Chapter III

Results

Extraction of total DNA from C. mydas

Total DNA from *C. mydas* was prepared using proteinase K/phenol extraction method. The quality and semiquantity of extracted total DNA was examined by electrophoresed through 0.7% agarose gel (Figure 3.1). It was found that the smear pattern was observed in total DNA extracted from soft tissues (approximately 70%) because the soft tissues needed to be ground to a fine powder with occasional adding of liquid nitrogen before the extraction. Preparation of DNA from soft tissues needed to be ground to a fine powder with occasional adding of liquid nitrogen before the extraction. To prepare DNA template for PCR amplification, the initial DNA stock solution was diluted to a final concentration of 50-100 ng/µl.

Optimisation of PCR condition for amplification of C. mydas microsatellite DNA

The amplification condition originally reported by FiztSimmons *et. al* (1995) did not work well for all three chosen primers. Fifty-five degree celsius annealing temperature originally used for all primers gave nonspecific PCR products which were not consistent. This indicated that further optimisation of the PCR condition was required. Therefore, different annealing temperature and Mg^{2+} concentration were then optimised for each locus.

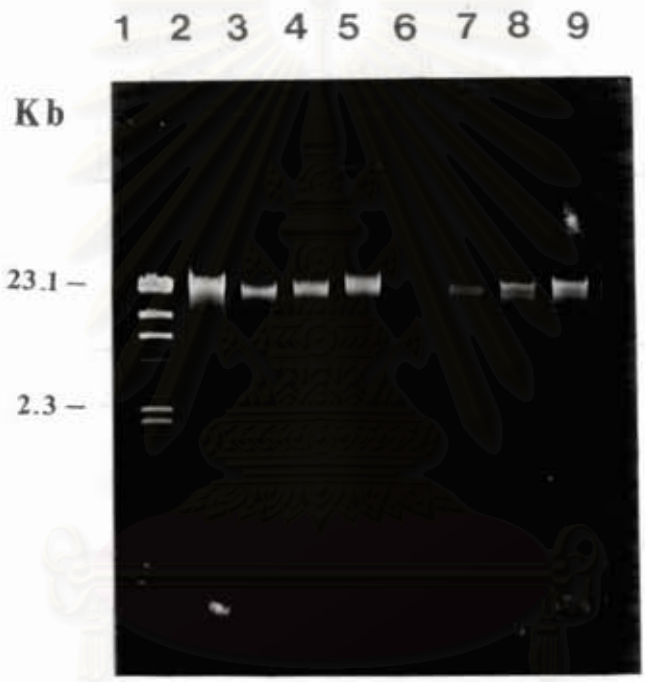

Figure 3.1 Agarose gel electrophoresis showing DNA quality extracted from *C. mydas*

lane 1 : λ/*Hin*dIII standard marker

lane 2-5: DNA extracted from blood.

lane 6-9: DNA extracted from soft tissues (heart, kidney and liver).

Four different anneanling temperature was employed (56, 58, 60 and 62°C for locus Cm3; 62, 64, 66 and 68°C for locus Cm72 and 57, 59, 61 and 63°C for locus Cc117) in the PCR reaction containing 2.5 mM Mg^{2+}. All other conditions were maintained as in the original publication (see in amplification of microsatellite DNA using the Polymerase Chain Reaction, chapter II). The optimal annealing temperature for each locus was selected from that provided the most intense band of PCR product which was 60°C for Cm3, 66°C for Cm72 and 61°C for Cc117.

An example for optimisation of suitable annealing temperature and Mg^{2+} concentration for locus Cm72 is shown in Figure 3.2. It should be noted that agarose gel was employed for determination of the amplification quality rather than the use of a proper polyacrylamide gel. The reason of this is that using agarose gel is much more convenient and an accurate size of the product was not required at this stage. The optimal Mg^{2+} level was also examined by varying its concentration in a series of 2.0, 2.5, 3.0 and 3.5 mM for Cm72 and Cc117 loci while higher concentrations (2.5, 3.0, 3.5, 4.0 and 4.5 mM) were tested for locus Cm3. As can be seen in Figure 3.2, the optimal concentrations of Mg^{2+} for Cm3 Cm72 and Cc117 loci were 4.0, 3.0 and 2.5 mM, respectively.

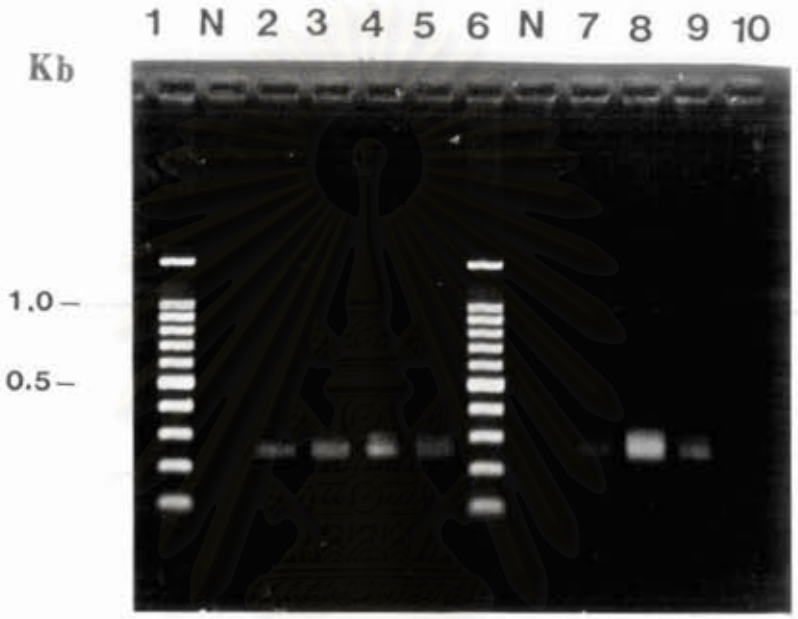

Figure 3.2 The optimal annealing temperature and Mg^{2+} concentration for microsatellite locus Cm72 detected by agarose gel electrophoresis

lane N : negative control

lane 1 and 6 : 100 bp DNA ladder standard marker

lane 2-5 : Cm72 product with a constant Mg^{2+} concentration of 2.5 mM at different annealing temperature of 62°, 64°, 66° and 68° C, respectively

lane 7-10 : Cm72 product amplified at annealing temperature 66° C using 2.0, 2.5, 3.0 and 3.5 mM of Mg^{2+}, respectively

Moreover, primers of Cm84 and Cm58 were originally included in the present study. However, scoring of amplified product of locus Cm84 was ambiguous because of its large size (>328 bp). Whereas the latter did not give polymorphic results from 8 individuals used in the pilot study. Therefore, it was not further utilised these 2 microsatellite loci for analysis of genetic population structure of *C. mydas*.

Genetic variation in *C. mydas*

The microsatellite products were able to amplify using both homologous (Cm3 and Cm72) and heterologous (Cc117) primers as shown in Figure 3.4, 3.5, 3.6. All primers generated groups of stutter bands. The allelic sizes were determined from the most intense band within a group of stutter bands. Individual generated 1 band and 2 bands were classified as homozygous (1 allele) and heterozygous (2 alleles), respectively. This classification was used for calculation of observed heterozygosity derived from direct count. All *C. mydas* specimens (90 individuals) could be amplified by Cm3 primers but only eighty-five individuals was able to be analysed for Cm72. It should be emphasised that much lower number of specimens (77 individuals) was successfully amplified using heterologous primers Cc117.

Thirty-one polymorphic alleles ranged between 144 and 182 bp were observed for locus Cm3 (Table 3.1). The number of alleles found in the Gulf of Thailand was higher than those in the Andaman Sea (26 and 23 alleles, respectively).

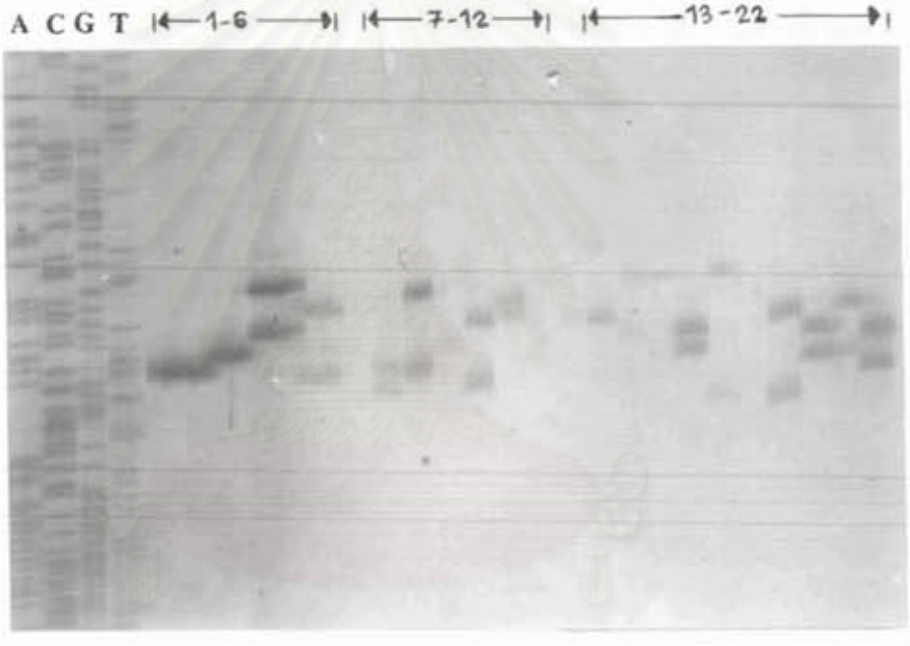

Figure 3.3 A 6% silver-stained denaturaing polyacrylamide gel illustrating amplified microsatellite products of locus Cm84

lane A, C, G and T: sequencing ladder pGEM-3Zf (+) using M13 forward primer

lane 1 - 22 : amplified microsatellite DNA at locus Cm 84

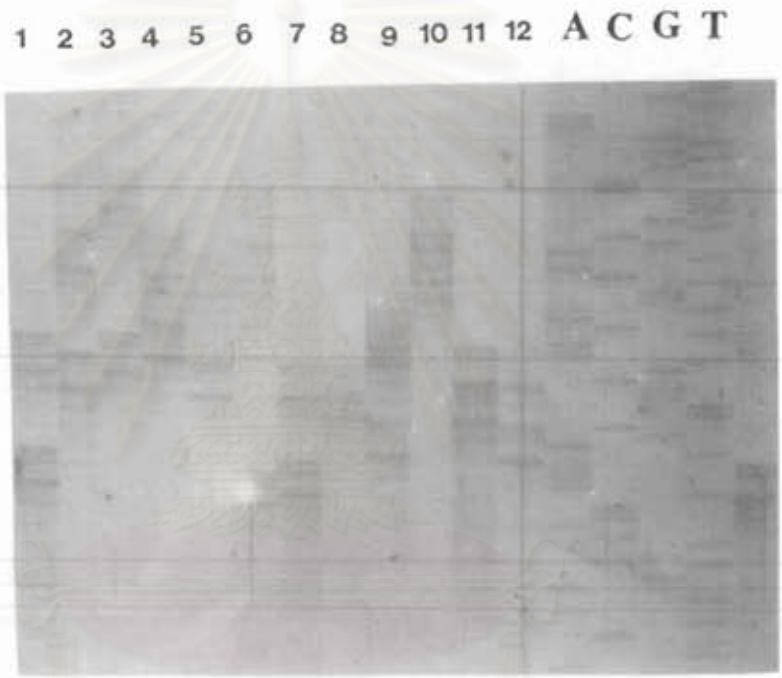

Figure 3.4 A 6% silver-stained denaturing polyacrylamide gel illustrating amplified microsatellite products of locus Cm3

lane A, C, G and T: sequencing ladder amplified from pGEM-3Zf (+) using M13 forward primer

lane1-12 : amplified microsatellite DNA at locus Cm3

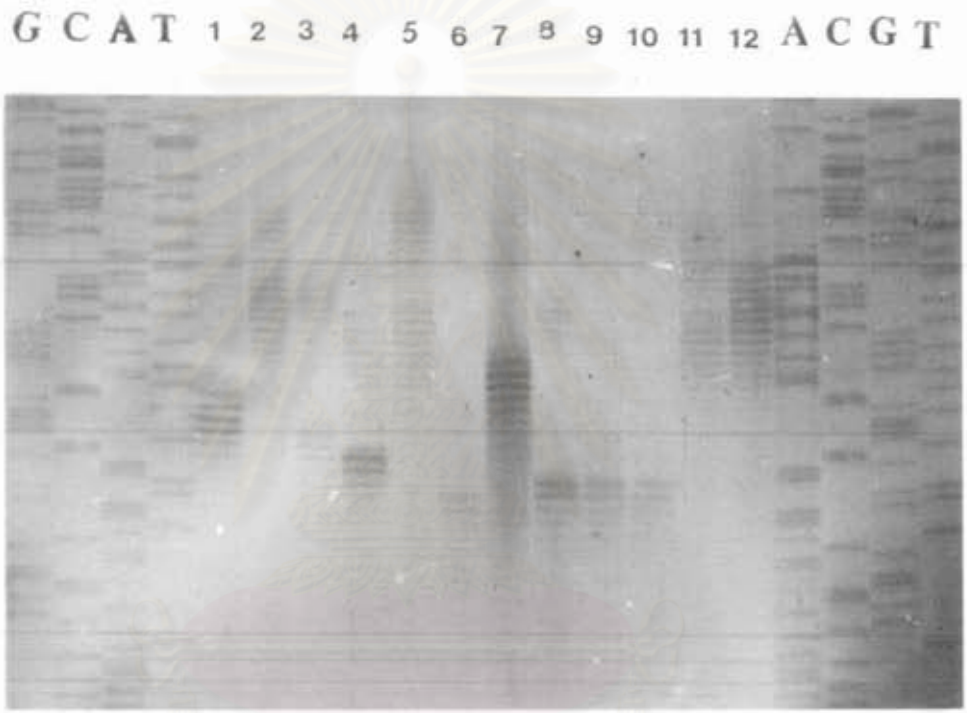

Figure 3.5 A 6% silver-stained denaturing polyacrylamide gel illustrating amplified microsatellite products of locus Cm72

lane A, C, G and T: sequencing ladder amplified from pGEM-3Zf (+) using M13 forward primer

lane1-12 : amplified microsatellite DNA at locus Cm72

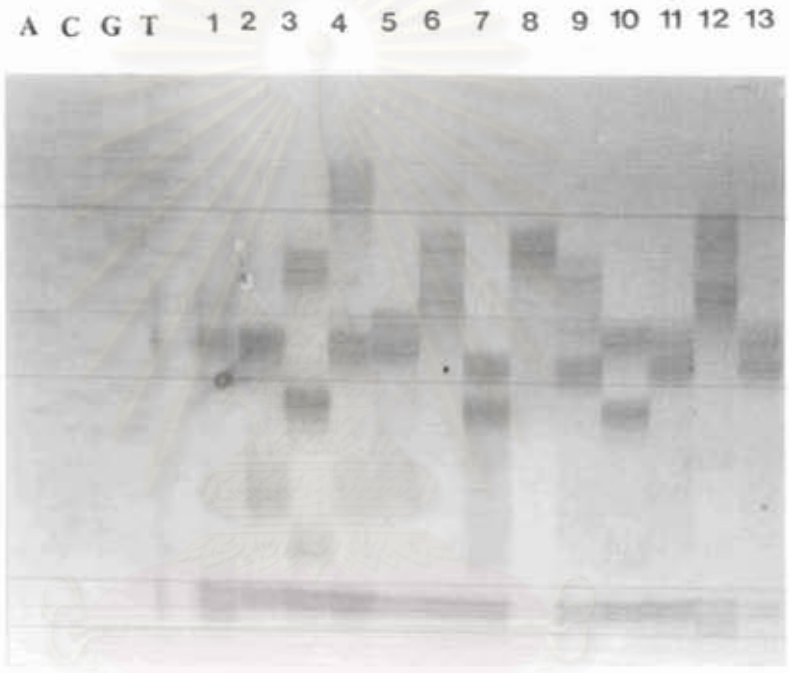

Figure 3.6 A 6% silver-stained denaturing polyacrylamide gel illustrating amplified microsatellite products of locus Cc117

lane A, C, G and T: sequencing ladder amplified from pGEM-3Zf (+) using M13 forward primer.

lane1-13 : amplified microsatellite of *C. mydas* at locus Cc117

Table 3.1 Allele frequencies distribution of *C. mydas* analysed at locus Cm3 in the Andaman Sea and the Gulf of Thailand samples

Allele (base pairs)	Locus Cm3	
	the Gulf of Thailand	the Andaman Sea
144	0.010	0.000
146	0.010	0.012
147	0.010	0.000
148	0.000	0.012
150	0.094	0.012
151	0.063	0.000
152	0.031	0.000
154	0.094	0.073
155	0.010	0.024
156	0.010	0.024
158	0.000	0.098
159	0.000	0.025
160	0.094	0.073
161	0.010	0.000
162	0.135	0.061
163	0.010	0.024
164	0.052	0.037
165	0.010	0.000
166	0.052	0.049
167	0.000	0.012
168	0.021	0.024
170	0.021	0.024
172	0.073	0.110
173	0.021	0.000
174	0.094	0.195
175	0.010	0.000
176	0.021	0.037
178	0.010	0.024
180	0.010	0.012
181	0.000	0.012
182	0.021	0.024

Eighteen polymorphic alleles were shared between both areas. A total of five (148, 158, 159, 167 and 181 bp) and eight (144, 147, 151, 152, 161, 165, 173 and 175 bp) alleles were respectively found in the Andaman Sea and the Gulf of Thailand.

Cm 72 was the most polymorphic microsatellite locus in this study. A total of 40 alleles covering 229-298 bp was observed (Table 3.2). Of which, twenty-eight alleles were detected in the Gulf of Thailand and thirty-two alleles were observed in the Andaman Sea. Twelve alleles (239, 242, 249, 255, 262, 264, 266, 267, 273, 290, 293 and 296 bp) were population specific and found only in the Andaman. A total number of eight alleles (229, 230, 268, 274, 277, 286, 289 and 292 bp) were only observed in the Gulf of Thailand.

The lowest polymorphic locus was Cc117 (Table 3.3). Only 19 alleles were observed from all investigated individuals. From total 90 individuals, PCR product sizes was ranged between 232 and 268 bp. Like Cm3, the observed alleles in the Gulf of Thailand were higher than the Andaman Sea with 18 and 16 alleles, respectively. A total number of 15 alleles were shared between both geographic samples. Four alleles were found in only specific region, 241 bp for the Andaman Sea as well as 262, 264 and 268 bp for the Gulf of Thailand. Allele distribution frequencies from Cm3, Cm72 and Cc117 for the Andaman Sea and the Gulf of Thailand *C. mydas* were compared and illustrated in Figure 3.7.

Table 3.2 Allele frequencies distribution of *C. mydas* analysed at locus Cm72 in the Andaman Sea and the Gulf of Thailand samples

Allele (base pairs)	Locus Cm72	
	the Gulf of Thailand	the Andaman Sea
229	0.011	0.000
230	0.023	0.000
239	0.000	0.024
240	0.011	0.024
242	0.000	0.024
244	0.057	0.024
246	0.034	0.037
248	0.068	0.049
249	0.000	0.012
250	0.023	0.037
251	0.011	0.012
252	0.011	0.037
254	0.034	0.024
255	0.000	0.012
256	0.045	0.012
260	0.023	0.037
262	0.000	0.012
264	0.000	0.012
266	0.000	0.012
267	0.000	0.012
268	0.023	0.000
270	0.034	0.049
272	0.057	0.049
273	0.000	0.012
274	0.023	0.000
276	0.023	0.037
277	0.011	0.000
278	0.057	0.098
280	0.102	0.049
282	0.091	0.024
284	0.023	0.073
286	0.068	0.000
288	0.023	0.085
289	0.034	0.000
290	0.000	0.024
292	0.034	0.000
293	0.000	0.012
294	0.011	0.012
296	0.000	0.037
298	0.034	0.024

Table 3.3 Allele frequencies distribution of *C. mydas* analysed at locus Cc117 in the Andaman Sea and the Gulf of Thailand samples

Allele (base pairs)	Locus Cc117	
	the Gulf of Thailand	the Andaman Sea
232	0.013	0.014
234	0.051	0.014
236	0.038	0.041
238	0.077	0.068
240	0.218	0.216
241	0.000	0.108
242	0.051	0.189
244	0.077	0.068
246	0.141	0.095
248	0.038	0.014
250	0.077	0.041
252	0.064	0.054
254	0.038	0.014
256	0.026	0.041
258	0.013	0.014
260	0.038	0.014
262	0.013	0.000
264	0.013	0.000
268	0.013	0.000

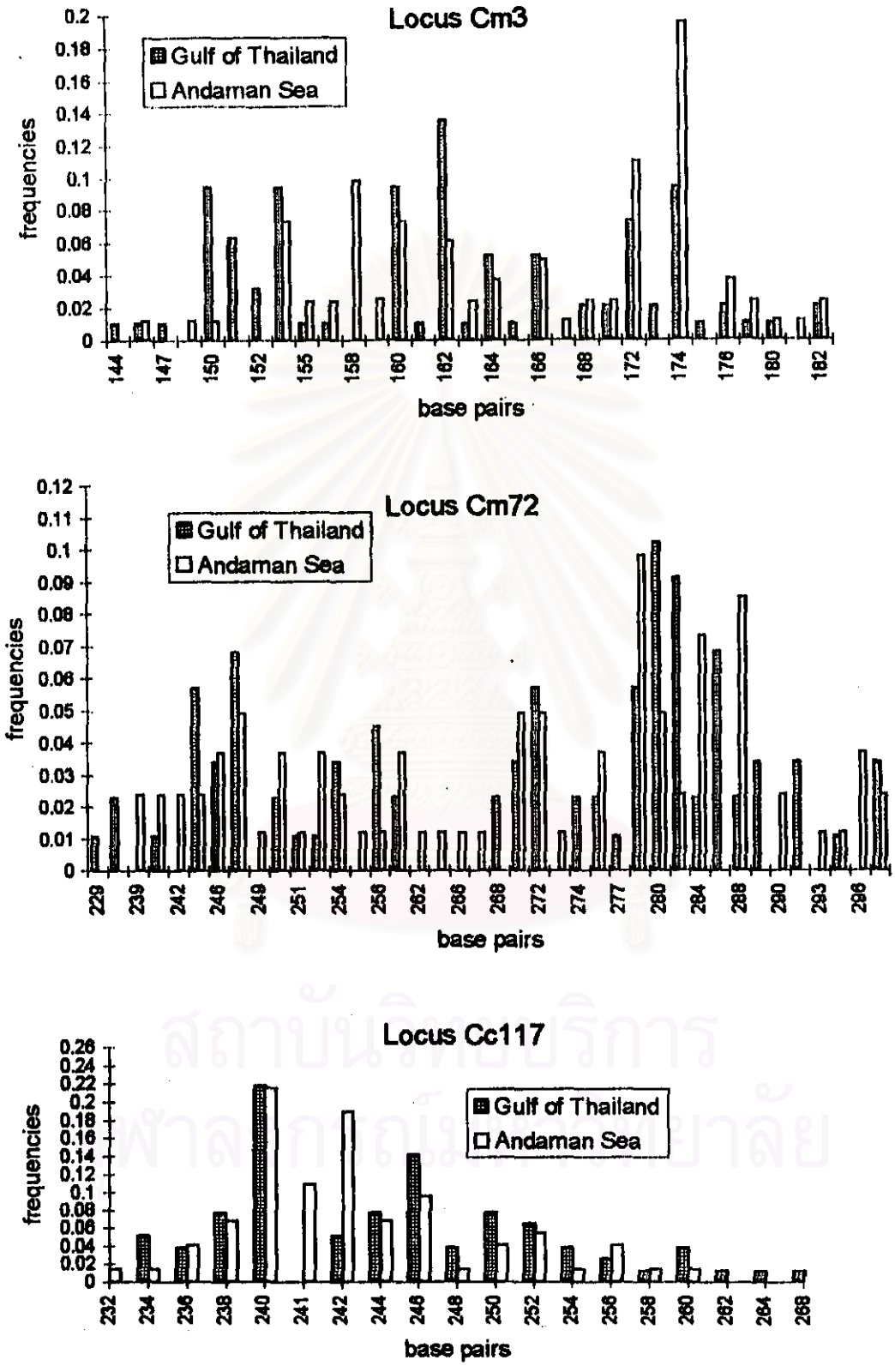

Figure 3.7 Histogram showing allele frequencies of three microsatellite loci (Cm3, Cm72 and Cc117) in two populations (the Gulf of Thailand and the Andaman Sea) of *C. mydas*

The direct count heterozygosity (H_o) and the expected heterozygosity (H_e) (calculated from data statistical analysis, chapter II) of each locus were shown in Table 3.4. Direct count heterozygosity (range = 0.74-0.87) indicated high genetic variation levels in *C. mydas*. The highest polymorphic locus found in the present study was Cm3 (H_o = 0.87, H_e = 0.93) following by Cm 72 (H_o = 0.85, H_e = 0.95) and Cc117 (H_o = 0.74, H_e = 0.89). Heterozygosity of the Gulf of Thailand samples resulted from Cm3 was slightly greater than that of the Andaman Sea samples whereas heterozygosity observed from Cm 72 was in the opposite. Cc117 gave nearly same heterozygosity between both sample area. In comparison, the observed heterozygosities were lower than the expected values (range = 0.89-0.95). An examination of the fixation indices calculated from the formula $F = (H_e-H_o)/H_e$ showed high values.

Hardy-Weinberg expectation was carried out using an exact test. As can be seen from Table 3.5, only the Andaman samples conformed such expectation for locus Cm3 whereas all others deviated from the Hardy-Weinberg equilibrium. However, the average P-values for all loci and all population were significantly deviated from the equilibrium for *C. mydas* in Thailand (highly significant P-value).

Table 3.4 Sample size (N), number of alleles per locus (A), observed heterozygosity (H_o), expected heterozygosity (H_e), fixation indices (F) at three loci for two *C. mydas* populations.

Locus	the Gulf of Thailand	the Andaman Sea	Total
N	49	41	90
Cm3			
A	26	23	31
H_o	0.88	0.83	0.87
H_e	0.93	0.92	0.93
F	-	-	0.065
Cm72			
A	28	32	40
H_o	0.77	0.90	0.85
H_e	0.95	0.95	0.95
F	-	-	0.105
Cc117			
A	18	16	19
H_o	0.73	0.71	0.74
H_e	0.90	0.88	0.89
F	-	-	0.169

Table 3.5 Hardy-Weinberg expectation of *C. mydas* from the Andaman Sea and the Gulf of Thailand samples

Locus	the Gulf of Thailand[a]	the Andaman Sea[b]
Cm3	0.0436	0.1258
Cm72	0.0000	0.0001
Cc117	0.0000	0.0002

[a] χ^2 = infinity, d.f. = 6 and p = highly significant (all loci)
[b] χ^2 = 39.2, d.f. = 6 and p = 0.0000 (all loci)

Nei's genetic distance was calculated as described in data analysis chapter II. Genetic distance between the Andaman Sea and the Gulf of Thailand was 0.2693 when determined from all loci. Overestimation of genetic distance was observed when either one or two loci were included (Table 3.6). Large genetic difference between *C. mydas* from the east and the west coast of the peninsula indicated that genetic population structure does exist in *C. mydas*.

Wright's F_{ST} values were 0.0034, 0.0047 and 0.0104 for Cm72, Cc117 and Cm3, respectively (Table 3.7). The average F_{ST} for overall loci was 0.0062 ($p = 0.0275$). The probability value for this parameter was significantly higher than zero indicated the existence of population differentiation in this taxon.

Gene flow (the number of migrants among different populations per generation, N_m) was then estimated. It was found that N_m of *C. mydas* between the Andaman Sea and the Gulf of Thailand were approximately 40 individuals per generation.

Genotypic disequilibrium was examined for all pairwise comparisons to test whether they are linked. If the linked loci will be found, it were less tedious to use only one rather than both of those to examine population structure analysis when equivalent level of discrimination power from markers are still the same. As can be seen from Table 3.8, all pairwise comparisons indicated linkage equilibrium among the three loci ($p = 0.4730$-1.0000).

Geographic heterogeneity in distribution of allele frequencies between the Andaman Sea and the Gulf of Thailand samples were illustrated by Table 3.9. Significant differences in allele frequencies were observed in *C. mydas*

Table 3.6 Genetic distance between the Andaman Sea and the Gulf of Thailand samples computed from one, two or overall loci of *C. mydas*

Locus	Genetic distance
loci Cm3, Cm72 and Cc117	0.2693
loci Cm3 and Cm72	0.3493
locus Cm3	0.3116

Table 3.7 F_{ST} and gene flow for *C. mydas* between the Andaman Sea and the Gulf of Thailand

Locus	F_{ST}	P-value
Cm3	0.0104	0.0232
Cm72	0.0034	0.1690
Cc117	0.0047	0.2105
Overall	0.0062	0.0275
Gene flow (*Nm*)	40.40	

$\chi^2 = 14.1981$, d.f. = 6

for loci Cm3 and Cm72 (p = 0.0030 and 0.0153, respectively). The remaining locus (Cc117) yielded geographic homogeneity between the two samples (p = 0.0891). However, the average for overall loci indicated highly significant geographic heterogeneity between the Andaman Sea and the Gulf of Thailand in *C. mydas* from Thailand (p = 0.0012).

Multiple paternity in C. mydas

Prior to the present study, it was not clear whether *C. mydas* was either mono- or polygamous. To verify the mating system of *C. mydas*, nine newly hatching *C. mydas* individuals (three of these were non-relative control and the remaining were the same clutch corrected from the Andaman Sea) were analysed for their genotypes for Cm 3, Cm72 and Cc117 loci. For locus Cm3, all related individuals showed 179/179 bp alleles while three non-relative specimens had 150/154, 158/160 and 158/170 genotypes (Fig 3.9 and Table 3.10). It can be then concluded that both parents for the investigated *C. mydas* are homozygous at this locus.

When analysed these nine samples with locus Cm72 (Figure 3.10 and Table 3.10), it was able to concluded that the female parent occupied genotype 238/276 whereas three different alleles were contributed from males. Three unrelated samples showed 272/272, 258/260 and 248/272 genotypes. At this stage, it was clear that female *C. mydas* were multiple-copulated but the number of males could be three (if all of them were homozygous) or two (if one of them were heterozygous) individuals.

Table 3.8 Pairwise comparisons of genotypic disequilibrium between Cm3, Cm72 and Cc117 loci observed in *C. mydas*

Locus pair	P-value
Cm3 & Cm72[a]	1.00000
Cm3 & Cc117[b]	1.00000
Cm72 & Cc117[c]	0.47303

[a] $\chi^2 = 0.000$, d.f. = 4
[b] $\chi^2 = 0.000$, d.f. = 4
[c] $\chi^2 = 3.532$, d.f. = 4

Table 3.9 Geographic heterogeneity test using a Monte Carlo simulation for 10,000 times

Locus	P-value
Cm3	0.0030±0.0005
Cm72	0.0153±0.0012
Cc117	0.0891±0.0028
overall [a]	0.0012

[a] The average geographic heterogeneity for overall loci was examined using the exact test implemented in Genepop version2

Using the genotypes of six newly hatching individuals obtained from locus Cc117 (Figure 3.11 and Table 3.10), the inferred genotype for the female parent at this locus was 242/252 whereas three alleles (240, 242 and 258 bp) were contributed by males. Genotypes observed from three non-relative control were 234/240, 242/242 and 242/242.

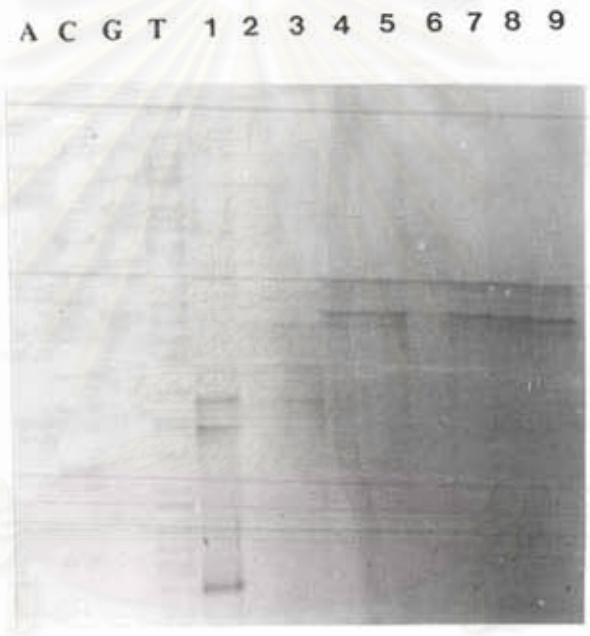

Figure 3.8 A 6% silver-stained denaturing polyacrylamide gel of microsatellite products of locus Cm3 used to determine multiple-paternity in *C. mydas*

lane A, C, G and T: sequencing ladder amplified from pGEM-3Zf (+)

lane 1-3 : three non-relative control *C. mydas*

lane 4-9 : six related juveniles *C. mydas*

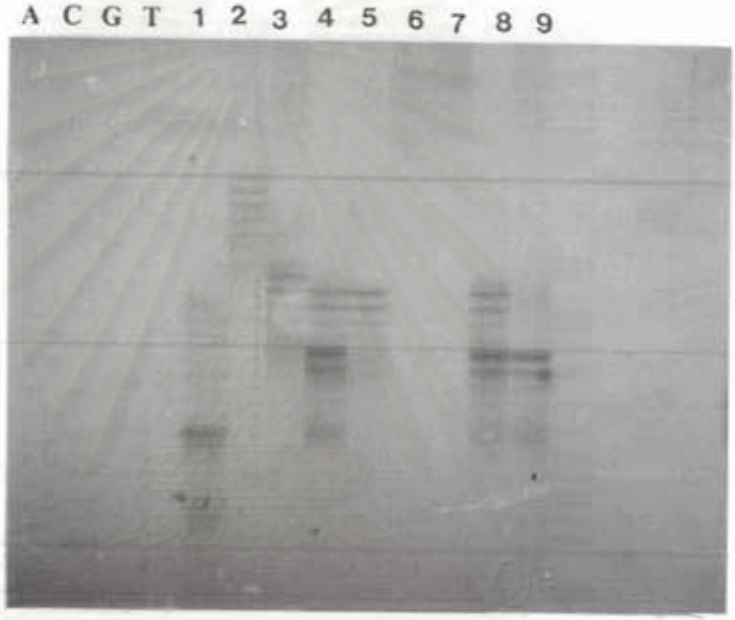

Figure 3.9 A 6% silver-stained denaturing polyacrylamide gel of microsatellite products of locus Cm72 used to determine multiple-paternity in *C. mydas*

lane A, C, G and T: sequencing ladder amplified from pGEM-3Zf (+)

lane 1-3 : three non-relative control *C. mydas*

lane 4-9 : six related juveniles *C. mydas*

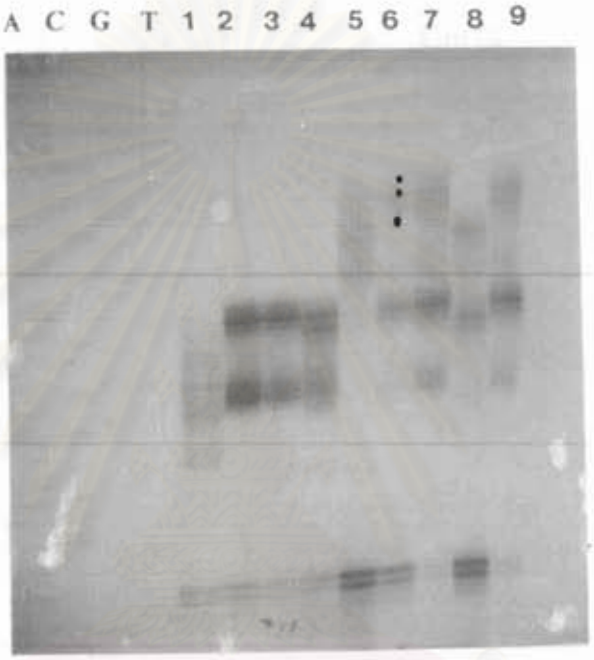

Figure 3.10 A 6% silver-stained denaturing polyacrylamide gel of microsatellite products of locus Cc117 used to determine multiple-paternity in *C. mydas*

lane A, C, G and T: sequencing ladder amplified from pGEM-3Zf (+)

lane 1-3 : three non-relative control *C. mydas*

lane 4-9 : six related juveniles *C. mydas*

Table 3.10 Determination of genotypes from three non-relative and six relative newly hatching *C. mydas* individuals using three microsatellite loci (Cm3, Cm72 and Cc117)

Individual No.	Genotype		
	Cm3	Cm72	Cc117
Non-relative control 1	150/154	226/230	234/240
Non-relative control 2	158/160	258/260	242/242
Non-relative control 3	158/170	240/272	242/242
Individual 1	179/179	230/238	240/242
Individual 2	179/179	238/292	252/258
Individual 3	179/179	276/292	242/242
Individual 4	179/179	276/292	242/242
Individual 5	179/179	230/238	240/252
Individual 6	179/179	230/276	242/242