CHAPTER III

RESULTS

Giant Tiger Prawn DNA Extraction

Total DNA was extracted from the pleopods using a proteinase K-phenol-chloroform extraction procedure of Davis et al. (1986) with slight modifications. From agarose gel electrophoresis as compared with standard DNA markers (λ/ *Hin*dIII), the individual extracted DNA migrated as high molecular weight fragments of greater than 23.1 kb (Figure 3.1). The $O.D_{260/280}$ ratio was higher than 1.8 indicating high purity. The DNA yield was about 1.5-3.0 μg/pleopod. The quality of obtained DNA was suitable for molecular procedures for the development of microsatellite DNA markers, e.g. restriction endonuclease digestion, cloning and being a template for PCR amplification in order to test the assessment of microsatellite alleles as described in Chapter II.

Preparation of Giant Tiger Prawn Genomic DNA for Cloning

To test the activity of restriction enzymes, the genomic DNA was separately digested with each of 4 restriction enzymes; *Alu*I, *Hae*III, *Rsa*I, and *Hin*cII. The agarose gel electrophoresis analysis of digested DNA in Figure 3.2

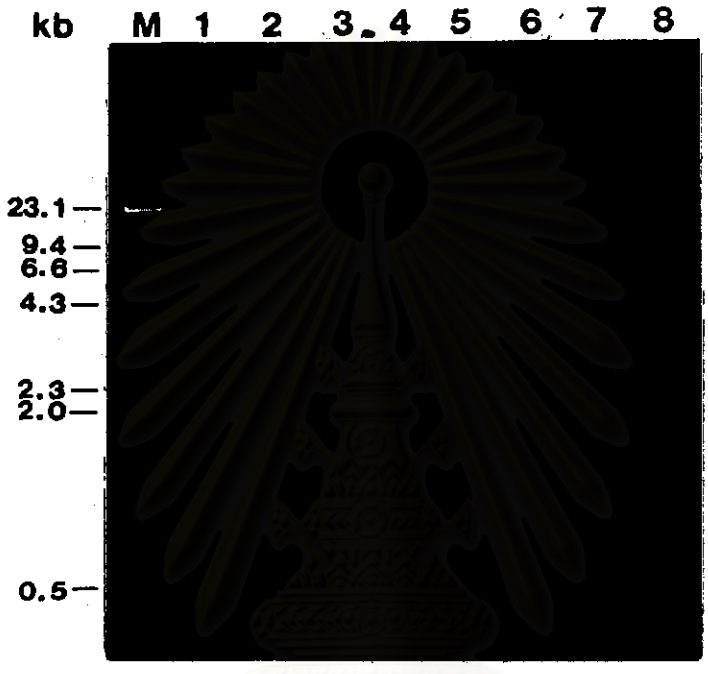


Figure 3.1 Ethidium bromide staining of extracted DNA from *P. monodon* pleopods. The DNA were subjected to electrophoresis on 0.8% agarose gel at 100 volt for 2 hours.

Lane M : DNA markers (λ /*Hin*dIII)

Lanes 1 - 8 : extracted prawn DNA

Figure 3.2 Ethidium bromide staining patterns of one individual *P. monodon* DNA separately digested with various 4 restriction enzymes. The DNA was subjected to electrophoresis on 1.5% agarose gel at 100 volt for 2 hours.

Lane M : DNA markers (λ/*Hin*dIII)

Lane 1 : undigested prawn DNA

Lane 2-5 : prawn DNA digested with *Alu*I, *Hae*III, *Rsa*I and *Hin*cII, respectively

showed that the DNA pattern resulted from 6-cutter enzyme *Hin*cII (GTPy/PuAC) digestion still had a high molecular weight band indicating an incomplete digestion, while 4-cutter enzymes; *Alu*I (AG/CT), *Hae*III (GG/CC), and *Rsa*I (GT/AC) gave the smear pattern of DNA lower than 23.1 kb.

For cloning, genomic DNA from one *P. monodon* (50 μg) was digested with a mixture of 4 restriction enzymes including *Alu*I, *Hae*III, *Rsa*I, and *Hin*cII (25 units each), all of which produced blunt ended DNA fragments. The DNA fragments were separated on a 1.5% low melting agarose gel with a 100 bp size standard ladder. Genomic DNA fragments of 300-700 bp were recovered from the gel by using a phenol freeze fracture procedure of Qian and Wilkinson (1996). The yield of recovery DNA was estimated to be 100 ng/μl using agarose gel electrophoresis as compared with standard DNA markers (λ/ *Hin*dIII) in Figure 3.3.

Library Construction and Screening

Genomic DNA fragments of 300-700 bp were blunt-end ligated with *Sma*I - digested and phosphatased pUC18 DNA vector (Pharmacia). Portions of the ligation mixture were transformed into *E. coli* DH5α cells by two procedures; $CaCl_2$ method and Electroporation. Transformants were selected with LB agar plates containing 50 μg/ml ampicillin. Partial genomic libraries of *P. monodon* from both transformation procedures consisted of about 18,000

Figure 3.3 Ethidium bromide staining of the recovery yield of genomic DNA fragments of 300 to 700 bp from low melting agarose gel. The DNA was run on 1.5% agarose gel at 100 volt for 2 hours.

Lane M : DNA markers (100 bp ladder)

Lane 1 : An individual *P. monodon* DNA digested with a mixture of *Alu*I, *Hae*III, *Rsa*I and *Hin*cII.

Lane 2 : recovered DNA fragments

transformant clones. They were lifted onto whatman paper filters and screened for microsatellites by colony hybridization with a ^{32}P end-labeled $(GT)_{15}$ oligonucleotide probe. A total of about 1,500 clones (8.3%) were tested positive for hybridization signal. An example of the results of colony hybridization was shown in Figure 3.4. Individual positive clones which gave strong hybridization signal were picked up for DNA sequencing.

Characterization of Microsatellites

1. Type of microsatellite arrays found in *P. monodon*

Plasmids from 184 of the 1,500 clones which were identified as putatively positive to a $(GT)_{15}$ probe were isolated and subjected to DNA sequencing. One hundred and two clones (55.4% of all sequenced clones) were found to contain microsatellites, twenty-three of which contained two or more microsatellite regions (separated by three or more bases). A total of 131 microsatellite loci were observed. Although the library was screened for $(GT/AC)_n$ microsatellites, several other microsatellite motifs were also picked up by chance (Tables 3.1 to 3.3). A sequence was scored as a microsatellite when the numbers of repeats were ≥ 10 for mononucleotide repeats, ≥ 6 for dinucleotide repeats, ≥ 4 for trinucleotide repeats, and ≥ 3 for tetra, penta and hexanucleotide repeats (Stalling et al., 1991; Estoup et al., 1993). In this study, one clone contained hexanucleotide microsatellite, two clones contained

Figure 3.4 Autoradiographs of colony hybridization screening for microsatellites from *P. monodon* genomic library with a ^{32}P-labeled $(GT)_{15}$ oligonucleotide probe. Each filter was from different plates. Colonies which gave strong hybridization signal as indicated by the arrows were picked up for sequencing.

Table 3.1 Perfect microsatellites in *P. monodon* genome

Type	Characteristic of repeat unit	No. of loci
mononucleotide	$(A/T)_n$	3
dinucleotide	$(GT/AC)_n$	23
	$(AG/CT)_n$	1
	$(AT/TA)_n$	8
	$(TC/GA)_n$	1
trinucleotide	$(ATT/TAA)_n$	1

Table 3.2 Imperfect microsatellites in *P. monodon* genome

Type	Characteristic of repeat unit	No. of loci
mononucleotide	$(A/T)_n$	1
	$(C/G)_n$	1
dinucleotide	$(GT/AC)_n$	52
	$(AT/TA)_n$	7
	$(CT/AG)_n$	5
	$(CA/TG)_n$	2

Table 3.3 Compound microsatellites in *P. monodon* genome

Type	Characteristic of repeat unit	No. of loci
mono - mono	$(A)_{10}(G)_{32}$	1
mono - di	$(A)_{31}(GT)_{54}$	1
	$(T)_{37}(GT)_{35}$	1
	$(T)_{30}(AC)_{60}$	1
di - di	$(AC)_{10}(GT)_{25}$	1
	$(AG)_{8}(AC)_{32}$	1
	$(AG)_{15}(GT)_{20}$	1
	$(AT)_{21}(AG)_{23}$	1
	$(AT)_{12}(CT)_{25}$	1
	$(AT)_{n}(GT)_{n}$	8
	$(CT)_{14}(AC)_{70}$	1
di - tetra	$(GT)_{8}(GAGT)_{6}$	1
	$(GT)_{16}(CGTT)_{8}$	1
tri - di	$(AAC)_{20}(AC)_{6}$	1
di - di - di	$(AT)_{35}(AC)_{80}(AG)_{20}$	1
	$(CT)_{37}(AC)_{17}(CT)_{15}$	1
	$(TG)_{16}(AT)_{31}(AG)_{30}$	1
di - tri - di	$(TG)_{44}(ATG)_{5}(TG)_{45}$	1
mix	$(AAGTGT)_{8}AA(GT)_{3}(AGTGTG)_{4}A(GT)_{31}(AG)_{26}$	1

tetranucleotide microsatellites, three clones contained trinucleotide microsatellites and six clones contained mononucleotide microsatellites. Ninety-six clones contained dinucleotide microsatellites, 77 of which had $(GT/AC)_n$ repeats as the main dinucleotide repeat units and 23 clones contained $(AT/TA)_n$ motifs.

One hundred and thirty-one isolated microsatellite sequences were classified into three categories according to Weber (1990), namely, perfect, imperfect and compound repeats. Perfect repeats are uninterrupted stretches of the repeat units, while imperfect repeats contain one to three intervening bases within the stretches. Compound repeats have different motifs adjacent to each other. Most of the *P. monodon* microsatellites found in this study were imperfect repeats (68 loci, 51.91%, Table 3.2), thirty-seven loci were perfect repeats (28.24%, Table 3.1) and twenty-six loci were compound repeats (19.85%, Table 3.3). Examples of the sequences of perfect, imperfect and compound microsatellite repeats and a false positive clone were shown in Figures 3.5, 3.6, 3.7 and 3.8, respectively.

2. Distribution of $(GT/CA)_n$ sequence

A total of 97 $(GT/AC)_n$ microsatellite arrays were identified from 77 clones. A rough estimate of the average distance between arrays $(GT/AC)_n$ arrays occuring in the genome was calculated. The partial genomic library of approximately 18,000 clones containing an average insert size of 500 bp was

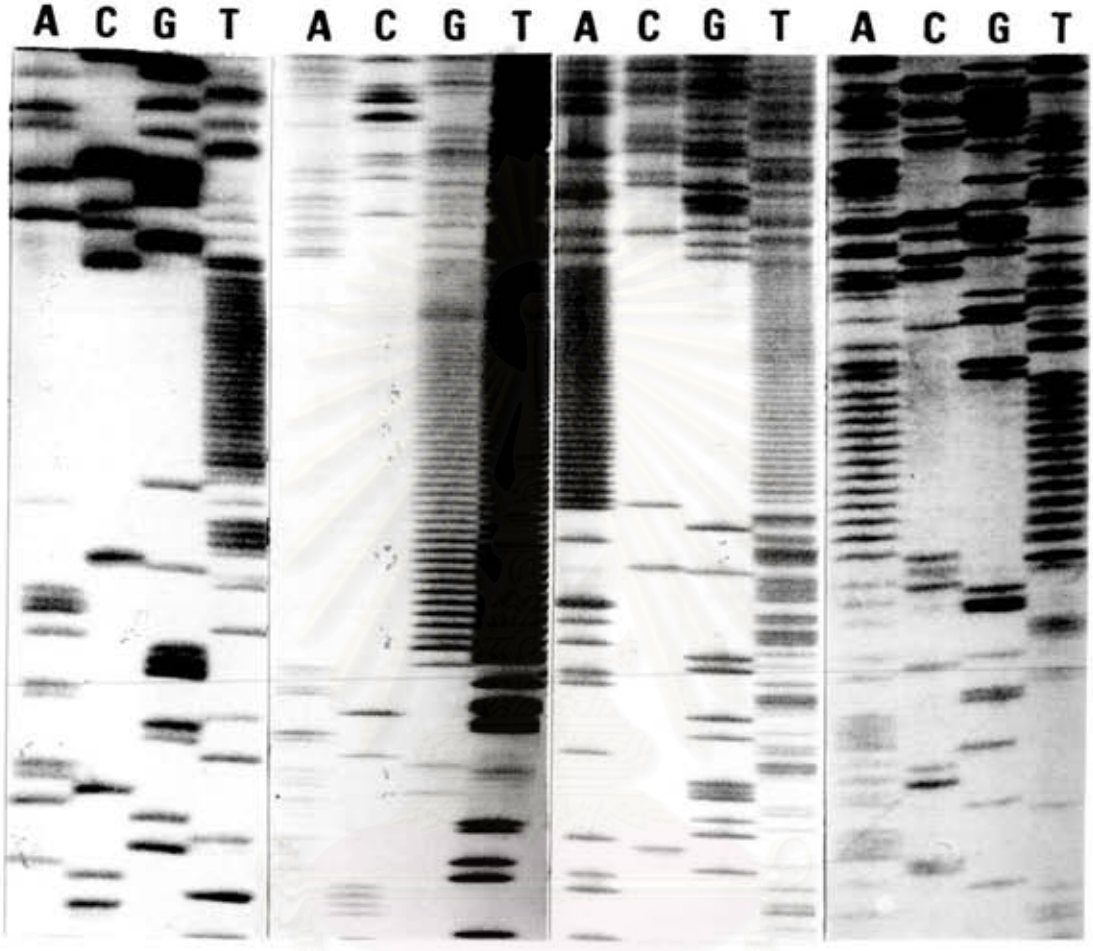


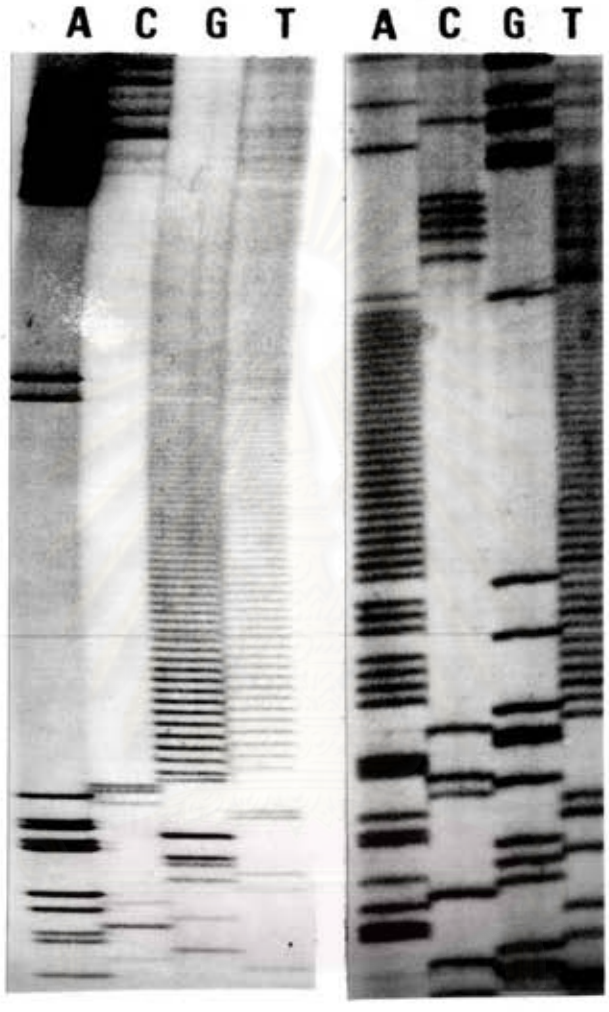
Figure 3.5 DNA sequences of perfect microsatellites

a. Clone No. 61 : $(T)_{32}$

b. Clone No. 380 : $(GT)_{50}$

c. Clone No. 14 : $(AT)_{35}$

d. Clone No. 388 : $(ATT)_{12}$

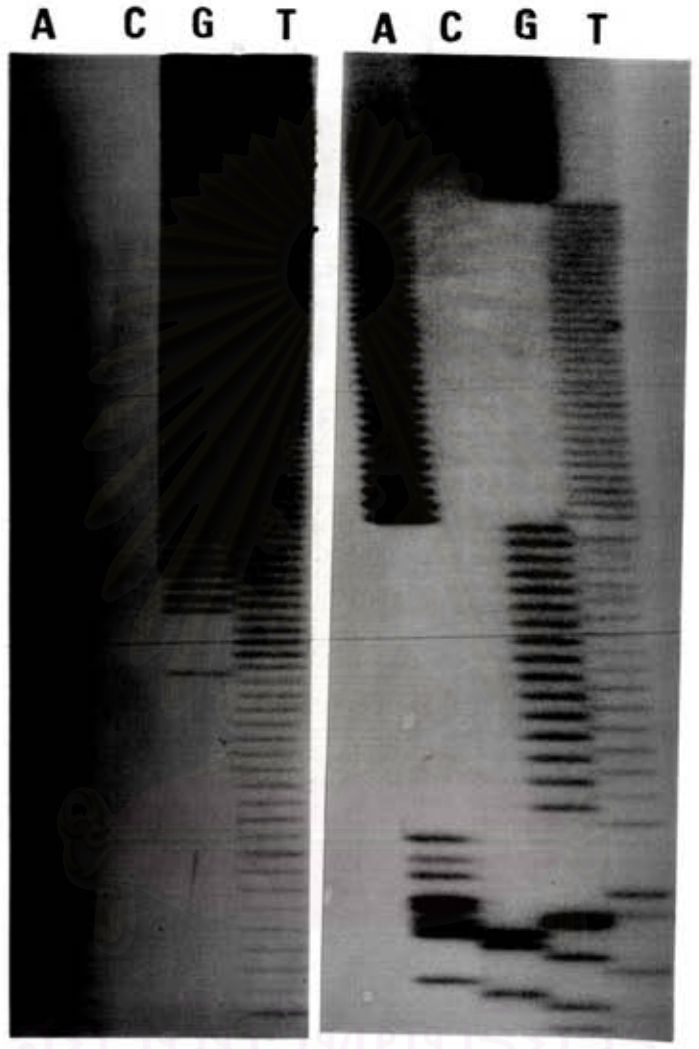


a. b.

Figure 3.6 DNA sequences of imperfect microsatellites

a. Clone No. 93-1 : $(GT)_{46}AT(GT)_3AT(GT)_{57}$

b. Clone No. 439 : $(AT)_4GT(AT)_3GT(AT)_{27}$



a. b.

Figure 3.7 DNA sequences of compound microsatellites

a. Clone No. 437 : $(AT)_{20}G(AT)_4(GT)_{38}T(GT)_{47}$

b. Clone No. 93-2 : $(TG)_{16}(AT)_{31}(AG)_{30}$

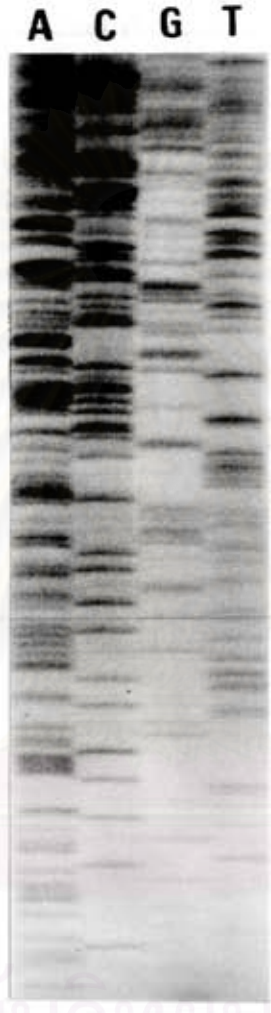


Figure 3.8 DNA sequence of a false positive clone

constructed, representing 18,000 x 500 = $9x10^6$ bp of giant tiger prawn genomic DNA. The assumption was made that $(GT/AC)_n$ sequences were distributed evenly throughout the prawn genome. The crude average distance between neighbouring microsatellites can be estimated by dividing the total length of screened DNA by the number of isolated microsatellites. Thus, in the cloned fraction of the giant tiger prawn genome, $(GT/AC)_n$ microsatellites occurred on an average of every 92.8 kb. The average distance of $(GT/AC)_n$ microsatellite in the genomes of various invertebrate species, fish and mammals was compared with that of giant tiger prawn as shown in Table 3.4.

3. Characteristics of the isolated $(GT/AC)_n$ microsatellites

The longest perfect stretch of ninety-seven of $(GT/AC)_n$ microsatellite arrays which were identified from the 77 clones was plotted according to classes and numbers of uninterrupted repeat units as shown in Figure 3.9. The number of (GT/AC) repeats ranged from 6 to more than 66 bp. Most of the arrays of $(GT/AC)_n$ were imperfect motifs which made up 53.6% of a total of 97 loci. The most common size class for $(GT/AC)_n$ microsatellites in giant tiger prawn genome was 30-35 repeats.

Proportion of each category, the most common category class, and the longest size category for giant tiger prawn were compared with microsatellites from other species: honey bee (Estoup et al., 1993), human (Weber, 1990), pig (Wintero et al., 1992), Atlantic salmon (Slettan et al., 1993), rainbow trout

Table 3.4 Average distance in kb between $(GT)_n$ microsatellites in genome of invertebrate, various fish and mammal species compared to giant tiger prawn.

Species	Average distance (kb)	Source
Invertebrates		
Giant tiger prawn (*Penaeus monodon*)	92.8	The present study
Honey bee (*Apis mellifera*)	34	Estoup et al., 1993
European flat oyster (*Ostrea edulis*)	139	Naciri et al., 1995
Fish		
Atlantic cod (*Gadus morhua*)	7	Brooker et al., 1994
Atlantic salmon (*Salmo salar*)	90	Slettan et al., 1993
Brown trout (*Salmo trutta*)	23	Morris et al., 1996
Mammals		
Human (*Homo sapiens*)	28	Weber, 1990
Porcine (*Sus sp.*)	46	Wintero et al., 1992

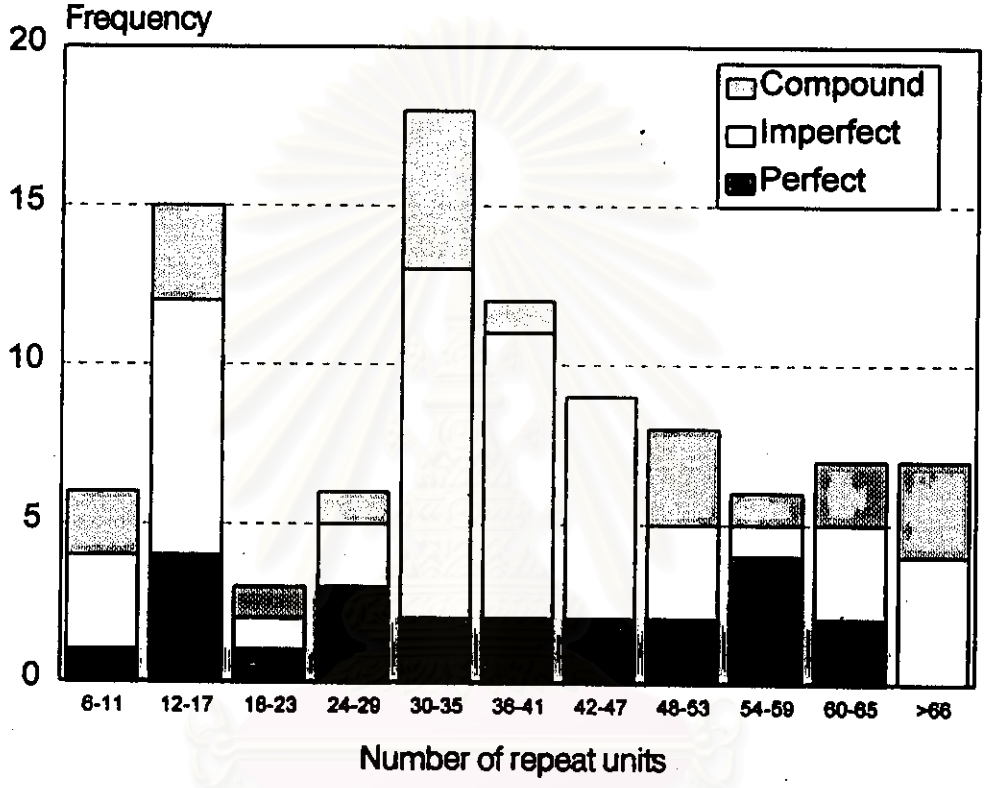


Figure 3.9 Frequency of different size classes of the longest uninterrupted $(GT/AC)_n$ microsatellite arrays from *P. monodon*. Size classes are represented as number of repeat units.

Table 3.5 Percentage of different categories and sizes of $(GT)_n$ microsatellites in honey bee, human, pig, Atlantic salmon, rainbow trout and Atlantic cod compared with giant tiger prawn genome.

	Giant tiger prawn	Honey bee	Human	Pig	Atlantic salmon	Rainbow trout	Atlantic cod
n	97	23	114	105	45	51	64
% perfect	23.7	48	64	71	80	56.9	48.4
% imperfect	53.6	22	25	19	20	31.4	45.3
% compound	22.7	10	11	10	0	11.7	6.3
Most common size class	30-35	7-9	12-15	16-18	6-9 (21-24)*	24-29	6-11 (30-35)*
Largest size class	>66	85-87	27-30	28-30	>33	>60	>60

* The criterion which define an imperfect repeat were altered to incorporate four or five intervening bases rather than three.

(Morris et al., 1996) and Atlantic cod (Brooker et al., 1994) as shown in Table 3.5.

Primer Design, Optimal PCR Conditions and Variability of Microsatellite Loci

PCR primers were designed from the unique flanking sequences of 7 microsatellites; 2 pairs for $(AT)_n$ microsatellites and 5 pairs for $(GT)_n$ microsatellites. Nucleotide sequences of *P. monodon* microsatellites and primers for amplification of loci by PCR were shown in Table 3.6. Although partial or complete sequence data were obtained from 131 loci, several of them (more than 80%) had at least one cloning site located adjacent to or very close to the microsatellite sequence, thus preventing the design of primer from unique flanking sequences. Most of the flanking regions of microsatellites in clones that contained 2 or more microsatellite regions were too short to use for primer design. Some microsatellites were flanked by sequences consisting mainly of the same bases contained in the microsatellite, making the primer design difficult.

By testing 8 individual prawns from Kruntung (the Andaman Sea) and Angsila (the Gulf of Thailand), it was found that 3 of 7 microsatellite primer sets, namely, Pmo 14, Pmo 18 and Pmo 386, produced fragments whose sizes were as expected, the others gave nonspecific amplifications. The PCR

Table 3.6 Nucleotide sequences of *P. monodon* microsatellites and primers for amplification of loci by PCR.

Locus	Repeat unit	5'-3' Primer	Annealing temperature (°C)	Expected size (bp)
Pmo 1	$(AT)_{32}$	[a]CTATTAACCAACATGCTCTG	51	120
		[b]TGTAATGAGGTTCAGCGTG		
Pmo 2	$(GT)_{18}$	[a]CTACCTGCGACCATCGTGAC	53	90
		[b]GGTACCCCGAATACGAGC		
Pmo 14	$(AT)_{35}$	[a]GCTTATCTTCATTCTGCGTCAT	57	190
		[b]AATGCTATATCCACTACAAACTG		
Pmo 18	$(GT)_{60}$	[a]TGTCATTCTTCTATTACGTGTC	55	160
		[b]GACTGACATCAACCATATACC		
Pmo 62	$(GT)_{30}$	[a]GTTACCGTAACCTTTGTGCAG	55	110
		[b]CCTTAGCGTTTGTTTGCGTC		
Pmo 380	$(GT)_{50}$	[a]TCACACTCACACTCTTGGTC	55	200
		[b]ATCATGCTGTACACGCGCTC		
Pmo 386	$(GT)_{30}$	[a]CGGTATCGGGTTAAAGAGT	47	120
		[b]TACAATGTTCATAATTCCTG		

a = forward primer

b = reverse primer

conditions for each primer set such as annealing temperatures, ratios of unlabeled to radioactive labeled primer and magnesium concentrations were optimized as follows.

Pmo 1 locus

Under the standard PCR condition as described in Materials and Methods, two different PCR procedures for amplification of Pmo 1 locus, i.e. one used ^{32}P-labeled reverse primer and the other used labeled forward primer, were performed at 51°C for the initial annealing temperature. Several nonspecific amplification fragments were detected from 8 individual templates as shown in Figure 3.10. Although manipulations of PCR conditions were done to get rid of the nonspecific products by raising the annealing temperature to 53°C and varying the concentrations of Tween20 in PCR amplifications (0.05% (normal), 0.025% and without Tween20), nonspecific products were still observed and alleles could not be scored as shown in Figure 3.11.

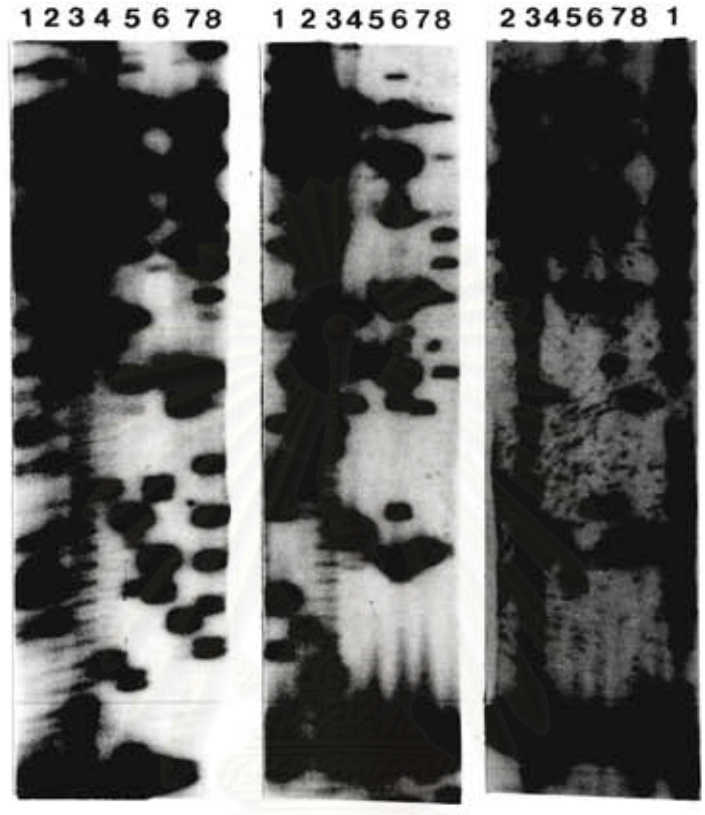
Pmo 2 locus

The initial annealing temperatures for Pmo 2 locus were estimated to be 59°C and 53°C for forward and reverse primers, respectively. PCR amplifications of Pmo 2 locus were performed at 2 different annealing temperatures, 53°C and 58°C, under the standard PCR condition for both labeled primers. Nonspecific amplification fragments were detected from 8 individual templates as shown in Figures 3.12 and 3.13.

Figure 3.10 PCR amplification patterns of Pmo 1 locus from eight individual *P. monodon* DNA (1-4: from Kruntrung, the Andaman sea and 5-8: from Angsila, the Gulf of Thailand) at 51°C for the initial annealing temperature under the standard PCR condition.

a. Forward primer labeled

b. Reverse primer labeled



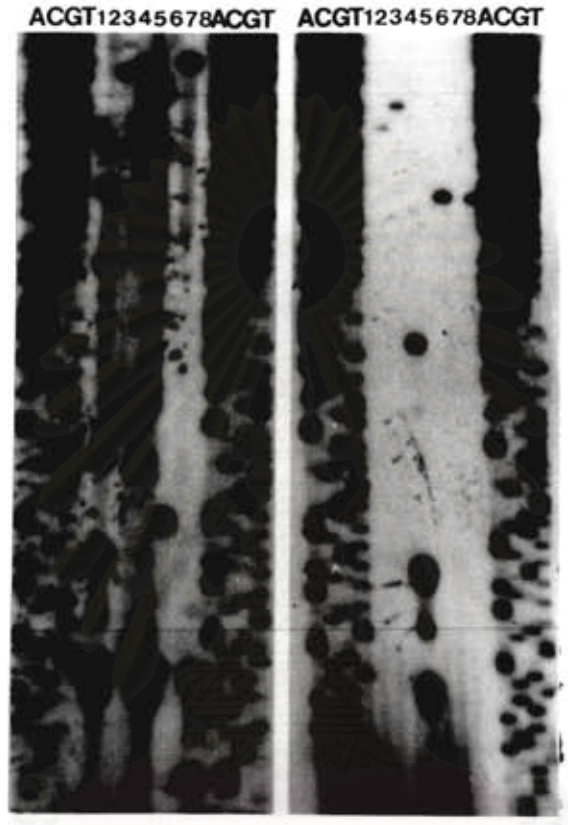
a. b. c.

Figure 3.11 PCR amplification patterns of Pmo 1 locus from eight individual *P. monodon* DNA (1-4: from Kruntrung, the Andaman sea and 5-8: from Angsila, the Gulf of Thailand) at the annealing temperature of 53°C and varying the concentrations of Tween20 in PCR amplifications by using labeled forward primer.

a. normal condition (0.05% Tween20)

b. 0.025% Tween20

c. without Tween20

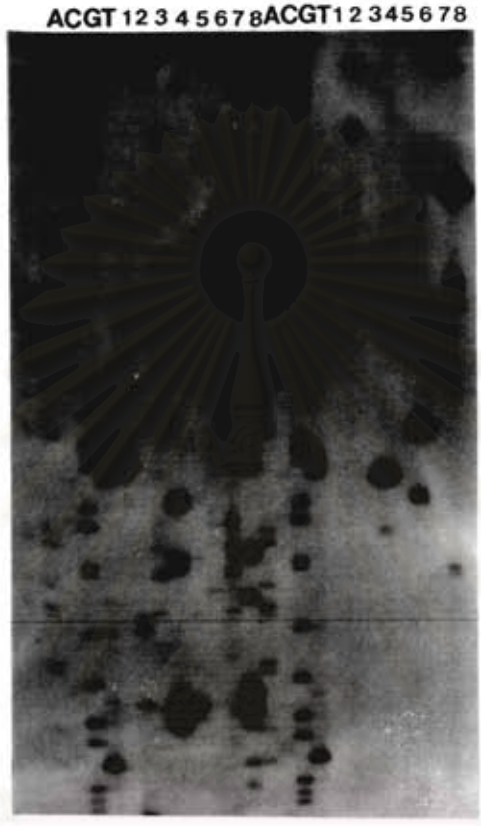


a. b.

Figure 3.12 PCR amplification patterns of Pmo 2 locus from eight individual *P. monodon* DNA (1-4: from Kruntrung, the Andaman sea and 5-8: from Angsila, the Gulf of Thailand) at 53°C for the initial annealing temperature under the standard PCR condition. The M13 sequence was used as size marker.

a. Reverse primer labeled

b. Forward primer labeled



a. b.

Figure 3.13 PCR amplification patterns of Pmo 2 locus from eight individual *P. monodon* DNA (1-4: from Kruntrung, the Andaman sea and 5-8: from Angsila, the Gulf of Thailand) at the annealing temperature of 58°C under the standard PCR condition. The M13 sequence was used as size marker.

a. Reverse primer labeled

b. Forward primer labeled

Pmo 62 locus

The initial annealing temperatures for Pmo 62 locus were estimated to be 57°C and 55°C for forward and reverse primers, respectively. PCR amplifications of Pmo 62 locus were initially performed at 55°C under the standard PCR condition for both labeled primers. The PCR patterns in Figure 3.14 showed nonspecific amplifications from 8 individual templates. Two different annealing temperatures, 53°C and 58°C, were used on subsequent trial. Nonspecific amplification fragments were still detected while expected fragments were not amplified as shown in Figure 3.15.

Pmo 380 locus

The initial annealing temperatures for Pmo 380 locus were estimated to be 55°C and 57°C for forward and reverse primers, respectively. PCR amplifications of Pmo 380 locus were initially performed at 55°C under the standard PCR condition for both labeled primers. The PCR patterns in Figure 3.16 showed nonspecific amplifications from 8 individual templates. The bands were faint due to low activity of radioisotope. Decreasing annealing temperature to 53°C was tried but the expected fragments were not amplified as shown in Figure 3.17.

Pmo 18 locus

PCR amplifications of Pmo 18 locus were performed at the initial annealing temperature of 55°C under the standard PCR condition for both

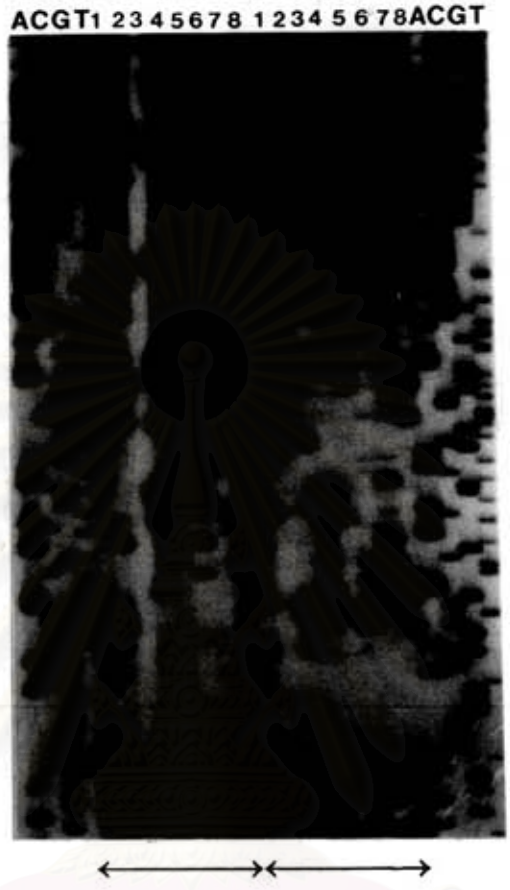


Figure 3.14 PCR amplification patterns of Pmo 62 locus from eight individual *P. monodon* DNA (1-4: from Kruntrung, the Andaman sea and 5-8: from Angsila, the Gulf of Thailand) at 55°C for the initial annealing temperature under the standard PCR condition. The M13 sequence was used as size marker.

a. Forward primer labeled

b. Reverse primer labeled

a. b.

Figure 3.15 PCR amplification patterns of Pmo 62 locus from eight individual *P. monodon* DNA (1-4: from Kruntrung, the Andaman sea and 5-8: from Angsila, the Gulf of Thailand) at 2 different annealing temperatures under the standard PCR condition by using labeled reverse primer. The M13 sequence was used as size marker.

a. 53°C

b. 58°C

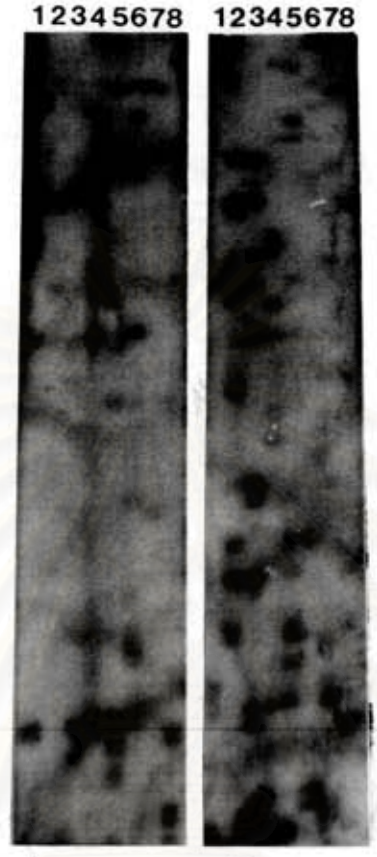


Figure 3.16 PCR amplification patterns of Pmo 380 locus from eight individual *P. monodon* DNA (1-4: from Kruntrung, the Andaman sea and 5-8: from Angsila, the Gulf of Thailand) at 55°C for the initial annealing temperature under the standard PCR condition.

a. Forward primer labeled

b. Reverse primer labeled

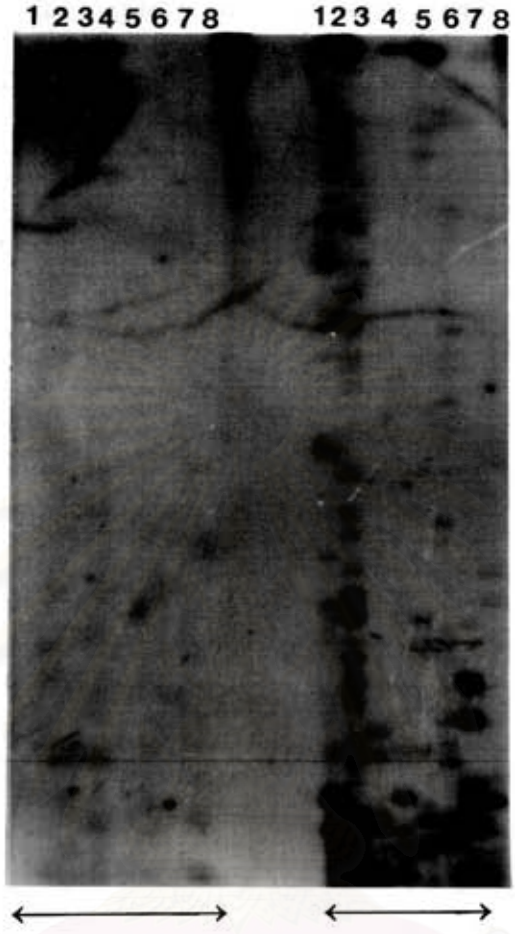


Figure 3.17 PCR amplification patterns of Pmo 380 locus from eight individual *P. monodon* DNA (1-4: from Kruntrung, the Andaman sea and 5-8: from Angsila, the Gulf of Thailand) at the annealing temperature of 53°C under the standard PCR condition.

a. Forward primer labeled

b. Reverse primer labeled

labeled primers. Polymorphic fragmjuents were obtained from 8 individual templates as shown in Figure 3.18. It was found that 11 alleles were unambiguously scored from a total of 16 possible alleles. All 8 templates were heterozygous. The sizes of 11 obseved alleles ranged from 108 to 160 bp. The sizes of the first band of each allele which were obtained from the PCR amplifications with the labeled reverse primer were determined using the M13 sequence markers as shown in Table 3.7.

Pmo 386 locus

The initial annealing temperatures for Pmo 386 locus were estimated to be 51°C and 47°C for forward and reverse primers, respectively. PCR amplifications of Pmo 386 locus were performed at two different annealing temperatures, 47°C and 45°C, under the standard PCR condition for both labeled primers. The results in Figure 3.19 revealed intense bands of expected PCR amplification products with stutter bands. However, the alleles from PCR amplifications of the labeled reverse primer were not scorable because of the interference from nonspecific amplification products. On the other hand, faint bands with stutters were obtained from PCR amplifications with the labeled forward primer. Optimization of PCR conditions were done in order to get the scorable products by changing the annealing temperatures and the concentration of Tween20 as summarized in Table 3.8. The results from trials No. 3 and 4 indicated that PCR conditions without Tween20 gave better results

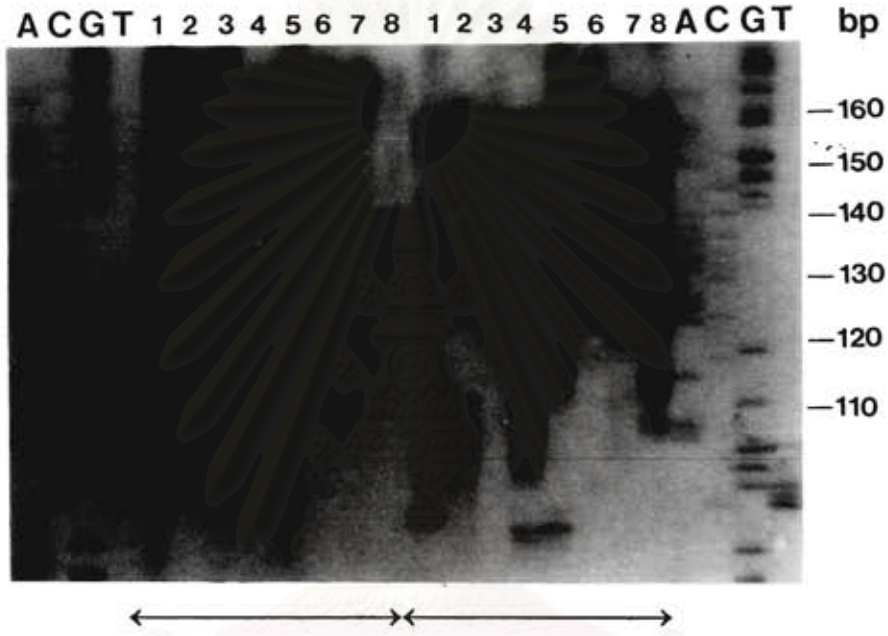


Figure 3.18 PCR amplification patterns of Pmo 18 locus from eight individual *P. monodon* DNA (1-4: from Kruntrung, the Andaman sea and 5-8: from Angsila, the Gulf of Thailand) at the annealing temperature of 55°C under the standard PCR condition. The M13 sequence was used as size marker.

a. Forward primer labeled

b. Reverse primer labeled

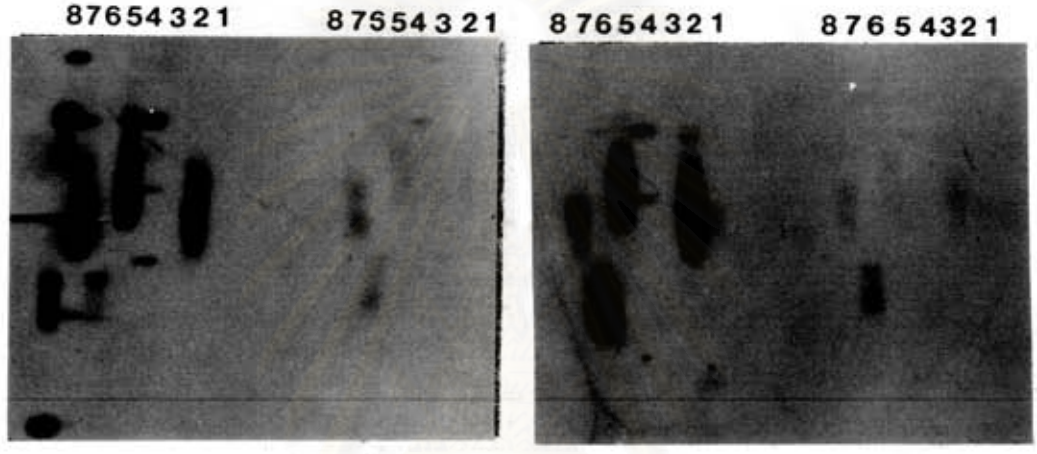
Table 3.7 Microsatellite variation of Pmo 18 locus in 8 individual prawns

Origin	Individual	Genotype
Kruntrung, the Andaman sea	1	160/116
	2	160/108
	3	154/142
	4	124/120
Angsila, the Gulf of Thailand	5	146/138
	6	160/148
	7	160/126
	8	160/130

Table 3.8 Optimization of PCR conditions for Pmo 386 locus

Trial No.	Labeled primer	PCR Conditions		Description	Note
		T_A (°C)	Tweeen20*		
1	R	47	+	Figure 3.19a	
	F	47	+	Figure 3.19b	
2	R	45	+	Figure 3.19c	T_A was lowered 2°C
	F	45	+	Figure 3.19d	from the initial T_A.
3	R	48	-	Figure 3.20a	T_A was raised 1°C to
	R	48	+	Figure 3.20b	reduce nonspecific background product.
	F	43	+	Figure 3.20c	T_A was lowered 4°C to
	F	43	-	Figure 3.20d	increase the intensity of band.
4	R	50	+	Figure 3.20e	T_A was raised 2°C
	R	50	-	Figure 3.20f	from the initial T_A.
	F	45	+	Figure 3.20g	T_A was lowered 2°C
	F	45	-	Figure 3.20h	from the initial T_A.
5	R	49	-	Figure 3.21a	19 other templates
	F	43	-	Figure 3.21b	were added.

* The plus sign (+) indicates the conditions with Tween20 and the minus sign (-) indicates the conditions without Tween20



a. b. c. d.

Figure 3.19 PCR amplification patterns of Pmo 386 locus from eight individual *P. monodon* DNA (1-4: from Kruntrung, the Andaman sea and 5-8: from Angsila, the Gulf of Thailand) at two annealing temperatures, 47°C and 45°C, under the standard PCR condition.

a. Reverse primer labeled, 47°C

b. Forward primer labeled, 47°C

c. Reverse primer labeled, 45°C

d. Forward primer labeled, 45°C

Figure 3.20 PCR amplification patterns of Pmo 386 locus from eight individual *P. monodon* DNA (1-4: from Kruntrung, the Andaman sea and 5-8: from Angsila, the Gulf of Thailand) at various annealing temperatures with or without Tween20. The M13 sequence was used as size marker.

a. Reverse primer labeled, 48°C, without Tween20

b. Reverse primer labeled, 48°C, with Tween20

c. Forward primer labeled, 43°C, with Tween20

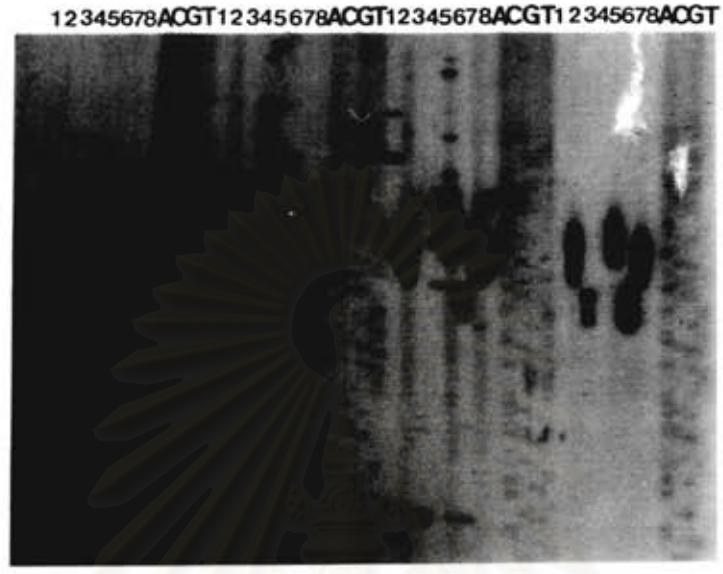
d. Forward primer labeled, 43°C, without Tween20

e. Reverse primer labeled, 50°C, with Tween20

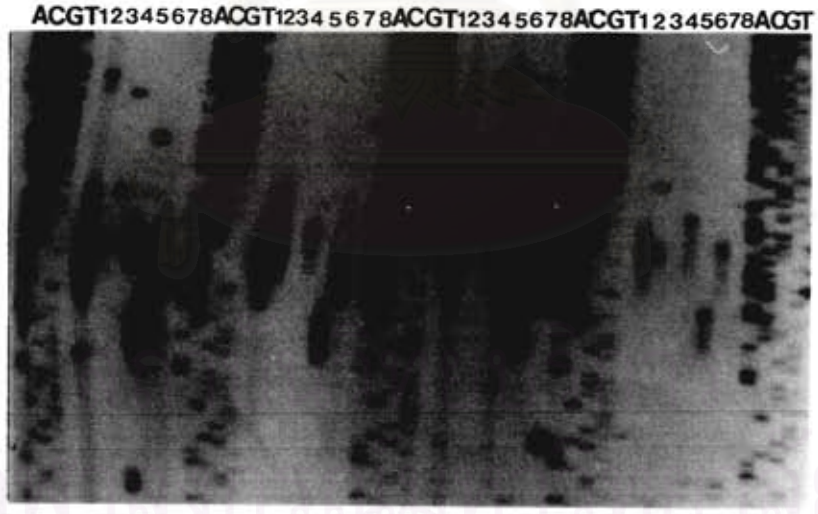
f. Reverse primer labeled, 50°C, without Tween20

g. Forward primer labeled, 45°C, with Tween20

h. Forward primer labeled, 45°C, without Tween20


12345678ACGT12345678ACGT12345678ACGT12345678ACGT

a. b. c. d.

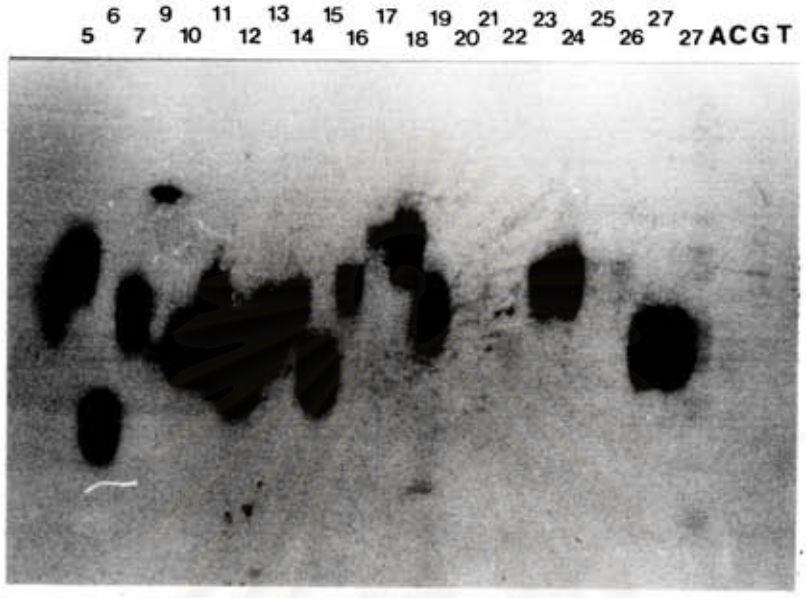

ACGT12345678ACGT12345678ACGT12345678ACGT12345678ACGT

e. f. g. h.

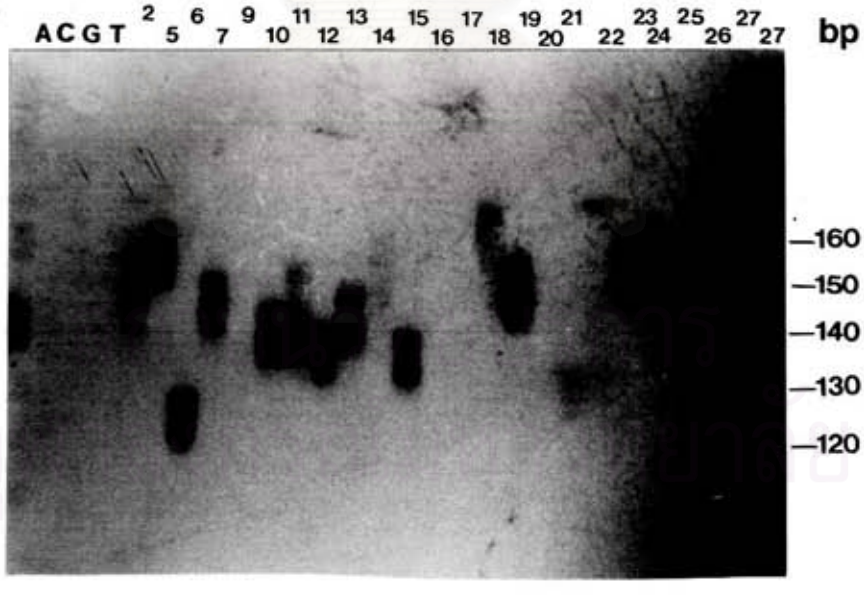
Figure 3.21 PCR amplification patterns of Pmo 386 locus from 23 individual *P. monodon* DNA (2, 14-16: from Kruntrung, the Andaman sea; 5-7, 9-13: from Angsila, the Gulf of Thailand; 17-22: from Trat, the Gulf of Thailand and 23-27: from Indonesia) under the optimal PCR conditions from Table 3.8. The M13 sequence was used as size marker.

a. Reverse primer labeled, 49°C, without Tween20

b. Forward primer labeled, 43°C, without Tween20


5 6 7 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 27 A C G T

a.


A C G T 2 5 6 7 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 27
bp
160
150
140
130
120

b.

Table 3.9 Microsatellite variation of Pmo 386 locus in 19 individual prawns

Origin	Individual	Genotype
Kruntrung	2	150/144
Angsila	5	158/158
Angsila	6	124/124
Angsila	7	146/146
Angsila	9	124/124
Angsila	10	140/140
Angsila	11	148/138
Angsila	12	136/136
Angsila	13	144/144
Kruntrung	14	154/144
Kruntrung	15	136/136
Trat	18	164/164
Trat	19	152/152
Trat	21	132/132
Trat	22	142/142
Indonesia	23	156/156
Indonesia	24	158/158
Indonesia	26	140/140
Indonesia	27	144/144

with the annealing temperature between 48 and 50°C for PCR amplifications of the labeled reverse primer and 43°C for PCR amplifications of the labeled forward primer. Since the PCR patterns were composed of only homozygous alleles in all 8 templates, other 19 individual templates, 5 individual prawns from Angsila, the Gulf of Thailand, 3 individual prawns from Kruntrung, the Andaman sea, 6 individual prawns from Trat, the Gulf of Thailand and 5 individual prawns from Indonesia, were tested to verify other alleles of heterozygosity. Four positive templates were used as control reactions in the experiment of trial No. 5. After 15 hours of exposure time with one intensifying screen, 4 templates were not amplified which may be due to the inappropriate concentration of templates. The other 19 templates showed polymorphic fragments which were scored for the allelic genotypes as shown in Table 3.9. The sizes of the first band of each allele which were obtained from the PCR amplification with the labeled forward primer were estimated with those of the M13 sequencing markers. It was found that 15 alleles were unambiguously scored from a total of 38 possible alleles. Sixteen templates from 19 scorable templates were homozygous. The sizes of 15 observed alleles ranged from 124 to 164 bp.

Pmo 14 locus

PCR amplifications of Pmo 14 locus were performed at the initial annealing temperature of 57°C under the standard PCR condition for both

labeled primers. It was found that there was only a signal of the primer-dimers after one day of exposure (data not shown). This was suspected as a result from the low activity of the radioisotope, thus the longer exposure of one week was done. The results were shown in Figure 3.22. There were PCR amplifications of microsatellite products with stutter bands but some were faint. Judging from the results, however, one week of exposure time was set for all later trials. Adjustment of the concentrations of unlabeled primers was done to increase the incorporation of the labeled primer. It was found that the reduction of unlabeled reverse and forward primers in PCR amplifications gave better results (Figures 3.23a, b). However, the intensity of the PCR amplified primer-dimers at the bottom of the X-ray film was very high. The production of these primer-dimers was a significant artifact which greatly reduced the efficiency of template amplification. The adjustment of PCR conditions were done in order to get the scorable products and get rid of the primer-dimers as summarized in Table 3.10. Magnesium concentration may also affect the formation of primer-dimer artefacts (Innis, 1990). The optimization of the magnesium ion concentration was done in trial no. 3. Increasing the annealing temperature and adjusting a ratio of unlabeled primer to 1:8 should enhance discrimination against incorrectly annealed primers (Doug Cook, personnal communication). Results from trial No. 4, however, still showed the problem. PCR amplifications in trial No. 5 with 50% reduction of both unlabeled primers and increasing the

concentration of $MgCl_2$ to 15 mM at the annealing temperature of 52°C gave better results. However, the alleles from all 8 individual prawns were not clearly scorable. To get better results, 17 other individual prawns were retested with the latter condition. Unfortunately, the polymorphic fragments from one week of exposure time were too faint and ambiguous to score as shown in Figure 3.25.

Table 3.10 Optimization of PCR conditions for Pmo 14 locus

Trial No.	Labeled primer	PCR conditions	Description	Note
1	F	T_A = 57°C	Figure 3.22a	PCR amplification of labeled reverse primer gave better results.
	R	T_A = 57°C	Figure 3.22b	
2	R	T_A = 57°C and		
		(a) decrease unlabeled reverse primer by 50%	Figure 3.23a	The reaction in (b) gave better results than a control standard condition in figure 3.23e.
		(b) decrease unlabeled reverse and forward primer by 50%	Figure 3.23b	
		(c) without Tween20	Figure 3.23c	(The signal was faint due to the low activity of the radioisotope.)
		(d) increase concentration of template to 25 ng	Figure 3.23d	
3	R	T_A = 57°C and adjust the concentration of $MgCl_2$ to		With 15 mM of $MgCl_2$, the results were better.
		(a) 5 mM $MgCl_2$	Data not shown	(The standard $MgCl_2$ concentration in PCR amplification was 10 mM)
		(b) 15 mM $MgCl_2$	Data not shown	
4	R,F	T_A = 65°C and adjust the unlabeled primer conc^n (F:R, R:F) to the ratio of 1:8 with 15mM $MgCl_2$	See only the primer-dimers (data not shown)	The annealing temperature was increased 8°C.
5	R	T_A = 52°C and		The annealing temperature was decreased 5°C.
		(a) decrease unlabeled reverse and forward primer 50%	Figure 3.24a	
		(b) decrease unlabeled reverse and forward primer 50% and set $MgCl_2$ to 15 mM	Figure 3.24b	
6	R	Use the same condition as in 5 (b) with 17 other templates	Figure 3.25	

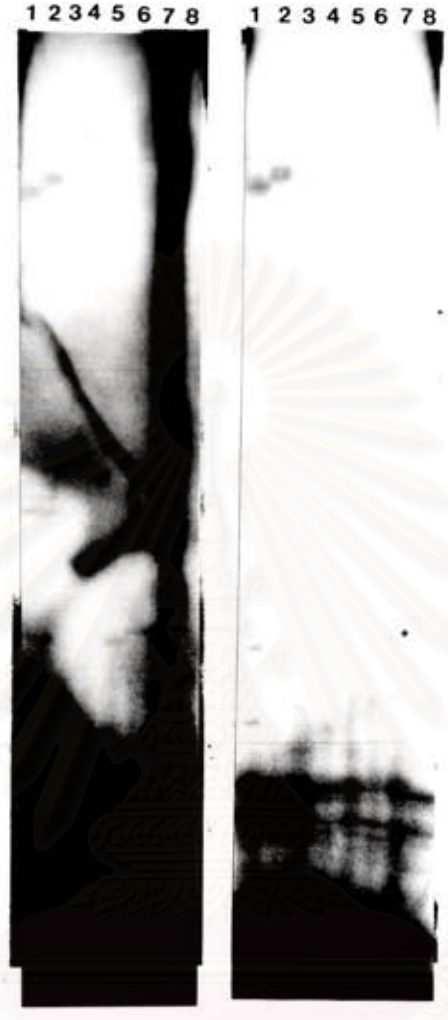


Figure 3.22 PCR amplification patterns of Pmo 14 locus from eight individual *P. monodon* DNA (1-4: from Kruntrung, the Andaman sea and 5-8: from Angsila, the Gulf of Thailand) at the initial annealing temperature of 57°C under the standard PCR condition.

a. Forward primer labeled

b. Reverse primer labeled

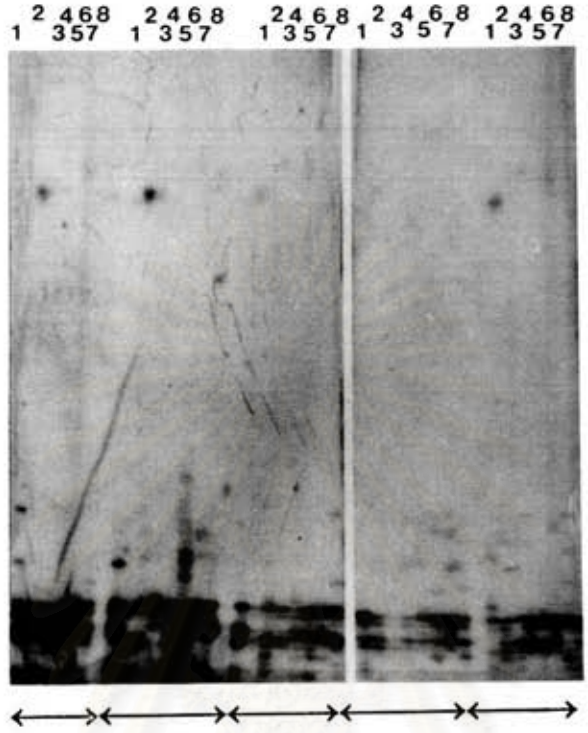


Figure 3.23 PCR amplification patterns of Pmo 14 locus from eight individual *P. monodon* DNA (1-4: from Kruntrung, the Andaman sea and 5-8: from Angsila, the Gulf of Thailand) by using labeled reverse primer at the annealing temperature of 57°C with:

a. Decreasing unlabeled reverse primer by 50%

b. Decreasing either unlabeled reverse or forward primer by 50% each

c. Without Tween20

d. Increasing the concentrations of templates to 25 ng

e. Standard conditions

a. b.

Figure 3.24 PCR amplification patterns of Pmo 14 locus from eight individual *P. monodon* DNA (1-4: from Kruntrung, the Andaman sea and 5-8: from Angsila, the Gulf of Thailand) by using labeled reverse primer at the annealing temperature of 52°C with:

a. Decreasing either unlabeled reverse or forward primers by 50% each

b. Decreasing either unlabeled reverse or forward primers by 50% each and 15 mM $MgCl_2$

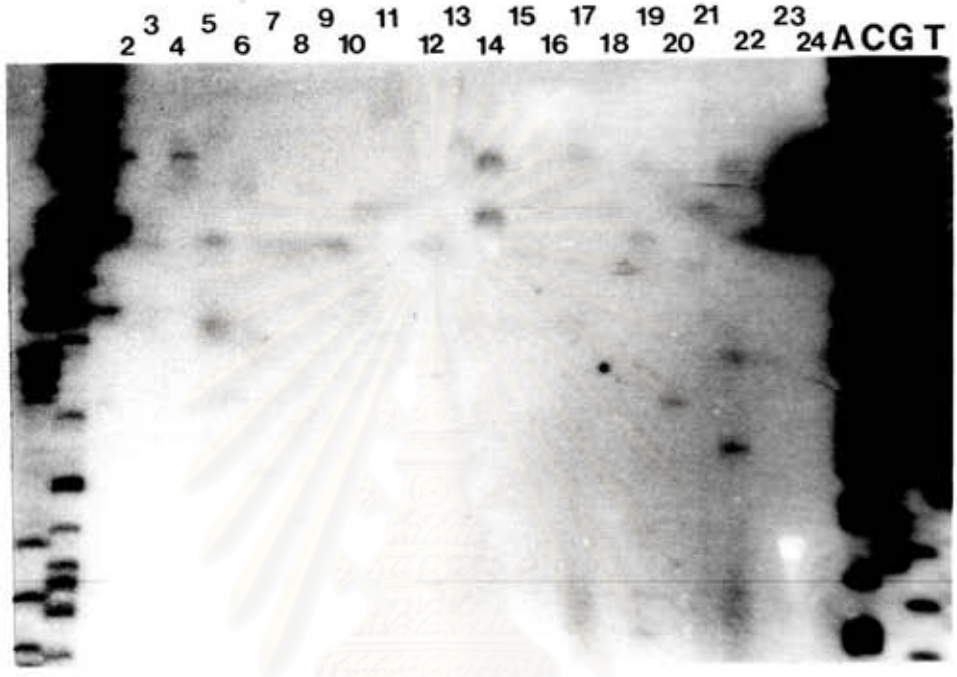


Figure 3.25 PCR amplification patterns of Pmo 14 locus from 24 individual *P. monodon* DNA (2-4, 15-18: from Kruntrung, the Andaman sea; 5-14: from Angsila, the Gulf of Thailand and 19-25: from Trat, the Gulf of Thailand) at the annealing temperature of 52°C by using labeled reverse primer with 50% decreasing of either unlabeled reverse or forward primer and 15 mM $MgCl_2$. The M13 sequence was used as size marker.