# Chapter IV

## Discussion

Genetic variation has the potential to play an important role in shrimp selective breeding programs. So far, however, only allozyme variability has been extensively used in penaeid shrimp and the few DNA studies that have been performed have shown that more genetic variation was detected using the DNA techniques (Garcia et al., 1994). In this study, RAPD technique was used to evaluate the extent of genetic variability in wild populations of *P. monodon* collected from four different geographic locales. These samples covered the main supplying sources of *P.monodon* broodstock used for hatchery purpose in Thailand.

The RAPD technique has been used to detect polymorphism in many organisms. It has uncovered cryptic genetic variability in organisms that distinguishing closely related taxa (Narang et al., 1994). Theoretically, the RAPD technique is expected to scan the whole target genome more randomly than other conventional methods. Since primers are constructed at random, both coding and non coding regions including VNTR loci should be targeted for PCR amplification. As a result, an unbiased used of DNA markers from significantly

different evolutionary rate is, at least theoretically, compromised.

Any parts of the PCR program can change RAPD-PCR patterns (Yu et al., 1993). Therefore, it is useful to spend some time testing various PCR when developing a new RAPD application (Weising et al., 1995). In this study, the reaction buffer components were similar to those described in several protocols but temperature profile, shorter time interval at each step was used. Too long a time at each step unnecessarily prolongs the procedure and may decrease the effectiveness of Taq polymerase. The length of time also depends on the thermocycler's construction and thermal control; therefore the length of time could differ from one brand to another (Yu et al., 1993). The optimized PCR temperature profile for RAPD analysis of the *P.monodon* DNA was denaturation at 94°C for 5 sec; annealing at 36°C for 45 sec and extension at 72°C for 90 sec. During optimization of PCR program parameters, only denaturation for 5 sec yielded consistent amplifications at all three DNA concentrations because shorter periods at 94°C, of course, prolonged the life time of the polymerase. Moreover, short denaturation for 5 sec is indicated that shrimp DNAs consisted of AT rich sequences. Hedgecock et al.(1982) had reported that within the Crustacea, satellite DNAs isolated from species of Cancer consisted of 90-97% alternating A-T. Too much DNA resulted in a smear presumably because DNA might contain some inhibitors (Yu et al., 1993). Annealing at 36°C for 30 sec reduced the yield of PCR products

indicated that insufficient primer annealing may occur, particularly when primer concentrations decline in the later cycles of a long PCR run. Extension for 75 sec and 105 sec resulted in faint bands and smearing respectively. In general, the length of extension step longer than might be expected to get a good yield of PCR products (Rosalind and Stamps, 1993). At the optimal temperature, rate of nucleotide incorporation for Taq polymerase is up to 150 nucleotides per second (Geland, 1989). Benter et al. (1995) had found that 60 sec was enough for extension step for human DNA (about 400-3,000 bp amplification products) but more than 240 sec increased smearing. The 30 cycles of amplifications were not sufficient to gave intense bands. Corresponding to several reports, it was suggested that above 35 cycles were enough for sufficient amplifications (Benter et al., 1995). In this study, the RAPD conditions were optimized and samples were amplified on the same machine. Thus, any differences in banding patterns among individuals should not be attributable to differences in PCR; only polymorphic loci that gave high intensity, easily scorable were used for data collection.

Three hundred RAPD primers (primer 101-300 and 401-500) were screened for their ability to prime PCR amplifications. On the basis of a preliminary results, primers 101, 174, 268, 428, 456, 457 and 459 were chosen. These primers produced strongly reproducible amplification products and gave several fragments. All seven ten-base oligonucleotide primers used in this study

have 60-80% GC content. Generally, primer with ten nucleotides and GC content of at least 50% are generally used (Weising et al., 1995). William et al.(1990) found that the minimum of a primer length to detect amplification in ethidium bromide-stained agarose-gel was 9 bases.

To assess the usefulness of RAPD markers in the determination of genetic diversity of *P.monodon*, samples from the Andaman sea were compared with those from the Gulf of Thailand. The samples from the Andaman Sea were collected from Satun-Trang and Medan (Indonesia), whereas those of the Gulf of Thailand were from Chonburi (Angsila district) and Trad. In Thailand, the Satun-Trang and Trad samples are the most commonly used as spawners in shrimp farming. However, broodstock from the Satun-Trang sample tends to have much bigger size and exhibit different color than those from Trad. Moreover, although get controversial, the shrimp farmers claim that the Andaman Sea broodstock produces more eggs and better quality seeds making the price of the Andaman Sea broodstock higher than that of the Gulf of Thailand.

Decapods are known to display relatively low level of enzyme polymorphism. Mean heterozygosities of 0.048 were reported for decapods, 0.082 for crustaceans (D'Amato and Corach, 1996). A high level of diversity was detected in *P. vannamai* Boone by RAPD analysis, being higher than the level of polymorphism with allozyme markers (Garcia et al., 1994). The

percentages of polymorphic bands range from 39-77%.

In this study, the percentages of polymorphic bands in *P.monodon* varied from 45.6-57.7%. The percentages of polymorphic bands were comparable in the Satun-Trang, Trad, and Medan samples, suggesting similar level of polymorphisms among these three geographic samples. The Angsila sample was the least polymorphic. These correspond to the fact that broodstock from Satun-Trang, Trad and Medan is commonly used as spawners in shrimp farming but broodstock from Angsila has never been used for shrimp industry. Moreover, there was no *P.monodon* found in Angsila until recently when farming of the species is common in such an area and a few were caught by fishermen (Menasveta, personal communication) suggesting that they might be the escapees from the farm. Thus, low level of polymorphism was expected for the Angsila samples. Recently the RAPD analysis of the six crossing families of *P. monodon* had shown a much low level of polymorphism (6.2%) (Garcia and Benzie, 1995). This may be due to the different in the total number of scoring bands, evolutionary rate of the primer's target per se, and the fact that the individuals from wild-caught populations would exhibit higher level of genetic differences than those of the breeding families.

Various RAPD primers used in this study make the possibility to resolve genetic variability between samples of Thai and Indonesian *P.monodon*. Some of RAPD markers identified here are monomorphic in *P.monodon* from

particular geographic samples. For example, primer 459 produced a major band that appeared to be monomorphic and fixed in all individuals from Medan. Other RAPD primers, primer 101, 428, and 457 also produced specific markers. Although Thai *P.monodon* from Trad and Angsila are geographically separated from Satun-Trang, the primers which gave geographically-specific fragments were rare. Only primer 428 gave a specific band which was found only in Satun-Trang. Band with size about 950 bp which was amplified by primer 428, was present in 28 out of 29 individuals from Satun-Trang. This band was also found in all individuals from Medan suggesting that this band should be specific for the populations of the Andaman Sea. Far more samples would need to be screened to establish whether a band at 950 bp would provide the real region-specific marker. After that, this marker can be cloned and sequenced to design a specific set of primers to be used as a region-specific marker or alternatively, the 950 bp can be reamplified by a "touch down" PCR and used as a DNA probe through Southern analysis for the same purpose. The lack of a band at 950 bp in samples from the Gulf of Thailand can also be explained by the lack of primer binding site within the window of size ranges.

For data analysis, the RAPD patterns were used to score for the presence or absence of the amplified DNA fragments. These allow comparisons of genetic similarity among individuals or populations. The similarity index and genetic distance which are described by Lynch (1991), were used in analysis of

the RAPD data. The S and $S_{ij}$ values are estimators of genetic variation within and between populations respectively. They indicate the level of similarity between the individuals or populations being compared. Thus, if S or $S_{ij}$ is high, the individuals or populations share a high level of sequence similarity. In the present study, intrapopulation RAPD variation was detected with all primers. It is noteworthy that samples from Angsila showed the highest S value, indicated that these individuals were more similar among themselves than those in other populations. This value corresponded to the lower percentage of polymorphic bands observed in this sample. It is not surprising that the Angsila sample has the lowest genetic diversity within population in comparison to others. This may be due to *P.monodon* fry which escaped from farms, were recaptured for the analysis in this study as discussed above. The values of $S_{ij}$ and genetic distance indicated a high genetic similarity between samples of Thai *P.monodon*, but indicated a lower genetic similarity between Thai and Indonesian *P.monodon*. Genetic distances were used to cluster the data using the UPGMA algorithum. Most notable, in this presentation, dendrograms implied that the Thai *P.monodon* (Satun-Trang, Trad, and Angsila) shared an ancestral gene pool. More importantly, the dendrograms illustrate a clear "genetic brake" between the Thai and Indonesian *P. monodon* at the Strait of Malacca.

The Satun-Trang and Medan samples are located on the Andaman Sea, so, it was expected to form a cluster. For this reason, the samples from Satun-

Trang, which were kindly provided by Banchong Farm, were suspected for the real locality. New samples, (12 individuals) were then collected from fishermen in Satun-Trang to further tested. The new Satun-Trang sample showed similar level of genetic variation as from the previous sample (see Appendix C). The similarity index within population was 0.8791 which was comparable to that of the previous one (S = 0.8655). The index of similarity between populations, genetic distances and UPGMA dendrograms also indicated that they were the similar samples (see Appendix C). This suggested that the previous Satun-Trang specimens were collected from the real locale.

The similarity index is assumed that any comigration of non allelic markers can be resolved either by differences in band intensity or from other information (Lynch, 1990). The RAPD bands of the same size may not actually have the same or similar sequence (Narang et al., 1994). This may be leading to lose of genetic informations.

A RAPD pattern is referred to as a genotype. A genotype, which specifies for particular population or geography, can be developed a genetic brand. Such as in this study, a genotype B of primer 101 was a genetic brand of *P. monodon* from Medan. This genetic brand can be used to detect the Medan sample. Thus, the advantages of genetic brands are the same as the specific-markers. Consequently, it is not possible to assign individuals taken at random to any specific population. Although the presence of private genotype appears to

be a characteristic of the population or region, this observation requires a larger sampling to confirm.

A chi-square ($\chi^2$) analysis, a Monte Carlo simulation, was also used to suggest genetic differentiation. Chi-square enabled discrimination with highly significant among Thai and Indonesian *P.monodon* as dendrograms. Moreover, chi-square can provided the geographic heterogeneity among Thai *P.monodon*, the Andaman Sea and the Gulf of Thailand. Using a chi-square analysis, several primers can discriminate among groups of Thai *P.monodon*. Although the UPGMA dendrograms clustered Satun-Trang with Angsila and Trad, the chi-square enabled the separation of Satun-Trang samples from those of the Gulf of Thailand. These results suggested the existence of differentiation between 2 geographically seperated population of Thai *P.monodon*. The UPGMA dendrograms and values of chi-square suggested clearly the genetic differences between Thai and Indonesian *P.monodon*.

The genetic population structure of *P.monodon* had also been analyzed by using mtDNA RFLP (Sirawut Klinbunga, 1995). Geographic heterogeneity based on chi-square test and UPGMA dendrogram showed that the Satun was clustered with Aceh (North of Sumatra island) and these two samples were isolated from Trad. Since Aceh locates nearly to Medan, on the Andaman Sea, the result of mtDNA RFLP was different from that of the RAPD analysis. However, there are many factors that effect the analyses of geographic

heterogeneity in this 2 studies. The collection of samples is one factor. The samples used in mtDNA RFLP analysis were collected in 1993, while in this study the samples were collected between 1995-1996. The population structure may be different in this 2 periods of time. Because, in Thailand, most female broodstock were transferred from the Andaman Sea to other parts of the country. *P.monodon* fries of these female broodstock were released to the Gulf of Thailand leading to stock mixing. The weak differentiation found among Thai *P.monodon* by the RAPD analysis may be the consequent of the mixing of stocks of Thai *P.monodon.* Therefore, determination of genetic polymorphism in Thailand should employ more than one technique to the same samples (Wilkerson et al., 1995).

To increase the reliability of the RAPD technique, a more vigorously search of polymorphism would most likely have to be performed. This may involve, more RAPD primers and more samples. A large number of primers must specific be tested with the expectation that at least some of the primers may amplify population-specific DNA fragments. Better resolution and more sensitive detection of RAPD fragments can be achieved by using polyacrylamide gel electrophoresis followed by silver staining. Polyacrylamide gel electrophoresis is known to increase the resolution of band separation whereas silver staining is more sensitive than ethidium bromide staining (Stothard and Rollinson, 1995).

RAPD markers inherited in a dominant fashion, because the presence of a given RAPD band does not distinguish whether its respective locus is homozygous or heterozygous. On the one hand less information is obtained from dominant markers (RAPD) than from codominant markers, caused loss of basic estimators for population genetic studies (Williams et al., 1993). On the other hand, the great advantage of RAPD technology is the ability to obtain DNA polymorphisms without having sequence or otherwise characterize genetic DNA of interest. The possibility of carrying out compatibility analysis with unlimited numbers of primers, each detecting variation of several regions in the genome, provides an advantage over other techniques. Even if some primers amplify identical regions of the genome or if the technique itself is noisy, it should be possible to build up a quickly consensus from patterns of interpopulation variation (Bardakcl and Skibinski, 1994; Wilkerson et al., 1995). This is contrast to other DNA based methods (e.g., southern blotting, DNA sequencing) which may take several days for completion and often require elaborate steps such as cloning. RAPD does not require the use of hazardous-radioactive chemicals. RAPD also allows for the processing of many samples simultaneously. The number of samples that can be processed is a function of the number of tubes that a thermalcycler will hold and the number of thermalcyclers that are available. Whereas the time required to obtain results from RAPD and allozyme analyses is comparable, allozyme analyses has certain limitations (Kambhampati, Black IV, and Rai, 1992).