CHAPTER IV

Discussion

Data on genetic diversity levels and population differentiation within any exploited species are basically important and help deciding whether such a species should be conserved or exploited. The first and necessary step is to identify that an interested species are panmictic (large single group) or reproductively isolated (the existence of several stocks). Homogeneous exploitation of a fisheries composed of unidentified different breeding populations can either destroy the population structure of locally exploited stocks or more seriously, cause the permanent extinction of the portion of the resources (Ovenden, 1990).

If population differentiation does exist, each stock within a species must be managed separately. Therefore, less abundance stocks should be protected and overexploitation of these must be avoided (Carvalho and Hauser, 1994).

The low levels of genetic polymorphism at both inter- and intraspecific levels in decapod crustaceans have been reported based on allozyme electrophoresis. This indicated by extremely low level of the number of allele per locus and heterozygotic levels (Hedgecock, 1987). The allozyme-based genetic variation and differentiation of tropical and eastern penaeid shrimp species including *Metapenaeus ensis*, *M. endeavouri*, *M. bennettae*, *M. macleayi*, *Penaeus latisulcatus*, *P. semisulcatus*, *P. esculentus*, *P. merguiensis*, *P. plebegus*, were extensively investigated. Intraspecific genetic differentiation was observed in *M. bennettae*, *M. macleayi*, *M. endeavouri*, *P.latisulcatus* but not in the remaining species (Mulley and Latter, 1981 a and 1981b).

The caramote prawn (*P. kerathurus*) is distributed along the Mediterranean coasts and is a candidate species for aquaculture. An electrophoretic survey for 18 loci of different geographic samples from Italy, Tunisia, France and Spain revealed low degree of heterozygosity (H_e = 0.046 - 0.095). Lack of population differentiation was observed in all Mediterranean populations of *P. kerathurus* (Mattoccia et al., 1987).

The existence of geographic heterogeneity of *P. monodon* was reported by Benzie et al.(1992). The direct count heterozygosity inferred from eletrophoretical allozyme analysis was between 0.046 - 0.103. Nevertheless, the average Nei's genetic distances between seven *P. monodon* samples was 0.000 - 0.015. Notably, there were no differences of genetic distances among pairs of the east *P. monodon* samples (d = 0.000 or 0.001). Nevertheless, population differentiation was observed between major coastal regions (west with both north and east, $P<0.001$).

Genetic variation and differentiation in Thai *P. monodon* was initial established by Sodsuk et al. (1992) based on allozymes. Subsequently, mtDNA-RFLP was further carried out in three geographic samples (Satun, Surat and Trat) of *P. monodon* and indicated strong genetic discontinuity between the Andaman and Gulf of Thailand *P. monodon* (Klinbunga, 1996 ; Klinbunga et al., 1999).

RAPD analysis has been increasingly used for determination of intraspecific population differentiation and identification of genetic markers at different taxonomic levels in marine organisms. This is possible due mainly to its flexibility and less time consuming (Williams et al., 1990). Based on the fact that arbitrary sequences of oligonucleotides (usually 10 - 12 mers) are required for this technique, the most suitable RAPD primers for a particular application can be selected from unlimited number of arbitrary oligonucleotides (Weising et al., 1995).

Garcia et al. (1994) used three molecular genetic techniques; RFLP of cytochrome oxidase subunit I (COI), RAPD and allozymes, to investigate genetic diversity of their cultured *P. vannamei*. Six informative RAPD primers (OPA9, OPA10, OPA20, OPB11, OPB14 and OPB20) generated 73 scored fragments. The percentage of polymorphic band in representative population 1 (families 1.5 and 1.6) was 55% which was greater than that in population 2 (48%). The highest percentage of polymorphic bands was 77% observed in population 4.

Tassanakajon et al. (1997 and 1998) demonstrated the potential use of RAPD for determination of genetic structure in *P. monodon*. Large genetic differences between *P. monodon* from the Andaman Sea and Gulf of Thailand were observed ($P < 0.0001$). Within each region, the homogeneous stock was observed in the west coast ($P > 0.05$) whereas Trat and Chumphon are reproductively isolated from each other ($P < 0.05$). The results indicated reduced genetic diversity in the Angsila sample reflecting the possibility of inbreeding in this geographic sample.

This thesis used samples originating from the same locales as did Tassanakajon et al., (1998) but collected at different time (Satun, Trang and Trat) therefore temporal genetic effects can be compared between experiments.

Generally, calculation of RAPD data for population genetic studies can be performed by regarding genotypes (or more specifically characters of genotypes) in either combined or composite approaches.

For the former, genotypes of an individual generated from a set of primers are considered as unlinked and independent between each primer. The results (similarity and/or distance) are calculated from each primer before combined and are averaged by the number of primers used in the experiment. This scoring method is particular suitable for primers giving significantly different number of scorable bands

and more importantly, for primers generated fingerprinting patterns rather than those allocate individuals to groups.

On the other hand, the latter regards relationships of genotypes generated between different RAPD primers. Therefore, this approach is particularly suitable for RAPD primers providing similar number of RAPD fragment, showing dependence of patterns between different primers and for primers pooling individuals into groups.

Practically, repeatability of a RAPD pattern from the same primer and the ability to analyzed DNA from each individual by all investigated primers (with consistent and reliable results) are momentous and should not be overlooked when RAPD was used.

Nevertheless, data from this thesis failed to follow that criterion because the member of individual successfully analysed by each primer varied enormously (89, 100 and 136 for primer UBC268, UBC273 and UBC299, resspectively). This unfortunately indicated lower degree of reproducibility of the experiments. Due to the limiting of time, this problem was not should by repeating of RAPD-PCR resulting in sampling error effects (large level of variance of results caused from missing data). Therefore, more number of specimen from the same location should be included in the experiment. Almost all of the specimens should be successfully examined across all primers.**Which such problem, level of genetic diversity reported by this thesis should be regarded as "a guide" rather than an accurate estimate.** Nevertheless, geographic heterogeneity analysis can be carried out separately for each primer ; as the result, population differentiation of *P. monodon* in Thailand did not largely violate by this test. The results from this thesis are in consonant to previous population concerning relevance topic in this species (Pongsomboon, 1996; Tassanakajon et al., 1997; Klinbunga et al., 1998; Supungul., 1998; Tassanakajon et al., 1998; Klinbunga et al., 1999).

Theoretically, mutation inhibiting binding of the primer or alternatively, interfering with amplification can be detected as the absence of the band in individuals. Similarily indices (or distances) can be calculated from the presence / absence of RAPD bands across individuals (Bowditch et al., 1993).

All investigated primers (UBC268, UBC273 and UBC299) yields similar number of scored fragments (17 -18 fragment). The size distribution of amplified RAPD fragment was comparable allowing the use of agarose gels at the same concentration. Considering the polymorphic levels, high polymorphic bands were found in all primers (approximately 80%) but this estimate cannot be directly compared between experiments as it is sensitive to different sample sizes.

Generally, the number of amplified bands per primer in this experiment was greater than those from Tassanakajon et al. (1998). For instance, the amplified bands from the primer UBC268 in that publication were 9 fragments for which 8 bands were polymorphic. In contrast, this experiment showed that 18 RAPD bands (14 polymorphic fragments) from the same primers were observed. The explanation for this circumstance was probably due to the use of different thermocyclers because the critical time switching from denaturation to annealing for a Perkin Elmer 2400 used in their experiment and that of an Omnigene Hybaid system used in this experiment might be drastically different.

The explanatory relationships between common genotypes for each of all investigated primers based on loss and gain of amplification RAPD fragments minimising errors from misscoring of the RAPD results in this study. Disregarding differences in sample sizes, the average number of genotypes per primer observed in this study was 26 genotypes which were significantly lower than 36 genotypes described in Tassanakajon et al (1998). Nevertheless, both studies illustrated comparable level of genetic diversity of *P. monodon* in Thailand.

The large number of unique genotypes generated by the UBC268 indicated high diversity within Trat compared to others. Additionally, the primer UBC299 generated 8 genotypes in Trat and four of which (accounting for 44% of this geographic sample) were not observed in the remaining samples. Both evidences implied that this *P. monodon* sample should be dissociated from the rest.

Geographically specific markers (at populations, regions or subspecies) are useful to trace the origin of suspected organisms under investigation. Moreover these markers can be used to compare the performance of commercially important phenotypes of *P. monodon* from different stocks, for example to verify whether progeny of breeding females from different stocks exhibit different survival and/or growth rates when reared in a large communal condition.

Garcia and Benzie (1995) determined RAPD patterns in six families of *P. monodon* using 14 RAPD primers and found three polymorphic markers that may be used as marker-assisted selection in this species. However, those markers seemed to be family-specific implying the need to identify other useful markers in selective breeding programme. Additionorlly, Garcia et al. (1994) found a population-specific marker in all individuals of population 2 of domesticated *P. vannamei* using the primer OPB20. However, it was not surprised to find this marker in that population because it was established from the cultured adult stock in Ecuador originally resulted from possibly 3 individuals of parents (Wolfus et al., 1997).

The population-specific fragment generated by the OPB20 was cloned into PCR Script vector and cycle-sequenced. The result showed that a 1259 bp clone contained two perfect microsatellites; CTTT and GAA which can be used to determine genetic polymorphisms of the cultured *P. vannamei* stocks (Garcia et al., 1996). This result indicated that RAPD can generate useful DNA markers for breeding programmes in penaeid shrimps.

Likewise, a population specific marker in wild *P. monodon* was also reported. A 950 bp RAPD fragment was found in over 95% of the Andaman *P. monodon* but was absent in *P. monodon* originating from the Gulf of Thailand. This maker also completely discriminated *P. monodon* originating from Medan from that of Thailand (Tassanakajon et al., 1997). As described previously, this genetic marker can be used to follow the performance on economically important traits of *P. monodon* from the Andaman Sea and the Gulf of Thailand.

The primer UBC268 used in the present study generated a fragment of 260 bp found in 90.9% of *P. monodon* from Trat and 8% of *P. monodon* from Chumphon. This marker can be employed in combination with a 950 bp marker generated from the UBC428 to assist the comparison of various phenotypes of regional different *P. monodon*.

On the basic of this study, between geographic sample similarity was basically lower than within sample similarity implying degrees of population subdivision in Thai *P. monodon*. A clearer conclusion can be drawn from genetic distances between compared samples. The anomalous Chumphon sample surprisingly showed closed genetic relationships to the Andaman samples. Although level of genetic distances among samples from this and Tassanakajon et. al. (1998) studies can not be directly compared (due to the large experimental error in this study), the same surprising status was observed.

In random mating organisms, RAPD is useful for determination of intraspecific genetic relationships of various species. The RAPD genetic distance based solely from band sharing coefficiency seems to be acceptable for construction of intraspecific phylogeny. However, it should be noted that the used of RAPD analysis for inferring of phylogenetic relationships at interspecific levels should be limited to extremely closely related organisms. The reason is that a particular RAPD

band (at certain molecular weight) is considered as a character for systematic studies. The presence or absence of such a fragment reflects the character state of the informative band. While the band presence showed the successfully amplified sequence of a specific length, the absence of such an amplified band is unfortunately resulted from several phenomena constituting of point mutations at the priming site (s), inversion, different secondary structure of the template, therefore, the likelihood of transitions between absence or presence of the bands are asymmetrical (Bowditch et al., 1993).

Moreover, comigrating RAPD bands analysed by gel electrophoresis might not be actually homologous across all investigated specimens(Weising et al.,1995). This situation seems to be worse when comparing distantly related taxa as the homology between comigrating bands is questionable. Accordingly, RAPD should not be used for phylogenetic and systematic studies at higher taxonomic levels (Bowditch et al., 1993).

A UPGMA dendrogram constructed from genetic distance converted from Nei's band sharing coefficiency indicated two genetically isolated groups. While the Trat sample was placed to the tree as a separated group (group A), all of the Gulf of Thailand samples were regarded as an another group (group B). The problematic Chumphon was clustered with Phangnga (the Gulf of Thailand). The topology of this phenogram are generally in agreement with that reported by Supungul (1998) who evaluated the genetic status of *P. monodon* using the same sample set of the present study by three microsatellite loci (CUPmo18, Di25 and Di27). The observed heterozygosity was between 0.66 - 0.80. Differences in allele frequencies between Trat and Chumphon were statistically significant at CUPmo18 and Di25 loci. All the Andaman samples were grouped by the neighbor-joining approach and Trat were located on the other extreme indicating its genetic dissociation to the Andaman *P. monodon*. The Chumphon *P. monodon* are allocated between these groups.

In Thailand, large amounts of *P. monodon* postlarvae (typically at PL_{15}) were released into several places in the Gulf of Thailand annually for stock enhancement purpose. More importantly, most female broodstock used to produce the released PL_{15}'s were usually transferred from the opposite side of the peninsula (Klinbunga, 1996; Sodsuk. 1996). On the other hand, the origin of founder *P. monodon* used for stock enhancement may have been originated from the Gulf of Thailand but lack of basic genetic data might promote the possible use of *P. monodon* individuals carrying genotypes that predominantly found in the Andaman *P. monodon*.

Chumphon is one of the most important culture areas of *P. monodon* in the east coast of Thailand. The farming area in this province estimated in 1995 accounted for 12,764 rai (Fisheries Economics Division, Department of Fisheries, 1998). Apparently, an anomalous gene pool observed in the Chumphon *P. monodon* might have been affected from transplantation as described above and from unintentional introduction of escapees from the farms (see below).

Practically, fishery managers have not concerned any effects of escapees released into the new environments either accidentally or intentionally. Poor management practices include immediate terminate of culture if postlarvae in hatcheries show any serious symptoms of infection and most farmers released infected *P. monodon* larvae directly into the sea. In the population genetic point of view, the anomalously genetic status of *P. monodon* needs to be clarified. Therefore, more *P. monodon* samples from the further south of Chumphon, for instance Surat and Songkla need to be genetically determined for a clear conclusion of the genetic status of *P. monodon* in the east coast of Thailand. Nevertheless, a larger number of RAPD primers used in this study must be included for reliable conclusions.

Geographic heterogeneity analysis using a Monte Carlo simulation showed the agreement of results from this study and those of Tassanakajon et al., (1998) and

Supungul (1998) using seven RAPD primers and three microsatellite loci, respectively. Each RAPD primer used in this study did not give contradectory heterogeneity results with the exception that Trat and Trang was not genetically different when analyzed with the primer UBC268 (Table 3.11). The difference in allele (or genotype) distribution frequencies between Trat and Chumphon was highly significant for all three studies ($P < 0.001$) indicating that *P. monodon* from these two geographic regions are reproductively isolated and should be regarded as different stocks. Therefore, five geographic sample of *P. monodon* in Thailand could be grouped into 3 separated stocks; A (Trat), B (the Andaman Sea) and C (Chumphon). The basic data on population differentiation of Thai *P. monodon* from this and previous studies indicated that *P. monodon* is not a panmictic species. In contrast, it fragmented into several isolated gene pools. In terms of population genetic point of view, the less abundant stocks need to be managed properly and unintentional mixing of *P. monodon* from different stocks must be avoid (Altukhov and Salmenkova, 1987).

To be safe, all commercially exploited species should be premarily recognized to represent in nature as genetically differentiated populations even when less sensitive analysis (e.g. allozymes) showed homogeneous of allele distribution geographically. The information on geographic population subdivision in one of the most commercial important cultured species in Thailand like *P. monodon* should lead to increase awareness for broodstock collection and exploitation for farming activities (Klinbunga, 1996) and must be taken into account by fisheries managers and government organizations. Careful records on the number and origin of cultivated or hatchery *P. monodon* stocks used must be carried out.

At present, the culture activity of *P. monodon* is still open, as a result the wild broodstock has been heavily fished. Genetic improvement and eventually, domestication of *P. monodon* are currently required. Under the concept of

domestication selection, the founder population (s) is suffered from the absence of gene flow leading to strong pressure on artificial selection. Genetic propensity of the cultured (or domesticated) stocks at consecutive generations is expected. Moreover, correlation between performance of commercial important trait (e.g. growth rate and/or disease resistance) and level of genetic diversity of artificially propagated stocks should be tested whether they are positivily related.

Accordingly, genetic diversity of *P. monodon* in both natural and domesticated stocks should be regularly monitored by various molecular approaches (e.g. microsatellites, mtDNA-RFLP and RAPD).