Chapter II

Materials and Methods

2.1 Equipments

- Autoclave LS-2D (Rexall industries Co. Ltd., Taiwan)
- Automatic micropipettes P20, P100, P200 and P1000 (Gilson Medical Electronic S.A., France)
- Autoradiography cassettes (Research Products International corp., USA)
- A -20 °C Freezer
- A -80 °C Freezer
- Gel dryer Model 583 (Bio-RAD Laboratories, USA)
- Heating block BD 1761G-26 (Sybron Thermermolyne Co.,USA)
- Horizontal gel electrophoresis apparatus
- Hyperfilm MP (Amersham International, England)
- Incubator BM-600 (Memmert GambH, Germany)
- Microcentrifuge tube 0.5, 1.5 ml (Bio-RAD Laboratories, USA)
- PCR Thermal cyclers: Omn-E (Hybaid Limited, England), Model 2400 (Perkin-Elmer Cetus, USA)
- PCR Workstation Model#P-036 (Scientific Co., USA)
- Pentax superA camera
- Plastic Trays
- Plastic wedges
- Pipette tips 100, 1000 µl (Bio-RAD Laboratories, USA)
- Power supplies: PowerPac300 Power Supply (Bio-Rad Laboratories, USA)

- Refrigerated microcentrifuge Kubota 1300 (Kubota, Japan)
- Spectrophotometer DU 650 (Beckman, USA)
- Vertical sequencing gel electrophoresis apparatus (Hoefer, USA)
- UV transilluminator (UVP Inc.)
- Whatman®3 MM Chromatography paper (Whatman International Ltd., England)
- Light box
- UV transilluminator and UVP ImageStore 7500 (UVP Inc.)

2.2 Chemicals

- Absolute ethanol (Merck, Germany)
- Acrylamide (Merck, Germany)
- Acetic acid gracial (Merck, Germany)
- Ammonium persulfate (Promega, USA)
- Boric acid (Merck, Germany)
- Bromophenolblue (Merck, Germany)
- Chelex®100 Resin (Bio-Rad Laboratories, USA)
- Choroform (Merck, Germany)
- Citric acid (Fluka, Switzerland)
- Developer (Eastman Kodak Company, USA)
- Ethidium bromide (Sigma Chemical Co., USA)
- Ethylene diamine tetraacetic acid, disodium salt dihydrate (Fluka, Switzerland)
- 100 mM dATP, dCTP, dGTP, dTTP, (Promega Corporation Medison, Wisconsin)
- Fixer (Eastman Kodak Co., USA)
- Formaldehyde solution, 37 to 41% HCOOH (BDH Chemical Ltd., England)

- Formamide (Gibco BRL Technologies Co., USA)
- GeneAmp PCR core reagents (Perkin-Elmer Cetus, USA)
  : 10X PCR buffer (100 mM Tric-HCl, pH 8.3 , 500 mM KCl)
  : 25 mM $MgCl_2$
- Hydrochloric acid (Merck, Germany)
- Isoamyl alcohol (Merck, Germany)
- MetaPhor® agarose (FMC Bioproducts, USA)
- Methanol (Merck, Germany)
- N,N-methylene-bis-acrylamide (Amersham, England)
- Nitric acid, 70% w/w (Ajax Chemicals, UNIVAR, Australia)
- Oligonucleotide primers (Biosynthesis)
- Potassium acetate (Merck, Germany)
- Phenol crystal (Fluka, Germany)
- Proteinase K (Gibco BRL Technologies Inc., USA)
- RNase A (Sigma Chemical Co., USA)
- SeaKem®LE agarose (FMC Bioproducts, USA)
- T7 Sequencing kit (Pharmacia Biotech, USA)
  : Mix-Short for each dATP, dCTP, dGTP, dTTP
  : Enzyme dilution buffer (20 mM Tris-HCl, pH 7.5, 5 mM DTT, 100 μM BSA/ml 5% glycerol)
  : Universal Primers
  : Annealing buffer (1 M Tris-HCl, 100 mM $MgCl_2$, 160 mM DTT)
  : Labelling Mix- dATP (1.375 μM each dCTP, dGTP and dTTP, 333.5 mM NaCl)
  : Control DNA template
- Silver nitrate (BDH Chemical Ltd., England)

- Sodium carbonate (Ajax Chemicals, UNIVAR, Australia)
- Sodium acetate (Merck, Germany)
- Sodium chloride (Merck, Germany)
- Sodium dodecyl sulfate (SDS, Sigma Chemical Co., USA)
- Sodium hydroxide (Merck, Germany)
- Sodium thiosulfate, 10 mg/ml
- N,N,N',N'-tetramethylethylenediamine (Gibco BRL Technologies Inc., USA)
- Tris-(hydroxy methyl)-aminomethane (Fluka, Switzerland)
- Urea (Ajax Chemicals, Australia)
- Xylene cyanol (Sigma, USA)

2.3 Radioisotopic

- [γ-^{32}P]dATP specific activity 3000 Ci/mmol (Amersham International, England)
- [α-^{32}P]dATP specific activity 800 Ci/mmol (Amersham International, England)

2.4 Enzymes

- Ampli*Taq* DNA polymerase (Perkin-Elmer Cetus, USA)
- T4 Polynucleotide kinase (Pharmacia Biotech, USA)
- T7 DNA Polymerase (Pharmacia Biotech, USA)

2.5 Samples

The black tiger prawn (*P. monodon*) broodstock was wild-caught alive from Trad (N = 51) in February 1998. Genomic DNA of the specimens was prepared by Supungul (1998). These DNA samples were used to determine polymorphism at eight microsatellite loci using primers developed by Pongsomboon et al. (2000).

For comparing of DNA from different extraction methods, pleopods were excised from freshly killed *P. monodon* individuals and immediately placed on dry ice. Alternatively, dissected pleopods or the whole postlarvae from hatcheries were immediately placed into the tubes containing an enough amount of absolute ethanol. Blood samples were drawn from live prawns and mixed with an equal volume of absolute ethanol.

Postlarvae (PL 10-15) from three control crossed families were provided by Dr. P. Jarayabhand, Aquatic Resources Research Institute (ARRI), Chulalongkorn University. These postlarvae were analyzed for Mendelianly inherited fashion of microsatellite loci in *P. monodon*. The specimens were collected and kept in ethanol before transported back to the laboratory at the Department of Biochemistry, Faculty of Science, Chulalongkorn University and stored at -80°C until required.

2.6 DNA Isolation

A pleopod of each frozen black tiger prawn was divided into two parts as the tip of pleopod (tPleopod) and the thigh muscle of pleopod (mPleopod). Each was weighed to approximately 10-30 mg or 2 mm^2 in size. The postlarvae (PL 10-15) with 10-30 mg in weight were also collected. Blood sample usually 20 µl was also used for DNA isolation. Genomic DNA of *P. monodon* specimens were isolated using six different methods described below.

2.6.1 **Phenol-chloroform extraction** (Supungul, 1998)

An aliquot of 20 µl of the blood/ethanol was mixed with 500 µl of high TE (100 mM Tris-HCl, 40 mM EDTA, pH 8.0) and spun to pellet the cells. The supernatant was discarded and the pellet was resuspended with 200 µl of extraction buffer (100mM Tris-HCl, pH 9.0, 50 mM Na_2EDTA, pH 8.0, 100 mM NaCl, 200 mM sucrose). Prawn tissues were carved and briefly ground in 200 µl of extraction buffer with pestle to disperse tissue in the buffer. The homogenate from either blood or tissue was added with 20 µl of 10% (w/v) SDS to a final concentration of 1% and incubated at 65 °C for 10 minutes. After incubation, 2.5 µl of 10 mg/ml RNaseA were added to a final concentration of 111 µg/ml and 5 µl of 20 mg/ml proteinase K to a final concentration of 667 µg/ml, and further incubated at 65 °C for 1 hour. Forty-six microlitres of 5M KOAc were added to a final concentration of 1 M and incubated on ice for 30 to 45 minutes. The reaction mixture was spun at 10,000 rpm for 10 minute. The aqueous layer was carefully removed to a new sterile microcentrifuge tube using a wide-bore pipette tip (about 3 mm). The sample was gently but thoroughly extracted once with an equal volume of buffer-saturated phenol, once with phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform-isoamyl alcohol (24:1). The aqueous layer was transferred to a new tube and 1/10 volume of 3M CH_3COONa, pH 5.2 was added. DNA was precipitated with 2 volumes of ice-cold absolute ethanol.

2.6.2 **Cell lysis / ProteinaseK extraction** (Cook et al., unpublished)

An aliquot of 20 µl of the blood/ethanol was mixed with 500 µl of high TE whereas the tissue was craved and ground in 500 µl of high TE. Samples were vortexd and spun for 10 to 15 second to pellet the cells. The supernatant was removed. The cell pellet was resuspended with 125 µl of MGPL lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 200 mM LiCl, pH 8.0 and 0.8%SDS). A 20 mg/ml proteinase K solution (1.25

μl) were added to a final concentration of 200 μg/ml. The mixture was then incubated at 65 °C until the cells were completely lysed (10-20 minutes). Following incubation, 250 μl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and 3M of NaCl (6.5 μl) were added to a final concentration of 50 mM. The reation mixture was centrifuged at 10,000 rpm for 10 minutes. The supernatant was transferred to a new microcentrifuge tube and DNA was precipitated with the addition of 400 μl of cold isopropanol.

2.6.3 Salting out extraction (Aljanabi and Martinez, 1997)

An aliquot of 20 μl of the blood/ethanol was mixed with 500 μl of high TE and spun to pellet the cells. The supernatant was discarded and resuspended in 200 μl of sterile salt homogenizing buffer (10 mM Tris-HCl, pH 8.0, 2 mM EDTA, pH 8.0 and 400 mM NaCl). Alternatively, the tissue was cut and ground in 200 μl of sterile salt homogenizing buffer. Samples were added and mixed well with 20 μl of 20%SDS and 4 μl of 20 mg/ml proteinase K to final concentrations of 2% and 400 μg/ml, respectively. The mixture was then incubated at 65 °C for 1 hour or until completely lysed. After which, 150 μl of 6M NaCl was added. The mixture was mixed and spun at 10,000 rpm 10 minutes. The supernatant was transferred to a sterile microcentrifuge tube. DNA was precipitated with the addition of an equal volume of cold isopropanol.

2.6.4 Salting out extraction (Martinez et al., 1998)

An aliquot of 20 μl of the blood/ethanol was mixed with 500 μl of high TE and spun to pellet the blood cells. The supernatant was discarded and the pellet was resuspended in 150 μl of resuspension buffer (100 mM Tris-HCl, 100 mM EDTA, 10 mM NaCl, pH 8.0). Alternatively, the tissue was carved and ground in 150 μl of resuspension buffer. The samples were immediately lysed by adding 150 μl of cell lysis buffer (100 mM Tris-HCl, 100 mM EDTA, 10 mM NaCl, 1%SDS, pH 8.0) and gently inverting the tube to mix the mixture thoroughly. The cell lysate was treated

with proteinase K (150 μg/ml) at 65 °C for 1 hour or until completely lysed. One hundred microlitres of 6 M NaCl was added to a final concentration of 1.5 M. The solution was vortex-mixed vigorously for 15 seconds, chilled on ice for 5 to 10 minutes, followed by centrifugation at 10,000 rpm for 10 minutes to partially precipitate hydrolysed polypeptides. If the supernatant was not clear, it was transferred to a new tube and recentrifuged for another 15 minutes. An equal volume of isopropanol was added. The tube was inverted gently several times, causing long strands of high-molecular weight DNA to appear.

The DNA pellet from 2.6.1 to 2.6.4 was washed with 70%ethanol and dried. The dried DNA pellet was resuspended in 20 to 200 μl of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and kept at 4°C until required. The volume of TE buffer added depended on the size of DNA pellet that was obtained.

2.6.5 Chelex-based DNA extraction (Walsh et al., 1991 and Altschmied et al., 1997)

An aliquot of 20 μl of the blood/ethanol was mixed with 500 μl of high TE (or sterile distilled water) and spun to pellet the cells. The supernatant was discarded and resuspended with 200 μl of a chelex working solution (5 to 10%, w/v, in sterile distilled water). Alternatively, the tissue was carved and ground in 200 μl of a chelex working solution. After the sample has been ground, the tube was mixed briefly. The reaction mixture was incubated at 65°C for 1 hour. After incubation, the reaction tube was placed in a boiling water bath for 5 minutes to stop the process and centrifuged to pellet the cell debris and resin. This supernatant contained template DNA used for PCR.

2.6.6 **Alkaline extraction** (described by Rudbeck and Dissing, 1998)

Five microlitres of blood/ethanol were mixed with 20 µl of 0.2 M NaOH and incubated at room temperature for 5 minutes while approximately 10 mg of tissue samples were carved and ground in 500 µl of sterile distilled water. The cells were spun down and the supernatant was discarded. The cell pellet was resuspended with 20 µl of 0.2 M NaOH and incubated at 75 °C for 10 minutes. Reactions were stopped by adding 180 µl of 0.04 M Tris-HCl, pH 7.5. After centrifugation, and the supernatant was transferred to a new sterile tube.

The DNA solution from 2.6.5 and 2.6.6 can be kept at -20 °C for a long storage until required. One microlitre of the extract was usually adequate for a 15-µl PCR reaction. The outlines of DNA extraction methods are illustrated in Figure 2.1.

2.7 **Measurement of DNA concentration**

The concentration of extracted DNA was spectrophotometrically measured at the optical density of 260 nanometre (OD_{260}). An OD_{260} of 1.0 corresponds to a concentration of 50 µg/ml double-stranded DNA. Therefore, the DNA concentration of each sample (in µg/ml) was calculated by ;

$$[\text{DNA}] = OD_{260} \times \text{a dilution factor} \times 50$$

Basically, the concentration of DNA sample used for PCR was diluted to 15 ng/µl in a total volume of 100 µl. Accordingly, the original volume required from each DNA sample can be calculated as follows;

$$\text{Volume required from the DNA sample} = (15 \times 100) / X$$

Where X is the concentration of DNA (in µg/ml) of a particular sample.

Figure 2.1 Outlines of DNA extraction methods used in this thesis

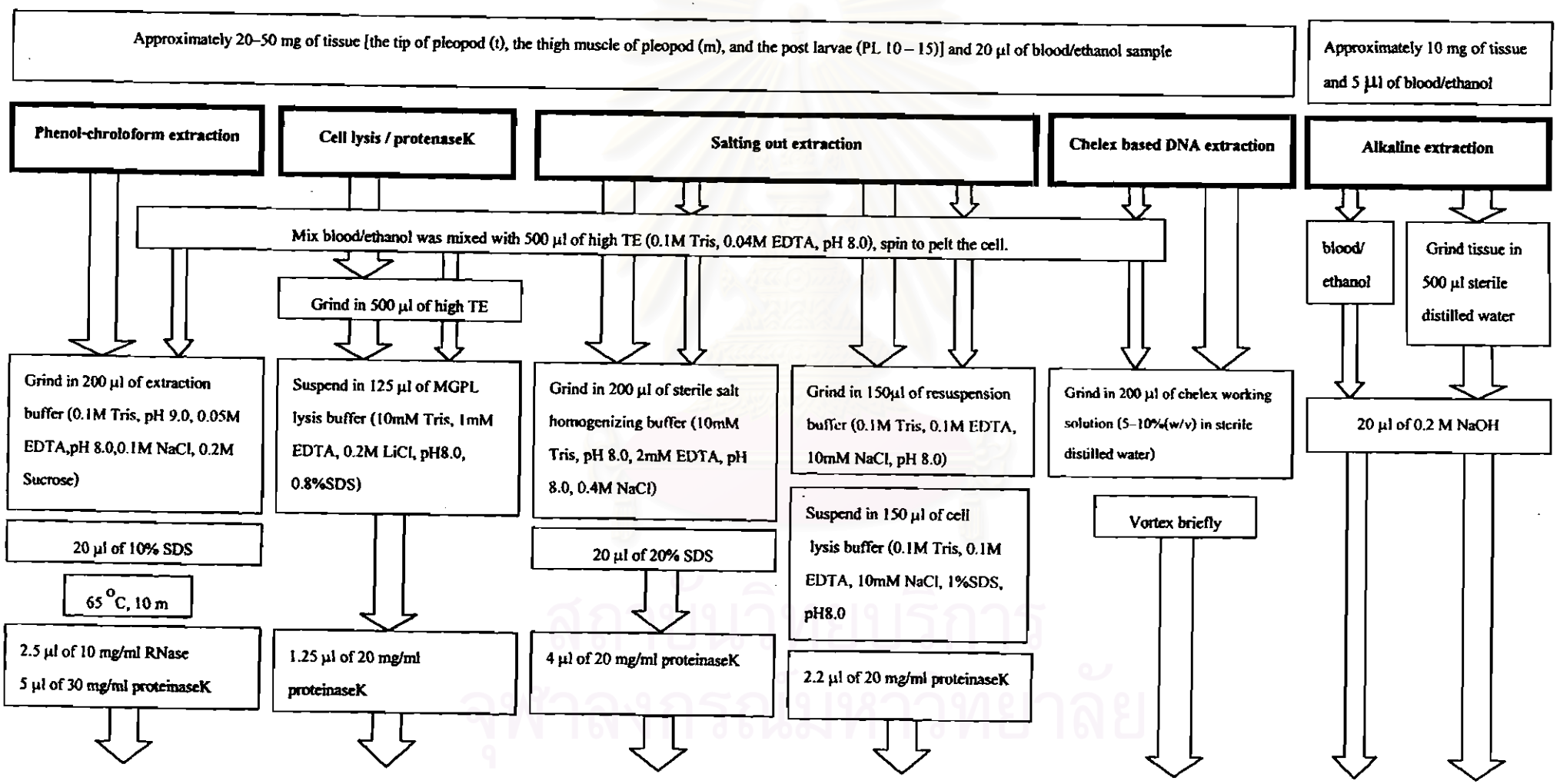
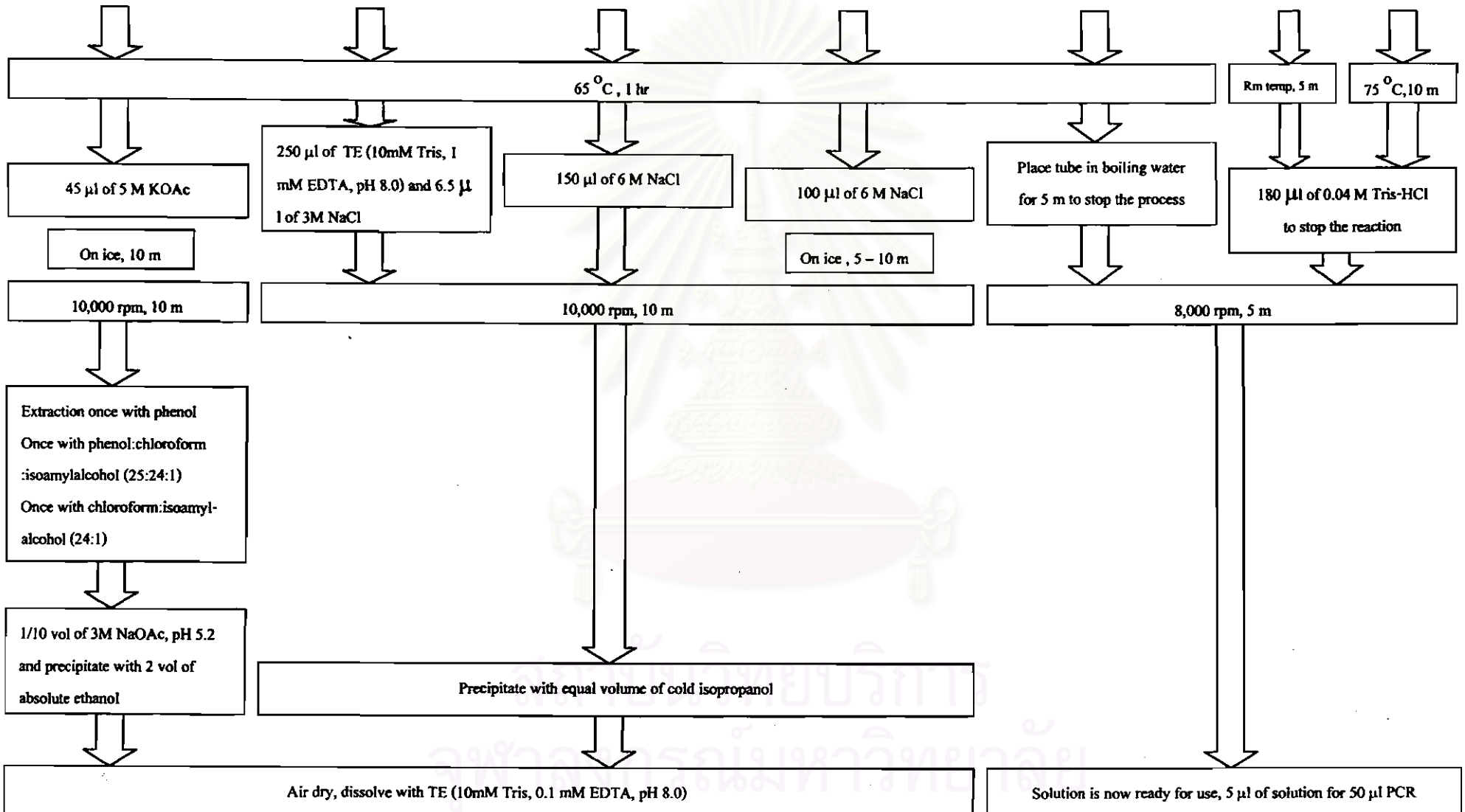


Figure 2.1 continued



The purity of extracted DNA can be examined by the ratio of OD_{260} and OD_{280}. A ratio of 1.8 indicates high quality DNA whereas much higher and lower values of this ratio indicate RNA or protein contamination of isolated DNA samples, respectively (Kirby, 1992).

2.8 PCR primers

The amplification success at the locus Di25 was used to check the quality of isolated genomic DNA from different extraction methods. These primers were kindly provided by Dr. F. Bonhomme, Labortoire Genome et Populations, CNRS URA 193, Universite de Montpellier II, C. C. 63, 34095, Monpellier cedes 5, France through Dr. J. A. H. Benzies, Australian Institute of Marine Science, PMB 3, Townsville, Qld 4810, Australia. Oligonucleotide primers for loci CSCUPmo1, CSCUPmo2, CSCUPmo3, CSCUPmo4, CSCUPmo6, CSCUPmo7, CSCUPmo9 and CSCUPmo11 were developed by Pongsomboon et al. (2000).

2.9 Microsatellite amplification

Microsatellite loci were amplified by the polymerase chain reaction (PCR). PCR products were size-fractionated by denaturing polyacrylamide sequencing gels. If PCR primers were radioactive-labeled, amplified alleles were detected by autoradiography. For nonradioactive-labeled PCR amplification, products were detected by silver staining.

2.9.1 5′-end labeling of primer with T4 polymucleotide kinase

The forward primer for each microsatellite locus was end-labeled using polynucleotide kinase. Approximately 10 pmole of the forward primer of each microsatellite locus (free 5′-OH groups) was used as a substrate for a 10 µl end-labeling reaction containing 1 µl of 10XT4 polymucleotide kinase buffer (0.5 M Tris-

HCl, pH 7.6, 0.1 M $MgCl_2$, 50 mM dithiothreitol, 1 mM spermidine), 30 μCi of [γ-^{32}P] dATP (3,000 μCi/mmol), 10 U of T4 polynucleotide kinase. Sterile distilled water was added to the reaction mixture to make the final volume to 10 μl. The end-labeled mixture was incubated at 37 °C for 30 minutes. At the end of the incubation period, the reaction was terminated by heat-inactivation of T4 polynucleotide kinase at 65 °C for 15 minutes.

2.9.2 PCR of microsatellite loci

Genomic DNA isolated from each part of *P. monodon* such as the tip of pleopod, the top of the thigh of pleopod, blood cells and the postlarvae of prawn (PL 10-15) with six extraction methods were tested against microsatellite locus Di25 following the protocol described by Supungul (1998). For radioactive-labeled methods of the other microsatellite loci, approximately 15 ng of genomic DNA was used in a 5 μl PCR reaction volume containing 0.425 μM of the forward primer, 0.45 μM of the reverse primer, 0.025 μM of the labelled forward primer, 0.5 μl of 10X PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 1.5 mM $MgCl_2$, 200 μM each of dNTPs (dATP, dCTP, dGTP, and dTTP) and 0.15 U of *Taq* polymerase.

For non-isotopic method, approximately 45 ng of genomic DNA were amplified in a 15 μl PCR reaction volume containing 0.45 μM each of forward and reverse primers, 1X PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 1.5 mM $MgCl_2$, 200 μM each of dNTPs and 0.45 U of *Taq* polymerase. The reaction mixture were then overlaid with a drop of RNase- and DNaes-free mineral oil before subjected to the amplification process in a thermal cycler (Omn-E, Hybaid).

The PCR cycling program began with 94 °C for 3 minutes (hot start), followed by 35 cycles of 94 °C for 30 seconds, annealing at 56 °C (except for CSCUPmo4 and

CSCUPmo9 where the annealing temperature was at 54 °C) for 1 minute and extension at 72 °C for 30 seconds followed by the last extension at 72 °C for 10 minutes.

2.9.3 Denaturation polyacrylamide gel electrophoresis

After the amplification process was complete, 4 µl of a formamide dye mixed solution (10 mM NaOH, 99% formamide, 0.1% bromophenol blue and 0.1% xylene cyanol) was added into each amplification reaction. The mixture was heated at 94 °C for 15 minutes and immediately snap-cooled on ice. Three and a half microlitres of the denatured mixture was loaded onto a 6 % denaturing polyacrylamide gel (57% acrylamide, 3% N,N' methylenebisacrylamide, 7.66 M Urea) prepared in 1X TBE (89 mM Tris-HCl, 89 mM Boric acid, 2 mM EDTA, pH 8.3) and then electrophoresed at 45 W for approximately 3 hours.

For non-isotopic method, the denatured mixture was loaded onto 8% denaturing polyacrylamide sequencing gel (76% acrylamide, 4% N,N'methylenebisacrylamide, 7.66 M Urea) and electrophoresed with the same condition for the isotopic method. Separation of microsatellite alleles with 15% denaturing polyacrylamide minigel (10x10 cm, 1 mm thick) was performed by electrophoresis at 200 V for approximately 5 hours. Both gel types were stained with silver staining method.

2.9.4 Detection of microsatellite alleles

Electrophoresed radioactive-labeled products were exposed with the X-ray film to determine sizes of microsatellite alleles. Silver staining procedure described by Bassam et al. (1991) was used to detect non-isotopic amplified products. After electrophoresis, gels were submerged in 10% acetic acid for 10 minutes at room temperature (RT). The gels were briefly rinsed three times (2 minutes each) with

ultrapure water at RT and incubated in a staining solution (0.1% $AgNO_3$ containing 1.5 ml 37% formaldehyde/liter) for 15 minutes. Excess silver ions were eliminated by rinsing the gel with a steam of ultrapure water. Gels were developed in the developing solution (300 ml of 3% sodium carbonate that chilled on ice and immediately before used, 450 µl of 37% formaldehyde and 60 ml of 10 mg/ml sodium thiosulfate were added) at 8-10 °C and agitated until the first bands were visible. The developing solution was poured off. The gels were covered again with the new developing solution and continue developing until the desired band intensity was achieved. This developing reaction was stopped with 10% acetic acid (already used in the fix step) and shaken for 5 minutes. The gels were rinsed two times (2 minutes each) with ultrapure water and kept on a piece Whatman® 3MM paper. The gel was dried at 80 °C under vacuum. The gel was photographed, if desired, for a permanent record.

2.9.5 MetaPhor® agarose gel electrophoresis

MetaPhor agarose gels were also used to size-fractionate PCR products. The MetaPhor agarose solution was prepared at 4% in 1X TBE. The horizontal gel was prepared at approximately 3 mm thick with 40 ml of MetaPhor® agarose solution in a 8 x 12 cm tray. The gel was allowed to solidify at room temperature for 20-30 minutes and then placed at 4 °C for 20 minutes to ensure sieving efficiency of the gel.

The samples were electrophoresed for 8 hours at a constant voltage (8–10 V/cm) using 1XTBE running buffer. The buffer was prechilled to 4-6°C to maintain uniformity of the band patterns across the gel. The buffer temperature typically rose to 18-20°C during electrophoresis. The nominal voltage gradients were determined by dividing the applied voltage by the length of the chamber.

Following electrophoresis, the gel was carefully transferred to a container and stained with 1 µg/ml ethidium bromide prepared in distilled water for 10 minutes. The

gel was rinsed with distilled water, visualized with the UV-transilluminator and photographed using a camera covered with the red filter. A UVP ImageStore 7500 system was also used to visualize and photograph the gel.

2.9.6 Size estimation of amplified microsatellite alleles

Sizes of microsatellite alleles of each locus were estimated by comparing with the standard markers (in base pair). Allele sizes of microsatellite loci were estimated by comparing with a M13 mp18 sequencing ladder in the isotopic method. For the non-isotopic method, allele sizes were estimated by allelic ladders that were constructed from known allele sizes.

2.9.6.1 M13 mp18 sequencing ladder

For the isotopic method, a DNA standard used for estimation of microsatellite alleles was the M13 sequencing marker prepared using T7 DNA sequencing kit. The M13 control template was annealed with the M13 sequencing primer in a 14 µl reaction mixture containing 5 µl of the template, 5 µl of sterile distilled water, 2 µl of annealing buffer (200 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl) and 2 µl of 4 ng/µl M13 forward primer. The annealing mixture was incubated at 65 °C for 15 minutes and allowed to cool at room temperature for 10 minutes. During the annealing period, the labeling mixture including 1 µl of sterile distilled water, 3 µl of Labelling Mix-dATP (1.37M dCTP, dGTP and dTTP), 1.7 µl of 5x sequencing buffer, 0.3 µl of T7 DNA polymerase and 1 µl of 800 Ci/mmol [γ-^{32}P] dATP, was prepared. Six microlitres of this mixture was then added to the annealing mixture. The labelling/extension mixture was then incubated at room temperature for 5 minutes. Five microlitres of this was dispensed to each of the 4 tubes containing appropriate termination mixture (each mixture contains all four dNTPs at the suitable concentrations and the appropriate ddNTP at a concentration of 14 µM). The reaction

tubes were incubated at 37 °C for 5 minutes. Thirty microlitres of a formamide containing dye solution and one drop of the mineral oil were added.

2.9.6.2 Allelic ladders

Allelic ladders of each microsatellite locus with the non-labeling method and allelic ladder of CSCUPmo1+2 (multiplex PCR of CSCUPmo1 and CSCUPmo2 loci) with the radiolabeled method00 were constructed for estimating allele sizes of investigated samples. An equal amount of approximately 45 ng each of known microsatellite alleles covering allele size ranges of each locus from different DNA samples was pooled. This mixture was amplified in a 15 µl PCR volume containing 0.6 µM each of forward and reverse primers, 1X PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 2.5 mM $MgCl_2$, 300 µM each of dNTPs and 0.45 U of *Taq* polymerase. PCR cycling programs were similar to those described in 2.9.2 except the annealing temperature of each locus was decreased by 6 °C to increase amplification efficiency of large alleles.

2.10 Multiplex analysis of microsatellite loci

To construct multiplex PCR, loci having compatible amplification protocols and exhibiting non-overlapping allele size ranges were simultaneously amplified in one reaction tube. The amplification products were separated by denaturing polyacrylamide gel electrophoresis and detected by either autoradiography or silver staining. The selected loci in each multiplex set must allow independent interpretation of each locus.

Multiplex PCR was designed for simultaneous amplification of multiple loci in a single reaction. This approach saves both sample materials and manipulation time conventional. Four sets of diplex PCR including CSCUPmo1 and CSCUPmo2

(CSCUPmo1+2), CSCUPmo4 and CSCUPmo9 (CSCUPmo4+9), CSCUPmo4 and CSCUPmo11 (CSCUPmo4+11), CSCUPmo6 and CSCUPmo11 (CSCUPmo6+11) and one set of triplex PCR consisting of CSCUPmo4, CSCUPmo9 and CSCUPmo11 (CSCUPmo4+9+11) were amplified by radioactive-labeled PCR. Approximately 15 ng of genomic DNA were included in a 5 μl PCR reaction volume containing 0.45 μM of the reverse primer, 0.425 μM of the forward primer, 0.025 μM of labeled forward primer, 1 μl of 10X PCR buffer, 2.5 mM of $MgCl_2$, 300 μM each of dNTPs and 0.15 U of *Taq* polymerase. These multiplex sets were amplified using the similar PCR cycling program for conventional PCR of each locus. The annealing temperature of a CSCUPmo1+2 set was 56 °C and that of other sets was 54 °C.

An another approach reaching the multiplex analysis involves mixing amplified samples from each locus prior to loading them on the gel. Three sets of combined diplex analysis including CSCUPmo1 and CSCUPmo2 (CSCUPmo1+ CSCUPmo2), CSCUPmo4 and CSCUPmo9 (CSCUPmo4+CSCUPmo9), CSCUPmo6 and CSCUPmo11 (CSCUPmo6+CSCUPmo11) and one set of triplex analysis, CSCUPmo4, CSCUPmo9 and CSCUPmo11 (CSCUPmo4+CSCUPmo9+ CSCUPmo11) were loaded onto the gel to determine microsatellite alleles. The alleles were size-estimated by comparing with the M13 mp18 sequencing ladder.

For the non-isotopic method, multiplex PCR of CSCUPmo1+2 and CSCUPmo4+11 were tested using similar PCR conditions of the radioactive-labeled method.

2.11 Data analysis

A genotype of each *P. monodon* individuals was scored from an electrophoretically observed pattern for each locus. Therefore, the genotypes could be divided into homo- or heterozygotic states. Based on the fact that stutter bands were

commonly observed in dinucleotide microsatellite, scoring of a particular band can be unambiguously carried out by making an assumption that an actual band of a given allele was the most intense band among the neighboring group of stuttered bands. Sizes of alleles, in bp, were estimated by relatively compared to the M13 sequencing marker. Each *P. monodon* individual was recorded to be either homo- and heterozygous for a particular locus. Allelic stages were also recorded from each individual for each locus.

2.11.1 Allele frequencies and genetic variation

For diploid taxa, the frequency of a particular allele in a population can be calculated as

$$p = (2N_{AA} + N_{Aa})/2N$$

where p is the frequency of the A allele, N is the total number of individuals in investigated population, and N_{AA} and N_{Aa} are the number of homo- and heterozygotes for such a locus. The unbiased estimate of heterozygosity (expected heterozygosity, h_{exp}) for each locus was estimated using the equation;

$$h_{exp} = 1 - \sum p_i^2$$

where p_i is the frequency of i^{th} allele (Nei, 1978).

2.11.2 Analysis of Mendelian inherited fashion of CSCUPmo1, CSCUPmo2, CSCUPmo4, CSCUPmo6, CSCUPmo9, and CSCUPmo11 microsatellites in *P. monodon*.

To determine whether six microsatellite sets used in this study segregated in a Mendelian fashion, fifty representative offspring from three full-sib families were

examined. Offspring genotype frequencies were subjected to goodness of fit test for Mendelian segregation using the typical χ^2-method (Sokal and Rohlf, 1981).