เครื่องหมายพันธุกรรมระดับโมเลกุลเพื่อระบุชนิด เพศ และประชากรของกุ้งกุลาดำ *Penaeus monodon*

นางสาวบวรลักษณ์ คำน้ำทอง

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MOLECULAR GENETIC MARKERS FOR IDENTIFICATION OF SPECIES, SEX, AND POPULATION OF GIANT TIGER SHRIMP *Penaeus monodon*

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บวรลักษณ์ คำน้ำทอง : เครื่องหมายพันธุกรรมระดับโมเลกุลเพื่อระบุชนิด เพศ และประชากรของกุ้งกุลาดำ *Penaeus monodon* (MOLECULAR GENETIC MARKERS FOR IDENTIFICATION OF SPECIES, SEX, AND POPULATION OF GIANT TIGER SHRIMP *Penaeus monodon*) อ. ที่ปรึกษา : ศ.ดร. เปี่ยมศักดิ์ เมนะเศวต, อ. ที่ปรึกษาร่วม : ดร. ศิราวุธ กลิ่นบุหงา 226 หน้า. ISBN 974-14-2336-5.

ทำการพัฒนาเครื่องหมายพันธุกรรมระดับโมเลกุลเพื่อระบุชนิดของกุ้ง 5 ชนิดประกอบด้วย *Penaeus monodon, P. semisulcatus, Litopenaeus vannamei, Fenneropenaeus merguiensis* และ *Marsupenaeus japonicus* ด้วยการวิเคราะห์ PCR-RFLP และ SSCP ของยีน 16S $rDNA_{560}$ พบว่า *P. monodon, L. vannamei* และ *F. merguiensis* สามารถจำแนกออกจากกันได้อย่างชัดเจน โดยการตัดยีน 16S $rDNA_{560}$ ด้วยเอนไซม์ตัดจำเพาะ *Alu* I, *Mbo* I, *Ssp* I และ *Vsp* I ขณะที่ *P. semisulcatus* และ *M. japonicus* ไม่สามารถจำแนกออกจากกันได้ เนื่องจากมีจีโนไทป์เหมือนกัน (BABB) เมื่อวิเคราะห์ชิ้น 16S $rDNA_{560}$ ด้วยวิธี SSCP พบว่าสามารถจำแนกกุ้งทั้งสองชนิดนี้ออกจากกันได้ อย่างไรก็ตาม เมื่อขยายตัวอย่างเพิ่มขึ้นพบปัญหาในการทำพีซีอาร์ในกุ้ง *L. vannamei* และ *F. merguiensis* จึงทำการโคลนยีน 16S $rDNA_{560}$ จากตัวแทนกุ้งทั้ง 5 ชนิดที่แสดง common mitotype ในกุ้งแต่ละชนิด หาลำดับนิวคลีโอไทด์และออกแบบไพรเมอร์ที่สามารถให้ผลิตภัณฑ์พีซีอาร์ขนาด 312 bp ได้ในกุ้งทั้ง 5 ชนิด เมื่อวิเคราะห์ด้วย PCR-RFLP ในตัวอย่างจำนวน 185 ตัว พบว่าให้ผลเช่นเดียวกับผลที่ได้จาก 16S $rDNA_{560}$ และพบรูปแบบของ SSCP ที่สามารถจำแนกกุ้งทั้ง 5 ชนิดออกจากกันได้อย่างถูกต้อง

จากการศึกษาพันธุศาสตร์ประชากรของกุ้งกุลาดำ *P. monodon* ในประเทศไทยจาก 5 แหล่ง (ตราด ชุมพร สตูล ตรังและพังงา) ด้วยการวิเคราะห์ PCR-RFLP และ SSCP ของยีน16S $rDNA_{312}$ พบว่ามีระดับความหลากหลายทางพันธุกรรมต่ำและไม่พบโครงสร้างประชากรทางพันธุกรรมของกุ้งกุลาดำที่ทำการศึกษา ($P > 0.05$) และเมื่อวิเคราะห์ด้วย AFLP จำนวน 320 คู่ไพรเมอร์ พบชิ้น AFLP ที่ polymorphic จึงทำการโคลนและหาลำดับนิวคลีโอไทด์ชิ้น AFLP จำนวน 22 ชิ้น ทำการออกแบบไพรเมอร์ 14 คู่และเลือก 4 คู่ไพรเมอร์ (P6M2-370, P6M6-470, E4M6-295 และ E7M10-450) ที่ให้ผล polymorphic มาศึกษาพันธุศาสตร์ประชากรของกุ้งกุลาดำ พบระดับความหลากหลายทางพันธุกรรมต่ำและไม่พบโครงสร้างประชากรทางพันธุกรรม ($P > 0.05$) เช่นเดียวกับผลจาก 16S $rDNA_{312}$ นอกจากนี้ ทำการหาลำดับนิวคลีโอไทด์ของยีน COI_{614} จากกุ้งจำนวน 100 ตัวอย่าง ผลจาก neighbor-joining tree สามารถจัดกลุ่มทางพันธุกรรมของกุ้งกุลาดำในประเทศไทยได้ 3 กลุ่ม โดยพบความแตกต่างทางพันธุกรรมระหว่างกลุ่มในระดับสูงแต่ภายในกลุ่มเดียวกันมีระดับต่ำ และพบการกระจายตัวของแฮพโพไทด์แต่ละกลุ่มในแต่ละประชากรมีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ ($P < 0.05$)

ทำการค้นหาเครื่องหมายโมเลกุลที่จำเพาะกับเพศและเครื่องหมายที่มีระดับการแสดงออกจำเพาะและแสดง ออกแตกต่างกันในรังไข่และอัณฑะกุ้งกุลาดำโดย RAPD (100 ไพรเมอร์) และ RAP-PCR (150 คู่ไพรเมอร์) ทำการโคลนเครื่องหมาย RAPD และเครื่องหมาย RAP-PCR จำนวน 8, 21 (รังไข่) และ 14 (อัณฑะ) เครื่องหมายตามลำดับ หาลำดับนิวคลีโอไทด์และออกแบบไพรเมอร์จำนวน 4 คู่จากชิ้น RAPD พบว่าให้ผลไม่จำเพาะกับเพศ และ 25 คู่จาก RAP-PCR เมื่อทดสอบการแสดงออกของยีนดังกล่าวกับกุ้งอายุประมาณ 3 เดือนและกุ้งโตเต็มวัยเพศเมีย ($N = 5$, $N = 7$ - 10) และเพศผู้ ($N = 4$, $N = 5$ - 7) พบเครื่องหมายที่แสดงออกจำเพาะกับกุ้งเพศเมียจำนวน 5 เครื่องหมาย (FI-4, FI-44, FIII-4, FIII-39 และ FIII-58) และเครื่องหมายที่แสดงออกจำเพาะกับกุ้งเพศผู้จำนวน 2 เครื่องหมาย (M457-A01 และ MII-51) นอกจากนี้ MII-5 ซึ่งพัฒนามาจากเครื่องหมาย RAP-PCR ที่แสดงออกในอัณฑะกลับให้ผลระดับการแสดงออกสูงในรังไข่มากกว่าในอัณฑะของกุ้งอายุ 3 เดือน และแสดงออกจำเพาะในรังไข่ของกุ้งเพศเมียในระยะโตเต็มวัย

ลายมือชื่อนิสิต.....นางสาวบวรลักษณ์ คำน้ำทอง.....

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

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

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BAVORNLAK KHAMNAMTONG: MOLECULAR GENETIC MARKERS FOR IDENTIFICATION OF SPECIES, SEX, AND POPULATION OF GIANT TIGER SHRIMP *Penaeus monodon*. THESIS ADVISOR: PROF. PIAMSAK MENASVETA, Ph.D., THESIS CO-ADVISOR: SIRAWUT KLINBUNGA, Ph.D. 226 pp. ISBN 974-14-2336-5.

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

Population genetic studies of *P. monodon* in Thailand were examined by PCR-RFLP and SSCP analysis of 16S $rDNA_{312}$. Low genetic diversity and a lack of intraspecific population subdivisions of *P. monodon* were illustrated ($P > 0.05$). Additionally, 320 AFLP primer combinations were screened against bulked genomic DNA of *P. monodon*. Twenty two polymorphic AFLP fragments were cloned and sequenced. Fourteen pairs of sequence-specific primers were designed. Four markers (P6M2-370, P6M6-470, E4M6-295 and E7M10-450) were used for population genetic studies of *P. monodon*. Like results from 16S $rDNA_{312}$, low genetic diversity and a lack of population differentiation was found ($P > 0.05$). Moreover, a COI_{614} gene segment of 100 individuals of *P. monodon* were unidirectional sequenced. A neighbor-joining tree indicated three phylogenetic lineages of *P. monodon*. Large nucleotide divergence was observed between inter-lineage haplotypes but limited divergence was found between intra-lineage haplotypes. Distribution frequencies of haplotype clusters indicating the existence of population subdivisions of *P. monodon* based on COI polymorphism ($P < 0.05$).

Sex determination and differentiation markers of *P. monodon* were analyzed by RAPD (100 primers) and RAP-PCR (150 primer combinations). Eight candidate genomic sex-specific RAPD bands and twenty-one and fourteen RAP-PCR fragments specifically/differentially expressed in ovaries and testes of *P. monodon* were successfully cloned and sequenced. Four RAPD-derived markers did not reveal sex-specificity when tested against genomic DNA of *P. monodon*. Therefore, genomic sex determination markers were not successfully developed in *P. monodon*. Expression patterns of 25 RAP-PCR derived markers were tested against the first strand cDNA of ovaries and testes of 3-month-old and broodstock-sized *P. monodon* ($N = 5$ and $N = 7 - 10$ for females and $N = 4$ and $N = 5 - 7$ for males, respectively). Five (FI-4, FI-44, FIII-4, FIII-39 and FIII-58) and two (M457-A01 and MII-51) derived RAP-PCR markers revealed female- and male-specific expression patterns in *P. monodon*. Surprisingly, MII-5 originally found in testes showed a higher expression level in ovaries than did testes of juvenile shrimps but a temporal female-specific pattern in *P. monodon* adults.

Student's signature. Bavornlak Khamnamtong

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

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

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LIST OF ABBREVIATIONS

bp	base pair
°C	degree Celcius
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DNA	deoxyribonucleic acid
HCl	hydrochloric acid
IPTG	isopropyl-thiogalactoside
Kb	kilobase
M	Molar
$MgCl_2$	magnesium chloride
mg	Milligram
ml	Millilitre
mM	Millimolar
ng	Nanogram
OD	optical density
PCR	polymerase chain reaction
RNA	Ribonucleic acid
RNase A	ribonuclease A
rpm	revolution per minute
SDS	sodium dodecyl sulfate
Tris	tris (hydroxyl methyl) aminomethane
µg	Microgram
µl	Microlitre
µM	Micromolar
UV	ultraviolet

CHAPTER I

INTRODUCTION

Members of the genus *Penaeus* are a large and diverse group of marine shrimps (or prawns) that have primarily distributed in tropical and subtropical regions. It is the most economically important group among shrimps and prawns, and perhaps among all crustaceans worldwide (Chan, 1998; Baldwin *et al*., 1998). The main species under cultivation are black tiger shrimp *Penaeus monodon*, *Litopenaeus vannamei*, *P. stylirosis*, *Feneropenaeus chiensis*, *F. indicus*, *F. merguiensis*, *Marsupenaeus japonicus* and *Metapenaeus ensis* (Fast and Lester, 1992; Pérez *et al*., 2004).

The black tiger shrimp (*P. monodon*) is one the most economically culture species in Thailand. Thailand has been regarded as the leading shrimp producer (previously *P. monodon* and presently *L. vannamei* as the main cultured species) for more than a decade with the production of approximately 200,000 metric tons providing an income of over US$ 2 billion annually (Rosenberry, 2001). Farming of *P. monodon* has achieved a considerable economic and social importance in the region, constituting a significant source of income and employment.

In Thailand, *P. monodon* have been intensively cultured for more than two decades. Approximately 60% of the total harvest shrimp comes from cultivation. Number of farms and area under marine shrimp culture during 1985-2004 was increased as show in Figure 1.1. Shrimp farms and hatcheries are scattered along the coastal areas of Thailand. Southern provinces (Nakorn Sri Thammarat and Surat Thani) account for the majority while those in the East (Chanthaburi) and Central regions (Samut Sakhon and Samut Songkhran) comprise the minority in terms of number. The intensive farming system (85%) has been used for *P. monodon* farming activity resulting in the consistent increase in the outcome production (Department of fisheries, 1999).

The success of tiger shrimp industry in Thailand has resulted in the steadily increased income for the nation. This has also elevated the quality of life for Thai farmers. 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 seawater during seasons, and ideal soils for pond construction.

The United States of America is traditionally the largest importer of Thai shrimp products (Table 1.1). Its imports accounted for 48.56% of all Thai shrimp exports in 2004, worthing 14,998.81 million baht. Japan is the second largest importing country with imports account for 18.37%, worthing 8,165.94 million baht. The remaining important markets are Canada, Australia, Europe, Asian countries and others brining of income about 32,529.62 million baht to the country (Figure 1.2).

Farming of *P. monodon* has consistently encountered production losses from infectious diseases, particularly from white spot syndrome virus (WSSV), yellow head virus (YHV) and *Vibrio* sp.. Genetic improvement and other biotechnology applications are crucial to the future development of this industry (Benzie, 1998; Brody, 1998). Besides problems from diseases, the lack of high quality wild and/or domesticated broodstock of *P. monodon* has possibly caused an occurrence of a large portion of stunted shrimps at the harvest time (3-5 g body weight at 4 month cultivation period). As a result, the Pacific white shrimp (*L. vannamei*) has been introduced to Thailand as an alternative cultured species and initially contributed approximately 20000 MT of the production in 2002 and dramatically increased to 170000 and 220000 MT in 2003 and 2004, respectively (Limsuwan, 2004).

Owing to morphological similarity at the larval stages, larvae of *F. merguiensis* are intentionally traded as those of *L. vannamei*. In addition, the external morphology of *P. monodon* and *P. semisulcatus* is resembled at all stages of development but the growth rate of *P. semisulcatus* is approximately 3 times lower than that of *P. monodon*. Once the shrimp is processed (e.g. leaving only the shrimp meat), species identification becomes problematic. Accordingly, species-diagnostic markers play important roles to prevent supplying incorrect shrimp larvae for the industry and for quality control of shrimps exported from Thailand.

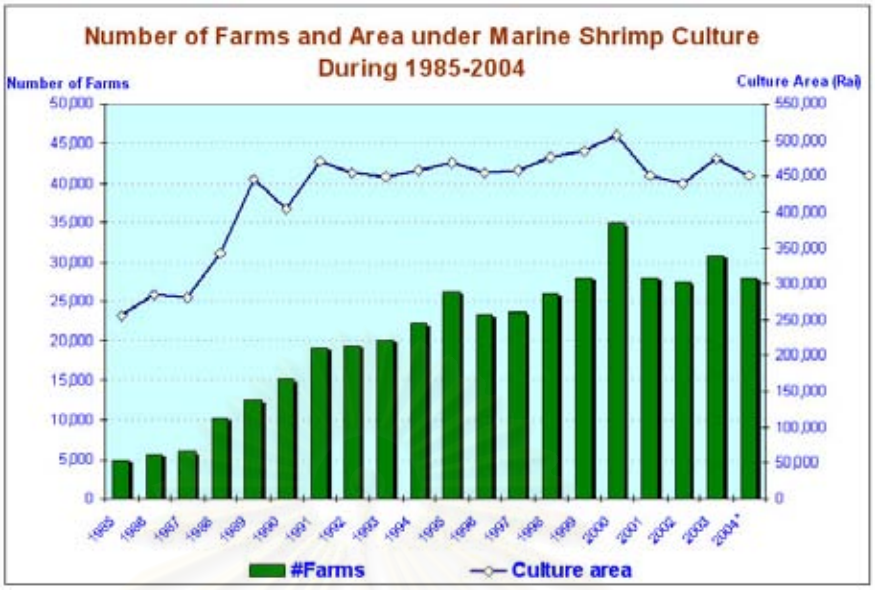

Figure 1.1 Number of farms and area under marine shrimp culture in Thailand during 1985-2004

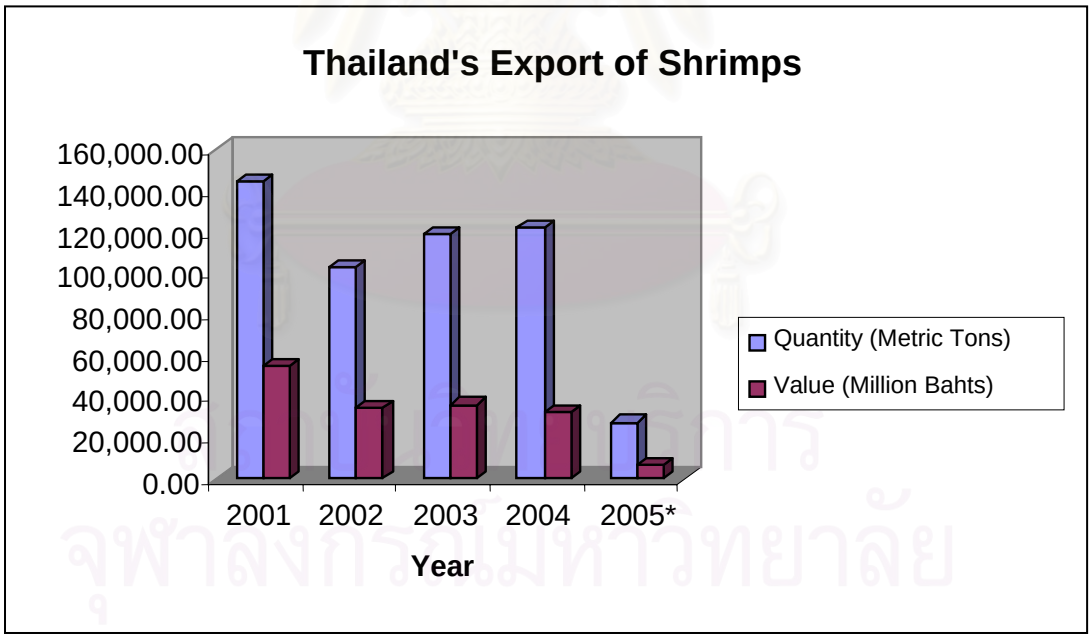

Figure 1.2 Quantity and value of export fresh, chilled or frozen shrimps of Thailand (2001 – March 2005)

Table1.1 Export fresh, chilled or frozen shrimps of Thailand between 2001 -2005

No.	Country	2001		2002		2003		2004		2005*	
		Q	V	Q	V	Q	V	Q	V	Q	V
1.	U.S.A	67,167.00	27,245.43	42,296.00	15,547.13	62,920.00	18,705.80	59,480.94	14,998.81	13,453.93	3,102.15
2.	Asia	57,366.00	20,314.58	47,672.00	14,712.92	41,459.00	13,066.30	44,435.39	12,882.40	9,394.79	2,415.80
	China	6,329.00	1,400.97	2,631.00	516.95	2,781.00	690.10	2,837.24	638.63	1,266.03	201.27
	Hong Kong	5,691.00	1,440.62	3,008.00	679.41	2,082.00	508.60	2,503.39	673.73	770.58	159.45
	Japan	24,878.00	11,671.07	26,445.00	9,619.55	22,380.00	8,524.10	22,494.71	8,165.94	4,127.61	1,387.16
	S. Korea	4,176.00	1,282.12	3,924.00	1,111.03	6,431.00	1,670.20	1,173.75	252.57	383.42	104.30
	Malaysia	250.00	34.21	185.00	13.34	250.00	24.50	8,318.12	1,931.06	1,804.40	365.83
	Singapore	9,367.00	2,655.21	6,675.00	1,535.67	4,936.00	967.10	3,777.20	632.45	596.78	89.95
	Taiwan	6,675.00	1,830.38	4,804.00	1,236.97	2,599.00	681.70	3,210.40	565.40	268.55	73.22
	Vietnam	-	-	-	-	-	-	120.58	22.62	177.42	34.62
3.	Canada	5,802.00	2,265.20	4,901.00	1,819.27	6,696.00	2.071.10	7,975.19	1,998.43	2,379.02	588.92
4.	Australia	3,643.00	1,407.73	1,407.73	3,209.00	4,033.00	1,067.50	4,517.55	1,073.92	1,052.75	212.19
5.	EU	7,059.00	2,422.45	1,814.00	536.12	692.00	235.50	2,431.51	716.09	446.34	116.86
	Belgium	302.00	118.58	313.00	96.90	140.00	41.70	369.32	86.47	130.02	26.54
	Denmark	107.00	47.66	17.00	6.38	-	-	1.75	1.43	0.35	0.28
	France	1,556.00	496.51	364.00	106.10	87.00	43.90	139.70	61.39	24.98	8.49
	French Polynesia (TAHITI)	-	-	-	-	-	-	319.17	84.77	78.71	20.02
	Germany	1,242.00	474.34	292.00	106.78	182.00	60.70	343.46	131.34	77.49	26.23
	Italy	876.00	161.52	170.00	25.20	74.00	21.60	603.09	118.42	78.10	17.68
	Netherlands	1,333.00	509.60	509.60	106.00	48.00	15.70	123.02	36.06	14.27	4.74
	Spain	55.00	16.67	16.67	65.00	29.00	10.10	1.39	0.94	0.01	0.01
	Utd Kingdom	1,588.00	597.57	597.57	487.00	132.00	41.80	530.61	195.27	42.41	12.87

Table1.1 (Continued)

No.	Country	2001		2002		2003		2004		2005*	
		Q	V	Q	V	Q	V	Q	V	Q	V
6.	New Zealand	337.00	115.25	115.25	454.00	-	-	750.51	200.52	117.94	32.83
7.	Middle East	-	-	-	-	-	-	589.36	62.06	132.98	13.50
	Saudi Arabia	-	-	-	-	-	-	202.98	26.79	39.29	3.90
	Arabs Emirates	-	-	-	-	-	-	386.38	35.27	93.69	9.60
8.	Switzerland	235.00	110.02	110.02	77.00	158.00	56.40	177.43	59.15	25.58	7.12
9.	Russian	-	-	-	-	-	-	86.32	34.71	8.44	3.39
10.	Norway	-	-	-	-	-	-	-	-	11.82	2.55
11.	Others	2,997.00	867.37	867.37	3,330.00	3,478.00	748.50	2,032.74	503.53	359.30	73.68
	Total	**144,606.00**	**54,748.03**	**102,753.00**	**34,424.53**	**119,436.00**	**35,951.10**	**122,476.94**	**32,529.62**	**27,382.89**	**6,568.99**

Source: The Customs Department/Thai Frozen Foods Association
Q = Quantity
V = Value (million baht)
* = January – March

The production cycle of *P. monodon* has yet to be completed. Farming of *P. monodon* presently relies almost entirely on wild-caught broodstock for the seed supply because breeding of *P. monodon* in captivity is extremely difficult. This open reproductive cycle and reliance on wild stocks of *P. monodon* results in heavy exploitation of female broodstock from wild populations. Genetic-based stock enhancement programs of natural *P. monodon* stocks are also important for sustainable aquaculture activity of this species. Polymorphic DNA markers are required for examining genetic diversity and population subdivisions of *P. monodon* in Thai waters and for identifying genotypes of shrimps in genetic-based selective breeding programs of *P. monodon*.

Domestication of *P. monodon* has been carried out for several generations (Withyachumnarnkul *et al*., 1998). Nevertheless, the use of spermatozoa from captive males yielded low survival rates of offspring. Using wild males instead of domesticated males with either wild or domesticated females has resolved this problem successfully (Withyachumnarnkul, personal communication). As a result, genes expressed in different stages of testicular development of wild and domesticated *P. monodon* should be studied.

In *P. monodon*, females exhibit approximately 10-20% greater growth rate than do males at all stages of development (Browdy, 1998). The diploid chromosome numbers of penaeid shrimps have been reported in *P. esculentus, P. monodon, Farfantepenaeus aztecus, Fenneropenaeus chinensis, Fenneropenaeus merguiensis, Fenneropenaeus penicillatus* and *Marsupenaeus japonicus* ($2N = 88$), *P. semisulcatus* and *Litopenaeus setiferus* ($2N = 90$), and *Farfantepenaeus californiensis* and *Litopenaeus occidentalis* ($2N = 92$) (Benzie, 1998). Nevertheless, a lack of obvious heteromorphic sex chromosomes in this species has been causing limited knowledge on sex chromosomal system (XY or ZW etc.) and their segregation patterns. In addition, sex determination cascades and sex-diagnostic markers have not been reported in penaeid shrimps. This has prevented the possibility to increase aquacultural production through a monosex culture approach.

Reduced spawning potential and low degree of maturation of *P. monodon* in captivity crucially prohibits several possible applications including development of

the effective breeding programs in this species. Genetic improvement of *P. monodon* cannot be achieved without knowledge on the control of reproduction. Mechanisms controlling ovarian maturation and sex differentiation processes at the molecular level are important and can be significantly applied to the industry of *P. monodon*. Isolation and characterization of genes specifically/differentially expressed in ovaries or testes of *P. monodon* is the initial step for understanding differentiation of sexes in this economically important species (Leelatanawit *et al*., 2004).

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

Infraorder Penaeidea

Superfamily Penaeoidea

Family Penaeidae Rafinesque, 1985

Genus Penaeus Fabricius, 1798

Subgenus Penaeus

Species monodon

Scientific name: *Penaeus monodon* (Fabricius), 1798 (Figure 1.3)

Common name: giant tiger prawn or black tiger shrimp

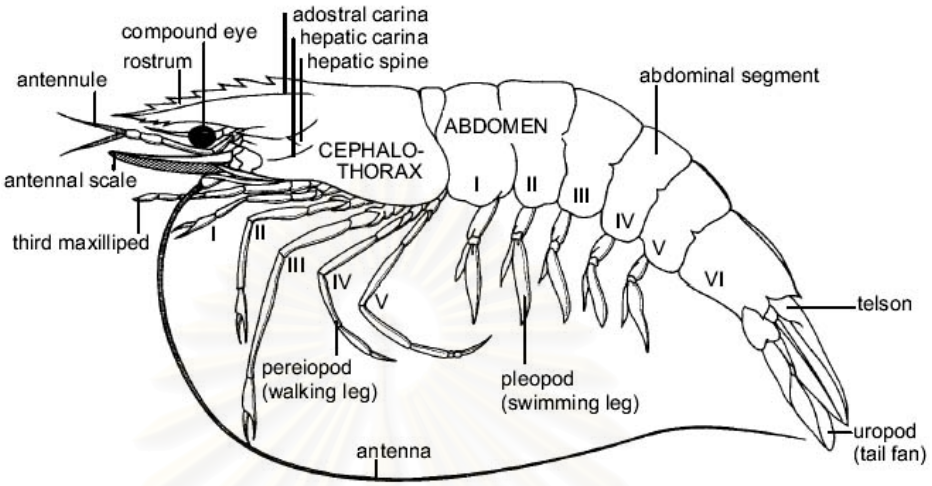

Figure 1.3 External anatomy of *P. monodon*

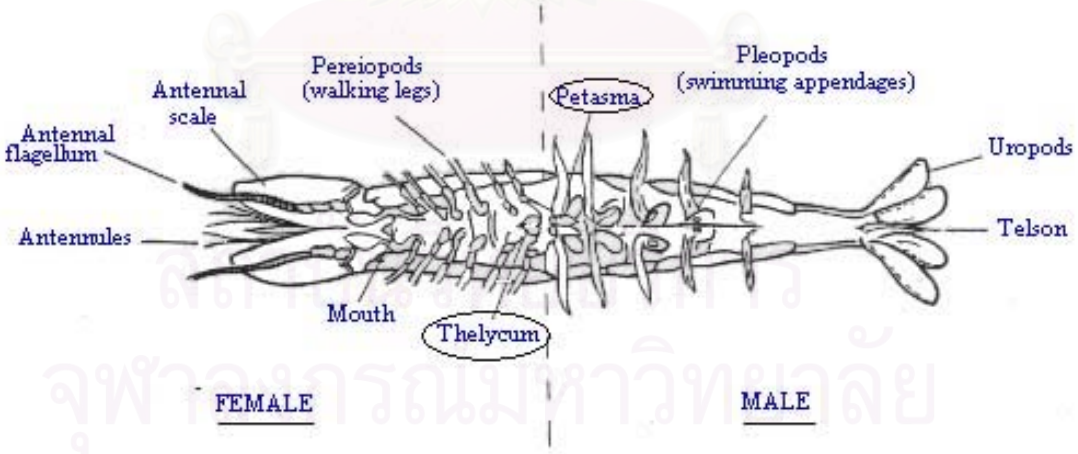

Figure 1.4 External characters; petasma and thelycum can be used to identify male and female *P. monodon* since the juvenile stages.

1.2 Molecular genetic markers used in this thesis

Molecular genetic markers are useful for genetic and systematic studies of natural and culture species. Genetic markers used for population genetic and systematic studies should exhibit suitable polymorphic levels for desired application and be selectively neutral. Genetic markers that can characterize relative levels of population variation in both wild and cultured stocks are needed to aid the development of the aquaculture industry and to assist in the conservation of wild stock.

Several molecular genetic techniques can be used at the genomic DNA and the cDNA levels. The former level generally includes polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), single strand conformational polymorphism (SSCP) and DNA sequencing. The latter level includes reverse transcription (RT)-PCR, differential display (DD-PCR), RNA arbitrary primed PCR (RAP-PCR), Expressed Sequence Tags (ESTs), cDNA subtraction and rapid amplification of cDNA ends-polymerase chain reaction (RACE-PCR) analysis.

The introduction of the polymerase chain reaction (PCR) by Mullis *et al.* (1987) has opened a new approach for molecular genetic studies. PCR is a method for an *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. Million copies of the target DNA sequence can be synthesized from a low amount of starting DNA template within a few hours.

The PCR reaction components are consisting of DNA template, a pair of primers for the target sequence, dNTPs (dATP, dCTP, dGTP and dTTP), buffer and heat-stable DNA polymerase (usually *Taq* polymerase). The amplification reaction usually consists of three steps; denaturation of double stranded DNA at high temperature, annealing to allow primers to form hybrid molecules at the optimal temperature, and extension of the annealed primers by heat-stable DNA polymerase.

The cycle is repeated for 30-40 times (Figure 1.5). The amplification product is electrophoretically analyzed.

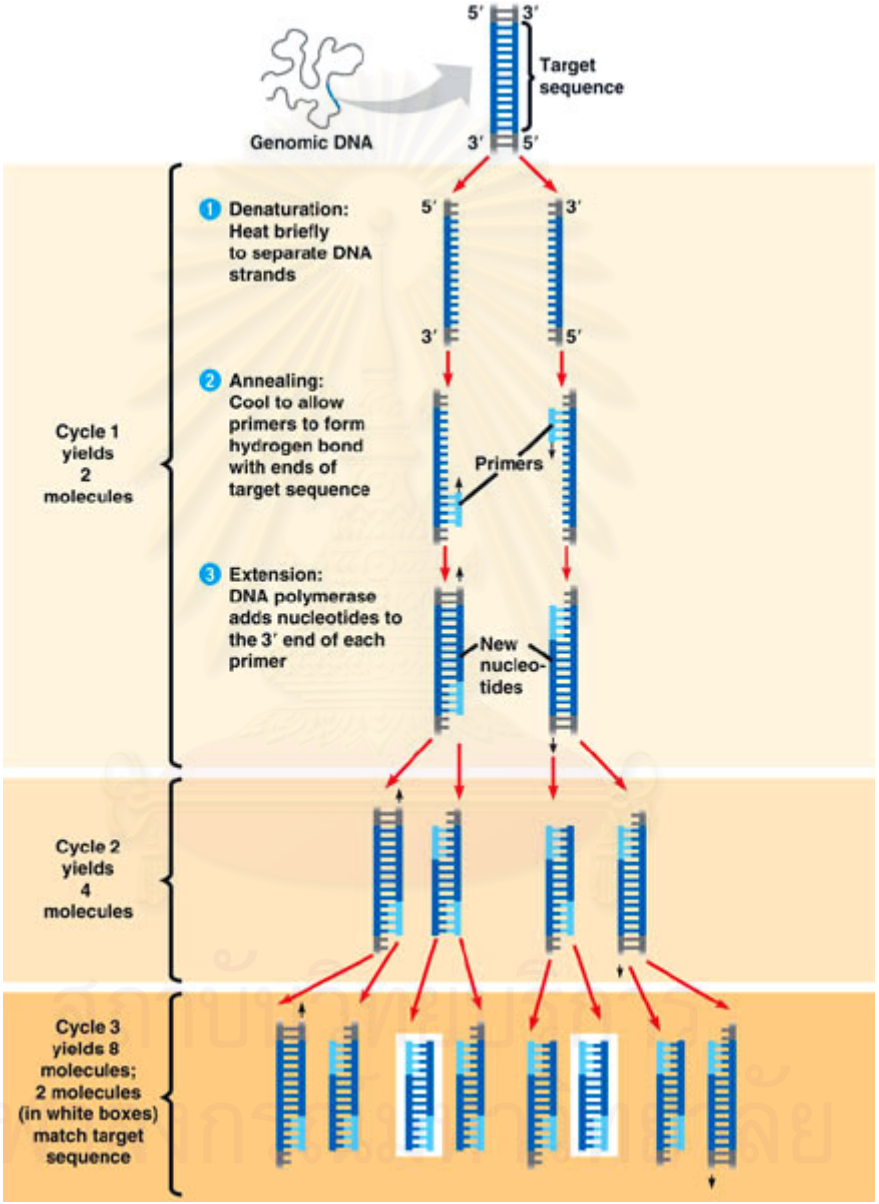

Figure 1.5 General illustration of the polymerase chain reaction (PCR) for amplifying DNA (http://campus.queens.edu/facult/jannr/Genetics/images/dnatech/pcr.gif).

1.2.1 PCR-restriction fragment length polymorphism (PCR-RFLP)

Restriction fragment length polymorphism (RFLP) analysis is indirectly used to determine genetic variation at the DNA level by comparison of shared restriction fragments or sites. Basically, variation in restriction enzyme cleavage sites generates size differences of the resulting fragments.

PCR-RFLP analysis is one of the initial techniques widely used to indirectly detect genetic variation at the DNA level (Figures 1.6 and 1.7). It examines size variation of specific DNA fragments due to base substitutions (transitions or transversions), indels or rearrangements at the recognition sites of a particular restriction endonuclease. Different restriction patterns are created and can be used for evaluation of genetic

For PCR-RFLP, the DNA fragment is amplified by PCR followed by digestion with restriction endonuclease (restriction site or fragment length polymorphism). The restricted fragments are fractionated in the agarose gel (or polyacrylamide gel), stained with ethidium bromide (or silver) and visualized by a UV transilluminator.

This technique has successfully been used for determination of genetic diversity and population differentiation of several marine species in Thai waters including; *P. monodon* (Klinbunga *et al*., 2001), cupped oyster of the genera *Crassostrea* and *Saccostrea* (Klinbunga *et al*., 2003) and abalone, *Haliotis asinina*, *H. ovina* and *H. varia* (Klinbunga *et al*., 2003).

1.2.2 Randomly amplified polymorphic DNA (RAPD)-PCR analysis

RAPD was first independently developed by Welsh and McClelland, 1990 and William *et al*., 1990. It is a PCR-based method but using a single short oligonucleotide primer (typically 10 bp long) of arbitrary sequence and G+C content grater than 50% instead a pair of specific primers in the reaction under low stringency conditions (annealing temperature often 36-40^{o}C) (Figure 1.8). The amplified fragments are those regions of the genome that are flanked by “inward-oriented”

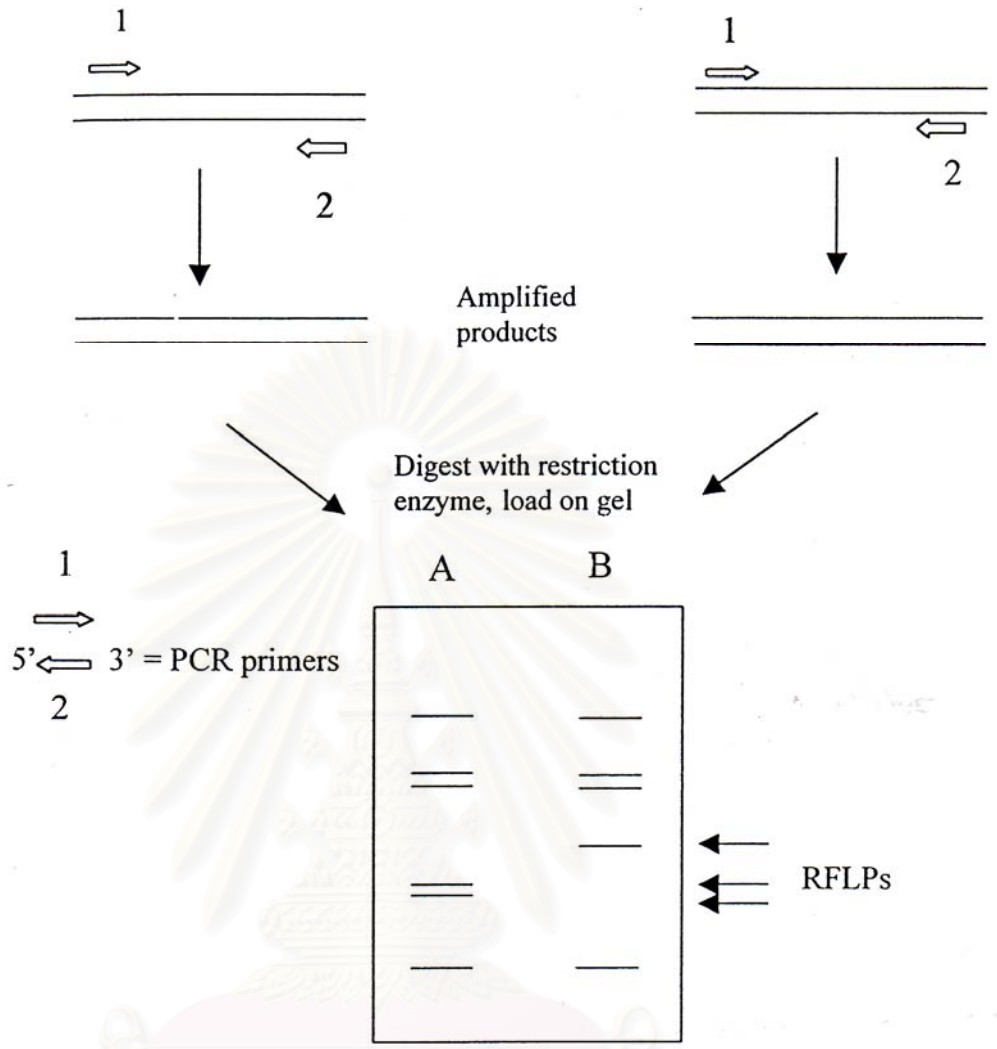

Figure 1.6 Diagram illustrating a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) approach.

Source: Seminar of the conference on the use of molecular markers in crop improvement (1992)

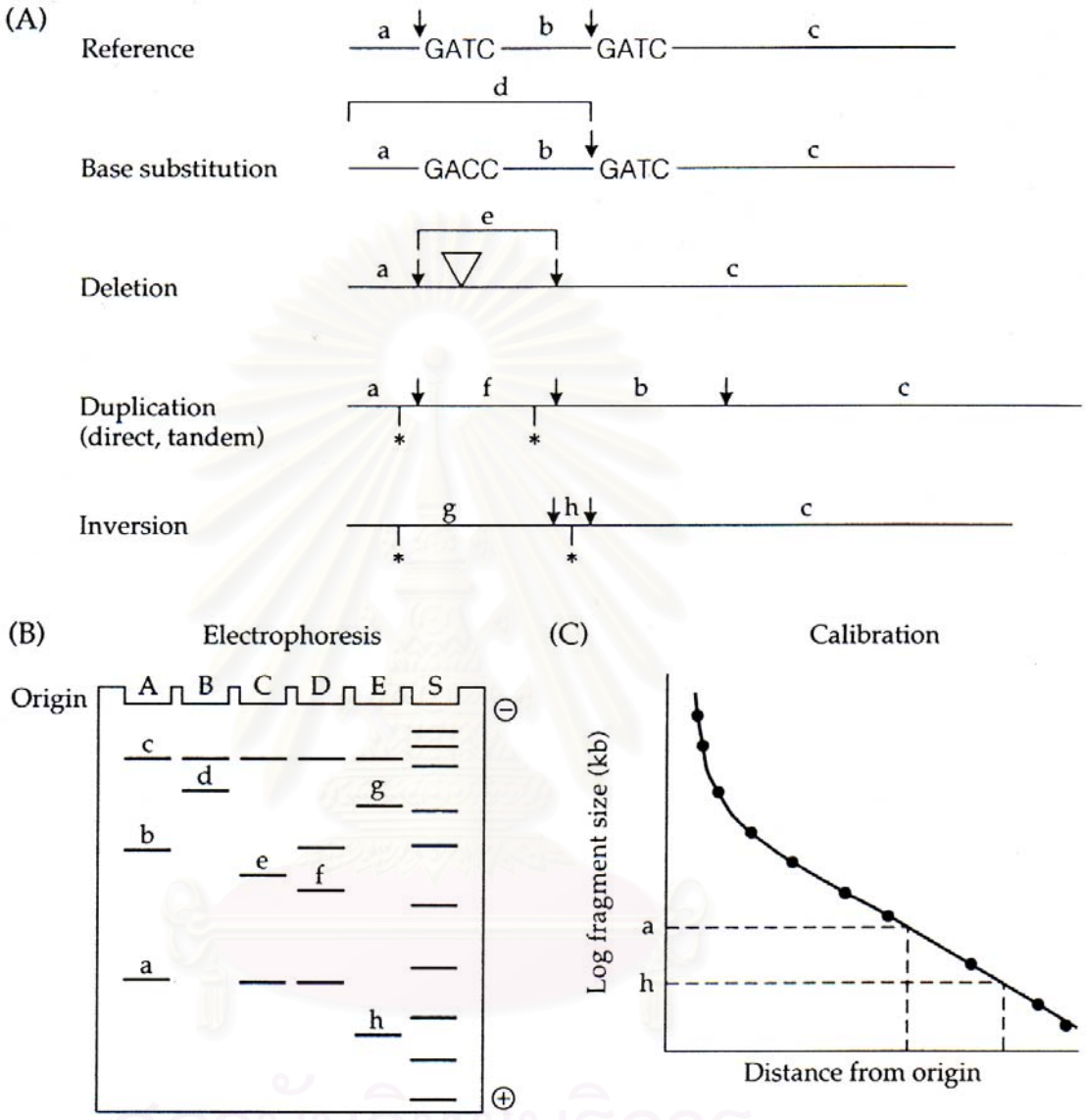

Figure 1.7 The effect of different kinds of sequence change on RFLPs. (A) DNA fragments (a-h) are generated by RE digestion and (B) electrophoretically separated by size. (C) Fragment sizes are determined using a calibration curve based on a sample with fragments of known size run on each gel (lane S = size standard). Vertical arrows indicate cleavage sites and asterisks indicate the boundaries of rearrangements.

Source: Dowling, Moritz, Palmer and Rieseberg (1996).

sequences complementary to the primer and a number of PCR products are generated from random locations within the genome.

Genetic variation and divergence within or between the interested samples of RAPD-PCR are assessed by the presence or absence of the particular amplification products, which can be separated on agarose gels stained with ethidium bromide. Polymorphic alleles may result from mutations at the primer binding sites which prevent its amplification or from indels that change the size of DNA segment (William *et al*., 1990; Lui and Cordes, 2004).

RAPD markers are mostly inherited in a dominant fashion. As a result, information on the parental origin of alleles may be inaccessible. Owing to short length of primer and low stringency of PCR conditions, RAPD may produce some artifact of amplification products therefore careful control of DNA quality and amplification conditions is necessary to ensure reproducible banding patterns (Carlson *et al*. 1991, Scott *et al*., 1993).

Several advantages of RAPD-PCR are reported. First, RAPD analysis is a simple, rapid and inexpensive method for detecting DNA polymorphism at different taxonomic levels. Second, RAPD does not require prior knowledge of the genome under investigation. Third, RAPD is a PCR-based method and, therefore, requires only small quantity of DNA template per reaction. Forth, RAPD-PCR does not require the use of radiolabelled probes for hybridization. Finally, unlimited numbers of RAPD primers can be screened for suitable molecular markers of various applications within a short period of time.

The disadvantage of RAPD-PCR is that more than 90% of polymorphisms segregate as dominant alleles. Moreover, reproducibility of amplification results is quite low. Accordingly, RAPD markers found from the experiments are usually converted to sequence-characterized amplified region (SCAR) markers through cloning and sequencing of the original marker. A pair of primer is designed and used for specific amplification of the target fragment.

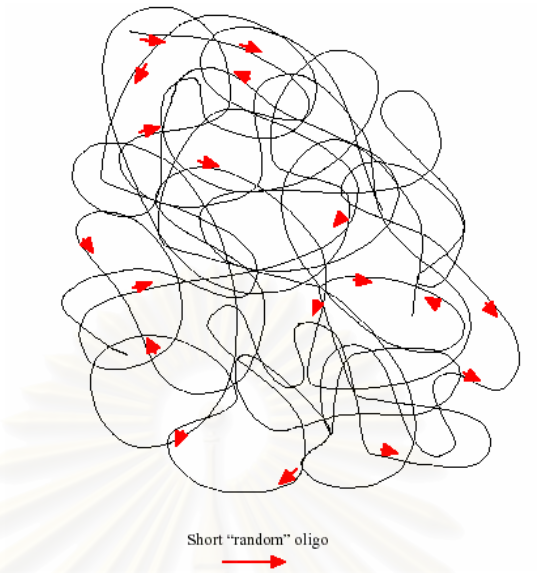

Figure 1.8 Schematic presentation of the RAPD procedure. Genomic DNA (indicated by long strings of lines) is used as template for PCR using an arbitrary short primers of identical sequences (indicated by the arrows annealing to their complementary sites in the genome either perfectly or non-perfectly) under low annealing temperatures. When the two primers bind to sites close enough (often less than 2000 base pairs) on opposite strands of DNA, a PCR product results.

1.2.3 Amplified fragment length polymorphism (AFLP) analysis

Amplified fragment length polymorphism (AFLP) is a technique developed for genetic DNA fingerprinting (Vos *et al.*, 1995). It combines restriction endonuclease digestion and PCR amplification of restriction fragments, and thus possesses the advantages of both RFLP and RAPD. AFLP has higher potentiality than RFLP and higher resolution and sensitivity than RAPD. Initially, genomic DNA is digested with a rare-cut restriction enzyme (usually *Eco*R I) and a frequent-cut restriction enzyme (usually *Mse* I) and ligated with double-stranded DNA adaptors to generate template DNA for amplification and used for the priming sites of PCR amplification.

A. Base substitutions at the primer binding sites

B. Insertion/deletion between two RAPD primers

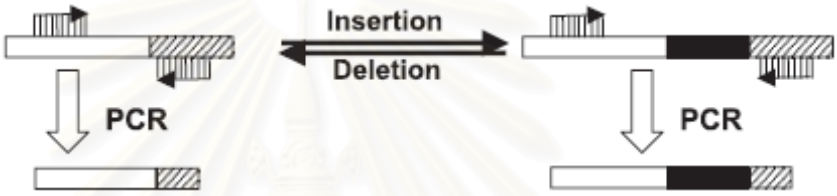

Figure 1.9 Molecular basis of RAPD polymorphism. (A) Base substitutions in the primer binding sites, especially at the 3′ end of the primer binding sites may lead to decrease (as shown) or increase of the number of RAPD bands. (B) Insertion or deletion between two primers may lead to increase or decrease of fragment sizes.

PCR amplification was carried out twice; preselective and selective amplification. The former was carried out by adding a single known base to the 3’ end of the primer complementary to either adaptor. The product from the primary amplification is diluted and further amplified by primers having 3 added nucleotides of the 3’ end. The numbers of amplified fragments are significantly reduced and can be simply analyzed by polyacrylamide gel electrophoresis (Figure 1.10).

The main advantages of AFLP are its reproducibility due to specificity of the PCR primer and high stringency of the amplification reaction. Like RAPD-PCR, AFLP analysis does not require the prior knowledge about genome sequences of species under investigation. The high numbers of potential polymorphic fragments

detected in a single AFLP reaction make this technique ideal for various applications, for example, studying genetic diversity, genotyping, population differentiation, quantitative trait loci (QTL) mapping and construction the genetic linkage maps of species that their genome are not well studied.

Similar to other dominant markers, heterozygosity can not be deduced from AFLP data. This reduces its potential for examination population genetic parameters of artificially propagated stocks in the hatcheries. Conversion of specific AFLP markers into single locus PCR markers (SCAR markers) is necessary when specificity and/or frequencies of a particular marker need to be investigated.

1.2.4 DNA sequencing

Polymorphism at the DNA level can be directly studied by determination of nucleotide sequences of a defined region. DNA sequencing is the most optimal method for several genetic applications particularly phylogenetic studies of organisms. 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. This eliminates the need to establish a genome library and searching of a particular gene in the library.

At present, automated DNA sequencing has been introduced and commonly used (Figure 1.11). DNA sequences can be detected using a fluorescence-based system following labeling with a fluorescence dye. PCR allow the possibility to isolate homologous DNA sequences from any organism of interest with unprecedented speed. This greatly allows wider application of DNA sequencing analysis for population genetic and systematic studies.

1.2.5 Single-stranded conformation polymorphism (SSCP) analysis

Single-stranded conformation polymorphism (SSCP) analysis was originally described by Orita *et al*. (1989). SSCP is one of the effective techniques widely used for the detection of mutations and variation of the DNA (deletions, insertions and single nucleotide polymorphism, SNP). The amplified PCR product (usually less than

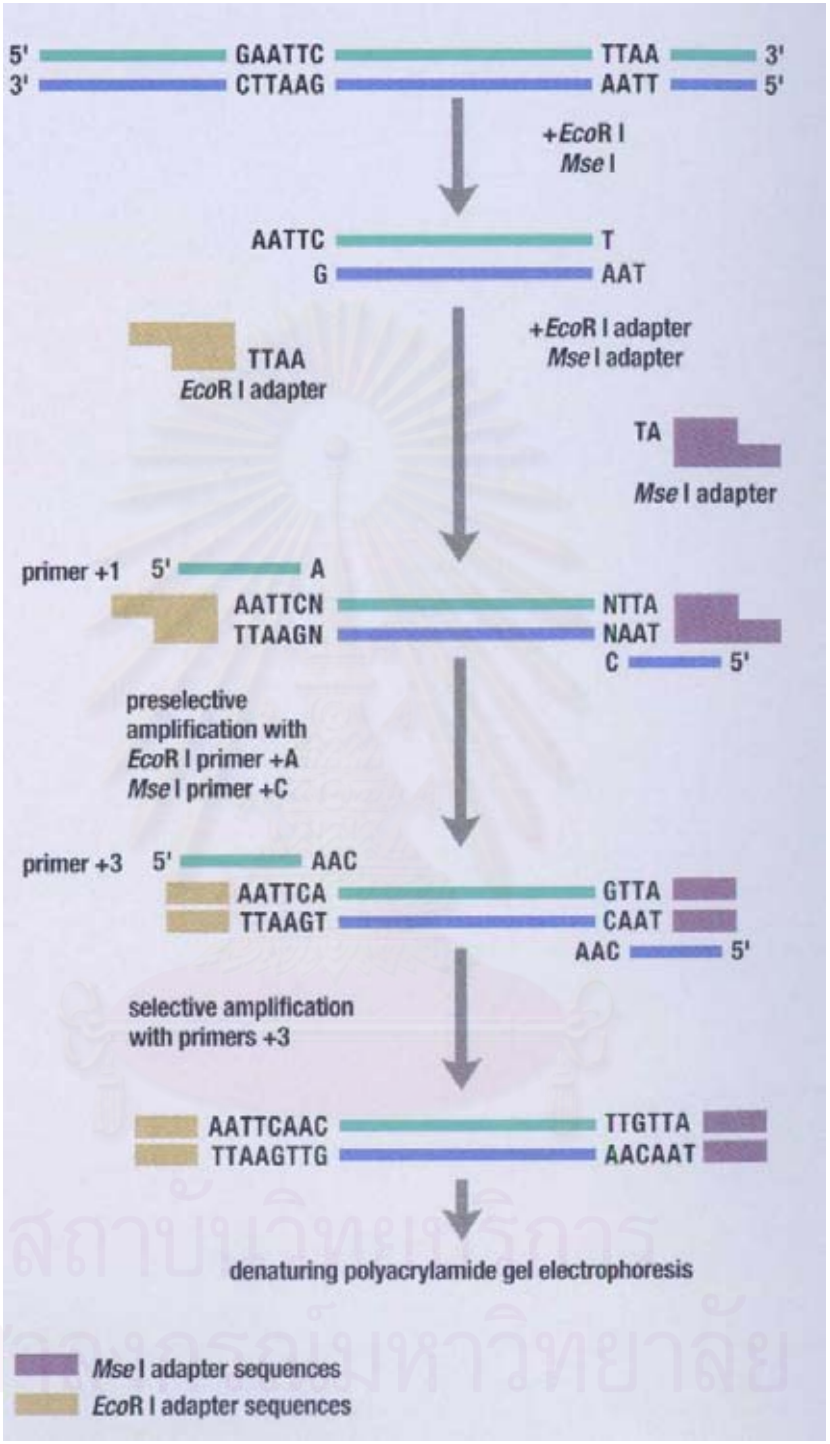

Figure 1.10 A schematic diagram illustrating principles of AFLP analysis (http://www.msu.edu/course/mmg/835/DNAmarkers/aflp.jpg).

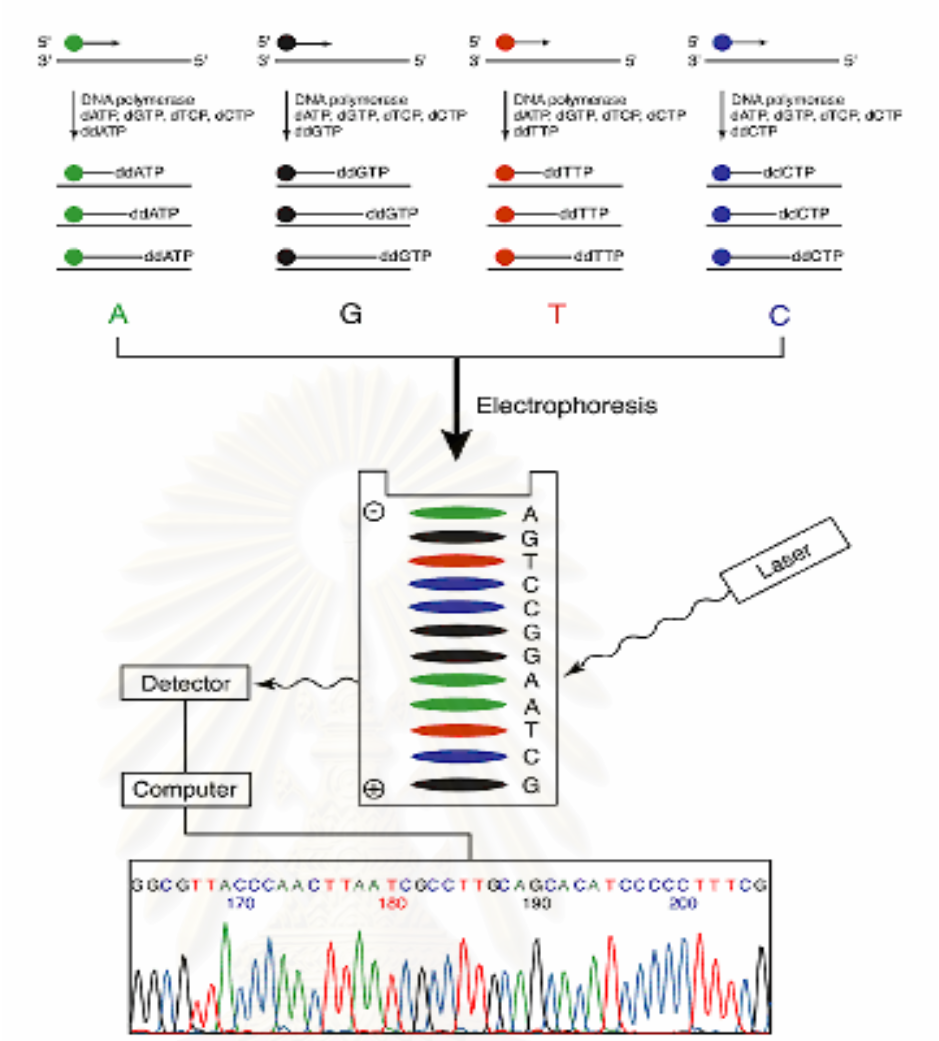

Figure 1.11 Automated DNA sequencing.

300 bp in length) is denatured and loaded into low crosslink non-denaturing polyacrylamide gel (with or without glycerol supplementation). The principle of this technique relies on different mobility due to differential folding of the single stranded DNA (Figure 1.12).

Single-stranded molecules take on secondary and tertiary structures (conformations) due to base pairing between nucleotides within each strand. These conformations depend on the length of the strand, and the location and number of regions of base pairing. They also depend on the primary sequence of the molecule,

such that a nucleotide change at a particular position can alter its conformation. Accordingly, molecules differing in their conformations (e.g. due to a single nucleotide change) can be separated.

The major advantage of SSCP is that many individual PCR products may be genotyped simultaneously. Heteroduplexes can occasionally resolve from homoduplexes 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 (Hayashi, 1996). The other advantage of SSCP is that small PCR amplicons (100-400 bp) are required. This small sizes of PCR products are relative easy to amplify.

1.2.6 Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR is a comparable method of conventional PCR but the first strand cDNA template rather than genomic DNA was used as the template in the amplification reaction (Figure 1.13). It is a direct method for examination of gene expression of known sequence transcripts in the target species. Alternatively, RT-PCR can also be used to identify homologues of interesting genes by using degenerate primers and/or conserved gene-specific primers from the original species and the first strand cDNA of the interesting species as the template. The amplified product is further characterized by cloning and sequencing.

1.2.7 RNA arbitrary prime-PCR (RAP-PCR)

RAP-PCR is a comparable method of conventional RAPD but the first strand cDNA rather than genomic DNA is used as the template in the amplification reaction (Welsh *et al*., 1992). The technique required reverse transcription of the target total RNA (or mRNA) to the first strand cDNA by oligo d(T) or short random nucleotides. The synthesized cDNA is included as the template in the PCR reaction using either the single primer or a combination of random primers. The amplification products are size-fragtionated through agarose or denaturing polyacrylamide gels and detected by either radiolabeled or non-radiolabeled (EtBr or silver staining) detection methods.

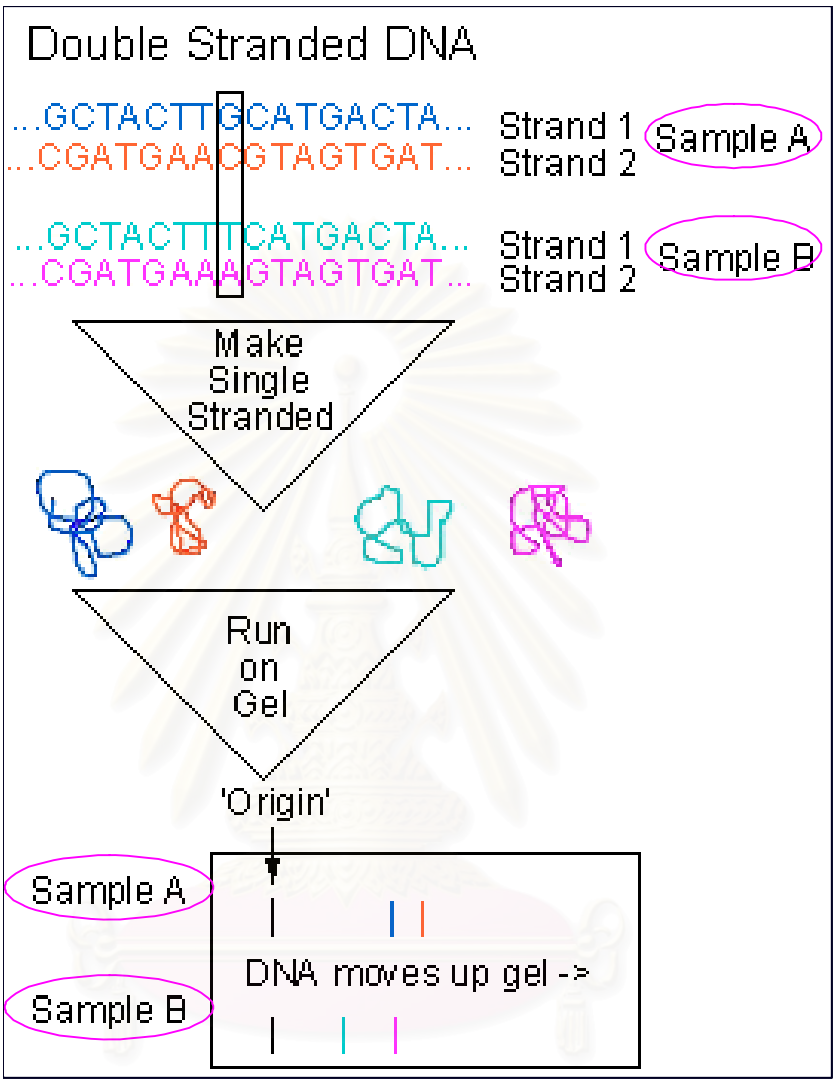

Figure 1.12 A schematic diagram of SSCP analysis (http://www.amonline.net.au/evolutionary_biology/images/sscp.gif).

RAP-PCR bands that are present in one sample and absent in another or bands that exhibit large differences in the intensity across the experimental treatments should represent potentially differentially expressed mRNA transcripts and required further characterization. The fragments can be cloned and sequenced. The expression levels of interesting bands are then examined using specific primers.

1.3 Development of species-specific markers in aquatic species

Klossa-Kilia *et al.* (2002) used PCR-RFLP of mitochondrial 16s rRNA gene segment to authenticate Messolongi (Greece) fish roe. The PCR products from five species (fresh fish and fish roe) was digested with *Bst*N I, *Taq* I and *Hin*f I and electrophoretically analyzed. Species specific restriction patterns clearly discriminated the fish roe of Messolongi, manufactured from the ovaries of *M. cephalus*, from that originating from the other four Mugilidae species coexisting in the same area. No intra-specific variation was detected in any species suggesting that the developed marker is reliable.

Identification of species origin of different processed products of billfish meats was reported. Hsieh *et al.* (2005) distinguished five billfish species *Xiphias gladius, Makaira nigricans, M. indica, Istiophorus platypterus* and *Tetrapturus audax* in raw, frozen and heat-treated meats by digestion of cytochrome b (cyt b, 348 bp) with *Bsa*JI, *Cac*8I and *Hpa*II. Species origins of 10 commercial samples including raw fish fillets, frozen fish meats and fried fish meats were identified. The results also indicated that two commercial samples of declared billfish products were actually not made from the billfish.

Species identification of red snapper in commercial salted products was analyzed by Zhang *et al*. (2006). PCR-RFLP of 12S rRNA (450 bp) could discriminate morphologically similar fishes; *Lutjanus sanguineus, L. erythopterus* from *L. argentimaculatus, L. malabarius, Lethrinus leutjanus* and *Pinjalo pinjalo* by *Hae* III, *Sca* I and *Sna*B I. *L. sanguineus* and *L. erythopterus* were further discriminated by *Mae* II.

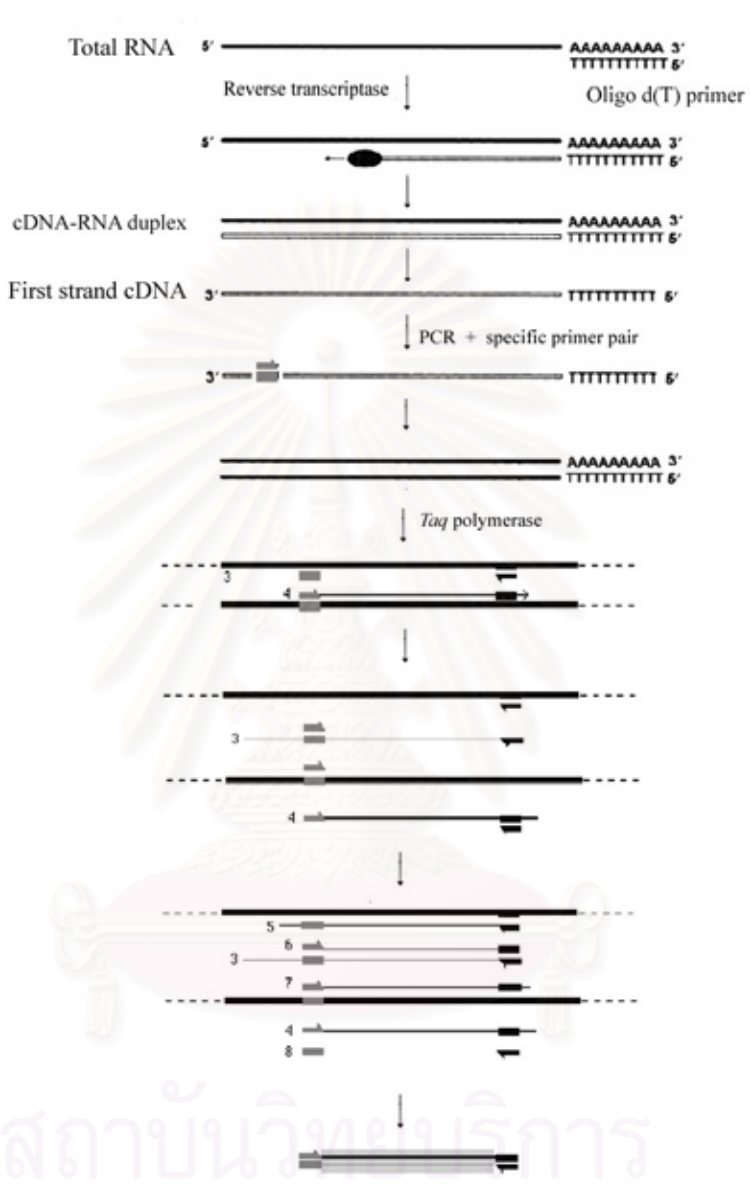

Figure 1.13 Overall concepts of the RT-PCR procedure. During first-strand cDNA synthesis an oligo d(T) primer anneals and extends from sites present within the mRNA. Second strand cDNA synthesis primed by the 18 – 25 base specific primer proceeds during a single round of DNA synthesis catalyzed by *Taq* polymerase. These DNA fragments serve as the template for PCR amplification.

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

Comi *et al.* (2005) developed a molecular approach to differentiate eight species currently used in the production of cod-fish; *Gadus morhua*, *G. macrocephalus*, *G. ogac*, *Molva molva*, *Melanogrammus aeglefinus*, *Brosmi brosme*, *Pollachius virens* and *Theragra calchograma*. The amplfied cyt b (N = 12) was amplified and further analyzed by RFLP, SSCP and DGGE. A combination of *Nla* III and *Rsa* I allowed the differentiation of six out of eight species in this study. Species belonging to the genera *Gadus* and *T. calchograma* gave identical restriction profiles and SSCP patterns. However, DGGE was able to produce different patterns across considered species.

Short segments (123 bp) of cyt b four species of tuna fish (*Thunnus albacares, T. obesus, T. alalunga* and *E. pelamis*) were analyzed by PCR-SSCP to get species-specific patterns of single-stranded DNA. All four species were clearly differentiated. Nevertheless, small differences of SSCP profiles of *T. albacares* and *T. obesus* were observed reflecting the small difference between sequences of cyt b of these two species (Colombo *et al*., 2005).

Thaewnon-ngiw *et al.* (2004) investigated species-diagnostic markers in the introduced apple snail (*Pomacea canaliculata*) and in the four native apple snails *Pila ampullaceal*, *P. angelica*, *P. pesmei*, and *P. polita* in Thailand by PCR-RFLP of COI (710 bp). The results showed that digestion of COI with *Dde* I could differentiated *P. canaliculata* from native *Pila* species, accurately. Twenty-one composite haplotypes showing non-overlapping distributions among species indicating that simple and reliable species identification method were successfully developed.

Klinbunga *et al*. (2003) successful developed species-diagnostic markers of the tropical abalone (*Haliotis asinina*, *H. ovina* and *H. varia*) in Thai waters based on PCR-RFLP of 16S rDNA (*Alu* I, *Bam* HI, *Eco* RI and *Hae* III). Non-overlapping mitotypes were found in *H. asinina* (AAAA and AAAE, *N* = 115), *H. ovina* (ABBB, AAAB and AABB, *N* = 71) and *H. varia* (BABG, BABC, BABD, BABF and AABG, *N* = 23), respectively.

In addition, species-diagnostic markers of 5 oyster species of genera *Crassostrea* and *Saccostrea*; *Crassostrea belcheri* (Sowerby, 1871), *C. iredalei* (Faustino, 1932), *Saccostrea cucullata* (Born, 1778), *S. forskali* (Chemnitz, 1785) and *Striostrea (Parastriostrea) mytiloides* (Lamarck, 1819), were investigated by PCR-RFLP of 16S (*Acs* I, *Alu* I, *Dde* I, *Dra* I, *Rsa* I and *Taq* I) and 18S (*Hin*f I) rDNAs and COI (*Acs* I, *Dde* I and *Mbo* I). A total of 54 composite haplotypes were observed. No overlapping haplotypes were found between different oyster species. Species-diagnostic composite haplotypes were specifically found in each commercially cultured oyster species (*C. belcheri*, *C. iredalei* and *S. cucullata*) (Klinbunga *et al*., 2005).

To date, there have been no publications concerning species-diagnostic markers of indigenously important shrimp species (*P. monodon*, *P. semisulcatus* and *F. merguiensis*) and the introduced species (*L. vannamei*) in Thailand. Due to morphological similarity between *F. merguiensis* and *L. vannamei* at the larval stages and between *P. monodon* and *P. semisulcatus* at all stages of development, reliable molecular markers for identification of species origin of shrimp species need to be developed.

1.4 Population genetic studies of Penaeid shrimps

Molecular phylogeny of penaeid shrimps has been reported based on nucleotide sequences of COI (Baldwin *et al*., 1998), 16S rDNA and COI (Lavery *et al*., 2004) and AFLP (Wang *et al*., 2004). Phylogenetic trees revealed close genetic relationships between *P. monodon* and *P. semisulcatus* (subgenera Penaeus) but distant relationships were observed among economically important shrimps from

different genera (*P. monodon*, *F. merguiensis*, *L. vannamei* and *Marsupenaeus japonicus*).

Baldwin *et al.* (1998) studied molecular phylogeny and biogeography of 13 species of Penaeid shrimps representing all six subgenera using COI (558 bp). No insertions or deletions across taxa were observed and the results showed that this gene section contained 204 variation sites. Genetic diversity within species was low (d = 0 - 3%) while that between species showed high level (d = 8 - 24%). Phylogenetic analysis represented an unambiguous grouping of western Atlantic species with the eastern Pacific species to form a monophyletic group relative to Indo-Pacific forms. Relationships between *P. canaliculatus* and *P. japonicus* and between *P. indicus* and *P. merguiensis* were closely related whereas *P. monodon* and *P. semisulcatus* could not resolve their relationships based on the parsimonious approach.

Larvery *et al*. (2004) reconstructed the phylogeny of *Penaeus* (26 of the extant 28 species including all the Indo-West Pacific, eastern Atlantic and western Atlantic species) using 16S rRNA (474 bp) and COI (414 bp). Results provided the evidence for division of the genus into two true natural groups; *Melicertus* plus *Marsupenaeus* and another group comprising of *Penaeus*, *Fenneropenaeus*, *Farfantepenaeus* and *Litopenaeus*. Previous molecular study concluded that subgenera *Farfantepenaeus* and *Litopenaeus* were paraphyletic (Baldwin *et al*., 1998 and Gusmão *et al*., 2000) but the results from this study suggested that both of them were monophyletic groups Moreover, this study supported an Indo-West Pacific origin of the genus, with a single relatively recent colonization of the Western Hemisphere, and subsequent subdivision into two clades prior to the emergence of the Panamanian isthmus.

Species identification and phylogenetic analysis of six penaeus shrimps; *P. monodon*, *P. chinensis*, *P. merguiensis*, *P. latisulcatus*, *P. canaliculatus* and *P. japonicus*, were explored by Wang *et al.* (2004) using AFLP technology. A total of 443 bands (size range 80-550 bp) were generated from 26 individuals from six species using three sets of selective primers. Eight bands (1.8%) were found in all species, with three of them (0.7%) scored in all individuals (i.e. monomorphic) and the other five polymorphic. Within a single species, 24.6% (*P. canaliculatus*) to 60.8% (*P. japonicus*) of the fragments were polymorphic. Species-specific AFLP markers were identified and would be converting to sequence characterized amplified region

(SCAR) markers that usefulness in genetic identification of larvae and post-larvae. Average genetic distances among individuals of the same species varied from 0.0023 in *P. chinensis* to 0.0068 in *P. japonicus*, while the average distances between species varied from 0.0207 to 0.0324. The phylogenetic tree indicated that individuals from each species cluster together and six investigated species were segregated into two major clades. While *P. monodon*, *P. chinensis* and *P. merguiensis* were classified in the first clade with the latter two species more closely related, *P. latisulcatus, P. canaliculatus* and *P. japonicus* were classified in the other clade with the former two species more closely related. Results were consistent with the previous study by Larvery et al. (2004) based on mitochondrial DNA analysis (16S rRNA and COI) on 26 of the extant 28 *Penaeus* species.

Population genetic structure of the kuruma prawn (*P. japonicus*) in East Asia was elucidated by sequence analyses on the complete mtDNA control region (992 bp). Five populations (N = 95) originating from the Japan Sea (JS), the north and south of the East China Sea (NECS and SECS), the Taiwan Strait (TS), and the north of the South China Sea (NSCS) were collected. Two hundred and ninety two variable sites without any insertions and deletions were observed. Nucleotide diversity in the total populations was 2.51 ± 0.07%, and the variations within populations ranged from 2.61 ± 0.93% (SECS) to 2.29 ± 0.16% (JS). The F_{ST} values across all populations showed a significant amount of genetic variation between five populations (F_{ST} = 0.0434, $p<0.01$) as the same as those between the JS and the remaining populations, between the NECS and NSCS populations, and between the SECS and NSCS populations. The UPGMA tree and analysis of molecular variance (AMOVA) indicated that three distinct genetic populations were existed in East Asia; one was in the JS; another was in the NECS; and the third was distributed in SECS, TS and NSCS (Tzeng *et al*., 2004).

Hualkasin *et al.* (2003) studied molecular phylogenies of white shrimp species in Thailand using variation observed a 558 bp COI gene segment. Three morphologically similar species; *P. merguiensis*, *P. silasi* and *P. indicus*, were clearly differentiated which very close relationship was observed between *P. merguiensis* and *P. silasi* (d = 8.61%). A neighbor-joining tree separated specimens into four clades; A, B, C and D. *P. silasi* and *P. indicus* were monophyletic wherein *P. merguiensis*

was paraphyletic. The clade A consisted of *P. merguiensis* from the Gulf of Thailand and Taiwan (Pacific Ocean) whereas the clade B consisted of the Andaman Sea sample. The average sequence divergence between these groups was 5%. Results point toward the possibility of *P. merguiensis* being a complex of two cryptic species or a single species with strong phylogeographic subdivision.

Wanna *et al.* (2005) examined sequence variation of the ITS1 region (range 499-772 bp in length) in four species of penaeid shrimps in Thailand; *P. merguiensis*, *P. silasi*, *P. monodon* and *P. semisulcatus*, and in two populations of *P. merguiensis*; the Gulf of Thailand and the Andaman Sea. They found that ITS1 variation was informative in estimating phylogenies. Four species of Penaeid shrimps could be differentiated and *P. merguiensis* species were divided into two clusters; A (Gulf of Thailand) and B (Andaman Sea) with a 2.03% divergence. The divergence within cluster A and B was 0.87% and 0.6%, respectively. Pairwise nucleotide sequence divergence in the ITS1 region ranged from 7.53% between *P. merguiensis* and *P. silasi* to 30.16% between *P. silasi* and *P. semisulcatus*. The divergences between *Litopenaeus* and *P. merguiensis*, *P. silasi* and *P. indicus*) range from 7.53% to 17.35% indicating that these shrimps were closely related species which *P. merguiensis* and *P. silasi* were more closely related to each other than they were to *P. indicus*. Moreover, *P. monodon* displayed the lowest degree of divergence from *P. semisulcatus* with a value 17.29%.

Xu *et al.* (2001) studied genetic diversity of wild and cultured black tiger shrimp (*P. monodon*) in the Philippines using six microsatellites. All six microsatellites were polymorphic (100%) and a total of 184 different alleles were found over all loci with allele size ranging from 159-400 bp. The observed heterozygosity (H_o) was high (0.47 to 1.00). F_{ST} showed significant genetic differentiation among overall populations at all six loci. The Negros Occidental-W population was significantly different from the other three populations (Quezon, Capiz and Palawan) based on the pairwise F_{ST} values, allelic and genotypic frequencies. No pairwise differentiation among Quezon, Capiz and Palawan was observed. The average number of alleles per locus in wild populations was significantly higher than that observed in the cultured populations ($P < 0.05$) while the average frequency of the most common allele in both wild and culture populations

was not different. Unfortunately, two cultured populations showed less genetic diversity and were significantly different from the four wild populations genetically.

Tong *et al.* (2002) developed polymorphic expressed sequence tags (EST) markers in *P. monodon* for genome mapping and other genetic studies of these species. Forty seven pairs of primers were designed based on ESTs from a *P. monodon* cephalothorax cDNA library. Thirty four of the primer pairs; representing 12 distinct genes and 22 unknown gene products, were successfully amplified from genomic DNA of *P. monodon*. PCR products from 6 primer pairs were larger than the expected sizes due to the presence of introns. Ten polymorphic ESTs markers were observed based on SSCP analysis. Mendelian inheritance of the EST-derived markers was examined in two international reference mapping families of *P. monodon*. Statistic tests on genotype distribution in the progeny confirmed Mendelian inheritance (χ^2 <1.2, P > 0.05). Some of the markers were successfully mapped in a genetic linkage map. In addition, some ESTs were successfully amplified in *P. chinensis*, *P. japonicus* and *P. vannamei* indicated that EST markers could be applied in genetic analysis of closely related species and facilitated further efforts towards construction of a syntenic gene map in *Penaeus*.

MtDNA-RFLP analysis was used to determine intraspecific genetic diversity of *P. monodon*. A total of 212 wild *P. monodon* individuals collected from ten sites (Lamu in Kenya, Medan, North and South Java in Indonesia, Satun, Surat and Trat in Thailand, Dungun and Kedah, Malaysia and Lingayen in the Philippines) were analysed with eleven restriction endonucleases (*Ava* II, *Bam*H I, *Cla* I, *Dra* I, *Eco*R V, *Hind* III, *Pvu* II, *Sac* I, *Sca* I and *Xba* I). Sixty-three mtDNA composite haplotypes were identified and 28 of these were found in Thai samples. These could be placed into one or other of two clonal lineages, A and B. The most easterly sites (Lingayen, Philippines) were fixed for cluster A haplotype and the most westerly site (Lamu, Kenya) was fixed for a B haplotype. At the other sites, both clusters were present with the A haplotypes generally more common in the South China and Java Sea than from samples in the Andaman Sea (Figure 1.14). Haplotype diversity of *P. monodon* mtDNA was 0.7689 ± 0.00186. The average nucleotide diversity within and between populations was 1.7276 ± 0.0007% and 2.7230 ± 0.0003% respectively. Significant population differentiation was observed with a major discontinuity between the

Andaman Sea, Java and South China Sea and West African samples of *P. monodon* (Klinbunga 1996; Klinbunga *et al*., 1999)

Benzie *et al.* (2002) surveyed mtDNA variation in 5 geographic samples of Southeast African, 5 of Australian and 3 of Southeast Asian *P. monodon* using RFLP. The results indicated that the Indo-West Pacific region is the site of accumulation of genetic diversity rather than the site of origin of genetic diversity. The dominant haplotype was different in the Australian and Southeast Asian samples. Genetic diversity was greatest in Indonesia, less in the Philippines and Australia and markedly less in the Southeast African and West Australia. The high diversity in the Southeast Asian samples resulted from the occurrence of a set of haplotypes found only in the Southeast Asian samples derived from the Southeast African haplotypes. These genetic variants were evolved in the Indian Ocean and subsequently migrated into the Indo-West Pacific region. Low genetic variation in geographically marginal samples in Southeast African and West Australia is possibly resulted from the consequence of bottleneck effects but mismatch haplotype distributions suggest that large population sizes have been maintained in Indonesian samples for long periods.

Genetic diversity of *P. monodon* in Thailand based on PCR-RFLP of 16S rDNA and COI-II was analyzed using specimens from Satun (N = 30), Trang (N = 30), and Phangnga (N = 31) in the Andaman Sea; and Chumphon (N = 39) and Trad (N = 24) in the Gulf of Thailand (Klinbunga *et al*., 2001). Digestion of *P. monodon* 16S rDNA (560 bp in length) with *Mbo*I and COI-COII (1700 bp) with *Alu*I, *Mbo*I, *Taq*I, *Hin*fI and *Dde*I produced 48 restriction fragments with an average of 8.0 fragments per enzyme. Linkage disequilibrium analysis indicated that haplotypes from the 16S rDNA and an intergenic COI-COII were associated nonrandomly (P < .0001).

In total, 37 composite haplotypes were identified among the 5 samples of Thai *P. monodon*. Of these, 22 composite haplotypes were carried by single individuals. Only 2 composite haplotypes (I, ABBBBA; and VII, BAAAAB) were commonly found, in 16.23% and 28.57% of overall specimens, respectively. These haplotypes were not population-specific but found in all geographic samples. The UPGMA cluster analysis based on sequence divergence estimated among composite haplotypes

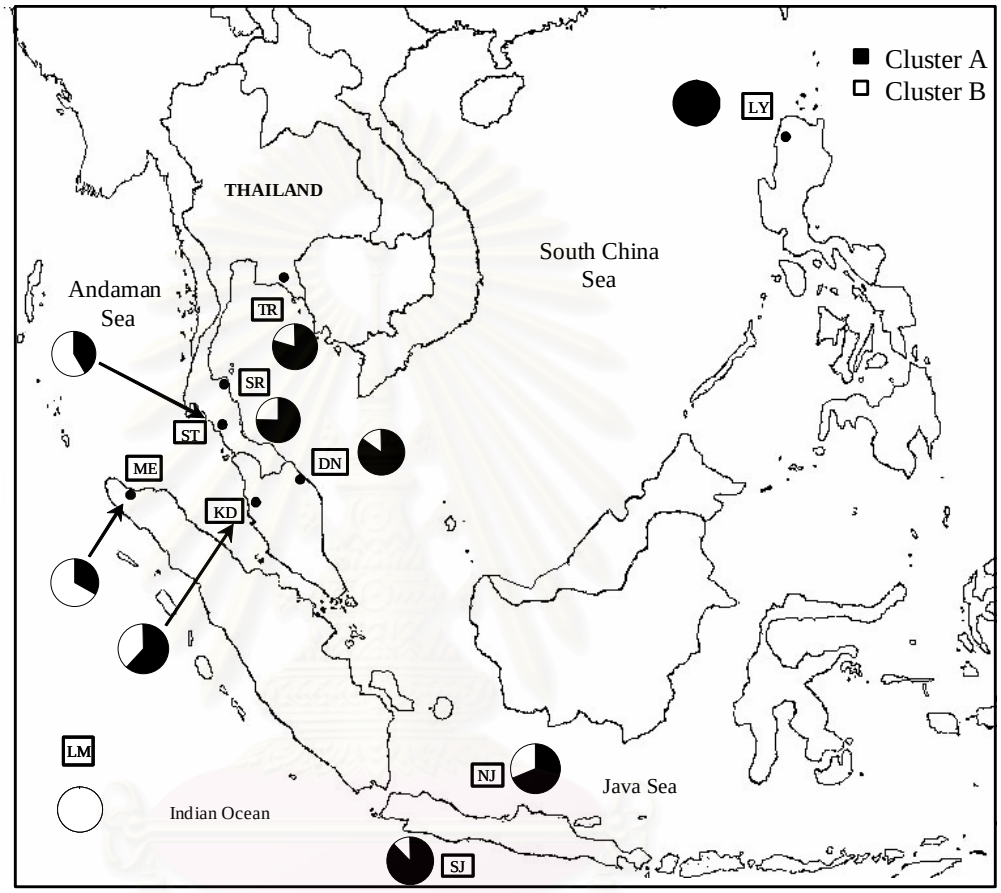

Figure 1.14 Geographic distribution of mtDNA phylogenetic clusters A and B among fourteen geographic samples of *P. monodon* analyzed by 11 polymorphic restriction enzymes.

LY = Lingayen, Philippines; DN = Dungun, KD = Kedah, Malaysia; SR = Surat, ST = Satun, TR = Trat, Thailand; ME = Medan, NJ = North Java, SJ = South Java, Indonesia; LM = Lamu, Kenya. Noted that Kenya is located in Africa.

indicated 2 major phylogenetic clusters of mtDNA haplotypes (clusters I and II) joined in the dendrogram with a genetic distance of 3.028%.

The distributions of these 2 clusters overlapped in all geographic samples with different proportions. The highest frequency of cluster I (identical to cluster B in the previous study using specimens covering the south-East region) was observed in Satun (0.600) followed by Chumphon (0.564), Trang (0.500), Phangnga (0.433), and Trad (0.375). The distribution of cluster II genotypes in a particular sample was reversed. Considering digestion patterns obtained from restriction enzymes in their study, patterns from digestion of an intergenic COI-COII with *Alu*I and *Taq*I could represent the frequency of each phylogenetic cluster accurately. This simplification opens the possibility of genetic-based stock enhancement (restocking) programs of *P. monodon* in Thailand without significant disturbance of its local gene pools.

RAPD analysis was used to examine genetic variation in *P. monodon* in Thai waters (Chumphon and Trad from the Gulf of Thailand and Satun, Trang and Phangnga from the Andaman Sea; Klinbunga et al., 2001). Results also indicated the existence of differentiation between *P. monodon* from the Andaman Sea and Gulf of Thailand ($P < 0.0001$). Surprisingly, paired comparisons of distances between Chumphon and each of the Andaman Sea samples (d_{RAPD} = -0.002 to 0.003) indicated closer genetic relationships than between Chumphon and Trad (d_{RAPD} = 0.034). Genetic distances between samples within the Andaman Sea samples were -0.001 to 0.005. Overall comparisons of RAPD genotype frequencies revealed significant geographic heterogeneity among Thai *P. monodon* ($P < .0001$), and between coastal regions ($P < .0001$). Not all RAPD primers used in Klinbunga et al. (2001) indicated geographic heterogeneity for all possible comparisons between Chumphon and each of the Andaman Sea samples ($P > 0.05$), but all primers revealed highly significant heterogeneity between Chumphon and Trad ($P < .0001$). This indicated that the Chumphon *P. monodon* showed genetically closer relationship to each of the Andaman populations than Trad located in the same coastal area.

Microsatellites in Thai *P. monodon* were also characterized (Tassanakajon *et al*., 1998a). Using these markers, high levels of genetic diversity were observed in five different geographic samples of *P. monodon* in Thailand (as also studied by

mtDNA polymorphism and RAPD analysis). The average observed heterozygosity in *P. monodon* was 0.78. A number of microsatellite alleles found in the Andaman sample were not available in Trat, but some of those existed in Chumphon. The failure to detect significant allele distribution frequencies between Chumphon and each of the west coast samples might have resulted from mixing of the gene pools of different *P. monodon* stocks as a consequence of extensive transplantation of *P. monodon* in Thailand.

Although genetic diversity of *P. monodon* in Thai waters has been studied by several approached, data from single copy nuclear (scn) DNA and those from a DNA sequencing approach have not been reported. SSCP analysis of AFLP-derived markers and DNA sequencing of COI was then carried out in this thesis. The information obtained is important for genotyping of artificially propagated *P. monodon* and genetic-based stock enhancement programs of natural *P. monodon* stocks in Thailand.

1.5 Sex-determination/differentiation markers

Sex determination is problematic in researches of many species. This problem is usually arisen when dealing with embryonic or juvenile forms of interesting species. One effective solution is to exploit DNA markers to diagnose sex of each individual. Such markers are present in the genome whenever sex determination is genetically controlled. In many organisms sexual differentiation is governed by chromosomal sex determination, where the sex determination genes are carried on a specialized pair of sex chromosomes. The two main forms are male heterogamy, where the male has XY chromosomes and the female is XX, and female heterogamy, where the female is WZ and the male is ZZ. The Y or W chromosomes are, thus, unique to one sex, so their presence or absence in a sample of genomic DNA is indicative of sex.

In mammals, sex determining region-Y chromosome (SRY) has been discovered for more than a decade. The gene is structurally conserved and Y-linked across the class. Therefore, identification of sexes in mammals at the DNA level is well established (O'Neill and O'Neill, 1999). Nevertheless, the homologue of mammalian SRY has not been identified in non-mammalian species. Although sex-

specific markers have also been described in several non-mammalian species, they are not usually conserved. As a result, sex specific-marker isolated from one species may not exist outside the genus (Griffiths and Tiwari, 1993).

Sex determination may not be controlled by sex chromosomes but is controlled autosomally. Some species of fish do not have sex chromosome as a result sex in these species is determined by male or female genes located on the autosomal chromosomes.

An understanding of sexual biology of any sexual-reproducing species is important for designing breeding programmes in that species. However, studies of sex determining mechanisms in insects and crustaceans are not well advanced. Previous researches has shown that most of the isopod species display chromosomal sex determination in both XX/XY and ZW/ZZ systems but heteromorphism of the sex chromosomes were observed in very few cases (Barzotti *et al*., 2000).

Karyological studies in the giant freshwater prawn (*Macrobrachium rosenbergii*) were conducted. The chromosome number of the antennal gland in males reveal a diploid number of 2n = 118. This was further confirmed by the haploid chromosome number (n = 59) from testes. While most of investigated females exhibit identical number of chromosome, a certain number of cells having 117 and 111 chromosomes were also observed. Therefore, it was not possible to conclude the chromosome number of female *M. rosenbergii* unambiguously. Moreover, sex chromosomes in *M. rosenbergii* could not be cytological identified (Justo *et al*., 1991).

Maleacha *et al*. (1992) examined sex-ratio and sex determination in progeny of crosses between masculinised genotypic females and normal females of the giant freshwater prawn (*M. rosenbergii*). Cumulative sex-ratios in the progeny support a hypothesis that sex of prawns is differentiated with female heterogamous (ZW) and male homogamous (ZZ). Nevertheless, variation of sex-ratios among different crosses implied that sex determination in *M. rosenbergii* is more complex than the simple ZW/ZZ system.

In *P. monodon*, females exhibit greater growth rate than do males at all stages of development. Nevertheless, a lack of obvious heteromorphic sex chromosomes in this species causing limited knowledge on sex chromosome (XY, ZW or the other system) and their segregation patterns. This prohibits the possibility to elevate culture efficiency of *P. monodon* through monosex farming.

Sex determination in the crustacean has been reviewed by Legrand *et al*. (1987) who note that the genetic basis for sex determination had been studied in only a few species and none of which were decapods. Korpelainen (1990) has reviewed the strong influence of environmental factors (including temperature, food supply and social environment) on sex determination in some crustacean groups. Neither sex chromosome in penaeids, nor any environmental sex determination has been observed.

Preechaphol (2004) analyzed pooled DNA of small orange claw (SOC, *N* = 10) and blue-claw (BC, *N* = 5) males and females (*N* = 10) of the giant freshwater prawn (*Macrobrachium rosenbergii*) using 64 AFLP primer combinations and found 90 and 42 AFLP markers in male and female *M. rosenbergii*, respectively. Additional sample sets of SOC (*N* = 5), orange claw (OC, *N* = 15) and BC (*N* = 10) males and females (*N* = 20) of *M. rosenbergii* were reanalyzed by 46 informative primers previously screened. In total, 5 candidate male-specific and 4 candidate female-specific AFLP fragments were cloned and characterized. Nevertheless, the developed SCAR markers did not reveal sex-specificity in *M. rosenbergii*.

In *P. japonicus*, sex of progeny from the mapping family (*N* = 54) was tightly mapped into the linkage group 28 of the female map (LOD = 5.0). However, specificity of the AFLP fragments has not been tested for the direct application as genomic sex-specific SCAR markers in this species. Although sex chromosomes or environmental sex determination have not been reported in *Penaeus* shrimps, the ability to identify only sex-linked markers on the female but not the male map suggested the possibility of female heterogamy in this species (Li *et al*., 2003).

Gonad development characteristics and sex ratio of the triploid shrimp (*Fenneropenaeus chinensis*) were recently reported. The development of gonad in

triploid *F. chinensis* is impaired especially in females. Interestingly, triploidy affected the sex ratio in *F. chinensis* (approximately 4 : 1 towards females) implying that sex chromosomal systems of *Penaeus* shrimps may be more complicated than simple X/Y or Z/W systems (Li *et al*., 2003).

Poonlaphdecha (2004) carried out genomic DNA subtraction between male and female *P. monodon* using the PERT and RDA approaches. A total of 13 (9 and 4 clones from male and female subtraction) and 8 (4 clones from each subtraction) were obtained. SCAR markers developed from a subtractive male (PERT) and 2 subtractive female (RDA) clones did not reveal the sex-specific amplification product in *P. monodon*.

Thumrungtanakit (2004) studied genomic sex-specific markers of male and female *P. monodon*. Five female- and one male-specific AFLP fragments identified from screening of 256 primer combinations against 6 (PmMB1-2, PmMJ1, PmFB1-2 and PmFJ1) or 10 (PmMB1-2, PmMJ1-3, PmFB1-2 and PmFJ1-3) bulked genomic DNA of *P. monodon* were cloned and sequenced. Comparing of DNA sequences derived from candidate female-specific AFLP fragments with data in the GenBank indicated that they were newly unidentified sequences ($P > 10^{-4}$). Four of six SCAR markers (FE10/9P1, FE10/10P1, FE10/10P2 and FE14/16P1) yielded the expected PCR product in both male and female *P. monodon* suggesting the loss of the original sex-specificity of AFLP fragments. Results from SSCP indicated that all SCAR markers except FE10/9P1 were polymorphic but not sex-linked.

Theoretically, the lack of genomic sex determination markers in *P. monodon* may have resulted from weak correlation between the genotypic sex and phenotypic sex due to autosomal modifier genes or genetic diversity between investigated individuals used for screening of markers is greater than the optimal level (Griffiths and Orr, 1999). Alternatively, the lack of sex chromosomes in *P. monodon* and other penaeid shrimps implied that sex chromosomes may not be present or they are not well differentiated in the genome of penaeid shrimps. Therefore, development of genomic sex determination markers in *P. monodon* may not be possible.

Sex differentiation mechanisms (to males and females) and genes involving oocyte maturation in penaeid shrimps were still not known. Isolation and

characterization of genes specifically/differentially expressed in ovaries or testes of *P. monodon* is therefore important and can be used to understanding differentiation of sexes and oocyte maturation process in *P. monodon*.

An isopod crustacean, *Asellus aquaticus*, consists of 8 homomorphic chromosomes in both sexes but a heteromorphic sex chromosome is present in one-quarter of the males in natural populations. The sex chromosomes in this species cannot be differentiated by conventional staining techniques (G- or R-banding). Genomic *in situ* hybridization cannot reveal any sex chromosome differentiation between homomorphic males and females whereas males exhibiting heteromorphic chromosomes showed differentially labeled regions with male-derived DNA probe (Barzotti *et al*., 2000).

Gender-specific gene expression has been recently reported in a mosquito-borne filarial nematode (*Brugia malayi*) isolated by differential display (DD) PCR and *in silico* subtraction of EST cluster database and further confirmed by RT-PCR. Six of twelve (27%) and seven of fifteen (47%) initially identified EST revealed gender-specific expression in *B. malayi* (Michalski and Weil, 1999).

In the silkworm (*Bombyx mori*), sex-specific mRNA isoforms were found in the double sex (*dsx*) gene, where the male-specific cDNA lacked the sequence between 713 and 961 nucleotides of the female specific cDNA (Ohbayashi *et al*., 2001).

Boag *et al.* (2000) successfully isolated and characterized sex-specific gene expression from the nodule worm; *Oesophagostomum dentatum* using RAP-PCR. A total of 31 bands showing differential expression between sexes were cloned and sequenced. Northern blot analysis indicated that ten ESTs were exclusively expressed in males (adults and fourth-stage larvae) while two ESTs were expressed solely in females. Three ESTs were expressed in both sexes, but at higher levels in females, and five ESTs could not be detected by Northern blotting analysis suggesting that they were rare transcripts. Sequence analysis revealed that two male-specific and two female-specific ESTs were significantly matched with a protein containing EGF-like cysteine motif and a serine/threonine phosphatase and to vitellogenin-5 and endonuclease III deduced from *C. elegans* sequences. Another two male-specific

ESTs significantly matched with non-nematode sequences. The remaining ESTs had no similarity to sequences in the GenBank.

1.6 Objectives of the thesis

The objectives of this thesis are development of molecular markers for identifying species origin of five penaeid species (*Penaeus monodon, P. semisulcatus, Feneropenaeus merguiensis, Litopenaeus vannamei* and *Marsupenaeus japonicus*) using PCR-RFLP and SSCP analyses, identification of additional polymorphic markers for population genetic studies of *P. monodon* in Thai waters using AFLP-derived markers and DNA sequencing of COI and isolation and characterization of sex-specific/differential expression markers in *P. monodon* using RAP-PCR.

CHAPTER II

MATERIALS AND METHODS

2.1 General Materials and Methods

The experimental procedures of this thesis were divided to 3 major parts including

- Identification of species origin of 5 penaeid shrimps (*P. monodon*, *P. semisulcatus*, *F. merguiensis*, *L. vannamei* and *M. japonicus*) by PCR-RFLP and SSCP analysis of 16S rDNA.

- Population genetic studies of *P. monodon* in Thailand by AFLP-derived SCAR markers and COI polymorphism

- Isolation and characterization of genomic sex determination markers using RAPD-PCR and sex-specific/differential expression markers of *P. monodon* using RAP-PCR analyses.

2.2 Identification of species origins of 5 penaeid shrimps (*P. monodon, P. semisulcatus, F. merguiensis, L. vannamei* and *M. japonicus*) by PCR-RFLP and SSCP analyses of 16S rDNA

2.2.1 Sampling

Broodstock-sized penaeid shrimps including the giant tiger shrimp; *P. monodon* (*N* = 86), the green tiger shrimp; *P. semisulcatus* (*N* = 15), the banana shrimp; *F. merguiensis* (*N* = 38), the introduced white shrimp; *L. vannamei* (*N* = 30) and the kuruma shrimp; *M. japonicus* (*N* = 16) were collected (Figure 2.1 and Table 2.1). Specimens were transported back to the laboratory at the Center of Excellence for Marine Biotechnology, Faculty of Science, Chulalongkorn University and kept at -30^{o}C until required.

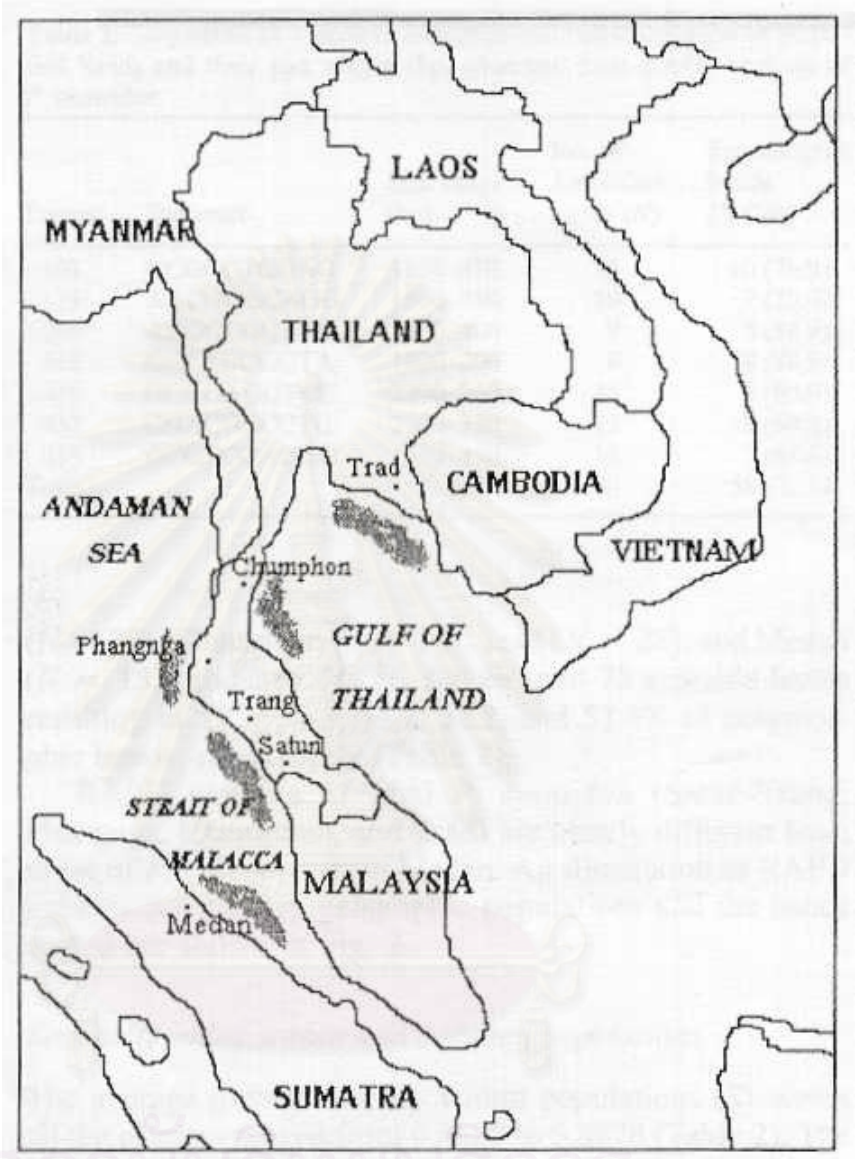

Figure 2.1 Map of Thailand indicating sample collection sites of *P. monodon* used in this study. Detailed information and abbreviations of sample sites are shown by Table 2.1.

Table 2.1 Sample collection sites and sample sizes of penaeid shrimps used in this study

Species	Sample location	Sample size[a]
P. monodon	Chumphon (GOT)	15 (4)
	Trat (GOT)	15 (4)
	Satun (west of PT)	15 (4)
	Trang (west of PT)	15 (4)
	Phangnga (west of PT)	15 (4)
	Ranong (west of PT)	11 (9)
P. semisulcatus	Chumphon (GOT)	11 (11)
	Phuket (west of PT)[b]	4 (4)
F. merguiensis	Samyan Market	7 (7)
	Chonburi (GOT)	17 (7)
	Indonesia (west of PT)	14 (0)
L. vannamei	Mexico*	6 (2)
	Ratchaburi (central Thailand)*	12 (2)
	Rangsit (central Thailand)*	12 (10)
M. japonicus	Japan*	16 (7)

[a]Sample sizes used for SSCP analysis of 16S rDNA (16S $rDNA_{312}$). The numbers in parentheses were individuals analyzed by universal primers of 16S rDNA (16S $rDNA_{560}$).

[b]unknown origin

*cultivated stocks

GOT = Gulf of Thailand, PT = peninsular Thailand

2.2.2 Genomic DNA extraction

Genomic DNA was extracted from a piece of a pleopod of each shrimp using a phenol-chloroform-proteinase K method (Klinbunga *et al*., 1996). The tissue was placed in a prechilled microcentrifuge tube containing 500 µl of extraction buffer (100 mM Tris-HCl, pH 9.0, 250 mM NaCl, 100 mM EDTA, pH 8.0) and briefly homogenized with a micro pestle. SDS (10%) and RNase A (10 mg/ml) solution 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 following by an addition of a proteinase K solution (10 mg/ml) to a final concentration of 200 µg/ml. The mixture was further incubated at 55^{o}C for 3-4 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 and the upper aqueous phase was transferred to a new sterile microcentrifuge tube. This extraction process was then repeated once with phenol:chloroform:isoamylalcohol (25:24:1) and twice with chloroform:isoamylalcohol (24:1). The resulting upper phase was transferred to a new sterile microcentrifuge tube. One-tenth volume of 3 M sodium acetate (pH 5.2) was added. DNA was precipitated by an addition of two volume of ice-cold absolute ethanol and incubated at -80^{o}C for 30 minutes. The precipitated DNA was recovered by centrifugation at 12,000 rpm for 15 minutes at room temperature and washed twice with 1 ml of 70% ethanol (15 and 5 minutes washed, respectively). After centrifugation, the supernatant was removed and the DNA pellet was air-dried and resuspended in 100 µl of TE buffer (10 mM Tris-HCl, pH 8.0 and 0.1 mM EDTA). The DNA solution was incubated at 37^{o}C for 1-2 hours for complete solubilization and kept at 4^{o}C until further needed.

2.2.3 Measuring concentrations of extracted DNA using spectrophotometry and minigel electrophoresis

The concentration of extracted DNA was spectrophotometrically estimated by measuring the optical density at 260 nanometer (OD_{260}). An OD_{260} of 1.0 corresponds to a concentration of 50 µg/ml double stranded DNA. Therefore, the concentration of DNA samples is estimated in µg/ml by multiplying an OD_{260} value with a dilution

factor and 50. The purity of DNA samples can be evaluated by a ratio of OD_{260}/OD_{280}. The ratio that much lower than 1.8 indicates contamination of residual proteins or organic solvents in the DNA solution whereas that much greater than 1.8 implies contamination of RNA (Kirby, 1992).

Agarose gel electrophoresis can also be used for rough estimation of DNA on the basis of the direct relationship between the amount of DNA and the level of fluorescence after ethidium bromide staining. DNA was run in a 0.8% agarose gel prepared in 1xTBE buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0) at 100 V. The gel was stained with ethidium bromide. DNA concentration was estimated by comparing the fluorescent intensity of a given band with that of undigested λDNA.

2.2.4 PCR using universal primers of COI-COII and 16S rDNA and restriction analysis

The COI-COII gene segment (approximately 1550 bp) of each *P. monodon* (*N* = 29) and *P. semisulcatus* (*N* = 15) was amplified using COI-COII-F (5'-TTG ATT TTT TGG TCA TCC AGA AGT-3') and COI-COII-R (5'-CCA CAA ATT TCT GAA CAT TGA CC-3'; Roehrdanz, 1993). In addition, the 16S rDNA (560 bp, hereafter called 16S $rDNA_{560}$) fragment of *P. monodon* (*N* = 29), *P. semisulcatus* (*N* = 15), *F. merguiensis* (*N* = 14), *L. vannamei* (*N* = 14) and *M. japonicus* (*N* = 7) was amplified using $16S_{F1}$ (5'-CGC CTG TTT AAC AAA AAC AT-3') and $16S_{R1}$ (5'-CCG GTC TGA ACT CAG ATC ATG T-3'; Palumbi *et al*., 1991).

PCR were performed in 50 µl reaction volume containing 25 ng template DNA, 1X PCR buffer (10 mM Tris-HCl; pH 8.8, 50 mM KCl, 0.1% Triton X-100), 200 µM of each dATP, dCTP, dTTP and dGTP, 2.5 mM (COI-COII) or 2.0 mM (16S $rDNA_{560}$) $MgCl_2$, 0.2 µM (COI-COII) or 0.5 µM (16S $rDNA_{560}$) of each primer and 1 unit of DyNazyme^{TM} II DNA polymerase (Finnzymes, Finland). The reaction mixture was performed in a PCR ThermoHybaid PxE thermal cycler. The amplification cycles were composed of predenaturation at 94^{o}C for 3 minutes, followed by 5 cycles of a low stringent condition of 94^{o}C for 1 minute, 42^{o}C for 1 minute and 72^{o}C for 1 minute and 35 cycles of a higher stringent condition at 94^{o}C for 1 minute, 53^{o}C (16S $rDNA_{560}$) and 55^{o}C (COI-COII) for 1 minute. The final extension was carried out at

72°C for 7 minutes. Five microliters of the amplification reaction were electrophoresed through 1% agarose gel to determine whether the PCR reaction was successful. Specimens showing expected product sizes were subjected to restriction analysis.

2.2.5 Agarose gel electrophoresis

Agarose gel electrophoresis separates DNA fragments on the basis of their molecular sizes. PCR products were analyzed by 1% standard agarose gels. An appropriate amount of agarose was weighed out and mixed with 1X TBE buffer (Maniatis *et al.*, 1982). The solution was heated in a microwave oven until complete solubilization and allowed to cool below 60°C before poured into the gel mould. The comb was then inserted. The gel was left to solidify at room temperature for 30-45 minutes. When needed, the comb was carefully removed. The gel was submerged in a chamber containing an enough amount of 1xTBE buffer covering the gel for approximately 0.5 cm.

PCR-amplified products or restriction enzyme digested products were mixed with the loading dye solution (0.25% bromophenol blue and 25% Ficoll 400 in H_2O). The mixture was carefully loaded into the well. DNA marker (λ-*Hin*d III and/or 100 bp ladder) were included as DNA standards. Electrophoresis was operated at 90 volts until bromophenol blue moved to approximately 2 cm from the bottom of the gel. The electrophoresed gel was stained with a 0.5 μg/ml ethidium bromide solution for 15 minutes and destained twice to remove unbound ethidium bromide in distilled water for 15 minutes each. DNA fragments were visualized under a UV transilluminator.

2.2.6 Restriction endonuclease digestion

The COI-COII and 16S $rDNA_{560}$ amplification products were singly digested with *Dra* I (TTT/AAA), *Ssp* I (AAT/ATT) and *Vsp* I (AT/TAAT) and *Alu* I (AG/CT), *Mbo* I (/GATC), *Ssp* I and *Vsp* I, respectively. The digestion was performed in a 15 μl reaction volume composing of 6 μl of the PCR product, 1X of restriction enzyme buffer, 0.1 μg/ml BSA, 4 mM spermidine trihydrochloride, 1 unit of a restriction endonuclease and appropriate amount of sterile deionized water. The mixture was incubated at 37°C for 3 hours. At the end of incubate period, 2 μl of a loading dye was added and mixed. The restricted products were electrophoresed through 1.0% (COI-

COII) and 1.5% (16S $rDNA_{560}$) agarose gels as described previously. Results of restriction enzyme digestion were recorded by photographed through a red filter using the Formapan film.

2.2.7 Recover of the amplified 16S $rDNA_{560}$ product from agarose gels

The 16S $rDNA_{560}$ gene region was subjected to ligated to pGEM®-T easy vector (Hoelzel and Green, 1992) and SSCP analysis. Therefore, the 16S $rDNA_{560}$ fragment was purified by fractionated through 1.5% agarose gels. The 560 bp fragment was excised from the electrophoresed gel and placed into a pre-weight 1.5 ml microcentrifuge tube. DNA was then eluted from agarose gels using QIAquick Gel Extraction Kit (QIAGEN). Three volumes of DNA purification binding buffer (QG buffer) was added to the microcentrifuge tube containing a gel slice. The mixture was incubated at 50°C for 10 minutes with occasional agitating for complete dissolving of the agarose gel slice every 2-3 minutes. The solution was applied to the QIAquick spin column placed into a collection tube and centrifuged at 13,000 rpm for 1 minute at room temperature. The supernatant was discarded. 500 µl of DNA binding buffer (QG buffer) was added and centrifuged for 1 minute. The supernatant was discarded. The column was washed once with 750 µl of wash buffer (PE buffer), centrifuged for 1 minute and recentrifuged once. QIAquick column was placed into a clean 1.5 ml microcentrifuge tube. 50 µl of the elution buffer (EB buffer) was added, let the column stand for 1 minute and then centrifuged for 1 minute. The gel-eluted DNA was kept at 4°C until further needed.

2.2.8 SSCP analysis of 16S $rDNA_{560}$

2.2.8.1 Preparation of glass plate

The glass plate was thoroughly wiped with 2 ml of 95% commercial grade ethanol in one direction with a tissue paper. This process was then repeated twice. Afterwards, the long glass plate was coated with 1 ml of freshly prepared binding solution (4 µl of Bind silane; Pharmacia, USA, 995 µl of 95% ethanol and 5 µl of glacial acetic acid) and left for 10 minutes. Excess binding solution was removed with 95% ethanol 1 time. The shot glass plate was treated as described above with the exception that the binding solution was replaced by the Repel silane (2%

dimethyldichlorosilane in octamethylcyclotetra-sitoxone). The cleaned glass plates were assembled with a pair of 0.4 mm spacers and a pair of the gel clamps.

The 12.5 - 15% low crosslink (37.5:1 acrylamide:bis-acrylamide, containing 1X TBE buffer equivalent to 2.66% crosslink) non-denaturing polyacrylamide gels were prepare by dilution of a 40% stock solution to the required concentration. The acrylamide gel solution (30-40 ml) was mixed with 240 µl of 10% APS and 24 µl of TEMED after degassed for 15 minutes using vacuum. The analytical comb was inserted into the prepared gel and polymerization was allowed for at lease 4 hours or overnight.

For SSCP analysis, 8 µl of the gel-eluted DNA of gene fragment was mixed with 4 volumes of the loading dye (98% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol and 10 mM NaOH), denatured at 95°C for 5 minutes and immediately cooled on ice for 3 minutes. Electrophoresis was carried out at 200 V for 12-16 hours at 4°C. As a control, the non-denatured gel-eluted DNA of gene fragment was also included in the gel. SSCP bands are visualized by silver staining.

2.2.8.2 Silver staining

The glass plates were carefully separated. The glass plate with the gel was placed in a plastic tray containing 1.5 liters of the fix/stop solution and agitated for 25-30 minutes. Then the gel was washed 3 times by shaking in deionized water for 3 minutes each. After washing, the gel was transferred to 0.1% silver nitrate solution (1.5 g of silver nitrate and 2.25 ml of 37% formaldehyde in 1.5 liters of deionized water) and stained with agitation for 30 minutes. The gel was soaked in 1.5 liters of deionized water with shaking and immediately placed in the tray containing 1.5 liter of the chilled developing solution (90 g of sodium carbonate in 3 liters of deionized water and 2.25 ml of 37% formaldehyde and 300 µl of a 10 mg/ml sodium thiosulfate solution were added before used). This step is crucial and the time taken to soak the gel in the water and transfer it to the chilled developing solution should not be longer than 10 seconds. The gel was well agitated until the first bands were visible (usually 1.5-2 minutes). The gel was then transferred to the other tray containing 1.5 liter of the chilled developing solution and shaken until every bands were observed (usually 2-3 minutes). Then, one liter of the fix/stop solution was directly added to the

developing solution and shaking was continued for 2 minutes. The stained gel was soaked in deionized water for 3 minutes and left drying at room temperature.

2.2.9 Cloning and sequencing of 16S rDNA$_{560}$ of penaeid shrimps

2.2.9.1 Amplification of 16S rDNA$_{560}$ from 5 penaeid shrimps

The 16S rDNA$_{560}$ segment was amplified from an individual representing the most common mitotype of *P. monodon, P. semisulcatus, F. merguiensis, L. vannamei* and *M. japoniccus* using conditions described in section 2.2.4. After electrophoresis, a 560 bp band was excised and isolated DNA from agarose gel using QIAquick Gel Extraction Kit (QIAGEN) as described in section 2.2.7.

2.2.9.2 Ligation of 16S rDNA$_{560}$ fragment to the pGEM®-T Easy vector

Taq I polymerase have a terminal transferase activity which results in the non-template addition of nucleotides to the 3' end of PCR products for which deoxyadenosine is almost preferentially added. This allows cloning of PCR-amplified fragments to the modified vector containing a single 3'-overhang thymidine residue (a T-A cloning approach).

A 560 bp 16S rDNA fragment was ligated to pGEM®-T easy vector (Promega, USA) in a 10 μl ligation reaction constituting of 5 μl of 2X rapid ligation buffer (60 mM Tris-HCl, pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP, 10% polyethylene glycol; MW 8000), 3 weiss units of T4 DNA ligase, 25 ng of pGEM®-T easy vector and 25 ng of the DNA insert. The reaction mixture was incubated overnight at 4^{o}C before transformed into *E. coli* strain JM 109 (Cohen *et al*., 1972).

2.2.9.3 Transformation of ligated products into *E. coli* (Cohen *et al*., 1972)

2.2.9.3.1 Preparation of competent cells

A single colony of *E.coli* JM109 was inoculated into 3 ml of LB medium (1% Bacto-tryptone, 0.5% Bacto-yeast extract and 0.5% NaCl, pH 7.0) and incubated with vigorous shaking at 37^{o}C overnight. The starting culture (1 ml) was inoculated into 100 ml of LB broth and continued culture at 37^{o}C with vigorous shaking to the OD_{600} of 0.4 to 0.6. The cells were chilled briefly on ice for 15 to 30 minutes and transferred

into a microcentrifuge tube and harvested by centrifugation in a prechilled rotor at 2,700xg for 10 minutes at 4^{o}C. The supernatant was discarded and the pellets were resuspended in 30 ml of sterile, ice-cold $MgCl_2$-$CaCl_2$ solution (80 mM $MgCl_2$ and 20 mM $CaCl_2$). The pellets were chilled on ice for 45 minutes and then centrifuged at 2,700 g for 10 minutes at 4^{o}C. After centrifugation, the pellets were resuspended in 4 ml of sterile, ice-cold 100 mM $CaCl_2$ (containing 15% glycerol) and divided to 100 µl aliquots. These concentrated cells could be used immediately or stored at -70^{o}C for subsequently used.

2.2.9.3.2 Transformation

The competent cells (JM109) were thawed on ice for 5 minutes. Approximately, 1 - 2 µl of the ligation product was added. The mixture was gently mixed by pipetting and left on ice for 30 minutes. At the end of period, the mixture was precisely inculated at 42^{o}C for 45 seconds and immediately placed on ice for 5 minutes. The mixture was transferred into a microcentrifuge tube containing 1 ml of SOC medium and incubated with shaking at 37^{o}C for 1 to 2 hours. Afterwards, cell suspension were spread on the LB agar plate containing 50 µg/ml of ampicillin, 25 µg/ml of IPTG and 20 µg/ml of X-Gal and further incubated at 37^{o}C overnight (Sambrook *et al*., 1989). The recombinant clones are selected using a *lac Z* system following a standard protocol (Maniatis *et al*., 1982) which recombinant clones containing inserted DNA are usually white while those without inserted DNA are blue.

2.2.9.4 Colony PCR

Colony PCR was performed in a 25 µl reaction volume containing 1xPCR buffer (10 mM Tris-HCl; pH 8.8, 50 mM KCl, 0.1% Triton X-100), 100 µM of each dATP, dCTP, dTTP and dGTP, 2 mM $MgCl_2$, 0.1 µM each of pUC1 (5'-CCG GCT CGT ATG TTG TGT GGA-3') and pUC2 (5'-GTG GTG CAA GGC GAT TAA GTT GG-3') and 1 unit of DyNazyme^{TM} II DNA polymerase (Finnzymes). A white colony was gently picked by a pipette tip and mixed well with the mixture. PCR was performed including predenaturation at 94^{o}C for 3 minutes followed by 35 cycles of denaturation at 94^{o}C for 1 minute, annealing at 55^{o}C for 1 minute and extension at 72^{o}C for 1.5 minutes. Final extension was performed at 72^{o}C for 7 minutes. The

colony PCR products were electrophoresed through 1.2% agarose gel as described previously.

2.2.9.5 Isolation of recombinant plasmid DNA

The recombinant plasmid DNA was isolated using QIAprep® Miniprep Kit (QIAGEN, Germany). A single white colony was inoculated into 3 ml of LB medium supplementing with 50 µg/ml of ampicillin and incubated with vigorous shaking at 37°C overnight. The culture was transferred into a new 1.5 ml microcentrifuge tube and centrifuged for 1 minute at 10,000 rpm. The supernatant was carefully poured off. Recombinant plasmid was extracted according the conditions recommended by the manufacturer. Recombinant plasmid DNA was stored at -20°C until used.

2.2.9.6 Detection of recombinant clones

The recombinant plasmid DNA was examined by digested with *E*coR I. The reaction was carried out in a 15 µl reaction volume containing 1X restriction enzyme buffer, 0.1 µg/ml BSA, 4 mM spermidine trihydrochloride, 2 units of *E*co RI, 2 µl of recombinant plasmid and appropriate amount of sterile deionized water. The mixture was incubated at 37°C for 3 hours. The resulting product was electrophoretically analyzed by 1.2% agarose gels. The size of insert was compared with that of λ-*Hin*d III and 100 bp DNA ladder.

2.2.9.7 DNA sequencing and data analysis

Five recombinant clones (Pmo4, Psemi1, Lvan28, Fmer44 and Mjapo12) were unidirectional sequenced using the M13 forward primer (5'-TTT TCC CAG TCA CGA C-3') on an automated MEGABACE1000 sequencer (Amersham Biosciences). The obtained sequences were blasted against previously deposited sequences in the GenBank (NCBI) using Blast*N* and Blast*X* (available at http://www.ncbi.nlm.nih.gov). Significant probabilities were considered when the probability (E) value was less than 10^{-4}.

2.2.10 Primer design, PCR-RFLP and SSCP of 16S rDNA$_{312}$

Nucleotide sequences of 16S rDNA$_{560}$ were aligned using Clustal W (Thompson *et al*., 1994). A pair of primers primed at the conserved regions of 16S

$rDNA_{560}$ was designed using the Primer Premier 5.0 program (Primer Premier, PREMIER Biosoft international) to generate a 312 bp fragment in representative individuals and tested against 185 shrimp individuals (Table 2.1). PCR was performed in 50 µl reaction volume containing 25 ng template DNA, 1X PCR buffer (10 mM Tris-HCl; pH 8.8, 50 mM KCl, 0.1% Triton X-100), 100 µM of each dATP, dCTP, dTTP and dGTP, 2 mM $MgCl_2$, 0.2 µM each of $16Pmo_{312F}$ (5'-GAA GGC TTG TAT GAA TGG TTG-3') and $16Pmo_{312R}$ (5'-AAA GAA GAT TAC GCT GTT ATC CCT A-3') and 1 unit of DyNazyme^{TM} II DNA polymerase (Finnzymes) (Klinbunga *et al.*, 2003).

The reaction was carried out by predenaturation at 94^{o}C for 3 minutes followed by 2 cycles of 94^{o}C for 45 seconds, 65^{o}C for 1 minute and 72^{o}C for 1 minute. Eight cycles of a touchdown phase with lowering of the annealing temperature for 2^{o}C in every 2 cycles was performed. Additional 28 cycles of 94^{o}C for 45 seconds, 56^{o}C for 1 minute and 72^{o}C for 1 minute and the final extension at 72^{o}C for 7 minutes were carried out. Five microliters of the amplification products were electrophoretically analyzed through 1.2% agarose gel.

Eight microliters of the product was subjected to PCR-RFLP (*Alu* I, *Ssp* I and *Vsp* I) and electrophoretically analyzed. Additionally, 5 µl of gel-eluted DNA of 16S $rDNA_{312}$ of each shrimp was analyzed by SSCP analysis as described in section 2.2.8 using 15.0% non-denaturing polyacrylamide gels (37.5:1) and electrophoresed at 200 V for 16 hours at 4°C.

2.3 Population genetic studies of *P. monodon* in Thailand by AFLP-derived SCAR markers and COI polymorphism

2.3.1 AFLP analysis

2.3.1.1 Restriction enzyme digestion and adapter ligation

Genomic DNA was individually extracted from wild *P. monodon* originating from Chumphon (*N* = 10), Trat (*N* = 5), Satun (*N* = 10), Trang (*N* = 10) and Pangnga (*N* = 10). Genomic DNA of different individual of each group was equally pooled 250 ng per group and used for screening of candidate polymorphic AFLP markers. AFLP

analysis (320 primer combinations) was carried out as essentially described by Vos *et al*. (1995).

Each pooled genomic DNA was simultaneously digested with 4 units of *Eco*R I (or *Pst* I) in a 40 µl reaction mixture containing 10 mM Tris-HCl, pH 7.5, 10 mM Mg-acetate, 50 mM K-acetate at 37^{o}C for 2 hours. The reaction was heat-terminated at 65^{o}C for 15 minutes before 2.5 units of *Mse* I was added and incubated at 65^{o}C for 2 hours. The reaction was stored at 4^{o}C until used.

The *Eco*R I (or *Pst* I) and *Mse* I adapters (Table 2.2) were ligated to restricted genomic DNA by adding 10 µl of the adapter-ligation solution (*Eco*R I or *Pst* I and *Mse* I adapters, 1 mM ATP, 10 mM Tris-HCl, pH 7.5, 10 mM Mg-acetate, 50 mM K-acetate and 1 unit of T4 DNA ligase. The ligation reaction was incubated at 12^{o}C for 16 hours.

2.3.1.2 Preamplification

The pre-amplification reaction was carried out in 25 µl reaction volume containing 1X PCR buffer (10 mM Tris-HCl; pH 8.8, 50 mM KCl, 0.1% Triton X-100), 200 µM of each dATP, dCTP, dTTP and dGTP, 1.5 mM $MgCl_2$, 37.5 ng of each adapter-specific primers with a single selective base on each primer; *Eco*R I primer (5'-GAC TGC GTA CCA ATT C**A**-3') and *Mse* I primer (5'-GAT GAG TCC TGA GTA A**C**-3'), 1.5 unit of DyNazyme^{TM} II DNA polymerase (Finnzymes, Finland) and 1 µl of the ligation product.

PCR was performed in a Perkin Elmer 9700 thermocycler consisting of 20 cycles 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. The final extension was carried out at 72^{o}C for 5 minutes. Three microliters of the amplification products were electrophoretically analyzed through 1.2% agarose gel. The pre-amplification product was 5 fold diluted and subjected to selective amplification.

2.3.1.3 Selective amplification

Selective amplification was carried out in a 20 µl reaction volume containing 1X PCR buffer (10 mM Tris-HCl; pH 8.8, 50 mM KCl, 0.1% Triton X-100), 200 µM of each dATP, dCTP, dTTP and dGTP, 1.5 mM $MgCl_2$, 30 ng of a combination pair

of E_{+3} and M_{+3} primers (additional 2 selective bases at the 3' terminus of each primer, Table 2.2), 1.5 unit of DyNazyme^{TM} II DNA polymerase (Finnzymes, Finland) and 5 µl of diluted pre-amplification product. The amplification reaction was carried out by denaturation at 94^{o}C for 30 seconds, annealing at 65^{o}C for 45 seconds and extension at 72^{o}C for 1.5 minute for 2 cycles followed by 12 cycles of a touchdown phase with lowering of the annealing temperature for 0.7^{o}C in every cycle. Additional 25 cycles of 94^{o}C for 30 seconds, 53^{o}C for 45 seconds and 72^{o}C for 1.5 minute was performed. The final extension was carried out at 72^{o}C for 5 minutes. AFLP fragments were analyzed on 4.5% denaturing polyacrylamide gels and visualized by silver staining.

2.3.2 Denaturing Polyacrylamide Gel Electrophoresis

The short and long glass plate was cleaned and prepared as described in section 2.2.8.1. The bottom and both sides of assembled glass plates were sealed with the plastic tape and spring clips.

A low percentage (4.5%) of denaturing polyacrylamide gel was prepared by combining 40 ml of the degassed acrylamide solution with 240 µl of freshly prepared 10% ammonium persulphate and 24 µl of TEMED. The assembled plate sandwich was held at a 45 degree angle on the bottom corner and the acrylamide solution was then gently applied into one side of the assembled plates using a 100 ml plastic bottle. 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 water-soaked tissue paper and left at room temperature for at least 4 hours for complete polymerization.

When required, the spring clips and the sealing tapes were carefully removed. The top of the gel was rinsed with 1X TBE buffer. The shark-tooth comb was rinsed. 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 side of the sequencing apparatus. The upper and lower buffer chambers were filled with approximately 300 ml of 1X TBE. The shark-tooth comb was inserted into the gel until the teeth just touched the surface of the gel.

Table 2.2 Sequences of AFLP adapters and primers used for identification of polymorphic AFLP markers in *P. monodon*

Primer	Sequences
Adapter sequences	
*Eco*R I adapter	5'-CTC GTA GAC TGC GTA CC-3'
	5'-AAT TGG TAC GCA GTC TAC-3'
Mse I adapter	5'-GAC GAT GAG TCC TGA G-3'
	5'-TAC TCA GGA CTC AT-3'
Pst I adapter	5'-CTC GTA GAC TGC GTA CAT GCA-3'
	5'-TGT ACG CAG TCT AC-3'
Preamplification primers	
E_{+A}	5'-GAC TGC GTA CCA ATT CA-3'
M_{+C}	5'-GAT GAG TCC TGA GTA AC-3'
Pre-*Pst*	5'-GAC TGC GTA CAT GCA GG-3'
Selective amplification primers	
E_{+3}-1	E_{+A}AC
E_{+3}-2	E_{+A}AG
E_{+3}-3	E_{+A}CA
E_{+3}-4	E_{+A}CT
E_{+3}-5	E_{+A}CC
E_{+3}-6	E_{+A}CG
E_{+3}-7	E_{+A}GC
E_{+3}-8	E_{+A}GG
E_{+3}-9	E_{+A}GT
E_{+3}-10	E_{+A}GA
E_{+3}-11	E_{+A}TG
E_{+3}-12	E_{+A}TC
E_{+3}-13	E_{+A}TA
E_{+3}-14	E_{+A}TT
E_{+3}-15	E_{+A}AA
E_{+3}-16	E_{+A}AT

Table 2.2 (continued)

Primer	Sequences
M_{+3}-1	M_{+C}AA
M_{+3}-2	M_{+C}AC
M_{+3}-3	M_{+C}AG
M_{+3}-4	M_{+C}AT
M_{+3}-5	M_{+C}TA
M_{+3}-6	M_{+C}TC
M_{+3}-7	M_{+C}TG
M_{+3}-8	M_{+C}TT
M_{+3}-9	M_{+C}GA
M_{+3}-10	M_{+C}GT
M_{+3}-11	M_{+C}GC
M_{+3}-12	M_{+C}GG
M_{+3}-13	M_{+C}CA
M_{+3}-14	M_{+C}CT
M_{+3}-15	M_{+C}CG
M_{+3}-16	M_{+C}CC
Pst I-1	Pre-*Pst*+AG
Pst I-2	Pre-*Pst*+AC
Pst I-3	Pre-*Pst*+GA
Pst I-4	Pre-*Pst*+GT
Pst I-5	Pre-*Pst*+CG
Pst I-6	Pre-*Pst*+CT
Pst I-7	Pre-*Pst*+TC
Pst I-8	Pre-*Pst*+TT

Six microliters of the acrylamide gel loading dye (98% formamide, 200 µl EDTA, 0.25% bromophenol blue and 0.25% xylene cyanol) were loaded into each well. The gel was prerun at 30-40 W for 20 minutes.

Six microliters of the amplification products were mixed with 3 µl of the loading buffer and heated at 95^{o}C for 5 minutes before snap cooling on ice for 3 minutes. The sample was carefully loaded into the well. Electrophoresis was carried out at 30-40 W for approximately 2.5 hours (xylene cyanol moved out from the gel for approximately 30 minutes). The gel was visualized by silver staining as described in section 2.2.8.2.

2.3.3 Cloning and sequencing of polymorphic AFLP markers

The polymorphic AFLP bands that were not uniquely or commonly found in investigated *P. monodon* were excised from the gel. DNA was eluted out from the polyacrylamide gels using a sterile razor blade. The polyacrylamide gel sliced was washed twice for 2 hours each at room temperature with 500 µl of sterile deionized water. After that, twenty microliters of ultrapure water was added and incubated at 50^{o}C for 30 minutes and at 37^{o}C overnight. These eluted AFLP products were kept at 4^{o}C until used.

The eluted AFLP products were reamplified using the original conditions of each technique. The resulting product was electrophoretically analyzed through 1.5% agarose gel at 7.5 volts/cm for approximately 1 hour. The reamplified DNA fragment was excised from the agarose gel, gel-eluted, cloned into pGEM®-T Easy vector and sequenced. Nucleotide sequences of 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 $<10^{-4}$.

2.3.4 Primer design and PCR amplification

Fourteen primer pairs were designed from AFLP-derived markers using Primer Premier 5.0 and tested against genomic DNA of each shrimp (Table 2.3). PCR was performed in 50 µl reaction volume containing 25 ng of genomic DNA, 1X PCR buffer (10 mM Tris-HCl; pH 8.8, 50 mM KCl, 0.1% Triton X-100), 200 µM of each

dATP, dCTP, dTTP and dGTP, 2.0 mM $MgCl_2$, 0.2 μM of each primer and 1 unit of DyNazyme^{TM} II DNA polymerase (Finnzymes, Finland).

The amplification reaction was carried out by predenaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at the appropriate temperature for 1 min, and extension at 72°C for 1 min. The final extension was performed at 72°C for 7 min.

Five microliters of the product were analyzed for the amplification success through 1.2% agarose gel eleectrophoresis. Polymorphism (SNP or indels) of the amplification product of different shrimp individuals were analyzed by single-stranded conformation polymorphism (SSCP) analysis.

Five microliters of the PCR product of each shrimp were mixed with 4 volumes of the loading dye (95% formamide, 0.25% bromophenol blue, 0.25% xylene cyanol and 10 mM NaOH), denatured at 95°C for 5 minutes, immediately cooled on ice for 3 minutes and electrophoretically analyzed through 12.5% - 17.5% low crosslink non-denaturing polyacrylamide gels (37.5:1 or 75:1 of acrylamide:bis-acrylamide) at 12.5 V/cm for 16 hours at 4°C. SSCP bands were visualized by silver staining.

2.3.5 Genetic diversity of *P. monodon* in Thailand examined by AFLP-derived markers

Four primer pairs (P6M2-370, P6M6-470, E4M6-295, E7M10-450) generating clear and easy scoring polymorphic SSCP patterns were selected and tested against genomic DNA of *P. monodon* (see Table 2.4 for sample sizes) originating from Satun, Trang and Phangnga located in the Andaman Sea and Chumphon and Tart located in the Gulf of Thailand. PCR amplification were performed and electrophoretically analyzed by SSCP analysis as described above.

2.3.6 Genetic diversity of *P. monodon* in Thailand examined by SSCP analysis of 16S rDNA$_{312}$

The 16S rDNA segment of *P. monodon* was amplified using primers 16Pmo$_{312F}$ and 16Pmo$_{312R}$, and conditions described in section 2.2.10 against 185

shrimp individuals (Table 2.4). The gel-eluted 16S $rDNA_{312}$ was analyzed by SSCP as described in section 2.2.8 using 15.0% non-denaturing polyacrylamide gel (37.5:1) and electrophoretically analyzed at 200 V for 16 hours at 4^{o}C. SSCP patterns were visualized by silver staining.

Table 2.3 Sequences and the melting temperature of primers derived from AFLP markers of *P. monodon*

Primer	Sequence (5´→ 3´)	Tm (^{o}C)
1. E4M6-295	F: TTC TTA CGG CAC TTG GAA AAT G	62
	R: TCC CCT CCT ATG CTA ACG CTA C	68
2. E8M7-323	F: AGA CTT TTT ATA CAT TCC TTC CC	62
	R: AGG TCC GCC CAC AAT CAT AC	62
3. E6M9-318	F: ACC GTA TTT CCA TCT ATC TC	56
	R: TTG TCT CGT TTT ATT TCT TG	52
4. E7M10-450	F: TGG TTG CGT TCA TCT TAT CTC	60
	R: TGT TAC AAT GCT GTC GTG GA	58
5. P2M5-295	F: TAA GCC TTT TTA CCA ATA GAC C	60
	R: TCA GAA GAA TAG CAA GAG AAC G	62
6. P2M6-270	F: ACT GGT CAC CTT AGG ATG CT	60
	R: CCT GGT TCT TTG CTG GAT	54
7. P2M7-285	F: GCA GAA CCG ATA CCC AAG GC	64
	R: GGT CCG TGG CTG AAA TGT AAA TA	72
8. P2M7-310	F: CAA GCC TCC AGA ACT CCT CA	58
	R: CTT TCA TCC CTC AAC ATC ACT	60
9. P6M2-370	F: CGG TGA AAG TAA GTC AAA TGT C	62
	R: TCA GGC AGA GTG GAG GGA A	60
10. P6M6-470	F: TTC GCT AAC TTT CTC CCC TAA	60
	R: CTC AGA CTC CCG CCT AAT CC	64
11. P2M6-385	F: CAG AAT CTT ACT CTC AGC CAC ATA CAC	78
	R: GCT TTC AGC AAC CCT TCC AGT A	66
12. P2M6-465	F: TTA CGA GCA GGG ATT CAG GTT C	66
	R: CAT TGG CAT CTG GCT TGG AG	62

Table 2.3 (continued)

Primer	Sequence (5´ → 3´)	Tm (^{o}C)
13. P2M6-850	F: ACC GCT ATG GAG TTA TGT TCT GC	68
	R: TCC TAT CTA TCT CAC CTG CTT ACC CT	76
14. P2M8-300	F: ACA CTT CCA AGC ATT TAC CTC GC	68
	R: TAC CAA CGC CCC ATC TAT TCT G	66

Table 2.4 Sample sizes of *P. monodon* in Thai waters used for population genetic studies using AFLP-derived markers and 16S $rDNA_{312}$

Sample	P6M2-370	P6M6-470	E4M6-15	E7M10-450	16S $rDNA_{312}$
Trat	18	16	16	12	37
Chumphon	32	30	26	25	32
Satun	26	28	21	21	39
Trang	26	30	20	22	33
Phangnga	32	32	29	25	33
Ranong	-	-	-	-	11
Total (*N*)	134	136	112	105	185

2.3.7 Genetic diversity of *P. monodon* in Thailand analyzed by sequencing of COI-COII

2.3.7.1 Amplification, purification and cloning of COI-COII gene (1550 bp)

The COI-COII (1550 bp) gene segment was amplified from an individual of *P. monodon* origination from Trat, Chumphon, Satun and Trang using primers; COI-COII-F and COI-COII-R using conditions described in section 2.2.4. After electrophoresis, a 1550 bp band was excised and eluted from the agarose gel using a QIAquick Gel Extraction Kit (QIAGEN) as described in section 2.2.7. The COI-COII

fragment was ligated to pGEM®-T easy vector (Hoelzel and Green, 1992) and cloned into *E. coli* JM109 as described in section 2.2.9.

2.3.7.2 DNA sequencing

Four recombinant clones (Td13, C20, S3 and T13) were sequenced by an automated DNA sequencer using M13-F and M13-R as the sequencing primers (Macrogen Inc., Korea). The obtained sequences were blasted against previously deposited sequences in the GenBank using Blast*N* and Blast*X* programs (available at http://www.ncbi.nlm.nih.gov). Significant probabilities were considered when the probability (E) value was less than 10^{-4}.

2.3.7.3 Primer design and PCR amplification

Nucleotide sequences of COI-COII (1550 bp) from different individuals were aligned using Clustal W (Thompson *et al*., 1994). Two pair of primers; T13COI-F2/ R2 and Td13COII-F3/R3 primed at the conserved regions of COI and COII genes was designed using Primer Premier 5.0 (Primer Premier, PREMIER Biosoft international) and tested for the amplification successes with genomic DNA of *P. mondon* (Table 2.5).

In addition, newly designed primers were also used to amplify genomic DNA of *P. monodon* in combination of with the universal primer COI-COII-F/R (COI-COII-F+T13COI-R2, COI-COII-F+Td13COII-R3, T13COI-F2+COI-COII-R, Td13COII-F3+COI-COII-R). The COI-COII-F+T13COI-R2 yielded a discrete 614 bp PCR product without non-specific products. This primer pair was used to amplify the COI_{614} gene segment from genomic DNA of *P. monodon* from Trat (*N* = 20), Chumphon (*N* = 20), Satun (*N* = 19), Trang (*N* = 21) and Phangnga (*N* = 20).

PCR was performed in 50 µl reaction volume containing 25 ng of genomic DNA, 1xPCR buffer (10 mM Tris-HCl; pH 8.8, 50 mM KCl, 0.1% Triton X-100), 200 µM of each dATP, dCTP, dTTP and dGTP, 2 mM $MgCl_2$, 0.2 µM each of primers (Table 2.5) and 1 unit of *Taq* DNA polymerase (Fermentas). The reaction was carried out by predenaturation at 94°C for 3 minutes followed by 5 cycles of 94°C for 45 seconds, 42°C for 60 seconds and 72°C for 90 seconds and additional 35 cycles of 94°C for 45 seconds, 55°C for 60 seconds and 72°C for 90 seconds. The final

extension was carried out at 72^{o}C for 7 minutes. Five microliters of the amplification products were electrophoretically analyzed through 1.2% agarose gel to examine whether the amplification of COI_{614} was successful.

Table 2.5 Newly designed primers from COI-COII gene, their sequences and Tm

Primer	Sequence (5´ → 3´)	Tm (^{o}C)
T13COI	F2: TCG TGC TTA CTT TAC ATC TGC TA	64
	R2: ATC GCC GAG GTA TTC CAT TA	58
Td13COII	F3: CGT ATT AGA GGG TGG GCG TC	64
	R3: CGA AAA TGT GGT TCC CGT CC	62

2.3.7.4 Recover of the amplified COI (614 bp) product from agarose gels

The amplified 614 bp COI fragment was size-fractionated through 1.5% agarose gel. After electrophoresis, a 614 bp fragment of each individual was excised from the gel and placed into a pre-weight 1.5 ml microcentrifuge tube. DNA was then eluted from the agarose gel using a HiYield Gel/PCR Mini Kit using the protocol recommended by the manufacture (Real Biotech Corporation, Taiwan).

2.3.7.5 DNA sequencing and data analysis

The gel-eluted COI (614 bp) of 100 individuals of *P. monodon* was unidirectionally sequenced using the M13 forward primer on an automated DNA sequencer (Macrogen Inc., Korea). The obtained sequences were blasted against previously deposited sequences in the GenBank using Blast*N* and Blast*X* programs (available at http://www.ncbi.nlm.nih.gov).

2.3.8 Data analysis

For population genetic analysis, SSCP fragments of AFLP and 16S $rDNA_{312}$ 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 in each geographic sample. Pairwise genetic identity and genetic distance were determined (Nei, 1972). A neighbor-joining tree (Saitou and Nei, 1987) 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}-based statistics (θ) of overall samples across all investigated primers were calculated. The chi-square value was calculated and tested using $\chi^2 = 2N\theta(k-1)$ and df = (k-1) (s-1) where N = the number of investigated individuals, k = the number of alleles per locus and s = the number of geographic samples. Population genetic parameters described above were computationally analyzed by Tool for Population Genetic Analysis (TFPGA, Miller, 1997) and POPGENE.

For nucleotide sequence analysis, COI_{614} sequences of 100 shrimps were multiple aligned. Different individuals exhibited identical nucleotide sequences were grouped to generate sequence haplotypes. Nucleotide sequence divergence between pairs of haplotypes were calculated based on the two parameter method (Kimura, 1980) and subjected to phylogenetic reconstruction using a neighbor-joining method (Saitou and Nei, 1987). Frequencies of different haplotype clusters in between pairs of geographic samples were statically tested using a Monte Carlo simulation using a Restriction Enzyme Analysis Package (REAP; McElroy et al., 1991).

2.4 Isolation and characterization of genomic sex determination markers using RAPD-PCR and sex-specific/differential expression markers of *P. monodon* using RAP-PCR analyses.

2.4.1 RAPD analysis

One hundred RAPD primers were screened against 6 bulked genomic DNA of representative individuals (pooled genomic DNA of 3, 5 or 10 individuals of male and female *P. monodon*) (Table 2.6). PCR was carried out according to conditions

Table 2.6 Arbitrary primers (10 bp in length) and their sequences used in this study

Primer	Sequence (5´ → 3´)
UBC 101	GCGCCTGGAG
UBC 110	TAGCCCGCTT
UBC 111	AGTAGACGGG
UBC 112	GCTTGTGAAC
UBC 115	TTCCGCGGGC
UBC 119	ATTGGGCGAT
UBC 120	GAATTTCCCC
UBC 122	GTAGACGAGC
UBC 128	GCATATTCCG
UBC 135	AAGCTGCGAG
UBC 138	GCTTCCCCTT
UBC 140	GTCGCATTTC
UBC 141	ATCCTGTTCG
UBC 146	ATGTGTTGCG
UBC 153	GAGTCACGAG
UBC 158	TAGCCGTGGC
UBC 159	GAGCCCGTAG
UBC 160	CGATTCAGAG
UBC 165	GAAGGCACGT
UBC 168	CTAGATGTGC
UBC 169	ACGACGTAGG
UBC 174	AACGGGCAGC
UBC 175	TGGTGCTGAT
UBC 191	CGATGGCTTT
UBC 193	TGCTGGCTTT
UBC 195	GATCTCAGCG
UBC 196	CTCCTCCCCC
UBC 197	TCCCCGTTCC
UBC 200	TCGGGATATG

Table 2.6 (continued)

Primer	Sequence (5´ → 3´)
UBC 210	GCACCGAGAG
UBC 217	ACAGGTAGAC
UBC 220	GTCGATGTCG
UBC 222	AAGCCTCCCC
UBC 228	GCTGGGCCGA
UBC 235	CTGAGGCAAA
UBC 237	CGACCAGAGC
UBC 259	GGTACGTACT
UBC 263	TTAGAGACGG
UBC 267	CCATCTTGTG
UBC 268	AGGCCGCTTA
UBC 270	TGCGCGCGGG
UBC 271	GCCATCAAGA
UBC 273	AATGTCGCCA
UBC 299	TGTCAGCGGT
UBC 428	GGCTGCGGTA
UBC 456	GCGGAGGTCC
UBC 457	CGACGCCCTG
UBC 459	GCGTCGAGGG
M13	GAGGGTGGNGGNTCT
PERI	GACNGGNACNGG
HRU18	ACCCGGCGCTTATTAGAG
HRU33	CCCAAGGTCCCCAAGGTCAGGGAGGCG
YN73	CCCGTGGGGCCGCCG
YNZ22	CTCTGGGTGTCGTGC
OPA 01	CAGGCCCTTC
OPA 02	TGCCGAGCTG
OPA 03	AGTCAGCCAC
OPA 05	AGGGGTCTTG

Table 2.6 (continued)

Primer	Sequence (5´→ 3´)
OPA 06	GGTCCCTGAC
OPA 07	GAAACGGGTG
OPA 08	GTGACGTAGG
OPA 09	GGGTAACGCC
OPA 10	GTGATCGCAG
OPA 11	CAATCGCCGT
OPA 12	TCGGCGATAG
OPA 13	CAGCACCCAC
OPA 14	TCTGTGCTGG
OPA 15	TTCCGAACCC
OPA 16	AGCCAGCGAA
OPA 17	GACCGCTTGT
OPA 18	AGGTGACCGT
OPA 19	CAAACGTCGG
OPA 20	GTTGCGATCC
OPB 02	TGATCCCTGG
OPB 03	CATCCCCCTG
OPB 04	GGACTGGAGT
OPB 05	TGCGCCCTTC
OPB 06	TGCTCTGCCC
OPB 07	GGTGACGCAG
OPB 08	GTCCACACGG
OPB 09	TGGGGGACTC
OPB 10	CTGCTGGGAC
OPB 11	GTAGACCCGT
OPB 12	CCTTGACGCA
OPB 13	TTCCCCCGCT
OPB 14	TCCGCTCTGG
OPB 15	GGAGGGTGTT

Table 2.6 (continued)

Primer	Sequence (5´→ 3´)
OPB16	TTTGCCCGGA
OPB 17	AGGGAACGAG
OPB 18	CCACAGCAGT
OPB 19	ACCCCCGAAG
OPB 20	GGACCCTTAC
OPZ 09	CACCCCAGTC
OPM 09	GTCTTGCGGA
$(CA)_8$	CACACACACACACACA
$(CAC)_5$	CACCACCACCACCAC
$(CT)_8$	CTCTCTCTCTCTCTCT
$(GTG)_5$	GTGGTGGTGGTGGTG
$(GACA)_4$	GACAGACAGACAGACA
$(GATA)_8$	GATAGATAGATAGATA

described by Klinbunga *et al*. (2004). PCR was performed in a 25 µl reaction mixture containing 1X buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100), 4 mM $MgCl_2$, 100 µM of each dATP, dCTP, dTTP and dGTP, 0.4 µM of an arbritary primer, 25 ng of genomic DNA and 1 unit of DyNazyme^{TM} II DNA polymerase (Finnzymes, Finland).

PCR was performed in a PCR ThermoHybaid PxE thermal cycler. The amplification cycles were composed of predenaturation at 94^{o}C for 3 minutes followed by 45 cycles of denaturation at 94^{o}C for 20 seconds, annealing at 36^{o}C for 1 minute and extension at 72^{o}C for 1.5 minutes. The final extension was performed at 72^{o}C for 7 minutes. Five microliters of the amplification reaction were electrophoresed through 1.6% agarose gel and visualized by a UV transilluminator after ethidium bromide staining.

2.4.2 RAP-PCR

2.4.2.1 Experimental shrimps

Broodstock-sized female and male *P. monodon* were live-caught from Angsila, Chonburi and transported back to the laboratory at the Center of Excellence for Marine Biotechnology (CEMB), National Center for Genetic Engineering and Biotechnology (BIOTEC) located at Chulalongkorn University. The gender and weight of each *P. monodon* individual were recorded.

2.4.2.2 Total RNA extraction

Total RNA was extracted from ovaries and testes of each shrimp using TRI Reagent®. A piece of tissue were dissected out, weighed and immediately placed in liquid N_2 and ground to the fine powder. The tissue powder was transferred to a microcentrifuge tube containing 500 µl of TRI Reagent (50-100 mg of starting tissue/1 ml) and homogenized. Additional 500 µl of TRI Reagent were added, homogenized and left for 5 minutes and 0.2 ml of chloroform was added. The homogenate was vortexed for 30 - 60 seconds and left at room temperature for 2 - 15 minutes and centrifuged at 12,000 g for 15 minutes at 4^{o}C. The mixture was separated into the lower red, phenol-chloroform phase, the interphase, and the colorless upper aqueous phase. The aqueous phase (inclusively containing RNA) was transferred to a new 1.5 ml microcentrifuge tube. RNA was precipitated by an addition of 0.5 ml of isopropanol and mixed thoroughly. The mixture was left at room temperature for 10-15 minutes and centrifuged at 12,000 g for 10 minutes at 4-25^{o}C. The supernatant was removed. The RNA pellet was washed with 1 ml of 75% ethanol and centrifuged at 7,500 g for 5 minutes at 4^{o}C. The ethanol was removed and the RNA pellet was air-dried for 5-10 minutes. RNA was dissolved in DEPC-treated water for immediate use. Alternatively, the RNA pellet was kept under absolute ethanol at -80^{o}C for long storage.

2.4.2.3 Measuring concentrations of extracted total RNA by spectrophotometry

The concentration of total RNA was spectrophotometrically estimated by measuring the optical density at 260 nanometer (OD_{260}). An OD_{260} of 1.0 corresponds

to a concentration of 40 µg/ml single stranded RNA. The purity of RNA samples can be evaluated by a ratio of OD_{260}/OD_{280}. The ratio of purified RNA was approximately 2.0 (Sambrook, *et al*., 1989).

2.4.2.4 Bulked Segregant Analysis (BSA)

Two bulked total RNA of male (*N* = 3) and female (*N* = 3) *P. monodon* broodstock were generated by pooling an equal amount of total RNA (0.5 µg) from each shrimp. One and a half micrograms of bulked total RNA of each sex was reversed-transcribed using an Improm-II™ Reverse Transcription System Kit (Promega). One of the five selected arbitrary primers (UBC 119, 299, 428, 456, 457) was used as the synthesizing primers. Bulked total RNA was combined with synthesizing primer (2.5 µM final concentration in a final volume of 20 µl of reverse transcription reaction) and appropriates DEPC-treated water in a final volume of 5 µl. The reaction was incubated at 70°C for 5 minutes and immediately placed on ice for 5 minutes. After that, 5X reaction buffer, $MgCl_2$, dNTP mix and RNasin were added to the final concentrations of 1X, 3.125 mM, 0.5 mM and 20 units, respectively. Finally, 1 µl of an Impromp-II™ reverse transcriptase was added and gently mixed by pipetting. The reaction mixture was incubated at 25°C for 10 minutes and at 42°C for 60 minutes. The reaction mixture was then incubated at 70°C for 15 minutes to terminate reverse transcriptase activity. Concentration and rough quality of the first stranded cDNA was spectrophotometrically examined (OD_{260} and OD_{260}/OD_{280}) and electrophoretically analyzed (1.0% agarose gel).

2.4.2.5 Screening of primers

First strand cDNA representing male and female *P. monodon* was used as template for PCR. RAP-PCR was carried out (Welsh *et al*., 1992) in a 25 µl reaction mixture containing 1X PCR buffer (10 mM Tris-HCl; pH 8.8, 50 mM KCl, 0.1% Triton X-100), 4 mM $MgCl_2$, 100 µM of each dATP, dCTP, dTTP and dGTP, 0.5 µM of an arbitrary primer (using the synthesizing primer or a new arbitrary primer for a total of 30 primers, Table 2.2), 1 µg of the first strand cDNA and 1 unit of DyNazyme™ II DNA polymerase (Finnzymes, Finland). PCR was carried out by predenaturing at 94° C for 3 minutes followed by 40 cycles of denaturation at 94°C for 20 seconds, annealing at 36°C for 60 seconds and extension at 72°C for 90

seconds. The final extension was carried out at the same temperature for 7 minutes. Five microliters of each amplification products were electrophoretically analyzed through 2.0% agarose gels and visualized under a UV transilluminator after ethidium bromide staining.

2.4.3 Cloning and sequencing of candidate sex-specific RAPD fragments and sex-specific/differential expression RAP-PCR fragments

RAPD fragments found only in the amplification products of male or female *P. monodon* and RAP-PCR fragments exhibiting sex-specific or differential expression between ovaries and testes of *P. monodon* were eluted out from agarose gel using QIAquick Gel Extraction Kit (QIAGEN), reamplified and ligated to pGEM®-T easy vector. The ligation product was transformed to *E. coli* strain JM109 and recombinant clone was identified by colony PCR. Plasmid DNA was extracted by QIAprep® Spin Miniprep Kit (QIAGEN) and unidirectional sequenced using an automated DNA sequencer (ABI310) at the Bioservice unit (BSU), National Center for Genetic Engineering and Biotechnology (BIOTEC). In total. 8 RAPD fragments and 35 sex-specific/differential expression RAP-PCR fragments from ovaries or testes of *P. monodon* were characterized. DNA sequence of each clone was blasted against data in the GenBank using Blast*N* and Blast*X*. Significant similarity was considered when the probability (E) value < 10^{-4}.

2.4.4 Primer design and PCR

2.4.4.1 RAPD-derived markers

Four primer pairs were designed from sequences of cloned RAPD fragments (Table 2.7) and tested against genomic DNA of each shrimp. PCR was performed in 50 µl reaction volume containing 25 ng template DNA, 1X PCR buffer (10 mM Tris-HCl; pH 8.8, 50 mM KCl, 0.1% Triton X-100), 200 µM of each dATP, dCTP, dTTP and dGTP, 2.0 mM $MgCl_2$, 0.2 µM of each primer and 1 unit of DyNazyme™ II DNA polymerase (Finnzymes, Finland). The amplification reaction was carried out by predenaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at the appropriate temperature for 1 min, and extension at 72°C for 1 min. The final extension was performed at 72°C for 7 min. Five microliters of the product were analyzed for the amplification success through 1.2% agarose gel

eleectrophoresis. Additionally, the amplification products of RAPD-derived SCAR markers were further analyzed by single-stranded conformation polymorphism (SSCP).

Five microliters of the PCR product of each shrimp were mixed with 4 volumes of the loading dye (95% formamide, 0.25% bromophenol blue, 0.25% xylene cyanol and 10 mM NaOH), denatured at 95°C for 5 minutes, immediately cooled on ice for 3 minutes and electrophoretically analyzed through 12.5% - 17.5% low crosslink non-denaturing polyacrylamide gels (37.5:1 or 75:1 of acrylamide:bis-acrylamide) at 12.5 V/cm for 16 hours at 4°C. SSCP bands were visualized by silver staining.

2.4.4.2 RAP-PCR-derived markers

Twenty-five primer pairs were designed (Table 2.7). Expression patterns of interesting transcripts were investigated using the first strand cDNA of ovaries and testes of both juvenile and broodstock-sized *P. monodon* by RT-PCR following conditions described by Leelatanawit *et al*. (2004).

PCR was performed in a 25 µl reaction volume containing 300 ng of the first strand cDNA or 25 ng genomic DNA, 1X PCR buffer (10 mM Tris-HCl; pH 8.8, 50 mM KCl, 0.1% Triton X-100), 200 µM of each dATP, dCTP, dTTP and dGTP, 2.0 mM $MgCl_2$, 0.2 µM of each primer and 1 unit of DyNazyme^{TM} II DNA polymerase (Finnzymes, Finland). The reaction mixture was performed in a PCR ThermoHybaid PxE thermal cycler. The amplification cycles were composed of predenaturation at 94^{o}C for 3 minutes followed by 30 cycles of denaturation at 94^{o}C for 45 seconds, annealing at 53^{o}C for 1 minute and extension at 72^{o}C for 1 minute. Final extension was performed at 72^{o}C for 7 minutes. Five microliters of the amplification reaction were electrophoresed through 1.5% agarose gel.

β-actin and elongation facter 1 alpha (EF-1α) were included as the positive control in RT-PCR. They are housekeeping genes exhibiting relatively constant expression levels across various experimental conditions. A 315 bp (β–actin) and 500 bp (EF-1α) segment were successfully amplified by using heterospecific primers Actin-F: 5'-GGT ATC CTC ACC CTC AAG TA-3' and Actin-R: 5'-AAG AGC

GAA ACC TTC ATA GA-3' and EF-1αF: 5'-ATG GTT GTC AAC TTT GCC CC-3' and EF-1αR: 5'-TTG ACC TCC TTG ATC ACA CC-3'.

Table 2.7 Sequences of primers derived from candidate sex-specific RAPD fragments and sex-specific/differential expression RAP-PCR fragments of *P. monodon*

Primer	Sequence (5´→ 3´)	Tm (^{o}C)
RAPD		
1. 119-sx	F: TGA TAG TCT CGC AGT CCA AAG G	66
	R: CAC CAA TCG TAA CAA GGA AAG TAA C	70
2. OPA01-sx	F: AGC AGT TTC AAA GCA TAA CCC AG	66
	R: TAT ATT GAC TGT AGG CAT CTA T	58
3. 135-sx	F: TTA CTG GTG ATA ATG ATG CCT T	60
	R: AAC GCT AAC AAT GAG AGT GGT C	64
4. OPB20-sx	F: CTT GTT GAA CTC AAT GTC TTT AT	58
	R: TCC ATC GCT CCA TCC TCC TT	62
RAP-PCR		
1. FI-4	F: CAA TAC GGG AAA GAA GAA GCA	60
	R: AGA CAA CCC ATA CTG GAG GAG	64
2. FI-44	F: CCA TTG CCA AGA ATA AGT G	62
	R: GTA ACG CCA AAT CTC AAC CA	58
3. FIII-4	F: GCA ATC TCG CAC AGC CAA TAC T	66
	R: CGG AAA GAC AGG GCA GCA AC	64
4. FIII-39	F: ATC TCG CCA GGA GGA AAT AA	58
	R: CCT TGT TCA GTT CTT GCC AC	60
5. FIII-58	F: CCA ACC AAG AAA TAA CAG GCA CA	72
	R: TCC GAG GGC ACC ACC AAG	52
6. MII-5	F: TGT AGA CAA GCG ACT GGA AG	60
	R: GCT TCT GGC TAC CAA TCT TC	60
7. MII-51	F: CCT GAT GAA ATC GGG TCA AAA C	64
	R: ATA CTC TCC TCT GCC GCT CG	64
8. M457-A01	F: CTT CTT ATG TCT GTC CTT TGA TGA	66
	R: TTC TTA GGG AAA CTG CTT GC	58

Table 2.7 (continued)

Primer	Sequence (5´ → 3´)	Tm (^{o}C)
9. FI-1	F: GTA TTC CAT CCT CAA CAA CTG	60
	R: ACT GGG AGC ACT ACC ATC TT	60
10. FI-6	F: AGA TGG AGA TGG GTT AGG A	56
	R: AGG AGT AGA CGC CGT TTG T	58
11. FI-40	F: AAT AAC CGT TCT CAG CAG CA	58
	R: ATT CAA GGC GTT CAC ATC C	56
12. FII-17	F: TGG AGG AGT AGA TGC CGT T	58
	R: GTG ATG GGT TAG GTG ATG C	58
13. FII-18	F: TCC AGC AGA AAG AAG GTG AC	60
	R: TCG GAC AAG TGG GTA GTA TG	60
14. FII-22	F: ATT TCT CAG GGT GTC CAG	54
	R: AGA TTA CGC AGT CAT TAG GT	56
15. FIII-8	F: CCT CAT AAA CCA GGC ACT AA	58
	R: AGA ATC ATC CCA GGA ATC AC	58
16. FIV-1	F: GAA CAG GTC AGA GCA GGT A	58
	R: ATG TGC CTC TTC AGT TTG T	54
17. FIV-2	F: CGT ATG CCA CAT CCC ACA	56
	R: TTC TTT TCT GAA GGA GGT CG	58
18. FIV-20	F: GCC AAG CAG TAA CAA AGA CCA	62
	R: GGC TCA ACC TCC AGG AAC AG	64
19. FIV-33	F: TGG GAC TGT TTG TTT CTT G	54
	R: TCT CTT GGT TAG GTG TTG GT	58
20. FV-1	F: CGT ATG CCA CAT CCC ACA GA	62
	R: GAG CCC GTA CCA TTG AGA AA	60
21. FV-3	F: ATT TGC CTC TAT GCT CCT TC	58
	R: GTC GCA GAC TTC TTG GTG A	58
22. FV-27	F: GCC CTG GCA CAG CAC TTA G	62
	R: GTC CCA ATC TTC CTC TTT CA	58

Table 2.7 (continued)

Primer	Sequence (5´ → 3´)	Tm (^{o}C)
23. FV-42	F: AAG TGA CCT TGA TAT GAG T	56
	R: ATC CCT TCG TTG TAA GTA GA	56
24. MI-36	F: ATG TAT GTT TGT GTA TGT AGG TGT G	68
	R: AGA CGG CAA GGA AAG ATG AG	60
25. 428-OPB17	F: CTC TGA CTG GTG GAG GGA AT	62
	R: CTG GCT CGT GGG AGT GTA AT	62

CHAPTER III

RESULTS

3.1 Development of molecular markers for identification of species origin of five penaeid species (*Penaeus monodon, P. semisulcatus, Feneropenaeus merguiensis, Litopenaeus vannamei* and *Marsupenaeus japonicus*) by PCR-RFLP and SSCP analyses

3.1.1 DNA Extraction

Genomic DNA of each shrimp was extracted from a piece of pleopod using a phenol-chloroform-proteinase K method (Klinbunga *et al*., 1996). The quality and quantity of extracted DNA was electrophoretically determined using a 0.8 % agarose gel (w/v) and spectrophotometrically estimated, respectively. High molecular weight DNA at 23.1 kb was obtained (Figure 3.1). The ratio of OD_{260}/OD_{280} of extracted DNA ranged from 1.8 – 2.0 indicating that DNA samples were relatively pure. DNA samples showing the ratio much lower then 1.8 was possibly contaminated with residual protein or phenol. In contrast, those with the ratio greater than 2.0 may be contaminated with RNA. Extracted DNA was stored at 4^{o}C until needed.

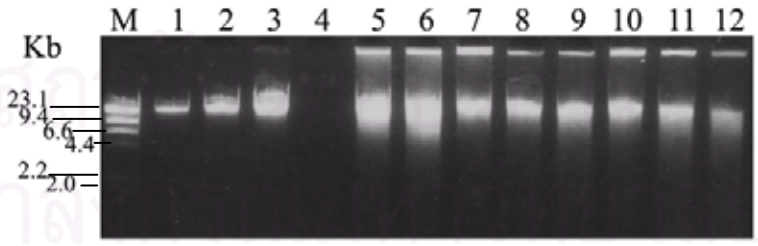

Figure 3.1 A 0.8% ethidium bromide stained agarose gel showing the quality of genomic DNA individually extracted from the pleopod of *P. monodon* (lanes 5 - 6), *P. semisulcatus* (lanes 7 - 8), *F. merguiensis* (lanes 9 - 10), *L. vannamei* (lane 11) and *M. japonicus* (lane 12). Lane M = λDNA digested with *Hind* III. Lanes 1 - 3 = 50, 100 and 200 ng of undigested λDNA, respectively.

3.1.2 PCR-RFLP and SSCP analysis

The amplification product of COI-COII and 16S $rDNA_{560}$ generated from universal primers was approximately 1550 and 560 bp in length (hereafter called COI-$COII_{1550}$ and 16S $rDNA_{560}$). This former fragment was successfully amplified in *P. monodon* and *P. semiosulcatus* but not in other species whereas the latter was obtained from all five shrimp species. Polymorphism of the COI-$COII_{1550}$ gene segment of *P. monodon* (*N* = 29) and *P. semisulcatus* (*N* = 15) was then further examined. In addition, the 16S $rDNA_{560}$ fragment of *P. monodon* (*N* = 29), *P. semisulcatus* (*N* = 15), *F. merguiensis* (*N* = 14), *L. vannamei* (*N* = 14) and *M. japonicus* (*N* = 7) was also analyzed by PCR-RFLP (Figure 3.2).

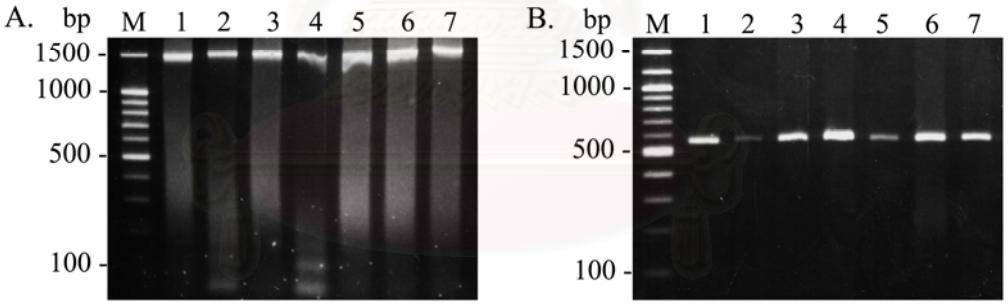

Figure 3.2 A 1.0% ethidium bromide stained agarose gel illustrating the amplification products of COI-COII (approximately 1550 bp, A) and 16S rDNA (approximately 560 bp, B) against genomic DNA of *P. monodon* (lanes 1 and 2), *P. semisulcatus* (lane 3 and 4), *F. merguiensis* (lane 5), *L. vannamei* (lane 6) and *M. japonicus*.(lane 7). Lanes M = 100 bp DNA marker.

The amplified COI-COII$_{1550}$ and 16S rDNA$_{560}$ products were singly digested with 15 restriction endonucleases including *Alu* I, *Bam*H I, *Bgl* II, *Dra* I, *Dde* I, *Eco*R I, *Hae* III, *Hind* III, *Hin*f I, *Kpn* I, *Mbo* I, *Nde* I, *Sal* I, *Ssp* I and *Vsp* I. The restricted products were electrophoresed through 1.0% (COI-COII$_{1550}$) and 1.5% (16S rDNA) agarose gels as shown in Figures 3.3 - 3.4, respectively. Three (*Dra* I, *Ssp* I and *Vsp* I) and four (*Alu* I, *Mbo* I, *Ssp* I and *Vsp* I) restriction endonucleases yield polymorphic patterns in COI-COII$_{1550}$ and 16S rDNA$_{560}$ of investigated shrimps, respectively (Table 3.1).

PCR-RFLP analysis of COI-COII$_{1550}$ generated 2, 5 and 4 restriction patterns from *Dra* I, *Ssp* I and *Vsp* I. Shared restriction patterns were not observed. Accordingly, species origin of *P. monodon* and *P. semisulcatus* can be simply examined.

Combined restriction patterns generated four mitotypes (ABA, AAA, AEA and AAD) in *P. monodon* and four mitotypes (BCB, BDB, BDC and BCC) in *P. semisulcatus* (Table 3.2). These mitotypes was not overlapped between *P. monodon* and *P. semisulcatus*. As a result, these two morphologically similar shrimps could simply be differentiated by restriction patterns and mitotypes generated from PCR-RFLP of COI-COII$_{1550}$.

Four, four, three and four restriction patterns were generated from digestion of 16S rDNA$_{560}$ of 5 penaeid shrimps with *Alu* I, *Mbo* I, *Ssp* I, *Vsp* I. Species origins of investigated shrimps cannot be authenticated using restriction patterns of each restriction enzyme.

A total of 11 mitotypes were found from combining restriction patterns of the 16S rDNA$_{560}$ of each shrimp individual digested with *Alu* I, *Mbo* I, *Ssp* I, *Vsp* I. Five mitotypes were found in *P. monodon* (AAAA, ACBA, ADCB, DDBD and DDCD), three mitotypes were found in *L. vannamei* (CBBC, CBBB and AABC) and only one mitotype found in *P. semisulactus* (BABB), *M. japonicus* (BABB) and *F. merguiensis* (BAAB). Therefore, morphologically similar shrimps for example, between *P. monodon* and *P. semisulcatus* and between *P. vannamei* and *P. merguiensis*, could

unambiguously differentiate. Nevertheless, shared mitotype (BABB) was found in *P. semisulactus* and *M. japonicus*.

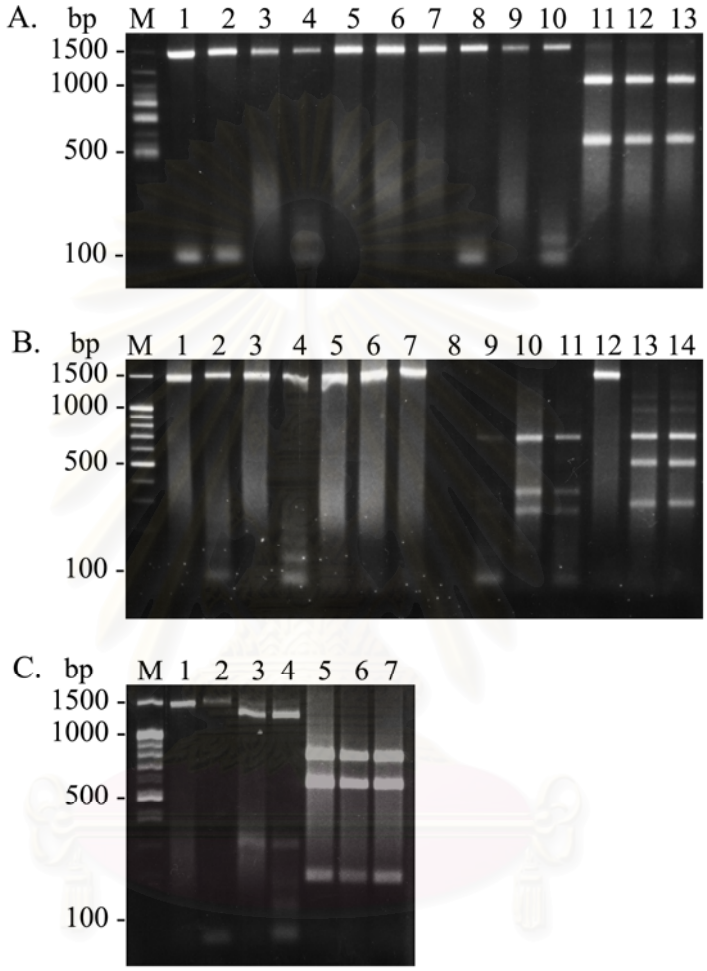

Figure 3.3 Restriction analysis of COI-COII$_{1550}$ of *P. monodon* and *P. semisulcatus* various restriction enzymes.

Panel A: Lanes 2 - 4 and 8 - 10 =COI of *P. monodon*, lanes 5 - 7 and 11 - 13 = COI of *P. semisulcatus* digested with *Bam* HI and *Dra* I, respectively.

Panel B: Lanes 2 - 4 and 9 - 11 = COI of *P. monodon*, lanes 5 - 7 and 12 - 14 = COI of *P. semisulcatus* digested with *Kpn* HI and *Vsp* I, respectively.

Panel C: Lanes 2 - 4 = COI of *P. monodon* and lanes 5 - 7 = COI of *P. semisulcatus* digested with *Ssp* I.

Lanes M and 1 are 100 bp DNA marker and undigested PCR product, respectively.

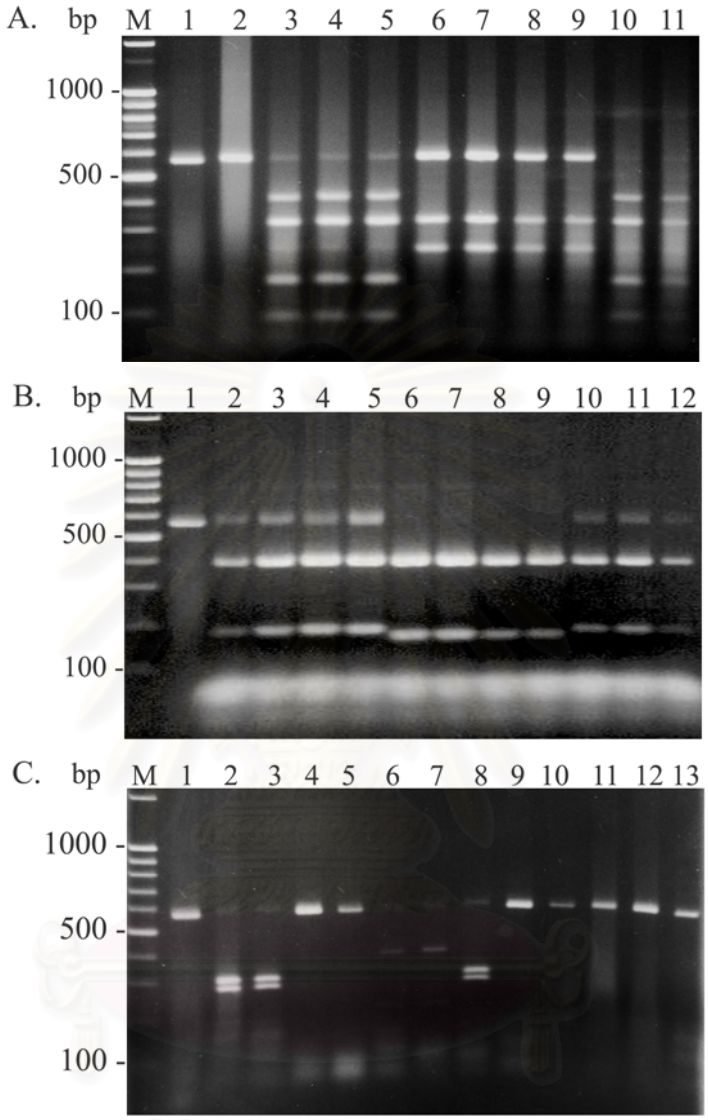

Figure 3.4 Restriction analysis of 16S rDNA$_{560}$; *P. monodon* (lanes 2, panel A and 2-3, panel C), *P. semisulcatus* (lanes 3 – 5, panel A, 2 – 3, panel B and 4 – 5, panel C), *F. merguiensis* (lanes 6 – 7, panel A, 6 – 9 and panel B), *L. vannamei* (lanes 8 – 9, panel A and 10 – 11, panel B) and *M. japonicus* (lanes 10 – 11, panel A and 12, panel B) with *Alu* I (panel A), *Mbo* I (panel B) and *Vsp* I (panel C). Lanes M and 1 are a 100 bp DNA marker and undigested PCR product, respectively.

Table 3.1 Restriction fragment patterns resulted from digestion of mitochondrial gene segments (COI-$COII_{1550}$ and 16S $rDNA_{560}$) of *P. monodon* (PM), *P. semisulcatus* (PS), *F. merguiensis* (FM), *L. vannamei* (LV) and *M. japonicus* (MJ) with various restriction enzymes

Gene/Enzyme	Pattern observed (bp)	PM	PS	FM	LV	MJ
$COI\text{-}COII_{1550}$						
Dra I	A: 1550	+	-	ND	ND	ND
	B: 1000, 500	-	+	ND	ND	ND
Ssp I	A: 1550	+	-	ND	ND	ND
	B: 1250, 280	+	-	ND	ND	ND
	C: 800, 600	-	+	ND	ND	ND
	D: 800, 500, 100	-	+	ND	ND	ND
	E: 960, 580	+	-	ND	ND	ND
Vsp I	A: 700, 380, 300	+	-	ND	ND	ND
	B: 700,550, 350	-	+	ND	ND	ND
	C: 1550	-	+	ND	ND	ND
	D: 1100, 380	+	-	ND	ND	ND
16S $rDNA_{560}$						
Alu I	A: 560	+	-	-	+	-
	B: 320, 160, 80	-	+	+	-	+
	C: 320, 240	-	-	-	+	-
	D: 260, 100, 70, 70	+	-	-	-	-
Mbo I	A: 380, 170	+	+	+	+	+
	B: 380, 160	-	-	-	+	-
	C: 280, 170, 110	+	-	-	-	-
	D: 320, 270	+	-	-	-	-
Ssp I	A: 340, 220	+	-	+	-	-
	B: 560	+	+	-	+	+
	C: 470, 70	+	-	-	-	-

Table 3.1 (continued)

Gene/Enzyme	Pattern observed (bp)	PM	PS	FM	LV	MJ
16S $rDNA_{560}$						
Vsp I	A: 290, 270	+	-	-	-	-
	B: 560	+	+	+	+	+
	C: 370, 190	-	-	-	+	-
	D: 460, 100	+	-	-	-	-

+ = found in a particular species, - = not found, ND = not determined

SSCP which is an effective non-sequencing-based method for detection of sequence variation of in DNA fragments due to single strand conformation were then applied. Results illustrated that shrimps exhibiting a BBAB mitotype was able to be consistently differentiated by this approach (Figure 3.5).

Amplification of the 16S $rDNA_{560}$ was carried out using larger sample sizes of each species. However, amplification of 16S $rDNA_{560}$ in *L. vannamei* and *F. merguiensis* using the original universal primers was not consistently successful. As a result, more specific and reliable primers need to be developed. The 16S $rDNA_{560}$ of a representative individual exhibiting the most common mitotype of each species; AAAA in *P. monodon*, BABB in *P. semisulcatus*, CBBC in *L. vannamei*, BAAB in *F. merguiensis* and BABB in *M. japonicus*, was amplified. The amplification product was gel-eluted, cloned and sequenced for both directions (Figure 3.6). The nucleotide sequences of 5 penaeid shrimps were blasted against data in the GenBank and results from Blast *N* indicating that they are 16S rDNA gene segment.

The actual length of this fragment was 561 bp in *P. semisulcatus* and 562 bp in other species. After multiple alignment, the conserved and polymorphic regions were observed (Figure 3.7).

Table 3.2 Mitotypes of 5 penaeid shrimps; *P. monodon* (PM), *P. semisulcatus* (PS), *F. merguiensis* (FM), *L. vannamei* (LV) and *M. japonicus* (MJ), analysed by PCR-RFLP of COI-COII$_{1550}$ and 16S rDNA$_{560}$ gene segments

Gene	Mitotype	Frequency				
		PM	PS	FM	LV	MJ
COI-COII$_{1550}$						
	ABA	0.481	-	ND	ND	ND
	AAA	0.407	-	ND	ND	ND
	AEA	0.074	-	ND	ND	ND
	AAD	0.037	-	ND	ND	ND
	BCB	-	0.667	ND	ND	ND
	BDB	-	0.200	ND	ND	ND
	BDC	-	0.067	ND	ND	ND
	BCC	-	0.067	ND	ND	ND
16S rDNA$_{560}$						
	AAAA	0.414	-	-	-	-
	ACBA	0.345	-	-	-	-
	ADCB	0.034	-	-	-	-
	DDBD	0.034	-	-	-	-
	AABB	0.069	-	-	-	-
	DDCD	0.103	-	-	-	-
	BABB	-	1.000	-	-	1.000
	CBBC	-	-	-	0.643	-
	CBBB	-	-	-	0.214	-
	AABC	-	-	-	0.143	-
	BAAB	-	-	1.000	-	-

ND = not determined

Sequence divergence (Kimura, 1980) between pairs of 16S $rDNA_{560}$ was 5.76% (between *P. semisulcatus* and *F. merguiensis*) - 10.23% (between *M. japonicus* and *L. vannamei*). The divergence between taxonomically problematic species was 6.15% between *P. monodon* and *P. semisulcatus* and 9.80% between *L. vannamei* and *F. merguiensis*, respectively.

Due to high intraspecific genetic diversity previously reported in *P. monodon* (Klinbunga *et al.*, 1999 and 2001b) and *F. merguiensis* (Hualkasin *et al.*, 2003; Wanna *et al.*, 2004), species-specific PCR (presence/ absence of the amplification band) for each species was not directly developed because serious false negative may be occurred when specimens from new geographic samples are analyzed. Alternatively, a pair of primers primed ($16Pmo_{312}$-F/R) at the conserved region of 16S rDNA providing a 312 bp fragments (hereafter called $16Pmo_{312}$) was designed.

Restriction analysis of 16S $rDNA_{312}$ with *Alu* I, *Ssp* I and *Vsp* I generated 3, 2 and 3 digestion patterns from *Alu* I, *Ssp* I and *Vsp* I, respectively. The *Alu* I-16S $rDNA_{312}$ patterns of *P. monodon* and *L. vannamei* were A and C whereas the B pattern was found in *P. semisulcatus*, *F. merguiensis* and *M. japonicus*. Moreover, the pattern C was restrictively observed in 16S $rDNA_{312}$ of *L. vannamei* digested with *Vsp* I. As a result, species-origin of *P. monodon* and *L. vannamei* can be identified by restriction patterns of *Alu* I and *Vsp* I (Table 3.3).

When the restriction pattern of each shrimp generated from *Alu* I, *Ssp* I and *Vsp* I was combined, 3, 1, 2, 1, 1 mitotypes were found in *P. monodon* (AAA, ABA, ABB), *P. semisulcatus* (BBB), *L. vannamei* (CBC, BBC), *F. merguiensis* (BAB) and *M. japonicus* (BBB), respectively (Figure 3.9 and Table 3.4).

Non-overlapping mitotypes were observed in *P. monodon*, *L. vannamei* and *F. merguiensis* (Table 3.4). As a result, morphologically similar shrimps; between *P. monodon* and *P. semisulcatus* and between *L. vannamei* and *F. merguiensis* were successfully differentiated. However, shared mitotype (BBB) was observed in *P. semisulcatus* and *M. japonicus*. SSCP analysis was then applied for further diagnosis.

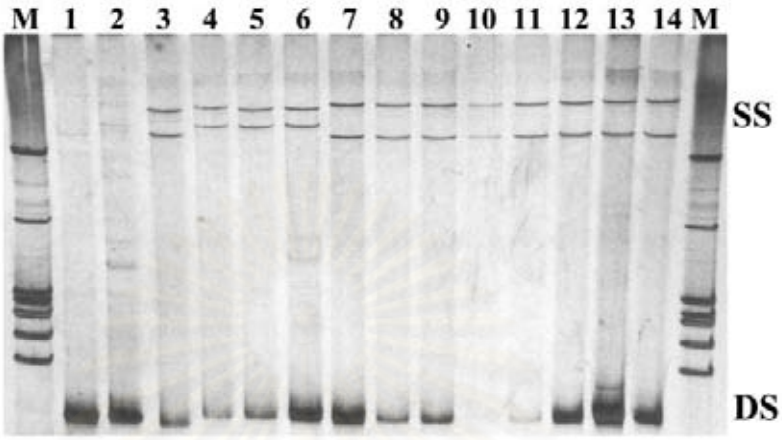

Figure 3.5 SSCP patterns of a 560 bp fragment of 16S rDNA$_{560}$ of *P. semisulcatus* (lanes 7-14) and *M. japonicus* (lanes 4-6) that shared a BABB mitotype electrophoresed through a 12.5% non-denaturing polyacrylamide gel (37.5:1 crosslink). Lane M, 1 and 2 are 100 bp DNA marker and the non-denatured PCR products, respectively. The PCR product of *P. monodon* (lane 3) was included. DS and SS are double-stranded and single-stranded PCR product, respectively.

A.

CGCCTGTTTAACAAAAACATGTCTATATGATTGTTATATAAAGTCTAGCCTGCCCACTGAATTATTTTTAAAGGGC
CGCGGTATACTGACCGTGCGAAGGTAGCATAATCATTAGTCTTTTAATTGAAGGCTTGTATGAATGGTTGGACAAA
AAGTAATCTGTCTCAGTTATAATAGTTGAACTTAACTTTTAAGTGAAAAGGCTTAAATACTTTAAGGGGACGATAA
GACCCTATAAAACTTAACAATAATTTGATTAAATTATAAATTGTTAGTATAACTTGATTTTAATTAATGTTTGTTG
CGTTGGGGCGACGGGAATATAATTAGTAACTGTTCTTAAATATTTTATTAACAAGTATAATTGAAGAATAATTGAT
CCTTTATTAAAGATTAAAAGATTAAGTTACTTTAGGGATAACAGCGTAATCTTCTTTGAGAGTCCTCATCGACAAG
AAGGTTTGCGACCTCGATGTTGAATTAAGGTATCCTTATAATGCAGCAGTTATAAAGGAAGGTCTGTTCGACCTTT
AAATCCTT**ACATGATCTGAGTTCAGACCGG**

B.

CGCCTGTTTAACAAAAACATGTCTATATGATTGTTATATAAAGTCTAGCCTGCCCACTGATTGAAATTTAAAGGGC
CGCGGTATACTGACCGTGCGAAGGTAGCATAATCATTAGTCTTTTAATTGAAGGCTTGTATGAATGGTTGGACAAA
AAGTAAGCTGTCTCAGTTATAATAATTGAATTTAACTTTTAAGTGAAAAGGCTTAAATGGATTAAGGGGACGATAA

GACCCTATAAAGCTTGACAATAAGTTAATTATATTATAAATTGTTAGTATAACTTGATTTTAATTGACGTTTGTTA
CGTTGGGGCGACGAGAATATAATGAGTAACTGTTCTTAAATGTTTATTGACAAATATAATTGGTATTTGATTGATC
CTTTATTAAAGATTAAAAGATTAAGTTACTTTAGGGATAACAGCGTAATCTTCTTTGAGAGTTCATATCGACAAGA
AGGTTTGCGACCTCGATGTTGAATTAAGGTATCCTTATGATGCAGCAGTTATAAAGGAAGGTCTGTTCGACCTTTA
AATCCTT**ACATGATCTGAGTTCAGACCGG**

C.

CGCCTGTTTAACAAAAACATGTCTATATGATTGGTATGTAAAGTCTGGCCTGCCCACTGATTTATTTTAAAGGGCC
GCGGTATACTGACCGTGCGAAGGTAGCATAATCATTAGTCTCTTAATTGGAGGCTTGTATGAATGGTTGGACAAAA
AGCAAACTGTCTCAATTATATTTATTGAATTTAACTTTTAAGTGAAAAGGCTTAAATAAATTAAGGGGACGATAAG
ACCCTATAAAGCTTTACAATAAGTTACCTATATTATAAATTGTTAGTATAACTTGAGTTTAGGTAACGTTTGTTGC
GTTGGGGCGACGAGAATATAATAAGTAACTGTTCTTAAGTTATTTAATGACAGAAATTTCTGGAAAATTAATGATC
CTCTACTAGAGATCATAAGATTAAGTTACTTTAGGGATAACAGCGTAATCTTCTTTGAGAGTCCACATCGACAGGA
AGGGTTGCGACCTCGATGTTGAATTAAGGGTTCCTTATAATGCAGCAGTTATAAAGGAGGGTCTGTTCGACCTTTA
AATCCTT**ACATGATCTGAGTTCAGACCGGA**

D.

CGCCTGTTTAACAAAAACATGTCTATATGATTGTTATATAAAGTCTAGCCTGCCCACTGATTTAGTTTAAAGGGCC
GCGGTATATTGACCGTGCGAAGGTAGCATAATCATTAGTCTTTTAATTGAAGGCTTGTATGAATGGTTGGACAAAA
AGTAAGCTGTCTCAATTATAATGATTGAACTTAACTTTTAAGTGAAAAGGCTTAAATAAATTAAGGGGACGATAAG
ACCCTATAAAGCTTGACAATAATTTAATTATACTATCAATTGTTAGTATAACTTGGTTTTAATTAAGATTTGTTGC
GTTGGGGCGACGAGAATATAATAGGTAACTGTTCTTAAATATTTAATAACAAATATAATTGAAAATTAGTGTGATC
CTCTATTAGCGATTAAAAGATTAAGTTACTTTAGGGATAACAGCGTAATCTTCTTTGAGAGTCCCTATCGACAAGA
AGGTTTGCGACCTCGATGTTGAATTAAGGTATCCTTATGATGCAGCAGTTATANAGGAAGGTCTGTTCGACCTTTA
AATCCTT**ACATGATCTGAGTTCAGACCGGA**

E.

CGCCTGTTTAACAAAAACATGTCTATATGATTGTTATATAAAGTCTAGCCTGCCCACTGATTTGTTTTAAAGGGCC
GCGGTATATTGACCGTGCGAAGGTAGCATAATCATTAGTCTTTTAATTGGAGGCTTGTATGAATGGTTGGACAAAA
AGTAAGCTGTCTCGATTATAATAATTGAACTTAACTTTTAAGTGAAAAGGCTTAAATGTTTCAGGGGGACGATAAG
ACCCTATAAAGCTTGACAATAACTTCGTTATATTATAAATTGTTAGTATAACTTGATTTTAACGGGGGTTTGTTTC
GTTGGGGCGACGGGAATATAATAAATAACTGTTCTTTTAAATATAATTACAAAAATGTTTGGTAAATAATTGATCC
TCTATTAGAGATTAAAAGATTAAGTTACTTTAGGGGATAACAGCGTAATCTTCTTTGAGAGTCCACATCGACAAGA
AGGTTTGCGACCTCGATGTTGAATTAAGGTATCCTTATAATGCAGCAGTTATAAAGGAGGGTCTGTTCGACCTTTA
AATCCTT**ACATGATCTGAGTTCAGACCGGA**

Figure 3.6 Nucleotide sequences of 16S rDNA$_{560}$ of *P. monodon* (A), *P. semisulcatus* (B), *L. vannamei* (C), *F. merguiensis* (D) and *M. japonicus* (F). Sequences and positions of the forward primer and those complementary to the reverse primer are underlined and boldfaced.

```
P. semisulcatus     CGCCTGTTTAACAAAAACATGTCTATATGATTGTTATATAAAGTCTAGCCTGCCCACTGA
P. monodon          CGCCTGTTTAACAAAAACATGTCTATATGATTGTTATATAAAGTCTAGCCTGCCCACTGA
F. merguiensis      CGCCTGTTTAACAAAAACATGTCTATATGATTGTTATATAAAGTCTAGCCTGCCCACTGA

M. japonicus        CGCCTGTTTAACAAAAACATGTCTATATGATTGTTATATAAAGTCTAGCCTGCCCACTGA
L. vannamei         CGCCTGTTTAACAAAAACATGTCTATATGATTGGTATGTAAAGTCTGGCCTGCCCACTGA
                    ********************************** *** ******** *************

P. semisulcatus     TTGAAATTTAAAGGGCCGCGGTATACTGACCGTGCGAAGGTAGCATAATCATTAGTCTTT
P. monodon          ATTATTTTTAAAGGGCCGCGGTATACTGACCGTGCGAAGGTAGCATAATCATTAGTCTTT
F. merguiensis      TTTAG-TTTAAAGGGCCGCGGTATATTGACCGTGCGAAGGTAGCATAATCATTAGTCTTT
M. japonicus        TTTGT-TTTAAAGGGCCGCGGTATATTGACCGTGCGAAGGTAGCATAATCATTAGTCTTT
L. vannamei         TTTAT-TTTAAAGGGCCGCGGTATACTGACCGTGCGAAGGTAGCATAATCATTAGTCTCT
                    *    ****************** ******************************** *

                              16Pmo312-F
P. semisulcatus     TAATTGAAGGCTTGTATGAATGGTTGGACAAAAAGTAAGCTGTCTCAGTTATAATAATTG
P. monodon          TAATTGAAGGCTTGTATGAATGGTTGGACAAAAAGTAATCTGTCTCAGTTATAATAGTTG
F. merguiensis      TAATTGAAGGCTTGTATGAATGGTTGGACAAAAAGTAAGCTGTCTCAATTATAATGATTG
M. japonicus        TAATTGGAGGCTTGTATGAATGGTTGGACAAAAAGTAAGCTGTCTCGATTATAATAATTG
L. vannamei         TAATTGGAGGCTTGTATGAATGGTTGGACAAAAAGCAAACTGTCTCAATTATATTTATTG
                    ****** **************************** ** *******  ***** *  ***

P. semisulcatus     AATTTAACTTTTAAGTGAAAAGGCTTAAATGGATTAAGGGGACGATAAGACCCTATAAAG
P. monodon          AACTTAACTTTTAAGTGAAAAGGCTTAAATACTTTAAGGGGACGATAAGACCCTATAAAA
F. merguiensis      AACTTAACTTTTAAGTGAAAAGGCTTAAATAAATTAAGGGGACGATAAGACCCTATAAAG
M. japonicus        AACTTAACTTTTAAGTGAAAAGGCTTAAATGTTTCAGGGGGACGATAAGACCCTATAAAG
L. vannamei         AATTTAACTTTTAAGTGAAAAGGCTTAAATAAATTAAGGGGACGATAAGACCCTATAAAG
                    ** ***************************  * * **********************

P. semisulcatus     CTTGACAATAAGTTAATTATATTATAAATTGTTAGTATAACTTGATTTTAATTGACGTTT
P. monodon          CTTAACAATAATTTGATTAAATTATAAATTGTTAGTATAACTTGATTTTAATTAATGTTT
F. merguiensis      CTTGACAATAATTTAATTATACTATCAATTGTTAGTATAACTTGGTTTTAATTAAGATTT
M. japonicus        CTTGACAATAACTTCGTTATATTATAAATTGTTAGTATAACTTGATTTTAACGGGGGTTT
L. vannamei         CTTTACAATAAGTTACCTATATTATAAATTGTTAGTATAACTTGAGTTTAGGTAACGTTT
                    *** ******* **   ** * *** ******************  ****      ***

P. semisulcatus     GTTACGTTGGGGCGACGAGAATATAATGAGTAACTGTTCTTAAATGTTT-ATTGACAAAT
P. monodon          GTTGCGTTGGGGCGACGGGAATATAATTAGTAACTGTTCTTAAATATTTTATTAACAAGT
F. merguiensis      GTTGCGTTGGGGCGACGAGAATATAATAGGTAACTGTTCTTAAATATTT-AATAACAAAT
M. japonicus        GTTTCGTTGGGGCGACGGGAATATAATAAATAACTGTTCTTTTAAATAT-AATTACAAAA
L. vannamei         GTTGCGTTGGGGCGACGAGAATATAATAAGTAACTGTTCTTAAGTTATTTAATGACAGAA
                    *** ************* *********   ***********       * * * ***
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P. semisulcatus     ATAATTGGTATTTGAT-TGATCCTTTATTAAAGATTAAAAGATTAAGTTACTTTAGGG-A
P. monodon          ATAATTGAAGAATAAT-TGATCCTTTATTAAAGATTAAAAGATTAAGTTACTTTAGGG-A
F. merguiensis      ATAATTGAAAATTAGTGTGATCCTCTATTAGCGATTAAAAGATTAAGTTACTTTAGGG-A
M. japonicus        ATGTTTGGTAAATAAT-TGATCCTCTATTAGAGATTAAAAGATTAAGTTACTTTAGGGGA
L. vannamei         ATTTCTGGAAAATTAA-TGATCCTCTACTAGAGATCATAAGATTAAGTTACTTTAGGG-A
                    **   **     *    ******* ** **  *** * ******************* *

                          16Pmo312-R
P. semisulcatus     TAACAGCGTAATCTTCTTTGAGAGTTCATATCGACAAGAAGGTTTGCGACCTCGATGTTG
P. monodon          TAACAGCGTAATCTTCTTTGAGAGTCCTCATCGACAAGAAGGTTTGCGACCTCGATGTTG
F. merguiensis      TAACAGCGTAATCTTCTTTGAGAGTCCCTATCGACAAGAAGGTTTGCGACCTCGATGTTG
M. japonicus        TAACAGCGTAATCTTCTTTGAGAGTCCACATCGACAAGAAGGTTTGCGACCTCGATGTTG
L. vannamei         TAACAGCGTAATCTTCTTTGAGAGTCCACATCGACAGGAAGGGTTGCGACCTCGATGTTG
                    ************************ *  ******* ***** ****************

P. semisulcatus     AATTAAGGTATCCTTATGATGCAGCAGTTATAAAGGAAGGTCTGTTCGACCTTTAAATCC
P. monodon          AATTAAGGTATCCTTATAATGCAGCAGTTATAAAGGAAGGTCTGTTCGACCTTTAAATCC
F. merguiensis      AATTAAGGTATCCTTATGATGCAGCAGTTATANAGGAAGGTCTGTTCGACCTTTAAATCC
M. japonicus        AATTAAGGTATCCTTATAATGCAGCAGTTATAAAGGAGGGTCTGTTCGACCTTTAAATCC
L. vannamei         AATTAAGGGTTCCTTATAATGCAGCAGTTATAAAGGAGGGTCTGTTCGACCTTTAAATCC
                    ********  ******* ************** **** **********************

P. semisulcatus     TTACATGATCTGAGTTCAGACCGG-
P. monodon          TTACATGATCTGAGTTCAGACCGG-
F. merguiensis      TTACATGATCTGAGTTCAGACCGGA
M. japonicus        TTACATGATCTGAGTTCAGACCGGA
L. vannamei         TTACATGATCTGAGTTCAGACCGGA
                    ************************
```

Figure 3.7 Multiple alignments of 16S rDNA$_{560}$ sequences of *P. monodon*, *P. semisulcatus*, *F. merguiensis, L. vannamei* and *M. japonicus*. Asterisks indicated identical bases among different sequences. A new pair of forward and reverse primers generating the expected 312 bp product was designed. Sequences and positions of the forward primer and those complementary to the reverse primer are underlined and boldfaced.

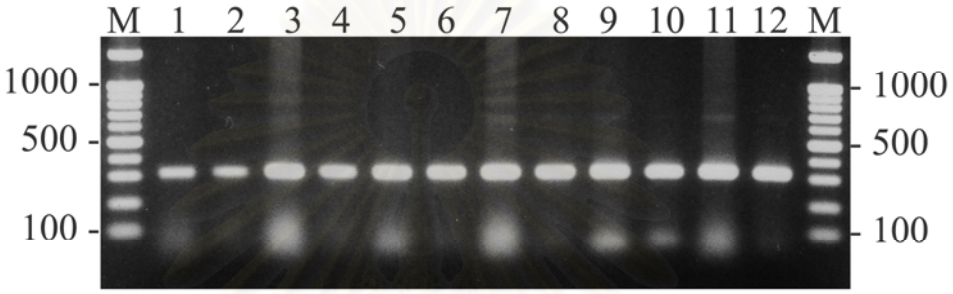

Figure 3.8 A 1.2% ethidium bromide stained agarose gel illustrating the amplification products of 16 rDNA gene segment (312 bp in length) using genomic DNA of *P. monodon* (lanes 1 – 4), *P. semisulcatus* (lanes 5 – 6), *F. merguiensis* (lanes 7 – 8), *L. vannamei* (lanes 9 – 10) and *M. japonicus*.(lanes 11 – 12) using newly designed primers; $16SPmo_{312}$–F/R. The PCR products were electrophoretically analyzed through a 1.2% agarose gel. Lane M = 100 bp DNA markers.

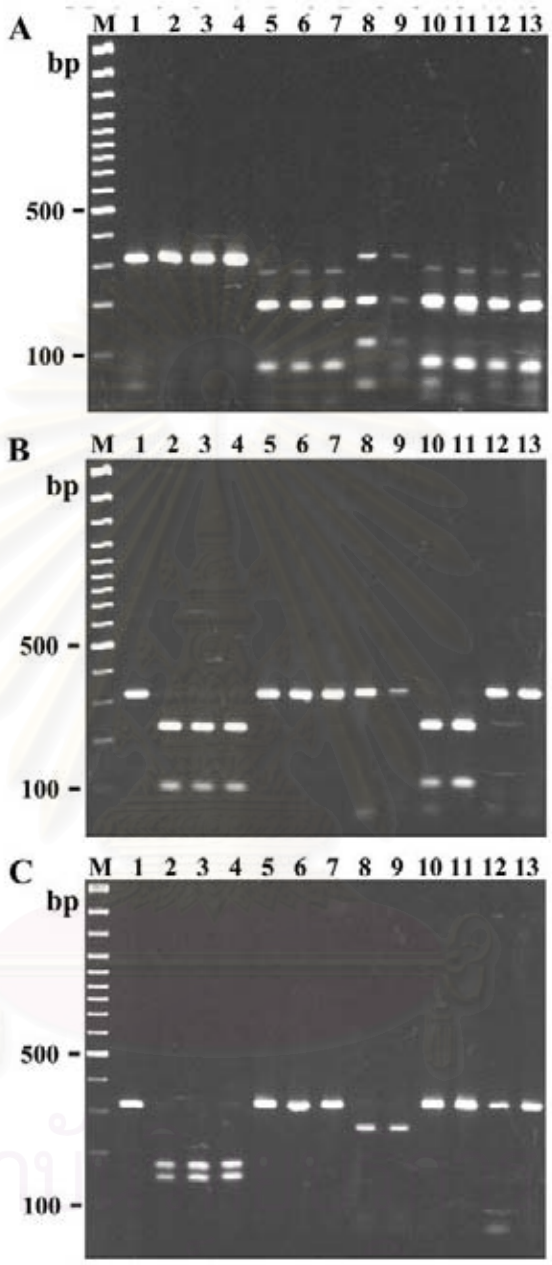

Figure 3.9 Restriction analysis of 16S rDNA$_{312}$ of *P. monodon* (lanes 2 – 4), *P. semisulcatus* (lanes 5 – 7), *F. merguiensis* (lanes 8 – 9), *L. vannamei* (lanes 10 – 11) and *M. japonicus* (lanes 12 – 13) with *Alu* I (panel A), *Ssp* I (panel B) and *Vsp* I (panel C). Lanes M and 1 are 100 bp DNA marker and the undigested PCR product, respectively.

Table 3.3 Restriction fragment patterns resulted from digestion of 16S $rDNA_{312}$ gene segments of *P. monodon* (PM), *P. semisulcatus* (PS), *F. merguiensis* (FM), *L. vannamei* (LV) and *M. japonicus* (MJ) with *Alu* I, *Ssp* I and *Vsp* I

Enzyme	Pattern observed (bp)	PM	PS	FM	LV	MJ
Alu I	A: 312	+	-	-	-	-
	B: 200, 90	-	+	+	+	+
	C: 200, 110	-	-	-	+	-
Ssp I	A: 220, 100	+	-	+	-	-
	B: 312	+	+	-	+	+
Vsp I	A: 170, 150	+	-	-	-	-
	B: 312	+	+	+	-	+
	C: 250, 70	-	-	-	+	-

Table 3.4 Mitotype frequencies from restriction analysis of the 16S $rDNA_{312}$ gene segments of 5 penaeid shrimps

Samples	Mitotype (*Alu* I, *Ssp* I, *Vsp* I)	No. of sample (*N*)	Location	Frequency
P. monodon	AAA	44	All geographic samples examined	0.512
	ABA	37	All geographic samples examined	0.430
	ABB	5	Satun (1), Trang (1), Trad(1) and Ranong(2)	0.058
P. semisulcatus	BBB	15	Chumphon(11), Phuket(4)	1.000
L. vannamei	CBC	29	All samples examined	0.967
	BBC	1	Ratchaburi	0.033
F. merguiensis	BAB	38	Samyan market(7) Chonburi (17) Indonesia(14)	1.000
M. japonicus	BBB	16	Japan	1.000

Species-specific SSCP patterns were verified against the same sample set of each shrimp species (N = 185) analyzed by PCR-RFLP of 16S $rDNA_{312}$. Non-overlapping SSCP patterns were found indicating successful development of species-diagnostic markers across all taxa (Figure 3.10 and Table 3.5). Therefore, reliable methods for identifying species origins of morphological similar shrimps were successfully developed based on PCR-RFLP and SSCP analysis of 16S $rDNA_{312}$.

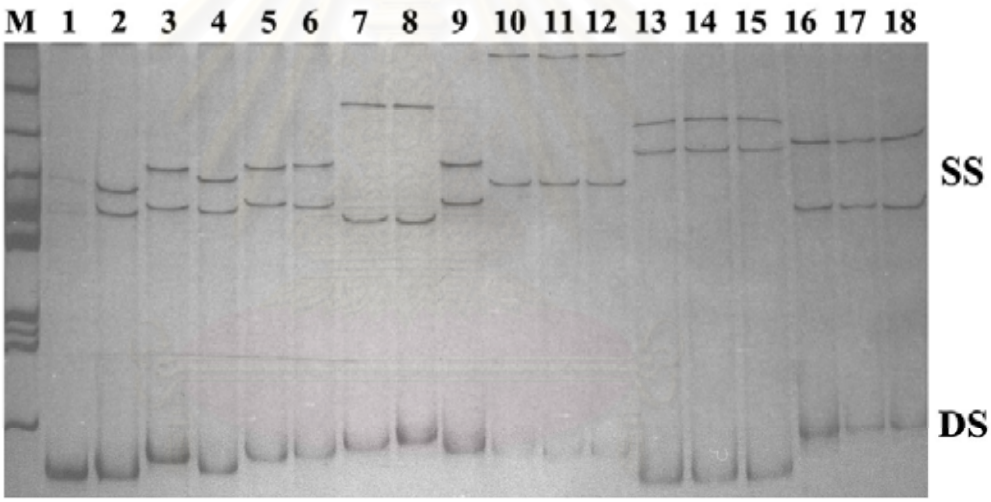

Figure 3.10 SSCP patterns of a 312 bp fragment generated from 16S $rDNA_{312}$-F/R using genomic DNA of *P. monodon* (lanes 2 - 6 and 9), *P. semisulcatus* (lanes 7 – 8), *L. vannamei* (lanes 10 – 12), *F. merguiensis* (lanes 13 – 15) and *M. japonicus* (lanes 16 – 18) and electrophoresed through a 15% non-denaturing polyacrylamide gel (37.5:1 crosslink). Lanes M and 1 are 100 bp DNA marker and the non-denatured PCR products, respectively. DS and SS are double and single strand PCR products.

Table 3.5 SSCP patterns of the 16S $rDNA_{312}$ gene segment of *P. monodon*, *P. semisulcatus*, *F. merguiensis*, *L. vannamei* and *M. japonicus*)

Mitotype/SSCP patterns	Frequency				
	PM	**PS**	**FM**	**LV**	**MJ**
AAA					
9/15	0.383	-	-	-	-
10/15	0.086	-	-	-	-
4/14	0.037	-	-	-	-
9/14	0.012	-	-	-	-
10/14	0.012	-	-	-	-
10/17	0.012	-	-	-	-
ABA					
11/16	0.395	-	-	-	-
10/16	0.025	-	-	-	-
13/17	0.025	-	-	-	-
10/15	0.012	-	-	-	-
ABB					
13/17	0.037	-	-	-	-
11/16	0.025	-	-	-	-
BBB					
3/18	-	1.000	-	-	-
7/17	-	-	-	-	1.000
CBC					
2/12	-	-	-	0.933	-
1/12	-	-	-	0.033	-
BBC					
2/12	-	-	-	0.033	-
BAB					
5/8	-	-	0.445	-	-
4/6	-	-	0.421	-	-
5/9	-	-	0.053	-	-
5/11	-	-	0.053	-	-
6/8	-	-	0.026	-	-

3.1.3 Application of developed species-diagnostic markers for species identification of suspected shrimps and frozen shrimp meat

Authentication of the correct seed species for the industry is essential for successful aquaculture of economically important species because wrong seed species may be intentionally supplied.

Two groups of cultured juvenile shrimps were sent to the Center of Excellence of Marine Biotechnology for identification of species. Farmer was told when purchased the larvae that the Group 1 sample was *F. merguiensis* from Indonesia and the Group 2 sample was hybrid offspring between *P. monodon* (sire) and *M. japonicus* (dam).

The amplification product of 16S $rDNA_{312}$ of Group 2 larvae was digested with *Alu* I and *Ssp* I and compared to restriction patterns of *P. monodon*, *F. merguiensis* and *M. japonicus*. Restriction patterns of specimens from both groups were identical to that of *F. merguiensis* indicated that larvae of both groups were *F. merguiensis* (BAB mitotype, Figure 3.11). SSCP analysis of the amplified 16S $rDNA_{312}$ further confirmed results from PCR-RFLP analysis (Figure 3.12).

In the other case, the frozen shrimp meat was introduced to Thailand from Vietnam and claimed as the product of the white shrimp (*P. orientalis*) and the pink shrimp (*Metapenaeus affinis*) from fisheries.

Results from PCR-RFLP analysis of 16S $rDNA_{312}$ revealed that the suspected *P. orientalis* exhibited a BAB mitotype restrictively found in *F. merguiensis* whereas the suspected *M. affinis* showed a DBB mitotype which were not found in *Penaeus* shrimps previously examined (Figure 3.13).

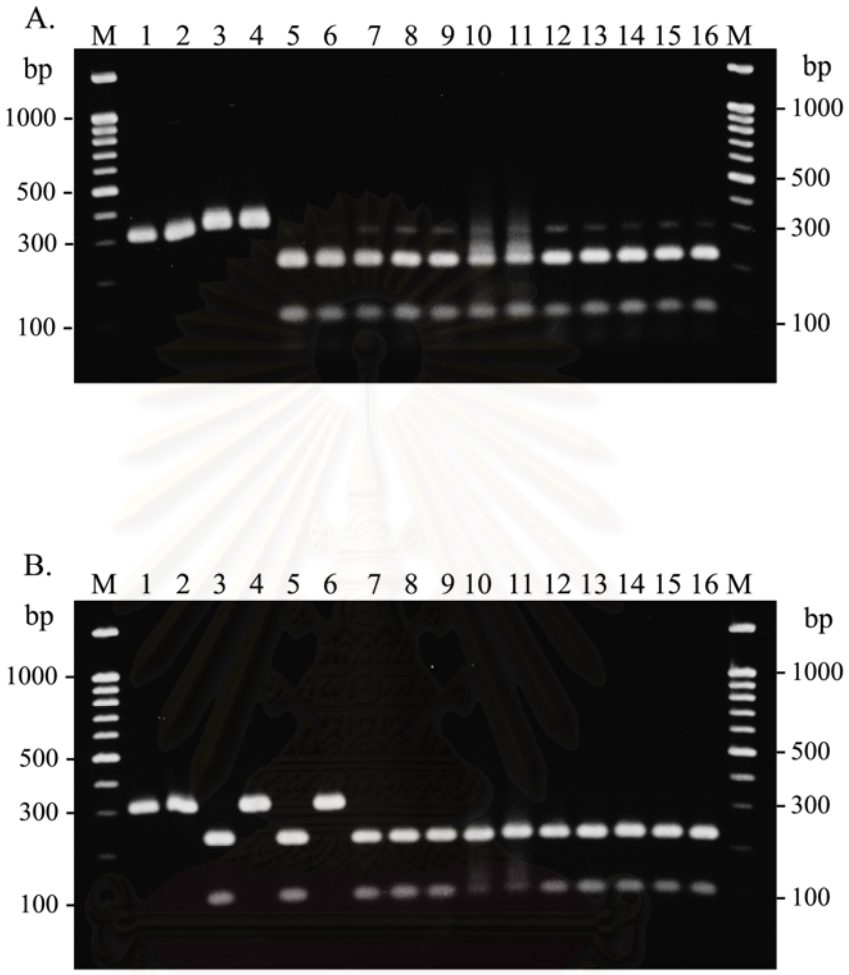

Figure 3.11 Restriction patterns of 16S $rDNA_{312}$ digested with *Alu* I (panel A) and *Ssp* I (panel B) of suspected cultured shrimps. Lanes M and 1 - 2 are 100 bp DNA marker and undigested PCR product group 1, group 2, respectively. Lanes 3 - 4 = *P. monodon*; lane 5 = *F. merguiensis*; lanes 6 = *M. japonicus*, lanes 7 - 11 = *F. merguiensis* offspring group 1 and lanes 12 - 16 = offspring group 2 claimed as hybrids between *P. monodon* (sire) and *M. japonicus* (dam).

SSCP analysis further confirmed that suspected *P. orientalis* was actually *F. merguiensis* (Figure 3.14). In addition, suspected *M. affinis* exhibited clearly different SSCP patterns with that of *P. semisulcatus*, *L. vannamei*, *F. merguiensis* and *M. japonicus*. Its polymorphic SSCP patterns were similar, but not identical, to that of *P. monodon* exhibiting an AAA mitotype. Based on large interspecific sequence divergence of 16S $rDNA_{560}$ found in the present study (5.76 - 10.23%), it could be concluded that suspected *M. affinis* should be a member of *Penaeus* rather than *Metapenaeus*.

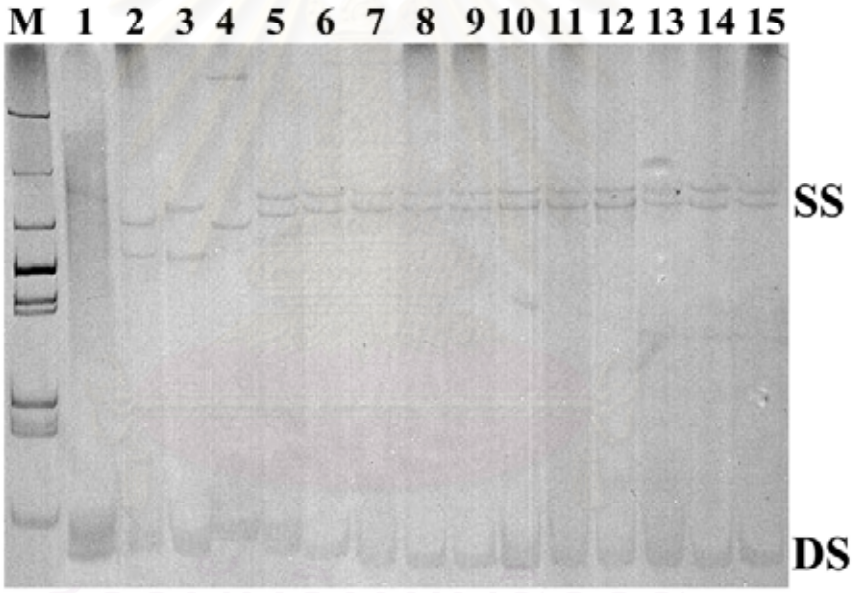

Figure 3.12 SSCP patterns of 16S $rDNA_{312}$ of suspected cultured shrimp. Lanes M and 1 are 100 bp DNA marker and non-denatured PCR product, respectively. Lanes 2 - 5 = 16S $rDNA_{312}$ of *P. monodon*, *M. japonicus*, *L. vannamei* and *F. merguiensis*. Lanes 6 - 10 = 16S $rDNA_{312}$ of *F. merguiensis* offspring group 1 and lanes 11 - 15 = 16S $rDNA_{312}$ of offspring group 2 which was claimed as hybrids between *P. monodon* (sire) and *M. japonicus* (dam).

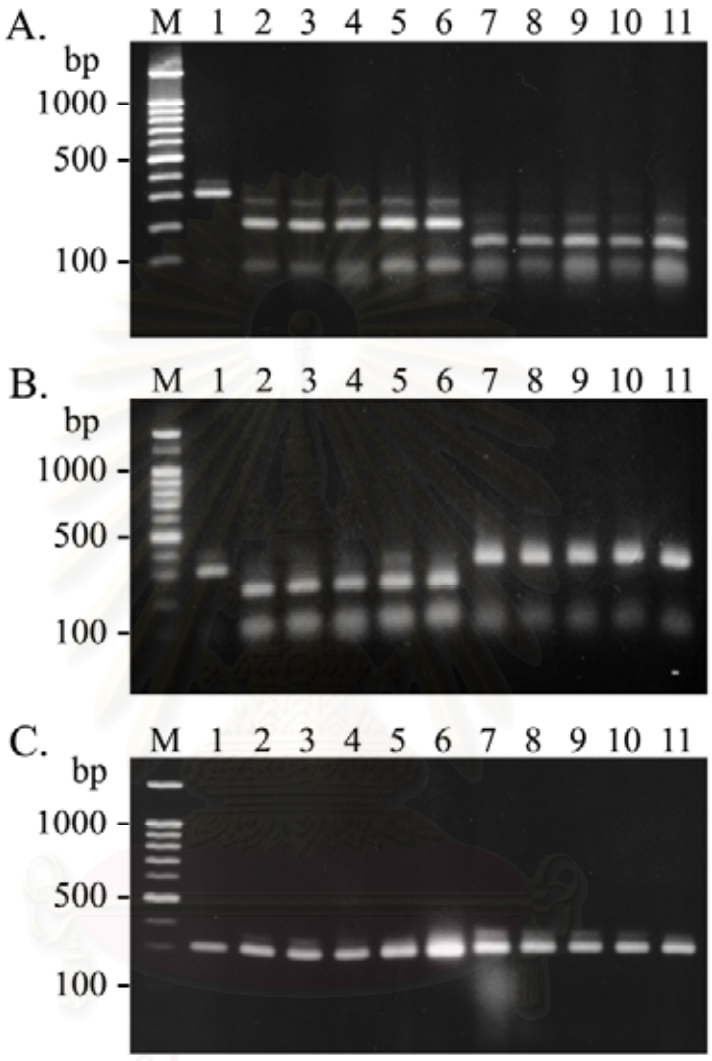

Figure 3.13 Restriction patterns of 16S rDNA$_{312}$ digested with *Alu* I (panel A), *Ssp* I (panel B) and *Vsp* I (panel C) of introduced frozen shrimps meat. Lanes M and 1 are 100 bp DNA marker and undigested PCR product, respectively. Lanes 2 - 6 = suspected white shrimps (*P. orientaris*) and lanes 7 - 11 = suspected pink shrimps (*Metapenaeus affinis*)

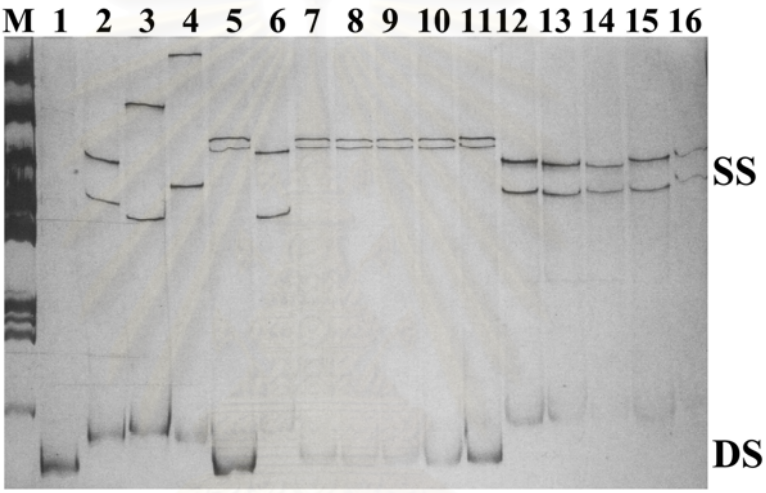

Figure 3.14 SSCP patterns of suspected frozen shrimp meat (claimed as *P. orientalis*, lanes 7 - 11 and *Metapenaeus affinis*, lanes 12 - 16. SSCP patterns of *P. monodon* (lane 2), *P. semisulcatus* (lane 3), *L. vannamei* (lane 4), *F. merguiensis* (lane 5) and *M. japonicus* (lane 6) were also included. Lanes M and 1 are a 100 bp DNA ladder and non-denatured 16S $rDNA_{312}$, respectively.

3.2 Genetic diversity and genotyping of *P. monodon* in Thai waters

3.2.1 Determination of 16S $rDNA_{312}$ polymorphism by PCR-RFLP and PCR-SSCP

Apart from the required information on genetic diversity and population subdivisions of *P. monodon* in Thai waters, the other major goal of genetic diversity studies in this thesis is identification of suitable molecular genetic markers that can be applied for rapid genotying of *P. monodon*. The developed markers will be used in combination with microsatellites for determination of genetic diversity levels of domesticated *P. monodon*. The rapid and simple genotying of *P. monodon* allow practical applications of polymorphic markers for management of both natural and domesticated broodstock of *P. monodon*.

The amplified 16S $rDNA_{312}$ of 86 individuals was initially analyzed by PCR-RFLP with 3 restriction endonucleases (*Alu* I, *Ssp* I and *Vsp* I). Only 3 mitotypes (AAA, ABA and ABB, Table 3.6) were observed. These PCR-RFLP mitotypes were distributed at approximately equal frequencies across different geographic samples. Geographic heterogeneity analysis using the exact test indicated that frequencies of PCR-RFLP mitotypes between pairs of geographic samples were not significantly different ($P > 0.05$, Table 3.7).

Table 3.6 Distribution of 16S $rDNA_{312}$ mitotypes across six geographic samples of *P. monodon* in Thai waters

Geographic sample	Mitotype			No. of sample
	AAA	ABA	ABB	
Trat	6	8	1	15
Chumphon	9	6	-	15
Satun	10	4	1	15
Trang	8	6	1	15
Phangnga	8	7	-	15
Ranong	3	6	2	11
Total	**44**	**37**	**5**	**86**

Table 3.7 Genetic heterogeneity analysis between pairs of geographic samples of *P. monodon* based on restriction analysis of 16S $rDNA_{312}$ with 3 restriction endonucleases analyzed by a Monte Carlo simulation

	Trat	**Chumphon**	**Satun**	**Trang**	**Phangnga**	**Ranong**
Trat	-					
Chumphon	0.4495^{ns}	-				
Satun	0.8428^{ns}	0.7001^{ns}	-			
Trang	0.8519^{ns}	1.0000^{ns}	0.8428^{ns}	-		
Phangnga	0.7229^{ns}	1.0000^{ns}	0.4478^{ns}	1.0000^{ns}	-	
Ranong	0.5434^{ns}	0.1135^{ns}	0.1758^{ns}	0.4542^{ns}	0.1950^{ns}	-

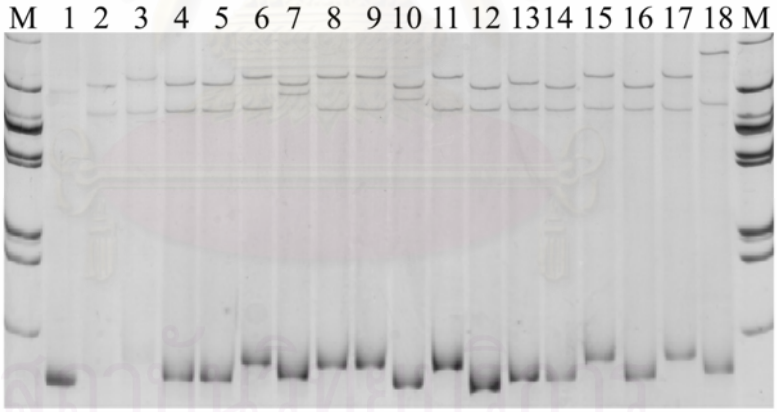

Figure 3.15 SSCP patterns of 16S $rDNA_{312}$ of *P. monodon* originating from Trang (lanes 2 and 13-15), Satun (lanes 3 and 10-12), Trat (lanes 4-6), Chumphon (lanes 7-9) and Phangnga (lanes 16-18). Lanes M and 1 are a 100 bp DNA marker and non-denatured 16S $rDNA_{312}$, respectively. DS and SS are double-stranded and single-stranded PCR product, respectively.

Table 3.8 SSCP patterns resulted from analysis of 16S $rDNA_{312}$ of *P. monodon* originating from different geographic samples in Thai waters

SSCP band pattern	**No. of individuals**					
	Trat (*N* = 32)	**Chumphon (*N* = 37)**	**Satun (*N* = 39)**	**Trang (*N* = 33)**	**Phangnga (*N* = 33)**	**Ranong (*N* = 11)**
9,15	11	14	15	13	13	1
11,16	10	11	10	9	9	5
4,14	1	3	-	1	1	-
10, 15	5	2	3	6	6	1
10,16	1	1	3	-	1	-
13,17	1	1	1	-	-	2
9,16	1	-	-	-	-	1
11,15	1	2	4	2	3	-
10,14	-	1	-	-	-	-
10,17	-	1	-	-	-	-
10,12	-	1	-	-	-	-
9,17	-	-	1	-	-	-
11,13	-	-	2	-	-	-
12,16	-	-	-	1	-	-
12,15	-	-	-	1	-	-
9,14	-	-	-	-	-	1
9,10,15	1	-	-	-	-	-
Nei's (1973) gene diversity	**0.0983**	**0.0996**	**0.0964**	**0.0946**	**0.0939**	**0.0985**
No. of polymorphic loci	**5**	**6**	**5**	**5**	**5**	**6**
Percentage of polymorphism	**50.0**	**60.0**	**50.0**	**50.0**	**50.0**	**60.0**

PCR-RFLP of 16S $rDNA_{312}$ generated only 3 mitotypes implying low polymorphism in this gene region. One of the possible approach to resolve such a problem is the use of a more sensitive technique for detection of genetic diversity studies or alternatively the use of other DNA segments exhibiting higher levels of polymorphism.

PCR-SSCP (15% polyacrylamide gel with crosslink 37.5:1 and electrophoresed at 200 V for 20 hrs.) was then applied to analyze polymorphism of the same fragment of 185 individuals.

A total of 17 SSCP patterns were found (Figure 3.15 and Table 3.8). The average number of individuals per SSCP pattern was 10.88. Nine, ten, seven, eight, seven and eight fragments (treated as loci due to the dominant segregation patterns of this data set) were found in Trat, Chumphon, Satun, Trang, Phanganga and Ranong. The gene diversity was comparatively low in all geographic samples (0.0946 – 0.0996).

Genetic distance between pairs of geographic sample was limited (Table 3.9). The lowest genetic distance was found between *P. monodon* from Trang and Phangnga (0.0001) whereas the greatest distance was observed between Ranong and Trang samples (0.0100).

Genetic heterogeneity analysis based on the exact test did not reveal significant differentiation between pairs of investigated samples (Table 3.10). This indicated the lack of intraspecific genetic differentiation of *P. monodon* analyzed by polymorphism of 16S $rDNA_{312}$. Results also indicated that levels of 16S $rDNA_{312}$ polymorphism were not high enough to detect genetic differentiation in this species.

Table 3.9 Pairwise genetic distance between geographic samples of *P. monodon* based on PCR-SSCP of 16S $rDNA_{312}$

	Trat	**Chumphon**	**Satun**	**Trang**	**Phangnga**	**Ranong**
Trat	-					
Chumphon	0.0005	-				
Satun	0.0003	0.0006	-			
Trang	0.0007	0.0014	0.0008	-		
Phangnga	0.0006	0.0014	0.0007	0.0001	-	
Ranong	0.0062	0.0048	0.0059	0.0100	0.0099	-

Table 3.10 Pairwise geographic heterogeneity analysis between *P. monodon* samples based on PCR-SSCP of 16S $rDNA_{312}$

	Trat	**Chumphon**	**Satun**	**Trang**	**Phangnga**	**Ranong**
Trat	-					
Chumphon	$P=1.0000^{ns}$	-				
Satun	$P=1.0000^{ns}$	$P=0.9992^{ns}$	-			
Trang	$P=1.0000^{ns}$	$P=1.0000^{ns}$	$P=0.9996^{ns}$	-		
Phangnga	$P=1.0000^{ns}$	$P=1.0000^{ns}$	$P=1.0000^{ns}$	$P=1.0000^{ns}$	-	
Ranong	$P=0.9862^{ns}$	$P=0.9986^{ns}$	$P=0.9914^{ns}$	$P=0.8140^{ns}$	$P=0.8473^{ns}$	-

3.2.2 Development of SCAR markers from polymorphic AFLP fragments and application for population genetic studies

A total of 320 (256 of *Eco*R I_{+ANN}/*Mse* I_{+CNN} and 64 of *Pst* I_{+GNN} / *Mse* I_{+CNN}) primer combinations were screened against pooled genomic DNA of *P. monodon* originating from Satun, Trang, Phangnga, Chumphon and Trat (Figure 3.16). Fourteen fragments found in only one geographic sample but not others were cloned and sequenced (Appendix C). A pair of primers was designed and applied for detection of population specificity.

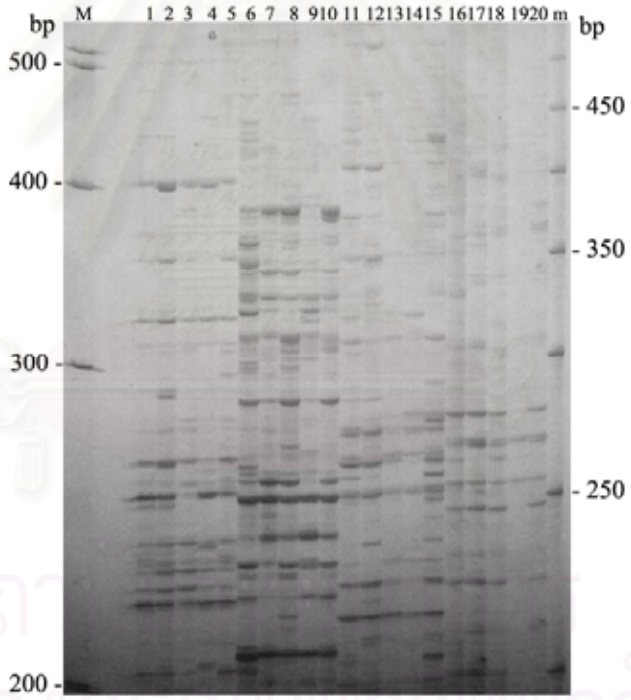

Figure 3.16 AFLP patterns generated from primers P2M5 (lanes 1 - 5), P2M6 (lanes 6 - 10), P2M7 (lanes 11 - 15) and P2M8 (lanes 16 - 20) using pooled genomic DNA of *P. monodon* from Trat (lanes 1, 6, 11 and 16), Chumphon (lanes 2, 7, 12 and 17), Satun (lanes 3, 8, 13 and 18), Trang (lanes 4, 9, 14 and 19) and Phangnga (lanes 5, 10, 15 and 20).

Almost all of the primers generated the expected amplification product (Table 3.11). Nevertheless, results indicated that SCAR markers developed from candidate AFLP fragments were not population-specific (Table 3.11 and Figure 3.17 – 3.20).

Four SCAR markers origination from E4M6-295, E7M10-450, P6M2-370, and P6M6-470 were selected for further analysis of genetic diversity of *P. monodon* and tested across 112, 105, 134 and 136 individuals, respectively. Eighty-one individuals were successfully genotyped by all primers.

GATGAGTCCTGAGTAACTCTAGTGAATCATTTCCTGAAACT**TCCCCTCCTATGCTAA**
CGCTACTTACACCACTTTGTCGCACCAACTGTTGTAATTTTGCTATTCTAAGATGTT
GCAATCGTCCAAGTTCTTCCGTCATGCGTTTCTTTTCTCTCATAAGCTCATGGATTT
CTTGGTCAA**CATTTTCCAAGTGCCGTAAGAA**TTCTGTTTCTTGCTCACTGAACACCA
TCATTTGTTGTAGTGCTACTCCTGCATTGTGGCTGAACTCAAATTCACTGAGTGAAT
TGGTACGCAGTCA

Figure 3.17 Nucleotide sequences of E4M6-295. Sequences and positions of the forward primer and those complementary to the reverse primer are underlined and boldfaced.

GATGAGTCCTGAGTAACGTGTGAATGGGTATGCAAGAAATTCATTCATAATGGACAA
GGTTCTCGAAGAAACGCGCGGC**TGTTACAATGCTGTCGTGGA**AAGGTACTTTTCAAA
CTTTACTCTACACTCACCATTCATGGAGATGCTATTGCTTACAGCGTCAGGTAAGTG
TACGCTAAATGTAATGAGGCAAAGAGTAATTCTTTGTTTTGCTATCGCGCACACACA
CACTTACAAAAAAAGTGATGTATTATTATGATGCATCCTTTGCATCTTAGACTTCCA
TACCTTTTTCAAAAATTCCTTTCCAAACATTTCTTTAAATTTCCATTTTCAGGCGAT
ATCGCCTTCGGAGCTGAATTCT**GAGATAAGATGAACGCAACCA**TGGTTGACTTCAAA
AAGTTTCCTGATTTCCCCTGGATATTCTACGGCGTTGCTGAATTGGTACGCAGTC

Figure 3.18 Nucleotide sequences of E7M10-450. Sequences and positions of the forward primer and those complementary to the reverse primer are underlined and boldfaced.

GACTGCGTACATGCAGACTCCCCAGCTCGAGCGGACGATTCGGAACGGTTTCGAAG**C**
GGTGAAAGTAAGTCAAATGTCTTTATTTCCATATCGAGACCATTTCTGTTTTCCATT
CTTTGATATTTTCTTTTAAGTCGTTAGTGTAGCGTATGCGTTCTCTGATATATAAAT
ATTTGTATTCTTAAATGTATTTGCGAATATACTACGGTTAAACACCGGTCTATCTTC
T**TTCCCTCCACTCTGCCTGA**AATGTTATTCACTTAGGAATATTTTTTAAATAATTTC
ATTACAGCGTTAACGCTTTCTTGATACATATAAAATTCATACAAAAATAAGATGCTA
ATATAGTGTTACTCAGGACTCATCA

Figure 3.19 Nucleotide sequences of P6M2-370. Sequences and positions of the forward primer and those complementary to the reverse primer are underlined and boldfaced.

GATGAGTCCTGAGTAACTCTACCAGTCATAAATAGTGATTCTCCATCTGCCTCGAGA
ATGTTGAAGTGAAAATGCTTTTACATTCTGTACAACTCAGGATTTCATGGTCAAGTT
ACATTCTGGCTGCCACGAGGCACGAAGTAGCAAAATG**CTCAGACTCCCGCCTAATCC**
ATGAATGTATTACTGTCTTCCACATCCGCAAAGAATTGGTCAACAGAGACGCTAGAG
CCCGAGAACTTTGACGCTACATTGTACATCGTGGGAACCATGCAGGCACCCATGCTT
GATGCAGCCGTAACAAGGGTGAGGGCAGAGGAGGGGAGTGGAGTAGGAGAGAGAATG
GTTTCGGTTTGTGTGGCGAGAAAAAGATGGAGACTGATTCAGAAATAGATTGTGAAG
AAGATGAGTGAGAAATAAGAGAAGTCAGTCC**TTAGGGGAGAAAGTTAGCGAA**GTCTG
CATGTACGCAGTCA

Figure 3.20 Nucleotide sequences P6M6-470. Sequences and positions of the forward primer and those complementary to the reverse primer are underlined and boldfaced.

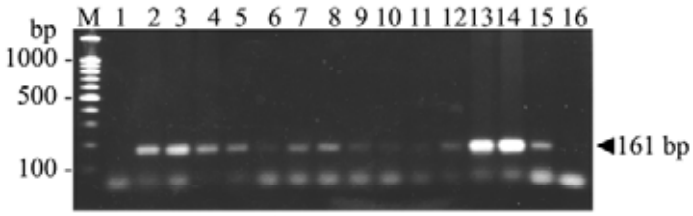

Figure 3.21 Agarose gel elctrophoresis illustrating the amplification product of E4M6-295 (161 bp) of *P. monodon* from Trat (lanes 1 - 3), Chumphon (lanes 4 - 6), Satun (lanes 7 - 9), Trang (lanes 10 - 12) and Phangnga (lanes 13 - 15). Lanes M and 16 are 100 bp DNA marker and negative control (no DNA template).

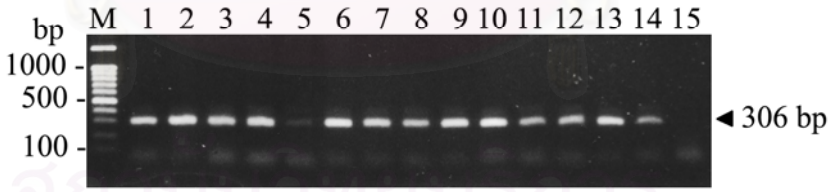

Figure 3.22 Agarose gel elctrophoresis illustrating the amplification product of E7M10-450 (306 bp) of *P. monodon* from Trat (lanes 1 - 3), Chumphon (lanes 4 - 5), Satun (lanes 6 - 8), Trang (lanes 9 - 11) and Phangnga (lanes 12 - 14). Lanes M and 15 are 100 bp DNA marker and negative control (no DNA template).

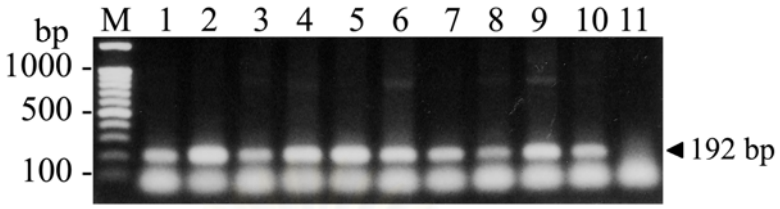

Figure 3.23 Agarose gel elctrophoresis illustrating the amplification product of P6M2-370 (192 bp) of *P. monodon* from Trat (lanes 1 - 2), Chumphon (lanes 3 - 4), Satun (lanes 5 - 6), Trang (lanes 7 - 8) and Phangnga (lanes 9 - 10). Lanes M and 11 are 100 bp DNA marker and negative control (no DNA template).

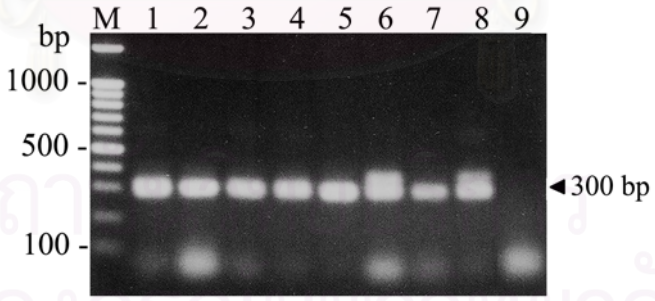

Figure 3.24 Agarose gel elctrophoresis illustrating the amplification product of P6M6-470 (300 bp) of *P. monodon* from Trat (lane 1), Chumphon (lane 2), Satun (lanes 3 - 4), Trang (lanes 5 - 6) and Phangnga (lanes 7 - 8). Lanes M and 9 are 100 bp DNA marker and negative control (no DNA template).

Table 3.11 Amplification results of SCAR markers derived from polymorphic AFLP fragments

Primer	Expected size (bp)	Amplification results	SSCP
1. E4M6-295	**161**	**+**	**Polymorphism**
2. E8M7	258	+	Polymorphism
3. E6M9-318	148	-	ND
4. E7M10-450	**306**	**+**	**Polymorphism**
5. P2M5-295	160	+	Polymorphism
6. P2M6-270	156	-	ND
7. P2M7-280	217	-	ND
8. P2M7-310	197	+	Polymorphism
9. P6M2-370	**192**	**+**	**Polymorphism**
10. P6M6-470	**300**	**+**	**Polymorphism**
11. P2M6-850	319	-	ND
12. P2M6-465	238	+	Polymorphism
13. P2M8-300	204	+	Polymorphism
14. P2M6-385	250	+	Polymorphism

- = no amplification product, ND = not determined.

Genetic diversity of each *P. monodon* sample was relatively low. The average gene diversity of each geographic samples was 0.1452 (Trat) – 0.1742 (Trang). The number of polymorphic loci was 31, 40, 38, 43 and 47 accounting to the percentage of polymorphic loci of 53.54%, 68.97%, 65.52%, 74.14% and 81.03% for *P. monodon* from Trat, Chumphon, Satun, Trang and Phangnga, respectively (Table 3.12).

Genetic distance between pairs of geographic samples analyzed by SSCP of SCAR markers was low. The lowest genetic distance was observed between Chumphon and Trang (0.0064). The greatest genetic distance was found between Trat and Phangnga (Tables 3.13).

Genetic heterogeneity analysis indicated a lack of intraspecific population subdivisions in these *P. monodon* when analyzed by low polymorphic AFLP derived markers (Table 3.14).

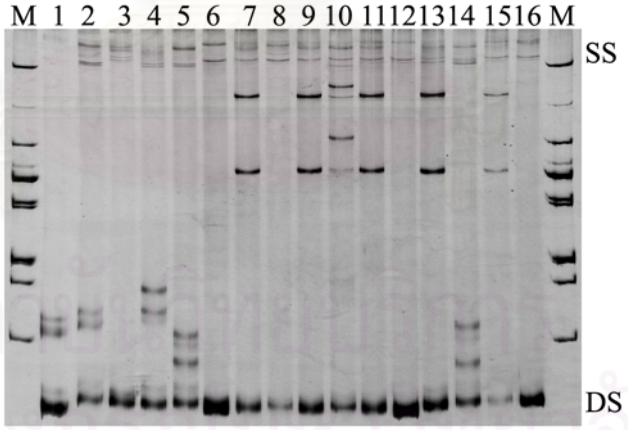

Figure 3.25 Polymorphic SSCP patterns generated from primers P6M6-470 analyzed against genomic DNA of *P. monodon* from Satun (lanes 2-4), Phangnga (lanes 5-7), Trang (lanes 8-10), Chumphon (lanes 11-13) and Trat (lanes 14-16). Lanes M are 100 bp ladder.

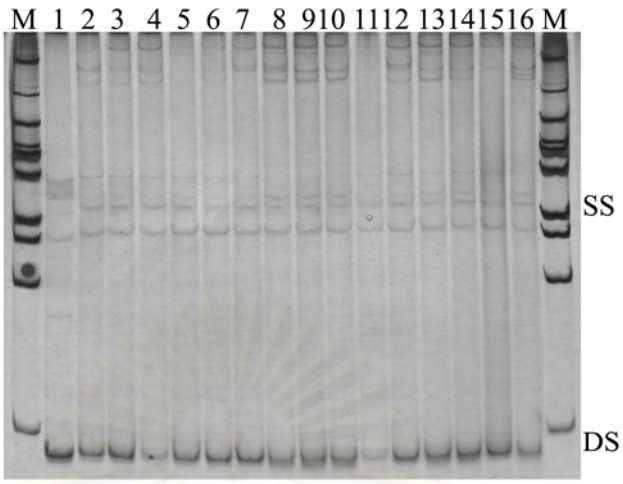

Figure 3.26 Polymorphic SSCP patterns generated from primers E4M6-295 analyzed against genomic DNA of *P. monodon* from Trat (lanes 2-4), Chumphon (lanes 5-7), Satun (lanes 8-10), Trang (lanes 11-13) and Phangnga (lanes 14-16). Lanes M are 100 bp ladder.

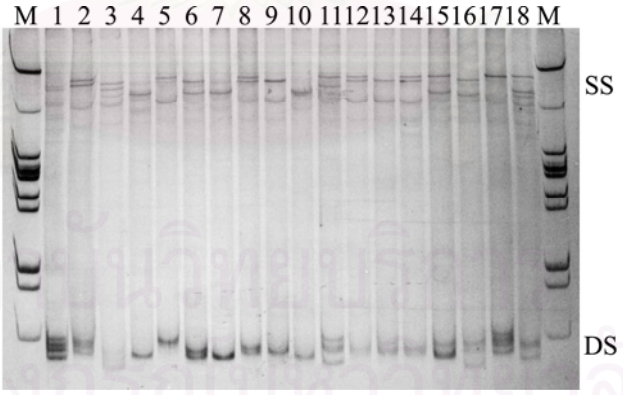

Figure 3.27 Polymorphic SSCP patterns generated from primers E7M10-450 analyzed against genomic DNA of *P. monodon* from Trat (lanes 2-4), Chumphon (lanes 5-8), Satun (lanes 9-12), Trang (lanes 13-15) and Phangnga (lanes 16-18). Lanes M are 100 bp ladder.

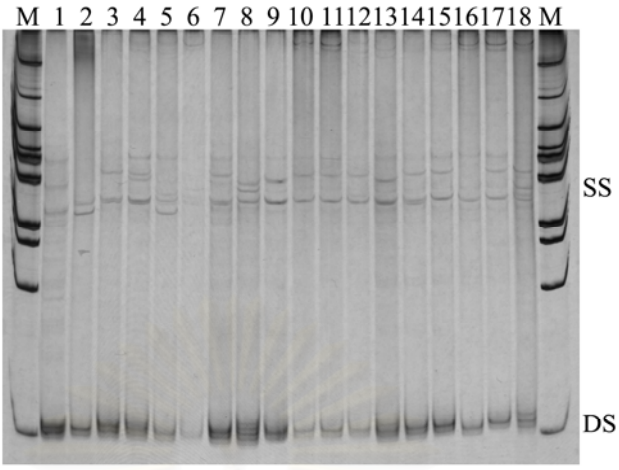

Figure 3.28 Polymorphic SSCP patterns generated from primers P6M2-370 analyzed against genomic DNA of *P. monodon* from Trat (lanes 4-6), Chumphon (lanes 2, 7-9), Satun (lanes 10-12), Trang (lanes 13-15) and Phangnga (lanes 3, 16-18). Lanes M are 100 bp ladder.

Table 3.12 A summary of gene diversity index (Nei, 1973) of all AFLP-derived loci in each geographic sample and overall samples

Locus	Trat (*N* = 9)	Chumphon (*N* = 20)	Satun (*N* = 13)	Trang (*N* = 14)	Phangnga (*N* = 25)	Overall samples (*N* = 81)
SSCP1	0.4444	0.4954	0.3907	0.4809	0.4998	0.4960
SSCP2	0.0000	0.0000	0.0000	0.0000	0.0396	0.0124
SSCP3	0.0000	0.0494	0.0000	0.1374	0.3200	0.1485
SSCP4	0.4880	0.4954	0.4940	0.4809	0.4998	0.4963
SSCP5	0.0000	0.4746	0.4940	0.4972	0.4914	0.4778
SSCP6	0.0000	0.0974	0.0754	0.0000	0.0000	0.0372
SSCP7	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
SSCP8	0.1078	0.2321	0.2157	0.2014	0.1889	0.1980
SSCP9	0.4444	0.5000	0.3382	0.4522	0.3492	0.4383
SSCP10	0.2083	0.0494	0.1474	0.1374	0.1530	0.1316
SSCP11	0.0000	0.0000	0.0754	0.0000	0.0396	0.0248
SSCP12	0.0000	0.0494	0.2157	0.0701	0.0783	0.0857
SSCP13	0.4444	0.4416	0.2795	0.3690	0.4256	0.4052
SSCP14	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
SSCP15	0.0000	0.3125	0.2795	0.2617	0.2892	0.2629
SSCP16	0.1078	0.0494	0.2157	0.1374	0.1530	0.1318
SSCP17	0.2083	0.0494	0.0000	0.0701	0.0783	0.0736

Table 3.12 (continued)

Locus	Trat (*N* = 9)	Chumphon (*N* = 20)	Satun (*N* = 13)	Trang (*N* = 14)	Phangnga (*N* = 25)	Overall samples (*N* = 81)
SSCP18	0.2083	0.2733	0.1474	0.2617	0.1889	0.2197
SSCP19	0.1078	0.0000	0.0000	0.0000	0.1530	0.0622
SSCP20	0.1078	0.0000	0.0000	0.0000	0.0000	0.0126
SSCP21	0.0000	0.0000	0.0000	0.0701	0.1162	0.0495
SSCP22	0.1078	0.1889	0.3382	0.2014	0.1889	0.2100
SSCP23	0.0000	0.0000	0.0000	0.0000	0.0396	0.0124
SSCP24	0.2083	0.4142	0.0754	0.4142	0.3200	0.3249
SSCP25	0.2997	0.1889	0.4940	0.2617	0.3492	0.3367
SSCP26	0.0000	0.0000	0.0000	0.0701	0.0000	0.0125
SSCP27	0.1078	0.1439	0.2157	0.1374	0.1530	0.1538
SSCP28	0.2083	0.1889	0.1474	0.2617	0.1162	0.1767
SSCP29	0.0000	0.0974	0.0000	0.0701	0.0396	0.0491
SSCP30	0.2083	0.0974	0.3382	0.2617	0.2571	0.2319
SSCP31	0.1078	0.0000	0.0000	0.0000	0.0000	0.0126
SSCP32	0.2083	0.1889	0.2795	0.2014	0.1162	0.1878
SSCP33	0.0000	0.3125	0.1474	0.0701	0.3492	0.2358
SSCP34	0.2997	0.2321	0.2157	0.4522	0.0783	0.2487

Table 3.12 (continued)

Locus	Trat (*N* = 9)	Chumphon (*N* = 20)	Satun (*N* = 13)	Trang (*N* = 14)	Phangnga (*N* = 25)	Overall samples (*N* = 81)
SSCP35	0.2083	0.1889	0.2157	0.1374	0.1162	0.1652
SSCP36	0.4984	0.4844	0.4940	0.4976	0.4914	0.4993
SSCP37	0.2997	0.3822	0.3907	0.3179	0.3767	0.3636
SSCP38	0.0000	0.0494	0.0754	0.2014	0.0000	0.0623
SSCP39	0.0000	0.0000	0.0000	0.0000	0.0396	0.0124
SSCP40	0.4444	0.3832	0.2157	0.0701	0.1889	0.2680
SSCP41	0.0000	0.0000	0.0754	0.0000	0.0000	0.0125
SSCP42	0.1078	0.0494	0.0000	0.1374	0.1162	0.0851
SSCP43	0.0000	0.0974	0.0000	0.1374	0.0000	0.0497
SSCP44	0.0000	0.0000	0.0754	0.0701	0.0783	0.0491
SSCP45	0.0000	0.0494	0.0754	0.0000	0.1530	0.0737
SSCP46	0.4444	0.4954	0.4711	0.4522	0.4022	0.4582
SSCP47	0.0000	0.0000	0.1474	0.2014	0.1162	0.0979
SSCP48	0.4984	0.4142	0.2795	0.2617	0.3767	0.3871
SSCP49	0.0000	0.1889	0.2157	0.2014	0.0783	0.1439
SSCP50	0.0000	0.0000	0.0754	0.0701	0.0396	0.0369
SSCP51	0.1078	0.0000	0.0000	0.0000	0.0783	0.0372

Table 3.12 (continued)

Locus	Trat (*N* = 9)	Chumphon (*N* = 20)	Satun (*N* = 13)	Trang (*N* = 14)	Phangnga (*N* = 25)	Overall samples (*N* = 81)
SSCP52	0.2997	0.4142	0.2157	0.2617	0.3767	0.3397
SSCP53	0.4984	0.3832	0.4357	0.4809	0.4022	0.4419
SSCP54	0.0000	0.0494	0.0000	0.1374	0.0783	0.0613
SSCP55	0.3796	0.4142	0.3907	0.3690	0.3767	0.3878
SSCP56	0.0000	0.0494	0.0000	0.0000	0.0000	0.0124
SSCP57	0.0000	0.0000	0.0000	0.0000	0.0396	0.0124
SSCP58	0.0000	0.0000	0.0000	0.0701	0.0000	0.0125
Average gene diversity	**0.1452**	**0.1677**	**0.1616**	**0.1742**	**0.1723**	**0.1729**
No. of polymorphic loci	**31**	**40**	**38**	**43**	**47**	**100**
Percentage of polymorphic loci	**53.45**	**68.97**	**65.52**	**74.14**	**81.03**	**100**

Table 3.13 Pairwise genetic distance between geographic samples of *P. monodon* based on PCR-SSCP of four AFLP-derived markers

	Trat	**Chumphon**	**Satun**	**Trang**	**Phangnga**
Trat	-				
Chumphon	0.0118	-			
Satun	0.0156	0.0110	-		
Trang	0.0148	0.0064	0.0080	-	
Phangnga	0.0160	0.0066	0.0088	0.0067	

Table 3.14 Pairwise geographic heterogeneity analysis between *P. monodon* from different geographic samples based on PCR-SSCP of four AFLP-derived markers

	Trat	**Chumphon**	**Satun**	**Trang**	**Phangnga**
Trat	-				
Chumphon	$P=1.0000^{ns}$	-			
Satun	$P=1.0000^{ns}$	$P=0.9962^{ns}$	-		
Trang	$P=1.0000^{ns}$	$P=1.0000^{ns}$	$P=1.0000^{ns}$	-	
Phangnga	$P=1.0000^{ns}$	$P=0.9979^{ns}$	$P=0.9981^{ns}$	$P=1.0000^{ns}$	-

3.2.3 Determination of COI_{614}, polymorphism in *P. monodon* by DNA sequencing

The COI-COII segment (1550 bp) of representative *P. monodon* originating from Chumphon, Satun, Trang and Trad was amplified using universal primers COI-COII-F/R. The amplification product was direct sequenced one only one direction. Nucleotide sequences obtained was blasted against data in the GenBank to verify whether they were part of COI or COII gene segment.

Sequences of individuals from Chumphon, Satun, Trang were significantly matched the COI gene segment whereas those of the Trad sample were regarded as the COII fragment. Multiple sequence alignment of the COI gene segment of those individuals resulted in 790 characters and high intraspecific sequence divergence was observed (Figure 3.29). Conversely, the COII segment of the Trad individual showed low polymorphism when aligned with the sequence of *P. monodon* COII retrieved from the GenBank (Figure 3.30).

Two primers pairs ($T13COI_{F2}$, and $T13COI_{R2}$ for COI and $T13COI_{F3}$ and $T13COI_{R3}$ for COII, Table 3.15) were designed from nucleotide sequence of a specimen from Trang. These primers were used in combination of Universal COI-COII-F and Universal COI-COII-R. The discrete 614 bp (from Universal COI-COII-F+ $T13COI_{R2}$) and 416 bp (from $T13COI_{F2}$ +$T13COI_{R2}$) fragments were successfully amplified (Figure 3.31 and 3.32). The former product was chosen for direct sequencing analysis due to its longer fragment length which provides more informative sites.

The COI_{614} gene segment amplified from 100 individuals; Trat (N = 20), Chumphon (N = 20), Satun (N = 19), Trang (N = 21) and Phangnga (N = 20) were analyzed. After multiple alignment, high genetic polymorphism was observed and 43 haplotypes were identified (Figure 3.33). Therefore, nucleotide sequences of an individual representing each haplotype were reanalyzed and multiple aligned (Figure 3.34). Polymorphic sites of COI_{614} of *P. monodon* can be used as DNA barcodes (string of sequences) for effective genotyping of this economically important species (Appendix D).

```
C20-M13F-trim.txt   1:TTGATTTTTTGGTCATCCAGAAGTATATATTTTAATT-TTACCTGGCTTTTGGTACAATT 59
S3-M13F-trim.txt    1:TTGATTTTTTGGTCATCCAGAAGTATATATTTTAATTCTT-CCT-GCCTTTGGGATAATC 58
T13-M13F-trim.txt   1:TTGATTTTTTGGTCATCCAGAAGTATATATTTTAATTCTT-CCT-GCATTTGGAATAATT 58

C20-M13F-trim.txt  60:TCCCATATTATTAGTCAAGAATCTGGTAAAAAAGAAGCATTCGGAACAC-TCGGGATAAT 118
S3-M13F-trim.txt   59:TCACATATTATTAGTCAAGAGTCTGGTAAAAAAGAAGCTTTTGGAACA-TTAGGAATAAT 117
T13-M13F-trim.txt  59:TCACACATTATTAGTCAAGAATCTGGTAAAAAAGAAGCGTTCGGAACACTT-GGAATAAT 117

C20-M13F-trim.txt 119:CTATGCTATACTTGCTATTGGTGTCCTAGGATTCGTAGTATGAGCACACCATATATTTAC 178
S3-M13F-trim.txt  118:CTATGCTATACTAGCCATTGGTGTTCTAGGATTTGTAGTATGAGCTCATCATATATTTAC 177
T13-M13F-trim.txt 118:TTATGCTATACTAGCTATTGGTGTTCTAGGATTTGTGGTATGAGCTCATCATATATTTAC 177

C20-M13F-trim.txt 179:AGTAGGTATGGATGTTGATACTCATGCTTATTTTACATCTGCAACAATGATTATTGCCGT 238
S3-M13F-trim.txt  178:TGTAGGTATAGACGTTGATACTCGTGCTTACTTTACATCTGCTACGATAATTATTGCTGT 237
T13-M13F-trim.txt 178:TGTAGGGATAGATGTTGATACTCGTGCTTACTTTACATCTGCTACAATAATTATTGCTGT 237

C20-M13F-trim.txt 239:TCCTACAGGTATTAAAATTTTCAGTTGACTAGGTA-C-TCTTCATGGTACTCAACTT-AA 295
S3-M13F-trim.txt  238:CCCGACGGGTATTAAGATCTTCAGCTGACTAGG-AGCAT-TACACGGTACTC-AATTGAA 294
T13-M13F-trim.txt 238:ACCAACAGGTATTAAAATCTTCAGTTGATTAGG-AACAT-TACATGGTACTC-AGTTGAA 294

C20-M13F-trim.txt 296:CTATAGTCCTTCTCTAATTTGAGCGCTAGGATTTGTATT-TCTATTTACTGTGGGTGGTT 354
S3-M13F-trim.txt  295:TTATAGTCCTGCTTTAATTTGGGCATTAGGGTTTGTATTTT-TATTTACAGTTGGGGGTC 353
T13-M13F-trim.txt 295:TTATAGTCCTTCTTTAATTTGAGCCTTAGGGTTCGTATTCT-TATTTACAGTAGGAGGTT 353

C20-M13F-trim.txt 355:TAACCGGTGTTGTACTAGCTAATTCTTCAATTGACATTATTTTACATGATACATACTACG 414
S3-M13F-trim.txt  354:TAACAGGAGTTGTCCTTGCTAATTCATCTATTGATATTATCTTGCACGATACTTATTATG 413
T13-M13F-trim.txt 354:TAACAGGAGTTGTACTTGCTAACTCATCTATTGACATCATCTTGCACGATACTTATTATG 413

C20-M13F-trim.txt 415:TAGTAGCACATTTCCACTATGTTCTTTCAATAGGAGCTGTATTTGGTATTTTTGCAGGTA 474
S3-M13F-trim.txt  414:TAGTAGCCCACTTCCACTATGTTCTTTCAATAGGAGCCGTATTTGGTATTTTTGCAGGTA 473
T13-M13F-trim.txt 414:TAGTAGCCCATTTTCACTACGTCCTTTCAATAGGAGCAGTATTTGGTATTTTTGCAGGTA 473

C20-M13F-trim.txt 475:TTGCCCACTGATTCCCT-TTATTTACGGGGCTT-ACCTTA-TT-TTAAACCCTAAATGAT 530
S3-M13F-trim.txt  474:TTGCCCACTGATTTCCTCTT-TTTAC-CGGTTTAACCCT-GTTCTT-AACCCAAAATGAT 529
T13-M13F-trim.txt 474:TTGCTCACTGATTTCCTCTT-TTTAC-TGGTTTAACCTTAATTC-T-AACCCAAAATGAT 529

C20-M13F-trim.txt 531:TAAAAATTCACTTTCTTGTTATATTCATTGGAGTAAATATTACATTCTTCCCCCAACATT 590
S3-M13F-trim.txt  530:TAAAAATCCACTTTCTAGTTATATTTATTGGGGTAAACATTACATTTTTCCCTCAACATT 589
T13-M13F-trim.txt 530:TAAAAATCCACTTTTTAGTTATATTTATTGGGGTAAACATTACATTTTTCCCACAACATT 589

C20-M13F-trim.txt 591:TCTTAGGTCTTAATGGAATACCTCGACGATACTCAGACTACCCAGATGCTTATTCAGCAT 650
S3-M13F-trim.txt  590:TTTTAGGGCTTAATGGTATGCCTCGACGCTATTCAGATTATCCAGACGCCTACACAGCAT 649
T13-M13F-trim.txt 590:TCTTAGGACTTAATGGAATACCTCGGCGATATTCAGATTATCCAGACGCTTATACAGCAT 649

C20-M13F-trim.txt 651:GAAATGTTGTATCTTCCATTGGATCAACGGTATCCCTGATTGCCGTATTANGCTTTGTTA 710
S3-M13F-trim.txt  650:GAAATGTTATATCATCTATTGGATCTACAGTATCATTAATTGCAGTACTAGGTTTTGTTA 709
T13-M13F-trim.txt 650:GAAATGTTGTATCATCTATTGGATCTACAGTATCATTAATTGCTGTTTTAGGTTTTGTTA 709

C20-M13F-trim.txt 711:TAATCGTCTGAGAAGCATTAACTGCT-GCTCGGCCTGTAGTT-T-TT-TCACTATTCTTA 766
S3-M13F-trim.txt  710:TAATTGTATGAGAAGCCTTAACTG-TAGCTCGACC---AGTTATATTTTCTTTATTTTTA 765
T13-M13F-trim.txt 710:TAATTGTGTGAGAAGCCTTAACTG-TTGCACGACC---AGTTATATTCTCTTTATTTTTA 765

C20-M13F-trim.txt 767:CAAAC-TTCAATCGAATGACAGC-                                   788
S3-M13F-trim.txt  766:CCTACTTTCGA-TGAATGACAGCA                                   788
T13-M13F-trim.txt 766:CCTAC-TTCGATTGAATGACAACA                                   788
```

Figure 3.29 Multiple alignments of the amplified COI-COII (only sequences of COI are shown). High sequence divergence between individuals was observed in this gene region.

```
COX2 gene(Pmonodon)   1:ACATCGAGCCGGTCAATATAAAAGAAATAAAAATGGATGAAGCTAACTTACTGTTGTATT 60
Td13(rev.)            1:ACATCGAGCTGGTCAATATAAAAGAAATAAAAATGGATGAAGCTAACTTACTGTCGTATT 60

COX2 gene(Pmonodon)  61:AGAGGGTGGGCGTCTAGTATCAATATATCTTTAAGGAAATTAATGATTAAAGATTTTACC 120
Td13(rev.)           61:AGAGGGTGGGCGTCTAGTATCAATATATCTTTAAGGAAATTAATGATTAAAGATTTTACC 120

COX2 gene(Pmonodon) 121:GTCTATCTACGTATCCTAAATTCGAGGATGGTCCTTCTTATAGAAGAAGAAAATCTTTTG 180
Td13(rev.)          121:GTCTATCTACGTATCCTAAATTCGAGGATGGTCCTTCTTATAGAAGAAGAAAATCTTTTG 180

COX2 gene(Pmonodon) 181:AATACGGTTGTACTCCAGTGAATCCAAATGTTCTATCTCGAAGAGGAGAATATCTTGTTA 240
Td13(rev.)          181:AATACGGTTGTACTCCAGTGAATCCAAATGTTCTATCTCGAAGAGGAGAATATCTTGTTA 240

COX2 gene(Pmonodon) 241:ATTAAAAAAAGGTACTAGTGCGATATCATCATAATTAAAATTAATGTTGTAATCAACCTA 300
Td13(rev.)          241:ATTAAAAAAAGGTACTAGTGCGATATCATCATAATTAAAATTAATGTTGTAATCAACCTA 300

COX2 gene(Pmonodon) 301:TATATTATAGTTGAAATAAAAAATTATTAAAATGTTTAGCGAAGAATAACCTTCCTGTTT 360
Td13(rev.)          301:TATATTATAGTTGAAATAAAAAATTATTAAAATGTTTAGCGAAGAATAACCTTCCTGTTT 360

COX2 gene(Pmonodon) 361:GATAACTTTAACATACCTGTCATGAAGGGCGAGATTAAAATTAAAAATAACGTGAAGGAA 420
Td13(rev.)          361:GATAACTTTAACATACCTGTCATGAAGGGCGAGATTAAAATTAAAAATAACGTGAAGGAA 420

COX2 gene(Pmonodon) 421:GAAATGCTAATGATATAGATAATCTACTTCAATTATTAGGTTCACAATGGAACTTTTGAT 480
Td13(rev.)          421:GAAATGCTAATGATATAGATAATCTACTTCAATTATTAGGTTCACAATGGAACTTTTGAT 480

COX2 gene(Pmonodon) 481:AACCTGTGGTTACTATGACTTCAATGCTCATTAAAAGAGTCTGAAAAATGTTTAACTTAA 540
Td13(rev.)          481:AACCTGTGGTTACCATGACTTCAATGCTCATAAAATGAGTCTGAAAAATGTTTAACTTAA 540

COX2 gene(Pmonodon) 541:ACTGAGAATATATTAAGGTAGTTTACTTAATAGTCTTCTGCCTAAAGCTGAAAATCTACA 600
Td13(rev.)          541:ACTGAGAATATATTAAGGTAGTTTACTTAATAGTCTTCTGTCTAAAGCTGAAAATCTGCA 600

COX2 gene(Pmonodon) 601:CCTATTAGCTTGACAAGATGGATACTTATGAGTTTAAGCTCATAATTAATCTCGACGACT 660
Td13(rev.)          601:CCTATTAGCTTGACAAGATGGATACTTATGAGTTTAAGCTCATAATTAATCTCGACGACT 660

COX2 gene(Pmonodon) 661:ACAATAAGTAAGTACTTGTCATGGACGGGAACCACATTTTCGTCTACGTTAAGGTCCCGC 720
Td13(rev.)          661:ACAATAAGTAAGTACTTGTCATGGACGGGAACCACATTTTCGTCTACGTTAAGGT-CCGC 719

COX2 gene(Pmonodon) 721:TGAATTAGTTCATTCAAAAAATTATTTAGCTGGTCCTAATAAAATACCAGTTACAAGTCT 780
Td13(rev.)          721:TGAA-TAGTTCATTCAAAAAATTATTTAGCTGGTCCTAATAAAATACCAGTTACAAGTCT 778

COX2 gene(Pmonodon) 781:TTAAACACC                                                  840
Td13(rev.)          781:TTAAACACC                                                  838
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Figure 3.30 Nucleotide sequence alignments of the amplified COI-COII (only sequences of COII are shown). Low sequence divergence between a specimen from Trat and that in the GenBank was observed in this gene region.

Table 3.15 Newly designed primers from amplification of COI and COII gene segments, the expected product and amplification results

Primer	Expected product	Amplification result
Universal COI-COII-F/R	1550 bp	1550 bp
Universal COI-COII-F+ $T13COI_{R2}$	614 bp	An intense band at 614 bp
Universal COI-COII-F+ $Td13COII_{R3}$	1423 bp	No amplification product
$T13COI_{F2}$ + Universal COI-COII-R	1310 bp	Faint band at approximately 1200 bp
$Td13COII_{F3}$ + Universal COI-COII-R	728 bp	No amplification product
$T13COI_{F2}$ +$T13COI_{R2}$	416 bp	An intense band at 416 bp
$Td13COII_{F3}$ +$Td13COII_{R3}$	643 bp	No amplification product

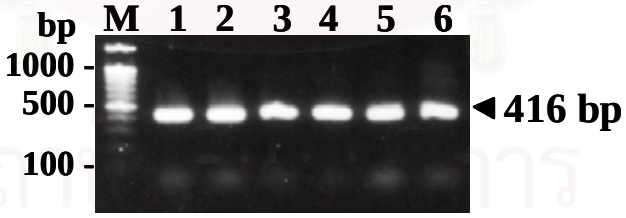

Figure 3.31 Agarose gel elctrophoresis illustrating the amplification product of COI (416 bp) of *P. monodon* from Trat (lane 1), Chumphon (lanes 2 and 3), Satun (lane 4), Trang (lane 5) and Phangnga (lane 6). Lane M is 100 bp DNA marker.

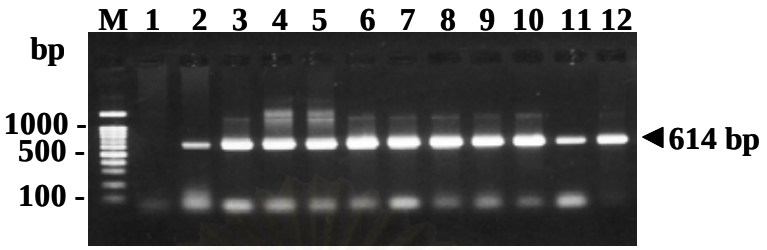

Figure 3.32 Agarose gel elctrophoresis illustrating the amplification product of COI (614 bp) of *P. monodon* from Trat (lanes 2 - 4), Chumphon (lanes 5 - 6), Satun (lanes 7 - 8), Trang (lanes 9 - 10) and Phangnga (lanes 11 - 12). Lanes M and 1 are 100 bp DNA marker and negative control (no DNA template).

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P28     GCTTGGATATTTCACACATTATTAGTCAAGAATCTGGTAAAAAAGAAGCGTTCGGAA
S25     GCTTGGAAATTTC-CACATTATTAGTCAAGAATCTGGTAAAAAAGAAGCGTTCGGAA
S7      GATTGGATATTTCACACATTATTAGTCAAGAATCTGGTAAAAAAGAAGCGTTCGGAA
s11     GCTTGGATATTTCACACATTATTAGTCAAGAATCTGGTAAAAAAGAAGCGTTCGGAA
P29     GCTTGGATATTTC-CACATTATTAGTCAAGAATCTGGTAAAAAAGAAGCGTTCGGAA
TD18    GCTTGGATATTTC-CATATTATTAGTCAAGAATCTGGTAAAAAAGAAGCGTTCGGAA
p21     TTTGGATAATTTCACACATTATTAGTCAAGAATCTGGTAAAAAAGAAGCGTTCGGAA
t16     TTTGGATAATTTCACACATTATTAGTCA-GAATCTGGTAAAAAAGAAGCGTTCGGAA
s10     TTTGGATAATTTCACACATTATTAGTCAAGAATCTGGTAAAAAAGAAGCGTTCGGAA
p17     TTTGGATAATTTCACACATTATTAGTCAAGAATCTGGTAAAAAAGAAGCGTTCGGAA
C10     TTTGGAAAATTTCACACATTATTAGTCA-GAATCTGGTAAAAAAGAAGCGTTCGGAA
S33     CTTTGGATATTTCACACATTATTAGTCAAGAATCTGGTAAAAAAGAAGCGTTCGGAA
c9      GCTTGGATATTTCACACATTATTAGTCAAGAATCTGGTAAAAAAGAAGCGTTCGGAA
C41     GATTGGATATTTCACACATTATTAGTCAAGAATCTGGTAAAAAAGAAGCGTTCGGAA
T30     CTTGGGATATCTCACATATTATTAGTCAAGAGTCTGGTAAAAAAGAAGCTTTTGGAA
C21     CTTGGGATATCTC---CATATATAGTCA-GAGTCTGGTAAAAAAGAAGCTTTTGGAA
P7      CTTGGGATATCTC-CATATTATTAGTCA-GAGTCTGGTAAAAAAGAAGCTTTTGGAA
C18     TTCGGAAT--CTC-CATATTATTAGTCA-GAATCTGGTAAAAAAGAAGCTTTTGGAA
p24     TTGGATAT--CTCACATATTATTAGTCAAGAATCTGGTAAAAAAGAAGCTTTTGGAA
P39     CTCGGGATATCTCACATATTATTAGTCAAGAATCTGGTAAAAAAGAAGCTTTTGGAA
S18     TTTGGGATATCTCACATATTATTAGTCAAGAATCTGGTAAAAAAGAAGCTTTTGGAA
c4      TTTGGGATATCTC-CATATTATTAGTCAAGAGTCTGGTAAAAAAGAAGCTTTTGGAA
C51     TTTGGGATATCTC-CATATTATTAGTCA-GAGTCTGGTAAAAAAGAAGCTTTTGGAA
S1      TTTGGGATATCTCACATATTATTAGTCAAGAGTCTGGTAAAAAAGAAGCTTTTGGAA
P2      CTTGGGATATCTCACATATTATTAGTCAAGAGTCTGGTAAAAAAGAAGCTTTTGGAA
c6      TTTGGGATATCTCACATATTATTAGTCAAGAGTCTGGTAAAAAAGAAGCTTTTGGAA
T43     TTTGGGATATCTCACATATTATTAGTCAAGAGTCTGGTAAAAAAGAAGCTTTTGGAA
t11     TTTGGGATATCTC-CATATTATTAGTCAAGAATCTGGTAAAAAAGAAGCTTTTGGAA
C1      TTTGGGATATCTCACATATTATTAGTCAAGAATCTGGTAAAAAAGAAGCTTTTGGAA
Td17    TTTGGGATATCTCACATATTATTAGTCAAGAATCTGGTAAAAAAGAAGCTTTTGGAA
t18     TTTGGGATATCTCACATATTATTAGTCAAGAATCTGGTAAAAAAGAAGCTTTCGGAA
p10     TTTGGGATATCTC-CATATTATTAGTCAAGAGTCTGGTAAAAAAGAAGCTTTTGGAA
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c13     GCTTGGATATCTCACATATTATTAGTCAAGAATCTGGTAAAAAAGAAGCTTTTGGAA
C26     GCTTGGATATCTCACATATTATTAGTCAAGAATCTGGTAAAAAAGAAGCTTTTGGAA
P8      CTTTGGATATCTCACATATTATTAGTCAAGAATCTGGTAAAAAAGAAGCTTTTGGAA
S43     TTGGATAT---TTCCATATTATTAGTCAAGAATCTGGTAAAAAAGAAGCATTCGGAA
S30     TTGGATAT---TTCCATATTATAGTC--AGAATCTGGTAAAAAAGAAGCATTCGGAA
S38     TTGGATAT---TTCCATATTATTAGTCAAGAATCTGGTAAAAAAGAAGCATTCGGAA
S48     TTGGATAT---TTCCATATTATTAGTCAAGAATCTGGTAAAAAAGAAGCATTCGGAA
td20    TTGGATAT---TTCCCTATTATTAGTCAAGAATCTGGTAAAAAAGAAGCATTCGGAA
TD24    TTTGGTATATTTCCCATATTATTAGTCAAGAATCTGGTAAAAAAGAAGCATTCGGAA
C20     TTTGGTATATTTCCCATATTATTAGTCAAGAATCTGGTAAAAAAGAAGCATTCGGAA
t17     ATTTGGATAATTTCCACATTATTAGTCAAGAATCTGGTAAAAAAGAAGCGTTCGGAA
                  *     **          ** ***************** ** ****

P28     CACTTGGAATAATTTATGCTATATTAGCTATTGGTGT-TCTAGGATTTGTGGTATGAGCT
S25     CACTTGGAATAATTTATGCTATATTAGCTATTGGTGT-TCTAGGATTTGTGGTATGAGCT
S7      CACTTGGAATAATTTATGCTATATTAGCTATTGGTGT-TCTAGGATTTGTGGTATGAGCT
s11     CACTTGGAATAATTTATGCTATATTAGCTATTGGTGT-TCTAGGATTTGTGGTATGAGCT
P29     CACTTGGAATAATTTATGCTATATTAGCTATTGGTGT-TCTAGGATTTGTGGTATGAGCT
TD18    CACTTGGAATAATTTATGCTATATTAGCTATTGGTGT-TCTAGGATTTGTGGTATGAGCT
p21     CACTTGGAATAATTTATGCTATATTAGCTATTGGTGT-TCTAGGATTTGTGGTATGAGCT
t16     CACTTGGAATAATTTATGCTATATTAGCTATTGGTGT-TCTAGGATTTGTGGTATGAGCT
s10     CACTTGGAATAATTTATGCTATATTAGCTATTGGTGT-TCTAGGATTTGTGGTATGAGCT
p17     CACTTGGAATAATTTATGCTATATTAGCTATTGGTGT-TCTAGGATTTGTGGTATGAGCT
C10     CACTTGGAATAATTTATGCTATATTAGCTATTGGTGT-TCTAGGATTTGTGGTATGAGCT
S33     CACTTGGAATAATTTATGCTATATTAGCTATTGGTGT-TCTAGGATTTGTGGTATGAGCT
c9      CACTTGGAATAATTTATGCTATATTAGCTATTGGTGT-TCTAGGATTTGTGGTATGAGCT
C41     CACTTGGAATAATTTATGCTATATTAGCTATTGGTGT-TCTAGGATTTGTGGTATGAGCT
T30     CATTAGGAATAATCTATGCTATACTAGCCATTGGTGT-TCTAGGATTTGTAGTATGAGCT
C21     CATTAGGAATAATCTATGCTATACTAGCCATTGGTGT-TCTAGGATTTGTAGTATGAGCT
P7      CATTAGGAATAATCTATGCTATACTAGCCATTGGTGT-TCTAGGATTTGTAGTATGAGCT
C18     CATTAGGAATAATCTATGCTATACTAGCCATTGGTGT-TCTAGGATTTGTAGTATGAGCT
p24     CATTAGGAATAATCTATGCTATACTAGCCATTGGTGT-TCTAGGATTTGTAGTATGAGCT
P39     CATTAGGAATAATCTATGCTATACTAGCCATTGGTGT-TCTAGGATTTGTAGTATGAGCT
S18     CATTAGGAATAATCTATGCTATACTAGCCATTGGTGT-TCTAGGATTTGTAGTATGAGCT
c4      CATTAGGAATAATCTATGCTATACTAGCCATTGGTGT-TCTAGGATTTGTAGTATGAGCT
C51     CATTAGGAATAATCTATGCTATACTAGCCATTGGTGT-TCTAGGATTTGTAGTATGAGCT
S1      CATTAGGAATAATCTATGCTATACTAGCCATTGGTGT-TCTAGGATTTGTAGTATGAGCT
P2      CATTAGGAATAATCTATGCTATACTAGCCATTGGTGT-TCTAGGATTTGTAGTATGAGCT
c6      CATTAGGAATAATCTATGCTATACTAGCCATTGGTGT-TCTAGGATTTGTAGTATGAGCT
T43     CATTAGGAATAATCTATGCTATACTAGCCATTGGTGT-TCTAGGATTTGTAGTATGAGCT
t11     CATTAGGAATAATCTATGCTATACTAGCCATTGGTGT-TCTAGGATTTGTAGTATGAGCT
C1      CATTAGGAATAATCTATGCTATACTAGCCATTGGTGT-TCTAGGATTTGTAGTATGAGCT
Td17    CATTAGGAATAATCTATGCTATACTAGCCATTGGTGT-TCTAGGATTTGTAGTATGAGCT
t18     CATTAGGAATAATCTATGCTATACTAGCCATTGGTGT-TCTAGGATTTGTAGTATGAGCT
p10     CATTAGGAATAATCTATGCTATACTAGCCATTGGTGT-TCTAGGATTTGTAGTATGAGCT
c13     CATTAGGTATAATTTATGCTATACTGGCTATTGGTGT-TCTAGGATTTGTAGTATGAGCT
C26     CATTAGGTATAATTTATGCTATACTGGCTATTGGTGT-TCTAGGATTTGTAGTATGAGCT
P8      CATTAGGTATAATTTATGCTATACTGGCTATTGGTGT-TCTAGGATTTGTAGTATGAGCT
S43     CACTCGGGATAATCTATGCTATACTTGCTATTGGTGT-CCTAGGATTCGTAGTATGAGCA
S30     CACTCGGGATAATCTATGCTATACTTGCTATTGGTGT-CCTAGGATTCGTAGTATGAGCA
S38     CACTCGGGATAATCTATGCTATACTTGCTATTGGTGT-CCTAGGATTCGTAGTATGAGCA
S48     CACTCGGGATAATCTATGCTATACTTGCTATTGGTGT-CCTAGGATTCGTAGTATGAGCA
td20    CACTCGGGATAATCTATGCTATACTTGCTATTGGTGT-CCTAGGATTCGTACTATGAGCA
TD24    CACTCGGGATAATCTATGCTATACTTGCTATTGGTGT-CCTAGGATTCGTAGTATGAGCA
C20     CACTCGGGATAATCTATGCTATACTTGCTATTGGTGT-CCTAGGATTCGTAGTATGAGCA
t17     CACTTGGAATAATTTATGCTATATAAGCTATTGGAGCATCCAGGACGCGGGGTGTGAACA
        ** * ** ***** *********   ** ***** *   * ****   *   * *** *

P28     CATCATATATTTAC-TGTAGGGATAGATGTTGATACTCGTGCTTACTTTACATCTGCTAC
S25     CATCATATATTTAC-TGTAGGGATAGATGTTGATACTCGTGCTTACTTTACATCTGCTAC
S7      CATCATATATTTAC-TGTAGGGATAGATGTTGATACTCGTGCTTACTTTACATCTGCTAC
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s11     CATCATATATTTAC-TGTAGGGATAGATGTTGATACTCGTGCTTACTTTACATCTGCTAC
P29     CATCATATATTTAC-TGTAGGGATAGATGTTGATACTCGTGCTTACTTTACATCTGCTAC
TD18    CATCATATATTTAC-TGTAGGGATAGATGTTGATACTCGTGCTTACTTTACATCTGCTAC
p21     CATCATATATTTAC-TGTAGGGATAGATGTTGATACTCGTGCTTACTTTACATCTGCTAC
t16     CATCATATATTTAC-TGTAGGGATAGATGTTGATACTCGTGCTTACTTTACATCTGCTAC
s10     CATCATATATTTAC-TGTAGGGATAGATGTTGATACTCGTGCTTACTTTACATCTGCTAC
p17     CATCATATATTTAC-TGTAGGGATAGATGTTGATACTCGTGCTTACTTTACATCTGCTAC
C10     CATCATATATTTAC-TGTAGGGATAGATGTTGATACTCGTGCTTACTTTACATCTGCTAC
S33     CATCATATATTTAC-TGTAGGGATAGATGTTGATACTCGTGCTTACTTTACATCTGCTAC
c9      CATCATATATTTAC-TGTAGGGATAGATGTTGATACTCGTGCTTACTTTACATCTGCTAC
C41     CATCATATATTTAC-TGTAGGGATAGATGTTGATACTCGTGCTTACTTTACATCTGCTAC
T30     CATCATATATTTAC-TGTAGGTATAGACGTTGATACTCGTGCTTACTTTACATCTGCTAC
C21     CATCATATATTTAC-TGTAGGTATAGACGTTGATACTCGTGCTTACTTTACATCTGCTAC
P7      CATCATATATTTAC-TGTAGGTATAGACGTTGATACTCGTGCTTACTTTACATCTGCTAC
C18     CATCATATATTTAC-TGTAGGTATAGACGTTGATACTCGTGCTTACTTTACATCTGCTAC
p24     CATCATATATTTAC-TGTAGGTATAGACGTTGATACTCGTGCTTACTTTACATCTGCTAC
P39     CATCATATATTTAC-TGTAGGTATAGACGTTGATACTCGTGCTTACTTTACATCTGCTAC
S18     CATCATATATTTAC-TGTAGGTATAGACGTTGATACTCGTGCTTACTTTACATCTGCTAC
c4      CATCATATATTTAC-TGTAGGTATAGACGTTGATACTCGTGCTTACTTTACATCTGCTAC
C51     CATCATATATTTAC-TGTAGGTATAGACGTTGATACTCGTGCTTACTTTACATCTGCTAC
S1      CATCATATATTTAC-TGTAGGTATAGACGTTGATACTCGTGCTTACTTTACATCTGCTAC
P2      CATCATATATTTAC-TGTAGGTATAGACGTTGATACTCGTGCTTACTTTACATCTGCTAC
c6      CATCATATATTTAC-TGTAGGTATAGACGTTGATACTCGTGCTTACTTTACATCTGCTAC
T43     CATCATATATTTAC-TGTAGGTATAGACGTTGATACTCGTGCTTACTTTACATCTGCTAC
t11     CATCATATATTTAC-TGTAGGTATAGACGTTGATACTCGTGCTTACTTTACATCTGCTAC
C1      CATCATATATTTAC-TGTAGGTATAGACGTTGACACTCGTGCTTACTTTACATCTGCTAC
Td17    CATCATATATTTAC-TGTAGGTATAGACGTTGATACTCGTGCTTACTTTACATCTGCTAC
t18     CATCATATATTTAC-TGTAGGTATAGACGTTGATACTCGTGCTTACTTTACATCTGCTAC
p10     CATCATATATTTAC-TGTAGGTATAGACGTTGATACTCGTGCTTACTTTACATCTGCTAC
c13     CATCATATATTTAC-TGTAGGTATAGACGTCGATACTCGTGCTTACTTTACATCTGCTAC
C26     CATCATATATTTAC-TGTAGGTATAGACGTCGATACTCGTGCTTACTTTACATCTGCTAC
P8      CATCATATATTTAC-TGTAGGTATAGACGTCGATACTCGTGCTTACTTTACATCTGCTAC
S43     CACCATATATTTAC-AGTAGGTATGGATGTTGATACTCGTGCTTATTTTACATCTGCAAC
S30     CACCATATATTTAC-AGTAGGTATGGATGTTGATACTCGTGCTTATTTTACATCTGCAAC
S38     CACCATATATTTAC-AGTAGGTATGGATGTTGATACTCGTGCTTATTTTACATCTGCAAC
S48     CACCATATATTTAC-AGTAGGTATGGATGTTGATACTCGTGCTTATTTTACATCTGCAAC
td20    CACCATATATTTAC-AGTAGGTATGGATGTTGATACTCGTGCTTATTTTACATCTGCAAC
TD24    CACCATATATTTAC-AGTAGGTATGGATGTTGATACTCGTGCTTATTTTACATCTGCAAC
C20     CACCATATATTTAC-AGTAGGTATGGATGTTGATACTCGTGCTTATTTTACATCTGCAAC
t17     CTTATTATTTCTCTGTGTGGTAAGAATTGGGTATACTCTCATCT-CTCTTTATCTGTTCG
        *    *** * *    ** *  *     *   * ****     *  * *  *****

P28     AATAATTATTGCTGTACCAACAGGTATTAAAATCTTCAGTTGATTAGGAACATTACATGG
S25     AATAATTATTGCTGTACCAACAGGTATTAAAATCTTCAGTTGATTAGGAACATTACATGG
S7      AATAATTATTGCTGTACCAACAGGTATTAAAATCTTCAGTTGATTAGGAACATTACATGG
s11     AATAATTATTGCTGTACCAACAGGTATTAAAATCTTCAGTTGATTAGGAACATTACACGG
P29     AATAATTATTGCTGTACCGACAGGTATTAAAATCTTCAGTTGATTAGGAACATTACATGG
TD18    AATAATTATTGCTGTACCAACAGGTATTAAAATCTTCAGTTGATTAGGAACATTACATGG
p21     AATAATTATTGCTGTACCAACAGGTATTAAAATCTTCAGTTGATTAGGAACATTACATGG
t16     AATAATTATTGCTGTACCAACAGGTATTAAAATCTTCAGTTGATTAGGAACATTACATGG
s10     AATAATTATTGCTGTACCAACAGGTATTAAAATCTTCAGTTGATTAGGAACATTACATGG
p17     AATAATTATTGCTGTACCAACAGGTATTAAAATCTTCAGTTGATTAGGAACATTACATGG
C10     AATAATTATTGCTGTACCAACAGGTATTAAAATCTTCAGTTGATTAGGAACATTACATGG
S33     AATAATTATTGCTGTACCAACAGGTATTAAAATCTTCAGTTGATTAGGAACATTACATGG
c9      AATAATTATTGCTGTACCAACAGGTATTAAAATCTTCAGTTGATTAGGAACATTACATGG
C41     AATAATTATTGCTGTACCAACAGGTATTAAAATCTTCAGTTGATTAGGAACATTACATGG
T30     GATAATTATTGCTGTCCCGACGGGTATTAAGATCTTCAGCTGACTAGGAACATTACACGG
C21     GATAATTATTGCTGTCCCGACGGGTATTAAGATCTTCAGCTGACTAGGAACATTACACGG
P7      GATAATTATTGCTGTCCCGACGGGTATTAAGATCTTCAGCTGACTAGGAACATTACACGG
C18     GATAATTATTGCTGTCCCGACGGGTATTAAGATCTTCAGCTGACTAGGAACATTACACGG
p24     GATAATTATTGCTGTCCCGACGGGTATTAAGATCTTCAGCTGACTAGGAACATTACACGG
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P39     GATAATTATTGCTGTCCCGACGGGTATTAAGATCTTCAGCTGACTAGGAACATTACACGG
S18     GATAATTATTGCTGTCCCGACGGGTATTAAGATCTTCAGCTGACTAGGAACATTACACGG
c4      GATAATTATTGCTGTCCCGACGGGTATTAAGATCTTCAGCTGACTAGGAACATTACACGG
C51     GATAATTATTGCTGTCCCGACGGGTATTAAGATCTTCAGCTGACTAGGAACATTACACGG
S1      GATAATTATTGCTGTCCCGACGGGTATTAAGATCTTCAGCTGACTAGGAACATTACACGG
P2      GATAATTATTGCTGTCCCGACGGGTATTAAGATCTTCAGCTGACTAGGAACATTACA-GG
c6      GATAATTATTGCTGTCCCGACGGGTATTAAGATCTTCAGCTGACTAGGAACATTACACGG
T43     GATAATTATTGCTGTCCCGACGGGTATTAAGATCTTCAGCTGACTAGGAACATTACACGG
t11     GATAATTATTGCTGTCCCGACGGGTATTAAGATCTTCAGCTGACTAGGAACATTACACGG
C1      GATAATTATTGCTGTCCCGACGGGTATTAAGATCTTCAGCTGACTAGGAACATTACACGG
Td17    GATAATTATTGCTGTCCCGACGGGTATTAAGATCTTCAGCTGACTAGGAACATTACACGG
t18     GATAATTATTGCTGTCCCGACGGGTATTAAGATCTTCAGCTGACTAGGAACATTACACGG
p10     GATAATTATTGCTGTCCCGACGGGTATTAAGATCTTCAGCTGACTAGGAACATTACACGG
c13     AATAATTATTGCTGTCCCAACGGGTATTAAAATCTTCAGCTGACTAGGAACATTACACGG
C26     AATAATTATTGCTGTCCCAACGGGTATTAAAATCTTCAGCTGACTAGGAACATTACACGG
P8      AATAATTATTGCTGTCCCAACGGGTATTAAAATCTTCAGCTGACTAGGAACATTACACGG
S43     AATGATTATTGCCGTTCCTACAGGTATTAAAATTTTCAGTTGACTAGGTACTCTTCATGG
S30     AATGATTATTGCCGTTCCTACAGGTATTAAAATTTTCAGTTGACTAGGTACTCTTCATGG
S38     AATGATTATTGCCGTTCCTACAGGTATTAAAATTTTCAGTTGACTAGGTACTCTTCATGG
S48     AATGATTATTGCCGTTCCTACAGGTATTAAAATTTTCAGTTGACTAGGTACTCTTCATGG
td20    AATGATTATTGCCGTTCCTACAGGTATTAAAATTTTCAGTTGACTAGGTACTCTTCATGG
TD24    AATGATTATTGCCGTTCCTACAGGTATTAAAATTTTCAGTTGACTAGGTACTCTTCATGG
C20     AATGATTATTGCCGTTCCTACAGGTATTAAAATTTTCAGTTGACTAGGTACTCTTCATGG
t17     CATAATGAATGTTGGACCAACAGGTAGGAATAGATACTGTTGCTTTCGAACATTCCATCC
          ** ** * **  *  ** ** ****  ** *  * * * **  *  * **  * **

P28     TAC-TCAGTTGAATTATAGTCCTTCTTTAATTTGAGCCTTAGGGTTTGTATTCTTATTTA
S25     TAC-TCAGTTGAATTATAGTCCTTCTTTAATTTGAGCCTTAGGGTTTGTATTCTTATTTA
S7      TAC-TCAGTTGAATTATAGTCCTTCTTTAATTTGAGCCTTAGGGTTTGTATTCTTATTTA
s11     TAC-TCAGTTGAATTATAGTCCTTCTTTAATTTGAGCCTTAGGGTTTGTATTCTTATTTA
P29     TAC-TCAGTTGAATTATAGTCCTTCTTTAATTTGAGCCTTAGGGTTTGTATTCTTATTTA
TD18    TAC-TCAGTTGAATTATAGTCCTTCTTTAATTTGAGCCTTAGGGTTTGTATTCTTATTTA
p21     TAC-TCAGTTGAATTATAGTCCTTCTTTAATTTGAGCCTTAGGGTTTGTATTCTTATTTA
t16     TAC-TCAGTTGAATTATAGTCCTTCTTTAATTTGAGCCTTAGGGTTTGTATTCTTATTTA
s10     TAC-TCAGTTGAATTATAGTCCTTCTTTAATTTGAGCCTTAGGGTTTGTATTCTTATTTA
p17     TAC-TCAGTTGAATTATAGTCCTTCTTTAATTTGAGCCTTAGGGTTTGTATTCTTATTTA
C10     TAC-TCAGTTGAATTATAGTCCTTCTTTAATTTGAGCCTTAGGGTTTGTATTCTTATTTA
S33     TAC-TCAGTTGAATTATAGTCCTTCTTTAATTTGAGCCTTAGGGTTTGTATTCTTATTTA
c9      TAC-TCAGTTGAATTATAGTCCTTCTTTAATTTGAGCCTTAGGGTTTGTATTCTTATTTA
C41     TAC-TCAGTTGAATTATAGTCCTTCTTTAATTTGAGCCTTAGGGTTTGTATTCTTATTTA
T30     TAC-TCAATTGAATTATAGTCCTTCTTTAATTTGGGCATTAGGGTTTGTATTTTTATTTA
C21     TAC-TCAATTGAATTATAGTCCTTCTTTAATTTGGGCATTAGGGTTTGTATTTTTATTTA
P7      TAC-TCAATTGAATTATAGTCCTTCTTTAATTTGGGCATTAGGGTTTGTATTTTTATTTA
C18     TAC-TCAATTGAATTATAGTCCTTCTTTAATTTGGGCATTAGGGTTTGTATTTTTATTTA
p24     TAC-TCAATTGAATTATAGTCCTTCTTTAATTTGGGCATTAGGGTTTGTATTTTTATTTA
P39     TAC-TCAATTGAATTATAGTCCTTCTTTAATTTGGGCATTAGGGTTTGTATTTTTATTTA
S18     TAC-TCAATTGAATTATAGTCCTTCTTTAATTTGGGCATTAGGGTTTGTATTTTTATTTA
c4      TAC-TCAATTGAATTATAGTCCTTCTTTAATTTGGGCATTAGGGTTTGTATTTTTATTTA
C51     TAC-TCAATTGAATTATAGTCCTTCTTTAATTTGGGCATTAGGGTTTGTATTTTTATTTA
S1      TAC-TCAATTGAATTATAGTCCTTCTTTAATTTGGGCATTAGGGTTTGTATTTTTATTTA
P2      TAC-TCAATTGAATTATAGTCCTTCTTTAATTTGGGCATTAGGGTTTGTATTTTTATTTA
c6      TAC-TCAATTGAATTATAGTCCTTCTTTAATTTGGGCATTAGGGTTTGTATTTTTATTTA
T43     TAC-TCAATTGAATTATAGTCCTTCTTTAATTTGGGCATTAGGGTTTGTATTTTTATTTA
t11     TAC-TCAATTGAATTATAGTCCTTCTTTAATTTGGGCATTACGGTTTGTATTTTTATTTA
C1      TAC-TCAATTGAATTATAGTCCTTCTTTAATTTGGGCATTAGGGTTTGTATTTTTATTTA
Td17    TAC-TCAATTGAATTATAGTCCTTCTTTAATTTGGGCATTAGGGTTTGTATTTTTATTTA
t18     TAC-TCAATTGAATTATAGTCCTTCTTTAATTTGGGCATTAGGGTTTGTATTTTTATTTA
p10     TAC-TCAATTGAATTATAGTCCTTCTTTAATTTGGGCATTAGGGTTTGTATTTCTATTTA
c13     TAC-TCAATTGAATTATAGTCCTTCTTTAATTTGGGCATTAGGGTTTGTATTTTTATTTA
C26     TAC-TCAATTGAATTATAGTCCTTCTTTAATTTGGGCATTAGGGTTTGTATTTTTATTTA
P8      TAC-TCAATTGAATTATAGTCCTTCTTTAATTTGGGCATTAGGGTTTGTATTTTTATTTA
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S43     TAC-TCAACTTAACTATAGTCCTTCTCTAATTTGAGCGCTAGGATTTGTATTTCTATTTA
S30     TAC-TCAACTTAACTATAGTCCTTCTCTAATTTGAGCGCTAGGATTTGTATTTCTATTTA
S38     TAC-TCAACTTAACTATAGTCCTTCTCTAATTTGAGCGCTAGGATTTGTATTTCTATTTA
S48     TAC-TCAACTTAACTATAGTCCTTCTCTAATTTGAGCGCTAGGATTTGTATTTCTATTTA
td20    TAC-TCAACTTAACTATAGTCCTTCTCTAATTTGAGCGCTAGGATTTGTATTTCTATTTA
TD24    TAC-TCAACTTAACTATAGTCCTTCTCTAATTTGAGCGCTAGGATTTGTATTTCTATTTA
C20     TAC-TCAACTTAACTATAGTCCTTCTCTAATTTGAGCGCTAGGATTTGTATTTCTATTTA
t17     CTTGTCTATTGAATTAAATTACCTACTCACCATGGGTGGAACGGATTGTATTCTTCTCCA
            **   * ** ** * * * *    *   ** *    * *  *******  * *  *

P28     CAGTAGGAGGTTTAACAGGAGTTGTACTTGCTAACTCATCTATTGACATCATCTTGCACG
S25     CAGTAGGAGGTTTAACAGGAGTTGTACTTGCTAACTCATCTATTGACATCATCTTGCACG
S7      CAGTAGGAGGTTTAACAGGAGTTGTACTTGCTAACTCATCTATTGACATCATCTTGCACG
s11     CAGTAGGAGGTTTAACAGGAGTTGTACTTGCTAACTCATCTATTGACATCATCTTGCACG
P29     CAGTAGGAGGTTTAACAGGAGTTGTACTTGCTAACTCATCTATTGACATCATCTTGCACG
TD18    CAGTAGGAGGTTTAACAGGAGTTGTACTTGCTAACTCATCTATTGACATCATCTTGCACG
p21     CAGTAGGAGGTTTAACAGGAGTTGTACTTGCTAACTCATCTATTGACATCATCTTGCACG
t16     CAGTAGGAGGTTTAACAGGAGTTGTACTTGCTAACTCATCTATTGACATCATCTTGCACG
s10     CAGTAGGAGGTTTAACAGGAGTTGTACTTGCTAACTCATCTATTGACATCATCTTACACG
p17     CAGTAGGAGGTTTAACAGGAGTTGTACTTGCTAACTCATCTATTGACATCATCTTGCACG
C10     CAGTAGGAGGTTTAACAGGAGTTGTACTTGCTAACTCATCTATTGACATCATCTTGCACG
S33     CAGTAGGAGGTTTAACAGGAGTTGTACTTGCTAACTCATCTATTGACATCATCTTACACG
c9      CAGTAGGAGGTTTAACAGGAGTTGTACTTGCTAACTCATCTATTGACATCATCTTACACG
C41     CAGTAGGAGGTTTAACAGGAGTTGTACTTGCTAACTCATCTATTGACATCATCTTACACG
T30     CAGTTGGGGGTCTAACAGGAGTTGTCCTTGCTAATTCATCTATTGATATTATCTTGCACG
C21     CAGTTGGGGGTCTAACAGGAGTTGTCCTTGCTAATTCATCTATTGATATTATCTTGCACG
P7      CAGTTGGGGGTCTAACAGGAGTTGTCCTTGCTAATTCATCTATTGATATTATCTTGCACG
C18     CAGTTGGGGGTCTAACAGGAGTTGTCCTTGCTAATTCATCTATTGATATTATCTTGCACG
p24     CAGTTGGGGGTCTAACAGGAGTTGTCCTTGCTAATTCATCTATTGATATTATCTTGCACG
P39     CAGTTGGGGGTCTAACAGGAGTTGTCCTTGCTAATTCATCTATTGATATTATCTTGCACG
S18     CAGTTGGGGGTCTAACAGGAGTTGTCCTTGCTAATTCATCTATTGATATTATCTTGCACG
c4      CAGTTGGGGGTCTAACAGGAGTTGTCCTTGCTAATTCATCTATTGATATTATCTTGCACG
C51     CAGTTGGGGGTCTAACAGGAGTTGTCCTTGCTAATTCATCTATTGATATTATCTTGCACG
S1      CAGTTGGGGGTCTAACAGGAGTTGTCCTTGCTAATTCATCTATTGATATTATCTTGCACG
P2      CAGTTGGGGGTCTAACAGGAGTTGTCCTTGCTAATTCATCTATTGATATTATCTTGCACG
c6      CAGTTGGGGGTCTAACAGGAGTTGTCCTTGCTAATTCATCTATTGATATTATCTTGCACG
T43     CAGTTGGGGGTCTAACAGGAGTTGTCCTTGCTAATTCATCTATTGATATTATCTTGCACG
t11     CAGTTGGGGGTCTAACAGGAGTTGTCCTTGCTAATTCATCTATTGATATTATCTTGCACG
C1      CAGTTGGGGGTCTAACAGGAGTTGTCCTTGCTAATTCATCTATTGATATTATCTTGCACG
Td17    CAGTTGGGGGTCTAACAGGAGTTGTCCTTGCTAATTCATCTATTGATATTATCTTGCACG
t18     CAGTTGGGGGTCTAACAGGAGTTGTCCTTGCTAATTCATCTATTGATATTATCTTGCACG
p10     CAGTTGGGGGTCTAACAGGAGTTGTCCTTGATCATTCATCTATTGATACTATCGTGCGAG
c13     CAGTTGGGGGTCTAACAGGAGTTGTCCTTGCCAATTCATCTATTGATATTATTTTACATG
C26     CAGTTGGGGGTCTAACAGGAGTTGTCCTTGCCAATTCATCTATTGATATTATCTTACATG
P8      CAGTTGGGGGTCTAACAGGAGTTGTCCTTGCCAATTCATCTATTGATATTATCTTACATG
S43     CTGTGGGTGGTTTAACCGGTGTTGTACTAGCTAATTCTTCAATTGACATTATTTTACATG
S30     CTGTGGGTGGTTTAACCGGTGTTGTACTAGCTAATTCTTCAATTGACATTATTTTACATG
S38     CTGTGGGTGGTTTAACCGGTGTTGTACTAGCTAATTCTTCAATTGACATTATTTTACATG
S48     CTGTGGGTGGTTTAACCGGTGTTGTACTAGCTAATTCTTCAATTGACATTATTTTACATG
td20    CTGTGGGTGGTTTAACCGGTGTTGTACTAGCTAATTCTTCAATTGACATTATTTTACATG
TD24    CTGTGGGTGGTTTAACCGGTGTTGTACTAGCTAATTCTTCAATTGACATTATTTTACATG
C20     CTGTGGGTGGTTTAACCGGTGTTGTACTAGCTAATTCTTCAATTGACATTATTTTACATG
t17     CTGCAGGATGTTCAATAGGAGATGCACTTGGCTACTCATCTCCTGCCGTCTTCTTGCTCG
        * *  **  **  **  ** * **  ** *   * ** **   **      *  * *  *

P28     ATACTTATTATGTAGTAGCCCATTTTCACTACGT-CCTTTCAATAGGAGCAGTATTTGGT
S25     ATACTTATTATGTAGTAGCCCATTTTCACTACGT-CCTTTCAATAGGAGCAGTATTTGGT
S7      ATACTTATTATGTAGTAGCCCATTTTCACTACGT-CCTTTCAATAGGAGCAGTATTTGGT
s11     ATACTTATTATGTAGTAGCCCATTTTCACTACGT-CCTTTCAATAGGAGCAGTATTTGGT
P29     ATACTTATTATGTAGTAGCCCATTTTCACTACGT-CCTTTCAATAGGAGCAGTATTTGGT
TD18    ATACTTATTATGTAGTAGCCCATTTTCACTACGT-CCTTTCAATAGGAGCAGTATTTGGT
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p21     ATACTTATTATGTAGTAGCCCATTTTCACTACGT-CCTTTCAATAGGAGCAGTATTTGGT
t16     ATACTTATTATGTAGTAGCCCATTTTCACTACGT-CCTTTCAATAGGAGCAGTATTTGGT
s10     ATACTTATTATGTAGTAGCCCATTTTCACTATGT-CCTTTCAATAGGAGCAGTATTTGGT
p17     ATACTTATTATGTAGTAGCCCATTTTCACTACGT-CCTTTCAATAGGAGCAGTATTTGGT
C10     ATACTTATTATGTAGTAGCCCATTTTCACTACGT-CCTTTCAATAGGAGCAGTATTTGGT
S33     ATACTTATTATGTAGTAGCCCATTTTCACTATGT-CCTTTCAATAGGAGCAGTATTTGGT
c9      ATACTTATTATGTAGTAGCCCATTTTCACTATGT-CCTTTCAATAGGAGCAGTATTTGGT
C41     ATACTTATTATGTAGTAGCCCATTTTCACTATGT-CCTTTCAATAGGAGCAGTATTTGGT
T30     ATACTTATTATGTAGTAGCCCACTTCCACTATGT-TCTTTCAATAGGAGCCGTATTTGGT
C21     ATACTTATTATGTAGTAGCCCACTTCCACTATGT-TCTTTCAATAGGAGCCGTATTTGGT
P7      ATACTTATTATGTAGTAGCCCACTTCCACTATGT-TCTTTCAATAGGAGCCGTATTTGGT
C18     ATACTTATTATGTAGTAGCCCACTTCCACTATGT-TCTTTCAATAGGAGCCGTATTTGGT
p24     ATACTTATTATGTAGTAGCCCACTTCCACTATGT-TCTTTCAATAGGAGCCGTATTTGGT
P39     ATACTTATTATGTAGTAGCCCACTTCCACTATGT-TCTTTCAATAGGAGCCGTATTTGGT
S18     ATACTTATTATGTAGTAGCCCACTTCCACTATGT-TCTTTCAATAGGAGCCGTATTTGGT
c4      ATACTTATTATGTAGTAGCCCACTTCCACTATGT-TCTTTCAATAGGAGCCGTATTTGGT
C51     ATACTTATTATGTAGTAGCCCACTTCCACTATGT-TCTTTCAATAGGAGCCGTATTTGGT
S1      ATACTTATTATGTAGTAGCCCACTTCCACTATGT-TCTTTCAATAGGAGCCGTATTTGGT
P2      ATACTTATTATGTAGTAGCCCACTTCCACTATGT-TCTTTCAATAGGAGCCGTATTTGGT
c6      ATACTTATTATGTAGTAGCCCACTTCCACTATGT-TCTTTCAATAGGAGCCGTATTTGGT
T43     ATACTTATTATGTAGTAGCCCACTTCCACTATGT-TCTTTCAATAGGAGCCGTATTTGGT
t11     ATACTTATTATGTAGTAGCCCACTTCCACTATGT-TCTTTCAATAGGAGCCGTATTTGGT
C1      ATACTTATTATGTAGTAGCCCACTTCCACTATGT-TCTTTCAATAGGAGCCGTATTTGGT
Td17    ATACTTATTATGTAGTAGCCCACTTCCACTATGT-TCTTTCAATAGGAGCCGTATTTGGT
t18     ATACTTATTATGTAGTAGCCCACTTCCACTATGT-TCTTTCAATAGGAGCCGTATTTGGT
p10     ATACGTATTATGTAGGAGCCCACTTCCACTATGT-TCTTTCAATAGGAGCCGAATTTGCT
c13     ATACTTATTATGTAGTAGCCCATTTTCACTATGT-TCTTTCAATAGGAGCCGTATTTGGT
C26     ATACTTATTATGTAGTAGCCCATTTTCACTATGT-TCTTTCAATAGGAGCCGTATTTGGT
P8      ATACTTATTATGTAGTAGCCCATTTTCACTATGT-TCTTTCAATAGGAGCCGTATTTGGT
S43     ATACATACTACGTAGTAGCACATTTCCACTATGT-TCTTTCAATAGGAGCTGTATTTGGT
S30     ATACATACTACGTAGTAGCACATTTCCACTATGT-TCTTTCAATAGGAGCTGTATTTGGT
S38     ATACATACTACGTAGTAGCACATTTCCACTATGT-TCTTTCAATAGGAGCTGTATTTGGT
S48     ATACATACTACGTAGTAGCACATTTCCACTATGT-TCTTTCAATAGGAGCTGTATTTGGT
td20    ATACATACTACGTAGTAGCACATTTCCACTATGT-TCTTTCAATAGGAGCTGTATTTGGT
TD24    ATACATACTACGTAGTAGCACATTTCCACTATGT-TCTTTCAATAGGAGCTGTATTTGGT
C20     ATACATACTACGTAGTAGCACATTTCCACTATGT-TCTTTCAATAGGAGCTGTATTTGGT
t17     AGAGAGACTAAGGAGCACTCTATTTGCACTACGTCTCTTTCCATAGGAACAATACTTGTC
        * *   * ** * ** *    * ** ***** **  ***** ****** *   * ***

P28     ATTTTTGCAGGTATTGCTCACTGATTTCCTCTTTTTACTGGTTTAACCTT-AAACCCAAA
S25     ATTTTTGCAGGTATTGCTCACTGATTTCCTCTTTTTACTGGTTTAACCTT-AAACCCAAA
S7      ATTTTTGCAGGTATTGCTCACTGATTTCCTCTTTTTACTGGTTTAACCTT-AAACCCAAA
s11     ATTTTTGCAGGTATTGCTCACTGATTTCCTCTTTTTACTGGTTTAACCTT-AAACCCAAA
P29     ATTTTTGCAGGTATTGCTCACTGATTTCCTCTTTTTACTGGTTTAACCTT-AAACCCAAA
TD18    ATTTTTGCAGGTATTGCTCACTGATTTCCTCTTTTTACTGGTTTAACCTT-AAACCCAAA
p21     ATTTTTGCAGGTATTGCTCACTGATTTCCTCTTTTTACTGGTTTAACCTT-AAACCCAAA
t16     ATTTTTGCAGGTATTGCTCACTGATTTCCTCTTTTTACTGGTTTAACCTT-AAACCCAAA
s10     ATTTTTGCAGGTATTGCTCACTGATTTCCTCTTTTTACTGGTTTAACCTT-AAACCCAAA
p17     ATTTTTGCAGGTATTGCTCACTGATTTCCTCTTTTTACTGGTTTAACCTT-AAACCCAAA
C10     ATTTTTGCAGGTATTGCTCACTGATTTCCTCTTTTTACTGGTTTAACCTT-AAACCCAAA
S33     ATTTTTGCAGGTATTGCTCACTGATTTCCTCTTTTTACTGGTTTAACCTT-AAACCCAAA
c9      ATTTTTGCAGGTATTGCTCACTGATTTCCTCTTTTTACTGGTTTAACCTT-AAACCCAAA
C41     ATTTTTGCAGGTATTGCTCACTGATTTCCTCTTTTTACTGGTTTAACCTT-AAACCCAAA
T30     ATTTTTGCAGGTATTGCCCACTGATTTCCTCTTTTTACCGGTTTAACCCT-GAACCCAAA
C21     ATTTTTGCAGGTATTGCCCACTGATTTCCTCTTTTTACCGGTTTAACCCT-GAACCCAAA
P7      ATTTTTGCAGGTATTGCCCACTGATTTCCTCTTTTTACCGGTTTAACCCT-GAACCCAAA
C18     ATTTTTGCAGGTATTGCCCACTGATTTCCTCTTTTTACCGGTTTAACCCT-GAACCCAAA
p24     ATTTTTGCAGGTATTGCCCACTGATTTCCTCTTTTTACCGGTTTAACCCT-GAACCCAAA
P39     ATTTTTGCAGGTATTGCCCACTGATTTCCTCTTTTTACCGGTTTAACCCT-GAACCCAAA
S18     ATTTTTGCAGGTATTGCCCACTGATTTCCTCTTTTTACCGGTTTAACCCT-GAACCCAAA
c4      ATTTTTGCAGGTATTGCCCACTGATTTCCTCTTTTTACCGGTTTAACCCT-GAACCCAAA
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C51     ATTTTTGCAGGTATTGCCCACTGATTTCCTCTTTTTACCGGTTTAACCCT-GAACCCAAA
S1      ATTTTTGCAGGTATTGCCCACTGATTTCCTCTTTTTACCGGTTTAACCCT-GAACCCAAA
P2      ATTTTTGCAGGTATTGCCCACTGATTTCCTCTTTTTACCGGTTTAACCCT-GAACCCAAA
c6      ATTTTTGCAGGTATTGCCCACTGATTTCCTCTTTTTACCGGTTTAACCCT-GAACCCAAA
T43     ATTTTTGCAGGTATTGCCCACTGATTTCCTCTTTTTACCGGTTTAACCCT-GAACCCAAA
t11     ATTTTTGCAGGTATTGCCCACTGATTTCCTCTTTTTACCGGTTTAACCCT-GAACCCAAA
C1      ATTTTTGCAGGTATTGCCCACTGATTTCCTCTTTTTACCGGTTTAACCCT-GAACCCAAA
Td17    ATTTTTGCAGGTATTGCCCACTGATTTCCTCTTTTTACCGGTTTAACCCT-GAACCCAAA
t18     ATTTTTGCAGGTATTGCCCACTGATTTCCTCTTTTTACCGGTTTAACCCT-GAACCCAAA
p10     ATTTTTGCCCGTATCGCCCACTGATATCCTCTTTTTACTGCTTTAACCCCAGAACCCAAA
c13     ATTTTTGCAGGTATTGCTCACTGATTTCCTCTTTTTACCGGTTTAACCCT-GAACCCAAA
C26     ATTTTTGCAGGTATTGCTCACTGATTTCCTCTTTTTACCGGTTTAACCCT-GAACCCAAA
P8      ATTTTTGCAGGTATTGCTCACTGATTTCCTCTTTTTACCGGTTTAACCCT-GAACCCAAA
S43     ATTTTTGCAGGTATTGCCCACTGATTCCCTTTATTTACGGGGCTTACCTT-AAACCCTAA
S30     ATTTTTGCAGGTATTGCCCACTGATTCCCTTTATTTACGGGGCTTACCTT-AAACCCTAA
S38     ATTTTTGCAGGTATTGCCCACTGATTCCCTTTATTTACGGGGCTTACCTT-AAACCCTAA
S48     ATTTTTGCAGGTATTGCCCACTGATTCCCTTTATTTACGGGGCTTACCTT-AAACCCTAA
td20    ATTTTTGCAGGTATTGCCCACTGATTCCCTTTATTTACGGGGCTTACCTT-AAACCCTAA
TD24    ATTTTTGCAGGTATTGCCCACTGATTCCCTTTATTTACGGGGCTTACCTT-AAACCCTAA
C20     ATTTTTGCAGGTATTGCCCACTGATTCCCTTTATTTACGGGGCTTACCTT-AAACCCTAA
t17     AATTTTGCTTGTCTTGCTCGCTGTCTACCTCTTATTGTTGGGTAAACCCT-ACACCCACA
        * ******  ** * ** * ***    *** *  **   *     ***     ****  *

P28     ATGATTAAAAATCCACTTTTTAGTTATATTTATTGGGGTAAACATTACATTTTTCCCACA
S25     ATGATTAAAAATCCACTTTTTAGTTATATTTATTGGGGTAAACATTACATTTTTCCCACA
S7      ATGATTAAAAATCCACTTTTTAGTTATATTTATTGGGGTAAACATTACATTTTTCCCACA
s11     ATGATTAAAAATCCACTTTTTAGTTATATTTATTGGGGTAAACATTACATTTTTCCCACA
P29     ATGATTAAAAATCCACTTTTTAGTTATATTTATTGGGGTAAACATTACATTTTTCCCACA
TD18    ATGATTAAAAATCCACTTTTTAGTTATATTTATTGGGGTAAACATTACATTTTTCCCACA
p21     ATGATTAAAAATCCACTTTTTAGTTATATTTATTGGGGTAAACATTACATTTTTCCCACA
t16     ATGATTAAAAATCCACTTTTTAGTTATATTTATTGGGGTAAACATTACATTTTTCCCACA
s10     ATGATTAAAAATCCACTTTTTAGTTATATTTATTGGGGTAAACATTACATTTTTCCCACA
p17     ATGATTAAAAATCCACTTTTTAGTTATATTTATTGGGGTAAACATTACATTTTTCCCACA
C10     ATGATTAAAAATCCACTTTTTAGTTATATTTATTGGGGTAAACATTACATTTTTCCCACA
S33     ATGATTAAAAATCCACTTTTTAGTTATATTTATTGGGGTAAACATTACATTTTTCCCACA
c9      ATGATTAAAAATCCACTTTTTAGTTATATTTATTGGGGTAAACATTACATTTTTCCCACA
C41     ATGATTAAAAATCCACTTTTTAGTTATATTTATTGGGGTAAACATTACATTTTTCCCACA
T30     ATGATTAAAAATCCACTTTCTAGTTATATTTATTGGGGTAAACATTACATTTTTCCCTCA
C21     ATGATTAAAAATCCACTTTCTAGTTATATTTATTGGGGTAAACATTACATTTTTCCCTCA
P7      ATGATTAAAAATCCACTTTCTAGTTATATTTATTGGGGTAAACATTACATTTTTCCCTCA
C18     ATGATTAAAAATCCACTTTCTAGTTATATTTATTGGGGTAAACATTACATTTTTCCCTCA
p24     ATGATTAAAAATCCACTTTCTAGTTATATTTATTGGGGTAAACATTACATTTTTCCCTCA
P39     ATGATTAAAAATCCACTTTCTAGTTATATTTATTGGGGTAAACATTACATTTTTCCCTCA
S18     ATGATTAAAAATCCACTTTCTAGTTATATTTATTGGGGTAAACATTACATTTTTCCCTCA
c4      ATGATTAAAAATCCACTTTCTAGTTATATTTATTGGGGTAAACATTACATTTTTCCCTCA
C51     ATGATTAAAAATCCACTTTCTAGTTATATTTATTGGGGTAAACATTACATTTTTCCCTCA
S1      ATGATTAAAAATCCACTTTCTAGTTATATTTATTGGGGTAAACATTACATTTTTCCCTCA
P2      ATGATTAAAAATCCACTTTCTAGTTATATTTATTGGGGTAAACATTACATTTTTCCCTCA
c6      ATGATTAAAAATCCACTTTCTAGTTATATTTATTGGGGTAAACATTACATTTTTCCCTCA
T43     ATGATTAAAAATCCACTTTCTAGTTATATTTATTGGGGTAAACATTACATTTTTCCCTCA
t11     ATGATTAAAAATCCACTTTCTAGTTATATTTATTGGGGTAAACATTACATTTTTCCCTCA
C1      ATGATTAAAAATCCACTTTCTAGTTATATTTATTGGGGTAAACATTACATTTTTCCCTCA
Td17    ATGATTAAAAATCCACTTTCTAGTTATATTTATTGGGGTAAACATTACATTTTTCCCTCA
t18     ATGATTAAAAATCCACTTTTTAGTTATATTTATTGGGGTAAACATTACATTTTTCCCTCA
p10     ATGATTAAGAATCCACCTTCTATTTATATTTCTGGGGGTAAACATTGCCTTCCTCCCTCG
c13     ATGATTAAAAATCCACTTTCTAGTTATATTTATTGGGGTAAATATTACATTTTTCCCACA
C26     ATGATTAAAAATCCACTTTCTAGTTATATTTATTGGGGTAAATATTACATTTTTCCCACA
P8      ATGATTAAAAATCCACTTTCTAGTTATATTTATTGGGGTAAATATTACATTTTTCCCACA
S43     GTGATTAAAAATTCACTTTCTTGTTATATTCATTGGAGTAAATATTACATTCTTCCCCCA
S30     GTGATTAAAAATTCACTTTCTTGTTATATTCATTGGAGTAAATATTACATTCTTCCCCCA
S38     GTGATTAAAAATTCACTTTCTTGTTATATTCATTGGAGTAAATATTACATTCTTCCCCCA
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S48     ATGATTAAAAATTCACTTTCTTGTTATATTCATTGGAGTAAATATTACATTCTTCCCCCA
td20    ATGATTAAAAATTCACTTTCTTGTTATATTCATTGGAGTAAATATTACATTCTTCCCCCA
TD24    GTGATTAAAAATTCACTTTCTTGTTATATTCATTGGAGTAAATATTACATTCTTCCCCCA
C20     ATGATTAAAAATTCACTTTCTTGTTATATTCATTGGAGTAAATATTACATTCTTCCCCCA
t17     ACGATTAAAAATCCACTGCTTGCTCACATTTATTGGGCTAAATGTTAAATATTTTCACAC
          ****** *** ***    *  * * ***  * **  ****  **   *   * *

P28     ACATTTCTT-AGGACTTAATGGAATACCTCGGCGAT
S25     ACATTTCTT-AGGACTTAATGGAATACCTCGGCGAT
S7      ACATTTCTT-AGGACTTAATGGAATACCTCGGCGAT
s11     ACATTTCTT-AGGACTTAATGGAATACCTCGGCGAT
P29     ACATTTCTT-AGGACTTAATGGAATACCTCGGCGAT
TD18    ACATTTCTT-AGGACTTAATGGAATACCTCGGCGAT
p21     ACATTTCTT-AGGACTTAATGGAATACCTCGGCGAT
t16     ACATTTCTT-AGGACTTAATGGAATACCTCGGCGAT
s10     ACATTTCTT-AGGACTTAATGGAATACCTCGGCGAT
p17     ACATTTCTT-AGGACTTAATGGAATACCTCGGCGAT
C10     ACATTTCTT-AGGACTTAATGGAATACCTCGGCGAT
S33     ACATTTCTT-AGGACTTAATGGAATACCTCGGCGAT
c9      ACATTTCTT-AGGACTTAATGGAATACCTCGGCGAT
C41     ACATTTCTT-AGGACTTAATGGAATACCTCGGCGAT
T30     ACATTTTTT-AGGGCTTAATGGAATACCTCGGCGAT
C21     ACATTTTTT-AGGGCTTAATGGAATACCTCGGCGAT
P7      ACATTTTTT-AGGGCTTAATGGAATACCTCGGCGAT
C18     ACATTTTTT-AGGGCTTAATGGAATACCTCGGCGAT
p24     ACATTTTTT-AGGGCTTAATGGAATACCTCGGCGAT
P39     ACATTTTTT-AGGGCTTAATGGAATACCTCGGCGAT
S18     ACATTTTTT-AGGGCTTAATGGAATACCTCGGCGAT
c4      ACATTTTTT-AGGGCTTAATGGAATACCTCGGCGAT
C51     ACATTTTTTTAGGGCTTAATGGAATACCTCGGCGA-
S1      ACATTTTTT-AGGGCTTAATGGAATACCTCGGCGAT
P2      ACATTTTTT-AGGGCTTAATGGAATACCTCGGCGAT
c6      ACATTTTTT-AGGGCTTAATGGAATACCTCGGCGAT
T43     ACATTTTTT-AGGGCTTAATGGAATACCTCGGCGAT
t11     ACATTTTTT-AGGGCTTAATGGAATACCTCGGCGAT
C1      ACATTTTTT-AGGGCTTAATGGAATACCTCGGCGAT
Td17    ACATTTTTT-AGGGCTTAATGGAATACCTCGGCGAT
t18     ACATTTTTT-AGGGCTTAATGGAATACCTCGGCGAT
p10     TCATTCATGTAGGGCTTAATGGAATACCTCGGCGAT
c13     ACATTTTCT-AGGGCTTAATGGAATACCTCGGCGAT
C26     ACATTTTCT-AGGGCTTAATGGAATACCTCGGCGAT
P8      ACATTTTCT-AGGGCTTAATGGAATACCTCGGCGAT
S43     ACATTTCTT-AGGTCTTAATGGAATACCTCGGCGAT
S30     ACATTTCTT-AGGTCTTAATGGAATACCTCGGCGAT
S38     ACATTTCTT-AGGTCTTAATGGAATACCTCGGCGAT
S48     ACATTTCTT-AGGTCTTAATGGAATACCTCGGCGAT
td20    ACATTTCTT-AGGTCTTAATGGAATACCTCGGCGAT
TD24    ACATTTCTT-AGGTCTTAATGGAATACCTCGGCGAT
C20     ACATTTCTT-AGGTCTTAATGGAATACCTCGGCGAT
t17     TACCTTCCTTATGACTTAATGGAATACCTCGGCGAT
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Figure 3.33 Multiple alignments of 43 shrimp individuals representing each haplotype of the COI_{614} gene segment. Asterisks indicate identical nucleotide at a particular position.

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I	P28								
II	S25	0.0018							
III	S7	0.0018	0.0035						
IV	s11	0.0018	0.0035	0.0035					
V	P29	0.0018	0.0035	0.0035	0.0035				
VI	TD18	0.0018	0.0035	0.0035	0.0035	0.0035			
VII	p21	0.0107	0.0089	0.0107	0.0125	0.0125	0.0125		
VIII	t16	0.0107	0.0089	0.0107	0.0125	0.0125	0.0125	0.0018	
IX	s10	0.0143	0.0125	0.0143	0.0161	0.0161	0.0161	0.0035	0.0035
X	p17	0.0107	0.0089	0.0107	0.0125	0.0125	0.0125	0.0035	0.0035
		0.0071							
XI	C10	0.0089	0.0071	0.0089	0.0107	0.0107	0.0107	0.0053	0.0053
		0.0089	0.0018						
XII	S33	0.0071	0.0089	0.0071	0.0089	0.0089	0.0089	0.0142	0.0142
		0.0106	0.0142	0.0125					
XIII	c9	0.0035	0.0053	0.0053	0.0053	0.0053	0.0053	0.0143	0.0143
		0.0107	0.0143	0.0125	0.0035				
XIV	C41	0.0053	0.0071	0.0035	0.0071	0.0071	0.0071	0.0143	0.0143
		0.0107	0.0143	0.0125	0.0035	0.0018			
XV	T30	0.0895	0.0917	0.0896	0.0875	0.0877	0.0877	0.0932	0.0934
		0.0932	0.0933	0.0914	0.0853	0.0895	0.0896		
XVI	C21	0.0944	0.0964	0.0945	0.0924	0.0924	0.0964	0.0982	0.0982
		0.0982	0.0982	0.0962	0.0902	0.0944	0.0945	0.0071	
XVII	P7	0.0898	0.0919	0.0899	0.0879	0.0879	0.0879	0.0935	0.0935
		0.0935	0.0937	0.0916	0.0856	0.0898	0.0899	0.0000	0.0071
XVIII	C18	0.0921	0.0942	0.0922	0.0901	0.0901	0.0901	0.0919	0.0919
		0.0919	0.0879	0.0858	0.0919	0.0921	0.0922	0.0089	0.0162
		0.0089							
XIX	p24	0.0939	0.0962	0.0940	0.0919	0.0921	0.0921	0.0957	0.0959
		0.0957	0.0916	0.0897	0.0937	0.0939	0.0940	0.0107	0.0181
		0.0107	0.0053						
XX	P39	0.0895	0.0917	0.0896	0.0875	0.0877	0.0877	0.0952	0.0953
		0.0952	0.0911	0.0892	0.0873	0.0895	0.0896	0.0053	0.0125
		0.0053	0.0035	0.0071					
XXI	S18	0.0875	0.0897	0.0876	0.0856	0.0857	0.0857	0.0913	0.0914
		0.0913	0.0872	0.0853	0.0873	0.0875	0.0876	0.0053	0.0125
		0.0053	0.0035	0.0053	0.0035				
XXII	c4	0.0896	0.0917	0.0897	0.0877	0.0877	0.0877	0.0934	0.0936
		0.0934	0.0893	0.0874	0.0895	0.0896	0.0897	0.0035	0.0107
		0.0035	0.0053	0.0071	0.0053	0.0018			
XXIII	C51	0.0900	0.0920	0.0901	0.0880	0.0880	0.0880	0.0937	0.0937
		0.0937	0.0896	0.0876	0.0898	0.0900	0.0901	0.0035	0.0107
		0.0035	0.0053	0.0071	0.0053	0.0018	0.0000		
XXIV	S1	0.0895	0.0917	0.0896	0.0875	0.0877	0.0877	0.0932	0.0934
		0.0932	0.0891	0.0873	0.0893	0.0895	0.0896	0.0035	0.0107
		0.0035	0.0053	0.0071	0.0053	0.0018	0.0000	0.0000	
XXV	P2	0.0877	0.0899	0.0878	0.0877	0.0859	0.0859	0.0934	0.0936
		0.0934	0.0893	0.0874	0.0856	0.0877	0.0878	0.0018	0.0089
		0.0018	0.0071	0.0089	0.0035	0.0035	0.0018	0.0018	0.0018
XXVI	c6	0.0895	0.0917	0.0896	0.0875	0.0877	0.0877	0.0910	0.0912
		0.0910	0.0912	0.0893	0.0893	0.0895	0.0896	0.0035	0.0107
		0.0035	0.0071	0.0089	0.0071	0.0035	0.0018	0.0018	0.0018
		0.0035							
XXVII	T43	0.0895	0.0917	0.0896	0.0875	0.0877	0.0877	0.0910	0.0912
		0.0910	0.0931	0.0913	0.0893	0.0895	0.0896	0.0035	0.0107
		0.0035	0.0089	0.0107	0.0088	0.0053	0.0035	0.0035	0.0035
		0.0053	0.0018						
XXVIII	t11	0.0897	0.0918	0.0898	0.0878	0.0878	0.0878	0.0913	0.0914
		0.0913	0.0914	0.0896	0.0896	0.0897	0.0898	0.0071	0.0144
		0.0071	0.0071	0.0089	0.0071	0.0035	0.0053	0.0053	0.0053
		0.0071	0.0035	0.0053					

XXIX	C1	0.0895	0.0917	0.0896	0.0875	0.0877	0.0877	0.0910	0.0912
		0.0910	0.0912	0.0893	0.0893	0.0895	0.0896	0.0071	0.0143
		0.0071	0.0071	0.0089	0.0071	0.0035	0.0053	0.0053	0.0053
		0.0071	0.0035	0.0053	0.0035				
XXX	Td17	0.0875	0.0897	0.0876	0.0856	0.0857	0.0857	0.0891	0.0892
		0.0891	0.0892	0.0873	0.0873	0.0875	0.0876	0.0053	0.0125
		0.0053	0.0053	0.0071	0.0053	0.0018	0.0035	0.0035	0.0035
		0.0053	0.0018	0.0035	0.0018	0.0018			
XXXI	t18	0.0836	0.0858	0.0837	0.0817	0.0818	0.0818	0.0852	0.0853
		0.0852	0.0853	0.0835	0.0835	0.0836	0.0837	0.0089	0.0162
		0.0089	0.0089	0.0107	0.0088	0.0053	0.0071	0.0071	0.0071
		0.0089	0.0053	0.0070	0.0053	0.0053	0.0035		
XXXII	p10	0.1510	0.1533	0.1511	0.1489	0.1489	0.1489	0.1524	0.1527
		0.1524	0.1527	0.1507	0.1507	0.1510	0.1511	0.0631	0.0714
		0.0632	0.0672	0.0691	0.0668	0.0630	0.0611	0.0612	0.0611
		0.0631	0.0590	0.0609	0.0629	0.0628	0.0609	0.0647	
XXXIII	c13	0.0815	0.0837	0.0835	0.0796	0.0836	0.0798	0.0936	0.0937
		0.0897	0.0936	0.0917	0.0816	0.0777	0.0797	0.0397	0.0476
		0.0399	0.0419	0.0437	0.0397	0.0379	0.0398	0.0400	0.0397
		0.0398	0.0397	0.0397	0.0398	0.0397	0.0379	0.0416	0.1007
XXXIV	C26	0.0796	0.0818	0.0816	0.0777	0.0817	0.0778	0.0916	0.0918
		0.0877	0.0916	0.0897	0.0797	0.0758	0.0778	0.0379	0.0457
		0.0380	0.0400	0.0418	0.0379	0.0361	0.0380	0.0381	0.0379
		0.0380	0.0379	0.0379	0.0380	0.0379	0.0361	0.0397	0.0987
		0.0018							
XXXV	P8	0.0835	0.0857	0.0836	0.0816	0.0856	0.0818	0.0933	0.0935
		0.0894	0.0892	0.0873	0.0776	0.0797	0.0798	0.0360	0.0438
		0.0361	0.0380	0.0398	0.0341	0.0341	0.0360	0.0361	0.0359
		0.0341	0.0378	0.0396	0.0379	0.0378	0.0359	0.0396	0.0983
		0.0053	0.0035						
XXXVI	S43	0.1697	0.1700	0.1698	0.1718	0.1676	0.1655	0.1688	0.1692
		0.1645	0.1713	0.1693	0.1650	0.1653	0.1655	0.1733	0.1817
		0.1716	0.1691	0.1639	0.1729	0.1708	0.1709	0.1716	0.1729
		0.1733	0.1702	0.1681	0.1684	0.1702	0.1681	0.1681	0.2257
		0.1698	0.1720	0.1735					
XXXVII	S30	0.1795	0.1799	0.1797	0.1817	0.1775	0.1753	0.1786	0.1790
		0.1742	0.1812	0.1791	0.1748	0.1751	0.1753	0.1832	0.1918
		0.1815	0.1789	0.1736	0.1828	0.1806	0.1807	0.1815	0.1828
		0.1832	0.1800	0.1778	0.1782	0.1800	0.1778	0.1778	0.2365
		0.1797	0.1819	0.1834	0.0071				
XXXVIII	S38	0.1697	0.1700	0.1698	0.1718	0.1676	0.1655	0.1688	0.1692
		0.1645	0.1713	0.1693	0.1650	0.1653	0.1655	0.1733	0.1817
		0.1716	0.1691	0.1639	0.1729	0.1708	0.1709	0.1716	0.1729
		0.1733	0.1702	0.1681	0.1684	0.1702	0.1681	0.1681	0.2257
		0.1698	0.1720	0.1735	0.0000	0.0071			
XXXIX	S48	0.1675	0.1678	0.1677	0.1697	0.1655	0.1633	0.1667	0.1670
		0.1624	0.1692	0.1671	0.1628	0.1631	0.1633	0.1711	0.1795
		0.1694	0.1669	0.1618	0.1708	0.1686	0.1688	0.1694	0.1708
		0.1711	0.1681	0.1659	0.1663	0.1681	0.1659	0.1659	0.2234
		0.1677	0.1698	0.1713	0.0018	0.0089	0.0018		
XL	td20	0.1722	0.1726	0.1724	0.1744	0.1702	0.1680	0.1714	0.1717
		0.1670	0.1739	0.1719	0.1675	0.1679	0.1680	0.1758	0.1819
		0.1742	0.1716	0.1665	0.1755	0.1733	0.1735	0.1742	0.1755
		0.1758	0.1728	0.1706	0.1710	0.1728	0.1706	0.1706	0.2285
		0.1724	0.1746	0.1761	0.0053	0.0125	0.0053	0.0035	
XLI	TD24	0.1620	0.1623	0.1622	0.1641	0.1600	0.1579	0.1612	0.1615
		0.1570	0.1637	0.1617	0.1574	0.1577	0.1579	0.1677	0.1760
		0.1661	0.1664	0.1681	0.1696	0.1653	0.1654	0.1661	0.1674
		0.1677	0.1648	0.1627	0.1630	0.1648	0.1627	0.1627	0.2194
		0.1643	0.1665	0.1680	0.0053	0.0125	0.0053	0.0071	0.0107
XLII	C20	0.1599	0.1602	0.1600	0.1620	0.1579	0.1557	0.1591	0.1594
		0.1548	0.1615	0.1595	0.1553	0.1556	0.1558	0.1656	0.1738
		0.1639	0.1643	0.1659	0.1674	0.1631	0.1633	0.1639	0.1653
		0.1656	0.1627	0.1605	0.1608	0.1627	0.1605	0.1605	0.2171
		0.1622	0.1643	0.1658	0.0071	0.0143	0.0071	0.0053	0.0089
		0.0018							

XLIII	t17	0.3743	0.3752	0.3748	0.3771	0.3746	0.3746	0.3899	0.3908
		0.3957	0.3831	0.3806	0.3801	0.3800	0.3805	0.4735	0.4829
		0.4720	0.4687	0.4747	0.4685	0.4654	0.4658	0.4670	0.4685
		0.4665	0.4723	0.4754	0.4627	0.4723	0.4692	0.4629	0.5251
		0.4584	0.4553	0.4506	0.5402	0.5587	0.5402	0.5368	0.5454
		0.5359	0.5326						

Figure 3.34 Pairwise nucleotide sequence divergence between different haplotypes of COI_{614} of *P. monodon*.

Nucleotide sequence divergence of COI_{614} of investigated *P. monodon* varied greatly. Basically, each shrimp could be allocated into one of three groups (lineages I, II, III in a neighbor-joining tree, Figure 3.35). Low nucleotide divergence was observed between members of the same lineages but large divergence was found between individuals from different lineages.

Surprisingly, t17 (Trang) revealed large sequence divergence with the remaining specimens (37.43 – 55.87%). This divergence level is enormously greater than the typical level at the intraspecific level. The nucleotide of t17 sequence was then blasted and the E value from Blast *N* was 4×10^{-32} for which *P. monodon* is the closet species (E-values for most of other sequences was 0.0). This open three interesting issues; the untypical high genetic diversity of COI in *P. monodon*, the amplification of a nuclear gene possessing similar sequences with mitochondrial COI or the existence of interspecific hybridization between male *P. monodon* and a female of the other species. It is premature at this stage to conclude that which possibility is correct. Therefore, this suspected circumstance should be further analyzed.

The haplotype distribution frequencies of COI could not directly analyze statistically because a large number of haplotypes with low frequencies and singletons were found. Therefore, frequencies of members of phylogenetic lineages in each geographic sample (Table 3.16) were subjected to a Monte Carlo simulation.

Significant genetic heterogeneity was found across overall investigated samples (P = 0.0006). Geographic heterogeneity analysis between pairs of samples indicated significant population differentiation between Trang and Phangnga from the Andaman Sea and Trat from the Gulf of Thailand (P = 0.0034 and 0.0017, respectively). Surprisingly, Chumphon showed significant genetic heterogeneity with Trat located in the same coastal region (P = 0.0113). Additionally, Satun (the

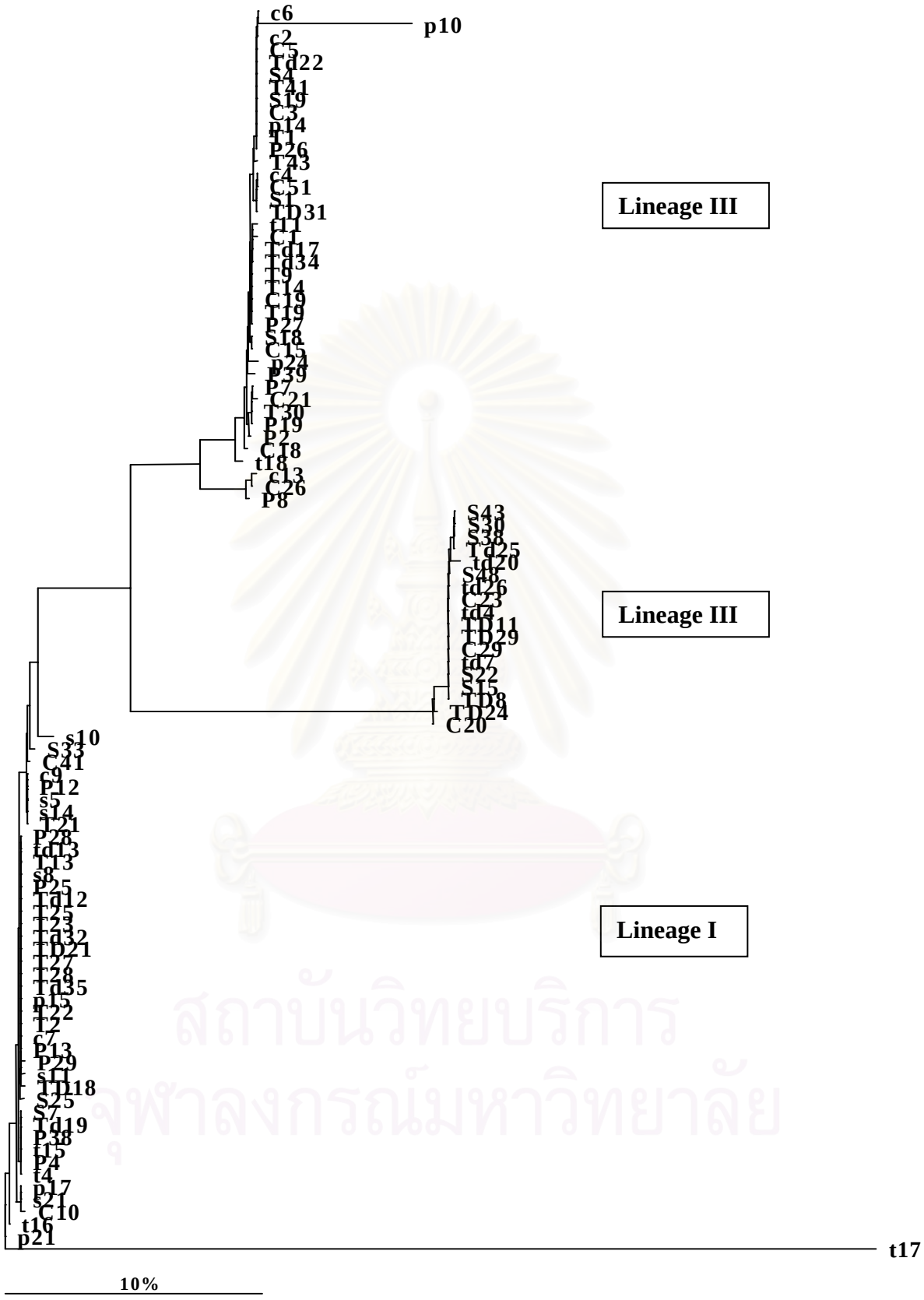

Figure 3.35 A neighbor-joining tree illustrating relationships of *P. monodon* in Thai water based on nucleotide sequence divergence of the COI_{614} gene segment.

Andaman Sea) which did not show significant genetic differentiation with Trat (located in the Gulf of Thailand, P = 0.6538) exhibited significant differentiation with other geographic samples from the Andaman Sea (Trang and Phangnga, P = 0.0125 and 0.0122, respectively).

Unlike results from PCR-RFLP and SSCP analysis of 16S $rDNA_{312}$ and SSCP analysis of AFLP-derived markers, nucleotide sequences of the COI fragment showed high genetic diversity intraspecifically and revealed that the gene pool of *P. monodon* in Thai waters is not panmictic but reproductively isolated into several populations. Significant genetic heterogeneity was observed both between samples from different coastal region (Trang and Phangnga with Trat) and within coastal regions (between Trat and Chumphon and between Trang/Phangnga and Satun).

Table 3.16 Distribution of members of different phylogenetic lineages of the NJ tree constructed from nucleotide sequence divergence of the COI_{614} gene segment

Geographic sample	Phylogenetic lineage		
	I	II	III
Trat	7	4	9
Chunphon	4	13	3
Satun	9	4	6
Trang	11	9	-
Phangnga	10	10	-

Table 3.17 Pairwise geographic heterogeneity analysis between *P. monodon* from different geographic samples based on nucleotide sequence divergence of the COI_{614} gene segment analyzed by a Monte Carlo simulation

	Trat	**Chumphon**	**Satun**	**Trang**	**Phangnga**
Trat	-				
Chumphon	P=0.0113	-			
Satun	P=0.6538^{ns}	P=0.0219	-		
Trang	P=0.0034	P=0.0260	P=0.0125	-	
Phangnga	P=0.0017	P=0.0506^{ns}	P=0.0122	P=1.0000 ^{ns}	-

3.3 Isolation and characterization of sex-related markers in *P. monodon*

3.3.1 Identification of genomic sex markers in *P. monodon* by RAPD and SCAR markers

Bulked segregrant analysis (BSA) and RAPD approaches are potential for isolation of phenotype-specific markers in various organisms when used in combination. To isolate sex-specific RAPD markers in *P. monodon*, 100 RAPD primers were screened. Eighteen RAPD primer provided candidate sex-specific RAPD markers. As a results, they were subjected for further analysis against three bulks of DNA (*N* = 3, 5 and 10 individuals) for each sex. Nevertheless, only eight primers (Table 3.18 and 3.19 and Figures 3.36 – 3.40) generated ten promising sex-specific RAPD fragments.

Seven RAPD fragments are existent in one sex but absent in the other whereas three fragments were found in both sexes of *P. monodon* but were found as the very faint and intense bands between different sexes. As a result, they may be different sequences having similar sizes when analyzed by agarose gel electrophoresis.

Basically, three different types of products are expected; 1) identical RAPD fragments found in both male and female *P. monodon*, 2) amplified fragments from polymorphic loci among different individuals having the same sex and 3) RAPD-amplified fragments derived from the heterogametic sex.

Using multiple bulks for each sex, the first and second types of RAPD band should have been eliminated. Nevertheless, the third types of markers should be examined against separate individuals of male and female *P. monodon* to eliminate products of a rare polymorphism.

A total of 10 candidate sex-specific RAPD fragments were found. The target band were excised from the gels and eluted. The reamplified product was gel-eluted and cloned. Eight of these were successfully cloned and sequenced (Table 3.19). Two RAPD fragments (650 bp and 440 bp) from OPM09 illustrating the possible male-specific nature did not give reproducible amplification results when amplification was repeated. Seven recombinant clones (pPMF650, pPMF530, pPMF500, pPMM350, pPMM800, pPMM600 and pPMM650) were unidirectional sequenced whereas pPMM1100 were sequenced for both directions (Figure 3.41).

Table 3.18 Amplification results of RAPD primers for identification of genomic sex-specific markers in *P. monodon*

Primers	Stringency	Sex-specific marker (bp)	
		Male	Female
1.UBC 101	+ +	-	-
2.UBC 115	+ +	-	-
3.UBC 119	+ + +	800*	-
4.UBC 120	++	-	-
5.UBC 122	+ +	-	-
6.UBC 128	+ +	-	-
7.UBC 135	+ ++	350*	-
8.UBC 138	+ +	-	-
9.UBC 146	+	-	-
10.UBC 153	+ +	-	-
11.UBC 158	+ +	-	-
12.UBC 159	+ +	-	-
13.UBC 160	+	-	-
14.UBC 165	+ +	-	-
15.UBC 168	+ +	-	-
16.UBC 169	+ +	-	-
17.UBC 174	+ +	-	-
18.UBC 175	+ +	-	-
19.UBC 191	+ +	-	-
20.UBC 193	-	-	-
21.UBC 195	-	-	-
22.UBC 196	+ +	-	-
23.UBC 197	-	-	-
24.UBC 200	+ +	-	-
25.UBC 210	+ +	-	-

Table 3.18 (continued)

Primers	Stringency	Sex-specific marker (bp)	
		Male	Female
26.UBC 217	+	-	-
27.UBC 220	+	-	-
28.UBC 222	+ +	-	-
29.UBC 228	+	-	-
30.UBC 235	++	-	-
31.UBC 237	++	-	-
32.UBC 259	+	-	-
33.UBC 263	++	-	-
34.UBC 267	+	-	-
35.UBC 268	+	-	-
36.UBC 270	-	-	-
37.UBC 271	-	-	-
38.UBC273	+ +	-	-
39.UBC299	++	-	-
40.UBC428	+++	-	650*
41.UBC456	++	-	-
42.UBC457	++	-	-
43.UBC459	++	-	-
44.M13	+	-	-
45.PERI	-	-	-
46.HRU18	+	-	-
47.HRU33	-	-	-
48.YN73	+	-	-
49.YNZ22	++	-	-
50. $(CA)_8$	-	-	-
51. $(CAC)_5$	-	-	-
52. $(CT)_8$	-	-	-

Table 3.18 (continued)

Primers	Stringency	Sex-specific marker (bp)	
		Male	Female
53. $(GTG)_5$	-	-	-
54. $(GACA)_4$	-	-	-
55. $(GATA)_8$	-	-	-
56.OPA01	+++	-	530*
57.OPA02	++	-	-
58.OPA03	+	-	-
59.UBC140	-	-	-
60.OPA05	++	-	-
61.OPA06	++	-	-
62.OPA07	++	-	-
63.OPA08	++	-	-
64.OPA09	++	-	-
65.OPA10	++	-	-
66.OPA11	+ +	-	-
67.OPA12	++	-	-
68.OPA13	++	-	-
69.OPA14	+++	600	-
70.OPA15	+++	650*	500
71.OPA16	+ +	-	-
72.OPA17	++	-	-
73.OPA18	++	-	-
74.OPA19	+	-	-
75.OPA20	+	-	-
76.UBC141	-	-	-
77.OPB02	+	-	-
78.OPB03	+ +	-	-
79.OPB04	++	-	-
80.OPB05	++	-	-

Table 3.18 (continued)

Primers	Stringency	Sex-specific marker (bp)	
		Male	Female
81.OPB06	++	-	-
82.OPB07	++	-	-
83.OPB08	++	-	-
84.OPB09	-	-	-
85.OPB10	++	-	-
86.OPB11	++	-	-
87.OPB12	+	-	-
88.OPB13	+	-	-
89.OPB14	+	-	-
90.OPB15	++	-	-
91.OPB16	-	-	-
92.OPB17	++	-	-
93.OPB18	++	-	-
94.OPB19	++	-	-
95.OPB20	+++	1,100*	-
96.OPM09	+++	650*, 400	-
97.OPZ09	++	-	-
98.UBC110	-	-	-
99.UBC111	-	-	-
100.UBC112	-	-	-

Abbreviations;

+ + + = Successful amplification with clear fragments and providing a band difference between male and female *P. monodon*

++ = Successful amplification with clear fragments but did not provide a band difference between male and female *P. monodon*

+ = Successful amplification but amplified RAPD fragments were not clear

- = no amplification product

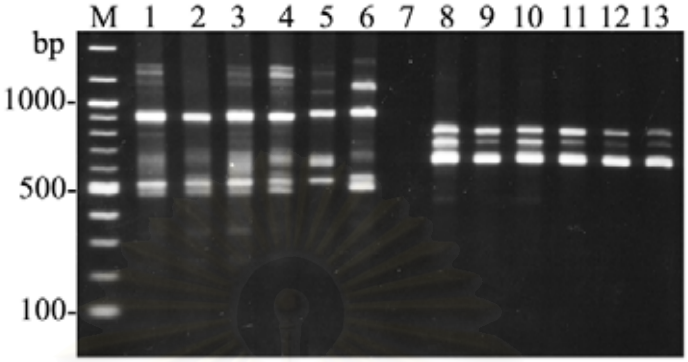

Figure 3.36 RAPD patterns from amplification of bulked male (*N* = 10, lanes 1 & 8; *N* = 5, lanes 3 & 10 and *N* = 3, lanes 5 & 12) and female (*N* = 10, lanes 2 & 9; *N* = 5, lanes 4 & 11 and *N* = 3, lanes 6 & 13) using primers OPA05 and OPA17. A 100 bp ladder (lane M) was used as a DNA marker.

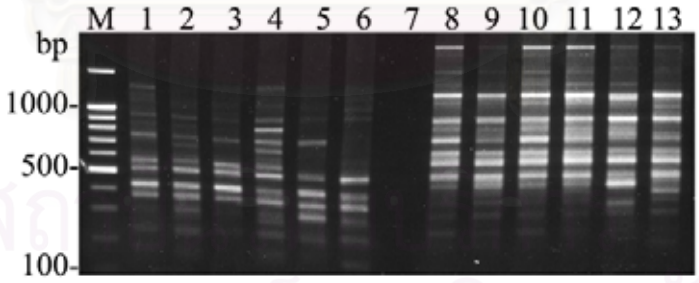

Figure 3.37 RAPD patterns from amplification of bulked male (*N* = 10, lanes 1 & 8; *N* = 5, lanes 3 & 10 and *N* = 3, lanes 5 & 12) and female (*N* = 10, lanes 2 & 9; *N* = 5, lanes 4 & 11 and *N* = 3, lanes 6 & 13) using primers UBC428 and UBC456. A 100 bp ladder (lane M) was used as a DNA marker.

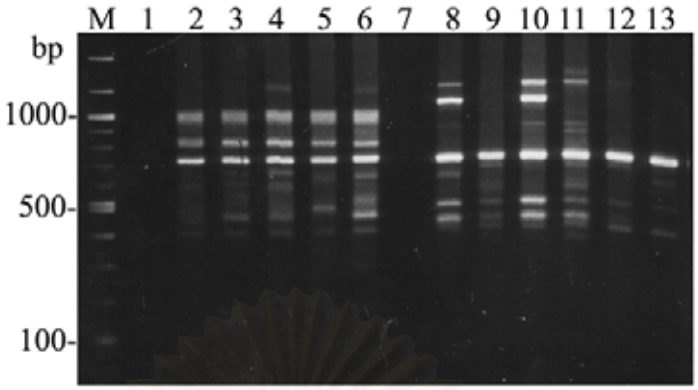

Figure 3.38 RAPD patterns from amplification of bulked male (*N* = 10, lanes 1 & 8; *N* = 5, lanes 3 & 10 and *N* = 3, lanes 5 & 12) and female (*N* = 10, lanes 2 & 9; *N* = 5, lanes 4 & 11 and *N* = 3, lanes 6 & 13) using primers OPB19 and OPB20. A 100 bp ladder (lane M) was used as a DNA marker.

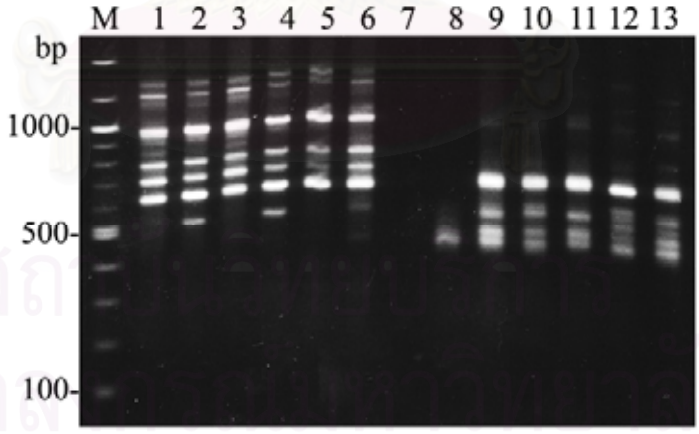

Figure 3.39 RAPD patterns from amplification of bulked male (*N* = 10, lanes 1 & 8; *N* = 5, lanes 3 & 10 and *N* = 3, lanes 5 & 12) and female (*N* = 10, lanes 2 & 9, *N* = 5 lanes 4 & 11 and *N* = 3, lanes 6 & 13) using primers OPA01 and OPB06. A 100 bp ladder (lane M) was used as a DNA marker.

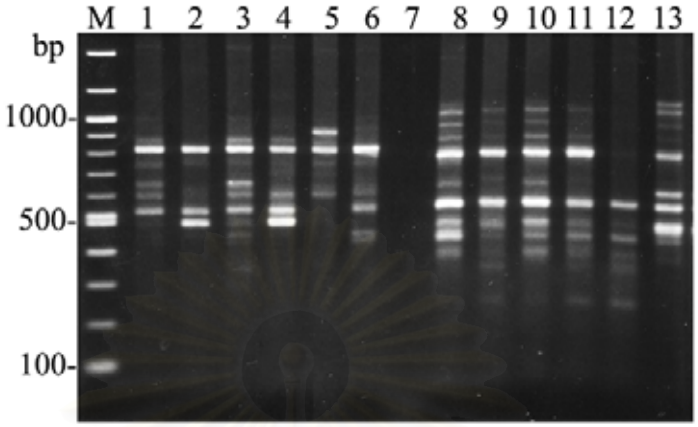

Figure 3.40 RAPD patterns from amplification of bulked male (*N* = 10, lanes 1 & 8, *N* = 5, lanes 3 & 10 and *N* = 3, lanes 5 & 12) and female (*N* = 10, lanes 2 & 9, *N* = 5, lanes 4 & 11 and *N* = 3, lanes 6 & 13) using primers OPA15 and OPM09. A 100 bp ladder (lane M) was used as a DNA marker.

Table 3.19 Candidate sex-specific RAPD markers found in this study

Primer	**Number of RAPD (size in bp)**	
	Male	**Female**
UBC119	1 (**800**)*	-
UBC135	1 (**350**)*	-
UBC428	-	1 (**650**)*
OPA01	-	1 (**530**)*
OPA14	1 (**600** [a])	-
OPA15	1 (**650**)*	1 (**500**[a])
OPB20	1 (**1100**)*	-
OPM09	2 (650*, 400 [a])	-

*indicate fragments found in one sex but were absent from the other sex of *P. monodon.* [a]indicate fragments that are existent in both males and females but the intensity of the bands was different between sexes. Fragments which were successfully cloned are illustrated in boldface.

These clones did not exhibit significant similarity with nucleotide (Blast*N*) or translated protein (Blast*X*) sequences previously deposited in the GenBank (E values $> 10^{-4}$) and were then regarded as unknown DNA segments.

Four pairs of primers were designed (PMF530-F/R, PMM350-F/R, PMM1100-F/R, PMM800-F/R) from the obtained sequence and converted to sequence-characterized amplified region (SCAR) markers. These sequence-specific primers were tested against genomic DNA of five representative male and female *P. monodon*. Two primers pairs (PMF530-F/R, PMM350-F/R) yielded positive amplification products in both sexes. Neither sex-specific amplification patterns nor length polymorphism between the PCR product of male and female *P. monodon* were observed (Figure 3.42, 3.43).

The PCR product from each shrimp was then further characterized to verify whether sex-specific single nucleotide polymorphism (SNP) was existent in the amplification product using SSCP analysis. Results showed polymorphism between single stranded DNA of these shrimps but was not relevant to sex specificity in this species (Figure 3.44 and 3.45).

A.pPMF650

GATTTGTCAGCGGTTGGCAAAACGTGTCATTAATATACAACACATAGCGGTATCTCTTTCTT
ACTGCATAAACACCGATACATAATTTTCGAACAAAAAATTTGCCGTCGGATATATCCATTCT
TTTACATGAATTTCTCAACAATAACATCTTGAGGCGGTATTCGACTTTGATCCTTATCTGCT
CTTGGCTTAAGGCACAGATACCATCGCTAATTACATTCATAGTTCACCGTTTAGTCTTCGAG
CCTTACCAGCCAAAAATACCCATTAATTATTCCTGGCAAAGGAACTGGATTGTGTGCTTGGT
GCCTCCTAGTCTGACCCTAATGTACCTTCAGTGCAAGAATGATATTAGAATAATAACTCTGA
GGNACAGGTTGGTTGAAATAATGACCCTTATGGCGACGAAAGATAGATGCGGAGTTGCTTTA
AGAGGCTCGAACAGGAAGCAAATCTTCACGGTGAGAAAACGTTTTTCCGCCAAGATTTAATC
TCTCTTGTTTTGTCGTCTTAATCACCCGCCTCCAGCTGNACTCATCTGTGCTAATGCAGATG
AAAATGTCTAGTAAATAAATTAATGATGAATATTAGTTGAAAAATAATGGAACATGCTAGTC
AATGAGTAAACCCCTGGACAAATCACTANT

B.pPMF530

ACTAGTGATTCAGGCCCTTCGAAGTTTTACCAAAGCAAGTTTCACACACACAAAAAAGTCTG
TTTCTGTTCTAG**AGCAGTTTCAAAGCATAACCCAG**TTTTTTCCCCACGTTGAACCAAAGAGG
CATGTGCTGGGGATTAGAAGCACTGTGAAGCTCTGTTTACTTTGCTCTGTGCAAATTTAGCA
GCAGCATACACAATTTTCTAGTAAATGAGACTAAAACAAGGCCTTAACTCCATTTTGAGGGT
ATATGAATACTGCACCCATGGCCATACTAACTAGCAAACCACTTTTNGGGATTGGTTTAGAA
TGGCAAAA**ATAGATGCCTACAGTCAATATA**TTAATTTTGCAGTTGCATGAGCAAAAATTA

C.pPMF500

ACTAGTGATTTTCCGAACCCCTAGGTTTAAAACGTATCTCTCTCTCTCTTTCTTCTCTCTCT
TTCTTCTCTCTCTTTCTCTCTCTCTCTCCTCCCACCCCTCTCTCTCTCTATCTCTATCTCTA
TCTCTATCTCTATCTCTCTATCTCTATCTCTATCTCTCTCTCTGTTTCTTCCCTGTTCATAC
CTGTGTGTATCATTTTGTATATATTGTATATATAAAATAGAGACAGAAACAGATAAATCTAA
GCTGATAGATAGCTAAAGCATGGG

D.pPMM1100

GGACCCTTACTAAGATTGACAATGTTCGCATTACGTAAATATAAGACAAAAATATATGCATA
TGACACTATCCATTTCTCACTCCATATGCATATATATAAAAAAAAAAAAAAATACTTGCAAA
GACCATAATCAATATCTTTACAAATGTTTACAAATGCATAATATAACAAAGATGCAACTCTA
ATAAGAATATCTCAACAGAAATTTCTTAACTTCGCGGGAACGATTGATAAACTTGCGAACTC
CTCTCTTCTTTTAACATATTATACTAGTCGTTCTTACATCCCAAAGGCATCTTCATAATAAA

AATACAATTCAGTGAAAAGGATCATGAAACTCTTGTTGTACCCAACATGCCTTTAATATGAC
TTTCAAGCTTACACAAACACGCGTCCTAACAGGTGATAACACAAATAAGATAAAAATAACAG
GCATATATTTTTTGTGTAAATGTAAATGCGATAACTACCATTACGCACCTTTGAAAATTGCA
GGCTTATACAACAGGTGCAACCCTAATAAGAATAACAACAGAAATTTCTTAACTTCGCGGGA
CGATTGATAAGCTATCTTACATCCCAAAGGCATCTTTCTAATAAAAATACAATTCACTCAAA
AGGATCATGAAACT**CTTGTTGNACTCAATGTCTTTAT**TATGACTTTCACAAACACACATCCT
AACAGGCGATAATACAAATAAAATAAAAATAACAGGCTTAAATTTTCTGTGTAAATGTAAAT
GCGATAACCACTATCTCATACGTTTGAAAATTTCAGGCTTATACAACAGTTTAATTACCTAG
CAGGTTGAAAAGTCGCCGGTTTTATTCTATTTAGAGAGTATTAATAGGGTTTTCAACGCACC
TGCGCTGAA**AAGGAGGATGGAGCGATGGA**AAGTATGAGATGTTACTGGACTCCTGAAAGAGG
CAAAAAACCTACAGGAAAGACACAGAAAAACAATTGCAACAAGAGAAGAGATGGACAAATCA
AGGAAATTTGCCAGTCTAATGAAACAAGGCAAAGTGACAAAGGCAGTAAGGGTCCA

E.pPMM350

GATTAAGCTGCGAGGCGTACGAGTCAGTTTCTGAGATCGGGTTTTGTGATATCTAGAAAAAA
AACCGGTATAATCCAGTATATTTTATCTAGATTAACGGATTTA**TTACTGGTGATAATGATGC**
CTTATTGCTATAATTATTGAGATTTTGTTATTGCTAAAATTTTCACTCCTGTTCTATACGGT
CGTTATCAGCATTTCATTTATTATCAATGATATTATCAAAATTCTCTAT**GACCACTCTCATT**
GTTAGCGTTTGTATTTCTAGTGGCAATAGGTATGGTGAGCGATAGCGGTATTAACTGTGCAT
TTTACTCAACGAGTTAATGGTATCTCGCAGCTTAATCACTAGT

F.pPMM800

ATTGGGCGATAGTTATTTATATCCTCGATGTCAGTGAGGGATTTTCCAAAAGTTAGGGAATG
TGTTGGTAACAATAGAAGTGTTTACAACAATCATAATGTAAAATGTAATAACTGGCAACGAG
TCTCTTATGAATCGAAATGGAACCCCATTAAAACCATAGGAATTAGAAAATGTTAAACGTTT
TACAGTTAAAA**TGATAGTCTCGCAGTCCAAAGG**ATCAAGTCTAAAGATATTTTTGGGACCTT
CATTTGAAGTGTAAATTCCTTCGTCAATAGCTTCAGTAATATGAGAGTTTAGATTCACTTGT
GACTTTTCATAAGCATCTTTGCCAGCATTTGCAAAAAACATATTAGATTCCTCTGCTCTCTC
ACGTAAGTTATCGCTACCATTTCTTTTGTTCTGGAATTCTCTGTAGTTTTTTTTTTTTTTT
TTTTTTTTTTTTTTGCCTGTGCCAATAAATAAATTGAGTCGTTTCTTCTCTTCTTTGTAAAG
GTCATATTGTACCTATTACTCTTAATCCTTTTTCTAAGCTGGTCCCCAGTCTTCACTGCAGC
CAG**GTTACTTTCCTTGTTACGATTGGTG**CACAGTTGTCCATGCATTTCAGGAAAGCATTAGT
TAACTTTTTTATTTGTACACTAACAGTTTGAAGAATTTCTGTAAAAGATTAAGAGCTTCATT

TAATAGTGATTAAATGAAGCTCCTACTTGAGTCAACTTCACATTCTTCATTAATTTAGCAAT
TTCATTAATTGATGCTAATAAATTATCATTGAAATCGCCCAATAAT

G.pPMM600

TCTGTGCTGGTGGTAAACAAAATGCCTATCTGTGCTGGACTAAAGATGATATTAATGGGGAG
GCTAAGAGTACCTATCAATATGTATTAGATCTGAGATCTAGATTAGAACAATTGGCTCAACT
AGCAGACGTGAAAAGCAGAATGTACAAGACGTACTTCGACAGGAAAGCTAGAGCTCGAACAT
TAAAAGAAGGGGATGAAGTACTTGTGCTGTTGCCAACCTCGTATAACAAACTAACTGTTCAG
TGGAAGGGTCCCTATTCTGTTGTATGCAAACATGAAAATGGAGTAGACTATGAAATAAAAAT
AAAAGGGAAAATGAAGCTTTATCATGTAAACATGCTGAAGAAATATGAAAGACGCGAAAATG
ACATTTCTCACTCTCAGGTGTGCCAAGCTTGCGTGATAGATGCTTCTGATCCCATAGATAAG
AAGCTAATGGCGTATGTGATATACCCGAATTGTATCCGCTTGGTAAATATGATTTTAATTTT
AACTCTGAACTCTCAAATGAACAAACCTCAGAGTTAAACCATCTAATCGCTGATTTTCTTGA
CGTATTTGTCGATAAACCAGGCGTAACCAGCACAGAA

H.pPMM650

TTCCGAACCCATAATAAAAACAAAGCGATGAACCCATATGCGCGAGCTGTTGACGTCACCTA
TTTTCCGAACCCCGGAGGCCATGAAGTCCCAATCTTCCCGTGACCTCAACACTGAAGGAGGG
ACGTCTGGAGAACCCGCTGGAGTGATAGCGATGTCGTAGTGTGAAGATCTCTCTGTCGTTAA
TAGAATCCTACAGGGAGTAAGGTTTACGTGTTTGTGAGGTTGAATTTCGGTGCGTAGAAAAT
AGATAAGCTGTTATTTCAAGAATAGCATGATTATTCAGTTGTGTTTTTTTCCTGTAGAGTTG
ATGATTTCGTTTGATTCTTACCTACTCGCGGCATTATCTTCATCAAAGTGCTAATATTTTAC
CGTGCGAACAGTGATTATTAAACGGCTGTGTATTGCCATCAATTATTGTTTTAATCTACTGT
TAGATGTCTGGCACCGCGTAAACGGTTTTTGTTTTCAGTATTTATAGAGATCTTACACTAAA
CAAAGCAAGATAAACACAAAAAAATATATATAAAAACGGTAAAATAAAAAACTGAAGATAAA
GGACACTGAATTGCCTATCTTACAAATAATCAGAGCAACTGCTGGCAGTGAACTTATTACAG
TTATCTGGGTTCGGAAA

Figure 3.41 Nucleotide sequences of candidate sex-specific RAPD fragments; pPMF650 (A), pPMF530 (B), pPMF500 (C), pPMM1100 (D), pPMM350 (E), pPMM800 (F), pPMM600 (G), pPMM650 (H) using M13 reverse primer as a sequencing primer with the exception that pPM1100 sequence combined from M13 forward and M13 reverse primers.

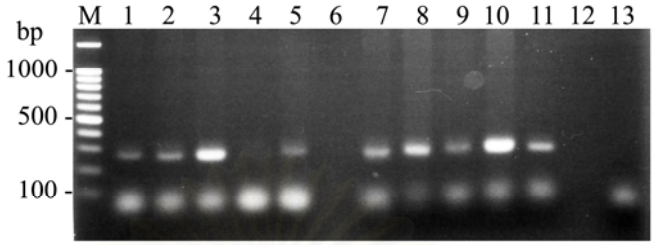

Figure 3.42 Agarose gel electrophoresis illustrating the product resulted from amplification of genomic DNA of 5 individuals of males (lanes 1 – 5) and females (lanes 7 – 11) *P. monodon* with primers PMF530-F/R. Lanes M and 13 are a100 bp DNA markers and the negative control (no DNA template), respectively.

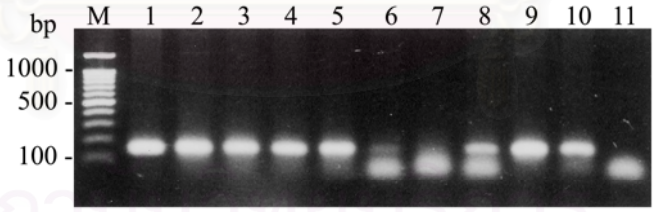

Figure 3.43 Agarose gel electrophoresis illustrating the product resulted from amplification of genomic DNA of 5 individuals of males (lanes 1 – 5) and females (lanes 6 – 10) *P. monodon* with primer PMM350-F/R. Lanes M and 11 are a 100 bp DNA markers and the negative control (no DNA template), respectively.

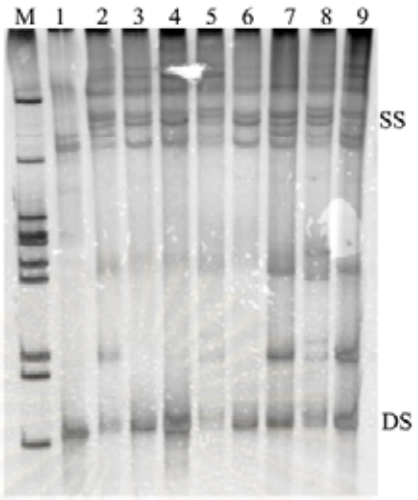

Figure 3.44 SSCP analysis of the product resulted from amplification of genomic DNA of males (lanes 2 – 5) and females (lanes 6 – 9) of *P. monodon* using PMF530-F/R. Lanes M and 1 are a 100 bp DNA marker and non-denatured PCR product, respectively.

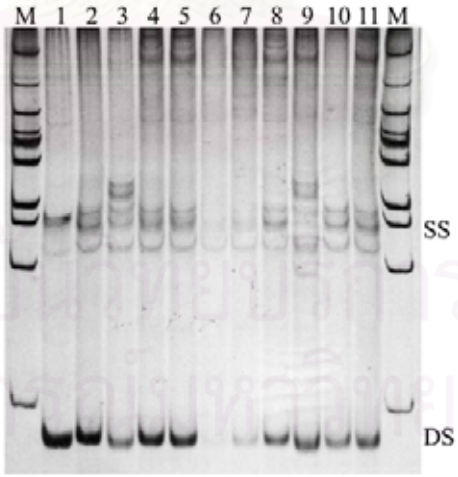

Figure 3.45 SSCP patterns of the product resulted from amplification of genomic DNA of males (lanes 2 – 6) and females (lanes 7 – 11) *P. monodon* using PMM350-F/R. Lanes M and 1 are a 100 bp DNA marker and non-denatured PCR product, respectively.

3.3.2 Isolation and characterization of sex-specific/differential expression markers of *P. monodon* using RAP-PCR analysis

3.3.2.1 Total RNA Extraction and Bulked Segregant Analysis (BSA)

Total RNA was isolated from ovaries and testes of each *P. monodon* broodstock. The ratio of OD_{260}/OD_{280} was 1.7 - 2.0 indicating the accepted quality of total RNA. An agarose gel electrophoresis revealed predominantly discrete bands along with smear high molecular weight RNA of total RNA from ovaries and testes (Figure 3.46A). Two bulked total RNA of male (*N* = 3) and female (*N* = 3) *P. monodon* were generated from an equal amount (0.5 μg) of each individual. The first strand cDNA was synthesized from those total RNA and electrophoretically analyzed. The synthesized first strand cDNA covered <0.5 to 7 kb in length (Figure 3.46B).

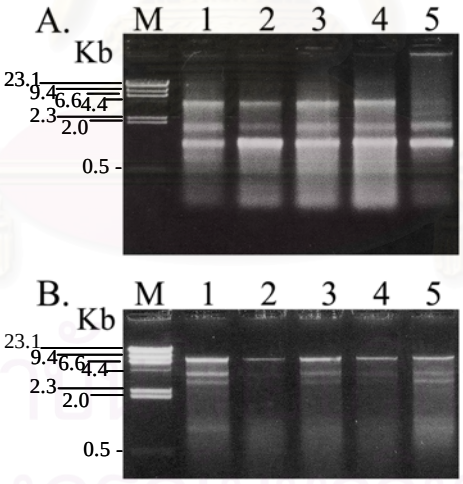

Figure 3.46 A 1.0% agarose gel electrophoresis showing the quality of total RNA extract from ovaries (lanes 1 – 3) and testes (lanes 4 – 5) of *P. monodon* (A) and the corresponding first strand cDNA (B). Lane M = λ-*Hind* III DNA marker.

First strand cDNA representing male and female *P. monodon* was used as template for identified sex-specific/differential expression markers by RAP-PCR. Several candidate markers were identified after being electrophoretically analyzed by 1.6% agarose gel (Figures 3.47 - 3.48).

After preliminary screening (150 primer combinations), a large number of candidate markers were divided into 2 groups (Table 3.20). The group 1 was RAP-PCR fragments specifically expressed in testes and ovaries composing of 46 and 110 candidate fragments, respectively. The group 2 was differential expression fragments between testes and ovaries composing of 45 and 44 candidate fragments, respectively.

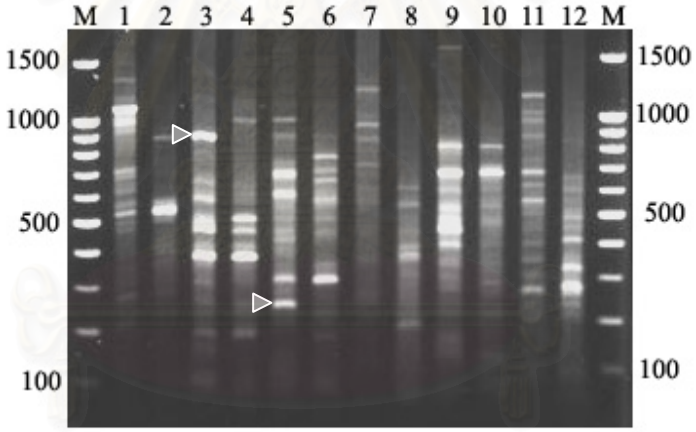

Figure 3.47 A 1.6% agarose gel illustrating RAP-PCR fragments of ovaries (lanes 1, 3, 5, 7, 9 and 11) and testes (lanes 2, 4, 6, 8, 10 and 12) generated from UBC299 (1st primer) and UBC459 (lanes 1 and 2), OPB04 (lanes 3 and 4), OPB07 (lanes 5 and 6), OPB11 (lanes 7 and 8), OPB14 (lanes 9 and 10) and OPB15 (lanes 11 and 12). Lanes M are a 100 bp DNA marker. Arrowheads indicate a candidate female-specific RAP-PCR fragment of *P. monodon*.

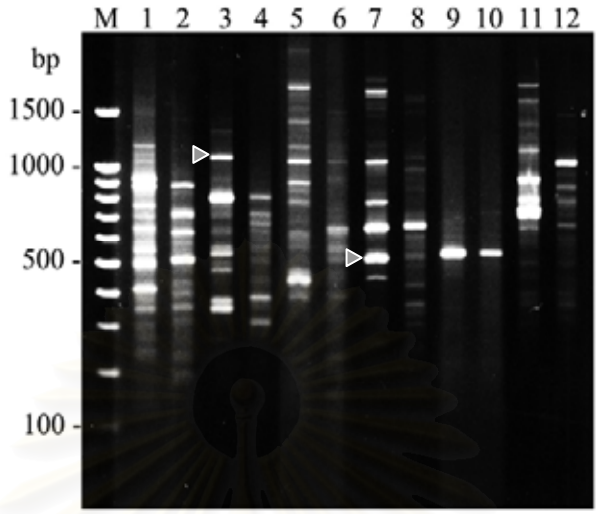

Figure 3.48 A 1.6% agarose gel illustrating RAP-PCR fragments of ovaries (lanes 1, 3, 5, 7, 9 and 11) and testes (lanes 2, 4, 6, 8, 10 and 12) generated from UBC428 (1st primer) and OPB10 (lanes 1 and 2), OPB15 (lanes 3 and 4), UBC101 (lanes 5 and 6), UBC119 (lanes 7 and 8), UBC138 (lanes 9 and 10) and UBC191 (lanes 11 and 12). Lanes M are a 100 bp DNA marker. Arrowheads indicate a candidate female-specific RAP-PCR fragment of *P. monodon*.

Table 3.20 Candidate sex-specific/differential expression RAP-PCR fragments in ovaries and testes of *P. monodon*

1st Primer	RAP-PCR group I		RAP-PCR group II	
	Male	**Female**	**Male**	**Female**
UBC 119	20	18	22	17
UBC 299	11	19	8	6
UBC 428	6	29	3	6
UBC 456	3	20	6	8
UBC 457	6	24	6	7
Total	**46**	**110**	**45**	**44**

A total of 25 and 20 fragments from candidate female and male RAP-PCR fragment were chosen. Generally, five fragments (from each sex) having different sizes except M119-M09, M428-191, M428-B17 and M457-A01 were pooled and simultaneously cloned. Generally, 3-5 different inserts were able to recover after cloning. The PCR products exhibiting similar sizes were further characterized by digestion of plasmid DNA or colony PCR products with restriction enzymes.

A total of 21 and 14 types of sequences originating from 24 female and 15 male cloned fragments were obtained. Nucleotide sequences of these 35 transcripts were blasted against data in the GenBank (Table 3.21). The major transcripts found were unknown transcripts (51.43%), 18S rRNA (17.14%) and thrombospondin (TSP, 11.43%) homologues. Rare transcripts encoding NADPH ferrihemoprotein reductase, Ran-binding protein, G-protein pathway suppressor 1, nucleoporin Nup153 and hypothetical protein were also identified (Table 3.22).

A pair of primers was design from each of 16 unknown transcripts and 9 known transcripts. The expression patterns of these 25 transcripts were tested against the first strand cDNA synthesized from total RNA of ovaries (N = 5) and testes (N = 4) of 3-month-old *P. monodon* (Table 3.23, Figure 3.49). Five derived RAP-PCR markers (FI-4, FI-44, FIII-4, FIII-39 and FIII-58) revealed sex-specific expression in females of juvenile *P. monodon*. These markers were further tested against ovaries (N = 7) and testes (N = 7) of *P. monodon* broodstock and the results revealed that the expression pattern of these markers was consistent in *P. monodon* broodstock (Figure 3.50).

Table 3.21 Blast results of sex-specific/differential expression RAP-PCR fragments in ovaries and testes of *P. monodon*

Clone*	No. of nucleotides sequenced (bp)	Closest species	E-values	Blast analysis
1. MI-1	280	*Litopenaeus vannamei*	10^{-121}	18S rRNA
2. MI-36	391	-	$> 10^{-4}$	Unknown
3. MI-56	570	-	0.0	Shrimp WSSV
4. MII-5	894	*Musca domesticus*	$2x10^{-77}$	NADPH ferrihemoprotein reductase
5. MII-51	413	-	$> 10^{-4}$	Unknown
6. MII-57	358	*Litopenaeus vannamei*	10^{-131}	18S rRNA
7. MIII-6	579	*Litopenaeus aztecus*	10−89	18S rRNA
8. MIII-7	412	-	> 10−4	Unknown
9. MIII-8(1)	485	-	> 10−4	Unknown
10. MIII-8 (2)	726	*Litopenaeus vannamei*	0.0	18S rRNA
11. FI-1	744	*Drosophila melanogaster*	$3x10^{-32}$	Ran-binding protein
12. FI-4	625	-	$> 10^{-4}$	Unknown
13. FI-6	660	*Penaeus monodon*	$2x10^{-49}$	Thrombospondin
14. FI-40	401	*Mus musculus*	$4x10^{-43}$	Hypothetical protein
15. FI-44	693	-	$> 10^{-4}$	Unknown
16. FII-17	681	*Bos Taurus*	$3x10^{-40}$	Thrombospondin
17. FII-18	1008	*Drosophila pseudoobscura*	10^{-105}	GA16635-PA
18. FII-22	921	-	$> 10^{-4}$	Unknown
19. FIII-4	745	-	$> 10^{-4}$	Unknown
20. FIII-8	868	-	$> 10^{-4}$	Unknown

Table 3.21 (continued)

Clone*	No. of nucleotides sequenced (bp)	Closest species	E-values	Blast analysis
21. FIII-39	493	-	$> 10^{-4}$	Unknown
22. FIII-58	1058	-	$> 10^{-4}$	Unknown
23. FIV-1	952	-	$> 10^{-4}$	Unknown
24. FIV-2	399	-	$> 10^{-4}$	Unknown
25. FIV-20	755	*Strongylocentrotus purpuratus*	$3x10^{-66}$	G protein pathway suppressor 1 isoform 1
26. FIV-33	706	-	$> 10^{-4}$	Unknown
27. FV-1	399	-	$> 10^{-4}$	Unknown
28. FV-3	587	-	$> 10^{-4}$	Unknown
29. FV-11	851	*Mus musculus*	5x10−55	Thrombospondin 1
30. FV-27	660	*Danio rerio*	4x10−32	Thrombospondin 4
31. FV-42	639	-	$> 10^{-4}$	Unknown
32. M119-M09	559	*Litopenaeus aztecus*	$6x10^{-79}$	18S rRNA
33. M428-191	900	*Litopenaeus vannamei*	0.0	18S rRNA
34. M428-B17	755	*Xenopus laevis*	$2x10^{-8}$	Nucleoporin Nup153
35. M457-A01	599	-	$> 10^{-4}$	Unknown

*F and M were RAP-PCR fragments specifically found (or differentially expressed) in ovaries or testes of *P. monodon*, respectively (GenBank accession no. DV018448-DV018482).

Table 3.22 Gene homologues in ovaries and testes of *P. monodon* identified through RAP-PCR analysis

Transcript	Redundancy	Percentage of expressed transcripts
Unknown genes	18	51.43
18S rRNA	6	17.14
Thrombospondin homologues (TSP)	4	11.43
NADPH-cytochrome P450 reductase	1	2.86
Hypothetical proteins	1	2.86
White Spot Syndrome Virus	1	2.86
Ran-binding protein	1	2.86
G protein pathway suppressor 1	1	2.86
Nucleoporin Nup153 (Xenopus laevis)	1	2.86
Drosophila pseudoobscura (GA16635-PA)	1	2.86
Total	**35**	**100**

Surprisingly, MII-5 originally identified in testes by RAP-PCR analysis showed sex-differential expression with a higher level in ovaries. Temporal female specific expression of MII-5 was observed when tested against ovaries (N = 7) and testes (N = 7) of *P. monodon* broodstock (Figure3.50).

M457-A01 and MII-51 revealed male-specific expression in juvenile *P. monodon* (N = 5 and 4, respectively). Results were consistent when tested with *P. monodon* broodstock (N = 10 and 5, respectively; Figure 3.51).

Table 3.23 Amplification of newly designed primers derived from candidate sex-specific/differential expression fragments of *P. monodon*

Primers	Expected product size (bp)	Amplification result			
		Ovaries	Testes	Female DNA	Male DNA
1. FI-4	**415**	+++	-	-	-
2. FI-44	**261**	+++	-	-	-
3. FIII-4	**342**	+++	-	+I	+I
4. FIII-39	**362**	+++	-	+I	+I
5. FIII-58	**397**	+++	-	-	-
6. MII-5	**266**	+++	-	-	-
7. MII-51	**123**	-	+++	NS	NS
8. M457-A01	**324**	-	+++	NS	NS
9. FI-1	189	+++	+	+	+
10. FI-6	265	+++	+	+I	+I
11. FI-40	220	+++	++	NS	NS
12. FII-17	263	NS	NS	NS, I	NS, I
13. FII-18	155	-	-	-	-
14. FII-22	309	-	-	-	-
15. FIII-8	333	+++	+	+	+
16. FIV-1	317	+++	+	-	-
17. FIV-2	197	+	-	NS	NS
18. FIV-20	270	+++	++	-	-
19. FIV-33	158	+++	+	+	+
20. FV-1	226	+++	+	+	+
21. FV-3	146	+++	+	-	-
22. FV-27	233	+++	+	+I	+I
23. FV-42	207	+++	+	+I	+I
24. MI-36	182	+++	+++	NS	NS
25. 428-OPB17	238	+++	+	+	+

*F and M were RAP-PCR fragments specifically found (or differentially expressed) in ovaries or testes of *P. monodon*, respectively. Sex-specific expression markers are illustrated in boldface. NS = non-specific amplification, - = no product, + = positively expected amplification product, +I = positive amplification product exhibiting a larger size.

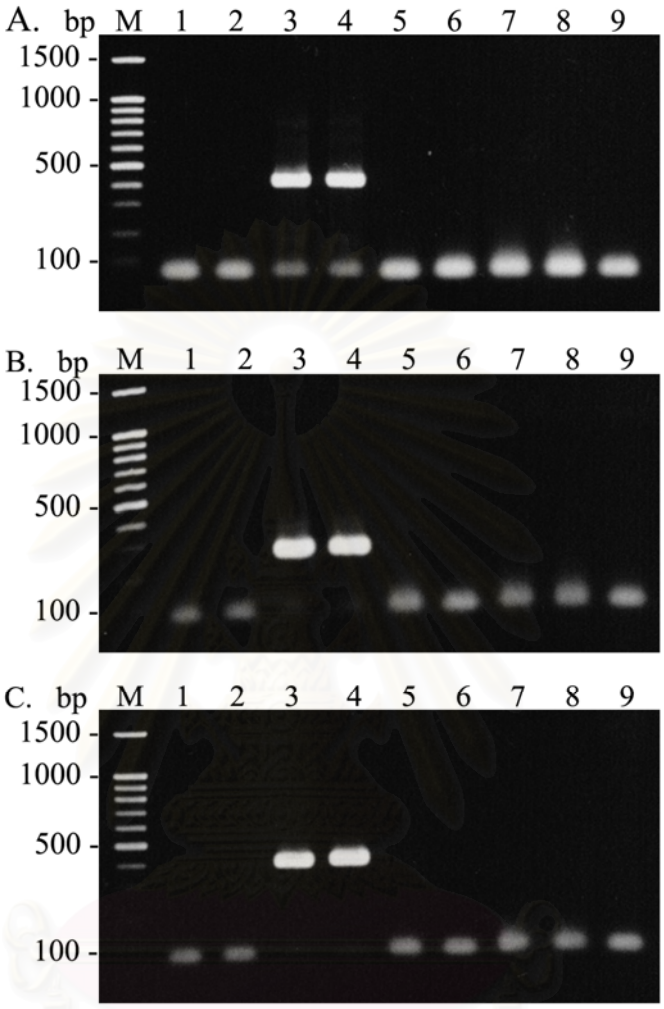

Figure 3.49 RT-PCR of sex-specific expression markers derived from FI-4 (A), FI-44 (B) and FIII-58 (C) against the first strand cDNA of testes (lanes 1 – 2), ovaries (lanes 3 – 4), hemocytes (lanes 5 – 6) and genomic DNA of male (lane 7) and female (lane 8) of 3-month old shrimps. Lanes M and 9 are a 100 bp DNA ladder and the negative control (without the first strand cDNA template).

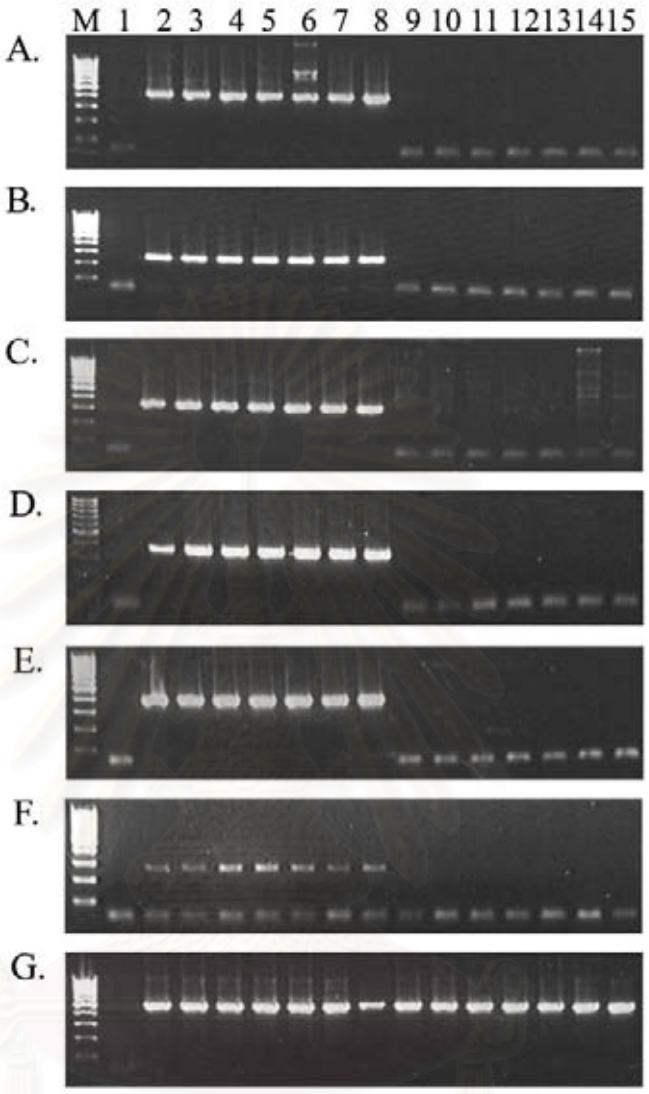

Figure 3.50 RT-PCR of female-specific expression markers derived from FI-4 (A), FI-44 (B), FIII-4 (C), FIII-39 (D), FIII-58 (E) and MII-5 (F) against the first strand cDNA of ovaries (lanes 2 - 8) and testes (lanes 9 - 15) of *P. monodon* broodstock. Lanes M and 1 are a 100 bp DNA ladder and the negative control (without the first strand cDNA template). *EF-1α* (G) amplified from the same template was included as the positive control.

In addition, sixteen primer pairs gave positive amplification products against genomic DNA of *P. monodon*. Although six primers generated the positive amplification product, nonspecific amplification products were also observed. The remaining primer pairs generated the discrete amplification product. Five of which generated the expected product sizes whereas other five primer pairs yielded larger product sizes due to the existence of intron in the amplified DNA segments.

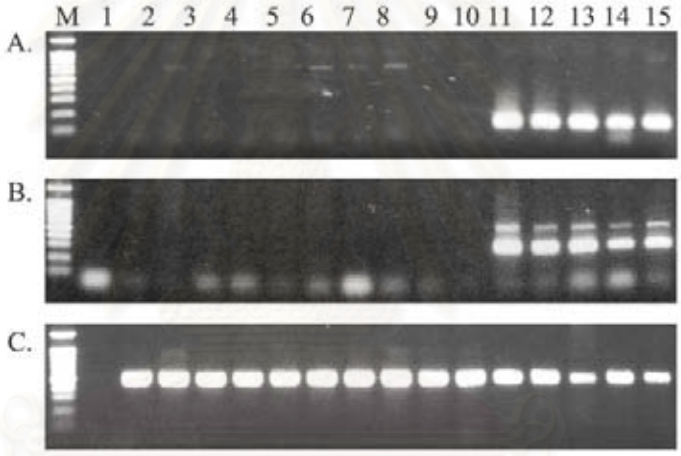

Figure 3.51 RT-PCR of male-specific expression markers derived from MII-51 (A) and M457-A01 (B) against the first strand cDNA of ovaries (lanes 2 - 11) and testes (lanes 12 - 16) of *P. monodon* broodstock. Lanes M and 1 are a 100 bp ladder and the negative control (without the first strand cDNA template). *EF-1α* (C) amplified from the same template was included as the positive control.

Seven pairs of primers (FIII-4, FIII-39, FIV-2, FIV-33, FV-1, FV-27 and FV-42) were further tested against genomic DNA of wild *P. monodon* originating from geographically different locations in Thai waters (*N* = 16) by SSCP analysis. Sequence polymorphism was observed in these markers (Figure 3.53).

Low polymorphism was found from SSCP patterns of FIII-4, FIII-39, FIV-2., FIV-33 and FV-1. On the other hand, those FV-27 and FV-42 showed highly polymorphic SSCP patterns. Results indicated the successful development of SNP through polymorphic RAP-PCR fragment of *P. monodon* owing to SNP or small indels of a particular gene region. The developed markers may be used for detection of mutation, construction of the genetic linkage map and/or population genetic studies of *P. monodon*.

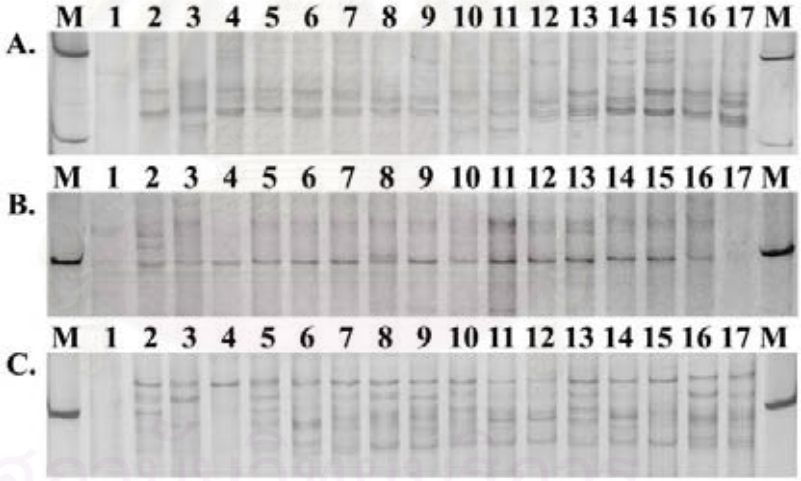

Figure 3.52 SSCP patterns of a SNP by EST (SBE) marker derived from FV-42 (A), FIII-4 (B) and FV-27 (C) amplified against genomic DNA of *P. monodon* originating from Trat (lanes 2 - 5), Chumphon (lanes 6 - 9), Satun (lanes 10 - 13), Trang (lanes 14 - 15) and Phangnga (lanes 16 - 17). Lane M and 1 are 100 bp ladder and a non-denatured PCR product (double strand DNA control).

CHAPTER IV

DISCUSSIONS

Development of molecular markers for identification of species origin of five penaeid species (*P. monodon*, *P. semisulcatus*, *F. merguiensis*, *L. vannamei* and *M. japonicus*) by PCR-RFLP and SSCP analyses

PCR-RFLP of COI-COII (1550 bp) of *P. monodon* (*N* = 29) and *P. semisulcatus* (*N* = 15) digested with *Dra* I, *Ssp* I and *Vsp* I generated eight composite restriction patterns (mitotypes) and exhibiting non-overlapping distribution between these taxa. Both single enzyme digestion patterns and mitotypes of COI-COII$_{1550}$ could discriminate *P. monodon* and *P. semisulcatus* unambiguously. The experiments were extended to cover *L. vannamei*, *F. merguiensis* and *M. japonicus* but COI-COII primers did not generate the positive amplification product in those taxa. The universal primers of 16S rDNA$_{560}$ previously used for population genetic studies of *P. monodon* were then applied (Klinbunga *et al*., 2001b).

The 16S rDNA$_{560}$ gene segment was successfully amplified across small sample sizes of each shrimp (overall specimens = 79). Fifteen restriction patterns (4, 4, 3 and 4 from *Alu* I, *Mbo* I, *Ssp* I and *Vsp* I, respectively) were found. Differentiation between *P. monodon* and *P. semisulcatus* and between *L. vannamei* and *F. merguiensis* by single enzyme digestion patterns was interfered by shared restriction patterns from other species.

A total of 11 mitotypes were observed. Non-overlapping mitotypes of 16S rDNA$_{560}$ were found in *P. monodon*, *L. vannamei* and *F. merguiensis*. Three mitotypes were found in *L. vannamei* indicating that *L. vannamei* has possibly been introduced from more than a single stock. Nevertheless, *P. semisulcatus* and *M. japonicus* shared a BABB genotype. Phylogenetic relationships regard these shrimps to be distantly related taxa (Baldwin *et al*., 1998; Lavery *et al*., 2004). Therefore, a shared mitotype should be resulted from the use of limited number of restriction endonucleases. SSCP analysis of 16S rDNA$_{560}$ indicated clear distinction between *P. semisulcatus* and *M. japonicus*.

Species identification is necessary for quality control of cultured species particularly when wrong species are intentionally supplied. Moreover, labeling and traceability of the cultured product are important matter owing to an increase in trade and the need to maintain confidence in the quality of the products.

Oysters have been classified based principally on morphology. However, they show ecomorphological variation. Therefore, two sympatric species or allopatric populations of a single species inhabit different habitats may be misidentified. Species-diagnostic markers of *Crassostrea belcheri*, *C. iredalei*, *Saccostrea cucullata*, *S. forskali* and *Striostrea* (*Parastriostrea*) *mytiloides* were examined using PCR-RFLP of 16S rDNA (*Acs* I, *Alu* I, *Dde* I, *Dra* I, *Rsa* I and *Taq* I), 18S rDNA (*Hin*f I) and COI (*Acs* I, *Dde* I and *Mbo* I). A total of 54 mitotypes were found. Species-diagnostic PCR-RFLP markers were specifically found in *C. belcheri*, *C. iredalei* and *S. cucullata* but not in *S. forskali* and *Striostrea* (*Parastriostrea*) *mytiloides* (Klinbunga *et al*., 2003a).

Likewise, species-diagnostic markers of the tropical abalone (*Haliotis asinina*, *H. ovina* and *H. varia*) in Thai waters were successfully developed based on PCR-RFLP of 16S rDNA (*Alu* I, *Bam*H I, *Eco*R I and *Hae* III). Non-overlapping mitotypes were found in *H. asinina* (AAAA and AAAE, $N = 115$), *H. ovina* (ABBB, AAAB and AABB, $N = 71$) and *H. varia* (BABG, BABC, BABD, BABF and AABG, $N = 23$), respectively. The 16S rDNA from an individual representing each mitotype was cloned and sequenced. Species-specific PCR was further developed in *H. asinina* and *H. varia* without any false negative or false positive results. The sensitivity of detection was approximately 25 pg and 50 pg of the DNA template, respectively (Klinbunga *et al*., 2003b).

Species-diagnostic markers should be established from DNA segments exhibiting low genetic polymorphism within a particular species but showing high genetic divergence between different species (Thaewnon-ngiw *et al*., 2004).

Overlapping patterns (in this case, PCR-RFLP and SSCP) between different species should not be observed. Although the nature of 16S $rDNA_{560}$ fulfilled the requirement, the amplification success in *L. vannamei* and *F. merguiensis* was quite

low when sample sizes were increased. Therefore, more reliable primers were required.

The 16S $rDNA_{560}$ gene segment was then amplified from a representative individual possessing the most common mitotype of each species, cloned and sequenced. The actual length of this fragment was 561 bp in *P. semisulcatus* and 562 bp in other species. Sequence divergence (Kimura, 1980) between pairs of 16S $rDNA_{560}$ was 5.76% (between *P. semisulcatus* and *F. merguiensis*) - 10.23% (between *M. japonicus* and *L. vannamei*). The divergence between taxonomically problematic species was 6.15% between *P. monodon* and *P. semisulcatus* and 9.80% between *L. vannamei* and *F. merguiensis*, respectively.

Due to high intraspecific genetic diversity previously reported in *P. monodon* (Klinbunga *et al*., 1999 and 2001b) and *F. merguiensis* (Hualkasin *et al*., 2003; Wanna *et al*., 2004), species-specific PCR (presence/ absence of the amplification band) for each species was not developed because serious false negative may be occurred when specimens from new geographic samples are analyzed.

Alternatively, a pair of primers primed at the conserved region of 16S $rDNA_{560}$ providing a 312 bp fragments was designed. Three, two and three restriction patterns were found from digestion of 16S $rDNA_{312}$ with *Alu* I, *Ssp* I and *Vsp* I, respectively. All restriction patterns observed for each enzyme could be related to one another by the loss or gain of a single or double restriction sites. Restriction patterns of *Alu* I- and *Vsp* I-digested 16S $rDNA_{312}$ clearly differentiate both *P. monodon* and *L. vannamei* from the remaining species. Seven mitotypes were found. Like results from 16S $rDNA_{560}$, *P. monodon*, *L. vannamei* and *F. merguiensis* can be unambiguously differentiated while a shared BBB mitotype was observed in *P. semisulcatus* and *M. japonicus*.

Although nucleotide sequences of 16S $rDNA_{560}$ indicated that *Aha* III, *Apo* I, *Mae* II, *Mae* III, *Mse* I and *Tsp*E I could differentiate *P. semisulcatus* and *M. japonicus*, all restriction enzymes except *Mse* I are not common. Moreover, the predicted restriction products of 16S $rDNA_{312}$ digested with *Mse* I are too small (7-83 bp) for conveniently analyzed by agarose gel electrophoresis. Using SSCP analysis, non-overlapping SSCP patterns were found indicating successful development of

species-diagnostic markers across all taxa. Typically, a single mitotype from PCR-RFLP (e.g. AAA, ABA and ABB in *P. monodon* and BAB in *F. merguiensis*) possessed several SSCP patterns. This indicated that SSCP analysis is more sensitive than PCR-RFLP when the same DNA fragment is analyzed. Accordingly, cost and time consuming based on restriction analysis particularly when a large number of restriction enzymes are needed, can be significantly reduced. Additional gene segments can then be included to the analysis favored species identification based on SSCP analysis.

Applications for species identification of suspected shrimps and frozen shrimp meat

Two groups of cultured juvenile shrimps were sent to a laboratory at the Center of Excellence for Marine Biotechnology, Chulalongkorn University for authentication of the species origin. Farmer was told when purchased the larvae that the Group 1 sample was *F. merguiensis* from Indonesia and the Group 2 sample was hybrid offspring between *P. monodon* (sire) and *M. japonicus* (dam). PCR-RFLP of 16S $rDNA_{312}$ indicated that both samples were *F. merguiensis* (BAB mitotype). SSCP analysis of the amplified 16S $rDNA_{312}$ further confirmed results from PCR-RFLP analysis.

In the other case, the frozen shrimp meat was introduced to Thailand and claimed as the product of the white shrimp (*P. orientalis*) and the pink shrimp (*Metapenaeus affinis*) from fisheries. The suspected *P. orientalis* exhibited a BAB mitotype restrictively found in *F. merguiensis* whereas the suspected *M. affinis* showed a DBB mitotype which were not found in our database.

SSCP analysis of 16S $rDNA_{312}$ indicated that suspected *P. orientalis* was actually *F. merguiensis*. In addition, suspected *M. affinis* exhibited clearly different SSCP patterns with that of *P. semisulcatus*, *L. vannamei*, *F. merguiensis* and *M. japonicus*. Its polymorphic SSCP patterns were similar, but not identical, to that of *P. monodon* exhibiting an AAA mitotype. Based on large interspecific sequence divergence of 16S $rDNA_{560}$ found in the present study (5.76 - 10.23%), we concluded that suspected *M. affinis* should be a member of *Penaeus* rather than *Metapenaeus*.

Reliable PCR-based methods for identifying species origins of morphological similar shrimps (between *P. monodon* and *P. semisulcatus* and between *L. vannamei* and *F. merguiensis*) were successfully developed based on PCR-RFLP and SSCP analyses of the amplified rDNA gene segment. These simple methods can be used to prevent supplying incorrect shrimp larvae for the industry and for quality control of shrimp products from Thailand. Population genetic studies of local *Penaeus* species (e.g. *P. semisulcatus* and *F. merguiensis*) can be conveniently carried out. The techniques can be applied for rapid genotyping of captured shrimp released from the stock enhancement programs of *P. monodon* in Thai waters.

Population genetic studies of *P. monodon* in Thai waters using polymorphism of 16S rDNA$_{312}$, AFLP-derived SCAR markers and COI

Population genetic studies of *P. monodon* have been reported based on PCR-RFLP of mitochondrial DNA (Klinbunga *et al.*, 1999 and 2001; Benzie *et al*., 2002), RAPD (Tassanakajon *et al*., 1998; Klinbunga *et al*., 2001), and type II microsatellites (Supungul *et al*., 2000; Xu *et al*., 2001). Recently, Klinbunga *et al*. (2006) reported population genetic studies of *P. monodon* based on EST-derived markers (nuclear DNA polymorphism). However, information based on sequencing of mitochondrial DNA gene segments on population genetics of *P. monodon* has not been reported.

Several studies have assessed genetic heterogeneity in *P. monodon* using various genetic markers over distances of hundreds or thousands of kilometers (Tassanakajon *et al.* 1998; Benzie *et al*. 2002) but genetic differentiation have generally been found to be low except where major biogeographic boundaries act to disrupt gene flow (Benzie 2000).

PCR-RFLP of 16S rDNA$_{312}$ implied low polymorphism in this gene region limiting its ability for differentiation of genetic populations of *P. monodon*. The use of enzymes generating a low number of restriction patterns (1, 2 and 2 digestion patterns for *Alu* I, *Ssp* I and *Vsp* I, respectively) may responsible for providing biased analyzing results.

SSCP analysis which is theoretically more sensitive technique than PCR-RFLP was applied for determination of genetic diversity of the same gene segment (16S rDNA$_{312}$) of *P. monodon*. Low genetic distance (0.0001 – 0.0100) between pairs

of geographic samples was found. Moreover, distribution frequencies of common SSCP genotypes were nearly identical in each sample. As a result, a lack of intraspecific population differentiation was found in *P. monodon* ($P > 0.05$).

More recently, Klinbunga *et al*. (2006) identified and sequenced 90 and 157 ESTs from normal and subtractive ovarian cDNA libraries of the giant tiger shrimp (*Penaeus monodon*). SSCP analysis of disulfide isomerase (*DSI*), zinc finger protein (*ZFP*), *PMO920*, and *PMT1700* was carried out for population genetic studies of the same sample set of *P. monodon* used in this thesis. The number of co-dominant alleles per locus for overall samples was 6 for *PMO920*, 5 for *PMT1700*, and 12 for *ZFP* and there were 19 dominant alleles for *DSI*. The observed heterozygosity of each geographic sample was 0.3043 - 0.5128 for *PMO920*, 0.3462 - 0.4643 for *PMT1700*, and 0.5000 - 0.8108 for *ZFP*. Low genetic distance was found between pairs of geographic samples (0.0077 - 0.0178). Geographic differentiation was not significantly different except that between Satun-Trat and Satun-Phangnga at the *ZFP* locus ($P < 0.05$), suggesting low degrees of genetic subdivision of Thai *P. monodon*.

Noncoding DNA segments (e.g. AFLP-derived markers) possibly exhibiting higher levels of polymorphism than that of the coding gene segments (EST derived markers) was then analyzed. Four AFLP-derived markers (E4M6-295, E7M10-450, P6M2-370, and P6M6-470) were genotyped against genomic DNA of *P. monodon*. Unlike EST-derived marker, all AFLP-derived SCAR markers showed more complicated patterns and therefore were treated as dominant segregation markers. The deficiency of fixed population- or region-specific genotypes and the presence of shared genotypes with relatively comparable allele frequencies across geographic samples were found. Low genetic distance was also observed (0.0064 – 0.1060) between paired geographic samples. Genetic heterogeneity was not observed in all possible pairwise comparisons of overall primers ($P > 0.05$).

The failure to detect genetic heterogeneity in Thai *P. monodon* in this thesis by 16S $rDNA_{312}$ and AFLP-derived markers may result from the use of a lower polymorphic level of coding and non-coding nuclear DNA than that of microsatellites and mtDNA gene segments against mixed populations of *P. monodon*. However, the conclusion on the panmictic gene pool of Thai *P. monodon* should be treated with

cautions because it is different from all population genetic studies previously reported for *P. monodon*.

Supungul *et al.* (2000) examined genetic diversity of *P. monodon* in Thailand by microsatellites (*CUPmo1, CUPmo18, Di25, CSCUPmo1*, and *CSCUPmo2*) using the same sample set as in this study. The average observed heterozygosity was relatively high in each geographic sample (0.71 – 0.82). Low genetic distance between pairs of samples was observed. The greatest genetic distance was observed between Trat and Satun (d = 0.030), and the shortest distance was between Satun and Trang (d = 0.024). Three microsatellite loci (*Di27, CSCUPmo1*, and *CSCUPmo2*) did not show any significant geographic differences among all pairwise comparisons. F_{ST} between pairs of geographic samples was usually < 0.01. Low degrees of differentiation were observed between shrimps from Satun and Trat (F_{ST} = 0.0124, P < 0.01), and Trat and Chumphon (F_{ST} = 0.0118, P < 0.01) but not from the remaining comparisons.

Likewise, high diversity but low genetic differentiation was also reported in wild (Palawan, Quezon, Capiz, and Negros Occidental-W) and cultured (Negros Occidental-C and Antique) *P. monodon* in the Philippines analyzed by six microsatellites (*TUZXPm*2.41, *TUZXPm*4.45, *TUZXPm*4.55, *TUZXPm*4.82, *TUZXPm*4.85, and *TUZXPm*4.9). The observed heterozygosity of these samples was 0.47 – 1.00. A low but significant degree of population differentiation was found between Negros Occidental-W and other samples (F_{ST} = 0.009 – 0.013), but not between the remaining comparisons (F_{ST} = 0.000 – 0.001) (Xu *et al*., 2001).

In contrast, strong genetic differentiation of *P. monodon* from Satun (the Andaman Sea) and Surat and Trat (the Gulf of Thailand) was illustrated using restriction analysis of the entire mtDNA. Twenty-eight composite haplotypes were generated from 52 restriction profiles of *P. monodon* mtDNA digested with 11 restriction enzymes. The size of the entire *P. monodon* mitochondrial genome was estimated to be 15.913 ± 0.177 kb. The average haplotype diversity in *P. monodon* was 0.864, whereas the mean nucleotide diversity within populations was 2.51%, 2.22%, and 1.91% for Satun, Trat, and Surat, respectively. Geographic heterogeneity analysis indicated strong population differentiation between *P. monodon* from the

Andaman Sea and *P. monodon* from the Gulf of Thailand ($P < 0.0001$) (Klinbunga *et al*., 1999).

In addition, high haplotype diversity (0.855) and nucleotide diversity (3.328%) of Thai *P. monodon* were observed based on PCR-RFLP of 16S rDNA and COI. Population differentiation and large genetic distance of *P. monodon* between the Andaman Sea and Gulf of Thailand was clearly illustrated ($P < 0.0001$) (Klinbunga *et al*., 2001).

In the present study, high genetic diversity of *P. monodon* was observed based on COI sequences. A total of 266 polymorphic sites were found after multiple alignments of these sequences. Forty-three haplotypes were found from 100 investigated individuals. The average number of individuals that share identical haplotypes was 2.33. The percentage of nucleotide divergences between pairs of COI sequences was 0.00 – 23.65% whereas that of T17 and the remaining sequences was 37.43 – 55.87%. A neighbor-joining tree revealed 3 different phylogenetic lineages. Distribution frequencies of members of theses lineages in overall samples was statistically significant ($P = 0.0006$). All pairwise comparisons except Satun - Trat ($P = 0.6538$), Trang - Phangnga ($P = 1.0000$) were statistically significant ($P = 0.0006$ and $P < 0.05$).

Genetic differentiation patterns of *P. monodon* in this study were different from those previously reported. Result from PCR-RFLP (Klinbunga *et al*., 2001) revealed strong differentiation between *P. monodon* from the Andaman (Satun, Trang and Phangnga) and Gulf of Thailand (Trat and Chumphon) samples but not within each coastal region. Further within coastal region differentiation between Chumphon and Trat was subsequently reported based on RAPD (Klinbunga *et al*., 2001) and microsatellites (Supungul *et al*., 2000).

In the present study, within coastal region subdivisions were observed in both the Andanan Sea (between Satun and Trang and Phangnga) and Gulf of Thailand (between Chumphon and Trat). Surprisingly, specimens from Trat ($N = 20$) did not reveal significant genetic heterogeneity with those of Satun ($N = 19$, $P > 0.05$). Although numbers of specimens seem to be sufficient for DNA sequencing analysis, more specimens are perhaps required for the unambiguous conclusion.

Under a presumption of selective neutrality for genetic markers (RAPD, microsatellites and mtDNA), one of the possible explanations for anomalous patterns of genetic differentiation of Thai *P. monodon* is the mixing of captive and native stocks of *P. monodon* which may have resulted from a small scale consistent release of unclear origin of *P. monodon* in Thai waters.

Tassanakajon *et al.* (1998) found the maximum genetic distance of 0.334 for population genetic studies of *P. monodon* from the Gulf of Thailand (Trat and Chummphon) and the Andaman Sea (Satun-Trang and Phangnga, Thailand and Medan, Indonesia) where geographic sites separated by up to 1,000 km. A lower level of genetic distance was observed when the Medan sample was excluded (genetic distance of 0.0029 – 0.0661). Subsequently, Klinbunga *et al*. (2001) found similar low genetic distance values ranging from -0.002 - 0.037 (geographic ranges separated by up to 650 km) from the same approach using the different sample set originating from the same locations in Thai waters.

Evidence of the mixing of wild and hatchery-raised shrimp has been reported for *P. monodon* in Thailand (Supungul *et al*., 2000) and the Philippines (Xu *et al*., 2001) based on microsatellite analysis. Deviations from the Hardy-Weinberg expectation were observed in 19 of 25 possible tests in the former owing to an excess of homozygotes. Similarly, at least one of six investigated microsatellite loci of each wild geographic sample in the Phillipines exhibited the deficiency of heterozygotes resulting in Hardy-Weinberg disequilibrium of *P. monodon*.

In addition, Klinbunga *et al*. (2001) reported contradictory results between nuclear (RAPD) and mtDNA markers in patterns of genetic differentiation of *P. monodon* populations across the Thai-Malaysian peninsula as a result of localized wild stock displacement by aquaculture activity. Due to potential adaptive differences between natural *P. monodon* populations and hatchery-reared larvae, the pollution of locally-adapted gene pools is a matter of national concern in the potential loss of a valuable genetic resource for future farming practices of *P. monodon*.

Apart from data about population genetics of *P. monodon,* the other major goal of this study is to identify molecular genetic markers that can be applied for genotying of *P. monodon*. Sequencing of COI can be used in combination with

microsatellites for determination of genetic diversity levels of domesticated *P. monodon*. The rapid and simple genotying of *P. monodon* by direct sequencing of COI would allow practical applications of polymorphic markers for management of both natural and domesticated broodstock of *P. monodon*.

Molecular population genetic studies provide necessary information required for elevating culture and management efficiency of *P. monodon*. In the present study, the potential of SSCP analysis of 16S $rDNA_{312}$ and AFLP-derived markers and DNA sequencing of the COI gene segment was demonstrated for evaluation of genetic diversity of Thai *P. monodon*. Although data from SSCP (relatively low genetic diversity and a lack of population differentiation) and DNA sequencing of COI (high genetic diversity and the existence of population subdivisions in this species) were contradictory, *P. monodon* should not be regarded as a panmictic species.

For the conservation point of view, each geographic sample of *P. monodon* in Thailand should be treated different populations and managed separately. This basic knowledge on levels of genetic diversity and population differentiation of *P. monodon* not only yields critical information on historical and evolutionary aspects of *P. monodon* but also allows the ability to construct effective breeding programs and stock enhancement projects in this species.

Isolation and characterization of genomic sex determination markers and sex-specific/differential expression markers of *P. monodon* using RAPD-PCR and RAP-PCR analysis

Identification of sex determination markers of P. monodon by RAPD-PCR

Sex-specific markers should be developed from fixed polymorphism in genomic DNA of male and female *P. monodon* to avoid destruction of specimens and for direct application of sex determination markers (e.g. determination of sex-specific survival and early growth rates) in this economically important species.

From bulked segregant analysis (BSA) and RAPD analysis, 10 candidate sex-specific RAPD fragments were found and only 8 fragments were successfully cloned and sequenced. Four pairs of primers were designed (PMF530-F/R, PMM350-F/R, PMM1100-F/R, PMM800-F/R) and tested against genomic DNA of male and female

P. monodon. Two primers pairs (PMF530-F/R, PMM350-F/R) yielded positive amplification products in both sexes whereas the other two primer pairs did not amplified product. Neither sex-specific amplification patterns nor length polymorphism between the PCR product of male and female *P. monodon* from PMF530-F/R and PMM350-F/R were observed. Further analysis with SSCP revealed polymorphism between single stranded DNA of these shrimps but was not relevant to sex specificity in this species. Therefore, genomic sex determination markers were not successfully developed in *P. monodon*.

Li *et al*. (2003) constructed a genetic linkage map in *P. japonicus* using polymorphic AFLP markers in a pseudo testcross strategy. Fifty-six progeny (top and bottom 8 % of the body weight at 6 months) of the intermediate F_2 cross family were genotypes by 54 primer combinations. A total of 502 polymorphic AFLP fragment were found and 359 and 138 of these segregated in 1:111 and 3:1 ratios. The markers with a 1:1 segregation ratio were combined with those previously examined (Moore *et al*., 1999) and 217 AFLP markers were ordered into 43 linkage groups of the paternal map and 125 AFLP markers were in 31 linkage groups of the maternal map. A female-linked AFLP marker was tightly linked to the linkage group 28 of the female map. This implies that the female of *P. japonicus* exhibits the heterogametic sex. Notably, no sex chromosome has been reported in penaeid shrimps and the authors of that publication are still not able to develop a SCAR marker derived from the sex-linked AFLP markers in *P. japonicus*.

Thumrungtanakit (2004) used AFLP analysis to isolate genomic sex-specific markers in *P. monodon*. A total of 256 primer combinations were tested against 6-10 bulked genomic DNA of *P. monodon*. Five (FE10M9520, FE10M10725, FE14M16340, FE15M14400 and FE16M8350) and one (ME10M8420) candidate female and male-specific AFLP markers were identified. The former markers were cloned and further characterized. SCAR markers derived from FE10M9520, FE10M10725.1, FE10M10725.2, FE14M16340 did not retain the original sex specificity. SSCP analysis was applied to identify whether fixed SNP was existent in SCAR markers amplified from male and female *P. monodon*. Polymorphic but not sex-linked pattern were found from FE10M10725.1 and FE14M16340-derived SCAR markers.

Likewise, genomic DNA subtraction between male and female *P. monodon* was performed by the PERT and RDA approaches, SCAR markers developed from a subtractive male (PERT) and 2 subtractive female (RDA) clones did not reveal the sex-specific amplification product in *P. monodon* (Poonlaphdecha, 2004).

Preechaphool (2004) analyzed pooled DNA of small orange claw (SOC, *N* = 10) and blue-claw (BC, *N* = 5) males and females (*N* = 10) of the giant freshwater prawn (*Macrobrachium rosenbergii*) using 64 AFLP primer combinations and found 90 and 42 AFLP markers in male and female *M. rosenbergii*, respectively. Additional sample set of SOC (*N* = 5), OC (*N* = 15) and BC (*N* = 10) males and female (*N* = 20) *M. rosenbergii* originating from different geographic locations with that of the first sample set were reanalyzed by 46 informative primers previously used. In a total, 5 candidate male-specific and 4 candidate female-specific AFLP markers were finally identified. These markers were cloned and characterized. A PCR-based method for sex determination of *M. rosenbergii* was developed but sex-specificity of AFLP-derived markers was not observed.

Identification of sex determination markers based on AFLP analysis was also carried out in the tropical abalone (*Haliotis asinina*). Seven female- and male-specific AFLP fragments were identified from screening 224 primer combinations with 4 bulked DNA of male and female *H. asinina*. SCAR markers developed from candidate sex-specific AFLP fragments did not reveal their initial specificity. Further characterization of the PCR products by SSCP analysis did not provide fixed polymorphism between male and female abalone (Amparyup, 2004).

Li *et al*. (2002) identified genomic sex markers of the green spotted pufferfish (*Tetraodon nigroviridis*, *N* = 83) using RAPD (600 primers and 1700 primers for the first and the second set of pooled DNA), AFLP (64 primer combinations) and RDA (1 set of adaptors). A total of 59, 126, 16 and 16 putative sex-specific markers were found after the primary screening. Nevertheless, the secondary screening (re-testing of DNA from individuals for RAPD and AFLP and using the putative RDA markers as the probes for genomic Southern analysis of male and female DNA) did not demonstrate the presence of sex-specific markers in *T. nigroviridis*.

Sex determination is problematic in researches of many species and can usually be resolved by the application of DNA-based technology but this is only possible if a sex-specific marker located on unique sex chromosomes is available. Theoretically, the lack of genomic sex determination markers in *P. monodon* may have resulted from weak correlation between the genotypic sex and phenotypic sex due to autosomal modifier genes or genetic diversity between investigated individuals used for screening of markers is greater than the optimal level (Griffiths and Orr, 1999).

Although these possibilities cannot be excluded, they are unlikely. Alternatively, the lack of sex chromosomes in *P. monodon* and other penaeid shrimps implied that sex chromosomes may not be present or they are not well differentiated in the genome of penaeid shrimps. Therefore, development of genomic sex determination markers in *P. monodon* may not be possible.

Identification of sex-specific/differential expression markers of P. monodon by RAP-PCR analysis

The most important step towards understanding molecular mechanisms of sex differentiation (development undifferentiated gonads to ovaries or testes) in *P. monodon* is the identification and characterization of sex-specific/differential expression markers. Such molecular markers could be used to determine the initial expression of those transcripts following ovarian and testicular development

Gender-specific gene expression has been recently reported in a mosquito-borne filarial nematode (*B. malayi*) isolated by differential display (DD) PCR and *In silico* subtraction of EST cluster database and further confirmed by RT-PCR. Six of 12 (27%) and seven of 15 (47%) initially identified by DD-PCR and *In silico* subtraction revealed gender-specific expression in that species (Michalski and Weil, 1999).

In the silkworm (*Bombyx mori*), sex-specific mRNA isoforms were found in double sex (*dsx*) gene where the male-specific cDNA lacked the sequence between 713 – 961 nucleotides of the female-specific cDNA (Ohbayashi *et al*., 2001).

On the basis of preliminary screening, a large number of RAP-PCR fragments specifically or differentially expressed in testes and ovaries of *P. monodon* were identified. A total of 25 and 20 fragments from candidate female and male RAP-PCR fragments were cloned. Finally, 21 and 14 types of sequences originating from 24 female and 15 male cloned fragments were obtained. Blast results indicated that the major transcripts found were unknown transcripts (51.43%), 18S rRNA (17.14%) and thrombospondin (TSP, 11.43%) homologues. Shrimp ovarian peritrophin (SOP) and TSP are major component of cortical rods in mature ovaries of penaeid shrimps (Kayat *et al.*, 2001; Yamano *et al.*, 2004). As a result, these transcripts may involve with the final stages of oocyte maturation in shrimps.

Five (FI-4, FI-44, FIII-4, FIII-39 and FIII-58) and two (M457-A01 and MII-51) derived RAP-PCR markers revealed sex-specific expression in females and males *P. monodon* of both juvenile and broodstock. Surprisingly, MII-5 exhibited temporal female specific expression when tested against ovaries and testes of *P. monodon* broodstock.

Leelatanawit *et al*. (2004) constructed subtractive cDNA libraries of ovaries and testes of *P. monodon*. Most of the expressed genes in the subtractive cDNA library of ovaries encoded unknown transcripts (78 clones accounting for 49.7% of total characterized cDNA), TSP (45 clones, 28.7%), peritrophin (17 clones, 10.8%), respectively. Homologues of elongation factor-2, oxidoreductase, transketolase, hypothetical protein FLJ23251, and sex-linked ENSANGP00000010123 and X-linked protein 1 (XNP-1) were also isolated. Gender-specific expression of candidate sex-linked gene homologues was examined by RT-PCR. While XNP-1 and peritrophin were expressed in both ovaries and testes, TSP and ENSANGP00000010123 homologues revealed sex-specific expression in female *P. monodon*.

Oogenesis is the preparation for embryogenesis. It is characterized by the progressive accumulation of reserve materials and diversity mRNA used later in fertilization and embryonic development. Additionally, more than 100 genes have been identified to be essential for spermatogenesis. Some of these genes are ubiquitously expressed, and some are expressed in a testis-specific fashion.

In the present study, sex-specific expression markers of *P. monodon* were successfully developed at the cDNA level. Five and two RAP-PCR derived markers showed sex-specific expression in ovaries and testes of both 3-month-old and broodstock-sized *P. monodon*. This opens the possibility to study the initial expression and localization of sex-specific expression markers in undifferentiated gonads of *P. monodon* by *in situ* hybridization. Sex differentiation processes in *P. monodon* can then be initially examined intensively.

CHAPTER V

CONCLUSIONS

1. PCR-RFLP and SSCP analysis of 16S $rDNA_{312}$ was succerssfully developed for authentication the species origin of five penaeid shrimps (*P. monodon, P. semisulcatus, F. merguiensis, L. vannamei* and *M. japonicus*). PCR-RFLP of 16S $rDNA_{312}$ successfully differentiated *P. monodon, F. merguiensis* and *L. vannamei* but not *P. semisulcatus* and *M. japonicus* which shared a BABB mitotype. All shrimp species could be unambiguously discriminated by SSCP analysis of 16S $rDNA_{560}$.

2. Genetic diversity and population differentiation of *P. monodon* originating from the Andaman Sea (Satun, Trang and Phangnga) and Gulf of Thailand (Chumphon and Trat) was examined by PCR-RFLP and SSCP analysis of 16S $rDNA_{312}$. Low genetic diversity and a lack of intraspecific population subdivisions of *P. monodon* were illustrated ($P > 0.05$).

3. A total of 320 AFLP primer combinations were screened against bulked genomic DNA of *P. monodon*. Twenty two polymorphic AFLP fragments were cloned and sequenced. Fourteen pairs of sequence-specific primers were designed and 4 AFLP-derived SCAR markers (P6M2-370, P6M6-470, E4M6-295 and E7M10-450) were applied for population genetic studies of *P. monodon*. Like results from 16S $rDNA_{312}$, low genetic diversity and a lack of population differentiation was found ($P > 0.05$).

4. The COI-$COII_{1550}$ fragments of representatives of *P. monodon* was cloned and sequenced. A COI_{614} gene segment was successfully amplified using Universal COI-COII-F+ $T13COI_{R2}$ primers. Nucleotide sequences of 100 shrimp individuals were examined. A neighbor-joining tree indicated three phylogenetic lineages of *P. monodon*. Large nucleotide divergence was observed between inter-lineage haplotypes but limited divergence was found between intra-lineage haplotypes. Distribution frequencies of haplotype clusters in overall samples and pairs of geographic samples were statistically significant ($P < 0.05$) indicating the existence of population subdivisions based on mtDNA COI polymorphism.

5. Genomic sex determination markers were developed by RAPD analysis. A total of 100 primers were screened. Eight candidate sex-specific RAPD fragments were cloned and sequenced. Four primer pairs were designed and tested for sex-specificity. Results indicated that RAPD-derived SCAR markers did not showed sex-specificity in investigated *P. monodon*.

6. Sex-specific/differential expression markers of *P. monodon* were identified by RAP-PCR. Twenty-one and fourteen RAP-PCR fragments specifically/differentially expressed in ovaries and testes of *P. monodon* were successfully cloned and sequenced.

7. Expression patterns of 25 transcripts were tested against the first strand cDNA of ovaries and testes of 3-month-old and broodstock-sized *P. Monodon*. Five (FI-4, FI-44, FIII-4, FIII-39 and FIII-58) and two (M457-A01 and MII-51) derived RAP-PCR markers revealed female- and male-specific expression patterns in *P. monodon*. In addition, MII-5 originally found in testes showed a higher expression level in ovaries than did testes of juvenile shrimps but a temporal female-specific pattern in *P. monodon* adults.

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APPENDICES

APPENDIX A

PCR-RFLP patterns of 16S $rDNA_{312}$ gene segments digested with *Alu* I, *Ssp* I, *Vsp* I of 5 penaeid shrimps; *P. monodon*, *P. semisulcatus*, *F. merguiensis*, *L. vannamei* and *M. japonicus* and their SSCP patterns

Species	Population	Specimens	PCR-RFLP pattern (*Alu* I, *Ssp* I, *Vsp* I)	SSCP pattern
P. monodon	Trat	1. Td1	ABA	11,16
		2. Td3	AAA	9,15
		3. Td4	AAA	9,15
		4. Td5	AAA	9,15
		5. Td6	ABA	11,16
		6. Td7	ABA	10,16
		7. Td8	AAA	9,15
		8. Td9	ABA	11,16
		9. Td11	ABA	11,16
		10. Td12	ABA	11,16
		11. Td13	-	9,10,15
		12. Td14	-	9,15
		13. Td15	ABA	11,16
		14. Td16	AAA	4,14
		15. Td17	-	9,16
		16. Td18	-	10,15

Species	Population	Specimens	PCR-RFLP pattern (*Alu* I, *Ssp* I, *Vsp* I)	SSCP pattern
		17. Td19	-	11,16
		18. Td20	AAA	10,15
		19. Td21	-	10,15
		20. Td22	-	9,15
		21. Td23	-	9,15
		22. Td24	ABB	13,17
		23. Td25	-	11,16
		24. Td26	-	9,15
		25. Td29	ABA	11,16
		26. Td30	-	9,15
		27. Td31	-	11,16
		28. Td32	-	10,15
		29. Td33	-	10,15
		30. Td34	-	9,15
		31. Td35	-	11,15
		32. Td37	-	9,15
	Chumphon	33. C1	AAA	10,14
		34. C2	-	11,16
		35. C3	-	10,15
		36. C4	AAA	9,15
		37. C5	-	10,15

Species	Population	Specimens	PCR-RFLP pattern (*Alu* I, *Ssp* I, *Vsp* I)	SSCP pattern
		38. C6	AAA	9,15
		39. C7	ABA	11,16
		40. C9	-	9,15
		41. C10	-	11,15
		42. C13	AAA	4,14
		43. C14	ABA	13,17
		44. C15	-	11,16
		45. C17	ABA	11,16
		46. C18	AAA	9,15
		47. C19	AAA	9,15
		48. C20	AAA	4,14
		49. C21	AAA	9,15
		50. C22	AAA	10,17
		51. C23	ABA	11,16
		52. C24	-	11,16
		53. C25	-	9,15
		54. C26	-	4,14
		55. C27	-	9,15
		56. C29	ABA	11,16
		57. C31	-	11,16
		58. C34	-	11,16

Species	Population	Specimens	PCR-RFLP pattern (*Alu* I, *Ssp* I, *Vsp* I)	SSCP pattern
		59. C35	-	9,15
		60. C36	-	11,16
		61. C37	-	10,12
		62. C40	ABA	11,16
		63. C41	-	11,15
		64. C43	-	9,15
		65. C44	-	9,15
		66. C48	-	9,15
		67. C49	-	9,15
		68. C51	-	9,15
		69. C55	-	10,16
	Satun	70. S1	AAA	9,15
		71. S2	AAA	9,15
		72. S3	AAA	9,15
		73. S4	-	10,16
		74. S5	AAA	9,15
		75. S6	AAA	9,15
		76. S7	-	11,16
		77. S8	-	11,16
		78. S10	-	11,15
		79. S11	ABA	11,16

Species	Population	Specimens	PCR-RFLP pattern (*Alu* I, *Ssp* I, *Vsp* I)	SSCP pattern
		80. S12	AAA	9,15
		81. S13	AAA	9,15
		82. S14	ABA	11,16
		83. S15	AAA	9,15
		84. S16	-	10,15
		85. S17	AAA	9,15
		86. S18	-	9,15
		87. S19	-	9,17
		88. S20	ABB	13,17
		89. S21	ABA	11,16
		90. S22	ABA	11,16
		91. S23	AAA	10,15
		92. S24	-	11,16
		93. S25	-	11,16
		94. S26	-	9,15
		95. S27	-	11,15
		96. S28	-	9,15
		97. S29	-	9,15
		98. S30	-	11,16
		99. S33	-	11,13
		100. S35	-	9,15

Species	Population	Specimens	PCR-RFLP pattern (*Alu* I, *Ssp* I, *Vsp* I)	SSCP pattern
		101. S36	-	11,15
		102. S38	-	10,16
		103. S39	-	11,16
		104. S43	-	11,13
		105. S46	-	10,16
		106. S47	-	10,15
		107. S49	-	9,15
		108. S50	-	11,15
	Trang	109. T1	-	9,15
		110. T2	-	9,15
		111. T3	-	9,15
		112. T4	-	10,15
		113. T8	AAA	9,15
		114. T9	AAA	10,15
		115. T10	AAA	9,15
		116. T11	AAA	9,15
		117. T12	AAA	10,15
		118. T13	-	10,15
		119. T14	-	9,15
		120. T15	ABA	11,16
		121. T16	-	11,16

Species	Population	Specimens	PCR-RFLP pattern (*Alu* I, *Ssp* I, *Vsp* I)	SSCP pattern
		122. T17	ABB	11,16
		123. T18	AAA	9,15
		124. T19	AAA	9,15
		125. T21	-	10,15
		126. T22	ABA	11,16
		127. T23	ABA	11,16
		128. T24	ABA	11,16
		129. T25	ABA	11,16
		130. T26	-	9,15
		131. T27	-	11,16
		132. T28	ABA	11,16
		133. T29	-	10,15
		134. T30	AAA	9,15
		135. T32	-	11,15
		136. T41	-	9,15
		137. T43	-	9,15
		138. T46	-	12,16
		139. T47	-	4,14
		140. T49	-	12,15
		141. T52	-	11,15

Species	Population	Specimens	PCR-RFLP pattern (*Alu* I, *Ssp* I, *Vsp* I)	SSCP pattern
	Phangnga	142. P1	-	11,15
		143. P2	AAA	10,15
		144. P3	AAA	9,15
		145. P4	ABA	11,16
		146. P5	-	9,15
		147. P6	ABA	11,16
		148. P7	AAA	9,15
		149. P8	-	4,14
		150. P10	AAA	9,15
		151. P11	ABA	11,16
		152. P12	-	11,16
		153. P13	ABA	10,16
		154. P14	AAA	9,15
		155. P15	-	10,15
		156. P16	-	9,15
		157. P17	ABA	11,16
		158. P18	-	9,15
		159. P19	AAA	10,15
		160. P21	ABA	11,16
		161. P23	AAA	9,15

Species	Population	Specimens	PCR-RFLP pattern (*Alu* I, *Ssp* I, *Vsp* I)	SSCP pattern
		162. P24	-	9,15
		163. P25	ABA	11,16
		164. P26	-	9,15
		165. P27	AAA	10,15
		166. P28	-	11,16
		167. P29	-	10,15
		168. P30	-	9,15
		169. P34	-	9,15
		170. P35	-	10,15
		171. P36	-	11,16
		172. P37	-	11,15
		173. P38	-	11,15
		174. P39	-	9,15
	Ranong	175. R1	AAA	9,14
		176. R2	ABB	11,16
		177. R3	ABA	11,16
		178. R4	ABA	13,17
		179. R5	ABA	11,16
		180. R6	ABA	11,16
		181. R7	AAA	9,15
		182. R8	ABA	10,15

Species	Population	Specimens	PCR-RFLP pattern (*Alu* I, *Ssp* I, *Vsp* I)	SSCP pattern
		183. R9	ABA	11,16
		184. R10	ABB	13,17
		185. R11	AAA	9,15
P. semisulcatus	Chumphon	186. Se.1	BBB	3,18
		187. Se.2	BBB	3,18
		188. Se.3	BBB	3,18
		189. Se.4	BBB	3,18
		190. Se.5	BBB	3,18
		191. Se.6	BBB	3,18
		192. Se.7	BBB	3,18
		193. Se.8	BBB	3,18
		194. Se.9	BBB	3,18
		195. Se.10	BBB	3,18
		196. Se.11	BBB	3,18
	Phuket	197. Se.P1	BBB	3,18
		198. Se.P2	BBB	3,18
		199. Se.P3	BBB	3,18
		200. Se.P4	BBB	3,18
L. vannamei	Mexico	201. V1	CBC	2,12
		202. V2	CBC	2,12
		203. V3	CBC	2,12

Species	Population	Specimens	PCR-RFLP pattern (*Alu* I, *Ssp* I, *Vsp* I)	SSCP pattern
		204. V4	CBC	2,12
		205. V5	CBC	2,12
		206. V6	CBC	2,12
	Ratchaburi	207. VR1	CBC	2,12
		208. VR2	CBC	2,12
		209. VR3	CBC	2,12
		210. VR4	CBC	2,12
		211. VR5	CBC	2,12
		212. VR6	BBC	2,12
		213. VR7	CBC	2,12
		214. VR8	CBC	2,12
		215. VR9	CBC	2,12
		216. VR10	CBC	2,12
		217. VR11	CBC	2,12
		218. VR12	CBC	2,12
	Rangsit	219. VF1	CBC	1,12
		220. VF3	CBC	2,12
		221. VF4	CBC	2,12
		222. VF5	CBC	2,12
		223. VF6	CBC	2,12
		224. VF7	CBC	2,12

Species	Population	Specimens	PCR-RFLP pattern (*Alu* I, *Ssp* I, *Vsp* I)	SSCP pattern
		225. VF8	CBC	2,12
		226. VF9	CBC	2,12
		227. VF11	CBC	2,12
		228. VF12	CBC	2,12
		229. VF13	CBC	2,12
		230. VF14	CBC	2,12
F. merguiensis	Market	231. M1	BAB	5,8
		232. M2	BAB	5,8
		233. M3	BAB	5,11
		234. M4	BAB	5,8
		235. M5	BAB	5,11
		236. M6	BAB	4,6
		237. M7	BAB	5,8
	Chonburi	238. BP1	BAB	5,8
		239. BP2	BAB	5,9
		240. BP3	BAB	5,8
		241. BP4	BAB	5,8
		242. BP5	BAB	5,8
		243. BP6	BAB	6,8
		244. BP7	BAB	5,8
		245. BP8	BAB	5,8

Species	Population	Specimens	PCR-RFLP pattern (*Alu* I, *Ssp* I, *Vsp* I)	SSCP pattern
		246. BP9	BAB	5,8
		247. BP10	BAB	5,8
		248. BP11	BAB	5,8
		249. BP12	BAB	5,8
		250. BP13	BAB	5,8
		251. BP14	BAB	5,8
		252. BP15	BAB	4,6
		253. BP16	BAB	5,8
		254. BP17	BAB	5,9
	Indonesia	255. BP18	BAB	4,6
		256. BP19	BAB	4,6
		257. BP20	BAB	4,6
		258. BP21	BAB	4,6
		259. BP22	BAB	4,6
		260. BP23	BAB	4,6
		261. BP24	BAB	4,6
		262. BP25	BAB	4,6
		263. BP26	BAB	4,6
		264. BP27	BAB	4,6
		265. BP28	BAB	4,6
		266. BP29	BAB	4,6

Species	Population	Specimens	PCR-RFLP pattern (*Alu* I, *Ssp* I, *Vsp* I)	SSCP pattern
		267. BP30	BAB	4,6
		268. BP31	BAB	4,6
M. japonicus	Australia	269. J1	BBB	7,17
		270. J2	BBB	7,17
		271. J3	BBB	7,17
		272. J4	BBB	7,17
	Japan	273. MK1	BBB	7,17
		274. MK2	BBB	7,17
		275. MK3	BBB	7,17
		276. MK4	BBB	7,17
		277. MK5	BBB	7,17
		278. MK6	BBB	7,17
		279. FK1	BBB	7,17
		280. FK2	BBB	7,17
		281. FK3	BBB	7,17
		282. FK4	BBB	7,17
		283. FK5	BBB	7,17
		284. FK6	BBB	7,17

APPENDIX B

Genetic distances of 5 penaeid shrimps (*P. monodon, P. semisulcatus, L. vannamei, F. merguiensis and M. japonicus*) based on SSCP analysis of 16S rDNA$_{312}$

NEI'S (1972) IDENTITIES/DISTANCES
Nei's original (1972) identity

Population	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	*****														
2	0.9995	*****													
3	0.9997	0.9994	*****												
4	0.9993	0.9986	0.9992	*****											
5	0.9994	0.9986	0.9993	0.9999	*****										
6	0.9939	0.9952	0.9941	0.9901	0.9901	*****									
7	0.8693	0.8723	0.8691	0.8674	0.8668	0.8718	*****								
8	0.8693	0.8723	0.8691	0.8674	0.8668	0.8718	1.0000	*****							
9	0.8693	0.8739	0.8691	0.8709	0.8668	0.8718	0.7778	0.7778	*****						
10	0.8990	0.9037	0.8988	0.9006	0.8964	0.9015	0.8053	0.8053	0.9940	*****					
11	0.8693	0.8739	0.8691	0.8709	0.8668	0.8718	0.7778	0.7778	1.0000	0.9940	*****				
12	0.9591	0.9625	0.9594	0.9568	0.9565	0.9627	0.8576	0.8576	0.8576	0.8869	0.8576	*****			
13	0.9418	0.9449	0.9416	0.9397	0.9391	0.9437	0.8433	0.8433	0.8433	0.8724	0.8433	0.9948	*****		
14	0.8712	0.8755	0.8722	0.8674	0.8668	0.8827	0.7778	0.7778	0.7778	0.8053	0.7778	0.8576	0.8433	*****	
15	0.8712	0.8755	0.8722	0.8674	0.8668	0.8827	0.7778	0.7778	0.7778	0.8053	0.7778	0.8576	0.8433	1.0000	*****

Nei's original (1972) distance

Population	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	*****														
2	0.0005	*****													
3	0.0003	0.0006	*****												
4	0.0007	0.0014	0.0008	*****											
5	0.0006	0.0014	0.0007	0.0001	*****										
6	0.0062	0.0048	0.0059	0.0100	0.0099	*****									
7	0.1400	0.1366	0.1402	0.1423	0.1429	0.1372	*****								
8	0.1400	0.1366	0.1402	0.1423	0.1429	0.1372	0.0000	*****							
9	0.1400	0.1348	0.1402	0.1382	0.1429	0.1372	0.2513	0.2513	*****						
10	0.1064	0.1013	0.1067	0.1047	0.1093	0.1037	0.2166	0.2166	0.0060	*****					
11	0.1400	0.1348	0.1402	0.1382	0.1429	0.1372	0.2513	0.2513	0.0000	0.0060	*****				
12	0.0418	0.0382	0.0414	0.0442	0.0444	0.0381	0.1536	0.1536	0.1536	0.1200	0.1536	*****			
13	0.0599	0.0566	0.0602	0.0622	0.0628	0.0579	0.1704	0.1704	0.1704	0.1366	0.1704	0.0052	*****		
14	0.1379	0.1329	0.1368	0.1423	0.1429	0.1248	0.2513	0.2513	0.2513	0.2166	0.2513	0.1536	0.1704	*****	
15	0.1379	0.1329	0.1368	0.1423	0.1429	0.1248	0.2513	0.2513	0.2513	0.2166	0.2513	0.1536	0.1704	0.0000	*****

Populations 1 – 6 = *P. monodon* from Trat, Chumphon, Satun, Trang, Phangnga and Ranong, respectively,
Populations 7 – 8 = *P. semisulcatus* from Chumphon and Phuket, respectively,
Populations 9 – 11 = *L. vannamei* from Mexico, Rangsit and Ratchaburi, respectively,
Populations 12 – 13 = *F. merguiensis* from Market and Chonburi, respectively,
Populations 14 – 15 = *M. japonicus* from Australia and Japan, respectively

APPENDIX C

Nucleotide sequences of 14 cloned AFLP fragments of *P. monodon* and positions of the forward primer and those complementary to the reverse primer (underlined and boldfaced)

> E4M6-295

GATGAGTCCTGAGTAACTCTAGTGAATCATTTCCTGAAACT**TCCCCTCCTATGCTAACGCTAC**TTACAC
CACTTTGTCGCACCAACTGTTGTAATTTTGCTATTCTAAGATGTTGCAATCGTCCAAGTTCTTCCGTCA
TGCGTTTCTTTTCTCTCATAAGCTCATGGATTTCTTGGTCAA**CATTTTCCAAGTGCCGTAAGAA**TTCTG
TTTCTTGCTCACTGAACACCATCATTTGTTGTAGTGCTACTCCTGCATTGTGGCTGAACTCAAATTCAC
TGAGTGAATTGGTACGCAGTCA

> E7M10-450

GATGAGTCCTGAGTAACGTGTGAATGGGTATGCAAGAAATTCATTCATAATGGACAAGGTTCTCGAAGA
AACGCGCGGC**TGTTACAATGCTGTCGTGGA**AAGGTACTTTTCAAACTTTACTCTACACTCACCATTCAT
GGAGATGCTATTGCTTACAGCGTCAGGTAAGTGTACGCTAAATGTAATGAGGCAAAGAGTAATTCTTTG
TTTTGCTATCGCGCACACACACACTTACAAAAAAAGTGATGTATTATTATGATGCATCCTTTGCATCTT
AGACTTCCATACCTTTTTCAAAAATTCCTTTCCAAACATTTCTTTAAATTTCCATTTTCAGGCGATATC
GCCTTCGGAGCTGAATTCT**GAGATAAGATGAACGCAACCA**TGGTTGACTTCAAAAAGTTTCCTGATTTC
CCCTGGATATTCTACGGCGTTGCTGAATTGGTACGCAGTC

> P6M2-370

GACTGCGTACATGCAGACTCCCCAGCTCGAGCGGACGATTCGGAACGGTTTCGAAG**CGGTGAAAGTAAG
TCAAATGTC**TTTATTTCCATATCGAGACCATTTCTGTTTTCCATTCTTTGATATTTTCTTTTAAGTCGT
TAGTGTAGCGTATGCGTTCTCTGATATATAAATATTTGTATTCTTAAATGTATTTGCGAATATACTACG
GTTAAACACCGGTCTATCTTCT**TTCCCTCCACTCTGCCTGA**AATGTTATTCACTTAGGAATATTTTTTA
AATAATTTCATTACAGCGTTAACGCTTTCTTGATACATATAAAATTCATACAAAAATAAGATGCTAATA
TAGTGTTACTCAGGACTCATCA

> P6M6-470

GATGAGTCCTGAGTAACTCTACCAGTCATAAATAGTGATTCTCCATCTGCCTCGAGAATGTTGAAGTGA
AAATGCTTTTACATTCTGTACAACTCAGGATTTCATGGTCAAGTTACATTCTGGCTGCCACGAGGCACG
AAGTAGCAAAATG**CTCAGACTCCCGCCTAATCC**ATGAATGTATTACTGTCTTCCACATCCGCAAAGAAT
TGGTCAACAGAGACGCTAGAGCCCGAGAACTTTGACGCTACATTGTACATCGTGGGAACCATGCAGGCA
CCCATGCTTGATGCAGCCGTAACAAGGGTGAGGGCAGAGGAGGGGAGTGGAGTAGGAGAGAGAATGGTT
TCGGTTTGTGTGGCGAGAAAAAGATGGAGACTGATTCAGAAATAGATTGTGAAGAAGATGAGTGAGAAA
TAAGAGAAGTCAGTCC**TTAGGGGAGAAAGTTAGCGAA**GTCTGCATGTACGCAGTCA

> E8M7-323

GATGAGTCCTGAGTAACTGACGAAACGCTGG**AGGTCCGCCCACAATCATAC**AGCTCCACCCACTGTGGA
CCGTGTATATAAAGAGCCATCGCCTTTGGGATGAGCACACTGTACTGCACAGCCATCAGAATGCAACGT
CTTCTTGTTGTGAGCGTTTTGACCTCTTGTTGGAGGACAAATTTATGGTCATGTCTGGAAATAGAGTGT
ATTGTGTCGAAACGGAAACTTGTTCAGAAAAAAGACGTTTTTATTAGCTGTCCTCCATA**GGGAAGGAAT
GTATAAAAAGTCT**ATATTTGTGATTCTGATACCTGAATTGGTACGCAGTCA

> E6M9-318

GATGAGTCCTGAGTAACGACTAAGAAGACAATGTCCATACTATTTTATCTTCAAAAACAGAGCTAAAAA
CATTTTATGACGACAAAGAAACAGCTTAAAGAAACAGCTTATGGTACATTACTTTTGAGAAGAATGACG
GGATTTCTT**TTGTCTCGTTTTATTTCTTG**CTCCGCTGATGCGATTTTTTTTCTTTCAGATGTTTATAAA

GACACAAGATTCAACTCCTTTAGCCAGGTAGTTTTTTTTTTAAGAATTGAGAATTAGAAAAAAATATT**GAGATAGATGGAAATACGGT**GCGTGAATTGGTACGCAGTC

> P2M5-295

GACTGCGTACATGCAGAACAGTACAGCATATACATAAGCACGATTTCTG**TAAGCCTTTTTACCAATAGACC**ACATGGCTCTAAGTCAAGAGCTTCTTTCCTTTCCTTCTTCTCCCATAGAAGCCGTATGTGCAGTCATTCTCACACTGGTTTTGTTGGGTAAAAGTGCTAAGTAACAGCAAATGGCA**CGTTCTCTTGCTATTCTTCTGA**CTTAGATCGTATTACTGTGTGATCACAGTATGTGGTCAACAAACATGAATCTTGTTCATTAGCTAAGTAGTTACTCAGGACTCATCA

> P2M6-270

GACTGCGTACATGCAGA**ACTGGTCACCTTAGGATGCT**GTGGGAAGGACATCATCGGAAGATCGCCATAGGCCTGTGGACCAGGATGTTTGTCAGAGCAGGTGTTAAGCTGTCCTATGATGTAGTGGACGGGCGCTGCCTGTCGAGACGCCCGTTG**ATCCAGCAAAGAACCAGG**AACTCGCCAGCGCAGGTACCAGTCACCAAGAAGGACCTGGACGGGATCTGTAAGGACGTGTGGACGAGGACGCCGCCGAGTTACTCAGGACTCATCA

> P2M7-285

GACTGCGTACAT**GCAGAACCGATACCCAAGGC**ATTCGTTATTACATCAGAGTTACGCTACGAAGTTTGTATTTTCAAACCGGTTTCGCGGTGTTATGATAACGTCAAACTTCATAATAACAGTAAACACTTCTTGGGAAAAATAGACTTCTTTTTCATACGTTTCGTAAATCTAATCCAGTCTTGACAACAATGCCCCAAACTACT**TATTTACATTTCAGCCACGGACC**GAGATATGAATCGCAACCTTGAGGGATTCTAAATCTACCGTTCAGTTACTCAGGACTCATCA

> P2M7-310

GATGAGTCCTGAGTAACTGTCTTGACTGTGTATTTCGGATCGCAGCGCAACACGGAGTTGGGGCGACGCACGAACTTTAAAGCTCCCCCCCACCAGTCTGAAAGTG**CTTTCATCCCTCAACATCACT**TTCTTTCAATCGGACAGTATCTATCGTCGATACTTCTTGCAAAATTTATTTTCTCTTTCATAGCTTTAGTGAGCAGAGGCTTCACAGTTACGTGCCGAGTCGGTAAACCGAGGCCTTTCTGAAGGCGCTGCTGGATGGTCCTTATAGACACGTCCA**TGAGGAGTTCTGGAGGCTTG**TTCTGCATGTACGCAGTCA

> P2M6-850

GATGAGTCCTGAGTAACTCTGGGGCTGCGGTTGCCTTCCACCCTCCCCCTCTGCCACGCCGCGGTCGTGTGAATAATGTGCGAAGCAAGGAGGGGGGATGTCAACTCACGGTCGTCAGCTGTGGATCTGCGCCCTCGTGCCGATTCCCAGATCAGTTATAGGTTTGTAACGTACAGGTATACGGGCACCTGATTAGGTAGCTTGGTCAATGATATAAGCAGCTGAGGATGAAGTCA**ACCGCTATGGAGTTATGTTCTGC**CTCCTGTATTTTTTTGGTGAATTTTGTTACATACAGATGACTCCACATATGCTCAGCCAGCAAGGAGTCTATCAGGAGACCCTACTTACCGATCCTGATTTCCCCATTTCCTTGAATTGGCGGGAAAATGTGTTTTTCCTTTTAATACAATTAATATCGATACGGTTATTATTGTTATTGATGTTATGATTATTAAATTGTTATTAAAATCTTGATAACATGTAAGACAATGAAATGATTACAAAAGACCCTCTCCAAAAGTTCAGGAAA**AGGGTAAGCAGGTGAGATAGATAGGA**CTGATGACTGACTGACTTGGTGACAAAGCACTTGCACAGCCATCTATGTGCAAATGCAATAAATAAACTTGCGTTACAGTAGGCATGGCATGTATTCCTGCCATACGGGGCGATTGGGTTAATTTCAATTTCTAACAAGTTGGTTATAAGGCTGGATCAGGGACTCTTGGTTGGCGCTGTTACCGACGAGACAATAACAATGGCAACATGGAAGGTAATTATCGTTGAATATCATTCGAAGAAATGTTATCCTTAATCTTATTCTTTGAAAAGTATATATCAATTGCCCTCAACTAGCTCAATCAGTCTGCATGTACGCAGTCA

> P2M6-465

GACTGCGTACATGCAGAACTAC**TTACGAGCAGGGATTCAGGTTC**TGGATTAACATGACAGCTATCCCATTAGAGAGCTTGTCTGTGAAGCTCATGGCCCCATAGACAAAAGCTCCTGACTCAATATTTGGGCCAATGAGGTCTGCCGTGATGGAAAGGGATGTGATCAGCATGATAGAGCCACCTGCCCCTAACATGGCTGTCAGCACATACAGGATGTACTTGCGGTAGATCTCACCCT**CTCCAAGCCAGATGCCAATG**CAGGCTGCTCCACCTATGAGGGTGCCCAGGATGAAGGACGCCTTGGGAGATGATGAAAGTACATGAACTGATTTCAGAGGGGGCATTTATGTCACAAAGAAAATGATCACCTCCAGTATGAAAATCTTCAGGGGAAAAGAATACATTTCCAAGTCATATAAAAAGCACAAAAGCTAAAATAGAAACCATTAGAGTTACTCAGGACTCATCA

> P2M8-300

GACTGCGTACATGCAGA**ACACTTCCAAGCATTTACCTCGC**AAATGTTTGCTGTCTCACTAACAGAGTAC
ACGAATTCGACGTCACAATAAATCAAAATAATGTCGGCATTGCAGTAATCACAAAAACATGGTTCAAAG
ACGGTACACTCAACTTCGGAACCATACAGGGGTACCATTCATACAACAAAAGTCGTGAGGG**CAGAATAG**
ATGGGGCGTTGGTATTTATATCAAAAACACAATCGCCAAACAAAATGTTAATCAACACCAAGAAGTGCG
CAGAAATAAGTTACTCAGGACTCATCA

> P2M6-385

GACTGCGTACATGCAGAA**CAGAATCTTACTCTCAGCCACATACAC**GGAATTCTCCTTTCAAACGACATG
CGAATCAGATGCGGAATCTTCCAGGGCGACTCTTTTTTCCCTCTTTTATTTGCATGGCACTTATCCCAC
TCTCCCAACTTCTTAACAACACAGGGTATAGATATAAGATCATGGACAAGAAGATCAATCATCTTTCCT
ATATGGATGACTTGAAACTTTACACTCAGAATGATGGTG**TACTGGAAGGGTTGCTGAAAGC**CGTAAAAA
ATTTTAGTGATGACATAGGTATGAAATTTGGTCTCGATAAATGTGCTAAAGGAACCTTTAAGCAAGGAA
AACTAGTTGCATCTGACAATATGGAGTTACTCAGGACTCATCA

APPENDIX D

Polymorphic sites of the COI_{614} gene segment of *P. monodon*

	Nucleotide position																							
	2	3	4	5	6	7	9	11	13	15	17	18	20	21	24	25	26	27	33	36	54	57	64	66
P28	C	-	T	T	-	G	-	T	-	T	C	A	A	C	T	A	T	T	A	A	G	C	C	T
S7	A	-	T	T	-	G	-	T	-	T	C	A	A	C	T	A	T	T	A	A	G	C	C	T
P29	C	-	T	T	-	G	-	T	-	T	C	-	A	C	T	A	T	T	A	A	G	C	C	T
S11	C	-	T	T	-	G	-	T	-	T	C	A	A	C	T	A	T	T	A	A	G	C	C	T
S25	C	-	T	T	-	G	-	A	-	T	C	-	A	C	T	A	T	T	A	A	G	C	C	T
Td18	C	-	T	T	-	G	-	T	-	T	C	-	A	T	T	A	T	T	A	A	G	C	C	T
P21	C	A	T	T	T	G	-	T	A	T	C	A	A	C	T	A	T	T	A	A	G	C	C	T
T16	C	A	T	T	T	G	-	T	A	T	C	A	A	C	T	A	T	T	-	A	G	C	C	T
P17	C	-	T	T	T	G	-	T	A	T	C	A	A	C	T	A	T	T	A	A	G	C	C	T
C10	C	-	T	T	T	G	-	A	A	T	C	A	A	C	T	A	T	T	-	A	G	C	C	T
C9	C	-	T	T	-	G	-	T	-	T	C	A	A	C	T	A	T	T	A	A	G	C	C	T
C41	C	A	T	T	-	G	-	T	-	T	C	A	A	C	T	A	T	T	A	A	G	C	C	T
S33	C	-	T	T	T	G	-	T	-	T	C	A	A	C	T	A	T	T	A	A	G	C	C	T
S10	C	A	T	T	T	G	-	T	A	T	C	A	A	C	T	A	T	T	A	A	G	C	C	T
C6	C	C	T	T	T	G	G	T	-	C	C	A	A	T	T	A	T	T	A	G	T	T	T	T
Td17	C	C	T	T	T	G	G	T	-	C	C	A	A	T	T	A	T	T	A	A	T	T	T	T
T43	C	T	T	T	T	G	G	T	-	C	C	A	A	T	T	A	T	T	A	G	T	T	T	T
S1	C	-	T	T	T	G	G	T	-	C	C	A	A	T	T	A	T	T	A	G	T	T	T	T
C4	C	-	T	T	T	G	G	T	-	C	C	-	A	T	T	A	T	T	A	G	T	T	T	T
C51	C	-	T	T	T	G	G	T	-	C	C	-	A	T	T	A	T	T	-	G	T	T	T	T
P2	C	-	C	T	T	G	G	T	-	C	C	A	A	T	T	A	T	T	A	G	T	T	T	T
S18	C	-	T	T	T	G	G	T	-	C	C	A	A	T	T	A	T	T	A	A	T	T	T	T
T11	C	C	T	T	T	G	G	T	-	C	C	-	A	T	T	A	T	T	A	A	T	T	T	T
C1	C	C	T	T	T	G	G	T	-	C	C	A	A	T	T	A	T	T	A	A	T	T	T	T
T30	C	-	T	T	-	G	G	T	-	C	C	A	A	T	T	A	T	T	A	G	T	T	T	T
P7	C	-	T	T	-	G	G	T	-	C	C	-	A	T	T	A	T	T	-	G	T	T	T	T
C21	C	-	T	T	-	G	G	T	-	C	C	-	A	T	-	-	A	T	-	G	T	T	T	T

C18	C	-	T	T	-	C	G	-	-	C	C	-	A	T	T	A	T	T	-	A	T	T	T	T
T18	C	C	T	T	T	G	G	T	-	C	C	A	A	T	T	A	T	T	A	A	T	C	T	T
P39	C	C	T	C	-	G	G	T	-	C	C	A	A	T	T	A	T	T	A	A	T	T	T	T
P24	C	-	T	T	-	G	-	T	-	C	C	A	A	T	T	A	T	T	A	A	T	T	T	T
C13	C	-	T	T	-	G	-	T	-	C	C	A	A	T	T	A	T	T	A	A	T	T	T	T
C26	C	-	T	T	-	G	-	T	-	C	C	A	A	T	T	A	T	T	A	A	T	T	T	T
P8	C	C	T	T	T	G	-	T	-	C	C	A	A	T	T	A	T	T	A	A	T	T	T	T
P10	C	C	T	T	T	G	G	T	-	C	C	-	A	T	T	A	T	T	A	G	T	T	T	T
S48	C	-	T	T	T	G	-	T	-	-	T	C	A	T	T	A	T	T	A	A	A	C	C	C
S43	C	-	T	T	T	G	-	T	-	-	T	C	A	T	T	A	T	T	A	A	A	C	C	C
S30	C	-	T	T	T	G	-	T	-	-	T	C	A	T	T	A	T	-	-	A	A	C	C	C
S38	C	-	T	T	T	G	-	T	-	-	T	C	A	T	T	A	T	T	A	A	A	C	C	C
Td20	C	-	T	T	T	G	-	T	-	-	T	C	C	T	T	A	T	T	A	A	A	C	C	C
Td24	C	T	T	T	T	G	T	T	-	T	C	C	A	T	T	A	T	T	A	A	A	C	C	C
C20	C	T	T	T	T	G	T	T	-	T	C	C	A	T	T	A	T	T	A	A	A	C	C	C
T17	C	A	T	T	T	G	-	T	A	T	C	-	A	C	T	A	T	T	A	A	G	C	C	T

	69	75	85	86	87	90	96	98	99	100	102	107	108	109	111	112	113	115	119	121	123	124	125	126
P28	A	T	T	T	A	T	T	T	-	T	T	T	T	T	T	G	G	A	G	T	A	T	C	A
S7	A	T	T	T	A	T	T	T	-	T	T	T	T	T	T	G	G	A	G	T	A	T	C	A
P29	A	T	T	T	A	T	T	T	-	T	T	T	T	T	T	G	G	A	G	T	A	T	C	A
S11	A	T	T	T	A	T	T	T	-	T	T	T	T	T	T	G	G	A	G	T	A	T	C	A
S25	A	T	T	T	A	T	T	T	-	T	T	T	T	T	T	G	G	A	G	T	A	T	C	A
Td18	A	T	T	T	A	T	T	T	-	T	T	T	T	T	T	G	G	A	G	T	A	T	C	A
P21	A	T	T	T	A	T	T	T	-	T	T	T	T	T	T	G	G	A	G	T	A	T	C	A
T16	A	T	T	T	A	T	T	T	-	T	T	T	T	T	T	G	G	A	G	T	A	T	C	A
P17	A	T	T	T	A	T	T	T	-	T	T	T	T	T	T	G	G	A	G	T	A	T	C	A
C10	A	T	T	T	A	T	T	T	-	T	T	T	T	T	T	G	G	A	G	T	A	T	C	A
C9	A	T	T	T	A	T	T	T	-	T	T	T	T	T	T	G	G	A	G	T	A	T	C	A
C41	A	T	T	T	A	T	T	T	-	T	T	T	T	T	T	G	G	A	G	T	A	T	C	A

S33	A	T	T	T	A	T	T	T	-	T	T	T	T	T	T	G	G	A	G	T	A	T	C	A
S10	A	T	T	T	A	T	T	T	-	T	T	T	T	T	T	G	G	A	G	T	A	T	C	A
C6	A	C	C	T	A	C	T	T	-	T	T	T	T	T	T	A	G	A	G	T	A	T	C	A
Td17	A	C	C	T	A	C	T	T	-	T	T	T	T	T	T	A	G	A	G	T	A	T	C	A
T43	A	C	C	T	A	C	T	T	-	T	T	T	T	T	T	A	G	A	G	T	A	T	C	A
S1	A	C	C	T	A	C	T	T	-	T	T	T	T	T	T	A	G	A	G	T	A	T	C	A
C4	A	C	C	T	A	C	T	T	-	T	T	T	T	T	T	A	G	A	G	T	A	T	C	A
C51	A	C	C	T	A	C	T	T	-	T	T	T	T	T	T	A	G	A	G	T	A	T	C	A
P2	A	C	C	T	A	C	T	T	-	T	T	T	T	T	T	A	G	A	G	T	A	T	C	A
S18	A	C	C	T	A	C	T	T	-	T	T	T	T	T	T	A	G	A	G	T	A	T	C	A
T11	A	C	C	T	A	C	T	T	-	T	T	T	T	T	T	A	G	A	G	T	A	T	C	A
C1	A	C	C	T	A	C	T	T	-	T	T	T	T	T	T	A	G	A	G	T	A	T	C	A
T30	A	C	C	T	A	C	T	T	-	T	T	T	T	T	T	A	G	A	G	T	A	T	C	A
P7	A	C	C	T	A	C	T	T	-	T	T	T	T	T	T	A	G	A	G	T	A	T	C	A
C21	A	C	C	T	A	C	T	T	-	T	T	T	T	T	T	A	G	A	G	T	A	T	C	A
C18	A	C	C	T	A	C	T	T	-	T	T	T	T	T	T	A	G	A	G	T	A	T	C	A
T18	A	C	C	T	A	C	T	T	-	T	T	T	T	T	T	A	G	A	G	T	A	T	C	A
P39	A	C	C	T	A	C	T	T	-	T	T	T	T	T	T	A	G	A	G	T	A	T	C	A
P24	A	C	C	T	A	C	T	T	-	T	T	T	T	T	T	A	G	A	G	T	A	T	C	A
C13	T	T	C	T	G	T	T	T	-	T	T	T	T	T	T	A	G	A	G	T	A	T	C	A
C26	T	T	C	T	G	T	T	T	-	T	T	T	T	T	T	A	G	A	G	T	A	T	C	A
P8	T	T	C	T	G	T	T	T	-	T	T	T	T	T	T	A	G	A	G	T	A	T	C	A
P10	A	C	C	T	A	C	T	T	-	T	T	T	T	T	T	A	G	A	G	T	A	T	C	A
S48	G	C	C	T	T	T	T	T	-	C	T	T	T	C	T	A	G	A	G	A	A	C	C	A
S43	G	C	C	T	T	T	T	T	-	C	T	T	T	C	T	A	G	A	G	A	A	C	C	A
S30	G	C	C	T	T	T	T	T	-	C	T	T	T	C	T	A	G	A	G	A	A	C	C	A
S38	G	C	C	T	T	T	T	T	-	C	T	T	T	C	T	A	G	A	G	A	A	C	C	A
Td20	G	C	C	T	T	T	T	T	-	C	T	T	T	C	T	A	C	A	G	A	A	C	C	A
Td24	G	C	C	T	T	T	T	T	-	C	T	T	T	C	T	A	G	A	G	A	A	C	C	A
C20	G	C	C	T	T	T	T	T	-	C	T	T	T	C	T	A	G	A	G	A	A	C	C	A
T17	A	T	T	A	A	T	A	C	A	T	C	C	G	C	G	G	G	G	A	A	T	T	A	T

	130	132	134	135	136	137	140	142	143	145	146	147	148	149	151	152	153	155	160	161	162	163	164	166
P28	A	T	A	C	-	T	A	G	G	T	A	G	A	T	T	T	G	T	G	T	G	C	T	A
S7	A	T	A	C	-	T	A	G	G	T	A	G	A	T	T	T	G	T	G	T	G	C	T	A
P29	A	T	A	C	-	T	A	G	G	T	A	G	A	T	T	T	G	T	G	T	G	C	T	A
S11	A	T	A	C	-	T	A	G	G	T	A	G	A	T	T	T	G	T	G	T	G	C	T	A
S25	A	T	A	C	-	T	A	G	G	T	A	G	A	T	T	T	G	T	G	T	G	C	T	A
Td18	A	T	A	C	-	T	A	G	G	T	A	G	A	T	T	T	G	T	G	T	G	C	T	A
P21	A	T	A	C	-	T	A	G	G	T	A	G	A	T	T	T	G	T	G	T	G	C	T	A
T16	A	T	A	C	-	T	A	G	G	T	A	G	A	T	T	T	G	T	G	T	G	C	T	A
P17	A	T	A	C	-	T	A	G	G	T	A	G	A	T	T	T	G	T	G	T	G	C	T	A
C10	A	T	A	C	-	T	A	G	G	T	A	G	A	T	T	T	G	T	G	T	G	C	T	A
C9	A	T	A	C	-	T	A	G	G	T	A	G	A	T	T	T	G	T	G	T	G	C	T	A
C41	A	T	A	C	-	T	A	G	G	T	A	G	A	T	T	T	G	T	G	T	G	C	T	A
S33	A	T	A	C	-	T	A	G	G	T	A	G	A	T	T	T	G	T	G	T	G	C	T	A
S10	A	T	A	C	-	T	A	G	G	T	A	G	A	T	T	T	G	T	G	T	G	C	T	A
C6	A	T	A	C	-	T	A	G	T	T	A	G	A	C	T	T	G	T	G	T	G	C	T	A
Td17	A	T	A	C	-	T	A	G	T	T	A	G	A	C	T	T	G	T	G	T	G	C	T	A
T43	A	T	A	C	-	T	A	G	T	T	A	G	A	C	T	T	G	T	G	T	G	C	T	A
S1	A	T	A	C	-	T	A	G	T	T	A	G	A	C	T	T	G	T	G	T	G	C	T	A
C4	A	T	A	C	-	T	A	G	T	T	A	G	A	C	T	T	G	T	G	T	G	C	T	A
C51	A	T	A	C	-	T	A	G	T	T	A	G	A	C	T	T	G	T	G	T	G	C	T	A
P2	A	T	A	C	-	T	A	G	T	T	A	G	A	C	T	T	G	T	G	T	G	C	T	A
S18	A	T	A	C	-	T	A	G	T	T	A	G	A	C	T	T	G	T	G	T	G	C	T	A
T11	A	T	A	C	-	T	A	G	T	T	A	G	A	C	T	T	G	T	G	T	G	C	T	A
C1	A	T	A	C	-	T	A	G	T	T	A	G	A	C	T	T	G	C	G	T	G	C	T	A
T30	A	T	A	C	-	T	A	G	T	T	A	G	A	C	T	T	G	T	G	T	G	C	T	A
P7	A	T	A	C	-	T	A	G	T	T	A	G	A	C	T	T	G	T	G	T	G	C	T	A
C21	A	T	A	C	-	T	A	G	T	T	A	G	A	C	T	T	G	T	G	T	G	C	T	A
C18	A	T	A	C	-	T	A	G	T	T	A	G	A	C	T	T	G	T	G	T	G	C	T	A
T18	A	T	A	C	-	T	A	G	T	T	A	G	A	C	T	T	G	T	G	T	G	C	T	A
P39	A	T	A	C	-	T	A	G	T	T	A	G	A	C	T	T	G	T	G	T	G	C	T	A
P24	A	T	A	C	-	T	A	G	T	T	A	G	A	C	T	T	G	T	G	T	G	C	T	A
C13	A	T	A	C	-	T	A	G	T	T	A	G	A	C	T	C	G	T	G	T	G	C	T	A

C26	A	T	A	C	-	T	A	G	T	T	A	G	A	C	T	C	G	T	G	T	G	C	T	A
P8	A	T	A	C	-	T	A	G	T	T	A	G	A	C	T	C	G	T	G	T	G	C	T	A
P10	A	T	A	C	-	T	A	G	T	T	A	G	A	C	T	T	G	T	G	T	G	C	T	A
S48	A	T	A	C	-	A	A	G	T	T	G	G	A	T	T	T	G	T	G	T	G	C	T	A
S43	A	T	A	C	-	A	A	G	T	T	G	G	A	T	T	T	G	T	G	T	G	C	T	A
S30	A	T	A	C	-	A	A	G	T	T	G	G	A	T	T	T	G	T	G	T	G	C	T	A
S38	A	T	A	C	-	A	A	G	T	T	G	G	A	T	T	T	G	T	G	T	G	C	T	A
Td20	A	T	A	C	-	A	A	G	T	T	G	G	A	T	T	T	G	T	G	T	G	C	T	A
Td24	A	T	A	C	-	A	A	G	T	T	G	G	A	T	T	T	G	T	G	T	G	C	T	A
C20	A	T	A	C	-	A	A	G	T	T	G	G	A	T	T	T	G	T	G	T	G	C	T	A
T17	T	C	C	T	G	T	G	T	A	G	A	A	T	T	G	G	T	T	T	C	A	T	C	-

	167	169	171	172	178	179	180	181	182	185	188	190	193	194	196	197	200	203	208	209	212	214	215	217
P28	C	T	A	C	C	T	A	C	A	A	T	T	C	T	T	A	A	A	T	T	A	T	C	T
S7	C	T	A	C	C	T	A	C	A	A	T	T	C	T	T	A	A	A	T	T	A	T	C	T
P29	C	T	A	C	C	T	A	C	A	A	T	T	C	T	T	A	G	A	T	T	A	T	C	T
S11	C	T	A	C	C	T	A	C	A	A	T	T	C	T	T	A	A	A	T	T	A	T	C	T
S25	C	T	A	C	C	T	A	C	A	A	T	T	C	T	T	A	A	A	T	T	A	T	C	T
Td18	C	T	A	C	C	T	A	C	A	A	T	T	C	T	T	A	A	A	T	T	A	T	C	T
P21	C	T	A	C	C	T	A	C	A	A	T	T	C	T	T	A	A	A	T	T	A	T	C	T
T16	C	T	A	C	C	T	A	C	A	A	T	T	C	T	T	A	A	A	T	T	A	T	C	T
P17	C	T	A	C	C	T	A	C	A	A	T	T	C	T	T	A	A	A	T	T	A	T	C	T
C10	C	T	A	C	C	T	A	C	A	A	T	T	C	T	T	A	A	A	T	T	A	T	C	T
C9	C	T	A	C	C	T	A	C	A	A	T	T	C	T	T	A	A	A	T	T	A	T	C	T
C41	C	T	A	C	C	T	A	C	A	A	T	T	C	T	T	A	A	A	T	T	A	T	C	T
S33	C	T	A	C	C	T	A	C	A	A	T	T	C	T	T	A	A	A	T	T	A	T	C	T
S10	C	T	A	C	C	T	A	C	A	A	T	T	C	T	T	A	A	A	T	T	A	T	C	T
C6	C	T	A	C	C	T	A	C	G	A	T	T	C	T	T	C	G	G	T	T	G	T	C	T
Td17	C	T	A	C	C	T	A	C	G	A	T	T	C	T	T	C	G	G	T	T	G	T	C	T
T43	C	T	A	C	C	T	A	C	G	A	T	T	C	T	T	C	G	G	T	T	G	T	C	T
S1	C	T	A	C	C	T	A	C	G	A	T	T	C	T	T	C	G	G	T	T	G	T	C	T
C4	C	T	A	C	C	T	A	C	G	A	T	T	C	T	T	C	G	G	T	T	G	T	C	T

C51	C	T	A	C	C	T	A	C	G	A	T	T	C	T	T	C	G	G	T	T	G	T	C	T
P2	C	T	A	C	C	T	A	C	G	A	T	T	C	T	T	C	G	G	T	T	G	T	C	T
S18	C	T	A	C	C	T	A	C	G	A	T	T	C	T	T	C	G	G	T	T	G	T	C	T
T11	C	T	A	C	C	T	A	C	G	A	T	T	C	T	T	C	G	G	T	T	G	T	C	T
C1	C	T	A	C	C	T	A	C	G	A	T	T	C	T	T	C	G	G	T	T	G	T	C	T
T30	C	T	A	C	C	T	A	C	G	A	T	T	C	T	T	C	G	G	T	T	G	T	C	T
P7	C	T	A	C	C	T	A	C	G	A	T	T	C	T	T	C	G	G	T	T	G	T	C	T
C21	C	T	A	C	C	T	A	C	G	A	T	T	C	T	T	C	G	G	T	T	G	T	C	T
C18	C	T	A	C	C	T	A	C	G	A	T	T	C	T	T	C	G	G	T	T	G	T	C	T
T18	C	T	A	C	C	T	A	C	G	A	T	T	C	T	T	C	G	G	T	T	G	T	C	T
P39	C	T	A	C	C	T	A	C	G	A	T	T	C	T	T	C	G	G	T	T	G	T	C	T
P24	C	T	A	C	C	T	A	C	G	A	T	T	C	T	T	C	G	G	T	T	G	T	C	T
C13	C	T	A	C	C	T	A	C	A	A	T	T	C	T	T	C	A	G	T	T	A	T	C	T
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P8	C	T	A	C	C	T	A	C	A	A	T	T	C	T	T	C	A	G	T	T	A	T	C	T
P10	C	T	A	C	C	T	A	C	G	A	T	T	C	T	T	C	G	G	T	T	G	T	C	T
S48	T	T	A	C	C	A	A	C	A	G	T	T	C	C	T	T	T	A	T	T	A	T	T	T
S43	T	T	A	C	C	A	A	C	A	G	T	T	C	C	T	T	T	A	T	T	A	T	T	T
S30	T	T	A	C	C	A	A	C	A	G	T	T	C	C	T	T	T	A	T	T	A	T	T	T
S38	T	T	A	C	C	A	A	C	A	G	T	T	C	C	T	T	T	A	T	T	A	T	T	T
Td20	T	T	A	C	C	A	A	C	A	G	T	T	C	C	T	T	T	A	T	T	A	T	T	T
Td24	T	T	A	C	C	A	A	C	A	G	T	T	C	C	T	T	T	A	T	T	A	T	T	T
C20	T	T	A	C	C	A	A	C	A	G	T	T	C	C	T	T	T	A	T	T	A	T	T	T
T17	C	C	T	T	T	T	C	G	C	A	G	A	T	T	G	A	A	A	G	G	T	G	A	A

	219	221	224	225	227	228	230	233	234	236	239	240	241	242	243	244	245	248	249	250	252	255	258	260
P28	A	T	A	T	A	G	A	A	T	A	T	G	G	T	A	C	-	A	G	T	G	T	T	G
S7	A	T	A	T	A	G	A	A	T	A	T	G	G	T	A	C	-	A	G	T	G	T	T	G
P29	A	T	A	T	A	G	A	A	T	A	T	G	G	T	A	C	-	A	G	T	G	T	T	G
S11	A	T	A	T	A	G	A	A	T	A	C	G	G	T	A	C	-	A	G	T	G	T	T	G
S25	A	T	A	T	A	G	A	A	T	A	T	G	G	T	A	C	-	A	G	T	G	T	T	G
Td18	A	T	A	T	A	G	A	A	T	A	T	G	G	T	A	C	-	A	G	T	G	T	T	G

P21	A	T	A	T	A	G	A	A	T	A	T	G	G	T	A	C	-	A	G	T	G	T	T	G
T16	A	T	A	T	A	G	A	A	T	A	T	G	G	T	A	C	-	A	G	T	G	T	T	G
P17	A	T	A	T	A	G	A	A	T	A	T	G	G	T	A	C	-	A	G	T	G	T	T	G
C10	A	T	A	T	A	G	A	A	T	A	T	G	G	T	A	C	-	A	G	T	G	T	T	G
C9	A	T	A	T	A	G	A	A	T	A	T	G	G	T	A	C	-	A	G	T	G	T	T	G
C41	A	T	A	T	A	G	A	A	T	A	T	G	G	T	A	C	-	A	G	T	G	T	T	G
S33	A	T	A	T	A	G	A	A	T	A	T	G	G	T	A	C	-	A	G	T	G	T	T	G
S10	A	T	A	T	A	G	A	A	T	A	T	G	G	T	A	C	-	A	G	T	G	T	T	G
C6	A	C	A	C	A	G	A	A	T	A	C	G	G	T	A	C	-	A	A	T	G	T	T	G
Td17	A	C	A	C	A	G	A	A	T	A	C	G	G	T	A	C	-	A	A	T	G	T	T	G
T43	A	C	A	C	A	G	A	A	T	A	C	G	G	T	A	C	-	A	A	T	G	T	T	G
S1	A	C	A	C	A	G	A	A	T	A	C	G	G	T	A	C	-	A	A	T	G	T	T	G
C4	A	C	A	C	A	G	A	A	T	A	C	G	G	T	A	C	-	A	A	T	G	T	T	G
C51	A	C	A	C	A	G	A	A	T	A	C	G	G	T	A	C	-	A	A	T	G	T	T	G
P2	A	C	A	C	A	G	A	A	T	A	-	G	G	T	A	C	-	A	A	T	G	T	T	G
S18	A	C	A	C	A	G	A	A	T	A	C	G	G	T	A	C	-	A	A	T	G	T	T	G
T11	A	C	A	C	A	G	A	A	T	A	C	G	G	T	A	C	-	A	A	T	G	T	T	G
C1	A	C	A	C	A	G	A	A	T	A	C	G	G	T	A	C	-	A	A	T	G	T	T	G
T30	A	C	A	C	A	G	A	A	T	A	C	G	G	T	A	C	-	A	A	T	G	T	T	G
P7	A	C	A	C	A	G	A	A	T	A	C	G	G	T	A	C	-	A	A	T	G	T	T	G
C21	A	C	A	C	A	G	A	A	T	A	C	G	G	T	A	C	-	A	A	T	G	T	T	G
C18	A	C	A	C	A	G	A	A	T	A	C	G	G	T	A	C	-	A	A	T	G	T	T	G
T18	A	C	A	C	A	G	A	A	T	A	C	G	G	T	A	C	-	A	A	T	G	T	T	G
P39	A	C	A	C	A	G	A	A	T	A	C	G	G	T	A	C	-	A	A	T	G	T	T	G
P24	A	C	A	C	A	G	A	A	T	A	C	G	G	T	A	C	-	A	A	T	G	T	T	G
C13	A	C	A	C	A	G	A	A	T	A	C	G	G	T	A	C	-	A	A	T	G	T	T	G
C26	A	C	A	C	A	G	A	A	T	A	C	G	G	T	A	C	-	A	A	T	G	T	T	G
P8	A	C	A	C	A	G	A	A	T	A	C	G	G	T	A	C	-	A	A	T	G	T	T	G
P10	A	C	A	C	A	G	A	A	T	A	C	G	G	T	A	C	-	A	A	T	G	T	T	G
S48	A	T	A	C	A	G	T	T	C	T	T	G	G	T	A	C	-	A	A	C	T	C	T	G
S43	A	T	A	C	A	G	T	T	C	T	T	G	G	T	A	C	-	A	A	C	T	C	T	G
S30	A	T	A	C	A	G	T	T	C	T	T	G	G	T	A	C	-	A	A	C	T	C	T	G
S38	A	T	A	C	A	G	T	T	C	T	T	G	G	T	A	C	-	A	A	C	T	C	T	G
Td20	A	T	A	C	A	G	T	T	C	T	T	G	G	T	A	C	-	A	A	C	T	C	T	G

Td24	A	T	A	C	A	G	T	T	C	T	T	G	G	T	A	C	-	A	A	C	T	C	T	G
C20	A	T	A	C	A	G	T	T	C	T	T	G	G	T	A	C	-	A	A	C	T	C	T	G
T17	T	T	C	T	T	C	A	A	T	C	T	C	C	C	T	T	G	T	A	T	G	T	A	T

	262	264	266	267	268	269	271	272	273	276	278	279	280	281	283	285	286	294	295	297	299	300	303	305
P28	C	T	C	T	T	T	A	T	T	A	C	C	T	T	G	G	T	C	T	A	T	T	A	T
S7	C	T	C	T	T	T	A	T	T	A	C	C	T	T	G	G	T	C	T	A	T	T	A	T
P29	C	T	C	T	T	T	A	T	T	A	C	C	T	T	G	G	T	C	T	A	T	T	A	T
S11	C	T	C	T	T	T	A	T	T	A	C	C	T	T	G	G	T	C	T	A	T	T	A	T
S25	C	T	C	T	T	T	A	T	T	A	C	C	T	T	G	G	T	C	T	A	T	T	A	T
Td18	C	T	C	T	T	T	A	T	T	A	C	C	T	T	G	G	T	C	T	A	T	T	A	T
P21	C	T	C	T	T	T	A	T	T	A	C	C	T	T	G	G	T	C	T	A	T	T	A	T
T16	C	T	C	T	T	T	A	T	T	A	C	C	T	T	G	G	T	C	T	A	T	T	A	T
P17	C	T	C	T	T	T	A	T	T	A	C	C	T	T	G	G	T	C	T	A	T	T	A	T
C10	C	T	C	T	T	T	A	T	T	A	C	C	T	T	G	G	T	C	T	A	T	T	A	T
C9	C	T	C	T	T	T	A	T	T	A	C	C	T	T	G	G	T	C	T	A	T	T	A	T
C41	C	T	C	T	T	T	A	T	T	A	C	C	T	T	G	G	T	C	T	A	T	T	A	T
S33	C	T	C	T	T	T	A	T	T	A	C	C	T	T	G	G	T	C	T	A	T	T	A	T
S10	C	T	C	T	T	T	A	T	T	A	C	C	T	T	G	G	T	C	T	A	T	T	A	T
C6	C	T	C	T	T	T	A	T	T	G	C	A	T	T	G	G	T	T	T	A	T	T	A	T
Td17	C	T	C	T	T	T	A	T	T	G	C	A	T	T	G	G	T	T	T	A	T	T	A	T
T43	C	T	C	T	T	T	A	T	T	G	C	A	T	T	G	G	T	T	T	A	T	T	A	T
S1	C	T	C	T	T	T	A	T	T	G	C	A	T	T	G	G	T	T	T	A	T	T	A	T
C4	C	T	C	T	T	T	A	T	T	G	C	A	T	T	G	G	T	T	T	A	T	T	A	T
C51	C	T	C	T	T	T	A	T	T	G	C	A	T	T	G	G	T	T	T	A	T	T	A	T
P2	C	T	C	T	T	T	A	T	T	G	C	A	T	T	G	G	T	T	T	A	T	T	A	T
S18	C	T	C	T	T	T	A	T	T	G	C	A	T	T	G	G	T	T	T	A	T	T	A	T
T11	C	T	C	T	T	T	A	T	T	G	C	A	T	T	G	G	T	T	T	A	T	T	A	T
C1	C	T	C	T	T	T	A	T	T	G	C	A	T	T	G	G	T	T	T	A	T	T	A	T
T30	C	T	C	T	T	T	A	T	T	G	C	A	T	T	G	G	T	T	T	A	T	T	A	T
P7	C	T	C	T	T	T	A	T	T	G	C	A	T	T	G	G	T	T	T	A	T	T	A	T
C21	C	T	C	T	T	T	A	T	T	G	C	A	T	T	G	G	T	T	T	A	T	T	A	T

C18	C	T	C	T	T	T	A	T	T	G	C	A	T	T	G	G	T	T	T	A	T	T	A	T
T18	C	T	C	T	T	T	A	T	T	G	C	A	T	T	G	G	T	T	T	A	T	T	A	T
P39	C	T	C	T	T	T	A	T	T	G	C	A	T	T	G	G	T	T	T	A	T	T	A	T
P24	C	T	C	T	T	T	A	T	T	G	C	A	T	T	G	G	T	T	T	A	T	T	A	T
C13	C	T	C	T	T	T	A	T	T	G	C	A	T	T	G	G	T	T	T	A	T	T	A	T
C26	C	T	C	T	T	T	A	T	T	G	C	A	T	T	G	G	T	T	T	A	T	T	A	T
P8	C	T	C	T	T	T	A	T	T	G	C	A	T	T	G	G	T	T	T	A	T	T	A	T
P10	C	T	C	T	T	T	A	T	T	G	C	A	T	T	G	G	T	T	C	A	T	T	A	T
S48	C	T	C	T	C	T	A	T	T	A	C	G	C	T	G	A	T	T	C	A	T	T	T	T
S43	C	T	C	T	C	T	A	T	T	A	C	G	C	T	G	A	T	T	C	A	T	T	T	T
S30	C	T	C	T	C	T	A	T	T	A	C	G	C	T	G	A	T	T	C	A	T	T	T	T
S38	C	T	C	T	C	T	A	T	T	A	C	G	C	T	G	A	T	T	C	A	T	T	T	T
Td20	C	T	C	T	C	T	A	T	T	A	C	G	C	T	G	A	T	T	C	A	T	T	T	T
Td24	C	T	C	T	C	T	A	T	T	A	C	G	C	T	G	A	T	T	C	A	T	T	T	T
C20	C	T	C	T	C	T	A	T	T	A	C	G	C	T	G	A	T	T	C	A	T	T	T	T
T17	A	C	A	C	T	C	C	C	A	G	T	G	G	A	C	G	A	C	T	C	C	C	T	C

	306	309	310	313	314	317	318	321	323	326	327	330	332	333	334	336	339	342	343	344	347	348	349	350
P28	A	A	G	T	T	C	A	A	T	T	A	T	C	T	A	C	A	T	A	T	A	C	A	T
S7	A	A	G	T	T	C	A	A	T	T	A	T	C	T	A	C	A	T	A	T	A	C	A	T
P29	A	A	G	T	T	C	A	A	T	T	A	T	C	T	A	C	A	T	A	T	A	C	A	T
S11	A	A	G	T	T	C	A	A	T	T	A	T	C	T	A	C	A	T	A	T	A	C	A	T
S25	A	A	G	T	T	C	A	A	T	T	A	T	C	T	A	C	A	T	A	T	A	C	A	T
Td18	A	A	G	T	T	C	A	A	T	T	A	T	C	T	A	C	A	T	A	T	A	C	A	T
P21	A	A	G	T	T	C	A	A	T	T	A	T	C	T	A	C	A	T	A	T	A	C	A	T
T16	A	A	G	T	T	C	A	A	T	T	A	T	C	T	A	C	A	T	A	T	A	C	A	T
P17	A	A	G	T	T	C	A	A	T	T	A	T	C	T	A	C	A	T	A	T	A	C	A	T
C10	A	A	G	T	T	C	A	A	T	T	A	T	C	T	A	C	A	T	A	T	A	C	A	T
C9	A	A	G	T	T	C	A	A	T	T	A	T	C	T	A	C	A	T	A	T	A	C	A	T
C41	A	A	G	T	T	C	A	A	T	T	A	T	C	T	A	C	A	T	A	T	A	C	A	T
S33	A	A	G	T	T	C	A	A	T	T	A	T	C	T	A	C	A	T	A	T	A	C	A	T
S10	A	A	G	T	T	C	A	A	T	T	A	T	C	T	A	C	A	T	A	T	A	C	A	T

C6	T	G	G	C	T	C	A	A	T	T	C	T	C	T	A	T	A	T	A	T	A	T	A	T
Td17	T	G	G	C	T	C	A	A	T	T	C	T	C	T	A	T	A	T	A	T	A	T	A	T
T43	T	G	G	C	T	C	A	A	T	T	C	T	C	T	A	T	A	T	A	T	A	T	A	T
S1	T	G	G	C	T	C	A	A	T	T	C	T	C	T	A	T	A	T	A	T	A	T	A	T
C4	T	G	G	C	T	C	A	A	T	T	C	T	C	T	A	T	A	T	A	T	A	T	A	T
C51	T	G	G	C	T	C	A	A	T	T	C	T	C	T	A	T	A	T	A	T	A	T	A	T
P2	T	G	G	C	T	C	A	A	T	T	C	T	C	T	A	T	A	T	A	T	A	T	A	T
S18	T	G	G	C	T	C	A	A	T	T	C	T	C	T	A	T	A	T	A	T	A	T	A	T
T11	T	G	G	C	T	C	A	A	T	T	C	T	C	T	A	T	A	T	A	T	A	T	A	T
C1	T	G	G	C	T	C	A	A	T	T	C	T	C	T	A	T	A	T	A	T	A	T	A	T
T30	T	G	G	C	T	C	A	A	T	T	C	T	C	T	A	T	A	T	A	T	A	T	A	T
P7	T	G	G	C	T	C	A	A	T	T	C	T	C	T	A	T	A	T	A	T	A	T	A	T
C21	T	G	G	C	T	C	A	A	T	T	C	T	C	T	A	T	A	T	A	T	A	T	A	T
C18	T	G	G	C	T	C	A	A	T	T	C	T	C	T	A	T	A	T	A	T	A	T	A	T
T18	T	G	G	C	T	C	A	A	T	T	C	T	C	T	A	T	A	T	A	T	A	T	A	T
P39	T	G	G	C	T	C	A	A	T	T	C	T	C	T	A	T	A	T	A	T	A	T	A	T
P24	T	G	G	C	T	C	A	A	T	T	C	T	C	T	A	T	A	T	A	T	A	T	A	T
C13	T	G	G	C	T	C	A	A	T	T	C	T	C	C	A	T	A	T	A	T	A	T	A	T
C26	T	G	G	C	T	C	A	A	T	T	C	T	C	C	A	T	A	T	A	T	A	T	A	T
P8	T	G	G	C	T	C	A	A	T	T	C	T	C	C	A	T	A	T	A	T	A	T	A	T
P10	T	G	G	C	T	C	A	A	T	T	C	T	A	T	C	T	A	T	A	T	A	T	A	C
S48	G	T	G	T	T	C	C	T	T	T	A	A	C	T	A	T	T	A	A	T	A	C	A	T
S43	G	T	G	T	T	C	C	T	T	T	A	A	C	T	A	T	T	A	A	T	A	C	A	T
S30	G	T	G	T	T	C	C	T	T	T	A	A	C	T	A	T	T	A	A	T	A	C	A	T
S38	G	T	G	T	T	C	C	T	T	T	A	A	C	T	A	T	T	A	A	T	A	C	A	T
Td20	G	T	G	T	T	C	C	T	T	T	A	A	C	T	A	T	T	A	A	T	A	C	A	T
Td24	G	T	G	T	T	C	C	T	T	T	A	A	C	T	A	T	T	A	A	T	A	C	A	T
C20	G	T	G	T	T	C	C	T	T	T	A	A	C	T	A	T	T	A	A	T	A	C	A	T
T17	A	A	T	T	C	T	A	A	A	C	A	T	G	C	T	C	A	T	C	C	C	C	G	T

	351	352	354	355	357	359	360	363	365	366	367	369	372	374	377	379	380	381	382	384	387	393	396	397
P28	C	A	C	T	G	A	C	T	C	T	T	T	T	T	T	G	C	C	C	T	T	C	-	C
S7	C	A	C	T	G	A	C	T	C	T	T	T	T	T	T	G	C	C	C	T	T	C	-	C
P29	C	A	C	T	G	A	C	T	C	T	T	T	T	T	T	G	C	C	C	T	T	C	-	C
S11	C	A	C	T	G	A	C	T	C	T	T	T	T	T	T	G	C	C	C	T	T	C	-	C
S25	C	A	C	T	G	A	C	T	C	T	T	T	T	T	T	G	C	C	C	T	T	C	-	C
Td18	C	A	C	T	G	A	C	T	C	T	T	T	T	T	T	G	C	C	C	T	T	C	-	C
P21	C	A	C	T	G	A	C	T	C	T	T	T	T	T	T	G	C	C	C	T	T	C	-	C
T16	C	A	C	T	G	A	C	T	C	T	T	T	T	T	T	G	C	C	C	T	T	C	-	C
P17	C	A	C	T	G	A	C	T	C	T	T	T	T	T	T	G	C	C	C	T	T	C	-	C
C10	C	A	C	T	G	A	C	T	C	T	T	T	T	T	T	G	C	C	C	T	T	C	-	C
C9	C	A	C	T	A	A	C	T	C	T	T	T	T	T	T	G	C	C	C	T	T	T	-	C
C41	C	A	C	T	A	A	C	T	C	T	T	T	T	T	T	G	C	C	C	T	T	T	-	C
S33	C	A	C	T	A	A	C	T	C	T	T	T	T	T	T	G	C	C	C	T	T	T	-	C
S10	C	A	C	T	A	A	C	T	C	T	T	T	T	T	T	G	C	C	C	T	T	T	-	C
C6	T	A	C	T	G	A	C	T	C	T	T	T	T	T	T	G	C	C	C	C	C	T	-	T
Td17	T	A	C	T	G	A	C	T	C	T	T	T	T	T	T	G	C	C	C	C	C	T	-	T
T43	T	A	C	T	G	A	C	T	C	T	T	T	T	T	T	G	C	C	C	C	C	T	-	T
S1	T	A	C	T	G	A	C	T	C	T	T	T	T	T	T	G	C	C	C	C	C	T	-	T
C4	T	A	C	T	G	A	C	T	C	T	T	T	T	T	T	G	C	C	C	C	C	T	-	T
C51	T	A	C	T	G	A	C	T	C	T	T	T	T	T	T	G	C	C	C	C	C	T	-	T
P2	T	A	C	T	G	A	C	T	C	T	T	T	T	T	T	G	C	C	C	C	C	T	-	T
S18	T	A	C	T	G	A	C	T	C	T	T	T	T	T	T	G	C	C	C	C	C	T	-	T
T11	T	A	C	T	G	A	C	T	C	T	T	T	T	T	T	G	C	C	C	C	C	T	-	T
C1	T	A	C	T	G	A	C	T	C	T	T	T	T	T	T	G	C	C	C	C	C	T	-	T
T30	T	A	C	T	G	A	C	T	C	T	T	T	T	T	T	G	C	C	C	C	C	T	-	T
P7	T	A	C	T	G	A	C	T	C	T	T	T	T	T	T	G	C	C	C	C	C	T	-	T
C21	T	A	C	T	G	A	C	T	C	T	T	T	T	T	T	G	C	C	C	C	C	T	-	T
C18	T	A	C	T	G	A	C	T	C	T	T	T	T	T	T	G	C	C	C	C	C	T	-	T
T18	T	A	C	T	G	A	C	T	C	T	T	T	T	T	T	G	C	C	C	C	C	T	-	T
P39	T	A	C	T	G	A	C	T	C	T	T	T	T	T	T	G	C	C	C	C	C	T	-	T
P24	T	A	C	T	G	A	C	T	C	T	T	T	T	T	T	G	C	C	C	C	C	T	-	T
C13	T	A	T	T	A	A	T	T	C	T	T	T	T	T	T	G	C	C	C	T	T	T	-	T

C26	T	A	C	T	A	A	T	T	C	T	T	T	T	T	T	G	C	C	C	T	T	T	-	T
P8	T	A	C	T	A	A	T	T	C	T	T	T	T	T	T	G	C	C	C	T	T	T	-	T
P10	T	A	C	G	G	G	A	T	C	G	T	T	T	T	T	G	C	C	C	C	C	T	-	T
S48	T	A	T	T	A	A	T	T	C	A	T	C	C	T	T	G	C	A	C	T	C	T	-	T
S43	T	A	T	T	A	A	T	T	C	A	T	C	C	T	T	G	C	A	C	T	C	T	-	T
S30	T	A	T	T	A	A	T	T	C	A	T	C	C	T	T	G	C	A	C	T	C	T	-	T
S38	T	A	T	T	A	A	T	T	C	A	T	C	C	T	T	G	C	A	C	T	C	T	-	T
Td20	T	A	T	T	A	A	T	T	C	A	T	C	C	T	T	G	C	A	C	T	C	T	-	T
Td24	T	A	T	T	A	A	T	T	C	A	T	C	C	T	T	G	C	A	C	T	C	T	-	T
C20	T	A	T	T	A	A	T	T	C	A	T	C	C	T	T	G	C	A	C	T	C	T	-	T
T17	C	T	C	T	G	T	C	G	G	A	G	C	A	G	C	C	T	C	T	T	G	C	C	T

	403	410	412	413	414	416	420	421	423	430	431	434	436	439	441	445	446	447	448	452	454	455	458	459
P28	A	G	A	G	T	T	G	T	T	A	G	A	T	T	A	A	T	T	T	C	T	T	A	C
S7	A	G	A	G	T	T	G	T	T	A	G	A	T	T	A	A	T	T	T	C	T	T	A	C
P29	A	G	A	G	T	T	G	T	T	A	G	A	T	T	A	A	T	T	T	C	T	T	A	C
S11	A	G	A	G	T	T	G	T	T	A	G	A	T	T	A	A	T	T	T	C	T	T	A	C
S25	A	G	A	G	T	T	G	T	T	A	G	A	T	T	A	A	T	T	T	C	T	T	A	C
Td18	A	G	A	G	T	T	G	T	T	A	G	A	T	T	A	A	T	T	T	C	T	T	A	C
P21	A	G	A	G	T	T	G	T	T	A	G	A	T	T	A	A	T	T	T	C	T	T	A	C
T16	A	G	A	G	T	T	G	T	T	A	G	A	T	T	A	A	T	T	T	C	T	T	A	C
P17	A	G	A	G	T	T	G	T	T	A	G	A	T	T	A	A	T	T	T	C	T	T	A	C
C10	A	G	A	G	T	T	G	T	T	A	G	A	T	T	A	A	T	T	T	C	T	T	A	C
C9	A	G	A	G	T	T	G	T	T	A	G	A	T	T	A	A	T	T	T	C	T	T	A	C
C41	A	G	A	G	T	T	G	T	T	A	G	A	T	T	A	A	T	T	T	C	T	T	A	C
S33	A	G	A	G	T	T	G	T	T	A	G	A	T	T	A	A	T	T	T	C	T	T	A	C
S10	A	G	A	G	T	T	G	T	T	A	G	A	T	T	A	A	T	T	T	C	T	T	A	C
C6	A	G	C	G	T	T	G	T	T	A	G	A	T	C	A	A	T	T	T	C	T	T	A	C
Td17	A	G	C	G	T	T	G	T	T	A	G	A	T	C	A	A	T	T	T	C	T	T	A	C
T43	A	G	C	G	T	T	G	T	T	A	G	A	T	C	A	A	T	T	T	C	T	T	A	C
S1	A	G	C	G	T	T	G	T	T	A	G	A	T	C	A	A	T	T	T	C	T	T	A	C
C4	A	G	C	G	T	T	G	T	T	A	G	A	T	C	A	A	T	T	T	C	T	T	A	C

C51	A	G	C	G	T	T	G	T	T	A	G	A	T	C	A	A	T	T	T	C	T	T	A	C
P2	A	G	C	G	T	T	G	T	T	A	G	A	T	C	A	A	T	T	T	C	T	T	A	C
S18	A	G	C	G	T	T	G	T	T	A	G	A	T	C	A	A	T	T	T	C	T	T	A	C
T11	A	G	C	G	T	T	G	T	T	A	G	A	T	C	A	A	T	T	T	C	T	T	A	C
C1	A	G	C	G	T	T	G	T	T	A	G	A	T	C	A	A	T	T	T	C	T	T	A	C
T30	A	G	C	G	T	T	G	T	T	A	G	A	T	C	A	A	T	T	T	C	T	T	A	C
P7	A	G	C	G	T	T	G	T	T	A	G	A	T	C	A	A	T	T	T	C	T	T	A	C
C21	A	G	C	G	T	T	G	T	T	A	G	A	T	C	A	A	T	T	T	C	T	T	A	C
C18	A	G	C	G	T	T	G	T	T	A	G	A	T	C	A	A	T	T	T	C	T	T	A	C
T18	A	G	C	G	T	T	G	T	T	A	G	A	T	C	A	A	T	T	T	C	T	T	A	C
P39	A	G	C	G	T	T	G	T	T	A	G	A	T	C	A	A	T	T	T	C	T	T	A	C
P24	A	G	C	G	T	T	G	T	T	A	G	A	T	C	A	A	T	T	T	C	T	T	A	C
C13	A	G	C	G	T	T	G	T	T	A	G	A	T	T	A	A	T	T	T	C	T	T	A	C
C26	A	G	C	G	T	T	G	T	T	A	G	A	T	T	A	A	T	T	T	C	T	T	A	C
P8	A	G	C	G	T	T	G	T	T	A	G	A	T	T	A	A	T	T	T	C	T	T	A	C
P10	A	G	C	G	A	T	G	T	T	C	C	A	C	C	A	A	T	A	T	C	T	T	A	C
S48	A	G	T	G	T	T	G	T	T	A	G	A	T	C	A	A	T	T	C	T	A	T	A	C
S43	A	G	T	G	T	T	G	T	T	A	G	A	T	C	A	A	T	T	C	T	A	T	A	C
S30	A	G	T	G	T	T	G	T	T	A	G	A	T	C	A	A	T	T	C	T	A	T	A	C
S38	A	G	T	G	T	T	G	T	T	A	G	A	T	C	A	A	T	T	C	T	A	T	A	C
Td20	A	G	T	G	T	T	G	T	T	A	G	A	T	C	A	A	T	T	C	T	A	T	A	C
Td24	A	G	T	G	T	T	G	T	T	A	G	A	T	C	A	A	T	T	C	T	A	T	A	C
C20	A	G	T	G	T	T	G	T	T	A	G	A	T	C	A	A	T	T	C	T	A	T	A	C
T17	C	A	A	A	T	C	T	C	A	T	T	C	T	T	G	T	C	T	A	C	T	A	G	T

	460	462	463	464	465	466	470	471	472	473	474	479	480	482	483	490	494	498	499	500	501	503	504	506
P28	T	G	T	T	T	A	T	T	-	A	A	A	A	A	T	A	C	T	T	T	T	A	G	T
S7	T	G	T	T	T	A	T	T	-	A	A	A	A	A	T	A	C	T	T	T	T	A	G	T
P29	T	G	T	T	T	A	T	T	-	A	A	A	A	A	T	A	C	T	T	T	T	A	G	T
S11	T	G	T	T	T	A	T	T	-	A	A	A	A	A	T	A	C	T	T	T	T	A	G	T
S25	T	G	T	T	T	A	T	T	-	A	A	A	A	A	T	A	C	T	T	T	T	A	G	T
Td18	T	G	T	T	T	A	T	T	-	A	A	A	A	A	T	A	C	T	T	T	T	A	G	T

P21	T	G	T	T	T	A	T	T	-	A	A	A	A	A	T	A	C	T	T	T	T	A	G	T
T16	T	G	T	T	T	A	T	T	-	A	A	A	A	A	T	A	C	T	T	T	T	A	G	T
P17	T	G	T	T	T	A	T	T	-	A	A	A	A	A	T	A	C	T	T	T	T	A	G	T
C10	T	G	T	T	T	A	T	T	-	A	A	A	A	A	T	A	C	T	T	T	T	A	G	T
C9	T	G	T	T	T	A	T	T	-	A	A	A	A	A	T	A	C	T	T	T	T	A	G	T
C41	T	G	T	T	T	A	T	T	-	A	A	A	A	A	T	A	C	T	T	T	T	A	G	T
S33	T	G	T	T	T	A	T	T	-	A	A	A	A	A	T	A	C	T	T	T	T	A	G	T
S10	T	G	T	T	T	A	T	T	-	A	A	A	A	A	T	A	C	T	T	T	T	A	G	T
C6	C	G	T	T	T	A	C	T	-	G	A	A	A	A	T	A	C	T	T	T	C	A	G	T
Td17	C	G	T	T	T	A	C	T	-	G	A	A	A	A	T	A	C	T	T	T	C	A	G	T
T43	C	G	T	T	T	A	C	T	-	G	A	A	A	A	T	A	C	T	T	T	C	A	G	T
S1	C	G	T	T	T	A	C	T	-	G	A	A	A	A	T	A	C	T	T	T	C	A	G	T
C4	C	G	T	T	T	A	C	T	-	G	A	A	A	A	T	A	C	T	T	T	C	A	G	T
C51	C	G	T	T	T	A	C	T	-	G	A	A	A	A	T	A	C	T	T	T	C	A	G	T
P2	C	G	T	T	T	A	C	T	-	G	A	A	A	A	T	A	C	T	T	T	C	A	G	T
S18	C	G	T	T	T	A	C	T	-	G	A	A	A	A	T	A	C	T	T	T	C	A	G	T
T11	C	G	T	T	T	A	C	T	-	G	A	A	A	A	T	A	C	T	T	T	C	A	G	T
C1	C	G	T	T	T	A	C	T	-	G	A	A	A	A	T	A	C	T	T	T	C	A	G	T
T30	C	G	T	T	T	A	C	T	-	G	A	A	A	A	T	A	C	T	T	T	C	A	G	T
P7	C	G	T	T	T	A	C	T	-	G	A	A	A	A	T	A	C	T	T	T	C	A	G	T
C21	C	G	T	T	T	A	C	T	-	G	A	A	A	A	T	A	C	T	T	T	C	A	G	T
C18	C	G	T	T	T	A	C	T	-	G	A	A	A	A	T	A	C	T	T	T	C	A	G	T
T18	C	G	T	T	T	A	C	T	-	G	A	A	A	A	T	A	C	T	T	T	T	A	G	T
P39	C	G	T	T	T	A	C	T	-	G	A	A	A	A	T	A	C	T	T	T	C	A	G	T
P24	C	G	T	T	T	A	C	T	-	G	A	A	A	A	T	A	C	T	T	T	C	A	G	T
C13	C	G	T	T	T	A	C	T	-	G	A	A	A	A	T	A	C	T	T	T	C	A	G	T
C26	C	G	T	T	T	A	C	T	-	G	A	A	A	A	T	A	C	T	T	T	C	A	G	T
P8	C	G	T	T	T	A	C	T	-	G	A	A	A	A	T	A	C	T	T	T	C	A	G	T
P10	T	C	T	T	T	A	C	C	A	G	A	A	A	A	T	G	C	C	T	T	C	A	T	T
S48	G	G	G	C	T	T	T	T	-	A	A	T	A	A	T	A	T	T	T	T	C	T	G	T
S43	G	G	G	C	T	T	T	T	-	A	A	T	A	G	T	A	T	T	T	T	C	T	G	T
S30	G	G	G	C	T	T	T	T	-	A	A	T	A	G	T	A	T	T	T	T	C	T	G	T
S38	G	G	G	C	T	T	T	T	-	A	A	T	A	G	T	A	T	T	T	T	C	T	G	T
Td20	G	G	G	C	T	T	T	T	-	A	A	T	A	A	T	A	T	T	T	T	C	T	G	T

Td24	G	G	G	C	T	T	T	T	-	A	A	T	A	G	T	A	T	T	T	T	C	T	G	T
C20	G	G	G	C	T	T	T	T	-	A	A	T	A	A	T	A	T	T	T	T	C	T	G	T
T17	T	G	G	T	A	A	C	T	-	A	C	A	C	A	C	A	C	T	G	C	T	G	C	C

	508	512	513	515	518	519	524	525	528	529	530	532	534	535	538	540	542	543	545	548	549	550	551	552
P28	T	T	A	T	G	G	C	A	A	C	A	-	T	T	C	A	A	A	A	T	C	T	T	-
S7	T	T	A	T	G	G	C	A	A	C	A	-	T	T	C	A	A	A	A	T	C	T	T	-
P29	T	T	A	T	G	G	C	A	A	C	A	-	T	T	C	A	A	A	A	T	C	T	T	-
S11	T	T	A	T	G	G	C	A	A	C	A	-	T	T	C	A	A	A	A	T	C	T	T	-
S25	T	T	A	T	G	G	C	A	A	C	A	-	T	T	C	A	A	A	A	T	C	T	T	-
Td18	T	T	A	T	G	G	C	A	A	C	A	-	T	T	C	A	A	A	A	T	C	T	T	-
P21	T	T	A	T	G	G	C	A	A	C	A	-	T	T	C	A	A	A	A	T	C	T	T	-
T16	T	T	A	T	G	G	C	A	A	C	A	-	T	T	C	A	A	A	A	T	C	T	T	-
P17	T	T	A	T	G	G	C	A	A	C	A	-	T	T	C	A	A	A	A	T	C	T	T	-
C10	T	T	A	T	G	G	C	A	A	C	A	-	T	T	C	A	A	A	A	T	C	T	T	-
C9	T	T	A	T	G	G	C	A	A	C	A	-	T	T	C	A	A	A	A	T	C	T	T	-
C41	T	T	A	T	G	G	C	A	A	C	A	-	T	T	C	A	A	A	A	T	C	T	T	-
S33	T	T	A	T	G	G	C	A	A	C	A	-	T	T	C	A	A	A	A	T	C	T	T	-
S10	T	T	A	T	G	G	C	A	A	C	A	-	T	T	C	A	A	A	A	T	C	T	T	-
C6	T	T	A	T	G	G	C	A	A	C	A	-	T	T	C	T	A	A	A	T	T	T	T	-
Td17	T	T	A	T	G	G	C	A	A	C	A	-	T	T	C	T	A	A	A	T	T	T	T	-
T43	T	T	A	T	G	G	C	A	A	C	A	-	T	T	C	T	A	A	A	T	T	T	T	-
S1	T	T	A	T	G	G	C	A	A	C	A	-	T	T	C	T	A	A	A	T	T	T	T	-
C4	T	T	A	T	G	G	C	A	A	C	A	-	T	T	C	T	A	A	A	T	T	T	T	-
C51	T	T	A	T	G	G	C	A	A	C	A	-	T	T	C	T	A	A	A	T	T	T	T	T
P2	T	T	A	T	G	G	C	A	A	C	A	-	T	T	C	T	A	A	A	T	T	T	T	-
S18	T	T	A	T	G	G	C	A	A	C	A	-	T	T	C	T	A	A	A	T	T	T	T	-
T11	T	T	A	T	G	G	C	A	A	C	A	-	T	T	C	T	A	A	A	T	T	T	T	-
C1	T	T	A	T	G	G	C	A	A	C	A	-	T	T	C	T	A	A	A	T	T	T	T	-
T30	T	T	A	T	G	G	C	A	A	C	A	-	T	T	C	T	A	A	A	T	T	T	T	-
P7	T	T	A	T	G	G	C	A	A	C	A	-	T	T	C	T	A	A	A	T	T	T	T	-
C21	T	T	A	T	G	G	C	A	A	C	A	-	T	T	C	T	A	A	A	T	T	T	T	-

C18	T	T	A	T	G	G	C	A	A	C	A	-	T	T	C	T	A	A	A	T	T	T	T	-
T18	T	T	A	T	G	G	C	A	A	C	A	-	T	T	C	T	A	A	A	T	T	T	T	-
P39	T	T	A	T	G	G	C	A	A	C	A	-	T	T	C	T	A	A	A	T	T	T	T	-
P24	T	T	A	T	G	G	C	A	A	C	A	-	T	T	C	T	A	A	A	T	T	T	T	-
C13	T	T	A	T	G	G	T	A	A	C	A	-	T	T	C	A	A	A	A	T	T	C	T	-
C26	T	T	A	T	G	G	T	A	A	C	A	-	T	T	C	A	A	A	A	T	T	C	T	-
P8	T	T	A	T	G	G	T	A	A	C	A	-	T	T	C	A	A	A	A	T	T	C	T	-
P10	T	T	C	G	G	G	C	A	G	C	C	-	C	C	C	T	G	T	A	C	A	T	G	T
S48	T	C	A	T	A	G	T	A	A	C	A	-	C	T	C	C	A	A	A	T	C	T	T	-
S43	T	C	A	T	A	G	T	A	A	C	A	-	C	T	C	C	A	A	A	T	C	T	T	-
S30	T	C	A	T	A	G	T	A	A	C	A	-	C	T	C	C	A	A	A	T	C	T	T	-
S38	T	C	A	T	A	G	T	A	A	C	A	-	C	T	C	C	A	A	A	T	C	T	T	-
Td20	T	C	A	T	A	G	T	A	A	C	A	-	C	T	C	C	A	A	A	T	C	T	T	-
Td24	T	C	A	T	A	G	T	A	A	C	A	-	C	T	C	C	A	A	A	T	C	T	T	-
C20	T	C	A	T	A	G	T	A	A	C	A	-	C	T	C	C	A	A	A	T	C	T	T	-
T17	C	T	A	T	G	C	T	G	A	A	A	A	T	T	A	A	T	A	C	C	C	T	T	-

	554	556
P28	G	A
S7	G	A
P29	G	A
S11	G	A
S25	G	A
Td18	G	A
P21	G	A
T16	G	A
P17	G	A
C10	G	A
C9	G	A
C41	G	A
S33	G	A
S10	G	A

C6	G	G
Td17	G	G
T43	G	G
S1	G	G
C4	G	G
C51	G	G
P2	G	G
S18	G	G
T11	G	G
C1	G	G
T30	G	G
P7	G	G
C21	G	G
C18	G	G
T18	G	G
P39	G	G
P24	G	G
C13	G	G
C26	G	G
P8	G	G
P10	G	G
S48	G	T
S43	G	T
S30	G	T
S38	G	T
Td20	G	T
Td24	G	T
C20	G	T
T17	T	A

APPENDIX E

Award and Publications from This Thesis

Award

The Best Student Abstract, 7th Asian Fisheries Forum. 30 November - 4 December 2004. Penang, Malaysia, The Asian Fisheries Society

Publications from this study

1. **Khamnamtong, B.**, Thumrungtanakit, S., Klinbunga, S., Hirono, T. Aoki, T. and Menasvetas, P. (2006). Identification of sex-specific expression markers of the giant tiger shrimp (*Penaeus monodon*). *J. Biochem. Mol. Biol.* 39: 37-45.

2. **Khamnamtong, B.**, Klinbunga, S. and Menasveta, P. (2005). Species identification of five penaeid shrimps using PCR-RFLP and SSCP analyses of 16S ribosomal DNA. *J. Biochem Mol. Biol.* 38: 491-499.

3. **Khamnamtong, N.**, Klinbunga, S., Tassanakajon, A., Puanglarp, N. and Menasveta, P. (2002). Isolation and characterization of sex-specific markers of the black tiger shrimp (*Penaeus monodon*). 28th Congress on Science and Technology of Thailand. 24-26 October 2002, Bangkok, Thailand, p.434 (Oral presentation).

4. Klinbunga, S., **Khamnamtong, B.**, Tassanakajon, A. and Menasveta, P. (2003). Molecular genetic markers as a tool for conservation and broodstock management of penaeid shrimps. 6th International Conference on the Environmental Management of Enclosed Coastal Seas. 18-21 November 2003. Bangkok, Thailand. p. 135 (Oral presentation).

5. **Khamnamtong, B.**, Klinbunga, S., Puanglarp, P. and Menasveta, P. (2004). Identification of morphological similar penaeid shrimps using PCR-RFLP and SSCP analyses. The 15th Annual Meeting of the Thai Society for Biotechnology. 3-6 February 2004, Chiang Mai, Thailand (Oral presentation).

6. Klinbunga, S., Pongsomboon, S., **Khamnamtong, B.**, Tassanakajon, A. and Menasveta, P. (2004). Molecular Genetic Markers As a Tool for Broodstock Management and Breeding Programmes of the Giant Tiger Shrimp (*Penaeus monodon*). 5th National Symposium on Marine Shrimps. 29-30 March 2004, Bangkok, Thailand (Oral presentation).

7. **Khamnamtong, B.**, Klinbunga, S. and Menasveta, P. (2004). Development of species-diagnostic markers for differentiation of morphological similar penaeid shrimps. 5th National Symposium on Marine Shrimps. 29-30 March 2004, Bangkok, Thailand (Oral presentation).

8. **Khamnamtong, B.**, Klinbunga, S., Tassanakajon, A. and Menasveta, P. (2004). Identification of differentially expressed genes in ovaries and testes of the giant tiger shrimp (*Penaeus monodon*). 30th Congress on Science and Technology of Thailand, 19-21 October 2004, Bangkok (Oral presentation).

9. Klinbunga, S., Pongsomboon, S., Supungul, P., **Khamnamtong, B**., Thumrungtanakit, S., Tassanakajon, A. and Menasveta, P. (2004). Genetic diversity and population structure of the giant tiger shrimp *Penaeus monodon*. Advances in Shrimp Biotechnology II, 7th Asian Fisheries Forum. 30 November-4 December 2004. Penang, Malaysia (Oral presentation).

10. **Khamnamtong, B.**, Klinbunga, S. and Menasveta, P. (2004). Development of molecular markers for differentiation of morphological similar *Penaeus* shrimps. Advances in Shrimp Biotechnology II, 7th Asian Fisheries Forum. 30 November-4 December 2004. Penang, Malaysia (Oral presentation).

11. Klinbunga, S., **Khamnamtong, N.**, Thumrungtanakit, S., Puanglarp, N. and Menasveta, P. (2005). Molecular genetic markers as a tool for aquatic biotechnology. The 8 th Applied Biology Symposium, 22 -23 June 2005, Marriott Putrajaya Hotel, Malaysia (Plenary lecture).

12. **Khamnamtong, B.**, Klinbunga, S. and Menasveta, P. (2005). Isolation and characterization of sex-specific expression markers in ovaries of the giant tiger shrimp *Penaeus monodon.* 31st Congress on Science and Technology of Thailand, 18–20 October 2005, Nakornratchasrima, Thailand (Oral presentation).

13. **Khamnamtong, B.**, Thumrungtanakit, S., Klinbunga, S. and Menasveta, P. (2005). Development of Female- and Male-Specific Expression Markers in Ovaries and Testes of the Giant Tiger Shrimp (*Penaeus monodon*). International Shrimp Symposium, BIOTHAILAND 2005, 2-5 November 2005, Bangkok, Thailand (Oral presentation).

Biography

Miss Bavornlak Khamnamtong was born on July 23, 1975 in Ubonratchathani Province, Thailand. She graduated with the degree of Bachelor of Science (Biochemistry) and Master of Science (Biotechnology) from Chulalongkorn University in 1997 and in 2000, respectively. She has studied for the degree of Doctoral of Science (Biotechnology) at the Program of Biotechnology, Chulalongkorn University since 2001.