CHAPTER 4
DISCUSSION

The primary objective of this work is to examine genetic differentiation between normal and viral disease tolerance in *P. monodon* using molecular markers. Genetic analysis of many plants and vertebrates related to important traits have been demonstrated. In 1987, Landry et al. used two types of molecular markers, RFLP and isozyme, to construct a genetic map involving disease resistant and morphology of Lettuce. Michelmore et al. (1991) could identify three RAPD markers linked to disease resistance gene using DNA-bulk segregant analysis. Cheng et al. (1995) used micorsatellites and RAPD markers to develop a genetic map concerning important genes in chicken (resistant genes to Marek's disease, genes involved in abdominal fat and production trait). RFLP analysis has been used extensively as the most efficient method to generate a genetic markers for important traits (Cheng et al., 1995). But RFLP analysis is laborious and time-consuming (Paran and Michelmore, 1993). The wide spread use of minisatellite and microsatellite markers is limited, however, because they rely on predetermined variation or on the genomic distribution and organization of tandem repeats. Many minisatellite alleles are too large to be amplified and the microsatellites identified so far are only sparsely distributed in some species (Weber et al., 1991). Therefore, methods are required for rapidly obtaining markers involved tolerance genes for genetic analysis such as a randomly amplified polymorphic DNA (RAPD) techniques (Williams et al., 1990). This method uses arbitrary primers without prior knowledge on the genome sequences and allows the quick construction of genetic markers for several species, especially in plant (Agwanda et al., 1997; Alcivar-Warren et al., 1997; Kelly and Miklas, 1997; Conner et al., 1998; Morgan et al., 1998). Because RAPD analysis is simple, fast, does not used the radioactive isotopes and can be scaled up to analyze large amount of samples, this technique is used to find genetic

markers that could differentiate between normal and viral disease tolerance *P. monodon* in Thailand.

The white spot disease outbreaks in *P. monodon* have great impact in shrimp cultivation and farming. Epizootics of disease depend on the season. It seems to be serious in winter. Because of widespread epizootics of this disease, the shrimp cultured production in Thailand has decreased since 1995. Rosenberry (1997) reported a drop of shrimp production in Thailand from 225,000 metric tons in 1995 to 160,000 tons in 1996. Of the high price, shrimp farming areas increased while the production did not. Lack of prevention and pathology study in tissue of the disease, the farmer lost income and ecology of coast was destroyed. Diagnosis of white spot disease in shrimp had been developed. Polymerase chain reaction (PCR) technique was used to test white spot virus infection in post-larvae shrimps. Shrimps which showed negative PCR results would be further cultured (Tongchuea, 1996). However, shrimp farms in Thailand has reported successful harvests in spite of the presence of white spot virus in their ponds throughout the cultivation cycle (Flegel, 1997). The shrimps seemed to be able to survive and grow normally while infected. These shrimps were called tolerance shrimps rather than resistance (Pasharawipas et al., 1997), because the shrimp had active but innocuous infections. In this thesis, viral tolerance samples were white spot viral tolerance shrimps which were kindly provided by the Shrimp Culture Research Center, Charoen Pokphand group of companies. These shrimps were WSV infected shrimps by PCR test, further cultured in farm and still survived. According to Withyachumnarnkul (Personnel comm.), most of infected shrimps died and caused widespread epizootics within populations with high mortalities. However, there were approximately 0.1 % of infected shrimps that survived.

To compare RAPD patterns between normal and viral tolerance shrimps, it is important that they are in the same population otherwise the RAPD markers could be population specific markers. According to the Shrimp Culture Research Center,

Charoen Pokphand group of companies, their viral tolerance shrimps were offspring of wild broodstocks caught off the Andaman Sea in 1996. The fishermen claimed that the broodstocks were from Satun-Trang provinces. To avoid mixing of populations, normal and tolerance shrimps were collected from the same ponds. However, due to the problem of sample collection, most of the normal shrimps could not be immediately stored frozen after dead resulted in low quality DNA (data not shown). Thus uninfected shrimps from Satun-Trang which were collected alive were used as normal shrimps in this study with the assumption that 99.9 % of them were normal shrimps which would die if infected with WSV. RAPD patterns of normal and viral tolerance shrimps using 26 selected primers showed similar patterns. These result suggested that they were from the same populations. Moreover, Pongsomboon (1996) and Supankul (1998) also reported that the genetic of the Andaman samples (Satun-Trang, Phuket and Medan) were not significant different using 7 selected RAPD primers and 3 microsatellite loci, respectively. In addition, Pongsomboon had reported the presence of the 950 bp RAPD marker specific to the Andaman Sea populations and this fragment was found in the RAPD patterns of the normal and viral tolerance shrimps as reported by Pongsomboon (1996) (see Appendix 2.2). These results suggested that the normal and viral tolerance samples were likely from the same source, Satun-Trang.

From RAPD pattern using primer OPA-04, it was found that the 800 bp DNA fragment was present only in normal shrimps but absent in viral tolerance shrimps. The presence of this fragment was consistently observed in normal samples in 1996 collection and showed a faintly DNA band in samples collected in 1997 (Figs 3.6 and 3.8). When specific primers were designed from the partial sequence of the 800 bp fragment, a 173 bp PCR product was found in 80 % of normal samples in 1996 collection and 15 % in 1997 collected shrimps. Therefore, it was unlikely that samples were collected from the same area, Satun-Trang. The problem concerning the source of samples must be solved. However, because wild *P. monodon*,

particularly adults and sub-adults live in deep sea water, samples were collected by fishermen and true identity of animal was always doubtful.

Several methods were attempted for cloning RAPD marker in this study. First was the blunt-end cloning but not successful. The second method was performed by ligation into T-overhang vector (pGEM® T- vector). Hadjeb and Berkowitz (1996) reported low efficiency of transformation when cloning PCR product and many false positive clones were found. Extension by *Taq* DNA polymerase may be results in the addition of a single adenosine residue to the 3' ends of most of the PCR-generated DNA fragments. Since most of the PCR-generated DNA fragments have an A-overhang. Therefore, Hadjeb and Berkowitz presented a strategy to further optimize PCR product cloning by modifying the T-overhang cloning protocol. Although using T-overhang cloning, the ligation and transformation into pGEM® T- vector was still not successful. The last method used the adapter-RAPD primer linkage. New oligonucleotide primer containing 10 bases of *Bam*H I adapter and 10 bases of RAPD primer (OPA-04) was synthesized. This oligonucleotide was used to reamplify the 800 bp DNA fragment and the resulted PCR product was further ligated into pUC18/*Bam*H I/BAP as described in 2.8.3. The last method successfully yielded positive clones containing inserted fragments. From these experiments, it is suggested that cloning of PCR product was easily succeed by making sticky-end ligation using proper restriction enzyme.

The 800 bp RAPD fragment was partially sequenced by the ABI-PRISM automated sequencer. Approximately 469 bases (58.6 %) were sequenced. The sequences (nucleotides and amino acids) were then aligned to other genes in the GenBank using BLAST program as mentioned in 3.6. The sequence comparisons showed no similarity to any known genes or proteins. These nucleotide sequences may be the introns (non-coding regions) of genes. Benzie (1998) reported the genome size of four species of penaeid shrimp was approximately 70 % that of human genome. The size of introns, normally found in eukaryotes, are broadly

distributed from <50 to >200,000 bp (80 % of structural genes). In human genome, protein-coding sequences of hemoglobin genes represented less than 5 % of globin gene cluster (Voet, 1995). It is estimated that only 1 % of billions of nucleotides in the mammalian genome codes for essential proteins (Carvalho and Pitcher, 1995). So it is likely that the 800 bp could be the introns rather than parts of the coding region of a new gene.

Generally, a detection method based on RAPD analysis suffered from non-reproducible RAPD patterns (Garcia et al., 1994 and 1996). Thus, specific primers designed from the sequences of the RAPD marker will provide a more specific detection. The specific primers (upper and lower) were designed from nucleotides of the 800 bp DNA fragment using Oligo4.0 software program. The PCR amplification yielded a 173 bp product and the presence of this fragment in geographically separated *P. monodon* in Thailand was examined. Amplifications of 16 normal shrimps and 3 individuals of Satun samples (in 1997 collection) yielded a 173 bp amplified fragment while those of other groups did not showed any PCR product suggested specificity of this method. The result suggested that the 800 bp DNA fragment was specific to normal shrimp and the specific amplification using PCR technique could be used to further examine linkage of this marker to normal and tolerance phenotype. Whether or not, this marker could be used to identify normal and viral tolerance shrimps required further investigation using a larger number of samples and different generations of shrimps. The latter would depend on the domestication in this important species.

The maximum sample sizes was required for correctly and confidentially detection (Hillis et al., 1996). This research was limited in the number of samples. Therefore, increasing the sample sizes might be useful to find different DNA band between normal and viral tolerance *P. monodon*.

From the large number of chromosomes and relatively large size of the genome and the considerable divergence in species of penaeid shrimp, it suggests

that the considerable effort will be required to develop the basic information to assist the shrimp genetic improvement such as genome mapping. Knowledge of genome information can easily assist in the important trait research in penaeid shrimps. Therefore, the increasing application of molecular techniques and a concerted effort on the virology of penaeids will lead to more rapid diagnosis and a greater understanding of the epidemiology of the diseases and to more rapid development of effective control of these disease.