CHAPTER III

MATERIALS AND METHODS

3.1 Taxonomic Reviews of *Amphidromus*

A range of forest types were surveyed and specimens of shell and living individuals sampled across Thailand, Laos, Malaysia, Singapore and Indonesia. Fieldwork focused on localities for which there were previous records but included some additional localities. Specimens were drowned in water and preserved in 70% ethanol prior to anatomical study and eventual storage in our general collection. For molecular study, portions were frozen at -80 °C and immediately transferred to 95% ethanol. Genital morphology and any available spermatophores were critically examined. Intact adult shells were measured for whorl number, shell height (h), major diameters or shell width (d) using digital vernier calipers (Mitutoyo, CD-6 CS) (Fig. 3.1). Shell height/shell width ratios (h/d ratio) were calculated as a measure of shape to reduce the dominating effects of overall size (Pilsbry, 1939; Kerney and Cameron, 1979).

The buccal mass was removed and soaked in 10% potassium hydroxide solution for 3 to 5 hours before extracting the radula, which was cleaned in distilled water and preserved in 95% ethanol. Radulae were examined under a Scanning Electron Microscope (JEOL, JSM-5410 LV); the formula and shape of radula teeth were recorded.

The following abbreviations are newly introduced for spermatophore: ces, coiled expanded section; ss, sperm sac (Fig. 4.6), and others are as defined by Collinge (1901), Pilsbry (1939) and Solem (1983): ag, albumin gland; ap, appendix; at, atrium; e, epiphallus; ep, epiphallic pilaster; evd, entrance of vas deferens; fl, flagellum; fo, free oviduct; gd, gametolytic duct; gs, gametolytic sac; hd, hermaphroditic duct; hg, hermaphroditic gland; ov, oviduct; p, penis; pp, penial pilaster; pr, penial retractor muscle; pv, penial verge; pvo, penial verge orifice; ta, talon; v, vagina; vd, vas deferens; vp, vaginal pilaster. The direction of shell coiling for the material examined is indicated by: D, dextral and S, sinistral

Registration numbers all refer to collections of the Chulalongkorn University, Museum of Zoology, Bangkok, Thailand (CUMZ), unless otherwise stated.

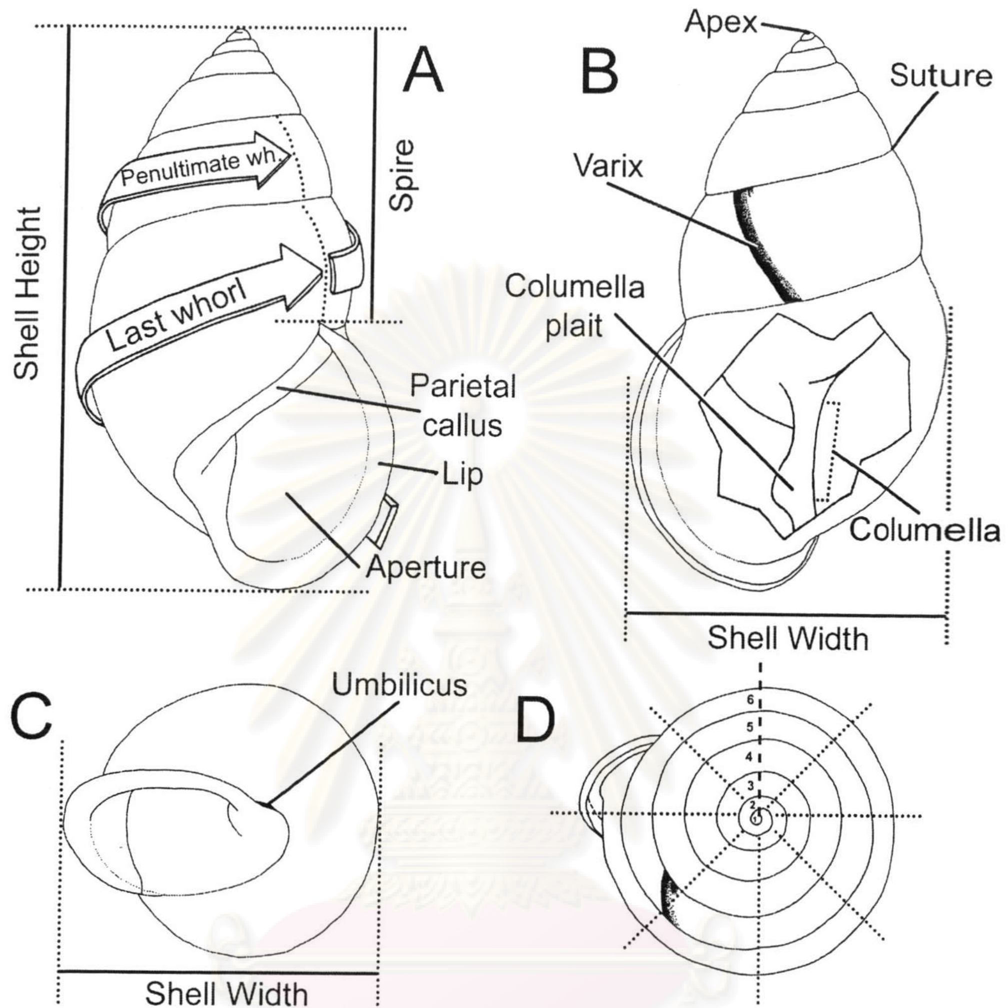

Figure 3.1 *Amphidromus* shell terminology and its measurement: (**A**) shell height, (**B, C**) shell height and (**D**) whorls count.

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3.2 Molecular Phylogeny of *Amphidromus*

3.2.1 Sample analyzed

We obtained samples from 30 localities from Indonesia, Laos, Malaysia, Singapore and Thailand (Fig. 6.1). Those samples included 51 individuals of 11 taxa in 8 species of the subgenus *Amphidromus* and 33 individuals of 9 species in the subgenus *Syndromus* (Table 1). We stored foot tissues of those individuals at -80 °C or in 95% ethanol. We used species of three camaenid genera as outgroups for phylogenetic analysis, *Camaena illustris*, *Chloritis siamensis* and *Beddomea albizonatus,* because they have been suggested as the basal to the genus *Amphidromus* (Pilsbry, 1900; 1901; Gude, 1914; Laidlaw and Solem, 1961). We identified all the samples based on Pilsbry (1900), Fulton (1901) and Solem (1965).

3.2.2 DNA Sequencing

We extracted a total DNA sample from each tissue specimen by a standard protocol with phenol-chloroform (Hillis *et al.*, 1996). We also used DNeasy DNA Extraction Kit (Qiagen) for old tissue specimens. Approximately 0.01-0.5 g of tissue was grounded in 500 μl of STE extraction buffer with 25 μl of proteinase K (10 mg/ml), 50 μl of 5M NaCl and 25 μl of 20% SDS. After incubation at 55 °C for 2-3 hours, lysates were extracted 2-3 times in phenol-chloroform. DNA molecules were precipitated with 1/10 volume of 3 M sodium acetate and 2 volumes of ethanol, followed by 70% ethanol purification. The dry pellet was dissolved in 200-300 μl of TE buffer and stored in -20 °C.

We amplified a fragment of about 800 bp of mtDNA coding 16S rRNA by PCR with primers 16Scs1 5'-AAACATACCTTTTGCATAATGG-3' (Chiba, 1999) and 16Sbd1 5'-CTGAACTCAGATCATGTAGG-3' (Seki, 2002). The amplification reaction-mixture contained 0.2 mM of dNTP, 0.5 μM of each primer, 100-500 ng of template DNA and 0.5 unit of *Taq* DNA polymerase (Takara). Template DNA in each reaction mixture was initially denatured at 95 °C for 4 min, and then amplified through 35 cycles of denaturation for 35 sec. at 94 °C, annealing for 45 sec. at 50-55 °C, extension for 55 sec. at 72 °C and extra extension for 10 min at 72 °C. PCR products were electrophoresed in 1% agarose gels. We excised the target fragments from the gels and purified with Gene Clean Kit III (Bio 101). We directly sequenced the purified PCR products with an automate DNA sequencer (LI-COR, Gene reader 4200) in both directions by the dideoxynucleotide dye-

primer method following the protocol for Thermo Sequencease Cycle Sequencing Kit (Amersham Bioscience).

3.2.3 Sequences Analysis.

We aligned nucleotide sequences primarily with software AlignIR (Li-Cor). We then completed alignments based on maximum nucleotide similarity using CLUSTRAL W 1.4 (Thompson *et al.*, 1994) and confirmed manually. We excluded ten regions of ambiguous alignment from the final data set, which reduced the sequence alignment to 845 bp regions. All members of the ingroup had a 12 bp deletion which was not detected in the outgroups. A single taxon in the ingroup exhibited a 6 bp insertion. We did not exclude these insertions or deletions from the analysis because the tree topologies remained virtually identical even though those regions were excluded. For phylogenetic analysis, we used sequences of approximately 780 bp of partial 16S rRNA gene. Those sequences have been deposited in Genbank (Table 6.1). Gaps were treated as missing data.

We conducted maximum parsimony (MP), maximum likelihood (ML) and neighbor-joining (NJ) analyses with Jukes-Cantor and Kimura 2-parameter model using PAUP* 4.0 (Swofford, 1998). For the MP analysis, we applied equal weighting and a heuristic search option with tree bisection reconnection branch-swapping and 100 random additions. We assumed the transition:transversion bias as 2:1 in all analyses according to the observed ratio in the ingroup taxa (transition/transversion = 1.59). We used the bootstrap probability to test the reliability of each node based on 1000 replications for MP and NJ methods, and 100 replications for ML method.

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Table 3.1 Specimens examined.

Taxon	Locality	Chirality	N	Accession number
A. (A.) atricallosus atricallosus	16, 17, 18	dimorphic	5D, 6S	**AB112365, AB112393-94**
A. (A.) atricallosus leucoxanthus	10, 23	dimorphic	5D, 1S	**AB112369, AB112392, AB112395**
A. (A.) atricallosus perakensis	27	dimorphic	4D, 1S	**AB112368**
A. (A.) inversus inversus	27, 28	dimorphic	6D, 2S	**AB112367, AB112400**
A. (A.) inversus annamiticus	19, 20	dextral	15	**AB112366, AB112391**
A. (A.) schomburgki	6, 12	dimorphic	3D	**AB112373, AB112396**
A. (A.) perversus natunensis	24	dimorphic	2D	**AB112375**
A. (A.) palaceus	29	dimorphic	1D, 2S	**AB112374**
A. (A.) martensi	26	dimorphic	1D	**AB112376**
A. (A.) similis	25	dimorphic	2D, 2S	**AB112371**
A. (A.) givenchyi	6, 7, 8	dextral	7	**AB112372, AB112398-99**
A. (S.) pictus	26	sinistral	1	**AB112381**
A. (S.) adamsii	26	sinistral	1	**AB112370**
A. (S.) areolatus	18, 22	sinistral	6	**AB112387, AB112405**
A. (S.) xiengensis	2,3,17,19	sinistral	9	**AB112377, AB112397, AB112401-02**
A. (S.) semitessellatus	9, 10, 11	sinistral	6	**AB112379, AB112403-04**
A. (S.) flavus	4	sinistral	1	**AB112386**
A. (S.) porcellanus	30	sinistral	3	**AB112380**
Amphidromus (S.) sp.[a]	21	sinistral	1	**AB112378**
A. glaucolarynx	13,14,15,16	dimorphic	4D, 1S	**AB112382-85**
Outgroup				
Beddomea albizonatus	1	dextral	1	**AB112388**
Camaena illustris	5	dextral	1	**AB112389**
Chloritis siamensis	13	dextral	2	**AB112390**

Notes. Locality numbers correspond to those in Fig. 6.1. N indicates the number and chirality of individuals examined. D: dextral; S: sinistral. All sequences used in the present study are registered to GenBank. ([a]) undescribed species.

3.3 Morphological Phylogeny of *Amphidromus*

3.3.1 Characters Determination

The ingroup taxa for the present study comprise of two subgenera; there are 9 species of *Amphidromus (Amphidromus)* and the less 7 species from *Amphidromus (Syndromus)*. All ingroup taxa were selected on the basis of availability of specimens with preserved bodies suitable for anatomical studies. Characters used in the cladistic analysis were defined on the basis of anatomical studies. A total of 17 morphological characters included shell (12), radula (2) and genital system (3) are selected from the 18 species examined. Characters and character states are fully explained below. Shell and genitalia terminology mainly follows Pilsbry (1900), Collinge (1901, 1902), Solem (1983).

The selected characters were coded in a matrix. From a total of 17 characters, 4 were coded as multistage and the remaining 13 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 means 'character state unknown' or 'not applicable'. For the cladistic analysis of the character matrix, the computer program PAUP. The ingroup taxa were rooted in *Camaena illustris* and *Chloritis siamensis*, representing the confamily closest to *Amphidromus* based on Pilsbry (1900) mentioned and Scott (1996) phylogeny.

3.3.2 Characters

Characters 1-12: Shell structure

1. Shell structure: 0 = thin and fragile
   1 = heavy and solid

2. Shell outline: shell height: 0 = less than shell width
   1= less than or equal 35 mm
   2 = larger than 35 mm

3. Apertural shape: 0 = sub-circular
   1 = ovate
   2 = elongate ovate

4. Lip developed: 0 = simple expanded, not thicken and reflected
   1 = expanded, thickened and reflected

5. Coiling direction: 0 = dextral (right-handed coiling)
   1 = sinistral (left-handed coiling)
   2 = dimorphic coiling (both left- and right-handed individuals found in a population)

6. Periostracum: 0 = corneous transparent
   1 = translucent and colored

7. Varix: 0 = absent
   1 = present

8. Protoconch color: 0 = colorless or white
   1 = brown or black
   2 = purple

9. Peristome color: 0 = white
   1 = brown or purplish

10. Parietal callus: 0 = very thin or transparent
    1 = thicken

11. Parietal callus color: ? = not applicable because parietal callus very thin and transparent (see character 10)
    0 = white
    1 = deep purplish
    2 = brown or black

12. Shell color pattern: 0 = monochrome
    1 = with radial streak
    2 = with spiral band
    3 = with spiral band and radial streak or blotch

Characters 13-14:Radula

13. Radula: teeth shape: 0 = triangular
    1 = spatula

14. Radula: central tooth: 0 = monocuspid
    1 = tricuspid

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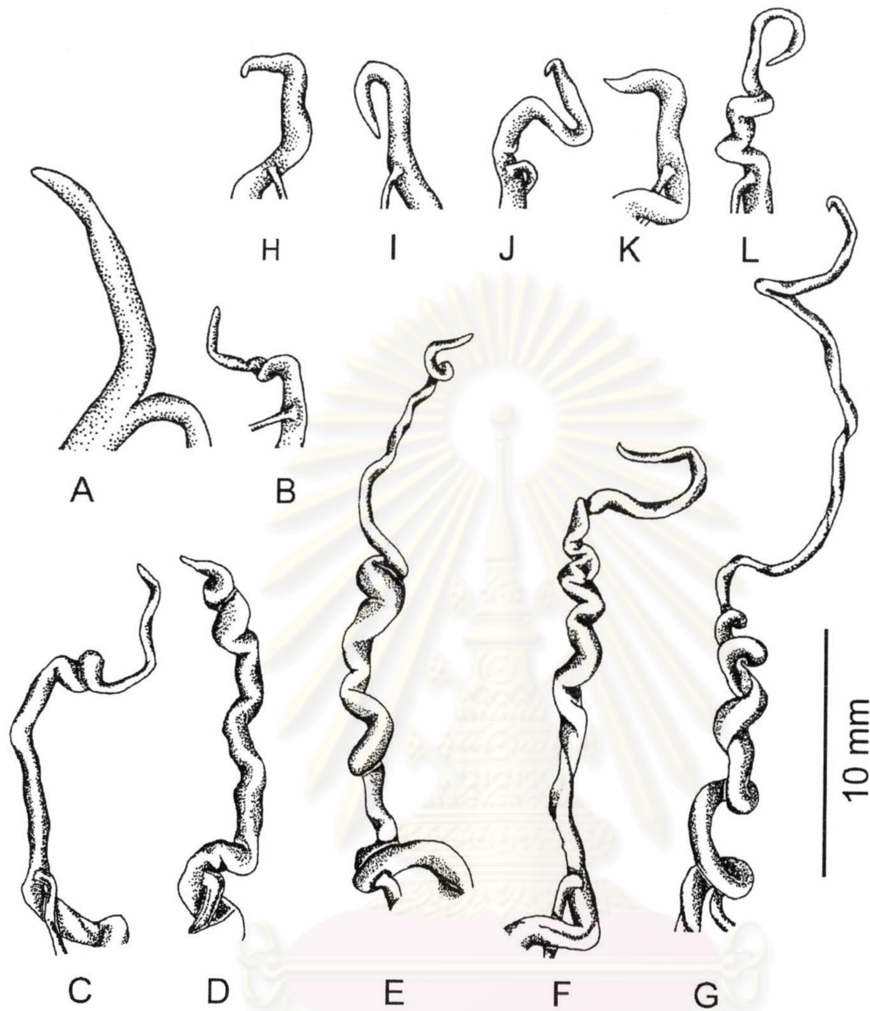

Figure 3.2 Flagellum of (**A**) *Camaena illustris* and (**B**) *Chloritis siamensis*. Flagellum and appendix of (**C**) *A. (A.) perversus natunensis*, (**D**) *A. (A.) inversus*, (**E**) *A. (A.) atricallosus*, (**F**) *A. (A.) givenchyi* and (**G**) *A. (A.) schomburgki*. Flagellum of (**H**) *A. (S.) flavus*, (**I**) *A. (S.) xiengensis*, (**J**) *A. (S.) semitessellatus*, (**K**) *Amphidromus (Syndromus)* sp. and (**L**) *A. (S.) glaucolarynx*.

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Characters 15-17: Reproductive system

15. Flagellum: 0 = long, straight, thin, distally with enlarge coiled
    1 = short, thick and apical portion abruptly thinner

Distal portion of penial complex is above the junction of the vas deferens (Fig. 3.2). The flagellum in the *Amphidromus (Amphidromus)* is always present and shows a variety of shapes and length. In the one hand, *Amphidromus (Syndromus)* always have a short flagellum.

16. Appendix: 0 = absent
    1 = present, slightly short to long.

Most of the distal portion which beyond the coiled portion of flagellum (Fig. 3.2). The appendix in the *Amphidromus* is explicitly smaller size than flagellum, and it shows a wide variety length ranging from absent or vestigial in *A. (A.) inversus* to longer than the flagellum length. In the one hand, *Amphidromus (Syndromus)* usually have greatly short of flagellum and lacked of an appendix; excepted in *A. (S.) glaucolarynx*, the flagellum is slightly short; appendix is smaller size and similar length with flagellum.

17. Internal sculpture of vagina: 0 = proximally with simple ridges
    1 = proximally with specific folding ridges closed to the atrium.

Rounded protuberance pouch located closed to the vagina, atrium and penis junction. When it presence, the vaginal sculpture inside has a transverse and swollen ridges. This characteristic is absent in the member of *Amphidromus (Amphidromus)*, but *Amphidromus (Syndromus)* vary greatly from absent to well developed.

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