

MOLECULAR PHYLOGENY OF LAND SNAIL *LEPTOPOMA* (GASTROPOD: CYCLOPHORIDAE)
IN SABAH, MALAYSIA

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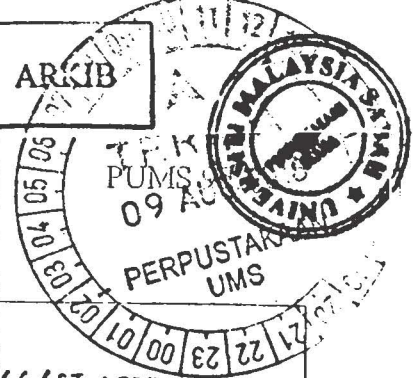
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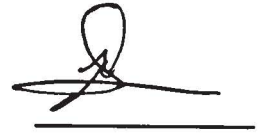
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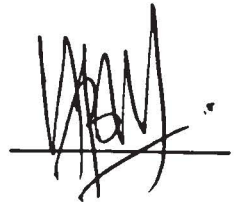
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ABSTRACT

Arboreal land snail genus *Leptopoma* has extraordinary high distribution in Sabah, Malaysia. Despite their wide distribution, taxonomy of genus *Leptopoma* still largely depended on traditional morphological classification. The reliability of morphology-based classification is controversial because snail's morphology is highly influence by environmental factors due to land snail's low mobility. As a contribution solving these problem, I reconstructed the phylogeny of genus *Leptopoma* using molecular approach. Two mitochondrial genes (COI and 16S) were obtained from *Leptopoma* specimens and the phylogenetic trees were revealed by performing maximum likelihood and bayesian inference analysis. Besides, shell morphometric also investigated and mapped onto phylogenetic trees to test phylogenetic signal. All the DNA sequences and specimens details were established to Barcoding of Life Data Systems (BOLD), at the same time barcode gap within *Leptopoma* also investigated. A total of 17 *Leptopoma* specimens, which representing three nominal species and one related-outgroup species were included in this study. All phylogenetic trees strongly supported the monophyly of *L. sericatum* and *L. perlucidum* from Sabah. This results congruent with their morphological classification. Among the nine shell morphometrics, presence of inner ring in aperture exhibited significant phylogenetic signal. This suggested that presence of inner ring can be the reliable diagnostic character in delimitating *Leptopoma* species. Based on Kimura-2-parameter distance model, barcode gap is present within genus where intra-specific distance is below 10% while inter-specific distance is above 10%. In overall, this study has contributed 38% of all the *Leptopoma* records in BOLD which enable others to access and make use of in other related research. Further study which include more specimens from every distribution range in Sabah is needed to elucidate a comprehensive taxonomic status of *Leptopoma*.

Keywords: *Leptopoma*, molecular phylogeny, phylogenetic signal, barcoding of life database, Sabah

ABSTRAK

MOLEKUL FILOGENI BAGI SIPUT DARATAN GENUS *LEPTOPOMA* (GASTROPODA: CYCLOPHORIDAE) DI SABAH, MALAYSIA

Siput daratan genus *Leptopoma* mempunyai kadar taburan yang luas di Sabah, Malaysia. Walaupun dengan taburan yang luas, sebahagian besar taksonomi genus *Leptopoma* masih bergantung kepada klasifikasi morfologi tradisional tetapi ia kadangkala menimbulkan kontroversi kerana morfologi siput adalah senang dipengaruhi oleh faktor-faktor alam sekitar. Bagi menyelesaikan masalah ini, saya mengkaji filogeni genus *Leptopoma* dengan menggunakan pendekatan molekul. Dua gen mitokondria (COI dan 16S) telah diperolehi daripada spesimen-spesimen *Leptopoma* dan pokok-pokok filogenetik dibina dengan menggunakan persamaan maksimum dan analisis inferens Bayesian. Selain itu, morfometrik cangkerang juga dikaji dan dipetakan ke pokok filogenetik untuk tujuan pengujian isyarat filogenetik. Semua urutan DNA dan maklumat spesimen telah dimuat naik ke *Barcoding of Life Data Systems* (BOLD). Pada masa yang sama jarak barcod antara *Leptopoma* juga dikaji. Sebanyak 17 *Leptopoma* spesimen, yang mewakili tiga spesies nominal, dan satu spesies berkaitan-*outgroup* telah dimasukkan dalam kajian ini. Semua pokok filogenetik menunjukkan sokongan yang tinggi terhadap monofili *L. sericatum* dan *L. perlucidum* dari Sabah. Hasil ini adalah selaras dengan klasifikasi morfologi mereka. Antara sembilan morfometrik cangkerang, kehadiran cincin dalaman mempamerkan isyarat filogenetik yang ketara. Ini mencadangkan bahawa kehadiran cincin dalaman boleh dijadikan karakter diagnostik yang membezakan antara spesies *Leptopoma*. Berdasarkan Kimura-2-parameter model, terdapat jarak barcod untuk gen COI, di mana jarak antara spesimen-spesimen untuk satu spesies adalah di bawah 10%, dan jarak antara spesies melebihi 10%. Kajian ini telah menyumbang 38% daripada semua rekod *Leptopoma* di BOLD, membolehkan orang lain untuk mengakses dan menggunakan dalam penyelidikan lain yang berkaitan. Kajian lanjut berkenaan dengan melibatkan lebih spesimen dari setiap rangkaian pengedaran di Sabah diperlukan untuk memperoleh status taksonomi yang komprehensif bagi *Leptopoma*.

Kata Kunci: *Leptopoma*, filogeni molekul, isyarat filogenetik, *barcoding of life database*, Sabah

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LIST OF SYMBOLS, UNITS AND ABBREVIATIONS

°C	Degree Celsius
m	Meter
mm	Millimeter
mM	Millimolar
μl	Microliter
%	Percent
rpm	Rotation per minute
V	Volt
x	Magnification
dNTP	Deoxyribonucleotide triphosphate
DNA	Deoxyribonuclei acid
TBE	Tris Borate-EDTA
MP	Maximum parsimony
ML	Maximum likelihood
BS	Bayesian Inference
PCR	Polymerase Chain Reaction
<i>Taq</i>	<i>Thermus aquaticus</i>
sp.	species
spec.	specimen
BOLD	Barcode of Life Data System
BP	Bootstrap
P.P	Posterior Probability

CHAPTER 1

INTRODUCTION

1.1 Background

The Mollusca is the second largest phylum of animals, only to the Arthropoda, with an estimated 200,000 living species. Molluscs have seven classes of living molluscs documented and at least two extinct class-rank taxa (Ponder & Lindberg, 2008). Gastropoda, commonly known as snails and slugs, is the largest class among the seven classes of molluscs, which accounts for about 80% of the extant fauna (Bieler, 1992). The total number of living gastropod species was estimated to be at least 40,000 and perhaps more than 100,000, with about 13,000 named genera (Bieler, 1992). Caenogastropods consist of about 60% of living gastropod species and comprise a large number of ecologically important families. Bouchet and Rocroi (2005) stated that Caenogastropoda comprise about 136 extant families and thousands of genera arranged in 41 superfamilies including superfamilies Cyclophoroidae.

Superfamily Cyclophoroidae are dioecious terrestrial caenogastropod snails and also one of the species-rich taxon which shows a wide variety of lifestyles. This operculate land snail distributed in tropical parts of Southeastern Asia, Africa, Australia, Southern Europe, and various Pacific islands (Kobelt, 1902). Currently, the family Cyclophoridae comprises of about 870 species arranged in three subfamilies and 35 genera (Bouchet & Rocroi, 2005; Lee *et al.*, 2008a). Kobelt (1902) had established the most broadly used Cyclophoridae's classifications based on shell morphology and anatomy. Among the 35 genera of Cyclophoridae, only a small number of genera have been investigated using molecular phylogenetic approach. For example, Lee *et al.* (2008a) investigated phylogeny among cyclophorids and within *Cyathopoma* species in Taiwan. On the other hand, Nantararat *et al.* (2014a) conducted first molecular

phylogenetic study of genus *Cyclophorus* in Thailand. Therefore, the phylogenetic relationship between and within families and superfamilies still remain largely unsolved.

One of the genus from family Cyclophoridae, genus *Leptopoma* are arboreal and many are found in rocky habitats, most are restricted to forest leaf litter. They are an Old World taxon which ranging through Asia and Indonesia (Little, 1983). Genus *Leptopoma* are described by most authors based on shell morphology solely. According to their morphological classification, *Leptopoma* consists of four subgenera and 105 species (Kobelt, 1902). Recently, taxonomic revisions on two land snail genera (*Everettia* and *Plectostoma*) in Malaysia proposed that genetic data such as phylogenetic relationships could help in clarifying the taxonomy status of morphological ill-defined taxa (Liew *et al.*, 2009; 2014). The phylogenetic relationship of genus *Leptopoma* was not yet investigate before. Hence, molecular approach could help in revealing the complete taxonomy of genus *Leptopoma*.

Sabah is one of the two Malaysia's states on the island of Borneo. It is located on the northern portion of Borneo and known as the second largest state in Malaysia. At the moment, Sabah has recorded about 300 species of land snails (Vermeulen *et al.*, in preparation). Among genus *Leptopoma* in Sabah, there are four species recorded which included *L. atricapillum* (Sowerby, 1843), *L. pellucidum* (Grateloupe, 1840), *L. sericatum* (Pfeiffer, 1851), and *L. undatum* (Metcalf, 1851) (<http://borneanlandsnails.myspecies.info/>). The land snail fauna of Borneo is rich and complex but the taxonomic literature is scattered and was mostly 19th-century (Schilthuizen & Rutjes, 2001). In this case, *Leptopoma* snails are widely distributed in Sabah but the phylogenetic relationships among the genus still remain unclear.

The aims of this project are to reconstruct a molecular phylogeny of *Leptopoma* and to examine the phylogenetic signal of the shell's characters. Besides, barcode gap also has been examined between *Leptopoma* sp. Although *Leptopoma* has wide distribution throughout Asia, this project focuses on *Leptopoma* land snail that found in Sabah. Despite of shell morphological study (Kobelt, 1992; Vermeulen, 1999), a well-supported phylogeny is lacking for the genus. Therefore, molecular approach such as DNA sequencing was used to reveal phylogenetic relationships among *Leptopoma*

species and to compare with phylogenetic signal of the shell's characters. 16S rRNA and COI genes were used in the molecular study. All the data were deposited in Barcode of Life Data System (BOLD) for the purpose of DNA barcoding.

1.2 Justification

Land snails remain a popular group for scientific investigation as well as having a large amateur interest due to their diverse morphology. As most classifications of land snails are still depending on traditional morphological approach, the taxonomic status of these morphological-diverse taxa are complicated. Investigations on land snail phylogeny using molecular approaches have suggested that such approaches are potentially useful (Colgan *et al.*, 2007; Lee *et al.*, 2008a; 2008b). Genus *Leptopoma* is one of the examples which lack of updated molecular phylogeny. Phylogenetic knowledge about the *Leptopoma* is limited and thus there is great interest in resolving their phylogenetic relationship.

1.3 Objectives

This study consists of three objectives in total:

- a. To evaluate the phylogeny of *Leptopoma* sp. from Sabah.
- b. To examine the shell traits and their phylogenetic signal of the *Leptopoma* sp. from Sabah.
- c. To establishing Barcoding of Life Database and inferring barcode gap within *Leptopoma* sp. from Sabah.

1.4 Hypothesis

The molecular phylogeny of *Leptopoma* species is in agreement with their current species classification that based on the shell morphological characteristics.

CHAPTER 2

LITERATURE REVIEW

2.1 Family Cyclophoridae

Family Cyclophoridae are dioecious terrestrial operculate land snails with earliest known records extending from Mesozoic era (Kongim *et al.*, 2006). The Cyclophoridae are one of the most diverse terrestrial gastropods and possess a wide variety of habitats and lifestyles, which comprise of ground dwelling species, tree climbing species and also arboreal species that occupy the canopy (Sutcharit *et al.*, 2014). Commonly known as the largest group of terrestrial prosobranch snails, Cyclophoridae occupy a wide current of geographical distribution. It has extremely wide distribution in Tropical Asia, and also found in Africa, Australia, Southern Europe, Central America, peninsular India, Sri Lanka, Indonesian archipelagoes and various Pacific Islands (Kobelt, 1902; Solem, 1959; Laidlaw, 1928). Cyclophorids have been recorded from karstic tropical forest with alkaline soils (Tweedie, 1961; Schilthuizen *et al.*, 2003) , non-karstic tropical forest (Schilthuizen & Rutjes, 2001; bin Marzuki & Clement, 2013), and from sea level up to 3000m (Laidlaw, 1937; Little, 1983; Liew *et al.*, 2010). Recently, 13 genera consisting of 70 species of Cyclophoridae have been recorded from Peninsular Malaysia and this family has becomes the most diverse terrestrial prosobranch family after Diplommatinidae in this region (bin Marzuki & Clement, 2013). The Cyclophoridae shows extremely variety of shell form and size (Little, 1983). The shell usually turreted (Figure 2.1a; e.g. genus *Japonia*) to disk-shaped (Figure 2.1b; e.g. genus *Cyclotus*), either dextral (e.g. genus *Leptopoma*) or sinistral (Figure 2.2; e.g. some specimens of *Neocyclotus prominulus*), occasionally with partly detached whorls. They have either smooth or sculpture shell surface (Figure 2.3) with round aperture and simple or multiple margin. Some of them possess an indentation or a breathing tube (e.g. *Rhiostoma smithi*) as shown in figure 2.4 (Aldrich, 1889). Their operculum usually circular, may be a simple

chondroid plate or may bear lamellae or various types of calcifications (Torre *et al.*, 1942). The Cyclophoridae usually of large or moderate size where the big *Cyclophorus* sp. may reach 38 millimeter in diameter, and minute *Diplommatina* sp. barely a millimeter and a half long (Figure 2.5) (Laidlaw, 1928).

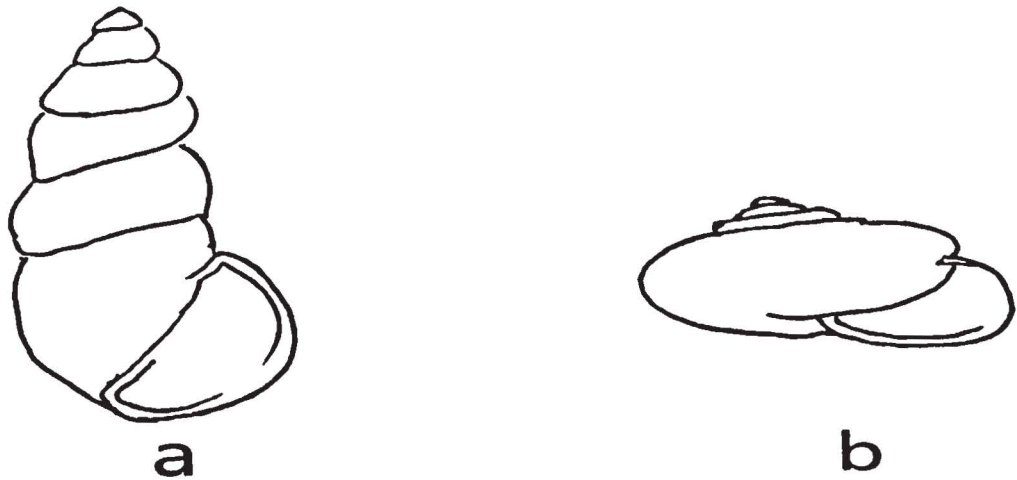


Figure 2.1 Shell shape. (a) Turreted; (b) disk-shaped.

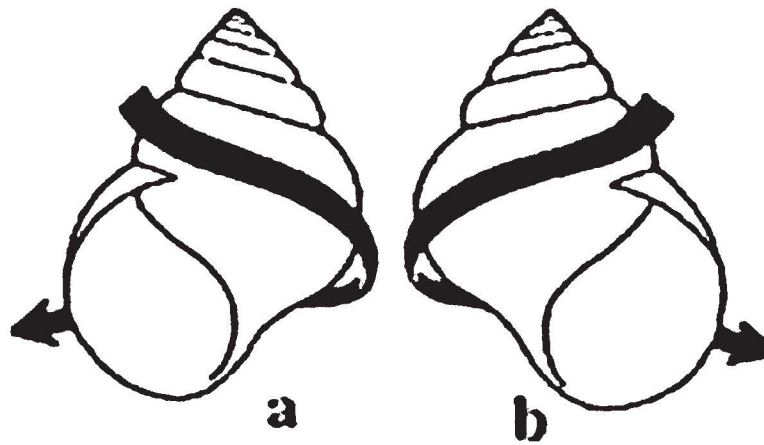


Figure 2.2 Shell's coiling direction. (a) sinistral; (b) dextral. (Source: Panha & Burch, 2005)

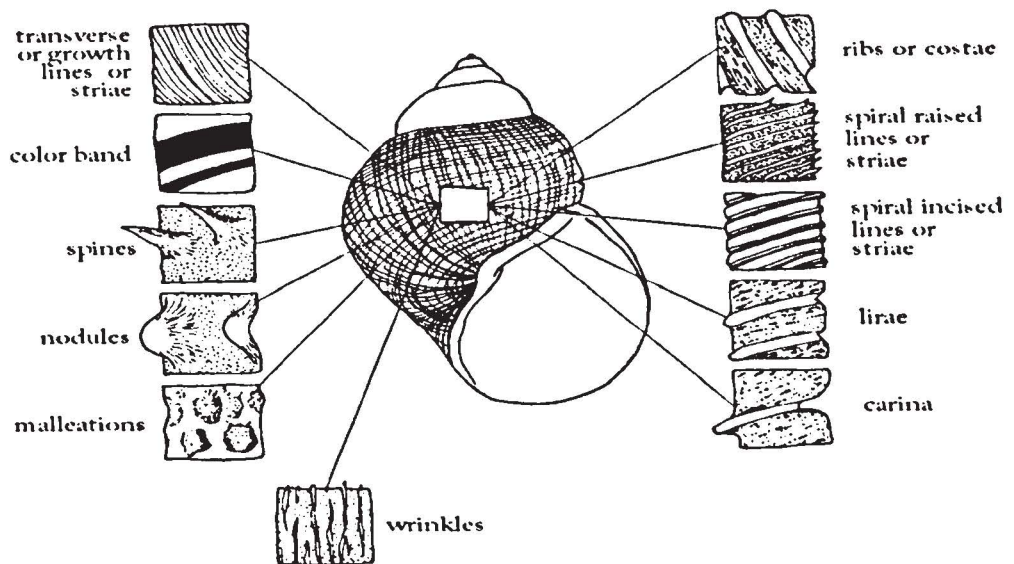


Figure 2.3 Various types of shell surface.

(Source: <http://www.northamericanlandsnails.com/>)

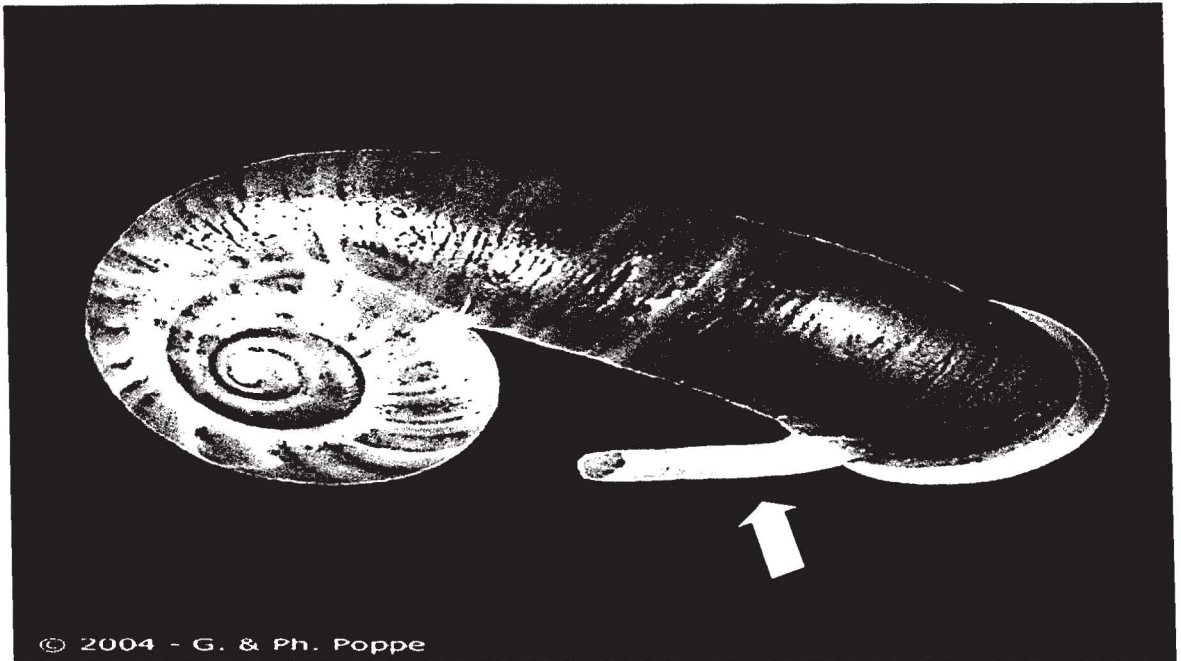


Figure 2.4 Breathing tube of *Rhiostoma Smithi*.

(Source: <http://www.biodiversitylibrary.org>)

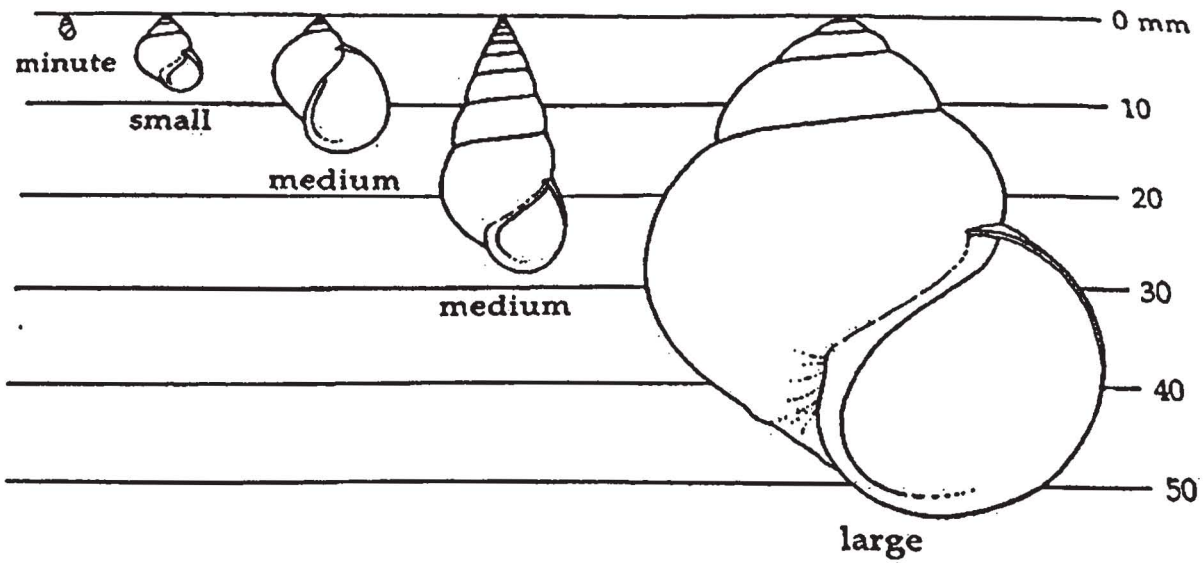


Figure 2.5 Shell sizes. (Source: Panha & Burch, 2005)

The morphological-based classification of family Cyclophoridae was initially established based on hard body (e.g. shell) and then later based on soft body (e.g. genitalia). Kobelt (1902) has classified Cyclophoridae based on shell, opercular and radular characters and his works remain the broadly accepted reference for the group based on shell morphology (Nantarat *et al.*, 2014a; 2014b). Later, this classification has been modified by Thiele (1929) and further modification was done by Tielecke (1940) on the basis of the structures of male and female genitalia, nervous system and pallial region (Solem, 1956). Three subfamilies have been recognised which are Cyclophorinae, Alycaeinae and Spirostomatinae (Bouchet & Rocroi, 2005) where subfamily Cyclophorinae is the most morphologically diverse taxa compared to other subfamilies (Lee *et al.*, 2008b). The Cyclophoridae currently consists of about 810 species arranged into 35 genera (Nantarat *et al.*, 2014a).

Besides morphological classification, there are only very few studies on the phylogenetic relationship of Cyclophoridae by utilizing molecular approach. The first phylogenetic analysis which focus on family Cyclophoridae was conducted by Lee *et al.* (2008b) by examined 32 species from 10 genera. According to the result, the genera *Cyclophorus*, *Cyclotus*, *Leptopoma* and *Cyathopoma* are monophyletic while the monophyly of genus *Japonia* is rejected. The taxonomy of a sister taxa to *Leptopoma*, genus *Cyclophorus*, were investigated by Nantarat and co-authors (2014a) in Thailand for the first time. They came out a conclusion that phylogenetic placement of most *Cyclophorus* species was in agreement with traditional morphology-based classification. Most species were monophyletic but at least three species were polyphyletic. Besides, phylogeny of genus *Cyathopoma* and *Cyclotus taivanus ssp.* also have been carried out (Lee *et al.*, 2008a, 2012).

2.2 Genus *Leptopoma*

Leptopoma is one of the 35 genera of family Cyclophoridae. Kobelt (1902) proposed 4 subgenera (*Trocholeptopoma*, *Entochilus*, *Leucoptychia*, *Leptopoma*) in *Leptopoma* and 105 species based on their morphological characteristics. This large genus is exclusively arboreal species and found abundant in rocky habitat and restricted to forest leaf litter (Little, 1983). Genus *Leptopoma* has wide distribution, ranging from Japan, Taiwan,

Philippines, Malaysia, Indonesia, southwards to Australia, eastwards to Pacific (Vermeulen, 1999).

The shell characteristics of genus *Leptopoma* have been described by several authors. Baird (1850) illustrated that the shell of *Leptopoma* sp. are globose, turbinated or conical. Their operculum is membranous (Figure 2.6b), closely whorled, entirely corneous and flat. *Leptopoma* sp. have simple and reflexed lip (Figure 2.6d). Their lip has no notch in the angular edge and there is widely spaced spiral ringlets present in first whorls (Vermeulen & Whitten, 1998). Their shell size ranging from 9.5-24 mm wide; whitish to corneous; with or without colour markings (Vermeulen, 1999).

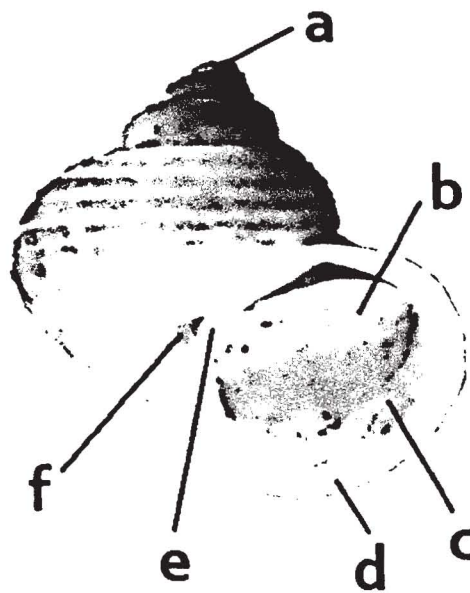


Figure 2.6 Shell of *Leptopoma* sp. (a) Apex; (b) Membranous Operculum; (c) Aperture; (d) reflexed lip; (e) columella; (f) umbilicus.

The taxonomy of genus *Leptopoma* has been reviewed only by morphological-based classification. For example, review by Kobelt (1902) based on shell morphology, and review by Sarasin & Sarasin (1898) based on radula and operculum. Besides, Jonges (1980) had examined genital ducts in several species of the *Leptopoma* and suggested that this can be a useful diagnosis character for taxonomy of genus *Leptopoma*. In 1999,

Vermeulen have carried out extensive revision on the taxonomy of *Leptopoma* sp. from Borneo based on morphological characters. However, phylogenetic relationship of genus *Leptopoma* has not been investigated before. Genus *Leptopoma* always examined as outgroup in molecular approach of their sister taxa (e.g. genus *Cyathopoma* and *Japonia* in Lee *et al.* 2008a, 2008b; genus *Cyclophorus* in Nantarat *et al.*, 2014). Genus *Leptopoma* also included in molecular analysis carried by Lee *et al.* (2012) which aims to reveal phylogenetic relationship within the family Cyclophoridae. All these three researches give high support on monophyly of genus *Leptopoma*.

Genus *Leptopoma* was found in Malaysia with exceptional high abundance in Sabah, Borneo. In west Malaysia, *Leptopoma* sp. were discovered mostly from limestone hills in Ipoh, Perak (e.g. *L. aspirans* in Skyes, 1903; *L. lowi* in Van Benthem Jutting, 1949). The published records of this genus in Sabah started in the eighteen century and all these records suggest that *Leptopoma* sp. are widely distributed in Sabah. During 1889, Godwin-Austen recorded many *Leptopoma* species (*L. bicolor*, *L. sericatum*, *L. signatum*, *L. lowi*, *L. whiteheadi*, *L. duplicatum* and *L. undatum*) from Pulau Tiga, Pulau Mantanani and several places in Sabah. Dr. Hanitsch has recorded two *Leptopoma* species (*L. undatum* and *L. whiteheadi*) at height 1,280m and 1,066m respectively from Mount Kinabalu during year 1899 (Laidlaw, 1937). *Leptopoma* species also found in summit of Mount Ambun in Sabah, at elevation of 1,158m which collected by Mr. S.B.J. Skertchly (Smith, 1895). In addition, Vermeulen (1999) documented three *Leptopoma* species (*L. undatum*, *L. pellucidum*, *L. sericatum*) from a number of places in Sabah for instance Mount Kinabalu National Park, Poring Hot Spring, Sandakan, Tawau, Lahad Datu, Pulau Gaya, Kinabatangan Valley, Sukau and also Sapulut. In addition, limestone areas in Danum Valley also housed some *Leptopoma* species (Schilthuizen & Rutjes, 2001). On the other hand, a recent record of diversity of *Leptopoma* species in Sabah was conducted by Uchidal *et al.* (2013) where they found *L. pellucidum*, *L. sericatum*, *L. undatum* in UMS peak, Sepilok, Sukau, Gomantong Caves and Tabin Wildlife Reserve.

2.3 Morphology versus molecular classification

In the past, taxonomists were discriminating species mainly based on visible characters (Hirano *et al.*, 2014). However, morphology-based classification has limitations in

describing all the life forms on Earth. First, presence of phenotypic plasticity (e.g. brown seaweed, Lane *et al.*, 2007) and genetic variability in characters which lead to misidentification. Morphologically cryptic taxa which are common in many groups (Jarman & Elliott, 2000) which could be difficult to discriminate. In many cases, immature specimens (e.g. fish larvae, Pegg *et al.*, 2006) and gender (e.g. bird, Dubiec & Zagalska-Neubauer, 2006) cannot be discriminated by just referring to external characteristics.

In land snails' taxonomic studies, the reliability of morphological-based classification has been questioned due to enormous amount of phylogenetic studies had discovered incongruence between morphological and molecular classification (Plam *et al.*, 2008; Kameda *et al.*, 2007; Correa *et al.*, 2011). This is mainly because land snails' classification was depended heavily on shell characteristics (Fiorentina *et al.*, 2008) which were easily influence by environmental conditions. Convergence in shell morphology can occur in species that live in similar environment condition. For example, homoplasy occurs in shell morphology of Australian Camaenidae which occupied similar ecological niches (Köhler & Criscione, 2014).

In addition, shell morphology in same species can be highly diverge due to geographical variation. For instance, Irie (2006) revealed high morphological variation within and among populations of *Cypraea annulus* along different geographic gradients. He also proposed that shell morphology was influence by some ecological factors such as density of population and temperature. Besides, predation pressure can be the factor for causing variation in shell morphology (DeWitt, 2000; Schilthuizen *et al.*, 2006).

Over past decade, molecular phylogenetic analysis has been proven more reliable than morphology-based analysis in resolving land snails' taxonomy (Harasewych *et al.*, 1998; Colgan *et al.*, 2007; Correa *et al.*, 2011,). By using DNA sequencing, the phylogeny of species where morphology is ambiguous can be solved (Vogler & Monaghan, 2007; Latiolais *et al.*, 2006). Most studies have combined both molecular and morphological-based classification to produce better taxonomic and phylogenetic understanding of taxa (Douris *et al.*, 1998; Pola *et al.*, 2007; Wang *et al.*, 2014).

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