Chapter IV

Discussion

Several DNA extraction methods were used to isolate genomic DNA from pleopods of *P. monodon*. Of which, the extraction protocol modified from Davis et al., (1986) and that described by Cook et al., (submitted) are suitable as high molecular weight genomic DNA was consistently obtained. Although the former gave better quality and higher yields of extracted DNA, it was tedious, time consuming and hazardous. Accordingly, the alternative method described by Cook et al., (submitted) was more appropriate when dealing with a large number of specimens. Practically, these methods yielded the same degree of amplification success, as a result, a more tedious method was subsequently replaced by that of Cook et al., (submited).

It is accepted that the information on levels of genetic variation and population differentiation of the black tiger shrimp, *P. monodon* are crucial for formulation of the most appropriate conservation and management programs in this species. Additionally, these basic data is also applicable to effective breeding programs. Based on the fact that natural *P. monodon* broodstock, particularly mature females, has been heavily exploited by the culture activity, a supplement of local founders from artificially propagated programs may be necessary in the future. Alternatively, a particular stock having economically important traits can be developed through selective breeding programs. In this circumstance, highly polymorphic genetic markers are substantially required.

The genetic structure of penaeid shrimp populations inferred from allozyme analysis has been reported in several species in Australia. Intraspecific population structure was observed in *Metapenaeus bennettae*, *M. endeavouri*, and *P. latisulcatus* whereas no geographical subdivision was observed even over large geographic distances of *M. ensis*, *P. esculentus*, *P. merguiensis*, *P. plebejus*, and *P. semisulcatus* (Mulley and Latter, 1981).

Geographical differentiation of the tiger shrimp, *P. monodon*, based on allozyme analysis was first investigated in populations in Australia (Benzie et al., 1992). *P. monodon* samples obtained from seven locales throughout the Australian waters were surveyed electrophoretically at eight polymorphic loci. Three loci (*GPI*, PGM* and MPI**) contributed to significance in the distribution of allele frequencies among populations. Geographic differentiation between western compared with northern and eastern populations was observed ($P < 0.001$ from χ^2 analysis and FST = 0.0311). Population subdivision of Australian *P. monodon* based on mtDNA variability was also reported (Benzie et al., 1993). The mtDNA genotype frequencies were significantly different between the eastern and the western populations ($P < 0.05$). The results were concordant to data previously established by allozymes.

Tassanakajon et al., (1997) reported the potential use of RAPD markers for population genetic studies of *P. monodon*. A total of 200 octanucleotide primers was empirically screened. Six informative primers were obtained and used for analysis of genetic variation level of *P. monodon* originating from three different sites (Satun-Trang, Trad and Angsila). The highest polymorphic bands was observed in the Satun-Trang (48%) followed by the Trad (45%) and Angsila (25 %) samples. Additional genetic population structure analysis using RAPD was subsequently published (Tassanakajon et al., 1998). One hundred *P. monodon* individuals collected from 5 geographically separated locations constituting of Satun-Trang, Phangnga and Medan in the Andaman Sea and Chumphon and Trad in the Gulf of Thailand were examined. Significant differences in distribution of genotype frequencies between all Thai *P. monodon* and that of Medan (Indonesia) were observed ($P < 0.0001$). Geographic heterogeneity between *P. monodon* from the Andaman Sea and the Gulf of Thailand were found ($P < 0.001$).

Both protein and DNA markers have been used for determination of genetic population structure of *P. monodon*. Nevertheless, each genetic markers has some limitations and cannot be singly used for all population genetic and systematic applications. At present, a genetic maker suitable to be used as marker assisted selection (MAS) for breeding programs of this taxa are concerned. Accordingly, the allozyme markers seem to be inappropriate for this purpose due to its low variability

such as previously reported in extensive survey of *P. monodon* collected from 11 geographically separated samples throughout its distribution from Kenya to Indo-China and the Philippines (average heterozygosity was 0.027) (Sodsuk, 1996). Although high level of heterotype diversity in this species was found from mtDNA polymorphisms, the mtDNA markers can not detect F1 hybrid because of the maternally inherited property therefore these markers need to be used in coupling with the other having co-dominant segregation nature. RAPD analysis has been increasingly used in several applications but the limitation to apply the RAPD markers for pedigree analysis of large progeny samples is resulted from an inability to evaluate the actual status of a given allele in as much as homozygotic cannot be distinguished from heterozygotic states.

Basically, highly polymorphic markers with abundance, even distibution throughout the genome and transmitting according to the Mendelian fashion are ideally required for wider applications. Microsatellite loci fall into these requirements, therefore they have been elevating used for population genetic studies in several taxa. Microsatellites are short DNA stretches composed of mono-, di-, tri-, or tetranucleotides repeated tandemly (Wright, 1993, 1994 ; Park and Moran, 1994). Generally, they are highly abundant and dispersed at 7 - 100 kb intervals in eukaryotic genome (Wright, 1993).

Tassanakajon et al., (1998) characterized dinucleotide-motif microsatellite in *P. monodon* and reported their potential utility for parental determination and stock structure analysis. The average distance between neighboring (GT)n microsatellites in this species was 92.8 kb (Tiptawonnukul, 1996) which was much less abundant than those in mammals (every 18 - 46 kb) (Weber, 1990) and the honeybee, *Apis mellifera* (every 34 kb) (Estoup et al., 1993). On the other hand, the abundance of (GT)n microsatellites in *P. monodon* were approximately comparable to those of the Atlantic salmon, *Salmo salar* and the European flat oyster, *Ostrea edulis* where the (GT) motifs are found every 90 kb and 139 kb, respectively (Brooker et al., 1994; Naciri et al., 1995). The most common size-class in all microsatellite categories in *P monodon* was the sequences containing 30 - 35 tandemly repeated units (Tiptawonnukul, 1996).

This value is comparatively identical to that previously reported in the Atlantic cod, *Gadus morhua* (Brooker et al., 1994).

This thesis emphasized the use of three homologous microsatellite loci constituting of CUPmo18, Di25 and Di27 for extensive investigation on levels of genetic variability and differentiation of *P. monodon* in the Thai waters. Highly polymorphic levels (indicating by high observed heterozygosity) were found in all investigated *P. monodon* samples eliminating the suspected possibility on the occurrence of bottleneck effects in this taxon. The number of alleles per locus was 37 (mean sample size 35.6 individuals per sample), 34 (n = 36.8 per sample) and 32 (n = 35.6 per sample) for CUPmo 18, Di25 and Di27 corresponding to high observed heterozygosity (0.68 - 0.81). The within sample diversity found in this study was slightly lower than that previously reported by mtDNA-RFLP of three geographical samples in Thailand; 0.851 $\pm$ 0.031 in Satun, 0.862 $\pm$ 0.037 in Surat and 0.879 $\pm$ 0.027 in Trad (Klinbunga et al., 1998).

All previous publications based on allozymes showed extremely low level of genetic diversity in *P. monodon*. While Benzie et al., reported the heterozygosity of the Australian *P. monodon* to be 0.045 - 0.103, a lower diversity was observed in Sodsuk (1996) who studied the genetic variation level of *P. monodon* at macrogeographic scale and estimated that heterozygosity in such a species was 0.027. This indicated that microsaltellite DNA are more powerful than allozymes for differentiation and identification wild populations.

The effective number of alleles (averaged for three loci) was relatively high in all sample. However, The Gulf of Thailand *P. monodon* possessed higher n_e (16.36) than did the Andaman Sea (15.15). This circumstance was supported by the overall heterozygosity averaged for all investigated loci (0.79 and 0.80 in Chumphon and Trad and 0.66, 0.70 and 0.73 in Satun, Trang and Phang-nga).

Generally, several microsatellite alleles found in the Andaman samples were not available in Trad. However, some of which did exist in Chumphon. At the locus CUPmo 18, a total of 15 alleles found in the Andaman was not observed in Trad but

10 of these was found in Chumphon. Twelve alleles of Di25 which were seen in the Andaman was not found in Trad but five of which were possessed by the Chumphon sample. The results from Di27 was not clear for this aspect. The basic distribution data from the two loci (CUPmo 18 and Di25) and an inability to detect any significant difference in allele frequency distribution between Chumphon and Satun by all three microsatellite implied that the status of Chumphon *P. monodon* might be a result of mixing between different gene pools as it related to geographic samples from either side of the peninsula.

One important reasons to explain this phenomenon is that part of the Chumphon *P. monodon* gene pool might has been disturbed by escapees originating from the Andaman Sea. It should be emphasized that the farms usually released *P. monodon* larvae into the sea when an outbreak of serious diseases was occurred. Besides this, juveniles and broodstock of this species have been released for a restocking purpose (Department of Fisheries). Intraspecific hybridization between different *P. monodon* stocks may be occurred.

As can be seen from Table 3.6, large differences between observed and expected heterozygosity were observed for all samples overall loci, therefore the analysis of Hardy-Weinberg conformations were carried out. Surprisingly, all geographic *P. monodon* samples deviated from all investigated microsatellite loci with the exception of Trad at locus Di27.

Sodsuk (1996) examined genetic diversity of *P. monodon* collected from 11 sample sites (Kenya; the Gulf of Thailand, Trad and Surat; the east, Dungun and the east of the Malaysian peninsula, Phuket, Satun, Kedah and Medan; Java; north ar.d south Java; and Philippines) using 40 allozyme loci but only *IDHP** (Surat) and *PGM** (Phuket) did not conform Hardy-Weinberg expectations.

Significant deviation from the expectations found in the present study may be explained by several reasons including first, null allele (non-amplifying alleles) at these loci may be present. This crucial circumstance is observed for amplification of microsatellites in human and other mammals studies (e.g. Callen et al., 1993 ; Koorey et al. , 1993 ; Pemberton et al., 1995). Non-amplification alleles can be detected

through mismatched patterns between parents and offspring whose their genotypes were already known (Pemberton et al., 1995). Null-alleles can cause underestimate level of heterozygosity as heterozygote individuals are scored as homozygous and thus either showing non-Mendelian inheritance or resulting in significant deviation from Hardy-Weinberg equilibrium. Second, most dinucleotide microsatellite show stuttered bands so mis-scoring of a particular allele may be occurred(O'Reilly &Wright, 1995).

In the present study, the distribution of allele showed that allele 168 in locus CUPmo18, alleles 138, 178 and 200 in locus Di25 and allele 116 in locus Di27 were not observed. Thus, chance of detecting null allele was low. Besides this, each locus showed many alleles having low frequencies and several unique alleles therefore the greater sample sizes are required to ensure true representatives. On the one hand, significant departures from Hardy-Weinberg expectations may reveal the mixture of genetically different population in a local samples, through an observed homozygote excess (Wahlun, 1928 : cited in Hedrick, 1985). On the other hand, it may indicate the influence of selection on a particular locus.

Segregation analysis of CUPmo 18, Di25 and Di27 in *P. monodon* using 20 progeny from a representative full-sib family revealed non-significant deviations from gametic segregation expectation for all loci ($P > 0.05$) indicating Mendelian inheritance nature of these three microsatellite loci. Therefore, departures of Hardy-Weinberg equilibrium should be caused by low number of sample sizes and sampling errors rather than from effects of selection in as much as no common allele (s) with the extremely high frequency was observed. Based on the basic biology of *P. monodon*, the important life-historical generalization may be drawn. The *P. monodon* females may spawn more than once a year. Each wild breed female may produce 248,000 to 811,000 eggs per single spawn (Motoh, 1981 : cited in Solid, 1988) to compensate for an enormous gamete wastage in the planktonic larval stages. In this circumstance, the enormous number of eggs may be sufficient to replace part of the entire adult population. Sampling errors could then be happened.

Geographic homogeneity was observed all pairwise comparison between sites and regions ($P > 0.05$) when Di27 was employed indicating the limitation to use this

locus for population genetic analysis of *P. monodon* in Thailand where low level of genetic differentiation among population was exist. Nevertheless, this microsatellite yielded the highest level of observed heterozygosity (0.71-0.95; mean = 0.81) with the Mendelian segregation nature so it is useful in selective breeding program in which pedigree analysis of large number of families are required.

Generally, CUPmo18 and Di25 were equally good for determination of population subdivisions in this study as indicated by six significant pairs of all possible comparisons. There were no significant differences in allele distribution frequencies of Satun to other samples except Trad (Phanag-nga and Chumphon) for both loci implying their sympatric status. In contrast, geographic heterogeneity between Chumphon and Trad located in the same coastal site was observed ($P < 0.001$). The contradictory results of CUPmo18 and Di25 was observed between Phang-nga and Trang and Chumphon ($P = 0.004$ and 0.084 for the former and $P = 0.169$ and 0.004 for the latter locus). After non-significant geographic samples were combined and reanalyzed, the significant differences in allele frequencies among different sites (between Andaman - Chumphon, Andaman-Trad) were observed. Based on this analysis, *P. monodon* in Thailand could be genetically separated into three stocks; the Andaman (stock A), Chumphon (stock B) and Trad (stock C).

Disregarding estimation of evolutionary time (the molecular clock concept), it was recently reported that the probability to obtained the correct topology of a phylogenetic tree based on both the infinite allele and the stepwise mutation models was high when the Cavalli-Sforza and Edwards' chord distance was employed (Takezaki and Nei, 1996). Apparently, the genetic distance between each pair of geographic samples was not largely different implying low level of differentiation in *P. monodon*. The neighbor-joining tree allocated five investigated samples into three phylogenetically related lineages as the same as that from geographic heterogeneity test. While Satun, Trang and Phang-nga were placed at one extreme, Trad was placed at the other. Chumphon was located in the middle and could possible placed in either the Gulf of Thailand or Andaman samples.

The degree of population differentiation in this species was weak but still significant (θ = 0.009) as differentiation due to between samples were only approximately 1%. This results indirectly indicated that *P. monodon* in Thailand were not suffered from reduced heterozygosity indicating by high genetic diversity within each investigated samples.

Microsatellite DNA are useful for not only identification of genetically distinct stocks but for examination of individuality and parentage in the commercial important species like *P. monodon*. The present study indicated the existence of population differentiation in this species. Although the level of subdivisions may be low, three different *P. monodon* stocks found should be treated, from a management point of view, as separate exploitation stocks (Carvalho and Hauser, 1994). The basic information obtained from this study implied the possibility to used microsatellite markers for selective breeding programs. For example, representatives from each wild stocks can be randomly chosen. Intraspecific hybridization among different strains can be carried out. The progeny from all random crosses are reared together obviating significantly common environmental effects. After reaching the market size, the family selection approach can then be performed. For population genetic studies, all microsatellite loci (CUPmo18, Di 25, Di27) in this study can be used to identify genetic variation level in geographic samples which are inbred and/or reduced heterozygosity was observed from other genetic markers.