Phylogenetic relationships of North American nymphophiline gastropods based on mitochondrial DNA sequences

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Phylogenetic relationships of 36 nymphophiline species representing 10 genera were inferred from mtCOI sequence data and compared to recent morphology-based classifications of this group. Parsimony and maximum likelihood analyses of the molecular data set suggested monophyly of the North American nymphophilines and a sister or otherwise close relationship between this fauna and a European species assigned to the subfamily. Results also supported a previously hypothesized close relationship between the predominantly freshwater nymphophilines and the brackish-water genus *Hydrobia*. Our analyses resolved a North American nymphophiline subclade composed of *Floridobia*, *Nymphophilus*, and *Pyrgulopsis*, and depicted the remaining North American genera (*Cincinnatia*, *Marstonia*, *Notogillia*, *Rhapinema*, *Spilochlamys*, *Stiobia*) as either a monophyletic or paraphyletic group. Two of the large North American genera (*Floridobia*, *Marstonia*) were supported as monophyletic groups while monophyly of *Pyrgulopsis*, a western North American group containing > 100 species, was equivocal. North American nymphophiline phylogeny implies that vicariance of eastern and western North American groups was followed by a secondary invasion of eastern coastal areas from the west. We attribute this to dispersal of salt-tolerant progenitors along the Gulf of Mexico coast

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Introduction

The Nymphophilinae (family Hydrobiidae) is one of the largest groups of aquatic molluscs in North America, currently comprising 159 species in 10 genera. These small, gill-breathing gastropods are locally abundant in lotic and lentic habitats throughout much of the continent. Nymphophilines have limited dispersal abilities and most species are narrowly distributed in local drainage systems. Endemic faunas of these snails have been a focus of various biogeographical studies (Thompson 1968; Johnson 1973; Taylor 1985, 1987; Hershler & Pratt 1990). Members of the subfamily often live in small springs and other fragile habitats. Five species are federally listed as endangered (USFWS 2001), while 43 currently are on the IUCN Red List of threatened species (IUCN 2000). However, despite these important and compelling features, the phylogenetic relationships of the nymphophilines have been little studied and are poorly understood.

The subfamily Nymphophilinae was erected by Taylor (1966) for *Nymphophilus minckleyi* on the basis of the large trochoidal shell, multispiral operculum, and distinctive penis of this snail, which is endemic to the Cuatro Cienegas basin in north-eastern Mexico. Thompson (1968) disputed recognition of a monotypic Nymphophilinae based on these characters and subsequently allocated seven other North American (and six central European) genera to the subfamily, which he diagnosed by 10 shell and anatomical characters (Thompson 1979). However, none of these characters provide unequivocal support for nymphophiline monophyly. Although the Nymphophilinae continues to be recognized as a distinct hydrobiid subfamily in several recent classifications (e.g. Ponder & Warén 1988; Vaught 1989), others have disagreed and suggested that it is scarcely differentiated from the subfamily Hydrobiinae (Giusti & Bodon 1984; Giusti & Pezzoli 1984; Davis & Mazurkiewicz 1985; Bodon & Giusti 1991).

Most of the North American nymphophilines (125 of 159 species, 79%) have been described only recently (after 1960) and many remain incompletely studied. Five genera contain between one and three species, and are endemic to portions

of the south-eastern United States (*Notogillia*, *Rhapinema*, *Spilochlamys*, *Stiobia*) and north-eastern Mexico (*Nymphophilus*). All of these genera have had uncomplicated taxonomic histories following their fairly recent descriptions. Monotypic *Birgella*, which is broadly distributed in eastern North America, has not been further treated following its recent transfer to the Nymphophilinae (Thompson 1984). While the systematics of these small genera may be considered stable, that of the remaining genera, which together contain 149 species, has been problematic, reflecting, in part, conflict between traditional generic concepts based on shell and penial characters and alternative groupings suggested by newly discovered features of female reproductive anatomy. The genus *Cincinnatia* traditionally was considered a cohesive group diagnosed by a simple conical shell and complex pattern of lobes and glandular fields on the penis (Thompson 1968). However, based on female reproductive anatomy the genus was recently restricted to its type species, *Cincinnatia integra*, while other congeners were transferred to *Marstonia* and a newly erected genus, *Floridobia* (Thompson & Hershler, 2002). The genus *Marstonia* was utilized early for a small, well differentiated group of eastern American species (Thompson 1977). Hershler & Thompson (1987) subsequently placed this genus in synonymy with *Pyrgulopsis* based on similarities of penial morphology and female reproductive anatomy, only to later resurrect *Marstonia* to generic status and allocate to it all of the eastern North American species that were previously placed in *Pyrgulopsis* (Thompson & Hershler, 2002).

Modern study of the systematics of the western North American nymphophilines began with Gregg & Taylor (1965), who erected *Fontelicella* (with three subgenera) for a group of eight species. Taylor (1987) later described 11 new species as members of *Fontelicella* and erected two monotypic genera (*Apachecoccus*, *Yaquicoccus*) for species from southern Arizona. Hershler & Thompson (1987) radically revised the western fauna by placing all of these genera in synonymy with *Pyrgulopsis*, which originally was utilized for a small group of carinate-shelled species from the eastern and western United States (Call & Pilsbry 1886). Many newly discovered species have subsequently been described as members of *Pyrgulopsis* (e.g. Hershler 1998). The genus currently consists of 122 species, representing the entire western American nymphophiline fauna (aside from two Mexican species belonging to *Nymphophilus*), and contains as much morphological diversity as the rest of the nymphophiline genera combined. A recent morphology-based phylogenetic analysis which included 51 species of *Pyrgulopsis* provided little resolution and did not include appropriate taxa to rigorously test monophyly and phylogenetic relationships of these snails relative to other North American nymphophilines (Hershler 1994). The only other explicitly phylogenetic studies which sampled nymphophilines included only one or two species of the subfamily (Hershler 1996; Wilke *et al*. 2000, 2001; Liu *et al*. 2001). Herein we use DNA sequences of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene to examine the phylogenetic relationships of the North American nymphophilines in a more comprehensive manner. This gene was chosen for analysis because it has proved useful in previous phylogenetic studies of hydrobiids (e.g. Hershler *et al*. 1999; Wilke & Davis 2000; Liu *et al*. 2001). We seek: (1) to determine whether the North American nymphophilines are a monophyletic group; (2) to evaluate the systematic placement of these snails relative to other hydrobiid groups, and (3) to explore relationships within the subfamily and contrast these with recent generic classifications and anatomical variation (based on the literature and our unpublished studies). Although our focus is on the North American nymphophilines, we use available COI sequence data for a European member of the subfamily (Wilke *et al*. 2000) to test the previously proposed *trans*-Atlantic distribution of the group (Thompson 1979).

Materials and methods

Specimens

Specimen samples utilized in this study, together with voucher depositions, are listed in the Appendix. Thirty-five species of North American nymphophilines, representing nine of the 10 currently recognized genera, were sequenced. We were unable to collect fresh material of the remaining North American genus (monotypic *Birgella*) for inclusion in this study. We sampled multiple species of each of two large North American nymphophiline genera (*Floridobia*, 5 of 14 species; *Marstonia*, 7 of 11 species) to enable testing of their monophyly. For the largest genus, *Pyrgulopsis*, we sampled a much smaller fraction of its species (21 of 122 species) that nonetheless spans the broad range of morphological variation within this huge group. We included other North American (and two European) hydrobiids in our analysis to test nymphophiline monophyly and to determine placement of this group within the family. These outgroups consisted of species of three other subfamilies that occur in North America (Cochliopinae [recently elevated to family status; Wilke *et al*. 2000, 2001), Hydrobiinae, Lithoglyphinae), and one of the two species of *Probythinella*, a North American genus whose systematic position has been closely associated with the nymphophilines (Hershler 1996). A species of *Phrantela*, an Australian genus which was hypothesized by Ponder *et al*. (1993 : 734) to be the most basal group of hydrobiids, was used to root all trees.

DNA isolation, amplification, and sequencing

Genomic DNA was extracted from individual snails using a CTAB protocol (Bucklin 1992). Amplifications were conducted in a 25-μL total volume, containing 5 μL of Invitrogen optimizer buffer (Invitrogen, Inc.), 2.5 μL of dNTPs

(125 μM each), 1.25 μL of each primer (0.5 μM each), 1 unit *Taq* polymerase, 1 μL of template (*c.* 100 ng double-stranded DNA), and 13.8 μL of sterile water. Approximately 710 bp or 900 bp of the COI gene were amplified as a single product using primer pairs COIL1490 and COIH2198 (Folmer *et al.* 1994) or COIL1492 and COIH2390 (Liu *et al.* 2001). The standard PCR profile consisted of 30 cycles of 94 °C for 1 min, 50–55 °C for 1 min, and 72 °C for 2 min. Amplified DNA was examined by electrophoresis on 1.5% agarose gel stained with ethidium bromide. The amplified PCR product was incubated at 37 °C for 15 min and then at 85 °C for another 15 min with 0.5 unit Shrimp Alkaline Phosphatase (SAP, Amersham) and five units Exonuclease I (ExoI, Amersham) to remove excess primers and nucleotides. Approximately 10–30 ng of cleaned PCR product was used as a template in a cycle sequencing reaction using the Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystem, Inc.). The following cycling conditions were used: 96 °C for 2 min, followed by 30 cycles of 94 °C for 15 s, 50 °C for 5 s, and 60 °C for 4 min. The cycle-sequenced product was cleaned using the ethanol precipitation method, then run on an ABI 310 sequencer. Sequences were determined for both strands.

Data analysis

Sequences were edited and aligned using Sequencher. Mutational saturation for each codon was examined by plotting the absolute number of transitions and transversions against uncorrected genetic distance (p-distance), and by plotting p-distance against inferred distance (TrN-distance, HKY distance) (Berbee *et al.* 1995; Griffiths 1997; Siemer *et al.* 1998).

Parsimony trees were generated using the maximum parsimony option of PAUP* 4.0b8 (Swofford 2001). Minimum length trees were determined using weighted parsimony in which transversions were weighted more than transitions. Weightings were varied across codon positions to reflect differences in rates across sites. Several schemes were employed, including assigning equal weights across the sites, down-weighting TS in the third codon position, and weighting based on the maximum value of the rescaled consistency index (successive approximations-weighted analyses; Carpenter 1988). Results from only one substitution model are reported: that using minimum transition to transversion ratios at each codon position (25 : 3 : 22), where the numbers indicate the weights applied to transversions relative to transitions for the first, second, and third codon positions, respectively. The heuristic search option with 10 replications of random stepwise additions was used to search for minimum length trees.

Maximum-likelihood analyses were performed using PAUP* 4.0b8 (Swofford 2001). Modeltest 3.06 (Posada & Crandall 1998) was used to evaluate 56 'models of evolution' in order to obtain an appropriate substitution model and parameter values for maximum-likelihood analyses. In pairwise comparisons, if the improvement in likelihood scores by a more complex model was not found to be significant then the simpler model was chosen. The parsimony tree found to be most likely under the adopted model was used as the initial topology for branch-swapping.

Robustness of the phylogenetic analyses was tested using the bootstrap (Felsenstein 1985; as implemented in PAUP* 4.0b8; Swofford 2001). One thousand pseudoreplications were used for the parsimony analyses and 100 pseudoreplications were used for the maximum likelihood analyses.

Results

The alignment of COI sequences produced 609 bp, of which 266 sites were variable (43.7%) and 236 were parsimony informative (38.8%). Average base frequencies for the total data set were 25% A, 34.9% T, 20.4% C and 19.7% G, typical of gastropod mitochondrial genes (e.g. Collins *et al.* 1996; Hershler *et al.* 1999; Liu *et al.* 2001). No mutational saturation was evident at the first (Fig. 1A) and second codon

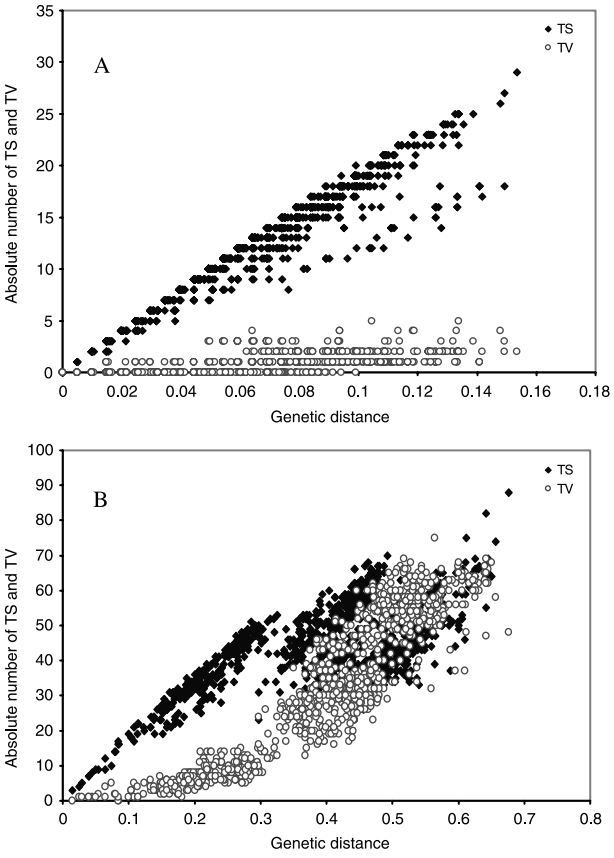

Fig. 1 A, B. Plots of total number of transitions and transversions vs. sequence divergence for all pairwise comparisons. —A. First codon position. —B. Third codon position.

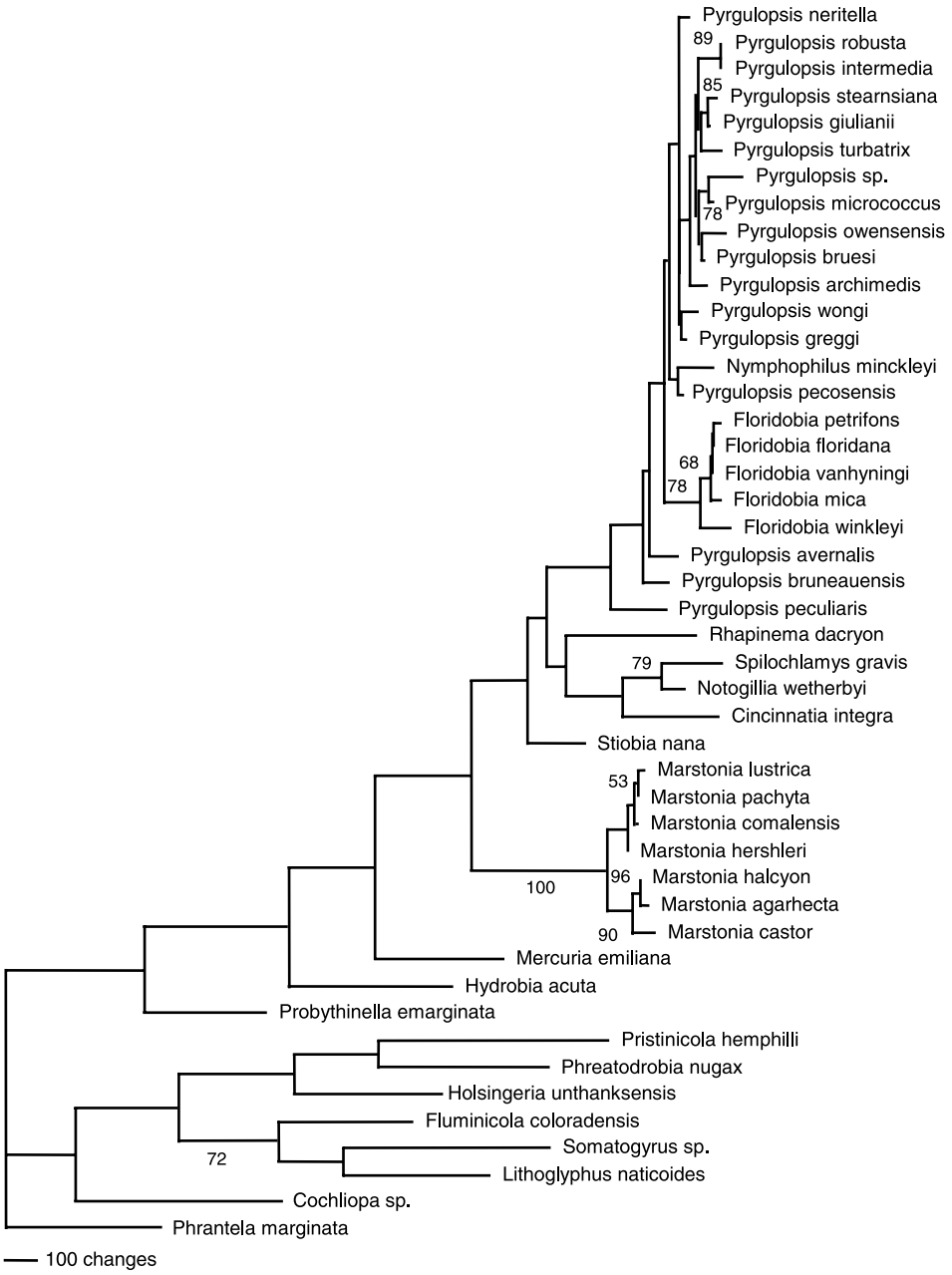

Fig. 2 One of 23 shortest length trees based on maximum-parsimony analysis of mtCOI sequence data. Bootstrap percentages are given when ≥ 50%.

positions. For the third codon position, multiple hits occurred — transitions levelled off in the first test (Fig. 1B) and the plotted line deviated from the identity line in the second and third tests. Thus, transitions in the third codon position do not appear to provide much information regarding phylogenetic relationships among lineages whose divergence is more than 35% in this position.

Percent sequence differences between species (uncorrected for multiple hits) ranged from 0.5 to 6.1% for *Floridobia*, 1.0–8.5% for *Marstonia*, and 2.8–11.2% for *Pyrgulopsis*. Comparisons among nymphophiline genera ranged from 5.8 to 18.9%.

Parsimony analyses using the 25 : 3 : 22 weighting scheme yielded 23 trees of 12733 steps (CI, 0.28; RI, 0.58). One of these is shown in Fig. 2. Other topologies varied in basal relationships within the clade composed of *Nymphophilus*, *Pyrgulopsis*, and *Floridobia*; and in relationships within the *Floridobia* and *Marstonia* clades. Additionally, in two of the 23

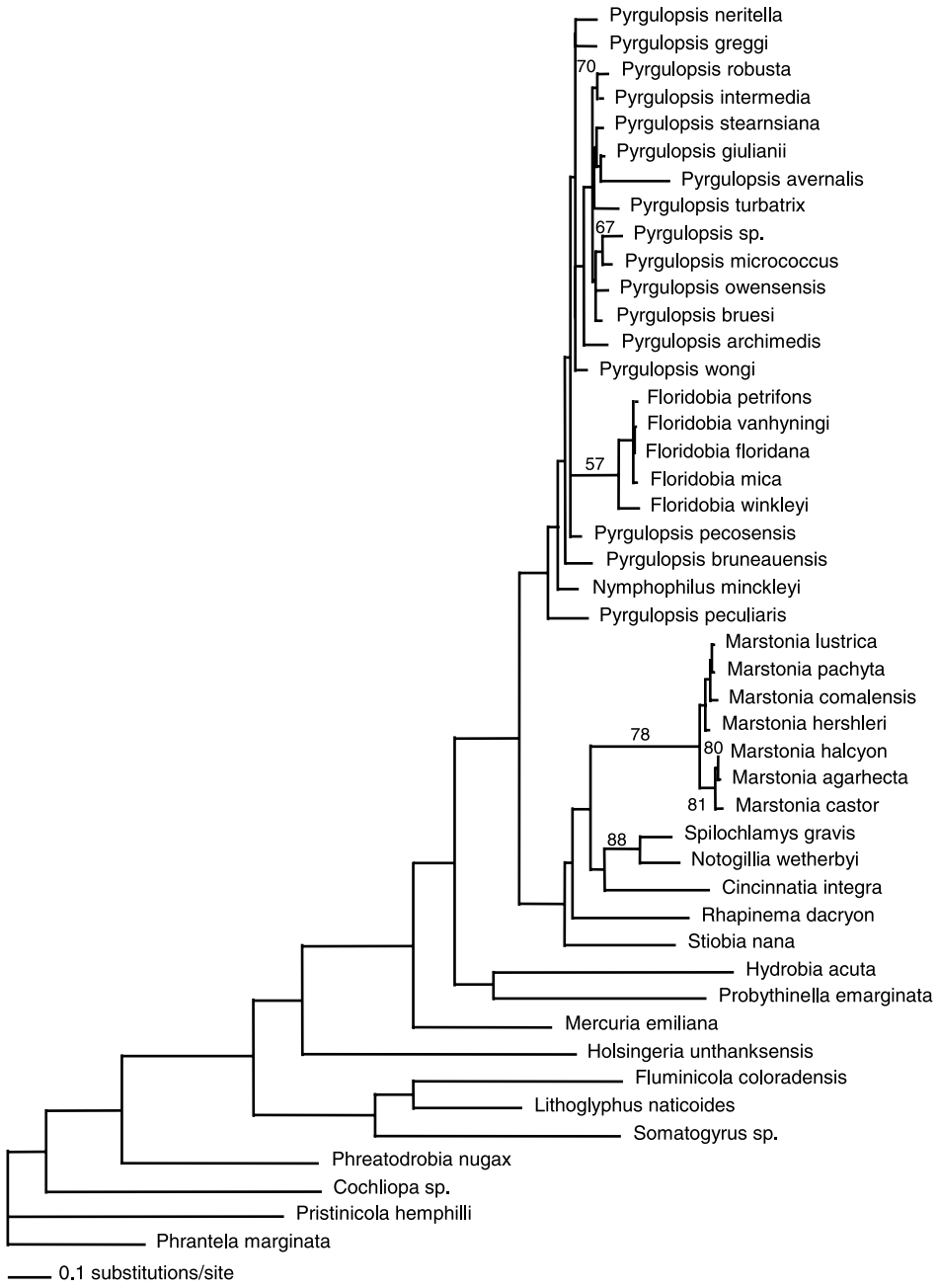

Fig. 3 Tree based on maximum likelihood analysis. Bootstrap percentages are given when ≥ 50%.

trees the clade composed of *Nymphophilus minckleyi* and *Pyrgulopsis pecosensis* was positioned as sister to *Floridobia* (instead of as in Fig. 2).

When the 23 most parsimonious trees were evaluated using Modeltest the most parameter-rich model (GTR + G + I) was found to be significantly better fitting than the next best model (GTR + G) ($P < 0.001$), and tree 7 was found to be the most likely of the 23 most parsimonious trees. Using this as the initial tree for branch-swapping, maximum likelihood analysis ultimately identified a single most likely tree with a log likelihood score of 7291.35 (Fig. 3).

The North American nymphophilines form a monophyletic group in both the parsimony and likelihood analyses, although bootstrap support for this clade is weak (< 50%). In the parsimony trees this group is most closely related to a European nymphophiline, *Mercuria emiliana*, and this more

inclusive clade is sister to another European species, *Hydrobia acuta*. In the maximum likelihood tree *Hydrobia acuta* and North American *Probythinella emarginata* form a clade which is sister to the North American nymphophilines while *Mercuria emiliana* is positioned as sister to this more inclusive group. In the maximum likelihood tree the North American nymphophilines are divided into two monophyletic groups, one consisting of six eastern North American genera ('eastern clade') and the other containing the two western North American genera (*Nymphophilus*, *Pyrgulopsis*) and one eastern genus (*Floridobia*). In the latter clade *Floridobia* species form a weakly supported (57%) monophyletic group which is nested (along with *Nymphophilus minckleyi*) within *Pyrgulopsis*. Although *Pyrgulopsis* is depicted as paraphyletic, only two additional steps are required to achieve monophyly of this group in the parsimony analysis. The constrained and unconstrained trees are not significantly different (based on the Wilcoxon sign-rank test; Templeton 1983) and thus we cannot reject monophyly of *Pyrgulopsis*. Within the eastern clade, *Marstonia* forms a moderately supported (78%) monophyletic unit which is most closely related to a subclade composed of *Cincinnatia*, *Notogillia wetherbyi*, and *Spilochlamys gravis*, while *Rhapinema* and *Stiobia* are basal to these clades. The parsimony analyses produced the same set of results, except that *Stiobia* and the *Marstonia* clade are positioned outside of the eastern group and instead occupy basal positions relative to the rest of the North American nymphophilines.

Discussion

Monophyly and relationships of the Nymphophilinae

Our finding of a North American nymphophiline clade is consistent with the unique presence of surficial glandular fields (see Hershler 1994: fig. 2a–c) on the penis in this subfamily (Thompson 1979). All North American nymphophilines have one or more of these glands except for a few species of *Pyrgulopsis* (including *Pyrgulopsis bruesi* and *Pyrgulopsis greggi*, which were included in our analyses). In other species of *Pyrgulopsis* the glandular fields are well developed, consistently weak, or weak and occasionally absent. This variation, together with the distal position of *Pyrgulopsis* within the nymphophiline clade, suggests to us that this structure has been secondarily lost (perhaps iteratively) within the genus.

Our finding that *Probythinella emarginata* is excluded from the nymphophilines conflicts with an earlier hypothesis that this genus is closely related to *Cincinnatia* based on radular morphology and coiling of the vas deferens (Hershler 1996), but is consistent with the absence of penial glands in *Probythinella*. Our results were equivocal with respect to whether the North American nymphophilines and a European species assigned to this subfamily (*Mercuria emiliana*) form a monophyletic group. Morphological evidence pertinent to this issue also is inconclusive. While some European nymphophilines (e.g. *Avenionia brevis*, Boeters 1970: fig. 1; *Litthabitella elliptica*, Boeters 1974: figs 6, 7) have lobate penes with glandular ornament that resembles the glandular fields of North American taxa, the different styles of description and illustration used by students of these two faunas do not enable a confident assessment of the possible homology of these structures. However, a recently published analysis based on sequences from COI and two other genes did not depict a sister relationship between two North American nymphophilines and three (other) European species of the subfamily (Wilke *et al.* 2001). The sum total of molecular evidence suggests to us that while the North American nymphophilines may be more closely related to European hydrobiids than to other North American taxa, these two faunas are strongly differentiated. This would be expected if (per Thompson's 1979 hypothesis) their biogeographical history reflects fragmentation of ancestral biota into modern continental components in association with the opening and widening of the North-Central Atlantic Ocean beginning in the middle-late Jurassic (150–170 Ma; Klitgord & Schouten 1986).

Our results are consistent with prior hypotheses suggesting a close relationship between *Hydrobia* (Hydrobiinae) and the Nymphophilinae. As mentioned in the Introduction, some have argued that these two groups are scarcely differentiated morphologically and may not merit recognition as separate subfamilies. However, as discussed above, nymphophiline monophyly is supported by the unique presence of glandular fields on the penis (see Davis & Mazurkiewicz 1985, for a different interpretation of the significance of these glands) while a clade composed of *Hydrobia* and closely similar genera may be supported by a different suite of reproductive anatomical characters (Ponder *et al.* 1993: 735). Additional analyses incorporating DNA sequences of other taxa assigned to the Hydrobiinae will be necessary to further explore this issue and to examine the biogeographical implications of a possible sister relationship between progenitors of *Hydrobia*, a trans-Atlantic genus whose species live in coastal marshes and estuaries, and the predominantly freshwater nymphophilines.

Phylogenetic structure of the North American nymphophilines

Our finding of a clade composed of *Floridobia*, *Nymphophilus*, and *Pyrgulopsis* is consistent with the superficial position of the female bursa copulatrix and its duct on the albumen gland (Fig. 4A & B), which is unique to these taxa within the North American nymphophilines. In all other members of this fauna (including *Birgella*, which is not included in this analysis; Thompson 1984) these structures are imbedded within the albumen gland (Fig. 4C & D). Our finding that *Floridobia* forms a monophyletic group is consistent with the unique

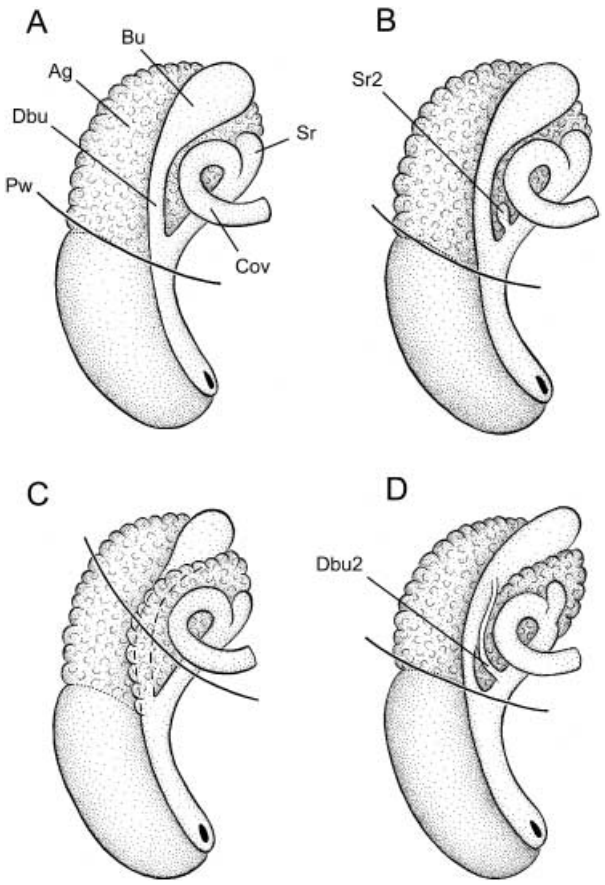

Fig. 4 A–D. Schematic drawings showing variation in distal female gentalic groundplans of nymphophiline snails. —A. *Nymphophilus*, *Pyrgulopsis*. Bursa copulatrix and duct superficial upon albumen gland (see Thompson 1979; Hershler 1994). —B. *Floridobia*. Bursa copulatrix and duct superficial upon albumen gland, second seminal receptacle present (Thompson 2000). —C. *Marstonia*. Bursa copulatrix and duct imbedded in albumen gland, pallial section of albumen gland large (Thompson 1977; Hershler 1994). —D. *Cincinnatia*, *Notogillia*, *Spilochlamys*, *Stiobia*. Bursa copulatrix and duct imbedded in albumen gland, second bursa copulatrix duct present (Hershler & Thompson 1996; unpublished data). Abbreviations: Ag, albumen gland; Bu, bursa copulatrix; Cov, coiled section of oviduct; Dbu, bursal duct; Dbu2, second bursal duct; Pw, posterior wall of pallial cavity; Sr, seminal receptacle; Sr2, second seminal receptacle.

presence of a second seminal receptacle (albeit sometimes only weakly developed as a small bulge) in species of this genus among the North American nymphophilines (Fig. 4B). The exclusion of *Cincinnatia integra* from this clade, despite the close similarity of the penes in these two groups (species of *Floridobia* were previously placed in *Cincinnatia* on this basis), suggests that this aspect of anatomy may not accurately define nymphophiline relationships. Our finding that *Pyrgulopsis* may be paraphyletic conflicts with an earlier treatment of this genus as a monophyletic group (Hershler 1994) and suggests a need to study the relationships of the large western North American fauna in more detail.

Our findings support the recent resurrection of *Marstonia* for eastern North American species that were previously placed in *Pyrgulopsis* (Thompson & Hershler, 2002). Monophyly of *Marstonia*, which is supported by our results, is consistent with the large extension of the albumen gland into the pallial roof (Fig. 4C) that is unique to this genus within the nymphophilines (Hershler 1994). Our finding of a well-supported sister relationship between *Notogillia* and *Spilochlamys* is congruent with a previously proposed hypothesis based on penial morphology (Thompson 1968: 109). Although not supported as a monophyletic group in our results these genera, together with two other eastern North American nymphophilines (*Cincinnatia*, *Stiobia*), are united by the presence of two well developed ducts connecting the oviduct and the bursa copulatrix (Fig. 4D), which is unique among hydrobiid snails. (For the maximum parsimony trees, forcing of these four taxa into a clade requires four additional steps.) All other nymphophilines have a single bursal duct except *Pyrgulopsis peculiaris*, whose second duct is weakly developed and opens to a different part of the bursa copulatrix than in the above genera (Hershler 1998: fig. 43E). Monotypic *Rhapinema*, which is shown to be most closely related to these genera in our analyses, has a completely different female anatomical groundplan which includes the unique absence of a bursa copulatrix (within the Nymphophilinae) and a more complex pattern of oviduct coiling than in any other member of the subfamily (unpublished data).

Biogeographical implications

The North American nymphophilines consist of geographically nonoverlapping western and eastern faunas. The former, consisting of *Nymphophilus* and *Pyrgulopsis*, is distributed from the Rio Grande basin and headwaters of the Missouri River across the continental divide to the Pacific margin. The latter, composed of the remaining eight genera, ranges from the Mississippi River basin eastwards to the Atlantic margin. Our phylogenetic results imply biogeographical subdivision into western and eastern North American components consistent with the distribution of these modern faunas, but are complicated by the inclusion within the western clade of *Floridobia*, which is distributed in Florida and coastal Maine. This implies a secondary invasion of eastern North America by progenitors of *Floridobia* from the West, which we interpret within the context of the ecological attributes of these snails. Although most nymphophilines live in freshwater habitats, there is evidence of salt tolerance within the group. *Floridobia winkleyi* lives in brackish estuaries (Davis *et al.* 1982; Davis & Mazurkiewicz 1985) while another congener, *Floridobia helicogyra*, lives in an occasionally brackish coastal lagoon (Thompson 1968 : 131). *Nymphophilus* (Minckley &

Cole 1968) and various species of *Pyrgulopsis* (e.g. Hershler 1998; Hershler & Sada 2000) live in concentrated springs. These features, together with the distribution of *Floridobia* in close proximity to modern or Pleistocene coastlines, suggest to us that progenitors of this genus may have dispersed eastward through brackish water coastal habitats along the northern Gulf of Mexico. Subsequent vicariance of the *Floridobia* clade presumably reflected perturbation of Gulf Coastal habitats, and perhaps occurred only recently as a result of the Laurentide meltwater flooding through the Mississippi River basin 14–11 ka (Teller 1990; Brown & Kennett 1998). A more comprehensive and robust phylogenetic hypothesis for the North American nymphophilines, which can be achieved by denser sampling of species and sequencing of additional genes, will be necessary to further evaluate this and other intriguing aspects of nymphophiline biogeography, including the apparent invasion of the central United States by multiple lineages (e.g. *Cincinnatia*, *Marstonia*).

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Appendix 1. Species, collection localities and voucher information

Abbreviations: UF, Florida Museum of Natural History; USNM, National Museum of Natural History, Smithsonian Institution. For previously sequenced material, only GenBank accession numbers are provided, while this information, together with locality and voucher (in parentheses) details are provided for species that were newly sequenced for this study.

Class Gastropoda
Superfamily Rissooidea
Family Hydrobiidae

Subfamily Hydrobiinae

Hydrobia acuta (Draparnaud, 1805), AF 354773.

Subfamily Lithoglyphinae

Fluminicola colouradensis Morrison, 1940, Provo River, Wasatch Co., UT, AF520931. *Holsingeria unthanksensis* Hershler, 1989, Skyline Caverns, Warren Co., VA, AF520947 (USNM 883928). *Lithoglyphus naticoides* (Pfeiffer, 1828), AF 354770. *Phreatodrobia nugax* (Pilsbry & Ferriss, 1906), artesian well, South-west Texas State University, Hays Co., TX, AF520927 (USNM 883784). *Pristinicola hemphilli* (Pilsbry, 1890), springs, 1.8 km E of Lower Kalama Hatchery, Cowlitz Co., WA, AF520940. *Somatogyrus* sp., Choctawhatchee River, 3.2 km N of Geneva, Geneva Co., AL, AF520942 (USNM 854736).

Subfamily Nymphophilinae

Cincinnatia integra (Say, 1821), stream, 5.6 km N of Fredericksburg, Gillespie Co., TX, AF520948 (UF 271729).

Floridobia floridana (Frauenfeld, 1863), Juniper Springs outflow, HWY 19 crossing, Marion Co., FL, AF520916 (UF 281698). *Floridobia mica* (Thompson, 1968), Coffee Spring, *c.* 1.6 km NE of US Hwy 27, Suwanee Co., FL, AF520914 (UF 281412). *Floridobia petrifons* (Thompson, 1968), Rock Springs, Kelly Park, Orange Co., FL, AF520920 (UF 280766). *Floridobia vanhyningi* (Vanatta, 1934), Seminole Springs, Lake Co., FL, AF520915 (UF 280812). *Floridobia winkleyi* (Pilsbry, 1912), Dunstan River salt marsh, Scarborough, ME, AF520917. *Marstonia agarhecta* Thompson, 1970, Bluff Creek, Hwy 129, Pulaski Co., GA., AF520934 (UF 279023). *Marstonia castor* Thompson, 1977, Mercer Mill Creek, Hwy 300, Worth Co., GA, AF520938 (UF 278962). *Marstonia comalensis* (Pilsbry & Ferriss, 1906), Old Faithful Spring, 1.0 km N of Camp Wood, Real Co., TX, AF520933 (UF 283565). *Marstonia halcyon* (Thompson, 1977), Ogeechee River, 2.1 km SW of Rocky Ford, Screven Co., GA, AF520935 (UF 279039). *Marstonia hershleri* (Thompson, 1995), Coosa River, *c.* 9.6 km N of Wetumpka, Elmore Co., AL, AF520946 (UF 279578). *Marstonia lustrica* (Pilsbry, 1890), Stockbridge Bowl, northwest portion, Berkshire Co., MA, AF520945 (USNM 894700). *Marstonia pachyta* Thompson, 1977, Limestone Creek, *c.* 1.6 km NE of Morresville, Limestone Co., AL, AF520939 (UF 279586). *Mercuria emiliana* (Paladilhe, 1869), AF 213346. *Notogillia wetherbyi* (Dall, 1885), Rainbow Springs, Marion Co., FL, AF520918 (UF 263135). *Nymphophilus minckleyi* Taylor, 1966, AF 354771. *Pyrgulopsis archimedis* Berry, 1947, Upper Klamath Lake at Hagelstein Park outlet, Klamath Co., OR, AF520950 (USNM 894697). *Pyrgulopsis avernalis* (Pilsbry, 1935), Muddy Spring, Moapa Valley, Lincoln Co., NV, AF520930 (USNM 903988). *Pyrgulopsis bruesi* Hershler & Sada, 2000, Fly Geyser, Black Rock Desert, Washoe Co., NV, AF520926 (USNM 892584). *Pyrgulopsis bruneauensis* Hershler, 1990, Bruneau Hot Springs, Owyhee Co., ID, AF520941. *Pyrgulopsis giulianii* Hershler & Pratt, 1990, stream, Sand Canyon, Kern Co., CA, AF520937 (USNM 894352). *Pyrgulopsis greggi* Hershler, 1995, Grapevine Creek, Fort Tejon Historical State Park, Kern Co., CA, AF520943 (USNM 903984). *Pyrgulopsis intermedia* (Tryon, 1865), Crooked Creek Spring State Wayside, Harney Co., OR, AF520928 (USNM 863511). *Pyrgulopsis micrococcus* (Pilsbry, 1893), spring just S of Springdale, Nye Co., NV, AF520944 (USNM 894330). *Pyrgulopsis neritella* Hershler, 1998, Big Spring, Steptoe Ranch, White Pine Co., NV, AF520951 (USNM 894720). *Pyrgulopsis owensensis* Hershler, 1989, spring, Graham Ranch, *c.* 8.0 km E of Big Pine, Inyo Co., CA, AF520922 (USNM 894691). *Pyrgulopsis pecosensis* (Taylor, 1987), Blue Spring, Eddy Co., NM, AF520929 (USNM 892588). *Pyrgulopsis peculiaris* Hershler, 1998, spring above Swasey Spring, Whirlwind Valley, Millard Co., UT, AF520912 (USNM 894883). *Pyrgulopsis robusta* (Walker, 1908), Polecat Creek, Teton Co. WY, AF520949 (USNM 905297). *Pyrgulopsis stearnsiana* (Pilsbry, 1899), springs, Wild Cat Canyon, El Sobrante, Contra Costa Co., CA, AF520925 (USNM 894694). *Pyrgulopsis turbatrix* Hershler, 1998, Horseshutem Springs, Pahrump Valley, Nye Co., NV, AF520936 (USNM 903989). *Pyrgulopsis wongi* Hershler, 1989, spring, lower Pine Creek Canyon, Inyo Co., CA, AF520923 (USNM 894692). *Pyrgulopsis* sp., spring, Amargosa Canyon, *c.* 3.2 km S of Tecopa, Inyo Co., CA, AF520924 (USNM 894693). *Rhapinema dacryon* Thompson, 1969, Chipola River, Florida Caverns State Park, Jackson Co., FL, AF520932 (UF 283994). *Spilochlamys gravis* Thompson, 1968, Alexander Springs, Lake Co., FL, AF520919 (USNM 854816). *Stiobia nana* Thompson in Thompson & McCaleb, 1978, Coldwater Spring, 10.7 km W of Oxford, Calhoun CO., AL, AF520921 (USNM 854934).

Subfamily uncertain

Phrantela marginata (Petterd, 1889), AF 129331. *Probythinella emarginata* (Küster, 1852), Cedar Creek, 4.0 km S of Cedar Point, Chase Co., KS, AF520913.

Family Cochliopidae

Cochliopa sp., AF 354762.