Chapter III

Results

3.1 DNA extraction

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

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

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

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

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

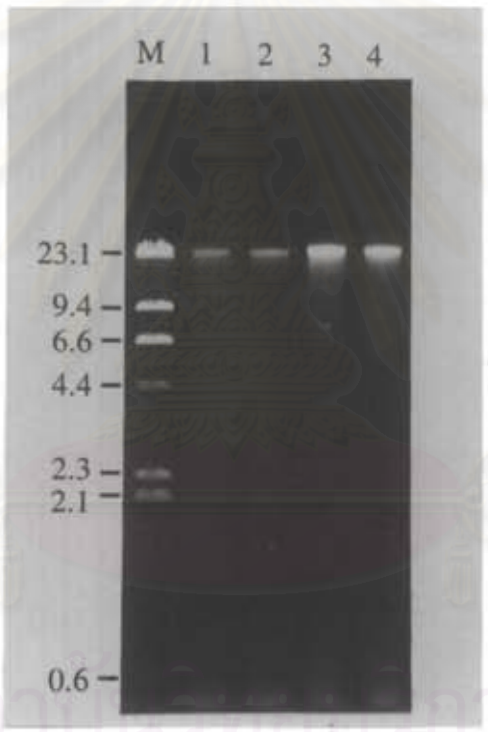


Figure 3.1 Ethidium bromide staining of 0.7% agarose gel showing DNA extracted from pleopods of *P. monodon* .

Lane M : DNA/*Hind* III digested size markers.

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

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

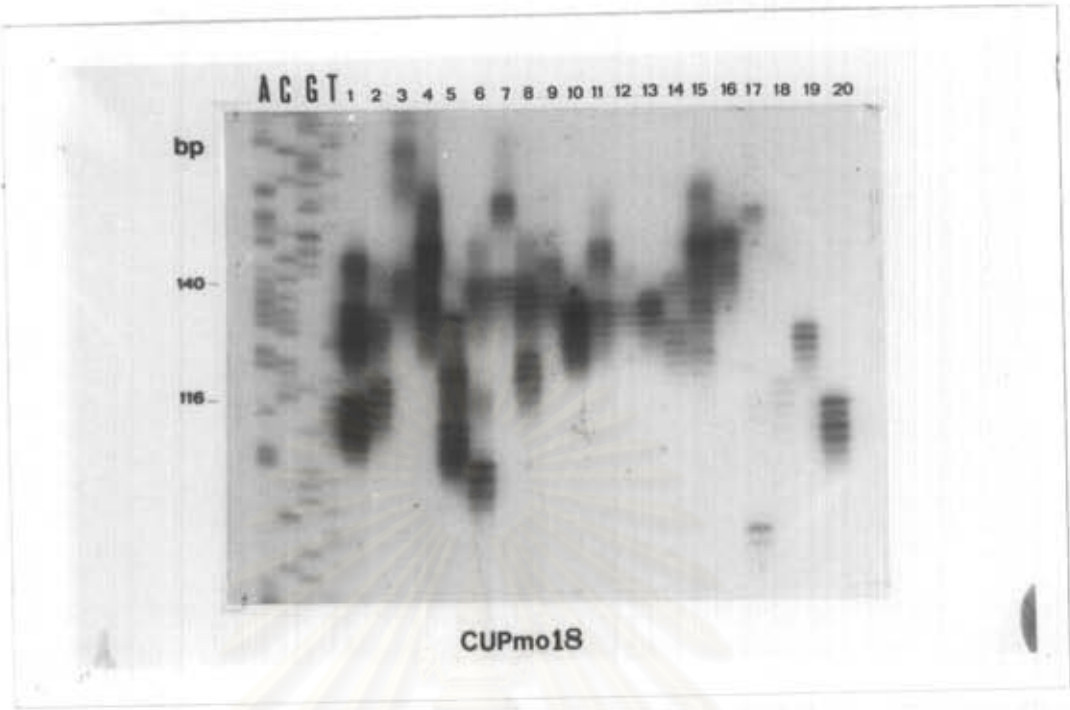


Figure 3.2 PCR amplification patterns of the CUPmo18 locus from 20 individuals *P. monodon* DNA (lanes 1-20) under the optimal PCR conditions with annealing temperature at 55 °C. The size standard is a sequencing ladder of M13 mp 18.

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

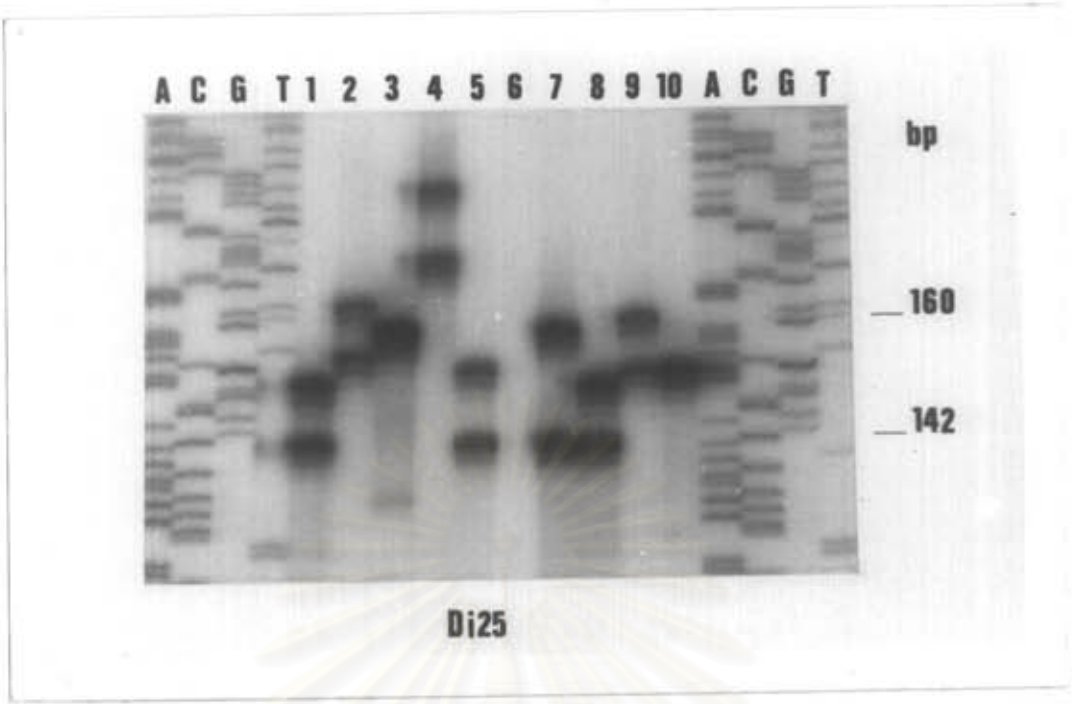


Figure 3.3 PCR amplification patterns of the Di25 locus from 10 individuals *P. monodon* DNA (lanes 1-10) under the optimal PCR conditions with annealing temperature at 65 °C. The size standard is a sequencing ladder of M13 mp 18.

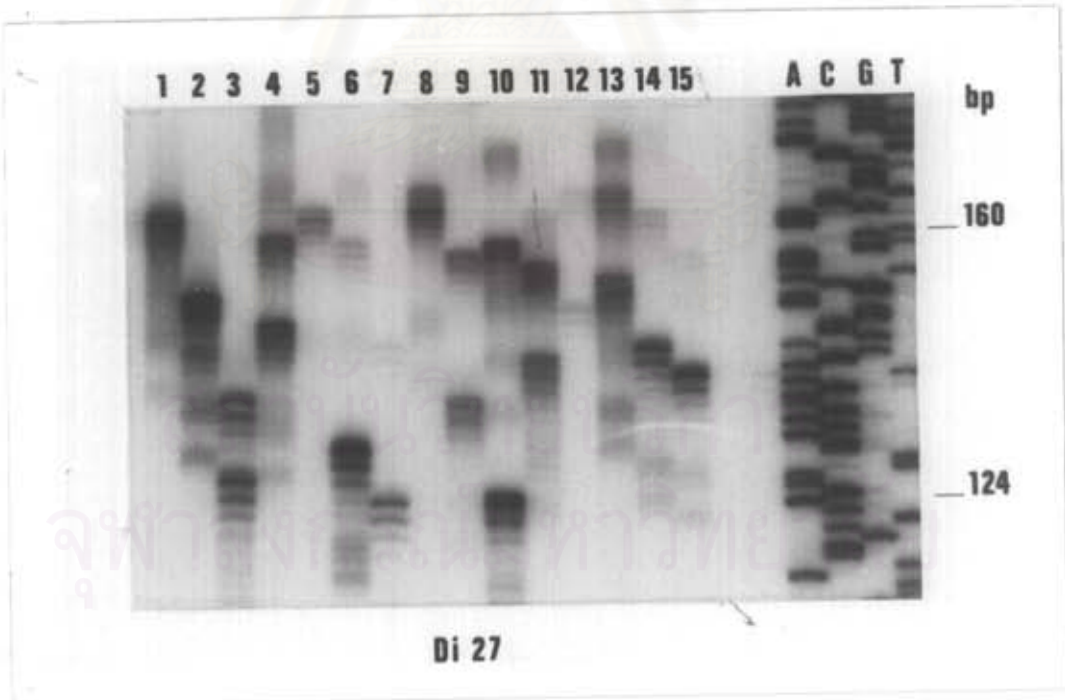


Figure 3.4 PCR amplification patterns of the Di27 locus from 15 individuals *P. monodon* DNA under the optimal PCR conditions with annealing temperature at 54 °C. The size standard is a sequencing ladder of M13 mp 18.

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

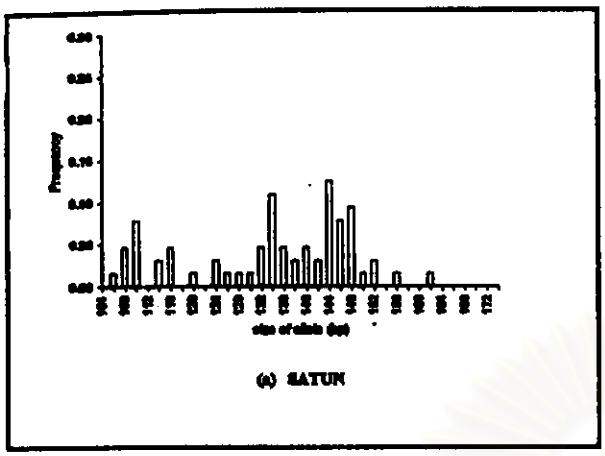
3.3 Variability of three microsatellites

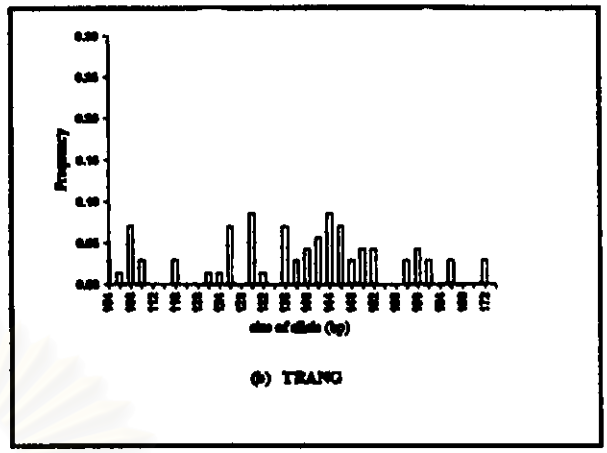
3.3.1 Diversity within samples

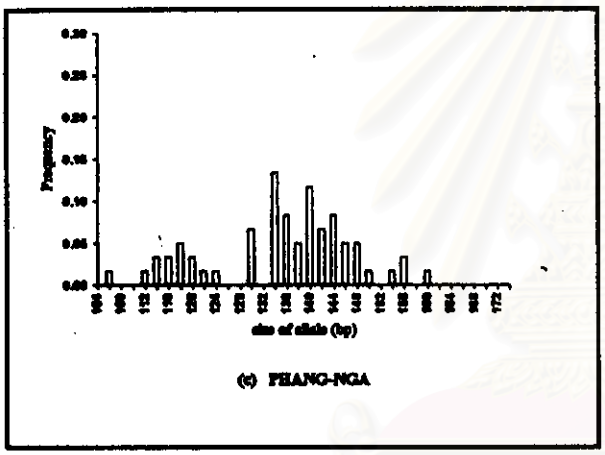
All three investigated loci were highly polymorphic. The most polymorphic locus was CUPmo 18 followed by Di25 and Di27. From, 184 individual *P. monodon* examined, a total of 37, 34 and 32 alleles was observed at CUPmo 18, Di25, and Di27 with the allele sizes ranged from 104 - 172 bp, 136 - 208 bp, and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

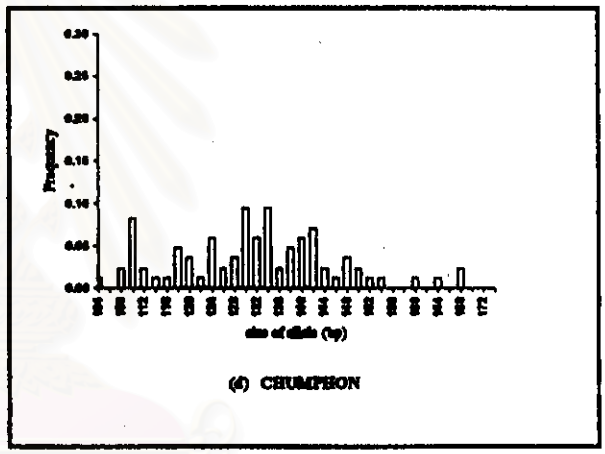
The allele distribution frequencies of the microsatellite CUPmo 18, Di25, and Di27 loci could be summarized and shown in Fig. 3.5, 3.6 and 3.7, Table 3.2, 3.3 and 3.4 respectively. As typically observed in the distribution pattern of microsatellite DNA , several rare alleles were found in each geographically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

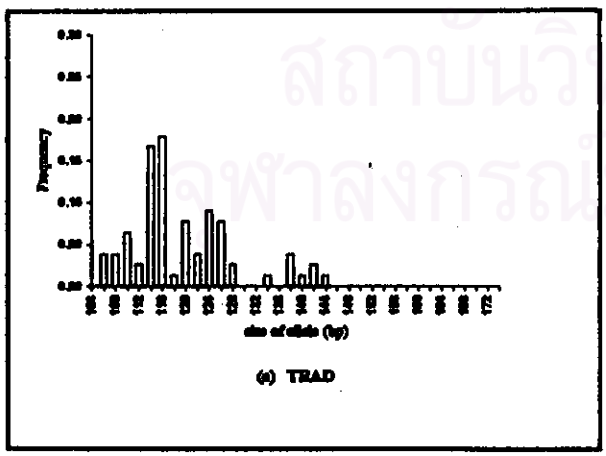
For CUPmo 18, only two alleles (114 and 116 bp) in Trad possessed the frequency slightly higher than 0.15. The Di25 locus also showed allele frequencies higher than 0.15 for a 152 bp allele in Trang and a 156 bp allele in Chumphon. For Di27, only a 162 bp allele in Satun showed the allele frequency greater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











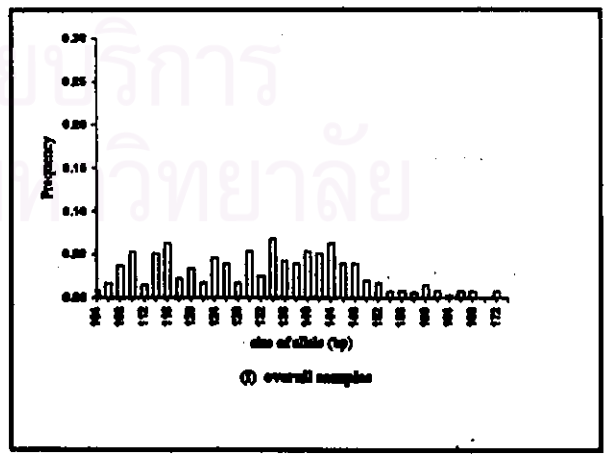


Fig 3.5 Distribution frequencies of alleles at the CUPmo18 locus from Satun (n=32),Trang(n=35),Phang-nga(n=30),Chumphon(n=42),Trad(n=39)and overall samples(n=178)

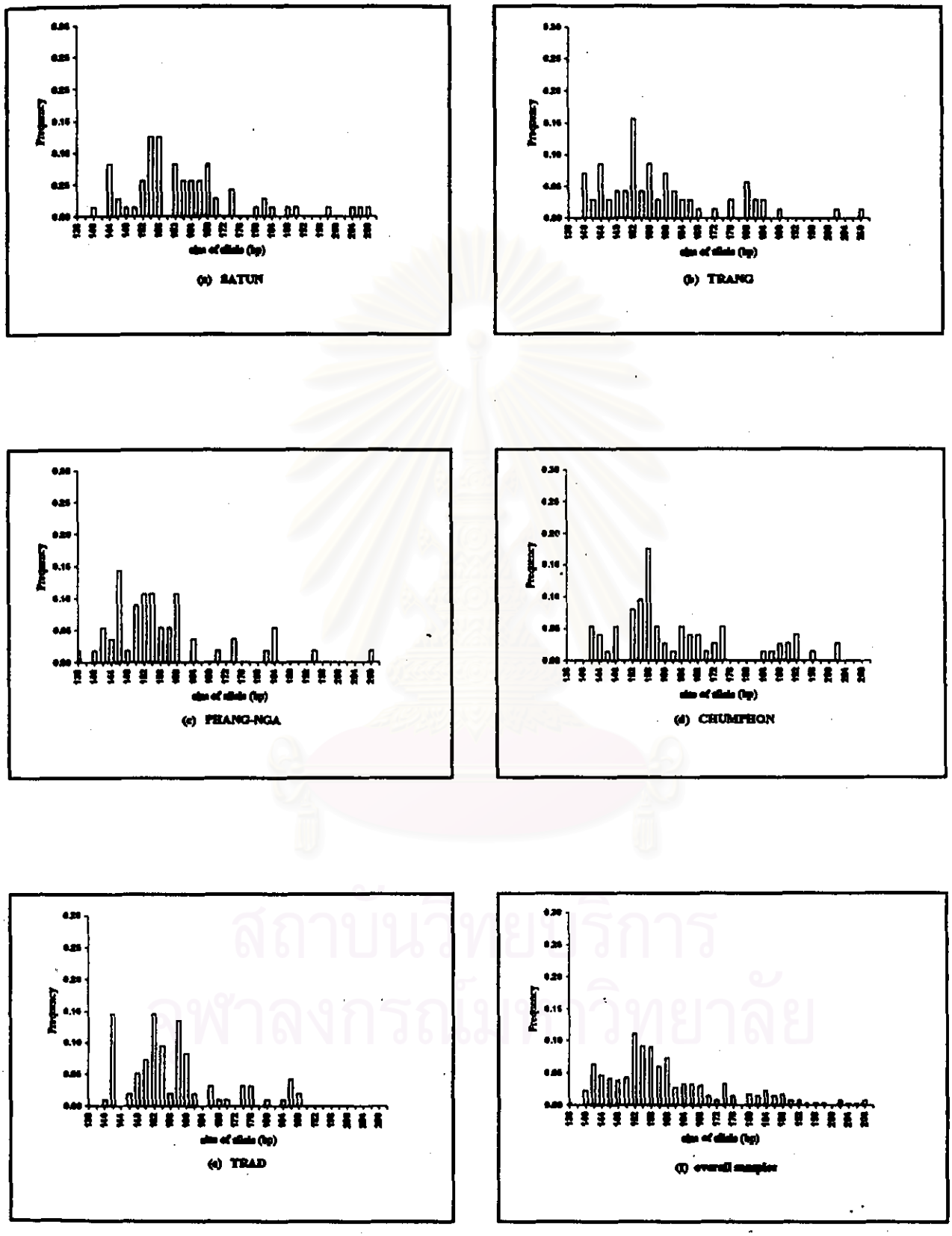
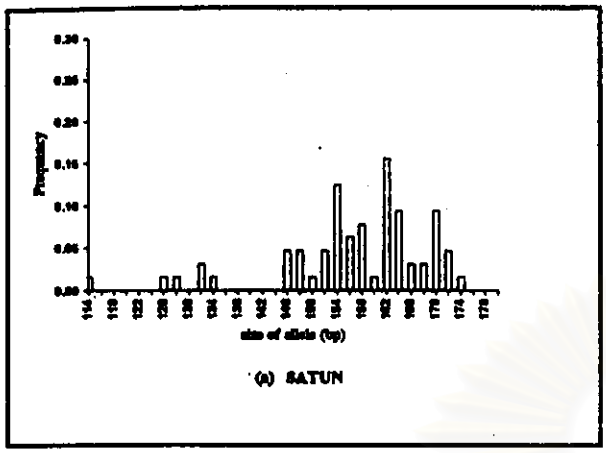
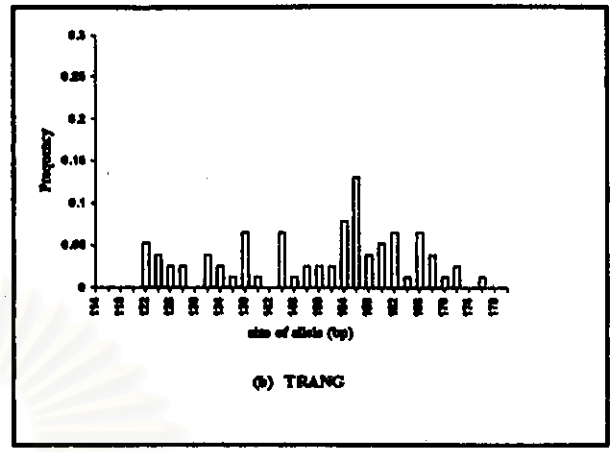


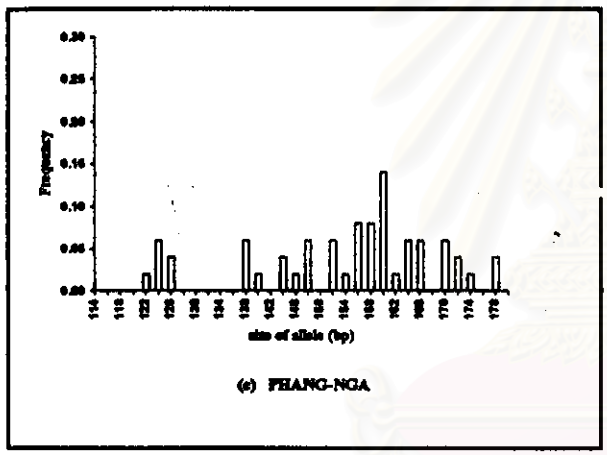
Fig 3.6 Distribution frequencies of alleles at the Di25 locus from Satun(n=36), Trang (n=35), Phang-nga(n=28), Chumphon(n=37), Trad(n=48) and overall samples(n=184)



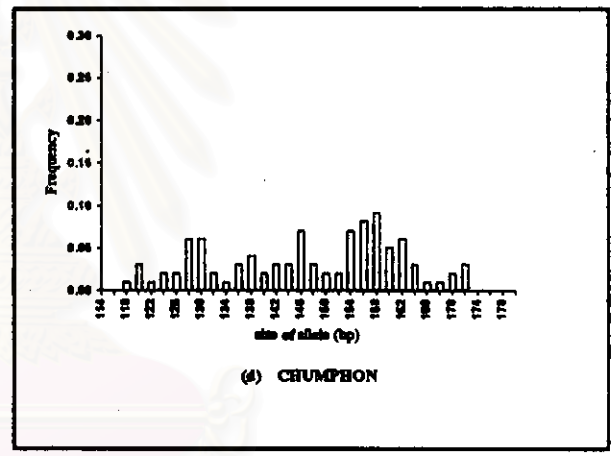
(a) SATUN



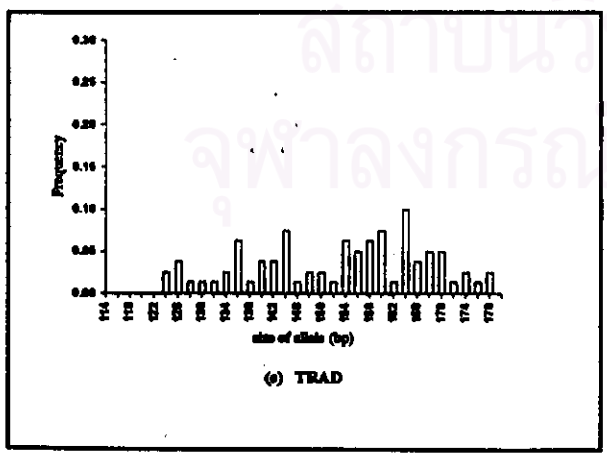
(b) TRANG



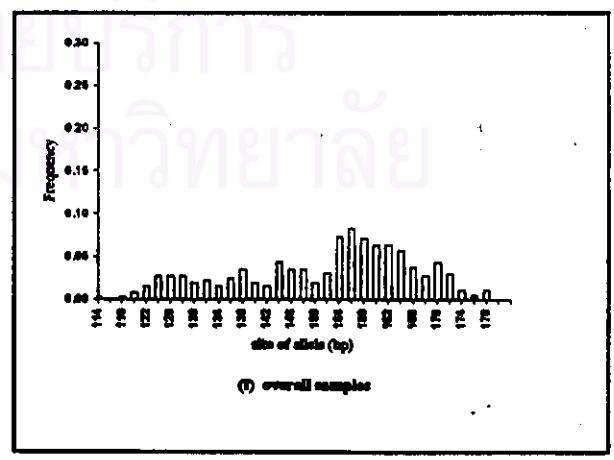
(c) PHANG-NGA



(d) CHUMPHON



(e) TRAD



(f) overall samples

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

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

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

Table 3.2 (continued)

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

N = Number of sample examined

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

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

Table 3.3 (continued)

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

N = number of sample examined

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

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

Table 3.4 (continued)

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

N = number of sample examined

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

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

loci were shown in Table 3.6. Chumphon showed the highest genetic diversity followed by Trang, Trad, Satun and Phang-nga respectively.

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

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

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

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

3.3.2 Hardy - Weinberg disequilibrium

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

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

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

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

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

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

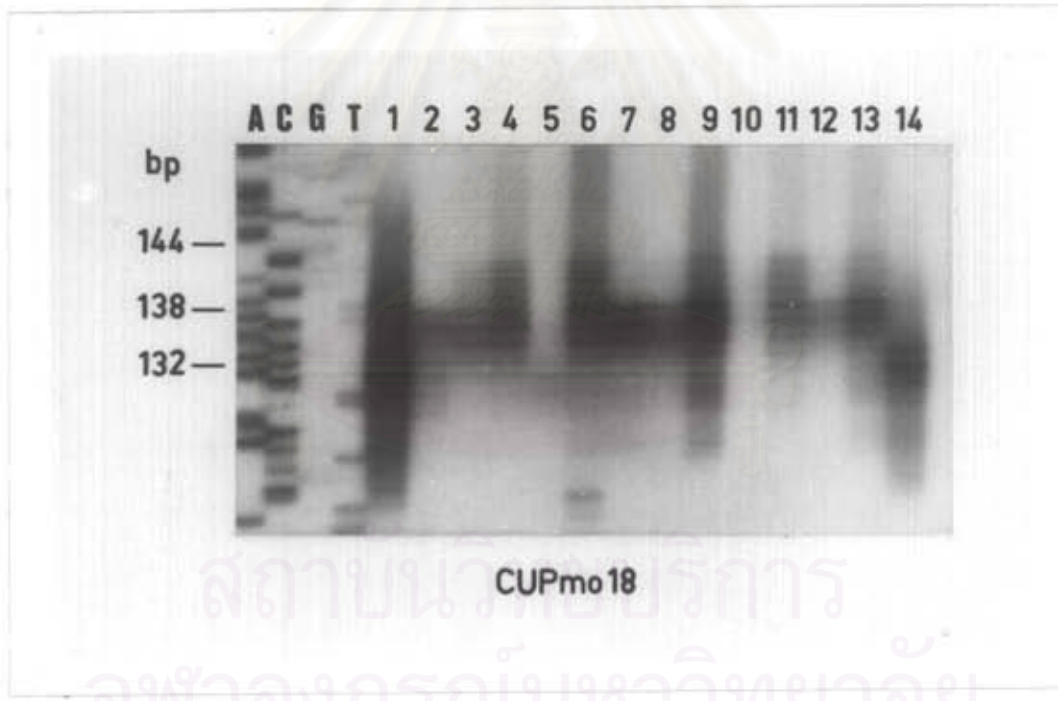


Figure 3.8 PCR amplification patterns of microsatellite locus CUPmo18 from progeny of a representative full-sib family. The size standard is a sequencing ladder of M13 mp 18.

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

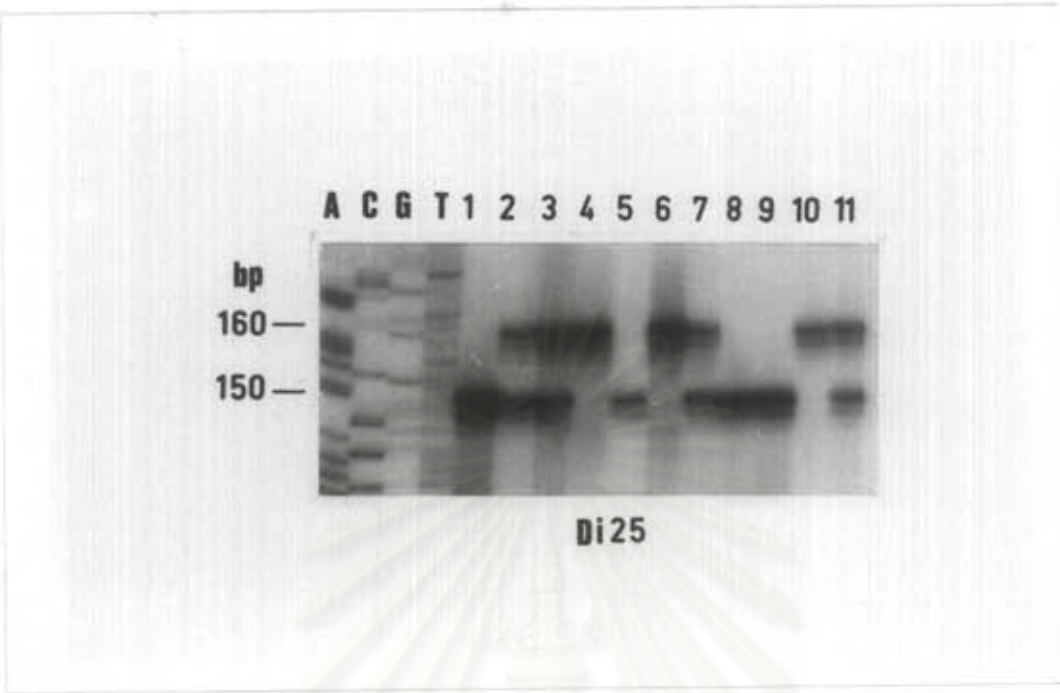


Figure 3.9 PCR amplification patterns of microsatellite locus Di25 from progeny of a representative full-sib family. The size standard is a sequencing ladder of M13 mp 18.

The progeny have 3 genotypes : 160/160 in lanes 4, 6, and 10.
: 160/150 in lanes 2, 3, 7, and 11.
: 150/150 in lanes 1, 5, 8, and 9.

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

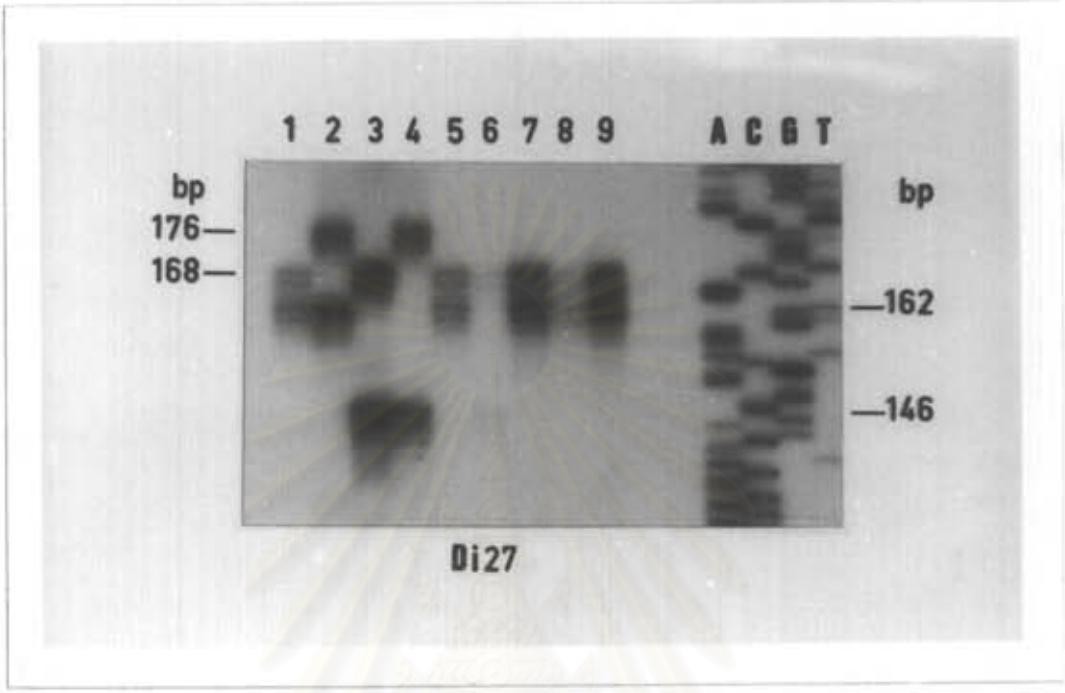


Figure 3.10 PCR amplification patterns of microsatellite locus Di27 from progeny of a representative full-sib family. The size standard is a sequencing ladder of M13 mp 18.

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

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

Locus	Parents[a]		Genotypes of the offspring			
	1	2	Expected ratio	Genotypes	observed individual	χ^2
CUPmo18	132/144	138/138	1:1	132/138	8	
				144/138	12	0.8^{ns}
Di25	160/150	160/150	1:2:1	160/160	5	
				160/150	6	
				150/150	9	4.8^{ns}
Di27	162/146	168/176	1:1:1:1	162/168	8	
				162/176	3	
				146/168	5	
				146/176	4	2.8^{ns}

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

ns = not significant

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

for $p< 0.05$ df = 2 = 5.99

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

3.3.4 Estimation of genetic distance, intraspecific phylogency and population differentiation

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

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

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

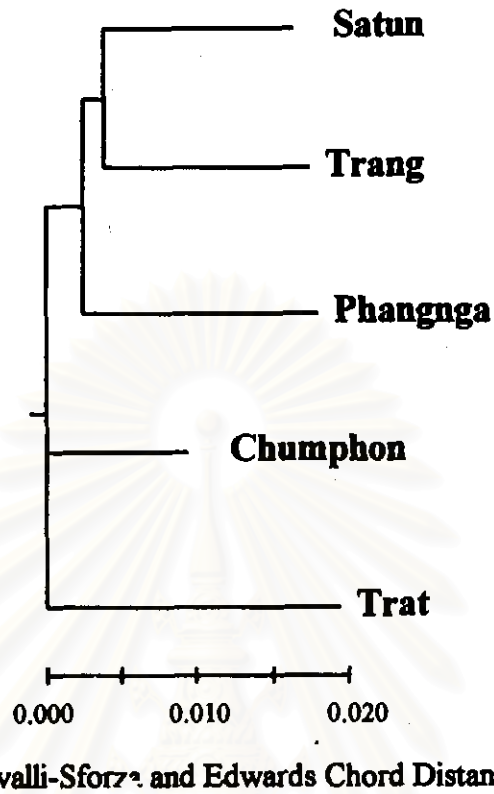


Figure 3.11 A neighbor-joining tree illustrating relationships among 5 geographic samples of *P. monodon* in Thailand based on Cavalli - Sforza and Edwards chord distance.

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

genetically different from Chumphon when Di25 was employed. Within the Andaman samples, the heterogeneity was observed between a pairwise comparison of Trang and Phang-nga at the CUPmo18 ($p = 0.004$) loci. More importantly, high significant difference in allele frequencies was observed between Trad and Chumphon located in the same coast ($p < 0.001$ for CUPmo 18 and $p < 0.001$ for Di25).

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

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

a = Significant levels were adjusted using a Bonferroni technique.

ns = not significant

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

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

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

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