Final Report

Taxonomy and secondary metabolites of endophytic actinobacteria isolated from Acanthaceae plants in Thailand

By WONGSAKORN PHONGSOPITANUN

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Final Report

Taxonomy and secondary metabolites of endophytic actinobacteria isolated from Acanthaceae plants in Thailand

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บทคัดย่อ

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โครงการ : อนุกรมวิธานและสารเมแทบอไลต์ทุติยภูมิของเอนโดไฟติกแอคติโนแบคทีเรียที่คัดแยกได้วงศ์ Acanthaceae ในประเทศไทย

หัวหน้าโครงการ : ดร. วงศกร พงศ์โสภิตานันท์

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บทคัดย่อ

"จุลินทรีย์" โดยเฉพาะอย่างยิ่งแอคติโนแบคทีเรีย เป็นแหล่งของสารออกฤทธิ์ทางชีวภาพขั้นต้นที่ช่วยในการขับเคลื่อนกระบวนการค้นพบยา ในศตวรรษที่ผ่านมาแอคติโนแบคทีเรียจำนวนมากได้ถูกคัดแยกจากดินและได้ถูกใช้ในการผลิตยาที่สำคัญหลายชนิด อย่างไรก็ตามในปัจจุบันการค้นพบสารต้นแบบที่จะนำมาพัฒนาเป็นยาใหม่มีแนวโน้มลดลงอย่างต่อเนื่องทั้งนี้เนื่องจากการคัดแยกจุลินทรีย์จากแหล่งที่อยู่เดิม ดังนั้นการค้นหาแหล่งที่อยู่ใหม่ของจุลินทรีย์ที่เป็นผู้ผลิตสารออกฤทธิ์ทางชีวภาพจึงเป็นสิ่งสำคัญ "เอนโดไฟต์" คือจุลินทรีย์ที่อาศัยอยู่ในเนื้อเยื่อพืชโดยไม่ก่อให้เกิดโทษกับพืช และมีการศึกษาพบว่าจุลินทรีย์กลุ่มนี้มีศักยภาพในการผลิตสารชนิดใหม่ที่สามารถนำไปประยุกต์ใช้งานได้หลากหลาย ต้นไม้ในวงศ์ Acanthaceae ได้ถูกใช้เป็นยาพื้นบ้านของประเทศไทยมาอย่างช้านาน ในประเทศไทยมีรายงานว่ามีพืชกลุ่มนี้อยู่ถึง 40 สกุล และมีความหลากหลายประมาณ 200 สปีชีส์ อย่างไรก็ตามการศึกษาแอคติโนแบคทีเรียที่สัมพันธ์กับพืชกลุ่มนี้ยังมีอยู่น้อยมาก ในการศึกษานี้สามารถคัดแยกแอคติโนแบคทีเรียได้ 52 ไอโซเลต จากตัวอย่างใบ ลำต้น และรากของ พืชในวงศ์ Acanthaceae ที่แตกต่างกันจำนวน 6 สปีชีส์ จากผล BLAST และการวิเคราะห์ความสัมพันธ์ทางวิวัฒนาการพบว่าแอคติโนแบคทีเรียที่คัดแยกได้นี้สามารถจำแนกได้เป็น 4 วงศ์ คือ *Nocardiaceae*, *Micromonosporaceae*, *Streptosporangiaceae* และ *Streptomycestaceae* และสามารถจำแนกในระดับสกุลได้เป็น 6 สกุล คือ *Actinomycetospora* (1 ไอโซเลต), *Dactylosporangium* (1 ไอโซเลต), *Nocardia* (3 ไอโซเลต), *Microbispora* (5 ไอโซเลต), *Micromonospora* (10 ไอโซเลต) and *Streptomyces* (32 ไอโซเลต) จากผลการคัดกรองฤทธิ์ต้านจุลชีพพบว่าแอคติโนแบคทีเรีย จำนวน 8 ไอโซเลต คือ *Actinomycetospora* จำนวน 1 ไอโซเลต และ *Streptomyces* จำนวน 7 ไอโซเลต สามารถยับยั้งจุลินทรีย์ที่ใช้ในการทดสอบได้ ในการศึกษานี้ไอโซเลต 3R004 ซึ่งแสดงฤทธิ์ต้านจุลชีพที่ดีที่สุดได้ถูกคัดเลือกเพื่อใช้ในการศึกษาต่อ จากการวิเคราะห์ความสัมพันธ์ทางวิวัฒนาการโดยใช้จีโนมพบว่าไอโซเลต 3R004 มีความสัมพันธ์ใกล้เคียงกับ *Streptomyces antibioticus* และ *Streptomyces auilus* จากการศึกษา average nucleotide identity และ DNA-DNA hybridization พบว่าจีโนมของไอโซเลต 3R004 แตกต่างจากสายพันธุ์ใกล้เคียงดังนั้นจึงสามารถสรุปได้ว่าไอโซเลต 3R004 มีความเป็นไปได้สูงที่จะเป็นแอคติโนแบคทีเรียสายพันธุ์ใหม่ จากผลการศึกษาสารเมแทบอไลต์ทุติยภูมิโดยการวิเคราะห์จีโนมและคัดแยกสารโดยวิธีทางเคมีพบว่า สาร

ออกฤทธิ์ทางชีวภาพของไอโซเลต 3R004 คือ actinomycin D และพบว่าเมื่อเลี้ยงเชื้อในอาหาร 54 medium จะได้ผลผลิตของ actinomycin D สูงสุดที่ 378.3 mg/L ที่ระยะเวลา 12 วัน และจากผลการศึกษาในงานวิจัยนี้พบว่า actinomycin D ออกฤทธิ์ในการฆ่าเชื้อแบคทีเรียแกรมบวก *Bacillus cereus* และสามารถฆ่าแบคทีเรียแกรมลบ *Acenitobacter baumannii* ในสภาวะที่มีการเติม efflux inhibitor นอกจากนี้ยับพบฤทธิ์ยับยั้งเซลล์มะเร็ง คือ KB, MCF-7 and NCI-H187 ดังนั้นจึงอาจสรุปได้ว่าพืชในวงศ์ Acanthaceae เป็นแหล่งที่อยู่ที่สำคัญของแอคติโนแบคทีเรียและแอคติโนแบคทีเรียที่คัดแยกได้จากพืชวงศ์นี้สามารถนำไปใช้ต่อยอดทางเภสัชกรรมต่อไปในอนาคตได้

คำสำคัญ: เอนโดไฟติกแอคติโนแบคทีเรีย, actinomycin D, แอคติโนแบคทีเรียสายพันธุ์ใหม่, สารเมแทบอไลต์ทุติยภูมิที่มีฤทธิ์ทางชีวภาพ

Abstract

Project Code : MRG6180011

Project Title : Taxonomy and secondary metabolites of endophytic actinobacteria isolated from Acanthaceae plants in Thailand

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Abstract:

Microorganisms, especially actinobacteria, are the primary source of the bioactive natural products which drive drug discovery processes. In the past century, a numerous actinobacteria have been isolated from soil and used as the producer of key drugs. However, the discovery of novel lead compounds has been decreased because of the redundancy of the samples. Thus, it is necessary to investigate the untapped microorganism. Endophytes, the microorganisms that live inside the plant tissues without having negative impact, have a massive potential to produce a numbers of novel compounds that find wide-range application. Plants in family Acanthaceae have been used for Thai traditional medicines for a long time. Approximately 40 genera and 200 species of Acanthaceae plants have been reported in Thailand. However, the actinobacteria associated with the members of this plant family are rarely studied. In this study, 52 actinobacteria were isolated from leaves, stems and roots of six species of Acanthaceae plants. BALST result and phylogenetic tree analysis, actinobacteria obtained in this study were identified and categorized into 4 families (*Nocardiaceae*, *Micromonosporaceae*, *Streptosporangiaceae*, and *Streptomycetaceae*) and 6 genera including *Actinomycetospora* (1 isolate), *Dactylosporangium* (1 isolate), *Nocardia* (3 isolates), *Microbispora* (5 isolates), *Micromonospora* (10 isolates) and *Streptomyces* (32 isolates). Based on antimicrobial activity screening results, 8 isolates, including one *Actinomycetospora* and seven *Streptomyces*, exhibited antimicrobial activity against tested microorganisms. In this study, the strain 3R004 which produced the best antimicrobial activity against Gram-positive bacteria was selected for further study. Based on phylogenomic analysis, strain 3R004 are closely related to *Streptomyces antibioticus* and *Streptomyces aquilus*. Based on, the average nucleotide identity (ANI) and digital DNA-DNA hybridization analysis, the genome of strain 3R004 was different from those known species. Thus, strain 3R004 is the candidate of novel actinobacterial species. The secondary metabolites analysis results including genome BLAST and chemical isolation

confirm that the bioactive compound of strain 3R004 is actinomycin D. The maximum yield of actinomycin D, 378.3 mg/L, was observed after culture the strain in 54 medium for 12 days. Based on the result obtained in this study, actinomycin D showed antimicrobial activity against Gram-positive bacterial, *Bacillus cereus*, antimalarial activities against *Plasmodium falciparum*. The antigram-negative bacteria against *Acinetobacter baumannii* was observed when the efflux inhibitor was presented. The cytotoxicity against cancer cell was observed in KB, MCF-7 and NCI-H187. It can be concluded from this study that Acanthaceae plant is one of the actinobacterial habitat and the actinobacteria obtained from these plants could be used for further pharmaceutical applications.

Keywords: Endophytic actinobacteria, Actinomycin D, Novel actinobacterial species, Bioactive secondary metabolites

2. Executive summary

52 actinobacteria were isolated from leaves, stems and roots of six species of Acanthaceae plants. BLAST result and phylogenetic tree analysis, actinobacteria obtained in this study were identified and categorized into 4 families (*Nocardiaceae*, *Micromonosporaceae*, *Streptosporangiaceae*, and *Streptomycetaceae*) and 6 genera including *Actinomycetospora* (1 isolate), *Dactylosporangium* (1 isolate), *Nocardia* (3 isolates), *Microbispora* (5 isolates), *Micromonospora* (10 isolates) and *Streptomyces* (32 isolates). Based on antimicrobial activity screening results, 8 isolates, including one *Actinomycetospora* and seven *Streptomyces*, exhibited antimicrobial activity against tested microorganisms. The strain 3R004 which produced the best antimicrobial activity against Gram-positive bacteria was selected for further study. Based on phylogenomic analysis, strain 3R004 are closely related to *Streptomyces antibioticus* and *Streptomyces aquilus*. Based on the average nucleotide identity (ANI) and digital DNA-DNA hybridization analysis, the strain 3R004 is the candidate of novel actinobacterial species. The secondary metabolites analysis results including genome BLAST and chemical isolation confirm that the bioactive compound of strain 3R004 is actinomycin D. The maximum yield of actinomycin D, 378.3 mg/L, was observed after culture the strain in 54 medium for 12 days. Based on the result obtained in this study, actinomycin D showed antimicrobial activity against Gram-positive bacterial, *Bacillus cereus*, antimalarial activities against *Plasmodium falciparum*. The anti gram-negative bacteria against *Acinetobacter baumannii* was observed when the efflux inhibitor was presented. The cytotoxicity against cancer cell was observed in KB, MCF-7 and NCI-H187.

3. Objective

3.1To isolate novel endophytic actinobacteria from the Acanthaceae plants

3.2To identify the selected endophytic actinomycete isolates based on phenotypic, chemotaxonomic and genotypic characteristics.

3.3To screen, isolate and elucidate the new secondary metabolites from the selected novel actinobacterial isolates.

4. Research methodology

The experiment is designed to isolate the endophytic actinobacteria from the Acanthaceae plants collected in Thailand. The 16S rRNA gene of all isolates was determined to find out the candidate of novel species. All candidates of novel actinobacterial species were

selected for further taxonomic studies using a polyphasic approach. In addition, those novel strains were screened for their ability to produce antimicrobial agents as well as other new secondary metabolites. The selected isolates, based on the novel species which best antimicrobial activity, was cultivated in the large scale condition to obtain enough crude extract. The compounds in the crude extract were separated by chromatographic methods. The chemical structures of the pure compounds were elucidated using spectroscopic methods including ultraviolet spectroscopy, infrared spectroscopy, nuclear magnetic resonance spectroscopy (H^1-NMR, C^{13}-NMR) and mass spectrometry. Finally, the pure compound was tested for other biological activities. The experimental design is shown in the **Fig 4.1**

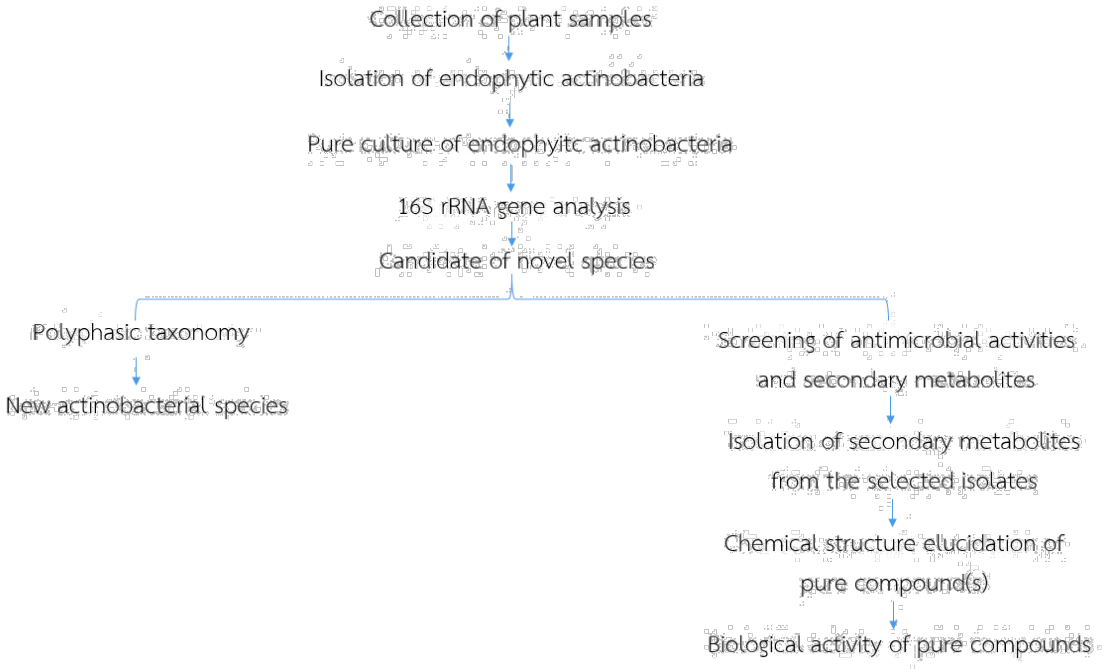


Fig 4.1 Experimental design for this research

4.1 Collection of Acanthaceae plants and isolation of endophytic actinobacteria

Plant samples were collected and identified by Assist. Prof. Dr. Kanokorn Rueangsawag, the expert of Acanthaceae plants. The surface of plant samples was sterilized using 70% alcohol and 1% sodium hypochlorite (NaOCl) and washed by sterile water. The clean samples were ground using a sterile mortar. Then, the suitable sterile buffer was added to the sample and made the serial dilution. The resultant of solution was spread on the suitable isolation media including water proline agar, starch casein nitrate agar and humic acid vitamin agar. The isolation plates were incubated at 30^{o}C and observed for the actinobacterial colonies for 7, 14 and 21 days. The actinobacterial colony was purified on ISP2 agar. For long term

preservation, all actinobacterial isolates were preserved by freezing at -20^{o}C. In addition, the candidate of novel actinobacterial species was preserved in the national culture collections (TBRC and/or TISTR).

4.2 Identification of actinobacteria

All endophytic actinobacterial isolates was identified using the 16S rRNA gene analysis. To find out the novel actinobacterial species, the isolates showing the value of 16S rRNA gene lower than 99% similarity and/or representing some unique characteristics were selected for further taxonomic studies.

4.2.1 16S rRNA gene amplification and sequencing

The PCR amplification of the 16S rRNA gene was performed using the universal primers including 20F (5-GAGTTTGATCCTGGCTCAG-3) and 1500R (5-GTTACCTTGTTACGACTT-3) (Lane, 1991; Suriyachadkun *et al*. 2009). The PCR products were verified using agarose gel electrophoresis and purified using DNA purification kit (Geneaid^{TM}). The sequencing of PCR products was carried out by sequencing service (Macrogen, Korea).

4.2.2 16S rRNA gene analysis and phylogenetic construction

BLASTn analysis of the sequences was determined using the EzTaxon-e server (http://www.ezbiocloud.net; Yoon *et al*., 2017). The 16S rRNA gene sequences were aligned with the selected sequences from the GenBank/EMBL/DDBJ database based on CLUSTAL W (Thompson *et al*., 1994) using BioEdit software (Hall, 1999). The neighbor-joining (NJ; Saitou & Nei, 1987), maximum-parsimony (MP; Fitch, 1971) and maximum-likelihood (ML; Felsenstein, 1981) phylogenetic trees were constructed using MEGA version 7.0 (Kumar *et al*., 2016).

4.2.3 Morphological and cultural characteristics

Morphological characteristics were observed using light microscope and scanning electron microscopy. The cultural characteristics were determined according to the standard methods (Shirling & Gottlieb, 1966). The color of the aerial mass, substrate mycelia and diffusible pigment were determined using the ISCC-NBS colour system (Kelly, 1964).

4.2.4 Biochemical and physiological properties

Biochemical and physiological properties were tested using standard methods (Williams & Cross, 1971; Arai, 1975). Temperature and pH for growth and NaCl tolerance were determined using ISP2 agar.

4.2.5 Chemotaxonomy

All chemotaxonomic studies were analyzed using freeze-dried cells. Standard thin layer chromatography (TLC) procedures was used to determine the isomer of diaminopimelic

acid, sugars (Staneck & Roberts, 1974) and polar lipids (Minnikin *et al*., 1984). The acyl type of muramic acid was determined using the method of Uchida & Aida (1984). Menaquinones was analyzed by HPLC (Collins *et al*., 1977). Cellular fatty acid methyl esters was extracted following the method described by Sasser (1990) and analyzed using gas chromatography (GC) according to the Microbial Identification System (MIDI, Sherlock Microbial Identification System, USA).

4.3 Screening of antimicrobial activity and secondary metabolites

All candidates of novel actinobacterial species was cultured in 10 ml of the production media at 30^{o}C for 7-14 days. In this study, the ISP2 broth [1.0 g glucose, 4.0 g malt extract 1.0 g yeast extract, added distilled water up to 100 mL, pH 7.0] was used as the production medium.

After incubation, 10 ml of 95% ethanol was added into the culture broth and shaked at 180 r.p.m. for 2 hours. The extract solution was centrifuged at 3,000 r.p.m. for 15 minutes and preserved at -20 ^{o}C. The production medium without the culture was used as a negative control.

The screening of antimicrobial activity was determined using agar disc diffusion method. Each of paper disc (8 mm) was soaked into the extract solution and air-dried. After drying, the discs was put onto the surface of the agar plate containing a tested microorganism and cooled at 4 ^{o}C for 30 minutes before incubation. Four bacteria, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853, one yeast, *Candida albicans* ATCC 10231 were used as the tested microorganisms.

For screening of the secondary metabolites, the culture broth was analyzed by HPLC. The chromatographic bioassay guided isolation was used to isolate the bioactive compounds.

7.4 Fermentation and extraction

The selected isolate was cultured in suitable production media using shaking flask condition for 7-14 days. The cultured broth was extracted using ethyl acetate three times. The ethyl acetate layer was collected and evaporated to dryness. The crude extract(s) was used for further chemical isolation.

7.5 Isolation, chemical structure elucidation and biological activities of pure compounds

The chemical structure of pure compounds was elucidated using various spectroscopic analyses including ultraviolet spectroscopy, infrared spectroscopy, nuclear magnetic resonance spectroscopy (H^{1}-NMR, C^{13}-NMR) and mass spectrometry. The pure compound was tested for others biological activities (Bioassay laboratory, The National Center for Genetic Engineering and Biotechnology (BIOTEC)).

5. Result and Dicussion

5.1 Diversity of endophytic actinobacteria isolated from Acanthaceae plants

In this study, 52 actinobacteria were isolated from leaves, stems and roots of six species of Acanthaceae plants. In this number, 49 isolates were obtained from roots followed by 2 and 1 isolate were obtained from leaves and stem, respectively. The results of this study are similar to previous studies showing that nearly all the plants harbor endophytes. Janso and Carter discussed that actinobacteria could be isolated from every tissue type of samples; however, root and bark had the highest isolate-to-sample ratio.

On the basis of BALST result and phylogenetic tree analysis, actinobacteria obtained in this study were identified and categorized into 4 families (*Nocardiaceae*, *Micromonosporaceae*, *Streptosporangiaceae*, and *Streptomycetaceae*) and 6 genera including *Actinomycetospora* (1 isolate), *Dactylosporangium* (1 isolate), *Nocardia* (3 isolates), *Microbispora* (5 isolates), *Micromonospora* (10 isolates) and *Streptomyces* (32 isolates) (Fig. 5.1; Fig 5.2 ;Table 5.1). Based on this study, the most abundant genus found in *Acanthaceae* plants were *Streptomyces* (61%) followed by *Micromonospora* (19%) and *Microbispora* (10%) (Fig 5.1). The pattern of the diversity of culturable actinobacteria of this study, which *Streptomyces* are the predominant species, is similar to the previous report. In 2012, Kim et al isolated 61 endophytic actinobacteria, comprised 15 genera including *Streptomyces*, *Micromonospora*, *Rhodococcus*, *Microbispora*, *Micrococcus*, *Microbacterium*, *Streptacidiphilus, Arthrobacter*, *Dietzia*, *Kitasatospora*, *Herbiconiux*, *Mycobacterium*, *Nocardia*, *Rathayibacter* and *Tsukamurella*, from the native herbaceous plant species of Korea. In that study, they found that members of the genus *Streptomyces* comprised 45.9% of the total isolates and was followed by *Micromonospora* (18.8%). In the study of Janso and Carter, 123 isolates of endophytic actinobacteria, including 17 genera, were isolated from the tropical native plants in Papus New Guinea and Mborokua Island, Solomon Island. The community of endophytic actinobacteria may be varied according to the host plant. Jiang et al isolated 101 endophytic actinobacteria from five different mangrove plants including *Avicennia marina, Aegiceras corniculatum, Kandelia obovota, Bruguiera gymnorrhiza*, and *Thespesia populnea*. Based on 16S rRNA gene these actinobacteria distributed in 15 families and 28 genera including *Actinoplanes, Agrococcus, Amnibacterium, Brachybacterium, Brevibacterium, Citricoccus, Curtobacterium, Dermacoccus, Glutamicibacter, Gordonia, Isoptericola, Janibacter, Kineococcus, Kocuria, Kytococcus, Leucobacter, Marmoricola, Micrococcus, Microbacterium, Micromonospora, Mycobacterium, Nocardioides, Nocardia, Nocardiopsis, Pseudokineococcus, Sanguibacter, Streptomyces,* and *Verrucosispora*. In addition, Widiantini and Franco reported that the

dominant endophytic actinobacteria species isolated from rice plants of Australia is *Microbispora*. The variable of endophytic actinobacterial species in the different plants may depending on factors such as host specificity, stage of the host, type of sample, geographical condition, season, surface sterilant, culture condition and selective media

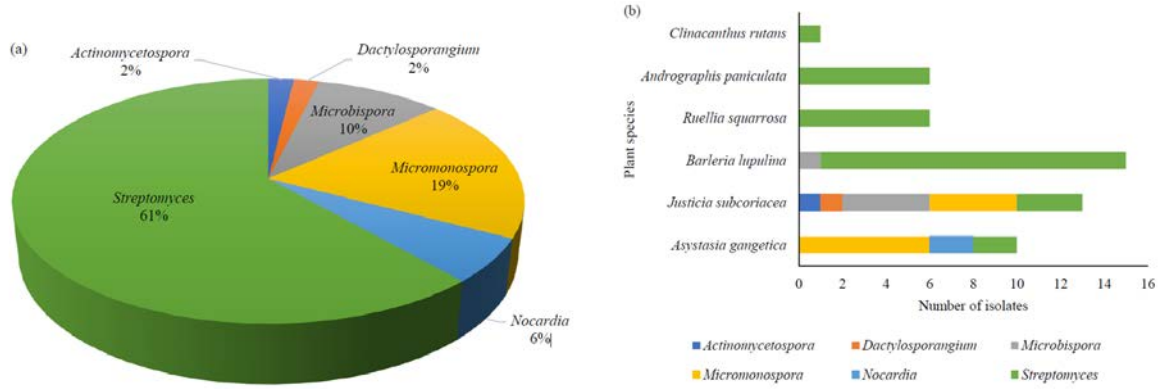


Fig. 5.1 Diversity of actinobacteria isolated from Acanthaceae plant species. (a) Pie chart represented the percentage of actinobacterial genera within the total number of isolates. (b) The number of actinobacteria isolated from different plant species.

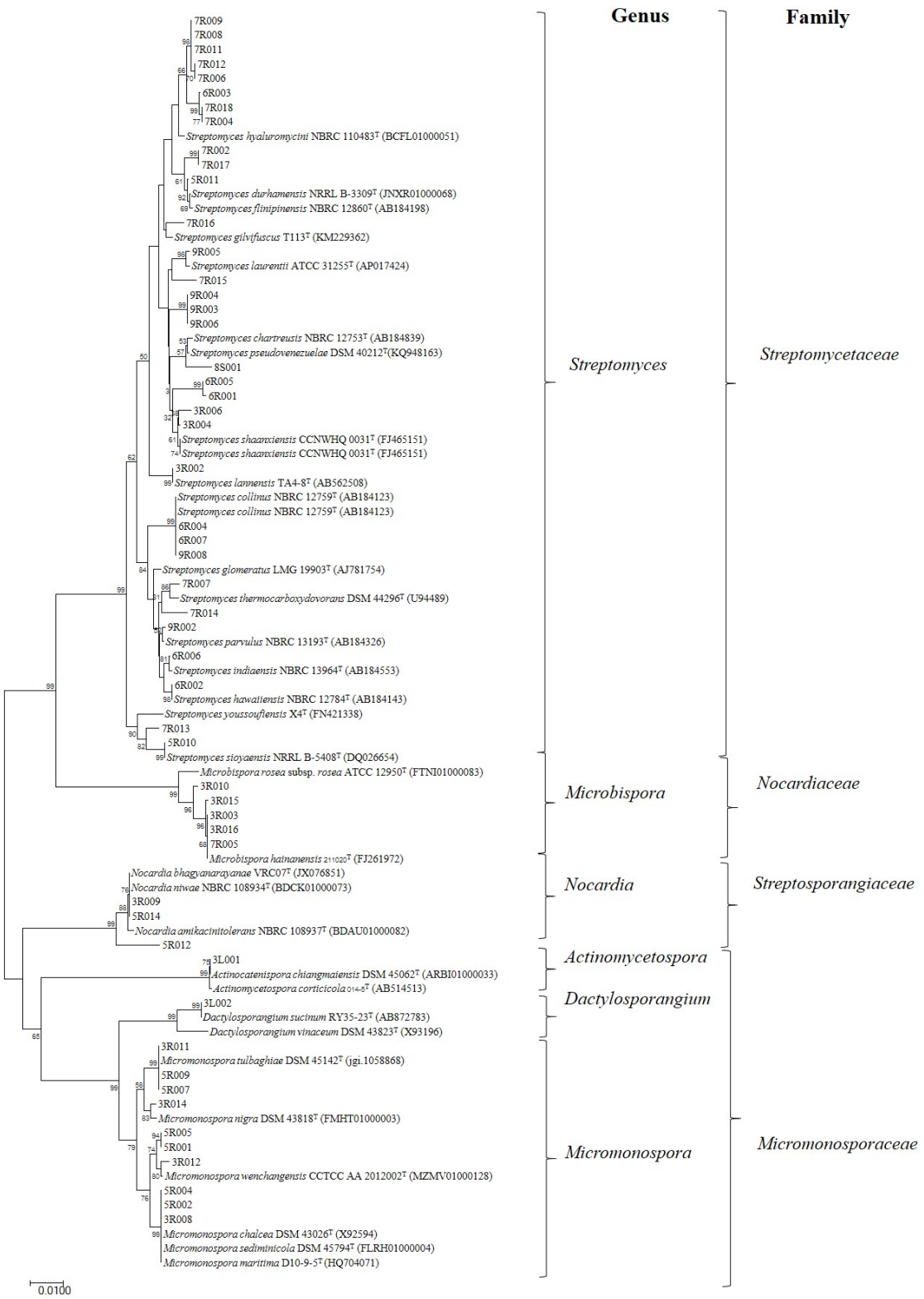


Fig. 5.2 Neighbour-Joining phylogenetic tree based on 16S rRNA gene of the actinobacterial isolates and closely related actinobacterial type strains shown that the isolates were clustered within four families and six genera. Numbers at the nodes indicate bootstrap values based on 1,000 replicates.

5.2 Antimicrobial activity screening of endophytic actinobacteria

In this study 8 isolates, including one *Actinomycetospora* and seven *Streptomyces*, exhibited antimicrobial activity against tested microorganisms. Most of the active isolates showed antimicrobial activity against Gram-positive bacteria but no activity was observed against Gram-negative bacteria (Fig. 3; Table 1). The antimicrobial activity of endophytic *Streptomyces* against Gram-positive bacteria has been documented in previous studies. Zhang et al. study of antimicrobial activity of 65 endophytic actinobacteria, isolated from *Achyranthes bidentata, Paeonia lactiflora, Radix Platycodi and Artemisiae argyi,* against *penicillin resistant Staphylococcus aureus*. They found that 12 strains, the majority were *Streptomyces* spp., showed activity against this pathogen. Although no actinobacteria obtained from this study showed antiGram-negative bacterial activity. Mingma et al isolated 317 actinobacteria from root and rhizospheric soils of leguminous plants and 64 of the isolates (20.2%) showed antagonistic activity against soybean pathogen *Xanthomonas campestris* pv. glycine. In addition, 21 endophytic actinobacteria isolated by Jiang et al showed activity against *P. aeruginosa*. This evidence showed that antiGram-negative bacteria could be observed in some endophytic actinobacteria.

The production of novel antimicrobial metabolites from endophytic actinobacteria has been documented in the various reports. The example is maklamicin, misamycin and diastaphenazine.

Maklamicin, a new spirotetronate-class polyketide, isolated from *Micromonospora* sp. GMKU326, the endophytic actinobacteria isolated from root nodule of the legume *Lupinus angustifolius*, showed strong to moderate antimicrobial activity against Gram-positive bacteria including *Micrococcus luteus, Bacillus subtilis, B. cereus, Staphylococcus aureus* and *Enterococcus faecalis* with MIC values of 0.2, 1.7, 6.5, 13 and 13 µg/ml, respectively.

In this study, the isolate 5R010, closely related to *Streptomyces sioyaensis* NRRL-B5408^{T}, showed antifungal activity against *C. albicans*. This isolate was selected to test the antagonistic activity against phytopathogenic fungi.

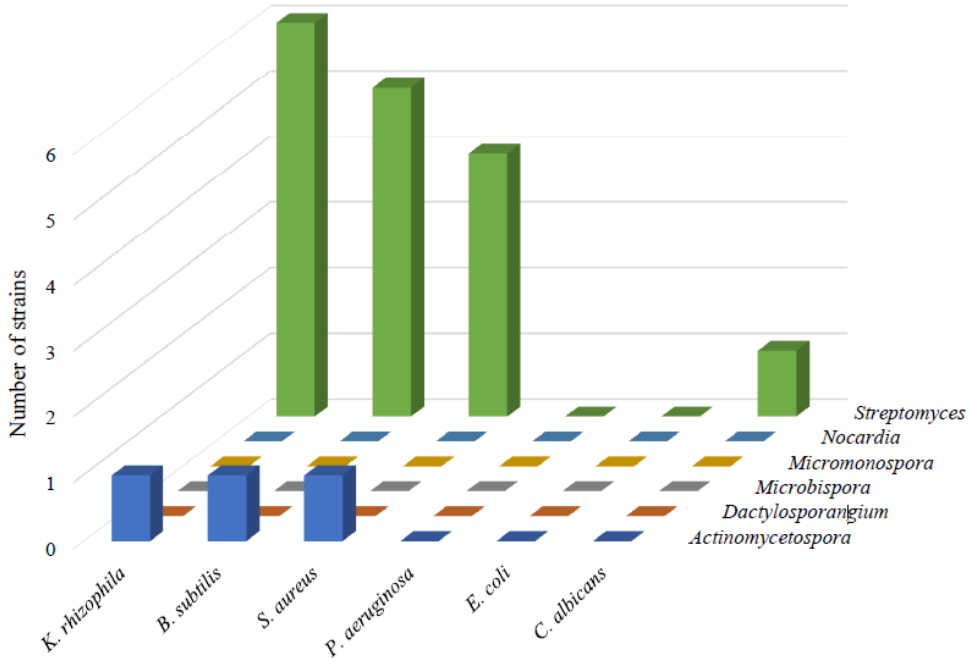


Fig. 5.3 Antimicrobial activity of the actinobacteria against tested microorganisms

5.3 Antiphytopathogenic fungi activity

Based on the co-cultivation method, the strain 5R010 showed antagonistic activity against *Fusarium* sp., *Colletotrichum* sp., *Sclerotium* sp. but no activity was observed on *Colletotrichum gloerosporiodes*, *Curvularia oryzae* and *Lasiodiplodia theobromae* (Fig. 5.4). It has been reported in several studies that the endophytic actinobacteria can be used to control plant diseases. Álvarez-Pérez et al used endophytic actinobacteria isolated from the root system of the grapevine plants, *Vitis vinifera*, to reduce nursery fungal graft infections caused by *Diplodia seriata Dactylonectria macrodidyma Phaeomoniella chlamydospora* and *Phaeoacremonium minimum*. Taechowisan et al reported that three endophytic *Streptomyces* sp. showed strongly inhibited *Colletotrichum musae* and five were very active against *Fusarium oxysporum*. The *Streptomyces* strain CEN6, isolated from *Centella asiatica*, showed good antagonistic activity against *Alternaria brassicicola*-the pathogen causes leaves spot of cabbage. The fungal treated by this stain showed abnormal characteristics including swelling and frequent septa. The used of endophytic *Streptomyces platensis* F-1, isolated from *Oryza sativa*, as biofumigation to control plant fungal disease was reported by Wan et al. The volatile substance produced by the strain F-1 could effectively reduce the incidence and the severity of the disease caused by *Botrytis cinerea*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*. Besides the application as biocontrol, the novel antifungal compounds,

such as dehydroxyaquayamycin B and fistupyrone, were isolated from endophytic actinobacteria. Dehydroxyaquayamycin B, a new C-glycosylated benz[α]anthraquinone, was isolated from endophytic *Streptomyces blastomycetica* F4-20. The compound showed fungicidal activity against *Valsa mali, Colletotrichum orbiculare* and *Fusarium graminearum*. Fistupyrone, a new microbial compound, isolated from the culture broth of endophytic *Streptomyces* sp. TP-A0569 can inhibit the *in vivo* infection of the seedlings of Chinese cabbage by *Alternaria brasicicola*, the cause of *Alternaria* leaf spot. The antagonistic activity of the strain 5R010 found in this study revealed that this strain may be used for the fungal biocontrol in the future. In addition, the active compounds produced by this strain should be characterized in further study.

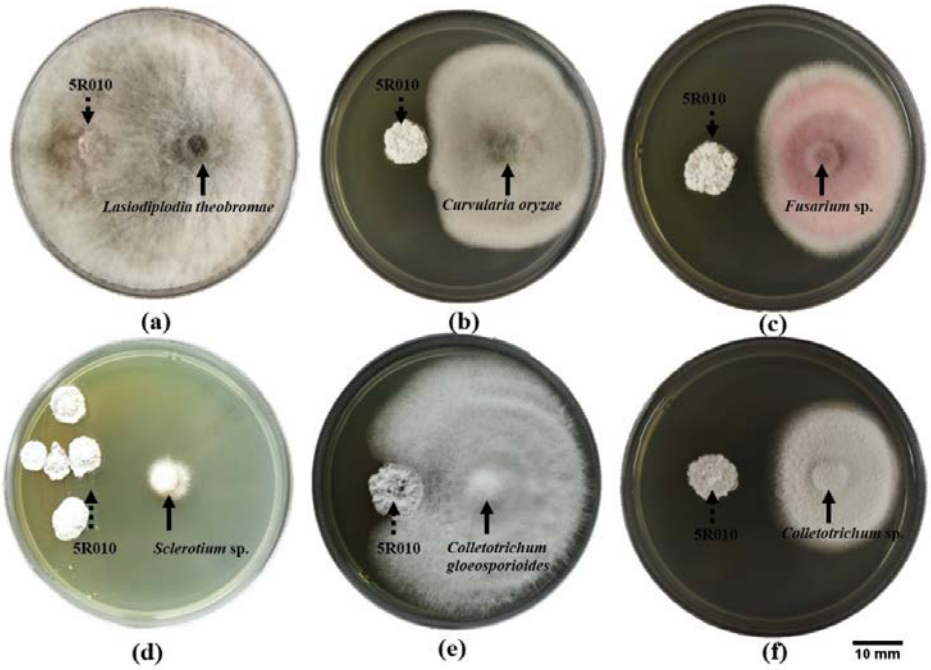


Fig. 5.4 Antagonistic activity of the isolate 5R010 against phytopathogenic fungi (a) *Lasiodiplodia theobromae*, (b) *Curvularia oryzae* (c) *Fusarium* sp. (d) *Sclerotium* sp. (e) *Colletotrichum gloeosporioides* (f) *Colletotrichum* sp. The arrows ⋯▶ and ⟶ indicate the colony of isolate 5R010 and fungal pathogens, respectively. The scale bar is 10 mm.

Table 5.1 Closest BLASTN matches for the 16S rDNA sequence and antimicrobial activity of the actinobacterial isolates

Plant host	plant material	isolation no.	accession number	BLAST match result		isolation media	Inhibition zone (mm)					
				Closest species	Similarity %		K	B	S		E	C
Asystasia gangetica	Root	5R001	LC497879	*Micromonospora chokoriensis* DSM45160^T	99.51	SCN	-	-	-	·	-	·
		5R002	LC497878	*Micromonospora maritima* D10-9-5^T	100	SCN	-	-	-	·	-	·
		5R004	LC497877	*Micromonospora maritima* D10-9-5^T	99.79	SCN	-	-	-	·	-	·
		5R005	LC497876	*Micromonospora chokoriensis* DSM45160^T	99.51	SCN	-	-	-	·	-	·
		5R007	LC497873	*Micromonospora tulbaghiae* DSM 45142^T	100	SCN	-	-	-	·	-	·
		5R009	LC497875	*Micromonospora tulbaghiae* DSM 45142^T	100	Proline	-	-	-	·	-	·
		5R010	LC500018	*Streptomyces sioyaensis* NRRL-B5408^T	99.72	HV	-	-	-	·	-	16
		5R011	LC497872	*Streptomyces durhamensis* NRRL-B3309^T	99.41	HV	26	15	17	·	-	·
		5R012	LC497874	*Nocardia xishanensis* NBRC 101358^T	99.93	HV	-	-	-	·	-	·
		5R014	LC497871	*Nocardia bhagyanarayanae* VRC07^T	98.68	SCN	-	-	-	·	-	·
Justicia subcoriacea	Root	3R002	LC497888	*Streptomyces lannensis* TA4-8^T	99.93	SCN	-	-	-	·	-	·
		3R003	LC497882	*Microbispora hainanensis* 211020^T	100	HV	-	-	-	·	-	·
		3R004	LC497890	*Streptomyces shaanxiensis* CCNWHQ0031^T	99.3	HV	21	21	27			
		3R006	LC497880	*Streptomyces cyaneus* NRRL B-2296^T	98.91	HV	-	-	-	·	-	·
		3R008	LC497886	*Micromonospora chalcea* DSM 43026^T	99.65	proline	-	-	-	·	-	·
		3R009	LC497889	*Nocardia bhagyanarayanae* VRC07^T	99.72	proline	-	-	-	·	-	·
		3R010	LC497887	*Microbispora hainanensis* 211020^T	99.17	proline	-	-	-	·	-	·
		3R011	LC497885	*Micromonospora tulbaghiae* DSM 45142^T	99.44	SCN	-	-	-	·	-	·
		3R012	LC497891	*Micromonospora wenchangensis* CCTCCAA 2012002^T	99.44	SCN	-	-	-	·	-	·
		3R014	LC497881	*Micromonospora nigra* DSM 43818^T	98.25	SCN	-	-	-	·	-	·
		3R015	LC497884	*Microbispora hainanensis* 211020^T	99.72	HV	-	-	-	·	-	·
		3R016	LC497883	*Microbispora hainanensis* 211020^T	99.72	HV	-	-	-	·	-	·
	Leaf	3L001	LC497892	*Actinomycetospora corticicola* 014-5^T	99.62	Proline	22	19	18	·	-	·
		3L002	LC497920	*Dactylosporangium sucinum* RY35-23^T	99.58	Proline	-	-	-	·	-	·
Barleria lupulina	Root	7R002	LC497919	*Streptomyces shenzhenensis* 172115^T	99.38	HV	-	-	-	·	-	·

		7R004	LC497918	*Streptomyces shenzhenensis* 172115^T	99.65	Proline	-	-	-	·	-	·
		7R005	LC497917	*Microbispora hainanensis* 211020^T	99.86	Proline	-	-	-	·	-	·
		7R006	LC497916	*Streptomyces graminisoli* JR-19^T	99.93	HV	-	-	-	·	-	·
		7R007	LC497908	*Streptomyces chiangmaiensis* T4A-1^T	98.75	HV	-	-	-	·	-	·
		7R008	LC497915	*Streptomyces graminisoli* JR-19^T	99.51	HV	-	-	-	·	-	·
		7R009	LC497914	*Streptomyces graminisoli* JR-19^T	99.86	HV	-	-	-	·	-	·
		7R011	LC497913	*Streptomyces graminisoli* JR-19^T	99.86	starch	-	-	-	·	-	·
		7R012	LC497912	*Streptomyces graminisoli* JR-19^T	99.79	proline	-	-	-	·	-	·
		7R013	LC497911	*Streptomyces lilacinus* NRRL 1968^T	99.21	proline	15	-	-	·	-	·
		7R014	LC497910	*Streptomyces lusitanus* NBRC 13464^T	99.65	HV	-	-	-	·	-	·
		7R015	LC497906	*Streptomyces neopeptinius* KNF 2047^T	98.64	HV	-	-	-	·	-	·
		7R016	LC497907	*Streptomyces gilvifuscus* KM229362^T	98.21	HV	-	-	-	·	-	·
		7R017	LC500017	*Streptomyces shenzhenensis* 172115^T	99.45	HV	-	-	-	·	-	·
		7R018	LC497909	*Streptomyces shenzhenensis* 172115^T	99.58	HV	-	-	-	·	-	·
Ruellia squarrosa	Root	9R002	LC497898	*Streptomyces parvulus* NBRC 13193^T	99.72	HV	-	-	-	·	-	·
		9R003	LC497897	*Streptomyces chartreusis* NBRC 12753^T	99.31	SCN	9.5	8	7	·	-	·
		9R004	LC497895	*Streptomyces chartreusis* NBRC 12753^T	99.38	proline	8.5	8	-	·	-	·
		9R005	LC497896	*Streptomyces laurentii* ATCC 31255^T	99.31	HV	-	-	-	·	-	·
		9R006	LC497894	*Streptomyces chartreusis* NBRC 12753^T	99.38	proline	-	-	-	·	-	·
		9R008	LC497893	*Streptomyces collinus* NBRC 12759^T	99.93	HV	19	12	14	·	-	·
Andrographis paniculata	Root	6R001	LC497905	*Streptomyces deccanensis* DAS-139^T	99.78	HV	-	-	-	·	-	·
		6R002	LC497904	*Streptomyces hawaiiensis* NBRC 12784^T	99.72	HV	-	-	-	·	-	·
		6R003	LC497903	*Streptomyces shenzhenensis* 172115^T	99.79	HV	-	-	-	·	-	·
		6R004	LC497902	*Streptomyces collinus* NBRC 12759^T	99.93	HV	-	-	-	·	-	·
		6R005	LC497901	*Streptomyces deccanensis* DAS-139^T	99.79	HV	-	-	-	·	-	·
		6R006	LC497900	*Streptomyces indiaensis* NBRC 13964^T	99.29	HV	-	-	-	·	-	·
Clinacanthus rutans	Stem	8S001	LC500016	*Streptomyces cavourensis* NBRC 13026^T	100	HV	-	-	-	·	-	·

Abbreviations: K = *Kocuria rhizophila*; B = *Bacillus subtilis*; S. *Staphylococcus aureus*; P = *Pseudomonas aeruginosa*; E = *Escherichia coli*; C = *Candida albicans*

5.4 Taxonomic study of the selected endophytic actinobacteria

According to the antimicrobial activity screening, strain 3R004 showed good activity against most tested Gram-positive bacteria. Because of this criteria, the strain 3R004 was chosen for furthcuer study.

5.4.1 Phenotypic characteristics of strain 3R004

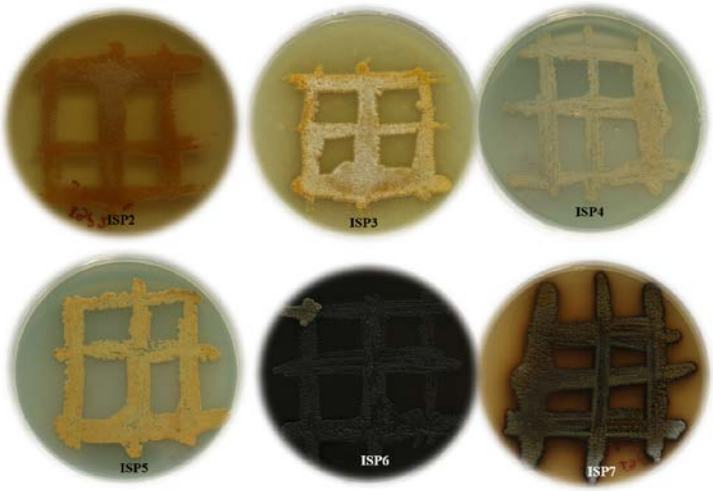
Strain 3R004 grew well on ISP2-ISP7 media. The colony color was vivid yellow to vivid greenish yellow tone onISPi, ISP3, ISP4 and ISP6 media. The olive black colony was observed when the strain grew on ISP6 and ISP7 media. White to light grey aerial mass was observed on ISP2, ISP3 and ISP7 media. The strain produced yellow pigment on ISP2 and ISP3 and ISP5 media. Melanin pigment was obserbed on ISP6 and ISP7 (Fig 5.5a).

The microscopic observation revealted that the strain produced long chain of spore on aerial mycelia. The cylindrical spore shape with smooth surface was observed when using scanning electronmicroscope (Fig 5.5b). Growth was observed at pH 5-9. No growth was observed at 45^{o}C. The maximum NaCl for growth is 7% (w/v).

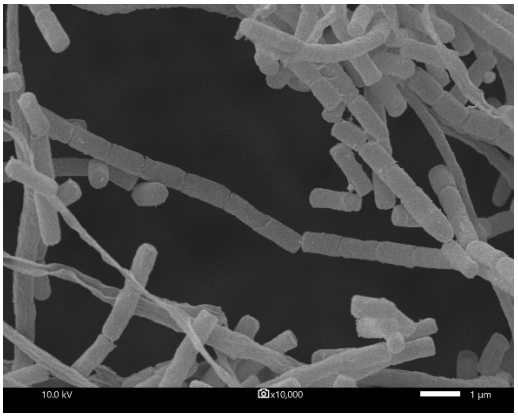
According to chemotaxonomic study, LL-diaminopimelic acid, glucose and ribose was detected in the whole-cell hydrolysate. Based on chemotaxonomic study and morphology, strain 3R004 could be classified in the members of the genus *Streptomyces*.

On the basis of16S rRNA gene analysis, strain 3R004 showed the highest similarity to *Streptomyces cyaneochromogenes* MK-45^{T} with a value of 99.45%. However, the phylogenetic analysis based on neighbor-joining and maximum likelihood using the 16S rRNA gene alone cannot conclude the evolutionary relationship of this strain because of the low bootstrap values in all trees (Fig. 5.6 and 5.7). Because of this reason, the genome of strain 3R004 was sequenced to overcome this problem.

The draft genome of strain 3R004 has the total base of 10,454,561 nucleotides with 64 contigs. Strain 3R004 contained 70.8 mol% of GC content. The summarized data of the draft genome assembly were shown in table 5.2



(a)



(b)

Fig 5.5 (a) Cultural characteristics of strain 3R004 when the culture grown on tested agar media at 30°C for 14 days. (b) The scanning electron micrograph of the strain 3R004 when culture on ISP2 agar at 30°C for 14 days produced the long chain of cylindrical spore on the aerial mycelia

Table 5.2 Summary of sequence reads assembly of strain 3R004

Number of contigs	64
Largest contig	945,532 bp
GC (%)	70.8
N50	449,515
L50	8
Average coverage	150X
Total bases	10,454,561

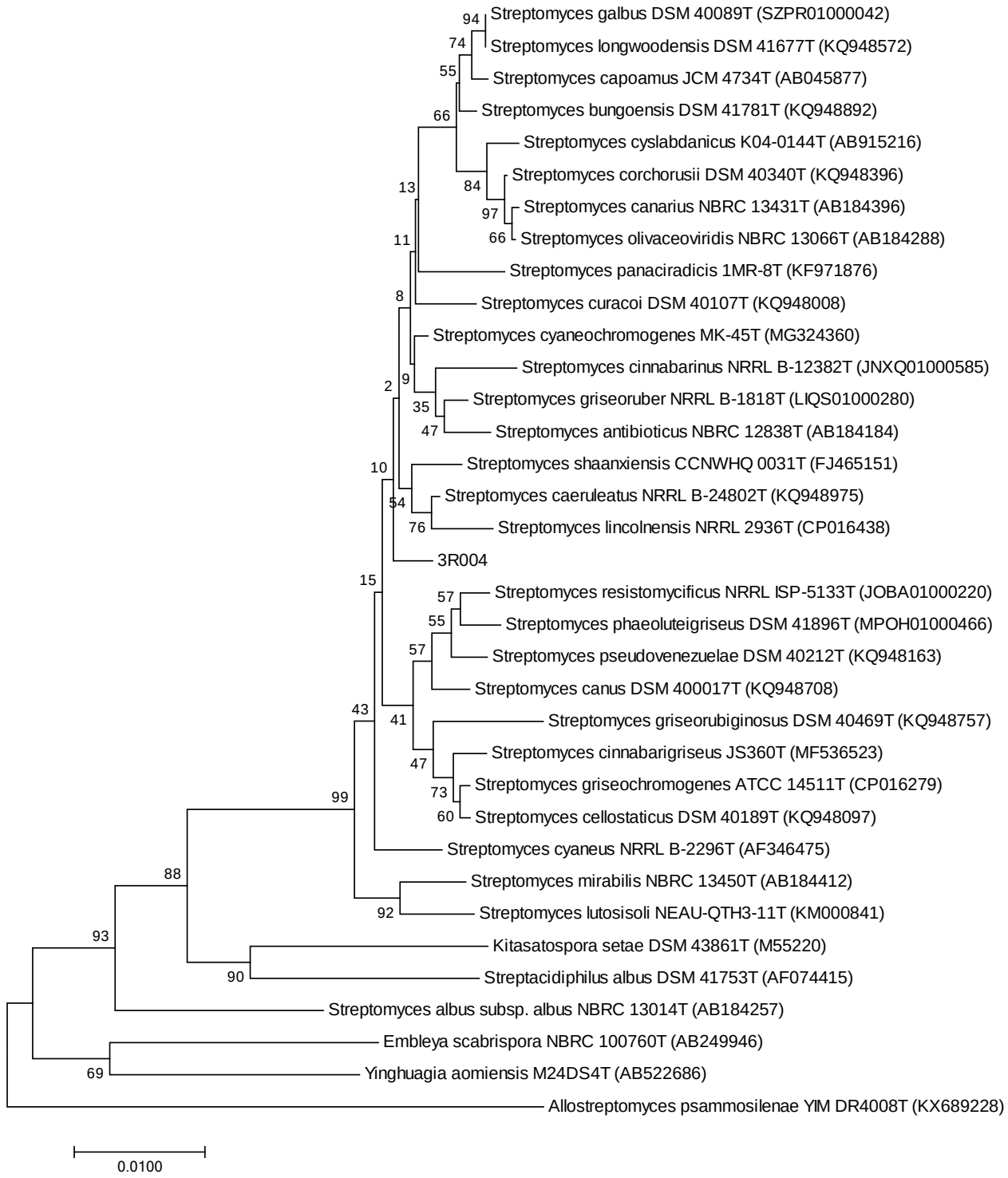


Fig 5.6 Phylogenetic based on neighbor-joining method using 16S rRNA gene sequences of strain 3R004 and related *Streptomyces* species as well as the type species of the family *Streptomycetaceae*. The number at branch nodes indicate bootstrap percentages derived from 1000 replication. Bar, 0.01 substitutions per nucleotide position.

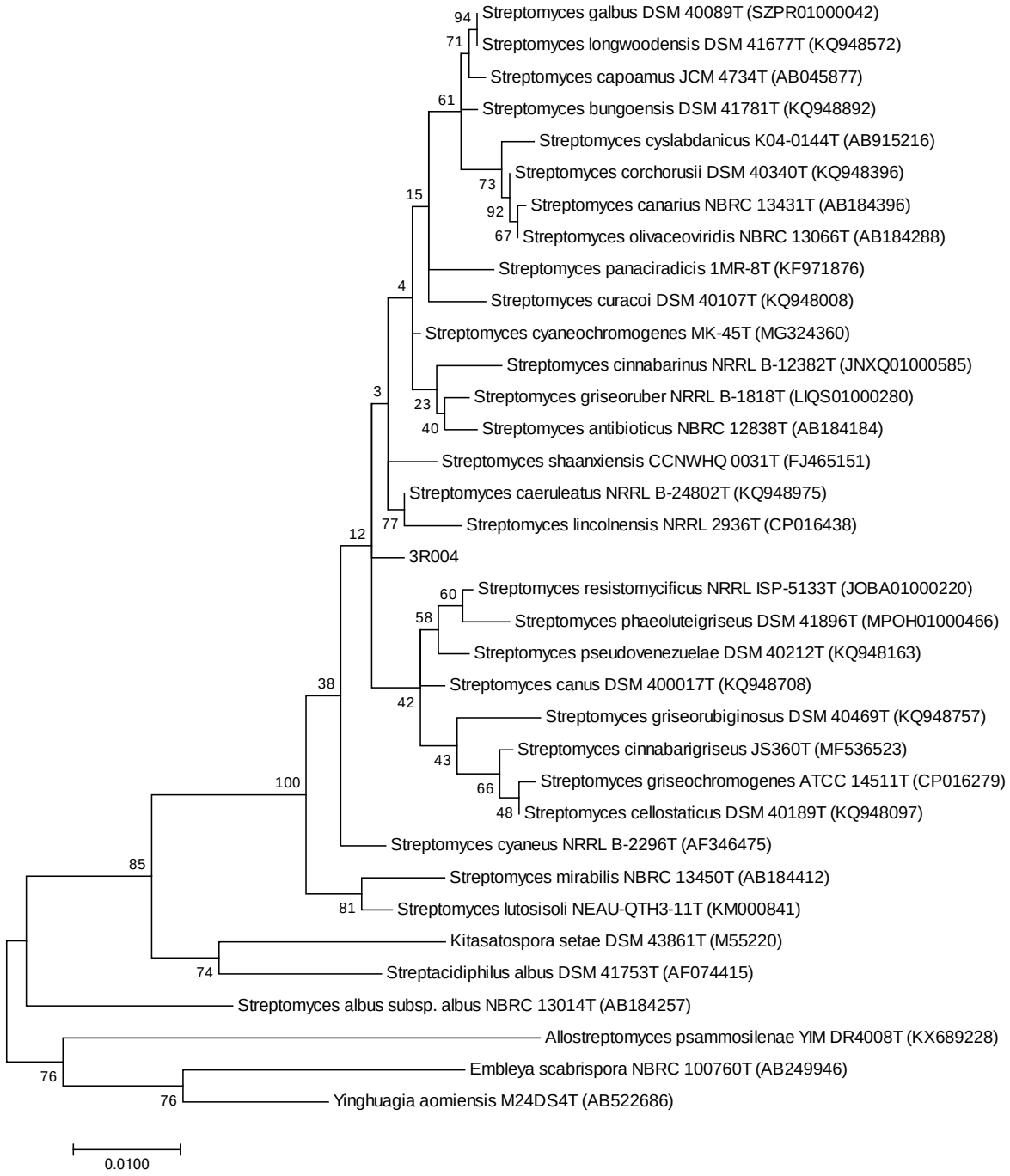


Fig 5.7 Phylogenetic based on maximum-likelihood method using 16S rRNA gene sequences of strain 3R004 and related *Streptomyces* species as well as the type species of the family *Streptomycetaceae*. The number at branch nodes indicate bootstrap percentages derived from 1000 replication. Bar, 0.01 substitutions per nucleotide position.

Based on the phylogenomic analysis, strain 3R004 shared the cluster with *Streptomyces aquilus* GGCR-6^{T} and *Streptomyces antibioticus* DSM 40234^{T} (Fig 5.8). Therefore, these two type strains were selected for further taxonomic comparison with strain 3R004.

In the classical taxonomy, the value of 70% DNA-DNA hybridization is a threshold for the cut off the strain to the same species. In the genomic era, according to Kim et al (2014), an ANI value of 95% is a threshold for cut off level. To confirm the hypothesis that stran 3R004 shold be the new actinobacterial species or not, the digital DNA-DNA hybridization and the average nucleotide identity (ANI) were calculated. The result in table 5.3 shown that both ANI and dDDH values of among close related *Streptomyces* type strain and strain 3R004 was lower than the threshold level. This indicate that strain 3R004 is the candidate of novel actinobacterial speices. However, the type strain of *S. aquilus, S. antibioticus* and *S. cyaneochromogenes* shold be order to confirm the phenotypic difference in the future study.

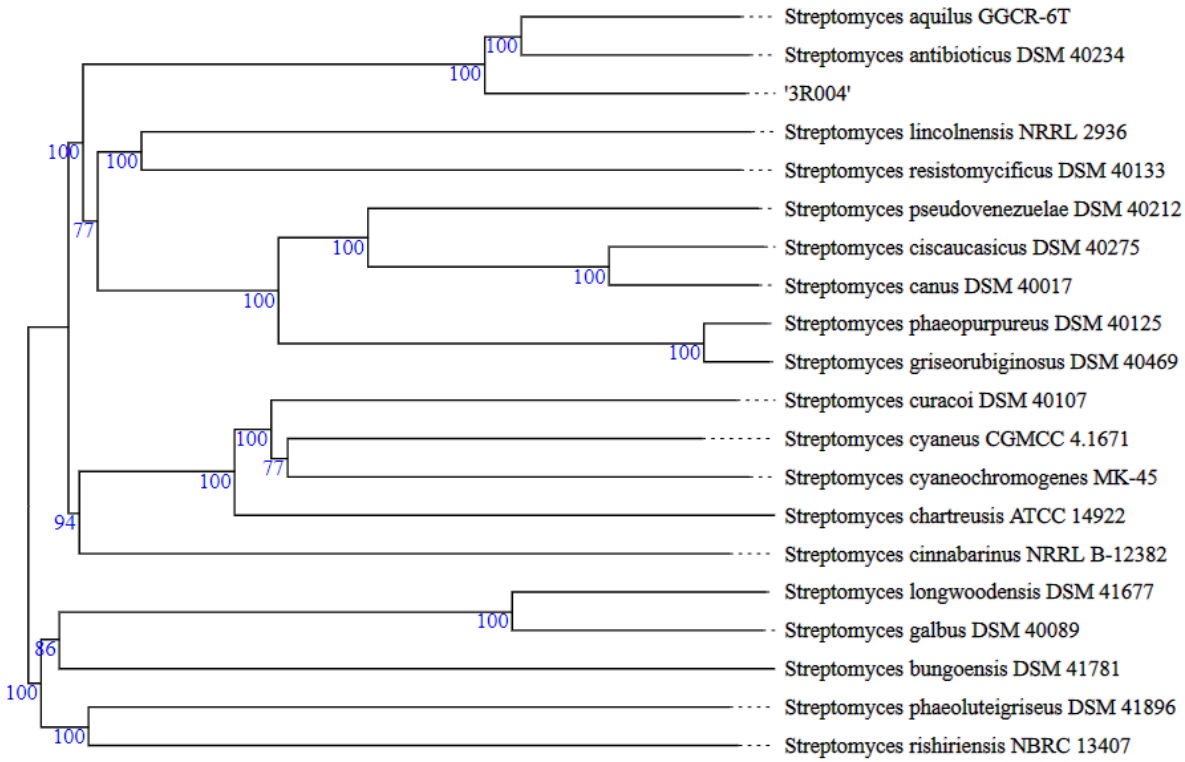


Fig. 5.8 The phylogenomic tree based on the genome of the strain 3R004 and closely related *Streptomyces* type strains.

Table 5.3 ANIb and ANIm values (%) and the digital DNA-DNA hybridization (dDDH) values between the draft genomes of strain CR1-09^T and its closest related type strains

strain	Genome size	Accession number	% GC content	% dDDH	ANIb	ANIm
Streptomyces antibioticus DSM 40232^T	10,133,328	GCA_001514065	70.77	62.1	92.21	93.99
Streptomyces aquilus GGCR-6^T	10,393,987	CP034463	10.85	63.1	92.70	94.29
Streptomyces cyaneochromogenes MK-45^T	10,677,137	CP034539	70.63	35.5	82.67	86.98

5.4.2 Secondary metabolites produced by strain 3R004

A total of 363.9 mg of the deep red solid crude ethyl acetate was obtained from 2.4 L of 14-day culture of ISP2 broth.

5.4.2.1 Antimicrobial activity of the crude extract

The agar disc diffusion was used to determine the antimicrobial activity. The concentration of 2 mg of crude extract/disc was used. The crude extract shown activity against tested-Gram positive bacteria including *Micrococcus luteus*, *Staphylococcus aures* and *Bacillus subtilis*. No activity was observed on Gram-negative bacteria and yeast. This result consistent with the primary screening that the strain 3R004 showed very good activity only Gram-positive bacteria (Table 5.4).

Table 5.4 Antimicrobial activity of the crude extract of strain 3R004

Compound	Inhibition zone (mm)					
	M. luteus	*S. aureus*	*B. subtilis*	*E. coli*	*P. aeruginosa*	*C. albicans*
Crude extract	29	24	25	-	-	-
Chloramphenicol 30 µg	36	30	26	22	nd	nd
Amphotericin B 20 µg	nd	nd	nd	nd	nd	18

The reverse phase HPLC, Agilent 1100 series equipped with 5C18-MS-II COSMOSIL ∅ 4.6 x 150 mm water type, was used to screen the chemical profile in the crude extract. The gradient mode of 50 – 100% of water-acetonitrile, flow rate 1 ml/min with the injection volume of 20 µL, was used in this study. The HPLC profile revealed the main major peak at retention time of 15.4 minute. Compared with the known compound the the in-houaw library, this retention time was same as actinomycin D (Fig 5.9). To confirm the structure of this compound, the bioassay-guided chromatographic fractionation was carried out to isolate this compound. The open column, Sephadex LH-20, 2.5 x 20 cm elueted with 100% methanol was used in this study. The isolation scheme was shown in Fig. 5.10 Finally, 153.4 mg of the compound 1 was obtained.

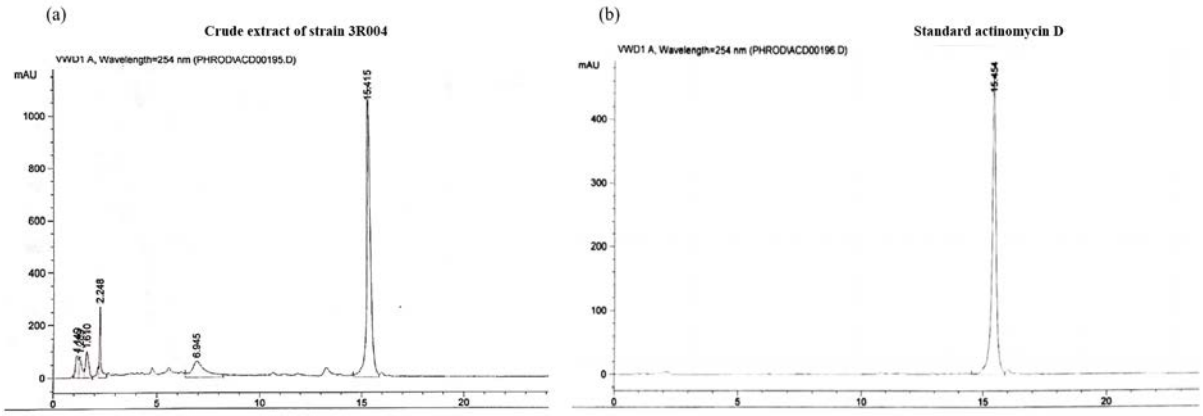


Fig 5.9 HPLC chromatrogram of the crude extract of strain 3R004 (a) and actinomycin D standard (b)

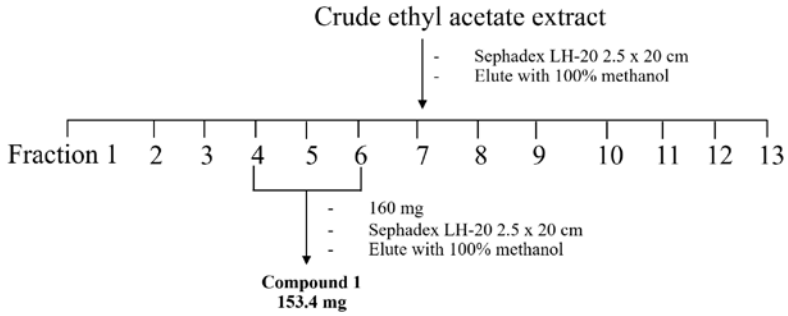


Fig. 5.10 Isolation scheme of the compound 1

Compound 1 was a red amorphous powder. The HRESIMS m/z $[M+ Na]^+$ showed the molecular ion of 1277.6176 (cal for $C_{62}H_{86}N_{12}O_{16}Na$). The H^1-NMR and C^{13}-NMR of the compound 1 shown the consistent spectrum to the known actinomycin D (Appendix A). Based

on HPLC chromatogram, H^1-NMR, C^{13}-NMR, and mass spectrometry, the compound was identified as actinomycin D (Fig 5.11).

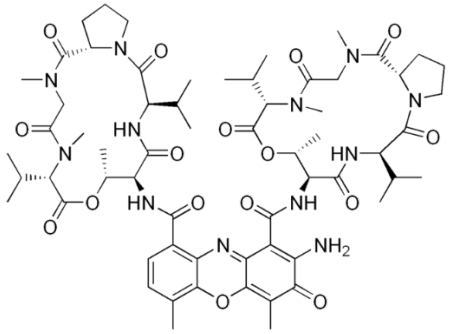


Fig 5.11 The chemical structure of actinomycin D, the bioactive compound produced by the strain 3R004

5.4.2.2 Secondary metabolite biosynthesis gene cluster of strain 3R004

The biosynthesis gene cluster in the genome of strain 3R004 was determined using antiSMASH version 5.1.2 server. BLAST result of known cluster was summarized in Table 5.5. The presence of actinomycin D biosynthesis gene cluster in the genome of strain 3R004 support the ability to produced actinomycin D of the strain 3R004.

Table 5.5 BLAST result of the known biosynthesis gene cluster of strain 3R004. Only the cluster that shown similarity value more than 80% was shown.

Type	Most similar known cluster	Similarity
Siderophore	Desferrioxamin B/ desferrioxamine E	83%
NRPS, melanin	Melanin	80%
NRPS, NRPS-like, T1PKS	coelichelin	100%
T2PKS	setomimycin	93%
Terpene	Albaflavenone	100%
Ectoine	Ectoine	100%
Terpene	Geosmin	100%
Terpene	hopene	92%
NRPS	Actinomycin D	89%
T2PKS	Spore pigment	83%
Lanthipeptide, bacteriocin	Informatipeptin	100%

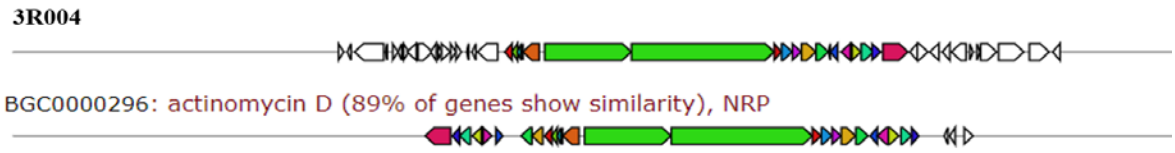


Fig 5.12 Actinomycin D biosynthesis gene cluster of strain 3R004 and known actinomycin D biosynthesis gene cluster. BGC0000296 is the actinomycin D biosynthetic gene cluster from *Streptomyces anulatus*.

5.4.2.3 Biological activity of the actinomycin D

The biological activity of actinomycin D were determined by Bioassay Labolaroty, Microbial Biotechnology and Biochemicals research unit (BIOTEC). Based on this study, actinomycin D exhibited antimicrobial activity against *Mycobacterium tuberculosis* and *Bacillus cereus* at the MIC value of 12.5 and 1.56 µg/ml, respectively. The antimicrobial activity against Gram-negative bacteria and yeast was not observed in this study. However, the antigram-negative bacterial activity of was observed when test actinomycin D with *Acenetobacter baumanii* in the presence of phenylalanine-arginine-β-naphtylamide (PaβN), the efflux pump inhibitor. Antimalarial activity against *Plasmodium falciparum* was observed at the IC_{50} of 0.015 µg/ml. No antifungal activity and antiviral activity were observed in all tested in this study.

Actinomycin D shown the good activity against lung cancer cell line including NCI-H187 (lung cancer cell line), KB (oral caviry cancer cell line), and MCF-7 (breast cancer cell line) with IC_{50} of 0.7 ng/ml, 18.4 ng/ml and 22.0 µg/ml, respectively. The biological activities were summarized in table 5.6

Table 5.6 Biological activity of the actinomycin D

Compound	Antimicrobial activity						Antimalaria	Anti-plant pathogenic fungi			Cytotoxicity				Antivirus	
	M. Tuberculosis MIC(μg/ml)	*B. cereus* MIC (μg/ml)	*C. albicans* IC_{50}	*A. baumannii* MIC_{90} (μg/ml)	*A. Baumannii* + PAβN MIC_{90}(μg/ml)	*P. aeruginosa* IC_{50} (μg/ml)	*Pl. falciparum* IC_{50} (μg/ml)	*Cur. lunata* MIC_{90} (μg/ml)	*Magnaporthe grisea* MIC (μg/ml)	*Alternaria brassicicola* MIC (μg/ml)	Vero cell IC_{50} (μg/ml)	NCI-H187 (lung cancer) IC_{50}(μg/ml)	KB (oral cavity cancer) IC_{50}(μg/ml)	MCF7 (breast cancer) IC_{50}(μg/ml)	Anti HSV-1 IC_{50}(μg/ml)	Neuramidinase inhibitor IC_{50}(μg/ml)
Actinomycin D	12.5	1.56	-	-	6.25	-	0.0148	-	-	-	0.125	0.000694	0.0184	21.94	-	-
Rifampicin	0.025	nd	nd	3.13	0.19	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Streptomycin	0.625	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Isoniazid	0.047	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Ofloxacin	0.781	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Ethambutol	1.88	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Vancomycin	nd	2.00	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Amphotericin B	nd	nd	0.0946	nd	nd	nd	nd	3.13	3.13	3.13	nd	nd	nd	nd	nd	nd
Erythromycin	nd	nd	nd	12.5	0.78	>32.0	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Dihydroartemisinine	nd	nd	nd	nd	nd	nd	3.46 nM	nd	nd	nd	nd	nd	nd	nd	nd	nd
Mefloquine	nd	nd	nd	nd	nd	nd	70.68 nM	nd	nd	nd	nd	nd	nd	nd	nd	nd
Chlororamphenicol	nd	nd	nd	nd	nd	>8	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Ellipticine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.959	2.95	2.81	nd	nd	nd
Doxorubicin	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.1333	1.35	8.98	nd	nd
Tamoxifen	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	7.91	nd	nd
Acyclovir	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	4.67	nd
Oseltamivir carboxylate	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.625nM

5.4.2.3 Production of the actinomycin D by strain 3R004

To find out the suitable production media for actinomycin D, strain 3R004 was culture in four different production media including 54 broth, ISP2 broth, ISP3 broth and 301 broth in shaking condition at 180 rpm 30°C for 14 days. The culture broth of each media was sampling every 24 hour until the end of fermentation. The actinomycin D in culture broth was analyzed using reverse phase HPLC. The cell ans solid particle in sample was eliminated by filtration (nylon, ∅ 0.45 µm). The standard curve was constructed using pure actinomycin D perchased from Sisco Research Laboratories PVt, Ltd (SRL). Three independent experiments were carried out for this experiment.

The results demonstrated that among four different production media, the 54 medium is the best production medium for strain 3R004 to produce the actinomycin D. The maximum yield of actinomycin D, 378.3 mg/L, was observed after culture the strain in 54 medium for 12 days. After the maximum production the actinomycin D in culture broth trend to dramatically decreasing (Fig 5.13).

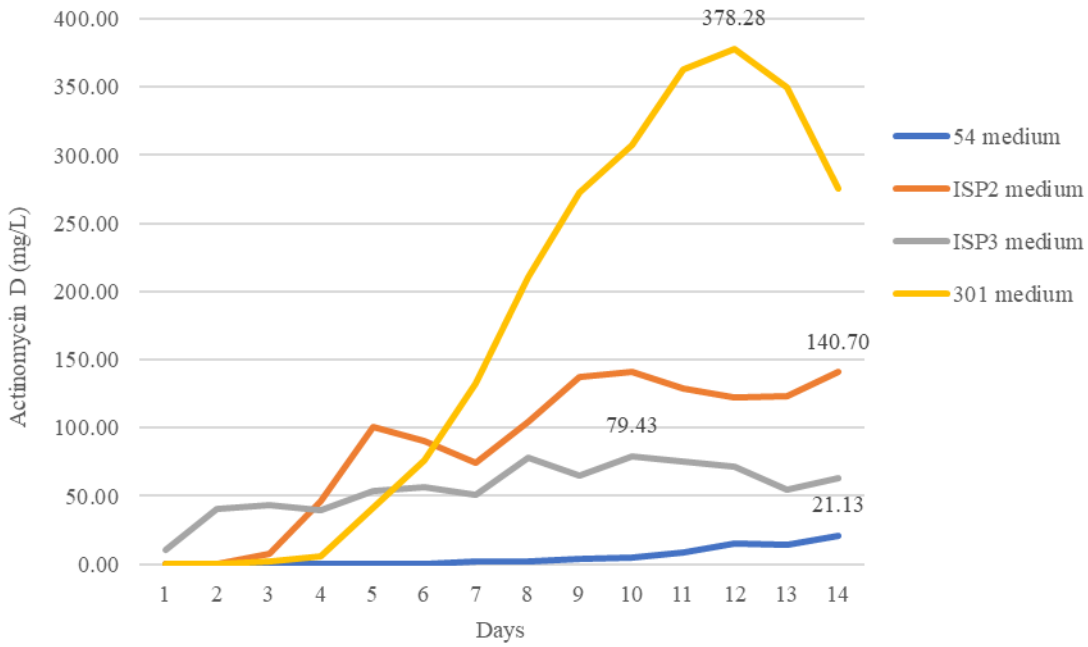


Fig. 5.13 The time course production of actinomycin D by strain 3R004.

6. Conclusion and Discussion

This study revealed that Acanthaceae plants are the good source of actinobacterial isolation which can be used for further study.

7. Appendix

Accepted Manuscript

Phongsopitanun W., Sripreechasak P., Rueangsawang K., Panyawut R., Pittayakhajonwut P., Tanasupawat S., (2020). Diversity and antimicrobial activity of culturable endophytic actinobacteria associated with *Acanthaceae* plants. Science Asia, (accepted manuscript)

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<u>รายงานสรุปการนำผลงานวิจัยไปใช้ประโยชน์</u>

สัญญาเลขที่ MRG6180011 **ชื่อโครงการ** อนุกรมวิธานและสารเมแทบอไลต์ทุติยภูมิของเอนโดไฟติกแอคติโนแบคทีเรียที่คัดแยกได้จากพืชวงศ์ Acanthaceae ในประเทศไทย

หัวหน้าโครงการ...ดร. วงศกร พงศ์โสภิตานันท์.....**หน่วยงาน** คณะวิทยาศาสตร์ มหาวิทยาลัยรามคำแหง

โทรศัพท์ 0892529781 **โทรสาร**.....**อีเมล์** Wongsakorn_P@outlook.com

สถานะผลงาน ☑ **ปกปิด** □ **ไม่ปกปิด**

ความสำคัญ / ความเป็นมา

"จุลินทรีย์" โดยเฉพาะอย่างยิ่งแอคติโนแบคทีเรีย เป็นแหล่งของสารออกฤทธิ์ทางชีวภาพขั้นต้นที่ช่วยในการขับเคลื่อน กระบวนการค้นพบยา ในศตวรรษที่ผ่านมาแอคติโนแบคทีเรียจำนวนมากได้ถูกคัดแยกจากดินและได้ถูกใช้ในการผลิตยาที่ สำคัญหลายชนิด อย่างไรก็ตามในปัจจุบันการค้นพบสารต้นแบบที่จะนำมาพัฒนาเป็นยาใหม่มีแนวโน้มลดลงอย่างต่อเนื่องทั้งนี้ เนื่องจากการคัดแยกจุลินทรีย์จากแหล่งที่อยู่เดิม ดังนั้นการค้นหาแหล่งที่อยู่ใหม่ของจุลินทรีย์ที่เป็นผู้ผลิตสารออกฤทธิ์ทาง ชีวภาพจึงเป็นสิ่งสำคัญ "เอนโดไฟต์" คือจุลินทรีย์ที่อาศัยอยู่ในเนื้อเยื่อพืชโดยไม่ก่อให้เกิดโทษกับพืช และมีการศึกษาพบว่า จุลินทรีย์กลุ่มนี้มีศักยภาพในการผลิตสารชนิดใหม่ที่สามารถนำไปประยุกต์ใช้งานได้หลากหลาย ต้นไม้ในวงศ์ Acanthaceae ได้ ถูกใช้เป็นยาพื้นบ้านของประเทศไทยมาอย่างช้านาน ในประเทศไทยมีรายงานว่ามีพืชกลุ่มนี้อยู่ถึง 40 สกุล และมีความ หลากหลายประมาณ 200 สปีชีส์ อย่างไรก็ตามการศึกษาแอคติโนแบคทีเรียที่สัมพันธ์กับพืชกลุ่มนี้ยังมีอยู่น้อยมากจึงเป็นที่มา ของโครงการนี้

วัตถุประสงค์ของโครงการ

1. เพื่อคัดแยกแอคติโนแบคทีเรียจากพืชวงศ์ Acanthaceae
2. เพื่อระบุชนิดและศึกษาอนุกรมวิธานของเชื้อไอโซเลตที่ถูกคัดเลือก
3. เพื่อคัดกรอง คัดแยก และระบุโครงสร้างสารออกฤทธิ์ทางชีวภาพของเชื้อสายพันธุ์ที่ถูกคัดเลือก

ผลการวิจัย

ในการศึกษานี้สามารถคัดแยกแอคติโนแบคทีเรียได้ 52 ไอโซเลต จากตัวอย่างใบ ลำต้น และรากของ พืชในวงศ์ Acanthaceae ที่แตกต่างกันจำนวน 6 สปีชีส์ จากผล BLAST และการวิเคราะห์ความสัมพันธ์ทางวิวัฒนาการพบว่าแอคติโนแบคทีเรียที่คัดแยกได้นี้สามารถจำแนกได้เป็น 4 วงศ์ คือ *Nocardiaceae*, *Micromonosporaceae*, *Streptosporangiaceae* และ *Streptomycestaceae* และสามารถจำแนกในระดับสกุลได้เป็น 6 สกุล คือ *Actinomycetospora* (1 ไอโซเลต), *Dactylosporangium* (1 ไอโซเลต), *Nocardia* (3 ไอโซเลต), *Microbispora* (5 ไอโซเลต), *Micromonospora* (10 ไอโซเลต) and *Streptomyces* (32 ไอโซเลต) จากผลการคัดกรองฤทธิ์ต้านจุลชีพพบว่าแอคติโนแบคทีเรีย จำนวน 8 ไอโซเลต คือ *Actinomycetospora* จำนวน 1 ไอโซเลต และ *Streptomyces* จำนวน 7 ไอโซเลต สามารถยับยั้งจุลินทรีย์ที่ใช้ในการทดสอบได้ ในการศึกษานี้ไอโซเลต 3R004 ซึ่งแสดงฤทธิ์ต้านจุลชีพที่ดีที่สุดได้ถูกคัดเลือกเพื่อใช้ในการศึกษาต่อ จากการวิเคราะห์ความสัมพันธ์ทางวิวัฒนาการโดยใช้จีโนมพบว่าไอโซเลต 3R004 มีความสัมพันธ์ใกล้เคียงกับ *Streptomyces antibioticus* และ *Streptomyces auilus* จากการศึกษา average nucleotide identity และ DNA-DNA hybridization พบว่าจีโนมของไอโซเลต 3R004 แตกต่างจากสายพันธุ์ใกล้เคียงดังนั้นจึงสามารถสรุปได้ว่าไอโซเลต 3R004 มีความเป็นไปได้สูงที่จะเป็นแอคติโนแบคทีเรียสายพันธุ์ใหม่ จากผลการศึกษาสารเมแทบอไลต์ทุติยภูมิโดยการวิเคราะห์จีโนมและคัดแยกสารโดยวิธีทางเคมีพบว่าสารออกฤทธิ์ทางชีวภาพของไอโซเลต 3R004 คือ actinomycin D และพบว่าเมื่อเลี้ยงเชื้อในอาหาร 54 medium จะได้ผลผลิตของ actinomycin D สูงสุดที่ 378.3 mg/L ที่ระยะเวลา 12 วัน และจากผลการศึกษาในงานวิจัยนี้พบว่า actinomycin D ออก

ฤทธิ์ในการฆ่าเชื้อแบคทีเรียแกรมบวก *Bacillus cereus* และสามารถฆ่าแบคทีเรียแกรมลบ *Acenitobacter baumannii* ในสภาวะที่มีการเติม efflux inhibitor นอกจากนี้ยังพบฤทธิ์ยับยั้งเซลล์มะเร็ง คือ KB, MCF-7 and NCI-H187 ดังนั้นจึงอาจสรุปได้ว่าพืชในวงศ์ Acanthaceae เป็นแหล่งที่อยู่ที่สำคัญของแอคติโนแบคทีเรียและแอคติโนแบคทีเรียที่คัดแยกได้จากพืชวงศ์นี้สามารถนำไปใช้ต่อยอดทางเภสัชกรรมต่อไปในอนาคตได้

คำสืบค้น (Keywords)

Endophytic actinobacteria, Actinomycin D, Novel actinobacterial species, Bioactive secondary metabolites

การนำผลงานวิจัยไปใช้ประโยชน์ *(ดูคำจำกัดความ และตัวอย่างด้านหลังแบบฟอร์ม)*

☐ **ด้านนโยบาย โดยใคร** *(กรุณาให้ข้อมูลเจาะจง)*.....

มีการนำไปใช้อย่างไร

.....

☐ **ด้านสาธารณะ โดยใคร** *(กรุณาให้ข้อมูลเจาะจง)*

มีการนำไปใช้อย่างไร

.....

☐**ด้านชุมชนและพื้นที่ โดยใคร***(กรุณาให้ข้อมูลเจาะจง)*

มีการนำไปใช้อย่างไร

.....

☑ **ด้านพาณิชย์ โดยใคร** ศูนย์เก็บรักษาสายพันธุ์จุลินทรีย์

มีการนำไปใช้อย่างไร

สามารถจำหน่าย Type strain ได้

☑ **ด้านวิชาการ** **โดยใคร** นักอนุกรมวิธาน นักจุลชีววิทยา นักเคมี ฯลฯ

มีการนำไปใช้อย่างไร

นักวิทยาศาสตร์ที่ต้องการนำเชื้อสายพันธุ์ใหม่ที่ค้นพบไปใช้ประโยชน์สามารถนำเชื้อไปต่อยอดได้โดยผ่านศูนย์เก็บเชื้อมาตรฐาน

☐ **ยังไม่มีการนำไปใช้** (โปรดกรอกในกรอบถัดไป)

(กรณีที่ยังไม่มีการใช้ประโยชน์) **ผลงานวิจัยมีศักยภาพในการนำไปใช้ประโยชน์**

☐ **ด้านนโยบาย** ☐ **ด้านสาธารณะ** ☐ **ด้านชุมชนและพื้นที่** ☐ **ด้านพาณิชย์** ☐ **ด้านวิชาการ**

ข้อเสนอแนะเพื่อให้ผลงานถูกนำไปใช้ประโยชน์

.....

.....

การเผยแพร่/ประชาสัมพันธ์ *(กรุณาให้รายละเอียด พร้อมแนบหลักฐาน)*

1. สิ่งพิมพ์ หรือสื่อทั่วไป

   ☐ **หนังสือพิมพ์** ☑ **วารสาร** ☐ **โทรทัศน์** ☐ **วิทยุ** ☐ **เว็บไซต์** ☐ **คู่มือ/แผ่นพับ** ☐ **จัดประชุม/อบรม** ☐ **อื่น ๆ**

   .....

   .....

2. สิ่งพิมพ์ทางวิชาการ

   Phongsopitanun W., Sripreechasak P., Rueangsawang K., Panyawut R., Pittayakhajonwut P., Tanasupawat S., (2020). Diversity and antimicrobial activity of culturable endophytic actinobacteria associated with *Acanthaceae* plants. Science Asia, (accepted manuscript)

Diversity and antimicrobial activity of culturable endophytic actinobacteria associated with Acanthaceae plants

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Abstract. In this study, a total of 52 endophytic actinobacteria were isolated from six species of Acanthaceae plants collected in Thailand. Most actinobacteria were obtained from the root part. Based on 16S rRNA gene analysis and phylogenetic tree these actinobacteria were classified into 4 family (*Nocardiaceae*, *Micromonosporaceae*, *Streptosporangiaceae*, and *Streptomycetaceae*) and 6 genera including *Actinomycetospora* (1 isolate), *Dactylosporangium* (1 isolate), *Nocardia* (3 isolates), *Microbispora* (5 isolates), *Micromonospora* (10 isolates) and *Streptomyces* (32 isolates). The result of antimicrobial activity screening indicated that eight isolates, including one *Actinomycetospora* and seven *Streptomyces*, exhibited antimicrobial activity against tested microorganisms. In addition, the selected *Streptomyces* sp. 5R010 showed antagonistic activity against fungal plant pathogens including *Fusarium* sp., *Colletotrichum* sp. and *Sclerotium* sp. Therefore, this study demonstrated that the Acanthaceae plant species harbored the endophytic actinobacteria which can be used for the source of the antimicrobial compound.

Keyword: Endophytic actinobacteria; antimicrobial activity; Acanthaceae; phytopathogenic fungi

INTRODUCTION

Microorganisms, especially actinobacteria, are the primary source of the bioactive natural products which is driving drug discovery[1]. In the past century, numerous actinobacteria have been isolated from soil and used as the producer of key drugs such as actinomycin, avermectin, erythromycin, gentamicin, neomycin, platensimycin, streptomycin, vancomycin, etc. Although many drugs are developed from the actinobacteria, the discovery of novel lead compounds has decreased because of the redundancy of the samples. Consequently, it is extremely necessary to investigate the untapped microorganisms to drive natural product research.

Actinobacteria are well known to contain valuable economically important microorganisms for a long time because of their ability to produce a large number of bioactive secondary metabolites[2]. Actinobacteria are one of the major soil microbiota. However, they are widely distributed in other various environments such as marine sediment, freshwater, insects, and plants. In the past decade, the untapped habitats, especially endophytic, have become a promising source of novel actinobacteria[3].

Endophytes are the microorganisms that spend at least parts of their life cycle inside the plant tissues without having a negative impact on the host plants[4]. These microbes, especially actinobacteria, have a massive potential to produce a number of novel compounds that find wide-range application as agrochemicals, antibiotics, immunosuppressants, antiparasitics, and anticancer agents[5]. A huge diversity of secondary metabolites of actinobacteria may occur because of the natural adaptation to the environments[6]. Recently, many of novel actinobacteria such as *Asanoa endophytica*, *Phytoactinopolyspora endophytica*, *Phytohabitans kaempferiae* and *Streptomyces oryzae,* have been isolated from various plants species[7, 8, 9, 10]

Acanthaceae is a family of dicotyledonous flowering plants containing approximately 210 genera and nearly 4,000 species. These plants are widely distributed in tropical and subtropical regions[11]. At present, many plant species in this family, for example, *Andrographis paniculata*, *Barlelia lupulina*, *Clinacanthus nurans*, *Thunbergia laurifolia,* etc., have been used for Thai traditional medicines. However, the actinobacteria associated with this plant family are rarely reported. Therefore, the objectives of this study were to study the diversity of endophytic actinobacteria associated with the Acanthaceae plant and to screen the antimicrobial activity of the actinobacterial isolates

MATERIAL AND METHOD

Plant collections and isolation of actinobacteria

Plant samples were collected and planted in the botanical garden of the Department of Biology, Faculty of Science, Ramkhamhaeng University prior to isolation. In this study, six species of plants in the Family Acanthaceae including *Andrographis paniculata, Asystasia gangetica, Berleria lupulina, Clinacanthus nutans, Justicia subcoriacea* and *Ruellia squarrosa*, were collected.

Actinobacteria were isolated from leaves, stems, and roots of each plant sample. Plant samples were washed to remove soil from the samples. The three-step surface sterilization was used to eliminate the surface microbes. Briefly, a 5-min wash in 3% NaOCl, followed by a 1-min wash in 95% ethanol and a final wash with a sterile distilled water 2 times. 0.5 gram of the surface-sterilized materials was aseptically ground with 5 ml of extraction solution[12]. Then, 0.1 mL of plant suspension was spread on humic acid-vitamin agar[13], starch casein nitrate agar[14], proline agar[15] supplemented with nalidixic acid (25 mgL^{-1}) and cycloheximide (50 mgL^{-1}) to control the growth of Gram-negative bacteria and fungi, respectively. The plates were incubated at 30 ^{o}C for 14 days. The colonies of actinobacteria were collected and purified on ISP2 medium.

Identification of actinobacteria

The identification of actinobacteria was performed by 16S rRNA gene analysis. The genomic DNA of actinobacteria was extracted from the mycelia grown in yeast-dextrose broth (1 g glucose; 1 g yeast extract; 100 ml water, pH 7.0-7.2) at 30 ^{o}C for 3-7 days[16]. The amplification was carried out using standard primers (5´-GAGTTTGATCCTGGCTCAG-3´) and 1530R (5´-GTTACCTTGTTACGACTT-3´) with the initial incubation of 3 min at 94 °C followed by 30 cycles of 1 min at 94 °C, 1 min at 50 °C and 2 min at 72 °C, followed by a 3 min final extension at 72 °C[17, 18]. The nucleotide of the PCR product was sequenced using the sequencing service (Macrogen, Korea). The nucleotide sequence was manually analyzed using BioEdit software (Ibis Biosciences). BLAST was determined using the EzbioCloud database[19]. Phylogenetic analysis was constructed using MEGA 7.0 software[20]. The tree topology was evaluated using the bootstrap test[21].

Antimicrobial activity screening

Antimicrobial activity of actinobacterial isolates was determined using the agar disc diffusion method. Briefly, each actinobacterium was cultured in ISP2 broth pH 7.0 in shaking condition at 180 rpm 30 ^{o}C for 14 days. Then, one volume (equivalent to culture broth volume) of 95% ethanol was added and shook at 180 rpm for 1 hour followed by centrifuge at 4500 rpm for 10 min. The supernatant was collected and preserved at -20 ^{o}C. To

prepare the tested disc, the sterile paper disc was dipped into each broth library and air-dried in the biosafety cabinet. The sterile ISP2 broth added with one volume of ethanol was used as the negative control.

Six microorganisms including three Gram-positive bacteria, *Staphylococcus aureus, Bacillus subtilis* and *Kocuria rhizophila*, and two Gram-negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa*, and one yeast, *Candida albicans*, were used as the tested microorganisms. The tested bacteria and yeast were activated on Mueller-Hinton agar (MHA) and sabouraud dextrose agar (SDA) for 27 hr at 37 ºC and 30 ºC, respectively. To prepare a microbial suspension, the turbidity of each tested microorganism in normal saline solution was adjusted to 0.5 McFarland standards. Then, the tested bacteria and yeast were swabbed on the surface of MHA and SDA, respectively. The prepared paper disc was put on the surface of media swabbed with the tested microorganisms and incubated at 37 ºC and 30 ºC 24 hr for bacteria and yeast, respectively. The inhibition zone was observed and documented.

Antagonistic activity against phytopathogenic fungi activity of the selected strain

The co-cultivation method was used to determine the antagonistic activity of the selected actinobacteria against six phytopathogenic fungi including *Colletotrichum gloeosporioides, Colletotrichum* sp., *Curvularia oryzae, Fusarium* sp., *Lasiodiplodia theobromae* and *Sclerotium* sp.

The selected actinobacterium was cultured on one side of the ISP2 agar plate and incubated at 30 ºC for 7 days. Then, the 7 day-olds of the tested phytopathogenic fungi grown on SDA agar were cut by the cork borer (6 mm in diameter) and transferred to the opposite of the prepared actinobacterium plate and incubated at 30 ºC for 7-10 days. The inhibition zone around the actinobacterial colony indicated fungal inhibition. The fungi grown on ISP2 agar without actinobacteria were used as the growth control of the fungi.

RESULT AND DISCUSSION

Diversity of actinobacteria

In this study, 52 actinobacteria were isolated from leaves, stems and roots of six species of Acanthaceae plants. In this number, 49 isolates were obtained from roots followed by 2 and 1 isolate were obtained from leaves and stem, respectively. The results of this study are similar to previous studies showing that nearly all the plants harbor endophytes[22]. Janso and Carter[23] discussed that actinobacteria could be isolated from every tissue type of samples; however, root and bark had the highest isolate-to-sample ratio.

On the basis of BALST result and phylogenetic tree analysis, actinobacteria obtained in this study were identified and categorized into 4 families (*Nocardiaceae*, *Micromonosporaceae*, *Streptosporangiaceae*, and *Streptomycetaceae*) and 6 genera including *Actinomycetospora* (1 isolate), *Dactylosporangium* (1 isolate), *Nocardia* (3 isolates), *Microbispora* (5 isolates), *Micromonospora* (10 isolates) and *Streptomyces* (32 isolates) (Fig. 1; Fig. 2 ;Table 1). Based on this study, the most abundant genus found in *Acanthaceae* plants were *Streptomyces* (61%) followed by *Micromonospora* (19%) and *Microbispora* (10%) (Fig. 1). The pattern of the diversity of culturable actinobacteria of this study, which *Streptomyces* are the predominant species, is similar to the previous report[24]. In 2012, Kim et al[25] isolated 61 endophytic actinobacteria, comprised 15 genera including *Streptomyces*, *Micromonospora*, *Rhodococcus*, *Microbispora*, *Micrococcus*, *Microbacterium*, *Streptacidiphilus*, *Arthrobacter*, *Dietzia*, *Kitasatospora*, *Herbiconiux*, *Mycobacterium*, *Nocardia*, *Rathayibacter* and *Tsukamurella*, from the native herbaceous plant species of Korea. In that study, they found that members of the genus *Streptomyces* comprised 45.9% of the total isolates and was followed by *Micromonospora* (18.8%). In the study of Janso and Carter[23], 123 isolates of endophytic actinobacteria, including 17 genera, were isolated from the tropical native plants in Papus New Guinea and Mborokua Island, Solomon Island. The community of endophytic actinobacteria may be varied according to the host plant. Jiang et al[26] isolated 101 endophytic actinobacteria from five different mangrove plants including *Avicennia marina*, *Aegiceras corniculatum*, *Kandelia obovota*, *Bruguiera gymnorrhiza*, and *Thespesia populnea*. Based on 16S rRNA gene these actinobacteria distributed in 15 families and 28 genera including *Actinoplanes, Agrococcus, Amnibacterium, Brachybacterium, Brevibacterium, Citricoccus, Curtobacterium, Dermacoccus, Glutamicibacter, Gordonia, Isoptericola, Janibacter, Kineococcus, Kocuria, Kytococcus, Leucobacter, Marmoricola, Micrococcus, Microbacterium, Micromonospora, Mycobacterium, Nocardioides, Nocardia, Nocardiopsis, Pseudokineococcus, Sanguibacter, Streptomyces,* and *Verrucosispora*. In addition, Widiantini and Franco[27] reported that the dominant endophytic actinobacteria species isolated from rice plants of Australia is *Microbispora*. The variable of endophytic actinobacterial species in the different plants may depending on factors such as host specificity, stage of the host, type of sample, geographical condition, season, surface sterilant, culture condition and selective media[28, 29]

Antimicrobial activity

In this study 8 isolates, including one *Actinomycetospora* and seven *Streptomyces*, exhibited antimicrobial activity against tested microorganisms. Most of the active isolates showed antimicrobial activity

against Gram-positive bacteria but no activity was observed against Gram-negative bacteria (Fig. 3; Table 1). The antimicrobial activity of endophytic *Streptomyces* against Gram-positive bacteria has been documented in previous studies. Zhang et al.[30] study of antimicrobial activity of 65 endophytic actinobacteria, isolated from *Achyranthes bidentata, Paeonia lactiflora, Radix Platycodi and Artemisiae argyi*, against *penicillin resistant Staphylococcus aureus.* They found that 12 strains, the majority were *Streptomyces* spp., showed activity against this pathogen. Although no actinobacteria obtained from this study showed antiGram-negative bacterial activity. Mingma et al[31] isolated 317 actinobacteria from root and rhizospheric soils of leguminous plants and 64 of the isolates (20.2%) showed antagonistic activity against soybean pathogen *Xanthomonas campestris* pv. glycine. In addition, 21 endophytic actinobacteria isolated by Jiang et al[26] showed activity against *P. aeruginosa*. This evidence showed that antiGram-negative bacteria could be observed in some endophytic actinobacteria.

The production of novel antimicrobial metabolites from endophytic actinobacteria has been documented in the various reports. The example is maklamicin, misamycin and diastaphenazine.

Maklamicin, a new spirotetronate-class polyketide, isolated from *Micromonospora* sp. GMKU326, the endophytic actinobacteria isolated from root nodule of the legume *Lupinus angustifolius*, showed strong to moderate antimicrobial activity against Gram-positive bacteria including *Micrococcus luteus, Bacillus subtilis, B. cereus, Staphylococcus aureus* and *Enterococcus faecalis* with MIC values of 0.2, 1.7, 6.5, 13 and 13 µg/ml, respectively[32].

Misamycin, a new anthracycline antibiotic, was isolated from the culture broth of endophytic *Streptomyces* sp. YIM66403. The compound exhibited moderate antibacterial activity against *S. aureus* with MIC value of 64 µg/ml. Besides antibacterial activity, it showed cytotoxicity against various human cell lines including human promyelocytic leukemia HL-60, human hepatoma SMMC-7721, non-small cell long cancer A-549, breast cancer MCF-7 and human colorectal carcinoma SW4801 with IC_{50} values of 15.37, 16.34, 25.98, 20.71 and 9.75, µM respectively[33].

Diastaphenazine, a new dimeric phanazine, was isolated from the culture broth of endophytic *Streptomyces diastacicus* subsp. *ardesiacus* isolated from sterile tissue of *Artemisia annua*. The compound showed antimicrobial activity against *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 10231. In addition, it showed weak cytotoxicity against five human tumor cell lines including BGC-823, Hela, HCT116, HepG2 and H460 with IC_{50} values of 14.9, 28.8, 65.2, 82.5 and >100 µM, respectively[34].

In this study, the isolate 5R010, closely related to *Streptomyces sioyaensis* NRRL-B5408^T, showed antifungal activity against *C. albicans*. This isolate was selected to test the antagonistic activity against phytopathogenic fungi.

Antiphytopathogenic fungi activity

Based on the co-cultivation method, the strain 5R010 showed antagonistic activity against *Fusarium* sp., *Colletotrichum* sp., *Sclerotium* sp. but no activity was observed on *Colletotrichum gloerosporiodes*, *Curvularia oryzae* and *Lasiodiplodia theobromae* (Fig. 4). It has been reported in several studies that the endophytic actinobacteria can be used to control plant diseases. Álvarez-Pérez et al[35] used endophytic actinobacteria isolated from the root system of the grapevine plants, *Vitis vinifera*, to reduce nursery fungal graft infections caused by *Diplodia seriata Dactylonectria macrodidyma Phaeomoniella chlamydospora* and *Phaeoacremonium minimum.* Taechowisan et al[36] reported that three endophytic *Streptomyces* sp. showed strongly inhibited *Colletotrichum musae* and five were very active against *Fusarium oxysporum*. The *Streptomyces* strain CEN6, isolated from *Centella asiatica*, showed good antagonistic activity against *Alternaria brassicicola*-the pathogen causes leaves spot of cabbage. The fungal treated by this stain showed abnormal characteristics including swelling and frequent septa[37]. The used of endophytic *Streptomyces platensis* F-1, isolated from *Oryza sativa*, as biofumigation to control plant fungal disease was reported by Wan et al[38]. The volatile substance produced by the strain F-1 could effectively reduce the incidence and the severity of the disease caused by *Botrytis cinerea*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*. Besides the application as biocontrol, the novel antifungal compounds, such as dehydroxyaquayamycin B and fistupyrone, were isolated from endophytic actinobacteria. Dehydroxyaquayamycin B, a new C-glycosylated benz[α]anthraquinone, was isolated from endophytic *Streptomyces blastomycetica* F4-20. The compound showed fungicidal activity against *Valsa mali*, *Colletotrichum orbiculare* and *Fusarium graminearum*[39]. Fistupyrone, a new microbial compound, isolated from the culture broth of endophytic *Streptomyces* sp. TP-A0569 can inhibit the *in vivo* infection of the seedlings of Chinese cabbage by *Alternaria brasicicola*, the cause of *Alternaria* leaf spot[40]. The antagonistic activity of the strain 5R010 found in this study revealed that this strain may be used for the fungal biocontrol in the future. In addition, the active compounds produced by this strain should be characterized in further study.

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CONFLICTS OF INTEREST

On behalf of all authors, the corresponding author states that there is no conflict of interest**.**

CONTRIBUTION OF AUTHORS

We declare that the present study was performed by the authors named in this article. W. Phongsopitanun designed the study and performed experiments on isolation, identification of actinobacteria and screening of antimicrobial activity of the actinobacterial isolates; P. Sripreechasak performed experiments on data analysis; K. Rueangsawang and R. Panyawut performed experiments on plant sample collection and identification of the plant species; P. Pittayakhajonwut and S. Tanasupawat gave conceptual advice.

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FIGURE LEGENDS

Fig. 1 Diversity of actinobacteria isolated from Acanthaceae plant species. (a) Pie chart represented the percentage of actinobacterial genera within the total number of isolates. (b) The number of actinobacteria isolated from different plant species.

Fig. 2 Neighbour-Joining phylogenetic tree based on 16S rRNA gene of the actinobacterial isolates and closely related actinobacterial type strains shown that the isolates were clustered within four families and six genera. Numbers at the nodes indicate bootstrap values based on 1,000 replicates.

316 **Fig. 3** Antimicrobial activity of the actinobacteria against tested microorganisms

317 **Fig. 4** Antagonistic activity of the isolate 5R010 against phytopathogenic fungi (a) *Lasiodiplodia theobromae*, (b)
318 *Curvularia oryzae* (c) *Fusarium* sp. (d) *Sclerotium* sp. (e) *Colletotrichum gloeosporioides* (f) *Colletotrichum* sp.
319 The arrows ⇢ and → indicate the colony of isolate 5R010 and fungal pathogens, respectively. The scale bar
320 is 10 mm.

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Table 1 Closest BLASTN matches for the 16S rDNA sequence and antimicrobial activity of the actinobacterial isolates

Plant host	plant material	isolation no.	accession number	BLAST match result		isolation media	Inhibition zone (mm)					
				Closest species	Similarity %		K	B	S	P	E	C
Asystasia gangetica	Root	5R001	LC497879	*Micromonospora chokoriensis* DSM45160^T	99.51	SCN	-	-	-	-	-	-
		5R002	LC497878	*Micromonospora maritima* D10-9-5^T	100	SCN	-	-	-	-	-	-
		5R004	LC497877	*Micromonospora maritima* D10-9-5^T	99.79	SCN	-	-	-	-	-	-
		5R005	LC497876	*Micromonospora chokoriensis* DSM45160^T	99.51	SCN	-	-	-	-	-	-
		5R007	LC497873	*Micromonospora tulbaghiae* DSM 45142^T	100	SCN	-	-	-	-	-	-
		5R009	LC497875	*Micromonospora tulbaghiae* DSM 45142^T	100	Proline	-	-	-	-	-	-
		5R010	LC500018	*Streptomyces sioyaensis* NRRL-B5408^T	99.72	HV	-	-	-	-	-	16
		5R011	LC497872	*Streptomyces durhamensis* NRRL-B3309^T	99.41	HV	26	15	17	-	-	-
		5R012	LC497874	*Nocardia xishanensis* NBRC 101358^T	99.93	HV	-	-	-	-	-	-
		5R014	LC497871	*Nocardia bhagyanarayanae* VRC07^T	98.68	SCN	-	-	-	-	-	-
Justicia subcoriacea	Root	3R002	LC497888	*Streptomyces lannensis* TA4-8^T	99.93	SCN	-	-	-	-	-	-
		3R003	LC497882	*Microbispora hainanensis* 211020^T	100	HV	-	-	-	-	-	-
		3R004	LC497890	*Streptomyces shaanxiensis* CCNWHQ0031^T	99.3	HV	21	21	27			
		3R006	LC497880	*Streptomyces cyaneus* NRRL B-2296^T	98.91	HV	-	-	-	-	-	-
		3R008	LC497886	*Micromonospora chalcea* DSM 43026^T	99.65	proline	-	-	-	-	-	-
		3R009	LC497889	*Nocardia bhagyanarayanae* VRC07^T	99.72	proline	-	-	-	-	-	-
		3R010	LC497887	*Microbispora hainanensis* 211020^T	99.17	proline	-	-	-	-	-	-

		3R011	LC497885	*Micromonospora tulbaghiae* DSM 45142^{T}	99.44	SCN	-	-	-	-	-	-
				Micromonospora wenchangensis CCTCCAA								
		3R012	LC497891	2012002^{T}	99.44	SCN	-	-	-	-	-	-
		3R014	LC497881	*Micromonospora nigra* DSM 43818^{T}	98.25	SCN	-	-	-	-	-	-
		3R015	LC497884	*Microbispora hainanensis* 211020^{T}	99.72	HV	-	-	-	-	-	-
		3R016	LC497883	*Microbispora hainanensis* 211020^{T}	99.72	HV	-	-	-	-	-	-
	Leaf	3L001	LC497892	*Actinomycetospora corticicola* 014-5^{T}	99.62	Proline	22	19	18	-	-	-
		3L002	LC497920	*Dactylosporangium sucinum* RY35-23^{T}	99.58	Proline	-	-	-	-	-	-
Barleria lupulina	Root	7R002	LC497919	*Streptomyces shenzhenensis* 172115^{T}	99.38	HV	-	-	-	-	-	-
		7R004	LC497918	*Streptomyces shenzhenensis* 172115^{T}	99.65	Proline	-	-	-	-	-	-
		7R005	LC497917	*Microbispora hainanensis* 211020^{T}	99.86	Proline	-	-	-	-	-	-
		7R006	LC497916	*Streptomyces graminisoli* JR-19^{T}	99.93	HV	-	-	-	-	-	-
		7R007	LC497908	*Streptomyces chiangmaiensis* T4A-1^{T}	98.75	HV	-	-	-	-	-	-
		7R008	LC497915	*Streptomyces graminisoli* JR-19^{T}	99.51	HV	-	-	-	-	-	-
		7R009	LC497914	*Streptomyces graminisoli* JR-19^{T}	99.86	HV	-	-	-	-	-	-
		7R011	LC497913	*Streptomyces graminisoli* JR-19^{T}	99.86	starch	-	-	-	-	-	-
		7R012	LC497912	*Streptomyces graminisoli* JR-19^{T}	99.79	proline	-	-	-	-	-	-
		7R013	LC497911	*Streptomyces lilacinus* NRRL 1968^{T}	99.21	proline	15	-	-	-	-	-
		7R014	LC497910	*Streptomyces lusitanus* NBRC 13464^{T}	99.65	HV	-	-	-	-	-	-
		7R015	LC497906	*Streptomyces neopeptinius* KNF 2047^{T}	98.64	HV	-	-	-	-	-	-
		7R016	LC497907	*Streptomyces gilvifuscus* KM229362^{T}	98.21	HV	-	-	-	-	-	-

		7R017	LC500017	*Streptomyces shenzhenensis* 172115^{T}	99.45	HV	-	-	-	-	-	-
		7R018	LC497909	*Streptomyces shenzhenensis* 172115^{T}	99.58	HV	-	-	-	-	-	-
		9R002	LC497898	*Streptomyces parvulus* NBRC 13193^{T}	99.72	HV	-	-	-	-	-	-
		9R003	LC497897	*Streptomyces chartreusis* NBRC 12753^{T}	99.31	SCN	9.5	8	7	-	-	-
Ruellia		9R004	LC497895	*Streptomyces chartreusis* NBRC 12753^{T}	99.38	proline	8.5	8	-	-	-	-
squarrosa	Root	9R005	LC497896	*Streptomyces laurentii* ATCC 31255^{T}	99.31	HV	-	-	-	-	-	-
		9R006	LC497894	*Streptomyces chartreusis* NBRC 12753^{T}	99.38	proline	-	-	-	-	-	-
		9R008	LC497893	*Streptomyces collinus* NBRC 12759^{T}	99.93	HV	19	12	14	-	-	-
		6R001	LC497905	*Streptomyces deccanensis* DAS-139^{T}	99.78	HV	-	-	-	-	-	-
		6R002	LC497904	*Streptomyces hawaiiensis* NBRC 12784^{T}	99.72	HV	-	-	-	-	-	-
Andrographis		6R003	LC497903	*Streptomyces shenzhenensis* 172115^{T}	99.79	HV	-	-	-	-	-	-
paniculata	Root	6R004	LC497902	*Streptomyces collinus* NBRC 12759^{T}	99.93	HV	-	-	-	-	-	-
		6R005	LC497901	*Streptomyces deccanensis* DAS-139^{T}	99.79	HV	-	-	-	-	-	-
		6R006	LC497900	*Streptomyces indiaensis* NBRC 13964^{T}	99.29	HV	-	-	-	-	-	-
Clinacanthus												
rutans	Stem	8S001	LC500016	*Streptomyces cavourensis* NBRC 13026^{T}	100	HV	-	-	-	-	-	-

Abbreviations: K = *Kocuria rhizophila*; B = *Bacillus subtilis*; S. *Staphylococcus aureus*; P = *Pseudomonas aeruginosa*; E = *Escherichia coli*; C = *Candida albicans*