CHAPTER II

Materials and Methods

2.1 Material and equipment

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, Germany)
8. Magnetic stirrer M21/1 (Franz Morat KG GambH, Germany)
9. Microcentrifuge: MicroCen 13D (Herolab, Germany)
10. Microcentrifuge tube (Bio – RAD Laboratories, USA)
11. Microwave oven: Power Boost 900 (Hitachi, Japan)
12. PCR Thermal Cycle: PCR Sprint (Hybaid Limited, England)
13. Pipette tips (Axygen Hayward, USA)
14. Power supply: Power Pac 300 (Bio – RAD Laboratories, USA)

    : Model 200/2.0
15. Polaroid camera: DS34 (Polaroid, USA)

16. Refrigerated microcentrifuge: 3K18 (Sigma, Germany)

17. Spectrophotometer: Spectronic GENESYS 5 (MiltonRoy, USA)

18. Standard film cassette: FOMAPAN DX100

: AGFA APX100

19. UV transilluminator : UVP (USA)

20. Waterbath: SBS 30 (STUART Scientific, UK)

2.2 Chemicals

1. Absolute ethanol (Merck, Germany)

2. Boric acid (Merck, Germany)

3. Bovine Serum Albumin Acetylated (Promega, USA)

4. Bromophenol blue (Merck, Germany)

5. Chloroform (Merck, Germany)

6. 100 mM dATP, dCTP, dGTP, dTTP, (New England Biolabs, USA)

7. Ethidium bromide (Sigma Chemical Company, USA)

8. Ficoll Type 400 (Sigma Chemical Co., USA)

9. 25 mM $MgCl_2$ (Perkin – Elmer Cetus, USA)

10. 10X PCR buffer; 100 mM Tris – HCl , pH 8.3 , 500 mM KCl (Perkin – Elmer Cetus, USA)

11. Phenol, redistilled (Aldrich Chemical Co., USA)

12. Potassium acetate (Merck, Germany)

13. Sodium acetate (Merck, Germany)

14. Sodium chloride (Merck, Germany)

15. Sodium dodecyl sulphate: SDS (Sigma Chemical Co., USA)

16. Spermidine trihydrochloride (Sigma Chemical Co., USA)

17. Sodium sulfite, Photographic Grade (Sigma Chemical Co., USA)

18. Tris (hydroxymethyl) aminomethane (Sigma Chemical Co., USA)

19. Ultrapure agarose (Gibco BRL Life Technology, Inc., USA, Seakem GTG, FMC)

: SeaKem LE Agarose

: MetaPhor Agarose

2.3 Enzymes

1. Ampli*Taq* DNA Polymerase (Perkin Elmer Cetus, USA)

2. Proteinase K (Gibco BRL Life Technology, Inc., USA)

3. Ribonuclease A (Sigma Chemical Co., USA)

4. Restriction endonuclease: *Eco* R I, *Bam* H I, *Hind* III, *Acs* I, *Bfr* I, *Swa* I, *Bgl* II,*Nde* I, *Ssp* I, *Hae* III, *Cla* I, *Dra* I, *Alu* I, *Mbo* I, *Dde* I, *Taq* I, *Rsa* I, and*Hinf* (Promega, USA)

2.4 Primers

Oligonucleotides

Bioservice Unit (BSU), National Center of Genetic Engineering and Biotechnology (BIOTEC), Thailand

Biosynthesis, Inc., USA

Vetrogen, Inc., Canada

2.5 Sampling

Two hundred and thirty-three individuals of *P.monodon* were wild–caught alive from Phangnga (*N* = 39), Satun (*N* = 50), Trang (*N* =54) in the Andaman Sea and from Chumphon (*N*= 55) and Trat (*N*=35) in the Gulf of Thailand during January to December 1997 (Appendix A). One hundred fifty-four individuals were included for mtDNA-RFLP analysis. Pleopods of each P. *monodon* individual were dissected out, placed on dry ice and transferred to a –80 °C freezer for long storage.

2.6 DNA extraction

Total DNA was prepared from each shrimp using a modification of the method of Klinbunga et al. (1996). Unless specified, the extraction process was carried out at room temperature. A pleopod was dissected out from individual *P. monodon*, homogenised with a micropestle in a prechilled 1.5 ml microcentrifuge tube containing 400 ml of TEN buffer (200 mM Tris-HCl, 100 mM Na_2EDTA and 250 mM NaCI; pH 8.0). The cells were lysed by an addition of 40 ml of 10% sodium dodecyl sulfate (SDS). RNA was removed by adding 5 μl of an RNaseA solution (10 mg/ml) to a final concentration of 100 μg/μl and incubated at 37°C for 30 min. A proteinase K solution (10 mg/ml) was added to a final concentration of 200 mg/ml

Figure 2.1 Map of Thailand illustrating five sample collection sites (Satun, Trang, Phangnga, Chumphon and Trat) of *P. monodon* used in this study.

and further incubated at 55 °C for 3-4 hours. At the end of incubation period, an equal volume of buffer equilibrated phenol was added and gently mixed for 15 min.

The sample was centrifuged at 10,000 rpm for 10 min. The aqueous phase was carefully removed to a new sterile microcentrifuge tube using a wide bore pipette tip. An equal volume of phenol/chloroform/isoamyl alcohol mixture (25:24:1, v/v) was added and mixed gently for 5 min and spun at 10,000 rpm for 10 min. The aqueous phase was transferred to a sterile microcentrifuge tube followed by the addition of an equal volume of chloroform/ isoamyl alcohol (24: 1 v/v). The sample was mixed gently for 5 min and spun at 10,000 rpm for 5 min. Two hundred microlitres of the upper layer was then transferred to a new sterilled tube. An equal volume of TE buffer (10 mM Tris-HCl, pH 8.0 and 0.1 mM EDTA pH 8.0) was added. One-tenth volume of 3 M sodium acetate, pH 5.2, was added followed by 2 volumes of cold absolute ethanol and mixed gently. The mixture was kept overnight in a -20 °C freezer. DNA was recovered by centrifugation at 12,000 rpm for 15 minutes and washed twice with 70 % ethanol, for 30 minutes each, air dried and resuspended in an appropriate amount of a TE solution. The DNA solution was incubated at 37 °C for l-2 hours and stored at 4 °C until further needed.

2.7 Quantitative analysis of DNA concentration

2.7.1 Spectrophotometry

The amount of DNA was spectrophotometrically measured at 260 nm. The OD value at 260 nm allows calculation of total nucleic acid while the value reading at

280 nm determines the amount of proteins in the DNA solution. An OD at 260 nm corresponds to approximately 50 mg/ml for double stranded DNA (Maniatis et al., 1982). The ratio between OD 260/280 provides a rough estimate for the purity of extracted DNA. A pure preparation of DNA has a 260/280 ratio of 1.8 - 2.0 (Kirby, 1992). The DNA concentration is estimated in mg/ml by the following equation.

$$[DNA] = OD_{260} \times \text{Dilution factors} \times 50$$

2.7.2 Mini - gel method

Total DNA concentration can also estimated by comparing the amount of fluorescence of the DNA with varying amount of undigested lambda DNA (25, 50, 100, 150, 200 ng) and λ-Hind III for PCR amplified fragments. DNA was electrophoresed through 0.8 % agarose gels in the presence of 0.5 μg/ml EtBr at 5 - 7.5 V/cm for approximately 60 min.

2.8 Screening of primers for poputation genetic study of *P. monodon*

Thirteen primers (COI-COII, 16S rDNA, 12S rDNA, ATPase6-ATPase8, ND1, ND2 (first primer set), ND4, ND5, ND2 (second primer set), sRNA gene, lrRNA gene, Hemocyanin, 18S rDNA) were screened for the amplification success in of *P. monodon* DNA (Table 2.1).

2.9 PCR amplification

Two different regions of *P. monodon* mitochondrial genome (an intergenic COI - COII and the 16S rDNA) were *in vitro* amplified by PCR. The amplification

Table 2.1 PCR primers and sequences initially screened for the amplification success in *P. monodon*

Primer	Sequence	Reference
COI-COII	COI : 5'TTG ATT TTT TGG TCA TCC AGA AGT 3' COII : 5'GGA CAA ATT TCT GAA CAT TGA CC3'	Sihanunthavong et al. (1999)
16S rDNA	F : 5'CGC CTG TTT AAC AAA AAC AT 3' R : 5' CCG GTC TGA ACT CAG ATC ATG T 3'	Palumbi et al. (1991)
12S rDNA	F : 5' AAA CTA GGA TTA TAT ACC CTA TTA 3' R : 5' AAG AGG GAC GGG CGA TTT GT 3'	Palumbi et al. (1991)
ATPase6-ATPase8	ATPase6 : 5' TTT AAT TCC TCA AAT AAT AAT AC 3' ATPase8 : 5' TTA ATT TGA TTC AGA GAA AT 3'	Crozier and Crozier (1993)
ND1	F : 5' ACC CCG CCT GTT TAC CAA AAA CAT 3' R : 5'GGT ATG AGC CCG ATA GCT TA 3'	*P. notolis*
ND2 (first primer set)	F : 5' AGA GCG TTG CAT TGA AG3' R : 5' AGG AGC ATT AGA GTG AT 3'	*P. notolis*
ND4	F : 5' TTA AAG CAG CCT CTG AGG AGT AC 3' R : 5'AGA CGC ATT GAT TTC GAC AAC G 3'	*P. notolis*
ND5	F : 5' ATA GAG CGT TGC ATT GAA GC 3' R : 5'TCA AGG AGC ATT AGA GTG AG 3'	*P. notolis*
ND2 (second primer set)	F : 5' CTA TTC AGG CAC CAA ACC AA 3' R : 5' AAC GAG AGC GAC GGG CGA TG 3'	*P. notolis*
sRNA gene	F : 5' AAA AAG CTT CAA ACT GGG ATT AGA TAC CCC 3' R : 5'TGA CTG CAG AGG GTG ACG GGC GGT GTG T 3'	Kocher et al. (1989)
lrRNA gene	F : 5' CTA TAG GGT CTT ATC GTC CC 3' R : 5'TTT TGT ACC TTT TGT ATC AGG GTT G 3'	Hall and Smith (1991)
Hemocyanin	F : 5' CAA CAT ACA AGG CAT AGA CA 3' R : 5' GAC GGA GAC TTG CTG GCT AC 3'	Moore et al. (1998)
18S rDNA	F : 5' TGG ATC CGG GCA AGT CTG GTG CC 3' R : 5' TGA AGT CAA GGG CAT CAC AGA CC 3'	Aoki, T (personal communication)

reaction was prepared in a 50 ml reaction volume containing 25 ng DNA template, 200 mM of each dNTP (dATP, dCTP, dGTP, dTTP), 1x PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl), 2.5 mM $MgCl_2$, 0.20 μM and 0.5 μM each of primers for COI - COII and 16S rDNA and 1 unit of *Taq* DNA polymerase.

The amplification reaction of an intergenic CO I-II was initially denatured at 94 °C for 3 min followed by 10 cycles of 92 °C for 1 min, 48 °C for 1 min and 72°C for 1.5 min and 35 cycles of 92 °C for 1 minute, 55 °C for 1.5 min and 72°C for 1.5 min. The final extension was carried out at 72 °C for 7 min.

The amplification reaction of 16S rDNA was initially denatured at 94 °C for 3 min followed by 10 cycles of 92 °C for 1 min, 45 °C for 1 min and at 72 °C for 1.5 min and 35 cycles of 92 °C for 1 minute, 52 °C for 1.5 min and 72°C for 1.5 min. The final extension was also carried out at 72 °C for 7 min.

Five microlitres of the PCR reaction were electrophoresed in 1.0% agarose gels to determine whether the reaction was successfully amplified. Samples showing positive results were subjected to restriction enzyme analysis.

2.10 Agarose gel electrophoresis

DNA fragments were separated by agarose gel electrophoresis on the basic of molecular sizes. Suitable amount of agarose (SeaKem LE or Metaphor agarose, FMC, Table2.2) was weighed out and mixed with 1X TBE buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.O). The agarose solution was heated to complete solubilisation in a microwave oven. The melted agarose was left at room

temperature to approximately 50 °C before poured into a gel mould. The gel was left at room temperature for 40-60 min to completely solidify.

For Metaphor agarose, the gel was prechilled in a refrigerator for at least 30 min to achieve sieving ability before being used. When needed, the gel was placed in the electrophoretic chamber containing 1x TBE buffer which is enough to cover the gel for approximately 2-3 mm. The comb was gently removed.

One-quarter volume of the loading buffer (0.25% bromphenol blue and 25% ficoll) was mixed with each DNA sample before loaded into the well. Eletrophoresis was carried out at 3-4 V/cm until bromphenol blue migrated to the bottom of the gel. The electrophoresed gel was stained with a 0.5 mg/ml ethidium bromide solution for 15 min and destained twice in distilled water for 15 min each to remove unbound ethidium bromide from the gel. The DNA was visualized under UV light. The gel was photographed through a red filter using a typical camera (K1000; Pentax) or a polaroid camera (Polaroid film type G65).

Table 2.2 Optimal concentration of Seakem LE and Metaphor agarose prepared in 1XTBE buffer for separation of double stranded DNA.

Gel percentages	Range of fragment size to be separated (bp)	
	Seakem LE agarose	MetaPhor agarose
0.50	1,000-23,000	-
0.70	800-10,000	-
0.85	400-8,000	-
1.00	300-7,000	-
1.25	200-4,000	
1.75	100-3,000	-
2.00	-	100-600
3.00	-	50-25
4.00	-	20-130
5.00	-	<80

Reference: FMC BioProducts, Denmark.

2.11 Digestion of amplified COI - COII and 16S rDNA with restriction endonucleases

Eighteen restriction enzymes *Dra* I (TTTAAA), *EcoR* I (GAATTC), *Alu* I (AGCT), *Mbo* I (GATC), *Dde* I (CTNAG), *Bam* HI (GGATCC), *Hind* III (AAGCTT), *Taq* I (TCGA) , *Acs* I (A/GAATTT/C), *Bfr* I (CTTAAG), *Swa* I (ATTT/AAAT), *Hinf* I (GANTC), *Bgl* II (AGATCT), *Rsa* I (GTAC), *Nde* I (CATATG), *Ssp* I (AATATT), *Cla* I (ATCGAT) and *Hae* III (GGCC) were screened

against ten *P. monodon* individuals to identify informative restriction endonucleases (enzymes showing polymorphic cutting sites).

After informative enzymes were selected, the amplified DNA fragments of each *P. monodon* was separately digested with a battery of restriction endonucleases (*Mbo* I for 16S rDNA ,and *Alu* I, *Mbo* I, *Dde* I, *Hinf* I, *Taq* I for an intergenic COI-COII, respectively). Digestion was performed in a 15 µl reaction volume containing, 1.5 µl of 10X restriction enzyme buffer, 1.5 µl of 40 mM spermidine, 0.15 µl of 100 mg/ml BSA, 2-5 units of each restriction endonuclease, 400 ng of DNA and appropriate amount of sterile deionised water. The reaction mixture was incubated at 37 °C for 6-12 hours for all enzymes except *Taq* I where the optimal temperature was at 65°C. At the end of digestion period one-fifth volume of a loading dye was added to the reaction mixture. The resulting mixture was thoroughly mixed and electrophoretically analysed in 2.5-3.5% Metaphor agarose gels.

2.12 Data analysis

2.12.1 Restriction pattern analysis

Restriction patterns generated from each restriction endonuclease were given letter designations according to their frequencies. Haplotype A refers to the most common digestion pattern in investigated specimens. The remaining alphabetical profile names (B, C, etc.) indicate variant digestion patterns reflecting their frequencies in order. Composite haplotypes were constructed from all enzymes used and arranged from patterns of amplified 16S rDNA digested with *Mbo* I followed by those of the intergenic COI -COII digested with *Alu* I, *Mbo* I, *Taq* I, *Dde* I, and *Hin*

fI, respectively. Binary characters including the presence (1) or absence (0) of a particular band in each composite haplotypes were recorded and used for further statistical analysis. All subsequent statistical analyses were carried out using Restriction Enzyme Analysis Package, REAP (McElroy, 1991).

2.12.1.1 Genetic distance

The genetic distance (percentage of sequence divergence between mtDNA haplotypes) was estimated by

$$d = -(2 / r)\log_e(G)$$

where r is the number of recognised sequences at the restriction site and G = [F (3-$2G_1$]$^{1/4}$. This iterative computation is repeated until G = G_1.

F is the similarity between haplotype patterns and is estimated by

$$F = 2m_{xy} /(m_x + m_y)$$

where m_x and m_y are the numbers of restriction fragments in the x^{th} and y^{th} haplotypes, respectively, and m_{xy} is the number of shared fragments between two haplotypes (Nei and Li, 1979).

2.12.1.2 Haplotype and nucleotide diversity with geographic samples

Genetic diversity within geographic samples was estimated from the haplotype and nucleotide diversity. The former is a function of the frequency of different haplotypes in the same sample which can be estimate by

$$\hat{h} = n(1 - \sum_{i=1}^{1} x_i^2) / (n - 1)$$

where n is the number of individuals investigated and x_i is the frequency of the i^{th} haplotype (Nei and Tajima, 1981).

Nucleotide diversity within sample (the average number of nucleotide substitutions within a sample) was calculated by

$$\hat{d}_x = \frac{n_x}{n_x - 1} \sum_{ij} \hat{x}_i \hat{x}_j d_{ij}$$

where n_x is the number of sequences sampled and d_{ij} is the number of nucleotide substitutions per site between the i^{th}. and j^{th} haplotype. The x_i and x_j values are the sample frequencies of the ith and jth haplotypes in geographic sample X (Nei, 1987).

2.12.1.3 Nucleotide divergence

Nucleotide diversity between two samples (the average number of nucleotide substitutions between DNA haplotypes from samples X and Y) was estimated by

$$\hat{d}_{xy} = \sum_{ij} x_i y_j d_{ij}$$

where d_{ij} is the nucleotide substitutions between the i^{th} and j^{th} haplotype from geographic sample X and Y, respectively. Nucleotide divergence between two samples (the average number of nucleotide substitution per site where the effect of within geographic sample polymorphism has been substracted) was then calculated by

$$\hat{d} = \hat{d}_{xy} - (\hat{d}_x + \hat{d}_y)/2$$

All statistical parameters described above were practically calculated using REAP, version 4.0 (D. McElroy, et al., 1991).

2.12.1.4 Geographic heterogeneity analysis

To determine whether there was significant heterogeneity in the distribution of mtDNA composite haplotypes among geographically isolated samples, a Monte Carlo simulations was carried out 10,000 times (Roff and Bentzen, 1989) using the MONTE routine from REAP. Results are expressed as the probability of homogeneity among compared samples or regions.

2.12.2 Phylogenetic reconstruction

A phenogram based on genetic distance (d) between composite haplotypes was constructed using the unweighted pair-group method with arithmetic mean (UPGMA) implemented in Phylip, version 3.56c (Felsenstein, 1993).