สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Chapter III

Results

3.1 PCR conditions for amplification of microsatellite loci in *P. monodon*

Eight microsatellite loci, CSCUPmo1, CSCUPmo2, CSCUPmo3, CSCUPmo4, CSCUPmo6, CSCUPmo7, CSCUPmo9 and CSCUPmo11 developed by Pongsomboon et al. (2000) were selected for this study. The repeat and primer sequences and annealing temperatures for these microsatellite loci are shown in Table 3.1.

PCR conditions and programs for amplification of all eight loci were identical except the annealing temperatures which were optimized for each locus to obtain the maximum yield. The optimal annealing temperature for amplification of six microsatellite loci; CSCUPmo1, CSCUPmo2, CSCUPmo3, CSCUPmo6, CSCUPmo7 and CSCUPmo11 was 56 °C. This annealing temperature resulted in reproducible and scorable results as illustrated by Figures 3.1-3.3, 3.5, 3.6 and 3.8. The most suitable annealing temperature for amplification of the CSCUPmo4 and CSCUPmo9 loci was at 54 °C. At a 56 °C annealing temperature, low yields of amplification products were obtained while at a 52 °C, non-specific amplification products were clearly observed. An increase of a temperature to 54 °C revealed much better results as can be seen in Figures 3.4 and 3.7.

3.2 Polymorphism of eight investigated microsatellites

DNA samples isolated from *P. monodon* originating from Trad province by Supungul (1998) were used to determine polymorphism of eight microsatellite loci. The number of alleles, size range of alleles and observed heterozygosity were illustrated in Table 3.2. The number of alleles ranged from 10-33 alleles per locus and heterozygosity ranged from 0.21- 0.90. Allelic sizes varied from the smallest allele at

132 bp (CSCUPmo11) to the largest allele at 380 bp (CSCUPmo9). PCR amplified microsatellites of eight loci were loaded in one gel to illustrate variation in allelic sizes (Figure 3.9).

Table 3.1 The repeat and primer sequences, and annealing temperatures for amplification of eight microsatellite loci in *P. monodon*.

Locus	Repeat sequence	Primer sequence	Annealing temperature (°C)
CSCUPmo1	$(GAA)_{43}$	F[a]=ATGATGGCTTTGGTAAATGC R[b]=CGTACTTCCTCTTCATAGGTATC	56
CSCUPmo2	$(ATCT)_{12}(TA)_{10}(TAGA)_3$	F[a]=CCAAGATGTCCCCAAGGC R[b]=CTGCAATAGGAAAGATCAGAC	56
CSCUPmo3	$(ATCT)_{12}(AT)_9T(GT)_9$	F[a]=TGCGTGATTCCGTGCATG R[b]=AGACCTCCGCATACATAC	56
CSCUPmo4	$(CT)_{10}TG(CT)_{17}(ATCT)_{10}$	F[a]=TTTCTTTCTTCTCGTGATCCC R[b]=GACGGCATGAGGAATAGAGG	54
CSCUPmo6	$(GATA)_6(GA)_{16}$	F[a]=TAGTGTTACTCAGGTGCAGC R[b]=GCGTGTATTTGTGATTTCAC	56
CSCUPmo7	$(CT)_{15}(ATCT)_9$	F[a]=ACGAATGAATGCGGTGGTGC R[b]=TCGGTGCCCAGTTGTATGAGAG	56
CSCUPmo9	$(TA)_{49}$	F[a]=TGCCGTTTTGCGAACATGCG R[b]=TGATGGTGAGCTTAGAACTACCAAC	54
CSCUPmo11	$(TAA)_9TGA(TAA)_3$	F[a]=TGCTATGACTCGCCTAACAG R[b]=GGTTATGAAAATTACCCTAA	56

F[a] : Forward primer

R[b] : Reverse primer

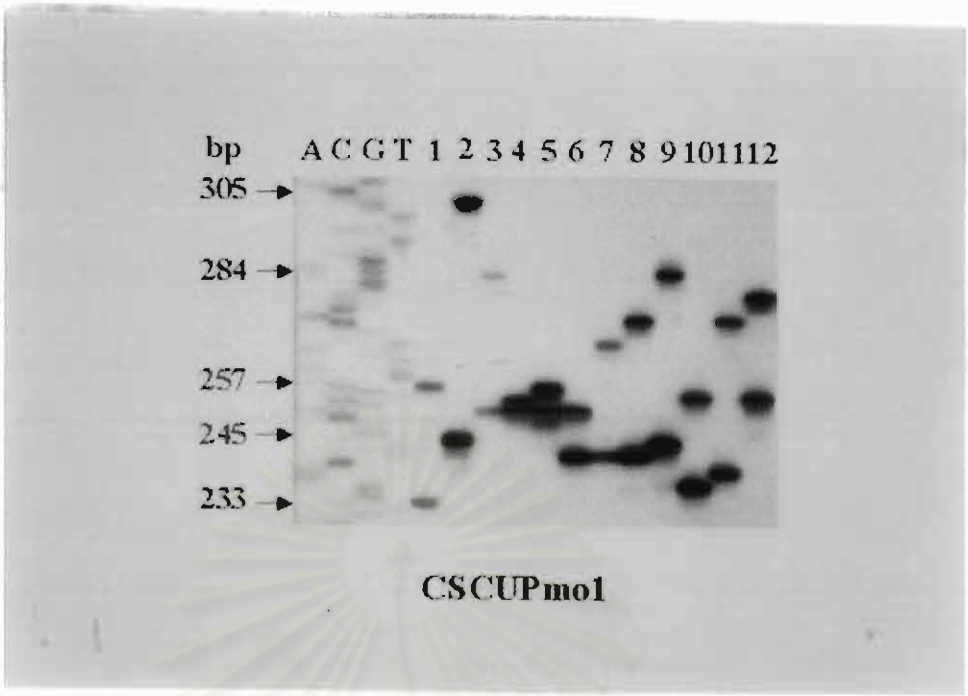


Figure 3.1 PCR amplification patterns of the CSCUPmo1 locus from 12 individuals of *P. monodon* (lanes 1-12). The size standard is a sequencing ladder of M13 mp18.

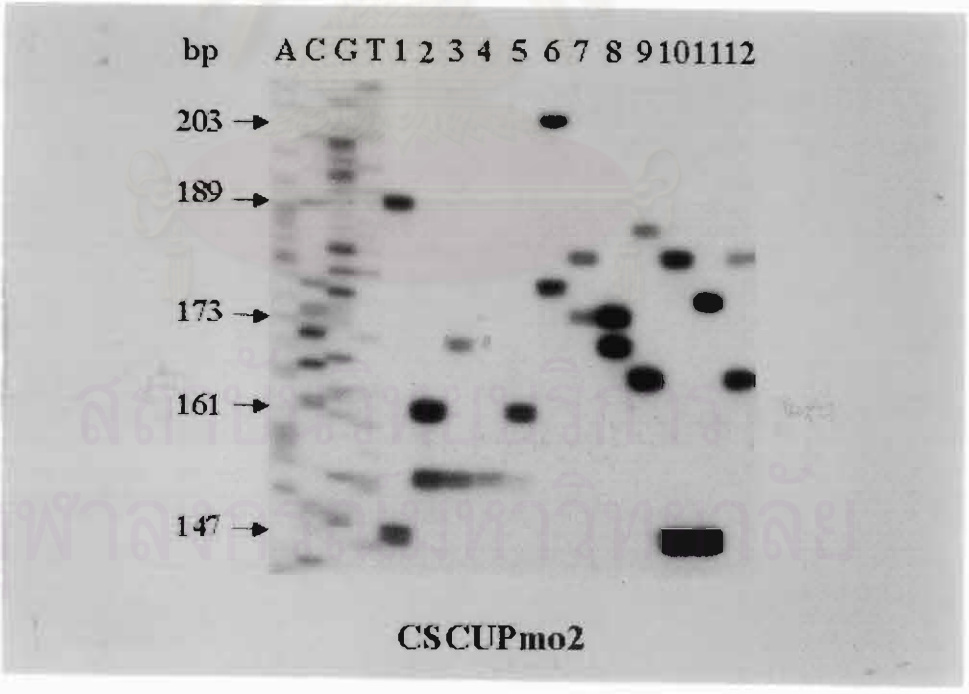


Figure 3.2 PCR amplification patterns of the CSCUPmo2 locus from 12 individuals of *P. monodon* (lanes 1-12). The size standard is a sequencing ladder of M13 mp18.

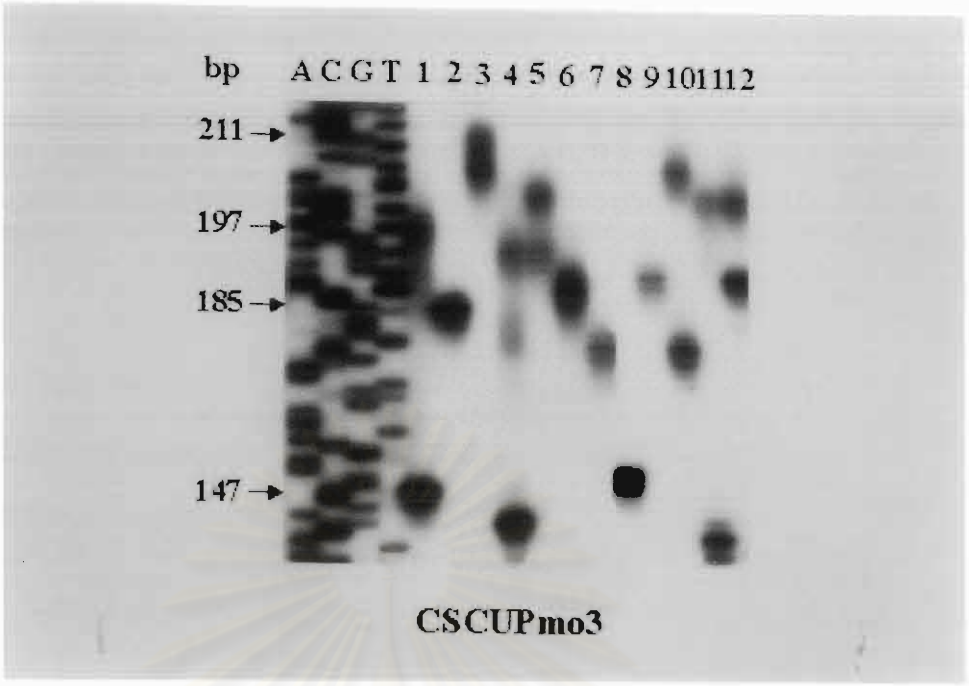


Figure 3.3 PCR amplification patterns of the CSCUPmo3 locus from 12 individuals of *P. monodon* (lanes 1-12). The size standard is a sequencing ladder of M13 mp18.

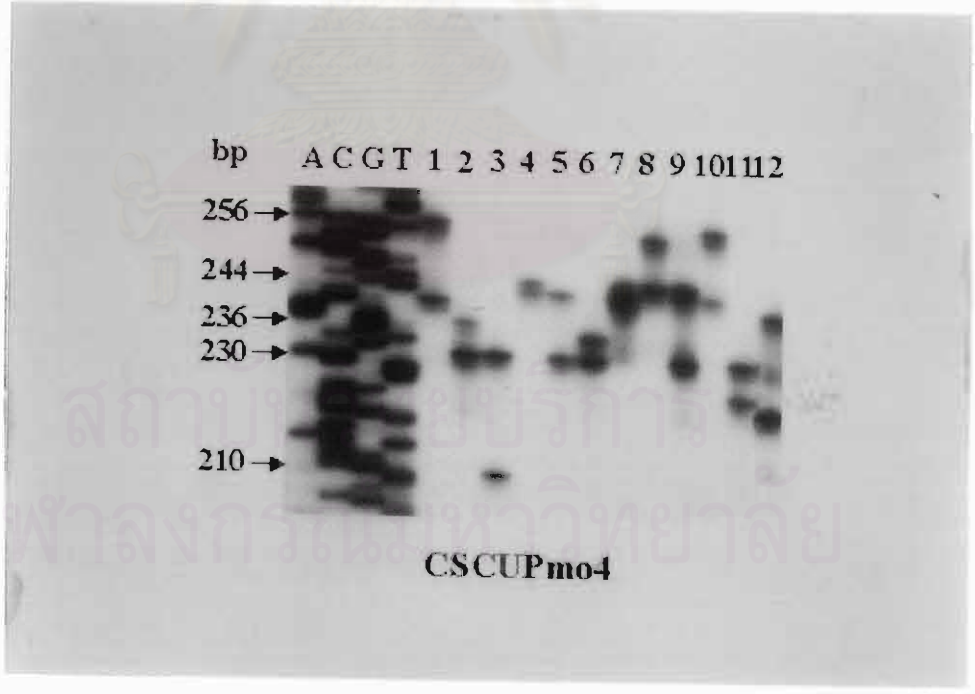


Figure 3.4 PCR amplification patterns of the CSCUPmo4 locus from 12 individuals of *P. monodon* (lanes 1-12). The size standard is a sequencing ladder of M13 mp18.

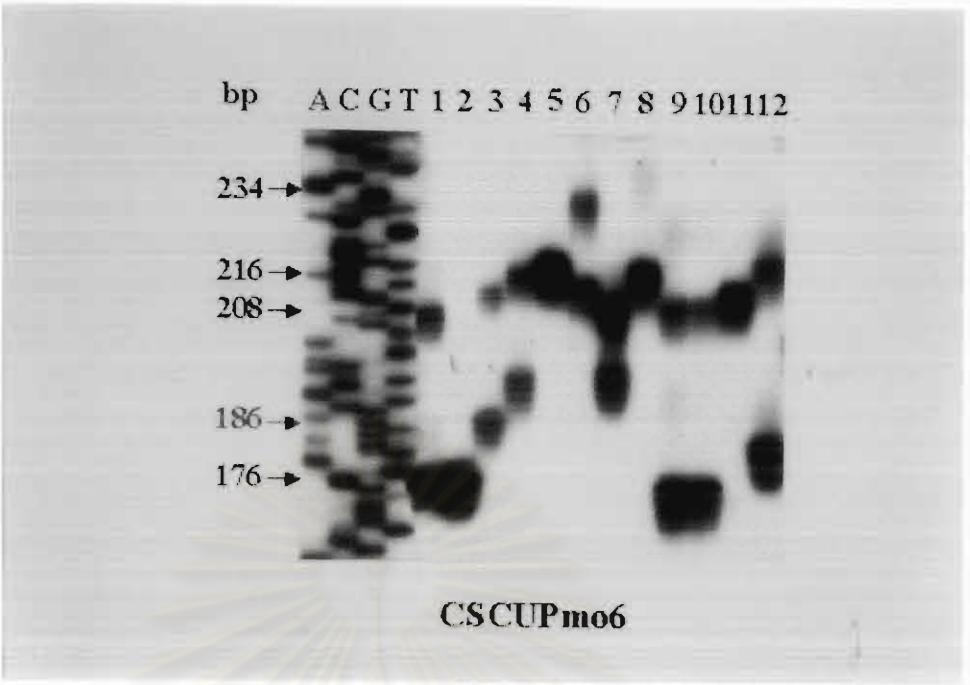


Figure 3.5 PCR amplification patterns of the CSCUPmo6 locus from 12 individuals of *P. monodon* (lanes 1-12). The size standard is a sequencing ladder of M13 mp18.

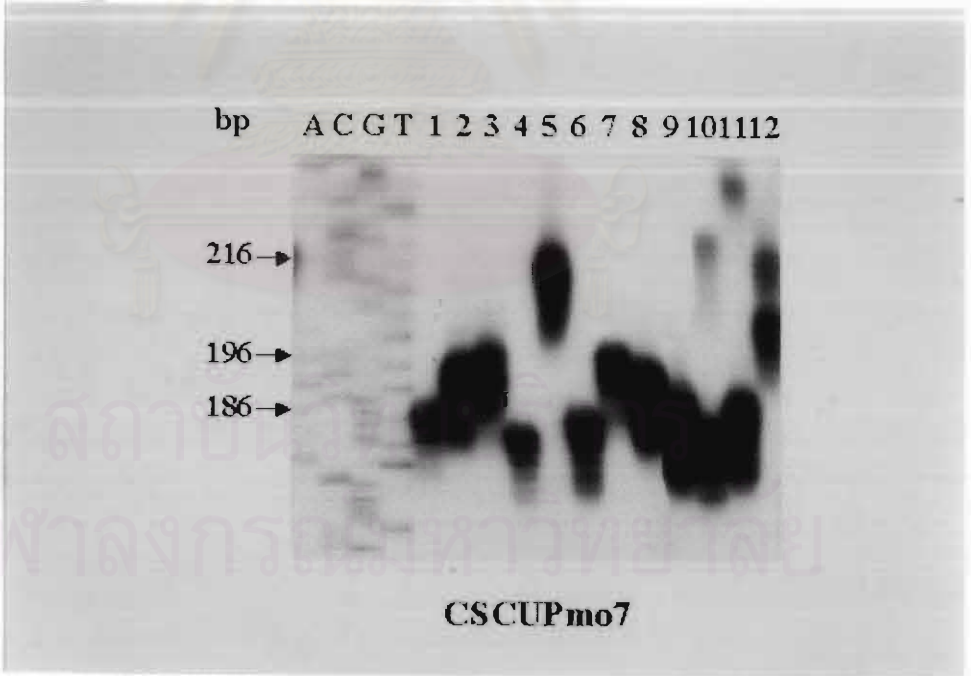


Figure 3.6 PCR amplification patterns of the CSCUPmo7 locus from 12 individuals of *P. monodon* (lanes 1-12). The size standard is a sequencing ladder of M13 mp18.

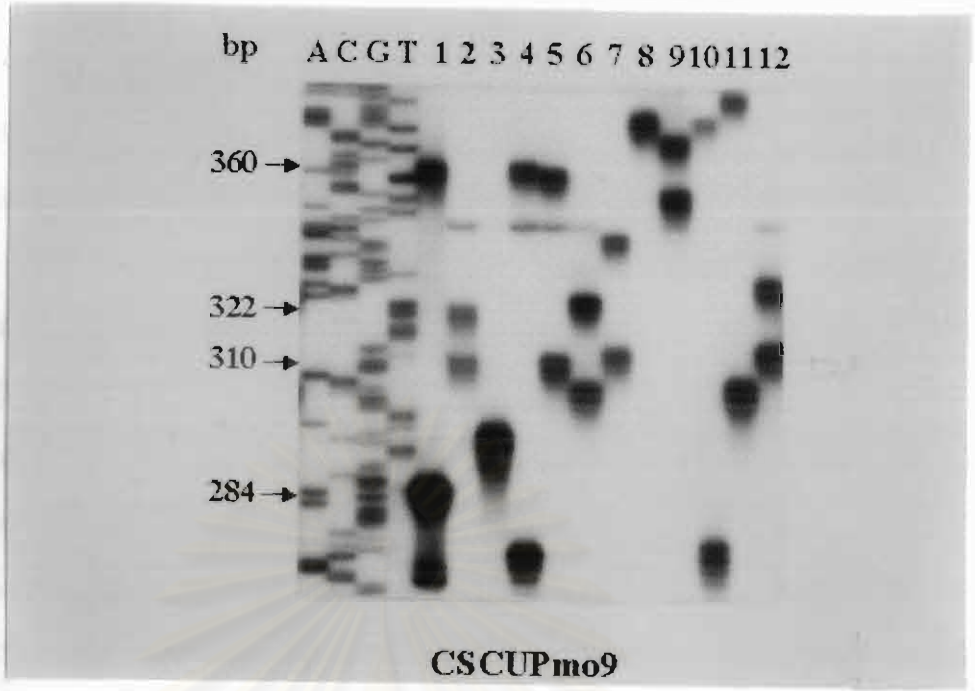


Figure 3.7 PCR amplification patterns of the CSCUPmo9 locus from 12 individuals of *P. monodon* (lanes 1-12). The size standard is a sequencing ladder of M13 mp18.

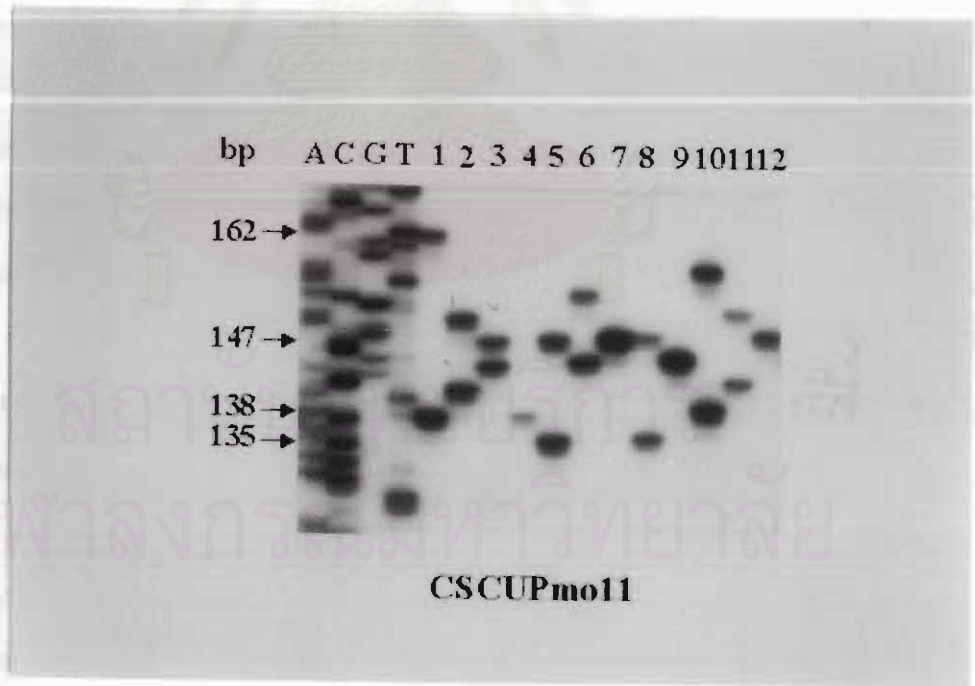


Figure 3.8 PCR amplification patterns of the CSCUPmo11 locus from 12 individuals of *P. monodon* (lanes 1-12). The size standard is a sequencing ladder of M13 mp18.

Allele distribution frequencies of *P. monodon* at eight microsatellites in the Trad population were summarized in Figure 3.10. The most common allele of each locus was found at frequency less than 0.2 except that of the locus CSCUPmo11. The allele differences between consecutive alleles depended on the repeat type of microsatellite loci. For CSCUPmo1 and CSCUPmo11 loci, consecutive alleles differ by 3 bases. For loci linked to various types of dinucleotide repeats, the 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 CSCUPmo3 jumped from 147 to 169 which was larger than other loci. Disappearance of the rare alleles with sizes larger than 220 bp for the CSCUPmo6 locus may result from less amplification efficiency of those alleles obviating an ability to score these alleles.

From DNA typing and polymorphic studies of eight microsatellites, six microsatellites including CSCUPmo1, CSCUPmo2, CSCUPmo4, CSCUPmo6, CSCUPmo9 and CSCUPmo11, were selected for further studies. The *P. monodon* samples from other places in Thailand (Trang and Chumporn) were included to obtain more reliable information on size ranges of alleles and observed heterozygosity of each locus as illustrated in Table 3.3. Different numbers of specimens for each locus reflected the efficiency of amplification success at a given locus. A total of 14 different alleles resulting in a larger size range of alleles were found at the locus CSCUPmo9 when the number of samples was increased to 87 samples. The number of alleles of CSCUPmo11 did not change even when the number of samples was increased to 97. The increases of 6, 3, 1 and 4 newly identified alleles were found at loci CSCUPMo1, CSCUPmo2, CSCUPmo4 and CSCUPmo6 when the number of samples was increased to 124, 121, 97 and 93 individuals, respectively. Although the number of alleles per locus at almost all of the loci was increased where larger sample sizes were analyzed,

observed heterozygosity levels were relatively constant suggesting that a sample size of approximately 50 individuals should be sufficient to represent the diversity level of a *P. monodon* population.

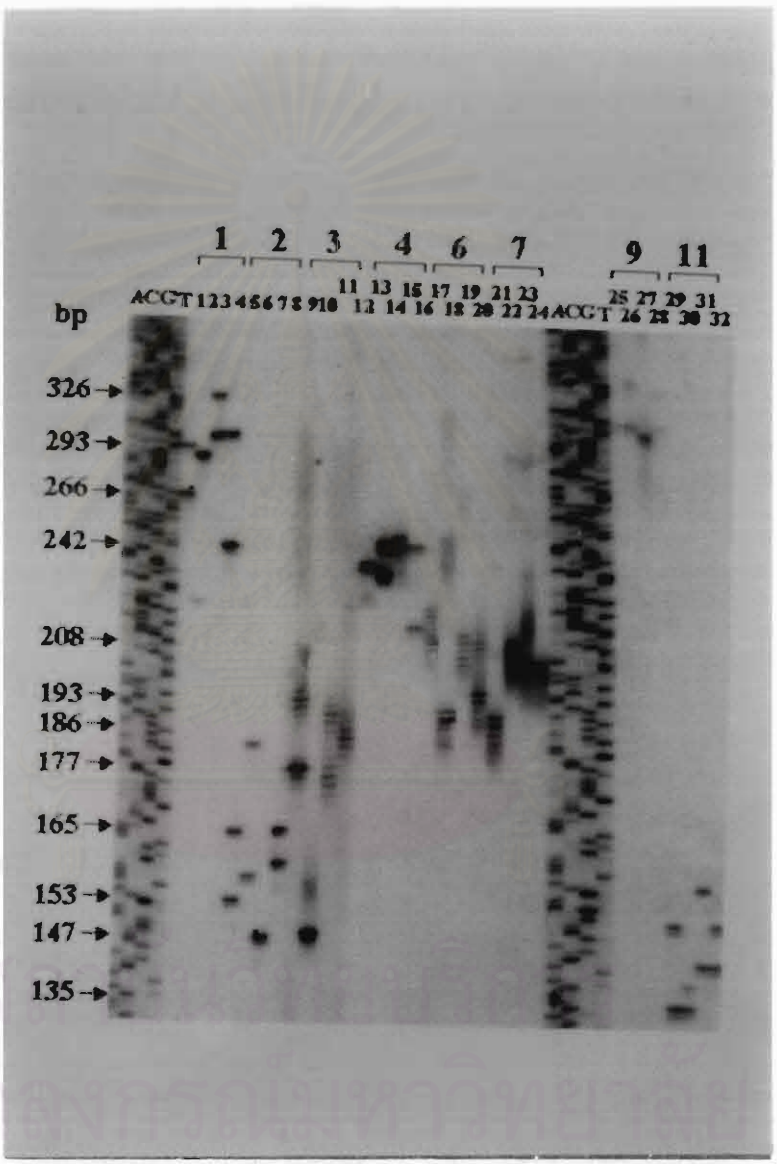


Figure 3.9 PCR amplified microsatellites of unrelated *P. monodon* for eight microsatellites: CSCUPmo1, CSCUPmo2, CSCUPmo3, CSCUPmo4, CSCUPmo6, CSCUPmo7, CSCUPmo9 and CSCUPmo11. Sizes of microsatellite alleles were estimated by comparing with a sequencing ladder of M13 mp18

Table 3.2 Number of alleles, size-range of alleles and observed heterozygosity at eight microsatellites of *P. monodon* from Trad

Locus	Number of samples	Number of alleles	Size range of alleles	Observed heterozygosity
CSCUPmo1	51	29	224 - 326	0.75
CSCUPmo2	51	27	147 - 217	0.78
CSCUPmo3	50	27	135 - 223	0.62
CSCUPmo4	51	21	206 - 256	0.90
CSCUPmo6	51	29	166 - 242	0.71
CSCUPmo7	43	22	172 - 234	0.21
CSCUPmo9	40	33	274 - 380	0.90
CSCUPmo11	47	10	132 - 162	0.81

Table 3.3 Number of alleles, size-range of alleles and observed heterozygosity at six microsatellites of *P. monodon* from Trad, Trang, and Chumporn

Locus	Number of samples (no. of successfully amplified samples)	Number of alleles	Size range of alleles	Observed heterozygosity
CSCUPmo1	124 (124)	35	218 - 332	0.76
CSCUPmo2	124 (121)	30	147 - 219	0.83
CSCUPmo4	108 (97)	22	206 - 256	0.92
CSCUPmo6	103 (93)	34	164 - 242	0.73
CSCUPmo9	103 (87)	47	258 - 380	0.90
CSCUPmo11	103 (97)	10	132 - 162	0.87

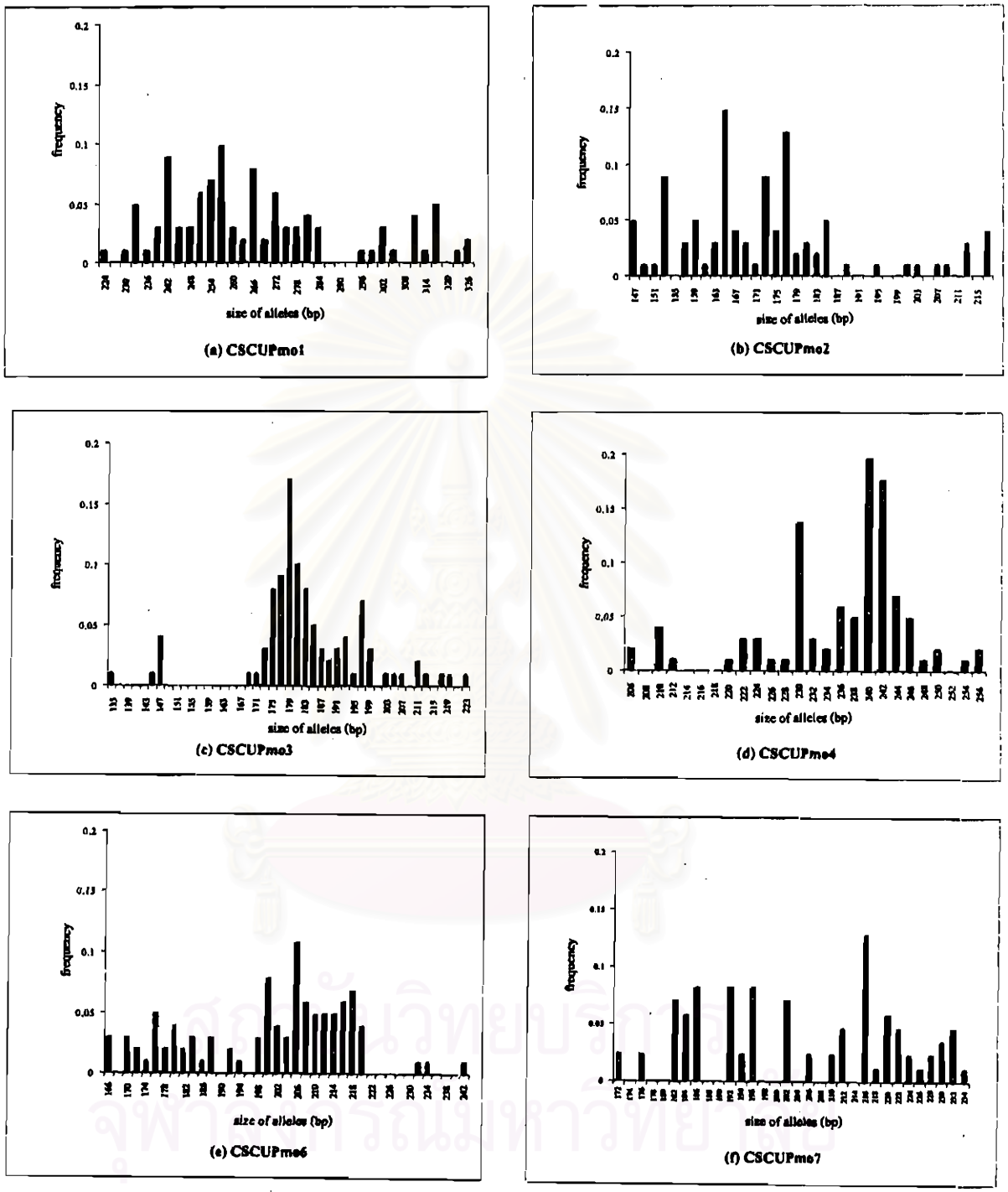
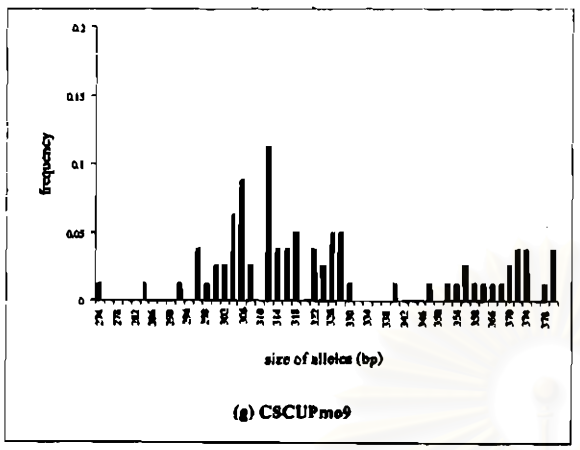


Figure 3.10 Allele distribution frequencies of *P. monodon* from Trad province at eight microsatellites: CSCUPmo1 (n=51), CSCUPmo2 (n=51), CSCUPmo3 (n=50), CSCUPmo4 (n=51), CSCUPmo6 (n=51), CSCUPmo7 (n=43), CSCUPmo9 (n=40) and CSCUPmo11 (n=47)



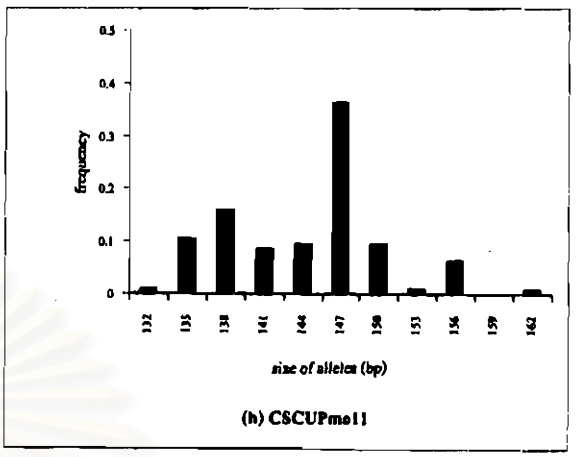


Figure 3.10 (continued)

3.3 Improved methods for the simple detection of microsatellites in *P. monodon*

Detection of microsatellites was simplified by improved DNA isolation method, followed by PCR amplification conditions at each locus and the detection method of microsatellite alleles.

3.3.1 DNA isolation

Six different DNA extraction methods were examined to select the most suitable method providing good DNA template for simple and reliable microsatellite amplification. These methods were: phenol-chloroform, cell lysis/proteinase K, two procedures of salting-out extraction described by Aljanabi and Martinez (1997) and Martinez et al. (1998), chelex and alkaline extraction methods. Specimens used to extract genomic DNA were: the tip of pleopod (tPleopod), the thigh muscle of pleopod (mPleopod), the whole body of postlarvae (PL 10-15) and the blood/ethanol sample. The yields of genomic DNA extracted from all specimens were determined by spectrophotometric measurement. Using one of the six methods, approximately 20 μg of DNA/mg of the postlavae were obtained. The quantity of genomic DNA extracted from the pleopod tissues (tPleopod and mPleopod) was approximately 0.4-1.5 μg of

DNA/mg of the specimen. Blood/ethanol samples gave low yield of DNA but the quality and quantity were sufficient for PCR. Genomic DNA isolated from all types of specimens using both chelex-based and alkaline extraction methods yielded approximately 20 μg of DNA/mg of the specimen. This level was higher than that isolated from other four methods which yielded approximately 0.4-1.5 μg of DNA/mg of the specimen.

The ratio of OD_{260}/OD_{280} was 1.8-2.0 reflecting good quality of obtained DNA. Four extraction methods (phenol-chloroform, cell lysis/proteinase K and two procedures of salting-out extraction methods) gave high molecular weight DNA with greater than 23.1 kb in size whereas chelex-based and alkaline extraction methods yielded degraded DNA. Although chelex-based and alkaline extraction methods gave low molecular weight DNA (<23.1 kb) as can be observed with the smear pattern (Figure 3.11), this DNA could be used as the DNA template for amplification at the at microsatellite locus Di25 like that from other extraction methods as illustrated in Figure 3.12. The alkaline extraction method which used only sodium hydroxide for lysing the cell and Tris-base for stopping the reaction was, therefore, the most suitable method to isolate DNA from a large number of samples.

3.3.2 Multiplex analysis of microsatellie loci

Genotyping of *P. monodon* at several microsatellite loci can be simultaneously detected by multiple analysis of PCR-amplified products. This minimizes materials and analysis time. Multiplex analysis can be performed in which multiple loci were simultaneously amplified in a single tube or a single loading of combined PCR-amplified products of each non-overlapped microsatellite locus.

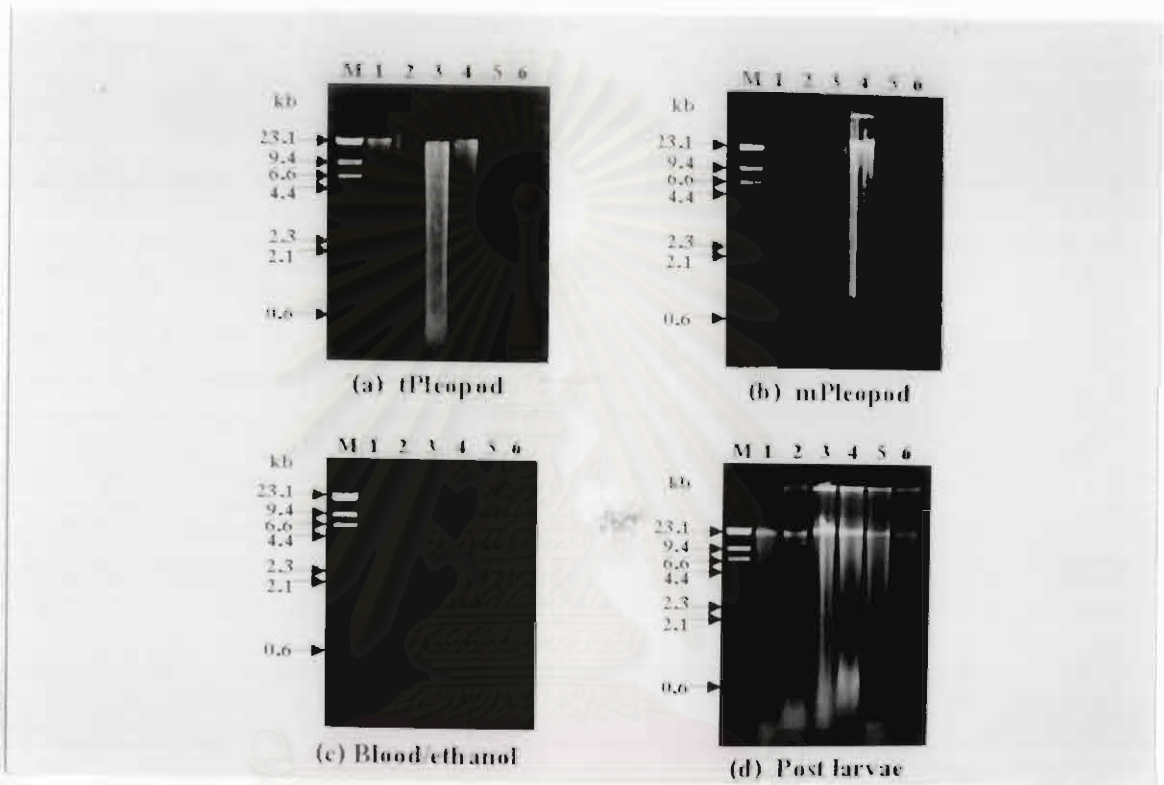


Figure 3.11 Ethidium bromide staining of 0.7% agarose gels showing genomic DNA isolated from the tip (a) and the thigh muscle (b) of pleopods and blood/ethanol (c) of adult *P. monodon* and the postlarvae (d), Lane M : λ DNA/*Hin*d III. Genomic DNA obtained from: phenol-chloroform extraction (1), cell lysis/proteinase K extraction (2), salting-out extraction (Alfanabi and Martinex, 1997) (3), salting-out extraction (Gonzalo et al., 1998) (4), chelex based DNA extraction (5) and alkaline extraction methods (6).

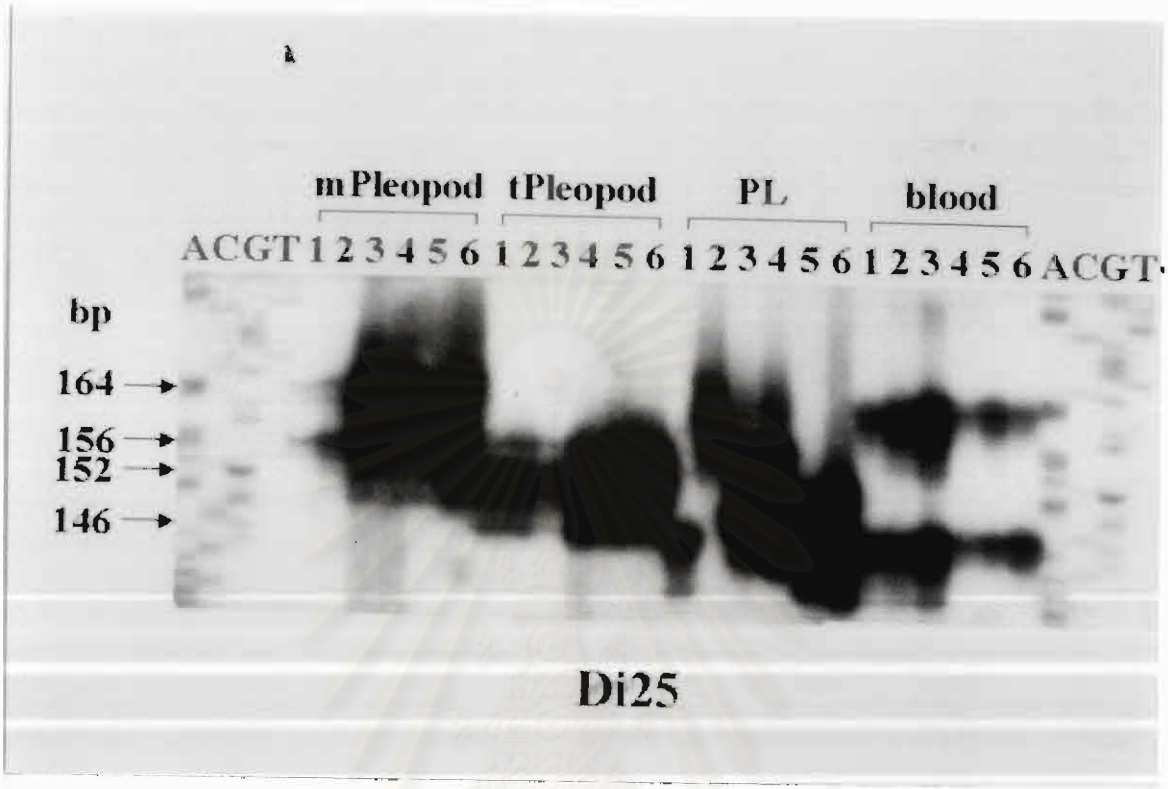


Figure 3.12 Microsatellite amplification patterns at the Di25 locus using genomic DNA isolated from different sources of *P. monodon* specimens and the postlarvae. Lanes 1-6 are resulting products when the DNA template obtained from six DNA extraction methods as shown in Figure 3.11 was included in the amplification reactions. The size standard is a sequencing ladder of M13mp18.

3.3.2.1 Multiplex PCR

Based on allelic size-ranges in Table 3.3, five sets of multiplex PCR were carried out. These were the diplex PCR of microsatellite loci CSCUPmo1 and CSCUPmo2 (CSCUPmo1+2); CSCUPmo4 and CSCUPmo11 (CSCUPmo4+11); CSCUPmo6 and CSCUPmo11 (CSCUPmo6+11); CSCUPmo4 and CSCUPmo9 (CSCUPmo4+9) and 1 set of triplex PCR of CSCUPmo4, CSCUPmo9 and

CSCUPmo11 (CSCUPmo4+9+11). Microsatellite patterns of CSCUPmo1+2 and CSCUPmo4+11 were easy to score and sizes of alleles are comparable with a sequencing ladder of M13 mp18 (Figures 3.13, 3.14).

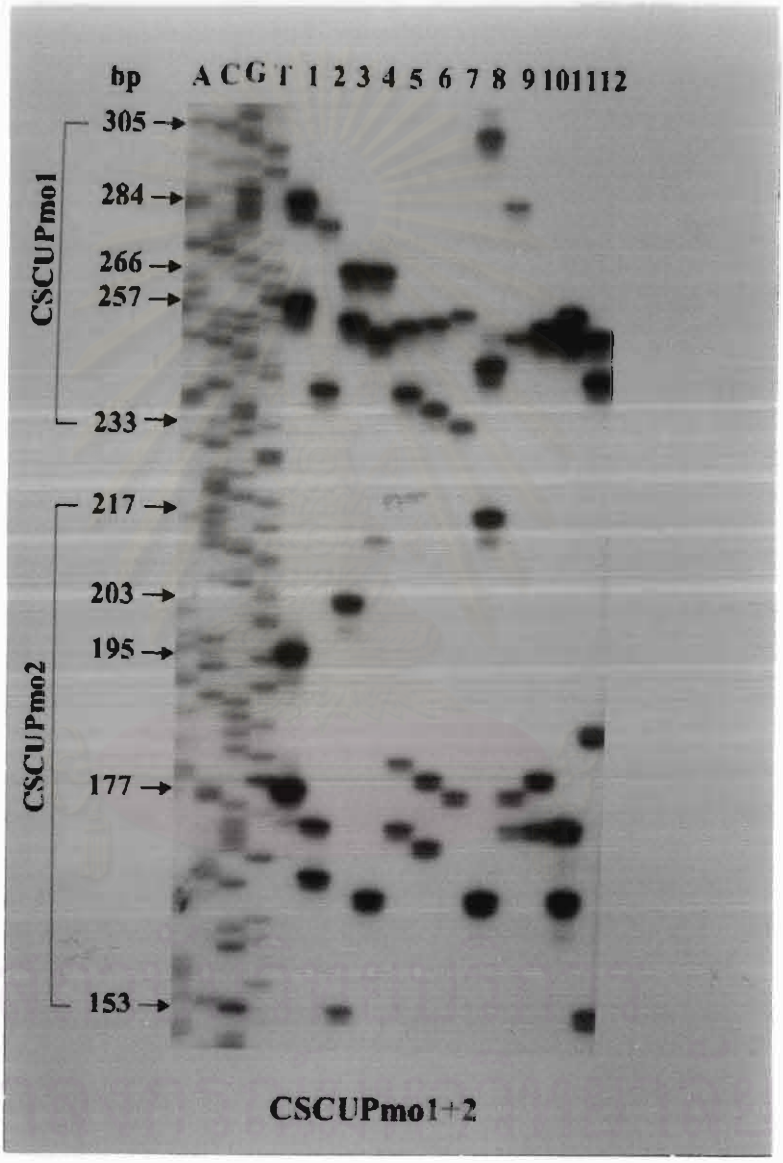


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Figure 3.13 Microsatellite patterns of the diplex CSCUPmo1+2 of 12 individuals of *P. monodon* (lanes 1-12) under the optimal PCR conditions with an annealing temperature at 56 °C. The size standard is a sequencing ladder of M13 mp18.

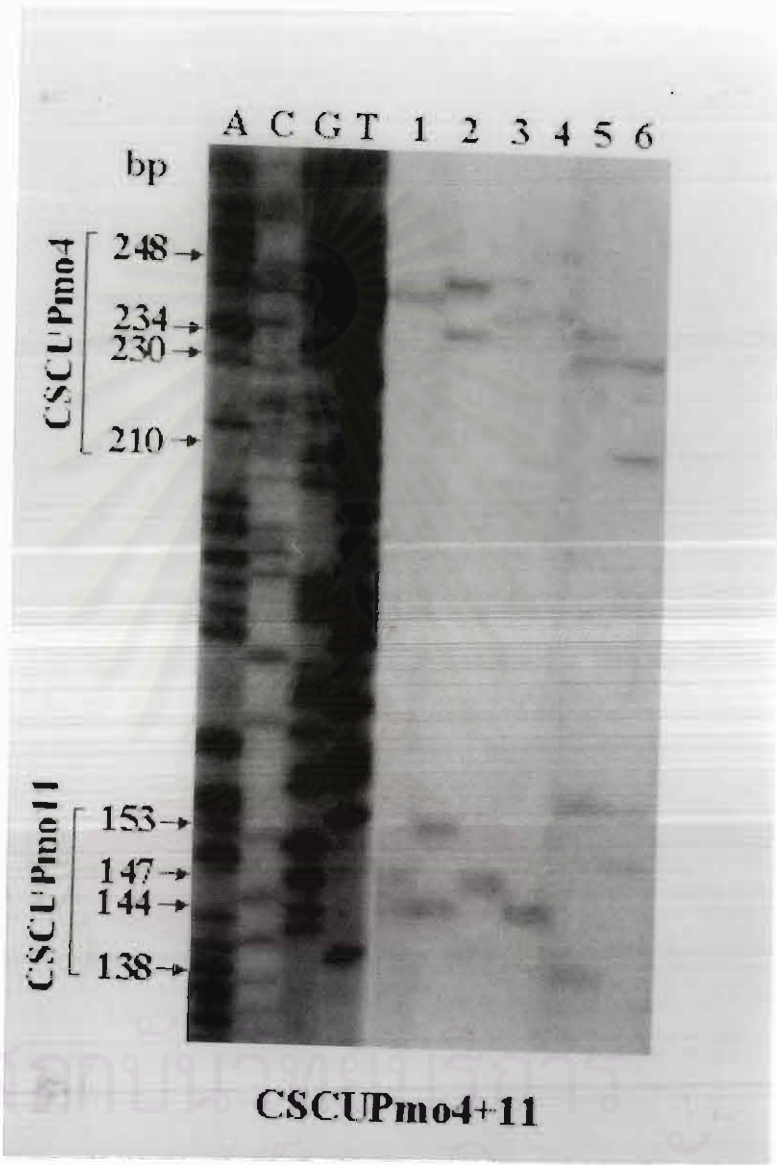

Figure 3.14 Microsatellite patterns of the diplex CSCUPmo4+11 of 6 individuals of *P. monodon* (lanes 1-6) under the optimal PCR conditions with an annealing temperature at 54 °C. The size standard is a sequencing ladder of M13 mp18.

The low intensity of allelic bands of CSCUPmo4+11 was solved by increasing loading volume of amplified samples or the exposure time during autoradiography whereas those of CSCUPmo6+11 showed decreasing amplification efficiency of each locus (Figure 3.15). The diplex of CSCUPmo4+9 and the triplex of CSCUPmo4+9+11 were unsuccessfully amplified while amplification of each locus separately yielded scorable microsatellite patterns (Figure 3.16).

3.3.2.2 Single loading of combined PCR amplified products of different microsatellite loci

The size-ranges of each microsatellite from Table 3.3 were used to choose for a suitable single loading of combined amplification products in 6% polyacrylamide sequencing gels. Microsatellite alleles and their artifacts of each locus should not overlap with each other. PCR amplified alleles of selected loci were mixed prior to loading on a single lane of the denatured polyacrylamide gel. The results were illustrated in Figures 3.17-3.20. Microsatellite allelic patterns of a single loading gel of three sets of combined two microsatellite loci, CSCUPmo1 and CSCUPmo2 (CSCUPmo1+CSCUPmo2), CSCUPmo4 and CSCUPmo9 (CSCUPmo4+ CSCUPmo9), CSCUPmo6 and CSCUPmo11 (CSCUPmo6+CSCUPmo11) that illustrated in Figure 3.17-3.19 were easily scored. This was due to different microsatellite allelic patterns of each particular locus. The other combination of these microsatellite loci including CSCUPmo1+CSCUPmo11; CSCUPmo2+CSCUPmo9; CSCUPmo4+CSCUPmo11; CSCUPmo6+CSCUPmo9 could be used for some appropriate situations (data not shown).

Unresolvable banding patterns were found for the locus CSCUPmo9 in the single loading of combined three microsatellite loci, CSCUPmo4, CSCUPmo9 and CSCUPmo11 (CSCUPmo4+CSCUPmo9+CSCUPmo11). This problem was due to a

large difference in allele size range between CSCUPmo9 and CSCUPmo11 (Figure 3.20). Using wedged spacer, density gradient gel or increasing the proportion of increasing PCR products at the locus CSCUPmo11 may solve this problem.

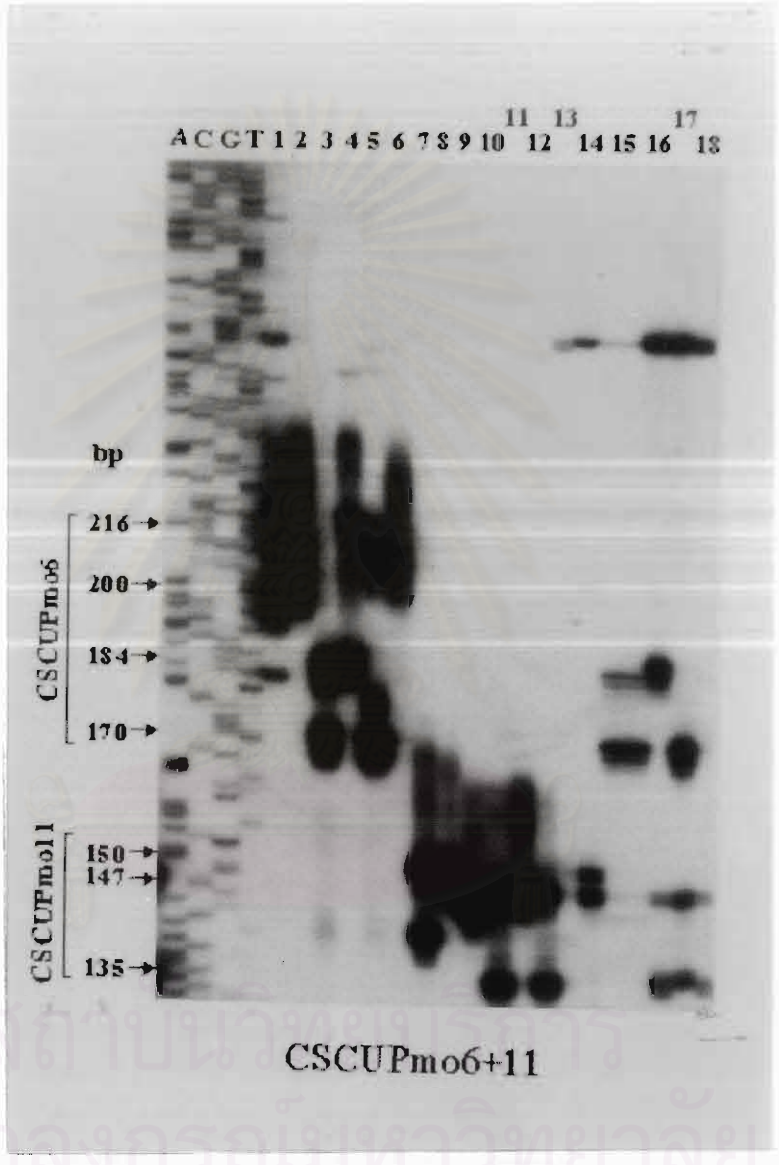

Figure 3.15 Microsatellite patterns of the diplex CSCUPmo6+11 of 6 individuals of *P. monodon* (lanes 13-18) under the optimal PCR conditions with an annealing temperature at 54 °C. Lanes 1-6 and lanes 7-12 are the PCR-amplified fragments of CSCUPmo6 and CSCUPmo11 of those individuals, respectively. The size standard is a sequencing ladder of M13 mp18.

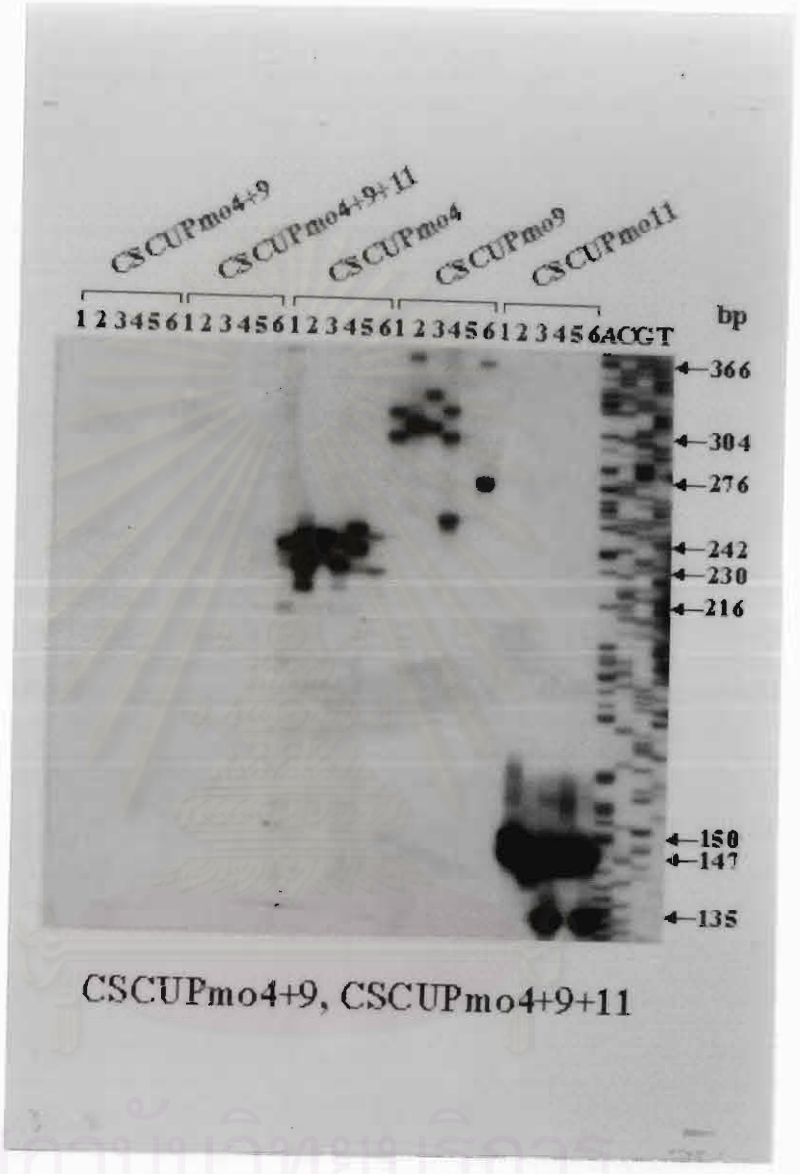

Figure 3.16 Microsatellite patterns of the diplex CSCUPmo4+9 and the triplex CSCUPmo4+9+11 of 6 *P. monodon* individuals under the optimal PCR conditions with an annealing temperature at 54 °C. The same samples were separately amplified with each microsatellie locus. The size standard is a sequencing ladder of M13 mp18.

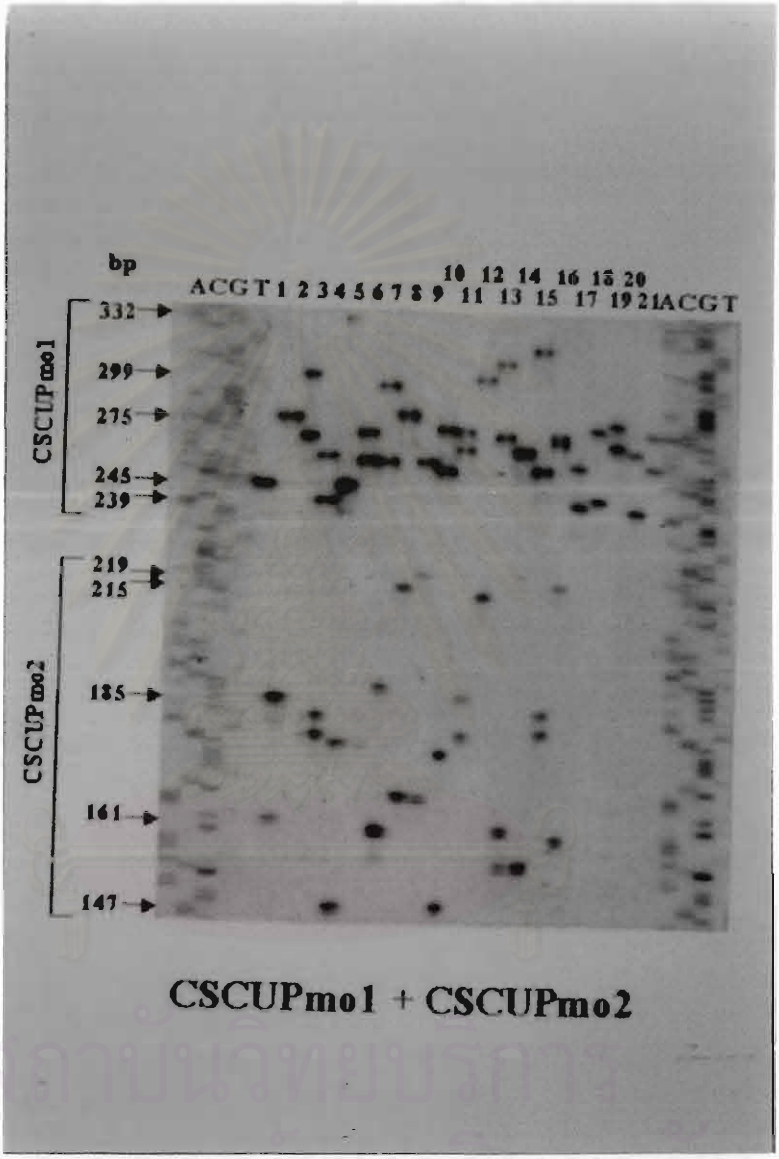

Figure 3.17 Single loading of PCR amplified alleles of 21 individuals of *P. monodon* for loci CSCUPmo1+CSCUPmo2 (lanes 1-21) was electrophoresed in a 6% polyacrylamide sequencing gel. The size standard is a sequencing ladder of M13 mp18.

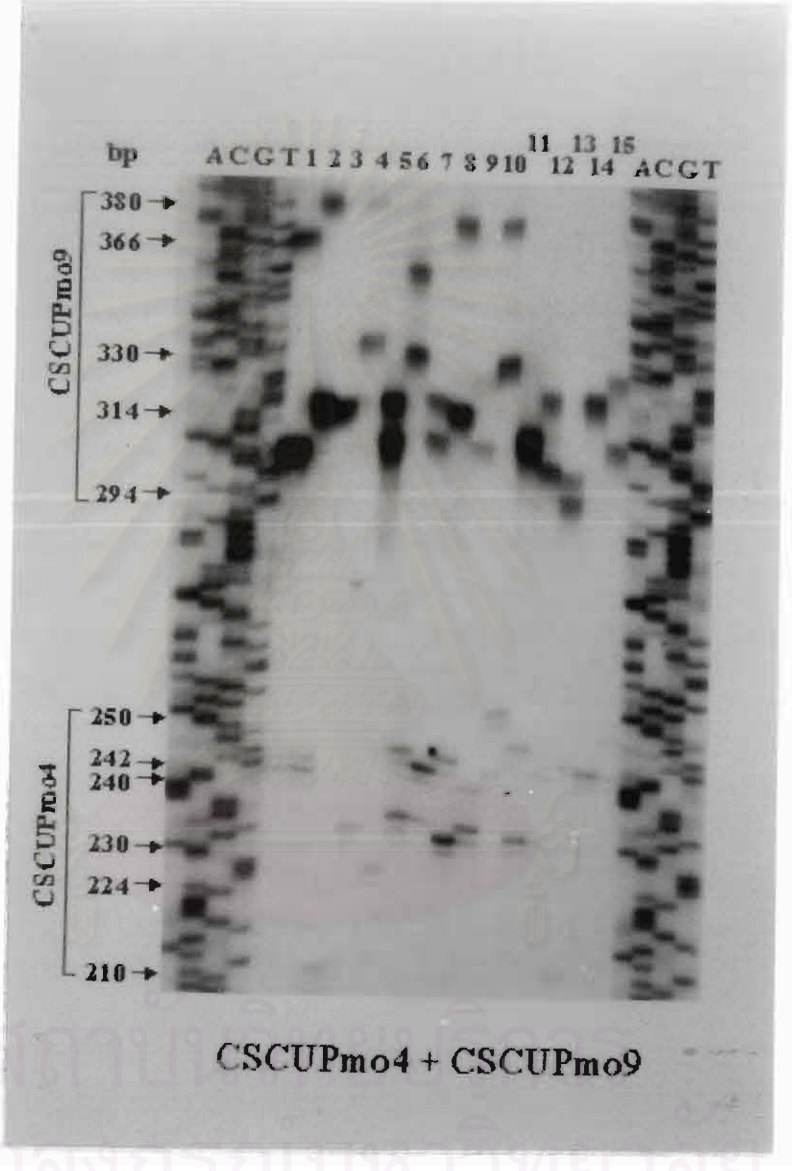

Figure 3.18 Single loading of PCR amplified alleles of 15 individuals of *P. monodon* for loci CSCUPmo4+CSCUPmo9 (lanes 1-15) was electrophoresed in a 6% polyacrylamide sequencing gel. The size standard is a sequencing ladder of M13 mp18.

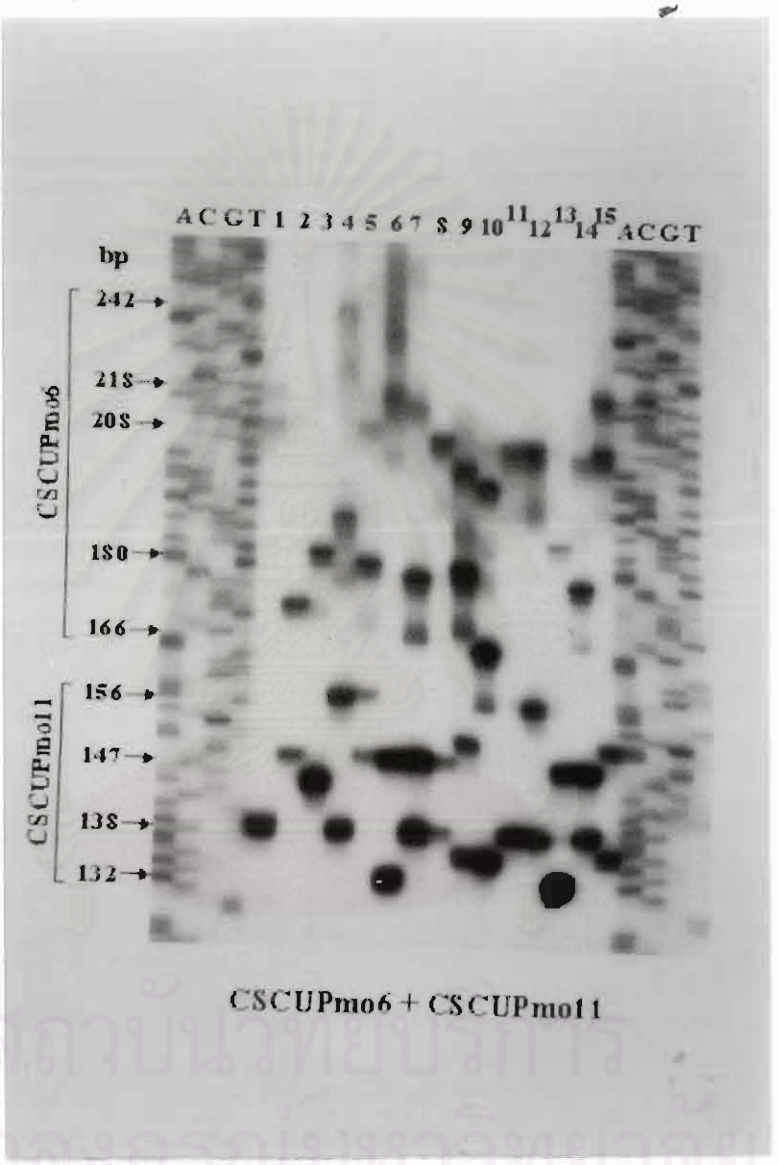

Figure 3.19 Single loading of PCR amplified alleles of 15 individuals of *P. monodon* for loci CSCUPmo6+CSCUPmo11 (lanes 1-15) was electrophoresed in a 6% polyacrylamide sequencing gel. The size standard is a sequencing ladder of M13 mp18.

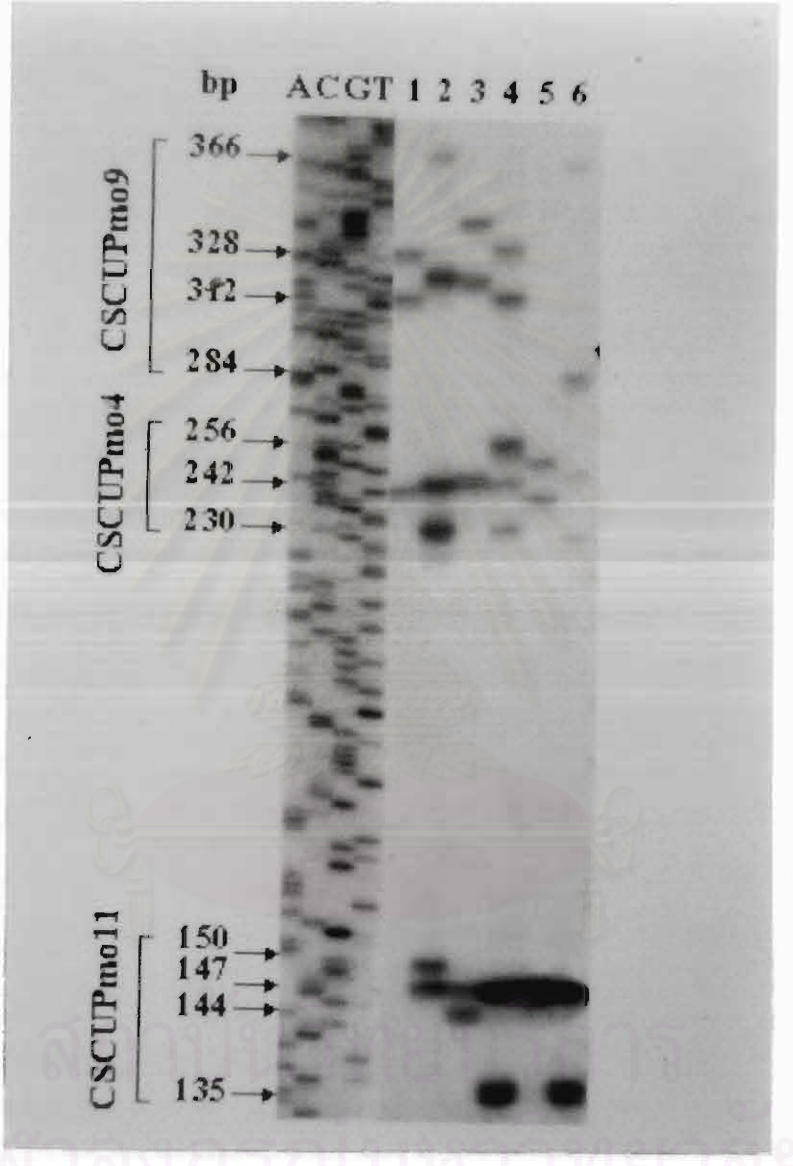

Figure 3.20 Single loading of PCR amplified alleles of 6 individuals of *P. monodon* for loci CSCUPmo4+CSCUPmo9+CSCUPmo11 (lanes 1-6) was electrophoresed in a 6% polyacrylamide sequencing gel. The size standard is a sequencing ladder of M13 mp18.

3.3.3 Non-isotopic detection of microsatellite alleles

Detections of microsatellite alleles with non-isotopic methods such as ethidium bromide and silver staining were also used in this study. Microsatellite alleles from loci CSCUPmo1 and CSCUPmo2 that differ in 3 and 2 bases, respectively and have low stutter bands were chosen to separate by MetaPhor agarose gel electrophoresis and detected by ethidium bromide staining. Amplified products from all six microsatellite loci were separated by denaturing polyacrylamide sequencing gel electrophosis and detected by silver staining. Size estimation of these microsatellite alleles visualized with non-isotopic detection was improved by including of their allelic ladders.

3.3.3.1 MetaPhor agarose gel electrophoresis and detection of microsatellite by ethidium bromide staining

MetaPhor agarose gel electrophoresis followed by ethidium bromide staining is the simplest method to determine microsatellite alleles that differ in 2 bases (White and Dusukawa, 1997). MetaPhor (4%) agarose gel electrophoresis was then tested for separation of PCR-amplified fragments of two microsatellites CSCUPmo1 and CSCUPmo2. These two loci were chosen because their amplified alleles were easy to score with low stutter bands when separated by polyacrylamide gel followed by autoradiography. However, PCR-amplified alleles of these loci could not be scored when separated by MetaPhor (Figure 3.21). The *P. monodon* samples with previously known heterozygous alleles showed three bands instead of two bands which suspected the allele sizes. Therefore, size-fractionation of microsatellite alleles using MetaPhor agarose gels was not further attempted.

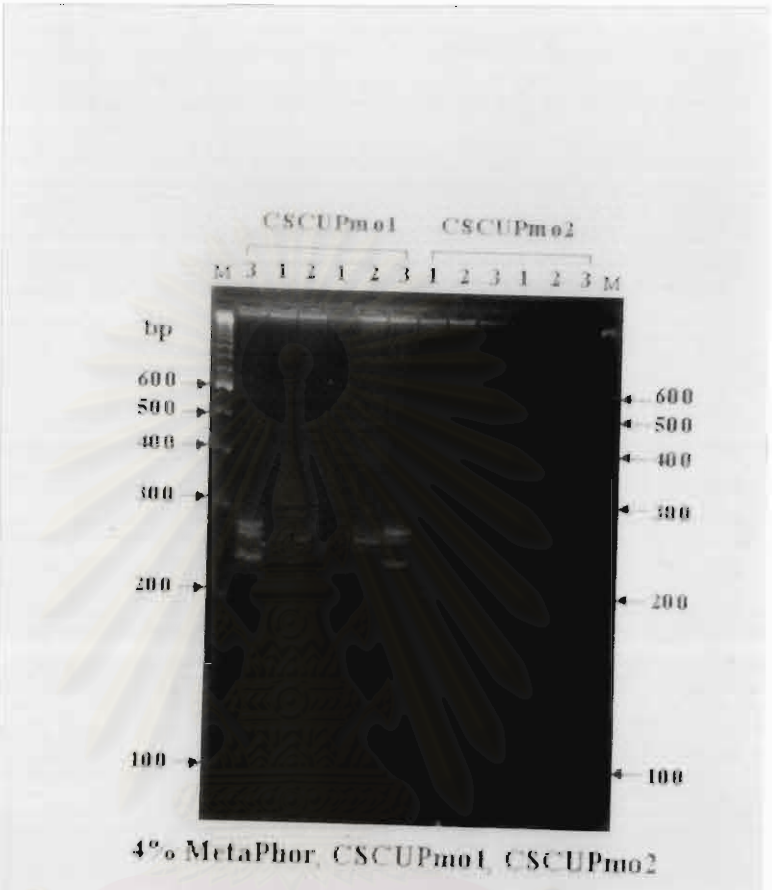

Figure 3.21 A 4% ethidium bromide stained MetaPhor agarose gel showing patterns of CSCUPmo1 and CSCUPmo2. Lanes 1-3 are *P. monodon* heterozygotes at CSCUPmo1 and CSCUPmo2 loci which were previously identified using denaturing polyacrylamide gel. The size standard marker is a 100bp ladder (lane M).

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3.3.3.2 Denaturing polyacrylamide gel electrophoresis and silver staining

Detection of amplified microsatellite alleles by non-isotopic method was also performed by separation of amplified products in 8% denaturing polyacrylamide gels followed by silver staining. Similar allelic patterns were found when compared with those of the isotopic method (Figure 3.22 showed three loci, CSCUPmo1, CSCUPmo2 and CSCUPmo6). However, high background was appeared with silver staining particularly for loci CSCUPmo4, CSCUPmo6 and CSCUPmo9 that contained stutter bands. Two fragments were revealed for each allele as can be seen at loci CSCUPmo1, CSCUPmo2 and CSCUPmo11. This is caused by differential strand migration for products of the same length exhibiting different sequence in polyacrylamide gels. Detection of microsatellite which were interfered with their authentic alleles were improved further with their allelic ladder to estimate the precise allele size.

Multiplex PCR-amplified fragments of CSCUPmo1+2 were tested to separate in 8% polyacrylamide sequencing gels and detected with silver staining (Figure 3.23). Similar allelic patterns were found when compared with those of the isotopic method. There were the same problems of detection of each amplified microsatellite. Although, some artifacts were revealed and gave difficulty in scoring allele sizes, the authentic alleles could be differentiated. This result demonstrated that separation of these microsatellites by polyacrylamide gel electrophoresis and silver staining could be used for genotyping of *P. monodon*.

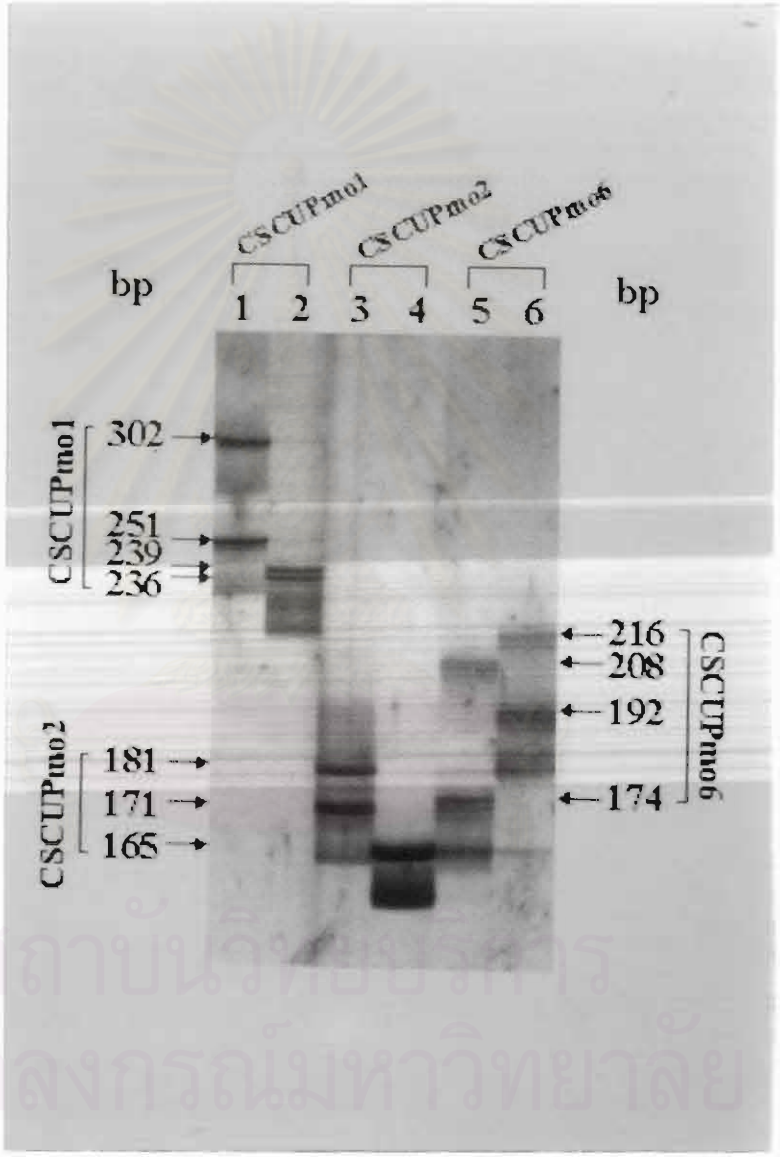

Figure 3.22 Microsatellite patterns of 2 *P. monodon* individuals (lanes 1-2) of three microsatellites (CSCUPmo1, CSCUPmo2 and CSCUPmo6) separated in a 8% polyacrylamide sequencing gel and detected by silver staining.

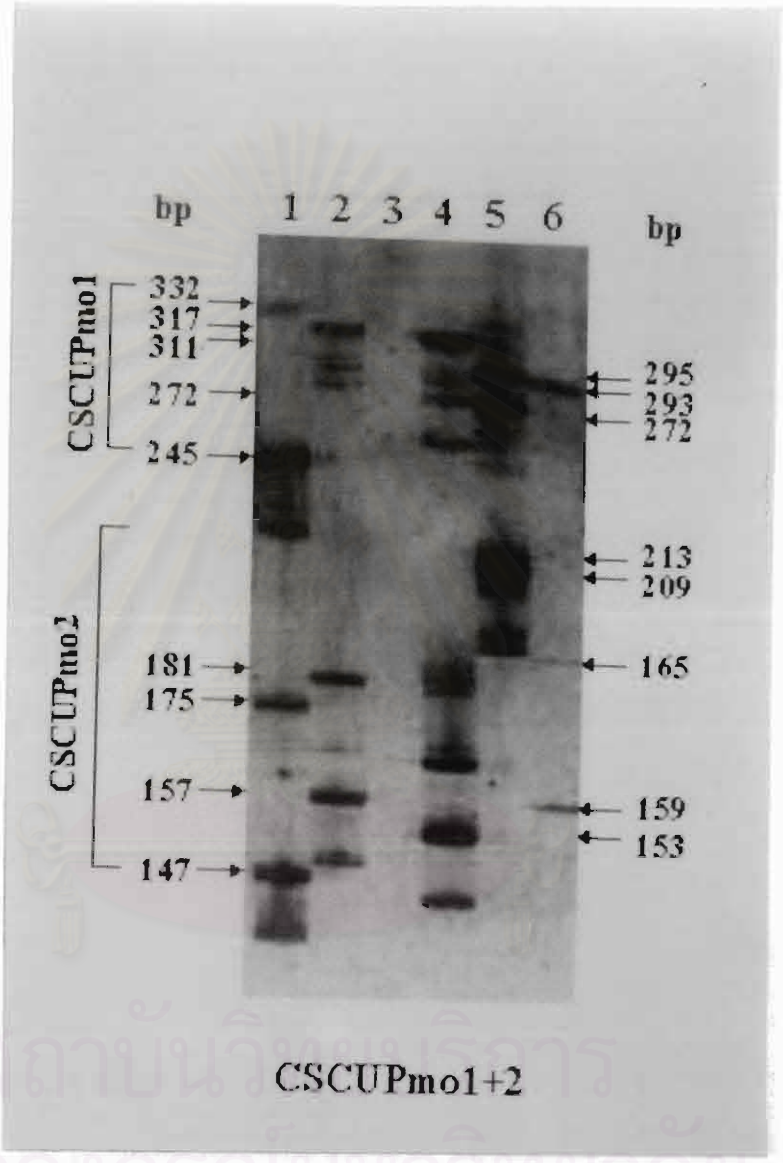

Figure 3.23 Multiplex PCR-amplified fragments of CSCUPmo1+2 (lanes 1-6) were separated in a 8% polyacrylamide sequencing gel and detected by silver staining.

3.3.3.3 Allelic ladders

Typically, size estimation of microsatellite alleles detected by isotopic method was comparing with the sequencing M13 mp18 marker. This marker is expensive, thus the allelic ladder of each locus was constructed to substitute this marker. An allelic ladder consisted of amplified fragments of several alleles has been employed s a standard marker for relatively accurate assignment of microsatellite alleles. In this study, allelic ladders were constructed for six microsatellite loci (CSCUPmo1, CSCUPmo2, CSCUPmo4, CSUPmo6, CSCUPmo9 and CSCUPmo11). DNA samples of homo and heterzygous individuals of *P. monodon* were pooled and re-amplified to generate fragments of several known alleles for a particular locus. The allelic ladder for the CSCUPmo11 gave the best resolvable allelic pattern among overall loci (Figure 3.24). PCR-amplified fragments of the allelic ladder and its components of CSCUPmo11 could be re-amplified when diluted to a 1: 100 concentration with sterile distilled water. This result makes it suitable for using as a reference across laboratories.

The allelic ladders and their parts of components for the CSCUPmo1 and CSCUPmo2 showed high background (Figure 3.25 and 3.26). Similar results were found in other microsatellite loci including CSCUPmo4, CSCUPmo6 and CSCUPmo9 (data not shown). The problems for construction of allelic ladders had caused from the limitation on number of known microsatellite alleles that can be used for PCR amplifying and their intense artifacts below authentic bands. These made their allelic ladders difficult to identify the allele size. The sensitivity of the proper amount of each template DNA in the mixture caused difficulties to adjust for finding the suitable allelic ladder. Re-amplification of these allelic ladders gave blurred and unscorable microsatellite fragments. Thus the allelic ladders of there loci (CSCUPmo4, CSCUPmo6 and CSCUPmo9) were not studied further.

The M13 mp18 sequencing ladder constructed from the non-labeled method or specimens exhibiting known allele were used to estimate allele sizes of microsatellite that the allelic ladder could not be constructed.

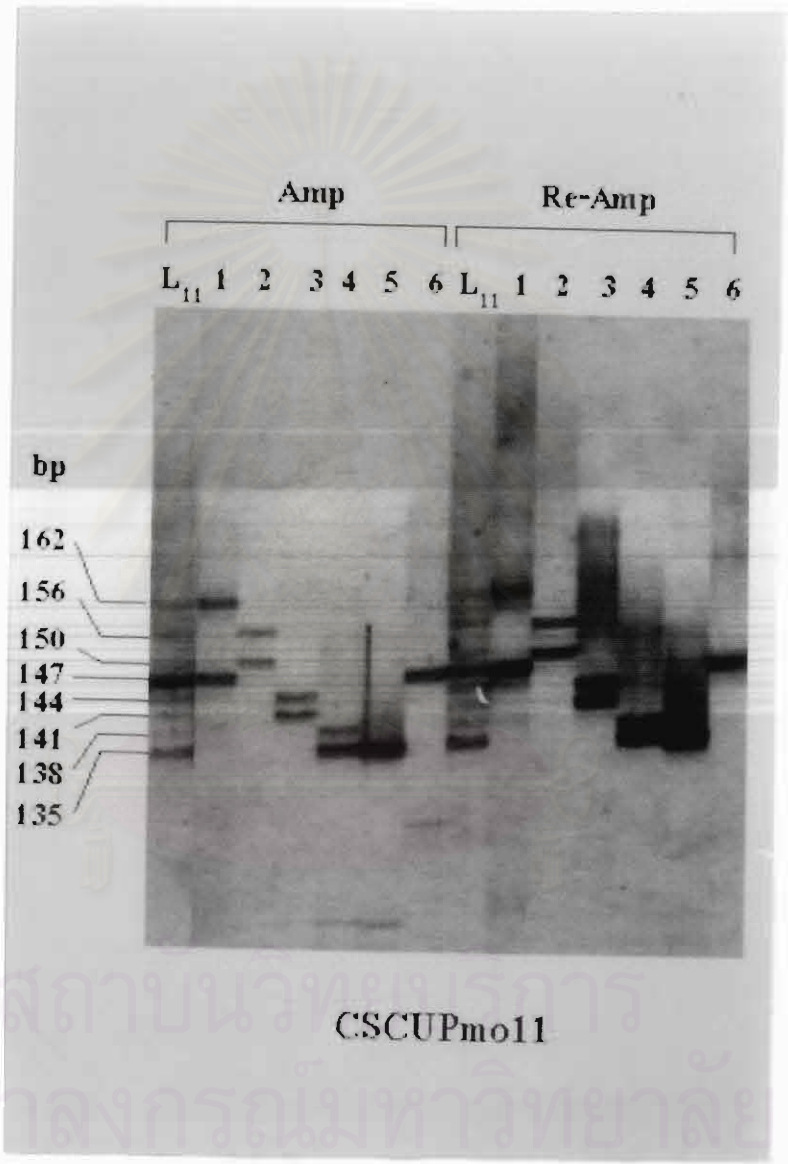

Figure 3.24 PCR amplified fragments of the allelic ladder (lane L_{11}) and its components (lanes 1-6) of the microsatellite locus CSCUPmo11. Both were diluted 1: 100 with sterile distilled water and re-amplified, separated by 8% polyacrylamide sequencing gel electrophoresis and detected by silver staining. The results gave identical amplification patterns.

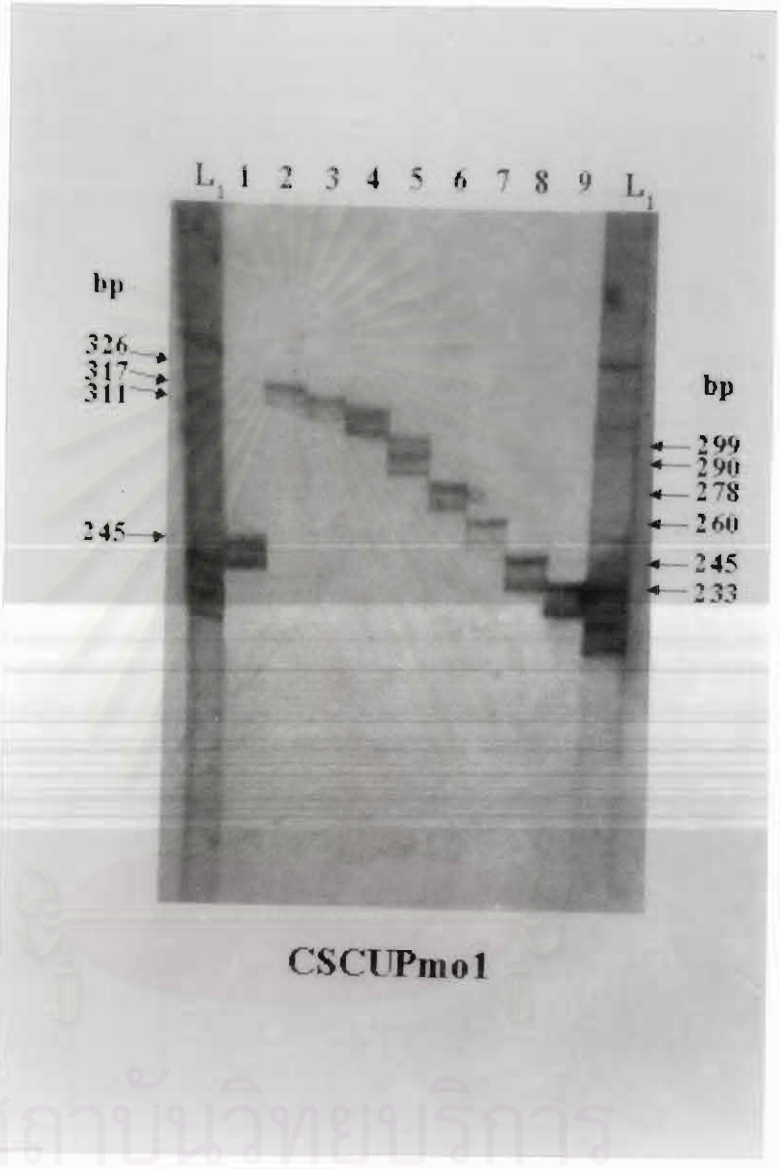

Figure 3.25 PCR amplified fragments of the allelic ladder (lane L_1) and its components (lanes 1-9) of the microsatellite locus CSCUPmo1 were separated by 8% polyacrylamide sequencing gel electrophoresis and detected by silver staining.

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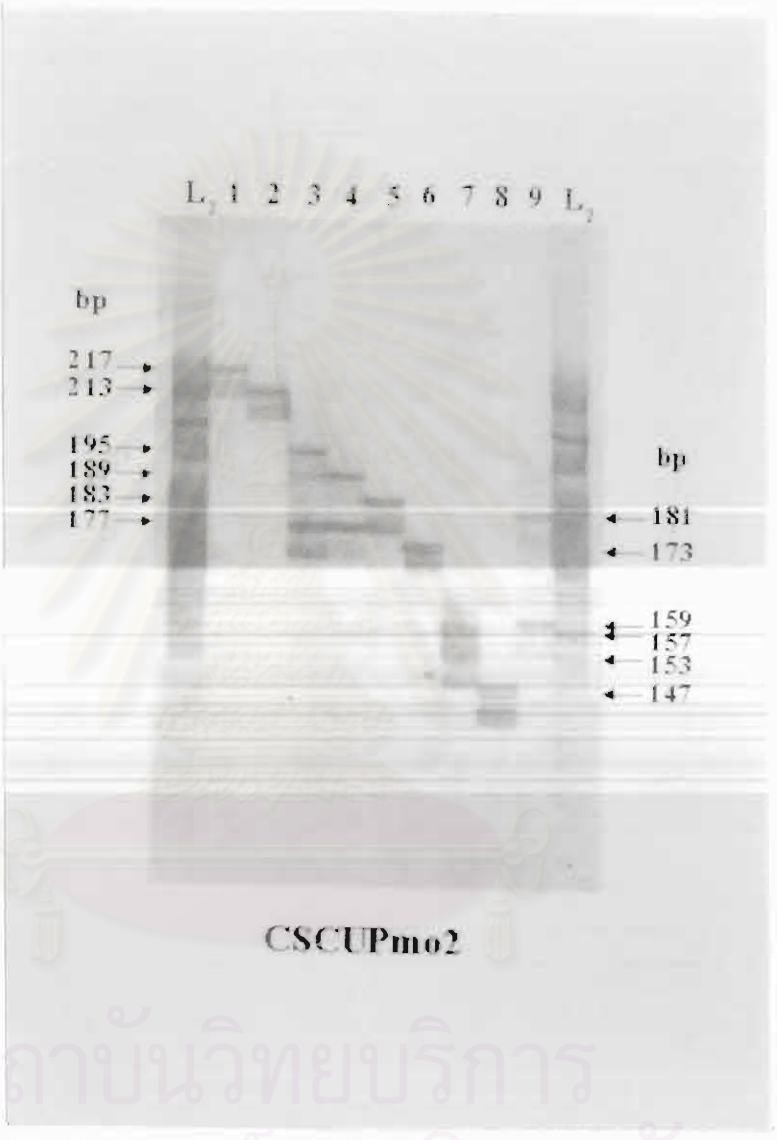

Figure 3.26 PCR amplified fragments of the allelic ladders (lane L_2) and its components (lanes 1-9) of the microsatellite locus CSCUPmo2 were separated by 8% polyacrylamide sequencing gel electrophoresis and detected by silver staining.

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To reduce the amount of work and cost, denaturing polyacrylamide minigel electrophoresis was chosen to separate amplified fragments of the CSCUPmo11 followed by silver staining. Because of smaller amount of reagent in gel preparation and staining, the 8% polyacrylamide minigel was used. As can be seen in Figure 3.27, its concentration was not enough to separate allelic bands of this microsatellite. The concentration of the gel was thus increased to 15% (Figure 3.28). Three base-differences of allele sizes were seen in the allelic ladder and two samples with known allele having genotypes 141/144 and 135/138 (lanes 3 and 4, respectively). An artifact band that appeared below the authentic band was stood out. This resulted from a better resolution of this gel.

The allelic ladder of the locus CSCUPmo11 was applied for studying Mendelian's segregation of *P. monodon* family B2 and B26 where their genotypes were previously known. The samples were separated in the 8% polyacrylamide sequencing gel and the 15% polyacrylamide minigel, respectively (Figure 3.29 and 3.30). In the former, allelic bands were easily scored by comparing with allelic ladder that have the clear size. This made it suitable to be used across laboratories. Results of separation using the latter system showed non-added alleles (153 bp and 159 bp). These were resulted from artifacts of alleles 156 bp and 162 bp. The allelic 159 bp band of the CSCUPmo11 was not observed in investigated *P. monodon* whereas a 153 bp allele exhibited very faint intensity suggesting that they are not the true alleles. The ability to estimate the correct alleles even though the space between loading wells made difficulty for scoring when compared with the allelic ladder allowing much simples and more rapid detection of microsatellite using the non-isotopic method.

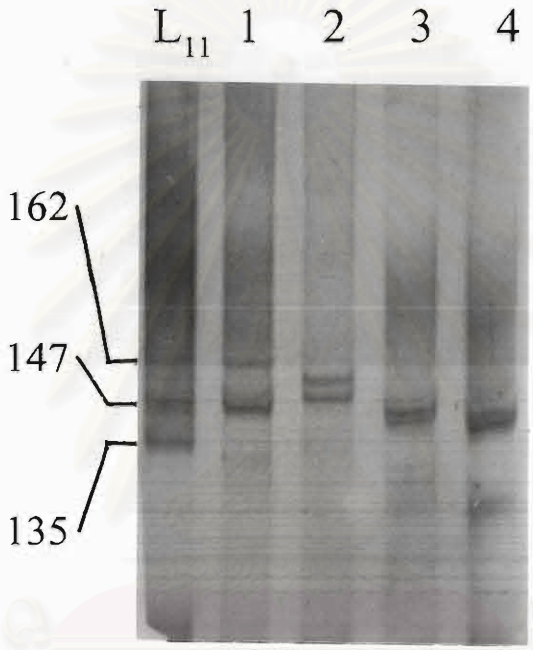

Figure 3.27 Microsatellite amplified fragments of the CSCUPmo11 of 4 *P. monodon* individuals (lanes 1 - 4) were separated along with its allelic ladder (lane L_{11}) in a 8% polyacrylamide minigel (10x10 cm) and detected by silver staining. Genotypes of these individuals were previously typed as (147/162), (150/156), (141/144) and (138/135), respectively but they could not be accurately analyzed at this concentration of the gel.

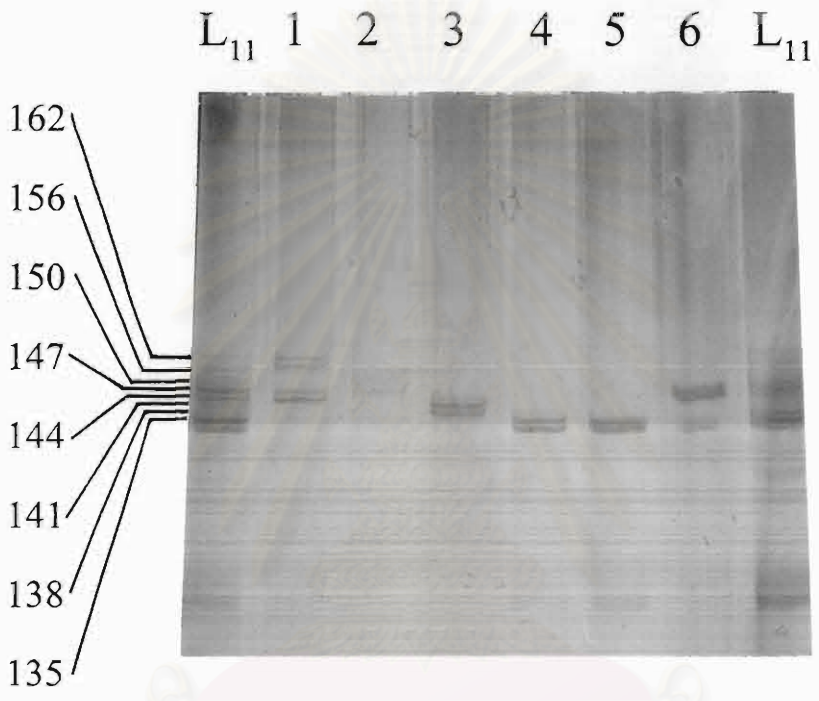

Figure 3.28 Microsatellite amplified fragments of the CSCUPmo11 of 6 *P. monodon* individuals (lanes 1-6) were separated with its allelic ladder (lane L_{11}) in a 15% polyacrylamide minigel (10x10 cm) and detected by silver staining. Genotypes of lanes 1-6 are (147/162), (150/156), (141/144), (138/135),(135/135) and (147/147), respectively.

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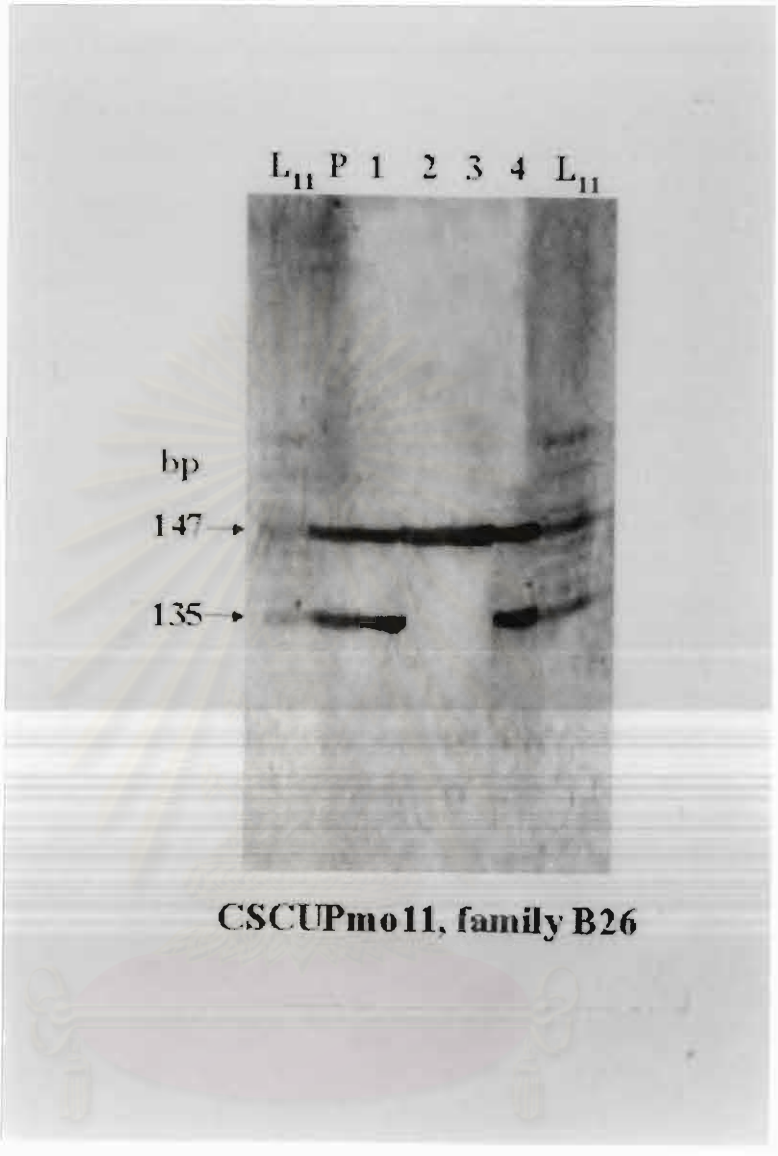

Figure 3.29 Application of microsatellite techniques of the locus CSCUPmo11 for studying Mendelian's segregation of 4 individuals progeny of *P. monodon*, family B26 (lanes 1-4) and the dam (lane P) that were separated with its allelic ladder (lane L_{11}) in a 8% polyacrylamide sequencing gel and detected by silver staining. Genotypes of lanes 1-4 and P are (135/147), (147/147), (147/147), (135/147) and (135/147), respectively.

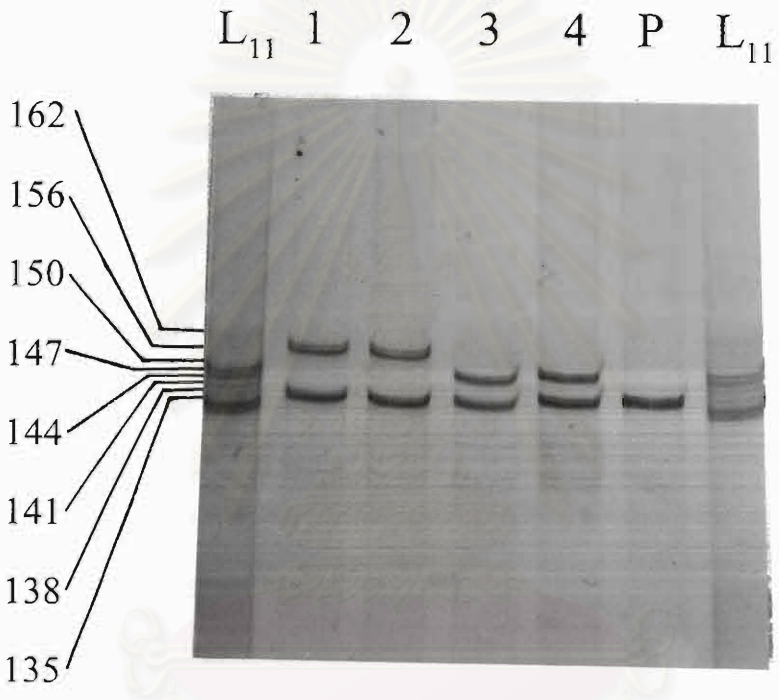

Figure 3.30 Application of microsatellite techniques of the locus CSCUPmo11 for studying Mendelian's segregation of 4 individuals progeny of *P. monodon*, family B2 (lanes 1-4) and the dam (lane P) that were separated with its allelic ladder (lane L_{11}) in a 15% polyacrylamide minigel and detected by silver staining. Genotypes of lanes 1-4 and P are (141/159), (141/159), (141/150), (141/150) and (141/141), respectively.

3.4 Application of microsatellite techniques

The improved microsatellite technique previously described was applied to determine genotypes of three families of *P. monodon* at six microsatellite loci. Alkaline extraction method was used to isolate DNA from approximately fifty progeny and a dam of each family. Radioactive-labeled detection was used to determine results of all six microsatellite loci.

Allele segregation patterns of six microsatellite loci used in the present study were determined whether they are Mendelianly inherited. The radioactive-labeled multiplex PCR of CSCUPmo1+2 and combined CSCUPmo4+CSCUPmo9 and CSCUPmo6+CSCUPmo11 products were analyzed against three families of *P. monodon*. Genotypes at loci CSCUPmo1+2 and CSCUPmo6+CSCUPmo11 of families B2 and B26 were unambiguously identified as can be seen in Figure 3.31 and 3.33. The combined CSCUPmo4+CSCUPmo9 products indicated non-specific amplification products from CSCUPmo9 appeared along with actual alleles (Figure 3.32). Nevertheless, this did not interfere the ability to score microsatellite alleles at this locus because the sizes of non-specific bands were constant.

Fifty progeny of each family and their dam were genetically analyzed. Non-related progeny were found in families B2 (Table 3.4) and B26 (Table 3.6). A total of eleven non-family progeny (4, 7, 16, 35, 37, 40, 44, 45, 46, 47 and 48) in the B2 family were observed. There are some indications showing relationships among these non-family progeny. This indicated contamination of the B2 family with the other rather than the existence of multiple-mating of *P. monodon* or high mutation rate of microsatllite loci between a generation. The microsatellite genotypes of family B26 indicated three non-family progeny. These specimens are genetically related at all loci. Therefore, three contaminating specimens should be from the same family. Results

from microsatellite analysis suggested that care must be taken during preparation of pedigree samples otherwise genotype results could not be concluded.

Data on allelic inheritances of six microsatellites for three families of *P. monodon* were illustrated in Table 3.7 – 3.12. The number of observed genotypes in each locus were subjected to a goodness of fit test using a chi-square methods. The mendelianly segregated fashion was observed in all families at CSCUPmo4, CSCUPmo6, CSCUPmo9 and CSCUPmo11 ($p>0.05$). Deviation of allele segregation was observed at loci CSCUPmo1 and CSCUPmo2 in B10 and B26 family, respectively ($p<0.05$). Non-amplification of the other allele for individuals possessing the 275/275 genotype in the family B10 should have caused this phenomenon. The non-Mendelian segregation observed in the B26 family should be resulted from sampling errors. However, this should be further confirmed using control crosses with the large sample size within a family.

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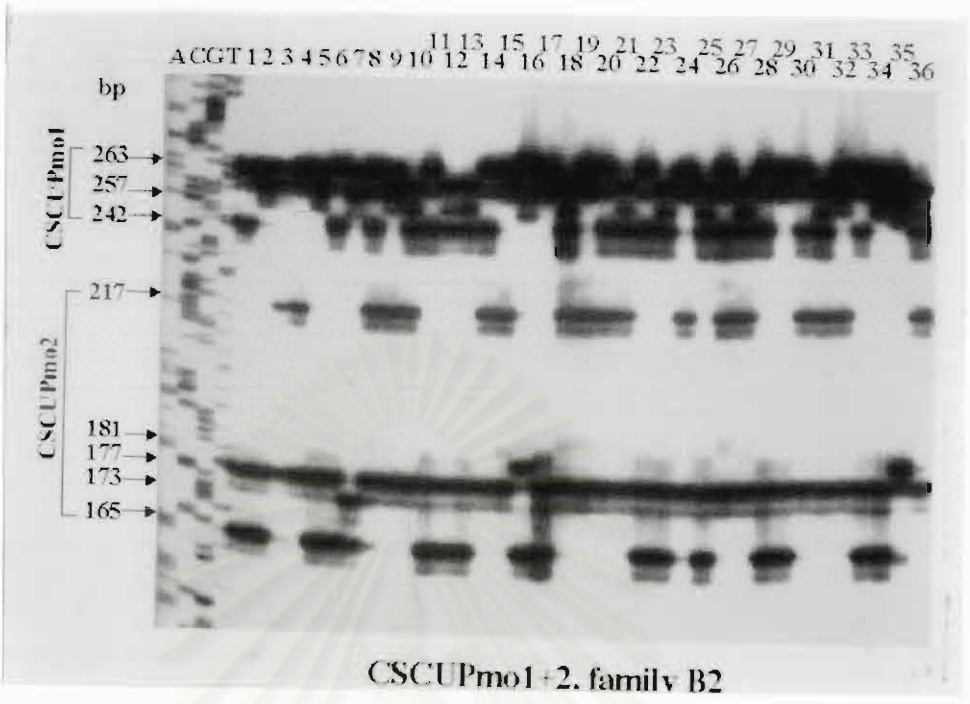


*Assuming the non-family progeny

Figure 3.31 Multiplex PCR of the CSCUPmo1+2 from representative progeny of the full-sib family B2. The size standard is a sequencing ladder of M13 mp18.

At the CSCUPmo1 locus, the progeny have 4 genotypes :

242/257 in lanes 10, 12, 13, 18, 21, 23, 25, 27, 31 and 36

242/263 in lanes 1, 6, 8, 11, 14, 20, 22, 26, 28, 30 and 33

257/263 in lanes 2, 5, 7*, 9, 16*, 32, 34 and 35*

263/263 in lanes 3, 4, 15, 17, 19, 24 and 29

At the CSCUPmo2 locus, the progeny have 2 genotypes :

165/177 in lanes 1, 2, 5, 6, 11, 12, 13, 17, 22, 23, 25, 28, 29, 33 and 34

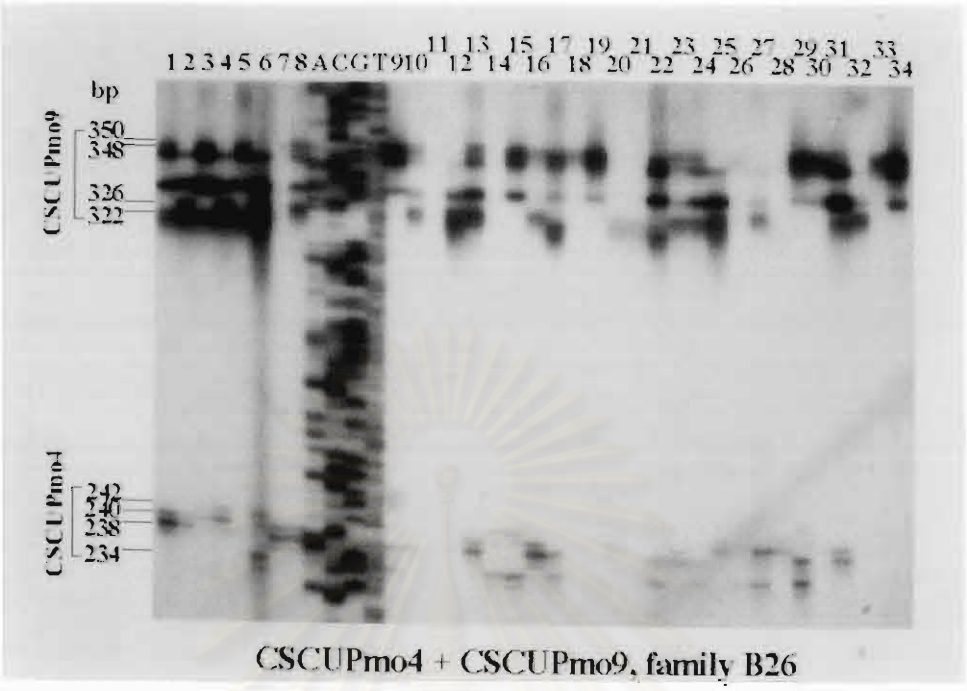
177/217 in lanes 3, 4, 8, 9, 10, 14, 15, 18, 19, 20, 21, 24, 26, 27, 30, 31, 32 and 36

the non-family progeny have :

165/173 in lane 7*

165/181 in lane 16*

181/181 in lane 35*



*Assuming the non-family progeny

Figure 3.32 Single loading of loci CSCUPmo4+CSCUPmo9 from representative progeny of the full-sib family B26. The size standard is a sequencing ladder of M13 mp18.

At the CSCUPmo4 locus, the progeny have 4 genotypes :

234/238 in lanes 9, 17, 21, 22, 24 and 29

234/242 in lanes 6, 14, 15 and 26

238/240 in lanes 1, 2, 7, 8, 10, 13, 16, 23 and 31

240/242 in lanes 3, 4, 20, 25 and 28

the non-family progeny have : 234/240 in lanes 27*

At the CSCUpmo9 locus, the progeny have 4 genotypes :

322/326 in lanes 2, 4, 6, 12, 20, 21 and 25

322/348 in lanes 1, 3, 5, 17 and 22

326/350 in lanes 8, 10, 13, 16, 23, 24 and 31

348/350 in lanes 9, 15, 19, 29, 30, 33 and 34

the non-family progeny has : 328/380 in lane 32*

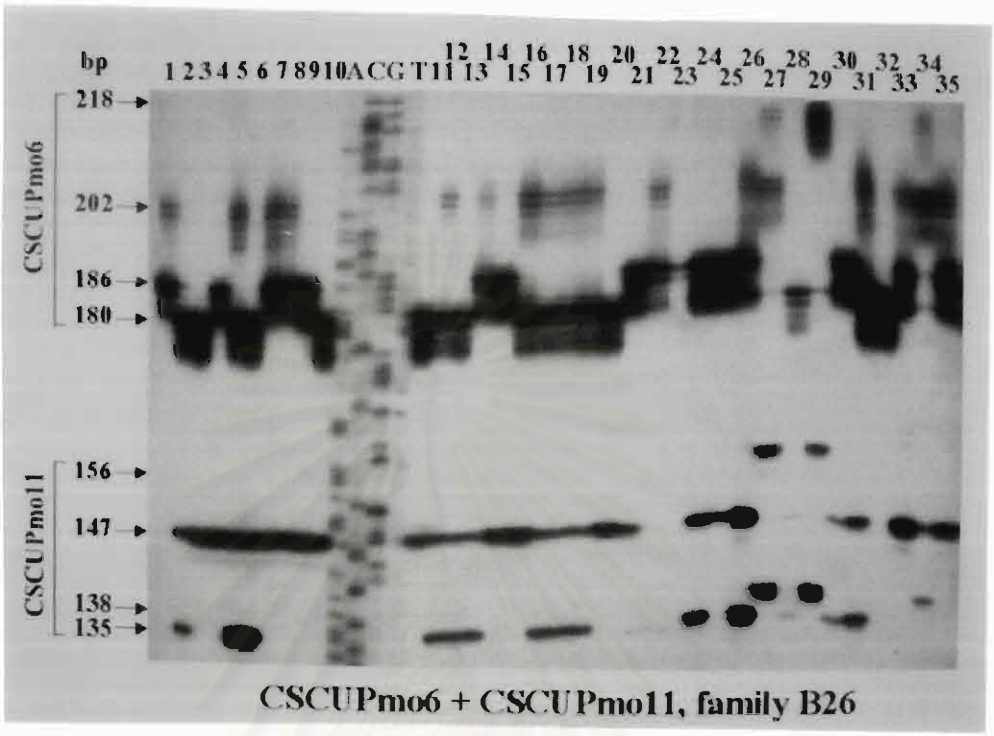


Figure 3.33 Single loading of loci CSCUPmo6+CSCUPmo11 from progeny of a representative full-sib family B26. The size standard is a sequencing ladder of M13 mp18.

At the CSCUPmo6 locus, the progeny have 4 genotypes :

180/180 in lanes 2, 3, 6, 10, 11, 13, 20, 28 and 32

180/202 in lanes 5, 12, 16, 17, 18, 19 and 31

186/186 in lanes 4, 9, 15, 21, 23, 24, 25 and 30

186/202 in lanes 1, 7, 8, 22, 26, 33 and 35

the non-family progeny have : 202/218 in lanes 27 and 34; 218/218 in lane 29

At the CSCUPmo11 locus, the progeny have 2 genotypes :

135/147 in lanes 2, 5, 6, 12, 13, 14, 17, 18, 19, 24, 26, 28, 30 and 31

147/147 in lanes 3, 4, 7, 8, 9, 10, 11, 15, 16, 20, 21, 25, 33 and 35

the non-family progeny have : 138/156 in lanes 27 and 29; 138/147 in lane 34

Table 3.4 Genotypes of 50 individuals *P. monodon* family B2 for six microsatellite loci

Sample	Genotype					
	CSCUPmo1	CSCUPmo2	CSCUPmo4	CSCUPmo6	CSCUPmo9	CSCUPmo11
(1)	242/263	165/177	230/240	172/212	302/310	141/159
(2)	257/263	165/177	240/240	172/178	380/380	141/150
(3)	257/263	177/217	230/240	212/212	310/380	141/159
X (4)	263/263	177/217	230/240	204*/210* #	310/380	138*/141 #
(5)	257/263	165/177	230/240	212/212	380/380	141/159
(6)	242/263	165/177	240/240	172/212	302/380	141/150
X (7)	254*/263 #	165/173* #	214*/246* #	204*/210* #	306*/350* #	138*/144* #
(8)	242/263	177/217	230/240	212/212	310/380	141/150
(9)	257/263	177/217	240/240	212/212	310/380	141/150
(10)	242/257	177/217	230/240	212/212	302/310	141/159
(11)	242/263	165/177	230/240	212/212	310/380	141/159
(12)	242/257	165/177	240/240	172/212	380/380	141/159
(13)	242/257	165/177	230/240	178/212	302/310	141/150
(14)	242/263	177/217	230/240	212/212	302/380	141/150
(15)	263/263	177/217	240/240	172/212	310/380	141/150
X (16)	254*/266* #	165*/181* #	246*/258* #	204*/210* #	306*/350* #	138*/141 #
(17)	263/263	165/177	240/240	212/212	380/380	141/159
(18)	242/257	177/217	230/240	178/212	302/310	141/150
(19)	263/263	177/217	230/240	172/178	380/380	141/150
(20)	242/263	177/217	230/240	172/212	302/310	141/159
(21)	242/257	177/217	240/240	178/212	380/380	141/159
(22)	242/263	165/177	230/240	172/178	302/380	141/150
(23)	242/257	165/177	240/240	172/178	310/380	141/150
(24)	263/263	177/217	240/240	178/212	302/380	141/150
(25)	242/257	165/177	230/240	172/178	302/310	141/150

* unrelated allele

\# unrelated genotype

X Assuming the non-family progeny

Table 3.4 continued

Sample	Genotype					
	CSCUPmo1	CSCUPmo2	CSCUPmo4	CSCUPmo6	CSCUPmo9	CSCUPmo11
(26)	242/263	177/217	240/240	172/178	380/380	141/159
(27)	242/257	177/217	230/240	172/212	380/380	141/159
(28)	242/263	165/177	240/240	172/212	380/380	141/159
(29)	263/263	165/177	230/240	178/212	310/380	141/159
(30)	242/263	177/217	240/240	212/212	380/380	141/159
(31)	242/257	177/217	230/240	172/212	310/380	141/150
(32)	257/263	177/217	240/240	172/212	302/310	141/150
(33)	242/263	165/177	230/240	212/212	380/380	141/159
(34)	257/263	165/177	240/240	172/178	380/380	141/159
X (35)	254*/263 #	181*/181* #	214*/242* #	176*/202* #	310/340* #	138*/144* #
(36)	242/257	177/217	240/240	178/212	380/380	141/159
X (37)	221*/263 #	153*/211* #	240/248* #	208*/208* #	350*/354* #	138*/147* #
(38)	242/263	177/217	240/240	212/212	302/380	141/150
(39)	242/257	165/177	240/240	212/212	302/310	141/159
X (40)	257/263	165/173* #	214*/242* #	200*/210* #	306*/324* #	138*/141 #
(41)	242/257	177/217	240/240	172/178	302/310	141/150
(42)	257/263	177/217	230/240	172/212	302/380	141/150
(43)	242/257	165/177	240/240	172/212	302/310	141/159
X (44)	242/257	165/177	230/240	172/212	298*/320* #	141/150
X (45)	-	-	230/240	172/212	320*/320* #	141/159
X (46)	242/263	165/177	240/240	178/212	298*/320* #	141/159
X (47)	254*/266* #	165/173* #	240/240	204*/212 #	298*/320* #	138*/144* #
X (48)	263/263	165/177	230/240	-	298*/320* #	141/150
(49)	242/263	177/217	-	178/212	-	141/150
(50)	257/263	165/177	-	212/212	-	141/150

* unrelated allele

unrelated genotype

X Assuming the non-family progeny

Table 3.5 Genotypes of 50 individuals *P. monodon* family B10 for six microsatellite loci

Sample	Genotype					
	CSCUPmo1	CSCUPmo2	CSCUPmo4	CSCUPmo6	CSCUPmo9	CSCUPmo11
(1)	275/293	165/177	240/240	186/202	310/344	147/147
(2)	245/275	165/183	240/240	186/216	298/310	147/147
(3)	275/275	147/183	228/240	186/216	320/344	147/147
(4)	275/275	147/183	240/240	186/202	298/320	147/147
(5)	275/275	147/177	240/240	174/202	310/344	147/147
(6)	245/275	165/183	228/240	174/202	298/320	147/147
(7)	275/275	147/183	228/240	174/216	298/310	147/147
(8)	275/275	147/183	228/240	174/202	320/344	147/147
(9)	275/293	147/177	240/240	174/216	320/344	147/147
(10)	275/275	165/183	228/240	186/216	298/310	147/147
(11)	245/293	147/183	240/240	174/202	298/310	147/147
(12)	245/293	147/183	228/240	186/202	298/310	147/147
(13)	275/275	147/183	228/240	186/216	320/344	147/147
(14)	275/293	165/177	228/240	186/216	298/310	147/147
(15)	245/293	165/183	228/240	174/216	320/344	147/147
(16)	245/275	165/183	228/240	174/202	298/320	147/147
(17)	275/293	147/177	240/240	186/202	310/344	147/147
(18)	275/275	165/183	228/240	186/202	310/344	147/147
(19)	275/275	147/183	228/240	186/216	310/344	147/147
(20)	275/275	147/183	240/240	174/202	298/320	147/147
(21)	275/275	147/177	240/240	186/202	298/320	147/147
(22)	245/275	165/177	240/240	174/216	298/320	147/147
(23)	275/275	147/177	240/240	186/216	298/310	147/147
(24)	275/275	147/183	240/240	186/202	298/320	147/147
(25)	275/275	147/177	240/240	174/202	310/344	147/147

Table 3.5 continued

Sample	Genotype					
	CSCUPmo1	CSCUPmo2	CSCUPmo4	CSCUPmo6	CSCUPmo9	CSCUPmo11
(26)	275/275	165/183	240/240	174/202	298/320	147/147
(27)	275/275	165/183	240/240	174/216	298/310	147/147
(28)	275/275	165/177	228/240	174/216	298/310	147/147
(29)	245/275	147/183	28/240	174/202	298/310	147/147
(30)	245/293	147/183	240/240	174/202	298/320	147/147
(31)	245/275	147/177	228/240	186/202	298/320	147/147
(32)	275/293	165/177	228/240	174/202	298/320	147/147
(33)	275/275	165/177	240/240	174/216	310/344	147/147
(34)	245/293	165/183	228/240	186/216	298/320	147/147
(35)	275/275	147/183	228/240	186/202	298/320	147/147
(36)	275/293	165/177	228/240	186/216	298/310	147/147
(37)	245/275	147/183	240/240	174/216	320/344	147/147
(38)	275/275	165/183	240/240	186/216	298/310	147/147
(39)	275/275	165/177	240/240	174/216	310/344	147/147
(40)	245/293	165/177	240/240	186/216	310/344	147/147
(41)	245/293	147/183	240/240	186/216	310/344	147/147
(42)	245/275	147/183	240/240	186/216	298/320	147/147
(43)	275/293	165/183	240/240	186/202	320/344	147/147
(44)	245/275	147/177	240/240	174/202	298/320	147/147
(45)	245/293	147/177	240/240	186/202	320/344	147/147
(46)	245/293	165/183	240/240	186/216	310/344	147/147
(47)	245/293	165/183	240/240	174/202	298/320	147/147
(48)	245/293	165/177	240/240	174/202	298/310	147/147
(49)	245/275	165/183	240/240	186/216	310/344	147/147
(50)	245/275	165/183	228/240	186/216	298/320	147/147

Table 3.6 Genotypes of 50 individuals *P. monodon* family B26 for six microsatellite loci

Sample	Genotype					
	CSCUPmo1	CSCUPmo2	CSCUPmo4	CSCUPmo6	CSCUPmo9	CSCUPmo11
(1)	242/299	153/177	234/242	186/202	322/326	147/147
(2)	254/335	153/217	240/242	180/180	-	135/147
(3)	242/299	153/177	238/240	180/180	322/348	147/147
(4)	242/299	213/217	238/240	186/186	22/326	147/147
(5)	254/299	153/217	240/242	180/202	322/348	135/147
(6)	242/299	153/217	240/242	180/180	322/326	135/147
(7)	242/335	153/217	-	186/202	322/348	147/147
(8)	254/335	153/217	234/242	186/202	322/326	147/147
(9)	242/335	153/217	238/240	186/186	-	147/147
(10)	242/299	213/217	238/240	180/180	326/350	147/147
(11)	254/299	153/177	234/238	180/180	348/350	147/147
(12)	242/355	153/177	238/240	180/202	326/350	135/147
(13)	242/299	153/217	234/240	180/180	-	135/147
(14)	254/299	213/217	240/242	186/202	322/326	135/147
(15)	242/335	153/217	238/240	186/186	326/350	147/147
(16)	242/335	153/217	234/242	180/202	-	147/147
(17)	242/299	213/217	234/242	180/202	348/350	135/147
(18)	242/335	153/177	238/240	180/202	326/350	135/147
(19)	242/299	153/177	234/238	180/202	322/348	135/147
(20)	254/299	213/217	240/242	180/180	348/350	147/147
(21)	242/335	153/177	240/242	186/186	348/350	147/147
(22)	242/299	213/217	240/242	186/202	322/326	135/147
(23)	242/335	153/217	234/238	186/186	322/348	-
(24)	254/335	153/217	234/238	186/186	322/348	135/147
(25)	254/335	153/217	238/240	186/186	326/350	147/147

* unrelated allele

\# unrelated genotype

X Assuming the non-family progeny

Table 3.6 continued

Sample	Genotype					
	CSCUPmo1	CSCUPmo2	CSCUPmo4	CSCUPmo6	CSCUPmo9	CSCUPmo11
(26)	242/299	153/217	234/238	186/202	326/350	135/147
X (27)	263*/287* #	159*/159* #	240/242	202/218* #	322/328* #	138*/156* #
(28)	254/299	177/213	234/242	180/180	348/350	135/147
X (29)	263*/281* #	159*/209* #	234/240 #	218*/218* #	328*/380* #	138*/156* #
(30)	254/299	177/213	240/242	186/186	322/348	135/147
(31)	242/299	153/217	234/238	180/202	348/350	135/147
(32)	254/299	177/213	240/242	180/180	348/350	135/147
(33)	242/299	213/217	238/240	186/202	326/350	147/147
X (34)	287*/299 #	159*/209* #	240/242	202/218* #	328*/380* #	138*/147 #
(35)	254/335	213/217	234/242	186/202	348/350	147/147
(36)	242/299	153/217	234/238	186/186	348/350	135/147
(37)	254/299	153/217	234/242	180/180	-	147/147
(38)	254/299	213/217	238/240	180/180	322/348	147/147
(39)	242/335	153/177	234/238	186/202	322/348	135/147
(40)	254/335	153/217	234/242	180/180	322/348	147/147
(41)	242/335	153/177	240/242	186/186	-	147/147
(42)	254/335	153/177	234/238	186/202	348/350	135/147
(43)	242/299	213/217	234/242	186/186	322/326	147/147
(44)	254/299	213/217	240/242	180/180	322/348	135/147
(45)	254/299	153/217	234/238	186/202	322/326	147/147
(46)	254/335	213/217	234/242	186/186	322/326	147/147
(47)	254/299	153/217	234/238	186/186	322/326	135/147
(48)	242/299	153/217	234/238	186/202	322/348	147/147
(49)	242/335	213/217	238/240	186/186	-	147/147
(50)	242/299	153/177	240/242	186/186	-	147/147

* unrelated allele

unrelated genotype

X Assuming the non-family progeny

Table 3.7 Segregation analysis of the microsatellitee locus CSCUPmo1 resulted from randomly chosen progeny of three *P. monodon* families

Family	Parental genotypes (female x male[a])	Observed no. of progeny in each genotypic class				Expected individuals in each class[b]	*n*	χ^2
B2	242/263 x 257/263	242/257	242/263	257/263	263/263	9.75	39	4.78
		13	13	8	5			
B10	245/275 x 275/293	245/275	245/293	275/275	275/293	12.50	50	**8.56**
		11	11	21	7			
B26	299/335 x 242/254	242/299	242/335	254/299	254/335	11.75	47	2.82
		16	11	12	8			

[a] Inferred from the offspring.

[b] Assuming codominant inheritance.

For $p < 0.05$, the critical χ^2 value is 7.81 for d.f. = 3 and 3.84 for d.f. =1

Table 3.8 Segregation analysis of the microsatellite locus CSCUPmo2 resulted from randomly chosen progeny of three *P. monodon* families

Family	Parental genotypes (female x male[a])	Observed no. of progeny in each genotypic class				Expected individuals in each class[b]	*n*	χ^2
B2	177/177 x 165/217	165/177		177/217		19.50	39	0.24
		18		21				
B10	177/183 x 147/165	147/177	147/183	165/177	165/183	12.50	50	2.96
		9	16	10	15			
B26	153/213 x 177/217	153/177	153/217	177/213	213/217	11.75	47	**12.49**
		11	20	3	13			

[a] Inferred from the offspring.

[b] Assuming codominant inheritance.

For $p < 0.05$, the critical χ^2 value is 7.81 for d.f. = 3 and 3.84 for d.f. =1

Table 3.9 Segregation analysis of the microsatellite locus CSCUPmo4 resulted from randomly chosen progeny of three *P. monodon* families

Family	Parental genotypes (female x male[a])	Observed no. of progeny in each genotypic class				Expected individuals in each class[b]	*n*	χ^2
B2	240/240 x 230/240	230/240		240/240		18.50	37	0.02
		18		19				
B10	240/240 x 228/240	228/240		240/240		25.00	50	2.00
		20		30				
B26	238/242 x 234/240	234/238	234/242	238/240	240/242	11.50	46	0.43
		12	10	11	13			

[a] Inferred from the offspring.

[b] Assuming codominant inheritance.

For $p < 0.05$, the critical χ^2 value is 7.81 for d.f. = 3 and 3.84 for d.f. =1

Table 3.10 Segregation analysis of the microsatellite locus CSCUPmo6 resulted from randomly chosen progeny of three *P. monodon* families

Family	Parental genotypes (female x male[a])	Observed no. of progeny in each genotypic class				Expected individuals in each class[b]	*n*	χ^2
B2	178/212 x 172/212	172/178	172/212	212/212	178/212	9.75	39	2.33
		8	11	13	7			
B10	174/186 x 202/216	174/202	174/216	186/202	186/216	12.50	50	1.68
		13	10	11	16			
B26	180/202 x 180/186	180/180	180/202	186/186	186/202	1125	45	1.49
		13	8	13	11			

[a] Inferred from the offspring.

[b] Assuming codominant inheritance.

For $p < 0.05$, the critical χ^2 value is 7.81 for d.f. = 3 and 3.84 for d.f. =1

Table 3.11 Segregation analysis of the microsatellite locus CSCUPmo9 resulted from randomly chosen progeny of three *P. monodon* families

Family	Parental genotypes (female x male[a])	Observed no. of progeny in each genotypic class				Expected individuals in each class[b]	*n*	χ^2
B2	310/380 x 302/380	302/310	302/380	310/380	380/380	9.25	37	2.89
		10	6	8	13			
B10	310/320 x 298/344	298/310	298/320	310/344	320/344	12.50	50	3.28
		13	17	12	8			
B26	322/350 x 326/348	322/326	322/348	326/350	348/350	9.75	39	1.11
		11	11	7	10			

[a] Inferred from the offspring.

[b] Assuming codominant inheritance.

For $p < 0.05$, the critical χ^2 value is 7.81 for d.f. = 3 and 3.84 for d.f. =1

Table 3.12 Segregation analysis of the microsatellite locus CSCUPmo11 resulted from randomly chosen progeny of three *P. monodon* families

Family	Parental genotypes (female x male[a])	Observed no. of progeny in each genotypic class		Expected individuals in each class[b]	*n*	χ^2
B2	141/141 x 150/159	141/150	141/159	19.50	39	0.02
		20	19			
B10	147/147 x 147/147	147/147		50.00	50	0.00
		50				
B26	135/147 x 147/147	135/147	147/147	23.00	46	0.34
		21	25			

[a] Inferred from the offspring.

[b] Assuming codominant inheritance.

For $p < 0.05$, the critical χ^2 value is 7.81 for d.f. = 3 and 3.84 for d.f. =1