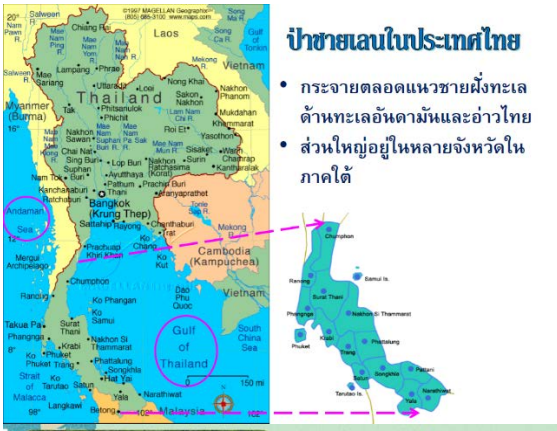


Isolation from plant tissue (cont.)






ป่าชายเลน
ป่าชายเลน หรือ ป่าโกงกาง (Mangrove forest หรือ Intertidal forest)
• สังคมพืชทนเค็มซึ่งขึ้นอยู่ในบริเวณชายฝั่งทะเล ปากแม่น้ำ หรืออ่าว หรือ
• สังคมพืชที่ประกอบด้วยพันธุ์ไม้หลายชนิดหลายสกุล และเป็นพวกที่มี ใบเขียวตลอดปี (evergreen species) ซึ่งมีลักษณะทางสรีรวิทยาและความต้องการสิ่งแวดล้อมที่คล้ายกัน ส่วนใหญ่ประกอบด้วยพันธุ์ไม้สกุลโกงกาง (Rhizophora spp.)
(สมิท อักษรแก้ว, 2542 ; http://th.wikipedia.org)

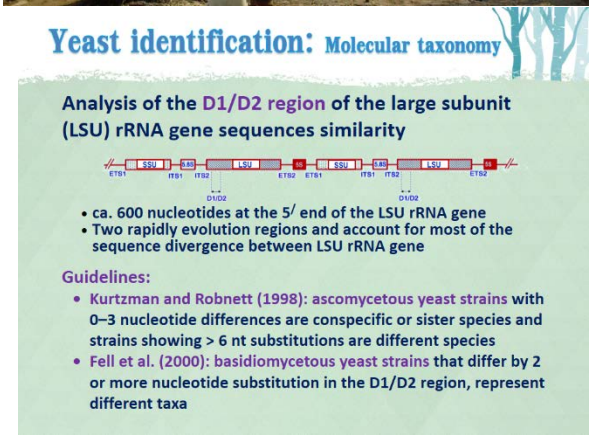

ป่าชายเลนในประเทศไทย
• กระจายตลอดแนวชายฝั่งทะเลด้านทะเลอันดามันและอ่าวไทย
• ส่วนใหญ่อยู่ในหลายจังหวัดในภาคใต้
Laos
Thailand
Vietnam
Cambodia (Kampuchea)
Gulf of Thailand
Andaman Sea
Bangkok (Krung Thep)
Malaysia

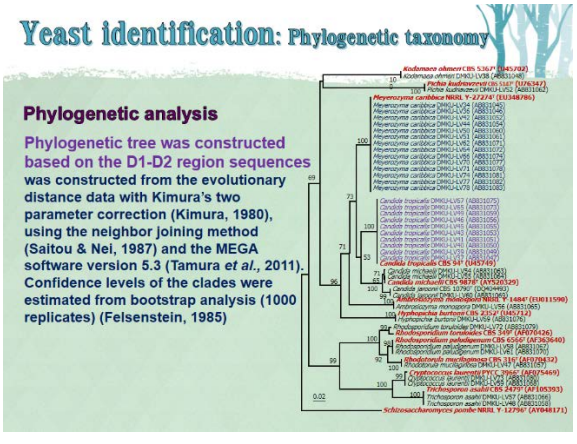

ป่าชายเลนทางด้านทะเลอันดามัน
• จังหวัดระนอง
• อุทยานแห่งชาติแหลมสน อำเภอสุขสำราญ
• จังหวัดพังงา
• อุทยานแห่งชาติเขาสำปี-หาดท้ายเหมือง อำเภอท้ายเมือง
• อุทยานแห่งชาติหมู่เกาะระ-เกาะพระทอง อำเภอคุระบุรี และอำเภอตะกั่วป่า
Phang-nga
Phatong
Ranong
Phangnga


การเก็บตัวอย่าง


ออกเก็บตัวอย่าง

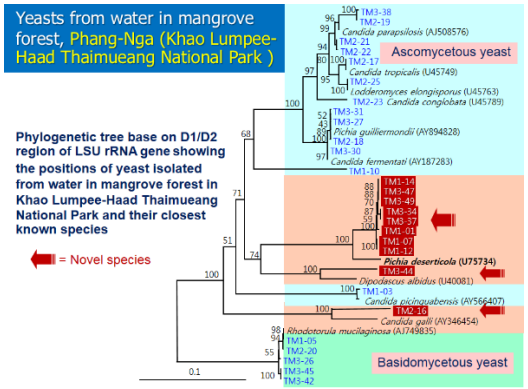

การเก็บตัวอย่างและการแยกยีสต์
ยีสต์


Yeast identification: Molecular taxonomy
Analysis of the D1/D2 region of the large subunit (LSU) rRNA gene sequences similarity
SSU
LSU
ITS1
ITS2
D1/D2
• ca. 600 nucleotides at the 5′ end of the LSU rRNA gene
• Two rapidly evolution regions and account for most of the sequence divergence between LSU rRNA gene
Guidelines:
• Kurtzman and Robnett (1998): ascomycetous yeast strains with 0–3 nucleotide differences are conspecific or sister species and strains showing > 6 nt substitutions are different species
• Fell et al. (2000): basidiomycetous yeast strains that differ by 2 or more nucleotide substitution in the D1/D2 region, represent different taxa

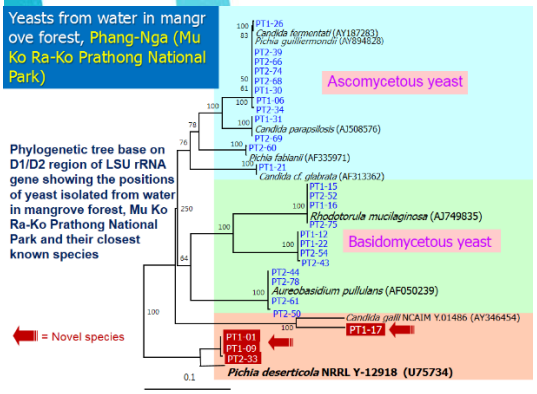

Yeast identification: Phylogenetic taxonomy
Phylogenetic analysis
Phylogenetic tree was constructed based on the D1-D2 region sequences was constructed from the evolutionary distance data with Kimura's two parameter correction (Kimura, 1980), using the neighbor joining method (Saitou & Nei, 1987) and the MEGA software version 5.3 (Tamura et al., 2011). Confidence levels of the clades were estimated from bootstrap analysis (1000 replicates) (Felsenstein, 1985)



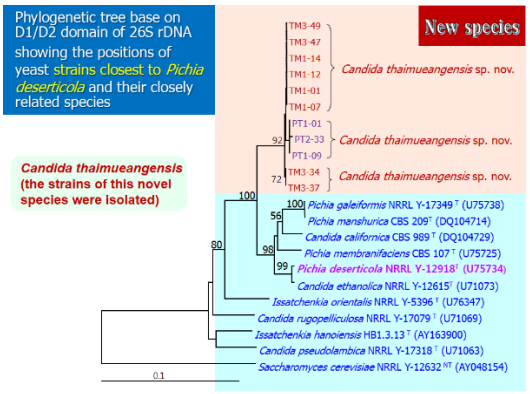
Yeasts from water in mangrove forest, Phang-Nga (Khao Lumpee-Haad Thaimueang National Park)



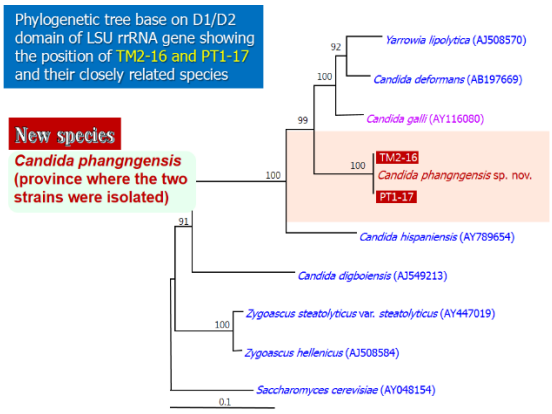
Yeasts from water in mangrove forest, Phang-Nga (Mu Ko Ra-Ko Prathong National Park)



Phylogenetic tree base on D1/D2 domain of 26S rDNA showing the positions of yeast strains closest to *Pichia deserticola* and their closely related species



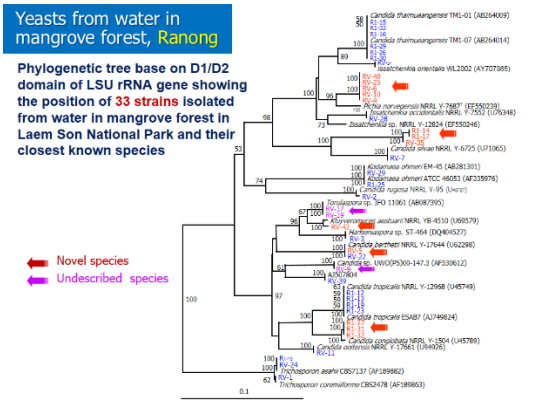
Phylogenetic tree base on D1/D2 domain of LSU rrRNA gene showing the position of TM2-16 and PT1-17 and their closely related species



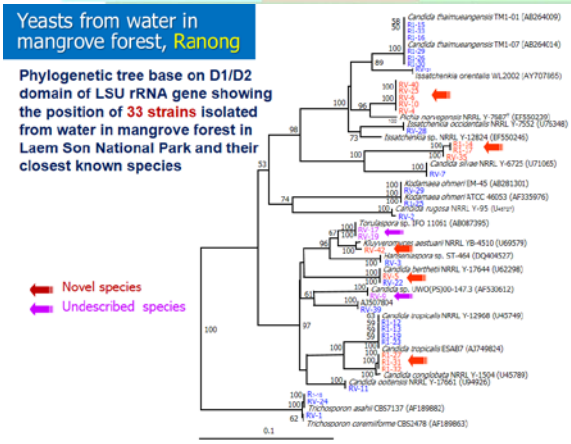
Comparison of known yeast species isolated from water in mangrove forests in Phang-Nga province

Known Species	Khao Lumpee-Haad Thaimueang	Mu Ko Ra-Ko Prathong
Aureobasidium pullulans	-	PT2-44, PT2-50, PT2-61, PT2-78, PT2-80
Candida conglobata	TM2-23	-
Candida fabianii	-	PT2-60
Candida fermentati	TM3-30	PT1-06, PT1-26, PT1-30, PT2-39, PT2-68, PT2-74, PT2-75
Candida cf. *glabrata*	-	PT1-21
Candida membranifeciens	TM1-10	-
Candida parapsilosis	TM2-19, TM2-21, TM2-22, TM3-38	PT1-31, PT2-69
Candida picinguabensis	TM1-03	-
Candida tropicalis	TM2-17	PT2-34, PT2-66
Lodderomyces elongisporus	TM2-25	-
Pichia guilliermondii	TM2-18, TM3-27, TM3-31	-
Rhodotorula mucilaginosa	TM3-26, TM3-42, TM3-45	PT1-15, PT1-16, PT2-52, PT2-76

Yeasts from water in mangrove forest, Ranong

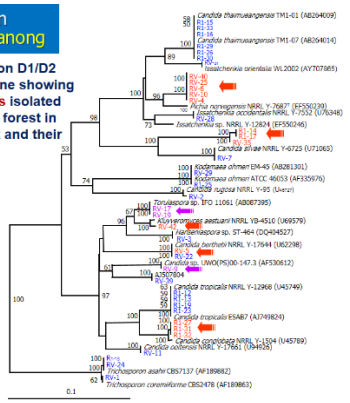


Yeasts from water in mangrove forest, Ranong



Yeasts from water in mangrove forest, Ranong

Phylogenetic tree base on D1/D2 domain of LSU rRNA gene showing the position of 33 strains isolated from water in mangrove forest in Laem Son National Park and their closest known species



Comparison of known yeast species isolated from water in mangrove forests in Phang-Nga province and Ranong province

Known Species	Phang-Nga province	Ranong province
Aureobasidium pullulans	PT2-44, PT2-50, PT2-61, PT2-78, PT2-80	-
Candida berthetii	-	RV22, RV62
Candida conglobata	TM2-23	-
Candida fabianii	PT2-60	-
Candida fermentati	TM3-30, PT1-06, PT1-26, PT1-30, PT2-39, PT2-68, PT2-74, PT2-75	-
Candida cf. glabrata	PT1-21	-
Candida membranifeciens	TM1-10	-
Candida ooitensis	-	RV11
Candida parapsilosis	TM2-19, TM2-21,TM2-22, TM3-38, PT1-31, PT2-69	-
Candida pseudolambica	-	RV08, RV63
Candida picinguabensis	TM1-03	-
Candida rugosa	-	RV02

Comparison of known yeast species isolated from water in mangrove forests in Phang-Nga province and Ranong province

Known Species	Phang-Nga province	Ranong province
Candida silvae	-	RV07
Candida tropicalis	TM2-17, PT2-34, PT2-66	RV13
Debaryomyces nepalensis	-	RV61, RS11
Hanseniaspora opuntiae	-	RS07, RS10
Issatchenkia occidentalis	-	RV12, RV15, RV18, RS05
Issatchenkia orientalis	-	RS06
Lodderomyces elongisporus	TM2-25	-
Pichia galeiformis	-	RS03
Pichia guilliermondii	TM2-18, TM3-27, TM3-31	-
Rhodotorula mucilaginosa	TM3-26,TM3-42,TM3-45, PT1-15, PT1-16, PT2-52, PT2-76	-
Saccharomyces cerevisiae	-	RV23
Trichosporon asahii	-	RV24
Trichosporon coremiiforme	-	RV01
Trichosporon japonicum	-	RV59

สรุป

- Investigation of yeast diversity in water in mangrove forests was carried out and both known and new species were found.
- Yeast strains from waters in the mangrove forests were described as 3 novel species *Candida thaimueangensis* sp. nov., *Candida phangngensis* sp. nov. and *Torulaspora maleeae* sp. nov.

Summary of yeast diversity study in mangrove forest

- เริ่มทำการวิจัยความหลากหลายของยีสต์ในปี พ.ศ. 2541 โดยใช้พื้นที่ป่าชายเลนที่อยู่ในอุทยานแห่งชาติแหลมสน ส่วนที่อยู่ในความดูแลของสถานีวิจัยเพื่อการพัฒนาชายฝั่งอันดามัน จังหวัดระนอง มหาวิทยาลัยเกษตรศาสตร์
- แหล่งที่อยู่ที่ศึกษา
  - น้ำและตะกอนดิน
  - ชิ้นส่วนของพืช ใบไม้ร่วง กิ่งไม้ร่วง เปลือกไม้ร่วง และลูกไม้ร่วงที่กำลังย่อยสลายและแช่น้ำในป่าชายเลน
- ป่าชายเลนในที่จังหวัดที่ศึกษา จังหวัดระนอง พังงา ภูเก็ต ตราด จันทบุรี เพชรบุรี ประจวบคีรีขันธ์ ชุมพร และสุราษฎร์ธานี
- พบและรายงานยีสต์สปีชีส์ใหม่ 16 สปีชีส์ ในวาสารนานาชาติ 10 เรื่อง

New yeast species from mangrove forest

1. ***Candida asiatica*** (Limtong et al. 2010b)
2. ***Candida andamanensis*** (Am-in et al. 2011)
3. ***Candida chanthaburiensis*** (Limtong et al. 2010a)
4. ***Candida kungkrabaensis*** (Limtong et al. 2010a)
5. ***Candida laemsonensis*** (Am-in et al. 2011)
6. ***Candida phangngensis*** (Limtong et al. 2008)
7. ***Candida prachuapensis*** (Nitiyon et al. 2011)
8. ***Candida ranongensis*** (Am-in et al. 2011)
9. ***Candida sanitii*** (Limtong et al. 2011)
10. ***Candida sekii*** (Limtong et al. 2011)
11. ***Candida siamensis*** (Boonmak et al. 2009)
12. ***Candida suratensis*** (Limtong et al. 2010a)
13. ***Candida suwanaritii*** (Limtong et al. 2011)
14. ***Candida thaimueangensis*** (Limtong et al. 2007)
15. ***Kluyveromyces siamensis*** (Am-in et al. 2008)
16. ***Torulaspora maleeae*** (Limtong et al. 2008)



ตัวอย่างผลงานตีพิมพ์



Candida thaimueangensis sp. nov., an anamorphic yeast species from estuarine water in a mangrove forest in Thailand

Candida phangngensis sp. nov., an anamorphic yeast species in the *Yarrowia* clade, isolated from water in mangrove forests in Phang-Nga Province Thailand

Torulaspora maleeae **sp. nov., a novel ascomycetous yeast species from Japan and Thailand**

Candida siamensis **sp. nov., an anamorphic yeast species in the *Saturnispora* clade isolated in Thailand**

Candida andamanensis sp. nov., *Candida laemsonensis* sp. nov. and *Candida ranongensis* sp. nov., anamorphic yeast species isolated from estuarine waters in a Thai mangrove forest

Kluyveromyces siamensis **sp. nov., an ascomycetous yeast isolated from water in a mangrove forest in Ranong Province, Thailand**

Candida asiatica sp. nov., an anamorphic ascomycetous yeast species isolated from natural samples from Thailand, Taiwan, and Japan

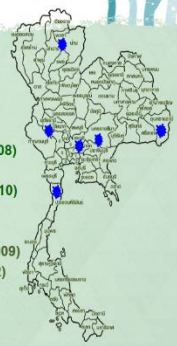
Three anamorphic yeast species *Candida sanitii* sp. nov., *Candida sekii* sp. nov. and *Candida suwanaritii*, three novel yeasts in the *Saturnispora* clade isolated in Thailand

Summary of yeast diversity study in soil and decaying materials

- เริ่มในปี พ.ศ. 2540 โดยทำการวิจัยความหลากหลายของยีสต์ในเขตรักษาพันธุ์สัตว์ป่าห้วยขาแข้ง โดยศึกษาในดินจากป่า 3 ชนิด คือ ดินป่าเต็งรัง (dry dipterocarp forest) ป่าเบญจพรรณ (mixed deciduous forest) และป่าดิบแล้ง (dry evergreen forest)
- ดินในป่า 4 ชนิด คือ ป่าดิบเขา ป่าเบญจพรรณ ป่าเต็งรัง และป่าสนในเขตอุทยานแห่งชาติน้ำหนาว ในเขตจังหวัดเพชรบูรณ์
- ดินในป่า บริเวณสถานีฝึกนิสิต คณะวนศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ อำเภอวังน้ำเขียว จังหวัดนครราชสีมา
- ดินในป่า ในวนอุทยานแห่งชาติ 3 แห่ง อุทยานแห่งชาติ 9 แห่ง เขตรักษาพันธุ์สัตว์ป่า 2 แห่ง และป่าไม้อื่นอีก 8 แห่ง ในจังหวัดของภาคตะวันออกเฉียงเหนือของประเทศไทย
- พบและรายงานยีสต์สปีชีส์ใหม่ 13 สปีชีส์ ในวาสารนานาชาติ 10 เรื่อง

New yeast species from soil and decaying materials

1. *Barnettozyma siamensis* A (Polburee et al. 2014)
2. *Candida asiatica* A (Limtong et al. 2010b)
3. *Candida phayaonensis* A (Junyapate et al. 2012)
4. *Candida saraburiensis* A (Nitiyon et al. 2011)
5. *Candida uthaithanina* A (Limtong et al. 2011)
6. *Candida wangnamkhiaoensis* A (Limtong et al. 2008)
7. *Geotrichum siamensis* A (Kaewwichia et al. 2010)
8. *Geotrichum phurueaensis* A (Kaewwichia et al. 2010)
9. *Kazachstania siamensis* A (Limtong et al. 2007)
10. *Pichia jaroonii* A (Limtong et al. 2009)
11. *Wickerhamomyces edaphicus* A (Limtong et al. 2009)
12. *Wickerhamomyces xylosica* A (Limtong et al. 2012)
13. *Yamadazyma ubonensis* B (Junyapate et al. 2014)



Summary of yeast diversity study in phylloplane/phyllosphere

- Phyllophane หรือ phylloshere คือ ผิวด้านนอกของพืช ซึ่งส่วนใหญ่มักเป็นผิวด้านนอกของใบพืช ดังนั้นจึงมักหมายของผิวใบพืช
- ยีสต์ส่วนใหญ่ที่พบเป็น basidiomycetous yeast
- ประเทศไทยมีการศึกษาน้อย
- เริ่มทำวิจัยเมื่อ พ.ศ. 2552
- ใบพืชที่ศึกษา พืชเศรษฐกิจ องุ่น ข้าว ข้าวโพด อ้อย และใบพืชอื่น ๆ
- พบและรายงานยีสต์สปีชีส์ใหม่แล้ว 19 สปีชีส์ ในวาสารนานาชาติ 10 เรื่อง

New yeast species from phylloplane/phyllosphere

1. *Candida phyllophila* A (Limtong et al. 2012)
2. *Candida sakaeoensis* A (Limtong et al. 2012)
3. *Candida sirachaensis* A (Limtong et al. 2012)
4. *Candida vitiphila* A (Limtong et al. 2012)
5. *Metschnikowia saccharicola* A (Kaewwichian et al. 2012)
6. *Metschnikowia lopburiensis* A (Kaewwichian et al. 2012)
7. *Nakazawaea siamensis* A (Kaewwichian et al. 2014)
8. *Occultifur tropicalis* A (Khunnamwong et al. 2014)
9. *Wickerhamiella siamensis* A (Kaewwichian et al. 2013a)
10. *Hannaella siamensis* B (Kaewwichian et al. 2015)
11. *Hannaella phetchabunensis* B (Kaewwichian et al. 2015)
12. *Hannaella phyllophila* B (Surussawade et al. 2015)
13. *Papiliotrema siamense* B (Surussawade et al. 2014)
14. *Pseudozyma vetiver* B (Chamanpa et al. 2013)
15. *Yamadazyma epiphylla* B (Jindamorakot et al. 2015)
16. *Yamadazyma insecticola* B (Jindamorakot et al. 2015)
17. *Yamadazyma paraphyllophila* B (Kaewwichian et al. 2013a)
18. *Yamadazyma phyllophila* B (Kaewwichian et al. 2013a)
19. *Yamadazyma siamensis* B (Kaewwichian et al. 2013a)



Methyltrophic yeast diversity study in Thailand

- เมทิลโลโทรฟิกยีสต์ (methylotrophic yeasts) หรือ ยีสต์ที่ใช้เมทานอล (methanol utilizing yeast) คือ ยีสต์ที่สามารถใช้เมทานอล (คาร์บอนหนึ่งอะตอม) เป็นแหล่งคาร์บอนและแหล่งพลังงานได้ด้วย
- ยีสต์ทั่วไปสามารถเจริญและเพิ่มจำนวนได้เป็นอย่างดีในอาหารที่มีน้ำตาลและคาร์โบไฮเดรตซึ่งเป็นสารประกอบที่มีคาร์บอนตั้งแต่สองอะตอมขึ้นไปเป็นแหล่งคาร์บอนและแหล่งพลังงาน

Methyltrophic yeast diversity study in Thailand (cont.)

ประโยชน์ได้หลายด้าน เช่น

- ใช้ในการผลิตโปรตีนเซลล์เดี่ยว (single cell protein) และกรดอะมิโน โดยใช้เมทานอล (ราคาถูก) เป็นวัตถุดิบ
- ใช้เป็นเจ้าบ้านในการแสดงออกของยีนจากแหล่งอื่นเพื่อผลิตโปรตีนลูกผสม (recombinant protein)
- สามารถในการย่อยสลายเมทานอลและฟอร์มาลดีไฮด์ (formaldehyde) นำไปใช้เพื่อการบำบัดของเสียและฟื้นฟูสภาพแวดล้อมโดยวิธีทางชีวภาพ โดยเฉพาะกับน้ำเสียจากโรงงาน

Taxonomy of methyltrophic yeasts

Methylotrophic yeast อยู่ใน Phylum Ascomycota สกุล:

- *Komagataella*
- *Ogataea*
- *Kuraishia* และ
- บางสปีชีส์ของ *Candida*

(Yamada *et al.*, 1994; Yamada *et al.*, 1995; Mikata & Yamada, 1995; Kurtzman and Fell, 1998; Barnett *et al.*, 2000; Dlauchy *et al.*, 2003; Kurtzman, 2005; Kurtzman *et al.*, 2011)

Objectives

- To study diversity of cultivable thermotolerant methylotrophic yeasts in Thailand
- To collect thermotolerant methylotrophic yeast strains for further utilization

Selection of thermotolerant methylotrophic yeasts from the strains in private collection

1,032 strains in private culture collection
(Dept. Microbiology, Fac. Sciences, Kasetsart University)

↓

determination of growth on
1% methanol-YNB agar at 30°C

↓

5 strains: positive growth at 30°C

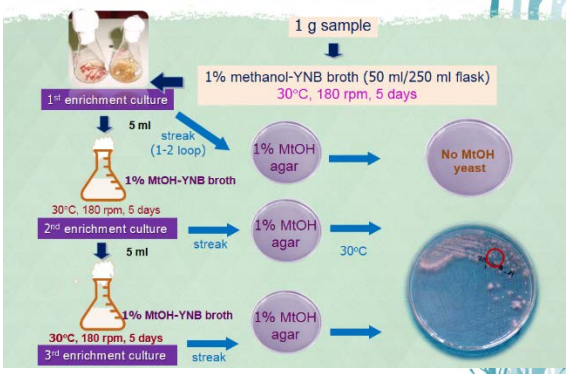
↓

determination of growth on
1% methanol-YNB agar at 37°C

↓

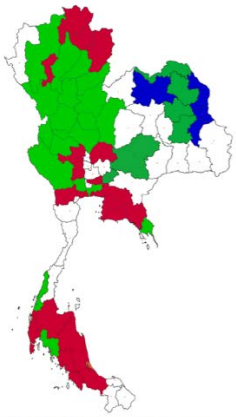
1 strain: positive growth at 37°C

Enrichment isolation of methylotrophic yeasts



Sample collection

- Soil
- Plants (flowers, tree bark, tree exudate)
- 634 samples from 45 provinces in Thailand



Yeast identification: Molecular taxonomy

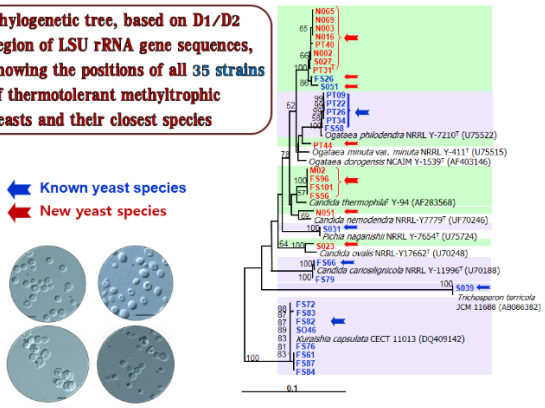
Analysis of the D1/D2 region of the large subunit (LSU) rRNA gene sequences similarity



Guidelines: Kurtzman and Robnett (1998): ascomycetous yeast strains with 0–3 nucleotide differences are conspecific or sister species and strains showing > 6 nt substitutions are different species

Phylogenetic analysis: tree was constructed based on the D1-D2 region sequences was constructed from the evolutionary distance data with Kimura's two parameter correction (Kimura, 1980), using the neighbor joining method (Saitou & Nei, 1987). Confidence levels of the clades were estimated from bootstrap analysis (1000 replicates) (Felsenstein, 1985)

Phylogenetic tree, based on D1/D2 region of LSU rRNA gene sequences, showing the positions of all 35 strains of thermotolerant methyltrophic yeasts and their closest species



5 known methylotrophic yeast species (17 strains)

Species	Soil	Plants	No. of strains
Candida cariosilignicola	-	FS66, FS79	2
Kuraishia capsulata	S046	FS61, FS72, FS78, FS82, FS83, FS84, FS87	8
Ogataea naganishi	S031	-	1
Ogataea philodendra	PT09, PT22, PT26, PT34	FS58	5
Trichosporon terricola	S039	-	1

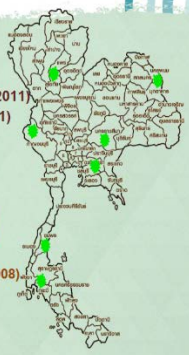
7 novel methylotrophic yeast species (18 strains)

Species	Soil	Plants	No. of strains
Candida krabiensis	N051	-	1
Candida sithepensis	S023	-	1
Ogataea chonburiensis	PT44	-	1
Ogataea nakonphanomensis	S051	-	1
Ogataea (Pichia) siamensis	-	M02, FS56, FS95, FS96, FS101	5
Ogataea (Pichia) thermomethanolica	N002, N003, N016, N065, N069, PT31, PT40, S027	-	8

New methylotrophic yeast species in Thailand

พบและรายงานยีสต์สปีชีส์ใหม่ 11 สปีชีส์ ในวารสารนานาชาติ 6 เรื่อง

1. *Candida chumphonensis* (Koowadjanakul et al. 2011)
2. *Candida mattranensis* (Koowadjanakul et al. 2011)
3. *Candida krabiensis* (Limtong et al. 2004)
4. *Candida sithepensis* (Limtong et al. 2004)
5. *Ogataea chonburiensis* (Limtong et al. 2008)
6. *Ogataea kanchanaburiensis* (Limtong et al. 2013)
7. *Ogataea nakhonphanomensis* (Limtong et al. 2008)
8. *Ogataea phyllophila* (Koowadjanakul et al. 2011)
9. *Ogataea siamensis* (Limtong et al. 2004, 2008)
10. *Ogataea thermomethanolica* (Limtong et al. 2005, 2008)
11. *Ogataea wangdongensis* (Limtong et al. 2013)







สรุป

- การวิจัยความหลากหลายของยีสต์ในแหล่งที่อยู่ธรรมชาติในประเทศไทย พบว่ามีประเทศไทยมีความหลากหลายของยีสต์มากเช่นเดียวกับจุลินทรีย์อื่น
- จากการวิจัยทำให้พบและรายงานยีสต์สปีชีส์ใหม่จำนวนมาก อย่างไรก็ตามยังมีแหล่งที่อยู่ และระบบนิเวศน์ที่ยังไม่ได้มีการวิจัย ดังนั้นจึงเชื่อว่ายังมียีสต์สปีชีส์ใหม่หรือหน่วยอนุกรมวิธานอื่นใหม่อีกจำนวนมาก
- ยีสต์สายพันธุ์ที่รวบรวมได้จากการวิจัยความหลากหลายทางชีวภาพโดยเทคนิคการเลี้ยงยีสต์นั้นสามารถนำมาวิจัยต่อเพื่อการประโยชน์ต่อไปได้

หน่วยงานที่สนับสนุนทุนวิจัย

- มหาวิทยาลัยเกษตรศาสตร์
- สถาบันวิจัยและพัฒนา แห่งมหาวิทยาลัยเกษตรศาสตร์
- ศูนย์วิทยาการขั้นสูงทรัพยากรธรรมชาติเขตร้อน สถาบันวิทยาการขั้นสูง แห่งมหาวิทยาลัยเกษตรศาสตร์
- สำนักงานคณะกรรมการวิจัยแห่งชาติ (วช)
- สำนักงานกองทุนสนับสนุนการวิจัย (สกว)
- โครงการพัฒนาองค์ความรู้และศึกษานโยบายการจัดการทรัพยากรชีวภาพในประเทศไทย

ขอบคุณ

ทีมวิจัย



ขอบคุณ

ภาคผนวก 3

การเสนอผลงานในการประชุมวิชานานาชาติในต่างประเทศ

สำเนาบทคัดย่อ โปสเตอร์ และ power point

1. **Limtong,S., P.Khunnamwong.** Diversity of endophytic yeasts in sugarcane leaves in Thailand. เสนอใน 14th International Congress of Bacteriology and Applied Microbiology, the 14thInternational Congress of Mycology and Eukaryotic Microbiology and the 16th International Congress of Virology (IUMS 2014) ระหว่าง 27 กรกฎาคม - 1 สิงหาคม 2557 ณ เมือง มอนทรีออน ประเทศแคนาดา

2. **Jindamorakot, S., S. Am-In, S. Limtong.** Diversity of epiphytic yeasts on rice leaves in Thailand. เสนอใน 14th International Congress of Bacteriology and Applied Microbiology, the 14thInternational Congress of Mycology and Eukaryotic Microbiology and the 16th International Congress of Virology (IUMS 2014) ระหว่าง 27 กรกฎาคม - 1 สิงหาคม 2557 ณ เมือง มอนทรีออน ประเทศแคนาดา

3. **Kaewwichian, R.**, W. Yongmanitchai, **S. Limtong.** Diversity of yeasts in corn phylloplanes and their antagonistic activities against pathogenic fungi. เสนอใน14th International Congress of Bacteriology and Applied Microbiology, the 14thInternational Congress of Mycology and Eukaryotic Microbiology and the 16th International Congress of Virology (IUMS 2014) ระหว่าง 27 กรกฎาคม - 1 สิงหาคม 2557 ณ เมือง มอนทรีออน ประเทศแคนาดา

4. **Limtong, S., J. Surussawadee** and **N. Srisuk.** Diversity of cultivable yeast in sugarcane phyllosphere in Thailand, a tropical country. เสนอ ใน ISSY 32 International Specialized Symposium on Yeasts biodiversity and Biotechnology in the Twenty-first Century ระหว่าง 13 - 17 กันยายน 2558 ณ เมือง Perugia ประเทศอิตาลี

5. **Tantirungkij, M., R. Nasanit** and **S. Limtong.** Diversity of culture-independent endophytic yeasts from sugarcane leaves in Thailand (ดร.มณี ตันติรุ่งกิจ นำเสนอ) เสนอ ใน ISSY 32 เสนอ ใน ISSY 32 International Specialized Symposium on Yeasts Biodiversity and Biotechnology in the Twenty-first Century ระหว่าง 13 - 17 กันยายน 2558 ณ เมือง Perugia ประเทศอิตาลี

6. **Nasanit, R., S. Jaibangyang, M. Tantirungkij** and **S. Limtong.** Diversity of epiphytic yeasts in phyllosphere of corn in Thailand by culture-independent approach (ดร. รุจิกาญจน์ นาสนิท นำเสนอ) เสนอ ใน ISSY 32 เสนอ ใน ISSY 32 International Specialized Symposium on Yeasts Biodiversity and Biotechnology in the Twenty-first Century ระหว่าง 13 - 17 กันยายน 2558 ณ เมือง Perugia ประเทศอิตาลี



International Union of Microbiological Societies Congresses

July 27 – August 1, 2014

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27 juillet – 1er août 2014

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XIVe Congrès international de bactériologie and Applied Microbiology | XIVe Congrès international de mycologie et microbiologie eukaryote | XVIe Congrès international de virologie

ABSTRACTS
RÉSUMÉS

Organized with the Canadian Society of Microbiologists and the National Research Council of Canada
Organisé avec la Société canadienne des microbiologistes et le Conseil national de recherches du Canada

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Poster Session

MEM-PT3011 - Diversity of endophytic yeasts in sugarcane leaves in Thailand

Savitree Limtong[1], Pannida Khunnamwong[1]
[1]*Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok, Thailand*

Endophytic microorganism have been defined as the microorganism that reside the whole or part of their life cycle inside plant tissues without causing any damage or showing negative effect on host plant. This work aimed to study the diversity of endophytic yeasts in tissues of sugarcane leaf in Thailand. One hundred and two samples of sugarcane leaf were collected from thirty-two sampling sites in 12 provinces during January to April 2012. These 12 provinces were in the central and north-eastern parts of Thailand, where the average maximum temperature was 32-36°C and average minimum temperature was 22-25°C throughout the year. Endophytic yeasts were isolated from surface-sterilized sugarcane leaves and 130 isolates were obtained from 83 sugarcane leaf samples. On the basis of the D1/D2 region of the large subunit rRNA gene sequence analysis, 114 strains were identified to 27 known species in 12 genera belonging to Basidiomycota *viz. Bullera coprosmaensis, Bullera japonica, Bullera pseudoalba, Bullera sinensis, Cryptococcus cellulolyticus, Cryptococcus flavescens, Cryptococcus flavus, Cryptococcus laurentii, Cryptococcus nemorosus, Cryptococcus rajasthanensis, Dioszegia takashimae, Jaminaea angkoriensis, Occultifur externus, Pseudozyma antarctica, Pseudozyma aphidis, Pseudozyma hubeiensis, Pseudozyma prolifica, Pseudozyma rugulosa, Pseudozyma tsukubaensis, Rhodosporidium toruloides, Rhodotorula marina, Rhodotorula minuta, Rhodotorula mucilaginosa, Sporidiobolus pararoseus, Sporobolomyces vermiculatus, Tremella globispora* and *Trichosporon asahii* and six species in four genera of Ascomycota *viz. Candida tropicalis, Cyberlindnera rhodanensis, Meyerozyma caribbica, Meyerozyma guilliermondii, Pichia kudriavzevii and Pichia rhodanensis*. Eight strains had similar or identical D1/D2 region sequences with the five undescribed species in the genus *seudozyma, Cryptococcus* and *Sporobolomyces*. Eight strains represented three new yeast species in the genus *Occultifur, Wickerhamiella and Tremella*. The most prevalent species was *Meyerozyma guilliermondii* with a 16% frequency of occurrence followed by *Cryptococcus flavescens* (10%) and then *Pichia kudriavzevii* and *Pseudozyma aphidis*, each with a frequency of occurrence of 6%.

Diversity of endophytic yeasts in sugarcane leaves in Thailand

Savitree Limtong[1] (fscistl@ku.ac.th), Pannida Khunnamwong[1], Sasitorn Jindamorakot[2]

[1] Department of Microbiology Faculty of Science, Kasetsart University, Thailand;
[2] National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand

Introduction

Endophytic microorganisms are microorganisms that colonize healthy plant tissues without causing any damage or developing external structure on host plant (Petrini, 1991). Endophytic fungi in leaf tissue have been extensive study during the past 30 years and evidence has shown that fungal endophytes are ubiquitous in tissue of all plant species (Hyde and Soytong 2008). However few studies of endophytic yeasts in leaf tissue have been carried out. Therefore, we had conducted a research to investigate the diversity of endophytic yeasts in leaf tissue of sugarcane in Thailand, due to the fact that sugarcane is the second most important agronomic crop in Thailand.

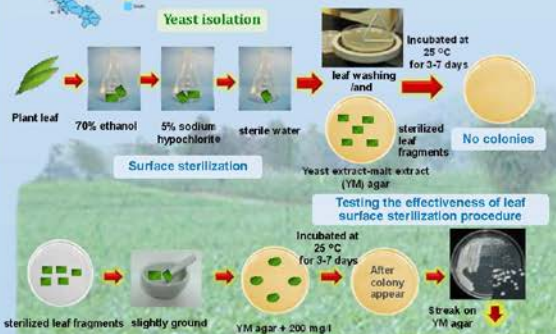
Methods

Sample collection



Province that sample were collected
Buri Ram
Nakhon Ratchasima
Nakhon Pathom
Suphan Buri
Kanchanaburi
Chai Nat
Nakhon Sawan
Phichit
Phitsanulok
Phetchabun
Lop Buri
Saraburi

Yeast isolation



Yeast identification

Analysis of the D1/D2 domain of the large subunit (LSU) rRNA gene sequences similarity



- D1/D2: ca. 600 nucleotides at the 5' end of the LSU rRNA gene
- Two rapidly evolution regions and account for most of the sequence divergence between LSU rRNA gene

Guideline of Kurtzman and Robnett (1998) that yeast strains with 0–3 nucleotide differences are conspecific or sister species and strains showing > 6 nt substitutions are different species

Phylogenetic analysis

Phylogenetic tree was constructed from the evolutionary distance data with Kimura's two parameter correction (Kimura, 1980), using the neighbor joining method (Saitou & Nei, 1987) and the MEGA software version 5.03 (Tamura *et al.*, 2011). Confidence levels of the clades were estimated from bootstrap analysis (1000 replicates) (Felsenstein, 1985).

References

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Hyde KD, Soytong K (2008). Fungal Divers 33: 163-167.
Kimura M (1980). J Mol Evol 16: 111-120.
Manamgoda DS, et al. (2013). Fungal Divers 61:107-115
Petrini, O (1991). Fungal endophytes of tree leaves. In: Andrews, J.H., Hirano S.S., (eds) Microbial Ecology of the Leves Springer-Verlag, New York. pp. 179-197.
Saitou N, Nei M (1987). Mol Biol Evol 4: 406–425.
TamuraK, et al. (2011). Mol Biol Evol 28: 2731–2739.

Results

One hundred and two samples of sugarcane leaf were collected from thirty-two sampling sites in 12 provinces during January to April 2012 (Fig. 1). These 12 provinces were in the central and north-eastern parts of Thailand, where the average maximum temperature was 32-36°C and average minimum temperature was 22-25°C throughout the year. Endophytic yeasts were isolated from surface-sterilized sugarcane leaves and 130 isolates were obtained from 83 sugarcane leaf samples.

All strains were identified n the basis of the D1/D2 region of the large subunit rRNA gene sequence analysis and result showed that 114 strains were identified as known yeast species (Table 1, 2).

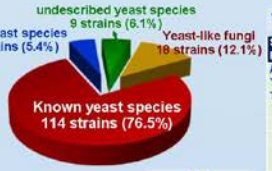


Fig. 1 Identification

The 114 strains of know yeast species were identified as 27 known species in 12 genera belonging to Basidiomycota (Table 1) and six species in four genera of Ascomycota (Table 2)

Table 1 Known species in phylum Basidiomycota

Subphylum/Class/Order/ Family	Species	No of strain
Agaricomycotina/ Tremellomycetes/ Tremellales/Tremellaceae	*Bullera coprosmaensis*	4
	Bullera pseudoalba	1
	Bullera japonica	4
	Dioszegia takashimae	1
	Cryptococcus laurentii	2
	Cryptococcus cellulolyticus	3
	Cryptococcus flavescens	11
	Cryptococcus flavus	5
	Cryptococcus nemorosus	3
	Cryptococcus rajasthanensis	2
	Tremella globispora	2
	Trichosporon asahii	1
Agaricomycotina/ Tremellomycetes/Tremellales	*Hannaella sinensis*	1
Pucciniomycotina/	*Occultifur externus*	4
Cystobasidiomycetes/	*Rhodotorula minuta*	1
Erythrobasidiales/	*Rhodotorula marina*	1
Erythrobasidiaceae	*Sporobolomyces vermiculatus*	2
Pucciniomycotina/	*Rhodosporidium toruloides*	2
Microbotryomycetes/	*Rhodotorula mucilaginosa*	4
Sporidiobolales	*Sporidiobolus pararoseus*	3
Ustilaginomycotina/ Exobasidiomycetes/ Microstromales	*Jaminaea angkoriensis*	1
Ustilaginomycotina/	*Pseudozyma antarctica*	5
Ustilaginomycetes/	*Pseudozyma aphidis*	7
Ustilaginales/	*Pseudozyma hubeiensis*	1
Ustilaginaceae	*Pseudozyma prolifica*	4
	Pseudozyma rugulosa	3
	Pseudozyma tsukubaensis	1

Table 2 Known species in phylum Asomycota

Class/ Order/ Family	Species	Strains
Saccharomycetes/ Saccharomycetales	*Candida tropicalis*	5
Saccharomycetes/ Saccharomycetales/ Wickerhamomycetaceae	*Cyberlindnera rhodanensis*	1
Saccharomycetes/ Saccharomycetales/ Debaryomycetaceae	*Meyerozyma caribbica*	20
Saccharomycetes/ Saccharomycetales/ Pichiaceae	*Pichia kudriavzevii*	6
Saccharomycetes/ Saccharomycetales/ Pichiaceae	*Pichia rhodanensis*	3



Fig. 2 Frequency of occurrence

The most prevalent species was *Meyerozyma caribbica* with 17.5% frequency of occurrence followed by *Cryptococcus flavescens* (9.6%), *Pseudozyma aphidis* (6.1%) and *Pichia kudriavzevii* (5.3%) (Fig. 2).

Eight strains had similar or identical D1/D2 region sequences with the five undescribed species in three genera *viz. Pseudozyma*, *Cryptococcus* and *Sporobolomyces*. Eight strains represented three new yeast species in the three genera *viz. Wickerhamiella*, *Occultifur* and *Tremella* (Table 3).

Table 3 Identification of new yeast species based on the analysis of the D1/D2

Strain	Closest Species	Nucleotide Similarity %	Substitution / total	Substitution %	Result of identification
DMKU-SE106	*Candida infanticola* NRRL Y-17858[T] (DQ438230)	94.9	25/556	4.5	New species in *Wickerhamiella* clade
DMKU-SE110	*Candida infanticola* NRRL Y-17858[T] (DQ438230)	94.9	25/556	4.5	
DMKU-SE112	*Candida infanticola* NRRL Y-17858[T] (DQ438230)	94.7	26/556	4.6	
DMKU-SE132	*Candida infanticola* NRRL Y-17858[T] (DQ438230)	94.7	26/556	4.6	
DMKU-SE38	*Occultifur externus* CBS 8732[T] (AY745723)	98.5	8/563	1.7	New species of the genus *Occultifur*
DMKU-SE59	*Occultifur externus* CBS 8732[T] (AY745723)	98.4	8/599	1.5	
DMKU-SE89	*Tremella globispora* CBS 6972 (AF189869)	98.6	9/638	1.6	New species of the genus *Tremella*
DMKU-SE147	*Tremella globispora* CBS 6972 (AF189869)	97.6	12/567	2.1	

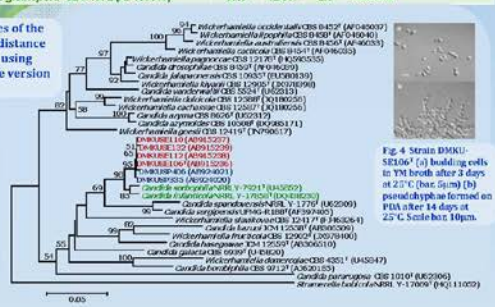


Fig. 3 Phylogenetic tree based on the sequences of the D1/D2 region, constructed from evolutionary distance data with Kimura's two-parameter correction, using the neighbor joining method by MEGA software version 5.03.

Fig. 4 Strain DMKU-SE106[T] (a) budding cells in YM broth after 3 days at 25°C (bar, 5μm) (b) pseudohyphae formed on PDA after 14 days at 25°C. Scale bar, 10μm.

Acknowledgements

This work was supported by the Thailand Research Fund through the TRF Research-Team Promotion Grant (RTA5480009), the Royal Golden Jubilee Ph.D. program grant no. PHD/0128/2554 and the Higher Education Research Promotion and National Research University Project of Thailand. Travel grant for the presenting author was supported by the TRF and KURDI, Kasetsart University.

Poster Session

MEM-PT3016 - Diversity of Epiphytic Yeasts on Rice Leaves in Thailand

Sasitorn Jindamorakot[1], Somjit Am-In[1], Savitree Limtong[2]
[1]*National Center for Genetic Engineering and Biotechnology, Pathumthani, Thailand,* [2]*Department of Microbiology, Facculty of Science, Kasetsart University, Bangkok, Thailand*

Three hundred and seventy nine isolates of epiphytic yeasts were isolated from 112 rice leaves from 12 provinces in Thailand. Among them, 354 isolates were identified based on the sequences of D1/D2 domain of 26S rDNA. Two hundred and ninety eight isolates (78.6%) were identified as 50 known species in 18 genera of Bullera (18 isolates), Candida (24 isolates), Cryptococcus (22 isolates), Debaryomyces (2 isolates), Hyphopichia (1 isolates), Jaminaea (1 isolates), Kodamaea (8 isolates), Lodderomyces (1 isolates), Meyerozyma (10 isolates), Pichia (3 isolates), Pseudozyma (125 isolates), Rhodosporidium (10 isolates), Rhodotorula (30 isolates), Sporidiobolus (5 isolates), Sporobolomyces (27 isolates). Torulaspora (2 isolates), Trichosporon (4 isolates) and Wickerhamomyces (5 isolates). Twenty one isolates (5.5%) were identified as undescribed species in Candida (1 isolate), Cryptococcus (9 isolates), Pseudozyma (4 isolates) and Rhodotorula (7 isolates). Furthermore, thirty five isolates (9.2%) differed from the nearest species in 4 nucleotides or more in the D1/D2 domain and were considered to represent 20 new species of Blastobotrys (1 isolate), Bullera (2 isolates), Candida (1 isolate), Cryptococcus (13 isolates), Occultifur (2 isolates), Pseudozyma (8 isolates), Rhodosporidium (4 isolates), Rhodotorula (3 isolates) and Sakaguchia (1 isolates) and the remaining 25 isolates (6.6%) are wating for identify. Yeast-like fungi in the genera Aureobasidium, Acremonium, Cephalosporium, Hyponectria, Melanopsichium, Moesziomyces, Sarocladium, Sporisorium, Syncephalastrum and Ustilago also found on the rice leaves. These epiphytic yeasts will be screened for promising auxins producing strains emphasize on indole-3-acetic acid auxin.

Diversity of Epiphytic Yeasts on Rice Leaves in Thailand

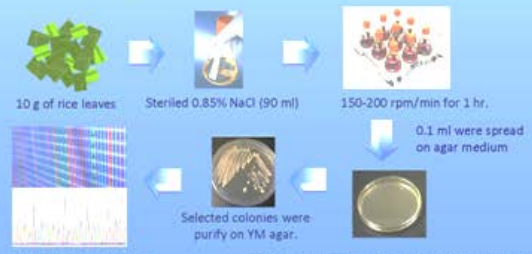
Sasitorn Jindamorakot[1], Somjit Am-In[1] and Savitree Limtong[2]

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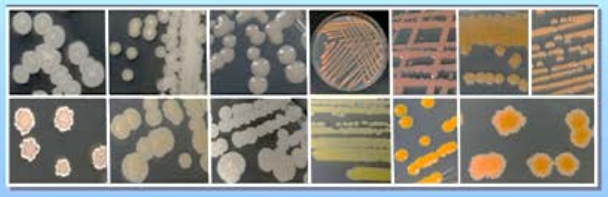
Abstract

Three hundred and seventy nine isolates of epiphytic yeasts were isolated from 112 rice leaves from 12 provinces in Thailand. Among them, 354 isolates were identified based on the sequences of D1/D2 domain of 26S rDNA. Two hundred and ninety eight isolates (78.6%) were identified as 50 known species in 18 genera of *Bullera* (18 isolates), *Candida* (24 isolates), *Cryptococcus* (22 isolates), *Debaryomyces* (2 isolates), *Hyphopichia* (1 isolate), *Jaminaea* (1 isolate), *Kodamaea* (1 isolate), *Lodderomyces* (1 isolate), *Meyerozyma* (10 isolates), *Pichia* (3 isolates), *Pseudozyma* (125 isolates), *Rhodosporidium* (10 isolates), *Rhodotorula* (30 isolates), *Sporidiobolus* (5 isolates), *Sporobolomyces* (27 isolates), *Torulaspora* (2 isolates), *Trichosporon* (4 isolates), and *Wickerhamomyces* (5 isolates). Twenty-one isolates (5.5%) were identified as undescribed species in *Candida* (1 isolate), *Cryptococcus* (9 isolates), *Pseudozyma* (4 isolates) and *Rhodotorula* (7 isolates). Furthermore, thirty five isolates (9.2%) differed from the nearest species in 4 nucleotides or more in the D1/D2 domain and were considered to represent 20 new species of *Blastobotrys* (1 isolate), *Bullera* (2 isolates), *Candida* (1 isolate), *Cryptococcus* (13 isolates), *Occultifur* (2 isolates), *Pseudozyma* (8 isolates), *Rhodosporidium* (4 isolates), *Rhodotorula* (3 isolates) and *Sakaguchia* (1 isolate) and the remaining 25 isolates (6.6%) are waiting for identify. Yeast-like fungi in the genera *Aureobasidium*, *Acremonium*, *Cephalosporium*, *Hyponectria*, *Melanopsichium*, *Moesziomyces*, *Sarocladium*, *Sporisorium*, *Syncephalastrum* and *Ustilago* also found on the rice leaves. These epiphytic yeasts will be screened for potential auxins producing strains emphasize on indole-3-acetic acid auxin.

Materials and Methods



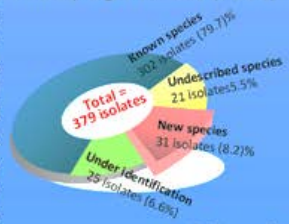
- Epiphytic yeasts isolation: YM + 0.02% chloramphenical, incubation at 25°C for 2-3 days.
- Epiphytic fungi count: PDA + 0.02% chloramphenical, incubation at 25°C for 5 days.
- Epiphytic bacteria count: PCA, incubation at 25°C for 2 days.



Group	No. of identified isolates	Known species	New species	Undescribed species	Pending for identify
Ascomycetous	58 (16%)	56 isolates	1 isolates	1 isolates	?
		19 species	1 species	1 species	
		9 genera	1 genera	1 genera	
Basidiomycetous	296 (84%)	242 isolates	34 isolates	20 isolates	?
		31 species	19 species	9 species	
		9 genera	8 genera	3 genera	
Total	**354**	**298 isolates**	**35 isolates**	**21 isolates**	**25 isolates**

Results

Number of epiphytic yeasts: $1.9x10^3$-$5.3x10^7$ CFU/g rice leave. Three hundred and fifty four isolates were isolated and identified based on the sequences of D1/D2 domain of 26S rDNA. Among them, 84 % were classified to Basidiomycetous yeasts and 16% were ascomycetous yeast. Two hundred and ninety eight isolates (78.6%) were identified as 50 known species in 18 genera, 21 siolates (5.5%) were identified as undescribed species, furthermore, 35 isolates (9.2%) differed from the nearest species in 4 nucleotides or more in the D1/D2 domain and were considered to represent ca. 20 new species and the remaining 25 isolates (6.6%) are waiting for identify.



Conclusions

Three hundred and fifty four isolates were isolated and identified as 50 known species of 18 genera, 35 isolates were identified as 20 new species of 9 genera which were polyphasic taxonomy to confirm their taxonomy and propose as novel species. *Pseudozyma antarctica* is the dominant species and fallow by *Pseudozyma rugulosa*. Phylloplane in Thailand are a good habitat for yeasts, many new species and undescribed species are widely distributed in various places of Thailand.

Acknowledgement

This work was supported by Thailand Research Fund (TRF), Kasetsart University and National Center for Genetic Engineering and Biotechnology (BIOTEC)

Poster Session

MEM-PT3013 - Diversity of yeasts in corn phylloplanes and their antagonistic activities against pathogenic fungi

Rungluk Kaewwichian[1,2], Wichien Yongmanitchai[1], Savitree Limtong[1]
[1]Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok, Thailand, [2]National Center for Genetic Engineering and Biotechnology, PathumThani, Thailand

One hundred and twenty-six yeast strains were isolated by the enrichment technique from the phylloplane of 65 corn leaf samples collected from eight districts in four provinces (Lop Buri, Nakhon Ratchasima, Nakhon Sawan and Phetchabun) of Thailand. On the basis of the D1/D2 domain of the large subunit rRNA gene sequences analysis and phylogeny 121 strains were identified as 38 known species in 17 genera belonging to Ascomycota i.e. Candida catenulate, Candida conglobate, Candida glabrata, Candida mesorugosa, Candida orthopsilosis, Candida pseudointermedia, Candida quercitrusa, Candida rugosa, Candida sojae, Candida sorboxylosa, Candida tropicalis, Cyberlindnera rhodanensis, Geotrichum phurueaensis, Hanseniaspora guilliermondii, Hanseniaspora opuntiae, Hanseniaspora thailandica, Kluyveromyces marxianus, Kodamaea ohmeri, Meyerozyma caribbica, Meyerozyma guilliermondii, Millerozyma acaciae, Millerozyma koratensis, Pichia galeiformis, Pichia kluyveri, Pichia kudriavzevii, Torulaspora globosa, Wickerhamomyces edaphicus and Yamadazyma philogaea and Basidiomycota i.e. Cryptococcus flavescens, Cryptococcus heveanensis, Cryptococcus laurentii, Rhodosporidium fluviale, Rhodosporidium paludigenum, Rhodosporidium toruloides, Rhodotorula taiwanensis, Sporidiobolus pararoseus, Sporidiobolus ruineniae and Sporobolomyces nylandii. Two strains had identical sequences with an undescribed Candida species the other two strains were identical with Cryptococcus aff. laurentii. One strain represented a novels species in the genus Yamadazyma. The prevalent species were Kodamaea ohmeri, Meyerozyma caribbica and Rhodotorula taiwanensis, each of which having a frequency of occurrence of 7.9%, followed by Candida tropicalis, Hanseniaspora opuntiae, Pichia kudriavzevii and Rhodosporidium fluvial, each with a frequency of occurrence of 6.4%, and then Cryptococcuslaurentii (5.6%). Only one strain namely Torulaspora globosa DMKU-RK547 significantly inhibited Fusarium moniliforme, Rhizoctonia solani and Helminthosporium oryzae, which were pathogenic fungi of rice and its biocontrol activity seem to be based on the production of siderophore and volatile compounds. The nutrient concentrations had significant different effect on fungal growth inhibition by T. globosa DMKU-RK547 and its living cells are necessary for the biological control activity.

Diversity of yeasts in corn phylloplanes and their antagonistic activities against pathogenic fungi

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Abstract

One hundred and twenty-six yeast strains were isolated by the enrichment technique from the phylloplane of 65 corn leaf samples collected from eight districts in four provinces (Lop Buri, Nakhon Ratchasima, Nakhon Sawan and Phetchabun) of Thailand. On the basis of the D1/D2 domain of the large subunit rRNA gene sequences analysis and phylogeny 121 strains were identified as 38 known species in 17 genera belonging to Ascomycota i.e. *Candida catenulate*, *Candida conglobate*, *Candida glabrata*, *Candida mesorugosa*, *Candida orthopsilosis*, *Candida pseudointermedia*, *Candida quercitrusa*, *Candida rugosa*, *Candida sojae*, *Candida sorboxylosa*, *Candida tropicalis*, *Cyberlindnera rhodanensis*, *Geotrichum phurueaensis*, *Hanseniaspora guilliermondii*, *Hanseniaspora opuntiae*, *Hanseniaspora thailandica*, *Kluyveromyces marxianus*, *Kodamaea ohmeri*, *Meyerozyma caribbica*, *Meyerozyma guilliermondii*, *Millerozyma acaciae*, *Millerozyma koratensis*, *Pichia galeiformis*, *Pichia kluyveri*, *Pichia kudriavzevii*, *Torulaspora globosa*, *Wickerhamomyces edaphicus* and *Yamadazyma philogaea* and Basidiomycota i.e. *Cryptococcus flavescens*, *Cryptococcus heveanensis*, *Cryptococcus laurentii*, *Rhodosporidium fluviale*, *Rhodosporidium paludigenum*, *Rhodosporidium toruloides*, *Rhodotorula taiwanensis*, *Sporidiobolus pararoseus*, *Sporidiobolus ruineniae* and *Sporobolomyces nylandii*. Two strains had identical sequences with an undescribed *Candida* species the other two strains were identical with *Cryptococcus* aff. *laurentii*. One strain represented a novels species in the genus *Yamadazyma*. The prevalent species were *Kodamaea ohmeri*, *Meyerozyma caribbica* and *Rhodotorula taiwanensis*, each of which having a frequency of occurrence of 7.9%, followed by *Candida tropicalis*, *Hanseniaspora opuntiae*, *Pichia kudriavzevii* and *Rhodosporidium fluvial*, each with a frequency of occurrence of 6.4%, and then *Cryptococcus laurentii* (5.6%). Only one strain namely *Torulaspora globosa* DMKU-RK547 significantly inhibited *Fusarium moniliforme*, *Rhizoctonia solani* and *Helminthosporium oryzae*, which were pathogenic fungi of rice and its biocontrol activity seem to be based on the production of siderophore and volatile compounds. The nutrient concentrations had significant different effect on fungal growth inhibition by *T. globosa* DMKU-RK547 and its living cells are necessary for the biological control activity.

Introduction

The external surfaces of plant leaves, often referred to the phylloplane or phyllosphere, have been recognized as an important habitat for microorganisms. Thailand is agricultural country and rice, corn and sugarcane are the important agricultural crops. Though there were some investigations on yeast associate with plants but the investigations were conducted mainly with flowers and fruits of non-agricultural plants and almost no work was focused on yeast associate with surface of leaves of agricultural crops. Although application of fungicides is still the effective method to control plant diseases cause by fungi. The wide spread use of the chemical fungicides has become a subject of concern due to their harmful effect on non-target organisms. Biological control, which is the use of living agents to control plant diseases, is increasing considered to be a reliable alternative to chemical fungicides. Among the organisms to be studied for their biological control activities yeast is one of the promising biological control agent. Therefore we are interesting to investigate diversity of yeast on phylloplane of corn and to determine their biocontrol mechanisms on plant pathogenic fungi.

Materials & Methods

❖ Sample collection & Yeast Isolation



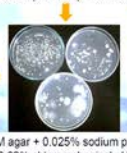
Green and healthy leaves of corn (*Zea mays* Linn.) were random collected from corn fields.



3 g of cut leaves



YM broth + 0.025% sodium propionate + 0.02% chloramphenicol pH 3.7-3.8



YM agar + 0.025% sodium propionate + 0.02% chloramphenicol pH 3.7-3.8



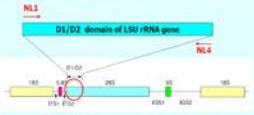
Purification on YM agar



Freezing at -80 °C

❖Yeast Identification

❖ Analysis of the D1/D2 domain of the large subunit (LSU rRNA) gene sequence similarities (Kurtzman and Robnett 1998)



❖ Phylogenetic analysis (Kimura, 1980; Felsenstein, 1985; Saitou & Nei, 1987)

❖ Evaluation of the in vitro antagonistic activity against plant pathogenic fungi of phylloplane yeasts

Method: dual cultivation (Rosa *et al*., 2010) on PDA, incubated at 25°C for 7 days

Rice pathogenic fungi
- *Fusarium moniliforme*
- *Helminthosporium oryzae*
- *Rhizoctonia solani*

Corn pathogenic fungi
- *Bipolaris maydis*
- *Bipolaris zeicola*
- *Exserohilium turcica*
- *Fusarium moniliforme*
- *Macrophomina phaseolina*



❖Evaluation of the in vitro antagonistic mechanism

- ❖ Evaluation of antagonism in solid medium
- ❖ Production of antifungal volatile compounds
- ❖ Siderophores production
- ❖ Detection of killer activity
- ❖ Production of hydrolytic enzymes (β-glucanase and chitinase)
- ❖ Effect of nutrient concentration on yeast-fungal pathogen interaction
- ❖ Effect of yeast cell suspension, culture filtrate and autoclaved yeast cell suspension on pathogenic fungal growth

Conclusion

- ❖ High diversity of yeast on phylloplane of corn were observed.
- ❖ Both ascomycete and basidiomycete yeasts were detected, though higher frequency of isolation was ascomycete yeast, which may due to enrichment isolation technique was used.
- ❖ *Torulaspora globosa* DMKU-RK547 significatly inhibited *Fusarium moniliforme*, *Rhizoctonia solani*, *Helminthosporium oryzae*, which are the rice pathogenic fungi.

Acknowledgement

This work was supported by the Thailand Research Fund through Royal Golden Jubilee Ph.D. Program (PHD/0215/2551) and TRF Research-Team Promotion Grant (RTA5480009).

Results

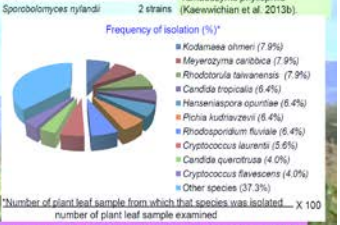
➢ Sample collection and yeast isolation

Sixty-five samples of corn leaf were collected from eight sampling sites in four provinces in Thailand between September 2009 and May 2010. Yeasts were isolated by the enrichment technique, and 126 isolates were obtained.

➢ Yeast identification and diversity of yeasts in corn phylloplanes

Ascomycota		Basidiomycota	
28 species in 12 genera		10 species in 5 genera	
Candida catenulata	1 strain	*Cryptococcus flavescens*	5 strains
Candida conglobata	1 strain	*Cryptococcus heveanensis*	1 strain
Candida glabrata	2 strains	*Cryptococcus laurentii*	7 strains
Candida pseudointermedia	3 strains	*Rhodosporidium fluviale*	8 strains
Candida quercitrusa	5 strains	*Rhodosporidium paludigenum*	3 strains
Candida tropicalis	8 strains	*Rhodosporidium toruloides*	2 strains
Candida mesorugosa	1 strain	*Rhodotorula taiwanensis*	10 strains
Candida orthopsilosis	2 strains	*Sporidiobolus pararoseus*	1 strain
Candida rugosa	1 strain	*Sporidiobolus ruineniae*	1 strain
Candida sojae	1 strain	*Sporobolomyces nylandii*	2 strains
Candida sorboxylosa	1 strain		
Cyberlindnera rhodanensis	1 strain		
Geotrichum phurueaensis	1 strain		
Hanseniaspora guilliermondii	2 strains		
Hanseniaspora opuntiae	8 strains		
Hanseniaspora thailandica	2 strains		
Kluyveromyces marxianus	1 strain		
Kodamaea ohmeri	10 strains		
Meyerozyma caribbica	10 strains		
Meyerozyma guilliermondii	3 strains		
Millerozyma acaciae	1 strain		
Millerozyma koratensis	1 strain		
Pichia galeiformis	2 strains		
Pichia kluyveri	1 strain		
Pichia kudriavzevii	8 strains		
Torulaspora globosa	1 strain		
Wickerhamomyces edaphicus	2 strains		
Yamadazyma philogaea	1 strain		

Among four strains of undescribed species two strains had identical sequences of the D1/D2 region with an undescribed species which the sequence has been deposit in GenBank, *Candida* sp. 05-7-186T, and two strains were identical with *Cryptococcus* aff. *laurentii* APSS 823.One strain representing one new yeast species was in the genus *Yamadazyma* in Asomycota and previously proposed as *Yamadazyma phyllophila* (Kaewwichian et al. 2013b).



*Number of plant leaf sample from which that species was isolated / number of plant leaf sample examined X 100

➢ Evaluation of the in vitro antagonistic activity against plant pathogenic fungi of phylloplane yeasts



Table 1 Mycelium growth inhibition of *Fusarium moniliforme*, *Rhizoctonia solani* and *Helminthosporium oryzae* by *Torulaspora globosa* DMKU-RK547 by dual cultivation on PDA at 25°C for 7 days

	Diameter of fungal colony (cm)*	% Inhibition
Fusarium moniliforme	6.50±0.10	-
DMKU-RK547 + *Fusarium moniliforme*	4.63±0.06	28.72
Rhizoctonia solani	5.90±0.10	-
DMKU-RK547 + *Rhizoctonia solani*	4.17±0.06	29.38
Helminthosporium oryzae	5.10±0.10	-
DMKU-RK547 + *Helminthosporium oryzae*	4.50±0.10	11.76

* Data were averaged from three replicates.

Fig. 1 Mycelium growth inhibition of *Fusarium moniliforme* (A), *Rhizoctonia solani* (B) and *Helminthosporium oryzae* (C) by *Torulaspora globosa* DMKU-RK547 by dual cultivation on PDA at 25°C for 7 days.

➢ Evaluation of the in vitro antagonistic mechanism

Only one strain namely *Torulaspora globosa* DMKU-RK547 significantly inhibited the three pathogenic fungi of rice and its biocontrol activity seem to be based on the production of siderophore and volatile compounds. The nutrient concentrations had significant different effect on fungal growth inhibition by *T. globosa* DMKU-RK547 and its living cells are necessary for the biological control activity.

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Diversity of cultivable yeast in sugarcane phyllosphere in Thailand, a tropical country

Savitree Limtong[1,2], Janjira Surussawadee[1], Nantana Srisuk[1,2]

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The phyllosphere, which is usually referred to the external surface of plant leaf, has been recognized as an important habitat for epiphytic microorganisms that are capable of surviving, growing and reproducing in it (Fonseca and Inacio 2006). This study aimed to investigate the diversity of cultivable yeast in the phyllosphere of sugarcane in Thailand. Yeasts were isolated by plating of leaf washing from 84 leaf samples and 262 strains that revealed yeast type colonies were collected. On the basis of sequence analysis of the D1/D2 region of the large subunit (LSU) rRNA gene or the D1/D2 and internal transcribed space (ITS) regions, 168 strains were found to be yeasts while 94 strains were fungi. Among yeasts strains, 83 strains were identified to be 24 known species in 11 genera of Basidiomycota *viz. Bullera sinensis*, *Cryptococcus flavescens*, *C. flavus*, *C. heveanensis*, *C. rajasthanensis*, *Dioszegia zsoltii*, *Hannaella pagnoccae*, *Occutifur externus*, *Pseudozyma alboarmeniaca*, *P. aphidis*, *P. hubeiensis*, *P. jejuensis*, *P. rugulosa*, *P. siamensis*, *P. vetiver*, *Rhodotorula benthica*, *R. mucilaginosa*, *R. taiwanensis*, *Rhodosporidium paludigenum*, *Sporobolomyces blumeae*, *S. carnicolor*, *S. vermiculatus*, *Jaminaea angkoriensis* and *Tremella globispora*, and four species in three genera of Ascomycota *viz.* and *Candida parapsilosis*, *C. nymphaea*, *Kodamaea ohmeri* and *Meyerozyma caribbica*. As many as 85 strains represented new or may be new yeast species. Seventeen strains were confirmed to be 11 new species in seven genera *viz. Cryptococcus* (2 species), *Hannaella* (2 species), *Occultifur* (1 species), *Papiliotrema* (1 species), *Pseudozyma* (2 species), *Rhodotorula* (1 species), *Tremealla* (1 species) and *Wickerhamilla* (1 species). Sixty-eight strains were possible to be new yeast species in the phylum Basidiomycota, however, further analysis is needed. Number of basidiomycetous yeast strains and species were found to be higher than that of ascomycetous strains and species. The most prevalent species among known species was *M. caribbica* with a 22.9% frequency of occurrence.

KEYWORDS: Yeast, Phyllosphere, Sugarcane, Thailand, *Meyerozyma caribbica*

REFERENCES:

Fonseca A, Inacio J (2006.) Phylloplane yeasts. In: Rosa C, Peter G (eds) Biodiversity and ecophysiology of yeasts. Springer-Verlag, Berlin Heidelberg 263–301

Table 1 Strains confirmed to be new yeast species in phylum Basidiomycota

Strain	Closest Species (GenBank Accession number)	% nt substitution	
		D1/D2	ITS
DMKU-SP67	*Cryptococcus laurentii* (AF075469)	2.1	1.7
DMKU-SP105	*Cryptococcus laurentii* (AF075469)	1.3	4.6
DMKU-SP423	*Cryptococcus laurentii* (AF075469)	2.2	3.5
DMKU-SP85	*Cryptococcus nemorosus* (AF472625)	1.2	2.5
DMKU-SP56	*Hannaella coprosmaensis* (AF363660)	5.5	0.8
DMKU-SP385	*Occultifur externus* (AF131062)	1.7	3.4
DMKU-SP71	*Pseudozyma churashimaensis* (AB548955)	1.5	0.5
DMKU-SP76	*Pseudozyma rugulosa* (AJ235300)	1.2	0.6
DMKU-SP427	*Rhodotorula calyptogenae* (AB025996)	0.4	5.6
DMKU-SP200	*Rhodotorula marina* (AF189944)	1.1	4.3
DMKU-SP213	*Rhodotorula marina* (AF189944)	0.9	3.5
DMKU-SP273	*Rhodotorula marina* (AF189944)	1.3	3.2
DMKU-SP322	*Rhodotorula marina* (AF189944)	1.1	3.5
DMKU-SP332	*Rhodotorula marina* (AF189944)	0.9	3.5
DMKU-SP23	*Tremella globispora* (AF189869)	1.8	3.3
DMKU-SP40	*Tremella globispora* (AF189869)	1.7	3.5
DMKU-SP403	*Tremella globispora* (AF189869)	1.3	3.4

Diversity of Cultivable Yeast in Sugarcane Phyllosphere in Thailand, a Tropical Country

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Introduction:

The phyllosphere, which is usually referred to the external surface of plant leaf, has been recognized as an important habitat for epiphytic microorganisms that are capable of surviving, growing and reproducing in it (1). There were only a few reports on the diversity of yeasts in phyllosphere of plants in Thailand, which is in tropical region. Recently, our group studied the diversity of sugarcane phyllosphere because sugarcane is the second most important crop in Thailand. The study was based on enrichment isolation and resulted in higher number of ascomycete yeasts than basidiomycete yeasts (2), which is in contrast to the reported of studies in the countries in temperate zone. Therefore, we carried out this work to check the different.

Objective:

This study aimed to investigate the diversity of cultivable yeast in the phyllosphere of sugarcane in Thailand based on plating of leaf washing.

Methods:

Yeast isolation from surface of plant leaves by plating of leaf washing:



Yeast identification



- Analysis of the D1/D2 region of the large subunit (LSU) rRNA gene and internal transcribe spacer (ITS) region sequence similarity
- Phylogenetic analysis based on the D1/D2 region of the LSU rRNA gene

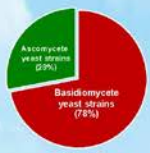


Fig. 4

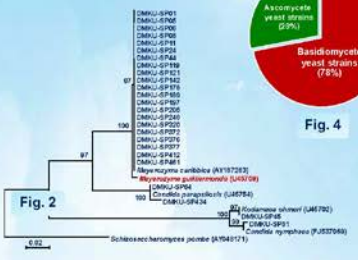


Fig. 2

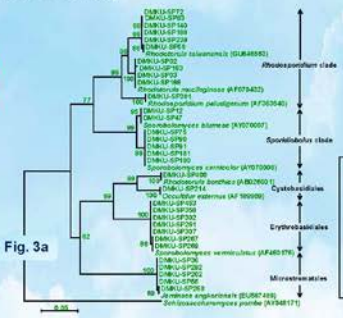


Fig. 3a

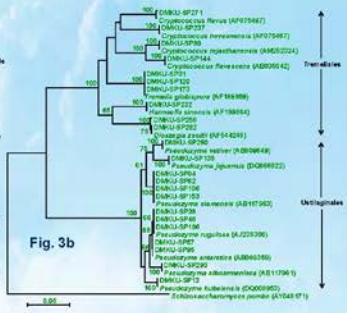


Fig. 3b

Results:

- Isolation of yeast from surface of 84 sugarcane leaf samples resulted in collection of 262 yeast type colonies.
- Identification revealed that 168 strains were yeasts and 94 strains were fungi.
- Among 168 strains, 83 strains (49%) were identified to be known yeast species (Table 1) , 17 strains (10%) were confirmed to be new 11 yeast species (Table 2) and 86 strains (41%) may be new species in Basidiomycota but need to be confirmed (Fig. 1) .

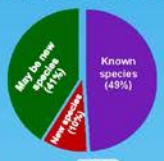


Fig. 1

- 83 strains of known species consisted of 24 known species in 11 genera in Basidiomycota and 4 species in 3 genera in Ascomycota (Table 1, Fig. 2, 3). *M. caribbica* was the most prevalent species with a 22.9% frequency of occurrence.

Table 1: Know yeasts species in phylum Asomycota (blue) and Basidiomycota (green), S = number of strains, F = frequency of occurrence (no. of leaf sample contain each yeast species/ total leaf sample x100)

Species	S	F
Candida parapsilosis	2	2.4
Candida nymphaea	1	1.2
Kodamaea ohmeri	1	1.2
Meyerozyma caribbica	19	22.9
Cryptococcus flavescens	1	1.2
Cryptococcus flavus	1	1.2
Cryptococcus heveanensis	1	1.2
Cryptococcus rajasthanensis	1	1.2
Dioszegia zsoltii	2	2.4
Hannaella sinensis	1	1.2

Species	S	F
Hannaella pagnoccae	6	7.2
Tremella globispora	3	3.6
Rhodotorula benthica	1	1.2
Occultifur externus	1	1.2
Rhodotorula mucilaginosa	3	3.6
Rhodotorula taiwanensis	6	7.2
Sporobolomyces blumeae	2	2.4
Sporobolomyces carnicolor	4	6.0
Sporobolomyces vermiculatus	7	8.4
Jaminaea angkorlensis	5	6.0

Species	S	F
Rhodosporidium paludigenum	1	1.2
Pseudozyma alboarmeniaca	1	1.2
Pseudozyma aphidis	2	2.4
Pseudozyma hubeiensis	1	1.2
Pseudozyma jejuensis	1	1.2
Pseudozyma rugulosa	3	3.6
Pseudozyma siamensis	4	4.8
Pseudozyma vetiver	1	1.2

Table 2: Strains confirmed to be new yeast species in phylum Basidiomycota

Strain	Closest species (type strain)	% nt substitution D1/D2	% nt substitution ITS
DMKU-SP67	*Cryptococcus laurentii*	2.1	1.7
DMKU-SP105	*Cryptococcus laurentii*	1.3	4.6
DMKU-SP423	*Cryptococcus laurentii*	2.2	3.5
DMKU-SP85	*Cryptococcus nemorosus*	1.2	2.5
DMKU-SP56	*Hannaella coprosmaensis*	5.5	0.8
DMKU-SP385	*Occultifur externus*	1.7	3.4
DMKU-SP71	*Pseudozyma churashimaensis*	1.5	0.5
DMKU-SP76	*Pseudozyma rugulosa*	1.2	0.6

Strain	Closest species (type strain)	% nt substitution D1/D2	% nt substitution ITS
DMKU-SP427	*Rhodotorula calyptogenae*	0.4	5.6
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DMKU-SP40	*Tremella globispora*	1.7	3.5
DMKU-SP403	*Tremella globispora*	1.3	3.4

Conclusions:

- The result indicates the dominance of basidiomycete yeasts in the phyllosphere of sugarcane in Thailand (Fig. 4).
- The most prevalent species was *M. caribbica*, the ascomycete yeast.

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Diversity of culture-independent endophytic yeasts from sugarcane leaves in Thailand

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Endophytic microorganisms inhabit internal plant tissues without causing any symptoms or negative effects in the host plant. Diversity of endophytes has been evaluated due to their ability to produce various beneficial bioactive, however, only a few reports have focused on yeast community. Therefore, in this study culture-independent method was used to investigate the endophytic yeasts associated with sugarcane leaves in Thailand via semi-nested PCR and amplified rDNA restriction analysis (ARDRA) techniques. Based on sequence analysis of the D1/D2 domain of the LSU rDNA, the results indicated that the colonization frequency (CF) and the relative species frequency (RF) of endophytic yeast phylotypes were 0.52 and 0.21, respectively. The closest related species to the clone sequences determined by BLAST search showed eight (77.2% RF) and nine (22.8% RF) phylotypes belonged to the Ascomycota and Basidiomycota, respectively. The phylotypes were designated as three known species (*Candida palmioleophila, Debaryomyces hansenii* and *Kodamaea ohmeri*), together with ten phylotypes closest to *D. hansenii*, *Cryptococcus flavus*, *Malassezia restricta*, *Pseudozyma aphidis, Rhodotorula cassiicola, R. marina* and *Sporobolomyces vermiculatus.* The most prevalent phylotypes were *K. ohmeri* (44.2% RF) followed by *D. hansanii* (20.5% RF) and *C. palmioleophila* (11.6% RF). Moreover, the most of novel phylotypes were the phylotypes in the Basidiomycota. Consequently, our findings suggest that sugarcane leaves are a potential choice for the discovery of novel yeast species.

KEYWORDS: Endophyte, Yeast, Sugarcane, Culture-independent diversity, PCR

Session 1A: Yeasts in the environment: ecology and taxonomy

Diversity of Culture-Independent Endophytic Yeasts from Sugarcane Leaves in Thailand

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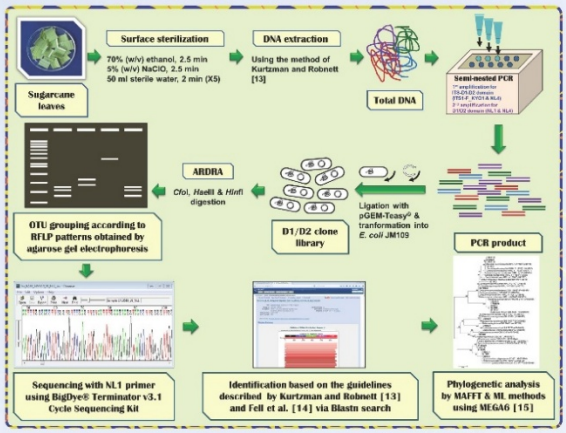
ABSTRACT

Endophytic microorganisms inhabit internal plant tissues without causing any symptoms or negative effects in the host plant. Diversity of endophytes has been evaluated due to their ability to produce various beneficial bioactive, however, only a few reports have focused on yeast community. Therefore, in this study culture-independent method was used to investigate the endophytic yeasts associated with sugarcane leaves in Thailand via semi-nested PCR and amplified rDNA restriction analysis (ARDRA) techniques. Based on sequence analysis of the D1/D2 domain of the LSU rDNA, the results indicated that the colonization frequency (CF) and the relative species frequency (RF) of endophytic yeast phylotypes were 0.52 and 0.21, respectively. The closest related species to the clone sequences determined by BLAST search showed six (77.2% RF) and nine (22.8% RF) OTU belonged to the Ascomycota and Basidiomycota, respectively. The phylotypes were designated as three known species (*Candida palmioleophila, Debaryomyces hansenii* and *Kodamaea ohmeri*), together with ten phylotypes closest to *D. hansenii, Cryptococcus flavus, Malassezia restricta, Pseudozyma aphidis, Rhodotorula cassiicola, R. marina* and *Sporobolomyces vermiculatus*. The most prevalent phylotypes were *K. ohmeri* (44.2% RF) followed by *D. hansanii* (20.5% RF) and *C. palmioleophila* (11.6% RF). Moreover, the most of novel phylotypes were the phylotypes in the Basidiomycota. Consequently, our findings suggest that sugarcane leaves are a potential choice for the discovery of novel yeast species.

INTRODUCTION

Endophytic microorganisms, inhabitng internal plant tissues without causing any symptoms or negative effects in the host plant [1-7], have been receiving attention due to their potential use in agricultural, industrial and pharmaceutical fields. In Thailand sugarcane is an important agronomic crops used to produce sugar for both local consumption and export. According to plant-microbe interaction, endophytes associated with sugarcane have been investigated, however, focusing on bacterial and fungal community structures and their biological activities [8-9]; only a few reports have described the diversity and the application of endophytic yeasts [10-11]. Therefore, in this study we aimed to evaluate the diversity of endophytic yeasts in sugarcane leaves using a culture-independent approach via semi-nested PCR and amplified rDNA restriction analysis (ARDRA) techniques.

MATERIALS & METHODS



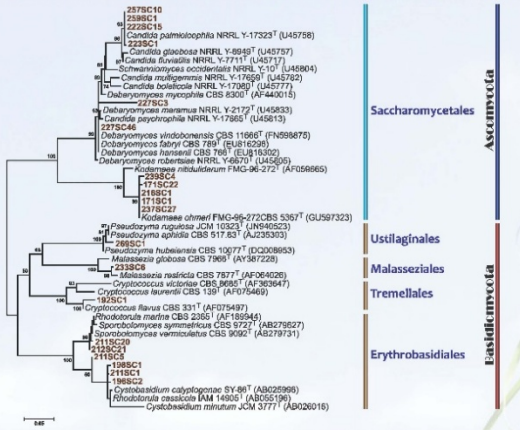


Fig. 1 Phylogenetic tree, based on the sequences of the D1/D2 region of the LSU rDNA, showing the placement of endophytic yeast phylotypes with respect to closely related species. The phylogenetic tree was constructed from evolutionary distance data with Kimura's two-parameter correction, using the maximum likelihood method and MEGA version 6.0. Numbers at nodes indicate percentages of bootstrap sampling derived from 1000 replicates and bootstrap percentages higher than 50% are shown. Bar, 0.05 Knuc. Sequences obtained in the present study are printed in bold letters and the sequences of type strains are indicated by superscript T.

RESULTS & DISCUSSION

Total DNA were extracted from 29 sugarcane leaf samples collected in the Central Region: Chainat (n = 2), Lop Buri (n = 5), Nakhon Sawan (n = 1), Phetchabun (n = 5), Phichit (n = 2), Phitsanulok (n = 3), Suphan Buri (n = 2) and Saraburi (n = 3), and the Northeastern region: Burirum (n = 3) and Nakhon Ratchasima (n = 3) and were used as DNA templates. The sterilization process could significantly reduce the contamination of epiphytic yeast DNA and sufficiently prevent the amplification of the D1/D2 domain of the epiphytic yeast DNA. However, a small amount of residual DNA may remain on the leaf surface. The D1/D2 domain of the LSU rDNA was amplified by semi-nested PCR using ITS1-F_KYO1 [12] and NL4 as the outer set and NL1/NL4 [13] as the inner set. Therefore, 46 D1/D2 libraries were constructed and a total of 1144 randomly picked clones were grouped into operational taxonomic units (OTUs), according to the ARDRA patterns obtained from the independent digestion by three restriction endonucleases: *Cfo*I, *Hae*III and *Hin*fI. The representative clones from each OTU were sequenced and analyzed. Through the BLAST analysis, 224 clones (21%) were closely related to yeasts, whereas the others belonged to filamentous fungi (692 clones, 65%) and the sugarcane sequence (147 clones, 14%). Since NL1 and NL4, the universal primers for amplification of the D1/D2 domain of yeast cultures, could also amplify the LSU rDNA of sugarcane, semi-nested PCR combined with the highly fungal specific primers (ITS1-F_KYO1) was used to enhance the specific amplification of small amounts of fungal DNA compared to sugarcane DNA in the reaction, which could reduce the proportion of sugarcane clones from 99% to 14%. Moreover, the number of yeast phylotypes in sugarcane leaves ranged from 0 to 3 phylotypes per sample. Among 29 leaf samples, yeast phylotypes were found in 15 leaf samples, whereas all leaf samples contained filamentous fungal phylotypes, revealing that yeasts were in a minority among endophytic fungi in sugarcane leaves. The colonization frequency (CF) and the relative frequency (RF) of endophytic yeast phylotypes in sugarcane leaves were 0.52 and 0.21, respectively.

Based on sequence analysis of the D1/D2 domain of the LSU rDNA, the D1/D2 clone sequences of endophytic yeasts derived from sugarcane leaves were identified and affiliated by their similarity and phylogenetic relationships to the sequences of the closest related strains in NCBI and related type strains. A total of 224 sequences of yeast clones showed that 15 OTUs were composed of 17 different phylotypes. The closest related species of the clone sequences showed eight (77.2%) and nine (22.8%) phylotypes belonged to the Ascomycota and Basidiomycota, respectively. The phylogenetic tree showed that the analyzed sequences were distributed in 5 taxonomic orders, namely, Erythrobasidiales, Malasseziales, Tremellales and Ustilaginales in Basidiomycota and Saccharomycetales in Ascomycota (Fig. 1). However, the majority of novel phylotypes belonged to the Basidiomycota. The tree also revealed that most of the novel sequences were affiliated in the Erythrobasidiales. The phylotypes were designated as three known species (*Candida palmioleophila, Debaryomyces hansenii* and *Kodamaea ohmeri*, whereas the others may be novel phylotypes closest to *D. hansenii, Cryptococcus flavus, Malassezia restricta, Pseudozyma aphidis, Rhodotorula cassiicola, R. marina* and *Sporobolomyces vermiculatus*. The most prevalent phylotypes were *K. ohmeri* (44.2%) followed by *D. hansanii* (20.5%) and *C. palmioleophila* (11.6%) (Fig. 2).

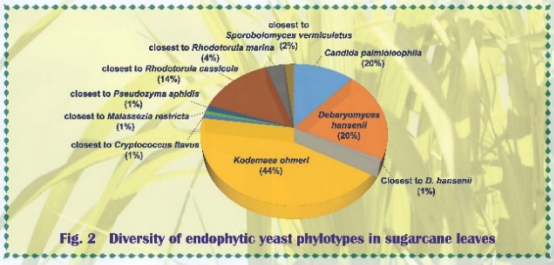


Fig. 2 Diversity of endophytic yeast phylotypes in sugarcane leaves

The yeast phylotypes in the present study belonged to the yeast species recovered from various sources, e.g., the phylloplane, soil, flower, insect frass and osmophilic samples and known to be the attractive microbes, as enzyme [2] and sugar alcohol producers [3], indole-3-acetic acid producer [4, 11], biocontrol agents [4] and azo dye decolorizing agents [6], for biotechnological and agricultural applications. Although it is not possible to specify the roles of these phylotypes, the reviewed information suggests that there are potentially beneficial endophytic yeast strains in sugarcane leaves.

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Diversity of epiphytic yeasts in phyllosphere of corn in Thailand by culture-independent approach

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The epiphytic yeast diversity in corn phyllosphere in Thailand was investigated by culture-independent technique based on the sequence of the D1/D2 domain of the large subunit rRNA gene. Thirty-seven samples of corn leaf were collected randomly from 10 provinces in Thailand. The DNA was extracted from leaf washing samples and the D1/D2 domain was amplified using PCR technique. The PCR products were cloned and then screened by colony PCR. The restriction analysis was used to preliminarily cluster the PCR products from each clone library. Of total 1,041 clones, 357 clones (33.7%) revealed the D1/D2 domain sequences closely related to sequences of yeasts in GenBank, and they were clustered into 108 operational taxonomic units (OTUs) at 99% homology cut off. The majority of yeasts presented in corn leaf surfaces were members of phylum Basidiomycota (98.0%). Of total yeast related clones, 98 clones (27.5%, 14 OTUs) were identified as 12 known yeast species including *Bullera derxii*, *B. oryzae*, *Cryptococcus flavescens*, *Hannaella sinensis*, *Jaminaea angkoriensis*, *Pseudozyma antarctica*, *P. aphidis*, *P. hubeiensis*, *P. prolifica*, *Sporidiobolus pararoseus*, *Sporobolomyces carnicolor* and *S. odoratus*. The D1/D2 sequences (259 clones) that could not be identified as known yeast species were closest to 1 and 26 species in Ascomycota and Basidiomycota, respectively, some of which may be new yeast species. Interestingly, yeasts in subphylum Ustilaginomycotina were mostly detected (72.3%). *Pseudozyma* was the most prevalent yeast genus and it seems to represent the asexual counterpart of Ustilaginales that parasitize monocotyledonous plants. Some species of this genus has been found in the gut of several pests of corn. The most common yeast species detected were *P. antarctica* and *P. hubeiensis* with 24.3 % frequency of occurrence in corn phyllosphere samples.

KEYWORDS: Corn, Phyllosphere, D1/D2 domain, Epiphytic yeast, Culture-independent

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INTRODUCTION

Epiphytic Yeasts

- Epiphytic yeasts colonize plant surface and their association on the plant surface ranges from saprophytic, commensal symbiotic (one organism benefits without affecting the other) to parasite.
- Few investigations of diversity of epiphytic yeasts especially from phyllosphere were undertaken in Thailand
- Consequently the diversity and ecology of phyllosphere yeasts in Thailand cannot be concluded so far.

INTRODUCTION

- rRNA-coding DNA has been widely used in taxonomic studies
  - Ribosomes are present in all cellular organisms
  - Appear to share a common evolutionary origin
  - Some rRNA/rDNA sequences are sufficiently conserved that they are homologous for all organisms and serve as reference points
  - Enable alignment of the less conserved areas used to measure evolutionary relationships



Ribosome gene cluster in tandem repeats, separated by intergenic spacers (IGS)

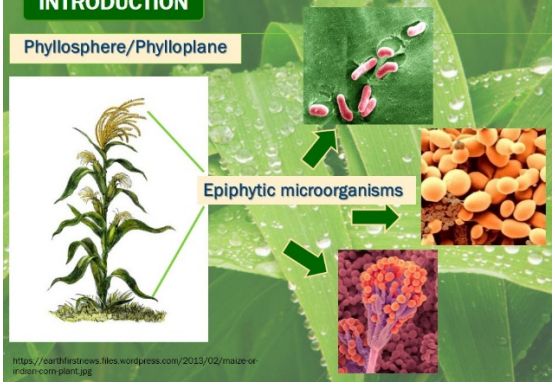
OBJECTIVE

To investigate the diversity of epiphytic yeasts associated with corn phyllospheres by culture-independent method

- PCR-RFLP techniques
- Sequence analysis of D1/D2 region of large subunit (LSU) rRNA gene

INTRODUCTION

Phyllosphere/Phylloplane



https://earthfirstnews.files.wordpress.com/2013/02/maize-or-indian-corn-plant.jpg

Study on Diversity of Microorganisms

Culture-dependent method



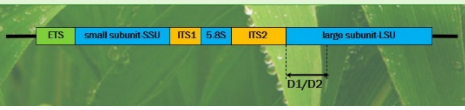
Culture-independent method

- Extraction of DNA directly from cell pellets without the requirement to culture microorganisms in the laboratory
- Less time consuming
- High accuracy (study on rRNA gene)
- Circumvent the obstracle due to the unculturable strains

http://www.afh-ef.de/hygiene-mikrobiologie.html
http://www.abiotec.com/microbiology/
https://www.rmjonline.com/site/sections/232
http://www.anamobasystems.com/home/prepubbed-media/peptone-yeast

Yeast Identification Based on LSU rDNA partial sequence

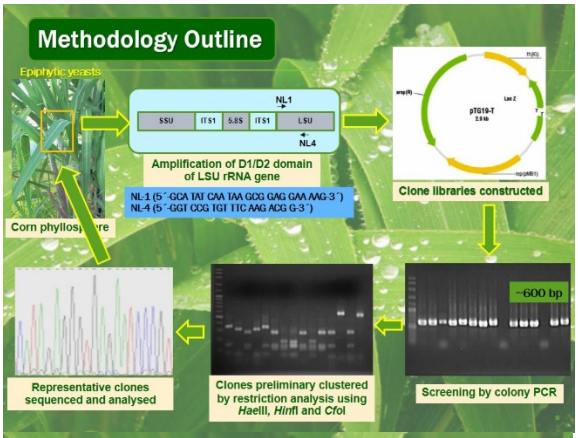
- Analysis of areas of homology for the D1/D2 domain of LSU rRNA gene sequences and those of type strains.
- Ascomycetous yeasts: (Kurtzman and Robnett, 1998)
  - Sequences showing greater than 1 % nucleotide substitutions (6 nucleotides) in the ca. 600-nucleotide D1/D2 domain are likely to be of different species
  - strains with 0-3 nucleotide differences are either conspecific or sister species
- Basidiomycetous yeasts: Sequences that differ by two or more nucleotides in the D1/D2 region, represent different taxa (Fell et al., 2000)

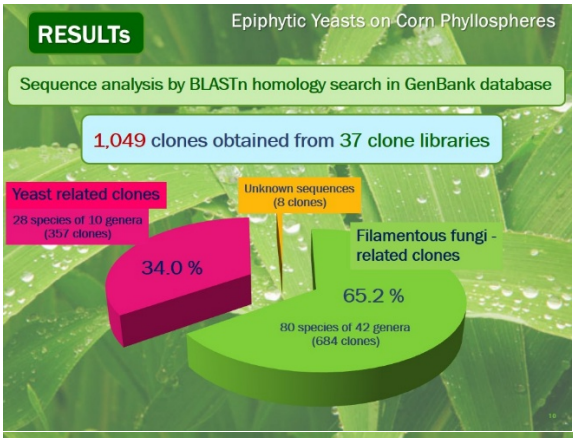


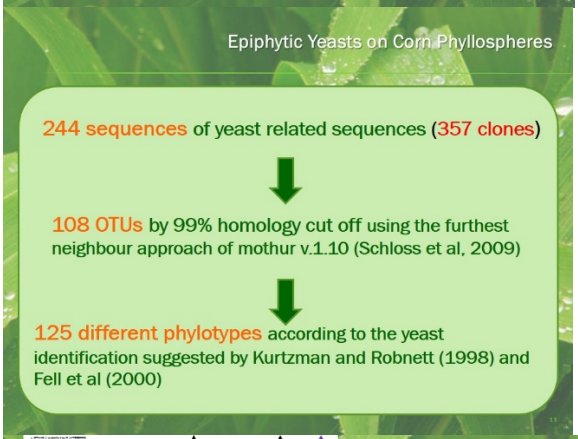


Corn leaf samples : 37 samples from 10 provinces in Thailand during Jan – May 2012

Province	No. of samples
Phitsanulok	1
Phetchabun	16
Chaiyaphum	2
Nakhon Ratchasima	5
Sisaket	1
Nakhon Pathom	4
Nakhon Sawan	2
Lopburi	3
Saraburi	1
Suphanburi	2

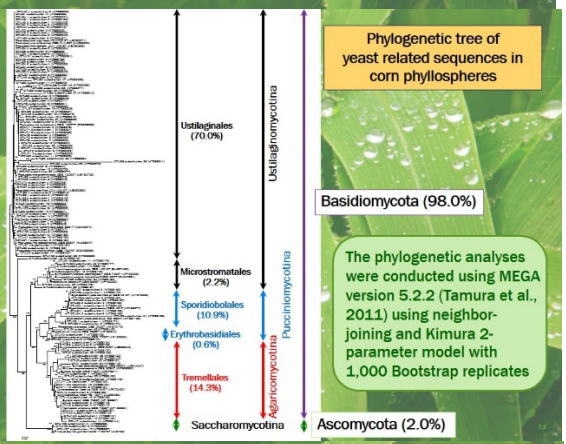


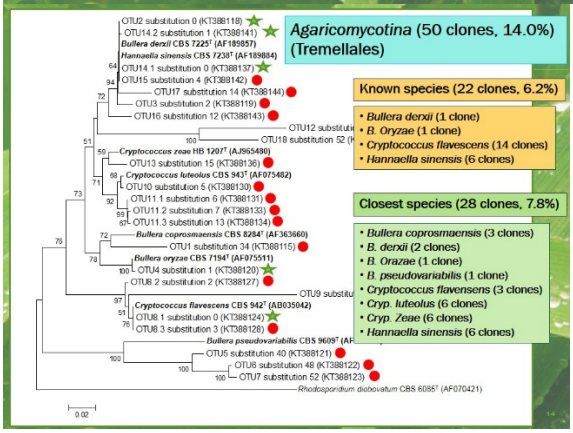


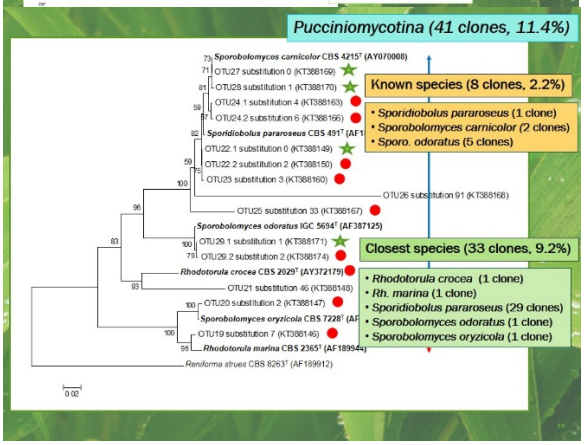


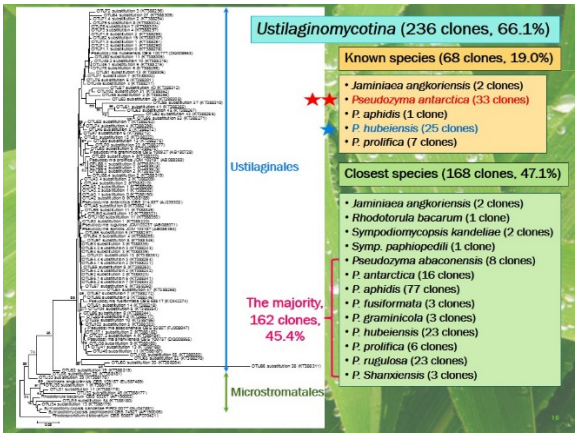
Examples of yeast identification

OTU	No of clones	No of sequences	Closest species	Nucleotide substitution (gap)	Identity (%)	Identification
1	3	3	*Bullera coprosmaensis* CBS 8284^T (AF363660)	34(2,4)	93.5-93.9	Closest to *Bullera coprosmaensis*
2	1	1	*Bullera derxii* CBS 7225^T (AF189857)	0(0)	100.0	*Bullera derxii*
3	2	1	*Bullera derxii* CBS 7225^T (AF189857)	2(0)	99.6	Closest to *Bullera derxii*
4	1	1	*Bullera oryzae* CBS 7194^T (AF075511)	1(1)	99.6	*Bullera oryzae*
5	1	1	*Bullera oryzae* CBS 7194^T (AF075511)	40(13)	89.4	Closest to *Bullera oryzae*
6	1	1	*Bullera pseudovariabilis* CBS 9609^T (AF544247)	48(8)	90.2	Closest to *Bullera pseudovariabilis*
7	1	1	*Bullera pseudovariabilis* CBS 9609^T (AF544247)	52(7)	89.7	Closest to *Bullera pseudovariabilis*
8.1	14	3	*Cryptococcus flavescens* CBS 942^T (AB035042)	0(0)	100.0	*Cryptococcus flavescens*
8.2	1	1	*Cryptococcus flavescens* CBS 942^T (AB035042)	2(0)	99.6	Closest to *Cryptococcus flavescens*
8.3	1	1	*Cryptococcus flavescens* CBS 942^T (AB035042)	3(1)	99.3	Closest to *Cryptococcus flavescens*

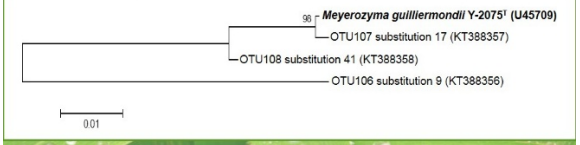






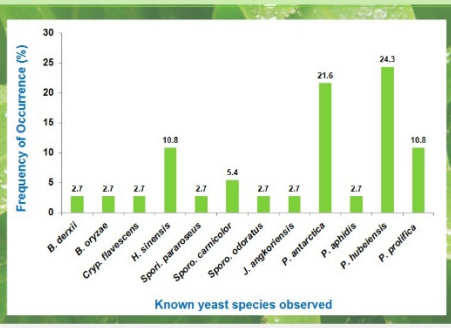


Saccharomycotina (7 clones, 2.0%) (*Saccharomycetales*)



Closest species • *Meyerozyma guilliermondii* (7 clones)

FREQUENCY OF OCCURRENCE IN CORN PHYLLOSPHERES



Frequency of occurrence (%) was calculated as the number of samples, where a particular species was observed, as a propotion of the total number of samples.

Epiphytic Yeasts on Corn Phyllospheres

Yeast related clones (357 clones, 244 sequences)

- Known yeast species (98 clones, 29.4%, 59 sequences)
  - Basidiomycota
    - Agaricomycotina
    - Pucciniomycotina
    - Ustilaginomycotina
- Unknown yeast taxa (259 clones, 70.6%, 185 sequences)
  - Ascomycota (2.0%)
    - *Meyerozyma guilliermondii*
  - Basidiomycota (68.6%)
    - Agaricomycotina
    - Pucciniomycotina
    - ★★ Ustilaginomycotina



ORIGINAL PAPER

Assessment of epiphytic yeast diversity in rice (*Oryza sativa*) phyllosphere in Thailand by a culture-independent approach

Rujikan Nasanit · Kultara Krataithong · Manee Tantirungkij · Savitree Limtong

Fungal Biology

The assessment of epiphytic yeast diversity in sugarcane phyllosphere in Thailand by culture-independent method

Rujikan Nasanit*, Apirat Tangwong-o-thai*, Manee Tantirungkij*, Savitree Limtong*,*

Plant phyllosphere	Rice (Nasanit et al.,2015)	Sugarcane (accepted in Fungal Biology)	Corn (present study)
No. of samples	44	45	37
Total no. of clones	451	426	357
Total number of phylotypes	57	38	125
Shannon equitability index (H')	2.19	2.33	4.03
Community evenness (E_H)	0.54	0.64	0.83
Dominant species	*P. antarctica*, *B. japonica*	*Pseudozyma -P. prolifica*	*P. antarctica*, *P. hubeiensis*

Discussion and Conclusion

- Yeasts in the order *Ustilaginales* were the most prevalently detected in corn phyllospheres in Thailand
- *Pseudozyma* was the most prevalent yeast genus
  - This genus seems to represent the asexual counterpart of *Ustilaginales* that parasitize grasses (Begerow et al., 2014; Begerow et al., 2006).
- *Pseudozyma prolifica* and *P. aphidis* have also been found in the gut of several pests of maize (Molnar et al., 2008).
  - Therefore it can be hypothesized that pathogenic yeasts found in corn fields in Thailand may have been introduced or spread by the crop pests.

Discussion and Conclusion

The presence of the phylotypes related to species known for their agricultural and industrial application together with the occurrence of novel phylotypes, should encourage extensive studies to discover novel yeast species and to understand their interaction to the agronomic crops.

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Acknowledgement

Biodiversity and ecology of endophytic and epiphytic yeasts from leaves of agronomic crops in Thailand and production of plant growth promoting auxins by the selected promising strain with the elucidation of it biosynthetic pathway



Prof. Dr. Savitree Limtong*
Dr. Manee Tantirungkij
Asst.Prof. Natana Srisuk
Dr. Sasitorn Jindamorakot
Dr.Panarat Arunrattiyakorn
Dr.Ruthada Chanklan
Dr. Rungluk Kaewwichian
Somjit Am-In
Chommanat Kerdkhong
Ph.D. students
M.S. students

•The Thailand Research Fund through the TRF Research-Team Promotion Grant (RTA54800009)
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•The Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission.

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Thank you for your attention

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- Zhang et al. (2007) Biological Control 42: 308-315.

ภาคผนวก 4

การเสนอผลงานในการประชุมวิชาการในประเทศไทย

(Names in bold are researchers and students of the project)

1. **Kaewwichian, R.**, W. Yongmanitchai **and S. Limtong**. Description of six novel yeast species isolated from phylloplane of rice, corn and sugarcane เสนอใน RGJ Seminar Series ครั้งที่ 88 หัวข้อ Microbial Resources: Their Biodiversity and Utilization จัดโดยโครงการปริญญาเอกกาญจนาภิเษก ร่วมกับภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ วันที่ 24 สิงหาคม 2555 ณ คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์
2. **Nutaratat, P., N. Srisuk** and **S. Limtong.** Screening for a promising yeast capable on plant growth promotion เสนอใน RGJ Seminar Series ครั้งที่ 88 หัวข้อ Microbial Resources: Their Biodiversity and Utilization จัดโดยโครงการปริญญาเอกกาญจนาภิเษก ร่วมกับภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ วันที่ 24 สิงหาคม 2555 ณ คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์
3. **Kaewwichian, R.**, W. Yongmanitchai and **S. Limtong**. Diversity of yeasts on phylloplane of rice, corn and sugarcane in Thailand and description of seven novel ascomycetous yeast species เสนอในการประชุมวิชาการ The 6th Thai Mycological Conference and Annual Meeting of Thai Mycological Association (TMA) 2012, 6 March 2012 at Maruay Garden Hotel, Bangkok, Thailand
4. **Krataithong, K., R. Nasanit, M. Tantirungkij** and **S. Limtong.** Identification of epiphytic yeast on rice population in Thailand based on molecular biological techniques. TSB 2013 Conference Proceedings. The 25th Annual Meeting of the Thai Society for Biotechnology and International Conference. 16-19 Ocotober 2013, The Emerald Hotel, Bangkok, Thailand.
5. **Tangwong, A., R. Nasanit, M. Tantirungkij** and **S. Limtong.** Identification of epiphytic yeast from sugarcane phyllospheres using PCR and RFLP techniques. TSB 2013 Conference Proceedings. The 25th Annual Meeting of the Thai Society for Biotechnology and International Conference. 16-19 Ocotober 2013, The Emerald Hotel, Bangkok, Thailand.
6. **Jindamorakot, S., S. Am-In** and **S. Limtong.** Investigation on diversity of epiphytic yeasts on rice leaves in Thailand. เสนอใน The 1st ASEAN Microbial Biotechnology Conference. 19-21 Febuary 2014. BITEC, Bangkok, Thailand**.**
7. **Am-In S, S Jindamorakot and S Limtong.** Biodiversity of epiphytic yeasts from corn leaves by culture dependent technique. ในThe 1st ASEAN Microbial Biotechnology Conference. BITEC, Bangkok Thailand**.** 19-21 February 2014.
8. **Jaibangyang, S., R. Nasanit, M. Tantrirungkij.** and **S. Limtong**. Study on epiphytic yeasts in corn phylloplane from Phetchabun province, Thailand by culture-independent approach. เสนอใน The 26th Annual Meeting of the Thai Society for Biotechnology and International Conference is organized jointly by the Thai Society for Biotechnology (TSB) and Mae Fah Lunag University (School of Science), Chiang Rai, Thailand, 26th and 29th November, 2014.

9. **Thaicharoena, P., R. Chanklana** and **S. Limtong**. **Selection of Indole-3-acetic Acid Producing-epiphytic Yeasts Isolated from Corn Leave**. เสนอใน The 4th International Biochemistry and Molecular Biology Conference. April 2-3, 2014, Bangkok, Thailand**.**

10. **จันทร์จีรา สุรัสวดี, นันทนา สีสุข และสาวิตรี ลิ่มทอง** ความหลากหลายของยีสต์อีพิไฟต์บนใบอ้อยและการอธิบายยีสต์สปีชีส์ใหม่ เสนอใน การประชุมซิสเทมาติคส์ในประเทศไทยครั้งที่ 5 ซึ่งจัดที่ คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ในระหว่าง 25-27 พฤษภาคม 2558

11. **ศศิธร จินดามรกฎ, สมจิต อ่ำอินทร์และสาวิตรี ลิ่มทอง** ความหลากหลายของยีสต์อีพิไฟต์บนใบข้าว เสนอในการประชุมซิสเทมาติคส์ในประเทศไทยครั้งที่ 5 ซึ่งจัดที่ คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ในระหว่าง 25-27 พฤษภาคม 2558

12. **สมจิต อ่ำอินทร์, ศศิธร จินดามรกฎ และสาวิตรี ลิ่มทอง** การวิจัยความหลากหลายของยีสต์อีพิไฟต์บนใบข้าวโพดในประเทศไทย เสนอใน การประชุมซิสเทมาติคส์ในประเทศไทยครั้งที่ 5 ซึ่งจัดที่ คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ในระหว่าง 25-27 พฤษภาคม 2558

Seminar Program

RGJ Seminar Series LXXXVIII
Microbial Resources: Their Biodiversity and Utilization
Organized by
Department of Microbiology, Faculty of Science, Kasetsart University
and The Royal Golden Jubilee Ph.D.
Friday 24th August 2012
At Room 202, SCL building, Faculty of Science, Kasetsart University, Chatuchak, Bangkok

Master of Ceremonies:
Dr. Noppon Lertwattanasakul
Dr. Churapa Teerapatsakul

8.30-9.00	Registration and poster set up
9.00-9.15	Welcome address Dean, Faculty of Science, Kasetsart University Opening address Director, RGJ Ph.D. Program
9.15-9.55	Chair: Assoc. Prof. Captain Chaivat Kittigul Keynote Speaker **Usefulness of thermotolerant microbes** By *Prof. Dr. Mamoru YAMADA*, Applied Molecular Bioscience, Graduate School of Medicine, Yamaguchi University, Yamaguchi, Japan
9.55-10.15	Group photo
10.15-10.30	Break
10.30-11.10	Chair: Assoc. Prof. Dr. Lerluck Chitradon Secretary: Dr. Noppon Lertwattanasakul Invited Speaker I **Glycan structural study and its application to glycoprotein production in yeast** By *Asst. Prof. Dr. Takao OHASHI* International Center for Biotechnology (ICBiotech), Osaka University, Osaka, Japan
11.10-11.55	Chair: Prof. Dr. Somboon Tanasupawat Secretary: Dr. Ingorn Kimkong Experts: Assoc. Prof. Dr. Chuenchit Boonchird and Assoc. Prof. Dr. Sunee Nitisinprasert
(11.10-11.25)	**Soil fungal communities and enzymes in a sandy tropical soil treated with biochemically contrasting organic inputs** By *Bhanudacha Kamolmanit et al.*; Khon Kaen University
(11.25-11.40)	**Description of six novel yeast species isolated from phylloplane of rice, corn and sugarcane** By *Rungluk Kaewwichian et al.*; Kasetsart University

Seminar Program (cont.)

(11.40-11.55)	**Selection and taxonomic characterization of oleaginous yeast for lipid production from glycerol** By *Pirapan Polburee et al*.; Kasetsart University
11.55-13.00	Lunch
13.00-13.30	Chair: Prof. Dr. Mamoru Yamada Secretary: Dr. Churapa Teerapatsakul Invited Speaker II **Actinomycetes from rice field and their antifungal activities** By *Asst. Prof. Dr. Kannika DUANGMAL* Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok, Thailand
13.30-15.00	Poster Discussion 13 poster presentations Experts: Assoc. Prof. Dr. Chuenchit Boonchird and Assoc. Prof. Dr. Sunee Nitisinprasert (students are requested to summarize their poster within 3 min each, question and comments will be contribute by the experts, lecturers and other students)
15.00-15.15	Break
15.15-16.00	Chair: Assoc. Prof. Dr. Vichien Kitpreechavanich Secretary: Dr. Patcharaporn Siwayaprahm Experts: Assoc. Prof. Dr. Chuenchit Boonchird and Assoc. Prof. Dr. Sunee Nitisinprasert
(15.15-15.30)	**Screening of nonpolyenic actinomycetes for the production biocontrol agents on orchid pathogens, *Erwinia* sp. and *Phytophthora* sp.** By *Chatsuda Sakdapetsiri et al*.; Kasetsart University
(15.30-15.45)	**Novel secondary xylan-binding site within a single-domain of family 11 xylanase from *Bacillus firmus* K-1** By *Puangpen Limsakul et al*.; King Mongkut's University of Technology Thonburi
(15.45-16.00)	**Process development of lactic acid production from cassava starch by *Rhizopus oryzae* in airlift fermenter** By *Srisakul Trakarnpaiboon et al*.; Kasetsart University
16.00-16.15	Conclusion by Prof. Dr. Savitree Limtong
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Kaewwichian, R., W. Yongmanitchai and S. Limtong. Description of six novel yeast species isolated from phylloplane of rice, corn and sugarcane เสนอใน RGJ Seminar Series ครั้งที่ 88 หัวข้อ Microbial Resources: Their Biodiversity and Utilization

RGJ Seminar Series LXXXVIII Oral presentation

Microbial Resources: Their Biodiversity and Utilization

Description of Six Novel Yeast Species Isolated From Phylloplane of Rice, Corn and Sugarcane

Rungluk Kaewwichian,[a] Wichien Yongmanitchai,[a] Savitree Limtong [a]

[a]*Department of Microbiology, Faculty of Science, Kasetsart University, BangKhan, Bangkok, 10900*

Introduction and Objective

The external surfaces of plant leaves, often referred to the phylloplane or phyllosphere, have been recognized as an important habitat for microorganisms (Fonseca et al. 2006). Phylloplane from various are found to be colonized by members of both basidiomycete and ascomycete yeasts. Although most common phylloplane yeasts are reported to be basidiomycete species, various ascomycete species have also been detected on the phylloplane. During an investigation of yeasts on phylloplane of rice, corn and sugarcane in Thailand, various known yeast species were obtained and nine strains were found to represent six novel yeast species. In this study we described six novel yeast species namely, *Metschnikowia saccharicola* sp. nov.,*Metschnikowia lopburiensis* sp. nov., *Yamadazyma siamensis* sp. nov., *Yamadazyma phyllophila*sp. nov., *Wicherhamomycessiamensis* sp. nov. and*Nakazawaea siamensis* sp. nov.

Methods

Eighty-five samples of rice leaves,65 samples of corn leaves and95 samples of sugarcane leaveswere collected from 28 districts in nine provinces of Thailand. Yeast isolation from phylloplane was carried out by an enrichment technique in yeast extract-malt extract broth supplemented with 0.025% sodium propionate and 0.02% chloramphenicol. Yeast strains were identified on the basis of sequences analysis of the D1/D2 domain of the large subunit rRNA gene and phylogeny. New yeast species were characterized by polyphasic taxonomy.

Results

A total of 454 yeast strains were collected from the 245 leaf samples including154 yeast strains from rice leaves, 134 yeast strains from corn leaves and 166 yeast strains from sugarcane leaves. On the basis of the D1/D2 domain of the large subunit rRNA gene sequences analysis153 yeast strains from rice leaves were identified as 35 known species in 14 genera, 125 strains from corn leaves were identified as 37 known species in 13 genera and 153 strains from sugarcane leaves were identified as 36 known species in 15 genera. Moreover, we found nine strains represented six novel ascomycete yeast species. On the basis of polyplasic taxonomy four strains (DMKU-RK16, DMKU-RK24, DMKU-RK198 and DMKU-RK500^T) from phylloplane of sugarcane were described as *Metschnikowia saccharicola* sp. nov.,strain DMKU-RK277^T from phylloplane of rice was described as *Metschnikowia lopburiensis* sp. nov., strain DMKU-RK254^T from phylloplane of sugarcane was described as *Yamadazyma siamensis* sp. nov., strain DMKU-RK548^T from phylloplane of corn was described as *Yamadazyma phyllophila* sp. nov., strain DMKU-RK359^T from phylloplane of sugarcane was described as *Wicherhamomycessiamensis* sp. nov. andstrain DMKU-RK467^T from phylloplane of sugarcane was described as *Nakazawaea siamensis* sp. nov.

Conclusion

In conclusion, from 454 yeast strains obtained from phylloplane of rice, corn and sugarcane. Four hundred and thirty one strains (94.9%) were identified as described species, fourteen strains (3.1%) were assigned asundescribed species and nine strains (2.2%) were identified to be novel species. On the basis of polyphasic taxonomy nine strains were described as six novel ascomycete yeast species namely, *Metschnikowia saccharicola* sp. nov.,*Metschnikowia lopburiensis* sp. nov.,

Yamadazyma siamensis sp. nov., *Yamadazyma phyllophila* sp. nov., *Wicherhamomycessiamensis* sp. nov. and*Nakazawaea siamensis* sp. nov.

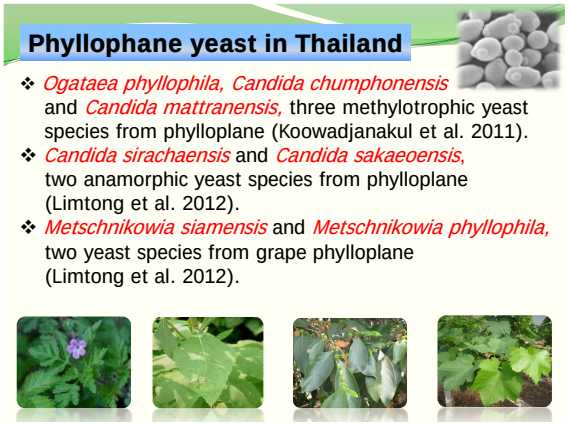
Keywords:phylloplane yeast, yeast in Thailand, ascomycetous yeast

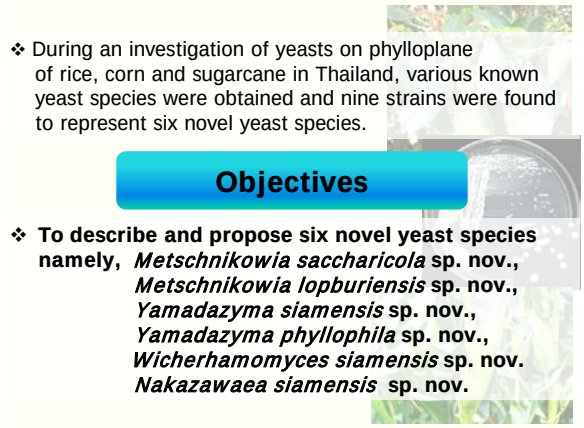
Selected Reference:

1. Fonseca, A. and Inácio, J. (2006) Phylloplane Yeasts. In C. Rosa and G. Péter (Eds.), *Biodiversity and Ecophysiology of Yeasts.* (pp. 263–301).Springer-Verlag Berlin Heidelberg, Springer.









Materials & Method

1. Collection of leaves samples in Thailand





Rice leaves



Corn leaves



Sugarcane leaves

9 provinces (245 samples)

- rice leaves 85 samples
- corn leaves 65 samples
- sugarcane leaves 95 samples

Materials & Method

2. Isolation of yeast from leaves samples by enrichment technique (Limtong *et al.*, 2007)



YM broth + 0.025% sodium propionate* + 0.02% chloramphenicol**



YM agar + 0.025% sodium propionate* + 0.02% chloramphenicol**



Culture collection in 10% glycerol at -80°C

*Inhibiter to filamentous fungi
**Inhibiter to bacteria

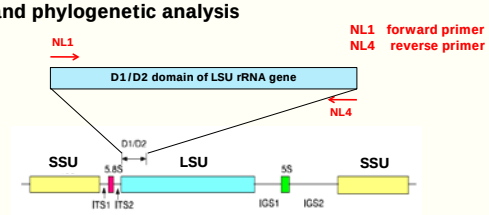
Results

A total of 454 yeast strains (245 leaves samples)

rice leaves	85 samples	→ yeast 154 strains
corn leaves	65 samples	→ yeast 134 strains
sugarcane leaves	95 samples	→ yeast 166 strains
total	245 samples	→ yeast 454 strains

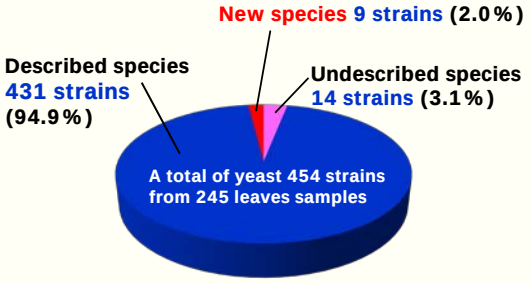
Materials & Method

3. Identification of phylloplane yeast by sequencing analysis of the D1/D2 domain of the LSU rRNA gene and phylogenetic analysis



- ~600 nucleotides at the 5′ end of LSU rRNA gene
  Nucleotide substitution ≥ 1% → new species
  (Kurtzman & Robnett, 1998)

Results

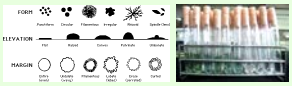


Materials & Method

4. New yeast species

Conventional taxonomy

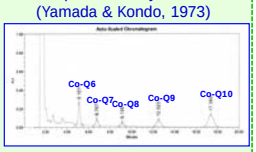
- Morphological Characteristics (Yarrow, 1998) mode of vegetative reproduction, cell & colony morphology, filamentation, ascospore formation etc.



- Physiological & Biochemical characteristics (Yarrow, 1998) carbon & nitrogen assimilation, fermentation of sugars, etc.

Chemotaxonomy

- Ubiquinone analysis (Yamada & Kondo, 1973)



- %GC content analysis (Tamaoka & Komagata, 1984)

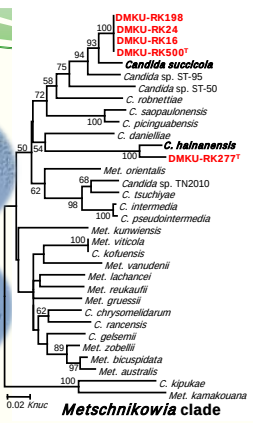
Results

DMKU-RK16, DMKU-RK24, DMKU-RK198 & DMKU-RK500^T

- Closely related to ***Candida succicola*** but with **3.5% nucleotide substitutions** in the D1/D2 domain
- **Ascospores are not produced**
- **Pseudohyphae are formed**
- **Major ubiquinone is Q9**
- **GC content is 42%**

DMKU-RK277^T

- Closely related to ***Candida hainanensis*** but with **3.8% nucleotide substitutions** in the D1/D2 domain
- **Ascospores are not produced**
- **Pseudohyphae are not formed**
- **Major ubiquinone is Q9**
- **GC content is 51%**



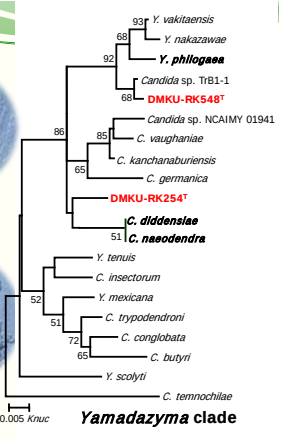
Results

DMKU-RK254^T

- Closely related to ***Candida diddensiae & C. naeodendra*** but with **1.8% nucleotide substitutions** in the D1/D2 domain
- **Ascospores are not produced**
- **Pseudohyphae are formed**
- **Major ubiquinone is Q9**
- **GC content is 46%**

DMKU-RK548^T

- Closely related to ***Yamadazyma philogaea*** but with **1.1% nucleotide substitutions** in the D1/D2 domain
- **Ascospores are not produced**
- **Pseudohyphae are formed**
- **Major ubiquinone is Q9**
- **GC content is 39%**

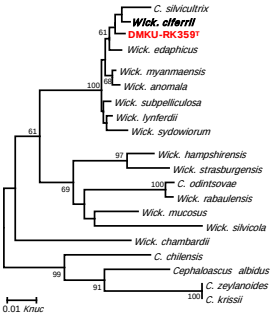


Results

DMKU-RK359^T

- Closely related to ***Wickerhamomyces ciferrii*** but with **1.2% nucleotide substitutions** in the D1/D2 domain
- **Ascospores are 2-4 hat shape**
- **Pseudohyphae are formed**
- **Major ubiquinone is Q7**
- **GC content is 36%**





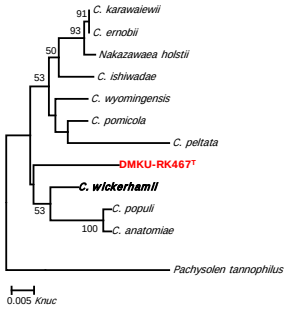
***Wickerhamomyces* clade**

Results

DMKU-RK467^T

- Closely related to ***Candida wickerhamii*** but with **1.4% nucleotide substitutions** in the D1/D2 domain
- **Ascospores are not produced**
- **Pseudohyphae are not formed**
- **Major ubiquinone is Q7**





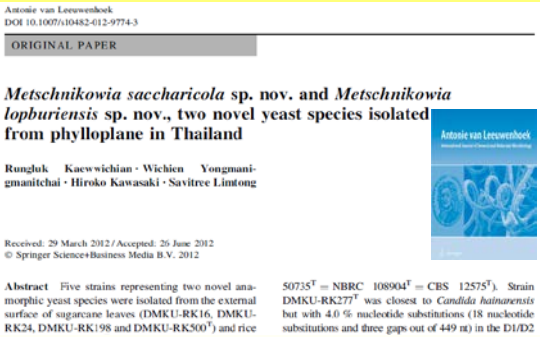
***Nakazawaea* clade**

Conclusion

- **On the basis of polyphasic taxonomy (molecular taxonomy, conventional taxonomy and chemotaxonomy) 9 yeast strains were described as 6 novel ascomycete yeast species namely,**
  - ***Metschnikowia saccharicola* sp. nov. (DMKU-RK16,DMKU-RK24,DMKU-RK198 & DMKU-RK500^T)**
  - ***Metschnikowia lopburiensis* sp. nov. (DMKU-RK277^T)**
  - ***Yamadazyma siamensis* sp. nov. (DMKU-RK254^T)**
  - ***Yamadazyma phyllophila* sp. nov. (DMKU-RK548^T)**
  - ***Wicherhamomyces siamensis* sp. nov. (DMKU-RK359^T)**
  - ***Nakazawaea siamensis* sp. nov. (DMKU-RK467^T)**

Describe and propose new species

- **DMKU-RK16, DMKU-RK24, DMKU-RK198, DMKU-RK277^T and DMKU-RK500^T**



Antonie van Leeuwenhoek
DOI 10.1007/s10482-012-9774-3

ORIGINAL PAPER

Metschnikowia saccharicola sp. nov. and *Metschnikowia lopburiensis* sp. nov., two novel yeast species isolated from phylloplane in Thailand

Rungluk Kaewwichian · Wichien Yongmanitchai · Hiroko Kawasaki · Savitree Limtong

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Abstract Five strains representing two novel anamorphic yeast species were isolated from the external surface of sugarcane leaves (DMKU-RK16, DMKU-RK24, DMKU-RK198 and DMKU-RK500^T) and rice 50735^T = NBRC 108904^T = CBS 12575^T). Strain DMKU-RK277^T was closest to *Candida hainanensis* but with 4.0 % nucleotide substitutions (18 nucleotide substitutions and three gaps out of 449 nt) in the D1/D2

Describe and propose new species by publication

- ***Yamadazyma siamensis* sp. nov. and *Yamadazyma phyllophila* sp. nov., two novel yeast species isolated from phylloplane in Thailand and Taiwan (DMKU-RK254^T , K129, DMKU-RK548^T and TrB1-1)**

Acknowledgements

- **Thailand Research Fund through Royal Golden Jubilee Ph.D. program**
- **Prof. Dr. Savitree Limtong (Advisor)**
- **Dr. Wichien Yongmanitchai (Co-advisor)**
- **Dr. Hiroko Kawasaki**
- **NITE Biological Resource Center (NBRC)**
- **Department of Microbiology, Faculty of Science, Kasetsart University**

Nutaratat, P., N. Srisuk and S. Limtong. Screening for a promising yeast capable on plant growth promotion เสนอใน RGJ Seminar Series ครั้งที่ 88 หัวข้อ Microbial Resources: Their Biodiversity and Utilization

RGJ Seminar Series LXXXVIII
Microbial Resources: Their Biodiversity and Utilization

Screening for a Promising Yeast Capable on Plant Growth Promotion

Pumin Nutaratat, Nantana Srisuk and Savitree Limtong
Department of Microbiology, Faculty of Science, Kasetsart University, Chatuchak, Bangkok, Thailand

Introduction and Objective

Plant growth promoting microbes including bacteria, actinomycetes, fungi and yeasts have been isolated and identified to elucidate the crucial role of such microbes to plant growth. Several plant growth promoting characters such as indole-3-acetic acid (IAA) and siderophore production as well as phosphate solubilization have been shown in other microbes but only few studies have been conducted in plant associated yeasts. This present work was therefore aimed to search for a promising isolate of yeast that possesses these key characters.

Methods

All yeast isolates obtained from rice and sugar cane plant leaves were spectrophotometrically screened for IAA production [1] respective to yeast dry weight. High level of IAA producing yeasts were selected for further screening for siderophore production as well as phosphate solubilizing ability on Chrome Azurol S (CAS) agar medium [2] and Pikovskaya's agar medium [3], respectively.

Results

Six hundred and sixty eight yeast isolates comprises of 125 endophytic yeasts and 308 epiphytic yeasts from rice plant leaves and 235 epiphytic yeasts from sugar cane leaves were screened for IAA production. Five yeast isolates namely RE67, RP105, RP163, RP198 and RP206 obtained from rice plant leaves showed high level of IAA production (42.7-117.8 mg/g dry weight). Among these, only one epiphytic yeast RP206 appeared to be the best IAA producer under the culture conditions used in this study. However, these six yeast isolates were subjected to further screening i.e. siderophore production and phosphate solubilizing ability on agar medium. A promising yeast isolate with high potency to produce IAA, siderophore and solubilize insoluble phosphate will be further focused on yeast identification.

Conclusion

Five yeast isolates namely RE67, RP105, RP163, RP198 and RP206 obtained from rice plant leaves possessed IAA production of between 42.7 and 117.8 mg/g dry weight. These isolates were determined for siderophore production and phosphate solubilization on agar medium.

Keywords:yeast, IAA, siderophore, phosphate solubilization

Selected References

1. Xin, G., D. Glawe and S.L. Doty. 2009. Mycol. Res. 3: 973-980.
2. Schwyn, B. and J.B. Neilands. 1987. Anal. Chem. 160: 47-56.
3. Zaidi, S., S. Usmani, B.R. Singh and J. Musarrat. 2006. Chemosphere. 64: 991-997

Screening for a Promising Yeast Capable on Plant Growth Promotion

Pumin Nutaratat, Nantana Srisuk and Savitree Limtong

Department of Microbiology, Faculty of Science, Kasetsart University, Chatuchak, Bangkok, Thailand

Abstract

Six hundred and sixty eight yeasts were isolated from rice and sugar cane leaves collected in Thailand. Those yeasts were screened for IAA production and five isolates namely RE67, RP105, RP163, RP198 and RP206 obtained from rice leaves showed high level of IAA production (42.7-117.8 mg/g dry weight). Among these, only one epiphytic yeast namely RP206, the best IAA producer under the culture conditions used in this study. Strain RP206 possesses both phosphate solubilization and siderophore production.

Introduction and Objective

Plant growth promoting microbes including bacteria, actinomycetes, fungi and yeasts have been isolated and identified to elucidate the crucial role of such microbes to plant growth. Several plant growth promoting characters such as indole-3-acetic acid (IAA) and siderophore production as well as phosphate solubilization have been shown in other microbes but only few studies have been conducted in plant associated yeasts. This present work was therefore aimed to search for a promising isolate of yeast that possesses these key characters.

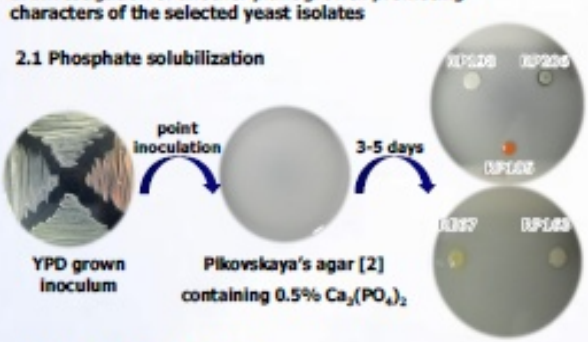
Methods

1. Screening for potent yeast isolates capable on IAA production

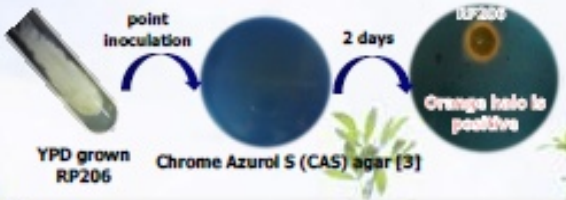


2. Investigation of another plant growth promoting characters of the selected yeast isolates

2.1 Phosphate solubilization



2.2 Siderophore production



Results

One hundred twenty five endophytic yeasts , 308 epiphytic yeasts from rice plant leaves and 235 epiphytic yeasts from sugar cane leaves were screened for IAA production. Five isolates namely RE67, RP105, RP163, RP198 and RP206 obtained from rice plant leaves showed high level of IAA production ranging from 42.7-117.8 mg/g dry weight. These five isolates were screened for phosphate solubilization and only the isolate RP206 showed capability on calcium phosphate solubilization. In addition, RP206 possesses siderophore production, an important character for plant growth promotor.

Conclusions

The yeast RP206, isolated from rice phyllosphere, was selected as a promising plant growth promoting yeast according to the highest IAA production (117.8 mg/g dry weight) under conditions studied as well as capability on phosphate solubilization and siderophore production.

References

[1] Xin, G., D. Glawe and S.L. Doty. 2009. Mycol. Res. 3: 973-980.

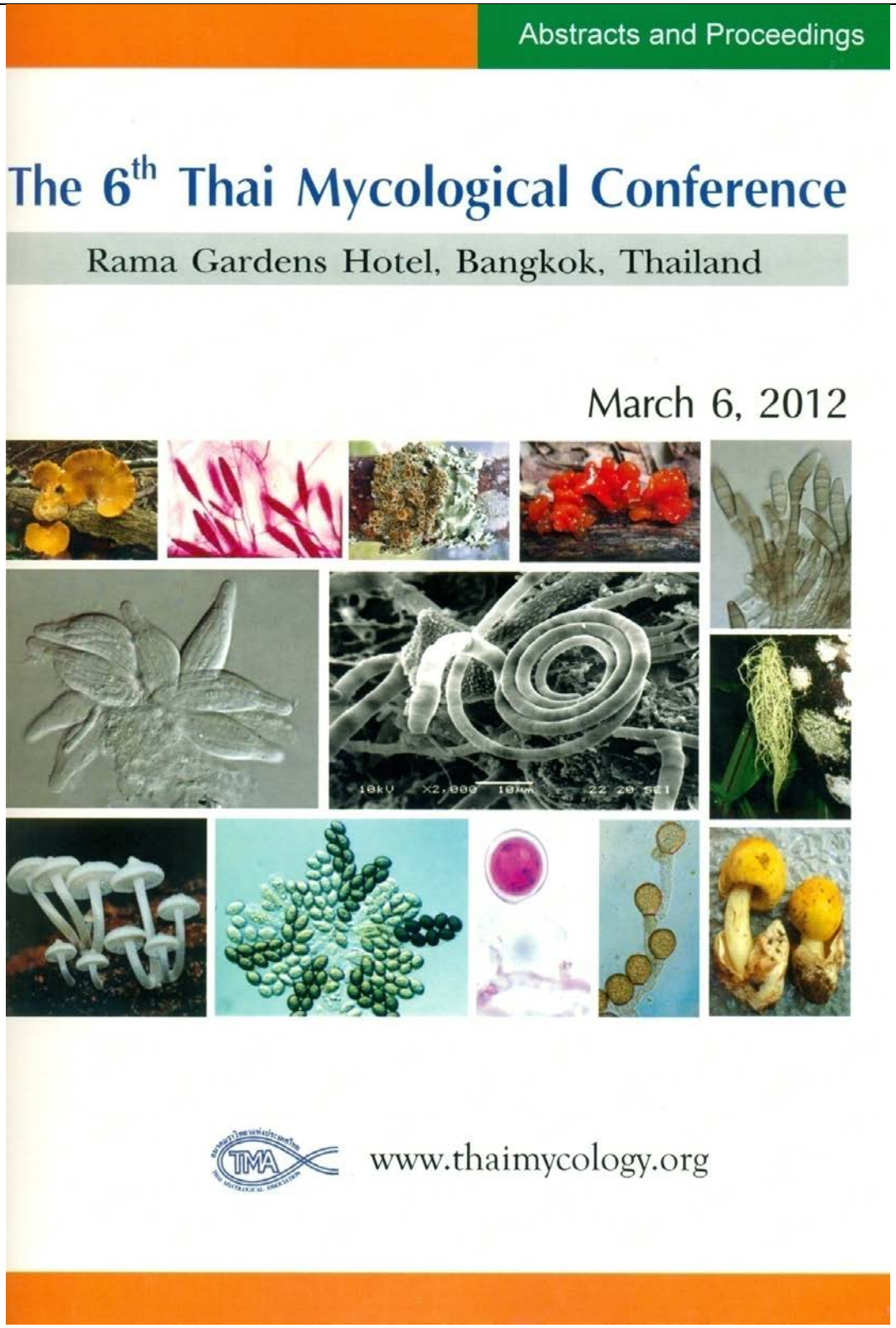
[2] Zaidi, S., S. Usmani, B.R. Singh and J. Musarrat. 2006. Chemosphere. 64: 991-997.

[3] Schwyn, B. and J.B. Neilands. 1987. Anal. Chem. 160: 47-56.

Acknowledgemnet

This work was supported by TRF Research-Team Promotion Grant (RTA5480009).

Kaewwichian, R., W. Yongmanitchai and S. Limtong. Diversity of yeasts on phylloplane of rice, corn and sugarcane in Thailand and description of seven novel ascomycetous yeast species เสนอในการประชุมวิชาการ The 6th Thai Mycological Conference and Annual Meeting of Thai Mycological Association (TMA) 2012



P1-02

Diversity of Yeasts on Phylloplane of Rice, Corn and Sugarcane in Thailand and Description of Seven Novel Ascomycetous Yeast Species

Rungluk Kaewwichian[1*], Wichien Yongmanitchai[1], Savitree Limtong[1]
[1]Department of Microbiology, Faculty of Science, Kasetsart University, BangKhan, Bangkok, 10900.
*E-mail: runglukk@gmail.com

Ninety two samples of rice leaves, 69 samples of corn leaves and 100 samples of sugarcane leaves were collected from 28 districts in nine provinces of Thailand. Yeast isolation was carried out by an enrichment technique in yeast extract-malt extract broth supplemented with 0.025% sodium propionate and 0.02% chloramphenicol. Yeast strains were identified on the basis of sequences analysis of the D1/D2 domain of the large subunit rRNA gene and phylogeny. A total of 454 yeast strains were collected from the 261 leaf samples including 154 yeast strains from rice leaves, 134 yeast strains from corn leaves and 166 yeast strains from sugarcane leaves. On the basis of the D1/D2 domain of the large subunit rRNA gene sequences analysis 153 yeast strains from rice leaves were identified as 35 known yeast species in 14 genera i.e. *Brettanomyces*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Kodamaea*, *Kluyveromyces*, *Metschnikowia*, *Meyerozyma*, *Pichia*, *Pseudozyma*, *Rhodotorula*, *Trichosporon*, *Sporobolomyces* and *Yarrowia*. One hundred and twenty five strains from corn leaves were identified as 37 known yeast species in 13 genera i.e. *Candida*, *Cryptococcus*, *Geotrichum*, *Hanseniaspora*, *Kluyveromyces*, *Kodamaea*, *Meyerozyma*, *Pichia*, *Rhodosporidium*, *Rhodotorula*, *Sporidiobolus*, *Sporobolomyces* and *Torulaspora*. Eight strains were similar to undescribed species. One hundred and fifty two yeast strains from sugarcane leaves were identified as 35 known yeast species in 15 genera i.e. *Bullera*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Hanseniaspora*, *Kluyveromyces*, *Lodderomyces*, *Metschnikowia*, *Meyerozyma*, *Pichia*, *Rhodosporidium*, *Rhodotorula*, *Sporidiobolus*, *Sporobolomyces* and *Torulaspora*. Six strains were similar to undescribed species. Moreover, in this research were found ten strains represented seven novel species. In conclusion, 454 yeast strains were identified as described species 430 strains (94.7%), undescribed species 14 strains (3.1%) and novel species 10 strains (2.2%). The prevalent species were *Rhodotorula mucilaginosa* (30 strains) on rice leaves, *Kodamaea ohmeri* (12 strains) on corn leaves and *Meyerozyma guilliermondii* (26 strains) on sugarcane leaves.

Key words: Phylloplane Yeast, Diversity of Yeast, Yeast in Thailand, Ascomycetous Yeast

Identification of epiphytic yeast on rice leaves population in Thailand based on molecular biological techniques

Kultara Krataithong[1], Rujikan Nasanit[1], Manee Tantirungkij[2] and Savitree Limtong[3*]

ABSTRACT

Phyllosphere, the external surface of the plant leaf, has been recognized as an important habitat for epiphytic microorganisms. Yeast is one of the microorganisms found as a phyllosphere colonizer which could promote plant growth or biocontrol activity. Rice is an essential food plant and the important Thai's industrial crop. Recently, diversity of microorganisms has been determined by molecular biological methods since it requires labors and time less than traditional methods. In this study, 33 samples of rice leave were collected from seven provinces (Surindra, Burirum, Srisakade, Chachoengsao, Nonthaburi, Nakornpathom and Suphanburi) in the north-east and central part of Thailand. DNA samples of the epiphytes from rice leaves were extracted and then the D1/D2 region of large subunit (LSU) rRNA gene was amplified with polymerase chain reaction (PCR) using NL1 and NL4 primers. The amplified products were cloned into pTG19-T cloning vector and transformed into *E. coli* DH5α. The recombinant clones were screened by colony PCR. The PCR products were then analyzed with restriction fragment length polymorphism (RFLP) and the different RFLP patterns were grouped in operational taxonomic units (OTUs). From 33 rice leaves samples, 331 OTUs from 798 positive clones were classified. Representative of each OTUs was sequenced and compared to the nucleotide sequences in the NCBI database. Most of which were identified as filamentous fungi, however we found 11 genera of yeast which can be identified into 18 different species.

Key words: rice, epiphytic yeast, PCR, RFLP, large subunit rRNA gene

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The studies of epiphytic yeast diversity from phyllosphere were undertaken. However, until present there is less information of the diversity and ecology of epiphytic yeasts in Thailand. Therefore this research aimed to determine the diversity of epiphytic yeasts on sugarcane phyllosphere collected from several areas in Thailand and carried out with culture-independent techniques, PCR and RFLP. The results of nucleotide sequence were compared to the GenBank database, National Center for Biotechnology Information (NCBI) using Blastn to specify the yeast species, in order to evaluate the diversity of epiphytic yeasts on the sugarcane phyllospheres.

MATERIALS AND METHODS

Samples

Twenty-seven samples of sugarcane leaves were collected from the agricultural fields in several areas in Thailand, such as Nakorn Pathom, Nakhon Sawan, Phitsanulok, Suphan Buri, Chai Nat, Phetchabun and Phichit provinces (Table 1) and stored at 4 °C.

Table 1 Sugarcane leave samples collected from the farms in seven provinces.

Area	Number of sample	Code No.
NakhonPathom (NP)	3	S01NP,S04NP, S09NP
SuphanBuri (SP)	6	S06SP, S07SP, S08SP, S21SP, S22SP, S23SP
Chai Nat (CN)	3	S24CN, S25CN, S26CN
Nakhon Sawan (NK)	3	S27NK, S28NK, S29NK
Phichit (PC)	1	S30PC
Phitsanulok (PS)	1	S31PS
Phetchabun (PB)	10	S38PB, S44PB, S45PB, S46PB, S47PB, S48PB, S49PB, S50PB, S51PB, S52PB

DNA extraction

Sugarcane leaves (6g) were cut to small size and washed with washing buffer (1X Phosphate buffered saline, Tween 20 by sonicating for 7 min, then centrifuged at 5000 xg for 5 min. The total genomic DNA was extracted from the cell pellet by adding lysis buffer (100 mM Tris-HCl, 0.5% SDS (w/v), 30 mM EDTA, pH 7.5)and incubated for 15 min at 95 °C. Then 2.5 M potassium acetate was added and incubated on ice for 60 min then centrifuged at 12000 xg for 5 min. Genomic DNA was precipitated with isopropanol then washed with 70% ethanol and centrifuged at 12,000 xg for 5 minute. The DNA was dried and resuspended with 30 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) then stored at -20 °C.

PCR amplification and DNA cloning

The D1/D2 domain of the 26S rDNA was amplified using NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') primers. PCRs were performed in a total volume of 25µl, containing 20 pmol of each primer, 200 µM of dNTPs, and 1 U of DNA polymerase. Amplification was performed in a thermal cycler programmed as followed: 94°C for 5 min, then 35 cycles of 94 °C for 45 s, 52 °C for 30 s, and 72°C for45 s, followed by 72°C for 10 min. PCR products were separated by gel electrophoresis on a 0.8% (w/v) agarose gel in 1X TAE buffer (Tris-acetate buffer, EDTA, pH 8.0), detected by ethidium bromide staining. The amplified DNA was purified with GF-1 AmbiClean Kit

INTRODUCTION

Rice is an important industrial crop cultivated throughout Thailand. Plant leaves has been recognized as a source of energy for plant due to the photosynthesis occurs also an important habitat for micro-organisms (Kaewwichian et al., 2013). Among the epiphytes, yeast has been considered as an important microorganism because some can promote plant growth (Limtong and Koowadjanakul, 2012). In addition, they can stimulate the colonization of other epiphytes to suppress infection by plant pathogen (Costa et al., 2008; El-Tarabily and Sivasithamparam, 2006). The diversity of yeast species may change depending on season and crop characteristic (Glushakova and Chernov, 2010). Some yeasts are unculturable, therefore the important members of the composting microbial community may have been missed when using culture-dependent method (Takaku et al, 2006). Epiphytic yeasts have been studied intensively; however, investigation on diversity of epiphytic yeasts of rice based on culture-independent method has less informations. The molecular based methods permit direct evaluation of yeast community, new information of yeast diversity may obtain by characterization of their rRNA gene using cultured-independent techniques. Recently, the molecular biological techniques such as polymerase chain reaction (PCR) (Makimura et al., 1994; Inacio et al., 2005), denaturing gradient gel electrophoresis (DGGE) (Takaku et al, 2006) and restriction fragment length polymorphism (RFLP) (Kachalkin et al., 2008) have been applied to examine microbial community DNA to determine the presence of novel microorganisms (Takaku et al., 2006). Therefore, this study aimed to investigate the diversity of epiphytic yeasts on rice leave samples in Thailand by molecular based methods including PCR, RFLP and sequence analysis based on LSU rRNA gene in the D1/D2 domain.

MATERIALS AND METHODS

Rice leave samples collection

Thirty-three samples of rice leaves were randomly collected from seven provinces in Thailand which are common cultivated rice areas : Surindra (R01SR, R02SR, R03SR), Burirum (R04BR), Srisakade (R05SK, R06SK, R07SK, R08SK, R09SK, R11SK, R15SK), Chachoengsao (R25CC, R26CC, R28CC, R30CC), Nonthaburi (R41NT), Nakornpathom (R16NP, R17NP, R18NP, R42NP, R43NP, R44NP, R46NP, R63NP, R64NP, R65NP, R68NP) and Suphanburi (R57SP, R58SP, R59SP, R60SP, R61SP, R62SP) and kept at 4°C.

DNA extraction of epiphytic yeasts from rice phyllosphere

Rice leaves (6 g) were cut and submerged in 40 ml of washing buffer (1X Phosphate buffered saline, Tween 20), then sonicated for 7 min using ultrasonic cleaning bath (Bransonic, USA) to take out microbes from sample surfaces. The suspension was centrifuged at 5,000 x g for 5 min and the supernatant was discarded. DNA was extracted from the cell pellet according to the method of Makimura et al. (1994) with slight modification. Briefly, the cell pellet was suspended in 100 µl of lysis buffer (100 mM Tris-HCl, 0.5% w/v SDS, 30 mM EDTA, pH 7.5) and incubated at 95°C for 15 min. 2.5 M

potassium acetate (100 µl) was added and incubated on ice for 60 min, then centrifuged at 12,000 x g for 5 min. The supernatant was transfered to new tube and DNA was precipitated with an equal volume of isopropanol, then washed with 70% ethanol. Precipitated DNA was air dried, resuspended in 30 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and stored at -20 °C.

Amplification of D1/D2 domain of LSU rRNA gene

DNA sequence of D1/D2 domain of LSU rRNA gene was amplified by PCR using the following primers : NL-1 (5´-GCA TAT CAA TAA GCG GAG GAA AAG-3´) and NL-4 (5´-GGT CCG TGT TTC AAG ACG G-3´) (Kurtzman and Robnett, 1998). The amplification was carried out in 25 µl of the reaction mixture containing 50 ng of genomic DNA, 1X PCR buffer (Vivantis, USA), 1.5 mM $MgCl_2$ (Vivantis, USA), 100 µM of each dNTP (Vivantis, USA), 20 pmol of each primer, and 1 U of *Taq* DNA polymerase (Vivantis, USA) The reaction was performed as followed: initial DNA denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 52 °C for 30 s and extension at 72°C for 45 s and final extension at 72°C for 10 min. Determination of amplified DNA was performed by electrophoresis using 0.8% (w/v) agarose gel in 1X TAE buffer (Tris-acetate buffer, EDTA, pH 8.0), then stained with ethidium bromide and visualized under UV illuminator. The amplified DNA was purified with Nucleic acid Extraction kit (Vivantis, USA) according to the manufacturer's instruction.

Cloning, colony PCR and RFLP analysis

The purified PCR products were ligated into pTG19-T cloning vector (Vivantis, USA) performed with manufacturer's protocol, and transformed into *E.coli* DH5α competent cells using method of Sambrook et al. (1989). The recombinant clones were screened by colony PCR, manipulated with the same PCR condition described above excepted using cell suspension (2 µl) as template. PCR products with expected size were analyzed with RFLP by digested with the restriction enzymes, *Hin*fI and *Hae*III, then separated in 2% (w/v) agarose gel electrophoresis in 1X TAE buffer. The different RFLP patterns were grouped in operational taxonomic units (OTUs). The inserted DNA of D1/D2 from the representative transformants of each OTUs were subjected to sequence.

Sequence analysis

The sequences of D1/D2 region were submitted to BLASTn homology search in National Centre of Biotechnology Information (NCBI) database, and aligned with the sequences of related species retrieved from GenBank using CLUSTALW 1.6 multiple alignment program. The phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5.2.2 (Tamura et al., 2011) by Kimura 2-parameter model and neighbor-joining method. Bootstrap analysis was performed 1,000 random replications.

RESULTS

The genomic DNA of 33 samples of rice leave phyllosphere were extracted and the D1/D2 domain of LSU rRNA gene were amplified by PCR. The expected size was approximately 600 bp (Figure 1). The PCR products were then cloned into pTG19-T vector and transformed into *E.coli* DH5α and screened by colony PCR. Seven hundred and ninety-eight clones had expected size of inserted DNA (data not shown). RFLP patterns of these PCR products from all positive clones were grouped into 331 OTUs after digested with 2 restriction enzymes, *Hin*fI and *Hae*III.

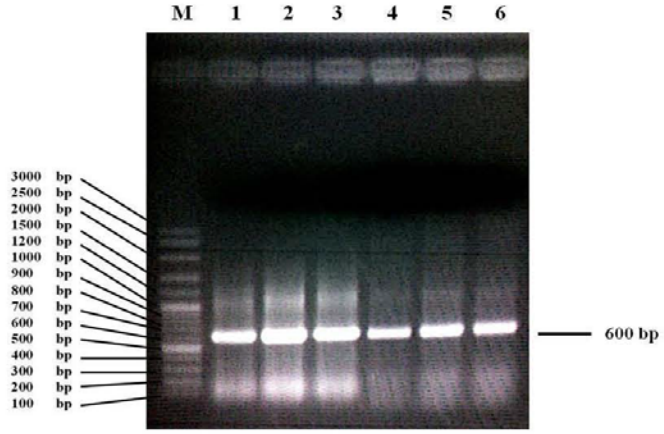


Figure 1 PCR products of D1/D2 domain of LSU rRNA gene of rice phyllosphere samples: lane M, 100 bp DNA ladder (Vivantis, USA); lanes 1-6, PCR products amplified from genomic DNA of rice phyllosphere samples no. R16NP, R17NP, R18NP, R42NP, R43NP and R44NP, respectively.

The sequences of representative transformants of each OTU were analyzed and found that most of them were identified as filamentous fungi (data not shown), while 66 OTUs were identified as yeasts, which were found in 11 genera such as *Bullera, Cryptococcus, Hannaella, Hyphopichia, Metschnikowia, Meyerozyma, Pichia, Pseudozyma, Sporobolomyces, Sporidiobolus* and *Wickerhamomyces*, which could be identified into 18 different species (Table 1). However, yeast was not detected in some samples such as R05SK, R41NT and R44NP. Yeast in the genus *Cryptococcus* had more variable species than others (5 species). These include *Cryptococcus curvatus, C. flavescens, C. flavus, C.cf.celluloyticus* and *C.cf.luteolus*; followed by genus *Pseudozyma* that had 4 species such as *P. antarctica*, *P. aphidis*, *P. hubeiensis* and *P.rugulos.* While, *P. antarctica* was detected in most samples (22 out of 33 samples) followed by *B. japonica* which was found in 9 samples of rice phyllospheres. The percentage of nucleotide substitutions of D1/D2 domain can be used to differentiate the yeast strains (Kurtzman and Robnett, 1998). The conspecific strains generally had fewer than 1% nucleotide substitutions in this domain. From our results, there were 9 sequences that had greater than 1% nucleotide substitutions which were closely related to *Hannaella zeae* (R01SR: 1.67%, R59SP: 5.00% and R65NP: 1.64%), *Pseudozyma aphidis* (R03SR: 7.32%), *Cryptococcus* sp. (R03SR: 1.68%, R43NP: 6.66% and R63NP: 9.20%), *Hyphopichia burtonii* (R16NP: 2.63%), *Bullera japonica* (R16NP: 6.17%), *Sporidiobolus pararoseus* (R25CC: 1.96%), *Pseudozyma antarctica* (R30CC: 1.48%) and *Cryptococcus cf. cellulolyticus* (R60SP: 8.03% and R65NP: 9.57%).

Table 1 Epiphytic yeast on rice phyllosphere samples based on D1/D2 domain of LSU rRNA gene.

Sample no. (+ clone)	Yeast OTUs/ Total OTUs	Number of yeast species	Closet relative species (no. of clone)	Accession no.	%Identity	Substitution	
						No.	%
R01SR (17)	3/10	3	*Cryptococcus flavus* (2)	EU177572.1	99 (597/601)	2	0.33
			Pseudozyma antarctica (1)	JQ650240.1	99 (612/615)	0	0.00
			Hannaella zeae CMT28	JQ754112.1	98 (587/599)	10	1.67
R02SR (18)	2/11	2	*Pseudozyma antarctica* (3)	JQ650240.1	99 (612/615)	0	0.00
			Metschnikowia koreensis (3)	AB435077.1	99 (519/524)	2	0.38
R03SR (14)	5/8	5	*Bullera japonica* (1)	AF444760.2	99 (596/599)	2	0.33
			Pseudozyma aphidis (3)	GU373766.1	92 (552/601)	44	7.32
			Sporobolomyces blumeae (1)	AB279628.1	99 (568/569)	1	0.18
			Pseudozyma sp. (1)	DQ178644.1	99 (609/617)	6	0.97
			Cryptococcus sp. (2)	GQ231513.1	98 (351/357)	6	1.68
R04BR (38)	1/18	1	*Bullera japonica* (6)	AF444760.2	99 (591/595)	1	0.17
R05SK (22)	0/16	-					
R06SK (18)	1/5	1	*Pseudozyma antarctica* (1)	JQ650240.1	99 (599/605)	5	0.83
R07SK (38)	2/16	2	*Pseudozyma antarctica* (7)	JQ650240.1	99 (605/610)	2	0.33
			Pseudozyma hubeiensis (1)	AB566327.1	99 (602/609)	5	0.82
R08SK (24)	1/11	1	*Cryptococcus flavescens* (1)	JQ964205.1	99 (599/607)	4	0.66
R09SK (28)	2/11	2	*Pichia guilliermondii* (2)	EF532298.1	99 (576/580)	2	0.34
			Meyerozyma guilliermondii (3)	HM988686.1	99 (564/566)	1	0.18
R11SK (21)	1/6	1	*Pseudozyma antarctica* (1)	AB566343.1	99 (604/608)	1	0.16
R15SK (34)	4/12	4	*Cryptococcus flavus* (6)	EU177572.1	99 (592/597)	3	0.50
			Wickerhamomyces anomalus (2)	JX049429.1	99 (565/566)	1	0.18
			Cryptococcus curvatus (2)	HQ323253.1	99 (591/596)	2	0.34
			Pseudozyma antarctica (1)	JQ650240.1	99 (603/605)	1	0.17
R16NP (43)	5/30	4	*Pseudozyma antarctica* (2)	AB566343.1	99 (594/599)	2	0.33
			Hyphopichia burtonii (1)	HF952839.1	97 (369/379)	10	2.63
			Wickerhamomyces anomalus (1)	KC494721.1	99 (543/545)	2	0.37
			Bullera japonica (4)	AF444760.2	92 (522/567)	35	6.17
R17NP (31)	2/19	2	*Bullera japonica* (2)	AF444760.2	99 (583/587)	3	0.51
			Cryptococcus flavus (2)	EU177572.1	99 (597/601)	3	0.50
R18NP (27)	3/9	3	*Pseudozyma antarctica* (1)	JQ650240.1	99 (608/612)	3	0.49
			Bullera japonica (16)	AF444760.2	99 (592/597)	2	0.33
			Wickerhamomyces anomalus (1)	JX049429.1	99 (577/579)	0	0.00
R25CC (22)	1/2	1	*Sporidiobolus pararoseus* (21)	HE802531.1	98 (451/460)	9	1.96
R26CC (12)	1/8	1	*Bullera japonica* (1)	AF444760.2	99 (570/571)	0	0.00
R28CC (17)	1/7	1	*Cryptococcus* sp.(4)	AY422719.1	99 (343/348)	3	0.86
R30CC (20)	2/5	2	*Bullera japonica* (12)	AF444760.2	99 (592/594)	0	0.00
			Pseudozyma antarctica (3)	AB566343.1	99 (596/605)	9	1.48
R41NT (18)	0/1	-					
R42NP (17)	1/10	1	*Pseudozyma antarctica* (1)	AB566343.1	99 (611/614)	3	0.49
R43NP (26)	6/13	4	*Pseudozyma rugulosa* (1)	JN940523.1	99 (606/613)	5	0.82
			Pseudozyma antarctica (14)	JQ650240.1	99 (580/582)	1	0.17
			Bullera japonica (1)	AF444760.2	99 (567/572)	4	0.70
			Cryptococcus sp.BT195(1)	EU200789.2	91 (547/601)	40	6.66
R44NP (18)	0/6	-					
R46NP (16)	1/11	1	*Pseudozyma aphidis* (2)	GU373766.1	99 (602/605)	3	0.50
R57SP (15)	1/9	1	*Pseudozyma antarctica* (9)	AB566343.1	99 (598/600)	0	0.00
R58SP (19)	1/3	1	*Pseudozyma antarctica* (11)	JQ650240.1	99 (599/602)	0	0.00
R59SP (21)	3/8	2	*Pseudozyma antarctica* (14)	JQ650240.1	99 (558/561)	3	0.53
			Hannaella zeae (2)	KC494736.1	94 (566/599)	30	5.00
R60SP (35)	2/6	2	*Pseudozyma antarctica* (11)	JQ650240.1	91 (595/601)	3	0.50
			Cryptococcus cf. cellulolyticus (1)	FJ743601.1	99 (531/585)	47	8.03
R61SP (19)	1/5	1	*Pseudozyma antarctica* (11)	AB566343.1	99 (611/612)	1	0.16
R62SP (17)	1/6	1	*Pseudozyma antarctica* (5)	AB566343.1	99 (610/614)	2	0.33
R63NP (19)	2/11	2	*Pseudozyma antarctica* (4)	AB566343.1	96 (579/601)	2	0.33
			Cryptococcus sp.HX-2006C (1)	DQ333279.1	90 (538/598)	55	9.20
R64NP (35)	2/8	2	*Pseudozyma aphidis* (9)	GU373766.1	99 (580/583)	3	0.51
			Pseudozyma antarctica (2)	JQ650240.1	99 (579/581)	2	0.34
R65NP (35)	4/12	4	*Hannaella zeae* (1)	KC494736.1	98 (597/609)	10	1.64
			Cryptococcus cf. luteolus CSDX3-Y1(1)	EF370392.1	88 (533/606)	58	9.57
			Pseudozyma antarctica (3)	JQ650240.1	99 (607/608)	0	0.00
			Sporobolomyces sp.HB1056 (1)	AJ510199.1	99 (600/603)	1	0.17
R68NP (44)	4/18	3	*Pseudozyma antarctica* (8)	AB566343.1	99 (610/613)	1	0.16
			Sporidiobolus pararoseus (1)	JN940727.1	99 (577/585)	2	0.34
			Bullera japonica (1)	AF444760.2	99 (601/609)	5	0.82

The phylogenetic result shown in Figure 2 revealed that the epiphytic yeasts were classified into 4 groups (Group I, II, III and IV). Group I, II and III were identified as basidiomycetous yeasts, while the group IV was ascomycetous yeast which were less common found on rice leaves in this study.

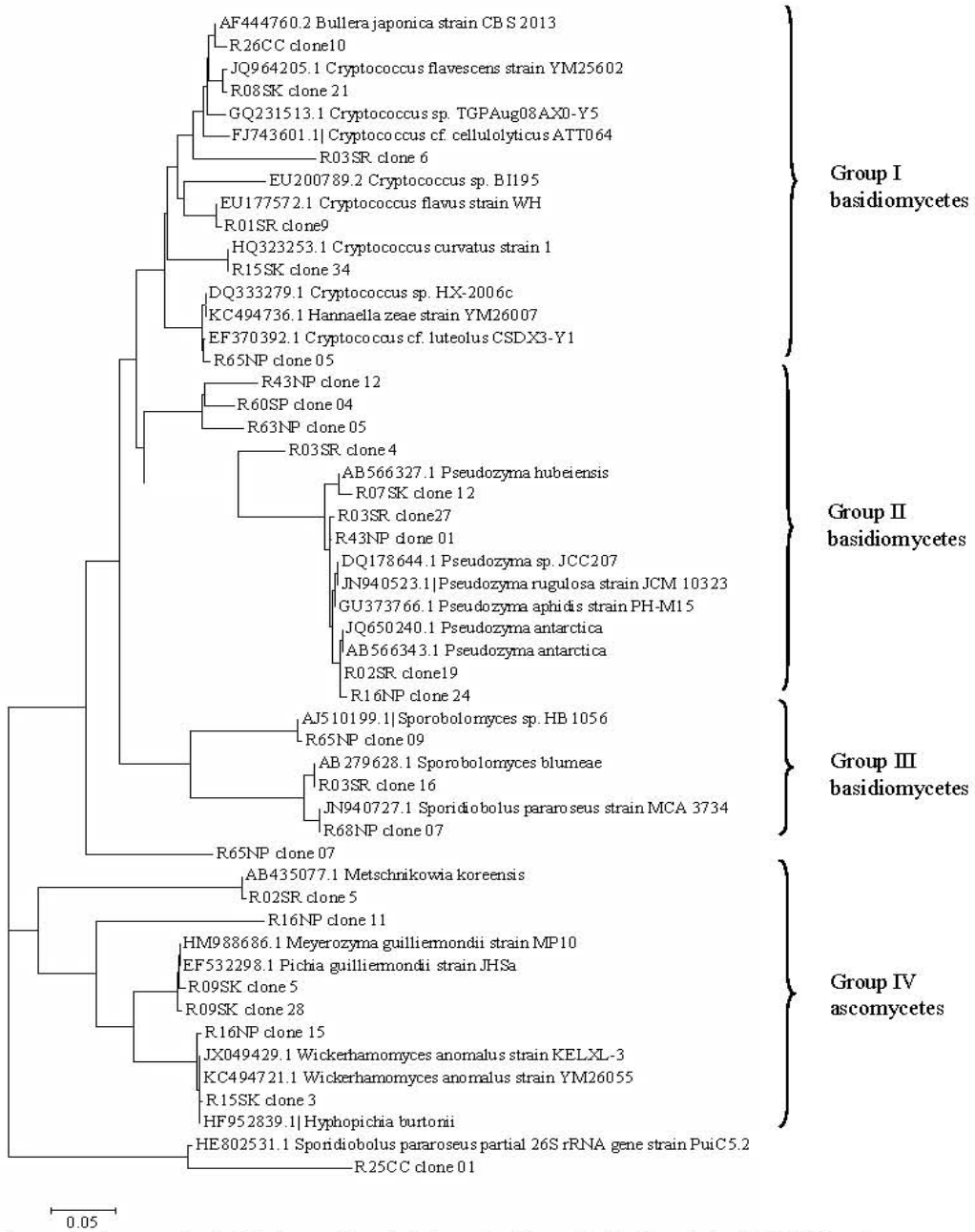


Figure 2 Phylogenetic tree constructed between the related species from GenBank and the D1/D2 domain sequences of representative clones. The scale bar corresponded to a genetic distance of 0.05 substitution per position.

DISCUSSION

From the results of this study we found that most species of epiphytic yeasts identified from rice leave phyllosphere were basidiomycetes which corresponded to the previous study by Glushakova and Chernov (2010) which reported that basidiomycetous yeasts were predominate on plant leaves. The common phylloplane yeasts were basidiomycetous species belonging to the genera *Bullerra, Cryptococcus, Dioszegia, Rhodotorula, Sporobolomyces, Tilletiopsis* and *Trichosporon*. While the other study reported that ascomycetes were most detected from plant phyllosphere by enrichment technique (Limtong and Koowadjanakul, 2012). These yeasts usually inhabit substrates with high concentrations of simple sugars from plant (Glushakova et al., 2007). Whipp (2008) reported that *Cryptococcus*, *Sporobolomyces* and *Rhodotorula* were limited genera found by culture-dependent method. However, they presented in this study by using culture-independent method. This could be an advantage of this technique over the traditional method that can identify the unculturable or hardly-culturable species of microorganisms (Giraffa et al., 2001). In this study, *Pseudozyma* was the most common genus found in rice phyllosphere, which had been reported that it could produce biocontrol agent such as fungicide (Hammami et al., 2010). Furthermore, the nucleotide sequences of D1/D2 domain in some samples were greater than 1% which were likely to be different species compared to the closely related strains. However, this should be investigated for more information.

CONCLUSION

Rice phyllosphere samples were collected from the north-eastern, central and eastern area in Thailand. All of samples were grouped into 331 OTUs and were identified into 11 genera and 18 different species of yeast. They were classified into 4 groups with phylogenetic tree analysis, however, most of them were basidiomycetous yeast. *Pseudozyma* was the most common genera detected in most samples, which may protect plant againt fungal pathogen by producing fungicide. However, this needs to be studied in more detailed.

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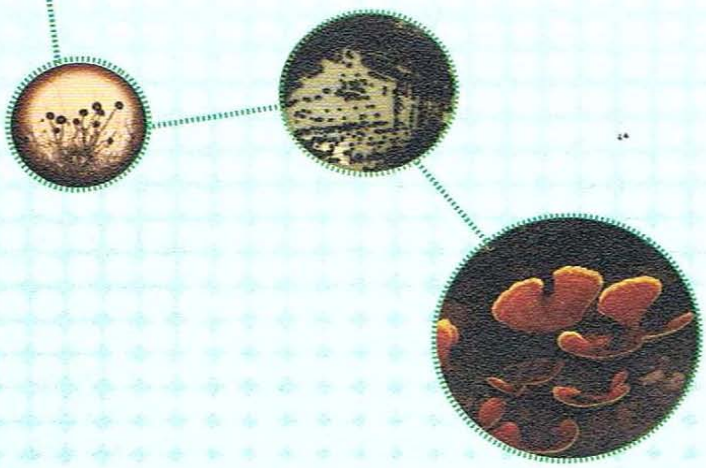
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Investigation on diversity of epiphytic yeasts on rice leaves by culture-dependent method

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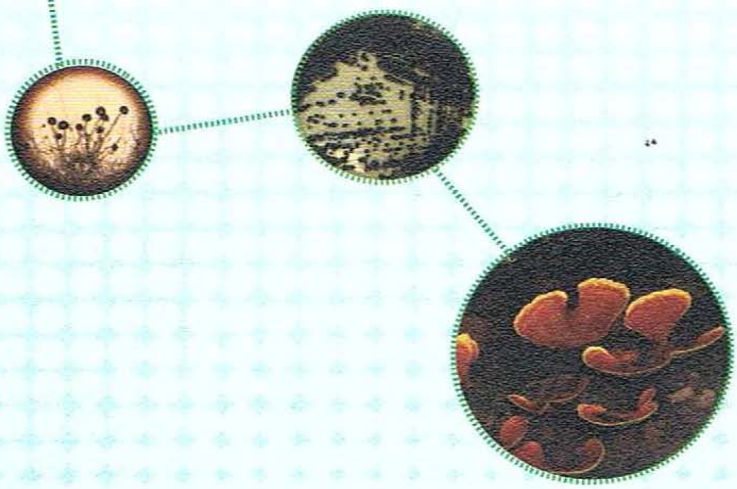
Abstract:
Three hundred and seventy nine isolates of epiphytic yeasts were isolated from 112 rice leaves from 12 provinces in Thailand. Among them, 354 isolates were identified based on the sequences of D1/D2 domain of 26S rDNA. Two hundred and ninety eight isolates (78.6%) were identified as 50 known species in 18 genera of *Bullera* (18 isolates), *Candida* (24 isolates), *Cryptococcus* (22 isolates), *Debaryomyces* (2 isolates), *Hyphopichia* (1 isolates), *Jaminaea* (1 isolates), *Kodamaea* (8 isolates), *Lodderomyces* (1 isolates), *Meyerozyma* (10 isolates), *Pichia* (3 isolates), *Pseudozyma* (125 isolates), *Rhodosporidium* (10 isolates), *Rhodotorula* (30 isolates), *Sporidiobolus* (5 isolates), *Sporobolomyces* (27 isolates). *Torulaspora* (2 isolates), *Trichosporon* (4 isolates) and *Wickerhamomyces* (5 isolates). Twenty one isolates (5.5%) were identified as undescribed species in Candida (1 isolate), *Cryptococcus* (9 isolates), *Pseudozyma* (4 isolates) and *Rhodotorula* (7 isolates). Furthermore, thirty five isolates (9.2%) differed from the nearest species in 4 nucleotides or more in the D1/D2 domain and were considered to represent 20 new species of *Blastobotrys* (1 isolate), *Bullera* (2 isolates), *Candida* (1 isolate), *Cryptococcus* (13 isolates), *Occultifur* (2 isolates), *Pseudozyma* (8 isolates), *Rhodosporidium* (4 isolates), *Rhodotorula* (3 isolates) and *Sakaguchia* (1 isolates) and the remaining 25 isolates (6.6%) are wating for identify. Yeast-like fungi in the genera *Aureobasidium, Acremonium, Cephalosporium, Hyponectria, Melanopsichium, Moesziomyces, Sarocladium, Sporisorium, Syncephalastrum* and *Ustilago* also found on the rice leaves. These epiphytic yeasts will be screened for promising auxins producing strains emphasized on indole-3-acetic acid auxin. The IAA production will also be performed after optimization by response surface methodology.

Keywords: Yeast, epiphytic yeast, diversity

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Biodiversity of epiphytic yeast from corn leaves by culture dependent technique

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Abstract:

Yeast is diverse microorganism and can be found in natural environment. The biodiversity of epiphytic yeasts was investigated from 109 corn leaves which were collected from 11 provinces of Thailand. Two hundred and eighty four isolates of epiphytic yeasts were isolated by culture dependent technique and identification based on comparative sequence analysis of the D1/D2 domain of the LSU rRNA gene. Among them, 149 isolates were identified as described species belonged to 43 species of 15 genera namely *Bandoniozyma* (1 isolate), *Bullera* (19 isolates), *Candida* (10 isolates), *Cryptococcus* (31 isolates), *Dioszegia* (1 isolate), *Hanseniaspora* (2 isolates), *Jaminaea* (1 isolate), *Kodamaea* (3 isolates), *Meyerozyma* (5 isolates), *Pseudozyma* (20 isolates), *Rhodotorula* (3 isolates), *Rhodosporidium* (15 isolates), *Sporidiobolus* (23 isolates), *Sporobolomyces* (14 isolates) and *Wickerhamomyces* (1 isolate). *Sporidiobolus pararoseus* (17 isolates) is the dominant species. Twenty four isolates were identified to be undescribed species in the 6 genera namely *Cryptococcus* (9 isolates), *Occultifur* (2 isolates), *Pseudozyma* (2 isolates), *Rhodotorula* (7 isolates), *Sporidiobolus* (2 isolates), *Sporobolomyces* (2 isolates). From this study, 16 isolates were identified as novel yeast species belonged to genus *Bullera* (4 isolates), *Cryptococcus* (7 isolates), *Occultifur* (1 isolate), *Rhodotorula* (2 isolates), *Rhodosporidium* (1 isolate) and *Sporidiobolus* (1 isolate). These yeasts were studied by polyphasic approaches for described and proposed novel yeast species. The remaining 42 isolates are in the process of identification. Furthermore, 53 isolates are expected to be yeast by cells and colony morphological characteristics but the sequence analysis of the D1/D2 region supported to identified as fungi that are known or new species in 7 genera namely *Acremonium* or *Cephalosporium* (1 isolate), *Aureobasidium* (8 isolates), *Kabatiella* (1 isolate), *Moesziomyces* (2 isolates), *Sarocladium* (1 isolate), *Sporisorium* (8 isolates) and *Ustilago* (32 isolates).

Keywords: Biodiversity, epiphytic yeasts, corn leaves

O-07-011

Identification of Epiphytic Yeast from Sugarcane Phyllospheres Using PCR and RFLP Techniques

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ABSTRACT

Phyllosphere, the external surface of plant leaves, has been recognized as an important habitat for microorganisms. The phyllosphere is the well-suited model system to study the basic principles of ecology and oxygen exposure for plant photosynthesis. Molecular genetics technique has been widely used to study microbial diversity due to high accuracy and less time consuming. In this research, we aimed to study the diversity of epiphytic yeast on the sugarcane phyllosphere. The sugarcane leaves were collected from several areas in Thailand, such as Nakorn Pathom, NakhonSawan, Phitsanulok, Suphan Buri, Chai Nat, Phetchabun and Phichit provinces. The genomic DNA samples were extracted from phyllospheres of 27 sugarcane leaf samples. Yeast strains were identified based on sequencing analysis of the D1/D2 region on 26S rRNA gene with polymerase chain reaction (PCR). The recombinant clones of amplified products were screened by colony PCR method. The restriction fragment length polymorphism (RFLP) patterns of PCR products digested with *Hae*III and *Hin*fI were analyzed. The results showed that the RFLP patterns of all samples were grouped into 189 operational taxonomic units (OTUs). The nucleotide sequence of each OTU was analyzed and compared to the NCBI database using Blastn. From 27 samples of sugarcane leaves, there were three yeast genera, *Cryptococcus*, *Pseudozyma* and *Jaminaea* which were classified into 6 different species.

Keywords: phyllosphere, D1/D2, RFLP, sugarcane leaves, epiphytic yeast

INTRODUCTION

Microorganisms can inhabit external surface of plant leaves by using leaf surface as their nutrients such as carbohydrates, amino acids, organic acids and sugar alcohols. Yeast is a microorganism found in sugarcane. Some of them can promote plant growth and plant protection from pathogen (Vorholt, 2012) which can be used as bio-control for agriculture (De Curtis *et al.*, 2012).

Culture-independent technique has been at present used to study microbial diversity due to its high accuracy and less time consuming by extraction of DNA directly from cells without the requirement to culture microorganisms in the laboratory, also avoiding problems associated with the differential growth rates (Hirsch *et al.*, 2010). The nucleic acid-based molecular methods, in particular the polymerase chain reaction (PCR) has been widely used to investigate the microbial diversity by amplifying the target DNA sequence such as the large subunit ribosomal DNA (Saksinchai *et al.*, 2012). In addition, the DNA sequences were differentiated by restriction fragment length polymorphism (RFLP) (Mu *et al.*, 2012). The large subunit (LSU) of rRNA gene provides identification of yeasts with varying degrees of certainty to the genus, species or sub-species level (Hirsch *et al.*, 2010).

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The studies of epiphytic yeast diversity from phyllosphere were undertaken. However, until present there is less information of the diversity and ecology of epiphytic yeasts in Thailand. Therefore this research aimed to determine the diversity of epiphytic yeasts on sugarcane phyllosphere collected from several areas in Thailand and carried out with culture-independent techniques, PCR and RFLP. The results of nucleotide sequence were compared to the GenBank database, National Center for Biotechnology Information (NCBI) using Blastn to specify the yeast species, in order to evaluate the diversity of epiphytic yeasts on the sugarcane phyllospheres.

MATERIALS AND METHODS

Samples

Twenty-seven samples of sugarcane leaves were collected from the agricultural fields in several areas in Thailand, such as Nakorn Pathom, Nakhon Sawan, Phitsanulok, Suphan Buri, Chai Nat, Phetchabun and Phichit provinces (Table 1) and stored at 4 °C.

Table 1 Sugarcane leave samples collected from the farms in seven provinces.

Area	Number of sample	Code No.
NakhonPathom (NP)	3	S01NP,S04NP, S09NP
SuphanBuri (SP)	6	S06SP, S07SP, S08SP, S21SP, S22SP, S23SP
Chai Nat (CN)	3	S24CN, S25CN, S26CN
Nakhon Sawan (NK)	3	S27NK, S28NK, S29NK
Phichit (PC)	1	S30PC
Phitsanulok (PS)	1	S31PS
Phetchabun (PB)	10	S38PB, S44PB, S45PB, S46PB, S47PB, S48PB, S49PB, S50PB, S51PB, S52PB

DNA extraction

Sugarcane leaves (6g) were cut to small size and washed with washing buffer (1X Phosphate buffered saline, Tween 20 by sonicating for 7 min, then centrifuged at 5000 ×g for 5 min. The total genomic DNA was extracted from the cell pellet by adding lysis buffer (100 mM Tris-HCl, 0.5% SDS (w/v), 30 mM EDTA, pH 7.5)and incubated for 15 min at 95 °C. Then 2.5 M potassium acetate was added and incubated on ice for 60 min then centrifuged at 12000 ×g for 5 min. Genomic DNA was precipitated with isopropanol then washed with 70% ethanol and centrifuged at 12,000 ×g for 5 minute. The DNA was dried and resuspended with 30 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) then stored at -20 °C.

PCR amplification and DNA cloning

The D1/D2 domain of the 26S rDNA was amplified using NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') primers. PCRs were performed in a total volume of 25µl, containing 20 pmol of each primer, 200 µM of dNTPs, and 1 U of DNA polymerase. Amplification was performed in a thermal cycler programmed as followed: 94°C for 5 min, then 35 cycles of 94 °C for 45 s, 52 °C for 30 s, and 72°C for45 s, followed by 72°C for 10 min. PCR products were separated by gel electrophoresis on a 0.8% (w/v) agarose gel in 1X TAE buffer (Tris-acetate buffer, EDTA, pH 8.0), detected by ethidium bromide staining. The amplified DNA was purified with GF-1 AmbiClean Kit

(Vivantis, USA). The purified DNA was ligated into pTG19-T cloning vector (Vivantis, USA) and transformed into competent *E. coli* DH5α cells.

Colony PCR analysis

Cell suspension of recombinant clones were attenuated with 25 µl of DI water, and 2 µl of cell suspension was used as template. NL1 and NL4 primers were used. A thermo-cycling amplification program as followed: 94°C for 10 min, then 35 cycles of 94 °C for 45 s, 52 °C for 30 s, and 72 °C for 45 s, followed by 72 °C for 10 min. Colony PCR products were separated by gel electrophoresis on a 0.8% (w/v) agarose gelin 1X TAE buffer, and detected by ethidium bromide staining.

RFLP analysis

Colony PCR products were separately digested with 10U of restriction enzymes *Hae*III, and *Hin*fI (Biolabs, USA). The resulting DNA fragments were visualized by electrophoresis on 2% (w/v) agarose gel in 1X TAE buffer. The operational taxonomic units (OTUs) were determined from the RFLP results.

Sequencing analysis

The sequences of each OTU were analyzed and compared to the GenBank nucleotide data library using the Blastn software at the NCBI website (http://www.ncbi.nlm.nih.gov).The phylogenetic tree was constructed using MEGA5.2.2 (Tamura *et al.*, 2011) by Kimura 2-parameter model and neighbor-joining method. Bootstrap analysis was performed 1,000 random replications.

RESULTS

D1/D2 domain of LSU rDNA gene amplification

The expected DNA fragment by PCR was approximately 600 bp (Figure 1). The purified DNA fragment were ligated with pTG19-T cloning vector and transformed into competent *E. coli* DH5α cell and the recombinant clones were further screened by colony PCR using the same primers.

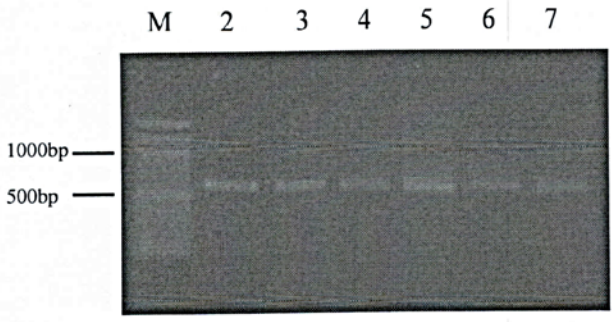


Figure 1 PCR products of the D1/D2 region of LSU rRNA gene: lane M; 100 bp DNA marker, lanes 2-7;S01NP, S04NP, S06SP, S07SP, S08SP, S09NP respectively.

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RFLP and sequence analysis

RFLP patterns of PCR products digested with *Hae*III and *Hin*fI were analyzed. The results showed that the RFLP patterns of all samples were grouped into 189OTUs. However, only 21OTUs were identified as yeast species which related to three genera such as, *Cryptococcus*, *Pseudozyma* and *Jaminaea*. These genera were classified into 6 different species, which were *C. flavus*, *P. rugulosa, P. prolifica*, *P. aphidis*, *P. hubeiensis* and *J. angkoriensis* as shown in Table 2. The common yeast genus found in sugarcane samples was *Pseudozyma* which classified into 4 different species. These included *P. rugulosa, P. prolifica, P. aphidis and P. hubeiensis. P. prolific* was detected in most samples (8 out of 12 samples). The nucleotide substitution of the D1/D2 domain on LSU rRNA gene was used to distinguish the different yeast strains. In general, the nonspecific strains had less than 1% nucleotide substitution (Kurtzman and Robnett, 1998). In this study, there were 9 sequences that had percentage of nucleotide substitutions greater than 1%. These sequences were closely related to *P. prolifica* (S21SP: 10.6%, S23SP: 5.7%, S24CN: 1.2%, S25CN:1.5%, S28NK: 1.9%, S30PC: 1.7%, S38PB: 1.2% and S44PB: 1.2%) and *C. flavus* (S22SP: 1.3%). These sequences may represent the sequences of new yeast species.

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Table 2 Epiphytic yeasts from sugarcane phyllospheres by sequencing analysis of the D1/D2 region on LSU rRNA gene

Sample no. (no. of clone)	Yeast OUT /total OTU	No. of yeast species	Closest relative yeast (no. of clone)	Accession no.	Identity (%)	Substitution No.	%
S01NP(25)	0/3	0	-	-	-	-	-
S04NP(18)	0/5	0	-	-	-	-	-
S06SP(16)	0/7	0	-	-	-	-	-
S07SP(18)	0/6	0	-	-	-	-	-
S08SP(13)	0/4	0	-	-	-	-	-
S09NP(24)	0/10	0	-	-	-	-	-
S21SP(18)	4/8	2	*Cryptococcus flavus*CBS 331^{T}(3)	AF075497.1	99(586/589)	3	0.5
			*Pseudozymaprolifica*CBS 319.87^{T}(1)	AJ235298.1	88(507/576)	61	10.6
S22SP(18)	3/5	3	*Cryptococcus flavus*CBS 331^{T}(9)	AF075497.1	98(593/603)	8	1.3
			*Pseudozymaaphidis*JCM10318^{T}(1)	AB089363.1	99(565/571)	4	0.7
			*Pseudozymarugulosa*CBS 170.88^{T}(2)	AJ235300.1	99(558/563)	4	0.7
S23SP(26)	1/8	1	*Pseudozymaprolifica*CBS 319.87^{T}(10)	AJ235298.1	94(540/577)	33	5.7
S24CN(19)	1/2	1	*Pseudozymaprolifica*CBS 319.87^{T}(15)	AJ235298.1	99(566/574)	7	1.2
S25CN(37)	3/11	3	*Pseudozymaaphidis* JCM10318^{T}(6)	AB089363.1	99(560/563)	2	0.4
			*Jaminaea angkoriensis*CBS 10918^{T}(2)	EU587489.1	99(509/514)	4	0.8
			*Pseudozymaprolifica*CBS 319.87^{T}(1)	AJ235298.1	98(572/582)	9	1.5
S26CN(21)	0/12	0	-	-	-	-	-
S27NK(19)	1/8	1	*Pseudozymaaphidis*JCM10318^{T}(3)	AB089363.1	97(560/563)	2	0.4
S28NK(30)	2/19	2	*Pseudozymahubeiensis* CBS 10077^{T}(2)	DQ008953.1	99(587/590)	3	0.5
			Pseudozymaprolifica CBS 319.87^{T}(1)	AJ235298.1	98(575/587)	11	1.9
S29NK(18)	0/5	0	-	-	-	-	-
S30PC(27)	2/17	2	*Pseudozymaaphidis*JCM10318^{T}(1)	AB089363.1	97(560/563)	2	0.4
			*Pseudozymaprolifica*CBS 319.87^{T}(2)	AJ235298.1	98(570/582)	10	1.7
S31PC(21)	0/11	0	-	-	-	-	-
S38PB(22)	1/7	1	*Pseudozymaprolifica* CBS 319.87^{T}(1)	AJ235298.1	99(571/578)	7	1.2
S44PB(24)	1/3	1	*Pseudozymaprolifica*CBS 319.87^{T}(1)	AJ235298.1	98(577/586)	7	1.2
S45PB(36)	1/4	1	*Jaminaea angkoriensis*CBS 10918^{T}(6)	EU587489.1	99(587/595)	4	0.7
S46PB(28)	0/8	0	-	-	-	-	-
S47PB(25)	0/4	0	-	-	-	-	-
S48PB(34)	1/3	1	*Pseudozymaprolifica*CBS 319.87^{T}(13)	AJ235298.1	99(567/574)	5	0.9
S49PB(39)	0/3	0	-	-	-	-	-
S50PB(24)	0/7	0	-	-	-	-	-
S51PB(33)	0/4	0	-	-	-	-	-
S52PB(25)	0/5	0	-	-	-	-	-

Phylogenetic analyses

Phylogenetic tree of recombinant sequences and the related yeast species selected from Genbank was constructed using neighbor-joining analysis based on the 26S rDNA D1/D2 region sequence data (Figure 2). Epiphytic yeasts identified in this study were classified into 3 groups, which were identified as basidiomycetous yeasts, such as *Pseudozyma*, *Jaminaea* and *Cryptococcus*.

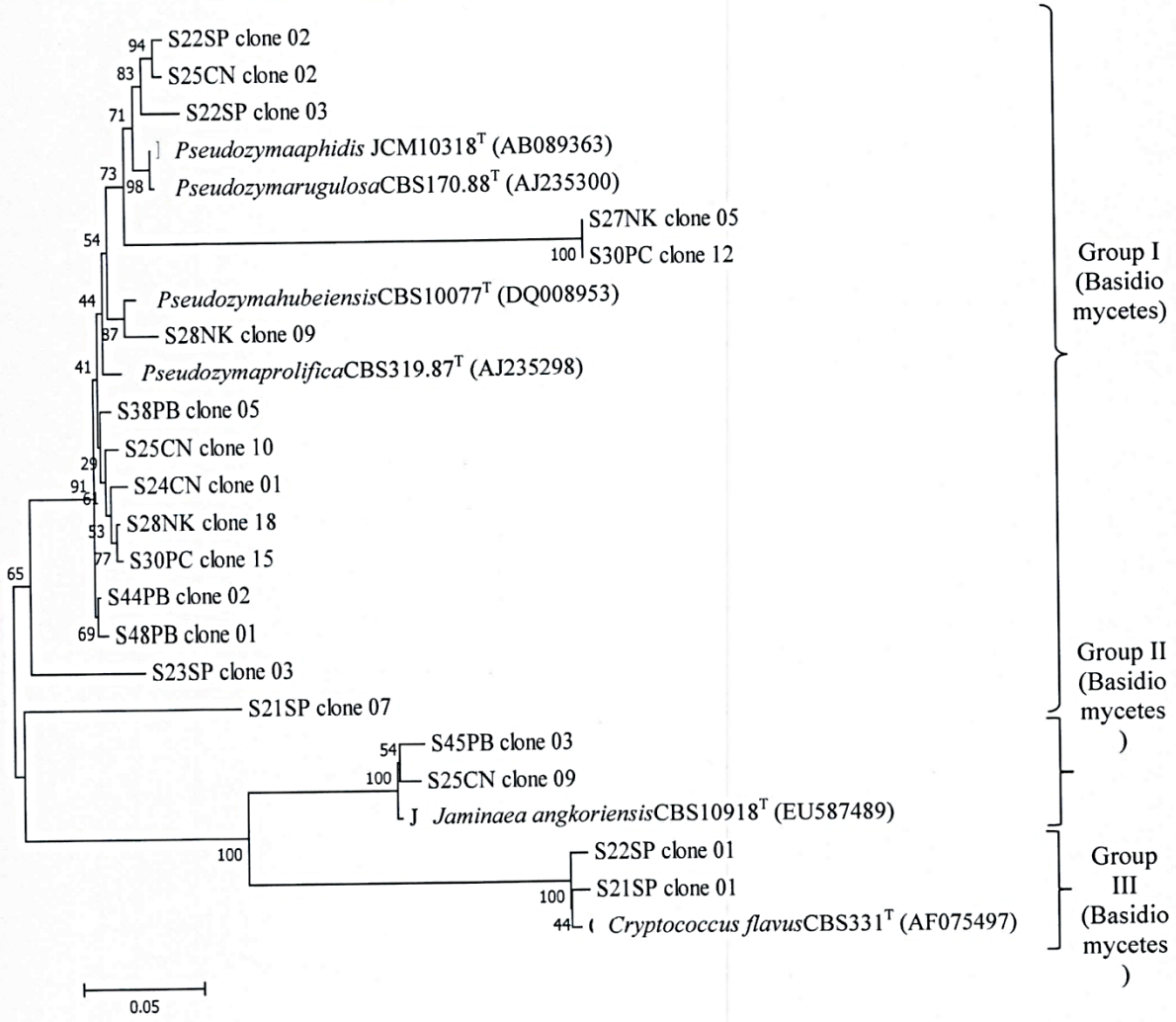


Figure 2 Phylogenetic tree of recombinant sequences and the related yeast species selected from Genbank using MEGA 5.2.2 Phylogeny Analysis software. The bar indicates the sequence dissimilarity value of 0.05 substitutions per site.

DISCUSSION

In this study, twenty-seven samples of sugarcane leaves were randomly collected from several areas in Thailand to determine the diversity of epiphytic yeasts. Among the total samples, the epiphytic yeasts were detected from 12 samples. However, most of the microorganism found was identified as filamentous fungi (data not shown). All of the identified yeast was in basidiomycota based on the sequence of D1/D2 domain on 26S rRNA gene. Three genera were found in this study such as *Cryptococcus*, *Jaminaea* and *Pseudozyma*. This result corresponded to the previous study by Glushakova *et al.* (2010) which reported that the dominant yeasts on plant phyllospheres were basidiomycetous yeast. The common epiphytic yeasts reported by Sampaio *et al.* (2012) were in genera *Cryptococcus, Rhodotorula and Sporobolomyces*. Recently, the epiphytic yeasts from sugarcane phyllosphere in Thailand were investigated by the culture-dependent technique, which could isolate the novel yeast species such as *Pseudozymavetiver* sp. nov. (Chamnanpa *et al.*, 2013) and *Wickerhamomyces siamensis*sp. nov. (*Kaewwichian et al.* 2013). In this research, *Pseudozyma* was the most common genus detected in most sugarcane samples. This yeast may have a potential for bio-control as there were the reports indicated that yeast in genera *Cryptococcus* and *Pseudozyma* could be used as bio-control for agriculture (Hammami *et al.*, 2010; Zhang *et al.*, 2013). Furthermore, Rau *et al.* (2005) reported that *P. aphidis* could produce mannosylerythritol which is biosurfactant that could be used for industrial purposes. However, the culture-dependent technique needs to be incorporated if the promising cultures would be preferred for their further applications. Moreover, we detected 9 sequences of D1/D2 domain with greater than 1% nucleotide substitution compared to those of the closet relative yeast strains. These could be new yeast strains detected in this study.

CONCLUSION

In conclusion, we found three genera of yeast such as *Cryptococcus*, *Pseudozyma* and *Jaminaea* which were classified into 6 different species such as *C. flavus*, *P. rugulosa, P. prolifica*, *P. aphidis*, *P. hubeiensis* and *J. angkoriensis* from 27 samples of sugarcane phyllospheres. *Pseudozyma* was the most common genera in samples. Phylogenetic tree divided into three groups, which all of them were classified in basidiomycota.

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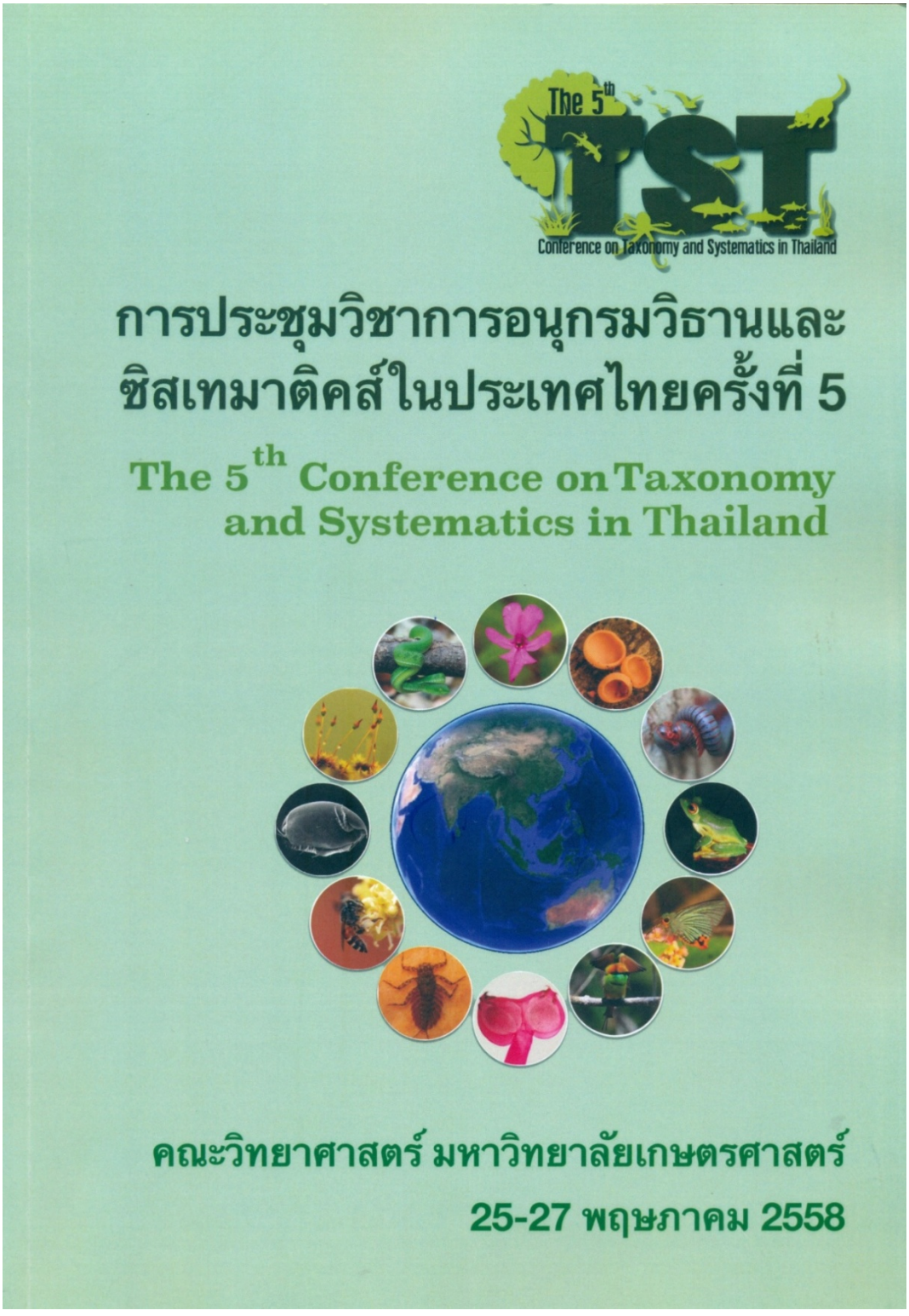
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การประชุมซิสเทมาติคส์ในประเทศไทยครั้งที่ 5

คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ในระหว่าง 25-27 พฤษภาคม 2558

1. จันทร์จีรา สุรัสวดี, นันทนา สีสุข และสาวิตรี ลิ่มทอง ความหลากหลายของยีสต์อีพิไฟต์บนใบอ้อยและการอธิบายยีสต์สปีชีส์ใหม่ความ
2. ศศิธร จินดามรกฎ, สมจิต อ่ำอินทร์และสาวิตรี ลิ่มทอง ความหลากหลายของยีสต์อีพิไฟต์บนใบข้าว
3. สมจิต อ่ำอินทร์, ศศิธร จินดามรกฎ และสาวิตรี ลิ่มทอง การวิจัยความหลากหลายของยีสต์อีพิไฟต์บนใบข้าวโพดในประเทศไทย
4. 

ตารางการประชุมและการนำเสนอแบบบรรยาย
วันอังคารที่ 26 พฤษภาคม 2558 (ห้อง 307 อาคารวิทยาศาสตร์ 45 ปี)

กลุ่มจุลินทรีย์

ดำเนินการ โดย อ.ดร. ปราโมทย์ ชำนาญปืน

อ.ดร. นพรัตน์ สระแก้ว

		หน้า
09.15-09.45 น.	**การค้นพบสายพันธุ์ใหม่ สารออกฤทธิ์ทางชีวภาพชนิดใหม่ และการส่งเสริมการเจริญของพืชจากแอคติโนมัยสีทเอนโดไฟต์** อรินทิพย์ ธรรมชัยพิเนต	22
09.45-10:00 น.	**การอธิบายลักษณะทางอนุกรมวิธานของ *Aliquandostipite manochii*: ราน้ำจืดชนิดใหม่ในประเทศไทย** วีระ ศรีอินทร์สุทธิ์, นัฐวุฒิ บุญยืน, สาทินี ซื่อตรง, Ka Lai Pang และ E.B. Gareth Jones	8
10:00-10:15 น.	**การศึกษาความหลากหลายทางชีวภาพของราไมคอร์ไรซาที่อาศัยอยู่บริเวณรากของต้นยางนาด้วยเทคนิค PCR-DGGE** ณัฐวุฒิ วิริยะธนาวุฒิวงษ์, กฤษชนะ นิสสะ และสมฤดี ตะเคียนเกลี้ยง	10
10:15-10:30 น.	**รับประทานอาหารว่าง**	

ดำเนินการ โดย รศ.ดร. วรรณวิภา วงศ์แสงนาค

อ.ดร. นิตยา สมทรัพย์

10:30-10:45 น.	**ความหลากหลายของยีสต์อีพิไฟต์บนใบอ้อยและการอธิบายยีสต์สปีชีส์ใหม่ความหลากหลายของยีสต์อีพิไฟต์บนใบอ้อยและการอธิบายยีสต์สปีชีส์ใหม่** จันทร์จีรา สุรัสวดี, นันทนา สีสุข และสาวิตรี ลิ่มทอง	12
10:45-11:00 น.	**ความหลากหลายของยีสต์อีพิไฟต์บนใบข้าว** ศศิธร จินดามรกฎ, สมจิต อ่ำอินทร์ และสาวิตรี ลิ่มทอง	14
11:00-11:15 น.	**การวิจัยความหลากหลายของยีสต์อีพิไฟต์บนใบข้าวโพดในประเทศไทย** สมจิต อ่ำอินทร์, ศศิธร จินดามรกฎ และสาวิตรี ลิ่มทอง	16
11:15-11:30 น.	***Graphis koratensis* Pitakpong, Kraichak, Lücking sp. nov ไลเคนส์ชนิดใหม่ของสกุล *Graphis* (Graphidaceae, Ascomycota) ในประเทศไทย** อรัณย์ภัค พิทักษ์พงษ์, เอกพันธ์ ไกรจักร์, ขวัญเรือน บุตรสาทร พาป้อง, หนูเดือน เมืองแสน, พงศ์เทพ สุวรรณวารี, เอช โทรสเทน ลุมช และโรเบิร์ต ลุคกิง	18

25 – 27 May 2015
Faculty of Science, Kasetsart University
OM03

Diversity of epiphytic yeasts in sugarcane phylloplane and description of new yeast species

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This study aimed to investigate the diversity of epiphytic yeasts in sugarcane phylloplane in Thailand by culture-dependent technique. A total of 84 sugarcane leaf samples were collected from 10 provinces. Yeasts were isolated from the surface of leaves by dilution plating of leaf washings and 173 strains were obtained. Identification based on molecular taxonomy by analysis of the D1/D2 region of the large subunit rRNA gene sequence revealed that 87 strains (50.3%) were identified as known yeast species consisted of 61 basidiomycetous yeast strains (35.3%) and 26 ascomycetous yeast strains (15%). Known basidiomycetous yeast species were in 10 genera including *Pseudozyma*, *Cryptococcus*, *Rhodotorula*, *Sporobolomyces*, *Hannaella*, *Dioszegia*, *Jaminaea*, *Occultifur*, *Rhodosporidium* and *Tremella*, and ascomycetous yeast were in *Candida, Kodamaea* and *Meyerozyma.* Sixty seven strains could new or known yeast species. Moreover, 20 strains were found to represent 12 novel yeast species. So far based on polyphasic taxonomy they were proposed as *Bulleromyces siamensis* sp. nov., *Hannaella phyllophyla* sp. nov., *Occultifur tropicalis* sp. nov., *Papiliotrema siamense* sp. nov., *Sporobolomyces siamensis* sp. nov., *Tremella phyllophyla* sp. nov., and *Wickerhamiella siamensis* sp. nov. The most prevalent yeast species was *Meyerozyma carribbica* (20.2% of sugarcane leaf samples).

25 – 27 May 2015
Faculty of Science, Kasetsart University
OM04

Diversity of epiphytic yeasts on rice leaves

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Three hundred and fifty-four isolates of epiphytic yeasts were isolated from 112 rice leaves from 12 provinces in Thailand. They were identified based on the sequences of D1/D2 domain of 26S rDNA. Three hundred and two isolates (85.3%) were identified as 51 known species in 19 genera of *Blastobotrys*, *Bullera*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Hyphopichia*, *Jaminaea*, *Kodamaea*, *Lodderomyces*, *Meyerozyma*, *Pichia*, *Pseudozyma*, *Rhodosporidium*, *Rhodotorula*, *Sporidiobolus*, *Sporobolomyces*, *Torulaspora*, *Trichosporon* and *Wickerhamomyces*. *Pseudozyma antractica* is the dominant species. Twenty-one isolates (5.9%) were identified as undescribed species in 4 genera of *Candida*, *Cryptococcus*, *Pseudozyma* and *Rhodotorula*. Furthermore, thirty-one isolates (8.8%) differed from the nearest species by 1% nucleotide substitutions or more in the D1/D2 domain and were considered to represent 20 new species of *Bullera*, *Candida*, *Cryptococcus*, *Occultifur*, *Pseudozyma*, *Rhodosporidium*, *Rhodotorula* and *Sakaguchia*. The new species were characterized using polyphasic taxonomy and the name *Yamadazyma nontaburiensis* sp. nov. was proposed for the strain YE170. These epiphytic yeasts will be screened for promising auxins producing strains emphasize on indole-3-acetic acid. IAA production will also be measured after optimization by response surface methodology.

25 – 27 May 2015
Faculty of Science, Kasetsart University
OM05

Investigation on Diversity of Epiphytic Yeasts on Corn Leaves in Thailand

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The diversity of epiphytic yeasts was investigated from 109 corn leaves which were collected from 11 provinces in Thailand. One hundred and ninety isolates of epiphytic yeasts, isolated using a culture-dependent method were identified based on comparative sequence analysis of the D1/D2 domain of the LSU rRNA gene. Among them, 151 isolates were identified as known species belonging to 43 species of 15 genera including *Bullera*, *Candida*, *Cryptococcus*, *Dioszegia*, *Hanseniaspora*, *Jaminaea*, *Kodamaea*, *Meyerozyma*, *Occultifur*, *Pseudozyma*, *Rhodosporidium*, *Rhodotorula*, *Sporidiobolus*, *Sporobolomyces* and *Wickerhamomyces*. The dominant species was *Sporidiobolus pararoseus* (17 isolates). Eighteen isolates were identified as undescribed species of *Cryptococcus*, *Pseudozyma* and *Rhodotorula*. In this study, 21 isolates were identified as novel yeast species belonging to six genera including *Bullera*, *Cryptococcus*, *Occultifur*, *Rhodosporidium*, *Rhodotorula* and *Sporidiobolus*. These yeasts were studied by polyphasic taxonomy for describe and propose as novel yeast species.

ภาคผนวก 5

การจัดประชุมเมธีวิจัยอาวุโส สกว

1. จัดการประชุมเมธีวิจัยอาวุโส สกว ครั้งที่ 1 วันที่ 23 สิงหาคม 2556 คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ จตุจักร กรุงเทพมหานคร มีผู้เข้าประชุม 72 คน
2. จัดการประชุมเมธีวิจัยอาวุโส สกว ครั้งที่ 2 วันที่ 13 มิถุนายน 2557 คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ จตุจักร กรุงเทพมหานคร มีผู้เข้าประชุม 60 คน
3. จัดการประชุมเมธีวิจัยอาวุโส สกว ครั้งที่ 2 วันที่ 3 กันยายน 2558 คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ จตุจักร กรุงเทพมหานคร มีผู้เข้าประชุม 81 คน

การประชุมวิชาการ เมธีวิจัยอาวุโส สกว.

ศาสตราจารย์ ดร.สาวิตรี ลิ่มทอง

เรื่อง ความหลากหลายและนิเวศวิทยาของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์ จากใบของพืชเศรษฐกิจในประเทศไทย และการผลิตสารส่งเสริมการเจริญพืชออกซินโดยสายพันธุ์ที่คัดเลือกว่ามีประสิทธิภาพสูงพร้อมกับการศึกษาชีววิถีการสังเคราะห์

(โครงการวิจัยเลขที่ RTA5480009)

วันศุกร์ที่ 23 สิงหาคม 2556 เวลา 8.45-16.30 น.

ณ ห้อง 202 อาคารทวี ญาณสุคนธ์ คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ จตุจักร กรุงเทพมหานคร

8.45-9.00 น.	ลงทะเบียน
9.00-12.00	ผศ. ดร. นันทนา สีสุข ผู้ดำเนินรายการ
9.00–9.15 น.	สรุปภาพรวมของโครงการเลขที่ RTA5480009 An overview of Project No. RTA5480009 ศ.ดร.สาวิตรี ลิ่มทอง ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์
9.15–9.45 น.	ประธาน: ดร.มณี ตันติรุ่งกิจ นางสาวรุจิราลัย พูลทวี เลขานุการ ความหลากหลายของยีสต์บนผิวใบพืช การค้นพบ และการเสนอยีสต์สปีชีส์ใหม่ (Diversity of phylloplane yeasts, discovery and proposal of novel yeast species) ดร.รุ่งลักษณ์ แก้ววิเชียร ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ และศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ
9.45–10.15 น.	การวิจัยความหลากหลายของยีสต์อีพิไฟต์บนใบข้าวด้วยวิธีการเพาะเลี้ยงเชื้อ (Investigation on diversity of epiphytic yeasts on rice leaves by culture-dependent method) ดร.ศศิธร จินดามรกฎ ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ
10.15-10.30น.	พักรับประทานอาหารว่าง
10.30 –11.00 น.	ประธาน: ดร.มณี ตันติรุ่งกิจ นางสาวรุจิราลัย พูลทวี เลขานุการ การวิจัยความหลากหลายของยีสต์อีพิไฟต์บนใบข้าวโพดด้วยวิธีการเพาะเลี้ยงเชื้อ (Investigation on diversity of epiphytic yeasts on corn leaves by culture-dependent method) นางสาวสมจิต อ่ำอินทร์ ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ
11.00– 11.30 น.	การวิจัยความหลากหลายของยีสต์อีพิไฟต์บนใบอ้อยด้วยวิธีการเพาะเลี้ยงเชื้อและการประเมินความสามารถในการควบคุมราโรคพืช (Investigation on diversity of epiphytic yeasts on sugarcane leaves by culture-dependent method and evaluate to control plant pathogenic fungi) นางสาวจันทร์จีรา สุรัสวดี ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์

11.30-12.00 น.	การวิจัยความหลากหลาย ของยีสต์เอนโดไฟต์ ในใบข้าว ข้าวโพด และอ้อย ด้วยวิธีการเพาะเลี้ยงเชื้อและการประเมินความสามารถในการควบคุมราโรคพืช (Investigation on diversity of endophytic yeasts from rice, sugarcane and corn leaves by culture-dependent method and evaluate to control plant pathogenic fungi) นางสาวพัณณิดา ขุนนามวงษ์ ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์
12.00-13.00 น.	พักรับประทานอาหารกลางวัน
13.00-15.30	นางสาววนัชพร บุญธรรม ผู้ดำเนินรายการ
13.00-13.30 น.	ประธาน: ศ.ดร.สาวิตรี ลิมทอง นางสาวพัณณิดา ขุนนามวงษ์ เลขานุการ การวิจัยความหลากหลายของยีสต์เอนโดไฟต์ไฟต์ในใบพืชเศรษฐกิจด้วยวิธีการไม่เพาะเลี้ยงเชื้อ (Investigation on diversity of endophytic yeasts from leaves of agronomic crops by culture-independent method) ดร.มณี ตันติรุ่งกิจ ฝ่ายปฏิบัติการวิจัยและเรือนปลูกพืชทดลอง สถาบันวิจัยและพัฒนา กำแพงแสน มหาวิทยาลัยเกษตรศาสตร์ วิทยเขตกำแพงแสน
13.30–14.00 น.	การวิจัยความหลากหลายของยีสต์อีพิไฟต์บนผิวใบพืชเศรษฐกิจด้วยด้วยวิธีการไม่เพาะเลียงเชือ (Investigation on diversity of epiphytic yeast on agronomic crop phyllospheres by culture-independent method) ดร.รุจิกาญจน์ นาสนิท ภาควิชาเทคโนโลยีชีวภาพ คณะวิศวกรรมศาสตร์และเทคโนโลยีอุตสาหกรรม มหาวิทยาลัยศิลปากร
14.00 -14.30 น.	ปัจจัยที่มีผลต่อการผลิตกรดอินโดล-3-อะซิติกในห้องปฏิบัติการโดยยีสต์บนใบข้าว (Factors affecting laboratory scale production of indole-3-acetic by rice leaf associated yeast) ผศ. ดร.นันทนา สีสุข ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์
14.30-15.00 น.	การคัดเลือกยีสต์ที่สามารถผลิตกรดอินโดล-3-อะซิติกจากผิวใบข้าวโพด (Screening of indole-3-acetic acid producing-yeasts isolated from phylloplane of corn) นางสาวพรพรรณ ไทยเจริญ ภาควิชาเทคโนโลยีชีวภาพ คณะวิทยาศาสตร์และเทคโนโลยี มหาวิทยาลัยธรรมศาสตร์
15.00 - 15.30 น.	อิทธิพลของแหล่งคาร์บอนและไนโตรเจนต่อการผลิตกรดอินโดล-3-อะซิติกโดยยีสต์จากผิวใบข้าวโพด (Effect of carbon and nitrogen sources on indole-3-acetic acid production by corn phylloplane yeast) ดร.รัฐดา จันทร์กลั่น ภาควิชาเทคโนโลยีชีวภาพ คณะวิทยาศาสตร์และเทคโนโลยี มหาวิทยาลัยธรรมศาสตร์
15.30–16.00 น.	พักรับประทานอาหารว่าง
16.00–16.30 น.	ประชุมโต๊ะกลม

เรื่อง ความหลากหลายและนิเวศวิทยาของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์จากใบของพืชเศรษฐกิจในประเทศไทยและการผลิตสารส่งเสริมการเจริญพืชออกซินโดยสายพันธุ์ที่คัดเลือกว่ามีประสิทธิภาพสูง พร้อมกับการศึกษาชีววิถีการสังเคราะห์
(โครงการวิจัยเลขที่ RTA5480009)

ความหลากหลายของยีสต์บนผิวใบพืช การค้นพบ และการเสนอยีสต์สปีชีส์ใหม่

รุ่งลักษณ์ แก้ววิเชียร[1,2], วิเชียร ยงมานิตชัย[1] และ สาวิตรี ลิ่มทอง[1]

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ศึกษาความหลากหลายของยีสต์บนผิวใบข้าว ใบข้าวโพด และใบอ้อย โดยเก็บตัวอย่างมาจาก 28 อำเภอ ใน 9 จังหวัดของประเทศไทย รวม 244 ตัวอย่าง นำมาแยกยีสต์ด้วย enrichment technique ในอาหารเหลว yeast extract-malt extract (YM) ที่เติมคลอแรมฟินิคอล 0.02 เปอร์เซ็นต์ และโซเดียมโพรพิโอเนต 0.025 เปอร์เซ็นต์ ได้ยีสต์จากใบข้าว 156 สายพันธุ์ ยีสต์จากใบข้าวโพด 126 สายพันธุ์ และยีสต์จากใบอ้อย 159 สายพันธุ์ เมื่อจัดจำแนกโดยอาศัยอนุกรมวิธานระดับโมเลกุลด้วยการเปรียบเทียบลำดับนิวคลีโอไทด์ในบริเวณ D1/D2 ของ large subunit rRNA gene และวิเคราะห์ความสัมพันธ์เชิงวิวัฒนาการ พบว่ายีสต์จากใบข้าว 155 สายพันธุ์ จัดจำแนกเป็นแอสโคมัยซีตัสยีสต์ 23 สปีชีส์ใน 13 สกุล คือ *Candida*, *Clavispora*, *Cyberlindnera*, *Debaryomyces*, *Kodamaea*, *Lachancea*, *Metschnikowia*, *Meyerozyma*, *Millerozyma*, *Pichia*, *Wickerhamomyces*, *Yamadazyma* และ *Yarrowia* และเป็นแบสิดิโอมัยซีตัสยีสต์ 8 สปีชีส์ใน 5 สกุล ได้แก่ *Cryptococcus*, *Pseudozyma*, *Rhodotorula*, *Sporobolomyces* และ *Trichosporon* ยีสต์จากใบข้าวโพด 123 สายพันธุ์ จัดจำแนกเป็นแอสโคมัยซีตัสยีสต์ถึง 28 สปีชีส์ใน 12 สกุล คือ *Candida*, *Cyberlindnera*, *Geotrichum*, *Hanseniaspora*, *Kluyveromyces*, *Kodamaea*, *Meyerozyma*, *Millerozyma*, *Pichia*, *Torulaspora*, *Wickerhamomyces* และ *Yamadazyma* และเป็นแบสิดิโอมัยซีตัสยีสต์ 10 สปีชีส์ใน 5 สกุล คือ *Cryptococcus*, *Rhodosporidium*, *Rhodotorula*, *Sporobolomyces* และ *Sporidiobolus* และยีสต์จากใบอ้อย 146 สายพันธุ์ จัดจำแนกเป็นแอสโคมัยซีตัสยีสต์ 23 สปีชีส์ใน 13 สกุล คือ *Candida*, *Cyberlindnera*, *Debaryomyces*, *Hanseniaspora*, *Kluyveromyces*, *Lachancea*, *Lodderomyces*, *Metschnikowia*, *Meyerozyma*, *Millerozyma*, *Pichia*, *Torulaspora* และ *Wickerhamomyces* และเป็นแบสิดิโอมัยซีตัสยีสต์ 12 สปีชีส์ใน 6 สกุล ได้แก่ *Hannaella*, *Cryptococcus*, *Rhodosporidium*, *Rhodotorula*, *Sporidiobolus* และ *Sporobolomyces* ส่วนยีสต์อีก 8 สายพันธุ์ เหมือนกับยีสต์สปีชีส์ที่ยังไม่มีการอธิบาย นอกจากนี้ยังพบยีสต์ 9 สายพันธุ์ จัดจำแนกเป็นยีสต์สปีชีส์ใหม่ สำหรับรายงานนี้เสนอเป็นยีสต์สปีชีส์ใหม่โดยอาศัยอนุกรมวิธานพอลิฟาซิกรวม 6 สปีชีส์ คือ *Metschnikowia saccharicola* sp. nov., *Metschnikowia lopburiensis* sp. nov., *Yamadazyma siamensis* sp. nov., *Yamadazyma phyllophila* sp. nov., *Wicherhamomyces siamensis* sp. nov. และ *Nakazawaea siamensis* sp. nov. ผลการศึกษาความหลากหลายบนผิวใบข้าว ใบข้าวโพด และใบอ้อย มีความหลากหลายของยีสต์สูง โดยเฉพาะยีสต์ในสกุล *Candida* มีมากถึง 21 สปีชีส์ ส่วนสปีชีส์ที่พบบ่อยที่สุดคือ *Rhodotorula taiwanensis* (14.3 เปอร์เซ็นต์ของยีสต์ทั้งหมด) *Meyerozyma caribbica* (12.0 เปอร์เซ็นต์ของยีสต์ทั้งหมด) และ *Candida tropicalis* (10.9 เปอร์เซ็นต์ของยีสต์ทั้งหมด) โดยส่วนใหญ่ตัวอย่างใบพืชทั้งสามชนิดจะพบยีสต์สองสปีชีส์ ซึ่งในใบข้าวพบ 56.5 เปอร์เซ็นต์ของตัวอย่างใบข้าวทั้งหมด ใบข้าวโพดพบมากถึง 60.0 เปอร์เซ็นต์ของตัวอย่างใบข้าวโพดทั้งหมด และใบอ้อยพบ 48.9 เปอร์เซ็นต์ของตัวอย่างใบอ้อยทั้งหมด

เรื่อง ความหลากหลายและนิเวศวิทยาของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์จากใบของพืชเศรษฐกิจในประเทศไทยและการผลิตสารส่งเสริมการเจริญพืชออกซินโดยสายพันธุ์ที่คัดเลือกว่ามีประสิทธิภาพสูง พร้อมกับการศึกษาชีววิถีการสังเคราะห์
(โครงการวิจัยเลขที่ RTA5480009)

การวิจัยความหลากหลายของยีสต์อีพิไฟต์บนใบข้าวด้วยวิธีการเพาะเลี้ยงเชื้อ

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จากการศึกษาความหลากหลายของยีสต์อีพิไฟต์บนใบข้าวด้วยวิธีการเพาะเลี้ยงเชื้อ โดยทำการเก็บตัวอย่างใบข้าว 112 ตัวอย่าง จาก 12 จังหวัด ในภาคต่างๆ ของประเทศไทย คัดแยกได้ยีสต์ 379 ไอโซเลต จากนั้นนำมาจัดจำแนกโดยอาศัยอนุกรมวิธานระดับโมเลกุลด้วยการวิเคราะห์ลำดับนิวคลีโอไทด์ในบริเวณ D1/D2 ของ large subunit rRNA gene ผลการจัดจำแนกยีสต์จำนวน 354 ไอโซเลต พบว่ามียีสต์จำนวน 299 ไอโซเลต จัดจำแนกเป็นยีสต์ที่ทราบชื่อแล้ว (Known species) 50 สปีชีส์ ใน 18 สกุล คือ *Bullera* (18 ไอโซเลต), *Candida* (24 ไอโซเลต), *Cryptococcus* (22 ไอโซเลต), *Debaryomyces* (2 ไอโซเลต), *Hyphopichia* (1 ไอโซเลต), *Jaminaea* (1 ไอโซเลต), *Kodamaea* (8 ไอโซเลต), *Lodderomyces* (1 ไอโซเลต), *Meyerozyma* (10 ไอโซเลต), *Pichia* (3 ไอโซเลต), *Pseudozyma* (125 ไอโซเลต), *Rhodosporidium* (10 ไอโซเลต), *Rhodotorula* (30 ไอโซเลต), *Sporidiobolus* (5 ไอโซเลต), *Sporobolomyces* (27 ไอโซเลต), *Torulaspora* (2 ไอโซเลต), *Trichosporon* (4 ไอโซเลต) และ *Wickerhamomyces* (5 ไอโซเลต) พบยีสต์ที่จำแนกเป็นยีสต์สปีชีส์ใหม่ (New species) 35 ไอโซเลต ใน 9 สกุล คือ *Blastobotrys* (1 ไอโซเลต), *Bullera* (2 ไอโซเลต), *Candida* (1 ไอโซเลต), *Cryptococcus* (13 ไอโซเลต), *Occultifur* (2 ไอโซเลต), *Pseudozyma* (8 ไอโซเลต), *Rhodosporidium* (4 ไอโซเลต), *Rhodotorula* (3 ไอโซเลต), และ *Sakaguchia* (1 ไอโซเลต) คาดว่าเป็นยีสต์สปีชีส์ใหม่ประมาณ 20 สปีชีส์ และยีสต์จำนวน 21 ไอโซเลตจัดจำแนกเป็นสปีชีส์ที่ยังไม่มีการอธิบาย (undescribed species) ใน 4 สกุล คือ *Candida* (1 ไอโซเลต), *Cryptococcus* (9 ไอโซเลต), *Pseudozyma* (4 ไอโซเลต) และ *Rhodotorula* (7 ไอโซเลต) ส่วนยีสต์ที่เหลืออีก 25 ไอโซเลต ยังไม่ได้ทำการจัดจำแนก และในการศึกษาครั้งนี้ยังพบยีสต์ที่มีลักษณะโคโลนีคล้ายราอีกด้วยเช่น *Aureobasidium, Acremonium* หรือ *Cephalosporium, Hyponectria, Melanopsichium, Moesziomyces, Sarocladium, Sporisorium, Syncephalastrum* และ *Ustilago* ยีสต์อีพิไฟต์บนใบข้าวที่คัดแยกได้นั้นได้นำไปศึกษาความสามารถในการสร้างสารเร่งการเจริญพืชและกำลังจะศึกษาการควบคุมราโรคพืชเพื่อการใช้ประโยชน์ต่อไป

เรื่อง ความหลากหลายและนิเวศวิทยาของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์จากใบของพืชเศรษฐกิจในประเทศไทยและการผลิตสารส่งเสริมการเจริญพืชออกซินโดยสายพันธุ์ที่คัดเลือกว่ามีประสิทธิภาพสูง พร้อมกับการศึกษาชีววิถีการสังเคราะห์
(โครงการวิจัยเลขที่ RTA5480009)

การวิจัยความหลากหลายของยีสต์อีพิไฟต์บนใบข้าวโพด ด้วยวิธีการเพาะเลี้ยงเชื้อ

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ยีสต์เป็นจุลินทรีย์ที่มีความหลากหลาย และพบได้ทั่วไปในสิ่งแวดล้อม สำหรับการศึกษาความหลากหลายของยีสต์อีพิไฟต์บนใบข้าวโพดที่เก็บจากแหล่งต่างๆ ในประเทศไทย จำนวน 109 ตัวอย่าง แล้วนำไปแยกยีสต์ด้วยการเพาะเลี้ยงยีสต์จากน้ำล้างใบ และจัดจำแนกยีสต์โดยอาศัยอนุกรมวิธานระดับโมเลกุลด้วยการวิเคราะห์ลำดับนิวคลีโอไทด์ในบริเวณ D1/D2 ของ large subunit rRNA gene พบว่ายีสต์ 149 ไอโซเลต จัดจำแนกเป็นยีสต์สปีชีส์ที่รู้จักแล้ว โดยจำแนกเป็น 15 สกุล คือ *Bandoniozyma*, *Bullera*, *Candida*, *Cryptococcus*, *Dioszegia, Hanseniaspora*, *Jaminaea*, *Kodamaea*, *Meyerozyma, Pseudozyma, Rhodotorula, Rhodosporidium, Sporidiobolus, Sporobolomyces* และ *Wickerhamomyces* โดยพบยีสต์สปีชีส์ *Sporidiobolus pararoseus* มากที่สุด (17 ไอโซเลต) ส่วนยีสต์จำนวน 24 ไอโซเลต จัดจำแนกเป็นสปีชีส์ที่ยังไม่มีการอธิบายใน 6 สกุล คือ *Cryptococcus* (9 ไอโซเลต), *Occultifur* (2 ไอโซเลต), *Pseudozyma* (2 ไอโซเลต), *Rhodotorula* (7 ไอโซเลต), *Sporidiobolus* (2 ไอโซเลต) และ *Sporobolomyces* (2 ไอโซเลต) นอกจากนี้ยังพบยีสต์ที่มีลักษณะโคโลนีและเซลล์ที่คาดว่าจะเป็นยีสต์ 53 ไอโซเลต แต่เมื่อจัดจำแนกพบว่าเป็นราสปีชีส์ที่รู้จักแล้วหรือราสปีชีส์ใหม่ใน 7 สกุล คือ *Acremonium* หรือ *Cephalosporium* (1 ไอโซเลต), *Aureobasidium* (8 ไอโซเลต), *Kabatiella* (1 ไอโซเลต), *Moesziomyces* (2 ไอโซเลต), *Sarocladium* (1 ไอโซเลต), *Sporisorium* (8 ไอโซเลต) และ *Ustilago* (32 ไอโซเลต)

สำหรับยีสต์ 16 ไอโซเลต ถูกจัดจำแนกเป็นยีสต์สปีชีส์ใหม่ใน 6 สกุล คือ *Bullera* (4 ไอโซเลต), *Cryptococcus* (7 ไอโซเลต), *Occultifur* (1 ไอโซเลต), *Rhodotorula* (2 ไอโซเลต), *Rhodosporidium* (1 ไอโซเลต) และ *Sporidiobolus* (1 ไอโซเลต) สำหรับยีสต์สปีชีส์ใหม่ที่ค้นพบนั้นจะนำไปศึกษาลักษณะต่างๆตามเกณฑ์อนุกรมวิธานแบบโพลีฟาซิก เพื่ออธิบายพร้อมทั้งเสนอตั้งชื่อเป็นยีสต์สปีชีส์ใหม่ต่อไป

การวิจัยความหลากหลายของยีสต์อีพิไฟต์บนใบอ้อยด้วยวิธีการเพาะเลี้ยงเชื้อและการประเมินความสามารถในการควบคุมราโรคพืช

จันทร์จีรา สุรัสวดี และ สาวิตรี ลิ่มทอง

ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ กรุงเทพฯ 10900

ศึกษาความหลากหลายของยีสต์อีพิไฟต์ (epiphytic yeast) บนผิวใบอ้อยที่ปลูกในภาคกลางและภาคตะวันออกเฉียงเหนือของประเทศไทย รวม 10 จังหวัด โดยเก็บใบอ้อย 102 ตัวอย่าง นำมาแยกยีสต์ด้วยการเพาะเลี้ยงยีสต์จากน้ำล้างใบ จากนั้นเลือกเชื้อที่มีลักษณะเซลล์และโคโลนีแบบยีสต์ได้ 267 สายพันธุ์ และจากการจำแนกโดยอาศัยอนุกรมวิธานระดับโมเลกุลด้วยการเปรียบเทียบลำดับนิวคลีโอไทด์บริเวณ D1/D2 ของ large subunit rRNA gene พบว่า114 สายพันธุ์ (42.7เปอร์เซ็นต์) เป็นยีสต์สปีชีส์ที่มีการอธิบายแล้วโดยส่วนใหญ่ (91สายพันธุ์) เป็นแบสิดิโอมัยซีตัสยีสต์ 26 สปีชีส์ ใน 9 สกุล ได้แก่ *Rhodotorula marina*, *R. mucilaginosa*, *R. minuta*, *Cryptococcus flavescens*, *C. laurentii*, *C. nemorosus*, *C. rajasthanensis*, *C. flavus*, *C.* aff. *laurentii*, *C. luteolus*, *C. heveanensis*, *Tremella globispora*, *Jaminaea angkoriensis*, *Pseudozyma aphidis*, *P. Antarctica*, *P. shanxiensis*, *P. hubeiensis, P. rugulosa*, *P. jejuensis*, *Sporobolomyces carnicolor*, *S. blumeae*, *S. nylandii*, *S. vermiculatus*, *Sporidiobolus ruineniae* var. *ruineniae*, *Occultifur externus* และ *Bullera sinensis* และ 23 สายพันธุ์ เป็นแอสโคมัยซีตัสยีสต์ใน 5 สปีชีส์ ของ 3 สกุล ได้แก่ *Meyerozyma caribbica*, *M. guilliermondii*, *Kodamaea ohmeri*, *Candida fermentati* และ *C. parapsilosis* ส่วนยีสต์ 57 สายพันธุ์ ใน 19 สปีชีส์ของ 4 สกุล เหมือนยีสต์สปีชีส์ที่ยังไม่มีการอธิบาย นอกจากนี้พบยีสต์ 59 สายพันธุ์ เป็นราคล้ายยีสต์ 21 สปีชีส์ และที่สำคัญพบว่ามี 31 สายพันธุ์ จัดจำแนกเป็นยีสต์สปีชีส์ใหม่ที่ใกล้เคียงกับ *Pseudozyma* sp., *Candida* sp., *Occultifur* sp., *Tremella* sp., *Jaminaea* sp., *Cryptococcus* sp. และ *Rhodotorula sp.* นอกจากนี้เมื่อวิเคราะห์ความถี่ของยีสต์บนผิวใบอ้อยพบว่า*Jaminaea angkoriensis* เป็นสปีชีส์ที่พบมากที่สุด คือพบ 14 สายพันธุ์ รองมา คือ *Meyerozyma guilliermondii* พบ 12 สายพันธุ์ และ *Sporobolomyces vermiculatus* พบ 8 สายพันธุ์

การตรวจความสามารถในการควบคุมราโรคพืช พบว่า *K. ohmeri* DMKU-SP45 สามารถยับยั้งการเจริญของราโรคข้าว 3 สปีชีส์ คือ *Helminthosporium oryzae*, *Rhizoctonia solani* และ *Fusarium moniliforme* ส่วนสายพันธุ์ DMKU-SP81 ที่เหมือนกับ *Candida* sp.05-7-186^{T} สามารถยับยั้งราโรคข้าวโพดได้ 1 สปีชีส์ คือ *F. moniliforme* นอกจากนี้สายพันธุ์ DMKU-SP85 ซึ่งเหมือนกับ *Cryptococcus* sp. CBS8363 สามารถยับยั้งราโรคข้าวโพดได้ 1 สปีชีส์ คือ *Bipolaris maydis* และสายพันธุ์DMKU-SP290 ที่เหมือนกับ *Pseudozyma* sp. DMST 17137 สามารถยับยั้งการเจริญของราโรคในข้าวโพดได้ 2 สปีชีส์ คือ *Bipolaris maydis* และ *Exserohilium turcica* ขณะนี้กำลังศึกษากลไกการยับยั้งการเจริญของราโรคพืช

ความหลากหลายของยีสต์เอนโดไฟต์จากใบพืชเศรษฐกิจ และความสามารถในการควบคุมราโรคพืช

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ยีสต์เอนโดไฟต์ (endophytic yeast) เป็นจุลินทรีย์ที่อาศัยอยู่ในเนื้อเยื่อของพืชโดยไม่ทำให้พืชเกิดโรค หรือความเสียหาย หรือการเปลี่ยนแปลงทางสรีรวิทยาที่ผิดปกติแก่พืชชนิดนั้นๆ เมื่อเทียบกับจุลินทรีย์เอนโดไฟต์อื่นๆ พบว่ายีสต์เอนโดไฟต์ยังมีการศึกษาน้อย ในประเทศไทยยังไม่มีผู้ใดศึกษายีสต์เอนโดไฟต์ทั้งด้านความหลากหลาย และการใช้ประโยชน์ ดังนั้นงานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาความหลากหลายของยีสต์เอนโดไฟต์จากใบพืชเศรษฐกิจ และตรวจสอบความสามารถในการควบคุมราโรคพืช โดยเก็บตัวอย่างใบพืช 3 ชนิด ประกอบไปด้วย ใบข้าว, ใบอ้อย และใบข้าวโพด จาก 30 อำเภอใน 11 จังหวัด จากนั้นนำมาแยกยีสต์โดยนำตัวอย่างใบพืชมาฆ่าเชื้อบนพื้นผิวใบ (surface steriliz ation) บดใบพืชให้ละเอียด แล้วนำไปวางบน yeast extract – malt extract (YM) agar ที่เติมคลอแรมฟินิคอล 0.02 เปอร์เซ็นต์ บ่มที่ 25 องศาเซลเซียส 3-7 วัน พบว่าจากใบข้าว 100 ตัวอย่าง แยกยีสต์เอนโดไฟต์ได้ 125 สายพันธุ์ ใบอ้อย 102 ตัวอย่าง แยกยีสต์เอนโดไฟต์ได้ 149 สายพันธุ์ และใบข้าวโพด 109 ตัวอย่างแยกยีสต์เอนโดไฟต์ได้ 143 สายพันธุ์ นำยีสต์เอนโดไฟต์ที่แยกได้ทั้งหมด 418 สายพันธุ์ มาจัดจำแนกโดยอาศัยอนุกรมวิธานระดับโมเลกุลด้วยการหาลำดับนิวคลีโอไทด์ของ D1/D2 domain ของ Large subunit (LSU) rRNA gene ผลการจัดจำแนกพบว่า 345 สายพันธุ์ (82.5 เปอร์เซ็นต์) เป็นสปีชีส์ที่มีการอธิบายแล้วหรือรู้จักแล้ว โดยส่วนใหญ่คือ 256 สายพันธุ์ (74.2 เปอร์เซ็นต์) เป็นเบสิดิโอมัยซีตัสยีสต์ ได้แก่ *Bullera derxii, B. japonica, B. pseudoalba, B. sinensis, B. coprosmaensis, Cryptococcus albidosimilis, C. cellulolyticus, C. flavescens, C. flavus, C. liquefaciens, C. nemorosus, C. rajasthanensis, C. luteolus, C. laurentii, C. heveanensis, C. taiwanensis, Cyberlindnera rhodanensis, Jaminaea angkoriensis, Occultifur externu, Pseudozyma aphidis, P. prolifica, P. rugulosa , P. tsukubaensis, P. hubeiensis , P. antarctica, Rhodosporidium toruloides, R. paludigenum, Rhodotorula marina, R. minuta, R. mucilaginosa, R. oryzicola, R. taiwanensis, R. slooffiae, Sporobolomyces vermiculatus, S. blumeae, Sporidiobolus pararoseus, Trichosporon asahii* และ *Tremella globispora* ในขณะที่ 89 สายพันธุ์ (25.8 เปอร์เซ็นต์) เป็นแอสโคมัยซีตัสยีสต์ ได้แก่ *Candida tropicalis, C. citri, C. nivariensis, C. intermedia, C. glabrata, C. pseudointermedia, C. parapsilosis*, *Dioszegia takashimae, Kodamaea ohmeri, Pichia kudriavzevii, P. caribbica, P. rhodanensis, P. anomalus, Meyerozyma guilliermondii, M. caribbica* และ *Wickerhamomyces anomalus* ส่วน 22 สายพันธุ์ (6.4 เปอร์เซ็นต์) เป็นยีตส์ที่ยังไม่มี

การอธิบาย และ 15 สายพันธุ์ (3. 5 เปอร์เซ็นต์) จัดจำแนกเป็นยีต์สปีชีส์ใหม่ที่ใกล้เคียงกับ *Candida* sp., *Pseudozyma* sp., *Cryptococcus* sp., *Occultifur* sp. และ *Tremella* sp. นอกจากนี้ยังพบ 36 สายพันธุ์ (8.6 เปอร์เซ็นต์) จำแนกเป็นราคล้ายยีสต์

เมื่อนำยีสต์เอนโดไฟต์ทั้ง 408 สายพันธุ์มาตรวจสอบความสามารถในการยับยั้งราโรคข้าวและข้าวโพดพบว่า มีเพียง 8 สายพันธุ์ที่มีความสามารถยับยั้งราก่อโรค โดยเป็นยีสต์ที่แยกได้จากใบข้าว 4 สายพันธุ์ คือ *Pichia anomalus* DMKU-RE13, *P. anomalus* DMKU-RE62, *Kodamaea ohmeri* DMKU-RE27 และ *K. ohmeri* DMKU-RE65 และเป็นยีสต์ที่แยกได้จากใบข้าวโพด 4 สายพันธุ์ คือ *Candida nivariensis* DMKU-CE18, *Candida* sp. DMKU-CE23, *Wickerhamomyces anomalus* DMKU-CE52 และ *W. anomalus* DMKU-CE55 ทั้งนี้กำลังศึกษากลไกการยับยั้งการเจริญของราโรคพืชต่อไป

การวิจัยความหลากหลายของยีสต์เอนโดไฟต์ในใบพืชเศรษฐกิจด้วยวิธีการไม่เพาะเลี้ยงเชื้อ

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ในปัจจุบันเทคนิคด้านชีวโมเลกุลช่วยให้การศึกษาด้านความหลากหลายของจุลินทรีย์ในระบบนิเวศต่างๆ ทำได้รวดเร็วขึ้นทั้งในกลุ่มของจุลินทรีย์ที่สามารถเพาะเลี้ยงได้บนอาหารเลี้ยงเชื้อและกลุ่มที่ไม่สามารถเพาะเลี้ยงได้ ดังนั้นในการวิจัยนี้จึงใช้เทคนิคด้านชีวโมเลกุลในการตรวจหายีสต์เอนโดไฟต์ในใบพืชเศรษฐกิจโดยไม่เพาะเลี้ยงเชื้อ (culture-independent method) เพื่อวิเคราะห์และเปรียบเทียบผลความหลากหลายของยีสต์เอนโดไฟต์ในใบพืชเศรษฐกิจกับวิธีการเพาะเลี้ยงเชื้อ (culture-dependent method) โดยสกัดดีเอ็นเอของยีสต์เอนโดไฟต์จากตัวอย่างใบพืชที่ทำ surface-sterilization จำนวน 321 ตัวอย่าง (ใบข้าว 112 ตัวอย่าง ใบอ้อย 102 ตัวอย่าง และใบข้าวโพด 107 ตัวอย่าง) เพื่อใช้เป็นดีเอ็นเอต้นแบบในการทำปฏิกิริยา PCR เพื่อเพิ่มปริมาณดีเอ็นเอบริเวณ D1/D2 ของ large subunit (LSU) rRNA geneด้วยคู่ไพรเมอร์ NL1 และ NL4 และสร้าง D1/D2 library ของดีเอ็นเอที่สกัดได้จากตัวอย่างใบข้าวคัดเลือก clone ที่มีลำดับนิวคลีโอไทด์ในบริเวณ D1/D2 ที่แตกต่างกันในแต่ละ library ด้วยวิธี PCR-RFLP โดยใช้ เอนไซม์ *Hae*III, *Hin*fI และ *Cfo*I จากนั้นนำ PCR product ของ clone ที่มีลำดับนิวคลีโอไทด์ในบริเวณ D1/D2 ที่แตกต่างกันในแต่ละ library ไปวิเคราะห์หาลำดับนิวคลีโอไทด์และเปรียบเทียบกับลำดับนิวคลีโอไทด์ในฐานข้อมูลเพื่อระบุชนิดและหาความสัมพันธ์ทางพันธุกรรม พบว่า D1/D2 library จำนวน 92 library ส่วนใหญ่มี clone ที่ PCR product มี PCR-RFLP pattern เพียง 1 แบบ และ PCR productที่ได้เป็นดีเอ็นเอส่วนของ LSU rRNA gene ของข้าวอ้อย และข้าวโพดสำหรับ library ของข้าวที่มี PCR-RFLP pattern มากกว่า 1 แบบจำนวน 11 library พบว่ามี 1 phylotype ที่มีลำดับนิวคลีโอไทด์ใกล้เคียงกับยีสต์สกุล *Debaromyces* 12 phylotype ที่มีลำดับนิวคลีโอไทด์ใกล้เคียงกับราเส้นใยในสกุล *Bipolaris, Dendryphiella, Neurospora* และ *Passalora* และ 1 phylotype ที่มีลำดับนิวคลีโอไทด์ใกล้เคียงกับราในไฟลัม *Ascomycota* ส่วน D1/D2 library ของอ้อยและข้าวโพดจำนวน 14 และ 7 library ไม่พบ PCR product ที่เป็นส่วนของ D1/D2 ของยีสต์หรือราเส้นใยแสดงว่าไพร์เมอร์ NL1 และNL4 ซึ่งเป็นคู่ไพรเมอร์ที่นิยมใช้ในการเพิ่มปริมาณดีเอ็นเอบริเวณ D1/D2 ของ LSU rRNA gene ของยีสต์บริสุทธิ์สามารถเพิ่มปริมาณของ LSUrRNA gene ของข้าวอ้อยและข้าวโพดได้ ดังนั้นจึงทดลองปรับเปลี่ยน forward primer จาก NL1 เป็น ITS1-F_KYO1ซึ่งเป็นไพรเมอร์ที่ออกแบบให้มีความจำเพาะต่อการเพิ่มปริมาณดีเอ็นเอบริเวณ ITS ของราทั้ง Ascomycetes และ Basidiomycetes จาก

ตัวอย่างพืช และใช้วิธีการเพิ่มปริมาณดีเอ็นเอบริเวณ D1/D2 แบบ semi-nested PCR พบว่าสามารถสร้าง D1/D2 library ของข้าว อ้อย และข้าวโพดที่ clone ส่วนใหญ่มีส่วนของ D1/D2 ของยีสต์หรือราเส้นใยอยู่ และจากD1/D2 library ของข้าว จำนวน 4 library พบว่ามี 5 phylotype ที่มีลำดับนิวคลีโอไทด์ใกล้เคียงกับยีสต์ในสกุล *Candida, Debaryomyces* และ *Cryptococcus* 25 phylotype ที่มีลำดับนิวคลีโอไทด์ใกล้เคียงกับราเส้นใยในสกุล*Bipolaris, Cochliobolus, Coniosporium, Nigrospora, Phaeosphaeria, Ramularia, Sarocladium, Setosphaeria, Lentinula, Xylaria* และ Uncultured fungal clone และ 1 phylotype ที่มีลำดับนิวคลีโอไทด์ใกล้เคียงกับราในไฟลัม *Ascomycota* สำหรับ D1/D2 library ของข้าวโพดจำนวน 3 library พบว่ามี 5 phylotype ที่มีลำดับนิวคลีโอไทด์ใกล้เคียงกับยีสต์ในสกุล*Trichosporon* 13 phylotype ที่มีลำดับนิวคลีโอไทด์ใกล้เคียงกับราเส้นใยในสกุล*Bipolaris, Cladosporium, Cochliobolus, Curvularia, Fusarium, Persiciospora* และ Uncultured fungal clone และ 2 phylotype ที่มีลำดับนิวคลีโอไทด์ใกล้เคียงกับราในไฟลัม *Ascomycota* ส่วน D1/D2 library ของอ้อย จำนวน 4 library พบว่ามี 2 phylotype ที่มีลำดับนิวคลีโอไทด์ใกล้เคียงกับยีสต์ในสกุล *Kodameae* และ 7phylotype ที่มีลำดับนิวคลีโอไทด์ใกล้เคียงกับราในสกุล *Cladosporium, Khuskia* และ *Penicillium* ซึ่งผลการทดลองแสดงให้เห็นว่าวิธีการเพิ่มปริมาณดีเอ็นเอบริเวณ D1/D2 แบบ semi-nested PCR โดยใช้ไพรเมอร์ ITS1-F_KYO1 และ NL4 เพิ่มปริมาณส่วนของ ITS และ LSU rDNAเพื่อใช้เป็นดีเอ็นเอต้นแบบสำหรับการเพิ่มปริมาณดีเอ็นเอบริเวณ D1/D2 ด้วยไพรเมอร์ NL1 และ NL4 สามารถแก้ไขปัญหาการเพิ่มปริมาณดีเอ็นเอบริเวณ D1/D2 ของข้าว อ้อยและข้าวโพดในปฏิกิริยา PCR ได้ดี

เรื่อง ความหลากหลายและนิเวศวิทยาของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์จากใบของพืชเศรษฐกิจในประเทศไทยและการผลิตสารส่งเสริมการเจริญพืชออกซินโดยสายพันธุ์ที่คัดเลือกว่ามีประสิทธิภาพสูง พร้อมกับการศึกษาชีววิถีการสังเคราะห์
(โครงการวิจัยเลขที่ RTA5480009)

การวิจัยความหลากหลายของยีสต์อีพิไฟต์บนพืชเศรษฐกิจด้วยวิธีการไม่เพาะเลี้ยงเชื้อ

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งานวิจัยนี้ทำเพื่อศึกษาความหลากหลายของยีสต์บนผิวใบพืช ได้แก่ ใบข้าว และใบอ้อย โดยเก็บตัวอย่างใบพืชจากพื้นที่เพาะปลูกในหลายจังหวัดของประเทศไทย ได้แก่ ฉะเชิงเทรา ชัยนาท นครปฐม นครสวรรค์ นนทบุรี บุรีรัมภ์ พิจิตร พิษณุโลก เพชรบูรณ์ ศรีสะเกษ สุพรรณบุรี และสุรินทร์ และใช้เทคนิคระดับโมเลกุลในการจัดจำแนกยีสต์ ได้แก่ วิธีปฏิกิริยาลูกโซ่ (Polymerase chain reaction, PCR) และการวิเคราะห์ด้วยเอนไซม์ตัดจำเพาะ (Restriction fragment length polymorphism, RFLP) 2 ชนิด (*Hae* III และ *Hinf* I) โดยศึกษาลำดับนิวคลีโอไทด์ในบริเวณ D1D2 บนยีน large subunit (LSU) rRNA จากผลการวิเคราะห์รูปแบบขนาดดีเอ็นเอจากการตัดด้วยเอนไซม์ตัดจำเพาะจากจำนวนโคลนทั้งสิ้น 798 โคลน ของใบข้าว 33 ตัวอย่าง สามารถจัดกลุ่มโคลนเบื้องต้นได้ 331 OTUs (Operational taxonomic units) และจากการวิเคราะห์ลำดับนิวคลีโอไทด์พบว่าสามารถจัดจำแนกเป็นยีสต์ได้เพียง 65 OTUs โดยยีสต์ที่จำแนกได้จัดอยู่ใน 11 สกุล และจัดจำแนกในระดับสปีชีส์ได้ทั้งสิ้น 18 สปีชีส์ โดยตัวอย่างใบข้าวส่วนใหญ่พบยีสต์ในไฟลัมเบสิดิโอไมโคตา ได้แก่ สกุล *Bullera, Cryptococcus, Hannaella, Pseudozyma* และ *Sporobolomyces* โดยตรวจพบ *Pseudozyma antarctica* ในตัวอย่างใบข้าวส่วนใหญ่ (22 ตัวอย่าง) สำหรับยีสต์แอสโคมัยซีสที่ตรวจพบบนใบข้าวจัดอยู่ในสกุล *Hyphopichia, Metschnikowia, Meyerozyma, Pichia, Sporidiobolus* และ *Wickerhamomyces* ผลการจัดจำแนกยีสต์อีพิไฟต์ในใบอ้อยทั้งสิ้น 27 ตัวอย่าง พบว่าสามารถจัดกลุ่มโคลนเบื้องต้นได้ 189 OTUs จาก 658 โคลน และสามารถจัดจำแนกเป็นยีสต์ได้เพียง 21 OTUs จากใบอ้อย 13 ตัวอย่าง โดยยีสต์ที่ตรวจสอบได้จัดอยู่ใน 3 สกุล และจัดจำแนกในระดับสปีชีส์ได้ 6 สปีชีส์ ซึ่งยีสต์ทั้งหมดที่จำแนกได้อยู่ในไฟลัมเบสิดิโอไมโคตา ได้แก่ สกุล *Cryptococcus*, *Pseudozyma* และ *Jaminaea* โดยตรวจพบ *Pseudozyma prolifica* ในตัวอย่างใบอ้อยส่วนใหญ่ จากผลการทดลองเบื้องต้นนี้สามารถสรุปได้ว่ายีสต์อีพิไฟต์ที่พบบนผิวใบพืชส่วนใหญ่เป็นเบสิดิโอไมซีส รวมทั้งความหลากหลายของยีสต์บนใบพืชทั้งสองชนิดมีความแตกต่างกัน โดยจากงานวิจัยนี้พบว่ายีสต์อีพิไฟต์บนผิวใบข้าวมีความหลากหลายมากกว่ายีสต์บนใบอ้อย และยีสต์ส่วนใหญ่ที่พบบนใบพืชทั้ง 2 ชนิด จัดอยู่ในสกุล *Cryptococcus* และ *Pseudozyma*

ปัจจัยที่มีผลต่อการผลิตกรดอินโดล-3-อะซิติกในห้องปฏิบัติการโดยยีสต์บนใบข้าว

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กรดอินโดล-3-อะซิติกเป็นฮอร์โมนพืชที่จัดอยู่ในกลุ่มของออกซิน มีบทบาทหน้าที่ที่สำคัญต่อพืชหลายประการ เช่น กระตุ้นการแบ่งเซลล์ การยืดขยายตัวของเซลล์ กระตุ้นให้เซลล์เกิดความแตกต่าง ควบคุมการร่วงของใบและการสุกของผลไม้ และยังช่วยการตอบสนองของแสงและแรงโน้มถ่วง จากรายงานทางวิชาการที่ผ่านมานอกจากพืชที่สามารถผลิตกรดอินโดล-3-อะซิติกได้แล้ว ยังพบว่าจุลินทรีย์ทั้งแบคทีเรีย รา สาหร่าย รวมทั้งยีสต์ ก็สามารถผลิตกรดอินโดล-3-อะซิติกได้เช่นกัน สำหรับยีสต์นั้นมีรายงานเกี่ยวกับการศึกษาการผลิตกรดอินโดล-3-อะซิติกไม่มากนัก และยังไม่พบการศึกษาการคัดเลือกปัจจัยที่มีอิทธิพลต่อการผลิตกรดอินโดล-3-อะซิติกในยีสต์ งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาปัจจัยที่มีอิทธิผลต่อการผลิตกรดอินโดล-3-อะซิติกโดยยีสต์ที่คัดเลือกได้ โดยศึกษาเกี่ยวกับปัจจัยที่เคยมีรายงานมาก่อนหน้านี้แล้ว ว่าส่งผลต่อการผลิตกรดอินโดล-3-อะซิติก ได้แก่ ระยะเวลาการเพาะเลี้ยง อุณหภูมิ ความเร็วของการเขย่า พีเอชของอาหารเลี้ยงเชื้อ แหล่งคาร์บอน แหล่งไนโตรเจน ความเข้มข้นของโซเดียมคลอไรด์และแอล-ทริปโตเฟน โดยใช้วิธีดั้งเดิม (one factor at a time) ศึกษาระยะเวลาการเพาะเลี้ยง ชนิดของแหล่งคาร์บอนและแหล่งไนโตรเจนที่เหมาะสมต่อการผลิตกรดอินโดล-3-อะซิติก ซึ่งพบว่าระยะเวลาการเพาะเลี้ยงยีสต์ 7 วัน ร่วมกับการใช้ซูโครสและยีสต์เอ็กซ์แทร็กซ์ ให้กรดอินโดล-3-อะซิติกสูง (281.79 มก.ต่อลิตร) หลังจากนั้นจึงใช้วิธีการทางสถิติเข้ามาช่วยออกแบบการทดลองโดยใช้แผนการทดลอง Plackett-Burman design เพื่อระบุปัจจัยที่มีอิทธิผลต่อการผลิตกรดอินโดล-3-อะซิติกโดยยีสต์สายพันธุ์ที่คัดเลือกได้ ปัจจัยที่ศึกษาคือ อุณหภูมิ ความเร็วของการเขย่า พีเอชของอาหารเลี้ยงเชื้อ ซูโครส ยีสต์เอ็กซ์แทร็กซ์ ความเข้มข้นของโซเดียมคลอไรด์และแอล-ทริปโตเฟน ซึ่งจากการศึกษาพบว่าความเร็วของการเขย่า อุณหภูมิ พีเอชของอาหารเลี้ยงเชื้อ ซูโครส ยีสต์เอ็กซ์แทร็กซ์ และความเข้มข้นของแอล-ทริปโตเฟน มีอิทธิพลต่อการผลิตกรดอินโดล-3-อะซิติกโดยยีสต์ที่คัดเลือกได้อย่างมีนัยสำคัญ ซึ่งจะได้นำมาศึกษาสภาวะที่เหมาะสมของปัจจัยเหล่านี้ต่อไป

เรื่อง ความหลากหลายและนิเวศวิทยาของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์จากใบของพืชเศรษฐกิจในประเทศไทยและการผลิตสารส่งเสริมการเจริญพืชออกซินโดยสายพันธุ์ที่คัดเลือกว่ามีประสิทธิภาพสูง พร้อมกับการศึกษาชีววิถีการสังเคราะห์
(โครงการวิจัยเลขที่ RTA5480009)

การคัดเลือกยีสต์ผิวใบข้าวโพดที่มีความสามารถในการผลิต indole-3-acetic acid (IAA) ได้สูง

พรพรรณ ไทยเจริญ[1] สาวิตรี ลิ่มทอง[2] และ รัฐดา จันทร์กลั่น[1]
[1]ภาควิชาเทคโนโลยีชีวภาพ คณะวิทยาศาสตร์และเทคโนโลยี มหาวิทยาลัยธรรมศาสตร์ ปทุมธานี 12120
[2]ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ กรุงเทพฯ 10900

คัดเลือกยีสต์ผิวใบข้าวโพดที่ผลิต indole-3-acetic acid (IAA) ได้โดยคัดเลือกขั้นต้นในอาหารเหลว YPD พีเอช 6.0 ที่มีกลูโคส 2 เปอร์เซ็นต์ เปปโตน 2 เปอร์เซ็นต์ ยีสต์เอกซ์แทรค 1 เปอร์เซ็นต์ และแอล-ทริปโตเฟน 0.1 เปอร์เซ็นต์ เป็นส่วนประกอบ ใช้อาหารปริมาตร 5 มิลลิลิตร บรรจุในหลอดทดสอบขนาด 18x180 มิลลิเมตร บ่มบนเครื่องเขย่าด้วยความเร็ว 250 รอบต่อนาที ที่อุณหภูมิห้อง เก็บตัวอย่างที่เวลา 72 ชั่วโมง วิเคราะห์การเจริญโดยวัดน้ำหนักเซลล์แห้ง และวิเคราะห์ปริมาณ IAA โดยวิธีการวัดสี (Colorimetric method) เปรียบเทียบกับยีสต์อ้างอิง *Rhodosporidium fluviale* RK 253 พบว่า จากยีสต์ผิวใบข้าวโพดจำนวนทั้งหมด 335 ไอโซเลต มีเพียง 16 ไอโซเลต ที่สามารถผลิต IAA ได้มากกว่า 7 มิลลิกรัมต่อกรัมน้ำหนักแห้ง โดยที่ยีสต์อ้างอิงผลิต IAA ได้ 5-7 มิลลิกรัมต่อกรัมน้ำหนักแห้ง ที่สภาวะเดียวกัน และคัดเลือกขั้นที่สองโดยเพาะเลี้ยงในอาหารเหลว YPD สูตรเดียวกัน ปริมาตร 150 มิลลิลิตร บรรจุในฟลาสก์ขนาด 500 มิลลิลิตร โดยปรับให้มีค่าความขุ่น(OD_{600}) ของเชื้อเริ่มต้นเท่ากับ 1 บ่มบนเครื่องเขย่าที่ความเร็ว 200 รอบต่อนาที ที่อุณหภูมิ 30 องศาเซลเซียส เก็บตัวอย่างทุก 24 ชั่วโมง จนครบ 96 ชั่วโมง วิเคราะห์การเจริญโดยการวัดค่าความขุ่น และวัดน้ำหนักเซลล์แห้ง วิเคราะห์ปริมาณ IAA โดยวิธีการวัดสี และยืนยันผลเชิงคุณภาพและปริมาณโดยการวิเคราะห์ด้วย HPLC พบว่าไอโซเลต DMKU-CP 293 ผลิต IAA ได้สูงสุดเมื่อเทียบกับไอโซเลตอื่นๆและยีสต์อ้างอิง โดยผลิตได้เท่ากับ 141.50±1.63 มิลลิกรัมต่อลิตร คิดเป็น 31.90±1.31 มิลลิกรัมต่อกรัมน้ำหนักแห้ง ที่เวลา 72 ชั่วโมง ในขณะที่ยีสต์อ้างอิง ผลิตได้ 98.73±2.60 มิลลิกรัมต่อลิตร คิดเป็น 27.43±0.86 มิลลิกรัมต่อกรัมน้ำหนักแห้งที่เวลาเดียวกัน

การประชุมวิชาการ เมธีวิจัยอาวุโส สกว.
ศาสตราจารย์ ดร.สาวิตรี ลิ่มทอง

เรื่อง ความหลากหลายและนิเวศวิทยาของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์จากใบของพืชเศรษฐกิจในประเทศไทยและการผลิตสารส่งเสริมการเจริญพืชออกซินโดยสายพันธุ์ที่คัดเลือกว่ามีประสิทธิภาพสูง พร้อมกับการศึกษาชีววิถีการสังเคราะห์
(โครงการวิจัยเลขที่ RTA5480009)
วันศุกร์ที่ 23 พฤษภาคม 2557 เวลา 9.00-16.30 น.
ณ ห้อง 202 อาคารทวี ญาณสุคนธ์ คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์
จตุจักร กรุงเทพมหานคร

9.00–9.15 น. ลงทะเบียน

9.15-12.15 น. ผู้ดำเนินรายการ: ดร. พนารัตน์ อรุณรัติยากร

9.15–9.45 น. กล่าวต้อนรับ และสรุปภาพรวมของโครงการเลขที่ RTA5480009
ศ.ดร.สาวิตรี ลิ่มทอง
ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์

9.45-12.15 น. ประธาน: ดร.มณี ตันติรุ่งกิจ เลขานุการ: นางสาววนัชพร บุญธรรม

9.45–10.15 น. ความหลากหลายของยีสต์บนผิวใบพืชและความสามารถในการผลิตกรดอินโด-3-อะซิติก (Diversity of phylloplane yeasts and their capability to produce indole-3-acetic acid)
ดร.รุ่งลักษณ์ แก้ววิเชียร และคณะ
ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ และศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ

10.15–10.45 น. การวิจัยความหลากหลายของยีสต์อีพิไฟต์บนใบข้าวด้วยวิธีการเพาะเลี้ยงเชื้อ (Investigation on diversity of epiphytic yeasts on rice leaves by culture-dependent method)
ดร.ศศิธร จินดามรกฎ และคณะ
ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ
สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ

10.45-11.15 น. พักรับประทานอาหารว่าง

11.15–11.45 น. การวิจัยความหลากหลายของยีสต์อีพิไฟต์บนใบข้าวโพดด้วยวิธีการเพาะเลี้ยงเชื้อ (Investigation on diversity of epiphytic yeasts on corn leaves by culture-dependent method)
นางสาวสมจิต อ่ำอินทร์ และคณะ
ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ

สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ

11.45–12.15 น. การวิจัยความหลากหลายของยีสต์อีพิไฟต์บนใบอ้อยด้วยวิธีการเพาะเลี้ยงเชื้อและการอธิบายยีสต์สปีชีส์ใหม่
(Investigation on diversity of epiphytic yeasts on sugarcane leaves by culture-dependent method and description of new yeast species)
นางสาวจันทร์จีรา สุรัสวดี และคณะ
ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์

12.15-13.15 น. พักรับประทานอาหารกลางวัน

13.15-17.00 น. ผู้ดำเนินรายการ: อาจารย์รุจิราลัย พูลทวี
ประธาน: ผศ.ดร.นันทนา สีสุข เลขานุการ: ดร.รุ่งลักษณ์ แก้ววิเชียร

13.15-13.45 น. การวิจัยความหลากหลายของยีสต์เอนโดไฟต์ในใบข้าว ข้าวโพด และอ้อย ด้วยวิธีการเพาะเลี้ยงเชื้อและการอธิบายยีสต์สปีชีส์ใหม่
(Investigation on diversity of endophytic yeasts from rice, sugarcane and corn leaves by culture-dependent method and description of new yeast species)
นางสาวพัณณิดา ขุนนามวงษ์ และคณะ
ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์

13.45-14.15 น. การวิจัยความหลากหลายของยีสต์เอนโดไฟต์ในใบข้าวและใบอ้อยด้วยวิธีการไม่เพาะเลี้ยงเชื้อ
(Investigation on diversity of endophytic yeasts from rice and sugarcane leaves by culture-independent method)
ดร.มณี ตันติรุ่งกิจ และคณะ
ฝ่ายปฏิบัติการวิจัยและเรือนปลูกพืชทดลอง คณะเกษตร กำแพงแสน
มหาวิทยาลัยเกษตรศาสตร์ วิทยาเขตกำแพงแสน

14.15 -14.45 น. การวิจัยความหลากหลายของยีสต์อีพิไฟต์บนผิวใบข้าวและใบอ้อยด้วยวิธีทางอณูชีววิทยา
(Investigation on diversity of epiphytic yeasts on rice and sugarcane phyllospheres by molecular biological techniques)
ดร.รุจิกาญจน์ นาสนิท และคณะ
ภาควิชาเทคโนโลยีชีวภาพ คณะวิศวกรรมศาสตร์และเทคโนโลยีอุตสาหกรรม
มหาวิทยาลัยศิลปากร

14.45-15.15 น. พักรับประทานอาหารว่าง

15.15-15.45 น. การศึกษาสภาวะที่เหมาะสมของการผลิตกรดอินโดล 3 แอซิติก โดยยีสต์สีแดง *Rhodosporidium paludigenum* DMKU-RP301
(Optimization of indole-3-acetic acid production by red yeast *Rhodosporidium paludigenum* DMKU-RP301)
นายภูมิน นุตรทัต และคณะ
ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์
ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ

15.45–16.15 น. ปัจจัยที่มีผลต่อการผลิตกรดอินโดล-3-แอซิติกของยีสต์อีพิไฟต์สายพันธุ์ DMKU-CP293 จากผิวใบข้าวโพด
(Factors effecting indole-3-acetic-acid production of epiphytic yeast strain DMKU-CP293 from corn leaves)
ดร. รัฐดา จันทร์กลั่น และคณะ
ภาควิชาเทคโนโลยีชีวภาพ คณะวิทยาศาสตร์และเทคโนโลยี มหาวิทยาลัยธรรมศาสตร์

16.15–17.00 น. การศึกษาสภาวะที่เหมาะสมต่อการผลิตกรดอินโดล-3-แอซิติกแอซิดจากยีสต์อีพิไฟต์สายพันธุ์ DMKU-CP293 โดยวิธีพื้นผิวตอบสนอง
(Optimization for indole-3-acetic acid production from epiphytic yeast strain DMKU-CP293 using response surface methodology)
นางสาวพรพรรณ ไทยเจริญ และคณะ
ภาควิชาเทคโนโลยีชีวภาพ คณะวิทยาศาสตร์และเทคโนโลยี มหาวิทยาลัยธรรมศาสตร์

17.00-17.30 น. ประชุมโต๊ะกลม ประธาน ศ.ดร. สาวิตรี ลิ่มทอง

TRF Senior Research Symposium
Professor Dr. Savitree Limtong

Title: Biodiversity and ecology of endophytic and epiphytic yeasts from leaves of agronomic crops in Thailand and production of plant growth promoting auxins by the selected promising strain with the elucidation of it biosynthetic pathway (Research number RTA5480009)

Diversity of phylloplane yeasts and their capability to produce indole-3-acetic acid

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Samples of rice leaves (n=85), corn leaves (n=65) and sugarcane leaves (n=94) were collected from 28 districts within nine provinces throughout Thailand. A total of 441 yeast strains were isolated from the phylloplane of leaf samples (n=244), which included 156 yeast strains from rice leaves, 126 yeast strains from corn leaves and 159 yeast strains from sugarcane leaves. Strain and species were identified using gene sequence analysis of the D1/D2 domain of the large subunit rRNA and phylogeny. 155 yeast strains were identified from rice leaves from 31 known species in 18 genera belonging to Ascomycota i.e. *Candida*, *Clavispora*, *Cyberlindnera*, *Debaryomyces*, *Kodamaea*, *Lachancea*, *Metschnikowia*, *Meyerozyma*, *Millerozyma*, *Pichia*, *Wickerhamomyces*, *Yamadazyma* and *Yarrowia*, and Basidiomycota i.e. *Cryptococcus*, *Pseudozyma*, *Rhodotorula*, *Sporobolomyces* and *Trichosporon*; 123 strains were identified from corn leaves from 38 known species in 17 genera belonging to Ascomycota i.e. *Candida*, *Cyberlindnera*, *Geotrichum*, *Hanseniaspora*, *Kluyveromyces*, *Kodamaea*, *Meyerozyma*, *Millerozyma*, *Pichia*, *Torulaspora*, *Wickerhamomyces* and *Yamadazyma*, and Basidiomycota i.e. *Cryptococcus*, *Rhodosporidium*, *Rhodotorula*, *Sporobolomyces* and *Sporidiobolus*; and 146 strains were identified from sugarcane leaves from 35 known species in 19 genera belonging to Ascomycota i.e. *Candida*, *Cyberlindnera*, *Debaryomyces*, *Hanseniaspora*, *Kluyveromyces*, *Lachancea*, *Lodderomyces*, *Metschnikowia*, *Meyerozyma*, *Millerozyma*, *Pichia*, *Torulaspora* and *Wickerhamomyces*, and Basidiomycota i.e. *Hannaella*, *Cryptococcus*, *Rhodosporidium*, *Rhodotorula*, *Sporidiobolus* and *Sporobolomyces*. Eight strains showed homology to undescribed species. In total, nine strains were found to represent six novel species. The prevalent species were *Rhodotorula taiwanensis*, *Meyerozyma caribbica* and *Candida tropicalis* with low frequencies, 14.3, 12.0 and 10.9% of the total number of yeast strains, respectively. Individual leaf sample was generally accommodated by two yeast species (56.5, 60.0 and 48.9% of total samples of rice, corn and sugarcane phylloplane, respectively). One hundred and forty eight strains from 40 species, four strains of an undescribed species and two strains of a novel species showed the capability to produce IAA when cultivated in yeast extract peptone dextrose broth supplemented with 0.1% L-tryptophan. *Rhodosporidium fluviale* DMKU-RK253 produced the highest IAA concentration at 417.8 mg/L.

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Investigation on diversity of epiphytic yeasts on rice leaves by culture dependent method

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Three hundred and seventynine isolates of epiphytic yeasts were isolated from 112 rice leaves from 12 provinces in Thailand. Among them, 354 isolates were identified based on the sequences of D1/D2 domain of 26S rDNA. Three hundred and two isolates (85.3%) were identified as 51 known species in 19 genera of *Blastobotrys* (1 isolates), *Bullera* (18 isolates), *Candida* (24 isolates), *Cryptococcus* (22 isolates), *Debaryomyces* (2 isolates), *Hyphopichia* (1 isolates), *Jaminaea* (1 isolates), *Kodamaea* (8 isolates), *Lodderomyces* (1 isolates), *Meyerozyma* (10 isolates), *Pichia* (3 isolates), *Pseudozyma* (128 isolates), *Rhodosporidium* (10 isolates), *Rhodotorula* (30 isolates), *Sporidiobolus* (5 isolates), *Sporobolomyces* (27 isolates). *Torulaspora* (2 isolates), *Trichosporon* (4 isolates) and *Wickerhamomyces* (5 isolates). Twenty-one isolates (5.9%) were identified as undescribed species in 4 genera of *Candida* (1 isolate), *Cryptococcus* (9 isolates), *Pseudozyma* (4 isolates) and *Rhodotorula* (7 isolates). Furthermore, thirty-one isolates (8.8%) differed from the nearest species by 1% nucleotide substitutions or more in the D1/D2 domain and were considered to represent 20 new species of *Bullera* (2 isolates), *Candida* (1 isolate), *Cryptococcus* (13 isolates), *Occultifur* (2 isolates), *Pseudozyma* (5 isolates), *Rhodosporidium* (4 isolates), *Rhodotorula* (3 isolates) and *Sakaguchia* (1 isolate). The new species were characterized using polyphasic taxonomy and the name *Yamadazyma nontaburiensis* sp. nov. was proposed for the strain YE170. The remaining 25 isolates are currently being identified. Yeast-like fungi in genera *Aureobasidium, Acremonium, Cephalosporium, Hyponectria, Melanopsichium, Moesziomyces, Sarocladium, Sporisorium, Syncephalastrum* and *Ustilago* were also found on the rice leaves. These epiphytic yeasts will be screened for promising auxin producing strains prioritising production of indole-3-acetic acid. IAA production will also be measured after optimization by response surface methodology.

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Investigation on diversity of epiphytic yeasts on corn leaves by culture-dependent method

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The diversity of epiphytic yeasts was investigated from 109 corn leaves which were collected from 11 provinces in Thailand. Two hundred and ninety six isolates of epiphytic yeasts, isolated using a culture dependent technique, were identified based on comparative sequence analysis of the D1/D2 domain of the LSU rRNA gene. Among them, 218 isolates were identified as described species belonging to 39 species of 14 genera including *Bullera* (11 isolates), *Candida* (15 isolates), *Cryptococcus* (21 isolates), *Debaryomyces* (1 isolate), *Jaminaea* (1 isolate), *Kodamaea* (7 isolates), *Meyerozyma* (10 isolates), *Pseudozyma* (97 isolates), *Rhodosporidium* (7 isolates), *Rhodotorula* (23 isolates), *Sporidiobolus* (2 isolates), *Sporobolomyces* (19 isolates), *Torulaspora* (2 isolates) and *Wickerhamomyces* (2 isolates). The dominant species was *Pseudozyma antarctica* (58 isolates). Nine isolates were identified as undescribed species of *Cryptococcus* (4 isolates) and *Rhodotorula* (5 isolates). In this study, 24 isolates were identified as novel yeast species belonging to 7 genera including *Bullera* (2 isolates), *Cryptococcus* (9 isolates), *Occultifur* (2 isolates), *Pseudozyma* (3 isolates), *Rhodotorula* (3 isolates), *Rhodosporidium* (4 isolates) and *Sakaguchia* (1 isolate). These yeast isolates will be characterized by polyphasic taxonomy in order to describe and propose as novel yeast species. The remaining 25 isolates have not yet been identified. Furthermore, 20 isolates initially identified as yeast by cells and colony morphological characterization, were further characterised by sequence analysis of the D1/D2 domain of the LSU rRNA gene and were in fact indentified as fungi belonging to eight genera including *Acremonium, Aureobasidium, Melanopsichium, Moesziomyces, Sporisorium, Syncephalastrum, Urocystis* and *Ustilago.*

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Investigation on diversity of epiphytic yeasts on sugarcane leaves by culture-dependent method and description of new yeast species

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This study aimed to investigate epiphytic yeast diversity isolated from the phylloplane of sugarcane in Thailand. A total of 102 sugarcane leaf samples were collected from 10 provinces. Yeasts were isolated from the surface of leaves by dilution plating of leaf washings. A total of 267 yeast strains were obtained and identified by molecular taxonomy using sequence analysis of the D1/D2 region of the large subunit (LSU) rRNA gene. 127 strains (47.6 %) were identified as known yeast species including 91 strains (34.1 %) of basidiomycetous yeast and 23 strains (8.6 %) of ascomycetous yeast. The known basidiomycetous yeast species were identified as 26 species in 9 genera including *Cryptococcus* (8 species), *Pseudozyma* (6 species), *Sporobolomyces* (4 species), *Rhodotorula* (3 species), *Bullera* (1 species), *Jaminaea* (1 species), *Occultifur* (1 species), *Sporidiobolus* (1 species) and *Tremella* (1 species). The known ascomycetous species were identified as 4 species in 3 genera including *Candida* (2 species), *Kodamaea* (1 species) and *Meyerozyma* (1 species). Fifty-seven strains were identical or similar to 19 undescribed yeast species in 4 genera. In addition, 31 strains were found to represent novel yeast species showing homology to thirteen species including *Candida infanticola*, *Cryptococcus laurentii*, *Cryptococcus* aff. *laurentii*, *Cryptococcus nemorosus*, *Jaminaea angkoriensis*, *Occultifur externus*, *Pseudozyma jejuensis*, *Pseudozyma pruni*, *Pseudozyma rugulosa*, *Pseudozyma shanxiensis*, *Rhodotorula marina*, *Rhodotorula taiwanensis* and *Tremella globispora*. The most prevalent yeast species from the phylloplane of sugarcane was *Meyerozyma caribbica* (15.7 %) followed by *Jaminaea angkoriensis* (9.4 %) and *Sporobolomyces vermiculatus* (6.3 %). Among the strains of novel yeast species strain DMKU-SP85^{T} was studied based on polyphasic taxonomy together with strain (DMKU-RE97) isolated from the tissue of rice leaf. Using morphological, biochemical, physiological and chemotaxonomic characteristics, and sequence analysis of the D1/D2 region of the large subunit (LSU) rRNA gene and the internal transcribed spacer (ITS) region, these strains were identified as a novel *Papiliotrema* species although sexual reproduction was not observed. The sequences of the D1/D2 region of the LSU rRNA gene and ITS region of these strains were identical but different from the type strain of *Cryptococcus nemorosus* by 0.6% nucleotide substitutions (4 nt substitutions out of 597 nt) in the D1/D2 region of the LSU rRNA gene and 1.8% nucleotide substitution (9 nt substitutions out of 499 nt) in the ITS region. Therefore, the name *Papiliotrema siamensis* f.a., sp. nov. was proposed (type strain DMKU-SP85^{T} = BCC 69499^{T} = CBS 13330^{T}).

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Investigation on diversity of endophytic yeasts from rice, sugarcane and corn leaves by culture-dependent method and description of new yeast species

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Endophytic microorganisms have been defined as the microorganism that reside the whole or part of their life cycle inside plant tissues without causing any damage or showing negative effect on a host plant. This work aimed to study the diversity of endophytic yeasts in plant leaf tissue. A total of 311 plant leaf samples were collected from cultivation fields in central and north-eastern parts of Thailand from October 2011 to April 2012. The samples consisted of 100 samples of rice leaf, collected from 30 districts in 11 provinces, 109 samples of corn leaf collected from 27 districts in 12 provinces and 102 samples of sugarcane leaf collected from 32 districts in 13 provinces. Four hundred and seventeen endophytic yeast strains were isolated from the tissue of plant leaves including 125 strains from rice leaves, 143 strains from corn leaves and 149 strains from sugarcane leaves. Identification on the basis of the D1/D2 region of the large subunit (LSU) rRNA gene sequence analysis revealed that 116 strains from rice leaves were identified to be known species. Eighty-nine strains were in Phylum Basidiomycota, 20 species in 8 genera *viz. Pseudozyma* (5 species), *Cryptococcus* (1 species), *Rhodotorula* (4 species), *Rhodosporidium*(2 species), *Sporobolomyces*(2species), *Bullera* (1 species), *Cyberlindnera* (1 species), *Sporidiobolus* (1 species) and twenty-seven strains were in Phylum Ascomycota, 9 species in 5 genera *viz. Candida* (3 species), *Meyerozyma* (2 species), *Kodamaea* (1 species), *Pichia* (2 species), *Wickerhamomyces* (1 species). One strain was similar to an undescribed species. In addition, seven strains were identified as 4 new species. One hundred and twenty endophytic yeast strains from corn leaves were identified to be known species including 90 strains from 23 species of 8 genera belonging to Phylum Basidiomycota *viz. Cryptococcus* (8 species), *Pseudozyma* (5 species), *Rhodosporidium* (2 species), *Rhodotorula* (2 species), *Sporobolomyces* (2 species), *Sporidiobolus* (2 species), *Bullera* (1 species), *Occultifur* (1 species) and thirty strains from 11 species of 5 genera belonging to Phylum Ascomycota *viz. Candida* (6 species), *Pichia* (2 species), *Kluyveromyces* (1 species), *Meyerozyma* (1 species) and *Wickerhamomyces* (1 species). Four strains were similar to four undescribed species and one strain represented a new species. Among endophytic strains from sugarcane leaf, 114 strains were identified as known species including79 strains from 27 species of 12 genera belonging to Phylum Basidiomycota *viz. Cryptococcus* (6 species), *Pseudozyma* (6 species), *Rhodotorula* (3 species), *Bullera* (4 species), *Cyberlindnera* (1 species), *Jaminaea* (1 species), *Occultifur* (1 species), *Rhodosporidium* (1 species), *Sporobolomyces* (1 species), *Sporidiobolus* (1 species), *Tremella* (1 species) and *Trichosporon* (1 species), and 35 strains from 6 species of 4 genera belonging to

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Ascomycota *viz. Meyerozyma* (2 species), *Pichia* (2 species), *Candida* (1 species) and *Dioszegia* (1 species). Nine strains were similar to 7 undescribed species and 8 strains represented 3 new species. The most prevalent species of endophytic yeasts from rice leaves was *Pseudozyma aphidis* with a frequency of 22 %, while the most prevalent species of endophytic yeast from corn leaves were *Pseudozyma rugulosa* (12 %) and *Meyerozyma guilliermondii* (12 %). The most prevalent species of endophytic yeasts from sugarcane leaves was *Meyerozyma guilliermondii* with a frequency of 16%. Identification on the basis of morphological, biochemical, physiological and chemotaxonomic characteristics, sequence analysis of the D1/D2 region of LSU rRNA gene and the internal transcribed spacer (ITS) region, the 16 strains represented 8 new yeast species in the genera *Candida*, *Cryptococcus*, *Occultifur*, *Pseudozyma*, *Rhodosporidium*, *Tremella* and *Wickerhameilla*. We proposed three strains isolated from the tissue of sugarcane leaves represent a novel species of the genus *Wickerhamiella*. The new species were most closely related to the type strain of *Candida infanticola* NRRL Y-17858^{T} (DQ438230) but with 25-26 nucleotide substitutions (4.5- 4.6 %) in the D1/D2 region of the LSU rRNA gene and 11-13 nucleotide substitution (3.1-3.6 %) in the ITS region. The name *Wickerhamiella siamensis* sp. nov. will be proposed and the type strain will be DMKU-SE106^{T} (=BCC61185^{T} = NBRC 109697^{T} = CBS 13331^{T}).

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Investigation on diversity of endophytic yeasts from rice and sugarcane leaves by culture-independent method

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Recent studies have employed molecular biology techniques to rapidly investigate microbe diversity within and between ecosystems. Therefore, in this study a culture-independent method was used to examine endophytic yeasts associated with rice and sugarcane leaves using a semi-nested PCR technique. The primers used were ITS1-F_KYO1/NL4, the outer set, to amplify the ITS - D1/D2 region of large subunit (LSU) rRNA gene and NL1/NL4, the inner set, to amplify the D1/D2 region. The D1/D2 libraries of rice and sugarcane were characterized by PCR-RFLP using a single digestion with *Hae*III, *Hin*fI and *Cfo*I, and the selected clones were subjected to sequence and phylogenetic analysis. The results revealed that 22 OTUs from 12 rice libraries belonged to 5 genera and 9 species of yeast as follows: *Candida metapsilosis, Cryptococcus foliicola, Cryptococcus laurentii, Cryptococcus victoriae, Debaryomyces hansenii, Meyerozyma guilliermondii, Pseudozyma antarctica, Pseudozyma aphidis* and *Pseudozyma rugulosa*. Moreover, OTUs having more than 1% substitution (n=3) were found to be closely related to *Candida metapsilosis* and *Cryptococcus laurentii.* Sugarcane samples produced 20 OTUs from 15 libraries which belonged to 8 genera and 10 species of yeast as follows: *Candida palmioleophila, Cryptococcus flavus, Debaryomyces hansenii, Kodamaea ohmeri, Malassezia restricta, Rhodotorula calyptogenae, Rhodotorula cassiicola, Rhodotorula marina, Pseudozyma aphidis* and *Sporobolomyces vermiculatus.* Additionally, 6 OTUs with more than 1% nucleotide substitutions were closely related to *Cryptococcus flavus, Debaryomyces hansenii, Malassezia restricta, Rhodotorula calyptogenae, Rhodotorula cassiicola* and *Pseudozyma aphidis.*

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Investigation on diversity of epiphytic yeasts on rice and corn phyllospheres by molecular biological techniques

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This research aimed to study the diversity of epiphytic yeasts from the phyllospheres of economic crops in Thailand such as rice and sugarcane. The samples of plant leaf were collected randomly from agronomic areas in Thailand. Molecular biology techniques were used to identify yeasts including polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) and nucleotide sequence analysis of the D1/D2 domain of the large subunit (LSU) rRNA gene. The results showed that the most common yeasts detected in both rice (47 samples) and sugarcane (48 samples) phyllospheres were members of basidiomycota. Yeasts found on rice phyllospheres were identified as 17 described species in 9 genera including *Bullera*, *Cryptococcus*, *Hannaella*, *Pseudozyma*, *Sporidiobolus*, *Sporobolomyces*, *Metschnikowia*, *Meyerozyma* and *Wickerhamomyces*, and 7 undescribed species in 5 genera. *Pseudozyma antarctica* and *Bullera japonica* were the most common species detected on rice phyllosphere samples, 34 samples and 11 samples, respectively. Furthermore, yeasts detected on sugarcane phyllospheres were identified as 8 described species in 5 genera including *Jaminaea*, *Pseudozyma*, *Malassezia*, *Rhodotorula* and *Sporobolomyces*, and 8 undescribed species in 4 genera. All of them were members of basidiomycota. The most common genus on sugarcane phyllospheres was *Pseudozyma* including *P. rugulosa*, *P. hubeiensis* and *P. prolifica*. In conclusion, we can reveal that most of epiphytic yeasts on rice and sugarcane phyllospheres from this study were

basidiomycetous yeasts. Moreover, the epiphytic yeasts on rice leaves were more diverse than those on sugarcane leaves.

Optimization of indole-3-acetic acid production by red yeast *Rhodosporidium paludigenum* DMKU-RP301

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Indole-3-acetic acid (IAA), a plant hormone classified as an indole derivative of the auxin family, has been widely studied and is the dominant auxin found in plants. IAA can be produced not only from plants, but also from various kinds of microorganisms such as bacteria and fungi, including yeast and algae. There is a long history of IAA production research in yeast. However, no reports of the optimization of yeast IAA production has been published to date. Therefore, this study aimed to investigate the optimization of IAA production in yeast. In this study optimisation of the production of IAA by red yeast *Rhodosporidium paludigenum* DMKU-RP301 (AB920314) using one factor at a time (OFAT) and response surface methodology (RSM) i.e. Plackett-Burman and Box-Behnken designs was investigated. The study determined the effects of incubation time, carbon and nitrogen sources, growth factor, tryptophan, temperature, shaking speed, NaCl and pH on the production of IAA. The results showed that incubation time, carbon and nitrogen sources, growth factor, tryptophan, temperature, shaking speed and pH affected IAA production by *R. paludigenum* DMKU-RP301. Maximum IAA production of 1,464.7 and 1,623.9 mg/l were observed as a result of the studies using OFAT and RSM, respectively. The optimal medium and conditions observed in this study resulted in an increase of IAA production 4.6-5.0 times compared to unoptimized conditions, i.e. when YPD broth supplemented with 0.1% L-tryptophan was used as a production medium.

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Factors Effecting Indole-3-Acetic-Acid Production of DMKUCP-293 Epiphytic Yeast from Corn Leaves

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Factors influencing the production of Indole-3-Acetic-Acid (IAA) of DMKUCP-293, epiphytic yeast isolated from corn leaves, were studied using the conventional method (one factor at a time). A 500 ml Erlenmeyer flask containing 150 ml YPD broth varying carbon and nitrogen source and concentration, L-tryptophan concentration, pH, and agitation speed, initial cell concentration was used. The initial cell concentration was adjusted to an optical density at 600 nm of 1.0 and then incubated on a rotary shaker at 30 °C and 200 rpm. Yeast growth and the production of IAA were observed by sampling 24 h intervals. Growth was evaluated by the determination of dry cell weight and IAA concentration was quantitatively determined using High Pressure Liquid Chromatography (HPLC). The results revealed that growth of DMKUCP-293 was 8.10 g/l DCW and the highest IAA concentration obtained was 1,559.36 mg/L (with 192.46 mg/g DCW) when cultivated in culture medium that composed of 3.0% galactose, 1.0% yeast extract, 2.0% peptone, 0.5% tryptophan, pH 6.0 and incubated for 9 days. Effect of agitation speed on IAA production for DMKUCP-293 was investigated using 5L batch fermentation and with 2L of working volume of the same culture medium composition, pH 6.0 with 1.0 vvm aeration rate at 30 °C, varying the agitation speed as 400, 500 and 600 rpm, respectively. The results showed that an agitation speed of 500 rpm allowed DMKUCP-293 to produce the highest concentration of IAA. These conditions provided 8.18 g/L DCW and 2,339.87mg/L of IAA production with 286.02 mg/g DCW yield when cultivated for 9 days.

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Optimization for Indole-3-acetic acid production from DMKU-CP293 epiphytic yeast using response surface methodology

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Study of factors affecting the interaction of galactose, yeast extract, and tryptophan to produce IAA from DMKUCP-293epiphytic yeast using response surface methodology with Box-benhken statistical experimental design. Experiments were performed in 250 ml Erlenmeyer flasks containing 50 ml culture broth. The initial cell concentration was adjusted as optical density at 600 nm of 1.0 and incubated at 30 °C on 200 rpm rotary shaker. Samples were collected for analysis at regular intervals. Growth was determined using the dry weight of the cells and IAA production was quantitatively analyzed using high pressure liquid chromatography (HPLC). Results indicated that all 3 factors affected IAA production. The optimal medium consisted of 2.41, 1.52, and 0.41% galactose, yeast extract, and L-tryptophan, respectively. IAA production of DMKUCP-293 with 8 days of cultivation resulted in an IAA yield of 2,402.12±48.08 mg/L, which matched well with the predicted values of 2,311.79 mg/L over the same period of time. Optimized medium composition will be further investigated for IAA production by batch fermentation using a 5L bioreactor.

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การประชุมวิชาการ เมธีวิจัยอาวุโส สกว.
ศาสตราจารย์ ดร. สาวิตรี ลิ่มทอง

ความหลากหลายและนิเวศวิทยาของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์จากใบของพืชเศรษฐกิจในประเทศไทย และการผลิตสารส่งเสริมการเจริญพืชออกซินโดยสายพันธุ์ที่คัดเลือกว่ามีประสิทธิภาพสูง พร้อมกับการศึกษาชีววิถีการสังเคราะห์
(โครงการวิจัยเลขที่ RTA5480009)

วันที่ 3 กันยายน 2558 เวลา 9.00-16.30 น.
ณ ห้อง 202 อาคารทวี ญาณสุคนธ์ คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์
จตุจักร กรุงเทพมหานคร

9.00–9.30 น.	**ลงทะเบียน**
9.30-10.00 น.	ผู้ดำเนินรายการ: ดร. พนารัตน์ อรุณรัติยากร **เปิดประชุม โดย คณบดี คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์** ถ่ายภาพหมู่
10.00-10.20 น.	**สรุปภาพรวมและผลสำเร็จของโครงการเลขที่ RTA5480009** โดย ศ.ดร. สาวิตรี ลิ่มทอง ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์
10.20–10.40 น.	พักรับประทานอาหารว่าง (เชิญผู้ร่วมประชุมทั้งหมด)
10.40-12.00 น.	**นำเสนอผลงาน** ประธาน: ผศ.ดร. นันทนา สีสุข เลขานุการ: นางสาวพิรพรรณ พลบุรี
10.40–11.00 น.	**ความหลากหลายของยีสต์อีพิไฟต์บนใบข้าวด้วยวิธีการเพาะเลี้ยงเชื้อ** (Diversity of epiphytic yeasts on rice leaves by culture-dependent method) โดย ดร. ศศิธร จินดามรกฎ ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ
11.00-11.20 น.	**ความหลากหลายของยีสต์อีพิไฟต์บนใบข้าวโพดด้วยวิธีการเพาะเลี้ยงเชื้อ** (Diversity of epiphytic yeasts on corn leaves by culture-dependent method) โดย นางสาวสมจิต อ่ำอินทร์ ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ
11.20–11.40 น.	**ความหลากหลายของยีสต์อีพิไฟต์บนใบอ้อยด้วยวิธีการเพาะเลี้ยงเชื้อและความสามารถในการควบคุมราโรคพืชในหลอดทดสอบ** (Diversity of epiphytic yeasts on sugarcane leaves by culture-dependent method and their biocontrol activity against plant pathogenic fungi in vitro) โดย นางสาวจันทร์จีรา สุรัสวดี ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์

11.40-12.00 น. **ความหลากหลายของยีสต์บนผิวใบพืชและความสามารถในการผลิตกรดอินโด-3-อะซิติก**
(Diversity of phylloplane yeasts and their capability to produce indole-3-acetic acid)
โดย ดร. รุ่งลักษณ์ แก้ววิเชียร
สาขาจุลชีววิทยา คณะวิทยาศาสตร์วิทยาศาสตร์และเทคโนโลยี
มหาวิทยาลัยราชภัฏบ้านสมเด็จเจ้าพระยา

12.00-13.00 น. **พักรับประทานอาหารกลางวัน** (เชิญผู้ร่วมประชุมทั้งหมด)

13.00-16.00 น. ผู้ดำเนินรายการ: ดร. ชนิตา บุญมาก
การนำเสนอผลงาน
ประธาน: ดร. ศศิธร จินดามรกฎ เลขานุการ: นางสาวสมจิต อ่ำอินทร์

13.00-13.20 น. **ความหลากหลายของยีสต์อีพิไฟต์บนผิวใบข้าวโพดด้วยวิธีการไม่เพาะเลี้ยงเชื้อ**
(Diversity of epiphytic yeasts on corn phyllospheres by culture-independent method)
โดย ดร. รุจิกาญจน์ นาสนิท
ภาควิชาเทคโนโลยีชีวภาพ คณะวิศวกรรมศาสตร์และเทคโนโลยีอุตสาหกรรม
มหาวิทยาลัยศิลปากร

13.20-13.40 น. **ความหลากหลายของยีสต์เอนโดไฟต์ในใบข้าว อ้อย และข้าวโพดด้วยวิธีการไม่เพาะเลี้ยงเชื้อ**
(Diversity of endophytic yeasts from rice, sugarcane and corn leaves by culture-independent method)
โดย ดร. มณี ตันติรุ่งกิจ และคณะ
ศูนย์ปฏิบัติการวิจัยและเรือนปลูกพืชทดลอง คณะเกษตร กำแพงแสน
มหาวิทยาลัยเกษตรศาสตร์ วิทยาเขตกำแพงแสน

13.40-14.00 น. **ความหลากหลายของยีสต์เอนโดไฟต์ในใบข้าว อ้อย และข้าวโพดด้วยวิธีการเพาะเลี้ยงเชื้อและการอธิบายยีสต์สกุลและสปีชีส์ใหม่**
(Investigation on diversity of endophytic yeasts from rice, sugarcane and corn leaves by culture-dependent method and description of new yeast genus and species)
โดย นางสาวพัณณิดา ขุนนามวงษ์
ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์

14.00-14.20 น. ***Rhodosporidium paludigenum*** **DMKU-RP301 ยีสต์ผิวใบข้าวที่มีคุณลักษณะสนับสนุนการเจริญของพืชรวมทั้งการผลิตกรดอินโดล 3 แอซิติก**
(*Rhodosporidium paludigenum* DMKU-RP301 rice phyllospheric yeast possesses plant growth promoting characters including indole-3-acetic acid production)
โดย ผศ.ดร. นันทนา สีสุข
ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์

14.20-14.40 น. **พักรับประทานอาหารว่าง** (สำหรับผู้ร่วมประชุมทุกคน)

14.40-15.40 น. **การนำเสนอผลงาน (ต่อ)**

15.00-15.20 น. **การผลิตกรดอินโดล 3 แอซิติก โดยยีสต์ผิวใบข้าว *Rhodosporidium paludigenum* DMKU-RP301 และการศึกษาชีววิถีการสังเคราะห์**
(Production of indole-3-acetic acid by rice phyllospheric yeast *Rhodosporidium paludigenum* DMKU-RP301 and investigation of its IAA biosynthetic pathway)
โดย นายภูมิน นุตรทัต
ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์

15.20-15.40 น. ความสามารถในการควบคุมราโรคพืชโดยยีสต์อีพิไฟต์บนผิวใบพืช
(Biocontrol activity of epiphytic yeasts from plant leaves against plant pathogenic fungi)
โดย นางสาวปาริฉัตร อินโต
ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์

15.40–16.00 น. **ประชุมโต๊ะกลม**

ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

โครงการทุนส่งเสริมกลุ่มวิจัย สัญญาเลขที่ RTA5480009
เรื่อง "ความหลากหลายและนิเวศวิทยาของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์จากใบของพืชเศรษฐกิจ ในประเทศไทย และการผลิตสารส่งเสริมการเจริญพืชออกซิน โดยสายพันธุ์ที่คัดเลือกว่ามีประสิทธิภาพสูง พร้อมกับการศึกษาชีววิถีการสังเคราะห์"

1. **Kaewwichian R.**, W. Yongmanitchai, H. Kawasaki and **S. Limtong***. 2012. *Metschnikowia saccharicola* sp. nov. and *Metschnikowia lopburiensis* sp. nov., two novel yeast species isolated from phylloplane in Thailand. *Antonie van Leeuwenhoek*. 102: 743–751. (IF 2012: 2.072) (*Corr)
2. **Kaewwichian, R.**, W. Yongmanitchai, H. Kawasaki, P.-H. Wang, S.-H. Yang and **S. Limtong***. 2013. *Yamadazyma siamensis* sp. nov., *Yamadazyma phyllophila* sp. nov. and *Yamadazyma paraphyllophila* sp. nov., three novel yeast species isolated from phylloplane in Thailand and Taiwan. *Antonie van Leeuwenhoek.* 103: 777-788. (IF 2013: 2.137) (*Corr)
3. **Kaewwichian, R.**, H. Kawasaki and **S. Limtong*.** 2013. *Wickerhamomyces siamensis* sp. nov., a novel yeast species isolated from phylloplane in Thailand. *International Journal of Systematic and Evolutionary Microbiology.* 63: 1568-1573. (IF 2014: 2.511) (*Corr)
4. Chamnanpa T., P. Limtong, N. Srisuk and S. Limtong*. 2013. *Pseudozyma vetiver* sp. nov., a novel anamorphic ustilaginomycetous yeast species isolated from the phylloplane in Thailand. *Antonie van Leeuwenhoek*. 104: 637-644. (IF 2014: 2.137) (*Corr)
5. **Nutaratat, P., N. Srisuk*, P. Arunrattiyakorn** and **S. Limtong**. 2014. Plant growth promoting traits of epiphytic and endophytic yeasts isolated from rice and sugar cane leaves in Thailand. *Fungal Biology.* 118: 683-694. (IF 2014: 2.342) (*Corr)
6. **Kaewwichian, R.** and **S. Limtong***. 2014. *Nakazawaea siamensis* f.a., sp. nov., a novel yeast species isolated from phylloplane in Thailand. *International Journal of Systematic and Evolutionary Microbiology.* 64: 266–270. (IF 2014: 2.511) (*Corr)
7. **Limtong, S.*, R. Kaewwichian**, W. Yongmanitchai and H. Kawasaki. 2014. Yeasts in phylloplanes of sugarcane in Thailand and their capability to produce indole-3-acetic Acid. *World Journal of Microbiology* and *Biotechnology*. 30:1785–1796. (IF2014: 1.779) (*Corr)
8. **Surussawadee, J.**, **P. Khunnamwong**, **N. Srisuk** and **S. Limtong*.** *Papiliotrema siamensis* f.a., sp. nov., a novel yeast species isolated from plant leaf in Thailand. 2014. *International Journal of Systematic and Evolutionary Microbiology.* 64: 3058-3062. (IF 2014: 2.511) (*Corr)
9. **Khunnamwong P., J. Surussawadee, S. Jindamorakot** and **S. Limtong*** 2014. *Wickerhamiella siamensis* f.a., sp. nov., a novel endophytic and epiphytic yeast species isolated from sugarcane leaf in Thailand. *International Journal of Systematic and Evolutionary Microbiology*. 64: 3849-3855. (IF 2014: 2.511) (*Corr)

10. **Nutaratat, P.**, W. Amsri, **N. Srisuk***, **P. Arunrattiyakorn** and **S. Limtong***. 2015. Indole-3-acetic acid production by newly isolated red yeast *Rhodosporidium paludigenum. Journal of General and Applied Microbiology.* 61: 1–9. (IF 2014: 0.943) (*Corr)
11. **Limtong, S***. and **R. Kaewwichian**. 2015. The diversity of culturable yeasts in the phylloplane of rice in Thailand. *Annals of Microbiology*. 65: 667–675. (IF 2014: 0.990) (*Corr)
12. **Jindamorakot S., S. Am-In, R. Kaewwichian** and **S. Limtong***. 2015. *Yamadazyma insecticola* f.a., sp. nov. and *Yamadazyma epiphylla* f.a., sp. nov., two novel yeast species isolated in Thailand". *International Journal of Systematic and Evolutionary Microbiology.* 65: 1290–1296. (IF 2014: 2.511) (*Corr)
13. **Kaewwichian R., S. Jindamorakot, S. Am-In**, M. Sipiczki and **S. Limtong***. (2015). *Hannaella siamensis* sp. nov. and *Hannaella phetchabunensis* sp. nov., two new anamorphic basidiomycetous yeast species isolated from plants. *International Journal of Systematic and Evolutionary Microbiology.* 65: 1297–1303. (IF 2014: 2.511) (*Corr)
14. **Nasanit R., K. Krataithong, M. Tantirungkij** and **S. Limtong*.** (2015). Assessment of epiphytic yeast diversity in rice (*Oryza sativa*) phyllosphere in Thailand by a culture-independent approach. *Antonie van Leeuwenhoek*. 107:1475–1490. DOI 10.1007/s10482-015-0442-2 (IF 2014: 1.806) (*Corr)
15. **Surussawadee, J., S. Jindamorakot**, T. Nakase, C.-F. Lee and **S. Limtong*.** (2015). *Hannaella phyllophila* sp. nov., a novel basidiomycetous yeast species associated with plants in Thailand and Taiwan. *International Journal of Systematic and Evolutionary Microbiology*.65 (7): 2135-2140. (IF 2014: 2.511) (*Corr)
16. **Khunnamwong, P., J. Surussawadee, S. Jindamorakot,** J. R. A. Ribeiro, A. N. Hagler and **S. Limtong*.** (2015).2 *Occultifur tropicalis* f.a., sp. nov., a novel Cystobasidiomycetous yeast species isolated from tropical regions. *International Journal of Systematic and Evolutionary Microbiology*. 65 (5): 1578–1582. (IF 2014: 2.511) (*Corr)
17. **Tantirungkij M., R. Nasanit** and **S. Limtong***. (2015). Assessment of endophytic yeast diversity in rice leaves by a culture-independent approach. *Antonie van Leeuwenhoek*. 108 (3): 633–647. (IF 2014: 1.806) (*Corr)

หากสนใจสามารถขอสำเนาเอกสารการตีพิมพ์แต่ละเรื่องได้ที่ ศ.ดร. สาวิตรี ลิ่มทอง (fscistl@ku.ac.th**)**

เรื่อง ความหลากหลายและนิเวศวิทยาของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์จากใบของพืชเศรษฐกิจในประเทศไทยและการผลิตสารส่งเสริมการเจริญพืชออกซินโดยสายพันธุ์ที่คัดเลือกว่ามีประสิทธิภาพสูง พร้อมกับการศึกษาชีววิถีการสังเคราะห์
(โครงการวิจัยเลขที่ RTA5480009)

ความหลากหลายของยีสต์อีพิไฟต์บนใบข้าวด้วยวิธีการเพาะเลี้ยงเชื้อ

ศศิธร จินดามรกฎ[1], สมจิต อ่ำอินทร์[1], และสาวิตรี ลิ่มทอง[2]
[1] *ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ ปทุมธานี 12120*
[2] *ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ กรุงเทพฯ 10900*

จากการศึกษาความหลากหลายของยีสต์อีพิไฟต์บนใบข้าวด้วยวิธีการเพาะเลี้ยงเชื้อ โดยทำการเก็บตัวอย่างใบข้าวจำนวน 112 ตัวอย่าง จาก 12 จังหวัด ในภาคต่างๆ ของประเทศไทยและนำมาจัดจำแนกโดยอาศัยอนุกรมวิธานระดับโมเลกุลด้วยการวิเคราะห์ลำดับนิวคลีโอไทด์ในบริเวณ D1/D2 ของ large subunit rRNA gene ผลการจัดจำแนกยีสต์จำนวน 354 ไอโซเลต พบว่ามียีสต์จำนวน 302 ไอโซเลต จัดจำแนกเป็นยีสต์ที่ทราบชื่อแล้ว (Known species) 51 สปีชีส์ ใน 19 สกุล คือ *Blastobotrys* (1 ไอโซเลต) *Bullera* (18 ไอโซเลต) *Candida* (24 ไอโซเลต) *Cryptococcus* (22 ไอโซเลต) *Debaryomyces* (2 ไอโซเลต) *Hyphopichia* (1 ไอโซเลต) *Jaminaea* (1 ไอโซเลต) *Kodamaea* (8 ไอโซเลต) *Lodderomyces* (1 ไอโซเลต) *Meyerozyma* (10 ไอโซเลต) *Pichia* (3 ไอโซเลต) *Pseudozyma* (128 ไอโซเลต) *Rhodosporidium* (10 ไอโซเลต) *Rhodotorula* (30 ไอโซเลต) *Sporidiobolus* (5 ไอโซเลต) *Sporobolomyces* (27 ไอโซเลต) *Torulaspora* (2 ไอโซเลต) *Trichosporon* (4 ไอโซเลต) และ *Wickerhamomyces* (5 ไอโซเลต) ยีสต์จำนวน 21 ไอโซเลตจัดจำแนกเป็นสปีชีส์ที่ยังไม่มีการอธิบาย (undescribed species) 16 สปีชีส์ ใน 4 สกุล คือ *Candida* (1 ไอโซเลต) *Cryptococcus* (9 ไอโซเลต) *Pseudozyma* (4 ไอโซเลต) และ *Rhodotorula* (7 ไอโซเลต) นอกจากนี้ยังพบยีสต์ที่มีลำดับนิวคลีโอไทด์ต่างจากสายพันธุ์ใกล้เคียงตั้งแต่ 1% ขึ้นไปจำแนกเป็นยีสต์สปีชีส์ใหม่ 31 ไอโซเลต ซึ่งจัดอยู่ใน 8 สกุล คือ *Bullera* (2 ไอโซเลต) *Candida* (1 ไอโซเลต) *Cryptococcus* (13 ไอโซเลต) *Occultifur* (2 ไอโซเลต) *Pseudozyma* (5 ไอโซเลต) *Rhodosporidium* (4 ไอโซเลต) *Rhodotorula* (3 ไอโซเลต) และ *Sakaguchia* (1 ไอโซเลต) โดยคาดว่าเป็นยีสต์สปีชีส์ใหม่ประมาณ 20 สปีชีส์ และได้นำยีสต์เหล่านี้มาศึกษาลักษณะต่างๆของยีสต์สปีชีส์ใหม่ด้วยวิธี polyphasic taxonomy โดยในการศึกษาครั้งนี้ได้เสนอยีสต์สายพันธุ์ YE-170 เป็นยีสต์สปีชีส์ใหม่ที่ให้ชื่อว่า *Yamadazyma epiphylla* sp. nov.

เรื่อง ความหลากหลายและนิเวศวิทยาของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์จากใบของพืชเศรษฐกิจ
ในประเทศไทยและการผลิตสารส่งเสริมการเจริญพืชออกซินโดยสายพันธุ์ที่คัดเลือกว่ามีประสิทธิภาพสูง
พร้อมกับการศึกษาชีววิถีการสังเคราะห์
(โครงการวิจัยเลขที่ RTA5480009)

Diversity of epiphytic yeasts on rice leaves by culture-dependent method

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Three hundred and seventy-nine isolates of epiphytic yeasts were isolated from 112 rice leaves from 12 provinces in Thailand. Among them, 354 isolates were identified based on the sequences of D1/D2 domain of large subunit rDNA. Three hundred and two isolates (85.3%) were identified as 51 known species in 19 genera of *Blastobotrys* (1 isolates), *Bullera* (18 isolates), *Candida* (24 isolates), *Cryptococcus* (22 isolates), *Debaryomyces* (2 isolates), *Hyphopichia* (1 isolates), *Jaminaea* (1 isolates), *Kodamaea* (8 isolates), *Lodderomyces* (1 isolates), *Meyerozyma* (10 isolates), *Pichia* (3 isolates), *Pseudozyma* (128 isolates), *Rhodosporidium* (10 isolates), *Rhodotorula* (30 isolates), *Sporidiobolus* (5 isolates), *Sporobolomyces* (27 isolates), *Torulaspora* (2 isolates), *Trichosporon* (4 isolates) and *Wickerhamomyces* (5 isolates). Twenty-one isolates (5.9%) were identified as undescribed species in 4 genera of *Candida* (1 isolate), *Cryptococcus* (9 isolates), *Pseudozyma* (4 isolates) and *Rhodotorula* (7 isolates). Furthermore, thirty-one isolates (8.8%) differed from the nearest species by 1% nucleotide substitutions or more in the D1/D2 domain and were considered to represent 20 new species of *Bullera* (2 isolates), *Candida* (1 isolate), *Cryptococcus* (13 isolates), *Occultifur* (2 isolates), *Pseudozyma* (5 isolates), *Rhodosporidium* (4 isolates), *Rhodotorula* (3 isolates) and *Sakaguchia* (1 isolate). These new species were characterized using polyphasic taxonomy and *Yamadazyma epiphylla* sp. nov. was described for the strain YE-170.

เรื่อง ความหลากหลายและนิเวศวิทยาของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์จากใบของพืชเศรษฐกิจในประเทศไทยและการผลิตสารส่งเสริมการเจริญพืชออกซินโดยสายพันธุ์ที่คัดเลือกว่ามีประสิทธิภาพสูง พร้อมกับการศึกษาชีววิถีการสังเคราะห์
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ความหลากหลายของยีสต์อีพิไฟต์บนใบข้าวโพดด้วยวิธีการเพาะเลี้ยงเชื้อ

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จากการศึกษาความหลากหลายของยีสต์อีพิไฟต์บนใบข้าวโพด โดยเก็บตัวอย่างใบข้าวโพดจำนวน 109 ตัวอย่าง จาก 11 จังหวัดของประเทศไทย และนำมาคัดแยกยีสต์ด้วยวิธีการเพาะเลี้ยงเชื้อ สามารถคัดแยกยีสต์ได้จำนวน 190 ไอโซเลต จากนั้นนำมาจัดจำแนกโดยอาศัยการเปรียบเทียบลำดับนิวคลีโอไทด์บริเวณ D1/D2 ของ LSU rRNA gene ผลการจัดจำแนกพบว่ายีสต์ 151 ไอโซเลต จัดจำแนกเป็นยีสต์สปีชีส์ที่รู้จักแล้ว 43 สปีชีส์ ใน 15 สกุล คือ *Bullera* (19 ไอโซเลต) *Candida* (10 ไอโซเลต) *Cryptococcus* (31 ไอโซเลต) *Dioszegia* (2 ไอโซเลต) *Hanseniaspora* (2 ไอโซเลต) *Jaminaea* (1 ไอโซเลต) *Kodamaea* (3 ไอโซเลต) *Meyerozyma* (5 ไอโซเลต) *Occultifur* (2 ไอโซเลต) *Pseudozyma* (21 ไอโซเลต) *Rhodosporidium* (15 ไอโซเลต) *Rhodotorula* (3 ไอโซเลต) *Sporidiobolus* (23 ไอโซเลต) *Sporobolomyces* (13 ไอโซเลต) และ *Wickerhamomyces* (1 ไอโซเลต) โดยพบยีสต์สปีชีส์ *Sporidiobolus pararoseus* มากที่สุด (17 ไอโซเลต) ส่วนยีสต์ 18 ไอโซเลต จัดจำแนกเป็นยีสต์สปีชีส์ที่ยังไม่มีการอธิบายในสกุล *Cryptococcus* (9 ไอโซเลต) *Pseudozyma* (2 ไอโซเลต) และ *Rhodotorula* (7 ไอโซเลต) จากการศึกษานี้พบยีสต์ 21 ไอโซเลต ที่จัดจำแนกเป็นยีสต์สปีชีส์ใหม่ใน 6 สกุล คือ *Bullera* (4 ไอโซเลต) *Cryptococcus* (8 ไอโซเลต) *Occultifur* (1 ไอโซเลต) *Rhodosporidium* (1 ไอโซเลต) *Rhodotorula* (4 ไอโซเลต) และ *Sporidiobolus* (3 ไอโซเลต) สำหรับยีสต์สปีชีส์ใหม่ที่ค้นพบนี้จะนำไปศึกษาลักษณะต่างๆ ตามเกณฑ์อนุกรมวิธานแบบโพลีฟาซิก เพื่ออธิบายพร้อมทั้งเสนอตั้งชื่อเป็นยีสต์สปีชีส์ใหม่ต่อไป

เรื่อง ความหลากหลายและนิเวศวิทยาของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์จากใบของพืชเศรษฐกิจ
ในประเทศไทยและการผลิตสารส่งเสริมการเจริญพืชออกซินโดยสายพันธุ์ที่คัดเลือกว่ามีประสิทธิภาพสูง
พร้อมกับการศึกษาชีววิถีการสังเคราะห์
(โครงการวิจัยเลขที่ RTA5480009)

Diversity of epiphytic yeasts on corn leaves by culture-dependent method

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The diversity of epiphytic yeasts was investigated from 109 corn leaves which were collected from 11 provinces in Thailand. One hundred and ninety isolates of epiphytic yeasts isolated using a culture-dependent method, were identified based on comparative sequence analysis of the D1/D2 domain of the LSU rRNA gene. Among them, 151 isolates were identified as known species belonging to 43 species of 15 genera including *Bullera* (19 isolates), *Candida* (10 isolates), *Cryptococcus* (31 isolates), *Dioszegia* (2 isolates), *Hanseniaspora* (2 isolates), *Jaminaea* (1 isolate), *Kodamaea* (3 isolates), *Meyerozyma* (5 isolates), *Occultifur* (2 isolates), *Pseudozyma* (21 isolates), *Rhodosporidium* (15 isolates), *Rhodotorula* (3 isolates), *Sporidiobolus* (23 isolates), *Sporobolomyces* (13 isolates) and *Wickerhamomyces* (1 isolate). The dominant species was *Sporidiobolus pararoseus* 17 isolates). Eighteen isolates were identified as undescribed species of *Cryptococcus* (9 isolates), *Pseudozyma* (2 isolates) and *Rhodotorula* (7 isolates). In this study, 21 isolates were identified as novel yeast species belonging to six genera including *Bullera* (4 isolates), *Cryptococcus* (8 isolates), *Occultifur* (1 isolate), *Rhodosporidium* (1 isolate), *Rhodotorula* (4 isolates) and *Sporidiobolus* (3 isolates). These yeasts were studied by polyphasic taxonomy for describe and propose as novel yeast species.

เรื่อง ความหลากหลายและนิเวศวิทยาของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์จากใบของพืชเศรษฐกิจ
ในประเทศไทยและการผลิตสารส่งเสริมการเจริญพืชออกซินโดยสายพันธุ์ที่คัดเลือกว่ามีประสิทธิภาพสูง
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ความหลากหลายของยีสต์อีพิไฟต์บนใบอ้อยด้วยวิธีการเพาะเลี้ยงเชื้อและความสามารถในการควบคุมราโรคพืชในจานเพาะเชื้อ

จันทร์จีรา สุรัสวดี นันทนา สีสุข และ สาวิตรี ลิ่มทอง

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ศึกษาความหลากหลายของยีสต์บนผิวใบอ้อยในประเทศไทยด้วยวิธีการเพาะเลี้ยงเชื้อ โดยเก็บตัวอย่างใบอ้อยรวม 84 ตัวอย่าง จาก 10 จังหวัด นำมาแยกยีสต์ด้วยการเพาะเลี้ยงยีสต์จากน้ำล้างใบเจือจางลำดับส่วน ได้ยีสต์ 173 สายพันธุ์ เมื่อจัดจำแนกโดยอนุกรมวิธานระดับโมเลกุลด้วยการเปรียบเทียบลำดับนิวคลีโอไทด์บริเวณ D1/D2 ของ large subunit rRNA gene พบว่า 87 สายพันธุ์ (50.3 เปอร์เซ็นต์) เป็นยีสต์สปีชีส์ที่อธิบายแล้ว ประกอบด้วยแบสิดิโอมัยซีตัสยีสต์ 61 สายพันธุ์ (35.3 เปอร์เซ็นต์) และแอสโคมัยซีตัสยีสต์ 26 สายพันธุ์ (15 เปอร์เซ็นต์) โดยแบสิดิโอมัยซีตัสยีสต์จัดจำแนกเป็น 24 สปีชีส์ ใน 10 สกุล ได้แก่ *Cryptococcus*, *Dioszegia*, *Hannaella*, *Jaminaea*, *Occultifur*, *Pseudozyma*, *Rhodotorula*, *Rhodosporidium*, *Sporobolomyces* และ *Tremella* ส่วนแอสโคมัยซีตัสยีสต์จัดอยู่ใน *Candida*, *Kodamaea* และ *Meyerozyma* มี 67 สายพันธุ์ ที่อาจเป็นยีสต์สปีชีส์ใหม่หรือสปีชีส์ที่มีการอธิบายแล้ว นอกจากนั้น 20 สายพันธุ์ จัดจำแนกเป็นยีสต์สปีชีส์ใหม่ 12 สปีชีส์ โดยสปีชีส์ที่ศึกษาแล้วคือ *Bulleromyces siamensis* sp. nov., *Hannaella phyllophyla* sp. nov., *Occultifur tropicalis* sp. nov., *Papiliotrema siamense* sp. nov., *Sporobolomyces siamensis* sp. nov., *Tremella siamensis* sp. nov. และ *Wickerhamiella siamensis* sp. nov. ยีสต์ที่พบบ่อยที่สุดคือ *Meyerozyma caribbica* (20.2 เปอร์เซ็นต์ของตัวอย่างใบอ้อย)

การตรวจสอบความสามารถของยีสต์ทั้ง 173 สายพันธุ์ ในการยับยั้งการเจริญของราโรคพืชได้แก่ ราโรคข้าว (*Helminthosporium oryzae*, *Rhizoctonia solani* และ *Fusarium moniliforme*) ราโรคข้าวโพด (*Bipolaris maydis*, *Bipolaris zeicola*, *Exserohilium turcicum*, *Fusarium moniliforme* และ *Macrophomina phaseolina*) และราโรคอ้อย (*Collectotrichum falcatum* และ *Fusarium moniliforme*) พบว่า มีเพียง 4 สายพันธุ์ ยับยั้งการเจริญของราโรคพืชได้คือ *Papiliotrema siamense* DMKU-SP85^{T} ยับยั้งการเจริญของ *B. maydis* (ราโรคข้าวโพด) ได้สูงที่สุดเท่ากับ 50.9 เปอร์เซ็นต์

Kodamaea ohmeri DMKU-SP45 ยับยั้งการเจริญของ *H. oryzae* และ *F. moniliforme* ซึ่งเป็นราโรคข้าว เท่ากับ 28.6 เปอร์เซ็นต์และ 20.3 เปอร์เซ็นต์ตามลำดับ และยับยั้ง *C. falcatum* (ราโรคอ้อย) เท่ากับ 39.8 เปอร์เซ็นต์ *Candida* sp. 05-7-186^{T} (DMKU-SP81) ยับยั้งการเจริญของ *F. moniliforme* (ราโรคข้าวโพด) และ *C. falcatum* (ราโรคอ้อย) เท่ากับ 12.9 เปอร์เซ็นต์และ 29.3 เปอร์เซ็นต์ตามลำดับ และ *Pseudozyma alboarmeniaca* DMKU-SP290 ยับยั้งการเจริญของ *R. solani* (ราโรคข้าว) *B. maydis* และ *E. turcica* (ราโรคข้าวโพด) โดยยับยั้งได้เท่ากับ 50 เปอร์เซ็นต์ 40.2 เปอร์เซ็นต์และ 41.1 เปอร์เซ็นต์ตามลำดับ จากการตรวจสอบกลไกในการยับยั้งการเจริญของราโรคพืชในจานเพาะเชื้อพบว่ายีสต์ทั้ง 4 สายพันธุ์ สามารถสร้างสารประกอบไซเดอโรฟอร์ ในขณะที่ *P. alboarmeniaca* DMKU-SP290 สามารถสร้างสารระเหยและแก่งแย่งสารอาหารเพื่อยับยั้งการเจริญของราโรคพืชได้ ส่วน *Candida* sp. 05-7-186^{T} (DMKU-SP81) สามารถสร้างเอนไซม์บีตา-กลูคาเนสได้สูงที่สุดและ *K. ohmeri* DMKU-SP45 สามารถสร้างเอนไซม์ไคติเนสได้สูงที่สุด

เรื่อง ความหลากหลายและนิเวศวิทยาของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์จากใบของพืชเศรษฐกิจ
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(โครงการวิจัยเลขที่ RTA5480009)

Diversity of epiphytic yeasts on sugarcane leaves by culture-dependent method and their biocontrol activity against plant pathogenic fungi *in vitro*

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This study aimed to investigate the diversity of epiphytic yeasts in sugarcane phylloplane in Thailand by culture-dependent technique. A total of 84 sugarcane leaf samples were collected from 10 provinces. Yeasts were isolated from leaf surfaces by dilution plating of leaf washings and 173 strains were obtained. Identification based on molecular taxonomy by analysis of the D1/D2 region of the large subunit rRNA gene sequence revealed that 87 strains (50.3%) were identified as known yeast species consisting of 61 basidiomycetous yeast strains (35.3%) and 26 ascomycetous yeast strains (15%). Known basidiomycetous yeast species were identified as 24 species in 10 genera including *Cryptococcus*, *Dioszegia*, *Hannaella*, *Jaminaea*, *Occultifur*, *Pseudozyma*, *Rhodotorula*, *Rhodosporidium*, *Sporobolomyces* and *Tremella*, and ascomycetous yeasts were in 3 genera including *Candida*, *Kodamaea* and *Meyerozyma*. Sixty seven strains could be new or known yeast species. Moreover, 20 strains were found to represent 12 novel yeast species. So far based on polyphasic taxonomy they were proposed as *Bulleromyces siamensis* sp. nov., *Hannaella phyllophyla* sp. nov., *Occultifur tropicalis* sp. nov., *Papiliotrema siamense* sp. nov., *Sporobolomyces siamensis* sp. nov., *Tremella siamensis* sp. nov., and *Wickerhamiella siamensis* sp. nov. The most prevalent yeast species in phylloplane of sugarcane was *Meyerozyma carribbica* (20.2% of sugarcane leaf samples).

Determination of antagonistic activity of all 173 strains against plant pathogenic fungi including rice pathogenic fungi (*Helminthosporium oryzae*, *Rhizoctonia solani* and *Fusarium moniliforme*), corn pathogenic fungi (*Bipolaris maydis*, *Bipolaris zeicola*, *Exserohilium turcicum*, *Fusarium moniliforme* and *Macrophomina phaseolina*) and sugarcane pathogenic fungi (*Collectotrichum falcatum* and *Fusarium moniliforme*) was carried out by dual cultivation on potato dextrose agar plate. Only four yeast strains showed inhibition of growth of some pathogenic fungi with various percentages of inhibition. *Papiliotrema siamense* DMKU-SP85^{T} revealed the highest inhibition (50.9%) of growth of *B. maydis*. *Kodamaea ohmeri* DMKU-SP45 could inhibit growth of *H. oryzae* (28.6%), *F. moniliforme* (20.3%) and *C. falcatum* (39.8%). *Candida* sp. 05-7-186^{T} (DMKU-SP81) inhibited growth of *F. moniliforme* and *C. falcatum* by 12.9% and 29.3%, respectively. *Pseudozyma alboarmeniaca* DMKU-SP290 showed growth inhibition of *R. solani* (50%), *B. maydis* (40.2%) and *E. turcica* (41.1%). All four antagonistic yeast strains had ability toproduce siderophore while *P. alboarmeniaca* DMKU-SP290 could produce volatile compounds and inhibited growth of pathogenic fungi by competition of nutrients. *Candida* sp. 05-7-186^{T} (DMKU-SP81) produced the highest ß-glucanase enzyme and *K. ohmeri* DMKU-SP45 produced the highest chitinase enzyme.

เรื่อง ความหลากหลายและนิเวศวิทยาของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์จากใบของพืชเศรษฐกิจในประเทศไทยและการผลิตสารส่งเสริมการเจริญพืชออกซินโดยสายพันธุ์ที่คัดเลือกว่ามีประสิทธิภาพสูง พร้อมกับการศึกษาชีววิถีการสังเคราะห์
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ความหลากหลายของยีสต์บนผิวใบพืชและความสามารถในการผลิตกรดอินโดล-3-แอซีติก

รุ่งลักษณ์ แก้ววิเชียร[1,2], วิเชียร ยงมานิตชัย[1] และ สาวิตรี ลิ่มทอง[1]

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ศึกษาความหลากหลายของยีสต์บนผิวใบข้าว ใบข้าวโพด และใบอ้อย โดยเก็บตัวอย่างมาจาก 28 อำเภอ ใน 9 จังหวัดของประเทศไทย รวม 244 ตัวอย่าง นำมาแยกยีสต์ด้วย enrichment technique ในอาหารเหลว yeast extract-malt extract (YM) ที่เติมคลอแรมฟินิคอล 0.02 เปอร์เซ็นต์ และโซเดียมโพรพิโอเนต 0.025 เปอร์เซ็นต์ ได้ยีสต์จากใบข้าว 156 สายพันธุ์ ยีสต์จากใบข้าวโพด 126 สายพันธุ์ และยีสต์จากใบอ้อย 159 สายพันธุ์ เมื่อจัดจำแนกโดยอาศัยอนุกรมวิธานระดับโมเลกุลด้วยการเปรียบเทียบลำดับนิวคลีโอไทด์ในบริเวณ D1/D2 ของ large subunit rRNA gene และวิเคราะห์ความสัมพันธ์เชิงวิวัฒนาการ พบว่ายีสต์จากใบข้าว 155 สายพันธุ์ จัดจำแนกเป็นแอสโคมัยซีตัสยีสต์ 23 สปีชีส์ใน 13 สกุล คือ *Candida*, *Clavispora*, *Cyberlindnera*, *Debaryomyces*, *Kodamaea*, *Lachancea*, *Metschnikowia*, *Meyerozyma*, *Millerozyma*, *Pichia*, *Wickerhamomyces*, *Yamadazyma* และ *Yarrowia* และเป็นแบสิดิโอมัยซีตัสยีสต์ 8 สปีชีส์ใน 5 สกุล ได้แก่ *Cryptococcus*, *Pseudozyma*, *Rhodotorula*, *Sporobolomyces* และ *Trichosporon* ยีสต์จากใบข้าวโพด 123 สายพันธุ์ จัดจำแนกเป็นแอสโคมัยซีตัสยีสต์ถึง 28 สปีชีส์ใน 12 สกุล คือ *Candida*, *Cyberlindnera*, *Geotrichum*, *Hanseniaspora*, *Kluyveromyces*, *Kodamaea*, *Meyerozyma*, *Millerozyma*, *Pichia*, *Torulaspora*, *Wickerhamomyces* และ *Yamadazyma* และเป็นแบสิดิโอมัยซีตัสยีสต์ 10 สปีชีส์ใน 5 สกุล คือ *Cryptococcus*, *Rhodosporidium*, *Rhodotorula*, *Sporobolomyces* และ *Sporidiobolus* และยีสต์จากใบอ้อย 146 สายพันธุ์ จัดจำแนกเป็นแอสโคมัยซีตัสยีสต์ 23 สปีชีส์ใน 13 สกุล คือ *Candida*, *Cyberlindnera*, *Debaryomyces*, *Hanseniaspora*, *Kluyveromyces*, *Lachancea*, *Lodderomyces*, *Metschnikowia*, *Meyerozyma*, *Millerozyma*, *Pichia*, *Torulaspora* และ *Wickerhamomyces* และเป็นแบสิดิโอมัยซีตัสยีสต์ 12 สปีชีส์ใน 6 สกุล ได้แก่ *Hannaella*, *Cryptococcus*, *Rhodosporidium*, *Rhodotorula*, *Sporidiobolus* และ *Sporobolomyces* ส่วนยีสต์อีก 8 สายพันธุ์ เหมือนกับยีสต์สปีชีส์ที่ยังไม่มีการอธิบาย นอกจากนี้ยังพบยีสต์ 9 สายพันธุ์ จัดจำแนกเป็นยีสต์สปีชีส์ใหม่ 6 สปีชีส์ ผลการศึกษาความหลากหลายบนผิวใบข้าว ใบข้าวโพด และใบอ้อย มีความ

หลากหลายของยีสต์สูง โดยสปีชีส์ที่พบบ่อยที่สุดคือ *Rhodotorula taiwanensis* (14.3 เปอร์เซ็นต์ของยีสต์ทั้งหมด) *Meyerozyma caribbica* (12.0 เปอร์เซ็นต์ของยีสต์ทั้งหมด) และ *Candida tropicalis* (10.9 เปอร์เซ็นต์ของยีสต์ทั้งหมด) โดยส่วนใหญ่ตัวอย่างใบพืชทั้งสามชนิดจะพบยีสต์สองสปีชีส์ ซึ่งในใบข้าวพบ 56.5 เปอร์เซ็นต์ของตัวอย่างใบข้าวทั้งหมด ใบข้าวโพดพบมากถึง 60.0 เปอร์เซ็นต์ของตัวอย่างใบข้าวโพดทั้งหมด และใบอ้อยพบ 48.9 เปอร์เซ็นต์ของตัวอย่างใบอ้อยทั้งหมด จากยีสต์ที่แยกได้ทั้งหมด พบว่ายีสต์สปีชีส์ที่มีการอธิบายแล้ว (40 สปีชีส์) 148 สายพันธุ์ ยีสต์สปีชีส์ที่ยังไม่มีการอธิบาย 4 สายพันธุ์ และยีสต์สปีชีส์ใหม่ 2 สายพันธุ์ สามารถผลิตกรดอินโดล-3-แอซีติกได้ เมื่อเพาะเลี้ยงในอาหารเหลว yeast extract peptone dextrose ที่เติม L-tryptophan 0.1 เปอร์เซ็นต์ โดย *Rhodosporidium fluviale* DMKU-RK253 ผลิตกรดอินโดล-3-แอซีติกได้สูงที่สุดคือ 417.8 มิลลิกรัมต่อลิตร

Diversity of phylloplane yeasts and their capability to produce indole-3-acetic acid

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Eighty five samples of rice leaves, 65 samples of corn leaves and 94 samples of sugarcane leaves were collected from 28 districts in nine provinces of Thailand. Yeast isolation from phylloplane was carried out by an enrichment technique. A total of 441 yeast strains were isolated from the 244 leaf samples including 156 yeast strains from rice leaves, 126 yeast strains from corn leaves and 159 yeast strains from sugarcane leaves. On the basis of the D1/D2 domain of the large subunit rRNA gene sequence analysis and phylogeny 155 yeast strains from rice leaves were identified as 31 known species in 18 genera belonging to Ascomycota i.e. *Candida*, *Clavispora*, *Cyberlindnera*, *Debaryomyces*, *Kodamaea*, *Lachancea*, *Metschnikowia*, *Meyerozyma*, *Millerozyma*, *Pichia*, *Wickerhamomyces*, *Yamadazyma* and *Yarrowia*, and Basidiomycota i.e. *Cryptococcus*, *Pseudozyma*, *Rhodotorula*, *Sporobolomyces* and *Trichosporon*, 123 strains from corn leaves were identified as 38 known species in 17 genera belonging to Ascomycota i.e. *Candida*, *Cyberlindnera*, *Geotrichum*, *Hanseniaspora*, *Kluyveromyces*, *Kodamaea*, *Meyerozyma*, *Millerozyma*, *Pichia*, *Torulaspora*, *Wickerhamomyces* and *Yamadazyma*, and Basidiomycota i.e. *Cryptococcus*, *Rhodosporidium*, *Rhodotorula*, *Sporobolomyces* and *Sporidiobolus*, and 146 strains from sugarcane leaves were identified as 35 known species in 19 genera belonging to Ascomycota i.e. *Candida*, *Cyberlindnera*, *Debaryomyces*, *Hanseniaspora*, *Kluyveromyces*, *Lachancea*, *Lodderomyces*, *Metschnikowia*, *Meyerozyma*, *Millerozyma*, *Pichia*, *Torulaspora* and *Wickerhamomyces*, and Basidiomycota i.e. *Hannaella*, *Cryptococcus*, *Rhodosporidium*, *Rhodotorula*, *Sporidiobolus* and *Sporobolomyces*. Eight strains were similar to undescribed species. Moreover, nine strains were found to represent six novel species. The prevalent species were *Rhodotorula taiwanensis*, *Meyerozyma caribbica* and *Candida tropicalis* with low frequencies, 14.3, 12.0 and 10.9% of the total number of yeast strains, respectively. Individual leaf sample was generally accommodated by two yeast species (56.5, 60.0 and 48.9% of total samples of rice, corn and sugarcane phylloplane, respectively). One hundred and forty eight strains in 40 species, four strains of an undescribed species and two strains of a novel species showed the capability to produce IAA when cultivated in yeast extract peptone dextrose broth supplemented with 0.1% L-tryptophan. *Rhodosporidium fluviale* DMKU-RK253 produced the highest IAA concentration at 417.8 mg/L.

เรื่อง ความหลากหลายและนิเวศวิทยาของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์จากใบของพืชเศรษฐกิจในประเทศไทยและการผลิตสารส่งเสริมการเจริญพืชออกซินโดยสายพันธุ์ที่คัดเลือกว่ามีประสิทธิภาพสูง พร้อมกับการศึกษาชีววิถีการสังเคราะห์
(โครงการวิจัยเลขที่ RTA5480009)

ความหลากหลายของยีสต์อีพิไฟต์บนผิวใบข้าวโพดด้วยวิธีการไม่เพาะเลี้ยงเชื้อ

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งานวิจัยนี้ทำเพื่อศึกษาความหลากหลายของยีสต์อีพิไฟต์บนผิวใบข้าวโพดด้วยวิธีการไม่เพาะเลี้ยงเชื้อโดยอาศัยการเปรียบเทียบลำดับนิวคลีโอไทด์บริเวณ D1/D2 บนยีน large subunit rRNA โดยสกัดดีเอ็นเอจากน้ำล้างตัวอย่างใบข้าวโพดจำนวน 37 ตัวอย่างซึ่งสุ่มเก็บจาก 10 จังหวัดในประเทศไทยและเพิ่มปริมาณดีเอ็นเอบริเวณ D1/D2 ด้วยวิธีปฏิกิริยาลูกโซ่ (Polymerase chain reaction, PCR) ทำการโคลนผลผลิต PCR และคัดเลือกโคลนที่มีดีเอ็นเอบริเวณ D1/D2 สอดแทรกอยู่ด้วยวิธี colony PCR จากนั้นทำการจัดกลุ่มโคลนเบื้องต้นของแต่ละ clone library โดยการวิเคราะห์ด้วยเอนไซม์ตัดจำเพาะ (Restriction fragment length polymorphism, RFLP) ผลจากการวิเคราะห์ลำดับนิวคลีโอไทด์บริเวณ D1/D2 ในฐานข้อมูล GenBank พบว่าจำนวนโคลน 357 โคลน (34.0%) จากทั้งสิ้น 1,049 โคลน มีลำดับนิวคลีโอไทด์ จัดอยู่ในกลุ่มยีสต์ และลำดับนิวคลีโอไทด์เหล่านี้สามารถจัดกลุ่มได้เป็น 108 OTUs ที่มีความเหมือนกันอย่างน้อย 99% ยีสต์ส่วนใหญ่ที่พบบนผิวใบข้าวโพดจัดอยู่ในไฟลัมเบสิดิโอไมโคตา (98%) ซึ่งสามารถระบุสปีชีส์ได้ 12 สปีชีส์ ใน 7 สกุล จาก 98 โคลน (27.5%, 14 OTUs) ได้แก่ *Bullera derxii*, *B. oryzae*, *Cryptococcus flavescens*, *Hannaella sinensis*, *Jaminaea angkoriensis*, *Pseudozyma Antarctica*, *P. aphidis*, *P. hubeiensis*, *P. prolifica*, *Sporidiobolus pararoseus*, *Sporobolomyces carnicolor* และ *S. odoratus* ส่วนลำดับนิวคลีโอไทด์ที่เหลือจาก 259 โคลนนั้นไม่สามารถระบุสปีชีส์ได้ ซึ่งลำดับนิวคลีโอไทด์เหล่านี้มีความใกล้เคียงกับยีสต์ในไฟลัมแอสโคมัยโคต้า 1 สปีชีส์ และ แบสิดิโอมัยโคต้า 26 สปีชีส์ โดยลำดับนิวคลีโอไทด์เหล่านี้อาจเป็นลำดับนิวคลีโอไทด์ของยีสต์ชนิดใหม่ ยีสต์ส่วนใหญ่ที่ตรวจสอบพบในงานวิจัยนี้จัดอยู่ในซับไฟลัม Ustilaginomycotina (72.3%) และมีความใกล้เคียงกับยีสต์ในสกุล *Pseudozyma* เป็นส่วนใหญ่ โดยยีสต์ที่สามารถระบุสปีชีส์ได้ที่พบบนตัวอย่างผิวใบข้าวโพดส่วนใหญ่คือ *P. hubeiensis* และ *P. antarctica* ด้วยความถี่ที่พบบนผิวใบข้าวโพด 24.3% และ 21.6% ตามลำดับ

เรื่อง ความหลากหลายและนิเวศวิทยาของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์จากใบของพืชเศรษฐกิจ
ในประเทศไทยและการผลิตสารส่งเสริมการเจริญพืชออกซินโดยสายพันธุ์ที่คัดเลือกว่ามีประสิทธิภาพสูง
พร้อมกับการศึกษาชีววิถีการสังเคราะห์
(โครงการวิจัยเลขที่ RTA5480009)

Diversity of epiphytic yeasts on corn phyllospheres by culture-independent method

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The epiphytic yeast diversity in corn phyllosphere in Thailand was investigated by culture-independent technique based on the sequence of the D1/D2 domain of the large subunit rRNA gene. Thirty-seven samples of corn leaf were collected randomly from 10 provinces in Thailand. The DNA was extracted from leaf washing samples and the D1/D2 domain was amplified using PCR technique. The PCR products were cloned and then screened by colony PCR. The restriction analysis was used to preliminarily cluster the PCR products from each clone library. Of total 1,049 clones, 357 clones (34.0%) revealed the D1/D2 domain sequences closely related to sequences of yeasts in GenBank, and they were clustered into 108 operational taxonomic units (OTUs) at 99% homology cut off. The majority of yeasts presented in corn leaf surfaces were members of phylum Basidiomycota (98.0%). Of total yeast related clones, 98 clones (27.5%, 14 OTUs) were identified as 12 known yeast species including *Bullera derxii*, *B. oryzae*, *Cryptococcus flavescens*, *Hannaella sinensis*, *Jaminaea angkoriensis*, *Pseudozyma antarctica*, *P. aphidis*, *P. hubeiensis*, *P. prolifica*, *Sporidiobolus pararoseus*, *Sporobolomyces carnicolor* and *S. odoratus*. The D1/D2 sequences (259 clones) that could not be identified as known yeast species were closest to 1 and 26 species in Ascomycota and Basidiomycota, respectively, some of which may be new yeast species. Interestingly, yeasts in subphylum Ustilaginomycotina were mostly detected (72.3%). *Pseudozyma* was the most prevalent yeast genus. The most common yeast species detected were *P. hubeiensis* and *P. antarctica* with 24.3 % and 21.6% frequency of occurrence in corn phyllosphere samples, respectively.

เรื่อง ความหลากหลายและนิเวศวิทยาของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์จากใบของพืชเศรษฐกิจในประเทศไทยและการผลิตสารส่งเสริมการเจริญพืชออกซินโดยสายพันธุ์ที่คัดเลือกว่ามีประสิทธิภาพสูง พร้อมกับการศึกษาชีววิถีการสังเคราะห์
(โครงการวิจัยเลขที่ RTA5480009)

ความหลากหลายของยีสต์เอนโดไฟต์ในใบข้าว อ้อย และข้าวโพดด้วยวิธีการไม่เพาะเลี้ยงเชื้อ

มณี ตันติรุ่งกิจ[1] ชมนาถ เกิดคง[1] รุจิกาญจน์ นาสนิท[2] และสาวิตรี ลิ่มทอง[3]

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[2] *ภาควิชาเทคโนโลยีชีวภาพคณะวิศวกรรมศาสตร์และเทคโนโลยีอุตสาหกรรม มหาวิทยาลัยศิลปากร วิทยาเขตพระราชวังสนามจันทร์ นครปฐม 73000*

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ในปัจจุบันเทคนิคด้านชีวโมเลกุลช่วยให้การศึกษาด้านความหลากหลายของจุลินทรีย์ในระบบนิเวศต่างๆ ทำได้รวดเร็วขึ้น ดังนั้นในการวิจัยนี้จึงใช้เทคนิคด้านชีวโมเลกุลในการตรวจหายีสต์เอนโดไฟต์ในใบข้าว อ้อยและข้าวโพดโดยไม่เพาะเลี้ยงเชื้อ (culture-independent method) ด้วยวิธี semi-nested PCR โดยใช้คู่ไพรเมอร์ ITS1-F_KYO1 และ NL4 ในการทำปฏิกิริยา PCR เพื่อเพิ่มปริมาณดีเอ็นเอบริเวณ ITS - D1/D2 และใช้คู่ไพรเมอร์ NL1 และ NL4 เพื่อเพิ่มปริมาณดีเอ็นเอบริเวณ D1/D2 ของ large subunit (LSU) rRNA gene และสร้าง D1/D2 library ของข้าวอ้อยและข้าวโพด เมื่อคัดเลือก clone ที่มีลำดับนิวคลีโอไทด์ในบริเวณ D1/D2 ที่แตกต่างกันในแต่ละ library ด้วยวิธี PCR-RFLP โดยใช้ เอนไซม์ *Hae*III, *Hin*fI และ *Cfo*I และวิเคราะห์หาลำดับนิวคลีโอไทด์และเปรียบเทียบกับลำดับนิวคลีโอไทด์ในฐานข้อมูลเพื่อระบุชนิดและหาความสัมพันธ์ทางพันธุกรรม โดยในใบข้าว 46 ตัวอย่าง พบ phylotype ของยีสต์ 5 สปีชีส์ใน 4 สกุล คือ *Cryptococcus victoriae, Debaryomyces hansenii, D. Vindobonensis, Meyerozyma guilliermondii* และ *Pseudozyma antarctica* และพบ phylotype ของยีสต์ที่อาจเป็นยีสต์สปีชีส์ใหม่ 17 phylotype ที่มีความใกล้เคียงกับ *Candida metapsilosis, Cryp. foliicola, Cryp. laurentii, P. abaconensis, P. aphidis* และ *Trichosporon asahii* ส่วนในใบอ้อย 29 ตัวอย่าง พบ phylotype ของยีสต์ 3 สปีชีส์ คือ *C. palmioleophila, D. hansenii* และ *Kodamaea ohmeri* และพบ phylotype ของยีสต์ที่อาจเป็นยีสต์สปีชีส์ใหม่ 10 phylotype ที่มีความใกล้เคียงกับ *D. hansenii, Cryp. flavus, Malassezia restricta, P. aphidis, Rhodotorula cassiicola, R. marina* และ *Sporobolomyces vermiculatus* และจากใบข้าวโพด 35 ตัวอย่าง พบ phylotype ของยีสต์ 6 สปีชีส์ คือ *Hannaella sinensis, Mal. restricta, P. parantarctica, R. taiwanensis, T. asahii* และ *Yarrowia lipolytica* และพบ phylotype ของยีสต์

ที่อาจเป็นยีสต์สปีชีส์ใหม่ 26 phylotype ที่มีความใกล้เคียงกับ *Mal. restricta*, *P. antarctica*, *P. parantarctica*, *R. colostri* และ *T. faecale* โดย phylotype ที่พบมากที่สุดในใบข้าว อ้อย และข้าวโพด ได้แก่ phylotype ที่มีความใกล้เคียงกับ *Cryp. foliicola* phylotype ของ *K. ohmeri* และ phylotype ของ *Y. lipolytica* ตามลำดับ และยังพบ phylotype ของยีสต์ที่อาจเป็นยีสต์สปีชีส์ใหม่มากถึง 56.4 32.6 และ 69.2 เปอร์เซนต์ของ phylotype ของยีสต์ทั้งหมด ตามลำดับ ซึ่ง phylotype ของยีสต์เอนโดไฟต์ที่พบในพืชเศรษฐกิจทั้ง 3 ชนิด ส่วนหนึ่งเป็นของยีสต์ที่มีศักยภาพในด้านการเกษตรและอุตสาหกรรม รวมทั้งการพบ phylotype ของยีสต์ที่คาดว่าจะเป็นยีสต์สปีชีส์ใหม่ ชี้ให้เห็นว่าใบข้าว อ้อย และข้าวโพดเป็นแหล่งอาศัยที่น่าสนใจแหล่งหนึ่งในการค้นหายีสต์สายพันธุ์ใหม่หรือสปีชีส์ใหม่และศึกษาถึงบทบาทของยีสต์ที่มีต่อพืชอาศัย

เรื่อง ความหลากหลายและนิเวศวิทยาของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์จากใบของพืชเศรษฐกิจ
ในประเทศไทยและการผลิตสารส่งเสริมการเจริญพืชออกซินโดยสายพันธุ์ที่คัดเลือกว่ามีประสิทธิภาพสูง
พร้อมกับการศึกษาชีววิถีการสังเคราะห์
(โครงการวิจัยเลขที่ RTA5480009)

Diversity of endophytic yeasts from rice, sugarcane and corn leaves by culture-independent method

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Recent studies have employed molecular biology techniques to rapidly investigate microbe diversity within and between ecosystems. Therefore, in this study a culture-independent method was used to examine endophytic yeasts associated with rice, sugarcane and corn leaves using a semi-nested PCR and amplified rDNA restriction analysis (ARDRA) techniques. The primers used were ITS1-F_KYO1/NL4, the outer set, to amplify the ITS - D1/D2 region of large subunit (LSU) rRNA gene and NL1/NL4, the inner set, to amplify the D1/D2 region. The D1/D2 libraries of rice, sugarcane and corn were characterized by PCR-RFLP using a single digestion with *Hae*III, *Hin*fI and *Cfo*I, and the selected clones were subjected to sequence and phylogenetic analysis. Based on sequence analysis of the D1/D2 domain of the LSU rDNA, the results showed that endophytic phylotypes in rice leaves (46 samples) were designated as 5 known species (*Cryptococcus victoriae, Debaryomyces hansenii, D. vindobonensis, Meyerozyma guilliermondii* and *Pseudozyma antarctica*), together with 17 novel phylotypes closest to *Candida metapsilosis, Cryp. foliicola, Cryp. laurentii, P. abaconensis, P. aphidis* and *Trichosporon asahii*, whereas the endophytic phylotypes in sugarcane leaves (29 samples) were designated as 3 knon species (*Candida palmioleophila, Debaryomyces hansenii* and *Kodamaea ohmeri*), together with 10 novel phylotypes closest to *D. hansenii, Cryp. flavus, Malassezia restricta, P. aphidis, Rhodotorula cassiicola, R. marina* and *Sporobolomyces vermiculatus.* In case of corn leaves, the endophytic phylotypes were designated as 6 known species (*Hannaella sinensis, Mal. restricta, P. parantarctica, R. taiwanensis, T. asahii* and *Yarrowia lipolytica*), together with 26 novel phylotypes closest to *Mal. restricta, P. antarctica, P. parantarctica, R. colostri* and *T. faecale*. The most prevalent phylotypes in rice, sugarcane and corn leaves were novel phylotypes closest to *Cryp. foliicola*, phylotypes identified as *K. ohmeri* and phylotypes identified as *Y. lipolytica, respectively*. Moreover, the novel phylotypes were found at high frequency as 56.4%, 32.6% and 69.2% in rice, sugarcane and corn leaves, respectively. The results revealed the differences of endophytic yeast phylotype among these agronomic crop and most of novel phylotypes were the phylotypes in the Basidiomycota. The presence of the phylotypes related to species known for their agricultural and industrial application together with the occurrence of novel phylotypes, should encourage extensive studies to discover novel yeast species and to understand their roles in the leaves of agronomic crops.

เรื่อง ความหลากหลายและนิเวศวิทยาของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์จากใบของพืชเศรษฐกิจในประเทศไทยและการผลิตสารส่งเสริมการเจริญพืชออกซินโดยสายพันธุ์ที่คัดเลือกว่ามีประสิทธิภาพสูง พร้อมกับการศึกษาชีววิถีการสังเคราะห์
(โครงการวิจัยเลขที่ RTA5480009)

ความหลากหลายของยีสต์เอนโดไฟต์ในใบข้าว อ้อย และข้าวโพดด้วยวิธีการเพาะเลี้ยงเชื้อและการอธิบายยีสต์สกุลและสปีชีส์ใหม่

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งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาความหลากหลายของยีสต์เอนโดไฟต์จากเนื้อเยื่อใบพืชเศรษฐกิจ (ข้าว อ้อย และข้าวโพด) โดยเก็บตัวอย่างใบพืชทั้งหมด 311 ตัวอย่าง จากพื้นที่เพาะปลูกในภาคกลางและภาคตะวันออกเฉียงเหนือของประเทศไทยระหว่างเดือนตุลาคม 2011 ถึงเมษายน 2012 ประกอบด้วย ใบข้าว 100 ตัวอย่าง จาก 11 จังหวัด ใบอ้อย 102 ตัวอย่าง จาก 13 จังหวัด และใบข้าวโพด 109 จาก 12 จังหวัด แยกยีสต์เอนโดไฟต์จากใบพืชที่ผ่านการฆ่าเชื้อบนผิวใบทั้งสามชนิดได้รวม 417 สายพันธุ์ โดยแยกจากใบข้าว ใบอ้อย และใบข้าวโพด ได้ 125 149 และ 143 สายพันธุ์ ตามลำดับ จากการจัดจำแนกโดยอาศัยอนุกรมวิธานระดับโมเลกุลด้วยการหาลำดับนิวคลีโอไทด์บริเวณ D1/D2 ของ large subunit (LSU) rRNA gene พบว่ายีสต์เอนโดไฟต์จากใบข้าว 116 สายพันธุ์ จัดจำแนกเป็นสปีชีส์ที่รู้จักแล้วอยู่ใน 12 สกุล โดย 27 สายพันธุ์ อยู่ในไฟลัมแอสโคมัยโคตา (5 สกุล) ได้แก่ *Candida* (3 สปีชีส์) *Cyberlindnera* (1 สปีชีส์) *Kodamaea* (1 สปีชีส์) *Meyerozyma* (1 สปีชีส์) และ *Wickerhamomyces* (1 สปีชีส์) และอีก 90 สายพันธุ์ อยู่ในไฟลัมเบสิดิโอมัยโคตา (7 สกุล) ได้แก่ *Bullera* (1 สปีชีส์) *Cryptococcus* (1 สปีชีส์) *Pseudozyma* (5 สปีชีส์) *Rhodotorula* (4 สปีชีส์) *Rhodosporidium* (2 สปีชีส์) *Sporobolomyces* (2 สปีชีส์) และ *Sporidiobolus* (1 สปีชีส์) และ 1 สายพันธุ์ จัดจำแนกเป็นสปีชีส์ที่ยังไม่มีการอธิบาย นอกจากนี้ยังพบว่า 4 สายพันธุ์ จัดจำแนกเป็นยีสต์สกุลและสปีชีส์ใหม่ โดย 1 สายพันธุ์ได้ศึกษาเพื่ออธิบายเป็นสปีชีส์ใหม่โดยอาศัยอนุกรมวิธานพอลีฟาซิกและได้เสนอตั้งชื่อเป็น *Papiliotrema siamense* และ 3 สายพันธุ์ อยู่ในระหว่างการเสนอเป็นยีสต์สกุลและสปีชีส์ใหม่โดยการตีพิมพ์ในวารสารนานาชาติ และ 3 สายพันธุ์ จัดจำแนกเป็นราคล้ายยีสต์ (2 สกุล) ได้แก่ *Sporisorium* (2 สปีชีส์) และ *Ustilago* (1 สปีชีส์) การจัดจำแนกยีสต์เอนโดไฟต์จากใบอ้อยพบว่า 99 สายพันธุ์ จัดจำแนกเป็นสปีชีส์ที่รู้จักแล้วอยู่ใน 14 สกุล โดย 35 สายพันธุ์ อยู่ในไฟลัมแอสโคมัยโคตา (4 สกุล) ได้แก่ *Candida* (1 สปีชีส์) *Cyberlindnera* (1 สปีชีส์) *Meyerozyma* (2 สปีชีส์) และ *Pichia* (2 สปีชีส์) และ อีก 65 สายพันธุ์ อยู่ในไฟลัมเบสิดิโอมัยโคตา (10 สกุล) ได้แก่ *Bullera* (4 สปีชีส์) *Cryptococcus* (5 สปีชีส์) *Dioszegia* (1 สปีชีส์) *Jaminaea* (1 สปีชีส์) *Pseudozyma* (5 สปีชีส์) *Rhodotorula* (3 สปีชีส์) *Rhodosporidium* (1 สปีชีส์) *Sporobolomyces* (1 สปีชีส์) *Sporidiobolus*

(1 สปีชีส์) และ *Trichosporon* (1 สปีชีส์) นอกจากนี้มี 6 สายพันธุ์จัดจำแนกเป็นยีสต์สปีชีส์ใหม่ 2 สปีชีส์ ซึ่งได้ศึกษาเพื่ออธิบายเป็นสปีชีส์ใหม่โดยอาศัยอนุกรมวิธานพอลีฟาซิกและได้เสนอตั้งชื่อเป็น *Wickerhamiell*

siamensis และ *Occultifur tropicalis* นอกจากนี้ 22 สายพันธุ์ จัดจำแนกเป็นราคล้ายยีสต์ (3 สกุล) ได้แก่ *Aureobasidium* (1 สปีชีส์) *Sporisorium* (2 สปีชีส์) และ *Ustilago* (1 สปีชีส์) ส่วนยีสต์เอนโดไฟต์จากใบข้าวโพด 119 สายพันธุ์ จัดจำแนกเป็นสปีชีส์ที่รู้จักแล้วอยู่ใน 12 สกุล โดย 29 สายพันธุ์ อยู่ในไฟลัมแอสโคมัยโคตา (5 สกุล) ได้แก่ *Candida* (5 สปีชีส์) *Kluyveromyces* (1 สปีชีส์) *Meyerozyma* (1 สปีชีส์) *Pichia* (1 สปีชีส์) และ *Wickerhamomyces* (1 สปีชีส์) และอีก 90 สายพันธุ์ อยู่ในไฟลัมเบสิดิโอมัยโคตา (7 สกุล) ได้แก่ *Bullera* (1 สปีชีส์) *Cryptococcus* (9 สปีชีส์) *Rhodotorula* (2 สปีชีส์) *Pseudozyma* (3 สปีชีส์) *Rhodosporidium* (2 สปีชีส์) *Sporobolomyces* (2 สปีชีส์) และ *Sporidiobolus* (2 สปีชีส์) นอกจากนี้ 1 สายพันธุ์ (DMKU-CE23) จัดจำแนกเป็นยีสต์สปีชีส์ใหม่ใน *Yamadazyma* clade และมี 3 สายพันธุ์ จัดจำแนกเป็นสปีชีส์ที่ยังไม่มีการอธิบาย 3 สปีชส์ และอีก 17 สายพันธุ์ จัดจำแนกเป็นราคล้ายยีสต์ (2 สกุล) ได้แก่ *Aureobasidium* (1 สปีชีส์) และ *Sporisorium* (2 สปีชีส์) ยีตส์เอนโดไฟต์ที่มีความถี่ในการพบมากที่สุดในเนื้อเยื่อใบข้าว ใบอ้อย และใบข้าวโพด คือ 25.0 17.6 และ 20.0% ตามลำดับ

เรื่อง ความหลากหลายและนิเวศวิทยาของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์จากใบของพืชเศรษฐกิจ
ในประเทศไทยและการผลิตสารส่งเสริมการเจริญพืชออกซินโดยสายพันธุ์ที่คัดเลือกว่ามีประสิทธิภาพสูง
พร้อมกับการศึกษาชีววิถีการสังเคราะห์
(โครงการวิจัยเลขที่ RTA5480009)

Investigation on diversity of endophytic yeasts from rice, sugarcane and corn leaves by culture-dependent method and description of new yeast genus and species

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This work aimed to study the diversity of endophytic yeasts in plant leaf tissues (rice, sugarcane and corn). A total of 311 samples of plant leaf were collected from fields in central and north-eastern parts of Thailand during October 2011 to April 2012. The samples consisted of rice leaf (n=100) collected from 11 provinces, sugarcane leaf (n=102) collected from 13 provinces and corn leaf (n=109) collected from 12 provinces. Four hundred and seventeen endophytic yeast strains isolated from tissue of surface sterilized plant leaves, which obtained from rice leaves, sugarcane leaves and corn leaves were 125, 149 and 143 strains, respectively. Yeast identification on the basis of the D1/D2 region of the large subunit (LSU) rRNA gene sequence analysis revealed that 116 strains from rice leaves were known species in 12 genera. Twenty-seven strains were in Phylum Ascomycota (5 genera) *viz.* *Candida* (3 species), *Cyberlindnera* (1 species), *Kodamaea* (1 species), *Meyerozyma* (1 species) and *Wickerhamomyces* (1 species), and 90 strains were in Phylum Basidiomycota (7 genera) *viz.* *Bullera* (1 species), *Cryptococcus* (4 species), *Pseudozyma* (5 species), *Rhodotorula* (4 species), *Rhodosporidium* (2 species), *Sporobolomyces* (2 species) and *Sporidiobolus* (1 species). One strain was the same as undescribed species. In addition, four strains were found to represent two novel species and one genus, which one strain was already proposed on the basis of polyphasic taxonomy as *Papiliotrema siamense* and three strains representing a single novel genus and species is being proposed by submitting the manuscript to international journal. Three strains were identified to be yeast-like fungi (2 genera) *viz.* *Sporisorium* (2 species) and *Ustilago* (1 species). Among endophytic yeasts from sugarcane leaf, 99 strains were identified as known species in 14 genera. Thirty-four strains were in Phylum Ascomycota (4 genera) *viz.* *Candida* (1 species), *Cyberlindnera* (1 species), *Meyerozyma* (2 species) and *Pichia* (2 species), and 65 strains were in Phylum Basidiomycota (10 genera) *viz.* *Bullera* (4 species), *Cryptococcus* (5 species), *Dioszegia* (1 species), *Jaminaea* (1 species), *Pseudozyma* (5 species), *Rhodotorula* (3 species), *Rhodosporidium* (1species), *Sporobolomyces* (1 species), *Sporidiobolus* (1 species) and *Trichosporon* (1 species). Furthermore, six strains were found to represent two novel yeast species and proposed on the basis of polyphasic taxonomy as *Wickerhamiella siamensis* and *Occultifur tropicalis*. Moreover, 22 strains were identified to be yeast-like fungi (3 genera)

viz. Aureobasidium (1 species), *Sporisorium* (2 species) and *Ustilago* (1 species). A total of 119 yeast strains obtained from corn leaves were identified to be known species in 12 genera. Twenty-nine strains were in Phylum Ascomycota (5 genera) *viz. Candida* (5 species), *Kluyveromyces* (1 species), *Meyerozyma* (1 species), *Pichia* (1 species) and *Wickerhamomyces* (1 species). Other 90 strains were identified to be known species in Phylum Basidiomycota (7 genera) *viz. Bullera* (1 species), *Cryptococcus* (9 species), *Rhodotorula* (2 species), *Pseudozyma* (3 species), *Rhodosporidium* (2 species), *Sporobolomyces* (2 species) and *Sporidiobolus* (2 species). One strain (DMKU-CE23) was found to be a novel strain in *Yamadazyma* clade, and three strains were similar to three undescribed species. In addition, 17 strains were yeast-like fungi (2 genera) *viz. Aureobasidium* (1 species) and *Sporisorium* (2 species). The most prevalent species of endophytic yeasts from rice leaves, sugarcane leaves and corn leaves were *Pseudozyma aphidis*, *Meyerozyma caribbica* and *Pseudozyma churashimaensis* with frequency of 25.0, 17.6 and 20.0%, respectively.

Rhodosporidium paludigenum DMKU-RP301 ยีสต์ผิวใบข้าวที่มีคุณลักษณะสนับสนุนการเจริญของพืชรวมทั้งการผลิตกรดอินโดล-3-แอซีติก

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ยีสต์สีแดง *Rhodosporidium paludigenum* DMKU-RP301 เป็นแบสิดิโอมัยซีตัสยีสต์ที่แยกได้จากผิวใบข้าวในประเทศไทยและผลิตกรดอินโดล-3-แอซีติกได้สูงที่สุด คือ 321.7 มิลลิกรัมต่อลิตร จากยีสต์ที่นำมาคัดเลือกทั้งหมด 1,035 ไอโซเลต ยีสต์สายพันธุ์ดังกล่าวยังผลิตแอมโมเนียและไซเดอโรฟอร์ซึ่งเป็นลักษณะที่สนับสนุนการเจริญของพืชนอกเหนือไปจากการผลิตกรดอินโดล-3-แอซีติกด้วย การศึกษาสภาวะที่เหมาะสมของอาหารเลี้ยงเชื้อและสภาวะการเพาะเลี้ยงเพื่อเพิ่มการผลิตกรดอินโดล-3-แอซีติกของยีสต์ *R. Paludigenum* DMKU-RP301 โดยวิธีการศึกษาทีละปัจจัยและวิธีพื้นผิวตอบสนอง พบว่าระยะเวลาการเพาะเลี้ยง แหล่งคาร์บอน แหล่งไนโตรเจน growth factor ทริปโตเฟน อุณหภูมิการเพาะเลี้ยง ความเร็วรอบการเขย่าและพีเอชเริ่มต้นของอาหารเลี้ยงเชื้อ ส่งผลต่อการผลิตกรดอินโดล-3-แอซีติกของยีสต์ *R. Paludigenum* DMKU-RP301 โดยเมื่อเลี้ยงแบบเขย่าฟลาส์กภายใต้สภาวะที่เหมาะสม ที่ได้จากการศึกษาด้วยวิธีพื้นผิวตอบสนอง พบว่ายีสต์ผลิตกรดอินโดล-3-แอซีติกได้เพิ่มขึ้น 5 เท่า คือ ผลิตได้ 1,623.9 มิลลิกรัมต่อลิตร และเมื่อนำสภาวะดังกล่าวมาศึกษาในถังหมักขนาด 2 ลิตร พบการผลิตกรดอินโดล-3-แอซีติกได้เท่ากับ 1,627.1 มิลลิกรัมต่อลิตร แสดงให้เห็นว่าสภาวะเหมาะสมที่ได้จากการศึกษาในการเพาะเลี้ยงระดับเล็กสามารถนำไปใช้สำหรับการเพาะเลี้ยงระดับใหญ่เพื่อเพิ่มการผลิตกรดอินโดล-3-แอซีติกโดยยีสต์นี้ได้ การศึกษาครั้งนี้เป็นการรายงานครั้งแรกเกี่ยวกับการศึกษาสภาวะที่เหมาะสมต่อการผลิตกรดอินโดล-3-แอซีติกโดยยีสต์ *Rhodosporidium* ด้วย

เรื่อง ความหลากหลายและนิเวศวิทยาของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์จากใบของพืชเศรษฐกิจ
ในประเทศไทยและการผลิตสารส่งเสริมการเจริญพืชออกซินโดยสายพันธุ์ที่คัดเลือกว่ามีประสิทธิภาพสูง
พร้อมกับการศึกษาชีววิถีการสังเคราะห์
(โครงการวิจัยเลขที่ RTA5480009)

Rhodosporidium paludigenum DMKU-RP301 rice phyllospheric yeast possesses plant growth promoting characters including indole-3-acetic acid production

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A basidiomycetous red yeast *Rhodosporidium paludigenum* DMKU-RP301 isolated from a rice phyllosphere in Thailand was shown to be the best indole-3-acetic acid (IAA) producer (321.7 mg/l) among 1,035 yeast isolates screened. This yeast also showed the ability to produce NH_3 and siderophore which are additional plant growth promoting characters. An optimization of culture medium and culture conditions to improve IAA production by *R. paludigenum* DMKU-RP301 was carried out. The studies were conducted using one factor at a time (OFAT) and response surface methodology (RSM). The results showed that incubation time, carbon and nitrogen sources, growth factor, tryptophan, temperature, shaking speed and pH affected IAA production by *R. paludigenum* DMKU-RP301. Maximum IAA production of 1,623.9 mg/l was obtained as a result of the studies using RSM. The optimal medium and growth conditions observed in this study resulted in an increase of IAA production up to 5.0 times compared to an unoptimized condition, i.e. when yeast extract peptone dextrose (YPD) broth supplemented with 0.1% L-tryptophan was used as a production medium. When the production of IAA was scaled up in a 2-l stirred tank fermenter, the maximum IAA of 1,627.1 mg/l was obtained. This experiment indicated that the obtained optimal medium and growth conditions (pH and temperature) from shaking flask production can be used for production of IAA in a larger size production. In addition, the present research is the first report on optimization of IAA production by the yeast *Rhodosporidium*.

การผลิตกรดอินโดล-3-แอซีติกโดยยีสต์ผิวใบข้าว *Rhodosporidium paludigenum* DMKU-RP301 และการศึกษาชีววิถีการสังเคราะห์

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กรดอินโดล-3-แอซีติกเป็นฮอร์โมนพืชออกซินที่สำคัญ การสังเคราะห์กรดอินโดล-3-แอซีติกมี 2 ชีววิถีสังเคราะห์หลัก คือ วิถีที่ใช้ทริปโตเฟน และวิถีที่ไม่ใช้ทริปโตเฟน การสังเคราะห์กรดอินโดล-3-แอซีติกที่ใช้ทริปโตเฟนมีหลายวิถี ได้แก่ วิถีอินโดล-3-แอซีตาไมด์ วิถีกรดอินโดล-3-ไพรูวิก วิถีอินโดล-3-*แอซีโตไนไทร*ล์ วิถีทริปตามีน และวิถีทริปโตเฟนไซด์เชนออกซิเดส มีการเสนอว่าการสังเคราะห์กรดอินโดล-3-แอซีติกที่ไม่ใช้ทริปโตเฟนนั้นมาจากอินโดลหรืออินโดล-3-กลีเซอรอลฟอสเฟต ยีสต์เป็นหนึ่งในจุลินทรีย์ที่น่าสนใจสำหรับการผลิตกรดอินโดล-3-แอซีติก เนื่องจากการผลิตผลิตภัณฑ์จากการหมักในปริมาตรมากโดยยีสต์นั้นมีการศึกษาเป็นอย่างดี ยีสต์มีผนังเซลล์หนาซึ่งช่วยปกป้องเซลล์ยีสต์จากความเสียหายทางกายภาพในระหว่างการหมัก ยิ่งกว่านั้นเทคโนโลยีการเก็บเกี่ยวเซลล์ยีสต์ก็ทำได้ง่ายกว่าการใช้แบคทีเรียเนื่องจากขนาดของเซลล์ยีสต์มีขนาดใหญ่กว่า ดังนั้นงานวิจัยนี้จึงมุ่งศึกษาการผลิตกรดอินโดล-3-แอซีติกในถังหมักขนาด 2 ลิตร และชีววิถีสังเคราะห์กรดอินโดล-3-แอซีติกโดยยีสต์ *Rhodosporidium paludigenum* DMKU-RP301 ที่แยกได้จากผิวใบข้าว โดยศึกษาการหมักแบบแบตช์และเฟด-แบตช์ จากการหมักแบบแบตช์พบว่าเมื่อบ่มที่อัตราการกวน 200 รอบต่อนาที และอัตราการให้อากาศ 3 ลิตรต่อนาที ยีสต์ผลิตกรดอินโดล-3-แอซีติกได้สูงสุดเท่ากับ 1,627.1 มิลลิกรัมต่อลิตร (9.7 มิลลิกรัมต่อลิตรต่อชั่วโมง) ส่วนการหมักแบบเฟด-แบตช์พบว่ายีสต์ผลิตกรดอินโดล-3-แอซีติกได้สูงกว่าการหมักแบบแบตช์ คือ ผลิตได้เท่ากับ 2,707.9 มิลลิกรัมต่อลิตร (25.1 มิลลิกรัมต่อลิตรต่อชั่วโมง) แสดงว่าการหมักแบบเฟด-แบตช์สามารถเพิ่มประสิทธิภาพการผลิตกรดอินโดล-3-แอซีติกทั้งในแง่ของความเข้มข้นและความสามารถในการผลิต ยิ่งกว่านั้นยังพบกรดอินโดล-3-แลคติกและอินโดล-3-เอทานอลในอาหารเลี้ยงเชื้อซึ่งเป็นผลของปฏิกิริยารีดักชันของกรดอินโดล-3-ไพรูวิกและอินโดล-3-แอซีทัลดีไฮด์ในวิถีกรดอินโดล-3-ไพรูวิก แสดงว่า *R. paludigenum* DMKU-RP301 ผลิตกรดอินโดล-3-แอซีติกผ่านทางวิถีกรดอินโดล-3-ไพรูวิก

Production of indole-3-acetic acid by rice phyllospheric yeast *Rhodosporidium paludigenum* DMKU-RP301 and investigation of its IAA biosynthetic pathway

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Indole-3-acetic acid (IAA) is the most important auxin of plant hormones. Two major pathways for IAA biosynthesis have been proposed: tryptophan-dependent and tryptophan-independent pathways. Several pathways using tryptophan as a precursor for IAA biosynthesis have been proposed, including an indole-3-acetamide pathway, an indole-3-pyruvic acid pathway, an indole-3-acetonitrile pathway, a tryptamine pathway and a tryptophan side-chain oxidase pathway. The tryptophan-independent IAA biosynthetic pathway has been proposed as a branch from either indole or indole-3-glycerol phosphate. Yeast is one of the microbial resources for IAA production. Production of fermentation products by yeasts is well-developed for large scale. The presence of a thick cell wall protects yeast cells from physical damage during fermentations. Moreover, yeast cells harvest technology has been shown to be simpler than that used for bacteria due to the large cell size of yeast. This study, therefore, investigated IAA production in a 2-l stirred tank fermenter and the IAA biosynthetic pathway in *Rhodosporidium paludigenum* DMKU-RP301, isolated from rice phyllosphere. Batch and fed-batch fermentation of *R. paludigenum* DMKU-RP301 were conducted. In batch fermentation, we found that when cultivated at a 200 rpm agitation speed and 3 l/min aeration rate, this yeast produced IAA at its maximum level as 1,627.1 mg/l (9.7 mg/l/h). In fed-batch fermentation, a higher level of maximum IAA production than that found in batch fermentation was observed i.e. 2,707.9 mg/l (25.1 mg/l/hr). We therefore suggested that fed-batch fermentation improved the efficiency of IAA production in terms of product concentration and IAA productivity. In addition, indole-3-lactic acid and indole-3-ethanol were found in the culture medium as a result of the enzymatic reduction of indole-3-pyruvic acid and indole-3-acetaldehyde, in IAA biosynthesis via an indole-3-pyruvic acid pathway. We hence concluded that *R. paludigenum* DMKU-RP301 produces IAA through an indole-3-pyruvic acid pathway.

เรื่อง ความหลากหลายและนิเวศวิทยาของยีสต์เอนโดไฟต์และยีสต์อีพิไฟต์จากใบของพืชเศรษฐกิจในประเทศไทยและการผลิตสารส่งเสริมการเจริญพืชออกซินโดยสายพันธุ์ที่คัดเลือกว่ามีประสิทธิภาพสูง พร้อมกับการศึกษาชีววิถีการสังเคราะห์
(โครงการวิจัยเลขที่ RTA5480009)

ความสามารถในการควบคุมราโรคพืชโดยยีสต์อีพิไฟต์บนผิวใบพืช

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งานวิจัยนี้มีวัตถุประสงค์เพื่อตรวจหายีสต์สายพันธุ์สำหรับใช้เป็นตัวควบคุมทางชีวภาพของโรคพืชที่เกิดจากรา จากการประเมินความสามารถของยีสต์ที่แยกได้จากผิวใบข้าวและข้าวโพด 221 สายพันธุ์ในการควบคุมการเจริญของรา 10 ชนิดที่เป็นสาเหตุของโรคข้าว (*Fusarium moniliforme*, *Helminthosporium oryzae* และ *Rhizoctonia solani*) โรคข้าวโพด (*Fusarium moniliforme*, *Exserohilum turcicum*, *Bipolaris maydis*, *Bipolaris zeicola* และ *Macrophomina phaseolina*) และโรคอ้อย (*Fusarium moniliforme* และ *Colletotrichum falcatum*) โดยวิธีเพาะเลี้ยงร่วมกัน สามารถคัดเลือกยีสต์ที่สามารถควบคุมราโรคพืชได้ 37 สายพันธุ์ ใน17 สปีชีส์ 12 สกุล โดย *Torulaspora globosa* DMKU-RP31 และ *T. globosa* DMKU-RP35 ยับยั้งราสาเหตุโรคพืชได้ 8 ชนิด *Wickerhamomyces anomalus* DMKU-RP04 ยับยั้งราสาเหตุโรคพืชได้ 7 ชนิด *Kodamaea ohmeri* DMKU-RP06 และ *W. anomalus* DMKU-RP25 ยับยั้งราสาเหตุโรคพืชได้ 5 ชนิด ยีสต์ 3 สายพันธุ์ ได้แก่ *W. anomalus* DMKU-CP122 *W. anomalus* DMKU-CP127 และ *K. ohmeri* DMKU-RP34 ยับยั้งราสาเหตุโรคพืชได้ 4 ชนิด แต่ละสายพันธุ์ของยีสต์ที่เหลือยับยั้งราสาเหตุโรคพืชได้น้อยกว่า 4 ชนิด จากการทดลองนี้ยีสต์ปฏิปักษ์ทั้ง 37 สายพันธุ์ พบว่า สายพันธุ์ DMKU-CP149, DMKU-CP295, DMKU-CP367, DMKU-CP437, DMKU-RP04, DMKU-RP25, DMKU-RP31, DMKU-RP34 และ DMKU-RP35 ยับยั้งราโรคพืชสูงกว่า 50 เปอร์เซ็นต์ ส่วนที่เหลือยับยั้งได้ต่ำกว่า 50 เปอร์เซ็นต์

จากการศึกษากลไกควบคุมทางชีวภาพของยีสต์ทั้ง 37 สายพันธุ์ที่คัดเลือกได้ ในการควบคุมราสาเหตุโรคพืช พบยีสต์ที่ผลิตไซเดอโรฟอร์ได้เมื่อทดสอบโดยการเพาะเลี้ยงบนอาหารแข็ง Chrome Azurol S blue จำนวน 8 สายพันธุ์ ยีสต์ที่ผลิตสารระเหยเพื่อยับยั้ง *Rhizoctonia solani* ได้ จำนวน 7 สายพันธุ์ โดยมีการยับยั้งในช่วง 57.0-73.9 เปอร์เซ็นต์ ยีสต์ที่ผลิตเอนไซม์เบต้า-กลูคาเนสในช่วง 0.5-42 mU/ml จำนวน 33 สายพันธุ์ โดย *Wickerhamomyces anomalus* DMKU-CP122, *Rhodosporidium paludigenum* DMKU-CP170, *Hanseniaspora guilliermondii* DMKU-CP295, *W. anomalus* DMKU-RP04 และ *Kodamaea ohmeri* DMKU-RP34 มีกิจกรรมของเบต้า–กลูคาเนสสูงกว่า 25 mU/ml และยีสต์ที่ผลิตไคติเนสในช่วง 2-249 mU/ml จำนวน 21 สายพันธุ์ โดย *H. guilliermondii* DMKU-CP295 *Torulaspora globosa* DMKU-RP35 และ *Kodamaea ohmeri* DMKU-RP57 มีกิจกรรมของไคติเนส 166.7, 166.8 และ

249 mU/ml ตามลำดับ ความสามารถในการแข่งขันใช้สารอาหารและพื้นที่โดยวิธีเพาะเลี้ยงร่วมกันบนอาหารแข็ง PDA ที่มีธาตุอาหารความเข้มข้นต่างๆ พบว่ายีสต์ 12 สายพันธุ์ ยับยั้งราสาเหตุโรคพืชสูงเมื่อมีธาตุอาหารความเข้มข้นต่ำลง

การทดสอบการควบคุมโรคกาบใบแห้ง (*Rhizoctonia solani*) ในเรือนทดลองโดยยีสต์ปฏิปักษ์ที่คัดเลือกได้ 7 สายพันธุ์ (*Candida floricola* DMKU-CP149, *H. guilliermondii* DMKU-CP295, *C. floricola* DMKU-CP367, *Bullera sinensis* DMKU-CP437, *W. anomalus* DMKU-RP25, *T. globosa* DMKU-RP31 และ *T. globosa* DMKU-RP35) พบว่า การพ่นเซลล์แขวนลอยของยีสต์ *H. guilliermondii* DMKU-CP295 กดการเกิดโรคกาบใบแห้งในข้าวได้อย่างมีนัยสำคัญ สูงกว่า 65 เปอร์เซ็นต์ ในขณะที่ยีสต์สายพันธุ์อื่นแสดงการกดการเกิดโรคต่ำในช่วง 12.0-53.9 เปอร์เซ็นต์

Biocontrol activity of epiphytic yeasts from plant leaves against plant pathogenic fungi

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This study aimed to investigate for yeast strains to be used as biocontrol agents of fungal plant diseases. A total of 221 epiphytic yeast strains, which were isolated from rice and corn leaf surfaces, were determined for their biocontrol activities against 10 pathogenic fungi causing diseases of rice (*Fusarium moniliforme, Helminthosporium oryzae* and *Rhizoctonia solani*), corn (*Fusarium moniliforme, Exserohilum turcicum, Bipolaris maydis, Bipolaris zeicola and Macrophomina phaseolina*) and sugarcane (*Fusarium moniliforme* and *Colletotrichum falcatum*) by dual cultivation. Thirty seven epiphytic yeast strains belonging to 17 species in 12 genera, which inhibited growth of these pathogenic fungi, were selected for further studies. *Torulaspora globosa* DMKU-RP31 and *T. globosa* DMKU-RP35 could inhibit 8 plant pathogenic fungi (PPF). *Wickerhamomyces anomalus* DMKU-RP04 inhibited 7 PPF. *Kodamaea ohmeri* DMKU-RP06 and *W. anomalus* DMKU-RP25 inhibited 5 PPF. Three epiphytic yeast strains namely *W. anomalus* DMKU-CP122, *W. anomalus* DMKU-CP127 and *K. ohmeri* DMKU-RP34 inhibited 4 PPF. The other strains showed biocontrol activities against less than 4 PPF. Among the 37 antagonistic yeast strains, strains DMKU-CP149, DMKU-CP295, DMKU-CP367, DMKU-CP437, DMKU-RP04, DMKU-RP25, DMKU-RP31, DMKU-RP34 and DMKU-RP35 inhibited growth of PPF greater than 50% while the others showed growth inhibition lower than 50%.

Biocontrol mechanisms of the 37 selected yeast strains against plant pathogenic fungi were investigated. Eight yeast strains produced siderophore tested by cultivated on Chrome Azurol S blue agar. Seven biocontrol yeast strains produced volatile compounds, which inhibited *Rhizoctonia solani* in the range of 57.0-73.9 %. Thirty three strains produced β-glucanase in the range of 0.5-42 mU/ml, where *Wickerhamomyces anomalus* DMKU-CP122, *Rhodosporidium paludigenum* DMKU-CP 170, *Hanseniaspora guilliermondii* DMKU-CP295, *W. anomalus* DMKU-RP04 and *Kodamaea ohmeri* DMKU-RP34 produced higher than 25 mU/ml. Twenty one strains produced chitinase in the range of 2-249 mU/ml, where *Hanseniaspora guilliermondii* DMKU-CP295, *Torulaspora globosa* DMKU-RP35 and *Kodamaea ohmeri* DMKU-RP57 produced relatively higher activities of 166.7, 166.8 and 249 mU/ml, respectively. The competition of nutrient and space determined by dual cultivation on PDA with various nutrient concentrations revealed that 12 yeast strains showed high percentage of inhibition when the PDA nutrient concentration was low.

Rice sheath blight disease (*Rhizoctonia solani*) control was tested in greenhouse by the seven antagonistic yeast strains selected (*Candida floricola* DMKU-CP149, *H. guilliermondii* DMKU-CP295, *Candida floricola* DMKU-CP367, *Bullera sinensis* DMKU-CP437, *W. anomalus* DMKU-RP25, *T. globosa* DMKU-RP31 and *T. globosa* DMKU-RP35).

Cell suspension of *H. guilliermondii* DMKU-CP295 applied by spraying significantly suppressed the rice sheath blight disease by up to 65 %, whereas the other yeast strains expressed the low disease suppression of 12.0-53.9 %.