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GENETIC DIVERSITY OF THE BLUE SWIMMING CRAB *Portunus pelagicus* IN THAILAND ANALYZED BY AFLP AND RAPD

Miss Kannika Khetpu

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ทำการศึกษาความหลากหลายทางพันธุกรรมและความแตกต่างประชากรพันธุศาสตร์ของปูม้า (*Portunus pelagicus*) ด้วยเทคนิค RAPD และ AFLP สำหรับการวิเคราะห์ด้วยเทคนิค RAPD พบแถบ RAPD จำนวนทั้งหมด 112 แถบ จากตัวอย่างปูม้าที่ทำการศึกษาจำนวน 109 ตัว ด้วยไพรเมอร์ OPA02, OPA14, OPB10, UBC122 และ UBC158 โดยมีเปอร์เซ็นต์ของแถบที่ polymorphic ในแต่ละกลุ่มตัวอย่างเท่ากับ 72.23% - 85.05% โดยมีค่าเฉลี่ยระยะห่างทางพันธุกรรมระหว่างกลุ่มตัวอย่างในทุกไพรเมอร์ คือ 0.1151 - 0.2440 ผลจากการวิเคราะห์ geographic heterogenity โดยวิธี exact test และค่าสถิติ F_{ST} แสดงความแตกต่างอย่างมีนัยสำคัญทางสถิติ (P<0.001) ระหว่างทุกคู่ตัวอย่างที่ทำการเปรียบเทียบ โดยเป็นความแตกต่างทางพันธุกรรมในระดับกลุ่มตัวอย่างที่ทำการศึกษา ไม่ใช่ระดับฝั่งทะเลอันดามันและอ่าวไทยดังที่พบในสัตว์ทะเลชนิดอื่นๆที่ทำการศึกษาก่อนหน้านี้ นอกจากนี้ยังพบแถบ RAPD จำนวน 15 แถบที่บ่งชี้ถึงความจำเพาะต่อปูม้า ซึ่งทำการโคลนและหาลำดับนิวคลีโอไทด์ของชิ้น RAPD จำนวน 8 แถบ และทำการออกแบบไพรเมอร์จำนวน 5 คู่ไพรเมอร์ โดยพบว่าเครื่องหมายพันธุกรรมจำนวน 3 เครื่องหมาย (pPP122-510, pPP158-1200 และ pPP158-1500) ให้ผลิตภัณฑ์ลูกโซ่โพลิเมอเรสตรงตามที่คาดหมายไว้ (152, 397 และ 262 bp) 95%, 100% และ 100% ตามลำดับในปูม้าและไม่พบผลิตภัณฑ์ดังกล่าวในปูชนิดอื่นที่ทำการศึกษา เมื่อทำการทดสอบความว่องไวของปฏิกิริยา พบว่าไพรเมอร์ดังกล่าวสามารถใช้ตรวจสอบปริมาณดีเอ็นเอในระดับต่ำสุดที่ 400 pg, 200 pg และ 12.21 pg ตามลำดับ สำหรับการทดสอบความคงที่ของปฏิกิริยากับ genomic DNA ที่ได้จากเนื้อปูม้าแช่แข็ง, เนื้อปูม้าในน้ำเกลือ และเนื้อปูม้าที่ผ่านการต้ม โดยทำการสกัดด้วยวิธี phenol-chloroform และ การใช้ chelex สามารถพบผลิตภัณฑ์ลูกโซ่โพลิเมอเรสในทุกตัวอย่าง แม้ว่าแหล่งที่มาของ genomic DNA และวิธีการสกัดที่ใช้จะแตกต่างกัน

จากการศึกษา AFLP ในตัวอย่างจำนวน 72 ตัว พบความหลากหลายทางพันธุกรรมสูงในปูม้า ด้วย 4 คู่ไพรเมอร์ (P_{+3}-2/M_{+3}-1, P_{+3}-4/M_{+3}-1, P_{+3}-4/M_{+3}-2 และ P_{+3}-7/M_{+3}-1) โดยพบแถบ AFLP จำนวน 227 แถบ และมีเปอร์เซ็นต์ของแถบที่ polymorphic ในแต่ละกลุ่มตัวอย่างเท่ากับ 66.19% - 94.38% โดยมีค่าเฉลี่ยระยะห่างทางพันธุกรรมระหว่างกลุ่มตัวอย่างในทุกไพรเมอร์ คือ 0.0929 - 0.2471 ผลวิเคราะห์ค่า geographic heterogenity ของข้อมูล AFLP ด้วยวิธี exact test และค่าสถิติ F_{ST} แสดงความแตกต่างทางพันธุกรรมอย่างมีนัยสำคัญทางสถิติ (P<0.001) ระหว่างทุกคู่ตัวอย่างที่ทำการเปรียบเทียบเช่นเดียวกับผลจาก RAPD นอกจากนี้ยังพบแถบ AFLP จำนวน 13 แถบที่บ่งชี้ถึงความจำเพาะต่อสปีชีส์ ซึ่งทำการโคลนและหาลำดับนิวคลีโอไทด์ของชิ้น AFLP จำนวน 2 แถบ จากนั้นทำการออกแบบไพรเมอร์จากลำดับนิวคลีโอไทด์ดังกล่าว โดยไพรเมอร์จาก pP4M1-300 ให้ผลิตภัณฑ์ลูกโซ่โพลิเมอเรสในปูม้าคิดเป็น 30% สำหรับไพรเมอร์จาก pP4M2-420 ให้ผลิตภัณฑ์ลูกโซ่โพลิเมอเรสในปูม้าคิดเป็น 97% อย่างไรก็ตาม เครื่องหมาย pP4M2-420 สามารถให้ผลิตภัณฑ์ได้ในปูม้าลาย (N = 6) แต่ไม่ให้ผลิตภัณฑ์ดังกล่าวในปูทะเล (N = 38) จากการวิเคราะห์ด้วย SSCP พบว่าสามารถแสดงให้เห็นถึงรูปแบบที่เฉพาะของปูม้าได้ โดยความว่องไวของปฏิกิริยาจาก pP4M2-420 สามารถตรวจสอบปริมาณดีเอ็นเอต่ำสุดได้ในระดับ 400 pg อีกทั้งยังมีความคงที่ของปฏิกิริยาในระดับสูง เช่นเดียวกับเครื่องหมายโมเลกุลที่พัฒนามาจาก RAPD

ลายมือชื่อนิสิต.....กรรณิการ์ เกตุภู่.....

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

ปีการศึกษา.....2548.....ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

4672203623 : MAJOR BIOTECHNOLOGY
KEY WORD: *Portunus pelagicus* / RAPD / AFLP / GENETIC DIVERSITY / SCAR MARKERS

KANNIKA KHETPU: GENETIC DIVERSITY OF THE BLUE SWIMMING CRAB *Portunus pelagicus* IN THAILAND ANALYZED BY AFLP AND RAPD. THESIS ADVISOR: PROF. PIAMSAK MENASVETA, Ph.D., THESIS CO-ADVISOR: SIRAWUT KLINBUNGA, Ph.D, 115 pp. ISBN 974-14-2314-4.

Genetic diversity and population differentiation of the blue swimming crab (*Portunus pelagicus*) was analyzed by RAPD and AFLP analyses. A total of 112 RAPD fragments were generated from analysis of 109 individuals of *P. pelagicus* with OPA02, OPA14, OPB10, UBC122 and UBC158. The percentage of polymorphic bands in each sample was 72.73% - 85.05% The mean genetic distance between samples across overall primers was 0.1151 - 0.2440. Results from geographic heterogeneity analysis using the exact test and F_{ST}-based statistics (θ) indicated statistically significant differences between all pairwise comparisions ($P < 0.001$) indicating microgeographic differentiation of investigated samples. Fifteen RAPD fragments were regarded as candidate species-specific fragments. Eight of these were cloned and sequenced. Five pairs of primers were designed. Three RAPD-derived SCAR markers (pPP122-510, pPP158-1200 and pPP158-1500) generated the expected product (152, 397 and 262 bp) in 95%, 100% and 100% in the target species without any false positive in the non-target species. The sensitivity of detection was approximately 400 pg, 200 pg and 12.21 pg, respectively. Stability of *P. pelagicus*-specific markers was tested against genomic DNA of frozen, saline-preserved and boiled P. pelagicus meat using either a standard phenol/chloroform or a chelex-based methods. The positive amplification product was consistently found in all specimens regardless sources of genomic DNA and extraction methods.

High genetic diversity was also observed when analyzed 72 individuals of *P. pelagicus* with 4 primer combinations (P_{+3}-2/M_{+3}-1, P_{+3}-4/M_{+3}-1, P_{+3}-4/M_{+3}-2 and P_{+3}-7/M_{+3}-1). A total of 227 AFLP fragment were generated and the percentage of polymorphic band in each samples was 66.19% - 94.38%. The mean genetic distance between paired sample was 0.0929 - 0.2471. Like results from RAPD analysis, geographic heterogeneity analysis of AFLP data using the exact test and F_{ST}-based statistics (θ) indicated statistically significant differences between all pairwise comparisions ($P < 0.001$). This also indicated a fine scale genetic differentiation in this species. Thirteen candidate species-specific AFLP fragments were found. Two of these were cloned and sequenced. A primer pair was designed from nucleotide sequence of each fragment. One AFLP-derived SCAR marker (P4M1-300) gave the positive amplification product in 30% of investigated *P. pelagicus* individuals while the other (pP4M2-420) consistently generated the expected product in 97.0% of the target species. Nevertheless, the positive amplification product of pP4M2-420 was cross amplified with genomic DNA of *C. crucifera* ($N = 6$) but not mud crabs ($N = 30$). SSCP analysis was successfully applied to further authenticate the species origin of *P. pelagicus*. The sensitivity of detection of pP4M2-420 was approximately 400 pg of the target DNA template. High stability of the developed markers was also observed in frozen, saline-preserved and boiled *P. pelagicus* specimens.

Student's signature K. Khetpu

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

Academic year……2005……Co-advisor's signature……

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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
DNA	deoxyribonucleic acid
HCl	hydrochloric acid
IPTG	isopropyl-thiogalactoside
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
Tris	tris (hydroxyl methyl) aminomathane
μg	microgram
μl	micromolar
UV	ultraviolet

CHAPTER I

INTRODUCTION

1.1 General introduction

The blue swimming crab (*Portunus pelagicus*) is a large, edible species inhabiting coastal areas throughout the Indo-West Pacific region, from east Africa to Japan and northern New Zealand (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.

The blue swimming crab is one of economically important aquatic species in Thailand. Commercial fisheries of the blue swimming have rapidly expanded since the last few years. The export volume of the blue swimming crab was approximately 2883 metric tones in 2002 and increased to 4217, 6544 and 6978 metric tons in 2003, 2004 and 2005, respectively producing the income of approximately 1000 million baht annually (Table 1.1 and Fig. 1.1). The occurrence of a large number of smaller sizes in the catch at present suggested that the blue swimming crab has been overexploited. Therefore, the development of aquaculture of this species would significantly reduce the harvest of natural resources. To sustain both the fisheries and aquaculture production, several disciplines including effective fisheries management and appropriate breeding programs need to be established.

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). Moreover, fishery management also requires a basic understanding of biological principles e.g. ecology, population structure and dynamics.

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

Table 1.1 Production volume and value of export crab meat in 2002-2005

Years	Volume (tones)	Value (million Baht)
2002	2,883	1,012.3
2003	4,217	988.5
2004	6,544	1,237.8
2005	6,978	1,228.0

Source: Ministry of Commerce Thailand

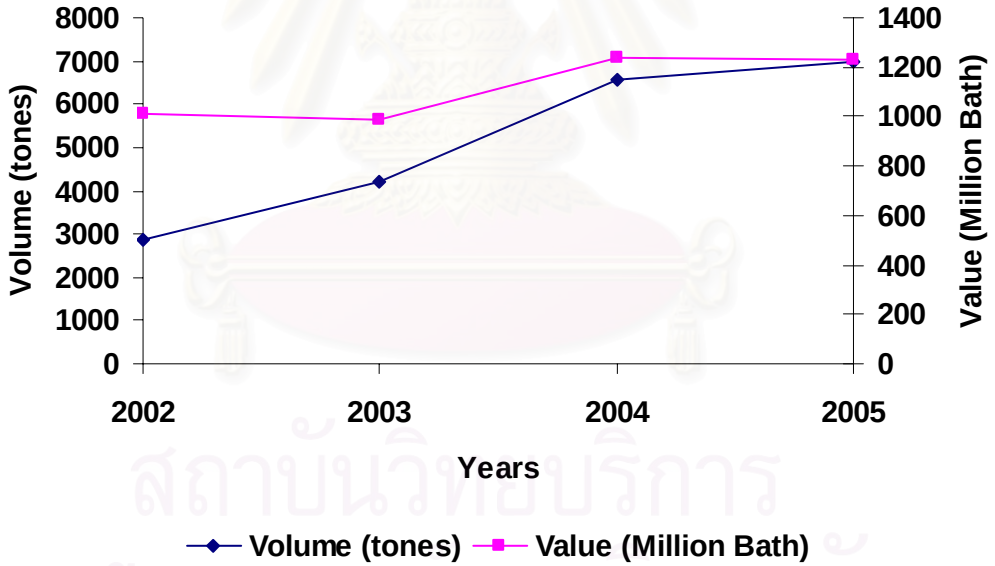


Figure 1.1 Production volume and value of export crab meat in 2002-2005

for its effective genetic-based management. Fishery managers need information on size and number of populations in an 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. Appropriate genetic markers can be used to elevate culture and management efficiency of economically important species in Thailand. However, there have been no publications concerning genetic diversity and population structure of *P. pelagicus*. This information is essential for the construction of appropriate breeding programmes and for broodstock selection and management scheme (stock structure analysis and phylogenetic studies) leading to sustainable culturing activity of *P. pelagicus* in Thailand. Additionally, species-specific markers can be used for quality control to prevent supplying incorrect species for canned products.

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*, Species *pelagicus*. The scientific name of this species is *Portunus pelagicus* (Linnaeus),1766 (Figure 1.2) and common name is blue swimming crab or sand crab.

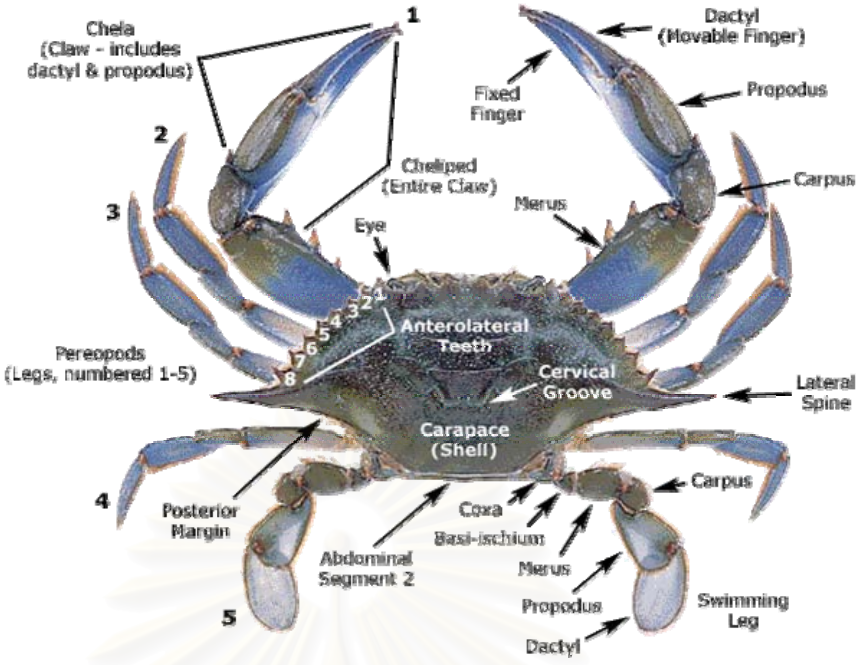
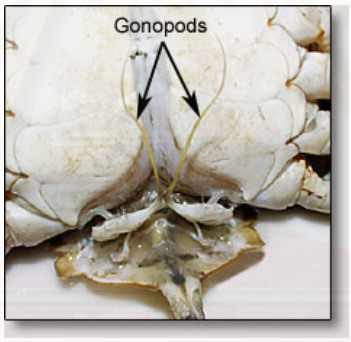
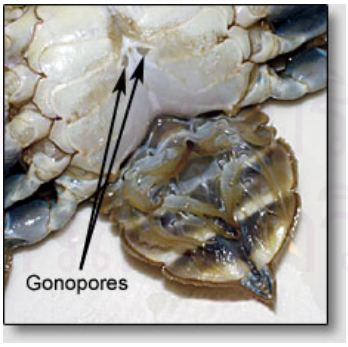


Figure 1.2 External anatomy of *P. pelagicus*



Male



Female

Figure 1.3 Sexes of male and female *P. pelagicus* identified by positions of gonopores and characters of abdomen.

1.3 Molecular technique commonly used for studying genetic diversity at the DNA level

All organisms are subject to mutation as a result of normal cellular operations or interactions with the environment, leading to genetic variation (polymorphism). 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 at present), insertions or deletions of nucleotide sequence (indels) within a locus, and rearrangement of DNA segments around a locus of interest. Genetic variation is evolutionary accumulated through time and should exits in any given species.

After the introduction of the polymerase chain reaction (PCR), several molecular techniques for determination of DNA polymorphism have been developed and have been successfully used for population genetics and systematics of various organisms.

PCR is a method for an *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. 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 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 heat-stable DNA polymerase. The cycle is repeated for 30-40 times (Fig.1.4). The amplification product is determined by gel electrophoresis.

DNA marker technology can be applied to examine mutations. Large deletions and insertions (indels) cause shift in the size of DNA fragments. Smaller indels require DNA sequencing or more elaborate electrophoretic techniques to determine smaller changes in size. Base substitutions can be examined by restriction enzymes

after PCR amplification. Inversions and rearrangements that involve restriction sites can be easy to detect 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.

Several marker types are commonly used in population genetics. Previously, allozymes has been popular in genetic research. Nevertheless, allozyme analysis underestimates the genetic diversity level due to synonymous mutations and nonsynonymous mutations from non-charged to the other non-charged amino acid residues. Presently, PCR based techniques for instance, PCR-restriction fragment length polymorphism (PCR-RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), single strand conformational polymorphism (SSCP) are commonly used for genetic diversity and population differentiation in various taxa.

1.3.1 Randomly amplified polymorphic DNA (RAPD)

RAPD procedures were independently developed by Welsh and McClelland (1990) and Williams et al. (1990) by using PCR to randomly amplify anonymous segments of nuclear DNA with a single primers (10 - 12 bp in length, Fig.1.5). Because the short primers exhibiting relatively low annealing temperatures (often 36-40^{o}C) are used, the likelihood of successful amplifying multiple products is high, with each product (presumably) representing a different locus. Because most of the nuclear genome in vertebrates is non-coding, it is presumed that most of the amplified loci are selectively neutral.

Genetic variation and divergence within and between interesting taxa are assessed by the presence or absence of each amplification product. RAPD polymorphisms can occur due to base substitutions at the primer binding sites or to indels in the regions between the sites (Fig.1.6). The potential power of RAPD for detection of polymorphism is relatively high; typically, 5-20 bands can be produced using a given primer, and multiple sets of random primers can be used to scan the

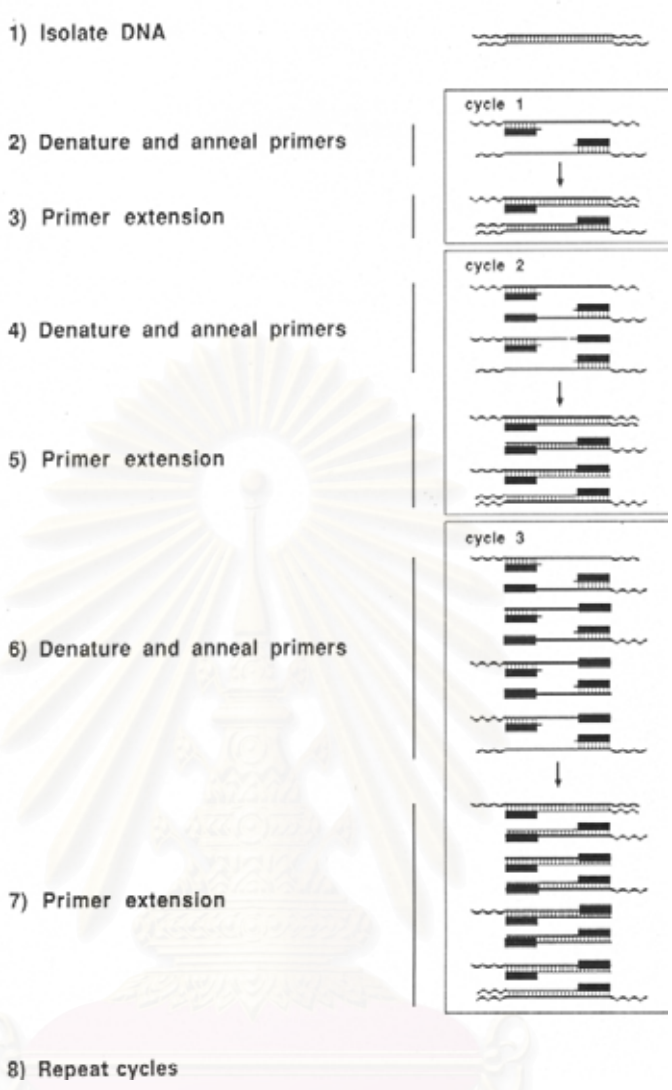


Figure 1.4 Polymerase chain reaction (PCR) for amplifying DNA.

entire genome for differential RAPD bands. RAPD markers are inherited as Mendelian marker in a dominant fashion and each band is considered a bi-allelic locus (presence or absence of an amplified product).

Distinguishing homozygous dominant from heterozygous individuals is not generally possible by RAPD analysis owing to the efficiency of PCR amplification. In addition, it is difficult to determine whether bands represent different loci or alternative alleles of a single locus, so that the number of loci under study can be

erroneously assessed particularly when polymorphism caused by deletion or insertion within the locus rather than at the primer binding sites.

The main advantages of RAPD is that unlimited number of primers available commercially. In addition, prior knowledge of the target DNA sequence or gene organization under investigation is required. Multilocus amplification product can be simply separated on agarose gels and stained with ethidium bromide. Alternatively, higher resolution of RAPD bands can be achieved by polyacrylamide gel electrophoresis and silver staining but they are more tedious and time consuming.

Other advantages of RAPD are a large number of loci with different evolutionary rates and a large number of individuals can be screened. RAPD has been successfully simultaneously used for determination of genetic diversity and for species identification in the giant tiger shrimp (Tassanakajon et al., 1998), oysters (Klinbunga et al., 2000 and 2001), and abalone (Klinbunga et al., 2003 and 2004) in Thai waters.

Shortcomings of RAPD include the difficulty of demonstrating Mendelian inheritance of the loci and the inability to distinguish between homozygotes and heterozygotes. In addition, the presence of paralogous PCR product (different DNA regions which have the same lengths and thus appear to be a single locus) limits the use of this marker. Finally, RAPD markers exhibit low reproducibility due to the low annealing temperature used in the PCR amplification.

Accordingly, RAPD markers are usually converted to sequence-characterized amplified region (SCAR) markers through cloning and sequencing of the original marker. A pair of sequence-specific primer is designed and used for amplification of the target fragment.

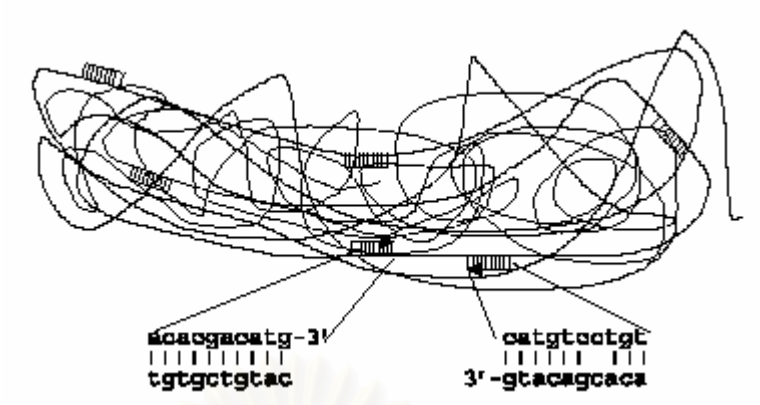


Figure 1.5 Schematic presentation of the RAPD procedure. Genomic DNA (indicated by long strings of lines) is used for PCR using an arbitrary short primers of identical sequences (indicated by short segments annealing to their complementary sites in the genome either perfectly or non-perfectly) under low annealing temperatures. When the two primers bind to sites close enough (often less than 2000 base pairs) on opposite strands of DNA (indicated by arrowed segments with base pairing), a PCR product results.

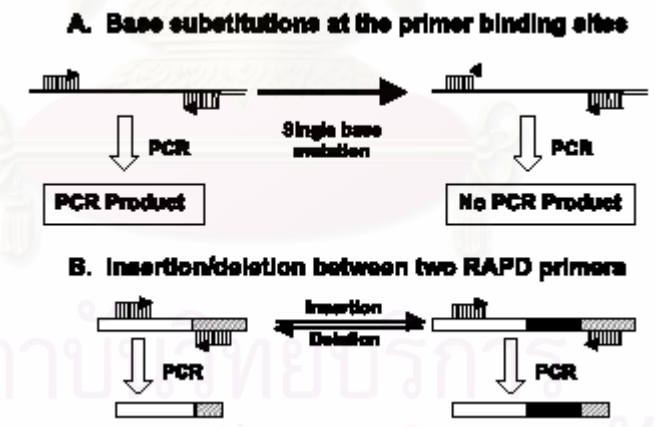


Figure 1.6 Molecular basis of RAPD polymorphism. (A) Base substitutions in the primer binding sites, especially at the 3′ end of the primer binding sites may lead to decrease (as shown) or increase of the number of RAPD bands. (B) Insertion or deletion between two primers may lead to increase or decrease of fragment sizes.

1.3.2 Amplified fragment length polymorphism (AFLP)

AFLP is a PCR-based, multi-locus fingerprinting technique that combines the strengths and overcomes the weaknesses of the RFLP and RAPD methods (Fig.1.7). The molecular basis of AFLP polymorphisms includes indels between restriction sites and base substitutions at restriction sites like RFLP analysis. Moreover, it also includes base substitution at PCR primer binding sites like RAPD. 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 (Vos et al., 1995) begins with digestion of the whole genomic DNA with two enzyme (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 adding known bases to the 3′ of the PCR primers. 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 4-fold. Therefore, adding three bases to each PCR primer should result in a 4096-fold reduction during selective amplification.

Like RAPD, AFLP markers treated as bi-allelic dominant markers, 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, but is difficult for population studies. This can be overcome by converting a band of interest from an AFLP gel to a SACR marker.

The major strengths of the AFLP method include large (over 100) numbers of polymorphic loci screened, high reproducibility due to high PCR annealing temperatures, and relatively cost effectiveness. Like RAPD, it dose not require any prior molecular information about sequence under investigation and thus is applicable to species with the genome sequences are not well characterized.

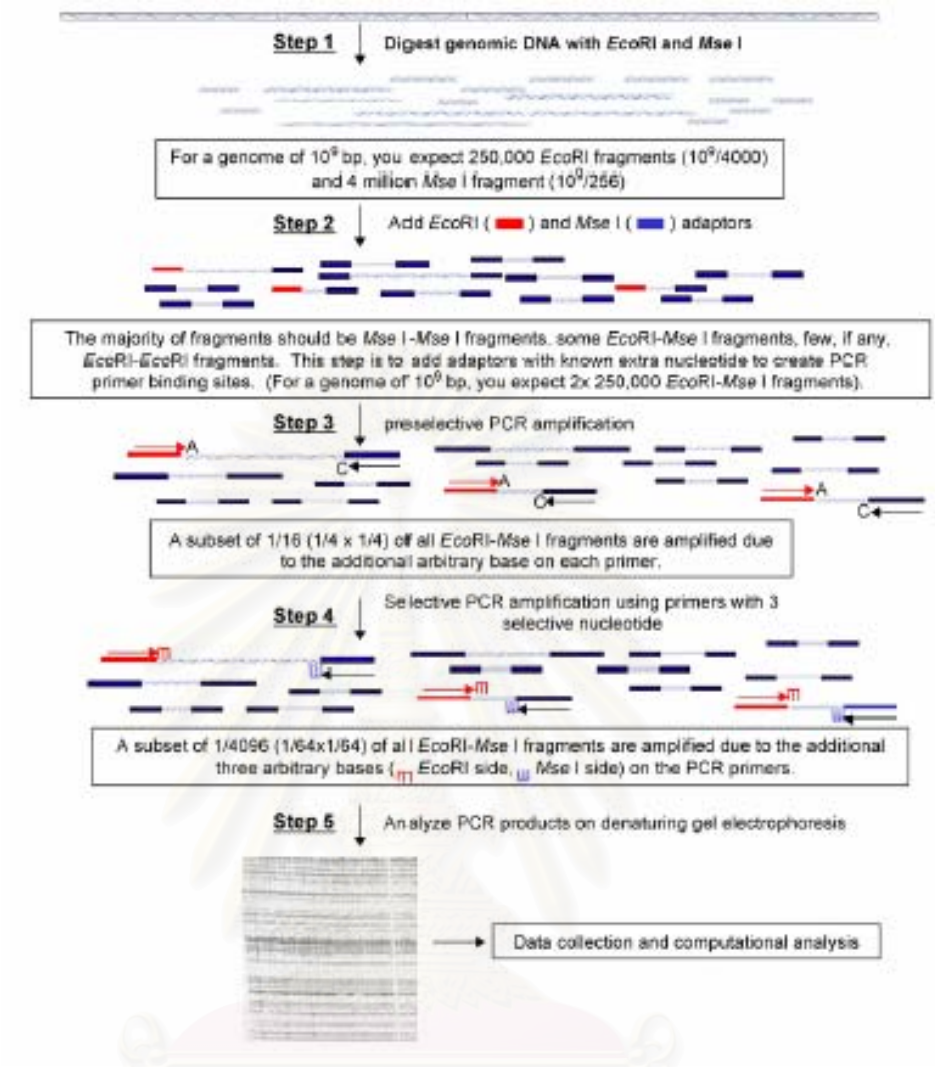


Figure 1.7 A schematic diagram illustrating principles of AFLP analysis.

1.3.3 Single-stranded conformation polymorphism (SSCP) analysis

SSCP is one of the most widely used for the detection of mutations and variation of the DNA (deletions, insertions and single nucleotide polymorphism, SNP). The amplified PCR product is denatured and loaded into low crosslink non-denaturing polyacrylamide (with or without glycerol supplementation).The principle of this technique relies on different mobility due to differential folding of the single stranded DNA (Fig.1.8).

The major advantage of SSCP is that a large number of individual may be simultaneously genotyped. Heteroduplexes can occasionally resolve from heteroduplexs and 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. 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.

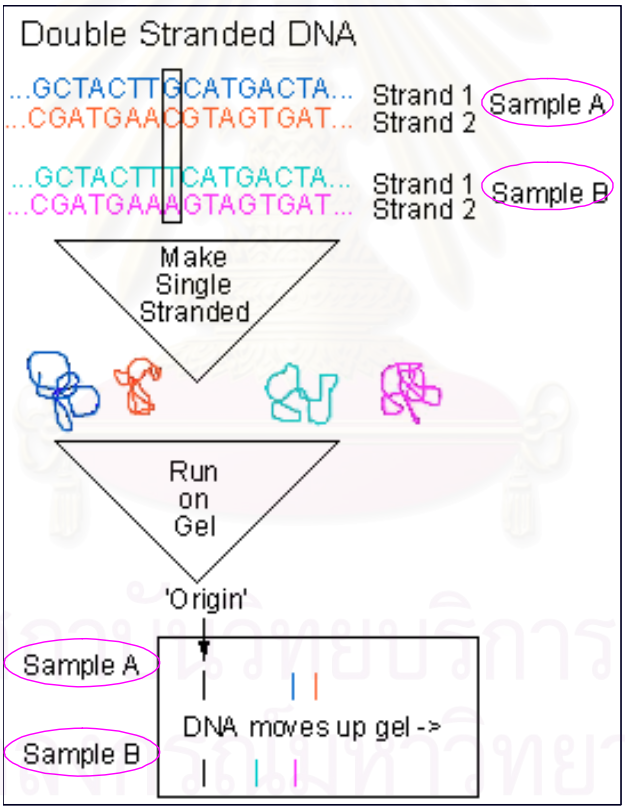


Figure 1.8 A schematic diagram of SSCP analysis.

1.3.4 DNA sequencing

DNA sequencing is the process of determining the exact order of the bases A, T, C and G in a piece of DNA. In essence, the DNA is used as a template to generate a set of fragments that differ in length from each other by a single base. The fragments are then separated by size, and the bases at the end are identified, recreating the original sequence of the DNA.

There are two general methods for sequencing of DNA segments: the “chemical cleavage” procedure was described by Maxam and Gilbert, 1977 and the “chain termination” procedure was described by Sanger, 1977. Nevertheless, the latter method is more popular because chemical cleavage procedure requires the use of several hazardous substances. 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.

DNA sequencing is the molecular technique for determining sequence of a piece of DNA. This technique provides high resolution and facilitating interpretation. However, sequencing of a large number of individuals using conventional method is extremely tedious and prohibitively possible. The sequencing method has been facilitated by the direct and indirect use of DNA fragments generated through PCR. At present, automatic DNA sequencing has been introduced (Fig. 1.9) and commonly used. This greatly allows wider application of DNA sequencing analysis for population genetic and systematic studies.

1.4 Population genetic studies of marine organism

One of the most important goals for fishery management is to primarily obtain detailed information of genetically different stocks and understand interactions among stocks. The basic information on the number of genetic populations of exploited species in a particular area is of importance for broodstock selection and breeding programs (Carvalho and Hauser, 1994)**.** Knowledge of the genetic diversity of blue swimming crab is essential to construct an appropriate management scheme in these taxa.

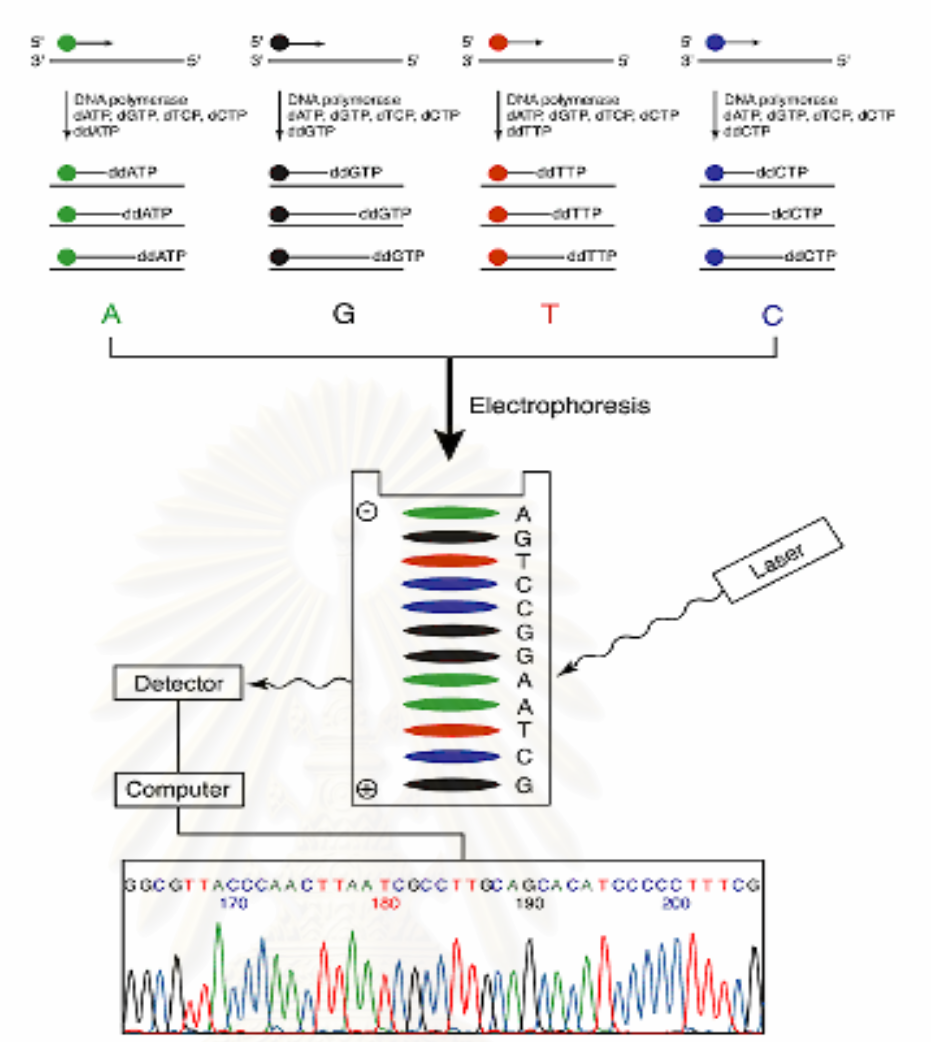


Fig. 1.9 Automated DNA sequencing.

1.4.1 Genetic diversity studies and development of species-specific marker of marine species based on RAPD analysis

Zhou et al., (2001) converted 3 (RA1-PA, RA2-EF and RA4-D) produced by OPJ01, and 2 RAPD fragments (RA3-PAD and RA5-D) produced by OPJ07 to SCAR markers and used for identification of the specific fragments in gynogenetic clones of silver crucian carp. Only individuals from clones P and A amplified a specific band using a pair of primers from RA1-PA, whereas no product was detected in individuals from clones D, E and F. The expected PCR product was successfully amplified from primers designed from RA2-EF and RA3-PAD. Furthermore, a pair of primers from

RA4-D amplified specific bands only in individuals from clone D, although weak bands could be produced in all individuals of the five clones when lower annealing temperatures were used. Primers from RA5-D amplified genomic DNA of fish from clones P, A, D and E but not F. A 385 bp RA5-D marker showed significant matching with the 5' end of *gonadotropin I-β subunit 2* and *growth hormone* genes. SCAR markers identified in that study offer a powerful and rapid method for discrimination of different fish clones and for genetic analyses that examine their origins and unique reproductive modes in the crucian carp.

Khoo et al., (2002) examined genetic variability within and among feral populations and cultured strains of the guppy (*Poacilia reticulate*) RAPD fingerprinting using 7 primers (GEN1-60-09, GEN2-60-12, OPF-11, OPH-15, OPJ-04, TAA1 and TAA2). Feral guppies were collected from 6 isolated populations (BT, Bukit Timah; NS, Nee Soon; TS, Tuas; MF, Mount Faber; KR, Kranji; LI, laboratory-inbred feral line), while the Tuxedo and Green Variegated strains were sample from 2 guppy farms in Singapore. were employed in this study. Of 228 RAPD fragments detected, 204 (89.47%) were polymorphic and GEN1-6-09 produced the highest number of polymorphic bands. A UPGMA dendogram constructed from pairwise RAPD genetic distances revealed distinct clustering of guppy individuals into their respective populations and strains. The percentage of polymorphic loci ranged from 54.96% (TS) to 68.70% (KR), while the average heterozygosity ranged from 0.220 (GV) to 0.217 (KR). Among populations and strains, *S* ranged from 0.703 (between GV and Li) to 0.809 (between NS and MB). The GV strain *S* was closer to TX (0.784) than to the feral guppies. Bootstrapped genetic distance trees depicted 3 major nodes comprising BT-TS, NS-MF, TX-GV. Principle coordinate analysis also differentiated the 6 feral populations from 2 cultured strains.

Barman et al., (2003) evaluated genetic relationships and diversity of four species of Indian major carps (Family *Cyprinidae*) by RAPD. A total of 40 primers were screened and 34 of which generated reproducible profiles and could be used to identify species-specific RAPD markers among rohu (*Labeo rohita*), kalbasu (*L. calbasu*), catla (*Catla catla*) and mrigal (*Cirrhinus mrigala*). In general, the number of resolved amplified varied from 3 to 10 (0.25 to 1.50 kb in size). The average number of fragments was 7.4 bands for the rohu, 6.5 for the kalbasu and 6.3 for the

catla. The average intraspecific and interspecific genetic similarity indices are 0.893 - 0.975 and 0.190 - 0.336, respectively. The low levels of within-species genetic variation exhibited in farmed stocks of the rohu and the kalbasu are due to their repeated propagation and maintenance with limited number of individuals sampled from the wild for long period of time. Relatively higher levels of intraspecific genetic variation exhibited in the catla and the mrigal may be due to comparatively lower rate of propagation than to either rohu or kalbasu.

Wang et al., (2004) analyzed DNA fingerprinting of 33 selected *Laminaria* (Phaeophyta) male and female gametophytes by RAPD. A total of 233 polymorphic bands were generated from 18 selected primers and 27 RAPD markers were selected for to construct a fingerprinting map for discrimination of each gametophyte. Seven markers ($S30_{500}$, $S51_{1100}$, $S51_{960}$, $S104_{1450}$, $S1501_{2100}$, $S1501_{1450}$, $S1520_{1070}$) were finally used to construct a DNA fingerprint map. Three gametophytes specific RAPD markers ($S1520_{620}$ of 6m, $S1520_{1880}$ of 20m and $S1501_{750}$ of 9m) were cloned and sequenced. The re-examined PCR results were consistent with the original RAPD band, except that a weak band of 3m also was amplified with the SCAR primer of $S1520_{1880}$.

Klinbunga et al., (2001) studied genetic diversity and species-diagnostic markers of 5 oysters in Thailand, *Crassostrea belcheri*, *Crassostrea iredalei*, *Saccostrea cucullata*, *Saccostrea forskali*, and *Striostrea (Parastriostrea) mytiloides*, were investigated by RAPD analysis using OPA09, OPB10, OPB08, UBC210 and UBC220). In a total, 135, 127, and 108 genotypes were observed from primers OPA09, OPB01, and OPB08 and 131 and 122 genotypes from primers UBC210 and UBC220, respectively. Two hundred fifty-four reproducible and polymorphic fragments (200–2500 bp in length) were generated across the 5 investigated species. The average number of bands per primer varied between 12.4 and 32.2. The percentage of polymorphic bands within *Saccostrea* and *Striostrea* (86.21%–99.36%) oysters were much greater than those of *Crassostrea* (53.23%–77.67%) oysters, indicating the lowest level of genetic diversity in the last group. Nine, species-specific markers were found in *C. belcheri*, 4 in *C. iredalei*, and 2 in *S. cucullata* 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. The mean of a ratio

between the number of genotypes generated by each primer and the number of investigated specimens of *C. belcheri* (0.58) was lower than that of the remaining species (0.90–1.00). Genetic distances between pairs of oyster samples were between 0.105 and 0.811. A neighbor-joining tree indicated distant relationships between *Crassostrea* and *Saccostrea* oysters, but closer relationships were observed between the latter and *Striostrea mytiloides*.

1.4.2 Genetic diversity studies and development of species-specific marker of marine species based on AFLP analysis

Han and Ely, (2002) assessed genetic variation across two *Morone* and three *Thunnus* species using AFLP. The AFLP profiles from 23 primers revealed high polymorphic levels in all 5 species. The percentage of primers showing polymorphism across investigated samples was 9%-26% in *Morone* and. 57% - 65% in *Thunnus* whereas the percentage of polymorphic bands was 1% - 6% in *Morone* and 11% - 19% in *Thunnus*. Therefore, there is a higher level of genetic variation within each of the three *Thunnus* species than within either of the *Morone* species. However, very different band patterns were observed between the 2 species by AFLP analysis; many AFLP bands were present in the striped bass but absent in the white bass and *vice versa*. Twenty of the 23 primers revealed informative polymorphism for discriminating between these species. In contrast, all *Thunnus* species were highly polymorphic intraspecifically but there was much less variation among the 3 species. These data provide evidence that *Thunnus* species are closely related throughout their entire genome. In addition, the Atlantic northern bluefin tuna AFLP patterns are more similar to those of the yellowfin tuna than those of the albacore.

Mickett et al., (2003) analyzed genetic diversity of 16 populations of the channel catfish (*Ictalulus punctatus*) in Alabama by 5 primer combination. In total, 454 bands were identified from 282 individuals. All 454 bands were polymorphic, with an average heterozygosity of 0.135. The percentage of polymorphic loci varied greatly within distinct populations (18.6% - 100%). Research populations tended to have lower percentage of loci polymorphic than farm populations. The estimated average F_{ST} statistics across all loci was 0.4456. However, this value dropped to 0.1763 when the Hicks Farm stock was excluded from the analysis. The dendodram constructed from a Jaccard's pairwise similarity coefficient indicated high similarity

among individuals, with some population-specific clusters and with the Hicks Farm stock being highly distinctive. Genetic identity between pairs of populations was high (96%) except with the Hick's Farm population (34% similar to other populations). Results of this study suggested that many channel catfish farm strains in Alabama are genetically similar, but some very distinct differences exist. Such information has implications for future broodstock selection and management and for further analysis of interactions between domestic and wild populations of the channel catfish.

Zhang et al., (2004) characterized 11 *Lutjanus* species captured in Nansha coral reefs by AFLP analysis. The AFLP patterns for the larvae and juveniles of the same species were 95.45%-100% similar. IN total, there were 132 AFLP loci based on the size of AFLP fragments, and the number of AFLP fragments for each species varied from 44 to 69, but only 7 markers were fixed in all specimens. The levels of interspecific hetorogenenity are sufficient to classify the specie origin of all larvae. The high levels of intraspecific homogeneity demonstrated the ongoing gene flow among individuals of same *Lutjanus* species in Nansha coral reefs. Owing to the high genetic information, phylogenetic trees (neighbor-joining and UPGMA) constricted from AFLP fingerprints has been used to infer phylogenetic relationships of these closely related species based on measures of genetic distance. The phenetic analyses of phylogenic relationships correspond well to the grouping of species in the Allen's morphology-based system.

Wang et al., (2004) explored the use of an AFLP technology in species identification and phylogenetic analysis of penaeid shrimp. AFLP fingerprinting of six *Penaues* species, *P. monodon, P. chinensis, P. merguiensis, P. latisulcatus, P. canaliculatus and P. japonicus* was compared using three sets of selective primers for PCR amplification. A total of 443 bands (size range 80 - 550 bp) were generated. Eight bands (1.8%) were found in all species, but only three of them (0.7%) were existent in all individuals. The level of polymorphic band differed among species and ranged from 24.6% in *P. canaliculatus* to 60.8% in *P. japonicus*. Species-specific markers with potential for genetic identification of species were identified. The average intraspecific genetic distances varied from 0.0023 in *P. chinensis* to 0.0068 in *P. japonicus*, while the average distances between species were 0.0207 to 0.0324. Phylogenetic analysis allocated six species into two major clades. The first clade

consisted of *P. monodon, P. chinensis* and *P. merguiensis*, with the latter two species more closely related. The other clade comprised *P. latisulcatus, P. canaliculatus* and *P. japonicus*. This result based on AFLP is consistent with the segregation of two distinct clades of *Penaeus* as previously documented by mitochondrial DNA analysis.

Liu et al., (2005) used 10 different AFLP primer combinations to evaluate genetic characteristics of one common population and two selective hatchery populations of the flounder (*Paralichthys olivaceus*). Within populations, 60.92 - 65.78% of fragments were polymorphic. The average heterozygosity of common, susceptible and resistant populations showed no significant differences (*H*: 0.1586 - 0.1656). The two selective hatcheries populations, susceptible and resistant, showed significant genetic differences with the common population including a smaller ($P < 0.05$) number of total loci, a smaller ($P < 0.05$) number of total polymorphic loci and smaller ($P < 0.05$) percentage of low frequency (0 - 0.2) polymorphic loci. The UPGMA dendrogram showed a closer relationship of the susceptible population and the common population than did the resistant population.

Simmons et al., (2006) analyzed genetic diversity of wild channel catfish (*Ictalurus punctatus*) populations by AFLP and compared to that of domestic catfish populations to determine the genetic impact of domestic catfish on wild catfish populations. Fourteen wild populations within various watersheds of Alabama were analyzed using five AFLP primer combinations. A total of 396 polymorphic bands were detected from 269 individuals, with an expected mean heterozygosity of 0.16. The percentage of polymorphic bands varied greatly (32.2% - 85.0%) among populations. The estimated level of population differentiation as measured by average F_{ST} value across all loci was 0.36. The 14 tested wild populations were exhibited Nei's unbiased population-wise identity of 88%. In order to analyze the interactions between domestic and wild populations, 191 polymorphic bands were used to evaluate 569 individuals from 31 populations. The wild populations exhibited higher levels of polymorphism and heterozygosities than did the domestic populations. Strong genetic structures were associated with the geographical distribution of sample, with all samples from a single watershed being closely related. The domestic populations were all related to one another, while all but the Tennessee River populations of many wild populations were more related to one another than to

domestic populations. Genetic identities of wild fish from proximal and distal sites were similar, while both wild populations differed from the nearby domestic catfish populations.

14.3 Molecular genetic studies of marine crabs

Klinbunga et al. (2000) determined genetic diversity of three mud crab species, *Scylla serrata*, *S. oceanica*, and *S. tranquebarica*, collected from two locations in eastern Thailand by RAPD-PCR. Ninety-one reproducible RAPD fragments, generated by UBC456, UBC457, and YNZ22, were polymorphic. The percentage of polymorphic bands for each primer was extremely high (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* and *S. oceanica* could be indicated by the existence of a 725-bp band from YNZ22 in the former but absence in the latter species. 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 this area should be recognized as three different species rather than a single panmictic species exhibiting different morphs.

Fratini and Vannini (2002) determined genetic differentiation in the mud crab *S. serrata* (Decapoda: Portunidae) within the Indian Ocean (N = 77) using sequences of cytochrome oxidase subunit I (COI, 535 bp) gene segment of *S. serrata* 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. Analysis of molecular variance (AMOVA), F_{ST} statistics and χ^2 contingency analysis of spatial distribution of mtDNA haplotype frequencies revealed a significant genetic differentiation among populations. These results indicated that gene flow might be reduced, even between geographically close sites, despite the high potential for dispersal. The recorded level of divergence and the abundance of rare haplotypes and

singletons in our data set suggested that repeated sampling over time is necessary to establish whether the recorded pattern of genetic differentiation is stable and biologically significant.

Steven et al., (2005) isolated and characterized microsatellites in the blue crab (*Callinectes sapidus*) by hybridization of dinucleotide and tetranucleotide oligonucleotide probes to AFLP fragments (Fast Isolation by AFLP of Sequences Containing repeats, FIASCA). A total of 34 microsatellite loci flanking unique microsatellite sequences were obtained. Tem primer pairs of microsatellites were genotyped across a captive-mated pair and their 30 offspring and eight loci inherited a Mendelian fashion. Other two loci were monomorphic. The developed primers were fluorescently labeled and genotyped three sets of the blue crab. The first two sets of crabs collected from two different portions of the Chesapeake Bay (N = 73 from Maryland and 29 from Virginia). Heterozygosities between two populations were similar at most loci. Allele frequencies at CSC-001 CSC-004 and CSC-007 of the Maryland population and CSC-094 and CSA-073 of the Virginia population deviated from Hardy-Weinberg equilibrium. No significant differences in allele frequency distribution was observed at CSC-007, CSA-073 and CSA-0121 or MIH-SSR but significant genetic heterogeneity was found at CSC-001 (P = 0.00086), CSC-004 (P = 0.00843), CSC-094 (P < 0.00001) and CSA-035 (P = 0.00005). Seven loci displayed private alleles between compared populations. However, these may be resulted from limited sample sizes used in the experiment.

Place et al., (2005) determined complete mitochondria genome sequence of the blue crab (*C. sapidus*). The mitochondrial genome of *C. sapidus* is 16,263 bp in length, and contains the genes for the same 13 proteins, 22 tRNAs, and two rRNAs as found in other metazoans. In addition, there is a 1435 bp non-coding AT-rich region (78.2%), between the *rrnS* and *trnI* genes. Gene order and arrangement is similar to other arthropods but dramatically different from the hermit crab, which has a unique gene order among arthropods. As in the mtDNA of the swimming crab (*P. trituberculatus*), *trnH* is located between the *trnE* and *trnF* genes, rather than at its primitive position upstream of *nad5*. 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 11,319 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 with similar bootstrap values.

Yamauchi et al. (2003) study complete mitochondrial DNA sequence of the swimming crab, *P. trituberculatus* (Crustacea: Decapoda: Brachyura). The mitochondrial genome of *P. trituberculatus* was 16,026 bp in length, and the genome content 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 sr*RNA* and *tRNA* ^{Ile} genes. The overall A + T content of the L-strand of *P. trituberculatus* mtDNA was 70.2% (A = 33.3%; C =18.5%; G =11.3%; T = 36.9%). 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).

Prior to this study, there were no publications concerning genetic variation and intraspecific population subdivisions of the blue swimming crab (*P. pelagicus*). This basic information is essential for the construction of suitable management schemes of this economically important species leading to sustainable fisheries and aquaculture of *P. pelagicus* in Thailand. Additionally, species-specific markers can be used for quality control to prevent intentionally use of the wrong species in canning. Therefore, it was desired to explore the possibility of using RAPD and AFLP analysis to evaluate genetic variability and population structure of *P. pelagicus* in Thai waters and to develop species-diagnostic markers for this species.

1.5 Objectives of the thesis

The aim of this thesis is determination of genetic diversity of *P. pelagicus* in Thailand by RAPD and AFLP analysis and development of species-specific SCAR and SSCP marker for quality control of *P. pelagicus* products.

CHAPTER II

MATERIALS AND METHODS

2.1 Experimental animals

The blue swimming crabs (*P. pelagicus*) were collected from Ranong (*N* = 23) and Krabi (*N* = 22) located in the Andaman Sea and Chantaburi (*N* = 23), Prachup Khiri Khan (*N* = 20) and Suratthani (*N* = 21) located in the Gulf of Thailand (Fig.2.1). Whole specimens were transported to the laboratory at the Center of Excellence for Marine Biotechnology, Faculty of Science, Chulalongkorn University on ice. Alternatively, muscle was dissected out from the first walking leg of each individual. Specimens were kept in a -80^{o}C freezer until used for DNA extraction.

2.2 Genomic DNA extraction

2.2.1 A proteinase K/phenol-chloroform method

Genomic DNA was extracted from the muscle of each blue swimming crab using a phenol-chloroform-proteinase K method. The muscle tissue 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^{o}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 300 μg/ml and further incubated at 55^{o}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 transferred to a new sterile microcentrifuge tube. This extraction process was then repeated one with phenol and twice with chloroform:isoamyalcohlo (24:1). The aqueous phase was transferred into

Figure 2.1 The sampling collection sites for blue swimming crab (*P. pelagicus*) used in this study.

a sterile microcentrifuge. 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 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, pH8.0). 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.

2.2.2 A chelex-based method

A piece of the muscle tissue was homogenized in 200 μl of a 5% chelex (w/v). The mixture was incubated at 56^{o}C for 30 minutes and in the boiling water for 10 minutes. The mixture was briefly centrifuged at 14,000 rpm. The supernatant was transferred to a new tube and kept at 4^{o}C. This solution was used as the template for PCR.

2.3 Measuring DNA concentration of extracted DNA using spectrophotometry and electrophoresis

2.3.1 Spectrophotometry

The concentration of extracted DNA can be estimated by measuring the optical density at 260 nanometre (OD_{260}). 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 solution. The ratio between OD_{260}/OD_{280} provides an estimate on the purity of extracted DNA. A pure preparation of DNA has and OD_{260}/OD_{280} ratio of 1.8-2.0. The ratio that much lower than 1.8 indicates contamination of residual proteins or phenol (Kirby, 1992). An OD_{260} of 1.0 corresponds to a concentration of 50 μg/ml of double stranded DNA, therefore the DNA concentration is estimated in μg/ml by the following equation;

$$[DNA] = OD_{260} \times \text{Dilution factors} \times 50$$

2.3.2 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.

2.4 Randomly amplified polymorphic DNA (RAPD) analysis

2.4.1 RAPD primers

Thirty-one RAPD primers were screened for the amplification success against genomic DNA of three individuals. Five primers (OPA 02, OPA 14, OPB 10, UBC122 and UBC 158) were selected for population genetic analysis of *P. pelagicus* in Thai waters (Table 2.1).

2.4.2 RAPD-PCR

PCR components and the amplification conditions of RAPD-PCR were optimized until clear intensity of the PCR products and reproducible results were obtained. RAPD-PCR was performed in a 25 μl reaction volume containing 10 mM Tris-HCl; pH 8.8, 0.01% Triton X-100, 50 mM KCl, 2 mM $MgCl_2$, 100 μM of each dNTP (dATP, dCTP, dGTP and dTTP), 0.2 μM of a primer, 1 unit of DyNazyme^{TM}II DNA Polymerase (Finnzymes) and 25 ng of DNA template. PCR was performed in a Omnigene-E thermocycler cocsisting by 40 cycles of denaturation at 94^{o}C for 15 seconds, annealing at 36^{o}C for 60 seconds and extension at 72^{o}C for 90 seconds. The final extension was carried out at 72^{o}C for 7 minutes. After amplification, PCR products were electrophoretically analyzed as soon as possible.

Table 2.1 RAPD primer and their sequence used for determining genetic diversity in *P. pelagicus*

Primer	Sequence (5′ → 3′)	Reference
OPA-01	CAGGCCCTTC	Operon technologies
OPA-02	TGCCGAGCTG	Operon technologies
OPA-06	GGTCCCTGAC	Operon technologies
OPA-09	GGGTAACGCC	Operon technologies
OPA-14	TCTGTGCTGG	Operon technologies
OPA-15	TTCCGAACCC	Operon technologies
OPA-16	AGCCAGCGAA	Operon technologies
OPA-17	GACCGCTTGT	Operon technologies
OPB-10	CTGCTGGGAC	Operon technologies
OPZ-09	CACCCCAGTC	Operon technologies
UBC 119	ATTGGGCGAT	University of British Columbia
UBC 122	GTAGACGAGC	University of British Columbia
UBC 128	GCATCTTCCG	University of British Columbia
UBC 135	AAGCTGCGAG	University of British Columbia
UBC 138	GCTTCCCCTT	University of British Columbia
UBC 158	TAGCCGTGGC	University of British Columbia
UBC 159	GAGCCCGTAG	University of British Columbia
UBC 169	ACGACGTAGG	University of British Columbia
UBC 174	AACGGGCAGC	University of British Columbia
UBC 191	CGATGGCTTT	University of British Columbia
UBC 222	AAGCCTCCCC	University of British Columbia
UBC 228	GCTGGGCCGA	University of British Columbia
UBC 263	TTAGAGACGG	University of British Columbia
UBC 268	AGGCCGCTTA	University of British Columbia
UBC 273	AATGTCGCCA	University of British Columbia
UBC 299	TGTCAGCGGT	University of British Columbia
UBC 456	GCGGAGGTCC	University of British Columbia
UBC 457	CGACGCCCTG	University of British Columbia
UBC 458	CTCACATGCC	University of British Columbia
UBC 459	GCGTCGAGGG	University of British Columbia
YNZ 22	CTCTGGGTGTCGTGC	Daniel, D.H., George, K.L.,& Robert, H.D. (1993)

2.4.3. Agarose gel electrophoresis

The amplification products were electrophoresed through 1.6% agarose gels. The appropriate amount of agarose was weighed and mixed with 1xTBE (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. 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.

2.5 Amplified fragment length polymorphism (AFLP) analysis.

2.5.1 Restriction enzyme digestion and adaptor ligation

Each individual DNA (500 ng) was digested with 6 units of *Eco*RI or 6 units of *Pst*I in a 40 μl reaction mixture containing 1xOPA 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, 3 units of *Tru*9I (isoschizomer of *Mse*I) were added. The reaction was incubated at 65^{o}C for approximately 3 hours. The *Eco*RI, or *Pst*I and *Mse*I adaptors (Table 2.2) were ligated to restricted genomic DNA in a 20 μl reaction volume composing of 10 μl of the adaptor ligation solution, 1X OPA buffer, *Eco*RI, or *Pst*I, *Mse*I adaptors, 1 mM ATP and 1 U of T4 DNA ligase. The reaction was incubated at 4^{o}C for approximately 16 hours.

2.5.2 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 of E_{+A} (5′-GAC TGA GTA CCA ATT CA-3′) M_{+C} (5′-GAT GAG TCC TGA GTA AC-3′) primers or P_{+A} (5′-GAC TGC GTA CAT GCA GA-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 minute and extension at 72^{o}C for 1 minute.

2.5.3 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 (or P_{+3} and M_{+3}) primers or E_{+2} and M_{+3} primers (or P_{+2} and M_{+2}) primers (Table 2.2), 1.5 units of DyNazyme^{TM}II DNA Polymerase (Finnzymes) and 1 μl of the preamplification product. PCR was performed in a Perkin Elmer 9700 thermocycler 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 25-28 cycles. The final extension was carried out at 72^{o}C for 5 minutes.

Table 2.2 AFLP primers and primer sequences used for determining genetic diversity in *P. pelagicus*.

Primer	Sequences
Adaptor sequences	
Eco RI adaptor	5′-CTC GTA GAC TGC GTA CC-3′
	5′-AAT TGG TAC GCA GTC TAC-3′
Pst I adaptor	5′-CTC GTA GAC TGC GTA CAT GCA-3′
	5′-TGT ACG CAG TCT AC-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 TGA GTA CCA ATT CA-3′
P_{+A}	5′-GAC TGC GTA CAT GCA GA-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
P_{+3}-1	P_{+A}AG
P_{+3}-2	P_{+A}AC
P_{+3}-3	P_{+A}GA
P_{+3}-4	P_{+A}GT
P_{+3}-5	P_{+A}CG
P_{+3}-6	P_{+A}CT
P_{+3}-7	P_{+A}TC
P_{+3}-8	P_{+A}TT
M_{+3}-1	M_{+C}AA
M_{+3}-2	M_{+C}AC
M_{+3}-3	M_{+C}AG
M_{+3}-4	M_{+C}AT

Table 2.2 (cont.)

Primer	Sequence
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
E_{+2}-1	E_{+A}G
E_{+2}-2	E_{+A}A
P_{+2}-1	P_{+A}G
P_{+2}-2	P_{+A}A
M_{+2}-1	M_{+A}G
M_{+2}-2	M_{+A}A

2.5.4 Agarose gel electrophoresis

The selective amplification AFLP products were electrophoresed through 1.8-2.0% agarose gels as described previously.

2.6 Denaturing Polyacrylamide Gel Electrophoresis

2.6.1 Preparation of Glass Plate

The long glass plate was thoroughly wiped with 2 ml of 95% commercial grade ethanol in one direction with a tissue. 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, 995 μl of 95% ethanol and 10 μl of 5%

glacial acetic acid) and left for approximately 10 - 15 minutes. Excess binding solution was removed with a piece of tissue. The long glass plate was further cleaned 3 times with 95% ethanol. 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 0.4 mM spacer. The bottom and both sides of assembled glass plates were sealed with the plastic tape.

2.6.2 Preparation of denaturing polyacrylamide gel electrophoresis

Six percentage 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 240 µl of freshly prepared 10 % ammonium persulphate and 24 µl of TEMED. The acrylamide solution was gently swirled and degassed for 20 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 using a 50 ml syringe. 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 1x TBE.

2.6.3 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 sharktooth comb was reinserted into the gel until the teeth just touched the surface of the gel. Six microlitres of the polyacrylamide gel loading dye (98 % formamide, 200 ul EDTA, 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 move out from the gel for approximately 30 minutes).

2.7 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 and agitated well for 30 minutes. The gel was 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 4 seconds. The gel was transferred to 0.1% silver nitrate (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 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 dried at 80°C for 2 – 3 hours.

2.8 Cloning of AFLP fragments

2.8.1 Elution of DNA from agarose gels

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 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 minute. The eluted sample was stored at -20°C until further required.

2.8.2 Elution of DNA from polyacrylamide gels

Candidate species-specific AFLP fragments were excised from the gel using a sterile razor blade and washed 3 times for 30 minutes each at room temperature with 200 µl of sterile deionized water. Twenty microlitres of water was then added and incubated overnight at 37°C. Reamplification of the target fragment was carried out 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 5 cycles of 94°C for 30 seconds, 42°C for 1 minute and 72°C for 1 minute followed by additional 35 cycles at a higher stringent annealing temperature at 50°C. The final extension was performed at 72°C for 7 minutes. The reamplified product was electrophoretically analysed through a 1.5 - 1.75 % agarose gel at 7.5 V/cm for approximately 1 hour.

2.8.3 Ligation of PCR product to vector

The ligation reaction was set up in the total volume of 10 μl containing of 3 ul 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.

2.8.4 Preparation of competent cells

A single colony of *E. coli* JM109 was innoculated 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 OD600 of 0.5 - 0.8. The cells were chilled on ice for 10 minutes before centrifuged at 3,000 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 subsequent used.

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

The competent cells were thawed on ice for 5 minutes. Two microlitres of the ligation mixture were added and gently mixed by pipetting. The mixtures were incubated on ice for 30 minutes. The reaction tube was then placed in a 42°C water bath for 45 seconds without shaking. The 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 1.5 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/mlof IPTG and 20 μg/mlof X-gal. The plate was left until

the cell suspension was absorbed and further incubated at 37°C overnight. The recombinant clones containing inserted DNA are white whereas those without inserted DNA are blue.

2.8.6 Detection of recombinant clone by colony PCR

Colony PCR was performed in a 25 μl reaction volume containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 200 μM each of dATP, dCTP, dGTP and dTTP, 1.5 mM $MgCl_2$, 0.2 μM of pUC1 (5'-TCCGGCTCGTATGT TGTGTGGA-3/) and pUC2 (5/-GTGGTGCAAGGCGATTAAGTTGG-3') primers and 0.5 unit of DyNAzymeTM II DNA Polymerase. 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 30 cycles of 94°C for 30 seconds, 55°C for 60 seconds and 72 °C for 90 seconds. The final extension was carried out at 72°C for 7 minutes. The resulting PCR products were electrophoretically analyzed through agarose gels.

2.8.7 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 12,000 rpm for 1 min. The cell pellet was collected and resuspended with 200 μl of the PD1 buffer. The mixture was vortexed and treated with 200 μl of the P2 buffer and mix gently by inverting the tube 10 times. After that 300 μl of the buffer PD3 was added and gently mixed. To separate the cell debris, the mixture was centrifuged at 15,000 rpm for 15 minutes. The supernatant was transferred into a new microcentrifuge tube and to the PD column and centrifuged at 8,600 rpm for 1 minute. The flow-through was discarded. The PD column was washed by adding 400 μl of the W1 buffer and centrifuged for 1 minute. The flow-through was discarded. The spin tube was centrifuge for an additional 1 minute to remove the residual wash buffer. The PD column was placed in a new 1.5 ml microcentrifuge tube and 40 μl of the elution buffer was added to elute the

extracted plasmid DNA. The column was left at room temperature for 2 minutes and centrifuge at 14,000 rpm for 2 minute.

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 15 μl containing 1x restriction buffer (90 mM Tris-HCl; pH 7.5, 10 mM NaCl and 50 mM $MgCl_2$), 1 μg of recombinant plasmid and 2 - 3 unit *Eco* RI and incubated at 37°C for 3 hours before elctrophoretically analyzed by agarose gel elctrophoresis.

2.8.8 Digestion of the amplified DNA insert

Clones showing corresponded DNA insert size were separately digested with *Hind* III and *Rsa* I to verify whether a single insert contained only one type of sequence. Typically, the digestion reaction was set up in the total volume of 15 μl containing appropriate restriction enzyme buffer (buffer E; 6 mM Tris-HCl; pH 7.5, 6 mM $MgCl_2$, 100 mM NaCl and 1 mM DTT for *Hind* III and buffer C; 10 mM Tris-HCl; pH 7.9, 10 mM $MgCl_2$, 50 mM NaCl and 1 mM DTT for *Rsa* I), 5 μl of the amplified product and 2 units of either *Hind* III or *Rsa* I. The reaction mixture was at incubated at 37°C for 3 – 4 hours. Digestion patterns were analyzed by agarose gel electrophoresis.

2.8.9 DNA sequencing

The recombinant plasmid was unidirectional sequenced for undirectional sequenced using a Thermo Sequence Fluorescent Labelled Primer Cycle Sequencing Kit (Amersham Biosciences, Sweden) with the M13 reverse or M13 forward primers on an automated DNA sequencer.

2.9 Primer design, sensitivity and species-specific tests

2.9.1 Primer design from candidate species-specific marker of RAPD fragment.

Night candidate species-specific RAPD markers were cloned and sequenced. Five pairs of primers (Table 2.2) were designed using Primer premier 5.0. They were preliminarily tested for species specificity against genomic DNA of *P. pelagicus*.

Five pairs of primers (pPP14-500, pPP122-510, pPP158-500, pPP158-1200 and pPP158-1500) were further examined against larger specimens of *P. pelagicus* (*N* = 100) and other non-target species; the mud crabs (*Scyllal serrata*, *N* = 8; *S. oceanica*, *N* = 19 and *S. tranquebarica*, *N* = 11) and the swimming crab (*Charyddis crucifera*, *N* = 6).

The amplification reaction were performed in a 25 μl reaction volume containing 10 mM Tris-HCl; pH 8.8, 0.01% Triton X-100, 50 mM KCl, 2 mM $MgCl_2$, 100 μM of each of dNTPs (dATP, dCTP, dGTP and dTTP), 0.2 μM of primer, 1 unit of DyNazyme^{TM}II DNA Polymerase (Finnzymes) and 25 ng of DNA template. PCR was performed in a Perkin Elmer 9700 thermocycler by predenaturation at 94^{o}C for 3 minutes followed by 35 cycles of 94^{o}C for 30 seconds, 58^{o}C for 45 seconds and 72^{o}C for 30 seconds. The final extension was carried out at 72^{o}C for 7 minutes. Five to eight microliters of the amplification products were electrophoretically analyzed by 1.2 to 1.8% agarose gels and visualized under a UV transilluminator after ethidium bromide staining.

Table 2.3 Nucleotide sequence, length and melting temperature of primers designed from candidate species-specific RAPD fragmentts of *P. pelargicus*.

Primer	Sequence	Length (bp)	Tm (^{o}C)
pPP14-500-F	5′ CAC AGG TCT GGC ACA ACT CT 3′	20	55.3
pPP14-500-R	5′ AAC ATT TGC TCG GAA TCA CA 3′	20	56.2
pPP122-510-F	5′ CAC GAA ACC TCC ACG ACC AA 3′	20	61.4
pPP122-510-R	5′ CAA CGA ACA GTG ACG ACC GA 3′	20	59.9
pPP158-500-F	5′ ATT TCA GCC CAC ACG CAC CT 3′	20	62.6
pPP158-500-R	5′ TCA CAG TTT TTC CCC TTT CCG 3′	21	61.6
pPP158-1200-F	5′ GAC GAT GGT GGT CTG GTG AA 3′	20	58.4
pPP158-1200-R	5′ CGT GGA GAG GAA CAG AGT GC 3′	20	57.7
pPP158-1500-F	5′ CCT TCC ATT GCC TCC ATC TA 3′	20	57.5
pPP158-1500-R	5′ TTC CAA CAC CCC AGT TCG TC 3′	20	60.3

Table 2.4 PCR conditions used for amplification of SCAR markers of *P. pelagicus* using primers listed in Table 2.2

SCAR marker	dNTPs (μM)	$MgCl_2$ (mM)	Primer (μM)	PCR condition
1. pPP14-500	100	1.5	0.2	94°C for 3 min followed by 94°C for 45 s, 55°C for 60 s, 72°C for 60 s for 5 cycles and 94°C for 45 s, 53°C for 60 s, 72°C for 60 s for additional 35 cycles and 72°C for 7 min; 1 cycle
2. pPP122-510	100	1.5	0.2	94°C for 3 min followed by 3 cycles of 94°C for 30 s, 63°C for 45 s and 72°C for 30 s. The annealing temperature was lowered to 60 °C for 3 cycles and 57 °C for additional 3 cycles followed by 28 cycles of 94°C for 30 s, 53°C for 45 s and 72°C for 30 s and 1 cycle of 72°C for 7 min.
3. pPP158-500	100	1.5	0.2	94°C for 3 min followed by 35 cycles of 94°C for 45 s, 50°C for 60 s, 72°C for 60 s and 1 cycle of 72°C for 7 min
4. pPP158-1200	100	1.5	0.2	94°C for 3 min followed by 35 cycles of 94°C for 30 s, 58°C for 45 s, 72°C for 30 s and 1 cycle of 72°C for 7 min.
5. pPP158-1500	100	1.5	0.2	As described in 4

2.9.2 Primer design from candidate species-specific marker of AFLP fragment.

Six candidate species-specific AFLP markers were cloned and sequenced. Two pairs of primers (Table 2.4) were designed using Primer premier 5.0. They were preliminarily tested for specificity against genomic DNA of *P.pelagicus*.

Two pairs of primers (pP4M1-300 and pP4M2-410) were further examined against a large sample size of *P. pelagicus* (*N*=100). In addition, cross-species amplification was also tested in other non-target species; the mud crabs (*Scylla serrata*, *N* = 8; *S. oceanica*, *N* = 19 and *S. tranquebarica*, *N* = 11) and the swimming crab (*C. crucifera*, *N* = 6).

The amplification reaction were performed in a 25 μl reaction volume containing 10 mM Tris-HCl; pH 8.8, 0.01% Triton X-100, 50 mM KCl, 2 mM $MgCl_2$, 200 μM of each of dNTPs (dATP, dCTP, dGTP and dTTP), 0.2 μM of primer, 1 unit of DyNazyme^{TM}II DNA Polymerase (Finnzymes) and 25 ng of DNA template. PCR was performed in a Perkin Elmer 9700 thermocycler by predenaturation at 94^{o}C for 3 minutes followed by 35 cycles of 94^{o}C for 30 seconds, 58^{o}C for 45 seconds and 72^{o}C for 30 seconds. The final extension was carried out at 72^{o}C for 7 minutes. Five to eight microliters of the amplification products were electrophoretically analyzed.

Table 2.5 Nucleotide sequence, length and melting temperature of primers designed from candidate species-specific AFLP fragments of *P. pelargicus*.

Primer	**Sequence**	**Length (bp)**	**Tm (^{o}C)**
pPP4M1-300-F	5′ GTG CGT AAC TCT GTC TGG ATT 3′	21	54.4
PP4M1-300-R	5′ TAA CAA CAA ACC GAA GTG GAA 3′	21	56.0
pP4M2-410-F	5′ CAA AAC AAG ACA GAA GAG AA 3′	20	56.3
pP4M2-410-R	5′ AGC ACA GAGG CAA TAG AAC CA 3′	20	55.8

Table 2.6 PCR conditions used for amplification of SCAR markers of *P. pelagicus* using primers listed in Table 2.4

SCAR marker	dNTPs (μM)	$MgCl_2$ (mM)	Primer (μM)	PCR conditions
1. pP4M1-300	200	2	0.2	94°C for 3 min; 1 cycle followed by 94°C for 30 s, 42°C for 45 s, 72°C for 30 s for 5 cycles and 35 cycles of 94°C for 30 s, 53°C for 45 s and 72°C for 30 s and 72°C for 7 min; 1 cycle
2. pP4M2-410	200	1.5	0.2	94°C for 3 min;1 cycle followed by 35 cycles of 94°C for 30 s, 58°C for 45 s and 72°C for 30 s and 1 cycle of 72°C for 7 min.

2.9.3 Sensitivity and stability tests

Sensitivity of pPP158-1200, pPP158-1500, pPP122-510 and pP4M2-410 primers were examined against varying concentrations of the target DNA template (12.21 pg-25 ng) using the same conditions as were used for the specificity test. The stability of pPP158-1200, pPP158-1500, pPP122-510 and pP4M2-410 markers was determined using genomic DNA extracted from frozen, boiled and saline-preserved *P. pelagicus* by both typical phenol-chloroform and chelex-based methods.

2.10 Single strand conformational polymorphism (SSCP) analysis

The glass plates (PROTEAN II Xi Cell) were cleaned and prepared as described previously. Low crosslink non-denaturing polyacrylamide gels (37.5:1 of acrylamide and bis-acrylamide) were prepared. The acrylamide gel solution (30-40 ml) was mixed with 240 μl of 10% APS and 24 μl of TEMED. The analytical comb was inserted into the prepared gel and allow for polymerization for 4 hours or overnight.

The PCR product of P4M2F410 (8 μl) were mixed with 32 μl of the 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 10 minutes. The denatured products were electrophoretically analyzed in native polyacrylamide gels at 250 volts for 16 hours at 4^{o}C. SSCP band were visualized by silver staining.

2.11 Data analysis

Nucleotide sequences of RAPD and AFLP fragments were compared with those previously deposited in the GenBank using BLAST*N* and BLAST*X* (Altschul et.al., 1990 available at http://www.ncbi/nlm/nih.gov). Significant probabilities of matched nucleotides/proteins were considered when the E-value was $<10^{-4}$.

For population genetic analysis, RAPD and AFLP bands were treated as dominant markers. Each band was treated as a locus and scored for presence (1) or absence (0), and transformed into 0/1 binary character matrix without consideration of band intensity differences between homo- and heterozygotes.

Gene diversity and percentage of monomorphic and polymorphic loci was estimated for each geographic sample. Pairwise unbiased genetic identity and genetic distance were determined (Nei, 1978). A neibour-joining tree was constructed using genetic distance between pairs of geographic samples.

Genetic heterogeneity of overall sample and between pairs of samples was examined using the exact test. F_{ST} statistics (θ) of overall samples and between pairs of geographic 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 allele per locus and s = the number of geographic samples. Population genetic parameters described above were computationally analyzed by Tool for Population Genetic Analysis (TFPGA, Miller, 1997) and POPGENE. Genetic exchange among population per generation (gene flow) was estimated from θ using $N_m = (1-F_{ST}/4F_{ST})$.

CHAPTER III

RESULTS

3.1 DNA extraction

High molecular weight DNA at approximately 23.1 kb was obtained from DNA extraction using a proteinase K-phenol-chloroform method (Fig 3.1). DNA concentrations were spectrophotometrically determined by measuring the optical density at 260 nm (1 OD_{260} unit was equipment to 50 μg DNA/ml). The ratio of OD_{260}/OD_{280} was 1.8 - 2.0 suggesting that quality of extracted genomic DNA was acceptable for further used.

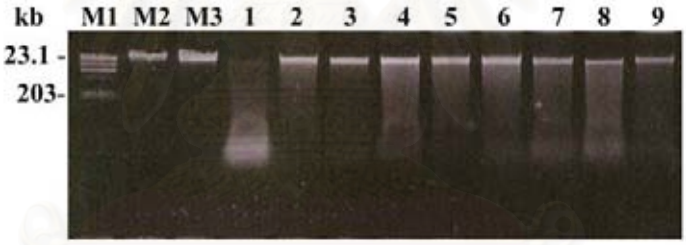


Figure 3.1 A 0.8% agarose gel showing the quality of total DNA extracted from the muscle of *P. pelagicus* (lanes 1-9). Lane M1 is λ/*Hin*d III DNA marker. Lanes M2 and M3 are 100 and 200 ng of undigested λDNA.

3.2 Genetic diversity of *P. pelagicus* in Thailand examined by RAPD analysis.

Among the 31 RAPD primers screened against 2 - 3 representatives of *P. pelagicus*, 26 primers successfully amplified genomic DNA of blue swimming crab. Five primers (OPA02, OPA14, OPB10, UBC122 and UBC158) exhibiting reproducible and easy scoring results were selected and further tested to evaluate genetic diversity and intraspecific population differentiation against a larger number of *P. pelagicus* originating from Chantaburi (*N* = 23), Ranong (*N* = 23), Suratthani (*N* = 21), Krabi (*N* = 22) and Prachuap Khiri Khan (*N* = 20) (Fig 3.2-3.6)

High genetic polymorphism was observed in all geographic samples. A total of 112 RAPD fragments ranging from 220 bp and 1500 bp in length were generated. The number of amplified bands in overall investigated sample was 25, 22, 19, 22 and 24 bands for primers OPA02, OPA14, OPB10, UBC122 and UBC158, respectively. The percentage of polymorphic bands (< 95.0% of all specimens) for each primer was 92.00%, 100.00%, 94.47%, 95.45% and 95.83%, respectively (Table 3.2).

The average polymorphic band of all primers across overall samples was 95.54%. Five monomorphic bands (found in > 95.0% of all specimens) were regarded as candidate species-specific fragments. Additional 10 fragments were found in slightly lower than 95% of specimens were also considered potential fragments.

Considering the number of monomorphic and polymorphic RAPD bands in each geographic sample, relatively high percentage of polymorphic bands were also observed. The Chantaburi sample exhibited the highest percentage of polymorphic bands (85.05%) followed by Prachuap Khiri Khan (83.52%), Ranong (82.73%), Krabi (82.0%) and Suratthani (72.73%). Population-specific RAPD fragments were not found in these geographic samples.

Table 3.1 Amplification results of screening RAPD primer for determination of genetic diversity in *P. pelagicus*

Primer	Amplification results	Primer	Amplification results
OPA-01	+	UBC 159	-
OPA-02	++	UBC 169	+
OPA-06	+	UBC 174	+
OPA-09	-	UBC 191	+
OPA-14	+++	UBC 222	+
OPA-15	+	UBC 228	+
OPA-16	-	UBC 263	+
OPA-17	+	UBC 268	+
OPB-10	++	UBC 273	+
OPZ-09	+	UBC 299	+
UBC 119	+	UBC 456	-
UBC 122	+++	UBC 457	+
UBC 128	-	UBC 458	+
UBC 135	+	UBC 459	+
UBC 138	+	YNZ 22	+
UBC 158	+++		

- = no amplification products, + = positive amplification products but amplification may be difficult or inconsistent, ++ and +++ = positive amplification products with easy scoring results

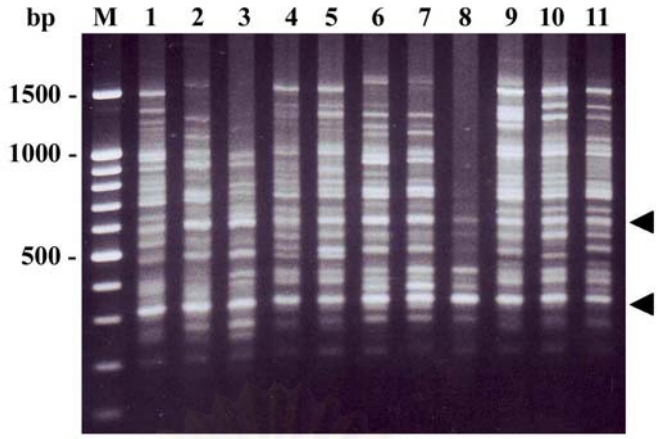


Figure 3.2 Agarose gel elctrophoresis showing RAPD patterns of *P. pelagicus* originating from Chantaburi (lane 1), Ranong (lanes 2 - 3), Suratthani (laness 4 - 5), Krabi (lanes 6 - 8) and Prachuap Khiri Khan (lanes 9 - 11) generated by OPA02. Lane M is a 100 bp ladder. Arrowheads indicate candidate species-specific RAPD fragments found in *P. pelagicus*.

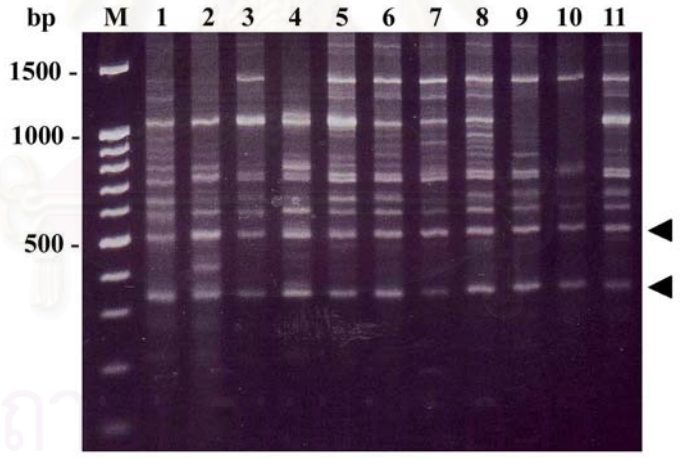


Figure 3.3 Agarose gel elctrophoresis showing RAPD patterns of *P. pelagicus* originating from Chantaburi (lane 1), Ranong (lane 2), Suratthani (lane 3), Krabi (lane 4) and Prachuap Khiri Khan (lanes 5-11) generated by OPA14. Lane M is a 100 bp ladder. Arrowheads indicate candidate species-specific RAPD fragments found in *P. pelagicus*.

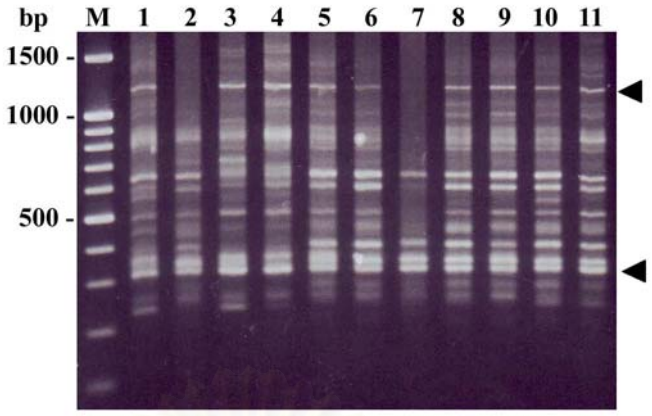


Figure 3.4 Agarose gel electrophoresis showing RAPD patterns of *P. pelagicus* originating from Chantaburi (lane 1), Ranong (lane 2), Suratthani (lane 3), Prachuap Khiri Khan (lane 4) and Krabi (lanes 5-11) generated by OPB10. Lane M is a 100 bp ladder. Arrowheads indicate candidate species-specific RAOD fragments found in *P. pelagicus*.

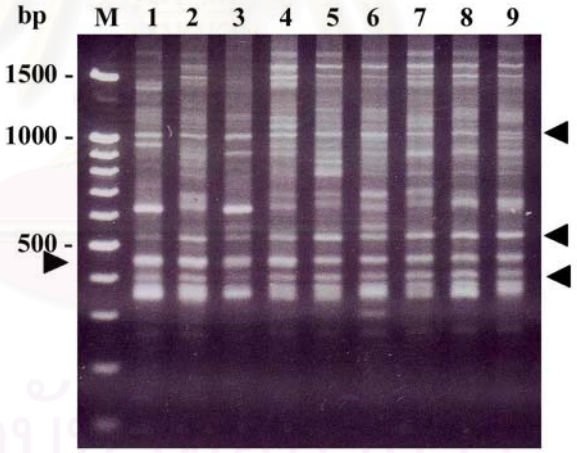


Figure 3.5 Agarose gel electrophoresis showing RAPD patterns of *P. pelagicus* originating from Chantaburi (lane 1), Ranong (lane 2), Suratthani (lane 3), Krabi (lane 4) and Prachuap Khiri Khan (lanes 5 - 11) generated by UBC122. Lane M is a 100 bp ladder. Arrowheads indicate candidate species-specific RAPD fragments found in *P. pelagicus*.

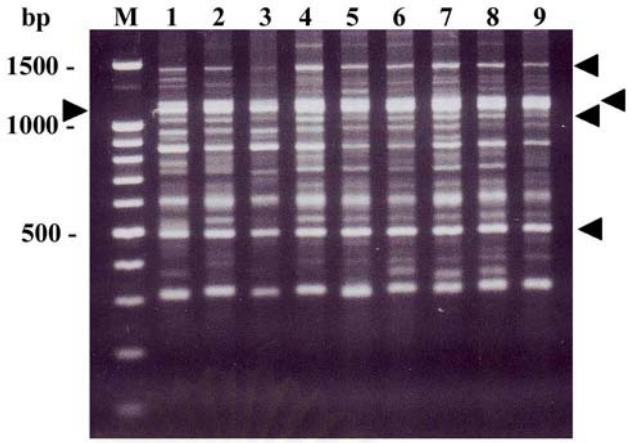


Figure 3.6 Agarose gel electrophoresis showing RAPD patterns of *P. pelagicus* originating from Chantaburi (lane 1), Ranong (lane 2), Suratthani (lane 3) and Krabi (lanes 4 - 9) generated by UBC158. Lane M is a 100 bp ladder. Arrowheads indicate candidate species-specific RAPD fragments found in *P. pelagicus*.

Table 3.2 Sequences of oligonucleotide primers, sizes and number of amplified bands, and percentage of polymorphic bands resulting from RAPD analysis using primers OPA02, OPA14, OPB10, UBC122 and UBC158.

Primer	**Sequence**	**Size-range (bp)**	**No. of RAPD bands**	**Polymorphic band (%)**
OPA02	TGCCGAGCTG	250-1500	25	23 (92.00%)
OPA14	TCTGTGCTGG	340-1400	22	22 (100.00%)
OPB10	CTGCTGGGAC	220-1500	19	18 (94.74%)
UBC122	GTAGACGAGC	320-1200	22	21 (95.45%)
UBC158	TAGCCGTGGC	310-1500	24	23 (95.83%)
Overall primers	-	220-1500	112	107 (95.54%)

Table 3.3 Total number of bands, percentage of polymorphic and monomorphic bands of each geographic sample revealed by RAPD analysis using primer OPA02, OPA14, OPB10, UBC122 and UBC158.

Primer	Chantaburi (*N* = 23)			Ranong (*N* = 23)			Suratthani (*N* = 21)		
No.	No. of bands	No. of polymorphic bands	No. of monomorphic bands	No. of bands	No. of polymorphic bands	No. of monomorphic bands	No. of bands	No. of polymorphic bands	No. of monomorphic bands
OPA02	23	19	4	25	23	2	24	21	3
OPA14	22	19	3	22	21	1	22	13	9
OPB10	18	16	2	17	15	2	19	15	4
UBC122	21	18	3	22	17	5	21	17	4
UBC158	23	19	4	24	15	9	24	14	10
Total	107	91 (85.05%)	16 (14.95%)	110	91 (82.73%)	19 (17.27%)	110	80 (72.73%)	30 (27.27%)

Table 3.3 (Continued).

Primer No.	Krabi (*N* = 22)			Prachuap Khiri Khan (*N* = 20)		
	No. of bands	No. of polymorphic bands	No. of monomorphic bands	No. of bands	No. of polymorphic bands	No. of monomorphic bands
OPA02	24	22	2	23	19	4
OPA14	16	12	4	13	9	4
OPB10	19	17	2	16	14	2
UBC122	19	14	5	19	16	3
UBC158	22	17	5	20	18	2
Total	100	82 (82.00%)	18 (18.00%)	91	76 (83.52%)	15 (16.48%)

Genetic distance between pair of geographic sample was 0.0059 – 0.1546, 0.0713 – 0.2966, 0.0329 – 0.2039, 0.0497 – 0.2661 and 0.0677 – 0.4563 for OPA02, OPA14, OPB10, UBC122 and UBC185, respectively (Table 3.4). Generally, relatively large genetic distance was observed between all pairwise comparison and implied intraspecific population differentiation at the microgeographic scale (e.g. between locations) rather than at the macrogeographic scale (e.g. between coastal regions).

The average genetic identity between pairs of geographic samples across overall primers was 0.7811 (Prachuap Khiri Khan and Ranong) – 0.9113 (Prachuap Khiri Khan and Krabi). The mean genetic distance between sample across overall primers was 0.1151 - 0.2440 (Table 3.5). This reflected clear genetic subdivisions between pairs of geographic samples of *P. pelagicus*.

Table 3.4 Pairwise genetic distance between geographic sample of Thai *P. pelagicus* based on RAPD analysis using OPA02, OPA14, OPB10, UBC122 and UBC158

Pairs of samples	**Primer**				
	OPA02	**OPA14**	**OPB10**	**UBC122**	**UBC185**
CHN-SUT	**0.0228**	**0.1078**	**0.0550**	**0.0497**	**0.2240**
CHN-PKK	**0.0059**	**0.1199**	**0.0329**	**0.2661**	**0.1187**
CHN-RNG	0.1546	0.1375	0.1905	0.0622	0.3650
CHN-KRB	0.0827	0.2033	0.1429	0.1380	0.1562
SUT-PKK	**0.0147**	**0.1750**	**0.0608**	**0.2080**	**0.2537**
SUT-RNG	0.0914	0.2257	0.2039	0.0619	0.0677
SUT-KRB	0.0509	0.2966	0.1616	0.1320	0.2489
PKK-RNG	0.1470	0.2051	0.1923	0.2317	0.4563
PKK-KRB	0.0873	0.0713	0.1413	0.1132	0.0678
RNG-KRB	**0.0294**	**0.2291**	**0.0137**	**0.0974**	**0.4172**

Within region genetic distance is illustrated in boldface. Abbreviations: CHN = Chantaburi, RNG = Ranong, SUT = Suratthani, KRB = Krabi, PKK = Prachuap Khiri Khan.

A neighbor-joining (NJ) tree constructed from the average unbiased genetic distance between pairs of geographic samples of *P. pelagicus* based on RAPD analysis divided 5 investigated samples to 3 groups: Prachuap Khiri Khan, Chantaburi and Krabi (Group I), Suratthani (Group II) and Ranong (Group III, (Fig. 3.7).

Significant geographic heterogeneity was observed in almost all of the pairwise comparisons of investigated samples analyzed by each primer ($P < 0.01$ according to the sequential Bonferroni adjustment method, Table 3.6). Result did not imply intraspecific population subdivisions between coastal regions but reveal fine scale genetic heterogeneity of *P. pelagicus*. Data from all primers were combined and analyzed. Results from the exact test (geographic heterogeneity analysis) and F_{ST}-based statistics (θ) indicated statistically significant differences between all pairwise comparisions. Therefore, the gene pool of *P. pelagicus* in this study is reproductively isolated and each geographic sample should be regarded as different genetic populations ($P < 0.0001$, Table 3.7).

Table 3.5 Pairwise Nei's (1978) genetic distance (below diagonal) and genetic identity (above diagonal), between pairs of geographic samples of *P. pelagicus.*

	Chantaburi	**Suratthani**	**Prachuap Khiri Khan**	**Ranong**	**Krabi**
Chantaburi	-	0.9109	0.8974	0.8351	0.8645
Suratthani	0.0933	-	0.8666	0.8842	0.8363
Prachuap Khiri Khan	0.1083	0.1432	-	0.7811	0.9113
Ranong	0.1802	0.1230	0.2471	-	0.8525
Krabi	0.1456	0.1788	0.0929	0.1596	-

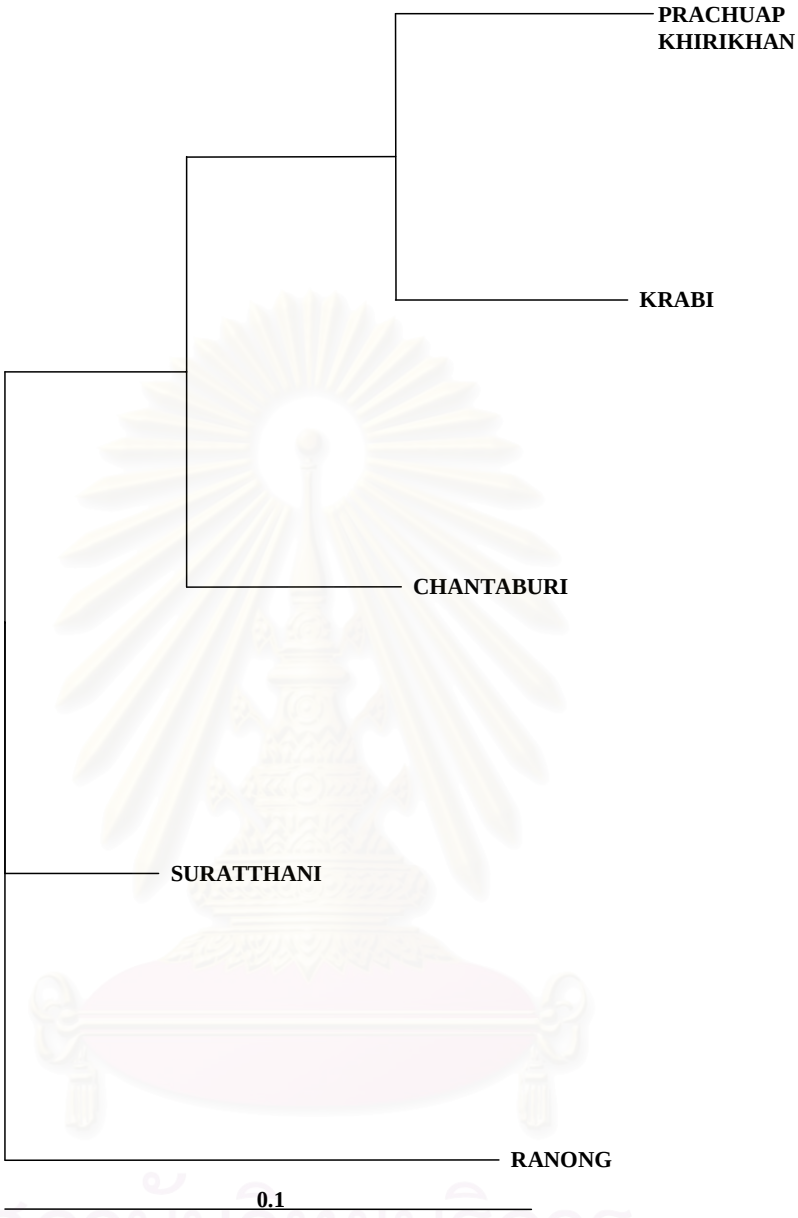


Figure 3.7 A neighbor-joining tree indicating relationships of *P. pelagicus* in Thai waters based on genetic distance between pairs of geographic samples using RAPD-PCR analysis.

Table 3.6 Geographic heterogeneity analysis between pairs of geographic samples of *P. pelagicus* resulted from RAPD analysis (OPA02, OPA14, OPB10, UBA122 and UBC 158)

	OPA02	**OPA14**	**OPB10**	**UBC122**	**UBC185**
CHN-SUT	0.1217^{ns}	< 0.0001*	0.4837^{ns}	0.0253^{ns}	< 0.0001*
CHN-PKK	0.8360^{ns}	< 0.0001*	0.4449^{ns}	< 0.0001*	< 0.0001*
CHN-RNG	< 0.0001*	< 0.0001*	< 0.0001*	0.0041*	< 0.0001*
CHN-KRB	<0.0001*	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*
SUT-PKK	0.9785^{ns}	< 0.0001*	0.0422^{ns}	< 0.0001*	< 0.0001*
RNG-SUT	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*	0.0045*
SUT-KRB	0.0001*	< 0.0001*	< 0.0001*	< 0.0001*	< 0.000*1
RNG-PKK	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*
KRB-PKK	< 0.0001*	0.0017*	< 0.0001*	< 0.0001*	< 0.0001*
RNG-KRB	0.0019*	< 0.0001*	0.1398^{ns}	< 0.0001*	< 0.0001*

CHN = Chantaburi, RNG = Ranong, SUT = Suratthani, KRB = Krabi, PKK = Prachuap Khiri Khan

The gene flow level between pairs of geographic samples was estimated and nearly all comparisons exhibited restrictive gene flow level (< 1 individual per generation) reflecting strong genetic subdivisions in Thai *P. pelagicus*.

3.2.1 Development of a sequence–characterized amplified region (SCAR) marker for detection of *P.* pelagicus.

Although high polymorphic levels were observed from RAPD analysis of Thai *P. pelagicus*, several candidate species-specific RAPD markers observed. Five fragments (300 bp and 590 bp from OPA02, 310 bp from OPB10, 400 bp from UBC122 and 1150 bp from UBC158) were found in all individuals, 4 fragments (500 bp from OPA14, 1050 bp from UBC122 and 500 bp and 1200 bp from UBC158) were found in more than 95.0% of all individuals and 6 fragments (340 bp from OPA14, 1200 bp from OPB10, 440 bp and 510 bp from UBC122 and 1050 bp and 1500 bp

from UBC 158) were found in more than 90.0% (Fig. 3.2-3.6 and Table 3.8). The candidate species-specific RAPD bands were cloned and further characterized to develop species-specific SCAR markers in this species.

In total, 9 candidate RAPD fragments were cloned and sequenced. After blast analysis, they did not significantly matched with those previously deposited in the GenBank (E value>10^{-4}) and were regarded as unknown DNA segments (Fig. 3.8-3.16).

A primer pair was designed from pPP14-500, pPP122-510, pPP158-500, pPP158-1200 and pPP158-1500 (Fig. 3.12 – 3.16) and was subjected to the initial amplification and specificity test. The preliminary results indicated possible species-specific nature of pPP122-510F/R, pPP158-1200F/R and pPP158-1500F/R while pPP14-500F/R yielded cross-species amplification in the mud crab (*S. serrata*, *S. oceanica* and *S. transcuebarica*) and the other swimming crab (*C. crucifera*). The pPP158-500F/R did not generate the positive amplification product in any species (Fig. 3.17).

Three SCAR markers (pPP122-510F/R, pPP158-1200F/R and pPP158-1500F/R) were then tested against the larger number of *P. pelagicus* (*N* = 100), *S. serrata* (*N*= 8), *S. oceanica* (*N* = 19), *S. transcuebarica* (*N* = 11) and *C. crurifera* (*N* = 6).

The expected product sizes (152 bp, 397 bp and 262 bp for pPP122-510F/R, pPP158-1200F/R and pPP158-1500F/R) were found in 95%, 100% and 100% in the target species without the false positive in the non-target species (Table 3.9 and Fig. 3.18 – 3.20). Heterozygotes that exhibited two different sizes (alleles) of the amplification products were not observed across overall specimens when analyzed by these primers. This implies the retention of a dominant segregated fashion of the original RAPD markers of these species-specific SCAR markers.

Table 3.7 F_{ST}-based statistics (θ) and geographic heterogeneity analysis between pairs of 5 geographic samples of *P. pelagicus* based on RAPD analysis

Geographic Sample	Theta test		Gene flow	Exact test (P-value)
	Theta P (θ)	χ^2		
CHN-RNG	0.2750	25.3*	0.66	<0.0001*
CHN-SUT	0.1732	15.24*	1.19	<0.0001*
CHN-KRB	0.2508	22.57*	0.75	<0.0001*
CHN-PKK	0.2026	17.42*	0.98	<0.0001*
SUT-PKK	0.2618	21.47*	0.70	<0.0001*
SUT-RNG	0.2215	19.49*	0.88	<0.0001*
SUT-KRB	0.3024	26.01*	0.58	<0.0001*
PKK-RNG	0.3617	31.11*	0.44	<0.0001*
PKK-KRB	0.1987	16.69*	1.01	<0.0001*
RNG-KRB	0.2758	24.82*	0.66	<0.0001*

$\chi^2 = 2N\theta(k-1)$, df = (s-1)(k-1), was used N is the number of individuals used in the analysis, k is the number of alleles and s the number of populations. * = significant at P < 0.01. The χ^2 value at P = 0.05, 0.01 and 0.001, df = 4 is 9.49, 13.28 and 18.74, respectively.

Table 3.8 Candidate species-specific RAPD fragments of *P. pelagicus* in Thailand revealed by RAPD analysis.

Primer	RAPD marker
OPA02	**300*** and **590***
OPA14	340 and **500***
OPB10	**310*** and 1200
UBC122	400, **440***, **510*** and 1050
UBC158	**500***, 1050, 1150, **1200*** and **1500***

Fragments that were cloned, sequenced and tested for species-specificity.

```
TGCCGAGCTG TTTAGGGGTG TGAGGGGAAG GTAATTAGAG GATGAAGAAT AGCAGAAAGT 60
GTGTGTAGTA AAACGTTATA CAGCCGTATT TGGAGTGACA CGAACGAAAT GACAAAGGAG 120
TAAGGAGATG AATAGAGGGA ATGGCCTGTG GAGGAAAGAT ATTGAGCAGT GTTTTGAAGG 180
ACTTAGATGC TACAGGTAAA GAAGAAGATA GAGGAATAGC GCTGGATGTT TTTTGTATTA 240
TTTGAAAGAT TTGGTTGGCA AAATAACAAG AAACAAGAAA TAGGCAAAAT AGTAAGTAAG 300
TCAGCTCGGC AA                                                     312
```

Figure 3.8 Nucleotide sequence of pPPOPA02-300.

```
TGCCGAGCTG TGTGAGAGAA TCAAGTTTAA GACGGAGAAC TCAAACTATC AAAGCTTCCA 60
CTCCGTCTTG TCCATCCTGA GCTACCTGTG CAAGGCGCCT CTTGTCCCTC CTGGCACACC 120
AATCATCAAC TCGCTCTTCA GACAACGATC ATGCATTGAG AACATTCTAA GGGCGTGTCT 180
CGGTCTGCCG CCCCTGAACC ACATGCTGCT GGAGTACAAG CATGAGCAGG TGGAGTGTGG 240
CAAGGTGAAG CGTGCAGCTG AGGCGGTGAA GCGGCCAGCG GAAGTGATGA ACGGCTCAAC 300
TAAACACCAA GCGCCACAAC GCATCCGCCG ACTGTCCGAT GAGATTGATA AGTGACTGTG 360
AGGTGATGGT GACTGTCTGG TGATGTGTGT GTGTGTGGTG GTGTTGTGGT GATCTCCTCC 420
TCCTCACCCT TCGCTTCTAC TTTGCAGTTC CTCCTCGTGT TCTCCTTCTT CTCCTCCTCT 480
CCATTTTCCT TCTTCTTTAA CCTTCTTTTC TCGTTTTTTA TCTTCTTATC CGTCCTTTGA 540
ATATTCCTAA GCCTCTTCCT CTTCTTTCAG CTCGGCAA                         588
```

Figure 3.9 Nucleotide sequence of pPPOPA02-590.

```
CTGCTGGGAC CAACCTCAAC ACACCAGGAG AGACACCATC AACACCACAA GGTTTTTCAG 60
GTCTCATTTT CTGAATCTGT ATTGATAATT CAACTGTGAT AGCGTCATCT AAAATTGGTA 120
CAAAAGGAGA ATCGTTAGAA TAATTGAAAT CTTCGTGTAA GCCGGGATTA CAAAATGTCT 180
CATAATAAGA TTTGAATTCA TCATCAGATG GGGCGGTACT GTTTGTAGGA CCATCCTGGA 240
ATTTTCCTTG CCAATCGATG GCTTTGCATA CTCTTGTATC ATCAGGGTCA CTCAGCAACC 300
TGTCCCAGCA GA                                                     312
```

Figure 3.10 Nucleotide sequence of pPPOPB10-310.

```
GAATTCGACT GTAGACGAGC ACGACGCGGG ACACACACAC GCCTAACAGT GTTCCCTTTA 60
AATCATTACT ATTATGCGCT GACTATATCA AGAGGAAGCT CAGATACATT TTCTTAGTTT 120
ATTTTTTTGT GTGAATAAAA CAAACTTTAA TGTCTCTGCT TTTTACAAAC GAATATCTAA 180
CCTTTACAAT CATCTGTCCA TTTGTTCCCG TTTAGTGTAT GTAAATGGAT GTCCCTGTGA 240
ATTTCTGCTT CTTATTTCTT AACATTTCTG AGCGTAAATG GAGTATCCAA TTTGCTTTCA 300
ACCACTTTGC TCTACACCAA CTGAGGGTGT GTCTCGAAGC ATCAACCATC ATTATTTTAT 360
TTAATACGGT CACGCGTAGG GACACATTTA AACGACAATA GAGGAGAAGA AAAAAACGTT 420
TCTTGCATCT TTTTCCTCAC ACCAGCTCGT CTACAATCAC TAGT                   464
```

Figure 3.11 Nucleotide sequence of pPPUBC122-440.

```
TCTGTGCTGG CACGAAAGGC AAAAATAGAA TCCAAGGCTG CTAGAGTGAA ACAAACACTT 60
CATTTATACT GTGTTTCTAT TATAATCAGT TACTTTCATT TGTAAAATAT GTGAAACTTA 120
                          pPP14-500F
CACATGTGTG TGCCCAGTGC ACAGGTCTGG CACAACTCTG CATTCCCTTT CATTTTACGT 180
GAAGTGCCGT AGCATATGAA CATGTAGAAC TATACTGTGT TTAATCTGAA CCATTCGCAA 240
AAAGCAAATA TGATATGAAA TCAGTATCAG GGTCGAGTGG CTAATGACAA GTGTGGTGCT 300
GGTACAAGCC ATCGATGAGG CCAAACTATA GTGAGTAAAG AAGCTATTCA GACAAGAAAT 360
                                      pPP14-500R
TTAAAATTGT TTAGACACAT AAGATAAACA ACATTTGCTC GGAATCACAT GGTTTACATT 420
TGTGATTCCG AGCAAATGTT ACAGAGGTGG AAGAGTAGTC ATGAGTGTAA AGTGATTTCA 480
CAACCACAAG TCCATCA                                               497
```

Figure 3.12 Nucleotide sequence of pPP14-500. The location and sequence of a forward primer (pPP14-500F) and those complementary to a reverse primer (pPP14-500R) are illustrated in boldface and underlined.

```
GTAGACGAGC CGAGTTTGTC ATGTAACTAT ATCATGTTTA GGAGCTCAAG TATTACATAT 60
AGTTAGTAGA GAAAAGCTAT AACAAAGTAA GCAATGCAGG GAAGGTATTA TAAGCTACTT 120
AGTCACGGGA TGGATGGCAA CACATCAGAG GGTGGTGCAG NTAGNNTATT TNCNTCTCAC 180
CNCCCCCCCC CCCAACAACC AAAGATTACA CAGGATAAAA CAACGGACGT AGCGGTGGGT 240
TGGTTATGAC ATGGCATGGC TTGACAATGA GGACTCAATG AATGCTCGGC TAGTGATCAC 300
pPP122-510F
GAAACCTCCA CGACCAATTG ACACGTGAAG TGGGTGCGTA TTTTCTCTAT TATGGTTGAG 360
GTGCGGGACG AATCATGGTG GTGAAGAGTG AATAGCACGG GAAGGTTAGG TGGTTCAGTG 420
               pPP122-510R
GTTCAGTGGT TCGGTCGTCA CTGTTCGTTG AGACGGCTCT GTTTCGCCAC CGTCATAAAC 480
CCGAACCATT GTCTCGAGAC TGATCCGAAA TACTGCTCGT CTACA                 525
```

Figure 3.13 Nucleotide sequence of pPP122-510. The location and sequence of a forward primer (pPP122-510F) and those complementary to a reverse primer (pPP122-510R) are illustrated in boldface and underlined.

```
                                                    pPP158-500F
ACTAGTGATT TAGCCGTGGC GTGCAAATGG ACGACAAAAA TATCTTGCTG CTTTCAGAAC 60
GACTTTGGCA TGCAAATTTC AGCCCACACG CACCTCGTTT GCATAAATAC AAGGGTGTCT 120
ACGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTGTGTTT GTGTGTGTTT 180
GTGCGTGCGC ATGAGAGAGA AAAATAAGAG AGGACAGCTA GCCGATACGG AAAGGGGAAA 240
AACTGTGAAG AGAAAGGAGG AGGANGAGGA GGANGGAGAA AAAGGACANG AAGAAAGGGA 300
                    pPP158-500R
GTCTATTTTT GGGTACCTAG CATTAGCATC TCACTGGAGA GTTGATCAAT ACATATTTTT 360
AAACCCGANT ACTCCAAACA CACGGACAGA AAACACTANT AGACTTTAG             409
```

Figure 3.14 Nucleotide sequence of pPP158-500. The location and sequence of a forward primer (pPP158-500F) and those complementary to a reverse primer (pPP158-500R) are illustrated in boldface and underlined.

```
                                                          pPP158-1200F
TAGCCGTGGC CACGCGAGGC TGCAGCAATG AGAGATATTC AGCGTATCTA GACGATGGTG 60
GTCTGGTGAA TTATTACAAT ACTCAACTGC CAATTAGTTC ACGGCAATTC AGGGCTGACA 120
TTACATTATG CAACCTTGTT TATCCAATGG AAACACAACT GCCGGGAATT ATAATTGCCA 180
TCATGGCGGG TTTATCAAGG TTAATGACAA TCTTGCAGCA AAGCGTGGAA GAAATACGAT 240
TAATTATCTG AGGTGGAAAG AATACTCGTG CGCCGCATAA TGGTTGTTTA GTGGTGTCGT 300
GGATACCTCA CGGGGGACAA TTTAGCGTGT GGAGAACATC TCTCATTTTG CTATTAATTA 360
TTCGATGTAG CATTTTTCTT TCGCGTTTGT TGCATCACGG GAATGATTTA CGTCTGTATG 420
          pPP158-1200R
CTTAGATGCA CTCTGTTCCT CTCCACGCTA TGAGACAAGC GCCAAAAGAC ATTATCCAAT 480
GTGGTGCTGT CTTTTTTCTT TTCTTTTTTT TTCTTTTTTA TGGATTTACT GCACCACTAC 540
AGAATGATTT TTGTCTGTTA CGTTTAAGAC AAGCATTACA ACATGTGAAA GGAGGGAGCG 600
TGACGCGGTG TCCCTTTTCT GTCATTGTTA TGTCGCAAGA ATGGCAACGT TTCGTCTTCC 660
ATGGTATTGG TTATCAGAAG CTCTCAAACG AACGTGGCGT GGCATCTCTT TTTTCTGCGT 720
TTCCTATACC ACATCTCAAC GTACCACTTT ACTATGGCAC TGTTGTGATT GCTGAACGAC 780
ACACGAATTA TCCCGTTGTG TTGTAATCTC CATGTTATAN ATAGGAAAGG AGGAGGAGAA 840
AAGGATGCTC GCGGTATATT CATGAATCAG AGTGATGATA ACGTAATATG CAAGACGGAG 900
GTTAATTCCT TACAATTTTT CGGTGTAAAT TAGGAATGGG TTCAGAATGC CATTCCGTCC 960
TCTCGCCGCC ACGCAAGGAA TTACGTACAA ATCTGATGCC CTGCTTTGTG GTGTATTTGT 1020
TTCATGGTTC ACTTTACTTA GGGAGAAAAT AAGTTGGAGC TGCATAATTT TGAGAATGAT 1080
AGGAGTGAGC CACGGCTAA                                           1099
```

Figure 3.15 Nucleotide sequence of pPP158-1200. The location and sequence of a forward primer (pPP158-1200F) and those complementary to a reverse primer (pPP158-1200R) are labeled underlined.

```
TAGCCGTGGC CCCGAGTGAT CCTGAAATCC TGAAGCCAAC GCAAAAATTC TTATTGTTAC 60
CTGAGGGTGA GGCAGACAGA CACACACACG CGCACACACA CACACACACA GATGTAGTTT 120
AATATAAGAT CCATATTTTA TTTTCTCATT TACGTGAATT TTTGAGATAT TTGGTATTTT 180
AGGGATGTTT AGAAAGTTGA ATTTGAAGAC AGACAGACAG ACAGACAGAC AGATAGACAG 240
ACAGATAGAC ATATATAGAT GCAAAATGCC TACTTTCAAA TCTCATTACG TAAAGGTCAG 300
AGGTACATAC AAGGACAGAC AGAAAGACAG ACAGAGACTC AGACACACAG ACAGACTAAC 360
CGACATGGTA AAATAAAATC TCTGCTTTCA GATATCACAC ACACACACAC ACACACACAC 420
ACACACACAC ACACACACAC ACACACACAC ACACACACAC ACACACACAC ACACACACAC 480
AAAATGTCCC TAAACACACT CAAAACACCG CACAACCTCT CAATGTCCCC TAAACACACT 540
CAAAACACCA CACAACCTCT CAATGCCCCT AAAACACACT CAAACCACCA CACAACCTCT 600
CAATGTCCCC AAAACACACT CAAAACACCA CATAACCTCT CAAAATGTCC CCAAAACACA 660
CTCAAAACAC CACACAACCT CTCAAATTGT CCCAAAAACA CACTCAAAGC ACCACACAAT 720
CCCTCAATGT CCCCAAAACA CACTCAAAAC ATAAAATACA CCTAAAGGGC CTAAAACACA 780
CTGAAAACAC ACAAAAGGCA CCAAAGACAT ACTCATAACA TCCTAGAACA CTACTGAATA 840
CACTTGAAAT ACACCAAATA TACCCCAAAG CCAACAGTAC CGATCCATTA CAAAAAAGTT 900
AAGATTTAAA GGAACACTTA CCTAAATATT ACACCTTGGC TCCTTCTCCT ACTCGTATTC 960
CGCCTCTTTT TTTAATTTCT TCTTCATCCT CCTCCATTGT TTCTCCTCCT TCTTCCTTCC 1020
pPP158-1500F
ATTGCCTCCA TCTACACGCC TGAGCCTGGA AACACATCAA AACACTAGAT ATTAGACAAA 1080
ATATTGAGAA AATATTGTAG CTTGGCGGCT GCTCCTCTTC CTTCTACCTT CTCCTTCCTT 1140
CCTCCTTATC CTCCTTTATT TCTCCTTTCT CCCTCTTTCT GCTACCAATA ACTACACATC 1200
TGAATCTAGA AACACACGAA AACACGTAGA AATCACTAGA TATCAGGCAA AATATTGACG 1260
    pPP158-1500R
AACTGGGGTG TTGGAAAAGA GGCGCCAGCC TGGGGGCCAT TATGAATCAC TACCTGGGTG 1320
GGCTGCGGTC ATCGGAGAGA ACGGGAGCGG GGGTGACTCG GCTAGATTAC GTAAATCATT 1380
TCATTTTAAA AATGACTTTT AGAAAAAACG AAGCCACGGC TAA                   1423
```

Figure 3.16 Nucleotide sequence of pPP158-1500. The location and sequence of a forward primer (pPP158-1500F) and those complementary to a reverse primer (pPP158F1500-R) are labeled underlined.

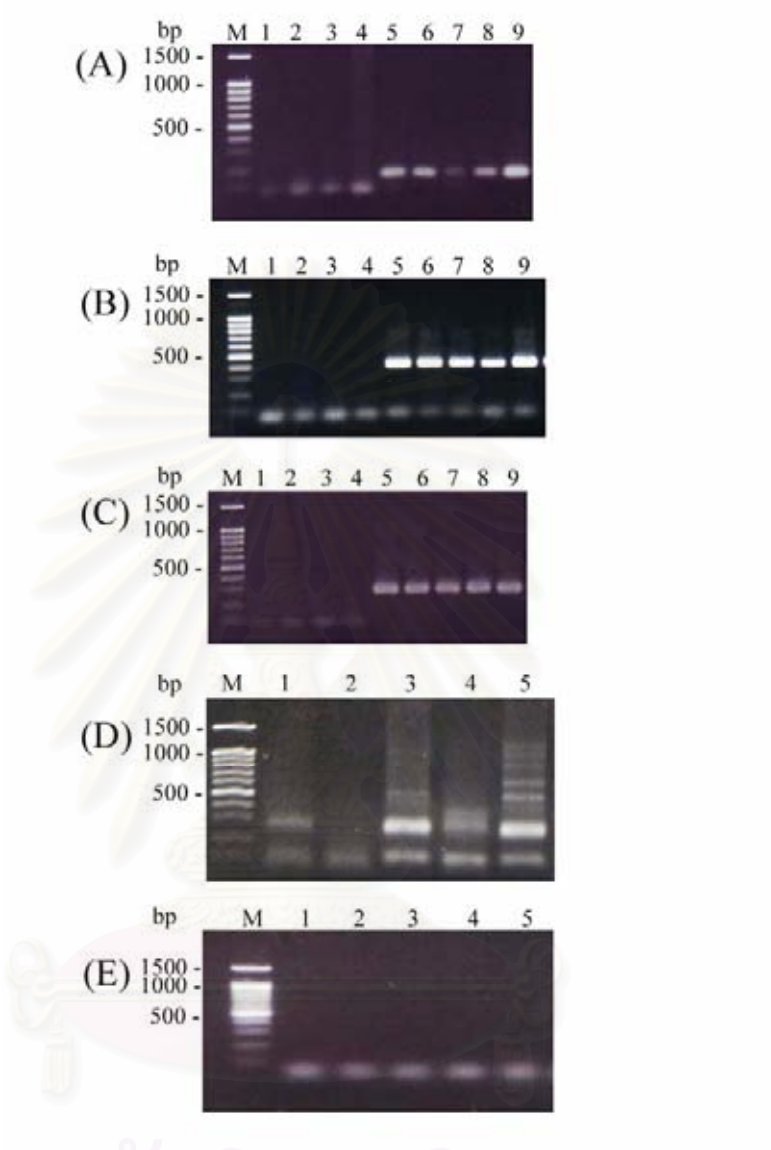


Figure 3.17 Agarose gel electrophoresis showing results from amplification of genomic DNA of *P. pelagicus* (lanes 5-9, A, B, C, and lanes 1-5, D, E), *S. serrata* (lane 1, A - C), *S. oceanica* (lane 2, A - C), *S. tranquebarica* (lane 3, A - C) and *C. crucifera* (lane 4, A - C) by pPP122-510F/R (A), pPP158-1200F/R (B), pPP158-1500F/R (C), pPP14-500F/R (D) and pPP158-500F/R (E). A 100 bp ladder (lanes M) were used as the DNA marker.

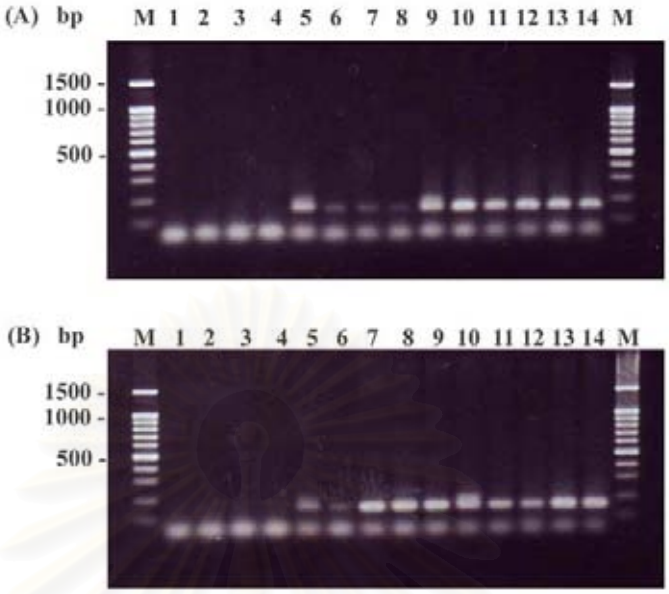


Figure 3.18 Agarose gel electrophoresis illustrating species-specificity of pPP122-510F/R against genomic DNA of *P. pelagicus* (lanes 5 - 14, A and B), *S. serrata* (lane 1, A and B), *S. oceanica* (lane 2, A and B), *S. tranquebarica* (lane 3, A, and B) and *C. crucifera* (lane 4, A and B). A 100-bp ladder (lanes M) was used as DNA markers.

Sensitivity of *P. pelagicus*-specific primers was examined against varying concentrations of the target DNA template (2-fold dilutions of 12.21 pg - 25 ng genomic DNA). All primer pairs revealed good correlations between the amount of DNA template and intensity PCR product. The sensitivity of detection was approximately 400 pg and 200 pg of the target DNA template for primer pPP122-510F/R and pPP158-1200F/R but greater sensitivity was observed with pPP158-1500F/R (12.21 pg) (Fig. 3.21).

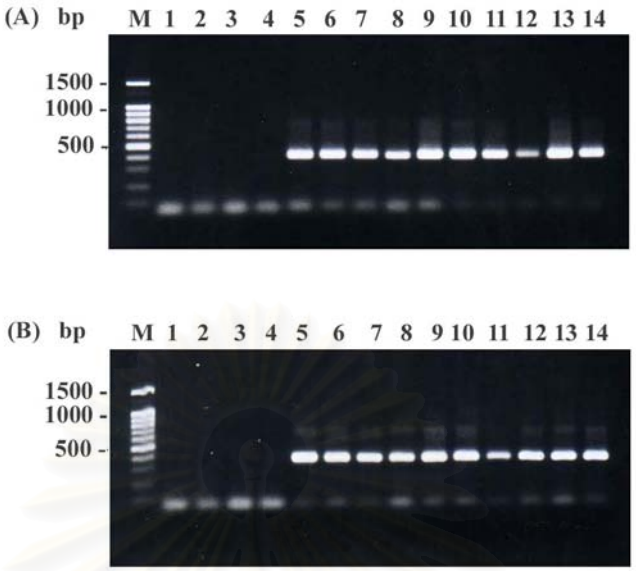


Figure 3.19 Agarose gel electrophoresis illustrating species-specificity of pPP158-1200F/R against genomic DNA of *P. pelagicus* (lanes 5 - 14, A and B), *S. serrata* (lane 1, A and B), *S. oceanica* (lane 2, A and B), *S. tranquebarica* (lane 3, A, and B) and *C. crucifera* (lane 4, A and B). A 100 bp ladder (lanes M) was used as the DNA marker.

Stability of *P. pelagicus*-specific markers was tested. The tedious and time-consuming phenol/chloroform extraction method was omitted by simplification of the extraction method to a rapid 5% chelex-based method. Species-specific PCR of the developed SCAR markers (PP122-510, PP158-1200 and PP158-1500) was tested against genomic DNA of frozen specimens, boiled and saline-preserved specimens (Fig. 3.22). Positive amplification products were consistently observed indicating reliability of these species-specific SCAR markers.

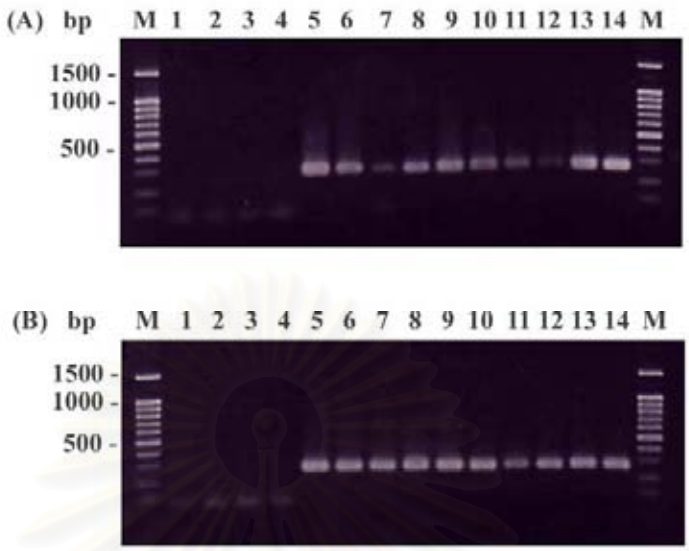


Figure 3.20 Agarose gel electrophoresis illustrating species-specificity of pPP158-1500F/R against genomic DNA of *P. pelagicus* (lane 5 - 14, A and B), *S. serrata* (lane 1, A and B), *S. oceanica* (lane 2, A and B), *S. tranquebarica* (lane 3, A, and B) and *C. crucifera* (lane 4, A and B). A 100 bp ladder (lanes M) was used as DNA markers.

Table 3.9 Sequences and amplification results of primers designed from recombinant clones possessing a candidate species-specific RAPD fragment of *P. pelagicus*.

SCAR marker	Primer name	Primer sequence	Amplification results
PP14-500	pPP14-500F/R	F: 5'CACAGGTCTGGCACAACTCT3' F: 5'AACATTTGCTGCGAATCACA3'	Cross-species amplification
PP122-510	pPP122-510F/R	F: 5'CACGAAACCTCCACGACCAA3' R: 5'TCGGTCGTCACTGTTCGTTG3'	A 152 bp product was observed in 95% of *P. pelagicus* but not in other species
PP158-500	pPP158-500F/R	F: 5'CTTGCTGCTTTCAGAACGAC3' R: 5'AGCATTAGCATCTCACTGGA3'	-
PP158-1200	pPP158-1200F/R	F: 5'GACGATGGTGGTCTGGTGAA3' R: 5'GCACTCTGTTCCTCTCCACG3'	A 397 bp product was observed in all individuals of P. pelagicus (*N* = 100) without a cross-species amplification result
PP158-1500	pPP158-1500F/R	F: 5'CCTTCCATTGCCTCCATCTA3' R: 5'GACGAACTGGGGTGTTGGAA3'	A 262 bp product was observed in all individuals of P. pelagicus (*N* = 100) without a cross-species amplification result

- , amplification was not successful

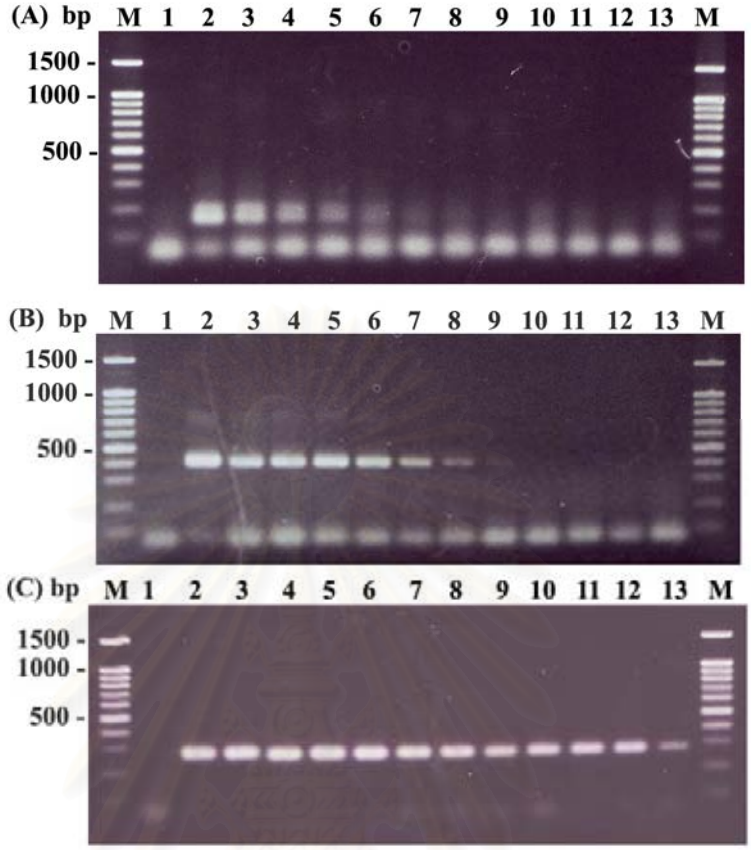


Figure 3.21 Sensitivity of pPP122-510F/R (A), pPP158-1200/R (B) and pPP158-1500F/R (C) was examined against varying concentrations of *P. pelagicus* DNA template (25 ng, 12.5 ng, 6.25 ng, 3.12 ng, 1.56 ng, 781.25 pg, 390.62 pg, 195.31 pg, 97.66 pg, 48.83 pg, 24.41 pg, and 12.21 pg corresponding to lanes 2 - 13, respectively). Lane 1 is a negative control (without genomic DNA template). A 100 bp ladder (lanes M) was used as a marker.

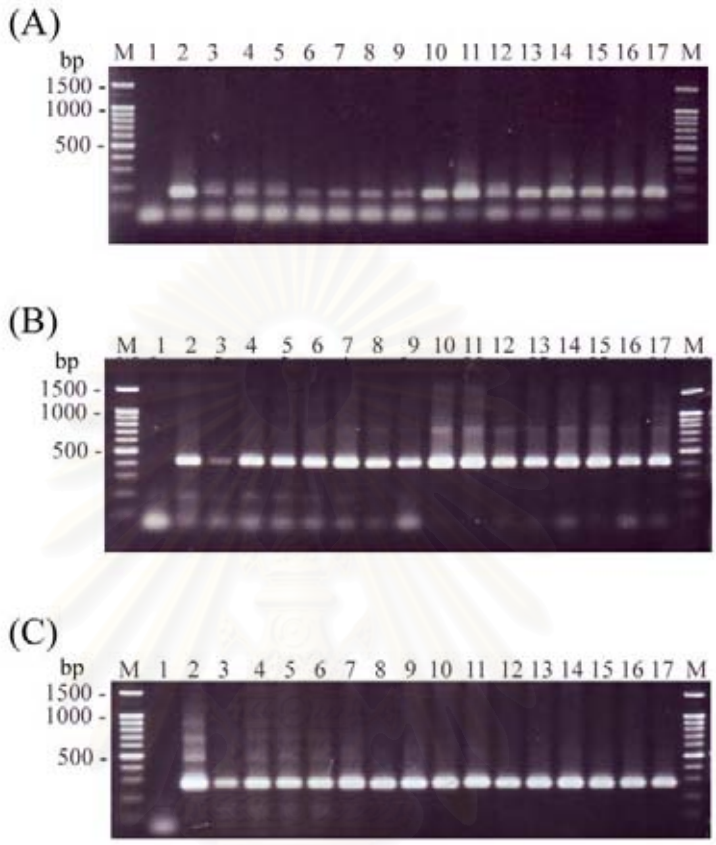


Figure 3.22 Agarose gel electrophoresis showing results from amplification of total DNA extracted with phenol/chloroform (lanes 2 - 9) and 5% chelex extraction method (lanes 10 - 17) of frozen (lanes 2 - 3 and 10-11), boiled (lanes 4 - 7 and 12 - 15) and saline-preserved (lane 8-9 and 15-16) specimens with primer pPP122-510, pPP158-1200 and pPP158-1500. Lane 1 is a negative control (without DNA template). A 100 bp ladder (lanes M) was used as a DNA marker.

3.3 Genetic diversity of *P. pelagicus* in Thailand examined by AFLP analysis.

The preamplification reactions using primers 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 (Fig. 3.17). This indicated successful restriction/ligation of genomic DNA of *P. pelagicus*. The preamplification products were diluted and subjected to selective amplification with a pair of 2 or three selective nucleotides. The amplification results were preliminary analyzed by agarose gel electrophoresis.

A total of 52 primer combinations were screened. No primers with 2 selective nucleotides provided the amplification bands. The combinations between E_{+3} and M_{+3} primers generated smear patterns of the amplification products. The band patterns were only found in primers from combination of P_{+3} and M_{+3} primers.

Disregarding the primer types, only one primer combination did not provide the amplification products. Twenty-four and fourteen primer combinations generated smear amplification patterns with the products sizes less than 500 bp and 300 bp, respectively. Nine primer combinations yielded discrete bands but the amplification product sizes were less than 300 bp. Four primer combinations (P_{+3}-2/M_{+3}-1, P_{+3}-4/M_{+3}-1, P_{+3}-7/M_{+3}-1, and P_{+3}-4/M_{+3}-2) provided reproducible and scorable amplification bands between 100 – 600 bp (Table 3.10) were further applied for population genetic studies of *P. pelagicus* in Thai waters (Fig. 3.18-3.21).

The amplification products from selected primers of *P. pelagicus* individuals from Chantaburi (*N* = 12), Ranong (*N* = 14), Suratthani (*N* = 16), Krabi (*N* = 14) and Prachuap Khiri Khan (*N* = 16) were electrophoresed through 6.0% denaturing polyacrylamide gel electrophoresis (crosslink 19:1, Fig. 22 – 25).

Table 3.10 Amplification results of screening AFLP primer combinations for determination of genetic diversity in *P. pelagicus*

Primer	Result	Primer	Result
E_{+2}-1/M_{+2}-1	Smear patterns < 300 bp	E_{+3}-2/M_{+3}-7	Smear patterns < 300 bp
E_{+2}-1/M_{+2}-2	Smear patterns < 500 bp	E_{+3}-2/M_{+3}-8	Smear patterns < 300 bp
E_{+2}-2/M_{+2}-1	Smear patterns < 500 bp	P_{+2}-1/M_{+2}1	Smear patterns < 300 bp
E_{+2}-2/M_{+2}-2	Smear patterns < 500 bp	P_{+2}-1/M_{+2}2	Smear patterns < 300 bp
E_{+3}-1/M_{+3}1	Smear patterns < 500 bp	P_{+2}-2/M_{+2}1	Smear patterns < 300 bp
E_{+3}-1/M_{+3}2	Smear patterns < 500 bp	P_{+2}-2/M_{+2}2	Smear patterns < 300 bp
E_{+3}-1/M_{+3}3	Smear patterns < 500 bp	P_{+3}-3/M_{+3}-1	Smear patterns < 500 bp
E_{+3}-1/M_{+3}4	Smear patterns < 500 bp	P_{+3}-4/M_{+3}-1	++
E_{+3}-1/M_{+3}5	Smear patterns < 500 bp	P_{+3}-5/M_{+3}-1	Smear patterns < 500 bp
E_{+3}-1/M_{+3}6	Smear patterns < 500 bp	P_{+3}-6/M_{+3}-1	Smear patterns < 500 bp
E_{+3}-1/M_{+3}7	Smear patterns < 500 bp	P_{+3}-7/M_{+3}-1	+++
E_{+3}-1/M_{+3}8	Smear patterns < 500 bp	P_{+3}-8/M_{+3}-1	Smear patterns < 500 bp
E_{+3}-1/M_{+3}9	Smear patterns < 500 bp	P_{+3}-1/M_{+3}-2	+
E_{+3}-1/M_{+3}10	Smear patterns < 500 bp	P_{+3}-2/M_{+3}-2	+
E_{+3}-1/M_{+3}11	Smear patterns < 500 bp	P_{+3}-3/M_{+3}-2	_
E_{+3}-1/M_{+3}12	Smear patterns < 500 bp	P_{+3}-4/M_{+3}-2	++
E_{+3}-1/M_{+3}13	Smear patterns < 500 bp	P_{+3}-1/M_{+3}-3	+
E_{+3}-1/M_{+3}14	Smear patterns < 500 bp	P_{+3}-2/M_{+3}-3	+
E_{+3}-1/M_{+3}15	Smear patterns < 500 bp	P_{+3}-3/M_{+3}-3	Smear patterns < 300 bp
E_{+3}-1/M_{+3}16	Smear patterns < 500 bp	P_{+3}-4/M_{+3}-3	+
E_{+3}-2/M_{+3}-1	Smear patterns < 300 bp	P_{+3}-5/M_{+3}-3	+
E_{+3}-2/M_{+3}-2	Smear patterns < 300 bp	P_{+3}-6/M_{+3}-3	+
E_{+3}-2/M_{+3}-3	Smear patterns < 300 bp	P_{+3}-7/M_{+3}-3	+
E_{+3}-2/M_{+3}-4	Smear patterns < 300 bp	P_{+3}-8/M_{+3}-3	-
E_{+3}-2/M_{+3}-5	Smear patterns < 300 bp	P_{+3}-1/M_{+3}-1	Smear patterns < 500 bp
E_{+3}-2/M_{+3}-6	Smear patterns < 300 bp	P_{+3}-2/M_{+3}-1	+++

- = no amplification products, + = positive amplification product but sizes were less than 300 bp, ++ and +++ = positive amplification products exhibiting appropriate size range and was further used for population genetic studies

Figure 3.23 A 1.8% agarose gel electrophoresis showing preamplification product of genomic DNA of *P. pelagicus*. A 100 bp (lane M) was used as the DNA marker.

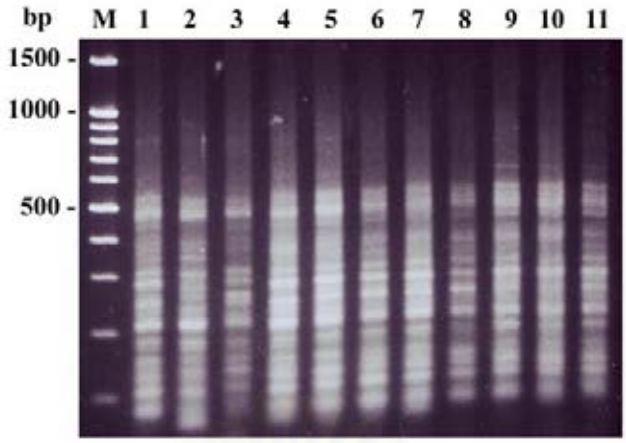


Figure 3.24 A 2.0% agarose gel electrophoresis showing selective amplification products of P_{+3}-2/M_{+3}-1 against genomic DNA of *P. pelagicus* originating from Chantaburi (lane 1), Ranong (lane 2), Krabi (lane 3), Prachuap Khiri Khan (lane 4) and Suratthani (lanes 5-11). A 100 bp ladder (lane M) was included as the DNA marker

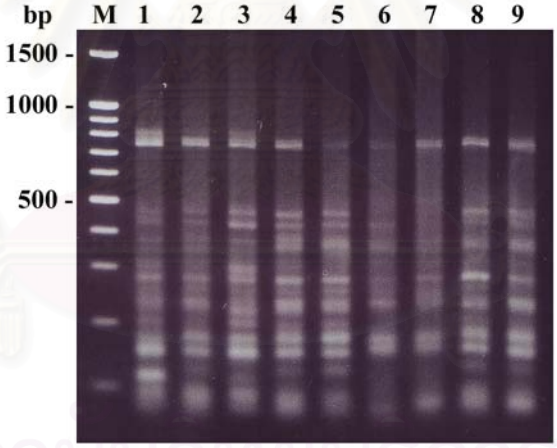


Figure 3.25 A 2.0% agarose gel electrophoresis showing selective amplification products of P_{+3}-4/M_{+3}-1 against genomic DNA of *P. pelagicus* originating from Ranong (lane 1), Suratthani (lane 2), Krabi (lane 3), Prachuap Khiri Khan (lane 4) and Chantaburi (lanes 5-9). A 100 bp ladder (lane M) was used as the DNA marker.

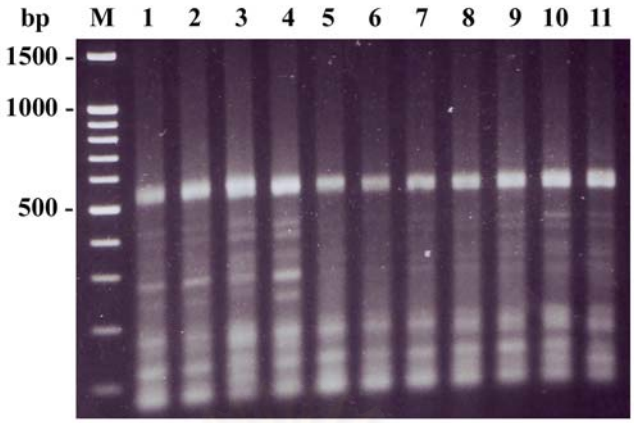


Figure 3.26 A 2.0% agarose gel electrophoresis showing selective amplification products of P_{+3}-4/M_{+3}-2 against genomic DNA of *P. pelagicus* originating from Chantaburi (lane 1), Suratthani (lane 2), Krabi (lane 3), Prachuap Khiri Khan (lane 4) and Ranong (lanes 5-11). A 100 bp ladder (lane M) was used as the DNA marker,

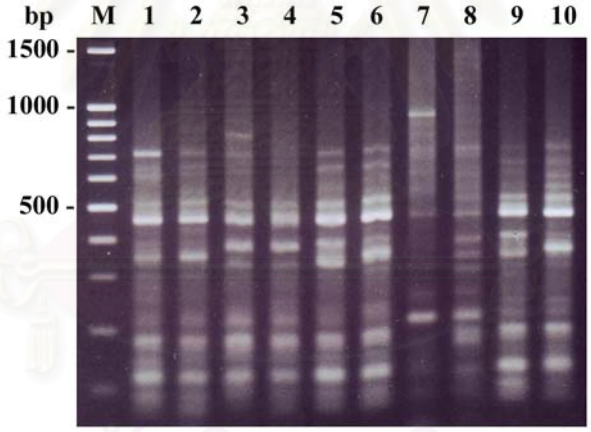


Figure 3.27 A 2.0% agarose gel electrophoresis showing selective amplification products of P_{+3}-7/M_{+3}-1 against genomic DNA of *P. pelagicus* originating from Chantaburi (lane 1), Ranong (lanes 3-4), Suratthani (lanes 5-6), Krabi (lanes 7-8), and Prachuap Khiri Khan (lanes 9-10). A 100 bp ladder (lane M) was usec as the DNA marker,

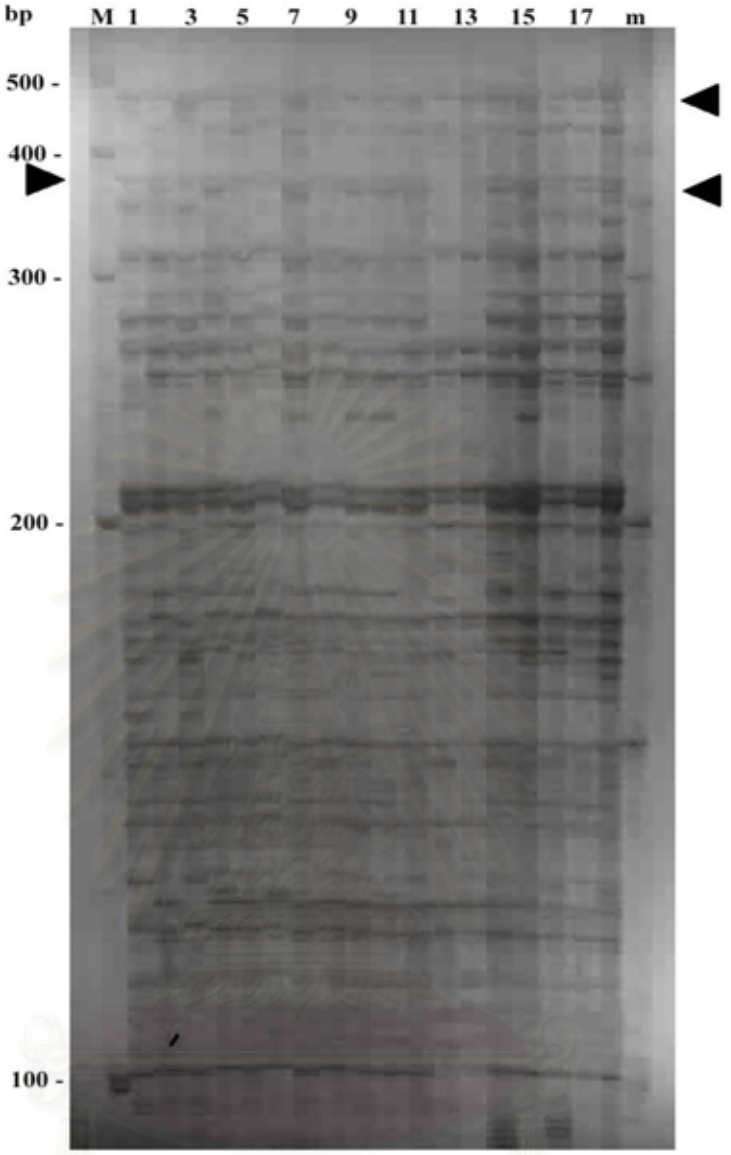


Figure 3.28 AFLP products of P_{+3}-2/M_{+3}-1 amplified from genomic DNA of *P. pelagicus* originating from Ranong (lane 1), Suratthani (lane 2), Krabi (lane 3), Prachuap Khiri Khan (lane 4) and Chanthaburi (lanes 5-18) were fractionated through a 6% denaturing polyacrylamide gel. Lane M and m are 100 bp and 50 bp DNA markers, respectively. Arrowheads indicate species-specific AFLP fragments in *P. pelagicus.*

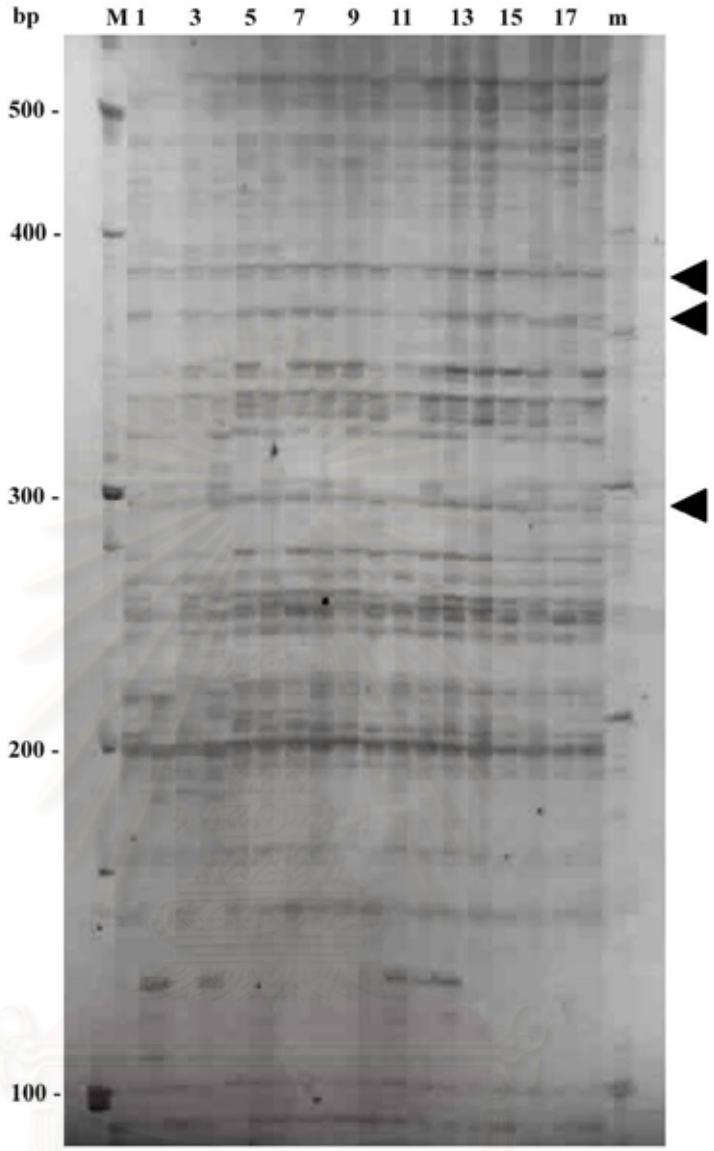


Figure 3.29 AFLP products P_{+3}-4/M_{+3}-1 amplified from genomic DNA of *P. pelagicus* originating from Chanthaburi (lane 1), Ranong (lane 2), Suratthani (lane 3), Krabi (lane 4) and Prachuap Khiri Khan (lanes 5-18) were fractionated through a 6% denaturing polyacrylamide gel. Lane M and m are 100 bp and 50 bp DNA markers, respectively. Arrowheads indicate candidate species-specific AFLP fragments in *P. pelagicus*.

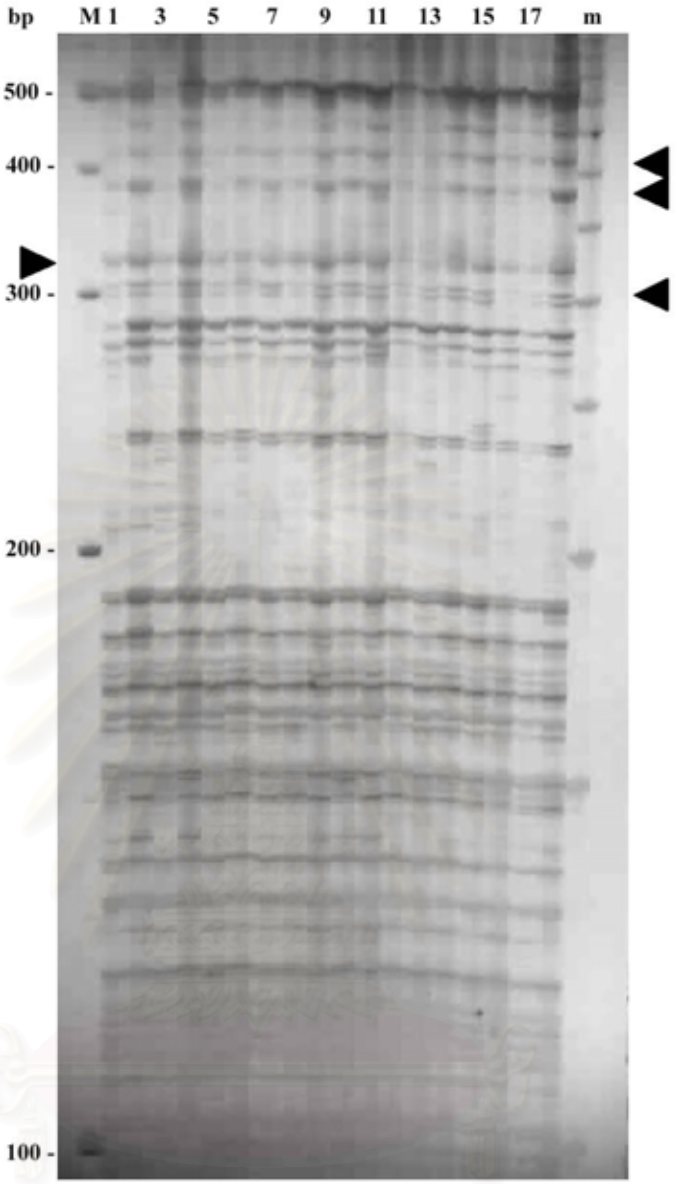


Figure 3.30 AFLP products of P_{+3}-4/M_{+3}-2 amplified from genomic DNA of *P. pelagicus* originating from Ranong (lane 1), Suratthani (lane 2), Krabi (lane 3), Prachuap Khiri Khan (lane 4) and Chanthaburi (lanes 5-18) were size-fractionated through a 6% denaturing polyacrylamide gel. Lane M and m are 100 bp and 50 bp DNA markers, respectively. Arrowheads indicate candidate species-specific AFLP fragments in *P. pelagicus*.

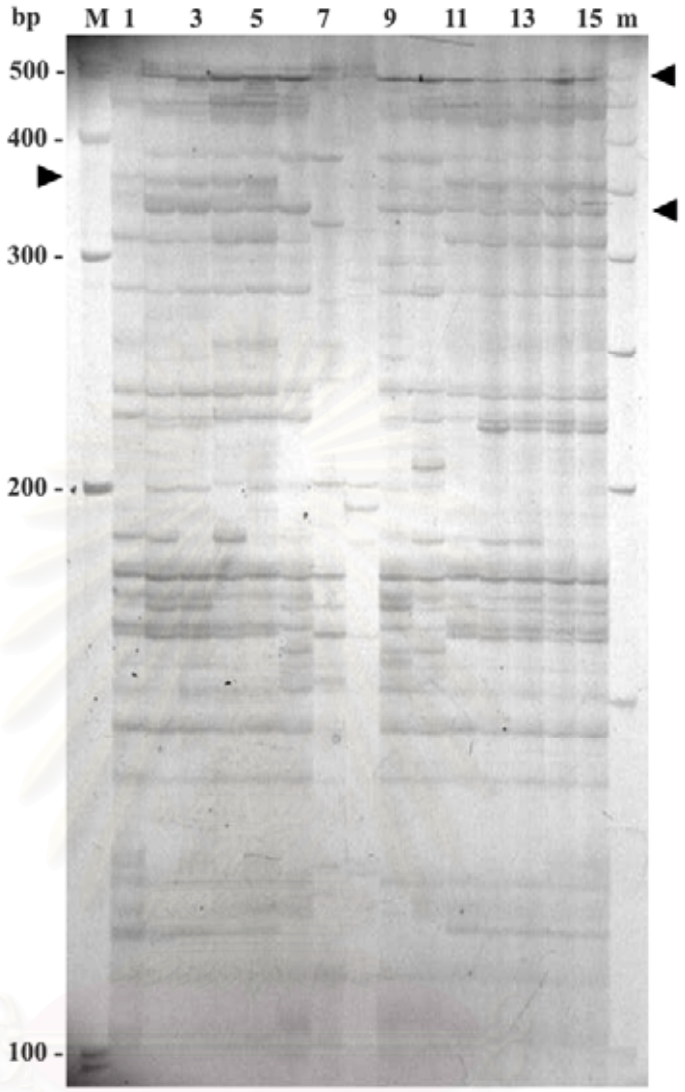


Figure 3.31 AFLP products of P_{+3}-7/M_{+3}-1 amplified from genomic DNA of *P. pelagicus* originating from Chanthaburi (lanes 1-5), Ranong (lanes 6-10), and Suratthani (lanes 11-15) were size-fractionated through a 6% denaturing polyacrylamide gel. Lane M and m are 100 bp and 50 bp DNA markers, respectively. Arrowheads indicate species-specific AFLP fragments in *P. pelagicus*.

High genetic diversity was observed in all geographic samples. A total of 61, 63, 44 and 59 bands from P_{+3}-2/M_{+3}-1, P_{+3}-4/M_{+3}-1, P_{+3}-4/M_{+3}-2 and P_{+3}-7/M_{+3}-1 primer combinations, respectively were observed in overall samples. Three and four monomprphic AFLP bands were found in P_{+3}-2/M_{+3}-1 (360, 370 and 470 bp), P_{+3}-4/M_{+3}-1 (300, 360 and 380 bp) and P_{+3}-7/M_{+3}-1 (340, 350 and 500 bp), and P_{+3}-4/M_{+3}-2 (300, 320, 390 and 420 bp). This may be regarded as species-diagnostic AFLP fragments in *P. pelagicus*. Like RAPD analysis, population and/or region specific fragments were not found.

The number of amplification for all primers was 160, 142, 139, 138 and 137 fragment in Krabi, Chantaburi, Prachuap Khiri Khan, Ranong and Suratthani, respectively. The average polymorphic bands (< 95.0% of all investigated specimens) of all primers in each of the Andaman sample (94.38% and 90.20% in Krabi and Ranong) was greater than that of the Gulf of Thailand (73.24% and 66.19% in Chantaburi and Prachuap Khiri Khan) where the Suratthani sample possessed a higher polymorphic band (93.84%) than other east-coast samples (Table 3.11).

The unbiased genetic distance between pair of geographic sample was 0.1097 – 0.3786, 0.0655 – 0.2577, 0.1218 – 0.3086 and 0.0180 – 0.2616 for P_{+3}-2/M_{+3}-1, P_{+3}-4/M_{+3}-1, P_{+3}-4/M_{+3}-2 and P_{+3}-7/M_{+3}-1, respectively (Table 3.12). Like RAPD analysis, relatively large genetic distance was observed between all pairwise comparison and implied intraspecific population differentiation at the microgeographic scale (e.g. between locations) rather than at the the macrogeographic scale (e.g. between coastal regions).

The average genetic identity between pairs of geographic samples across overall primers was 0.7834 (Prachuap Khiri Khan and Krabi) – 0.8913 (Suratthani and Ranong). The means between sample genetic distance were 0.0929 - 0.2471 (Table 3.5).

Table 3.11 Total number of bands, percentage of polymorphic and monomorphic bands of each geographic sample of *P. pelagicus* revealed by AFLP analysis by P_{+3}-2/M_{+3}-1, P_{+3}-4/M_{+3}-1, P_{+3}-4/M_{+3}-2 and P_{+3}-7/M_{+3}-1

Primer.	Chantaburi (*N* = 12)			Ranong (*N* = 14)			Suratthani (*N* = 16)		
	No. of bands	No. of polymorphic bands	No. of monomorphic bands	No. of bands	No. of polymorphic bands	No. of monomorphic bands	No. of bands	No. of polymorphic bands	No. of monomorphic bands
P_{+3}-2/M_{+3}-1	26	12	14	27	19	8	29	25	4
P_{+3}-4/M_{+3}-1	44	41	3	47	46	1	36	35	1
P_{+3}-4/M_{+3}-2	23	4	19	32	26	6	33	30	3
P_{+3}-7/M_{+3}-1	49	47	2	47	47	0	48	47	1
Total	142	104 (73.24%)	38 (26.76%)	153	138 (90.20%)	15 (9.80%)	146	137 (93.84%)	9 (6.16%)

Table 3.11 (cont.).

Primer	Krabi (*N* = 14)			Prachuap khiri khan (*N* = 16)		
	No. of bands	No. of polymorphic bands	No. of monomorphic bands	No. of bands	No. of polymorphic bands	No. of monomorphic bands
P_{+3}-2/M_{+3}-1	27	26	1	24	12	12
P_{+3}-4/M_{+3}-1	46	44	2	44	33	11
P_{+3}-4/M_{+3}-2	36	36	0	33	19	14
P_{+3}-7/M_{+3}-1	51	45	6	38	28	10
Total	160	151 (94.38%)	9 (5.62%)	139	92 (66.19%)	47 (33.81%)

Table 3.12 Pairwise genetic distance (Nei, 1978) between geographic samples of *P. pelagicus* in Thailand based on AFLP analysis using P_{+3}-2/M_{+3}-1, P_{+3}-4/M_{+3}-1, P_{+3}-4/M_{+3}-2 and P_{+3}-7/M_{+3}-1.

Pairs of samples	**Primer**			
	P_{+3}-2/M_{+3}-1	**P_{+3}-4/M_{+3}-1**	**P_{+3}-4/M_{+3}-2**	**P_{+3}-7/M_{+3}-1**
CHN-SUT	0.2108	0.0722	0.1677	0.0180
CHN-PKK	0.3786	0.1657	0.1492	0.1881
CHN-RNG	0.2774	0.0816	0.1482	0.0678
CHN-KRB	0.2945	0.1255	0.3086	0.1298
SUT-PKK	0.1863	0.2113	0.1218	0.2210
SUT-RNG	0.1921	0.0779	0.1584	0.0348
SUT-KRB	0.1097	0.0880	0.1509	0.1443
PKK-KRB	0.2388	0.2577	0.2740	0.2142
RNG-PKK	0.2368	0.2214	0.2200	0.2616
RNG-KRB	0.1783	0.0655	0.1472	0.0791

CHN = Chantaburi, RNG = Ranong, SUT = Suratthani, KRB = Krabi, PKK = Prachuap Khiri Khan

Table 3.13 The average genetic identity (above diagonal) and genetic distance (Nei's,1978; below diagonal) between pairs of geographic samples of *P. pelagicus.*

	Chantaburi	**Suratthani**	**Prachuap Khiri Khan**	**Ranong**	**Krabi**
Chantaburi	-	0.8889	0.7963	0.8611	0.8087
Suratthani	0.1177	-	0.8290	0.8913	0.8883
Prachuap Khiri Khan	0.2278	0.1876	-	0.7898	0.7834
Ranong	0.1495	0.1151	0.2360	-	0.8890
Krabi	0.2123	0.1184	0.2440	0.1176	-

A neighbor-joining (NJ) tree constructed from the average AFLP genetic distance between pairs of geographic samples of *P. pelagicus* divided 5 geographic samples into 3 phylogenetically related group; I (Krabi, Ranong and Suratthani), II (Chantaburi) and III (Prachuap Khiri Khan) (Fig. 3.26).

Significant geographic heterogeneity was observed in all pairwise comparisons of samples analyzed by P_{+3}-2/M_{+3}-1, P_{+3}-4/M_{+3}-1 and P_{+3}-4/M_{+3}-2 ($P < 0.0001$, Table 3.14). Only P_{+3}-7/M_{+3}-1 did not reveal significant genetic differences between *P. pelagicus* from Chantaburi and Prachuap Khiri Khan ($P = 0.6812$) but the remaining comparisons were significant differences statistically ($P < 0.0001$ except between Chantaburi and Suratthani where the P-value was $P = 0.0001$).

Result did not imply intraspecific population subdivisions between coastal regions but reveal microgeographic genetic heterogeneity of *P. pelagicus*. The combined data for all primers was reanalyzed by the exact test (geographic heterogeneity analysis) and was analyzed by F_{ST}-based statistics (θ). Results indicated significantly genetic differences between all pairwise comparisons. Therefore, the gene pool of *P. pelagicus* in Thai waters is reproductively isolated and each geographic sample should be regarded as different genetic populations ($P < 0.0001$, Table 3.7).

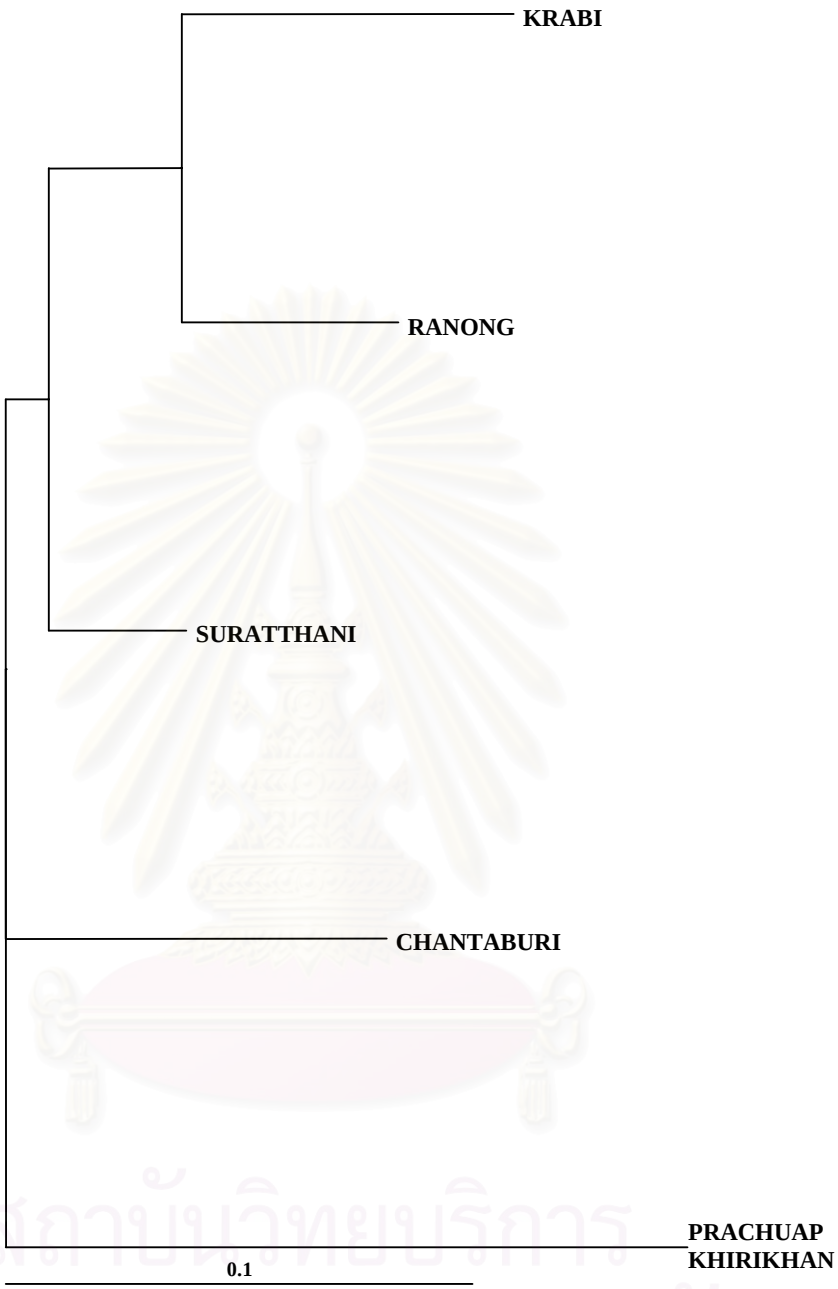


Figure 3.32 A neighbor-joining tree illustrating relationships of *P. pelagicus* in Thai waters constructed from genetic distance between pairs of samples resulted from AFLP analysis.

Table 3.14 Geographic heterogeneity analysis between pairs of geographic samples of *P. pelagicus* resulted from AFLP analysis (P_{+3}-2/M_{+3}-1, P_{+3}-4/M_{+3}-1, P_{+3}-4/M_{+3}-2 and P_{+3}-7/M_{+3}-1)

	P_{+3}-2/M_{+3}-1	**P_{+3}-4/M_{+3}-1**	**P_{+3}-4/M_{+3}-2**	**P_{+3}-7/M_{+3}-1**
CHN-SUT	<0.0001*	<0.0001*	<0.0001*	0.0001*
CHN-PKK	<0.0001*	<0.0001*	<0.0001*	0.6812^{ns}
CHN-RNG	<0.0001*	<0.0001*	<0.0001*	<0.0001*
CHN-KRB	<0.0001*	<0.0001*	<0.0001*	<0.0001*
SUT-PKK	<0.0001*	<0.0001*	<0.0001*	<0.0001*
SUT-RNG	<0.0001*	<0.0001*	<0.0001*	<0.0001*
SUT-KRB	<0.0001*	<0.0001*	<0.0001*	<0.0001*
PKK-KRB	<0.0001*	<0.0001*	<0.0001*	<0.0001*
RNG-PKK	<0.0001*	<0.0001*	<0.0001*	<0.0001*
RNG-KRB	<0.0001*	<0.0001*	<0.0001*	<0.0001*

CHN = Chantaburi, RNG = Ranong, SUT = Suratthani, KRB = Krabi, PKK = Prachuap Khiri Khan

The gene flow level between pairs of geographic samples was estimated and all pairs of geographic samples exhibited the restrictive gene flow level (< 1 individual per generation) indicating strong genetic subdivisions in Thai *P. pelagicus*.

3.2.1 Development of a sequence–characterized amplified region (SCAR) marker for detection of *P.* pelagicus.

In a total, 11 candidate species-specific AFLP markers were observed from genotying of *P. pelagicus* (*N* = 72) with 4 primer combinations. Name of informative primers and sizes of candidate AFLP fragments are illustrated by Table 3.16.

Table 3.15 F_{ST}-based statistics (θ) and geographic heterogeneity analysis between pairs of 5 geographic samples of *P. pelagicus* based on AFLP analysis

Geographic sample	Theta test		Gene flow	Exact test
	Theta P (θ)	χ^2		P
CHN-SUT	0.3052	17.09*	0.57	<0.0001*
CHN-PKK	0.4050	27.50*	0.37	<0.0001*
CHN-RNG	0.3437	17.87*	0.48	<0.0001*
CHN-KRB	0.2627	21.06*	0.70	<0.0001*
SUT-PKK	0.4076	26.09*	0.36	<0.0001*
0SUT-RNG	0.2480	15.76*	0.76	<0.0001*
SUT-KRB	0.4474	16.12*	0.31	<0.0001*
PKK-RNG	0.4910	26.84*	0.26	<0.0001*
PKK-KRB	0.4421	26.53*	0.32	<0.0001*
RNG-KRB	0.2686	13.89*	0.68	<0.0001*

$\chi^2 = 2N\theta(k-1)$, df = (s-1)(k-1), was used *N* is the number of individuals used in the analysis, k is the number of alleles and s the number of populations. * = significant at P < 0.01. The χ^2 value at P = 0.05, 0.01 and 0.001, df = 4 is 9.49, 13.28 and 18.74, respectively.

Table 3.16 Candidate species-specific RAPD fragments of *P. pelagicus* in Thailand revealed by AFLP analysis

Primer	AFLP marker (bp)
P_{+3}-2/M_{+3}-1	360, 370 and 470
P_{+3}-4/M_{+3}-1	**300***, 360 and 380
P_{+3}-4/M_{+3}-2	300, 320, 390 and **420***
P_{+3}-7/M_{+3}-1	340, 350 and 500

*Fragments that were cloned, sequenced and tested for species-specificity.

Two candidate species-diagnistic AFLP fragments composing of a 300 bp fragment from $P_{+3}4/M_{+3}$-1 and a 410 bp fragment from P_{+3}-4/M_{+3}-2 were reamplified and cloned by a TA cloning approach. The recombinant plasmid was isolated and sequence for both direction (Figs. 3.28 and 3.28). Nucleotide sequences of these recombinant cloned were blasted against data in the GenBank and were not significantly matched with sequences previously deposited in the database (E value>10^{-4}). Therefore, they were regarded as newly unidentified sequences.

Two pairs of primers were designed from pP4M1-300 and pP4M2-420. Specificity of primers were tested against genomic DNA of the targer (*P. pelagicus*, *N* = 100) and nontarget species (*S. scyllata*, *N* = 8; *S. oceanica*, *N* =11; *S. transquebarica*, *N* = 11 and *C. crucifera*, *N* = 6). Although the false positive results of pP4M1-300 were not observed, the amplification success of this SCAR marker was very low (30%). As a result, it cannot be used as species-diagnostic markers in *P. pelagicus* (Fig. 3.29).

Conversely, pP4M2-420 generated consistently amplification results in the target species (97 from 100 individuals accounting for 97.0% investigated specimens, Fig. 3.30). No positive amplification product (176 bp) was found in mud crabs (*S. serrata*, *S. oceanica* and *S. transquebarica*). Nevertheless, the positive amplification product was found in all individuals of *C. crucifera* (*N* = 6).

SSCP analysis was then applied to verify whether sequences of the PCR product of *P. pelagicus* and *C. crucifera* were different. A single SSCP pattern was observed in *C. crucifera* whereas several SSCP patterns were found in *P. pelagicus* suggesting high polymorphism of this DNA segment. SSCP analysis can then be applied to identify homologous DNA segments exhibiting identical sizes but different sequences (Fig.3.31). An additional species-diagnostic SCAR marker of *P. pelasgicus* was successfully developed

```
GACTGCGTAC ATGCAGAGTG GCGCTCAACA TCAAACCACG CAGATCACGC CACGCCCACC 60
                                                  pP4M1F300-F
CCTACCTAAC CAGGTCCTGT GCTGCGACCA GAGGGGAGGG GTGCGTAACT CTGTCTGGAT 120
TATCTAGCCT TTGCACAAAT ATGTAGTGTT TCGTAGTCAG TATTTTCACA AAACTCACAA 180
TTATGAATTC GCATCAAGAA AGCTTTTTTC TCTTTTCTCC CGTTCATTCC CTAATTCTTC 240
       pP4M1F300-R
TTTTTCCACT TCGGTTTGTT GTTACTCAGG ACTCATCA                         278
```

Figure 3.33 Nucleotide sequence of pP4M1-300. The location and sequence of a *P. pelagicus*-specific forward primer (P4M1-300F) and those complementary to a reverse primer (P4M1-300R) are illustrated in boldface and underlined.

```
GACTGCGTAC ATGCAGAGTG TGTTGCAGCA ACAAGAGGTT AGGTTTGCCG GCGACGGCGA 60
TCTGCTGTCG ACTCTTCACG AGCAACACGT GATGGCCGGC CAGGGATCCA TGAACAAGAA 120
                                  pP4M2-420F
GCAGGGTGTG TCCGGAGAGT CGTGCTTCCA AAACAAGACA GCCGAGAAGA TACACATCCA 180
GAGATTCGAT AAAGATTTCA GGTACGTGAT GATGTGGCGC TGTGGTGTTG TGTTTGACTC 240
GTCGTGGGTG AAGGTTGTGG GTTTGACTCA CGACTCACTG CCGTTTCTTA GCAGGAGAGG 300
                    pP4M2420-R
TAACGTGTTC CTGTTGTGGT TCTATTGCCT TGTGCTTTTG TGGTGATGTT GCCCAGCTAG 360
TACGTTAGTT TTATTGATTT TGTATCTCTC CTGTTGTGTT ACTCAGGACT CATCA      415
```

Figure 3.34 Nucleotide sequence of pP4M2-420. The location and sequence of a *P. pelagicus*-specific forward primer (P4M2-420F) and those complementary to a reverse primer (P4M2-420R) are illustrated in boldface and underlined.

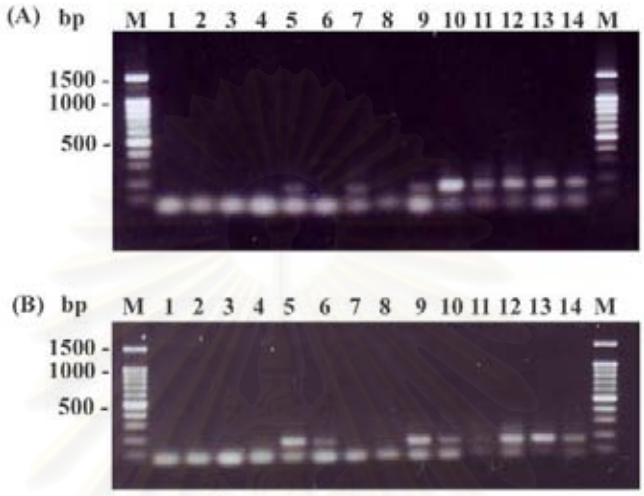


Figure 3.35 Agarose gel electrophoresis illustrating the specificity test of pP4M1-300 against genomic DNA of *S. serrata* (lanes 1, A and B), *S. oceanica* (lanes 2, A and B), *S. tranquebarica* (lanes 3, A, and B), *C. crucifera* (lanes 4, A and B) and *P. pelagicus* (lanes 5 - 14, A and B). A 100 bp ladder (lanes M) were used as the DNA marker.

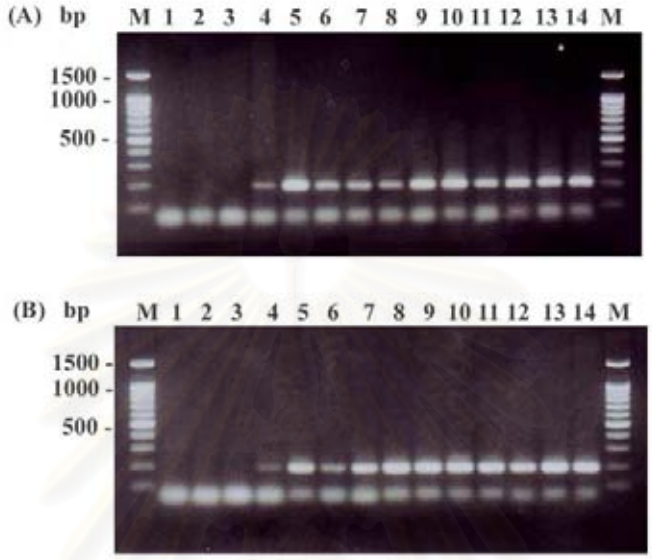


Figure 3.36 Agarose gel electrophoresis illustrating results from the specificity test of pP4M2-420 against genomic DNA *S. serrata* (lane 1, A and B), *S. oceanica* (lane 2, A and B), *S. tranquebarica* (lane 3, A, and B), *C. crucifera* (lane 4, A and B) and *P. pelagicus* (lanes 5 - 14, A and B) A 100-bp ladder (lanes M) were used as the DNA marker.

Table 3.17 Sequences and amplification results of primers designed from recombinant clones possessing a candidate species-specific AFLP fragment of *P. pelagicus*.

SCAR marker	Primer name	Primer sequence	Amplification results
pP4M1-300	pP4M1-300F/R	F:5'GTCCGTAACTCTGTCTGGATT3' R:5'TCCCACTTCGGTTTGTTGTTA3'	High false negative results (30% amplification success)
pP4M2-420	pP4M2-420F/R	F:5'CAAAACAAGACAGCCGCGAA3' R 5'TGGTTCTATTGCCTTGTGCT3'	97% in *P. pelagicus* and cross-amplified in *C. crucifera* (*N* = 6) but not in mud crabs (*N* = 38)

Sensitivity of *P. pelagicus*-specific primers was examined against varying concentrations of the target DNA template (2-fold dilutions of 12.21 pg - 25 ng). pP4M2-420 primer pairs revealed good correlation between the amount of DNA template and intensity PCR product. The sensitivity of detection was approximately 400 pg of the target DNA template (Fig. 3.32).

Stability of *P. pelagicus*-specific SCAR markers derived from AFLP was tested. The tedious and time-consuming phenol/chloroform extraction method was omitted by simplification of the extraction method to a rapid 5% chelex-based method without significant disturbance of the amplification success (Fig. 3.33). The developed SCAR marker (pP4M2-420) was found in frozen, boiled and saline-preserved specimens suggesting high stability of this marker.

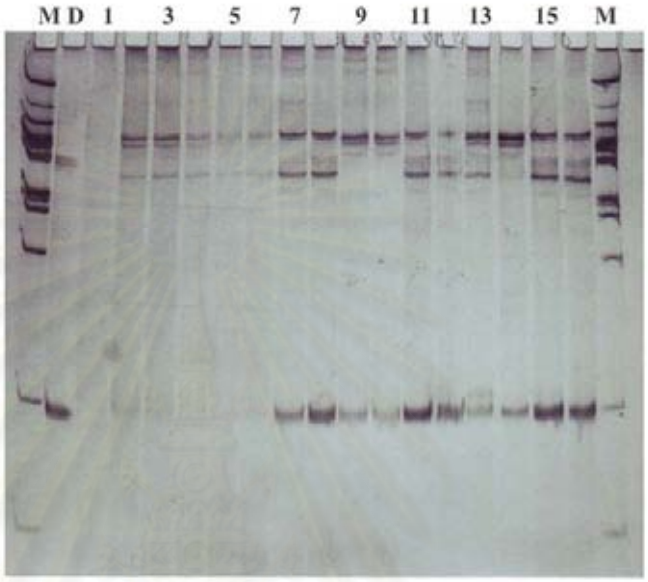


Figure 3.37 SSCP patterns of the PCR product of P4M2-420 against genomic DNA of *C. crucifera* (lanes 2-6) and *P. pelagicus* (lanes 7-16). The PCR product was size-fractionated through native 17.5% PAGE (crosslink = 37.5:1) at 200 V for 18 hours. Lanes M and D are a 100 bp DNA marker and the non-denatured PCR product, respectively.

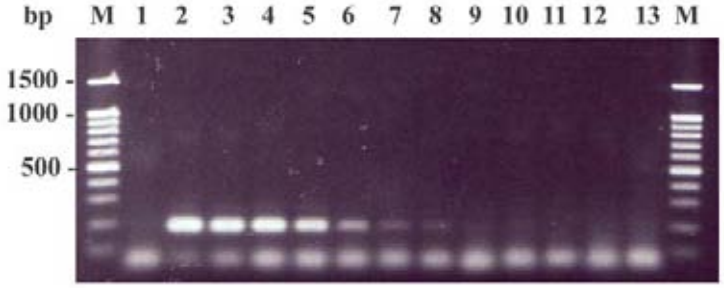


Figure 3.38 Sensitivity of P4M2-420 was examined against varying concentrations of *P. pelagicus* DNA template (25 ng, 12.5 ng, 6.25ng, 3.12 ng, 1.56 ng, 781.25 pg, 390.62 pg, 195.31 pg, 97.66 pg, 48.83 pg, 24.41 pg, and 12.21 pg corresponding to lanes 2 - 13, respectively). Lanes 1 and M are the negative control (without the genomic DNA template) and 100 bp DNA marker.

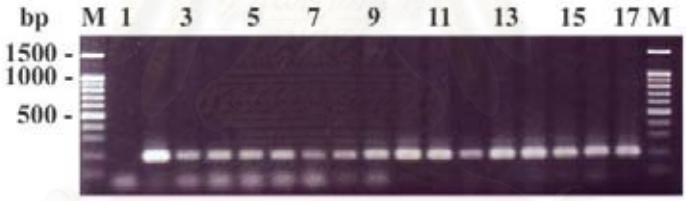


Figure 3.39 Agarose gel electrophoresis showing results from amplification of total DNA extracted with phenol/chloroform (lanes 2 - 9) and 5% chelex-based extraction methods (lanes 10 - 17) of frozen (lanes 2 - 3 and 10 - 11), boiled (lanes 4 - 7 and 12 - 15) and saline-preserved (lanes 8 - 9 and 15 - 16) specimens with P4M2-420F/R. Lanes 1 and M are the negative control (without genomic DNA template) and a 100 bp DNA marker.

CHAPTER IV

DISCUSSION

Genetic diversity of *Portunus pelagicus* in Thailand

Analysis of genetic diversity and differentiation of various organisms is essential for genetic research (individuality and parentage, population genetics, phylogenetics, molecular taxonomy and systematics, and evolutionary studies) of various organisms (Avise, 1994).

RAPD-PCR has been widely used for population genetic and systematic studies (Garcia et al., 1994; Heipel et al., 1998; Tassanakajon et al., 1998). This method has also been used for identification of molecular genetic markers in various applications including determination of mode of reproduction in the bivalve mollusk *Lasaea rubra* (Tyler-Walters and Hawkins, 1995), differentiation of two species of the freshwater mussels *Pleurobema clava* and *P. sintixia* (White et al., 1996), and generation of microsatellite markers in the blacklip abalone *Haliotis rubra* (Huang and Hanna, 1998).

AFLP is a new approach that combines advantages of RAPD-PCR and PCR-RFLP. Although it is promising for population genetic studies, the use of AFLP analysis for genetic diversity and population differentiation of marine organisms is still limited. Most studies have used this approach for construction of the genetic linkage maps, for example in the kuruma shrimp, *Penaeus japonicus* (Moore et al., 1999; Li et al., 2003) and in black tiger shrimp, *P. monodon* (Wilson et al., 2002).

There are no publications concerning genetic diversity and population structure of *P. pelagicus*. A total of 112 RAPD fragments were observed from analysis of *P. pelagicus* (N = 109) in this study with 5 decanucleotide primers indicating that inbreeding is not a major concerned in this species.

The percentage of polymorphic bands in each population of blue swimming crab was 72.73% - 85.05% which was slightly greater than that previously reported in

the giant tiger shrimp *Penaeus monodon* from five geographic locations in Thailand (51.1% to 57.7%; Tassanakajon et al., 1998) but lower than that of the tropical abalone, *Haliotis asinina* from 3 wild samples and 2 hatchery stocks in Thailand and 1 hatchery stock from the Philippines (81.25% to 90.48%, Tang et al., 2005).

For gene diversity studies of marine crabs in Thai waters, Klinbunga et al. (2000) examined genetic diversity of three mud crab species, *Scylla serrata, S. oceanica*, and *S. tranquebarica*, collected from two locations in eastern Thailand (Chanthaburi and Trat) by RAPD-PCR. Ninety-one reproducible RAPD fragments, generated by UBC456, UBC457, and YNZ22, were polymorphic. The percentage of polymorphic bands within populations ranged from 47.92% to 77.59%. Species-specific RAPD markers were also observed and used to construct a molecular diagnostic key in these taxa. Large genetic differences between species were found (*Dij* = 0.425 to 0.751), whereas those between populations within each species were much lower (*Dij* = 0.171 to 0.199). The neighbor-joining tree based on genetic distances among pairs of individuals indicated three distinct groups, corresponding to *S. serrata, S. oceanica,* and *S. tranquebarica.* No genotypes were shared among these three species. This suggests the absence of genetic exchanges between sympatric mud crab species in eastern Thailand. Therefore, mud crabs in this area should be recognized as three different species rather than a single panmictic species exhibiting different morphs.

Although RAPD-PCR is sensitive to several reaction factors, it is quite useful, when used with caution, for applications in many organisms. Barman et al., (2003) evaluated genetic diversity and phylogenetic relationships of four species of Indian major carps (Family *Cyprinidae*) including the rohu (*Labeo rohita*), the kalbasu (*L. calbasu*), the catla (*Catla catla*) and the mrigal (*Cirrhinus mrigala*). The average intraspecific and interspecific genetic similarity indices are 0.893 - 0.975 and 0.190 - 0.336, respectively. The low levels of within-species genetic variation was found in farmed stocks of the rohu and the kalbasu is possibly due to the use of limited number of wild founders for propagation and maintenance of the cultured stock of each species for a long period of time. In contrast, relatively higher levels of intraspecific genetic variation exhibited in the catla and the mrigal. This may be due to the lower rate of propagation in these species than rohu and kalbasu.

Genetic diversity of *P. pelagicus* in Thai water was also studied by AFLP analysis. Four primer combinations (P_{+3}-2/M_{+3}-1, P_{+3}-4/M_{+3}-1, P_{+3}-7/M_{+3}-1, and P_{+3}-4/M_{+3}-2) were analyzed against 72 individuals of *P. pelagicus*. Although sample sizes of *P. pelagicus* from each geographic sample in this thesis were relatively small, specimens were collected from different geographic locations on both the Andaman Sea and the Gulf of Thailand. This should be sufficient and compensated by the use of a more sensitive approach like RAPD.

Like RAPD analysis, high genetic diversity within each geographic sample was also observed from AFLP analysis. The average polymorphic bands in each geographic sample was 66.19% (Prachuap Khiri Khan) - 94.38% (Krabi). The Krabi and Ranong (94.38% and 90.20%) samples exhibited greater levels of within sample variation than these from Chantabuti and Prachuap Khiri Khan (73.24% and 66.19%). Nevertheless, the Suratthani sample possessed a higher level of polymorphic bands (93.84%) than other east-coast samples.

Han and Ely, (2002) assessed genetic variation across two *Morone* and three *Thunnus* species using AFLP. AFLP profiles from 23 primer combinations reveled higher polymorphic levels of each *Thunnus* (tuna) species than the *Morone* (striped bass) species. The average number of polymorphic bands was 11 – 19% in *Thunnus* species and 1 – 6% in *Morone* species. The percentage of primers showing polymorphism across investigated samples was 9%-26% in *Morone* and. 57% - 65% in *Thunnus*. Notably, underestimation of genetic diversity of investigated species may adiitinally promoted by employing agarose gel electrophoresis rather than denaturing polyacrylamide gel electrophoresis typically used for AFLP fingerprinting.

Genetic diversity of a highly endangered Asian arowana (*Scleropages formosus*) was investigated by AFLP analysis. Thirty-two individuals from each of the green, the red and the golden stocks were genotyped by six primer combinations. A total of 324 bands were found across three sample sets. The green stock showed the highest percentage of polymorphic bands (15.6%) and expected heterozygosity (0.26) followed by the red (13.)% and 0.24) and the golden (12.7% and 0.22) stocks (Yue et al., 2004).

Recently, AFLP analysis was also applied for evaluation of genetic diversity of domestic populations of the channel catfish, *Ictalurus punctatus* (Mickett et al., 2003), determination of genetic impact of domestic channel catfish on wild channel catfish populations (Simmons et al., 2006) and evaluation of genetic characteristics of one common population and two selectively hatcheries populations of the founder, *Paralichthys olivaceus* (Liu et al., 2005).

The levels of genetic diversity of *P. pelagicus* reflected from the percentage of polymorphic bands indicated that AFLP may be more powerful than RAPD analysis. On the other hand, RAPD-PCR is less tedious and time consuming than AFLP analysis. Additionally, the electrophoresed patterns of RAPD are also easier to score than those of AFLP. As a result, both approaches should be equally effective for evaluation species exhibiting relatively large genetic diversity like *P. pelagicus*.

Intraspecific genetic population differentiation and gene flow of *P. pelagicus*

Hartl and Clark (1997) suggest that the structuring of populations occurs in almost all organisms. Many species organize themselves into natural subpopulations. When a population is subdivided, some degree of differentiation is virtually inevitable in the acquisition of the allelic frequencies that vary in that population. Factors determine the structure of a population may be the system of reproduction, natural selection favoring different genotypes in different populations, degree of endogamy, gene flow and/or genetic drift within and between populations.

Frattini and Vannini (2002) examined genetic differentiation of mud crab (*S. serrata*) within the Indian Ocean using DNA sequencing of COI (535 bp, $N = 77$ from 4 mangrove swamps of the African topics). Twenty-four haplotypes were found. A single most frequent haplotype shared among all geographic samples and rare haplotypes were typically found in only one or two individuals of a specific population. Analysis of molecular variance (AMOVA), F_{ST} statistics and χ^2 contingency analysis of mtDNA haplotype frequencies revealed significant genetic differentiation between geographic samples.

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 and AFLP polymorphism. 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. For AFLP analysis, no significant genetic heterogeneity was found between *E. sinensis* and *E. japonicus*. This indicates that AFLP analysis may not be more sensitive than RAPD-PCR for detection of genetic diversity and differentiation at least in the mitten crabs.

Large genetic distance between pairs of geographic samples were observed in both RAPD (0.1151 - 0.2440) and AFLP (0.0929 - 0.2471) analyses. This indirectly reflected within species differentiation of *P. pelagicus* in this study. Generally the levels of pairwise genetic distance did not correlate with either coastal regions (low genetic distance within the same region but larger distance between samples from different regions) or geographic distance (larger genetic distance with more geographic distance according to the isolation by distance model).

A neighbor-joining tree constructed from RAPD genetic distance allocated the Kribi sample into the same cluster as Prachuap Khiri Khan and Chantaburi whereas *P. pelagicus* from Ranong (Andaman Sea) and Suratthani (Gulf of Thailand) were considered as separate genetic groups.

On the other hand, the topology of a neighbor-joining tree constructed from AFLP genetic distance divided all investigated samples to different groupings. Suratthani, Krabi and Ranong were regarded as the same group whereas Prachuap Khiri Khan and Chantaburi samples were independently related from that group and one another.

The disagreement of the NJ tree toplogy from different molecular approaches may have resulted from analysis of different regions of genome amplified by RAPD and AFLP. This possibly caused by the use of limited numbers of primers for genetic analysis of *P. pelagicus* in this study. Although phylogenetic trees constructed from different data sets did not agree well, both trees strongly suggested the existence of reproductively fragmented gene pooled of *P. pelagicus* in Thai waters.

Geographic heterogeneity analysis by each RAPD primer indicated significant genetic heterogeneity among 43 of 50 possible comparisons even after a sequential Bonferroni method was applied ($P < 0.01$). The combined data indicated microgeographic population subdivisions among all pairwise comparisons of *P. pelagicus* based on the exact test ($P < 0.0001$) and F_{ST}-based statistics (θ, $P < 0.01$).

Likewise, significant geographic heterogeneity was observed between pairs of samples analyzed by each AFLP primer combination. Almost all of the comparisons (49 of 50 possible comparisons) were statistically significant ($P < 0.0001$). The combined data indicated intraspecific population differentiation among all pairwise comparisons of *P. pelagicus* based on the exact test ($P < 0.0001$) and F_{ST}-based statistics (θ, $P < 0.01$).

The information indicated that the gene pool of *P. pelagicus* was not homogeneous but microgeographically fragmented intraspecifically. This reasonably explains different phylogenetic grouping of neighbor-joining trees constructed from RAPD and AFLP data.

Patterns of genetic differentiation at the micorgeographic scale in this study (e.g. between geographic samples) were different from those of marine species (e.g. the giant tiger shrimp and abalone) previously reported where a lack of genetic differentiation or significant genetic heterogeneity was found between coastal region (e.g. between Andaman Sea and Gulf of Thailand).

In *P. monodon*, Klinbunga et al., (2001) examined genetic diversity of specimens collected from 5 areas, Chumphon and Trad (Gulf of Thailand), and Pangnga, Satun and Trang (Andaman Sea) by RAPD and mitochondria DNA (16S ribosomal DNA and an intergenic COI-COII) polymorphism. A total of 53 polymorphic fragments from UBC299, UBC273 and UBC268 were consistently scored across all samples. From the respective primer 26, 32 and 30 genotypes were generated. In addition, 37 mtDNA composite haplotypes were observed from restriction analysis for the same *P. monodon* samples. High haplotype diversity (0.855) and nucleotide diversity (3.328%) of Thai *P. monodon* were observed. Strong population differentiation of *P. monodon* between the Andaman Sea and Gulf of Thailand was clearly illustrated by both techniques ($P<0.0001$).

Population genetic studies of *P. monodon* using microsatellites (Supungul et al., 2000) and sequencing of the mitochondrial COI gene segment (Khamnamtong, personal communication) were carried out and also provided a similar differentiation pattern (between different coastal regions).

In Thai abalone, Klinbunga et al. (2003) examined genetic diversity of *Haliotis asinina*, *H. ovina*, and *H. varia*, by PCR-RFLP of 18S and 16S rDNAs and RAPD. Restriction analysis of 18S (nuclear) ribosomal DNA with *Alu* I, *Taq* I, and *Hae* III and 16S (mitochondrial) rDNA with *Bam* HI, *Eco* RI, *Hae* III, and *Alu* I gave 12 and 13 digestion patterns, respectively. A total of 49 composite haplotypes were found. A UPGMA dendogram constructed from divergence between pairs of composite haplotypes, revealed reproductively isolated gene pools of these abalone and indicated that *H. asinina* and *H. ovina* are genetically closer than H. varia. Geographic heterogeneity analysis and F_{ST} estimate indicated clear genetic differentiation between *H. ovina* originating from the Andaman Sea (west) and the Gulf of Thailand (east, $P < 0.0001$), whereas partial differentiation was observed between the Philippines and the remaining *H. asinina* samples ($P < 0.0021$). Nevertheless, the nucleotide divergence between geographic samples within each species was extremely low, implying a low degree of intraspecific population differentiation in abalone.

Subsequently, population genetic studies of *H. asinina* in Thai waters were carried out with microsatellites and results indicated significant genetic differentiation between specimens from the Andaman Sea (west) and the Gulf of Thailand (east, $P < 0.05$, Tang et al., 2005) but not within each coastal region.

Several factors are responsible for intraspecific differentiation: migration or gene flow, random genetic drift, modes of natural selection, mutation and genetic recombination through mating systems. In addition to such 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 mobility is the most important factor reflecting the apparent magnitude of intraspecific subdivisions. Therefore, it is not surprising that vagile organisms (high degree of gene flow) for example insects and

birds, have lower level of population differentiation than do relatively sedentary species (Ward *et al.*, 1992).

Gene flow is the exchange of genetic material between populations caused by movement of individuals or their successfully fertilizing gametes. Basically, it is possible to estimate only the parameter N_em. This is interpreted as the absolute number of successfully reproducing individuals exchanged between populations per generation (Ovenden, 1990; Avise, 1994). Generally, 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 (e.g. the sea urchins, *S. purpuratus* and *Hediocedaris tuberculata* and the red rock lobster, *Jasus edwardsii*) (Palumbi and Wilson, 1990; McMillan, 1992; Ovenden *et al*., 1992).

On the basis of both RAPD and AFLP analyses, strong population differentiation was observed in this species. As a result, *P. pelagicus* is regarded as a low gene flow species ($N_em < 1$ for almost all of the pairwise comparisons). A restricted level of gene flow during larval stages of *P. pelagicus* may be responsible this differentiation. Moreover, *P. pelagicus* migrates inwards and outwards of the estuaries into the open ocean for spawning and as a reaction to lowered salinities (Meagher 1971, Potter *et al.* 1983, 1998). Due to their strong swimming ability, *P. pelagicus* adults are able to actively travel approximately 20 km a day (Sumpton and Smith 1991).

Nevertheless, the geographic distance within the same coastal region, for instance, between Chantaburi to Prachub Khiri Khan, Prachub Khiri Khan to Suratthani and Ranong to Krabi was 526 and 364 and 296 km, respectively. As a result, this geographic parameter may promote levels of population differentiation in *P. pelagicus*.

On the basis of the present study, five *P. pelagicus* samples were regarded as five different genetic populations. From the management point view, these isolated populations should be treated as separately exploited stocks.

Molecular population genetic techniques provide necessary information required for elevating culture and management efficiency of *P. pelagicus*. knowledge

on genetic diversity and population differentiation of *P. pelagicus* not only yields critical information on historical and evolutionary aspects of *P. pelagicus* in Thailand but also the ability to construct effective breeding programs and restocking projects in this species.

Additionally, 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, genetic improvement through selective breeding programs and enhancement of natural *P. pelagicus* stocks. Nevertheless, general considerations for genetic management of *P. pelagicus* should emphasize conservation of its genetic diversity as much as possible. An overexploitation of wild *P. pelagicus* should be compensated by the stock enhancement programmes where local populations are required as the founders.

Species-specific SCAR markers in *Portunus pelagicus*

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 markers 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 polymerase chain reaction 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.

Zhou et al., (2001) converted 3 (RA1-PA, RA2-EF and RA4-D) produced by OPJ01, and 2 RAPD fragments (RA3-PAD and RA5-D) produced by OPJ07 to SCAR markers and used for identification of the specific fragments in gynogenetic clones of silver crucian carp (*Carassius auratus gibelio*). Only individuals from clones P and A generated a specific band using a pair of primers from RA1-PA, whereas no products

were detected in individuals from clones D, E and F. The expected PCR product was successfully amplified from primers designed from RA2-EF and RA3-PAD. Furthermore, a pair of primers from RA4-D amplified specific bands only in individuals from clone D even though weak bands could be found in all individuals of the five clones when lower annealing temperatures were used. Primers from RA5-D amplified genomic DNA of fish from clones P, A, D and E but not F. SCAR markers identified in that study offer a powerful and rapid method for discrimination of different fish clones and for genetic analyses that examine their origins and unique reproductive modes in the crucian carp.

AFLP analysis has also applied for larval identification of fish of the genus *Lutjanus* which is important for relative ecological studies and mariculture (Zhang et al., 2004). AFLP was used to identify and characterize 11 *Lutjanus* species captured in Nansha coral reefs. A total of 132 AFLP fragments were found across all species. Seven markers were fixed in all specimens and used for diagnosis of fish larvae of this genus.

Species-specific markers play important roles for quality control of products from *P. pelagicus*. Using RAPD and AFLP analyses, several candidate species-specific fragments were observed in *P. pelagicus*. However, population-specific RAPD and AFLP marker were not observed.

RAPD-PCR is sensitive to reaction conditions. This technique also requires high-quality DNA template for reliable and consistent results, which may not be possible for field specimens. This may cause significant false-negative results from suspected specimens. Although AFLP analysis is more reproducible than RAPD-PCR, this technique requires several tedious steps (digestion, ligation and amplifications of DNA) limiting the ability to analyze a large number of specimens within a short period of time.

Sweijd et al. (1998) developed species-specific markers for differentiation of a commercially exploited abalone in South Africa, *H. midae*, and a sympatrically congeneric species, *H. spadicea*, for quality control of exported abalone based on species-specific PCR and PCR-RFLP approaches. PCR primers designed from cDNA of the lysin gene sequences specifically amplified a 1300-bp fragment from genomic

DNA of dried, cooked, and fresh abalone tissues. A 146-bp fragment was used to verify whether canned abalone was *H. midae*. Discrimination of these abalone could also be carried out by restriction analysis of a 1300-bp fragment with *Cfo* I, *Dra* I, *Taq* I, and *Hinf* I.

Nine RAPD and two AFLP fragments exhibiting *P. pelagicus*-specific nature were cloned and sequenced. Five primer pairs were designed and further tested for specificity against genomic DNA of the target and non-target species. Three RAPD-derived SCAR markers (pPP122-510F/R, pPP158-1200F/R and pPP158-1500F/R) showed species-specificity (95%, 100% and 100% amplification success) and can be used as species diagnosis markers for *P. pelagicus*. In contrast, P4M2-410F/R provided cross species amplification in *C. crucifera* and required further analysis by SSCP.

Primer sensitivity was tested by PCR amplification using homologous DNA template. The sensitivity of detection was approximately 400 and 200 pg of the target DNA template for primer pPP122-510F/R and pPP158-1200F/R but greater sensitivity (12.21 pg) was observed with pPP158-1500F/R. The sensitivity levels of species-specific PCR developed in this study were sufficient for the identification of the species-origins of *P. pelagicus* beginning with the early development stages.

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. The positive fragment of pPP122F510 (152 bp), pPP158F1200 (397 bp) and pPP158F1500 (262 bp) was consistently obtained from frozen, saline-preserved and boiled *P. pelagicus*. This reduces the operation time and effort for authentication of *P. pelagicus* products.

In this thesis, RAPD and AFLP analyses was successfully used to generate species-specific markers of *P. pelagicus* in Thailand. SCAR markers derived from RAPD provided species-specificity and can simply detected by PCR and agarose gel electrophoresis while the AFLP-derived SCAR marker required further SSCP analysis for *P. pelagicus*-specific detection.

CHAPTER V

CONCLUSIONS

1. RAPD (OPA02, OPA14, OPB10, UBC122 and UBC158) and AFLP (P_{+3}-2/M_{+3}-1, P_{+3}-4/M_{+3}-1 and P_{+3}-4/M_{+3}-2). analyses indicated high genetic diversity within each geographic sample of *P. pelagicus*. The percentage of polymorphic bands in each sample was 72.73% - 85.05% and 66.19% - 94.38%, respectively.

2. Large genetic distance between pairs of conspecific samples of *P. pelagicus* was observed from both RAPD and AFLP analyses. Pairwise genetic differentiation was statistically significant based on geographic heterogeneity analysis ($P < 0.0001$) and F_{ST}-based statistics ($P < 0.01$). This indicated that the gene pool of *P. pelagicus* in Thai waters is not panmictic but fragmented at the microgeographic scale.

3. Fifteen RAPD and thirteen AFLP candidate species-specific fragments were identified. Three RAPD derived SCAR markers (pPP122-510, pPP158-1200F and pPP158-1500) generated the expected product in 95%, 100% and 100% in the target species without the false positive in the non-target species. In addition, one SCAR marker derived from the candidate AFLP fragment gave the expected product in 97% of investigated *P. pelagicus* individuals. This marker also cross-amplified genomic DNA of *C. crucifer* ($N = 6$) but not in mud crabs ($N = 30$). Authentication of *P. pelagicus* could be further carried out by SSCP of the amplified product.

4. Sensitivity of detection of *P. pelagicus*-specific SCAR primers was approximately 400 pg, 200 pg, 12.21 pg and 400 ng of pPP122-510, pPP158-1200, pPP158-1500 and pP4M2-410, respectively.

5. Stability of *P. pelagicus*-specific markers was tested. Positive amplification products were consistently observed against genomic DNA of frozen, saline-preserved and boiled *P. pelagicus* meat extracted from both the standard phenol/chloroform and chelex-based extraction methods.

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APPENDICES

Appendix A

Chemicals for Preparation of Polyacrylamide Gels and Silver Staining

1. 6% Denaturing acrylamide solution, 600 ml

Acrylamide	34.2	g
Bis-aclylamide	1.8	g.
7 M urea	252	g.

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

Acrylamide	194.80	g.
Bis-aclylamide	5.19	g.

3. Bind silane

95% Ethanol	995	µl
Bind silane	4	µl
Acetic acid	5	µl

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 (10mg/ml)	600	µl

Appendix B

Pairwise genetic identity between geographic sample of Thai *P. pelagicus* based on RAPD analysis using OPA02, OPA14, OPB10, UBC122 and UBC158

Pairs of samples	Primer				
	OPA02	**OPA14**	**OPB10**	**UBC122**	**UBC185**
CHN-SUT	0.9775	0.8978	0.9465	0.9515	0.7993
CHN-PKK	0.9941	0.8870	0.9676	0.7664	0.8881
CHN-RNG	0.8568	0.8716	0.8265	0.9397	0.6966
CHN-KRB	0.9207	0.8160	0.8669	0.8711	0.8554
SUT-PKK	0.9854	0.8395	0.9410	0.8122	0.7760
SUT-RNG	0.9126	0.7979	0.8155	0.9400	0.9346
SUT-KRB	0.9504	0.7433	0.8508	0.8764	0.7797
PKK-RNG	0.8633	0.8145	0.8251	0.7932	0.6336
PKK-KRB	0.9164	0.9312	0.8683	0.8929	0.9346
RNG-KRB	0.9710	0.7952	0.9864	0.9072	0.6589

Pairwise genetic identity (Nei, 1978) between geographic samples of *P. pelagicus* in Thailand based on AFLP analysis using P_{+3}-2/M_{+3}-1, P_{+3}-4/M_{+3}-1, P_{+3}-4/M_{+3}-2 and P_{+3}-7/M_{+3}-1.

Pairs of samples	Primer			
	P_{+3}-2/M_{+3}-1	P_{+3}-4/M_{+3}-1	P_{+3}-4/M_{+3}-2	P_{+3}-7/M_{+3}-1
CHN-SUT	0.8110	0.9303	0.8456	0.9822
CHN-PKK	0.6848	0.8473	0.8614	0.8285
CHN-RNG	0.7578	0.9216	0.8623	0.9344
CHN-KRB	0.7449	0.8821	0.7345	0.8783
SUT-PKK	0.8300	0.8095	0.8856	0.8017
SUT-RNG	0.8252	0.9251	0.8535	0.9658
SUT-KRB	0.8961	0.9157	0.8599	0.8656
PKK-KRB	0.7875	0.7729	0.7603	0.8072
RNG-PKK	0.7891	0.0.8014	0.8025	0.7698
RNG-KRB	0.8367	0.8366	0.8631	0.9239

Biography

Miss Kannika Khetpu was born on September 21, 1977 in Bangkok, Thailand. She graduated with degree of Bachelor of Science (Biotechnology) from Ramkhamhaeng University in 1999. She has studied for the degree of Master of Science (Biotechnology) at the Program of Biotechnology, Chulalongkorn University since 2003.

Publication during graduate study

1. **Khetpu, K**., Wongphayak, S., Klinbunga, S. and Menasveta, P. (2005). Genetic diversity and species-specific markers of the blue swimming crab *Portunus pelagicus*. 31st Congress on Science and technology of Thailand. 18-20 October 2005, Nakhon Ratchasima, Thailand (Oral presentation)