

Molecular analyses of ostracod flocks from Lake Baikal and Lake Tanganyika

Isa Schön · Koen Martens

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Abstract Ancient lakes are excellent laboratories for evolutionary research, where species can be studied in the cradle where they originated. In this article, we investigate two endemic ostracod species flocks from the two oldest lakes in the world, Lake Baikal (LB) (ca. 28 myr) and Lake Tanganyika (LT) (ca. 12 myr), with DNA sequence data. Nuclear ITS1 failed to resolve the phylogeny of both flocks. Whilst most phylogenetic relationships of the Tanganyika flock are resolved with mitochondrial COI, the Baikalian tree contains multifurcations of up to seven different clades. The Tanganyikan *Cyprideis* flock shows higher genetic variability, which matches its higher morphological variability. A significant deviation from a constant divergence rate through time indicates that the *Cytherissa* flock most likely experienced explosive speciation events during its earlier

history. Comparative analyses of substitution rates furthermore revealed that they are not clock-wise for COI. Ancestral *Cytherissa* probably radiated in LB 5–8 myr ago, around the time when the cold, oxygenated abyss was formed in LB. The Tanganyikan *Cyprideis* flock is almost twice as old as the Baikalian *Cytherissa* flock, and possibly older than LT itself, ca. 15 myr. The *Cyprideis* flock has survived drastic lake level changes and resulting salinity crises during its entire history.

Keywords Ancient lakes · Ostracods · Genetic variability · Phylogeny · Divergence rates

Introduction

Ancient lakes are natural laboratories for evolutionary research, because of their exceptionally high biodiversity, the large number of endemics and the fact that most species are still found in the cradle where they first originated (Martens et al., 1994, Martens, 1997; Schön & Martens, 2004). Geological and climatic reconstructions provide further detailed information on the time of origin of these lakes and on changing environmental conditions over the millions of years of their history (e.g. for Lake Baikal (LB): Karabanov et al., 2004; Sherstyankin & Kuimova, 2006, for LT: Cohen et al., 1993, 1997, 2007; Lezzar et al., 1996; Nicholson, 1999; Scholz et al., 2003). Molecular research on evolution and speciation of ancient lake

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I. Schön (✉) · K. Martens
Royal Belgian Institute of Natural Sciences, Freshwater
Biology, Vautierstraat 29, 1000 Brussels, Belgium
e-mail: isa.schoen@naturalsciences.be

I. Schön
Research Group Zoology, University of Hasselt,
Agoralaan gebouw D, 3590 Diepenbeek, Belgium

K. Martens
Biology, University of Ghent, K. L. Ledeganckstraat 35,
9000 Ghent, Belgium

taxa has mainly focused on cichlid fish (see Seehausen, 2006, and Koblmüller et al., 2008, for more recent reviews on cichlids), although molecular analyses on invertebrates such as crabs (Marijnissen et al., 2006, 2008), snails (West & Michel, 2000; Wilson et al., 2004; Albrecht et al., 2006, 2008; Hauswald et al., 2008; Schultheiß et al., 2008), sponges (Schröder et al., 2002) and amphipods (Macdonald et al., 2005) have meanwhile also been published.

Ostracods, small, bivalved crustaceans with one of the best fossil records in the animal kingdom (Horne & Martens, 2000) form a significant part of ancient lake invertebrate faunas (Martens, 1994; Frogley et al., 2002). The two oldest lakes, the tropical Tanganyika (East Africa) and the boreal Baikal (Siberia), hold about 200 species each, of which 90% are endemic (Martens, 1994; Martens & Schön, 1999; Martens et al., 2008). The importance of ostracods from ancient lakes is also illustrated by the fact that ancient lakes contribute 25% of the known specific diversity of all Recent, non-marine ostracods in the world (Martens et al., 2008). However, up to now, they have not been investigated in depth with DNA sequencing analyses with the exception of a short note by Schön et al. (2000a).

In this article, we report on a comparative study of two ostracod species flocks from the same subfamily from the two most ancient lakes in the world: the boreal LB, the oldest lake of the world with 28–30 myr of age (Logatchev, 1993; Mats, 1993; Martin, 1994), and the tropical LT, being 9–12 myr old (Cohen et al., 1993, 1997). The Baikalian *Cytherissa* flock consists of about 50 endemic ostracod species and subspecies in a single genus (Mazepova, 1990, 1994). The *Cyprideis* flock from LT has more than 16 endemic species in at least five genera, with a higher morphological variability than the *Cytherissa* flock (Wouters & Martens, 1992, 1994, 1999, 2000, 2001, 2007, 2008).

We analyse DNA sequence data from the mitochondrial COI gene and the nuclear ITS1 region for both ostracod species flocks. Our main aim is to unravel phylogenetic relationships at the species and genus level and to use these phylogenies to compare the evolution of the two ostracod species flocks in two lakes with different limnological regimes (tropical and temperate).

More specifically, we will test the following hypotheses: (1) that both ostracod species flocks are monophyletic; (2) that the *Cytherissa* and the *Cyprideis* flocks differ in rates of speciation and

substitution; (3) that the age of the Baikalian flock coincides with the shift to cold, oxygenated water in LB ca. 5–6 myr ago, whilst (4) the Tanganyikan flock is about the same age as the lake.

Materials and methods

Sampling

Ostracods were sampled during one expedition to LB in 1996 and one expedition each to LB and LT in 1997. Samples were taken randomly from boats by Ekman and PONAR grabs and Reineck boxcorers, and with hand nets during SCUBA diving. Ostracods were sorted alive in the field with binocular microscopes and stored in absolute EtOH at 4°C.

Table 1 provides an overview of the analysed samples.

DNA extraction, PCR amplification and automatic sequencing

DNA was extracted with the Chelex method (Walsh et al., 1991), either from whole, individual ostracods or from soft parts if valves were kept for morphological analyses. 5–8 µl of the supernatant were used for PCR amplification with 0.5 U *Taq* DNA polymerase (Amersham Pharmacia Biotech.) with the provided buffer, 1.5 mM MgCl₂, 10 mM dNTPs, 10 pmol of each primer and mineral oil in 25 µl volumes in a 480 DNA thermal cycler (Applied Biosystems). A manual hot start (denaturation step of 5 min without *Taq* DNA polymerase prior to PCR cycles) was applied to increase PCR specificity. For more details on the PCR conditions and automatic sequencing, which was conducted on an ALF express[®] (Amersham Pharmacia Biotech.), see Schön (2001). PCRs and automatic sequencing were conducted with universal primers for COI (Folmer et al., 1994) and ITS (White et al., 1990) and specific primers (Schön, 2001).

Sequence analyses

Sequences were automatically processed using the ALFwin[®] Sequence Analyzer 2.11 (Amersham Pharmacia Biotech.) and checked by eye. After the forward and reverse strand had been aligned with ClustalX (Larkin et al., 2007) for each individual ostracod,

Table 1 Ostracod samples from which ITS or COI or both were amplified

DNA extraction abbreviation	Lake	COO	Species name	COI	ITS
25Clacu	Baikal		<i>Cytherissa lacustris</i> (Sars, 1863)		JN715664
132Clacu	Baikal		<i>Cytherissa lacustris</i>	JN715748	
135Clacu	Baikal		<i>Cytherissa lacustris</i>	JN715749	
141Csini	Baikal	53°51'N 108°40'E	<i>Cytherissa sinistrodentata</i> Bronshstein, 1930	JN715723	
137Csini	Baikal	53°50'N 108°37'E	<i>Cytherissa sinistrodentata</i>	JN715724	
138Csini	Baikal	53°50'N 108°37'E	<i>Cytherissa sinistrodentata</i>	JN715725	
140Csini	Baikal	53°50'N 108°37'E	<i>Cytherissa sinistrodentata</i>	JN715726	
304Csini	Baikal	52°45'N 106°33'E	<i>Cytherissa sinistrodentata</i>	JN715727	
143Csern	Baikal	53°50'N 108°37'E	<i>Cytherissa sernovi</i> Bronshstein, 1930	JN715730	
144Csern	Baikal	53°50'N 108°37'E	<i>Cytherissa sernovi</i>	JN715731	
146Csern	Baikal	53°50'N 108°37'E	<i>Cytherissa sernovi</i>	JN715732	
147Csern	Baikal	53°50'N 108°37'E	<i>Cytherissa sernovi</i>	JN715733	
281Csern	Baikal	53°0'N 106°56'E	<i>Cytherissa sernovi</i>	JN715734	JN715665
300Csern	Baikal	53°0'N 106°56'E	<i>Cytherissa sernovi</i>	JN715735	
326Csern	Baikal	53°0'N 106°56'E	<i>Cytherissa sernovi</i>	JN715736	
343Csern	Baikal	53°0'N 106°56'E	<i>Cytherissa sernovi</i>	JN715737	
355Csern	Baikal	53°0'N 106°56'E	<i>Cytherissa sernovi</i>	JN715738	
175Cplen	Baikal	51°43'N 103°44'E	<i>Cytherissa plena</i> Mazepova, 1985	JN715753	
176Cplen	Baikal	51°43'N 103°44'E	<i>Cytherissa plena</i>	JN715754	
177Cplen	Baikal	51°43'N 103°44'E	<i>Cytherissa plena</i>	JN715755	
164Cplsm	Baikal	51°43'N 103°44'E	<i>Cytherissa plena</i> small	JN715743	
171Cplsm	Baikal	51°43'N 103°44'E	<i>Cytherissa plena</i> small	JN715744	
172Cplsm	Baikal	51°43'N 103°44'E	<i>Cytherissa plena</i> small	JN715742	
273Cdonq	Baikal	53°51'N 108°40'E	<i>Cytherissa donquixotei</i> Mazepova, 1990		JN715673
274Cdonq	Baikal	53°51'N 108°40'E	<i>Cytherissa donquixotei</i>	AJ319740	JN715671
313Cdonq	Baikal	53°51'N 108°40'E	<i>Cytherissa donquixotei</i>	JN715751	JN715669
341Cdonq	Baikal	53°51'N 108°40'E	<i>Cytherissa donquixotei</i>	JN715752	
275Cinsu	Baikal	53°51'N 108°39'E	<i>Cytherissa sernovi insularis</i> Mazepova, 1990	JN715739	
276Cparv	Baikal	53°51'N 108°39'E	<i>Cytherissa parva</i> Mazepova, 1984		JN715680
282Cparv	Baikal	53°0'N 106°55'E	<i>Cytherissa parva</i>		JN715679
318Cparv	Baikal	53°0'N 106°55'E	<i>Cytherissa parva</i>	JN715750	
283Ctuber	Baikal	53°0'N 106°56'E	<i>Cytherissa tuberculata</i> Bronshstein, 1930		JN715676
296Ctuber	Baikal	52°45'N 106°33'E	<i>Cytherissa tuberculata</i>	JN715741	
285Ctria	Baikal	53°0'N 106°58'E	<i>Cytherissa triangulata</i> Bronshstein, 1947	JN715774	
286Ctrunc	Baikal	52°45'N 106°33'E	<i>Cytherissa truncata</i> Bronshstein, 1930	JN715778	JN715682
278Celon	Baikal	52°45'N 106°33'E	<i>Cytherissa elongata</i> Bronshstein, 1947	AJ319741	
287Celon	Baikal	52°45'N 106°33'E	<i>Cytherissa elongata</i>	JN715760	
289Celon	Baikal	53°12'N 107°21'E	<i>Cytherissa elongata</i>	JN715761	JN715681
314Celon	Baikal	52°56'N 106°55'E	<i>Cytherissa elongata</i>	JN715763	JN715677
319Celon	Baikal	53°12'N 107°21'E	<i>Cytherissa elongata</i>	JN715764	
340Celon	Baikal	52°45'N 106°33'E	<i>Cytherissa elongata</i>	JN715762	
290Cparal	Baikal	52°56'N 106°55'E	<i>Cytherissa parallela</i> Bronshstein, 1947		JN715670

Table 1 continued

DNA extraction abbreviation	Lake	COO	Species name	COI	ITS
301Cparal	Baikal	52°56'N 106°55'E	<i>Cytherissa parallela</i>	JN715758	
303Cparal	Baikal	52°56'N 106°55'E	<i>Cytherissa parallela</i>	JN715759	JN715666
279Cgoly	Baikal	53°51'N 108°40'E	<i>Cytherissa golyschkiniae</i> Mazepova, 1990	JN715777	
299Cgoly	Baikal	53°51'N 108°40'E	<i>Cytherissa golyschkiniae</i>		JN715683
302Clata	Baikal	53°0'N 106°56'E	<i>Cytherissa lata</i> Bronshtein, 1930	JN715765	JN715674
306Clata	Baikal	53°0'N 106°56'E	<i>Cytherissa lata</i>	JN715766	
321Clata	Baikal	53°0'N 106°56'E	<i>Cytherissa lata</i>	JN715767	
305Cpter	Baikal	53°0'N 106°56'E	<i>Cytherissa pterygota</i> Bronshtein, 1947	JN715775	JN715667
329Cpter	Baikal	53°0'N 106°56'E	<i>Cytherissa pterygota</i>	JN715776	
310Cpusi	Baikal	52°45'N 106°33'E	<i>Cytherissa pusilla</i> Mazepova, 1985	JN715745	JN715675
323Cpusi	Baikal	52°45'N 106°33'E	<i>Cytherissa pusilla</i>	JN715746	
334Cpusi	Baikal	52°45'N 106°33'E	<i>Cytherissa pusilla</i>	JN715747	JN715672
158Cnspe	Baikal	51°43'N 103°44'E	<i>Cytherissa nov. spec.1</i>	JN715728	
159Cnspe	Baikal	51°43'N 103°44'E	<i>Cytherissa nov. spec.1</i>	JN715729	
311Cnsp	Baikal	53°51'N 108°40'E	<i>Cytherissa nov. spec.2</i>	JN715772	JN715678
332Cnsp	Baikal	52°45'N 106°33'E	<i>Cytherissa nov. spec.2</i>	JN715773	
284Cverru	Baikal	53°51'N 108°40'E	<i>Cytherissa verrucosa</i> Mazepova, 1990	JN715769	
309Cverru	Baikal	53°51'N 108°40'E	<i>Cytherissa verrucosa</i>	JN715768	
325Cverru	Baikal	53°51'N 108°40'E	<i>Cytherissa verrucosa</i>	JN715770	JN715668
339Cverru	Baikal	53°51'N 108°40'E	<i>Cytherissa verrucosa</i>	JN715771	
322Ccrep	Baikal	52°12'N 107°21'E	<i>Cytherissa crepera</i> Mazepova, 1990	JN715740	
320Ccyth	Baikal	52°56'N 106°55'E	<i>Cytherissa cytheriformis</i> Bronshtein, 1947	JN715756	
327Ccyth	Baikal	52°45'N 106°33'E	<i>Cytherissa cytheriformis</i>	JN715757	
229Rampla	Tanganyika	4°46'S 29°36'E	<i>Romecytheridea ampla</i> Wouters, 1988		JN715687
212Rampla	Tanganyika	4°46'S 29°29'E	<i>Romecytheridea ampla</i>		JN715690
230Rampla	Tanganyika	4°46'S 29°29'E	<i>Romecytheridea ampla</i>	JN715780	JN715692
414Rampla	Tanganyika	3°41'S 55°29'E	<i>Romecytheridea ampla</i>		JN715693
434Rampla	Tanganyika	4°38'S 7°29'E	<i>Romecytheridea ampla</i>		JN715684
441Rampla	Tanganyika	4°38'S 29°37'E	<i>Romecytheridea ampla</i>		JN715686
455Rampla	Tanganyika	4°38'S 7°29'E	<i>Romecytheridea ampla</i>	JN715779	
457Rampla	Tanganyika	4°38'S 29°37'E	<i>Romecytheridea ampla</i>		JN715688
459Rampla	Tanganyika	4°38'S 7°29'E	<i>Romecytheridea ampla</i>		JN715685
470Rampla	Tanganyika	4°38'S 29°37'E	<i>Romecytheridea ampla</i>		JN715689
473Rampla	Tanganyika	4°38'S 7°29'E	<i>Romecytheridea ampla</i>		JN715691
219Rlong	Tanganyika	4°46'S 27°29'E	<i>Romecytheridea longior</i> Wouters & Martens, 1999		JN715694
231Rlong	Tanganyika	4°46'S 27°29'E	<i>Romecytheridea longior</i>	JN715785	JN715695
436Rtenis	Tanganyika	4°54'S 29°35'E	<i>Romecytheridea tenuisculpta</i> (Rome, 1962)		JN715712
472Rtenis	Tanganyika	4°54'S 29°35'E	<i>Romecytheridea tenuisculpta</i>		JN715711
475Rtenis	Tanganyika	4°54'S 29°35'E	<i>Romecytheridea tenuisculpta</i>	JN715788	JN715713
445Tcalj	Tanganyika	4°54'S 29°35'E	<i>Tanganyikacythere caljoni</i> Wouters & Martens, 1994	JN715789	JN715702

Table 1 continued

DNA extraction abbreviation	Lake	COO	Species name	COI	ITS
450Tcalj	Tanganyika	4°38'S 22°29'E	<i>Tanganyikacythere caljoni</i>		JN715698
476Tcalj	Tanganyika	4°38'S 22°29'E	<i>Tanganyikacythere caljoni</i>		JN715701
216Tburt	Tanganyika	4°46'S 27°29'E	<i>Tanganyikacythere burtonensis</i> Ducasse & Carbonel, 1993		JN715699
225Tburt	Tanganyika	4°46'S 27°29'E	<i>Tanganyikacythere burtonensis</i>		JN715703
226Tburt	Tanganyika	4°46'S 27°29'E	<i>Tanganyikacythere burtonensis</i>	JN715784	JN715704
227 Tburt	Tanganyika	4°46'S 27°29'E	<i>Tanganyikacythere burtonensis</i>	JN715783	
464Tburt	Tanganyika	4°38'S 22°29'E	<i>Tanganyikacythere burtonensis</i>	JN715790	JN715700
210Mirsa	Tanganyika	4°46'S 27°29'E	<i>Mesocyprideis irsacae</i> (Kiss, 1959)		JN715721
211Mirsa	Tanganyika	4°46'S 27°29'E	<i>Mesocyprideis irsacae</i>		JN715720
217Mirsa	Tanganyika	4°46'S 27°29'E	<i>Mesocyprideis irsacae</i>	AJ319742	JN715719
228Mirsa	Tanganyika	4°46'S 27°29'E	<i>Mesocyprideis irsacae</i>	JN715782	AJ534420
416Mirsa	Tanganyika	3°34'S 22°29'E	<i>Mesocyprideis irsacae</i>		JN715710
418Mirsa	Tanganyika	3°34'S 22°29'E	<i>Mesocyprideis irsacae</i>		JN715722
454Mirsa	Tanganyika	4°38'S 22°29'E	<i>Mesocyprideis irsacae</i>	JN715781	JN715708
453Mnsp	Tanganyika	4°38'S 22°29'E	<i>Mesocyprideis nov. spec.</i>		JN715718
471Mpi	Tanganyika	4°38'S 22°29'E	<i>Mesocyprideis pila</i> Wouters & Martens, 1999		JN715709
461Cspat	Tanganyika	4°38'S 22°29'E	<i>Cyprideis spatula</i> Wouters & Martens, 1999		JN715707
468Cspat	Tanganyika	4°38'S 7°29'E	<i>Cyprideis spatula</i>		JN715705
474Cspat	Tanganyika	4°38'S 22°29'E	<i>Cyprideis spatula</i>		JN715706
449Atub	Tanganyika	4°38'S 22°29'E	<i>Archaeocyprideis tuberculata</i> Ducasse & Carbonel, 1994		JN715716
463Atub	Tanganyika	4°46'S 27°29'E	<i>Archaeocyprideis tuberculata</i>		JN715715
469Atub	Tanganyika	4°46'S 27°29'E	<i>Archaeocyprideis tuberculata</i>		JN715714
477Atub	Tanganyika	4°46'S 27°29'E	<i>Archaeocyprideis tuberculata</i>		JN715717
413Kbrac	Tanganyika	4°46'S 27°29'E	<i>Kavalacythereis braconensis</i> Wouters, 1979	JN715787	JN715696
444Kbrac	Tanganyika	4°38'S 22°29'E	<i>Kavalacythereis braconensis</i>	JN715786	JN715697
Linopinata	Outgroup		<i>Limnocythere inopinata</i> (Baird, 1843)	AJ534412	AJ534423

Numbers are Genbank accession numbers

COO coordinates

chromatograms were further verified manually. Sequences were trimmed to equal lengths before any analyses. Genetic identity of all sequences was confirmed by BLAST searches (Altschul et al., 1990) in Genbank.

Phylogenetic analyses, rates of divergence, substitution rates and genetic distances

The sequences of both flocks were aligned for COI and ITS1, respectively, together with *Limnocythere inopinata*

as outgroup with CLUSTALX. jModeltest (Posada, 2008) was used to identify the appropriate model of molecular evolution. For Maximum-Likelihood analyses with PHYML 3.0 (Guindon & Gascuel, 2003) and estimates of genetic diversity with PAUP (Swofford, 1998), all possible 88 models were tested in jModeltest, whilst only 24 models were tested for Bayesian inference (BI) with Mr Bayes 3.1.2 (Huelsenbeck & Ronquist, 2001) and TREE-PUZZLE 5.0 (Schmidt et al., 2002).

Phylogenetic analyses were conducted using PHYML with the model identified by the AICc

criterion in jModeltest, a BIONJ starting tree and the following options: optimised tree topologies, slow, accurate tree topology search and 1,000 bootstrap replicates. Phylogenetic analyses with BI were conducted using Mr Bayes with two independent runs and the model identified by jModeltest for 4–10 million generations (depending on when the standard deviation of split frequencies was below 0.01 and the potential scale reduction factor was close to 1.0), and sampling frequencies of every 100th generation. The first 25% of trees were disregarded as burn-in.

We calculated the consensus tree of the phylogenies with bootstraps obtained with PHYML in MEGA 5.0 (Tamura et al., 2011). All obtained phylogenies were rooted with the outgroup and visualised with Tree-View (Page, 1996) and MEGA.

For comparisons of divergence rates and substitution rates, we pruned the original datasets by removing sequences from conspecifics. As described in “Results” section, several sequences from so-called conspecifics grouped separately in the phylogenetic trees. We kept these sequences in the pruned data sets because they most likely resemble cryptic species, novel species or misidentifications (see also discussion below).

To compare divergence rates between the two ostracod flocks, we tested for departures from the pure-birth model of cladogenesis. For this purpose, ultrametric trees were constructed with PATHd8 from COI sequence data for both lakes together and for Baikal and Tanganyika separately. We then randomly resolved tree polytomies in Ape (Paradis et al., 2004; R working environment) and conducted lineage-through-time plots for these trees. We also estimated gamma statistics of inter-node intervals in our COI phylogenies (Pybus & Harvey, 2000) and tested whether the obtained gamma statistics were significantly different from constant rates of clade diversification in Ape with one-tailed tests. Because our lake sampling was incomplete, we used Monte Carlo simulations in LASER (Rabosky, 2006) to check whether the gamma values remained statistically significant with our incomplete sampling. For this purpose, 10,000 replications were applied to the expected and sampled number of species for each ostracod flock, together with the estimated gamma values. Because of the low resolution of ITS (see below), we limited our analyses of divergence rates through time to the COI data.

We applied two kinds of comparative tests for deviations from clock-like substitution rates. First, we

used PATHd8 (Britton et al., 2007) to test for deviations from a molecular clock for individual edge lengths of nodes from the COI and ITS tree with the data sets including both flocks.

Second, we conducted likelihood ratio tests for the pruned data sets (without conspecifics) by constructing phylogenies with and without clock assumption in TREE-PUZZLE and MrBayes without the outgroup (Lemey et al., 2009). When using BI, we followed the procedure by Wilke et al. (2009).

We also estimated genetic distances as p distances between and within the two ostracod flocks with MEGA 5.0 to verify whether the Tanganyikan flock with its higher morphological variability was also genetically more variable.

Age estimates

Following Wilke et al. (2009), we first checked with DAMBE (Xia & Xie, 2001) whether the COI data set and tree were saturated. Because of the multifurcations in the Baikalian COI tree (see below), more sophisticated analyses for age estimates as for example with Multidiv (Thorne & Kishino, 2002) or mcmctree in PAML 4.4 (Yang, 2007) could not be used. Instead, we conducted age estimates of the Bayesian COI tree with PATHd8 (Britton et al., 2007), which uses non-parametric rate smoothing and can handle partly unresolved phylogenies. Rates are estimated sequentially by taking average path lengths from internodes to terminal taxa. Smoothing occurs locally and not simultaneously over the whole tree. The root age was fixed to 1 in ultrametric trees because we could only provide a single age constraint for each tree. Trees were subsequently scaled with four different calibration points (see below).

Although ostracods have one of the best fossil records of the animal kingdom, no fossil ostracods are yet available from LB and LT for absolute dating. We therefore had to revert to other approaches to obtain calibration points. Various ancient lake taxa have radiated within each of these two ancient lakes (Meyer, 1993; Kontula et al., 2003; Verheyen et al., 2003; Macdonald et al., 2005); thus, it seems reasonable to use the maximal geological age of LT and LB, of 12 myr (Cohen et al., 1993, 1997) and 28 myr (Logatchev, 1993; Mats, 1993; Martin, 1994), respectively, as calibration points. As a second approach, we used fossil data of *Cytherissa*. Twenty-five myr is the minimal age

of the genus, because its first fossil appearance is in the Palaeogene (Danielopol et al., 1990). We also took 8 myr as an alternative calibration point from the fossil record, when ca. 10 fossil *Cytherissa* species were widespread during the Upper Miocene in Eurasia (Danielopol et al., 1990). Recent species of *Cytherissa* are endemic to LB, with the exception of asexual populations of *Cytherissa lacustris* with a Holarctic distribution (which appear to be a recent spin-off of the sexual, Baikalian *Cytherissa lacustris*, Schön et al., 2000a), four (subfossil) *Cytherissa* species from the Caspian Sea (Boomer et al., 2005) and three (subfossil) from Chinese lakes (Yu et al., 2009), respectively, that occurred until recently. As a final approach, we also applied a general COI clock to the average, interspecific COI distances for each ostracod flock. For these relative age estimates, we calculated the interspecific genetic distances anew with the model parameters from jModeltest in PAUP and excluded the taxa for which PATHd8 had identified significant deviations from the molecular clock at their nodes.

We then divided the obtained interspecific genetic distances by two to obtain substitution rates and used the COI invertebrate clock of Wilke et al. (2009) with the HKY+I+ γ model (1.53% per myr) to calculate relative age estimates. Standard deviations of our relative age estimates were calculated from the standard deviations of the genetic distances.

Results

We obtained 68 new sequences for COI of 450 base pairs, 12 from LT (for seven species and four genera out of 16 species and six genera) and 56 from LB (for 22 species out of ca. 50 species) and 58 new sequences for ITS1 of 450 base pairs (38 from LT, representing 11 species and 6 genera, and 20 from LB representing 14 species), respectively. Thus, our COI phylogenies include approximately half of the Baikalian *Cytherissa* species, one third of the Tanganyikan *Cyprideis* species and two thirds of the Tanganyikan genera. For ITS, representatives of about 33% of the Baikalian and 75% of the Tanganyikan species and all Tanganyikan genera could be sequenced. Thus, our samples are representative for the overall ostracod diversity in both lakes.

All sequences gave low E-values in BLAST searches and matched with COI or ITS regions from

other ostracods or arthropods. Sequences have been submitted to Genbank (accession numbers AJ319740–AJ319742, AJ534412, AJ534420, AJ534423 and JN715664–JN715790; see Table 1). Because of the small size of ostracods and the fact that Chelex was used for DNA extractions, which prevents long-term storage, it was not possible to obtain a congruent data set for both markers from the two species flocks. Table 2 summarises the results of jModeltest for the two data sets.

Phylogenies

As we observed deviations in the clock-like rate of substitutions between both flocks (see below), we only show the Bayesian trees here, which we consider more accurate since BIs are less susceptible to rate differences (Huelsenbeck & Ronquist, 2001; see also below). The Bayesian consensus tree for COI, a phylogram with mean branch lengths and posterior probabilities for each split (Fig. 1), shows a clear separation between the two flocks according to the lake with high posterior probabilities. This is also supported by the bootstrap values from the ML tree (Fig. 1). Both flocks appear to be monophyletic in comparison to the outgroup (see Fig. 1 and below). The terminal branches of the *Cyprideis*-phylogenies from LT are supported with both methods for tree construction, whilst the interior, longer branches have lower posterior probabilities of 0.52–0.63 (Fig. 1). The ML tree (not shown) displays two sequences of *Tanganyikacythere burtonensis* as most basal, whereas the Bayesian analysis puts the two specimens of *Kavalacythereis braconensis* (with the most aberrant valve morphology) as basal (Fig. 1). Apart from the latter species, the *Tanganyikacythere* lineage is supported by a posterior probability of 1.0. The other two phylogenetic groups, with *Romecytheridea tenisculptata* and *R. longiformes* on one hand and *Mesocyprideis irsacae* and *R. ampla* on the other hand, are less well supported with posterior probabilities of 0.70 and 0.71, respectively, and lower bootstrap values.

In contrast to the Tanganyikan *Cyprideis* flock, the Baikalian *Cytherissa* flock shows many multifurcations and neither the Bayesian tree (Fig. 1) nor the ML tree (not shown) are fully resolved, except for the two specimens of *C. tuberculata* (296Ctuber) and *C. parva* (318Cparv) that cluster outside of the remaining flock with high statistical support. We can distinguish at

Table 2 Models identified with the AICc criterion in jModeltest (Posada, 2008)

Marker	Dataset	–Ln; Model	Parameters
COI	LB & LT; all 88 models	9187.7671; TPM1uf+ γ	freqA = 0.2417; freqC = 0.2271; freqG = 0.2101; freqT = 0.3211; [AC] = 1.0000; [AG] = 6.2730; [AT] = 1.2748; [CG] = 1.2748; [CT] = 6.2730; [GT] = 1.0000 Gamma shape = 0.3200
COI	LB & LT; 24 models	8041.0052; HKY+I+ γ	freqA = 0.2334; freqC = 0.2328; freqG = 0.2104; freqT = 0.3234 kappa = 6.6086 (ti/tv = 3.3304) p-inv = 0.3810 Gamma shape = 0.7560
ITS	LB & LT; all 88 models	2350.0290; TPM2+ γ	[AC] = 0.5669; [AG] = 2.1020; [AT] = 0.5669; [CG] = 1.0000; [CT] = 2.1020; [GT] = 1.0000 Gamma shape = 0.3650
ITS	LB & LT; 24 models	3329.7795; SYM+ γ	[AC] = 0.4861; [AG] = 2.2435; [AT] = 1.0500; [CG] = 1.5240; [CT] = 1.9388; [GT] = 1.0000 Gamma shape = 0.4100

LB Lake Baikal; LT Lake Tanganyika; –Ln log likelihood value

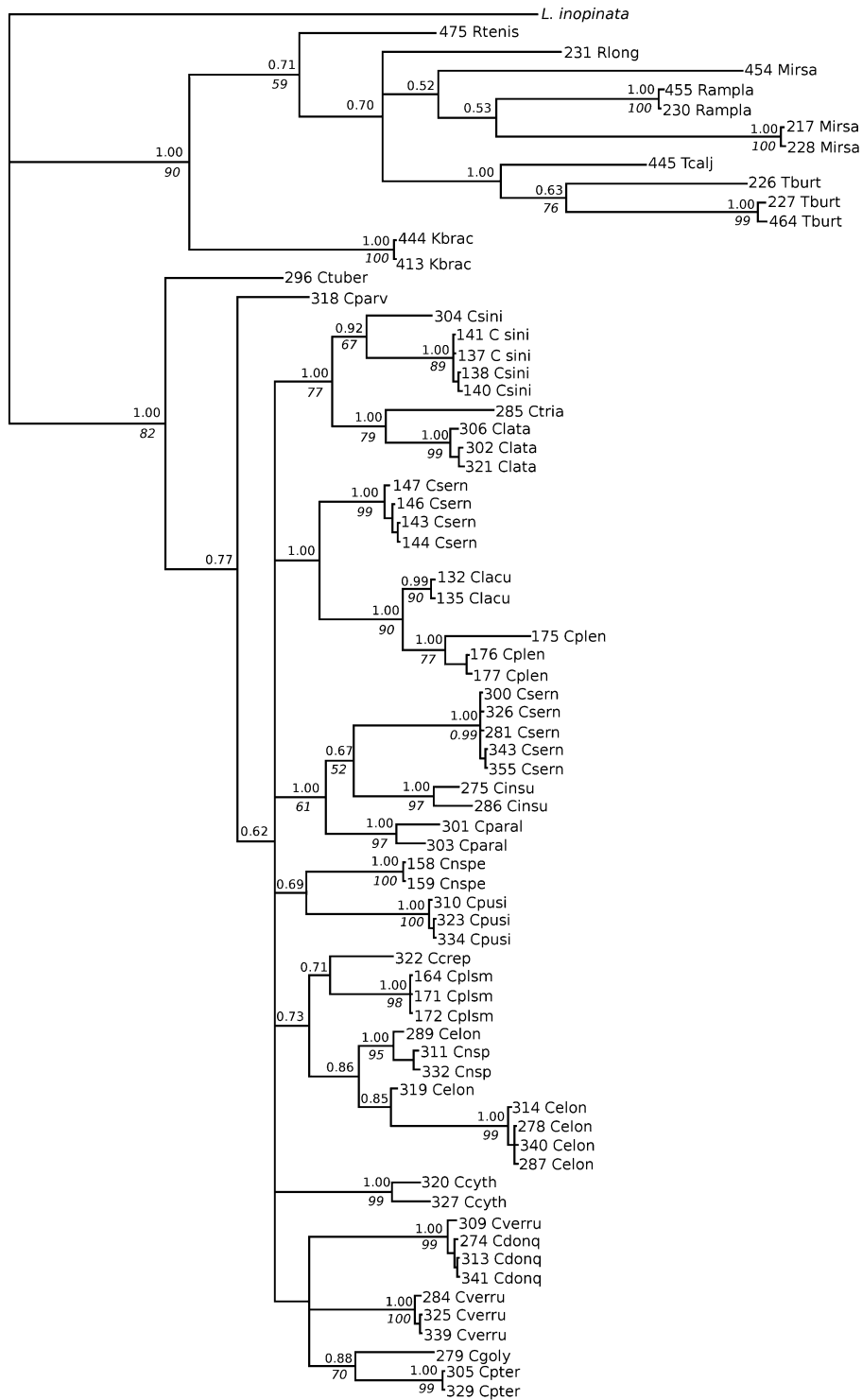
least four other clades with good support and posterior probabilities of 1.0: (1) *C. sinistrodentata/C. triangulata/C. lata*; (2) *C. sernovii/C. lacustris/C. plena*; (3) *C. sernovii/C. insularis/C. truncata/C. parallela*; and (4) *C. cytheriformes*. The remaining three phylogenetic clades with *C. nov. spec.1/C. pusilla*; *C. creperal/C. plena small/C. elongata/C. nov. spec.2*; *C. verrucosa/C. donquixotei/C. golyschkinal/C. pterygota* are statistically less well supported (posterior probabilities between 0.69 and 0.77; Fig. 1). How these lineages are related to each other, could not be derived because of the multifurcations. There are several examples where COI sequences from specimens tentatively identified as belonging to the same morphospecies did not cluster together, e.g. in *Mesocyprideis irsacae* and *Tanganyikacythere burtonensis* in the *Cyprideis* flock and *C. sernovi* and *C. elongata* in the *Cytherissa* flock (see Fig. 1). As described above, these sequences were kept in the pruned data sets.

With the ITS1 sequence data, the two flocks are clearly separated, but even more multifurcations are observed, making it more difficult to resolve the phylogenetic relationships within each ostracod flock (see Fig. 2). This fits with the overall lower genetic variability of ITS as compared to COI (see below). For the *Cyprideis* flock, ITS was more useful as seven different lineages could be identified with posterior probabilities of 1.0 within this flock, mostly following taxonomic identifications, and *Kavalacythereis braconensis* appeared again as most the basal branch, similarly

to the Bayesian COI tree. Most Tanganyikan species from the same genus clustered together with high posterior probabilities and bootstrap supports, although there were outsiders (such as one specimen of *Tanganyikacythere caljoni*) or tentative conspecifics that were divided into several groups (e.g. *Mesocyprideis irsacae* and *T. burtonensis*). Finally, *Romecytheridea tenuisculptata* and *Mesocyprideis nov. spec.* might represent new genera as they did not cluster with the other *Romecytheridea* and *Mesocyprideis* species. The branches in the Baikalian *Cytherissa* flock were even shallower, which made it impossible to verify any phylogenetic relationships with this marker (see Fig. 2).

Diversification rates of ostracod flocks

Lineage-through-time plots (Fig. 3) indicate that the Baikalian *Cytherissa* flock might have shown an increase in its diversification rate in its more distant past. This was confirmed by the negative gamma parameter for the Baikalian COI tree (see Table 3). Statistical analyses of the gamma parameters with Monte Carlo simulations revealed that the divergence rate of the Baikalian flock was significantly different from constant diversification rates through time (Table 3), even if our sampling was incomplete. The negative gamma parameter indicates that diversification was shifted towards the root, thus to older evolutionary times. Although the Tanganyikan *Cyprideis* flock showed some indications for an unequal diversification rate through time, the gamma parameter



0.1

Fig. 1 Consensus Bayesian tree, constructed from COI sequence data with MrBayes (Huelsenbeck & Ronquist, 2001). For species names, see Table 1. Numbers above branches are Bayesian

posterior probabilities, numbers below branches in italics are bootstrap values of 1,000 replicates from the Maximum-Likelihood analyses in PHYML (Guindon & Gascuel, 2003)

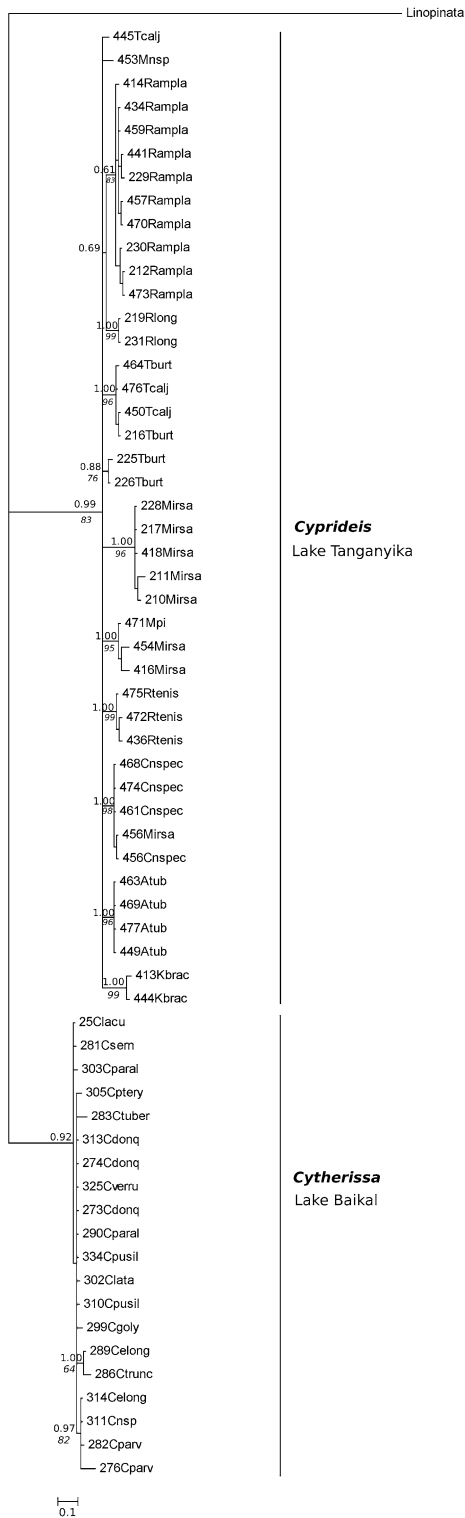


Fig. 2 Consensus Bayesian tree, constructed from ITS1 sequence data with MrBayes (Huelsenbeck & Ronquist, 2001). For species names, see Table 1. Numbers above branches are Bayesian posterior probabilities, numbers below branches in italics are bootstrap values of 1,000 replicates from the Maximum-Likelihood analyses in PHYML (Guindon & Gascuel, 2003)

did not remain statistically significant after the simulations for incomplete sampling (see Table 3).

Genetic distances and substitution rates

Regardless of the marker used to estimate genetic p distances, ostracod species within the Tanganyikan flock were genetically more variable than ostracods from LB (see Fig. 3a, b).

Comparative likelihood ratio tests based on the maximum-likelihood trees in TREE-PUZZLE showed significant deviations from a clock-like rate for all data sets except for ITS within the Tanganyikan flock (Table 4). When applying similar comparative tests to BI, we found evidence for a violation of clock-like substitution rates for COI when the two ostracod flocks were analysed together and the Baikalian flock alone (Table 4). Substitution rates of COI within the Baikalian flock were also not clock-like if the two Baikalian taxa with long branches in the COI tree, 316Cparva and 298Ctube (see Fig. 1) were excluded from the data set (Table 4). No ITS phylogeny showed deviations from clock-like substitution rates with comparative likelihood ratio tests based on BIs (Table 4).

Age estimates

We did not find significant evidence for saturation of the COI data set with DAMBE (see above). Because of the unresolved phylogenies (see above), we conducted the analysis with PATHd8 to allow for rate smoothing across the COI tree. In the ultrametric COI tree (see Fig. 5), the maximal relative age of the Baikalian flock between the two branches “296Ctuber” and “279Cgol” was half of the maximum age of the Tanganyikan flock (between “475Rtenis” and “413Kbrac”). Depending on which of the four calibration points was used, the absolute age of the Tanganyikan *Cyprideis* flock varied between 11.6 and 52.4 myr and the absolute age of the Baikalian *Cytherissa* flock between 6.2 and 28 myr (see Table 5). If we used the invertebrate COI clock of Wilke et al. (2009) as described above, the average age

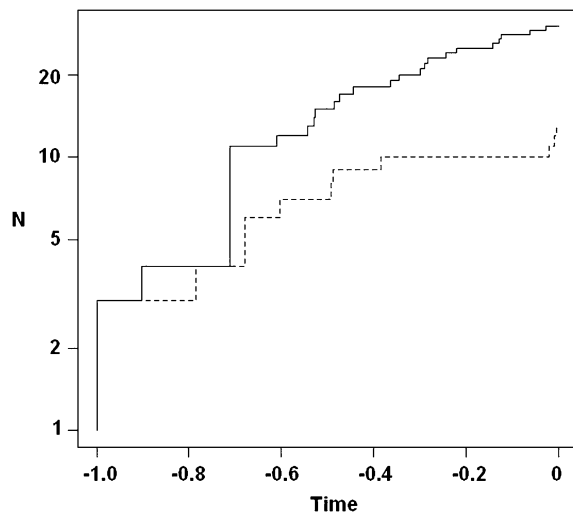


Fig. 3 Lineage-through-time plots of the two ostracod flocks for COI. Lineage-through-time plots were conducted with Ape (Paradis et al., 2004) from ultrametric trees. Multifurcations were randomly resolved in Ape. The *solid line* represents the plot for the *Cytherissa* flock from LB, the *dotted line* the plot for the *Cyprideis* flock from Lake Tanganyika

estimate for the Tanganyikan flock was 12.5 myr (minimum) to 16.9 myr (maximum) and for the Baikalian flock 7.2–10.4 myr.

The ultrametric tree for the complete COI data set was scaled with a maximum age of 15 myr (Fig. 5; see “Discussion” section below why we chose this age estimate). This would set the first appearance of a Baikalian species, *Cytherissa tuberculata*, to 8.4 myr, followed by *C. parva* 6 myr ago, whilst the majority of lineages would have originated 5.25 myr ago. In the ultrametric trees, Tanganyikan ostracods would have originated stepwise, starting with *Kavalacythereis braconensis* around 15 myr ago, followed by *Romeocythere tenisculptata* (12 myr), *R. longiformes*

(11 myr), and *Tanganyikacythere*, *R. ampla* and *Mesocyprideis* around 10 myr ago.

Discussion

Monophyly of species flocks and diversification rates

Our phylogenetic reconstructions based on COI and ITS reveal a monophyletic origin of both ostracod flocks. This result needs to be further confirmed in the future with more closely related outgroups. How the different species and genera of the Tanganyikan *Cyprideis* flock are related to each other, could be determined, except for the exact position of *Mesocyprideis irsacae* and *Romeocytherea ampla*. This flock, moreover, seems to have evolved with constant divergence rates through time (Fig. 3).

The relationships within the Baikalian *Cytherissa* flock could not be resolved (see the Bayesian COI tree in Fig. 1 and the Bayesian ITS tree in Fig. 2) and showed many multifurcations. Also with other methods of tree constructions such as ML or Maximum Parsimony (trees are available on request from the first author), we obtained unresolved COI phylogenies. Whether one can indeed identify seven different *Cytherissa* lineages as described above, remains to be further investigated with additional samples and more variable markers, which will hopefully provide phylogenetic resolutions with better statistical support.

The unresolved phylogenies could be owing to an early, explosive radiation of the Baikalian *Cytherissa* flock. Our lineage-through-time plots (Fig. 3) and the tests for differences in divergence rates of COI (Table 3) confirm this explosive radiation, as is obvious from the

Table 3 Tests for constant divergence rates through time with pure-birth models

Marker	Dataset	Number of taxa in pruned data sets	Gamma parameter & <i>P</i> value	Gamma parameter & <i>P</i> from mcr simulation
COI	LB, LT, outgroup	42	−0.876 (0.3801)	NA
COI	LB	32	−3.158** (0.0016)	−2.457 (0.0085)
COI	LT	9	1.997** (0.0003)	−1.982 (0.9951)

The table shows estimates of gamma parameters for divergence rates through time (Pybus & Harvey, 2000) and results of one-tailed statistical tests for constant divergence rates through time (*P*) that were conducted with Ape (Paradis et al., 2004)

LB Lake Baikal; LT Lake Tanganyika

mcr Monte Carlo simulations for incomplete data sets that were conducted in LASER (Rabosky, 2006)

** *P* < 0.01

Table 4 Results of comparative likelihood ratio tests for clock-wise rates of substitutions

Marker	Dataset	Phylogenetic method	Log <i>L</i> unconstrained (no clock)	Log <i>L</i> constraint (clock)	2 delta Log <i>L</i>
COI	LB & LT	ML, Quartet ^a	-8020.24	-8240.41	440.34***; df = 37; 73.88***; df = 37; 253.96***; df = 35; 65.56**; df = 35; 48.00**; df = 28; 99.96***; df = 28; 108.89***; df = 26; 46.17**; df = 26; 50.75***; df = 7; 12.09; df = 7
	LB & LT	Bayesian ^b	-7393.102	-7430.042	
	LB & LT [§]	ML, Quartet ^a	-8452.43	-8579.41	
	LB & LT [§]	Bayesian ^b	-7066.203	-7098.984	
	LB	Bayesian ^b	-5116.291	-5140.970	
	LB	ML, Quartet ^a	-5468.89	-5518.87	
	LB [§]	ML, Quartet ^a	-5051.69	-5106.13	
	LB [§]	Bayesian ^b	-4782.655	-4805.741	
	LT	ML, Quartet ^a	-3049.96	-3075.34	
	LT	Bayesian ^b	-2746.55	-2752.60	
	ITS	LB & LT	ML, Quartet ^a	-2009.74	
LB & LT		Bayesian ^b	-1983.52	-2002.80	
LB		ML, Quartet ^a	-834.05	-862.24	
LB		Bayesian ^b	-848.31	-853.88	
LT		ML, Quartet ^a	-1407.44	-1421.63	
LT		Bayesian ^b	-1446.07	-1454.98	

All the tests were conducted with pruned data sets (without conspecifics) and without outgroups

L Likelihood; *df* degrees of freedom for χ^2 distribution

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; [§] further pruned data set, without 316Cparva and 298Ctube

^a TREE-PUZZLE (Schmidt et al., 2002)

^b MrBayes (Huelsenbeck & Ronquist, 2001)

Table 5 Age estimates of both flocks

Calibration point	Maximal absolute age of the Tanganyikan <i>Cyprideis</i> flock (myr)	Maximal absolute age of the Baikalian <i>Cytherissa</i> flock (myr)
28 myr (maximal age of LB) ^a	52.4	28
25 myr (first fossil appearance of <i>Cytherissa tumula</i>) ^c	46.8	25
12 myr (maximal age of Lake Tanganyika) ^b	11.6	6.2
8 myr (wide fossil appearance of 10 <i>Cytherissa</i> species in Eurasia) ^c	15	8
	Relative age estimate of the Tanganyikan <i>Cyprideis</i> flock	Relative age estimate of the Baikalian <i>Cytherissa</i> flock
Invertebrate COI clock ^d	14.73 ± 2.21 myr	8.80 ± 1.57 myr

Absolute age estimates were obtained from ultrametric trees, for which the root age was set to 1 in PATHd8 (Britton et al., 2007) and scaling trees with four different calibration points. Relative age estimates are based on the COI clock for invertebrates by Wilke et al. (2009) with the HKY+I+ γ model \pm the standard deviations of the substitution rates of the two ostracod species flocks. Ancestral polymorphisms (Wilke et al., 2009) were not considered for relative age estimates

myr million of years

^a Logatchev (1993), Mats (1993), and Martin (1994); ^b Cohen et al. (1993, 1997); ^c Danielopol et al. (1990); ^d Wilke et al. (2009)

significant gamma parameter (Pybus & Harvey, 2000; see also Table 3). The negative gamma parameter furthermore indicates that the diversification might have occurred earlier on in the history of this species flock. An explosive radiation in LB has also been postulated for some cottoid fish species (Kontula et al., 2003).

Danielopol et al. (1990) suggested that the radiation of the Baikalian *Cytherissa* flock originated through several independent invasions of the lake by different ancestral species. A similar pattern was reported by MacDonald et al. (2005) for Baikalian amphipods and by Wilson et al. (2004) for Tanganyikan gastropods.

The genus *Cytherissa* is supposed to have had a wide distribution, especially in Eurasia and even North America, during the Upper Tertiary (Miocene, >5 myr), with at least 10 (now mostly extinct) species (Danielopol et al., 1990). *Cytherissa* furthermore colonised the Eastern Paratethys during the Pliocene (ca. 5.2–1.6 myr) and at least four species are supposed to have survived in the Caspian Sea (Boomer et al., 2005) and three in Chinese lakes (Yu et al., 2009) up to recently. There were thus several candidates for multiple invasions in LB at the time that its limnological regime changed to its present state, with a permanent stratification and oxygenated abyss, 5–3 myr ago (Lukin, 1986) (see also below for further discussions on age of flocks).

Danielopol et al. (1990) postulated that the *Cytherissa* species flock in LB was polyphyletic and they recognised at least the *C. tuberculata*, the *C. sernovi* and the *C. lacustris* groups. Danielopol et al. (loc.cit.) also thought that the former two lineages might have

colonised the lake earlier than the latter. Indeed, *C. tuberculata* is positioned well outside of the main flock (Fig. 1) and seems to be the oldest lineage of the analysed species (Fig. 5). It could thus in theory have originated from a different ancestor than the rest of the flock. This species is also basal in the Baikalian flock, and might thus be derived from one of the earliest invasions in this multiple invasion scenario. However, the same is not true for the postulated *C. lacustris* and *C. sernovi* groups (Danielopol et al., 1990). The sexual form of *C. lacustris* is nested in the same clade as *C. sernovi* (see Fig. 1), and could thus not result from a different invasion. In fact, its derived position in the tree indicates that *C. lacustris*, a common, Holarctic species, might have originated as part of the Baikalian radiation (see also Schön et al., 2000a), after which the parthenogenetic forms managed to adapt to extra-lacustrine conditions and invaded the Holarctic. This could have happened fairly early on, as the earliest fossils of parthenogenetic *C. lacustris* are known from the Upper Pliocene of North Alaska and Greece (ca. 2 myr, Danielopol et al., 1990). The phylogenetic position of *C. lacustris* moreover indicates that ancient lakes such as LB are not only local hotspots of biodiversity, but could also be engines for regional, continental and even global biodiversity (Schön et al., 2000a).

Differences in genetic distances and substitution rates

If we assess the *p* distances, COI is more variable than ITS (see Fig. 4a, b). This pattern is similar to other

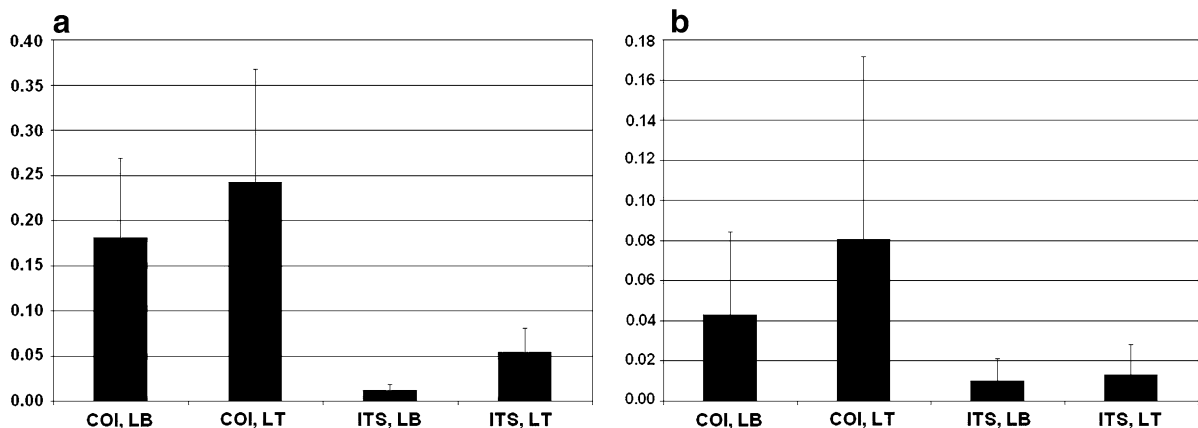


Fig. 4 Estimates of genetic distances at the inter- (a) and intra-specific (b) level. The figures show the average genetic *p* distances (y-axis) with the standard deviations. **a** At the interspecific level for LB and LT. **b** At the intraspecific level for LB and LT

studies on ostracods when both markers were used for phylogenetic (Schön et al., 1998, 2000b, 2003, 2010; Nunes Brandao et al., 2010) and phylogeographic (Schön, 2007) studies.

It is obvious from the branch lengths in both Bayesian trees (Figs. 1, 2) and from the estimated genetic distances (Fig. 4a, b) that the endemic *Cyprideis* flock from LT is more variable than the *Cytherissa* flock at both the inter- and the intraspecific levels. Thus, genetic variability matches the higher morphological variability in the *Cyprideis* flock (as exemplified by higher taxonomic resolution—see above). However, we consider the current taxonomic placement of Baikalian *Cytherissa* in the same genus as an underestimation of the real morphological variability. It would be more appropriate to place some species into different genera, because of their morphological variability, as was foreshadowed by Danielopol et al. (1990). As soon as the taxonomy of both ostracod flocks has been revised, they could present an interesting case study to test the genus concept of Martens et al. (2000) with genetic distances.

From our phylogenetic and distance analyses, it is obvious that ITS is less suitable as a molecular marker for the investigated time frames in ancient lake ostracods than COI to resolve phylogenetic relationships.

We observed deviations from clock-like substitution rates for both COI and ITS when conducting comparative likelihood ratio tests with the maximum-likelihood trees in TREE-PUZZLE (Table 4), except for ITS from the Tanganyikan flock. In contrast, comparative tests based on Bayesian trees identified deviations of COI from a clock-like rate for both flocks together, as well as for the Baikalian flock alone. These results confirm that BI is less sensitive to changes in substitution rates (Huelsenbeck & Ronquist, 2001). If the two oldest Baikalian *Cytherissa* taxa with the longest branches are excluded, there is still evidence that substitution rates of COI are not clock-like within the Baikalian flock and when comparing both ostracod species flocks. What could have triggered this possible deviation within the *Cytherissa* flock remains to be further investigated. Several factors could have caused the apparent deviation between the two flocks, and they are most likely related to the different limnological regimes of the two lakes.

Generation time might be the most obvious factor as other researchers have found that short generation times cause faster rates of molecular evolution in taxa

as different as plants (Smith & Donoghue, 2008), birds (Mooers & Harvey, 1994), primates (Sarich & Wilson, 1973), reptiles (Bromham, 2002), and 143 invertebrate species (Thomas et al., 2010). Cold, stenotherm *Cytherissa lacustris* has a generation time of 1.9–2.2 years in Austrian Mondsee, depending on the depths (Geiger, 1990) and it is reasonable to assume that this is similar in other *Cytherissa* species from cold LB. Belgian *Cyprideis torosa* take about one year to complete their life cycle, but their development has been shown to be strongly temperature-related. The species requires a minimum temperature of 15°C for development to adulthood, which is then completed within 129–152 days (Heip, 1976). From these data, we would expect that ostracods of the *Cyprideis* flock from tropical LT, with water temperatures generally between 23 and 27°C (Coulter, 1994), have at least 2–3 generations a year. This hypothesis needs to be further confirmed by life history experiments of ostracods from both ancient lakes, by estimates of substitution rates from larger genomic regions and by further investigations of the effects of selection on these ostracods.

Theoretical considerations suggest that explosive radiations, as might have occurred in the *Cytherissa* flock, could speed up substitution rates. This remains to be further tested with more variable markers, although Bromham & Woolfit (2004) did not find any evidence for faster rates when testing 19 independent instances of recent explosive radiations of island endemics from different taxa and genes.

Finally, the longer history of the *Cyprideis* flock (see below) could have caused rate changes in the distant or recent past. We did not (yet) find any evidence for this, but it is planned to verify this hypothesis in the near future by lineage-through-time plots of more extensive COI phylogenies than are currently available. Wilson et al. (2004) found no evidence for a significant acceleration of Tanganyikan gastropods with a similar approach.

Age estimates

Because there was evidence for differences in the substitution rates between the two analysed flocks (see above), it seems incorrect to apply a general COI clock without further corrections to estimate ages of the species flocks. We compensated for this using PATHd8 that accounts for rate heterogeneity. All

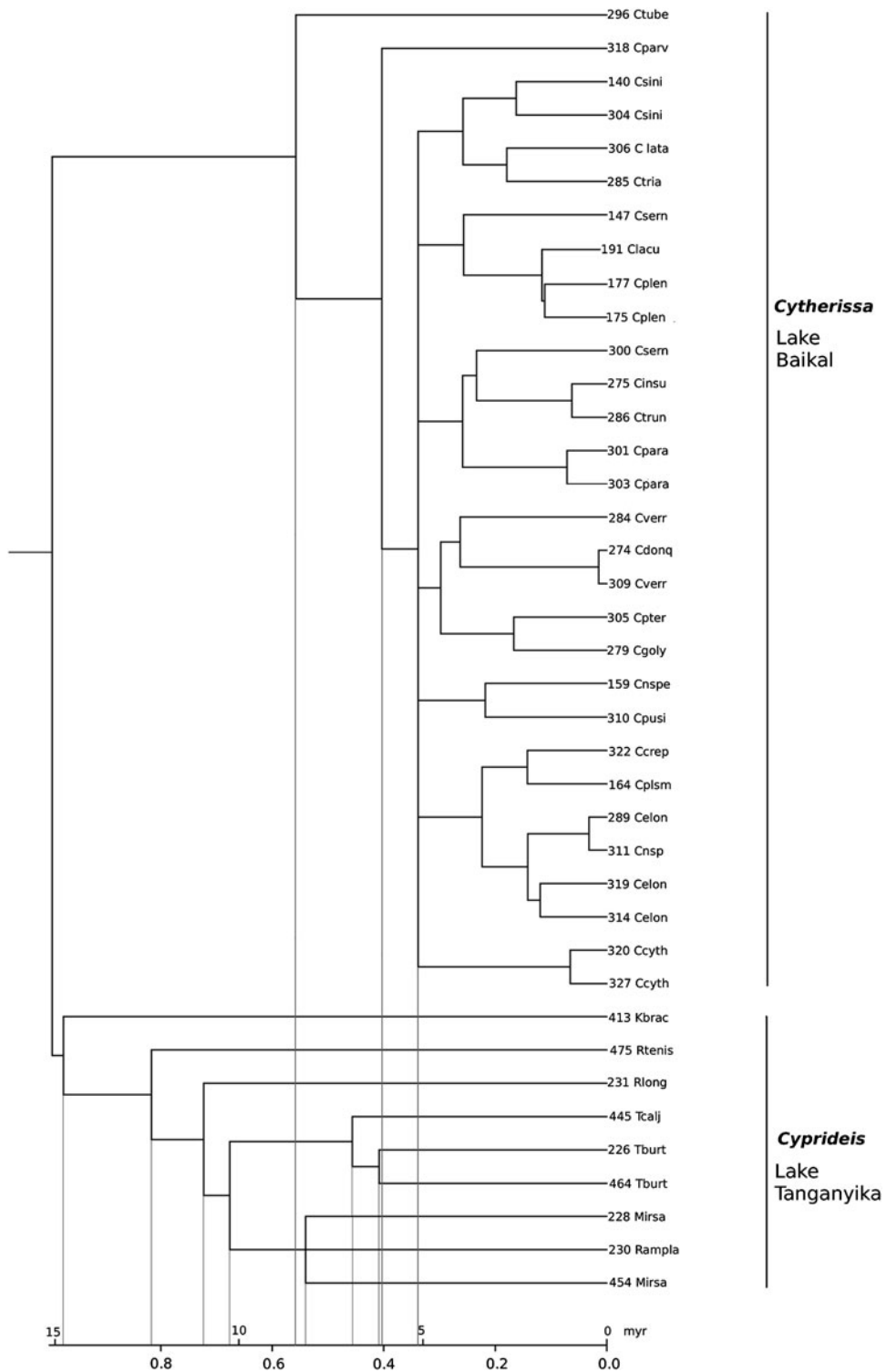
our relative age estimates with PATHd8 indicate that the Tanganyikan *Cyprideis* flock is ca. twice as old as the Baikalian *Cytherissa* flock. Our estimates thus confirm an earlier, preliminary result (Schön et al., 2000a), namely that the younger lake, Tanganyika, holds the older ostracod species flock.

However, it is difficult to obtain real time estimates from this relative difference. We used four different calibration points and two different methods to estimate the age of the ostracod flocks (see Table 5), resulting in age estimates that are not all equally likely. (1) When using the maximal age of LB as calibration point, the *Cytherissa* flock would become as old as LB itself, thus ca. 28 myr (see Table 4). Consequently, the Tanganyikan flock would then become 52 myr old, which is way above the fossil age of the genus *Cyprideis*, which appeared from the early Oligocene onwards, ca. 18 myr ago (Sandberg, 1964). (2) As indicated above (Danielopol et al., 1990), the earliest *Cytherissa* species was found ca. 25 myr ago resulting in an absolute age estimate of 47 myr for the Tanganyikan flock, which we consider equally doubtful. That the Tanganyikan flock would have originated 47 myr ago is as unlikely as the age estimate of 52 myr, given the fossil age of the genus *Cyprideis* of only 18 myr (Sandberg, 1964). Furthermore, in such time frames, we would expect COI to have saturated and the slower molecular marker ITS to resolve our phylogenies. None of this is the case and we therefore consider also this calibration point as unsuitable. (3) Using the maximum age of LT of 12 myr (Cohen et al., 1993, 1997) as calibration point would reduce the age of both flocks and imply that the *Cyprideis* flock is 11.6 myr old and the Baikalian *Cytherissa* flock 6.2 myr old (see Table 5). (4) The wide fossil appearance of *Cytherissa* ca. 8 myr ago (Danielopol et al., 1990) would make the Baikalian flock 8 myr old and put the age of *Cyprideis* flock at 15 myr. The latter estimate would mean that the *Cyprideis* flock is older than Lake Tanganyika itself. Thus, its ancestors must already have occurred in the Eastern African rift valley before LT was formed and colonised this lake from its very beginning. To test this hypothesis, additional fossil data from the area around LT would be required. (5) The average molecular COI invertebrate clock from Wilke et al. (2009) results in a maximum age of ca. 14.7 myr for the

Tanganyikan flock and of ca. 8.8 myr for the Baikalian *Cytherissa* flock. These estimates are rather similar to the absolute age estimates when using the Miocene age of *Cytherissa* (see (4), above) as calibration point and present at the moment in our opinion the most likely age estimates for both ostracod species flocks. This is also the reason why we used 15 myr as maximum to scale the ultrametric COI tree (Fig. 5).

These estimates of ca. 15 myr would make the *Cyprideis* flock younger than gastropods from LT, for which Wilson et al. (2004) estimated an age of 40 myr. With ca. 15 myr, the *Cyprideis* flock would be considerably older than most cichlids with an age of 5–6 myr (e.g. Danley & Kocher, 2001; Salzburger et al., 2002; Brandstätter et al., 2005; Duftner et al., 2005; Koblmüller et al., 2005; but see Koblmüller et al. (2008) and Genner et al. (2007) on a recent discussion of the age of the primary Tanganyikan cichlid radiation which might now be put back as far as 20 myr) and crabs (Marijnissen et al., 2006), for which ca. 3 myr were estimated.

Taking the habitat requirements of the extralacustrine relatives into account can provide additional confirmation of the age estimates of both flocks. *Cyprideis torosa* is a euryhaline species of shallow brackish waters (Heip, 1976). It is quite likely that it, or a related species, was already present in these kinds of habitats in the East African rift valley before LT originated. Lezzar et al. (1996) and Cohen et al. (1993, 1997) estimated that the central basin of LT was formed ca. 12 myr ago. Our age estimate of the Tanganyikan *Cyprideis* flock exceeds this presumed date for the origin of LT. Of course, the early stages in the ontogeny of this lake consisted of a series of shallow lakes and wetlands. A true lacustrine habitat was established only 5–6 myr ago in the northern basin (Tiercelin & Mondeuger, 1991). However, if these early lagoons were slightly saline, they would have constituted excellent habitats for species of *Cyprideis*. Another, similar *Cyprideis* radiation has been described from a fossil South American lake (Muñoz-Torres et al., 2006). Our age estimate of ca. 15 myr also means that the LT *Cyprideis* species flock must have survived the Pliocene regional aridifications 2–4 myr ago (Cohen et al., 1997) and other, more recent climate changes (Alin & Cohen, 2003; Cohen et al., 2007; McGlue et al., 2008), which might all have resulted in severe lake level drops and resulting



salinity crises. This might be possible, because of the relatively high salinity tolerance known for some extra-lacustrine species such as *Cyprideis torosa*.

The history of the Baikalian *Cytherissa* flock was clearly different. Sherbakov (1999) illustrated that evolutionary histories of Baikalian animal taxa are

◀ **Fig. 5** Ultrametric COI tree including the two ostracod flocks and the outgroup, *Limnocythere inopinata*. The tree is scaled to a maximum of 15 myr, in our opinion the most likely age estimate for the Tanganyikan *Cyprideis* flock for now. The tree is rooted with the outgroup, *Limnocythere inopinata*, but most of this branch has been omitted for technical reasons.

quite diverse as both old and young radiations can be distinguished. Thus, also other Baikalian groups are of young age, even in the same order of magnitude as, or younger than, our estimates for the Baikalian *Cytherissa* flock (ca. 8 myr). For Baikalian cottoids, age estimates are between 3 myr (Kirilchik & Slobodnyuk, 1997) to 6.5 myr (Kontula et al., 2003), whilst the age of Baicaliidae gastropods has been estimated to be 3 myr (Zubakov et al., 1997). The formation of the oxygenated abyss around 5–6 myr ago (Lukin, 1986) might have caused these younger radiations in LB (Martens, 1997). From our ultrametric tree (Fig. 5), it seems that only two *Cytherissa* species are ca. 8 myr old whilst the majority of Baikalian *Cytherissa* originated around 5.3 myr ago. It is known from Holarctic *Cytherissa lacustris* that this ostracod species requires cold, well-oxygenated habitats (Danielopol et al., 1990). Species of the Baikalian *Cytherissa* flock have similar habitat requirements and can only have successfully radiated in LB after these habitats had been formed (see above).

We hope that fossil data from ostracods in and around ancient lakes will soon become available, as these would allow us to improve the present, preliminary age estimates. In the future, additional ostracod samples, more suitable molecular markers and other analytical tools will hopefully all contribute to a deeper knowledge of the processes that have shaped the amazing diversity of ostracods in ancient lakes.

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