ความหลากหลายทางพันธุกรรมและโครงสร้างประชากรของปูม้า *Portunus pelagicus* ในประเทศไทย

นางสาว เนตรชนก ธรรมเนียมดี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปีการศึกษา 2550

GENETIC DIVERSITY AND POPULATION STRUCTURE OF THE BLUE SWIMMING CRAB *Portunus pelagicus* IN THAILAND

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biotechnology
Faculty of Science
Chulalongkorn University
Academic Year 2007

Thesis Title	GENETIC DIVERSITY AND POPULATION STRUCTURE OF THE BLUE SWIMMING CRAB *Portunus pelagicus* IN THAILAND
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เนตรชนก ธรรมเนียมดี : ความหลากหลายทางพันธุกรรมและโครงสร้างประชากรของปูม้า *Portunus pelagicus* ในประเทศไทย (GENETIC DIVERSITY AND POPULATION STRUCTURE OF THE BLUE SWIMMING CRAB *Portunus pelagicus* IN THAILAND)
อ. ที่ปรึกษา: ศ.ดร.เปี่ยมศักดิ์ เมนะเศวต, อ.ที่ปรึกษาร่วม: ดร.ศิราวุธ กลิ่นบุหงา 152 หน้า

วิเคราะห์ความหลากหลายทางพันธุกรรมและโครงสร้างประชากรของปูม้า *(Portunus pelagicus)*ในประเทศไทยด้วยเทคนิค SSCP โดยนำไพร์เมอร์จำนวน 38 คู่มาทำปฏิกิริยาพีซีอาร์กับดีเอ็นเอของปูม้า (N = 3) พบว่า *cytochrome oxidase subunit I* (*COI*), M122/135RAP และ $P_{13}4M_{13}1\_454$ ให้แถบดีเอ็นเอที่ชัดเจน จึงทำการโคลนและหาลำดับนิวคลีโอไทด์ของผลิตภัณฑ์พีซีอาร์ที่ได้และออกแบบไพร์เมอร์ที่จำเพาะกับลำดับนิวคลีโอไทด์ของชิ้นดีเอ็นเอดังกล่าว (PP-COI_{270}-F/R, PP-$SCARRAP_{318}$-F/R และ PP-$SCARAFLP_{300}$-F/R ตามลำดับ) ทำการทดสอบกับตัวอย่างปูม้าจากจันทบุรี (N = 29) ประจวบคีรีขันธ์ (N = 40) สุราษฎร์ธานี (N = 35) ระนอง (N = 35) และกระบี่ (N = 35) จำนวนรวมทั้งสิ้น 174 ตัว ด้วยเทคนิค single strand conformational polymorphism (SSCP) พบแถบดีเอ็นเอจำนวน 9, 19 และ 7 แถบ และพบรูปแบบ SSCP ทั้งหมดจำนวน 8, 56 และ 21 รูปแบบจาก *PP-*COI_{270}, *PP-*$SCARRAP_{318}$ และ *PP-*$SCARAFLP_{300}$ ตามลำดับ จากจำนวนรูปแบบของ SSCP ที่พบบ่งชี้ได้ว่าปูม้าในประเทศไทยมีความหลากหลายทางพันธุกรรมภายในชนิดสูง โดยพบค่าเฉลี่ยความเหมือนทางพันธุกรรมระหว่างกลุ่มตัวอย่างเท่ากับ 0.8871 - 0.9902 และพบความแตกต่างทางพันธุกรรมระหว่างกลุ่มตัวอย่างเท่ากับ 0.0099 - 0.1198 ซึ่งความแตกต่างของกลุ่มตัวอย่างระหว่างฝั่งทะเล (0.0346 - 0.1198) มีมากกว่ากลุ่มตัวอย่างที่อยู่ฝั่งทะเลเดียวกัน (0.0099 – 0.0198 และ 0.0135) และพบการแบ่งแยกทางพันธุกรรมของตัวอย่างปูม้าที่ทำการศึกษาอย่างมีนัยสำคัญทางสถิติ ($P < 0.0001$) แสดงว่าของปูม้าในประเทศไทยจากแหล่งต่างๆไม่ได้มีอีนพูลเดียวกัน แต่มีการแบ่งแยกเป็นหลายกลุ่มประชากรพันธุศาสตร์ ทั้งนี้สามารถคิดค่าอีนโฟลของปูม้าในประเทศไทยได้เท่ากับ 0.39 - 5.37 ตัวต่อรุ่น เมื่อนำค่าระยะห่างทางพันธุกรรมของแต่ละคู่กลุ่มตัวอย่างไปสร้างแผนภูมิ UPGMA พบว่าสามารถแยกตัวอย่างที่ศึกษาออกได้เป็น 2 กลุ่มวิวัฒนาการตามฝั่งทะเลของประเทศไทย คือ จันทบุรี สุราษฎร์ธานี และ ประจวบคีรีขันธ์ (ฝั่งทะเลอ่าวไทย กลุ่ม A) กับ ระนองและกระบี่ (ฝั่งทะเลอันดามัน กลุ่ม B)

นอกจากนี้ได้ทำการพัฒนาเครื่องหมายพันธุกรรมที่จำเพาะกับปูม้าชนิด *P. pelagicus* โดยโคลนและหาลำดับนิวคลีโอไทด์ของยีน *COI* ซึ่งมีขนาด 706 คู่เบส และ *12S rDNA* ซึ่งมีขนาด 406 คู่เบส ทำการออกแบบไพร์เมอร์จากลำดับนิวคลีโอไทด์ที่ได้และทดสอบความจำเพาะของไพรเมอร์ที่พัฒนา (PP-COI_{270}-F/R สำหรับ *COI* และ PP-$12S_{312}$-F/R สำหรับ *12S rDNA*) กับ genomic DNA ของปูครอบครัว *Portunidae* ชนิดต่างๆ โดยเครื่องหมาย *PP-*COI_{270} นั้นให้ผลิตภัณฑ์พีซีอาร์ขนาด 270 คู่เบส เฉพาะในปูม้า *P. pelagicus* (N = 174) แต่ไม่ให้ผลิตภัณฑ์ดังกล่าวในปูทะเล (*Scylla oceanica*, N = 18, *S. serrata*, N = 7, *S. tranquebarica*, N= 9) ปูลาย *Charybdis crucifera* (N = 20) และปูดาว *P. sanguinolentus* (N = 10) สำหรับเครื่องหมาย *PP-*$12S_{312}$ นั้นให้ผลิตภัณฑ์พีซีอาร์ขนาด 312 คู่เบส ในทุกตัวอย่างของปูม้า *P. pelagicus* (N = 174) และทุกตัวอย่างของปูชนิดอื่นๆ จึงนำผลิตภัณฑ์พีซีอาร์ของปูชนิดต่างๆ มาวิเคราะห์ความแตกต่างของลำดับนิวคลีโอไทด์ด้วยเทคนิค SSCP พบรูปแบบ SSCP จำนวน 5 แบบในปูม้า (N = 174) โดยรูปแบบที่พบมากที่สุดคิดเป็น 91.4% (159/174) ของตัวอย่างที่ศึกษา โดยรูปแบบ SSCP ของปูม้าแตกต่างไปจากรูปแบบที่พบในปูทะเล ปูลาย และปูดาว เครื่องหมายไมเลกุลที่พัฒนาได้สามารถนำไปตรวจสอบและยืนยันผลิตภัณฑ์ของปูม้าจากประเทศไทย

นอกจากนี้ได้ทำการสืบค้นยีนที่มีการตอบสนองต่ออุณหภูมิในเลือดของปูม้า *P. pelagicus* ด้วยเทคนิค cDNA-AFLP โดยทำการคัดเลือกไพร์เมอร์ทั้งสิ้น 110 คู่ และใช้ cDNA ที่สกัดจากเลือดปูม้าเป็นต้นแบบ โดยศึกษาเปรียบเทียบระหว่างกลุ่มควบคุม (N = 4) กับกลุ่มทดลองที่มีการกระตุ้นโดยอุณหภูมิที่ 33 องศาเซลเซียส เป็นเวลา 3 ชั่วโมงและทำการเก็บเลือดหลังถูกกระตุ้นไปแล้วเป็นเวลา 0, 3, 6, 12 และ 24 ชั่วโมงตามลำดับ (N = 4 ในแต่ละกลุ่มตัวอย่าง) ได้เลือกชิ้น cDNA-AFLP ที่มีการตอบสนองต่ออุณหภูมิจำนวนทั้งหมด 7 ชิ้นไปโคลนและหาลำดับนิวคลีโอไทด์ จากนั้นออกแบบไพร์เมอร์ที่จำเพาะ และศึกษาระดับการแสดงออกของยีน *PP-*$TSRT_{152}$, *PP-*$TSRT_{200}$ และ *PP-*$TSRT_{339}$ ด้วยวิธี semiquantitative RT-PCR พบว่า *PP-*$TSRT_{152}$ และ *PP-*$TSRT_{339}$ มีการแสดงออกลดลงอย่างมีนัยสำคัญทางสถิติที่ 12 และ 24 ชั่วโมง และ 12 ชั่วโมง หลังการกระตุ้นด้วยอุณหภูมิตามลำดับ ($P < 0.05$)

ลายมือชื่อ.....

สาขาวิชา.....เทคโนโลยีชีวภาพ.....ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา.....2550.....ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

4872340023 : MAJOR BIOTECHNOLOGY
KEY WORD: *Portunus pelagicus* / SSCP / GENETIC DIVERSITY / SCAR MARKERS

NATECHANOK THAMNIEMDEE: GENETIC DIVERSITY AND POPULATION STRUCTURE OF THE BLUE SWIMMING CRAB *Portunus pelagicus* IN THAILAND
THESIS ADVISOR: PROF. PIAMSAK MENASVETA, Ph.D.
THESIS CO-ADVISOR: SIRAWUT KLINBUNGA, Ph.D., 152 pp.

Genetic diversity and population differentiation of the blue swimming crab (*Portunus pelagicus*) in Thai waters was examined by SSCP analysis. Thirty eight primers pairs were initially tested against genomic DNA of *P. pelagicus* ($N = 3$). The positive amplification product of *cytochrome oxidase subunit I* (*COI*), M122/135RAP and $P_{+3}4M_{+3}1\_454$ was cloned and sequenced. A pair of primer was designed from each sequence (called PP-COI_{270}-F/R, PP-$SCARRAP_{318}$-F/R and PP-$SCARAFLP_{300}$-F/R, respectively) and tested against *P.pelagicus* from Chanthaburi ($N = 29$), Prachuap Kriri Khan ($N = 40$), Suratthani ($N = 35$), Ranong ($N = 35$) and Krabi ($N = 35$). SSCP analysis was carried out. A total of 9, 19 and 7 SSCP fragments were found across overall investigated individuals and generated 8, 56 and 21 SSCP genotypes, respectively. Large numbers of SSCP genotypes found in Thai *P. pelagicus* suggested high genetic diversity in this species. The average genetic identity between pairs of geographic samples was 0.8871 - 0.9902. Genetic distance between pairs of geographic samples was 0.0099 - 0.1198. Generally, larger genetic distance was observed between samples from different coastal regions (0.0346 - 0.1198) than that between geographic locations within coastal regions (0.0099 – 0.0198 and 0.0135 for Gulf of Thailand and Andaman samples, respectively). Significant geographic heterogeneity was observed across overall samples ($P < 0.01$ for F_{ST} based statistics, θ and $P < 0.0001$ for exact test) suggested that the gene pool of *P. pelagicus* in Thai waters is not panmictic but genetically fragmented at the microgeographic level. The estimated gene flow level of Thai *P. pelagicus* was 0.39 – 5.37 individuals per generation. A UPGMA dendrogram constructed from the average unbiased genetic distance between pairs of geographic samples allocated 5 geographic samples to 2 evolutionary lineages; Chanthaburi, Suratthani and Prachuap Kriri Khan (Gulf of Thailand, A) and Ranong and Krabi (Andaman Sea, B).

In addition, species-diagnostic markers for authentication of *P. pelagicus* were successfully developed. Initially, *cytochrome oxidase subunit I* (*COI*, 706 bp) and *12S ribosomal* (*r*) *DNA* (406 bp) gene segments of *P. pelagicus* were amplified using universal primers. These gene segments were cloned and sequenced. Gene-specific primers were designed (PP-COI_{270}-F/R for *COI* and PP-$12S_{312}$-F/R for *12S rDNA*) and tested for the species specificity against genomic DNA of various species. The expected product of *PP-COI_{270}* (270 bp) was specifically found in all individuals of *P. pelagicus* ($N = 174$) but not in the mud crabs (*Scylla oceanica*, $N = 18$, *S. serrata*, $N = 7$, *S. tranquebarica*, $N = 9$), the swimming crab, *Charybdis crucifera* ($N = 20$) and the three spot swimming crab, *P. sanguinolentus* ($N = 10$). The expected product of *PP-$12S_{312}$* (312 bp) was 100% successfully amplified in *P. pelagicus* ($N = 174$) and all non-target species. The PCR product of all investigated species was further analyzed by SSCP. Five SSCP genotypes were found in *P. pelagicus* and the most common genotype was observed in 91.4% (159/174 individuals) of investigated specimens. Non-overlapping SSCP genotypes were found in other crab species. These molecular markers can be directly applied to authenticate the products of *P. pelagicus* from Thailand.

Temperature-stress response transcripts (*TSRT*) in haemocytes of *P. pelagicus* were identified by cDNA-AFLP. A total of 110 primer combination were screened using cDNA of haemocytes of normal crab ($N = 4$) and those at 0, 3, 6, 12 and 24 hours ($N = 4$ for each group) after temperature stressed at 33°C for 3 hours. Seven *TSRT* fragments were cloned and sequenced. Expression levels of three *TSRTs* (*PP-$TSRT_{152}$*, *PP-$TSRT_{200}$* and *PP-$TSRT_{339}$*) were examined by semiquantitative RT-PCR. *PP-$TSRT_{152}$* and *PP-$TSRT_{339}$* were significantly down-regulated at 12 and 24 hpt and 12 hpt, respectively ($P < 0.05$).

Student's signature Natechanok Thamniemdee.

Field of study.....Biotechnology.....Advisor's signature.....

Academic year.....2007.....Co-advisor's signature.....

ACKNOWLEDGEMENTS

I would like to express my deepest sense of gratitude to my advisor, Prof. Dr. Piamsak Menasveta, and my co-advisor, Dr. Sirawut Klinbunga for their great helps, guidance, encouragement, valuable suggestion and supports throughout my study.

My gratitude is also extended to the thesis committee; Assist. Prof. Dr. Charoen Nitithamyong, Assist. Prof. Dr. Thaithaworn Lirdwitthayaprasit and Dr. Bavornlak Khamnamtong for their recommendations and useful suggestion.

I would like to acknowledge Center of Excellence for Marine Biotechnology (CEMB), Faculty of Science, Chulalongkorn University for providing facilities and National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA) for a studentship.

In addition, I would also like to extend my special thanks to Miss Kannika Khetpu, Miss Rungnapa Leelatanawit, Mr. Arun Buaklin and Miss Ratchanimuk Preechapol for their great suggestion about my works. I also thank Miss Kanchana Sittikhankeaw and Miss Sirikan Prasertlux for best friendship and great help during my research in our laboratory.

Many thanks are extended to members of CEMB and Program in Biotechnology, Chulalongkorn University for their helps and kindness that give a funny time during my study periods.

Finally, I would like to express my deepest gratitude to my beloved parents, my sister and my closest friends; Mr. Natthapong Pobsamai and Miss Chiraporn Waimittra, for their love, care, understanding and encouragement extended throughout my live.

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LIST OF ABBREVIATIONS

bp	base pair
^{o}C	degree Celcius
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DEPC	dethylpyrocarbonate
DNA	deoxyribonucleic acid
HCl	hydrochloric acid
IPTG	isopropyl-thiogalactoside
Kb	kilobase
M	Molar
$MgCl_2$	magnesium chloride
mg	milligram
ml	milliliter
mM	millimolar
ng	nanogram
OD	optical density
PCR	polymerase chain reaction
rpm	revolution per minute
RNA	ribonucleic acid
RNase A	ribonuclease A
RT	reverse transcription
SDS	sodium dodecyl sulfate

Tris	tris (hydroxyl methyl) aminomathane
μg	microgram
μl	microlitre
μM	micromolar
UV	ultraviolet

CHAPTER I

INTRODUCTION

1.1 General introduction

The blue swimming crab, *Portunus pelagicus* (Linnaeus, 1758), is a large, edible species inhabiting coastal marine and estuarine waters throughout the Indo-West Pacific region, from east Africa to Japan and northern New Zealand (Stephenson, 1962, Kailola *et al.,* 1993). In Thailand, *P. pelagicus* is continuously distributed along the coastal lines (< 50 meters in depth) of both the Andaman Sea and the Gulf of Thailand (Naiyanetr, 1998).

The blue swimming crab is one of economically important aquatic species in Thailand. The catch volumes and production values of the blue swimming crab in Thai waters were greater than those of mud crab and other marine crab species (Table 1.1 and 1.2). Nevertheless, commercial fisheries of this species have reduced since the last few years. The catch volume of the blue swimming crab in the Gulf of Thailand was reduced from 37219 metric tones (MT) in 2000 to 22113 MT in 2004 but that in the Andaman Sea was consistent during the same period (6652-9549 MT). The production value of this species was estimated to be at least 2000 million baht annually (Table 1.2). The main exported markets of the blue swimming crab are USA, Canada, France, Japan, Vietnam, Australia, Hong Kong and China.

An occurrence of an increasingly large proportion of small sizes of wild-caught *P. pelagicus* at present suggests overexploitation of this species. Currently, commercial cultivation of *P. pelagicus* has successfully been developed in Thailand (Klinbunga *et al*., 2007). This would significantly reduce the heavily commercial harvest of natural *P. pelagicus* that may result in stock collapse.

Fishery management has been defined as the application of scientific knowledge to the problems of providing the optimum yield, which is prescribed on the basis of maximum sustainable yield of commercial fisheries products (Allendorf *et al*., 1987). The proper management requires a basic understanding of biological

Table 1.1 Catch and value (in brackets) of marine crabs (including coastal aquaculture) from the Gulf of Thailand and Andaman Sea during 2000-2004

Species	Volume (ton) and value (million Baht)									
	Gulf of Thailand					Andaman Sea				
	2000	2001	2002	2003	2004	2000	2001	2002	2003	2004
Blue swimming crab	37219	29634	21407	22825	22113	6652	7171	7467	9549	7411
	(2046.076)	(1828.574)	(1630.317)	(1901.624)	(1947.084)	(353.273)	(456.868)	(566.584)	(724.551)	(616.514)
Mud crabs	3426	2955	703	596	2051	3495	2462	3120	663	808
	(237.295)	(188.705)	(89.363)	(60.537)	(203.161)	(254.068)	(184.647)	(224.690)	(43.926)	(51,073)
Other crabs	788	1003	1413	2459	3551	6528	7048	8011	7538	6288
	(20.894)	(33.512)	(52.519)	(122.882)	(179.202)	(162.355)	(227.778)	(269.006)	(360.209)	(324.419)

Source: Fisheries statistics 2000-2004, Department of Fisheries.

Table 1.2 Total production and value of marine crabs for both marine fisheries and coastal aquaculture during 2000-2004

Species	Production (x 1000 tons)					Value (million Baht)				
	2000	2001	2002	2003	2004	2000	2001	2002	2003	2004
Blue swimming crabs	43.9	36.8	28.9	32.3	29.5	2399.4	2285.4	2196.9	2644.2	2563.6
Mud crabs	6.9	5.4	3.8	1.3	2.9	492.4	374.2	315.8	106.1	257.9
Other crabs	7.3	8.1	9.4	10.0	9.8	183.2	261.3	312.5	483.1	503.6

Source: Fisheries statistics 2000-2004, Department of Fisheries.

principles e.g. ecology, population structure and dynamics which are exceptionally important for economically important species.

To sustain both fisheries and aquaculture production, several disciplines including effective fisheries management and appropriate breeding programs need to be established. One of the primary objectives of fishery management is to understand the population structure of the exploited species. The recognition of reproductively isolated and genetically differentiated populations within a species is of importance for its effective genetic-based management. Fishery managers need information on size and number of populations in the area so they can describe a strategy of management and exploitation. In the long term, the most important goal of fishery management for exploited species is to conserve the existing resources to ensure sustainable yield.

Mutations lead to genetic variation (polymorphism) of organisms. Genetic variation is evolutionary accumulated through time and should exit in any given species. Selection and genetic drift promote levels of genetic variation within and among individuals, species, and higher order taxonomic groups. At the DNA level, types of genetic variation include base substitutions (transitions and transversions collectively called single nucleotide polymorphism, SNP), insertions or deletions of nucleotide sequence (indels) within a locus, and rearrangement of DNA segments around a locus of interest.

DNA marker technology can be applied to examine levels of genetic variability. Large deletions and insertions (indels) that cause shift in the size of DNA fragments can be simply detected by agarose gel electrophoresis. Smaller indels require DNA sequencing or more elaborate electrophoretic techniques to determine smaller changes in size. Base substitutions can be examined by various techniques for example, restriction fragment length polymorphism of PCR products (PCR-RFLP), single-stranded conformation polymorphism (SSCP) and DNA sequencing. Inversions and rearrangements that involve restriction sites can also be detected because they disrupt the ability of a restriction enzyme to cut DNA at a given site and thus can produce relatively large changes in DNA fragment sizes.

Appropriate genetic markers can be used to elevate culture and management efficiency of economically important species. However, the information on genetic diversity and population structure of *P. pelagicus* is rather limited. This information is essential for the construction of appropriate breeding programmes and for broodstock selection and management scheme leading to sustainable culturing activity of *P. pelagicus* in Thailand. The blue swimming crab is the major species for the canned crab meat industry. Therefore, species-specific markers play important roles for quality control of products of this economically important species. Moreover, they can be used to verify species origins of various forms of the blue swimming crab products from Thailand to prevent intentionally use of the wrong species in canning.

1.2 Taxonomy of *P. pelagicus*

Taxonomic definition of the blue swimming crab, *P. pelagicus* is as follows Phylum Arthropoda, Subphylum Crustacea, Class Malacostraca, Subclass Eumalacostraca, Order Decapoda, Suborder Pleocyemata, Superfamily Portunoidea, Family Portunidae, Genus *Portunus*. The scientific name of this species is *Portunus pelagicus* (Linnaeus, 1758) (Figure 1.1). The English common name is blue swimming crab or sand crab.

Sex of *P. pelagicus* can be externally differentiated by the location of gonopores and characters of abdomen (Figure 1.2).

1.3 Taxonomy of three-spot swimming crab (*P. sanguinolentus*)

Taxonomic definition of the three-spot swimming crab, *P. sanguinolentus* is as follows Phylum Arthropoda, Subphylum Crustacea, Class Malacostraca, Subclass Eumalacostraca, Order Decapoda, Suborder Pleocyemata, Superfamily Portunoidea, Family Portunidae, Genus *Portunus*. The scientific name of this species is *Portunus sanguinolentus* (Herbst, 1798) and the English common name is three-spot swimming crab.

1.4 Taxonomy of swimming crab (*Charybdis crucifera*)

Taxonomic definition of the swimming crab, *C. crucifera* is as follows Phylum Arthropoda, Subphylum Crustacea, Class Malacostraca, Subclass

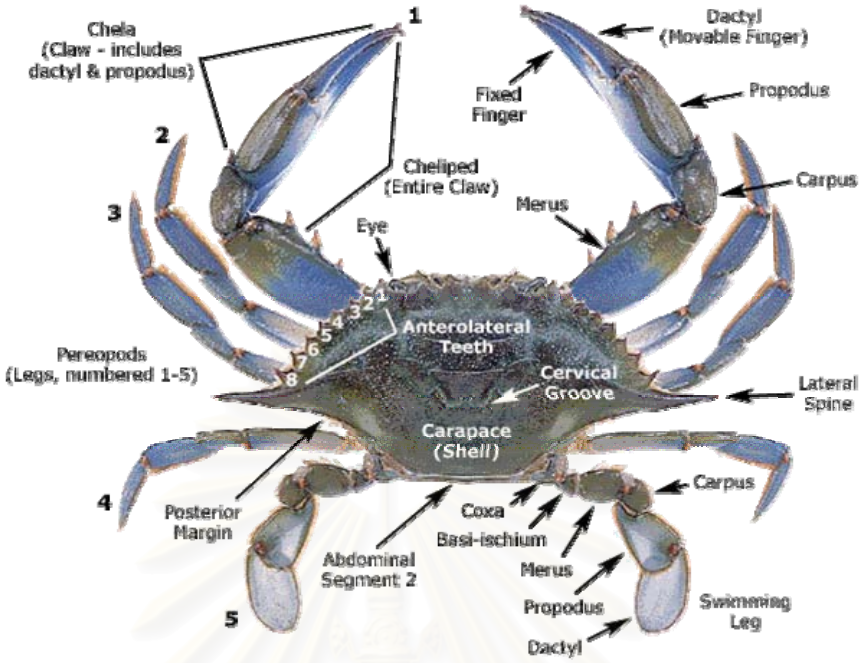
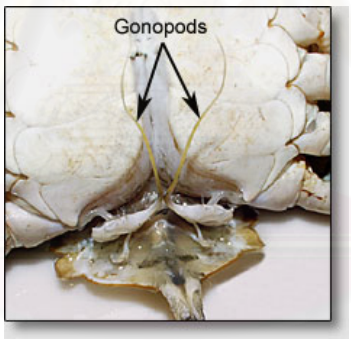
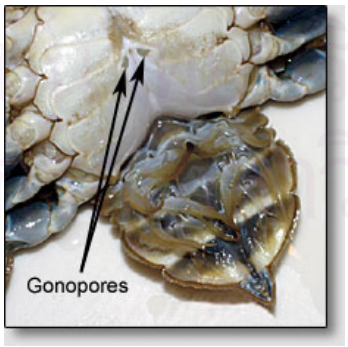


Figure 1.1 External morphology of *P. pelagicus*



Male



Female

Figure 1.2 Male and female *P. pelagicus* are identified by positions of gonopores and the external character of abdomen.

Eumalacostraca, Order Decapoda, Suborder Pleocyemata, Superfamily Portunoidea, Family Portunidae, Genus *Charybdis*. The scientific name of this species is *Charybdis crucifera* (Fabicius, 1798) and the English common name is swimming crab or musk crab.

1.5 Taxonomy of mud crab (*Scylla sp.*)

Taxonomic definition of the mud crab, *Scylla sp.* is as follows Phylum Arthropoda, Subphylum Crustacea, Class Malacostraca, Subclass Eumalacostraca, Order Decapoda, Suborder Pleocyemata, Superfamily Portunoidea, Family Portunidae, Genus *Scylla*. The English scientific names are *S. oceanica* (Dana), *S. serrata* (Forskal), *S. tranquebarica* (Fabricius) and the English common name is mud crabs or mangrove crabs.

1.6 Molecular genetic approaches used in this thesis

1.6.1 Polymerase Chain Reaction (PCR)

The introduction of the polymerase chain reaction (PCR) by Mullis *et al.* (1987) has opened a new approach for molecular genetic studies. This is a technique for enzymatically replicating DNA without using a living organism, such as *E. coli* or yeast. PCR is a method for an *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA by two oligonucleotide primers, usually 18-27 nucleotides in length. The target DNA sequence can be synthesized from a low amount of starting DNA template within a few hours.

The PCR reaction components constitute DNA template, a pair of primers for the target sequence, dNTPs (dATP, dCTP, dGTP and dTTP), appropriate buffer and heat-stable DNA polymerase (usually *Taq* polymerase). The amplification reaction usually consists of three steps; denaturation of double stranded DNA at high temperature, annealing to allow primers to form hybrid molecules at the optimal temperature, and extension of the annealed primers by the heat-stable DNA polymerase. The cycle is repeated for 30-40 times (Figure 1.3). The amplification product is determined by gel electrophoresis.

1.6.2 DNA sequencing

Polymorphism at the DNA level can be studied by several methods but the direct strategy is determination of nucleotide sequences of a defined region. DNA sequencing is the process of determining the exact order of the bases A, T, C and G in a piece of DNA. There are two general methods for sequencing of DNA segments: the “chemical cleavage” procedure (Maxam and Gilbert, 1977) and the “chain termination” procedure (Sanger, 1977). Nevertheless, the latter method is more popular because chemical cleavage procedure requires the use of several hazardous substances.

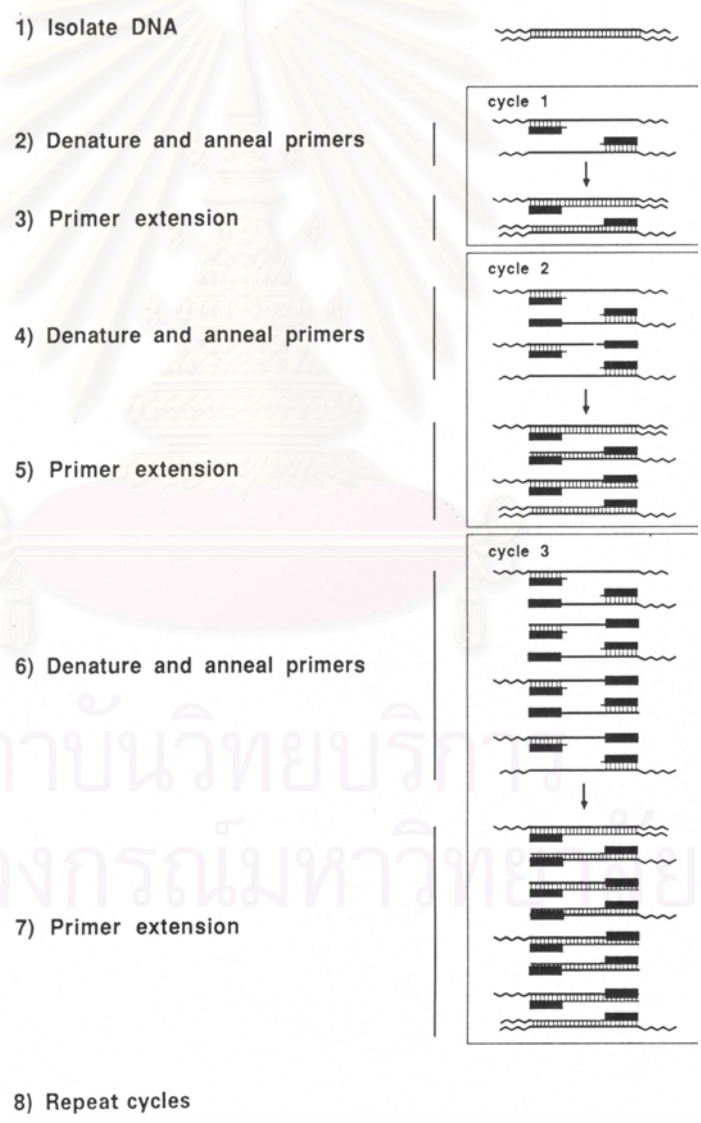


Figure 1.3 General illustration of the polymerase chain reaction (PCR) for amplifying DNA segments.

DNA sequencing provides high resolution and facilitating interpretation. DNA fragments generated from PCR can be directly sequenced or alternatively, those fragments can be cloned and sequenced. This eliminates the need to establish a genome library and searching of a particular gene in the library. However, sequencing of a large number of individuals using conventional method is extremely tedious and prohibitively possible. The enzymatic sequencing approach has presently been developed to the automated method (Figure 1.4). DNA sequences can be detected using a fluorescence-based system following labeling of a sequencing primer or incorporated nucleotides with a fluorescence dye. At present, automated DNA sequencing is commonly used. This greatly allows wider application of DNA sequencing analysis for population genetic and systematic studies.

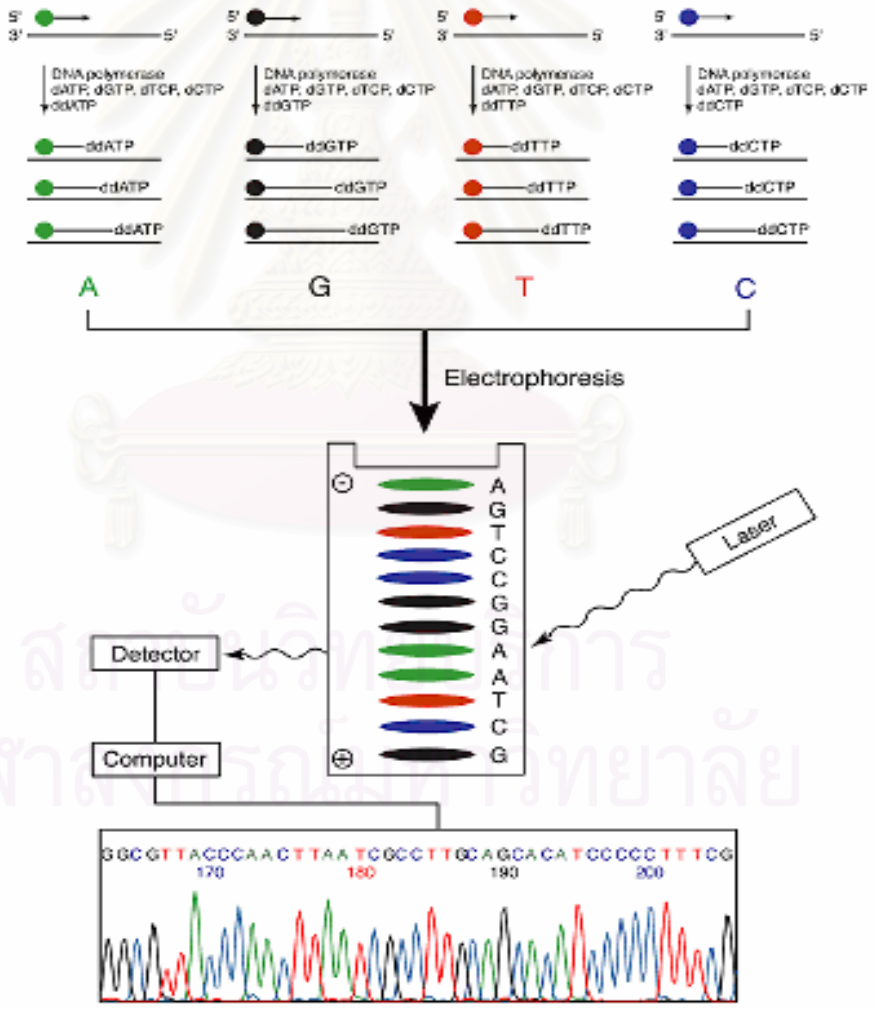


Figure 1.4 Automated DNA sequencing.

1.6.3 Single-stranded conformation polymorphism (SSCP) analysis

SSCP analysis is one of the most widely used techniques for inderectly detection of mutations and variation of DNA (deletions, insertions and single nucleotide polymorphism, SNP). The amplified PCR product (usually less than 300 bp in length) is denatured and loaded into a low crosslink non-denaturing polyacrylamide gel (with or without glycerol supplementation). The principle of this technique relies on different mobility due to differential folding of the single stranded DNA (Figure 1.5).

Single strand DNA molecules take on secondary and tertiary structures (conformations) due to base pairing between nucleotides within each strand. These conformations depend on the length of the strand, and the location and number of regions of base pairing. They also depend on the primary sequence of the molecule, such that a nucleotide change at a particular position can alter its conformation. Accordingly, molecules differing in their conformations (e.g. due to a single nucleotide change) can be separated.

The major advantage of SSCP is that a large number of individuals may be simultaneously genotyped. Heteroduplexes (products from misannealing of single stranded DNA from different alleles) can occasionally resolve from homoduplexs (products from annealing of single stranded cDNA of the same alleles) give additional information on the presence of variants. Therefore, SSCP is regarded as one of the potential techniques that can be used to detect low polymorphism in various species prior to confirmation of the results by nucleotide sequencing. In addition, SSCP required small PCR amplicons which are relative easy to amplify.

The disadvantages of SSCP are reproducibility of the technique because SSCP patterns are strongly affected by temperature and degree of cross-linking. Additionally, multi-allelic patterns of some nuclear DNA markers may cause the SSCP patterns too complicated for estimation of allele frequencies precisely.

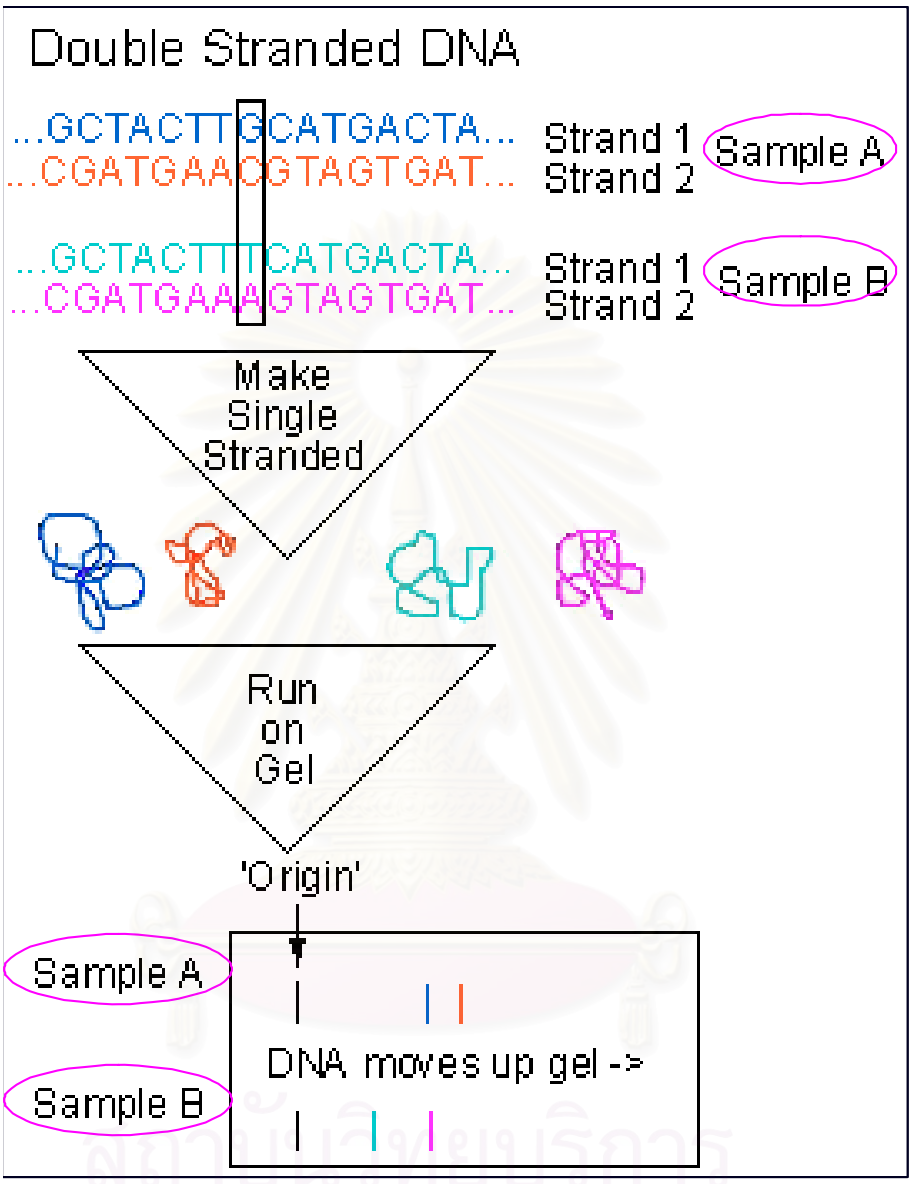


Figure 1.5 A schematic diagram of SSCP analysis. (http://www.amonline.net.au/evolutionary_biology/images/sscp.gif).

1.6.4 Amplified fragment length polymorphism (AFLP)

AFLP is a PCR-based, that combines the strengths and overcomes the weaknesses of the RFLP and RAPD methods for generation of multi-locus fingerprinting of organisms (Vos *et al*., 1995). It is a powerful technique, especially when combined with bulked segregrant analysis (BSA), for isolation of phenotypes affected by (or majority by) single locus markers. In addition, fingerprinting-band patterns of AFLP are effectively used to evaluate DNA polymorphism between samples.

The major strengths of the AFLP method include large (> 100) numbers of polymorphic loci screened, high reproducibility due to high PCR annealing temperatures, and relatively cost effectiveness.

The molecular basis of AFLP polymorphism includes indels between restriction sites and base substitutions at restriction sites for RFLP as well as indels in the amplification regions and base substitution at PCR primer binding sites for RAPD analyses (Liu and Cordes, 2004). The unique feature of the technique is the addition of adaptors of known sequence to DNA fragments generated by digestion of whole genomic DNA. This allows for the subsequent PCR amplification of a subset of the total fragments separated by gel electrophoresis.

AFLP begins with digestion of the whole genomic DNA with two restriction enzymes (most often *Eco*RI and *Mse*I). Since sequences for the resulting DNA fragments are unknown, adaptors of known sequence are ligated to the ends of the fragments and used as primer sites for PCR amplification. Since these would result in the production of millions of PCR fragments, the number of amplified fragments is reduced by selective amplification.

Selective nucleotides (usually a single base for preamplification and three bases for selective amplification) extending into the restriction fragments are added to the 3′ ends of PCR primers such that only a subset of restriction fragments are recognized. The primer will only anneal if the fragment has the correct sequence and adding one known base to one of the primers will reduce the number of amplified fragments for 4-fold. Therefore, adding three bases to each PCR primer should result

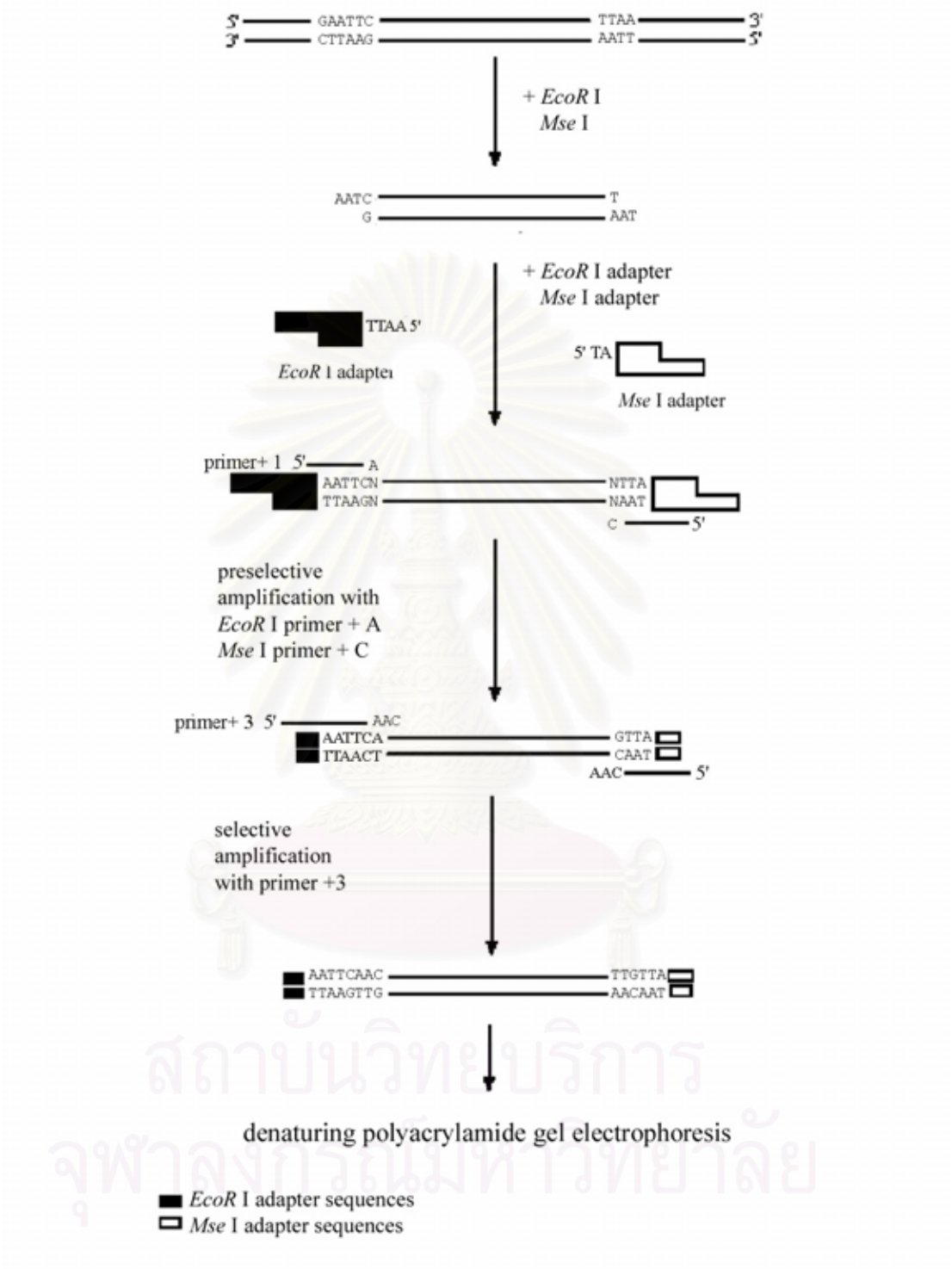


Figure 1.6 A schematic diagram illustrating an AFLP procedure. (AFLP Analysis System I; GibroBRL, USA)

in a 4096-fold reduction during selective amplification. The subsets of amplified fragments are then analyzed by denaturing polyacrylamide gel electrophoresis followed by radioactive (autoradiography) or non-radioactive (silver staining) detection (Figure 1.6).

AFLP markers are treated as the bi-allelic dominant marker, even though software packages are now available (e.g. from AFLP QuantaPro, Key Gene) for co-dominant scoring of AFLP bands. Co-dominant scoring is possible when-characterized families are used, but this is difficult for population genetic studies. This disadvantage can be overcome by converting an AFLP band of interest to a SCAR marker.

1.6.5 Techniques for detection of differentially expressed genes

1.6.5.1 Differential display-polymerase chain reaction (DD-PCR)

DD-PCR is one of the important approaches to identify differentially expressed transcripts in various organisms (Liang and Pardee, 1992). The identification of such “differentially expressed” RNAs is a powerful tool for studying differentiation, the cell cycle, carcinogenesis, inductive events, and other biological phenomena that involve changes in gene expression. The advantage of this technique is that differentially expressed bands are obtained before further characterized by cloning and sequencing of the target transcripts.

The mRNA is reverse-transcribed using oligo (dT) primers having 4-12 additional bases at the 3′ end. The first strand cDNA is amplified by PCR using a short arbitrary primer and anchored primer. The amplified products are size-fractionated through denaturing polyacrylamide electrophoresis. DD-PCR bands are detected by autoradiography.

-Delta® Differential Display Kit

The commercially available Delta® Differential Display Kit is commonly used to identify RNAs that are expressed in one RNA population but missing in another (Diachenko *et al*., 1996).

The protocol consists of two stages: cDNA synthesis and differential display PCR (Figure1.7 and 1.8). The first strand cDNA is synthesized using each of the RNAs of interest as a template and oligo (dT) as a primer. In contrast to the 9-12 syntheses required for each different RNA sample in other differential display protocols (Liang & Pardee, 1992; Liang *et al*., 1993), the Delta protocol requires only a single cDNA synthesis reaction for each RNA sample.

In differential display PCR, sequences are amplified in the presence of [α-^{33}P] dATP based on chance homology to arbitrary P primers. The PCR cycling program differs substantially from conventional PCR cycling programs. Three initial cycles are performed at low stringency (i.e., low annealing temperature) to allow the primers to anneal and initiate DNA synthesis. Because of the low stringency, each P primer will bind sites on many cDNAs with imperfect and/or incomplete matches. The products of these early cycles are then amplified (using the P primer and the downstream T primer) during 22–25 high stringency PCR cycles. When examined by polyacrylamide electrophoresis and autoradiography, these reactions produce characteristic banding patterns differentially expressed in the starting RNA.

1.6.5.2 RNA arbitrarily primed-polymerase chain reaction (RAP-PCR)

RNA arbitrarily primed PCR (RAP-PCR; Welsh *et al*., 1992) is a simple version of DD-PCR. This technique is generally comparable to RAPD-PCR with the exception that the first-strand cDNA synthesized from total RNA (or mRNA) was used as the template (Figure 1.9). It is believed that the first strand cDNA synthesized from a random primer allow better possibility to detect the inner part of the transcripts.

PCR is carried out using combination of two different arbitrary primers. Differentially expressed transcripts are detected after size-fractionated through denaturing polyacrylamide gels by silver staining. Fragments from DD-PCR and/or RAP-PCR should be characterized by cloning and sequencing. Primers can be designed and tested for the expression levels of interesting fragments.

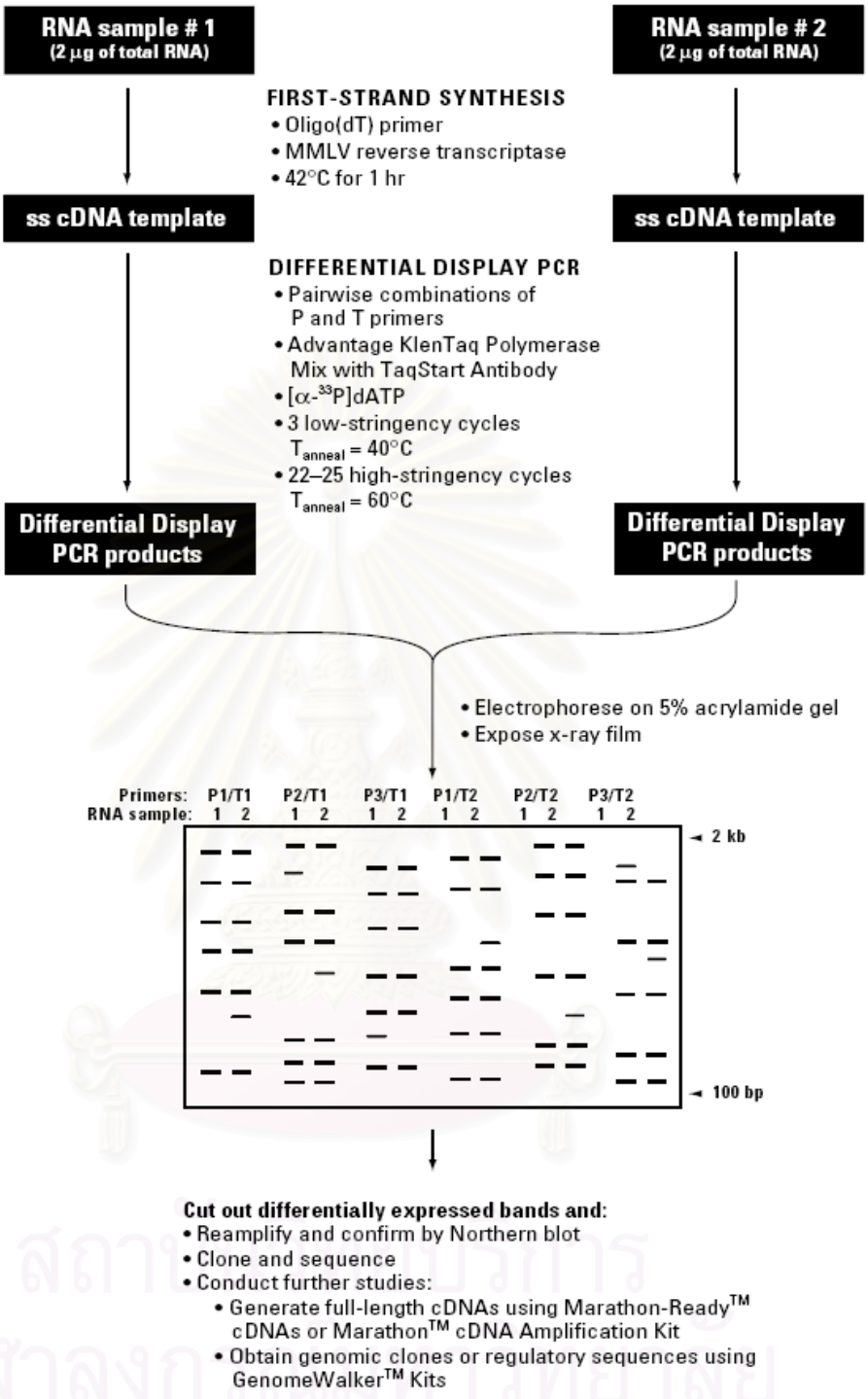


Figure 1.7 DD-PCR using the Delta Differential Display protocol. In the actual protocol, each differential display PCR is performed using two different dilutions of each cDNA sample. (http://www.clontech.com/images/pt/dis_manuals/PT1173-1.pdf)

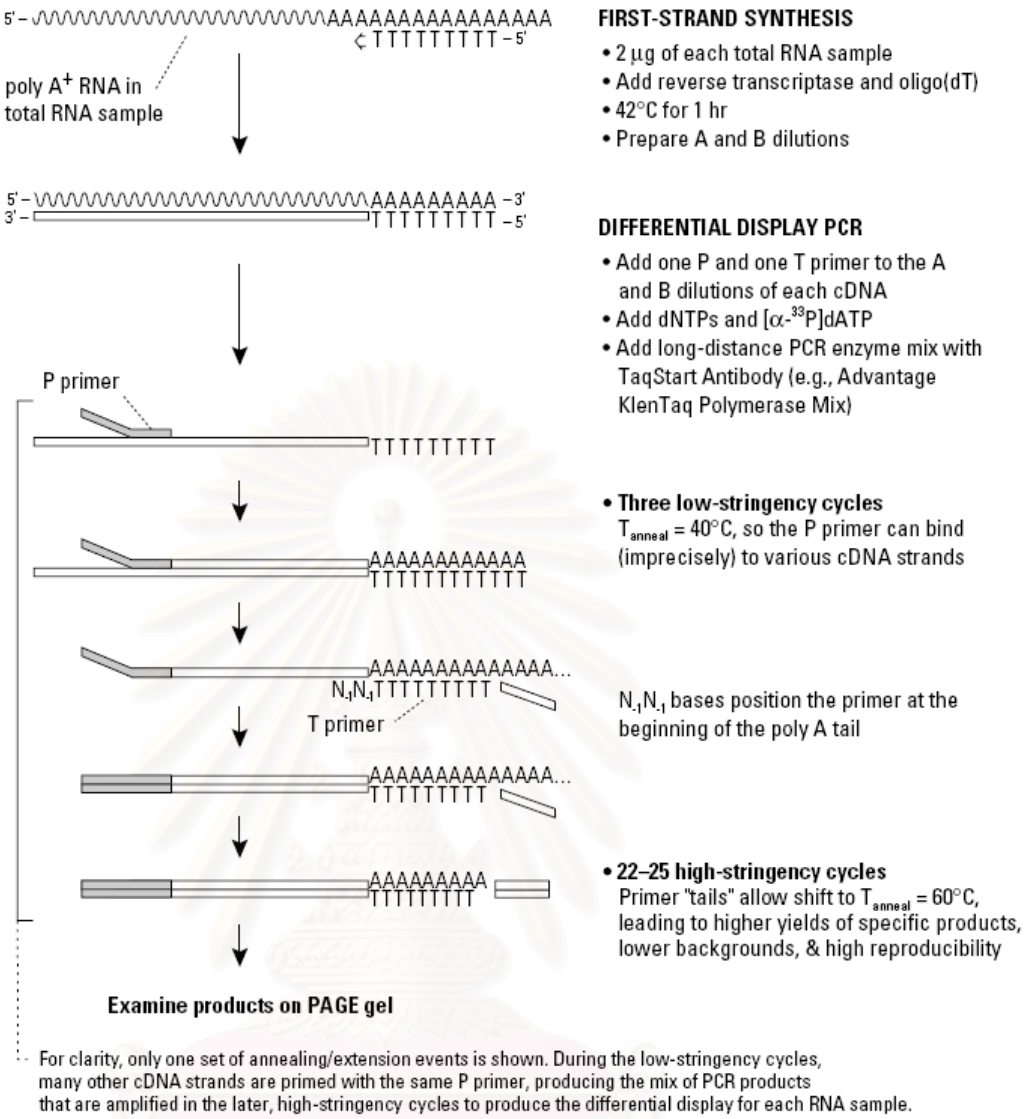


Figure 1.8 A detailed flow chart of the cDNA synthesis and PCR of the Delta Differential Display protocol. This figure shows differential display PCR using one P primer and one T primer. Although these are the most commonly performed reactions, DD-PCR can also be performed using P primers alone, T primers alone and pairs of P primers or pairs of T primers.

(http://www.clontech.com/images/pt/dis_manuals/PT1173-1.pdf)

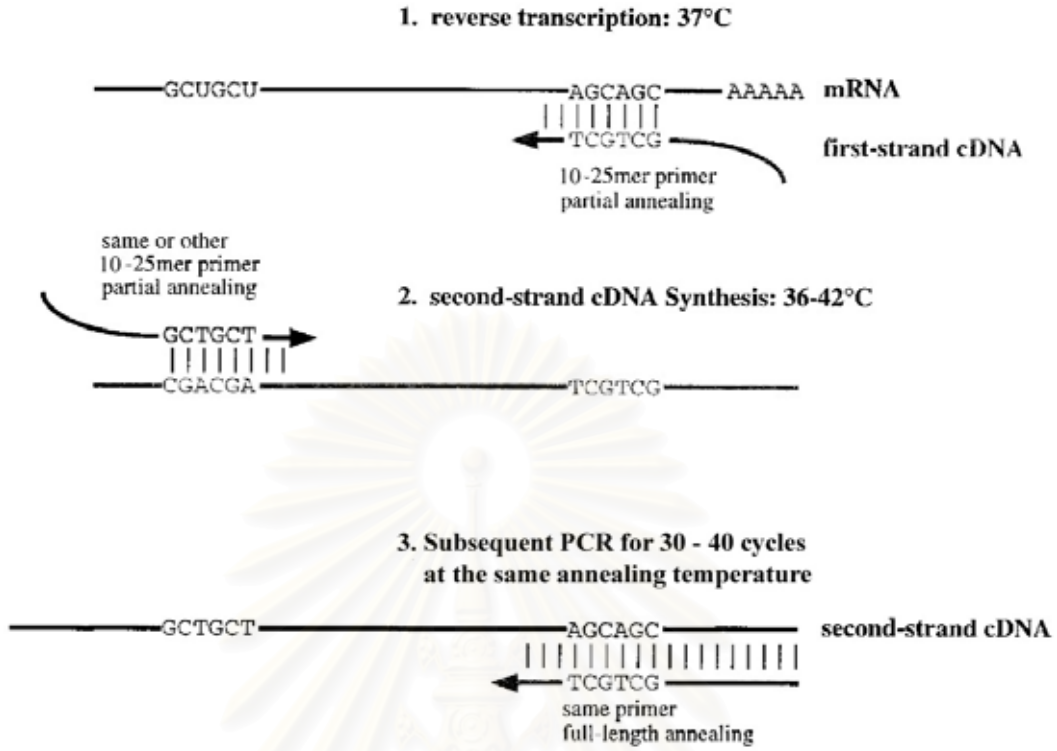


Figure 1.9 Overall concept of RAP-PCR. (1) During the first-strand cDNA synthesis, a single 10 – 25 base arbitrary primer anneals and extends from sites present within the mRNA. (2) Synthesis of the second strand cDNA primed by the same, or another, 10 – 25 base arbitrary primer proceeds during a single round of low-stringency DNA synthesis catalyzed by *Taq* polymerase. The result of the two enzymatic steps is the synthesis of a collection of molecules that are flanked at their 3′ and 5′ ends by sequences completely matching the sequences of the arbitrary primers. (3) These DNA fragments serve as templates for PCR amplification. (http://pubs.nrc-cnrc.gc.ca/ispmb/ispmb19/R00-060.pdf)

1.6.5.3 Complementary DNA-Amplified fragment length polymorphism (cDNA-AFLP)

The cDNA-AFLP is an improvement of traditional differential display techniques. It is a PCR-based method which starts with cDNA synthesis from total or mRNA followed by the conventional AFLP approach. Accordingly, the cDNA-AFLP

technique is essentially identical to AFLP with the exception that cDNA rather than genomic is used as the starting material. In this study, single strand cDNA was synthesized from total RNA of each sample using a SMART^{TM} PCR cDNA synthesis kit. The resulting single-stranded (ss) cDNA contains the 5' end of the mRNA, as well as sequences that are complementary to the SMART Oligonucleotide. The second strand cDNA was synthesized by PCR. The resulting cDNA was then manipulated using a typical AFLP approach described previously.

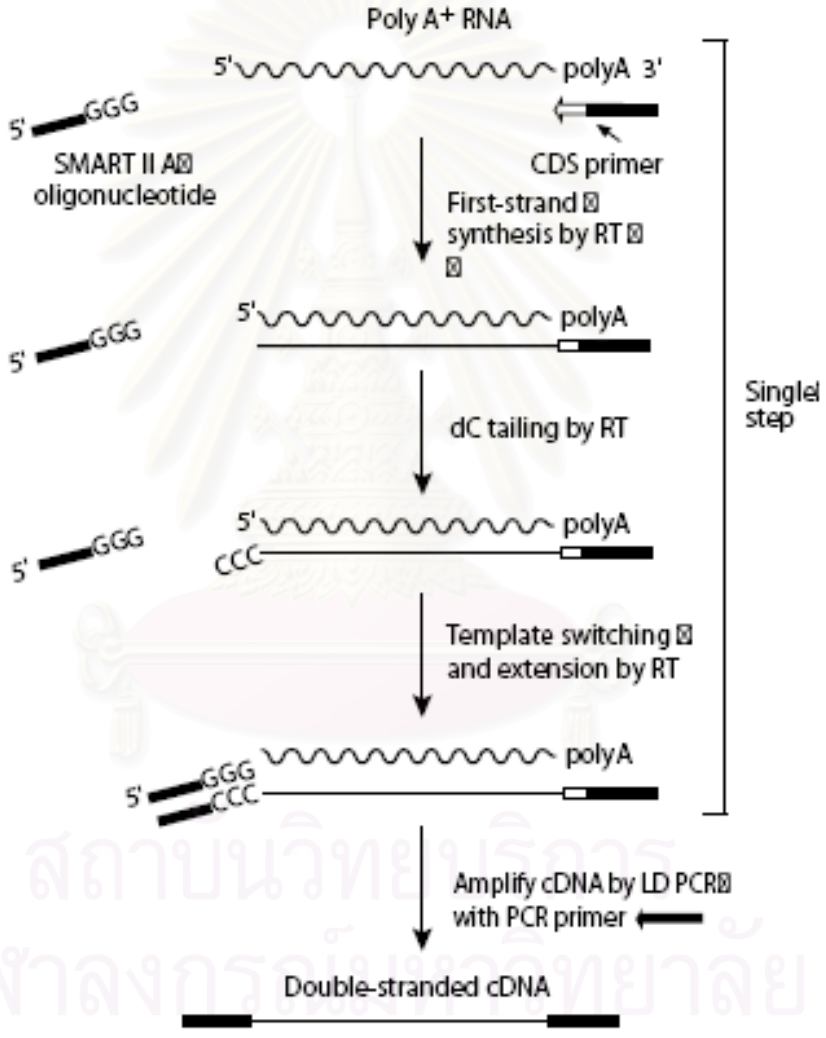


Figure 1.10 A flow chart illustrating cDNA synthesis using a SMART™ PCR cDNA Synthesis kit**.** The SMART II A Oligonucleotide, 3' SMART CDS Primer II A, and 5' PCR Primer II A all contain a stretch of identical sequence.
(http://www.clontech.com/images/pt/PT3041-1.pdf)

By increasing the stringency of the PCR amplification (adding more additional nucleotides to the primers), the sensitivity of the analysis can be increased. In this way, genes with a low expression level can also be detected. The fragments that are amplified are roughly 100-400 bp. These fragments are separated on high-resolution gels. The differences in the intensity of the bands that can be observed and provide a preliminary measure of the relative differences in the levels of gene expression. Further characterization of interesting transcripts is often required. The sensitivity and specificity of the method allows the detection of poorly expressed genes and the determination of subtle differences in transcriptional activity. Therefore, cDNA-AFLP can generate a global overview of gene expression under investigated conditions.

1.6.5.4 Reverse transcription-polymerase chain reaction (RT-PCR) and semiquantitative RT-PCR

RT-PCR is a comparable method of conventional PCR but the first strand cDNA instead of genomic DNA is used as the template in the amplification reaction (Figure 1.11). It is a direct method for examination of gene expression of known transcripts in the target species. The template for RT-PCR can be the first stranded cDNA synthesized from total RNA or poly A^+ RNA using oligo (dT) or random primers and reverse transcriptase. The product is then subjected to the second strand synthesis using gene-specific forward and reverse primers.

RT-PCR can also be used to identify homologues of interesting genes by using degenerate primers and/or conserved gene-specific primers from the original species and the first strand cDNA of the interesting species is used as the template. The amplified product is further characterized by cloning and sequencing**.**

Semi-quantitative RT-PCR is a quantitative approach where the target genes and the internal control (e.g. a housekeeping gene) were separately or simultaneously amplified using the same template. The internal control (such as *β-actin*; *elongationfactor*, *EF-1α* or *G3PDH*) is used under the assumption that those coding genes are transcribed constantly and independently from the extracellular environment stimuli and that their transcripts are reverse transcribed with the same efficiency as the product of interesting transcript.

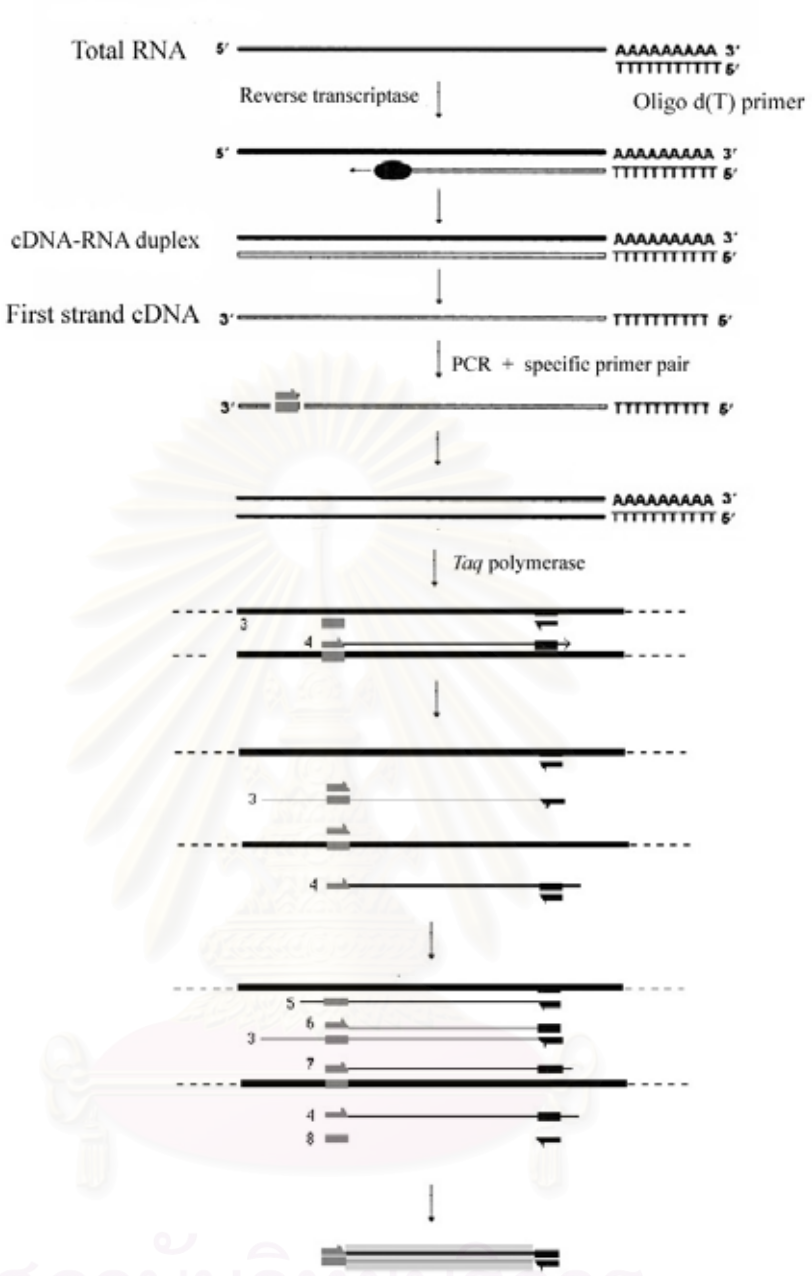


Figure 1.11 Overall concept of the RT-PCR procedure. During the first strand cDNA synthesis, an oligo d(T) primer anneals and extends from sites present within the total RNA. Second strand cDNA synthesis primed by the 18–25 base specific primer proceeds during a single round of DNA synthesis catalyzed by *Taq* polymerase. These DNA fragments serve as templates for PCR amplification.

1.7 Sources of DNA markers used for genetic diversity studies

1.7.1 Nuclear DNA

DNA in chromosomes of organisms is called chromosomal DNA, genomic DNA or nuclear DNA. Nuclear DNA is composed of coding genes (genes) and non-coding DNA. The genomic DNA of most eukaryotes contains large amounts of highly repetitive sequences located in the constitutive heterochromatin or scattered throughout the genome. The highly repetitive DNA sequences are divided into two groups on the basis of their arrangement in the genome. The first group is the repetitive sequences organized in tandemly reiterated clusters referred to as satellite DNA. The second class are those repeats scattered throughout the genome in an unrelated manner referred to as interspersed repeated sequences (minisatellites and microsatellites.

1.7.2 Mitochondrial DNA

The animal mitochondrial genome is a closed circular and double stranded DNA molecule contained in mitochondria. There are up to several thousand copies of the mitochondrial genomes per cell. This extra-chromosomal DNA is about 15700-19500 base pairs (bp) in length (Brown, 1983).

The mitochondrial genome of most animal species contains 13 protein coding genes, 2 genes coding for ribosomal RNAs (*12S and 16S ribosomal RNA*), 22 transfer RNA (*tRNA*) coding genes and the control region (D loop) containing initiation site for replication and transcription. The genes that code for proteins are subunits 1, 2, 3, 4, 4L, 5 and 6 of *NADH dehydrogenase* (*ND*), *cytochrome b* (*cyt b*), three subunits of *cytochrome c oxidase* (*COI*, *COII* and *COIII*) and two subunits of *ATP synthetase* (*ATPase 6* and *8*).

Ribosomal RNA (rRNA) genes found in the mitochondrial genome are different from those found in the nuclear genome. They are composed of *12S* (about 819-975 bp in vertebrates) and *16S* (about 1571-1640 bp in vertebrates) rather than *18S* and 28S genes in the nucleus (Meyer, 1994). The internal spacer (ITS) I and II as well as the nontranscribed spacer (NTS), the so called intergenic spacer (IGS), are not found in the mitochondrial genome. More importantly, they evolve approximately 100

times more rapidly than their nuclear counterparts. However, the mutation rate of these *rRNA* genes is about half of that of the protein coding genes. Transitions, which are predominant, and transversions are the common patterns of mutation. Deletions and insertion are usually about 1-5 bp. Length variation in 12S is basically less frequent than in the 16S subunit.

The secondary structure of the *12S* and *16S rRNA* genes is conserved across among distantly related species. Between coelacanth, lungfish, ray-finned fish and tetrapods, substitutions (transitions and transversions) in loops are about four times higher than in stems. Of these, transversions are about nine times higher in the loops than in the stems. (Meyer and Wilson, 1990; Meyer and Dolven, 1992; Meyer, 1994). The substitution rate mentioned above make the 12S and 16S rRNA genes useful for evolutionary studies between distantly related species.

The rate of evolution of protein coding genes in mtDNA varies from gene to gene and this depends upon factors such as functional constraints of the gene product and its base compositional biases (Avise, 1994; Meyer, 1994). Some genes for instance, *COI*, *COII*, *COIII* and *cyt b* are extremely conserved because of their functional constraints. On the other hand the seven subunits of *NADH dehydrogenase* and two subunits of *ATPase* are highly variable. Therefore, the *CO* subunits and *cyt b* are appropriate genes for the studies of population genetic questions in distantly related taxa. For such studies, PCR may be applied by using universal primers to amplify these gene regions. The PCR products may be used for either restriction endonuclease digestion or direct sequencing. For the reason mentioned above, *cyt b* is the most commonly used genes for direct DNA sequencing in many organisms. On the other hand, *NADH dehydrogenase* and *ATPase* subunits are, in general, suitable to investigate DNA polymorphism in both within species and closely related species. Mutations in the mitochondrial protein coding genes are transitions and transversions. Length polymorphism of these genes is less frequent than other genes in the genome.

Unlike the nuclear genome, the genes in the animal mitochondrial genome do not contain intervening sequences (introns) and they are usually separated by less than 10 bp. It is estimated that more than 90% of the mitochondrial genome is transcribed. The nontranscribed regions are in the control region sequences containing the heavy stranded origin of replication (O_H) and those located (in the human mitochondrial

genome) between proline and phenylalanine tRNA genes (Brown, 1983). This control region contains the D loop formed by the synthesis of a short piece of DNA which is displaced in this region (Clayton, 1991).

The number of genetic codes used for protein translation in the mitochondrial genome is less than those of the universal codes of the nuclear genome. The reason for this is that the mitochondrial DNA genetic codes are more degenerate and thus less constrained than the universal codes (Barrell *et al*, 1979; Attardi, 1985). Genome organization of mtDNA genes is generally different in every phylum which has been studied. Mitochondrial gene order is also slightly different among vertebrates (Meyer, 1994).

Considering organelle segregation, animal mtDNA is a haploid and non-recombinant molecule reflecting, generally, only one type of mtDNA in an organism. Therefore, intra-individual variation (heteroplasmy) would be expected to be very rare. However, mtDNA heteroplasmy has been reported in certain species; for example, mussel, *Mytilus edulis* (Hurst, 1993), shad (Bentzen *et al.*, 1989), deep-sea scallop, *Placopecten magellanicus* (Snyder *et al.*, 1987; La Roche *et al.* 1990)

Two properties of mtDNA which are suitable for stock identification are maternal inheritance and rapid evolution (Ferris and Berg, 1987). This property allows several applications, for instance determining the migration of female broodstock, evaluating the hybrid animals in natural hybrid zones. Nevertheless, based on the fact that the paternal mitochondria enter the fertilized eggs through spermatozoa, this may complicate genealogical analysis. However, this event is not a major factor for almost all of species (Lansman, 1983, Avise, 1994). It is proposed that paternal mitochondria are actively degraded during fertilization (Vaughn *et al.*, 1980) or may not be replicated thereafter (Meyer, 1994). However, a significant paternal contribution of mtDNA genotypes was reported in mice and marine mussel, *Mytilus edulis*.(Gylensten *et al*., 1991; Skibinski *et al*., 1994). Since there have been no publications concerning patterns of mtDNA transmission in *P. pelagicus,* therefore it is presumed that the mtDNA of *P. pelagicus* is transmitted maternally.

1.8 Genetic diversity studies in marine invertebrates

Mitochondrial DNA polymorphism is commonly used for determination of genetic variation in various species. Sato and Nagashima (2001) characterized and examined polymorphism of a mtDNA segment of the Japanese scallop (*Patinopecten yessoensis*). A 1.3-kb mtDNA segment of *P. yessoensis* containing the $tRNA^{Met}$ gene and partial sequences of 2 *rRNA*, together with 2 separate noncoding regions (designated NcR1 and NcR2) were cloned and sequenced. Seventy-eight individuals cultured in Lake Saroma or Matsu Bay, were sequenced, and they found 15 polymorphic positions including 13 substitutions, 1 deletion, and 1 insertion (1 position in NcR1, 14 positions in NcR2) resulting in a total of 17 haplotypes. Of these, 10 were found in the Saroma population only, 3 were found in the Mutsu population only, and 4 were found in both populations. The gene diversity and nucleotide diversity values were 0.87 and 0.0069 for the Saroma population, 0.63 and 0.0040 for the Mutsu population, and 0.83 and 0.0203 for overall samples.

Atlantic (*Pecten maximus*) and Mediterranean (*P. jacobaeus*) scallops have been traditionally considered as distinct species, but recent genetic studies have shown that they are races or subspecies separated by the Almeria-Oran oceanographic front in SE Spain. Nucleotide polymorphism of *16S rRNA gene* (511 bp, $N = 85$) from 4 populations of *P. maximus* and 3 populations of *P. jacobaeus* were examined. No significant differences in haplotype frequencies among populations or species were found. However, significant differentiation between taxa appeared when haplotypes were pooled in two groups according to their phylogenetic relationships and after analysis of molecular variance (AMOVA), in agreement with previous allozyme studies. Levels of within-population nucleotide diversity were similar in all populations except in *P. jacobaeus* from the northern Adriatic Sea, suggesting a smaller effective population size in that area which could be due to variable recruitment. An excess of rare haplotypes was observed in investigated populations. The mismatch distribution and several population genetic statistics indicate that the excess of rare variants is due to a population expansion which occurred in the second half of the Pleistocene period, less than 0.9 my before present, and probably well after the origin of the two scallop races (Saavedra and Pena, 2005)

Sequence variation of the mtDNA 16S rRNA region of the Asian moon scallop, *Amusium pleuronectes* in seven populations along the coast of Thailand was recently examined. A total of 16 unique haplotypes were detected among 174 individuals with 27 variable sites out of 534 bp sequenced. The haplotypes grouped into two distinct arrays (estimated to differ by about 2.62% to 2.99% nucleotide divergence) that characterized samples collected from the Gulf of Thailand versus the Andaman Sea. Low levels of intrapopulation variation were observed, while in contrast, significant divergence was observed between populations from the Gulf of Thailand and Andaman Sea. Results of AMOVA reveal a high F_{ST} value (0.765) and showed that the majority of the total genetic variance (76.03%) occurred among groups (i.e., Andaman Sea and the Gulf of Thailand) and little among populations within the group (0.52%) and within populations (23.45%). The genetic differentiation between the populations recorded in the present study is similar to that observed in a variety of marine species in the Indo-Pacific region (Mahidol, 2007).

Genetic diversity between cultured and wild populations the New Zealand greenshell mussel *Perna canaliculus* (Gmelin, 1791) was compared and examined whether the mussel obtained from Kaitaia, northern New Zealand, and transferred to mussel farms around the country, had introgressed into wild (naturally occurring) mussel stocks. The potential extent of genetic introgression of this mussel was assessed by analysis of allozyme, mitochondrial, SSCP and RFLP, and nuclear RAPD markers. Cultured and wild populations throughout New Zealand showed an absence of biochemical genetic differentiation at seven allozyme loci. For the DNA techniques, a haplotype specific to mussels originating from Kaitaia was identified only using mitochondrial composite markers. However, this composite marker occurred at low frequency in the Kaitaia population and not in any other populations and, therefore, could not be used to measure introgression of Kaitaia genes into wild populations. The mtDNA and RAPD analyses revealed that North Island and Greater Cook Strait mussels were genetically different from lower South Island, particularly South Island west coast mussels, and the wild Stewart Island population. Furthermore, the two marker types showed that cultured mussels were significantly differentiated from wild mussels. These two findings clearly indicate that at the molecular level, population subdivision exists in *P. canaliculus*. The SSCP and RFLP markers did not show any evidence of genetic introgression of spat moved from the north into

southern mussel populations. The RAPD markers, however, showed that the wild Stewart Island population (HSB) fell outside the northern and the southern clades of populations. The HSB population is located near an aquaculture site that has been seeded with mussels from Kaitaia for over a decade. Their RAPD data are consistent with the possibility of genetic introgression of genes from northern populations into this southern population (Apte *et al*., 2003).

Large-scale mortality events have been observed in the Pacific oyster, *C. gigas* on the west coast of France since the early 1980s, particularly during summer. In order to understand the causes of this mortality, genetic polymorphism of *glutamine synthetase* (amino-acid metabolism) and *delta-9 desaturase* (lipid metabolism) in families of *C. gigas* and their roles in susceptibility to summer mortality was determined (David *et al*., 2007). Those genes in two generations of oysters from single-pair mating were studied in three sites on the French Atlantic coast (Baie-des-Veys, Auray and Ronce-les-Bains) using PCR-SSCP. Three and two different alleles were detected for respective genes. Allele C of *glutamine synthetase* seemed to be counter-selected in some second generation families. Allele B of *delta-9 desaturase* was potentially counter-selected at Auray in the families showing higher mortality, and strong selection against BB homozygotes was observed. It was concluded that any selective effect of summer mortality on allele C of *glutamine synthetase* or allele B of *delta-9 desaturase* could be mediated either directly or via linkage to other loci involved in physiological pathways affecting susceptibility.

Genetic diversity and population differentiation of three populations of the giant tiger shrimp (*Penaeus monodon*) collected from Satun (the Andaman Sea) and Suratthani and Trat (the Gulf of Thailand) were examined by mtDNA polymorphism. Twenty-eight composite haplotypes were generated from 52 restriction profiles of the entire mtDNA digested with 11 restriction endonucleases. The average haplotype diversity in *P. monodon* was 0.864, whereas the mean nucleotide diversity within populations was 2.51%, 2.22% and 1.91% for Satun, Trat, and Suratthani, respectively. Geographic heterogeneity analysis indicated population differentiation between *P. monodon* from the Andaman Sea and *P. monodon* from the Gulf of Thailand ($P < 0.0001$) (Klinbunga *et al*., 1999).

Genetic diversity and molecular taxonomy of mud crabs, *Scylla serrata*, *S. oceanica*, and *S. tranquebarica*, collected from two locations in eastern Thailand were studied using RAPD-PCR. Ninety-one reproducible RAPD fragments, generated by UBC456, UBC457, and YNZ22, were polymorphic. The percentage of polymorphic bands for each primer was 97.22%-100%. The average number of genotypes per primer was 27.3 in *S. serrata*, 26.6 in *S. oceanica*, and 17.0 in *S. tranquebarica*. Shared genotypes between individuals of different mud crab species were not observed. A 600-bp band from YNZ22 was fixed in both *S. serrata* and *S. oceanica*, but it not found in *S. tranquebarica*. Further dissociation of *S. tranquebarica* (presence) and *S. oceanica* (absence) could be indicated by a 725-bp band from YNZ22. Species-specific RAPD markers were also observed and used to construct a molecular diagnostic key in these taxa. A neighbor-joining tree constructed from genetic distances between pairs of individuals indicated three separated groups corresponding to *S. serrata*, *S. oceanica* and *S. tranquebarica*. Therefore, mud crabs in eastern Thailand should be recognized as three different species rather than a single panmictic species exhibiting different morphs (Klinbunga et al., 2000).

Genetic differentiation in the mud crab, *S. serrata* within the Indian Ocean (N = 77) were studied using *COI* sequences (535 bp) of crabs from four representative mangrove swamps of the African tropics (Kenya and Zanzibar). A total of 24 different haplotypes were identified. A single most frequent haplotype shared among all four populations and a small number of rare haplotypes typically presented in only one or two individuals and represented a specific population. AMOVA, F_{ST} statistics and χ^2 contingency analysis of spatial distribution of mtDNA haplotype frequencies revealed a significant genetic differentiation among investigated populations. These results indicated that gene flow in this species might be reduced, even between geographically close sites, despite the high potential for dispersal (Fratini and Vannini, 2002).

The complete mitochondria genome sequence of the blue crab (*Callinectes sapidus*) was characterized and estimated to be 16263 bp in length. It contains genes for the same 13 proteins, 22 tRNAs, and two rRNAs as found in other metazoans. In addition, there is a 1435 bp putative control region (78.2% A/T), located between the *rrnS* and *trnI* genes. Gene order and arrangement of *C. sapidus* mtDNA is similar to

that of other arthropods but dramatically different from the hermit crab, which has a unique gene order among arthropods. Maximum parsimony (MP) analysis of the amino acid sequences from the concatenated 13 protein coding genes yield a single most parsimonious tree, with a length of 11319 steps (consistency index = 0.779; retention index = 0.462; rescaled consistency index = 0.359). Of the 3813 sites, 948 were constant and 1935 were parsimony informative. NJ analysis produced the same tree topology (Place *et al*., 2005).

The complete mitochondrial DNA sequence of the swimming crab, *P. trituberculatus* was recently reported. The mitochondrial genome of *P. trituberculatus* was 16026 bp in length, and the genome included the same 13 protein-coding, 22 tRNA, and two rRNA genes as found in other metazoan animals. In addition, there was a 1104-bp non-coding region between the *srRNA* and *tRNA* ^{Ile} genes. The overall A + T content of the L-strand of *P. trituberculatus* mtDNA was 70.2%. This pattern of the base composition held for the protein-coding, rRNA, and tRNA genes, as well as the control region. The gene order of *P. trituberculatus* was largely identical to those so far obtained in other arthropods but the *tRNA*^{His} gene in *P. trituberculatus* was found between the *tRNA*^{Glu} and the *tRNA*^{Phe} genes, the latter two tRNAs being located downstream of the *ND5* gene (rather than typically found between *ND4* and *ND5* in all other arthropods) (Yamauchi *et al*., 2003)

Population subdivisions of *P. pelagicus* in Australia were illustrated based on allozyme polymorphism. Initially, 57 individuals from 8 geographic sites were screened for 35 allozymatic loci. Subsequently, 609 individuals of *P. pelagicus* from 11 different locations covering three regions in South Australia and two regions in the Northern Territory were analyzed at 7 polymorphic loci (*ACYC*, *ALDH-2*, *ARGK*, *PEP-A*, *PEP-B2*, *PEP-D2* and *PGM*). Four differentiated subpopulations could be recognized including West Coast, Spencer Gulf, and Gulf St Vincent in South Australia and Darwin-Gove in the Northern Territory. Population substructuring among sites within each subpopulation was also found (Bryars and Adams, 1999).

Yap *et al*. (2002) isolated and characterized eight (seven dinucleotide and one tetranucleotide) microsatellites in *P. pelagicus*. All eight loci were polymorphic when tested against genomic DNA of *P. pelagicus* collected throughout Australia (N = 85–864). The mean observed heterozygosity (H_o) at each loci was 0.30–0.78

which was not significantly different from that of the expected heterozygosity (H_e) except at pPp05 where homozygote excess was observed.

More recently, Sezmis (2004) investigated population genetic structure of *P. pelagicus* collected from 16 different assemblages throughout the geographic range of this species in Australian waters (N = 4–57 per each sample site) using 6 microsatellite loci and a 342 bp fragment of *cytochrome oxidase subunit I* (*COI*). Significant genetic heterogeneity of Australian *P. pelagicus* was found (F_{ST}-based statistics for microsatellite and *COI* data = 0.098 and 0.375, respectively).

Genetic diversity and population differentiation of the blue swimming crab in Thai waters are rather limited. Klinbunga *et al*. (2007) examined genetic diversity and population differentiation of *P. pelagicus* in Thai waters originating from Ranong and Krabi located in the Andaman Sea (west) and Chantaburi, Prachup Khiri Khan and Suratthani located in the Gulf of Thailand (east) by AFLP analysis. High genetic diversity of *P. pelagicus* in Thai waters (N = 72) was found. A total of 227 AFLP fragments were generated from four primer combinations and the percentage of polymorphic bands in each geographic sample was 66.19% - 94.38%. The mean genetic distance between pairs of samples was 0.1151 - 0.2440. Geographic heterogeneity analysis between all pairwise comparisons were statistically significant ($P < 0.01$), indicating a fine-scale level of intraspecific population differentiation of Thai *P. pelagicus*. The estimated number of migrants per generation (N_em) was 0.26 - 0.76, suggesting the restricted gene flow levels of *P. pelagicus* in Thai waters.

Likewise, population genetics of *P. pelagicus* was also analyzed by RAPD analysis. A total of 112 RAPD fragments were generated (N = 109) from OPA02, OPA14, OPB10, UBC122 and UBC158. The percentage of polymorphic bands in each geographic sample was 72.73% - 85.05%. The average genetic distance between samples across overall primers was 0.0929 - 0.2471. Geographic heterogeneity analysis indicated statistically significant differences between all pairwise comparisons ($P < 0.0001$ for the exact test and $P < 0.01$ for F_{ST}-based statistics, θ) (Khetpu, 2005).

1.9 Species-specific markers in marine species

Species identification is necessary for the quality control of products, particularly when wrong species are intentionally supplied. In addition, labeling and traceability of the product are important matters owing to an increase in trade and the need to maintain confidence in the quality of the products (Weder *et al*. 2001).

Weder *et al*. (2001) used SSCP patterns of a 148 bp *cyt b* gene segment to identify species origins from raw materials of several fish and animal species. SSCP patterns of 2-4 bands were obtained from blue ling, carp, haddock, mackerel, mackerel shark, saithe, catfish, Alaska pollack and skipjack. The patterns were fish species-specific and the method could be used to identify Alaska pollack in surimi-based products.

Pardo *et al*., (2004) identified five different tuna species from commercial canned tuna by restriction site analysis of mtDNA products obtained by nested primer PCR. Species identification of commercial canned tuna by techniques based on PCR is rather difficult due to the presence of additives as well as to the fact that the DNA is usually severely degraded. The utilization of nested PCR, a technique which increases considerably the specificity and sensitive of the reactions, has allowed the ability to obtain an amplicon of 276 bp from commercial canned tuna. A very useful tool to authenticate canned tuna in brine, oil, pickled, sauced and spiced is presented here. This 276 bp amplicon is the longest fragment obtained so far from canned tuna. In this study, five diagnosis sites are described to discriminate the most common tuna species processed in the canning industry.

Rehbein (2004) identified the fish species of raw or cold-smoked salmon and salmon caviar by SSCP analysis. Differentiation between ten salmonid fish species belonging to the genera *Salmo, Oncorhynchus* and *Salvelinus* was achieved using PCR products of mitochondrial and nuclear genes. Amplicons (300–460 bp in size) of *cytochrome b*, *parvalbumin* and *growth hormone* gave species-specific patterns of single stranded DNA.

Klinbunga *et al*. (2000) identified species-specific markers of 5 oyster species in Thailand: *Crassostrea belcheri, Crassostrea iredalei, Saccostrea cucullata, Saccostrea forskali,* and *Striostrea* (*Parastriostrea*) *mytiloides.* Species-specific

RAPD fragments were found in *C. belcheri*, *C. iredalei*, and *S. cucullata* but not in *S. forskali* and *S. mytiloides.* Three *C. belcheri* specific RAPD fragments were cloned and sequenced. A primer set was designed from each of the recombinant clones (pPACB1, pPACB2, and pPACB3). The PCR products showed expected sizes of 536, 600, and 500 bp, respectively, with the sensitivity of detection approximately 30 pg of *C. belcheri* total DNA template. The specificity of pPACB1 was examined against 135 individuals of indigenous oyster species in Thailand and against outgroup references *S. commercialis* (*N* = 12) and *Perna viridis* (*N* = 12). Results indicated the species-specific nature of primers developed from pPACB1. These markers are thus useful for examination of the correct seed and broodstock species and for estimating the degree of hybridization in natural populations of Thai oysters.

Khamnamtong *et al*. (2005) developed DNA-based molecular markers for differentiation of five penaeid shrimps (*Penaeus monodon, P. semisulcatus, Feneropenaeus merguiensis, Litopenaeus vannamei* and *Marsupenaeus japonicus*) based on PCR-RFLP and SSCP of *16S rDNA.* Differentiation of *P. monodon, P. semisulcatus* and *L. vannamei* can be unambiguously carried out by PCR-RFLP of *16S* $rDNA_{560}$ whereas *P. semisulcatus* and *M. japonicus* shared a BABB mitotype. These shrimps were successfully discriminated by SSCP analysis of *16S* $rDNA_{560}$. Nevertheless, the amplification success for *L. vannamei* and *F. merguiensis* was not consistent when tested against larger sample sizes. As a result, *16S* $rDNA_{560}$ of an individual representing the most common mitotype of each species was cloned and sequenced. The new primer pair was designed and tested against the large sample sizes (312 bp product, *N* = 185). The amplification success was consistent across all species. PCR-RFLP of *16S* $rDNA_{312}$ was as effective as that of *16S* $rDNA_{560}$. Differentiation of all shrimp species were successfully carried out by SSCP analysis of *16S* $rDNA_{312}$.

Imai *et al*. (2004) differentiated four mud crab species (genus *Scylla*) using the first internal transcribed spacer (*ITS-1*) of nuclear rDNA and mtDNA *16S rDNA* markers. These gene segments were amplified by PCR using genomic DNA extracted from adult tissue of four species of *Scylla* spp. and the first zoeal stages of *S. serrata, S. paramamosain* and *S. olivacea* as template. Size polymorphism of the ITS-1 region was found to be useful for distinguishing *S. serrata* and *S. olivacea* from two other

species. The other two species (*S. paramamosain* and *S. tranquebarica*) could be identified using *Hha* I. All four species were identified using PCR-RFLP by double digestion of *16S rDNA* with *Dra*I and *Hin*dIII. These genetic markers can be used for hybridization breeding studies and in field studies of larval and juvenile mud crabs of the genus *Scylla*.

Khetpu (2005) identified fifteen candidate species-specific RAPD fragments in *P. pelagicus*. Nine of which were cloned and sequenced. Five pairs of primers were designed. Three RAPD-derived SCAR markers (PP122-510, PP158-1200 and PP158-1500) generated the expected product (152, 397 and 262 bp) in 95%, 100% and 100% in *P. pelagicus* without any false positive in four non-target species (*Scylla serrata*, *S. oceanica*, *S. tranquebarica* and *C. crucifera*).

In addition, Klinbunga *et al.* (2007) identified 13 AFLP fragments found in at least 95% of investigated specimens which were regarded as candidate species-specific markers for *P. pelagicus*. Two AFLP fragments (300 and 420 bp fragments from P_{AGT}/M_{CAA} and P_{AGT}/M_{CAC}) were cloned and sequenced. The specificity of BSCSCAR1 and BSCSCAR2 primer pairs were tested. The amplification success of BSCSCAR1 (164 bp) in *P. pelagicus* was low (30%). Conversely, BSCSCAR2 generated the expected product (188 bp) in 97.0% of *P. pelagicus* (N = 100; Fig. 1) but not in mud crabs; *S. serrata* (N = 8), *S. oceanica* (N = 19) and *S. tranquebarica* (N = 11). Nevertheless, cross-species amplification was found in *C. crucifera* (N = 6). SSCP analysis, which is favored for identifying species origins of various taxa due to its convenience and cost-effectiveness (Weder *et al*., 2001), was then applied to verify whether nucleotide sequences of the BSCSCAR2 in *P. pelagicus* and *C. crucifera* were different. A monomorphic SSCP pattern was observed in *C. crucifera*, whereas several nonoverlapping SSCP patterns were observed in *P. pelagicus*.

Klinbunga *et al.* (2000) identified species-specific markers to distinguish three mud crab species, *S. serrata*, *S. oceanica*, and *S. tranquebarica*, in eastern Thailand based on RAPD-PCR. Several RAPD fragments were fixed in each mud crab species and used to construct a molecular taxonomic key in those taxa. Therefore, RAPD markers (UBC456, UBC457 and YNZ22) can be used in coupling with BSCSCAR2, PP122-510, PP158-1200 and PP158-1500 to unambiguously differentiate species origin of *P. pelagicus* and *Scylla*.

1.10 Effects of thermal stress on expression of functionally important genes

Temperature is a critical abiotic factor affecting organisms on ecological, organismal, cellular and molecular levels. For many organisms, environmental temperature does not remain constant throughout life but may fluctuate yearly, seasonally, or daily, thus raising the question of how they contend with long term or acute temperature change.

To investigate the effect of stress on the giant tiger shrimp, *P. monodon*, juvenile shrimp were given short term exposure to hypoxic, hyperthermic and osmotic stress twice over a 1-week period and estimates of total haemocyte count (THC), *heat shock protein* (*HSP*) *70* expression and load of gill associated virus (GAV) were determined at different time points. While no significant differences were observed in survival and THC between stressed and control shrimp ($P > 0.05$), *HSP 70* expression and GAV load changed significantly ($P < 0.05$). *HSP 70* expression was higher in the hyperthermic treatment than in other treatments ($P < 0.05$). GAV load increased throughout the 21-day experiment for all groups and treatments except for hyperthermic stress. These findings suggest that a beneficial reduction in the rate of GAV replication, which cannot simply be attributed to holding the animals at a non-permissive temperature for GAV, results from short-term hyperthermic treatment (de la Vega *et al*., 2006).

Crabs of the Bythograeidae family (Crustacea: Brachyura: Bythogreoidea) are the only endemic crab family living in hydrothermal fields. The hydrothermal environment is characterized by unique ecological parameters, such as the high temperature gradient around the hydrothermal chimney (2-350°C), a fluid environment containing high levels of metals and numerous gases. *HSP70* of *Bythograea thermydron*, *Cyanagraea praedator* and *Segonzacia mesatlantica* was characterized. Results revealed that Bythograeidae possess genes which are similar with those present in Xanthids (coastal crabs). The deduced protein sequences displayed motifs distinct from those in the other crustacean *HSC70*/*HSP70*s available in the databases. Phylogenetic analysis showed that these members of *HSP70* family identified in Bythograeidae and Xanthidae constitute a new subgroup within this family (Leignel *et al*., 2007).

Intertidal zone organisms experience thermal stress during periods of low tide, and much work has shown that induction of heat shock proteins and ubiquitination occurs in response to this stress. However, little is known of other cellular pathways that are regulated following thermal stress in these organisms. A functional genomics approach was used to identify genes that were up- and downregulated following heat stress in the intertidal porcelain crab, *Petrolisthes cinctipes* using custom cDNA microarrays made from 13824 cloned *P. cinctipes* ESTs representing 6717 unique consensus sequences. Statistically significant differences in gene expression between heat stressed and control groups were determined with ANOVA. Genes which were upregulated following heat stress were involved with protein folding, protein degradation, protein synthesis and gluconeogenesis, suggesting that heat stress accelerated protein turnover. Genes which were downregulated following heat stress were involved with detoxification, oxygen transport, oxidative phosphorylation, and lipid metabolism, suggesting that the animals were avoiding the generation of reactive oxygen species. ESTs matching hypothetical proteins and ESTs that had no GenBank match were also found to have been both up- and downregulated following heat stress, suggesting that these novel genes may be involved in the heat stress response (Teranishi and Stillman, 2007).

Differential display PCR approaches have been successfully used for isolation of genes expressed in various conditions and/or various tissues of organisms. Khamnamtong *et al*., (2006) used bulked segregant analysis (BSA) and and RAP-PCR to identify sex-specific (or differential) expression markers in ovaries and testes of *P. monodon* (150 primer combinations). Twenty-one and fourteen RAP-PCR fragments specifically/differentially expressed in ovaries and testes of *P. monodon* were successfully cloned and sequenced. Expression patterns of 25 transcripts were tested against the first stranded cDNA of ovaries and testes of 3-month-old and broodstock-sized *P. monodon* (N = 5 and N = 7 - 10 for females and N = 4 and N = 5 - 7 for males, respectively). Five (FI-4, FI-44, FIII-4, FIII-39 and FIII-58) and two (M457-A01 and MII-51) derived RAP-PCR markers revealed female- and male-specific expression patterns in *P. monodon*. Surprisingly, MII-5 originally found in testes showed a higher expression level in ovaries than did testes of juvenile shrimps but a temporal female-specific pattern in *P. monodon* adults.

Matsumoto *et al*. (2007) elucidate the molecular mechanisms involved in oogenesis by applied a differential display method to identify genes whose expression was detected only in ovaries containing oocytes. One of the cDNA fragments isolated by mRNA differential display was similar to vitellogenin. The full-length cDNA encoding putative vitellogenin in the Pacific oyster *Crassostrea gigas* was further isolated by RACE-PCR. The open reading frame predicted 1583 amino acid residues. The deduced primary structure of putative vitellogenin in *C. gigas* was similar to vitellogenins of various other mollusk, fish, crustacean and nematode species, especially in the N-terminal region. RT-PCR revealed that mRNA encoding putative vitellogenin was expressed only in the ovary. *In situ* hybridization analysis revealed that putative vitellogenin mRNA was expressed strongly in the follicle cells in the ovary. It is concluded that the follicle cells are the site of putative vitellogenin synthesis.

Cui *et al*., (2006) identified sex markers by cDNA-AFLP in *Takifugu rubripes*. Sex markers in mature male and female gonads and tailfins were identified by cDNA-AFLP performed on pooled cDNA samples. Five sex markers (TDF1-5) were identified, cloned, characterized and confirmed on individual samples of cDNA and genomic DNA, showing good agreement with phenotypic sex. Among these, four markers (TDF1-4) derived from gonads were detected in immature *T. rubripes* (at months 6, 12, 18 and 24) testis or ovary cDNA, while the TDF5 from tailfins was detected in immature *T. rubripes* (at months 6, 12, 18 and 24) tailfin cDNA and *T. rubripes* tailfin genomic DNA. Therefore, the sex specific primer or probe of TDF5 could be used in quick detection of phenotypic sex in juvenile and larval stages. TDF5 could amplify a specific fragment from *Takifugu pseudommus* tailfin genomic DNA, indicating that homologues of sex markers might exist in other species of the *Takifugu* genus.

Presently, the blue swimming crab is the new promising species for aquaculture in Thailand. Nevertheless, the basic information about genetic diversity and intraspecific population differentiation of this species in Thai waters is still limited. This basic information is essential for the construction of suitable management schemes of this important species leading to sustainable fisheries and aquaculture of *P. pelagicus* in Thailand. Although population genetic studies of

P. pelagicus have been reported based on RAPD (Khetpu, 2005) and AFLP (Klinbunga *et al.*, 2007) analyses, information based on the type I markers (coding sequences) on population genetics of *P. monodon* has not been reported. In addition, species-specific markers play important roles for quality control to prevent intentionally use of the wrong species in canning. Therefore, it was desired to explore the possibility of using SSCP analysis to evaluate genetic variability and population structure of *P. pelagicus* in Thai waters and to develop species-diagnostic mtDNA markers for this species. Moreover, cDNA differentially expressed during thermal stress of *P. pelagicus* has not been reported. The differentially displayed approach based on cDNA-AFLP was then applied. Subsequently, single nucleotide polymorphism (SNP) found in the characterized gene segments may be further examined for correlation with thermal stress tolerance and applied for genetic improvement of cultured *P. pelagicus*.

1.11 Objectives of the thesis

The aims of this thesis were determination of genetic diversity of *P. pelagicus* in Thailand by SSCP analysis and development of reproducible species-diagnostic markers of Thai *P. pelagicus*. Moreover, expression profiles of various genes under a temperature stress induced condition were also examined.

CHAPTER II

MATERIALS AND METHODS

2.1 Experimental animals

The blue swimming crabs (*P. pelagicus*) were collected from Chanthaburi (*N* = 29), Prachup Khiri Khan (*N* = 40) and Suratthani (*N* = 35) located in the Gulf of Thailand and Ranong (*N* = 35) and Krabi (*N* = 35) located in the Andaman Sea (Table 2.1 and Figure 2.1). The whole specimens or muscle dissected out from the first walking leg of each crab were transported to the laboratory at the Center of Excellence for Marine Biotechnology, Faculty of Science, Chulalongkorn University on ice. Specimens were kept in a -80^{o}C freezer until further used for DNA extraction.

In addition, the mud crabs; *S. oceanica* (*N* = 18), *S. serrata* (*N* = 7) and *S. tranquebarica* (*N* = 9) were collected from Chanthaburi and Rayong. The swimming crab, *Charybdis crucifera* (*N* = 20) and the three spot swimming crab, *Portunus sanguinolentus* (*N* = 10) were collected from Chonburi. These specimens were included as non-target species for the specificity test of developed species-diagnostic markers.

For cDNA-AFLP analysis, twenty four individuals of *P. pelagicus* were purchased from Sam-Yan market with the average weight and carapace length of 221.4 g and 14.4 cm, respectively. The experimental animals were acclimatized at the laboratory conditions (30 ppt of seawater at the ambient temperature and normal light:dark conditions) for 3 days and subsequently divided into six groups. The control group was maintained at the normal conditions whereas the remaining samples were treated at 33^{o}C for 3 hours. The haemolymph of the control and the treatment were collected before treatment and at 0, 3, 6, 12 and 24 hours post treatment (hpt), respectively.

Table 2.1 Species, geographic samples and number of specimens used for genetic diversity studies and analysis of species-specific markers

Samples	Genetic diversity studies (*N* = 174)	Species specificity (*N* = 238)
Blue swimming crab; *P. pelagicus*		
Chanthaburi (Gulf of Thailand)	29	29
Prachup Khiri Khan (Gulf of Thailand)	40	40
Suratthani (Gulf of Thailand)	35	35
Ranong (Andaman Sea)	35	35
Krabi (Andaman Sea)	35	35
Mud crabs		
S. oceanica	-	18
S. serrata	-	7
S. tranquebarica	-	9
Swimming crab		
C. crucifera	-	20
Three spot swimming crab		
P. sanguinolentus	-	10

2.2 Nucleic acid extraction

2.2.1 Genomic DNA extraction

2.2.1.1 A proteinase K/phenol-chloroform extraction method

Genomic DNA was extracted from the muscle of each blue swimming crab using a phenol-chloroform-proteinase K method. The muscle tissue (approximately 200 mg) was placed in a mortar containing liquid nitrogen and ground to fine powder. The tissue powder was transferred to a centrifuge tube containing 2 ml of the extraction buffer (100 mM Tris-HCl, 100 mM EDTA, 250 mM NaCl; pH 8.0) and briefly homogenized with a micropestle and aliquoted into microcentrifuge tubes (500 μl). SDS (10%) and RNase A (10 mg/ml) solutions were added to a final

concentration of 1.0% (w/v) and 100 μg/ml, respectively. The resulting mixture was then incubated at 37°C for 1 hour. At the end of the incubation period, a proteinase K solution (10 mg/ml) was added to the final concentration of 200 μg/ml and further incubated at 55°C for 3 hours. An equal volume of buffer-equilibrated phenol was added and gently mixed for 15 minutes. The solution was centrifuged at 12,000 rpm for 10 minutes at room temperature. The upper aqueous phase was transfered to a new sterile microcentrifuge tube.

Figure 2.1 Map of Thailand indicating sampling collection sites for the blue swimming crab (*P. pelagicus*) used in this study.

This extraction process was then repeated once with phenol and twice with chloroform:isoamylalcohol (24:1). The aqueous phase was transferred into a sterile microcentrifuge. One-tenth volume of 3 M sodium acetate, pH 5.2 was added. DNA was precipitated by an addition of two volume of pre-chilled absolute ethanol and mixed thoroughly. The mixture was incubated at -80^{o}C for 30 minutes. The precipitated DNA was washed twice with 1 ml of 70% ethanol (10 minutes and 5 minutes, respectively). After centrifugation, the supernatant was removed. The DNA pellet was air-dried and resuspended in 30 - 50 μl of TE buffer (10mM Tris-HCl and 0.1 mM EDTA, pH 8.0). The DNA solution was incubated at 37^{o}C for 1 - 2 hours for complete solubilization and kept at 4^{o}C until further used.

2.2.1.2 A chelex-based method

A piece of the muscle tissue was homogenized in 200 μl of a 5% Chelex® (w/v). The homogenate was incubated at 60ºC for 3 hours. To stop the DNase activity, the homogenate was placed in the boiling water for 10 minutes, left to room temperature and briefly centrifuged at 13000 rpm for 10 minutes. The supernatant was transferred to a new tube and kept at 4ºC. This DNA solution (usually 1 μl) was used as the template for PCR.

2.2.2 RNA extraction

Haemolymph was withdrawn from the unsclerotized membrane between the carpus and the propodus of a cheliped and walking legs (2nd - 5th pereopod) of each crab. Approximately 0.5 ml of the haemolymph was withdrawn into a syringe containing an equal volume of the anticoagulant (10% sodium citrate). The haemocyte was centrifuged at 3600 *g* at 4^{o}C. The supernatant was discarded. Total RNA was extracted from hemocyte of each the crab using TRI-REAGENT (Molecular Research Center). Two hundred microlitres of TRI-REAGENT were added and briefly homogenized with a micropestle. Another 800 μl of TRI-REAGENT was added. The homogenate was left at room temperature for 5 minutes before the addition of 0.2 ml of chloroform. The homogenate was vortexed for at least 15 minutes, left at room temperature for 2 - 15 minutes and centrifuged at 12000*g* for 15 minutes at 4^{o}C. The mixture was separated into the lower phenol-chloroform phase (red), the interphase, and the upper aqueous phase (colorless).

The aqueous phase (exclusively containing RNA) was carefully transferred to a new 1.5 ml microcentrifuge tube. RNA was precipitated by an addition of 0.5 ml of isopropanol and mixed thoroughly. The mixture were left at room temperature for 10-15 minutes and centrifuged at 12000*g* for 10 minutes at 4^{o}C. The supernatant was removed. The RNA pellet was washed with 1 ml of 75% ethanol and centrifuged at 12000*g* for 10 minutes at 4^{o}C. Ethanol was removed. The RNA pellet was air-dried for 5 - 10 minutes. RNA was dissolved in DEPC-treated H_2O for immediately used. Alternatively, the RNA pellet was kept under absolute ethanol in a -80^{o}C freezer for long storage.

2.3 Measuring concentration of nucleic acids

2.3.1 Estimation of DNA and RNA concentration by spectrophotometry

The concentration of extracted DNA or RNA was estimated by measuring the optical density at 260 nanometre (OD_{260}). An OD_{260} of 1.0 corresponds to a concentration of 50 μg/ml double stranded DNA, 40 μg/ml single stranded RNA and 33 μg/ml oligonucleotide (Sambrook and Russell, 2001). Therefore the concentrations of DNA/RNA are estimated in μg/ml by the following equation;

$$[\text{Nucleic acid}] = OD_{260} \times \text{dilution factors} \times \text{nucleotide factor}$$

; nucleotide factor = 50, 40 or 33 for DNA RNA or oligonucleotides, respectively

The value at OD_{260} allows calculation of total nucleic acid whereas the value at OD_{280} determines the amount of proteins in the DNA and RNA solution. The ratio between OD_{260}/OD_{280} provides an estimate on the purity of extracted DNA/RNA. For the extracted DNA, a pure preparation of DNA has OD_{260}/OD_{280} ratio of 1.8 - 2.0. The ratio of approximately 2.0 indicates the good quality of the extracted RNA. The ratios that much lower than those values indicate contamination of residual proteins or phenol in the extracted DNA or RNA (Kirby, 1992).

2.3.2 Estimation of the amount of DNA by mini-gel electrophoresis

DNA concentration can also be estimated on the basis of its direct relationships between the amount of DNA and the level of fluorescence after ethidium bromide staining. DNA was electrophoresed through 0.8-1.0% agarose gel prepared

in 1xTBE buffer (89 mM Tris-HCl, 8.91 mM boric acid and 2.5 mM EDTA, pH 8.0) at 100 V. After electrophoresis, the gel was stained with ethidium bromide. DNA concentration was estimated from the intensity of the fluorescent band by comparing with that of undigested λ-DNA.

Part I. Studies of Genetic Diversity and Population Differentiation of *P. pelagicus* in Thai waters

2.4 Cross-species amplification using heterospecific primers

Thirty eight primer pairs composing of universal primers and those previously developed from other marine species (*Penaeus monodon* and *Macrobrachium rosenbergii*) were screened against genomic DNA of *P. pelagicus*. Generally, PCR was performed in a 25 μl reaction volume containing 75 mM Tris-HCl (pH 8.8 at 25^{o}C), 20 mM $(NH_4)_2SO_4$, 0.1 % Tween 20, 1.5 - 2.0 mM $MgCl_2$, 200 μM of each dATP, dTTP, dCTP and dGTP, 0.25 - 0.5 μM of each primer (Table 2.2), 1 unit of *Taq* DNA polymerase (Fermentus) and 50 ng of genomic DNA. PCR was carried out using the amplification conditions described in Table 2.3.

2.5 Agarose gel electrophoresis

The amplification products were electrophoresed through 1.2 - 1.5% agarose gels. The appropriate amount of agarose was weighed and mixed with 1x TBE (89 mM Tris-HCl, 8.91 mM boric acid and 2.5 mM EDTA, pH 8.0). The solution was boiled in a microwave to complete solubilization and left at room temperature to approximately 60^{o}C before poured into a gel mould. The gel was left at room temperature for 30-45 minutes to completely solidified. When needed, the gel was placed in the electrophoretic chamber containing and enough amount of 1xTBE buffer covering the gel for approximately 0.5 cm and the comb was gently removed.

The product were mixed with one-fourth volume of a loading dye solution (0.25% bromophenol blue and 25% ficoll) and then loaded into the well. A 100 bp DNA ladder was used as standard DNA marker. Electrophoresis was carried out at 4-5 V/cm until the tracking dye migrated about three-quartered of the gel.

Table 2.2 Sequences of PCR primer screened for population genetic analysis of Thai *P. pelagicus*

Gene/Primer	Sequence	Length (bp)
1. *COI*	LCO1490: 5' GGT CAA CAA ATC ATA AAG ATA TTG G 3'	25
	HCO2198: 5' TAA ACT TCA GGG TGA CCA AAA AAT CA 3'	26
2. *COI-COII*	F: 5' TTG ATT TTT TGG TCA TCC AGA AGT 3'	24
	R: 5' CCA CAA ATT TCT GAA CAT TGA CC 3'	23
3. *ND5*	F: 5' ATA GAG CGT TGC ATT GAA GC 3'	20
	R: 5' TCA AGG AGC ATT AGA GTG AG 3’	20
4. *18S rDNA*	F: 5' TGG ATC CGG GCA AGT CTG GTG CC 3'	23
	R: 5' TGA AGT CAA GGG CAT CAC AGA CC 3’	23
5. *16S rDNA*	F: 5' CGC CTG TTT AAC AAA AAC AT 3'	20
	R1: 5' CCG GTC TGA ACT CAG ATC ATG T 3'	22
	R2: 5' GGT CTG AAC TCA GAT CAG ATC ACG T 3'	25
	R3: 5' CCG GTC TGA ACT CAG ATC AGA TCA CGT 3'	27
6. *12S rDNA*	F: 5' AAA CTA GGA TTA TAT ACC CTA TTA 3'	24
	R: 5' AAG AGG GAC GGG CGA TTT GT 3’	20
7. *12S rDNA*(F)/ *16S rDNA*(R1)	F: 5' AAA CTA GGA TTA TAT ACC CTA TTA 3'	24
	R1: 5' CCG GTC TGA ACT CAG ATC ATG T 3'	22
8. *12S rDNA*(F)/ *16S rDNA*(R2)	F: 5' AAA CTA GGA TTA TAT ACC CTA TTA 3'	24
	R2: 5' GGT CTG AAC TCA GAT CAG ATC ACG T 3'	25
9. *12S rDNA*(F)/ *16S rDNA*(R3)	F: 5' AAA CTA GGA TTA TAT ACC CTA TTA 3'	24
	R3: 5' CCG GTC TGA ACT CAG ATC AGA TCA CGT 3'	27
Homologue of sex-related transcripts of *P. monodon*		
10. *Difulfide isomerase* (*DSI*)	F: 5' GCC GTT GCC AAT AAG GAC GA 3'	20
	R: 5' TCA CCC GCC TTG AGA TTG GT 3'	20
Homologue of *SERCA* in *Macrobrachium rosenbergii*		
11. *Sarco endoplasmic reticulum* Ca^{2+} *ATPase* (*SERCA*)	F: 5' CTG GCT CTT CTT CCG TTA CAT 3'	21
	R: 5' ACA AGC AGC GAC TGG TTC TCA 3'	21
Candidate sex-specific primers of *Macrobrachium rosenbergii* from AFLP analysis		
12. $ME_{+3}3M_{+3}7425.1$	F: 5' GGA TGT CTG ACG GCT TGC TG 3'	20
	R: 5' GAG GCG GAA AAG GAT ATG TG 3'	20
13. $ME_{+3}3M_{+3}7425.2$	F: 5' AAG CAC GCA CAT ACT CAC ACA T 3'	22
	R: 5' GCA TCT TTC CAT CCT CCA AC 3'	20
14. $ME_{+3}7M_{+3}6425$	F: 5' AGA CCA CAT CCC TGA AAC CT 3'	20
	R: 5' CAT ATC TTG CCC ATC CTG CT 3'	20
15. $ME_{+3}4M_{+3}5570$	F: 5' TCA ACC ATA GCC TGT ACC TTT C 3'	22
	R: 5' ACT TCA CCC CTG GAG AGA CTG T 3'	22
16. $ME_{+3}8M_{+3}1310.1$	F: 5' AGT GAG GTA GGT TAC TGA CA 3'	20
	R: 5' AGG ATG GAC TGA ATC GGG CA 3'	20
17. $ME_{+3}8M_{+3}1310.2$	F: 5' GTA CAT TAT GAA CTA AGA CAA G 3'	22
	R: 5' TGT ATT GCT GTT TCC ATA GGG C 3'	22
18. $ME_{+3}8M_{+3}1310.3$	F: 5' TTG TCA GAT GGC TAA TAG TGT C 3'	22
	R: 5' CAA ATG AGA AAT GAA GTG GAA G 3'	22
19. $ME_{+3}4M_{+3}8517$	F: 5' TGT TAT CCT TCG TTC CCT CC 3'	20
	R: 5' AAA GTG ACA GTC CTG GCA AA 3'	20
20. $FE_{+3}1M_{+3}7290$	F: 5' GCT TAG GGT TCT GGT CTT GTG A 3'	22
	R: 5' AAA AGG GAG GGA GCA AAG AGT G 3'	22

Table 2.2 (cont.)

Gene/Primer	Sequence	Length (bp)
21. $FE_{+3}5M_{+3}1390$	F: 5' ACC CTC TTT TCT GGA TGG CA 3'	20
	R: 5' CGG CAA ACC TCA AAT CAC TC 3'	20
22. $FE_{+3}8M_{+3}3270.1$	F: 5' ACT ATT ATT TCT TAT TTT TCT CTC 3'	24
	R: 5' CAA GCC TTT ACA AAT AGG GAA T 3'	22
23. $FE_{+3}8M_{+3}3270.2$	F: 5' CGC CTA TTC CTC AAT CGG TCA A 3'	22
	R: 5' ATG CTT CCA TTC CTC CGT CCT T 3'	22
24. $FE_{+3}8M_{+3}6710$	F: 5' GCT TTA TTT CCT GCG TTA TTG G 3'	22
	R: 5' ACG ATT GGC TCA TTC GCT CCT A 3'	22
Candidate sex-specific/differential markers of *Macrobrachium rosenbergii* from RAP-PCR analysis		
Female-specific expression markers		
25. FeRAP315.1	F: 5' CAA CGT ACT ACT CTT GTT CAT C 3'	22
	R: 5' ATT GTT CCA AAG TGC CTA TTA 3'	21
26. FeRAP315.2	F: 5' TCC TAC TAC TAG CCA AGA CGA TT 3'	23
	R: 5' CGA CCA GGC AGA TGA CCC AAG 3'	21
Male-specific expression markers		
27. M268/128RAP	F: 5' GTG GGG GGA GTG CCG TCA GT 3'	20
	R: 5' ACA AAG GTG TAA CAG GAG AAA AAC 3'	24
28. M428/228RAP	F: 5' AAC TCG TTT GCT CCG AAG AA 3'	20
	R: 5' AAA GAA CCT CAA GTA ATG CCT 3'	21
29. M122/135RAP	F: 5' TCA GAC TAC ATA CCC CTT CAA T 3'	22
	R: 5' TGG GCG ACC AAA ACA GCA G 3'	19
30. MA16/222RAP	F: 5' CAT AAG AGC GAA TGA ACG AAG CA 3'	23
	R: 5' TCC TTA TTT GTA ATG TTT CGT CT 3'	23
31. M122/159RAP.1	F: 5' CTG CTG CTA CCT TTG GCT ATT A 3'	22
	R: 5' GCG TCA GTT ACA GTT GTC ACC 3'	21
32. M122/159RAP.2	F: 5' CGA CGG TAA TGT ATG AAG CAG T 3'	22
	R: 5' CCC GAG GAG AAC CAC CAA AGT 3'	21
Candidate differential expression markers		
33. SOCRAP340.1	F: 5' GAG TTT CTT TTA CCT GTG CGT GG 3'	23
	R: 5' CCC TGA TGT TGC GAG CAT TGG A 3'	22
34. SOCRAP340.2	F: 5' GTT TAG CAA CCA AGT ATT TAG TG 3'	23
	R: 5' TTG ACC GTC CTC GCA ATC CGT 3'	21
35. SOC268/273RAP	F: 5' ATA TTT GGT GCT CCA TCG TA 3'	20
	R: 5' TGA AGT CCT CAC TCC ATT GC 3'	20
36. SOC122/228RAP	F: 5' ACG ACG ACG GCG ACG AAC G 3'	19
	R: 5' TTG CAG TCC ATA CTT GCT GAA T 3'	22
37. BC428/228RAP	F: 5' TAC ACT GCG GAA AGT AAT GCT 3'	21
	R: 5' AGG ACT TAG ACT TAT TTT ACG 3'	21
38. BC428/273RAP	F: 5' CAA GAC TGT GGG AAA TCG TGT 3'	21
	R: 5' TAA TGA GAT GCT GAA GAA AAG A 3'	22

After electrophoresis, the gel was stained with ethidium bromide (0.5 μg/ml) for 5 minutes and destained to remove unbound EtBr by submerged in water for 15 minutes. The DNA fragments were visualized under the UV light using a UV transilluminator.

Table 2.3 PCR profiles and compositions (dNTP, $MgCl_2$ and primer concentrations) for amplification of genomic DNA of *P. pelagicus*

Gene/Primer	[dNTP] (µM)	[$MgCl_2$] (mM)	[Primer] (µM)	PCR conditions
1. *COI*	200	2	0.5	94°C, 3 min for 1 cycle followed by 94°C, 1 min; 42°C, 1.5 min and 72°C, 1.5 min for 10 cycles and 94°C, 1 min; 53°C, 1.5 min and 72°C, 1.5 min for 35 cycles and 72°C, 7 min for 1 cycle
2. *COI-COII*	200	2	0.5	As described in 1.
3. *ND5*	200	2	0.5	As described in 1.
4. *18S rDNA*	200	2	0.5	As described in 1.
5. *16S rDNA*	200	2	0.5	As described in 1.
6. *12S rDNA*	200	2	0.5	As described in 1.
7. *12S rDNA*(F)/ *16S rDNA*(R1)	200	2	0.5	As described in 1.
8. *12S rDNA*(F)/ *16S rDNA*(R2)	200	2	0.5	As described in 1.
9. *12S rDNA*(F)/ *16S rDNA*(R3)	200	2	0.5	As described in 1.
10. *Difulfide isomerase* (*DSI*)	200	2	0.4	94°C, 3 min for 1 cycle followed by 94°C, 30 sec; 53°C, 1 min and 72°C, 45 sec for 35 cycles and 72°C, 7 min for 1 cycle
11. *Sarco endoplasmic reticulum* Ca^{2+} *ATPase* (*SERCA*)	100	2	0.25	As described in 10.
12. $ME_{+3}3M_{+3}7425.1$	200	2	0.25	As described in 10.
13. $ME_{+3}3M_{+3}7425.2$	200	2	0.25	As described in 10.
14. $ME_{+3}7M_{+3}6425$	200	2	0.25	As described in 10.
15. $ME_{+3}4M_{+3}5570$	200	1.5	0.25	As described in 10.
16. $ME_{+3}8M_{+3}1310.1$	200	1.5	0.25	As described in 10.
17.$ME_{+3}8M_{+3}1310.2$	200	2	0.25	As described in 10.
18.$ME_{+3}8M_{+3}1310.3$	200	1.5	0.25	As described in 10.
19. $ME_{+3}4M_{+3}8517$	200	2	0.25	As described in 10.
20. $FE_{+3}1M_{+3}7290$	200	2	0.25	As described in 10.
21. $FE_{+3}5M_{+3}1390$	200	2	0.25	As described in 10.
22. $FE_{+3}8M_{+3}3270.1$	200	1.5	0.25	As described in 10.
23. $FE_{+3}8M_{+3}3270.2$	200	1.5	0.25	As described in 10.
24. $FE_{+3}8M_{+3}6710$	200	1.5	0.25	As described in 10.
25. FeRAP315.1	200	2	0.25	As described in 10.
26. FeRAP315.2	200	2	0.25	As described in 10.
27. M268/128RAP	200	2	0.25	As described in 10.
28. M428/228RAP	200	2	0.25	As described in 10.
29. M122/135RAP	200	2	0.25	As described in 10.
30. MA16/222RAP	200	2	0.25	As described in 10.

Table 2.3 (cont.)

Gene/Primer	[dNTP] (μM)	[$MgCl_2$] (mM)	[Primer] (μM)	PCR conditions
31. M122/159RAP.1	200	2	0.25	As described in 10.
32. M122/159RAP.2	200	2	0.25	As described in 10.
33. SOCRAP340.1	200	2	0.25	As described in 10.
34. SOCRAP340.2	200	2	0.25	As described in 10.
35. SOC268/273RAP	200	2	0.25	As described in 10.
36. SOC122/228RAP	200	2	0.25	As described in 10.
37. BC428/228RAP	200	2	0.25	As described in 10.
38. BC428/273RAP	200	2	0.25	As described in 10.

2.6 Polymorphic AFLP fragments of *P. pelagicus*

Previously, genetic diversity of *P. pelagicus* in Thailand based on AFLP and RAPD (Khetpu, 2005). Six AFLP fragments that were found in fifteen to eighty percent of tested individuals were chosen (Table 2.4). The AFLP bands were excised from the gel using a sterile razor blade and washed twice for 2 hours each at room temperature with 500 μl of sterile deionized water. Twenty microlitres of TE buffer or water was then added and incubated overnight at 37°C. The target fragment was reamplified using the same PCR recipes as those for selective amplification with the exception that 100 ng of each primer and 5 μl of the eluted AFLP product were used. The amplification conditions were composed of 94°C for 3 minutes, followed by 35 cycles of 94°C for 30 seconds, 50°C for 1 minute and 72 °C for 1 minute. The final extension was performed at 72°C for 7 minutes.

2.7 Cloning of PCR product

2.7.1 Elution of DNA from agarose gel

The required DNA fragment was fractionated through an agarose gel in duplication. One was run side-by-side with a 100 bp DNA markers and the other was loaded into the distal well of the gel. After electrophoresis, lanes representing the DNA standard and its proximal DNA sample were cut and stained with ethidium bromide (0.5 μg/ml) for 5 minutes. Positions of the DNA markers and the EtBr-stained reamplified fragment were used to align the position of the non-stained target DNA fragment.

The DNA fragment was excised from the gel with a sterile razor blade. DNA was eluted out from the agarose gels using a Hiyield^{TM} gel Extraction kit (Real Genomics) according to the protocol recommended by the manufacture. The excised gel was transferred up to 200 - 300 mg into a microcentrifuge tube. Approximately 500 μl of DF buffer was added to the sample and vortexed. The mixture was incubated at 55°C for 10 - 15 minutes with briefly vortexing every 2 - 3 minutes. After the gel was completely dissolved, the mixture was applied to the DF column placed on a 2 ml collection tube and centrifuged at 8,600 rpm for 1 minute at room temperature. The flow-through was discarded and 0.5 ml of the wash buffer was added. The DF column was centrifuged at 8,600 rpm for 1 minute at room temperature. The flow-through was discarded. The column was further centrifuged at room temperature for an additional 2 minute at 14,000 rpm to remove trance amount of the washing buffer. The column was then placed in a new microcentrifuge tube and 15-30 μl of the Elution buffer was added to the center of the DF membrane. The column was incubated at room temperature for 2 minute before centrifuged at 14,000 rpm for 2 minutes. The eluted sample was stored at -20°C until further required.

2.7.2 Ligation of PCR product to pGEM®-T Easy vector

The ligation reaction was set up in the total volume of 10 μl containing approximately 50 ng of the gel-eluted PCR product, 25 ng of pGEM®-T easy vector, 5 μl of 2X rapid ligation buffer (60 mM Tris-HCl pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP and 10 % PEG 8000) and 3 Weiss units of T4 DNA ligase. The ligation mixture was gently mixed by pipetting and incubated at 4 °C overnight.

2.7.3 Transformation of the ligation product to *E.coli* host cells

2.7.3.1 Preparation of competent cells

A single colony of *E. coli* JM109 was inoculated in 10 ml of LB broth (1% Bacto tryptone, 0.5% Bacto yeast extract and 0.5% NaCl) with vigorous shaking at 37°C overnight. The starting culture was inoculated into 50 ml of LB broth and continued culture at 37°C with vigorous shaking to the OD_{600} of 0.5 to 0.8. The cells were briefly chilled on ice for 10 minutes before centrifuged at 2,700 g for 10 minutes at 4°C. The pellets were resuspended in 30 ml of ice-cold $MgCl_2$-$CaCl_2$ solution

(80 mM $MgCl_2$ and 20 mM $CaCl_2$) and centrifuged as above. The supernatant was discarded and the pellet was resuspended in 2 ml of ice-cold 0.1 M $CaCl_2$ and divided into 100 μl aliquots. These competent cells could be used immediately or stored at –80°C for subsequently used.

2.7.3.2 Transformation

The competent cells were thawed on ice for 5 minutes. Two to four microlitres of the ligation mixture were added and gently mixed by pipetting. The mixtures were incubated on ice for 30 minutes. During the incubation period, the ice box was gently moved forward and backward a few times every 5 minutes. The reaction tube was heat-shocked in a 42°C water bath for exactly 45 seconds without shaking. The reaction tube was then immediately snapped on ice for 2 – 3 minutes. One microlitre of SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) was added to the tube. The cell suspension was incubated with shaking at 37°C for 1 to 1.5 hours. At the end on the incubation period, the cultured cell suspension was centrifuged at 6,000 rpm for 1 minute at room temperature. The pellet was gently resuspended in 100 μl of SOC and spread on a LB agar plate containing 50 μg/ml of ampicillin, 25 μg/ml of IPTG and 20 μg/ml of X-gal. The plate was left until the cell suspension was absorbed and further incubated at 37°C overnight (Sambrook and Russell, 2001). The recombinant clones containing inserted DNA are white whereas those without inserted DNA are blue.

2.7.4 Colony PCR and digestion of the amplified inserts by restriction endonucleases

Colony PCR was performed in a 25 μl reaction volume containing 75 mM Tris-HCl (pH 8.8 at 25°C), 20 mM $(NH_4)_2SO_4$, 0.1 % Tween 20, 2 mM $MgCl_2$, 100 μM each of dATP, dCTP, dGTP and dTTP, 0.1 μM of pUC1 (5' TCC GGC TCG TAT GTT GTG TGG A 3') and pUC2 (5' GTG CTG CAA GGC GAT TAA GTT GG 3') primers and 0.5 unit of *Taq* DNA Polymerase (Fermentus). A recombinant colony was picked up by the micropipette tip and mixed well in the amplification reaction. The PCR profiles was predenaturing at 94°C for 3 minutes, followed by 35 cycles of 94°C for 30 seconds, 50°C for 1 minute and 72 °C for 1 minutes. The final extension

was carried out at 72°C for 7 minutes. The colony PCR products were electrophoretically analyzed through a 1.2 % agarose gel and visualized after ethidium bromide staining.

The colony PCR products containing the insert were separately digested with *Alu* I and *Rsa* I (Promega) to verify whether an insert contained only one type of sequence. Typically, the digestion reaction was set up in the total volume of 20 μl reaction volume containing 1x restriction enzyme buffer (6 mM Tris-HCl; pH 7.5, 6 mM MgCl2, 50 mM NaCl and 1 mM DDT for *Alu* I and 10 mM Tris-HCl; pH 7.9, 10 mM MgCl2, 50 mM NaCl and 1 mM DDT for *Rsa* I), 0.1 mg/ml BSA, 2 units of each enzyme and 5 μl of the colony PCR product. The reaction mixture was incubated at 37°C for 4 hours or overnight. Digestion patterns were electrophoretically analyzed by 1.2% agarose gels.

2.7.5 Isolation and digestion of recombinant plasmid DNA

A recombinant clone was inoculated into 3 ml of LB broth (1% tryptone, 0.5% yeast extract, 1.0 % NaCl) containing 50 μg/ml of ampicillin and incubated at 37°C with constant shaking at 250 rpm overnight. The culture was transferred into 1.5 ml microcentrifuge tube and centrifuged at 14,000 rpm for 1 min. The supernatant was discarded. The bacterial cell pellet was collected and resuspended with 200 μl of the PD1 buffer containing RNaseA and thoroughly mixed by vortexed. The resuspended cells were lysed by the addition of 200 μl of the PD2 buffer and mixed gently by inverting the tube 10 times. The mixture was stood for 2 minutes at room temperature. After that, 300 μl of the buffer PD3 was added to neutralize the alkaline lysis step and mixed immediately by inverting the tube for 10 times. To separate the cell debris, the mixture was centrifuged at 14,000 rpm for 15 minutes. The supernatant was transferred into a new microcentrifuge tube and to the PD column and centrifuged at 6,000*g* (8,000 rpm) for 1 minute. The flow-through was discarded. The PD column was placed back in the collection tube. The column was washed by adding 400 μl of the W1 buffer and centrifuged at 6,000*g* (8,000 rpm) for 1 minute. After discarding the flow-through, 600 μl of the ethanol-added Wash buffer was added and centrifuged as above. The flow-through was discarded. The spin tube was centrifuge for an additional 2 minute at full speed (14,000 rpm) to remove the residual Wash buffer.

The dried PD column was placed in a new 1.5 ml microcentrifuge tube and 30-50 µl of the Elution buffer or water was added at the center of the column to elute the extracted plasmid DNA. The column was left at room temperature for 2 minutes and centrifuge at 14,000 rpm for 2 minutes. The concentration of extracted plasmid DNA was spectrophotometrically measured.

The insert size of each recombinant plasmid was examined by digestion of the plasmid with *Eco R*I. The digest was carried out in a 12 µl containing 1x restriction buffer (90 mM Tris-HCl; pH 7.5, 10 mM NaCl and 50 mM $MgCl_2$), 3 units *EcoR*I (Promega) and 1 µl of recombinant plasmid and incubated at 37°C for 4 hours or overnight before electrophoretically analyzed by agarose gel elctrophoresis.

2.7.6 DNA sequencing

The recombinant plasmid was unidirectional sequenced using the M13 reverse or M13 forward primers on an automated DNA sequencer. Nucleotide sequences were blasted against data in the GenBank (http://www.ncbi.nlm.nih.gov/blast) using Blast*N* (nucleotide similarity) and Blast*X* (translated protein similarity).

2.8 Identification of polymorphic sequence-characterized amplified region (SCAR) markers

2.8.1 Primer design and screening

Four primer pairs were designed from gene homologues; *cytochrome oxidase subunit I, 12S ribosomal (r) DNA, Sarco endoplasmic reticulum* Ca^{2+} *ATPase (SERCA)* and M122/135RAP, using Primer premier 5.0. In addition, six primer pairs were also designed from sequences of polymorphic AFLP fragment of *P. pelagicus*. These primers were screened against genomic DNA of *P. pelagicus* (Table 2.5).

2.9 Single strand conformational polymorphism (SSCP) analysis

The PCR products from sequence-specific primers were successfully amplified using the amplification conditions described in Table 2.6 but did not exhibit size polymorphism or allelic variants between individuals. They were further characterized using single strand conformational polymorphism (SSCP). SSCP

analysis allows the identification of polymorphic sites in DNA. Single-stranded DNA is resolved based on both conformation and size.

2.9.1 Preparation of glass plates

The long glass plate was thoroughly wiped with 1 ml of 95% commercial grade ethanol with a piece of the tissue paper in one direction. This process was then repeated twice. Afterwards, the long glass plate was coated with 1 ml of freshly prepared Bind silane (10 µl of Bind silane, Amersham Biosciences, 980 µl of 95% ethanol and 10 µl of 5% glacial acetic acid) and left for approximately 10 - 15minutes. Excess binding solution was removed with a piece of the tissue paper. The long glass plate was further cleaned with 95% ethanol for 3 times.

The short glass plate was treated as described above with the exception that the binding solution was replaced by the Repel silane (2% dimethyldichlorosilane in octamethylcyclotetrasitoxone). The cleaned glass plates were assembled with a pair of 1.5 mm spacers.

Table 2.4 Names of AFLP fragments, AFLP primer sequence, abbreviations of primer names and sizes of AFLP fragment used for development of SCAR markers

Fragment	Sequence	Abbreviation	Size (bp)
1. PP-P2M1_352	P2 F: 5' GAC TGC GTA CAT GCA GAA G 3' M1 R: 5' GAT GAG TCC TGA GTA ACA A 3'	P2M1	352
2. PP-P2M1_360	P2 F: 5' GAC TGC GTA CAT GCA GAA G 3' M1 R: 5' GAT GAG TCC TGA GTA ACA A 3'	P2M1	360
3. PP-P2M1_388	P2 F: 5' GAC TGC GTA CAT GCA GAA G 3' M1 R: 5' GAT GAG TCC TGA GTA ACA A 3'	P2M1	387
4. PP-P4M1_418	P4 F: 5' GAC TGC GTA CAT GCA GAG T 3' M1 R: 5' GAT GAG TCC TGA GTA ACA A 3'	P4M1	418
5. PP-P4M1_454	P4 F: 5' GAC TGC GTA CAT GCA GAG T 3' M1 R: 5' GAT GAG TCC TGA GTA ACA A 3'	P4M1	454
6. PP-P4M2_515	P4 F: 5' GAC TGC GTA CAT GCA GAG T 3' M2 R: 5' GAT GAG TCC TGA GTA ACA C 3'	P4M2	515

Table 2.5 Nucleotide sequence, length and melting temperature of primers designed from amplification of various genes and AFLP-derived fragments in *P. pelagicus*

Primer	Sequence	Length (bp)	Tm (ºC)	Expected size (bp)
1. *PP-COI$_{270}$*	F: 5' TTC AGC AGC CAT CGC TCA CG 3'	20	64	270
	R: 5' AGG GTC AAA GAA TGA AGT AT 3'	20	58	
2. *PP-12S$_{312}$*	F: 5' TTG GCG GTG GTT TAG TCT TG 3'	20	60	312
	R: 5' CGG GCG ATG TGT ACA TGC TT 3'	20	62	
3. PP-SERCA	F: 5' CAC TGT GTT CGC TGC CTC CT 3'	20	64	292
	R: 5' TTC CAC TAT TTC CTT CAA TGT ATG 3'	24	64	
4. PP-M122/135RAP	F: 5' TGA GGG ATG TAA GGT CAG AGG A 3'	22	66	318
	R: 5' GGT TCA CAG GAG GAA ACA AAG T 3'	22	64	
5. PP-P2M1_352	F: 5' GGT GTC AGT CGT CAG GAA GA 3'	20	62	246
	R: 5' GAG ATA GGT GAA TCG TGG GT 3'	20	60	
6. PP-P2M1_360	F: 5' TCA ACT TCA TCA CAT CCT CCA C 3'	22	64	254
	R: 5' ACG GTC ATC ACC ACC ACT CA 3'	20	62	
7. PP-P2M1_388	F: 5' TGC TCC CTT CAC AAG ACA CC 3'	20	62	289
	R: 5' ACC GCT CTG GCT CAT CAC TC 3'	20	64	
8. PP-P4M1_418	F: 5' ACC GAC CAC TTA CAC CTC TAC AA 3'	23	68	286
	R: 5' TCC GTC ACT CGT CAC ATC CC 3'	20	64	
9. PP-P4M1_454	F: 5' TCT CAG ATT GGG CTC CTT GT 3'	20	60	300
	R: 5' GGT TCT TTG TGC CTC TTT CA 3'	20	58	
10. PP-P4M2_515	F: 5' GGA GTG GAC TTC CTG CTT GG 3'	20	64	281
	R: 5' CCG TCA CCG TCA GCC TCT AC 3'	20	66	

2.9.2 Non-denaturing polyacrylamide gel electrophoresis

Different concentrations of low closslink non-denaturing polyacrylamide gels (37.5:1 or 75:1 of acrylamide and bis-acrylamide) were prepared by dilution of a 40% stock solution to required gel concentration and degased *in vacuo*. The acrylamide gel solution (30 - 40 ml) was mixed with 300 µl of 10% ammonium persulphate and 30 µl of tetramethylethylenediamine (TEMED). The analytical comb was inserted into the prepared gel and allow for polymerization for at least 4 hours or overnight.

Table 2.6 PCR conditions used for amplification of genomic DNA of *P. pelagicus* using primers listed in Table 2.5

Primer	dNTP (µM)	$MgCl_2$ (mM)	Primer (µM)	PCR conditions
1. *PP-COI$_{270}$*	100	2	0.2	94°C, 3 min for 1 cycle followed by 94°C, 1 min; 55°C, 1 min and 72°C, 1 min for 35 cycles and 72°C, 7 min for 1 cycle
2. *PP-12S$_{312}$*	100	2	0.2	94°C, 3 min for 1 cycle followed by 94°C, 45 sec; 58°C, 1 min and 72°C, 45 sec for 35 cycles and 72°C, 7 min for 1 cycle
3. *PP-SERCA*	100	2	0.2	As described in 1.
4. PP-M122/153RAP	100	2	0.2	As described in 1.
5. PP-P2M1_352				94°C, 3 min for 1 cycle followed by 94°C, 1 min; 57°C, 1 min and 72°C, 1 min for 35 cycles and 72°C, 7 min for 1 cycle
6. PP-P2M1_360	100	2	0.2	As described in 5.
7. PP-P2M1_388	100	2	0.2	As described in 5.
8. PP-P4M1_418	100	2	0.2	94°C, 3 min for 1 cycle followed by 94°C, 45 sec; 63°C, 1 min and 72°C, 30 sec for 3 cycles and 94°C, 45 sec; 61°C, 1 min and 72°C, 30 sec for 3 cycles and 94°C, 45 sec; 59°C, 1 min and 72°C, 30 sec for 3 cycles and 94°C, 45 sec; 57°C, 1 min and 72°C, 30 sec for 3 cycles and 94°C, 45 sec; 55°C, 1 min and 72°C, 30 sec for 28 cycles and 72°C, 7 min for 1 cycle
9. PP-P4M1_454	100	2	0.2	As described in 8.
10. PP-P4M2_515	100	1.5	0.2	As described in 8.

2.9.3 PCR and electrophoresis

PCR was carried out against genomic DNA of *P. pelagicus* using conditions described in Table 2.6. Six microlitres of the amplified PCR product were mixed with four volumes of SSCP loading dye (95% formamide, 0.25% bromophenol blue,

0.25% xylene cyanol and 10 mM NaOH), denatured at 95^{o}C for 5 minutes and immediately cooled on ice for 2 minutes. The denatured products were electrophoretically analyzed in native polyacrylamide gels at 250-300 volts for 16 hours at 4^{o}C. SSCP band were visualized by silver staining.

2.9.4 Silver staining

The gel plates were carefully separated using a plastic wedge. The long glass plate with the gel was placed in a plastic tray containing 2 litres of the fix/stop solution (10% glacial acetic acid) and agitate well for 30 minutes. The gel was briefly soaked with shaking 3 times for 3 minutes with deionized water. The gel was lifted out from the tray between each wash and allowed the washed water draining out of the gel for 5 seconds. The gel was transferred to 0.1% silver nitrate solution (1.5 litres) and incubated with agitation at room temperature for 30 minutes. The gel was soaked in 1.5 litres of deionized water with shaking (10 forward and 10 backward agitation) and immediately placed in the tray containing 1.5 litres of the chilled developing solution. This step is crucial and the time taken to soak the gel in the water and transfer it to chilled developing solution (Sodium carbonate) should be no longer than 5 - 10 seconds. The gel was well agitated until the first bands are visible (usually 1.5 - 2 minutes). The gel was then transferred to another tray containing 1.5 litres of chilled developer and shaken until bands from every lane were observed (usually 2 – 3 minutes). One litre of the fix/stop solution was directly added to the developing solution and continued shaking for 3 minutes. The stained gel was soaked in deionized water twice for 3 minutes each. The gel was placed in the plastic bag and air-dried.

2.10 Data analysis

SSCP fragments of *PP-COI*$_{270}$, PP- M122/153RAP (called *PP-SCARRAP*$_{312}$) and PP-P4M1_454 (called *PP-SCARAFLP*$_{300}$) bands were treated as dominant markers. Each band was treated as a locus and scored for presence (1) or absence (2) and transformed into 1/2 binary character matrix without consideration of band intensity differences between homo- and heterozygotes.

Gene diversity and percentage of monomorphic and polymorphic loci was estimated in each geographic sample. Pairwise genetic identity and genetic distance

were determined (Nei, 1978). A UPGMA dendrogram (Sneath and Sokal, 1973) was constructed based on genetic distance between pairs of geographic samples using PHYLIP.

Genetic heterogeneity of overall sample and between pairs of samples was examined using the exact test. F_{ST}-based statistics (θ) of overall samples across all investigated primers were calculated. The chi-square value was calculated and tested using $\chi^2 = 2N\theta$ (k-1) and df = (k-1)(s-1) where N = the number of investigated individuals, k = the number of alleles per locus and s = the number of geographic samples. Population genetics parameters described above were computationally analyzed by Tool for Population Genetic Analysis (TFPGA, Miller, 1997).

PART II Development of Species-diagnostic Markers in the Blue Swimming Crab (*Portunus pelagicus*)

2.11 PCR amplification of derived candidate species-specific markers of the blue swimming crab (*P. pelagicus*)

Species-diagnostic markers for authentication of the blue swimming crab *P. pelagicus* were developed. Two mitochondrial gene segments; *COI* and *12S rDNA* were amplified using universal primer (LCO1490: 5′- GGT CAA CAA ATC ATA AAG ATA TTG G-3′ and HCO2198: 5′- TAA ACT TCA GGG TGA CCA AAA AAT CA-3′ and 12S-F: 5′- AAA CTA GGA TTA TAT ACC CTA TTA-3′and 12S-R: 5′- AAG AGG GAC GGG CGA TTT GT-3′). The PCR reactions were performed in a 25 μl reaction volume containing 75 mM Tris-HCl (pH 8.8 at 25^{o}C), 20 mM $(NH_4)_2SO_4$, 0.1 % Tween 20, 2.0 mM $MgCl_2$, 200 μM of each dATP, dTTP, dCTP and dGTP, 0.5 μM of each primer, 1 unit of *Taq* DNA polymerase (Fermentus) and 50 ng of genomic DNA. The amplification condition was carried out as described in Table 2.3. After amplification, the PCR products were electrophoretically analyzed as described previously.

2.11.1 Cloning of *COI* and *12S rDNA* gene segments

The PCR profile of *COI* and *12S rDNA* was gel-eluted and reamplified using the original primers. The target band was ligated to pGEM-T Easy vector and

transformed into *E. coli* JM109. Plasmid DNA was extracted and sequenced for both directions using an automated DNA sequencer as described previously.

2.11.2 Primer design from candidate species-specific markers

The nucleotide sequence of *COI* and *12S rDNA* was compared with those previously deposited in the GenBank using BLAST*N* analysis. A pair of sequence-specific primers was designed from each insert (PP-COI_{270}–F/R and PP-$12S_{312}$–F/R Table 2.6) using Primer premier 5.0.

2.11.3 PCR

PP-COI_{270}, *12S rDNA* and PP-$12S_{312}$ were further examined against specimens of *P. pelagicus* (*N* = 174 for PP-COI_{270}, *12S rDNA* and PP-$12S_{312}$) and other non-target species; the mud crabs (*S. oceanica*, *N* = 18; *S. serrata*, *N* = 7 and *S. tranquebarica*, *N* = 9), the swimming crab (*C. crucifera*, *N* = 20) and the three spot swimming crab (*P. sanguinolentus*, *N* = 10). The amplification reaction were performed in a 25 µl reaction volume containing 75 mM Tris-HCl (pH 8.8 at 25^{o}C), 20 mM $(NH_4)_2SO_4$, 0.1 % Tween 20, 1.5 - 2.0 mM $MgCl_2$, 100 - 200 µM of each dATP, dTTP, dCTP and dGTP, 0.2 µM of each primer, 1 unit of *Taq* DNA polymerase (Fermentus) and 50 ng of DNA template using the PCR profile described in Table 2.7. The amplification products were electrophoretically analyzed through 1.5% agarose gels.

2.11.4 SSCP analysis

The PCR product of *12S rDNA* and PP-$12S_{312}$ was positively amplified in non-target species and required further analysis by SSCP. Accordingly, 6 µl of the PCR product of *12S rDNA* and PP-$12S_{312}$ were mixed with four volumes of the SSCP loading dye (95% formamide, 0.25% bromophenol blue, 0.25% xylene cyanol and 10 mM NaOH), denatured at 95ºC for 5 minutes and immediately cooled on ice for 2 minutes. The denatured product was electrophoretically analyzed in 12.5% native polyacrylamide gels (37.5:1 crosslink) at 300 volts for 13.5 hr at 4ºC. SSCP bands were visualized by silver staining.

2.11.4.1 Sequencing of representative individuals representing different SSCP genotypes

The amplified *PP-12S*$_{312}$ of *P. pelagicus* (*N* = 5), *S. oceanica* (*N* = 1), *S. serrata* (*N* = 1), *S. tranquebarica* (*N* = 1), *C. crucifera* (*N* = 1) and *P. sanguinolentus* (*N* = 1) was cloned and sequenced. The nucleotide sequence divergence between pairs of sequences was examined using Kimura (1980) two parameter method routine in PHYLIP.

Table 2.7 PCR compositions and conditions used for the species-specificity test of candidate species-specific markers of *P. pelagicus*

Primer	**dNTP (µM)**	**$MgCl_2$ (mM)**	**Primer (µM)**	**Conditions for *P. pelagicus***	**Conditions for other non-target species**
1. PP-COI_{270}	100	1.5	0.2	94°C, 3 min for 1 cycle followed by 94°C, 1 min; 58°C, 1 min and 72°C, 1 min for 25 cycles and 72°C, 7 min for 1 cycle	Identical conditions
2. *12S rDNA*	200	2.0	0.2	94°C, 3 min for 1 cycle followed by 94°C, 45 sec; 55°C, 45 sec and 72°C, 45 sec for 35 cycles and 72°C, 7 min for 1 cycle	Identical conditions
3. PP-$12S_{312}$	200	2.0	0.2	94°C, 3 min for 1 cycle followed by 94°C, 45 sec; 62°C, 1 min and 72°C, 45 sec for 35 cycles and 72°C, 7 min for 1 cycle	94°C, 3 min for 1 cycle followed by 94°C, 45 sec; 55°C, 45 sec and 72°C, 45 sec for 35 cycles and 72°C, 7 min for 1 cycle

PART III Isolation and Characterization of Temperature Responsive Genes in Haemocytes of *P. pelagicus*

2.12 cDNA-AFLP analysis

2.12.1 First strand cDNA synthesis

One microgram of total RNA from haemocytes of *P. pelagicus* was reverse-transcribed to the first strand cDNA. Total RNA was combined with 1 µl of 3' SMART^{TM} CDS Primer II A (15 µM final concentration), 1 µl SMART II^{TM}A Oligonucleotide (15 µM final concentration) (Table 2.8) and appropriate DEPC-treated H_2O in final volume of 5.5 µl. The reaction was incubated at 72ºC for 2 minutes and cooled on ice for 5 minutes. Then 5x First-Strand Buffer, 20 mM dithiothreitol (DTT), 10 mM of each dNTP were added to final concentrations of 1x, 2 mM and 1 mM, respectively. Finally, 0.5 µl of BD Powerscript^{TM} Reverse Transcriptase (Clontech) was added and gently mixed by pipetting. The reaction mixture was incubated at 42ºC for 1.5 hours. The reaction was incubated on ice to terminate the reverse transcriptase activity and 2 µl of the reverse-transcribed product were subjected to the second strand cDNA synthesis by PCR.

2.12.2 Second strand cDNA amplification by long distance (LD) PCR

The reaction components containing 10x Advantage 2 PCR Buffer, 10 mM of each dNTP, 5' PCR Primer II A (Table 2.7) and 50x BD Advantage 2 Polymerase Mix (Clontech) was combined to final concentration of 1x, 0.2 mM, 0.6 µM and 1x, respectively. Appropriated amount of DEPC-treated H_2O was added to make the final volume up to 50 µl. Then, 2 µl of the first strand cDNA were added. The reaction mixture was incubated at 95ºC for 1 minute followed by 95°C, 15 seconds; 65°C, 30 seconds and 68°C, 6 minutes for 8, 10 and 12 cycles. The resulting product was electrophoresed through a 1.0% agarose gel. The second strand synthesis was carried out again using 9 PCR cycles.

Table 2.8 Sequences of primers used for cDNA synthesis

Primer	Sequence	Length (bp)
3' SMART^{TM} CDS Primer II A	5' AAG CAG TGG TAT CAA CGC AGA GTA CT 3'	26
SMART II^{TM}A Oligonucleotide	5' AAG CAG TGG TAT CAA CGC AGA GTA CGC GGG 3'	30
5' PCR Primer II A	5' AAG CAG TGG TAT CAA CGC AGA GT 3'	23

2.12.3 Purification of PCR products

The PCR product from second strand cDNA amplification was transferred to a 1.5 ml microcentrifuge tubes and TE buffer was added to final volume of 100 μl. An equal volume of buffer-equilibrated phenol:chloroform was added and gently mixed for 2 minutes. The solution was centrifuged at 10,000 rpm for 10 minutes at room temperature. The upper aqueous phase was transfered to a new sterile microcentrifuge tube. One-tenth volume of 3 M sodium acetated, pH 5.2 was added. DNA was precipitated by an addition of two volume of prechilled absolute ethanol and mixed thoroughly. The mixture was incubated at -80^{o}C for 40 minutes. The precipitated second strand cDNA was washed once with 1 ml of 70% ethanol for 10 minutes. After centrifugation, the supernatant was removed. The cDNA pellet was air-dried and resuspended in 10 μl of TE buffer (10mM Tris-HCl and 0.1 mM EDTA, pH8.0). The DNA solution was kept at -20^{o}C until further used.

2.12.4 Amplified fragment length polymorphism (AFLP) using cDNA as the template

2.12.4.1 Restriction enzyme digestion and adaptor ligation

The synthesized cDNA of each individual (1000 ng) was initially digested with 6 units of *Eco*RI or 6 units of *Pst*I in a 25 μl reaction mixture containing 1x OPA buffer (10 mM Tris-acetate; pH 7.5, 100 mM magnesium acetate, 500 mM potassium acetate) at 37^{o}C for 3 hours. At the end of the incubation period, the reaction mixture was adjust to a final volume of 40 μl by adding 1x OPA buffer, deionized H_2O and 4 units of *Tru*9I (an isoschizomer of *Mse*I). The reaction was further incubated at 65^{o}C

for approximately 3 hours. The *Eco*RI, or *Pst*I and *Mse*I adaptors (Table 2.9) were ligated to restricted cDNA in a 30 μl reaction volume composing of 20 μl of the restricted product, 1X OPA buffer, 0.25 μM of *Eco*RI and 2.5 μM of *Mse*I adaptors, 1 mM of ATP and 3 Weiss units of T4 DNA ligase. The reaction was incubated at 4^{o}C for 16 hours.

2.12.4.2 Preamplification

The preamplification reaction was carried out in a 25 μl reaction volume containing 10 mM Tris-HCl; pH 8.8, 50 mM KCl, 0.1% Triton X-100, 200 μM of each dNTP, 1.5 mM $MgCl_2$, 37.5 ng each of E_{+A} (5'-GAC TGA GTA CCA ATT CA-3') and M_{+C} (5'-GAT GAG TCC TGA GTA AC-3') primers, 1.5 units of DyNazyme^{TM}II DNA Polymerase (Finnzymes) and 1 μl of the ligation product. PCR was performed in a Perkin Elmer 9700 thermocycler consisting of denaturation at 94^{o}C for 30 seconds, annealing at 56^{o}C for 1 minutes and extension at 72^{o}C for 1 minute for 20 cycles and the final extension at 72^{o}C for 5 minutes.

2.12.4.3 Selective amplification

Selective amplification was carried out in a 25 μl reaction volume containing 10 mM Tris-HCl; pH 8.8, 50 mM KCl, 0.1% Triton X-100, 200 μM of each dNTP, 1.5 mM $MgCl_2$, 30 ng of a combination of E_{+3} and M_{+3} primers (Table 2.9), 1.5 units of DyNazyme^{TM}II DNA Polymerase (Finnzymes) and 5 μl of 10-fold diluted preamplified product. PCR was performed consisting of denaturation at 94^{o}C for 30 seconds (or 45 seconds), annealing at 65^{o}C for 45 seconds (or 60 seconds) and extension at 72^{o}C for 90 seconds (or 120 seconds) for 2 cycles followed by 12 cycles of a touchdown phase with lowering of the annealing temperature for 0.7^{o}C (or 1^{o}C) in every cycle. The amplification consisting of 94^{o}C for 30 seconds (or 45 seconds), 56^{o}C (or 53^{o}C) for 45 seconds (or 60 seconds) and 72^{o}C for 90 seconds (or 120 seconds) was performed for additional 23 - 28 cycles. The final extension was carried out at 72^{o}C for 5 minutes.

2.12.4.4 Agarose gel electrophoresis

The selective amplification AFLP products were electrophoresed through 1.5-1.8% agarose gels to determine whether the amplification was successful as described

previously. Primer combinations that yielded positive amplification products were further analyzed by polyacrylamide gel electrophoresis.

2.13 Denaturing Polyacrylamide Gel Electrophoresis

2.13.1 Preparation of Glass Plate

The long and short glass plates were treated and prepared as previously described for SSCP analysis. The cleaned glass plates were assembled with a pair of 0.4 mM spacer. The bottom of assembled glass plates were sealed with the plastic tape. The bottom and both sides of the assembled glass plate were securely clamped.

2.13.2 Preparation of denaturing polyacrylamide gel electrophoresis

Desired concentrations (4.5 – 6.0%) of denaturing polyacrylamide gels were prepared by combining 40 ml of the acrylamide solution (19:1 acrylamide: bisacrylamide with 7 M urea in 1x TBE buffer) with 300 μl of freshly prepared 10 % ammonium persulphate and 30 μl of TEMED. The acrylamide solution was gently swirled and degassed for 15 minutes. The assembled plate sandwich was hold at a 45 degree angle on the bottom corner. The acrylamide solution was then gently injected into one side of the assembled plates. The filled plate sandwich was left in the horizontal position. The flat edge of the shark-tooth comb was then inserted. The gel was left at room temperature for 1 hour. After that, the polymerized gel was covered by the water-soaked tissue papers and left at room temperature for 4 hours (or overnight) for complete polymerization. When required, the spring clips and the sealing tape were carefully removed. The top of the gel was rinsed with deionized H_2O.

2.13.3 Electrophoresis

The gel sandwich was placed in the vertical sequencing apparatus with the short glass plate inward. The gel sandwich was securely clamped with the integral gel clamps along the sides of the sequencing apparatus. The upper and lower buffer chambers were filled with approximately 300 ml of 1x TBE. The shark-tooth comb was reinserted into the gel until the teeth just touched the surface of the gel. Three microlitres of the polyacrylamide gel loading dye (98 % formamide, 200 μl EDTA,

Table 2.9 AFLP primers and primer sequences used for isolation of stress-response genes

Primer	Sequence
Adaptor sequences	
Eco RI adaptor	5′-CTC GTA GAC TGC GTA CC-3′
	5′-AAT TGG TAC GCA GTC TAC-3′
Mse I adaptor	5′-GAC GAT GAG TCC TGA G-3′
	5′-TAC TCA GGA CTC AT-3′
Preamplification primers	
E_{+A}	5′-GAC TGC GTA CCA ATT CA-3′
M_{+C}	5′-GAT GAG TCC TGA GTA AC-3′
Selective amplification primers	
E_{+3}-1	E_{+A}AC
E_{+3}-2	E_{+A}AG
E_{+3}-3	E_{+A}CA
E_{+3}-4	E_{+A}CT
E_{+3}-5	E_{+A}CC
E_{+3}-6	E_{+A}CG
E_{+3}-7	E_{+A}GC
M_{+3}-1	M_{+C}AA
M_{+3}-2	M_{+C}AC
M_{+3}-3	M_{+C}AG
M_{+3}-4	M_{+C}AT
M_{+3}-5	M_{+C}TA
M_{+3}-6	M_{+C}TC
M_{+3}-7	M_{+C}TG
M_{+3}-8	M_{+C}TT
M_{+3}-9	M_{+C}GA
M_{+3}-10	M_{+C}GT
M_{+3}-11	M_{+C}GC
M_{+3}-12	M_{+C}GG
M_{+3}-13	M_{+C}CA
M_{+3}-14	M_{+C}CT
M_{+3}-15	M_{+C}CG
M_{+3}-16	M_{+C}CC

0.25 % bromophenol blue and 0.25 % xylene cyanol) was loaded into each well. The gel was prerun at 35 W for 20 minutes.

Six microlitres of the amplification products were mixed with 3 μl of the loading buffer and heated at 95°C for 5 minutes before snapped cooled on ice for 3 minutes. The sample was carefully loaded into the well. Electrophoresis was carried out at 35 W for approximately 2 hours (XC moved out from the gel for approximately 30 minutes). AFLP band were visualized by silver staining.

2.14 Cloning of AFLP fragments

Candidate temperature-stress response cDNA-AFLP fragments were chosen. The cDNA-AFLP band was excised from the gel using a sterile razor blade and washed 2 times for 2 hours each at room temperature with 500 μl of sterile deionized water. Twenty microlitres of TE buffer or water was then added and incubated overnight at 37°C. The target fragment was reamplified using the same PCR recipes as those for selective amplification with the exception that 100 ng of each primer and 5 μl of the eluted AFLP product were used. The amplification conditions were composed of 94°C for 3 minutes, followed by 35 cycles of 94°C for 30 seconds, 50°C for 1 minute and 72 °C for 1 minute. The final extension was performed at 72°C for 7 minutes. The reamplified product was electrophoretically analyzed through a 1.5 - 1.8% agarose gel electrophoresis, cloned into pGEM®-T easy vector, sequenced and the new primer pair was designed.

2.15 Semiquantitative-Reverse Transcription Polymerase Chain Reaction (Semiquantitative RT-PCR)

2.15.1 Total RNA extraction and DNase treatment of RNA to RT-PCR

The haemocytes of the control and the treatment were collected as described previously. Total RNA was extracted from haemocytes of *P. pelagicus* (N = 24). Ten micrograms of total RNA was digested using RNase-free DNase (Promega). Total RNA was combined with 1x DNase buffer, DNase (0.5 unit per μg of total RNA) and appropriate Nuclease-free water in final volume of 40 μl. The reaction mixture was incubated at 37°C for 30 minutes. An equal volume of phenol:chloroform:isoamyl

alcohol (P:C:I, 25:24:1) was added and mixed for 10 minutes. The solution was centrifuged at 12,000 rpm for 10 minutes at room temperature. The upper aqueous phase was transferred to a new sterile microcentrifuge tube. This extraction process was then repeated once with chloroform:isoamylalcohol (24:1). The aqueous phase was transferred into a sterile microcentrifuge. One-tenth volume of 3 M DEPC-treated sodium acetated, pH 5.2 was added. RNA was precipitated by an addition of two and half volume of prechilled absolute ethanol and mixed thoroughly. The mixture was incubated at -80^{o}C for 30 minutes and centrifuged at 12000*g* for 10 minutes at 4^{o}C. The supernatant was removed. The RNA pellet was washed with 1 ml of 75% ethanol and centrifuged at 12000*g* for 10 minutes at 4^{o}C. After removing of the ethanol, the RNA pellet was air-dried for 5-10 minutes. RNA was dissolved in DEPC-treated H_2O for immediately used. Alternatively, the RNA pellet was kept under absolute ethanol in a -80^{o}C freezer for long storage.

2.15.2 Synthesis of the first strand cDNA

One micrograms of DNase-treated total RNA was reverse-transcribed. Total RNA was combined with 1.0 μg of oligo dT_{12-18} and appropriate DEPC-treated H_2O in final volume of 5 μl. The reaction was incubated at 70 °C for 5 minutes and immediately placed on ice for 5 minutes. After that 5x first strand buffer, $MgCl_2$, dNTP Mix were added to final concentrations of 1x, 3.0 mM and 0.5 mM, respectively. Finally, 1 μl of ImProm- II^{TM} Reverse Transcriptase was add and gently mixed by pipetting. The reaction mixture was incubated at 25°C for 5 minutes and at 42°C for 90 minutes. The reaction mixture was incubated at 70°C for 15 minutes to terminate the reverse transcriptase activity. Concentration and rough quality of the newly synthesized first strand cDNA was spectrophotometrically examined (OD_{260}/OD_{280}) and electrophoretically (1 μl) analyzed by 1.2% agarose gels.

2.15.3 Optimization of semiquantitative RT-PCR conditions

Initially, RT-PCR of the target genes (Table 2.10) and *elongation factor-1α* (F:5'-ATG GTT GTC AAC TTT GCC CC-3' and R:5'-TTG ACC TCC TTG ATC ACA CC-3') were amplified in a 25 μl reaction volume. The RT-PCR reaction composing of 10 mM Tris-HCl pH 8.8 at 25°C, 50 mM KCl and 0.1 % Triton X – 100, 1.5 mM MgCl2, 200 mM each of dATP, dCTP, dGTP and dTTP, 0.2 μM of each

primer, 2.5 μl of 10-fold diluted first strand cDNA and 1 unit of Dynazyme^{TM} DNA polymerase (FINNZYMES). RT-PCR was carried out with the temperature profile of predenaturation at 94°C for 3 minutes followed by 25 and 30 cycles of denaturation at 94°C for 30 seconds, annealing at 53°C for 30 seconds and extension at 72°C for 30 seconds. The final extension was carried out at the same temperature for 7 minutes.

Table 2.10 Nucleotide sequence, length and melting temperature of primers designed from candidate thermal-stress response genes resulted from cDNA-AFLP analysis of *P. pelagicus*

Primer	Sequence	Length (bp)	Tm (ºC)	Expected size (bp)
1. PP-E2M5_336*	F: 5' AAT CCA ACA TTG ACC GCC AG 3'	20	60	152
	R: 5' GCA CTT CAG GTT TGC TCT TCC 3'	21	64	
2. PP-E2M5_368	F: 5' TGA CCT CCA TAG ACC CTT CC 3'	20	62	214
	R: 5' TTC CCA ACA TTC AGC ACA GA 3'	20	58	
3. PP-E2M7_374-I*	F: 5' GGA AGA AGA AAG CCT TGT GC 3'	20	60	200
	R: 5' GCC TTA TCC GTC CCT GTA TG 3'	20	62	
PP-E2M7_374-II	F2: 5' CAG TTA GTC AAA GGA AGG GAG T 3'	22	64	189
	R2: 5' AAG TTA GGT GTT CTG AGG CAT C 3'	22	64	
4. PP-E2M7_476	F: 5' AAG GTT GGA CAG AAT AGG GA 3'	20	58	232
	R: 5' GCT TGA TTA GAT AGG GTG GA 3'	20	58	
5. PP-E3M7_360-I	F: 5' CGT AGC GTT ACT CCC GTT TA 3'	20	60	302
	R: 5' TTG ACC AGA TGC TTT ATT TG 3'	20	54	
PP-E3M7_360-II	F2: 5' AGT CTG TGT GAC GCC GCC 3'	18	60	178
	R2: 5' GCT GAA CGA CCA ATG AAA CG 3'	20	60	
6. PP-E3M7_409	F: 5' CCA GTA TCC CTT TCA TCC TTC A 3'	22	64	289
	R: 5' CTT TGG CTT CTT ACT GCC CTC T 3'	22	68	
7. PP-E5M9_481*	F: 5' CTT TCG CAG TAG TTC GTC TT 3'	20	58	339
	R: 5' GCT CGT AGT TGG ATT TCA GT 3'	20	58	

*Used for semiquantitative RT-PCR.

After electrophoresis, the amount of template was further adjusted to 1.5 μl for *EF-1α* and PP-E2M7_374-I and 5 μl for PP-E2M5_336. For semiquantitative RT-PCR analysis, primer and $MgCl_2$ and the cycle numbers used for amplification of these transcripts were further optimized.

2.15.3.1 Optimization of $MgCl_2$ concentrations

The optimal $MgCl_2$ concentration of each primer pair (0, 1, 1.5, 2, 2.5, 3 and 4 mM) was examined using the standard PCR conditions and the optimized primer concentration. The concentration of $MgCl_2$ that gave the highest yields and showed specificity for each PCR product was chosen.

2.15.3.2. Optimization of primer concentrations

The optimal primer concentration for each primer pair (between 0, 0.1, 0.15, 0.2, 0.3, 0.4 and 0.5 μM) was examined using the PCR conditions described above with an optimized $MgCl_2$ concentration. The resulting product was electrophoretically analyzed. The primer concentration that gave product specificity and clear results were selected for further optimization of PCR cycles.

2.15.3.3. Optimization of the number of amplification cycles

The PCR amplifications were carried out at different cycles (i.e. 25, 28 and 30 cycles) using the optimized concentration of primers and $MgCl_2$. The number of cycles that gave the highest yield before the product reached a plateau phase of amplification was chosen.

2.15.4 Gel electrophoresis and data analysis

The amplification product of genes under investigation and *EF-1α* were electrophoretically analyzed by the same gel and photographed by a gel documentation machine (BioRad). The intensity of the amplified target genes and that of *EF- 1α* was quantified from the photograph of the gels using the Quantity One programme (BioRad).

The expression level of each gene was normalized by that of *EF-1α*. Expression levels between different groups of *P. pelagicus* were statistically tested using one way analysis of variance (ANOVA) followed by a Duncan's new multiple range test. Significant comparisons were considered when the *P* value was < 0.05.

CHAPTER III

RESULTS

3.1 Genetic diversity and population structure of *P. pelagicus* determined by SSCP analysis

3.1.1 DNA extraction

Genomic DNA was extracted from the muscle of the first pereopod of each crab using a phenol-chloroform-proteinase K method (Klinbunga *et al*, 1996). The quality of extracted genomic DNA was electrophoretically determined using a 0.8% agarose gel. High molecular weight DNA at approximately 23.1 kb along with partially degraded DNA was observed (Figure 3.1).

The ratio of OD_{260}/OD_{280} of extracted genomic DNA was 1.8 -2.0 suggesting that the quality of extracted genomic DNA was acceptable for further used. DNA samples showing the ratio much lower then 1.8 was possibly contaminated with residual proteins or phenol. In contrast, those with the ratio greater than 2.0 may be contaminated with RNA as visualized by the smear at the bottom of gel (as can be seen in Figure 3.1). However, RNA contamination did not affect the PCR amplification. Therefore, the extracted DNA can still be used. Extracted DNA was stored at 4°C until further used.

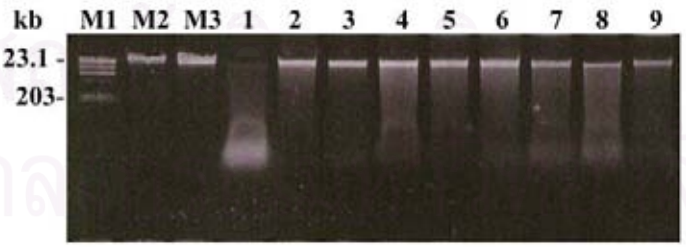


Figure 3.1 A 0.8% ethidium bromide stained agarose gel showing the quality of total DNA extracted from the muscle of *P. pelagicus* using a phenol-chloroform-proteinase K method (lanes 1 - 9). Lanes M1, M2 and M3 are λ/*Hin*d III DNA marker and 100 and 200 ng of undigested λ-DNA, respectively.

A Chelex®-based method was also used for the rapid extraction of genomic DNA. The rapid extracted DNA was subjected to amplification reaction as soon as possible after isolated.

3.1.2 Candidate primer for determination of genetic diversity in wild samples of *P. pelagicus*

In total, thirty eight primer pairs including universal primers and those previously developing from AFLP and RAP-PCR analyses of *P. monodon* or *M. rosenbergii*) were screened against genomic DNA of wild *P. pelagicus* originating from Chanthaburi, Suratthani and Ranong (N = 1 for each geographic sample).

Thirty-two primer pairs did not generate the amplification product whereas two primer pairs yielded nonspecific amplification products. These gene segments were *NADH dehydogenase 5 (ND5)* and $ME_{+3}3M_{+3}7425.2$ derived from AFLP fragments of of *M. rosenbergii*.

Four primer pairs provided positive amplification. These gene segments were *cytochrome oxidase subunit I* (*COI*, 706 bp), *12S ribosomal (r) DNA* (*12S rDNA*, 406 bp), *sarco endoplasmic recticulum* Ca^{++} *ATPase* (*SERCA*, 469 bp) and M122/135RAP (604 bp originally derived from RAP-PCR of *M. rosenbergii*). These gene segments were selected for further determination of genetic variation in wild samples of *P. pelagicus* in Thai waters.

The amplified fragment of each gene segment was cloned and sequenced (Figure 3.8–3.11). Nucleotide sequences were compared against data in the GenBank using Blast*N* and Blast*X* analyses. The closest similarity of those nucleotide sequences was *COI* of *Portunus pelagicus* voucher MaPrt001 (E-value = 0.0), *12S rDNA* of *Portunus pelagicus* voucher flh040815A (E-value = 0.0) and *SERCA* of the spiny lobster *Panulirus argus* ($2x10^{-60}$). In contrast, M122/135RAP did not significant match any previously deposited sequence in the GenBank (E-value > $1x10^{-4}$).

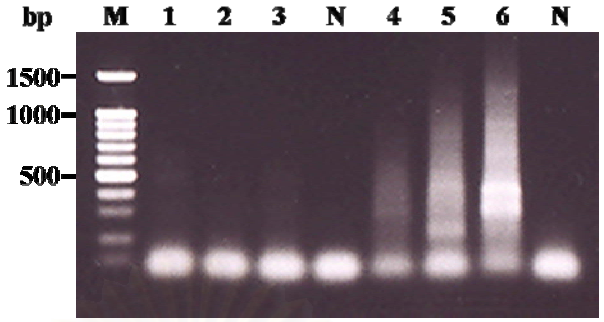


Figure 3.2 A 1.2% ethidium bromide stained agarose gel showing the amplification results of $ME_{+3}3M_{+3}7425.1$ primers (lanes 1-3) and $ME_{+3}3M_{+3}7425.2$ primers (lanes 4-6) against genomic DNA of *P. pelagicus* from different geographic locations in Thai waters. Lane M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.

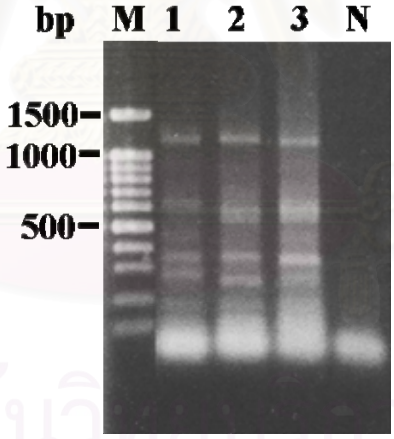


Figure 3.3 A 1.2% ethidium bromide stained agarose gel showing the non-specific amplification products resulted from *NADH dehydogenase 5 (ND5*) primers (lanes 1-3) against genomic DNA of *P. pelagicus* from different geographic locations in Thai waters. Lane M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.

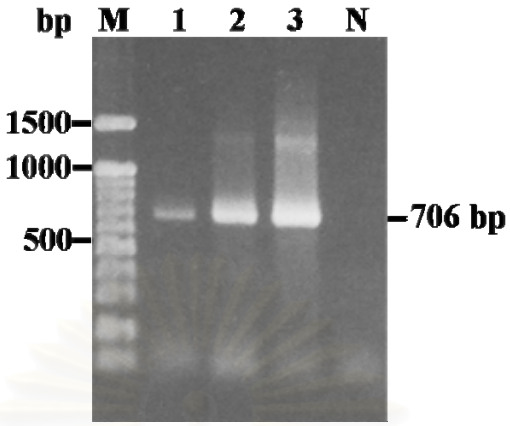


Figure 3.4 A 1.2% ethidium bromide stained agarose gel showing the positive amplification product of *COI* (706 bp, lanes 1-3) tested against genomic DNA of *P. pelagicus* from different geographic locations in Thai waters. Lane M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.

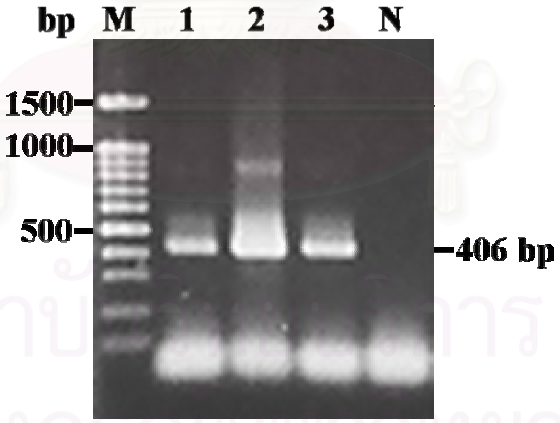


Figure 3.5 A 1.2% ethidium bromide stained agarose gel showing the positive amplification product of *12S rDNA* (406 bp, lanes 1-3) tested against genomic DNA of *P. pelagicus* from different geographic locations in Thai waters. Lane M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.

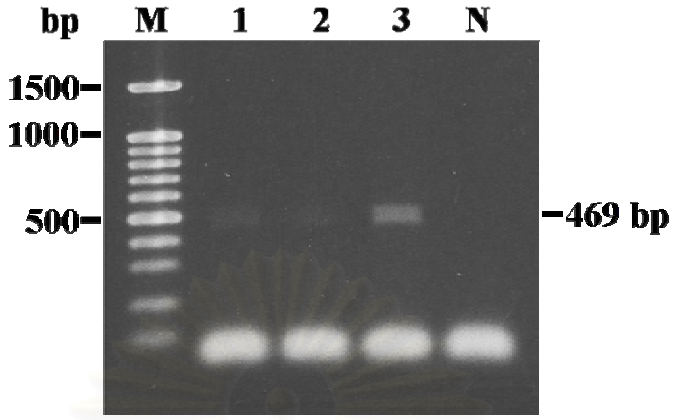


Figure 3.6 A 1.2% ethidium bromide stained agarose gel showing positive amplification product of *SERCA* (469 bp, lanes 1-3) tested against genomic DNA of *P. pelagicus* from different geographic locations in Thai waters. Lane M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.

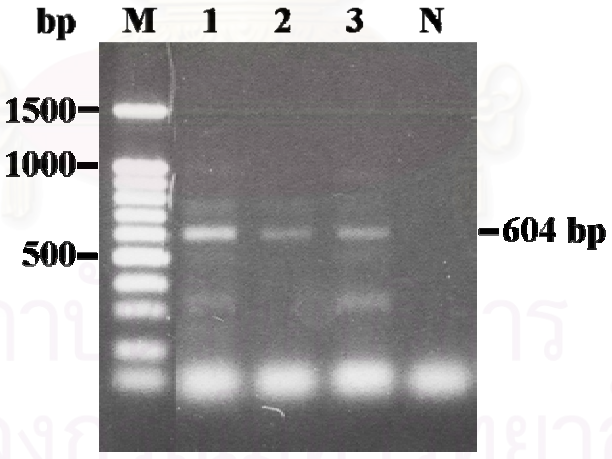


Figure 3.7 A 1.2% ethidium bromide stained agarose gel showing the positive amplification product of M122/135RAP primers (604 bp, lanes 1-3) tested against genomic DNA of *P. pelagicus* from different geographic locations in Thai waters. Lane M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.

Table 3.1 The amplification result of heterospecific primers of genes and DNA segments screened for genetic diversity studies of *P. pelagicus* in Thai waters

Primers of gene/DNA segment	Expected size (bp)	Observed size (bp)
1. *Cytochrome oxidase subunit I* (*COI*)	**710***	**706**
2. *COI-COII*	1500*	-
3. *NADH dehydogenase 5 (ND5)*	Not known	NS
4. *18S ribosomal (r) DNA*	900*	-
5. *16S rDNA*	500*	-
6. *12S rDNA*	**410***	**406**
7. *12S rDNA(F)/16S rDNA(R1)*	900*	-
8. *12S rDNA(F)/16S rDNA(R2)*	900*	-
9. *12S rDNA(F)/16S rDNA(R3)*	900*	-
10. *Difulfide isomerase (DSI)*	269*	-
11. *Sarco endoplasmic recticulum* Ca^{++} *ATPase* (*SERCA*)	**500****	**469**
12. $ME_{+3}3M_{+3}7425.1$	202**	-
13. $ME_{+3}3M_{+3}7425.2$	262**	NS
14. $ME_{+3}7M_{+3}6425$	333**	-
15. $ME_{+3}4M_{+3}5570$	384**	-
16. $ME_{+3}8M_{+3}1310.1$	210**	-
17. $ME_{+3}8M_{+3}1310.2$	235**	-
18. $ME_{+3}8M_{+3}1310.3$	219**	-
19. $ME_{+3}4M_{+3}8517$	277**	-
20. $FE_{+3}1M_{+3}7290$	143**	-
21. $FE_{+3}5M_{+3}1390$	277**	-
22. $FE_{+3}8M_{+3}3270.1$	152**	-
23. $FE_{+3}8M_{+3}3270.2$	147**	-
24. $FE_{+3}8M_{+3}6710$	385**	-
25. FeRAP315.1	203**	-
26. FeRAP315.2	209**	-
27. M268/128RAP	194**	-
28. M428/228RAP	240**	-
29. M122/135RAP	**284****	**604**
30. MA16/222RAP	147**	-
31. M122/159RAP.1	261**	-
32. M122/159RAP.2	292**	-
33. SOCRAP340.1	206**	-
34. SOCRAP340.2	265**	-
35. SOC268/273RAP	172**	-
36. SOC122/228RAP	166**	-
37. BC428/228RAP	184**	-
38. BC428/273RAP	247**	-

***** and ** = the expected size in *P. monodon* and *M. Rosenbergii*, respectively; - = no amplification product; NS = non-specific amplification products

GGTCAACAAATCATAAAGATATTGGTACATTATATTTTATTTTTGGAGCATGATCAGGAATA
GTAGGGACTTCTCTTAGTCTTATTATTCGAGCAGAACTAGGTCAACCTGGTACTCTTATTGG
TAATGACCAAATTTACAATGTTGTAGTTACAGCTCATGCTTTTGTAATAATTTTCTTTATAG
TTATACCAATTATAATTGGGGGATTTGGTAACTGACTAGTACCATTAATGTTAGGAGCCCCT
GNACATGGCTTTTCCTCGTTATAAACAACATAAGATTTTGACTTCTCCCTCCTTCTCTAACT
TTACTTCTTATAAGAGGTATAGTGGAAAGAGGTGTTGGTACAGGTTGAACCGTCTATCCTCC
TCT**TTCAGCAGCCATCGCTCACG**CAGGAGCTTCTGTAGATCTAGGTATTTTCTCTTTACATC
TAGCAGGTGTTTCCTCTATTCTAGGTGCAGTAAATTTCATGACCACCGTTATTAACATGCGA
TCTTTTGGTATAAGAATGGACCAAATGCCATTATTCGTTTGATCAGTATTTATCACTGCTAT
NCTTCTACTTTTATCTCTCCCGGTNCTGGCTGGAGCTATTACTATACTTCTTACAGACCGAA
ATCTA**ATACTTCATTCTTTGACCCT**GCCGGAGGTGGTGACCCTGTACNCTACNACCCTTATC
TGATTTTTGGGCCCCCGAAANTTA

Figure 3.8 Nucleotide sequence of *COI* (706 bp) amplified from genomic DNA of *P. pelagicus* using LCO1490+HCO2198 primers. The location and sequence of a forward primer (PP-COI_{270}-F) and those complementary to a reverse primer (PP-COI_{270}-R) are illustrated in boldface and underlined.

AAACTAGGATTAGATACCCTATTATACCAAGAAGTAATTATAAATACCTGAGTAGTAACAGC
TATGTTCTAAAAATTTGAAAAAT**TTGGCGGTGGTTTAGTCTTG**TCAGAGGAACCTGTCTTTT
AAACGATACACCACGAAATATCTTACTTGAGTTTGTATAGTATGTATACCATCATTATTAGG
TAATTTTTATAGAATAAATTACTGGAAAGTTTCGATAATGTTGAATATATTAGATCAAGGTG
CAGCTGATACTCAAGTTAAGGTGGGTTACAATAGTATTTACGCTATTACGGATAAGTAAATG
AAACTTTACTTTGAAGGAGGATTTGATTGTAAATTTAGTTTAATAAGCTAATTTGATATAAG
CTCTA**AAGCATGTACACATCGCCCG**TCGCTCTTA

Figure 3.9 Nucleotide sequence of *12S rDNA* (406 bp) amplified from genomic DNA of *P. pelagicus* using 12S-F/R primers. The location and sequence of a forward primer (PP-$12S_{312}$-F) and those complementary to a reverse primer (PP-$12S_{312}$-R) are illustrated in boldface and underlined.

CTGGCTCTTCTTCCGTTACATGGCCATCGGTGGCTATGTGGGCGCCGCCACTGTGTTCGCTG
CCTCCTGGTGGTTCATGTACGACCCCACCGGCCCTCAGCTCAACTACTACCA**GCTGTCCCAC**
CACCTCTCCTGCCTGGGTGACCCCGAGAACTTCGAGGGCCTGGACTGTGGCATCTTCAGCCA
CCCCGCCCCCATGACCATGGCCCTGNTCTGTCCTTGNTCACCATCGAGATGCTCAACGCCCT
CAACAGGTCAGCAACACACACACACACACACACACACACACACACACACACACACACACACA
CACACA**CATACATTGAAGGAAATAGTGGAA**TGAATATATTGATACTTGAGTCATATATTGCC
ACACACACACACACACACACACACANATACACAGNATGAGTACAATGAGTGTGTTGTCTCTG
TGCACAGCTTGTCTGANAACCANTCGNTGNTTGTN

Figure 3.10 Nucleotide sequence of *SERCA* (469 bp) amplified from genomic DNA of *P. pelagicus* using SERCA primers. The location and sequence of a forward primer (PP-SERCA-F) and those complementary to a reverse primer (PP-SERCA-R) are illustrated in boldface and underlined.

AGACTACATACCCCTTCAATCATGGGACACATTTTTACCTTGNAGATTTGTGTACCATCAGA
CCATTTTACTAATACTA**TGAGGGATGTAAGGTCAGAGGA**TTAATGGCTACAGTTTTCACTAT
TTTAATCCCCAATATAAGTATCTAAAACTATAAAATCACTAAATAGTAAGCAGAATGAATAT
AAAAACGCGTCCTGGTACTGAATGGGTTAAAGATGAGTTGCATAACACCGAACGAATAAAAA
AATGATACCACTAGTTAGTTGTAGACCATATGTACCACTCAAAGGGATCTAACCAAACTTTT
CTCCACAGTATTTCCCGAGTATAACATTACAGAAATCCCAATAGATGTGATTCCCTTACTTC
TCC**ACTTTGTTTCCTCCTGTGAACC**TCCGCGGACACTAAATGCTCGCAGGTTAAAGGGAGCT
GAGCAAAATTCTGATGTTGGAGACCGTCTCGTGTCCCTTCCTGCACCGCTCCCTCGAGTACT
GGTGTCAACACANGGCTCGTTTGGTGCTCCCAGTGTTTGGTGAGTGCAAGTATCCACTGCCA
GGAAGGAAGGCGTGCTGGTGCTTTGTTCTGCTGTTTNGGTCGCCNA

Figure 3.11 Nucleotide sequence of PP-M122/135RAP (604 bp) amplified from genomic DNA of *P. pelagicus* using M122/135RAP primers. The location and sequence of a forward primer (PP-M122/135RAP-F) and those complementary to a reverse primer (PP-M122/135RAP-R) are illustrated in boldface and underlined.

A pair of primers was designed from each sequence and tested against genomic DNA of wild *P. pelagicus*. The amplification product of *SERCA* of *P. pelagicus* revealed homo- and heterozygotic band patterns after analyzed by agarose gel electrophoresis (Figure 3.12). Appararently, *PP-SERCA* was difficult to amplify by PCR and band patterns cannot be reliably scored when analyzed by SSCP (Figure 3.13 and Table 3.2). *PP-12S*$_{312}$ was easily amplified (Figure 3. 14) but revealed low polymorphism following SSCP analysis (Figure 3.15 and Table 3.2). Therefore, it was not included in population genetic studies of Thai *P. pelagicus*.

The remaining gene segments (*PP-COI*$_{270}$, and *PP-M122/135RAP*) were consistently amplified (Figure 3.16 and 3.18). SSCP analysis indicated that *PP-COI*$_{270}$ and *PP-M122/135RAP* were highly polymorphic (Figures 3.17 and 3.19 and Table 2). These gene segments were then applied for population genetic studies of *P. pelagicus* in Thai waters.

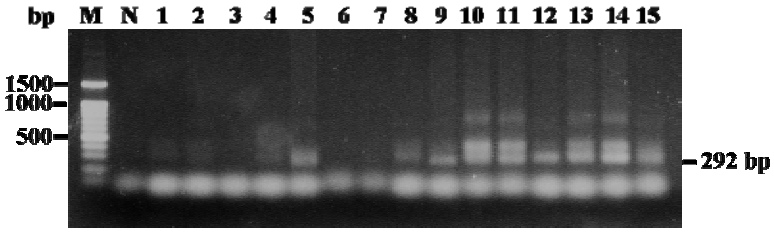


Figure 3.12 A 1.5% ethidium bromide stained agarose gel showing the PCR products generated from amplification of genomic DNA of *P. pelagicus* originating from Chanthaburi (lanes 1-4), Ranong (lanes 5-8), Suratthani (lanes 9-12) and Krabi (lanes 13-15) with PP-SERCA-F/R. Lane M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.

Figure 3.13 SSCP patterns of the PCR product of *P. pelagicus* from Chanthaburi (lanes 1-4), Ranong (lanes 5-8), Suratthani (lanes 9-12) and Krabi (lanes 13-16) amplified with PP-SERCA-F/R and electrophoresed through a 15% non-denaturing polyacrylamide gel (75:1 crosslink) at 250 volts for 15 hours at 4°C and silver stained. Lanes M and ds are a 100 bp DNA marker and the non-denatured PCR product, respectively.

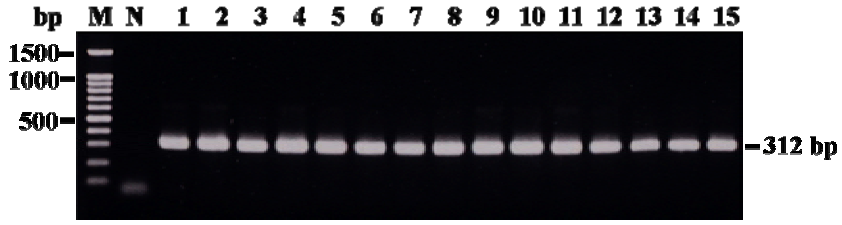


Figure 3.14 A 1.5% ethidium bromide stained agarose gel showing the PCR product generated from amplification of genomic DNA of *P. pelagicus* originating from Chanthaburi (lanes 1-3), Ranong (lanes 4-6), Suratthani (lanes 7-9), Krabi (lanes 10-12) and Prachuap Khiri Khan (lanes 13-15) with PP-12S_{312}-F/R. Lane M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.

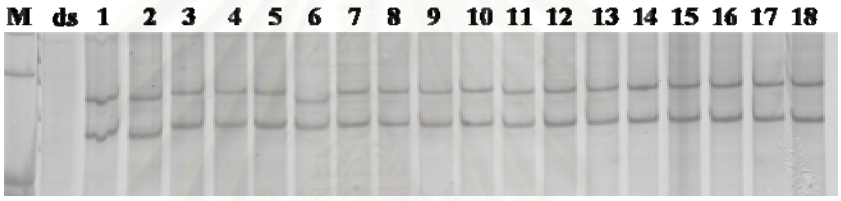


Figure 3.15 SSCP patterns of the PCR product of *P. pelagicus* from Chanthaburi (lanes 1-9) and Ranong (lanes 10-18) amplified with PP-12S_{312}-F/R and electrophoresed through 12.5% non-denaturing polyacrylamide gel (37.5:1 crosslink) at 250 volts for 15 hours at 4°C and silver stained. Lanes M and ds are a 100 bp DNA marker and the non-denatured PCR product, respectively.

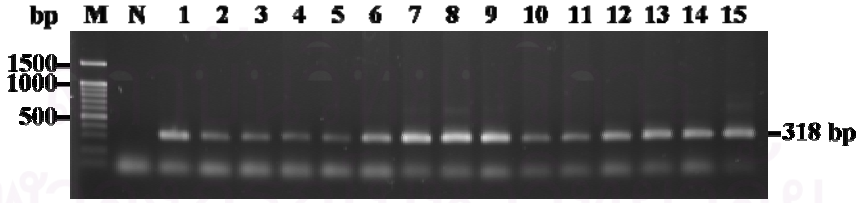


Figure 3.16 A 1.5% ethidium bromide stained agarose gel showing the PCR product generated from amplification of genomic DNA of *P. pelagicus* originating from Chanthaburi (lanes 1-3), Ranong (lanes 4-6), Suratthani (lanes 7-9), Krabi (lanes 10-12) and Prachuap Khiri Khan (lanes 13-15) with PP-M122/135RAP-F/R. Lane M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.

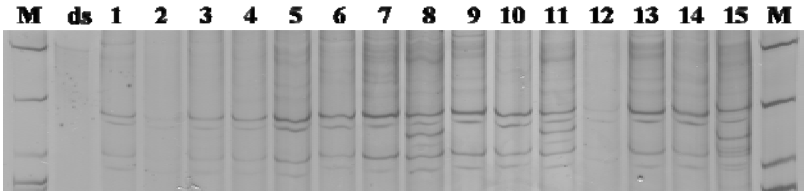


Figure 3.17 SSCP patterns of the PCR product of *P. pelagicus* from Chanthaburi (lanes 1-3), Ranong (lanes 4-6), Suratthani (lanes 7-9), Krabi (lanes 10-12) and Prachuap Khiri Khan (lanes 13-15) amplified with PP-M122/135RAP-F/R and electrophoresed through 15% non-denaturing polyacrylamide gel (37.5:1 crosslink) at 300 volts for 14 hours at 4°C and silver stained. Lanes M and ds are a 100 bp DNA marker and the non-denatured PCR product, respectively.

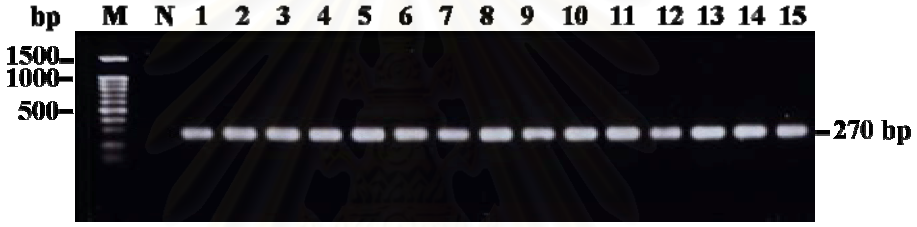


Figure 3.18 A 1.5% ethidium bromide stained agarose gel showing the PCR product generated from amplification of genomic DNA of *P. pelagicus* originating from Chanthaburi (lanes 1-3), Ranong (lanes 4-6), Suratthani (lanes 7-9), Krabi (lanes 10-12) and Prachuap Khiri Khan (lanes 13-15) with PP-COI_{270}-F/R. Lane M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.

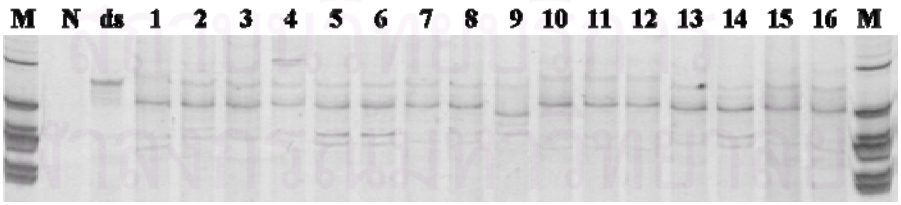


Figure 3.19 SSCP patterns of the PCR product of *P. pelagicus* from Ranong (lanes 1-8), Suratthani (lanes 9-12), Krabi (lanes 13-16) amplified with PP-COI_{270} and electrophoresed through 15% non-denaturing polyacrylamide gel (37.5:1 crosslink) at 250 volts for 15 hours at 4°C and silver stained. Lane M, N and ds are a 100 bp DNA ladder, the negative control (without genomic DNA template) and the non-denatured PCR product, respectively.

Table 3.2 The amplification success and SSCP results of primers designed from gene segments selected for population genetic studies of *P. pelagicus* in this study

Primer	Expected size (bp)	Amplification success	SSCP results
1. PP-SERCA	292	+	Difficult to score and inconsistent patterns
2. PP-$12S_{312}$	312	++	Low polymorphism
3. PP-COI_{270}	270	+++	High polymorphism
4. PP-M122/135RAP	318	+++	High polymorphism

\+ = amplification was inconsistent

++ = consistent amplification success but low polymorphism

+++ = consistent amplification success and high polymorphism

Previously, genetic diversity of the blue swimming crab (*P. pelagicus*) in Thai waters examined based on AFLP and RAPD analyses (Khetpu, 2005). Six polymorphic AFLP fragments found in 15 – 80% of tested individuals were chosen, cloned and sequenced (Figures 3.20 – 3.25). All of them did not match any previously deposited sequence and regarded as unidentified DNA segments

A pair of primers was designed from each sequence and tested against genomic DNA of wild *P. pelagicus*. PP-P2M1_388 did not generate the amplification product (Figure 3.26) whereas the amplification success of PP-P2M1_352 and PP-P2M1_360 (Figures 3.27 and 3.28) was inconsistent. Accordingly, polymorphism of these AFLP-derived markers was not further examined by SSCP analysis.

The amplification success of PP-P4M2_515 was relatively high (Fig. 3.29) but band patterns of this marker was not reliable when determined by SSCP analysis (Figure 3.30 and Table 3.3). Although the amplification success and results from SSCP analysis of PP-P4M1_418 was consistent (Figures 3.31 and 3.32 and Table 3.3), this AFLP-derived marker exhibited limited polymorphism. Therefore, they were not included in population genetic studies of Thai *P. pelagicus*.

In contrast, PP-P4M1_454 was consistently amplified by PCR (Figure 3.33). SSCP analysis indicated that PP-P4M1_454 were highly polymorphic (Figure 3.34 and Table 3.3). This gene segment along with *PP-COI*$_{270}$ and *PP-M122/135RAP* were then included for population genetic studies of Thai *P. pelagicus*.

GACTGCGTACATGCAGAACCGCGCCGATAGCAACAGAAGA**GGTGTCAGTCGTCAGGAAGA**GC
TGGGCATCAGGGTCAGGATACACTAGGAGAGGTAGCCAGGCGATGTCTTCTTTGATGGCCGA
GAAACTAGCAGCAGCCCTCTCAGTCCACCTCAGTGGGACGGAGGACCTGTAGGAGAGACCCT
TCACAAGCTCATATACAGTAGAATCTCGAATCTCGATCAATCTCATCTCGATATTTCGCATC
TCGATTGCACCATCAAAA**ACCCACGATTCACCTATCTC**GATCAAATTACACGAATCTCGATC
GCTATTTTTCATTTTGTTGTTGTTACTCAGGACTCATCA

Figure 3.20 Nucleotide sequence of P2M1_352 (352 bp) amplified from genomic DNA of *P. pelagicus* using P_{+3}-2/M_{+3}-1 primers. The location and sequence of a forward primer (PP-P2M1_352 -F) and those complementary to a reverse primer (PP-P2M1_352-R) are illustrated in bold and underlined.

GACTGCGTACATGCAGAACGACTCCTTCT**TCAACTTCATCACATCCTCCAC**CAGGTGGGTTC
TGTCAAGTGGTGACACCCCACATGTTGGGAGATTGTCCAGGAATAGATTGAGTCTATAAAAT
GCTATTTCTCAACAAGCAAAAAATTATGAGCAGTTGGGTTAGTTGAATTGTATCCAGAATGT
GTTTTATGAATTCAGTGTCACAGTGCTTAGGTCTATCTTGCTTTTCCTGGTACATGAAACCT
TGTGATATCCATACC**TGAGTGGTGGTGATGACCGT**GAATTGGTGTGTTTCAGTCCCTGAGGA
TCCCAATGAGGAGATGGATGAGGACACCCAGGCCTTGTTACTCAAGACTA

Figure 3.21 Nucleotide sequence of P2M1_360 (360 bp) amplified from genomic DNA of *P. pelagicus* using P_{+3}-2/M_{+3}-1 primers. The location and sequence of a forward primer (PP-P2M1_360 -F) and those complementary to a reverse primer (PP-P2M1_360-R) are illustrated in bold and underlined.

GATGAGTCCTGAGTAACAAGAGATTTAGTAACAGAGTTATCATAAACATTTGTACACGCG**TG**
CTCCCTTCACAAGACACCTTCTGCTACGGGTGGAACGACAGTAGTTGTCTAGTGGTCGTGGT
GACGATGAGTCCTGAGTAACAAATATAACTATCGGAGCAGCAGCGCGATGGTTCTGCATGTA
CGCAGTCATGATGAGTCCTGAGTAACAACGGTGTTCTGCATGTACGCAGTCATGATGAGTCC
TGAGTAACAAGGATCTGCATGTACGCAGTCATGATGAGTCCTGAGTAACAAGTGAGAGGTGA
AGTCTGTTATCACAGGGTC**GAGTGATGAGCCAGAGCGGT**GGGTGGAGAAGGTGACGTGGTTC
TGCATGTACGCAGTCA

Figure 3.22 Nucleotide sequence of P2M1_388 (388 bp) amplified from genomic DNA of *P. pelagicus* using P_{+3}-2/M_{+3}-1 primers. The location and sequence of a forward primer (PP-P2M1_388 -F) and those complementary to a reverse primer (PP-P2M1_388-R) are illustrated in bold and underlined.

GATGAGTCCTGAGTAACACTGTGGAGGAGTTCGTGACGGCTGAGGTGACGCTGTCATGTCTT
CTTGTCACTGCTACGGCTCAGGTAGCAGTGGCGGACGAACTGCCAGTCGCA**GGAGTGGACTT**
CCTGCTTGGGAACGATTTGGCTGGTGGTCGTGTATGGATCCCAACCTCTGATGAGGAGGAGG
TTGCTGAAGAGTCTGGGCAGGTGATGCAAGACTCTGTAAATGTTGTTACCTGCTCTCAGGCC
CGCTGGGTTGAGAGAGAGCCTGCTACTACCCAGGAGGCTGCTAGCATCCAGGTGGGATTGAC
TCCAGAAGGGAGTAGCAGTGCTGATGTGGTGGAGCTAGCGTTGGGCTCACCATTGAGTTCTG
GT**GTAGAGGCTGACGGTGACGG**CGTGGAGTCACCTGTTGATGCACCGGACTCGGCTTTGGGA
GTTGTTGCTGAGAGTGAGGACGGAGTGTTGAGTATAGATGAGTTGTTTGCTGGTTATCCTTA
CTCTGCATGTACGCAGTCA

Figure 3.23 Nucleotide sequence of P4M2_515 (515 bp) amplified from genomic DNA of *P. pelagicus* using P_{+3}-4/M_{+3}-2 primers. The location and sequence of a forward primer (PP-P4M2_515 -F) and those complementary to a reverse primer (PP-P4M2_515-R) are illustrated in bold and underlined.

GATGAGTCCTGAGTAACAACGAAGCCTCCATACACACACGAG**ACCGACCACTTACACCTCTA**
CAAGAACCATGGGCGACGGTACAACTTTTCTTTCCACTCGACCTTCTTTCTTTACTTTCTCT
TCCTCTCTGTGTCCTAACCTTCCCCTTTCTCGAGAAAAACGCCCCACACAAGAGTAACAAAA
GAAATAGCTACGGGCGCCACAGACCACGGACACAGGAGAACTGAAGGCTGTTTTGTTTGGAT
TTGAATACTCTGAAGTTGGGCGGAAGGAATAACGTCCTATATACAGAGAATGTGTGGGAG**GG**
GATGTGACGAGTGACGGACAACTGTGATCTATTGTTGACATCACTCACGAACACAGGAGAAC
TGGATGCTGTTATGTTTATATTTGAATACTCTGCATGTACGCAGTC

Figure 3.24 Nucleotide sequence of P4M1_418 (418 bp) amplified from genomic DNA of *P. pelagicus* using P_{+3}-4/M_{+3}-1 primers. The location and sequence of a forward primer (PP-P4M1_418 -F) and those complementary to a reverse primer (PP-P4M1_418-R) are illustrated in bold and underlined.

GACTGCGTACATGCAGAGTCGACAGATTTAGCTCAGCATAAGCAATCTAAGGTTTCTCCCGG
TACACATGATTGTGAGTCCTCCAGGGACCGCTCTGCTATGGTTTCTGCCCAGCCTTCTGTGG
CGCCCTCTG**TCTCAGATTGGGCTCCTTGT**GATGAGGATGGTTTTAGCATGAAGACGTCCGAT
GATATTGTCACAGCCGTCCATCTTGGACCCACTGAATCGAAAAGTGCAGTCCAGCCTGATCC
TCGTCCTCCGATGACCAGTAGAAGTAATGATGGTTCGCATCACTCACCGGTAGGCCGAAAGG
CAGCGGTCCAACGACCTGGCACATGTCAACTCCCGGTGTCTTCTCGTCGTTTGATTATTATT
ACTGATTGTAGTCAGTTGCCGAGCTCGAGCCCGCCGCGTTT**TGAAAGAGGCACAAAGAACC**T
CTTGTTACTCAGGACTCATCA

Figure 3.25 Nucleotide sequence of P4M1_454 (454 bp) amplified from genomic DNA of *P. pelagicus* using P_{+3}-4/M_{+3}-1 primers. The location and sequence of a forward primer (PP-P4M1_454 -F) and those complementary to a reverse primer (PP-P4M1_454-R) are illustrated in bold and underlined.

Table 3.3 The amplification success and SSCP results of primers designed from polymorphic AFLP fragments of *P. pelagicus*

Primer	Expected size (bp)	Amplification success	SSCP results
1. PP-P2M1_352	246	+	ND
2. PP-P2M1_360	254	+	ND
3. PP-P2M1_388	289	-	ND
4. PP-P4M2_515	281	++	Inconsistent
5. PP-P4M1_418	286	++	Low polymorphism
6. PP-P4M1_454	300	++	High polymorphism

- = no amplification products

+ = amplification was inconsistent.

++ = consistent amplification success but low polymorphism (or SSCP patterns were inconsistent).

+++ = consistent amplification success and high polymorphism.

ND = not determined.

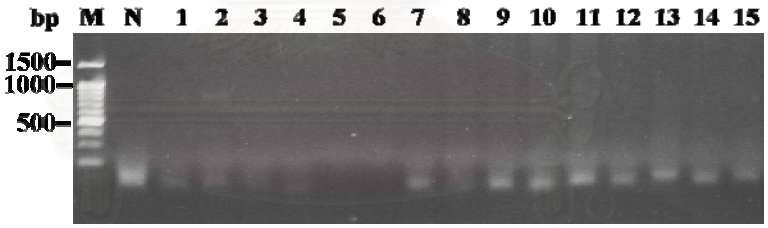


Figure 3.26 A 1.5% ethidium bromide stained agarose gel showing the PCR product generated from amplification of genomic DNA of *P. pelagicus* originating from Chanthaburi (lanes 1-3), Ranong (lanes 4-6), Suratthani (lanes 7-9) and Krabi (lanes 10-12) with PP-P2M1_388- F/R. Lane M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.

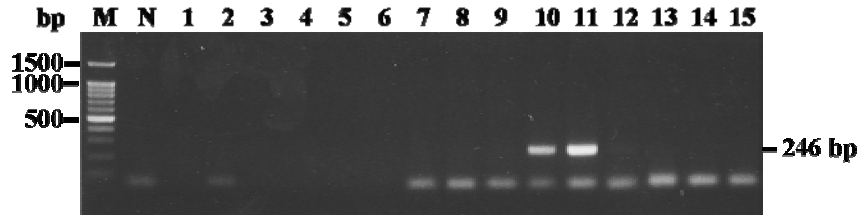


Figure 3.27 A 1.5% ethidium bromide stained agarose gel showing the PCR product generated from amplification of genomic DNA of *P. pelagicus* originating from Chanthaburi (lanes 1-3), Ranong (lanes 4-6), Suratthani (lanes 7-9) and Krabi (lanes 10-12) with PP-P2M1_352-F/R. Lane M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.

Figure 3.28 A 1.5% ethidium bromide stained agarose gel showing the PCR product generated from amplification of genomic DNA of *P. pelagicus* originating from Chanthaburi (lanes 1-3), Ranong (lanes 4-6), Suratthani (lanes 7-9) and Krabi (lanes 10-12) with PP-P2M1_360-F/R. Lane M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.

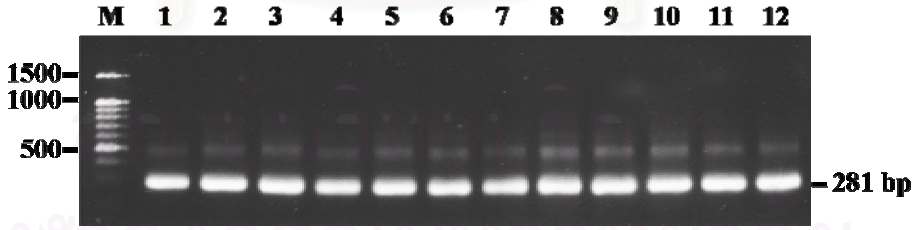


Figure 3.29 A 1.5% ethidium bromide stained agarose gel showing the PCR product generated from amplification of *P. pelagicus* originating from Suratthani (lanes 1-4), Krabi (lanes 5-8) and Prachuap Khiri Khan (lanes 9-12) with PP-P4M2_515-F/R. Lane M is 100 bp DNA ladder.

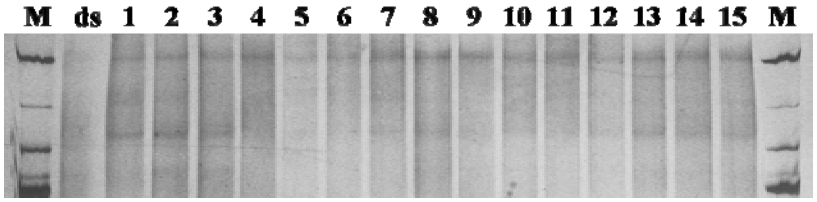


Figure 3.30 SSCP patterns of the PCR product of *P. pelagicus* from Chanthaburi (lanes 1-3), Ranong (lanes 4-6), Suratthani (lanes 7-9), Krabi (lanes 10-12) and Prachuap Khiri Khan (lanes 13-15) amplified with PP-P4M2_515-F/R and electrophoresed through 15% non-denaturing polyacrylamide gel (37.5:1 crosslink) at 250 volts for 15 hours at 4°C and silver stained. Lanes M and ds are a 100 bp DNA marker and the non-denatured PCR product, respectively.

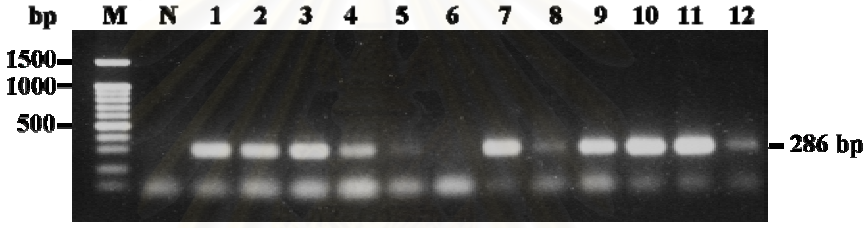


Figure 3.31 A 1.5% ethidium bromide stained agarose gel showing the PCR product generated from amplification of genomic DNA of *P. pelagicus* originating from Chanthaburi (lanes 1-3), Ranong (lanes 4-6), Suratthani (lanes 7-9) and Krabi (lanes 10-12) with PP-P4M1_418-F/R. Lane M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.

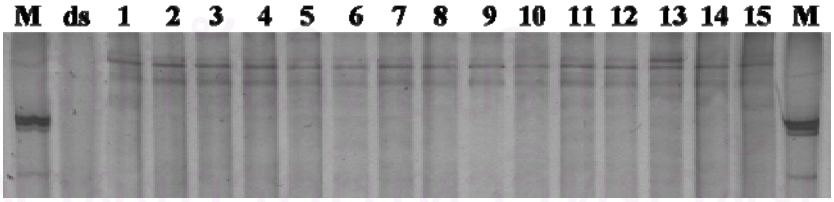


Figure 3.32 SSCP patterns of the PCR product of *P .pelagicus* from Chanthaburi (lanes 1-3), Ranong (lanes 4-6), Suratthani (lanes 7-9), Krabi (lanes 10-12) and Prachuap Khiri Khan (lanes 13-15) amplified with PP-P4M1_418-F/R and electrophoresed through 15% non-denaturing polyacrylamide gel (75:1 crosslink) at 250 volts for 15 hours at 4°C and silver stained. Lanes M and ds are a 100 bp DNA marker and the non-denatured PCR product, respectively.

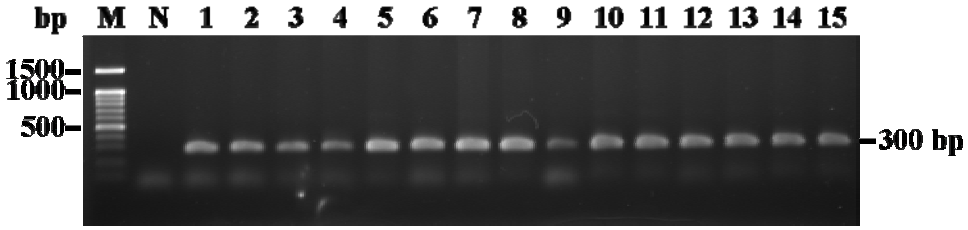


Figure 3.33 A 1.5% ethidium bromide stained agarose gel showing the PCR product generated from amplification of genomic DNA of *P. pelagicus* originating from Chanthaburi (lanes 1-3), Ranong (lanes 4-6), Suratthani (lanes 7-9), Krabi (lanes 10-12) and Prachuap Khiri Khan (lanes 13-15) with PP-P4M1_454-F/R. Lane M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.

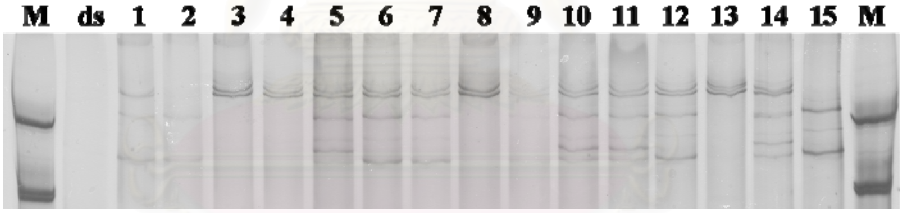


Figure 3.34 SSCP patterns of the PCR product of *P. pelagicus* from Chanthaburi (lanes 1-3), Ranong (lanes 4-6), Suratthani (lanes 7-9), Krabi (lanes 10-12) and Prachuap Khiri Khan (lanes 13-15) amplified with PP-P4M1_454-F/R and electrophoresed through 15% non-denaturing polyacrylamide gel (37.5:1 crosslink) at 300 volts for 13.5 hours at 4°C and silver stained. Lanes M and ds are a 100 bp DNA marker and the non-denatured PCR product, respectively.

3.1.3 Determination of *PP-COI$_{270}$*, *PP-M122/135RAP* and *PP-P4M1_454* polymorphism by PCR-SSCP

The blue swimming crab is the promising species for aquaculture. Nevertheless, the basic information about genetic diversity and differentiation of this species in Thai waters is still limited. Initially, polymorphic SCAR markers were developed from the positive amplification fragment of mtDNA gene; *cytochrome oxidase subunit I* (*COI*), and nuclear DNA gene; M122/135RAP and $P_{+3}4/M_{+3}1$_454. A pair of primers was designed from each sequence (hereafter called PP-COI_{270}-F/R, PP-$SCARRAP_{318}$-F/R and PP-$SCARAFLP_{300}$-F/R, respectively). These primer pairs were tested against genomic DNA of geographically different samples of *P. pelagicus* in Thailand (*N* = 174).

PP-COI_{270}-F/R provides the positive amplification product of 270 bp in all investigated individuals (100% amplification success, Figure 3.18). PP-$SCARRAP_{318}$-F/R and PP-$SCARAFLP_{300}$-F/R generated the positive amplification product of 318 bp and 300 bp in 168 (96.6%, Figure 3.16) and 166 (95.4%, Figure 3.33) tested individuals, respectively. No obvious length polymorphism or allelic variants were observed from this DNA segments when analyzed by agarose gel electrophoresis.

The amplification product of *PP-COI$_{270}$* from each crab was further genotyped by SSCP analysis. A total of 9 fragments were found across overall investigated individuals and 5 SSCP fragments accounting for 55.56% were polymorphic (< 95 % all examined specimens). These generated 8 SSCP genotypes (Tables 3.4). The discrimination capacity (average number of individuals that possibly share identical SSCP genotypes) of *PP-COI$_{270}$* was 21.75.

SSCP genotypes I and II of *PP-COI$_{270}$* predominated in crabs from the Gulf of Thailand (68/104 accounting for 65.38%) but each genotype was found in a single individual of the Andaman sample (1/70, 1.43%). In contrast, 92.86% (65/70) of the Andaman samples possessed the genotype IV whereas only 18 individuals of *P. pelagicus* from Prachuap Khiri Khan (17.31% of the Gulf of Thailand samples) exhibited that SSCP genotype.

Table 3.4 SSCP patterns resulted from analysis of *PP-COI*$_{270}$ of *P. pelagicus* originating from different geographic samples in Thai waters

SSCP pattern	No. of individuals					
	Chanthaburi (*N* = 29)	**Prachuap Kriri Khan (*N* = 40)**	**Suratthani (*N* = 35)**	**Ranong (*N* = 35)**	**Krabi (*N* = 35)**	**Total (*N* = 174)**
I. 1, 3, 5	17	5	23	-	1	46
II. 3, 5	10	10	3	-	1	24
III. 2, 5	-	4	-	-	-	4
IV. 2, 4	-	18	-	34	31	83
V. 6, 7	-	-	2	-	1	3
VI. 1, 2, 4	1	1	7	1	1	11
VII. 8, 9	1	-	-	-	-	1
VIII. 4, 6	-	2	-	-	-	2

The SSCP genotype VI was found across all geographic samples at low frequencies (6.32% of all investigated specimens). Likewise, genotypes III, V, VII and VIII were rare genotypes (< 5% of all investigated specimens, Table 3.4).

An individual representing each SSCP genotype of *PP-COI*$_{270}$ was direct sequenced and multiple aligned. Nucleotide sequences indicated that genotypes I and II shared identical sequence but the remaining genotypes were able to discriminate by nucleotide polymorphism. This indicated that SSCP analysis may overestimate level of genetic polymorphism of *P. pelagicus* when *PP-COI*$_{270}$ was analyzed.

Notably, sequencing errors from direct sequencing of the PCR product is quite high. Therefore, results from direct sequencing should be treated with cautions.

PP-SCARRAP$_{318}$-F/R generated the positive amplification product of 318 bp in 168 (96.55%, Figure 3.16) investigated individuals. Length polymorphism or allelic variants were not observed from the amplification segment when analyzed by agarose gel electrophoresis.

```
PP-COI270_2 TTCAGCAGCCATCGCTCACGCAGGAGCTTCTGTAGATCTAGGTATTTTCTCTTTACATCT
PP-COI270_1 TTCAGCAGCCATCGCTCACGCAGGAGCTTCTGTAGATCTAGGTATTTTCTCTTTACATCT
PP-COI270_3 TTCAGCAGCCATCGCTCACGCAGGAGCTTCTGTAGATCTAGGTATTTTCTCTTTACATCT
PP-COI270_4 TTCAGCAGCCATCGCTCACGCAGGAGCTTCTGTAGATCTAGGTATTTTCTCTTTACATCT
PP-COI270_5 TTCAGCAGCCATCGCTCACGCAGGAGCTTCTGTAGATCTAGGTATTTTCTCTTTACATCT
PP-COI270_6 TTCAGCAGCCATCGCTCACGCAGGAGCTTCTGTAGATCTAGGTATTTTCTCTTTACATCT
PP-COI270_7 TTCAGCAGCCATCGCTCACGCAGGAGCTTCTGTAGATCTAGGTATTTTCTCTTTACATCT
PP-COI270_8 TTCAGCAGCCATCGCTCACGCAGGAGCTTCTGTAGATCTAGGTATTTTCTCTTTACATCT
            ************************************************************60

PP-COI270_2 GGCAGGTGTTTCCTCTATTTTAGGTGCAGTAAATTTCATGACCACCGTTATTAACATGCG
PP-COI270_1 GGCAGGTGTTTCCTCTATTTTAGGTGCAGTAAATTTCATGACCACCGTTATTAACATGCG
PP-COI270_3 AGCAGGTGTTTCCTCTATTTTAGGTGCAGTAAATTTCATGACCACCGTTATTAACATGCG
PP-COI270_4 GGCAGGTGTTTCCTCTATTCTAGGTGCAGTAAATTTCATGACCACCGTTATTAACATGCG
PP-COI270_5 GGCAGGTGTTTCCTCTATTCTAGGTGCAGTAAATTTCATGACCACCGTCATTAACATGCG
PP-COI270_6 GGCAGGTGTTTCCTCTATTCTAGGTGCAGTAAATTTCATGACCACCGTTATTAACATGCG
PP-COI270_7 GGCAGGTGTTTCCTCTATTCTAGGTGCAGTAAATTTCATGACCACCGTTATTAACATGCG
PP-COI270_8 AGCAGGTGTTTCCTCTATTTTAGGTGCAGTAAATTTCATAACCACCGTTATTAACATACG
             ****************** ****************** ******** ******** **120

PP-COI270_2 ATCTTTTGGTATAAGAATGGACCAAATACCATTATTCGTTTGATCAGTTTTTATCACTGC
PP-COI270_1 ATCTTTTGGTATAAGAATGGACCAAATACCATTATTCGTTTGATCAGTTTTTATCACTGC
PP-COI270_3 ATCTTTTGGTATAAGAATGGACCAAATACCATTATTCGTTTGATCAGTTTTTATCACTGC
PP-COI270_4 ATCTTTTGGTATAAGAATAGACCAAATGCCATTATTCGTTTGATCAGTATTTATCACTGC
PP-COI270_5 ATCTTTTGGTATAAGAATGGACCAAATGCCATTATTCGTTTGATCAGTATTTATCACTGC
PP-COI270_6 ATCTTTTGGTATAAGAATGGACCAAATGCCATTATTCGTTTGATCAGTATTTATCACTGC
PP-COI270_7 GTCTTTTGGTATAAGAATGGACCAAATGCCATTATTCGTTTGATCAGTATTTATCACTGC
PP-COI270_8 ATCTTTTGGTATAAGAATGGACCAAATGCCATTATTCGTTTGATCAGTATTTATCACTGC
             ***************** ******** ******************** **********180

PP-COI270_2 TATTCTTCTACTTTTATCTCTCCCTGTTCTTGCTGGAGCTATTACTATACTTCTTACAGA
PP-COI270_1 TATTCTTCTACTTTTATCTCTCCCTGTTCTTGCTGGAGCTATTACTATACTTCTTACAGA
PP-COI270_3 TATTCTTCTACTTTTATCTCTCCCTGTTCTTGCTGGAGCTATTACTATACTTCTTACAGA
PP-COI270_4 TATTCTTCTACTTTTATCTCTCCCTGTTCTTGCTGGAGCTATTACTATACTTCTTACAGA
PP-COI270_5 TATTCTTCTACTTTTATCTCTCCCTGTTCTTGCTGGAGCTATTACTATACTTCTTACAGA
PP-COI270_6 TATTCTTCTACTTTTATCTCTCCCTGTTCTTGCTGGAGCTATTACTATACTTCTTACAGA
PP-COI270_7 TATTCTTCTACTTTTATCTCTCCCTGTTCTTGCTGGAGCTATTACTATACTTCTTACAGA
PP-COI270_8 TATTCTTCTACTTTTATCTCTCCCTGTTCTTGCTGGAGCTATTACTATACTTCTTACAGA
            ************************************************************240

PP-COI270_2 CCGAAATCTAAATACTTCATTCTTTGACCCT
PP-COI270_1 CCGAAATCTAAATACTTCATTCTTTGACCCT
PP-COI270_3 CCGAAATCTAAATACTTCATTCTTTGACCCT
PP-COI270_4 CCGAAATCTAAATACTTCATTCTTTGACCCT
PP-COI270_5 CCGAAATCTAAATACTTCATTCTTTGACCCT
PP-COI270_6 CCGAAATCTAAATACTTCATTCTTTGACCCT
PP-COI270_7 CCGAAATCTAAATACTTCATTCTTTGACCCT
PP-COI270_8 CCGAAATCTAAATACTTCATTCTTTGACCCT
            ******************************271
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Figure 3.35 Multiple alignments of 8 patterns of the *PP-COI*$_{270}$ gene segment. The location and sequence of a forward primer (PP-COI$_{270}$-F) and those complementary to a reverse primer (PP-COI$_{270}$-R) are illustrated in boldface and underlined.

The amplification product of *PP-SCARRAP$_{318}$* from each crab was further genotyped by SSCP analysis. A total of 19 fragments were found across overall investigated individuals and eight SSCP fragments accounting for 42.11% were polymorphic (< 95 % all examined specimens). These generated 56 SSCP genotypes (Table 3.5). The discrimination capacity of *PP-SCARRAP$_{318}$* was 3.00.

The SSCP genotype II of *PP-SCARRAP$_{318}$* were found in 21.43% (36/168) of all specimens. This genotype was distributed across all geographic samples. The remaining genotypes were found in less than 10% of investigated specimens. Genotypes I and VIII were region-specific and only found in the Gulf of Thailand samples. Several private genotypes (found in only one geographic sample) were observed in each location.

An individual representing each SSCP genotypes II, V, VII, VIII, IX, XIV, XXI, XXIX and XXXVIII of *PP-SCARRAP$_{318}$* was direct sequenced and multiple aligned. Nucleotide sequences indicated that each genotype did not share identical sequences could be discriminated by at least a single nucleotide polymorphism.

PP-SCARAFLP$_{300}$-F/R generated the positive amplification product of 300 bp in 167 (95.98%, Figure 3.33) tested individuals. No obvious length polymorphism or allelic variants were observed from *PP-SCARAFLP$_{300}$* when analyzed by agarose gel electrophoresis.

The amplification product of *PP-SCARAFLP$_{300}$* from each crab was further genotyped by SSCP analysis. A total of 7 fragments were found across overall investigated individuals and four polymorphic fragments accounting for 57.14% were found. These generated 21 SSCP genotypes (Table 3.6). The discrimination capacity of *PP-SCARAFLP$_{300}$* was 7.90.

SSCP genotypes I, IV and VII of *PP-SCARAFLP$_{300}$* were common. The genotype I predominated in crabs from the Gulf of Thailand (43/104 accounting for 41.35%) but IV and VII were distributed across each geographic samples with comparative frequencies. Genotypes X and XI were region-specific and only found in

Table 3.5 SSCP patterns resulted from analysis of *PP-SCARRAP*$_{318}$ of *P. pelagicus* originating from different geographic samples in Thai waters

SSCP pattern	Geographic sample (individuals)					
	Chanthaburi (***N*** **= 29)**	**Prachuap Kriri Khan** (***N*** **= 40)**	**Suratthani** (***N*** **= 35)**	**Ranong** (***N*** **= 35)**	**Krabi** (***N*** **= 35)**	**Total** (***N*****=174)**
I. 6, 7, 15	2	1	2	-	-	5
II. 6, 15	6	11	3	12	4	36
III. 4, 6	1	-	-	-	-	1
IV. 4, 5, 15	1	-	-	-	-	1
V. 6, 14	7	1	-	-	-	8
VI. 5, 9, 15	1	-	-	-	-	1
VII. 5, 15	3	10	-	-	-	13
VIII. 6, 10, 15	1	5	2	-	-	8
IX. 5, 14	2	1	-	2	-	5
X. 5, 6, 18, 19	1	-	-	-	-	1
XI. 4, 5, 15, 17	2	-	-	-	-	2
XII. 5, 15, 17	1	-	-	-	-	1
XIII. 6, 9, 11, 15	-	-	-	4	-	4
XIV. 5, 13	-	-	3	1	1	5
XV. 9, 11, 15	-	-	-	2	-	2
XVI. 6, 8, 15, 17	-	-	-	1	-	1
XVII. 6, 15, 17	-	-	-	1	-	1
XVIII. 6, 18	-	-	-	1	-	1
XIX. 3, 12, 13	-	-	-	2	-	2
XX. 2, 3, 11, 12	-	-	-	1	-	1
XXI. 4, 12	-	-	1	1	9	11
XXII. 4, 14, 15	-	-	-	1	-	1
XXIII. 5, 6, 14, 15	-	-	-	2	-	2
XXIV. 5, 9, 11, 13, 15	-	-	-	1	-	1
XXV. 3, 5, 13, 15	-	-	2	-	-	2
XXVI. 5, 12	-	-	1	-	-	1
XXVII. 1, 5, 12, 14	-	-	1	-	-	1
XXVIII. 6, 9, 10	-	-	1	-	-	1

Table 3.5 (cont.)

SSCP pattern	No. of individuals					
	Chanthaburi (*N* = 29)	**Prachuap Kriri Khan** (*N* = 37)	**Suratthani** (*N* = 35)	**Ranong** (*N* = 32)	**Krabi** (*N* = 35)	**Total** (*N*=168)
XXIX. 5, 7, 15, 17	-	-	5	-	-	5
XXX. 8, 15	-	-	1	-	-	1
XXXI. 6, 8, 15, 19	-	-	1	-	-	1
XXXII. 9, 15	-	-	1	-	-	1
XXXIII. 1, 7, 15	-	-	1	-	-	1
XXXIV. 3, 5	-	-	2	-	-	2
XXXV. 7, 15	-	-	2	-	1	3
XXXVI. 5, 6, 15	-	-	1	-	-	1
XXXVII. 5, 10, 14, 15	-	2	1	-	-	3
XXXVIII. 6, 10, 11, 15	-	-	4	-	-	4
XXXIX. 3, 6, 11, 14	-	-	-	-	1	1
XL. 2, 5, 11, 12	-	-	-	-	1	1
XLI. 4, 11	-	-	-	-	6	6
XLII. 3, 13	-	-	-	-	2	2
XLIII. 2, 12	-	-	-	-	1	1
XLIV. 2, 11	-	-	-	-	1	1
XLV. 3, 6, 8, 14	-	-	-	-	1	1
XLVI. 6, 13	-	-	-	-	1	1
XLVII. 5, 11, 12	-	-	-	-	2	2
XLVIII. 3, 4, 11	-	-	-	-	1	1
XLIX. 4, 9, 14	-	-	-	-	1	1
L. 3, 4, 12, 13	-	-	-	-	2	2
LI. 9, 11, 16	-	1	-	-	-	1
LII. 6, 14, 15	-	1	-	-	-	1
LIII. 4, 6, 14, 17	-	1	-	-	-	1
LIV. 1, 10, 12, 14	-	1	-	-	-	1
LV. 6, 11, 15	-	1	-	-	-	1
LVI. 10, 15	-	1	-	-	-	1

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PP-SCARRAP318_7  TGAGGGATGTAAGGTCAGAGGATTAATGGCTACAGTTTTCACTATTTTAATCCCCAACAT
PP-SCARRAP318_14 TGAGGGATGTAAGGTCAGAGGATTAATGGCTACAGTTTTCACTATTTTAATCCCCAACAT
PP-SCARRAP318_21 TGAGGGATGTAAGGTCAGAGGATTAATGGCTACAGTTATCTCTATTTTAATCCCCAACAT
PP-SCARRAP318_9  TGAGGGATGTAAGGTCAGAGGATTAATGGCTACAGTTATCTCTATTTTAATCCCCAACAT
PP-SCARRAP318_29 TGAGGGATGTAAGGTCAGAGGATTAATGGCTACAGTTTTCTCTATTTTAATCCCCAACAT
PP-SCARRAP318_38 TGAGGGATGTAAGGTCAGAGGATTAATGGCTACAGTTTTCACTATTTTAATCCCCAATAT
PP-SCARRAP318_5  TGAGGGATGTAAGGTCAGAGGATTAAGGGTT-CCGTTTTCCCTTTTTTTAT-CCCCACAT
PP-SCARRAP318_8  TGAGGGATGTAAGGTCAGAGGATTATGGGTT-CCGTTTTTCCTTTTTTTATTCCCCATAT
PP-SCARRAP318_2  TGAGGGATGTAAGGTCAGAGGATTAAGGGTTCCAGTTTTTCTTTTTTTATTCCCCACCTT
                 ************************  ** * * *** *   * **** * ***    *60
PP-SCARRAP318_7  AAGTATCTAAAACTATAAAATCACCAAATAGTAAGCAGAATGAATATAAAAACGCGTCCT
PP-SCARRAP318_14 AAGTATCTAAAACTATAAAATCACCAAATAGTAAGCAGAATGAATATAAAAACGCGTCCT
PP-SCARRAP318_21 AAGTATCTAAAACTATAAAATCACCAAATAGTAAGCAGAATGAATATAGAAACGCGTCCT
PP-SCARRAP318_9  AAGTATCTAAAACTATAAAATCACCAAATAGTAAGCAGAATGAATATAGAAACGCGTCCT
PP-SCARRAP318_29 AAGTATCTAAAACTATAAAATCACCAAATAGTAAGCAGAATGAATATAAAAACGCGTCCT
PP-SCARRAP318_38 AAGTATCTAAAACTATAAAATCCCCAAATAGTAAGCCGAATGAATATAAAAACGCGTCCT
PP-SCARRAP318_5  AAGTATCTAAAACTATAAAATCACTAAATAGTAAGCAGAATGAATATAAAAACGCGTCCT
PP-SCARRAP318_8  AAGTATCTAAAACTATAAAATCACCAAATAGTAAGCAGAATGAATATAAAAACGCGTCCT
PP-SCARRAP318_2  AGGTTTTTAAACTTTTAAATTTCCCAATTGGTA-GCCGGATGGATTTTAAAACGGGTTCT
                 * ** * ****  * **** *  * ** * *** ** * *** ** *  ***** ** **120
PP-SCARRAP318_7  GGTACTGAATGGGTT-AAAGATGAGTTGCATAACACCAAACGAATAAAA-AATGATACCA
PP-SCARRAP318_14 GGTACTGAATGGGTT-AAAGATGAGTTGCATAACACCAAACGAATAAAA-AATGATACCA
PP-SCARRAP318_21 GGTACTGAATGGGTT-AAAGATGAGTTGCATAACACCGAACGAATAAAAGAATGATACCA
PP-SCARRAP318_9  GGTACTGAATGGGTT-AAAGATGAGTTGCATAACACCGAACGAATAAAAGAATGATACCA
PP-SCARRAP318_29 GGTACTGAATGGGTT-AAAGATGAGTTGCATAACACCGAACGAATAAAAAAATGATACCA
PP-SCARRAP318_38 GGTACTGAATGGGTT-AAAGATGAGTTGCATAACACCGAACGAATAAAAAAATGATACCA
PP-SCARRAP318_5  GGTACTGAATGGGTT-AAAGATGAGTTGCATAACACCGAACGAATAAAAAATGGTACCCC
PP-SCARRAP318_8  GGTACTGAATGGGTT-AAAGATGAGTTGCATAACACCGAACGAATAAAAAAAGGATCCCC
PP-SCARRAP318_2  GGTTATGGATTGGTTTAAGGAGGAGTTGCATACCCCCAAAGGATTAAAA-AATGATACCA
                 ***  ** ** **** ** ** ********** * ** ** ** ***** *  *   ** 180
PP-SCARRAP318_7  CTAGTTAGTTGTAGACCATATGTACTACTCAAAGGGATCTAACCAAACTTTTCTCCACAG
PP-SCARRAP318_14 CTAGTTAGTTGTAGACCATATGTACTACTCAAAGGGATCTAACCGAACTTTTCTCCACAG
PP-SCARRAP318_21 CTAGTTAGTTGTAGACCATATGTACCACTCAAAGGGATCTACCCAAACTTTTCTCCACAG
PP-SCARRAP318_9  CTAGTTAGTTGTAGACCATATGTACCACTCAAAGGGATCTACCCAAACTTTTCTCCACAG
PP-SCARRAP318_29 CTAGTTAGTTGTAGACCATATGTACCACTCAAAGGGATCTAACCAAACTTTTCTCCACAG
PP-SCARRAP318_38 CTAGTTAGTTGTAGACCATATGTACCACTCAAAGGGATCTAACCAAACTTTTCTCCACAG
PP-SCARRAP318_5  CTATTTATTGGAAAACCTTTGGTCCACTTCAAAGGAATCTAACCAACTTTTTCCCCCGG
PP-SCARRAP318_8  CTATTTATTTGTAAACCATATGTCCAACTCAAAGGGATCTAACCAAATTTTTCTCCCCAG
PP-SCARRAP318_2  CTAGTTAGTTGTAGACCATATGTACTACTCAAAGGGATCTAACCAAACTTTTCTCCACAG
                 *** *** * * * *** *  ** *   ******* ***** ** *  ***** ** * *240
PP-SCARRAP318_7  TATTTCCCGAGTATAACATTACAGAAATCCCAATAGATGTGATTCCCTTACTTCTCCACT
PP-SCARRAP318_14 TATTTCCCGAGTATAACATTACAGAAATCCCAATAGATGTGATTCCCTTACTTCTCCACT
PP-SCARRAP318_21 TATTTCCCGAGGATAACATTACAGAAATCCCAATAGATGTGATTCCCTTACTTCTCCACT
PP-SCARRAP318_9  TATTTCCCGAGTATAACATTACAGAAATCCCAATAGATGTGATTCCCTTACTTCTCCACT
PP-SCARRAP318_29 TATTTCCCGAGTATAACATTACAGAAATCCCAATAGATGTGATTCCCTTACTTCTCCACT
PP-SCARRAP318_38 TATTTCCCGAGTATAACATTACAAAAATCCCAATAGATGTGATTCCCTTACTTCTCCACT
PP-SCARRAP318_5  ATTTTCCCAAGATTAACTTTCCAAAATCCCCATAAAAGGGAATCCCCTAACTCCCCCACT
PP-SCARRAP318_8  AATTTCCCAAGAATAACATTCCAAAATTCCCAAAAAAGGGAATTCCCTTACTTCCCCACT
PP-SCARRAP318_2  TATTTCCCAAGATTACCTTTCCGAAATTCCCATTAAAGGGATTCCCCTT-CCTCCCCACT
                   ****** **  ** * ** *  **  ****  * * *  * ****  *  * *****300
PP-SCARRAP318_7  TTGTTTCCTCCTGTGAACC
PP-SCARRAP318_14 TTGTTTCCTCCTGTGAACC
PP-SCARRAP318_21 TTGTTTCCTCCTGTGAACC
PP-SCARRAP318_9  TTGTTTCCTCCTGTGAACC
PP-SCARRAP318_29 TTGTTTCCTCCTGTGAACC
PP-SCARRAP318_38 TTGTTTCCTCCTGTGAACC
PP-SCARRAP318_5  TTGTTTCCTCCTGTGAACC
PP-SCARRAP318_8  TTGTTTCCTCCTGTGAACC
PP-SCARRAP318_2  TTGTTTCCTCCTGTGAACC
                 ******************319
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Figure 3.36 Multiple alignments of major SSCP genotypes of *PP-SCARRAP$_{318}$*. The location and sequence of a forward primer (PP-SCARRAP$_{318}$ -F) and those complementary to a reverse primer (PP-SCARRAP$_{318}$ -R) are illustrated in underlined.

Table 3.6 SSCP patterns resulted from analysis of *PP-SCARAFLP*$_{300}$ of *P. pelagicus* originating from different geographic samples in Thai waters

SSCP pattern	Geographic samples (No. of individuals)					
	Chanthaburi (***N*** **= 29)**	**Prachuap Kriri Khan** (***N*** **= 40)**	**Suratthani** (***N*** **= 35)**	**Ranong** (***N*** **= 28)**	**Krabi** (***N*** **= 35)**	**Total** (***N*****=167)**
I. 1, 3, 5, 7	11	23	9	7	8	58
II. 2, 4, 5, 7	2	2	-	-	-	4
III. 1, 5, 7	1	-	2	-	-	3
IV. 5, 7	8	8	12	9	10	47
V. 2, 4, 5, 6	1	-	-	-	-	1
VI. 3, 5, 7	2	-	1	-	-	3
VII. 1, 3	2	4	8	5	8	27
VII. 4, 5, 7	1	-	-	-	-	1
IX. 3, 4	1	-	-	-	-	1
X. 5, 6, 7	-	-	-	1	1	2
XI. 5, 6	-	-	-	2	3	5
XII. 1, 2	-	-	-	2	-	2
XIII. 1, 3, 5, 6	-	-	-	1	-	1
XIV. 3, 5, 6	-	-	-	1	-	1
XV. 3	-	-	3	-	1	4
XVI. 1	-	1	-	-	1	2
XVII. 1, 3, 5, 6, 7	-	-	-	-	1	1
XVIII. 4	-	-	-	-	1	1
XIX. 3, 6	-	-	-	-	1	1
XX. 1, 5, 6, 7	-	1	-	-	-	1
XXI. 2, 4	-	1	-	-	-	1

the Andaman samples. Several private genotypes were observed in each geographic location.

An individual representing each SSCP genotypes I, IV, VII and XI of *PP-SCARAFLP*$_{300}$ was direct sequenced and multiple aligned. Nucleotide sequences indicated that each genotype can be differentiated by single nucleotide polymorphism (SNP).

```
PP-SCARAFLP300_4    TCTCAGATTGGGCTCCTTGTGATGAGGATGGTTTTAGCATGAAGACGTCCGATGATATTG
PP-SCARAFLP300_11   TCTCAGATTGGGCTCCTTGTGATGAGGATGGTTTTAGCATGAAGACGTCCGATGATATTG
PP-SCARAFLP300_7    TCTCAGATTGGGCTCCTTGTGATGAGGATGGTTTTAGCATGAAGACGTCCGATGATATTG
PP-SCARAFLP300_1    TCTCAGATTGGGCTCCTTGTGATGAGGATGGTTTTAGCATGAAGACGTCCGATGATATTG
                    ************************************************************60

PP-SCARAFLP300_4    TCACAGCCGTCCATCTTGGACCCACTGAATCGAAAAGTGCAGTCCAGCCTGATCCTCGTC
PP-SCARAFLP300_11   TCACAGCCGTCCATCTTGGACCCACTGAATCGAAAAATGCAGTCCAGCCTGATCCTCGTC
PP-SCARAFLP300_7    TCACAGCCGTCCATCTTGGACCCACTGAATCGAAAAGTGCAGTCCAGCCTGATCCTCGTC
PP-SCARAFLP300_1    TCACAGCCGTCCATCTTGGACCCACTGAATCGAAAAGTGCAGTCCAGCCTGATCCTCGTC
                    ********************************** ***********************120

PP-SCARAFLP300_4    CTCCGATGACCAGTAGAAGTAATGATGGTTCGCATCACTCACCGGTAGGCCGAAAGGCAG
PP-SCARAFLP300_11   CTCCGATGACCAGTAGAAGTAATGATGGTTCGCATCACTCACCGGTAGGCCGAAAGGCAG
PP-SCARAFLP300_7    CTCCGATGACCAGTAGAAGTAATGATGGTTCGCATCACTCACCGGTAGGCCGAAAGGCAG
PP-SCARAFLP300_1    CTCCGATGACCAGTAGAAGTAATGATGGTTCGCTTCACTCACCGGTAGGCCGAAAGGCAG
                    ******************************** ************************180

PP-SCARAFLP300_4    CGGTCCAACGACCTGGCACATGTCAACTCCCGGTGTCTTCTCGTCGTTTGATTATTATTA
PP-SCARAFLP300_11   CGGTCCAACGACCTGGCACATGTCAACTCCCGGTGTCTTCTCGTCGTTTGATTATTATTA
PP-SCARAFLP300_7    CGGTCCAACGACCTGGCACATGTCAACTACCGGTGTCTTCTCGTCGTTTGATTATTATTA
PP-SCARAFLP300_1    CGGTCCAACGACCTGGCACATGTCAACTCCCGGTGTCTTCTCGTCGTTTGATTATTATTA
                    ************************** ******************************240

PP-SCARAFLP300_4    CTGATTGTAGTCAGTTGCCGAGCTCGAGCCCGCCGCGTTTTGAAAGAGGCACAAAGAACC
PP-SCARAFLP300_11   CTGATTGTAGTCAGTTGCCGAGCTCGAGCCCGCCGCGTTTTGAAAGAGGCACAAAGAACC
PP-SCARAFLP300_7    CTGATTGTAGTCAGTTGCCGAGCTCGAGCCCGCCGCGTTTTGAAAGAGGCACAAAGAACC
PP-SCARAFLP300_1    CTGATTGTAGTCAGTTGCCGAGCTCGAGCCCGCCGCGTTTTGAAAGAGGCACAAAGAACC
                    ************************************************************300
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Figure 3.37 Multiple alignments of major SSCP genotypes of *SCARAFLP*$_{300}$. The location and sequence of a forward primer (PP-SCARAFLP$_{300}$-F) and those complementary to a reverse primer (PP-SCARAFLP$_{300}$-R) are illustrated in underlined.

The average polymorphic loci and gene diversity in each geographic sample were 40.00% (Prachuap Khiri Khan) – 48.57% (Suratthani) and 0.1365 (Ranong) – 0.1781 (Suratthani). These parameters across overall samples were 48.57% and 0.1859, respectively (Table 3.7).

Large numbers of SSCP genotypes found in Thai *P. pelagicus* suggesting high genetic diversity in this species. The average genetic identity between pairs of geographic samples was 0.8871 (Chanthaburi and Ranong) to 0.9902 (Chanthaburi and Suratthani, Table 3.7). Genetic distance between pairs of geographic samples was calculated. The lowest distance was observed between *P. pelagicus* from Chanthaburi and Suratthani (0.0099). The greatest distance was observed between Chanthaburi and

Ranong (0.1198) samples. Generally, relatively larger genetic distance was observed between samples from different coastal regions than that between geographic locations within regions (Table 3.8).

Significant geographic heterogeneity was observed across overall samples ($P < 0.01$ for F_{ST} based statistics (θ) and $P < 0.0001$ for exact test). In addition, significant heterogeneity was also observed between all possible pairwise comparisons ($P < 0.05$, Table 3.9). Results indicated that the gene pool of *P. pelagicus* in Thai waters is not homogeneous but genetically fragmented at the microgeographic level (i.e. a few hundred kilometers within the same coastal regions).

The estimated gene flow level was low in this species (N_em = 0.39 – 5.37 individuals per generation). The level between geographic samples located in different coastal regions was 0.39 – 1.40 whereas that between samples within coastal regions was 1.46 – 5.37 for the Gulf of Thailand samples and 3.19 individuals per generation for the Andaman Sea samples. This indicated that *P. pelagicus* is a low gene flow species (Table 3.9).

A UPGMA dendrogram constructed from the average unbiased genetic distance between pairs of geographic samples of Thai *P. pelagicus* allocated 5 investigated samples to 2 evolutionarily related groups; Chanthaburi, Suratthani and Prachuap Kriri Khan (Gulf of Thailand, A) and Ranong and Krabi (Andaman Sea, B) (Figure 3.38).

Table 3.7 The average levels of polymorphic loci and gene diversity across geographic samples of *P. pelagicus* in Thai waters based on three gene segments

Geographic samples	Polymorphic loci (%)	Gene diversity (direct count)
Chanthaburi	42.86	0.1606
Prachuap Khiri Khan	40.00	0.1730
Suratthani	48.57	0.1782
Ranong	42.86	0.1365
Krabi	45.71	0.1527
All samples	48.57	0.1859

Table 3.8 Pairwise Nei's (1978) genetic distance (below diagonal) and genetic identity (above diagonal), between pairs of geographic samples of *P. Pelagicus* based on SSCP analysis of *PP-COI*$_{270}$, *PP-SCARRAP*$_{318}$ and *PP-SCARAFLP*$_{300}$

	CHN	**PKK**	**SUT**	**RNG**	**KRB**
CHN	-	0.9656	0.9902	0.8871	0.9005
PKK	0.0350	-	0.9804	0.9660	0.9639
SUT	0.0099	0.0198	-	0.9203	0.9344
RNG	0.1198	0.0346	0.0830	-	0.9866
KRB	0.1049	0.0368	0.0679	0.0135	-

Abbreviation: CHN = Chanthaburi, PKK = Prachuap Kriri Khan, SUT = Suratthani, RNG = Ranong, KRB = Krabi

Table 3.9 Geographic heterogeneity of five geographic samples of *P. pelagicus* based on SSCP analysis of *PP-COI*$_{270}$, *PP-SCARRAP*$_{318}$ and *PP-SCARAFLP*$_{300}$

Geographic sample	***F*$_{ST}$-based statistics**			**Exact test**
	Theta (θ)	**χ^2**	**$N_e m$**	**(*P*-value)**
CHN-PKK	0.1463	47.40*	1.46	< 0.0001
CHN-SUT	0.0445	14.42*	5.37	0.0021
CHN-RNG	0.3883	125.81*	0.39	< 0.0001
CHN-KRB	0.3518	113.98*	0.46	< 0.0001
PKK-SUT	0.0837	27.12*	2.74	< 0.0001
PKK-RNG	0.1514	49.05*	1.40	< 0.0001
PKK-KRB	0.1568	50.80*	1.34	< 0.0001
SUT-RNG	0.2920	94.61*	0.61	< 0.0001
SUT-KRB	0.2502	81.06*	0.75	< 0.0001
RNG-KRB	0.0726	23.52*	3.19	0. 0317

Geographic abbreviation as in Table 3.8

$\chi^2 = 2N\theta(k\text{-}1)$; df = $(s\text{-}1)(k\text{-}1)$; N is the number of individuals used in the analysis, k is the number of alleles per locus, and s is the number of geographic samples

* Significant at $P < 0.01$ following the sequential Bonferroni approach

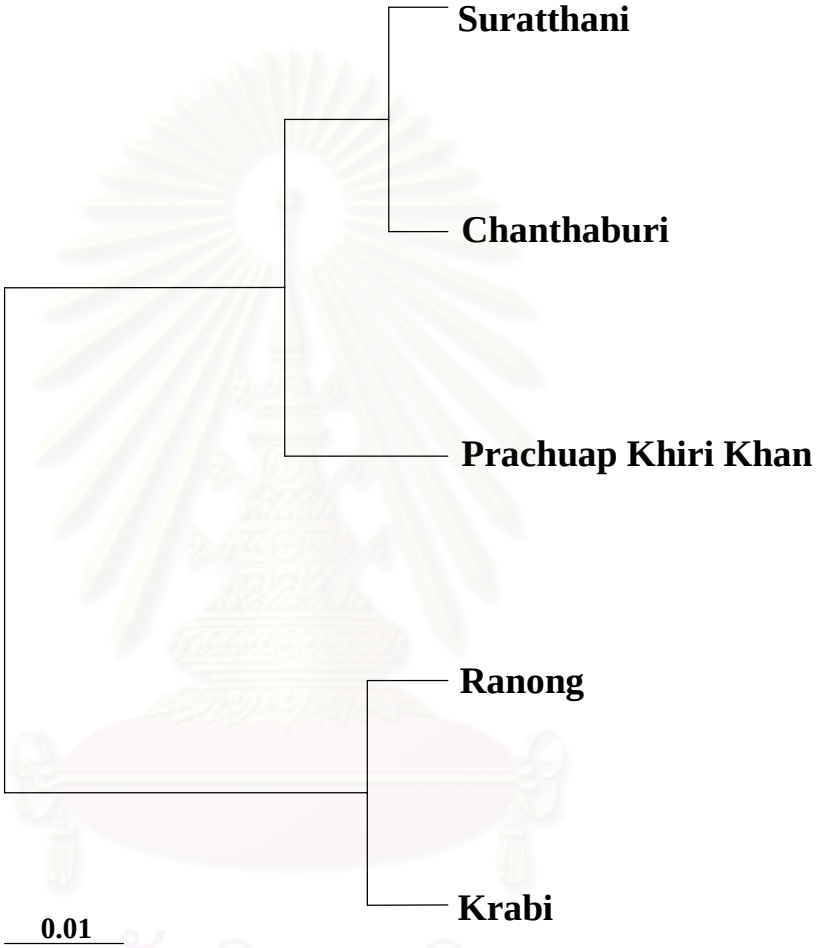

Figure 3.38 A UPGMA dendrogram indicating relationships of *P. pelagicus* in Thai water based on genetic distance between pairs of geographic samples using SSCP analysis

3.2 Development of Species-specific Markers in the Blue Swimming Crab (*Portunus pelagicus*)

The amplified product of 706 bp (*COI*, Figure 3.8*)* and 406 bp (*12S rDNA*, Figure 3.9) was cloned and sequenced for both directions. The nucleotide sequences obtained were searched for the best hit using Blast*N* and they significantly matched *COI* of *P. pelagicus* (E-value = 0.0, Figure 3.39) and *12S rDNA* of *P. pelagicus* (E-value = 0.0, Figure 3.40), respectively.

Sequence-specific primers were designed from the *COI* sequence (PP-COI_{270}-F/R). The specificity of PP-COI_{270}-F/R was tested against genomic DNA of a large sample set of *P. pelagicus*. This primer pair provides the positive amplification product (270 bp, called as *PP-COI_{270}*) in all investigated individuals of the target species without any false negative result (*N* = 174, Figure 3.41).

Cross species amplification of PP-COI_{270}-F/R was further examined against non-target crab species including the mud crabs, *S. oceanica* (*N* = 18), *S. serrata* (*N* = 7), *S. tranquebarica* (*N* = 9), the swimming crab, *C. crucifera* (*N* = 20) and the three-spot swimming crab, *P. sanguinolentus* (*N*= 10). The primer pair did not provide the positive amplification fragment in non-target species (Figure 3.41). Species-specific PCR (presence/absence of the amplification band) was then successfully developed based on *COI* polymorphism.

Although a species-specific PCR was successfully developed for identification the species-origin of *P. pelagicus*, application of the developed *PP-COI_{270}* marker for detection of crab meat in canned products is still limited. Practically, the meat of *P. pelagicus* meat may be mixed with that of a few non-target crab species. Therefore, molecular markers that can be used for amplification of the orthologous gene from various species should be firstly used. Sequence differences between mixed PCR products are then subsequently examined.

12S rDNA of *P. pelagicus* (406 bp) which was successfully amplified in *P. pelagicus* using universal primers (12S rDNA-F/R) was then tested in both target and non-target species (Figure 3.42). The amplification product was observed in all

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Query  27   ACATTATATTTTATTTTTGGAGCATGATCAGGAATAGTAGGGACTTCTCTTAGTCTTATT  86
            ||||||||||||||||||||||||||||||||||||||||||||||||| |||||||| |||
Sbjct  1    ACATTATATTTTATTTTTGGAGCATGATCAGGAATAGTAGGGACTTCACTTAGTCTAATT  60

Query  87   ATTCGAGCAGAACTAGGTCAACCTGGTACTCTTATTGGTAATGACCAAATTTACAATGTT  146
            ||||||||||||||| |||||||||||| |||||||||||||||| |||||||||||| |||
Sbjct  61   ATTCGAGCAGAACTGGGTCAACCTGGCACTCTTATTGGTAATGATCAAATTTACAACGTT  120

Query  147  GTAGTTACAGCTCATGCTTTTGTAATAATTTTCTTTATAGTTATACCAATTATAATTGGG  206
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct  121  GTAGTTACAGCTCATGCTTTTGTAATAATTTTCTTTATAGTTATACCAATTATGATTGGA  180

Query  207  GGATTTGGTAACTGACTAGTACCATTAATGTTAGGAGCCCCTGNACATGGCTTTTCCTCG  266
            |||||||||||||||||||| ||||||||| |||| ||||||| | ||||||||||||||
Sbjct  181  GGATTTGGTAACTGACTAGTTCCATTAATGCTAGGGGCCCCTG-ATATGGCTTTTCCTCG  239

Query  267  TTATAAACAACATAAGATTTTGACTTCTCCCTCCTTCTCTAACTTTACTTCTTATAAGAG  326
            | |||||||||||||||||||||||||||||||||||| ||||| ||||||||||||||||
Sbjct  240  T-ATAAACAACATAAGATTTTGACTTCTCCCTCCTTCTTTAACTCTACTTCTTATAAGAG  298

Query  327  GTATAGTGGAAAGAGGTGTTGGTACAGGTTGAACCGTCTATCCTCCTCTTTCAGCAGCCA  386
            |||| || |||||||||||||||| || |||||||| || |||||||||| ||||| |
Sbjct  299  GTATGGTAGAAAGAGGTGTTGGTACGGGCTGAACCGTATACCCTCCTCTTTCGGCAGCGA  358

Query  387  TCGCTCACGCAGGAGCTTCTGTAGATCTAGGTATTTTCTCTTTACATCTAGCAGGTGTTT  446
            ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  359  TCGCTCATGCAGGAGCTTCTGTAGATCTAGGTATTTTCTCTTTACATCTAGCAGGTGTTT  418

Query  447  CCTCTATTCTAGGTGCAGTAAATTTCATGACCACCGTTATTAACATGCGATCTTTTGGTA  506
            |||||||| |||||||||||||||||| |||||||||||||| |||||||||||||| |
Sbjct  419  CCTCTATTTTAGGTGCAGTAAATTTCATAACCACCGTTATTAATATGCGATCTTTTGGGA  478

Query  507  TAAGAATGGACCAAATGCCATTATTCGTTTGATCAGTATTTATCACTGCTATNCTTCTAC  566
            |||| || |||||||| |||||||||||||||||||| |||||||||||||| ||||| |
Sbjct  479  TAAGGATAGACCAAATACCATTATTCGTTTGATCAGTGTTTATCACTGCTATCCTTCTCC  538

Query  567  TTTTATCTCTCCCGGTNCTGGCTGGAGCTATTACTATACTTCTTACAGACCGAAATCT-A  625
            | |||||||||| || || ||||| |||||||||||||||||||||||||||||||| |
Sbjct  539  TCTTATCTCTCCCTGTTCTTGCTGGGGCTATTACTATACTTCTTACAGACCGAAATCTCA  598

Query  626  ATACTNCATTCTTTGACCCTGCCGGAGGTGGTGACCCTGTACNCTACNA  674
            ||||| | |||||||||||||||||| |||||||||||||||| |||| |
Sbjct  599  ATACTTCGTTCTTTGACCCTGCCGGTGGTGGTGACCCTGTACTCTACCA  647
```

Figure 3.39 Results from similarity search of cloned *COI* using Blast*N*. The nucleotide sequences obtained was significantly match *COI* of *Portunus pelagicus* (E-value = 0.0).

```
Query  26   ACCAAGAAGTAATTATAAATACCTGAGTAGTAACAGCTATGTTCTAAAAATTTGAAAAAT  85
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    ACCAAGAAGTAATTATAAATACCTGAGTAGTAACAGCTATGTTCTAAAAATTTGAAAAAT  60

Query  86   TTGGCGGTGGTTTAGTCTTGTCAGAGGAACCTGTCTTTTAAACGATACACCACGAAATAT  145
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   TTGGCGGTGGTTTAGTCTTGTCAGAGGAACCTGTCTTTTAAACGATACACCACGAAATAT  120

Query  146  CTTACTTGAGTTTGTATAGTATGTATACCATCATTATTAGGTAATTTTTATAGAATAAAT  205
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  CTTACTTGAGTTTGTATAGTATGTATACCATCATTATTAGGTAATTTTTATAGAATAAAT  180

Query  206  TACTGGAAAGTTTCGATAATGTTGAATATATTAGATCAAGGTGCAGCTGATACTCAAGTT  265
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  TACTGGAAAGTTTCGATAATGTTGAATATATTAGATCAAGGTGCAGCTGATACTCAAGTT  240

Query  266  AAGGTGGGTTACAATAGTATTTACGCTATTACGGATAAGTAAATGAAACTTTACTTTGAA  325
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  AAGGTGGGTTACAATAGTATTTACGCTATTACGGATAAGTAAATGAAACTTTACTTTGAA  300

Query  326  GGAGGATTTGATTGTAAATTTAGTTTAATAAGCTAATTTGATATAAGCTCTAAAGCATGT  385
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  GGAGGATTTGATTGTAAATTTAGTTTAATAAGCTAATTTGATATAAGCTCTAAAGCATGT  360
```

Figure 3.40 Results from similarity search of cloned *12S rDNA* using Blast*N*. The nucleotide sequences obtained was significantly match *12S rDNA* of *Portunus pelagicus* (E-value = 0.0).

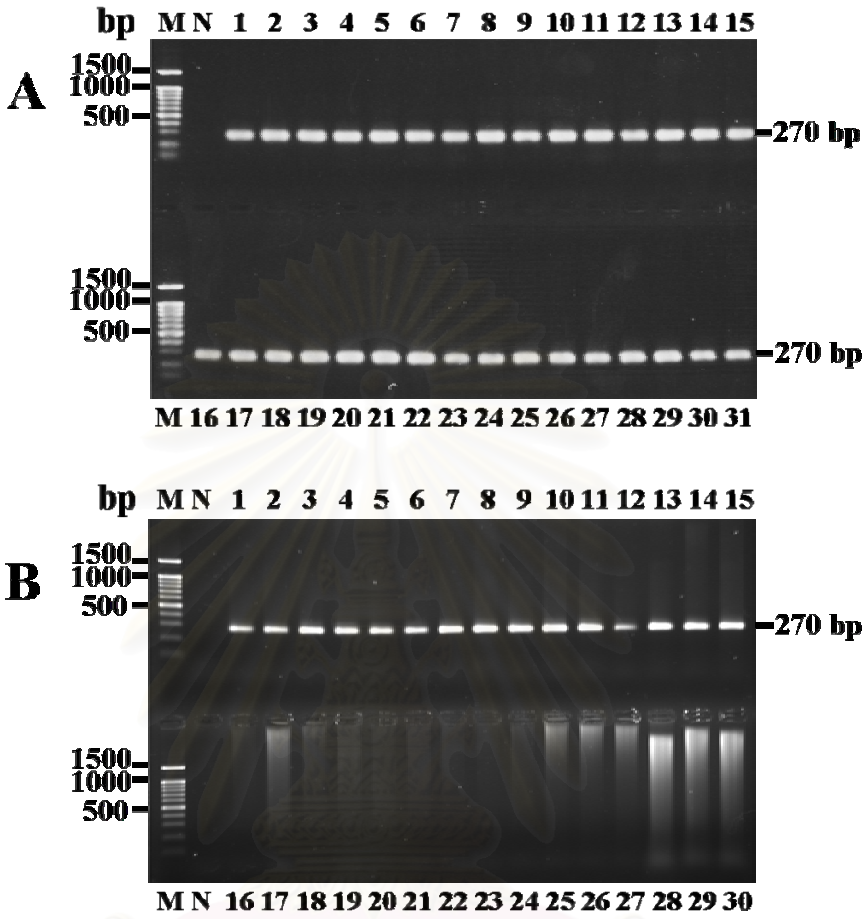

Figure 3.41 A 1.5% ethidium bromide stained agarose gel showing results from the primer specificity test by amplification of genomic DNA of *P. pelagicus* (lanes 1-31, panel A and lanes 1-15, panel B), *S. oceanica* (lanes 16-18, panel B), *S. serrata* (lanes 19-21, panel B), *S. transquebarica* (lanes 22-24, panel B), *C. crucifera* (lanes 25-27, panel B) and *P. sanguinolentus* (lanes 28-30, panel B) with PP-COI_{270}-F/R. Lane M and N are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.

examined species as required. SSCP analysis clearly distinguished non-target species from *P. pelagicus* (Figure 3.43). Nevertheless, the amplification success in *P. pelagicus* from different geographic locations varies greatly (57.1-100%, Table 3.9). As a result, false negative results may be seriously occurred.

Apparently, the *12S rDNA* gene segment fulfilled the requirement on the ability to amplify the orthologous gene from all investigated species. To resolve problems about inconsistent amplification, a new pair of primers was designed (Figure 3.9) and tested. Results indicated 100% amplification success in *P. pelagicus* and all non-target species (Figure 3.44).

SSCP analysis, which is favored for identifying species origins of various taxa due to their convenience and cost-effectiveness, was then applied to verify whether nucleotide sequences of *PP-12S*$_{312}$ in *P. pelagicus* and other crab species were different. The amplified *12S rDNA* of *P. pelagicus* (*PP-12S*$_{312}$) and other species; *SO-12S rDNA* (*N* = 18), *SS-12S rDNA* (*N* = 7), *ST-12S rDNA* (*N* = 9), *CC-12S rDNA* (*N* = 20)and *PT-12S rDNA* (*N* = 10) was analyzed.

Five SSCP genotypes were found in *P. pelagicus*. The SSCP genotype I is the most common genotype found in 159 individuals (accounting for 91.4%) of overall *P. pelagicus* specimens. Non-overlapping SSCP genotypes were found in other species as each non-target species exhibited a fixed SSCP genotype. SSCP patterns of *12S rDNA* were clearly observed and unambiguously differentiated these species consistently (Figure 3.45 and Table 3.11).

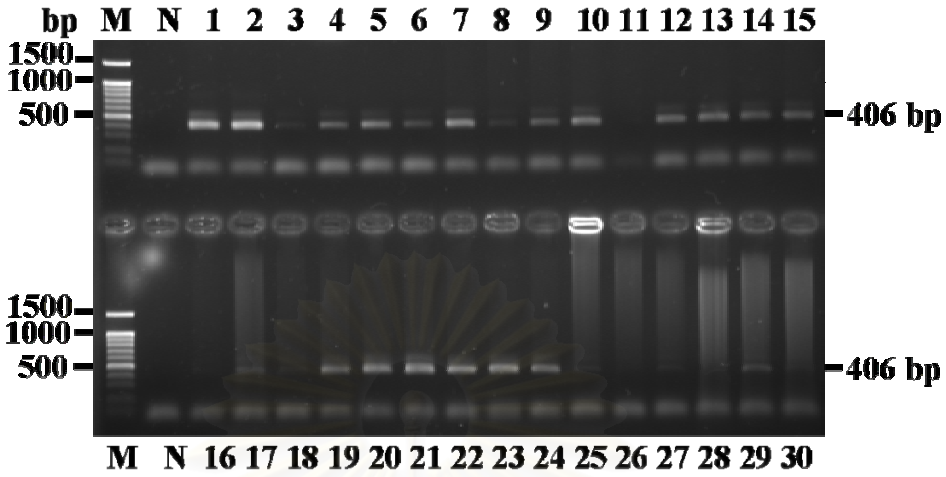

Figure 3.42 Agarose gel electrophoresis showing results from amplification of genomic DNA of *P. pelagicus* (lanes 1 - 15), *S. oceanica* (lanes 16-18), *S. serrata* (lanes 19-21), *S. tranquebarica* (lanes 22-24), *C. crucifera* (lanes 25-27) and *P. sanguinolentus* (lanes 28-30) with 12S rDNA-F/R. Lanes M and N are a 100 bp DNA marker and the negative control (without DNA template), respectively.

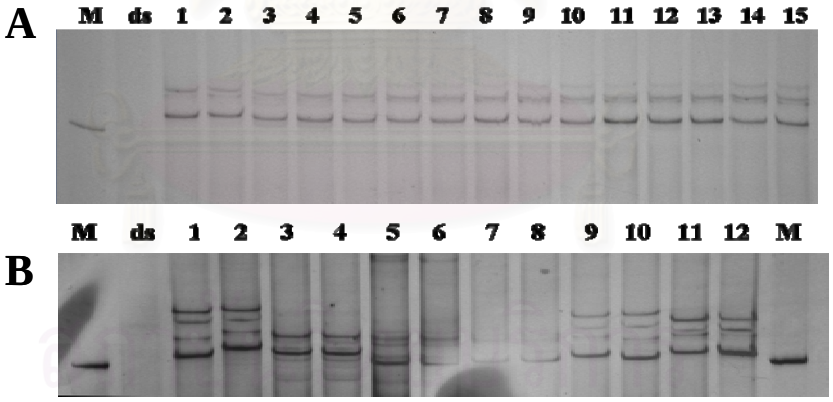

Figure 3.43 SSCP patterns of *12S rDNA* of *P. pelagicus* (lanes 1–15, panel A and lanes 1-2, panel B), *S. oceanica* (lanes 3-4, panel B), *S. serrata* (lanes 5-6, panel B), *S. tranquebarica* (lanes 7-8, panel B), *P. sanguinolentus* (lanes 11-12, panel B) and *C. crucifera* (lanes 9-10, panel B) electrophoretically analyzed through 12.5% non-denaturing polyacrylamide gel (300 V at 4ºC for 15 hr) and silver stained. Lanes M are a 100 bp DNA marker. The non-denatured PCR product was included as the double strand (ds) control.

Table 3.10 Amplification result and SSCP analysis of 12S rDNA of various crab species

Species	Positive amplification	Amplification success (%)	Analyzed by SSCP
Blue swimming crab; *P. pelagicus*			
Chantaburi (*N* = 29)	29	100.0	26
Prachup Khiri Khan (*N* = 40)	33	82.5	0
Suratthani (*N* = 35)	27	77.1	16
Ranong (*N* = 35)	20	57.1	18
Krabi (*N* = 35)	26	74.3	16
Mud crabs			
S. oceanica (*N* = 18)	5	27.8	2
S. serrata (*N* = 7)	5	71.4	2
S. tranquebarica (*N* = 9)	7	77.8	2
Swimming crab;			
C. crucifera (*N* = 20)	10	50.0	2
Three spot swimming crab;			
P. sanguinolentus (*N* = 10)	5	50.0	2

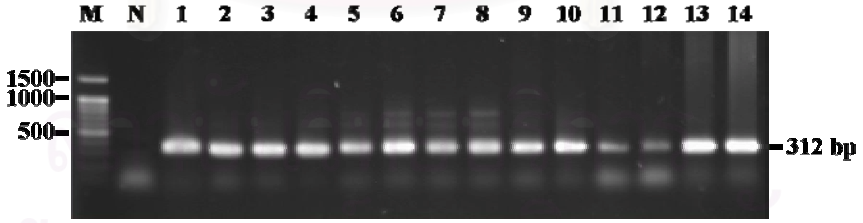

Figure 3.44 Agarose gel electrophoresis showing results from amplification of genomic DNA of *P. pelagicus* (lanes 1 - 4), *S. oceanica* (lanes 5 - 6), *S. serrata* (lanes 7 - 8), *S. tranquebarica* (lane 9 - 10), *C. crucifera* (lanes 11 - 12) and *P. sanguinolentus* (lanes 13 - 14) with PP-12S_{312}-F/R. Lanes M and N are a 100 bp DNA marker and the negative control (without DNA template), respectively.

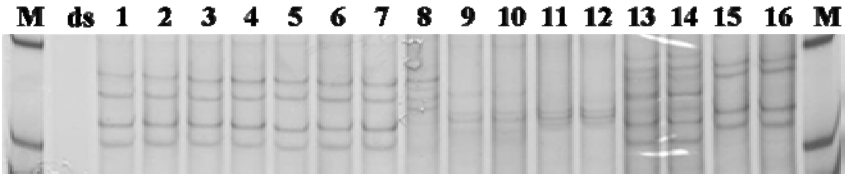

Figure 3.45 SSCP pattern of *PP-12S*$_{312}$ of *P. pelagicus* (lanes 1 - 7) and *12S rDNA* of *S. oceanica* (lane 8), *S. serrata* (lanes 9 - 10), *S. tranquebarica* (lanes 11 - 12), *P. sanguinolentus* (lanes 13 - 14) and *C. crucifera* (lanes 15 - 16) electrophoretically analyzed through a 15% non-denaturing polyacrylamide gel (300 V at 4ºC for 13.5 hr) and silver stained. The non-denatured PCR product was included as the double strand (ds) control.

Table 3.11 A summary of amplification results and SSCP analysis of primers designed from *PP-COI*$_{270}$, *12S rDNA* and *PP-12S*$_{312}$ and specificity-tested against genomic DNA of *P. pelagicus* and other non-target crab species

Marker	Primer name	Amplification results	SSCP analysis
PP-COI$_{270}$	PP-COI$_{270}$-F/R	100% amplification success in *P. pelagicus* without any false positive in non-target species	-
12S rDNA	12S-F1/R1	77.6% in *P. pelagicus* and cross-amplified in 27.8 – 77.8% of non-target species	Non-overlapping patterns between all investigated species
PP-12S$_{312}$	PP-12S$_{312}$-F/R	100% amplification success in all species	Non-overlapping patterns between all investigated species

To verify the accuracy of SSCP analysis, *PP-12S*$_{312}$ amplified from an individual of *P. pelagicus* exhibiting each SSCP pattern of *PP-12S*$_{312}$ and *12S rDNA* of a representative individual of each non-target species were cloned and sequenced. Results indicated that five different SSCP genotypes were different by one or a few nucleotide polymorphism (Figures 3.46). This suggested the accuracy of SSCP

analysis of *PP-12S*$_{312}$ for genotyping of *P. pelagicus*. Larger genetic variation between crab species was observed based on polymorphism of *12S rDNA*.

Nucleotide sequence divergence within *P. pelagicus* was quite low (0.32-1.29%). Three mud crab species were closely related reflected by relatively low interspecific nucleotide divergence (2.85-6.99%). Larger nucleotide divergence was clearly observed between *P. pelagicus* and mud crabs (15.48-16.65%) and between *P. pelagicus* and *C. crucifera* (17.70 - 18.11%), respectively.

```
PP-12S312_1     TTGGCGGTGGTTTAGTCTTGTCAGAGGAACCTGTCTTTTAAACGATACACCACGAAATAT
PP-12S312_2     TTGGCGGTGGTTTAGTCTTGTCAGAGGAACCTGTCTTTTAAACGATACACCACGAAATAT
PP-12S312_4     TTGGCGGTGGTTTAGTCTTGTCAGAGGAACCTGTCTTTTAAACGATACACCACGAAATAT
PP-12S312_5     TTGGCGGTGGTTTAGTCTTGTCAGAGGAACCTGTCTTTTAAACGATACACCACGAAATAT
PP-12S312_3     TTGGCGGTGGTTTAGTCTTGTCAGAGGAACCTGTCTTTTAAACGATACACCACGAAATAT
                ************************************************************60

PP-12S312_1     CTTACTTGAGTTTGTATAGTATGTATACCATCATTATTAGGTAATTTTTATAGAATAAAT
PP-12S312_2     CTTACTTGAGTTTGTATAGTATGTATACCATCATTATTAGGTAATTTTTATAGAATAAAT
PP-12S312_4     CTTACTTGAGTTTGTATAGTATGTATACCATCATTATTAGGTAATTTTTATAGGATAAAT
PP-12S312_5     CTTACTTGAGTTTGTATAGTATGTATACCATCATTATTAGGTAATTTTTATAGAATAAAT
PP-12S312_3     CTTACTTGAGTTTGTATAGTATGTATACCATCATTATTAGGTAATTTTTATAGAATAAAT
                ************************************************** ******120

PP-12S312_1     TACTGGAAAGTTTCGATAATGTTGAATATATTAGATCAAGGTGCAGCTGATACTCAAGTT
PP-12S312_2     TACTGGAAAGTTTCGATAATGTTGAATATATTAGATCAAGGTGCAGCTGATACTCAAGTT
PP-12S312_4     TACTGGAAAGTTTCGATAATGTTGAATATATTAGATCAAGGTGCAGCTGATACTCAAGTT
PP-12S312_5     TACTGGAAAGTTTCGATAACGTTGAATATATTAGATCAAGGTGCAGCTGATATTCAAGTT
PP-12S312_3     TACTGGAAAGTTCCGATAATGTTGAATACATTAGATCAAGGTGCAGCTGATACTCAAGTT
                ************ ****** ******** ********************** *******180

PP-12S312_1     AAGGTGGGTTACAATAGTATTTACGCTATTACGGATAAGTAAATGAAACTTTACTTTGAA
PP-12S312_2     AAGGTGGGTTACAATAGTATTTACGCTATTATGGATAAGTAAATGAAACTTTACTTTGAA
PP-12S312_4     AAGGTGGGTTACAATAGTATTTACGCTATTACGGATAAGTAAATGAAACTTTACTTTGAA
PP-12S312_5     AAGGTGGGTTACAATAGTATTTACGCTATTACGGATAAGTAAATGAAACTTTACTTTGAA
PP-12S312_3     AAGGTGGGTTACAATAGTATTTACGCTATTACGGATAAGTAAATGAAACTTTACTTTGAA
                ****************************** ****************************240

PP-12S312_1     GGAGGATTTGATTGTAAATTTAGTTTAATAAGCTAATTTGATATAAGCTCTAAAGCATGT
PP-12S312_2     GGAGGATTTGATTGTAAATTTAGTTTAATAAGCTAATTTGATATAAGCTCTAAAGCATGT
PP-12S312_4     GGAGGATTTGATTGTAAATTTAGTTTAATAAGCTAATTTGATATAAGCTCTAAAGCATGT
PP-12S312_5     GGAGGATTTGATTGTAAATTTAGTTTAATAAGCTAATTTGATATAAGCTCTAAAGCATGT
PP-12S312_3     GGAGGATTTGATTGTAAATTTAGTTTAATAAGCTAATTTGATATAAGCTCTAAAGCATGT
                ************************************************************300

PP-12S312_1     ACACATCGCCCG
PP-12S312_2     ACACATCGCCCG
PP-12S312_4     ACACATCGCCCG
PP-12S312_5     ACACATCGCCCG
PP-12S312_3     ACACATCGCCCG
                ************312
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Figure 3.46 Multiple alignments of 5 patterns of *PP-12S*$_{312}$. The location and sequence of a forward primer (PP-12S$_{312}$-F) and those complementary to a reverse primer (PP-12S$_{312}$-R) are underlined.

```
PP-12S312_2       TTGGCGGTGGTTTAGTCTTGTCAGAGGAACCTGTCTTTTAAA-CGATACACCACGAAATA
PP-12S312_4       TTGGCGGTGGTTTAGTCTTGTCAGAGGAACCTGTCTTTTAAA-CGATACACCACGAAATA
PP-12S312_1       TTGGCGGTGGTTTAGTCTTGTCAGAGGAACCTGTCTTTTAAA-CGATACACCACGAAATA
PP-12S312_3       TTGGCGGTGGTTTAGTCTTGTCAGAGGAACCTGTCTTTTAAA-CGATACACCACGAAATA
PP-12S312_5       TTGGCGGTGGTTTAGTCTTGTCAGAGGAACCTGTCTTTTAAA-CGATACACCACGAAATA
PS-12SrDNA        TTGGCGGTGGTTTAGTCTTGTCAGAGGAACCTGTCTTTTAAA-CGATACACCACGAAATA
SO-12SrDNA        TTGGCGGTGGTTTAGTCTTGTCAGAGGAACCTGGTTTTTAAATCGATACACCACGAAAAA
ST-12SrDNA        TTGGCGGTGGTTTAGTCTTGTCAGAGGAACCTGGTTTTTAAATCGATACACCACGAAGAA
SS-12SrDNA        TTGGCGGTGGTTTAGTCTTGTCAGAGGAACCTG-TTTTTGAATCGATACACCACCAAGAA
CC-12SrDNA        TTGGCGGTGGTTTAGTCTTGTCAGAGGAACCTG-TTTTTGAATCGATAAACCACGAATAA
                  ********************************  **** ** ***** ***** **  *60

PP-12S312_2       TCTTACTTGAGTTTGTAT---AGTATGTATACCATCATTATTAGGTAATTTTTATAGAAT
PP-12S312_4       TCTTACTTGAGTTTGTAT---AGTATGTATACCATCATTATTAGGTAATTTTTATAGGAT
PP-12S312_1       TCTTACTTGAGTTTGTAT---AGTATGTATACCATCATTATTAGGTAATTTTTATAGAAT
PP-12S312_3       TCTTACTTGAGTTTGTAT---AGTATGTATACCATCATTATTAGGTAATTTTTATAGAAT
PP-12S312_5       TCTTACTTGAGTTTGTAT---AGTATGTATACCATCATTATTAGGTAATTTTTATAGAAT
PS-12SrDNA        TCTTACTTAAGTTAGTAG---AGTATGTATACCATCATTATTAGGTAATTTTTATAGAAT
SO-12SrDNA        TCTTACTTAGGCTTGTAGAGTAGTATGTATACCATCATTATTAGGTAATTTTTATAGAAT
ST-12SrDNA        TCTTACTTAAACTTGTAAAGTAGTATGTATACCATCATTATTAGGTAATTTTTATAGAAT
SS-12SrDNA        TCTTACTTAAGCTTGTTA--TAGTATGTATACCATCATTATTAGGTCATTTTTATAGAAT
CC-12SrDNA        TCTTGCTCAATTTTGTAA---AGTATGTATACCATCATTATTAGGTAATTTTTATAGAAT
                  **** **     * **     ************************ ********** **120

PP-12S312_2       AAATTACTGGAA---AGTT-TCGATAATGTTGAATATATTAGATCAAGGTGCAGCTGATA
PP-12S312_4       AAATTACTGGAA---AGTT-TCGATAATGTTGAATATATTAGATCAAGGTGCAGCTGATA
PP-12S312_1       AAATTACTGGAA---AGTT-TCGATAATGTTGAATATATTAGATCAAGGTGCAGCTGATA
PP-12S312_3       AAATTACTGGAA---AGTT-CCGATAATGTTGAATACATTAGATCAAGGTGCAGCTGATA
PP-12S312_5       AAATTACTGGAA---AGTT-TCGATAACGTTGAATATATTAGATCAAGGTGCAGCTGATA
PS-12SrDNA        AAATTACTGTAT---TACGATTAATAATGTTAAATATATTAGATCAAGGTGCAGCTTATA
SO-12SrDNA        AAATTACTAAAA-ATAATTATTAATAGTGTTAAATATATTAGATCAAGGTGCAGCTAATG
ST-12SrDNA        AAATTACTGAAAGATAGTTATTAATAGTGTTAAATATATTAGATCAAGGTGCAGCTAATG
SS-12SrDNA        AAATTACTGTA---TATTTGTTAATAACGTTAAATATATTAGATCAAGGTGCAGCTAATG
CC-12SrDNA        AAATTACTGAA-----TTTGCTAATAATGTTAAATATATTAGATCAAGGTGCAGCTTATA
                  ********  *           ***  *** **** ********************** ** 180

PP-12S312_2       ----CTCAAGTTAAGGTGGGTTACAATAGTATTTACGCTATTATGGATAAGTAAATGAAA
PP-12S312_4       ----CTCAAGTTAAGGTGGGTTACAATAGTATTTACGCTATTACGGATAAGTAAATGAAA
PP-12S312_1       ----CTCAAGTTAAGGTGGGTTACAATAGTATTTACGCTATTACGGATAAGTAAATGAAA
PP-12S312_3       ----CTCAAGTTAAGGTGGGTTACAATAGTATTTACGCTATTACGGATAAGTAAATGAAA
PP-12S312_5       ----TTCAAGTTAAGGTGGGTTACAATAGTATTTACGCTATTACGGATAAGTAAATGAAA
PS-12SrDNA        ----CTTAAGTCAAGATGGGTTACAATAGAATTTATGCTAT-ACGAATGAGTAACTGAAA
SO-12SrDNA        ----CTTAAGTTAAAATGGGTTACAATAAAAATTTATTTAATACGGAATATTAATTTAAA
ST-12SrDNA        ----TTTAAGTTAAAATGGGTTACAATAAAAATTTATTTAATACGGAATATTAATTTAAA
SS-12SrDNA        AATGCTCAAGTTAAAATGGGTTACAATAAAA-TTCATTTAATACGGAATATTAATTTAAA
CC-12SrDNA        ----GTTGAGTTAAAATGGGTTACAATAAGT-TTTATTTATCACGGAGATATAGTTTAAA
                       *  *** **  ***********   **    **  * * *    **  * ***240

PP-12S312_2       CTTT-ACTTTGAAGGAGGATTTGATTGTAAATTTAGTTTAATAAGCT-AATTTGATATAA
PP-12S312_4       CTTT-ACTTTGAAGGAGGATTTGATTGTAAATTTAGTTTAATAAGCT-AATTTGATATAA
PP-12S312_1       CTTT-ACTTTGAAGGAGGATTTGATTGTAAATTTAGTTTAATAAGCT-AATTTGATATAA
PP-12S312_3       CTTT-ACTTTGAAGGAGGATTTGATTGTAAATTTAGTTTAATAAGCT-AATTTGATATAA
PP-12S312_5       CTTT-ACTTTGAAGGAGGATTTGATTGTAAATTTAGTTTAATAAGCT-AATTTGATATAA
PS-12SrDNA        TTGTGACTCGGAAGGAGGATTTGATTGTAAATCTAGTTTAATAAGCT-AGTTAGATATAA
SO-12SrDNA        TAATTAATTAGAAGGAGGATTTGATTGTAAATTAAATTTAATATGTTTAGTTAGATATAA
ST-12SrDNA        TAGTTAATTAGAAGGAGGATTTGATTGTAAATTAAATTTAATATGTTTAATTAGATATAA
SS-12SrDNA        TAGTTAATTAGGAGGAGGATTTGATTGTAAAATAAGTTTAATAAGCTTAGTTAGATATAA
CC-12SrDNA        CACTAGTTATGAAGGAGGATTTGATTGTAAGATAAGTTTAACACGCTT-GTTAGATATAA
                     *  *  * ******************   * ***** * * *   ** *******300

PP-12S312_2       GCTCTAAAGCATGTACACATCGCCCG
PP-12S312_4       GCTCTAAAGCATGTACACATCGCCCG
PP-12S312_1       GCTCTAAAGCATGTACACATCGCCCG
PP-12S312_3       GCTCTAAAGCATGTACACATCGCCCG
PP-12S312_5       GCTCTAAAGCATGTACACATCGCCCG
PS-12SrDNA        GCTCTAAAGCATGTACACATCGCCCG
SO-12SrDNA        GCTCTAAAGCATGTACACATCGCCCG
ST-12SrDNA        GCTCTAAAGCATGTACACATCGCCCG
SS-12SrDNA        GCTCTAAAGCATGTACACATCGCCCG
CC-12SrDNA        GCTCTAAAGCATGTACACATCGCCCG
                  *************************326
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Figure 3.47 Multiple alignments of *PP-12S*$_{312}$ of *P. pelagicus* and *12S rDNA* of *S. oceanica* (SO), *S. serrata* (SS), *S. tranquebarica* (ST), *P. sanguinolentus* (PS) and *C. crucifera* (CC). The location and sequence of a forward primer (PP-12S$_{312}$-F) and those complementary to a reverse primer (PP-12S$_{312}$-R) are underlined.

Table 3.12 The percentage of nucleotide sequence divergence between *12S rDNA* of *P. pelagicus*, mud crabs (*S. oceanica*, *S. serrata* and *S. tranquebarica*), *P. sanguinolentus* and *C. crucifera*

	PP-I	**PP-II**	**PP-III**	**PP-IV**	**PP-V**	**PS**	**SO**	**ST**	**SS**	**CC**
PP-I	-									
PP-II	0.32	-								
PP-III	0.64	0.97	-							
PP-IV	0.32	0.64	0.97	-						
PP-V	0.64	0.97	1.29	0.97	-					
PS	9.65	10.01	10.38	10.01	10.38	-				
SO	15.87	16.26	16.65	16.26	16.65	14.24	-			
ST	15.48	15.87	15.48	15.87	16.26	15.39	2.85	-		
SS	15.68	16.08	15.68	16.08	16.48	15.14	6.99	6.30	-	
CC	17.70	18.11	18.11	18.11	17.70	18.07	14.64	13.87	13.59	-

Abbreviations: PP-I = *P. pelagicus* showing SSCP pattern I, PP-II = *P. pelagicus* showing pattern II, PP-III = *P. pelagicus* showing pattern III, PP-IV = *P. pelagicus* showing pattern IV, PP-V = *P. pelagicus* pattern V, SO = *S. oceanica*, SS = *S. serrata*, ST = *S. tranquebarica*, PS = *P. sanguinolentus* and CC = *C. crucifer*

3.3 Isolation and characterization temperature-stress response transcripts (*TSRT*) in haemocytes of *P. pelagicus* using cDNA-AFLP

3.3.1. RNA extraction and first strand synthesis

Total RNA from haemocytes of *P. pelagicus* collected at different time intervals after temperature stress revealed several discrete bands along with smear high and low molecular weight RNA (Figure 3.48). The ratios of extracted RNA were 1.9-2.1. The first strand cDNA was reverse-transcribed using by BD Powerscript™ Reverse Transcriptase (Clontech). Agarose gel electrophoresis indicated that the resulting products covered the products between 100 bp - >2 kb indicating the acceptable quality of the synthesized first stand cDNA (Figure 3.49).

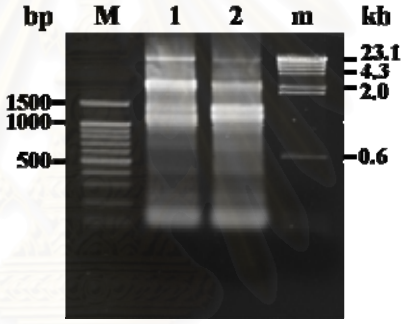

Figure 3.48 A 1.0% ethidium bromide-stained agarose gel showing the quality of total RNA extracted from haemocytes of different individuals of *P. pelagicus* (lanes 1 and 2). Lane M and m are a 100 bp DNA ladder and λ-*Hin*d III, respectively.

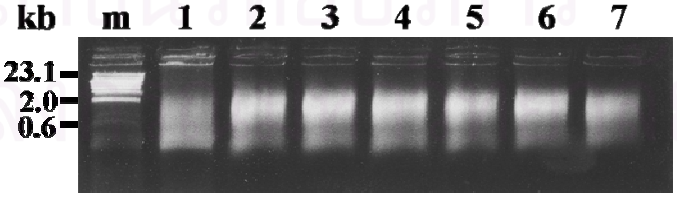

Figure 3.49 A 1.0% ethidium bromide-stained agarose gel showing the first strand cDNA reverse-transcribed from haemocyte total RNA of different *P. pelagicus* individuals (Lanes 1-7) using Powerscript™ Reverse Transcriptase. Lane m is the λ-*Hin*d III DNA marker.

The second strand cDNA was synthesized by long distance PCR. Initially, the synthesis was carried out for 8, 10 and 12 cycles (Figure 3.50). After electrophoresis, the amplification products from 8 and 10 amplification cycles were still not saturated. Accordingly, the second strand synthesis of all specimens was performed for 9 cycles.

The preamplification reactions with one selective nucleotide yielded smear amplification products with the molecular length up to 1000 bp but the size range of the major products was 300 - 600 bp in size (Figure 3.51). This indicated successful restriction/ligation of cDNA of *P. pelagicus*. The preamplification products were diluted and subjected to selective amplification with a pair of three selective nucleotides. The amplification results were preliminary analyzed by agarose gel electrophoresis.

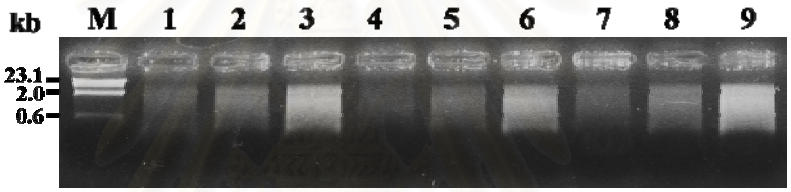

Figure 3.50 A 1.0% ethidium bromide-stained agarose gel showing the synthesized second strand cDNA of haemocytes of *P. pelagicus* by LD-PCR using Advantage2 DNA polymerase against the first strand cDNA of normal haemocytes for 8 cycles(lanes 1, 4 and 7), that of haemocytes of *P. pelagicus* at 6 hpt for 10 cycles (lanes 2, 5 and 8) and that of *P. pelagicus* haemocytes at 24 hpt for 12 cycles (lanes 3, 6 and 9). Lane M is the λ-*Hin*d III DNA marker.

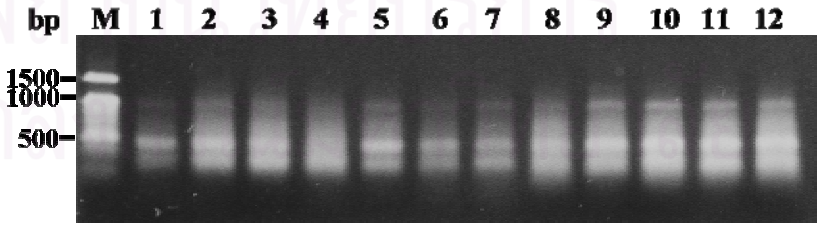

Figure 3.51 A 1.0% agarose gel electrophoresis showing preamplification products of haemocyte cDNA of male (lanes 1, 3, 5, 7, 9 and 11) and female (lanes 2, 4, 6, 8, 10 and 12) *P. pelagicus* of the control group (lanes 1-2), 0 (lanes 3-4), 3 (lanes 5-6), 6 (7-8), 12 (lanes 9-10 and 24 (lane 11 and 12) hpt. Lane M is a 100 bp DNA ladder.

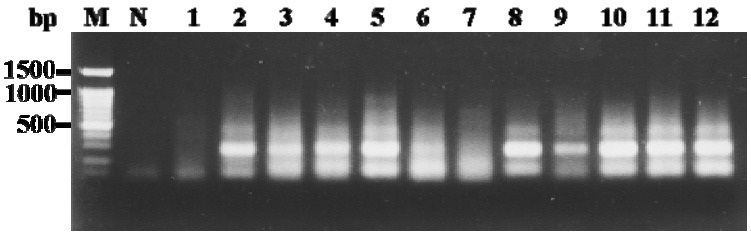

Figure 3.52 A 1.5% agarose gel electrophoresis showing selective amplification products of E_{+3}-2/ M_{+3}-5 against cDNA of male (lanes 1, 3, 5, 7, 9 and 11) and female (lanes 2, 4, 6, 8, 10 and 12) *P. pelagicus* of the control group (lanes 1-2), 0 (lanes 3-4), 3 (lanes 5-6), 6 (7-8), 12 (lanes 9-10) and 24 (lanes 11-12) hpt. Lanes M and N are a 100 bp DNA ladder and the negative control (without DNA template), respectively.

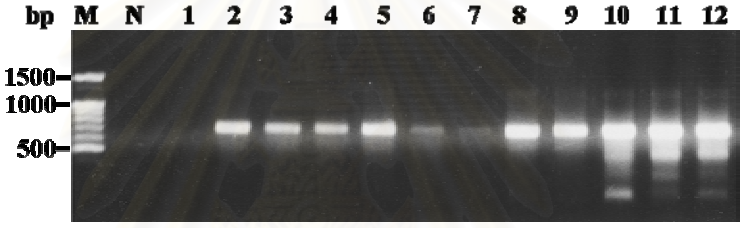

Figure 3.53 A 1.5% agarose gel electrophoresis showing selective amplification products of E_{+3}-2/ M_{+3}-7 against cDNA of male (lanes 1, 3, 5, 7, 9 and 11) and female (lanes 2, 4, 6, 8, 10 and 12) *P. pelagicus* of the control group (lanes 1-2), 0 (lanes 3-4), 3 (lanes 5-6), 6 (7-8), 12 (lanes 9-10) and 24 (lanes 11-12) hpt. Lanes M and N are a 100 bp DNA ladder and the negative control (without DNA template), respectively.

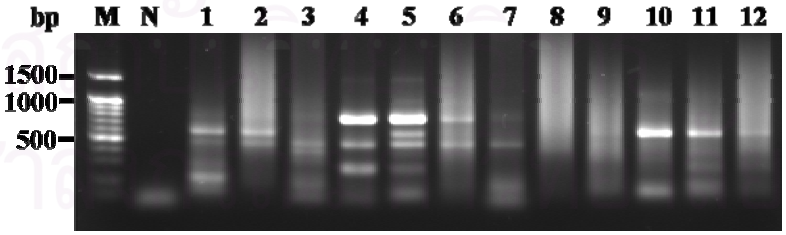

Figure 3.54 A 1.5% agarose gel electrophoresis showing selective amplification products of E_{+3}-5/ M_{+3}-9 against cDNA of male (lanes 1, 3, 5, 7, 9 and 11) and female (lanes 2, 4, 6, 8, 10 and 12) *P. pelagicus* of the control group (lanes 1-2), 0 (lanes 3-4), 3 (lanes 5-6), 6 (7-8), 12 (lanes 9-10) and 24 (lanes 11-12) hpt. Lanes M and N are a 100 bp DNA ladder and the negative control (without DNA template), respectively.

Table 3.13 Amplification results from screening cDNA-AFLP primer combinations

Primer	Result	Primer	Result
E_{+3}-1/ M_{+3}-1	Smear pattern < 400 bp	E_{+3}-3/ M_{+3}-1	+
E_{+3}-1/ M_{+3}-2	Smear pattern < 400 bp	E_{+3}-3/ M_{+3}-2	+
E_{+3}-1/ M_{+3}-3	Smear pattern < 400 bp	E_{+3}-3/ M_{+3}-3	+
E_{+3}-1/ M_{+3}-4	Smear pattern < 400 bp	**E_{+3}-3/ M_{+3}-4***	++
E_{+3}-1/ M_{+3}-5	Smear pattern < 300 bp	E_{+3}-3/ M_{+3}-5	+
E_{+3}-1/ M_{+3}-6	Smear pattern < 300 bp	E_{+3}-3/ M_{+3}-6	Smear pattern < 300 bp
E_{+3}-1/ M_{+3}-7	Smear pattern < 200 bp	**E_{+3}-3/ M_{+3}-7***	+++
E_{+3}-1/ M_{+3}-8	Smear pattern < 200 bp	E_{+3}-3/ M_{+3}-8	Smear pattern < 300 bp
E_{+3}-1/ M_{+3}-9	Smear pattern < 300 bp	E_{+3}-3/ M_{+3}-9	Smear pattern < 500 bp
E_{+3}-1/ M_{+3}-10	Smear pattern < 200 bp	E_{+3}-3/ M_{+3}-10	Smear pattern
E_{+3}-1/ M_{+3}-11	Smear pattern < 200 bp	E_{+3}-3/ M_{+3}-11	Smear pattern
E_{+3}-1/ M_{+3}-12	Smear pattern < 400 bp	E_{+3}-3/ M_{+3}-12	Smear pattern
E_{+3}-1/ M_{+3}-13	Smear pattern < 400 bp	E_{+3}-3/ M_{+3}-13	Smear pattern
E_{+3}-1/ M_{+3}-14*	+++	E_{+3}-3/ M_{+3}-14	Smear pattern
E_{+3}-1/ M_{+3}-15*	+++	E_{+3}-3/ M_{+3}-15	Smear pattern
E_{+3}-1/ M_{+3}-16	Smear pattern < 400 bp	E_{+3}-3/ M_{+3}-16	Smear pattern
E_{+3}-2/ M_{+3}-1	Smear pattern < 500 bp	**E_{+3}-4/ M_{+3}-1***	+++
E_{+3}-2/ M_{+3}-2	Smear pattern < 500 bp	E_{+3}-4/ M_{+3}-2	Smear pattern
E_{+3}-2/ M_{+3}-3*	+++	E_{+3}-4/ M_{+3}-3	Smear pattern < 500 bp
E_{+3}-2/ M_{+3}-4	Smear pattern < 500 bp	E_{+3}-4/ M_{+3}-4	Specific band at 700 bp
E_{+3}-2/ M_{+3}-5*	+++	E_{+3}-4/ M_{+3}-5	Smear pattern
E_{+3}-2/ M_{+3}-6	Smear pattern < 500 bp	E_{+3}-4/ M_{+3}-6	Smear pattern < 500 bp
E_{+3}-2/ M_{+3}-7*	+++	E_{+3}-4/ M_{+3}-7	Smear pattern < 200 bp
E_{+3}-2/ M_{+3}-8	+	E_{+3}-4/ M_{+3}-8	Smear pattern < 200 bp
E_{+3}-2/ M_{+3}-9*	++	E_{+3}-4/ M_{+3}-9	+
E_{+3}-2/ M_{+3}-10	Smear pattern < 300 bp	E_{+3}-4/ M_{+3}-10	Smear pattern < 500 bp
E_{+3}-2/ M_{+3}-11	Smear pattern < 300 bp	E_{+3}-4/ M_{+3}-11	Smear pattern < 500 bp
E_{+3}-2/ M_{+3}-12*	++	E_{+3}-4/ M_{+3}-12	Smear pattern < 500 bp
E_{+3}-2/ M_{+3}-13*	++	**E_{+3}-4/ M_{+3}-13***	+++
E_{+3}-2/ M_{+3}-14	Smear pattern < 300 bp	E_{+3}-4/ M_{+3}-14	Smear pattern < 500 bp
E_{+3}-2/ M_{+3}-15	Smear pattern < 500 bp	E_{+3}-4/ M_{+3}-15	+
E_{+3}-2/ M_{+3}-16*	++	E_{+3}-4/ M_{+3}-16	Smear pattern < 300 bp

Table 3.13 (cont.)

Primer	Result	Primer	Result
E_{+3}-5/ M_{+3}-1*	++	E_{+3}-6/ M_{+3}-8	Smear pattern
E_{+3}-5/ M_{+3}-2	Smear pattern < 500 bp	E_{+3}-6/ M_{+3}-9	Smear pattern
E_{+3}-5/ M_{+3}-3	Specific band at 600 bp	E_{+3}-6/ M_{+3}-10	Smear pattern
E_{+3}-5/ M_{+3}-4	Specific band at 700 bp	E_{+3}-6/ M_{+3}-11	Smear pattern
E_{+3}-5/ M_{+3}-5	Smear pattern < 200 bp	E_{+3}-6/ M_{+3}-12	Smear pattern
E_{+3}-5/ M_{+3}-6	Specific band at 500 bp	E_{+3}-6/ M_{+3}-13	Smear pattern < 300 bp
E_{+3}-5/ M_{+3}-7	-	E_{+3}-6/ M_{+3}-14	Smear pattern < 300 bp
E_{+3}-5/ M_{+3}-8	Smear pattern < 500 bp	E_{+3}-6/ M_{+3}-15	Smear pattern
E_{+3}-5/ M_{+3}-9*	++	E_{+3}-6/ M_{+3}-16	Smear pattern
E_{+3}-5/ M_{+3}-10	Smear pattern < 500 bp	**E_{+3}-7/ M_{+3}-1***	++
E_{+3}-5/ M_{+3}-11	Smear pattern < 500 bp	E_{+3}-7/ M_{+3}-2	Smear pattern < 200 bp
E_{+3}-5/ M_{+3}-12	+	**E_{+3}-7/ M_{+3}-3***	++
E_{+3}-5/ M_{+3}-13	+	E_{+3}-7/ M_{+3}-4	specific band at 600 bp
E_{+3}-5/ M_{+3}-14	Smear pattern	**E_{+3}-7/ M_{+3}-5***	++
E_{+3}-5/ M_{+3}-15	Smear pattern	E_{+3}-7/ M_{+3}-6	Smear pattern < 200 bp
E_{+3}-5/ M_{+3}-16	Smear pattern	E_{+3}-7/ M_{+3}-7	Smear pattern < 200 bp
E_{+3}-6/ M_{+3}-1	Smear pattern	E_{+3}-7/ M_{+3}-8	Smear pattern < 500 bp
E_{+3}-6/ M_{+3}-2	Smear pattern < 300 bp	E_{+3}-7/ M_{+3}-9	Smear pattern
E_{+3}-6/ M_{+3}-3	Smear pattern	E_{+3}-7/ M_{+3}-10	Smear pattern
E_{+3}-6/ M_{+3}-4	Smear pattern	E_{+3}-7/ M_{+3}-11	Smear pattern
E_{+3}-6/ M_{+3}-5	Smear pattern	E_{+3}-7/ M_{+3}-12	Smear pattern
E_{+3}-6/ M_{+3}-6	Smear pattern	E_{+3}-7/ M_{+3}-13	Smear pattern
E_{+3}-6/ M_{+3}-7	Smear pattern	E_{+3}-7/ M_{+3}-14	Smear pattern

*The amplification products that were further analyzed by denaturing gel electrophoresis.

- = no amplification products, + = positive amplification product but sizes were less than 300 bp, ++ and +++ = positive amplification products exhibiting appropriate size range but the latter showed more intense amplification products

A total of 110 primer combinations were screened (Table 3.13). Eighteen primer combinations that yielded positive amplification products were further analyzed by denaturing polyacrylamide gel electrophoresis. The expression patterns of cDNA AFLP fragments were classified to 3 different groups; those exhibiting up- or down regulation patterns and those present in the control (normal crab) but not the treatment groups and *vice versa*.

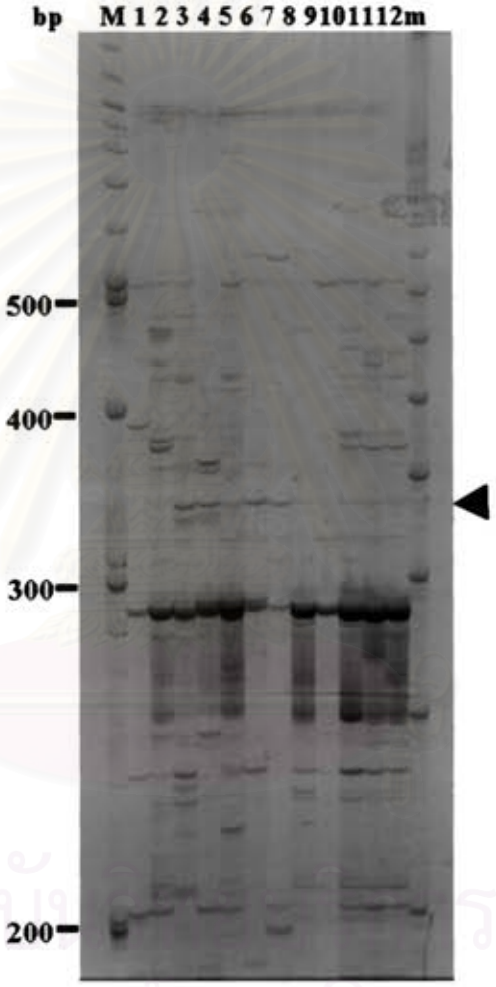

Figure 3.55 AFLP products of E_{+3}-2/ M_{+3}-5 amplified against cDNA of male (lanes 1, 3, 5, 7, 9 and 11) and female (lanes 2, 4, 6, 8, 10 and 12) *P. pelagicus* of the control group (lanes 1-2), 0 (lanes 3-4), 3 (lanes 5-6), 6 (7-8), 12 (lanes 9-10 and 24 (lanes 11-12) hpt. An arrowhead indicates a candidate differentially expressed cDNA-AFLP fragment (down-regulation) of *P. pelagicus* further characterized and used for semiquantitative RT-PCR. Lanes M and m are 100 bp and 50 bp DNA ladders, respectively.

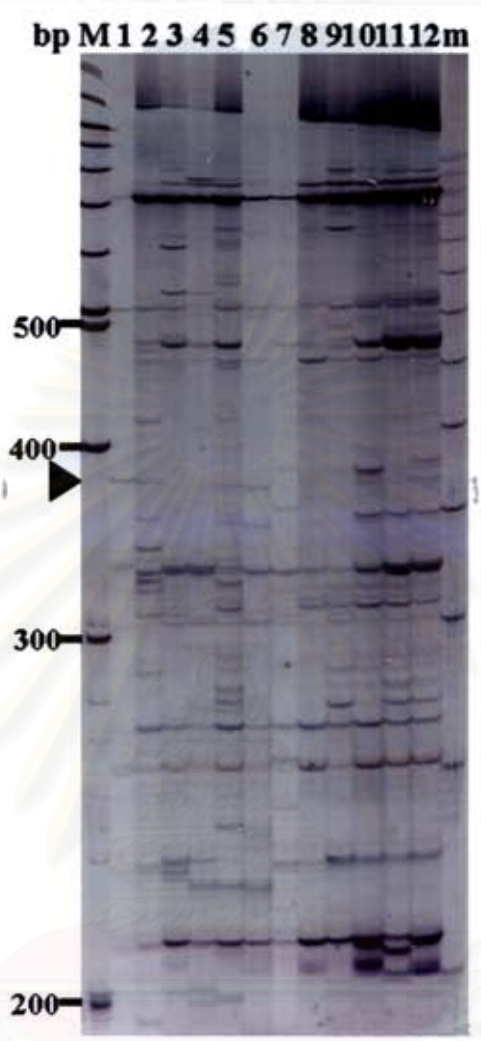

Figure 3.56 AFLP products of E_{+3}-2/ M_{+3}-7 amplified against cDNA of male (lanes 1, 3, 5, 7, 9 and 11) and female (lanes 2, 4, 6, 8, 10 and 12) *P. pelagicus* of the control group (lanes 1-2), 0 (lanes 3-4), 3 (lanes 5-6), 6 (7-8), 12 (lanes 9-10 and 24 (lanes 11-12) hpt. An arrowhead indicates a candidate differentially expressed cDNA-AFLP fragment (down-regulation) of *P. pelagicus* further characterized and used for semiquantitative RT-PCR. Lanes M and m are a 100 bp and 50 bp DNA ladders, respectively.

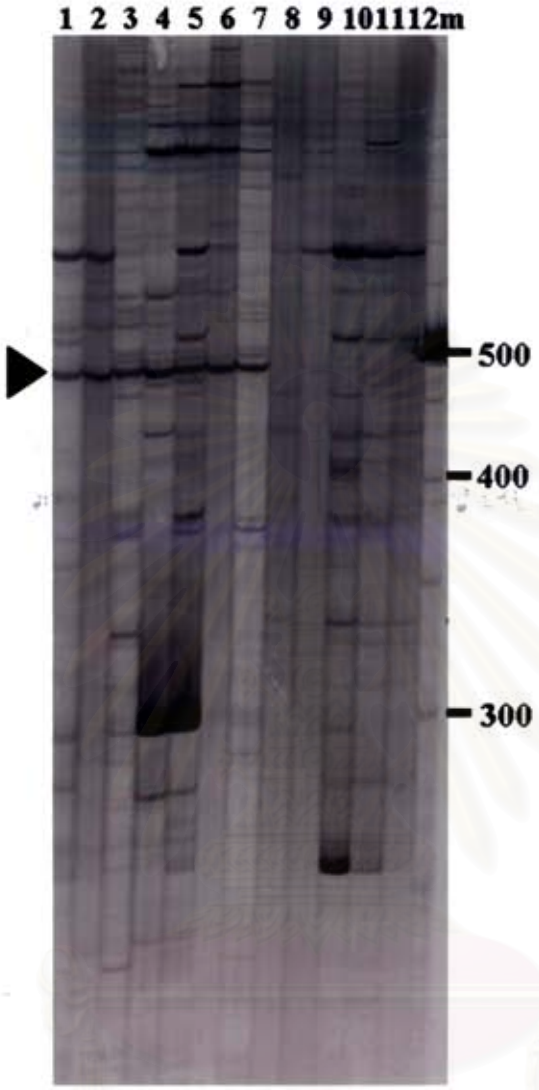

Figure 3.57 AFLP products of E_{+3}-5/ M_{+3}-9 amplified against cDNA of male (lanes 1, 3, 5, 7, 9 and 11) and female (lanes 2, 4, 6, 8, 10 and 12) *P. pelagicus* of the control group (lanes 1-2), 0 (lanes 3-4), 3 (lanes 5-6), 6 (7-8), 12 (lanes 9-10 and 24 (lanes 11-12) hpt. An arrowhead indicates a candidate differentially expressed cDNA-AFLP fragment (down-regulation) of *P. pelagicus* further characterized and used for semiquantitative RT-PCR. Lanes M and m are a 100 bp and 50 bp DNA ladders, respectively.

As can be seen from Table 3.14, only a single fragment (350 bp) from E_{+3}-2/ M_{+3}-9 was present in the control but not the treatment (0, 3, 6, 12 and 24 hour post treatment, hpt) groups. In contrast, six cDNA-AFLP bands were only found in the treatment groups. A large number of differentially expressed bands were found. Seven fragments were cloned and sequenced (Figures 3.58–3.64).

Table 3.14 Examples of candidate differential expressed cDNA-AFLP fragments of *P. pelagicus*

Primer	Expression pattern		
	Presence in the control	Presence in the treatment	Differential expression
E_{+3}-1/ M_{+3}-14		-	360, 380, 390, 400, 500, 550
E_{+3}-1/ M_{+3}-15		-	520, 570, 580, 670
E_{+3}-2/ M_{+3}-3		-	310, 460, 500
E_{+3}-2/ M_{+3}-5		-	**350***, **380**, 560, 620, 700
E_{+3}-2/ M_{+3}-7		-	310, 320, 360, 370, **380***, 490, **500**, 580
E_{+3}-2/ M_{+3}-9	350	-	360, 440, 450, 460, 480, 720
E_{+3}-2/ M_{+3}-12		-	300, 400, 460, 500, 720, 880
E_{+3}-2/ M_{+3}-13		650, 670	440
E_{+3}-2/ M_{+3}-16		340	320, 360, 550, 700
E_{+3}-3/ M_{+3}-4		-	320, 360, 550, 600
E_{+3}-3/ M_{+3}-7		**350**, **400**, 450	500, 560, 700
E_{+3}-4/ M_{+3}-1		-	360
E_{+3}-4/ M_{+3}-13		-	330, 480, 580, 590
E_{+3}-5/ M_{+3}-1		-	420, 460, 500, 640, 680
E_{+3}-5/ M_{+3}-9		-	380, **500***, 520, 620, 800
E_{+3}-7/ M_{+3}-1		-	320, 440, 460
E_{+3}-7/ M_{+3}-3		-	300, 420, 440, 450, 480
E_{+3}-7/ M_{+3}-5		-	300, 320, 330, 400, 430

Fragment that were cloned and sequenced were boldfaced. * = fragments that were used for semiquantitative RT-PCR analysis. - = not found.

Similarity search was carried out using Blast*N* and Blast*X* but they did not significantly match any previously deposited sequence in the GenBank (E-value > 10^{-4}). Therefore, these fragments were recognized as newly unidentified transcripts expressed in haemocytes of *P. pelagicus*.

TGACTGCGTACCAATTCAAGAAAGACTTGAAGAGACTGAGCCTAGACATGCAAA**AATCCAAC**
ATTGACCGCCAGTGGAAGTTTGACTGGTTGGACCCAACTATTGTGCCAAACTCAATCAGTGT
GTAAGAACTGAAAGAAGCAGGGGAGACAGTTCAAGACTGGTGGCTAGCTTTTCATCTCAAA**G**
GAAGAGCAAACCTGAAGTGCTTCCTGTTCACCAGATACTCTGCACTGTCACTGTACCAAGAG
TTGTGTGAACAATGTACGAGTATAGTTACTCAGGACCCATCGTGACTGAGTGCCAATTCAAG
TGGTTGGTAGTTACTCAGGACTCATC

Figure 3.58 Nucleotide sequence of E2M5_336 (336 bp) amplified from cDNA of *P. pelagicus*. The location and sequence of a forward primer (PP-E2M5_336-F) and those complementary to a reverse primer (PP-E2M5_336-R) are illustrated in boldface and underlined.

GACTGCGTACCAATTCAAGTAGTGAAGACTCTAGCCATCAATATTC**TGACCTCCATAGACCC**
TTCCTAATGTAAATAAAATGGTTTTATCATGCCCAAGAATCATGGTAAAAAATGTATCACAG
TACTGAAGAGAATGAGAGAAGTGTGAGTGTTGAGGTGATGTGATAGCATTGTCTAGACCTGA
GTGCATTGGTCACACACTACACCAACAACTCTGGCATCTGTTTTCTTCCTGTGG**TCTGTGCT**
GAATGTTGGGAAAAGAACCAAGAGAAGTGAAGAGAGAGGGAGAGAGAGACTCGTATTCTCAA
ACATTTGTGCGTTTCACCTCCACTATTTCAAGAGGGTTTAGTTACTCAGGACTCATCA

Figure 3.59 Nucleotide sequence of E2M5_368 (368 bp) amplified from cDNA of *P. pelagicus*. The location and sequence of a forward primer (PP-E2M5_368-F) and those complementary to a reverse primer (PP-E2M5_368-R) are illustrated in boldface and underlined.

A.
GACTGCGTACCAATTCAAGGGTGTGGAAAGGACAGATAGGGATGAGA**GGAAGAAGAAAGCCT**
TGTGCAGTGAGGCTGCAGGAGGTGGTGAGGCAGGCATTCTGGTGGTGAGAAAGAGGCTGAAG
ACAGTTAGTCAAAGGAAGGGAGTTGATAAGAGGAAGAGCTTTTGATTCCACCCTATCTAGTA
AAACAGTGTGACTGGAACCCCCAAACATCCGAAGAGCACTC**CATACAGGGACGGATAAGGC**C
CTAATACAGAGTAAGCAGTTGGAGGGGCGAGAAAAACTGGCAGAGATGCCTCAGAACACCTA
ACTTCATAGAAGCTGTTTTAGCAAGAGATGAGATGTGAAGTTTCCAGTTACTCAGGACTCAT
CA
B.
GACTGCGTACCAATTCAAGGGTGTGGAAAGGACAGATAGGGATGAGAGGAAGAAGAAAGCCT
TGTGCAGTGAGGCTGCAGGAGGTGGTGAGGCAGGCATTCTGGTGGTGAGAAAGAGGCTGAAG
A**CAGTTAGTCAAAGGAAGGGAGT**TGATAAGAGGAAGAGCTTTTGATTCCACCCTATCTAGTA
AAACAGTGTGACTGGAACCCCCAAACATCCGAAGAGCACTCCATACAGGGACGGATAAGGCC
CTAATACAGAGTAAGCAGTTGGAGGGGCGAGAAAAACTGGCAGA**GATGCCTCAGAACACCTA**
ACTTCATAGAAGCTGTTTTAGCAAGAGATGAGATGTGAAGTTTCCAGTTACTCAGGACTCAT
CA

Figure 3.60 Nucleotide sequence of E2M7_374 (374 bp) amplified from cDNA of *P. pelagicus*. The location and sequence of a forward primer (PP-E2M7_374-F1, A. and PP-E2M7_374-F2, B) and those complementary to a reverse primer (PP-E2M7_374-R1, A and PP-E2M7_374-R2, B) are illustrated in boldface and underlined.

GACTGCGTACCAATTCAAGGGTACATAAGATTGAAAAGGTTGAGAACCCCTGTATTAG**AAGG**
TTGGACAGAATAGGGATGAGAGGAAGAAAAAAGCCTTGTGCAGCGAGGCAGCAGGAAGAGGG
GAGGCATGCAGTTAGTAAGATCAGTAGTGCAGTTGGCATGAAAATAGCGATAACAGATAGAA
AGAGACGCAACATTTCAGCGGTGAGAAAGAGGCTGAAGACAGTCAGTCAGAGGAGTGGAGTT
GATGAGACGAAAAGCTTTTGAT**TCCACCCTATCTAATCAAGC**TGTATGAGTGGAACCCCCGA
AACATGCGAAGAATACTCCATACAAGGAGGGATAAGGCCCTTGTACAGTTAGCAGTTGAAGG
GCGACAAAAACTGGCGGAGATGCCACAGAACACTTGATTTCATAAAAAAAACTATTTTAGCA
AGAGATGAGATGTGAAGTTTTCCAGTTACTCAGGACTCATCA

Figure 3.61 Nucleotide sequence of E2M7_476 (476 bp) amplified from cDNA of *P. pelagicus*. The location and sequence of a forward primer (PP-E2M7_476-F) and those complementary to a reverse primer (PP-E2M7_476-R) are illustrated in bold and underlined.

A.
GACTGCGTACCAATTCACAGATAGA**CGTAGCGTTACTCCCGTTTA**TTTTAGTCTGTGTGACG
CCGCCTGCAGTAAATTTTCCATGTTCTTTTTCCTTTTCCTCGCCAAAGCTTTCACGCCCACT
CAGTCAATCGATCAGCTCTGCTCTAAAAGTCATTCTTTCATTTATCAGTCAGTCAGTCAATC
TCTCAGGCTCATTCACTCAGTCGTTTCATTGGTCGTTCAGCCAATCAGGGAGCCCATCCATC
AGTCAGAAATATATTCACTCGCTCAGCCGGTCAGCAAGTCTTTCGCTCCACTCAGTCAT**CAA**
ATAAAGCATCTGGTCAATCAGTTTGCCAGACAGTTACTCAGGACTCATCA
B.
GACTGCGTACCAATTCACAGATAGACGTAGCGTTACTCCCGTTTATTTT**AGTCTGTGTGACG**
CCGCCTGCAGTAAATTTTCCATGTTCTTTTTCCTTTTCCTCGCCAAAGCTTTCACGCCCACT
CAGTCAATCGATCAGCTCTGCTCTAAAAGTCATTCTTTCATTTATCAGTCAGTCAGTCAATC
TCTCAGGCTCATTCACTCAGT**CGTTTCATTGGTCGTTCAGC**CAATCAGGGAGCCCATCCATC
AGTCAGAAATATATTCACTCGCTCAGCCGGTCAGCAAGTCTTTCGCTCCACTCAGTCATCAA
ATAAAGCATCTGGTCAATCAGTTTGCCAGACAGTTACTCAGGACTCATCA

Figure 3.62 Nucleotide sequence of E3M7_360 (360 bp) amplified from cDNA of *P. pelagicus* using E_{+3}-3. The location and sequence of a forward primer (PP-E3M7_360-F1, A and PP-E3M7_360-F2, B) and those complementary to a reverse primer (PP-E3M7_360-R1, A and PP-E3M7_360-R2, B) are illustrated in bold and underlined.

GACTGCGTACCAATTCACATCGCATCAACTTTTCTTGTGCTNTACATGCTTTTATTA**CCAGT**
ATCCCTTTCATCCTTCATCTCTATCACCAATGACATCATCTCTCATCGCTTCCCAATCTGTG
TTCTTTTGGTATGATCTTATTTCAGAGTTCCTTTGCAGTTACTCAGGACTCATCATGACTGC
GTACCAATTCAACACATTCATCCAAGTCTGGGTTACTCAGGACTCATCACGACTGCGTACCA
ATTCAACACATTCATCCAAGTCTGGGTTACTCAGGACTCATCGTGACTGCGTACCAATTCAC
AAGAACCTGCGGAA**AGAGGGCAGTAAGAAGCCAAAG**AAAGACAATATTGCTAATGATAACAA
AGAAAGCAAAGTTGACACAGTTACTCAGGACTCATCA

Figure 3.63 Nucleotide sequence of E3M7_409 (409 bp) amplified from cDNA of *P. pelagicus*. The location and sequence of a forward primer (PP-E3M7_409-F) and those complementary to a reverse primer (PP-E3M7_409-R) are illustrated in bold and underlined.

GATGAGTCCTGAGTAACGAAAACATCCTTGGCAAATG**CTTTCGCAGTAGTTCGTCTT**GCGAC
GGTCCAAGAATTTCACCTCTAGCGTCGCAATACGAATGCCCCCGCCTGTTCCTATTAGTCAT
TACCTAGGGTTCAGAAAACCAACAAAATAGAACCGAGGTCCTATTCCATTATTCCATGCATA
GGCATTCAGGTCGTCAATGTAGCCTGCTTTGAGCACTCTAATTTTTCTCAAAGTAAACTCTG
CCGGCCACGCGGGACACTCGATGAAGAGCACCCCGTGCGAGCCAGCGGGCGGTTAAAAACTG
TTCGGAGCGTGACAGTGTGCACCGGGCGGTGAACCGTCAGTCCAGA**ACTGAAATCCAACTAC**
GAGCTTTTTAACCGCAACAACTTTAATATACGCTATTGGAGCTGGAATTACCGCGGCTGCTG
GCACCAGACTTGCCCTCCAATAGATCCTCGTTACTCAGGACTCATCA

Figure 3.64 Nucleotide sequence of E5M9_481 (481 bp) amplified from cDNA of *P. pelagicus*. The location and sequence of a forward primer (PP-E5M9_481-F) and those complementary to a reverse primer (PP-E5M9_481-R) are illustrated in boldface and underlined.

3.3.2 End point RT-PCR

Nine primer pairs were designed from sequences of 7 cDNA-AFLP fragments. Conventional RT-PCR was carried out. PP-E3M7_360-F1/R1 and PP-E3M7_409 did not generate the amplification product. Another primer pair for amplification of PP-E3M7_360 (PP-E3M7_360-F2/R2) was designed but did not give the PCR product (Figure 3.65 and Table 3.15).

PP-E2M5_368 and PP-E2M7_374-F2/R2 yielded faint amplification products and possible required the large amount of the first strand cDNA template for semiquantitative analysis. In addition, PP-E2M7_476-F/R provided the intense bands but this marker showed homo- and heterozygous band patterns (Figure 3.65 and Table 3.15) and is difficult to be used for quantitative analysis. As a result, those cDNA-AFLP-derived markers were not included for determination of the gene expression levels using semiquantitative analysis.

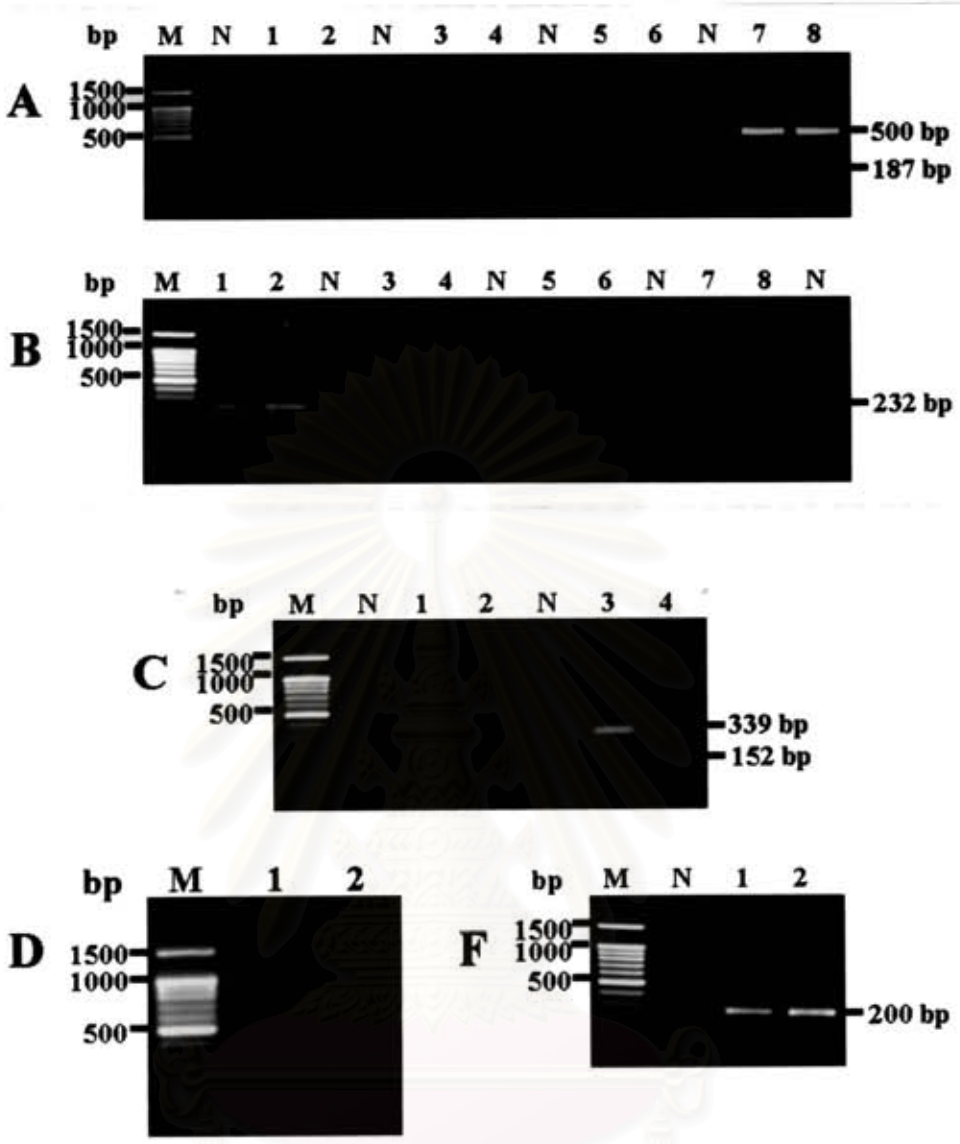

Figure 3.65 A 1.5% ethidium bromide stained agarose gel showing amplification results of PP-E2M7_374-F2/R2 (187 bp; lanes 1-2; panel A), PP-E2M5_368 (214 bp; lanes 3-4; panel A), PP-E2M7_360-F1/R1 (302 bp; lanes 5-6; panel A), PP-E2M7_476 (232 bp; lanes 1-2; panel B), PP-E3M7_360-F2/R2 (178 bp; lanes 3-4; panel B), PP-E3M7_360-F1/R2 (lanes 5-6; panel B), PP-E3M7_360-F2/R1 (lanes 7-8; panel B), PP-E2M5_336 (152 bp; lanes 1-2; panel C), PP-E5M9_481 (339 bp; lanes 3-4; panel C), PP-E3M7_409 (289 bp; lanes 1-2; panel D), PP-E2M7_374-F1/R1 (200 bp; lanes 1-2; panel F) and *EF-1α* (500 bp, lanes 7-8, panel A) against cDNA of *P. pelagicus* haemocytes. Lane M and N are a 100 bp DNA ladder and the negative control (without cDNA template), respectively.

Table 3.15 Amplification results of candidate differentially expressed cDNA-AFLP fragments

Primer	Expected size (bp)	Amplification success
1. PP-E2M5_336-F/R*	**152**	**+++**
2. PP-E2M5_368-F/R	214	+
3. PP-E2M7_374-F1/R1*	**200**	**+++**
4. PP-E2M7_374-F2/R2	189	+
5. PP-E2M7_476-F/R	232	H
6. PP-E3M7_360-F1/R1	302	-
7. PP-E3M7_360-F2/R2	178	-
8. PP-E3M7_409-F/R	289	-
9. PP-E5M9_481-F/R*	**339**	**+++**

- = no amplification products, + = faint amplification band, ++ = intense amplification product that show homo- and heterozygous states, +++ = intense single amplification band that were used for semiquantitative RT-PCR.

In contrast, PP-E2M5_336-F/R, PP-E2M7_374-F1/R1 and PP-E5M9_481-F/R generate the intense amplification product of 152, 200 and 339 bp in size (Figure 3.65). These cDNA-AFLP derived markers were easily amplified and, therefore, were used for semiquantitative RT-PCR analysis.

3.3.3 Examination of temperature-stress response transcripts by semiquantitative RT-PCR

Total RNA was extracted from haemocytes of *P. pelagicus* collected from different time intervals (N = 4 each of the control and 0, 3, 6, 12 and 24 hpt). After DNA treatment, DNA-free total RNA of each crab was reversed transcribed (Figure 3.66). Conditions for semiquantitative RT-PCR were then optimized.

3.3.3.1 Optimization of semiquantitative RT-PCR conditions

Expression levels of PP-E2M5_336, PP-E2M7_374-I and PP-E5M9_481; (hereafter called temperature stress response transcript, *PP-TSRT*$_{152}$; *PP-TSRT*$_{200}$ and *PP-TSRT*$_{339}$, respectively) were examined using semiquantitative RT-PCR. This technique requires optimization of several parameters including concentrations of $MgCl_2$, and primers and the number of PCR cycles. A house keeping gene, *elongation factor-1α* (*EF-1α*) was used as the control. The preliminary RT-PCR was carried out using the annealing temperature of 53°C as described previously.

Figure 3.66 A 1.0% ethidium bromide-stained agarose gel showing the synthesized first strand cDNA from haemocytes of *P. pelagicus* using an ImProm- II™ Reverse Transcriptase. Lane M and m are a 100 bp DNA ladder and λ-*Hin*d III. Lanes 1-6 are the first strand cDNA from haemocytes of different *P. pelagicus* individuals.

3.3.3.1.1 Optimization of the $MgCl_2$ concentration

RT-PCR of each gene was carried out with fixed components as for the end point RT-PCR except that $MgCl_2$ concentrations was varied (0, 1.0, 1.5, 2.0, 2.5, 3.0 and 4.0 mM). The concentration of $MgCl_2$ that gave the highest yields and specificity of each PCR product was chosen (Table 3.16).

3.3.3.1.2 Optimization of the primer concentration

The optimal concentration of each primer (between 0.10, 0.15, 0.20, 0.30, 0.40 and 0.50 μM) was carefully examined using the optimized $MgCl_2$ concentration of each transcript. Lower concentrations may result in non-quantitative amplification whereas higher concentrations of primer may leave a large amount of unused primers

which could give rise to non-specific amplification products. The suitable concentration of primers for each primer of each gene was then chosen (Table 3.16).

3.3.3.1.3 Optimization of the cycle numbers

The number of amplification cycles was important because the product reflecting the expression level should be measured quantitatively before reaching a plateau amplification phase. At the plateau stage, transcripts initially present at different level may give equal intensity of the amplification products.

In this experiment, RT-PCR of each gene was performed using the conditions that $MgCl_2$ and primer concentrations were optimized for 25, 28 and 30 cycles. The number of cycles that gave the highest yield before the product of each transcript reached a plateau phase of amplification was chosen (Table 3.13).

Table 3.16 Optimized $MgCl_2$ and primer concentrations and the number of PCR cycles for semiquantitative analysis of candidate thermal-stress response genes resulted from cDNA-AFLP analysis of *P. pelagicus* and the positive control (*EF1-α*)

Primer	Expected amplicon (bp)	$MgCl_2$ concentration (mM)	Primer concentration (μM)	PCR cycles
PP-E2M5_336	152	2.0	0.4	28
PP-E2M7_374-1	200	1.5	0.4	25
PP-E5M9_481	339	2.0	0.4	29
Elongation factor 1-α	500	1.5	0.3	25

3.3.3.2 Semiquantitative RT-PCR analysis

3.3.3.2.1 *PP-TSRT$_{152}$*

The expression patterns of *PP-TSRT$_{152}$* in haemocytes of *P. pelagicus* upon temperature-stress at 33°C for 3 hours revealed were concordant with preliminary results obtained from cDNA-AFLP. This transcript was significant down regulated at 12 hpt (0.626 ± 0.066, $P< 0.05$). The relative expression level at 24 hpt (0.647 ± 0.043) was also significantly different from that of the normal crab (0.849±0.180, $P < 0.05$).

3.3.3.2.2 *PP-TSRT$_{200}$*

The relative expression level of *PP-TSRT$_{200}$* was comparable at all time intervals after temperature treatment. Therefore, this transcript did not reveal any significant change between the normal and treatment group ($P > 0.05$).

3.3.3.2.3 *PP-TSRT$_{339}$*

The expression patterns of *PP-TSRT$_{339}$* in haemocytes of *P. pelagicus* upon temperature-stress at 33°C for 3 hours were concordant with preliminary results obtained from cDNA-AFLP. This transcript was initially down-regulated and significantly different from that of the control at 12 hpt (0.915 ± 0.102, $P < 0.05$). The expression levels returned to the normal level at 24 hpt (1.107 ± 0.132, $P < 0.05$)

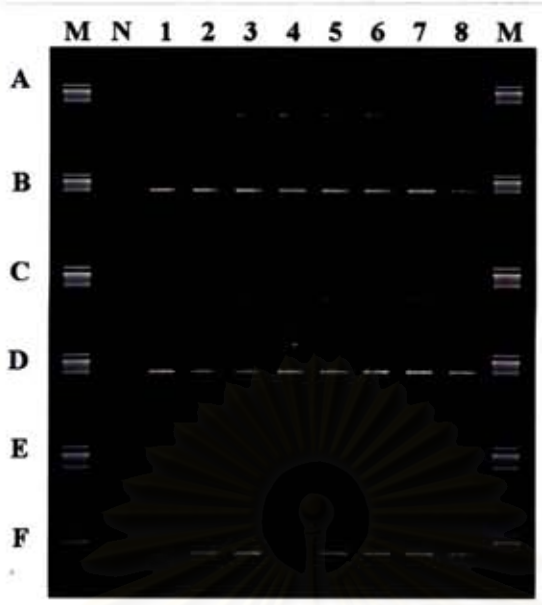

Figure 3.67 A 1.8% ethidium bromide-stained agarose gel showing the expression level of *PP-TSRT*$_{152}$ (lanes 1-8; A, C, E) and *EF-1α* (lanes 1-8; B, D, F) in haemocytes of the normal crab (lanes 1-4, A and B) and the temperature-treated crab at 0 (lanes 5-8, A and B), 3 (lanes 1-4, C and D), 6 (lanes 5-8, C and D), 12 (lanes 1-4, E and F) and 24 (lanes 5-8, E and F) hpt. Lanes M and N are a 100 bp DNA ladder and the negative control (without DNA template), respectively.

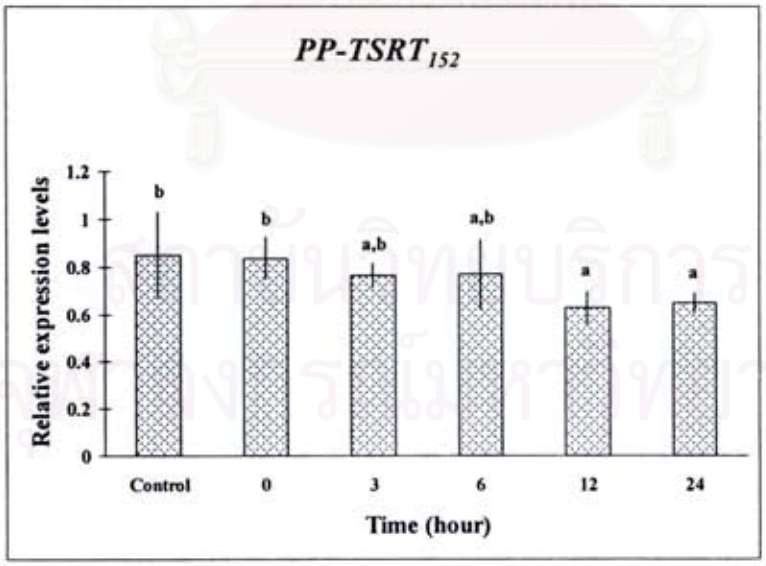

Figure 3.68 Histograms showing the time-course relative expression levels of *PP-TSRT*$_{152}$ in haemocytes of the normal crab and the temperature-treated crab at 0, 3, 6 12 and 24 hpt, respectively.

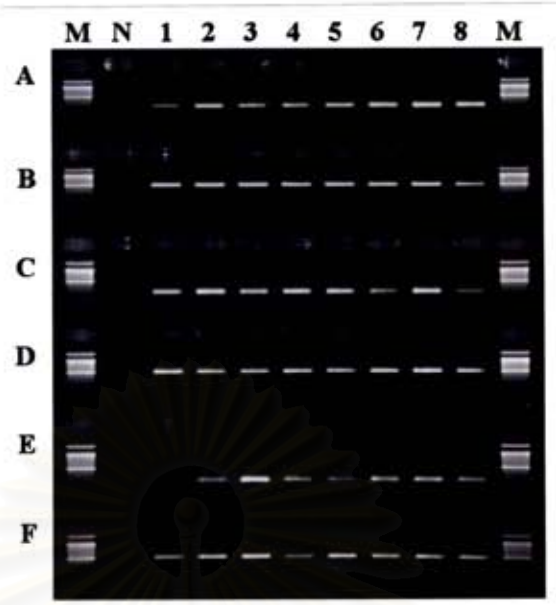

Figure 3.69 A 1.8% ethidium bromide-stained agarose gel showing the expression level of *PP-TSRT*$_{200}$ (lanes 1-8; A, C, E) and *EF-1α* (lanes 1-8; B, D, F) in haemocytes of the normal crab (lanes 1-4, A and B) and the temperature-treated crab at 0 (lanes 5-8, A and B), 3 (lanes 1-4, C and D), 6 (lanes 5-8, C and D), 12 (lanes 1-4, E and F) and 24 (lanes 5-8, E and F) hpt. Lanes M and N are a 100 bp DNA ladder and the negative control (without DNA template), respectively.

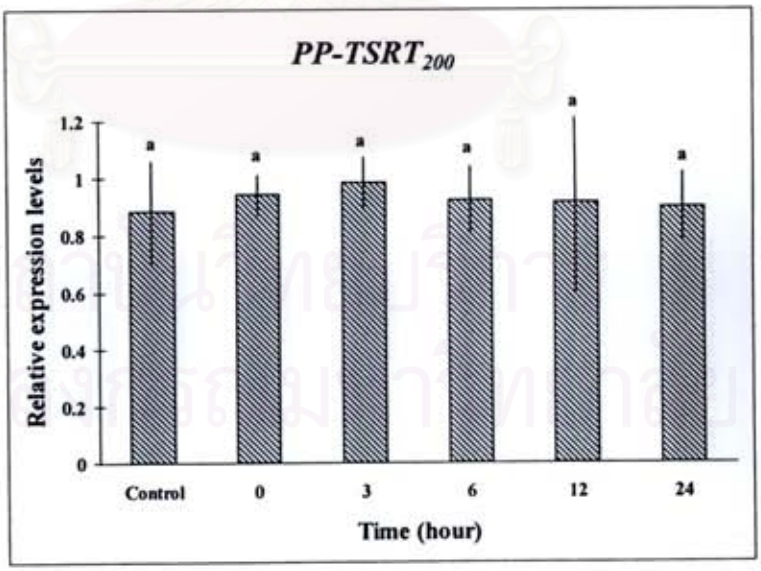

Figure 3.70 Histograms showing the time-course relative expression levels of *PP-TSRT*$_{200}$ in haemocytes of the normal crab and the temperature-treated crab at 0, 3, 6 12 and 24 hpt, respectively.

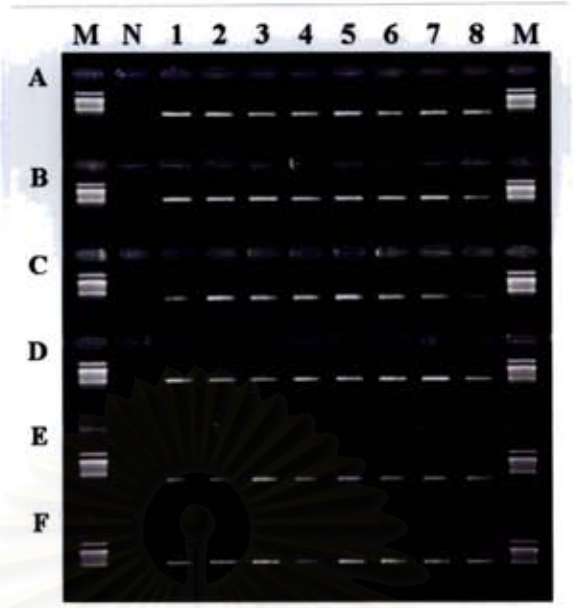

Figure 3.71 A 1.8% ethidium bromide-stained agarose gel showing the expression level of *PP-TSRT*$_{339}$ (lanes 1-8; A, C, E) and *EF-1α* (lanes 1-8; B, D, F) in haemocytes of the normal crab (lanes 1-4, A and B) and the temperature-treated crab at 0 (lanes 5-8, A and B), 3 (lanes 1-4, C and D), 6 (lanes 5-8, C and D), 12 (lanes 1-4, E and F) and 24 (lanes 5-8, E and F) hpt. Lanes M and N are a 100 bp DNA ladder and the negative control (without DNA template), respectively.

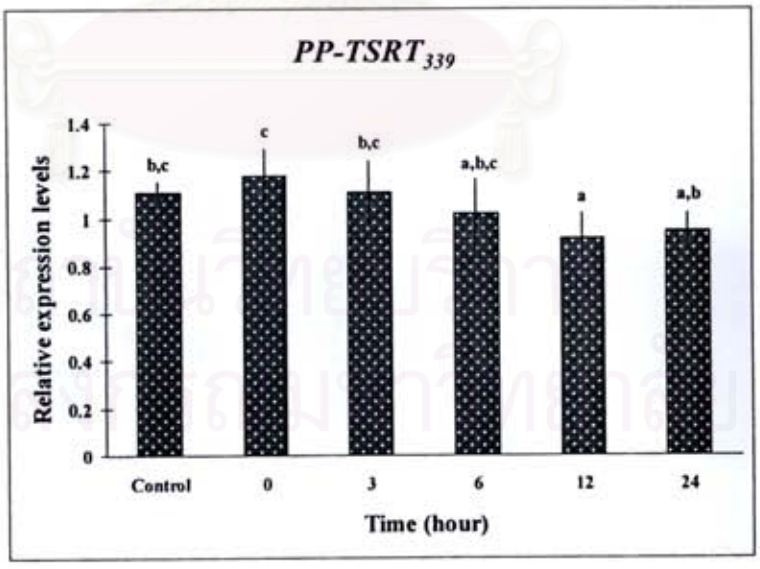

Figure 3.72 Histograms showing the time-course relative expression levels of *PP-TSRT*$_{339}$ in haemocytes of the normal crab and the temperature-treated crab at 0, 3, 6 12 and 24 hpt, respectively.

Table 3.17 A time-course analysis of candidate thermal-stress response genes (*PP-TSRT*$_{152}$, *PP-TSRT*$_{200}$ and *PP-TSRT*$_{339}$) of *P. pelagicus* using semiquantitative RT-PCR

Primer	Mean relative expression level*					
	Normal	0 hpt	3 hpt	6 hpt	12 hpt	24 hpt
PP-TSRT$_{152}$	0.849 ± 0.180^{b}	0.836 ± 0.087^{b}	$0.761 \pm 0.050^{a,b}$	$0.770 \pm 0.146^{a,b}$	0.626 ± 0.066^{a}	0.647 ± 0.043^{a}
PP-TSRT$_{200}$	0.883 ± 0.177^{a}	0.941 ± 0.068^{a}	0.985 ± 0.084^{a}	0.921 ± 0.117^{a}	0.910 ± 0.310^{a}	0.900 ± 0.118^{a}
PP-TSRT$_{339}$	$1.109 \pm 0.044^{b,c}$	1.178 ± 0.112^{c}	$1.107 \pm 0.132^{b,c}$	$1.017 \pm 0.143^{a,b,c}$	0.915 ± 0.102^{a}	$0.944 \pm 0.074^{a,b}$

*The same superscripts between different time interval data are not significantly different ($P > 0.05$).

CHAPTER V

DISCUSSION

I. Genetic Diversity of *P. pelagicus* in Thailand

The estimation and partition of the level of intraspecific genetic variation in any species is fundamental for establishing rational management of natural resources (Avise, 1994). In recent years, DNA analysis has been commonly used to determine the levels of intraspecific genetic variation and population differentiation of several species (Benzie, 2000). Genetic markers are potentially useful for several applications in economically important species like *P. pelagicus*, for instance, in the investigation of genetic variability of wild stocks, maintaining genetic characteristics of an artificially propagated stock, and enhancement of local *P. pelagicus* stocks.

DNA sequencing of informative DNA fragments is the direct approach for analysis of genetic diversity and differentiation of organisms. Nevertheless, several indirect approached (e.g. PCR-RFLP, RAPD, AFLP and SSCP etc.) are available at present and are cost-effective for studies of diversity of species that the knowledge about genome and genome sequences are not well studied. Of these, SSCP is one of the most widely used techniques for detection of mutations and variation of DNA (deletions, insertions and single nucleotide polymorphism, SNP). The major advantage of SSCP is that a large number of individual may be simultaneously genotyped. In addition, SSCP required small PCR amplicons which are relative easy to amplify. Therefore, SSCP is regarded as one of the potential techniques that can be used to detect low polymorphism in various species prior to confirmation of the results by nucleotide sequencing.

In this thesis, polymorphic SCAR markers were developed from the positive amplification fragment of mtDNA *COI* (*PP-COI*$_{270}$) and coding (*PP-SCARRAP*$_{318}$) and non-coding (*PP-SCARAFLP*$_{300}$) nuclear DNA segments and tested against genomic DNA of geographically different samples of *P. pelagicus* in Thai waters (N = 174). Relatively high genetic diversity within each geographic sample of Thai *P. pelagicus* was observed from SSCP analysis. A total of 35 bands (loci) were found.

The average polymorphic loci and gene diversity in this species were 48.57% and 0.1859.

Khetpu (2005) analyzed genetic diversity of *P. pelagicus* in Thai waters originating from the same geographic locations but smaller sample sizes (N = 109) by RAPD analysis. A total of 112 RAPD fragments were generated from OPA02, OPA14, OPB10, UBC122 and UBC158. The percentage of polymorphic bands in each geographic sample was 72.73% - 85.05%. The average genetic distance between samples across overall primers was 0.0929 - 0.1802.

In addition, genetic variation of Thai *P. pelagicus* was recently examined by AFLP analysis (Klinbunga *et.al*., 2007). High genetic diversity of *P. pelagicus* in Thai waters (N = 72) was found. A total of 227 AFLP fragments was generated from four primer combinations and the percentage of polymorphic bands in each geographic sample was 66.19% - 94.38%.

Taking all information together, Thai *P. pelagicus* exhibited high genetic diversity within each geographic sample suggesting that inbreeding is not a major concern for this economically important species. The percentage of polymorphic bands in *P. pelagicus* based on SSCP analysis was comparable with that of penaeid shrimp (*Penaeus monodon, P. chinensis, P. merguiensis, P. latisulcatus, P. canaliculatus and P. japonicus* with N = 3 – 8 for each species) analyzed with three AFLP primer combinations where the level of polymorphic bands ranged from 24.6% in *P. canaliculatus* to 60.8% in *P. japonicus* (Wang *et al*., 2004) but was slightly lower than that (60.92 - 65.78%) of the flounder (*Paralichthys olivaceus*) analyzed by 10 AFLP primer combinations (Liu *et al.*, 2005).

PP-COI$_{270}$ exhibited 8 SSCP patterns whereas a higher number of polymorphism was observed from *PP-SCARRAP*$_{318}$ (56 genotypes) and *PP-SCARAFLP*$_{300}$ (21 genotypes). Theoretically, mitochondrial DNA evolves 5-10 times faster than single copy nuclear DNA (Brown *et al*., 1979). This important property provides the magnifying ability to distinguish and identify the differences between populations and between closely related taxa. Nevertheless, the mitochondrial gene segment, *PP-COI*$_{270}$ exhibited lower genetic polymorphism than that of a coding gene, *PP-SCARRAP*$_{318}$. In addition, *PP-COI*$_{270}$ was also showed a lower level of

genetic polymorphism than did the non-coding *PP-SCARAFLP*$_{300}$. These results suggested that female founder effects may be occurred in Thai *P. pelagicus* limiting mtDNA but not nuclear DNA polymorphism.

II. Population Differentiation and Limited Gene Flow Levels of *P. pelagicus*

Several factors are responsible for intraspecific differentiation. These include migration or gene flow, random genetic drift, modes of natural selection, mutation and genetic recombination through mating systems. In addition to those general categories, biological factors related to individual groups of organisms, for instance ecological factors and life history also play a partitioning role in population differentiation (Avise, 1994).

It is generally accepted that gene flow is the most important factor reflecting the apparent magnitude of intraspecific subdivisions. Gene flow is the exchange of genetic material between populations caused by movement of individuals or their successfully fertilizing gametes. Marine species with long larval phases are thought to have high levels of genetic variation within populations (Féral, J-P, 2002). In addition, a long-duration planktonic larval stage influences the opportunity for a high degree of gene flow as evidenced by a near absence or lack of genetic differentiation over vast geographic areas in several taxa for example, in the sea urchins, *Strongylogentrotus purpulatus* and *S. droebachiensis* (Palumbi and Wilson, 1990) and the red rock lobster, *Jasus edwardsii* (Ovenden *et al*., 1992).

Theoretically, the extended planktonic larval stages of *P. pelagicus* suggest high dispersal potential and the possibility of extensive gene flow between conspecific samples at least on a geographic mesoscale (tens to hundreds of kilometers) (Yap *et al*., 2002). The findings of shared common SSCP genotypes with different frequencies across geographic samples and several private genotypes in each sample implied genetic differentiation between *P. pelagicus* from different geographic origins in Thai waters.

Large genetic distance between pairs of geographic samples of *P. pelagicus* was observed based on SSCP analysis (0.0099 - 0.1198). Generally, relatively larger genetic distance was observed between samples from different coastal regions than

that between geographic locations within regions. This indirectly reflected strong intraspecific genetic differentiation of *P. pelagicus* in Thai waters.

Geographic heterogeneity between geographic samples using three polymorphic DNA marker (*PP-COI*$_{270}$, *PP-SCARRAP*$_{318}$ and *PP-SCARAFLP*$_{300}$) indicated significant population differentiation among all pairwise comparisons of Thai *P. pelagicus* based on the exact test ($P < 0.0001$) and F_{ST}-based statistics (θ, $P < 0.01$). Moreover, the lower 95% confidence limit of overall samples and paired geographic samples bootstrapped overall loci was much greater than zero (0.0958 and 0.0131–0.1825, respectively). This further suggested the existence of significant genetic divergence at a fine-scale level between conspecific samples under investigation.

The present study indicated that the gene pool of *P. pelagicus* was not homogeneous but microgeographically fragmented intraspecifically. Patterns of genetic differentiation at the fine-scale level in *P. pelagicus* (e.g. between geographic samples located approximately a few hundred kilometers in distance) were different from those of other marine species; e.g. the giant tiger shrimp (*P. monodon*; Supungul *et al*., 2000; Klinbunga *et al*., 2001), the banana shrimp (*P. merguiensis*; Hualkasin *et al*., 2003) and the abalone (*Haliotis asinina* and *H. ovina*; Klinbunga *et al*., 2003) in Thai waters previously reported where significant genetic heterogeneity was found between geographic samples from different coastal regions (i.e. between the Andaman Sea and Gulf of Thailand).

Basically, *P. pelagicus* is regarded as a potential vagile species because adults are able to travel approximately 20 km daily (Kangas, 2000). Both adult and juvenile *P. pelagicus* inhabited sheltered benthic coastal environments and females migrate outwards into the open ocean for spawning and return into the estuaries for a time after spawning. Both males and females migrate from the estuaries as a reaction to lowered salinities (Meagher 1971, Potter *et al.* 1983, 1991). Nevertheless, the present study indicated strong population differentiation was observed in this species as reflected by the large genetic divergence and restricted levels of gene flow (N_em = 0.39 – 5.37 individuals per generation) between pairs of geographic samples of Thai *P. pelagicus*. Geographic distance between investigated samples *per se* and migratory

behavior of *P. pelagicus* may have promoted degrees of genetic differentiation of this species.

Population genetic structure of *P. pelagicus* was previously reported by RAPD (Khetpu, 2005) and AFLP (Klinbunga *et.al*., 2007) analyses. The average genetic distance between samples across overall primers was 0.0929 - 0.1802 and 0.1151 - 0.2440, respectively. Geographic heterogeneity analysis using the exact test and F_{ST}-based statistics (θ) indicated statistically significant differences between all pairwise comparisons ($P < 0.0001$ and $P < 0.001$) indicating a fine scale genetic differentiation of investigated samples. The estimated number of migrants per generation (N_em) was 0.44 – 1.19 for RAPD analysis and 0.26 - 0.76 for AFLP analysis, suggesting the restricted gene flow levels of *P. pelagicus* in Thai waters.

Lu *et al*. (2000) analyzed three species of mitten crabs (*Eriocheir sinensis*, *E. japonicus* and *E. japonicus hepuensis* and three geographic sample of *E. sinensis* by RAPD. No species diagnostic markers for each subspecies were found but significantly genetic differences were existent between taxa ($P < 0.001$) and geographic samples ($P < 0.001$) where intraspecific similarities were larger than interspecific similarity and intrapopulational similarity was larger than interpopulational similarities.

The blue swimming crab in Thailand, at present, is perceived and managed as a single stock. On the basis of the present study, five *P. pelagicus* samples were regarded as different stocks. From management points of view, these genetically isolated stocks should be treated as separate management units (Avise, 1994, Carvalho and Hauser, 1994).

The basic genetic information found in *P. pelagicus* are applicable for several disciplines including selection of appropriate broodstock for domestication of wild *P. pelagicus*, comparison on the performance of economical important traits between different stocks and genetic improvement through selective breeding programs. An overexploitation of wild *P. pelagicus* reflected by the occurrence of the increasing proportion of small sizes of captured *P. pelagicus* may be compensated by enhancement of natural *P. pelagicus* where a fine scale level of local stocks is required as the founders.

In terms of aquaculture, domestication and subsequently, selective breeding programs of *P. pelagicus* should be established and use the advantage about strong intraspecific genetic differentiation between geographically different samples of *P. pelagicus* found in the present study. The proper source to be used as the founder population for breeding programs of *P. pelagicus* should be established from different genetic stocks and they should be maintained separately. Crosses between stocks may be carried out to possibly promote heterosis of economically important traits in this species (Klinbunga *et al*., 2007).

Molecular population genetics provide necessary information required for elevating culture and management efficiency of *P. pelagicus*. Knowledge on genetic diversity and population differentiation of *P. pelagicus* found in this study not only yields critical information on biogeographic and evolutionary aspects of *P. pelagicus* in Thailand but also allows the ability to construct effective breeding programs and restocking projects in this species.

III. Species-specific SCAR markers in *P. pelagicus*

Species-specific markers play important roles for quality control of products from economically important species. Generally, species-diagnostic markers should be established from DNA segments exhibiting low genetic polymorphism within a particular species but showing high genetic divergence between different species. Overlapping patterns between different species should not be observed (Thaewnon-ngiw *et al.*, 2004).

Several thousands copies of mtDNA are found in each eukaryotic cell resulted in high sensitivity of mitochondrial gene segment when applied as species-diagnostic markers in animals. Results from population genetic studies indicated that *COI* and *12S rDNA* gene segments exhibited relatively low polymorphism and suggested the possible occurrence of female founder effects in *P. pelagicus*. This may result in limited polymorphism of mitochondrial DNA genes but open the possibility to develop species-diagnostic markers based on mitochondrial DNA polymorphism in this species.

Sequence-specific primers were designed from *COI* (PP-COI_{270}-F/R) and *12S rDNA* (12S rDNA-F/R). The specificity of both primer pairs was tested against

genomic DNA of 174 individuals of *P. pelagicus* and several non-target species. For rapid species-identification of *P. pelagicus*, the tedious and time-consuming phenol/chloroform extraction method was simplified to a rapid 5% chelex-based method. This reduces the operation time and effort for authentication of *P. pelagicus* products particularly when dealing with a large number of specimens.

PP-COI_{270}-F/R provided the positive amplification product in all investigated individuals of the target species without any false negative result. Cross species amplification of PP-COI_{270}-F/R was further examined and it did not generate the false positive in other crab species (*S. oceanica*, *S. serrata*, *S. tranquebarica*, *C. crucifera* and *P. sanguinolentus*). Accurate and convenient species-specific PCR based on *COI* polymorphism (presence/absence of the amplification band) was successfully developed in *P. pelagicus*.

In contrast, *12S rDNA* of *P. pelagicus* amplified using universal primers (12S rDNA-F/R) provided the positive amplification product of 406 bp in both target and non-target species. Although SSCP could differentiate all investigated species unambiguously, the amplification success in *P. pelagicus* was quite low. As a result, this marker was not directly applied for species identification of *P. pelagicus*.

Although a species-specific PCR was successfully developed for identification the species-origin of *P. pelagicus*, application of the developed *PP-COI_{270}* marker for detection of crab meat in canned products is limited in practice. In contrast to other commercially important species (e.g. shrimp, oysters and abalone) where the major part or the whole individuals of wrong species are intentionally supplied, the meat of *P. pelagicus* meat may be mixed with that of a few non-target crab species. Therefore, molecular markers that can be used for amplification of a gene of various species should be firstly amplified and sequence polymorphism should be further differentiated by the appropriate approach.

A pair of primer for generating a 312 bp fragment (*PP-$12S_{312}$*) was designed and tested. Non-overlapping SSCP genotypes were found between *P. pelagicus* and other crab species. Therefore, a species-diagnostic marker for identification of contaminated crab meat using SSCP analysis was also successfully developed. The suspected specimens should be collected from different positions in the can to ensure

that specimens are appropriately collected. This sampling strategy would promote the practical use of the newly developed markers.

Previously, species-diagnostic SCAR markers were successfully derived from RAPD (PP122-510, PP158-1200 and PP158-1500, Khetpu, 2005) and AFLP (BSCSCAR2, Klinbunga *et al*., 2007) fragments. In this study, additional species-diagnostic SCAR markers of *P. pelagicus* were successfully developed using species-specific PCR of *PP-COI*$_{270}$ and PCR-SSCP analysis of *PP-12S*$_{312}$. Results from this and previous studies indicated that sufficient numbers of species-diagnostic markers have been developed in *P. pelagicus*. Nevertheless, only *PP-12S*$_{312}$ could be used to illustrate contamination of the meat from different crab species within the same cans.

IV. Isolation of temperature stress response transcripts using cDNA-AFLP

Thermal stress has been considered as one of the serious factors that affect various immune responses and capacities of aquatic animals. Hence, the development of early warning bioindicators to detect the metabolic stress in *P. pelagicus* would be important for improving the efficiency of farming practice for crab culture. Stress-responsive gene transcripts have been given increasing attention as molecular indicators to detect and quantify the stress and risks occurring in aquatic animals. Moreover, little information is available on the expression of genes in *P. pelagicus*.

Picard and Schulte (2004) used differential display (DD) PCR to identify hepatic genes responsive to handling stress and genes that differ in expression between populations of a killifish, *Fundulus heteroclitus*, from different thermal environments. 20 putatively stress-regulated bands from Northern fish were cloned and 10 of which had high similarity to genes of known function. Five of these genes were selected for further analysis based on their known roles in the stress response. Three of these (*glucokinase*, *serine-threonine kinase 10* and *cRAF*) were confirmed as stress-responsive genes using real-time PCR. These genes increased in expression in response to a 7-day chronic stress protocol in fish from the Southern population of *F. heteroclitus*, but did not change significantly in fish from the Northern population. These three genes also differed in expression between populations in control fish, suggesting a link between the response to chronic stress and inter-population

differences in gene expression in unstressed laboratory-acclimated fish. Two genes, *glycogen synthase kinase* and *warm acclimation-related protein* (*WAP*) also differed between populations. Expression of *WAP* was eight-fold higher in Southern than in Northern fish, consistent with a previously suggested role for this gene in thermal acclimation or adaptation in fish.

Gaobeidian Lake, located in Beijing, China, derives its water mainly from the effluent of the Gaobeidian Wastewater Treatment Plant, which is moderately polluted. Additionally, as this water is used as a coolant in the nearby thermal power plant, the water of this lake has an elevated temperature. Suppressive subtractive hybridization (SSH) was performed on RNA in hepatic tissues of the goldfish, *Carassius auratus* from the Gaobeidian Lake using specimens from Huairou Reservoir as the reference. A total of 768 candidate clones were studied. Of these, 264 clones were differentially expressed between the goldfish from the two sites, 124 of which were then subjected to DNA sequencing. Consequently, 36 different genes with known functions were obtained, and some of these differential genes were further confirmed by semiquantitative RT-PCR. Many genes related to detoxification, stress and immune response, and metabolism, such as *glutathione S-transferase* (*GST*), *cytochrome P450, family 2, subfamily b, polypeptide 10* (*CYP2B10*), *CYP2X10*, *α-1-antitrypsin*, and *apolipoprotein A-I* (*Apo-AI*), had higher expression levels in goldfish hepatic tissue from Gaobeidian Lake than those from the reference site. A set of nine genes with known functions were downregulated in Gaobeidian Lake compared to the reference site. The results provided evidence that organisms inhabiting Gaobeidian Lake were suffering a complex stress process and showing metabolism changes and disturbance of homeostasis (Wang *et al*., 2007).

In this thesis, the cDNA-AFLP approach was applied for identification of stress-responsive transcripts in haemocytes of *P. pelagicus*. The clear and attractive advantages of cDNA-AFLP is that very small amounts of total RNA can be used as starting materials. Additionally, the method is highly versatile in that a wide representation of differentially expressed gene fragments can be achieved for multiple conditions by visualization of side-by-side comparisons and that both up-regulation and down-regulation of gene expression can be detected.

The disadvantage of cDNA-AFLP is that samples should be collected with caution because contamination of nucleic acids (both DNA and RNA) from other organisms may interfere the results by generating additional false positive of the desired fragments.

A total of 110 primer combinations were screened. Eighteen primer combinations yielded positive amplification products. On the basis of preliminary screening, several fragments exhibited up- and down-regulated expression patterns. Seven fragments showing differential expression between the normal and the treated crabs were cloned and sequenced. Only 4 newly unidentified transcripts were successfully amplified.

Three cDNA-AFLP fragments that showed down-regulation during preliminary were further analyzed by semiquantitative RT-PCR. The expression patterns of *PP-TSRT*$_{152}$ in haemocytes of *P. pelagicus* upon temperature-stress at 33°C for 3 hours was significant down regulated at 12 hpt ($P< 0.05$). The relative expression level at 24 hpt (0.647 ± 0.043) was also significantly different from that of the normal crab. In contrast, *PP-TSRT*$_{200}$ did not show significantly differential expression at all time intervals after temperature treatment ($P> 0.05$). *PP-TSRT*$_{339}$ exhibited the concordant expression patterns in haemocytes of *P. pelagicus* as previously identified through cDNA-AFLP. *PP-TSRT*$_{339}$ was initially down-regulated and significantly different from that of the control at 12 hpt ($P< 0.05$). The expression levels then returned to the normal level at 24 hpt ($P< 0.05$).

In the present study, temperature stress response transcripts were isolated and characterized using cDNA-AFLP. The primer pairs that generated the positive amplification product against the first strand cDNA template should be tested for the amplification success using genomic DNA of *P. pelagicus*. Polymorphism of the developed markers can be simply tested using SSCP analysis. If the polymorphic levels are sufficiently high, they can be used for population genetic studies. However, polymorphic markers may also be applied for association analysis between SNP in the stress-responsive genes and biologically important parameters (e.g. expression levels of the corresponding transcripts or phenotypes). The information can be applied for selective breeding programs of *P. pelagicus* in the future.

CHAPTER V

CONCLUSIONS

1. SSCP analysis using *PP-COI*$_{270}$, *PP-SCARRAP*$_{318}$ and *PP-SCARAFLP*$_{300}$ indicated relatively high genetic diversity of *P. pelagicus* in Thai waters (*N* = 174). The average polymorphic loci and gene diversity across overall samples were 48.57% and 0.1859, respectively.

2. Genetic distance between pairs of geographic samples of Thai *P. pelagicus* was 0.0099 - 0.1198. Larger genetic distance was observed between samples from different coastal regions (0.0346 - 0.1198) than that between geographic locations within coastal regions (0.0099 – 0.0198 and 0.0135 for Gulf of Thailand and Andaman samples, respectively).

3. Significant geographic heterogeneity was observed across overall samples ($P < 0.01$) and between all possible pairwise comparisons of conspecific samples of *P. pelagicus* ($P < 0.01$). Results indicated that the gene pool of *P. pelagicus* in Thai waters is not homogeneous but genetically fragmented at the microgeographic level.

4. The estimated gene flow levels between pairs of Thai *P. pelagicus* samples was 0.39 – 5.37 individuals per generation suggesting that *P. pelagicus* is a low gene flow species.

5. A UPGMA dendrogram constructed from the average unbiased genetic distance between pairs of geographic samples of Thai *P. pelagicus* allocated 5 investigated samples to two evolutionary groups; Chanthaburi, Suratthani and Prachuap Kriri Khan (Gulf of Thailand, A) and Ranong and Krabi (Andaman Sea, B).

6. Species-specific PCR for authentication of *P. pelagicus* were successfully developed. PP-COI$_{270}$-F/R provides the positive amplification product in all investigated individuals of *P. pelagicus* (*N* = 174) without any false positive in other crab species; *S. oceanica* (*N* = 18), *S. serrata* (*N* = 7), *S. tranquebarica* (*N* = 9), *C. crucifera* (*N* = 20) and *P. sanguinolentus* (*N* = 10).

7. PP-12S$_{312}$-F/R showed 100% amplification success in *P. pelagicus* (*N* = 174) and all non-target species; *S. oceanica* (*N* = 18), *S. serrata* (*N* = 7), *S. tranquebarica* (*N* = 9), *C. crucifera* (*N* = 20) and *P. sanguinolentus* (*N* = 10). SSCP analysis was further carried out and unambiguously differentiated all crab species accurately.

8. Temperature-stress response transcripts (*TSRT*) in haemocytes of *P. pelagicus* were identified by cDNA-AFLP. Seven cDNA-AFLP fragments were cloned and sequenced. Expression levels of three *TSRTs* (*PP-TSRT$_{152}$*, *PP-TSRT$_{200}$* and *PP-TSRT$_{339}$*) were examined by semiquantitative RT-PCR. *PP-TSRT$_{152}$* and *PP-TSRT$_{339}$* were significantly downregulated at 12 and 24 hpt and 12 hpt, respectively ($P < 0.05$).

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APPENDIX

APPENDIX

Chemicals for Preparation of Polyacrylamide Gels and Silver Staining

1. 4.5% Denaturing acrylamide solution, 500 ml

Acrylamide	21.375	g
Bis-aclylamide	1.125	g.
7 M urea	210	g.

2. 40% acrylamide solution (crosslink = 37.5:1), 500 ml

Acrylamide	194.80	g.
Bis-aclylamide	5.19	g.

3. 40% acrylamide solution (crosslink = 75:1), 500 ml

Acrylamide	78.94	g.
Bis-aclylamide	1.052	g.

4. Fix/stop solution (10% glacial acetic acid), 2 liters

Glacial acetic acid	200	ml
Ultrapure or deionized water	1800	ml

5. Staining solution, 1.5 liters

$AgNO_3$	1.5	g.
37% Formaldehyde	2.25	ml

6. Developing solution, 3 liters

Na_2CO_3	90	g.
37% Formaldehyde	5	ml
Sodium thiosulfate (10 mg/ml)	600	µl

BIOGRAPHY

Miss Natechanok Thamniemdee was born on November 27, 1981 at Angthong Province, Thailand. She graduated with degree of Bachelor of Science (Biology) from Mahidol University in 2003. She has studied for the degree of Master of Science (Biotechnology) at the Program in Biotechnology, Faculty of Science, Chulalongkorn University since 2005.

Publications from this thesis

1. **Thamniemdee, N.**, Khamnamtong, B., Klinbunga, S. and Menasveta, S. (2006). Genetic diversity and population structure of the blue swimming crab *Portunus pelagicus* determined by SSCP analysis. 32nd Congress on Science and Technology of Thailand, 10–12 October 2006, Queen Sirikit National Convention Hall, Bangkok, Thailand (Oral presentation).

2. **Thamniemdee, N.**, Khamnamtong, B., Klinbunga, S. and Menasveta, S. (2007). Development of species-specific markers of the blue swimming crab (*Portunus pelagicus*) determined by SSCP analysis. 33rd Congress on Science and Technology of Thailand, 18–20 October 2007, Walailak University, Nakhon Si Thammarat, Thailand (Oral presentation).