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EVALUATING THE MONOPHYLY OF *EULIMNADIA* AND THE LIMNADIINAE (BRANCHIOPODA: SPINICAUDATA) USING DNA SEQUENCES

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ABSTRACT

The evolutionary relationships among the six nominal genera within the spinicaudate clam shrimp family Limnadiidae, as well as the validity of the limnadiid genus *Eulimnadia*, have been much debated in the literature with little consensus emerging. The lack of resolution on these topics impedes evaluations of limnadiid biodiversity, phylogeny, and character evolution. To address these issues, we used Bayesian and parsimony methods to analyze DNA sequences from three genetic loci (28S, 12S, cytb) that were obtained from representatives of five nominal limnadiid genera and one undescribed limnadiid species. These analyses confirm the monophyly of *Eulimnadia* and the most taxonomically inclusive phylogenetic analysis (28S) produces the following tree topology: (((((*Eulimnadia*, *Metalimnadia*), undescribed limnadiid), *Imnadia*), *Limnadopsis*), *Limnadia*). This topology is inconsistent with prior estimates of limnadiid inter-generic relationships. Maximum likelihood-based constraint analyses demonstrate that the above topology is significantly better than prior hypotheses of limnadiid relationships, and support the monophyly of *Eulimnadia*. Morphological character optimization on this topology suggests that the postulated close relationship between *Limnadia* and *Eulimnadia* is based on shared ancestral characteristics rather than synapomorphies. Furthermore, the discovery of the genetically distinct undescribed limnadiid lineage strongly supports the necessity of efforts to better evaluate limnadiid biodiversity, especially those in poorly collected zoogeographic regions.

INTRODUCTION

Recent DNA-based phylogenetic analyses of branchiopod crustaceans (Spears and Abele, 2000; Braband et al., 2002) support the hypothesis that a monophyletic suborder Spinicaudata (Martin and Davis, 2001) comprises three families: Cyzicidae, Leptestheriidae, and Limnadiidae. Previous morphological analyses anticipated this view (Olesen et al., 1997; Olesen, 1998, 2000). The speciose and morphologically variable Limnadiidae comprises six genera: *Eulimnadia*, *Imnadia*, *Limnadia*, *Limnadiopsium*, *Limnadopsis*, and *Metalimnadia* (Straskraba, 1965; Pereira and Garcia, 2001), and relatively recent morphology-based taxonomic treatments support the monophyletic status of this family (Straskraba, 1965; Martin, 1989). According to Straskraba (1965), four limnadiid subfamilies have been erected which suggest some intrafamilial phylogenetic relationships: Imnadiinae (*Imnadia*), Limnadiinae (*Eulimnadia*+*Limnadia*), Limnadiopsinae (*Limnadiopsium*+*Limnadopsis*), and Metalimnadiinae (*Metalimnadia*). However, the monogeneric nature of two of these categories limits indications of evolutionary affinities. Furthermore, the lack of a clear-cut diagnosis has called into question the validity of the genus *Eulimnadia* (Webb and Bell, 1979; Brtek, 1997). Nonetheless, there has been little doubt expressed regarding the phylogenetic propinquity of its species with those of *Limnadia*. These morphology-based evaluations assume that the similarity of *Eulimnadia* and *Limnadia* specimens is due to recent common ancestry, rather than symplesiomorphy and/or convergence.

The phylogenetic analyses of Spears and Abele (2000) and Braband et al. (2002), while principally focused on clarifying higher-level branchiopod evolutionary relationships, also provide some insights regarding the status of the Limnadiidae and its inter-generic relationships. However, these insights are inherently limited due to each study's restricted taxonomic sampling within the Limnadiidae. Spears and Abele's analyses of 18S rDNA included sequences from individuals representing *Eulimnadia texana* Packard, 1871 and *Limnadia lenticularis* (Linnaeus, 1761), while the Braband et al. study, analyzing the 12S and EF1α loci, sequenced DNA from representatives of *Imnadia yeyetta* Hertzog, 1935 and *Limnadopsis birchii* (Baird, 1860), as well as from the two aforementioned species. Both of these studies offer weak support for the monophyly of the Limnadiidae as judged by relatively low Bremer support and/or nonparametric bootstrap values. However, the Braband et al. analyses present an explicit, if not taxonomically complete, hypothesis of limnadiid intergeneric relationships: ((*Imnadia*, *Limnadia*) (*Eulimnadia*, *Limnadopsis*)). Implicit in this topology is the rejection of a close evolutionary relationship between *Eulimnadia* and *Limnadia*, i.e., evidence against a monophyletic Limnadiinae *sensu* Straskraba, 1965.

The above observations suggest fundamental questions that must be addressed to facilitate a deeper understanding of limnadiid phylogenesis and character evolution: (1) Is the Limnadiidae monophyletic? (2) Was there a relatively recent evolutionary divergence between *Eulimnadia* and *Limnadia*,

i.e., a monophyletic Limnadiinae *sensu* Straskraba, 1965? (3) Is *Eulimnadia* a monophyletic genus? (4) Is the Braband et al. (2002) limnadiid topology supported by additional data and analyses? We will evaluate these hypotheses by constructing robust estimates of evolutionary relationships for five limnadiid genera using DNA sequences from both nuclear and mitochondrial loci, and discuss the implications of these estimates for limnadiid character evolution and classification.

Materials and Methods

Study Organisms

Species evaluated in this report, including GenBank accession numbers and locality information, are given in Table 1. In all of the phylogenetic analyses presented herein, the ingroup includes all available limnadiid taxa, while members of Cyzicidae and Leptestheridae were used as outgroups. The undescribed limnadiid specimen listed in Table 1 (collected from Mauritius Island by N. Rabet) shows some morphological affinity to *Eulimnadia* by sharing the characteristic dorsal organ and telson spine of this genus, but also has the caudal claw shape and antennal segment numbers more characteristic of *Limnadia* (C. Sassaman, personal communication).

Methods

Clam shrimp were preserved in 95-100% ethanol when collected from the field while lab-reared specimens were frozen at −70°C. Total DNA was isolated from individual clam shrimp using the Qiagen DNeasy Plant Kit. Portions of 28S and 12S rDNA and cytb were polymerase chain reaction (PCR; Mullis and Faloona, 1987) amplified using the primer pairs presented in Table 2. Each PCR reaction consisted of 5 μL 10X Qiagen PCR buffer, 1 μL dNTPs (0.2 mM each), 2.5 μL each primer (0.5 μM), between 1-5 μL of template DNA, 0.2 μL Qiagen *Taq* polymerase (1 unit), and enough H_2O to bring the total volume to 50 μL. PCR reactions were carried out in a PTC-100 thermal cycler (MJ Research, Inc., Waltham, MA). The thermal cycler programs consisted of forty cycles, each containing three steps: denaturing at 94°C for 1 minute, annealing at 40°C for 28S rDNA, 45°C for cytb and 46.5°C for 12S rDNA for 1 minute, and extending at 72°C for 1-1.5 minutes depending on the size of the fragment. PCR products were purified using 1.5% NuSieve (GTG agarose, FMC Bioproducts, Rockland, ME) low-melting point gels. Sequencing-template purification was done using the Wizard PCR preps DNA purification system (Promega, Madison, WI).

The mitochondrial (mt) and nuclear amplicons were characterized by cycle sequencing using the PCR amplification primers. The protocols for cycle sequencing of the amplicons are as presented in Folmer et al. (1994). These protocols include cycle-sequencing of both strands of each purified template using labeled primers. The separation of cycle-sequencing-reaction products was done in 3.7% and 5.5% polyacrylamide gels on LI-COR (Omaha, Nebraska) 4200L-2 and 4200S-2 automated DNA sequencers, respectively. The resulting sequences were aligned initially using AlignIR (AlignIR v2.0, LI-COR, Inc.) with subsequent refinement done manually using MacClade v. 4.0 (Maddison and Maddison, 2000). All sequences generated for this project have been deposited in the GenBank database (see Table 1 for accession numbers). The alignment of the cytb sequences utilized herein was straightforward since no indels have been detected at this locus in the spinicaudatan sequences we have generated to date. However, both the 28S and 12S rDNA sequences contained indels and areas of ambiguous alignment were deleted prior to phylogenetic analyses. The cytb multiple-sequence alignment contained 401 characters, while the 12S and 28S rDNA matrices contained 436 and 987 characters, respectively. These data sets are not identical regarding taxonomic representation due to our inability to amplify *Imnadia* and *Metalimnadia* with the 12S rDNA and cytb primers.

Phylogenetic analyses were conducted using the maximum parsimony (MP) and Bayesian inference (BI) algorithms in PAUP* (v.4.0b10; Swofford, 2001) and Mr. Bayes v3.0b4 (Huelsenbeck and Ronquist, 2003), respectively. Trees were constructed based on analyses of three datasets: (1) 28S rDNA sequences only, (2) 12S rDNA and cytb sequences, i.e., mtDNA only, and (3) sequences from all three gene regions, i.e., total evidence. The 28S rDNA only analyses are the most inclusive taxonomically (as indicated above). Combined analyses typically included only those specimens for which genetic data from each locus was available (to minimize potential topological distortions due to relatively large amounts of missing data). Thus, the multigene analyses have fewer terminals than do the 28S rDNA analyses. *Limnadopsis parvispinus* (NS44) 28S and *L. birchii* (W108) cytb and 12S sequences were combined so that *Limnadopsis* could be included in the combined analysis. All analyses that included cytb sequences were conducted using nucleotides representing all three codon positions. However, for the MP analyses, only transversions were coded since transitions were either saturated, e.g., at the third position, or beginning to show saturation, e.g., at the first position, at this level of divergence (analysis not shown). MP analyses were conducted using multiple runs and randomizing taxon addition order (1000 replicates) with tree-bisection-reconnection (TBR) branch swapping. For each MP analysis, consistency (CI), rescaled consistency (RI) and retention (RI) indices were calculated. The GTR+I+G model, denoted as appropriate for each matrix by ModelTest (v. 3.06: Posada and Crandall, 1998), was used in the BI analyses (5 chains, 5 million generations, 2 million generation burn-in using default priors). The robustness of the resulting MP and BI trees was evaluated using 100,000 fast-heuristic nonparametric bootstrap (Felsenstein, 1985) replicates (expressed as bootstrap percentages (BSP)) and posterior probabilities (PP), respectively. Pair-wise uncorrected proportional distances (p-distances) were calculated using PAUP*.

Alternative topologies were evaluated by analyzing the 28S rDNA and total evidence data sets using PAUP's maximum likelihood (ML) algorithm (100 random addition order replicates), with the model specifications (GTR+I+G) and parameters from the output of ModelTest. In the former instance, the Braband et al. (2002) limnadiid topology was compared to the best unconstrained ML topology using the approximately unbiased (AU, Shimodaira, 2002), Kishino-Hasegawa (KH), Shimodaira-Hasegawa (SH; Shimodaira and Hasegawa, 1999), weighted Kishino-Hasegawa (WKH), and weighted Shimodaira-Hasegawa (WSH; Shimodaira, 2002) tests as implemented in CONSEL (Shimodaira and Hasegawa, 2001). These tests were also used to compare the best unconstrained ML topology obtained from the total evidence data set with one in which *Limnadia+Eulimnadia* was constrained to be monophyletic and another in which *Eulimnadia* was constrained to be non-monophyletic.

Results

28S rDNA Analyses

Topologies generated by BI and MP were generally concordant, as indicated by the positive correlation between the nodal-support values produced by the two methods (Fig. 1). The MP analysis resulted in 18 equally parsimonious trees (315 steps each; CI = 0.7175; RC = 0.6883; RI = 0.9593; trees not shown). The Limnadiidae is supported as a monophyletic group by PP and BSP of 100 and 99, respectively. *Limnadia* is not the sister taxon of *Eulimnadia* but rather the sister taxon of a clade containing all other limnadiids (PP = 79, BSP = 74). *Limnadopsis* is the sister taxon to the clade of *Imnadia*+undescribed limnadiid+*Metalimnadia*+*Eulimnadia* (PP = 97, BSP = 73). The sister taxon to the undescribed limnadiid+*Metalimnadia*+*Eulimnadia* clade is *Imnadia* (PP = 100, BSP = 91).

The BI and MP analyses of the 28S rDNA matrix both indicate that the undescribed limnadiid is the sister lineage to *Metalimnadia*+*Eulimnadia* (PP = 100, BSP = 100), and that *Metalimnadia* is sister to a monophyletic *Eulimnadia* (PP = 85, BSP = 68). *Eulimnadia* is monophyletic and contains three subclades: *E. texana*+*E. cylindrova* (PP = 77, BSP = 77), *E. braueriana* (PP = 100, BSP = 98), and *E. diversa*+*E. magdalensis*+*E. agassazii*+*E. colombiensis* (PP = 91, BSP = 54). The *E. braueriana* lineage is sister to the remaining two subclades (PP = 91). Comparisons of 28S rDNA uncorrected p-distances indicate that the

Table 1. GenBank accession numbers and locality information for specimens utilized in this study. Abbreviations: nuclear locus: 28S ribosomal DNA (28S); mitochondrial loci: cytochrome b (cytb); 12S ribosomal DNA (12S). More specific locality data are available, upon request, from Steve Weeks (scw@uakron.edu).

Family	Species	Specimen No.	28S	cytb	12S	Locality
Limnadiidae	*Eulimnadia agassizii* Packard, 1874	NS58	AY851430	—	—	United States: MA
		NS59	AY851431	—	—	United States: MA
	E. braueriana Ishikawa, 1895	NS40	AY851425	—	—	Japan
		NS41	AY851426	—	—	Japan
		NS85	AY851443	AY779726	AY779679	Japan
	E. cylindrova Belk, 1989	NS11	AY851418	AY779697	AY779647	Mexico
		NS16	AY851422	AY779699	AY779650	Mexico
		NS17	AY851419	AY779698	AY779651	Mexico
		NS64	—	AY779701	AY779669	Ecuador: Galapagos Islands
		NS65	AY851432	AY779700	AY779670	Ecuador: Galapagos Islands
		NS103	AY851412	—	—	Venezuela
		NS104	AY851413	AY779716	AY779644	Venezuela
		NS105	AY851414	—	—	Venezuela
	E. colombiensis Roessler, 1989	NS106	AY851415	—	—	Venezuela
	E. diversa Mattox, 1937	NS4	—	AY779720	AY779664	United States: AZ
		NS8	AY851441	AY779721	AY779678	United States: AZ
		NS22	AY851420	—	—	United States: AZ
		NS23	AY851421	—	—	United States: AZ
		NS66	AY851433	AY779722	AY779671	Mexico
		NS67	AY851434	—	—	Mexico
	E. magdalensis Roessler, 1990	NS99	AY851445	AY779731	AY779681	Venezuela
		NS100	AY851411	AY779728	AY779643	Venezuela
		NS107	AY851416	—	—	Venezuela
		NS108	—	AY779730	AY779646	Venezuela
	E. texana Packard, 1871	JT5	AY851410	—	—	United States: NM
		NS9	AY851444	AY779708	AY779680	United States: NM
		NS5	AY851429	AY779714	AY779667	United States: NM
		NS34	AY851423	—	—	United States: NM
		NS35	AY851424	AY779712	AY779659	United States: NM
		NS50	AY851427	—	—	Mexico: Baja California
		NS51	AY851428	AY779703	AY779666	Mexico: Baja California
		NS70	AY851435	—	—	United States: NM
		NS71	AY851436	—	—	United States: NM
		NS72	AY851437	AY779711	AY779674	United States: NM
		NS73	AY851438	—	—	United States: NM
	Eulimnadia sp.	NS79	AY851440	AY779717	AY779676	Japan
		NS80	AY851442	AY779718	AY779677	Japan
		W132	AY851455	—	—	United States: IN
	Limnadia lenticularis (Linnaeus, 1758)	NS24	AY851399	—	—	United States: FL
		NS25	AY851400	AY779733	AY779655	United States: FL
		W66	AY851401	AY779732	AY779682	United States: FL
	Limnadopsis parvispinus Henry, 1924	NS44	—	AY779734	AY779665	Australia
	Limnadopsis birchii (Baird, 1860)	W108	AY851453	—	—	Australia
		W109	AY851451	—	—	Australia
		W116	AY851454	—	—	Australia
		W126	AY851452	—	—	Australia
	Imnadia yeyetta Hertzog, 1935	W125	AY851449	—	—	Austria
		W128	AY851446	—	—	Austria
		W129	AY851450	—	—	Austria
		W130	AY851447	—	—	Austria
		W131	AY851448	—	—	Austria
	Metalimnadia sp.	NS109	AY851417	—	—	Brazil
	Undescribed limnadiid	NS74	AY851439	AY779719	AY779675	Mauritius Island
Cyzicidae	*Cyzicus gynecia* (Mattox, 1950)	NS30	AY851402	—	—	United States: PA
		NS31	AY851403	AY779694	AY779656	United States: PA
		NS36	AY851404	AY779692	AY779660	United States: PA
		NS37	AY851405	AY779693	AY779661	United States: PA
	Eocyzicus digueti (Richard, 1895)	NS52	AY851406	—	—	Mexico: Baja California
		NS53	AY851407	AY779696	AY779668	Mexico: Baja California
Leptestheriidae	*Leptestheria compleximanus* (Packard, 1877)	NS14	AY851391	AY779683	AY779648	United States: NM
		NS15	AY851392	—	—	United States: NM
		NS20	AY851393	AY779684	AY779652	United States: NM
		NS21	AY851394	AY779685	AY779653	United States: NM
		NS32	AY851395	AY779687	AY779657	United States: NM
		NS33	AY851396	AY779688	AY779658	United States: NM
		NS38	AY851397	AY779686	AY779662	United States: NM
		NS39	AY851398	AY779689	AY779663	United States: NM
	L. dahalensis (Rüppell, 1837)	NS68	AY851408	AY779690	AY779672	Austria
		NS69	AY851409	AY779691	AY779673	Austria

Table 2. Primer pairs used in this study. Abbreviations: nuclear locus: 28S ribosomal DNA (28S); mitochondrial loci: cytochrome b (cytb); 12S ribosomal DNA (12S).

Gene	Primer	Primer sequence (5′-3′)	Amplicon size/Citation
28S	D1F	GGGACTACCCCCTGAATTTAAGCAT	~1100 bp
	D6R	CCAGCTATCCTGAGGGAAACTTCG	Park and O'Foighil, 2000
12S	12S30F	CTACTTTGTTACGACTTATCTC	~450 bp
	12S501R	AACCAGGATTAGATACCCT	Designed by Hoeh & Smallwood
cytb	UcytB151F	TGTGGRGCNACYGTWATYACTAA	~400 bp
	UcytB270R	AANAGGAARTAYCAYTCNGGYTG	Merritt et al., 1998

undescribed limnadiid is more divergent from all species of *Eulimnadia* than is the morphologically divergent genus *Metalimnadia* (Table 3). This finding is consistent with the topology presented in Figure 1. All topology test results (Table 4) indicate that the 28S rDNA limnadiid topology (Fig. 1) is significantly better ($P = 1e^{-04}$) than the topology presented in Braband et al. (2002).

mtDNA Analyses (12S rDNA+cytb)

Topologies generated by BI and MP were generally concordant as indicated by the positive correlation between the nodal support values produced by the two methods (Fig. 2). The MP analysis resulted in 141,333 equally parsimonious trees (568 steps each; CI = 0.6162; RC = 0.5449; RI = 0.8843; trees not shown). The Limnadiidae is supported as a monophyletic group by PP and BSP of 100 and 98, respectively. *Limnadia* is not the sister taxon of *Eulimnadia* but rather represents the sister taxon of a clade containing all other limnadiids (PP = 89, BSP = 71). *Limnadopsis*, the undescribed limnadiid, and *Eulimnadia* form a trichotomy. *Eulimnadia* is monophyletic (PP = 100, BSP = 89) and contains three major lineages: *E. texana*+*E. cylindrova* (PP = 100, BSP = 96), *E. diversa*+*E. magdalensis* (PP = 75, BSP = 88), and the *E. braueriana* lineage.

The *E. diversa*+*E. magdalensis* clade is the sister group to the remaining two lineages (PP = 100, BSP = 93). Comparisons of 12S rDNA+cytb uncorrected p-distances (using non-transformed cytb sequences) indicate that the undescribed limnadiid is more divergent from all other species of *Eulimnadia* than are the *Eulimnadia* species from each other (Table 3). This finding is consistent with the topology presented in Figure 2.

Total Evidence Analyses (28S rDNA+12S rDNA+cytb)

Topologies generated by BI and MP were generally concordant as indicated by the positive correlation between the nodal support values produced by the two methods (Fig. 3). The MP analysis resulted in 2,106 equally parsimonious trees (836 steps each; CI = 0.6603; RC = 0.5987; RI = 0.9067; trees not shown). The Limnadiidae is supported as a monophyletic group by PP and BSP of 100. *Limnadia* is not the sister taxon of *Eulimnadia* but rather represents the sister taxon of a clade containing all other limnadiids (PP = 100, BSP = 92). *Limnadopsis* is sister to the clade of *Eulimnadia*+undescribed limnadiid (PP = 100, BSP = 94). *Eulimnadia* is monophyletic (PP = 100, BSP = 100) and is sister to the undescribed limnadiid. The genus *Eulimnadia* contains three major sublineages: *E. texana*+*E. cylindrova* (PP = 100, BSP = 100), *E. diversa*+*E. magdalensis* (PP = 95, BSP = 87), and the *E. braueriana* lineage. The *E. diversa*+*E. magdalensis* clade is sister to the remaining two lineages (PP = 100, BSP = 93). All topology test results (Table 4), except the Shimodaira-Hasegawa ($P = 0.094$) test, indicate that the 28S rDNA+12S rDNA+cytb topology in Figure 3 is significantly better ($P \leq 0.016$) than one in which *Limnadia*+*Eulimnadia* is constrained to be monophyletic. Furthermore, all topology test results indicate that a tree topology containing a monophyletic *Eulimnadia* is significantly better than a tree with *Eulimnadia* constrained as non-monophyletic ($P \leq 0.017$; Table 4).

In summary, all of our best trees support both limnadiid and *Eulimnadia* monophyly while rejecting sister-taxon status for *Limnadia* and *Eulimnadia*. Thus, the Limnadiinae (*sensu* Straskraba, 1965) is not a monophyletic taxon. Furthermore, analyses of 28S rDNA sequences reject the Braband et al. (2002) hypothesis that *Imnadia*+*Limnadia* and *Eulimnadia*+*Limnadopsis* represent sister-taxon pairs.

Discussion

Taxonomic Status of *Eulimnadia*

The taxonomic status of the genus *Eulimnadia* has been an ongoing controversy in limnadiid systematics since its description by A. S. Packard in 1874 (Sars, 1895; Sayce, 1903; Daday, 1925; Ueno, 1927; Barnard, 1929; Brehm, 1933; Mattox, 1954; Straskraba, 1965; Webb and Bell, 1979; Belk, 1989; Martin, 1989; Martin and Belk, 1989; Brtek, 1997; Pereira and Garcia, 2001). The disagreement is founded on strongly differing opinions regarding the ability to morphologically differentiate specimens of *Eulimnadia* from those of *Limnadia*. Currently, the principal characteristic used to assign specimens to either *Eulimnadia*, or *Limnadia* is the presence or absence (respectively) of a well-developed spine on the posteroventral border of the caudal somite, e.g., Martin and Belk (1989). Webb and Bell (1979) question the usefulness of this characteristic to assign specimens generically and listed *Eulimnadia* as a junior synonym of *Limnadia*. The evidence used to support this position was the purported existence of character-state intermediates when comparisons were made using published figures of specimens representing multiple species of *Limnadia* and *Eulimnadia*. Nevertheless, the most recent taxonomic treatment of the Limnadiidae (Pereira and Garcia, 2001) supported the use of the caudal spine as a character diagnostic for *Eulimnadia*.

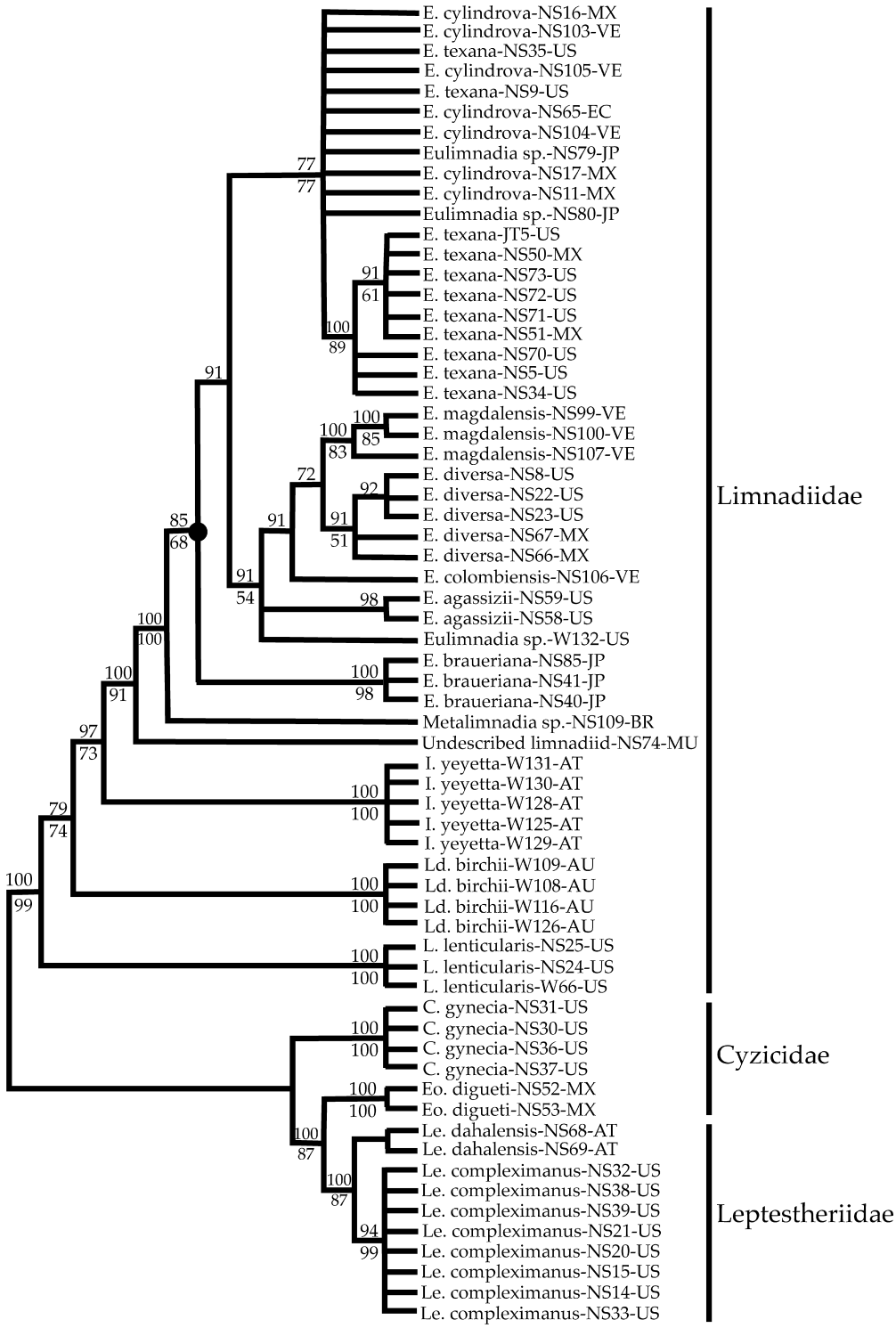


Fig. 1. Bayesian (BI) consensus tree generated from 28S ribosomal DNA sequences with BI posterior probabilities above the branches and MP bootstrap proportions (BSP) below the branches (BSP $\leq$ 50 not shown). The solid circle indicates the root of the clade containing all *Eulimnadia*. Genus designations: *Cyzicus* (C.); *Eocyzicus* (Eo.); *Eulimnadia* (E.); *Imnadia* (I.); *Leptestheria* (Le.); *Limnadia* (L.); *Limnadopsis* (Ld.). Country designations: Australia (AU); Austria (AT); Brazil (BR); Ecuador (EC); Mauritius Island (MU); Mexico (MX); Japan (JP); United States (US); Venezuela (VE).

The phylogenetic analyses presented herein strongly support the validity of the genus *Eulimnadia*. The BI analyses produced posterior probability values of 85 (28S rDNA), 100 (12S rDNA+cytb), and 100 (28S rDNA+12S rDNA+cytb) in support of a *Eulimnadia* clade. Similarly, the MP analyses supported the monophyly of *Eulimnadia* with values of 68, 89, and 100, respectively, for the same three datasets. The lower values supporting monophyly

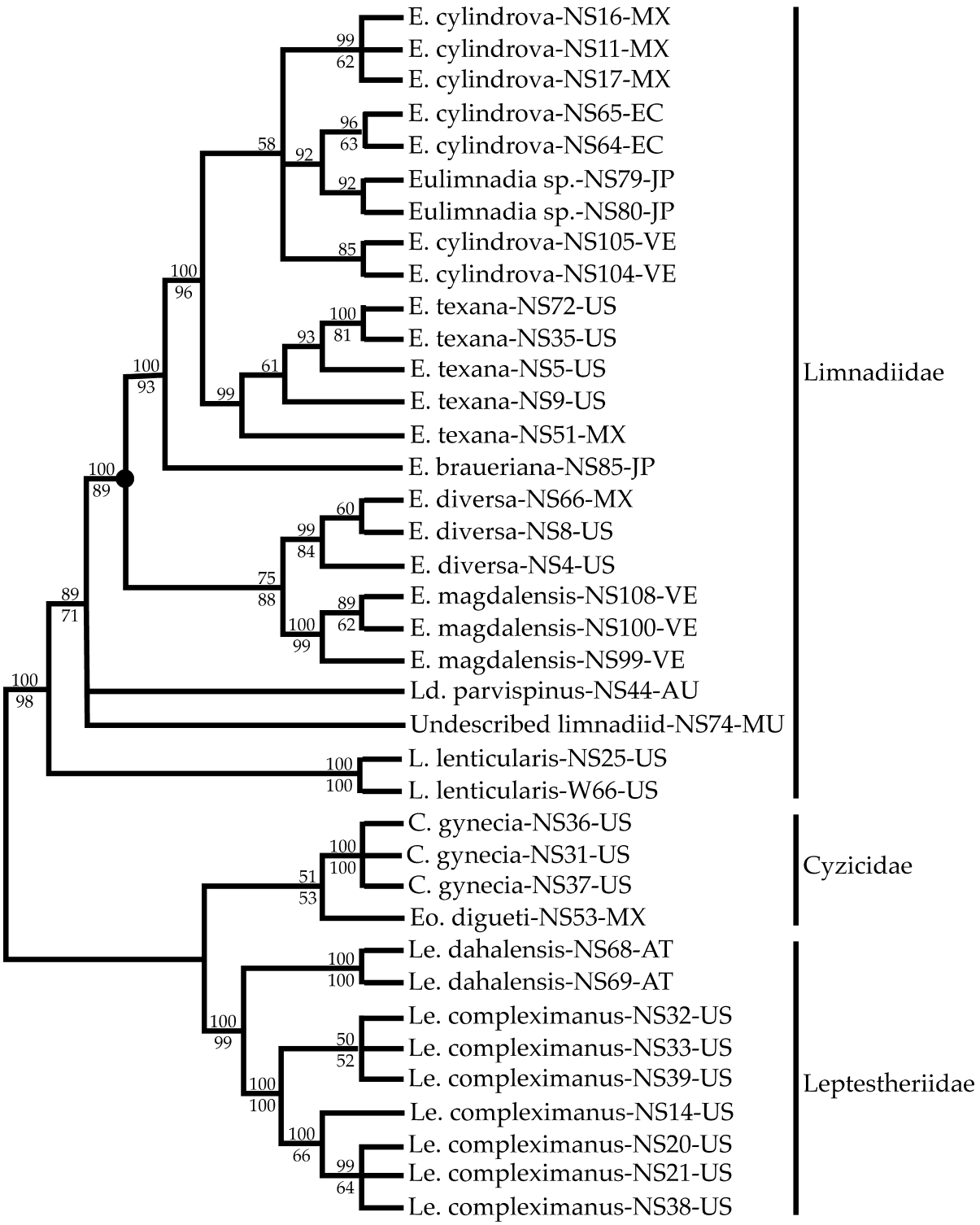


Fig. 2. Bayesian (BI) consensus tree from analysis of concatenated 12S rDNA and cytb mtDNA sequences with BI posterior probabilities above the branches and MP bootstrap proportions (BSP) below the branches (BSP $\leq$ 50 not shown). The solid circle indicates the root of the clade containing all *Eulimnadia*. Genus designations: *Cyzicus* (C.); *Eocyzicus* (Eo.); *Eulimnadia* (E.); *Imnadia* (I.); *Leptestheria* (Le.); *Limnadia* (L.); *Limnadopsis* (Ld.). Country designations: Australia (AU); Austria (AT); Ecuador (EC); Mauritius Island (MU); Mexico (MX); Japan (JP); United States (US); Venezuela (VE).

produced by analyses of the 28S rDNA dataset are likely due, in part, to a relatively reduced number of parsimony-informative characters in that particular dataset relative to the mtDNA dataset (136 vs. 305). Nevertheless, the independent and congruent support for *Eulimnadia* monophyly produced by the 28S rDNA and mtDNA analyses, combined with the corroborating results from the total evidence analyses, offer robust confirmation of *Eulimnadia* as

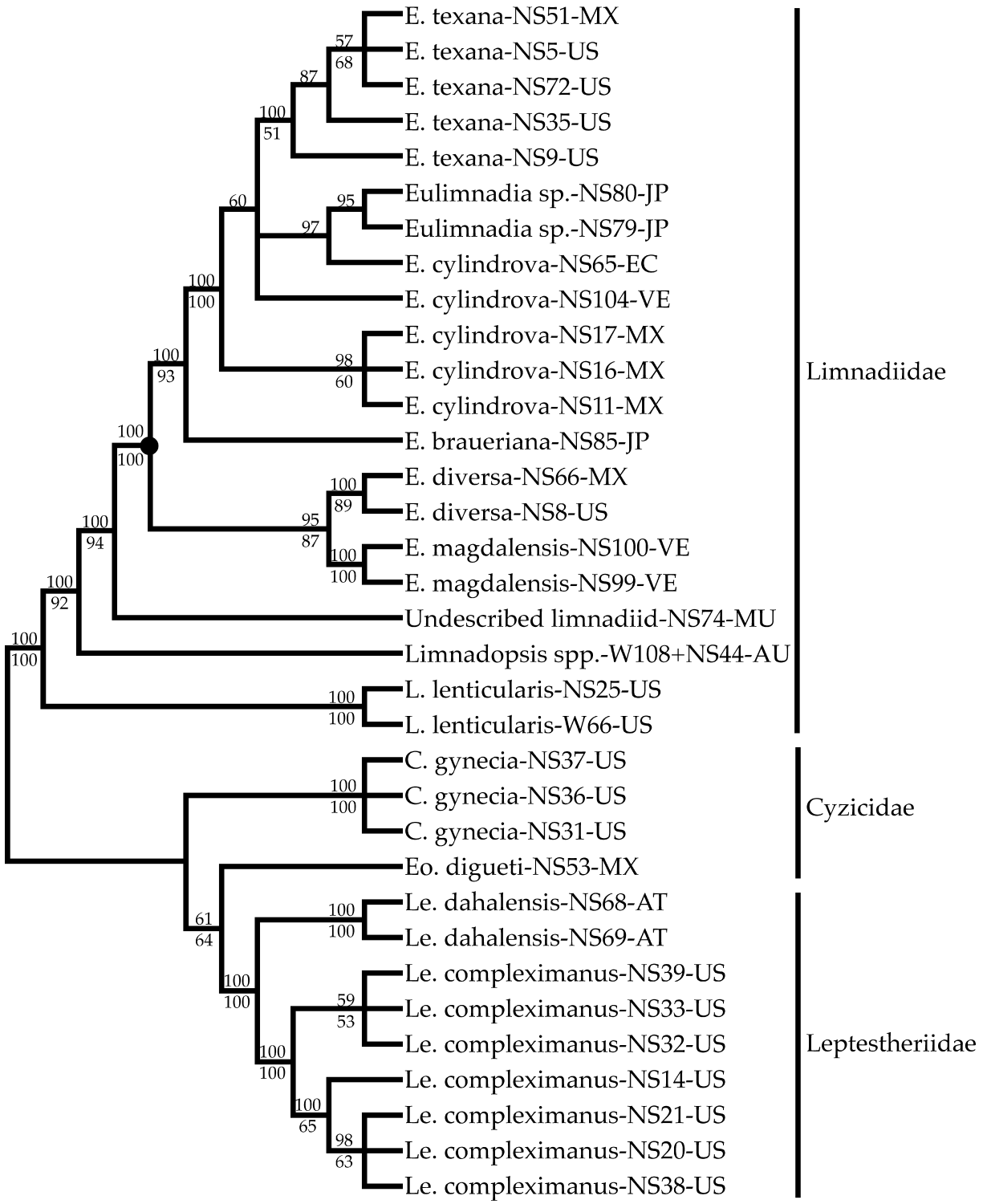
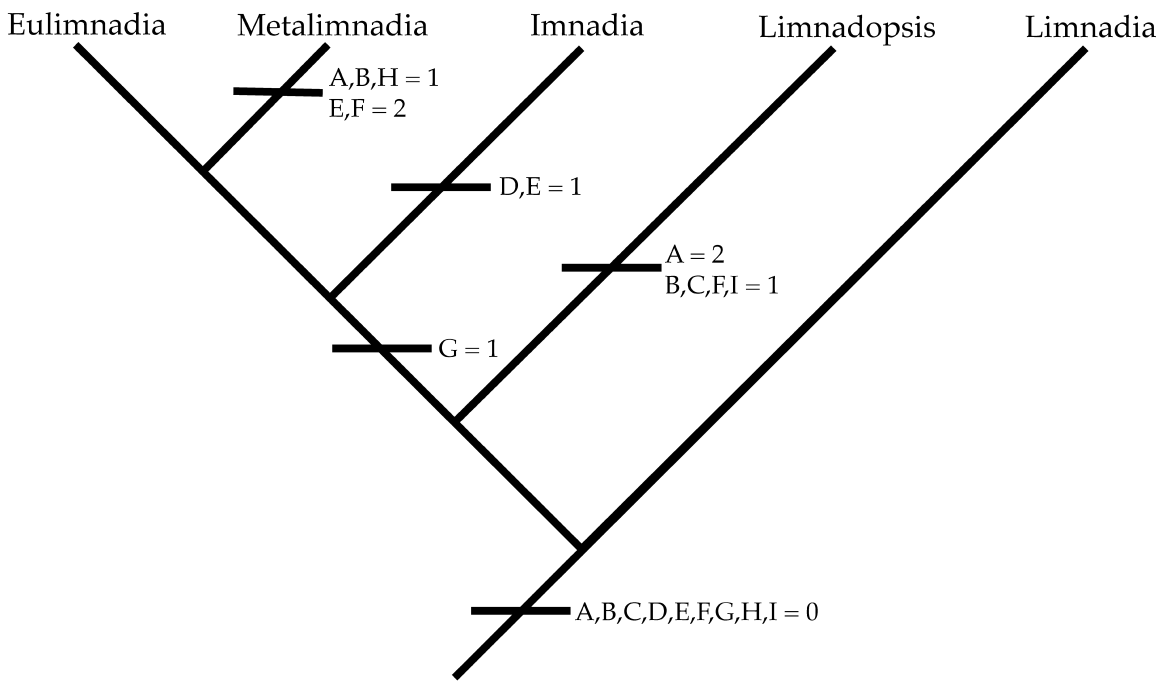


Fig. 3. Bayesian (BI) consensus tree generated from analysis of concatenated 28S rDNA, 12S rDNA and cytb sequences with BI posterior probabilities above the branches and MP bootstraps below the branches (BSP $\leq$ 50 not shown). The solid circle indicates the root of the clade containing all *Eulimnadia*. The *Limnadopsis* sequences analyzed are a composite of *L. birchii* (W108) and *L. parvispinus* (NS 44). Genus designations: *Cyzicus* (C.); *Eocyzicus* (Eo.); *Eulimnadia* (E.); *Imnadia* (I.); *Leptestheria* (Le.); *Limnadia* (L.); *Limnadopsis* (Ld.). Country designations: Australia (AU); Austria (AT); Ecuador (EC); Mauritius Island (MU); Mexico (MX); Japan (JP); United States (US); Venezuela (VE).



Designation	Character	State 0	State 1	State 2
A	Shell covered by striae	partly, few striae	partly, many striae	whole
B	Umbo	absent	present	--------
C	Dorsum	smooth	carinate	--------
D	Occiput	rounded	prominent	--------
E	Dorsal organ	on a pear-shaped appendage	directly on head	on a reduced appendage
F	Dorsal armiture by	setae	hooks	setae and spines
G	Upper corner of postabdomen	rounded	prominent	--------
H	First antennae	many-segmented, bar-shaped	two-segmented	--------
I	Terminal claws	long setae	smooth	--------

Fig. 4. Simplified topology from Figure 1 showing relationships among the limnadiid genera, with morphological character states (largely from Table 1 in Straskraba, 1965) parsimoniously plotted thereon.

a natural taxon. Furthermore, constraining *Eulimnadia* to be non-monophyletic using the total evidence dataset produces a tree that is significantly worse ($P \leq 0.017$) than the unconstrained analysis, as judged by the topology tests (Table 4).

All phylogenetic analyses support the hypothesis that three distinct sublineages exist within our sampled *Eulimnadia* specimens: (1) an *E. texana*+*E. cylindrova* clade, (2) an *E. diversa*+*E. magdalensis* clade, and (3) an *E. braueriana* lineage. The 28S rDNA analyses place *E. colombiensis* and *E. agassizii* in the *E. diversa*+*E. magdalensis* clade. The total evidence analyses suggest that *E. braueriana* and the *E. texana*+*E. cylindrova* clade are sister lineages with the *E. diversa*+*E. magdalensis*

Table 3. Pairwise uncorrected p-distances between clam shrimp species appearing in Table 1 (lower left: 28S rDNA p-distances; upper right: 12S rDNA+cytb p-distances). *Limnadopsis* (taxon 12) is the only taxon for which we have both nuclear and mitochondrial sequences, but with no overlap among individuals, thus the 28S rDNA sequence is from *L. birchii* (W108) and the 12S rDNA+cytb sequences are from *L. parvispinus* (NS44). Genus designations: *Cyzicus* (C.); *Eocyzicus* (Eo.); *Eulimnadia* (E.); *Imnadia* (I.); *Leptestheria* (Le.); *Limnadia* (L.).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1 *E. cylindrova*. NS104	—	0.03323	0.10189	0.09722	—	—	—	0.08505	—	0.16405	—	0.15760	0.17596	0.22901	0.18256	0.21077	0.19302
2 *E. texana*. NS72	0.00316	—	0.09479	0.10193	—	—	—	0.07634	—	0.15849	—	0.15869	0.18520	0.23748	0.18602	0.20661	0.18623
3 *E. magdalensis*. NS100	0.01690	0.02006	—	0.05193	—	—	—	0.09084	—	0.14785	—	0.15737	0.19572	0.22984	0.17185	0.18309	0.18510
4 *E. diversa*. NS8	0.01584	0.01900	0.00845	—	—	—	—	0.10308	—	0.14950	—	0.15304	0.19536	0.21998	0.19285	0.19277	0.18883
5 *E. colombiensis*. NS106	0.01274	0.01593	0.00530	0.00529	—	—	—	—	—	—	—	—	—	—	—	—	—
6 *E. agassazii*. NS59	0.00950	0.01267	0.00845	0.00845	0.00530	—	—	—	—	—	—	—	—	—	—	—	—
7 *Eulimnadia*. sp. W132	0.00845	0.01161	0.00950	0.00739	0.00424	0.00106	—	—	—	—	—	—	—	—	—	—	—
8 *E. braueriana*. NS85	0.01268	0.01584	0.01903	0.01798	0.01697	0.01585	0.01479	—	—	0.16634	—	0.14636	0.18812	0.23515	0.18725	0.19532	0.20044
9 *Metalimnadia*. sp. NS109	0.02436	0.02855	0.02642	0.02008	0.02331	0.02330	0.02224	0.02018	—	—	—	—	—	—	—	—	—
10 Undesc. limnadiid. NS74	0.03299	0.03504	0.03724	0.03406	0.03746	0.03936	0.03830	0.03517	0.03935	—	—	0.16188	0.20703	0.23830	0.18954	0.21403	0.20682
11 *I. yeyetta*. W125	0.05317	0.05519	0.05529	0.05532	0.05769	0.05957	0.05851	0.05227	0.05315	0.04351	—	—	—	—	—	—	—
12 *Limnadopsis*. W108/NS44	0.04697	0.04474	0.04485	0.03736	0.04292	0.04481	0.04375	0.04171	0.04593	0.02964	0.04039	—	0.16953	0.22647	0.20116	0.19391	0.19759
13 *L. lenticularis*. W66	0.04627	0.04829	0.05067	0.04532	0.05088	0.05062	0.04956	0.04540	0.05279	0.02894	0.03747	0.02251	—	0.22385	0.20660	0.20979	0.20141
14 *C. gynecia*. NS31	0.08696	0.08890	0.08489	0.08065	0.08535	0.08701	0.08594	0.08072	0.08165	0.06482	0.07356	0.06144	0.05322	—	0.21335	0.22394	0.23498
15 *Eo. digueti*. NS53	0.06972	0.07280	0.07512	0.07086	0.07441	0.07402	0.07296	0.07320	0.07520	0.06114	0.06952	0.06019	0.05300	0.06030	—	0.18022	0.18252
16 *Le. dahalensis*. NS69	0.06941	0.07252	0.07155	0.06623	0.07191	0.07368	0.07262	0.06852	0.06941	0.05347	0.05861	0.04481	0.04058	0.05008	0.03408	—	0.14760
17 *Le. complexmanus*. NS20	0.07244	0.07551	0.07458	0.06929	0.07494	0.07669	0.07563	0.07160	0.07249	0.05650	0.06067	0.04686	0.03940	0.05101	0.03407	0.00844	—

clade sister to the *E. braueriana*+*E. texana*+*E. cylindrova* clade.

Higher-level Evolutionary Relationships Within the Limnadiidae

The higher-level evolutionary relationships within the Limnadiidae have a history of instability and published articles on the subject offer relatively few explicit hypotheses for evaluation. Spencer and Hall (1896) described the genus *Limnadopsis*, which was given family-level status by Tasch (1969). However, the Limnadopsidae has not been generally accepted by subsequent authors (Martin and Davis, 2001). The genus *Imnadia* was described by Hertzog (1935) and subsequently this lineage was given family-level status by Botnariuc and Orghidan (1941). This taxonomic view was modified by Straskraba (1965) who gave the lineage subfamilial status within the Limnadiidae. Mattox (1952) described the genus *Metalimnadia* and, although stating that its distinctive morphology might be consistent with subfamilial or familial status, retained it as a genus within the Limnadiidae. This lineage was subsequently given subfamilial status by Straskraba (1965). Roessler (1995) proposed the elevation of the Metalimnadiinae to familial status based on distinctive features that include valve structure, head morphology, and reproductive traits. Pereira and Garcia (2001), however, listed *Metalimnadia* as a genus within the Limnadiidae.

Explicit hypotheses of inter-generic evolutionary relationships for the limnadiids are limited to Straskraba's (1965) morphology-based subfamilies and Braband et al.'s (2002) DNA sequence-based estimates of phylogeny. The former suggests a sister-taxon relationship for *Limnadia* and *Eulimnadia* (defined as Limnadiinae), while the latter offer the same for *Imnadia*+*Limnadia* and *Eulimnadia*+*Limnadopsis*. These two explicit hypotheses offer mutually exclusive views of limnadiid inter-generic relationships, but are limited in scope due to the low resolution inherent in Straskraba's use of two monogeneric subfamilies (Imnadiinae and Metalimnadiinae) and the absence of *Metalimnadia* from the Braband et al. (2002) analyses.

Our phylogenetic analyses (Figs. 1-3) robustly support the monophyly of the Limnadiidae, which encompasses *Limnadopsis*, *Imnadia*, and *Metalimnadia* (Fig. 1), as well as reject close relationships between (1) *Limnadia* and *Eulimnadia*, as suggested by Straskraba's (1965) and Tasch's (1969) Limnadiinae concept, and (2) *Imnadia*+*Limnadia* and *Eulimnadia*+*Limnadopsis* as suggested by Braband et al. (2002).

Character Evolution Within the Limnadiidae

Given the inter-generic evolutionary relationships suggested by our 28S rDNA phylogenetic analyses, the history of morphological character evolution within the Limnadiidae can be evaluated using parsimony optimization. A simplified version of our 28S rDNA topology (Fig. 1) is presented in Figure 4 with the morphological characteristics for the five limnadiid genera (largely from Table 1 in Straskraba,

Table 4. Results of the approximately unbiased (AU), Kishino-Hasegawa (KH), Shimodiara-Hasegawa (SH), weighted Kishino-Hasegawa (WKH), and weighted Shimodiara-Hasegawa (WSH) tests calculated using CONSEL.

Tree	−ln L	Difference	Test				
			AU	KH	SH	WKH	WSH
28S data: constraint = ((*Limnadia*+*Imnadia*) (*Eulimnadia*+*Limnadopsis*)):							
Unconstrained tree (Fig. 1)	−6695.47						
Constrained tree	−6833.43	137.95	$p = 1e^{-04}$	$p = 1e^{-04}$	$p = 1e^{-04}$	$p = 1e^{-04}$	$p = 1e^{-04}$
Cytb+12S+28S data: constraint = (*Limnadia*+*Eulimnadia*):							
Unconstrained tree (Fig. 3)	−15917.52						
Constrained tree	−15981.70	64.19	$p = 0.003$	$p = 0.011$	$p = 0.094$	$p = 0.011$	$p = 0.016$
Cytb+12S+28S data: constraint = *Eulimnadia* not monophyletic:							
Unconstrained tree (Fig. 3)	−15917.52						
Constrained tree	−16018.24	100.72	$p = 5e^{-04}$	$p = 0.001$	$p = 0.017$	$p = 0.001$	$p = 0.002$

1965) parsimoniously plotted thereon. The undescribed limnadiid species is not represented in Figure 4 due to the currently incomplete characterization of its morphology. A brief discussion regarding the evolutionary histories of the parsimony-informative character states follows. Our topology suggests that a rounded upper corner of the postabdomen (Fig. 4; character "G") is the ancestral character state for the Limnadiidae with the prominent upper corner interpreted as a synapomorphy for *Imnadia*+*Metalimnadia*+*Eulimnadia*. The absence of an umbo (Fig. 4; character "B") appears ancestral for the family, but the shared presence of that characteristic in *Limnadopsis* and *Metalimnadia* is interpreted as convergent evolution. Similarly, a pear-shaped dorsal organ (Fig. 4; character "E") is hypothesized as the ancestral character state for the Limnadiidae with the distinctive states observed in *Imnadia* and *Metalimnadia* construed as being independently derived. The topology and character optimization presented in Figure 4 suggest that the shared character states used to support a close evolutionary relationship between *Limnadia* and *Eulimnadia*, e.g., lack of umbo, rounded occiput, represent symplesiomorphies rather than synapomorphies.

Concluding Remarks

The analyses included herein represent the first use of molecular systematic techniques to address explicit hypotheses of limnadiid phylogeny and character evolution. Some of the evolutionary relationships and interpretations contained herein appear very robust. For example, we have presented phylogenetic analyses based on multiple independent genetic loci, which strongly support the monophyly and, hence, validity of the family Limnadiidae and the genus *Eulimnadia*. The apparent non-monophyly of the Limnadiinae (*sensu* Straskraba, 1965; Tasch, 1969) is also supported by independent analyses. However, other evolutionary relationships and interpretations presented herein are more provisional in nature due to limitations in both taxonomic and genetic data sampling. For example, we analyzed specimens representing a single species from the polytypic genus *Limnadia*. In future studies, however, the monophyly of all polytypic limnadiid genera should be explicitly evaluated by analyses that would ultimately include representatives of all nominal species. Our most taxonomically inclusive estimate of limnadiid phylogeny, containing specimens representing five nominal genera, is that from analyses of 28S rDNA sequences. Independent confirmation/refutation of the 28S rDNA-based phylogeny was not possible due to the inability of our current mtDNA primer pairs to amplify *Imnadia* and *Metalimnadia* templates. Future phylogenetic studies of the Limnadiidae should employ multiple independent genetic markers that are comparable across all limnadiid taxa.

The description (Mattox, 1952) of the morphologically distinct limnadiid genus, *Metalimnadia*, suggests that other, currently unrecognized, major lineages within the family may yet require morphological and genetic characterization. The phylogenetically distinct nature of the undescribed limnadiid specimen from Mauritius Island, as reported herein, offers support for this view. The study of a greater number of limnadiid specimens from poorly sampled zoogeographic regions will likely be necessary to better comprehend the patterns and processes underlying limnadiid biodiversity, phylogeny, and character evolution.

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