GENETIC VARIATION AND POPULATION STRUCTURE OF THE BLACK TIGER PRAWN *Penaeus monodon* IN THAILAND DETERMINED BY MICROSATELLITE MARKERS

Miss Premruethai Supungul

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Biochemistry
Department of Biochemistry
Graduate School
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ความแปรผันทางพันธุกรรมและโครงสร้างประชากรของกุ้งกุลาดำ, *Penaeus monodon* ในประเทศไทย

โดยตัวตรวจสอบชนิด ไมโครแซทเทลไลต์

นางสาวเปรมฤทัย สุพรรณกูล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

สาขาวิชาชีวเคมี ภาควิชาชีวเคมี

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Thesis Title	Genetic variation and population structure of the black tiger prawn *Penaeus monodon* in Thailand determined by microsatellite markers
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BRT 540049

พิมพ์ต้นฉบับบทคัดย่อวิทยานิพนธ์ภายในกรอบสีเขียวนี้เพียงแผ่นเดียว

เปรมฤทัย สุพรรณกูล : ความแปรผันทางพันธุกรรมและโครงสร้างประชากรกุ้งกุลาดำ, *Penaeus monodon* ในประเทศไทย โดยตัวตรวจสอบชนิดไมโครแซทเทลไลต์ (GENETIC VARIATION AND POPULATION STRUCTURE OF THE BLACK TIGER PRAWN *Peneaue Monodon* IN THAILAND DETERMINED BY MICROSATELLITE MARKERS) อ. ที่ปรึกษา : ร.ศ. ดร. อัญชลี ทัศนาขจร, อ. ที่ปรึกษาร่วม : ดร. วงศ์ปฐม กมลรัตน์, 68 หน้า. ISBN 974-639-736-2

ในการศึกษาความแปรผันทางพันธุกรรมของประชากรกุ้งกุลาดำจากธรรมชาติ 5 แหล่งของประเทศไทย ทำการเก็บตัวอย่างกุ้งจากทะเลอันดามัน 3 แหล่ง คือ จังหวัดสตูล ตรัง และพังงา และจากอ่าวไทย 2 แหล่ง คือ จังหวัดชุมพร และตราด โดยทำการตรวจสอบไมโครแซทเทลไลต์ดีเอ็นเอ (microsatellite DNA) ซึ่งอาศัยขบวนการลูกโซ่โพลีเมอร์เรส (Polymerase chain reaction) พบว่าทุกตำแหน่งของไมโครแซทเทลไลต์ (CUPmo18, Di25, และ Di27) มีความหลากหลายสูง และให้ค่าจำนวนอัลลีล (allele) ต่อตำแหน่ง คือ 37, 34 และ 32 อัลลีล ตามลำดับ เมื่อศึกษาประชากร 5 กลุ่ม โดยใช้ไมโครแซทเทลไลต์ 3 ตำแหน่งนี้ให้ค่าเฮเทอโรไซโกซิตี้ (heterozygosity) ในช่วง 0.66 ถึง 0.80 และให้ค่าเฉลี่ยของจำนวนอัลลีลในแต่ละตำแหน่งอยู่ในช่วง 22.23-26.33 ค่า effective number of alleles ชี้ให้เห็นว่า กลุ่มประชากรจากจังหวัดชุมพร (17.57) มีความแปรผันทางพันธุกรรมสูงกว่าตรัง (16.97), ตราด (15.15), สตูล (14.3) และพังงา (14.18) ตามลำดับ ความถี่ของอัลลีลของกลุ่มประชากรไม่เป็นไปตามกฎของ Hardy-Weinberg เนื่องจากในทุกกลุ่มประชากรมีจำนวนของโฮโมไซโกต (homozygote) มาก อย่างไรก็ตามเมื่อศึกษาการถ่ายทอดทางพันธุกรรม พบว่าไมโครแซทเทลไลต์ทั้ง 3 ตำแหน่งมีการถ่ายทอดอัลลีลตามกฎของเมนเดล เมื่อตรวจสอบโดยใช้ไมโครแซทเทลไลต์ที่ตำแหน่ง CUPmo18 และ Di25 พบการกระจายตัวของความถี่ของอัลลีลของกลุ่มประชากรจากทะเลอันดามันแตกต่างอย่างมีนัยสำคัญกับกลุ่มประชากรจากอ่าวไทยและที่ตำแหน่งทั้ง 2 นี้ยังให้ความแตกต่างอย่างมีนัยสำคัญภายในกลุ่มประชากรจากอ่าวไทย คือ กุ้งจากจังหวัดชุมพรและจังหวัดตราด

เมื่อทำการวิเคราะห์ทางสถิติเพื่อดูโครงสร้างประชากร พบว่าค่าสัมประสิทธิ์ของความแตกต่างของยีนในกลุ่มประชากรทั้งหมด (θ) มีค่าเฉลี่ยเท่ากับ 0.009 (95% CI = 0.0011, 0.0175) แสดงให้เห็นว่ามีความแตกต่างระหว่างกลุ่มประชากรน้อย อย่างไรก็ตามเมื่อศึกษา geographic heterogeneity และนำมาแสดงความสัมพันธ์ในเชิงแผนภูมิ โดยใช้วิธีของ Neighbor-joining สามารถแบ่งกลุ่มกุ้งกุลาดำของไทยออกเป็น 3 กลุ่มคือ กลุ่มที่ 1 สตูล, ตรัง และ พังงา กลุ่มที่ 2 ชุมพร และกลุ่มสุดท้ายคือ ตราด

ภาควิชา ....ชีวเคมี........	ลายมือชื่อนิสิต ....เปรมฤทัย สุพรรณกูล........
สาขาวิชา ....ชีวเคมี........	ลายมือชื่ออาจารย์ที่ปรึกษา ....อัญชลี ทัศนาขจร........
ปีการศึกษา ....2541........	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม ....

#C826210 : MAJOR BIOCHEMISTRY

KEY WORD: *Penaeus monodon* / black tiger shrimp / microsatellite / population structure / genetic markers

PREMRUETHAI SUPUNGUL : GENETIC VARIATION AND POPULATION STRUCTURE OF THE BLACK TIGER PRAWN *Penaeus monodon* IN THAILAND DETERMINED BY MICROSATELLITE MARKERS. THESIS ADVISOR : ASSOC. PROF. ANCHALEE TASSANAKAJON, Ph.D. THESIS CO-ADVISOR : MR. WONGPATHOM KAMONRAT, Ph.D. 68 pp. ISBN 974-639-736-2

Three microsatellite loci, CUPmo18, Di25, and Di27, were used to study on population genetics of the black tiger prawn (*Penaeus monodon*) by using Polymerase Chain Reaction (PCR). Genetic variation were examined in 5 geographic samples, three from the Andaman Sea (Satun, Trang, and Phang-nga) and two from the Gulf of Thailand (Chumphon and Trad). All microsatellite loci were shown to be highly polymorphic with number of alleles at each locus of 37, 34, and 32 alleles respectively. Population analyses based on 3 loci revealed heterozygosities between 0.66-0.80 and average alleles per locus were 22.23-26.33. The effective number of alleles suggested that Chumphon (17.57) has highest genetic variation followed by Trang (16.97), Trad (15.15), Satun(14.3) and Phang-nga (14.18) respectively. Gene frequencies of each population did not conform Hardy-Weinberg equilibrium due to significant excesses of homozygotes in all samples. However, segregation analysis of three loci using 20 progeny from a representative full-sib family revealed Mendelion segregation nature of these microsatellites. Significant difference in allele frequency distributions were found for the Andaman Sea and the Gulf of Thailand populations at the CUPmo18 and Di25 loci. At those loci, the two populations from the Gulf of Thailand were also genetically distinct.

Population structures of *P. monodon* were assessed using the hierarchical F-statistics. The average θ value among 5 samples across the three loci was 0.009 (95% CI : 0.0011 to 0.0175) suggested low level of population subdivision in Thai *P. monodon*. Nevertheless, the analysis of geographic heterogeneity and phylogenetic reconstruction using the Neighbor-joining approach divided 5 geographic *P. monodon* samples to three different gene pools constituting of Satun, Trang, Phang-nga(A), Chumphon(B) and Trad(C).

ภาควิชา ชีวเคมี	ลายมือชื่อนิสิต
สาขาวิชา ชีวเคมี	ลายมือชื่ออาจารย์ที่ปรึกษา
ปีการศึกษา 2541	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม

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LIST OF ABBREVIATIONS

bp	Base pair
°C	Degree Celsius
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphosphate
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetic acid (disodium salt)
EtBt	Ethidium bromide
H	Mean heterozygosities
HCl	Hydrochloric acid
kb	Kilobase pair (10^3 base pair)
$MgCl_2$	Magnesium chloride
min	Minute
ml	Millilitre (10^{-3} litre)
mM	Millimolar
MT	Metric tonnes
mtDNA	Mitochondrial DNA
n_e	Effective number of allele
ng	Nanogram (10^{-9} gram)
nM	Nanometre
OD	Optical Density
PCR	Polymerase chain reaction
Rnase	Ribonuclease
SDS	Sodium dodecyl sulfate
sec	Second
T_A	Annealing temperature
TE	Tris EDTA

Tris	Tris(hydroxy methyl)aminomethane
V	Volt
VNTR	Variable number of tandem repeats
μl	Microlitre (10^{-6} litre)

Chapter I

Introduction

Farming of the black tiger shrimp (*Penaeus monodon*) is one of the common aquacultural industry in many tropical countries. In 1996 the world's shrimp production was increased, for approximately 10% compared with that in 1995 (Table 1.1). Indonesia, Ecuador and India showed dramatically higher shrimp production than that of the previous year for approximately 32%, 20%, and 33%, respectively.

In Thailand, due to an outbreak of the white spot disease across vast culturing areas, the total cultured shrimp (mainly, *P. monodon*) production was decreased from 225,000 metric tons (MT) in 1995 to 205,000 MT in 1996 corresponding to approximately 9% decreasing in the shrimp production. Although, Thailand has confronted difficulties resulted from diseases, it is still the biggest *P. monodon* producer for six consecutive years.

The world's leading importing countries of shrimps are the United States of America and Japan. Approximately two-third of *P. monodon* exported from Thailand is imported to these countries, the remaining market are Europe, Asian countries, Australia, and others (Table 1.2). The production sources of *P. monodon* are from the captured fisheries and from an aquaculture sector. Previously, the former was the main contributor for the total production. Nevertheless, the number of farmed *P. monodon* has consistently increased since the last decade and has become more importance contribution than that of the captured fisheries (Table 1.3).

The farming activity of *P. monodon* in Thailand has rapidly increased reflecting large annual production. The reasons for this are supported by several factors including the appropriate farming areas without the serious disturbing from typhoons or cyclone, small variable of sea water during seasons, and ideal soils and terrain for pond construction. Culture of *P. monodon* causes increasing national revenue, therefore this penaeid shrimp species is economically important species in Thailand.

Table 1.1 World Cultured Shrimp Production : 1994-1996.

Country	Head-on Production (MT)			Variance 96/95	
	1994	1995	1996	MT	%
Thailand	250,000	225,000*	205,000*	-20,000	-9
Indonesia	100,000	100,000*	132,000*	+32,000	+32
Ecuador	100,000	100,000	120,000	+20,000	+20
India	70,000	60,000	80,000*	+20,000	+33
Vietnam	50,000	45,000*	45,000*	-	-
Bangladesh	35,000	30,000	35,000	+ 5,000	+17
China	35,000	70,000	80,000	+10,000	+14
Philippines	30,000	20,000	25,000	+ 5,000	+25
Other	88,000	82,000	83,000	+ 1,000	+1
TOTAL	758,000	732,000	805,000	+73,000	+10

Source : ASIAN SHRIMP NEWS, 4th Quarter 1996

Table 1.2 The annual shrimp exports of Thailand between 1992-1995.

	1995		1994		1993		1992	
	MT	%	MT	%	MT	%	MT	%
- USA	77,955	39	80,955	42	66,955	45	53,955	38
- JAPAN	48,725	24	49,455	26	51,635	35	47,000	33
- EUROPE /OTHER	75,320	37	60,240	32	30,299	20	39,477	28
TOTAL	202,000*	100	190,650	100	148,889	100	140,432	100

* = estimated Source : ASIAN SHRIMP NEWS, 1st Quarter 1996

Table 1.3 Shrimp production from fisheries and aquaculture sectors and culture area in Thailand.

Year	Production by normal fisheries (MT)	Production by aquaculture (MT)	Total Production (MT)	Culture Area (Rai)*
1981	122,706	10,729	133,435	171,619
1982	156,523	10,091	166,614	192,453
1983	127,584	11,550	139,134	222,107
1984	104,394	13,007	117,401	229,949
1985	91,632	15,840	107,472	254,805
1986	102,227	17,886	120,113	283,548
1987	128,100	23,566	151,666	279,812
1988	110,200	55,633	165,833	342,364
1989	110,800	93,495	204,295	444,785
1990	107,400	118,227	225,627	403,787
1991	129,100	162,070	291,170	470,826
1992	116,800	184,884	301,684	454,975
1993	100,000	225,514	325,514	449,292

* 6.25 rai = 1 ha
Source : ASIAN SHRIMP NEWS, 3rd Quarter 1996

1.1 Taxonomy of *P. monodon*

The taxonomic definition of the giant tiger shrimp, *P. monodon* is as follows (Bailey-Brook and Moss, 1992):

Phylum Arthropoda

Subphylum Crustacea

Class Malacostraca

Subclass Eumalacostraca

Order Decapoda

Suborder Natantia

Infraoder Penaeidea

Superfamily Penaeoidea

Family Penaeidae Rafinesque, 1985

Genus Penaeus Fabricius, 1798

Subgenus Penaeus

Species monodon

Scientific name : *Penaeus monodon* (Fabricius),1798

Common name : giant tiger shrimp or prawn

1.2 Morphololgy

Externally, the shrimp can be basically divided into thorax and abdomen (Fig. 1.1). The thorax is covered by single inmobile carapace which protects internal organs and supports muscle origins. The internal organs in this part consist of eyes and eye stalks, sensory antennules, antennae, and walking legs (pereopods). The abdomen has segmentation commonly observed in invertebrates. It consists of swimming legs (pleopods), which arise from each of six abdominal segments, and a tail. The tail fans comprises a telson, which bears the anus, and two uropods attach to

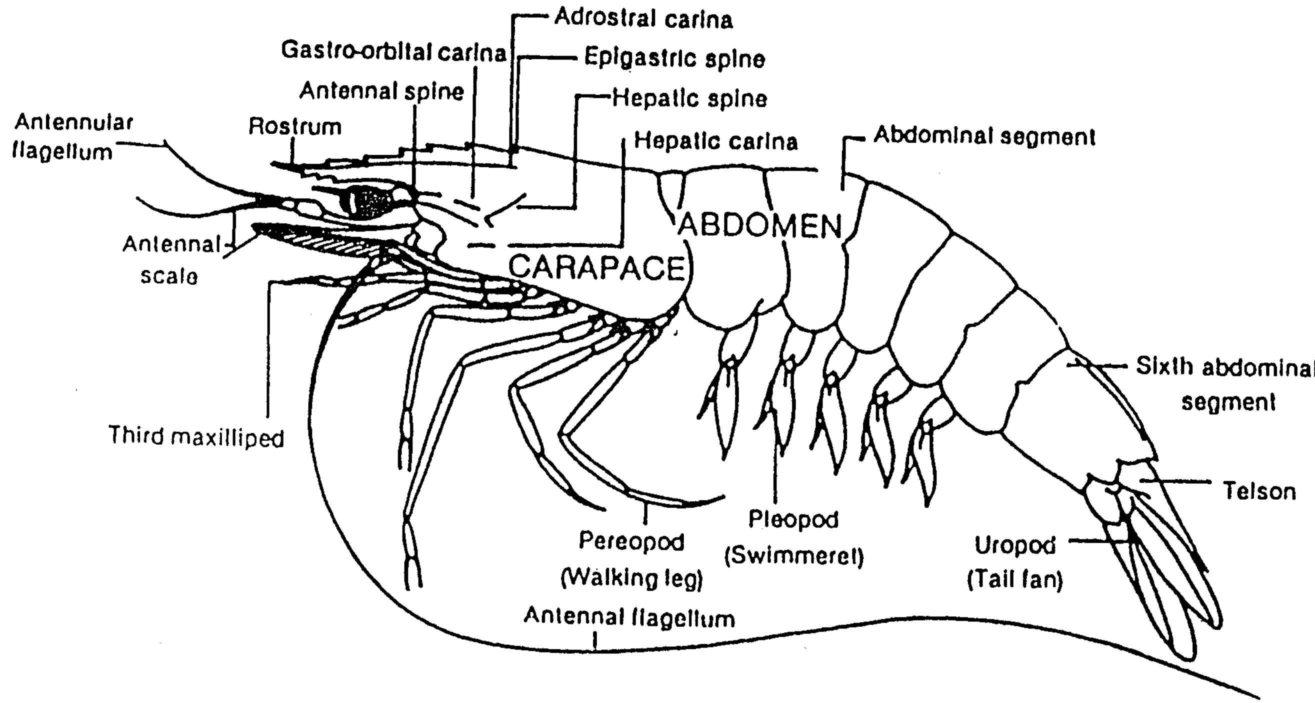

Fig.1.1 Lateral view of *P.monodon* showing important parts.

the last abdominal segment. A rapid ventral flexion of the abdomen with the tail fan produces the quick backward dart characteristic of prawns.

Shrimps grow by periodically releasing their cuticle secreted by the epidermal cell layer, consisting of chitin and proteins. Molting starts when the epidermis detaches from the cuticle layer and begins to secrete a new cuticle. The new cuticle is soft and is stretched to accommodate the increased size of the prawn immediately after molting(Anderson, 1993).

A live giant tiger shrimp has the following characteristic coloration : carapace and abdomen are transversely banded with red and white, the antennae are greyish brown, and the pereopods and pleopods are brown with crimson fringing setae. In shallow brackishwaters or when cultured in ponds, the color changes to dark and, often, to blackish brown (Motoh, 1981 : cited in Solis, 1988).

1.3 Life cycle

Development of penaeid shrimp is complex. It begins with a larvae hatching from the fertilized egg to the first stage, nauplius, followed by protozoea, mysis, and post larval stages (Fig 1.2). These require the developmental times about 1-5 days, 5 days, 4-5 days, and 6-15 days, respectively (Solis, 1988). Larvae exhibit planktonic behavior with antennal propulsion for swimming in nauplius, antennal and thoracic propulsion in mysis, and abdominal propulsion in megalopa. Nauplii utilize yolk granules within their body while the feeding starts in protozoea and mysis. At mysis stage, Lervae has five pairs of functioning pereiopods. The carapace now covers all the thoracic segments. The mysis swims like adults. After this stage, lervae metamorphoses to the post-larvae with a full complement of functioning appendages. The post-larvae continue to molting as they grow. They migrate shoreward and settle in nursery areas closed to shore or estuaries, before develop to juvenile and sub-adults, which more tolerate to variety of environmental factors. Sub - adults migrate back to the sea where they finally mature and have the first corpulation and spawn. The life span of penaeid shrimp are approximately 2 years (Anderson, 1993 ; Soils, 1988).

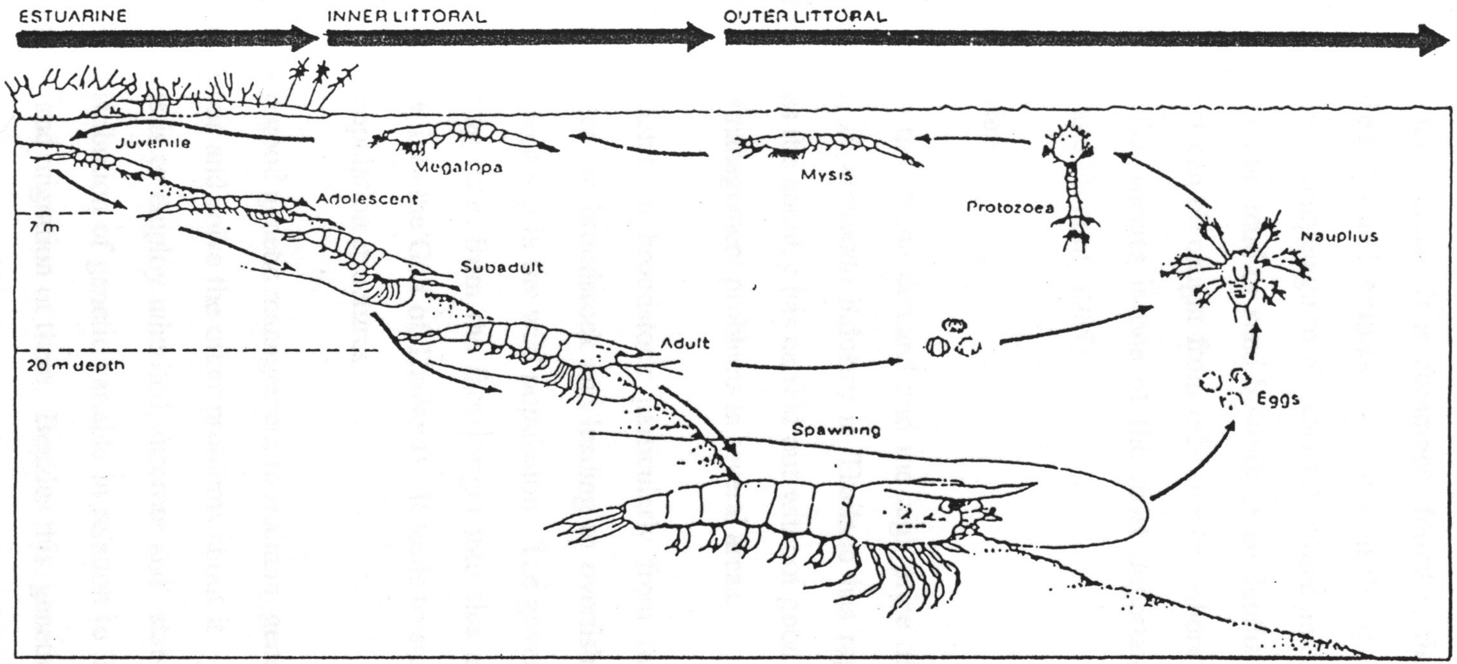

Figure 1.2 The life cycle of the giant tiger prawn, *P.monodon*, with stages in different habitats

1.4 Distribution

The black tiger prawn (*P. monodon*) is principally distributed in the major part of the Indo-West Pacific region. It is commonly found in the East and Southeast Africa, through the Red Sea and Arabian Gulf, around the Indian subcontinent, and throughout the Malasian Archipelago to Northern Australia and Japan (Fig. 1.3). It is a marine species inhabits in mud or sand bottoms at all depths from shallows to 110 meters (360 feet), so it can be caught from offshore or inshore as well as from tidal zones (or ponds). The species is one of the most important aquaculture shrimp species in Asia (Dore and Frimodt, 1987).

1.5 Exploitation

Due mainly to the strong demand and the high price of *P. monodon* in the international markets, *P. monodon* industry in Thailand has rapidly expanded. The farming activity across the country has carried out without good management practice producing serious mismanagement problems in several areas.

The high demand on broodstock particularly from the Andaman Sea has stimulated the movement of broodstock and leading to overfishing in this area. The broodstock was also declining in the wild population. The government try to increase the prawns by releasing them from the farm origin into this area (mainly from the Andaman Sea sources, into the Gulf of Thailand). It leads to stock mixing and breaks down of any natural population structures.

We must have good genetic management to maintain genetic variation, increase the prawns productions and solve the other problems about it. The basic principle of all molecular genetics is to employ inherited, discrete and stable markers to identify the levels and the distribution of genetic variable in relation to life history, population size, mating patterns and migration of them. Besides this, genetic information can help us to find or select the species which give more and more yields per area. It is better than the expanded the prawn farms which can destroy the mangrove forests.

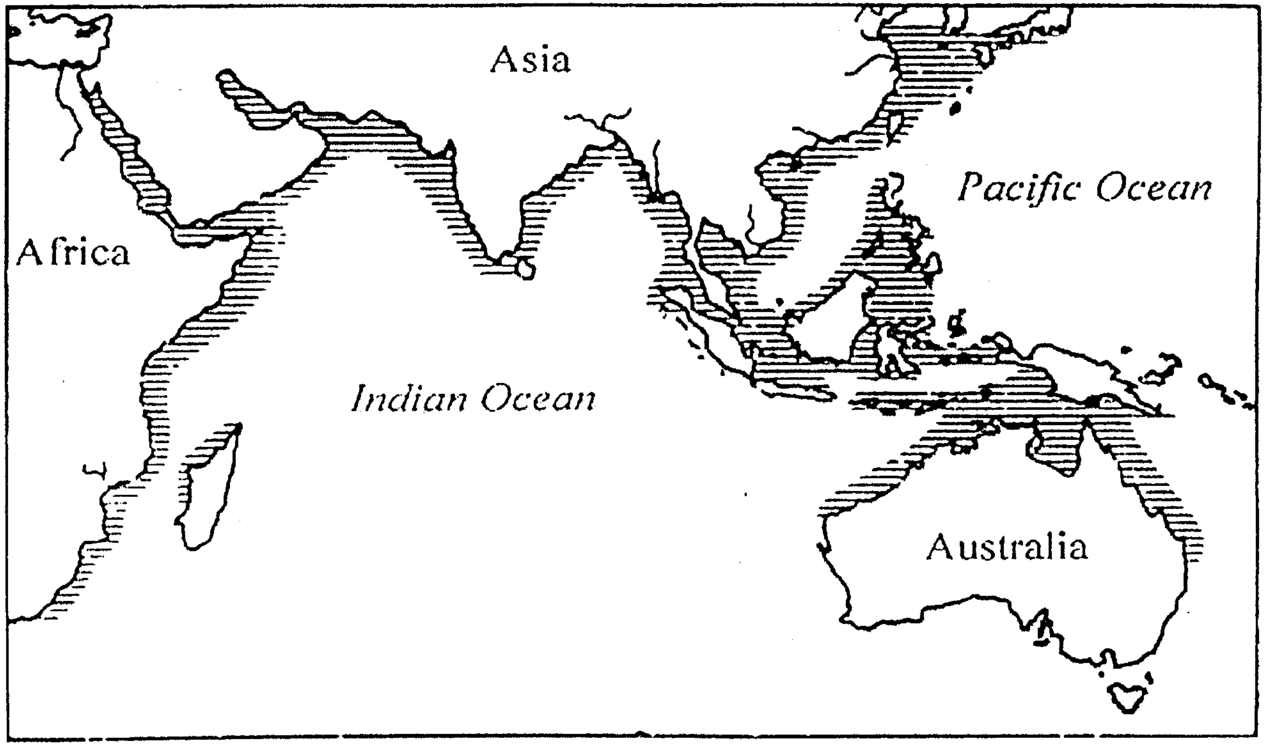

Figure 1.3 Geographic distribution of *P. monodon* in Indo-West Pacific region (Grey et al., 1983)

1.6 Genetic markers

Genetic markers especially those inherited in the Mendelian fashion are important for various population genetic studies. Based on the neutral theory, the polymorphisms observed are assumed to be primarily generated by mutation, migration, gene flow, and genetic drift. Therefore, analysis of population genetic problems involved with these factors can be answered using molecular markers. These include allozymes, mitochondrial DNA, and variable number of tandem repeat (VNTR).

1.6.1 Allozymes

Allozyme refers to the enzyme that is produced by different alleles at the same chromosomal DNA locus (Park and Moran, 1994). The polymorphic allozyme can be detected by electrophoresis of proteins (usually enzymes) observed based on the property that proteins with different net charges migrate at the different rate through a gel matrix when exposed to an electric field (Avise, 1994). The charge characteristics of proteins vary with pH of the running buffer reflecting their movements towards the positive or negative poles (Morizot and Schmidt, 1990). Therefore, proteins are electrically separated according to their net charges, sizes and shapes (Cooper, 1977). The advantages of allozyme analysis are that this technique is convenient and cost-effective. Large number of specimen can be analysed within a limitation of time. Moreover, several allozyme loci can be simultaneously examined. Accordingly, allozyme analysis is a technique of choice to begin with when the species under investigation has not been reported for any molecular data. The systems are generally useful across various taxonomic levels (Memzies, 1981 ; Mitton and Koehn, 1985 ; Todd and Hatcher, 1993 ; Ward and Elliott, 1993 all cited in Park and Moran, 1994). The allozyme markers are transmitted in a co-dominant manner. Unfortunately, this technique possesses the technical limitations. Only histochemical stains available for investigated enzymes can be carried out. The most serious problems to applied this approach to a practical selective breeding program are due mainly to the level of detected polymorphisms and the broodstock, in most cases, need to be sacrified for the analysis. Morever, isoloci can cause difficulties resulted from co-migration of bands originating from different loci (Buth, 1990). The products of protein coding genes have the lowest level of evolutionary rate so analysis of these may not be sensitive enough for descrimination of intraspecifically closely related populations which seem to be the case for population genetic studies of *P. monodon*.

1.6.2 Animal mitochondrial DNA

Mitochondrion is the cell organelle found in cytoplasm of eukaryotes. Each mitochondrion contains 5 - 10 copies of double - stranded circular DNA. The animal mitochondrial genome is approximately 16,000 - 20,000 bp in length coding for 13 protein coding genes (NADH dehydrogenase, ND, subunits 1, 2, 3, 4, 4L, 5 and 6 ; cytochrome b, three subunits of cytochrome oxidase, COI, II and III, and two subunits of ATP synthetase (ATPase 6 and 8), 2 genes coding for ribosomal RNAs (16S and 12S rRNA), 22 transfer RNA coding genes) (King and Stansfield, 1985; Park and Moran, 1994). Generally, mitochondria are inherited martiarchally except in some species (e.g. *Mytilus edulis, M. galloprovencialis*) whose contribution of paternal mitochondria is observed (Gyllensten et al., 1991 ; Margoulas and Zouros, 1993). Basically, the mutation rate of mtDNA is much more rapidly than that of single - copy nuclear genes reflecting its potential to be used for determination of intraspecific genetic variation among geographically different populations (Brown et al., 1979 ; Lynch and Jarrell, 1993).

Studies on mtDNA variation are usually by restriction analysis of the entire mtDNA or PCR - amplified mtDNA segment followed by RE digestion and/or sequencing. For restriction analysis, the digestion profile can be detected either by chemical staining or radioactive labeling (Chapman and Brown, 1990). Mutation occurring at a restriction site (either by substitutions or deletions) prevents or allows an investigated enzyme to cleave at such position and thus produces the different number of DNA fragments from investigated individuals. In addition, variation in copy number of localized tandem repeats, usually located in or near the control region of the mtDNA molecule, causes changes in size of mtDNA (length heteroplasmy)and, thus, creating differences in digestion profiles (Harrison et al. , 1985). At present, analysis of the entire mtDNA by restriction enzyme is increasingly replaced by PCR-RFLP which the specific regions of mtDNA are amplified through the polymerase chain reaction (PCR). The products are then digested with restriction endonuclease before electrophoretically analyzed. Alternatively, the PCR amplified products can be electrophoresed and further analyzed by direct sequencing (Chapman and Brown, 1990).

1.6.3. Variation number of tandem repeats (VNTR)

VNTR markers are characterized by a core sequence which consists of a number of identical repeated sequences. They can be divided into three categories; satellite, minisatellite, and microsatellite, based on the repeat length. (O'Reilly and Wright, 1995).

1.6.3.1 Satellites

Satellite DNA is a repetitive DNA that contains tandemly repeated short nucleotide sequences. The repeat unit may be from one to a few hundred nucleotides long. In some mammals, certain satellite DNAs may occur as millions of copies per genome (Alberts et al. , 1983). However, they are not as variable in size within populations as the other members of highly repetitive DNA family.

1.6.3.2 Minisatellites

Minisatellites is a repeating DNA sequence ranging between 15 - 70 bp per unit and 0.5 - 30 kb in size (Koreth et al., 1996). Minisatellites are found within noncoding regions of genomic DNA. Increases and decreases in the lengths of these result from changes in the number of repeat copies residing in the region and, hence, it is called variable numbers of tandem repeats (Avise, 1994). The variation of this DNA can be detected which is due to differences in length between conserved restriction sites. The mechanisms generating variability in minisatellites are still inconclusive. Several models have been suggested including unequal crossing over between homologous chromosomes during meiosis, replication slippage, and gene conversion (Wolff et al., 1989). Differences in length of minisatellites can be not only from the number of copies of repeats, but from the interspersion patterns of different types of repeats for which comprise the array.

Jeffreys et al. (1991) first developed a more expedient PCR-based method from the repeat unit sequence variation within single molecules, termed minisatellite variant repeat-PCR (MVR-PCR also referred as digital DNA fingerprinting). In this strategy, the sequence of repeat units in both arrays is simultaneously determined by PCR using a primer complementary to different repeat unit types, and the other primed nearby unique flanking DNA. The result in the rapid attenuation of signal strength of bands representing increasingly distal repeat positions. The problem was circumvented by using "the tagged" repeat primers. In this strategy, initial PCR amplifications were performed using the flanking primer, and lower concentrations of a repeat unit primer with approximately 20 bp extensions. After several cycles, a series of PCR products differing in length by integral numbers of repeat units are generated.

Preliminary MVR-PCR surveys of the Atlantic salmon (*Salmo solar*) Ssa 197 minisatellite locus revealed limited variation resulted from repeat units in the first 10 repeats at one end of the tandem array. Due to internal annealing of repeat unit primers, band intensity diminishes rapidly when the repeat position increases (Jeffreys et al., 1991).

1.6.3.3 Microsatellites

Microsatellites are short DNA consisting of short repeats (1-6 nucleotides) which arrayed in tandemly repeated manner for approximately 10-50 copies (Hearne et al., 1992). Microsatellite are highly abundant and randomly dispersed in most eukaryote genomes (Valdes et al., 1993 ; Weissenbach et al. , 1992 ; Wright, 1993). It was estimated that one microsatellite locus may be found every 10 kb in eukaryotic genomes (Tautz, 1989). Due mainly to high mutation rate of the microsatellite loci (1×10^{-5}- 5×10^{-4} per generation), they exhibit high allelic variation and heterozygosity levels (Hearne et al., 1992; Wright and Bentzen, 1994). Like minisatellite, the variability in microsatellites regions arise from changing in the number of repeated sequences which is proposed to be from slipped - strand misparing or slippage during DNA replication (Schlotter and Taulz, 1992). Microsatellite alleles exhibit codominant inheritance fashion (Queller et al., 1993). As the result, microsatellites are potential for several applications particularly when homozygotes are needed to be dissociated from heterozygotes.

Allelic variations of a particular microsatellite locus are detected through polymerase chain reaction. After amplification, the products are fractionated for their length polymorphisms using agarose (usually for tetranucleotide microsatellites) or polyacrylamide gels (di or trimeric microsatellites) with either non-radioactive or radioactive methods. Generally, detection of amplified microsatellites by autoradiography (labeled 5' end of the primers, electrophoresed of the products and exposed the gel with the X-ray films) are more sensitive and common. Radioactive detection of microsatellite gives clean results but less cost-effective than does the non-radioactive approach.

Non-radioactive detection are composed of ethidium bromide, silver stain and fluorescence. Staining of the electrophoretic gels with ethidium bromide is the simplest visualization approach but the lowest sensitivity compared to the remaining techniques. At least 10 ng of double standard DNA fragments are required for unambiguous detection (Bethwaite et al., 1995). Silver staining offers better sensitivity over ethidium bromide staining (pg quantities of DNA) and has been widely used for qualitative assessment of microsatellite allelic bands (Love, 1990). However, silver stain produce high but variable background caused by non-linear deposition of the silver. Detection of microsatellites using fluorescence dyes in coupling with the automated DNA sequencer yield significantly more rapid and reliable results. This technique is suitable for detection of multiplex amplification of multiple microsatellite loci. Nevertheless, the use of this system is limited by the cost.

Several recent studies used microsatellite marker for studies of population structure for example Tam and Kornfield (1996) characterized microsatellite markers in the American lobster (*Homarus americanus*), Wolfus et al. (1997) studied application of the microsatellite loci for genetic diversity study in shrimp breeding program, while Lanzaro et al. (1995) studied microsatellite variability in a West African population of *Anopheles gambiae.* Furthermore, many microsatellite loci can be amplified in a variety of closely related species. For example, microsatellite developed in the rainbow trout (*Oncorhychus mykis*) and the Atlantic salmon (*S. salar*) were able to cross-analyze in several other *Salmo* and *Onchorynchus* species (Morris et al., 1996 ; McConnell et al.,1995a).

The aim of this thesis is to study genetic variation and population structures of the black tiger shrimp *P. monodon* in Thailand using microsatellite markers.

Chapter II

Materials and Methods

2.1 Equipments

- Autoclave LS-2D (Rexall industries Co. Ltd., Taiwan)
-Automatic micropipette P20, P100, P200 and P1000 (Gilson Medical Electronic S.A., Franc)
-Autoradiography cassette(Research Products International corp., USA)
- A -20 °C Freezer
- A -80 ° C Freezer
- Gel dryer Model 583 (Bio-RAD Laboratories , USA)
- Heating block BD 1761G-26 (Sybron Thermermolyne Co., USA)
- Hyperfilm MP (Amersham International, England)
- Incubator BM-600 (Memmert GambH, Germany)
- Microcentrifuge tube 0.5 , 1.5 ml(Bio-RAD Laboratories , USA)
- PCR Thermal cycler : Omnigene-E (Hybaid Limited, England)
- PCR Workstation Model#P-036 (Scientific Co., USA)
- Pipette tips 100 , 1000 μl (Bio-RAD Laboratories , USA)
- Power supply : Power PAC 3000 (Bio-RAD Laboratories,USA)
- Refrigerated microcentrifuge Kubota 1300 (Kubota, Japan)
- Spectrophotometer DU 650 (Beckman, USA)
- Vertical sequencing gel electrophoresis apparatus (Hoefer, USA)

2.2 Chemicals

- Absolute ethanol (Merck, Germany)
- Acrylamide (Merck, Germany)
- Ammonium persulfate(Promega, USA)
- Boric acid (Merck, Germany)

- Bromophenolblue (Merck, Germany)
- Chloroform (Merck, Germany)
- Developer (Eastman Kodak Company, USA)
- Ethylene diamine tetraacetic acid, disodium salt dihydrate (Fluka, Switzerland)
- 100 mM dATP , dCTP , dGTP , dTTP (Promega Corporation Medison, Wisconsin)
- Fixer (Eastman Kodak Co., USA)
- Formamide(Gibco BRL Technologies, Co., USA)
- GeneAmp PCR core reagents (Perkin Elmer Cetus, USA)
    : 10X PCR buffer (100mM Tris-HCL pH 8.3, 500 mM KCl)
    : 25 mM $MgCl_2$
- Hydrochloric acid (Merck, Germany)
- Isoamyl alcohol (Merck, Germany)
- N,N-methylene-bis-acrylamide (Amersham, England)
- Oligonucleotide primers (Bio synthesis)
- Phenol crystal (Fluka, Germany)
- Proteinase K (Gibco BRL Technologies, Inc., USA)
- RNase A (Sigma Chemical Co., USA)
- T7 Sequencing kit (Pharmacia Biotech, USA)
    : Mix-Short for each dATP , dCTP , dGTP , dTTP
    : Enzyme dilution buffer (20 mM Tris-HCl pH 7.5, 5 mM DTT, 100 μM BSA/ml 5% glycerol)
    : Universal Primers
    : Annealing buffer (1M Tris-HCl , 100 mM $MgCl_2$, 160 mM D DTT)
    : Labelling Mix-dATP (1.375 μM each dCTP , dGTP and dTTP , 333.5 mM NaCl)
    : Control DNA template
- Sodium acetate (Merck, Germany)
- Sodium chloride (Merck, Germany)

- Sodium dodecyl sulfate (Sigma Chemical Co., USA)
- Sodium hydroxide (Merck, Germany)
- N,N.N',N'-tetramethylethylenediamine (Gibco BRL Technologies, Inc., USA)
- Tris-(hydroxy methyl)-aminomethane (Fluka, Switzerland)
- Urea (Fluka, Switzerland)
- Xylene cyanol (Sigma, USA)

2.3 Radioactive

- [γ-^{32}P]dATP specific activity 3000 Ci/mmol (Amersham International, England)
- [α-^{32}P]dATP specific activity 800 Ci/mmol (Amersham International, England)

2.4 Enzymes

- Ampli*Taq* DNA polymerase (Perkin-Elmer Cetus, USA)
- T4 Polynucleotide kinase (Pharmacia Biotech, USA)
- T7 DNA Polymerase (Pharmacia Biotech, USA)

2.5 Samples

The black tiger shrimp broodstock (*P. monodon*) was wild-caught alive from Satun (N = 36), Trang (N = 35) and Phang-nga (N = 30) located in the Andaman Sea and from Chumphon (N = 49) and Trad (N = 48) located in the Gulf of Thailand during December 1997 - February 1998 (Fig. 2.1). Pleopods were excised from freshly killed *P. monodon* individuals and immediately placed on dry ice. Alternatively, dissected pleopods or the whole post larvae from the hatcheires were immediately placed into the tubes containing an enough amount of absolute ethanol and transported back to the laboratory at the Department of Biochemistry, Faculty of Science, Chulalongkorn University. Specimens were stored at -80 °C until required.

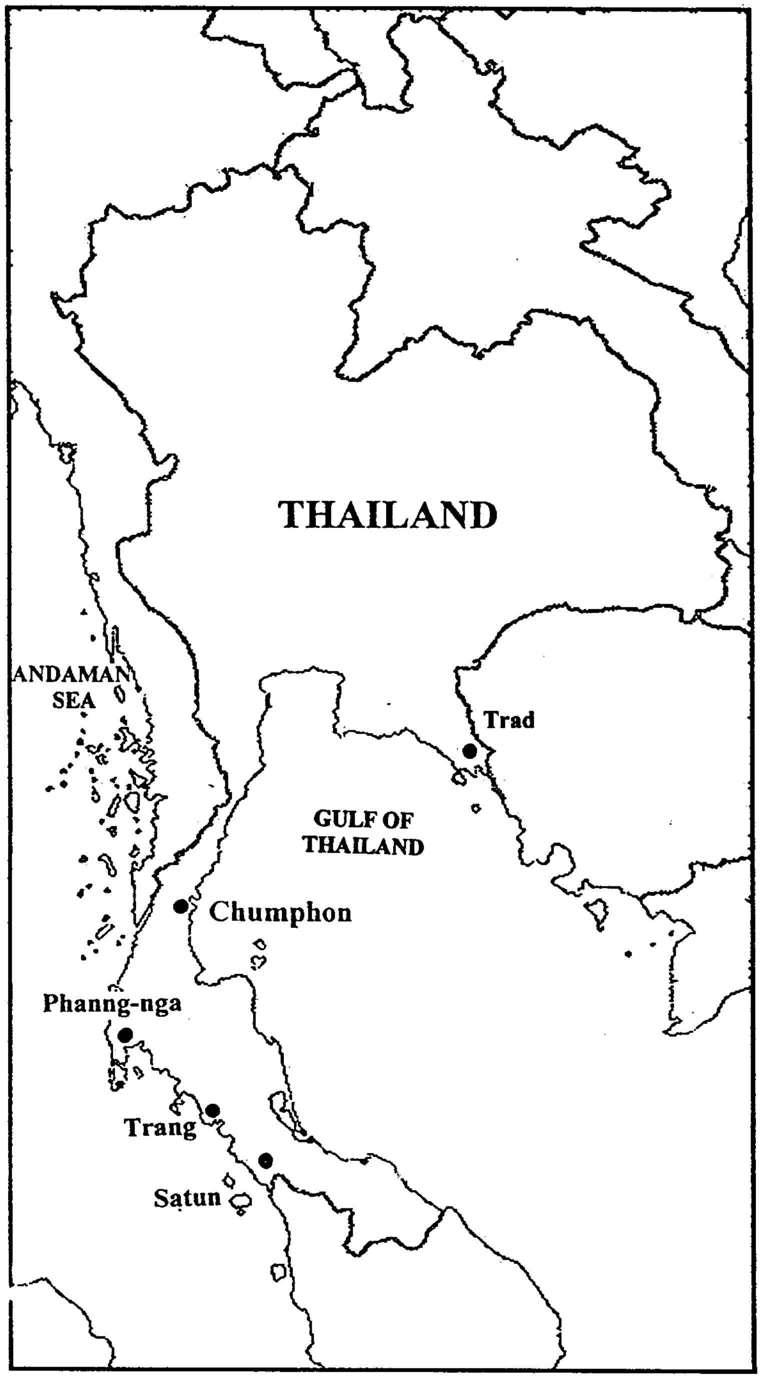

Fig 2.1 Map of Thailand illustrating sample collection sites including Satun, Trang, Phang-nga, Chumphon and Trad.

2.6 DNA extraction

Genomic DNA was extracted from a pleopod of each *P. monodon* individual using a phenol-chloroform modified from that of Davis et al. (1986). As soon as possible after removing from a -80 °C freezer, a pleopod was transferred into a 1.5 ml microcentrifuge tube containing 400 µl of pre-chilled extraction buffer (100 mM Tris-HCl, pH 9.0, 100 mM NaCl, 200 mM sucrose, 50 mM EDTA, pH 8.0) and briefly homogenized with a pre-chilled glass homogenizer. A 40% SDS solution was added to a final concentration of 1.0 % (w/v). The resulting mixture was then incubated at 65°C for 1 hour following by an addition of 10 µl of a Proteinase-K solution (30 mg/ml) and 5 µl of a RNase A solution (10 mg/ml). The mixture was further incubated at the same temperature for 3 hours. To remove proteins, ninety-one microliters of 5 M potassium acetate was added, thoroughly mixed and incubated at 4 °C for 10 minutes prior to centrifugation at 12,000 rpm for 10 minutes at 4 °C. The supernatant was decanted to a sterile microcentrifuge tube. An equal volume of buffer-equilibrated phenol-chloroform-isoamyl alcohol (25:24:1 v/v) was added and gently mixed. The mixture was then centrifuged at 12000 rpm for 10 minutes at room temperature. The upper aqueous phase was carefully transferred to a new microcentrifuge tube. One-tenth volume of 3 M sodium acetate pH 5.5 was added. DNA was precipitated by an addition of two volume of ice-cold absolute ethanol and kept at -20 °C overnight to ensure complete precipitation. The precipitated DNA pellet was recovered using a cut tip and briefly wash twice with 70% ethanol. The pellet was air-dried and resuspended in 300 µl of TE buffer (10 mM Tris-HCl, pH 7.4 and 1 mM EDTA). The DNA solution was incubated at 37 °C for 1-2 hours for complete redissolved and kept at 4 °C until further needed.

Alternatively, the rapid extraction method according to Cook, (submitted) was carried out. This method is much simpler but yielded comparable DNA quality to that from the Phenol-chloroform extraction method. A pleopod was chopped to small pieces (approximately 50 - 100 mg in weight) and added to a microcentrifuge tube containing 1 ml of high TE buffer (100 mM Tris-HCl, 40 mM EDTA, pH 8.0). The tube was briefly vortexed. The supernatant was discarded. The pellet was resuspended in 250 µl of MGPL lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 200 mM LiCi, pH 8.0 and 0.8% SDS). A proteinase-K solution was added to 200 µg/ml final concentration. The sample was incubated at 45 °C for 4 hours. During the incubation period, the sample was intermittently mixed until the residual tissue pieces were not observed. At the end of the incubation period, 500 µl of TE (10mM Tris-HCl, 1 mM EDTA, pH 8.0) was added and gently mixed prior to centrifugation at 8,000 rpm for 1 minute. The supernatant was then transferred to a new microcentrifuge tube before 9.4 µl of 4 mM NaCl and 750 µl of pre-chilled isopropanol were added. The solution was thoroughly mixed and incubated at -80 °C for 30 minutes. DNA was then recovered by centrifugation at 14,000 rpm for 1 minute at room temperature. The pellet was washed with 70% ethanol. The sample was spun at 12,000 rpm for 5 min at room temperature before removing of ethanol. DNA was then air-dried in a 37 °C for 5 - 10 min before resuspended with 100 µl of TE. The DNA solution was redissolved at 37 °C for 1-2 hours and kept at 4 °C until required.

2.7. Measurement of DNA concentration

The concentration of extracted DNA was spectrophotometrically measured at the optical density of 260 nanometres (OD_{260}). An OD_{260} of 1.0 corresponds to a concentration of 50 µg/ml double-stranded DNA. Therefore, the DNA concentration of each sample (in µg/ml) was calculated by;

$$[DNA] = OD_{260} \times \text{dilution factor} \times 50$$

Basically, the concentration of DNA samples used for PCR was diluted to 15 ng/µl in a total volume of 100 µl . Accordingly, the original volume required from each DNA sample can be calculated as follows;

Volume required from the DNA sample $= (15 \times 100) / X$

where X is the concentration of DNA (in µg/ml) of a particular sample.

The purity of extracted DNA sample can be examined by the ratio of OD_{260} and OD_{280}. A ratio of 1.8 indicates pure prepared DNA whereas much higher and lower values of this ratio indicate RNA or protein contamination of the isolated DNA samples, respectively (Kirby, 1992).

2.8 PCR primers

The oligonucleotide primers for locus CUPmo18 were developed by Tiptawonnukul (1996). The primer sequences for locus Di25 and Di27 which were also used in this study were kindly provided by Dr. F. Bonhomme, Laboratoire Genome et Populations, CNRS URA 193, Universite de Montpellier II, C.C. 63, 34095, Monpellier cedes 5, France through Dr. J.A.H. Benzies, Australian Institute of Marine Science, PMB 3, Townsville, Qld 4810, Australia.

2.9 Amplification of Microsatellite DNA using the Polymerase Chain Reaction (PCR)

Microsatellite DNA of investigated *P. monodon* individuals was *in vitro* amplified by PCR in which one of the primers was 5' radiolabeled with γ-^{32}P dATP. The resulting amplification product(s) was electrophoretically size-fractionated by denaturating polyacrylamide sequencing gels. The DNA patterns were then detected by autoradiography (Tam and Kornfield, 1996).

2.9.1 5'-end labelling with T4 polynucleotide kinase

The reverse primer for each microsatellite locus was end-labeled using polynucleotide kinase. Approximately 10 pmole of the reverse primer (free 5'-OH groups) were used as a substrate for a 10 µl end-labeling reaction containing 1 µl of 10x T4 Polynucleotide kinase buffer (0.5 M Tris-HCl pH 7.6, 0.1 M $MgCl_2$, 50 mM dithiothreitol, 1 mM spermidine), 3 µl of [γ-^{32}P] ATP (3,000 µCi/mmol), 10 U of T_4 polynucleotide kinase. An enough amount of sterile deionized H_2O was added to the reaction mixture to make the final volume to 10 µl. The end-labeled mixture was incubated at 37 °C for 30 minutes. At the end of the incubation period, the reaction was terminated by heat-inactivation of T_4 polynucleotide kinase activity at 65 °C for 15 minutes.

2.9.2 Amplification of microsatellite loci

Each of microsatellite loci was singly amplified from genomic DNA isolated from *P. monodon* individuals. Approximately 15 ng of genomic DNA isolated from each individual of *P. monodon* were used as the DNA template in a 5 µl PCR reaction volume containing 0.6 µM of the forward primer, 0.575 µM of the reverse primer, 0.025 µM of the lablled reverse primer, 1 µl of 10x PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl , 10 mM $MgCl_2$, 0.1% gelatin), 200 µM each of dNTPs (dATP, dCTP, dGTP and dTTP), 0.5 U of *Taq* polymerase. For reliable amplification results of CUPmo18, 0.05% of tween 20 was required in the amplification reaction (Tassanakajon et al., 1997). The reaction mixture were then overlaid with a drop of RNase- and DNase-free mineral oil and centrifuged briefly in a microcentrifuge before subjected to the amplification process in a thermal cycler (Omnigene, Hybaid).

To amplify the micrasatellite locus CUPmo18, PCR was carried out for seven cycles consisting of a 94 °C denaturation for 60 seconds, a 55 °C annealing for 30 seconds and a 72 °C extension for 60 seconds followed by thirty-eight cycles of a denaturation step at 94 °C for 30 seconds, an annealing step at 55 °C for 30 seconds and an extension step at 72 °C for 60 seconds.

For amplification of the loci Di25 and Di27, more complicated PCR amplification program were required. Firstly, the melting temperature (Tm) for each primer was examined using the Wallace's rules. As a result,

$$\text{Tm (°C)} = [\,4\,(\text{total number of G and C}) + 2\,(\text{total number of A and T})]$$

Theoretically, the annealing temperature (Ta) was approximately 5 °C below Tm of the primer. Based on the fact that the Tm value of the forward and reverse primers was not identical, the annealing temperature was set up from the primer having lower Tm. The most optimal annealing temperature for Di25 and Di27 was further adjusted from the autoradiography results.

To amplify the Di25 and Di27 loci, the multi-step cycling system, sometimes called a touch down PCR, were utilized. Initially, the reaction mixture was pre-denaturated at 94 °C for 4 minutes followed by a denaturating step at 94 °C for 1 minute, an annealing step at the most suitable Ta for 1 minutes and an extension step at 72 °C for 1 minutes. These were carried out for 3 cycles. Subsequently, the amplification was performed as described above with the exception that a pre-deaturation step was omitted and the annealing temperature were gradually decreased for 2 °C below the most optimal annealing temperature every 3, 3 and 4 cycles. At the end of these steps, the PCR reaction was performed for 20 cycles constituting of the typical denaturation temperature for 30 seconds, a -7 °C below the most optimal annealing temperature of each locus for 30 seconds and the typical extension temperature for 30 seconds.

2.9.3 Size estimation of amplified microsatellite allele using denaturating polyacrylamide gels

After the amplification process was complete, 5 μl of a formamide dry mix solution (10 mM NaOH, 99% formamide, 0.1% bromophenol blue and 0.1% xylene cyanol) was added into each amplification reaction. The mixture was heated at 94 °C for 15 minutes and immediately snap-cooled on ice. Three and a half microliters of the denatured mixture was loaded onto a 8% denaturing acrylaminde gel (76% acrylamide, 0.4% N,N' methylene bisacrylamide, 7.66 M Urea) prepared in 1x TBE (89 mM Tris-HCl, 89 mM Boric acid, 2 mM EDTA, pH 8.3) and electrophoresed at 50 W for approximately 2.5 hours.

A DNA standard used for estimation of microsatellite alleles was the M13 sequencing marker prepared using T7 DNA sequencing kit. The M 13 control template was annealed with the M13 sequencing primer in a 14 μl reaction mixture containing 5 μl of the template, 5 μl of sterile water, 2 μl of annealing buffer (200 mM

Tris-HCl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl) and 2 μl of 4 ng/μl M13 forward primer. The annealing mixture was incubated at 65 °C for 15 minutes and allowed to cool to room temperature for 10 minutes. During the annealing period, the labeling/extension mix including 1 μl of sterile H_2O, 3 μl of dNTP mix (2 μM each of dCTP, dGTP and dTTP), 1.7 μl of 5x Sequenase buffer, 0.3 μl of T7 DNA polymerase and 1 μl of 800 Ci/mmol [α-^{32}P] dATP), was prepared. Six microliters of this mixture was then added to the annealed template/primer mix. The labeling mixture was then incubated at room temperature for 5 minutes. Five microliters of this was dispensed to each of the 4 tubes containing appropriate termination mix (each mixtue contains all four dNTPs at the suitable concentrations and the appropriate ddNTP at a concentration of 14 μM). The reaction tubes were incubated at 37 °C for 5 minutes. Thirty microliters of a formamide containing dry solution and one drop of the mineral oil were added. The M13 sequencing marker was denatured and loaded onto a 8 % acrylamide gel as described above.

2.10 Data analysis

Assumption

A genotype of each *P. monodon* individuals was scored from an electrophoretically observed pattern for each locus. Therefore, the genotypes could be divided into homo- or heterozygotic states. Based on the fact that stutter bands were commonly observed in dinucleotide microsatellite, scoring of a particular band can be unambiguously carried out by making an assumption that an actual band of a given allele was the most intense band located at the biggest in size compared to the neighbor group of stuttered bands. The size of alleles, in bp, was estimated by relatively compared to the M13 sequencing marker. Each *P. monodon* individual was recorded to be either homo- and heteroygote. The allelic stages were also recorded from each individual for each locus.

2.10.1 Allele frequencies, genetic variation and effective number of alleles per locus

For diploid taxa, the frequency of a particular allele in a population can be calculated as

$$p = (2\,N_{AA} + N_{Aa}) / 2\,N$$

where p is the frequency of the A allele, N is the total number of individuals in the investigated sample, and N_{AA} and N_{Aa} are the number of homo- and heterozygotes for such a locus.

Practically, the number of alleles per locus, allelic frequency, the proportion of homo- and heterozygotes individuals (direct-count heterozygosity, h_{obs}) were estimated using GENEPOP Version 2.0 (Raymond and Rousset,1995) whereas the unbiased estimate of heterozygosity (expected heterozygosity, h_{exp}) for each locus was estimated using the equation

$$h_{exp} = 1 - \Sigma p_i^2$$

where p_i is the frequency of i^{th} allele, H_{obs} and H_{exp} is the average of h_{obs} and h_{exp} over all investigated loci, respectively (Nei,1978).

The effective number of alleles at each locus was calculated by

$$n_e = 1/\Sigma p_i^2$$

when p_i is the frequency of i^{th} allele (Crow and Kimura,1965).

2.10.2 Hardy-Weinberg equilibrium

Once allele and genotype frequencies of diploid organisms have been estimated, Hardy-Weinberg equilibrium of genotype frequency at a given locus for each investigated population should be examined. The observed genotype frequencies are concordant to Hardy-Weinberg expectation when there are no significantly disturbing forces e.g. selection, mutation or migration changing allele frequencies over time and mating is actually occurred at random in a large population.

In the present study, Hardy-Weinberg equilibrium (HWE) of each locus for each population were examined using a Markov chain "approximation to exact test" followed the algorithm of Guo and Thomsson (1992) (a test for determination of heterozygote deficiency) and implemented in GENEPOP version 2.0. The probability to reject null hypothesis (Ho: observed genotype frequencies of an investigated population at a given locus conform Hardy-Weinberg rule) were further adjusted using the Bonferroni technique (Hochberg,1988). This was carried out by dividing the initial significant level ($\alpha < 0.05$) by the number of tested loci.

2.10.3 Analysis of Mendelian inherited fashion of CUPmo18, Di25 and Di27 in P. monodon

To determine whether three microsatellite loci used in this study segregated in a Mandelian fashion, twenty representative offspring from a full-sib family were examined, at all loci. Offspring genotypes were subjected to goodness of fit test against Mendelian segregation using typical χ^2-method(Sokal and Rohlf,1981).

2.10.4 Genetic distance and phylogenetic reconstruction

Genetic distance based on Cavalli-Sforza and Edwards chord distance was calculated (Cavalli - Sforza and Edwards, 1967). This is the gene diversity among population expressed as a function of genotype frequency. The genetic distance estimated from this method are appropriate for microsatellite data obtained from various taxa whether they have undergone the bottleneck events or not (Takezaki et al.,1996). Practically, Cavalli-SforZa and Edwards chord distance was computational estimated using GENDIST(Felsenstein, 1993). The resulting genetic distance was subjected to phylogenetic reconstruction based on Neighbor-joining approach (Saitou and Nei , 1987) using NEIGHBOR. The NJ tree was plotted by DRAWTREE. All computational programs mentioned above are routinely implemented in Phylip 3.56c (Felsenstein, 1993).

2.10.5 Geographic heterogeneity analysis

The statistically significant differences in genotype frequencies between *P. monodon* from a pair of geographic sampling locations were tested using the exact test of Genic Differentiation of GENEPOP version 2.0. Results are expressed as the probability of homogeneity between compared populations or regions. To diminish type I error, level of significance was further adjusted using the Bonferroni test.

2.10.6 Interpopulation diversity and population differentiation

Although several approaches can be used to estimate intraspecific population structure, Wright's *F*-statistics (or allelic correlation's) are widely used. Nevertheless, Weir & Cockerham (1984) introduced the unbiased *F*st(or θ) for estimation of population subdivision for the random model and can be conveniently calculated using Diploid program (for diploid characteristic data) distributed by the author. This parameter (θ) is suitable to handle difficulties from multiple alleles at a locus, multiple loci and various sampling error from within and between populations (Weir, 1990). Accordingly, population differentiation of *P. monodon* in Thailand were examined using this approach.

Chapter III
Results

3.1 DNA extraction

Genomic DNA extracted from frozen pleopods using a phenol-chloroform modified from the procedure of Davis et al. (1986) and that of rapid extraction method of Cook et al. (submitted) yielded 250 - 400 ng/400 mg and 150 - 250 ng/100 mg of the pleopod, respectively (Fig. 3.1). The ratio of $OD_{260/280}$ was 1.8 - 2.0 reflecting good quality of the DNA obtained. Although both DNA extraction methods gave high molecular weight DNA which was greater than 23.1 kb, a rapid extraction protocol based on Cook et al. (submitted) provided slightly lower quality of DNA obtained as indicated by an available of contaminants e.g. the pigment of shrimp and cell debris. Moreover, slightly degradation of DNA obtained from this method was sometimes observed.

3.2 Optimization of PCR conditions for amplification of homologous microsatellite loci in *P. monodon*

Three microsatellite loci, CUPmo18, Di25 and Di27 were selected for this study. All loci contained dinucleotide repeats (GT)n. The annealing temperatures for CUPmo18, Di25, and Di27 microsatellite primers were shown in Table 3.1. The most suitable annealing temperature for the CUPmo18 locus was 55 °C. This annealing temperature resulted in unambiguously scorable results as illustrated by Fig. 3.2 .

Table 3.1 The primer annealing temperature for amplification of three microsatellites loci in *P. monodon*.

Locus	Repeat sequence	Annealing temperature (°C)
CUPmo18	(GT)60	55
Di25	(GT)43	65
Di27	(GT)21	54

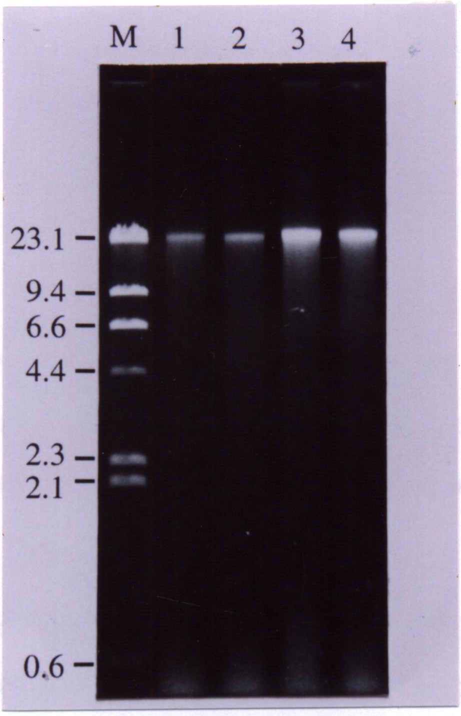

Figure 3.1 Ethidium bromide staining of 0.7% agarose gel showing DNA extracted from pleopods of *P. monodon* .

Lane M : DNA/*Hin*d III digested size markers.

Lane 1-2 : genomic DNA of *P. monodon* extracted by rapid extraction method (Cook et al., submitted)

Lane 3-4 : genomic DNA of *P. monodon* extracted by Phenol-Chloroform method (Davis et al., 1986)

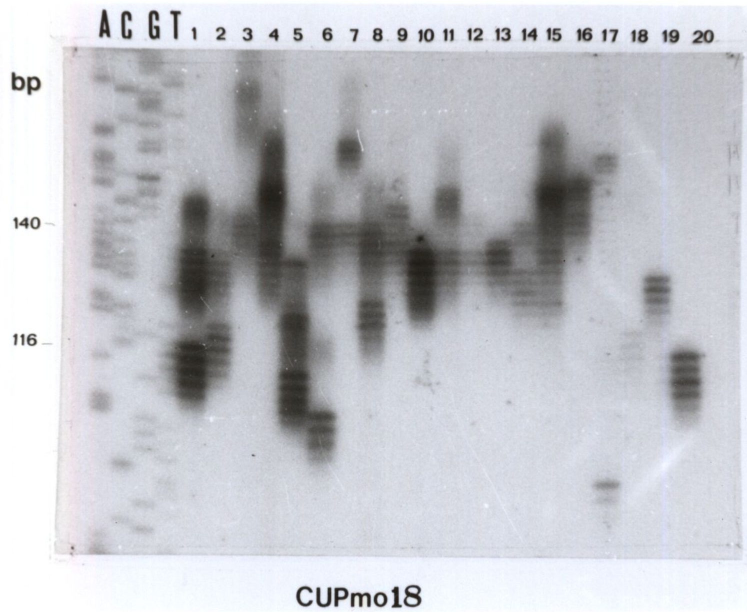

Figure 3.2 PCR amplification patterns of the CUPmo18 locus from 20 individuals *P. monodon* DNA (lanes 1-20) under the optimal PCR conditions with annealing temperature at 55 °C. The size standard is a sequencing ladder of M13 mp 18.

The PCR conditions previously used for loci Di25 and Di27 were not successful in the present study, as a result, the most optimal amplification conditions for each of these loci was further examined. For Di25, various annealing temperatures (52 °C , 56 °C, 60 °C and 65 °C) were tested whether they yielded the amplification success without the existence of non - specific products. All tested annealing temperatures gave the products. Nevertheless, the microsatellite DNA amplified at a 65 °C annealing temperature was free from interference causing the allele sizes to be examined unambiguously (Fig. 3.3)

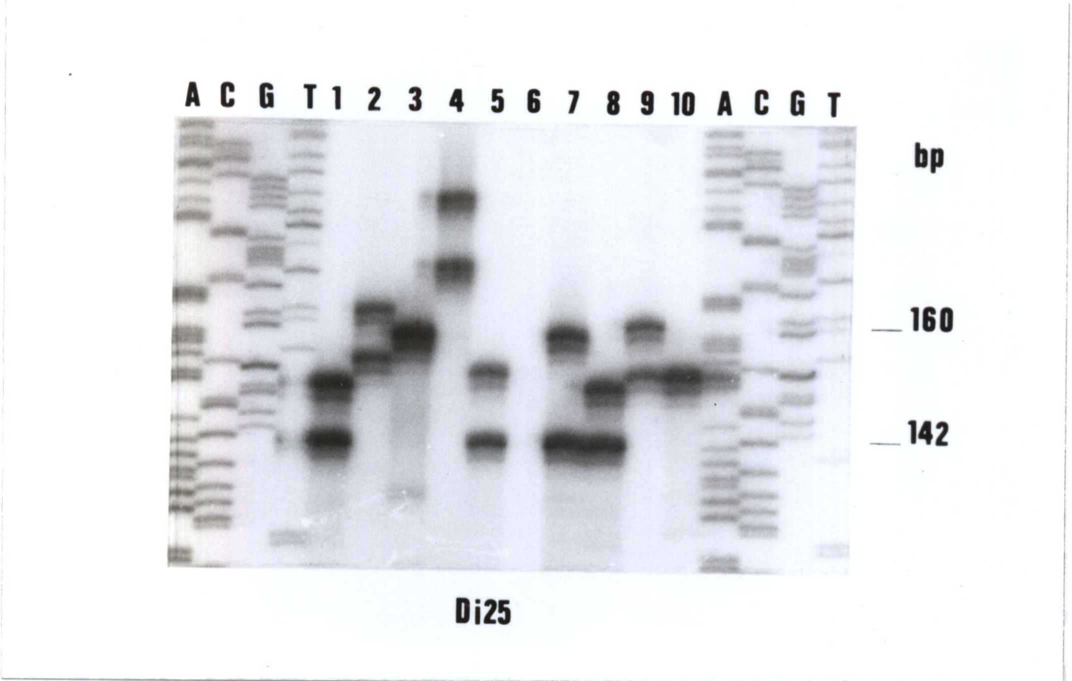

Figure 3.3 PCR amplification patterns of the Di25 locus from 10 individuals *P. monodon* DNA (lanes 1-10) under the optimal PCR conditions with annealing temperature at 65 °C. The size standard is a sequencing ladder of M13 mp 18.

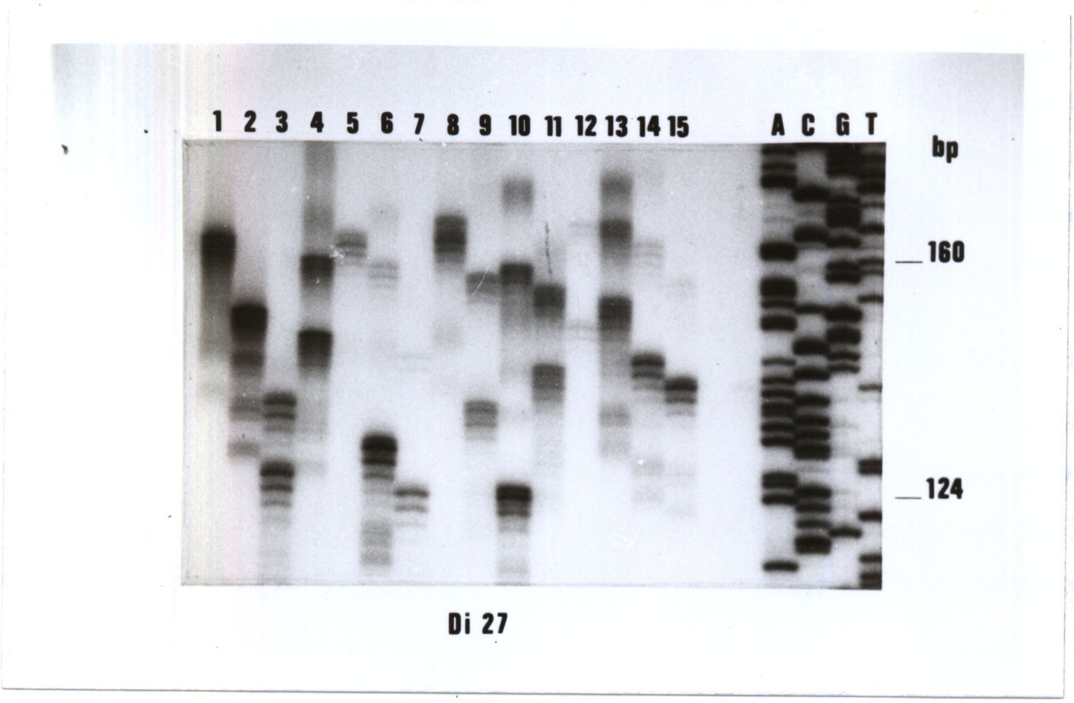

Figure 3.4 PCR amplification patterns of the Di27 locus from 15 individuals *P. monodon* DNA under the optimal PCR conditions with annealing temperature at 54 °C. The size standard is a sequencing ladder of M13 mp 18.

Likewise, the most optimal temperature to amplified the microsatellite fragments at the locus Di27 was also determined. At a 52°C annealing temperature, non - specific amplification products were clearly observed. An increase of such a temperature to 54 °C revealed much better results as can be seen in Fig. 3.4 .

3.3 Variability of three microsatellites

3.3.1 Diversity within samples

All three investigated loci were highly polymorphic. The most polymorphic locus was CUPmo 18 followed by Di25 and Di27. From, 184 individual *P. monodon* examined, a total of 37, 34 and 32 alleles was observed at CUPmo 18, Di25, and Di27 with the allele sizes ranged from 104 - 172 bp, 136 - 208 bp, and 114 - 178 bp, respectively. All observed alleles were smaller than 210 bp, therefore it was quite convenient to determine the actual allele status by the standard sequencing gel.

The allele distribution frequencies of the microsatellite CUPmo 18, Di25, and Di27 loci could be summarized and shown in Fig. 3.5, 3.6 and 3.7, Table 3.2, 3.3 and 3.4 respectively. As typically observed in the distribution pattern of microsatellite DNA , several rare alleles were found in each geographically investigated sample along with a few common (or none) alleles exhibiting the frequency greater than 0.15.

For CUPmo 18, only two alleles (114 and 116 bp) in Trad possessed the frequency slightly higher than 0.15. The Di25 locus also showed allele frequencies higher than 0.15 for a 152 bp allele in Trang and a 156 bp allele in Chumphon. For Di27, only a 162 bp allele in Satun showed the allele frequency greater than 0.15 whereas much lower frequencies of this was observed in the remaining geographically investigated samples.

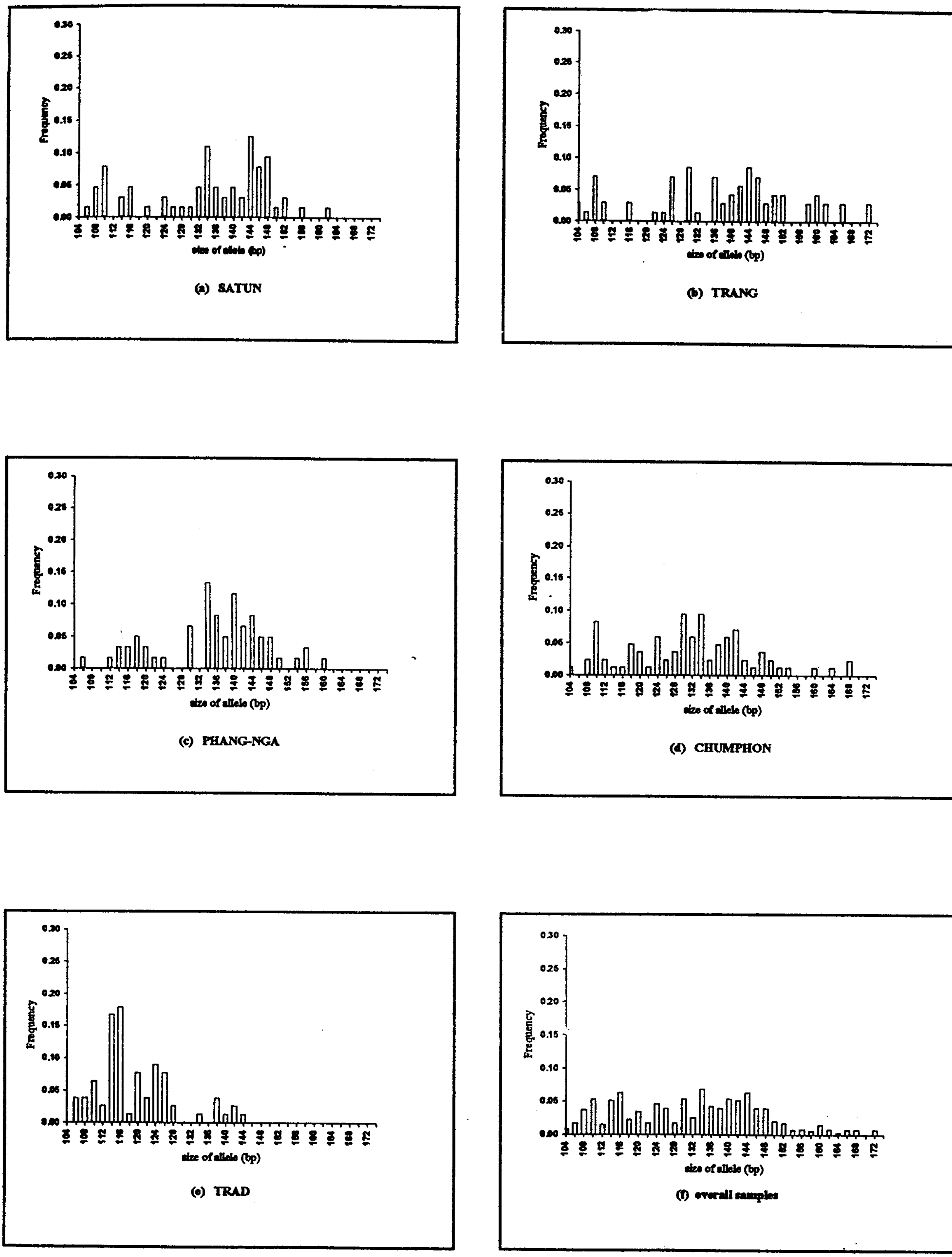

Fig 3.5 Distribution frequencies of alleles at the CUPmo18 locus from Satun (n=32),Trang(n=35),Phang-nga(n=30),Chumphon(n=42),Trad(n=39)and overall samples(n=178)

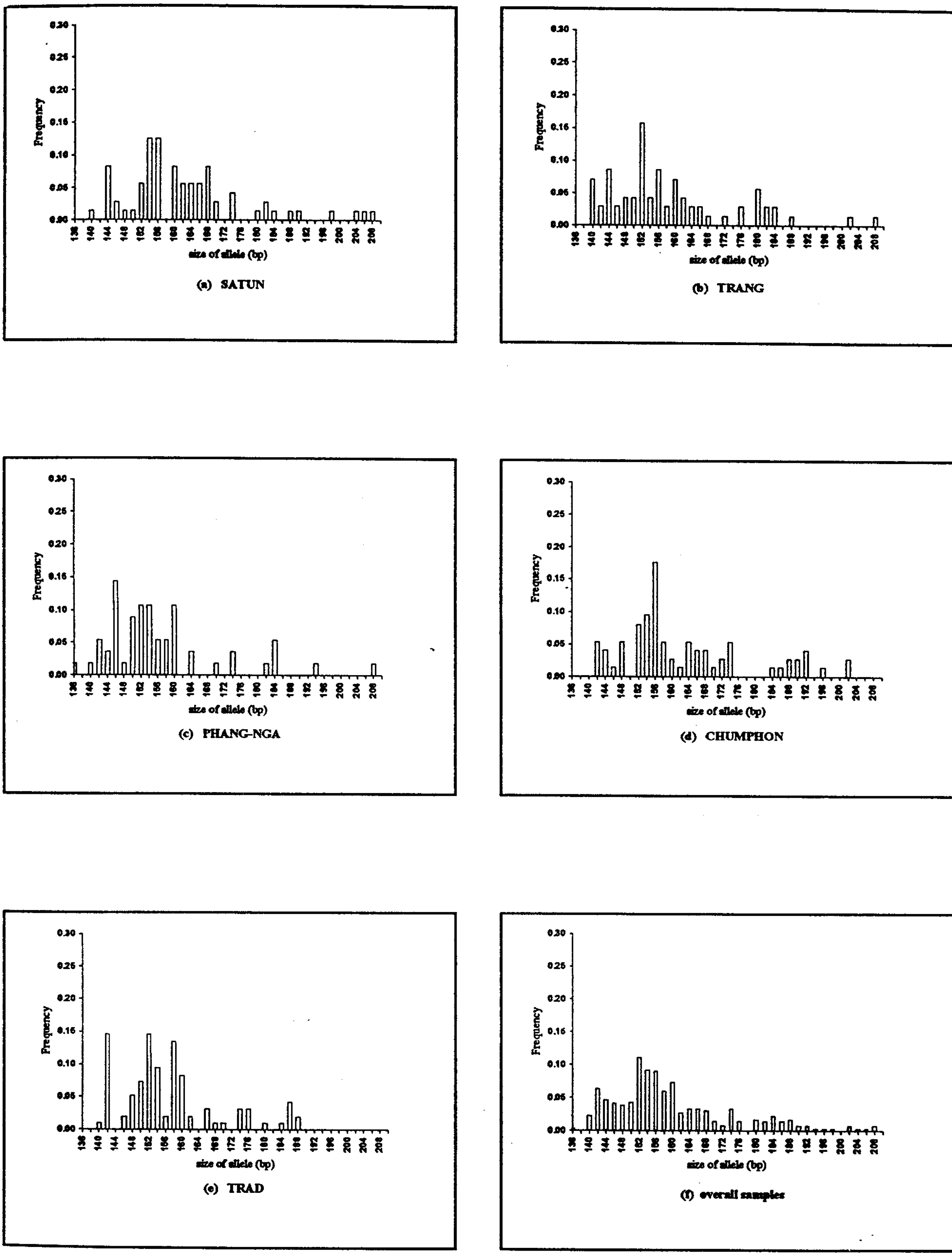

Fig 3.6 Distribution frequencies of alleles at the Di25 locus from Satun(n=36), Trang (n=35), Phang-nga(n=28), Chumphon(n=37), Trad(n=48) and overall samples(n=184)

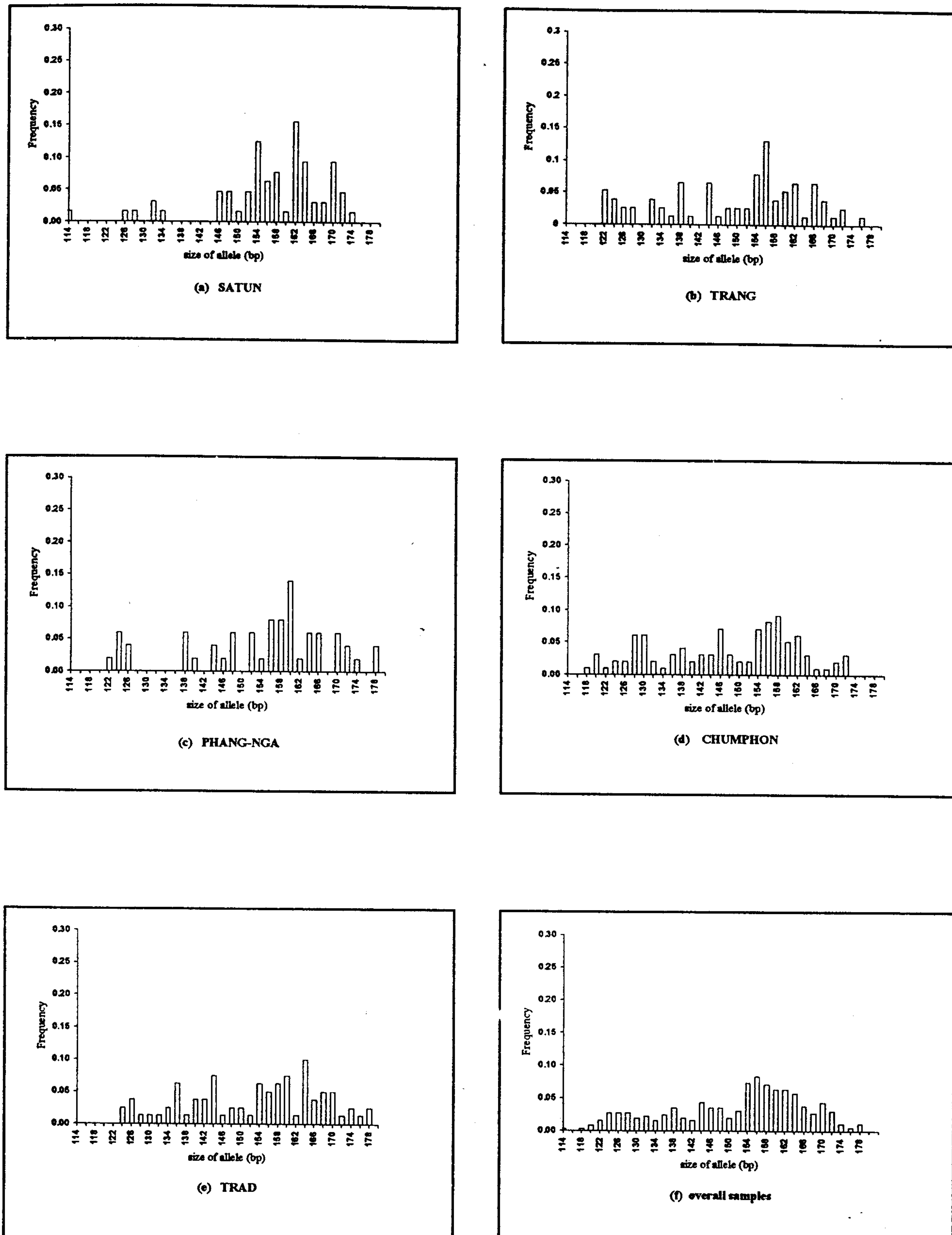

Fig 3.7 Distribution frequencies of alleles at the Di27 locus from Satun(n=32),Trang (n=38),Phang-nga(n=25),Chumphon(n=49),Trad(n=40)and overall samples(n=184)

Table 3.2 Allele sizes in base pairs at the CUPmo18 locus and its frequency distributions in five samples.

	Sampling locations				
Allele	Satun	Trang	Phang-nga	Chumphon	Trad
104	-	0.029	-	0.012	-
106	0.016	0.014	0.017	-	0.038
108	0.047	0.071	-	0.024	0.038
110	0.078	0.029	-	0.083	0.064
112	-	-	0.017	0.024	0.026
113	-	-	-	-	0.038
114	0.031	-	0.033	0.012	0.167
116	0.047	0.029	0.033	0.012	0.179
118	-	-	0.050	0.048	0.013
120	0.016	-	0.033	0.036	0.077
122	-	0.014	0.017	0.012	0.038
124	0.031	0.014	0.017	0.060	0.090
125	-	-	-	-	0.013
126	0.016	0.071	-	0.024	0.077
127	-	-	-	-	0.013
128	0.016	-	-	0.036	0.026
130	0.016	0.086	0.067	0.095	-
132	0.047	0.014	-	0.060	-
134	0.109	-	0.133	0.095	0.013
136	0.047	0.071	0.083	0.024	-
138	0.031	0.029	0.050	0.048	0.038
140	0.047	0.043	0.117	0.060	0.013
142	0.031	0.057	0.067	0.071	0.026
144	0.125	0.086	0.083	0.024	0.013
146	0.078	0.071	0.050	0.012	-

Table 3.2 (continued)

Allele	Sampling locations Satun	Trang	Phang-nga	Chumphon	Trad
148	0.094	0.029	0.050	0.036	-
150	0.016	0.043	0.017	0.024	-
152	0.031	0.043	-	0.012	-
154	-	-	0.017	0.012	-
156	0.016	-	0.033	-	-
158	-	0.029	-	-	-
160	-	0.043	0.017	0.012	-
162	0.016	0.029	-	-	-
164	-	-	-	0.012	-
166	-	0.029	-	-	-
168	-	-	-	0.012	-
172	-	0.029	-	-	-
N	32	35	30	42	39

N = Number of sample examined

Table 3.3 Allele sizes in base pairs at the Di25 locus and its frequency distributions in five samples.

Allele	Sampling locations Satun	Trang	Phang-nga	Chumphon	Trad
136	-	-	0.018	-	-
140	0.014	0.071	0.018	-	0.010
142	-	0.029	0.054	0.054	0.146
144	0.083	0.086	0.036	0.041	-
146	0.028	0.029	0.143	0.014	0.020
148	0.014	0.043	0..018	0.054	0.052
150	0.014	0..043	0.089	-	0.073
152	0.056	0.157	0.107	0.0881	0.146

Table 3.3 (continued)

	Sampling locations				
Allele	Satun	Trang	Phang-nga	Chumphon	Trad
154	0.125	0..043	0.107	0.095	0.094
156	0.125	0.086	0.054	0.1776	0.020
158	-	0.029	0.054	0.054	0.135
160	0.083	0.071	0.107	0.027	0.083
162	0.056	0.043	-	0.014	0.020
164	0.056	0.029	0.036	0.054	-
166	0.056	0.029	-	0.041	0.031
168	0.083	0.014	-	0.041	0.010
170	0.028	-	0.018	0.014	0.010
172	-	0.014	-	0.027	-
174	0.042	-	0.036	0.054	0.031
176	-	0.029	-	-	0.031
180	0.014	0.057	-	-	0.010
182	0.028	0.029	0.018	-	-
184	0.014	0.029	0.054	0.014	0.010
186	-	-	-	0.014	0.042
188	0.014	0.014	-	0.027	0.020
190	0.014	-	-	0.027	-
192	-	-	-	0.041	-
194	-	-	0.018	-	-
196	-	-	-	0.014	-
198	0.014	-	-	-	-
202	-	0.014	-	0.027	-
204	0.014	-	-	-	-
206	0.014	-	-	-	-
208	0.014	0.014	0.018	-	-
N	36	35	28	37	48

N = number of sample examined

Table 3.4 Allele sizes in base pairs at the Di27 locus and its frequency distributions in five samples.

	Sampling locations				
Allele	Satun	Trang	Phang-nga	Chumphon	Trad
114	0.016	-	-	-	-
118	-	-	-	0.010	-
120	-	-	-	0.031	-
122	-	0.053	0.020	0.010	-
124	-	0.039	0.060	0.020	0.025
126	0.016	0.026	0.040	0.020	0.038
128	0.016	0.026	-	0.061	0.014
130	-	-	-	0.061	0.014
132	0.031	0.039	-	0.020	0.014
134	0.016	0.026	-	0.010	0.025
136	-	0.013	-	0.031	0.063
138	-	0.066	0.060	0.041	0.014
140	-	0.013	0.020	0.020	0..038
142	-	-	-	0.031	0.038
144	-	0.066	0.040	0.031	0.075
146	0.047	0.013	0.020	0.071	0.014
148	0.047	0.026	0.060	0.031	0.025
150	0.016	0.026	-	0.020	0.0255
152	0..047	0.026	0.060	0.020	0.014
154	0.125	0.079	0.020	0.071	0.063
156	0.063	0.132	0.080	0.082	0.050
158	0.078	0.039	0.080	0.092	0.063
160	0.016	0.053	0.14	0.051	0.075
162	0.156	0.066	0.020	0.061	0.014
164	0.094	0.013	0.060	0.031	0.100
166	0.031	0.066	0.060	0.010	0.038

Table 3.4 (continued)

	Sampling locations				
Allele	Satun	Trang	Phang-nga	Chumphon	Trad
168	0.031	0.039	-	0.010	0.050
170	0.094	0.013	0.060	0.020	0.050
172	0.047	0.026	0.040	0.031	0.014
174	0.016	-	0.020	-	0.0255
176	-	0.013	-	-	0.014
178	-	-	0.040	-	0.025
N	32	32	25	49	40

N = number of sample examined

The number of allele, size range, heterozygosity and effective number of allele in 5 geographic sample for each microsatellite locus were shown in table 3.5

The highest allele number at the CUPmo 18 locus was observed in Chumphon (28) followed by Trang (24), Satun (23), Phang-nga (21) and Trad (20) whereas the Satun *P. monodon* showed the highest allele number at the Di25 locus. Trang and Chumphon samples had comparable polymorphic levels as did Satun. For the Di27 locus, the highest number of alleles was observed in Chumphon and Trad samples. Mean observed heterozygosities were 0.68, 0.71 and 0.81 for CUPmo18, Di25 and Di27 respectively. Difference between observed and expected heterozygosity was observed for all loci in all samples. Effective number of alleles (n_e) was lower than the actual number of alleles per locus because the n_e takes into account the frequencies of alleles, to which rare alleles negligible contribute to the estimates. As stated earlier, all loci in this study displayed a large number of rare alleles resulted in a much lower effective number of alleles. The average effective number of allele was positively correlated to the mean number of alleles per locus. Regarding to the mean heterozygosity, Chumphon and Trad shared considerably equivalent diversity level. The lowest heterozygosity averaged for 3 loci were 0.66±0.129(Trang) whereas the highest was 0.88±0.109(Trad). Genetic diversity of 5 samples averaged across all 3 loci were shown in Table 3.6. Chumphon showed the highest genetic diversity followed by Trang, Trad, Satun and Phang-nga respectively.

Table 3.5 Genetic polymorphisms resulted from analysis of three microsatellite loci (CUPmo 18, Di 25, and Di 27) on 5 geographical samples of *P. monodon* in Thailand.

Sample	Sample Size (n)	Number of alleles	size range (bp)	Heterozygosity		n_e
				observed	expected	
Locus CUPmo18						
Satun	32	23	106-162	0.56	0.94	15.06
Trang	35	24	104-172	0.49	0.95	18.53
Phang-nga	30	21	106-160	0.77	0.95	14.18
Chumphon	42	28	104-168	0.83	0.95	18.31
Trad	39	20	106-144	0.77	0.92	15.01
Mean	35.6	23.2	104-172	0.68	0.94	16.22
Locus Di25						
Satun	36	24	140-208	0.78	0.95	14.93
Trang	35	23	140-208	0.77	0.95	15.30
Phang-nga	28	19	136-208	0.61	0.94	13.29
Chumphon	37	23	142-202	0.70	0.94	14.05
Trad	48	20	140-188	0.69	0.92	11.10
Mean	36.8	21.8	136-208	0.71	0.94	13.73
Locus Di27						
Satun	32	20	114-174	0.75	0.94	12.91
Trang	38	25	122-176	0.71	0.95	17.08
Phang-nga	25	20	122-178	0.80	0.95	15.06
Chumphon	49	28	118-172	0.84	0.96	20.36
Trad	40	28	124-178	0.95	0.96	19.34
Mean	35.6	24.2	114-178	0.81	0.95	16.95

Table 3.6 The number of allele per locus, effective number of alleles and heterozygosity averaged over all loci and samples.

Population	Mean no. of alleles per locus	Effective no. of alleles (n_e)	Mean heterozygosity	
			observed (Ho ±SD)	expected (Ho ±SD)
Satun	22.33 ± 2.08	14.30	0.7 ± 0.097	0.94 ± 0.005
Trang	24.00 ± 1.00	16.97	0.66 ± 0.120	0.95 ± 0.000
Phang-nga	20.00 ± 1.00	14.18	0.73 ± 0.083	0.95 ± 0.006
Chumphon	26.33 ± 2.87	17.57	0.79 ± 0.064	0.95 ± 0.008
Trad	22.67 ± 4.62	15.15	0.80 ± 0.109	0.93 ± 0.018

3.3.2 Hardy - Weinberg disequilibrium

The differences between observed and expected heterozygosity of each *P. monodon* sample leading to suspicion that Hardy - Weinberg expectations might have not been conformed at these loci. As can be seen in Table 3.7, the frequency distribution of alleles was statistically significant deviated from Hardy - Weinberg expectations (following the correction for multiple test using the Bonferroni procedure), except for the Di27 locus in Trad.

Table 3.7 Estimation of Hardy-Weinberg expectations in each conspecific *P. monodon* sample for each microsatellite locus.

Population	P-value[a]		
	CUPmo18	Di25	Di27
Satun	< 0.0001	< 0.0001	0.0002
Trang	< 0.0001	< 0.0001	<0.0001
Phang-nga	< 0.0001	< 0.0001	0.0031
Chumphon	0.0003	< 0.0001	<0.0001
Trad	0.0004	< 0.0001	0.4748[ns]

[a]Significant level was further adjusted using a Bonferroni technique.
ns = not significant

3.3.3 Analysis of Mendelianly inherited fashions of microsatellite loci in P. monodon

Segregating patterns of alleles at three microsatellite loci used in the present study were determined whether they are Mandelianly inherited. A total number of 20 progeny from a representative full-sib family was examined for their genotypes at three microsatellite loci. Genotypes of the progeny were unambiguously identified as shown in Fig 3.8 – 3.10.

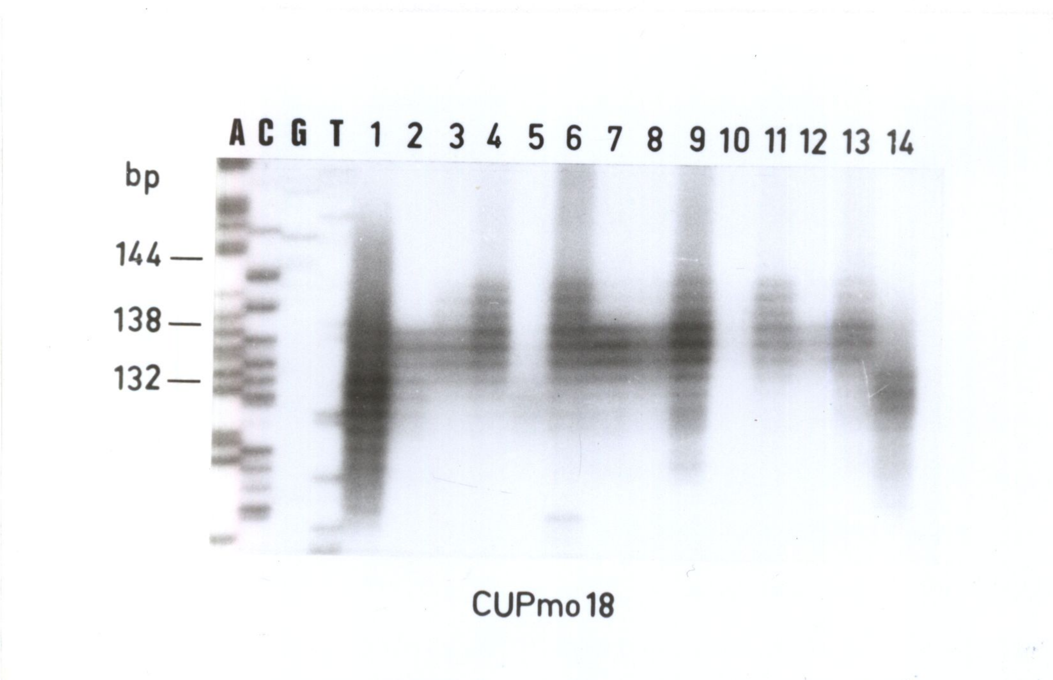

Figure 3.8 PCR amplification patterns of microsatellite locus CUPmo18 from progeny of a representative full-sib family. The size standard is a sequencing ladder of M13 mp 18.

The progeny have 2 genotypes : 132/138 in lanes 1, 2, 5, 7, and 14.
: 144/138 in lanes 3, 4, 6, 8, 9 , 11, 12, and 13

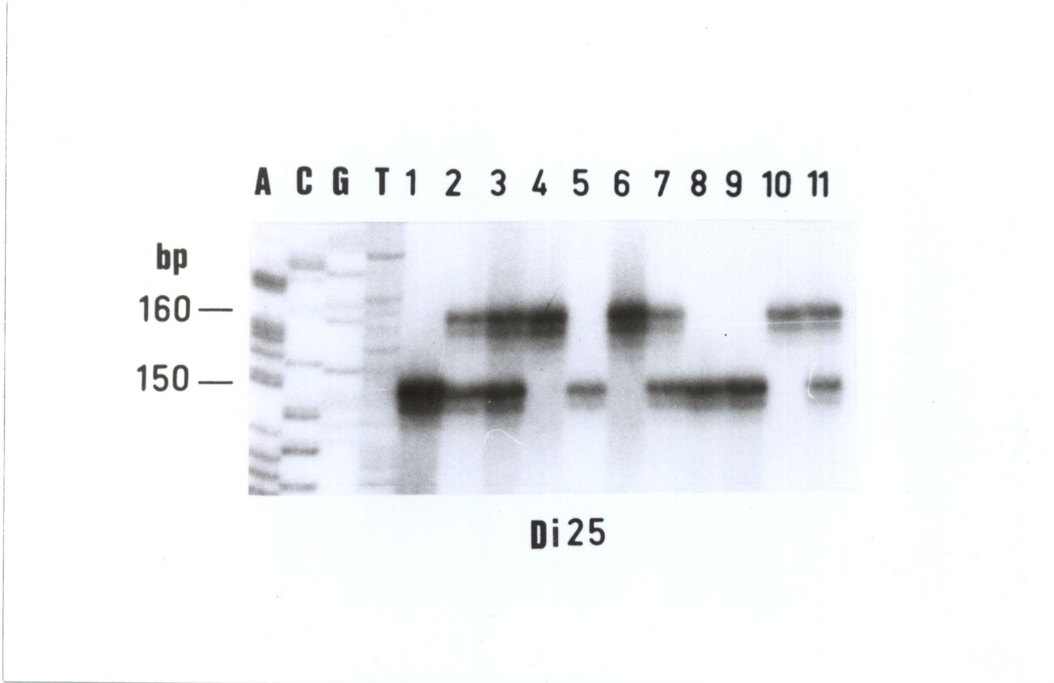

Figure 3.9 PCR amplification patterns of microsatellite locus Di25 from progeny of a representative full-sib family. The size standard is a sequencing ladder of M13 mp 18.

The progeny have 3 genotypes : 160/160 in lanes 4, 6, and 10.

: 160/150 in lanes 2, 3, 7, and 11.

: 150/150 in lanes 1, 5, 8, and 9.

For the CUPmo18 locus, 2 genotypes, 132/138 and 144/138, were observed so the inferred parental genotypes were 132/144 and 138/138. For the Di25 locus, 3 genotypes, 160/160, 160/150 and 150/150, were observed so the inferred both parental genotypes were 160/150. For the Di27 locus, 4 genotypes, 162/168, 162/176, 146/168 and 146/176, were observed thus inferred parental genotypes were 162/146 and 168/176. The number of observed genotypes in each locus were summarized in Table 3.8 and were subjected to goodness of fit test using typical χ^2 - method. The observed genotypes of offspring did not deviate from those of theoretical expectation ($p > 0.05$) therefore, the segregating nature of these microsatellite followed Mendel's law. (Table 3.8).

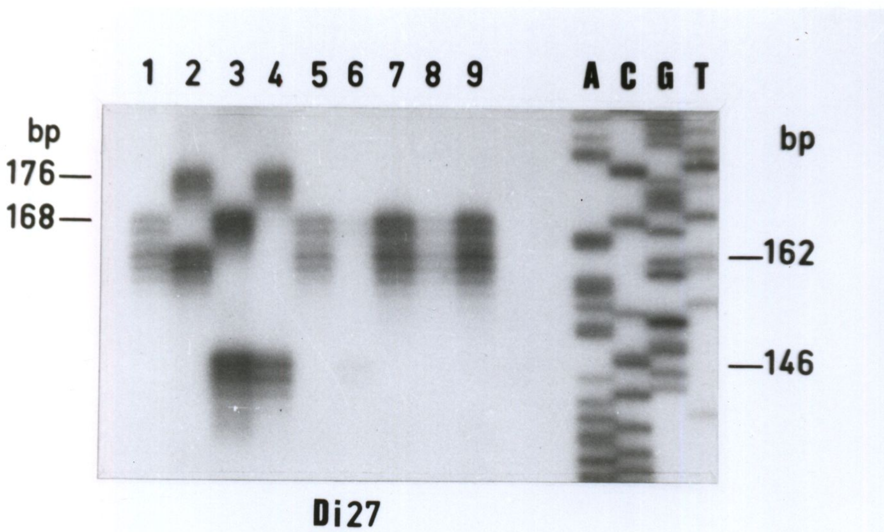

Figure 3.10 PCR amplification patterns of microsatellite locus Di27 from progeny of a representative full-sib family. The size standard is a sequencing ladder of M13 mp 18.

The progeny have 4 genotypes : 162/162 in lanes 1, 5, 7, 8, and 9.
: 162/176 in lane 2.
: 146/168 in lanes 3 and 6.
: 146/176 in lane 4

Table 3.8 Segregation analysis of three microsatellite loci(CUPmo18, Di25, Di27) resulted from twenty randomly chosen offspring from the same full-site family.

Locus	Parents[a]		Genotypes of the offspring			
	1	2	Expected ratio	Genotypes	observed individual	χ^2
CUPmo18	132/144	138/138	1:1	132/138	8	
				144/138	12	0.8^{ns}
Di25	160/150	160/150	1:2:1	160/160	5	
				160/150	6	
				150/150	[illegible]	4.8^{ns}
Di27	162/146	168/176	1:1:1:1	162/168	8	
				162/176	3	
				146/168	5	
				146/176	4	2.8^{ns}

a = genotype of parents were inferred from those of the offspring.

ns = not significant

The critical values for $p< 0.05$ df = 1 = 3.84

for $p< 0.05$ df = 2 = 5.99

and ,for $p< 0.05$ df = 3 = 7.82

3.3.4 Estimation of genetic distance, intraspecific phylogency and population differentiation

Allelic frequencies at 3 microsatellite loci in each pair of the samples were used to calculate genetic distance as described in 2.10.4 . Low level of genetic distance based on Cavalli-Sforza and Edwards' chord distance among each pairwise comparison of samples was observed. The lowest genetic distance was found between Chumphon and Satun (0.0229) whereas the highest was observed between Trad and Satun (0.0373)(Table 3.9). The neighbor - joining tree allocated all investigated samples to three different groups consisting of Satun, Trang and Phang-nga (group A), Chumphon (group B) and Trad (C)(Fig. 3.11).

Table 3.9 Cavalli-Sforza and Edwards chord distance between the 5 geographic sample of *P. monodon.*

	SAT	TNG	PHA	CHU	TRA
SAT	-				
TNG	0.0256	-			
PHA	0.0291	0.0296	-		
CHU	0.0229	0.0269	0.0296	-	
TRA	0.0373	0.0363	0.0348	0.0293	-

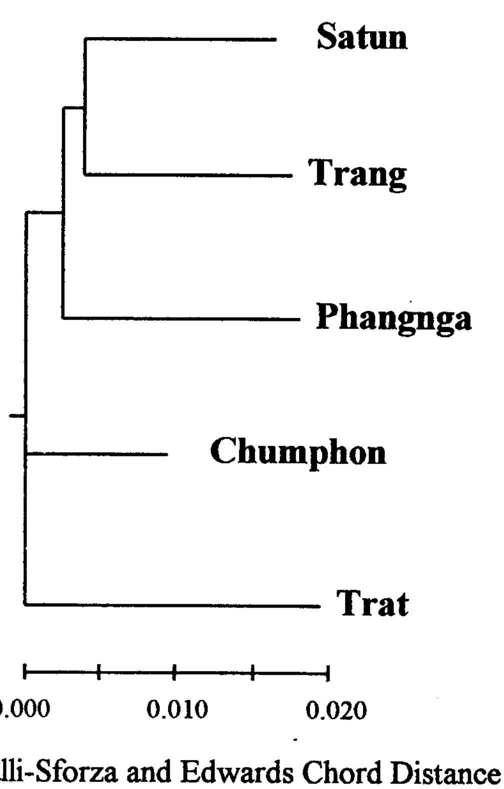

Figure 3.11 A neighbor-joining tree illustrating relationships among 5 geographic samples of *P. monodon* in Thailand based on Cavalli - Sforza and Edwards chord distance.

Hetreogeneity analysis of allele frequencies among samples was shown in Table 3.10. Although, significant difference in genotype distribution frequencies was found for overall populations ($p < 0.001$). The potential to illustrate heterogeneity among pairwise comparisons was not observed at the Di27 locus (all p - value > 0.05) . Geographic heterogeneity at the CUPmo 18 and Di25 loci was observed from 6 out of 10 possible pairwise comparisons of each locus. Interestingly, the Satun *P. monodon* was not different from all other samples except Trad at these two loci (Table 3.10). On the other hand, the allele distribution frequencies of *P. monodon* from Trad differed from did other samples. Surprisingly, geographic homogeneity between Satun and Chumphon was observed for all loci ($p = 0.0143$, $p = 0.180$ and $p = 0.212$ for CUPmo18 , Di25, and Di27 , respectively). Nonetheless, the CUPmo 18 loci could dissociate Chumphon from Trang ($p = 0.003$) whereas both Trang and Phang-nga was genetically different from Chumphon when Di25 was employed. Within the Andaman samples, the heterogeneity was observed between a pairwise comparison of Trang and Phang-nga at the CUPmo18 (p = 0.004) loci. More importantly, high significant difference in allele frequencies was observed between Trad and Chumphon located in the same coast (p < 0.001 for CUPmo 18 and p < 0.001 for Di25).

Table 3.10 Geographic hetrogeneity analysis of five conspecific samples of *P. monodon* using Three microsatellite loci (CUPmo18, Di25, Di27)

Population	P-value[a]		
	CUPmo18	Di25	Di27
Satun - Trang	0.060[ns]	0.200[ns]	0.030[ns]
Satun - Phang-nga	0.248[ns]	0.037[ns]	0.034[ns]
Satun - Chumphon	0.143[ns]	0.180[ns]	0.212[ns]
Satun - Trad	<0.001	<0.001	0.031[ns]
Trang - Phang-nga	0.004	0.169[ns]	0.368[ns]
Trang - Chumphon	0.003	0.015	0.407[ns]
Trang - Trad	<0.001	0.001	0.154[ns]
Phang-nga - Chumphon	0.084[ns]	0.004	0.186[ns]
Phang-nga - Trad	<0.001	0.008	0.573[ns]
Chumphon - Trad	<0.001	0.001	0.249[ns]
Andaman - Chumphon	0.042[ns]	0.021	0.040[ns]
Andaman - Trad	<0.001	<0.001	0.054[ns]

a = Significant levels were adjusted using a Bonferroni technique.

ns = not significant

Intraspecifically geographic differentiation of *P. monodon* in Thailand was further supported by θ (unbiased Fst). The average θ was 0.009 indicated relatively low (but significant) level of population differentiation (Table 3.11). This estimate indicated that 99 % of the microsatellite variation was within samples.

Table 3.11 F- statistics for microsatellite analysis of five geographic samples.

Locus	θ	SE	95%Ci[a]
CUPmo18	0.017	0.0016	(0.0141,0.0205)
Di25	0.002	0.0000	≅0.0021
Di27	0.010	0.0004	(0.0093,0.0109)
Overall	0.009	0.0041	(0.0011,0.0175)

a = Test for significant different from zero using 95 % confidential limits.

Chapter IV

Discussion

Several DNA extraction methods were used to isolate genomic DNA from pleopods of *P. monodon*. Of which, the extraction protocol modified from Davis et al., (1986) and that described by Cook et al., (submitted) are suitable as high molecular weight genomic DNA was consistently obtained. Although the former gave better quality and higher yields of extracted DNA, it was tedious, time consuming and hazardous. Accordingly, the alternative method described by Cook et al., (submitted) was more appropriate when dealing with a large number of specimens. Practically, these methods yielded the same degree of amplification success, as a result, a more tedious method was subsequently replaced by that of Cook et al., (submited).

It is accepted that the information on levels of genetic variation and population differentiation of the black tiger shrimp, *P. monodon* are crucial for formulation of the most appropriate conservation and management programs in this species. Additionally, these basic data is also applicable to effective breeding programs. Based on the fact that natural *P. monodon* broodstock, particularly mature females, has been heavily exploited by the culture activity, a supplement of local founders from artificially propagated programs may be necessary in the future. Alternatively, a particular stock having economically important traits can be developed through selective breeding programs. In this circumstance, highly polymorphic genetic markers are substantially required.

The genetic structure of penaeid shrimp populations inferred from allozyme analysis has been reported in several species in Australia. Intraspecific population structure was observed in *Metapenaeus bennettae*, *M. endeavouri*, and *P. latisulcatus* whereas no geographical subdivision was observed even over large geographic distances of *M. ensis*, *P. esculentus*, *P. merguiensis*, *P. plebejus*, and *P. semisulcatus* (Mulley and Latter, 1981).

Geographical differentiation of the tiger shrimp, *P. monodon,* based on allozyme analysis was first investigated in populations in Australia (Benzie et al., 1992). *P. monodon* samples obtained from seven locales throughout the Australian waters were surveyed electrophoretically at eight polymorphic loci. Three loci *(GPI*, PGM* and MPI*)* contributed to significance in the distribution of allele frequencies among populations. Geographic differentiation between western compared with northern and eastern populations was observed ($P < 0.001$ from χ^2 analysis and FST = 0.0311). Population subdivision of Australian *P. monodon* based on mtDNA variability was also reported (Benzie et al., 1993). The mtDNA genotype frequencies were significantly different between the eastern and the western populations ($P < 0.05$). The results were concordant to data previously established by allozymes.

Tassanakajon et al., (1997) reported the potential use of RAPD markers for population genetic studies of *P. monodon*. A total of 200 octanucleotide primers was empirically screened. Six informative primers were obtained and used for analysis of genetic variation level of *P. monodon* originating from three different sites (Satun-Trang, Trad and Angsila). The highest polymorphic bands was observed in the Satun-Trang (48%) followed by the Trad (45%) and Angsila (25 %) samples. Additional genetic population structure analysis using RAPD was subsequently published (Tassanakajon et al., 1998). One hundred *P. monodon* individuals collected from 5 geographically separated locations constituting of Satun-Trang, Phangnga and Medan in the Andaman Sea and Chumphon and Trad in the Gulf of Thailand were examined. Significant differences in distribution of genotype frequencies between all Thai *P. monodon* and that of Medan (Indonesia) were observed ($P < 0.0001$). Geographic heterogeneity between *P. monodon* from the Andaman Sea and the Gulf of Thailand were found ($P < 0.001$).

Both protein and DNA markers have been used for determination of genetic population structure of *P. monodon*. Nevertheless, each genetic markers has some limitations and cannot be singly used for all population genetic and systematic applications. At present, a genetic maker suitable to be used as marker assisted selection (MAS) for breeding programs of this taxa are concerned. Accordingly, the allozyme markers seem to be inappropriate for this purpose due to its low variability such as previously reported in extensive survey of *P. monodon* collected from 11 geographically separated samples throughout its distribution from Kenya to Indo-China and the Philippines (average heterozygosity was 0.027) (Sodsuk, 1996). Although high level of heterotype diversity in this species was found from mtDNA polymorphisms, the mtDNA markers can not detect F1 hybrid because of the maternally inherited property therefore these markers need to be used in coupling with the other having co-dominant segregation nature. RAPD analysis has been increasingly used in several applications but the limitation to apply the RAPD markers for pedigree analysis of large progeny samples is resulted from an inability to evaluate the actual status of a given allele in as much as homozygotic cannot be distinguished from heterozygotic states.

Basically, highly polymorphic markers with abundance, even distibution throughout the genome and transmitting according to the Mendelian fashion are ideally required for wider applications. Microsatellite loci fall into these requirements, therefore they have been elevating used for population genetic studies in several taxa. Microsatellites are short DNA stretches composed of mono-, di-, tri-, or tetranucleotides repeated tandemly (Wright, 1993, 1994 ; Park and Moran, 1994). Generally, they are highly abundant and dispersed at 7 - 100 kb intervals in eukaryotic genome (Wright, 1993).

Tassanakajon et al., (1998) characterized dinucleotide-motif microsatellite in *P. monodon* and reported their potential utility for parental determination and stock structure analysis. The average distance between neighboring (GT)n microsatellites in this species was 92.8 kb (Tiptawonnukul, 1996) which was much less abundant than those in mammals (every 18 - 46 kb) (Weber, 1990) and the honeybee, *Apis mellifera* (every 34 kb) (Estoup et al., 1993). On the other hand, the abundance of (GT)n microsatellites in *P. monodon* were approximately comparable to those of the Atlantic salmon, *Salmo salar* and the European flat oyster, *Ostrea edulis* where the (GT) motifs are found every 90 kb and 139 kb, respectively (Brooker et al., 1994; Naciri et al., 1995). The most common size-class in all microsatellite categories in *P monodon* was the sequences containing 30 - 35 tandemly repeated units (Tiptawonnukul, 1996).

This value is comparatively identical to that previously reported in the Atlantic cod, *Gadus morhua* (Brooker et al., 1994).

This thesis emphasized the use of three homologous microsatellite loci constituting of CUPmo18, Di25 and Di27 for extensive investigation on levels of genetic variability and differentiation of *P. monodon* in the Thai waters. Highly polymorphic levels (indicating by high observed heterozygosity) were found in all investigated *P. monodon* samples eliminating the suspected possibility on the occurrence of bottleneck effects in this taxon. The number of alleles per locus was 37 (mean sample size 35.6 individuals per sample), 34 (n = 36.8 per sample) and 32 (n = 35.6 per sample) for CUPmo 18, Di25 and Di27 corresponding to high observed heterozygosity (0.68 - 0.81). The within sample diversity found in this study was slightly lower than that previously reported by mtDNA-RFLP of three geographical samples in Thailand; 0.851 $\pm$ 0.031 in Satun, 0.862 $\pm$ 0.037 in Surat and 0.879 $\pm$ 0.027 in Trad (Klinbunga et al., 1998).

All previous publications based on allozymes showed extremely low level of genetic diversity in *P. monodon*. While Benzie et al., reported the heterozygosity of the Australian *P. monodon* to be 0.045 - 0.103, a lower diversity was observed in Sodsuk (1996) who studied the genetic variation level of *P. monodon* at macrogeographic scale and estimated that heterozygosity in such a species was 0.027. This indicated that microsaltellite DNA are more powerful than allozymes for differentiation and identification wild populations.

The effective number of alleles (averaged for three loci) was relatively high in all sample. However, The Gulf of Thailand *P. monodon* possessed higher n_e (16.36) than did the Andaman Sea (15.15). This circumstance was supported by the overall heterozygosity averaged for all investigated loci (0.79 and 0.80 in Chumphon and Trad and 0.66, 0.70 and 0.73 in Satun, Trang and Phang-nga).

Generally, several microsatellite alleles found in the Andaman samples were not available in Trad. However, some of which did exist in Chumphon. At the locus CUPmo 18, a total of 15 alleles found in the Andaman was not observed in Trad but

10 of these was found in Chumphon. Twelve alleles of Di25 which were seen in the Andaman was not found in Trad but five of which were possessed by the Chumphon sample. The results from Di27 was not clear for this aspect. The basic distribution data from the two loci (CUPmo 18 and Di25) and an inability to detect any significant difference in allele frequency distribution between Chumphon and Satun by all three microsatellite implied that the status of Chumphon *P. monodon* might be a result of mixing between different gene pools as it related to geographic samples from either side of the peninsula.

One important reasons to explain this phenomenon is that part of the Chumphon *P. monodon* gene pool might has been disturbed by escapees originating from the Andaman Sea. It should be emphasized that the farms usually released *P. monodon* larvae into the sea when an outbreak of serious diseases was occurred. Besides this, juveniles and broodstock of this species have been released for a restocking purpose (Department of Fisheries). Intraspecific hybridization between different *P. monodon* stocks may be occurred.

As can be seen from Table 3.6, large differences between observed and expected heterozygosity were observed for all samples overall loci, therefore the analysis of Hardy-Weinberg conformations were carried out. Surprisingly, all geographic *P. monodon* samples deviated from all investigated microsatellite loci with the exception of Trad at locus Di27.

Sodsuk (1996) examined genetic diversity of *P. monodon* collected from 11 sample sites (Kenya; the Gulf of Thailand, Trad and Surat; the east, Dungun and the east of the Malaysian peninsula, Phuket, Satun, Kedah and Medan; Java; north and south Java; and Philippines) using 40 allozyme loci but only *IDHP** (Surat) and *PGM** (Phuket) did not conform Hardy-Weinberg expectations.

Significant deviation from the expectations found in the present study may be explained by several reasons including first, null allele (non-amplifying alleles) at these loci may be present. This crucial circumstance is observed for amplification of microsatellites in human and other mammals studies (e.g. Callen et al., 1993 ; Koorey et al. , 1993 ; Pemberton et al., 1995). Non-amplification alleles can be detected through mismatched patterns between parents and offspring whose their genotypes were already known (Pemberton et al., 1995). Null-alleles can cause underestimate level of heterozygosity as heterozygote individuals are scored as homozygous and thus either showing non-Mendelian inheritance or resulting in significant deviation from Hardy-Weinberg equilibrium. Second, most dinucleotide microsatellite show stuttered bands so mis-scoring of a particular allele may be occurred(O'Reilly &Wright, 1995).

In the present study, the distribution of allele showed that allele 168 in locus CUPmo18, alleles 138, 178 and 200 in locus Di25 and allele 116 in locus Di27 were not observed. Thus, chance of detecting null allele was low. Besides this, each locus showed many alleles having low frequencies and several unique alleles therefore the greater sample sizes are required to ensure true representatives. On the one hand, significant departures from Hardy-Weinberg expectations may reveal the mixture of genetically different population in a local samples, through an observed homozygote excess (Wahlun, 1928 : cited in Hedrick, 1985). On the other hand, it may indicate the influence of selection on a particular locus.

Segregation analysis of CUPmo 18, Di25 and Di27 in *P. monodon* using 20 progeny from a representative full-sib family revealed non-significant deviations from gametic segregation expectation for all loci ($P > 0.05$) indicating Mendelian inheritance nature of these three microsatellite loci. Therefore, departures of Hardy-Weinberg equilibrium should be caused by low number of sample sizes and sampling errors rather than from effects of selection in as much as no common allele (s) with the extremely high frequency was observed. Based on the basic biology of *P. monodon,* the important life-historical generalization may be drawn. The *P. monodon* females may spawn more than once a year. Each wild breed female may produce 248,000 to 811,000 eggs per single spawn (Motoh, 1981 : cited in Solid, 1988) to compensate for an enormous gamete wastage in the planktonic larval stages. In this circumstance, the enormous number of eggs may be sufficient to replace part of the entire adult population. Sampling errors could then be happened.

Geographic homogeneity was observed all pairwise comparison between sites and regions ($P > 0.05$) when Di27 was employed indicating the limitation to use this locus for population genetic analysis of *P. monodon* in Thailand where low level of genetic differentiation among population was exist. Nevertheless, this microsatellite yielded the highest level of observed heterozygosity (0.71-0.95; mean = 0.81) with the Mendelian segregation nature so it is useful in selective breeding program in which pedigree analysis of large number of families are required.

Generally, CUPmo18 and Di25 were equally good for determination of population subdivisions in this study as indicated by six significant pairs of all possible comparisons. There were no significant differences in allele distribution frequencies of Satun to other samples except Trad (Phanag-nga and Chumphon) for both loci implying their sympatric status. In contrast, geographic heterogeneity between Chumphon and Trad located in the same coastal site was observed ($P < 0.001$). The contradictory results of CUPmo18 and Di25 was observed between Phang-nga and Trang and Chumphon ($P = 0.004$ and 0.084 for the former and $P = 0.169$ and 0.004 for the latter locus). After non-significant geographic samples were combined and reanalyzed, the significant differences in allele frequencies among different sites (between Andaman - Chumphon, Andaman-Trad) were observed. Based on this analysis, *P. monodon* in Thailand could be genetically separated into three stocks; the Andaman (stock A), Chumphon (stock B) and Trad (stock C).

Disregarding estimation of evolutionary time (the molecular clock concept), it was recently reported that the probability to obtained the correct topology of a phylogenetic tree based on both the infinite allele and the stepwise mutation models was high when the Cavalli-Sforza and Edwards' chord distance was employed (Takezaki and Nei, 1996). Apparently, the genetic distance between each pair of geographic samples was not largely different implying low level of differentiation in *P. monodon*. The neighbor-joining tree allocated five investigated samples into three phylogenetically related lineages as the same as that from geographic heterogeneity test. While Satun, Trang and Phang-nga were placed at one extreme, Trad was placed at the other. Chumphon was located in the middle and could possible placed in either the Gulf of Thailand or Andaman samples.

The degree of population differentiation in this species was weak but still significant (θ = 0.009) as differentiation due to between samples were only approximately 1%. This results indirectly indicated that *P. monodon* in Thailand were not suffered from reduced heterozygosity indicating by high genetic diversity within each investigated samples.

Microsatellite DNA are useful for not only identification of genetically distinct stocks but for examination of individuality and parentage in the commercial important species like *P. monodon*. The present study indicated the existence of population differentiation in this species. Although the level of subdivisions may be low, three different *P. monodon* stocks found should be treated, from a management point of view, as separate exploitation stocks (Carvalho and Hauser, 1994). The basic information obtained from this study implied the possibility to used microsatellite markers for selective breeding programs. For example, representatives from each wild stocks can be randomly chosen. Intraspecific hybridization among different strains can be carried out. The progeny from all random crosses are reared together obviating significantly common environmental effects. After reaching the market size, the family selection approach can then be performed. For population genetic studies, all microsatellite loci (CUPmo18, Di 25, Di27) in this study can be used to identify genetic variation level in geographic samples which are inbred and/or reduced heterozygosity was observed from other genetic markers.

Chapter V

Conclusions

1. Three microsatellite loci, CUPmo18, Di25 and Di27, were shown to be highly polymorphic with number of alleles at each locus of 37, 34 and 32 alleles respectively. Population analyses based on 3 loci revealed heterozygosities between 0.66-0.80 and average alleles per locus were ranged from 22.23-26.33.

2. The effective number of alleles and mean heterozygosity for overall loci indicated that the level of genetic polymorphisms of *P. monodon* originating from the Gulf of Thailand was slightly greater than that of the Andaman samples.

3. For determination of intraspecific population structure, CUPmo18 and Di25 were equally effective. Although Di27 did not provide an ability to detect geographic heterogeneity among Thai *P. monodon* samples, all microsatellite loci used in this study are useful for breeding programs of this economically important species.

4. Analysis of geographic heterogeneity and phylogenetic reconstruction using the Neighbor - joining approach divided 5 geographic *P. monodon* samples to three different gene pools (stocks) constituting of Satun, Trang and Phang-nga(A), Chumphon(B) and Trad(C).

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Biography

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