CHAPTER 2

Materials and Methods

2.1 Equipments

1. Autoclave : HVE-50 (Hirayama, Japan)
2. Automatic pipettes (Gilson Medical Electronic S.A., France)
3. Camera : K1000 (Pentax, Japan)
4. Electrophoresis apparatus (Bio-RAD Laboratories, USA)
5. –20 °C Freezer (Songserm intercool, Thailand)
6. –80 °C Freezer (Krungthai Limited, Thailand)
7. Incubator : BM-600 (Memmert GambH, Geramany)
8. Microcentrifuge : MicroCen 13D (Herolab, Geramany)
9. Microcentrifuge tube (Bio-RAD Laboratories, USA)
10. PCR Thermal Cycle : Omnigene-E (Hybaid Limited, England)
11. Power supply : Power Pac 300 (Bio-RAD Laboratories, USA)
12. Polaroid camera : DS 34 (Polaroid, USA)
13. Refrigerated microcentrifuge : 3K18 (Sigma, Geramany)
14. Spectophotometer : Spectronic GENESYS 5 (MiltonRoy, USA)
15. Shaker bath : SBS 30 (STUART Scientific, USA)
16. UV transilluminator : UVP (USA)

2.2 Chemicals

1. Absolute ethanol (Merck, Germany)
2. Boric acid (Merck, Germany)
3. Chloroform (Merck, Germany)
4. 100 mM dATP, dCTP, dGTP, dTTP (New England, Biolabs USA)
5. Ethylene diaminetetraacetic acid, disodium salt : dihydrate (Sigma Chemical CO., USA)
6. Ficoll Type 400 (Sigma Chemical CO.,USA)
7. Isoamyl alcohol (Merck, Germany)
8. 25 mM $MgCl_2$ (Perkin-Elmer Cetus, Norwalk, Connecticus, USA)
9. Mineral oil (Sigma Chemical CO.,USA)
10. 10 X PCR buffer ; 100 mM Tris-HCl, pH 8.3, 500 mM KCl (Perkin-Elmer Cetus, Norwalk, Connecticus, USA)
11. Phenol, redistilled (Aldrich Chemical Co., USA)
12. Potassium acetate (Merck, Germany)
13. Proteinase K (Gibco BRL Life Technology, Inc., USA)
14. Ribonuclease A (Sigma Chemical CO.,USA)
15. Sodium chloride (Merck, Germany)
16. Sodium dodecyl sulphate (Sigma Chemical CO.,USA)
17. Sodium sulfite, Photographic Grade (Sigma Chemical CO.,USA)
18. Tris (hydroxy - methyl) amino- methane (Sigma Chemical CO.,USA)
19. Ultrapure agarose (Gibco BRL Life Technology, Inc., USA, SeakemGTG, FMC)

2.3 Enzyme

AmpliTaq DNA Polymerase (Perkin- elmer Cetus, Norwalk, Connecticut, USA)

2.4 Oligonucleotide primers

Primers were purchased from Biosynthesis using the sequence from the Biotechnology Laboratory , University of British Columbia, Canada.

Primer	Sequence	% GC	Reference
UBC268	AGGCCGCTTA	50	Pongsomboon, (1996)
UBC273	AATGTCGCCA	50	Pongsomboon, (personal comunication)
UBC299	TGTCAGCGGT	60	Pongsomboon, (personal comunication)

2.5 Sampling

Samples of the black tiger shrimp (*P. monodon*) used in the experiments were wild captured broodstocks trawled from normal fisheries (Fig 2.1). Ninety individuals originating from Trat (N= 35) and Chumphon (N = 55) in the upper and lower Gulf of Thailand and one hundred and forty-three *P. monodon* individuals originating from Satun (N = 50), Trang (N = 54) and Phangnga (N = 39) in the Andaman Sea were collected during January to December 1997 (Appendix A).

All *P. monodon* specimens except those from Trat were transported alive to the laboratory at Marine Biotechnology Research Unit (MBRU), Chulalongkorn University. Pleopods of each individual were dissected out and placed on dry ice before transferred to a -80°C freezer for long storage. Specimens from Trat were processed in the field following the same process.

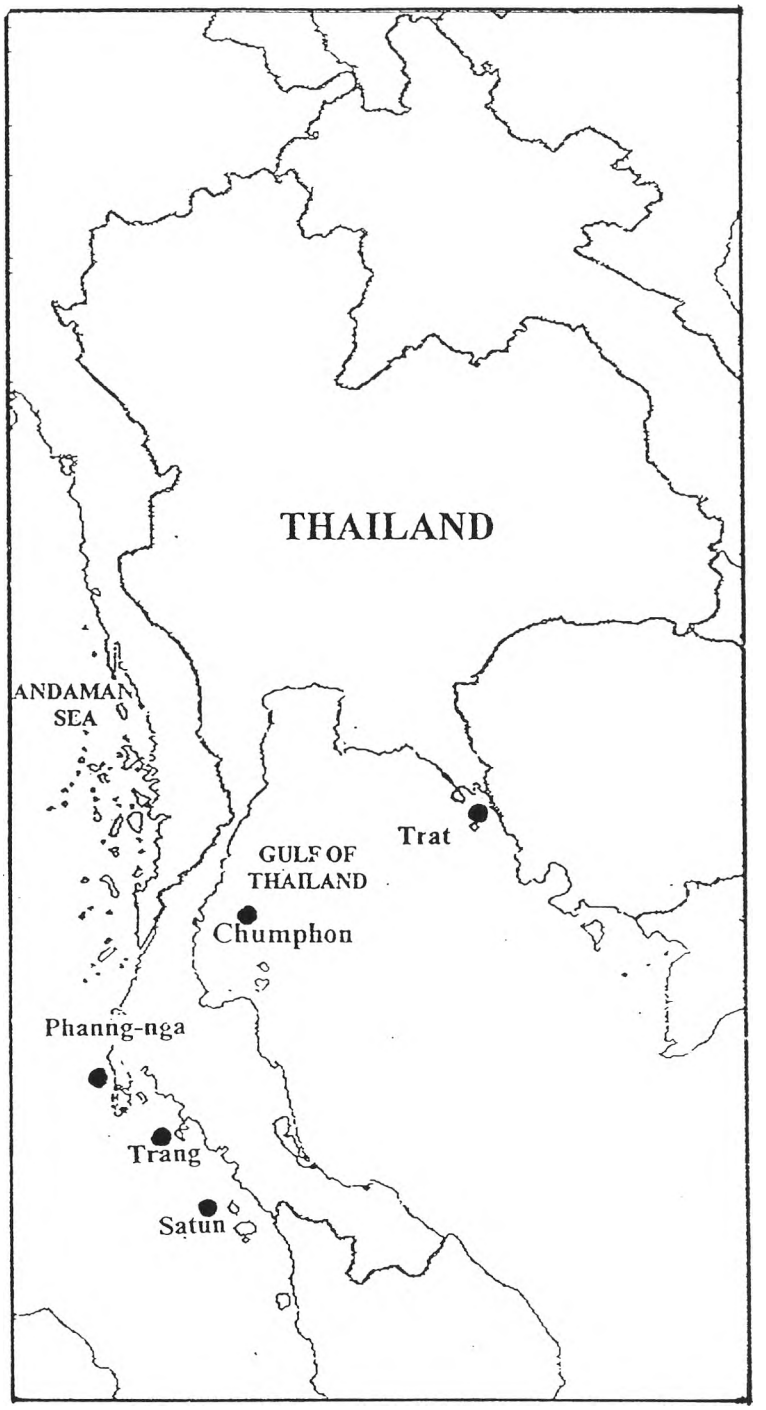

Figure 2.1 Map of Thailand illustrating sample collection sites including Satun, Trang, Phangnga, Chumphon and Trat

● = Sampling area

2.6 DNA extraction

A pleopod of individual specimen was homogenised with a micropestle in a prechilled 1.5 ml microcentrifuge tube containing 400 µl of TEN buffer (200 mM Tris-HCl, 100 mM Na_2EDTA and 250 mM NaCl; pH 8.0) The nucleated cells were lysed by an addition of 40% sodium dodecyl sulfate to the final concentration of 1.0 % (W/V). RNA was digested by adding 5 µl of an RNaseA solution (10 mg/ml) and incubated at 37°C for 30 min. Total proteins were digested by an addition of a proteinase K solution (10 mg/ml) to a final concentration of 200 µg/ml. The mixture was further incubated at 55 °C for 2 hours. To remove digested proteins, 5 M potassium acetate was added to 1M final cocentration. The sample mixture was chilled on ice for 30 min and centrifuged at 12,000 rpm for 10 min. The supernatant was transferred to a new 1.5 ml tube. An equal volume of buffered phenol was added and gently mixed for 15 min. The sample was then centrifuged at 12,000 rpm for 15 min. The upper aqueous phase was removed without disturbing the organic/aqueous interface. An equal volume of phenol/chloroform/isoamyl alcohol mixture (25:24:1, v/v) was added and mixed gently.

The mixture was spun as described earlier. The aqueous phase was transferred to a sterile microcentrifuge tube before being added with an equal volume of chloroform/ isoamyl alcohol (24 : 1 v/v) and mixed thoroughly.

After centrifugation, the upper phase was removed, one - half amount of TE buffer (10 mM Tris-HCl, pH 8.0 and 0.1 mM EDTA pH 8.0) was added. One - tenth volume of 3M sodium acetate, pH 5.2, was added, followed by 2 volumes ice - cold absolute ethanol. The mixture was thoroughly mixed by inverting of the tube.

If the DNA pellet could not be seen at this stage , the sample was kept in a - 20 °C freezer for 2 hours (or overnight). DNA was recovered by centrifugation at 12,000 rpm for 10 - 15 minutes. The DNA pellet was washed twice, at least 10 minutes each, with 70 % ethanol. The pellet was air dried and redissolved with appropriate amount of a TE solution (usually 50-100 µl), incubated at 37 °C for 1-2 hours and kept at 4 °C for further analysis.

2.7 Determination of DNA concentration

2.7.1 Spectrophotometry

The amount of DNA was estimated by determination of the optical density at 260 nm The OD valule at 260 allows calculation of total nucleic acids whereas the value reading at 280 nm determine the amount of protein in the sample.

An OD at 260 nm corresponds to approximately 50 µg/ml for double stranded DNA. (Maniatis et al., 1982). The ratio between OD 260/280 provides a roughly estimate for the purity of extracted DNA. A pure preparation of DNA has a 260/280 ratio of 1.8 - 2.0 (Kirby, 1992). To determine DNA concentration, 5 µl of extracted DNA was mixed with 995 µl of TE buffer in an Eppendorf tube and was shaken vigorously. The diluted DNA solution was transferred to a semimicro-UV cuvette and 1 ml of TE was placed in another cuvette to serve as a reagent blank. DNA sample concentration is estimated in µg/ml by the following equation.

$$[\text{DNA}] = OD_{260} \times \text{Dilution factors} \times 50 \qquad (2.1)$$

2.7.2 Mini – gel method

DNA concentration can be roughly estimated by comparing the amount of fluorescence of the DNA after electrophoresed through agarose mini – gels. One or two microlitres of the extracted DNA was diluted with an appropriate amount of a TE solution to make a 10 µl volume. Two microlitres of loading buffer (0.25% Bromophemol blue, 25% Ficoll) was added and mix thoroughly. A series of undigested DNA at different amount (25, 50, 100, 150, 200 ng) was prepared and included as the quantitative standards.

DNA was electrophoresed, usually through 0.8 % agarose gels in the presence of 0.5 µg/ml EtBr at 5 - 7.5 V/cm for approximately 30 mintutes. The approximate amount of DNA concentration were obtained by comparing the fluorescent level of an investigated band with those of a λ- *Hind* III marker. The DNA solution was then diluted to 25 µg/ml final concentration for convenient use in the PCR amplificaton.

2.8 Screening of primers used in this study

Originally, three hundred RAPD primers (UBC1 – UBC300) were screened for amplification of *P. monodon* DNA by Pongsomboon, (1996). Ten primers (UBC 101, UBC174, UBC228, UBC268, UBC273, UBC299, UBC428, UBC456, UBC457 and UBC459) were high strength for the amplification reactions. Only three of which were chosen on the basic that data from UBC273 and UBC299 have not been reported before. Although results from the UBC268 had been reported (Tassanakajon et al., 1998), it generated easy scoring patterns and provided highly informative results for population heterogeneity analysis.

2.9 RAPD method

The polymerase chain reaction was carried out in a final volume of 25 µl containing 0.2 µM of an appropriate primer (sequences were described previously), 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 100 µM each of dATP, dCTP, dGTP, dTTP, 1 unit of Ampli*Taq* DNA polymerase and approximately 50 ng of genomic DNA. The master mix without the DNA template (enough for desired number of reaction and a negative control) was prepared.

Aliquots of the master mix were dispensed. Fifty nanograms of DNA was then added to each tube. A drop of mineral oil was overlaid to prevent evaporation during amplification. The reaction tube were spun and subjected to RAPD–PCR using a Hybaid thermo–cycler.

The amplification reaction was operated for 35 cycles consisting of 15 sec at 94°C (denaturation), 60 sec at 36°C (annealing), 90 sec at 72°C (extension). After amplification, the resulting PCR mixture was electrophoretically analysed as soon as possible. If not, the reaction mixture was kept at - 20 C for long storage.

2.10 Agarose gel electrophoresis analysis

An appropriate amount of ultrapure agarose was weighed out and mixed with 1X TBE buffer (8.9 mM Tris - HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.3) to make desired concentrations of agarose gels used for analysis of the resulting RAPD products (1.6 % gel) and for detection of quality of extracted genomic DNA (0.8 % gel). The suspension was heated until complete solubilisation in a microwave. The melted agarose were left at room temperature to cool to

approximately 50°C and poured into a gel mould. The gel was left at room temperature for 40 - 60 minutes to completely solidify. The gel was then placed in the electrophoretic chamber containing 1X TBE buffer covering the gel for 1-2 mm. The comb was gently removed. Air bubbles trapped within the well were carefully removed. One-quarter volume of the gel loading dye (0.25 % bromophenolblue and 25 % Ficoll) was added and mixed. After briefly centrifugation, the sample was slowly loaded into the wells using an adjustable automatic pipette.

A 100 bp DNA ladder (Promaga) and the λ - *Hind* III DNA were used as DNA markers. The gel chamber was connected to a power supply and turned on. Electrophoresis was carried out at 4-5 V/cm until bromophenol blue migrated almost reaching the bottom of the gels. DNA was stained with ethidium bromide (0.5 μ g/ml) for 15 min and destained in distilled water for 15 min to leach out unbound ethidium bromide from the gel. Migration distance of marker was recorded. The RAPD bands were visualised under a UV transilluminator. The gel was directly photographed using a camera (K1000 ; Pentax) and a polariod camara (Polariod film type 665).

2.11 Statistical procedures of determination of genetic variation by RAPD analysis

Standard DNA markers were used to assign the size of each RAPD fragment. Only fragments that were 200 bp - 2 kb and bands that could be accurately scored throughout all lanes were chosen to score. Each RAPD fragment was assigned a molecular length and recorded in a binary matrix for each individual as presence (1) or absence (0) of a given band.

The percentages of polymorphic and monomorphic bands were evaluated. The RAPD pattern of individuals were compared within and between the 5 geographic samples.

2.11.1 Similarity index

Most commonly, a similarity index between individuals is calculated from band sharing data of each pair using the formula :

$$S_{xy} = 2\ N_{xy} / N_x + N_y \qquad (2.2)$$

where N_x and N_y represent the total number of band observed for compared individuals, and N_{xy} is the number of band which are present in both individual x and y (Lynch, 1990).

Similarity index within a population (S) is calculated as the average of S_{xy} across all possible comparisons between individuals within a population. Between population similarity, corrected for within population similarity effect (S'_{ij}) can be culculated using the formula :

$$S'_{ij} = 1 + S_{ij} - (S_i + S_j)/2 \qquad (2.3)$$

where S_i and S_j are the values of S for population i and j, respectively, and S_{ij} is the average similarity between randomly paired individuals from population i and j (Lynch, 1990)

2.11.2 Genetic distance

A genetic distance between pairs of genotypes or populations is a quantitative estimate of genetic divergence between two compared operational taxonomic unit (Avise, 1994). For RAPD, S'_{ij} is converted to the genetic distance (D_{ij}) using the formula :

$$D_{ij} = 1 - S'_{ij} \quad \text{(Lynch, 1990)} \qquad (2.4)$$

2.11.3 Phylogenetic reconstruction

The dendrograms based on genetic distance obtained was constructed using the unweighted pair group method with arithmetic averages (UPGMA) implemented in Phylip version 3.572 c (Felsenstein, 1993).

2.11.4 Geographic heterogeneity analysis

The statistically significant differences in genotype frequencies between *P. monodon* from a pair of geographic sampling location were tesed using pseudo chi-square (χ^2) test based on a Monte Carto simulation (McElroy et al., 1991), was performed on genotype to determine different allele distribution among the 5 geographic samples.