Chapter IV

Discussion

There are only two marine stations in Thailand that carry out conservation and enhancement programmes for *C. mydas*. Basically, fertilised eggs from breeding grounds were collected to the stations. Newly hatching *C. mydas* were nursed there until three to five months old. The total number of the juveniles maintained at Sattahip were approximately 6,000 individuals a year (Sea Turtle Conservation Center of the Royal Thai Navy, unpublished data) whereas the total juvenile *C. mydas* individuals nursed at Phangnga were about 3,000 (Sea Turtle Conservation Center of the Royal Thai Navy, unpublished data). Practically, more than one specimens from the same clutch may be sampling and used in this experiment. To minimise this sampling error, the juveniles used for genetic studies were obtained from proper records for the collection date and the original places. All juveniles used in the experiment were then obtained from individuals dying during their nursing and usually transported to the laboratory on ice.

The annealing temperature reported by FiztSimmons *et al.* (1995) for all loci (Cm3, Cm72 and Cc117) was 55° C. This was much lower than the suitable annealing temperature for PCR described by Hoelzel and Green (1992). As showed in Table 2.1, T_m of the primer locus Cm72 was 66 and 68° C as well as for locus Cc117. Theoretically, the optimal annealing temperature was much lower than the suitable annealing temperature ranges from 3° C to

12° C below T_m of the primers (Koreth *et al.*, 1996). Moreover, twenty rather than twenty-four nucleotides were used to amplified the microsatellite locus Cm3 in this study. The use of originally published conditions provided inconsistent results indicated a need to optimise the amplification condition in this study.

Based on the fact that the concentration of Mg^{2+} required in the PCR reaction was not reported in FitzSimmons *et al.* (1995), a series of Mg^{2+} concentration was then optimised and found that 4.0, 3.0 and 2.5 mM were optimum for Cm3, Cm72 and Cc117, respectively.

Visualisation of microsatellite bands usually employs the endlabelled primer by the radioisotope ($\gamma^{33}P$, $\gamma^{32}P$ or ^{35}S-NTP). The sensitivity to detect microsatellite bands on polyacrylamide gel was extremely high. However, radioisotopes are hazardous consequently, care must be taken to handle this chemical. The other disadvantage of radioisotopes is due to their stability as the half-life of ^{32}P and ^{35}S are 14.3 and 68 days so utilisation should be limited by short time. Furthermore, the waste need to be kept in the laboratory and subsequently disposed properly. In contrast, the use of silver staining is much more convenient because no hazardous substances are involved in the protocol even though the sensitivity to detect DNA fragments by this method is lower than radioisotope approach (Caetamo-Anolles, 1994). All chemical components are reasonably stable for example, a commercially available sequencing kit is recommended by the manufacturer to have at least 2 years shelf-life.

Basically, amplification of microsatellite loci using homologous primers (Cm3 and Cm72) was much more successful than the use of heterologous primers (i.e.Cc117). Microsatellite Cm3 was the easiest locus to amplify. The amplified products of this locus were usually much more intense than those from another loci when the same concentration of template are used. This probably due to its smallest size (144 - 182 bp) in comparison to the others (229 - 298 bp for Cm72 and 232 - 268 bp for Cc117 loci). Generally, the intensity of amplification products from Cc117 was slightly less intense than that from Cm72. The results on the weaker signal on the gel of PCR product from loci Cm72 and Cc117 is similar to those previously reported by FitzSimmons *et al.* (1995).

The ranges of allele sizes for all investigated loci of *C. mydas* from Thailand were comparable to those from the Australia. However, it is interesting, if possible, to collect the samples from the Australia and the Indo-Pacific regions for comparison of population structure and gene flow levels at macrogeographic scale.

It should be noted that an extra amplification fragment was always observed for locus Cc117 of almost all of the investigated individuals indicated that heterologous primers could anneal to and amplify another nonspecific sites. Scoring of microsatellite data is not possible if the nonspecific co-amplification product(s) is polymorphic. Nevertheless, the fragment co-amplified in the present study was monomorphic and identical in allele size so that it did not interfere the ability to examine the actual microsatellite alleles. Moreover, the

use of heterologous primers may not be appropriate at population level because homoplasy may be occurred and violated the estimation of population genetic parameters. Alternatively, such primers may be useful for pedigree analysis (FitzSimmons *et al.*, 1995 and Quattro and Weissman, 1997).

While Cm58 did not yield polymorphic results, Cm84 gave very large amplification products (approximately 300 to 400 bp). Extremely faint bands were observed in nearly forty per cent of investigated individuals (35 specimens) so that unambiguous results were not possible to deduce from this locus leading to exclusion of this from further analysis.

Considering the number of alleles found, Cm72 was the most polymorphic (40 alleles), following by Cm3 (31 alleles) and Cc117 (19 alleles), respectively. Population specific alleles were found for all three loci used. Twenty alleles of the locus Cm72 found from *C. mydas* were population-specific and very low frequencies (8 alleles for the Gulf of Thailand and 12 alleles for the Andaman Sea). Lower population specific alleles were observed in both Cm3 and Cc117. For Cm3, a total of thirteen alleles was available in only one population (8 alleles for the Gulf of Thailand and 5 alleles for the Andaman Sea). The lowest number of population specific alleles was found in Cc117 (4 alleles composed of 1 allele for the Andaman Sea and 3 alleles for the Gulf of Thailand).

The numbers of microsatellite alleles for Cm3, Cm72 and Cc117 analysed from *C. mydas* in Thailand were higher than the Australian *C. mydas* (45, 34 and 19 alleles in the Thai samples in comparison to 25, 21 and 17

alleles in *C. mydas* from Australian). The reason for this was that the number of investigated *C. mydas* used in this study was roughly 2.5 times greater than that in FitzSimmons *et al.* (1995).

Microsatellite locus Cm3, Cm72 and Cc117 are complete dinucleotides microsatellite (Table 2.1). Theoretically, the alleles size must be differed by 2 bases addition or deletion. However, the size of alleles obtained from microsatellite locus Cm72 (Table 3.2) are found to be increased or decreased for only one base. This kind of evidence are found in some microsatellite loci of many organisms such as *Botryllus schlosseri* (Stoner, Quattro and Weissman, 1997); honey bee *Apis mellifera* (Estoup *et al.*, 1995); grape *Vitis vinifera* (Bowers *et al.*, 1996) and even shrimp *Penaeus vannamei* (Wolfus *et al.*, 1997). There were 3 factors resulted in this appearance. Firstly, those polymorphic alleles were occurred by single-step mutation ($\pm$ 1 bp) in dinucleotides microsatellite DNA. The clear evidence was supported in *Botryllus schlosseri*. In addition, it has been hypothesized this mutation found in human microsatellite DNA (Stoner *et al.*, 1997). Secondly, deletions or insertions of the flanking regions was resulted in allelic diversity, consequently, homoplasy (e.g. convergence) (Hoelzel and Dover, 1991). Finally, slippage during *in vivo* DNA replication can yields a single nucleotide spacing for dinucleotides microsatellite (Schlotterer and Tautz, 1991).

Statistically significant deviation from Hardy-Weinberg were observed for the average P-value of all loci for both populations. Several biological parameters of *C. mydas* are not in agreement with basic assumptions of

Hardy-Weinberg test. These include extensive migration of the female over vast geographic areas, gender biased of *C. mydas* which is depended on incubation temperature of *C. mydas* eggs during their development. Significant deviation from Hardy-Weinberg expectation was revealed in several previous publication in marine organisms such as Turkish brown trout *Salmo trutta* (Arias *et al.*, 1995; Largiader *et al.*, 1995 and Togan *et al.*, 1995), tusk *Brosme brosmea* (Johansen *et al.*, 1995) and recently in whiting *Merlangius merlangus* (Rico *et al.*, 1997).

Because of deviation from Hardy-Weinberg expectation, direct count heterozygosity was used in estimation. The overall heterozygosity of each locus in the present study were lower than those of *C. mydas* from the Australia (0.93 compared to 0.87 for Cm3, 0.90 compared to 0.85 for Cm72 and 0.84 compared to 0.74 for Cc117). As previously discussed, overestimate of heterozygosity may be resulted from much smaller sample sizes (approximatly 40 individuals) used by FitzSimmons *et al.* (1995). The high heterozygosity in this study can be implied that *C mydas* in Thailand does not exist critical endangered species. However, extremely low genetic variation levels analysed by allozyme have been reported for several rare and endangered species. Though normal level of heterozygosity was also observed in several endangered species (Avise, 1994).

Historically, the northern elephant seal, *Mirounga augustirostris*, was believed to pass the bottleneck effect. Fewer than thirty individuals may have survived through the late nineteen century. Analysis of genetic variability in

159 *M. augustirotris* individuals using 24 allozyme loci indicated no genetic variation in this species. An increasing of allozyme loci to fifty-five (Hoelzel *et al.*, 1993 cited in Avise, 1994) did not provide any genetic variability. On the other hand, analysis of genetic variation in the southern elephant seal *M. leonina* displayed normal heterozygosity level (Bonnell and Selander, 1974 cited in Avise, 1994).

Heterozygosity in endangered species evaluated by microsatellite has been reported. High heterozygosities were also observed in the endangered coastal cutthroat trout *Oncorhynchus clarki*. Observed heterozygisities in this species were 0.26 - 0.97 (Wenburg, Olsen and Bentzen, 1996). Analysis of genetic variation in a West African population of *Anopheles gambiae* using allozyme analysis indicated low genetic diversity (the mean heterozygosity was 0.097) whereas much higher genetic variation level was observed from 3 microsatellite loci analysis (the average heterozygosity was 0.732) (Lanzaro *et al.*, 1995). This circumstance indicated the potential use of microsatellites to evaluate genetic variation level in conspecific population or congeneric species which low levels of genetic diversity were found from other markers. Several researches revealed high sensitivity of microsatellites in analysis of genetic variability of various species (Carvalho and Hauser, 1994; Lanzaro *et al.*, 1995; Ruzzante *et al.*, 1996 and Patton *et al.*, 1997).

Karl *et al.* (1992) investigated genetic diversity in *C. mydas* using five single copy nuclear DNA (CM-12, CM-14, CM-39, CM-45 and CM-67). Mean heterozygosity per population ranged from 0.44-0.77 with the overall average

of 0.30 $\pm$ 0.09 (SE). It was then concluded that inbreeding in this species, if any, was beyond the resolution of scnDNA polymorphism. On the basis of high level heterozygosity found from microsatellite studies, significant inbreeding should not be occurred.

Nei's genetic distance of *C. mydas* originating from the Andaman Sea and the Gulf of Thailand based on microsatellite DNA was 0.2693. This indicated that the former and the latter have a large genetic distance and should be regarded as a different stock. At the time of writing this thesis, it is believed that there have been no publications on population structure and genetic level using any molecular markers of *C. mydas* in Thailand. From direct sequencing of PCR-amplified control region in the mtDNA, the sequence divergence (genetic distance) of 0.044 was found in *C. mydas* sampled from the Atlantic and the Pacific Oceans (Encalada *et al.*, 1996). Karl *et al.* (1992) examined genetic distance among *C. mydas* macrogeographically collected from 15 *C. mydas* rookeries in the Atlantic-Mediterranean and Indian-Pacific areas using scnDNA polymorphism. A UPGMA dendrogram allocated *C. mydas* into two separate groups with a Nei's genetic distance of 0.13. Differences in levels of genetic distance of *C. mydas* are from the nature of different approaches but all previous publications and this study indicated high genetic distance among geographically separated populations implying the existence of population sub-division in this species.

The average F_{ST} - overall investigated loci in *C. mydas* was 0.0062 (0.0034-0.0104) which was extremely low compared to the humpback whale,

Megaptera novaeangliae which is well known to be the great voyager over vast geographic areas (Valsecchi *et al.*, 1997). The F_{ST} values (ranged between -0.006 - 0.048) estimated from four microsatellite loci of *M. novaeangliae* collected from four major oceanic areas (the north Atlantic, the north Pacific and two widely separated Antarctic samples) indicated that population structure of *M. novaeangliae* was greater than that of *C. mydas*. However, this conclusion should be treated with caution because the sampling areas of *M. novaeangliae* were in macrogeographic scale whereas those of *C. mydas* were significantly narrower.

Gene flow level inferred from microsatellite loci was high (40 individuals) indicated that *C. mydas* is a high gene flow species. However, this gene flow level is still not able to homogenise population differentiation between the Andaman Sea and the Gulf of Thailand as indicated by high difference in genetic distance between such areas. The result from this study is surprisingly contradictory to previously publication reported by Bowen *et al.* (1992) who surveyed mtDNA-RFLP from *C. mydas* rookeries around the world. It was found that a fundamental phylogenetic split disassociated all specimens in the Atlantic-Mediterranean from those in the Indo-Pacific region. Genetic substructure was evidenced by fixed or nearly fixed haplotypic differences among most rookeries leading to a conclusion that female *C. mydas* has a strong behaviour for natal homing. Allard *et al.* (1994) found a strong support for natal homing of female *C. mydas* using sequencing of mtDNA control region amplified from single representatives of each rookery (N = 39).

The restricted female gene flow was estimated to be 0.7-0.9 migrants per generation indicating that *C. mydas* experienced restricted level of gene flow. Moreover, it was also indicated population differentiation in a highly mobile species based on geographic constraints an a philopartic reproductive behaviour through a natal homing within oceans.

At this stage, the contradictory results on intraspecific gene flow in *C. mydas* inferred from microsatellite loci and mtDNA polymorphism (both RFLP and sequencing of the control region) may be resulted from a great contribution of male mediated gene flow.

Nevertheless, analysis of five polymorphic scnDNA loci using RFLP approach leaded to a conclusion of restricted gene flow (1.3 individuals per generation) in this species. The result strongly evidenced minor contribution of male mediated gene flow.

Further evidence to support limited male gene flow was reported by FitzSimmons *et al.* (1997) who sampling 82 breeding males from their three courtship areas and a total 174 female *C. mydas* individuals from the same locations. A 385 bp fragment amplified from the mtDNA control region followed by restriction analysis with *Hph* I and *Mae* I. The main objective of that experiment was to examine male natal homing in *C. mydas* on the basic assumption that if natal homing in males does exist, the analysed mtDNA patterns of breeding males at the courtship should be similar to those of the female from the same location. Similarity of composite haplotypes of *C. mydas* male within the same population should be greater than between populations.

F_{ST} was calculated separately between male and female within the same population. It was found that the haplotype frequencies of male and female were not significant. The average F_{ST} of male and female *C. mydas* were extremely high (F_{ST} = 0.78 and 0.73 for male and female, respectively) indicated the strong evidence on male philopatrically natal homing and restricted male mediated gene flow ($Nm < 1$). Therefore large differences between level of gene flow estimated from microsatellite and other techniques in *C. mydas* should be from the nature of microsatellite *per se.*

Theoretically, high mutation microsatellite loci may not be suitable for evaluation of gene flow because the island model of gene flow assumes the correlation between F_{ST} values and gene flow level if migration rate is much less than 1 and mutation rate is much lower than migration rate (Ward and Grewe, 1994). With this reason, microsatellites may not be appropriate for evaluation of some population genetic parameters.

The other reason to explain the high gene flow level in *C. mydas* is man-made disturbance between the two different gene pools of *C. mydas* in Thailand. Transplantation of juveniles from unknown origin of the parents may alternatively response for high gene flow estimated from the present study.

The Monte Carlo simulation indicated statistically significant different in allele frequency distribution between different geographic areas when Cm3 and Cm72 were analysed. The average geographic heterogeneity for overall loci also indicated population genetic structure in this species (P = 0.0012). Although a Cc117 locus were successfully amplified in *C. mydas*, it did not

show significant in allele frequency between the Andaman Sea and the Gulf of Thailand *C. mydas*.

Prior to sampling of died juvenile *C. mydas*, blood from 20 adult *C. mydas* were collected (13 individuals from the Gulf of Thailand and 7 individuals from the Andaman Sea). Because native place of individuals were unknown, furthermore, the number of sample was small. Thus *C. mydas* sampling trended to collect juvenile instead because large collected sample size was possible. In addition, place of birth can be known. But, it was difficult (or more specifically, it was not allowed) to collect blood from juveniles. Furthermore, marine turtles have been announced to be endangered species so that killing of *C. mydas* is illegal. As a result, died juvenile *C. mydas* were collected to used in this study as described in chapter II. However, 20 adult *C. mydas* were also used to determine genetic variation. Like juvenile, blood from adult *C. mydas* was extracted by proteinase K/phenol method. Comparison to soft tissue, DNA quality obtained from blood was better than that of because almost obtained DNA ralely were high molecular weight. After amplified by three primers (Cm3, Cm72 and Cc117), all microsatellite product PCR were also determined alleleic sizes by 6% sequencing acrylamide gel electrophoresis. Genotypes from 20 individuals were detected and then statistically calculated like those in juvenile. Because of small sample sizes, the erroneous results were obtained and completely contrasted to juveniles (See appendix II).

The basis knowledge on genetic population structure of *C. mydas* found in this study provides the data that essential for setting the appropriate conservation programme in this species. The government organisation should recognise that *C. mydas* originating from the Andaman Sea and the Gulf of Thailand are genetically difference stocks (genetic distance = 0.2693). Subsequently, the former conservation method should be changed. Basically, fertilised eggs have taken to the conservation stations without considering of their geographic origin. The nursed *C. mydas* juveniles were also released into the natural environment without the proper record therefore man-made disturbance of *C. mydas* gene pools may occur. Accordingly, only local individuals should be released for stock enhancement programme avoiding unnecessary mixing of the Andaman Sea and the Gulf of Thailand stocks. Based on the fact that genetic distance between two *C. mydas* stocks is extremely large, it will be wise to perform further studies on genetic population structure of proximate populations of the same coastal sites. If population differentiation is available at microgeographic scale for the highly disperse species such as *C. mydas*, the origin of collected eggs from the same coastal site should be further divided into several subpopulations on the basis of genetic data. Capture and recapture method (coupled with appropriate tagging methods) should be carried out to determine the actual census population size in *C. mydas*. Effective population size of *C. mydas* can be evaluated if the basic data on mutation rate of microsatellite loci used is available. Actually, collecting of the *C. mydas* eggs for consuming and killing of the adults are

illegal in Thailand. However, this law does not work properly in practice hence every organisation contributed in conservation of *C. mydas* should implement the regulation that practically work well as soon as possible.

In this study, all three microsatellite loci were utilised to investigate mating system of *C. mydas*. Prior to this thesis, there have been no formal reports on reproductive pattern of *C. mydas* to verify whether it is mono- or polygamous. This answer of this question was accidentally discovered as six newly hatching related *C. mydas* were primarily analysed to infer the genotype of their parents. Surprisingly, the results obtained from all three loci strongly supported multiple mating of females. Due to a limitation of *C. mydas* offspring used in the experiment, it is needed to carry out this experiment again which a larger number of related individuals to ensure that this basic behaviour is real rather than artifacts from the experiment. Harry and Briscoe, 1988 used allozyme marker including *Pgm, Mdh-C* and *Idh-C* gave strong evidence for multiple paternity in *C. caretta*. The use of microsatellite markers for determination of multiple paternity (and mating behaviour) in marine is much more sensitive than does the allozyme markers. This open a new approach for scientists in the field of biological behaviour.

Microsatellite loci are not only useful for population structure analysis but also important for determination of individuality and parentage as well as kinship analysis for various species. Appropriate loci should be selected based on a particular problem. For instance, loci with a few alleles (5-10 polymorphic alleles) may be useful for population genetic studies

whereas highly polymorphic loci with large numbers of alleles (usually heterozygosity higher than 0.7) can be used for pedigree analysis and/or markers linked to quantitative trait loci (e.g. disease resistance markers) in the genome project (Wright and Bentzen, 1994).