CHAPTER III

MATERIALS AND METHODS

3.1. Snail Collection

Sixteen populations of *Mekongia* snails were collected from five rivers and their tributaries in Thailand (Figure 3-1). The snails were collected in the field by hand and using bottom sampling net. The identification keys from Brandt (1974), Upatham *et al.* (1983), and Burch and Upatham (1989) were used to identify them. The specimens were transported to the laboratory at the Department of Biology, Faculty of Science, Chulalongkorn University and stored at -80 °C for allozyme electrophoresis analysis. After taken the soft bodies, shell specimens were used for morphological analysis.

Both shells and some snails with soft bodies have been collected systematically as voucher specimens and deposited in the Natural History Museum of Chulalongkorn University (Museum of Zoology).

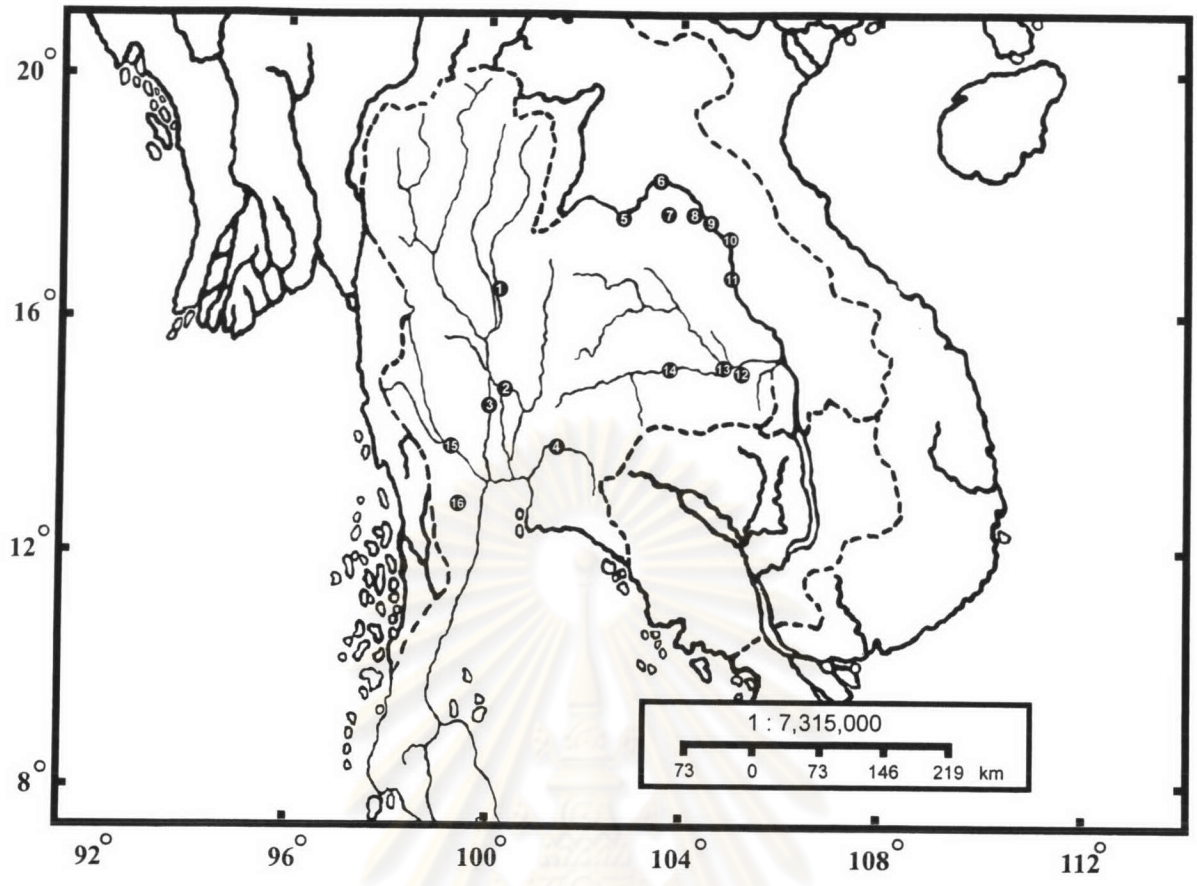

Figure 3-1. Map showing sixteen collecting sites from five rivers in Thailand. (1) = Vat Bot, Phitsanulok Province; (2) = Tha Ruea, Ayutthaya Province; (3) = Bang Ban, Ayutthaya Province; (4) = Kabin Buri, Prachin Buri Province; (5) = Mueang, Nong Khai Province; (6) = Bueng Kan, Nong Khai Province; (7) = Seka, Nong Khai Province; (8) = Sri Songkhram, Nakhon Phanom Province; (9) = Tha U-then, Nakhon Phanom Province; (10) = Mueang, Nakhon Phanom Province; (11) = That Phanom, Nakhon Phanom Province; (12) = Warin Chamrap, Ubon Ratchathani Province; (13) = Mueang, Ubon Ratchathani Province; (14) = Tha Tum, Surin Province; (15) = Muaeng, Ratchaburi Province; and (16) = Ban Lat, Phetchaburi Province.

3.2. Shell Morphological Variation Study

After taken the soft bodies for electrophoresis purposes, each shell was cleaned by removing the remaining tissues. The quantitative characters were chosen for morphometric measurements by following the method of

Davis (1969), Burch (1982), and Henriquez *et al.* (1993), *i.e.* shell height, shell width, body whorl height, body whorl width, aperture height, aperture width, apex height, spire height and penultimate whorl height (Figure 3-2).

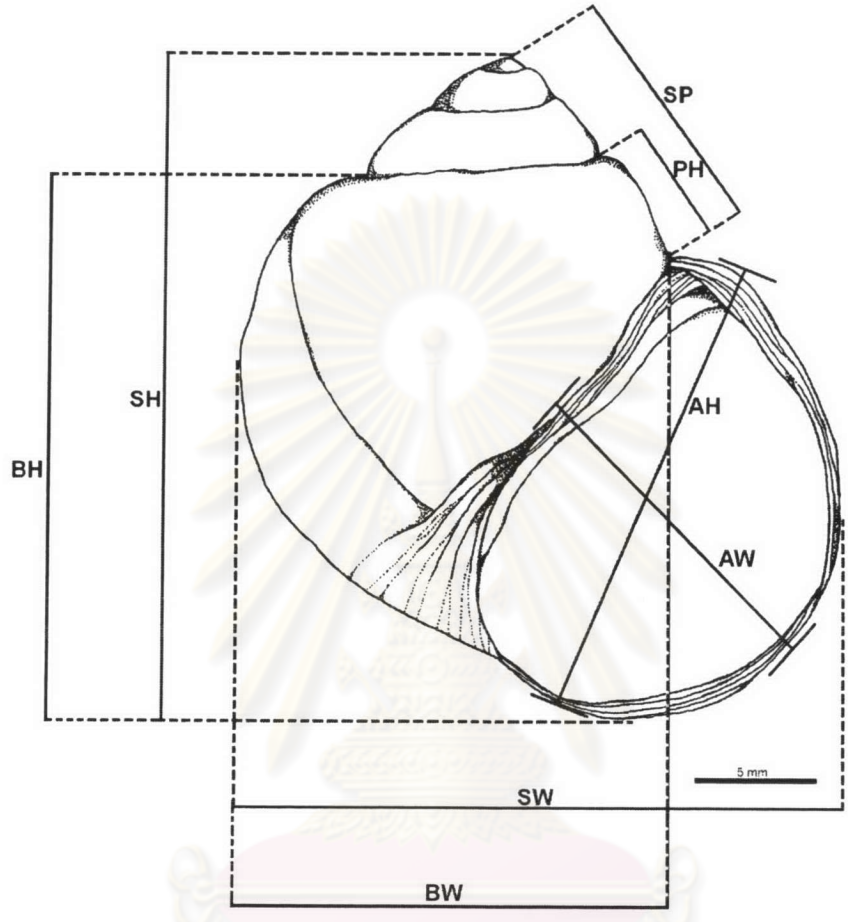

Figure 3-2. Morphometric measurements: shell height **SH**; shell width **SW;** body whorl height **BH**; body whorl width **BW**; aperture height **AH**; aperture width **AW**; spire height **SP** and penultimate whorl height **PH**.

The characters of the shell were measured by digital vernier caliper (Mitutoyo Company). The shell was oriented to standard position, *i.e.* aperture facing upward, and measured the following characters: shell height (SH), maximal length of shell axis; shell width (SW), maximal width of shell perpendicular to shell axis; body whorl height (BH), length from suture, at junction of penultimate and body whorl; body whorl width (BW), maximal diameter of first half-whorl of body whorl; aperture height (AH) maximal

length parallel to shell axis; aperture width (AW), maximal width perpendicular to shell axis; penultimate whorl height (PH) length from suture of penultimate whorl to apex ;and the apex height (SP) length from apex to suture of penultimate whorl. The comparative ratios of all measured characters were used for statistic analyses.

The raw data of shell measurements and the ratios were obtained using simple statistical, mean, standard deviation, and coefficient of variation. The parameters, especially the mean values were normally used to describe and to distinguish the shells of all populations. The measurement method and analysis was combination of some former publications for example Burch (1982), Mayr and Ashlock (1991), and Henriquez *et al.* (1993).

Discriminant analysis (Statistical Package for the Social Science for Windows: SPSS, version 11.0) was used to estimate the amounts of variation within and among populations for these characters. To evaluate group differences, the ratios of all measure characters of each group were computed. The discriminant functions obtained by this method were used to classify and cluster the individual, and interpreting the overlapping degree among groups.

3.3. Reproductive System Study

Reproductive system study was performed according to the method described by Berry (1974). Ten adult males and ten adult females of each population were selected to study their reproductive anatomy. All populations represented four species and six subspecies *i.e. Mekongia pongensis, M. swainsoni swainsoni, M. swainsoni braueri, M. swainsoni*

kmeriana, M. lamarcki, M. sphaericula sphaericula, M. sphaericula spiralis, and *M. sphaericula extensa*. The snails were fixed in 70 % ethylalcohol for dissection. The shells were gently but firmly crushed. Then, the animals were removed and placed in small dish containing normal saline solution sufficient to cover the animal. The soft bodies of snail were positioned on the wax-based dissecting dish, so that the right sides were upward and then firmly pinned the foot in place. These dissections were done under the stereoscopic binocular microscope with two stalked fiber optic lights. The size of various organs and part were measured at center regions from the camera lucida drawing and drawn the whole reproductive organs. An ocular micrometer was used to measure the size of each organ.

3.4. Allozyme Electrophoresis Study

Starch gel electrophoresis technique was performed following the methods described by Steiner and Joslyn (1979) and Murphy *et al.* (1990). The nomenclature for protein-coding loci and alleles followed the recommendations in a paper of Shaklee *et al.* (1990). The running buffers and staining procedures were used from a report by Richardson *et al.* (1986). The results of allozyme electrophoresis technique were interpreted and calculated the variability of individual and genetic variation and population structure. Thirteen enzyme loci were applied in this study.

3.4.1 Electrophoresis Technique

Three buffer systems were used (Table 3-1). The first buffer system (LiOH) is discontinuous; the bridge (electrode) buffer is different from gel buffer. The second (TC-7) and the third buffer system (AC-6) are continuous system, and have a lower ionic concentration and pH than the LiOH system.

Tissue Homogenization

For tissue homogenization, digestive glands were chosen to examine. The soft bodies were removed from their shells. The excess mucus and blood were absorbed by filter papers. The digestive glands were cut from each snail and placed in a 1.5 µl plastic centrifuge tube for stock tissues. Their digestive glands were transformed with homogenizing buffer keeping in homogenizing plastic spot plate, which laid on dry ice sheets. Enzymatic protein from tissues were extracted by hand grinding with the glass stirrer.

Gel Preparation

Starch gel was made in concentration of 12 % (12/100 m, w/v) Sigma starch (Sigma Chemical Co., St, Mo, USA) mixed with gel buffer in a slide-arm filter flask, and heated (gently shaking) to boiling point. Next, molten gel was then degreased with an aspirator about 30 seconds and rapidly poured the hot mixture into the Plexiglas mold 19.5 X 17.5 X 1 cm (about 400 ml in volume). Air bubbles may be occurred at this step. They were removed from molten gel by Pasteur pipette. After pouring, molten gel become cool at room temperature in one hour, and then covered it with plastic film to prevent gel dehydration, and then placed the gel in refrigerator for 30 minutes after wrapping.

Gel Loading

Homogenated crude enzymes were applied into a small pieces of filter paper (1.5 X 10 mm) wick (Whatman number 3), and excess sample was blotted off before the wick was inserted into origin area, a vertical slot cut 5.5 cm for LiOH buffer system and cut 6 cm for other TC-7 and AC-6. Twenty five samples and two markers - filter paper with dry maker (amino black: 1 mg/ml H_2O) - were horizontally placed on a gel.

Table 3-1. Gel and electrode chamber buffers.

System	Buffer type	Adjusted to pH	Chemical ingredients
LiOH	Gel	8.5	1. 0.03 M Trizma Base (3.63 g/L) 2. 0.005 M Citric Acid (0.96 g/L)
	Electrode	8.1	1. 0.06 M Lithium hydroxide (1.44 g/L) 2. 0.3 M Boric Acid (18.50 g/L)
TC-7	Gel	7.0	1. 0.135 M Trizma Base 2. 0.043 M Critric Acid *dilute 66.7 ml of electrode buffer to 1 L
	Electrode	7.0	1. 0.135 M Trizma Base (16.35 g/L) 2. 0.043 M Critric Acid (8.26 g/L)
AC-6	Gel	5.95	1. Citric Acid Anhydrous (19.2/ L) 2.Aminopropyl Morpholine (24 ml/ L) * 1/50 stock solution (1+2)
	Electrode	6.14	1. Citric Acid Anhydrous (19.2/ L) 2.Aminopropyl Morpholine (24 ml/ L) * 1/2.5 stock solution (1+2)

Table 3-2. Buffer combinations giving optimal resolution of enzyme in *Mekongia.*

Enzyme	Electrophoresis		
	Abbreviation	Buffer System	E. C. Number
Esterase	EST-α	LiOH	3.1.1.-
Glucose-6-phosphate isomerase	GPI	TC-7	5.3.1.9
Malate dehydrogenase	MDH	TC-7	1.1.1.37
Mannose-6-phosphate isomerase	MPI	LiOH	5.3.1.8
Peptidase-glycyl-L-luecine	PEP-GL	LiOH	3.4.-.-
Peptidase-luecyl-glycyl-glycine	PEP-LGG	LiOH	3.4.-.-
6-Phosphogluconate dehydrogenase	PGD	AC-6	1.1.1.44
Phosphoglucomutase	PGM	TC-7	5.4.2.2
Sorbitol dehydrogenase	SDH	LiOH	1.1.1.14

Electrophoresis

After enzymes were loaded, the gel was placed horizontally between two buffer chambers with platinum electrode. Cloth wicks (electric bridge) were placed to from a bridge between the gel and both of electrode buffer chambers in order to complete the electric circuit between gels and buffers well. The gel surface was completely covered with Saran Wrap, and then placed the system in refrigerator at 2-4 °C which kept the apparatus cooling into period of the electrophoretic run. An electrical power supply (Weatlec Crop Company: ELITE 300 PLUS) was connected to the two electrodes. Turn the power supply on - allow it warm up for ten minutes at 10 mA. Then tracking dye (1 % bromophenol blue maker dye) were checked by examining edge of gel to make sure that the gel will be properly oriented in the buffer well. The LiOH system, the buffer front was run for 10 cm at 20 mA in 16 hours, but TC-7 and AC-6 buffers were run for 10 cm at 25 mA in 15 hours. When tracking dye has reached the end of the gel, turned power supply off and removed gel from buffer chambers.

Slicing Gel

After electric running step was done, the end of the gel where the last samples wick were cut off (used as a marker for slicing). Then, the wick papers were removed from gel. The gel was cut horizontally into 9 gels (about 1 mm for a sliced gel) by used bow slicer (using a saw equipped with 0.009 mm diameter mandolin string) on slicing tray. Next, the sliced gels were laid on plastic plates for staining.

Gel Staining

Each slice was stained for a particular protein according to the specification (Table 3-3 and Table 3-4) in individual 50 ml beaker, and then mixed staining buffers with agar, pour the stain onto gel and gently shack the beaker freeing the gel from the bottom. Agar overlays were prepared by bring a 2 % agarose gel (w/v) mixture of agar/sterile water to a boiled, allowing it to set until all agar grain have disappeared, cooling to just below 50 °C. Cut filter paper labeled gel with the enzyme system and buffer condition. When staining was finished, the enzyme activity patterns (electromorphs) were documented immediately (NADP- and NAD-dependent enzymes faded at room temperature) by photography and drawing observed patterns on paper. The patterns were transformed into zymogram data sheets for data analysis.

3.4.2 Data Analysis

Electrophoretic data on single individual genotypes were loaded into the computer program BIOSYS-I (Swofford and Selander, 1981). The program computes allele frequencies and genetic variation were to test deviation of genotype frequencies from Hardy-Weinberg expectation and to calculate genetic distance and similarity. The dendrogram based on Nei's *D* was drawn using the unweighted-pair group method with arithmetic averaging (UPGMA) of Sneath and Sokal (1973).

Table 3-3. Stock solution for preparing stains and buffers (All water (H_2O) used was deionized and distilled).

Solution	Solution Name	Preparation
1	Fast Blue BB	20 mg/ml H_2O
2	Glucose-6-phosphate dehydrogenase (G6PDH)	32 u/ml 50% glycerol
3	L-amino acid oxidase	5 mg/ml
4	Malic acid (2 M)	26.82 g/100 ml H_2O
5	$Mgcl_2$ (0.1 M)	2.03 g/100 ml H_2O
6	$MnCl_2$ (0.1 M)	1.98 g/100 ml H_2O
7	MTT	2 mg/ml H_2O
8	O-dianisidine	5 mg/ml H_2O
9	Peroxidase	1100 u/ml
10	Phosphoglucose-isomerase (PGI)	0.7 u/ml
11	Phenazine methosulfate (PMS)	1 mg/ml H_2O
12	Tris-HCl 7.1 (0.2 M)	*0.4 M Trizma base 250 ml + **0.4 M HCl 200 ml + H_2O to 500 ml (adjust pH to 7.1)
13	Tris-HCl 7.5 (0.2 M)	*0.4 M Trizma base 250 ml + **0.4 M HCl 220 ml + H_2O to 500 ml (adjust pH to 7.5)
14	Tris-HCl 8.0 (0.2 M)	*0.4 M Trizma base 250 ml + **0.4 M HCl 135 ml + H_2O to 500 ml (adjust pH to 8.0)
15	α-naphthylacetate	1 g/25 ml acetone + 25 ml H_2O
16	Agarose gel	2 g Agarose gel + H_2O 100 ml
***17**	0.4 M Trizma base	Trizma base 48.44 + H_2O to 1 L
****18**	0.4 M HCl	HCl 33 ml (36.7) + H_2O to 1 L

Table 3-4. Solutions of preparing protein stains and buffers

Enzyme	Staining Buffer
EST-α	10 ml solution12 + 1.5 ml solution15 + 2.5 ml solution1 + 12.5 ml solution16.
GPI	12 mg Fructose-6-photphate + 5 mg NADP (Nicotinamide Adenine Dinucleotide Phosphate) + 10 ml solution14 + 0.25 ml solution5 + 1.25 ml solution7 + 1.25 ml solution11 + 0.25 ml solution2 + 10 ml solution16.
MDH	6 mg NAD (Nicotinamide Adenine Dinucleotide) + 10 ml solution14 + 0.5 ml solution4 + 1.25 ml solution7 + 1.25 ml solution11 + 12.5 ml solution16.
MPI	20 mg Mannose-6-phosphate + 6 mg NADP + 10 ml solution14 + 0.25 ml solution5 + 1.25 ml solution7 + 1.25 ml solution11 + 0.25 ml solution 2 + 25 µl solution10 + 10 ml solution16.
PEP-GL	10 mg Leucyl-glycine + 10 ml solution13 + 0.07 ml solution5 +0.07 ml solution6 + 0.5 ml solution3 + 0.5 ml solution9 + 1 ml solution 8 + 12.5 ml solution16.
PEP-LGG	10 mg Luecyl-glycyl-glycine + 10 ml solution13 + 0.07 ml solution5 +0.07 ml solution6 + 0.5 ml solution3 + 0.5 ml solution9 + 1 ml solution 8 + 12.5 ml solution16.
PGD	14 mg 6-phosphogluconate + 6 mg NADP + 10 ml solution14 + 0.25 ml solution5 + 1.25 ml solution7 + 1.25 ml solution + 10 ml solution16.
PGM	40 mg Glucose-1-phosphate + 6 mg NADP + 10 ml solution14 + 0.3 ml solution5 + 1.25 ml solution7 + 1.25 ml solution11 + 0.3 ml solution2 + 12.5 ml solution16.
SDH	150 mg Sorbital + 25 mg Pyruvic acid + 6 mg NAD+ 10 ml solution14 + 1.25 ml solution7 + 1.25 ml solution11 + 10 ml solution16.