CHAPTER 3

MATERIALS AND METHODS

Materials

1. Specimens

Specimens for this study were taken from two sorts, the first from the collection of Chulalongkorn University Museum of Zoology (CUMZ) and the second were collected from various parts of Thailand.

2. Materials

2.1. Collecting tools

- 2.1.1. Plastic bags
- 2.1.2. Zip lock plastic bags
- 2.1.3. Collecting boxes
- 2.1.4. Bottles in various size
- 2.1.5. Permanent labelling pens
- 2.1.6. Labelling papers

2.2. Laboratory tools

- 2.2.1. Vernier Calipper
- 2.2.2. 100 watt lamp
- 2.2.3. Dissecting tools
- 2.2.4. Tongs
- 2.2.5. Drawing papers
- 2.2.6. 2B pencils
- 2.2.7. Hot plate
- 2.2.8. Beakers
- 2.2.9. Test tubes
- 2.2.10. Test tube rack
- 2.2.11. Petridiscs
- 2.2.12. Vial 4 ml. and 10 ml.

2.2.13. Plaster pipette

2.2.14. Paintbrush

2.2.15. Camera and films

2.3. Microscope

2.3.1. Stereo Microscope

2.3.2. Scanning Electron Microscope (SEM)

2.4. Computer programs

2.4.1. Software program Adobe Photoshop 5.0 and SPSS 10.0

2.4.2. Software program Hennig86

2.5. Chemicals

2.5.1. 30%, 50%, 70%, and 95% Ethanol.

2.5.2. 10% NaOH

2.5.3. Water

Methods

1. Collecting

Specimen of rhiostomid snails were collected from 88 localities of mountain ranges, hills, natural forests, and islands throughout Thailand, since October 2000 to Febuary 2002. The collected places are presented in Appendix I.

2. Preservation

Collected specimens were separated into 2 groups: shell specimens and live specimens. Shells were cleaned, dry and process to cataloging. Living specimens were killed by baked in plastic box under 100 watt lamp for 15 minutes, and then preserved them in 70% ethanol.

3. Identification

Specimens of rhiostomid snails from the collection of CUMZ and present surveying were classified and identified by compared with the collection at Raffle Museum, National University of Singapore. Photographs of some type specimens from British Museum of Natural History were taken by Assoc. Prof. Somsak Panha. The

following main literatures, Moellendorff, 1894; Blanford, 1902; Sykes, 1903; Gude, 1921; Tomlin, 1931; Tomlin, 1938; Laidlaw, 1939; Salisbury, 1949, Habe, 1965, and Solem, 1966 were used for identification.

4. Cataloging

Specimens from the collections of CUMZ and present surveying, both shell and wet specimens were registered the collection number for each set, and individual number for each specimen in each set too. Other informations of each collection number were labeled on labeling paper for record including the collection number, scientific name, locality, collecting date, collector, number of specimens and habitat.

5. Description

The best specimens of each species were chosen for photographing, drawing and describing. Some important and unique characters of each species were drawn for identification key construction.

Collection number and individual number of described specimens are shown in appendix IV.

6. Shell morphometric study

Shell morphology including major diameter (MA), minor diameter (MI), shell width (SW), shell height (SH), anterior part of last whorl height (AH), spire height (SP), inner anterior part of last whorl length (IA), outer anterior part of last whorl length (OA), aperture width (AP) were measured in millimeters by vernier calipper, these parameters are illustrated in figure 3.1.

- Major diameter (MA) is measured from widest part of connected part of shell.
- Minor diameter (MI) is measured from narrowest part of connected part of shell.
- Shell width (SW) is measured from widest part of shell.
- Shell height (SH) is measured from apex to base of shell.
- Anterior part of last whorl height (AH) is measured from the origin of disconnected part of last whorl to the base of aperture.

- Spire height (SP) is calculated from shell height minus anterior part of last whorl height.

- Inner anterior part of last whorl length (IA) is measured from apertural notch along the interior keel to the origin of disconnected part of last whorl.

- Outer anterior part of last whorl length (OA) is measured along the peripheral band from the point as the same growth line with the origin of disconnected part of last whorl to the peristome.

- Aperture width (AP) is measured from the widest part of aperture.

All datas from measurement specimen were shown in Appendix III. Values from 8 characters were transformed to the ratio by used MI value with others, and then analyzed by used statistic ANOVA Duncan's multiple range test ($p<0.05$), and discriminant analysis.

7. Geographic distribution

One-hundred and forty localities were marked in distribution maps by using software program Adobe Photoshop 5.0.

8. Morphology of radula

Radula was removed from radula sac on posterior of buccal mass, placed it in warm 10% NaOH on the hot plate for 15 minutes, washed in water and dehydrated in 30%, 50%, 70%, 95% ethanol, kept in 95% ethanol and then proceed to the process for Scanning Electron Microscope (SEM).

9. Anatomical study

The soft parts were dissected under stereo microscope for separation of reproductive organ. Each specimens were investigated, sketched, and then photographed before drawing.

Collection number and number of dissected specimens were shown in appendix IV. The terminology used in this study was arranged and modified from Thompson (1969) and Kumprataung (1988). These organs were illustrated in figure 3.2.

10. Cladistic analysis

Twenty-four characters of shell, radula, and soft part anatomy were selected for cladistic analysis. The characters used are listed in Table 3.1, and the character states are given in Table 4.2. The analysis was performed using Hennig86 with unweighted, non-additive characters. The Thai cyclophorids snail *Cyclophorus volvulus* (Mueller, 1774) was selected to be outgroup, because it retains many ancestral characters, and placed in the same family Cyclophoridae.

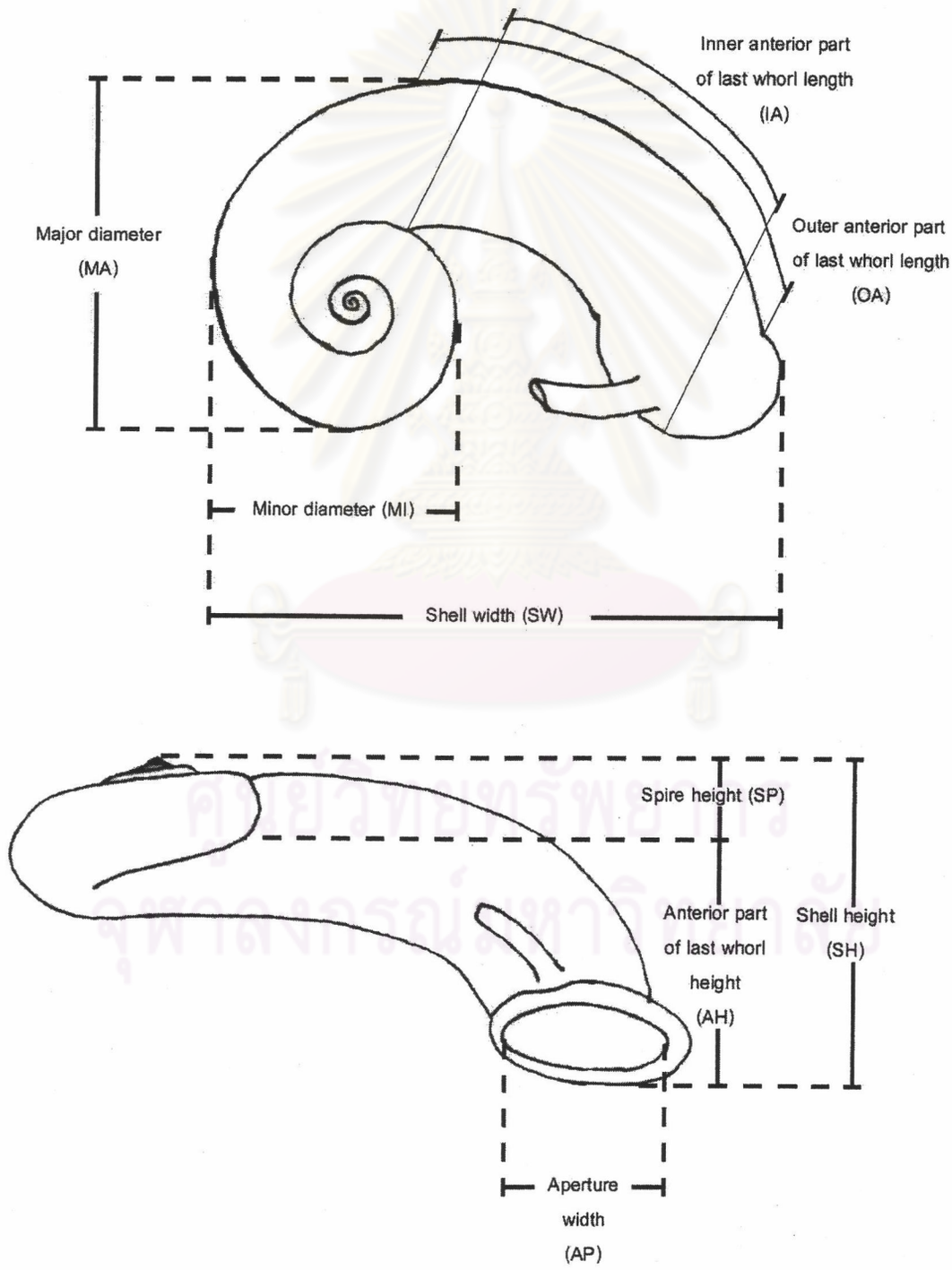

Figure 3.1 Terminology of *Rhiostoma* shell

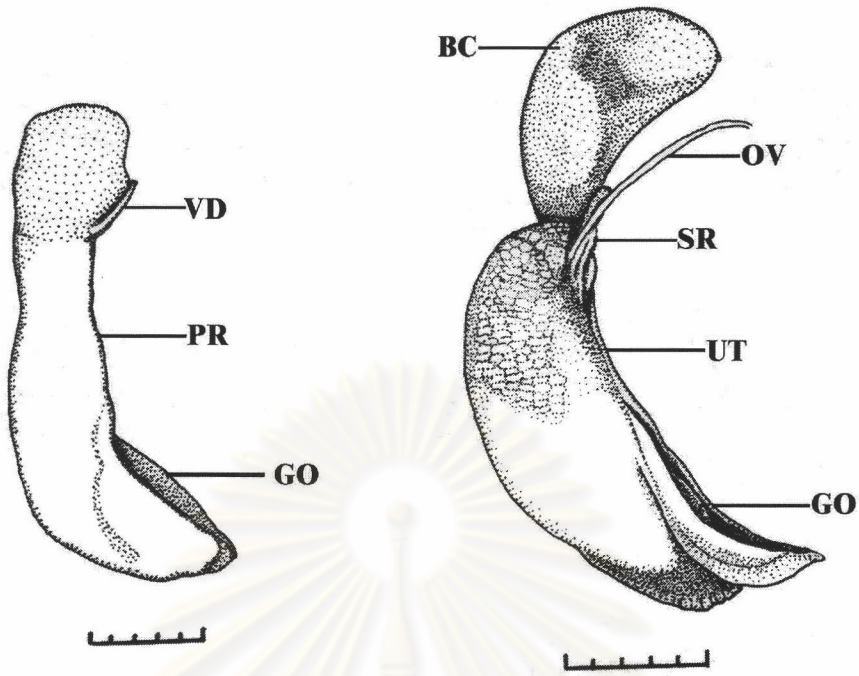

Figure 3.2 Genitalia terminology of *Rhiostoma hainesi*, male on left and female on right (abbreviation on page xii)

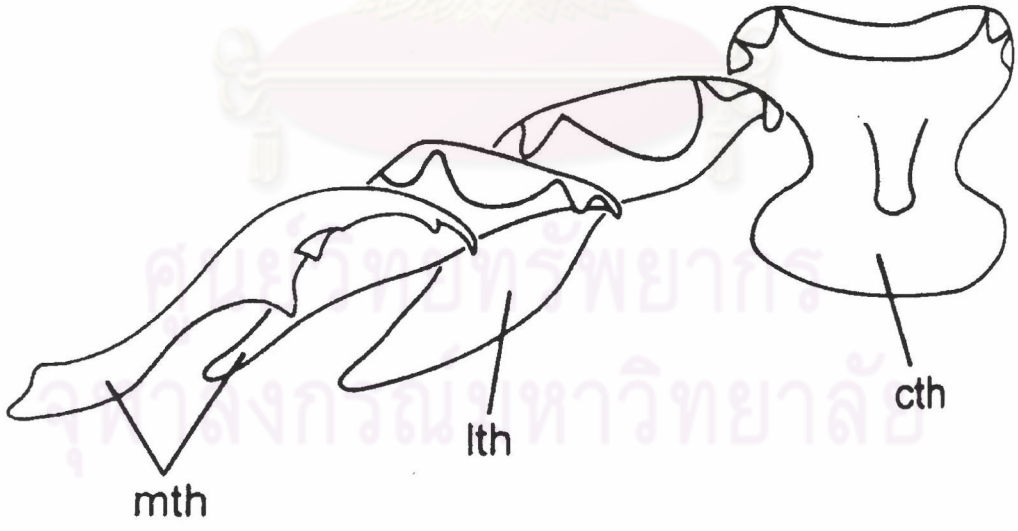

Figure 3.3 Radula terminology of Cyclophorid snails, *Leptopoma vitreum*, half of transverse row of radula (after Benthem Jutting, 1948) [abbreviation on page xii]

Table 3.1 Characters used in cladistic analysis

Characters	States
1. Shell shape	0 = depressed, 1 = sub-discoidal 2 = proboscis
2. Periostracum	0 = sub-diaphanuous, 1 = opaque
3. Dorsal side color form	0 = uniform, 1 = variegate, ? = uniform or variegate
4. Ventral side color form	0 = uniform, 1 = variegate, ? = uniform or variegate
5. Disconnected part of last whorl	0 = absent, 1 = present, ? = absent or present
6. Lip develop	0 = thicken, 1 = broadly expand
7. Apertural notch	0 = absent , 1 = present
8. Air breathing tube	0 = absent, 1 = present, ? = absent or present
9. Behind apertural projection keel	0 = blunt, 1 = sharp, ? = disconnected part of last whorl absent, present blunt or sharp keel
10. Apertural direction	0 = turn in, 1 = turn down
11. Swell ridge behind peristome	0 = absent, 1 = present
12. Operculum : structure	0 = corneous, 1 = calcarocorneous
13. Operculum : shape	0 = flat , 1 = cylindrical
14. Operculum : outside surface	0 = concave, 1 = convex
15. Radula : Central tooth head	0 = slightly convex, 1 = more convex, ? = non information
16. Radula : Width of central tooth	0 = shorter than the length, 1 = longer than the length, ? = non information
17. Radula : Ridge connecting of central tooth	0 = slender, 1 = stocky, ? = non information
18. Radula : 1st marginal teeth cusp	0 = 3 cusps, 1 = 4 cusps, ? = non information
19. Male : seminal vesicle	0 = absent, 1 = present, ? = non information
20. Male : Length of genital opening	0 = 1/8 of anterior part of prostate gland length 1 = 1/3 to ½ of anterior part of prostate gland length, ? = non information

Characters	States
21. Male : Penis length	0 = shorter than tentacle
	1 = longer than tentacle, ? = non information
22. Female : The end of seminal receptacle	0 = non over posterior end of uterus
	1 = long over posterior end of uterus
	? = non information
23. Female : Length of vagina	0 = reach to seminal receptacle
	1 = ¼ to ½ of uterus length
	?= non information
24. Female : Labial of vagina	0 = simple, 1 = thick and expand