4. MATERIALS AND METHODS

I. Materials

1. Animals

Two hundred and thirty-seven adult banana prawns were collected from the Andaman Sea (Phuket (PKW, 16), Ranong (RNW, 34), Satun (STW, 31) and Trang (TRW, 34) provinces) and the Gulf of Thailand (Songkhla (SKE, 34), Trad (TDE, 30), Surat-thani (SRE, 27) and Nakhon si thummarat (NKE, 31) provinces) (Figure 14).

2. Primers

The mitochondrial DNA primer oligonucleotide set (Table 4) was synthesized from the University of British Columbia Nucleic Acid-Protein Service Unit.

3. Plasmid Vector

pGEM®-TEasy Vectors were purchased from Promega (USA).

4. Bacterial strains

Escherichia coli strain Top 10F': {*lac*I^q, Tn10 (Tet ^{R})} *mcr*A Δ (*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*Z Δ M15 Δ *lac*X74 *deo*R *rec*A1 *ara*D139 Δ (*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G.

5. Chemicals

All chemicals and solvents (analytical grade) used in this study were purchased from Fluka, Switzerland; Sigma, US; and Promega, USA.

6. Enzymes

All of the enzymes and modifying enzymes were purchased from New England Biolabs, USA; Promega, USA; and QIAGEN, Germany.

▲ *P. merguiensis* (M)

■ *P. indicus* (I)

△ *P. silasi* (S)

□ Not determine species (ND)

○ *P. merguiensis* or *P. indicus* (M/I)

Figure 12. The eight sampling locations in Thailand for collection of specimen in this study.

Table 4. The invertebrate universal primers for amplification of the mitochondrial genome (Simon et al., 1994).

Name	Region in genome	Sequence (5'-3')
mtD-1	ND2	ATT TAC CCT ATC AAG GTA A
mtD-2	ND2	GCT AAA TAA GCT AAC AGG TTC AT
mtD-3	ND2	TGG GGT ATG AAC CCA GTA GC
mtD-4	ND2	TAC AAT TTA TCG CCT AAA CTT CAG CC
mtD-5	ND2	TGT TCC TAC TAT TCC GGC TCA
mtD-6	ND2	GGA GGA TTT GGA AAT TGA TTA GTT CC
mtD-7	ND2	GGA TCA CCT GAT ATA GCA TTC CC
mtD-8	COI	CAA CAT TTA TTT TGA TTT TTT GG
mtD-9	COI	CCC GGT AAA ATT AAA ATA TAA ACT TC
mtD-10	COI	TTG ATT TTT TGG TCA TCC AGA AGT
mtD-11	COI	ACT GTA AAT ATA TGA TGA GCT CA
mtD-12	COI	TCC AAT GCA CTA ATC TGC CAT ATT A
mtD-13	COII	AAT ATG GCA GAT TAG TGC A
mtD-14	COII	GGT CAA ACA ATT GAG TCT ATT TGA AC
mtD-15	COII	TCA TAA GTT CAR TAT CAT TG
mtD-16	COII	ATT GGA CAT CAA TGA TAT TGA
mtD-17	COII	GGT AAA ACT ACT CGA TTA TCA AC
mtD-18	COII	CCA CAA ATT TCT GAA CAT TGA CCA
mtD-19	COII	GAA ATT TGT GGA GCA AAT CAT AG
mtD-20	COII	GTT TAA GAG ACC AGT ACT TG
mtD-21	COIIIa	TTA TTT ATT GCA TCA GAA GT
mtD-22	COIIIb	TCA ACA AAG TGT CAG TAT CA
mtD-23	ND4rev	AAA GCT CAT GTT GAA GCT CC
mtD-24	ND4	GGA GCT TCA ACA TGA GCT TT
mtD-25	CB1L	CCA TCC AAC ATC TCA GCA TGA TGA AA
mtD-26	CB1	TAT GTA CTA CCA TGA GGA CAA ATA TC
mtD-27	CB2	CCC TCA GAA TGA TAT TTG TCC TCA
mtD-28	CB2	ATT ACA CCT CCT AAT TTA TTA GGA AT
mtD-29	ND1A	GGT CCC TTA CGA ATT TGA ATA TAT CCT
mtD-30	ND1	GTA GCA TTT TTA ACT TTA TTA GAA CG
mtD-31	16Sb	ACA TGA TCT GAG TTC AAA CCG G
mtD-32	16Sbr	CCG GTC TGA ACT CAG ATC ACG T
mtD-33	16Sa	ATG TTT TTG TTA AAC AGG CG
mtD-34	16Sar	CGC CTG TTT AAC AAA AAC AT
mtD-35	12Sbi	AAG AGC GAC GGG CGA TGT GT
mtD-36	12Sai	AAA CTA GGA TTA GAT ACC CTA TTA T
mtD-37	12S	TTA AAG TTT TAT TTT GGC

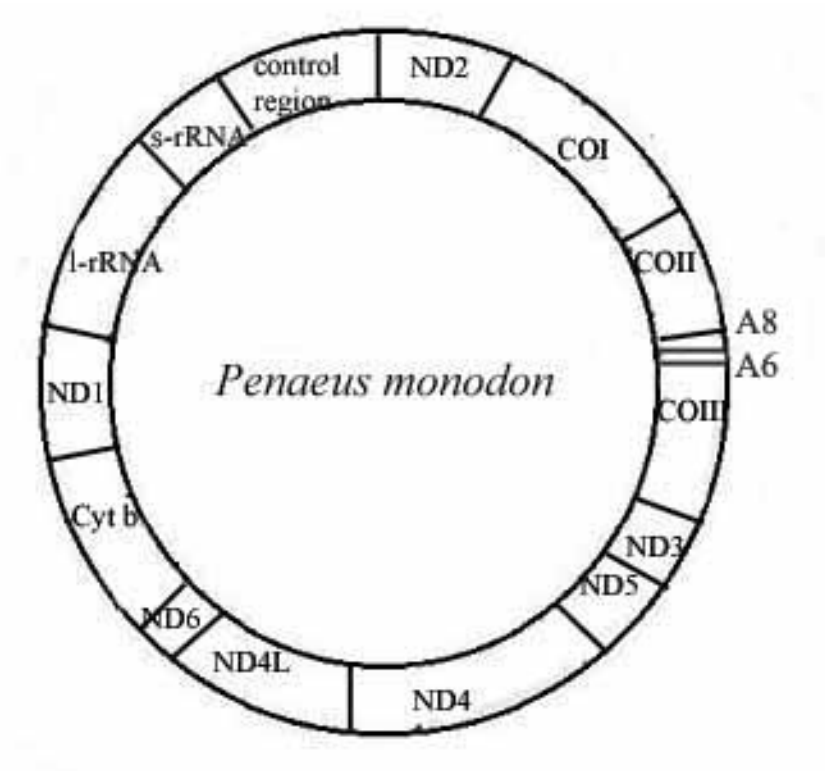

Figure 13. The mitochondrial genome of *Penaeus monodon*.

COI = Cytochrome oxidase subunit I, COII = Cytochrome oxidase subunit II, COIII = Cytochrome oxidase subunit III, Cyt b = Cytochrome b (Cyt b), ND1 = NADH dehydrogenase subunit 1, ND2 = NADH dehydrogenase subunit 2, ND3 = NADH dehydrogenase subunit 3, ND4 =NADH dehydrogenase subunit 4, ND4L = NADH dehydrogenase subunit 4L, ND5 = NADH dehydrogenase subunit 5, ND6 = NADH dehydrogenase subunit 6, 12S = The small subunit ribosomal RNA (s-rRNA) and 16S = The large subunit ribosomal RNA (l-rRNA).

II. Methods

1. Morphology and Morphometric Identification

The samples were identified individually according to taxonomic keys such as the rostrum, the distance between the hepatic spine and gastro-orbital ridge and the third maxilliped in males (Grey et al., 1983; Carpenter and Niem, 1998 and Chaitiamvong and Supongpun, 1992). The morphometric measurements followed (L_1: $L_{1,2}$ and carapace length according to Pendrey et al., 1999).

2. Isozyme Analysis

2.1 Protein extraction

Approximately 0.3 mg of muscle was dissected out into microcentrifuge tubes containing 70-100μL of electrode buffer solution (0.05 M Tris – malate, pH7.5). The sample was centrifuged at 8000 rpm for 5 min to separate the cellular debris from the protein-containing supernatant.

2.2 Electrophoresis

The protein-containing supernatant from the tissue extracts was applied to sephaphore cellulose polyacetate electrophoresis strips which were immersed in electrode buffer solution before use. The electrophoresis chamber was filled with electrode buffer. Power was applied (200V) to the strips for 2h.

2.3 Staining and Detection

The cellulose polyacetate strips were immersed within a solution of staining buffer (as modified by Richardson et al., 1986), containing malic acid 0.4 g as substrate, 20 ml of 0.2M Tris-HCl (pH 8.0), 150 μL of 0.5M $MgCl_2$, 1% NAD^+, 1% NBT, 1% PMS and 1% MTT. After the strips were incubated in the staining buffer in the dark for 30-60 min, the blue bands were appeared.

3. DNA markers

3.1 DNA Extraction

Total genomic DNA was individually extracted from frozen muscle of prawns (*P. merguiensis, P. silasi* and *P. indicus*). The tissue was chopped into small pieces and homogenized in 570 μl of the extraction solution (10 mM Tris-HCl, pH 7.5, 100 mM EDTA, 1% SDS and 30μl of 20μg/ml proteinase K). The homogenate was incubated at 37^o C for 1 h and 55^o C overnight. DNA was extracted twice using phenol/chloroform/isoamyl alcohol (25:24:1) and recovered by ethanol precipitation. DNA concentrations were spectro-photometrically determined (Sambrook, 1989).

3.2 Amplification of 12S rRNA and 16S rRNA gene

PCR was carried out in a 25 μl reaction mixture containing 200 μM each of dNTPs, 1.5 mM $MgCl_2$, 0.25 μM 12S primer pair; mtD-36 (12Sbi or SR-J-14233): 5'-AAG-AGC-GAC-GGG-CGA-TGT-GT-3' and mtD-35 (12Sai or SR-N-14588): 5'-AAA-CTA-GGA-TTA-GAT-ACC-CTA-TTA-T-3'and 16S primer pair; mtD-32 (16Sbr or LR-J-12887): 5'-CCG GTC TGA ACT CAG ATC ACG T-3' and

mtD-34 (16Sar or LR-N-13398): 5'-CGC CTG TTT AAC AAA AAC AT-3' (Simon et al., 1994), 1 U of *Taq* DNA Polymerase and 100 ng of total genomic DNA using a 2400 Perkin Elmer^{TM} thermal cycler. PCR conditions were predenaturation at 95^{o}C for 4 min, followed by 35 cycles of denaturation at 95^{o} C for 30 sec, annealing at 50^{o}C for 30 sec and extension at 72^{o}C for 30 sec and the final extension at 72^{o}C for 10 min.

3.3 Amplification of complete nucleotide sequence of COI

PCR was carried out in a 25 µl reaction mixture containing 200 µM each of dNTPs, 1.5 mM $MgCl_2$, 0.25 µM 5'end of COI primer pair; mtD-4 : 5'- TAC AAT TTA TCG CCT AAA CTT CAG CC -3' and mtD-9: 5'- CCC GGT AAA ATT AAA ATA TAA ACT TC -3'and 3' end of COI primer pair; mtD-8 : 5'- CAA CAT TTA TTT TGA TTT TTT GG -3' and mtD-12: 5'- TCC AAT GCA CTA ATC TGC CAT ATT A -3' (Simon et al., 1994), 1 U of *Taq* DNA Polymerase and 100 ng of total genomic DNA using a 2400 Perkin Elmer^{TM} thermal cycler. PCR conditions were predenaturation at 95^{o}C for 4 min, followed by 35 cycles of denaturation at 95^{o} C for 30 sec, annealing at 45^{o}C for 30 sec and extension at 72^{o}C for 30 sec and the final extension at 72^{o}C for 10 min.

3.4 Agarose gel electrophoresis

After the PCR products were amplified, they were analyzed by agarose gel electrophoresis. In this study, gel electrophoresis was used for determining the size of DNA of interest. For the gel, 1.8% (w/v) of agarose gel in 0.5x TAE buffer (40 mM Tris-borate, 1 mM EDTA) was melted and poured on a plastic tray, and a comb

was placed in the gel. After the agarose gel was completely set (30-45 min at room temperature), the comb was carefully removed and the gel was installed on a platform in the electrophoresis tank containing 0.5xTAE buffer. The PCR products were mixed with 30% (v/v) gel-loading buffer (25% (v/v) glycerol, 60 mM EDTA, 0.25% (w/v) Bromophenol Blue) and slowly loaded into the slots of the submerged gel using an automatic micropipette. The electrophoresis was carried out at a constant 100 V for 30 min. Then, the gel was stained with 2.5 μg/ml of Ethidium bromide (EtBr) solution for 5 min and de-stained with water for 15 min. Finally, the DNA fragments were observed under UV light box Gel Doc model 1000 (BIO-RAD, USA).

3.5 Purification of PCR product by using the QIAquick™ PCR Purification Kit (QIAGEN)

Five volumes of PB buffer were added to one volume of the PCR product reaction and mixed well. The sample was applied to the QIAquick spin column, inserted in a collection tube and centrifuged for 1 min at 12,000 rpm. The product in the column was then washed by adding 750 μl of PE buffer and centrifuge for 1 min. The flow through was discarded and the column was placed back in the same collection tube. Then this column was centrifuged at the same speed for 1 min to remove residual ethanol. The column was then inserted into a new microcentrifuge tube and the PCR products were eluted from the column with 50 μl of EB buffer or distilled water by centrifugation as described above. The DNA profile was determined by agarose gel electrophoresis.

3.6. Ligation of purified PCR product with pGEM ®-TEasy vector

The purified PCR products were ligated with pGEM®-TEasy Vector (Promega) according to the following condition. The 10 µl ligation mixture containing 5 µl of 2x Rapid ligation buffer (60 mM Tris-HCl (pH 7.8), 20 mM $MgCl_2$, 20 mM DTT, 20 mM ATP and 10% Polyethylene glycol), 0.5 µL of pGEM®-TEasy Vector (25 ng), 0.5 µl of T4 DNA ligase (3 U Weiss/µl) and 4 µl of purified PCR product was incubated at 4°C, overnight.

3.7 Transformation into *E. coli* strain Top 10 F' by $CaCl_2$ method

3.7.1 Preparation of *E. coli* strain Top 10 F'competent cells

An isolated single colony of *E. coli* strain Top 10 F'was inoculated into 3 ml of LB broth supplement with 100 µg/ml of tetracycline, incubated at 37°C overnight with shaking. The overnight culture was inoculated in 25 ml of fresh medium (1:100 dilution) and incubated at 37°C until OD_{600} reached 0.3-0.5. The cell pellet was harvested by centrifugation at 4,500 rpm for 6 min at 4°C and washed with 20 ml of ice-cold 0.1 M $MgCl_2$. The cell suspension was centrifuged at 4,500 rpm for 6 min at 4°C. The pellets were resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$, then incubated on ice for at least 15 min to establish competency. The cell suspension was centrifuged, then, resuspended in 1.7 ml of ice-cold 0.1 M $CaCl_2$. A volume of 0.3 ml of glycerol was added into the cell suspension to give 15% (v/v) of final concentration. The cell suspension was aliquoted in a volume of 100 µl per tube and kept at –80°C.

3.7.2 Transformation into *E. coli* strain Top 10 F' competent cells

A volume of 100 µl of *E. coli* competent cells was mixed gently with 0.1-1.0 µg of ligated DNA. The mixture was left on ice for 30 min to give higher transformation frequency. The cell mixture was incubated at 42°C for 90 sec and placed on ice for an additional 5 min. The transformed cells were mixed with 500 µl of LB low salt broth and incubated at 37°C for 1 h with constant shaking. Finally, 200 µl of transformed culture was spread on LB selective plate and incubated at 37°C for 16 h.

3.8 Plasmid extraction and purification from *E. coli* strain Top 10 F'

A single bacterial colony was inoculated into 3.0 ml of LB medium supplemented with 80 µg/ml of ampicillin and incubated overnight at 37°C with vigorous shaking. The cell culture was collected using centrifugation at 10,000 rpm for 1 min at room temperature in microcentrifuge tube. The supernatant was discarded, the bacterial pellet was resuspended in 350 µl of STET buffer (8% (w/v) glucose, 5% (v/v) Tritron X-100, 50 mM EDTA and 50 mM Tris-HCl, pH 8.0) and 25 µl of a 10 mg/ml lysozyme solution was added. The mixture was mixed and left at room temperature for 2 min, placed in the boiling-water bath for 40 sec and then incubated on ice for 3 min. The bacterial lysate was centrifuged at 14,000 rpm for 10 min at room temperature, then the pellet of bacterial debris was removed from the microcentrifuge tube with a sterile toothpick. 350 µl of isopropanol was added to this tube and mixed inversely, after which the mixture was incubated at -80°C for 10 min.

After centrifugation at 14,000 rpm for 10 min at 4°C, the supernatant was discarded. Finally, the tubes were placed in an inverted position on a paper towel to allow all fluid to drain off. Plasmid DNA was resuspended in 50 µl of 10 mM Tris-HCl, pH 8.0 containing DNase free pancreatic RNase A (2mg/ml) and stored at –20°C.

3.8.1 Purification of Plasmid DNA using QIAprep spin miniprep kit (QIAGEN)

A single colony of bacteria was inoculated in 6 ml of LB supplemented with 100 mg/ml of ampicillin. The cell culture was incubated at 37°C with shaking for 16-18 h. The bacterial cell pellets were harvested in 1.5 ml microcentrifuge tubes by centrifugation at 14,000 rpm for 15 sec and then resuspended gently with autopipette in 250 µl of P1 buffer. 250 µl of P2 buffer was added to the cell mixture and gently mixed by inverting the tube 4-6 times. Then 350 µl of N3 buffer was added and mixed gently. The cell mixture was centrifuged at 14,000 rpm for 10 min at room temperature. The supernatants were applied to the QIAprep column by decanting or pipetting. The flow through was discarded by centrifugation at 14,000 rpm for 1 min. The column was washed by adding 0.5 ml of PB buffer and centrifuged for 1 min at 14,000 rpm. After that 0.75 ml of PE buffer was added to the column, flow through was removed by centrifugation pellet and additionally centrifuged for 1 min to get rid of residual wash buffer. The QIAprep column was placed in clean microcentrifuge tube and then plasmid DNA was eluted by adding 50 µl of sterile distilled water, left at room temperature for 10 min and centrifuged at 14,000 rpm for 1 min.

3.9 Restriction endonuclease digestion

Restriction endonucleases are groups of enzymes that bind specifically and cleave double-stranded DNA at specific sequences. A typical reaction contains 1-2 µg of DNA or PCR product, 1 µl of restriction enzyme (1-10 U), 1x reaction buffer and sterile distilled water or TE (10 mM Tris-HCl pH 7.0, 1 mM EDTA pH 7.0) to give a total volume of 20 µl. The restriction enzymes used in this study, including their restriction sequences and optimal temperature, are shown in Table 6. After digestion had been completed, the digested products were analyzed by agarose gel electrophoresis.

Table 5. The restriction endonucleases with their recognition sequences and optimal temperatures.

Restriction enzyme	Recognition sequences	Optimal temperature
*Eco*RI	G↓AATTC	37°C
*Rsa*I	GT↓AC	37°C
*Bgl*II	A↓GATCT	37°C
*Mbo*I	↓GATC	37°C

3.10 Automated DNA sequencing

3.10.1 Preparation of 5% Acrylamide gel /6M Urea

18 g of urea and 5 ml of 50% acrylamide stock solution were combined in a 125 ml Erlenmeyer flask. De-ionized water was added to bring the

volume up to 40 ml. The solution was stirred and heated gently until the crystals just begin to dissolve. The heat was turned off and stirring continued until the crystals were dissolved completely. 5 ml of filtered 10 X TBE stock solution (40 mM Tris-borate, 1 mM EDTA) and de-ionized water were added to bring the volume to 50 ml. The acrylamide solution was vacuum filtered by using 0.2 μm unit for 5 min. 250 μl of 10% APS was added into the filtered gel solution and swirled for a few seconds. 25 μl of TEMED was added into the acrylamide solution and gently swirled for a few seconds. Then the solution was injected between the glass plates until the solution was up to the notch. The gel was polymerized at room temperature for at least 2 hr.

3.10.2 Preparation of the DNA samples for sequence analysis

The ABI PRISM™ BigDye Terminator Cycle Sequencing Kit was used to prepare the DNA samples for sequence analysis. The principle of the protocol is based on fluorescent-labeled terminator cycle sequencing. The PCR reaction was performed in a 20 μl reaction mixture containing a terminator ready reaction mix [A-dye terminator labeled with dichloro (R6G), C-dye terminator labeled with dichloro (ROX), G-dye terminator labeled with dichloro (R110), T-dye terminator labeled with dichloro (TAMR10), deoxynucleoside triphosphates (dATP, dCTP, dITP, dUTP), $MgCl_2$, Tris-HCl pH 9.0, Amplitaq DNA polymerase], 200-500 ng of plasmid DNA template, 3.2 pmol of primer and sterile water. The amplification was carried out in a DNA thermal cycler (GeneAmp System 2400, Perkin Elmer) with the following condition: 25 cycles of 96°C for 10 seconds, 55°C for 5 seconds and 70°C for 1 min. The PCR-based sequencing products were precipitated by

isopropanol after amplification and the dye-labeled DNA was analysed on 5% Long Ranger/6M Urea Acrylamide Gel by an Applied Biosystems 377 sequencer (Perkin-Elmer, Norwalk, CT, USA).

3.10.3 Precipitation of sequencing products

20 μl of dye-labeled DNA in 0.2 ml PCR tube were pipetted to 1.5ml microcentrifuged tubes and 80 μl of 75% isopropanol was added. The tubes were closed and then vortexed briefly. The reaction was incubated at room temperature for 15 min to precipitate the PCR products. The tubes were placed in a microcentrifuge and their orientations marked, followed by spinning for 20 min at maximum speed. The supernatants were aspirated and discarded by a separate pipette tip for each sample. 250 μl of 75% isopropanol was added to the tubes and vortexed briefly again. The tubes were placed in the microcentrifuge in the same orientation as the first time and spun for 5 min at maximum speed. The supernatants were aspirated carefully. The pellet was dried in a vacuum dryer for 10-15 min.

3.10.4 Preparing and loading the samples

6 μl of loading buffer (5 μl of deionized formamide, 1μl of 25 mM EDTA pH8 with 50 mg/ml blue dextran) were added to the dry pellet (product from 3.10.2). Vigorous vortex and spun to dissolve the pellet. This solution was heated at 95 ^{o}C for 2 min to denature the nucleotide. Then the tubes were placed on ice until ready to load on polyacrylamide gel.

3.11 Sequence Analysis

The 12S and 16S partial nucleotide sequences from *P.merguiensis* (AF401304, AF335280), *P. silasi* (AF401303, AF401305), *P. indicus* (AF401302, AF335279) and *P. monodon* (AF217843) were aligned by using Clustal W (Thompson et al., 1994). The sequence divergence between pairs of sequences was estimated using Kimura's (1980) two-parameter model.

The COI nucleotide and amino acide sequences from *P. merguiensis* and *P. silasi* from Thailand, *P. indicus* from the South Africa, and those retrieved from the GenBank including *P. merguiensis* (AF029390), *P. indicus* (AF014378), *P. monodon* (AF014377), *P. vannamei* (AF014383), and *P. japonicus* (AF014385), were aligned by using Clustal W (Thompson et al., 1994). The sequence divergence between pairs of sequences was estimated using Kimura's (1980) two-parameter model. 12S, 16S and COI nucleotide sequence data was bootstrapped into 1000 replications. The distance matrices were produced using DNAdist and Prodist in the PHYLIP package (Felsenstein, 1993). A tree based on the genetic distances was constructed by the Neighbor-Joining method (Saitou and Nei, 1987) using the NEIGHBOR program in the PHYLIP 3.5 package. A consensus neighbor-joining tree based on divergence between pairs of sequences was then constructed using Neighbor-Joining (Saitou and Nei, 1987) in PHYLIP (Felsenstein, 1993).