รายงานวิจัยฉบับสมบูรณ์

(1 ธันวาคม 2537 - 30 พฤศจิกายน 2540)

การศึกษาความแตกต่างแปรผันทางพันธุกรรมในประชากรกุ้งกุลาดำ โดยการตรวจลายพิมพ์ดีเอ็นเอ ด้วยตัวตรวจสอบที่มีลำดับเบสซ้ำแบบง่าย

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โครงการ การศึกษาความแตกต่างแปรผันทางพันธุกรรมในประชากรกุ้งกุลาดำ
โดยการตรวจลายพิมพ์ดีเอ็นเอ ด้วยตัวตรวจสอบที่มีลำดับเบสซ้ำแบบง่าย

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บทคัดย่อ

ในการศึกษาความแตกต่างแปรผันทางพันธุกรรมในประชากรกุ้งกุลาดำ ได้ทดสอบวิธีการตรวจลายพิมพ์ดีเอ็นเอในกุ้งกุลาดำและได้เลือก 2 วิธี เพื่อใช้ในการศึกษาความแตกต่างแปรผันทางพันธุกรรม คือ 1. การตรวจลายพิมพ์ดีเอ็นเอ โดยเทคนิค RAPD (Random Amplified Polymorphic DNA analysis) และ 2. การตรวจความแปรผันของ microsatellites DNA ในการศึกษานี้ได้เก็บตัวอย่างกุ้งจากแหล่งที่นิยมใช้เป็นพ่อ-แม่พันธุ์ในการเพาะเลี้ยงกุ้ง จำนวน 5 แหล่ง โดยเก็บตัวอย่างจากทะเลอันดามัน 3 แหล่ง ได้แก่ กุ้งบริเวณ จ.สตูล-ตรัง กุ้งจากจ.พังงา และ กุ้งจากเมดาน ประเทศอินโดนีเซีย และเก็บตัวอย่างกุ้งจากฝั่งอ่าวไทย 2 แหล่ง ได้แก่ กุ้งจาก จ.ตราด และ จากจ.ชุมพร โดยแต่ละวิธีได้ผลโดยสรุปดังนี้

การตรวจลายพิมพ์ดีเอ็นเอ โดยเทคนิค RAPD

จากการวิเคราะห์ RAPD patterns ของกุ้งทั้ง 5 กลุ่ม พบว่ามีจำนวนแถบดีเอ็นเอที่เกิดจาก RAPD primersทั้ง 7 ตัว รวม 80 แถบ ซึ่งมีขนาดอยู่ในช่วง 200-2000 คู่เบส เมื่อคำนวณค่าเปอร์เซ็นต์ความหลากหลายของแถบดีเอ็นเอ (%polymorphic bands) ในกุ้งแต่ละกลุ่มพบว่ามีค่าใกล้เคียงกันอยู่ในช่วง 51.5-57.7% ค่า similarity index ภายในกลุ่มประชากรอยู่ในช่วง 0.86-0.89 โดยตัวอย่างกุ้งจากพังงามีค่า similarity ภายในกลุ่มประชากรสูงสุด จากค่า similarlity index ระหว่างกลุ่มประชากร เมื่อนำมาคำนวณค่า genetic distance และ สร้าง dendrogram พบว่าตัวอย่างกุ้งกุลาดำจากเมดานมีลักษณะทางพันธุกรรมแตกต่างจากกุ้งของประเทศไทย (Dij=14.976%) และภายในกลุ่มตัวอย่างของกุ้งจากประเทศไทยด้วยกัน สามารถแยกกุ้งสตูล-ตรังออกจากกลุ่มตัวอย่างที่เหลือ (Dij=2.632%) ลายพิมพ์ดีเอ็นเอจากตัวอย่างกุ้งทั้ง 5 กลุ่ม มีลักษณะที่แตกต่างกันทั้งสิ้น 252 แบบ เมื่อนำมาวิเคราะห์ความแตกต่างโดยใช้ไคสแคว์ (chi-square analysis) และเปรียบเทียบระหว่างตัวอย่างกุ้งจากเมดานกับกลุ่มตัวอย่างของประเทศไทย และระหว่างกลุ่มตัวอย่างของประเทศไทยจากทะเลอันดามันและอ่าวไทย พบว่ามีความแตกต่างกันอย่างมีนัยสำคัญ ($P<0.0001$ และ $P=0.0000-0.0387$ ตามลำดับ)

การตรวจความแปรผันของ microsatellites DNA

สามารถแยก microsatellite clones จาก partial genomic library ของกุ้งกุลาดำได้ และจากการหาลำดับนิวคลีโอไทด์ของ microsatellite clones พบว่ามีลำดับเบสซ้ำเป็นแบบ (GT)n จำนวน 97 clones, (CT)n จำนวน 16 clones และเป็น microsatellites ชนิด perfect, imperfect และ compound ในสัดส่วน 24, 53, และ 23% ตามลำดับ แสดงว่า microsatellites ในกุ้งกุลาดำส่วนใหญ่

เป็นชนิด imperfect นอกจากนี้ยังพบว่าจำนวน repeat ในแต่ละ clone มีความยาวตั้งแต่ 6 repeats จนถึงมากกว่า 100 repeats โดยจำนวน repeat ที่พบมากที่สุดอยู่ในช่วง 30-35 repeats จากลำดับนิวคลีโอไทด์บริเวณ flanking regions ของ microsatellite clones นำมาออกแบบ PCR primers เพื่อตรวจความแปรผันของ microsatellite loci พบว่ามี 4 loci ที่ให้ PCR product ตามที่คาดไว้ และสามารถ score allele ได้ คือ CUPmo 18, CUPmo386, CUPmo131 และ CUPmo 195 เมื่อทำการทดสอบความแปรผันของ microsatellite loci เหล่านี้ โดยนำไปตรวจตัวอย่างกุ้งจากธรรมชาติ พบว่าทุก loci มีความแปรผันสูง คือมีจำนวน alleles ที่พบในแต่ละ locus อยู่ในช่วง 14-28 alleles เมื่อนำไปตรวจความแปรผันทางพันธุกรรมในตัวอย่างกุ้ง 4 กลุ่ม คือ สตูล ตรัง พังงา และชุมพร โดยการตรวจความแปรผันที่ตำแหน่ง CUPmo18 locus พบว่ากุ้งจากทะเลอันดามัน คือ สตูล ตรัง และพังงา มีความแตกต่างอย่างมีนัยสำคัญ (P<0.05) กับกุ้งจากอ่าวไทย คือ กุ้งชุมพร

Abstract

Two molecular methods, the Randomly Amplified Polymorphic DNA (RAPD) analysis and the microsatellite technique, were used in determining geneic variations in wild populations of the black tiger shrimps, *Penaeus monodon*, Fabricius. Specimens were collected from 5 geographically separated locations of *P. monodon* consisted of Satun-Trang, Phangnga, and Medan in the Andaman Sea and Chumphon and Trad in the Gulf of Thailand.

In the RAPD analysis using 7 arbitrarily selected primers, the percentages of polymorphic bands of 5 geographic populations investigated varied from 51.5-57.7%. The average similarity index within populations across all primers ranged from 0.86-0.89. The Phangnga *P. monodon* showed the highest level of within population similarity whereas the remaining populations showed slightly lower similarity index. The genetic distance between populations and UPGMA dendrograms indicated that the medan population was genetically different from Thai *P.monodon*. (Dij=14.976%). Within Thailand, the Satun-Trang *P. monodon* was separated from the remaining geographic populations with a genetic distance of 2.632%. RAPD analysis yielded a total of 252 genotypes. A Monte Carlo analysis illustrated geographic heterogeneity in genotype frequencies within this species suggesting that genetic population structure does exist in this taxon (P<0.001 for all primers). Significant differences in genotype frequencies between Thai and Indonesian(Medan) *P. monodon* were observed (P<0.0001). Within Thailand, The Andaman Sea *P. monodon* was significantly different from that of the Gulf of Thaialnd indicating population differentiation between *P. monodon* from these two main fishery regions of Thailand.

In the microsatellite technique, Ninety-seven (GT)n and 16 (CT)n microsatellites were isolated from the partial genomic library of *P. monodon*. The microsatellites sequences were classified into three categories, perfect, imperfect and compound. The predominant categories found in *P. monodon* microsatellites are imperfect repeats for both (GT)n and (CT)n. Very long repeat arrays with the common repeat of 30-35 were found in *P. monodon* microsatellite clones which resulted in difficulties in primer design. Four microsatellite loci, CUPmo18, CUPmo13, CUPmo195, and CUPmo386, were successfully amplified. The number of alleles of each locus ranged from 14-28 alleles. Genetic diversity among populations of Thai *P. monodon* were examined at CUPmo 18 locus. Differences in allelic distribution and heterozygosity were observed in all populations. A chi-square analysis using GENEPOP indicated a significant difference between the population of *P. monodon* from the Andaman Sea and the Gulf of Thailand ($P<0.05$).

วัตถุประสงค์

1.1 พัฒนาวิธีตรวจลายพิมพ์ดีเอ็นเอในกุ้งกุลาดำ ที่ง่าย รวดเร็ว และใช้ปริมาณดีเอ็นเอในการตรวจเพียงเล็กน้อย โดยเทคนิคการเพิ่มปริมาณดีเอ็นเอด้วยปฏิกิริยาลูกโซ่โพลีเมอเรส (Polymerase chain reaction) หรือ PCR

1.2 นำวิธีที่พัฒนาได้มาตรวจลายพิมพ์ดีเอ็นเอของกุ้งกลุ่มต่างๆ เพื่อดูความแตกต่างแปรผันทางพันธุกรรม

1.3 วิเคราะห์ความแปรผันและโครงสร้างประชากรกุ้งกุลาดำในประเทศไทย

ความสำคัญและปัญหาที่ทำการวิจัย

กุ้งกุลาดำ (*Penaeus monodon*) เป็นสัตว์น้ำเศรษฐกิจที่นำรายได้เข้าประเทศเป็น จำนวน4-5หมื่นล้านบาทต่อปี โดยผลผลิตกุ้งกุลาดำส่วนใหญ่มาจากการเพาะเลี้ยง จนทำให้เกิดเป็นอุตสาหกรรมการเพาะเลี้ยงกุ้งกุลาดำตามพื้นที่ชายฝั่งทะเล การขยายตัวอย่างรวดเร็วของอุตสาหกรรมนี้ ทำให้เกิดปัญหาต่างๆขึ้น เช่น ปัญหาการทำลายระบบนิเวศน์ชายฝั่งของนากุ้ง ปัญหาโรคระบาดในกุ้ง รวมทั้งปัญหาการขาดแคลน พ่อ-แม่พันธุ์กุ้งที่มีคุณภาพ การจัดการทางพันธุกรรมจึงเป็นแนวทางที่จะช่วยแก้ปัญหาเหล่านี้ได้ส่วนหนึ่ง การจัดโปรแกรมการคัดพันธุ์ (selective breeding program) จะนำไปสู่การพัฒนาปรับปรุงพันธุ์กุ้ง เพื่อเพิ่มผลผลิตและนำไปสู่การเพาะเลี้ยงกุ้งที่ยั่งยืนต่อไป

การศึกษาความแตกต่างแปรผันทางพันธุกรรม (genetic variation) ในประชากรกุ้งกุลาดำเป็นข้อมูลพื้นฐานทางพันธุกรรมที่จำเป็นต่อการคัดเลือกพันธุ์ ตลอดจนการควบคุมและจัดการพันธุกรรมในกุ้งกุลาดำ แต่ข้อมูลเหล่านี้ยังมีน้อยมากและขาด genetic markers ที่มีความละเอียด ชัดเจนในการจำแนกพันธุกรรม ความก้าวหน้าทางชีววิทยาระดับโมเลกุลทำให้สามารถพัฒนา genetic markers ที่สามารถตรวจความแตกต่างทางพันธุกรรมได้อย่างละเอียด การค้นพบวิธีตรวจลายพิมพ์ดีเอ็นเอในคน

เพื่อตรวจสอบบุคคล ตรวจความเป็นพ่อ-แม่-ลูก ไปสู่การตรวจสอบดีเอ็นเอในพืชและสัตว์ รวมทั้งสัตว์น้ำ เพื่อประโยชน์ในการคัดเลือกและปรับปรุงพันธุ์ ผู้วิจัยจึงใคร่จะทำการตรวจสอบความแตกต่างทางพันธุกรรมในประชากรกุ้งกุลาดำ โดยวิธีการตรวจลายพิมพ์ดีเอ็นเอ และพัฒนาวิธีการที่ง่าย รวดเร็วและใช้ปริมาณดีเอ็นเอในการตรวจสอบเพียงเล็กน้อย โดยไม่เป็นอันตรายต่อกุ้งที่ตรวจสอบ

ผลงานวิจัยที่ได้จะเป็นข้อมูลในการคัดเลือกพันธุ์ ซึ่งจะเป็นประโยชน์ต่อการพัฒนาปรับปรุงพันธุ์และการจัดการ breeding program ของกุ้งในบ่อเพาะเลี้ยง รวมทั้งในแง่ของการอนุรักษ์ไม่ให้มีการปะปนกันของกุ้งกลุ่มต่างๆ (mixed stocks)

ผลการดำเนินงาน

จากการทดสอบวิธีการตรวจลายพิมพ์ดีเอ็นเอในกุ้งกุลาดำ ได้เลือก 2 วิธี เพื่อใช้ศึกษาความแตกต่างแปรผันทางพันธุกรรมในประชากรกุ้งกุลาดำ คือ 1. การตรวจลายพิมพ์ดีเอ็นเอ โดยเทคนิค RAPD (Random Amplified Polymorphic DNA analysis) และ 2. การตรวจความแปรผันของ microsatellites markers โดยแต่ละวิธีได้ดำเนินการตามขั้นตอนต่างๆ และได้ผลโดยสรุปดังนี้

การตรวจลายพิมพ์ดีเอ็นเอ โดยเทคนิค RAPD

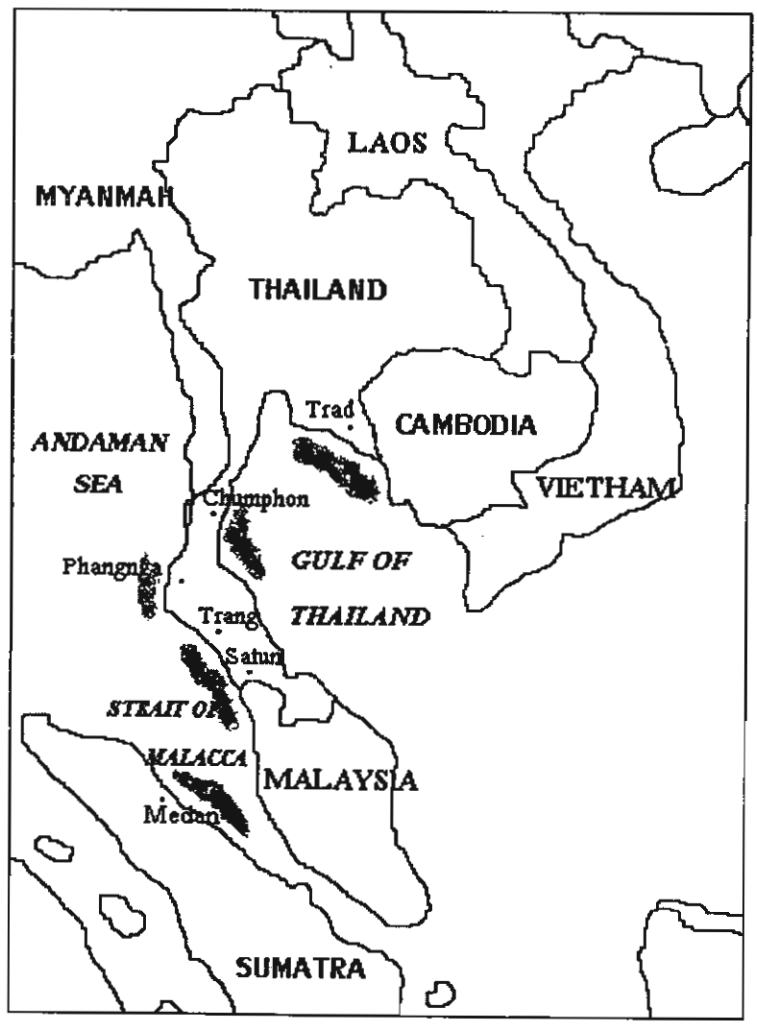
การคัดเลือก RAPD primers และสภาวะในการทำ PCR

จากการคัดเลือกหา RAPD primers ที่เหมาะสมจาก primer ขนาด 10 เบส จำนวน 300 ตัว (primers 101-400, 401-500 จาก Biotechnology laboratory, University of British Columbia) และเลือกไว้ 7 ตัว คือ primers 101, 174, 268, 428, 456, 457 และ 459 โดย primer ทั้ง 7 ตัว นี้ให้แถบดีเอ็นเอที่ชัดเจน วิเคราะห์ได้ง่าย และให้ผลไม่แปรปรวน (reproducible patterns)

จากการปรับหาสภาวะที่เหมาะสมสำหรับปฏิกิริยา PCR เพื่อตรวจลายพิมพ์ดีเอ็นเอของกุ้งกุลาดำพบว่าสภาวะที่เหมาะสม คือ ใน 25 µl PCR reaction ประกอบด้วย 10 mM Tris-HCl pH 9.0, 50 mM KCl, 2mM $MgCl_2$, 0.001% gelatin, 50 ng DNA, 0.4 mM RAPD primer, 100 ml dNTP, และ 1 unit เอนไซม์ *Taq* DNA polymerase ทำการ amplify จีโนมของกุ้งกุลาดำในสภาวะ denaturation 94^{o}C, 5s ; annealing 36^{o}C, 45s ; extension 72^{o}C, 90s จำนวนรอบในการทำ PCR เท่ากับ 35 รอบ

การตรวจความแตกต่างแปรผันทางพันธุกรรมในประชากรกุ้งกุลาดำ

ในการศึกษานี้ได้เก็บตัวอย่างกุ้งจากแหล่งที่นิยมใช้เป็นพ่อ-แม่พันธุ์ในการเพาะเลี้ยงกุ้ง จำนวน 5 แหล่ง โดยเก็บตัวอย่างจากทะเลอันดามัน 3 แหล่ง ได้แก่ กุ้งบริเวณ จ.สตูล-ตรัง กุ้งจากจ.พังงา และกุ้งจากเมดาน ประเทศอินโดนีเซีย และเก็บตัวอย่างกุ้งจากฝั่งอ่าวไทย 2 แหล่ง ได้แก่ กุ้งจาก จ.ตราด และจากจ.ชุมพร (รูปที่1) จากการวิเคราะห์ RAPD patterns ของกุ้งทั้ง 5 กลุ่ม พบว่ามีจำนวนแถบดีเอ็นเอที่เกิดจาก primers ทั้ง 7 ตัว รวม 80 แถบ ซึ่งมีขนาดอยู่ในช่วง 200-2000 คู่เบส เมื่อคำนวณเปอร์เซ็นต์ ความหลากหลายของแถบดีเอ็นเอ (%polymorphic bands) ในกุ้งแต่ละกลุ่ม พบว่ามีค่าใกล้เคียงกันอยู่ในช่วง 51.5-57.7% (ตาราง ที่1)



รูปที่ 1. บริเวณที่ทำการเก็บตัวอย่างกุ้งกุลาดำ (shaded areas)

จาก RAPD patterns ของกุ้งกลุ่มต่างๆนำมาหาค่า similarity จากสูตร

$$Sxy = 2n_{xy} / (n_x+n_y)$$

โดยที่ Sxy = similarity ภายในกลุ่ม (within population similarlity)

n_{xy} = จำนวน RAPD band ที่เหมือนกันในระหว่างกุ้ง x และ y ในกลุ่มเดียวกัน

n_x และ n_y = จำนวน RAPD band ในกุ้ง x และ y

เมื่อคำนวณค่า similarity idex ภายในกลุ่มประชากรพบว่า มีค่าอยู่ในช่วง 0.86-0.89 โดยตัวอย่างกุ้งจากพังงามีค่า similarity idex ภายในกลุ่มประชากรสูงสุด แสดงว่ามีความแปรผันทางพันธุกรรมน้อยที่สุด (ตารางที่ 1)

ตารางที่ 1. ค่า %ความหลากหลายของแถบดีเอ็นเอและ similarity index ภายในกลุ่มประชากร ตรวจสอบด้วย RAPD primer 7 ตัว

กลุ่มตัวอย่าง	จำนวนแถบดีเอ็นเอ	monomorphic/ polymorphic bands	%ความหลากหลาย ของแถบดีเอ็นเอ	ค่า similarity index ภายในกลุ่มประชากร
สตูล-ตรัง	71	30/41	57.7	0.8655
พังงา	66	32/34	51.5	0.8878
ชุมพร	64	29/35	54.7	0.8785
ตราด	69	33/36	52.2	0.8646
เมดาน	73	34/39	53.4	0.8626

และหาค่า similarlity ระหว่างกลุ่มประชากรจากสูตร

$$S_{ij} = 1 + S'_{ij} - 0.5 (S_i + S_j)$$

โดยที่ S_{ij} = similarity ระหว่างกลุ่ม i กับ j (between population similarlity)

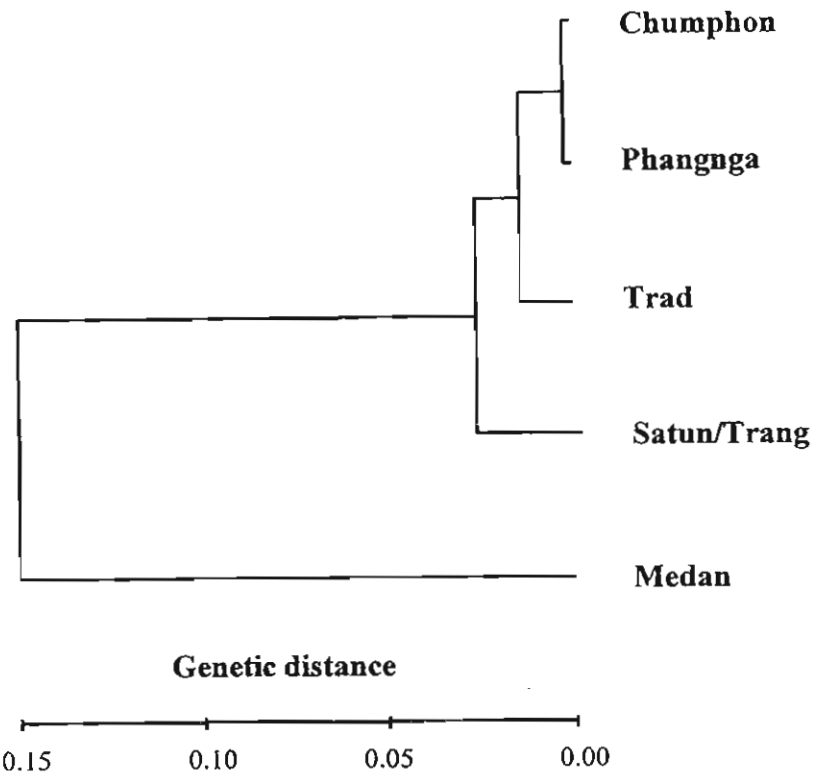
S'_{ij} = ค่า similarlity เฉลี่ยระหว่างกุ้งแต่ละคู่จากกลุ่ม i กับ j ที่เลือกมาแบบสุ่ม

S_i และ S_j = similarity ของ กลุ่ม i กับ j

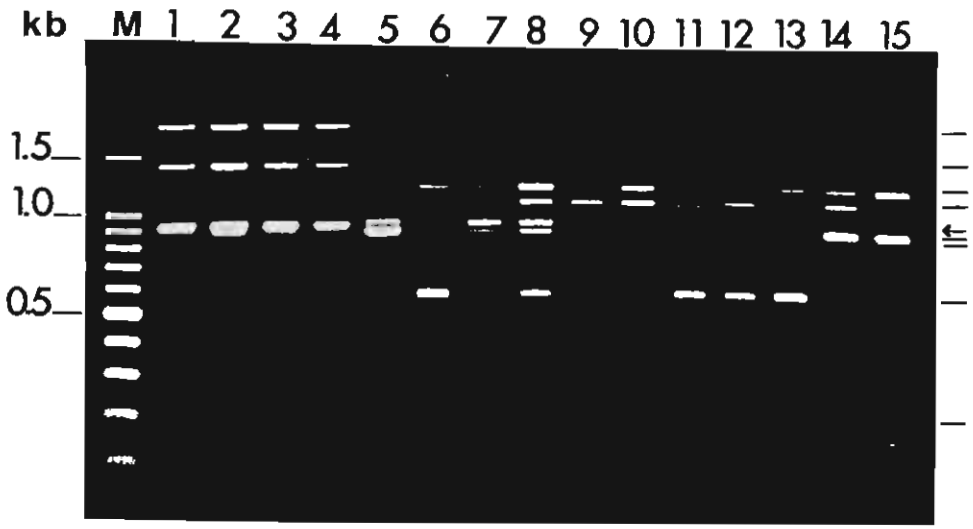
จากค่า similarlity index ระหว่างกลุ่มประชากร พบว่ากุ้งของไทยมีความเหมือนกันมากกว่าเมื่อเปรียบเทียบกับกุ้งอินโดนีเซีย (ตารางที่ 2) แม้ว่ากุ้งสตูล-ตรังและกุ้งเมดานจะอยู่ทางฝั่งอันดามันเหมือนกัน เมื่อนำมาคำนวณค่า genetic distance และ สร้าง dendrogram พบว่า พบว่าตัวอย่างกุ้งกุลาดำจากเมดานมีลักษณะทางพันธุกรรมแตกต่างจากกุ้งของประเทศไทย (Dij=14.976%) และภายในกลุ่มตัวอย่างของกุ้งจากประเทศไทยด้วยกัน สามารถแยกกุ้งสตูล-ตรังออกจากกลุ่มตัวอย่างที่เหลือ (Dij=2.632%) จึงสามารถแบ่งกุ้งออกเป็น 3 กลุ่ม โดย กลุ่มที่ 1 ประกอบด้วยกุ้งชุมพร ตราด และพังงา กลุ่มที่ 2 ได้แก่ กุ้งสตูล-ตรัง และ กลุ่มที่ 3 ได้แก่ กุ้งเมดาน (รูปที่ 2) นอกจากนี้พบว่า primer 428 ให้แถบดีเอ็นเอขนาด 950 bp ที่พบเฉพาะในกุ้งจากฝั่งอันดามัน แต่ไม่พบในกุ้งอ่าวไทย ซึ่งน่าจะนำมาใช้เป็น marker สำหรับจำแนกกุ้งจากฝั่งทะเลทั้งสองได้ (รูปที่ 3)

ตารางที่ 2 ค่า genetic distance (ค่าทแยงด้านบน) และ ค่า similarity index ระหว่างกลุ่มประชากร (ค่าทแยงด้านล่าง) กุ้งกุลาดำทั้ง 5 กลุ่ม

กลุ่มตัวอย่าง	สตูล-ตรัง	พังงา	ชุมพร	ตราด	เมดาน
สตูล-ตรัง	--	0.296	0.0661	0.0622	0.2812
พังงา	0.9744	--	0.0029	0.0299	0.2888
ชุมพร	0.9442	1.0026	--	0.0354	0.2941
ตราด	0.9478	0.9742	0.9697	--	0.3340
เมดาน	0.7882	0.7804	0.7782	0.7548	--



รูปที่ 2 UPGMA dendrogram แสดงความสัมพันธ์ระหว่างตัวอย่างกุ้งกุลาดำทั้ง 5 กลุ่ม



รูปที่ 3 RAPD pattern จาก primer 428
แถวที่ 1-4 กุ้งเมดาน; แถวที่ 5-8 กุ้งสตูล-ตรัง;
แถวที่ 9-15 กุ้งอ่าวไทย
ลูกศรแสดงแถบดีเอ็นเอที่พบเฉพาะในกุ้งจากทะเลอันดามัน

ลายพิมพ์ดีเอ็นเอจากตัวอย่างทั้ง 5 กลุ่ม มีลักษณะที่แตกต่างกันทั้งสิ้น 252 แบบ เมื่อนำมาวิเคราะห์ความแตกต่างโดยใช้ไคสแคว์ (chi-square analysis, ตารางที่3) พบว่ากลุ่มตัวอย่างจากเมดานมีความแตกต่างกับกลุ่มตัวอย่างทั้ง 4 กลุ่มของประเทศไทยอย่างมีนัยสำคัญ ($P<0.0001$) เมื่อเปรียบเทียบระหว่างตัวอย่างกุ้งของประเทศไทยด้วยกัน พบว่ากุ้งจากทะเลอันดามันและอ่าวไทยมีความแตกต่างกันอย่างมีนัยสำคัญ ยกเว้นลายพิมพ์ดีเอ็นเอที่เพิ่มขยายด้วยไพรเมอร์ 174 และ 456 ซึ่งไม่สามารถบอกความแตกต่างระหว่างกุ้งทั้ง 2 ฝั่งได้ (ค่า P-value =0.8810 และ 0.7604 ตามลำดับ)

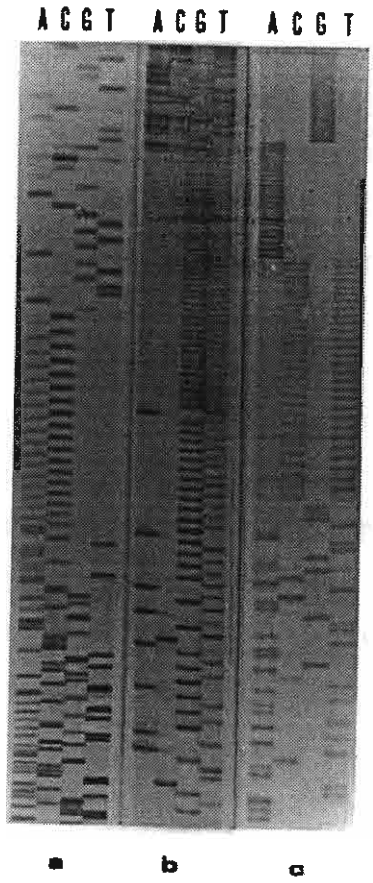
ตารางที่ 3 การวิเคราะห์ความแตกต่างของกลุ่มประชากร โดยการทดสอบการกระจายความถี่ของ genotype ด้วย chi-square analysis

	Primer						
	101	174	268	428	456	457	459
อันดามัน-อ่าวไทย	P=0.0201	p=0.8810	p=0.0001	P=0.0000	P=0.7604	P=0.0387	P=0.0000
อันดามัน-เมดาน	P=0.0000	P=0.0000	P=0.0000	P=0.0000	P=0.0000	P=0.0000	P=0.0000
อ่าวไทย-เมดาน	P=0.0000	P=0.0000	P=0.0000	P=0.0000	P=0.0000	P=0.0000	P=0.0000
ตัวอย่างทั้งหมด	P=0.0000	P=0.0001	P=0.0000	P=0.0000	P=0.0001	P=0.0000	P=0.0000

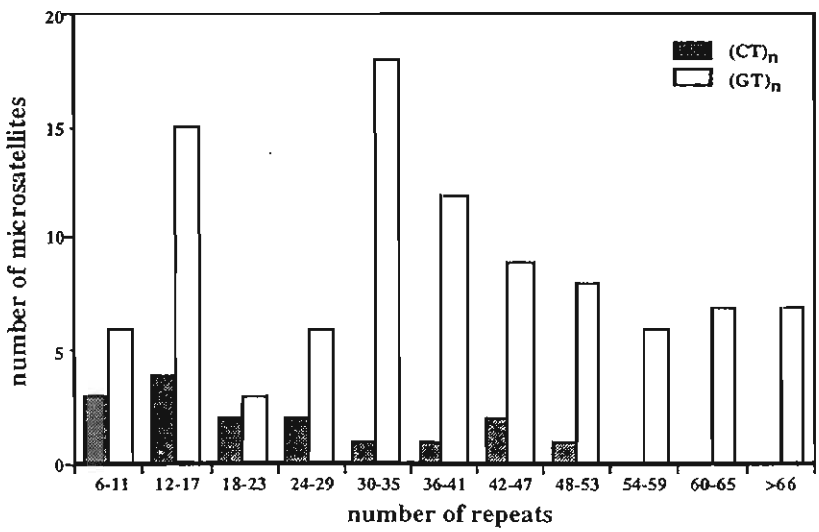
การตรวจความแปรผันของ microsatellites DNA

การแยกและวิเคราะห์ลักษณะ microsatellites ในจีโนมของกุ้งกุลาดำ

จาก partial genomic library ของกุ้งกุลาดำที่สร้างโดยการตัดดีเอ็นเอของกุ้งกุลาดำด้วย mix enzymes คือ *Alu*I, *Hae*III, *Hinc*II และ *Rsa*I เลือกชิ้นดีเอ็นเอขนาด 200-700 bp แล้วทำ blunt end ligation กับ pUC18 ที่ตัดด้วย *Sma*I จากนั้นจึง transform เข้าสู่ *E. coli* DH5α คัดเลือกด้วย ampicillin ได้ transformants ทั้งสิ้น 18,000 colonies เมื่อทำการตรวจสอบหา microsatellite clones โดยการ hybridization กับตัวตรวจสอบ $(GT)_{15}$ และ $(CT)_{15}$ พบว่ามีลำดับเบสซ้ำเป็น (GT)n 97 clones, (CT)n จำนวน 16 clones นอกนั้นเป็น microsatellites แบบอื่นๆ เช่น (GATA)n, (ATT)n, (AAC)n, (AT)n และ mononucleotides (A)n และ (G)n จากการหาลำดับนิวคลีโอไทด์ของ microsatellite clones พบว่าเป็น microsatellites ชนิด perfect, imperfect และ compound ในสัดส่วน 24, 53, และ 23% ตามลำดับ (ตัวอย่าง microsatellite ชนิดต่างๆแสดงในรูปที่ 4) แสดงว่า microsatellites ในกุ้งกุลาดำส่วนใหญ่เป็นชนิด imperfect นอกจากนี้ยังพบว่าจำนวน repeat ในแต่ละ clone มีความยาวตั้งแต่ 6 repeats จนถึงมากกว่า 100 repeats โดยจำนวน repeat ที่พบมากที่สุดอยู่ในช่วง 30-35 repeats (รูปที่ 5)



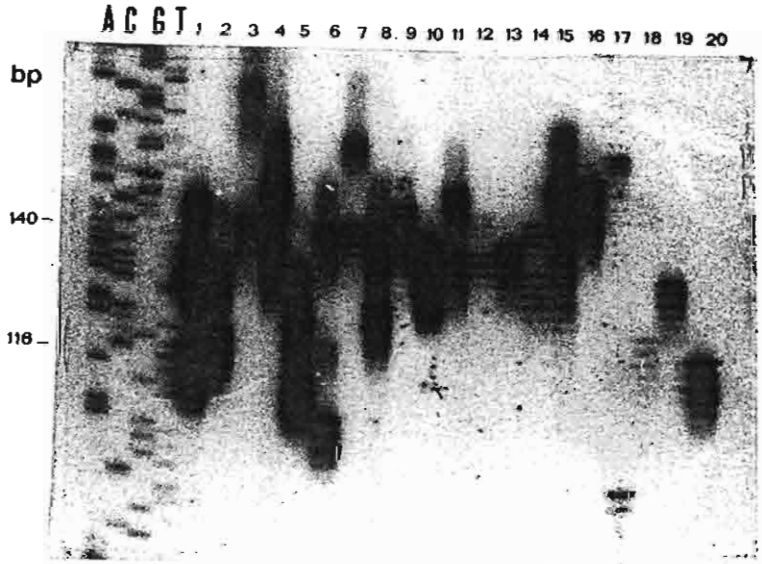
รูปที่ 4 ลำดับนิวคลีโอไทด์ของ a. perfect b. imperfect และ c. compound microsatellite clones



รูปที่ 5 ขนาดของ repeat ที่พบใน microsatellite clones ที่แยกได้จากจีโนมของกุ้งกุลาดำ

การออกแบบ PCR primers และ การวิเคราะห์ความแปรผันของ microsatellite loci

จากลำดับนิวคลีโอไทด์บริเวณ flanking regions ของ microsatellite clones นำมาออกแบบ PCR primers เพื่อตรวจความแปรผันของ microsatellite loci โดยใช้ computer software "Gene Runner" สภาวะในการทำ PCR คือ 1 PCR reaction ประกอบด้วย 15-25 ng DNA, 1 X PCR buffer, 200 mM dATP, dCTP, dGTP และ dTTP, 100 nM ของ primer แต่ละตัว และ PCR product จะตรวจสอบโดยการติดฉลาก upper หรือ lower primer ที่ตำแหน่ง 5' ด้วย g-32P dATP โดยใช้เอนไซม์ T4 polynucleotide kinase โปรแกรมสำหรับทำ PCR คือ 94o, 1 นาที; annealing temp (Tm-5), 30 วินาที; 72o, 1 นาที จำนวน 7 รอบ ต่อด้วย 90o, 30 วินาที; annealing temp (Tm-5), 30 วินาที; 72o, 1 นาที จำนวน 38 รอบ แล้ววิเคราะห์ผลด้วย 6% polyacrylamide gel (run gel ที่ 55 mA ประมาณ 2 ชม.) พบว่ามี 4 loci ที่ให้ PCR product ตามที่คาดไว้ และสามารถ score allele ได้ คือ CUPmo 18, CUPmo386, CUPmo131 และ CUPmo 195 เมื่อทำการทดสอบ loci เหล่านี้ โดยนำไปตรวจสอบกับตัวอย่างกุ้ง จากธรรมชาติ พบว่าทุก loci มีความแปรผันสูง คือมีจำนวน alleles ที่พบในแต่ละ locus อยู่ในช่วง 14-28 alleles ตัวอย่างการทดสอบความแปรผันของ microsatellite ที่ตำแหน่ง CUPmo18 locus แสดง ในรูปที่ 6



CUPmo18

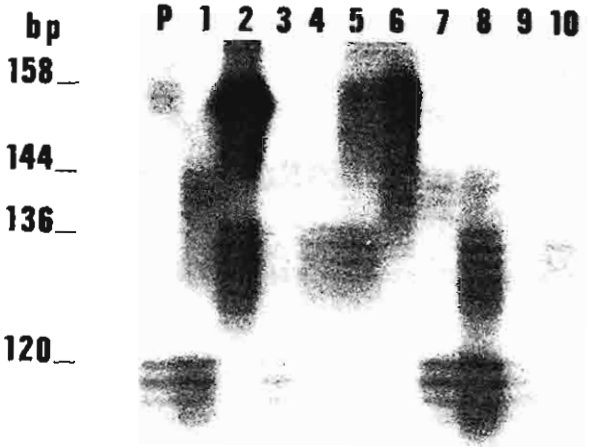
รูปที่ 6 ความแปรผันของ microsatellite ที่ตำแหน่ง CUPmo18 locus
แถว ACGT= M13 sequencing markers; แถวที่ 1-20 = กุ้งจากธรรมชาติ 20 ตัว

การตรวจสอบการถ่ายทอด allele จากพ่อ-แม่ ไปสู่ลูก

จากการตรวจสอบ allelic segregation ของลูกกุ้ง พบว่าทุก loci มีการถ่ายทอด allele จากพ่อ-แม่ ไปสู่ลูก เป็นไปตามกฎของเมนเดล ยกเว้นที่ตำแหน่ง CUPmo386 locus ซึ่งตรวจพบลักษณะของ null allele ดังนั้น microsatellite markers ที่พัฒนาได้จึงสามารถนำมาใช้เป็น genetic tags เพื่อตรวจสอบ genotype ของกุ้งได้ดี ตัวอย่างการถ่ายทอด allele จากพ่อ-แม่ ไปสู่ลูกที่ตำแหน่ง CUPmo18 locus แสดงในรูปที่ 7

การตรวจความแตกต่างแปรผันทางพันธุกรรมในประชากรกุ้งกุลาดำ

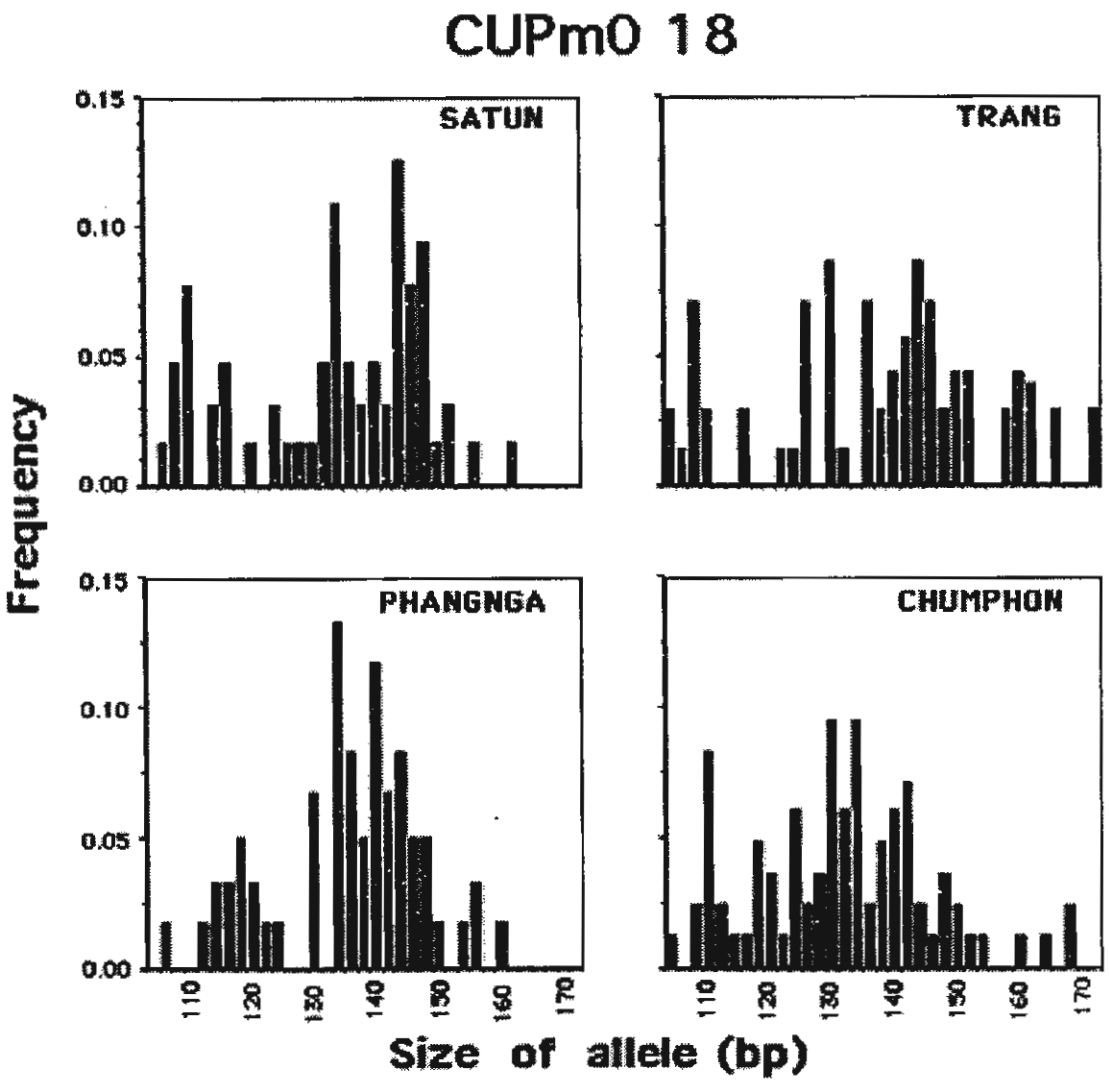
ทำการตรวจสอบความแปรผันทางพันธุกรรมในตัวอย่างกุ้ง 4 กลุ่มคือ สตูล ตรัง พังงา และชุมพร โดยการตรวจความแปรผันที่ locus CUPmo 18 พบว่ากุ้งกลุ่มต่างๆมีจำนวน allele และ การกระจายของ allele แตกต่างกัน (รูปที่ 8) และมีค่า heterozygosity อยู่ในช่วง 0.49-0.83 (ตารางที่ 3) เนื่องจากตัวอย่างที่ใช้ในการศึกษามี sample size ค่อนข้างเล็ก แต่ microsatellite locus ที่ตรวจสอบมีความแปรผันสูง ค่าที่จะบอกความแตกต่างทางพันธุกรรมได้ดีคือค่า effective number of allele (n_e) ที่คำนวณจากสูตร $n_e = 1/\Sigma P^2$ เมื่อ P = ค่า allelic frequencies จากค่า n_e พบว่ากุ้งตรัง และชุมพรมีความแตกต่างทางพันธุกรรมสูงสุด และกุ้งพังงามีความแตกต่างทางพันธุกรรมน้อยที่สุด และ จากค่า allelic frequencies เมื่อนำมาคำนวณเพื่อเปรียบเทียบความแตกต่างระหว่างกุ้งจากทะเลอันดามันกับกุ้งอ่าวไทย โดยใช้โปรแกรม GENEPOP พบว่ากุ้งจากทั้งสองฝั่งมีความแตกต่างอย่างมีนัยสำคัญ ($P < 0.05$)



รูปที่ 7 ความแปรผันของ microsatellite ที่ ตำแหน่ง CUPmo18 ใน F1 family

แถวที่ P : แม่กุ้ง; genotype คือ 158/120

แถวที่ 1-10 : ลูกกุ้ง 10 ตัวในระยะ post lavae; genotypes คือ 120/144, 158/136, 120/136, 120/144, 158/136, 158/144, 120/144, 120/136, 120/136 และ 158/136 ตามลำดับ



รูปที่ 8 การกระจายความถี่ของ allele ที่ ตำแหน่ง CUPmo18 ในกุ้งกลุ่มต่างๆ

ตารางที่ 3 การตรวจความแปรผันของกุ้งกลุ่มต่างๆ ที่ตำแหน่ง CUPmo18 locus

ตัวอย่างกุ้ง	จำนวนตัวอย่าง	ขนาดของ allele	จำนวน allele ที่พบ	observed heterozygosity	n_e
สตูล	32	106-162	23	0.56	15.06
ตรัง	35	104-172	24	0.49	18.53
พังงา	30	106-160	21	0.77	14.18
ชุมพร	42	104-168	28	0.83	18.31

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3. Tiptawonnukul, A., Rimphanitchayakit, V., Cook, D., Jarayabhand, Boonsaeng, V., and Tassanakajon, A. Microsatellite sequences in the black tiger prawn (*Penaeus monodon*). Sixth International Symposium on Genetic in Aquaculture, June 1997, Stirling, Scotland.

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การผลิตบัณฑิตปริญญาโท

ที่จบการศึกษาแล้ว

1. นส. สิริพร พงษ์สมบูรณ์ ปริญญาโทสาขาเทคโนโลยีชีวภาพ จบการศึกษาในปี พ.ศ. 2539 หัวข้อวิทยานิพนธ์เรื่อง "การตรวจหาความแปรผันทางพันธุกรรมในประชากรกุ้งกุลาดำ *Penaeus monodon* โดยการตรวจลายพิมพ์ดีเอ็นเอ"
2. นส. อมรรัตน์ ทิพถาวรนุกูล ปริญญาโทชีวเคมี จบการศึกษาในปี พ.ศ. 2539 หัวข้อวิทยานิพนธ์เรื่อง "การตรวจสอบลักษณะไมโครแซทเทลไลต์ดีเอ็นเอในจีโนมของกุ้งกุลาดำ *Penaeus monodon* "

ที่กำลังทำวิทยานิพนธ์

1. นส. เปรมฤทัย สุพรรณกูล นิสิตปริญญาโทชั้นปีที่ 3 ภาควิชาชีวเคมี กำลังทำวิทยานิพนธ์ในหัวข้อเรื่อง "การศึกษาความแปรผันทางพันธุกรรมและโครงสร้างประชากรของกุ้งกุลาดำ *Penaeus monodon* ในประเทศไทย โดยตัวตรวจสอบชนิดไมโครแซทเทลไลต์"
2. นส. ชวนชม มวลประสิทธิ์พร นิสิตปริญญาโทชั้นปีที่ 3 สาขาเทคโนโลยีชีวภาพ กำลังทำวิทยานิพนธ์ในหัวข้อเรื่อง "การแยกและวิเคราะห์ลักษณะของไมโครแซทเทลไลต์ ชนิดไดนิวคลีโอไทด์ในจีโนมของกุ้งกุลาดำ *Penaeus monodon* "

ผลงานที่ตีพิมพ์ในวารสารนานาชาติ

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Random amplified polymorphic DNA (RAPD) markers for determination of genetic variation in wild populations of the black tiger prawn (*Penaeus monodon*) in Thailand

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Abstract

Random amplified polymorphic DNA (RAPD) analysis was used to amplify the genome of black tiger prawns (*Penaeus monodon*) to detect DNA markers and assess the utility of the RAPD method for investigating genetic variation in wild *P. monodon* in Thailand. A total of 200 ten-base primers were screened, and 84 primers yielded amplification products. Six positive primers that gave highly reproducible RAPD patterns were selected for the analysis of three geographically different samples of Thai *P. monodon*. A total of 70 reproducible RAPD fragments ranging in size from 200 to 2000 bp were scored, and 40 fragments (57%) were polymorphic. The RAPD analysis of broodstocks from three different locales, Satun-Trang, Trat, and Angsila, revealed different levels of genetic variability among the samples. The percentages of polymorphic bands were 48% and 45% in Satun-Trang and Trat, respectively, suggesting a high genetic variability of the two samples to be used in selective breeding programs. Only 25% polymorphic bands were found in the Angsila sample, indicating the lowest polymorphic level among the three samples examined. Primer 428 detected a RAPD marker that was found only in *P. monodon* originating from Satun-Trang, suggesting the potential use of this marker as a population-specific marker in this species.

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Introduction

The black tiger prawn (*Penaeus monodon* Fabricius) is successfully cultured for food in many Asian countries. In Thailand, this species is the most economically important cultured shrimp, providing a substantial income of approximately US$2 billion in 1994 (Lin and Nash, 1996). The shrimp production of Thailand has increased rapidly during the last decade. The total amount of farm-raised black tiger prawns increased from 33,000 metric tons in 1987 to 220,000 metric tons in 1994. However, the farming of *P. monodon* relies entirely on broodstocks caught from wild populations for the supply of juveniles. Attempts to domesticate this species are now being undertaken (Benzie, 1994). As a first step toward the genetic improvement of this species, genetic variations among populations are being investigated.

Allozyme variability has been extensively used in Penaied prawns, but a low level of variation has been reported (Nelson and Hedgecock, 1980). Although a significant difference in allozyme frequency among Australian populations of *P. monodon* has been found (Benzie et al., 1992), more genetic variation has been detected using mitochondrial DNA (mtDNA) restriction fragment length polymorphism (RFLP) analysis (Benzie et al., 1993) and mtDNA sequence analysis (Palumbi and Benzie, 1991). These studies suggested that the DNA technique would provide a better source of useful markers in Penaeid prawns.

The random amplified polymorphic DNA (RAPD) analysis, developed by Williams et al. (1990), utilizes a single short primer of arbitrary sequence to amplify random segments of genomic DNA. The RAPD markers allow the examination of genome variation without previous knowledge of DNA sequences. The method has been used successfully to detect genetic variation in several plants and animals (Devos and Gale, 1992; Brummer et al.,

1995; Gwakisa et al., 1995). It has been used to examine genetic diversity in *P. vannamei* (Garcia et al., 1994), revealing a high level of genetic variation among populations. In *P. monodon*, the inheritance of RAPD banding patterns in six fullsib families has been demonstrated (Garcia and Benzie, 1995), indicating the possibility of using RAPD markers to track the progeny of pair mating in selective breeding programs.

In this study, we used RAPD analysis to detect polymorphic DNA markers in *P. monodon* and assess the utility of RAPD for investigating genetic variation of wild *P. monodon* in Thailand.

Results and Discussion

Optimization of the polymerase chain reaction assay for P. monodon

Experiments were carried out to optimize polymerase chain reaction (PCR) program parameters for the reproducible amplification of discrete DNA patterns. The length of each phase of the PCR program and the number of cycles were evaluated. Inconsistent amplification was minimized by including three concentrations of template DNA (100-fold span) in the program evaluations. Optimal program parameters were selected on the basis of consistent amplification at all DNA concentrations in addition to increased intensity and clarity of the banding pattern, as suggested by Bielawski et al. (1995). The optimal program parameters identified for *P. monodon* were 35 cycles of 5 seconds at 94°C, 45 seconds at 36°C and 1.5 minutes at 72°C. This program is considerably different from the standard protocol recommended by Williams et al. (1990) and that used by Garcia and Benzie (1995) for developing RAPD markers from the Australian *P. monodon*. We found that reducing the length of each phase of the program increased the intensity of amplification products, as also has been shown by Yu and Pauls (1992). This supports the proposal that decreasing the length of each phase of the PCR program helps maintain the activity of *Taq* polymerase. We also observed that increasing the number of PCR amplification cycles above 35 cycles did not significantly increase band intensity but produced smear patterns.

RAPD analysis

Two hundred 10-base primers were screened for their ability to prime PCR amplification of *P. monodon* genomic DNA. Only 84 RAPD primers (42%) yielded amplification products, while the rest of the primers did not amplify the DNA template or resulted in smear or faint bands. Six positive primers that gave reproducible RAPD patterns with resolvable fragments that stained intensely were selected for further analysis. A total of 70 scorable bands, ranging in size from 200 to 2000 bp, were produced from RAPD analysis of three geographically different samples of Thai *P. monodon*. Thirty of these bands were monomorphic, and 40 bands were polymorphic (Table 1). Each primer generated 8 to 15 scorable bands. An example of RAPD amplification patterns and the bands scored by primer 459 is shown in Figure 1. Only reproducible bands were scored for presence or absence. Primer 428 appears to identify more variable regions of the *P. monodon* genome, whereas primers 101 and 456 show less variable RAPD patterns.

To assess the usefulness of the RAPD markers in determining the genetic diversity of *P. monodon*, we studied genetic variation of three geographically different samples of *P. monodon* in Thailand. The most important shrimp fishing grounds in Thailand are the Gulf of Thailand and the Andaman Sea, and there are different locations along both coasts where broodstocks are caught for shrimp farming (Figure 2). In this study, we compared *P. monodon* of the Andaman Sea collected from Satun and Trang provinces with that of the Gulf of Thailand collected from Chon Buri (Angsila district) and Trat provinces. The tiger prawns collected from Satun-Trang and Trat are those most commonly used as spawners in *P. monodon* farming and are candidates for use in selective breeding. However, broodstocks from Satun-Trang tend to be much larger than those from Trat and exhibit different color. Moreover, although this is still controversial, the shrimp farmers claim that the Andaman Sea broodstocks produce more eggs and better quality seeds, making the price of the Andaman Sea broodstocks 5 to 10 times higher than those of the Gulf of Thailand.

RAPD analysis of 28 individuals in each of the Satun-Trang and Trat samples and 15 individuals of the Angsila sample revealed different levels of polymorphisms. The percentages of polymorphic bands were 47.8% for Satun-Trang, 45.3% for Trat, and 24.2% for Angsila (Table 2). The results suggest that *P. monodon* collected from Angsila are the least polymorphic among the three samples. The sample collected from Angsila was small because this population is rather homogeneous. Before farming activities, no *P. monodon* was found in this area, and not until recently was *P. monodon* present in fisheries

Table 1. Nucleotide sequences of six selected primers and number of amplified bands shown in the RAPD analysis in *P. monodon*.

Primer No.	Sequence	No. of amplified bands	No. of polymorphic bands
101	GCGCCTGGAG	9	4
174	AACGGGCAGG	15	9
428	GGCTGCGGTA	8	6
456	GCGGAGGTCC	15	7
457	CGACGCCCTG	15	10
459	GCGTCGAGGG	8	4
Total		70	40

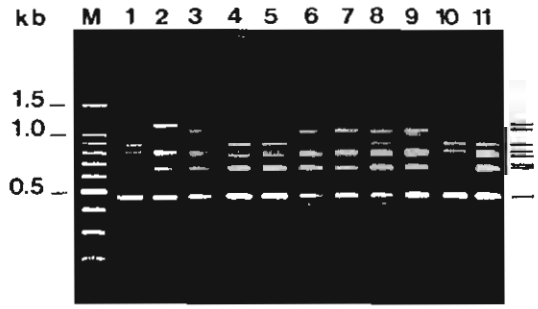

Figure 1. RAPD patterns using primer 459 of wild-caught individuals of *P monodon* collected from the Andaman Sea. Lane M: 100:bp DNA ladder. Lines indicate the bands that were scored.

catches, indicating that these shrimp may be escapees from farms in this area (K. Hassanai, personal communication). Although Angsila and Trat are a few hundred kilometers apart, prawns collected from these two areas are quite different. Broodstocks form Angsila will not be used for shrimp farming because of their lower fecundity and hatchability when compared with broodstocks from the Andaman Sea.

The percentages of polymorphic bands were comparable in Satun-Trang and Trat, suggesting similar levels of polymorphism of the two samples to be used for establishing selective breeding programs. This is in good agreement with mtDNA RFLP analysis of *P. monodon* collected from these two locations, which showed similar levels of mtDNA variations (Klinbunga, 1996). The RAPD analysis of the six fullsib families of *P. monodon* showed a much lower level of polymorphism (6.2%; Garcia and Benzie, 1995), which may be due the fact that wild-caught individuals would exhibit a higher level of genetic variation than that of the families. Studies of genetic variation in *P. vannamei* using the RAPD technique (Garcia et al., 1994) also indicated a higher genetic variability among populations; percentages of polymorphic bands ranging from 39% to 77% have been found.

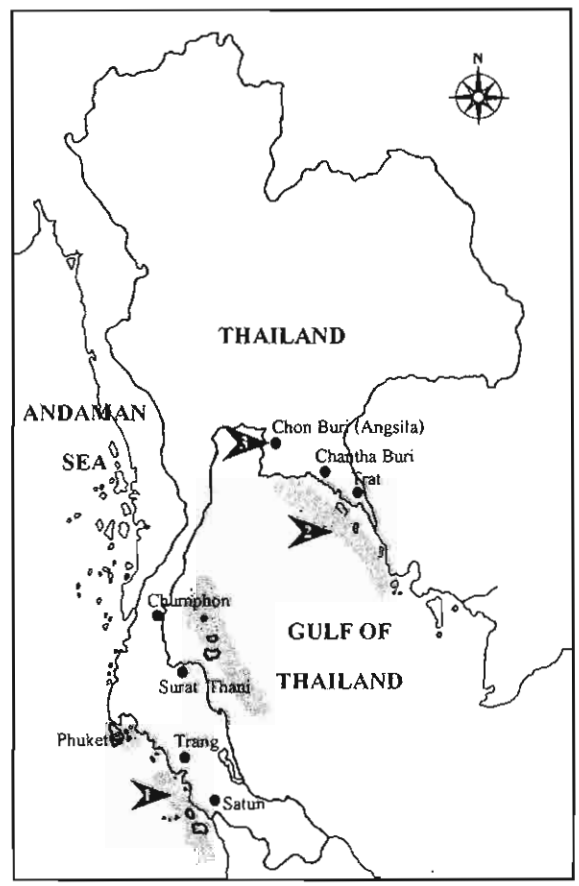

Figure 2. Map showing locations (shaded) where black tiger prawn broodstocks are commonly caught for shrimp farming in Thailand. Arrows indicate the three sample sites: 1, Satun-Trang: 2, Trat; and 3, Angsila.

The six RAPD primers used in this study appear to detect genetic variability among the three sam-

Table 2. Total numbers of bands and percentages of polymorphic bands found in populations of *P. monodon*.

Sample	Total no. of bands present out of the possible total of 70 bands	No. of monomorphic/ polymorphic bands	Percentage of polymorphic bands
Satun-Trang	67	35/32	47.8
Trat	64	35/29	45.3
Angsila	62	47/15	24.2

ples at different levels. Figure 3 shows the results of amplification using primer 428. Different patterns were seen between Satun-Trang and Trat. A 950-bp band (indicated by the arrow) was present in more than 70% of individuals from Satun-Trang (lanes 1–7) but absent in all individuals from Trat (lanes 8–14). This band was also absent in the Angsila sample (data not shown), suggesting a population-specific marker detected by primer 428. RAPD patterns produced by other primers such as primer 174 shows less variation, and different patterns among different samples were not clearly observed (Figure 4).

The total proportion of shared bands was used to calculate the similarity coefficient (*F*) between samples (Nei and Li, 1979). As shown in Table 3, genetic similarity coefficients between samples indicated that Trat is more similar to Angsila than to Satun-Trang. However, to obtain a more stable data analysis, a larger number of primers as well as larger sample sizes are required. Liu et al. (1994) suggested that the number of primers required is correlated with the level of genetic variation of the species. Six to seven primers were sufficient to assess genetic variation within and among populations of highly polymorphic species, but at least 10 to 15 primers were required for species with a low level of genetic diversity (Demeke et al., 1992; Huff et al., 1993). For marine species, a larger number of sample sizes was required to accurately detect genetic diversity in populations than for terrestrial species. Grewe et al. (1993) caculated that to be 95% confident of detecting mtDNA haplotypes that occur in a population of lake trout (*Salvelinus namaycush*) with a frequency of 5%, 60 fishes would have to be sampled. However, O'Connel et al. (1995) showed that a sample size of 25 to 30 individuals was adequate to identify all genotypes present in Atlantic salmon populations but a larger number of individuals would be required to differentiate between closely related populations.

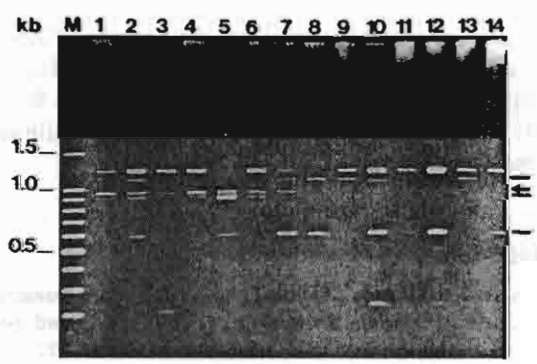

Figure 3. RAPD patterns using primer 428 Lane M: 100-bp DNA ladder; lanes 1–7, individuals from Satun-Trang; lanes 8–14, individuals from Trat. Lines indicate polymorphic bands; an arrow indicates the specific band that was found only in the Satun-Trang sample.

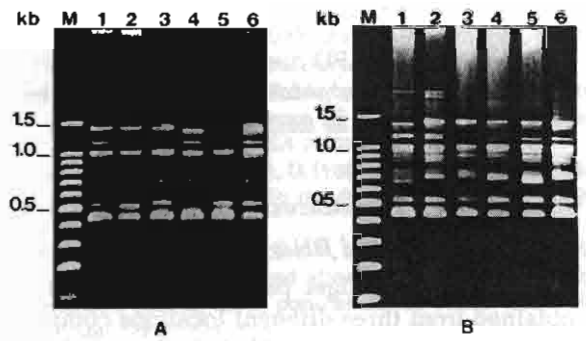

Figure 4. RAPD patterns using primer 174. Lane M: 100-bp DNA ladder; (A) lanes 1–6, individuals from Satun-Trang; (B) lanes 1–6 individuals from Trat.

Table 3. Similarity coefficients, *F* (top diagonal), and genetic distance, *d* (bottom diagonal), for the wild populations of *P. monodon* using RAPD analysis.

Sample	Satun-Trang	Trat	Angsila
Satun-Trang	—	0.931	0.930
Trat	0.069	—	0.968
Angsila	0.070	0.032	—

This work shows the usefulness of RAPD analysis in studying genetic variation in *P. monodon*. Genetic variability found in the wild populations will be useful in selecting particular populations for establishing a selective breeding program. The RAPD method provides a simple way to detect genetic polymorphisms with a nanogram of DNA, which allows the animals to be used further in the selective breeding program. DNA extracted from the juveniles as small as PL5 was also more than enough for genetic analysis using this method. Moreover, the number of DNA polymorphisms that can be detected by the RAPD method seems virtually unlimited because the number of primers can be increased effectively as desired.

Experimental Procedures

Prawn samples and DNA extraction

Samples of black tiger prawn (*P. monodon*) were obtained from three different locations comprising Trat and Angsila in the Gulf of Thailand and Satun-Trang in the Andaman Sea from December 1994 to June 1996. Specimens were kept frozen in liquid nitrogen or alternatively stored in ethanol and transported to the laboratory for further analysis. Genomic DNAs were extracted from pleopods, which were ground with plastic pestles in microcentrifuge tubes containing 500 µl of extraction buffer (100 mM Tris-HCl, pH9.0; 100 mM NaCl; 200 mM sucrose; 50 mM EDTA; and 1% SDS). Samples were incubated at 65°C for one hour. Subsequently, proteinase K (500µg/ml) and RNase solution (100µg/ml) were added to the reaction tubes, and the samples were further incubated at 37°C for one hour. Proteins were precipitated from nucleic acids by adding 5 M potassium acetate to the final concentration of 1 M, chilled on ice for 30 minutes, and centrifuged at 12,000 rpm for 10 minutes. The supernantants were then extracted once with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and twice with chloroform:isoamyl alcohol (24:1). The samples were precipitated with absolute ethanol and washed twice with 70% ethanol and resuspended in TE buffer. DNA concentration was determined by spectrophotometric method using DU 650 Spectrophotometer (Beckman Instrument Inc.).

PCR amplifications and product analysis

DNA amplifications were performed in a Perkin Elmer Cetus DNA Thermal Cycler (model 2400). Amplification reactions were carried out in 25-µl reaction volume containing 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 2 mM $MgCl_2$; 0.001% gelatin; 100 µM each dATP, dCTP, dGTP, and TTP; 0.2 µM primer; 25 ng genomic DNA; and 1 unit of *Taq* DNA polymerase (Williams et al. 1990). Ten-base oligonucleotides, numbers 101–200 and 401–500 (Biotechnology Laboratory, University of British Columbia), were used in the primer screening step. Amplification was perfomed for 35 cycles of 5 seconds at 94°C, 45 seconds at 36°C, and 1.5 minutes at 72°C.

Amplification products were analyzed by electrophoresis in 1.6% agarose gels and detected by ethidium bromide staining (Maniatis et al., 1982).

Data analysis

The RAPD patterns of individuals were scored based on band presence or absence. The index of similarity *(F)* between samples was calculated using the formula (Nei and Li, 1979):

$$F_{xy} = 2n_{xy}/(n_x + n_y),$$

where n_{xy} is the number of RAPD fragments shared by the two samples, and n_x and n_y are the numbers of RAPD fragments scored in each sample. The genetic distance (*d*) was calculated using the formula Hillis and Moritz, 1990):

$$d = 1 - F$$

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Isolation and characterization of microsatellite markers in the black tiger prawn, *Penaeus monodon*

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Running Title: microsatellite markers in *P. monodon*

Keywords: microsatellite, *Penaeus monodon*, genetic marker, tiger shrimp

Abstract

Isolation and characterization of microsatellite sequences from the genome of the black tiger prawn, *Penaeus monodon*, are described. Ninety-seven (GT)n and 16 (CT)n microsatellites were isolated from partial genomic libraries composed of 18,000 and 5,250 clones, respectively. The genomic library screening indicated that (GT)n microsatellites are more abundant than (CT)n in *P. monodon* genome. The microsatellites sequences were classified into three categories, perfect, imperfect and compound. The predominant categories found in *P. monodon* microsatellites are imperfect repeats for both (GT)n and (CT)n.

Very long repeat arrays were found in *P. monodon* microsatellite clones which resulted in difficulties in primer design. Two microsatellite loci, CUPmo18 and CUPmo386, were successfully amplified. The number of alleles of each locus was preliminary determined. For the CUPmo18 locus, Medelian inheritance was tested by analysis of genotypic ratios in F1 offspring and their parents. The results of this study firstly demonstrate the presence of highly polymorphic microsatellite markers in *P. monodon*. These markers will be useful in population studies and parental determination in *P. monodon*. However, the low abundance and difficulties in obtaining a large number of usable microsatellite loci indicated that these markers may not be appropriate for use in genome mapping of this species.

Introduction

Eukaryotic genomes contain a large number of interspersed tandemly repeated sequences called microsatellites or simple sequence repeats (Tautz, 1989; Weber and May, 1989). These refer to tandem repeats with a motif of less than 6 base pairs in length. The repeat number of microsatellites has been demonstrated to be highly variable in both plants and animals, providing an attractive source of genetic polymorphisms (Stallings et al., 1991, Roder et al. , 1995).

Microsatellite sequences have been obtained by searching databases such as GenBank (Weber,1990) and EMBL databank (Lagercrantz et al., 1993). However, this procedure is limited to a few species, mainly human and mouse (Beckmann and Weber, 1992). In species whose published sequences are not available, microsatellite sequences can be obtained by

screening genomic libraries with synthesized oligonucleotides and sequencing clones which gave positive hybridization (Cornall et al., 1991, Johansson et al., 1992). Based on the unique sequences flanking microsatellites, primers are designed to detect the microsatellite loci through polymerase chain reaction with genomic DNA. Alleles at microsatellite loci can be size fractionated on denaturing polyacrylamide gel electrophoresis. Allelic variants of a microsatellite locus are codominant and show Medelian inheritance.

Microsatellite markers have great potential use in aquaculture and fisheries biology. A number of studies have applied microsatellites to genetic tagging and population studies (Brooker et al., 1994; Garcia de Leon et al., 1995; Colbourne et al., 1996; Herbinger et al., 1997). The use of microsatellite markers to identify disease resistance has been emphasized in the European flat oyster, *Ostrea edulis* (Naciri et al., 1995). In this study, we have screened for the presence of microsatellite repeats in the genome of the black tiger prawn, *Penaeus monodon,* characterized and showed that they were polymorphic. The results suggest that *P. monodon* microsatellites will be valuable as genetic markers for use in population studies and parental determination.

Results and Discussion

Isolation of Microsatellites in P. monodon

A sequence is considered to be a microsatellite when the number of repeats is > 10 for mono, >6 for di, >4 for tri and >3 for tetra, penta and hexanucleotide repeats (Stalling et al., 1991, Estroup et al., 1993). In this study, a total of 156 microsatellites were isolated from a partial genomic library of *P. monodon.* Although the genomic library was screened for (GT)n and (CT)n microsatellites, several microsatellite motifs including one tetranucleotide: GAGT, two trinucleotide repeats: (ATT)n and (AAC)n, 27 dinucleotide repeats of (AT)n, and a few mononucleotides (A)n and (G)n, were coincidentally found.

Distribution of (GT)n and (CT)n microsatellites

The partial genomic libraries were screened separately with ^{32}P-labelled $(GT)_{15}$ and $(CT)_{15}$ synthetic probes. A larger proportion of the colonies screened gave positive hybridization with the $(GT)_{15}$ probe indicating that (GT)n were more abundant than (CT)n repeats in the *P.*

monodon genome. Ninety-seven (GT)n and 16 (CT)n microsatellites were isolated from 18,000 and 5,250 colonies screened, respectively. Assuming that all clones carried different inserts, and that the average insert size was 500 base pairs (bp), the total number of bp analyzed are estimated to be 9.0×10^6 bp for (GT)n and 2.6×10^6 bp for (CT)n. Considering that our partial library were representative of the whole *P. monodon* genome and assuming that the (GT)n and (CT)n sequences are evenly distributed throughout the genome, the average distance between neighboring microsatellites can be estimated by dividing the total length of screened DNA by the number of isolated microsatellites. Thus, in the clone fraction of the black tiger prawn genome, (GT)n microsatellites occur on average every 92.8 kb and (CT)n microsatellites every 164 kb. These values are based on several assumptions and, hence, must be treated with caution. On the basis of this estimate, it appears that the black tiger prawn microsatellites are less abundant than those previously reported in other species such as human (every 28 kb for (GT)n, Beckmann and Weber, 1992), rat, *rattus* sp. (every 21 kb for (GT)n, Stallings et al., 1991), honey bee, *Apis mellifera* (every 34 and 15 kb for (GT)n and (CT)n, respectively, Estroup et al., 1993) and Atlantic salmon, *Salmo sarla* (11-56 kb for (GT)n, McConnell et al., 1995).

Characterization of (GT)n and (CT)n microsatellites

Microsatellites of *P. monodon* described in this study were classified into three categories, perfect, imperfect and compound, as defined by Weber (1990). Perfect microsatellites are uninterrupted series of a repeat unit while imperfect microsatellite sequences differ from perfect ones by the presence of one to three bases that arrest an alternating tandem repeat. Compound repeats consist of several different repeat types and are separated by less than three bases. The predominant categories found in *P. monodon* microsatellites were imperfect repeats for both (GT)n and (CT)n microsatellites but higher percentages of imperfect (CT)n was found (Table 1). When compared with other species, the proportions of the different classes of microsatellites differ between species. Perfect repeats were the most abundant class found in other species, particularly in mammals. Surprisingly, the perfect repeats were found at very

low proportions in *P.monodon*, 24% and 12.5% for (GT)n and (CT)n microsatellites, respectively as compared to 64% for (GT)n in human

The longest perfect stretch of each of the (GT)n and (CT)n microsatellite arrays were plotted according to the number of uninterrupted repeat units (Fig. 1). The most common size class found in *P.monodon* microsatellites was 30-35 dinucleotide repeats for (GT)n. This is approximately twice the size of those reported in mammalian genome and about 4-5 times larger than those reported in Honey bees and Atlantic cod (Estroup et al, 1993; Brooker et al., 1994). The most common repeat unit of (CT)n microsatellites in *P. monodon* is 12-17 which is shorter than those of (GT)n repeats. However, it should be noted that the repeat lengths of the (CT)n microsatellites were actually extremely long but because there were many interrupted bases along (CT)n repeat arrays and only the largest perfect stretch of each repeats were counted. The maximum array size found in *P. monodon* microsatellites was 371 bp and the repeated arrays of greater than 200 bp were commom. Therefore, both (GT)n and (CT)n microsatellites found in *P. monodon* are significantly larger and more degenerate than those reported in other species.

(GT)n repeats were frequently associated with (AT) repeats in the compound microsatellites (37%, data not shown). (AT) repeats were also found in positive clones of perfect and imperfect categories suggested that this type of dinucleotide repeats may contribute at high density in the shrimp genome.

Microsatellite polymorphisms

Usable microsatellites were very difficult to obtain in *P. monodon* due to the very large and complex repeat arrays found in this species. Many clones contained only one side of the unique flanking sequences which made them not available for primer design. Insufficient flanking sequences obtained from many positive clones might be due to the strategy of digesting the same aliquot of genomic DNA with multiple restriction enzymes. This possibility will be addressed in the future work by first checking what enzyme(s) generated the clones which had little flanking sequence and when these have been identified, by using only the other enzymes to generate the fragments for future libraries. In some cases, even if sufficient flanking sequence was found in a microsatellite containing clones (which often was not the

case), the possible size of the PCR amplification products generated from the microsatellite would be larger than could be accurately separated and sized by the gel systems used.

In the present study, 9 primer sets were designed and PCR conditions were optimized. Only 2 primer sets yielded scorable amplification products. Most primer sets yielded multiple DNA bands which may be due to the fact that these microsatellite loci are embedded in some kinds of cryptic repeats which are dispersed throughout the genome (O'Reilly and Wright, 1995). The studies of repeated sequences in the genome of the marine shrimp, *Penaeus vannamei* have revealed a tandemly repeated pentanucleotide microsatellite embedded within a larger repeated satellite sequence (Bagshaw and Buckholt, 1997). The 2 usable microsatellite loci, CUPmo18 and CUPmo386, are both perfect (GT)n with 60 and 30 repeats units, respectively (Table 2).

To test the variability of *P. monodon* microsatellites, the 2 primer sets were used to amplify the loci from wild *P. monodon* (Figs. 2 and 3). For locus CUPmo18, we observed 28 alleles with the heterozygosity value of 0.66 . The locus CUPmo386 was also found to be polymorphic, showing 14 alleles with observed heterozygosity of 0.19. The expected heterozygosities estimated from allele frequencies for the CUPmo18 and CUPmo386 loci were 0.95 and 0.83, repectively (Table 3). The heterozygote deficits seen at both loci might be due to sampling errors. All shrimps used in this study were wild-caught broodstocks collected from trawlers at Satun province in The Andaman Sea and they might be widely caught from the sea around this area. Since the population structure of *P. monodon* in this area has not yet been extensively studied, the samples used in the variability test might consisted of more than one population causing the deficiencies of heterozygotes (Crow, 1986). The extreme heterozygote deficit found at locus CUPmo386 may also cause by the presence of null alleles. Information on the segregation and transmission of alleles within a large number of families of *P. monodon* is required for identification of null alleles at this locus. From allozyme analysis, homozygote excess has repeatedly been observed in marine mollusc which has been explained as a form of balancing selection, where the relative fitness of homozygotes and heterozygotes is different during different phases of the life cycle (Singh and Green, 1984, Smith, 1990). Moreover, the Australian Jack mackerel, *Trachurus declivis*, showed homozygote excess at

seven out of eight allozyme loci without any relations to geographically separated stocks (Richardson, 1982). Nonetheless, the results suggested that the microsatellite loci in *P. monodon* were highly polymorphic. Weber (1990) noted that the degree of microsatellite polymorphism in humans is positively correlated with the size of the microsatellite arrays. Therefore, the large microsatellite arrays found in *P. monodon* may indicate that microsatellites, when usable, could provide highly variable genetic markers for this species.

Allelic Inheritance

Mendelian inheritance of the microsatellite locus, CUPmo18, was tested in F1 progeny. The genotypes of 10 randomly selected fry and a parental female were examined. The parental female's genotype at locus CUPmo18 was 158/120. All fry inherited either the 158 or 120 alleles from the mother (Fig. 4). From the allelic segregation of the progeny, the inferred genotype of the parental male was 144/136. This indicates the feasibility of identifying progeny group by this microsatellite locus.

The results of this study demonstrate the presence of highly polymorphic microsatellite markers in the black tiger prawn, *P. monodon*. These and other microsatellite loci will be useful in breeding programme, population genetic studies and parental determination in *P. monodon*. However, a rather low abundance and difficulties in obtaining usable dinucleotide microsatellite markers indicated that the approach as outlined here may not be appropriate for generating sufficient markers for use in genome mapping or even for applications in aquaculture or fisheries biology requiring fewer markers. Enrichment procedures, now available, for the larger microsatlellites (tri and tetra nucleotide arrays) appear to be the direction that work should focus, to generate necessary nuclear markers for this or any other species which present the type of problems encountered.

Experimental Procedures

Samples and DNA extraction

Samples of black tiger prawn (*P. monodon*) were wild-caught broodstocks collected from Satun province (South of Thailand) in the Andaman Sea. Specimens were kept frozen in liquid nitrogen and transported to the laboratory for further analysis. Genomic DNA was extracted

from pleopods of individual shrimps as described by Tassanakajon et al, 1997. DNA concentration was determined by spectrophotometric method using DU 650 Spectrophotometer (Beckman Instrument Inc.).

Library construction

Fifty micrograms of genomic DNA was digested with 25 units each of *Alu* I, *Hae* III, *Hinc* II and *Rsa*I in 250 µl reaction, according to the manufacturer 's instructions. After digestion, DNA fragments were size fractionated on 1.5% low melting agarose (Sea Kem, FMC). Genomic DNA fragments of 300 to 700 bp were excised and recovered by using a phenol freeze fracture procedure (Qian and Wilkinson, 1991). Fifty nanograms of size-selected DNA fragments were ligated with 40 ng of *Sma* I-digested and phosphatased pUC18 DNA (Pharmacia) and ligation products were transformed into MAX Efficiency DH 5α competent cells (Gibco BRL). Transformed cells were grown on LB agar plates containing 50 µg/ml ampicillin according to standard protocol (Sambrook et al., 1989.)

Library screening

Following transformation, bacterial colonies were transferred to Whatman paper #42 (Ashless), denatured in 0.5 N NaOH, 1.5 M NaCl for 7 min, neutralized in 1 M Tris-HCl, pH 7.6, 1.5 M NaCl for 3 min twice and washed with 2 X SSC, 0.2% (w/v) SDS for 30s. DNA was immobilized onto the filters by baking at 80°C for 2 h. Libraries were screened separately for (GT)n and (CT)n microsatellite repeats using synthetic $(GT)_{15}$ and $(CT)_{15}$ oligonucleotides. The oligonucleotides were 5'end-labeled with [γ-^{32}P] ATP and T4 polynucleotide kinase (Pharmacia). The filters were hybridized to the labelled oligonucleotide probe at 60°C overnight using the reaction conditions of Westneat et al. (1988). The filters were washed twice with 2xSSC, 0.2% SDS at 60°C for 30 min. each and once with 0.2xSSC, 0.2% SDS at 60°C. Microsatellite clones were identified by autoradiography and positive clones were picked from plates and grown in LB medium.

DNA sequencing and primer design

Plasmid DNA was purified by a modification of the boiling lysis procedure of Holmes and Quigley and sequenced using a T7 Sequencing kit (Pharmacia) according to the manufacturers

recommendations. PCR primers were designed from the sequences flanking the microsatellite repeat arrays using Gene Runner V.3 (Hastings Software Inc.).

PCR Analysis

For PCR analyse of locus CUPmo18, the reverse primer was 5'end-labeled wih [γ-^{32}P]ATP using T4 polynucleotide kinase (Pharmacia). Amplification was performed in a 10 µl reaction volume containing 15 ng of DNA template, 0.6 µM labeled reverse primer, 0.6 µM forward primer, 10 mM Tris-HCl, pH8.3, 1mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 0.1% Tween-20, 200 µM dNTPs and 0.25 unit of Taq Polymerase (Perkin Elmer). PCR was carried out for 7 cycles consisting of 1 min at 94°C, 30s at 55°C and 1 min at 72°C, followed by 38 cycles of 30s at 90°C, 30s at 55°C and 1 min at 72°C.

For locus CUPmo386, the forward primer was labelled and PCR conditions were the same as those of locus CUPmo18 with the exception that annealing temperature was 43°C and Tween-20 was omitted. After PCR amplification, an equal volume of formamide loading buffer was added and the product was denatured for 15 min at 95°C before being subjected to electrophoresis on 6% denaturing acrylamide gel. A standard M13 sequencing reaction was used as DNA size marker. Gel was fixed, dried and exposed to an autoradiographic film at -80°C for 16 h.

Data analysis

The informativeness of the microsatellite loci were determined as follows. The number of alleles at each locus, the proportion of individual samples that are heterozygous (direct-count heterozygosity, H_{obs}) and the unbiased estimate of heterozygosity (H_{exp} Nei, 1978), were assessed for each locus using a modified version of BIOSYS-1 program (Swofford and Selander. 1981). Hardy-Weinberg expectation test for each locus was a Markov chain "approximation to exact test" following Guo and Thompson (1992) and carried out using the GENEPOP program (Raymond and Rousset,1995).

Table 1. Microsatellite sequences isolated from various species and categorized according to Weber (1990).

	Tiger shrimp[1]		Honey bee[2]		Human[3]	Porcine[4]	Atlantic cod[5]
motif	GT	CT	GT	CT	GT	GT	GT
n	97	16	23	52	114	105	64
% perfect	24	12.5	48	46	64	71	49
% imperfect	53	75	22	31	25	19	45
% compound	23	12.5	30	23	11	10	6.3
Most common size class	30-35	12-17	7-9	10-12	12-15	16-18	6-11
Largest size class	>66	48-53	>80	22-24	27-30	28-30	>60

[1] The present study

[2] Estroup et al, 1993.

[3] Beckmann and Weber, 1993.

[4] Wintero et al., 1992.

[5] Brooker et al., 1994.

Table 2. Microsatellite sequences, PCR primers and optimized annealing temperatures for *P. monodon* microsatellites loci

Locus	Repeat units	Primer sequences	Annealing Temp. (°C)
CUPmo18	$(GT)_{60}$	f* : 5' TGTCATTCTTCTATTACGTGTC	55
		r : 5' GACTGACATCAACCATATACC	
CUPmo386	$(GT)_{30}$	f : 5' CGGTATCGGGTTAAAGAGT	43
		r : 5' TACAATGTTCATAATTCCTG	

* f = forward, r = reverse

Table 3. Allele size and heterozygosity at *P. monodon* microsatellites loci detected by PCR amplification.

Locus	sample size	observed alleles	size-range (bp)	H_{obs}*	H_{exp}**	H-W*** (*P* -value)
CUPmo18	62	28	106-162	0.66	0.95	< 0.0001
CUPmo386	48	14	132-170	0.19	0.83	< 0.0001

*observed heterozygosity

**expected heterozygosity

***probability of agreement to Hardy-Weinberg equilibrium

Figure 1. Length distribution of (GT)n and (CT)n microsatellites in *P. monodon*. The three categories of microsatellites (perfect, imperfect and compound repeats) were pooled. Number of repeats were counted from the longest uninterrupted repeats.

Figure 2. Amplification pattern of microsatellite CUPmo18. Lanes 1-20 : 20 unrelated individual *P. monodon*. The size standard is a sequencing ladder of M13 mp18.

Figure 3. Amplification pattern of microsatellite CUPmo386. Lanes 1-14 : 14 unrelated individual *P. monodon*. The size standard is a sequencing ladder of M13 mp18.

Figure 4. Microsatellite alleles of a parental female (P) and 10 randomly selected offspring. The genotypes at locus CUPmo18 are as follows : (P) 120/158; postlarvae (1) 120/144; (2) 158/136; (3) 120/136; (4) 120/136 ; (5) 158/136; (6) 158/144; (7) 120/144; (8) 120/136; (9) 120/144; (10) 158/136; inferred parental male genotype: 136/144. Size of alleles were estimated using an M13mp18 sequence ladder (not shown).

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Fig. 1

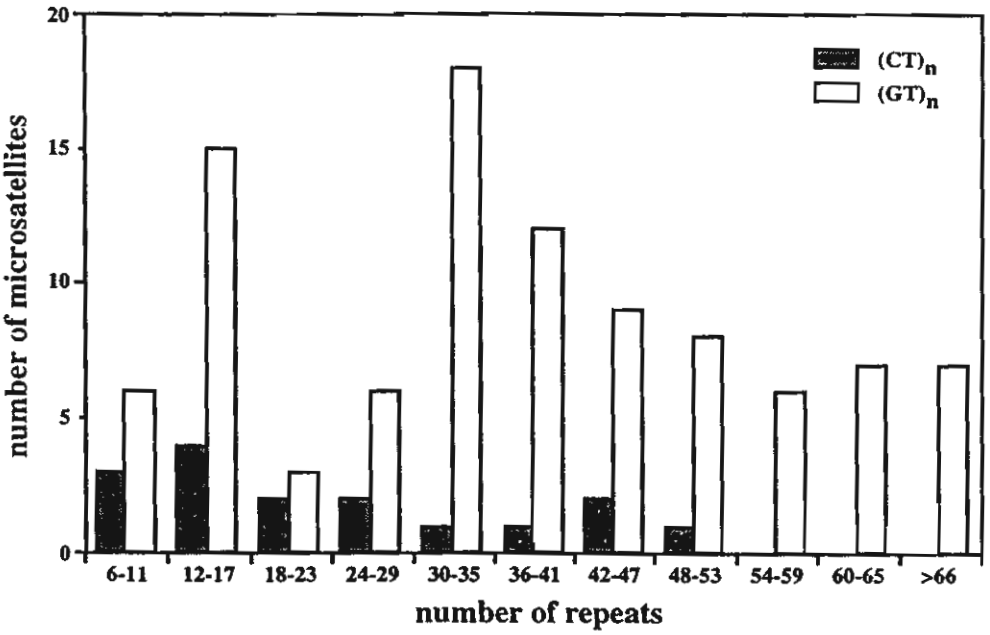
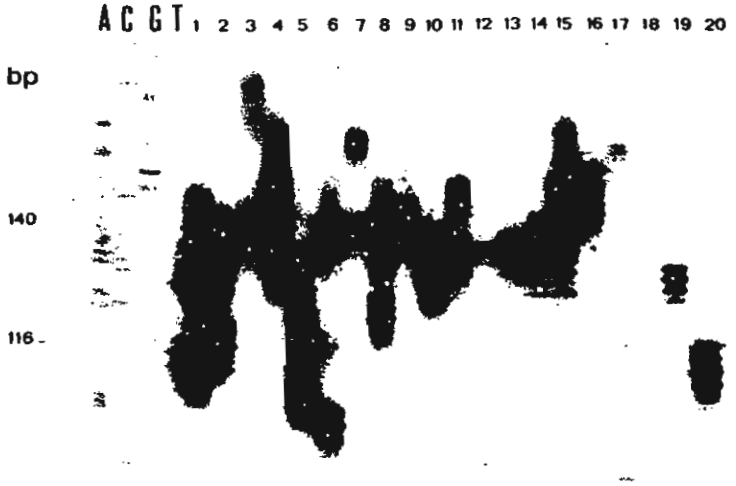
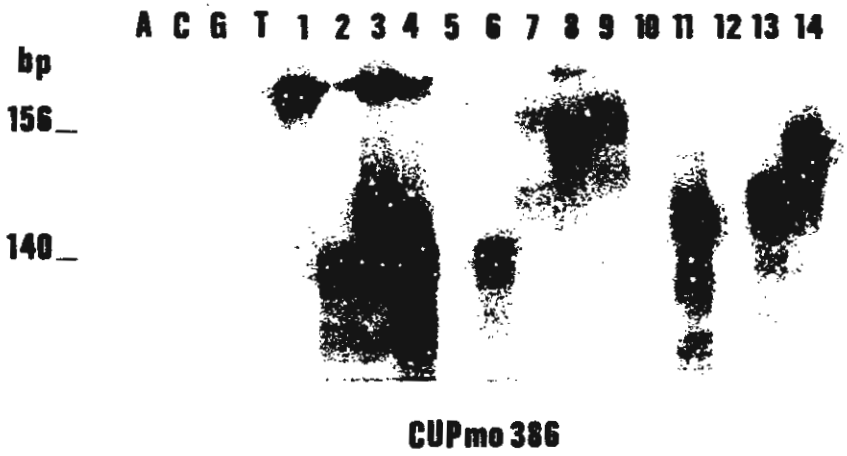

20
15
10
5
0
number of microsatellites
(CT)n
(GT)n
6-11
12-17
18-23
24-29
30-35
36-41
42-47
48-53
54-59
60-65
>66
number of repeats

Fig. 2



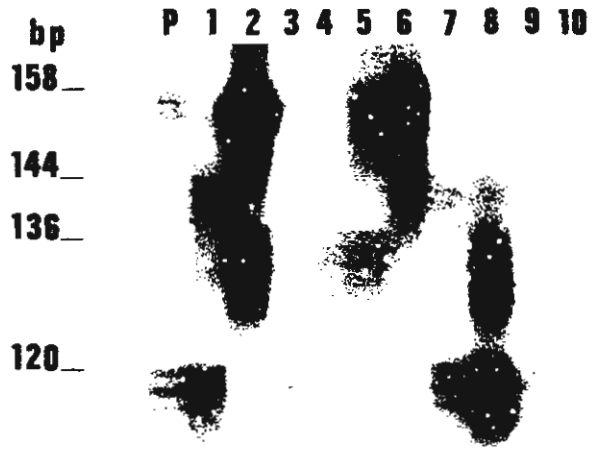
CUPmo18

Fig. 3



CUPmo 386

Fig. 4



Submitted

Genetic structure in wild populations of the black tiger shrimp (*Penaeus monodon*) using randomly amplified polymorphic DNA analysis

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Running Title : Genetic structure in *P. monodon*

Abstract. Randomly amplified polymorphic DNA analysis (RAPD) was used to examine genetic variation of wild black tiger shrimp, *Penaeus monodon*. Specimens were collected from 5 geographically separated locations of *P. monodon* consisting of Satun-Trang, Phangnga, and Medan in the Andaman Sea and Chumphon and Trad in the Gulf of Thailand.

A total of 100 *P. monodon* individuals were investigated using 7 arbitrarily selected primers. Fifty-eight (72.5%) out of 7 reproducible RAPD amplified fragments ranging in size from 200-2200 bp were polymorphic. The percentages of polymorphic bands of 5 geographic populations investigated varied from 51.5-57.7%. The genetic distance between populations and UPGMA dendrograms indicated that the Medan population was genetically different from Thai *P. monodon* (D_{ij} = 14.976%). Within Thailand, the Satun-Trang *P. monodon* was separated from the remaining geographic populations with a genetic distance of 2.632%.

RAPD analysis in the present study yielded a total of 252 genotypes. A Monte Carlo analysis illustrated geographic heterogeneity in genotype frequencies within this species suggesting that genetic population structure does exist in this taxon ($P < 0.001$ for all primers). Significant differences in genotype frequencies between Thai and Indonesian (Medan) *P.monodon* were observed ($P<0.0001$). Within Thailand, the Andaman Sea *P. monodon* was significantly different from that of the Gulf of Thailand (P-values between 0.0000 and 0.0387) indicating population differentiation between *P. monodon* from these two main fishery regions of Thailand.

Introduction

The black tiger shrimp, *Penaeus monodon* Fabricius, is one of the most economically important cultured *Penaeus* species. It accounts for 46 % of total world production for cultured marine shrimps (Hanpongkittikun et al. 1995). The main farming areas of *P. monodon* are mostly located in various tropical countries, particularly in the South East Asian region. In Thailand, the farming of *P. monodon* has expanded tremendously during the last decade and is still consistently increasing at present. As a result, Thailand has become the world's leading producer of *P. monodon* since 1991 with an average production of at least 200,000 metric tons per year (Lin and Nash 1996). However, the rapid expansion of the *P. monodon* farming activity has created several subsequent problems mainly originating from poor management resulting in an occurrence of environmental pollution and a spread of several epizootic diseases (Flegel et al. 1992). To maintain the national *P. monodon* production leading to its sustainable farming industry, proper management programs based on various scientific disciplines are required. Accordingly, a basic knowledge on genetic variation and population differentiation of *P. monodon* is essentially important for such a purpose.

Although a significant difference in allozyme frequency among Australian populations of *P. monodon* has been reported (Benzie et al. 1992), identification of genetic diversity in several Penaeid shrimp including *P. monodon* through allozyme analysis indicated a relatively low level of genetic polymorphisms and limited polymorphic allozyme markers (Lester 1978; Hedgecock et al. 1982). More genetic variation in *Penaeus* species has been detected using restriction fragment length polymorphism (RFLP) analysis of entire mitochondrial DNA

(mtDNA) (Benzie et al. 1993) and sequencing of amplified mtDNA fragments (Palumbi and Benzie 1991). Nevertheless, these approaches are tedious and time consuming.

Alternatively, randomly amplified polymorphic DNA analysis (RAPD) is a simple and rapid method to generate genetic markers without prior knowledge of the genome which is subjected to be analyzed (William et al. 1990). In this approach, a single random primer (usually 10-mers with GC content more than 50%) was utilized to randomly amplified segments of genomic DNA on the basis that the RAPD primer scans the genome for the small inverted sequences resulting in amplification of DNA segments of variable lengths. It has been successfully employed to determine genetic diversity in *P. vannamei* (Garcia et al. 1994) and *P. monodon* (Tassanakajon et al. 1997), suggesting the potential application of using this method for population genetic studies in other Penaeid species.

In the present study, genetic diversity in wild *P. monodon* was further investigated in 5 geographically separated populations of wild *P. monodon* from two main fishery regions: the Andaman Sea on the west and the Gulf of Thailand on the east coasts. The RAPD approaches were used to determine whether population differentiation actually occurs in Thai *P. monodon*.

Materials and methods

Sampling

One hundred *P. monodon* individuals were wild-caught alive from different sample sites during March 1995 to August 1996. These were from Satun-Trang (n = 17) and Phangnga (n = 20) of Thailand and Medan (n = 15) of Indonesia, which all are located in the Andaman Sea, and from Chumphon (n = 20) and Trad (n = 28) of the Gulf of Thailand (Fig. 1). Pleopods were dissected out and kept frozen in liquid nitrogen or alternatively in absolute ethanol during transportation. Specimens were stored individually at -80°C until required.

DNA extraction

Total DNA of *P. monodon* was extracted from abdominal pleopods using the proteinase-K/phenol-chloroform isolation method described in Tassanakajon et al. (1997). DNA concentration was determined spectrophotometrically using a DU 650 Spectrophotometer (Beckman Instruments Inc.).

PCR amplifications and analysis of the resulting products

DNA amplification was performed in a Perkin-Elmer Cetus DNA Thermal Cycler (model 2400). PCR amplification reactions were carried out in a 25 µl reaction volume containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatin, 100 µM each of dATP, dCTP, dGTP and dTTP, 0.2 µM RAPD primer, 50 ng genomic DNA and 1 unit of *Taq* DNA polymerase (William et al. 1990). Ten-base oligonucleotide primers were obtained from the

Biotechnology Laboratory, University of British Columbia. The PCR reaction was carried out for 35 cycles consisting of 5 s at 94°C, 45 s at 36°C and 90 s at 72°C. The amplification products were then electrophoretically analyzed in 1.6% agarose gels and visualized by ethidium bromide staining (Maniatis et al. 1982).

Data analysis

All reproducible and resolvable bands ranging from 200-2200 bp were scored from the gel. The presence (1) or absence (0) of an amplified fragment was treated in a dominant fashion without further consideration of the quantitative aspect of the results (i.e., band intensity differences between homo- and heterozygotes).

The index of similarity between individuals was calculated using the formula $S_{xy}=2n_{xy}/n_x+n_y$, where n_x and n_y represent the number of fragments scored for individual x and y, respectively, and n_{xy} is the number of fragments shared by both individuals. Within population similarity (S) is calculated as the average of S_{xy} across all pairwise comparisons between individuals within a population. Between population similarity with a correction for within population similarity is $S_{ij} = 1+S'_{ij}-0.5\ (S_i+S_j)$, where S_i and S_j represent the S estimates for population i and j, respectively, and S'_{ij} is the average similarity between random pairs of individuals across populations i and j (Lynch 1990).

S_{ij} was then converted to a measure of genetic distance (D_{ij}) using the equation $D_{ij} = -\ln[S'_{ij} /\sqrt{(S_iS_j)}]$, (Lynch 1991). A phenogram based on this estimate was constructed using the unweight pair-group method with arithmetic average (UPGMA) of Phylip version 3.57c (Felsenstein, 1991)

A RAPD pattern is referred to as a genotype. A geographic heterogeneity test of significance in genotype frequencies between populations or regions was carried out using a Monte Carlo simulation (Roff and Bentzen, 1989) implemented in REAP (McElroy et al. 1991). Results are expressed as the probability of homogeneity between compared populations or regions.

Results

RAPD analysis

Seven arbitrarily selected primers 101, 174, 268, 428, 456, 457 and 459, originated from the University of British Columbia Biotechnology Laboratory, were used in RAPD analysis of 100 *P. monodon* individuals collected from 5 geographically separated locales. A total of 80 scorable bands ranging from 200-2200 bp in size were unambiguously scored (Table 1). Twenty-two of these bands (27.5%) were monomorphic (present in at least 95% of all investigated individuals) and 58 bands (72.5%) were polymorphic (present in less than 95%). Each primer generated between 9-15 scorable bands. The complexity of the banding patterns varied dramatically between primers. It should be noted that primer 456 gave the highest number of amplified bands (15 bands) but yielded the least polymorphic level (60.0%). On the other hand, primers 268 and 428 yielded the highest level of polymorphism which was 88.9 %. The investigated *P. monodon* individuals from Satun-Trang (n = 17), Phangnga (n = 20), Chumphon (n = 20), Trad (n = 28) and Medan (n = 15) produced 71, 66, 64, 69 and 73 scorable bands resulting in 57.7, 51.5, 54.7, 52.2 and 53.4% of polymorphic bands, respectively (Table 2).

RAPD patterns of Thai *P. monodon* (Satun-Trang, Phangnga, Chumphon and Trad) are clearly different from those of *P. monodon* from Medan. An illustration of RAPD patterns for the 5 geographic populations and the bands scored are shown in Fig 2.

Similarity index within and between populations

The average genetic identity within populations (S) across all the primers ranged from 0.8626 to 0.8878 (Table 2). The highest similarity index within population was found in *P. monodon* from Phangnga (0.8878) whereas slightly lower similarity indices were observed in Chumphon, Trad, Satun-Trang and Medan at 0.8785, 0.8646, 0.8655 and 0.8626, respectively.

The average genetic similarity between populations (S_{ij}) across all the primers was from 0.7548 to 1.0026 (Table 3). While the S_{ij} among *P. monodon* originating from Thailand was not drastically different, such a value, however, indicated that the Thai and Medan *P. monodon* populations seem to be reproductively isolated.

Genetic distance and dendrogram

Genetic distance (D_{ij}) was converted from a measure of between-population similarity corrected by the within-population values (Nei 1972; Lynch 1990) and is shown in Table 3. These values were then subjected to phylogenetic reconstruction. The UPGMA dendrogram resulted from overall primers, indicating three distinctly phylogenetic-separated groups composed of group 1 (Phangnga, Chumphon, and Trad), group 2 (Satun-Trang) and group 3 (Medan) (Fig 3).

Large genetic differences were surprisingly observed between Medan and all of the Thai samples (14.976%). Within Thailand, the Satun-Trang *P. monodon* was separated from the remaining populations with a genetic distance of 2.6%.

Heterogeneity in genotype frequency distributions.

In the present study, 252 genotypes were observed from a total of 100 individuals using 7 decanucleotide RAPD primers. For overall populations, a Monte Carlo simulation showed statistically significant differences in RAPD genotype heterogeneity for all single primers, indicating that population subdivisions do exist in *P. monodon* ($P<0.0001$ for primers 101, 268, 428, 457 and 459, and $P = 0.0001$ for 174 and 456).

For each primer, highly significant differences in genotype frequencies were also observed between the Thai and Medan *P. monodon* ($P<0.0001$). Moreover, heterogeneity in genotype frequencies between Thai *P. monodon* from the Gulf of Thailand and the Andaman Sea was significantly observed for primers 101, 268, 428, 457 and 459 ($P = 0.0201$, 0.0001, <0.0001, 0.0387 and <0.0001, respectively). Interestingly, primer 428 gave significant results for geographic heterogeneity among all 4 geographic samples of the Thai *P. monodon* (P-values between 0.0000 and 0.0403). In contrast, primers 174 and 456 gave no significant geographic heterogeneity for all pairwise comparisons among all *P. monodon* populations from Thailand (P-values between 0.0924 and 0.9530).

Discussion

A basic knowledge of DNA polymorphisms and molecular markers of *P. monodon* is essential for its breeding programs (Tassanakajon et al. 1997). At present, only allozyme variability has been extensively used for population genetic studies in Penaeid species. Nevertheless, genetic heterogeneity detected by allozyme electrophoresis in these taxa, especially for *P. monodon*, is quite low (mean heterozygosity was 0.073 for 97 crustacean species and 0.0276 for *P. monondon* collected from 10 geographic locales in South East Asia), limiting its application to be further employed in selective breeding programs (Hedgecock et al. 1982; Daud 1995; Sodsuk 1996). A few genetic polymorphism studies based on the DNA approach have also been reported in *P. monodon* and generally shown a greater level of genetic variability than that from protein electrophoresis (Benzie et al 1993; Garcia et al. 1994; Sodsuk 1996).

In the present study, the RAPD technique was used to evaluate the extent of genetic variability in wild populations of *P. monodon* collected from 5 different geographic locales covering the main supplying sources of wild *P. monodon* broodstock for culture activity around the Malaysian Peninsula. Seven out of 300 RAPD 10-mers screened yielded high strength and consistent RAPD patterns with resolvable fragments (Tassanakajon et al. 1997). Nevertheless, to simplify the experiments, all primers were selected based on our "standard amplification program" and we did not attempt to further standardize such selected primers. This allows us, in practice, to employ the 7 selected primers in our selective breeding programme of *P. monodon* at Chulalongkorn University easily and conveniently. Theoretically, it may be argued that the RAPD approach can give a biased datum in population studies of *P. monodon* limiting its further application in "real practice".

Nevertheless, determination of the mode of inheritance (e.g., from our half-sib families) and verifying homology among co-migrating fragments from different populations of *P. monodon* will certainly result in more valid and stable data for breeding programs (Dowling et al. 1996).

The percentages of polymorphic bands of *P. monodon* in this study varied from 51.5-57.7%. This polymorphic level was about as high as those previously reported in *P. vannamei* (39-77%) (Garcia et al. 1994). Based on this estimate, Satun-Trang has the highest level of genetic diversity within a population. However, this estimate cannot be used as a standard among laboratories and should be treated with caution. The RAPD approach detected abundance polymorphisms in *P. monodon* revealed by a total of 252 genotypes from 7 different primers, of which 104 were private genotypes (found in only one population). The numbers of RAPD genotypes in Satun-Trang, Phangnga, Chumporn, Trad and Medan were 65, 58, 63, 85 and 50 genotypes, respectively.

The band sharing approach based on the presence or absence of the amplified DNA fragments was used to estimate similarity indices. The mean estimated similarity within each population was high, reflecting a high similarity of compared sequences. The Phangnga *P. monodon* showed the highest level of within population similarity whereas the remaining populations showed similar levels.

Similarity between populations (S_{ij}) was estimated with a correction value. As a result, the estimated level can be greater than 1 if sequence similarity between populations was higher than that within populations. The Medan population showed an extremely large difference in between population similarity when compared to all of the Thai *P. monodon*. This indirectly indicates that *P. monodon* from Medan should be regarded as a different stock.

The UPGMA dendrogram indicated an ancestral relationship shared among Phangnga, Chumphon and Trad whereas Satun-Trang from the Andaman Sea showed a reasonable genetic distance from the rest of Thai *P. monodon*. More importantly, Satun-Trang is located approximately 200 hundred kilometers far from Medan on the same coast, but a clear "genetic break" was observed between the former and the latter. This may be explained by the limitation of gene flow caused by the surface currents of the Strait of Malacca which serves as an overflow channel during both northeast and southwest monsoons (Dale 1956).

Geographic heterogeneity analysis illustrated highly significant differences in genotype frequencies among all Thai and Medan *P. monodon*. This was concordant with an extremely high F'estimate (0.4810 for overall populations and 0.2104 for Thai *P. monodon*). Moreover, it was clear that all but primers 174 and 456 was able to statistically significant distinguish *P. monodon* populations from the Andaman Sea and the Gulf of Thailand. These results suggested the existence of genetic population differentiation between two regions of Thai *P. monodon*. Surprisingly the Phangnga population located in the Andaman Sea was clustered as a sister taxon to Chumphon's from the Gulf of Thailand in the UPGMA dendrogram. Nonetheless, an inability to discriminate their genotype heterogeneity was also observed when using primer 174 (P = 0.1825), 268 (P = 1.0000), 456 (0.4409) and 457 (P = 0.0526). Sodsuk (1996) studied genetic polymorphisms of *P. monodon* in South East Asia using mtDNA-RFLP of 4 restriction enzymes (*Bam* HI, *Eco* RV, *Pvu* II and *Sac* I) with small sample sizes (N = 9.3 in average). His data indicated geographic homogeneity among the east and west coast populations (e.g., between Medan and Trad and between Surat of the east and Phuket of the west Malaysian Peninsula). However, significant geographic heterogeneity among these was observed with larger individuals of the same sample set (N = 20.6) and a higher number

of restriction endonucleases (10 hexameric and 1 pentameric restriction enzymes) (Klinbunga 1996). Accordingly, the unexpected historical and current relationships among geographically different *P. monodon* from Chumphon and Phangnga needs to be clarified by a larger number of RAPD primers. Moreover, to obtain more reliable results, a larger number of sample sizes may also be required.

Five out of 7 primers used in the present study showed significant differences between *P. monodon* from the Gulf of Thailand and the Andaman Sea. Only primer 428 showed genetic heterogeneity for all *P. monodon* populations used in this study implying the promising ability to developed population or region specific marker(s) through the RAPD approach. Moreover, the results from primer 428 strongly supported an occurrence of genetic population structure between the 4 geographically separated Thai *P. monodon* which is in accord with the overview results when microsatellite loci are employed (Tassanakajon, unpublished data). The Monte Carlo simulation analysis indicated that primers 174 and 456 did not yield any informative results which were possibly due to amplification of conserved regions in the *P. monodon* genome by such primers.

High levels of polymorphisms and the existence of population differentiation of *P. monodon* found in the present study showed that RAPD markers are one of the most suitable tools for population genetic studies and for establishing the breeding program in this economically important species.

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Table 1. Sequences of 7 decanucleotide primers used in this experiment, numbers of amplified bands and their size ranges (bp) observed from RAPD analysis of *P. monodon*.

Primer No.	Sequences	Size-range (bp)	No. of amplified bands	No. of polymorphic bands
101	GCGCCTGGAG	1800-420	13	10(76.9%)
174	AACGGGCAGG	1500-450	10	7(70.0%)
268	AGGCCGCTTA	1300-400	9	8(88.9%)
428	GGCTGCGGTA	1800-200	9	8(88.9%)
456	GCGGAGGTCC	2200-260	15	9(60.0%)
457	CGACGCCCTG	2000-350	13	9(69.2%)
459	GCGTCGAGGG	1550-430	11	7(63.6%)
Total			80	58(72.5%)

Table 2. Total numbers of bands, percentages of polymorphic bands and similarity estimates within populations (average for all primers) observed in 5 conspecific populations of *P. monodon*.

Sample site	Total no. of band observed	No. of monomorphic/ polymorphic bands	Percentage of polymorphic bands	Similarity index within populations (S ± SD)
Satun-Trang	71	30/41	57.7	0.8655 ± 0.0437
Phangnga	66	32/34	51.5	0.8878 ± 0.0691
Chumphon	64	29/35	54.7	0.8785 ± 0.0587
Trad	69	33/36	52.2	0.8646 ± 0.0557
Medan	73	34/39	53.4	0.8626 ± 0.0705

Table 3. Pairwise comparisons of genetic distance (above diagonal) and similarity between populations (below diagonal) across all investigated primers for 5 geographic populations of *P. monodon*

Population	Satun-Trang	Phangnga	Chumphon	Trad	Medan
Satun-Trang	-	0.0296	0.0661	0.0622	0.2812
Phangnga	0.9744	-	0.0029	0.0299	0.2888
Chumphon	0.9442	1.0026	-	0.0354	0.2941
Trad	0.9478	0.9742	0.9697	-	0.3340
Medan	0.7882	0.7804	0.7782	0.7548	-

Fig.1. Map showing sampling sites (shaded) for five geographic *P. monodon* populations used in this study.

Fig. 2. RAPD patterns using primer 268. Lane M: 100 bp DNA ladder; a, *P. monodon* individuals from Medan; b, *P. monodon* individuals from Satun-Trang; c, *P. monodon* individuals from Phangnga; d, *P. monodon* individuals from Chumphon; e, *P. monodon* individuals from Trad. Lines indicate bands that were unambiguously scored.

Fig. 3. UPGMA dendrogram showing the relationships among 5 geographically different populations of *P. monodon*, generated according the distance matrix in Table 3.

Fig. 1


LAOS
MYANMAH
THAILAND
Trad
CAMBODIA
ANDAMAN
SEA
VIETHAM
Chumphon
GULF OF
THAILAND
Phangnga
Trang
Satun
STRAIT OF
MALACCA
MALAYSIA
Medan
SUMATRA

Fig. 2

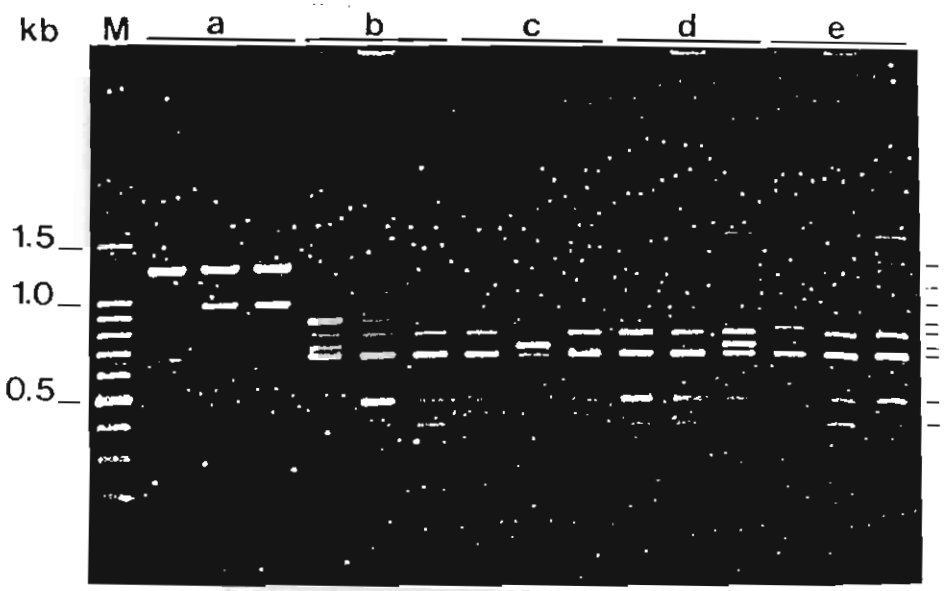
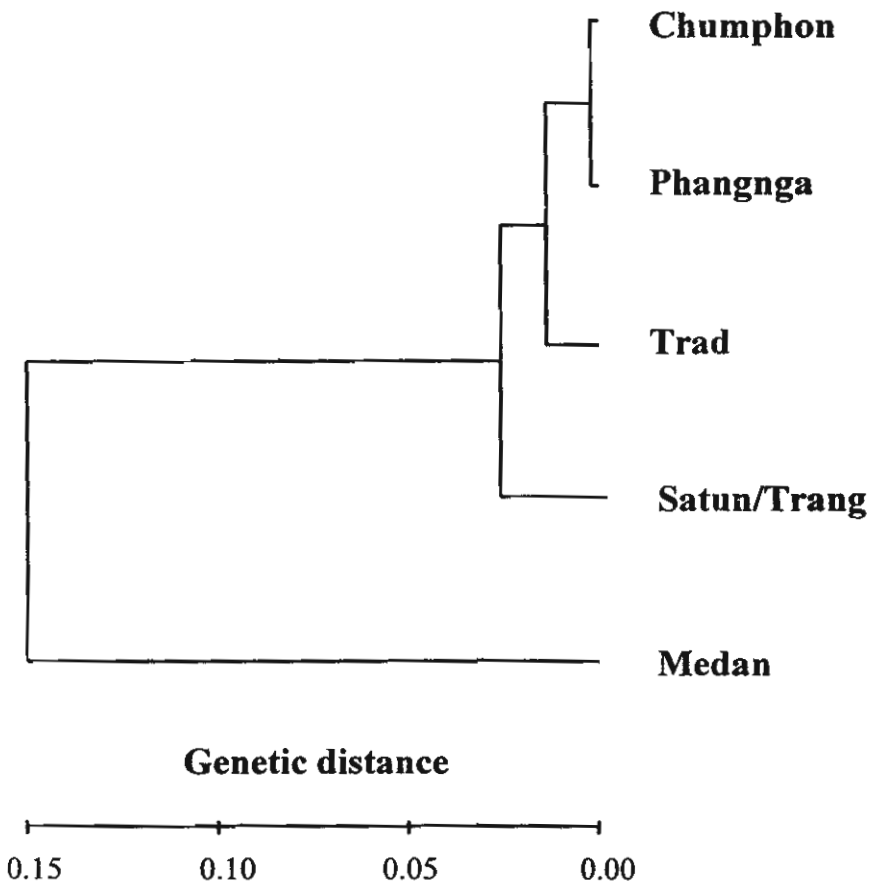


Fig. 3


Chumphon
Phangnga
Trad
Satun/Trang
Medan
Genetic distance
0.15
0.10
0.05
0.00