CHAPTER II

MATERIALS AND METHODS

2.1 Experimental animals and tissue collection

Domesticated *P. monodon* juveniles (3-month-old juveniles, SNP3A, BUM03 and 5-month-old juveniles, PM05) were collected from Burapha University, Chanthaburi (Table 2.1). For BUM03 and PM05 samples, the body weight and total length of each shrimp was measured. Pleopods of each shrimp were dissected out and kept at -20°C. For the SNP3A sample, the body weight, total length and hepatopancreatic weight of each shrimp was measured. Pleopods of each shrimp were dissected out and kept at -20 °C. Hepatopancreas of each shrimp was weighed. Hepatosomatic index (weight of hepatopancreas/the body weight X 100) of each shrimp was calculated. The hepatopancreas was snapped frozen in liquid N_2 and kept at -80°C. Pleopods of each shrimp were dissected out and kept at -20 °C.

Table 2.1 Mean and standard deviations of populations used in this study

Sample set	Age (months)	No. of individuals	Average body weight (mean ± SD)
SNP3A	3	350	11.80 ± 3.88g
BUM03	3	342	13.32 ± 2.42g
PM05	5	305	31.16 ± 6.06g

2.2 Nucleic acid extraction

2.2.1 Genomic DNA extraction

Genomic DNA was extracted from frozen pleopod of each shrimp using a phenol-chloroform-proteinase K method (Klinbunga et al., 1999). The pleopod tissue (approximately 100 mg) was transfer to a 1.5 ml microcentrifuge tube containing 500 µl of the extraction buffer (100 mM Tris-HCl, 100 mM EDTA, 250 mM NaCl; pH 8.0) and briefly homogenized with a micropestle. SDS (10%) and RNase A

(10 mg/ml) were add to a final concentration of 1.0 % (w/v) and 100 μg/ml, respectively. The mixture was incubated at 37°C in water bath for 1 hour until tissue has disintegrated. Afterward, proteinase K solution (10 mg/ml) was added to the final concentration of 200 μg/ml and the tube was left at 55°C in water bath for 3 – 4 hours until tissue is dissolved. After incubation, an equal volume of phenol: chloroform: isoamylalcohol in a 25:24:1 ratio was added and mixed gently (by invertion of the tube) for 10 minutes and centrifuged at 12,000 rpm for 10 minutes at room temperature. The top aqueous layer containing the DNA was transferred to a sterile microcentrifuge tube. The supernatant was repeated once with phenol–chloroform–isoamyl alcohol (25:24:1) and twice with chloroform:isoamylalcohol (24:1). The aqueous layer was transferred into a sterile microcentrifuge tube. One-tenth volume of 3 M sodium acetate, pH 5.2 was added. The DNA was precipitated with two volumes of cold absolute ethanol incubated at -80°C for 30 minutes. DNA was precipitated by centrifugation at 12,000 rpm, 4 °C for 10 minutes. The precipitated DNA was washed twice with 1 ml of cold 70% ethanol. The tube was inverted gently and centrifuged at 12,000 rpm at 4 °C for 10 minutes. The supernatant was removed. The pellet was dried at room temperature and dissolved in 30 – 50 μl of TE buffer (10 mM Tris-HCl, pH 8.0 and 0.1 mM EDTA). The DNA solution was incubated at 37 °C for 1 – 2 hours and kept at 4°C for immediately used or stored at –20°C for a long period of time. Genomic DNA concentrations were measured using spectrophotometer and DNA stocks were diluted to 12.5 ng/μl for use in PCR reactions.

2.2.2 RNA extraction

Total RNA was isolated from hepatopancreas of individual shrimp using TRI Reagent®. A piece of tissue was placed in a mortar containing liquid nitrogen and ground to fine powder. The tissue powder was transferred to a microcentrifuge tube containing 500 μl of TRI Reagent and homogenized. Additional 500 μl of TRI Reagent were added. The homogenate were left for 5 minutes at room temperature before adding 0.2 ml of chloroform. The homogenate was vortexed for at least 15 seconds, left at room temperature for 2-15 minutes and centrifuged at 12000g for 15 minutes at 4 °C. The mixture was separated into the lower red, phenol-chloroform phase, the interphase, and the colorless upper aqueous phase. The aqueous phase

(inclusively containing RNA) was transferred to a new 1.5 ml microcentrifuge tube. Total RNA was precipitated by an addition of 0.5 ml of isopropanol and mixed thoroughly. The mixture were left at room temperature for 10-15 minutes and centrifuged at 12000g for 10 minutes at 4°C. The supernatant was then removed. The RNA pellet was washed with 1 ml of cold 75 % ethanol prior to centrifugation at 12000g for 5 minutes at 4°C. The ethanol wash removed. The RNA pellet was air-dreid for 5-10 minutes and then dissolved in appropriate volume of DEPC-treated H_2O for immediately used. Alternatively, the total RNA pellet was kept under absolute ethanol in a -80°C freezer for long storage.

2.2.3 DNase I treatment of the extracted RNA

Ten micrograms of total RNA wash treated with DNase I (0.5 U/μg of total RNA, Promega) at 37°C for 30 minutes. After incubation, the sample was gently mixed with a sample volume of phenol–chloroform–isoamyl alcohol (25:24:1) for 10 minutes. The sample was centrifuged at 12000g for 10 minutes at 4°C, and the upper aqueous phase was collected. The extraction process was then repeated once with chloroform–isoamyl alcohol (24:1) and repeated again with chloroform. The final aqueous phase was mixed with one-tenth volume of 3 M sodium acetate, pH 5.2. The RNA was precipitated by adding two volume of cold absolute ethanol. The mixture was incubated at -80°C for 30 minutes. The precipitated RNA was recovered by centrifugation at 12000g for 10 minutes at 4°C. The RNA pellet was washed twice with 1 ml of cold 75 % ethanol before used or kept the RNA pellet in absolute ethanol at -80°C until required.

2.3 Measurement of nucleic acids concentrations using spectrophotometry and electrophoresis

2.3.1 Estimation of DNA and RNA concentrations using spectrophotometry

DNA or RNA quantification can be estimated by spectrophotometric measurement of UV absorption at wavelength 260 nm and 280 nm. An OD_{260} of 1.0 corresponds to a concentration of 50 μg/ml double stranded DNA, 40 μg/ml single stranded RNA and 33 μg/ml oligonucleotide (Sambrook et al., 2001). Therefore, the

concentration of DNA/RNA samples were estimated in μg/ml by using the following equation;

Nucleic acid concentration = OD_{260} x dilution factors x nucleic acid factor

; where nucleic acid factor = 50, 40 or 33 for DNA, RNA and oligonucleotides, respectively

An estimate of DNA or RNA purity can be made from OD_{260} / OD_{280} ratios. The ratio of OD_{260} / OD_{280} of a pure DNA solution is between 1.8 to 2.0. The ratio lower than 1.8 indicated contamination of protein or phenol whereas the ratio greater than this value indicate contamination of RNA in the DNA solution.

2.3.2 Estimation of the amount DNA using electrophoresis

Quantification of DNA can be roughly evaluated by running the DNA sample on 0.8 - 1.0% agarose gel stained with ethidium bromide (0.5 μg/ml). DNA extracts are loaded. A rough estimated of DNA content can be obtained by comparing bands intensity of DNA extract with undigest λDNA as standards. Genomic DNA is high molecular weight will appear as a well-resolved band alongside the λDNA band. The smearing below the band indicates shearing of DNA. A smeared band towards the bottom of the gel is an indication of the presence of RNA in the extract.

2.4 Identification of SNPs in the growth-related genes of *P. monodon* using polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP)

Homologues of gene functionally related with growth of *P. monodon* were identified by EST analysis. Primers were designed and used to amplified genomic DNA of *P. monodon* juveniles. The amplified gene segment that did not exhibit differences in size polymorphism or allelic variants of individuals were further characterized using SSCP analysis to examine whether single nucleotide polymorphism (SNP) caused the polymorphism in the same gene segments in different shrimp individuals was existent.

2.4.1 Design of primers from EST of *P. monodon*

Four primers pairs were designed from gene homologue including Cnn1-F/R, Cnn1-F3/R3, CyC-F/R and Cdc25-F/R using Primer Premier 5. The expected amplification size against the cDNA template from each primers is show in Table 2.2.

2.4.2 Polymerase chain reaction (PCR)

Generally, PCR amplification were carried out in 25 µl reaction mixtures containing 1X buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100), 100 µM dNTP, 1.5 mM $MgCl_2$, 0.1 µM of each primer (Table 2.2), 25 ng of genomic DNA of *P. monodon* and 0.5 unit of DyNAzyme^{TM} II DNA Polymerase (Finnzymes). The amplification profiles were carried out following conditions described in Table 2.2. The amplification products are separated by using 1.5% agarose gels electrophoresis. The migrated DNA is visualized under a UV transilluminator after ethidium bromide staining.

Table 2.2 Primer names, the expected size, PCR profiles and recipes for amplification of genomic DNA of *P. monodon*

Gene	Primer name	Size (bp)	dNTP (µM)	$MgCl_2$ (µM)	Primer (µM)	PCR conditions
Calponin1	Cnn1-F/R	316	100	1.5	0.1	94°C, 3 min for 1 cycle followed by 94°C, 45 sec; 55°C, 45 sec and 72°C, 45 sec for 35 cycles and 72°C, 7 min
	Cnn1-F3/R3	119	100	1.5	0.1	94°C, 3 min for 1 cycle followed by 94°C, 45 sec; 56°C, 45 sec and 72°C, 45 sec for 35 cycles and 72°C, 7 min.
Cyclin C	CyC-F/R	280	100	1.5	0.1	94°C, 3 min for 1 cycle followed by 94°C, 45 sec; 56°C, 30 sec and 72°C, 45 sec for 35 cycles and 72°C, 7 min
Cdc25	Cdc25-F/R	285	100	1.5	0.1	94°C, 3 min for 1 cycle followed by 94°C, 30 sec; 58°C, 30 sec and 72°C, 45 sec for 35 cycles and 72°C, 7 min

2.4.3 Agarose gel electrophoresis

The electrophoresis unit was prepared by cleaning the tray, running platform and combs. A measured quantity of 1x TBE buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.0 mM EDTA, pH 8.3) was added to a volumetric flask containing the desired amount of powered agarose. The gel slurry was heated in the microwave until the powered agarose completely dissolved. The gel solution was left at room temperature to approximately 55°C. The warm agarose solution was poured into a gel mould slowly without the air bubbles and the combs were inserted. The gel was allowed to set for about 30-45 minutes until the gel solidifies completely, then a small amount water (or TE) was poured on the top of the gel and the comb was carefully removed. The 1x TBE buffer was added in the gel chamber and the gel mould was placed in the gel chamber. Sample are mixed with the loading dye (0.25% bromphenol blue and 25% ficoll) and loaded into the well. A 100-bp DNA ladder was loaded alongside with the sample as a standard DNA marker. Electrophoresis was carried out at 4 - 5 V/cm until the tracking dye migrated about three-quartered of the gel. After electrophoresis, the gel was stained with ethidium bromide solution (0.5 μg/ml) for 5 min and destained to remove unbound EtBr by submerged in H_2O for 15-20 minutes. The migrated DNA were visualized under the UV light using a UV transilluminator.

2.4.4 SSCP analysis

2.4.4.1 Preparation of glass plates

The two glass plate consist of the short and long glass plate should be washed thoroughly using water with detergent and rinsed under running water until no remain of detergent and air-dried. The plates are wiped three times by 95% ethanol in one direction with tissue paper. The long glass plate was coated with 1 ml of freshly prepared Bind silane (4 μl of Bind silane, Amersaham Biosciences; 995 μl of 95% ethanol and 5 μl of 5% glacial acetic acid) and left for approximately 10 - 15 minutes. The short glass plate was coated with Repel silane (2% dimethyldichlorosilane in octamethylcyclotetrasitoxone). After coated, the two glass plates were further cleaned with 95% ethanol for 3 times. The cleaned glass plates were assembled with a pair of 0.4 mM spacer.

2.4.4.2 Preparation of non-denaturing polyacrylamide gels

Different concentration of low closslink non-denaturing polyacrylamide gels (37.5:1 or 75:1 of acrylamide and bis-acrylamide) were prepared for a 40% stock solution. The 40% stock solution was diluted by 10x TBE and sterile water to required percent gel concentration. The acrylamide gel solution of 40 ml was de-gassed under vacuum for 15 minutes and 300 µl of 10% APS (prepared freshly) and 30 µl of TEMED was add to the acrylamide solution. The mixture was mixed immediately and quickly poured to the casting gel cassette. After filling the solution, the analytical comb was inserted into the prepared gel and the gel was allowed to polymerize for at least 4 hours.

2.4.4 3 Preparation of samples

For SSCP analysis, 6 µl of the each amplification products were mixed with four volumes (24 µl) of the SSCP loading dye (95% formamide, 0.25% bromophenol blue, 0.25% xylene cyanol and 10 mM NaOH). The sample mixture was denatured at 95°C for 5 minutes and immediately cooled on ice for 2-3 minutes. The denatured products were electrophoretically analyzed in non-denaturing polyacrylamide gels at 200 volts for 16 hours at 4°C.

2.4.4.4 Silver staining

After electrophoresis, the short plate was carefully separated from the long plate. The gel should be strongly attached to the long glass plate. The long glass plates with the gel were transferred to a plastic tray containing 2 liters of the fix/stop solution (10% glacial acetic acid) and agitate well for 30 minutes. The gel may be stored in fix/stop solution overnight (without shaking). The fix/stop solution was kept into a beaker for terminate the developing reaction. The gel was soaked 3 times (10 minutes each) with deionized water with agitation. The gel was transferred to 1.5 liters staining solution (0.1% silver nitrate) and agitated well for 30 minutes. The developing solution was prepared by adding 2.25 ml of formaldehyde and 300 µl of sodium thiosulfate (10 mg/ml) to the chilled sodium carbonate solution (3 liters). A half of the chilled developing solution was poured into a tray. The gel was submerged into the tray containing 1.5 liters of deionized water with shaking (10

forward and 10 backward steps) and the gel was placed immediately in the tray containing 1.5 liters of the chilled developing solution. The timing of this step is very important. The time taken to soak the gel in the water and transfer it to chilled developing solution should be no longer than 5 – 10 seconds. The gel was well agitated by shaking until the marker band starts to develop or until the first bands are visible (usually 1.5 – 2 minutes). The gel was transferred to the remaining 1.5 liters of chilled developing solution and continues developing until all bands become visible. The fix/stop solution was directly added to the developing solution and continued shaking for 3 minutes. The stained gel was soaked in deionized water twice for 3 minutes each. The gel was left at room temperature for 2-3 hours.

2.5 Relationships between SSCP patterns and growth parameters of *P. monodon*

A SSCP pattern of each individual for each gene segment was recorded across overall specimens. SSCP patterns from different sample sets were named differently (patterns I, II and III, etc for the SNP sample, patterns A, B and C, etc. for the BUM03 sample and patterns 1, 2 and 3 etc. for the PM05 sample). Relationships between SSCP genotypes of each gene and body weight and total length (and hepatopancreatic weight and HSI for the SNP3A sample) of 3- and 5-month-old shrimp were statistically analyzed using one way analysis of variance (ANOVA) following by Duncan's new multiple range tests. When only 2 SSCP patterns were found in the examined sample, an independent t-test was applied. The significant differences were considered if P-value was less than 0.05 ($P < 0.05$).

2.6 Identification of SNPs of the cloned PCR products by DNA sequencing

2.6.1 PCR and electrophoresis

The amplification product of representative individuals exhibiting each SSCP patterns of *PmCnn1*$_{530}$ (F/R) and *PmCnn1*$_{425}$ (F3/R3), *PmCyC* and *PmCdc25* (N = 5 or 10 for each pattern for the total identified patterns of more than 3 and less than 3 patterns, respectively) was amplified by PCR. The amplification product was electrophoresed through 1.5% agarose gel. Electrophoresis of the PCR product was fractionated through agarose gels in duplication. One was run side-by-side with a 100 bp DNA markers and the other was loaded into the distal well of the gel. After electrophoresis, lanes representing the DNA standard and its proximal DNA sample

were cut and stained with ethidium bromide (0.5μg/ml) for 5 minutes. Positions of DNA markers and the EtBr-stained fragment were used to align the position of the non-stained target DNA fragment. The DNA fragment was excised from the gel with a sterile razor blade.

2.6.2 Elution of DNA from agarose gel

DNA was eluted out from the agarose gels using an illustra™ GFX PCR DNA and Gel Band Purification kit (GE Healthcare) according to the protocol recommended by the manufacture. The excised gel was transferred into a microcentrifuge tube and weighed. The 400-500 μl of capture buffer type 3 were added. The mixture was incubated at 60°C for 15-30 minutes and mixed by invertion of the tube every 3 minutes. After the agarose gel was completely dissolved, an illustra™ GFX MicroSpin column was place into the collection tube. The sample mixure was transferred onto the assembled GFX MicroSpin column which placed, incubated at room temperature for 1 minute and centrifuged at 13,000 rpm for 2 minutes. The flow through in the collection tube was discarded and the GFX MicroSpin column was place back inside the collection tube. The 500 μl of wash buffer type 1 were added to the GFX MicroSpin column and centrifuged at 13,000 rpm for 2 minutes. After the first centrifuge, the flow through was discarded and the tube was centrifuged at 13,000 rpm for an additional one minute to dry the column. The GFX MicroSpin column was transferred to a new microcentrifuge tube. The 10-15 μl of elution buffer type 4 was added to the center of membrane in the assembled GFX MicroSpin column and microcentrifuge tube. The tube was incubated at room temperature for 2 minute and centrifuged at 13,000 rpm for 2 minutes. The eluted sample was stored at -20°C until further required.

2.6.3 Ligation of eluted DNA to pGEM®-T easy vector

The gel-eluted DNA fragments was ligated to the pGEM®-T easy vector in a total volume of 10 μl containing 3 ul of the gel-eluted PCR product, 25 ng of pGEM®-T easy vector, 5 μl of 2X rapid ligation buffer (60 mM Tris-HCl pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP and 10 % PEG 8000) and 3 Weiss units of T4 DNA ligase. The ligation mixture was gently mixed by pipetting and incubated at 4°C overnight.

2.6.4 Tranformation of the ligation products into *E. coli* JM109

2.6.4.1 Preparation of competent cells

A single colony of *E. coli* JM109 was inoculated in 10 ml of LB broth (1% Bactotryptone, 0.5% Bacto yeast extract and 0.5% NaCl) with vigorous shaking at 37°C for 16 hours. The starting culture was inoculated into 100 ml of LB broth and continued culture at 37°C with vigorous shaking to the OD_{600} of 0.4 – 0.6. The cells was transferred to 50 ml tube and then chilled on ice for 30 minutes before centrifuged at 3,000 g for 15 minutes at 4°C. The cell medium was discarded. The pellets were resuspended in 30 ml of ice-cold $MgCl_2$-$CaCl_2$ solution (80 mM $MgCl_2$ and 20 mM $CaCl_2$) and chilled on ice for 45 minutes before centrifuged at 3,000 g for 15 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 2 ml of ice-cold 0.1 M $CaCl_2$ and divided into 100 µl aliquots. These competent cells could be used immediately or stored at –80°C for subsequent used.

2.6.4.2 Transformation of the ligation product to *E. coli* host cells

The competent cells were thawed on ice for 5 minutes. Three microliters of the ligation mixture was added and gently mixed by pipetting. The mixture was incubated on ice for 30 minutes. The cells were heat-shock for 45 seconds in a water bath at exactly 42°C without shaking. The tube was returned immediately to ice for 5 minutes. The cells transformed with ligation reactions were transferred to the tube containing 1 ml of room temperature SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose). The cell suspension was incubated with shaking at 37°C for 90 minutes. At the end on the incubation, the cultured cell suspension was centrifuged at 8,000 rpm for one minute at room temperature. The pellet was gently resuspended in 100 µl of SOC medium and spread on a LB agar plate containing 50 µg/ml of ampicillin, 25 µg/ml of IPTG and 20 µg/ml of X-gal. The spread agar plate was further incubated at 37°C overnight. The colonies containing inserted DNA are white while those without inserted DNA are blue.

2.6.4.3 Detection of recombinant clone by colony PCR

Colony PCR was performed in a 25 µl reaction volume containing 1X buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100), 100 µM dNTP, 1.5 mM $MgCl_2$, 0.2 µM of pUC1 (5'-TCC GGC TCG TAT GTT GTG TGG A-3') and pUC2 (5'-GTG GTG CAA GGC GAT TAA GTT GG-3') primers and 0.5 unit of DyNAzyme™ II DNA Polymerase. A recombinant colony was picked up by the a sterile toothpick and mixed well in the amplification reaction. The PCR profiles was predenaturing at 94°C for 3 minutes, followed by 35 cycles of 94°C for 30 seconds, 50°C for 60 seconds and 72 °C for 60 seconds. The final extension was carried out at 72°C for 7 minutes. The resulting PCR products were analyzed by agarose gel electrophoresis.

2.6.4.4 Isolation and digestion of recombinant plasmid DNA

A recombinant clone was inoculated into 3 ml of LB broth (1% Bacto-tryptone, 0.5% Bacto-yeast extract and 1.0 % NaCl) containing 50 µg/ml of amphicillin and incubated at 37°C with constant shaking at 250 rpm overnight. The recombinant plasmid was extracted using an illustra™ plasmidPrep Mini Spin kit (GE Healthcare). The culture was transferred into 1.5 ml microcentrifuge tube and centrifuged at 12,000 g for 1 minute. The cell pellet was collected and resuspended with 175 µl of the Lysis buffer type 7 (containing RNase A). The mixture was completely dispersed by vortexing. The mixture was then lysed with 175 µl of the Lysis buffer type 8 and gently mixed by inverting the tube approximately 10 times. Additionally, 350 µl of the Lysis buffer type 9 was added and gently mixed.

To separate the cell debris, the mixture was centrifuged at 12,000 g for 15 minutes. The supernatant was transferred into the the illustra™ plasmid mini column and centrifuged at 12,000 rpm for 2 minutes. The flow-through was discarded. The illustra™ plasmid mini-column was washed by adding 400 µl of the Wash buffer type 1 and centrifuged at 12,000 rpm for 2 minutes. The flow-through was discarded. The colum matrix was centrifuged for an additional 1 minute. The illustra™ plasmid mini column was placed in a new 1.5 ml microcentrifuge tube and 30 µl of the Elution buffer type 4 was added to elute the extracted plasmid DNA. The column was left at

room temperature for 2 minute and centrifuged at 12,000 g for 1 minute.

The insert size of each recombinant plasmid was examined by digestion of the plasmid with *Eco* RI. The digest was carried out in a 15 µl containing 1X restriction buffer (90 mM Tris-HCl; pH 7.5, 10 mM NaCl and 50 mM $MgCl_2$), 1 µg of recombinant plasmid and 2 – 3 units of *Eco* RI. The reaction was incubated at 37°C for 3-4 hours before analyzed by agarose gel elctrophoresis.

2.6.4.5 DNA sequencing

The nucleotide sequence of recombinant plasmid were examined by automated DNA sequencer using M13 forward and/or M13 reverse primer as the sequencing primer by MACROGEN (Korea). Nucleotide sequences were multiple-alignment using clustal W (http://www.genome.jp/tools/clustalw/)

2.7 Isolation and characterization of the full-length cDNA of *P. monodon cyclin c (PmCyC)* using Rapid Amplification of cDNA Ends-Polymerase Chain Reaction (RACE-PCR)

2.7.1 First strand cDNA synthesis

Total RNA was extracted from ovaries of *P. monodon* using TRI Reagent. Messenger (m) RNA was purified using a QuickPrep micro mRNA Purification Kit (Amersham Phamacia Biotech). The RACE-Ready cDNA template was synthesized using a BD SMART™ RACE cDNA Amplification Kit (BD Clontech) by combining 1.5 µg of ovarian mRNA with 1 µl of 5′CDS primer and 1 µl of 10 µm SMART II A oligonucleotide for 5′RACE-PCR and 1 µg of ovarian mRNA with 1 µl of 3′CDS primer A oligonucleotide for 3′ RACE-PCR (Table 2.3). The components were mixed and briefly centrifuged. The reaction was incubated at 70 °C for 2 minutes and snap-cooled on ice for 2 minutes. The reaction tube was briefly centrifuged. After that, 2 µl of 5X First-Strand buffer, 1 µl of 20 mM DTT, 1 µl of dNTP Mix (10 mM each) and 1 µl of PowerScript Reverse Transcriptase were added. The reactions were mixed by gently pipetting and briefly centrifuged. The tubes were incubated at 42 °C for 1.5 hours in an air incubator. The first-strand reaction products were diluted with 125 µl of Tris-TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) and heated at 72 °C for 7 minutes.

2.7.2 RACE-PCR

2.7.2.1 Primer design for 3′ RACE-PCR

A homologue of *PmCyC* was obtained from EST analysis of the hepatopancreas cDNA library of *P. monodon*. A gene-specific primer (GSPs) was designed using Primer Premier 5′ and 3′ RACE-PCR for isolation of the full-length cDNA of *PmCyC* amplification was carried out using the forward primer 3′CyC (24 bp, F: 5′-CCACCAGTGTCTTCCTCTCATCCA-3′, Tm = 70°C).

Table 2.3 Primer sequences for the first strand cDNA synthesis and RACE-PCR

Primer	Sequence
SMART II A Oligonucleotide	5′-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3′
3′-RACE CDS Primer A	5′-AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$N-1N-3′ (N=A, C, G or T; N-1=A, G or C)
5′-RACE CDS Primer	5′-(T)25N-1N-3′ (N=A, C, G or T; N-1=A, G or C)
10X Universal Primer A Mix (UPM)	Long: 5′-CTAATACGACTCACTATAGGGCAAGCAG TGGTATCAACGCAGAGT-3′ (0.4 μm)
	Short: 5′-CTAATACGACTCACTATAGGGC-3′ (2 μm)
Nested Universal Primer A (NUP)	5′-AAGCAGTGGTATCAACGCAGAGT-3′ (10 μm)

The master mix for 3′ RACE-PCR and the control reactions was prepared. For each amplification reaction, 14 μl of deionized H_2O, 2.5 μl of 10X Advantage 2 PCR buffer, 0.5 μl of dNTP mix (10 μM each) and 0.5 μl of 50X Advantage 2 polymerase mix were combined. 3′RACE-PCR were set up according to Table 2.4. The reaction was carried out for 20 cycles composing of a 94 °C for 30 seconds, 68 °C for 30 seconds and 72 °C for 3 minutes. The primary 3′RACE-PCR products were electrophoretically analyzed. The gel-eluted product was cloned into pGEM-TEasy and further characterized by DNA sequencing.

Table 2.4 Composition of 3′ RACE-PCR

Component	3′-RACE Sample	UPM only (Control)	GSP1 only (Control)
3′-RACE-Ready cDNA	1.5 µl	1.5 µl	1.5 µl
UPM(10X)	5.0 µl	5.0 µl	-
3′GSP(GSP2, 10µM)	1.0 µl	-	1.0 µl
H_2O	-	1.0µl	5.0 µl
Master Mix	17.5 µl	17.5 µl	17.5 µl
Final volume	25 µl	25 µl	25 µl

2.8 Development of PCR-RFLP for detection of SNP in *Calponin1* and *Cyclin C* of the giant tiger shrimp *Penaeus monodon*

2.8.1 PCR-RFLP

Restriction patterns of $PmCnn1_{530}$ and *PmCyC* of *P. monodon* juveniles previously analyzed by SSCP and DNA sequencing (N = 60 and 24) were examined. Restriction enzyme cutting sites within the amplified gene region were predicted using NEBcutter V2.0 (http://tools.neb.com/NEBcutter2/). The PCR products of *PmCnn1* and *PmCyC* were amplified. An aliquot of 5 µl of each PCR product was digested with 2.5 units of *Eco* RV (GATATC; $PmCnn1_{530}$) or *Dde* I (CTNAG; *PmCyC*) in a final volume of 15 µl containing 1X appropriate restriction enzyme buffer (6 mM Tris-HCl; pH 7.5, 6 mM $MgCl_2$, 50 mM NaCl and 1 mM DTT). The reaction mixture was incubated overnight at 37°C. The digestion product was size-fractionated by agarose gel electrophoresis and stained with EtBr.

2.9 Examination of expression levels of *PmCnn1* and *PmCdc25* in hepatopancreases of *P. monodon* by quantitative real-time PCR

2.9.1 Primer design

The partial exon/intron sequence of interested genes was characterized and sequenced. Several pairs of primers were designed from cDNA sequence of *PmCnn1* and *PmCdc25* by avoiding co-amplification of genomic DNA in reaction.

The specificity of primers to cDNA can increase the efficiency of amplification and prevent the false amplification of the residual genomic DNA in cDNA template solution. The sense or antisense primer covering intron-exon boundaries should be designed. Alternatively, the primer pairs sandwiching the large intron could be designed. An appropriate size of the expected amplification product was approximately 100-250 bp.

2.9.2 Construction of the standard curves

For construction of standard curve of each gene, the DNA fragment covering the target PCR product and *EF-1α* were amplified. The PCR product of each gene was cloned. The extracted recombinant plasmid DNA was used as the template for copy number estimation. A ten-fold serial dilution was prepared corresponding to 10^3-10^8 molecules/µl. Copy number of standard DNA molecules can be calculated according to the following equation;

X g/µl DNA / [plasmid length in bp x 660] x 6.022×10^{23} = Y molecules/µl

*(1 kb = 6.6×10^5 Dalton; 1 µg of 1 kb cDNA contains 0.91×10^{12} molecules)

The standard samples were performed in a 96-well plate and each standard point was run in duplicate. The appropriate standard curve having 0.995-1.00 of correlation coefficient or efficiency higher than 95% was generated from the run and used in normalization of each plate.

2.9.3 Quantitative real-time PCR analysis

The cDNA target transcripts and the internal control *EF-1α*, were amplified in a 10 µl reaction volume containing 2X LightCycler® 480 SYBR Green I Master (Roche, Germany) and gene specific primers at a final concentration of 0.2-0.3 µM. The amplification was carried out using a LightCycler® 480 Instrument II system (Roche). The amplification profile for SYBR Green real-time PCR was initially pre-denatured at 95°C for 10 minutes following by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 55-58°C for 30 seconds and extension at 72°C for 15-30 seconds. Melting curve analysis was subsequently performed at 95°C for 15 seconds, 65°C for 1 minute and at 98°C for continuation and cooling at 40°C for 30 seconds. The real-time PCR assay of each specimen were run in duplicate in a 96-well plate.

2.9.4 Statistical analysis

The relationships between SSCP patterns of each gene and the expression level of *PmCnn1* and *PmCdc25* were examined using ANOVA followed by a Duncan's new multiple range test and independent-sample t-test ($P < 0.05$), respectively.

2.10 *In vitro* expression of recombinant protein using a bacterial expression system

2.10.1 Primer design

The forward and reverse primers of *PmCnn1* overhang with *Bam* HI and *Xho* I+ 6 residues of His were designed to amplify the complete ORF of *PmCnn1* (Table 2.5).

Table 2.5 Nucleotide sequences of primers overhang used for *in vitro* expression of *Calponin1* of *P. monodon*

Primer	Sequence (5'-3')
Complete ORF containing restriction site	
Cnn1-BamHI-F	F:5'-CGGGGATCCATGAACCGTGCTACCAA-3'
Cnn1- XhoI-R	F: 5'-CTCGAGTTAATGATGATGATGATGATG CATGTGGCGAGTATTGCC-3'

2.10.2 Construction of recombinant plasmid in cloning and expression vectors

The complete ORF of *PmCnn1* was amplified by PCR. The amplification reaction was performed in a reaction volume of 25 µl including 100 ng of cDNA of hepatopancreases as template, 0.5 µM of each primers as described previously, 0.75-1.5 unit *Pfu* DNA polymerase (Promega) and 0.2 mM of each dNTP. The conditions for PCR were as followed; 2 minutes at 95°C followed by 30 cycles consisting of 45 second at 95°C, 30 second at 55°C and 2 minutes at 72°C. The final extension was carried out for 7 minutes at 72°C. The PCR product was separated on 1.5% agarose gel. The PCR fragments of the predicted size were excised from the agarose gel.

The electrophoresed fragment was eluted out from the gel using illustra^{TM} GFX PCR DNA and Gel Band Purification kit (GE Healthcare) before digested with appropriate restriction enzymes. The digested DNA fragment was again analyzed by agarose gel electrophoresis and the gel-eluted product was ligated with pET-29a expression vector and transformed into *E.coli* JM109. Plasmid DNA was extracted from a positive clone and carrying out the sequencing. The corrected direction of plasmid DNA of *Calponin1* was subsequently transformed into *E.coli* BL21-CodonPlus(DE3)-RIPL.

2.10.3 Expression of recombinant proteins

A single colony of recombinant *E. coli* BL21-CodonPlus (DE3)-RIPL carrying recombinant plasmid of *PmCnn1* was inoculated into 3 ml of LB medium containing 50 μg/ml chloramphinical at 37°C overnight. Fifty microliters of the culture was transferred to 50 ml of LB medium containing 50μg/ml kanamycin, 50 μg/ml chloramphinical and further incubated to an OD_{600} of 0.4-0.6. After IPTG induction (1.0 mM final concentration), appropriate volume of the culture corresponding to the OD of 1.0 was time-interval taken (0, 1, 2, 3, 6, 12 hours and overnight at 37°C) and centrifuged at 12000g for 1 minute. The pellet was resuspended in 1X PBS buffer and examined by 15% SDS-PAGE (Laemmli, 1970). In addition, 50 ml of the IPTG induced cells at the most suitable time-interval were taken (6 hours or overnight at 37°C or lower temperature), harvested by centrifugation 5000 rpm for 15 minutes and resuspended in the lysis buffer (0.05 M Tris-HCl; pH 7.5, 0.5 M Urea, 0.05 M NaCl, 0.05 M EDTA; pH 8.0 and I mg/ml lysozyme). The cell wall was disrupted by sonication using Digital Sonifier® sonicator Model 250 (BRANSON). The bacterial suspension was sonicated 2-3 times at 15-30% amplitude, pulsed on for 15 seconds and pulsed off for 15 second in period off 2-5 minutes. Soluble and insoluble portions were separated by centrifuged at 14000 rpm for 30 minutes. The protein concentration of both portions was measured using a dye-binding assay (Bradford, 1972). Expression of the recombinant protein was electrophoretically analyzed by 15% SDS-PAGE.

2.10.4 Detection of recombinant PmCnn1 protein

Recombinant PmCnn1 protein was separated with SDS-PAGE and electroblotted onto a PVDF membrane (Hybond P; GE Healthcare) (Towbin, 1979) in 25 mM Tris, 192 mM glycine (pH 8.3) containing 10% methanol at a constant current of 350 mA for 1 hour. The membrane was treated in the blocking solution (Roch) for 1 hour and incubated with the primary antibody (Anti-His (Biorad)) 1:1000 for 1 hour at room temperature. After washing with Tris-buffered saline Tween-20 (TBST; 50 mM Tris-HCl, 0.15 M NaCl, pH 7.5, 0.1% Tween-20). the membrane was incubated with a second antibody, goat anti-rabbit IgG (H+L) conjugated with alkaline phosphatase (Bio-Rad Laboratories) at 1:10,000 for 1 hour. The membrane was washed 3 times with 1X Tris-buffer saline Tween-20 TBST; 50 mM Tris-HCl, 0.15 M NaCl, pH 7.5, 0.1% Tween-20) and incubated and then washed 3 times with 1X TBST. Detection was performed using NBT/BCIP (Roch) as a substrate. The color reaction was stopped by transferring the membrane into water.

2.10.5 Purification of recombinant proteins

Recombinant PmCnn1 protein was purified using a His Gravitrap kit (GE Healthcare). The column was pre-equilibrated with binding buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4). Sample prepared from the previous step was harvested by centrifugation at 5000 rpm for 15 minutes. The pellet was resuspended in the binding buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4), sonicated and centrifuged at 14000 rpm for 30 minutes. The soluble and insoluble fractions were separated. Soluble fraction composed of the recombinant PmCnn1 protein was loaded into column. The column was washed with 10 ml of the binding buffer containing 20 mM imidazole (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4), 5 ml of the binding buffer containing 40 mM imidazole (20 mM sodium phosphate, 500 mM NaCl, 40 mM imidazole, pH 7.4) followed by 5 ml of the binding buffer containing 80 mM imidazole (20 mM sodium phosphate, 500 mM NaCl, 80 mM imidazole, pH 7.4) and 150 mM imidazole (20 mM sodium phosphate, 500 mM NaCl, 150 mM imidazole, pH 7.4). Finally, the recombinant protein was eluted with 6 ml of the elution buffer (20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4). Each fraction of the washing

and eluting step were analyzed by SDS-PAGE and western blotting. The purified proteins were stored at 4°C or 20°C for long term storage.

2.10.6 Polyclonal antibody production and western blot analysis

Polyclonal antibody against the rPmCnn1 protein was immunologically produced in a rabbit by Faculty of Associated Medical Sciences, Chiang Mai University. Western blot analysis was carried out to examine specificity and sensitivity of the antibody.

For western blot analysis of tissue proteins, hepatopancreas were homogenized in the sample buffer (50 mM Tris-HCl, 0.15 M NaCl, pH 7.4) complemented with protease inhibitors cocktail EDTA free (Roche) and homogenized by centrifugation at 12,000 g for 30 minutes at 4°C. The supernatant was collected and determined concentrations by the dye binding method (Bradford, 1976). Twenty-five microliters of proteins were heated at 100°C for 5 min and immediately cooled on ice. Proteins were size-fractionated on a 15% SDS-PAGE (Laemmli, 1970).

Proteins separated with SDS-PAGE were transferred onto a PVDF membrane (Hybond P; GE Healthcare) (Towbin, 1979) in 25 mM Tris, 192 mM glycine (pH 8.3) containing 10% methanol at a constant current of 350 mA for 1 hour. The membrane was treated in the blocking solution (Roch) for 1 hour and incubated with the primary antibody (Anti-PmCnn1PAb) 1:500 in the blocking solution for 1 hour at room temperature. The membrane was washed 3 times with 1X Tris-buffer saline Tween-20 (TBST; 50 mM Tris-HCl, 0.15 M NaCl, pH 7.5, 0.1% Tween-20) and incubated with goat anti rabbit IgG (H+L) conjugated with alkaline phosphatase (Bio-Rad Laboratories) at 1:3000 for 1 hour and washed 3 times with 1X TBST. Visualization of immunoreactive signals was carried out by incubating the membrane in NBT/BCIP (Roch) as a substrate. The color reaction was stopped by transferring the membrane into water.