CHAPTER III

MATERIALS AND METHODS

3.1 Sampling site and collection

The collection samples of *Babylonia areolata* in this study was divided into 3 areas: upper part (Trad to Prachuap Khiri Khan), lower part (Surat Thani to Narathiwat) of the Gulf of Thailand and Andaman sea (Figure 3.1). All of the samples used in this study were obtain from local fishery and a small part of foot tissues (5 cm^2) was cut from each sample a little and preserved in absolute ethanol, and then kept at -20 °C.

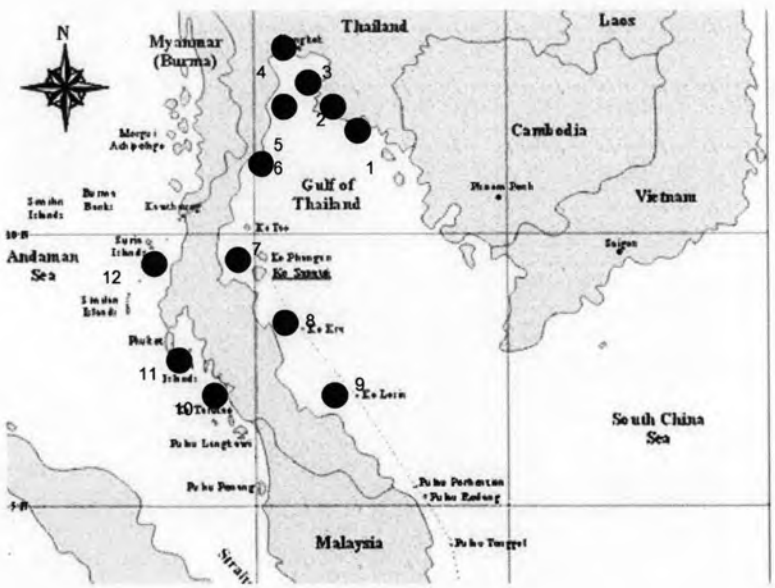

Figure 3.1 Sampling sites for samples collections. (1= Trad 2= Rayong 3= Chonburi 4= Samut Songkhram 5= Phetchaburi 6= Prachuap Khiri Khan 7= Surat Thani 8= Nakhorn Sri Thammarat 9= Songkhla 10= Satun 11= Krabi 12= Ranong)

3.2 DNA Extraction

Tissue was cut and immersed in 1.5 ml eppendorf tubes containing 230 µl of TEN buffer (0.1 M Tris-HCl (pH 8.0) 0.05 M EDTA, 0.2 M NaCl) with1% (w/v) SDS and 20 µl of proteinase K (10 mg/ml). The solution was then mixed gently and incubated at 55 °C for 3 hours. Then, 250 µl of 2% CTAB buffer (2% (w/v) 0.1 M Tris (PH 8.0) 1.4 M NaCl, 0.02M EDTA, with 0.002% (v/v) β-mercaptoethanol was added and incubated at

temperature 55 °C for 30 minutes. After that, 6 M NaCl 200 µl was added and centrifuged at 14,000 rpm for 10 minutes. After the centrifugation, the aqueous phase was carefully transferred to a new 1.5 ml microcentrifuge tube with 700 µl of phenol-chloroform: isoamyl alcohol (24:1) solution and the mixture were shaken vigorously, resulting milky solution. The solution was then centrifuged at 14,000 rpm for 10 minutes. It was turned to be two layers, and then the supernatant was removed carefully and transferred to a new 1.5 ml microcentrifuge tube. The DNA was precipitated by absolute ethanol 900 µl, inverted gently then kept at -20°C for 2 hours or overnight. The solution was centrifuge at 14,000 rpm for 20 minutes to collect the DNA pellet. The aqueous phase was discarded. The pellet was washed in 700 µl of 70% ethanol, after that brought it to centrifuge at 14,000 rpm for 10 minutes. The pellet was dried at room temperature. The DNA pellet was dissolved in 20 µl of TE buffer (10 mM Tris, 0.1 mM EDTA; pH 8.0) and stored at -20 °C until use.

3.2.1 Checking DNA quality and quantity

The DNA quality and quantity were determined using gel electrophoresis methods. DNA samples 1 µl of extracted DNA, consist of 2 µl of loading dye (standard stain orange G, 40% glycerol) and 7 µl of distilled water. They were loaded into 0.8% (w/v) agarose gel containing 0.4 g of agarose, 1X TBE buffer (0.89 M Tris-base, 0.89M boric acid, 0.02 M EDTA) and 4 µl of ethidium bromide solution (500 µg/ml). The gel was then run at 80 volt for 30 minutes. The DNA samples were visualized under UV spectrophotometer and took a digital picture by using gel document systems (BioRad) were compared the intensities of signals which obtained with λ *Hind* III, it was used as a molecular marker for quality the concentration of extracted DNA.

3.3 ISSR-PCR

3.3.1 The selection of ISSR primers

Forty eight ISSR primers were screened by using the template DNA which deriving from 5 randomly selected samples. The primers were optimized to get clearer ISSR profiles. Only were adjusted with annealing temperature and $MgCl_2$ concentration. PCR condition as the following: used initial temperature 45 °C for screen each primer.

3.3.2 ISSR-PCR amplification

Twenty five micro liter of PCR reaction mixtures contained 20 ng of template DNA, 10 pM of each primers, 0.25 mM DNTP's, 2.5 mM $MgCl_2$ and 1 unit *Taq* polymerase (Fermentus) in 1x PCR buffer (Fermentus). PCR amplifications were performed in a termal cycler with the following condition: initial denaturation at 95 °C of 5 minutes, followed by 45 cycle of denaturation at 95 °C 45 seconds, annealing at 45°C for 45 seconds and extension at 72 °C for 2 minutes a with 1 cycle final extension at 72 °C for 10 minutes.

3.3.3 Gel electrophoresis

Twenty five micro liter of PCR products were mixed with 5 µl of loading buffer (standard stain orange G, 40% glycerol). The mixtures were load onto 2.0% (w/v) agarose gel and run at 120 volt for 3 hours. The products were visualized under ultraviolet light. The size of the amplified fragments was determined using 100 bp DNA ladders (Hyper Ladder II (BIOLINE).

3.3.4 Data analysis

ISSR bans were scored as present (1) or absent (0) for each DNA sample. Only clearly reproducible bands were scored and differences in band intensity were not considered. The dendrogram was constructed base on Penny-Branch and bound method to find all members of the set of most parsimonios trees by using program PHYLIP version 3.67 (Felsenstein, 1993).

3.4 mtDNA PCR and DNA Sequencing

3.4.1 PCR amplification of 16S rRNA and COI fragment

DNA was selected from five shell color patterns which cover area the Gulf of Thailand. Five specimens were sequenced for partial 16S rRNA and fifteen specimens of COI, While *B. spirata* was used as out group. The primers pair (16Saf and 16Sbr) was used to amplify the partial 16S rRNA segment and COI primers were designed, based on sequence alignment with a range from invertebrates to mollusks (Table 3.1).

Table 3.1 Primers used to amplify fragments of mitochondrial.

Gene	Code	Sequence ($5'\rightarrow3'$)	References
16S	16Sar (F)	CGCCTGTTTATCAAAAACAT	Palumbi (1996)
	16Sbr (R)	CCGGTCTGAACTCAGATCACGT	Palumbi (1996)
COI	COIF	GCAGGVGCTATTACWTACT	
	COIR	TCTGARTANCGWCGAGGTAT	

PCR reactions were set up in a 25 µl volume and composed of 2 µM of each primer, 2 mM $MgCl_2$, 0.25 mM of dNTPs, 1X PCR buffer (Fermentus) and 1 unit of *Taq* DNA polymerase and 20-50 ng/ml of template DNA. The amplification cycles were started by denaturation at 95 °C for 5 minutes, Follow by 35 cycles 95 °C for 45 seconds for deneratuation 45°C and 50 °C for 45 seconds. For the annealing temperature of 16SrRNA and COI respectively and with *Taq* DNA polymerase (Real Biotech Corporation, RBC) at 72 °C for 1 minute, followed by a final extension at 72 °C for 10 minutes. In each set of reaction, a negative control was included one tube. The PCR products were checked quantification on 1% agarose gel eletrophoresis.

3.4.2 PCR product purification

The PCR products were purified using a MACHEREY-NAGEL PCR clean-up, Gel extraction kit. First, the DNA fragment was excised from an agarose gel carefully. The sliced gel was transferred to 1.5 ml microcentrifuge tube and was weight. Second, the gel was lysed by adding 200 µl buffer NT for each 100 mg of the gel. The mixture was incubated at 55 °C for 10 minutes or until the gel was completely dissolved. A Nucleospin® Extract II column was placed into a 2 ml collecting tube and the mixture was loaded into the column and then, was centrifuged for 1 minute at 11,000 × g to bind DNA. The aqueous phase was discarded then the Nucleospin® Extract II column was placed back into the collecting tube for wash silica membrane. Six hundreds micro liter of buffer NT3 was added and centrifuged for 1 minute at 11,000 × g 400 µl of buffer. NT3 was added and centrifuged at 11,000 × g for 1 minute.

The aqueous phase was discarded. The Nucleospin® Extract II column was placed back into the collecting tube and centrifuged for 2 minutes at 11,000 × g. After that The Nucleospin® Extract II column was placed into a new 1.5 ml microcentrifuge tube and incubated at 55 °C for 5 minutes. To eluted DNA, finally 20 µl of water (sigma) was added in it at room temperature for 1 minute, to increase the yield of DNA and was centrifuged for 1 minute at 11,000 × g. The purified PCR products were quantified again on 1% agarose gel eletrophoresis.

3.4.3 Sequencing and analysis

The amplification products of 16S rRNA and COI genes were sent to sequence at the sequencing laboratory Faculty of Reserch center, Ramathibodi Hospital, After that sequences were visualized and using Chromas 1.6 (Technelysium Pty Ltd.). To confirm the origin of DNA sequences, they were searched for similarity with known sequences in GenBank database using Blasts (http://www.ncbi.nlm.nih.gov/BLAST/). The sequences were aligned using ClustalX 1.81 analysis (Thompson et al., 1997). Each character was used to compare their pairwise percentage differences.

3.5 Preliminary cross breeding

3.5.1. Broodstock preparation

Various shell patterns of broodstock spotted babylon with average shell length of 5.0-7.0 cm were obtained from natural population in littoral region of Rayong and Phetchaburi (brown) and Nakhorn Sri Thammarat (orange and white) in the inner Eastern Gulf of Thailand, by mean of baited trap fishing. The broodstock composed of 3 categories depend on color of shell as: form 1 true spotted babylon or brown, form 2 orange patches on white basal shell and form 3 white basal shell without patches (Figure 4.1).

These broodstocks were then transported to mollusk hatchery of training Station, Chulalongkorn University at sub district Hajawsumran Phetchaburi province about 5 hours from the collecting site by car. The animals were held in 29 x 46 x 5 cm (length x width x high) concrete spawning tanks supplied with running ambient seawater at a rate of 150 l/hr. Salinity and temperature ranged from 29 to 30 ppt and 28 to 30°C

respectively, a 10-cm layer of coarse sand was provided as substratum. The animals were cultured until natural egg-laying occurred. The animals were fed to satiation once daily with fresh meat of carangid fish, *Selaroides leptolepis*. The adults were acclimated for 5-10 days to spawn naturally in the spawning tanks.

Figure 3.2 Sex identification of *Babylonia* by observing a penis (indicate by an arrow).

Figure 3.3 Male and female breeders at the same size of shell length.

3.5.2 Experimental design

The broodstocks were cultured in plastic spawning tanks with flow-through ambient seawater system and air pump. A ratio of male: female was 1:1. The experiment designs were divided into 9 experiments with 3 replicate as shown in Figure 3.4.

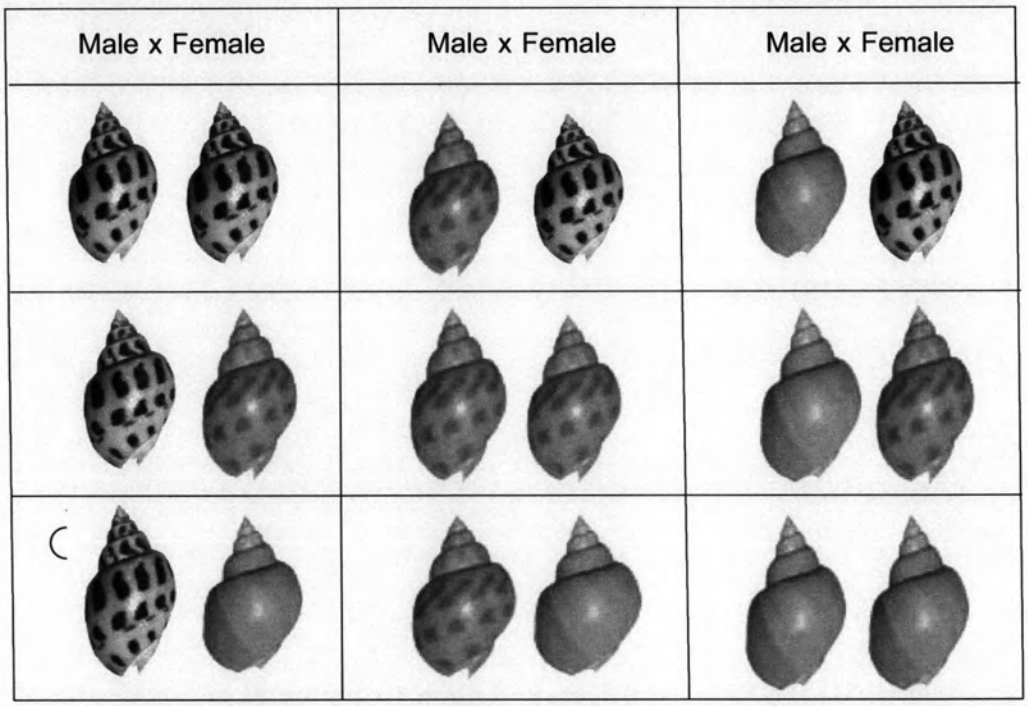

Figure 3.4 Cross breeding in 9 trials of male and female broodstocks with different color patterns in shell morphology.

(a)

(b)

(c)

Figure 3.5 Spawning tanks with bottom area of 1.42 m^2 equipped with running ambient seawater system (a, b) and 5-cm layer of coarse sand used as substratum (c).

(a)

(b)

(c)

Figure 3.6 Spawning tanks with bottom areas of 0.25 m^2 equipped with running seawater system.

3.5.3 Hatching

After laying eggs, eggs capsules were collected and rinsed with 1 μm filtered seawater. In order to remove the fouling contaminating the surface of egg capsules, the capsules were soaked in well water for 30–60 seconds. The capsules were then placed in plastic baskets of 1 cm mesh size and submerged in 500 liters semispherical hatching bowls containing 1-μm filtered and gently aerated ambient seawater. Water was replenished daily until hatching. The egg capsules per snail and fertilized eggs per capsule were counted and measured for morphology study. Egg capsules containing eggs and embryos in different stages of development were sampled daily, preserved in 5% neutral formalin and examined microscopically to evaluate intracapsular development (Chaitanawisuti and Kritsanapuntu, 1997).

(a)

(b)

Figure 3.7 Eggs capsules were collected and rinsed with filtered 1 μm seawater (a) and then placed in hatching tanks of 1.0-cm mesh size (b).

3.5.4. Larvae rearing

After hatching, the newly-hatched planktonic veliger larvae were collected with a 200-µm nylon mesh sieve and rinsed with 1-µm filtered, ambient seawater for three times. These veligers were transferred to 50 liters cylindrical rearing tanks containing 1-µm filtered, ambient, continuously aerated seawater. The initial stocking density was 10,000 larvae per liter. Larvae were primarily fed twice daily with $2.0x10^5$ cells ml^{-1} of mixed unicellular microalgae consisting of *Chaetoceros calcitrans* and *Tetraselmis* sp. at a ratio of 1:1). Water was changed every 2 days, and the rearing tank was primarily cleaned with 0.3 ppm clorox (sodium hypochlorite) for 10 minutes and rinsed two times with well water (Chaitanawisuti and Kritsanapuntu, 1997).

3.5.5 Juvenile rearing

Spotted babylon larvae were competent to metamorphose within 16-18 days after hatching, at which time they started settling on the bottom of the rearing tanks with no particular substrate provided. After settling, the newly-settled juveniles were then transferred into 500 liters cylindrical juvenile nursery tanks supplied with flow-through ambient seawater at a rate of 5 L min^{-1} and gently aerated. The nursery tank must be designed to prevent heavy post-set mortality of juveniles because the newly-settled juveniles crawled out of the water, desiccated and finally died. For this study, the nursery tanks must be equipped with porous pvc pipe provided with sprayed water jet to the wall of the nursery tanks. A 1-cm layer of very fine sand was provided as substrate. Initial stocking density was 100 juveniles m^{-2} to minimize detrimental effects of crowding on growth and survival. The food was changed from unicellular microalgae to fresh meat of carangid fish, *Selaroides leptolepis* and they were fed once daily at 09:00 AM. Food was offered until the animals stopped eating and the uneaten food was removed (Chaitanawisuti and Kritsanapuntu, 1997). Juveniles were cultured until the average shell length was 10 mm.