CHAPTER IV

DISCUSSION

4.1 Identification of polymorphism in *PmCnn1*, *PmCyC* and *PmCdc25* gene segments and relationships between their SSCP genotypes and growth parameters

Information on correlation between genotypic and phenotypic variations in domesticated penaeid shrimp is extremely limited. Analysis of gene-based single nucleotide polymorphism (SNP) is one of the efficient approaches for discovery of genes which significantly contribute in production traits of commercially important species (Liu and Cordes, 2004). This opens the possibility to locate major loci responsible for the difference in quantitative traits of *P. monodon* where this information is not available in this species at present (Tao and Boulding, 2003).

No validated marker linked to performance and production traits has been identified in penaeid shrimp up to date. Recently, a genetic linkage map of a full-sib F_2 intercross family of *M. japonicus* was constructed for identification of QTL influencing growth (weight, total length, and carapace length) using AFLP analysis. A homologue of gene encoding the elongation of very long chain fatty acids-like (ELOVL) protein family resided within the QTL peak was cloned and sequenced. However, association between SNP and growth rates or expression levels of this gene has not been examined (Lyons et al., 2007).

More recently, polymorphism of growth-related genes of *P. monodon* was found from SSCP analysis. Analysis of relationships between SSCP genotypes of *PmCHH1* (6 genotypes) and *PmIDGF* (10 genotypes), and the average body weight and total length of shrimp possessing different SSCP genotypes were statistically significant ($P < 0.05$) when data were analyzed with regarding and/or disregarding sexes of the BUM03 sample. Shrimp carrying SSCP genotypes A of *keratinocyte-associated protein 2* had greater average body weight and total length than those exhibiting genotype B. In the SNP3A sample, shrimp having different SSCP genotypes of *PmCHH1*, *PmIDGF*, *PmMIH*,

PmCGRP-RCP possessed differences in average body weight, total length and HP weight but those of *PmFAMet* was observed in shrimp exhibiting differences in average body weight and total length. Nucleotide sequences of representative individuals exhibiting different SSCP genotypes of these genes were examined. SNPs of these genes also showed significant relationships with growth parameters of *P. monodon* juveniles (Chumtong, 2011).

Growth traits are physiological functions under the control of several genes and regarded as a phenotype under the control of quantitative trait loci. Genotyping animals for all the genes encoding a polygenic trait seems impractical and so it is more realistic to focus on only a few genes having effects that account for a significant part of the genetic variation in growth traits (Li et al., 2010; Dekkers, 2004; Zhang et al., 2009).

Most studies investigating the effects of genes on growth have focused on hormones and peptides within either the somatotropic axis or the transforming growth factor superfamily. However, it is likely that genes outside these two pathways may also significantly influence growth. For instance, *parvalbumin (PVALB)* is one such gene candidate. Xu et al. (2006) demonstrated the association between polymorphism of *parvalbumin* and growth traits in Asian seabass (*Lates calcarjfer*). Expression of *PVALB2* was detected only in muscle, brain, and intestine, and was up to 10-fold lower than *PVALB1* expression. A $(CT)_{17}$ microsatellite was identified in the 3'UTR of *PVALB1* and three SNP were identified in the third intron of *PVALB2*. The microsatellite in *PVALB1* was significantly associated with body weight and body length at 90 days post-hatch ($P < 0.01$) whereas the SNPs in *PVALB2* were not associated with these important traits ($P > 0.05$).

In aquaculture, the primary goal of genetic selection is the growth improvement of economically important species. Although the genes that affect a polygenetic trait such as growth can typically be identified through the genetic linkage maps, a number of potential candidate genes can be selected based on a known relationship between physiological or biochemical processes and a particular trait (Kang et. al., 2002). These candidate genes are promising for the future development of gene-assisted selection (GAS) markers.

In *L. vannamei*, 1221 candidate SNP based on EST resource (7126 ESTs) were recently confirmed and an association analysis were performed between 211 SNP and phenotypic traits (i.e. disease resistance). Although significant relationships between genotype and a phenotype was observed, only 11% of any pair of SNP positions reveal the *r* value greater than 0.1. The information critically suggested that the idea of quantitative trail loci (QTL) may not be valid in shrimp and a particular trait may be mainly controlled by the single or a few gene loci. In this thesis, polymorphism of genes from transformation growth factor superfamily (*PmCnn1*) and cell cycle regulators (*PmCyC* and *PmCdc25*) were selected for studies on relationships between their SNP and growth parameters in *P. monodon*.

At present, various advanced molecular techniques have been applied in SNP discovery. PCR-SSCP analysis is a favorable technique due to its convenience and more cost-effectiveness than other techniques (Orita et al., 1989). Several publications indirectly examined SNP by PCR-SSCP analysis and evaluate the relationship with commercially important traits of examined samples.

In this study, two different parts of the *PmCnn1* gene segment ($PmCnn1_{530}$ and $PmCnn1_{425}$) were successfully amplified. In addition, the *PmCyC* (403 bp) and *PmCdc25* (285 bp) gene segments were also consistently amplified from genomic DNA of *P. monodon* juveniles. Sizes of the amplified $PmCnn1_{530}$, $PmCnn1_{425}$ and *PmCyC* were greater than those expected from their cDNA sequences. These fragments were cloned and sequenced and the results indicated that an intron was found in each gene segment.

SSCP analysis of $PmCnn1_{530}$ was carried out in the SNP3A and PM05 samples. The average BW and TL of 3-month old juveniles *P. monodon* (SNP3A) carrying genotypes I and II was significantly greater than those exhibiting genotypes III ($P < 0.05$). Interestingly, SSCP patterns of $PmCnn1_{530}$ can be scored in a co-dominant segregating fashion as patterns I and III are homozygotic while pattern II is heterozygotic states. In contrast, only male shrimp of the PM05 (5-month-old) sample set having SSCP pattern 1 showed a greater average BW and TL than those exhibiting patterns 3 and 4 ($P < 0.05$).

Polymorphism of *PmCnn1*$_{425}$ was also examined in 3 sample sets (BUM03, SNP3A and PM05). Results indicated that shrimp in the first sample set carrying *PmCnn1*$_{425}$ patterns B and C possessed a greater average BW and TL than those carrying patterns D and E ($P < 0.05$). Likewise, significant differences of growth parameters were observed in the SNP3A sample carrying different SSCP patterns. In contrast, these relationships was not observed in the PM05 sample.

In addition, PCR-SSCP of *PmCyC* was examined in BUM03, SNP3A and PM05. For the BUM03 and PM05 samples, shrimp exhibiting different SSCP patterns did not showed different growth parameters of the respective sample sets. Nevertheless, the SNP3A shrimp having pattern II had a greater average BW and HPW than those with patterns I and III ($P < 0.05$). Although results from *PmCyC* was less universal than those from *PmCnn1*, it is a promising growth-related gene in *P. monodon*.

Polymorphism of *PmCdc25* in BUM03, SNP3A and PM05 was also examined. A monomorphic SSCP patter was found in the BUM03 sample. Like *PmCyC*, the PM05 shrimp exhibiting different SSCP patterns did not show different growth parameters. However, the SNP3A sample, *P. monodon* juvenile with pattern I had a greater average BW, TL and HPW than those with pattern II ($P < 0.05$).

On the basis of SSCP analysis, it was indicated that polymorphism of *PmCnn1*, *PmCyC* and *PmCdc25* was significantly related with growth parameters in at least one examined sample set. Considering this criteria, *PmCnn1* was more potential growth-relate markers than *PmCyC* and *PmCdc25* in domesticated *P. monodon* of this study.

4.2 Identification of SNP genotypes in *PmCnn1*, *PmCyC* and *PmCdc25* gene segments and relationships between their SNP genotypes and growth parameters

Nucleotide sequences in representative individuals representing each genotype of these genes in the SNP3A sample was further examined. Shrimp exhibiting SSCP patterns I and III of *PmCnn1*$_{530}$ possessed homozygotic states of composite SNP diplotypes while shrimp having SSCP pattern II possessed heterozygtic states of diplotype which is similar as results inferred from SSCP analysis. Significant

relationships between genotypes of each SNP of *PmCnn1*$_{530}$ and growth parameter of the SNA3A sample was found.

SNPs in *PmCnn1*$_{425}$ of the SNP3A sample were examined. Three composite SNPs were generated from identified SNPs located in the intron and can be categorized into 3 diplotypes: D1$_{425}$; -/-$_{291}$-/-$_{292}$-/-$_{293}$A/A$_{294}$T/T$_{298}$-/-$_{315}$, D2$_{425}$; G/G$_{291}$T/T$_{292}$G/G$_{293}$C/C$_{294}$G/G$_{298}$G/G$_{315}$ and D3$_{425}$; (-/G)$_{291}$(-/T)$_{292}$(-/G)$_{293}$(A/C)$_{294}$ (T/G)$_{298}$ (-/G)$_{315}$ corresponding to SSCP patterns I+V, II+IV and III, respectively. Shrimp with each SNP of D1$_{425}$ diplotype showed a greater average BW than those with D3$_{425}$ diplotype. The results further confirm the potential of SSCP analysis for identification of polymorphic SNP and that polymorphism of *PmCnn1* is readily related of growth parameters in *P. monodon*.

Five SNPs were found in the amplified *PmCyC* gene segment. Of these three SNPs located in the exon but did not cause amino acid replacement. Two non-coding SNPs were also found. Five SNPs revealed that diplotypes (SSCP patterns) I and II possessed homozygotic states whereas diplotype (SSCP pattern) III possessed heterozygtic states. Like results from SSCP analysis, relationships between SNPs of *PmCyC* were observed.

Limited polymorphism was found in the amplified *PmCdc25* gene segment as only one SNP located in the exon region. The SNP3A shrimp A/C$_{243}$ had significantly greater average BW, TL and HPW than those with C/C$_{243}$. Results from DNA sequencing was similar as from SSCP analysis of the same gene region.

Generally, it is accepted that the efficiency to detect single base substitutions by SSCP was approximately 80% of those verified by DNA sequencing (Shastry, 2002). Results from this thesis suggested that SSCP and DNA sequencing are equally powerful for detection SNP in *PmCnn1*, *PmCyC* and *PmCdc25*.

4.3 Detection of SNPs in the amplified *PmCnn1* and *PmCyC* gene segment by PCR-RFLP

Simplification of the detection method of SNP is another important issues. On the basis of DNA sequences, the G/A$_{240}$ found in *PmCnn1*$_{530}$ and T/C$_{382}$ found in

PmCyC allows the development of PCR-RFLP using *Eco* RV (differentiation among G/A_{240}, A/A_{240} and G/G_{240}, N = 60) and *Dde* I (differentiation among C/C_{382}, T/T_{382} and C/T_{382} N = 24). To proof this concept, individuals of the SNP3A sample previously analyzed by SSCP was genotyped by RFLP analysis. Genotyping results were consistent. Therefore, PCR-RFLP which is convenient and cost-effective was successfully developed.

Previously, Glenn et al. (2005) studied association analysis of SNP of alpha-amylase (*AMY2*) and cathepsin-L (*CTSL*). The *AMY2* gene segment contained 4 intronic SNPs (nucleotides 340, 351, 415 and 501) in *L. vannamei* but not in *P. monodon* sequences. The *CTSL* gene segment contained one intronic SNP in *L. vannamei* (nucleotide 681) and *P. monodon* (nucleotide 178) sequences. PCR-RFLP was applied for detecting SNP of those genes (G351A of *AMY2* by *Sca* I, C618G of *CTSL* by *Pvu* II and G178C of *CTSL* by *Pst* I) in two populations of *L. vannamei* (LV1 and LV2, N = 75 and 30 with the mean body weight of 0.35 ± 0.06 and 2.52 ± 0.30 g, respectively) and a mapping population of *P. monodon* (n = 41 for which their body weight is not available). Neither polymorphism of *AMY2* (*Sca* I) nor *CTSL* (*Pvu* II) were found to be significantly associated with the body weight of LV1 and LV2 populations.

4.4 Determination of the expression levels of hepatopancreatic *PmCnn1* and *PmCdc25* in juvenile shrimp exhibiting different growth parameters

Quantitative real-time PCR has been applied to determine the association between the expression levels of interested gene and phenotypic traits of commercially important species. For instance, polymorphisms of 5′ flanking region of chicken prolactin (*cPRL*) gene were examined in several populations of Chinese chickens. Four SNPs were identified at position -2425(C/T), -2215(T/C), -2063(G/A) and -1967(A/G), a 24 bp indel and a poly A length polymorphism were identified from sequencing the 5′ flanking region (2638 bp) of *cPRL*. Quantitative real-time PCR and radioimmunology assay (RIA) was employed to investigate the potential association of the 24 bp indel locus with *cPRL* mRNA expression levels, plasma cPRL and brooding behaviors and observed that chickens with genotype AB (common genotype) had the highest *cPRL* mRNA levels, providing the possibility that

this polymorphic site might be related to the broodiness in chickens via modulating the transcriptional level of the *cPRL* gene (Liang et al., 2005).

Quantitative real-time PCR was carried out to determine whether the expression levels of *PnCnn1* and *PmCdc25* in juvenile shrimp exhibiting different SSCP patterns (SNP genotypes) are different or not. It has been reported that forced expression of chicken gizzard *calponin* in cultured smooth muscle cells and fibroblasts showed an inhibition of cell proliferation (Jiang et al., 1997). Accordingly, the calponin protein functions as a negative controlling factor for cytokinesis and the rate of cell proliferation. The expression level of *PmCnn1* in juvenile shrimp carrying SSCP pattern III was significantly greater than that of genotype I and II ($P < 0.05$). The result was in agreement as thr average body weight and total length of shrimp having SSCP pattern III were significantly lower than those of patterns I and II.

Cdc25 plays an important role in the signal transduction pathway of cell cycle progression. As a result, it is expected that the expression levels of *PmCdc25* should be positively correlated with the progression of cell cycles. Results in this study revealed that the expression level of *PmCdc25* in juvenile shrimp carrying SSCP pattern I was greater than that of genotype II ($P < 0.05$). Apparently, the average BW and TL of the former was significantly greater than that of the latter SSCP patterns. This is concordant with the functions of *cdc25* in the cell cycle.

Recently, a gene encoding prohormone convertase 2 (PC2), an enzyme responsible for post-translational modification of neuroendocrine hormones, has been isolated from the optic lobe of *P. monodon*. The full-length cDNA sequence of *PmPC2* has been identified. *PmPC2* was expressed in all larval developmental stages and in neuroendrocrine cells in the adult optic lobe. Its expression was found to be negatively related with shrimp body weight by qPCR ($P < 0.05$). By using the yeast two hybrid technique, PmPC2 was found to bind with *P. monodon* hyperglycemic hormone (Pem-CHH1) that plays an important role in glucose metabolism. *In vivo* injection of dsRNA-PmPC2 resulted in reduced transcripts for both PmPC2 and Pem-CHH1 on day 3 post injection, but there was no accompanying reduction of glucose level in the hemolymph. PmPC2 has a function(s) in the shrimp neuroendrocrine

system and that it may not only activate Pem-CHH1 but also affect its expression (Tangprasittipap et al., 2012).

In this study, rPmCnn1 protein and its polyclonal antibody were successfully produced. The preliminary results from Western blot analysis did not reveal the differences in immunoreactive band intensity between the SNP3A juveniles exhibiting large, moderate and small sizes. The association experiments at the protein level should be further carried out using a larger number of specimens.

The genetic improvement and other biotechnological applications are crucial to the future sustainable development of *P. monodon* industry. The findings about relationships between genotypes and phenotypes (commercially important traits like growth parameters and expression levels of *PmCnn1*, *PmCyC* and *PmCdc25*) can be applied to assist the ongoing domestication programs of this economically important species.