

Molecular evidence reveals a polyphyletic origin and chromosomal speciation of Lake Baikal's endemic asellid isopods

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Abstract

The six endemic isopod species of Lake Baikal have been regarded as a small species flock with uncertain affinities to related asellids. We provide evidence from 16S rRNA sequences for polyphyletic origins of Baikalian Asellidae. One clade of two species is related to the Eurasian genus *Asellus*. The other clade, *Baicalasellus*, shows affinities to North American asellids and may have a long evolutionary history within the lake basin. Some speciation events within *Baicalasellus* clearly have a chromosomal basis. In contrast with numerous taxa exhibiting monophyletic radiations in ancient lakes, the endemic Baikalian isopods arose by multiple invasions and chromosomal mechanisms.

Keywords: ancient lakes, biogeography, morphology, phylogeny, species flocks, 16S rRNA

Received 6 August 2002; revision received 5 January 2003; accepted 24 January 2003

Introduction

Lake Baikal in Siberia is renowned for its high species richness of endemic taxa including amphipods, gastropods, cottoid fish and oligochaetes (Sherbakov 1999), some of these radiations comprising hundreds of endemic species. Less well known are its endemic isopods, which have not undergone an extensive adaptive radiation. Four isopod species belong to the endemic genus *Baicalasellus* (*B. angarensis*, *B. minutus*, *B. korotnewi* and *B. baicalensis*) and two other species are from the monotypic genera *Limnoasellus* (*L. poberezhnii*) and *Mesoasellus* (*M. dybowskii*). All Baikalian isopods belong to the family Asellidae.

Phylogenetic relationships of Baikalian isopods are unknown. To our knowledge, no work on morphological cladistics or molecular systematics has been published on the Asellidae. An interesting overview of asellid taxonomy is given by Henry & Magniez (1993).

Asellid isopods are found in freshwater habitats on all continents in the northern hemisphere from 30° N to the Arctic (Birstein 1951). In total, approximately 360 species and subspecies from this family have been described. In the Palearctic, the highest diversity of epigeal (surface

water dwelling) asellids is found in the Mediterranean and eastern Asia. In southern Europe and North Africa species from the genus *Proasellus* dominate, whereas in eastern Asia most asellid isopods belong to a suprageneric group known as the 'Asellus pattern' group (Henry & Magniez 1995). The taxonomy of asellids has focused on pleopodal limbs as they provide clear diagnostic characters. As such, the 'Asellus pattern' is primarily based on the morphology of the male pleopod II, characterized by 'Asellus type' copulatory appendages. The Baikalian *L. poberezhnii* and *M. dybowskii* belong to this group, but the four described species of *Baicalasellus* do not, and occupy their own distinct position within the Asellidae, based on pleopod morphology.

Research on chromosomes, however, has revealed striking similarities in karyology between *B. angarensis* and *Asellus aquaticus* (Natyaganova *et al.* 1996). Both have a haploid chromosome number of 8 and the chromosomes have similar morphologies. This may counter the inferred morphological relationships and suggests close phylogenetic relationship between the two species. *B. minutus* has $n = 4$, *B. korotnewi* $n = 8$ and *B. baicalensis* $n = 12$ (Natyaganova 2000). Karyotypes of these congeners do not resemble those of *B. angarensis*. The 'Asellus pattern' endemics from Baikal both have $n = 6$. This variability is in sharp contrast with the karyotypical constancy found in Baikal's

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amphipods, with only one species known to deviate from the standard number $n = 26$. (Salemaa & Kamal'tynov 1994).

It has been suggested that Baikal's extreme age of 25–30 Mya (Martin 1994), its relative stability (Sherbakov 1999) and high biodiversity may have contributed significantly to the renewal of Palearctic species diversity. By investigating the phylogenetic relationship between *A. aquaticus* and *B. angarensis*, we test the hypothesis that Baikal is a source for biodiversity on the Eurasian continent for this specific group of organisms. A phylogeny may clarify whether *B. angarensis* or Baikalian asellids in general may be closely related to current Palearctic asellid isopods, in particular to *A. aquaticus*. Furthermore, we investigate whether the Baikalian isopod fauna is monophyletic, as suggested by Birstein (1951). By analysing 16S mitochondrial RNA across various genera, we address the phyletic status of the Baikalian isopods, and provide a basic phylogenetic framework for the Asellidae. We use this phylogeny to evaluate the phylogenetic usefulness of established diagnostic morphological characters.

Materials and methods

Specimens from Lake Baikal were collected on expeditions with the research vessels *Vereshagin* and *Obruchev* in the summers of 2000 and 2001 by dredging at 100 m depth near the biological station of the Irkutsk State University at Bol'shye Koty (*M. dybowskii*, *L. poberezhnii*, *B. minutus*). Isopods from shallow habitats, down to 20 m, were collected using SCUBA at Svyatoj Nos and Bol'shye Koty (*B. baicalensis*, *B. angarensis*, *B. korotnevi*). We collected *Asellus levandovororum* by hand from a pool near the Amur River outside the city of Khabarovsk in the far east of Russia. *Asellus aquaticus*, *Proasellus* sp. and *P. coxalis* were collected by hand outside Amsterdam (The Netherlands). All specimens were stored in 96% ethanol. Locality and sampling details are summarized in Table 1.

DNA was extracted from complete or partial specimens according to the CTAB/chloroform protocol (Sambrook *et al.* 1989) and diluted in 50–70 µL sterile water. The mitochondrial 16S fragment was amplified using the primers 16Sar-Dr 5'-CGCTGTTTAACAAAAACAT-3' and 16Sbr 5'-CCGGTCTGAACTCAAGATCACGT-3' (Palumbi *et al.* 1991) in 25 µL polymerase chain reaction (PCR) mixture consisting of 1 µL template DNA, 2.5 µL 10× PCR buffer (HT Biotechnology Ltd), 0.4 µL dNTP (10 mM), 0.2 µL *Taq* polymerase (5 units/µL, HTBiotechnology LTD), 20.5 µL sterile water and of both primers 0.2 µL (20 µM). Cycling conditions: 4 min at 94°; 35x: 45 s at 94°, 1 min at 54°, 90 s at 72°; final extension 5 min at 72°. Fragment length and quality were verified on a 1% agarose gel with ethidium bromide and cleaned with the GeneClean kit (Bio 101). Cleaned products of the same specimen from two reactions

Table 1 Sample details and GenBank Accession nos for analysed 16S rRNA sequences

Species	Author	Distribution	Depth (m)	Substrate	Sampling location	GenBank
<i>Asellus aquaticus</i>	Linnaeus (1758)	Europe	Shallow	Submerged plants, detritus	Zaandam, NL	AF532161
<i>Asellus levandovororum</i>	Henry & Magniez (1993)	Eastern Asia	Shallow	Submerged plants, detritus	Khabarovsk, RU	AF532160
<i>Mesosellus dybowskii</i>	Semenkevich (1924)	Lake Baikal	100 m +	Rocky	Bol'shye Koty, RU	AF532158
<i>Limnosellus poberezhnii</i>	Natyaganova & Hidding (in prep.)	Lake Baikal	8–100	Rocky	Bol'shye Koty, RU	AF532159
<i>Baicalasellus baicalensis</i>	Grube (1872)	Lake Baikal	3–50	Rocky	Svyatoj Nos, RU	AF532155
<i>Baicalasellus minutus</i>	Semenkevich (1924)	Lake Baikal	10–100	Rocky	Bol'shye Koty, RU	AF532153
<i>Baicalasellus korotnevi</i>	Semenkevich (1924)	Lake Baikal	10–50	Rocky	Svyatoj Nos, RU	AF532156
<i>Baicalasellus angarensis</i>	Dybowskii (1884)	Lake Baikal	3–10	Rocky	Bol'shye Koty, RU	AF532157
<i>Proasellus meridianus?</i>	Racovitza (1919)	Mediterranean and Central Europe	Shallow	Submerged plants, detritus	Amsterdam, NL	AF532161
<i>Proasellus coxalis</i>	Dollfus (1892)	Mediterranean and Central Europe	Shallow	Submerged plants, detritus	Zaandam, NL	AF532162
<i>Caecidotia</i> sp.	Packard (1871)	Europe	Shallow	Leaf litter, spring water	Washington DC, US	AF259534
<i>Jaera albifrons</i>	Leach (1814)	US and Canada Marine, North Atlantic coasts	—	Sandy shore	Loix en Re, France	AJ388078
<i>Janira</i> sp.	Leach (1814)	Marine cold waters	—	Sandy shore	Roscoff, France	AJ388079

were pooled if sequenced directly. Cycle sequence reaction were performed according to the manufacturer's protocols, and either sequenced directly on an ABI 377 automated sequencer, or first cloned into pGEM-T easy Vector (Promega). Recombinant plasmids were sequenced on Li-Cor or ABI automated sequencers using M13 primers.

Primer and vector parts were deleted from the sequences. BLAST searches were performed on the sequences to verify correct amplification of isopod DNA. Sequences were submitted to GenBank (See Table 1). The marine species *Jaera albifrons* (EMBL Accession no. AJ388078) and *Janira* sp. (AJ388079), belonging to the family Janiridae of the same suborder as the asellids (*Aselotta*), were added from GenBank as outgroup species. A sequence of the North American asellid species *Caecidotea* sp. was also included (AF259534). CLUSTAL W (Thompson *et al.* 1994) was used for initial alignment followed by manual fine-tuning.

The sequence alignment of 493 positions including gaps, was implemented in PAUP* 4.0b8 (Swofford 2001). Gaps were treated as missing characters. The 50 positions corresponding to an unalignable hypervariable region were deleted in the major analyses, as recommended for analyses of crustacean phylogeny (Held 2000; Michel-Salzat & Bouchon 2000; Schubart *et al.* 2000). A maximum parsimony analysis that included only transversions, and a neighbour-joining (NJ) analysis ('uncorrected P' distances) were applied to a data set containing all taxa. Bootstrap analyses were conducted using 1000 heuristic search replicates. A maximum likelihood analysis was performed from which the two least divergent *Baicalasellus* species were excluded. This was performed for the sake of computational speed and because resolution within the *Baicalasellus* clade is limited when the hypervariable region is omitted. For the maximum likelihood analysis, MODELTEST 3.06 (Posada & Crandall 1998) was run to test for the model best fitting the data from which the results following the minimum theoretical Aikake information criterion (AIC) were chosen. A likelihood ratio test was performed to test whether the data are consistent with a molecular clock. (Huelsenbeck & Crandall 1997; Huelsenbeck & Rannala 1997). Likelihoods under the two assumptions were calculated for a NJ ('uncorrected P') tree with the model suggested by MODELTEST. A χ^2 test did not reject the null hypothesis, which is based on a maximum likelihood analysis with a molecular clock ($\chi^2 = 13.4$, d.f. = 9, $P > 0.05$). The model thus became GTR + Γ + molecular clock. The two species appearing closest to the *Baicalasellus* clade were chosen as outgroups for the further detailed analysis of the *Baicalasellus* clade. Divergence in this clade is low and allows for a full alignment of the *Baicalasellus* species, without omission of the hypervariable fragment. For this data set the HKY85 + I model without molecular clock was applied, as suggested by a Likelihood Ratio Test.

Results

Based on sequences that were complete from primer to primer, fragment length varied from 467 base pairs (bp) (*Baicalasellus* spp.) to 475 bp (*Mesoasellus dybowskii*). The 443 bp data set, resulting from exclusion of the hypervariable region, contained 244 variable sites of which 187 sites were parsimony informative. Transition–transversion ratios, calculated for this data set, were found to vary between 13 and 9 among *Baicalasellus* species and 2 and 3 among other congeners. All intergeneric combinations within the ingroup give Ts–Tv ratios varying from 0.73 to 1.48, which may indicate saturation at variable sites.

The maximum parsimony analysis, which included only transversions (not depicted), resulted in four most parsimonious trees. A strict consensus of these trees offers no resolution within the *Baicalasellus* clade, but is congruent with the NJ tree in major topologies. The only conflict is the deep node representing the position of the *Proasellus* clade. Bootstrap support for its position in both trees is low. The ML tree (Fig. 1) is in agreement with the NJ tree (Fig. 2) on the position of the *Proasellus* clade but shows low bootstrap support. For the rest, the ML tree is in full agreement with the other trees.

From the obtained phylogenetic trees it is evident that the Baikalian isopod species are polyphyletic. *Mesoasellus dybowskii* and *Limnoasellus poberezhnii* group together with the genus *Asellus*, whereas the *Baicalasellus* species form a separate clade. Monophyly of the latter genus is highly supported in all analyses. The notion that the North American

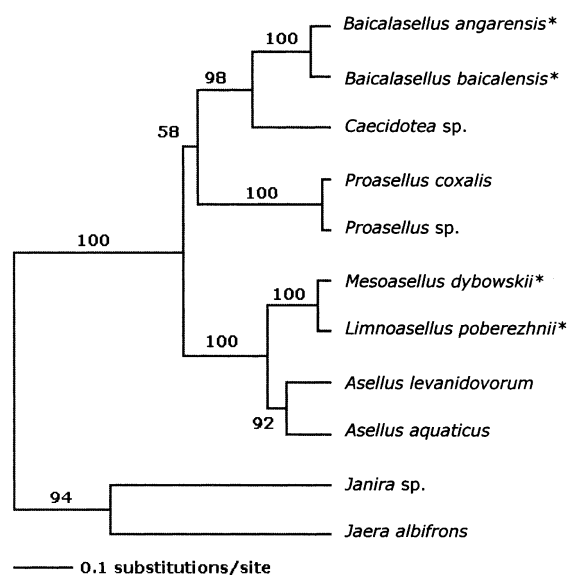


Fig. 1 Maximum likelihood phylogenetic tree of all genera. Evolutionary model: general time reversible with Γ -distribution and molecular clock. Bootstrap values given for 1000 search replicates. Species from Lake Baikal indicated with an asterisk.

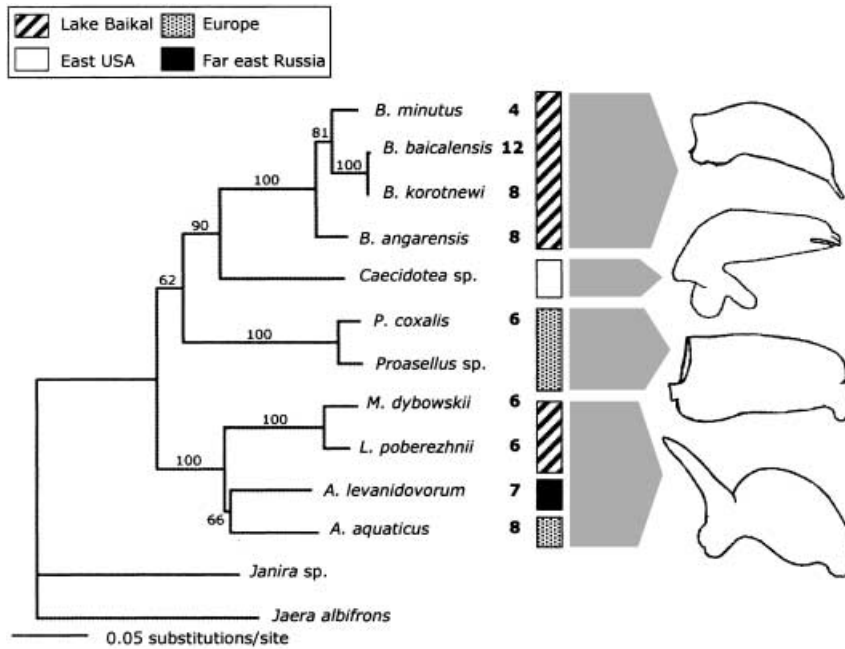


Fig. 2 NJ-tree with bootstrap values (1000×), depicted because of its full agreement with the ML trees. Plotted on the tree from left to right: the haploid chromosome numbers; bars indicating phylogeography; representative endopodites of the second male pleopods (the copulatory appendages) shown for each clade to indicate major morphological differences. From top to bottom: a representative of the *Baicalasellus* genus with a beaklike endopodite; *Caecidotea* sp. with the representative endopodite of *C. reddelli*, adapted from Lewis & Bowman (1996); *Proasellus* with short distal article, and without basal processes; *Asellus* with the typical elongated basal spur (adapted from Birstein 1951).

asellid *Caecidotea* is a sister taxon to the *Baicalasellus* species is also supported convincingly. The ML tree suggests that divergence between *Caecidotea* and *Baicalasellus* took place at approximately the same time as that between *Asellus* and *Mesoasellus*. Furthermore, divergences within the *Baicalasellus* clade and between *Limnoasellus* and *Mesoasellus* seem to have taken place at approximately the same time. The ML tree with molecular clock suggests that congeners *A. aquaticus* and *A. levanidovororum* diverged relatively long ago, compared to the Baikalian *M. dybowskii* and *L. poberezhnii* in the same clade (Fig. 1). The monophyly of the 'Asellus pattern' taxa supports the usefulness of the second male pleopods for taxonomic purposes, although wider taxon sampling is needed.

The relationships within the *Baicalasellus* species are as shown in Fig. 2. In the separate *Baicalasellus* analysis, *Caecidotea* and *Proasellus* serve as outgroups. *B. angarensis* is sister to the clade with the remaining three Baikalian species. 16S RNA sequences of two *B. korotnewi* specimens and one *B. baicalensis* individual showed no sequence difference, and this is due probably to a very recent divergence between these species. Interestingly, the tree topology found within these asellids coincides with their ploidy levels. In this perspective, *B. minutus* is diploid, *B. korotnewi* tetraploid, and *B. baicalensis* hexaploid.

Discussion

Our results indicate that the six Baikalian isopod endemics originated from two independent invasions, neither of which has radiated extensively based on extant representatives. This contrasts strikingly with the ecological

differentiation evident within the Baikalian amphipod fauna, which comprises an almost fully endemic species flock of more than 330 described species and subspecies (Takhteev 2000). We see little evidence for ecological divergence in the isopod endemics at present. Asellids are benthic scavengers or grazers, with limited swimming abilities, that perhaps limited their potential for radiation in competition with amphipods.

The only speciation event with a plausible ecological basis is between *L. poberezhnii* and *M. dybowskii*, as *M. dybowskii* has autapomorphic abyssal-type morphological features of long spines and reduced eyes and is only found at 100 m and deeper. Although *L. poberezhnii* can be found in deep water as well, it occurs typically at relatively shallow depths and lacks the abyssal characters. Both species share common karyotypical features and have six haploid chromosomes. The karyological characters of Baikalian asellids suggest that, in contrast to the endemic gammarid amphipod species flock from the same lake, speciation in *Baicalasellus* was caused by chromosomal mechanisms.

The genus *Mesoasellus* as established by Birstein was considered a widespread genus from the Tertiary (Birstein 1951; Vekhoff 1994) because of the disjunct geographical distribution of its original five species. Later revisions removed species from Japan, Siberia and California from *Mesoasellus*, leaving this a monotypic genus with the type *M. dybowskii* endemic to Baikal. Add to this the close relationship with *L. poberezhnii* and the statement about *Mesoasellus*' age becomes highly suspect. This is in agreement with the trend that many Baikalian species, once considered as 'ancient relicts', are currently considered relatively young (Timoshkin 1999). Our phylogenies indicate that the

time of divergence between *A. levanidovororum* and *A. aquaticus* occurred probably long before the divergence between *M. dybowskii* and *L. poberezhnii*. However, the two *Asellus* species are, in contrast with their Baikalian relatives, morphologically and ecologically very similar (Birstein 1951). Apparently, these vicariant species show parallelism in morphology.

Contrary to Birstein's idea that *Baicalasellus* evolved from a *Mesoasellus*-like predecessor (Birstein 1951; Vekhoff 1994), 16S analysis indicates that *Mesoasellus* and *Baicalasellus* are more distantly related (Fig. 1). The branch length for the *Baicalasellus* clade is long, indicating that the ancestor of the radiation might have been isolated in Lake Baikal for a long time. All the species dwelling in surrounding regions of Eastern Asia belong to the distantly related 'Asellus pattern' group, to which *L. poberezhnii* and *M. dybowskii* also belong. The latter species may therefore have invaded the lake relatively recently from adjacent water bodies.

In our results, the North American genus *Caecidotea* is sister to *Baicalasellus*, with strong branch support. This link was also noticed in morphology as some Appalachian *Caecidotea* species were once designated as *Pseudobaicalasellus* (Lewis 1980). In principle, a North American ancestor for the *Baicalasellus* clade is possible because the Bering land bridge existed until 5–7 Mya (Brigham-Grette 2001). Because *Caecidotea* and related species are not known from Northern Canada or Alaska, support for the Beringian scenario requires that the recorded natural distribution of *Caecidotea* is highly relictual and restricted. The common ancestor would have been ancient and distributed widely across Beringia, with extinction of sister taxa in the Pleistocene.

Alternatively, based on detailed work on pleopod morphology, Henry & Magniez (1995) suggested that ancestors of *Caecidotea* and *Baicalasellus* were marine and invaded freshwaters independently. Thus *Caecidotea* and *Baicalasellus* would not be sisters, rather the tree lacks intervening taxa. Testing this hypothesis would require very broad sampling of marine taxa, as no suggestions were made for potential marine relatives.

Asellids in general have a high diversity of haploid chromosome numbers (Salemaa & Kamal'tynov 1994), and this needs to be emphasized when considering the Baikalian isopod fauna. The *Baicalasellus* radiation, as depicted in Fig. 2, is likely to be a chromosomal phenomenon. The three tip taxa, *B. baicalensis*, *B. korotnewi* and *B. minutus*, share patterns of chromosomal banding and sizes, which supports their close relationship (Natyaganova 2000). Their haploid chromosome numbers range from $n = 4$ in the basal *B. minutus* to $n = 8$ and 12 in the other two species. This could be the result of polyploidization ($n = 4$ to $n = 8$, *B. minutus* and *B. korotnewi*) followed by hybridization between these taxa to derive $n = 12$ in *B. baicalensis*. *B. baicalensis* and *B. korotnewi* clearly diverged recently based on

their minimal 16S divergence and close morphological similarities. The difference in ploidy level, $n = 12$ and 8, respectively, probably keeps these species reproductively isolated. Studies of nuclear genes or allozymes may prove useful in elucidating the divergence history.

The species-rich genus *Proasellus* forms a distinct clade with a long branch, appearing as one of the three major lineages observed in the family, among the lineage *Asellus* and the clade with *Baicalasellus* and *Caecidotea*. The true position of this branch is not evident from mitochondrial 16S sequences, as branch support is very low for its placement. A slower evolving gene may give clues in this matter. As a phylogeny for the evolution of the whole family of asellids, the current tree gives merely an outline. Asellid distributions are currently disjunct around the globe. Henry & Magniez (1995) therefore concluded that the different evolutionary lines of asellids invaded continental freshwaters independently. They state that male copulatory organs (pleopod II) in each of these lineages are analogous in function, that is, protecting the sperm reservoir from the osmotic stress of a freshwater environment, but are not homologous. They suggest that the similar morphologies of somatic characters are homoplastic. If so, these similarities even confuse the asellids themselves as *Proasellus* and *Asellus* are sometimes found in precopula. Hybrids, however, are never found (Henry & Magniez 1983). We see this as an unresolved issue, and suggest that further morphological and molecular work should be conducted to test the origin of the divergent pleopod morphologies (Fig. 2) and, moreover, of freshwater habit for isopods.

Our results support the perspective that Baikal's diverse endemic fauna is young, but that it may stem from ancient lineages. The palaeohistory of Lake Baikal is characterized by strong fluctuations in primary production relating to Pleistocene glaciations, as documented in the lake's diatom palaeorecords (Khursevich *et al.* 2001). These circumstances, added to catastrophic cooling events in the Miocene and Pliocene (Antipin *et al.* 2001), may have induced major extinctions followed by new speciation events. Shallow divergences such as we found within the two Baikalian asellid isopod clades are also found among Baikal's endemic amphipods (Sherbakov *et al.* 1998), arguing for relatively recent intralacustrine speciation.

Acknowledgements

The authors are deeply indebted to Olya Savinova and Tanya Pudovkina, who challenged merciless autumn storms on expeditions and helped to extract DNA. Thanks to Dirk Platvoet and Marc Stiff for assistance with sampling, Oleg Timoshkin, Mikhail Grachev and Hans Breeuwer for providing facilities, and Dirk Erpenbeck, Tanya Sitnikova, Katya Mamonova, Irina Kaygorodova, Katja Peijnenburg, Martin Genner, Sven Lange, Peter Kuperus, Wil van Ginkel, Fred Schram, Steph Menken, Stefan Koenemann,

Jonathan Todd, Peter McIntyre, Andy Cohen, Saskia Marijnissen, Patrick Meirman, Regina Wetzler and Didier Bouchon for suggestions, useful comments and other assistance. Financial support was given by the Bekker la Bastide fund to B.H.

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This work is part of a research programme on comparative diversification patterns in the endemic faunas of ancient lakes. E. Michel and D. Sherbakov have collaborating research groups, and investigate molecular, morphological and ecological aspects of radiations in several taxa, aimed at developing rift lake faunas as models for ecology and evolution studies. B. Hidding completed this work as part of his MSc and A.V. Natyaganova is working towards her PhD.
