Chapter III

Results

3.1 Prawn DNA samples

Genomic DNA of *P. monodon* was prepared from pleopods for establishment of partial genomic DNA libraries using a standard phenol chloroform extraction (Supungul, 1998). The quality and quantity of genomic DNA were determined by agarose gel electrophoresis and UV spectrophotometry, respectively. Agarose gel electrophoresis (0.7%) showed the DNA to be primarily high molecular weight (>23.1 kb) with a modest amount of degradation (Figure 3.1). Spectrophotometry showed an OD_{260} to OD_{280} ratio higher than 1.8. The quality of DNA samples was suitable for development of microsatellite DNA markers. The DNA concentration of each pleopod sample was between 15-40 μg.

3.2 Preparation of prawn DNA for partial genomic library construction

Each partial genomic library was constructed using DNA from a single individual of *P. monodon*. Black tiger prawn genomic DNA was fragmented using either sonication or digestion with enzymes to obtain appropriate DNA fragments (300-1,500 bp) for cloning. Sonicated DNA gave the major smear pattern with a range of fragment between 500-1,900 bp (Figure 3.2 D). Enzymatic digestion of genomic DNA gave both optimal and undesirable fragment sizes so enzymatic-digested DNA

was size selected. *Sau*3 AI-, *Hin*c II/*Alu* I-, and Dnase I digested fragments ranging between 300-1,500 bp were recovered from the gels (Figure 3.2 A, B and C).

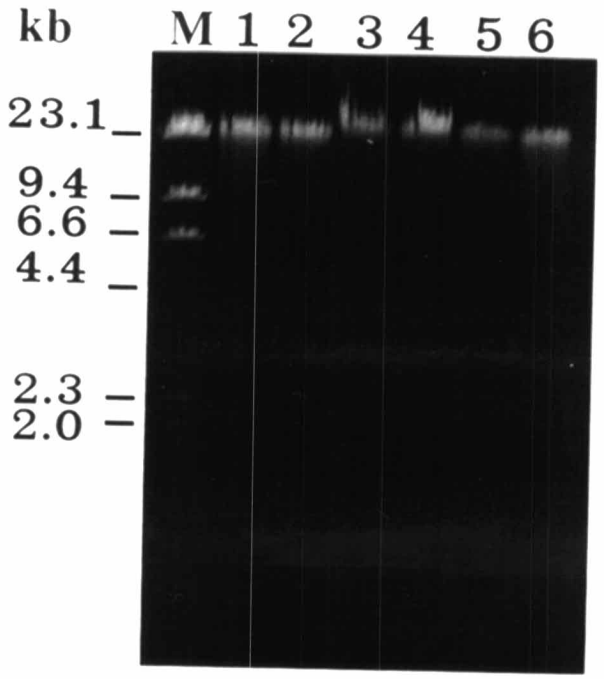
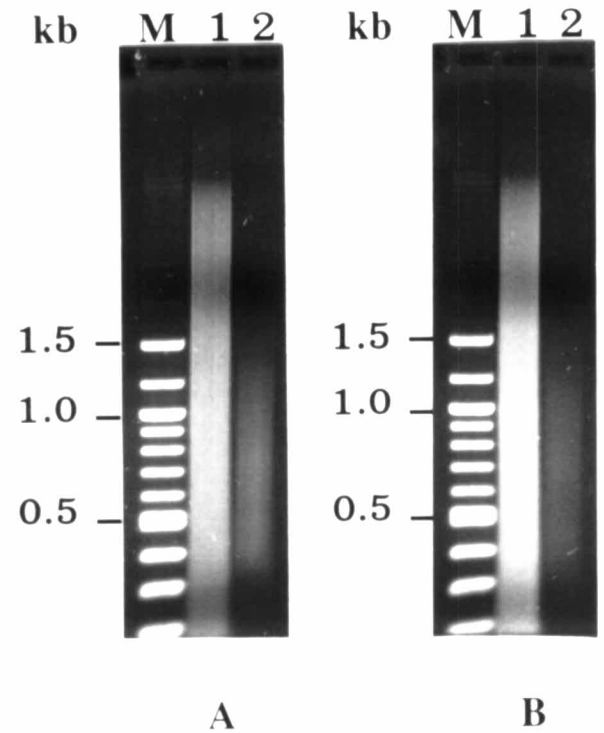


Figure 3.1 Ethidium bromide stained gel showing genomic DNA extracted from *P. monodon* pleopods (lanes 1-6). DNA was subjected to electrophoresis on 0.7% agarose gel at 100 V for 2 h. DNA molecular size markers is λ DNA/*Hin*d III (lane M).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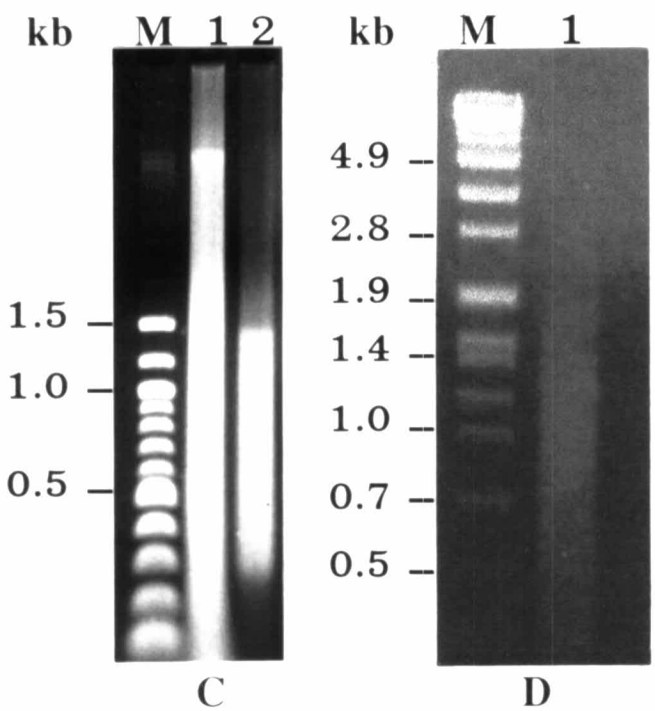
A B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M 1
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1.4
1.0
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0.5

C D

Figure 3.2 Ethidium bromide stained gels showing *P. monodon* DNA fragments used for library constructions. (A) DNA digested with *Hin*c II/*Alu* I (lane1) and the recovered DNA (lane 2). (B) DNA digested with *Sau3*A I (lane1) and the recovered DNA (lane 2). (C) DNA digested with Dnase I (lane1) and the recovered DNA (lane 2). (D) DNA fragmented with sonication (lane1). Lanes M (A, B, C) are a 100-bp ladders. Lane M (D) is a bacteriophage SSP1/*Eco*R I marker.

3.3 Construction of partial genomic libraries and isolation of microsatellites

3.3.1 Construction of conventional genomic libraries

To achieve a variety of DNA fragments for library construction, small insert genomic libraries were constructed from different DNA fragment types. Libraries A and C were constructed from sonicated DNA of *P. monodon* while libraries B and D were constructed from DnaseI- and *Hin*cII/*Alu*I-digested DNA fragments, respectively.

Previously, difficulties in obtaining usable microsatellite markers in *Penaeus* species have been reported (Tassanakajon et al., 1998; Moore et al., 1999) due to the complexity of microsatellite array and the extend length of repeats. High stringent conditions of hybridization and post-hybridization washes were applied in those previous reports. In this study, both high and low stringent conditions of hybridization and post-hybridization washes were applied for library screening. At low stringent conditions, short microsatellite repeats may be isolated resulting in a higher opportunity to develop usable microsatellite markers in *P. monodon*.

Tri- and tetranucleotide microsatellites were the target repeats to isolate from *P. monodon* genome. Unlike dinucleotide repeats, tri- and tetranucleotide microsatellites generally contain short repeat length and amplification products are easy to score (Crooijmans et al., 1997). In this study, nine microsatelltites; $(GAA)_n$, $(GATA)_n$, $(GGAT)_n$, $(GGAA)_n$, $(CACC)_n$, $(CAT)_n$, $(ATG)_n$, $(TCAG)_n$, $(CATA)_n$, were verified the prevalence in *P. monodon*. These tri- and tetranucleotide

microsatellites have been reported on the occurrence in plants, animals and humans (O'Reilly and Wright, 1995; Ma, Roder and Sorrells, 1996; Echt and May-Marquardt, 1997; Moore et al., 1999).

The conventional library A was preliminary produced to determine which microsatellites are relatively common in *P. monodon* genome. Ligated plasmid containing sonicated DNA fragments was transformed into commercial competent cells (Ultracompetent cells XL10-Gold^{TM}: Stratagene) which give high efficiency ($\geq 5x10^9$ transformants/μg of pUC 18 DNA). One transformation reaction was enough for screening with 5 different microsatellite probes (each probe/each screening). Approximately 9900, 4200, 7560, 1900 and 1900 recombinant clones were plated for screening with $(GAA)_n$, $(GATA)_6$, $(GGAT)_6$, $(GGAA)_5$ and $(CACC)_5$, respectively. Library screening was carried out at high stringent conditions. For oligonucleotide probes, high stringent conditions were hybridized at Tm-5°C whereas the $(GAA)_n$ concatamer was hybridization at 65°C. After hybridization, the unbound probe was washed out of the filters twice with 2X SSC, 0.1% SDS at the hybridization temperature for 10 min each and 0.2X SSC at the hybridization temperature for 10 min. The highest percentages of positive clones was obtained, when screening with $(GATA)_6$ followed by $(GAA)_n$ while $(GGAT)_6$, $(GGAA)_5$ and $(CACC)_5$ yielded either a few positive clones or no positive clones. The percentages of positive clones were 1.21% for $(GATA)_6$, 0.24% for $(GAA)_n$, 0.04% for $(GGAT)_6$, 0.05% for $(GGAA)_5$ and 0.00% for $(CACC)_5$ probes. Results of microsatellite isolation were summarized in Table 3.1.

The $(GATA)_n$ and $(GAA)_n$ repeats were found at high frequencies. To additionally isolate these abundant target microsatellite repeats, libraries B, C and D were produced and screened with the combination of $(GATA)_6$ and $(GAA)_8$ probes. Four additional repeat types, $(CAT)_n$, $(ATG)_n$, $(TCAG)_n$, $(CATA)_n$, were also verified the prevalence in *P. monodon* genome by screening libraries B and C with these repeat types.

For construction of libraries B, C and D, the ligated products were transformed into *E.coli* XL1-Blue using electrotransformation. All of the transformed-cell suspension from each transformation was plated out and screened with each set of microsatellite probes. Library screening was performed at low stringent conditions. Hybridization was carried out at Tm-10°C and unbound probe was washed off the filters twice with 2X SSC, 0.1% SDS at hybridization temperature for 10 min each. For libraries B and C, each transformation contained 2,100-2,640 recombinant colonies. For the library D, two ligation reactions were set up for transformation. Transformants from both ligation reactions were pooled for library screening. Approximately 5,080 recombinant clones were obtained. The library screening with combination of $(GAA)_8$ and $(GATA)_6$ probes showed high percentages of positive clones across all libraries. The percentages of positive clones were 1.16% for library B, 0.72% for library C and 1.32% for library D. The combination of $(CAT)_8$, $(ATG)_8$, $(TCAG)_5$ and $(CATA)_6$ probes was used to screen libraries B and C. Both libraries showed comparable percentages of positive clones, 0.38% for library B and 0.33% for library C (Table 3.1).

Results suggested that trinucleotide, $(GAA)_n$ and tetranucleotide, $(GATA)_n$ repeats presented in significant numbers of the *P. monodon* genome while $(GGAT)_n$, $(GGAA)_n$ and $(CACC)_n$ repeats were rare or did not exist. Examples of colony hybridization results were shown in Figure 3.3. Positive clone was picked. Plasmid DNA was extracted and digested with restriction enzymes to check the insert size. Clones with inserts ≥ 300 bp were selected for sequencing. Examples of plasmid DNA digestions were shown in Figure 3.4.

3.3.2 Construction of enriched genomic libraries of *P. monodon*

Because of the low abundance of tri- and tetranucleotide repeats in the genome, isolation of these type of microsatellites are successfully done by enrichment of the microsatellites. Two methods were used to enrich genomic libraries, the first method, microsatellites were enriched from recombinant clones of a genomic library whereas the second method, microsatellites were enriched from genomic DNA fragments before cloning. The libraries were enriched with either $(GAA)_n$ or $(GATA)_n$ repeats which were previously identified to be abundant in *P. monodon* genome by hybridization of conventional libraries.

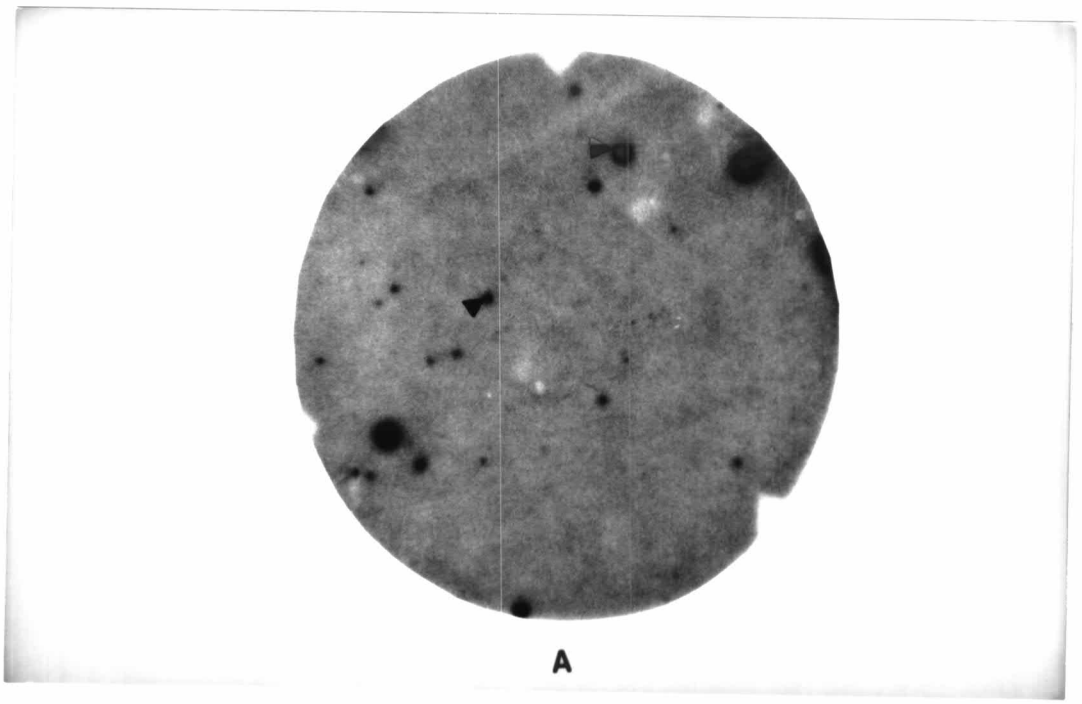
In the first method, the $(GATA)_n$ repeat was enriched. The initial library construction was done as in a standard protocol (non-enriched library) and the microsatellite-containing clones were isolated from pUC 18 genomic library with streptavidin-coated magnetic beads. Prawn DNA was sonicated and ligated into *Sma* I-digested pUC 18. The ligation mixture was transformed into commercial competent cells and transformed colonies were harvested from plates and plasmid DNA was extracted. Double-stranded plasmid DNA was then denatured and hybridized with 5′

Table 3.1 The number of colonies screened with different types of microsatellite probes and the percentages of positive clones obtained from conventional libraries.

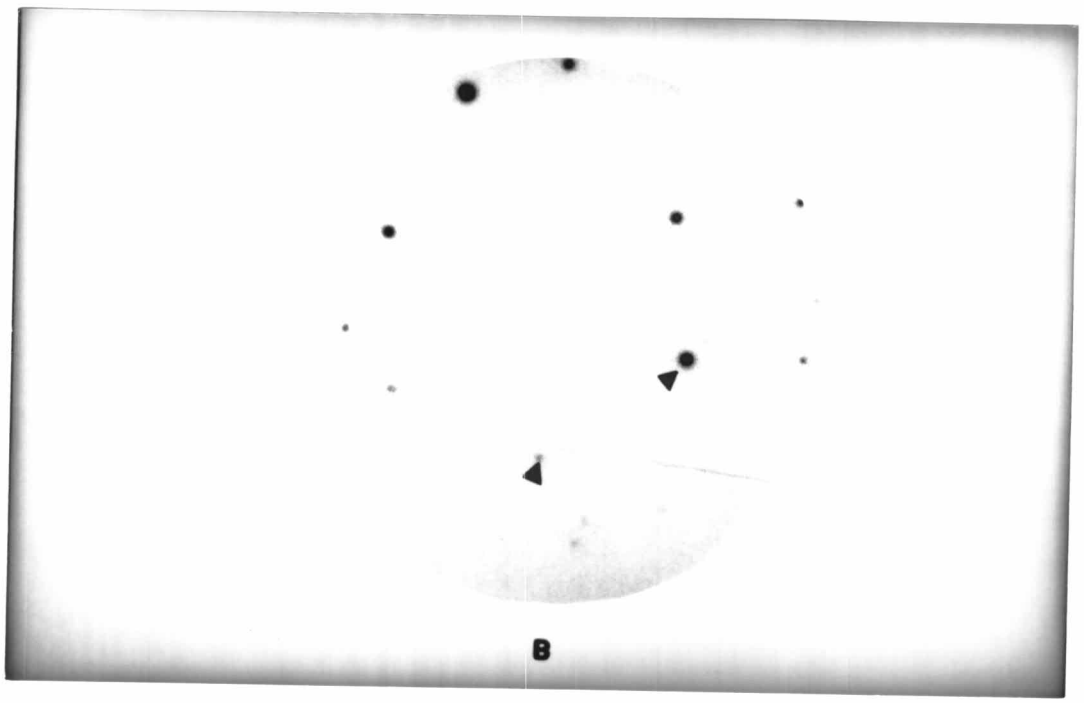
Library	Probe	Colonies screened	Positive clones (%)
A*	$(GAA)_n$	9,900	24 (0.24)
	$(GATA)_6$	4,200	51 (1.21)
	$(GGAT)_6$	7,560	3 (0.04)
	$(GGAA)_5$	1,900	1 (0.05)
	$(CACC)_5$	1,900	0 (0.00)
	Total	**25,460**	**79 (0.31)**
B#	$(GAA)_8$, $(GATA)_6$	2,500	29 (1.16)
	$(CAT)_8$, $(ATG)_8$, $(TCAG)_5$, $(CATA)_6$	2,400	9 (0.38)
	Total	**4,900**	**38 (0.78)**
C#	$(GAA)_8$, $(GATA)_6$	2,640	19 (0.72)
	$(CAT)_8$, $(ATG)_8$, $(TCAG)_5$, $(CATA)_6$	2,100	7 (0.33)
	Total	**4,740**	**26 (0.55)**
D#	$(GAA)_8$, $(GATA)_6$	5,080	67 (1.32)
	Total	**5,080**	**67 (1.32)**

*High stringency hybridization and washing conditions

#Low stringency hybridization and washing conditions



A



B

Figure 3.3 Autoradiograms of colonies hybridized with [γ-^{32}P]ATP labeled $(GAA)_8$ + $(GATA)_6$ oligonucleotide probes. (A) an autoradiogram of library C. (B) an autoradiogram of library D. Colonies indicated by arrows were picked up for sequencing.

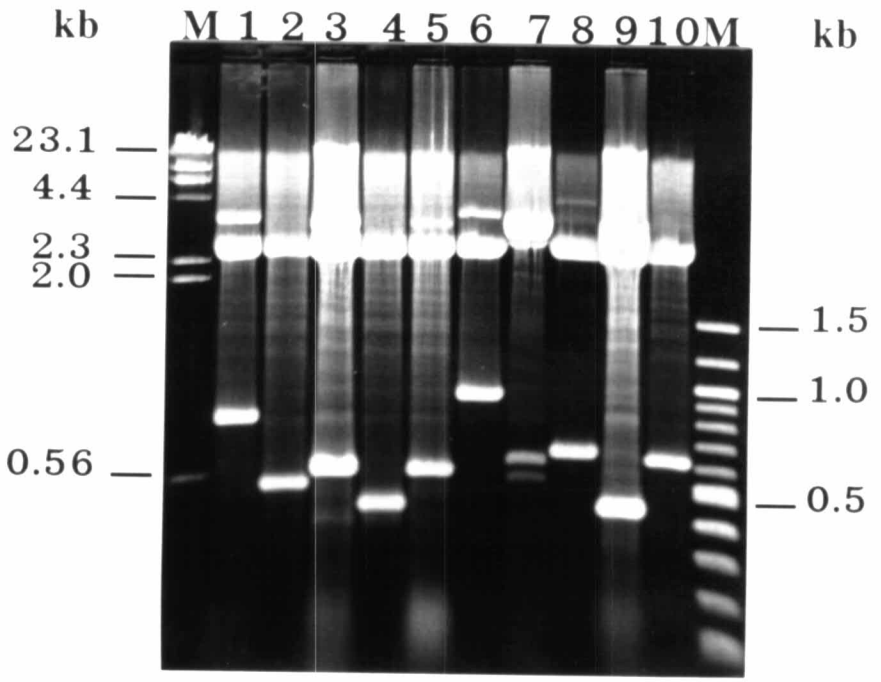


Figure 3.4 Agarose gel electrophoresis showing *Eco* RI/*Pst* I digested recombinant plasmids from the library D (lanes 1-10). Digested plasmid DNA was electrophoretically separated on 1% agarose gel at 100 V for 2 h, stained with ethidium bromide and visualized under a UV transilluminator. Lane M (on the left) is λDNA/*Hin*d III. Lane M (on the right) is 100-bp DNA ladder.

biotinylated $(GATA)_6$ probe at Tm-10°C. The biotinylated microsatellite probe-clone complexes were captured using streptavidin-coated magnetic beads. The complexes were washed with three different stringency wash solutions before the captured-DNA was eluted from the beads. Clones of the supernatant from the 2nd and 3rd washes and the final eluted DNA from beads were double-stranded and electroporated into *E. coli* XL1-Blue. The protocol of enrichment library was explained in Chapter II: Section 2.7.2.1 and Figure 2.1.

In total 169 and 1,036 colonies were obtained from transformation of clones from the 2nd and 3rd wash solutions, respectively while 339 colonies were obtained from transformation of the eluted clones from beads. Individual clones were grown in Terrific broth, plasmid DNA was extracted and digested with restriction enzymes. Two hundred and thirty-eight colonies were randomly chosen and digested with *Eco* RI and *Pst* I to check insert sizes. Only 34 colonies produced fragments of unique length whereas the rest produced fragments of similar length as can be seen in Figure 3.5. Thirty-four clones which produced fragments of unique length were selected for sequencing. Because of the high numbers of clones with similar insert size, several clones with similar insert size were selected for sequencing to verify redundant clones.

In the second method, microsatellites were enriched from genomic DNA fragments for $(GATA)_n$ and $(GAA)_n$ repeats. Prawn DNA was digested with *Sau*3 AI (Figure 3.2B) and ligated with adapters that contain a *Sau*3 AI restriction site. DNA fragments (300-1,500 bp) were subjected to magnetic bead capture using biotinylated microsatellite motif oligonucleotides, $(GATA)_6$ and $(GAA)_8$. Hybridization of DNA fragments with $(GATA)_6$ and $(GAA)_8$ was performed at Tm-5°C and Tm-10°C,

respectively. DNA fragments which had been pre-selected for the presence of microsatellite were amplified by PCR using sequences complementary to adapters as primers. The protocol of establishment of the enriched library was explained in Chapter II: Section 2.7.2.2 and Figure 2.1.

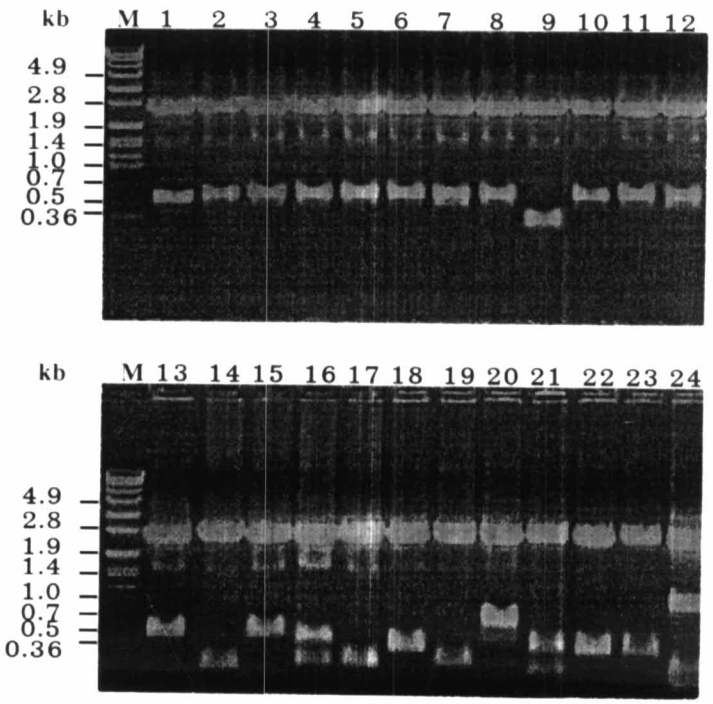


Figure 3.5 Agarose gel electrophoresis showing *Eco* RI/*Pst* I digested recombinant plasmid DNA from $(GATA)_n$ enriched library selected from the genomic library (lanes 1-24). Digested plasmid DNA was separated on 1% agarose gel at 100 V for 1 h, stained with ethidium bromide and visualized under UV transilluminator. Lanes M are the DNA size marker (Bacteriophage SSP1/*Eco* RI)

PCR products of the supernatant from the second and third washes, and final elution from the beads were shown in Figure 3.6. The predominant PCR products of each lane were smear between 300 to 1,500 bp, corresponding to the size selected DNA fragments. However, there was evidence of small amount of PCR products with unexpected size range (<300 and >1,500 bp) in each exponential amplification. The samples of *Sau*3 AI-digested PCR products from the 2nd and 3rd wash solutions and final eluted DNA from beads were ligated into *Bam* HI-digested pUC18 and electroporated into *E. coli* XL1-Blue.

For $(GATA)_n$ enriched library, recombinants were derived from the 2nd and 3rd wash solutions and final elution from beads containing 4,920; 5,860 and 5,610 colonies, respectively. Plasmid DNA was extracted from recombinant clones of each supernatant and digested with *Eco* RI/*Pst* I. This enrichment protocol provided clones with different insert sizes as can been seen in Figure 3.7. A total of 68 clones showed inserts with size ≥ 300 bp were selected for sequencing. For $(GAA)_n$ enriched library, recombinants were derived from the 2nd and 3rd wash solutions and final elution from beads containing 750, 933 and 1,000 clones, respectively. A total of 34 clones were selected for sequencing.

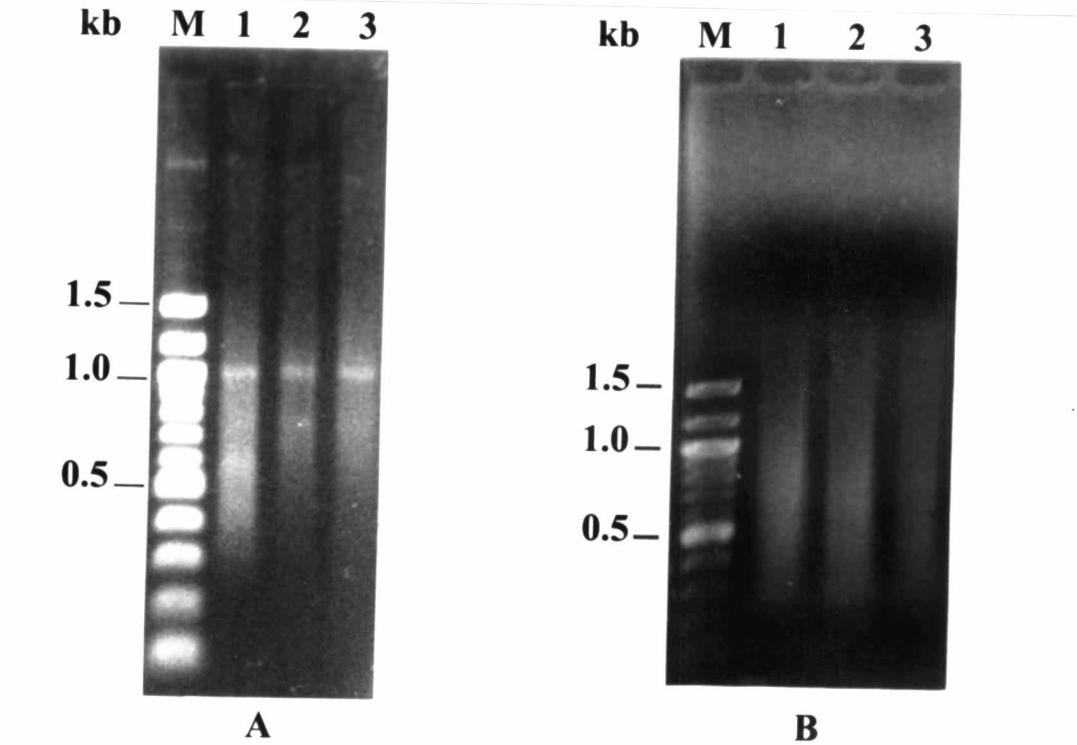


Figure 3.6 PCR amplification products of selected ssDNA fragments for microsatellite enrichment selected from DNA fragments. Amplification products were separated on 1.5% agarose gel and stained with ethidium bromide. (A) and (B) are the PCR amplification of selected ssDNA from $(GATA)_n$ and $(GAA)_n$ enrichment, respectively. Lanes M, a 100 bp ladder; lanes1-3, PCR products from 2nd wash (0.5X SSPE/0.1% tween 20), 3rd wash (0.5X SSPE/ 0.1% tween 20), and final elution with distilled water, respectively.

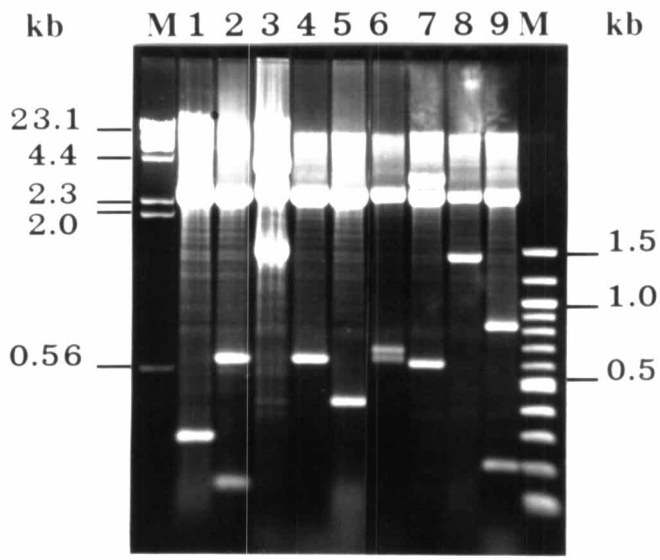


Figure 3.7 Agarose gel electrophoresis showing *Eco* RI/*Pst* I digested recombinant plasmid from $(GATA)_n$ enriched library selected from DNA fragments (lanes 1-9). Digested plasmid DNA was separated on 1% agarose gel at 100 V for 2 h, stained with ethidium bromide and visualized under UV transilluminator Lane M (on the left) is a λDNA/*Hin*d III marker. Lane M (on the right) is a 100-bp DNA ladder.

3.4 Determination of nucleotide sequences of clones isolated from *P. monodon* partial genomic libraries

3.4.1 Conventional libraries

According to Stalling et al. (1991) a sequence is considered to be a microsatellite when the number of the repeats is greater than 10 for mononucleotide, 6 for dinucleotide. 4 for trinucleotide and 3 for tetranucleotide, pentanucleotide and hexanucleotide. Microsatellites are interrupted by more than three bases considering separate loci. Out of 152 clones sequenced, 121 (79.6%) of these clones contained microsatellite loci. The highest percentages of positive clones containing microsatellites were found in library A (93.3%) following with library B (80.0%), C (73.7%) and D (51.5%), suggesting the specificity of library screening at high stringency conditions. The sequencing results of positive clones were summarized in Table 3.2.

As expected, most of microsatellite-containing clones of library A derived from screening of the library at high stringent conditions contained types of microsatellite repeats that were used to screen the library. Libraries B, C and D isolated microsatellite-containing clones at low stringent conditions yielded microsatellite repeats that were not used to screen the libraries at higher proportion when compared to the library A. For libraries B and C, clones containing microsatellite repeats that were used to screen the libraries were approximately 50% whereas that of library D yielded the lowest proportion, 17.65%.

Screening libraries B and C with combination of $(CAT)_8$, $(ATG)_8$, $(TCAG)_5$ and $(CATA)_6$, results of positive clones from the screening could not identify the percentage of positive clones for each repeat type. This parameter was calculated after these positive clones were sequenced. For the library B, 2 and 3 clones contained $(CAT)_n$ and $(ATG)_n$, respectively. This means screening of 2,400 clones yielded 0.08% positive clones for $(CAT)_n$ and 0.13% positive clones for $(ATG)_n$. For the library C, 1 clone contained $(CAT)_n$ and another contained $(ATG)_n$. This means screening of 2,100 clones yielded 0.05% positive clones for both probes. There was no clone containing either $(TCAG)_n$ or $(CATA)_n$. Results suggested that trinucleotide $(CAT)_n$ and $(ATG)_n$ were rare whereas tetranucleotide $(TCAG)_n$ and $(CATA)_n$ did not exist in *P. monodon* genome.

Table 3.2 The number of clones sequenced and the percentages of microsatellite-containing clones found from various conventional libraries.

Library	No. of clones Sequenced	Microsatellite-containing clones (%)	Clones without microsatellite (%)
A*	75	70 (93.3)	5 (6.7)
B#	25	20 (80.0)	5 (20.0)
C#	19	14 (73.7)	5 (26.3)
D#	33	17 (51.5)	16 (48.5)
Total	152	121 (79.6)	31 (20.4)

*High stringency hybridization and washing conditions

#Low stringency hybridization and washing conditions

3.4.2 Enriched libraries of *P. monodon*

One hundred and thirty-six clones from all enriched libraries were sequenced. One hundred and two clones (75%) contained microsatellite loci. The $(GATA)_n$ library selected from DNA fragments showed the highest percentages of microsatellite-containing clones (97.1%), followed by the $(GATA)_n$ library selected from genomic library (64.7%) and the $(GAA)_n$ library selected from DNA fragments (41.2%). The sequencing results of selected clones were summarized in Table 3.3.

The proportion of clones containing types of microsatellite repeats that were used to screen the libraries varied among each library. All of microsatellite-containing clones from the library of $(GATA)_n$ selected from genomic library contain $(GATA)_n$ while 89.4% from the library of $(GATA)_n$ selected from DNA fragments and 57.1% from the library of $(GAA)_n$ selected from DNA fragment contained $(GATA)_n$ and $(GAA)_n$, respectively.

Table 3.3 The number of clones sequenced and the percentages of microsatellite-containing clones found in enriched libraries.

Library	No. of clones Sequenced	Microsatellite-containing clones (%)	Clones without microsatellite (%)
$(GATA)_n$ selected from genomic library[#]	34	22 (66.7)	12 (32.3)
$(GATA)_n$ selected from DNA fragments[*]	68	66 (97.1)	2 (2.9)
$(GAA)_n$ selected from DNA fragments[#]	34	14 (41.2)	20 (58.8)
Total	136	102 (75.0)	34 (25.0)

*High stringent hybridization and washing conditions at Tm-5°C

#Low stringent hybridization and washing conditions at Tm-10°C

3.5 Characteristics of microsatellite sequences of *P. monodon*

3.5.1 Conventional libraries

Microsatellite repeat types

Upon sequencing, a total of 225 microsatellite loci were identified from 152 clones. Characteristics of microsatellite sequences in conventional libraries were

summarized in Table 3.4 and Figure 3.8. These microsatellites were 53 loci of $(GAA)_n$, 45 loci of $(GA)_n$, 40 loci of $(GATA)_n$, 18 loci of $(TG)_n$, 14 loci of $(TAA)_n$, 11 loci of $(ATG)_n$, 16 loci of $(TA)_n$, 9 loci of $(GGA)_n$, 7 loci of $(CAT)_n$, 4 loci of $(GGTA)_n$ and 1 locus of each of $(A)_n$, $(GTAT)_n$, $(GACT)_n$, $(TCG)_n$, $(TGC)_n$, $(GGAT)_n$, $(GGAA)_n$ and $(GGATA)_n$. Although clones were selected for either $(GATA)_n$, $(TCAG)_n$, $(CATA)_n$, $(GGAT)_n$, $(GGAA)_n$, $(CACC)_n$, $(GAA)_n$, $(CAT)_n$ or $(ATG)_n$, unexpected repeat types were detected. These unexpected microsatellites were revealed from either false-positive hybridization or the occurrence with expected microsatellite containing clones. Among microsatellite sequences which did not correspond to the probes used to screen, $(GA)_n$ was found at the highest frequency, following with $(TG)_n$.

Repeat length

There was a wide range of repeat numbers for microsatellite types. The length of dinucleotide microsatellites ranged from 6 to 87 repeats with an average of 25.5, that of trinucleotide microsatellites was from 5 to 88 with an average of 15.8 and that of tetranucleotide microsatellites from was 4 to 28 with an average of 7.9. The repeat length of dinucleotide was the longest. The maximum repeat number for di-, tri- and tetranucleotide repeats was 87 for $(TG)_n$, 88 for $(GAA)_n$ and 28 for $(GATA)_n$.

Microsatellite classes

Microsatellites were classified into 3 categories, perfect, imperfect and compound repeats (Weber, 1990). Perfect repeats are uninterrupted stretches of repeat units, while imperfect repeats have one to three intervening bases with repeat sequence on either side. Compound repeats consist of several different repeat types and are

separated by less than three bases. By these standard definitions, 103 (45.8%) of the repeat sequences were perfect, 70 (31.1%) were imperfect and 52 (23.1%) were compound microsatellites. Example of perfect, imperfect and compound microsatellite repeats were shown in Figure 3.9. Characteristics of compound microsatellite sequences were summarized in Table 3.5. Fifty-two loci of compound repeats associated with mono-, di-, tri-, tetra-, penta- or hexanucleotide repeats where those associated with dinucleotide repeats were the most frequent motifs. One to three repeat types of associated sequences were involved with each compound microsatellite locus. Out of 52 loci of compound microsatellites investigated, 41 loci associated with 1 type of repeat sequences, 7 with 2 types, 3 with 3 types and 1 with 4 types.

Table 3.4 Characteristics of microsatellite sequences in conventional libraries.

Library	Repeat type	No. of Loci	Range of repeat no.	Type of microsatellites		
				Perfect	Imperfect	Compound
A	$(GA)_n$	33	6-85	24	6	3
	$(TG)_n$	12	7-56	2	6	4
	$(AT)_n$	13	6-63	9	2	2
	$(GAA)_n$	39	5-35	15	21	3
	$(GGA)_n$	8	8-17	2	5	1
	$(ATG)_n$	3	5-17	1	2	0
	$(CAT)_n$	2	7, 17	1	0	1
	$(TAA)_n$	7	5-20	3	2	2
	$(GATA)_n$	37	4-16	13	6	18
	$(GTAT)_n$	1	5	1	0	0
	$(GGAT)_n$	1	4	1	0	0
	$(GGAA)_n$	1	6	1	0	0
	$(GGTA)_n$	4	4-16	4	0	0
	$(GGATA)_n$	1	6	1	0	0
	Total	**162**	-	**78 (48.2%)**	**50 (30.9%)**	**34 (21.0%)**
B	$(GA)_n$	2	24, 29	1	1	0
	$(TG)_n$	4	6-87	2	1	1
	$(AT)_n$	3	11-39	2	0	1
	$(GAA)_n$	6	9-30	1	2	3
	$(TGA)_n$	4	6-28	1	2	1
	$(TCA)_n$	2	24, 27	0	0	2
	$(TAA)_n$	1	38	1	0	0
	$(GATA)_n$	2	18, 28	1	0	1
	$(GACT)_n$	1	20	0	0	1
	Total	**25**	-	**9 (36.0%)**	**6 (24.0%)**	**10 (40.0%)**

Table 3.4 (continued).

Library	Repeat type	Loci	Range of repeat no.	Type of microsatellites		
				Perfect	Imperfect	Compound
C	$(GA)_n$	3	19-75	0	2	1
	$(GAA)_n$	5	15-88	1	3	1
	$(GGA)_n$	1	5	1	0	0
	$(TGA)_n$	3	6-24	1	0	2
	$(TCA)_n$	1	30	0	0	1
	$(TCG)_n$	1	12	1	0	0
	$(TAA)_n$	2	5, 14	2	0	0
	Total	**16**	-	**6 (37.5%)**	**5 (31.3%)**	**5 (31.3%)**
D	$(A)_n$	1	29	1	0	0
	$(GA)_n$	7	10-70	2	4	1
	$(TG)_n$	2	6, 30	0	2	0
	$(GAA)_n$	3	5-22	2	1	0
	$(TGA)_n$	1	8	1	0	0
	$(TCA)_n$	2	9, 11	1	1	0
	$(TGC)_n$	1	8	1	0	0
	$(TAA)_n$	4	9-26	2	1	1
	$(GATA)_n$	1	7	0	0	1
	Total	**22**	-	**10 (45.5%)**	**9 (40.9%)**	**3 (13.6%)**
Total (all categories)		**225**	-	**103 (45.8)**	**70 (31.1)**	**52 (23.1)**

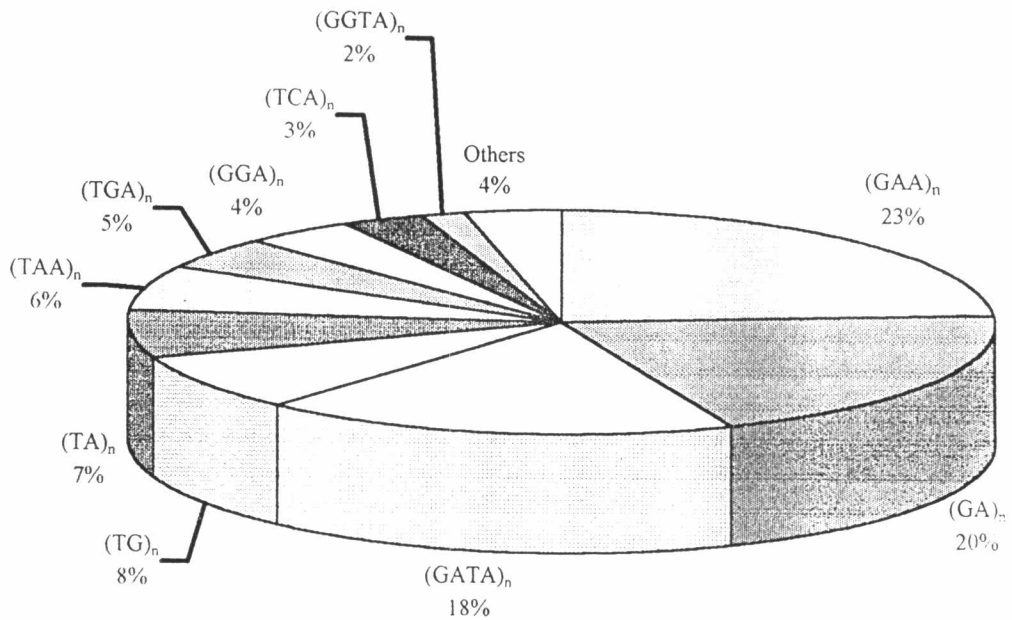


Figure 3.8 The percentages of various repeat types of microsatellite loci isolated from conventional genomic libraries of *P. monodon*.

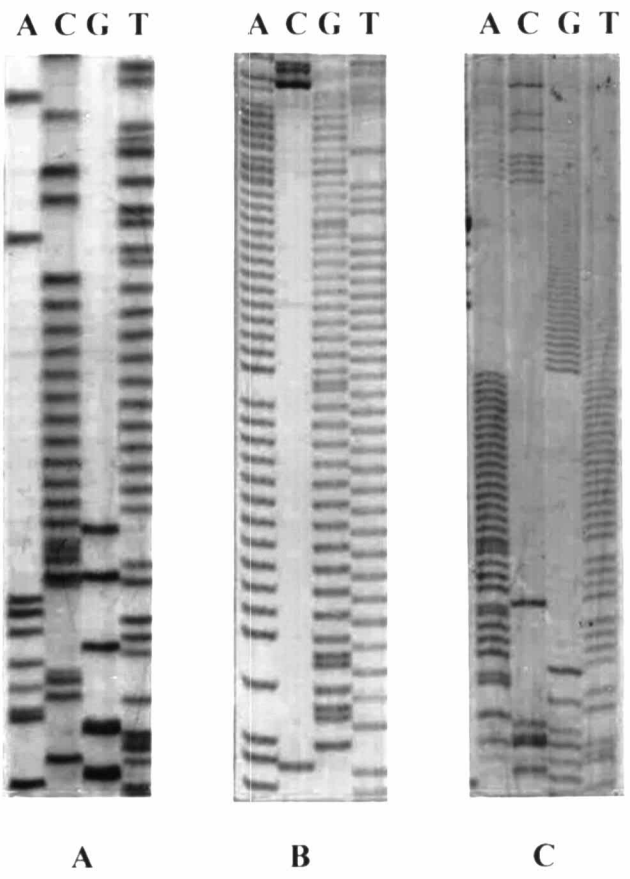


Figure 3.9 Autoradiograms showing sequences of different of microsatellite classes.

A. Perfect microsatellite, $(CT)_{12}$

B. Imperfect microsatellite, $(TGA)_{12}TGG(TGA)_{10}GAG(TGA)_3$

C. Compound microsatellite, $(AT)_{18}(GT)_n$

Table 3.5 Characteristics of compound microsatellite sequences in conventional libraries.

Type of compound microsatellites	No. of loci	Type of compound microsatellites	No. of loci
$(GA)_n(TG)_n$	3	$(GAA)_n(GA)_n$, $(GGA)_n(GAAA)_n$	1
$(GA)_n(AT)_n$	2	$(GAA)_n(GGA)_n$	1
$(TG)_n(AT)_n$	4	$(GAA)_n(TGA)_n$, $(TCA)_n(GGGA)_n$	1
$(TG)_n(CT)_n$	1	$(GAA)_n(GAAAA)_n$	1
$(AT)_n(GA)_n$	2	$(GGA)_n(GA)_n$	1
$(AT)_n(TG)_n$	1	$(GAT)_n(AT)_n$	1
$(GAA)_n(GA)_n$	1	$(GAT)_n(TG)_n(GGC)$	1
$(GAA)_n(TG)_n$	1	$(GAT)_n(TAA)_n$	1
$(GAA)_n(GA)_n(TG)_n$	1	$(CAT)_n(TAA)_n$	1
$(TAA)_n(TCATTA)_n$	1	$(CAT)_n(AT)_n(CT)_n$	1
$(TAA)_n(TGATTA)_n$	1	$(CAT)_n(GA)_n$	2
$(TAA)_n(A)_n$	1	$(GACT)_n(TGC)_n$	1
$(GATA)_n(GA)_n$	8	$(GATA)_n(AT)_n$	4
$(GATA)_n(TG)_n$	1	$(GATA)_n(GATA)_n(AT)_n$	3
$(GATA)_n(GA)_n(AT)_n$	1	$(GATA)_n(AT)_n$, $(TG)_n(GA)_n$	1
$(GATA)_n(TG)_n$	1	$(GATA)_n(GTAT)_n$, $(AT)_n$, $(TG)_n$ $(AT)_n$	1

3.5.2 Enrichment genomic libraries

Microsatellite repeat types

Among 136 sequenced clones across all 3 enriched libraries, 169 microsatellite loci were isolated. Characteristics of microsatellite sequences were summarized in Table 3.6 and Figure 3.10. These microsatellites were 111 loci of $(GATA)_n$, 14 loci of $(GA)_n$, 11 loci of $(TAA)_n$, 10 loci of $(GAA)_n$, 6 loci of $(AT)_n$, 6 loci of $(TG)_n$, 4 loci of $(ATG)_n$, 2 loci of $(GGAA)_n$, and 1 locus of each $(GGT)_n$, $(GGA)_n$, $(GACA)_n$, $(GACT)_n$. In addition, one octanucleotide repeat $(TCTATATA)_8$ was detected. Among microsatellite sequences that did not correspond to the oligonucleotide probes used to enrichment, $(GA)_n$ was found at the highest frequency, followed by $(TAA)_n$.

Repeat length

Length of dinucleotide microsatellites ranged from 6-94 with an average of 28, that of trinucleotide microsatellites was from 6-49 with an average of 19.1, and that of tetranucleotide microsatellites was from 3-58 with an average of 20.8. The maximum repeat number for di-, tri- and tetranucleotide repeats was 94 for $(GA)_n$, 49 for $(TAA)_n$ and 58 for $(GATA)_n$, respectively. Like conventional libraries, the length of dinucleotide repeats was the longest. Notably, the average repeat length of tetranucleotide repeats from the enriched libraries was unusually large. This was due to the large repeat length of tetranucleotide repeats which were derived from the library of $(GATA)_n$ selected from genomic DNA. Most tetranucleotide repeats from the library of $(GATA)_n$ selected from genomic DNA contained high number of repeat

units with an average of 31.1 repeats. The average repeat length of tetranucleotide repeats from the remaining 2 enriched libraries was 6.9 and 14.0 repeats.

Microsatellite classes

According to Weber Categories (1991), 117 (69.2%) loci of repeat sequences were perfect, 33 (19.5%) were imperfect and 19 (11.2%) were compound microsatellites. Characteristics of compound microsatellite sequences was summarized in Table 3.7. Nineteen loci of compound repeats associated with di-, tri- or tetranucleotide repeats where associated with dinucleotide repeats were the most frequent motif. Out of 19 loci of compound microsatellite investigated, 14 loci associated with 1 types of repeat sequences, 4 with 2 types and 1 with 3 types.

Table 3.6 Characteristics of microsatellite sequences in enriched libraries.

Library	Repeat type	No. of Loci	Range of repeat no.	Type of microsatellites		
				Perfect	Imperfect	Compound
$(GATA)_n$	$(GA)_n$	8	6-94	5	2	1
selected from	$(TG)_n$	2	20, 21	0	0	2
genomic library	$(AT)_n$	5	14-46	1	3	1
	$(TAA)_n$	10	6-49	4	5	1
	$(GGT)_n$	1	5	1	0	0
	$(GATA)_n$	46	3-23	34	4	8
	$(GGAA)_n$	2	4, 14	2	0	0
	$(TCTATATA)_n$	1	8	0	1	0
	Total	**75**	-	**47 (62.7%)**	**15 (20.0%)**	**13 (17.3%)**
$(GATA)_n$	$(GA)_n$	2	15, 22	1	1	0
selected from	$(TG)_n$	3	16-27	2	1	0
DNA fragments	$(AT)_n$	1	85	0	1	0
	$(GGA)_n$	1	22	0	1	0
	$(ATG)_n$	4	8-35	4	0	0
	$(GATA)_n$	65	5-58	50	11	4
	$(GACA)_n$	1	27	1	0	0
	Total	**77**	-	**58 (75.3%)**	**15 (19.5%)**	**4 (5.2%)**
$(GAA)_n$	$(GA)_n$	4	6-25	4	0	0
selected from	$(TG)_n$	1	25	1	0	0
DNA fragments	$(GAA)_n$	10	4-23	5	3	2
	$(TAA)_n$	1	40	1	0	0
	$(GACT)_n$	1	14	1	0	0
	Total	**17**	-	**12 (70.6%)**	**3 (17.7%)**	**2 (11.8%)**
Total (across all categories)		**169**	-	**117 (69.2%)**	**33 (19.5%)**	**19 (11.2%)**

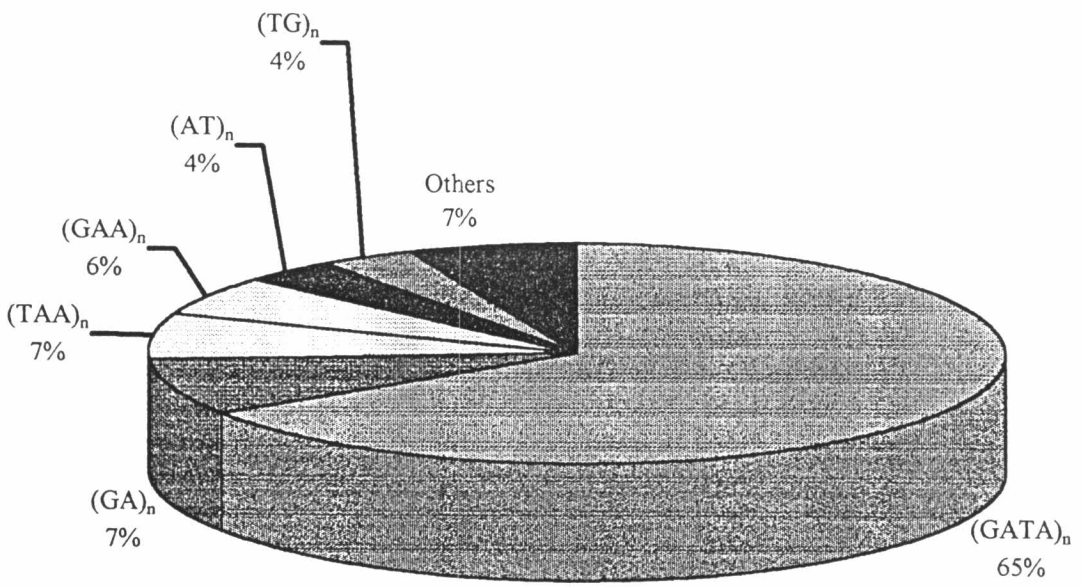


Figure 3.10 The percentages of various repeat types of microsatellite loci isolated from the enriched libraries of *P. monodon*.

Table 3.7 Characteristics of compound microsatellite sequences in enriched libraries.

Type of compound microsatellites	No. of loci	Type of compound microsatellites	No. of loci
$(GA)_n(TG)_n$	1	$(TAA)_n(AT)_n(GA)_n$	1
$(TG)_n(AT)_n$	2	$(GATA)_n(AT)_n$	2
$(AT)_n(TGA)_n(TG)_n$	1	$(GATA)_n(GA)_n$	6
$(GAA)_n(GA)_n$	1	$(GATA)_n(CAA)_n$	1
$(GAA)_n(GGA)_n$	1	$(GATA)_n(AT)_n$, $(TG)_n(TGA)_n$	1
$(GATA)_n(GA)_n(GACA)_n$	2		

3.6 Primer design and primer testing

From sequencing data, microsatellite loci that appeared to have flanking sequences with 18 bp or more were subjected to primer design using the software Oligo 4.0 (National Biosciences). Several parameters were considered in primer design as described in Chapter II: Section 2.10.

3.6.1 Conventional genomic libraries

The number of clones sequenced, primer pairs designed and primer pairs with successful amplification in expected size range are shown in Table 3.8. PCR primer pairs were designed from 27 different loci, while the flanking sequences of the remaining loci were unsuitable for designing primers. Although conventional libraries consisted of 225 microsatellite loci, only 27 primer pairs showed satisfactory stringency requirements for primer designing. There were several factors influencing

efficiency of marker development. The largest proportion of usable clones per sequenced recombinants was obtained from the library D (15 in 33) while those of libraries A and C were 7 in 15 and 3 in 19, respectively. Designing of primer from the library B was not attempted because of flanking sequences contained unsatisfied stringency requirements for primer design as many clones contained only one side of unique flanking sequences. Other microsatellites contained short repeat unit but those short loci were separated among each locus by short tract of non-tandem repeat array or degenerate microsatellite-like motifs.

The highest efficiency of usable clones was achieved for the library D that contained sequences with short repeat arrays and suitable flanking regions for designing primer pairs. The major problem of marker development for the library D was the isolation of clones without microsatellite sequences. Nevertheless, 17 primer pairs were designed from the library D.

Similar difficult circumstances occurring in the library B were also found in the library A. Only one and 6 primer pairs were designed from this library screened with $(GAA)_8$ and $(GATA)_6$, respectively. Efficiency of usable clones screened with $(GATA)_6$ was higher than $(GAA)_8$. This was possibly due to shorter microsatellite arrays from library screened with $(GATA)_6$. Moreover, most of the flanking regions of microsatellite loci from $(GAA)_n$ screening contained degenerate microsatellite-like motif and short tract of non-tandem repeat arrays (less than 18 bp).

Microsatellite loci from libraries B and C contained long repeat arrays and about 40% and 31% of microsatellite loci from libraries B and C were compound microsatellites while that of libraries A and D was about 17% and 13%, respectively.

Compound microsatellites were adjoined by other types of microsatellite repeats resulting in long repeat arrays. Moreover, many compound microsatellites consisted of several different repeat types. Many microsatellites were located adjacent to the cloning sites so unique sequences flanking the repeats for primer design were not available.

Examples of primers flanked microsatellites used for PCR amplification were shown in Figure 3.11. Primers were diluted to appropriate concentrations and tested for their ability to amplify *P. monodon* genomic DNA. Each primer pair was tested against 10 prawns. Amplification conditions, product separation and detection of primer testing were performed as described in Chapter II: Section 2.11. Among these, 23 loci yielded PCR products in expected size range. All of these were tested further for estimation of polymorphic levels against a representative sample of *P. monodon.*

Table 3.8 Efficiency of microsatellite marker isolated from conventional libraries.

Library	No. of clones sequenced	No. of primer pairs designed	No. of primer pairs with successful amplification in expected size range
A	17	7	7
B	25	0	-
C	19	3	2
D	33	17	14
Total	152	27	23

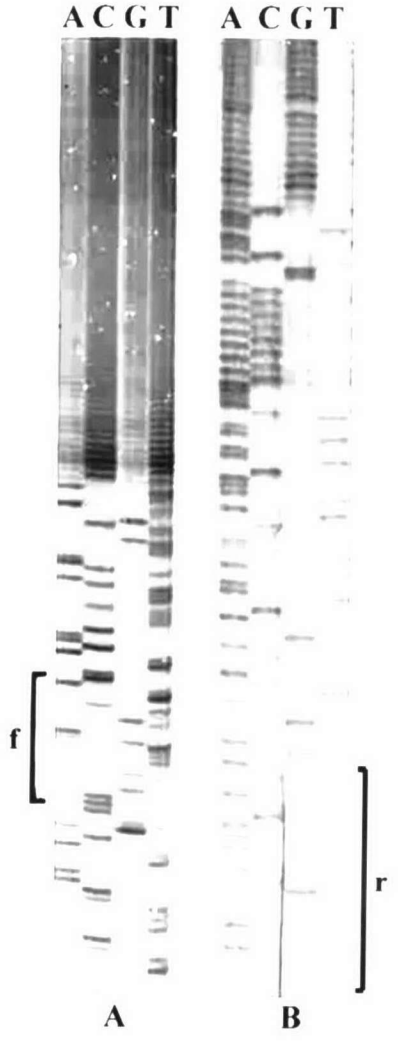


Figure 3.11 Autoradiogram of sequencing gels showing flanking regions of a microsatellite-containing clone. (A) and (B) showed sequencing data using universal and M13 reverse primers, respectively. ([ ) indicates the sequence region for primer design. (f) and (r) were forward and reverse microsatellite primers, respectively. A, C, G and T (on the top of each sequencing gel) were adenine, cytidine, guanine and thymine, respectively.

3.6.2 Enrichment libraries

PCR primers were designed from 12 different loci, while flanking sequences of the remaining loci were unsuitable for designing primers. The number of clones sequenced, primer pairs designed and primer pairs with successful amplification in expected size range are shown on Table 3.9. The largest proportion of usable clones per sequenced recombinants was obtained from the $(GAA)_n$ library selected from genomic DNA (4 in 34). The proportion of usable clones per sequenced recombinant of the $(GATA)_n$ library selected from the genomic library and DNA fragments was 3 in 34 and 4 in 68, respectively. The highest efficiency of usable clones was achieved from the $(GAA)_n$ library selected from DNA fragments.

The $(GAA)_n$ library selected from DNA fragments contained sequences with short repeat arrays and only 11% of microsatellite loci were compound microsatellites. For the $(GATA)_n$ library enriched from DNA fragments, the majority of microsatellite loci contained long repeat arrays and had at least one cloning site located adjacent to microsatellite sequences. These problems prevented the ability to design primers. Moreover sequences of either adapters used for cloning were found within sequence regions indicating the joining of DNA fragments in the clones. Figure 3.12 shows problems of microsatellite marker development from the $(GATA)_n$ library selected from DNA fragments.

For the $(GATA)_n$ library enriched from the genomic library, problems of microsatellite development were the same as that found in conventional library screened with $(GATA)_6$. Many microsatellites contained short repeat units but those short loci were separated among each locus by short tract of the non-tandem repeat

array or microsatellite-like motif. Most microsatellite sequences were located adjacent or very close to the cloning sites.

Table 3.9 Efficiency of microsatellite marker isolated from the enriched libraries.

Library	No. of clones sequenced	No. of primer pairs designed	No. of primer pairs with successful amplification in expected size range
$(GATA)_n$ selected from genomic library	34	3	2
$(GATA)_n$ selected from DNA fragments	68	4	0
$(GAA)_n$ selected from DNA fragments	34	5	1
Total	136	12	3

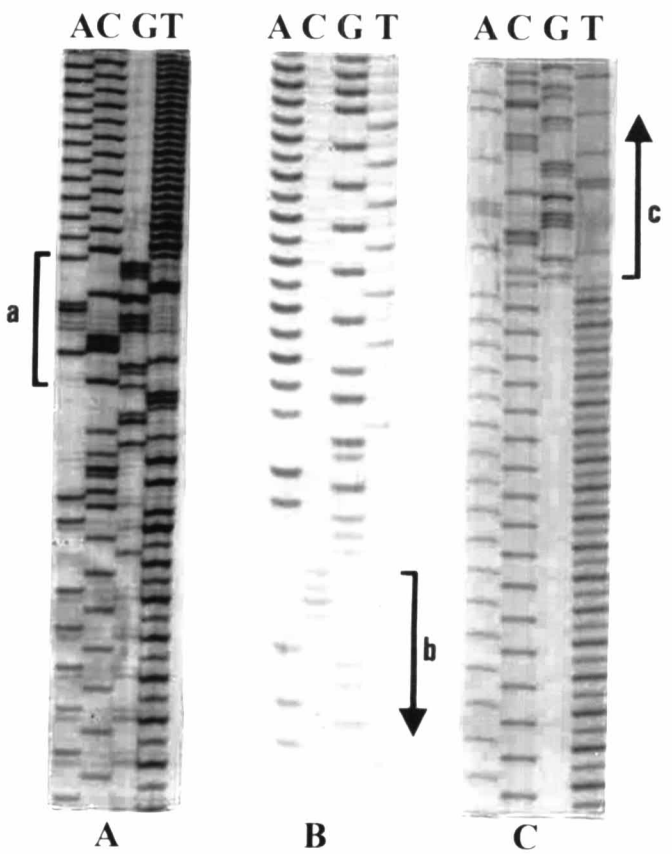


Figure 3.12 Autoradiogram of sequencing gels of microsatellite clones of $(GATA)_n$ selected from DNA fragments. (A) Sequences of adapter (a) were occurred within the clone. (B and C) Microsatellite sequences located adjacent to the cloning site. (b) is sequences of pUC 18 vector. (c) the sequences of adapter connecting with pUC 18 vector.

A total of 12 primer pairs were synthesized. Primer testing was done as described in Section 3.6.1 above. When the 12 loci were tested against 10 prawns, 3 loci yielded amplification products in expected size range. Of those loci, 2 were isolated from the $(GATA)_n$ library selected from the genomic library and the remaining locus was isolated from the $(GAA)_n$ library selected from DNA fragments. There was no successful amplification with primer developed from the $(GATA)_n$ library selected from DNA fragments. Only 1 in 5 designed primers from the $(GAA)_n$ library selected from DNA fragments succeeded to amplify genomic DNA. The unsuccessful amplification of primers showed no PCR products in expected size range.

The enriched library based on selection of DNA fragments from genomic DNA have to amplify selected DNA fragments with PCR before ligation into a vector and transformation into the host cells. In a PCR reaction, it was plenty of a population of microsatellite-containing DNA fragments which caused the amplification among these DNA. This phenomenon caused the unsuccessful amplification of primers derived from the enriched library based on selection of DNA fragments from genomic DNA. To overcome this, PCR conditions for amplification of selected DNA from the $(GAA)_n$ library enriched by selection from genomic DNA were adjusted. For the $(GATA)_n$ library, ten microlitres of ssDNA were amplified in a 25 μl PCR reaction but ssDNA from the $(GAA)_n$ library was reduceded to 2 μl instead. All sequenced clones from the $(GAA)_n$ library enriched by selection from genomic DNA contained no sequences of adapters. However, only 1 in 5 designed primers supported amplification of genomic DNA in expected size range. No attempt was made to improve on this type of the enriched libraries.

3.7 Polymorphism of investigated microsatellites

DNA sample isolated from *P. monodon* originating from Trad province by Supungul (1998) was used to determine polymorphism of 18 microsatellite loci. Polymorphism of the remaining 8 loci (CUPmo1, 2, 3, 4, 6, 7, 9, 11) were analyzed by Soot-anan (1999). For analysis of polymorphism, radioactive-labeled detection was used to detect genotypes of all 26 polymorphic markers. Radioactive-labeled PCR products were visualized by separating on denaturing polyacrylamide gel. The size of alleles was estimated by comparison sizes of PCR products with M13 sequencing ladder. Informations related to 26 microsatellite loci developed in this study are summarized in Table 3.10 and 3.11. These include locus designation, description of microsatellite array, sizes of PCR products, annealing temperatures and the genetic variability of microsatellite markers. PCR conditions and amplification profiles of all 26 loci were identical except the annealing temperatures which were optimized for each locus. Markers that produced polymorphic genotypes from primer testing (Section 3.6, above) were tested against 40-50 prawns to detect polymorphic levels. Markers that produced monomorphic genotypes from primer testing were further tested against 20 prawns.

PCR products displayed either one or two major fragments, consistent with single locus detection of either homozygotes or heterozygotes, respectively. Ladders of minor fragments of decreasing size were also present in some loci, especially dinucleotide microsatellites. Examples of microsatellite patterns were shown in Figures 3.13-3.20. Out of 26 primers investigated, 21 yielded polymorphic genotypes.

The number of alleles per a polymorphic locus varied from 4 to 33 with an average of 17.80 alleles. Sizes of allele ranged from 83-450 bp. The spectrum of allele lengths was very large at some loci such as CUPmo 9, 15, and 17 loci. The largest and smallest alleles of the CUPmo 17 locus differ about 165 bp. Allele distribution frequencies of *P. monodon* at 21 polymorphic markers were summarized in Figure 3.22. Allele of each locus was found at frequency less than 0.3 except that of CUPmo 11, 12, 16, 21, 23 and 24 loci. Differences between consecutive alleles depended on the repeat type of microsatellite loci. The allele differences for di-, tri and tetranucleotide repeats were 2, 3 and 4 bp, respectively. For loci linked to dinucleotide repeats, alleles were different by multiples of 2 bases. Each microsatellite locus did not exhibit all possible allele within it size-range resulting in gaps in the range of allele size. A gap of allele size of CUPmo 3 jumped from 147 to 169 which was larger than other loci.

The average observed heterozygosity of 21 polymorphic loci is 0.70 with a maximum of 0.95 for the CUPmo 22 and a minimum of 0.21 for the CUPmo 7. From 43 individuals of investigated *P. monodon* using the CUPmo 7 locus, only 9 individuals showed heterozygote genotypes. An example of microsatellite patterns derived from the CUPmo 7 locus was shown in Figure 3.21. The average expected heterozygosity was 0.84 with a maximum of 0.95 for loci CUPmo 1, 6, 9 and 20 and a minimum of 0.48 for the CUPmo 16 locus. Observed heterozygosity was usually lower than expected heterozygosity across overall loci leading to possible deviations from Hardy-Weinberg expectations of these microsatellites. As can be seen in Table 3.12, 9 mocrosatellite loci (CUPmo 1, 3, 6, 7, 14, 15, 17, 19 and 20) significantly deviated from Hardy-Weinberg expectations even after the correction for multiple test

using the Bonferroni procedure was applied. The average polymorphic information content (PIC) for all polymorphic markers was 0.82. Compound microsatellites had a greater average PIC and the number of alleles than perfect or imperfect microsatellite. PIC and the number of alleles tended to increase with increasing repeat numbers (Table 3.13). All 6 polymorphic loci developed from the library A showed high level of polymorphism (PIC $\geq$ 0.89) while the polymorphic microsatellite loci developed from the library D showed different degree of polymorphism (PIC = 0.42-0.95) because most microsatellite loci derived from the conventional library A contained large repeat length whereas the repeat length of microsatellite markers derived from the conventional library D vary in repeat numbers. The PIC values of remaining 3 loci developed from enriched libraries were 0.95, 0.78 and 0.77, respectively.

Table 3.10 Repeat sequences and annealing temperature of *P. monodon* microsatellite primers from various genomic libraries.

Locus	Repeat sequences	Annealing temp. (°C)
CUPmo 1[A]	$(GAA)_{43}$	56
CUPmo 2[A]	$(ATCT)_{12}(TA)_{10}(TAGA)_3$	56
CUPmo 3[A]	$(ATCT)_{12}(AT)_9(GT)_9$	56
CUPmo 4[A]	$(CT)_{10}TG(CT)_1$-$(ATCT)_{10}$	54
CUPmo 5[A]	$(GATA)_4$...$(GATA)_4$	56
CUPmo 6[A]	$(GATA)_6(GA)_{16}$	56
CUPmo 7[A]	$(CT)_{15}(ATCT)_9$	56
CUPmo 9*	$(TA)_{49}$	54
CUPmo 11*	$(TAA)_9TGA(TAA)_3$	56
CUPmo 12[#]	$(GA)_{18}$	54
CUPmo 13[D]	$(TCA)_9$	58
CUPmo 14[C]	$(GT)_9CAG(GT)_3$	58
CUPmo 15[D]	$(TAA)_{10}$	52
CUPmo 16[D]	$(CAT)_8$	52
CUPmo 17[C]	$(TAA)_3(TGA)_{16}(TAA)_2$	52
CUPmo 19[D]	$(TAA)_{10}$	54
CUPmo 20[D]	$(GA)_{46}$	50
CUPmo 21[D]	$(TTA)_5TTG(TTA)_5$	60
CUPmo 22[D]	$(AG)_9AAA(AG)_{52}$	50
CUPmo 23[D]	$(GATA)_{10}$	60
CUPmo 24[D]	$(ATT)_2GTT(ATT)_9$	50
CUPmo 25[D]	$(GA)_7GC(GA)_7AA(GA)_{18}$	62
CUPmo 26[D]	$(CA)_9$	62
CUPmo 27[D]	$(GGA)_2GAA(GGA)_5$	66
CUPmo 28[D]	$(GCA)_7$	58
CUPmo 29[D]	$(GA)_8$	62

[A] Conventional library A

[C] Conventional library C

[D] Conventional library D

* Enrichment library: $(GATA)_n$ selection from genomic library

[#] Enrichment library: $(GAA)_n$ selection from DNA fragments

Table 3.11 Polymorphism of 26 microsatellites loci of *P. monodon*.

Locus	No. of samples	No. of alleles	Size range of alleles	Observed heterozygosity	Expected heterozygosity	PIC*
CUPmo 1[#]	51	29	224-326	0.75	0.95	0.95
CUPmo 2[#]	51	27	147-217	0.78	0.93	0.92
CUPmo 3[#]	50	27	135-223	0.60	0.92	0.92
CUPmo 4[#]	51	21	206-256	0.90	0.89	0.89
CUPmo 5[#]	10	1	120	0	nd	nd
CUPmo 6[#]	51	28	166-242	0.69	0.95	0.95
CUPmo 7[#]	43	22	172-234	0.21	0.94	0.93
CUPmo 9[#]	40	33	274-380	0.90	0.95	0.95
CUPmo 11[#]	47	10	132-162	0.81	0.78	0.78
CUPmo 12	45	8	83-105	0.71	0.79	0.77
CUPmo 13	43	12	271-316	0.86	0.85	0.84
CUPmo 14	47	5	275-285	0.45	0.75	0.70
CUPmo 15	44	15	267-351	0.66	0.91	0.90
CUPmo 16	44	4	223-232	0.48	0.48	0.42
CUPmo 17	40	25	282-450	0.72	0.93	0.93
CUPmo 19	46	16	300-345	0.76	0.90	0.89
CUPmo 20	44	30	166-196	0.68	0.95	0.95
CUPmo 21	46	4	230-239	0.54	0.53	0.48
CUPmo 22	40	27	226-258	0.95	0.93	0.91
CUPmo 23	40	7	299-323	0.63	0.68	0.65
CUPmo 24	40	12	332-368	0.65	0.76	0.75
CUPmo 25	45	12	253-281	0.82	0.83	0.81
CUPmo 26	20	1	273	0	nd	nd
CUPmo 27	20	1	370	0	nd	nd
CUPmo 28	20	2	250, 253	1	nd	nd
CUPmo 29	20	1	181	0	nd	nd

* Polymorphic information content as described in Material and Method

[#] Genotyping, calculation of no. of allele and observed heterozygosity was done by Soot-anan, 1999.

nd = not determined

Table 3.12 Estimation of Hardy-Weinberg equilibrium against *P. monodon* from Trad for each polymorphic microsatellite locus.

Locus	P-value*
CUPmo 1	<0.0001
CUPmo 2	0.0030 ns
CUPmo 3	<0.0001
CUPmo 4	0.3294 ns
CUPmo 6	<0.0001
CUPmo 7	<0.0001
CUPmo 9	0.0039 ns
CUPmo 11	0.3521 ns
CUPmo 12	0.0942 ns
CUPmo 13	0.3377 ns
CUPmo 14	<0.0001
CUPmo 15	<0.0001
CUPmo 16	0.1473 ns
CUPmo 17	<0.0001
CUPmo 19	0.0007
CUPmo 20	<0.0001
CUPmo 21	0.3568 ns
CUPmo 22	0.5559 ns
CUPmo 23	0.1356 ns
CUPmo 24	0.0063 ns
CUPmo 25	0.1317 ns

*Significant level was further adjusted using a Bonferroni technique (Rice, 1989).

ns = not significant

Table 3.13 Polymorphism of 21 polymorphic markers for different microsatellite types of *P. monodon*.

Type	No. of markers	Average no. of alleles	Average PIC
All microsatellites	21	17.80	0.82
Microsatellite class			
Perfect	9	17.11	0.81
Imperfect	7	13.86	0.76
Compound	5	24.60	0.92
repeat number			
1-10 repeats	6	9.67	0.70
11-20 repeats	4	8.75	0.75
21-30 repeats	5	25.80	0.93
>31 repeats	6	25.33	0.91

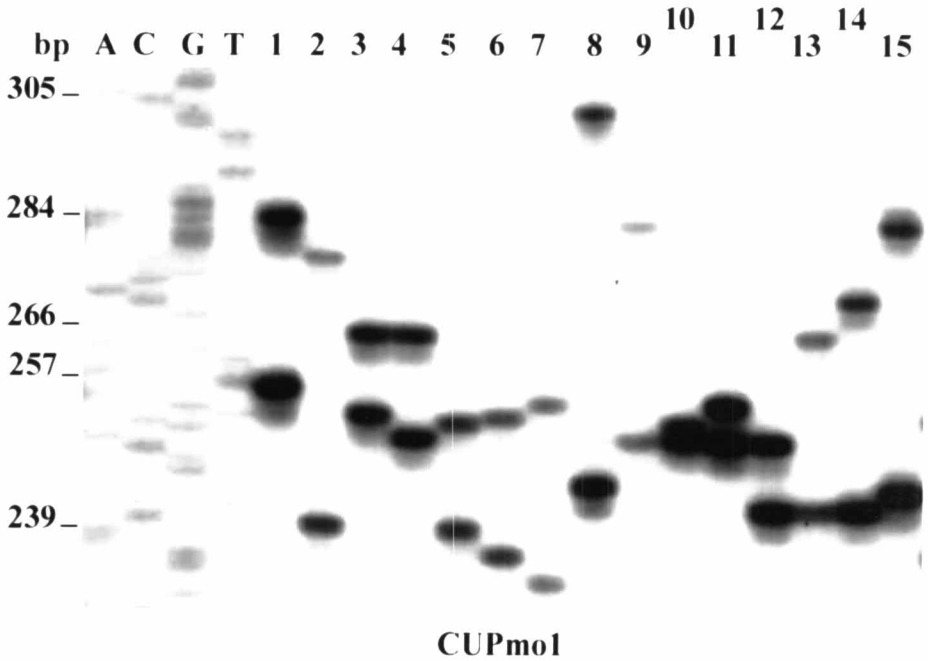


Figure 3.13 Genotype patterns at the CUPmo 1 locus of 15 individuals (lanes 1-15) of *P. monodon*. The size standard is a sequencing ladder of M13 mp18.

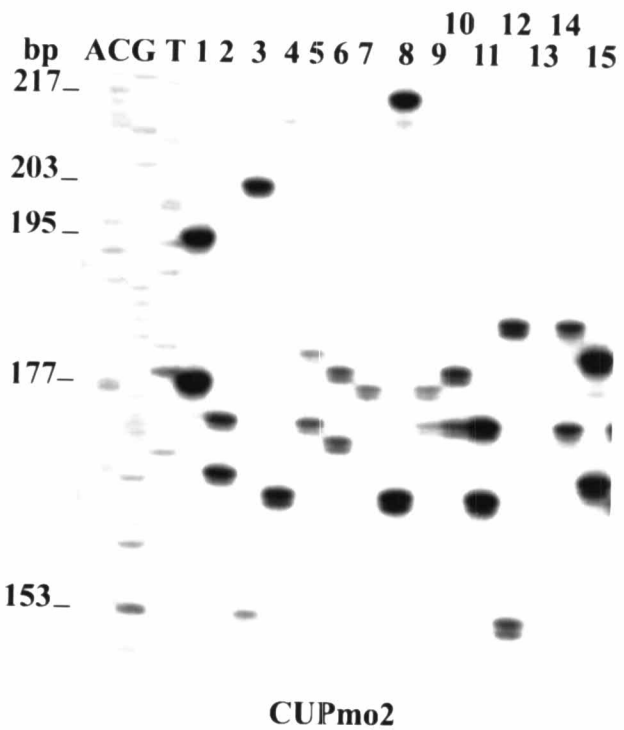
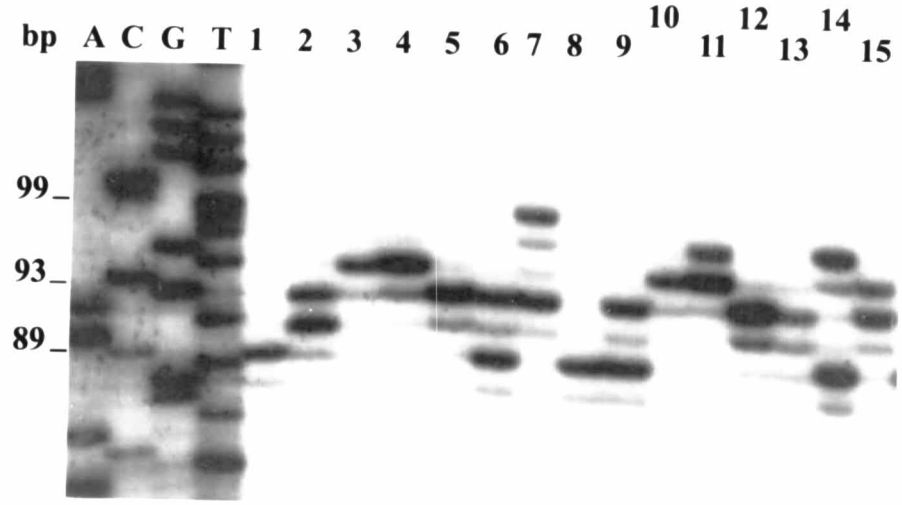
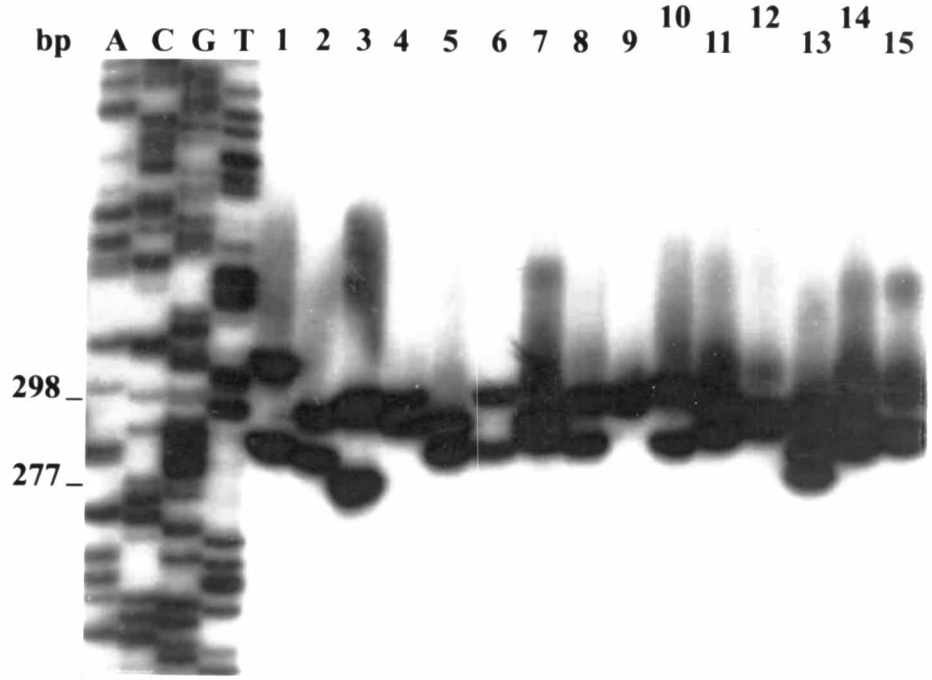


Figure 3.14 Genotype patterns at the CUPmo 2 locus of 15 individuals (lanes 1-15) of *P. monodon*. The size standard is a sequencing ladder of M13 mp18.



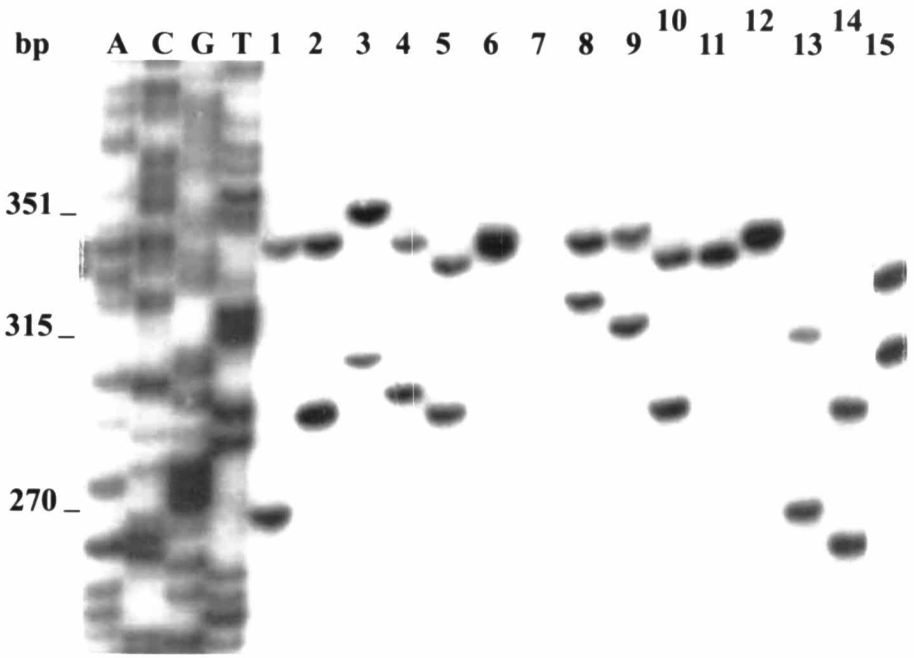
CUPmo12

Figure 3.15 Genotype patterns at the CUPmo 12 locus of 15 individuals (lanes 1-15) of *P. monodon*. The size standard is a sequencing ladder of M13 mp18.



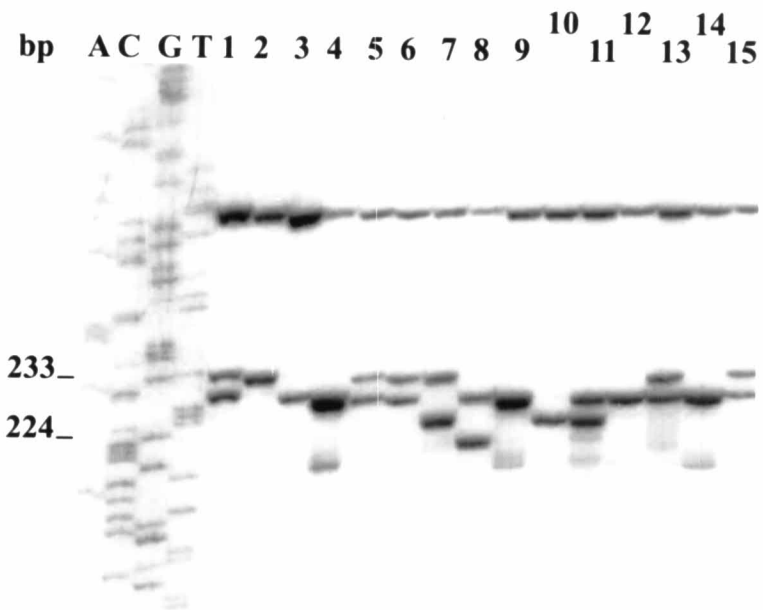
CUPmo13

Figure 3.16 Genotype patterns at the CUPmo 13 locus of 15 individuals (lanes 1-15) of *P. monodon*. The size standard is a sequencing ladder of M13 mp18.



CUPmo15

Figure 3.17 Genotype patterns at the CUPmo 15 locus of 15 individuals (lanes 1-15) of *P. monodon*. The size standard is a sequencing ladder of M13 mp18.



CUPmo16

Figure 3.18 Genotype patterns at the CUPmo 16 locus of 15 individuals (lanes 1-15) of *P. monodon*. The size standard is a sequencing ladder of M13 mp18.

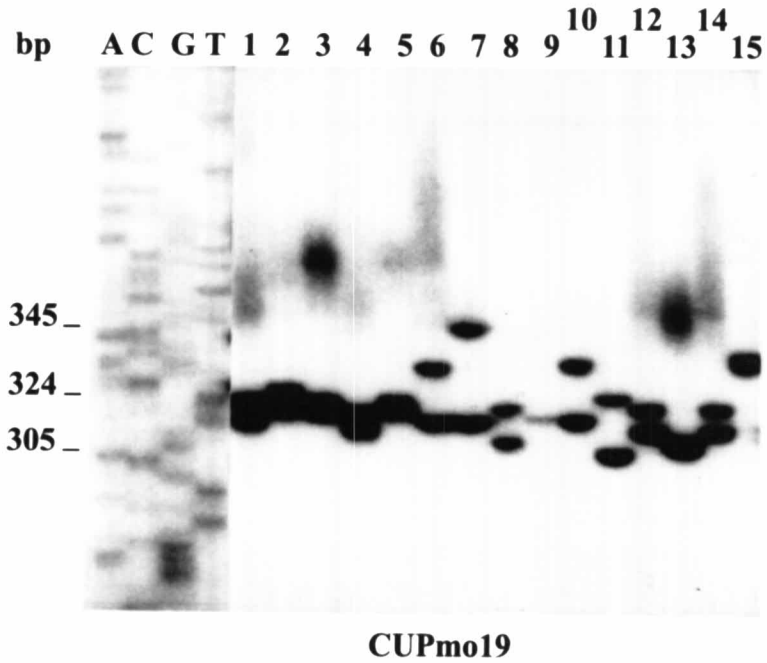


Figure 3.19 Genotype patterns at the CUPmo 19 locus of 15 individuals (lanes 1-15) of *P. monodon*. The size standard is a sequencing ladder of M13 mp18.

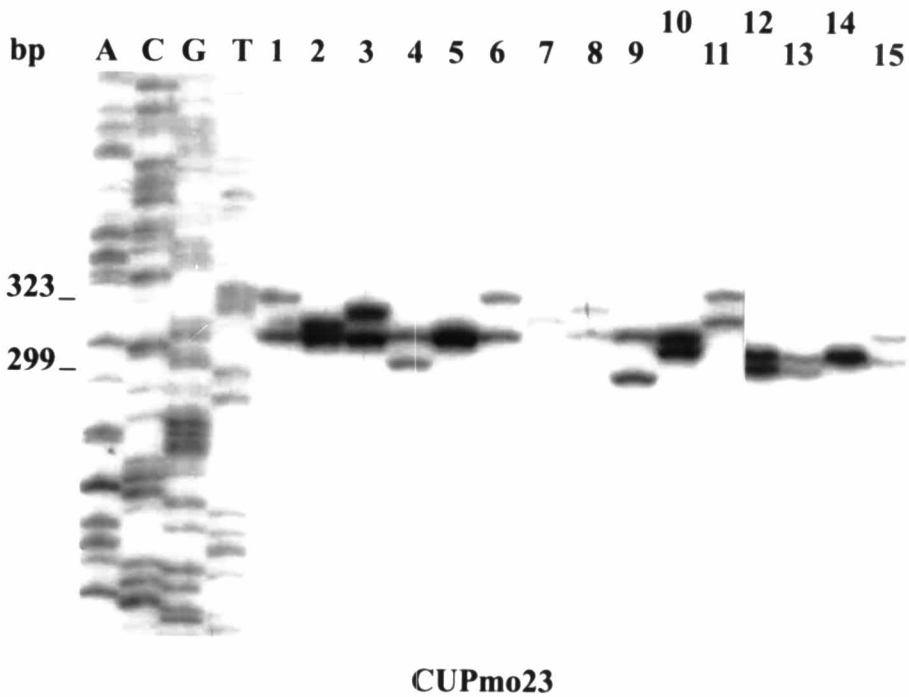


Figure 3.20 Genotype patterns at the CUPmo 23 locus of 15 individuals (lanes 1-15) of *P. monodon*. The size standard is a sequencing ladder of M13 mp18.

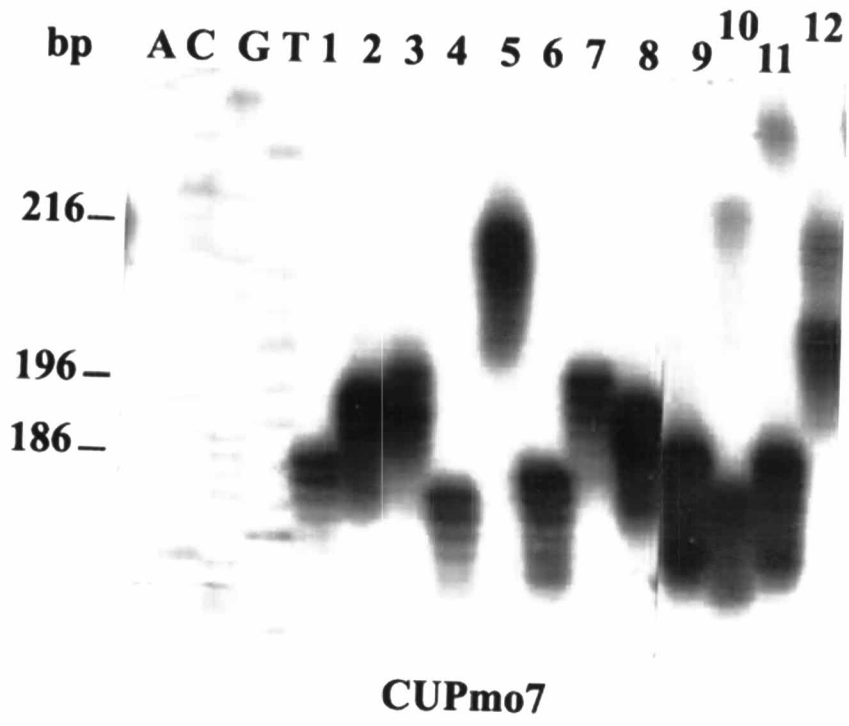


Figure 3.21 Genotype patterns at the CUPmo 7 locus of 12 individuals (lanes 1-12) of *P. monodon*. The size standard is a sequencing ladder of M13 mp18.

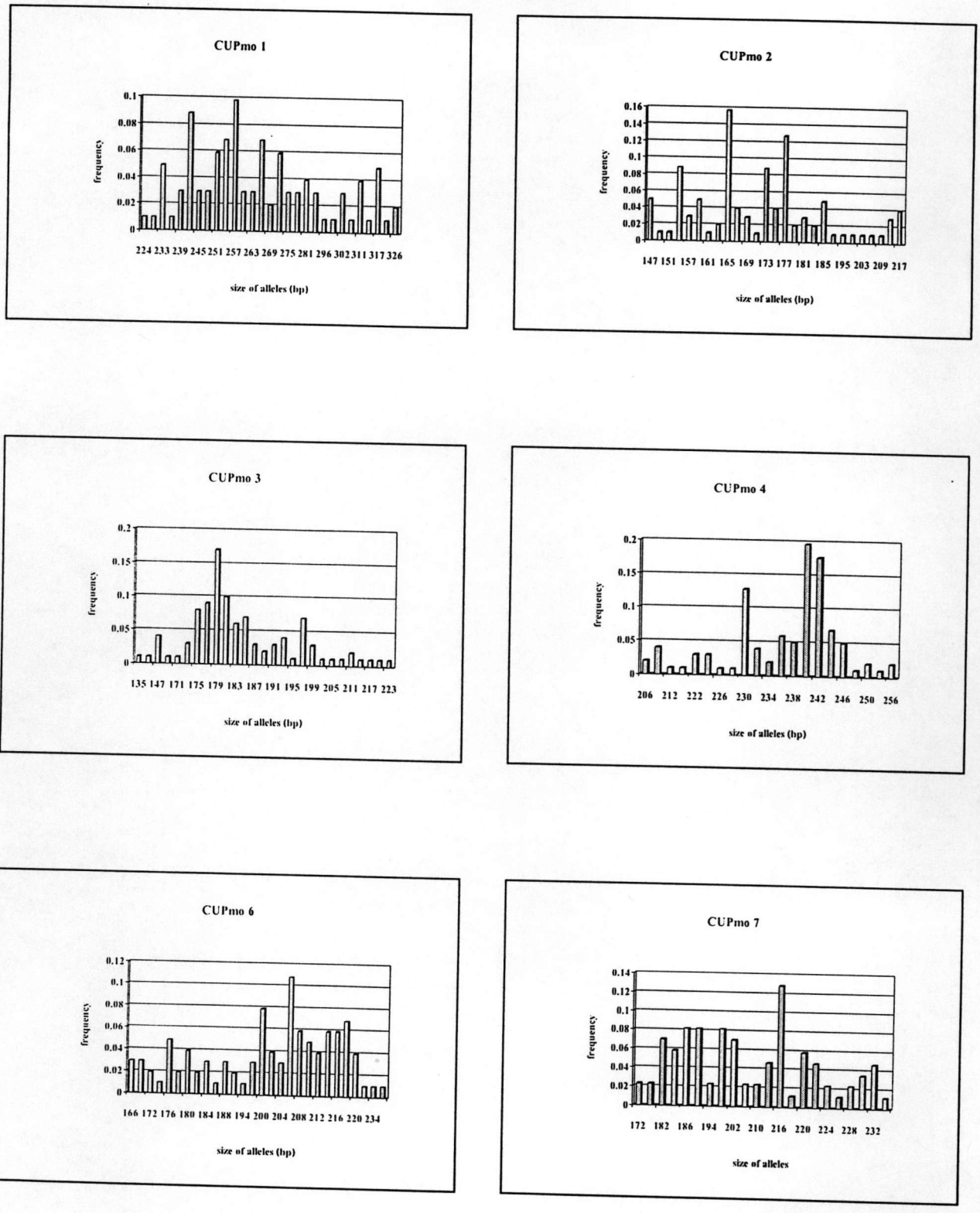
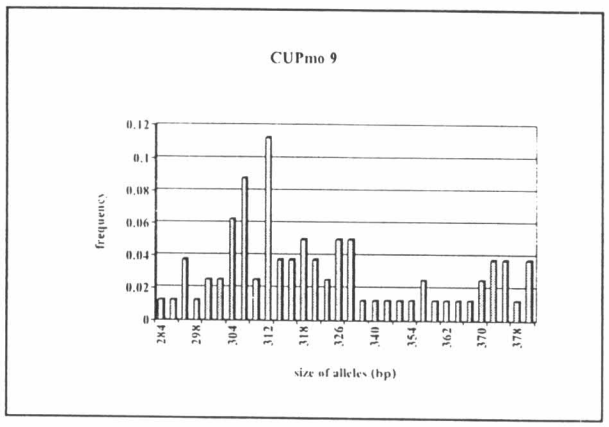
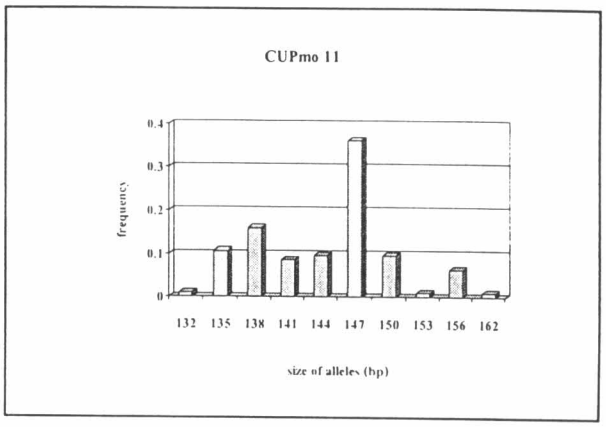
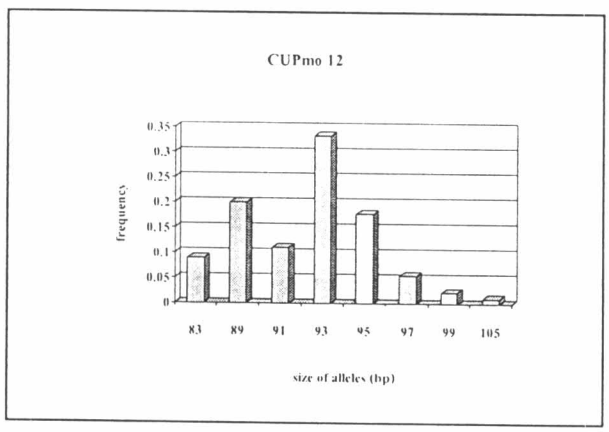


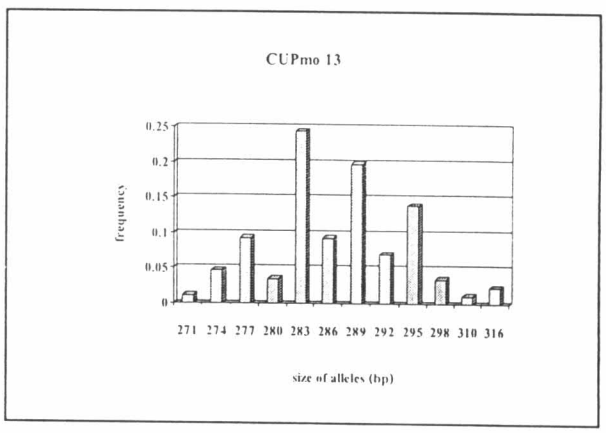
Figure 3.22 Allele distribution frequencies of *P. monodon* from Trad (Gulf of Thailand) at 21 microsatellite loci.

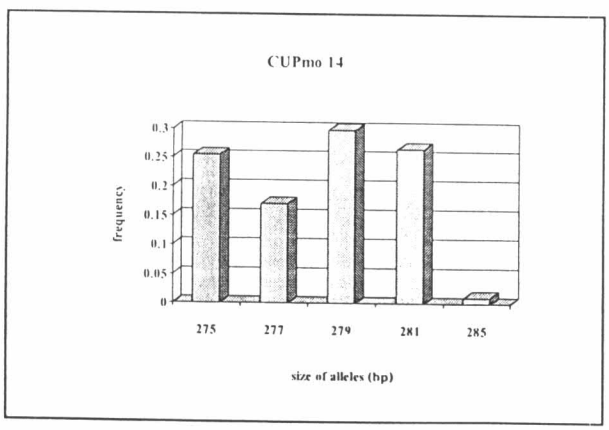
Figure 3.22 (Continued).











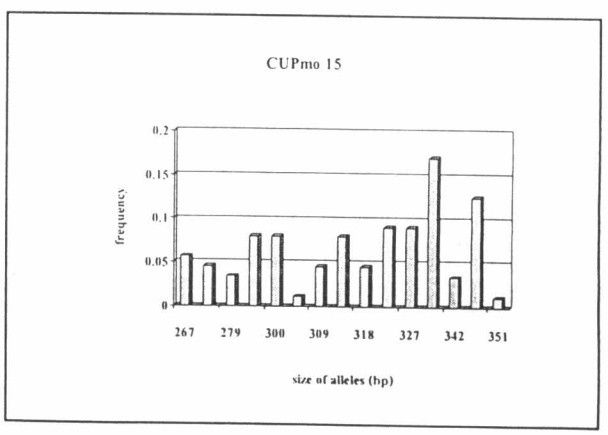
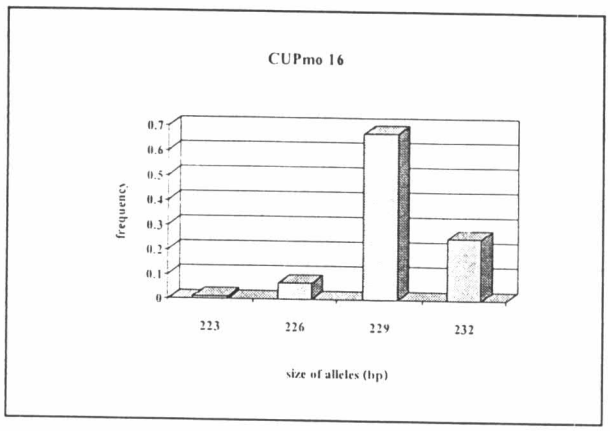
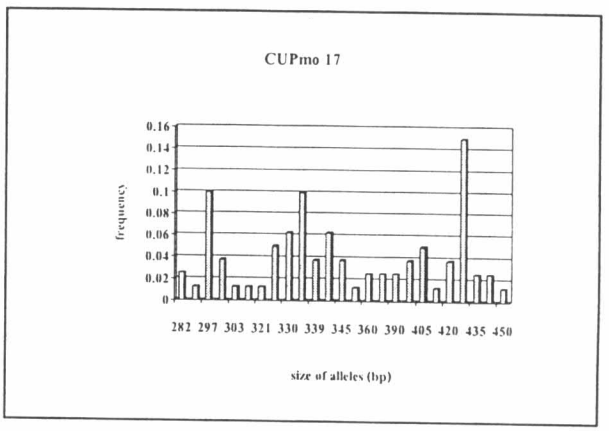
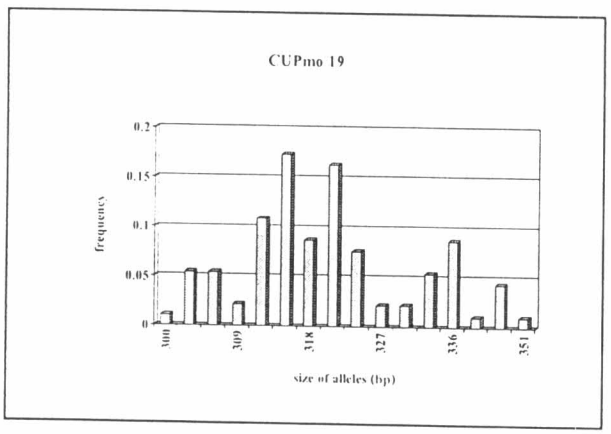
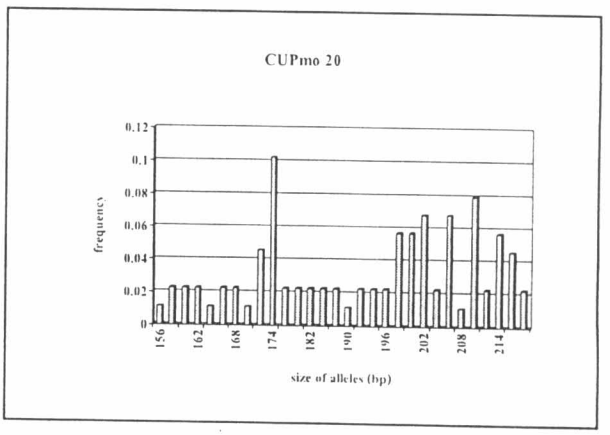


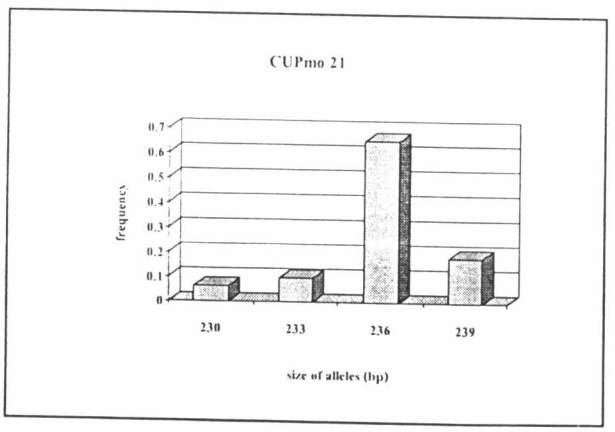
Figure 3.22 (Continued).











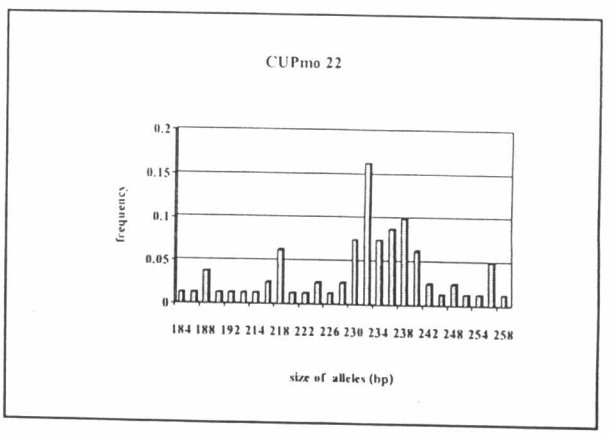
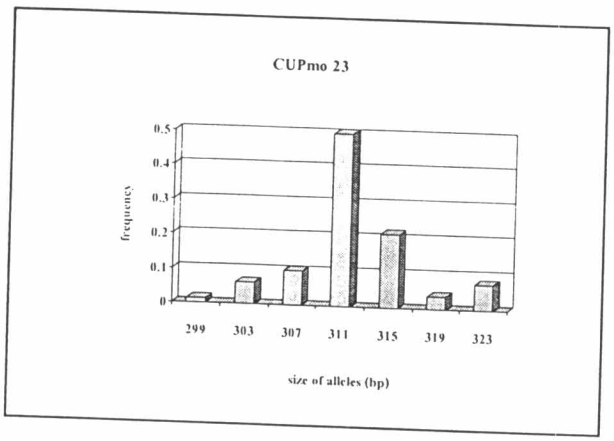
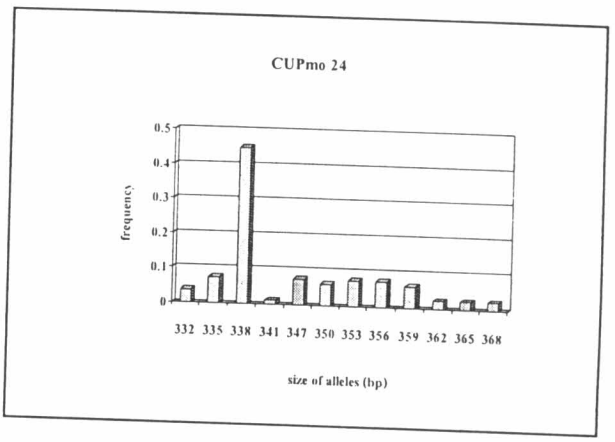
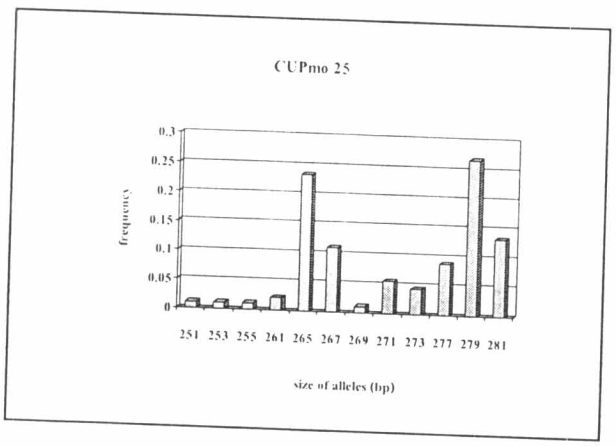


Figure 3.22 (Continued).







3.8 Multiplex analysis of microsatellite loci

Multiplex PCR is designed for simultaneous amplification of multiple loci in a single reaction. This approach saves both sample materials and manipulation time comparing with the single locus PCR. Co-amplification of microsatellite loci was selected on the basic of size range and ability to co-amplify in the reaction (Wright and O'Reilly, 1995). Basically, primer of loci co-amplified together should have similar primer annealing temperature and non-overlapping allele sizes. In addition, co-amplified loci were tested for primer compatibility and a minimum of interaction among primers by using the software Oligo 4.0 (National Biosciences).

In this study, 4 multiplex sets could be organized, containing the multiplex PCR set A (CUPmo 2+11+15+16), B (CUPmo 4 +13+19), C (CUPmo 14+21+23) and D (CUPmo 22+24). PCR amplification of these multiplex mixtures was performed first using the same amplification conditions as with a single locus PCR except annealing temperature. The lowest annealing temperature of a single locus PCR in each multiplex set was chosen to be the annealing temperature of each multiplex system. Using conditions of a single locus PCR, none of the 4 multiplex sets showed scorable genotype patterns. Multiplex amplifications showed non-specific products, preferential amplification or disappearance of some loci. Such a situation required further adjustment in PCR profiles and reaction components.

Optimization of multiplex PCR analysis of microsatellite loci is as follows. For the multiplex PCR set A, the optimization followed Henegariu et al. (1997) and Rithidech, Dunn and Gordon (1997). The multiplex PCR set A was co-amplification of CUPmo 2, 11, 15 and 16 loci. First, conditions were adjusted for co-amplification

of the duplex set of the loci 15 and 16 using the same amplification conditions as with a single locus PCR except the annealing temperature was chosen at 50°C (Figure 3.23). This resulted in disappearance of alleles of the CUPmo 15 locus. Non-specific products, which occurred from interaction between both primers, were appeared. These non-specific products have different size than alleles of both CUPmo 15 and 16 loci. Extension temperature and time were changed from 72°C for 1 min to 65°C for 2 min resulted in a better amplification of the CUPmo15 locus. However non-specific products still appeared strongly. To increase amounts of larger products, CUPmo 15 alleles, the extension time was increased from 2 to 4 min. To preserve *Taq* polymerase activity, both denaturing and annealing time were decreased to 30 sec thus the PCR profiles was first denaturing at 95°C for 3 min, followed by 30 cycles of denaturing at 95°C for 30 sec, annealing at 50°C for 30 sec, extension at 65°C for 4 min and a final extension at 65°C for 10 min. Using this PCR thermal profile, non-specific products disappeared and alleles of both CUPmo 15 and 16 loci could be scored (Figure 3.23). Then, CUPmo 11 primers were added into the duplex set. *Taq* polymerase, dNTP, $MgCl_2$ and DNA concentrations were increased to 0.36 U, 0.3 M, 3.0 mM and 20 ng, respectively. Amplification of the CUPmo 11 locus yielded weak bands of the alleles and many non-specific products (Figure 3.24). Generally, many primers producing longer amplification products worked better at low salt concentrations, whereas many primer pairs producing short amplification products worked better at high salt concentrations, where longer products become harder to denature. Henegariu et al. (1997) reported that the effect of KCl concentration was more important than any of the adjuvants tested (DMSO, glycerol or BSA). To improve the amplification of the CUPmo 11 locus, the concentration of KCl was tested at 75 and 100 mM. Results

showed that at both 75 and 100 mM of KCl, there was a dramatically increase in amplification efficiency of the CUPmo 11 alleles and the amplification reaction became more specific (non-specific products was disappeared) (Figure 3.24). Comparison of KCl concentration between 75 and 100 mM for amplification efficiency of this triplex system revealed more efficient when performed with 100 mM KCl. Then, CUPmo 2 primers was combined into this triplex set. The tetraplex set could be amplified successfully using the optimization protocol but PCR products still showed weak bands of non-specific products. To overcome these problems, a touchdown PCR was performed. An initial annealing temperature was 56°C. This temperature was 6°C higher than the target annealing temperature. This annealing temperature was decreased at the rate of 1°C for every PCR cycle (denaturation at 95°C for 30 sec, annealing for 30 sec and extension at 65°C for 4 min) until the targeted annealing temperature was reached, then 20 regular PCR cycles were performed (denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec and extension at 65°C for 4 min). Under these conditions, non-specific products were decreased or disappeared (Figure 3.25). Further adjustment was decreasing differences in the quantity of the products of each loci by increasing the amounts of primers for weak loci and at the same time with decreasing the amounts of primers for the strong loci. Genotypes of multiplex set A were obtained as shown in Figure 3.26.

The multiplex PCR set B was co-amplification of the CUPmo 4, 13 and 19 loci. The duplex set of loci 13 and 19 were first optimized by using the same amplification conditions as with a single locus PCR except that the annealing temperature was chosen at 50°C. Amplification of this duplex showed reliable genotype patterns. Then CUPmo 4 locus was combined into this duplex set. PCR

products were faint (Figure 3.27). For further adjustment, PCR profiles and reaction components were optimized similar to those described in the multiplex PCR set A. The reliable genotypes of the multiplex PCR set B were shown in Figure 3.28.

The multiplex PCR set C was co-amplification of CUPmo the 14, 21 and 23 loci. When the CUPmo 21 and 23 were co-amplified using amplification conditions as with a single locus PCR, result showed reliable genotypes. Then CUPmo 14 primers were combined into the duplex set. Non-specific products were appeared (Figure3.29). Further adjustment of the PCR profiles and conditions yielded reliable genotypes of the multiplex PCR set C as shown in Figure 3.30.

The multiplex PCR set D was co-amplification of CUPmo 22 and 24 loci. The multiplex PCR set D was duplex PCR, thus $MgCl_2$ and dNTP concentrations were decreased to 2 and 200 mM, respectively. Reliable genotypes of the multiplex PCR set D were shown in Figure 3.31. Lowering concentrations of $MgCl_2$ and dNTP did not affect the production of either locus. Genotypes of each multiplex PCR system were similar to those data obtained from the analysis by using a single locus PCR. The optimal PCR reaction components and thermal profiles of 4 multiplex sets were given in Chapter II: Table 2.2 and 2.3, respectively.

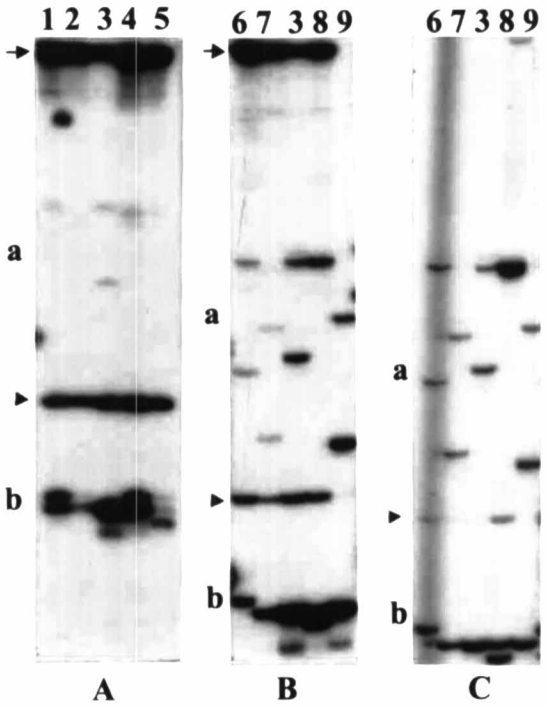


Figure 3.23 Optimization of multiplex PCR reactions with co-amplification of loci CUPmo 15 (a)+CUPmo 16 (b). The extension temperature and time were optimized. (A) Amplification products obtained from an extension at 72°C for 1 min. (B) Amplification products obtained from an extension at 65°C for 2 min. (C) Amplification products obtained from an extension at 65°C for 4 min. Arrows and arrowheads indicate fixed non-specific products. Lanes labeled with the same number indicated the same DNA template. Amplification cycles of (A) and (B) were 3 min 95°C denaturing step, followed by 30 cycles of 1 min at 95°C, 2 min at 50°C and extension conditions as indicated above. The reaction was terminated with a final extension of 10 min.

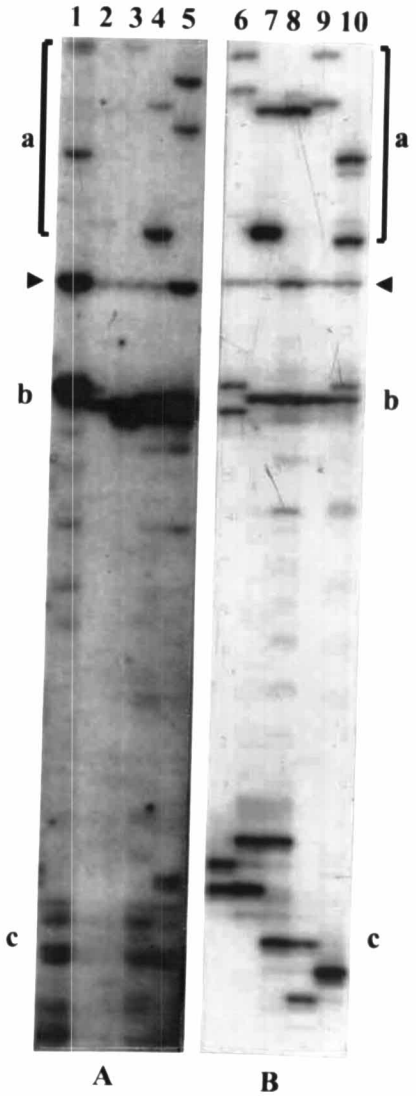


Figure 3.24 Optimization of multiplex PCR reactions with co-amplification of loci CUPmo15(a)+CUPmo16(b)+CUPmo11(c). (A) Amplification products were obtained at 50 mM KCl. (B) Amplification products were obtained at 100 mM KCl.

An arrowhead indicates fixed non-specific products.

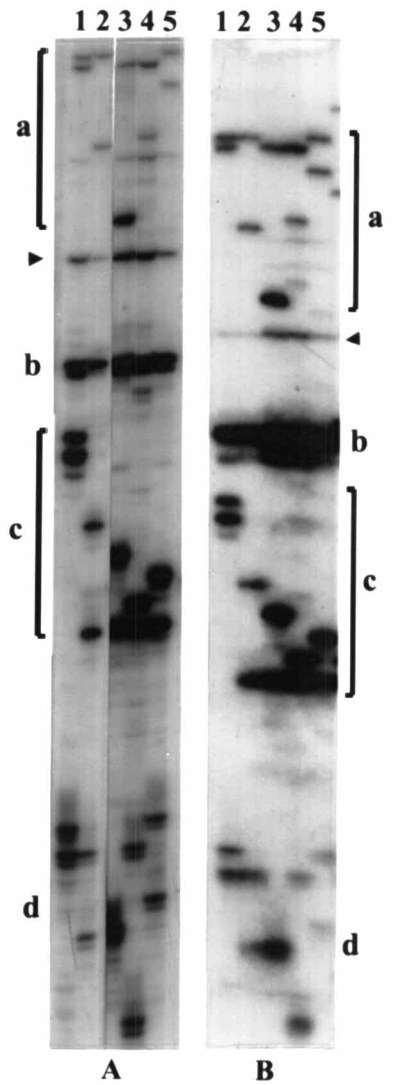


Figure 3.25 Optimization of multiplex PCR reactions with co-amplification of loci CUPmo 15 (a) + CUPmo 16 (b) + CUPmo 2 (c) + CUPmo 11 (d). (A) The PCR profile was performed using regular PCR while (B) the profile was a touchdown PCR. Amplification products of the CUPmo 15 (a) showed non-specific products with a regular PCR profile (A). Non-specific products were reduced with the touchdown PCR (B). Arrowhead indicated fixed non-specific products.

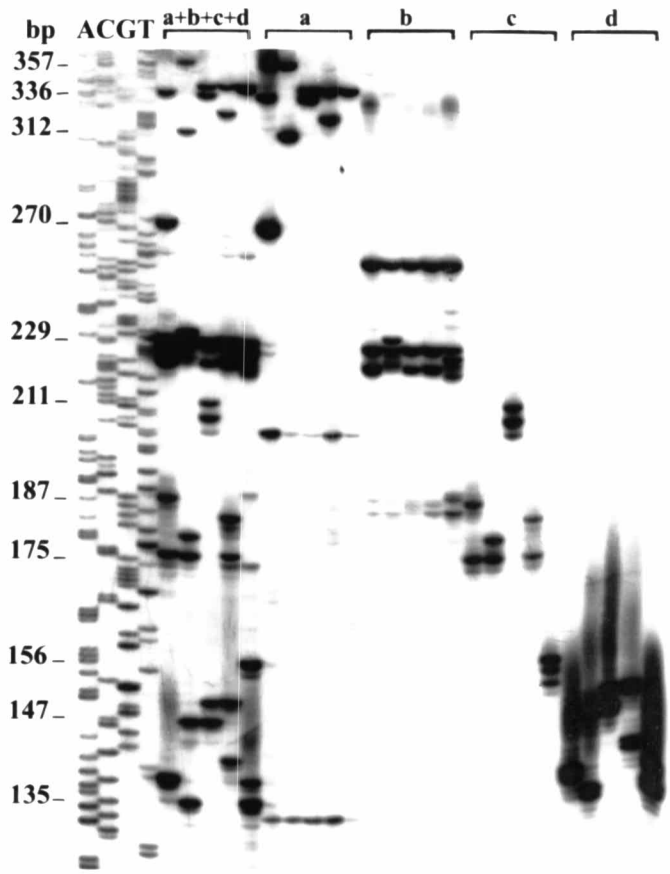


Figure 3.26 Comparison of microsatellite patterns between the multiplex PCR set A (CUPmo 15+16+2+11) and their single locus PCR (a, b, c and d, respectively). The size standard is a sequencing ladder of M13mp18.

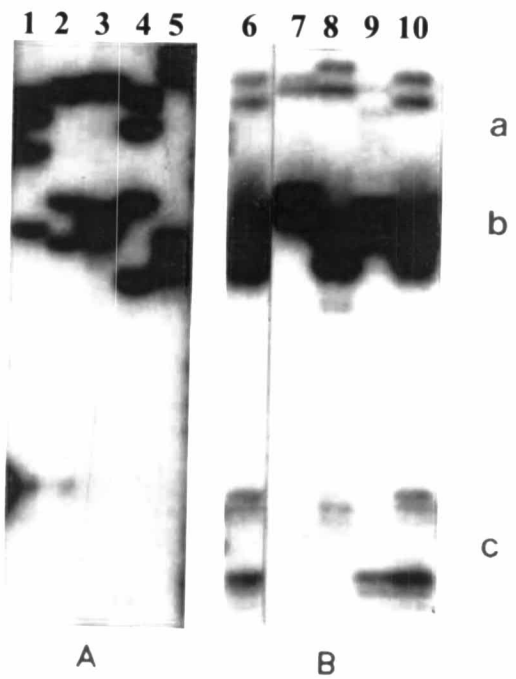


Figure 3.27 Optimization of multiplex PCR reactions with co-amplification of multiplex set B (CUPmo 19+13+4). (A) displays amplification products of CUPmo 19 (a) + CUPmo 13 (b). (B) displays amplification products of CUPmo 19 (a) + CUPmo 13 (b) + CUPmo 4 (c). The amplification reactions and PCR programs were the same as with a single locus PCR.

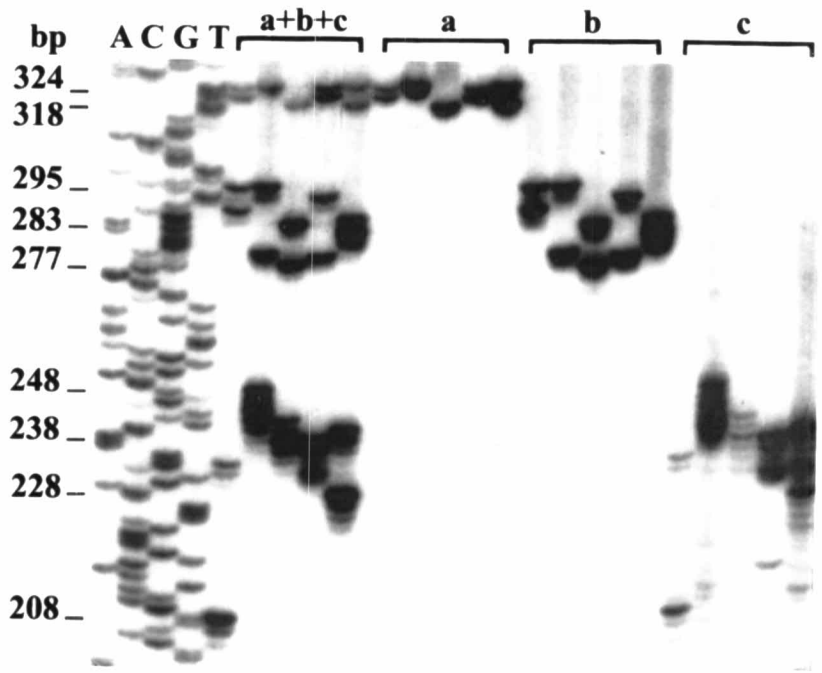


Figure 3.28 Comparison of microsatellite patterns between the multiplex PCR set B (CUPmo 19+13+4) and their single locus PCR (a, b and c, respectively). The size standard is a sequencing ladder of M13mp18.

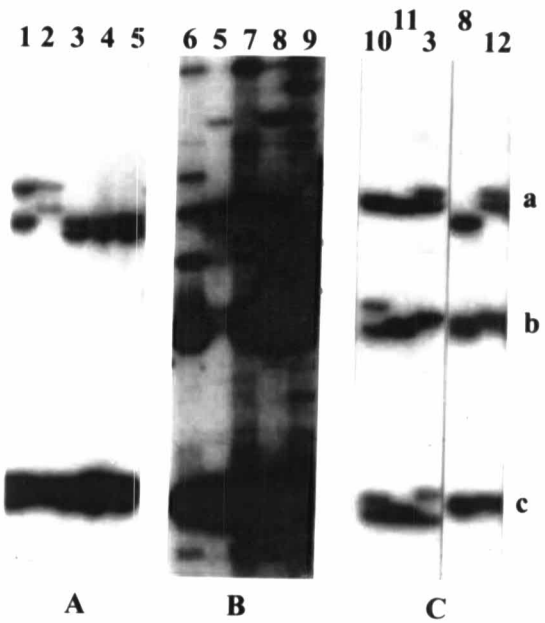


Figure 3.29 Optimization of multiplex PCR reactions with co-amplification of the multiplex set C (CUPmo 14+21+23). (A) displays amplification products of loci CUPmo 23 (a) + CUPmo 21 (c). (B and C) display amplification products of loci CUPmo 23 (a) + CUPmo 14 (b) + CUPmo 21 (c). The amplification reaction and PCR profiles of (A) and (B) were the same as a single locus PCR. (C) displays genotypes of the multiplex set C after amplification reactions and PCR profiles were adjusted as described for the multiplex set A. The size standard is a sequencing ladder of M13mp18.

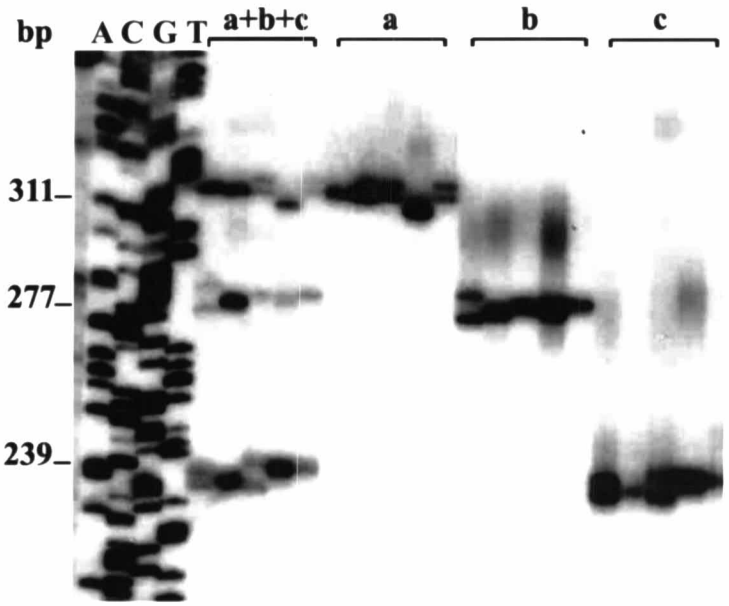


Figure 3.30 Comparison of microsatellite patterns between the multiplex PCR set C (CUPmo 23+21+14) and their single locus PCR (a, b and c, respectively). The size standard is a sequencing ladder of M13mp18.

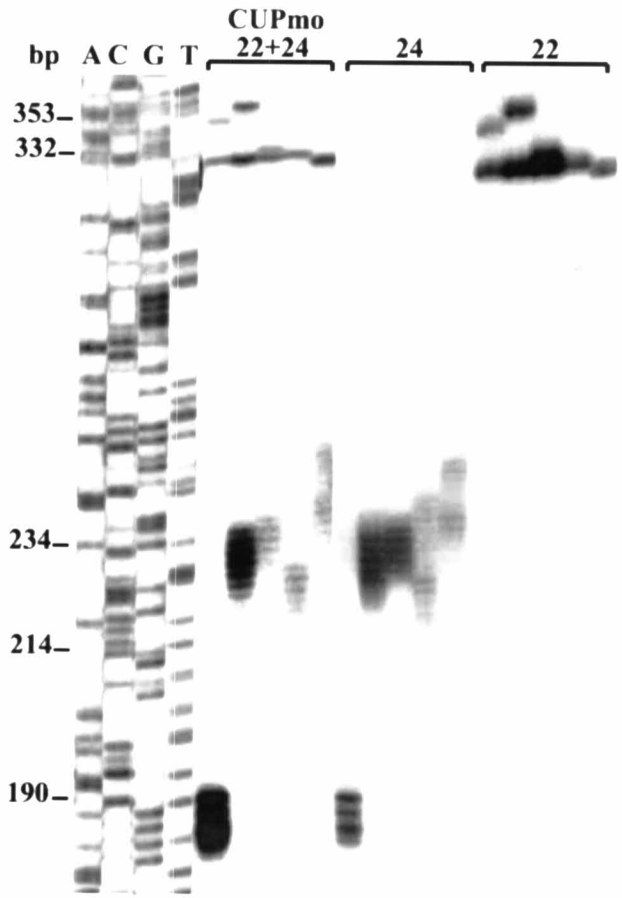


Figure 3.31 Comparison of microsatellite patterns between the multiplex PCR set D (CUPmo 22+24) and their single locus PCR. The size standard is a sequencing ladder of M13mp18.

3.9 Silver staining detection of microsatellite amplification

Traditionally, microsatellite analysis was performed based on radioactive detection of allelic patterns (PCR products). Despite its high sensitivity, radioactive detection requires the special care of harzadous radioisotopes. The silver staining detection method offers a non-harzadous and simple method for microsatellite analysis (Schlotterer, 1998). PCR amplifications were performed using the same amplification conditions as with radioactive-labeled PCR with exception that non labeling primer was added to the reaction. Detection of amplified microsatellite alleles was performed by separation of amplified products in 8% denaturing polyacrylamide gels, followed by silver staining.

The procedures for detection of microsatellite allelic patterns with silver staining system had been optimized by Soot-anan (1999). In this study, the multiplex PCR set B (CUPmo 4+13+19) was chosen for silver staining. The procedures for detection of allelic patterns for the multiplex PCR set B was followed Soot-anan (1999). The optimal procedures for microsatellite allele detection with silver staining system were described in Chaper II: Section 2.14.

As can be seen in Figure 3.32, allelic patterns of silver staining detection for the multiplex PCR set B were similar to those of radioisotopic detection except the numbers of amplified bands of each allele from silver staining detection were twice more than those from isotopic detection.

Two fragments were revealed for each allele at loci CUPmo 19 and 13 for silver staining detection while those of radioisotope detection were revealed 1

fragment for each allele (Schlotterer, 1998). However, the authentic alleles could be identified. The authentic alleles of CUPmo 4 from silver staining were difficult to identify because of allelic patterns of CUPmo 4 were low intensity and contained several stutter bands. CUPmo 4 is di-/tetranucleotide repeats. Dinucleotide repeats showed degree of stutter bands frequently. Results demonstrated that in general, separation of microsatellites by polyacrylamide gel electrophoresis and silver staining could be used for genotyping of *P. monodon.*

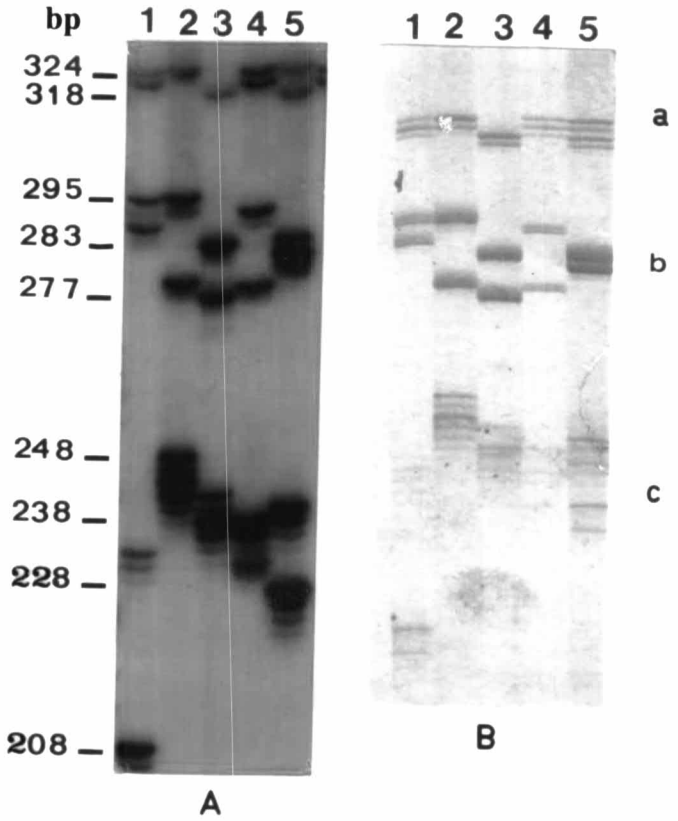


Figure 3.32 Comparison of microsatellite patterns of the multiplex PCR set B (CUPmo 19 (a) + 13 (b) + 4 (c) between radioisotope and silver staining detection methods. (A) displays the radio-labeled PCR products while (B) the PCR products were detected by silver staining. Lanes 1-5 contain individually amplified DNA samples.

3.10 Application in shrimp genome mapping

The aim of genetic mapping is to locate genetic markers at a particular position on the chromosomes. Two loci (or genetic markers) are thus said to be linked if the parental allele combinations are preserved more often than would be expected by random segregation. The degree of linkage, or genetic distance, between two loci is a function of the frequency of recombinations. The measurement of a map distance is expressed in Morgan (or cM for centiMorgan) and is defined as the expected number of crossover between two loci on chromosome (Boyd, 1998).

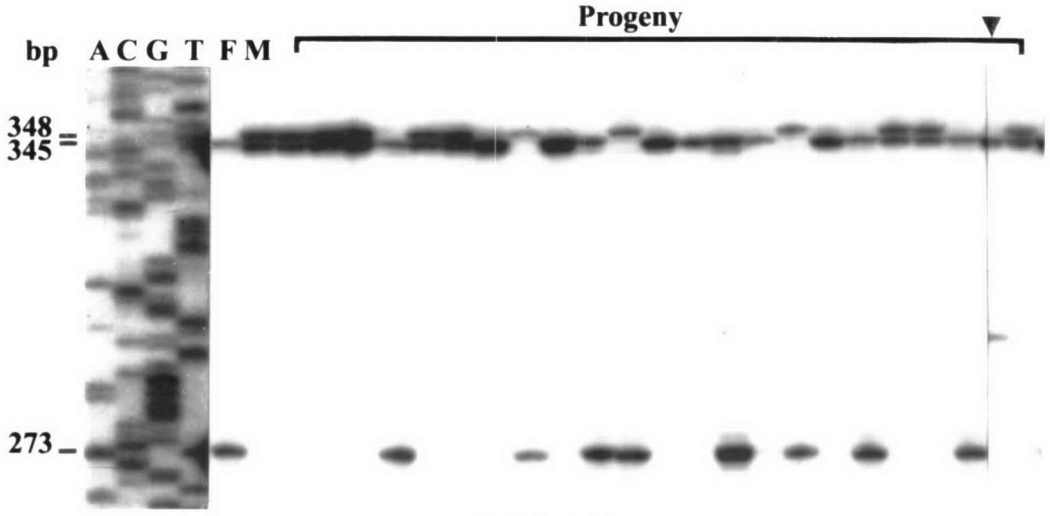
Due to difficulties in isolating microsatellites from penaeid species in a number of laboratories, the international collaboration between different parties on genetic mapping of *P. monodon* was initiated in 1997 (Wilson et al., 2002). Construction of the genetic map of *P. monodon* was carried out using a reference family produced at the Australian Institute of Marine Science (AIMS). The family was genotyped using 10 microsatellite loci, CUPmo 1, 2, 4, 9, 12, 13, 15, 19, 22 and 24. The amplification, separation and visualization of alleles, using M13 denaturing gel electrophoresis and radiolabeling technique were carried out as described in Chapter II: Section 2.11.

P. monodon family consisted of two parents and 42 offspring was genotyped with 10 microsatellite loci. Data on allelic inheritance of 10 microsatellites for the *P. monodon* family were illustrated in Table 3.14. Examples of allelic inheritance of CUPmo 15 and multiplex set B (CUPmo 4+13+19) were shown in Figures 3.33 and 3.34, respectively. Non-mendelianly inheritance observed in the progeny No.26. Not

all markers could identify non-family progeny of progeny No.26. This non-family progeny could be identified by markers CUPmo 2, 12, 13, 19, 22 and 24.

Genotypic data was sent to Molecular Animal Genetic Centre, University of Queensland, Australia for linkage mapping analysis. All 10 microsatellite markers were analyzed along with 29 AFLP primer combination. Seven markers were placed on the *P. monodon* linkage map. Information of *P. monodon* map is available on *P. monodon* shrimpmap web site (http://www.aims.gov.au/shrimpmap).

Due to difficulties in isolating microsatellites from penaeid species, the technology of Amplified Fragment Length Polymorphism (AFLPs) was applied to generate the preliminary genetic linkage map in *P. monodon* (Wilson et al., 2002). AFLPs can be applied without any prior development of markers and had allowed the generation of a preliminary genetic map in *P. japonicus* with a relative high efficiency (Moore, et al., 1999). In this study, ten microsatellite loci were analyzed linkage mapping with those AFLP markers. Only 7 loci were mapped on the existing AFLP marker(s) and the rest 3 did not. A summary of statistics for linkage map is shown in Table 3.15 and Figure 3.35. One hundred and sixty markers were mapped and formed 60 linkage groups with a total genome length of 1553 cM. The average density of markers was approximately one every 9.7 cM. Details of some individual linkage groups are shown in Table 3.16. CUPmo 2 and CUPmo 9 formed a linkage group whereas the remaining loci were mapped into linkage groups with one or more markers. CUPmo 1 and CUPmo 9 were mapped in more than 1 linkage group. This was due to the different alleles of the same microsatellite loci are coming from different parents (one group from male parent and other from the female parent).


Progeny
bp A C G T F M
348
345
273

CUPmo15

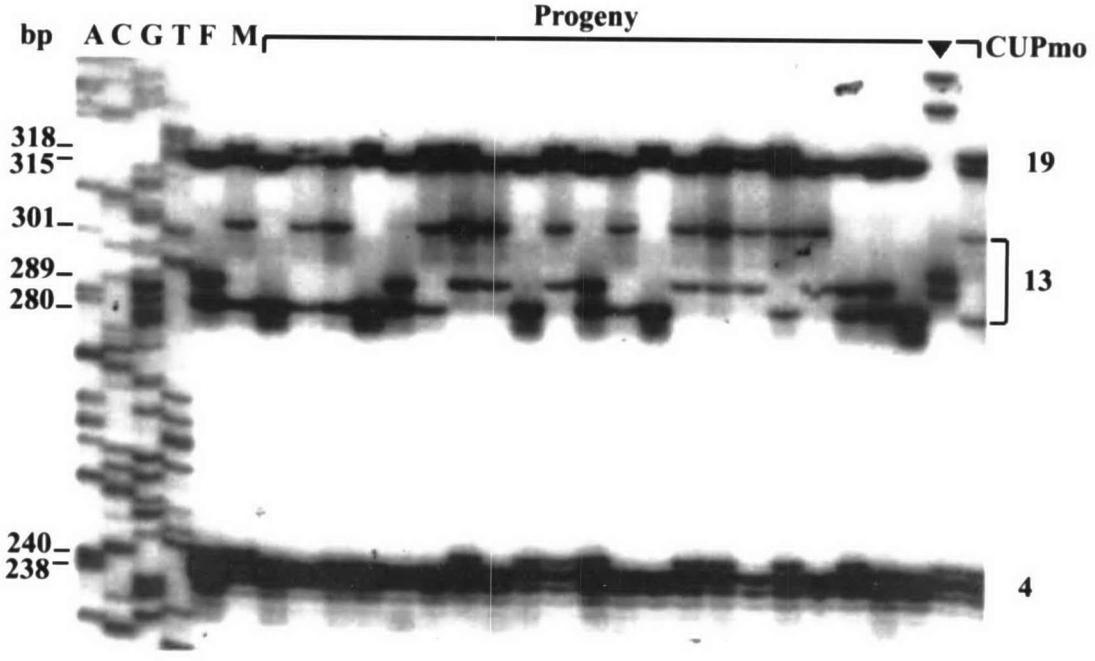

Progeny
bp A C G T F M
CUPmo
318
315
301
289
280
240
238
19
13
4

Figure 3.33 Allelic inheritance of CUPmo 15 for mapping analysis of *P. monodon* family. (F) and (M) are father and mother, respectively. An arrowhead indicates non-mendelian inheritance of progeny No.26. The size standard is a sequencing ladder of M13mp18.

Figure 3.34 Allelic inheritance of multiplex set B (CUPmo 19+13+4) for mapping analysis of *P. monodon*. (F) and (M) are father and mother, respectively. An arrowhead indicates non-mendelian inheritance of progeny No.26. The size standard is a sequencing ladder of M13mp18.

Table 3.14 Genotypes of *P. monodon* pedigree samples for 10 microsatellite loci.

Sample	Genotype									
	CUPmo 1	CUPmo 2	CUPmo 4	CUPmo 9	CUPmo 12	CUPmo 13	CUPmo 15	CUPmo 19	CUPmo 22	CUPmo 24
Father	BD	BB	BB	-	BC	BC	BC	BB	AA	AA
Mother	AC	AC	AB	-	AB	AC	AB	AB	BC	AB
Progeny										
1	AD	-	BB	-	BB	CC	AB	BB	AC	AB
2	AD	AB	BB	BD	AC	BC	AB	BB	AB	AA
3	BC	AB	BB	CD	BB	AC	AB	AB	AB	AB
4	AB	BC	BB	BD	AB	AC		BB	AB	AB
5	BC	AB	BB	-	AC	BC	AB	BB	AB	AA
6	AD	AB	BB	BD	AC	CC	AB	AB	AC	AA
7	CD	AB	BB	CA	AB	BC	BB	BB	AB	AB
8	BC	AB	BB	CA	AC	AC	AC	AB	AB	AA
9	AB	AB	AB	AB	AC	AB	BB	AB	AC	AB
10	BC	AB	BB	-	BB	AB	BC	BB	AC	AB
11	AD	BC	AB	AC	AB	CC	AC	BB	-	AA
12	AB	AB	AB	AC	BB	AB	BB	AB	AC	AB
13	CD	AB	AB	AC	BC	BC	BB	BB	AC	AB
14	BC	BC	BB	AC	AB	AC	BC	BB	AC	AB
15	-	BC	AB	-	AB	BB	BB	BB	AB	AA
16	CD	BC	AB	-	BC	AC	AC	AB	AB	AB
17	CD	AB	BB	-	BC	CC	BB	AB	AC	AB
18	CD	BC	AB	AC	BC	AB	BC	BB	AC	AA
19	BC	AB	AB	BD	BC	AB	AB	AB	AB	AB
20	BC	AB	BB	BD	AC	AB	AB	AB	AB	AB
21	AB	AB	AB	BD	BC	AC	BC	AB	AC	AB
22	CD	AB	BB	AB	BB	AB	BB	BB	AB	AB
23	AD	AB	AB	BD	AB	BC	BC	BB	AC	AB
24	BC	AB	BB	AC	AC	BC	AC	BB	AB	AA
25	AD	-	BB	-	BB	CC	AC	BB	AC	AA
26	AD	*XC	AB	AC	*XC	*XB	AB	*XY	*XX	*XY
27	CD	AB	AB	AB	BC	AC	AB	AB	AB	AA
28	-	BC	BB	BD	AC	BC	BC	AB	AC	AB
29	AB	AB	AB	BD	BC	CC	BB	AB	AC	AA
30	CD	BC	AB	BD	BC	BC	BB	BB	AC	AA
31	CD	AB	AB	BD	AB	AC	BC	AB	AC	AA
32	BC	AB	BB	BD	AB	AC	BB	BB	AB	AA

Table 3.14 (Continued)

Sample	Genotype									
	CUPmo 1	CUPmo 2	CUPmo 4	CUPmo 9	CUPmo 12	CUPmo 13	CUPmo 15	CUPmo 19	CUPmo 22	CUPmo 24
33	BC	BC	BB	-	AB	AC	BC	BB	AB	AA
34	AD	AB	AB	AB	BC	CC	BB	BB	AB	AA
35	AD	AB	BB	-	BB	AB	BB	BB	AC	AA
36	AB	BC	BB	BD	AB	AC	AB	BB	AB	AB
37	AD	AB	BB	AC	AB	BC	BB	AB	AC	AB
38	AB	BC	BB	AB	BB	AC	AB	BB	AC	AA
39	BC	BC	BB	CD	BC	AC	BB	AB	AB	AB
40	AD	AB	BB	AB	BB	AC	AB	BB	AC	AA
41	CD	BC	AB	AB	BB	CC	BB	AB	AC	AA
42	BC	AB	BB	-	AC	AC	AB	AB	AC	AA

* Unrelated genotype

CUPmo 1: A = 274 bp, B = 272 bp, C = 246 bp, D = 240 bp

CUPmo 2: A = 176 bp, B = 172 bp, C = 152 bp, X = 212 bp

CUPmo 4: A = 240 bp, B = 238 bp

CUPmo 9: A = 352 bp, B = 263 bp, C=300 bp, D=216 bp

CUPmo 12: A = 95 bp, B = 93 bp, C = 89 bp, X = 91 bp

CUPmo 13: A = 301 bp, B = 289 bp, C = 280 bp, X = 295 bp

CUPmo 15: A = 348 bp, B = 345 bp, C = 273 bp

CUPmo 19: A = 318 bp, B = 315 bp, X = 348 bp, Y = 330 bp

CUPmo 22: A = 236 bp, B = 204 bp, C = 202 bp, X = 240 bp

CUPmo 24: A = 356 bp, B = 344 bp, X = 352 bp, Y = 341 bp

Table 3.15 Summary of linkage map using AFLP and microsatellite data.

Total number of markers	324
No. of markers mapped	160
No. of linkage groups	60
Total length of genome mapped (cM)	1553
Average length of linkage group	25.9
Average number of markers per linkage group	2.7

Table 3.16 Linkage map data for each of microsatellite loci.

Markers	Distance (cM)
Linkage group 29	
CUPmo 9b	0.0
CUPmo 9cr	5.4
P072m	-----
	5.5 (3 markers)
Linkage group 23	
P025m	10.6
P063m	5.1
CUPmo 1d	2.5
CUPmo 1br	------
	18.2 (4 markers)
Linkage group 47	
CUPmo 1ar	2.5
CUPmo 1c	11.1
Z3158320	21.2
P035f	------
	34.8 (4 markers)
Linkage group 48	
P038f	25.5
CUPmo 12ar	------
	25.5 (2 markers)
Linkage group 53	
P044cr	25.5
P049f	14.4
CUPmo 4ar	------
	39.9 (3 markers)

Table 3.16 (Continued).

Markers	Distance (cM)
Linkage group 59	
CUPmo 2a	0.00
CUPmo 2cr	------
	0.0 (2markers)
Linkage group 60	
CUPmo 9a	0.0
CUPmo 9dr	------
	0.00 (2 markers)
Linkage group 32	
P015c	24.0
CUPmo 22br	5.0
CUPmo 22c	11.8
P001f	19.1
P031fr	-----
	59.9 (5 markers)
Linkage group 40	
P021f	18.1
B3160154	17.1
CUPmo 24b	-----
	35.3 (3 markers)

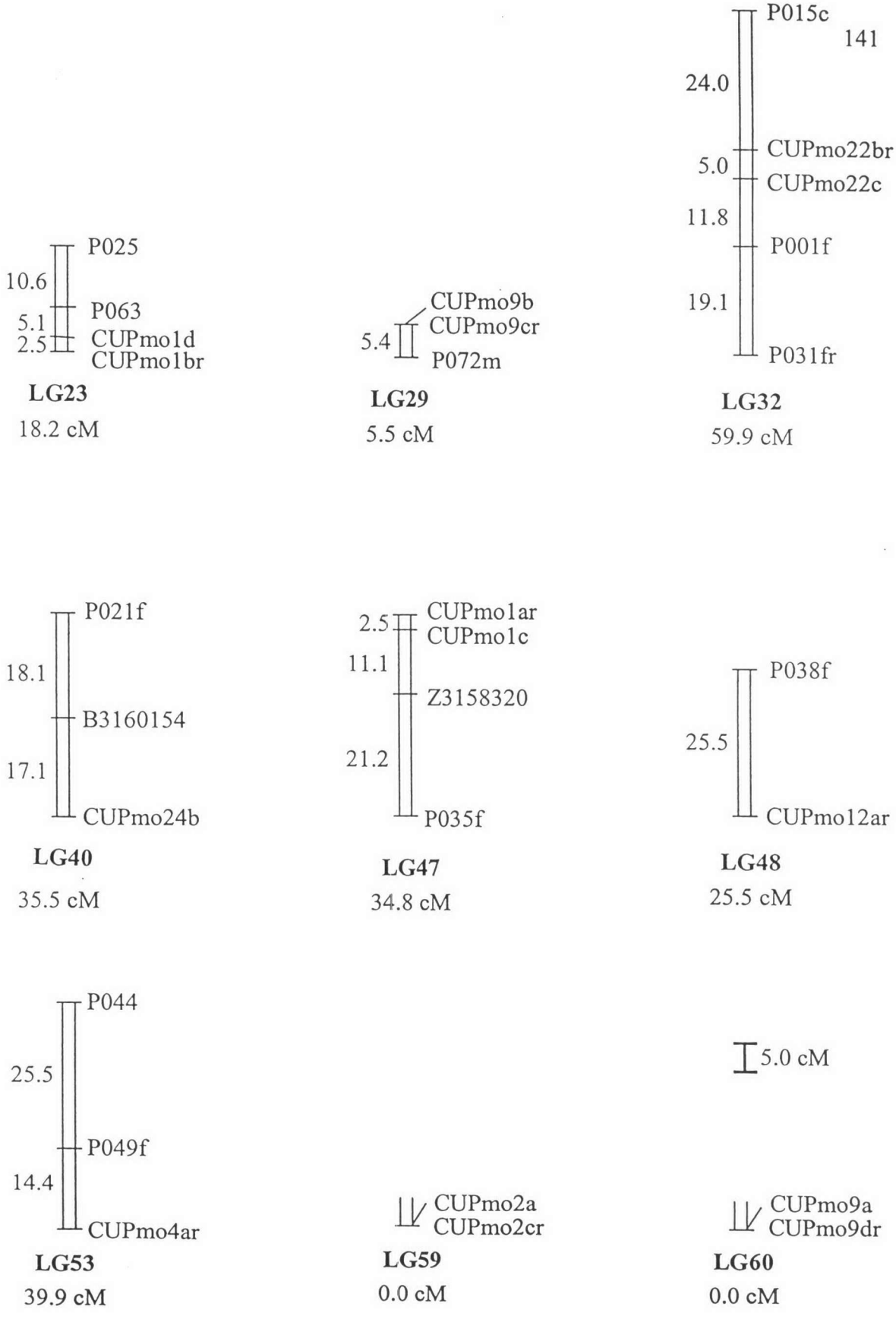


Figure 3.35 Linkage groups of 7 microsatellite loci derived from analyzing the data with AFLPs.