Chapter II

Materials and Methods

2.1 Equipments

- Autoclave LS-2D (Rexall industries Co. Ltd., Taiwan)
-Automatic micropipette P20, P100, P200 and P1000 (Gilson Medical Electronic S.A., Franc)
-Autoradiography cassette(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)
- Hyperfilm MP (Amersham International, England)
- Incubator BM-600 (Memmert GambH, Germany)
- Microcentrifuge tube 0.5 , 1.5 ml(Bio-RAD Laboratories , USA)
- PCR Thermal cycler : Omnigene-E (Hybaid Limited, England)
- PCR Workstation Model#P-036 (Scientific Co., USA)
- Pipette tips 100 , 1000 µl (Bio-RAD Laboratories , USA)
- Power supply : Power PAC 3000 (Bio-RAD Laboratories,USA)
- Refrigerated microcentrifuge Kubota 1300 (Kubota, Japan)
- Spectrophotometer DU 650 (Beckman, USA)
- Vertical sequencing gel electrophoresis apparatus (Hoefer, USA)

2.2 Chemicals

- Absolute ethanol (Merck, Germany)
- Acrylamide (Merck, Germany)
- Ammonium persulfate(Promega, USA)
- Boric acid (Merck, Germany)

- Bromophenolblue (Merck, Germany)
- Chloroform (Merck, Germany)
- Developer (Eastman Kodak Company, 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)
- Formamide(Gibco BRL Technologies, Co., USA)
- GeneAmp PCR core reagents (Perkin Elmer Cetus, USA)
  - : 10X PCR buffer (100mM Tris-HCL pH 8.3, 500 mM KCl)
  - : 25 mM $MgCl_2$
- Hydrochloric acid (Merck, Germany)
- Isoamyl alcohol (Merck, Germany)
- N,N-methylene-bis-acrylamide (Amersham, England)
- Oligonucleotide primers (Bio synthesis)
- Phenol crystal (Fluka, Germany)
- Proteinase K (Gibco BRL Technologies, Inc., USA)
- RNase A (Sigma Chemical Co., 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 (1M Tris-HCl , 100 mM $MgCl_2$, 160 mM D DTT)
  - : Labelling Mix-dATP (1.375 µM each dCTP , dGTP and dTTP , 333.5 mM NaCl)
  - : Control DNA template
- Sodium acetate (Merck, Germany)
- Sodium chloride (Merck, Germany)

- Sodium dodecyl sulfate (Sigma Chemical Co., USA)
- Sodium hydroxide (Merck, Germany)
- N,N.N',N'-tetramethylethylenediamine (Gibco BRL Technologies, Inc., USA)
- Tris-(hydroxy methyl)-aminomethane (Fluka, Switzerland)
- Urea (Fluka, Switzerland)
- Xylene cyanol (Sigma, USA)

2.3 Radioactive

- [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 shrimp broodstock (*P. monodon*) was wild-caught alive from Satun (N = 36), Trang (N = 35) and Phang-nga (N = 30) located in the Andaman Sea and from Chumphon (N = 49) and Trad (N = 48) located in the Gulf of Thailand during December 1997 - February 1998 (Fig. 2.1). Pleopods were excised from freshly killed *P. monodon* individuals and immediately placed on dry ice. Alternatively, dissected pleopods or the whole post larvae from the hatcheires were immediately placed into the tubes containing an enough amount of absolute ethanol and transported back to the laboratory at the Department of Biochemistry, Faculty of Science, Chulalongkorn University. Specimens were stored at -80 °C until required.

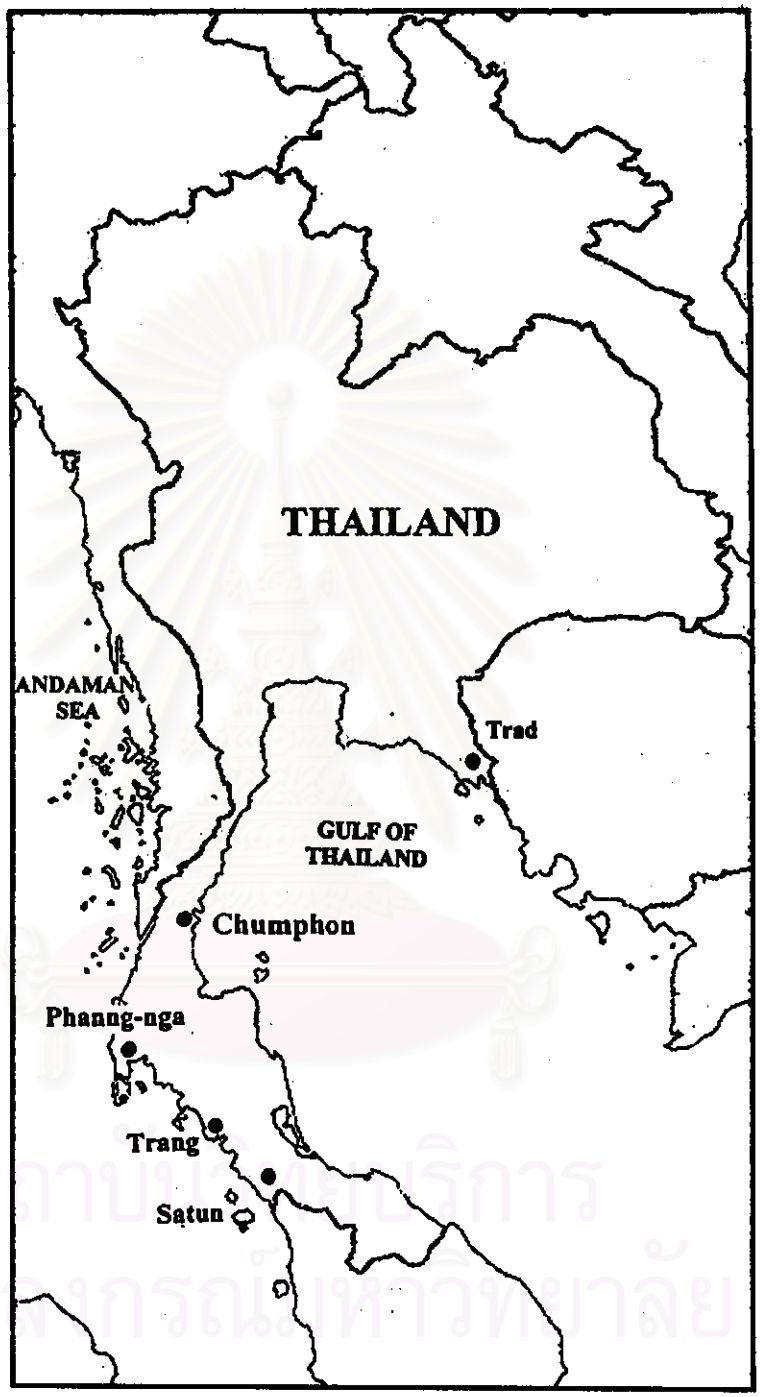

Fig 2.1 Map of Thailand illustrating sample collection sites including Satun, Trang, Phang-nga, Chumphon and Trad.

2.6 DNA extraction

Genomic DNA was extracted from a pleopod of each *P. monodon* individual using a phenol-chloroform modified from that of Davis et al. (1986). As soon as possible after removing from a -80 °C freezer, a pleopod was transferred into a 1.5 ml microcentrifuge tube containing 400 µl of pre-chilled extraction buffer (100 mM Tris-HCl, pH 9.0, 100 mM NaCl, 200 mM sucrose, 50 mM EDTA, pH 8.0) and briefly homogenized with a pre-chilled glass homogenizer. A 40% SDS solution was added to a final concentration of 1.0 % (w/v). The resulting mixture was then incubated at 65°C for 1 hour following by an addition of 10 µl of a Proteinase-K solution (30 mg/ml) and 5 µl of a RNase A solution (10 mg/ml). The mixture was further incubated at the same temperature for 3 hours. To remove proteins, ninety-one microliters of 5 M potassium acetate was added, thoroughly mixed and incubated at 4 °C for 10 minutes prior to centrifugation at 12,000 rpm for 10 minutes at 4 °C. The supernatant was decanted to a sterile microcentrifuge tube. An equal volume of buffer-equilibrated phenol-chloroform-isoamyl alcohol (25:24:1 v/v) was added and gently mixed. The mixture was then centrifuged at 12000 rpm for 10 minutes at room temperature. The upper aqueous phase was carefully transferred to a new microcentrifuge tube. One-tenth volume of 3 M sodium acetate pH 5.5 was added. DNA was precipitated by an addition of two volume of ice-cold absolute ethanol and kept at -20 °C overnight to ensure complete precipitation. The precipitated DNA pellet was recovered using a cut tip and briefly wash twice with 70% ethanol. The pellet was air-dried and resuspended in 300 µl of TE buffer (10 mM Tris-HCl, pH 7.4 and 1 mM EDTA). The DNA solution was incubated at 37 °C for 1-2 hours for complete redissolved and kept at 4 °C until further needed.

Alternatively, the rapid extraction method according to Cook, (submitted) was carried out. This method is much simpler but yielded comparable DNA quality to that from the Phenol-chloroform extraction method. A pleopod was chopped to small pieces (approximately 50 - 100 mg in weight) and added to a microcentrifuge tube containing 1 ml of high TE buffer (100 mM Tris-HCl, 40 mM EDTA, pH 8.0). The

tube was briefly vortexed. The supernatant was discarded. The pellet was resuspended in 250 µl of MGPL lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 200 mM LiCi, pH 8.0 and 0.8% SDS). A proteinase-K solution was added to 200 µg/ml final concentration. The sample was incubated at 45 °C for 4 hours. During the incubation period, the sample was intermittently mixed until the residual tissue pieces were not observed. At the end of the incubation period, 500 µl of TE (10mM Tris-HCl, 1 mM EDTA, pH 8.0) was added and gently mixed prior to centrifugation at 8,000 rpm for 1 minute. The supernatant was then transferred to a new microcentrifuge tube before 9.4 µl of 4 mM NaCl and 750 µl of pre-chilled isopropanol were added. The solution was thoroughly mixed and incubated at -80 °C for 30 minutes. DNA was then recovered by centrifugation at 14,000 rpm for 1 minute at room temperature. The pellet was washed with 70% ethanol. The sample was spun at 12,000 rpm for 5 min at room temperature before removing of ethanol. DNA was then air-dried in a 37 °C for 5 - 10 min before resuspended with 100 µl of TE. The DNA solution was redissolved at 37 °C for 1-2 hours and kept at 4 °C until required.

2.7. Measurement of DNA concentration

The concentration of extracted DNA was spectrophotometrically measured at the optical density of 260 nanometres (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;

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

Basically, the concentration of DNA samples 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;

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

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

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

2.8 PCR primers

The oligonucleotide primers for locus CUPmo18 were developed by Tiptawonnukul (1996). The primer sequences for locus Di25 and Di27 which were also used in this study were kindly provided by Dr. F. Bonhomme, Laboratoire 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.

2.9 Amplification of Microsatellite DNA using the Polymerase Chain Reaction (PCR)

Microsatellite DNA of investigated *P. monodon* individuals was *in vitro* amplified by PCR in which one of the primers was 5' radiolabeled with γ-^{32}P dATP. The resulting amplification product(s) was electrophoretically size-fractionated by denaturating polyacrylamide sequencing gels. The DNA patterns were then detected by autoradiography (Tam and Kornfield, 1996).

2.9.1 5'-end labelling with T4 polynucleotide kinase

The reverse primer for each microsatellite locus was end-labeled using polynucleotide kinase. Approximately 10 pmole of the reverse primer (free 5'-OH groups) were used as a substrate for a 10 µl end-labeling reaction containing 1 µl of 10x T4 Polynucleotide kinase buffer (0.5 M Tris-HCl pH 7.6, 0.1 M $MgCl_2$, 50 mM dithiothreitol, 1 mM spermidine), 3 µl of [γ-^{32}P] ATP (3,000 µCi/mmol), 10 U of T_4 polynucleotide kinase. An enough amount of sterile deionized H_2O 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 T_4 polynucleotide kinase activity at 65 °C for 15 minutes.

2.9.2 Amplification of microsatellite loci

Each of microsatellite loci was singly amplified from genomic DNA isolated from *P. monodon* individuals. Approximately 15 ng of genomic DNA isolated from each individual of *P. monodon* were used as the DNA template in a 5 µl PCR reaction volume containing 0.6 µM of the forward primer, 0.575 µM of the reverse primer, 0.025 µM of the lablled reverse primer, 1 µl of 10x PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl , 10 mM $MgCl_2$, 0.1% gelatin), 200 µM each of dNTPs (dATP, dCTP, dGTP and dTTP), 0.5 U of *Taq* polymerase. For reliable amplification results of CUPmo18, 0.05% of tween 20 was required in the amplification reaction (Tassanakajon et al., 1997). The reaction mixture were then overlaid with a drop of RNase- and DNase-free mineral oil and centrifuged briefly in a microcentrifuge before subjected to the amplification process in a thermal cycler (Omnigene, Hybaid).

To amplify the micrasatellite locus CUPmo18, PCR was carried out for seven cycles consisting of a 94 °C denaturation for 60 seconds, a 55 °C annealing for 30 seconds and a 72 °C extension for 60 seconds followed by thirty-eight cycles of a denaturation step at 94 °C for 30 seconds, an annealing step at 55 °C for 30 seconds and an extension step at 72 °C for 60 seconds.

For amplification of the loci Di25 and Di27, more complicated PCR amplification program were required. Firstly, the melting temperature (Tm) for each primer was examined using the Wallace's rules. As a result,

$$Tm\ (°C) = [4\ (\text{total number of G and C}) + 2\ (\text{total number of A and T})]$$

Theoretically, the annealing temperature (Ta) was approximately 5 °C below Tm of the primer. Based on the fact that the Tm value of the forward and reverse primers was not identical, the annealing temperature was set up from the primer having

lower Tm. The most optimal annealing temperature for Di25 and Di27 was further adjusted from the autoradiography results.

To amplify the Di25 and Di27 loci, the multi-step cycling system, sometimes called a touch down PCR, were utilized. Initially, the reaction mixture was pre-denaturated at 94 °C for 4 minutes followed by a denaturating step at 94 °C for 1 minute, an annealing step at the most suitable Ta for 1 minutes and an extension step at 72 °C for 1 minutes. These were carried out for 3 cycles. Subsequently, the amplification was performed as described above with the exception that a pre-deaturation step was omitted and the annealing temperature were gradually decreased for 2 °C below the most optimal annealing temperature every 3, 3 and 4 cycles. At the end of these steps, the PCR reaction was performed for 20 cycles constituting of the typical denaturation temperature for 30 seconds, a -7 °C below the most optimal annealing temperature of each locus for 30 seconds and the typical extension temperature for 30 seconds.

2.9.3 Size estimation of amplified microsatellite allele using denaturating polyacrylamide gels

After the amplification process was complete, 5 µl of a formamide dry mix 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 microliters of the denatured mixture was loaded onto a 8% denaturing acrylaminde gel (76% acrylamide, 0.4% N,N' methylene bisacrylamide, 7.66 M Urea) prepared in 1x TBE (89 mM Tris-HCl, 89 mM Boric acid, 2 mM EDTA, pH 8.3) and electrophoresed at 50 W for approximately 2.5 hours.

A DNA standard used for estimation of microsatellite alleles was the M13 sequencing marker prepared using T7 DNA sequencing kit. The M 13 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 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 to room temperature for 10 minutes. During the annealing period, the labeling/extension mix including 1 µl of sterile H_2O, 3 µl of dNTP mix (2 µM each of dCTP, dGTP and dTTP), 1.7 µl of 5x Sequenase buffer, 0.3 µl of T7 DNA polymerase and 1 µl of 800 Ci/mmol [α-^{32}P] dATP), was prepared. Six microliters of this mixture was then added to the annealed template/primer mix. The labeling mixture was then incubated at room temperature for 5 minutes. Five microliters of this was dispensed to each of the 4 tubes containing appropriate termination mix (each mixtue 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 microliters of a formamide containing dry solution and one drop of the mineral oil were added. The M13 sequencing marker was denatured and loaded onto a 8 % acrylamide gel as described above.

2.10 Data analysis

Assumption

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 located at the biggest in size compared to the neighbor group of stuttered bands. The size of alleles, in bp, was estimated by relatively compared to the M13 sequencing marker. Each *P. monodon* individual was recorded to be either homo- and heteroygote. The allelic stages were also recorded from each individual for each locus.

2.10.1 Allele frequencies, genetic variation and effective number of alleles per locus

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 the investigated sample, and N_{AA} and N_{Aa} are the number of homo- and heterozygotes for such a locus.

Practically, the number of alleles per locus, allelic frequency, the proportion of homo- and heterozygotes individuals (direct-count heterozygosity, h_{obs}) were estimated using GENEPOP Version 2.0 (Raymond and Rousset,1995) whereas 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, H_{obs} and H_{exp} is the average of h_{obs} and h_{exp} over all investigated loci, respectively (Nei,1978).

The effective number of alleles at each locus was calculated by

$$n_e = 1/\sum p_i^2$$

when p_i is the frequency of i^{th} allele (Crow and Kimura,1965).

2.10.2 Hardy-Weinberg equilibrium

Once allele and genotype frequencies of diploid organisms have been estimated, Hardy-Weinberg equilibrium of genotype frequency at a given locus for each investigated population should be examined. The observed genotype frequencies are concordant to Hardy-Weinberg expectation when there are no significantly disturbing forces e.g. selection, mutation or migration changing allele frequencies over time and mating is actually occurred at random in a large population.

In the present study, Hardy-Weinberg equilibrium (HWE) of each locus for each population were examined using a Markov chain "approximation to exact test" followed the algorithm of Guo and Thomsson (1992) (a test for determination of heterozygote deficiency) and implemented in GENEPOP version 2.0. The probability to reject null hypothesis (Ho: observed genotype frequencies of an investigated population at a given locus conform Hardy-Weinberg rule) were further adjusted using the Bonferroni technique (Hochberg,1988). This was carried out by dividing the initial significant level ($\alpha < 0.05$) by the number of tested loci.

2.10.3 Analysis of Mendelian inherited fashion of CUPmo18, Di25 and Di27 in P. monodon

To determine whether three microsatellite loci used in this study segregated in a Mandelian fashion, twenty representative offspring from a full-sib family were examined, at all loci. Offspring genotypes were subjected to goodness of fit test against Mendelian segregation using typical χ^2-method(Sokal and Rohlf,1981).

2.10.4 Genetic distance and phylogenetic reconstruction

Genetic distance based on Cavalli-Sforza and Edwards chord distance was calculated (Cavalli - Sforza and Edwards, 1967). This is the gene diversity among population expressed as a function of genotype frequency. The genetic distance estimated from this method are appropriate for microsatellite data obtained from various taxa whether they have undergone the bottleneck events or not (Takezaki et al.,1996). Practically, Cavalli-SforZa and Edwards chord distance was computational estimated using GENDIST(Felsenstein, 1993). The resulting genetic distance was subjected to phylogenetic reconstruction based on Neighbor-joining approach (Saitou and Nei , 1987) using NEIGHBOR. The NJ tree was plotted by DRAWTREE. All computational programs mentioned above are routinely implemented in Phylip 3.56c (Felsenstein, 1993).

2.10.5 Geographic heterogeneity analysis

The statistically significant differences in genotype frequencies between *P. monodon* from a pair of geographic sampling locations were tested using the exact test of Genic Differentiation of GENEPOP version 2.0. Results are expressed as the probability of homogeneity between compared populations or regions. To diminish type I error, level of significance was further adjusted using the Bonferroni test.

2.10.6 Interpopulation diversity and population differentiation

Although several approaches can be used to estimate intraspecific population structure, Wright's *F*-statistics (or allelic correlation's) are widely used. Nevertheless, Weir & Cockerham (1984) introduced the unbiased *F*st(or θ) for estimation of population subdivision for the random model and can be conveniently calculated using Diploid program (for diploid characteristic data) distributed by the author. This parameter (θ) is suitable to handle difficulties from multiple alleles at a locus, multiple loci and various sampling error from within and between populations (Weir, 1990). Accordingly, population differentiation of *P. monodon* in Thailand were examined using this approach.