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Molecular systematics of Hydrobiidae (Mollusca: Gastropoda: Rissooidea): testing monophyly and phylogenetic relationships

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ABSTRACT—The rissooidean family Hydrobiidae Troschel, 1857 is supposedly one of the largest gastropod families with more than 400 recent genera assigned. Due to the limited number of robust anatomical characters in hydrobiids, the high degree of intraspecific variation, the unknown phylogenetic significance of anatomical characters, and the high degree of homoplasy in anatomical characters, its systematic is confusing and phylogenetic relationships within the family and with other rissooidean groups are poorly understood.

We studied fragments of the COI and 18S genes from representatives of 40 genera to determine if the family Hydrobiidae (as defined by Kabat and Hershler, 1993) is monophyletic, if the family Hydrobiidae could be resolved using these genes, and whether the Cochliopidae are a distinct family as previously suggested.

The cluster patterns in the combined 18S + COI tree as well as in the individual 18S and COI trees show that the Hydrobiidae of Kabat and Hershler (1993) are polyphyletic. The analyses also confirm previous studies suggesting that the Cochliopidae are a family distinct from the Hydrobiidae. The following subfamilies are tentatively assigned to the Hydrobiidae: Hydrobiinae, Pseudamnicolinae, Nymphophilinae, Islamiinae, and Horatiinae. Three hydrobiid genera, *Mercuria*, *Hauffenia*, and *Graziana*, could not be assigned unambiguously to one of these subfamilies.

The phylogenetic relationships of the families studied are discussed in the light of the available anatomical data and the performance of the two gene fragments used.

INTRODUCTION

One of the most continuing and vexing problems in gastropod systematics is the definition of the rissooidean family Hydrobiidae and the criteria for classifying a taxon within the family. This is not a trivial concern because of the large numbers of taxa that have been relegated to the family over the years, and the importance of many of these taxa as in resolving problems involving ecology, biogeography, parasitism, and coevolution. In an overview of the Hydrobiidae, Kabat and Hershler (1993) provided a broad definition for the family based on comparative anatomical data. They relegated to the family 75 family-level names with 725 generic names. Hershler and Ponder (1998) illustrated all the characters and character-states found to be of use in differentiating taxa listed by Kabat and Hershler (1993) as far as anatomical data were available. Unfortunately, any cladistic application of the morphological data yields poorly resolved phylogenies (*e.g.,* Falniowski and Szarowska, 2000; Bodon et al., 2001). The primary problem is the considerable convergent evolution of many characters and character-states, and the paucity of unique characters to serve as synapomorphies.

Recent phylogenetic analyses of molecular data sets have clearly shown that certain anatomical characters and character-states have evolved independently and that there are parallel developments in distinctly different families of rissooidean taxa once classified as Hydrobiidae (Davis et al., 1998; Wilke et al. 2000). Given these early findings, our null hypothesis is that the Hydrobiidae defined by Kabat and Hershler (1993) are monophyletic.

The purposes of this paper are: 1) to test the null hypothesis, 2) to test whether the Cochliopidae are a distinct family as previously suggested in the literature, 3) to determine the extent to which the Hydrobiidae can be defined based on molecular data and given the taxa studied here, and 4) to determine if there are anatomical data that can be used to define the Hydrobiidae.

In order to answer these questions, we studied two independent gene fragments (the mitochondrial cytochrome c oxidase subunit I gene and the nuclear 18S rRNA gene) in representatives of 40 genera. They comprise, based on the classification of Kabat and Hershler (1993), species of 1) the subfamily Hydrobiinae, 2) taxa

of other putative hydrobiid subfamilies ("Amnicolinae", Cochliopinae, Islamiinae, Lithoglyphinae, "Moitessierinae", Sadlerianinae), and 3) taxa assigned to families currently not placed in the Hydrobiidae by recent authors (Bithyniidae, Pomatiopsidae, Truncatellidae, Rissoidae).

BACKGROUND

There have been numerous, often conflicting attempts, to define the Hydrobiidae and classify taxa within this family (Table 1). Kabat and Hershler (1993) provided an exhaustive catalog of supra-specific taxa of Hydrobiidae comprising 725 generic-level taxa of which they considered 405 to be nomenclaturally available and in current use. However, as different sets of anatomical characters can produce different phylogenies (Falniowski and Szarowska, 1995), the validity of numerous supra-specific taxa remained suspect.

Principal problems are 1) the morphological simplicity due to miniaturization and the limited number of robust anatomical characters in hydrobiids (if they exist at all), 2) a relatively high degree of intraspecific variation of anatomical characters (which may obscure interspecific variation), 3) the unknown phylogenetic significance of anatomical characters (*i.e.,* unknown signal: noise ratio), and 4) the high degree of homoplasy in anatomical characters.

The resolution of phylogenetic relationships among putative hydrobiid taxa requires the use of molecular characters. So far, most molecular studies of hydrobiids are restricted to the species or the genus level. First attempts to infer family-level relationships involving Truncatellidae, Pomatiopsidae, and Hydrobiidae using 28S rRNA sequencing data were done by Rosenberg et al. (1997). Davis et al. (1998) studied the mitochondrial cytochrome oxidase I (COI) gene from 20 individuals of four rissooidean families and confirmed the family–level status of the Pomatiopsidae, a group that was considered by some workers to belong to the Hydrobiidae. Wilke et al. (2000) used three gene fragments (COI, 16S rRNA, and 18S rRNA) to infer phylogenetic relationships among the Pomatiopsidae, Hydrobiidae, and Amnicolidae. They demonstrated the distinctness of the family Amnicolidae, previously considered a subfamily of the Hydrobiidae. Moreover, they suggested that the Cochliopinae, also previously considered to be a subfamily of the Hydrobiidae, required family status thus reflecting their phylogenetic relationships within the Rissooidea. However, as the number of taxa studied was limited, this hypothesis required further confirmation.

Here we use two independent genes to infer phylogenetic relationships among hydrobiid taxa: the mitochondrial, fast evolving, protein coding COI gene and the nuclear, non-protein coding, slow evolving 18S rRNA gene. Both gene fragments have been extensively used in reconstructing phylogenetic relationships in Mollusca and other animal groups. The variable COI gene shows a good phylogenetic signal from the population to family level, whereas the conservative 18S rRNA usually permits a good discrimination above the family level.

MATERIALS AND METHODS

Materials

Appendix I lists the species used in the present study, their collection sites, latitude and longitude information, DNA isolation numbers, GenBank accession numbers, and names of collectors. The genera and species are assigned to families and subfamilies chosen as a result of this study. Most specimens were fixed in 75% ethanol or 100% methanol prior to DNA isolation. For outgroup comparison we used an individual of *Setia turriculata* (family Rissoidae).

Terminology

Homogeneity-partition test: Significance test for incongruence between matrices (Farris et al., 1995).

Kimura 2-parameter-model (K2P): Model of DNA sequence evolution by Kimura (1980). It assumes two different rates, one for transitions and one for transversions.

Long-branch attraction: Attraction of taxa terminating long branches due to a disproportion of analogies and homologies (see Felsenstein, 1978; Hendy and Penny, 1989).

Maximum likelihood method (ML): Method of phylogenetic inference that maximizes the probability (expressed as log likelihood) of a tree based on the observed data and the assumed model of sequence evolution (Felsenstein 1981, 1983).

Model of DNA sequence evolution: Set of assumptions about the process of nucleotide substitution (also see Table 2).

Phylogenetic signal: A measure of phylogenetic information in data sets, here expressed as informative covariation among character states (t_{RASA}).

Relative apparent synapomorphy analysis (RASA): Topology-independent measure of phylogenetic signal (t_{RASA}) for discrete characters (Lyons-Weiler et al., 1996). To test whether a data set as a whole exhibits phylogenetic signal (t_{RASA}), the observed rate of increase in pairwise cladistic similarity per unit pairwise phenetic similarity (β observed) is compared to a null slope (β null) where cladistic support and phenetic similarities are randomly distributed among pairs of taxa.

SSU rRNA database: Aligned sequence database for the small subunit rRNA (18S) gene (Van de Peer et al., 2000).

Table 1. Previous classifications of the taxa studied in the present paper. Taxa assigned to the family Hydrobiidae are in bold. Taxa with a question mark were considered by the relevant workers to have unclear taxonomic status or could not be classified unambiguously.

Taylor (1966)	Davis (1979)	Radoman (1983)	Starobogatov and Sitnikova (1983)	Ponder and Warén (1988)	Bernasconi (1992)	Kabat and Hershler (1993)
Hydrobiidae	Bithyniidae	Bythinellidae	Amnicolidae	Bithyniidae	Bythiniidae	Bithyniidae
Amnicolinae	**Hydrobiidae**	**Hydrobiidae**	Bithyniidae	**Hydrobiidae**	**Hydrobiidae**	**Hydrobiidae**
Cochliopinae	**Hydrobiinae**	**Hydrobiinae**	Horatiidae	**Hydrobiinae**	**Hydrobiinae**	**Amnicolinae (?)**
Cochliopini	Pomatiopsidae	**Pseudamnicolinae**	Orientalininae	**Littoridininae**	**Horatiini**	**Cochliopinae**
Horatiini	Rissoidae	Lithoglyphidae	Horatiinae	**Lithoglyphinae**	**(= Islamiinae)**	**(= Littoridininae)**
Hydrobiinae	Truncatellidae	Orientalinidae	**Hydrobiidae**	**Nymphophilinae**	**Hydrobiini**	**Hydrobiinae**
Lithoglyphinae		Orientalininae	Islamiidae	**Moitessieriinae**	**(= Pseudamnicolinae)**	**Islamiinae (?)**
Littoridininae		Islamininae	Littoridinidae	**Amnicolinae**	**Nymphophilini**	**Lithoglyphinae**
Nymphophilinae			Moitessieriidae	Pomatiopsidae	**Lithoglyphini**	**Moitessieriinae (?)**
Pomatiopsinae			Pomatiopsidae	Rissoidae	**Moitessieriini**	**Nymphophilinae**
Bithyniidae			Sadlerianidae	Truncatellidae	**Amnicolinae**	**Sadlerianinae (?)**
			Pseudamnicolinae		**Littoridininae (?)**	Pomatiopsidae
			Sadlerianinae		Pomatiopsidae	Rissoidae
			Triculidae		Truncatellidae	Truncatellidae
			Pomatiopsidae			

Table 2. Parameters of DNA sequence evolution, phylogenetic reconstruction and bootstrap analyses used in PAUP for the COI, 18S, and COI+18S data sets.

	COI (638 bp)	18S (478 bp)	COI+18S (1116 bp)
DNA sequence evolution			
Model selected	TVM+I+G	TIMef+I+G	GTR+I+G
Rate matrix	[A − C] = 0.2208	[A − C] = 1.0000	[A − C] = 0.3968
	[A − G] = 12.7345	[A − G] = 1.3249	[A − G] = 8.6740
	[A − T] = 0.1388	[A − T] = 0.3254	[A − T] = 0.1722
	[C − G] = 1.6937	[C − G] = 0.3254	[C − G] = 1.0575
	[C − T] = 12.7345	[C − T] = 4.0016	[C − T] = 7.9843
	[G − T] = 1.0000	[G − T] = 1.0000	[C − T] = 1.0000
Base frequencies	freqA = 0.3552	equal frequencies	freqA = 0.3532
	freqC = 0.1215		freqC = 0.1365
	freqG = 0.0931		freqG = 0.1062
	freqT = 0.4303		freqT = 0.4040
Proportion of invariable sites (I)	0.5067	0.7279	0.6662
Gamma distribution shape parameter (G)	0.3582	0.6145	0.4187
Tree search			
Optimality criterion	max. likelihood	max. likelihood	max. likelihood
Search algorithm	heuristic	heuristic	heuristic
Branch-swapping	TBR	TBR	TBR
No. of random-addition-sequence replications	20	20	20
Character-weight assignment	none	none	COI:18S = 1:8
Boostrap analysis			
No. of bootstrap replications	100	100	100
Search algorithm	faststep	faststep	faststep

DNA Isolation and Sequencing

The methods of Spolsky et al. (1996) and Davis et al. (1998) were used for isolating DNA from individual snails. The primers to amplify a fragment of the COI gene with a target length of 658 base pairs (excluding 51 bp primer sequences) are those described by Folmer et al. (1994). To optimize the performance of the PCR the reverse primer was modified at position 24 (C→Y). The primers for amplification of a 500–625 bp long fragment (excluding 42 bp primer sequences) of 18S rDNA are as described by Holland et al. (1991). Each PCR reaction mixture, in a total volume of 50 μl, contained 20–100 ng of genomic DNA, 5 μl of 10× reaction buffer, 1 mM $MgCl_2$, 200 μM of each dNTP, 300 nM of each primer, 2 μl of 100× BSA solution, 0.5 μl TMAC, and 2.5 units of polymerase. Annealing temperatures were 47° C and 42° C for amplification of the COI and 18S fragment, respectively. The quality of PCR products was determined by electrophoresis in a 1% agarose gel. Amplified DNA products were separated by electrophoresis in a 1% low melting point agarose gel in TAE buffer. The bands corresponding to a fragment of the correct size were cut out and the DNA purified using Wizard PCR preps (Promega).

Sequences were determined by automated cycle sequencing using the DNA sequencer Long ReadIR 4200 (LI-COR) and the Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia) according to their protocols.

Sequence Alignment and Data Analyses

Sequences for each individual were assembled and edited using the computer program ESEE 3.0s (Cabot and Beckenbach, 1989). The protein-coding mitochondrial COI gene does not show insertions or deletions in the superfamily Rissooidea. Therefore, the sequences could be aligned easily. The initial alignment of 18S sequences was refined using secondary structure information from the SSU rRNA database (Van de Peer et al., 2000).

The first 2–10 base pairs behind the 3′ end of each primer are often difficult to read. We therefore uniformly excluded the first and last ten bp of each sequence, leaving a 638 bp-long fragment for the COI gene and a 480–605 bp-long fragment for the 18S gene corresponding to positions 180–1874 in the SSU rRNA sequence X94274 for the caenogastropod *Littorina obtusata* (Linnaeus, 1758). The sequences for the nonprotein-coding 18S gene contained highly variable regions (corresponding to positions 591–788 and 890–1017 in the aligned sequence for *Littorina obtusata*). In order to test whether these sections, which often cannot

be aligned easily and unambiguously, should be excluded from subsequent analyses to reduce background "noise" and to minimize the problem of long-branch attraction, we used RASA 2.5 (Lyons-Weiler et al., 1996) to examine the phylogenetic signal (t_{RASA}) of our 18S data set.

The phylogenetic signal in the 18S data set, with the highly variable regions excluded (sequences varied between 471 and 477 bp; the aligned sequences had 478 sites) and gaps treated as missing data, was considerably higher (t_{RASA} = 53.936) than in the 18S data set with the highly variable regions included (t_{RASA} = 11.469; also see Figs. 4a,b). Therefore 18S sequences with the variable regions excluded were used for subsequent analyses.

All sequences (18S, COI) are available from GenBank (Appendix I). Sixteen sequences were previously submitted to GenBank as part of related studies. Aligned 18S sequences are available from the senior author upon request.

Pairwise transition:transversion (Ts:Tv) ratios and pairwise distances (corrected according to the K2P-parameter-model) were calculated using MEGA 2 (Kumar et al., 2000). Parameters of DNA polymorphism were determined with DnaSP 3.50 (Rozas and Rozas, 1999).

Prior to the phylogenetic analyses we used the computer program Modeltest 3.0 (Posada and Crandall, 1998) in order to find the optimal model of DNA sequence evolution for each data set (COI, 18S, COI+18S). It performs hierarchical likelihood ratio tests among 56 possible models. The models selected are given in Table 2.

In order to reconstruct the phylogenetic relationships of the sequences sampled, maximum-likelihood (ML) analyses were conducted utilizing PAUP 4.0b5 (Swofford, 1998) with the parameters specified in Table 2. The ML approach was chosen because it shows one of the best performances of all major phylogenetic methods (Huelsenbeck, 1995). It also is one of the best methods to overcome long-branch attraction under conditions of extreme terminal branch-length variation (Cunningham et al., 1998).

RESULTS

Family names used in the result section in bold reflect the acceptance of that family based on previous anatomical and/or molecular studies. For species with controversial (synonymous) genus names, we used the potential synonym if type species were studied (*e.g., Adrioinsulana*, *Semisalsa*, *Ventrosia*, *Peringia*).

18S Sequence Polymorphism

From 470 sites (478 sites less 8 sites with alignment gaps) in the 40 individuals studied, 52 sites (11.1%) were polymorphic and 33 sites (7.0%) were parsimony informative. The pairwise K2P-distances ranged from 0.0 (among the cochliopids *Heleobops carrikeri*, *Semisalsa dalmatica*, *Littoridinops monroensis*, *Pyrgophorus platyrachis*, and *Spurwinkia salsa*; among the **pomatiopsids** *Gammatricula chinensis*, *Tricula* sp., and *Oncomelania h. hupensis*; among the **hydrobiids** *Horatia klecakiana*, *Sadleriana fluminensis*, *Belgrandia thermalis*, and *Orientalina callosa*; as well as between the **hydrobiids** *Fissuria boui* and *Alzoniella finalina*) to 0.0577 (between the **amnicolid** *Erhaia jianouensis* and the **hydrobiid** *Mercuria similis*). The average overall K2P-distance among the 18S sequences was 0.0276.

A plot of pairwise Ts:Tv ratios against pairwise K2P-distances does not indicate that the sequences approach transitional saturation (Fig. 1A).

COI Sequence Polymorphism

From 638 sites in 40 aligned sequences, 277 sites (43.4%) were polymorphic and 258 sites (40.4%) were parsimony informative. The average overall K2P-distance was 0.216 with a lowest distance of 0.046 (between the **hydrobiids** *Sadleriana fluminensis* and *Orientalina callosa*) and a highest distance of 0.292 (between the **amnicolid** *Amnicola limosa* and the **hydrobiid** *Peringia ulvae*).

The sequences approached transitional saturation (where Ts:Tv = 1; see Fig. 1B) at a pairwise K2P-distance of about 0.20.

18S Phylogeny

The t_{RASA}–value for the 18S data set (478 sites, t_{RASA} = 53.936, $p < 0.005$) indicated that cladistic support and phenetic similarity among pairs of taxa were not randomly distributed, suggesting the presence of a phylogenetic signal.

The ML analysis, using the rissoid *Setia turriculata* as outgroup, yielded a tree (Fig. 2A) that consists of six principal clades or lineages and several subclades (from top to bottom):

1) A distinct **hydrobiid** clade defined by clear apomorphies comprising 19 species. Six subclades and lineages can be assigned to this clade (1a–1f).
2) A **bithyniid** lineage with the genus *Bithynia*.
3) A clade with *Moitessieria* and *Bythiospeum* clustering together and *Lithoglyphus* being basal.

4a) An **amnicolid** subclade with *Erhaia*, *Amnicola*, and *Marstoniopsis*.
4b) A lineage with the **amnicolid** genus *Bythinella*.
4c) A well-defined but little differentiated cochliopid subclade containing six genera.

5a) A lineage with the **truncatellid** genus *Truncatella*.
5b) A lineage with the **truncatellid** genus *Geomelania*. Both truncatellid taxa cluster rather basal to all other ingroup taxa.

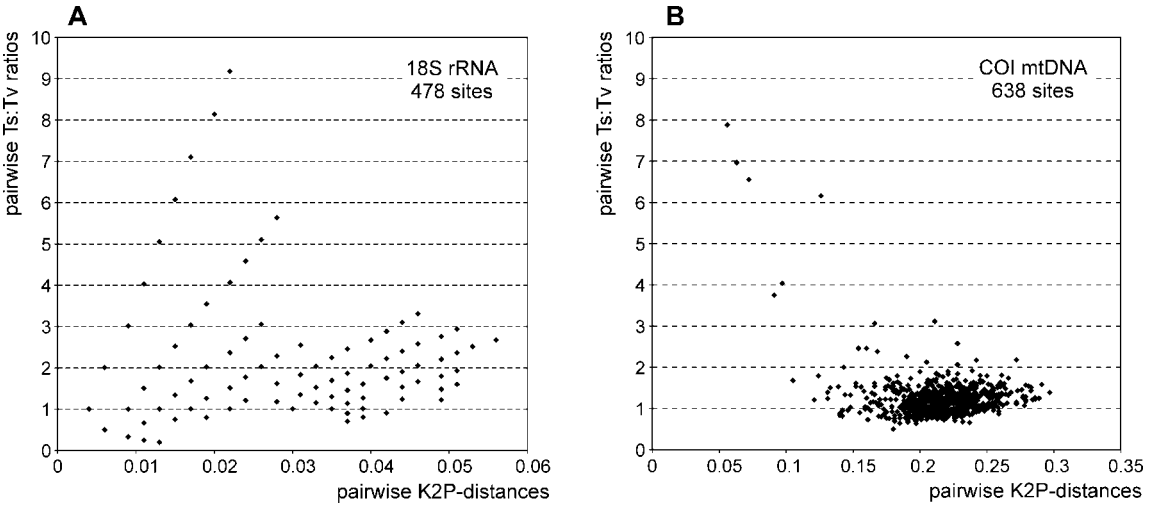

Fig. 1. Comparisons of transition:transversion ratios and pairwise K2P-distances in the 18S gene (A) and COI gene (B). The COI sequences approach transitional saturation (where Ts:Tv = 1) at a pairwise K2P-distance of about 0.20, whereas no effects of saturation can be detected in the 18S gene.

6) A **pomatiopsid** clade with the four genera studied. Interestingly three of the four pomatipsids (*Tricula*, *Gammatricula*, and *Oncomelania*) are homogenetic, whereas the fourth taxon, *Pomatiopsis* is genetically distinct although *Tricula* and *Gammatricula* belong to the subfamily Triculinae, whereas *Pomatiopsis* and *Oncomelania* belong to the subfamily Pomatiopsinae.

COI Phylogeny

The t_{RASA}–value for the COI data set (638 sites, t_{RASA} = 11.685, $p < 0.005$) suggested a significant degree of hierarchy and therefore the presence of a phylogenetic signal.

The ML analysis of COI sequences from 40 taxa yielded two trees, both with the same log likelihood score (9073.46). The only difference in the two trees was the arrangement of two clades (clades 3 and 4 in Fig. 2B). As these clades had branch lengths $<1^{-08}$, in PAUP they were collapsed by default, creating a polytomy. The resulting trees then had exactly the same topology.

The COI tree (Fig. 2B) comprises the outgroup taxon *Setia*, five dominant clades, and several subclades or lineages (from top to bottom):

1) Clade 1 has eight subclades:
1a) A well defined subclade with the two genera *Pseudamnicola* and *Adrioinsulana* (= 1a in Fig. 2A).
1b) A subclade containing the genera *Adriohydrobia*, *Ventrosia*, *Peringia*, and *Hydrobia* (= 1b in Fig. 2A).
1c) A subclade containing the two genera *Cincinnatia* and *Notogillia* (= 1c in Fig. 2A).
1d) A lineage with *Mercuria* being basal to subclades 1a–1c.
1e) A lineage with *Belgrandia* being basal to subclades 1f–1h.
1f) A subclade with the three genera *Horatia*, *Sadleriana*, and *Orientalina*.
1g) A well defined subclade with the taxa *Islamia*, *Avenionia*, *Alzoniella*, and *Fissuria* (= 1g in Fig. 2A).
1h) A subclade comprising *Hauffenia* and *Graziana*.
2a) A lineage with *Lithoglyphus* being basal to the other subclades of clade 2.
2b) A subclade with *Moitessieria* and *Bythiospeum*.
2c) A *Bithynia*-lineage (= 2 in Fig. 2A).
2d) An **amnicolid** subclade with *Amnicola*, *Erhaia*, *Marstoniopsis*, and *Bythinella* (= 4a,b in Fig. 2A).
3) A distinct **truncatellid** clade comprising *Truncatella* and *Geomelania* (= 5a, b in Fig. 2A).
4) A **Pomatiopsidae** clade with *Pomatiopsis*, *Gammatricula* + *Tricula*, and *Oncomelania* each forming subclades (= 6 in Fig. 2A).
5) A cochliopid clade, with six genera (= 4c in Fig. 2A). Within that clade three distinct subclades and lineages can be recognized (*Semisalsa* + *Heleobops*, *Littoridinops* + *Spurwinkia* + *Pyrgophorus*, and *Onobops*).

18S + COI Phylogeny

In order to test whether there are significant differences in incongruence length between the 18S and COI data sets we used the HOMPART command in PAUP to perform a homogeneity-partition test (Farris et al., 1995). As the test did not reveal significant differences (p = 1; 100 replicates), we used the two data sets in a combined analysis.

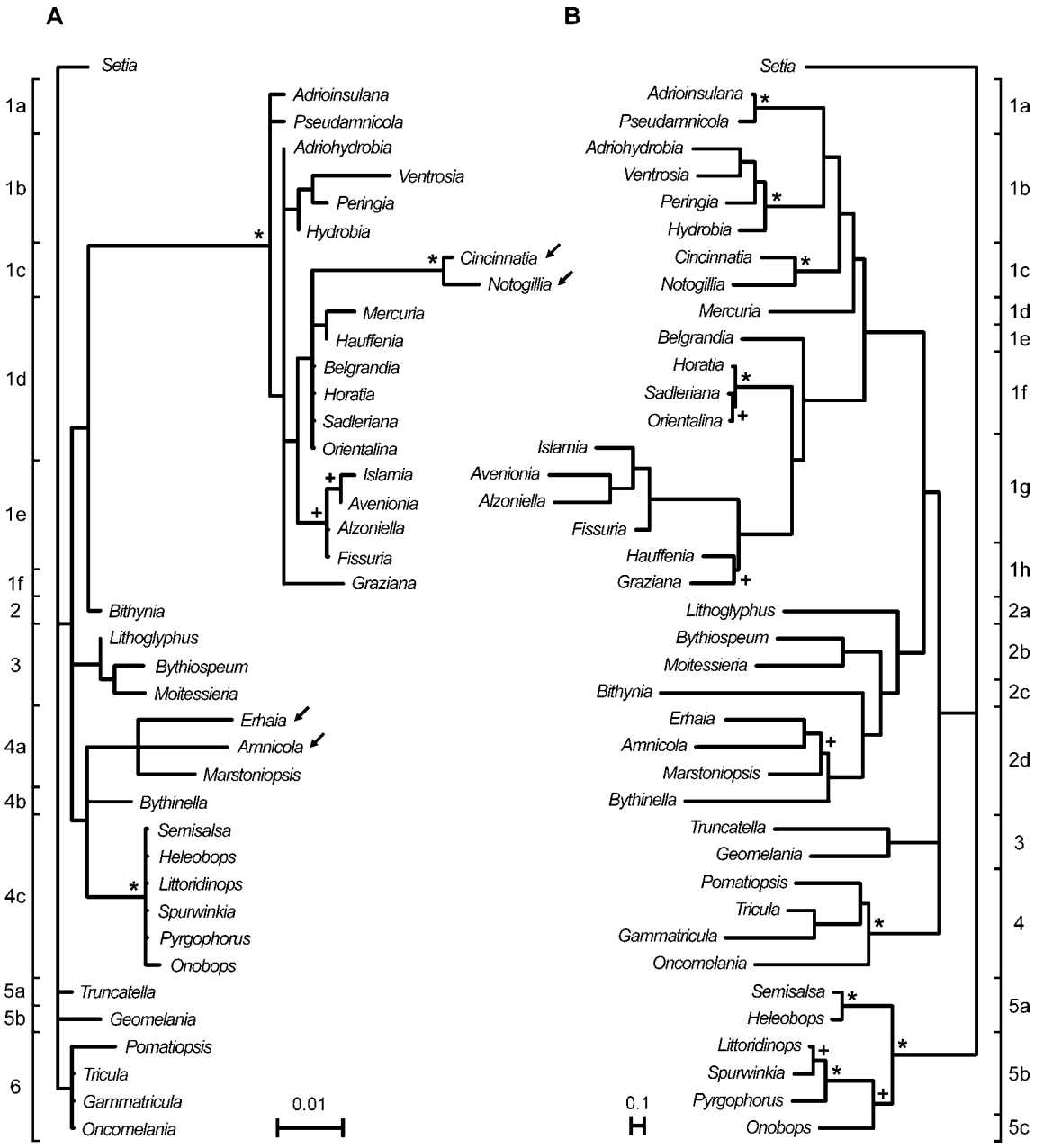

Fig. 2. ML trees for rissooidean species based on 18S (A) and COI (B) sequences. The scale bars indicate the expected substitution rate for each gene fragment. Bootstrap support is provided (* ≥70%; 50 ≤ + <70%; all other nodes are not resolved in a 50% majority-rule consensus tree). Note that the expected substitution rate is subject to the parameter selected in Table 2 and therefore different from K2P-distances. The rissoid *Setia turriculata* was used as outgroup. Taxa that are possibly subject to long-branch attraction are marked with an arrow. Major clades, subclades, and lineages are numbered.

The average K2P-distance among the 40 taxa in the COI portion of the combined data set is 0.216, whereas only 0.027 in the 18S portion. Therefore it is likely that the phylogenetic analysis of the combined data set would be biased towards the COI portion of the data set because of the higher substitution rate. In an attempt to compensate for the unequal rates of evolution in the COI and 18S genes, we weighted the 18S portion of the combined data set eight times over the COI portion using the WTSET assumption in PAUP.

The ML analysis yielded two trees. Similar to the COI analysis, the only difference in the trees was the arrangement of two clades (Bithyniidae and Moitessieriidae) that were collapsed by PAUP, creating a poly-

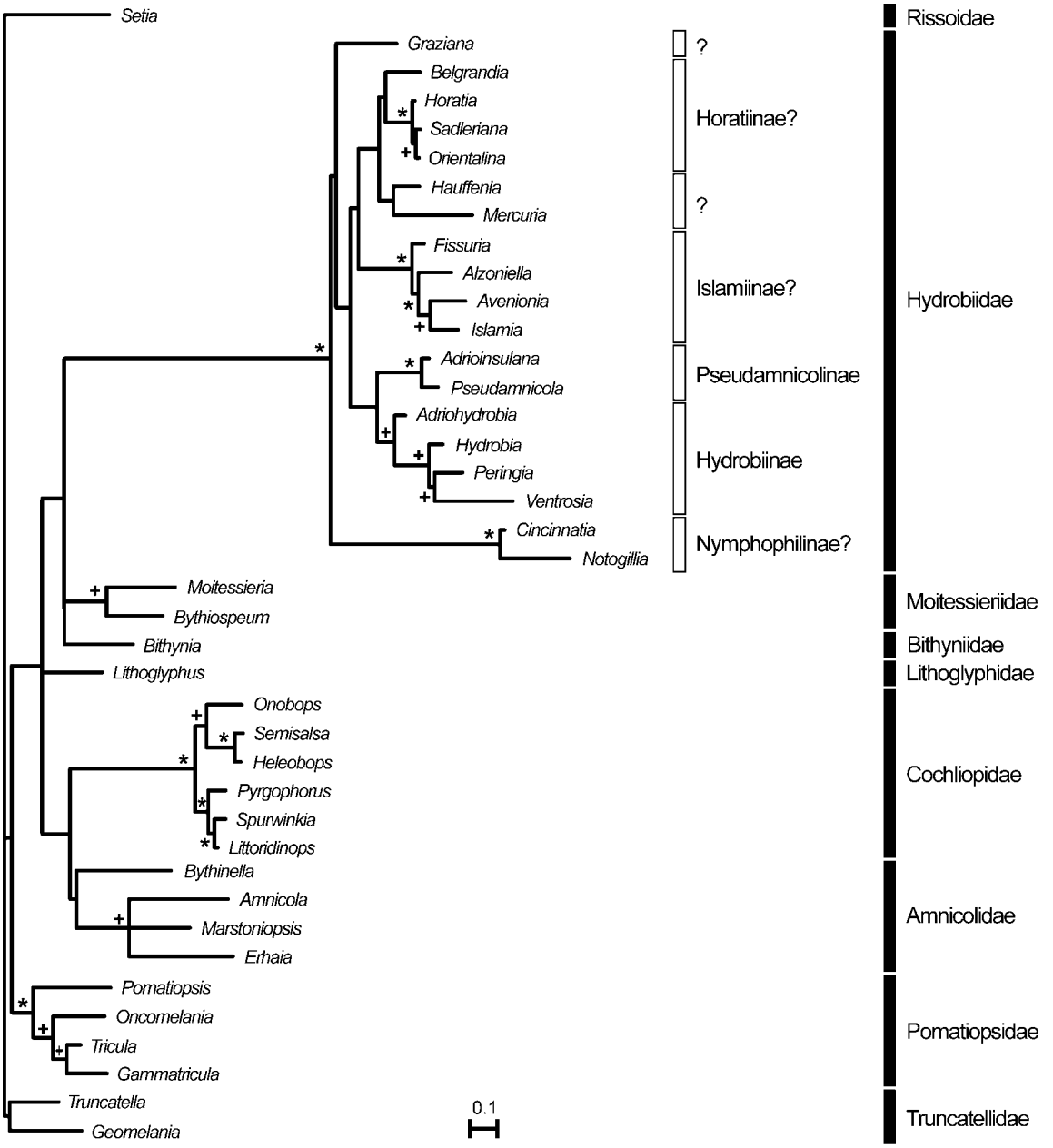

Fig. 3. ML tree for rissooidean species based on combined COI and 18S sequences. The scale bar indicates the expected substitution rate. Bootstrap support is provided (* ≥70%; 50 ≤ + <70%; all other nodes are not resolved in a 50% majority-rule consensus tree). The families involved are indicated with black bars; hypothetical subfamilies of the Hydrobiidae with white bars. These taxa are chosen as a result of this study.

tomy. The resulting trees then had exactly the same topology.

The ML tree based on the combined 18S and COI data sets (Fig. 3) rather well reflects the phylogenetic relationships found in the separate 18S and COI analyses (Fig. 2). Incongruencies among the three phylogenies mainly involve the arrangement of higher taxa that are characterized by few apomorphies in the 18S data and/or for which we have studied only one or few species each.

The cluster patterns in the combined 18S + COI tree as well as in the individual 18S and COI trees show that the Hydrobiidae *sensu* Kabat and Hershler (1993) are polyphyletic and that our initial null hypothesis has to be rejected. Therefore, from this point forward, taxa in clade 1 (Fig. 2 A, B) are considered to be Hydro-

Table 3. Average K2P-distances based on 1116 bp within (on diagonal line) and between (below diagonal line) selected families. Standard errors (estimated by 500 bootstrap replications) are given in parentheses.

	Hydrobiidae	Cochliopidae	Pomatiopsidae	Amnicolidae
Hydrobiidae (19 species)	0.111 (0.007)			
Cochliopidae (6 species)	0.144 (0.009)	0.070 (0.006)		
Pomatiopsidae (4 species)	0.137 (0.009)	0.126 (0.009)	0.094 (0.007)	
Amnicolidae (4 species)	0.149 (0.009)	0.143 (0.009)	0.140 (0.009)	0.117 (0.008)

biidae *s.s.* (see Fig. 3); taxa of subclade 4c (Fig. 2A) and clade 5 (Fig. 2B) are Cochliopidae (see Fig. 3). The distinctness of the Hydrobiidae and Cochliopidae clades are confirmed by a high bootstrap support (100% each!).

Besides the Cochliopidae, there are other groups of genera, previously considered to be subfamilies of the Hydrobiidae (Table 1), that clearly cluster distinctly outside the Hydrobiidae (*e.g.,* Moitessieriidae, Lithoglyphidae). It also has to be noted that the Cochliopidae cluster more closely with the now well established Amnicolidae than with the Hydrobiidae *s.s.*

As seen in Fig. 3, there are seven subclades and lineages within the Hydrobiidae, five of which correspond to nominal subfamilies (see Appendix II) that have previously been defined on the basis of anatomical data:

1) the Hydrobiinae (with *Hydrobia*, *Ventrosia*, *Peringia*, and *Adriohydrobia*),
2) the Pseudamnicolinae (with *Pseudamnicola* and *Adrioinsulana*)
3) the Nymphophilinae? (with *Cincinnatia* and *Notogillia*),
4) the Islamiinae? (with *Islamia*, *Fissuria*, *Avenionia*, and *Alzoniella*), and
5) the Horatiinae? (with *Horatia*, *Sadleriana*, *Belgrandia*, and *Orientalina*).

Three taxa cannot be assigned unambiguously to one of these subfamilies: *Mercuria*, *Hauffenia*, and *Graziana*. This is partly due to their low bootstrap support within the Hydrobiidae clade. They possibly belong to other putative subfamilies (*e.g., Hauffenia* to the Pseudohoratiinae and *Graziana* to the Belgrandiellinae, see Radoman, 1983), or to new subfamilies.

To further assess the distinctness of the Hydrobiidae and the Cochliopidae, we compared the K2P-distances within and between these groups with two other well-defined groups for which we have studied at least three species each, the Pomatiopsidae and the Amnicolidae (Table 3). Pairwise comparisons between these families yield similar average distances ranging from 0.126 (Cochliopidae vs. Pomatiopsidae) to 0.149 (Hydrobiidae vs. Amnicolidae). The average distance between the Hydrobiidae and Cochliopidae of 0.144 is well within this range. The K2P-distance within families ranges from 0.070 (Cochliopidae) to 0.117 (Amnicolidae). The average distance within the 19 Hydrobiidae is 0.111. All distances within the families are smaller than the distances between families, a further indication for the distinctness of the groups.

DISCUSSION

Methodological Issues

The information in the highly variable regions of the 18S gene is often used when studying relationships at lower systematic levels (*i.e.,* species and genera). When studying higher-level relationships (*i.e.,* family and above), those regions are often removed from analyses or downweighted. We applied a tree independent approach (RASA) to test whether the highly variable regions decrease the phylogenetic signal and/or increase the problem of long-branch attraction. The results of the RASA test are conclusive. The 18S data set with highly variable regions included had a relatively low ($t_{RASA} = 11.469$), but still significant ($p < 0.005$) phylogenetic signal (Fig. 4A). There are numerous outliers suspect to long-branch attraction with the taxon pairs *Pyrgophorus*: *Ventrosia* (Cochliopidae/Hydrobiidae), *Adriohydrobia*: *Ventrosia* (Hydrobiidae), *Bythiospeum*: *Ventrosia* (Moitessieriidae/Hydrobiidae), and *Onobops*: *Spurwinkia* (Cochliopidae) being the most severe. When removing the highly variable regions from the analysis (as done in the present study), the phylogenetic signal increases considerably to $t_{RASA} = 53.936$ Moreover, the RASA matrix (Fig. 4B) now shows only few pairs of taxa with slightly increased cladistic similarity (*e.g., Cincinnatia*: *Notogillia* and *Amnicola*: *Erhaia*). We retained those taxa in our study as we used a ML approach with an optimized model of sequence evolution (especially among-site rate variation) to infer phylogenetic relationships, which shows a high performance relative to overcoming long-branch attraction (Cunningham et al., 1998). Also, the two taxa pairs possibly subject to long-branch attraction in the 18S data set showed similar cluster patterns in the COI phylogeny where they are not subject to long-branch attraction (Fig. 4C).

Our study does not require *per se* the removal of the highly variable regions when studying higher-level phylogenetic relationships. Instead, each gene, each frag-

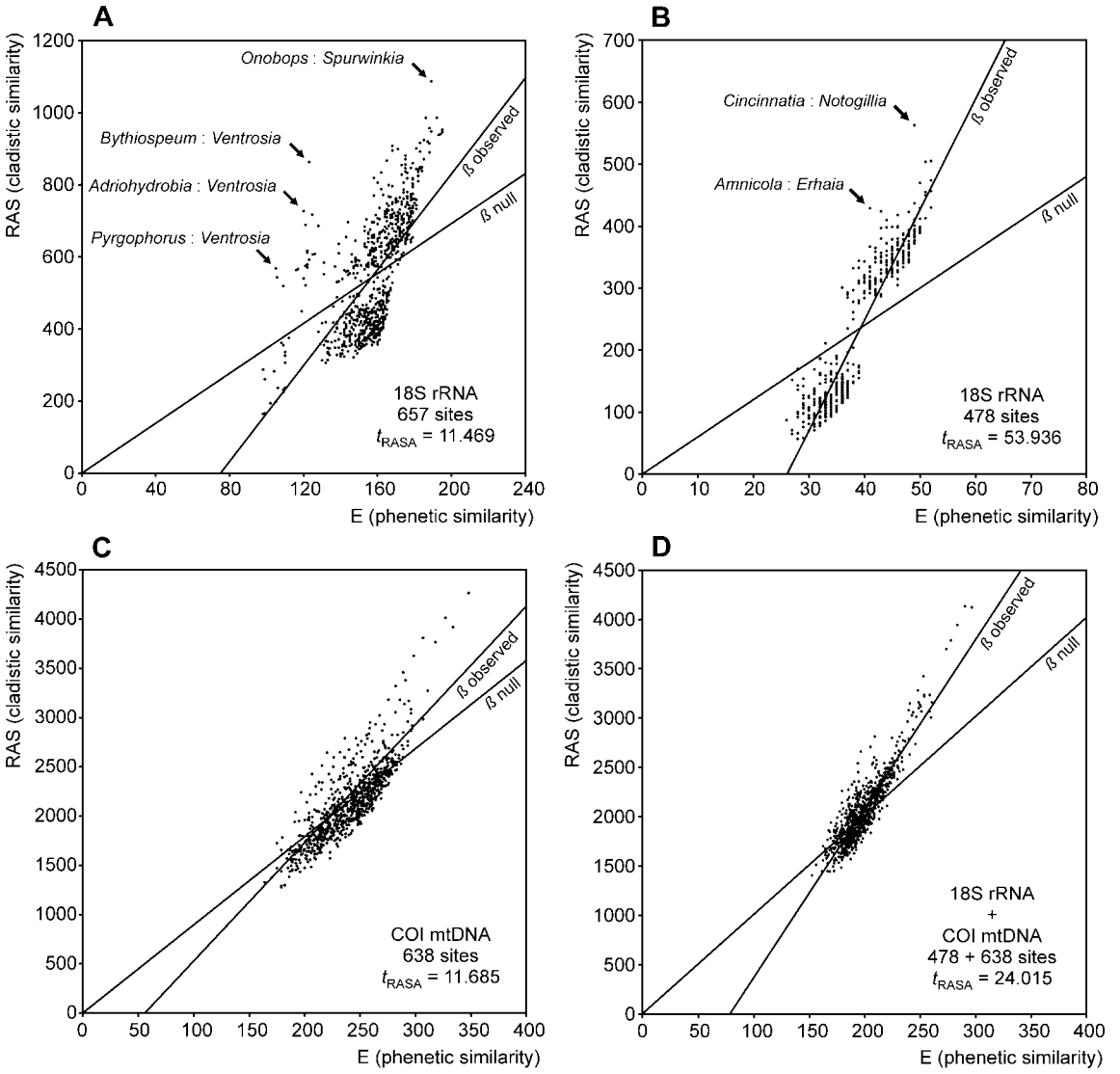

Fig. 4. RASA taxon variance plots for 18S (A: with highly variable regions; B: without highly variable regions), COI (C), and COI + 18S (D) data set.

ment, and each set of taxa should be tested for phylogenetic signal and possible effects of long-branch attraction and, if necessary, appropriate steps taken to minimize methodological problems. The tradeoff of removing the highly variable regions from our analysis is that the already low degree of polymorphism in the 18S gene fragment is further reduced. Thus, many relationships among closely related taxa (*e.g.,* within the Cochliopidae and Pomatiopsidae) could not be resolved. Moreover, even some higher level relationships among families (*e.g.,* Truncatellidae, Moitessieriidae, Lithoglyphidae, and Bithyniidae) remain ambiguous because of the low heterogeneity rate where random mutations can severely affect the tree topology. We studied only a small fragment (about 500 bp) of the 18S gene (total length about 1,800 bp). Thus, an analysis of the whole gene or a different (longer) fragment might well resolve these ambiguous relationships. An advantage of using the 18S gene is that there are no problems with saturation at the level we studied here (Fig. 1A).

In contrast to the 18S gene, the COI gene fragment is rather variable and shows a moderate phylogenetic signal in our data set (t_{RASA} = 11.685). No outliers, possibly subject to long-branch attraction, could be detected (Fig. 4C). The downside of the high sequence polymorphism is the high rate of transitional substitutions that are subject to saturation at or shortly below a pairwise K2P-distance of about 0.20. Thus, most relationships among families in our COI data set may be affected by transitional saturation, indicated by the low

bootstrap support for most deeper nodes in the ML analyses (Fig. 2B). However, we did not apply any measures to mitigate the effect of saturation (*e.g.,* eliminating 3rd position substitutions, considering transversions only) because most of the relationships within the two groups of main interest, the Hydrobiidae and the Cochliopidae, are not subject to saturation and the distinctness of these groups is confirmed by the 18S and the combined 18S + COI analyses.

In summary, both fragments used show a good performance in phylogenetic studies of rissooidean snails. The COI gene fragment shows a rather consistent performance on the genus and family level, but quickly approaches transitional saturation above the family level. The conservative 18S gene fragment has, with some exceptions and depending whether or not highly variable regions are used, a good phylogenetic signal on and above the family level. It is not subject to saturation, but shows an increased perception for long-branch attraction. Despite some differences, the two data sets are compatible (as shown by the homogeneity-partition test), and a combined analysis has likely improved the reliability of our phylogenetic hypothesis. This is indicated by the increased bootstrap support in the combined analysis, which is considerably higher than in the 18S or COI trees.

Hydrobiidae and Cochliopidae Resolved

The Family-level Taxon.—There is no universal definition for "family". It is the highest rank category whose nomenclature must follow the rules of the International Code of Zoological Nomenclature. It is a monophyletic group above the genus level comprising one or more genera. From a cladistic standpoint it is a clade clearly apart form other clades on the basis of synapomorphies (morphological, molecular, karyological, physiological, behavioral, etc.). In malacology, a family has previously been recognized because of major modifications in the anatomical ground plan seen most frequently in innovations of the reproductive, digestive, and respiratory systems. However, in the past decade, it has become increasingly clear that there are too few and/or wrongly identified morphology-based synapomorphies to clearly differentiate families and subfamilies of rissooidean snails.

Hydrobioid Snails and Morphological Discrimination.—Anatomical data from all organ systems are essential to differentiate genera and families of rissooidean snails (Davis 1967, 1968). Davis (1979) pointed out that the Hydrobiidae, as previously defined, were a loose assemblage of genera variously classified into poorly defined subfamilies, and that the current standard for classification was essentially that of Stimpson (1865), established a century ago. Davis (1979), in separating the Pomatiopsidae from the "Hydrobiidae", created the term "hydrobioid" for any rissooidean snail that had a similar shell, operculum, radula, and penis (the "Stimpson standard"). He listed 14 characters that were common to hydrobioids or excluded hydrobioids from other higher taxa (*e.g.,* the Assimineidae, Stenothyridae, Truncatellidae, and Bithyniidae).

Ponder (1988) provided the first comprehensive cladistic analysis of rissooidean (= truncatelloidean) families using 39 anatomical characters. As Davis (1979) had before, he justified removal of the Assimineidae, Bithyniidae, Stenothyridae, and Truncatellidae from the Hydrobiidae. However, as pointed out by Kabat and Hershler (1993), in Ponder's analysis, only two autapomorhpies serve to define the Hydrobiidae clade and of these, one is a reversal (loss of metapodial tentacles) that is paralleled in three other clades; the other, the reduction of the oesophageal gland, is paralleled in five situations. They concluded that, given the "enormous morphological variation of hydrobiids", the monophyly and scope of the Hydrobiidae had yet to be determined. The detailed definition of the Hydrobiidae they did provide is one that pertains to "hydrobioids" sensu Davis (1979).

Given that we used a limited number of taxa, a revision of the Hydrobiidae is beyond the scope of this paper. However, we can couple the molecular data with a judicious use of anatomical characters to define the core Hydrobiidae and the Cochliopidae.

The Hydrobiidae and Cochliopidae: Synthesis of Morphology and Molecular Data.—The results based on the molecular data make it possible to examine groups of morphological characters and character-states enabling the definition of families. At the same time the degree of parallelism, reversal, and convergence that make a cladistic analysis virtually impossible can be assessed. Davis et al. (1992) pointed out that about 33% of the characters and character-states used to distinguish among hydrobioid families and subfamilies were derived from the female reproductive system; about 23% from the male reproductive system, another 19% from the digestive system, 10% each for external features and mantle cavity structures. In all these systems homoplasy is rampant.

Once detailed studies of the ontogeny and histology of some organs are made, it may be possible to divide what is now considered one character-state into two or more and to find uniquely derived character states. One example is the hypothetical parallel pathways described by Ponder (1988) for the closure of the anterior pallial oviduct and the pathways that sperm could enter the female reproductive system. He included taxa with both the open ventral channel within the capsule gland to carry sperm and a closed tube (spermathecal duct) separated from the capsule gland within the Hydrobiidae. Molecular data show that taxa with a closed spermathecal duct belong to at least four families (Fig. 5; Amnicolidae, Cochliopidae, Pomatiopsidae and Truncatellidae), and no Hydrobiidae *s.s.* has a spermathecal duct.

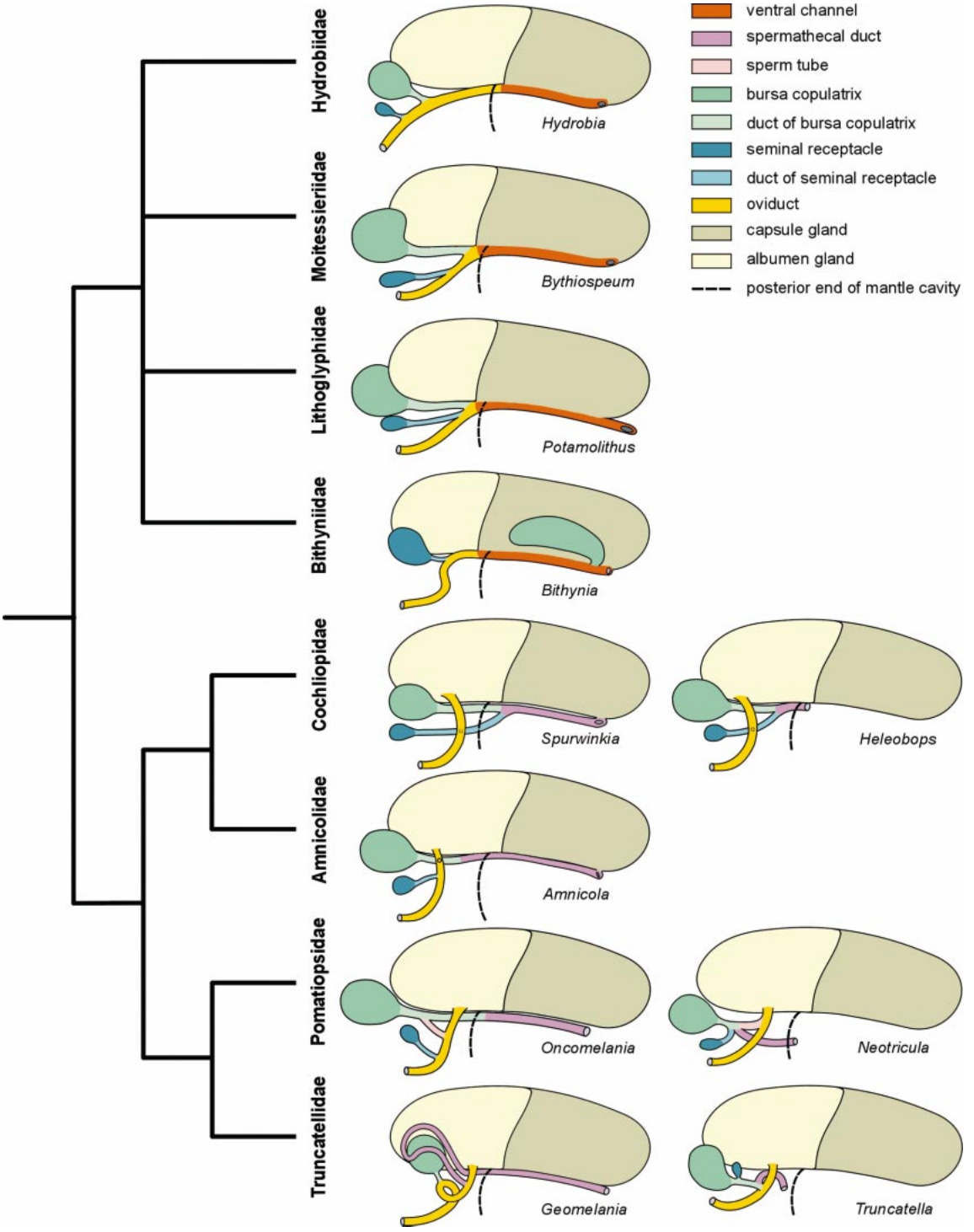

Fig. 5. Hypothetical phylogeny of selected rissooidean families based on an empirical synthesis of molecular and anatomical data. "Typical" anatomical ground plans are presented for the families. Note that these idealised ground plans do not reflect the enormous anatomical variation in some of the families. In families with the long and short condition of the spermathecal duct, both types are presented. The ground plan for *Amnicola* is adapted from Hershler and Thompson (1988), for *Truncatella* and *Geomelania* from Rosenberg (1996), and for *Bithynia* from Lilly (1953).

In comparing the Hydrobiidae and Cochliopidae, the ventral channel within the capsule gland is diagnostic for the Hydrobiidae, whereas the closed spermathecal tube is to be found in the Cochliopidae. One possible exception was reported by Hershler and Thompson (1992) for the genus *Subcochliopa*. In addition to a spermathecal duct, they also found a U-shape broadening of the ventral portion of the capsule gland, which resembled the ventral channel in the Hydrobiidae. However, the authors considered the condition in *Subcochliopa* to be a "new feature involving secondary modification of the typical simple lumen of the cochliopinids" and therefore not homologous to the ventral channel in the Hydrobiidae. We doubt that the U-shape feature in *Subcochliopa* is secondary. Alternatively, the condition could be a remnant of the closure of the spermathecal duct where parts of the "false ventral channel" (*sensu* Davis et al., 1982) formed a U-shape feature.

In addition to the presence of either a ventral channel or a spermathecal duct there are other anatomical characters that distinguish the Hydrobiidae from the Cochliopidae:

1. *Juncture of the anterior end of the oviduct.* In the Hydrobiidae the oviduct joins the ventral channel that is dorsally open to the capsule gland. In the Cochliopidae, the oviduct joins directly to the albumen gland (posterior pallial oviduct) as in most other hydrobioid families.

2. *Sperm passage between the seminal receptacle and the oviduct.* In the Hydrobiidae the duct of the seminal receptacle joins the oviduct. In the Cochliopidae the duct of the seminal receptacle joins the spermathecal duct where the duct of the bursa fuses with the spermathecal duct and sperm pass through an opening between the duct of the seminal receptacle and the oviduct, where the two ducts are fused, close to the opening of the oviduct into the albumen gland (Davis et al., 1982; Davis and McKee, 1989). Alternatively, the duct of the seminal receptacle enters a "fertilization duct" close to the juncture of the fertilization duct and the oviduct (Hershler and Thompson, 1992).

The three different clades (subclades 5a–5c in Fig. 2B) inside the Cochliopidae appear to be supported anatomically. *Littoridinops*, *Spurwinkia*, and *Pyrgophorus* (*Littoridina* group in Hershler and Thompson, 1992) are characterized by a spermathecal duct extending to the gonopore (Fig. 5) and by a penis with lobes or papillae. *Heleobops* and *Semisalsa* (*Heleobia* group in Hershler and Thompson, 1992) have a very short spermathecal duct (Fig. 5) and a penis with sucker-like glands. *Onobops* (assigned to the *Littoridina* group in Hershler and Thompson, 1992) has a very short spermathecal duct but a simple penis.

We have not studied *Cochliopa*, the type genus of the Cochliopidae, or other genera (*Aroapyrgus*, *Cochliopina*, *Mexithauma*, and *Subcochliopa*) that were assigned to the *Cochliopa* group by Hershler and Thompson (1992). It is well possible that these taxa belong to a distinct subfamily or even family. In the latter case, for nomenclatural reasons new family name(s) would have to be assigned to the cochliopid taxa studied in the present paper.

Notes on the Remaining Higher Taxa in Our Study

Despite the limitations of the genetic loci used in the present study, the great confusion that surrounds the current morphology-based systematics of rissooidean taxa, and the very limited number of family-level taxa studied in the present paper (*e.g.,* representatives of the Baicaliidae, Benedictiidae, Pyrgulidae, Emmericiidae are not included in the study), we feel it necessary to discuss our genetic data in the light of the available anatomical information (mainly data for the widely utilized female reproductive system).

The genetic data do not support the phylogenetic reasoning of Ponder (1988). We provide Fig. 5 as an empirical synthesis of the molecular and morphological data, with morphological data discussed below. Molecular and morphological data suggest that the pathway of evolution progresses from the open pallial oviduct (Rissoidae; *Setia* of this study) (grade I) to closure with an internal ventral channel to convey sperm (grade II) as seen in the Hydrobiidae, Lithoglyphidae (detailed anatomy based on *Potamolithus*; Davis and da Silva, 1984), Moitessieriidae, and Bithyniidae. The next evolutionary stage was separating the sperm-conducting function from the oviduct to be within an entirely closed tube (the spermathecal duct). This seems to have evolved two or three times (grade III, Fig. 5).

Considering grade II taxa, the Hydrobiidae, Moitessieriidae, Lithoglyphidae, and Bithyniidae are closely related families. Genera of these families have the simple internal ventral channel within the capsule gland and, except for the Bithyniidae, a simple penial structure. The seminal receptacle stems off the oviduct. To say more about the phylogenetic relationships among these groups would require more molecular and anatomical data for related genera and species of their respective families. However, it is instructive to inform that the radula of the Lithoglyphidae has a rather square central tooth with the basal cusps arising from the base of the tooth (convergent with the Pomatiopsidae), not the lateral angle as in the Hydrobiidae, Moitessieriidae, and Bithyniidae (convergent with the Amnicolidae and Cochliopidae).

The Bithyniidae have several unusual anatomical features that set them apart from the other groups with a ventral channel. The penis has a penial appendage through which a duct travels, having arisen as a coiled tube in the neck. This tube has been called an accessory prostate gland (Fretter and Graham, 1962). The ap-

pendage and coiled duct that terminates at an opening at the end of the penial appendage convergent with the Amnicolidae. In this regards, it is noteworthy that the COI data place *Bithynia* basal to the Amnicolidae cluster (Fig. 2 B) (the consensus molecular data place it in a tricotomy with the Hydrobiidae and Moitessieriidae). The type species of the genus *Bithynia*, *B. tentaculata*, is also unusual in having an anterior bursa positioned within the mantle cavity (Lilly, 1953), an epitaenial fold in the mantle cavity associated with filter feeding, and an operculum calcareous, with concentric striae and subcentral nucleus. Moreover, the taxon is distinguished by sperm dimorphism: the presence of both, typical (eupyrene) and atypical (oligopyrene and hyperpyrene) spermatozoa (Ankel, 1924; Fain-Maurel, 1966; Kohnert, 1980). Sperm dimorphism, which is known from the vast majority of the prosobranchs from the Archeogastropoda to the Caenogastropoda (for a review see Giusti and Selmi, 1982), has not been reported from other rissooidean groups studied so far (*e.g., Rissoa*, *Alvania*: Kohnert and Storch, 1984; *Truncatella*: Giusti and Mazzini, 1973; *Bythinella*, *Belgrandia*, *Hydrobia*, *Pseudamnicola*: Giusti, 1969, 1971).

We divide grade III taxa into three groups, A–C. The Amnicolidae are relegated to group A by having an elongated spermathecal duct and an opening between the spermathecal duct and the oviduct at the fusion of these ducts. The Amnicolidae are a sister clade to the Cochliopidae (III B) in the consensus tree (Fig. 3) as well as in the 18S-based tree (Fig. 2A). In the Amnicolidae the spermathecal duct is so fused to the capsule gland that the capsule gland-spermathecal duct complex resembles the capsule gland of the Hydrobiidae from external appearance (but see *Marstoniopsis* below). European *Bythinella* was originally portrayed as having the same female anatomy as *Hydrobia*, but a simple cross section of the capsule gland revealed the presence of a closed spermathecal duct (Giusti, unpublished data). One sees in the various species of the genus *Erhaia* (Asian Amnicolidae) a transitional series that bridges the Amnicolidae and Cochliopidae with respect to the sperm tube. In *Erhaia wufengensis* (China; Davis et al., 1985) the sperm duct arises from the oviduct anterior to the opening of the duct of the seminal receptacle and is relatively elongated, running anteriorly to enter the spermathecal duct at the posterior end of the mantle cavity. In *E. nainitalensis* (northeastern India; Davis and Rao, 1997) the duct of the seminal receptacle joins the oviduct precisely at the point where the very short sperm duct leaves the oviduct to attach to the spermathecal duct at the point of origin of the duct of the bursa copulatrix. Finally, in *E. wantanensis* (China, Davis and Kang, 1995), the oviduct, just anterior to the seminal receptacle, fuses to the oviduct with a small connecting opening. It is a very small step to derive the cochliopid situation where the condition in *E. nainitalensis* is minutely changed so that the sperm duct is continuous with the duct of the seminal receptacle and at the point they cross dorsal to the oviduct they fuse to the oviduct with a small opening between them.

Within the Amnicolidae one also finds characters and character-states that may indicate the pathway for the evolution of the spermathecal duct. In the genus *Marstoniopsis* the plesiomorphous state of the ventral channel is still retained. In *Amnicola*, the anterior end of the spermathecal duct fuses to the capsule gland, forming a very short ventral channel (Hershler and Thompson, 1988: Fig. 8c). This is somewhat similar to what is seen in some cochliopids. The Amnicolidae differ from the Cochliopidae by having the aforementioned penial appendage with its complex accessory duct (similar to the Bithyniidae). However, the penial appendage and duct are lost in the Asian genus *Erhaia*.

In groups III B (Cochliopidae) and III C (Truncatellidae; Pomatiopsidae), two character-states are obtained; 1) an elongated spermathecal duct, 2) a short spermathecal duct opening at the posterior end of the mantle cavity (or opening vial way of the pericardium, or the renal system). We postulate two, and possibly three different types of ontogeny (three independent origins) to accomplish this, the reality of which must be confirmed by developmental studies as well as detailed histological analyses. It would appear that the Pomatiopsidae-Truncatellidae have one mode (possibly two) and the Cochliopidae quite a different mode. In the latter group, we postulate that the spermathecal duct separated from anterior to posterior along the capsule gland (spatially separated) giving rise to the long-duct group of taxa of the Cochliopidae. Three stages of development occurred. The spermathecal duct maintains a connection with the capsule gland at the anterior end but separates from the oviduct for the rest of its length posteriorly (*e.g.,* in *Antrobia*, *Antroselates*, *Balconorbis*, *Coahuilix*, *Cochliopa*, *Littoridinops*, *Nanivitrea*, *Paludiscala*, *Spurwinkia*, *Pyrgophorus*, *Texapyrgus*). The next stage is the full-length separation of the spermathecal duct from the oviduct; spermathecal duct and oviduct now have separate openings (*e.g.,* in *Durangonella*, *Zetekina*). In the third stage the spermathecal duct shortens to open at the posterior end of the mantle cavity (*e.g.,* in *Heleobia*, *Heleobops*, *Littoridina*, *Cochliopina*, *Tryonia*, *Onobops*, *Mexipyrgus*, *Semisalsa*, *Subcochliopa*) (see Hershler and Thompson, 1992 for details). The molecular data, coupled with the long or short condition of the spermathecal duct, suggest that two subfamilies may be indicated such as in the Pomatiopsidae (group III B) where the Pomatiopsinae have a long spermathecal duct; the Triculinae have a short duct. The cochliopid genus *Aroapyrgu*s is reported to have species with both a long (*A. clenchi*) and short duct (*A. vivens*) (Hershler and Thompson, 1992). These species require additional study to ascertain if, indeed, both are *Aroapyrgus*.

Group III C taxa comprise the Truncatellidae and Pomatiopsidae. Davis et al. (1998) considered the Truncatellidae to be more closely allied to the Hydrobiidae than to the Pomatiopsidae. However, because of the low bootstrap support in their analysis, they suggested that more data would be required to resolve the relationships. This study based on two gene fragments and involving considerably more taxa, clearly shows that the Truncatellidae are more closely related to the Pomatiopsidae than to the Hydrobiidae. In his phylogenetic analysis, Ponder (1988) set apart a clade that included the Assimineidae (not studied in the present paper), Pomatiopsidae, and Truncatellidae. The synapomorphies are the step-like mode of progression and omniphoric grooves. He also made a very useful discussion of the unusual modes of reproduction obtained in the Pomatiopsidae and Truncatellidae. In the Pomatiopsidae: Pomatiopsinae the spermathecal duct is long, separated from the capsule gland by a small space. The sperm duct always arches from the distal end of the duct of the bursa to enter the oviduct just anterior to the opening of the duct of the seminal receptacle. In the Triculinae the spermathecal duct is shortened. It extends to the posterior end of the mantle cavity in some taxa, enters the pericardium in others with a separate opening from the pericardium to the mantle cavity, and in one genus, insemination is via the renal opening (reviewed in Ponder, 1988). The Truncatellidae also have the long and short condition of the spermathecal duct (= sperm tube *sensu* Rosenberg, 1996): in *Geomelania* the spermathecal duct is long, extending to the anterior end of the capsule gland. In *Truncatella* the short spermathecal duct ends within the pericardium. The latter condition is somewhat similar to the condition found in the pomatiopsid genus *Tricula*. In contrast to the anatomical data, molecular data from the 18S gene indicate that the Truncatellidae are a basal group, and it is quite feasible that the spermathecal duct and specialized alterations in the distal female reproductive system evolved independently from taxa in grades I and II.

We can conclude from our study that the Hydrobiidae are genetically different from all other groups thus far studied in terms of molecular phylogeny. The cohesive theme that unites the group morphologically is the ventral channel of the capsule gland. But few character-states separate the Hydrobiidae from the Moitessieriidae! Both families have virtually the same reproductive systems, and operculum. In an exhaustive study, Bodon and Giusti (1991) classified the key genera *Moitessieria* and *Paladilhia* in the Hydrobiidae:Hydrobiinae.

Within the Hydrobiidae are several distinct groups that may be the basis for discrete subfamilies. We have demonstrated the unique set of differences, morphological and genetic, that clearly set the Cochliopidae apart from the Hydrobiidae, and not by just a small amount of difference. The study serves well to point out major convergences in morphological characters and character-states, and these findings clearly indicate the need for detailed studies of the anatomy and ontogeny of the reproductive system. We simply have too few detailed accounts of the anatomy of numerous taxa, a situation that recalls the discussion that taxonomic discrimination requires a full suite of anatomical, cytological, and developmental characters (Davis, 1994).

Considering the fact that there are conflicts between morphology and molecular genetics (*e.g.,* the putative sister-group relationship of the Amnicolidae and Cochliopidae, the position of the Lithoglyphidae relative to the other groups with a ventral channel, and the distinctness of the Hydrobiidae), our conclusions are based on the interpretation of hypothetical character states, supported by available morphological data derived from the comparative anatomical studies of tiny organ systems, assisted by currently-available models of phylogenetic analysis. It is a new view of hydrobiid classification and a new contribution to work on rissooidean phylogeny. As more taxa are studied, more and longer gene fragments are used, and improvements are made in phylogenetic algorithms, we will expect greater clarification and improvement in our understanding of the relationships presented here.

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Appendix I. Locality information, DNA isolation, and GenBank accession numbers for the taxa studied. Sequences that were previously submitted to GenBank as part of related studies are marked with an asterisk.

Family/Subfamily	Species	Location	Latitude Longitude	DNA isolation #	GenBank # COI/18S	Leg.	Det.
Rissoidae (outgroup)	*Setia turriculata* Monterosato, 1884	Bulgaria, Nessebar, bay NW of dam to Nessebar Peninsula	42.660°N; 27.717°E	477	AF253084* AF367655	T. Wilke	T. Wilke
Hydrobiidae							
Hydrobiinae	*Hydrobia acuta* (Draparnaud, 1805)	France, Hérault, Etang du Prévost	43.513°N; 3.897°E	653	AF278808* AF367680	C. Casagranda	C. Casagranda
	Peringia ulvae (Pennant, 1777)	Russia, White Sea, logoon "Levin navolok"	66.53°N; 33.88°E	608	AF118302* AF367679	A. Gorbushin	T. Wilke
	Ventrosia ventrosa (Montagu, 1803)	United Kingdom, Norfolk, The Wash, Snettisham lagoon RSPB bird reserve	52.863°N; 0.460°E	717	AF118335* AF367681	B. James	B. James
	Adriohydrobia gagatinella (Küster, 1852)	Croatia, Krka River near Skradin	43.81712°N; 15.92353°E	2276	AF317857* AF367657	A. Falniowski, M. Szarowska	T. Wilke, A. Falniowski
Pseudamnicolinae	*Pseudamnicola lucensis* (Issel, 1866)	Italy, Tuscany, Lucca, Bagni di Lucca, Bagni Caldi, thermal spring	44.007°N; 10.585°E	2537	AF367651 AF367687	M. Bodon	M. Bodon
	Adrioinsulana conovula (Frauenfeld, 1863)	Croatia, Pag Island, Zubovici	44.52076°N; 14.97237°E	2466	AF367628 AF367656	A. Falniowski, M. Szarowska	A. Falniowsi, M. Szarowska
Horatiinae ?	*Horatia klecakiana* Bourguignat, 1887	Croatia, spring of Vrana River, between Vrana and Radosinovci	43.92532°N; 15.58799°E	2598	AF367637 AF367669	A. Falniowski, M. Szarwoska	A. Falniowski, M. Szarwoska
	Sadleriana fluminensis (Küster, 1853)	Croatia, Jadro River at Solin near Split	43.5453°N; 16.48780°E	2601	AF367647 AF367683	A. Falniowski, M. Szarowska	A. Falniowski, M. Szarowska
	Belgrandia thermalis (Linnaeus, 1767)	Italy, Tuscany, Pisa, S. Giuliano Terme, thermal channel near S. Giuliano	43.751°N; 10.440°E	2305	AF367648 AF367684	M. Bodon, E. Bo, M. Sosso	M. Bodon
	Orientalina callosa (Paulucci, 1881)	Italy, Abruzzo, Pescara, Caramanico Terme, spring stream on the left bank of Orfento River	42.1571°N; 14.0167°E	2791	AF367649 AF367685	M. Bodon	M. Bodon
Islamiinae ?	*Islamia piristoma* Bodon and Cianfanelli, 2001	Italy, Liguria, La Spezia, Arcola, spring on right bank of Magra River near Romito	44.1042°N; 9.9337°E	2327	AF367639 AF367671	M. Bodon, E. Bo, M. Sosso	M. Bodon
	Fissuria boui Boeters, 1981	France, Alpes Maritimes, Peymeinade, spring near La Prouveresse	43.64279°N; 6.88735°E	2435	AF367654 AF367690	M. Bodon, E. Bo, M. Sosso	M. Bodon
	Avenionia brevis berenguieri (Bourguignat, 1882)	France, Gard, spring of the fountain of St.-Victor-La Coste	44.057°N; 4.636°E	2241	AF367638 AF367670	M. Bodon, H. Girardi, B. Bomba	M. Bodon, H. Girardi
	Alzoniella finalina Giusti and Bodon, 1984	Italy, Liguria, Savona, Molino, spring at the Porra River	44.219°N; 8.255°E	2592	AF367650 AF367686	M. Bodon, S. Cianfanelli	M. Bodon

Appendix I (*continued*)

Family/Subfamily	Species	Location	Latitude Longitude	DNA isolation #	GenBank # COI/18S	Leg.	Det.
Nymphophilinae ?	*Cincinnatia winkleyi* (Pilsbry, 1912)	USA, Maine, Cumberland County, Spurwink River	43.57°N; 70.25°W	632	AF118370* AF212914*	G. M. Davis	G. M. Davis
	Notogillia wetherbyi (Dall, 1885)	USA, Florida, Hernando County, Weeki Wachee River, NW of Weeki Wachee Spring	28.5181°N; 82.5720°W	1635	AF367630 AF367660	P. L. Poland	P. L. Poland
?	*Mercuria similis* (Draparnaud, 1805)	Italy, Friuli-Venetia-Julia, Udine, Aquileia, Canale Panigai near Panigai	45.7415°N; 13.3408°E	2551	AF367646 AF367682	M. Bodon	M. Bodon
?	*Hauffenia tellinii* (Pollonera, 1898)	Italy, Friuli-Venetia Julia, Gorizia, Isonzo River near Sagrado, spring	45.8743°N; 13.4856°E	2566	AF367640 AF367672	M. Bodon, S. Cianfanelli	M. Bodon
?	*Graziana alpestris* (Frauenfeld, 1863)	Italy, Liguria, Savona, Molino, spring at the Porra River	44.219°N; 8.255°E	2562	AF367641 AF367673	M. Bodon	M. Bodon
Cochliopidae	*Littoridinops monroensis* (Frauenfeld, 1863)	USA, Maryland, Dorchester Co., marsh near the boatyard on Ragged Point Road	38.557°N; 76.251°W	624	AF367644 AF367677	G. M. Davis, M. McKee	G. M. Davis, M. McKee
	Pyrgophorus platyrachis Thompson, 1968	USA, Florida, Hillsborough County, Lithia Springs	27.8669°N; 82.2253°W	1643	AF367632 AF367662	P. L. Poland	P. L. Poland
	Spurwinkia salsa (Pilsbry, 1905)	USA, Maryland, Dorchester Co., Town Point at the end of Town Point Road	38.5418°N; 76.2080°W	779	AF367633 AF367663	G. M. Davis	G. M. Davis
	Onobops jacksoni (Bartsch, 1953)	USA, Maryland, Dorchester Co., Town Point at the end of Town Point Road	38.5418°N; 76.2080°W	614	AF367645 AF367678	G. M. Davis, M. McKee	G. M. Davis, M. McKee
	Semisalsa dalmatica Radoman, 1973	Croatia, Pirovac Spring near Pirovac	43.81670°N; 15.67658°E	2114	AF367631 AF367661	A. Falniowski, M. Szarowska	A. Falniowski, M. Szarowska
	Heleobops carrikeri Davis and McKee, 1989	USA, Maryland, Dorchester Co., Little Choptank River at the end of Ragged Point Road	38.5388°N; 76.2729°W	597	AF213347* AF212915*	G. M. Davis, M. McKee	G. M. Davis, M. McKee
Amnicolidae	*Amnicola limosa* (Say, 1817)	USA, Michigan, Washtenaw County, Lyndon Township, Blind Lake	42.38°N; 84.02°W	1060	AF213348* AF212916*	J. Burch, A. Romanski	J. Burch, A. Romanski
	Bythinella compressa (Frauenfeld, 1857)	Germany, Hessen, Altengronau	50.31756°N; 9.71186°E	2702	AF367653 AF367689	T. Wilke	T. Wilke
	Erhaia jianouensis (Liu and Zhang, 1979)	China, Fujian, Nanping, Tianxi	26.9925°N; 118.3874°E	652	AF367652 AF367688	G. M. Davis	G. M. Davis
	Marstoniopsis insubrica (Küster, 1853)	Germany, Rostock, Warnow River near the feeder of the Rostock waterworks	54.09°N; 12.12°E	2599	AF322408* AF367676	M. L. Zettler	M. L. Zettler, A. Falniowski
Moitessieridae	*Moitessieria* cf. *puteana* Coutagne, 1883	France, Alpes Maritimes, Peymeinade, spring near La Prouveresse	43.64279°N; 6.88735°E	2413	AF367635 AF367665	M. Bodon, E. Bo, M. Sosso	M. Bodon
	Bythiospeum sp.	France, Gard, Lirac, Source de la Nizon	44.03°N; 4.68°E	2328	AF367634 AF367664	M. Bodon, H. Girardi, B. Bomba	M. Bodon, H. Girardi

Appendix I (*continued*)

Family/Subfamily	Species	Location	Latitude Longitude	DNA isolation #	GenBank # COI/18S	Leg.	Det.
Bithyniidae	*Bithynia tentaculata* (Linnaeus, 1758)	Croatia, Cetina River estuary near Omiš	43.44253°N; 16.68964°E	2643	AF367643 AF367675	A. Falniowski, M. Szarowska	A. Falniowski, M. Szarowska
Lithoglyphidae	*Lithoglyphus naticoides* (C. Pfeiffer, 1828)	Poland, Narew River near Drozdowo	53.13853°N; 22.15180°E	1997	AF367642 AF367674	A. Falniowski, M. Szarowska	A. Falniowski, M. Szarowska
Truncatellidae	*Truncatella pulchella* (Pfeiffer, 1839)	Jamaica, Trelawny Perish, N of Falmouth	18.4910°N; 77.6577°W	479	AF253085* AF367658	G. Rosenberg, I. Muratov	G. Rosenberg
	Geomelania inornata Chitty, 1853	Jamaica, Trelawny Perish, Cocknit County, 2 km N or Quickstep	18.2580°N; 77.7075°W	800	AF367629 AF367659	G. Rosenberg, I. Muratov	I. Muratov
Pomatiopsidae	*Pomatiopsis lapidaria* (Say, 1817)	USA, Michigan, Washtenaw County, Bridgewater Township, near Raisin River at Allen Road	42.0893°N; 83.9725°W	1047	AF367636 AF367666	J. Burch, A. Romanski	J. Burch, A. Romanski
	Oncomelania h. hupensis Gredler, 1881	China, Anhui, Dalin	30.8780°N; 118.9143°E	792	AF254547* AF367667	G. M. Davis, T. Wilke	G. M. Davis, T. Wilke
	Tricula sp.	China, Sichuan, Chengdu, Huang Ba	30.54040°N; 103.24123°E	454	AF253071* AF411141	G. M. Davis	G. M. Davis
	Gammatricula chinensis Davis, Liu and Chen, 1990	China, Zhejiang Province, Kaiwa Co, Tong Cun Town, Bai Keng Village	29.0008°N; 118.2578°E	414	AF253067* AF367668	C.-E. Chen	G. M. Davis

Appendix II. Nomenclatural notes to putative subfamilies of the Hydrobiidae studied in the present paper.

Hydrobiinae Troschel, 1857/Pseudamnicolinae Radoman, 1977

The genus *Pseudamnicola* was long considered to belong to the subfamily Hydrobiinae (e.g. Radoman, 1973). In 1977 Radoman established the subfamily Pseudamnicolinae, which supposedly differs from the Hydrobiinae by a transverse opening of the capsule gland instead of a longitudinal opening and by a rather regular loop of the pigmented part of the oviduct instead of coiled loops. It remains open whether these anatomical differences justify the separation into different subfamilies. However, as both groups also cluster apart in our molecular analyses, we retain the subfamily assignment, pending further investigations.

Nymphophilinae Taylor, 1966

In our analysis, the North American genera *Cincinnatia* and *Notogillia* form a basal clade in the Hydrobiidae. Thompson (1979) placed the two genera into the Nymphophilinae, a subfamily that was originally erected by Taylor (1966) as a monogeneric taxon. We did not study its type species, *Nymphophilus minckleyi* Taylor, 1966. Therefore, the subfamily assignment of *Cincinnatia* and *Notogillia* needs further confirmation, especially in the light of the fact that Thompson (1979) also placed the European genera *Mercuria* and *Avenionia* in the Nymphophilinae, an assignment that is not supported by our data (see below).

Horatiinae Taylor, 1966

Four genera cluster together in our phylogenetic analysis: *Horatia, Sadleriana, Orientalina,* and *Belgrandia* with the former three taxa being distinct from the latter taxon. These taxa have been used to describe a number of tribes and subfamilies. The Horatiini were established by Taylor (1966) as a tribe of the Cochliopinae for 12 genera and subgenera (incl. *Horatia* and *Hauffenia*). Its placement into the Cochliopinae was later rejected by Hershler and Thompson (1992). Radoman (1973) placed *Horatia* into the subfamily Horatiinae of the family Orientaliidae, and *Sadleriana* and *Belgrandia* into the subfamily Sadlerianinae (Orientaliidae). In 1983 Radoman placed *Belgrandiella* and *Graziana* into the subfamily Belgrandiellinae (a replacement name for the Horatiinae) and moved *Horatia* and *Sadleriana* to the subfamily Orientalininae. This leaves us with at least three possible subfamily names: Horatiinae Taylor, 1966; Sadlerianinae Radoman, 1973; and Orientalininae Radoman, 1978. Pending further investigations we tentatively place our four genera studied into the Horatiinae, which seems to be the oldest available name.

Islamiinae Radoman, 1973

Four taxa form a distinct clade in our analysis, the genera *Islamia, Avenionia, Alzoniella,* and *Fissuria.* Radoman (1973, 1983) placed *Islamia* into the subfamily Islamiinae (Orientaliidae). Climo (1977) considered *Avenionia* to belong to the *Horatia*-group of the Hydrobiinae, whereas Thompson (1979) placed *Avenionia* into the Nymphophilinae. Giusti and Bodon (1984) provisionally included the genus *Alzoniella* into the subfamily Hydrobiinae. The subfamily assignment remains enigmatic, but based on the data presented here we follow the view of Radoman (1973, 1983) and tentatively consider them to belong to the Islamiinae.