Molecular Phylogeny of the Panpulmonate Groups Stylommatophora and Hygrophila

By

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Abstract

The phylogenetic relationships at the base of the stylommatophoran land snails and slugs have proven to be controversial, particularly the position of the Scolodontidae in relation to other stylommatophoran taxa. Likewise, the phylogenetic relationships within the hygrophilan freshwater snails and limpets are uncertain. This study provides molecular phylogenies for the Stylommatophora and Hygrophila at multiple scales including deep phylogenetic relationships of the major stylommatophoran and hygrophilan clades and family level relationships within the Orthurethra and Lymnaeoidea.

In **chapter 3**, four standard markers: almost the full-length large subunit (LSU) rRNA gene (also incorporating part of the 5.8S gene), almost the full-length small subunit (SSU) rRNA gene, part of the histone three (H3) gene and the 1st and 2nd codon positions of the CO1 gene were used with the aim of resolving the basal divisions within the Stylommatophora and evaluating the phylogenetic utility of these markers. The deep phylogenetic relationships at the base of the Stylommatophora are now clearly resolved. The Scolodontidae are shown categorically to be the sister group to all other stylommatophoran taxa with robust support and with all phylogeny reconstruction methods. Based on this result, a taxonomic revision for the basal groups within the Stylommatophora has been proposed by reclassifying the 'achatinoid' and 'non-achatinoid' clades at a lower rank than the Scolodontina in order to properly reflect the deep-level relationships within the Stylommatophora. The original LSU 1-5 fragment used extensively in studies of the Stylommatophora is found to be the most informative gene fragment among the genes used in this study. Expanding the length of the LSU gene and combining it with other genes is necessary to fully resolve the relationships within the Stylommatophora, particularly those at the base of the clade.

The family-group interrelationships within the Orthurethra were examined in **chapter 4** using the ribosomal (r) RNA gene cluster [including part of the 5.8S gene, the complete ITS-2 region, and almost the full-length large subunit (LSU; 28S) gene]. There is robust support for the basal division between the Chondrinidae and the rest of the Orthurethra in our tree. Likewise, the monophyly of the Chondrinidae, Cerastidae, Vertiginidae, Achatinellidae and Partulidae is well supported while the Pupillidae, Enidae, and Valloniidae are not recovered as monophyletic groups in our orthurethran tree.

Although there is relatively little support for the deep-level relationships among the main orthurethran groups, some groupings are strongly supported.

The validity of the families and subfamilies within the Hygrophila was investigated in **chapter 5** using the ribosomal (r) RNA gene cluster [including part of the 5.8S gene, the complete ITS-2 region, and almost the full-length large subunit (LSU; 28S) gene]. This study provides the most comprehensive sampling of the Hygrophila clade to date. The principle division of the Hygrophila into two monophyletic groups (the Chilinoidea and Lymnaeoidea) is robustly supported in our tree. The Acroloxidae, Lymnaeidae and Physidae are fully supported as monophyletic families. However, the Planorbidae are not supported as a monophyletic group due to the clustering of Bulinidae within the family. The subfamilies Amphipepleinae within the Lymnaeidae and both the Planorbinae and Ancylinae within the Planorbidae are strongly supported as monophyletic groups while the Aplexinae and Physinae within the Physidae and the Lymnaeinae within the Lymnaeidae are not recovered as monophyletic subfamilies. Based on the results of this study, a taxonomic revision for the hygrophilan families has been suggested by either retaining the Bulinidae as a family but reclassify the Ancylinae as a family outside the Planorbidae consistent with previous classifications or lowering the Bulinidae to subfamilial rank alongside the Ancylinae within the family Planorbidae.

The interrelationships among the lymnaeoidean families (Acroloxidae, Bulinidae, Burnupiidae, Lymnaeidae, Physidae and Planorbidae) are reasonably well resolved in **chapter 5** using a single standard marker, though they are not fully supported. Therefore, to better resolve these relationships we have undertaken a more detailed phylogenetic analyses in **chapter 6** using four standard molecular markers [LSU (incorporating 5.8S), SSU, H3 and 1st and 2nd codon positions of the CO1gene] for a representative taxa from the main hygrophilan groups within a framework of the Panpulmonata combined with a phylogenomic analysis based on transcriptome sequences data for a representative from each of the main lymnaeoidean families (Acroloxidae, Lymnaeidae, Physidae and Planorbidae). The observed interrelationships among the lymnaeoidean families based on the four standard markers are consistent with those obtained in **chapter 5**, but surprisingly support for the major division decreases raising uncertainty about the topology. However, the problematic interrelationships between the

lymnaeoidean families are well resolved in our phylogenomic analysis, with the Acroloxidae recovered as the sister group to a clade comprising the Lymnaeidae, Physidae and Planorbidae, the Physidae recovered as the sister group of a cluster comprising the Lymnaeidae and Planorbidae, and the Lymnaeidae and Planorbidae recovered as sister taxa.

Preface

This thesis comprises seven chapters. Chapter 1, chapter 2 and chapter 7 are written in normal thesis format while other chapters are written in journal paper format. A brief description is given below.

In Chapter 1 a brief introduction to the classification and phylogeny of the Stylommatophora and Hygrophila is given. Chapter 2 includes details of the general methods used throughout the thesis.

Chapter 3 entitled ‘‘Resolving the Basal Divisions in the Stylommatophoran Land Snails and Slugs with Special Emphasis on the Position of the Scolodontidae’’ published in Molecular Phylogenetics and Evolution journal.

Chapter 4 entitled ‘‘Molecular phylogeny of the Orthurethra (Panpulmonata: Stylommatophora)’’ is submitted to Zoological Journal of the Linnean Society.

Chapter 5 entitled ‘‘Molecular Phylogeny of the Freshwater Snails and Limpets (Panpulmonata: Hygrophila)’’ published in Zoological Journal of the Linnean Society.

Chapter 6 entitled ‘‘Resolving the Deep Relationships of the Families within the Hygrophila with special emphasis on the Lymnaeoidea’’.

Chapter 7 includes a summary of the main findings and conclusions.

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Chapter 1: Introduction

1.1 General Introduction

The Panpulmonata are a major group of snails and slugs within the Gastropoda. They are found globally (except in Antarctica) and in a wide range of habitats, including terrestrial, freshwater and marine (Schrödl *et al*., 2011). Panpulmonate taxa have been used as study organisms in phylogenetic and evolutionary studies since they have long and rich fossil records that reveal extinction periods (Ponder & Linberg, 1997; Klussmann-Kolb *et al*., 2008; Jörger *et al*., 2010; Kocot *et al*., 2013; Zapata *et al*., 2014). They have also proved to be excellent model systems in studies of biogeography, population genetics, biodiversity, physiology, neurophysiology and behavioural ecology (Goodacre & Wade 2001; Davison, 2002; Pinceel *et al*., 2005; Karanova & Ivlicheva, 2016; Bursztyka *et al*., 2018; Caro *et al*., 2019; Chukwuka & Ejere, 2019; Wendebourg & Hausdorf, 2019). Many panpulmonate species have considerable economic importance. Some are considered to be a valuable source of food such as *Achatina fulica* and *Helix pomatia* (Upatham *et al*., 1988; Gheoca, 2013), others such as *Limax cinereoniger* and *Helix aspersa* are used in pharmacological products (Thomas, 2015; Conte, 2016; Gonzalez, 2017; Laneri *et al*., 2019). Several panpulmonate species are of medical and veterinary importance as they act as vectors of disease, including *Biomphalaria* and *Bulinius* that transmit Schsitosomiasis, *Lymnaea* that transmits fascioliasis (Brown, 1994; Morgan *et al*., 2002) and *Achatina fulica* that transmits Angiostrongyliasis (Marquardt *et al*., 2000). They also include numerous invasive species such as *Achatina fulica*, *Arion lusitanicus* and *Deroceras reticulatum* which are considered as a major crop pest (Thiengo *et al*., 2007; Kozlowski & Kozlowska, 2002; Kozłowski & Kozłowski, 2011.) and *Physa acuta* which has a serious impact on the biodiversity of freshwater habitats (Albrecht *et al*., 2009; Ebbs *et al*., 2018).

Most panpulmonate taxa were originally placed within the Pulmonata. The Pulmonata was defined based on three anatomical characters: the pulmonary cavity opening by means of a pneumostome, the presence of a procerebrum and the existence of medio-dorsal (cerebral) bodies (Van Mol, 1967;

Haszprunar, 1988; Haszprunar & Huber, 1990). These characters were generally considered to be true synapomorphies of the Pulmonata (Dayrat & Tillier, 2002; Mordan & Wade, 2008). However, Ruthensteiner (1997) pointed out that the pulmonary cavity in pulmonates is homologous with the mantle (or pallial) cavities of other gastropods including the opisthobranchs. Likewise, the procerebrum in pulmonates is homologous to the rhinophoral ganglion of opisthobranchs. Although, the monophyly of the Pulmonata has been accepted in some morphological studies (Haszprunar, 1985; Haszprunar & Huber, 1990; Nordsieck, 1992; Dayrat & Tillier, 2002), they have not been recovered as a monophyletic group in most molecular analyses (Dayrat *et al*., 2001; Grande *et al*., 2004; Klussmann-Kolb *et al*., 2008; Dinapoli & Klussmann-Kolb, 2010; Jörger *et al*., 2010; Göbbeler & Klussmann-Kolb, 2011; Romero *et al*., 2016).

Jörger *et al*. (2010) in their molecular phylogeny of the Hetrobranchia, formally introduced the group Panpulmonata, uniting all previous pulmonate groups (the Siphonarioidea, Amphiboloidea, Hygrophila, Stylommatophora, Systellomatophora, Ellobioidea, Otinoidea and Trimusculoidea) with several lineages that were traditionally part of the Opisthobranchia (the Acochlidia and Sacoglossa) or part of the 'Lower Heterobranchia' (the Pyramidelloidea and Glacidorboidea). The results of subsequent molecular studies based on standard markers (Göbbeler & Klussmann-Kolb, 2011) and on phylogenomic analyses (Kocot *et al*., 2013; Zapata *et al*., 2014; Romero *et al*., 2016; Pabst & Kocot, 2018) have been consistent with Jörger *et al*. (2010) providing strong support for Panpulmonata monophyly. The taxonomy of Jörger *et al*. (2010) has been adopted in the most recent classification of Bouchet *et al*. (2017), placing the Panpulmonata at the rank of subcohort within the cohort Tectipleura.

Molecular techniques have established their value over the past decade in resolving the phylogenetic relationships and determining the time of evolutionary divergence within the Panpulmonata. However, the phylogenetic relationships of several subgroups within the Panpulmonata have not been well resolved particularly the basal relationships within the Stylommatophora and the deep relationships within the Hygrophila. Therefore, a robust phylogenetic framework is required to address the

shortcomings in these relationships. The work in this thesis focuses on two panpulmonate groups, the Stylommatophora and the Hygrophila.

1.2 Stylommatophora

The Stylommatophora are the largest panpulmonate group, comprising more than 100 families (Bouchet *et al*., 2017) and representing the vast majority of terrestrial gastropods with an estimated number of species between 30,000 and 35,000 (Solem, 1984). Stylommatophoran taxa primarily possess two pairs of tentacles on the head with eyes at the tips of the posterior pair and they do not have an operculum (Barker, 2001; Dayrat & Tillier, 2002). The first broad classification of the Stylommatophora was produced by Pilsbry (1900), who distinguished three primary divisions within the Stylommatophora (the Orthurethra, Heterurethra and Sigmurethra) based on differences in the anatomy of the main excretory organs (neprhidium and ureter). Of these divisions, the Orthurethra possess both orthureter and nephridium, but in most instances only the proximal part of the ureter developed as a groove along the rectal face of the nephridium (Figure 1.1). The Heterurethra lack an orthureter and the nephridium extends transversely with primary and secondary arms of the ureter developing at right angles to each other (Figure 1.1). Finally, the Sigmurethra were defined being without an orthureter but do have ureter that extends along the anterior part of the nephridium to the top of the pulmonary cavity and adjoining to the rectum until it reaches to the pneumostome (Figure 1.1). The taxonomy of Pilsbry (1900) was initially rejected by Simroth (1910) and Thiele (1931). However, Baker (1955) developed the taxonomy of Pilsbry further by adding a fourth division, the Mesurethra also based on the anatomy of the excretory system. The Mesurethra were defined by possessing neither a closed ureter nor an orthureter on the nephridium (Figure 1.1). He also placed all the stylommatophoran taxa that have a transverse nephridium under the term Heterurethra.

The division of the Stylommatophora into four primary divisions (commonly known as the Pilsbry-Baker System) was accepted by Zilch (1959-1960) and Solem (1978). However, the Pilsbry-Baker System was questioned by Schileyko (1976, 1979), Nordsieck (1986) and Tillier (1989). Schileyko (1979) produced a different classification for the Stylommatophora based mainly on the shell and reproductive morphology in which seven infraorders were recognised within the Stylommatophora: the

Pupillina, Achatinia, Oleacinina, Endodontinia, Helixinia, Zonitinia and Limaxinia, as well as two families: the Succineidae and Athoracophoridae. Nordsieck (1986) produced cladistic stylommatophoran taxonomy by reinterpreting the extensively published literature on the morphology of the shell and several organ systems within the Stylommatophora. In Nordsieck's classification, the Stylommatophora were divided into three main clades: the Orthurethra, Sigmurethra and Elasmognatha. A higher classification of the non-orthurethran taxa within the Stylommatophora was proposed later by Tillier (1989) based on differences within the renal organs. In this taxonomy, the Orthurethra were retained while the non-orthurethran stylommatophoran taxa were divided into two divisions Brachynephra and Dolichonephra. In the former division, the dedifferentiation of the kidney took place first and was then followed by a partial closure of the ureter while in the later division the closure of the ureter took place first and was then followed by a partial dedifferentiation of the kidney. However, this classification was later rejected by Nordsieck (1992) who produced an alternative taxonomy in which the Stylommatophora were divided into two clades (the Orthurethra and Sigmurethra) based on the excretory system.

Due to the highly conflicting results of the morphological studies (Schileyko, 1979; Nordsieck, 1986; Tillier, 1989; Nordsieck, 1992; Barker, 2001; Dayrat & Tillier, 2002) the relationships of the Stylommatophora have shown high controversy. This could be attributed to the relatively rapid radiations, the long evolutionary history and convergences of land snails as well as the insufficient taxon representation of these studies (Wade *et al*., 2001; Klussman-Kolb *et al*., 2008).

The use of molecular markers in the phylogenetic analyses of the Stylommatophora has proven to be essential for resolving the phylogenetic relationships within the group. The monophyly of the Stylommatophora has been supported by several molecular studies (Klussmann-Kolb *et al*., 2008; Dinapoli *et al*., 2010; Jörger *et al*., 2010; Göbbeler & Klussmann-Kolb, 2011; Romero *et al*., 2016; Teasdale *et al*., 2016). Wade *et al*. (2001, 2006) undertook the most comprehensive molecular studies of the Stylommatophora in term of species number. An interesting finding in these studies is the principle split of the Stylommatophora into two monophyletic groups: the 'achatinoid clade' and the

'non-achatinoid clade'. While the principle division of the Stylommatophora into the 'achatinoid' and 'non-achatinoid' clades has been accepted by Bouchet *et al*., (2017), the phylogenetic relationships at the base of the Stylommatophora have remained controversial particularly the position of the Scolodontidae. Only one molecular study (Ramírez *et al*., 2012) has addressed the phylogenetic position of the Scolodontidae and their relationship to the 'achatinoid' and 'non-achatinoid' clades, though their results showed inconsistent placement for the Scolodontidae. The deep phylogenetic relationships within the Stylommatophora are discussed in detail in chapter 3.

The phylogenetic relationships within the 'achatinoid clade' have been investigated thoroughly in recent molecular analyses by Fontanilla *et al*. (2017) where the taxonomic level of the family groups within the 'achatinoid clade' has been re-evaluated. Likewise, the monophyly of the major groups within the 'non-achatinoid clade' have been confirmed based on molecular studies (Wade *et al*., 2001, 2006; Teasdale *et al*., 2016). The deep phylogenetic relationships among the main groups of the 'non-achatinoid clade' (Limacoidea, Orthurethra, Helicoidea and Rhytididae) have been well supported in recent phylogenomic analyses by Teasdale *et al*. (2016). However, the interrelationships within several groups of the 'non-achatinoid clade' remain poorly resolved.

While the monophyly of the Orthurethra has been supported by several molecular analyses (Wade *et al*., 2001, 2006; Madeira *et al*., 2010; Nekola & Coles, 2015; Harl *et al*., 2016), the family-group interrelationships within the Orthurethra are still not clearly resolved particularly the basal division within the group. The phylogenetic relationships within the Orthurethra are discussed in detail in chapter 4.

In the most recent taxonomy of Bouchet *et al*. (2017), the Stylommatophora were divided into three suborders: the Achatinina ('achatinoid clade'), Scolodontina and Helicina ('non-achatinoid clade'). The suborder Achatinina includes two superfamilies: the Achatinoidea and Streptaxoidea while the Scolodontina includes one superfamily the Scolodontoidea. The suborder Helicina includes five infraorders: the Succineoidei [Elasmognatha], Rhytidoidei, Orthalicoidei, Pupilloidei [Orthurethra],

Clausilioidei, Arionoidei, Limacoidei [“Limacoid Clade”], Oleacinoidei and Helicoidei [“Helicoid Clade”] as well as it includes a group named ‘taxa of uncertain Position’.

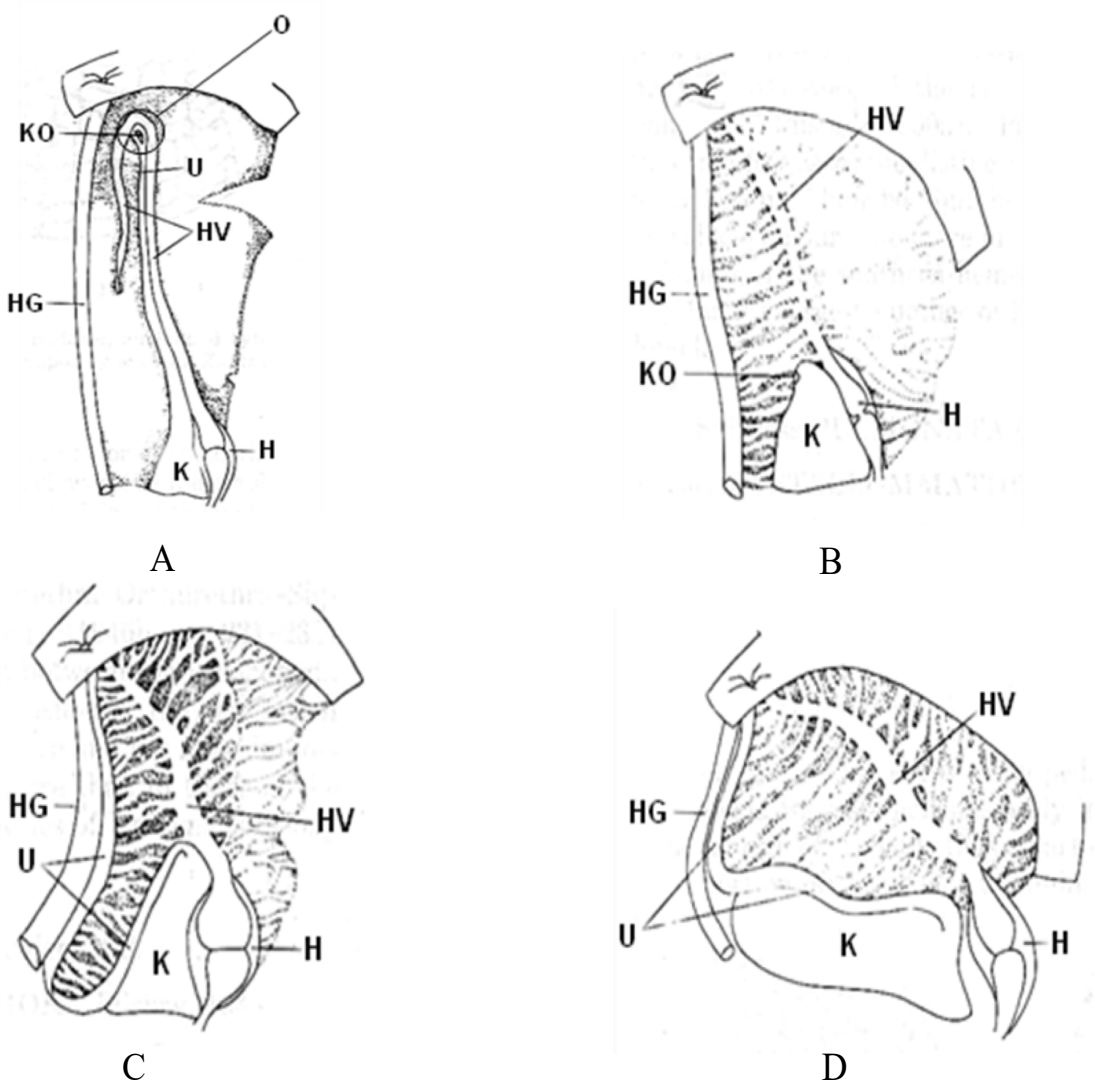


Figure 1.1. Pilsbry-Baker system of stylommatophoran classification based on the differences in the anatomy of the main excretory organs. The four primary divisions of the Stylommatophora are (A) Orthurethra, (B) Mesurethra, (C) Sigmurethra and (D) Heterurethra. The structures of the excretory organ are (K) nephridium, (U) ureter, (KO) renal orifice or nephropore, (O) orthureter, (HG) hindgut, (HV) principal pulmonary vein and H heart (From Solem, 1959).

1.3 Hygrophila

The Hygrophila represent the principle group of freshwater aquatic snails within the Panpulmonata, comprising eight families in the most recent classification of Bouchet *et al*. (2017). They are

characterised by the presence of a developed anterior gizzard, nephridial ureter and the eyes are present at the exterior bases of the tentacles within the Chilinidae while they are present at the interior bases of the tentacles in the lymnaeoidean families (Nordsieck, 1992). The absence of a free-swimming larval stage and the embryonic operculum are also features that characterise at least some of the hygrophilan taxa (Hubendick, 1978). Wide variations in shell form are found within the Hygrophila, not only among families but also within the individual families, including high-spired, discoidal (planispiral) or patelliform (limpet-like) shells, with both dextral (right-hand) or sinistral (left-hand) shell coiling (Hubendick, 1978).

Hygrophilan snails occupy a wide variety of habitats including large rivers, lakes, small ditches and ponds and they have a worldwide distribution. Some hygrophilan species are able to live in very unstable ponds or in human-made habitats such as irrigated areas in which water is available for only limited time per year (Jarne *et al*., 2010). The Hygrophila include several taxa that act as intermediate hosts of parasites of medical and veterinary importance, including *Biomphalaria* and *Bulinus* that transmit *Schistosoma* (blood flukes) and *Lymnaea* that transmits *Fasciola* (liver flukes) (Brown, 1994). Schistosomiasis is considered to be the second most important tropical parasitic disease after malaria with approximately 200 million infected individuals worldwide (Chitsulo *et al*., 2000). Similarly, Fascioliasis is considered as an important human parasitic disease with approximately 20 million infections around the world (Correa *et al*., 2010). Therefore, determining the phylogenetic relationships of some hygrophilan taxa that act as vectors of diseases are important to better understand the host-parasite relationships and their transition methods by mapping the phylogeny of the parasites onto the hygrophilan phylogenetic tree (Morgan *et al*., 2002).

Originally, all the freshwater panpulmonate snails were placed in the family Limnaeidae by Binney (1865) in which three subfamilies were recognised (the Limnaeinae, Planorbinae and Ancylinae) with *Physa* being included within the subfamily Limnaeinae. Fischer (1880-1887) then separated the Physidae as a family of their own. Likewise, Baker (1902) placed *Ancylus* in a separate family named the Ancylidae. In the classification of the Basommatophora of Dybowski (1903) six families were recognised (the Limnaeidae, Limnophysidae, Planorbidae, Ancylidae, Physidae and Amphipeplidae). Thiele (1931) ranked the Hygrophila as a stirps [=superfamily] within the order Basommatophora in

which six families: the Chilinidae, Latiidae, Physidae, Lymnaeidae, Planorbidae and Ancylidae were recognised. In Thiele's (1931) classification, the Acroloxidae were treated as a subfamily (Acroloxinae) within the Ancylidae. In Zilch's (1959-1960) classification of the Gastropoda, all the hygrophilan taxa were placed within the superfamily Lymnaeacea within the order Basommatophora. The Lymnaeacea were divided into ten families: the Chilinidae, Latiidae, Physidae, Lymnaeidae, Planorbidae, Neoplanorbidae, Rhodacmeidae, Ferrissiidae, Ancylidae and Acroloxidae. The Hygrophila were then ranked by Starobogatov (1970) as an order in which three superfamilies: the Chilinoidea, Latioidea and Lymnaeoidea were recognised. However, Hubendick (1978) treated the Hygrophila as a superfamily (Lymnaeacea) within the order Basommatophora with the Planorbidae and Ancylidae united in one family named the 'Ancyloplanorbidae' (= Planorbidae) and with the Hygrophila comprising six families: the Chilinidae, Latiidae, Acroloxidae, Physidae, Lymnaeidae, 'Ancyloplanorbidae' [= Planorbidae]. In Vaught's (1989) classification of living Mollusca, the term Hygrophila was not used, but instead the hygrophilan taxa were assigned to four superfamiles: the Chilinoidea (including the Chilinidae and Latiidae), Acroloxoidea (including the Acroloxidae), Lymnaeoidea (including the Lymnaeidae and Lancidae) Physiodea (including the Physidae) and Planorboidae (including the Planorbidae, Bulinidae and Ancylidae) within the Basommatophora order.

Nordsieck (1992) then ranked the Hygrophila as an order within the superorder Basommatophora comprising two suborders: the Chilinoidei and Lymnaeoidei. The latter suborder comprises three groups arranged sequentially as infraorders: the Acroloxoinei, Lymnaeoinei and Planorboinei. Moreover, Bouchet & Rocroi (2005) classified the Hygrophila as a clade within the informal group Basommatophora in which the Hygrophila were divided into four superfamilies: the Chilinoidea (including the Chilinidae and Latiidae), Acroloxoidea (including the Acroloxidae), Lymnaeoidea (including the Lymnaeidae) and Planorboidae (including the Planorbidae and Physidae). In Bouchet & Rocroi's (2005) classification, the Bulinidae were treated as a subfamily within the Planorbidae while the Ancylidae were classified as a tribe also within the Planorbidae. In the most recent classification of Bouchet *et al*. (2017), the name Basommatophora was not used and all their subgroups were moved to the subcohort Panpulmonata following the taxonomy of Jörger *et al*. (2010). In Bouchet *et al*. (2017),

the Hygrophila were ranked as a superorder comprising two superfamilies: the Chilinoidea (incorporating the Chilinidae and Latiidae) and the Lymnaeoidea (incorporating the Acroloxidae, Physidae, Lymnaeidae, Bulinidae, Burnupiidae and Planorbidae).

The monophyly of the Hygrophila has been supported in early morphological studies (Hubendick, 1978; Nordsieck, 1992; Barker, 2001) as well as in most molecular analyses (Dayrat *et al*., 2001; Klussmann-Kolb *et al*., 2008; Dinapoli *et al*., 2010; Jörger *et al*., 2010; Goebbeler & Klussmann-Kolb, 2011; Kocot *et al*., 2013). However, the interrelationships of the hygrophilan families remain poorly resolved. Likewise, the phylogenetic relationships and the subfamily ranks within the Lymnaeidae, Physidae and particularly the Planorbinae remain uncertain. Thus, a robust phylogenetic analysis is required to shed more light on the shortcomings in the phylogenetic relationships within the Hygrophila.

This study focuses on the family group relationships within the Hygrophila (chapter 5) and it focuses on the problematic interrelationships among the Acroloxidae, Physidae, Lymnaeidae and Planorbidae (chapter 6)

1.4 Summary of the Main Aims

Chapter 3 aims to resolve the basal divisions within the Stylommatophora by examining the position of the Scolodontidae and their relationship to the 'achatinoid' and 'non-achatinoid' clades. Phylogenetic analyses are based on multiple molecular markers. Additionally, the phylogenetic utility of the molecular markers used are examined by exploring and comparing the phylogenetic signals carried by the different genes.

Chapter 4 aims to examine the family-group interrelationships within the Orthurethra and to discuss the observe patterns of relationship more fully with special emphasis on the principle division within the Orthurethra.

In **chapter 5**, a comprehensive phylogeny of the Hygrophila is undertaken with the aims of examining the validity of the families and subfamilies within the Hygrophila and mapping the evolution of coiling direction onto the hygrophilan phylogenetic tree.

Chapter 6 aims to examine the position of the Hygrophila in relation to other panpulmonate groups and to determine the interrelationships among the hygrophilan families (based on multiple markers) with special emphases on the problematic interrelationships among the main lymnaeoidean families (Acroloxidae, Physidae, Lymnaeidae and Planorbidae) based on phylogenomic analyses.

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Chapter 2: Materials and Methods

2.1 Biological Materials

The gastropod specimens used in this study are shown in Table 2.1.

Table 2.1. Details of specimens, sampling localities and collectors. Classification of samples follows Bouchet *et al*. (2017).

Taxonomic rank	Species	Collection/Location	Collector
Phylum Mollusca, Class Gastropoda, Subclass Heterobranchia, Cohort Tectipleura, Subcohort Panpulmonata			
Superorder Sacoglossa **Superfamily Oxynooidea**			
Family Cylindrobullidae	*Cylindrobulla beauii* (P. Fischer, 1857)	FK-916, Jeffrey Key, NE tip of Jeffrey Key, Florida Keys, USA	Provided by Field museum Chicago_326526R
Volvatellidae	*Volvatella sp.* Pease, 1860	Madang Province, Astrolabe bay, Papua New Guinea	Provided by Muséum national d'Histoire naturelle (MNHN). MNHN-IM-2013-14281
Superfamily Plakobranchoidea			
Family Hermaeidae	*Hermaea bifida* (Montagu, 1816)	Connemara, Ireland	Bernard Picton
Family Limapontiidae	*Limapontia depressa* (Alder & Hancock, 1862)	Dorset, England, UK	Steve Trewhella
	Placida dendritica (Alder & Hancock, 1843)	Connemara, Ireland	Bernard Picton
	Ercolania margaritae (Burn, 1974)	Paterson, Cape, Victoria, Australia	Provided by Museums Victoria. T J. Hales. NMV F 133083
Family Plakobranchidae	*Elysia viridis* (Montagu, 1804)	Dorset, England, UK	Steve Trewhella
Superorder Siphonarimorpha, Order Siphonariida **Superfamily Siphonarioidea**			
Family Siphonariidae	*Siphonaria pectinata* (Linnaeus, 1758)	Zamara Los Atunes, Spain	S. Hawkins
	Siphonaria zelandica (Quoy and Gaimard, 1833)	Manu Bay, marine border shore, New Zealand	G. Barker
Superorder Pylopulmonata **Superfamily Pyramidelloidea**			
Family Pyramidellidae	*Boonea sp*. R. Robertson, 1978	South China sea	Provided by MNHN, MNHN-IM-2013-50057
	Turbonilla cummingi (Hori & Okutani, 1997)	St. John's Island, TMSI giant clam hatchery, Singapore	Lee Kong Chian Natural History MuseumL (ZRC.MOL.7379)
Superfamily Glacidorboidea			
Family Glacidorbidae	*Tasmodorbis punctatus* Ponder & Avern, 2000	Murchison Hwy, Deep Gully Creek, Tasmania, Australia	Australian museum (C.463949)
	Glacidorbis hedleyi Iredale, 1943	Blue Mountains, Boyd River, New South Wales, Australia	Australian museum (C.463947)
Superfamily Amphiboloidea			
Family Amphibolidae	*Salinator tecta* Golding, Ponder & Byrne, 2007	Boronia Park, Lane Cuve River, Sydney, Australia	R.Gozding
	Phallomedusa sp Golding, Ponder & Byrne, 2007	Boronia Park, Lane Cuve River, Sydney, Australia	R.Gozding
	Amphibola crenata (Gmelin, 1791)	Waihi Estuarine, New Zealand	G. Barker
Superorder Acochlidimorpha, **Superfamily Acochlidioidea**			
Family Hedylopsidae	*Hedylopsis spiculifera* (Kowalewsky, 1901)	Rovinj, Kleine Salinebucht, Istria	Provided by Zoologische Staatssammlung München

			(ZSM), M. Schroedl, ZSM Mol 20100450
Family Acochlidiidae	*Strubellia wawrai* Brenzinger, Neusser, Jörger & Schrödl, 2011	Papua New Guinea	Provided by MNHN, IM-2013-54739
Superfamily Parhedyloidea			
Family Parhedylidae = [Microhedylidae]	*Pontohedyle milatchevitchi* (Kowalewsky, 1901)	SW of Zadar, Pazman Island, Croatia	Provided by ZSM, M. Schroedl ZSM Mol 20130948
Superorder Hygrophila **Superfamily Chilinoidea**			
Family Chilinidae	*Chilina patagonica* (G. B. Sowerby II, 1874)	Golfo Elefantes, Region XI, Chile	David Reid
Family Latiidae	*Latia neritoides* (Gray, 1850)	Wainui, New Zealand	G. Barker
Superfamily Lymnaeoidea			
Family Acroloxidae	*Acroloxus lacustris* (Linnaeus, 1758)	West side of River Kozinka, Kievskaya Oblast, Ukraine	D.W. Taylor
	Pseudancylastrum sp. Lindholm, 1909	Lake Baikal, Russia	T. Sitmikova
Family Physidae	*Physa fontinalis* (Linnaeus, 1758)	Embayment from west side of River Desna, Kievskaya Oblast, Ukraine	D.W. Taylor
	Physella gyrina (Say, 1821*)*	Unknown	Unknown
	Haitia mexicana (Philippi, 1841)	pHake Lake, Los Angeles County, CA, USA	D.W. Taylor
	Mayabina sp. D. W. Taylor, 2003	Sacha Lodge, Sucumbíos, Ecuador	D.W. Taylor
	Stenophysa minor (d'Orbigny, 1837)	Departamento Canelones, Barra de Carrasco, Uruguay	D.W. Taylor
	Sibirenauta elongatus (Say, 1821)	Okanagan-Similkameen District, Penticton, British Columbia, Canada	D.W. Taylor
Family Lymnaeidae	*Bakerilymnaea bulimoides* (Lea, 1841) [= *Fossaria bulimoides*]	Corner of Alvadore Road, Lane County, Oregon, USA	D.W. Taylor
	Corvusiana sp. Servain, 1882	Pond of Institute of Fish Culture, Kievskaya Oblast, Kiev, Ukraine	D.W. Taylor
	Lymnaea stagnalis (Linnaeus, 1758)	Garden Pond, Morpeth, Northumberland, UK	Angus Davsion
	Omphiscola glabra (O. F. Müller, 1774)	Embayment from west side of River Desna, Kievskaya Oblast, Ukraine	D.W. Taylor
	Pseudosuccinea columella (Say, 1817)	Sacha Lodge, Sucumbíos, Ecuador	D.W. Taylor
	Stagnicola sp. Jeffreys, 1830	Pond of Institute of Fish Culture, Kievskaya Oblast, Kiev, Ukraine	D.W. Taylor
	Polyrhytis apicina (Meek, 1876)	Unknown	Unknown
	Myxas glutinosa (O. F. Müller, 1774)	Llyn Tegid, Gwynedd, UK	M. Willing
	Radix sp. Montfort, 1810	Pallang Stream, Gambia	Ebrima Joof
	Peregriana sp. Servain, 1882	Pond of Institute of Fish Culture, Kievskaya Oblast, Kiev, Ukraine	D.W. Taylor
	Lanx subrotundata (Tryon, 1865)	North Umpqua River, Singleton Park, Douglas County, Oregon, USA	D.W. Taylor
Family Bulinidae	*Bulinus natalensis* (Krauss in Kuster, 1841)	Lake Siboya, South Africa	Provided by Biomedical Parasitology Section, Natural History Museum (NHM) (from culture no. 272)
Family Burnupiidae	*Burnupia sp.* B. Walker, 1912	South Africa	Provided by Natal Museum
Family Planorbidae	*Anisus vortex* (Linnaeus, 1758)	Embayment from west side of river Desna, Kievskaya Oblast, Ukraine	D.W. Taylor
	Bathyomphalus contortus (Linnaeus, 1758)	Embayment from east side of river Desna, Kievskaya Oblast, Ukraine	D.W. Taylor
	Choanomphalus maacki Gerstfeldt, 1859	Lake Baikal, Berezoviy, Russia	T. Sitnikova

	Gyraulus parvus (Say, 1817)	Okanagan-Similkameen District, west end of Yellow Lake, British Columbia, Canada	D.W. Taylor
	Ceratophallus sp. D. S. Brown & Mandahl-Barth, 1973	Pacharr Irrigation Canal, Gambia	Ebrima Joof
	Planorbis planorbis (Linnaeus, 1758)	West side of River Kozinka, west of village Letki, Kievskaya Oblast, Ukraine	D.W. Taylor
	Segmentina nitida (O. F. Müller, 1774)	West side of River Kozinka, west of village Letki, Kievskaya Oblast, Ukraine	D.W. Taylor
	Biomphalaria glabrata (Say, 1818)	Puerto Rico	Provided by Mike Doenhoff, Biomedical Parasitology Section, School of Life Sciences, University of Nottingham
	Menetus callioglyphus (Vanatta, 1895)	Decker Creek at Matlock, Brady Road, Mason County, Washington, USA	D.W. Taylor
	Planorbarius corneus (Linnaeus, 1758)	Embayment from west side of River Desna, Kievskaya Oblast, Ukraine	D.W. Taylor
	Planorbella subcrenata (Carpenter, 1857)	Okanagan-Similkameen District, west end of Yellow Lake, British Columbia, Canada	D.W. Taylor
	Planorbula campestris (Dawson, 1875)	DNA 7, Teton County, Wyoming, USA	D.W. Taylor
	Promenetus exacuous (Say, 1821)	Okanagan-Similkameen District, NE side of Vaseaux Lake, British Columbia, Canada	D.W. Taylor
	Vorticifex effusus (I. Lea, 1856)	DNA 14, Deschutes River, Wasco county, Oregon, USA	D.W. Taylor
	Ancylus fluviatilis O. F. Müller, 1774	Broughton, Scotland, UK	G.M. Barker
	Ferrissia californica (Rowell, 1863)	T04-4204, Fern Ridge Reservoir, Lane County, Oregon, USA	D.W. Taylor
Superorder Eupulmonata, Order Ellobiida **Superfamily Ellobioidea**			
Family Ellobiidae	*Melampus luteus* (Quoy & Gaimard, 1832	Souilla, Mauritius	O. Griffiths
	Laemodonta sp.	Suralaya, West Java, Indonesia	B. Dharma
	Blauneria heteroclita (Montagu, 1808)		Provided by NHM
	Myosotella myosotis (Draparnaud, 1801)	Dorset, England, UK	Steve Trewhella
	Leucophytia bidentata (Montagu, 1808)	Dorset, England, UK	Steve Trewhella
	Cassidula nucleus (Gmelin, 1791)	East point, Darwin, Northern Territory, Australia	Provided by NHM. NHM_2394
	Ophicardelus sulcatus (H. Adams & A. Adams, 1854)	Australia, Nudgee, Brisbane	G. Barker
	Carychium tridentatum (Risso, 1826)	Abelheira, São Miguel, Azores	P. Mordan
Family Otinidae	*Otina ovata* (T. Brown, 1827)	Cave at West End of Freshwater Bay. Isle of Wight, UK	David Reid
Family Trimusculidae	*Trimusculus sp.*	Plage de Lavanono, Madagascar	Provided by MNHN. IM-2009-16399
Clade Geophila, Order Stylommatophora, Suborder Achatinina ['Achatinoid Clade'] **Superfamily Achatinoidea**			
Family Ferussaciidae	*Ferussacia folliculus* (Gmelin, 1791)	Los Alcornales, Prov Cadiz, Spain	M. Seddon
Family Subulinidae	*Subulina striatella* (Rang, 1831)	Kew Gardens (introduced)	F. Naggs
Family Glessulidae	*Glessula ceylanica* (Pfeiffer, 1845)	Colombo, Sri Lanka	P. Karunaratne
Family Achatinidae	*Lissachatina fulica* (Bowdich, 1822) [= *Achatina fulica*]	Unknown (Zool. Soc. Lond. Colln.)	P. Pearce-Kelly

Family Coeliaxidae	*Coeliaxis blandii* (Pfeiffer, 1852)	New Bradford, South Africa	N. Smith
Family Thyrophorellidae	*Thyrophorella thomensis* (Greeff, 1882)	Zampala, São Thomé, West Africa	A. Gascoigne
Superfamily Streptaxoidea			
Streptaxidae	*Gonaxis quadrilateralis* (Preston, 1910)	Reunion	O. Griffiths
Suborder Helicina ['Non-Achatinoid Clade'], Infraorder Pupilloidei [Orthurethra] Superfamily Pupilloidea			
Achatinellidae	*Elasmias luakahaense* Pilsbry and Cooke, 1915	Koolau Range, Oahu, Hawaii	R. Rundell & K. Olival
	Partulina proxima (Pease, 1862)	Maui, Hawaii	B. Holland
Family Cochlicopidae	*Cochlicopa lubricella* (Porro, 1838)	São Miguel, Azores	P. Mordan
Family Amastridae	*Leptachatina lepida* Cooke, 1910	Hawaii Island, Hawaii	P. Mordan & R. Cowie
Family Pupillidae	*Pupoides albilabris* (Adams, 1841)	Wilson County, Tennesse, USA	J. Slapcinsky & B. Coles
	Pupilla muscorum (Linnaeus 1758)	Garden, Victoria Grove, London, UK	Ellen Graubart
Family Lauriidae	*Lauria cylindracea* (da Costa, 1778)	Mullaghmore, Co. Sligo, Ireland	E. Platts
Family Valloniidae	*Vallonia excentrica* Sterki, 1892	São Miguel, Azores	P. Mordan
	Acanthinula aculeata (O. F. Müller, 1774)	Box Hill, Surrey, UK	J. Ablett
Family Vertiginidae	*Vertigo antivergo* (Draparnaud, 1801)	Chuett, Arnoldstein, Austria	P. Miltner
	Pronesopupa acanthinula Ancey, 1892	Koolau Range, Oahu, Hawaii	R. Rundell & K. Olival
Family Gastrocoptidae	*Gastrocopta armifera* (Say, 1821)	Wilson County, Tennesse, USA	J. Slapcinsky & B. Coles
Family Orculidae	*Orcula austriaca* Zimmerman, 1932	Kuhberg, Austria	P. Miltner
Family Strobilopsidae	*Eostrobilops nipponica* (Pilsbry, 1908)	Osaka, Japan	I. Matsumura
Family Pyramidulidae	*Pyramidula rupestris* (Draparnaud, 1801)	Mullaghmore, Co. Sligo, Ireland	E. Platts
Family Chondrinidae	*Chondrina clienta* (Westerlund, 1883)	Villach, Austria	P. Miltner
	Solatopupa similis (Bruguière, 1792)	Verdon Gorge, France	A. Davison
	Abida secale (Draparnaud, 1801)	Pulpit Down, Buckinghamshire, UK	P. Mordan
	Granaria frumentum (Draparnaud, 1801)	Pelsivec Plateau, Slovakia	J. Grego
Family Enidae	*Buliminus labrosus* (Olivier, 1804)	Saladin's Castle, Syria	P. Mordan
	Pene sidonensis (Férussac, 1821)	Saladin's Castle, Syria	P. Mordan
	Luchuena reticulata (Reeve, 1849)	Kikai Island, Ryukyu, Japan	S. Chiba
	Napaeus pruninus (Gould, 1846)	São Miguel, Azores	A. Polasczek
	Macaronapaeus vulgaris (Morelet & Drouet, 1857)	São Miguel, Azores	P. Mordan
	Ena obscura	Olcnava, Biela voda 260. Slovakia	leg. J. Steffek
	Mastus pupa (Bruguière, 1792)	Sicily	A. Davison
	Mirus stalix (Benson, 1863)	Agra, Sri Lanka	D. Raheem
	Chondrula albolimbata (Pfeiffer, 1848)	Spissky tlrad, Slovakia	J. Grego
Family Cerastidae	*Cerastus schweinfurthi* (Martens, 1895)	Al-Mahuit, N. Yemen	P. Mordan
	Pachnodus silhouettanus van Mol & Coppois, 1980	Silhouette Island, Seychelles	J. Gerlach
	Amimopina macleayi (Brazier, 1876)	Queensland, Australia	P. Mordan
Family Draparnaudiidae	*Draparnaudia singularis* Reeve, 1854	Mont Koghis, New Caledonia	C. Wade & K. Bowman
Family Partulidae	*Samoana conica* (Gould, 1847)	Samoa	R. Cowie
	Eua zebrina (Gould, 1847)	Samoa	R. Cowie
	Partula tohiveana Crampton, 1924	Moorea	Unkown
Infraorder Clausilioidei Superfamily Clausilioidea			
Family Clausiliidae	*Albinaria xanthostoma* (Boettger, 1883)	Crete	D. Thomaz
Infraorder Orthalicoidei			

Superfamily Orthalicoidea			
Family Orthalicidae	*Drymaeus discrepans* (Sowerby, 1833)	Guatemala	Unknown
Family Amphibulimidae	*Gaeotis nigrolineata* (Shuttleworth, 1854)	El Yunque, Puerto Rico	Unknown
Infraorder Oleacinoidei **Superfamily Oleacinoidea**			
Family Spiraxidae	*Euglandina rosea* (Férussac, 1821)	Moorea (Zool. Soc. Lond. Colln.)	P. Pearce-Kelly
Superfamily Haplotrematoidea			
Family Haplotrematidae	*Haplotrema vancouverense* (Lea, 1839)	Eugene, Oregon, USA	D. Taylor
Infraorder Rhytidoidei **Superfamily Rhytidoidea**			
Family Rhytididae	*Rhytida stephenensis* (Powell, 1930)	Manaaki Whenua, New Zealand	D. Gleeson
Family Megalobulimidae = Strophocheilidae	*Megalobulimus oblongus* (O. F. Müller, 1774)	Antigua (Zool. Soc. Lond. colln.)	P. Pearce-Kelly
Family Dorcasiidae	*Dorcasia alexandri* (Gray, 1938)	Windhoek, Namibia	C. Boix-Hinzen
Family Caryodidae	*Caryodes dufresnii* (Leach, 1815)	Mt Wellington, Hobart, Tasmania	B. Smith
Infraorder Limacoidei ['Limacoid Clade'] **Superfamily Limacoidea**			
Family Euconulidae	*Euconulus fulvus* (O. F. Müller, 1774)	New Forest, Hampshire, UK	P. Mordan
Superfamily Helicarionoidea			
Family Ariophantidae	*Cryptozona bistrialis* (Beck, 1837)	Sri Lanka	P. Karunaratne
Superfamily Limacoidea			
Family Vitrinidae	*Vitrina pellucida* (O. F. Müller, 1774)	Kirkdale, Derbyshire, UK	C. Wade
Superfamily Gastrodontoidea			
Family Oxychilidae	*Oxychilus alliarius* (Miller, 1822)	Deepdale, Derbyshire, UK	C. Wade
Infraorder Helicoidei ['Helicoid Clade'] **Superfamily Helicoidea**			
Family Camaenidae	*Satsuma japonica* (Pfeiffer, 1847)	Osaka City, Japan	P. Callomon
Family Hygromiidae	*Trochulus striolatus* (Pfeiffer, 1828)	Deepdale, Derbyshire, UK	C. Wade
Family Bradybaenidae =Camaenidae	*Bradybaena similaris* (Férussac, 1821)	Sri Lanka	P. Karunaratne
Family Helminthoglyptidae =Xanthonychidae.	*Monadenia fidelis* (Gray, 1834)	Oregon	D. Taylor
Infraorder Arionoidei **Superfamily Arionoidea**			
Family Arionidae	*Arion hortensis* (Férussac, 1819)	Kirkdale, Derbyshire, UK	C. Wade
Family Philomycidae	*Meghimatium bilineatum* (Benson, 1842)	Mauritius	O. Griffiths
Taxa of uncertain Position **Superfamily Testacelloidea**			
Family Testacellidae	*Testacella scutulum* (Sowerby, 1821)	North London, UK	R. Hurst
Superfamily Plectopyloidea			
Family Corillidae	*Corilla adamsi* (Gude, 1914)	Sri Lanka	D. Raheem
Suborder Scolodontina **Superfamily Scolodontoidea**			
Family Scolodontidae =Systrophiidae	*Guestieria sp.* Crosse, 1872	Ecuador	Hilary Kingston
	Systrophia sp. Pfeiffer, 1855	Ecuador	Hilary Kingston
Order Systellomatophora **Superfamily Onchidioidea**			
Family Onchidiidae	*Onchidella celtica* (Cuvier, 1817*)*	Polzeath Beach, Cornwall, England, UK	Mike Kent
Superfamily Veronicelloidea			
Family Veronicellidae	*Laevicaulis alte* (Férussac, 1823)	Dubai, United Arab Emirates	A. Green

	Semperula maculata (Templeton, 1858)	Los Baños, col, Philippines	M. Carandang
Cohort Acteonimorpha **Superfamily Acteonoidea**			
Family Aplustridae	*Hydatina sp*. (Schumacher, 1817)	Ambatobe, près Soamanitse, Madagascar	Provided by MNHN. IM-2009-16352
Subclass Caenogastropoda, Grade Architaenioglossa **Superfamily Ampullarioidea**			
Family Ampullariidae	*Pomacea sp*.	From London Zoo	Unknown
Superfamily Cyclophoroidea			
Family Cyclophoridae	*Cyclophorus herklotsi* (Martens, 1861)	Bosque Estatal de Guacataca, Peurto Rico	Angus Davison

2.2 Molecular Lab Methods

2.2.1 DNA and RNA Extraction

2.2.1.1 DNA Extraction of Snail Tissues Using the CTAB Protocol

This protocol is adapted from Hillis *et al*. (1996) and is described in Goodacre & Wade (2001). Genomic DNA was extracted from the whole snail (for small specimens) or from a small (1-2 mm^3) tissue sample taken from the foot of the snail. Material included living snails, fresh frozen snail tissue and ethanol-preserved specimens. Ethanol-preserved material was soaked in 1 ml TE buffer (10 mM Tris-HCl, 1mM EDTA) for about one hour before extraction.

1- The sample was placed in plastic tubes and ground using sterile glass beads and a plastic pestle. Then, immersed in 500 µl of CTAB solution [100 mM Tris-HCl pH 8, 20 mM EDTA pH 8, 1.4 mM NaCl, CTAB 2% (w/v)].

2- 10 µl of Proteinase K (10 mg/ml) and 1 µl β-mercaptoethanol were then added to each sample and the tubes were then incubated at 55 ^{0} C for at least two hours until the tissue slices were completely digested.

3- 500 µl of ice-cold chloroform-isoamyl alcohol (24:1) were added to remove proteins. The tubes were generally inverted for 5 minutes and the sample was then centrifuged for 10 minutes at 13,000 rpm. The aqueous phase (~400 µl) was transferred to a new tube.

4- Step 4 was repeated once and where necessary (excessive proteins) step 4 repeated a third time.

5- 2.5 volumes (~1 ml) of ice-cold 95% ethanol and 4 µ of 3 M-sodium acetate (NaOAc) were added to the mix followed by overnight incubation at -80 ^{0}C to precipitate the DNA.

6- The tubes were centrifuged for 15 minutes at 13,000 rpm, and then the supernatant was gently removed.

7- The remaining pellets were washed with 500 µl of ice-cold 70% ethanol and centrifuged for five minutes at 13,000 rpm. Afterward, the ethanol was gently removed.

8- The pellets were then air dried for 15 min at 45 °C and were then resuspended in 150-200 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.5).

9- Each tube was then boiled for 15 min at 95 °C to denature any residual enzymes in order to extend the life of the DNA extract. The DNA extracts were stored at -20 °C until use.

2.2.1.2 RNA Extraction

RNA was extracted from *Acroloxus lacustris* using ReliaPrep™ RNA Tissue Miniprep System provided by Promega. The protocol follows the manufactures instructions.

1- Before starting RNA extraction, the following four reagents were prepared:
   a- DNase I, 250 prep size (Cat.# Z6112): 275 µl of nuclease-free water (provided) as indicated on the vial label was added and then gently mixed.
   b- LBA + TG buffer, 250 prep size (Cat.# Z6112): 3,000 µl of 1-thioglycerol was added to 150 ml of LBA buffer.
   c- RNA wash solution, 250 prep size (Cat.# Z6112): 350 ml of 95% ethanol was added to a bottle containing 20 6ml of concentrated RNA wash solution (RWA).
   d- Column wash solution, 250 prep size (Cat.# Z6112): 36 ml of 95% ethanol was added to a bottle containing 24 ml of concentrated column wash solution (CWE).

2- 500 µl of LBA +TG buffer was added to a single *Acroloxus lacustris* specimen. The tissue was then ground using a sterile plastic pestle, followed by pipetting 7–10 times to shear the RNA using a P200 or P1000 pipettor.

3- To clear homogenates, the samples were centrifuged for 3 minutes at 14,000 × g and then, the homogenates were transferred to a clean tube and 170 µl of isopropanol was added to the tube.

4- A ReliaPrep™ Minicolumn was placed in a 2 ml collection tube and the sample was transferred to the Minicolumn and centrifuged at 12,000-14,000 × g for 1 minute at 20-25 °C. After that, the liquid was discarded from the collection tube.

5- 500µl of RNA wash solution was added to the ReliaPrep™ Minicolumn to wash the column and centrifuged at 12,000-14,000 × g for 30 seconds.

6- The DNase I incubation mix was prepared by combining three reagents (yellow core buffer 24 µl, MnCl2, 0.09 M and DNase I 3 µl) and mixing them by gentle pipetting. Then, 30 µl of this mix was applied to the minicolumn membrane and incubated for 15 minutes at 20-25 °C.

7- 200 µl of column wash solution was added to the minicolumn and centrifuged at 12,000-14,000 × g for 15 seconds. After that, 500 µl of RNA wash solution was added and centrifuged at 12,000-14,000 × g for 30 seconds.

8- The ReliaPrep™ minicolumn was placed into a new collection tube and 300 µl of RNA wash solution was added and centrifuged at high speed for 2 minutes.

9- In order to elute the RNA, the minicolumn was placed in a new collection tube and 15 µl of nuclease-free water was added to the membrane and centrifuged at 12,000-14,000 × g for 1 minute. Then, the column was removed and discarded, and the purified RNA was stored at -80 °C.

2.2.2 PCR Amplification and Sequencing

2.2.2.1 Large Subunit (LSU) rRNA Gene (incorporating 5.8s and ITS-2)

The amplified large subunit (LSU) ribosomal (r) RNA gene fragment included almost the whole length of the LSU rRNA gene as well as incorporating about 80 nucleotides of the 5.8S rRNA gene and the internal transcribed spacer 2 (ITS-2) region (see Figure 2.1). The LSU rRNA gene was amplified in a nested PCR reaction using primers LSU-1ii and LSU-12 in the first round, with the primary PCR products then used as template for the secondary PCR to amplify six internal fragments (A, B, C, D, E, and F) (see Table 2.2 for details of primers). Where necessary alternative primers were used. LSU 1ii and LSU 3ii were typically used for the Stylommatophora whereas LSU 1iii and LSU 3iii were used for the Hygrophila and other panpulmonate groups. Likewise, LSU 4ii and LSU 7 were typically used for Stylommatophora whereas LSU 4iii and LSU 7i were used for the Hygrophila and other

panpulmonate groups. LSU 6i and LSU 9i were typically used for the Stylommatophora whereas LSU 6ii and LSU 9ii were used for the Hygrophila and other panpulmonate groups. PCR amplification for the primary PCR was performed using the Qiagen Taq DNA polymerase and Q buffer system (1X buffer, 1X Q-solution, 0.3 mM dNTP, 1.5 mM magnesium chloride, 0.2 µM each primer and 1U Taq in a 50 µL final volume). Secondary PCR amplification was identical to the primary PCR with the exception that a lower 0.2 mM concentration of dNTPs was used. The cycling conditions (with a Perkin Elmer cycler) of the primary PCR were as follows: 96 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 50 °C for 30 sec, 72 °C for 3 min and then a final extension step at 72 °C for 5 min. The secondary PCR cycle conditions were as follows: 96 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 45 °C for 1 min, 72 °C for 2 min and then a final extension step at 72 °C for 5 min.

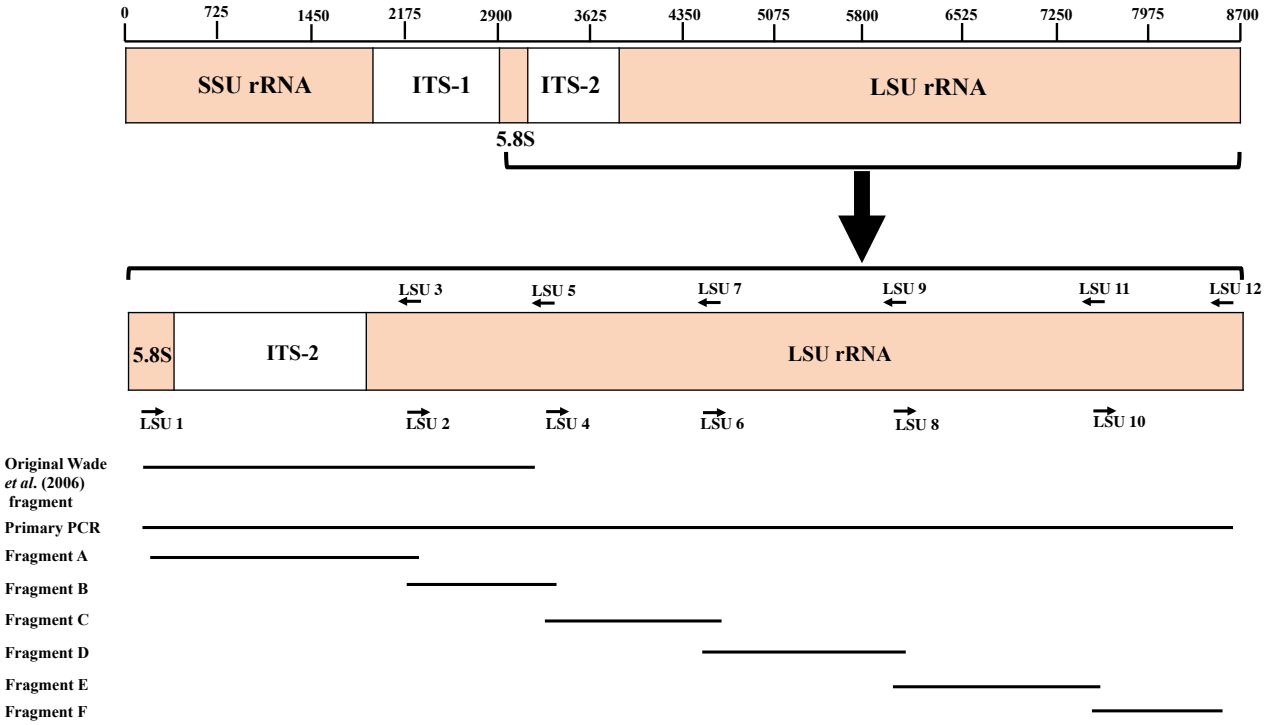


Figure 2.1. Schematic diagram of eukaryotic ribosomal RNA (rRNA) gene cluster showing the primers of the LSU rRNA gene (incorporating 5.8S and ITS-2) used in this study. The first line below the diagram corresponds to the length of the original LSU 1-5 fragment of Wade *et al*. (2006). The remaining lines correspond to the length of the fragments amplified with each primer pair.

2.2.2.2 Small Subunit (SSU) rRNA Gene

The small subunit (SSU) rRNA gene was amplified in a nested PCR reaction with primers 18E and 18P used for the first round and with the primary PCR products then used as template to amplify a single fragment with primers 1F and 9R (see Table 2.2 for details of primers). A series of six internal primers were used to sequence this fragment (see Figure 2.2 and Table 2.2 for details of primers). Some samples proved difficult to amplify as a single fragment and in these cases internal primer sets were used to amplify the SSU gene. Amplification was performed using BIOTAQ^{TM} DNA polymerase (1X reaction buffer, 0.2 mM dNTPs, 2.5 mM magnesium chloride, 0.2 µM each primer and 1U Taq in a 25 µL final volume). The PCR cycling conditions were as follows: 96 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 50 °C for 30 sec, 72 °C for 90 sec and then a final extension step of at 72 °C for 5 min for both primary and secondary PCR.

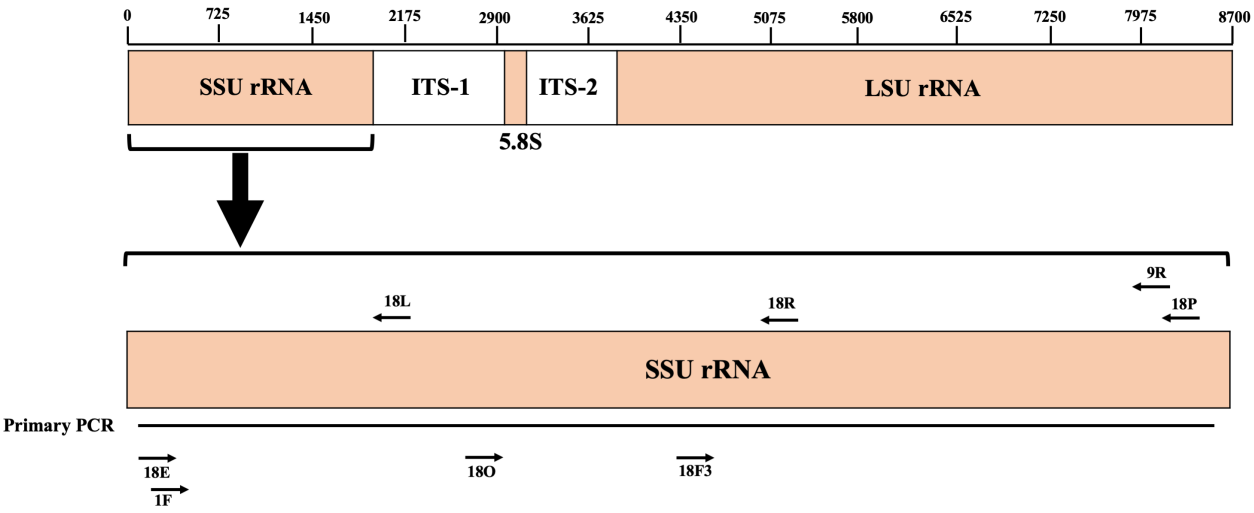


Figure 2.2. Schematic diagram of eukaryotic ribosomal RNA (rRNA) gene cluster showing the primers of the SSU rRNA gene used in this study. Line below the diagram corresponds to the length of the fragment amplified in this study.

2.2.2.3 Histone 3 Gene

A 331 bp fragment of the histone 3 (H3) gene was amplified as a single fragment using primers H3aF and H3aR as default primers, though different combinations of primers were used with some samples where they did not amplify with the default primes (see Table 2.2 for complete list of primers). The PCR amplification of the H3 gene was performed using BIOTAQ^{TM} DNA polymerase (1X reaction

buffer, 0.2 mM dNTPs, 2.5 mM magnesium chloride, 0.2 µM each primer and 1U Taq in a 25 µL final volume). The thermal cycling conditions were as follows: 96 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 50 °C for 30 sec, 72 °C for 3 min and then a final extension step at 72 °C for 5 min.

2.2.2.4 Cytochrome C Oxidase Subunit I Gene

The cytochrome oxidase 1 (CO1) gene was also amplified as a single fragment using primers LCO 1490 and HCO 2198 for the majority of samples. A few samples did not amplify with these primers thus different combinations of primer were used (see Table 2.2 for a complete list of primers). The PCR amplification of the CO1 gene was performed using BIOTAQ^{TM} DNA polymerase (1X reaction buffer, 0.2 mM dNTPs, 2.5 mM magnesium chloride, 0.2 µM each primer and 1U Taq in a 25 µL final volume). The thermal cycling conditions were as follows: 96 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 50 °C for 30 sec, 72 °C for 3 min and then a final extension step at 72 °C for 5 min.

Table 2.2. Primers used for PCR amplification

Primers		**Reference**	**Fragment Size (bp)**
Large Subunit rRNA (LSU) Incorporating 5.8S and ITS2			
Primary PCR	LSU-1ii (sense): 5'-CTAGCTGCGAGAATTAATGTGA-3' [Labelled as Primer LSU-1 in Wade et al. (2006)] LSU-12 (anti-sense): 5'-TTCTGACTTAGAGGCGTTCAG-3'	Wade & Mordan (2000); Wade *et al*. (2001, 2006) Fontanilla *et al*. (2017)	4000
A	LSU-1ii (sense): 5'-CTAGCTGCGAGAATTAATGTGA-3' [Labelled as Primer LSU-1 in Wade et al. (2006)] LSU-3ii (anti-sense): 5'-ACTTTCCCTCACGGTACTTG-3' [Labelled as Primer LSU-3 in Wade et al. (2006)] Alternative primers LSU-1iii (sense):5'-TGCGAGAATTAATGTGAATTGC-3' LSU-3iii (anti-sense): 5'-ACGGTACTTGTCCGCTATCG-3'	Wade & Mordan (2000); Wade *et al.* (2001, 2006) Wade & Mordan (2000); Wade *et al.* (2001, 2006) Fontanilla *et al*. (2017) Fontanilla *et al*. (2017)	~900-1200
B	LSU-2ii (sense): 5'-GGGTTGTTTGGGAATGCAGC-3' [Labelled as Primer LSU-2 in Wade et al. (2006)] LSU-5ii (anti-sense): 5'-GTTAGACTCCTTGGTCCGTG-3' [Labelled as Primer LSU-5 in Wade et al. (2006)]	Wade & Mordan (2000); Wade *et al.* (2001, 2006) Wade & Mordan (2000); Wade *et al.* (2001, 2006)	~580
C	LSU-4ii (sense): 5'-GTCGGCATTCCACCCGACC-3' LSU-7 (anti-sense): 5'- GCAGGTGAGTTGTTACACACTC-3' Alternative primers LSU-4iii (sense): 5'-CGGTGGCGAGTCTGTCGGC-3' LSU-7i (anti-sense): 5'-GTTGTTACACACTCCTTAGCGG-3'	Fontanilla *et al*. (2017) Fontanilla *et al*. (2017) Designed in this study Fontanilla *et al*. (2017)	~700
D	LSU-6i (sense): 5'-GTGCCAAACGCTGACGCTCA-3' LSU-9i (anti-sense): 5'-ACCCAGTCCTCAGAGCCAATC-3' Alternative primers LSU-6ii (sense): 5'-GTGCCAAACGCTGACGCTCA-3'	Fontanilla *et al*. (2017) Fontanilla *et al*. (2017) Fontanilla *et al*. (2017)	~850

	LSU-9ii (anti-sense): 5'-ACCCAGTCCTCAGAGCCAATC-3'	Fontanilla *et al*. (2017)	
E	LSU-8ii (sense): 5'-GTGCACAGCCTCTAGTCGATA-3' LSU-11ii (anti-sense): 5'-TCCTCCTGAGCTCGCCTTAG-3'	Fontanilla *et al*. (2017) Fontanilla *et al*. (2017)	~850
F	LSU-10i (sense): 5'-GGCCGCGATCCGTCTGAAGA-3' LSU-12i (anti-sense): 5'-GGCTTCTGACTTAGAGGCGTT-3'	Fontanilla *et al*. (2017) Fontanilla *et al*. (2017)	~500
Small Subunit rRNA (SSU)			
	18E (sense): 5'-CTGGTTGATCCTGCCAGT-3' 1F (sense): 5′-TAC CTG GTT GAT CCT GCC AGT AG-3′ 18L(anti-sense) 5'-GAATTACCGCGGCTGCTGGCACC-3' 18O (sense) 5'-GGAATRATGGAATAGGACC-3' 18R anti-sense) 5'-GTCCCCTTCCGTCAATTYCTTTAAG-3' 18F3 (sense) 5'-CGAAGACGATCAGATACCG-3' 9R (anti-sense) 5′-GATCCTTCCGCAGGTTCACCTAC-3 18P (anti-sense): 5'-TAA TGATCCTTCCGCAGGTTCACC T-3'	Hillis & Dixon (1991) Giribet *et al*. (1996) Halanych *et al*. (1998) Halanych *et al*. (1998) Passamaneck *et al*. (2004) Passamaneck *et al*. (2004) Giribet *et al*. (1996) Halanych *et al*. (1998)	~1801
Histone (H3)			
	H3aF (sense): 5'-ATGGCTCGTACCAAGCAGACVGC-3' H3aR (anti-sense): 5'-ATATCCTTRGGCATRATRAGTGAC-3'	Colgan *et al.* (1998) Colgan *et al.* (1998)	328
	Alternative H3 primers: H3Fm (sense): 5'-ATGGCTCGTACCAAGCAGAC-3' H3Fm1 (sense): 5'-ATGGCTAGAACGAAGCAGAC-3' H3Rm (anti-sense): 5'-TCCTTGGGCATGATGGTGAC-3' H3Rm1 (anti-sense): 5'-CCAACTGAATATCTTTGGGCAT-3'	 Fontanilla *et al*. (2017) Fontanilla *et al*. (2017) Fontanilla *et al*. (2017) Fontanilla *et al*. (2017)	331-340
Cytochrome Oxidase Subunit I (CO1)			
	LCO 1490 (sense): 5'-GGTCAACAAATCATAAAGATATTGG–3' HCO 2198 (anti-sense): 5'-TAAACTTCAGGGTGACCAAAAAATCA–3'	Folmer *et al.* (1994) Folmer *et al.* (1994)	655
	Alternative CO1 Primers: STY_LCOi (sense): 5'-TCAACGAATCATAAGGATATTGG-3' STY_LCOii (sense): 5'–ACGAATCATAAGGATATTGGTAC–3' STY_LCOiii (sense): 5'-TTTGGTATTTGATGTGGGTTAGT-3' STY_HCO (anti-sense): 5'-GAATTAAAATATATACTTCTGGGTG-3'	 Fontanilla *et al*. (2017) Fontanilla *et al*. (2017) Fontanilla *et al*. (2017) Fontanilla *et al*. (2017)	628-667

2.2.3 Agarose Gel Electrophoresis

To visualise the PCR products, the PCR products were run on an agarose gel. For the LSU rRNA gene, the whole volume of 50 µl was loaded in the gel in order to separate and extract the right band of the PCR products of this gene while for the SSU rRNA gene, H3 gene and CO1 gene, only 5 µl of 25 µl was loaded in the gel and the remaining PCR products were kept for PCR purification. The general protocol for agarose gel electrophoresis was as follows:

1- 1.5 g agarose was dissolved in 100 ml TBE buffer (54 g Tris, 27.5 g boric acid and 20 ml 0.5 EDTA dissolved in 1000 ml distilled water to make 5X TBE buffer and then diluted to 1X before use). The agarose was then heated in a microwave oven. Afterward, it was left to cool.

2- 3 µl of ethidium bromide (10 mg/ml) was added to the gel and mixed gently. The gel was then poured into a tray with combs set up to make the wells.
3- Once the gel had set, the combs were removed and the PCR products were loaded on the wells, and the gel submerged in a tank containing 1X TBE.
4- A current of 80-100 V was applied through the gel for 2 hours using a portable power supply.
5- When the PCR products had run their course through the gel, the gel was removed from the tank and placed on a UV transilluminator to visualize the bands.

2.2.4 Gel Extraction

As most of the PCR amplifications of the LSU rRNA gene produced more than one band, it was necessary to select the right band among these bands by gel electrophoresis prior to purification. QIAQuick gel extraction kit was used for gel extraction. The protocol follows the manufacturers' instructions for the QIAQuick gel extraction kit protocol.

1- PCR products were excised from the agarose gel with a clean, sharp scalpel and placed in a clean 1.5 ml microfuge tube.
2- The gel slice was weighed and three volumes of the buffer QG were added to each volume of the excised gel.
3- The gel slices were incubated at 50 ^{0}C for 10 min (or until the gel slices completely dissolved). To help dissolve the gel, tubes were inverted every 2-3 min during the incubation.
4- Once gel slices were completely dissolved, one gel volume of isopropanol was added and mixed.
5- A spin column with a membrane filter was placed in a 2 ml collection tube. Then, the sample was applied to the spin column and centrifuged at 13,000 rpm for 1 min to bind the PCR products. The flow-through was discarded and the column was placed back in the same collection tube. The maximum volume of the column reservoir is 800 µl. Therefore, the samples with volume more than 800 µl were loaded again in the same spin column until all the sample was used up.
6- To wash the samples, 750 µl of the washing buffer (Buffer PE) was added and centrifuged at 13,000 rpm for 1 min. The flow-through was discarded and the samples were centrifuged for an additional 1 min at 13,000 rpm to remove the residual ethanol from Buffer PE completely.

7- The spin column was placed into a clean 2 ml collection tube. 30 µl of Elution Buffer EB was added to the centre of the membrane filter, and the collection tube was centrifuged at 13,000 rpm for 1 min to elute the PCR products.

8- The spin column was discarded, and the DNA was stored at -20 ^{0}C until further use.

2.2.5 Purification of PCR Products

The remaining PCR products (20 µl) of the SSU rRNA gene, H3 gene and CO1 gene after the visualization in gel electrophoresis were purified using QIAquick PCR purification kit. The protocol follows the manufacturers' instructions for the QIAquick PCR purification kit protocol.

1- 5 volumes of the PB buffer were added to 1 volume of the PCR products.

2- A QIAquick spin column with a membrane filter was placed in a 2 ml collection tube. Then, the sample was applied to the spin column and centrifuged at 13,000 rpm for 30-60 seconds.

3- The flow-through was discarded and the spin column was placed back in the same collection tube.

4- 0.75 ml of PE buffer was added to wash the spin column and the spin column was centrifuged for 30–60 seconds. Then, the flow-through was discarded and the spin column was placed back in the same tube and centrifuged for an additional 1 minute to remove the residual ethanol completely.

5- The spin column was placed in a clean collection tube and 30 µl of buffer EB was added to the centre of the QIAquick membrane to elute the DNA and the spin column was centrifuged for 1 min.

2.2.6 DNA Quantification and Sequencing

PCR products were quantified using a NanoDrop® ND-1000 spectrophotometer before sending to Macrogen Inc for sequencing. Both sense and antisense strands were sequenced directly on an Applied Biosystems 3730 DNA sequencer using Big Dye terminator cycle sequencing chemistries at Macrogen Inc.

2.2.7 Transcriptome Sequencing

A novel transcriptome for *Acroloxus lacustris* was generated in this study. RNA quantity and quality were checked using the Qubit Fluorometer and the Qubit RNA BR Assay Kit (Thermo Fisher Scientific;

Q10211). The integrity of RNA was measured using the Agilent Bioanalyzer 2100 and the Agilent RNA 6000 Nano Kit (Agilent; 5067-1511). The NEBNext Poly (A) mRNA Magnetic Isolation Module (E7490) was used to purify mRNA from 0.5 μg of total RNA. Then, indexed sequencing libraries were prepared using the NEBNext Ultra Directional RNA Library Preparation Kit for Illumina (NEB; E7420) and NEBNext Multiplex Oligos for Illumina, Index Primer Set 1 (NEBNext; E7335). Libraries were quantified using the Qubit Fluorometer and the Qubit dsDNA HS Kit (ThermoFisher Scientific; Q32854). Library fragment length distributions were analysed using the Agilent Bioanalyzer 2100 and the Agilent High Sensitivity DNA Kit (Agilent; 5067-4626). Final library quantification was performed by using the KAPA Library Quantification Kit for Illumina (Roche; KK4824). The library was sequenced at Deep Seq (Centre for Genetics and Genomics at The University of Nottingham) on the Illumina NextSeq 500 using an Illumina NextSeq500 v2 Mid Output 300 cycle kit (Illumina; FC-404-2003) to generate 75 nucleotide sites paired-end reads.

2.2.8 Checking for Contamination

All precautions were taken to avoid contamination, which could happen during any step of sample preparations (DNA/RNA extraction, PCR amplification, Gel/PCR purifications and cycle sequencing). The precautions included keeping a clean working area in the lab, cleaning pipettes regularly with diluted acid (0.1 HCl) to destroy any possible DNA contamination in the pipettes and using sterile pipette tips, plastic pestles, gloves, racks, scalpel blades and reagents.

2.3 DNA Sequence Analyses

2.3.1 DNA Sequence Assembly and Alignment

The sequences of the LSU rRNA gene were assembled using the TED and XBAP programs in the STADEN package version 1.5.3 (Staden *et al*., 2000). TED was used to trim the primers at the 5' and 3' ends of the amplified sequences and XBAP was then used to assemble the trimmed sequences of the LSU rRNA gene fragments into a single contig. For the sequences of the SSU rRNA gene, H3 gene and CO1 gene, the TREV program was used to trim the primers at the 5' and 3' ends and the GAP4 program was then used to assemble the trimmed sequences into a single contig. All sequences

generated in this study were checked for contamination with Blast searches through NCBI. For the RNA cluster, each single fragment was checked before the assembly step.

After finishing the sequence assembly, the consensus sequences were brought into the Genetic Data Environment (GDE) package version 2.2 (Smith *et al*., 1994) and subsequently aligned manually. The LSU (incorporating 5.8S and ITS-2) and SSU rRNA sequences were aligned using the secondary structure as a guide. The alignment of the H3 and CO1 genes was guided by translating the DNA sequences to amino acid sequences.

Problem positions in forward and reverse strands were fixed manually within the Staden package by reference to the trace data for the sequences. Where necessary sequences were repeated to obtain better quality sequence data. However, ambiguity codes were assigned where necessary to positions for which the sequence data was contradictory or uninformative. In cases where one strand had a clear sequence and the other had a poor quality sequence, the sequence of the clear strand was used to decide the base at that position. In cases where both strands showed strong but contradictory peaks at a given position then this site was considered ambiguous and assigned to an ambiguity code. Likewise, where both strands had poor quality sequence at a given position then again, this site was assigned an appropriate ambiguity code. Codes for ambiguous sites used in this study followed Cornish-Bowden (1985) (see Table 2. 3).

Table 2.3. List of ambiguous codes for ambiguous sites in DNA sequences (Cornish-Bowden, 1985).

Base ambiguity	Code
A or C	M
A or G	R
A or T	W
C or G	S
C or T	Y
G or T	K
A or C or G (not T)	V
A or C or T (not G)	H
A or G or T (not C)	D
C or G or T (not A)	B
A or C or G or T	N

2.3.2 Phylogenetic Analyses

2.3.2.1 Choosing Unambiguously Aligned Sites

Prior to phylogenetic analyses, Gblocks v.0.91b (Castresana, 2000) with default settings was used as a guide to remove indels, poorly aligned positions and divergent regions of DNA sequences alignments.

2.3.2.2 Testing for Phylogenetic Signal

Tree length distribution skewness test was used to estimate the phylogenetic signal (Hillis & Huelsenbeck, 1992). In order to determine phylogenetic signal based on this method, a *g1* statistic was used. When the *g1* values are less (or more negative) than the critical values (values resulting from tree length distributions based on random datasets, see Table 2.4), significant phylogenetic signal will be presented (Hillis and Huelsenbeck, 1992). Critical values show very little change beyond 15 taxa, and therefore it is acceptable to use the critical value for 25 taxa for greater numbers of taxa (Hillis and Huelsenbeck, 1992). The *g1* statistic was performed in PAUP* using the randtrees command with 10,000 replicates.

Table 2.4. The critical values of Hillis & Huelsenbeck (1992) P=0.05.

Number of parsimony-informative sites	Number of Taxa							
	5	6	7	8	9	10	15	25
10	-0.95	-0.70	-0.59	-0.51	-0.44	-0.34	-0.23	-0.16
50	-0.78	-0.58	-0.45	-0.37	-0.25	-0.28	-0.19	-0.12
100	-0.66	-0.56	-0.40	-0.31	-0.25	-0.30	-0.20	-0.10
250	-0.81	-0.43	-0.39	-0.26	-0.22	-0.20	-0.16	-0.08
500	-0.73	-0.43	-0.27	-0.29	0.23	-0.16	-0.12	-0.07

2.3.2.3 Tree Construction

The maximum likelihood (ML) method for building phylogenetic trees (Felsenstein, 1981) is a character-state method that recovers the best tree with the maximum probability of observing the data using a specific model of DNA substitution. ML trees were constructed using the PhyML (version 3.0) package (Guindon *et al.*, 2010) with tree searching following a heuristic procedure with 10 random start trees and best of nearest-neighbour-interchange and subtree-pruning-regrafting branch swapping. The

general time reversible model incorporating gamma (GTR+Γ) (Lanave *et al*.,1984; Gu *et al*., 1995) was used to correct multiple substitutions for ML trees. Bootstrap resampling (Felsenstein, 1985) with 1000 replicates was undertaken to assign support to branches in the ML tree.

Bayesian inference (BI) (Larget & Simon, 1999) is similar to maximum likelihood in that it also employs likelihood values but unlike maximum likelihood, BI determines the optimal hypothesis that maximises the posterior probability (Holder & Lewis, 2003). BI analysis was undertaken using the MrBayes (version 3.1.2) package (Ronquist & Huelsenbeck, 2003). Two independent runs with four chains of a Markov Chain Monte Carlo (MCMC) algorithm were used to explore the tree space. BI analysis was conducted for 5 million generations with tree sampling every 100 generations. To ensure adequate chain swapping, a range of heating parameters were tested with the optimal parameter used to construct the final trees. Only after the Bayesian MCMC searches had reached a stationary phase (indicating convergence of the chains onto the target distribution) was the run ended. A consensus tree was built using the last 75% of trees (burnin=12501). The general time reversible model incorporating gamma (GTR+Γ) (Lanave *et al*.,1984; Gu *et al*., 1995) was used to correct multiple substitutions for BI trees.

The Neighbor-joining (NJ) method (Saitou & Nei, 1987) is an algorithmic distance tree construction method which is relatively fast and uses a matrix distances that adjusts the separation within each pair of sequence based on the average of divergence from all other sequences (Graur & Li, 2000). NJ analysis was performed using the PAUP* (version 4.0b10) package (Swofford, 2002). For NJ analysis, model parameters were estimated following an iteration process; for each tree the parameters were estimated and used to build the next tree until there was no further improvement of the likelihood score. The general time reversible model incorporating gamma (GTR+Γ) (Lanave *et al*.,1984; Gu *et al*., 1995) was used to correct multiple substitutions for NJ trees. Bootstrap resampling (Felsenstein, 1985) with 1000 replicates was undertaken for NJ trees.

In this thesis the bootstrap support values and Bayesian posterior probabilities are represented by four categories as follows:

1- Fully supported (ML=100%, NJ=100%, BI =1.00).

2- Strongly, highly, very well (ML>90%, NJ >90%, BI >0.99).

3- Well, moderate (ML>70%, NJ >70%, BI >0.90).

4- Weak, low (ML>50%, NJ >50%, BI >0.70).

Branches supported in >70% of bootstrap replicates are considered to be reliably supported based on simulation studies (Hedges, 1992; Hillis & Bull, 1993).

2.3.2.4 Optimality Test

Where phylogenetic trees do not yield expected topologies, optimality test is a useful tool to specifically test alternative hypotheses. This is particularly useful where a supposedly monophyletic group based on traditional taxonomical classifications is not resolved as a monophyletic group in inferred trees. By constraining the tree to reflect the expected topology and then comparing this tree to the inferred tree the significance of these alternative topologies can be assessed. If the optimal tree proves to be significantly better than the constrained tree, then the hypothesis being tested by the constrained tree is not robust and can be rejected. Alternative phylogenetic hypotheses were evaluated by likelihood using a Shimodaira–Hasegawa (RELL) test (Shimodaira & Hasegawa, 1999) on NJ generated topologies as implemented in PAUP*.

2.3.3 Transcriptome Sequences Analyses

2.3.3.1 Transcriptome Assembly

The transcriptome sequences and the transcriptome assembly were performed by Deep Seq staff. The first step of the transcriptome assembly was adopter trimming and read filtering. The program Skewer (Jiang *et al*., 2014) was used to trim and to remove adopter sequences and low-quality reads. Trimmed reads were then assembled by the program Trinity (Grabherr *et al*., 2011) with default settings that allow contigs with only 100 bp as a minimum length. To remove redundancy and cluster the assembled contigs, Cd-HIT (Li & Godzik, 2006) was used with 0.95 as the sequence identity threshold and 2 as the score of tolerance for redundancy. CAP3 (Huang & Madan, 1999) with default settings was then used to correct the assembled contigs and recreate new high quality contigs.

2.3.3.2 Additional Transcriptomes

In addition to **t**he novel transcriptome sequence of *Acroloxus lacustris*, transcriptome shotgun assemblies (TSAs) of *Biomphalaria pfeifferi* (GFMW01000001.1-GFMW01186204.1) (Buddenborg *et al*., 2018), *Lymnaea stagnalis* (FX180119.1- FX296473.1) (Sadamoto *et al*., 2012) and *Cepaea nemoralis* (GFLU01000001.1- GFLU01147386.1) (Kerkvliet *et al*., 2017) were downloaded from Genbank. Transcriptome assembly of *Physa acuta* which is published in Romiguier *et al*. (2014) was sent by Jonathan Romiguier (personal communication).

2.3.3.3 Finding Orthologs

The first step in ortholog identification is to determine the homology relationships among sequences. It is also essential to distinguish between gene copies that either originated from a common ancestor through a speciation event (orthologs) or originated through duplication event (paralogs). Only ortholog genes provide information on the evolutionary relationships of species, thus, the identification of ortholog genes is vital for any comparative genomic analysis (Nichio *et al*., 2017).

In this study, orthologous genes were identified in two datasets. The first dataset included four hygrophilan taxa (*Acroloxus lacustris*, *Biomphalaria pfeifferi*, *Lymnaea stagnalis* and *Physa acuta*), while the second dataset included the four hygrophilan taxa plus *Cepaea nemoralis*. Prior to ortholog identification, transcriptome sequences of all taxa were filtered by length using SeqKit Version: 0.10.1 to only allow contigs greater than 100 bp (Shen *et al*., 2016). The nucleotide sequences of all transcriptomes were then translated to amino acids using TransDecoder Release v5.5.0 (https://github.com/TransDecoder/TransDecoder). This program was run using the default settings which identifies open reading frames (ORFs) that are at least 100 amino acids long. Orthologous sequences were then identified among the translated genes using PorthoMCL (Tabari & Su, 2017) with default settings. Orthology prediction by PorthoMCL is similar to that of the more familiar OrthoMCL (Li *et al*., 2003) with both methods based on reciprocal best hits in 'All-Against-All BLAST' and Markov Clustering algorithm (MCL), but PorthoMCL is more efficient, ultrafast and highly scalable (Tabari & Su, 2017).

Ortholog prediction by PorthoMCL requires four programs (Perl 5.x, Python 2.x, NCBI Blast and MCL) and was conducted using the following steps.

1- Input protein sequences were prepared in Fasta format with protein identifiers.
2- Input protein sequences were then filtered based on minimum allowed protein lengths (25 amino acids) and maximum percent of stop codons (20%). Two files were produced: a good protein fasta file that includes the good quality sequences and a poor protein fasta file that includes the poor quality sequences.
3- A blast database was then created from the good protein Fasta file using 'makeblastdb'.
4- Parallel 'All-Against-All BLAST' searches were then implemented by undertaking individual-against-all BLAST searches for every transcriptome independently.
5- Blast results were then converted from NCBI BLAST tabular output format into a format that can be loaded into the orthomcl database using 'porthomclBlastParser'.
6- Best hits for all transcriptomes were then identified by parallel scanning of the individual-against-all BLAST. This step produced a single best-hit file for each transcriptome, which was later used to find orthologs and paralogs.
7- Orthologs were then identified using 'porthomclPairsOrthologs.py' and the outputs of this step for all transcriptomes were then merged together and fed to MCL.
8- Paralogs were then identified by normalising the scores calculated in step 7 using 'porthomclPairsInParalogs.py'. The output of this step is all the paralogs genes with normalized scores.
9- The final outputs of steps 8 and 9 were then assembled to construct a sequence similarity graph that was then cut by the MCL program to identify orthologous and paralogous gene groups.

The workflow of PorthoMCL is shown in Figure 2.3, with the final output of the MCL program two tabular text files, one that groups all the orthologous genes and one that groups all the paralogous genes. The Mcl output file that groups all the orthologous genes was converted to a text file (groups.txt) in which each orthologous group was given a unique id number using 'orthomclMclToGroups'. The custom Perl script 'CopyNumberGen.sh' (https://github.com/halexand/Ehux_HD/tree/master/orthoMCL_output) was then used to produce a

summary table including the number of genes that are present in each transcriptome for each ortholog group from groups.txt Likewise, the custom Perl script 'ExtractSCOs.sh' (https://github.com/halexand/Ehux_HD/tree/master/orthoMCL_output) was used to produce a table showing all the ortholog groups that have only one copy gene in every species from groups.txt. Seqkit (https://github.com/lh3/seqtk/blob/master/README.md) was then used to extract the sequences of the orthologous genes from the good protein fasta file (a file produced in step two of PorthoMCL protocol) and assigning each ortholog group to a single fasta file.

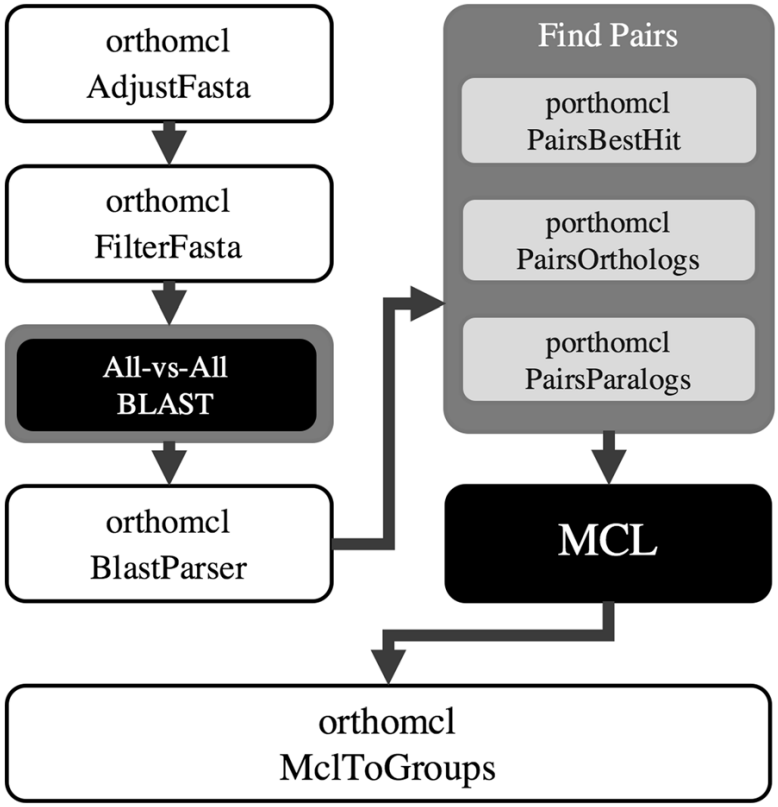


Figure 2.3. Flowchart of Porthomcl steps for orthology finding (Tabari & Su, 2017)

2.3.3.4 Phylogenetic Analyses

Amino acid sequences were aligned using MUSCLE v3.8.1551 (Edgar, 2004) with default settings. The alignments of all ortholog groups were then run through Gblocks v.0.91b (Castresana, 2000) with default settings to remove poorly aligned positions and divergent regions of amino acid alignments. Finally, trimmed alignments were filtered by length excluding any sequence shorter than 25 amino acids following Kocot *et al*. (2011).

Individual four taxon trees based on amino acid sequences of orthologous genes that were identified in the first dataset (four hygrophilan taxa) were constructed manually using ML. To correct for multiple substitutions, the best-fitting model was selected for each individual orthologous gene using Smart Model Selection (SMS) (Lefort *et al*., 2017). Bootstrap resampling (Felsenstein, 1985) with 1000 replicates was undertaken for all trees. Additionally, BI and ML trees were constructed based on a concatenated supermatrix of amino acid sequences of all orthologous genes identified in the first dataset (four hygrophilan taxa). The custom Perl script "catfasta2phyml.pl'' (https://github.com/nylander/catfasta2phyml/) was used to concatenate Fasta files. Once again to correct for multiple substitutions, the best-fitting model was selected using Smart Model Selection (SMS) (Lefort *et al*., 2017). Bootstrap resampling (Felsenstein, 1985) with 1000 replicates was undertaken for the ML tree. BI analysis was conducted for 5 million generations with tree sampling every 100 generations. A consensus tree was built using the last 75% of trees (burnin=12501).

In addition to the four taxon trees of the Hygrophila, BI and ML trees were constructed based on amino acid sequences of a concatenated supermatrix of all orthologs that were identified in the second dataset (comprising four hygrophilan taxa plus *Cepaea nemoralis*). To correct multiple substitutions for amino acid sequences of this supermatrix, the best-fitting model was selected using Smart Model Selection (SMS) (Lefort *et al*., 2017). Bootstrap resampling (Felsenstein, 1985) with 1000 replicates was undertaken for the ML tree. BI analysis was conducted for 5 million generations with tree sampling every 100 generations. A consensus tree was built using the last 75% of trees (burnin=12501).

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Chapter 3: Resolving the Basal Divisions in the Stylommatophoran Land Snails and Slugs with Special Emphasis on the Position of the Scolodontidae[1]

Abstract

The deep divisions at the base of the stylommatophoran land snails and slugs have proved to be controversial, with the phylogenetic position of the Scolodontidae remaining unresolved. Here we present a phylogenetic analysis of 34 stylommatophoran genera based on a combined dataset (5782 sites) of four loci with the aim of resolving the position of the Scolodontidae and their relationship to the 'achatinoid' and 'non-achatinoid' clades. We also evaluate the phylogenetic utility of different genes and gene partitions. The deep phylogenetic relationships within the Stylommatophora are now clearly resolved. The Scolodontidae are shown categorically to be the sister group to all other stylommatophoran groups with robust support and with all phylogeny reconstruction methods. The 'achatinoid' and 'non-achatinoid' clades are also strongly supported in the tree. The original LSU 1-5 fragment used extensively in studies of the Stylommatophora was found to be the most informative gene fragment and works well at resolving relationships at most levels in the Stylommatophora but does not reliably resolve the deep level relationships at the very base of the clade. The concatenated dataset of four genes employed in this study is not only informative at lower levels but also resolves the deep level relationships at the base of the Stylommatophora with robust support.

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3.1 Introduction

The stylommatophoran land snails and slugs are the largest group within the pulmonate gastropods, comprising approximately 30,000-35,000 species (Solem, 1984). They are the major group of terrestrial gastropods and are found in a diverse range of habitats (Mordan & Wade, 2008). Stylommatophoran taxa are characterised morphologically by the absence of an operculum and generally by two pairs of tentacles on the head (Barker, 2001; Dayrat & Tillier, 2002). The morphological complexities of the reproductive organs have proved to be valuable for recognizing species and genera in many stylommatophoran groups (Nordsieck, 1985; Tillier, 1989; Barker, 2001; Schileyko, 2003, 2004; Mead, 2004; Herbert & Mitchell, 2008; Sutcharit *et al*., 2010). The Stylommatophora were originally divided into three primary divisions by Pilsbry (1900), the Orthurethra, the Heterurethra and the Sigmurethra, based on the anatomy of the excretory system, with a fourth division, the Mesurethra, added by Baker (1955). Of these, only the Orthurethra are still universally recognized. Tillier (1989) proposed a three-way division of the Stylommatophora into Orthurethra, Brachynephra and Dolichonephra based on differences within their renal organ. Nordsieck (1992) produced an alternative taxonomy in which he divided the Stylommatophora into two clades, the Orthurethra and the Sigmurethra, based on the excretory system. Bouchet & Rocroi (2005) divided the Stylommatophora into the Elasmognatha, the Orthurethra and the Sigmurethra, based on the morphological and molecular data available at the time. In the most recent taxonomy (Bouchet *et al*., 2017), the Stylommatophora have been divided into three suborders; the Achatinina ('achatinoid clade'), Helicina ('non-achatinoid clade') and Scolodontina.

Molecular studies have significantly enhanced our recent understanding of stylommatophoran relationships. Most of these studies have focused on the phylogenetic relationships of a particular family (Goodacre & Wade, 2001; Holland & Hadfield, 2004; Sutcharit *et al*., 2010; Herbert *et al*., 2015; Moussalli & Herbert, 2016) or within a particular superfamily (Armbruster *et al.*, 2005; Wade *et al*., 2007; Breure *et al*., 2010; Köhler & Criscione, 2015; Razkin *et al*., 2015; Fontanilla *et al*., 2017). A handful of molecular studies (Wade *et al*., 2001, 2006; Ramirez *et al*., 2012; Lin *et al*., 2016; Teasdale *et al*., 2016) have focussed on the phylogenetic relationships of the Stylommatophora as a whole.

The most comprehensive molecular studies of the Stylommatophora were performed by Wade *et al*. (2001, 2006), based on partial sequences from the ribosomal RNA (rRNA) gene-cluster. Their analyses defined two groups within the Stylommatophora, the 'achatinoid' and the 'non-achatinoid' clades. These clades are now well accepted and have been adopted in the most recent taxonomy of the Stylommatophora by Bouchet *et al*. (2017).

The Scolodontidae (Baker, 1925), previously known as the Systrophiidae, has a complex taxonomic history; the placement of the family in stylommatophoran taxonomies has proven to be problematic due to the small size and relatively simple shells of snails within the family (Hausdorf, 2006). Ramírez *et al*. (2012) undertook a molecular phylogeny of the Scolodontidae in which they placed the Scolodontidae within the stylommatophoran phylogeny of Wade *et al*. (2006). Their phylogenetic analyses were inconsistent in the placement of the Scolodontidae. In their neighbor-joining analysis, the Scolodontidae fell at the very base of the Stylommatophora and outside of both the 'achatinoid' and 'non-achatinoid' clades. However, in their Bayesian analysis, the Scolodontidae fell between the 'achatinoid' and 'non-achatinoid' clades as the sister taxon to the 'non-achatinoid' clade. Bouchet *et al*. (2017) placed the Scolodontidae in a new suborder, Scolodontina, alongside the suborder Achatinina ('achatinoid clade') and the suborder Helicina ('non-achatinoid clade').

More recently, Teasdale *et al*. (2016) undertook a phylogenomic analysis of the Stylommatophora. Their study provided support for the monophyly of the Helicoidea, Limacoidea, Orthurethra and Rhytidoidea but did not address the basal divisions within the Stylommatophora as it did not include any representatives of either the 'achatinoid' clade or the Scolodontidae. Lin *et al*. (2016) included a single 'achatinoid' taxon, *Achatina fulica*, in their phylogenetic tree of the Stylommatophora based on 13 mitochondrial genes. The placement of *Achatina* in their tree is consistent with the Wade *et al* (2001, 2006) split of the Stylommatophora into 'achatinoid' and 'non-achatinoid' clades. They did not include the Scolodontidae.

The present study aims to resolve the basal divisions within the Stylommatophora by examining the position of the Scolodontidae and their relationship to the 'achatinoid' and 'non-achatinoid' clades.

Phylogenetic analyses are based on four molecular markers: almost the full-length large subunit (LSU) rRNA gene (also incorporating part of the 5.8S gene and the complete ITS-2 region), almost the full-length small subunit (SSU) rRNA gene, part of the histone three (H3) gene and part of the cytochrome oxidase subunit I (COI) gene. These represent a seven-fold increase in sequence data over the previous molecular phylogenetic studies of the Stylommatophora by Wade *et al*. (2006) and Ramírez *et al*. (2012). Additionally, we examine the phylogenetic utility of these genes by exploring and comparing the phylogenetic signals carried by the different genes.

3.2 Methods

3.2.1 Biological Material

A total of 34 genera of stylommatophoran land snails and slugs belonging to 33 families were included in this study. Four genera of non-stylommatophoran pulmonates were also included. *Siphonaria pectinata* was used as an outgroup to root the phylogenetic trees. Details of the specimens, sampling localities and the collectors are given in Table 3.1.

Table 3.1. Details of specimens, sampling localities and collectors. Classification of samples follows Bouchet *et al*. (2017).

Classification	Species	Collection Location	Collector
Order Stylommatophora			
Suborder Achatinina ['Achatinoid Clade']			
Superfamily Achatinoidea			
Family Ferussaciidae	*Ferussacia folliculus* (Gmelin, 1791)	Los Alcornales, Prov Cadiz, Spain	M. Seddon
Family Subulinidae	*Subulina striatella* (Rang, 1831)	Kew Gardens (introduced)	F. Naggs
Family Glessulidae	*Glessula ceylanica* (Pfeiffer, 1845)	Colombo, Sri Lanka	P. Karunaratne
Family Achatinidae	*Lissachatina fulica* (Bowdich, 1822) [= *Achatina fulica*]	Unknown (Zool. Soc. Lond. Colln.)	P. Pearce-Kelly
Family Coeliaxidae	*Coeliaxis blandii* (Pfeiffer, 1852)	New Bradford, South Africa	N. Smith
Family Thyrophorellidae	*Thyrophorella thomensis* (Greeff, 1882)	Zampala, São Thomé, West Africa	A. Gascoigne
Superfamily Streptaxoidea			
Family Streptaxidae	*Gonaxis quadrilateralis* (Preston, 1910)	Reunion	O. Griffiths
Suborder Helicina ['Non-Achatinoid Clade']			
Infraorder Pupilloidei [Orthurethra] **Superfamily Pupilloidea**			
Family Cochlicopidae	*Cochlicopa lubrica* (O. F. Müller, 1774)	Box Hill, Dorking, UK	P. Mordan & E. Platts
Family Valloniidae	*Vallonia excentrica* Sterki, 1892	São Miguel, Azores	P. Mordan
Family Chondrinidae	*Chondrina clienta* (Westerlund, 1883)	Villach, Austria	P. Miltner

Family Enidae	*Buliminus labrosus* (Olivier, 1804)	Saladin's Castle, Syria	P. Mordan
Infraorder Clausilioidei **Superfamily Clausilioidea**			
Family Clausiliidae	*Albinaria xanthostoma* (Boettger, 1883)	Crete	D. Thomaz
Infraorder Orthalicoidei **Superfamily Orthalicoidea**			
Family Orthalicidae	*Drymaeus discrepans* (Sowerby, 1833)	Guatemala	Unknown
Family Amphibulimidae	*Gaeotis nigrolineata* (Shuttleworth, 1854)	El Yunque, Puerto Rico	Unknown
Infraorder Oleacinoidei **Superfamily Oleacinoidea**			
Family Spiraxidae	*Euglandina rosea* (Férussac, 1821)	Moorea (Zool. Soc. Lond. Colln.)	P. Pearce-Kelly
Superfamily Haplotrematoidea			
Family Haplotrematidae	*Haplotrema vancouverense* (Lea, 1839)	Eugene, Oregon, USA	D. Taylor
Infraorder Rhytidoidei **Superfamily Rhytidoidea**			
Family Rhytididae	*Rhytida stephenensis* (Powell, 1930)	Manaaki Whenua, New Zealand	D. Gleeson
Family Megalobulimidae = Strophocheilidae	*Megalobulimus oblongus* (O. F. Müller, 1774)	Antigua (Zool. Soc. Lond. colln.)	P. Pearce-Kelly
Family Dorcasiidae	*Dorcasia alexandri* (Gray, 1938)	Windhoek, Namibia	C. Boix-Hinzen
Family Caryodidae	*Caryodes dufresnii* (Leach, 1815)	Mt Wellington, Hobart, Tasmania	B. Smith
Infraorder Limacoidei ['Limacoid Clade'] **Superfamily Trochomorphoidea**			
Family Euconulidae	*Euconulus fulvus* (O. F. Müller, 1774)	New Forest, Hampshire, UK	P. Mordan
Superfamily Helicarionoidea			
Family Ariophantidae	*Cryptozona bistrialis* (Beck, 1837)	Sri Lanka	P. Karunaratne
Superfamily Limacoidea			
Family Vitrinidae	*Vitrina pellucida* (O. F. Müller, 1774)	Kirkdale, Derbyshire, UK	C. Wade
Superfamily Gastrodontoidea			
Family Oxychilidae	*Oxychilus alliarius* (Miller, 1822)	Deepdale, Derbyshire, UK	C. Wade
Infraorder Helicoidei ['Helicoid Clade'] **Superfamily Helicoidea**			
Family Camaenidae	*Satsuma japonica* (Pfeiffer, 1847)	Osaka City, Japan	P. Callomon
Family Hygromiidae	*Trochulus striolatus* (Pfeiffer, 1828)	Deepdale, Derbyshire, UK	C. Wade
Family Bradybaenidae =Camaenidae	*Bradybaena similaris* (Férussac, 1821)	Sri Lanka	P. Karunaratne
Family Helminthoglyptidae =Xanthonychidae.	*Monadenia fidelis* (Gray, 1834)	Oregon	D. Taylor
Infraorder Arionoidei **Superfamily Arionoidea**			
Family Arionidae	*Arion hortensis* (Férussac, 1819)	Kirkdale, Derbyshire, UK	C. Wade
Family Philomycidae	*Meghimatium bilineatum* (Benson, 1842)	Mauritius	O. Griffiths
Taxa of uncertain Position **Superfamily Testacelloidea**			
Family Testacellidae	*Testacella scutulum* (Sowerby, 1821)	North London, UK	R. Hurst
Superfamily Plectopyloidea			
Family Corillidae	*Corilla adamsi* (Gude, 1914)	Sri Lanka	D. Raheem
Suborder Scolodontina **Superfamily Scolodontoidea**			
Family Scolodontidae =Systrophiidae	*Guestieria sp.* Crosse 1872	Ecuador	Hilary Kingston
	Systrophia sp. Pfeiffer 1855	Ecuador	Hilary Kingston
Non-Stylommatophoran Pulmonates: **Order Ellobiida** **Superfamily Ellobioidea**			
Family Ellobiidae	*Laemodonta sp.*	Suralaya, W. Java	B. Dharma

Family Carychiidae	*Carychium tridentatum* (Risso, 1826)	Abelheira, São Miguel, Azores	P. Mordan
Suborder Systellomatophora **Superfamily Veronicelloidea**			
Family Veronicellidae	*Laevicaulis alte* (Férussac, 1823)	Dubai, United Arab Emirates	A. Green
Order Siphonariida **Superfamily Siphonarioidea**			
Family Siphonariidae	*Siphonaria pectinata* (Linnaeus, 1758)	Zamara Los Atunes, Spain	S. Hawkins

3.2.2 DNA Extraction, PCR Amplification and Sequencing

DNA was extracted from a small (1-2 mm^3) tissue sample taken from the foot of the snail using a CTAB DNA extraction protocol (Goodacre & Wade, 2001). Four molecular markers were PCR amplified including three nuclear markers: approximately 4000 nucleotides of the LSU rRNA gene (including part of the 5.8S gene, the complete ITS-2 region, and almost the full-length large subunit (LSU; 28S) gene), approximately 1800 nucleotides of the small subunit (SSU, 18S) rRNA gene, approximately 370 nucleotides of the histone 3 (H3) gene; and one mitochondrial marker: approximately 650 nucleotides of cytochrome oxidase subunit I (COI).

The LSU rRNA gene (also incorporating part of the 5.8S gene and ITS-2), was amplified in a nested PCR reaction using primers LSU-1ii and LSU-12 in the first round, with the primary PCR products then used as template for the secondary PCR to amplify six internal fragments (A, B, C, D, E, and F) (see Table 2.2 for details of primers). PCR amplification for the primary PCR was performed using the Qiagen Taq DNA polymerase and Q buffer system (1X buffer, 1X Q-solution, 0.3 mM dNTP, 1.5 mM magnesium chloride, 0.2 μM each primer and 1U Taq in a 50 μL final volume). Secondary PCR amplification was identical to the primary PCR with the exception that a lower 0.2 mM concentration of dNTPs was used. The cycling conditions (with a Perkin Elmer cycler) of the primary PCR were as follows: 96 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 50 °C for 30 sec, 72 °C for 3 min and then a final extension step at 72 °C for 5 min. The secondary PCR cycle conditions were as follows: 96 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 45 °C for 1 min, 72 °C for 2 min and then a final extension step at 72 °C for 5 min.

Similarly, the SSU rRNA gene was amplified in a nested PCR with primers 18e and 18p used for the first round and with the primary PCR products then used as template to amplify a single fragment with

primers 1F and 9R. A series of 6 internal primers were used to sequence this fragment (see Table 2.2 for details of primers). Some samples proved difficult to amplify as a single fragment and in these cases the internal primers were used to amplify the SSU gene. Amplification was performed using BIOTAQ^{TM} DNA polymerase (1X reaction buffer, 0.2 mM dNTPs, 2.5 mM magnesium chloride, 0.2 µM each primer and 1U Taq in a 25 µL final volume). The PCR cycling conditions were as follows: 96 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 50 °C for 30 sec, 72 °C for 90 sec and then a final extension step of at 72 °C for 5 min for both primary and secondary PCR.

The H3 gene was amplified as a single fragment using primers H3aF and H3aR as default primers, though different combinations of primers were used with some samples where they did not amplify with the default primers (see Table 2.2 for a complete list of primers). The CO1 gene was also amplified as a single fragment using primers LCO 1490 and HCO 2198 for the majority of samples. A few samples did not amplify with these primers thus different combinations of primer were used (see Table 2.2 for a complete list of primers). The PCR amplification of the H3 and CO1 genes were performed using BIOTAQ^{TM} DNA polymerase (1X reaction buffer, 0.2 mM dNTPs, 2.5 mM magnesium chloride, 0.2 µM each primer and 1U Taq in a 25 µL final volume). The thermal cycling conditions were as follows: 96 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 50 °C for 30 sec, 72 °C for 3 min and then a final extension step at 72 °C for 5 min.

Amplification products were purified using either a Qiagen gel extraction kit or Qiagen PCR purification kit. Sense and antisense strands were sequenced directly on an Applied Biosystems 3730 DNA sequencer using Big Dye terminator cycle sequencing chemistries at Macrogen Inc.

3.2.3 Sequence Processing and Phylogenetic Analyses

DNA sequences were assembled using version 1.5.3 of the STADEN package (Staden *et al*., 2000). Subsequently, the sequences were manually aligned using version 2.2 of the Genetic Data Environment (GDE) package (Smith *et al*., 1994). The LSU (incorporating 5.8S and ITS-2) and SSU rRNA sequences were aligned using the secondary structure as a guide. The alignment of the H3 and CO1 genes was guided by translating the DNA sequences to amino acid sequences. Gblocks v.0.91b

(Castresana, 2000) with default settings was used as a guide to select the reliably aligned sites from the alignments of all genes.

Phylogenetic trees were constructed using maximum likelihood (ML) (Felsenstein, 1981), neighbor-joining (NJ) (Saitou & Nei, 1987) and Bayesian inference (BI) (Larget & Simon, 1999). The general time reversible model incorporating gamma (GTR+Γ) (Lanave *et al*., 1984; Gu *et al*., 1995) was used to correct for multiple substitutions. ML trees were constructed using the PhyML (version 3.0) package (Guindon *et al*., 2010) with tree searching following a heuristic procedure with 10 random start trees and best of nearest-neighbour-interchange and subtree-pruning-regrafting branch swapping. NJ analysis was performed using the PAUP* (version 4.0b10) package (Swofford, 2002). For NJ analysis, model parameters were estimated following an iteration process; for each tree the parameters were estimated and used to build the next tree until there was no further improvement of the likelihood score. Bootstrap resampling (Felsenstein, 1985) with 1000 replicates was undertaken for ML and NJ trees. BI analysis was undertaken using the MrBayes (version 3.1.2) package (Ronquist & Huelsenbeck, 2003). Two independent runs with four chains of a Markov Chain Monte Carlo (MCMC) algorithm were used to explore the tree space. BI analysis was conducted for 5 million generations with tree sampling every 100 generations. To ensure adequate chain swapping, a range of heating parameters were tested with the optimal parameter used to construct the final trees. Only after the Bayesian MCMC searches had reached a stationary phase (indicating convergence of the chains onto the target distribution) was the run ended. A consensus tree was built using the last 75% of trees (burnin=12501).

3.2.4 Assessing the Phylogenetic Utility of Different Genes

Three methods were used to explore the utility of each gene/ gene partition for phylogeny reconstruction: 1) Phylogenetic signal was measured using the tree length distribution skewness test (*g1* statistic) (Hillis & Huelsenbeck, 1992) implemented in PAUP* using the randtrees command with 10,000 replicates. 2) Total number of variable sites was determined in PAUP*. 3) Bootstrap support for key nodes in the phylogenetic tree was measured with maximum likelihood bootstrap support evaluated for the following clades: Stylommatophora monophyly (A), principle split of the stylommatophoran

tree between Scolodontidae and the remaining groups (B), 'achatinoid clade' (C), 'non-achatinoid clade' (D), Pupilloidei [=Orthurethra] (E), Limacoidei (F), Arionoidei (G), Orthalicoidei (H), Helicoidei (I) and Scolodontidae (J). Comparing phylogenetic signal based on nodal support has been used in several phylogenetic studies (Narechania *et al*., 2011; Botero-Castro *et al*., 2013; Sharma *et al*., 2014; López-Rubio *et al*., 2017).

3.3 Nucleotide Sequence Accession Numbers

Nucleotide sequences generated in this study have been given the GenBank accession numbers MN022656-MN022782. Full details of all sequence accessions are given in Appendix 3.1.

3.4 Results

Sequences of four genes: approximately 4000 nucleotides of the LSU rRNA gene (also incorporating approximately 80 nucleotides of the 5.8S and the complete ITS-2), approximately 1800 nucleotides of the SSU rRNA gene, approximately 370 nucleotides of the H3 gene and approximately 650 nucleotides of the CO1 gene have been generated for 34 stylommatophoran genera and 4 non-stylommatophoran genera.

3.4.1 Phylogenetic Analysis

Phylogenetic trees for the Stylommatophora were constructed using a concatenated dataset of 5782 unambiguously aligned nucleotide sites. This dataset consists of 3290 nucleotides of the LSU (and 5.8S) gene, 1748 nucleotides of the SSU gene, 328 nucleotides of the H3 gene and 416 nucleotides of the 1st and 2nd codon positions of the CO1 gene. The siphonariid *Siphonaria pectinata* was used as an outgroup to root the phylogenetic trees. The ITS-2 region of the rRNA cluster was removed completely from all analyses as it could not be aligned across all taxa due to its high variability. Likewise, the third codon positions of the CO1 gene were excluded from analyses due to saturation at these positions.

The Bayesian inference tree of the Stylommatophora based on the concatenated dataset is shown in Figure 3.1. Highly consistent topologies were observed with all phylogeny reconstruction methods used (BI, Figure 3.1; ML, Appendix 3.2; NJ, Appendix 3.3). The Stylommatophora are supported as a

monophyletic group in 100% ML, 99% NJ bootstrap replicates and with a Bayesian posterior probability of 1.00. The phylogenetic tree shows a principle division between the Scolodontidae on the one hand and all other stylommatophoran groups on the other. This division is strongly supported (93% ML, 75% NJ bootstraps and PP=1.00 BI) and with all phylogenetic analysis methods (Appendix 3.4). We also find an identical phylogenetic position for the Scolodontidae when all partial Scolodontidae sequences available on Genbank (from Ramirez *et al*., 2012) are included (Appendix 3.5). The monophyly of the Scolodontidae is also robustly supported (100% ML, 98% NJ bootstraps and PP=1.00 BI). Among the remaining stylommatophoran groups, the 'achatinoid' and the 'non-achatinoid' clades are clearly resolved with high support (100% ML, 92% NJ bootstraps and PP=1.00 BI for the 'achatinoid clade' and 82% ML, 93% NJ bootstraps and PP=1.00 BI for the 'non-achatinoid clade'). In the 'achatinoid clade' there is a basal dichotomy between the Streptaxoidea on the one hand and the Achatinoidea on the other (92% ML, 78% NJ bootstraps and PP=1.00 BI). The internal relationships within the Achatinoidea are not clear except for the position of the Ferussaciidae which is well resolved in the tree with 92% ML, 92% NJ bootstraps and PP=1.00 BI. Within the 'non-achatinoid clade' the Pupilloidei [=Orthurethra] are very well supported as a monophyletic group (92% ML, 97% NJ bootstraps, PP=1.00 BI). The Arionoidei and the Limacoidei are fully supported (100% ML, 100% NJ bootstraps, PP=1.00 BI) as monophyletic groups. The Arionoidei has a sister group relationship with the Limacoidei, though support for this is equivocal (66% ML bootstraps and PP=1.00 BI). The monophyly of the Orthalicoidei is very well supported (94% ML, 93% NJ bootstraps, PP=1.00 BI). The Helicoidei form a clear monophyletic group in the tree, fully supported with 100% ML, 100% NJ bootstraps and PP=1.00 BI. The Haplotrematoidea and Oleacinoidea cluster with the Helicoidea with strong support (87% ML, 96% NJ bootstraps and PP=0.99 BI and 84% ML, 84% NJ bootstraps and PP=1.00 BI respectively). The Rhytidoidea are not clearly resolved in the tree but there is weak support (53% ML bootstraps and PP=1.00 BI) for a group including the Caryodidae, Dorcasiidae and Rhytididae. Finally, the Pupilloidei, Arionoidei, Limacoidei, Plectopyloidea, Rhytidoidei, Testacelloidei and Clausiloidei seem to split from other 'non-achatinoid' taxa with moderate support (70% ML, 60% NJ bootstraps and PP=0.96 BI).

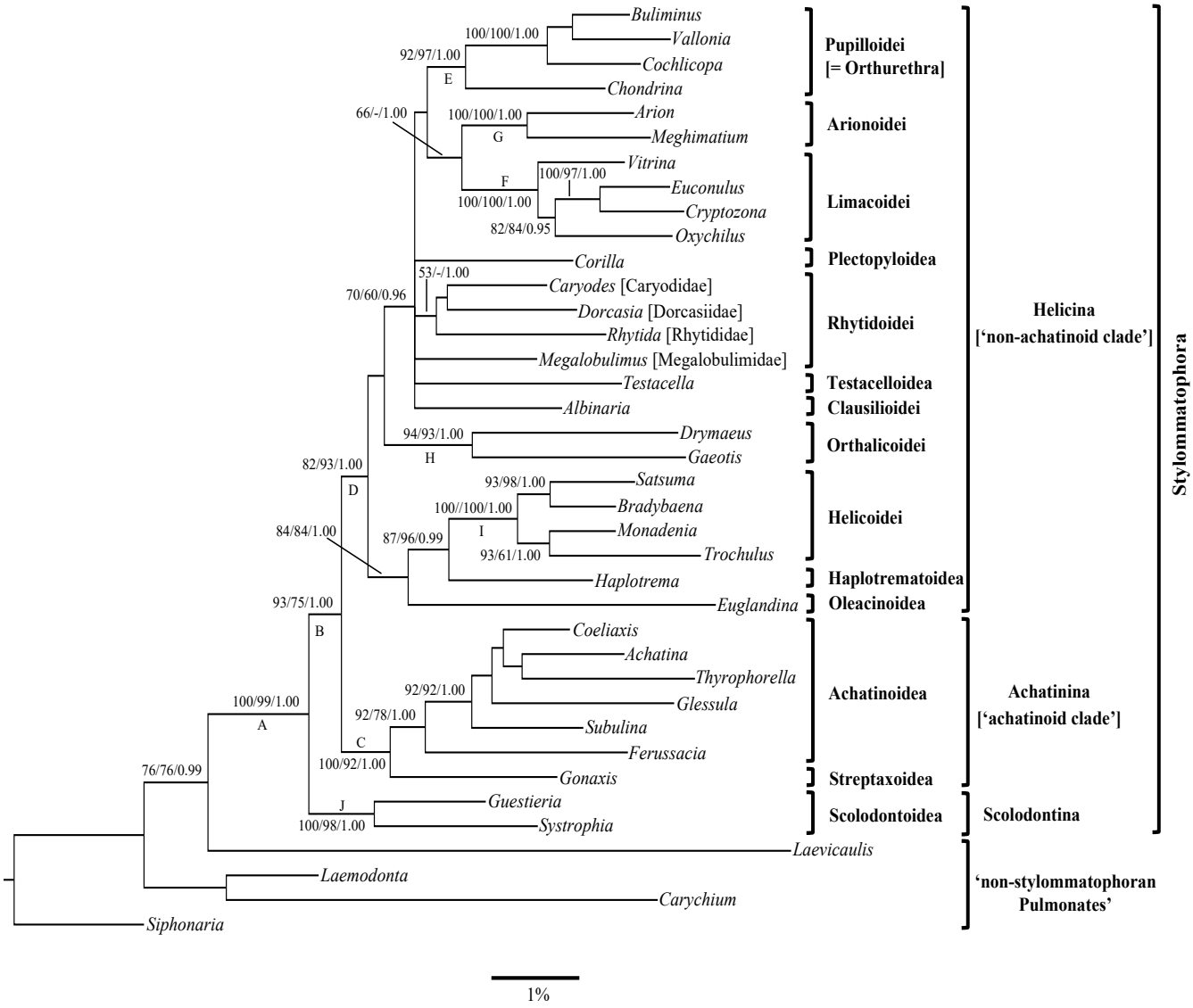


Figure 3.1. Bayesian phylogenetic tree of the Stylommatophora based on concatenated sequences of 5782 unambiguously aligned nucleotides from the combined dataset of the LSU (and 5.8S) gene, the SSU gene, the H3 gene and the 1st and 2nd codon positions of the CO1 gene. Values on the nodes represent bootstrap support (1000 replicates) for ML, NJ and posterior probabilities for BI (based on last 75% of trees), respectively. Bootstrap support values less than 50% and posterior probabilities less than 0.7 are not shown. The scale bar represents 1 substitutional change per 100 nucleotide positions. Nodes used to assess nodal support in analyses of the phylogenetic utility of different genes/ gene partitions are shown as letters on the corresponding nodes (A-J). Infraorder rank was used to label the phylogenetic tree for most groups. However, superfamily rank was also used with some groups if they were not assigned to infraorders or they belonged to non-monophyletic infraorders.

3.4.2 Phylogenetic Utility of Different Genes and Partitions

The phylogenetic utility of the different genes used in this study was evaluated to assess their usefulness for inferring stylommatophoran phylogeny. All four genes used in this study, the LSU (also incorporating the 5.8S), SSU, H3 and 1st and 2nd codon positions of CO1, as well as the concatenated dataset of all four genes were evaluated. Additionally, for the LSU gene, the utility of the full-length fragment (LSU 1-12) was compared with that of the original LSU 1-5 fragment used by Wade *et al*. (2001, 2006).

The values of the *g1* statistic for all genes and gene partitions (LSU 1-5, full-length LSU, SSU, H3, 1st and 2nd codon positions of CO1) as well as the concatenated dataset of all four genes were considerably lower than the corresponding critical values of Hillis & Huelsenbeck (1992) indicating the presence of strong phylogenetic signal (Table 3.2).

Examining the number of variable sites (Table 3.2) revealed that within the LSU gene, the majority of the variable sites (236 sites) came from the original LSU 1-5 fragment. Expanding the sequence length of the LSU fourfold, only brought in an additional 176 sites. The SSU gene brought in an additional 140 variable sites, with 132 variable sites coming from the H3 gene and 122 variable sites from the CO1 gene (1st and 2nd codon positions).

Based on the nodal support for clades in the phylogenetic tree (Table 3.2), the full-length LSU gene resolved all ten stylommatophoran clades under focus. The LSU 1-5 fragment resolved nine of the ten clades, albeit with slightly lower support for some clades. Of the other genes, only two of ten clades were resolved using the SSU, three of ten clades were resolved using H3 and two of ten clades were resolved using the 1st and 2nd codon positions of CO1. When all four genes were combined, all ten stylommatophoran clades were recovered using the concatenated dataset, with a significant increase in bootstrap support for the deep nodes within the Stylommatophora when compared to individual genes.

Table 3.2. *g1* statistic, total number of variable sites, and bootstrap support for nodes in the stylommatophoran phylogeny for each gene/ gene partition. Nodal support values were obtained for ten clades in the stylommatophoran tree; clade letters correspond to (A) Stylommatophora monophyly, (B)

principle split of the stylommatophoran tree between the Scolodontidae and all other stylommatophoran groups, (C) 'achatinoid clade', (D) 'non-achatinoid clade', (E) Pupilloidei [=Orthurethra], (F) Limacoidei, (G) Arionoidei, (H) Orthalicoidei, (I) Helicoidei and (J) Scolodontidae (clades shown on Figure 3.1). Where a clade is resolved the bootstrap support value is shown in the table. Clades not recovered using the gene under focus are shown as dashes (-). For the *g1* statistic, a critical value of -0.10 at P=0.05 level of significance was used for the LSU 1-5 fragment, SSU, H3 and CO1 (the 1st and the 2nd codon positions). For the full-length LSU gene a critical value of -0.08 at P=0.05 was used. For the combined dataset of the four genes, a critical value of -0.07 at P= 0.05 was used. Critical values are dependent on the number of taxa and the total number of variable sites (critical values from Hillis & Huelsenbeck, 1992).

Genes/ Gene Partitions	**Sequence Length**	***g1* Statistic**	**Variable Sites**	**Bootstrap Support For Clades (%)**									
				A	B	C	D	E	F	G	H	I	J
LSU1-5 fragment	800	-0.69	236	94	-	90	64	73	90	41	82	100	97
LSU full-length	3290	-0.76	412	100	58	91	65	89	93	55	88	100	99
SSU	1748	-0.52	140	56	-	-	-	-	-	36	-	-	-
H3	328	-0.34	132	-	-	-	-	-	97	87	-	-	24
1st & 2nd codon positions of CO1	416	-0.18	122	-	-	-		-	-	51	37	-	-
LSU+SSU	5038	-0.77	552	100	68	98	57	77	97	69	84	100	100
LSU+SSU+H3	5366	-0.61	684	100	85	100	75	86	100	96	93	100	99
LSU+SSU+H3+1st & 2nd codon positions of CO1	5782	-0.65	806	100	93	100	82	92	100	100	94	100	100

3.5 Discussion

We have examined the basal divisions in the stylommatophoran land snails and slugs and the validity of the major taxonomic groups within the clade based on a robust dataset of 5782 sites from four concatenated genes, representing a seven-fold increase in sequence data over the previous molecular phylogenetic studies of the Stylommatophora by Wade *et al*. (2006) and Ramírez *et al*. (2012).

3.5.1 Phylogenetic Relationships of the Stylommatophora

Our phylogenetic trees clearly show a basal dichotomy in the Stylommatophora between the Scolodontidae on one hand and a clade comprising all remaining stylommatophoran taxa on the other.

The taxonomic position of the Scolodontidae had previously remained controversial. Tillier (1980) placed the Scolodontidae with the Endodontidae in his morphological taxonomy of the Stylommatophora. Most other morphology-based taxonomic studies placed the Scolodontidae with the Rhytidoidea (Nordsieck, 1986; Vaught, 1989; Schileyko, 2000; Bouchet & Rocroi, 2005), while Barker (2001) placed the Scolodontidae as the sister group of a clade including the Haplotrematidae and Vitreidae. Previous molecular phylogenetic analyses had also failed to resolve the placement of the Scolodontidae, with Ramirez *et al*. (2012) showing a discrepancy in the position of the Scolodontidae according to the phylogeny reconstruction method used. Our molecular analyses now put this matter beyond doubt with the Scolodontidae falling as the sister group to all other stylommatophoran groups at the base of the stylommatophoran clade with all tree construction methods.

Our phylogenetic analyses are consistent with the previous 'achatinoid' and 'non-achatinoid' clades established by Wade *et al*. (2001, 2006). These groups remain stable in the current analyses, though with a significant increase in the bootstrap support for the 'non-achatinoid' clade. In earlier morphological studies, the 'achatinoid' and the 'non-achatinoid' clades were not predicted (Schileyko, 1978; Nordsieck, 1986; Tillier, 1989; Barker, 2001; Dayrat & Tillier, 2002). Pilsbry (1896, 1900) recognized two main divisions within the Stylommatophora on the basis of foot morphology: the holopod and the aulacopod. The current 'achatinoid' clade includes only holopod groups, but holopod taxa also incorporate with aulacopod groups (Limacoidea, Arionoidea and Endodontoidea) in the 'non-achatinoid' clade suggesting that the holopod foot is plesiomorphic. The 'achatinoid' and 'non-achatinoid' clades are well accepted and have been adopted in the most recent taxonomy of the Stylommatophora by Bouchet *et al*. (2017) at the same suborder level as Scolodontina, with the 'achatinoid' clade renamed as the suborder Achatinina and the 'non-achatinoid' clade renamed as the suborder Helicina. While our current phylogenetic analysis confirms the validity of these clades our findings would suggest that the 'achatinoid' and 'non-achatinoid' clades should be classified at a lower level than the Scolodontina in order to properly reflect the deep-level relationships within the Stylommatophora.

Within the ‘achatinoid clade’, the principle dichotomy between the Streptaxidae and Achatinoidea is well resolved with high bootstrap support. This finding agrees well with the previous molecular studies (Wade *et al*., 2001, 2006; Sutcharit *et al*., 2010). The internal relationships within the Achatinoidea are not examined here but have been examined in depth by Fontanilla *et al*. (2017). In the ‘non-achatinoid clade’, there is robust support for the Pupilloidei, Arionoidei, Limacoidei, Orthalicoidei and Helicoidei. The Arionoidei and the Limacoidei show a sister group relationship in the tree as previously observed (Wade *et al*., 2001, 2006) though support is equivocal. The Haplotrematoidea and Oleacinoidea fall as sister taxa of the Helicoidea as observed by Wade *et al*. (2006), though now with robust support. The Haplotrematoidea and Oleacinoidea were tentatively placed in a new infraorder named the Oleacinoidei by Bouchet *et al*. (2017) but the monophyly of this infraorder is not supported in the current phylogeny. The Rhytidoidea are represented by four families of which the Caryodidae, Dorcasiidae and Rhytididae form a clade that is poorly supported in BI and ML analyses, while in the NJ analysis the Rhytidoidea are resolved as a monophyletic group, but without significant support. The Rhytididae and all acavoid families were combined in one superfamily named Rhytidoidea by Bouchet *et al*. (2017) based on the molecular studies of Herbert *et al*. (2015) and Teasdale *et al*. (2016) as cited in Bouchet *et al*. (2017) where they showed the Rhytididae are nested in the Acavoidea. However, the monophyly of the Rhytidoidea in our phylogenetic trees is not clear.

3.5.2 Phylogenetic Utility of Different Genes and Partitions

The phylogenetic signal was examined directly in all genes/ gene partitions and in the combined dataset using the *g1* statistic. The results indicated the presence of significant phylogenetic signal in all genes/ gene partitions as well as in the concatenated dataset. Analysis of the number of variable sites reveals that within the LSU gene, the original LSU 1-5 fragment contains the majority of the variable sites. Although the LSU 1-5 fragment is only a quarter of the length of the full-length LSU gene, the LSU 1-5 fragment has more than 57% of the total number of variable sites. The LSU 1-5 fragment has been used extensively in phylogenetic studies of the Stylommatophora (Wade & Mordan 2000; Wade *et al*., 2001, 2006; Wade *et al*., 2007; Herbert & Mitchell, 2008; Breure *et al*., 2010; Breure & Romero, 2012;

Ramírez *et al*., 2012; Herbert *et al*., 2015; Nekola & Coles, 2015; Moussalli & Herbert, 2016) where its utility is well established in resolving phylogenetic relationships among this group.

It has been suggested that the number of variable sites in nuclear genes positively correlates with the phylogenetic signal (Meyer *et al*., 2015). However, Kimball and Braun (2014) demonstrated that expanding the sequence length to approximately threefold from their previous study (Kimball & Braun, 2008) resulted in only a 5% increase on average in the bootstrap support for branches. Similarly, in our analyses we found only a modest increase in bootstrap support values for the majority of branches when using the full-length LSU compared to the LSU 1-5 fragment. However, only the full-length LSU gene resolved all ten stylommatophoran clades evaluated in our study, with just nine of ten clades resolved with the LSU 1-5 fragment. This indicates that while the LSU 1-5 fragment is the most informative part of the LSU gene, analysis of the full-length LSU gene is necessary to fully resolve the relationships within the Stylommatophora, particularly those at the base of the clade.

The SSU, H3 and CO1 (1st and 2nd codon positions) genes have fewer variable sites than the LSU and resolve only a handful of stylommatophoran clades (two or three of the ten clades evaluated) indicating that the phylogenetic information carried by these genes on their own is not sufficient to construct a robust phylogeny. However, when these genes are combined with the LSU, phylogenetic trees built using this concatenated dataset comprising all four genes shows a marked improvement in resolution. All ten stylommatophoran clades evaluated are recovered and there is a marked improvement in bootstrap support with all clades resolved in over 80% of bootstrap replicates and many clades fully supported. This clearly shows a significant improvement in the phylogenetic signal in the concatenated dataset compared with the individual genes. The phylogenetic relationships at the base of the stylommatophoran tree (the principle split of the tree and the support for the 'achatinoid' and the 'non-achatinoid' clades) were either not recovered or poorly supported with the individual genes. In the combined dataset, the relationships at the base of Stylommatophora are resolved with high support. These findings are consistent with previous studies that show that the concatenation of individual genes improves phylogenetic signal and produces more accurate trees (Baldauf *et al*., 2000; Gontcharov *et al*., 2004; Gadagkar *et al*., 2005; de Queiroz & Gatesy, 2007; Botero-Castro *et al*., 2013) and it has been

suggested that using multiple independent loci is a more effective approach to increase bootstrap support than increasing the sequence length of an individual gene (Corl & Ellegren, 2013; Kimball & Braun, 2014).

3.6 Conclusion

This paper presents the phylogenetic relationships of the Stylommatophora based on 5782 sites from four concatenated genes with special emphasis on the position of the Scolodontidae and their relationship to the ‘achatinoid’ and ‘non-achatinoid’ clades. The Scolodontidae are now shown categorically to be the sister group to all other stylommatophoran groups, with robust support with all phylogeny reconstruction methods. The results also provide strong support for the ‘achatinoid’ and ‘non-achatinoid’ clades. The principle division in the stylommatophoran tree between the Scolodontidae and a clade comprising both the ‘achatinoid’ and ‘non-achatinoid’ groups would suggest that the ‘achatinoid’ and ‘non-achatinoid’ clades should be classified at a lower taxonomic level than the Scolodontina in order to properly reflect the deep-level relationships within the Stylommatophora.

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Chapter 4: Molecular phylogeny of the Orthurethra (Panpulmonata: Stylommatophora)[1]

Abstract

We have undertaken a molecular analysis of the Orthurethra, one of the major groups of stylommatophoran land snails and slugs based on 3370 sites of the large subunit and 5.8S ribosomal RNA genes. A primary division in the tree separates the Chondrinidae from the rest of the Orthurethra. For those families represented by more than one genus, the Chondrinidae, Cerastidae, Vertiginidae, Achatinellidae and Partulidae are recovered as strongly supported monophyletic units, whereas the Pupillidae, Enidae, and Valloniidae are unsupported in the tree. Although there is relatively little support for the deep-level relationships among the main orthurethran groups, some groupings are strongly supported. The sister-group relationship of the Cochlicopidae with the Amastridae is strongly supported in our molecular analyses, and there is also some support for the grouping of the Orculidae with the Pyramidulidae.

[1]*This chapter is submitted as "Saadi, A.J., Mordan P.B., Wade, C.M., 2019. Molecular phylogeny of the Orthurethra (Panpulmonata: Stylommatophora). Zool. J. Linn. Soc.".*

4.1 Introduction

The Orthurethra comprise approximately 6000 species, assigned to some 200 genera. The anatomy of the excretory system formed the basis of the classic Pilsbry-Baker system for the Stylommatophora (Pilsbry, 1900; Baker, 1955), with the Orthurethra characterised by having a simple, straight ureter that runs from the anterior end of the kidney towards the pneumostomal area of the lung, and opens as a backward-pointing pore. In most cases a secondary structure is developed which varies from a short groove in many species, a short tube (e.g. *Acanthinula*), a longer tube running most of the length of the kidney (e.g. *Abida*), or additionally back along the rectum (the so-called pseudosigmurethrous condition found in *Amimopina*). In this traditional classification, the Orthurethra are one of four primary divisions of the Stylommatophora. Pilsbry (1900) considered the Orthurethra to be an ancestral group to all other Stylommatophora. This was based on the belief that the orthurethran pallial system with its long kidney and direct ureter most closely resembled that of the supposedly more ancient Basommatophora, and this view has generally persisted (e.g. Schileyko, 1979; Nordsieck 1986; Pokryszko, 1994). The shape of the ureter does not allow water reabsorption, which was suggested by Solem (1978) as the reason the Orthurethra does not contain any slugs.

The Orthurethra range from Europe and regions bordering the Mediterranean to Japan and have reached most of Africa, India, much of Asia, North America, the islands of the Pacific and even Australia. In the Pacific islands, orthurethrans can represent as much as 60% of the land snail diversity. The four orthurethran families, Partulidae, Amastridae, Achatinellidae and Draparnaudiidae, are endemic to the islands of the Pacific. They have very narrow distributional ranges and are under severe threat from habitat destruction and introduced predators. Indeed, the Amastridae, which are endemic to Hawaii, have almost all become extinct and the loss of numerous achatinellid and partulid taxa has also been well documented (see Cowie, 1992; Coote & Loeve, 2003; Holland & Hadfield, 2004; Régnier *et al*., 2015 for a review).

In his classic paper dealing with the affinities of the Pacific genera *Achatinella* and *Partula*, Pilsbry (1900) recognised five families within his new group Orthurethra: the Achatinellidae, Partulidae, Pupidae (=Pupillidae), and questionably the Valloniidae and Cochlicopidae. Later he published a provisional list of subfamilies in his Pupillidae as follows: the Gastrocoptinae, Pupillinae, Pagodininae, Acanthulinae, Vertigininae, Orculinae and Strobilopsinae (Pilsbry, 1916-18). Subsequently, he added the Pagodulininae for two genera, *Spalaeodiscus* and *Pagodulina*, which would not fit conveniently elsewhere (Pilsbry, 1922-26). His work formed the basis of the Orthurethra as we know them today. At an early stage Pilsbry (1916-18) admitted that his Pupillidae had been given rather wide limits, but Watson (1920) was to go much further in suggesting that all but a few genera of orthurethrans should be combined into a single broad concept of the family Pupillidae.

In contrast, the detailed anatomical studies of Steenberg (1925) led him to recognise a total of 16 families in the Orthurethra as a whole. However, the limits of the group have not changed significantly since most of these additional families have resulted from the splitting of existing orthurethran groups, principally the Pupillidae *sensu lato*. Anatomical support for the various family-group taxa within the Orthurethra has always been poor. Baker (in Pilsbry, 1927-35) gave a comprehensive review of the limited anatomical information available at the time, but concluded that in respect of the Pupilloidea: 'any deductions based on the animal alone would be weak and this would be especially true of a group in which the shell characters … appear manifest while those of the soft parts are difficult to study and more so to evaluate'. As Nordsieck (1986) pointed out, the relationships within the Orthurethra established by Pilsbry and Baker (Pilsbry, 1927-35) are essentially based on conchological characters, which he believed overvalued the importance of the shell. This situation changed little until the work of Tillier (1989) who surveyed a fresh suite of characters of the pallial, nervous and alimentary systems. Tillier (1989) and Barker (2001) represent the only two attempts at a morphological phylogenetic analysis of the Orthurethra. Tillier considered the Orthurethra to be monophyletic and the sister group of all other Stylommatophora (Tillier, 1989; Emberton & Tillier, 1995). Though Tillier's tree divided the Orthurethra into two clades, his classification recognised three superfamilies: the Pupilloidea, Chondrinoidea and Partuloidea. However, Tillier (1989) considered his own classification of the

Orthurethra to be unsatisfactory. In Barker's unconstraint analysis of the Stylommatophora (including 12 orthurethran families) based on 57 mainly morphological characters, the orthurethrans fell into two distinct and remote clades, each of which showed close affinity with non-orthurethran families: one of these clades included the Sphincterocheilidae and Urocoptidae, the second included the Corillidae and Acavidae. These results appear to be even less satisfactory than Tillier's classification. In the classification of Bouchet & Rocroi (2005), the Orthurethra are considered as an informal group and subclade of the Stylommatophora, with the Orthurethra partitioned into five superfamilies: the Partuloidea, Achatinelloidea, Cochilicopoidea, Pupilloidea and Enoidea, and 20 families, one of which, the Cylindrellinidae, is extinct. Most recently, Bouchet *et al*. (2017) revised the earlier Bouchet & Rocroi (2005) classification, recognising a total of 26 families placed within a single superfamily, the Pupilloidea '(making Orthurethra and Pupilloidea synomyms)'.

Several molecular studies of the Orthurethra have been conducted. Most of them focused on phylogeography of a particular genus (Goodacre & Wade, 2001a; Goodacre, 2002; Tongkerd *et al*., 2004; Ketmaier *et al*., 2006; Sischo & Hadfield, 2017) or within a family (Goodacre & Wade, 2001b; Holland & Hadfield, 2004; Lee *et al*., 2009, 2014; Slapcinsky & Kraus, 2016; Köhler *et al*., 2017). The first comprehensive molecular studies of the Orthurethra aimed to solve both the relationships within the Orthurethra and to place them within the stylommatophoran phylogeny as a whole (Wade *et al*., 2001, 2006). However, these phylogenies were based on only 823 nucleotides of the rRNA gene cluster. The resulting orthurethran tree was poorly resolved and, except between closely related taxa, support for groupings was relatively low. The monophyly of the Orthurethra was recovered and the Orthurethra fell within the 'non-achatinoid' clade (Wade *et al*., 2001, 2006; Mordan & Wade, 2008). Armbruster *et al*. (2005) used histone genes to analyse the relationships of 13 stylommatophoran genera (including nine orthurethran genera). The Orthurethra were not recovered as a monophyletic group in their study, and three orthurethran families, the Cochlicopidae, Vertiginidae, and Valloniidae were paraphyletic in their tree. Madeira *et al*. (2010) examined the phylogenetic position of the orthurethran genera *Cryptazeca*, *Azeca* and *Hypnophila* (the Azecidae), essentially incorporating these taxa within the Wade

et al (2006) rRNA sequence dataset. Their analysis provided strong support for a monophyletic group comprising *Cryptazeca*, *Azeca* and *Hypnophila,* which led them to propose a new family, the Azecidae.

Nekola & Coles (2016) undertook a molecular analysis aimed at elucidating the supraspecific taxonomy of the Vertiginidae, but that also included a number of other orthurethran sequences, some new and some from earlier studies, principally those of Wade *et al*. (2001, 2006). Their phylogenetic conclusions were essentially based on 28S sequences of some 800 base pairs, and replicated many of the results of Wade *et al*. (2001, 2006); a 'vertiginid' clade was recovered which broadly corresponded with the Vertiginidae as outlined in Bouchet & Rocroi (2005). The overall conclusion was that their results 'strongly suggest that formal reconsideration of supraspecific concepts across the entire infraorder are warranted, based upon DNA sequence data'. *Truncatellina* and *Columella* were excluded from the Vertiginidae as they cluster with the Chondrinidae. The molecular study of Harl *et al*. (2017) targeted the family Orculidae, but included many non-orculid taxa; whilst clarifying the monophyly of the orculids, their other main contribution was the erection two new orthurethran families: the Agardhiellidae and Fauxulidae.

Here we present the results of a greatly enlarged molecular analysis that includes the sequences of 35 genera representing 16 families of Orthuretha, for the ribosomal (r) RNA gene cluster [including part of the 5.8S gene, the complete ITS-2 region, and almost the full-length large subunit (LSU; 28S) gene], and discuss more fully the observed patterns of relationship within the group.

4.2 Materials and Methods

4.2.1 Biological Material

A total of 35 genera, representing 16 orthurethran families were included in this study. Details of the specimens, sampling localities, and collectors are given in Table 4.1. We adopt the classification of Bouchet *et al*. (2017) throughout.

Table 4.1. Details of specimens, sampling localities and collectors. Classification of samples follows Bouchet *et al*. (2017).

Family	Species	Collection Location	Collector
Orthurethran Taxa			
Achatinellidae	*Elasmias luakahaense* Pilsbry and Cooke, 1915	Koolau Range, Oahu, Hawaii	R. Rundell & K. Olival
	Partulina proxima (Pease, 1862)	Maui, Hawaii	B. Holland
Cochlicopidae	*Cochlicopa lubricella* (Porro, 1838)	São Miguel, Azores	P. Mordan
Amastridae	*Leptachatina lepida* Cooke, 1910	Hawaii Island, Hawaii	P. Mordan & R. Cowie
Pupillidae	*Pupoides albilabris* (Adams, 1841)	Wilson County, Tennesse, USA	J. Slapcinsky & B. Coles
	Pupilla muscorum (Linnaeus 1758)	Garden, Victoria Grove, London	Ellen Graubart
Lauriidae	*Lauria cylindracea* (da Costa, 1778)	Mullaghmore, Co. Sligo, Ireland	E. Platts
Valloniidae	*Vallonia excentrica* Sterki, 1892	São Miguel, Azores	P. Mordan
	Acanthinula aculeata (Müller, 1774)	Box Hill, Surrey, UK	J. Ablett
Vertiginidae	*Vertigo antivertigo* (Draparnaud, 1801)	Chuett, Arnoldstein, Austria	P. Miltner
	Pronesopupa acanthinula (Ancey, 1892)	Koolau Range, Oahu, Hawaii	R. Rundell & K. Olival
Gastrocoptidae	*Gastrocopta armifera* (Say, 1821)	Wilson County, Tennesse, USA	J. Slapcinsky & B. Coles
Orculidae	*Orcula austriaca* Zimmerman, 1932	Kuhberg, Austria	P. Miltner
Strobilopsidae	*Eostrobilops nipponica* (Pilsbry, 1908)	Osaka, Japan	I. Matsumura
Pyramidulidae	*Pyramidula rupestris* (Draparnaud, 1801)	Mullaghmore, Co. Sligo, Ireland	E. Platts
Chondrinidae	*Chondrina clienta* (Westerlund, 1883)	Villach, Austria	P. Miltner
	Solatopupa similis (Bruguière, 1792)	Verdon Gorge, France	A. Davison
	Abida secale (Draparnaud, 1801)	Pulpit Down, Buckinghamshire, UK	P. Mordan
	Granaria frumentum (Draparnaud, 1801)	Pelsivec Plateau, Slovakia	J. Grego
Enidae	*Buliminus labrosus* (Olivier, 1804)	Saladin's Castle, Syria	P. Mordan
	Pene sidonensis (Férussac, 1821)	Saladin's Castle, Syria	P. Mordan
	Luchuena reticulata (Reeve, 1849)	Kikai Island, Ryukyu, Japan	S. Chiba
	Napaeus pruninus (Gould, 1846)	São Miguel, Azores	A. Polasczek
	Macaronapaeus vulgaris (Morelet & Drouet, 1857)	São Miguel, Azores	P. Mordan
	Ena obscura (Müller, 1774)	Biela voda, Olcnava, Slovakia	J. Steffek
	Mastus pupa (Bruguière, 1792)	Sicily	A. Davison
	Mirus stalix (Benson, 1863)	Agra, Sri Lanka	D. Raheem
	Chondrula albolimbata (Pfeiffer, 1848)	Spissky tlrad, Slovakia	J. Grego
Cerastidae	*Cerastus schweinfurthi* (Martens, 1895)	Al-Mahuit, N. Yemen	P. Mordan
	Pachnodus silhouettanus van Mol & Coppois, 1980	Silhouette Island, Seychelles	J. Gerlach
	Amimopina macleayi (Brazier, 1876)	Queensland, Australia	P. Mordan
Draparnaudiidae	*Draparnaudia singularis* Reeve, 1854	Mont Koghis, New Caledonia	C. Wade & K. Bowman

Partulidae	*Samoana conica* (Gould, 1847)	Samoa	R. Cowie
	Eua zebrina (Gould, 1847)	Samoa	R. Cowie
	Partula tohiveana Crampton, 1924	Moorea	Unknown
Non Orthurethran Taxa			
Ferussaciidae	*Ferussacia folliculus* (Gmelin, 1791)	Los Alcornales, Prov Cadiz, Spain	M. Seddon
Achatinidae	*Lissachatina fulica* (Bowdich, 1822) [= *Achatina fulica*]	Unknown (Zool. Soc. Lond. colln.)	P. Pearce-Kelly
Streptaxidae	*Gonaxis quadrilateralis* (Preston, 1910)	Réunion	O. Griffiths

4.2.2 DNA Extraction, PCR Amplification and Sequencing

DNA was extracted from a small (1-2 mm3) tissue sample taken from the foot of the snail or the whole snail (for small specimens) using a CTAB DNA extraction protocol (Goodacre & Wade, 2001b). Approximately 4000 nucleotides of the ribosomal (r) RNA gene cluster [including part of the 5.8S gene, the complete ITS-2 region, and almost the full-length large subunit (LSU; 28S) gene] were sequenced using a nested PCR approach, with the product of the primary PCR being used as a template for the secondary PCR. The primary PCR was performed using the primers LSU-1ii and LSU-12 followed by six internal fragments (A, B, C, D, E, and F) (see Table 2.2 for details of primers).

PCR amplification for the primary PCR was performed using the Qiagen Taq DNA polymerase and Q buffer system (1X buffer, 1X Q-solution, 0.3 mM dNTP, 1.5 mM magnesium chloride, 0.2 µM each primer and 1U Taq in a 50µL final volume). Secondary PCR amplification was identical to the primary PCR with the exception that a lower 0.2 mM concentration of dNTPs was used. The cycling conditions (with a Perkin Elmer cycler) of the primary PCR were as follows: 96 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 50 °C for 30 sec, 72 °C for 3 min and then a final extension step at 72 °C for 5 min. The secondary PCR cycle conditions were as follows: 96 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 45 °C for 1 min, 72 °C for 2 min and then a final extension step at 72 °C for 5 min. Amplification products were purified from an agarose gel using a Qiagen gel extraction kit. Both sense and antisense strands were sequenced directly on an Applied Biosystems 3730 DNA sequencer using Big Dye terminator cycle sequencing chemistries at Macrogen Inc.

4.2.3 Sequence Processing and Phylogenetic Analyses

DNA sequences were assembled using the STADEN package version 1.5.3 (Staden *et al*., 2000) and aligned using version 2.2 of the Genetic Data Environment (GDE) package (Smith *et al*., 1994). The LSU (incorporating 5.8S and ITS-2) rRNA sequences were aligned manually using the secondary structure as a guide. Gblocks v.0.91b (Castresana, 2000) with default settings was used as a guide to select the reliably aligned nucleotide sites.

Phylogenetic trees were constructed using maximum likelihood (ML) (Felsenstein, 1981), neighbor-joining (NJ) (Saitou & Nei, 1987) and Bayesian inference (BI) (Larget & Simon, 1999). The general time reversible model incorporating gamma (GTR+Γ) (Lanave *et al*., 1984; Gu *et al*., 1995) was used to correct for multiple substitutions for all model-based methods (ML, NJ and BI). ML trees were constructed using the PhyML (version 3.0) package (Guindon *et al*., 2010) with tree searching following a heuristic procedure with 10 random start trees and best of nearest-neighbour-interchange and subtree-pruning-regrafting branch swapping. NJ analysis was performed using the PAUP* (version 4.0b10) package (Swofford, 2002). For NJ analysis, model parameters were estimated following an iteration process; for each tree the parameters were estimated and used to build the next tree until there was no further improvement of the likelihood score. Bootstrap resampling (Felsenstein, 1985) with 1000 replicates was undertaken for ML and NJ trees. BI analysis was undertaken using the MrBayes (version 3.1.2) package (Ronquist & Huelsenbeck, 2003). Two independent runs with four chains of a Markov Chain Monte Carlo (MCMC) algorithm were used to explore the tree space. BI analysis was conducted for 5 million generations with tree sampling every 100 generations. To ensure adequate chain swapping, a range of heating parameters were tested with the optimal parameter used to construct the final trees. Only after the Bayesian MCMC searches had reached a stationary phase (indicating convergence of the chains onto the target distribution) was the run ended. A consensus tree was built using the last 75% of trees (burnin=12501). Alternative phylogenetic hypotheses were evaluated by likelihood using a Shimodaira–Hasegawa RELL test (Shimodaira & Hasegawa, 1999) on NJ generated topologies as implemented in PAUP*.

4.3 Results

Sequences of approximately 4000 nucleotides of the rRNA gene cluster were generated for 35 genera belonging to 16 orthurethran families. Phylogenetic trees for the Orthurethra were constructed using 3370 unambiguously aligned nucleotide sites of the LSU rRNA gene (also incorporating the 5.8S). Three species of the 'achatinoid' clade (Wade *et al*., 2001, 2006), *Lissachatina fulica*, *Ferussacia folliculus* and *Gonaxis quadrilateralis,* were used as an outgroup to root the phylogenetic trees.

The Bayesian inference tree of the Orthurethra is shown in Figure 4.1. Branches supported in >50% of bootstrap replicates and with Bayesian posterior probabilities >0.70 are consistent across all phylogeny reconstruction methods (BI, Figure 4.1; ML, Appendix 4.1; NJ, Appendix 4.2). The Orthurethra form a well resolved monophyletic group in the tree supported in 100% ML, 100% NJ bootstrap replicates and with a Bayesian posterior probability of 1.00. The first division in the Orthurethra separates the Chondrinidae from the remaining orthurethran families. The Chondrinidae are strongly supported (84% ML, 95% NJ bootstraps and PP=1.00 BI) and are themselves divided into a further two groups; the first comprises *Chondrina clienta* and *Abida secale* (100% ML, 100% NJ bootstraps and PP=1.00 BI) and the second comprises *Solatopupa similis* and *Granaria frumentum* (100% ML, 100% NJ bootstraps and PP=1.00 BI). The remaining orthurethrans (shaded area on tree, Figure 4.1) cluster together in a fully supported group (100% ML, 100% NJ bootstraps and PP=1.00 BI) though the phylogenetic relationships within this group are generally poorly resolved with just a few groupings strongly supported. The Cerastidae are well supported with 76% ML, 85% NJ bootstraps and PP=1.00 BI. Within the Cerastidae there is strong support for the sister relationship between *Pachnodus silhouettanus* and *Cerastus schweinfurthi* (96% ML, 95% NJ bootstraps and PP=1.00 BI). The Vertiginidae are also strongly supported in the tree (94% ML, 100% NJ and PP=1.00 BI). The Achatinellidae are fully supported in the tree with 100% ML, 100% NJ bootstraps and PP= 1.00 BI. The Partulidae also form a clear group in the tree, fully supported in 100% ML, 100% NJ bootstraps and PP=1.00 BI. Within the Partulidae, the sister relationship between *Samoana conica* and *Partula tohiveana* is supported in 75% ML, 68% NJ bootstraps and PP=0.89 BI.

The sister relationship between *Lauria cylindracea* (Lauriidae) and *Pupilla muscorum* (Pupillidae) is fully supported in the tree (100% ML, 100% NJ and PP=1.00 BI). Likewise, the Cochlicopidae (*Cochlicopa lubricella*) and Amastridae (*Leptachatina lepida*) are closely related with their sister group relationship strongly supported in 99% ML, 98% NJ bootstraps and PP=1.00 BI. There is also high support for the sister relationship of *Chondrula albolimbata* (Enidae) and *Mastus pupa* (Enidae) (89% ML, 99% NJ and PP=1.00 BI). Finally, the sister relationship between *Orcula austriaca* (Orculidae) and *Pyramidula rupestris* (Pyramidulidae) is also supported in the tree, though only weakly (55% ML, 67% NJ bootstraps and PP=0.96 BI).

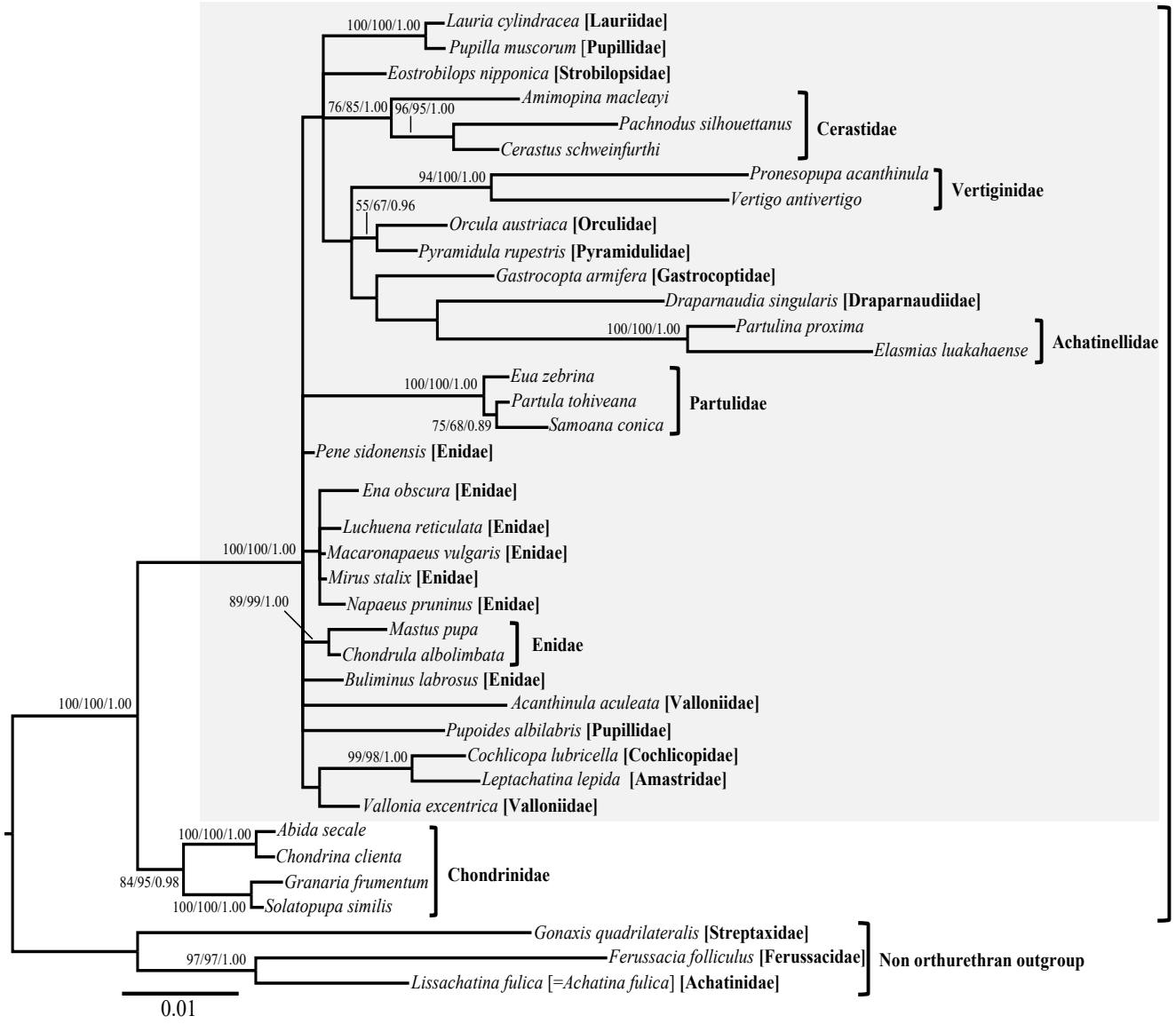


Figure 4.1. Bayesian phylogenetic tree of the Orthurethra based on 3370 unambiguously aligned nucleotide sites of the LSU rRNA (and 5.8S) gene. Values on nodes represent bootstrap support (1000 replicates) for ML, NJ and posterior probabilities (based on last 75% of the trees) for BI, respectively. Bootstrap support less than 50% and posterior probabilities less than 0.7 are not shown. The scale bar represents 1 substitutional change per 100 nucleotide positions.

4.4 Discussion

There is a clear basal dichotomy between the Chondrinidae on the one hand, and a clade comprising all the remaining orthurethran taxa on the other in our orthurethran tree. This is consistent with Wade *et al*. (2001, 2006) but here is based on a more robust data set. Madeira *et al*. (2010) showed a basal trichotomy within the Orthurethra between the Chondrinidae, Azecidae and the remaining orthurethran taxa. Our study is consistent with these findings in respect of the Chondrinidae but we did not include the Azecidae in our analyses. Likewise, Nekola & Coles (2016) and Harl *et al*. (2017) showed a principle division within the Orthurethra between the Chondrinidae plus Truncatellinidae and the remaining orthurethran taxa. Again, our study is consistent with these findings in respect of the Chondrinidae but we did not include the Truncatellinidae in our analyses.

Within the orthurethran tree presented here, the phylogenetic signal is very strong where it exists: the resolved groupings are strongly supported, and the unresolved ones are hardly supported at all. These areas of the tree lacking resolution reflect the extreme difficulty that morphological taxonomists have encountered over the years in classifying and understanding relationships within the Orthurethra. When Pilsbry (1900) first proposed his classification, he admitted that 'the group Orthurethra consists of families not separated by characters of great importance'. Watson (1920) emphasised the problem of defining orthurethran families anatomically. The only families excluded from his Pupillidae *sensu lato* were the Achatinellidae and Partulidae, and possibly also the Cerastidae. Baker (in Pilsbry, 1927-35) was in agreement with Watson, stating that 'the total range of anatomical differentiation seems much less than in most families of pulmonates'. Most of the orthurethrans were therefore included in a broad concept of the family referred to variously as the Pupillidae (e.g. Pilsbry, 1916-18), Pupilloidea (e.g. Pokryszko, 1994), or Pupillina (e.g. Schileyko, 1984). Recent molecular studies of orthurethrans have tended to concentrate on particular families: Nekola & Coles (2016) on supraspecific taxonomy in the family Vertiginidae; Madeira *et al*. (2010) on the phylogenetic position of the Azecidae; and Harl *et al*. (2017) on the Orculidae.

Of the 26 families recognised in the classification of Bouchet *et al*. (2017), 16 are represented in our tree more than in any other molecular analysis. A number of interesting points have emerged and are discussed below. The family groups discussed below are those recognised by Bouchet *et al*. (2017).

4.4.1 Chondrinidae

The chondrinids are small (shell < 10 mm) snails mainly restricted to the Iberian peninsula, south of France, Italy and the Balkans. They are strongly supported as a monophyletic family in our molecular analysis (Figure 4.1). The sister-group relationship of the Chondrinidae relative to the remaining Orthurethra is strongly supported in our molecular trees. Steenberg (1925) was the first to propose the Chondrinidae as a family in its own right. The only recent line of evidence supporting the sister relationship of the Chondrinidae to other Orthurethra, comes from a paper discussing the functional morphology of the orthurethran shell aperture (Suvorov, 1993). Suvorov concluded that the most ancestral pattern of orthurethran dentition (i.e. that most similar to the Ellobiidae) is to be found in members of the Chondrinidae. Pilsbry (1916-18) grouped the chondrinids and gastrocoptids on conchological characters, whilst Baker (in Pilsbry, 1927-35) later added several anatomical characters: short spermatheca, convoluted uterus, prostate as long as uterus, long and thin oviducal cul-de-sac, and simple penis. Nordsieck (1986) excluded the gastrocoptines from the chondrinids whilst Tillier (1989) united his Chondrinidae (including the gastrocoptids) in a clade with the Enidae, Partulidae, Vertiginidae, Orculidae and Cochlicopidae/Amastridae, based on a single nervous system character (the length of the cerebro-pedal connectives). The relationships proposed by Pilsbry and Tillier are not supported in our molecular trees.

4.4.2 Achatinellidae

The Achatinellidae are widely distributed on the islands of the Pacific, but reaching their highest diversity in the Hawaiian Islands. They are represented in the tree by *Partulina* and *Elasmias*, which were long treated as belonging in separate families, respectively the Achatinellidae and Tornatinellidae (Pilsbry & Cooke, 1912-14; Steenberg, 1925). *Partulina* belonged to the Achatinellidae whereas *Elasmias* was placed within the Tornatellinidae. Later Cook & Kondo (1960) studied the soft anatomy

of these two groups and concluded they should be united. The molecular analyses support Cook and Kondo's morphological interpretations with the Achatinellidae resolved as a strongly supported monophyletic family in our molecular trees (Figure 4.1).

4.4.3 Cerastidae

The Cerastidae were first recognised as a distinct group of orthurethran land snails by Watson (1920) and consists of approximately 15 genera and includes the largest genera exceeding 40 mm in shell height. They reach their greatest diversity in the Afrotropical zone and they also extend eastwards into the Indian subcontinent, with one genus, *Amimopina*, found in Australia and a probable cerastid, *Bulimus subangulatus* Pfeiffer recorded from Cambodia (Mordan, 1984, 1992; Nordsieck, 1986; Solem, 1978). The monophyly of the Cerastidae is well supported in our molecular analysis (Figure 4.1). Phylogenetic relationships within the Cerastidae were analysed by Mordan (1992) based on a morphologogical cladistic analysis. The family is characterised by what Solem (1978) in a discussion of the anatomy of *Amimopina* has called a 'pseudosigmurethrous' pallial system, in which the ureter initially runs straight towards the pneumostome from the anterior tip of the kidney, then extends back along the edge of the kidney and then returns towards the pneumostome along the side of the rectum. This additional structure takes the form of a groove or fully enclosed tube throughout its length. It is clearly a synapomorphic character of the family, and quite distinct from the true sigmurethrous condition found in most Stylommatophora.

4.4.4 Partulidae

The partulidae are medium-sized, ovoviviparous (live-bearing) tree snails endemic to the high islands of the Pacific Ocean (Cowie, 1992) from Palau and the Marianas to the Marquesas and Society Islands, but absent from Hawaii. Extensive evolutionary studies of the partulids have been undertaken to understand how these species colonised the different islands of the Pacific and how the speciation processes occurred (Cowie, 1992; Goodacre & Wade, 2001a; Goodacre & Wade 2001b; Goodacre, 2002; Lee *et al*., 2009, 2014; Sischo & Hadfield, 2017). Although, we now have a better understanding of partulid evolution, we still do not know where they came from. The anatomy of the Partulidae is

characterised by a relatively short, triangular kidney with a shortened ureter, a penis lacking an appendix and a spermatheca with a greatly thickened stalk, on which the narrow oviduct inserts laterally (Pilsbry, 1909-10). Schileyko (1979) also examined the anatomy of *Partula,* interpreting the group to be one of the most ancestral within the Stylommatophora. This conclusion was based on the anatomy of the kidney, foot and reproductive system. Nordsieck (1985) also excluded the Partulidae from the Orthurethra and included them in his 'achatinoid Sigmurethra' on the basis of the pallial system, as well as aspects of the male reproductive anatomy, which he considered to resemble more closely those of the Orthalicidae. The synthetic classification of Vaught (1989) follows Nordsieck in this respect, while Tillier (1989) followed Solem (1978) in uniting the Partulidae with the Enidae because of similarities in the length of the cerebral commissure. On the basis of similarities in both the nervous and reproductive systems, Tillier and Mordan (1995) suggested that the Draparnaudidae might be the sister group of the Partulidae. In the molecular tree (Figure 4.1), the partulids appear as a strong monophyletic family however, their position within the Orthurethra is not resolved.

4.4.5 Pupillidae/Lauriidae

The pupillids and lauriids are generally minute snails (<4 mm in length) living in both terrestrial and arboreal habitats (Solem, 1978). The taxonomy of the Pupillidae was mainly developed by Pilsbry (1916-18), Steenberg (1925) and Baker (1935), based on the shell morphology and several anatomical features. The Pupillidae are not resolved as a monophyletic group in the molecular tree (Figure 4.1) and their monophyly is not rejected in Shimodaira-Hasegawa likelihood testing [-ln Likelihood (L) 8902.50729 (best tree shown in Fig.1) versus -ln L=8993.78051 (Pupillidae monophyletic), $P< 0.05$]. The Lauriidae are distributed in Europe, Caucasus and Africa (Herbert, 2010). The family is represented by just one taxon, *Lauria cylindracea,* in our tree which has a sister group relationship with *Pupilla muscorum* (Pupillidae) with strong support (Fig.1). The systematic position of the Lauriidae has been interpreted variously. Bouchet & Rocroi (2005), for example, treated them as a family within the Pupilloidea and the molecular tree offers no justification for excluding them. However, in the H4/H3 tree of Harl *et al*. (2017) *P. muscorum* does not cluster with the putative lauriid genus *Leiostyla* suggesting that the position of the latter in the Lauriidae should be questioned.

4.4.6 Vertiginidae

The Vertiginidae are a large family of small snails that are widespread in the Northern Hemisphere. In the present analysis, the family is represented by two taxa, *Vertigo antivertigo* and *Pronesopupa acanthinula* which cluster together forming a monophyletic unit with robust support (Figure 4.1). This agrees with finding of Nekola & Coles (2016).

4.4.7 Gastrocoptidae

Gastrocopta has been generally considered as a vertiginid (Schileyko, 1984; Nordsieck, 1986; Bouchet & Rocroi, 2005) or chondrinid (Pokryszko *et al*., 2009) but, it was excluded by Nekola & Coles (2016) as it showed no clear affinity with either the vertiginids, or the chondrinids. The relationship of the Gastrocoptidae to other orthurethran taxa remains unclear in our tree (Figure 4.1).

4.4.8 Cochlicopidae/Amastridae

The Cochlicopidae are a small family of minute (shell <10 mm) snails found in Western Europe while the Amastridae are a more diverse family endemic to the Hawaiian Islands. Although these two families have always been considered to be distinct, their sister-group relationship has long been recognised. Pilsbry (1907-8) remarked that: 'no character of importance separates *Cochlicopa* from *Leptachatina*'. This led Watson (1920) to the conclusion that there was no justification in placing these genera in different families Confirmation of the sister-group relationship of the Amastridae and Cochlicopidae has been obtained in subsequent morphological studies (e.g. Tillier, 1989) and they have been grouped in the Cochlicopoidea. The molecular data (Figure 4.1) support these morphological analyses, with the Cochlicopidae and Amastridae clustering strongly in our trees.

4.4.9 Eniidae

Enidae

The Enidae are a large family of medium-sized snails, with a geographical range extending throughout much of the Palaearctic and North Africa to Japan, with a high diversity in the Far and Middle-east and around the Mediterranean Sea. They also extend in low diversity into East and South Africa and

surrounding islands, as well as India. The monophyly of this family has not been a particular point of debate and it is therefore surprising that the molecular data fail to recover it as a monophyletic unit. Although all phylogenetic methods fail to resolve the monophyly of the Enidae, none reject it unequivocally. Moreover, the monophyly of Enidae is not refuted in the Shimodaira-Hasegawa likelihood testing [-ln Likelihood (L) 8902.50729 (best tree shown in Figure 4. 1) versus -ln L=8905.50729 (Enidae monophyletic), P= 0.676].

4.4.10 Orculidae/Pyramidulidae

The Orculidae are a family of small (shell <10mm) snails with a main distribution in Europe. They extend in the western Palaearctic from Spain in the west to the Pamir Mountains in the East. They occur also in North Africa and have their highest diversity in Asia anterior (Hausdorf, 1996). The Pyramidulidae comprise a single genus *Pyramidula*, represented in the tree by its type species *Pyramidula rupestris*, a minute ovoviviparous (live-bearing) snail that lives in rocky areas in the southern Palaearctic from western Europe to Japan. The grouping of the Orculidae and Pyramidulidae has weak support (Figure 4.1). Both are generally included in the Pupillidae *sensu lato*, though the two were not explicitly paired as sister groups until the molecular analyses of Wade *et al*. (2001, 2006). Harl *et al*. (2017) also confirmed the sister grouping of the Orculidae with the Pyramidulidae, though using several orculid taxa and a single, but different, species of *Pyramidula*.

Baker (in Pilsbry, 1927-35) placed Orculidae and Pyramidulidae in separate subfamilies (the Pupillinae and Valloniinae respectively). Steenberg (1925) included *Pyramidula* in the Valloniidae, and the orculids in a distinct family of their own. Watson (1920) examined the anatomy of *Pyramidula* in detail and interpreted it as a paedomorphic member (Gould, 1977) of the Pupillidae, developed by progenesis (sexual maturity attained at a larval or juvenile stage). In the phylogenetic analysis of Tillier (1989), which used a rather different suite of anatomical characters, the Pyramidulidae fell as far from the Orculidae as was possible within the Orthurethra. It is difficult to establish any particular anatomical justification for uniting Orculidae and Pyramidulidae; certainly many of the reproductive characters differ, but these may have become modified in the latter as a result of ovoviviparity. The shell of *Pyramidula* lacks any internal lamella, but whilst orculids are characterised by lamellae in the neanic

shell (Pilsbry, 1922-26), it appears that they are absent from the embryonic shell of *Orcula* (Reinhardt, 1877), which would be consistent with a progenetic origin of the Pyramidulidae.

4.4.11 Valloniidae

The valloniids are a group of small land snails with diverse shell forms, principally distributed in North America, Europe, North Africa, central Asia and Japan, comprising approximately 25 species (Barker, 1985). Two genera, *Vallonia* and *Acanthinula* are represented in our tree but no clear sister-group relationship emerged. Acanthinulids were earlier recognised as a subfamily, either within the Pupillidae (Pilsbry, 1916-18) or Valloniidae (Steenberg, 1925), and there remains disagreement as to their placement; Tillier (1989) and Bouchet & Rocroi (2005) favoured Steenberg, classifying them in the Valloniidae. However, no sister-group relationship between *Acanthinula* and *Vallonia*, nor between either with any other group, was demonstrated in the analysis. The monophyly of the Valloniidae is not rejected in the Shimodaira-Hasegawa likelihood testing [-ln Likelihood (L) 8902.50729 (best tree shown in Figure 4.1) versus -ln L=8912.61913 (Valloniidae monophyletic), P= 1.00].

4.4.12 Draparnaudiidae

The Draparnaudiidae are a homogenous group of 13 recognised species endemic to the continental Pacific island of New Caledonia (Tillier & Mordan, 1995). The systematic position of the family is uncertain, although Tillier and Mordan suggested it appeared anatomically closest to the Partulidae. In the current analysis the relationship of *Draparnaudia* in the molecular tree is unresolved (Figure 4.1).

4.4.13 Strobilopsidae

The Strobilopsidae are defined by the peculiar structure of the last whorl of the shell, but it lacks sufficient anatomical characters to distinguish it from other families, such as the Pupillidae or Valloniidae. The Strobilopsidae have a wide geographical distribution (Americas, China, Japan, Philippines and New Guinea) with fossil species that date back to the early Tertiary of Europe

(Manganelli *et al*., 1989). Strobilopsids are represented by a single taxon, *Eostrobilops nipponica,* in the molecular tree (Figure 4. 1), the position of which remains unresolved.

4.4.14 Superfamilies

Of the five orthurethran superfamily groups described in Bouchet & Rocroi (2005), only the Achatinelloidea and the Cochlicopoidea are monophyletic in our tree. The superfamily Pupilloidea is not monophyletic, with members of this group falling separately in multiple locations of the tree and monophyly of the superfamily is strongly refuted in Shimodaira-Hasegawa likelihood testing [-ln Likelihood (L) 8902.50729 (best tree shown in figure 4.1) versus -ln L=8955.46430 (Pupilloidea monophyletic), P< 0.05]. The Enoidea is also not resolved as a monophyletic group in the tree but their monophyly is not rejected in Shimodaira-Hasegawa likelihood testing [-ln Likelihood (L) 8902.50729 (best tree shown in figure 4.1) versus -ln L=8912.37902 (Enoidea monophyletic), P= 0.547]. Likewise, the superfamily Partuloidea is not resolved as a monophyletic group in our tree, with the Draparnaudidae falling separately from the Partulidae. However, partuloid monophyly is not rejected in Shimodaira-Hasegawa likelihood testing [-ln Likelihood (L) 8902.50729 (best tree shown in figure 4.1) versus -ln L=8922.87819 (Partuloidea monophyletic), P= 0.091]. Harl *et al*. (2017) have argued against the designation of superfamilies in the Orthurethra in our present state of knowledge, and we would concur with this view; Bouchet *et al*. (2017) similarly refrain from designating superfamily groupings within the Orthurethra.

4.4.15 Biogeography

Orthurethrans are principally distributed in Eurasia, but significant radiations have also occurred in Africa (Cerastidae) and on the islands of the Pacific (Partulidae, Amastridae, Achatinellidae and Draparnaudiidae). The Pacific families are particularly well represented in our tree. Achatinellids have a predominantly Pacific distribution and some are endemic to Hawaii (Cooke & Kondo, 1960). The Amastridae are Hawaiian endemics, but their sister group, the Cochlicopidae (Figure 4.1), are thought to have a Holarctic origin (Eurasia and North America) and to have spread widely as a result of human activity. The Hawaiian orthurethran fauna would therefore appear to be diphyletic in origin. The

Draparnaudiidae (Draparnaudia) are endemic to New Caledonia, and the Partulidae are restricted to the high islands of the Pacific Ocean (Cowie, 1992). The tree suggests that the Pacific orthurethran families have evolved independently several times.

Both Nordsieck (1986) and Tillier (1989) offer biogeographical interpretations of orthurethran distribution patterns. Tillier distinguished northern and southern groups from his phylogenetic tree, associating them with Laurasian and Gondwanaland centres of origin. Nordsieck, on the other hand, believed the Orthurethra to be essentially a northern group with more recent radiations in the southern hemisphere. The tree does not show any strong evidence of any Gondwanan pattern of distribution, and the 'southern' groups from Africa (Cerastidae) and the Pacific can all be derived from Holarctic forms.

4.5 Conclusion

The general picture of family-group interrelationships within the Orthurethra which has emerged over the last hundred years is one of confusion: originally the system relied over-heavily on conchological characters (Nordsieck, 1986), but the steadily increasing body of anatomical knowledge has not, in general, greatly improved our understanding of orthurethran relationships. Whilst robust groupings have been established based on morphology, the evolutionary relationships of these groups have generally remained obscure. In most cases the existing family groupings have been supported, or at least not disproved, but some of the larger families such as the Enidae appear not to be robust. Exceptionally, as in the case of the Amastridae/Cochlicopidae pairing (e.g. Tillier, 1989) and the re-evaluation of the Achatinellidae/Tornatellinidae relationship (Cooke & Kondo, 1960), robust sister-group pairings have emerged as a result of anatomical studies, and where these exist, they have been strongly confirmed by the molecular tree. However, lack of resolution throughout other parts of the tree, and the absence of useful morphological characters to define relationships in many cases, is most probably due to a rapid evolutionary diversification of the group as a whole. Therefore, this study strongly advocates for the need of both extended morphological and molecular studies that will bring into light new or understated morphological characters as well as new, more variable molecular markers.

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Chapter 5: Molecular Phylogeny of the Freshwater Snails and Limpets (Panpulmonata: Hygrophila)[1]

Abstract

We have undertaken a comprehensive molecular phylogenetic analysis of the Hygrophila based on 3112 sites of the large subunit and 5.8S ribosomal RNA genes. A clear basal division between the Chilinoidea and Lymnaeoidea is observed. The monophyly of the Acroloxidae, Lymnaeidae and Physidae is also well established. However, the Planorbidae are not supported as a monophyletic group, because the Bulinidae cluster within the Planorbidae. The Amphipepleinae within the Lymnaeidae and both the Ancylinae and Planorbinae within the Planorbidae are strongly supported as monophyletic subfamilies. However, the Aplexinae within the Physidae and the Lymnaeinae within the Lymnaeidae are not recovered. A new taxonomic revision of the Hygrophila is proposed based on the findings of the molecular phylogeny, and the implications for the evolution of chirality are discussed.

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5.1 Introduction

The Hygrophila are the principle group of freshwater aquatic snails and are found almost worldwide (Mordan & Wade, 2008). Similar to other pulmonate gastropods, hygrophilan snails have a lung and lack an operculum, but unlike the terrestrial stylommatophoran pulmonates their eyes are present at the base of their tentacles rather than at the tips (Nordsieck, 1992). They act as intermediate hosts of parasites of medical and veterinary importance, including *Biomphalaria* and *Bulinus* that transmit *Schistosoma*, and *Lymnaea* that transmits *Fasciola* (Brown, 1994).

In the most recent classification (Bouchet *et al*., 2017), the Hygrophila were divided into two superfamilies: the Chilinoidea which comprise the Chilinidae and Latiidae, and the Lymnaeoidea which comprise the Acroloxidae, Bulinidae, Burnupiidae, Lymnaeidae, Physidae and Planorbidae. This principal division of the Hygrophila into Chilinoidea and Lymnaeoidea is well supported in molecular studies (Klussmann-Kolb *et al*., 2008; Dinapoli *et al*., 2010; Jörger *et al*., 2010; Goebbeler & Klussmann-Kolb, 2011; Davison *et al*., 2016).

The Chilinidae are distributed mainly in the southern parts of the Neotropical region where they inhabit a variety of freshwater habitats and comprise approximately 15 species (Strong *et al*., 2008). The Latiidae are distributed in New Zealand and are most abundant in lotic habitats with three described species (Wakerley, 2014). The Chilinidae and Latiidae are recovered as sister groups in the molecular studies of Klussmann-Kolb *et al*. (2008), Dinapoli *et al*. (2010), Jörger *et al*. (2010), Goebbeler & Klussmann-Kolb (2011) and Davison *et al*. (2016).

The Acroloxidae are a group of freshwater limpets distributed mainly in the Palaearctic region and comprise approximately 40 species that mostly inhabit lakes (Strong *et al*., 2008). In the morphological study of Hubendick (1978), the Acroloxidae were grouped with the Latiidae as both the Acroloxidae and Latiidae have a patelliform shape and the prostate gland has a distinctive arrangement. Limited studies have examined the Acroloxidae, with most of these based on morphological/ anatomical characteristics (Shirokaya *et al*., 2003; Shirokaya, 2007; Shirokaya, *et al*., 2011). The most comprehensive molecular phylogeny of the Acroloxidae was undertaken by Stelbrink *et al*. (2015) who

sampled 25 acroloxid species of five genera from Lake Baikal, demonstrating a sister group relationship between *Baicalancylus* and *Pseudancylastrum* and between *Gerstfeldtiancylus* and *Frolikhiancylus*.

The Physidae comprise about 80 species distributed mostly in the Holarctic region with extension into Central and South America (Wethington & Lydeard, 2007). They are found mainly in lentic habitats (Wethington & Lydeard, 2007). The Physidae were originally divided by Starobogatov (1967) into two subfamilies based on shell and anatomical characters: the Aplexinae, comprising three genera and the Physinae comprising four genera. Taylor (2003) provided an alternative classification scheme of the Physidae based on the penial complex in which he divided the Aplexinae and Physinae into seven new tribes including 11 new genera. The most comprehensive molecular analysis of the Physidae was undertaken by Wethington & Lydeard (2007) who examined 66 specimens representing 28 physid taxa. In their phylogeny, the monophyly of the Physidae was supported and six major clades corresponding to penial morphology were recovered.

The Lymnaeidae are the second largest family within the Hygrophila, with approximately 100 described species occurring mainly in the Palaearctic and Nearctic regions where they inhabit a wide variety of freshwater habitats (Strong *et al*., 2008). The phylogenetic relationships of the Lymnaeidae have long been controversial due to the morphological similarities of the lymnaeid taxa (Remigio & Blair, 1997). Initially, the Lymnaeidae were divided by Baker (1911) into two subfamilies, the Amphipepleinae and Lymnaeinae, based on differences in the anatomical structure of the reproductive organs. However, later studies (Colton, 1915; Hubendick, 1951; Kruglov, 2005) indicated that there were no significant differences among the reproductive organs of lymnaeid genera. Hubendick (1951) tentatively recognised only two structural types of lymnaeid reproductive organs and thus, he assigned all the lymnaeids into two genera, *Lymnaea* and *Lanx*. These two genera were later placed each into their own subfamily: the Lymnaeinae and the Lancinae (Zilch, 1959-1960). *Lanx* is a North American genus with an unusual internal structure (Vinarski, 2013) and has been assigned to a separate family Lancidae in several studies (Baker, 1925; Harry, 1964; Starobogatov, 1967; Taylor, 1981; Vaught, 1989). The

European lymnaeids were divided by Jackiewicz (1998) into two genera *Lymnaea* and *Omphiscola* again based on the reproductive organs, particularly the praeputium and prostate.

Although many molecular studies of the Lymnaeidae have been conducted, most of these have been limited by the number of taxa sampled (Bargues *et al*., 2003; Albrecht *et al*., 2008; Puslednik *et al*., 2009; Vinarski *et al*., 2011; Campbell *et al*., 2017; Aksenova *et al*., 2018). The most comprehensive was undertaken by Correa *et al*. (2010) and included 50 taxa belonging to 11 lymnaeid genera. This study demonstrated that there are three deep clades within the Lymnaeidae that correspond to the main geographic origin of species (America, Eurasia and the Indo-Pacific region). However, the analysis did not include any representatives of the subfamily Lancinae and two clades (C1 and C2) were not significantly supported in their maximum likelihood analysis. A new taxonomy of the Lymnaeidae was proposed in a review paper by Vinarski (2013) in which the Lymnaeidae were divided into two subfamilies: the Lymnaeinae with 18-19 chromosome pairs and Amphipepleinae (= Radicinae) with 16-17 chromosome pairs. However, in the most recent taxonomy of Bouchet *et al*. (2017), the Lymnaeidae were divided into three subfamilies, the Amphipepleinae, Lancinae and Lymnaeinae.

The Planorbidae are the largest and the most diverse group among the hygrophilan families with approximately 250 species distributed in a wide range of habitats (Strong *et al*., 2008). Originally, the Planorbidae and Ancylidae were two separate families (Thiele, 1931; Zilch, 1959-1960) but were united by Hubendick (1978) in a single new family named 'Ancyloplanorbidae' (= Planorbidae) based on gonad and prostate characters. This unification of the two families was not accepted by Malek (1985), Burch (1982), Burch (1989) or Brown (1994) who treated the Ancylidae as a separate family, but the inclusion of the Ancylidae within the Planorbidae was later adopted by Morgan *et al*. (2002), Jørgensen *et al*. (2004), Walther *et al*. (2006), Albrecht *et al*. (2007) and Soldatenko & Petrov (2019). The Planorbidae were divided by Hubendick (1978) into two major subfamilies, the Bulininae and Planorbinae, and one minor subfamily, the Rhodacmeinae based on the male copulatory organ and the radula. The Rhodacmeinae is represented by one genus only (*Rhodacmea*) which is enigmatic and very rare (Albrecht *et al*., 2007).

Most of the molecular studies of the Planorbidae have focused on the phylogenetic relationships of medically important genera (Pointier *et al*., 2005; Standley *et al*., 2011; Hassan *et al*., 2016), with few molecular studies addressing the phylogenetic relationships of the Planorbidae as a whole (Morgan *et al*., 2002; Jørgensen *et al*., 2004; Walther *et al*., 2006; Albrecht *et al*., 2007). Of these, the study of Albrecht *et al*. (2007), which included 26 planorbid genera, was the most comprehensive. *Burnupia* resolved as the sister taxon to all other Planorbidae in Albrecht's tree and it was suggested that *Burnupia* should be reclassified in a new family (Albrecht *et al*., 2007). Bouchet *et al.* (2017) adopted this suggestion in their most recent classification of the Gastropoda, with *Burnupia* placed within a new family Burnupiidae. Likewise, the Bulinini were reclassified as the family Bulinidae by Albrecht *et al.* (2007), with this adopted by Bouchet *et al*. (2017). The remaining Planorbidae were divided into three subfamilies, the Ancylinae, Miratestinae and Planorbinae (Bouchet *et al*., 2017).

Hygrophilan snails exhibit a wide variation in shell shape, including high-spired, discoidal (planispiral) or patelliform (limpet-like) shells, with both dextral (right-hand) or sinistral (left-hand) shell coiling (Hubendick, 1978). The Acroloxidae, Burnupiidae and Latiidae contain only patelliform species, while the Bulinidae, Chilinidae, Lymnaeidae, Physidae and Planorbidae have mainly coiled shells (Hubendick, 1978; Burch, 1982, 1989). Dextral and sinistral asymmetry can be traced back to the first division of the spiral cleavage as it originates from alternating rotations of sister blastomeres during cytokinesis (Crampton, 1894; Boycott & Diver, 1923). In dextral species, the shell spiral twists clockwise when viewed from the shell apex while in sinistral species, it twists anticlockwise (Schilthuizen & Davison, 2005). Likewise, in dextral species, the genital opening is on the right-hand side of the body, while in sinistral species, the genital opening is on the left-hand side (Schilthuizen & Davison, 2005). Within the Hygrophila, the Chilinidae and Lymnaeidae are dextral, while the Physidae, Bulinidae and Planorbidae are sinistral (Hubendick, 1978), although there are rare sporadic instances of sinistrality in dextral species and dextrality in sinistral species (Schilthuizen & Davison, 2005). A complete or almost complete reduction of spire and shell coiling occurs in the Acroloxidae, Burnupiidae and Latiidae, and in the subfamilies Lancinae in the Lymnaeidae and Ancylinae in the Planorbidae, leading to a more or less patelliform shell (Hubendick, 1978). However, the internal anatomy is dextral

in the Acroloxidae, Latiidae and the subfamily Lancinae of the Lymnaeidae (Suter, 1913; Burch, 1982), and sinistral in the Burnupiidae as well as in the Ancylinae and Planorbinae within the Planorbidae (Hubendick, 1978; Burch, 1982). In this study dextrality and sinistrality are defined based on shell coiling direction and/or based on directional asymmetry of the internal anatomy.

In this study, we aim to examine the validity of the families and subfamilies within the Hygrophila and to map the evolution of chirality onto the hygrophilan phylogenetic tree.

5.2 Methods

5.2.1 Biological Material

A total of 39 genera of hygrophilan freshwater snails belonging to eight families were included in this study. Two genera of non-hygrophilan panpulmonates from the family Glacidorbidae (*Glacidorbis hedleyi* and *Tasmodorbis punctatus*) were also included, with the Glacidorbidae used to root the phylogenetic trees. Details of the specimens sequenced during this study, sampling localities and the collectors are given in Table 5.1.

Table 5.1. Details of specimens sequenced in this study, sampling localities, collectors and snail chirality. Classification follows Bouchet *et al*. (2017). Snail chirality (dextrality and sinistrality) in Chilinidae, Burnupiidae, Lymnaeidae, Physidae follows Hubendick (1978), Bulinidae and Planorbidae follows Burch (1982), Acroloxidae follows Burch (1982) and Latiidae follows Suter (1913). Chirality is defined based on shell coiling direction and/or based on asymmetry of the internal anatomy.

Classification	Genus/ Species	Chirality	Collection Location	Collector
Superorder Hygrophila **Superfamily Chilinoidea**				
Family Chilinidae	*Chilina patagonica* (G. B. Sowerby II, 1874)	dextral	Golfo Elefantes, Region XI, Chile	David Reid
Family Latiidae	*Latia neritoides* (Gray, 1850)	dextral	Wainui, New Zealand	G. Barker
Superfamily Lymnaeoidea				
Family Acroloxidae	*Acroloxus lacustris* (Linnaeus, 1758)	dextral	West side of River Kozinka, Kievskaya Oblast, Ukraine	D.W. Taylor
	Pseudancylastrum sp. Lindholm, 1909	dextral	Lake Baikal, Russia	T. Sitmikova
Family Physidae Subfamily Physinae	*Physa fontinalis* (Linnaeus, 1758)	sinistral	Embayment from west side of River Desna, Kievskaya Oblast, Ukraine	D.W. Taylor
	Physella gyrina (Say, 1821)	sinistral	Unknown	Unknown
	Haitia mexicana (Philippi, 1841)	sinistral	pHake Lake, Los Angeles County, USA	D.W. Taylor

Subfamily Aplexinae	*Mayabina sp.* D. W. Taylor, 2003	sinistral	Sacha Lodge, Sucumbíos, Ecuador	D.W. Taylor
	Stenophysa minor (d'Orbigny, 1837)	sinistral	Departamento Canelones, Barra de Carrasco, Uruguay	D.W. Taylor
	Sibirenauta elongatus (Say, 1821)	sinistral	Okanagan-Similkameen District, Penticton, British Columbia, Canada	D.W. Taylor
Family Lymnaeidae Subfamily Lymnaeinae	*Bakerilymnaea bulimoides* (Lea, 1841) [= *Fossaria bulimoides*]	dextral	Corner of Alvadore Road, Lane County, Oregon, USA	D.W. Taylor
	Corvusiana sp. Servain, 1882	dextral	Pond of Institute of Fish Culture, Kievskaya Oblast, Kiev, Ukraine	D.W. Taylor
	Lymnaea stagnalis (Linnaeus, 1758)	dextral	Garden Pond, Morpeth, Northumberland, UK	Angus Davsion
	Omphiscola glabra (Müller, 1774)	dextral	Embayment from west side of River Desna, Kievskaya Oblast, Ukraine	D.W. Taylor
	Pseudosuccinea columella (Say, 1817)	dextral	Sacha Lodge, Sucumbíos, Ecuador	D.W. Taylor
	Stagnicola sp. Jeffreys, 1830	dextral	Pond of Institute of Fish Culture, Kievskaya Oblast, Kiev, Ukraine	D.W. Taylor
	Polyrhytis apicina (Meek, 1876)	dextral	Unknown	Unknown
Subfamily Amphipepleinae	*Myxas glutinosa* (O. F. Müller, 1774)	dextral	Llyn Tegid, Gwynedd, UK	M. Willing
	Radix sp. Montfort, 1810	dextral	Pallang Stream, Gambia	Ebrima Joof
	Peregriana sp. Servain, 1882	dextral	Pond of Institute of Fish Culture, Kievskaya Oblast, Kiev, Ukraine	D.W. Taylor
Subfamily Lancinae	*Lanx subrotundata* (Tryon, 1865)	dextral	North Umpqua River, Singleton Park, Douglas County, Oregon, USA	D.W. Taylor
Family Bulinidae Subfamily Bulininae	*Bulinus natalensis* (Krauss in Kuster, 1841)	sinistral	Lake Siboya, South Africa	Provided by Biomedical Parasitology Section, Natural History Museum (NHM) (from culture no. 272)
Family Burnupiidae	*Burnupia sp.* B. Walker, 1912	sinistral	South Africa	Provided by Natal Museum
Family Planorbidae Subfamily Planorbinae	*Anisus vortex* (Linnaeus, 1758)	sinistral	Embayment from west side of river Desna, Kievskaya Oblast, Ukraine	D.W. Taylor
	Bathyomphalus contortus (Linnaeus, 1758)	sinistral	Embayment from east side of river Desna, Kievskaya Oblast, Ukraine	D.W. Taylor
	Choanomphalus maacki Gerstfeldt, 1859	sinistral	Lake Baikal, Berezoviy, Russia	T. Sitnikova
	Gyraulus parvus (Say, 1817)	sinistral	Okanagan-Similkameen District, west end of Yellow Lake, British Columbia, Canada	D.W. Taylor
	Ceratophallus sp. D. S. Brown & Mandahl-Barth, 1973	sinistral	Pacharr Irrigation Canal, Gambia	Ebrima Joof
	Planorbis planorbis (Linnaeus, 1758)	sinistral	West side of River Kozinka, west of village Letki, Kievskaya Oblast, Ukraine	D.W. Taylor
	Segmentina nitida (O. F. Müller, 1774)	sinistral	West side of River Kozinka, west of village Letki, Kievskaya Oblast, Ukraine	D.W. Taylor
	Biomphalaria glabrata (Say, 1818)	sinistral	Puerto Rico	Provided by Mike Doenhoff, Biomedical Parasitology Section, School of Life Sciences, University of Nottingham
	Menetus callioglyphus (Vanatta, 1895)	sinistral	Decker Creek at Matlock, Brady Road, Mason County, Washington, USA	D.W. Taylor
	Planorbarius corneus (Linnaeus, 1758)	sinistral	Embayment from west side of River Desna, Kievskaya Oblast, Ukraine	D.W. Taylor

	Planorbella subcrenata (Carpenter, 1857)	sinistral	Okanagan-Similkameen District, west end of Yellow Lake, British Columbia, Canada	D.W. Taylor
	Planorbula campestris (Dawson, 1875)	sinistral	DNA 7, Teton County, Wyoming, USA	D.W. Taylor
	Promenetus exacuous (Say, 1821)	sinistral	Okanagan-Similkameen District, NE side of Vaseaux Lake, British Columbia, Canada	D.W. Taylor
	Vorticifex effusus (I. Lea, 1856)	sinistral	DNA 14, Deschutes River, Wasco county, Oregon, USA	D.W. Taylor
Subfamily Ancylinae	*Ancylus fluviatilis* O. F. Müller, 1774	sinistral	Broughton, Scotland, UK	G.M. Barker
	Ferrissia californica (Rowell, 1863)	sinistral	T04-4204, Fern Ridge Reservoir, Lane County, Oregon, USA	D.W. Taylor
Non-Hygrophilan panpulmonates: Superorder Pylopulmonata Superfamily Glacidorboidea				
Family Glacidorbidae	*Tasmodorbis punctatus* Ponder & Avern, 2000	-	Murchison Hwy, Deep Gully Creek, Tasmania, Australia	Australian Museum. C.463949
	Glacidorbis hedleyi Iredale, 1943	-	Blue Mountains, Boyd River, New South Wales, Australia	Australian Museum. C.463947

5.2.2 DNA Extraction, PCR Amplification and Sequencing

DNA was extracted from a small (1-2 mm^3) tissue sample taken from the foot of the snail or the whole snail (for small specimens) using a CTAB DNA extraction protocol (Goodacre & Wade, 2001). Approximately 4000 nucleotides of the ribosomal (r) RNA gene cluster [including part of the 5.8S gene, the complete ITS-2 region, and almost the full-length large subunit (LSU; 28S) gene] were sequenced using a nested PCR approach, with the product of the primary PCR being used as a template for the secondary PCR. The primary PCR was performed using the primers LSU-1ii and LSU-12 followed by six internal fragments (A, B, C, D, E, and F) (see Table 2.2 for details of primers).

PCR amplification for the primary PCR was performed using the Qiagen Taq DNA polymerase and Q buffer system (1X buffer, 1X Q-solution, 0.3 mM dNTP, 1.5 mM magnesium chloride, 0.2 µM each primer and 1U Taq in a 50µL final volume). Secondary PCR amplification was identical to the primary PCR with the exception that a lower 0.2 mM concentration of dNTPs was used. The cycling conditions (with a Perkin Elmer cycler) of the primary PCR were as follows: 96 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 50 °C for 30 sec, 72 °C for 3 min and then a final extension step at 72 °C for 5 min. The secondary PCR cycle conditions were as follows: 96 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 45 °C for 1 min, 72 °C for 2 min and then a final extension step at 72 °C for 5 min.

Amplification products were purified from an agarose gel using a Qiagen gel extraction kit. Both sense and antisense strands were sequenced directly on an Applied Biosystems 3730 DNA sequencer using Big Dye terminator cycle sequencing chemistries at Macrogen Inc.

5.2.3 Sequence Processing and Phylogenetic Analyses

DNA sequences were assembled using the STADEN package version 1.5.3 (Staden *et al*., 2000) and aligned using version 2.2 of the Genetic Data Environment (GDE) package (Smith *et al*., 1994). The LSU (incorporating 5.8S and ITS-2) rRNA sequences were aligned manually using the secondary structure as a guide. Gblocks v.0.91b (Castresana, 2000) with default settings was used as a guide to select the reliably aligned nucleotide sites.

Phylogenetic trees were constructed using maximum likelihood (ML) (Felsenstein, 1981), neighbor-joining (NJ) (Saitou & Nei, 1987) and Bayesian inference (BI) (Larget & Simon, 1999). The general time reversible model incorporating gamma (GTR+Γ) (Lanave *et al*., 1984; Gu *et al*., 1995) was used to correct for multiple substitutions for all model-based methods (ML, NJ and BI). ML trees were constructed using the PhyML (version 3.0) package (Guindon *et al*., 2010) with tree searching following a heuristic procedure with 10 random start trees and best of nearest-neighbour-interchange and subtree-pruning-regrafting branch swapping. NJ analysis was performed using the PAUP* (version 4.0b10) package (Swofford, 2002). For NJ analysis, model parameters were estimated following an iteration process; for each tree the parameters were estimated and used to build the next tree until there was no further improvement of the likelihood score. Bootstrap resampling (Felsenstein, 1985) with 1000 replicates was undertaken for ML and NJ trees. BI analysis was undertaken using the MrBayes (version 3.1.2) package (Ronquist & Huelsenbeck, 2003). Two independent runs with four chains of a Markov Chain Monte Carlo (MCMC) algorithm were used to explore the tree space. BI analysis was conducted for 5 million generations with tree sampling every 100 generations. To ensure adequate chain swapping, a range of heating parameters were tested with the optimal parameter used to construct the final trees. Only after the Bayesian MCMC searches had reached a stationary phase (indicating convergence of the

chains onto the target distribution) was the run ended. A consensus tree was built using the last 75% of trees (burnin=12501).

5.2.4 Nucleotide Sequence Accession Numbers

Nucleotide sequences generated in this study have been given the GenBank accession numbers MN644802-MN644842.

5.3 Results

Sequences of approximately 4000 nucleotides of the rRNA gene cluster were generated for 39 hygrophilan genera and two non-hygrophilan genera. Phylogenetic trees for the Hygrophila were constructed using 3112 unambiguously aligned nucleotide sites of the LSU and 5.8S gene. The ITS-2 region of the rRNA cluster was removed completely from all analyses as it could not be aligned across all taxa due to its high variability. The glacidorbids *Glacidorbis hedleyi* and *Tasmodorbis punctatus* were used as outgroups to root the phylogenetic tree.

The Bayesian inference tree of the Hygrophila is shown in Figure 5.1. Highly consistent topologies were observed for all phylogeny reconstruction methods used (BI, Figure 5.1; ML, Apendix 5.1; NJ, Apendix 5.2). The phylogenetic tree shows a clear basal division between the Chilinoidea on one hand and a clade comprising all the remaining hygrophilan taxa (Lymnaeoidea) on the other. This division is strongly supported in 100% ML, 99% NJ bootstrap replicates and with a Bayesian posterior probability of 1.00. The Chilinidae and Latiidae are fully supported (100% ML, 100% NJ bootstraps and PP=1.00 BI) as sister taxa.

The Acroloxidae are resolved as a monophyletic group in the tree, fully supported in 100% ML, 100% NJ bootstraps and PP=1.00 BI. This family has a sister group relationship to a clade comprising the Bulinidae, Burnupiidae, Lymnaeidae, Physidae and Planorbidae (100% ML, 99% NJ bootstraps and PP=1.00 BI). The Physidae are fully supported (100% ML, 100% NJ bootstraps and PP=1.00 BI) as a monophyletic group and resolve as a sister group to a clade comprising the Bulinidae, Burnupiidae, Lymnaeidae and Planorbidae (80% ML, 79% NJ bootstraps and PP=0.99 BI). The Physidae are split

into two lineages, the first comprising *Sibirenauta elongatus* that belongs to the subfamily Aplexinae and the second comprising *Stenophysa minor* and *Mayabina sp.* from Aplexinae in addition to *Physa fontinalis*, *Physella gyrina* and *Haitia mexicana* from the Physinae (100% ML, 100% NJ bootstraps and PP=1.00 BI). Of these genera, *Stenophysa minor* and *Mayabina sp.* cluster together as sister taxa with high support (98% ML, 92% NJ bootstraps and PP=1.00 BI).

The Lymnaeidae form a well resolved monophyletic group in the tree, fully supported in 100% ML, 100% NJ bootstraps and PP=1.00 BI. They have a sister group relationship with a clade comprising the Bulinidae, Burnupiidae and Planorbidae (72% ML, 83% NJ bootstraps and PP=0.97 BI). Within the Lymnaeidae, the subfamily Amphipepleinae are strongly supported (99% ML, 99% NJ bootstraps and PP=1.00 BI) as a monophyletic group within which the sister relationship between *Radix sp*. and *Peregriana sp*. is well supported in both the ML and BI analyses (86% ML bootstraps and PP=1.00 BI), although it is not supported in the NJ tree. The subfamily Lymnaeinae is not supported as a monophyletic group due to the nesting of Lancinae within the group. Within the Lymnaeinae, *Bakerilymnaea bulimoides* and *Polyrhytis apicina* cluster together as sister taxa (70% ML, 67% NJ bootstraps and PP=0.84 BI). Likewise, *Stagnicola sp.* has a sister taxon relationship to *Corvusiana sp.* with moderate support (75% ML, 73% NJ bootstraps and PP=0.96 BI).

The Burnupiidae have a sister group relationship with a clade comprising the Bulinidae and Planorbidae with robust support (100% ML, 98% NJ bootstraps and PP=1.00 BI). There is moderate support (70% ML, 60% NJ bootstraps and PP=0.98 BI) for a group that comprises the Planorbidae and Bulinidae. This group is divided into two further clades; one major and one minor. The major clade corresponds to the subfamily Planorbinae and is fully supported (100% ML, 100% NJ bootstraps and PP=1.00 BI). The minor clade comprises the family Bulinidae and the subfamily Ancylinae and is supported in 68% ML, 56% NJ bootstraps and PP=1.00 BI. The monophyly of the Ancylinae is fully supported (100% ML, 100% NJ bootstraps and PP=1.00 BI).

The tribe relationships within the Planorbinae are shown in Figure 5. 2. The monophyly of the tribe Planorbini is well supported in the tree (80% ML, 79% NJ bootstraps and PP=1.00 BI) and there is a

well resolved sister relationship between *Choanomphalus maacki* and *Planorbis planorbis* (86% ML, 88% NJ bootstraps and PP=1.00 BI) within this tribe. The monophyly of the tribe Helisomatini also received high support (91% ML, 98% NJ bootstraps and PP=1.00 BI) and within this tribe, there is a sister relationship between *Planorbula campestris* and *Promenetus exacuous* with moderate support (69% ML, 71% NJ bootstraps and PP=0.99 BI). The Segmentinini, represented by only a single species (*Segmentinina nitida*) clusters with the Planorbini in the tree with high support (100% ML, 98% NJ bootstraps and PP=1.00 BI). The Camptoceratini is also represented by just a single species (*Planorbarius corneus*) in the tree clusters with the Helisomatini with moderate support (66% ML, 80% NJ bootstraps and PP=0.88 BI).

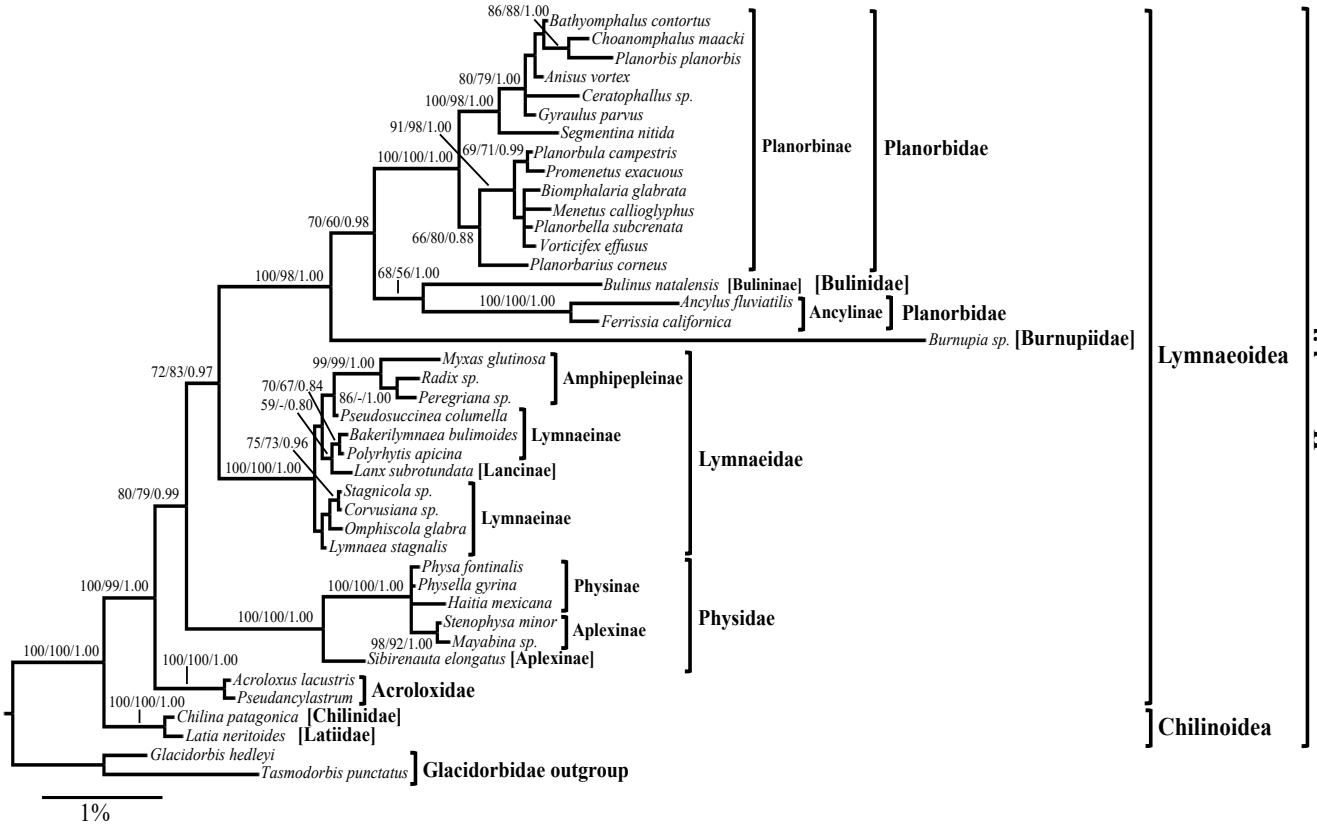


Figure 5.1. Bayesian phylogenetic tree of the Hygrophila based on 3112 unambiguously aligned nucleotide sites of the LSU (and 5.8S) gene. Values on nodes represent bootstrap support (1000 replicates) for maximum likelihood and neighbour-joining and posterior probabilities for Bayesian inference (based on the last 75% of trees), respectively. Bootstrap support values < 50% and posterior probabilities < 0.7 are not shown. The scale bar represents one substitutional change per 100 nucleotide positions. Classifications follow Bouchet *et al*. (2017).

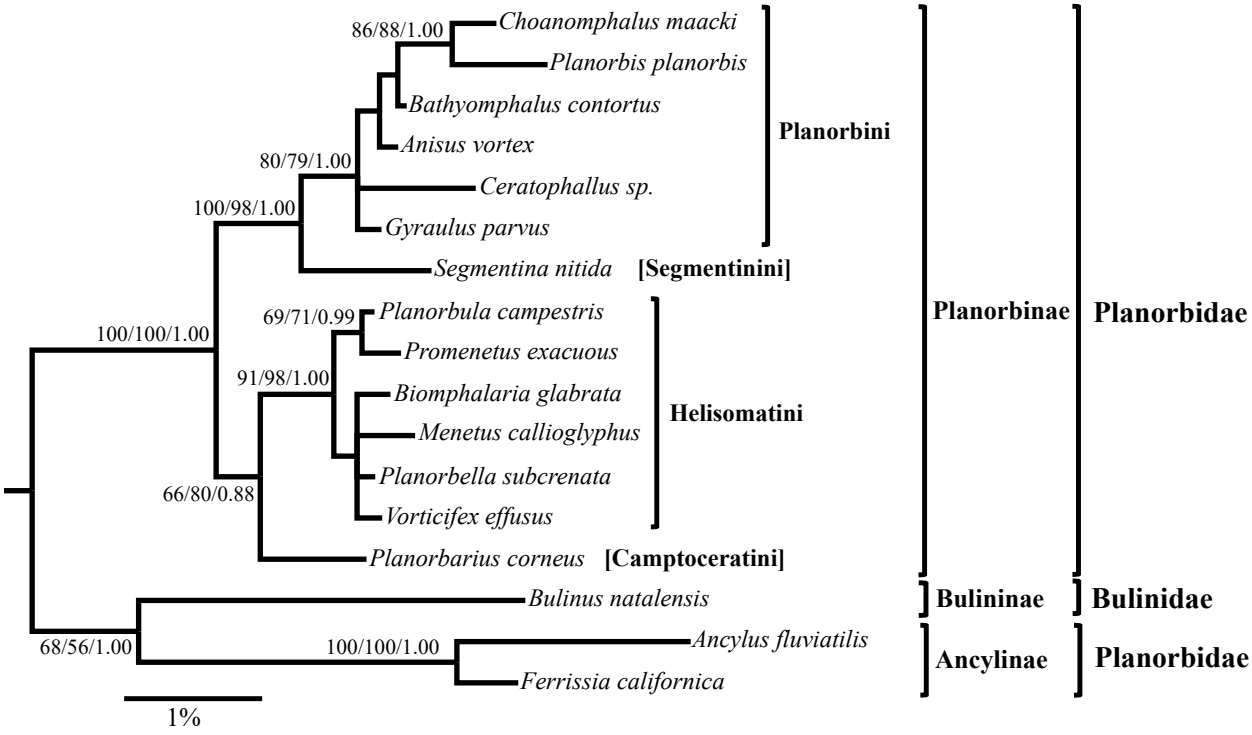


Figure 5.2. Bayesian phylogenetic tree of the Planorbidae and Bulinidae (derived from Figure 1) shows tribes within the subfamily Planorbinae. Values on nodes represent bootstrap support (1000 replicates) for maximum likelihood and neighbour-joining and posterior probabilities for Bayesian inference (based on last 75% of trees), respectively. Bootstrap support values less than 50% and posterior probabilities less than 0.7 are not shown. The scale bar represents 1 substitutional change per 100 nucleotide positions. Subfamily and tribe classifications follow Bouchet *et al*. (2017).

5.4 Discussion

The phylogenetic relationships of the Hygrophila and the validity of families and subfamilies within the Hygrophila were examined based on 3112 unambiguously aligned nucleotide sites of the LSU and 5.8S gene of 39 hygrophilan genera. The phylogenetic study provides the most comprehensive sampling of the hygrophilan clade to date.

5.4.1 Phylogenetic Relationships of the Hygrophila

In our phylogenetic analyses, there is a clear basal division between the Chilinoidea on one hand and a clade comprising all the remaining hygrophilan taxa (Lymnaeoidea) on the other. This principal division of the Hygrophila into Chilinoidea and Lymnaeoidea, as well as the sister group relationship of the Chilinidae and Latiidae is consistent with previous molecular studies (Klussmann-Kolb *et al*.,

2008; Dinapoli *et al*., 2010; Jörger *et al*., 2010; Goebbeler & Klussmann-Kolb, 2011; Davison *et al*., 2016). The interrelationships among the Acroloxidae, Bulinidae, Burnupiidae, Physidae, Lymnaeidae and Planorbidae are also reasonably well resolved. The monophyly of the Acroloxidae, Lymnaeidae and Physidae is well supported in our phylogenetic tree, consistent with previous molecular studies (Dayrat *et al*., 2011; Soldatenko & Petrov 2019). However, the Planorbidae as defined by Bouchet *et al*. (2017), are not supported as a monophyletic group because the Bulinidae cluster within the Planorbidae. The inclusion of the bulinids within the Planorbidae has been shown in previous molecular studies (Morgan *et al*., 2002; Albrecht *et al*., 2004; Jørgensen *et al*., 2004; Albrecht *et al*., 2007), but the taxonomic level of the bulinids is controversial. Baker (1945), Vaught (1989), Albrecht *et al*. (2007) and most recently Bouchet *et al*. (2017) classified them as a family, while Hubendick (1955), Zilch (1959-1960), Hubendick (1978) and Bouchet & Rocroi (2005) treated them as a subfamily. In our analyses, the Planorbidae would be a monophyletic group if the Bulinidae were classified as a subfamily rather than a family as they were in the previous classifications of Hubendick (1955), Zilch (1959-1960), Hubendick (1978) and Bouchet & Rocroi (2005).

The ancylids have also variously been classified as a family (Thiele, 1931; Zilch, 1959-1960; Burch, 1982, Burch, 1989; Vaught, 1989), subfamily (Hubendick, 1978) and tribe (Bouchet & Rocroi, 2005). In the most recent classification of Bouchet *et al*. (2017), the Ancylidae are now classified as a subfamily within the Planorbidae. In our tree, the deep clustering of *Ancylus* and *Ferrissia* relative to other planorbids and their sister group relationships to *Bulinus* is perhaps supportive of this group being distinct from the other Planorbidae, consistent with former classifications in which the Ancylidae were classified as a separate family to the Planorbidae.

Based on our phylogeny two alternative revisions to the taxonomy of the Hygrophila must be considered: (1) retain the Bulinidae as a family but reclassify the Ancylinae as a family outside the Planorbidae consistent with previous classifications; or (2) lower the Bulinidae to subfamilial rank alongside the Ancylinae within the family Planorbidae.

The Burnupiidae have a sister group relationship with the clade comprising the Bulinidae and Planorbidae. This placement is consistent with the findings of Albrecht *et al*. (2007).

We include six representatives from the Physidae belonging to two subfamilies, the Aplexinae and the Physinae. The Aplexinae are not recovered as a monophyletic group in our tree, with *Stenophysa minor* and *Mayabina sp*. resolving separately from *Sibirenauta elongatus*, and instead grouping with the Physinae. The clustering of *Stenophysa* (Aplexinae) with the Physinae was also reported in Wethington & Lydeard, (2007), who suggested that the separation of the Physidae into Aplexinae and Physinae is valid only if *Stenophysa* is included in the Physinae. In our phylogeny, *Stenophysa* is robustly resolved as the sister taxon to the aplexinid *Mayabina sp*. This is the first time that *Mayabina* has been included in a molecular phylogeny. A recent classification by Bank (2017) placed *Stenophysa* within the Physinae which is consistent with the results of this study. Our phylogeny suggests that *Mayabina* should also be reclassified and assigned to the subfamily Physinae instead of the Aplexinae.

Eleven lymnaeid genera belonging to three subfamilies (Amphipeplinae, Lancinae and Lymnaeinae) are included in this analysis. The Amphipeplinae are robustly supported as a monophyletic subfamily in our analyses consistent with the molecular studies of Correa *et al*. (2010) and Aksenova *et al*. (2018). However, the Lymnaeinae are not recovered as a clade in our tree. This is consistent with the findings of Aksenova *et al*. (2018) who also found *Lanx* (Lancinae) to cluster within the Lymnaeinae in their tree. The Lymnaeinae were recovered as monophyletic in the tree of Correa *et al*. (2010) but they did not include *Lanx* (Lancinae) in their study.

The relationship of *Lanx subrotundata* to the remaining lymnaeids within the subfamily Lymnaeinae is not well resolved in our tree. Campbell *et al*. (2017) similarly found that the phylogenetic relationships of lancines within the Lymnaeidae were not well recovered. In early morphological studies, Baker (1925) demonstrated that the anatomical characters of *Lanx* were not similar to the characters of other lymnaeid genera. While, Walter (1969) suggested that *Lanx* was closely related to other lymnaeids and the generic status of the lancines was not justified. Campbell *et al*. (2017) indicated that three

morphological synapomorphies could be recognised within the lancines, “the fully patelliform shell, shape of the penial complex and C-shaped to circular columellar muscle”.

Within the Planorbidae, the Ancylinae and Planorbinae are recovered as monophyletic subfamilies. Within the Planorbinae, the tribes Planorbini and Segmentinini cluster together as sister taxa. This supports the cladistic analysis in the morphological study of Meier-Brook (1984) which showed a sister group relationship between Planorbini and Segmentinini as both tribes have a separate prostate duct. Previous molecular studies (Morgan *et al*., 2002; Jørgensen *et al*., 2004; Albrecht *et al*., 2007) also supported this relationship. The phylogenetic relationships within the Planorbini are not well resolved and vary according to the phylogeny reconstruction method, with the exception of the sister group relationship between *Choanomphalus maacki* and *Planorbis planorbis* that is well resolved. This is consistent with the results of Albrecht *et al*. (2007) who showed a close relationship between *Planorbis* and *Choanomphalus*.

The other two tribes within the Planorbinae (Camptoceratini and Helisomatini) also cluster together as sister groups, although the support for this relationship is equivocal. This agrees with the molecular analyses of Morgan *et al*. (2002) and Jørgensen *et al*. (2004) who showed a close relationship between *Planorbarius* and Helisomatini, although without significant support. The phylogenetic position of *Planorbarius* in the molecular analysis of Albrecht *et al*. (2007) was not clear. Traditionally, *Planorbarius* was grouped with *Helisoma* by Hubendick (1955) based on morphological characters as they share a lateral penis pore, combination of long marginals in the radula and the lack of a convoluted pseudobranch. Within Helisomatini, the phylogenetic relationships are not well resolved except for the sister group relationship between *Promenetus exacuous* and *Planorbula campestris.* Based on the morphological characters, Hubendick (1955) indicated that there is no accessory duct at all in *Planorbula*, *Promenetus* and *Menetus*.

5.4.2 Chiral evolution

The construction of a comprehensive molecular phylogeny for the Hygrophila allows the mapping of the evolution of chirality within the group. Among the hygrophilan families, the Acroloxidae,

Chilinidae, Latiidae and Lymnaeidae have dextral coiling, while Bulinidae, Burnupiidae, Physidae and Planorbidae have sinistral coiling. By mapping chirality onto our phylogeny, two equally parsimonious hypotheses arise (Figure 5. 3). The first hypothesis supposes that sinistrality evolved once from a dextral common ancestor, with Lymnaeidae subsequently reverting back to dextral (Figure 5. 3A). The second hypothesis again assumes that sinistrality evolved from a dextral ancestor but on two separate occasions, in Physidae and Planorbidae (Figure 5. 3B).

Any discussion on chiral evolution hinges on the degree of mating succes between individuals with the same coiling direction (intrachiral mating), relative to that between individuals with the opposite coiling direction (interchiral mating). In dextral species, the genital opening locates on the right-hand side of the body while in sinistral individuals it locates on the left-hand side. It has been known for some time that this asymmetry could create 'insurmountable barriers' to interchiral mating (Schilthuizen & Davison, 2005). In snails with spherical shells, such as *Arianta arbustorum* and *Helix pomatia*, sinistral and dextral individuals may show total premating isolation (Gittenberger, 1988). Meisenheimer (1912) confined a dextral and a sinistral individual of *H. pomatia* in a container and observed that 'for days and weeks the animals fatigue each other in courtship, without achieving a final copulation'. Likewise, Hesse (1914) pointed out that interchiral copulation is impossible in *Helix aspersa* and *Helix pomatia*. Individuals (of a non-selfing species) with a coiling direction opposite to that of the majority of the population will be at a selective disadvantage as they will have difficulty in finding a mate. As a result, dimorphism in a single population is not a stable condition, and populations will have a tendency of being driven to monomorphism for the commonest morph (Van Batenburg & Gittenberger, 1996). The mating behaviour of hygrophilan snails such as *Lymnaea*, is relatively different from that of stylommatophorans. *Lymnaea* is dimorphic for shell coiling with mixed populations of dextral and sinistral individuals, though sinistral specimens are extremely rare in nature (Harada *et al*., 2004; Utsuno *et al*., 2011). Although a variation in coiling direction may not be a critical factor in snail speciation, 'it could act as a mechanism to reduce mating between closely related species' (Schilthuizen & Davison, 2005).

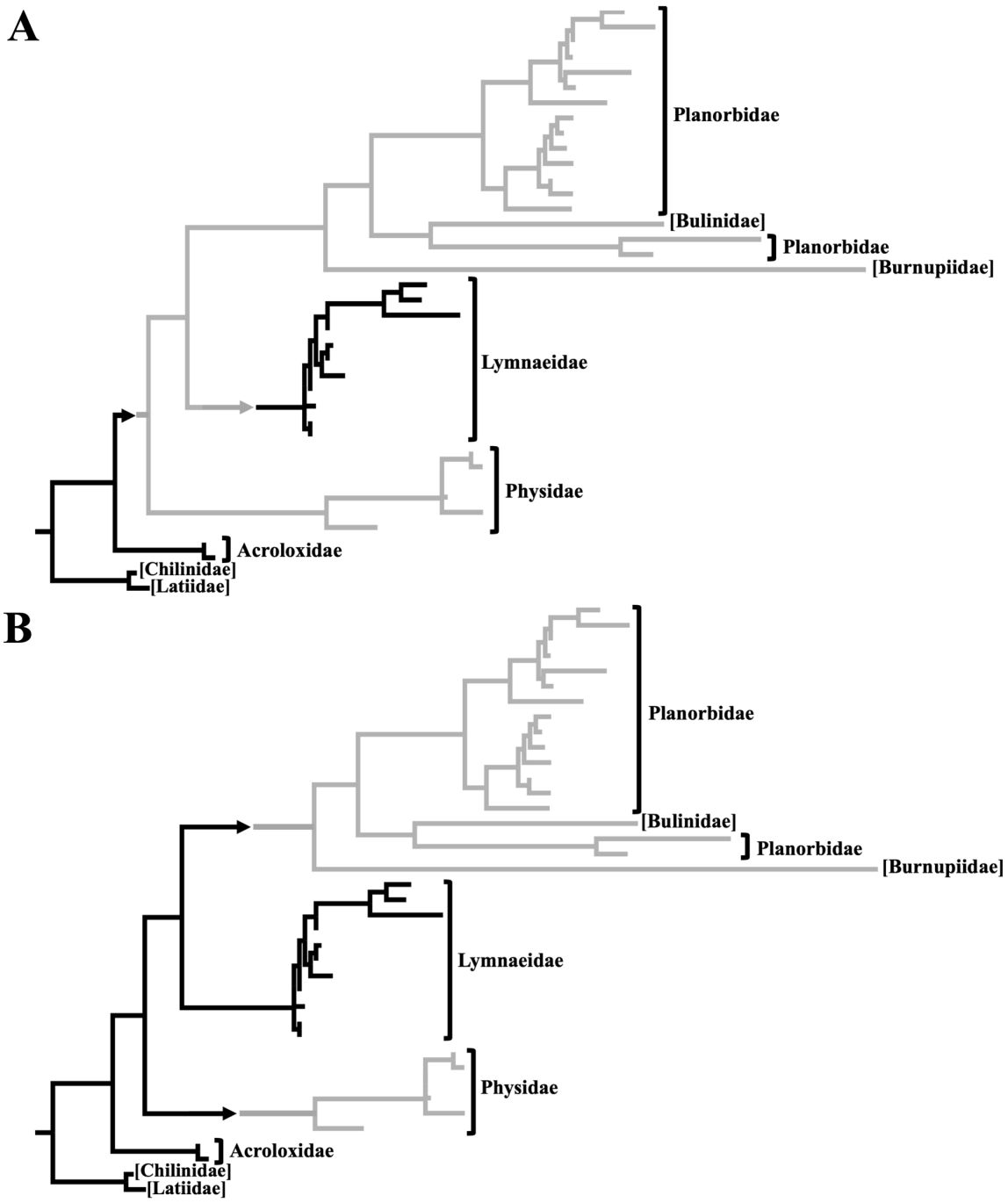


Figure 5.3. Two equally most parsimonious hypotheses for evolution of chirality within the Hygrophila (dextral, black; sinistral, grey). Either sinistrality evolved once, from a dextral ancestor, and then was lost in the Lymnaeidae (A) or sinistrality evolved twice, in Planorbidae and Physidae (B).

5.5 Conclusion

The phylogenetic study provides the most comprehensive sampling of the hygrophilan clade to date. The principle division in the Hygrophila is shown to be between the Chilinoidea and the Lymnaeoidea. The Acroloxidae, Lymnaeidae and Physidae are fully supported as monophyletic families. However, the Planorbidae are not supported as a monophyletic group due to the clustering of Bulinidae within the family. The Aplexinae are not recovered as a monophyletic subfamily within the Physidae. In the Lymneaidae, there is strong support for the monophyly of the Amphipepleinae, though the phylogenetic relationships within the remaining lymnaeids are not resolved. The Ancylinae and Planorbinae are recovered with full support as monophyletic subfamilies within the Planorbidae. Based on the result of the molecular phylogeny, a taxonomic revision must be considered for the hygrophilan families. By mapping chirality onto our phylogeny, two equally parsimonious hypotheses arise.

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Chapter 6: Resolving the Deep Relationships of the Families within the Hygrophila with special emphasis on the Lymnaeoidea

Abstract

The interrelationships among families within the Hygrophila are uncertain, with different studies providing conflicting results. We undertook a phylogenetic analysis of the Hygrophila based on four standard markers [LSU (and 5.8S) gene, SSU gene, the H3 gene and the 1st and 2nd codon positions of the CO1 gene] for representative taxa from the main hygrophilan groups within a framework of panpulmonate taxa combined with a phylogenomic analysis based on transcriptome data for a representative taxon from each of the four main lymnaeoidean families (Acroloxidae, Lymnaeidae, Physidae and Planorbidae). Both the monophyly of the Hygrophila and the principle division of the Hygrophila into two clades, the Chilinoidea and Lymnaeoidea are robustly supported in our analysis based on standard markers. However, the interrelationships among the lymnaeoidean families remained unclear. The interrelationships of the Acroloxidae, Lymnaeidae, Physidae and Planorbidae are well resolved in our phylogenomic analysis, with the Acroloxidae recovered as the sister group to a clade comprising the Lymnaeidae, Physidae and Planorbidae, the Physidae recovered as the sister group of a cluster comprising the Lymnaeidae and Planorbidae, and the Lymnaeidea and Planorbidae recovered as sister taxa.

6.1 Introduction

The Hygrophila are the principle group of freshwater aquatic snails within the Panpulmonata, comprising eight families, the Chilinidae, Latiidae, Acroloxidae, Lymnaeidae, Physidae, Planorbidae, Bulinidae and Burnupiidae, in the most recent classification of gastropod molluscs (Bouchet *et al*., 2017). They were originally placed within the Basommatophora alongside the Amphibolacea (Amphibolidae), Patelliformia (Siphonariidae and Gadiniidae (=Trimusculidae)) and Actophila (Otinidae and Ellobiidae) (Thiele, 1931). The Trimusculidae were later moved from the Basommatophora to the Eupulmonata by Haszprunar & Huber (1990). The Basommatophora were then divided by Nordsieck (1992) into two orders; the Thalassophila comprising the Amphiboloidei and Siphonariodei, and the Hygrophila comprising the Lymnaeoidei and Chilinoidei. Bouchet & Rocroi, (2005) classified the Basommatophora as an informal group comprising the clade Hygrophila and two superfamilies, the Amphiboloidea and Siphonarioidea, based on the morphological and molecular data available at the time. However, the monophyly of the Basomatophora has been rejected in the morphological studies of Barker (2001) and Dayrat *et al*. (2001) as well as in several molecular studies (Grande *et al*., 2004; Klussmann-Kolb *et al*., 2008; Dinapoli *et al*., 2010; Holznagel *et al*., 2010; Jörger *et al*., 2010; Göbbeler & Klussmann-Kolb, 2011) and Bouchet *et al*. (2017) dropped the Basommatophora from their gastropod classification, adopting instead the taxonomy of Jörger *et al*. (2010) in which all the basommatophoran subgroups were moved to the Panpulmonata subcohort.

The Hygrophila are the largest remnant of the Basommatophora. Their monophyly has been supported in early morphological studies (Hubendick, 1978; Nordsieck, 1992; Barker, 2001) as well as in most of the molecular analyses (Dayrat *et al*., 2001; Klussmann-Kolb *et al*., 2008; Dinapoli *et al*., 2010; Jörger *et al*., 2010; Goebbeler & Klussmann-Kolb, 2011; Kocot *et al*., 2013). However, the monophyly of the Hygrophila is rejected in the molecular phylogeny of Dayrat *et al*. (2011), which undertook broad taxon sampling of the Pulmonata for three molecular markers (18S, 16S and cytochrome oxidase subunit I) and in which the Chilinoidea fall separately to the Hygrophila as the sister group of the Amphiboloidea.

The position of the Hygrophila within the broader Panpulmonata has proven to be controversial. The Hygrophila were shown to be closely related to the Ellobiidae in the early morphological analysis of Hubendick (1978) while in the morphological classification of Nordsieck (1992), the Hygrophila were shown as the sister group to the Thalassophila. Moreover, in the morphological study of Barker (2001) based on a parsimony analysis of 72 characters, the Hygrophila were placed as the sister group to a clade comprising the Trimusculidae, Ellobiidae and Geophila. Similarly, in molecular analyses, the placement of the Hygrophila within the broader Panpulmonata also remains uncertain. The Hygrophila were shown as the sister group to a clade comprising Amphiboloidea and Pyramidelloidea in the analyses of Klussmann-Kolb *et al*. (2008) based on four markers (18S, 28S, 16S and CO1). Holznagel *et al*. (2010) using 3.8 kb of the 28S gene showed that the Hygrophila have a sister group relationship to a clade comprising Siphonariidae and Eupulmonata with moderate support while Jörger *et al*. (2010) using 28S, 18S, 16S and COI sequences showed that the Hygrophila have a sister group relationship with a clade comprising the Acochlidia and Eupulmonata, though without significant support. Moreover, two recent phylogenetic studies (Kocot *et al*., 2013; Romero *et al*., 2016) placed the Hygrophila as the sister group to the Stylommatophora. The first (Kocot *et al*., 2013) was based on a phylogenomic analysis of 102 nuclear protein-coding gene regions for 19 gastropods, though it had limited taxon sampling and did not include the key former basommatophoran groups (Glacidorboidea, Amphiboloidea or Pyramidelloidea). The second (Romero *et al*., 2016) included 50 gastropod taxa, though it was based only on mitochondrial sequence data.

The interrelationships of the Acroloxidae, Physidae, Lymnaeidae and Planorbidae within the Hygrophila remain uncertain with several studies showing conflicting results. In the morphological study of Hubendick (1978), the Hygrophila were divided into two main groups. The first included the Acroloxidae, Chilinidae and Latiidae. The second included the Lymnaeidae, Physidae and Planorbidae. Nordsiek (1992) also divided the Hygrophila into two groups (Chilinoidei and Lymnaeoidei) but this time the Acroloxinei were placed with the liminc basommatophoran families as the sister group to a clade comprising the Lymnaeoinei and Planorbinei in the Lymnaeoidei. Likewise, in the morphological study of Barker (2001), the Hygrophila were split into two lineages, the first comprising the Chilinidae

and the second comprising the remaining hygrophilan families but surprisingly the Latiidae were placed within the limnic basommatophoran families in Barker's tree.

Molecular phylogenetic studies of the Hygrophila have also provided contradictory results regarding the interrelationships among the Acroloxidae, Physidae, Lymnaeidae and Planorbidae (Walther *et al*., 2006; Klussmann-Kolb *et al*., 2008; Holznagel *et al*., 2010; Dayrat *et al*., 2011; Davison *et al*., 2016; Soldatenko & Petrov, 2019) and in many of these studies the interrelationships of the lymnaeoidean families were not precisely indicated (Walther *et al*., 2006; Klussmann-Kolb *et al*., 2008; Holznagel *et al*., 2010; Dayrat *et al*., 2011). However, the molecular phylogeny by Davison *et al*. (2016) using the 18S and 28S genes provided good support for the interrelationships of Acroloxidae, Physidae, Lymnaeidae and Planorbidae, though taxon sampling was limited. A more recent molecular analysis by Soldatenko & Petrov (2019) based on available hygrophilan 18S and CO1 sequences from GenBank also provided robust support for the interrelationships of the hygrophilan families, though only Bayesian analysis was used in this study. In both Davison *et al*. (2016) and Soldatenko & Petrov (2019), the Physidae and Lymnaeidae were recovered as sister groups, however the studies were inconsistent in their placement of the Acroloxidae and Planorbidae.

The interrelationships among the hygrophilan families are reasonably well resolved in chapter 5. However, they are not fully supported and inconsistent with the results of Davison *et al*. (2016) and Soldatenko & Petrov (2019). This chapter aims to better resolve the interrelationships among the lymnaeoidean families by undertaking a more detailed phylogenetic analyses of the Hygrophila based on four standard markers (LSU (incorporating 5.8S), SSU, H3 and CO1) for representative taxa from the main hygrophilan groups within a framework of panpulmonate taxa combined with a phylogenomic analysis based on transcriptome data for a representative taxon from each of the four main lymnaeoidean families (Acroloxidae, Physidae, Lymnaeidae and Planorbidae).

6.2 Methods

6.2.1 Biological Material

A total of 51 species belonging to 50 panpulmonate genera (including 14 hygrophilan genera) were included in this study. Three non-panpulmonate taxa were also included with the caenogastroods *Pomacea sp.* and *Cyclophorus herklotsi* used as outgroups to root the phylogenetic trees. Details of the specimens, sampling localities and the collectors are given in Table 6.1.

Table 6.1. Details of specimens, sampling localities and collectors. Classification of samples follows Bouchet *et al*. (2017).

Taxonomic rank	Species	Collection/Location	Collector
Phylum Mollusca, Class Gastropoda, Subclass Heterobranchia, Cohort Tectipleura, Subcohort Panpulmonata			
Superorder Sacoglossa **Superfamily Oxynooidea**			
Family Cylindrobullidae	*Cylindrobulla beauii* (P. Fischer, 1857)	FK-916, Jeffrey Key, NE tip of Jeffrey Key, Florida Keys, USA	Provided by Field museum Chicago_326526R
Volvatellidae	*Volvatella sp.* Pease, 1860	Madang Province, Astrolabe bay, Papua New Guinea	Provided by Muséum national d'Histoire naturelle (MNHN). MNHN-IM-2013-14281
Superfamily Plakobranchoidea			
Family Hermaeidae	*Hermaea bifida* (Montagu, 1816)	Connemara, Ireland	Bernard Picton
Family Limapontiidae	*Limapontia depressa* (Alder & Hancock, 1862)	Dorset, England, UK	Steve Trewhella
	Placida dendritica (Alder & Hancock, 1843)	Connemara, Ireland	Bernard Picton
	Ercolania margaritae (Burn, 1974)	Paterson, Cape, Victoria, Australia	Provided by Museums Victoria. T J. Hales. NMV F 133083
Family Plakobranchidae	*Elysia viridis* (Montagu, 1804)	Dorset, England, UK	Steve Trewhella
Superorder Siphonarimorpha, Order Siphonariida **Superfamily Siphonarioidea**			
Family Siphonariidae	*Siphonaria pectinata* (Linnaeus, 1758)	Zamara Los Atunes, Spain	S. Hawkins
	Siphonaria zelandica (Quoy and Gaimard, 1833)	Manu Bay, marine border shore, New Zealand	G. Barker
Superorder Pylopulmonata **Superfamily Pyramidelloidea**			
Family Pyramidellidae	*Boonea sp*. R. Robertson, 1978	South China sea	Provided by MNHN, MNHN-IM-2013-50057
	Turbonilla cummingi (Hori & Okutani, 1997)	St. John's Island, TMSI giant clam hatchery, Singapore	Lee Kong Chian Natural History MuseumL (ZRC.MOL.7379)
Superfamily Glacidorboidea Family Glacidorbidae	*Tasmodorbis punctatus* Ponder & Avern, 2000	Murchison Hwy, Deep Gully Creek, Tasmania, Australia	Australian museum (C.463949)
	Glacidorbis hedleyi Iredale, 1943	Blue Mountains, Boyd River, New South Wales, Australia	Australian museum (C.463947)
Superfamily Amphiboloidea Family Amphibolidae	*Salinator tecta* Golding, Ponder & Byrne, 2007	Boronia Park, Lane Cuve River, Sydney, Australia	R. Gozding

	Phallomedusa sp Golding, Ponder & Byrne, 2007	Boronia Park, Lane Cuve River, Sydney, Australia	R. Gozding
	Amphibola crenata (Gmelin, 1791)	Waihi Estuarine, New Zealand	G. Barker
Superorder Acochlidimorpha, Superfamily Acochlidioidea			
Family Hedylopsidae	*Hedylopsis spiculifera* (Kowalewsky, 1901)	Rovinj, Kleine Salinebucht, Istria	Provided by Zoologische Staatssammlung München (ZSM), M. Schroedl, ZSM Mol 20100450
Family Acochlidiidae	*Strubellia wawrai* Brenzinger, Neusser, Jörger & Schrödl, 2011	Papua New Guinea	Provided by MNHN, IM-2013-54739
Superfamily Parhedyloidea			
Family Parhedylidae [=Microhedylidae]	*Pontohedyle milatchevitchi* (Kowalewsky, 1901)	SW of Zadar, Pazman Island, Croatia	Provided by ZSM, M. Schroedl ZSM Mol 20130948
Superorder Hygrophila Superfamily Chilinoidea			
Family Chilinidae	*Chilina patagonica* (G. B. Sowerby II, 1874)	Golfo Elefantes, Region XI, Chile	David Reid
Family Latiidae	*Latia neritoides* (Gray, 1850)	Wainui, New Zealand	G. Barker
Superfamily Lymnaeoidea			
Family Acroloxidae	*Acroloxus lacustris* (Linnaeus, 1758)	West side of River Kozinka, Kievskaya Oblast, Ukraine	D.W. Taylor
	Pseudancylastrum sp. Lindholm, 1909	Lake Baikal, Russia	T. Sitmikova
Family Physidae	*Physa fontinalis* (Linnaeus, 1758)	Embayment from west side of River Desna, Kievskaya Oblast, Ukraine	D.W. Taylor
	Sibirenauta elongatus (Say, 1821)	Okanagan-Similkameen District, Penticton, British Columbia, Canada	D.W. Taylor
Family Lymnaeidae	*Bakerilymnaea bulimoides* (Lea, 1841) [= *Fossaria bulimoides*]	Corner of Alvadore Road, Lane County, Oregon, USA	D.W. Taylor
	Stagnicola sp. Jeffreys, 1830	Pond of Institute of Fish Culture, Kievskaya Oblast, Kiev, Ukraine	D.W. Taylor
	Lanx subrotundata (Tryon, 1865)	North Umpqua River, Singleton Park, Douglas County, Oregon, USA	D.W. Taylor
Family Bulinidae	*Bulinus natalensis* (Krauss in Kuster, 1841)	Lake Siboya, South Africa	Provided by Biomedical Parasitology Section, Natural History Museum (NHM) (from culture no. 272)
Family Burnupiidae	*Burnupia sp.* B. Walker, 1912	South Africa	Provided by Natal Museum
Family Planorbidae	*Planorbis planorbis* (Linnaeus, 1758)	West side of River Kozinka, west of village Letki, Kievskaya Oblast, Ukraine	D.W. Taylor
	Planorbella subcrenata (Carpenter, 1857)	Okanagan-Similkameen District, west end of Yellow Lake, British Columbia, Canada	D.W. Taylor
	Ferrissia californica (Rowell, 1863)	T04-4204, Fern Ridge Reservoir, Lane County, Oregon, USA	D.W. Taylor
Superorder Eupulmonata, Order Ellobiida Superfamily Ellobioidea			
Family Ellobiidae	*Melampus luteus* (Quoy & Gaimard, 1832	Souilla, Mauritius	O. Griffiths
	Laemodonta sp.	Suralaya, West Java, Indonesia	B. Dharma
	Blauneria heteroclita (Montagu, 1808)		Provided by NHM
	Myosotella myosotis (Draparnaud, 1801)	Dorset, England, UK	Steve Trewhella
	Leucophytia bidentata (Montagu, 1808)	Dorset, England, UK	Steve Trewhella

	Cassidula nucleus (Gmelin, 1791)	East point, Darwin, Northern Territory, Australia	Provided by NHM. NHM_2394
	Ophicardelus sulcatus (H. Adams & A. Adams, 1854)	Australia, Nudgee, Brisbane	G. Barker
	Carychium tridentatum (Risso, 1826)	Abelheira, São Miguel, Azores	P. Mordan
Family Otinidae	*Otina ovata* (T. Brown, 1827)	Cave at West End of Freshwater Bay. Isle of Wight, UK	David Reid
Family Trimusculidae	*Trimusculus sp.*	Plage de Lavanono, Madagascar	Provided by MNHN. IM-2009-16399
Clade Geophila, Order Stylommatophora, Suborder Achatinina ['Achatinoid Clade'] **Superfamily Achatinoidea**			
Family Subulinidae	*Subulina striatella* (Rang, 1831)	Kew Gardens (introduced)	F. Naggs
Family Achatinidae	*Lissachatina fulica* (Bowdich, 1822) [= *Achatina fulica*]	Unknown (Zool. Soc. Lond. Colln.)	P. Pearce-Kelly
Superfamily Streptaxoidea Family Streptaxidae	*Gonaxis quadrilateralis* (Preston, 1910)	Reunion	O. Griffiths
Suborder Helicina ['Non-Achatinoid Clade'], Infraorder Pupilloidei [Orthurethra] **Superfamily Pupilloidea**			
Family Valloniidae	*Vallonia excentrica* Sterki, 1892	São Miguel, Azores	P. Mordan
Infraorder Limacoidei ['Limacoid Clade'] **Superfamily Limacoidea, Superfamily Limacoidea**			
Family Vitrinidae	*Vitrina pellucida* (O. F. Müller, 1774)	Kirkdale, Derbyshire, UK	C. Wade
Infraorder Helicoidei ['Helicoid Clade'] **Superfamily Helicoidea**			
Family Helminthoglyptidae [=Xanthonychidae]	*Monadenia fidelis* (Gray, 1834)	Oregon	D. Taylor
Order Systellomatophora **Superfamily Onchidioidea**			
Family Onchidiidae	*Onchidella celtica* (Cuvier, 1817)	Polzeath Beach, Cornwall, England, UK	Mike Kent
Superfamily Veronicelloidea			
Family Veronicellidae	*Laevicaulis alte* (Férussac, 1823)	Dubai, United Arab Emirates	A. Green
	Semperula maculata (Templeton, 1858)	Los Baños, col, Philippines	M. Carandang
Cohort Acteonimorpha **Superfamily Acteonoidea**			
Family Aplustridae	*Hydatina sp*. (Schumacher, 1817)	Ambatobe, près Soamanitse, Madagascar	Provided by MNHN. IM-2009-16352
Subclass Caenogastropoda, Grade Architaenioglossa **Superfamily Ampullarioidea**			
Family Ampullariidae	*Pomacea sp*.	From London Zoo	Unknown
Superfamily Cyclophoroidea Family Cyclophoridae	*Cyclophorus herklotsi* (Martens, 1861)	Bosque Estatal de Guacataca, Peurto Rico	Angus Davison

6.2.2 DNA and RNA Extraction, PCR Amplification and Sequencing

Genomic DNA was extracted from the whole snail (for small specimens) or from a small (1-2 mm^3) tissue sample taken from the foot of the snail using a CTAB DNA extraction protocol (Goodacre & Wade, 2001). RNA was extracted from *Acroloxus lacustris* using ReliaPrep™ RNA Tissue Miniprep System provided by Promega.

Four molecular markers were PCR amplified including three nuclear markers: approximately 4000 nucleotides of the LSU rRNA gene (including part of the 5.8S gene, the complete ITS-2 region, and almost the full-length large subunit (LSU; 28S) gene), approximately 1800 nucleotides of the small subunit (SSU, 18S) rRNA gene, approximately 370 nucleotides of the histone 3 (H3) gene; and one mitochondrial marker: approximately 650 nucleotides of cytochrome oxidase subunit I (COI).

The LSU rRNA gene (also incorporating part of the 5.8S gene and ITS-2), was amplified in a nested PCR reaction using primers LSU-1ii and LSU-12 in the first round, with the primary PCR products then used as template for the secondary PCR to amplify six internal fragments (A, B, C, D, E, and F) (see Table 2.2 for details of primers). PCR amplification for the primary PCR was performed using the Qiagen Taq DNA polymerase and Q buffer system (1X buffer, 1X Q-solution, 0.3 mM dNTP, 1.5 mM magnesium chloride, 0.2 μM each primer and 1U Taq in a 50μL final volume). Secondary PCR amplification was identical to the primary PCR with the exception that a lower 0.2 mM concentration of dNTPs was used. The cycling conditions (with a Perkin Elmer cycler) of the primary PCR were as follows: 96 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 50 °C for 30 sec, 72 °C for 3 min and then a final extension step at 72 °C for 5 min. The secondary PCR cycle conditions were as follows: 96 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 45 °C for 1 min, 72 °C for 2 min and then a final extension step at 72 °C for 5 min.

Similarly, the SSU rRNA gene was amplified in a nested PCR with primers 18e and 18p used for the first round and with the primary PCR products then used as template to amplify a single fragment with primers 1F and 9R. A series of 6 internal primers were used to sequence this fragment (see Table 2.2 for details of primers). Some samples proved difficult to amplify as a single fragment and in these cases the internal primers were used to amplify the SSU gene. Amplification was performed using BIOTAQ^{TM} DNA polymerase (1X reaction buffer, 0.2 mM dNTPs, 2.5 mM magnesium chloride, 0.2 μM each primer and 1U Taq in a 25 μL final volume). The PCR cycling conditions were as follows: 96 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 50 °C for 30 sec, 72 °C for 90 sec and then a final extension step of at 72 °C for 5 min for both primary and secondary PCR.

The H3 gene was amplified as a single fragment using primers H3aF and H3aR as default primers, though different combinations of primers were used with some samples where they did not amplify with the default primers (see Table 2.2 for a complete list of primers). The CO1 gene was also amplified as a single fragment using primers LCO 1490 and HCO 2198 for the majority of samples. A few samples did not amplify with these primers thus different combinations of primer were used (see Table 2.2 for a complete list of primers). The PCR amplification of the H3 and CO1 genes were performed using BIOTAQ^{TM} DNA polymerase (1X reaction buffer, 0.2 mM dNTPs, 2.5 mM magnesium chloride, 0.2 µM each primer and 1U Taq in a 25 µL final volume). The thermal cycling conditions were as follows: 96 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 50 °C for 30 sec, 72 °C for 3 min and then a final extension step at 72 °C for 5 min.

Amplification products were purified using either a Qiagen gel extraction kit or Qiagen PCR purification kit. Sense and antisense strands were sequenced directly on an Applied Biosystems 3730 DNA sequencer using Big Dye terminator cycle sequencing chemistries at Macrogen Inc.

A novel transcriptome for *Acroloxus lacustris* was generated in this study. RNA quantity and quality were checked using the Qubit Fluorometer and the Qubit RNA BR Assay Kit (Thermo Fisher Scientific; Q10211). Indexed sequencing libraries were prepared using the NEBNext Ultra Directional RNA Library Preparation Kit for Illumina (NEB; E7420) and NEBNext Multiplex Oligos for Illumina, Index Primer Set 1 (NEBNext; E7335). The library was sequenced at Deep Seq (Centre for Genetics and Genomics, University of Nottingham) on the Illumina NextSeq 500 using an Illumina NextSeq500 v2 Mid Output 300 cycle kit (Illumina; FC-404-2003) to generate 75 nucleotide sites paired-end reads.

6.2.3 Sequence Processing and Phylogenetic Analysis of Standard Markers

DNA sequences were assembled using version 1.5.3 of the STADEN package (Staden *et al*., 2000). Subsequently, the sequences were manually aligned using version 2.2 of the Genetic Data Environment (GDE) package (Smith *et al*., 1994). The LSU (incorporating 5.8S and ITS-2) and SSU rRNA sequences were aligned using the secondary structure as a guide. The alignment of the H3 and CO1 genes was guided by translating the DNA sequences to amino acid sequences. Gblocks v.0.91b (Castresana, 2000)

with default settings was used as a guide to select the reliably aligned sites from the alignments of all genes.

Phylogenetic trees were constructed using maximum likelihood (ML) (Felsenstein, 1981), neighbor-joining (NJ) (Saitou & Nei, 1987) and Bayesian inference (BI) (Larget & Simon, 1999). The general time reversible model incorporating gamma (GTR+Γ) (Lanave *et al*., 1984; Gu *et al*., 1995) was used to correct for multiple substitutions. ML trees were constructed using the PhyML (version 3.0) package (Guindon *et al*., 2010) with tree searching following a heuristic procedure with 10 random start trees and best of nearest-neighbour-interchange and subtree-pruning-regrafting branch swapping. NJ analysis was performed using the PAUP* (version 4.0b10) package (Swofford, 2002). For NJ analysis, model parameters were estimated following an iteration process; for each tree the parameters were estimated and used to build the next tree until there was no further improvement of the likelihood score. Bootstrap resampling (Felsenstein, 1985) with 1000 replicates was undertaken for ML and NJ trees. BI analysis was undertaken using the MrBayes (version 3.1.2) package (Ronquist & Huelsenbeck, 2003). Two independent runs with four chains of a Markov Chain Monte Carlo (MCMC) algorithm were used to explore the tree space. BI analysis was conducted for 5 million generations with tree sampling every 100 generations. To ensure adequate chain swapping, a range of heating parameters were tested with the optimal parameter used to construct the final trees. Only after the Bayesian MCMC searches had reached a stationary phase (indicating convergence of the chains onto the target distribution) was the run ended. A consensus tree was built using the last 75% of trees (burnin=12501).

6.2.4 Sequence Processing and Phylogenetic Analyses of the Transcriptome Datasets

The transcriptome assembly for *Acroloxus lacustris* was undertaken by Deep Seq (Centre for Genetics and Genomics, University of Nottingham). Orthologous genes were identified in two distinct datasets. The first dataset included four hygrophilan taxa: *Acroloxus lacustris*, *Biomphalaria pfeifferi* (GFMW01000001-GFMW01186204) (Buddenborg *et al*., 2018), *Lymnaea stagnalis* (FX180119-FX296473) (Sadamoto *et al*., 2012) and *Physa acuta* (Romiguier *et al*., 2014, assembly provided by

Jonathan Romiguier). The second dataset included the four hygrophilan taxa plus a stylommatophoran outgroup *Cepaea nemoralis* (GFLU01000001-GFLU01147386) (Kerkvliet *et al*., 2017).

Prior to orthologue identification, transcriptome sequences of all taxa were filtered by length using SeqKit Version: 0.10.1 to only allow contigs greater than 100 bp (Shen *et al*., 2016). The nucleotide sequences of all transcriptomes were then translated to amino acids using TransDecoder Release v5.5.0 (https://github.com/TransDecoder/TransDecoder) run using default settings which identify open reading frames (ORFs) that are at least 100 amino acids long. Orthologous sequences were then identified among the translated genes using PorthoMCL (Tabari & Su, 2017) again with default settings.

Amino acid sequences of all orthologues were aligned using MUSCLE v3.8.1551 (Edgar, 2004) with default settings. The alignments were then run through Gblocks v.0.91b (Castresana, 2000) with default settings to remove poorly aligned positions and divergent regions of amino acid alignments. Finally, trimmed alignments were filtered by length excluding any sequence shorter than 25 amino acids following Kocot *et al*. (2011).

Individual four taxon trees based on amino acid sequences of each orthologue identified in the first dataset (four hygrophilan taxa) were constructed manually using ML analysis. Additionally, BI and ML trees were constructed based on a concatenated supermatrix of amino acid sequences of all orthologous genes identified in the first dataset (four hygrophilan taxa). The custom Perl script "catfasta2phyml.pl'' (https://github.com/nylander/catfasta2phyml/) was used to concatenate fasta files. Likewise, BI and ML trees were constructed based on amino acid sequences of a concatenated supermatrix of all orthologues that were identified in the second dataset (comprising four hygrophilan taxa plus *Cepaea nemoralis*). To correct for multiple substitutions, the best-fitting model was selected using Smart Model Selection (SMS) (Lefort *et al*., 2017). Bootstrap resampling (Felsenstein, 1985) with 1000 replicates was undertaken for the ML trees. BI analyses were conducted for 5 million generations with tree sampling every 100 generations. A consensus tree was built using the last 75% of trees (burnin=12501).

6.3 Results

6.3.1 Phylogenetic Analysis Based on Standard Makers

Sequences of four genes: approximately 4000 nucleotides of the LSU rRNA gene (also incorporating approximately 80 nucleotides of the 5.8S and the complete ITS-2), approximately 1800 nucleotides of the SSU rRNA gene, approximately 370 nucleotides of the H3 gene and approximately 650 nucleotides of the CO1 gene have been generated for 51 panpulmonate species (including 14 hygrophilan species) and 3 non-panpulmonate species.

Phylogenetic trees for the Panpulmonata were constructed using a concatenated dataset of 5502 unambiguously aligned nucleotide sites. This dataset consists of 3063 nucleotides of the LSU (and 5.8S) gene, 1695 nucleotides of the SSU gene, 328 nucleotides of the H3 gene and 416 nucleotides of the 1st and 2nd codon positions of the CO1 gene. The caenogastropods *Cyclophorus herklotsi* and *Pomacea sp.* were used as outgroups to root the phylogenetic trees. The ITS-2 region of the rRNA cluster was removed completely from all analyses as it could not be aligned across all taxa due to its high variability. Likewise, the third codon positions of the CO1 gene were excluded from analyses due to saturation at these positions.

The Bayesian inference tree of the Panpulmonata based on the concatenated dataset is shown in Figure 6.1. Highly consistent topologies were observed with the BI and ML analyses, though slight differences in topology were observed in the NJ analysis (BI, Appendix 6.1; ML, Appendix 6.2 and NJ, Appendix 6.3). The Hygrophila are robustly supported as a monophyletic group in all analyses (95% ML, 70% NJ bootstrap replicates and with a Bayesian posterior probability of 1.00). However, the position of the Hygrophila within the Panpulmonata remains unresolved. In the BI and ML analyses the Hygrophila are recovered as the sister group to a clade comprising the Pyramidelloidea, Glacidorboidea and Amphiboloidea (PP= 0.91 BI but only 42% ML bootstraps) but as the sister group to a clade comprising just the Glacidorboidea and Amphiboloidea in the NJ analysis (49% NJ bootstraps). Within the Hygrophila, there is a principle division between the Chilinioidea on one hand and a clade comprising all the remaining hygrophilan taxa (Lymnaeoidea) on the other. This division is strongly supported with

the Lymnaeoidea supported in 98% ML, 98% NJ bootstraps and PP=1.00 BI, but the NJ analysis does not support the sister group relationship between the Acroloxidae and a clade comprising the Physidae, Lymnaeidae, Burnupiidae and Planorbidae (also incorporating the Bulinidae). Within the Lymnaeoidea, the Acroloxidae have a sister group relationship to a clade comprising the Physidae, Lymnaeidae, Burnupiidae and Planorbidae (also incorporating the Bulinidae) in the BI and ML analyses support in 98% ML bootstraps and PP=1.00 BI. However, the Acroloxidae are recovered as the sister group to the Physidae in the NJ analysis, though without significant support. The Physidae are recovered as the sister group to a clade comprising the Lymnaeidae, Burnupiidae and Planorbidae (also incorporating the Bulinidae) in the BI and ML analyses with weak support (51% ML bootstraps and PP=0.79 BI) while the Physidae are recovered as the sister group to the Acroloxidae in the NJ analysis, though without significant support. The Lymnaeidae are recovered as the sister group to clade comprising the Burnupiidae and Planorbidae (also incorporating the Bulinidae) in the BI and ML analyses with weak support (50% ML bootstraps and PP=0.71 BI), though the Lymnaeidae are shown as an unresolved polytomy in the NJ analysis. Finally, the Burnupiidae are fully supported) as the sister group for a clade comprising the Bulinidae and Planorbidae (100% ML, 100 NJ bootstraps and PP=1.00 BI).

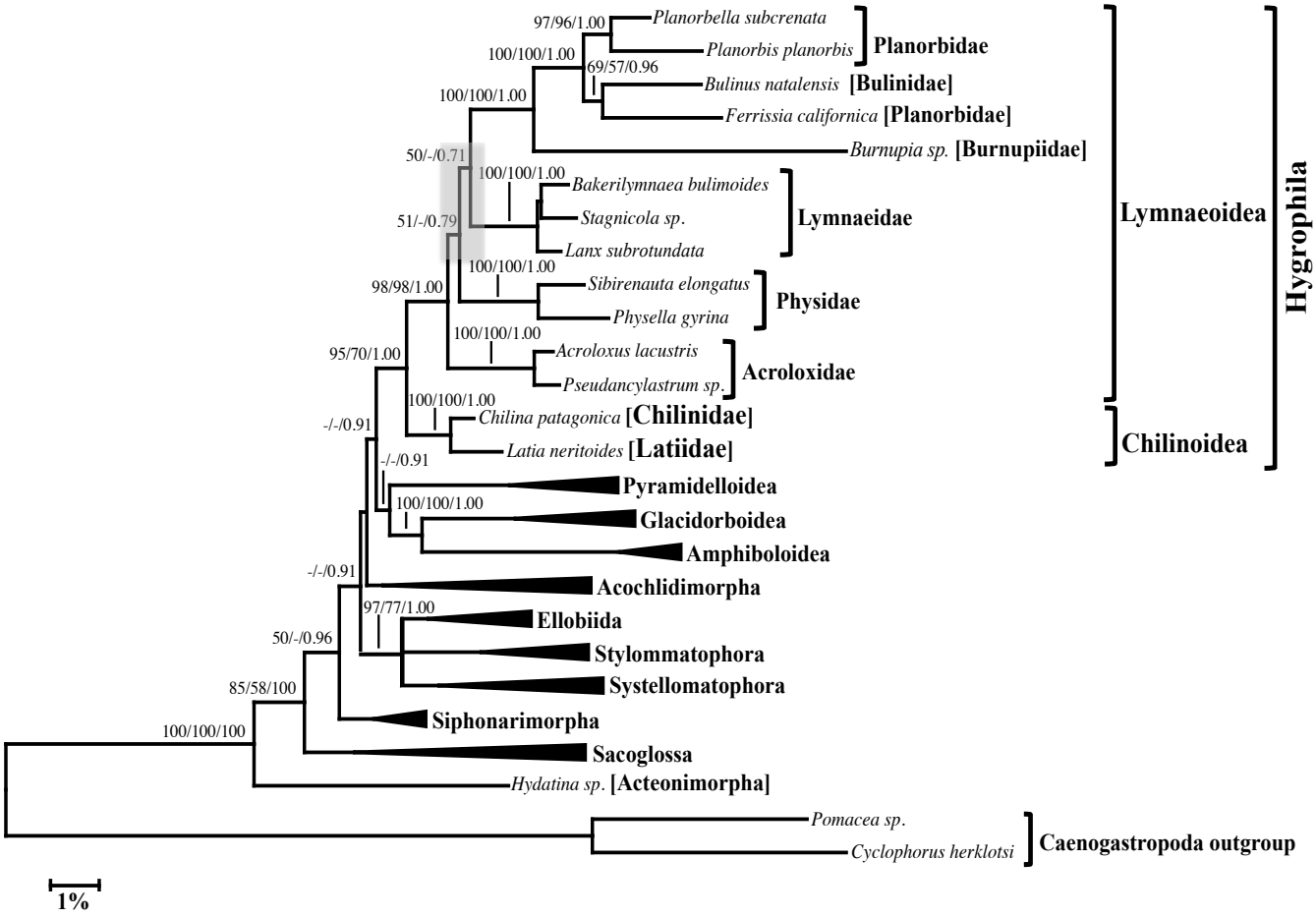


Figure 6.1. Bayesian phylogenetic tree of the Panpulmonata based on concatenated sequences of 5502 unambiguously aligned nucleotides from the combined dataset of the LSU (and 5.8S) gene, the SSU gene, the H3 gene and the 1st and 2nd codon positions of the CO1 gene. Values on the nodes represent bootstrap support (1000 replicates) for ML and NJ and posterior probabilities for BI (based on last 75% of trees) respectively. Bootstrap support values less than 50% and posterior probabilities less than 0.7 are not shown. The shaded area in the Hygrophila indicates that the interrelationships within the lymnaeoidean families are not well resolved. The scale bar represents 1 substitutional change per 100 nucleotide positions. All panpulmonate clades are collapsed except the Hygrophila clade to allow better visibility.

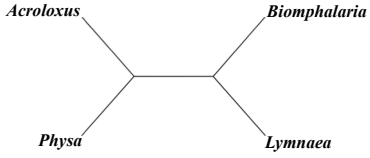
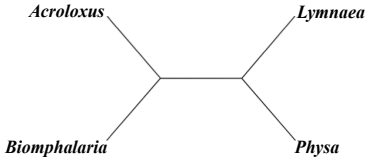
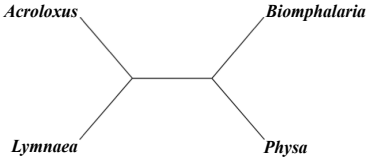
6.3.2 Phylogeny Based on Transcriptome Sequences

A total of 404 orthologues were identified in the transcriptome dataset of the four hygrophilan taxa, *Acroloxus* (Acroloxidae), *Physa* (Physidae), *Biomphalaria* (Planorbidae) and *Lymnaea* (Lymnaeidae) of which 201 orthologues produced significant trees (bootstrap support >70). Of the significant trees 96 trees support tree 1 in which *Acroloxus* (Acroloxidae) clusters with *Physa* (Physidae) and *Biomphalaria*

(Planorbidae) clusters with *Lymnaea* (Lymnaeidae), 57 trees support tree 2 in which *Acroloxus* (Acroloxidae) clusters with *Biomphalaria* (Planorbidae) and *Lymnaea* (Lymnaeidae) clusters with *Physa* (Physidae) and 48 trees support tree 3 in which *Acroloxus* (Acroloxidae) clusters with *Lymnaea* (Lymnaeidae) and *Biomphalaria* (Planorbidae) clusters with *Physa* (Physidae) (Table 6.2). BI and ML phylogenetic trees constructed based on a supermatrix of 60458 amino acids from the concatenation of all 404 orthologues supported tree 1 (100% ML bootstraps and PP=1.00 BI).

In the transcriptome dataset of five taxa (4 hygrophilan taxa plus outgroup), a total of 186 orthologues were identified. BI and ML phylogenetic trees were constructed based on a supermatrix of 25325 unambiguously aligned amino acids from the concatenation of all 186 orthologues, with the stylommatophoran *Cepaea nemoralis* used as the outgroup to root the trees. Identical topologies were observed with both BI and ML (Figure 6.2). In this phylogeny, *Acroloxus* (Acroloxidae) has a sister taxon relationship to a clade comprising *Physa* (Physidae), *Lymnaea* (Lymnaeidae) and *Biomphalaria* (Planorbidae) with full support (100% ML bootstraps and PP=1.00 BI). Likewise, *Physa* (Physidae) is strongly supported as the sister taxon to a clade comprising *Lymnaea* (Lymnaeidae) and *Biomphalaria* (Planorbidae) (85% ML bootstraps and PP=1.00 BI). Finally, *Lymnaea* (Lymnaeidae) and *Biomphalaria* (Planorbidae) are fully supported (100% ML bootstraps and PP=1.00 BI) as sister taxa.

Table 6.2: Three alterative tree topologies for the four hygrophilan taxa, showing the number of orthologues supporting each tree with bootstrap support >70%. Phylogenetic trees for each orthologue were constructed using maximum likelihood and based on amino acid sequences.

Trees	**Tree 1**	**Tree 2**	**Tree 3**
			
Number of orthologous	96	57	48

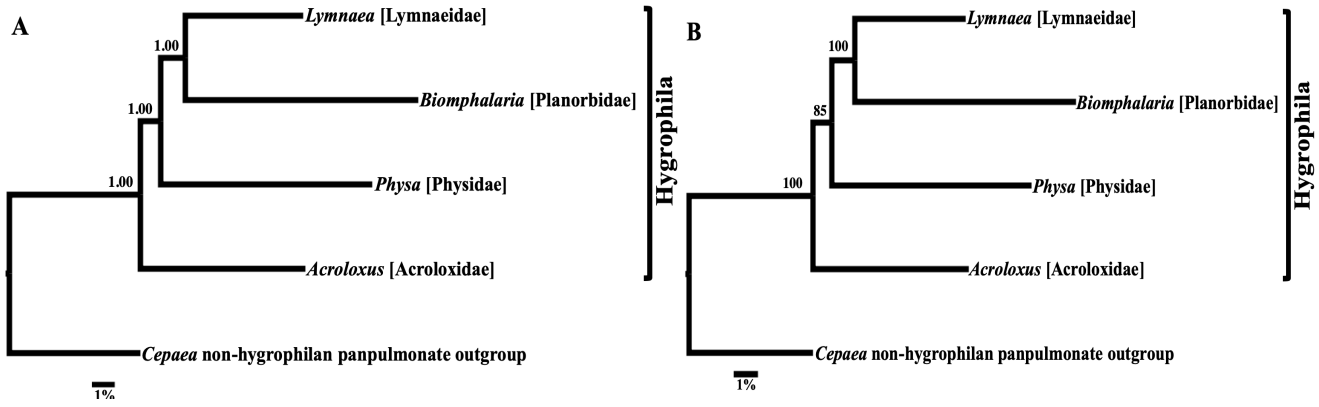


Figure 6.2. Bayesian (A) and maximum likelihood (B) phylogenetic trees of the transcriptome dataset of five taxa (4 hygrophila plus outgroup), based on a supermatrix of 25325 unambiguously aligned amino acids. Values on the nodes represent bootstrap support (1000 replicates) for ML and posterior probabilities for BI (based on last 75% of trees) respectively. Bootstrap support values less than 50% and posterior probabilities less than 0.7 are not shown. The scale bar represents 1 substitutional change per 100 nucleotide positions.

6.4 Discussion

The monophyly of the Hygrophila, their sister group relationship with other panpulmonate groups and the interrelationships among the hygrophilan families were examined based on 5547 unambiguously aligned nucleotide sites of four concatenated genes. The interrelationships of the four lymnaeoidean families (Acroloxidae, Physidae, Lymnaeidae and Planorbidae) were also examined based on two supermatrices of 60458 and 25325 amino acids of transcriptome sequences.

In our phylogeny based on four standard markers, the monophyly of the Hygrophila is robustly confirmed, consistent with previous morphological and molecular studies (Hubendick, 1978; Nordsieck, 1992; Barker, 2001; Dayrat *et al*., 2001; Klussmann-Kolb *et al*., 2008; Dinapoli *et al*., 2010; Jörger *et al*., 2010; Goebbeler & Klussmann-Kolb, 2011; Kocot *et al*., 2013). Although the Hygrophila are shown as the sister group of a clade comprising the Pyramidelloidea, Glacidorboidea and Amphiboloidea in the BI analysis, this relationship is not significantly supported by the ML analysis. This placement is consistent with the finding of Dinapoli *et al*. (2010), who showed a similar placement

for the Hygrophila with robust support based on Bayesian analysis. In the NJ tree, the Hygrophila again cluster with the Glacidorboidea and Amphiboloidea but the Pyramidelloidea fall elsewhere in the tree. Early morphological studies provided different placements for the Hygrophila (Hubendick, 1978; Nordsieck, 1992; Barker, 2001). Likewise, the position of the Hygrophila within broader panpulmonate trees remains uncertain based on previous molecular studies (Klussmann-Kolb *et al*., 2008; Dinapoli & Klussmann-Kolb, 2010; Jörger *et al*., 2010; Goebbeler & Klussmann-Kolb, 2011; Kocot *et al*., 2013; Romero *et al*., 2016).

Within the Hygrophila, there is a clear basal division between the Chilinioidea on one hand and a clade comprising all the remaining hygrophilan taxa (Lymnaeoidea) on the other. This finding supports previous morphological studies of Hubendick (1978) and Nordsieck (1992) and also supports previous molecular studies (Klussmann-Kolb *et al*., 2008; Goebbeler & Klussmann-Kolb, 2011; Davison *et al*. 2016). Within the Lymnaeoidea, a sister group relationship is recovered between the Acroloxidae and a clade comprising the Physidae, Lymnaeidae, Burnupiidae and Planorbidae (also incorporating the Bulinidae). This relationship received high support in the BI and ML analyses (though was not resolved in our NJ analysis) based on the four standard markers. There is high support for the sister group relationship between the Acroloxidae and a clade comprising the Physidae, Lymnaeidae and Planorbidae in the analyses of the Hygrophila based on transcriptome datasets. In the early morphological study of Hubendick (1978) the Acroloxidae were grouped with the Latiidae as both families have a patelliform shape and the prostate gland has a distinctive arrangement. However, Nordsiek (1992) placed the Acroloxinei with the Lymnaeoinei and Planorbinei. Our molecular phylogeny does not support Hubendick's placement of the Acroloxidae with the Latiidae but does support the association of the Acroloxidae with lymnaeoidean families (Acroloxidae, Lymnaeidae, Physidae and Planorbidae). The relationship of the Acroloxidae to other hygrophilan families in the molecular analyses of Klussmann-Kolb *et al*. (2008) was unclear and varied according to the phylogeny reconstruction method used. Recent molecular analyses by Davison *et al*. (2016) provided strong support for the sister group relationship of the Acroloxidae with a clade comprising Physidae, Lymnaeidae and Planorbidae which is consistent with the position of Acroloxidae in our BI and ML

analyses based on standard markers as well as with the position of Acroloxidae based on our analyses of the transcriptome dataset. In the more recent molecular tree of Soldatenko & Petrov (2019), the Acroloxidae were shown as the sister group to a clade comprising just the Lymnaeidae and Physidae and not including the Planorboidea, though only one phylogeny reconstruction method was used (Bayesian analysis). Our analyses do not support the Acroloxidae placement in Soldatenko & Petrov (2019).

The Physidae are recovered as the sister group to a cluster comprising the Lymnaeidae, Burnupiidae and Planorbidae (also incorporating the Bulinidae) in the BI and ML analyses and as the sister group to the Acroloxidae in the NJ analysis. Although the support for these relationships is equivocal in our analyses of the Hygrophila based on the four standard markers used, the Physidae are well supported as the sister group to a cluster comprising Lymnaeidae and Planorbidae in the analysis of the Hygrophila based on the transcriptome dataset. Hubendick (1978) grouped the Physidae with the Lymnaeidae and Planorbidae as they have a common origin and the male and female reproductive ducts in these families are completely separated below the albumen gland. In Hubendick's diagram of probable relationships among the basommatophoran families, the Physidae were shown to be closely related to 'Ancyloplanorbidae' (= Planorbidae). In our BI and ML trees, the Physidae, Lymnaeidae and Planorbidae cluster together as a monophyletic group consistent with Hubendick's finding. However, the Physidae in our BI and ML analyses fall as the sister group to both the Lymnaeidae and Planorbidae as opposed to the sister taxon of just the Planorbidae which contradicts Hubindick's placement of the Physidae. The phylogenetic position of the Physidae in the molecular studies of Klussmann-Kolb *et al*. (2008) and Holznagel *et al*. (2010) was inconsistent in both studies and varied according to the phylogeny reconstruction method used. Likewise, the Physidae in the molecular analyses of Dayrat *et al*. (2011) received low support as the sister group to the Acroloxidae which is consistent with our NJ analysis. The Physidae were recovered as the sister group to the Lymnaeidae with robust support in the molecular studies of Davison *et al*. (2016) and Soldatenko & Petrov (2019) which is inconsistent with the position of the Physidae in our analyses. However, in both of these studies taxon sampling was limited.

The Lymnaeidae are recovered as the sister group to a clade comprising the Burnupiidae and Planorbidae (also incorporating the Bulinidae) in our BI and ML analyses and as an unresolved polytomy in our NJ analysis based on the four standard markers used. The sister group relationship between the Lymnaeidae and Planorbidae is also supported by the highest number of trees in the analysis of Hygrophila based on four taxon trees of individual genes of the transcriptome dataset. It is also fully supported in the concatenated transcriptome datasets. In the morphological analyses of Barker (2001), the Lymnaeidae was shown as the sister group to the Physidae while the Planorbidae was shown as an unresolved polytomy. Likewise, the relationship between the Lymnaeidae and Planorbidae in the molecular studies of Klussmann-Kolb *et al*. (2008), Holznagel *et al*. (2010) and Dayrat *et al*. (2011) was uncertain. However, in the recent molecular analyses of Davison *et al*. (2016) and Soldatenko & Petrov (2019) the Planorbidae were shown as the sister group to a clade comprising the Lymnaeidae and Physidae, though in both the Davison *et al*. (2016) and Soldatenko & Petrov (2019) studies taxon sampling was limited and only two genes were used. In our analyses, the relationship between the Lymnaeidae and Planorbidae is well established as those families have a clear sister group relationship.

6.5 Conclusion

The monophyly of the Hygrophila, their sister group relationship with other panpulmonate groups and the interrelationships among the hygrophilan families were investigated based on 5547 sites from four concatenated genes. The monophyly of the Hygrophila and the basal split between the Chilinoidea and Lymnaeoidea within the Hygrophila are robustly supported in our phylogenetic trees. However, the interrelationships among the Acroloxidae, Physidae, Lymnaeidae, Burnupiidae and Planorbidae (also incorporating the Bulinidae) are not well resolved. To better resolve the interrelationships of the Lymnaeoidean families, a phylogeny based on transcriptome sequence data was also undertaken. Here, the relationships among the Lymnaeoidean families are well established with the Acroloxidae recovered as the sister group to a clade comprising the Physidae, Lymnaeidea, and Planorbidae, the Physidae recovered as the sister group of a cluster comprising the Lymnaeidae and Planorbidae, and the Lymnaeidea and Planorbidae recovered as sister taxa.

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Chapter 7: Summary and Conclusion

This work provides detailed molecular phylogenies for two panpulmonate groups: the Stylommatophora and Hygrophila at multiple scales including deep phylogenetic relationships of the major stylommatophoran and hygrophilan clades and family level relationships within the Orthurethra and Lymnaeoidea. The uncertain evolutionary relationships at the base of the Stylommatophora and the family group relationships of the Orthurethra have been addressed in this study. Likewise, the problematic interrelationships of the hygrophilan families and subfamilies have been investigated. A taxonomic revision for the Stylommatophora and Hygrophila has been suggested based on the results of this study.

In **chapter 3** of this thesis, the basal relationships of the stylommatophoran land snails and slugs have been investigated using four standard molecular markers (the LSU (and 5.8S) gene, the SSU gene, the H3 gene and the 1st and 2nd codon positions of the CO1 gene). The phylogenetic utility of these genes and gene partitions was evaluated to assess their usefulness for inferring stylommatophoran phylogeny. The basal division in the Stylommatophora tree is shown to be between the Scolodontidae and all other stylommatophoran groups with robust support. In previous morphology-based taxonomic studies, the phylogenetic position of the Scolodontidae had remained controversial (Thiele; 1931; Zilch, 1959-1960; Tillier 1980; Nordsieck, 1986; Vaught, 1989; Schileyko, 2000; Barker, 2001; Bouchet & Rocroi, 2005). The phylogenetic position of the Scolodontidae based on previous molecular analyses (Ramírez *et al*., 2012) also remained uncertain and varied according to the phylogeny reconstruction method used. Our molecular analyses provide robust support for the position of the Scolodontidae at the base of the Stylommatophora as the sister group to all other stylommatophoran taxa with all tree construction methods. The previous 'achatinoid' and 'non-achatinoid' clades of Wade *et al*. (2001, 2006) remained stable in our analyses with significant increase in the bootstrap support for the 'non-achatinoid' clade. In the most recent classification of Bouchet *et al*. (2017), the Scolodontidae have been given the suborder rank at the same level of the 'achatinoid' and 'non-achatinoid' clades. Based on the results of our molecular analyses, it is suggested that the 'achatinoid' and 'non-achatinoid' clades should be

classified at a lower rank than the Scolodontina in order to properly reflect the deep-level relationships within the Stylommatophora. Given that the Scolodontina are such an important clade in the styommatophoran tree because they resolve as the sister group to all other stylommatophoran taxa, it is important that future work examines this group in more depth with the inclusion of more taxa in order to better understand the evolutionary relationships within the group.

Based on the results of our study, it has been confirmed that the original LSU 1-5 fragment of Wade *et al*. (2001, 2006) is the most informative gene fragment among the genes used in this study and that it works well at resolving relationships at most levels in the Stylommatophora. However, it does not reliably resolve the deep level relationships at the very base of the stylommatophoran clade. Expanding the length of the LSU gene examined and combining it with other genes is necessary to fully resolve the relationships within the Stylommatophora, particularly those at the base of the clade.

The phylogenetic relationships within the Orthurethra have been investigated in **chapter 4** using almost the full-length of the LSU rRNA gene (incorporating part of the 5.8S). Within the Orthurethra, the Chondrinidae, Cerastidae, Vertiginidae, Achatinellidae and Partulidae are recovered as monophyletic units with robust support while the Pupillidae, Enidae, and Valloniidae are not recovered as monophyletic groups in the tree. The phylogenetic tree shows a clear basal division between the Chondrinidae and the rest of the Orthurethra. This supports the previous findings of Wade *et al*. (2001, 2006), though here is based on more data and with full support. Madeira *et al*. (2010) revealed a basal trichotomy between the Chondrinidae, Azecidae and the remaining orthurethran taxa. Our study is consistent with these findings in respect of the Chondrinidae but a lack of samples precluded the inclusion of the Azecidae in our analyses. Recent molecular studies by Nekola & Coles (2016) and Harl *et al*. (2017) showed that the principle division within the Orthurethra is between the Chondrinidae plus Truncatellinidae on the one hand and the remaining orthurethran taxa on the other. Again, our result is consistent with these findings in respect of the Chondrinidae but we did not include the Truncatellinidae in our analyses. The uncertainty in the basal relationships within the Orthurethra and the lack of resolution throughout other parts of the tree could be attributed to poor taxon sampling as well as probably due to a rapid evolutionary diversification whithi the main orthurethran group (shaded area

on tree, Figure 4.1). Therefore, based on the results of our study it is strongly suggested that adding taxa from the Azecidae and Truncatellinidae as well as using more variable molecular markers are necessary to better understand the phylogenetic relationships within the Orthurethra.

The family-group interrelationships within the Hygrophila have been investigated in **chapter 5** using almost the full-length of the LSU rRNA gene (incorporating part of the 5.8S). The principle division in the Hygrophila is shown to be between the Chilinoidea and a clade comprising all the remaining hygrophilan taxa (Lymnaeoidea). This supports the results of previous molecular studies (Klussmann-Kolb *et al*., 2008; Dinapoli *et al*., 2010; Jörger *et al*., 2010; Göebbeler & Klussmann-Kolb, 2011; Davison et al. 2016). The monophyly of the Acroloxidae, Physidae and Lymnaeidae are fully supported in our molecular trees. However, the Planorbidae as defined by Bouchet *et al*. (2017) are not recovered as a monophyletic unit due to the clustering of the Bulinidae within the Planorbidae. The inclusion of the Bulinidae within the Planorbidae has been shown in previous molecular studies (Morgan *et al*. 2002; Jørgensen *et al*. 2004; Albrecht *et al*., 2007), though the position of the Bulinidae in relation to other planorbid taxa have received low support in these studies. The Amphipepleinae within the Lymnaeidae and both the Planorbinae and Ancylinae within the Planorbidae are strongly supported as monophyletic subfamilies. However, the Lymnaeinae within the Lymnaeidae and both the Physinae and Aplexinae within the Physidae are not recovered as monophyletic subfamilies. Based on the results of our study, it is suggested that a taxonomic revision for the Hygrophila should be considered by either retaining the Bulinidae as a family but reclassifying the Ancylini as a family or by lowering the Bulinidae to a subfamily alongside the Ancylini within the family Planorbidae.

The interrelationships among the lymnaeoidean families (Acroloxidae, Physidae, Lymnaeidae, Bulinidae, Burnupiidae and Planorbidae) have been reasonably well resolved in **chapter 5** using a single standard marker. However, they are not fully supported and inconsistent with the results of recent studies by Davison *et al*. (2016) and Soldatenko & Petrov (2019). Therefore, we have undertaken a more detailed phylogenetic analyses in **chapter 6** using four standard molecular markers (LSU (incorporating 5.8S), SSU, H3 and the 1st and 2nd codon positions of the CO1gene) for representative taxa from the main hygrophilan groups within a framework of the Panpulmonata combined with a

phylogenomic analysis based on transcriptome sequences data for a representative from each of the main lymnaeoidean families (Acroloxidae, Physidae, Lymnaeidae and Planorbidae). The observed interrelationships among the Acroloxidae, Physidae, Lymnaeidae, Bulinidae, Burnupiidae and Planorbidae based on the four standard markers in the BI and ML analyses are consistent with those obtained in **chapter 5**, though they are shown as unresolved polytomy in the NJ analysis. Surprisingly, support for the major division decreases raising uncertainty about the topology. However, in our phylogenomic analyses of the four lymnaeoidean families, the interrelationships are well resolved, with the Acroloxidae recovered as the sister group to a clade comprising the Physidae, Lymnaeidae, and Planorbidae, the Physidae recovered as the sister group of a cluster comprising the Lymnaeidae and Planorbidae, and the Lymnaeidea and Planorbidae recovered as sister taxa.

During the course of this project, Bouchet *et al*. (2017) produced a revised classification of the Hygrophila. This combined with our findings necessitate greater sampling of the families Bulinidae and Burnupiidae as well as the subfamily Ancylinae. Future studies need to focus on these groups in more detail with better sampling of representatives from these groupings.

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Appendices

Appendix 3.1. Genbank sequence accessions of all sequences used in chapter 3.

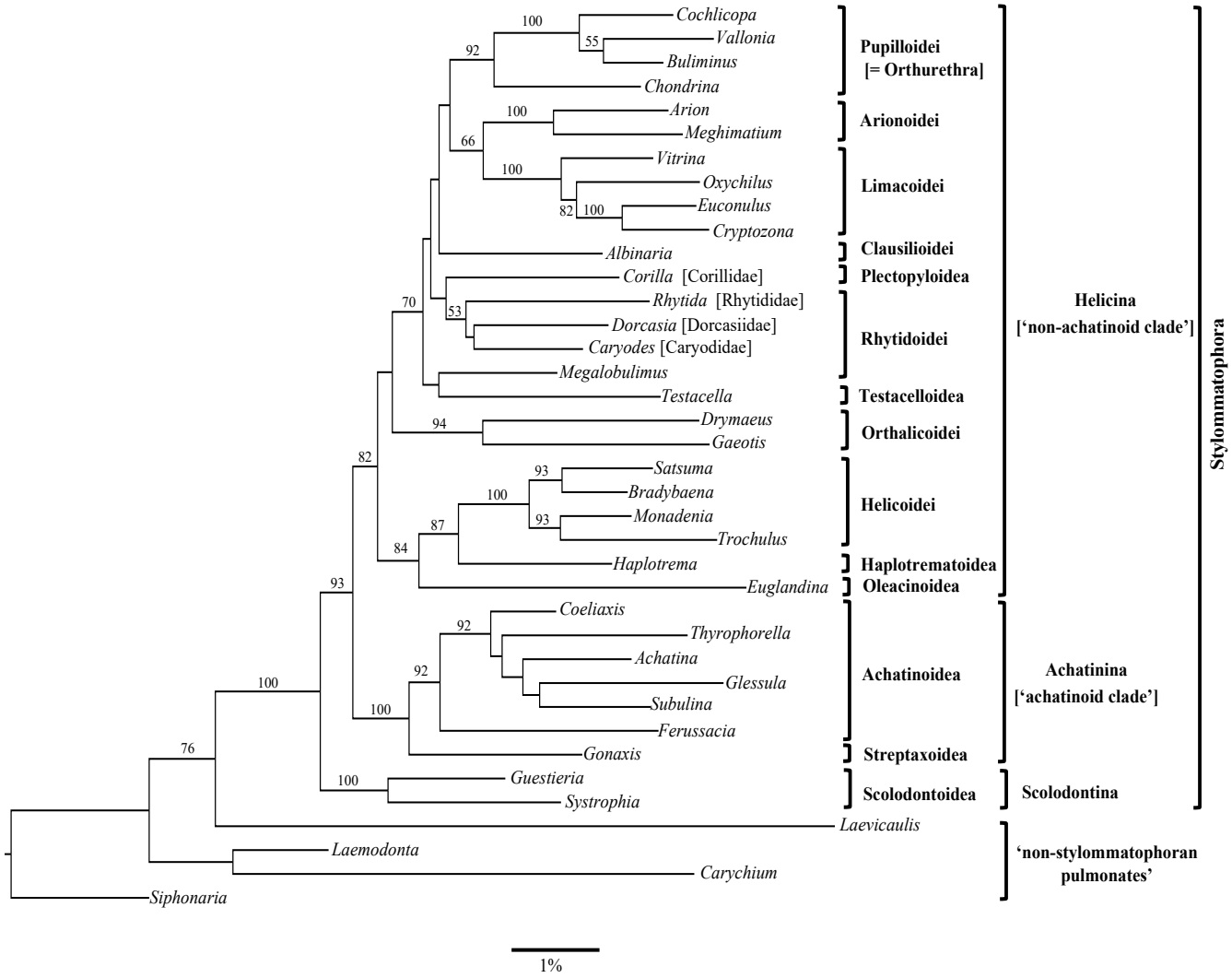
Taxa	**LSU rRNA gene (also incorporating partial 5.8S and complete ITS-2)**	**SSU rRNA**	**H3**	**CO1**
Ferussacia folliculus	MF444871[1]	MN022689	MF415338[1]	MF415348[1]
Subulina striatella	MF444888 [1]	MN022690	MF415330[I]	MF415358[1]
Glessula ceylanica	MF444872[1]-MF444873[1]	MN022691	MF415337[1]	MF415347[1]
Lissachatina fulica [= *Achatina fulica*]	MF444864[1]	MN022692	MF415320[1]	MF41534[1]
Coeliaxis blandii	MF444867[1]- MF444868[1]	MN022693	MF415335 [1]	MF415345[1]
Thyrophorella thomensis	MF444891[1]	MN022694	MF415339[1]	MF415349[1]
Gonaxis quadrilateralis	MF444893[1]	MN022697	MN022760	MN022729
Cochlicopa lubrica	KU341313 [2]	MN022682	MN022751	MN022720
Vallonia excentrica	MN022656	MN022683	MN022752	MN022721
Chondrina clienta	MN022657	MN022684	MN022753	MN022722
Buliminus labrosus	MN022658	MN022685	MN022754	MN022723
Albinaria xanthostoma	MN022659	MN022686	MN022755	MN022724
Drymaeus discrepans	MN022660	MN022687	MN022756	MN022725
Gaeotis nigrolineata	MN022661	MN022688	MN022757	MN022726
Euglandina rosea	MN022662	MN022695	MN022758	MN022727
Haplotrema vancouverense	MN022668	MN022702	MN022765	MN022734
Rhytida stephenensis	MN022667	MN022701	MN022764	MN022733
Megalobulimus oblongus	MN022664	MN022698	MN022761	MN022730
Dorcasia alexandri	MN022665	MN022699	MN022762	MN022731
Caryodes dufresnii	MN022666	MN022700	MN022763	MN022732
Euconulus fulvus	MN022670	MN022704	MN022767	MN022736
Cryptozona bistrialis	AY014106[3] & MN022671	MN022705	MN022768	MN022737
Vitrina pellucida	MN022672	MN022706	MN022769	MN022738
Oxychilus alliarius	MN022673	MN022707	MN022770	MN022739
Satsuma japonica	MN022674	MN022708	MN022771	MN022740
Trochulus striolatus	MN022675	MN022709	MN022772	MN022741
Bradybaena similaris	MN022676	MN022710	MN022773	MN022742
Monadenia fidelis	MN022677	MN022711	MN022774	MN022743
Arion hortensis	KU341315[2]	MN022712	MN022775	MN022744
Meghimatium bilineatum	MN022678	MN022713	MN022776	MN022745
Testacella scutulum	MN022663	MN022696	MN022759	MN022728
Corilla adamsi	AY014091[3] & MN022669	MN022703	MN022766	MN022735
Guestieria sp.	MN022679	MN022714	MN022777	MN022746
Systrophia sp.	MN022680 & MN022681	MN022715	MN022778	MN022747
Laemodonta sp.	KU341316[2]	MN022716	MN022779	KM281112[4]
Carychium tridentatum	KU341318[2]	MN022717	MN022780	MN022748
Laevicaulis alte	KU341319[2]	MN022718	MN022781	MN022749
Siphonaria pectinata	AY014149[3] & KU341320[2]	MN022719	MN022782	MN022750

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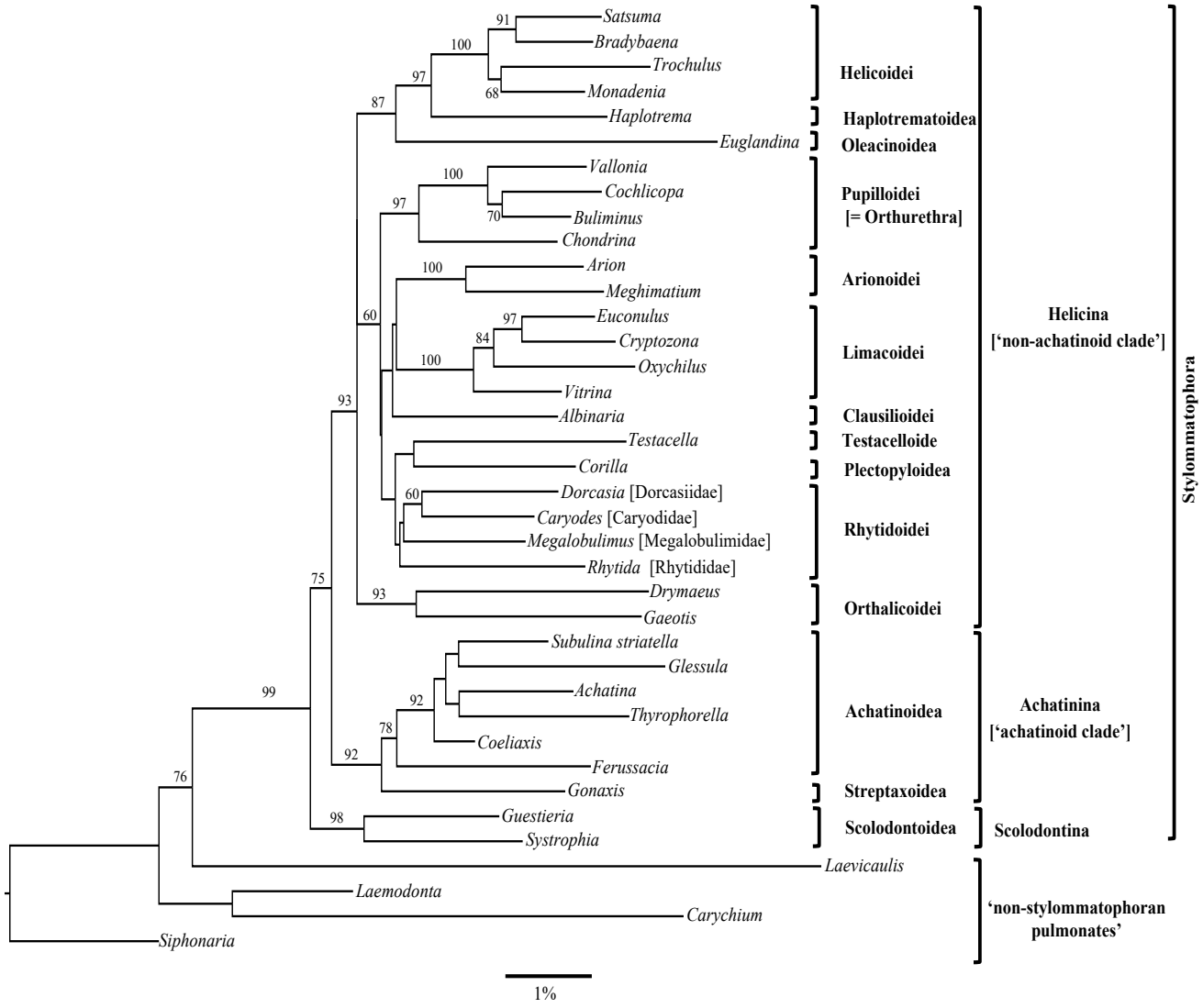
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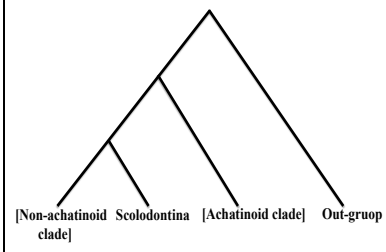
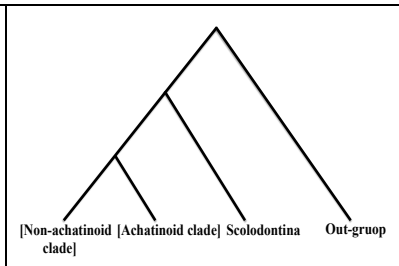
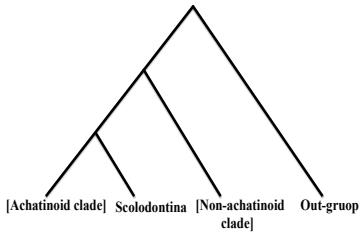
Appendix 3.2. Maximum likelihood phylogenetic tree of the Stylommatophora based on concatenated sequences of 5782 unambiguously aligned nucleotides from the combined dataset of the LSU (and 5.8S) gene, the SSU gene, the H3 gene and the 1st and 2nd codon positions of the CO1 gene. The optimal model GTR+Γ was used. The phylogeny is rooted on the siphonariid *Siphonaria pectinata*. Values on

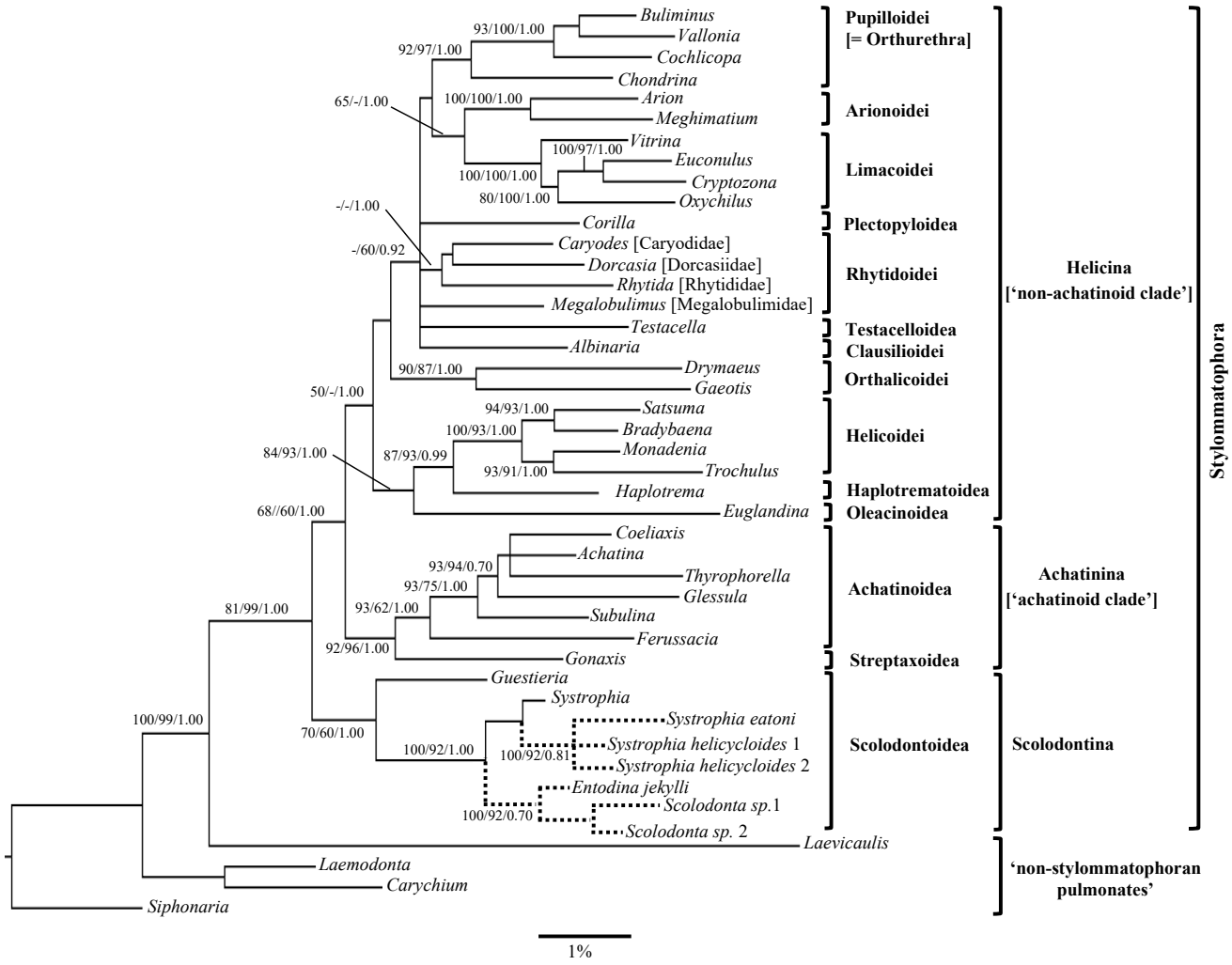
the nodes represent bootstrap support (1000 replicates). Bootstrap support values less than 50% are not shown. The scale bar represents 1 substitutional change per 100 nucleotide positions.



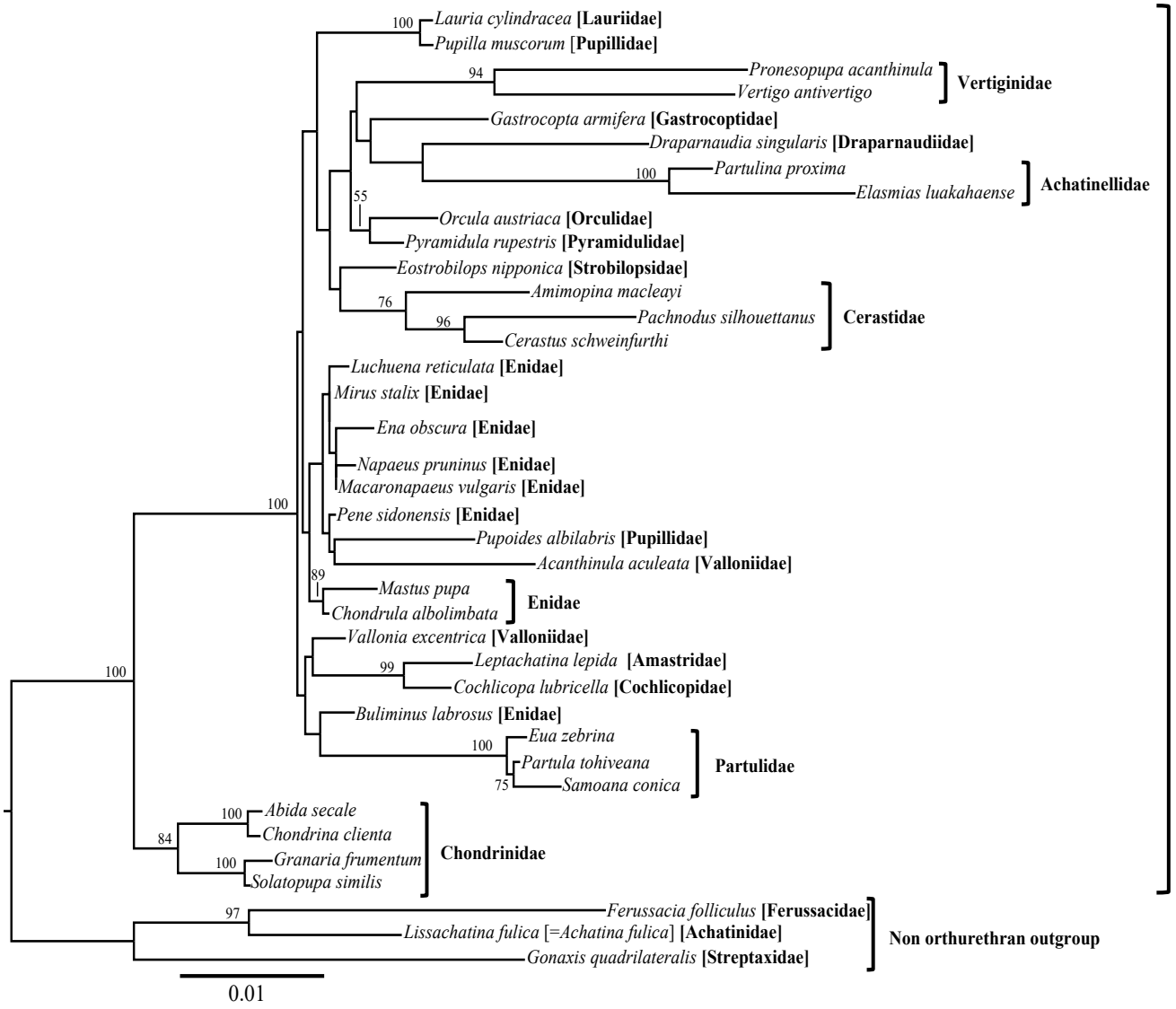
Appendix 3.3. Neighbor-joining phylogenetic tree of the Stylommatophora based on concatenated sequences of 5782 unambiguously aligned nucleotides from the combined dataset of the LSU (and 5.8S) gene, the SSU gene, the H3 gene and the 1st and 2nd codon positions of the CO1 gene. The phylogeny is rooted on the siphonariid *Siphonaria pectinata*. Values on the nodes represent bootstrap support (1000 replicates). Bootstrap support values less than 50% are not shown. The scale bar represents 1 substitutional change per 100 nucleotide positions.

Appendix 3.4. Three possible alternative phylogenetic trees showing the possible position of the Scolodontina within the Stylommatophora (tree one: 'non-achatinoid clade' clusters with Scolodontina, tree two: 'non-achatinoid clade' clusters with 'achatinoid clade' with Scolodontina falling as a sister group to both clades, tree three: 'achatinoid clade' clusters with Scolodontina. The reliability of the inferred trees was tested by bootstrap resampling of maximum likelihood (ML), neighbor-joining (NJ), minimum-evolution (ME), Fitch-Margoliash (FM) and differences in the number of supporting parsimony informative sites. The number of supporting parsimony informative sites were calculated based on four taxon trees incorporating *Siphonaria pectinata*, *Guestieria sp.*, *Lissachatina fulica* and *Monadenia fidelis*.

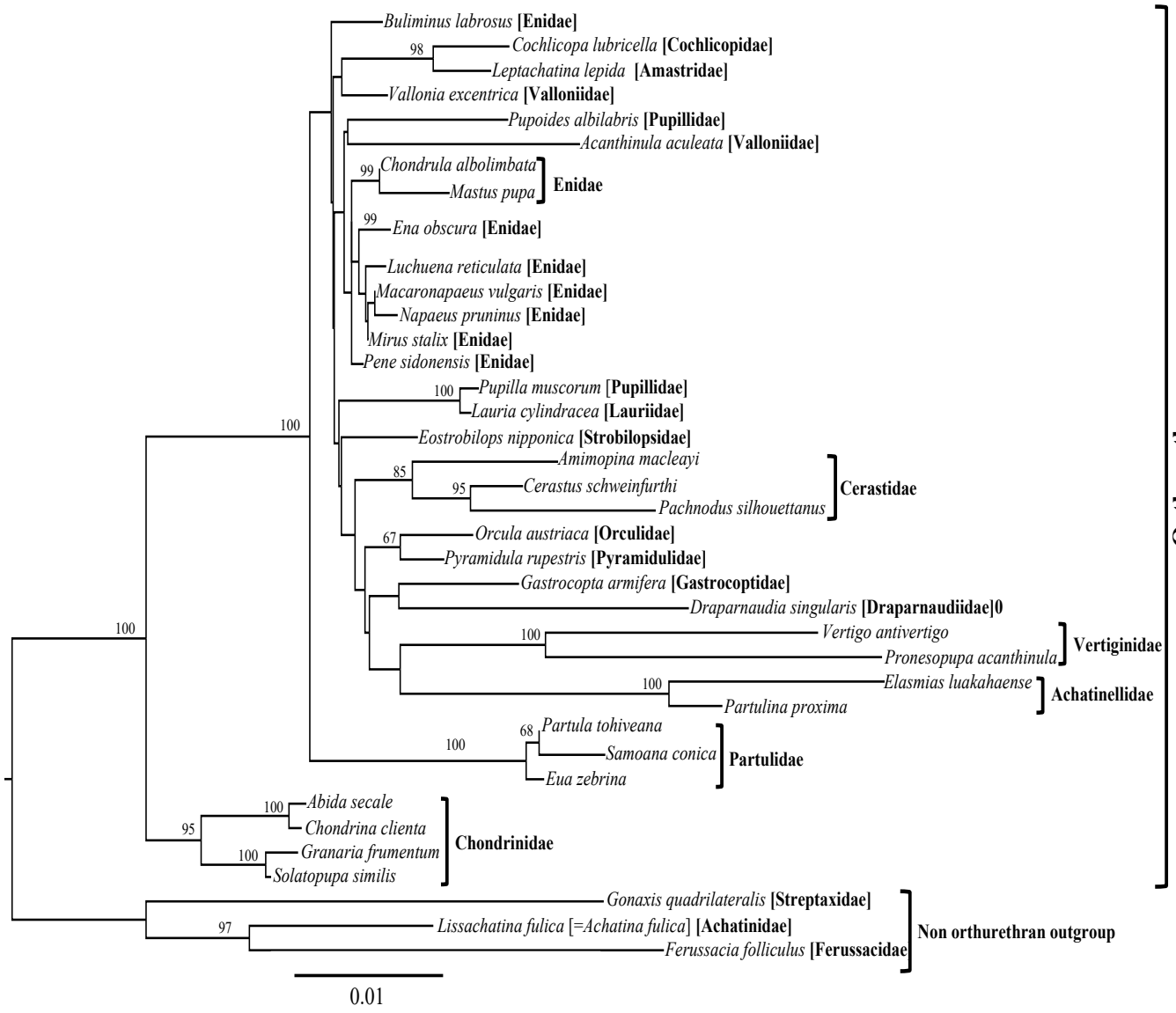
			
	Tree1	**Tree 2**	**Tree 3**
ML bootstrap	3	93	4
NJ bootstrap	22	75	3
ME bootstrap	8	88	4
FM bootstrap	5	84	11
Parsimony informative sites	11	22	11
Parsimony transversion/ sites	0	10	2



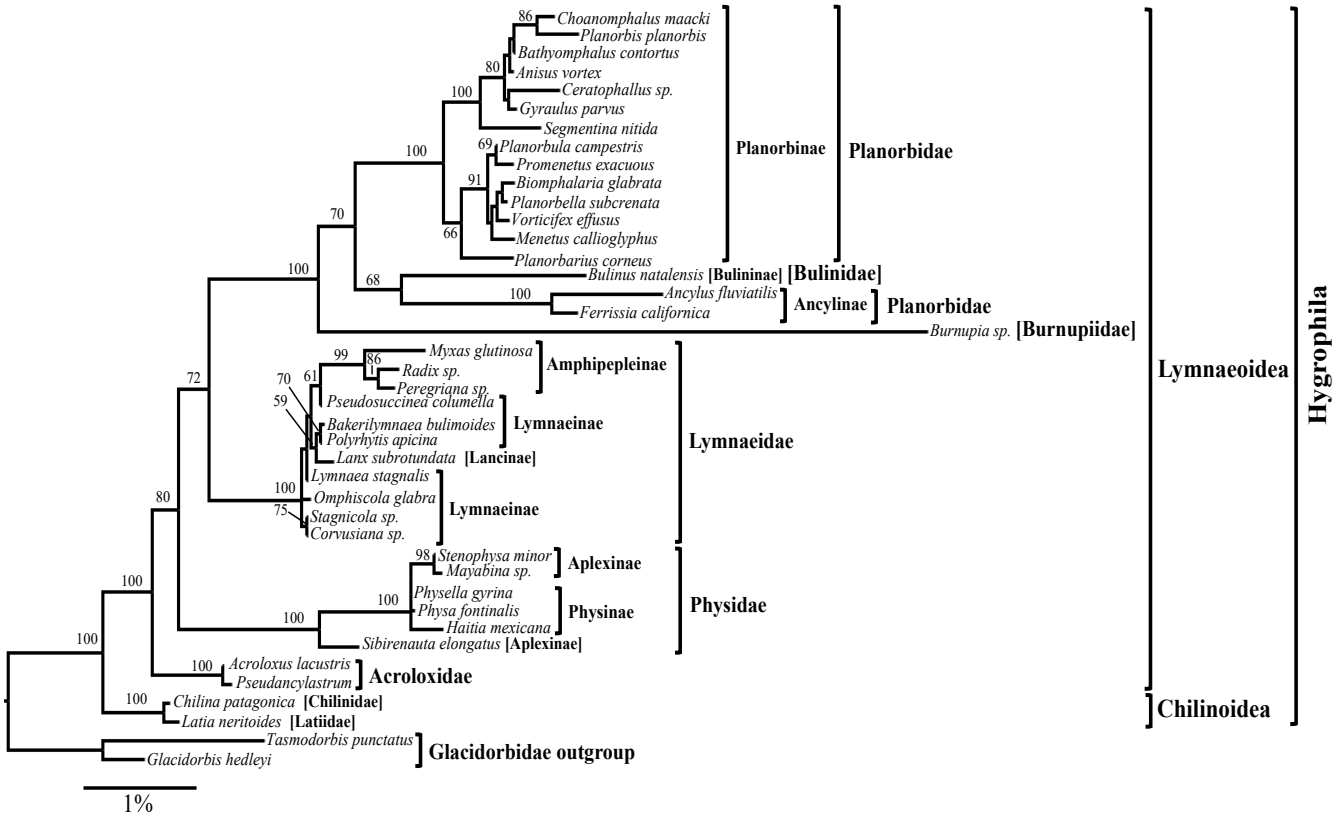
Appendix 3.5. Bayesian phylogenetic tree of the Stylommatophora based on concatenated sequences of 5782 unambiguously aligned nucleotides from the combined dataset of the LSU (and 5.8S) gene, the SSU gene, the H3 gene and the 1st and 2nd codon positions of the CO1 gene. The phylogeny also incorporates all published partial rRNA sequences (431bp) of the Scolodontidae from Ramirez *et al*. (2012) shown as dotted lines. The accession numbers of the published taxa are (*Systrophia eatoni* = HM067823.1, *Systrophia helicycloides* 1= JN604723.1, *Systrophia helicycloides* 2= JN604724.1, *Entodina jekylli* = HM067824.1, *Scolodonta sp.* = HM067825.1 and *Scolodonta sp.* 2 =HM116227.1). The optimal model GTR+Γ was used. The phylogeny is rooted on the siphonariid *Siphonaria pectinata*. Values on the nodes represent bootstrap support (1000 replicates) for ML, NJ and posterior probabilities for BI (based on last 75% of trees), respectively. Bootstrap support values less than 50% and posterior probabilities less than 0.7 are not shown. The scale bar represents 1 substitutional change per 100 nucleotide positions.



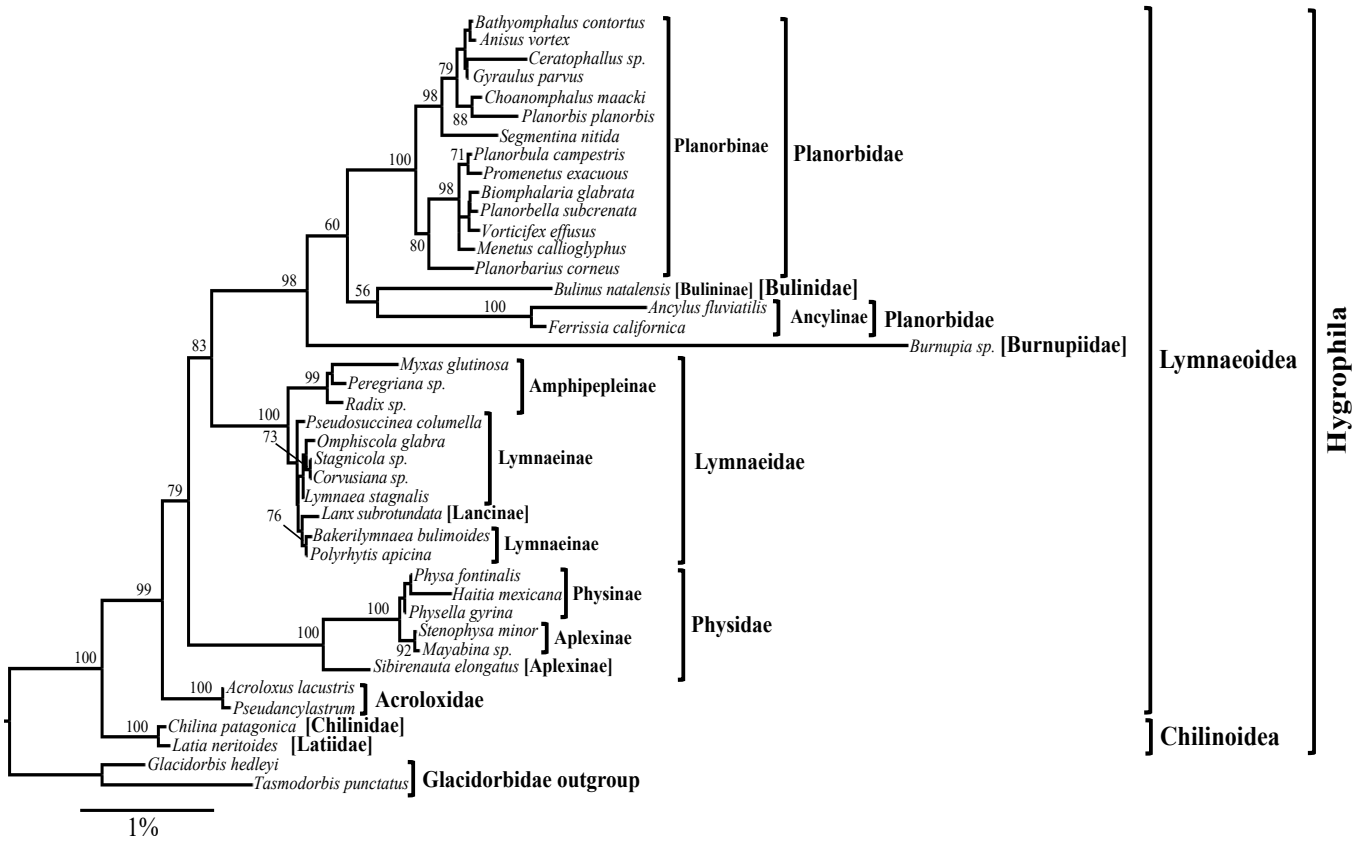
Appendix 4.1. Maximum likelihood tree of the Orthurethra based on 3370 unambiguously aligned nucleotide sites of the LSU (and 5.8S) gene. The optimal model GTR+Γ was used. The phylogeny is rooted on taxa of the 'achatinoid' clade: *Lissachatina fulica*, *Ferussacia folliculus* and *Gonaxis quadrilateralis*. Values on the nodes represent bootstrap support (1000 replicates). Bootstrap support values less than 50% are not shown. The scale bar represents 1 substitutional change per 100 nucleotide positions.



Appendix 4.2. Neighbor-joining phylogenetic tree of the Orthurethra based on 3370 unambiguously aligned nucleotide sites of the LSU (and 5.8S) gene. The optimal model GTR+Γ was used. The phylogeny is rooted on taxa of the 'achatinoid' clade: *Lissachatina fulica*, *Ferussacia folliculus* and *Gonaxis quadrilateralis*. Values on the nodes represent bootstrap support (1000 replicates). Bootstrap support values less than 50% are not shown. The scale bar represents 1 substitutional change per 100 nucleotide positions.



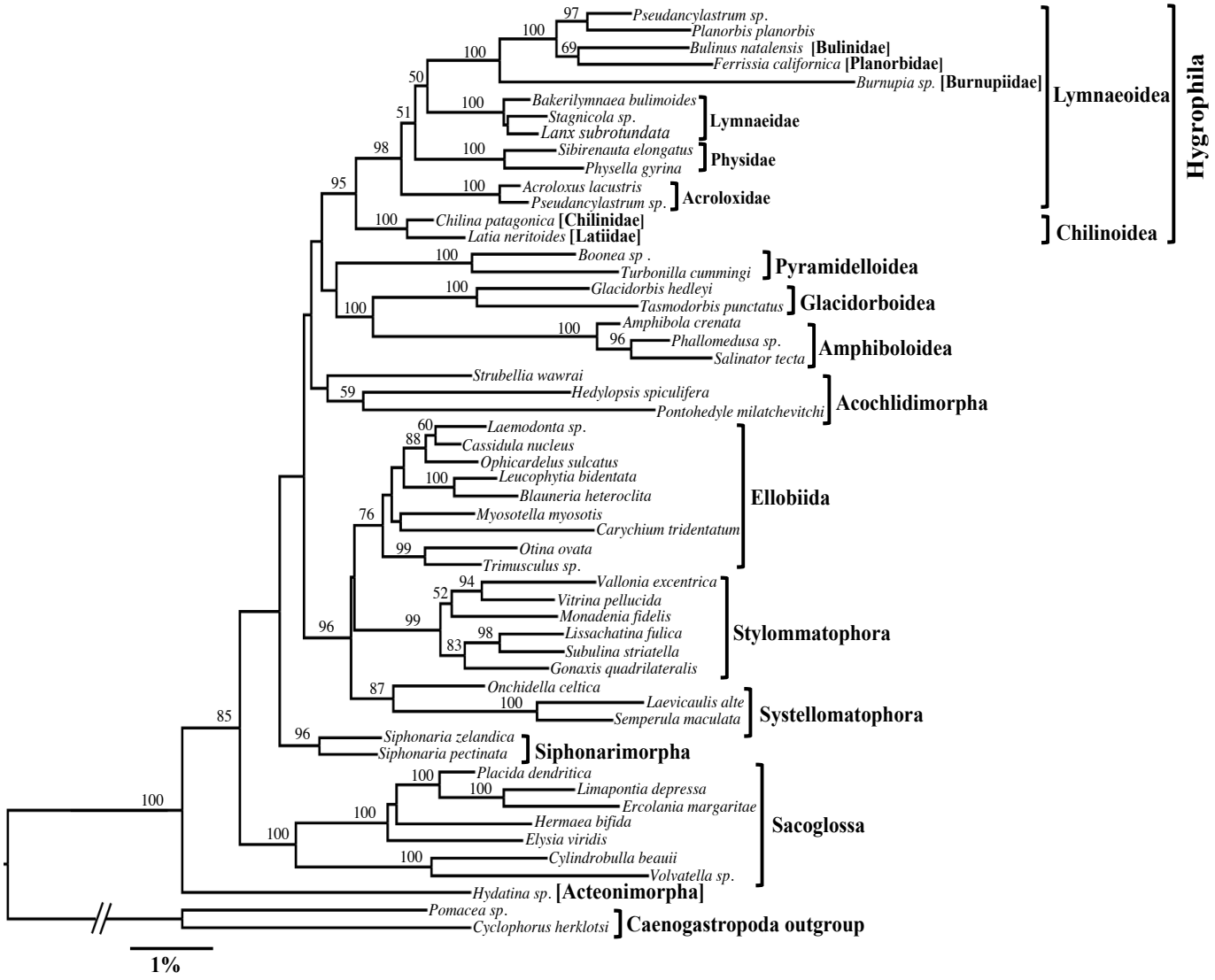
Appendix 5.1. Maximum likelihood phylogenetic tree of the Hygrophila based on 3112 unambiguously aligned nucleotide sites of the LSU (and 5.8S) gene. Values on nodes represent bootstrap support (1000 replicates). Bootstrap support less than 50 is not shown. The optimal model GTR+Γ was used. The phylogeny is rooted on the glacidorbids *Glacidorbis hedleyi* and *Tasmodorbis punctatus*. The scale bar represents 1 substitutional change per 100 nucleotide positions. Classification follows Bouchet *et al*. (2017).



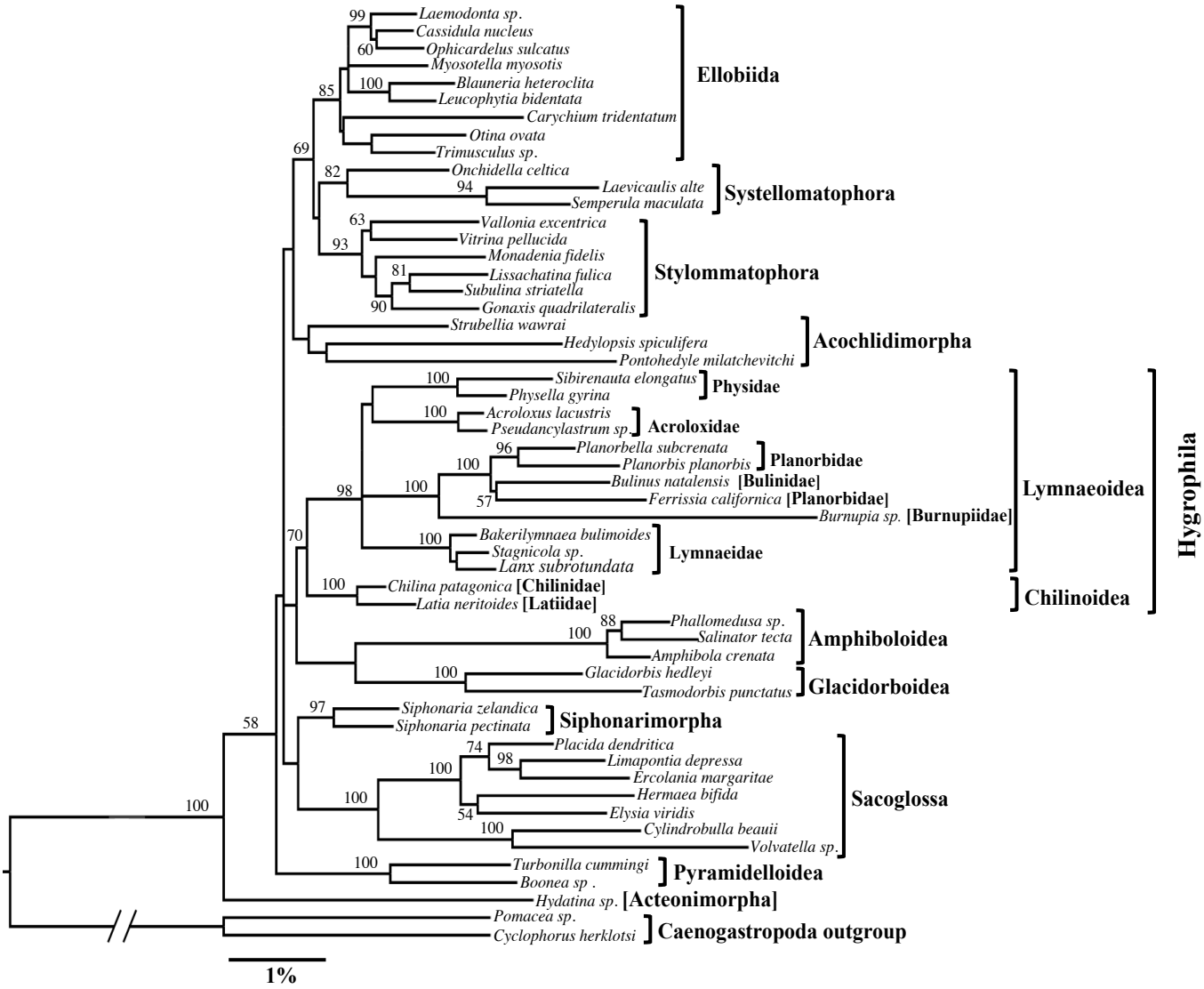
Appendix 5.2. Neighbor joining phylogenetic tree of the Hygrophila based on based on 3112 unambiguously aligned nucleotide sites of the LSU (and 5.8S) gene. Values on nodes represent bootstrap support (1000 replicates). Bootstrap support less than 50 is not shown. The optimal model GTR+Γ was used. The phylogeny is rooted on the glacidorbids *Glacidorbis hedleyi* and *Tasmodorbis punctatus*. The scale bar represents 1 substitutional change per 100 nucleotide positions. Classification follows Bouchet *et al*. (2017).



Appendix 6.1 Bayesian phylogenetic tree of the Panpulmonata based on concatenated sequences of 5502 unambiguously aligned nucleotides from the combined dataset of the LSU (and 5.8S) gene, the SSU gene, the H3 gene and the 1st and 2nd codon positions of the CO1 gene. Values on the nodes represent posterior probabilities (based on last 75% of trees). Posterior probabilities less than 0.7 are not shown. The scale bar represents 1 substitutional change per 100 nucleotide positions. The branch leading to the Caenogastropoda (outgroup) was shortened by about 50% to allow better visibility.



Appendix 6.2. Maximum likelihood tree of the Panpulmonata based on concatenated sequences of 5502 unambiguously aligned nucleotides from the combined dataset of the LSU (and 5.8S) gene, the SSU gene, the H3 gene and the 1st and 2nd codon positions of the CO1 gene. Values on the nodes represent bootstrap support (1000 replicates). Bootstrap support values less than 50% are not shown. The scale bar represents 1 substitutional change per 100 nucleotide positions. The branch leading to the Caenogastropoda (outgroup) was shortened by about 50% to allow better visibility.



Appendix 6.3. Neighbor-joining tree of the Panpulmonata based on concatenated sequences of 5502 unambiguously aligned nucleotides from the combined dataset of the LSU (and 5.8S) gene, the SSU gene, the H3 gene and the 1st and 2nd codon positions of the CO1 gene. Values on the nodes represent bootstrap support (1000 replicates). Bootstrap support values less than 50% are not shown. The scale bar represents 1 substitutional change per 100 nucleotide positions. The branch leading to the Caenogastropoda (outgroup) was shortened by about 50% to allow better visibility.