CHAPTER III

MATERIALS AND METHODS

3.1 Specimen collection and classification

The specimens of *Cyclophorus* were collected from various forest mountains throughout Thailand and nearby areas. Fieldworks were focused on localities of which there were previous records but included some additional localities. The position of each locality was recorded by GPS. Species classification and identification of specimens were made on the basis of Reeve (1861), Nevill (1881), Morlet (1891), Möllendorff (1894), Kobelt (1902), Blandford (1903), Gude (1921), Benthem Jutting (1948, 1949), Zilch (1956), Habe (1964), Solem (1966), Minato and Habe (1982) and Abbott (1989), and comparison with type material at the Senckenberg Museum, Frankfurt (SMF) and the Natural History Museum, London (NHM). Voucher specimens were deposited in the Museum of Zoology, Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand (CUMZ).

3.2 The specimens of each species were investigated as follows

3.2.1 Shell, Anatomy of genitalia and radula characters

The specimens were studied for shell morphology i.e. shape, size and colour pattern. Whole shells were photographed under digital camera. For anatomical examination, the specimens of each species were dissected following Thompson (1969) and Kasinathan (1975) to examine reproductive system in terms of shape and internal sculpture. Anatomical details were studied and drawn under stereoscopic magnifying with a camera lucida. The terminology of male and female *Cyclophorus* genitalia followed from

Tielecke (1940), Kasinathan (1975) and Girardi (1978) with male genital organs i.e. go, genital opening; pg, prostate gland; sv, seminal vesicle; vd, vas deferens and female i.e. ag, albumin gland; bc, bursa copulatrix; go, genital opening; ov, oviduct and sr, seminal receptacle.

Radula was studied according to Benthem Jutting (1948). The radula tooth was removed from radula sac, placed it in 10% NaOH, boiled for 15 minutes, then washed in water and dehydrated in 30%, 50%, 70%, 95% ethanol, kept in 95% and examined under Scanning Electron Microscope (SEM). Radula terminology was modified from Kasinathan (1975) i.e. c, central tooth; l, lateral teeth; im, inner marginal teeth and om, outer marginal teeth.

3. 2.2 Morphometric analysis

The measurement of the shells height (SH), spire height (SP), aperture height (AH), aperture width (AW), major diameter (MA) and minor diameter (MI) were accomplished with a vernier caliper (Fig. 3.1): according to techniques and illustrations in Pilsbry (1910), Solem & Climo (1985) and Emberton (1985). Number of whorls was determined according to the techniques of Diver (1931). Terminology applied to shell shape and the aperture direction were based on Pfeiffer (1845, 1848) and Pilsbry (1910). The statistical analysis of shell measurements ratio was analysed using one way analysis of variance (ANOVA). Duncan multiple range test (Steel & Tories, 1980) was also used to separate significant difference among means. Differences were considered significant at $P \leq 0.05$.

3.2.3 Cladistic analysis of morphological characters

The individuals of each recognized species were examined for a qualitative morphological character series as follows: shell, soft parts anatomy and radula. These characters were selected for cladistic analysis. The characters used were listed and the character states were analysed. The data were analyzed using *PAUP*4.0* (Swofford, 2002) under the maximum parsimony (MP) optimality criterion. The *Leptopoma* Pfeiffer, 1847 were selected as outgroups because it is closely related to the *Cyclophorus*.

Characters determination of ingroup taxa for the present study comprised of fifteen species of *Cyclophorus*. Twenty-two morphological characters included shell (15), habitat type (1), radula (3), and genital system (3) are selected from the 15 species examined (Table 3.1). Shell and genitalia terminology mainly follows Tielecke (1940), Kasinathan (1975), and Girardi (1978).

Data were obtained from recent descriptions of species from Thailand. These included fifteen species of *Cyclophorus*, one species of *Leptopoma* Pfeiffer, 1847. The selected characters were coded in a matrix (Table 3.2). From a total of 22 characters, 5 were coded as multistage and the remaining 17 as binary characters. All multistage characters were treated as unordered because this allows for all possible hypotheses of order to be tested simultaneously by character congruence following Hauser (1992) and Rognes (1997). Polymorphic characters were coded as a subset polymorphism. The symbol '-' in the matrix mean character state cannot be coded (structure does not exist) and '?' mean unknown (structure not available). For the cladistic analysis of the character matrix, the computer program PAUP. The ingroup taxa were rooted in *Leptopoma perlucidum* Benthem Jutting, 1963 representing the confamily closest to *Cyclophorus* based on Kobelt (1902) and Benthem Jutting (1948).

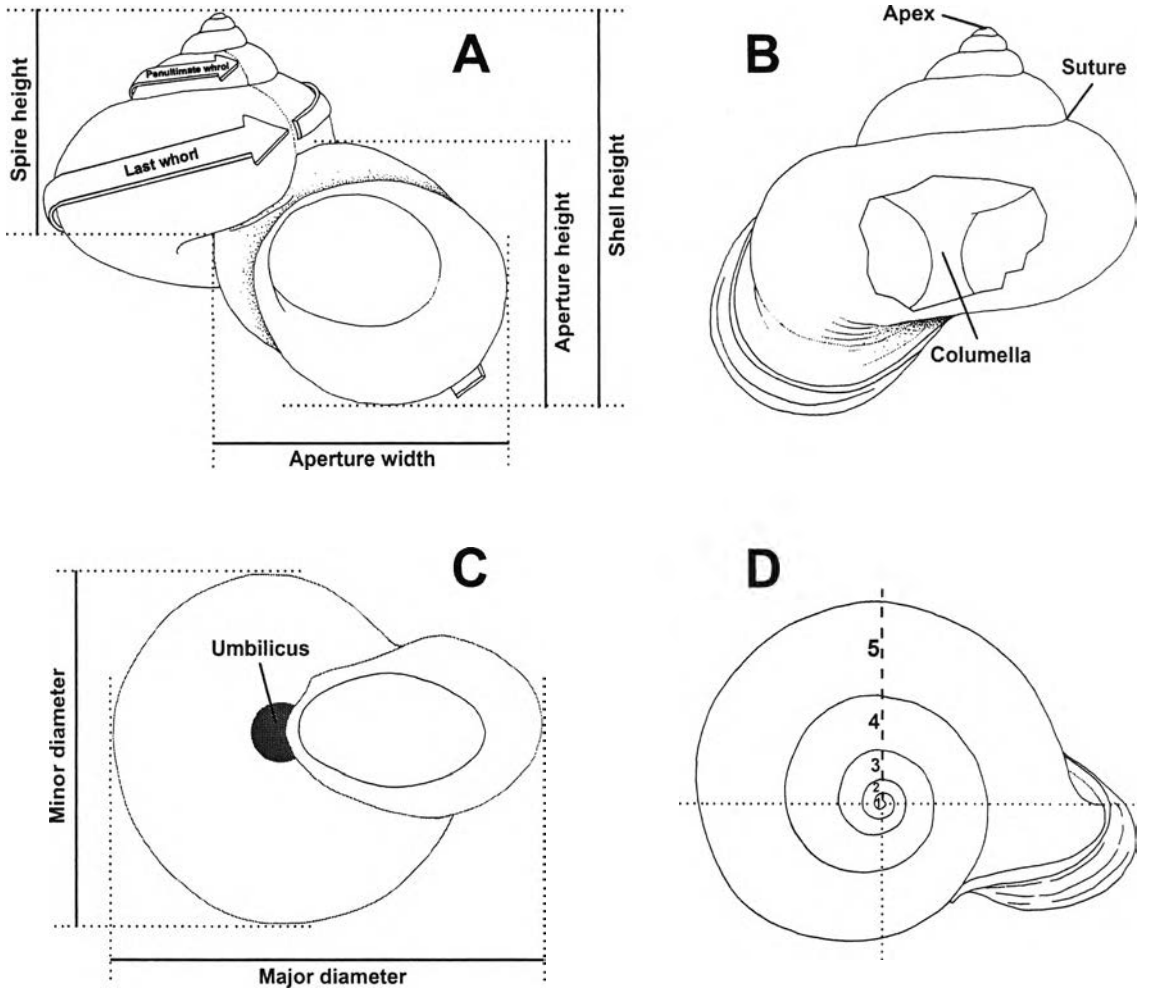

Figure 3.1 *Cyclophorus* shell terminology and its measurement: (**A**) shell height, spire height, aperture height and aperture width (**B**, **C**) major and minor diameter and (**D**) whorls count.

Table 3.1 Phylogenetic characters and character states for 15 *Cyclophorus* and outgroup *Leptopoma perlucidum* (Cyclophoridae).

Shell Characters

1. Structure: 0 = thin and fragile; 1 = thin and solid; 2 = heavy and solid
2. Shape (shell height/): 0 = depressed (≤0.75); 1 = elevated (>0.75)
3. Spire (spire height/shell height): 0 = depressed (≤0.55); 1 = elevated (>0.55)
4. Size (shell width in mm): 0 = small (10-26); 1 = medium (27-44); 2 = large (45-60)
5. Peripheral keel: 0 = absent; 1 = weak: 2 = strong
6. Transverse rib: 0 = absent; 1 = present
7. Ground colour: 0 = white; 1 = brown; 2 = transparence
8. Sculpture cover with gray colour: 0 = absent; 1= present
9. Black and white banded on periphery: 0 = absent; 1 = present
10. Aperture double layers: 0 = absent; 1 = present
11. Aperture strongly expanded: 0 = absent: 1 = present
12. Aperture (aperture width/shell width): 0 = narrow (≤0.55); 1 = broad (>0.55)
13. Aperture rosy colour: 0 = absent; 1 = present
14. Columellar aperture with umbilicus: 0 = free; 1 = locate over half
15. Angle near umbilicus: 0 = absent; 1 = present

Habitat type

16. Habitat: 0= ground; 1 = tree

Radula and Reproductive Characters

17. Central tooth structure: 0 = wide; 1 = narrow
18. Central denticle of central teeth: 0 = triangular; 1 = no triangular
19. Outer marginal teeth: 0 = two denticles; 1 = three denticles
20. Male seminal vesicle: 0 = not stalk; 1 = short stalk; 2 = long stalk
21. Female labial vagina: 0 = absent; 1 = present
22. Female bursa copulatrix shape: 0 = round; 1 = ovate; 2 = long pouch

Table 3.2 Character state matrix for phylogenetic analysis of 15 species of *Cyclophorus*, with *Leptopoma perlucidum* as outgroup. The definition of characters of characters and states. '-' cannot be coded (structure does not exist). ' ?' unknown (structure not available).

	Character				
Species	01-05	06-10	11-15	16-20	21-22
Cyclophorus volvulus	11110	00010	01100	01011	01
C. aurantiacus	20021	01000	00000	01011	02
C. semisulcatus	20010	11000	00001	0????	??
C. speciosus	21120	11100	01010	01011	01
C. cantori	11112	01000	11111	01111	01
C. fulguratus fulguratus	11110	0(01)010	01000	01010	01
C. fulguratus ssp.1	11110	01010	00000	01011	11
C. malayanus	20021	01000	00001	01011	10
C. saturnus saturnus	20020	01010	10000	01011	02
C. saturnus ssp.1	21021	01110	11000	01011	01
C. courbeti	21110	01000	01001	01011	01
C. subfloridus	10110	00000	01011	01011	01
C. diplochilus	11010	00011	11000	01011	02
C. orthostylus	11012	01000	00120	01012	01
Cyclophorus sp.	11111	01000	11001	01010	02
Leptopoma perlucidum	01100	02000	01100	1010-	11

3.2.4 Karyotype analysis

Chromosome preparations were made from male and female gonad tissue by an air-drying method modified from Patterson and Burch (1978) and Park (1994). Gonads were directly injected with 0.1 ml of 0.1% colchicine (Sigma D-89552), dissected after 4-5 hours and cut into small pieces in 0.07% hypotonic KCl solution. Separated cells were collected by centrifugation at 1,000 rpm for 10 minutes and fixed in fresh Carnoy's fixative (3 parts of absolute ethanol and 1 part glacial acetic acid). The

supernatant was replaced with fresh fixative for each of the two centrifugations. Cell suspensions were dropped onto clean glass slides pre-heated to 60°C. Slides were then air-dried and stained in 4% Giemsa solution for 15 minutes. Photomicrographs of ten well-spread metaphase cells were measured for relative length and centromeric index. Mitotic karyotypes were arranged and numbered for chromosome pairs in order to decrease mean relative length. Nomenclature of morphological chromosome types followed that of Levan *et al.* (1964).