

Testing paleolimnological predictions with molecular data: the origins of Holarctic *Eubosmina*

R. A. HANEY*† & D. J. TAYLOR*

*Department of Biological Sciences, University at Buffalo, Buffalo, NY, USA

Keywords:

Bosminidae;
Eubosmina;
glaciation;
Paleolimnology;
phylogeography;
speciation;
vicariance.

Abstract

Zooplankton of the family Bosminidae have a unique paleolimnological record in many Holarctic lakes that provides a near continuous record of morphological change for thousands of years. If this morphological change could be interpreted reliably, then a rarely achieved direct observation of macroevolution would be feasible. We tested paleolimnological predictions derived from morphological variation found in the genus *Eubosmina* using mtDNA and nuclear DNA sequence variation from geographically distant Holarctic sites. The mtDNA and nDNA trees were congruent but genetic divergence was inversely associated with morphological divergence. The three most genetically divergent groups belonged to *Eubosmina longispina*, whose phylogeography and genetic divergence was consistent with glacial vicariance. The genetic evidence also supported the hypothesis that at least two Nearctic species were recent European introductions. Finally, the genetic evidence was consistent with paleolimnology in the finding of several proposed species undergoing rapid morphological evolution and being post-glacially derived from European *E. longispina*. The results suggested that lacustrine bosminids are susceptible to geographic speciation processes, and that morphological interpretation of diversity in paleolimnology can be markedly improved by genetic studies.

Introduction

G. E. Hutchinson likened evolution to a play and ecology to a stage, but because of the epic length of the evolutionary play, short-lived humans are normally prevented from witnessing more than a single scene. Even in the rare cases where fossils exist for a group of organisms, the large time intervals among preserved specimens again usually limit study to a single scene of the evolutionary play. It is fitting, then, that Hutchinson's student, Deevey (1942), pioneered a unique and potentially valuable system where the transitions of animal macroevolution could be directly observed with high-resolution: bosminid paleolimnology. Bosminids are small (approximately 0.5 mm), elephant-shaped,

filter-feeding crustaceans that comprise a large portion of the zooplankton in Holarctic lakes. Their body parts and embryos are continuously preserved in layered, dateable lake sediments, enabling historical reconstruction (Hann, 1989; Finney *et al.*, 2000). Bosminid carapaces and head parts are usually the most common animal microfossils in lake sediments, with continuous records in many lakes back to the Late Pleistocene and early Holocene (Frey, 1962; Hofmann, 1984, 1991). Related water fleas, such as daphniids, also possess a paleolimnological record, but compared with bosminids, their carapaces and head parts are relatively thin and almost always absent in sediments older than a few hundred years (Frey, 1991). If bosminid paleolimnology could be reliably interpreted, then the promise of valuable cases with continuous records of biogeographic and macroevolutionary processes over millennia may be realized.

A common macroevolutionary theme is that bosminid species have been relatively unaffected by geographic dispersal barriers and arose largely via sympatric processes.

Correspondence: Derek J. Taylor, Department of Biological Sciences, University at Buffalo, Buffalo, NY 14260, USA.

Tel.: 716 645 6953;

e-mail: djtaylor@acsu.buffalo.edu

†Present address: Department of Ecology and Evolutionary Biology, Brown University, Providence, RI 02912, USA

Little *et al.* (1997), for example, provided evidence for sympatric speciation via allopolyploid speciation in Arctic bosminids. Kerfoot (1975) provided evidence for a sympatric reproductive isolating mechanism based on disruptive selection related to differential predation on littoral and limnetic forms of *Bosmina*. Likewise, De Melo & Hebert (1994a) suggested a role for disruptive selection between sympatric habitats to explain genotypic patterns in several bosminids. Geographic speciation has been thought to be less important because several species appear to have distributions spanning multiple continents with little apparent geographic structuring in morphology (Lieder, 1983b; De Melo & Hebert, 1994a, c). Also, aspects of bosminid life history, such as desiccation-resistant resting eggs and a cyclic parthenogenetic breeding system, should enhance migration and reduce the influence of geographic dispersal barriers. Holarctic bosminids as lacustrine taxa have been further subject to selection for strong dispersal and large range sizes because their habitats have been repeatedly eliminated by the waves of Pleistocene glaciation (Dynesius & Jansson, 2000).

Sympatric mechanisms have also been proposed to explain the apparent rapid evolution of the Holarctic subgenus *Eubosmina* (*Eubosmina*). Only one species, *Eubosmina longispina* (Leydig) has been reported from lacustrine sediments of the last interglacial and glacial periods (Frey, 1962; Hann & Karrow, 1984, 1987). After glacial retreat, *E. longispina* presumably colonized most of the newly formed glacial lakes because, in every documented case, it has been the earliest species detected in post-glacial lake sediments (Gunther & Lieder, 1993). Today *E. longispina* is distributed throughout the Holarctic, with a known gap only in the central Precambrian Shield lakes of the Nearctic (Keller & Pitbaldo, 1989). The post-glacial sediments from about 8000 years ago showed the first records of additional *Eubosmina* species. Indeed, the sediments and recent records provided evidence for the existence of at least three other morphologically distinct species, *Eubosmina coregoni* (Baird), *Eubosmina longicornis* (Schoedler), and *Eubosmina crassicornis* (Lilljeborg). These species and over a dozen subspecies were restricted until recently to Europe (Lieder, 1983a, b). How did the remarkable European diversity arise? Lieder (1983a) reasoned two plausible scenarios: (i) *in situ* rapid radiation from *E. longispina*, which is the scenario predicted from available paleolimnological evidence (Fig 1.) and (ii) Colonization by two or more preglacial species that formed several stabilized hybrid lineages in European glacial lakes. There is no paleolimnological evidence for the existence of more than one preglacial *Eubosmina* species, but some lake sediments show an abrupt post-glacial transition in morphology that has been interpreted as the replacement of *E. longispina* by immigrating species (Nauwerck, 1991; Gunther & Lieder, 1993; Lieder & Gunther, 1995). Lieder (1983a) and Hofmann (1984, 1991) also provided paleolimnological evidence for

post-glacial hybridization as morphological transitions among taxa in their sediment cores involved intermediates. For North American lakes, the replacement process of *E. longispina* with *E. coregoni* is proposed to have been very recent (1960s) and mediated by human shipping traffic in the Great Lakes (Lieder, 1991; De Melo & Hebert, 1994b). Initial population genetic studies (De Melo & Hebert, 1994b, c) revealed that at least two European forms, *Eubosmina maritima* (Muller) and *E. coregoni*, were distinct from the eastern North American *E. longispina*, but phylogeographic resolution awaits DNA sequence-based analysis.

The aim of this study is to examine the following questions in northern lacustrine *Eubosmina*: First, does the genetic evidence support the paleolimnological prediction that non *longispina* taxa arose via rapid morphological evolution from a *E. longispina*-like ancestor (Fig. 1)? Secondly, does the Holarctic species, *E. longispina*, possess shallow gene trees with sympatric lineages as the morphological and paleolimnological records suggest? If glacial vicariance has contributed to genetic divergence, then a specific spatio-temporal signature should be evident. Geographic isolates should be concordant with the positions of previously identified refugia, or with dispersal out of these refugia, and the timing of their segregation from putative sister taxa should be consistent with glacial events. Finally, is there phylogeographic evidence for the recent immigration of North American *E. maritima* and *E. coregoni*, which are undetected in the subfossils and proposed to be recent invaders of the continent?

Materials and methods

Sample collection

Bosminids were examined from 33 ponds and lakes in North America and Europe (Figs 2 and 3). Table 1 gives location and species assignment for each of the 46 specimens. Six of these samples formed a part of an earlier study on genus and subgenus level systematics (Taylor *et al.*, 2002) and the Lake Ontario 16S rDNA sequence was from Cristescu & Hebert (2002). We obtained representatives of all five of the named *Eubosmina* species (Lieder, 1983b; De Melo & Hebert, 1994c; Taylor *et al.*, 2002). Most of these species are unmistakable from morphology, but North American *E. maritima* is an exception. The oblique mucro spines thought to be diagnostic of this species appear to be common in North American *E. longispina*. Hence, *E. maritima* was sampled from the same population as the original description from North America (De Melo & Hebert, 1994c). The species *E. longispina* appears relatively invariant in morphology on a Holarctic scale, and hence we sampled as broadly as could be achieved across its range. The geographic centers of proposed freshwater refugia (Beringian and Atlantic) were a particular focus for collection.

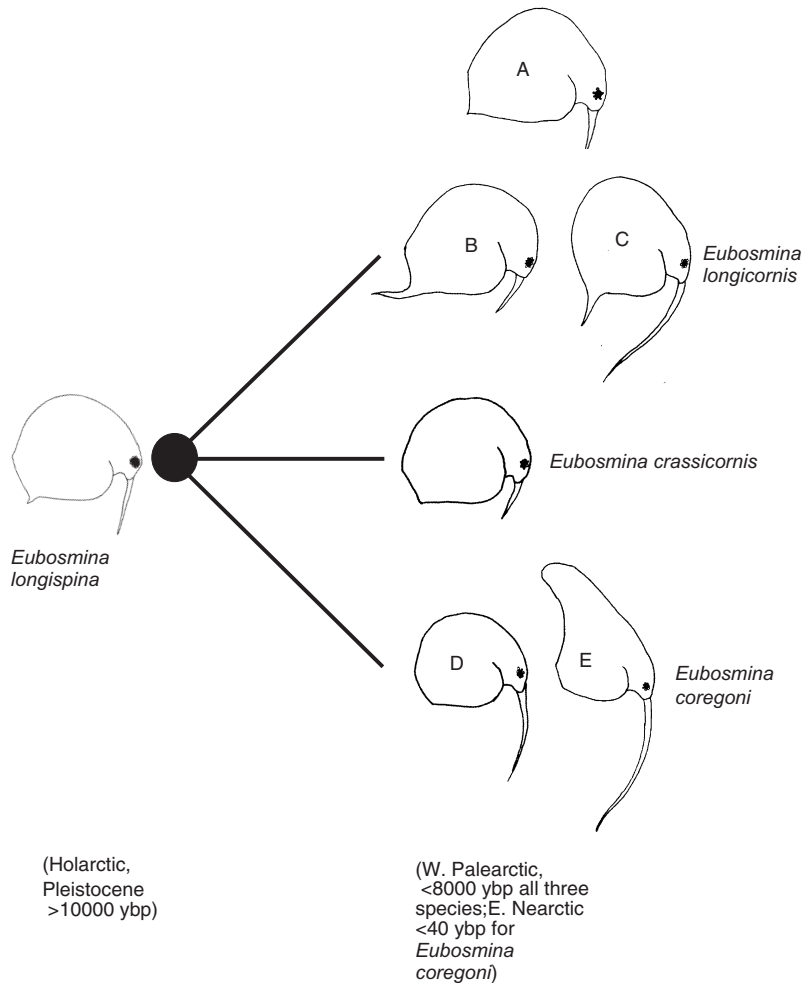


Fig. 1 One evolutionary hypothesis for the origins of diversity in Holarctic *Eubosmina* based on paleolimnological, morphological, and current distributional evidence of the taxa examined in this study. Drawings are outlines of the lateral view of mature females. *Eubosmina longispina* was drawn from Ullswater, England. The subspecies are lettered as (A) *Eubosmina longicornis kessleri* (redrawn from Lieder, 1991), (B) *E. longicornis reflexa* (redrawn from Lieder, 1983b), (C) *E. longicornis longicornis* (redrawn from Lieder, 1983b), (D) *E. coregoni coregoni* (redrawn from Lieder, 1983b), and (E) *E. coregoni thersites* (redrawn from Lieder, 1983b).

DNA extraction, amplification and sequencing

Total DNA of single individuals was extracted using variations on a cetyltrimethylammonium bromide (CTAB) protocol, with either chloroform/isoamyl alcohol being used alone or in conjunction with a second step involving phenol/chloroform/isoamyl alcohol. Precipitation was performed with isopropanol or 95% ethanol. Polymerase chain reactions (PCR) were performed in 25 or 50 μ L reactions, using 2–5 μ L of template, in one of two protocols. The first included 1.5 mM MgCl₂ buffer, 10 μ M primers, 10 mM dNTP's and 1 unit of *Taq* polymerase. The second included 2 mM MgCl₂ buffer, 10 μ M primers, 10 mM dNTP's, and 1 unit of Faststart *Taq* DNA polymerase (Roche, Mannheim, Germany). The PCR conditions for the two protocols were, respectively: (i) 94 °C for 1 min, 48–50 °C for 1 min, and 72 °C for 1 min and 30 s for 40 cycles, followed by one cycle of 72 °C for 7 min; (ii) 95 °C for 4–6 min for one cycle, followed by forty cycles of 95 °C for 1 min, 48–50 °C for 1 min and 72 °C for 1 min and 30 s, followed by 7–8 min at 72 °C for one cycle. Primers used to amplify and sequence both strands of a

fragment of the 16S gene were: 16Sar (5'-CGCCTGT-TTATCAAAAACATC-3') and 16Sbr (5'-CCGGTCTGAA-CTCAGATCACGT-3'). An additional *Bosmina* specific primer (16SarBosmina: 5'-CGCCTGTTTATCAAAAACAT-CGC-3') replaced 16Sar for some specimens. The fragment of the mitochondrial large ribosomal subunit sequenced corresponds to positions 12658–13132 in the *Daphnia pulex* complete mitochondrial genome (Crease, 1999). Four primers were utilized to amplify and sequence both strands of the nuclear ITS, 5.8S and D1 28S rDNA regions: 18SD, 28SD2R, 5.8SF and 5.8SR (Taylor *et al.*, 2002). Primer-free PCR products were generated by gel purification. Both strands were sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit and an ABI 377 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA).

DNA sequence alignment and analysis

Electrophoregrams were examined using Sequencher version 4.0.1 (Gene Codes Corporation, Ann Arbor, MI, USA) and consensus sequences were assembled.

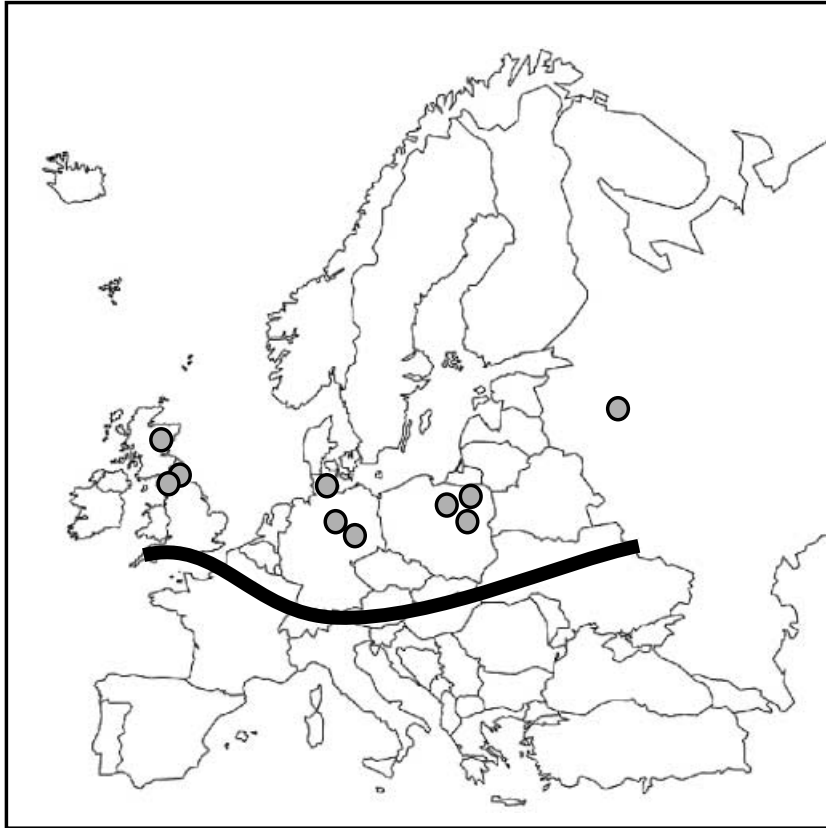


Fig. 2 European collection locations for phylogenetic analysis (see Table 1 for details). Gray circles represent lakes examined in the current study. The transcontinental line represents the southern limit of the *Eubosmina longispina* complex (from Lieder, 1983a).

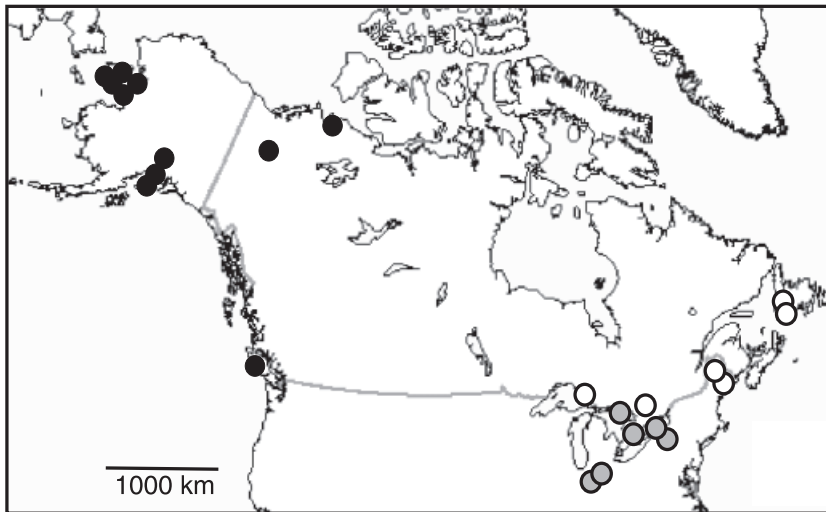


Fig. 3 North American collection sites for phylogenetic analysis (See Table 1 for details). The black circles represent western Nearctic sites, the white circles represent eastern Nearctic sites, and the gray circles represent proposed Palearctic invaders of Nearctic.

Consensus sequences were aligned in ClustalX (Thompson *et al.*, 1997). Sequences were deposited in Genbank under accession numbers AY264717–AY264773.

Pairwise sequence divergence estimates and average distances among groups based on the Kimura

two-parameter distance were computed using MEGA v2.0 (Kumar *et al.*, 2001). MODELTEST v.3.06 (Posada & Crandall, 1998) was used to discover the best-fit model of nucleotide substitution for each dataset by hierarchical likelihood-ratio test and maximum-likelihood trees were created in PAUP 4.0 (Swofford,

Table 1 Location of samples and species assignment.

Location	Species	Latitude	Longitude
Kuhn Lake, IN, USA	<i>E. coregoni coregoni</i>	N 41°10.2'	W 84°25.8'
Center Lake, IN, USA	<i>E. coregoni coregoni</i>	N 41°14.8'	W 85°51.4'
Guelph Lake, Ontario, Canada	<i>E. coregoni coregoni</i>	N 43°36.0'	W 80°15.0'
Lake Ontario, Ontario, Canada	<i>E. coregoni coregoni</i>	GenBank Accession AY075079	
Muggelsee, Germany	<i>E. coregoni thersites</i>	N 52°26.0'	E 13°39.0'
Lake Ros, Poland	<i>E. coregoni thersites</i>	N 53°40.4'	E 21°54.7'
Lake Dargin, Poland	<i>E. crassicornis</i>	N 54°08.0'	E 21°44.0'
Lake Glubokoe, Russia	<i>E. longicornis kessleri</i>	N 55°45.0'	E 36°30.0'
Deep Lake, NY, USA	<i>E. longicornis kessleri</i>	N 43°01.3'	W 77°34.3'
Selenter See, Germany	<i>E. longicornis longicornis</i>	N 54°18.5'	E 10°27.5'
Lake Hancza, Poland	<i>E. longicornis reflexa</i>	N 54°14.6'	E 22°48.0'
Mirror Lake, AK, USA	<i>E. longispina</i>	N 61°25.7'	W 149°24.9'
Jerome Lake, AK, USA	<i>E. longispina</i>	N 60°32.7'	W 149°34.3'
Pond 19, Teller, AK, USA	<i>E. longispina</i>	N 65°00.2'	W 166°09.9'
Paulatuk, Northwest Territories, Canada	<i>E. longispina</i>	N 69°00.0'	W 127°00.0'
Summit Lake, AK, USA	<i>E. longispina</i>	N 60°38.2'	W 149°30.4'
Pond 8 Nome, AK, USA	<i>E. longispina</i>	N 64°33.6'	W 165°28.3'
Pond 4, Nome, AK, USA	<i>E. longispina</i>	N 64°33.6'	W 165°28.3'
Pond 7, Taylor, AK, USA	<i>E. longispina</i>	N 65°14.0'	W 164°49.4'
Pond 9, Taylor, AK, USA	<i>E. longispina</i>	N 65°10.5'	W 164°49.6'
Sherbourne Lake, Ontario, Canada	<i>E. longispina</i>	N 45°11.8'	W 79°43.2'
Mijiniungshing Lake, Ontario, Canada	<i>E. longispina</i>	N 47°42.0'	W 84°42.0'
Ullswater, UK	<i>E. longispina</i>	N 54°34.0'	W 02°52.0'
Okersee, Germany	<i>E. longispina</i>	N 51°52.0'	E 10°29.0'
Derwent Water, UK	<i>E. longispina</i>	N 54°35.5'	E 03°08.5'
Fox Lake, Yukon Territory, Canada	<i>E. longispina</i>	N 61°10.7'	W 135°23.4'
Clatto Country Park, Dundee, UK	<i>E. longispina</i>	N 56°29.9'	W 03°01.7'
Unnamed Pond, Cornerbrook, Newfoundland	<i>E. longispina</i>	N 48°53.0'	W 58°14.5'
Unnamed Pond, Cornerbrook, Newfoundland	<i>E. longispina</i>	N 48°49.0'	E 57°59.0'
Phillips Lake, Maine, USA	<i>E. longispina</i>	N 44°41.9'	W 68°36.1'
Green Lake, Maine, USA	<i>E. longispina</i>	N 44°40.3'	W 68°33.1'
Croman Lake, British Columbia, Canada	<i>E. longispina</i>	N 50°12.1'	W 126°27.1'
Georgian Bay, Ontario, Canada	<i>E. maritima</i>	N 45°00.0'	W 81°15.0'

2002) using the substitution model selected by MODELTEST. Bayesian reconstruction of phylogenies was performed using MrBayes 3.0b3 (Huelsenbeck *et al.*, 2001). One million generations were run with four Markov chains using the model of substitution selected by MODELTEST. The results were examined to determine the generation at which a stable value of the summed likelihoods of the trees in each chain was attained, trees prior to that generation were discarded, and a majority-rule consensus of the remaining trees was created in PAUP 4.0. For the analysis with combined genes, parameters were assigned across the data partitions in MrBayes. Nonparametric bootstrapping (1000 iterations for nrDNA and 100 iterations for mtDNA) was carried out using maximum likelihood with heuristic searches (with tree bisection reconnection branch swapping) as an additional test of reliability. To test the significance of differences in hypothesized tree topologies chosen *a posteriori*, the Shimodaira-Hasegawa (SH) test with 1000 bootstrap pseudoreplicates and RELL optimization was employed (Shimodaira & Hasegawa, 1999; Goldman *et al.*, 2000).

Results

Features of sequence data

Alignment of the 46 *Eubosmina* 16S rDNA sequences was unambiguous and required only three gaps for completion. Twenty-seven sites were variable and 17 were parsimony informative from a total of 479. Kimura two-parameter pairwise sequence divergences ranged from 0 to 0.029. The sequences had an average base composition of A = 32.1%; C = 20.8%; G = 13.4%; T = 33.7%; A + T = 65.8% and nucleotide composition did not vary significantly among sequences ($\chi^2 = 1.7898$; d.f. = 123; $P = 1.00$). The transition/transversion ratio was 2.69. In cases where multiple sequences were obtained from the same body of water, variation was for the most part nonexistent. Exceptions to this rule were Center Lake, IN, USA where two species were identified, *Bosmina liederi* and *E. coregoni*, Phillips Lake, ME, USA where the two haplotypes differed by a single transition, Lake Ros, Poland where the two sequences obtained differed by two substitutions, and Sherbourne

Lake, Ontario, Canada where three distinct haplotypes were identified. Sherbourne Lake 1 differs from the other two samples by three substitutions, whereas Sherbourne Lake 3 has a two-basepair deletion relative to the other two sequences.

Nuclear rDNA data was collected for a subset of representative samples from each species or intraspecific clade. There was no evidence of multiple PCR bands or of within-individual variation from the electropherograms. The alignment required two gaps for completion (both in the sequence from Ullswater, UK) and totaled 1170 characters. The alignment contained a total of 11 variable and six parsimony informative sites. Pairwise sequence divergence ranged from 0 to 0.006 based on a Kimura two-parameter distance. The average base composition for the 20 sequences was as follows: A = 29.5%; C = 22.8%; G = 25.7%; T = 22.0%. Nucleotide composition does not vary significantly among gene sequences ($\chi^2 = 0.2671$; d.f. = 60; $P = 1.00$). The transition/transversion ratio was 4.51.

As shown previously, these two markers constituted an excellent combination with respect to phylogenetic reconstruction because of the complementary nature of

their signal (Taylor *et al.*, 2002). Divergences were lower in the nuclear rDNA region than in the mitochondrial marker, and hence provided increased resolution at deeper levels of the phylogeny. Divergences among sequences were higher for mitochondrial 16S (Taylor *et al.*, 2002), and multiple transitions occurring at the same site may bias the transition/transversion ratio downward for 16S to a level below that of the nuclear sequence data, as was evident here.

Phylogenetic reconstruction

Figure 4 displays the 16S rDNA maximum-likelihood phylogram for all populations in the subgenus *Eubosmina* ($-\ln L = 879.185$) under a substitution model of HKY85 + G (Hasegawa *et al.*, 1985; Yang, 1994). This model assumes different substitution rates for transitions and transversions, unequal base frequencies and a gamma distribution of rate variation among sites. The Bayesian majority-rule consensus and maximum-likelihood phylogenies were identical. Figure 5 shows the maximum-likelihood topology for the 23 specimens for which nuclear rDNA data were collected under

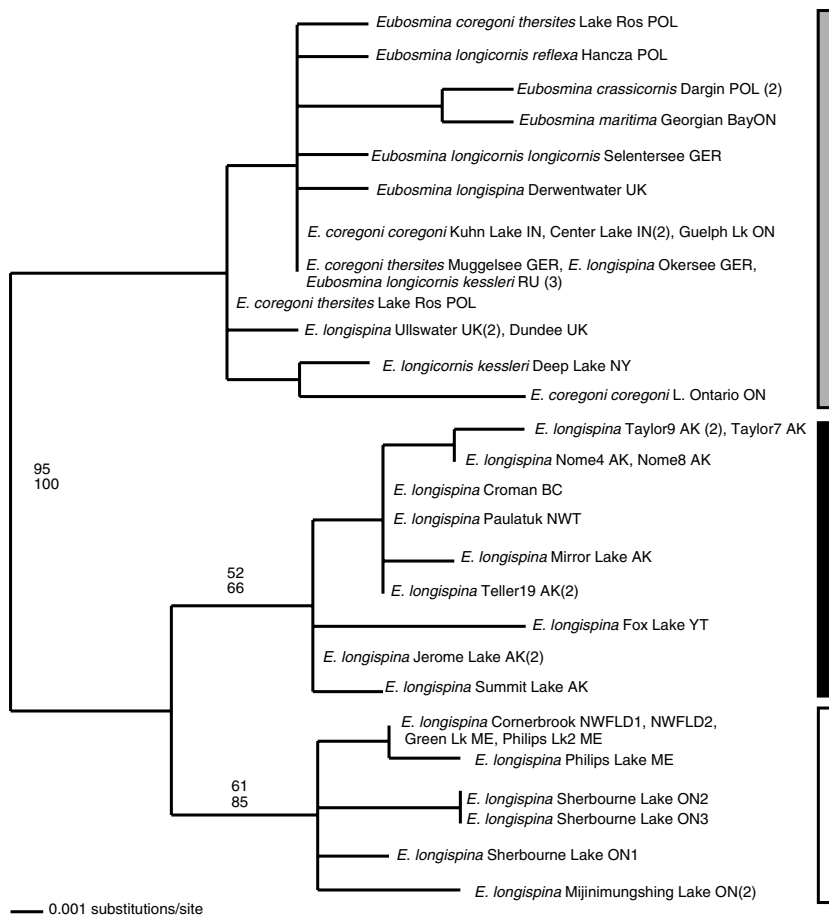
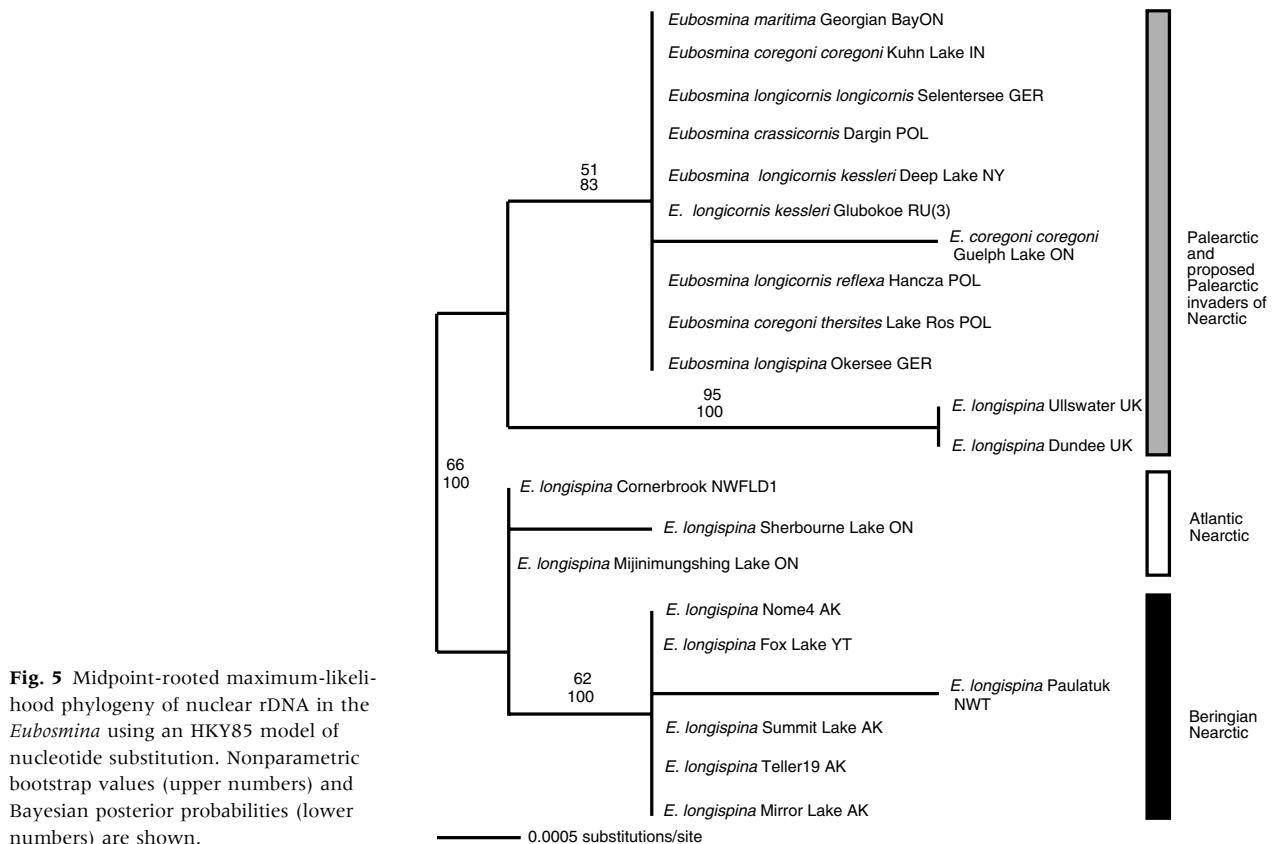


Fig. 4 Midpoint-rooted maximum-likelihood phylogeny of the mitochondrial 16S rDNA gene in 46 specimens of *Eubosmina* using an HKY85 model of nucleotide substitution with gamma distributed rate variation among sites. Nonparametric bootstrap values (upper numbers) and Bayesian posterior probabilities (lower numbers) are shown. The black bar represents western Nearctic sites, the white bar represents eastern Nearctic sites, and the gray bar represents Palearctic sites and proposed Palearctic invaders of Nearctic.



an HKY85 model of nucleotide substitution ($-\ln L = 1703.126$). Under this model, the transition/transversion ratio and base frequencies were estimated from the data. Again, the phylogeny derived from the Bayesian analysis was identical to that obtained using a maximum-likelihood approach. Figure 6 shows the combined 16S rDNA and nuclear rDNA tree obtained using Bayesian analysis. The combined tree was identical to the mtDNA tree in topology, but had stronger branch support.

Examination of Holarctic phylogeographic patterns

Individuals assigned by morphology to the Holarctic species *E. longispina* were affiliated with three genetically distinct geographic groups. Two of these groups appeared regionally restricted in distribution to eastern and western North America with the third containing primarily European populations of most members of the *Eubosmina* species flock, along with some recent invaders to the North American continent. The North American regional clades had weak reliability values for individual data sets but a strong support value from the combined analysis. The European group had strong support for its separation from North American clades in all analyses. This group was comprised of all European *E. longispina*, all non*longispina* taxa, and the proposed

European invaders from North America (*E. longicornis* and *E. maritima*). Pairwise averages of Kimura two-parameter distances were substantially lower among populations of multiple species within the European clade (0.4%) than within the North American *E. longispina* clade (0.8%).

Timing of phylogeographic divergences

As a prelude to the calculation of divergence dates, a maximum-likelihood 16S rDNA phylogeny was generated with a molecular clock enforced for *Eubosmina* ($-\ln L = 888.254$). The constrained topologies were not significantly different by likelihood-ratio test from unconstrained trees (d.f. = 40; $P > 0.5$), and we cannot reject clocklike behaviour in this sequence data.

As a calibration for a bosminid-specific molecular clock is lacking, we used an average value from available crustacean mitochondrial clock calibrations of 0.68% per million years (Cunningham *et al.*, 1992; Schubart *et al.*, 1998) to tentatively date these genetic fragmentations. The divergence between the North American Beringian and Atlantic clades was 1.1%, dating to 1.6 my bp, whereas that between the North American and European clades was 2%, for a date of 2.9 my bp. Although these estimates are a rather crude approximation, they give

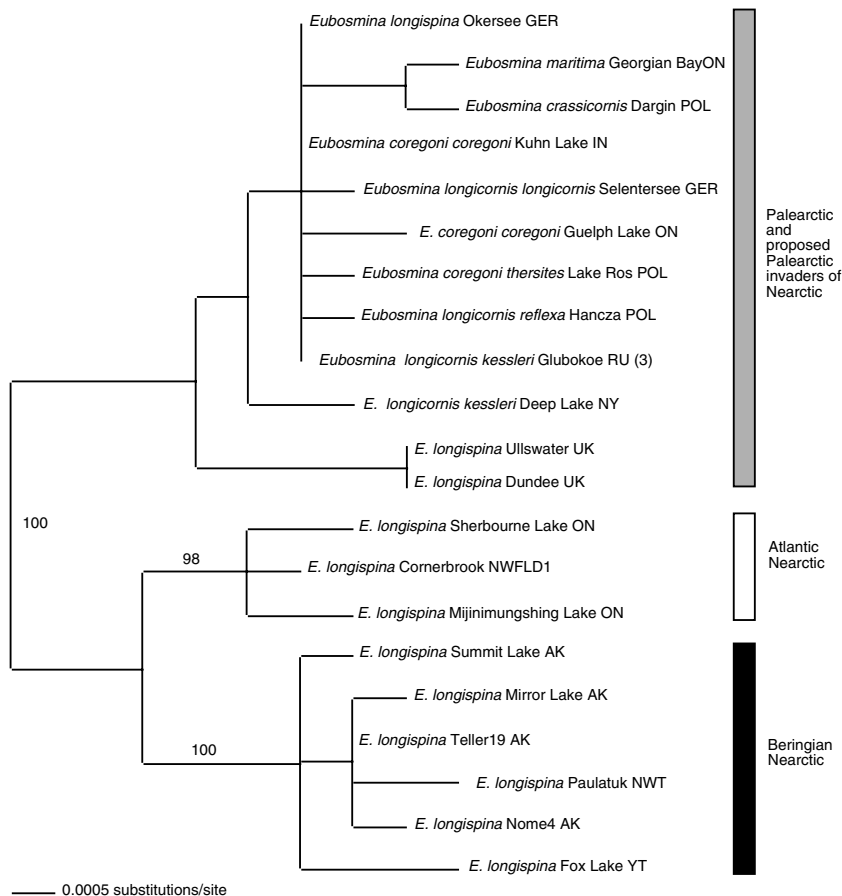


Fig. 6 Midpoint-rooted maximum-likelihood phylogeny of combined nuclear and mtDNA in the *Eubosmina* using a model with parameters partitioned across genes. Bayesian posterior probabilities (lower numbers) are shown.

some insight into the relative timing of divergence events within the Bosminidae.

Lack of morphological and genetic covariation in *Eubosmina*

Morphological and genetic variation in *Eubosmina* failed to correspond (Fig. 7). For example, individuals (Guelph Lake, Müggelsee, Okersee, Center Lake, Lake Glubokoe) with identical haplotypes, possessed the extremes of morphology in *Eubosmina* (*E. coregoni coregoni*, *E. coregoni thersites*, *E. longispina* and *E. longicornis kessleri*). In addition, *E. longispina* was clearly paraphyletic in both the mitochondrial and nuclear phylogenies, whereas the other *Eubosmina* taxa formed an unresolved clade with European *E. longispina*. We also observed that the oblique mucro spines thought to be diagnostic of *E. maritima* were common in North American *E. longispina*. Trees constraining the monophyly of *E. longispina* were significantly less likely than the maximum-likelihood topology as assessed by SH test ($-\ln L = 901.4$, Diff $-\ln L = 22.21$; $P = 0.013$), and monophyly of this species as currently defined can be rejected.

Discussion

The origins of Palearctic *Eubosmina*

The mtDNA, nuclear DNA, and paleolimnological evidence are consistent with the hypothesis that the European *Eubosmina* phylogroup is of post-glacial origin from a *E. longispina* – like ancestor (Fig. 7). The close genetic similarity and unresolved phylogenies of the European specimens are inconsistent with the existence of more than one preglacial species (where the expected pattern is a concordant reciprocal monophyly for the two genomes). We note that the near identity of European specimens does not necessarily indicate that European *Eubosmina* is comprised of just one polymorphic species. Cladoceran species can possess marked genetic differences when they are apparently identical at the conserved 16S rDNA gene (Taylor *et al.*, 1998). Most importantly, Hellsten & Sundberg (2000) have shown that co-existing *E. coregoni* and European *E. longispina* formed distinct genetic groups based on randomly amplified polymorphic DNA (RAPD) markers. Genetic cohesion under conditions of sympatry strongly suggests reproductive isolation. They also indicated that, in

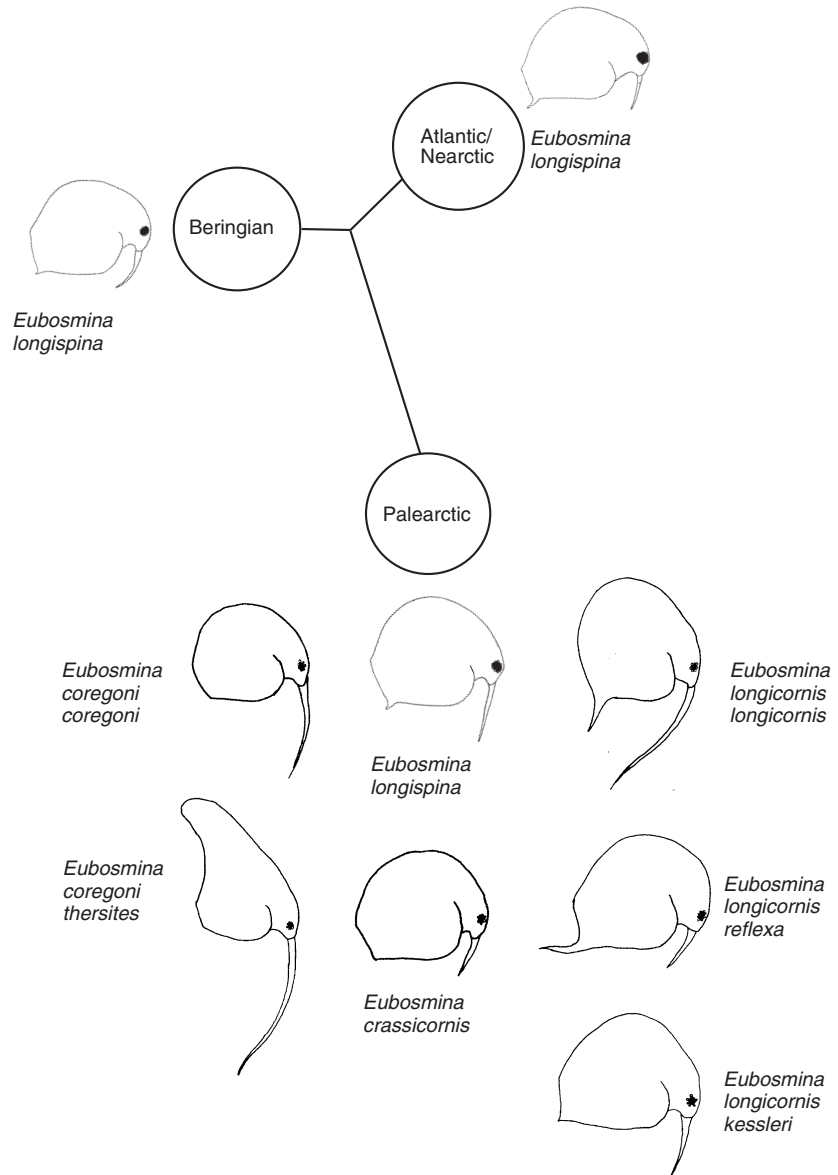


Fig. 7 Unrooted summary tree with divergences based on mtDNA sequence variation and the HKY + gamma distance for Holarctic *Eubosmina*. The evidence reveals the paraphyly of *Eubosmina longispina* and the close relationships of the Palearctic taxa. Drawings are outlines of the lateral view of mature females. *Eubosmina longispina* was drawn from Ullswater, England (Palearctic), Phillips Lake, Maine (Atlantic/Nearctic), and Summit Lake, Alaska (Beringian). The remaining females were redrawn Lieder (1983b, 1991).

culturing studies, *E. coregoni* and *E. longispina* maintained their distinct morphologies. More studies are needed using rapidly evolving genetic markers and culturing studies to address species boundaries for the remaining European taxa. The available evidence does suggest that there has been rapid morphological evolution in European *Eubosmina*, associated, in at least one case, with recent speciation.

With no evidence for multiple preglacial taxa, the problem of why so many forms of *Eubosmina* (*E.*) evolved in the western Palearctic, whereas a single form prevailed in the rest of the Holarctic, remains a mystery. We suggest that the dismissal of the old hypothesis of

variable predation regimes driving the morphological diversity of *Eubosmina* (Lieder, 1983a, b) is premature. Most of the morphological variation in *Eubosmina* involves modifications of the brood chamber, antennules, and posterior spines. There is now substantial evidence that these structures act as morphological defenses against predation (Kerfoot, 1978, 1987; Post *et al.*, 1995; Hellsten *et al.*, 1999; Chang & Hanazato, 2003). Invertebrate predators such as copepods, *Bythotrephes*, *Leptodora*, and *Cercopagis* can apparently profoundly reduce or eliminate bosminids that lack morphological defenses (Yan *et al.*, 2002) and are potentially strong selective agents for these structures. It is

plausible that *E. longispina* and various local Palearctic predators such as *Bythotrephes* colonized glacial lakes in Europe from different refugia resulting in rapid post-glacial evolution of defensive structures in *Eubosmina*. The tendency for lake districts to evolve as self-contained units (De Meester *et al.*, 2002) and to possess unique predation regimes may have permitted the origin and maintenance of multiple defensive solutions. Other cladoceran groups, such as *Daphnia*, seem to have undergone post-glacial radiations associated with analogous defensive structures and local invertebrate predators (e.g. *Daphnia longiremis* and *Heterocope*; Riessen & O'Brien, 1980). The role of invertebrate predation in driving bosminid radiations may be further tested by paleolimnology, because some invertebrate predator (*Bythotrephes* and *Leptodora*) and prey body parts are preserved in the sediments. Finally, part of the above evolutionary scenario is being replayed in the Nearctic as Palearctic predators such as *Bythotrephes* and *Cercopagis* have recently invaded Nearctic lakes with *E. longispina*.

Endemism and refugial inertia

Our mtDNA and nDNA phylogeographic evidence suggests an emerging genealogical concordance (Avice, 2000) that *E. longispina* was sundered into at least three refugial groups during the Pleistocene. First, there is a concordant pattern of three groups with strong to modest support in relatively conserved genes from the nuclear and mtDNA genomes. The among-genome concordance makes locus-specific selection an unlikely explanation for the pattern. Secondly, multiple co-distributed aquatic species, including fish, show a strikingly similar phylogeographic pattern (Bernatchez & Wilson, 1998; Cox & Hebert, 2001). Thirdly, there is independent geological evidence that glacier-free habitats existed in Beringia, the Atlantic coastal plain and Europe. Fourthly, there is a sizable distribution gap in Nearctic *E. longispina* in much of Ontario containing seemingly habitable lakes (Keller & Pitbaldo, 1989), perhaps indicating a region where Atlantic and Alaskan refugial clades have yet to colonize. Finally, although the estimates are crude, the timing of the divergences among the phylogroups is consistent with isolation during the Pleistocene. Although the absolute genetic distances are shallow, the divergence estimates for these genes are in the millions of years. These divergence times, the robust phylogroups, and the lack of an apparent association between geographic distance and genetic divergence within phylogroups suggests that recent (<10 000 YA) dispersal from a single refugium is an unlikely explanation for *E. longispina* phylogeography.

The results suggest that, contrary to theoretical and morphological predictions, northern lacustrine zooplankton are susceptible to gene flow barriers, and that glaciation could be the source of vicariance. Dynesius & Jansson (2000) proposed that glacial cycles reduced

diversity of northern lacustrine taxa through extinction or homogenization of glacial survivors shortly after deglaciation. Their assumption is that glacial cycles provided insufficient time for reproductive isolation. Nevertheless, water fleas have a unique biology that may implicate glaciation as a generator of diversity. One feature commonly unappreciated is that there are very few strictly lacustrine specialists among northern freshwater crustaceans. The dominant water flea taxa of many glacial lakes are common denizens of small ponds in the Arctic and in known refugia. Some taxa showing this pattern are *Daphnia longiremis*, *D. dentifera*, *D. pulicaria*, *D. dubia*, *D. umbra*, *Holopedium* sp., *B. longirostris*, and *E. longispina* (Riessen & O'Brien, 1980; Lieder, 1983b; Sars, 1993; Taylor & Hebert, 1994; Taylor *et al.*, 1998; Weider & Hobaek, 2003). The success of these taxa in ponds presumably aided in survival during glacial times when suitable lakes were certainly rare. Additional features that might buffer water fleas against glacial extinction are their resting egg bank and asexual phase of reproduction (Cox & Hebert, 2001). Also, homogenization of refugial populations after glaciation is unlikely in water fleas because they should possess pronounced refugial inertia. That is, successful colonization of an occupied water body is theoretically very difficult because of the strong priority effects from rapid lake-specific selection, massive egg banks and large existing populations (De Meester *et al.*, 2002). The effect can essentially extend the time of genetic isolation of refugia even in the face of dispersal. This refugial inertia could then provide more time for reproductive isolation to occur and thus promote species diversity. It is clear that after thousands of years of unglaciated conditions, the proposed refugial groups of *Eubosmina* remain distinct. Where they have met as a result of human introductions, there is no indication of interbreeding. That is, North American *E. maritima* and *E. longicornis kessleri* are proposed to be strictly of hybrid origin involving introduced European species (Lieder, 1983a; De Melo & Hebert, 1994a, b). However, we observed no heterozygosity at nrDNA, or discordance in mtDNA and nrDNA phylogenies for these taxa. Lastly, the current taxonomy needs revising as it fails to recognize the major genetic division of *Eubosmina* (*E.*). Paraphyly of *E. longispina* with nuclear and mtDNA gene evidence, and the likely reproductive isolation of North American *E. longispina* from European *E. longispina*, warrants that North American *Eubosmina* (*E.*) be assigned a unique species name. Herrick's (1882) description from Lake Minnetonka, MN, USA seems to have precedence, yielding the name *Eubosmina* (*Eubosmina*) *striata* (Herrick).

The recent invasion of Palearctic *Eubosmina* (*Eubosmina*) into Nearctic lakes

A possible exception to monopolization is the apparent colonization of North America by three European taxa: *E. maritima* and *E. coregoni*, and *E. longicornis kessleri* (Deep

Lake NY and Muskoka Lake). Our finding of the near identity of these species with Eurasian specimens indicates a very recent connection consistent with human-mediated introduction. *E. coregoni* has successfully colonized many lakes in North America, although most of these are highly eutrophic artificial water bodies south of the *E. longispina* distribution. The taxonomic status of *E. maritima* and *E. longicornis kessleri* remains dubious until more comparisons and culture studies are made with European types. Our results agree with De Melo & Hebert (1994c) that these taxa have a close Palearctic connection and that the present range of this clade in North America may best be assessed by genetic markers, with the sediment record perhaps allowing for limited genetic analysis of embryos to better assess the timing of its arrival.

We conclude that the *Eubosmina* system will be unusually valuable for studying macroevolutionary processes provided that the genetic basis for the morphological change is also studied. The paleolimnological record and genetic record agreed on the rapid morphological evolution in north Europe, and in the recent invasions into North America. However, the major genetic divergences in the *Eubosmina* are invisible using existing morphological schemes and indicate that detailed morphometrics of subfossils is warranted. Perhaps the most surprising result is the paraphyly of the morphologically uniform *E. longispina* with morphologically diverse Palearctic species. The genealogical concordance with known glacial refugia suggests that lacustrine Holarctic bosminids are susceptible to geographic speciation processes.

Acknowledgments

We thank Michael Boller, Sandra Connelly, Marketa Faustova, Andrew Giordano, Seiji Ishida, Nikolai Korovchinsky, Alexey Kotov, Angela Omilian, Adam Petrussek, Veronika Sacherova and Jonathan Witt for aid in obtaining specimens. Vladimir Korinek identified the Polish forms. Alan Seigel helped with microscopy and Bill Piel provided manuscript improvements. We thank Christine Ishikane for assistance with PCR and assembly of the ITS data, and Michelle Detwiler for help in electrophoresis of sequences. We also thank the Dept. of Hydrobiology, Warsaw University, for use of their field station at Pilchy. This research was supported by a National Science Foundation grant (OPP, Arctic Natural Sciences) to D.J.T.

References

- Avise, J.C. 2000. *Phylogeography: The History and Formation of Species*. Harvard University Press, London, England.
- Bernatchez, L. & Wilson, C.C. 1998. Comparative phylogeography of Nearctic and Palearctic fishes. *Mol. Ecol.* **7**: 431–452.
- Chang, K.H. & Hanazato, T. 2003. Seasonal and reciprocal succession and cyclomorphosis of two *Bosmina* species (Cladocera, Crustacea) co-existing in a lake: their relationship with invertebrate predators. *J. Plankton Res.* **25**: 141–150.
- Cox, A.J. & Hebert, P.D.N. 2001. Colonization, extinction, and phylogeographic patterning in a freshwater crustacean. *Mol. Ecol.* **10**: 371–386.
- Crease, T.J. 1999. The complete sequence of the mitochondrial genome of *Daphnia pulex* (Cladocera: Crustacea). *Gene* **233**: 89–99.
- Cristescu, M.E.A. & Hebert, P.D.N. 2002. Phylogeny and adaptive radiation in the Onychopoda (Crustacea, Cladocera): evidence from multiple gene sequences. *J. Evol. Biol.* **15**: 838–849.
- Cunningham, C.W., Blackstone, N.W. & Buss, L.W. 1992. Evolution of king crabs from hermit-crab ancestors. *Nature* **355**: 539–542.
- De Meester, L., Gomez, A., Okamura, B. & Schwenk, K. 2002. The monopolization hypothesis and the dispersal-gene flow paradox in aquatic organisms. *Acta Oecologica* **23**: 121–135.
- De Melo, R. & Hebert, P.D.N. 1994a. Allozymic variation and species diversity in North American Bosminidae. *Can. J. Fisheries Aquat. Sci.* **51**: 873–880.
- De Melo, R. & Hebert, P.D.N. 1994b. Founder effects and geographical variation in the invading cladoceran *Bosmina (Eubosmina) coregoni* Baird 1857 in North America. *Heredity* **73**: 490–499.
- De Melo, R. & Hebert, P.D.N. 1994c. A taxonomic reevaluation of North American Bosminidae. *Can. J. Zool.* **72**: 1808–1825.
- Deevey, E.S. 1942. Studies on Connecticut lake sediments 3. *Am. J. Sci.* **240**: 233–264; 313–338.
- Dynesius, M. & Jansson, R. 2000. Evolutionary consequences of changes in species' geographical distributions driven by Milankovitch climate oscillations. *Proc. Natl. Acad. Sci. U. S. A.* **97**: 9115–9120.
- Finney, B.P., Gregory-Eaves, I., Sweetman, J., Dougas, M.S.V. & Smol, J.P. 2000. Impacts of climatic change and fishing on Pacific salmon abundance over the past 300 years. *Science* **290**: 795–799.
- Frey, D.G. 1962. Cladocera from the Eemian interglacial of Denmark. *J. Paleolimnol.* **36**: 1133–1154.
- Frey, D.G. 1991. First subfossil records of *Daphnia* headshields and shells (Anomopoda, Daphniidae) about 10 000 years old from northernmost Greenland, plus *Alona guttata* (Chydoridae). *J. Paleolimnol.* **6**: 193–197.
- Goldman, N., Anderson, J.P. & Rodrigo, A.G. 2000. Likelihood-based tests of topologies in phylogenetics. *Syst. Biol.* **49**: 652–670.
- Gunther, J. & Lieder, U. 1993. Postglacial succession in the subgenus *Eubosmina* (Crustacea, Cladocera) in the region of the Unterhavel River (Near Berlin, Germany) – type changes or species immigration. *Int. Revue. Ges. Hydrobiol.* **78**: 1–19.
- Hann, B.J. 1989. Methods in quaternary ecology. 6. Cladocera. *Geosci. Can.* **16**: 17–26.
- Hann, B.J. & Karrow, P.F. 1984. Pleistocene paleoecology of the Don and Scarborough Formations, Toronto, Canada, based on Cladoceran microfossils at the Don Valley Brickyard. *Boreas* **13**: 377–391.
- Hann, B.J. & Warner, B.G. 1987. Late quaternary Cladocera from coastal British Columbia, Canada – a record of climatic or limnologic change. *Archiv f8r Hydrobiologie* **110**: 161–177.
- Hasegawa, M., Kishino, H. & Yano, T. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *J. Mol. Evol.* **22**: 160–174.

- Hellsten, M.E. & Sundberg, P. 2000. Genetic variation in two sympatric European populations of *Bosmina* spp. (Cladocera) tested with RAPD markers. *Hydrobiologia* **421**: 157–164.
- Hellsten, M., Lagergren, R. & Stenson, J. 1999. Can extreme morphology in *Bosmina* reduce predation risk from *Leptodora*? An experimental test. *Oecologia* **118**: 23–28.
- Herrick, C.L. 1882. Papers on the Crustacea of the fresh waters of Minnesota. 10th Annual Report, The Geological and Natural History Survey of Minnesota, pp. 235–252.
- Hofmann, W. 1984. Morphological variation in a late glacial population of *Bosmina longispina* (Leydig) (Crustacea, Cladocera) from the Lobsigensee (Swiss Plateau). Studies in the late-Quaternary of Lobsigensee 9. *Swiss J. Hydrol.* **46**: 139–146.
- Hofmann, W. 1991. The Late-Glacial/Holocene *Bosmina* (*Eubosmina*) fauna of Lake Constance (Untersee) (F.R.G.): traces of introgressive hybridization. *Hydrobiologia* **225**: 81–85.
- Huelsensbeck, J.P., Ronquist, F., Nielsen, R. & Bollback, J.P. 2001. Evolution–Bayesian inference of phylogeny and its impact on evolutionary biology. *Science* **294**: 2310–2314.
- Keller, W. & Pitbaldo, J.R. 1989. The distribution of crustacean zooplankton in northern Ontario, Canada. *J. Biogeogr.* **16**: 249–259.
- Kerfoot, W.C. 1975. The divergence of adjacent populations. *Ecology* **56**: 1298–1313.
- Kerfoot, W.C. 1978. Combat between predatory copepods and their prey – *Cyclops*, *Epischura*, and *Bosmina*. *Limnol. Oceanogr.* **23**: 1089–1102.
- Kerfoot, W.C. 1987. Translocation experiments – *Bosmina* responses to copepod predation. *Ecology* **68**: 596–610.
- Kumar, S., Tamura, K., Jakobsen, I.B., & Nei, M. 2001. MEGA2: Molecular evolutionary genetics analysis software. *Bioinformatics* **17**: 1244–1245.
- Lieder, U. 1983a. Introgression as a factor in the evolution of polytypical plankton Cladocera. *Int. Revue. Ges. Hydrobiol.* **68**: 269–284.
- Lieder, U. 1983b. Revision of the genus *Bosmina* (Baird, 1845) (Crustacea, Cladocera). *Int. Revue. Ges. Hydrobiol.* **68**: 121–139.
- Lieder, U. 1991. The *Bosmina kessleri*-like morphotype of *Eubosmina* in Lake Muskoka, Ontario, Canada, as putative interspecific hybrids. *Hydrobiologia* **225**: 71–80.
- Lieder, U. & Gunther, J. 1995. *Bosmina* (*Eubosmina*) *longicornis* Schoedler, 1866 (Crustacea, Cladocera, Bosminiidae) From the Postglacial Sediments of the Neuendorfer-See, East-Germany. *Int. Revue. Ges. Hydrobiol.* **80**: 111–120.
- Little, T.J., Demelo, R., Taylor, D.J. & Hebert, P.D.N. 1997. Genetic characterization of an arctic zooplankton: insights into geographic polyploidy. *Proc. R. Soc. Lond. B. Biol. Sci.* **264**: 1363–1370.
- Nauwerck, A. 1991. The history of the genus *Eubosmina* in Lake Mondsee (upper Austria). *Hydrobiologia* **225**: 87–103.
- Posada, D. & Crandall, K.A. 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14**: 817–818.
- Post, D.M., Frost, T.M. & Kitchell, J.F. 1995. Morphological responses by *Bosmina longirostris* and *Eubosmina tubicen* to changes in copepod predator populations during a whole-lake acidification experiment. *J. Plankton Res.* **17**: 1621–1632.
- Riessen, H. & O'Brien, W. 1980. Re-evaluation of the taxonomy of *Daphnia longiremis* Sars, 1862 (Cladocera) – description of a new morph from Alaska. *Crustaceana* **38**: 1–11.
- Sars, G.O. 1993. *On the Freshwater Crustaceans Occurring in the Vicinity of Christiania*. University of Bergen, Bergen, Norway.
- Schubart, C.D., Diesel, R. & Hedges, S.B. 1998. Rapid evolution to terrestrial life in Jamaican crabs. *Nature* **393**: 363–365.
- Shimodaira, H. & Hasegawa, M. 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. *Mol. Biol. Evol.* **16**: 1114–1116.
- Swofford, D.L. 2002. *Phylogenetic Analysis using Parsimony (and other Methods)*. Version 4. Sinauer Associates, Sunderland, MA.
- Taylor, D.J. & Hebert, P.D.N. 1994. Genetic assessment of species boundaries in the north-American *Daphnia longispina* complex (Crustacea, Daphniidae). *Zool. J. Linnean Soc.* **110**: 27–40.
- Taylor, D.J., Finston, T.L. & Hebert, P.D.N. 1998. Biogeography of a widespread freshwater crustacean: pseudocongruence and cryptic endemism in the North American *Daphnia laevis* complex. *Evolution* **52**: 1648–1670.
- Taylor, D.J., Ishikane, C.R. & Haney, R.A. 2002. The systematics of Holarctic bosminids and a revision that reconciles molecular and morphological evolution. *Limnol. Oceanography* **47**: 1486–1495.
- Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. & Higgins, D.G. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic. Acids. Res.* **25**: 4876–4882.
- Weider, L. & Hobaek, A. 2003. Glacial refugia, haplotype distributions, and clonal richness of the *Daphnia pulex* complex in arctic Canada. *Mol. Ecol.* **12**: 463–473.
- Yan, N.D., Girard, R. & Boudreau, S. 2002. An introduced invertebrate predator (*Bythotrephes*) reduces zooplankton species richness. *Ecol. Lett.* **5**: 481–485.
- Yang, Z. 1994. Maximum likelihood phylogenetic estimation from DNA sequences with variable rates over sites: approximate methods. *J. Mol. Evol.* **39**: 306–314.

Received 17 December 2002; revised 14 April 2003; accepted 19 May 2003