Chapter II

Materials and Methods

Materials and chemicals

-Absolute ethanol (Merck, Germany.)

-Agarose gel type 1-A Low (Sigma Chemical Co., U.S.A.)

-Ammonium acetate (Merck, Germany.)

-Boric acid (Merck, Germany.)

-Chloroform (Merck, Germany.)

-100mM dATP, dCTP, dGTP, dTTP (Promega Corporation Medison, Wisconsin.)

-Ethylene diaminetetraacetic acid disodium salt dihydrate (Fluka Chemika-Bio Chemika, Switzerland.)

-Isoamyl alcohol (Merck, Germany.)

-25 mM $MgCl_2$ (Perkin-elmer Cetus, Norwalk, Connecticus.)

-10 X PCR buffer ; 10 mM Tris-HCl, pH 8.3, 50 mM KCl (Perkin-elmer Cetus, Norwalk, Connecticus.)

- Phenol crystal (Fluka Chemika-Bio Chemika, Switzerland.)

- Potassium acetate (Merck, Germany.)

- Proteinase-K (Gibco BRL life Technologies, Inc., U.S.A.)
- RNase (Sigma Chemical Co., U.S.A.)
- Sodium chloride (Merck, Germany.)
- Sodium dodecyl sulfate (Sigma Chemical Co., U.S.A.)
- Sucrose (Sigma Chemical Co., U.S.A.)
- Tris-(hydroxy methyl)-amino methane (Fluka Chemika-Bio Chemika, Switzerland.)

Enzyme

AmpliTaq DNA polymerase (Perkin-elmer Cetus, Norwalk, Connecticus.)

DNA primers

Oligonucleotide primers (University of British Columbia)

Primer sequences were shown in table 3.1.

Shrimp samples

The wild populations of *Penaeous monodon* were obtained from different location sites on the Andaman Sea and the Gulf of Thailand during March 1995 to August 1996. The shrimps of the Andaman Sea were collected from Satun, Trang provinces (Thailand) and Medan (Indonesia) whereas the shrimps of the Gulf of Thailand were collected from Chonburi (Angsila district)

Figure 2.1 Map of collection sites for *P.monodon* samples.

and Trad provinces. The pleopods were dissected out from the live shrimps, kept on ice or absolute ethanol during transportation and stored individually at -80°C.

DNA extraction

The shrimp DNA were prepared from the pleopods by using proteinase-K / phenol-chloroform extraction. This method has been shown to be effective removing protein from nucleic acid and become a standard method for DNA extraction (Kirby, 1992).

A pleopod of individual specimen was homogenized with a micropestel in a 1.5 ml microcentrifuge tube, on ice, containing 400 µl of extraction buffer (100 mM Tris-HCl, pH 9.0, 100 mM NaCl, 200 mM sucrose and 50 mM Na_2EDTA, pH 8.0). The nucleated cell was lysed by incubating with 0.4 % sodium doducyl sulfate for 1 hour at 65°C. Subsequently protein and RNA were destroyed by incubating with proteinase-K (500 µg/ml) and RNase solution (100 µg/ml), respectively, at 65°C for 3 hours. Protein was precipitated from nucleic acid by adding 5 M potassium acetate to the final concentration of 1 M. The sample was chilled on ice for 30 min and centrifuged at 12,000 rpm for 10 min. The supernatant was added with an equal volume of redistilled phenol and mixed very gently. The sample was then spun in a microfuge for 5 min at 7,000 rpm, the upper aqueous phase was removed without disturbing the

organic/aqueous interface and added with and equal volume of phenol/chloroform/isoamyl alcohol mixture (25:24:1, by volume) and gently mixed. After centrifugation, the lower organic phase was discarded, the upper aqueous phase was added with an equal volume of chloroform/isoamyl alcohol (24:1, by volume) and mixed gently. After centrifugation, the upper aqueous was removed, added with 1/10 volume of 3M sodium acetate, pH 5.5, mixed gently and added with two volume of prechilled -20°C absolute ethanol. The mixture was kept at -20°C overnight. The DNA pellet was collected by hooking out and washed twice with 70% ethanol, air-dried and dissolved in 300 µl of TE buffer (10 mM Tris-HCl, pH 7.4 and 1 mM EDTA, pH 8.0). The DNA was stored over night at 4 °C for complete solubilization.

Spectrophotometric measuring of DNA concentration

DNA concentration is estimated by measuring the OD_{260}. An absorbance optical density (O.D.) of 1.0 corresponds to 50 µg double-stranded DNA per ml. DNA sample concentration is estimated in µg/ml by OD_{260} x dilution factor x 50. An estimation of the purity of a sample can be obtained by calculating the ratio of the O.D. at 260 and 280 nm. For a pure preparation of DNA, $OD_{260/280}$ should be ≥ 1.8 (Kirby, 1992).

RAPD analysis

RAPD-PCR conditions for amplification reactions were 35 cycles of 5 sec at 94°C (denaturation), 45 sec at 36°C (annealing) and 90 sec at 72°C (extension). Amplification reactions were carried out in a final volume of 25 µl containing to 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 100 µM each dATP, dCTP, dGTP, dTTP, 0.2 µM primer, 50 ng genomic DNA and 0.2 unit of AmpliTaq DNA polymerase. Reactions were performed in a Perkin-Elmer Cycler model 2400.

Agarose gel electrophoresis

DNA was analyzed by convection sub-marine gel electrophoresis. Agarose was mixed with Tris-Borate-EDTA (TBE) buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0) to make the concentration of 1.6% (W/V) and 0.7% (W/V) for detection of amplification products and quality of extracted genomic DNA respectively. The calculated amount of agarose was dissolved in 1XTBE buffer by heating until complete solubilization. The solution was allowed to cool at room temperature and poured into a chamber set with a comb. After the gel hardened, the comb was carefully withdrawn and the seal was removed from the ends of the plateform. Sufficient 1XTBE buffer was added to cover the gel for approximately 0.5 cm. An appropriate amount of RAPD-amplified DNA samples or extracted genomic DNA was mixed with 1/4

volume of the gel-loading dye (0.25% bromphenol blue, 0.25 xylene cyanol FF and 15% ficoll) and loaded into the well. The low molecular weight 100 bp DNA ladder (Promega) and Lambda *Hin*d III fragments were used as standard DNA markers. The electrophoresis was carried out in 1XTBE buffer from cathode to anode at 100 volts until the bromphenol blue marker dye migrated almost out of the gel. After finishing, the gel was stained in 2.5μg/ml ethidium bromide (EtBr) solution for 5 min and destained (to remove excessive EtBr) by submerged in an excessive amount of distilled water for 15 min. The nucleic acid bands were visualized as fluorescent bands under UV transilluminator and photographed through a red filter using Kodak Tri-X-Pan 400 film. The exposure time was usually about 10-15 seconds.

Statistical procedures

The photographs of RAPD patterns were evaluated directly by eye with the help of a transparent ruler. Only unambigous fragments that were 200 bp-2 Kbp and bands that could be accurately scored throughout all lanes were chosen to score. Standard DNA markers were used to assign the size of each RAPD fragment, and were markers for the accurate comparison of fragments among lanes, derived from the same or different gels. Each RAPD fragment was assigned a molecular length and recorded in a binary matrix for each individual as present (1) or absent (0) of a given band. The percentages of polymorphic bands and monomorphic bands were evaluated. The RAPD patterns of

individuals were compared within and between the 4 geographic samples.

1. Similarity index

The index of similarity between individuals was calculated using the formula: $S_{xy}=2n_{xy}/n_x+n_y$, where n_{xy} is the number of fragments shared by individuals x and y, and n_x and n_y are the number of fragments scored for each individual (Lynch, 1990). Within population similarity (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, is: $S_{ij} = 1+S'_{ij}-0.5\ (S_i+S_j)$, 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 populations i and j (Lynch, 1990).

2. Genetic distance

S'_{ij} is also converted to a measure of genetic distance (D_{ij}) using the equation: $D_{ij} = -\ln[S'_{ij} /\sqrt{(S_iS_j)}]$, (Lynch, 1991).

3. Dendrograms

The distance values were subjected to cluster analysis using the UPGMA method of Phylip version 3.57 c.

4. Chi-square analysis

A RAPD pattern is referred to as genotype which is generated from randomly amplified polymorphic DNA. Chi-square (χ^2) test, a Monte Carlo

simulation, was also performed on genotypes to ascertain the difference between the 4 geographic samples for the number and size of RAPD fragments.