CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipment

- Autoclave: HEV-50 (Hirayama, Japan)
- Automatic micropipettes P10, P20, P100, P200, and P1000 (Gilson S.A., France)
- Camera: K1000 (Pentax, Japan)
- -20 °C Freezer (Songserm Intercool, Thailand)
- -30 °C Freezer (Sanyo, Japan)
- -80 °C Freezer (Sanyo, Japan)
- Microcentrifuge: MicroCen 13D (Herolab, Germany)
- Microcentrifuge tube 0.5 and 1.5 ml (Bio-RAD Laboratories, USA)
- Microwave (Hitachi, Japan)
- pH meter (Orion, USA)
- PCR Thermal cycle: Omnigene-E (Hybaid Limited, England)
- PCR Thermal cycle: Sprint (Hybaid Limited, England)
- Pipette tips 0.2, 10, 200, 1000 µl (Bio-RAD Laboratories, USA)
- Power supply: Power Pac 300 (Bio-RAD Laboratories, USA)
- Refrigerated centrifuge: 3K18 (Sigma, Germany)
- Scuba gear
- Shaker bath: SBS30 (STUART Scientific, UK)
- Spectrophotometer: Spectronic GENESYS5 (MiltonRoy, USA)

- Sterilize syringe 1 ml
- Underwater camera
- UV transilluminator: UVP (USA)

2.1.2 Chemicals

- Absolute ethanol (Merck, Germany)
- Boric acid (Merck, Germany)
- Bromphenol blue (Merck, Germany)
- Chloroform (Merck, Germany)
- 100 mM dATP, dCTP, dGTP, dTTP (Promega Corporation Medison, Wisconsin)
- Ethidium bromide (Sigma Chemical Co., USA)
- Ethylene diamine tetraacetic acid, disodium salt dihydrate (Sigma Chemical Co., USA)
- Ficoll Type400 (Sigma Chemical Co., USA)
- GeneAmp PCR core reagents (Perkin Elmer Cetus, USA)

  : 10x PCR buffer (100mM Tris-HCl pH8.3, 500 mM KCl)

  : 25 mM $MgCl_2$
- Isoamyl alcohol (Merck, Germany)
- Mineral oil (Sigma Chemical Co., USA)
- Oligonucleotide primers: 10-mers (BSU, National Center for Genetic Engineering and Biotechnology)
- Phenol, redistilled (Aldrich Chemical Co., USA)
- Sodium acetate (Merck, Germany)
- Sodium chloride (Merck, Germany)
- Sodium dodecyl sulfate (Sigma Chemical Co., USA)
- Sodium hydroxide (Merck, Germany)

- Tris (hydroxy - methyl) amino-methane (Sigma Chemical Co., USA and Pharmacia)
- Ultrapure agarose (SeakemGTG, FMC)

2.1.3 Enzymes

- Ampli *Taq* DNA polymerase (Perkin Elmer Cetus, USA)
- DyNAzyme^{TM} II DNA Polymerase (Finnzymes, Finland)
- Proteinase K (Gibco BRL life technologies, Inc., USA)
- RNase A (Sigma Chemical Co., USA)

2.2 Methods

2.2.1 Choices for sample collection sites

The primary data for sources of abalone in Thailand was obtained by interviewing researchers, fishermen and seafarmers. The secondary data, about habitats and distributions of *H. asinina*, *H. ovina* and *H. varia* in Thai waters, was obtained through previous publications between 1986 to 1998 (Nateewathana and Hylleberg, 1986; Tookwinas *et al.*, 1986, Nateewathana and Bussarawit, 1988, Jarayabhand *et al.*, 1991; Kakhai and Petjamrat, 1992; Singhagriwan, 1992, Tanawansombat, 1992; Ngow and Jarayabhand, 1993; Jarayabhand and Paphavasit, 1996 and Jarayabhand *et al.*, 1998). Coral reef maps for the Gulf of Thailand and the Andaman Sea were used in order to choose suitable sampling sites of abalone (Department of Fisheries, 1999). Geographically different locations of *H. asinina* and *H. ovina* were selected covering both sides of peninsular Thailand. Additionally, *H. varia*, which has not been reported in the Gulf of Thailand, was collected only from the Andaman Sea.

2.2.2 Sources of specimens

2.2.2.1 Natural habitat

Abalone were collected by snorkeling and SCUBA diving covering their geographic distributions in Thailand (the upper Gulf of Thailand and the Andaman Sea). Live specimens collected from various locations in the Gulf of Thailand were brought back to the hatchery at the Angsila Marine Biological Research Station located at Chon Buri Province. Dead specimens were transported on ice to the laboratory at the Marine Biotechnology Research Unit (MBRU), Chulalongkorn University, as soon as possible.

To collect specimens from the Andaman Sea, a one-week trip was usually required due mainly to the long traveling distance. The collected specimens were sent back to the laboratory by plane as soon as possible. Alternatively, specimens collected from the first few days of the trip were kept alive in the sea prior to final transportation to the laboratory.

Abalone specimens of each species were collected from different locations depending on their habitats, for example, *H. asinina* and *H. ovina* were collected from the subtidal zones (*H. asinina* live under dead corals or in the crevices of the branching corals whereas *H. ovina* was collected from the crevices of the living corals). *H. varia* were collected from the intertidal areas as well as from the rock crevices in the subtidal zones.

Information regarding abalone habitats from some publications appeared to be out of date. Specimens were not present in several locations that were previously reported.

2.2.2.2 Abalone populations

Apart from wild specimens, *H. asinina* were also obtained from three hatchery locations. They are from the Angsila Marine Biological Research Station, Phuket Abalone Farm and SEAFDEC (the Philippines) for the hachery *H. asinina* 's stock. No hatchery stocks were available for *H. ovina* and *H. varia*, therefore only broodstock was collected.

A total number of collected specimens was three hundred and thirty-one individuals, comprising of *H. asinina* (*N*=163), *H. ovina* (*N*=135) and *H. varia* (*N*=33). Geopraphic locations of sampling sites were illustrated by Fig 2.1 and Table 2.1. The whole specimens were collected at -30°C while dissected foot tissue was kept at -80°C.

Figure 2.1 Map of Thailand indicating collection sites of abalone in the Gulf of Thailand and Andaman Sea used in this study.
(*Haliotis asinina* = ● , *H. ovina* = ▲ and *H. varia* = ■).

Table 2.1 Sample collection sites, code of populations and number of collected individuals (and number of individuals used in this study) for three species of abalone in Thailand.

Collection site	Code of populations	No. of individuals (No. used in this study)
H. asinina		
Samet Island, Rayong	HASM	19 (14)
Talibong Island, Trang	HALB	28 (19)
Cambodia	HACB	23 (19)
Hatchery stock, P_0 (Broodstock from Samet Island, Rayong)	HASH	28 (14)
Hatchery stock, P_0 (Broodstock from Cambodia)	HACH	15 (13)
Hatchery stock, F_1 (Broodstock from Philippines)	HAPH	30 (20)
H. ovina		
Khang Kao Island, Chon Buri	HOSC	29 (29)
Samet Island, Rayong	HOSM	43 (27)
Chuak Island, Trang	HOTR	47 (23)
Similan Island, Phang-nga	HOPG	16 (16)
H. varia		
Aeo Island, Phuket	HVPK	29 (28)
Similan Island, Phang-nga	HVPG	4 (4)
Total		**311 (206)**

2.2.3 DNA extraction

Genomic DNA was extracted from the frozen foot tissue or haemolymp of each abalone using a phenol-chloroform proteinase K method (Klinbuga *et al.*, 1999). A piece of foot tissue was dissected out, placed in a 1.5 ml microcentrifuge tube containing 360 µl of extraction buffer (200 mM Tris-HCl, 100 mM Na_2EDTA and 250 mM NaCl; pH 8.0) and 80 µl of 10% SDS, and homogenized with a micropestle. RNA was digested by adding 8 µl of RNaseA (10 mg/ml). Additional 360 µl of extraction buffer was added. The homogenate was further homogenized for a few strokes and incubated at 37°C for 1 hr. A proteinase K solution (10 mg/ml) was added to make a final concentration of 200 mg/ml. The mixture was further incubated at 55° C for 4 hours. An equal volume of buffer equilibrated phenol was added and gently mixed for 20 min. The sample was centrifuged at 12,000 rpm for 10 min at room temperature. The upper aqueous phase was removed without disturbing the organic/aqueous interface. Phenol extraction was repeated twice. An equal volume of chloroform/isoamyl alcohol (24:1, v/v) was added and mixed gently. The upper aqueous phase was transferred to a new microcentrifuge tube. This step was repeated twice. The upper phase from the final chloroform:isoamyl alcohol extraction was removed. One - half volume of TE buffer (10 mM Tris-HCl, pH 8.0 and 0.1 mM EDTA pH 8.0) was added followed by one-tenth volume of 3M sodium acetate, pH 5.2 and 2 final volumes of ice - cold absolute ethanol. The mixture was thoroughly mixed by invertion of the tube. If the DNA pellet is not observed at this stage, the sample was kept in a - 20°C freezer for at least 1 hours. DNA was recovered by centrifugation at 12,000 rpm for 10 minutes at room temperature. The DNA pellet

was washed twice, at least 30 minutes each, with 70 % ethanol. The pellet was air dried and redissolved with appropriate volume of a TE solution (usually 50-100 µl). The DNA solution was incubated at 37°C for 1-2 hours and kept at 4°C until further analysis.

2.2.4 Measurement of DNA concentrations

2.2.4.1 Spectrophotometry

The amount of DNA was estimated by determination of the optical density at 260 nm. The OD value 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 transferred to an Eppendorf tube containing 995 µl of TE buffer. The tube was shaken vigorously. The diluted DNA solution was transferred to a semimicro-UV cuvette where a cuvette containing 1 ml of TE was served as the reagent blank. DNA concentration is estimated in µg/ml using the following equation;

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

2.2.4.2 Mini-gel method

DNA concentration can be roughly estimated by comparing the amount of fluorescence of the DNA after electrophoresis through mini agarose gels. One or two

microlitres of the extracted DNA was diluted to a 10 µl final volume. Two microlitres of loading buffer (0.25% Bromophenol blue, 25% Ficoll) was added and mixed 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 through 0.8 % agarose gels in the presence of 0.5 µg/ml EtBr at 5 - 7.5 Volt/cm for approximately 30 minutes to 1 hr. 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 and undigested λ DNA. A portion of the DNA solution was then diluted to 25 µg/ml final concentration for using in RAPD-PCR analysis.

2.2.5 RAPD Analysis

One hundred and seventeen decatanucleotide primers, six microsatellites and seven minisatellites primers (Table 3.1) were screened for the successful amplification of 3 abalone species in Thai waters. An individual of each abalone were used for screening of primers.

RAPD - PCR was carried out in a final volume of 25 µl containing 0.2 µM of an appropriate primer, 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 25-50 ng of genomic DNA. The reagent mix without the DNA template was included as the negative control.

The amplification reaction was operated for 1 cycles at 94 ^{o}C for 3 min followed by 40 cycles at 94^{o}C for 30 sec (denaturation), 36^{o}C for 60 sec for all specimen except the primer UBC271 for which 40 ^{o}C was used (annealing) and 90 sec at 72^{o}C (extension) and finally, 7 min at 72^{o}C. After amplification, 5-10 µl of the resulting PCR mixture was electrophoretically analysed as soon as possible. The remaining reaction mixture was kept at - 20°C for long storage.

Five primers (UBC101, OPB11, UBC195, UBC197 and UBC271) producing reliable and reproducible results were chosen. Primers UBC101 and OPB11 were used for determination of genetic diversity and identification of species - specific markers among three abalone, whereas all 5 primers were used for determination of intraspecific genetic diversity in the economically promising species, *H. asinina*.

2.2.6 Agarose gel electrophoresis

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 1.6% and 0.8% of agarose gels for analysis of the resulting RAPD products and for detection of quality of extracted genomic DNA, respectively. The suspension was heated until complete solubilization in a microwave. The melted agarose were left at room temperature to cool to approximately 50^{o}C and poured into a gel mould. An appropriate comb was then inserted. The gel was left at room temperature for at least 30 minutes to completely solidify. The gel was placed in the electrophoretic chamber containing 1X TBE buffer covering the gel for 1-2 mm in depth. The comb was gently removed. Air bubbles trapped within the well were carefully removed.

One-quarter volume of the gel loading dye (0.25% Bromphenol blue 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 (BioLabs and SibEnzyme) and the λ - *Hind* III DNA were used as DNA markers. Electrophoresis was carried out at 5 V/cm until bromophenol blue migrated about three-quater of the gels. The gel was stained with ethidium bromide (0.5 μg/ml) for 10 min and destained in distilled water for 15 min to leach out unbound ethidium bromide from the gel. Migration distance of the DNA marker was recorded. The gel was photographed using a K1000 camera (Pentax, Japan).

2.2.7 Statistical analysis

Standard DNA markers (100 bp ladder and λ-*Hin*dIII) were used to assign the size of each RAPD fragment. Only fragments that could be accurately scored (250 bp - 2300 bp) were chosen. 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 base on the assumption that bands that are present in less than 95% of investigated individuals are polymorphic. The RAPD patterns of individuals between different populations of three species of Thai abalone were compare using primer UBC101 and OPB11. Intraspecific genetic diversity of *H. asinina* was estimated using primers UBC101, OPB11, UBC195, UBC197 and UBC271.

Similarity index

A similarity index between individuals within geographic samples was calculated from band sharing of each pair of individuals 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).

For overall species, similarity index within a geographic sample (S) and between samples (S_{ij}) were calculated as the average of S_{xy} across all possible comparisons between individuals within a geographic sample and between individuals from different geographic samples, respectively.

Between sample similarity, corrected for within sample similarity effect (S_{aij}) was used to estimate the similarity index among different samples of *H. asinina*, and can be calculated using the formula:

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

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

Genetic distance

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

$$D = 1 - S \quad \text{(Lynch, 1990)} \qquad (2.4)$$

Phylogenetic reconstruction

Phylogenetic relationships between investigated sample of abalone were constructed based on a neighbor-joining/UPGMA method using Neighbor in PHYLIP (Felsenstein, 1993) version 3.562c. The neighbor-joining trees were illustrated using Treeview.