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The systematics of Holarctic bosminids and a revision that reconciles molecular and morphological evolution

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Abstract

Bosminids are ubiquitous and abundant crustacean herbivores in freshwater ecosystems. They are among the best preserved zooplankters in limnological sediments, making them ideal paleolimnological indicators of ecosystem change. Moreover, their egg banks make possible the study of paleogenetics and resurrection ecology. A major limitation to these comparative disciplines is that the relatedness and identities of compared bosminid specimens or subfossils is often uncertain. We aimed to remedy this by developing the first robust molecular phylogeny of the group and inferring patterns of morphological evolution. We used sequence alignments of five nuclear rDNA genes (partial 18S rDNA, internal transcribed spacer 1 [ITS-1], 5.8S, ITS-2, and partial 28S rDNA) and one mitochondrial gene (partial 16S rDNA) from representative Holarctic species from all of the proposed subgenera and genera. No evidence of within–individual sequence variation in the gene regions analyzed was found for the genus *Bosmina* and the genus *Eubosmina.* Our nuclear and mitochondrial genetic results revealed a robust phylogeny and were congruent with morphological changes. Competing morphological schemes of bosminid systematics were reconciled by reassigning several species to different subgenera, resurrecting the genus *Eubosmina,* erecting a new subgenus, and revising the character coding systems that created paraphyletic groups. The results provide an evolutionary framework with diagnostic genetic and morphologic characters for studies of bosminid paleolimnology and paleogenetics.

Many of the processes that limnologists study, from ecosystem change to organic evolution, take place over a timescale from decades to millennia. Carrying out studies that involve direct observation of these processes is a formidable challenge. Three relatively new limnological disciplines provide hope in this regard because they permit direct analysis of biotic change from dated lake sediments (Jeppesen et al. 2001). Paleolimnology uses organismal remains, including body parts and their pigments, to examine historical limnological processes. Paleogenetics relies on polymerase chain reaction (PCR) technology to examine genetic changes in minute aquatic organisms over a broad timescale (Weider et al. 1997). Resurrection ecology involves comparing live specimens that have been grown from resting stages of different time zones in the sediments (Kerfoot et al. 1999). These are powerful approaches to examine long-term ecosystem change and the organismal evolution that result from disturbances such as eutrophication, salinization, differing UV exposures, biological invasions, and glaciation. Nevertheless, because these promising new approaches are exercises in comparative biology, their value depends on knowledge of how the compared organisms are related. To determine whether organisms have invaded, become extinct, hybridized, or adapted to a disturbed environment, a robust systematic biology is essential. It is also important that studies of ecosystem change be able to determine whether the data compared are historically independent.

Of course, only small subsets of aquatic biota are suitable subjects for historical limnology, and even fewer are suitable for all major methods of sediment analysis. Bosminid microcrustaceans belong to such a subset because they are already an important group in paleolimnology and have the potential to be important in paleogenetics and resurrection ecology. The body parts, embryos, and pigments of this cosmopolitan family preserve well in freshwater and marine sediments, and they are often the most abundant subfossils in lake sediments (Jeppesen et al. 2001). The resting embryos, or ephippia, have the potential to be resurrected for experiments or genotyped directly by PCR. In short, bosminids have the potential to become preeminent subjects for historical limnology.

Unfortunately, this potential is not yet realized, partly because the group has a problematic systematic biology. Whereas the systematic biology of many freshwater invertebrate groups is described as difficult or confused, the systematic biology of the Bosminidae has been called legendary, notorious, and in desperate need of critical attention (Deevey and Deevey 1971; Lieder 1983; De Melo and Hebert 1994*b*). One reason for the superlatives is that since Müller first described *Bosmina,* few morphological characters (e.g., the postabdominal claw structure [Fig. 1A] and the lateral head pore location [Fig. 1B]) have been identified as ontogenetically and environmentally invariant (Goulden and Frey 1963). The need is desperate because, as superabundant herbivores and occupants of prominent positions in freshwater food webs, bosminids are studied extensively in many disciplines of aquatic biology. Still, investigators rarely have confidence in the relationships or identities of the specimens that are being compared, so it is hardly an overstatement that the systematic biology of bosminids is important to an

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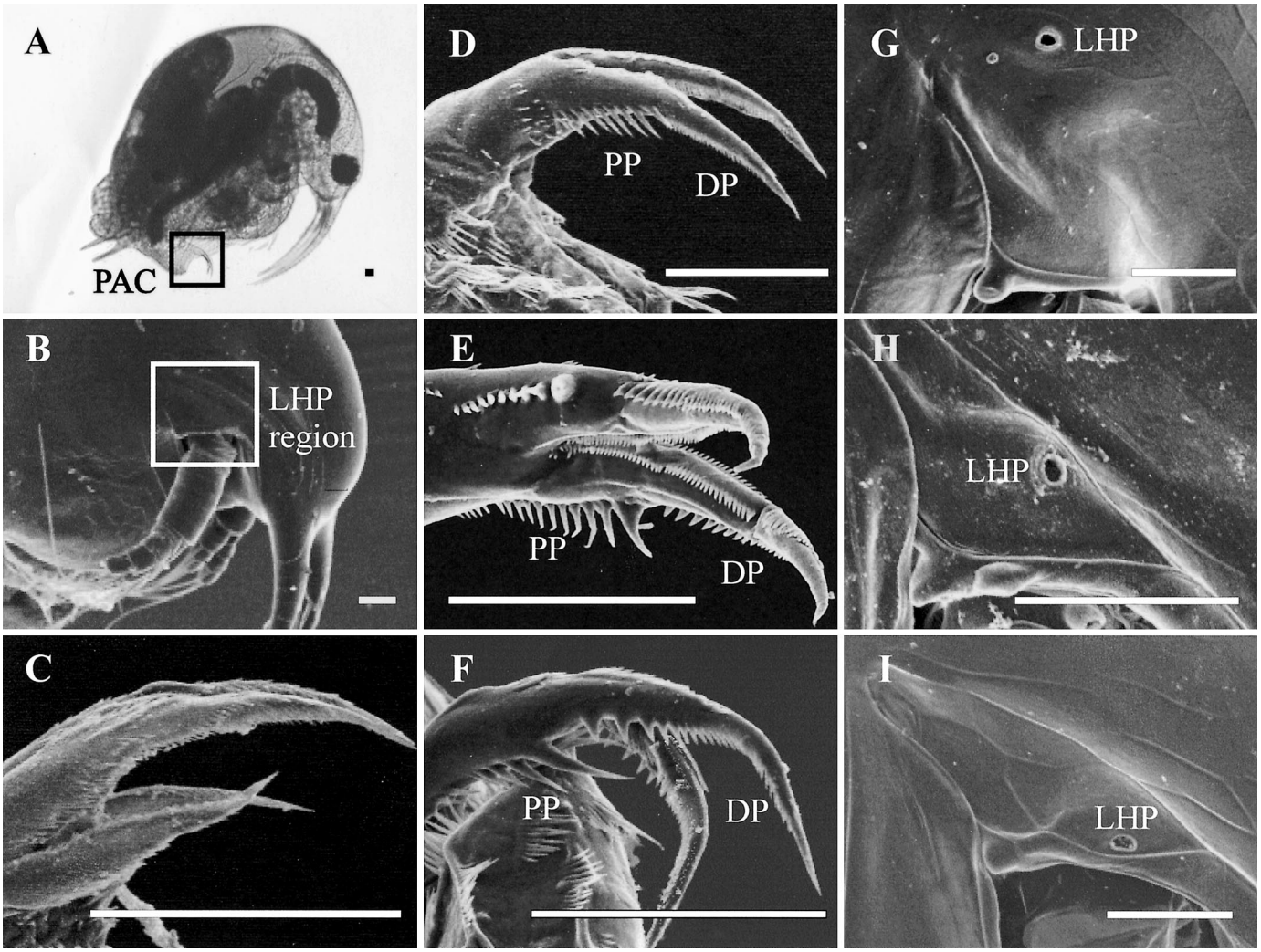

Fig. 1. Micrographs of bosminid body structures. Horizontal scale bars indicate 20 μm. (A) Lateral view of adult female of *Bosmina* (*Eubosmina*) *oriens* showing the boxed location of the postabdominal claw (PAC). (B) Lateral view of a mature *Bosmina* female with the boxed region marking the region of the lateral head pore (LHP) magnified in G–I. (C) Lateral view of postabdominal claw and basal claw from *Bosminopsis deitersi,* Lake Jack Lee, Arkansas. (D) Lateral view of postabdominal claws from a mature female of E showing the proximal (PP) and distal pectens (DP). (E) Ventral view of PAC from a female of *Bosmina* (*Bosmina*) *longirostris,* Alder Lake Alaska. (F) Lateral view of a female PAC from *Bosmina* (*Bosmina*) *liederi,* Halls pond, Massachusetts. (G) LHP region of *Bosmina* (*Neobosmina*) *hagmanni,* Halls Pond, Massachusetts. (H) LHP region of *Bosmina* (*Sinobosmina*) *fatalis* from Suwa Lake, Japan. (I) LHP region of *B.* (*B.*) *liederi* from Center Lake, Indiana.

improved understanding of many limnological disciplines, from ecosystems biology and invasion biology to paleolimnology.

There are three widely accepted but different schemes for assigning the bosminids to subgenera based on morphological characters (Table 1; Fig. 1). The scheme proposed by Deevey and Deevey (1971) recognized three genera of bosminids: *Eubosmina, Bosmina,* and *Bosminopsis.* Lieder (1983) recognized only *Bosmina* and *Bosminopsis* but suggested that there are at least four very divergent subgenera of *Bosmina* (*Bosmina, Eubosmina, Sinobosmina,* and *Neobosmina*) and identified four diagnostic morphological characters (Table 1). Later schemes (De Melo and Hebert 1994*b*; Kořínek et al. 1999) redefined Lieder's subgenera by scoring *Bosmina* (*Sinobosmina*) claw character states the same as (*Bosmina*) *Bosmina* and by scoring a third character state for the lateral head pore position (Table 1). Thus, the three new species described by De Melo and Hebert (1994*a*) and Kořínek et al. (1999) as belonging to the subgenus *Sinobosmina* (*B.* (*S.*) *freyi, B.* (*S.*) *liederi,* and *B.* (*S.*) *tripurae*) would be classified as the subgenus *Bosmina* by Lieder (1983) and the genus *Bosmina* by Deevey and Deevey (1971). For this paper, we initially used the recent scheme of De Melo and Hebert (1994*b*) with two bosminid genera (*Bosmina* and *Bosminopsis*) and four subgenera of *Bosmina.*

The generic and subgeneric assignments are unresolved, and because no one has proposed an explicit phylogenetic tree based on morphology, even less is known about the relationships of the bosminids to one another. Thus far, genetic studies (allozymes and randomly amplified polymor-

Table 1. Morphological schemes for higher level bosminid systematics.

Morphological character	Deevey and Deevey (1971)	Lieder (1983)	De Melo and Hebert (1994*b*)
Robustness and shape of postabdominal claw setation	0. Without distal pecten (*Eubosmina*) 1. With distal pecten (*Bosmina*)	0. Distal pecten with fine hairlike setae; proximal pecten with short robust spines of even thickness (subgenera *Eubosmina, Neobosmina, Sinobosmina*) 1. Distal pecten with 7–10 minute spines; proximal pecten with 4–12 fine, long spines (subgenus *Bosmina*)	0. Distal pecten with long, fine spines and length > width (subgenera *Eubosmina, Neobosmina*) 1. Distal pecten with short spines and length = width (subgenera *Bosmina, Sinobosmina*)
Lateral head pore location	1. Distant from shell margin near insertion of mandibles (*Eubosmina*) 2. Near shell margin and insertion of antenna (*Bosmina*)	1. Distant from shell margin near insertion of mandibles (subgenera *Eubosmina, Neobosmina*) 2. Near shell margin and insertion of antenna (subgenera *Bosmina, Sinobosmina*)	1. Distant from shell margin near insertion of mandibles (subgenera *Eubosmina, Neobosmina*) 2. Near shell margin but between forked fornix lines (subgenus *Sinobosmina*) 3. Abutting shell margin near insertion of second antennae (subgenus *Bosmina*)
Shape of anal margin on male postabdomen	0. Truncated (*Bosmina, Eubosmina*) 1. Beveled (*Eubosmina*)	0. Truncated (subgenera *Bosmina, Neobosmina, Sinobosmina*) 1. Beveled (subgenus *Eubosmina*)	Not scored
Location of carapace spine (mucro) serrations	0. Serrations ventral (*Eubosmina*) 1. Serrations dorsal (*Bosmina, Eubosmina*)	0. Serrations ventral (subgenera *Bosmina, Eubosmina, Sinobosmina*) 1. Serrations dorsal (subgenus *Neobosmina*)	0. Serrations ventral (subgenera *Bosmina, Eubosmina, Sinobosmina*) 1. Serrations dorsal (subgenus *Neobosmina*)

phic DNA) have made tremendous progress in the assessment of species boundaries and in the determination of hybridization but have failed to produce a robust phylogenetic tree (De Melo and Hebert 1994*a*; Hellsten and Sundberg 2000). De Melo and Hebert (1994*a*) presented a UPGMA (unweighted pair group method with arithmetic averages) tree based on eight allozyme loci, and it conflicted with their proposed subgenera. The reliability could not be assessed because of the small number of allozyme loci used. Lieder's (1983, p. 135) comment that ''one is forced to conclude that it is difficult to set up any reasonable sort of phylogenetic scheme of *Bosmina* subgenera altogether, at least with the knowledge we currently possess'' still rings true today.

Four morphological characters have been commonly used in *Bosmina* systematics and are generally well-preserved in lake sediments (Lieder 1983). The female postabdominal claw region (Fig. 1A) is markedly different in structure between the two bosminid genera. *Bosminopsis* possesses claws with uniform fine setation (Kotov 1997) and a large basal spine (Fig. 1C); *Bosmina* lacks a basal spine, but its claws contain at least two comblike areas of setation, often termed the proximal and distal pectens (Fig. 1D). Burkhardt (1899) noted that European *Bosmina* formed two main groups based on character states of the female postabdominal claw. Modern schemes separate claws into prominent and weak distal pecten character states (Fig. 1D vs. Fig. 1E,F). Goulden and Frey (1963) later discovered that bosminids could also be grouped based on the position of an approximately 2- × 6-μm lateral pore (Fig. 1G–I) on the head. The two remaining commonly used characters are more difficult to score because they are either restricted to rare males or juveniles. These are the location of serration on the posterior carapace spines (or mucros) of juveniles and the shape of the anal margin in the male postabdomens (Lieder 1983).

The purpose of this study is to provide the first molecular systematic investigation of bosminids. Using DNA sequence information from five genes of the nuclear ribosomal gene array (18S rDNA, internal transcribed spacer 1 [ITS-1], 5.8S, ITS-2, 28S rDNA) and one gene from the mitochondrial genome (16S rDNA), we test the agreement of molecular data with the recent subgeneric hypotheses and evaluate the phylogenetic utility of both morphological and molecular characters on a Holarctic scale. More specifically, we test the susceptibility of the morphological characters used in paleolimnology to parallel or convergent evolution and determine the minimum number of genetically divergent groups represented by the subtle morphological changes in *Bosmina*.

Methods

Sampling strategy—We sequenced specimens from 27 populations of bosminids from the three proposed genera (Table 1). Our sampling represents 10 of the 11 currently recognized North American species, several Eurasian species, and multiple members of the four proposed subgenera. Within a species, specimens were chosen to represent either geographically distant populations (often from different continents) or reference populations from prior studies (De Melo and Hebert 1994*b*). We used the macrothricid, *Ilyocryptus*, as an additional outgroup because it is, without controversy, closely related to, but not part of, the Bosminidae (Fryer 1995; Olesen 1998).

SEM methods—Specimens were prepared for scanning electron microscopy (SEM) by dehydration in either a graded acetone dilution series (of 60–100% with 5 min at each step) for live or frozen samples or placed directly into 100% acetone for solvent-preserved samples. The specimens were then treated with 100% hexamethyldisilazane (Sigma) for 20 min (Laforsch and Tollrian 2000). After complete evaporation of hexamethyldisilazane, individuals were placed onto an aluminum stub coated with double-sided sticky tape and then sputtered with carbon under vacuum. Images were taken with a Hitachi S-4000 field emissions scanning electric microscope at 5 kV.

Laboratory protocols—Specimens were examined for lateral head pore, claw, and mucro morphology and then DNA was extracted using the 2× CTAB buffer protocol (Doyle and Doyle 1987). Each 50-μl polymerase chain reaction (PCR) consisted of 40 μl irradiated H_2O, 5 μl 10× PCR buffer, 1.5 mM $MgCl_2$, 2 mM of each dNTP, 1 μM of each primer, 0.5–1 units of *Taq* DNA polymerase, and 1/15th of the DNA extract (2 μl of a 30-μl solution). The primers 18SD (Palumbi 1996) and 28SD2R (Omilian and Taylor 2001) were used for PCR of the nuclear gene regions, and two additional internal primers were used to complete the sequencing of both strands: 5.8SF (5′-ACCCTGAACGGTGGATCACTAGGCTC) and 5.8SR (5′-TAGGATTAGCGCACTTTGCTGC). The two primers 16Sar and 16Sbr (Kocher et al. 1989) were used to amplify the mitochondrial 16S rDNA (mtrDNA) fragment. The PCR conditions for the nuclear DNA amplification consisted of 40 cycles of 60 s at 94°C, 60 s at 55°C, 90 s at 72°C; followed by one cycle of 6 min at 72°C. The conditions were the same for the mtrDNA region but the annealing temperature was reduced to 50°C. PCR was conducted on a Stratagene RoboCycler.

PCR products were gel-purified using the Amicon kit for DNA extraction. We sequenced the fragments in both directions. For the initial seven specimens (MA1, AR1, CT, ON3, SC, MA4, NWT), the nuclear rDNA fragment was cloned with the Invitrogen TOPO TA Cloning Kit for Sequencing (Version B). More than three clones were sequenced per fragment. After detecting no within-individual variation, we directly sequenced the remainder of the ingroup specimens without cloning. The nuclear rDNA fragment of the outgroup, *Bosminopsis*, proved difficult to sequence from PCR product, and it was also cloned. The ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit or the Amersham Pharmacia Biotech Thermo Sequenase fluorescent-labeled primer cycle sequencing kit with 7-deaza-dGTP and the ABI 377 or a LI-COR 4200 automated DNA sequencer were used for sequencing.

Sequence assembly and alignment—Sequences were assembled and edited with Sequencher 4.0 (Gene Codes Corporation) and then aligned with Clustal X using the default

parameters (Thompson et al. 1994; Thompson et al. 1997). The 16S rDNA alignment was manually adjusted with Bioedit (Hall 1999) according to core region rRNA secondary structure (De Rijk et al. 2000). Nexus files were deposited in Treebase (study accession number = S717), and sequences were deposited to Genbank (AF482735–AF482756 and AF483999–AF484023). Gene boundaries were based on the proposal of De Rijk et al. (2000). Initial authenticity of the sequences was examined by BLAST comparisons.

Phylogenetics—Phylogenetic analyses were conducted in PAUP* 4.0 (Swofford 2000) and Mr. Bayes 3.0 (Huelsenbeck et al. 2000; Huelsenbeck and Ronquist 2001). Fifty-six maximum likelihood (ML) models were assessed by a series of likelihood ratio tests with the program Modeltest 3.06 (Posada and Crandall 1998). To find the best tree under the ML criterion, we used this model with a heuristic search, tree bisection–reconnection (TBR) branch swapping, and 10 random-sequence taxon additions. To test the hypothesis that the observed tree was more likely than the expected tree, we used the Shimodaira–Hasegawa (S-H) test with 1,000 bootstrap replicates and full optimization (Shimodaira and Hasegawa 1999; Goldman et al. 2000).

To estimate phylogenetic uncertainty, we used nonparametric bootstrapping and a Bayesian statistical method with Markov chain Monte Carlo (MCMC) sampling (Huelsenbeck et al. 2000). The MCMC method samples trees from the universe of possible trees in proportion to their probability given a model of DNA evolution. Our models were determined by hierarchical likelihood ratio tests. We sampled 10,000 trees and removed the first 100 found to account for variance due to convergence on the Markov chain. The remaining trees were exposed to 50% majority rule consensus tree analysis in PAUP. The proportion of trees containing a clade represents its posterior probability or the probability of being correct given the data and model of evolution.

Results

Data features—The alignments were 1,215 base pairs (bp) for the nuclear rDNA (nrDNA) sequences and 498 bp for the mtrDNA sequences. The gene boundaries for the nrDNA alignment were: 18S, 1–119; ITS-1, 120–399; 5.8S, 400–554; ITS-2, 555–930; and 28S, 931–1,215. Tests for among-taxon base composition bias revealed no significant differences among taxa (nrDNA $\chi^2_{42} = 5.55$, $P > 0.99$, with the average base composition being: A 0.29, C 0.23, G 0.26, T 0.22; mtDNA $\chi^2_{57} = 14.41$, $P > 0.99$, with the average base composition being: A 0.34, C 0.13, G 0.20, T 0.33).

Authenticity of each segment was analyzed by comparing BLAST results, where the expectation value (E) is the number of matches expected by chance between the query sequence and unrelated sequences from a database that is the same size as GenBank. For the nuclear rDNA PCR product, the 18S section of *B. freyi* was blasted, and the best 19 matches were branchiopod crustaceans, the group to which Bosminidae belong (best match *Eulimnadia texana,* score 163 bits; $E = 7 \times 10^{-38}$). For the mtDNA, *B. freyi* also had a best match with branchiopod crustaceans (*Daphnia longicephala,* score 208 bits; $E = 3 \times 10^{-51}$). These matches

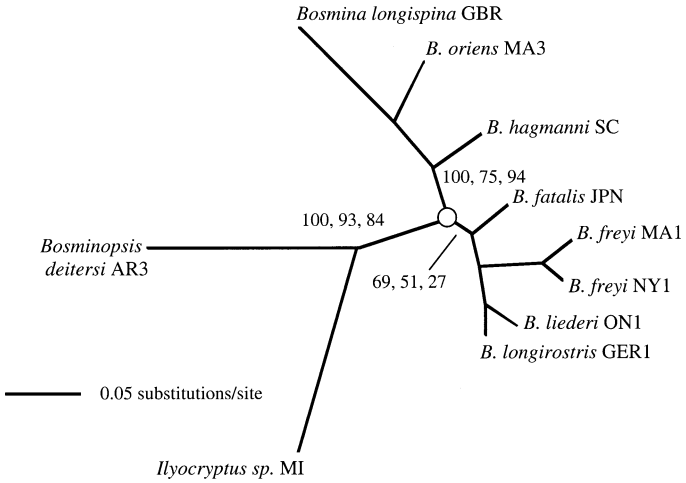

Fig. 2. Unrooted phylogram (drawn proportional to the amount of evolutionary change) showing the posterior probabilities of branches from Bayesian analysis of the 16S rDNA mitochondrial gene under differing models of DNA evolution. The tree is from the GTR model and the probability values are from the following models: GTR, GTR + invariable sites, GTR + invariable sites + gamma. The circle indicates the rooting position of the outgroups *Bosminopsis* and *Ilyocryptus* to representative ingroup taxa. Specimen location symbols are detailed in Web Appendix 1: http://www.aslo.org/lo/toc/vol_47/issue_5/1486a1.pdf.

are consistent with authenticity of bosminid crustacean PCR products and the lack of a systemic contaminant or artifact.

Hierarchical model fitting indicated that the transversion model with invariable sites and the gamma estimate of among-site rate variation (TVM + I + G) had the best fit to the mtDNA data, whereas the Hasegawa-Kishino-Yano (HKY) + G model had the best fit for the nuclear rDNA. The TVM has four base frequency parameters, four transversion substitution parameters, and one transition parameter. The HKY model has a transition–transversion ratio parameter and four base frequency parameters.

Rooting strategy—The use of *Bosminopsis* and *Ilyocryptus* as outgroups was problematic. For the nrDNA, *Bosminopsis* yielded a fragment about 60 bp larger than *Bosmina.* This outgroup proved to be so different in sequence that alignment was unreliable with the ingroup. For the mtDNA, support for the placement of the outgroup was highly model-dependent. More specifically, the addition of the gamma parameter to the model eroded the resolution of the root position and placed it weakly on the long branch leading to *B. freyi.* So the addition of the gamma parameter to the model swaps the positions of *B. freyi* and *B. fatalis* from those shown in Fig. 2. Without the gamma parameter, there is strong to moderate support for the root placement in Fig. 2 for both simple and complex substitution models (general time-reversible [GTR] and Jules Cantor [JC]) and the addition of invariable sites parameters does not change the root position. The rooting position in Fig. 2 was also found by ML, maximum parsimony (MP), and distance with minimum evolution (ME) optimality criteria. Finally, this rooting position and the position of *B. freyi* and *B. fatalis* were also

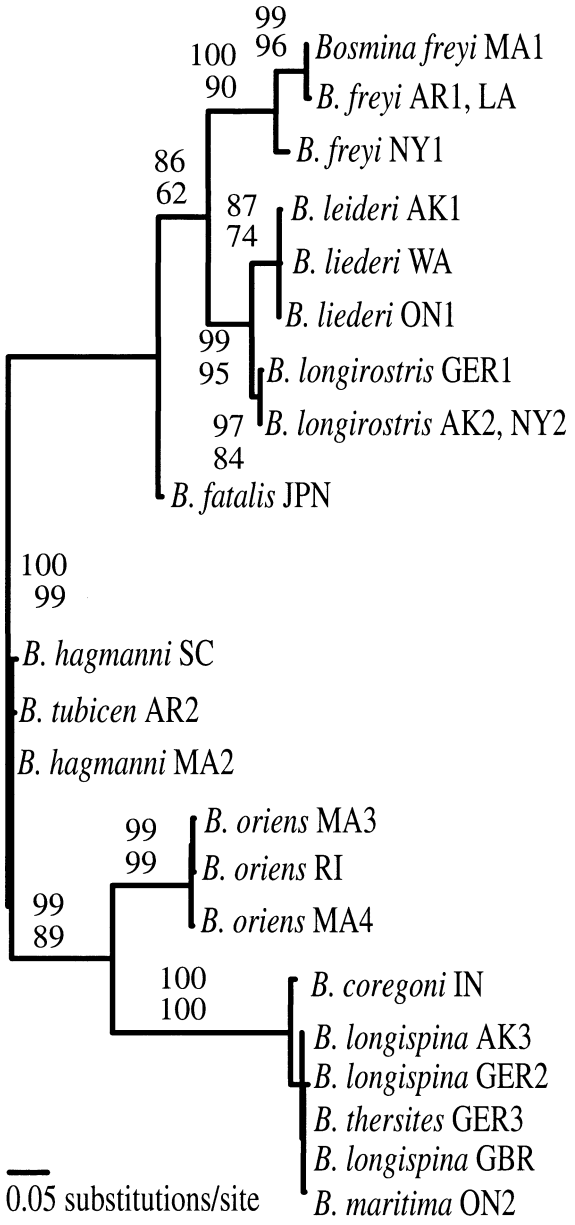

Fig. 3. Maximum likelihood phylogram based on sequence analysis of the 16S rDNA mitochondrial gene and rooted in the position indicated by outgroup analysis. Upper numbers on the branches indicate posterior probability of the branch being correct; lower numbers indicate maximum likelihood bootstrap support (250 pseudoreplicates). Specimen location symbols are detailed in Web Appendix 1.

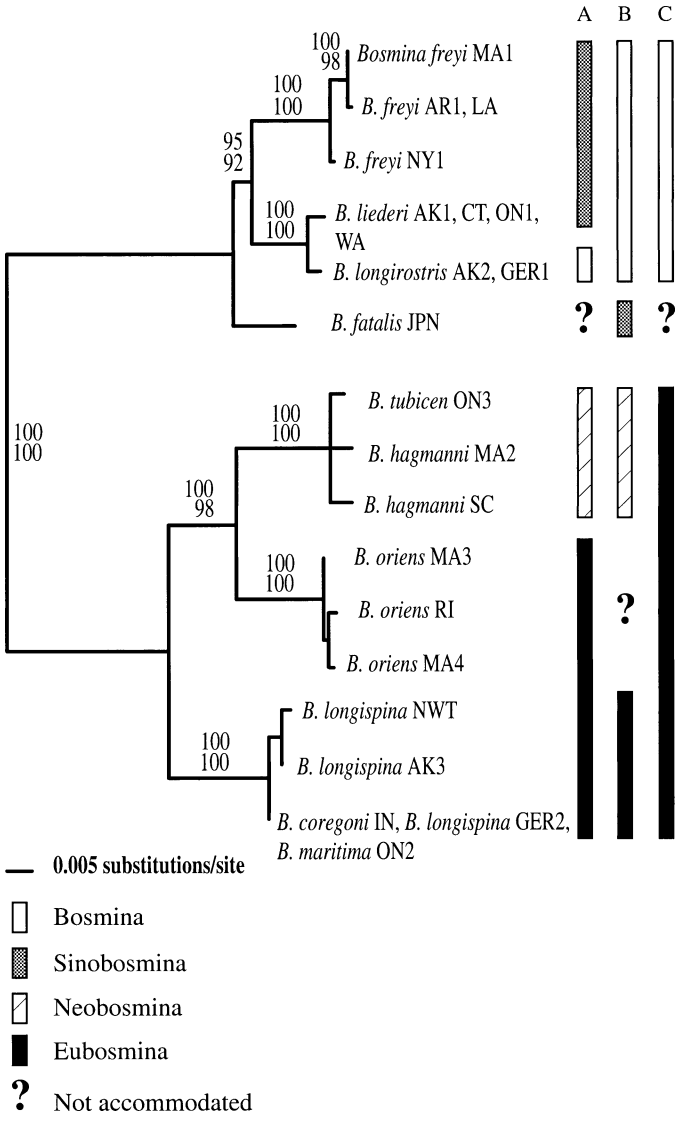

Fig. 4. Maximum likelihood phylogram based on sequence analysis of the partial 18S, ITS-1, 5.8S, ITS-2, and partial 28S nuclear rDNA genes and rooted in the position indicated by outgroup analysis (also the position indicated by midpoint rooting). Upper numbers on the branches indicate posterior probability of the branch being correct; lower numbers indicate maximum likelihood bootstrap support (1,000 pseudoreplicates). Taxa with identical sequences from different geographic locations are denoted with multiple location symbols after the taxon name. Specimen location symbols are detailed in Web Appendix 1. Shaded boxes represent coding of these species by the morphological schemes in Table 1. (A) De Melo and Hebert (1994*b*); (B) Lieder (1983); (C) Deevey and Deevey (1971).

found with midpoint rooting of the nuclear rDNA and is most consistent with the morphological characters (*see below*). Because this pattern suggests that accommodating a distantly related outgroup and ingroup with a single model is creating a topological artifact, we estimated model parameters and mtDNA trees with ingroup taxa only.

Phylogenetic results and support—Heuristic search with the 16S rDNA data found 21 haplotypes and a single ML tree with a score of $-\ln L = 1{,}780.69$ (Fig. 3). The nrDNA analysis revealed 15 different ingroup genotypes and a single ML tree of score $-\ln L = 3{,}045.56$ (Fig. 4.). In each data set, several individuals possessed identical sequences despite being separated by vast geographic distances (e.g., *B. longirostris* and *B. liederi*). With the exception of *B. oriens*, the mitochondrial (Fig. 3) and the nuclear ML (Fig. 4A–C) trees agreed in topology and were well supported by bootstrap and by posterior probability values. *B. oriens* grouped with

Lieder's subgenus *Eubosmina* for the mtDNA but with Lieder's subgenus *Neobosmina* with the nuclear DNA (Figs. 3, 4B). In general, the nrDNA tree had stronger branch support than the mtDNA tree, and the Bayesian posterior probabilities were slightly higher than the nonparametric ML bootstrap values. With the exception of the (*B. freyi, B. liederi, B. longirostris*) clade, which had a posterior probability of 95, all branches had a value of 100.

A strong genetic dichotomy is evident in both data sets that agrees with the lateral head pore morphology and, with the exception of *B. fatalis,* agrees with Deevey and Deevey's generic designations of *Eubosmina* and *Bosmina* (Fig. 4C). These two groups are separated by an average ML distance of 13.6% with the nrDNA and 44.2% with the mtDNA. Within each putative genus, the average ML distance is 3.6–5.6% (nrDNA) and 20–33.4% (mtDNA). In addition to the major dichotomy, there are at least five major groups apparent in each genetic data set. Four of these match Lieder's subgeneric groups (Fig. 4B), but there is strong support for the uniqueness of the Nearctic endemic *B. oriens* and of the Palearctic endemic *B. fatalis.*

The hypothesis of *Bosmina* (*Sinobosmina*) monophyly ($-\ln L = 3{,}069.16$) requires a significantly less likely tree than the observed tree (S-H test, $-\ln L = 3{,}045.56$, $P = 0.006$). The hypothesis of *Bosmina* (*Eubosmina*) monophyly (S-H test, $-\ln L = 3{,}054.19$, $P = 0.194$) was not rejected by the nuclear data. Neither the monophyly of the *Bosmina* (*Sinobosmina*) (S-H test, $-\ln L = 1{,}788.76$, $P = 0.098$) nor the placement of the *Bosmina* (*Eubosmina*) with the *Bosmina* (*Neobosmina*) by the nuclear data (S-H test, $-\ln L = 1{,}785.02$, $P = 0.123$) was rejected with the mitochondrial DNA data.

Discussion

The great advantage of morphological characters in the Bosminidae is that they are continuously preserved in the sediments, with some lakes having specimen records as much as tens of thousands of years old. Nevertheless, these characters will be less useful if they are not identical by descent. Our genetic results bode well for bosminid paleolimnology because there is complete concordance of the morphological changes with the genetic changes, and there is no case of a character state reversal (parallel or convergent changes). It is clear that the centuries of study and winnowing of morphological characters to minimize phenotypic plasticity by bosminologists has been fruitful. Although the true phylogeny of the Bosminidae is unknown, the concordance of independent molecular and morphological evidence suggests that a reasonably robust phylogenetic estimate is attainable.

Each of the recent major morphological schemes significantly advanced bosminid systematics at the continental level, but our study revealed that each is deficient at the Holarctic level (Fig. 4). North American schemes (Deevey and Deevey 1971; De Melo and Hebert 1994*b*) failed to accommodate the character combination of the Eurasian *Bosmina* (*Sinobosmina*), which has a *Bosmina* (*Eubosmina*) type of claw and a *Bosmina* (*Bosmina*) type of lateral head pore.

Table 2. Revised morphological taxonomic key to the genera and subgenera of the Bosminidae.

1a.	Accessory claw attached to base of postabdominal claw (Fig. 1C); no apparent lateral head pore; exopod of swimming antennae with three segments . . . . . *Bosminopsis*	
1b.	Accessory claw at base of postabdominal claw absent (Fig. 1D–F), lateral head pores apparent (Fig. 1G–I); exopod of swimming antennae with four segments . .	2
2a (1b).	Lateral head pore greater than nine pore diameters dorsal to the shell margin at the insertion site of the second antennae and dorsal to the fornix reticulation fork (Fig. 1G) . . . . . *Eubosmina*	3
2b.	Lateral head pore within five pore diameters dorsal to the shell margin at the insertion site of the second antennae and positioned within the fornix reticulation fork (Fig. 1H,I) . . . . . *Bosmina*	4
3a (2a).	Carapace spine (mucro) serrations on dorsal margin of spines in juvenile females (Fig. 5) . . . . . *Eubosmina* (*Neobosmina*)	
3b.	Carapace spine (mucro) serrations on ventral margin of spines or absent in juvenile females (Fig. 5)	5
4a (2a).	Postabdominal claw with fine hair-like spines (length > width) on distal pecten and evenly thick robust spines on proximal pecten (Fig. 1D); lateral head pore positioned in the dorsal half of the reticulation fork of the fornix (Fig. 1H) . . . . . *Bosmina* (*Sinobosmina*)	
4b.	Postabdominal claw with some sawtooth-like spines (length = width) on distal pecten and spines of distally increasing thickness and length on proximal pecten (Fig. 1E,F); lateral head pore positioned in the ventral half of the reticulation fork of the fornix (Fig. 1) . . . . . *Bosmina* (*Bosmina*)	
5a (3b).	Anal margin of male postabdomen beveled, forming obtuse angle with dorsal margin (Fig. 5); antennule has one evenly curved bow . . . *Eubosmina* (*Eubosmina*)	
5b.	Anal margin of male postabdomen truncated, forming acute or right angle with dorsal margin (Fig. 5); antennule has two evenly curved bows joined at central articulation . . . . . *Eubosmina* (*Lunobosmina*)	

The Eurasian scheme of Lieder (1983) failed to recognize the character combination of the North American specimens described by Deevey and Deevey (1971), which had the *Bosmina* (*Eubosmina*) head pore position, claw type, and mucro spine serration but the *Bosmina* (*Neobosmina*/*Bosmina*) type of male postabdomen. Higher level names are arbitrary, but ideally, a taxonomy should reflect monophyletic groups and preserve long-standing names in the literature. We present a revised Holarctic scheme and key (Table 2) below based on our genetic and morphological evidence that preserves long-standing names and reconciles the differences in the existing schemes.

Bosminopsis—The genus *Bosminopsis* is both genetically and morphologically very divergent from *Bosmina,* making its phylogenetic position in the water fleas uncertain. From our SEM analysis, we agree with Kotov (1997) that *Bosminopsis* lacks the lateral head pores that were once thought to be shared derived features of the bosminids. *Bosminopsis* does, however, share several derived features with at least

some members of the family Chydoridae: an accessory claw (Fig. 1C), position of anal opening, three-segmented exopod of the swimming antennae, and five thoracic limbs (six are found in other bosminids). Clearly, a higher level phylogenetic reassessment of water fleas is warranted to resolve the *Bosminopsis* position. It is quite possible that *Bosminopsis* represents a lineage of the mostly benthic and littoral chydorids that has colonized the open water and independently evolved a morphology similar to the pelagic bosminids.

Bosmina *and* Eubosmina *as genera*—Based on our evidence, we propose that a genus-level distinction between *Bosmina* and *Eubosmina* (Deevey and Deevey 1971) be resurrected. There is a deep phylogenetic dichotomy that largely agrees with the genus *Eubosmina* and the genus *Bosmina.* Indeed, for the nuclear genes, the divergence is two- to fourfold greater between *Bosmina* and *Eubosmina* than within each group. The among-group divergence is large, at 44% for the mtDNA, and this might be underestimated compared to the within-group divergence because of substitutional saturation. The major dichotomy warrants recognition, but we amend Deevey and Deevey (1971) to permit species with the *Eubosmina*-type claw (i.e., *Bosmina* (*S.*) *fatalis*) in the genus *Bosmina.* This means that the morphological synapomorphies for genera include the position of the lateral head pore but not the claw setation states.

We agree with those taxonomies that recognize the traditional four subgenera of bosminids. Nevertheless, we propose an additional fifth subgenus—our genetic evidence clearly indicates the existence of five divergent groups in multiple nuclear and mitochondrial genes, each possessing unique suites of diagnostic morphologies and very little within-group genetic variation. We comment on each subgenus below.

Bosmina (*Bosmina*)—Our genetic and morphological evidence rejects the monophyly hypothesis of the recent definitions of the subgenus *Sinobosmina* and the subgenus *Bosmina* by De Melo and Hebert (1994*b*) but are consistent with the monophyly of the scheme proposed by Lieder (1983). The data indicate that the *Bosmina* (*Bosmina*) type of claw evolved only once (Fig. 5) and that the scoring of *B.* (*S.*) *freyi* and *B.* (*S.*) *liederi* as having the subgenus *Sinobosmina* type of lateral head pore position by De Melo and Hebert (1994*b*) leads to a paraphyletic subgenus *Sinobosmina* (Fig. 4A). Although we disagree with the scoring, we agree that dividing the pore into the character states outlined by De Melo and Hebert (1994*b*) is phylogenetically useful. *B.* (*S.*) *freyi* and *B.* (*S.*) *liederi* have a pore position that is much closer to the carapace margin that does *B.* (*S.*) *fatalis* (Fig. 1H,I). Indeed, in the specimens that we have examined, the differences in pore position between *B.* (*S.*) *liederi, B.* (*S.*) *freyi,* and *B.* (*B.*) *longirostris* are extremely difficult to discern. Kořínek et al. (1997) also note that the lateral head pores of *B.* (*S.*) *liederi* and *B.* (*S.*) *freyi* are shifted more to the carapace margin than those of *B.* (*S.*) *fatalis.* Using our genetic information and Lieder's morphological definitions, we propose that there are no known North American members of the subgenus *Sinobosmina* and reassign *B.* (*S.*) *freyi* and *B.* (*S.*) *liederi* to the subgenus *Bosmina.* There are at least three members of the subgenus *Bosmina*: *Bosmina* (*Bosmina*) *freyi, Bosmina* (*Bosmina*) *liederi,* and *Bosmina* (*Bosmina*) *longirostris.* Our reassignment of the North American *Sinobosmina* is also consistent with the observed lack of allozyme divergence between *B.* (*B.*) *liederi* and *B.* (*B.*) *longirostris* (De Melo and Hebert 1994*a*). Also, the reassignment makes the character state of lateral head pore shape (Kořínek et al. 1997) a phylogenetically consistent character. *B.* (*B.*) *freyi, B.* (*B.*) *liederi,* and *B.* (*B.*) *longirostris* share an oval (often ventrally flattened) lateral head pore, whereas other species possess a more rounded lateral head pore (Fig. 1G–I). Finally, the reassignment means that the only genetically confirmed case of bosminid hybridization (Little et al. 1997) involves crossing within a subgenus rather than among subgenera. The near genetic identity of putative *Bosmina longirostris* populations from Alaska, New York, and Germany for the genes examined provides support for the existence of a Holarctic *B. longirostris.* Our results extend the geographic range of *B. liederi* markedly from its Great Lakes basin distribution (De Melo and Hebert 1994*b*) to the Bering Strait in Alaska. Given its presence in the heart of the Beringian refugium, it is very possible that *B. longirostris* populations from Asia will also belong to this spe-

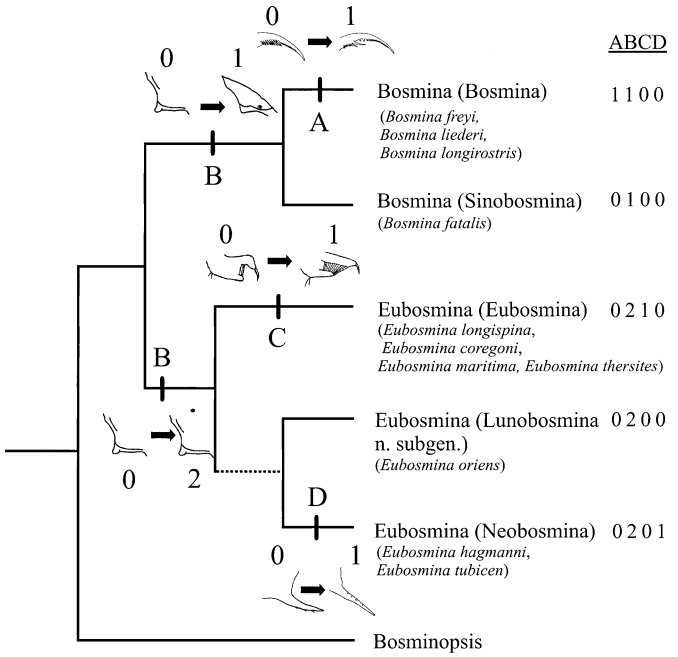

Fig. 5. Summary tree showing the estimated phylogenetic relationships of bosminid crustacean genera and subgenera based on nuclear (five genes of the rDNA array) and mitochondrial DNA sequences (16S rDNA) overlaid with morphological changes. The gray horizontal line represents a disagreement between the nuclear and mtDNA trees, with the total evidence ML tree result depicted. Letters indicate morphological characters (A) postabdominal claw setation, (B) lateral head pore location, (C) shape of anal margin on male postabdomen redrawn from Lieder (1983), and (D) posterior carapace spine (mucro) serration pattern redrawn from De Melo and Hebert (1994*b*). Numbers indicate the character state changes as described in Table 1. Genus and species designations are given below the subgeneric designations for species examined in this study.

cies. Until now, there has been no genetic evidence for the separation of *B. liederi* from *B. longirostris* (De Melo and Hebert 1994*a*). Our finding of reciprocal monophyly for nuclear and mtDNA genes, together with the widespread sympatric range, suggests that these are ''good'' species.

Bosmina (*Sinobosmina*)—Our data is largely consistent with the definition of the subgenus *Sinobosmina* by Lieder (1983). The sole species of this subgenus in our study is *Bosmina* (*Sinobosmina*) *fatalis.*

Eubosmina (*Eubosmina*)—Our evidence is in agreement with the morphological description of the subgenus *Eubosmina* by Lieder (1983). With the exception of *B.* (*E.*) *oriens* (*see below*), all of the subgenus *Eubosmina* species formed an exceptionally closely related group for both nuclear and mitochondrial genes. It appears that the exceptional carapace variation found in these groups occurred recently, perhaps postglacially. The lack of genetic variation does not mean that the subgenus *Eubosmina* is one polymorphic species—only that the taxa we sampled likely diverged very recently. Examination of more rapidly evolving gene regions will be necessary to address species boundaries within the subgenus *Eubosmina.*

Eubosmina *(*Lunobosmina *n. subgen.)—Bosmina* (*E.*) *oriens* fits into neither Lieder's morphological scheme nor a monophyletic subgenus *Eubosmina.* Kotov (1996) observed that the drawings of males by Deevey and Deevey (1971) do not represent males of European subgenus *Eubosmina.* That is, its males lack the characteristic beveled postabdomen, and possess the ancestral indented type. Moreover, the males possess unique double-arched antennules that are apparently observable in subfossils and found in no other subgenus. It is clear from the >100 μm mucro spines, bulging anterior rostrum, dystrophic habitat preference, and geographic distribution that Deevey and Deevey (1971) described *B.* (*E.*) *oriens* as *Eubosmina longispina.* Given the genetic, morphological, and geographic distinctness and the goals of maintaining long-standing names in the literature, the evidence presented here indicates that *B.* (*E.*) *oriens* belongs to a different subgenus than those described by Lieder (1983). We have assigned *B.* (*E.*) *oriens* to a new subgenus, *Eubosmina* (*Lunobosmina*) *oriens.* The name Luno refers to the swordlike extensions of mucros from *Eubosmina* (*Lunobosmina*) *oriens.* Baird named the genus *Bosmina* after Fingal's daughter in the poem by MacPherson. The Son of Luno is the name of the defensive sword used by Fingal.

Eubosmina (*Neobosmina*)—Whereas allozyme and prior morphological investigations have suggested that this group is artificial, our results support it as a genetically distinct subgenus. We retain the morphological definition of Lieder (1983). The relationships of its constituent species are more problematic. Southern hemisphere members of this group, such as *E.* (*N.*) *brehmi* and *E.* (*N.*) *meridionalis*, need to be studied before a complete picture of the relationships within the *Neobosmina* group can be clear. It is clear from the genetic data that the truncated antennules of *E.* (*N.*) *tubicen* are a polymorphic trait and that other morphological characters for this group should be sought.

Limitations of genetic data—In addition to the congruence of independent data sets, there are other reasons to rule out the common sources of phylogenetic error associated with the genes studied. We observed no discernible within-individual variation in any of the ingroup specimens. This would have been apparent as multiple peaks, or even in the loss of readability in the case of length variation. Indeed there is little, if any, within-species variation in the nuclear genes sequenced. We found no evidence of nuclear pseudogenes of the mitochondrial gene; that is, we found no multiple peaks, multiple PCR bands, or noncompensatory mutations in the conserved secondary structure. The authenticity of the sequences is verified by their closest matching sequences after a BLAST analysis. Finally, there are some areas of the alignment that are length variable and might contribute to error. Nevertheless, the exclusion of the gapped regions gives the same topology as reported. These results provide another case of the ITS regions being a useful phylogenetic marker in invertebrates despite the existence of potential sources of error. Such studies are still uncommon in animals compared to plants and fungi (Coleman and Vacquier 2002) but are important because the ITS gene region is one of the few variable nuclear regions that can easily be isolated by PCR for most eukaryotic groups.

An advantage of using a nuclear and a mitochondrial gene region in the present study is that these genes appear complementary with respect to signal. The 16S mtDNA tree contains more within-species information or haplotypes, whereas the nuclear ITS gene region contains more phylogenetic signal for the deeper branches. In addition to differing mutation rates, these complementary signals might be due in part to the increased rate of transitional saturation rate and the smaller effective population size of mtDNA versus nuclear DNA. Based on our present results, we predict that mtDNA genes will be highly informative in biogeographic studies of bosminids and that the ITS gene region will be most informative for studies that involve comparisons above the species level.

Although the genes that we used provide excellent resolution for *Bosmina* and *Eubosmina,* their analysis creates difficulty in accommodating distantly related outgroups. Our results show that *Bosminopsis* and *Ilyocryptus* are distantly related to *Bosmina* and *Eubosmina.* This level of divergence can lead to alignment error, long-branch attraction bias, and incorrect model estimation. Indeed, we did observe that *B. fatalis* moved to a position that is inconsistent with morphological and nuclear rDNA evidence in the mtDNA tree when *Bosminopsis* was used as an outgroup. *Bosminopsis* attached to the long branch leading to *B. freyi,* and unexpectedly, the optimal model with a gamma parameter was the most sensitive to this potential bias. We speculate that the increased branch length imparted by the gamma parameter created a long branch attraction scenario. Tarrio et al. (2000) reported a very similar situation with the *Xdh* gene of drosophilids but proposed that the gamma parameter and base composition differences were interacting to create the bias. The bosminid and drosophilid studies represent rare cases where

trees with uneven branch lengths are apparently more correctly recovered by simple models (and MP) than by ML with complex models. Covariotide models that accommodate parameter change between outgroup and ingroup might alleviate this bias (Galtier 2001).

Our results provide the first robust molecular phylogeny for bosminid crustaceans. They are inconsistent with the three commonly used morphological schemes, but with modest reassignments and changes in character state coding, a morphological scheme can be reconciled with the genetic data. We propose a revision for bosminids that recognizes three genera and five subgenera. One new subgenus is proposed. We identify morphological and genetic characters for the major groups of bosminids and an evolutionary framework for paleogenetic, paleolimnological, and ecological studies.

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