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SPECIATION IN ANCIENT LAKES 6

High cryptic diversity and persistent lineage segregation in endemic *Romecytheridea* (Crustacea, Ostracoda) from the ancient Lake Tanganyika (East Africa)

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Abstract Ostracods form a substantial part of the endemic fauna of ancient lakes. Here, we have investigated the phylogenetic and phylogeographic patterns and genetic diversities of species of the endemic genus *Romecytheridea* from the Southern and Central part of Lake Tanganyika. We found that ostracod populations from four different localities are genetically highly differentiated from each other when analyzing the mitochondrial 16S region, while they are almost identical with genetic markers from the nuclear genome (D1-D2 from the large ribosomal subunit (LSU) and ITS). The criteria of the K/θ method for the evolutionary species concepts are fulfilled when analyzing 16S DNA sequence data, indicating that these populations are in fact different (cryptic) species with allopatric distribution. We discuss various hypotheses on how this high diversity could have originated. The complete lineage segregation can partly be explained by geographic isolation during periods of low lake level stands. But, other factors must have contributed to genetic isolation and speciation, as the two closest populations (Chimba and Katoto) from shallow parts of the Southern basin of Tanganyika are also geographically fully segregated.

Keywords Ostracoda · Cryptic species · Ancient lakes · Speciation · Lake level fluctuations

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Introduction

Ancient lakes are natural laboratories for evolutionary research because of their high levels of endemic diversity and the fact that many species can be studied in the cradle where they originated (Martens, 1997). Non-marine ostracods are one of several crustacean groups that have formed endemic species flocks in ancient lakes (Martens, 1994; Schön & Martens, 2012). The fact that ostracods from ancient lakes contribute one quarter of the 2,000 recent non-marine ostracod morphospecies currently known on a global scale (Martens et al., 2008) further illustrates the importance of these lakes for generating diversity in non-marine ostracods.

Lake Tanganyika is at 9–12 millions of years (myr) the second oldest lake in the world (Cohen et al., 2007) and holds several endemic ostracod radiations. From the *Cyprideis* flock, about 23 morphospecies (all

endemic) are known from 6 genera (5 endemic) (Rome, 1962, Wouters, 1979, 1988a, b; Ducasse & Carbonel, 1993, Wouters & Martens, 1992, 1994, 1999, 2000, 2001, 2007, 2008). The flock is genetically and morphologically more diverse than the related *Cytherissa* flock from Lake Baikal. The age of the *Cyprideis* flock has been estimated at c. 15 myr, exceeding the age of Lake Tanganyika itself (Schön & Martens, 2012) and being older than other crustacean radiations such as, e.g. crabs (Marijnissen et al., 2006).

The application of molecular methods in the last decade has revealed a second level of hidden or so-called cryptic diversity, where species cannot be differentiated by morphological but only by genetic characters. Such cryptic species are meanwhile known from many animal taxa (Pfenninger & Schwenk, 2007; Trontelj & Fiser, 2009) including non-marine ostracods (e.g. Bode et al., 2010; Martens et al., 2012; Schön et al., 2012). In the ostracod morphospecies *Eucypris virens*, for example, close to 40 cryptic species have been described from Europe and North Africa (Bode et al., 2010), while Schön et al. (2012) found three and eight cryptic species each, respectively, in the darwinulid ostracods *Darwinula stevensoni* and *Penthesilenula brasiliensis*.

So far, speciation and evolution of endemic ostracods in Lake Tanganyika have only been investigated at higher taxonomic levels from the Central part of the lake (Schön & Martens, 2012). Here, we will focus on lower taxonomic levels (genus and species) and test with DNA sequence data whether cryptic species are also present in endemic *Romecytheridea* ostracods from Lake Tanganyika and to which extend they might contribute to the overall ostracod diversity in this ancient lake. On the one hand, this will provide more realistic estimates of biodiversity in ancient lakes, which is required for sustainable conservation strategies. On the other hand, the obtained genetic data will also be used for phylogenetic and phylogeographic analyses of *Romecytheridea* to shed more light on the evolutionary processes that might have caused the amazing endemic diversity of Lake Tanganyika ostracods.

Materials and methods

Ostracod samples

Ostracods were sampled during two expeditions to Lake Tanganyika in 2005 and 2006 by SCUBA diving and using a hand net with a mesh size of 160 µm. Samples were washed at the lake shore and living ostracods were sorted in situ with a binocular microscope and pipettes. They were stored in 95% EtOH at 4°C prior to DNA extraction. 61 specimens were thought to belong to the morphospecies *Romecytheridea ampla* (Wouters, 1988b) as defined by the carapace morphology (Table 1). They were sampled from the Southern and Central parts of the lake and were successfully analyzed genetically. They came from the following localities: Katoto, Chimba, and Kipili from the Southern part and Ekola from the Central part of Lake Tanganyika (Fig. 1, Table 1). In Katoto, ostracods were sampled from two different water depths of 15 and 20 m (see Table 1), while ostracods in the other localities came from the same water depth, namely 5, 18, or 20 m.

DNA extraction, PCR amplification, and automatic sequencing

DNA was extracted from individual ostracods with the Qiagen DNA Easy Blood and Tissue kit, following the manufacture's protocol. Part of the mitochondrial 16S region was amplified with the universal primers 16SH (5′-CGCCTGTTTAACAAAAACAT-3′) and 16SL (5′-CCGGTCTGAACTCAGATCACGT-3′) (Palumbi et al., 1991) and the following conditions: 5 min at 95°C, 35 cycles with 30 s at 95°C, 50 s at 42–52°C, 1 min at 72°C, and a final extension step for 10 min at 72°C in a T personal Thermoblock (Biometra). For several specimens, amplicons were cloned prior to sequencing with M13 primers as described in Schön & Martens (2003). The nuclear large ribosomal subunit (28S, LSU) was amplified with the universal primers LSU D1,D2 fw1 (5′-AGCGGAGGAAAAGAAA CTA-3′) and LSU D1,D2 rev1 (5′-TACTAGAAGGT TCGATTAGTC-3′) (Sonnenberg et al., 2007) and ITS with the universal primers ITS1L (5′-TCCGTAGGTG AACCTGCGGAAGGAT-3′) (Hillis & Dixon, 1991) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3) (White et al., 1990). The following PCR programs were used to amplify the D1–D2 fragment: 5 min at 95°C for initial denaturation; 45 cycles with 20 s 94°C, 20 s 52.5°C, and 90 s 72°C, followed by 10 min at 72°C for final extension, and ITS: 5 min at 95°C for initial denaturation; 35 cycles with 30 s 95°C, 50 s 50°C, and 90 s 72°C, followed by 10 min at 72°C for final extension.

Table 1 Overview of analyzed samples

DNA ID	Locality	Coordinates	Depths (m)	Genbank acc. # 16S	ITS[a]	LSU[a]
R bacata1	Chimba	8°25′29″S 30°27′27″E	18	KF061103	KF061165	
R bacata2	Chimba	8°25′29″S 30°27′27″E	18	KF061116		
R bacata3	Chimba	8°25′29″S 30°27′27″E	18	KF061111	+	
R bacata4	Chimba	8°25′29″S 30°27′27″E	18	KF061115		
R bacata5	Chimba	8°25′29″S 30°27′27″E	18	KF061114	+	
R bacata6	Chimba	8°25′29″S 30°27′27″E	18	KF061118		
R bacata7	Chimba	8°25′29″S 30°27′27″E	18	KF061104	+	
R bacata8	Chimba	8°25′29″S 30°27′27″E	18	KF061105		
R bacata9	Chimba	8°25′29″S 30°27′27″E	18	KF061112	+	
R bacata10	Chimba	8°25′29″S 30°27′27″E	18	KF061121	+	
R bacata11	Chimba	8°25′29″S 30°27′27″E	18	KF061107	+	+
R bacata12	Chimba	8°25′29″S 30°27′27″E	18	KF061110	+	+
R bacata13	Chimba	8°25′29″S 30°27′27″E	18	KF061120	+	
R bacata14	Chimba	8°25′29″S 30°27′27″E	18	KF061106	+	
R bacata15	Chimba	8°25′29″S 30°27′27″E	18	KF061119	+	+
R bacata16	Chimba	8°25′29″S 30°27′27″E	18	KF061113	+	+
R bacata17	Chimba	8°25′29″S 30°27′27″E	18	KF061117	+	
R bacata18	Chimba	8°25′29″S 30°27′27″E	18	KF061108	+	
R bacata20	Chimba	8°25′29″S 30°27′27″E	18	KF061109	+	+
R “ampla”21	Katoto	8°48′39″S 31° 1′44″E	20	KF061122	+	+
R “ampla”22	Katoto	8°48′39″S 31° 1′44″E	20	KF061135	+	KF061166
R “ampla”23	Katoto	8°48′39″S 31° 1′44″E	20	KF061136		
R “ampla”29	Katoto	8°48′39″S 31° 1′44″E	20	KF061124		
R “ampla”31	Katoto	8°48′39″S 31° 1′44″E	20	KF061137		
R “ampla”33	Katoto	8°48′39″S 31° 1′44″E	20	KF061130		
R “ampla”35	Katoto	8°48′39″S 31° 1′44″E	20	KF061127		
R “ampla”37	Katoto	8°48′39″S 31° 1′44″E	20	KF061129		
R “ampla”38	Katoto	8°48′39″S 31° 1′44″E	20	KF061123		
R “ampla”39	Katoto	8°48′39″S 31° 1′44″E	20	KF061132		
R “ampla”40	Katoto	8°48′39″S 31° 1′44″E	20	KF061125		
R “ampla”41	Katoto	8°48′39″S 31° 1′44″E	15	KF061134	+	+
R “ampla”44	Katoto	8°48′39″S 31° 1′44″E	15	KF061128		
R “ampla”45	Katoto	8°48′39″S 31° 1′44″E	15	KF061131		
R “ampla”46	Katoto	8°48′39″S 31° 1′44″E	15	KF061126		
R “ampla”47	Katoto	8°48′39″S 31° 1′44″E	15	KF061133		
R “ampla”54	Kipili	7°17′20″S 30°59′30″E	20	KF061140		
R “ampla”55	Kipili	7°17′20″S 30°59′30″E	20	KF061150		
R “ampla”56	Kipili	7°17′20″S 30°59′30″E	20	KF061149		
R “ampla”57	Kipili	7°17′20″S 30°59′30″E	20	KF061152		
R “ampla”58	Kipili	7°17′20″S 30°59′30″E	20	KF061148		
R “ampla”59	Kipili	7°17′20″S 30°59′30″E	20	KF061151	+	+
R “ampla”60	Kipili	7°17′20″S 30°59′30″E	20	KF061147		
R “ampla”61	Kipili	7°17′20″S 30°59′30″E	20	KF061155	+	+
R “ampla”62	Kipili	7°17′20″S 30°59′30″E	20	KF061153		

Table 1 continued

DNA ID	Locality	Coordinates	Depths (m)	Genbank acc. # 16S	ITS[a]	LSU[a]
R "ampla"63	Kipili	7°17′20″S 30°59′30″E	20	KF061143	+	+
R "ampla"64	Kipili	7°17′20″S 30°59′30″E	20	KF061141		
R "ampla"66	Kipili	7°17′20″S 30°59′30″E	20	KF061142		
R "ampla"67	Kipili	7°17′20″S 30°59′30″E	20	KF061138		
R "ampla"68	Kipili	7°17′20″S 30°59′30″E	20	KF061146		
R "ampla"69	Kipili	7°17′20″S 30°59′30″E	20	KF061154		
R "ampla"70	Kipili	7°17′20″S 30°59′30″E	20	KF061144		
R "ampla"71	Kipili	7°17′20″S 30°59′30″E	20	KF061139		
R "ampla"72	Kipili	7°17′20″S 30°59′30″E	20	KF061145		
R "ampla"75	Ekola	6°47′10″S 30°59′30″E	5	KF061159		
R "ampla"76	Ekola	6°47′10″S 30°59′30″ E	5	KF061160		
R "ampla"78	Ekola	6°47′10″S 30°59′30″ E	5	KF061156		
R "ampla"79	Ekola	6°47′10″S 30°59′30″ E	5	KF061163		
R "ampla"80	Ekola	6°47′10″S 30°59′30″ E	5	KF061157		+
R "ampla"81	Ekola	6°47′10″S 30°59′30″ E	5	KF061162	+	+
R "ampla"82	Ekola	6°47′10″S 30°59′30″ E	5	KF061161		
R "ampla"83	Ekola	6°47′10″S 30°59′30″ E	5	KF061158		
Cytherissa verrucosa	Lake Baikal			KF061164		

We refer to most samples as "*R. ampla*" because these specimens still require additional taxonomic verification

+ indicates that we obtained an ITS or LSU sequence for this specimen

[a] Only one Genbank accession number each is provided for LSU and ITS because all samples had the same sequence

Success of PCR amplifications was verified with agarose gel electrophoresis and subsequent Gelred staining (Biotium). PCR products were purified with the GFX© PCR DNA and gel band purification kit (GE Healthcare) according to the manufacture's protocol and bi-directionally sequenced using the PCR primers and the Big Dye 3.1 kit (Applied Biosystems). All sequences were generated on an ABI 3130X automatic sequencer.

Analyses of molecular data

Chromatograms were visualized with CodonCode Aligner, the Forward and Reverse strands for each sequence aligned with Muscle (Edgar, 2004) implemented in SeaView v4.4.0 (Gouy et al., 2010), and all ambiguities checked and corrected manually. Ostracod sequence identity was confirmed by BLAST searches (Altschul et al., 1990). Sequences from individual ostracods were assembled and used for phylogenetic reconstructions. All sequences have been submitted to Genbank (accession numbers KF061103-KF061166; see Table 1 for more details).

The model of molecular sequence evolution which fitted the 16S data best was identified using jModeltest (Posada, 2008). The TIM2+G model (according to both AICc and BIC criteria) was then used for phylogenetic tree constructions with Maximum Likelihood methods in PHYML 3.0 (Guindon & Gascuel, 2003) and the GTR+G model for Bayesian Inference in MrBayes 3.2 (Ronquist et al., 2012), the latter with and without clock assumption. We used a sequence from Baikalian *Cytherissa verrucosa* Mazepova, 1990 as the outgroup (see Table 1). Parsimonious networks were constructed from the 16S dataset with TCS v1.21 (Clement et al., 2000) at the 95% confidence limit, while F_{st} calculations and AMOVAs were conducted with Arlequine 3.5 (Excoffier & Lischer, 2010) in order to assess the structuration of the genetic variability. The significance of the obtained values was assessed through 1,000 permutations. We used MEGA 5.0 (Tamura et al., 2011) to estimate the genetic diversity across the Tanganyikan specimens for three genomic regions: mitochondrial 16S and nuclear ITS and LSU as (raw) *p* distances with the Maximum Composite Likelihood model (being based

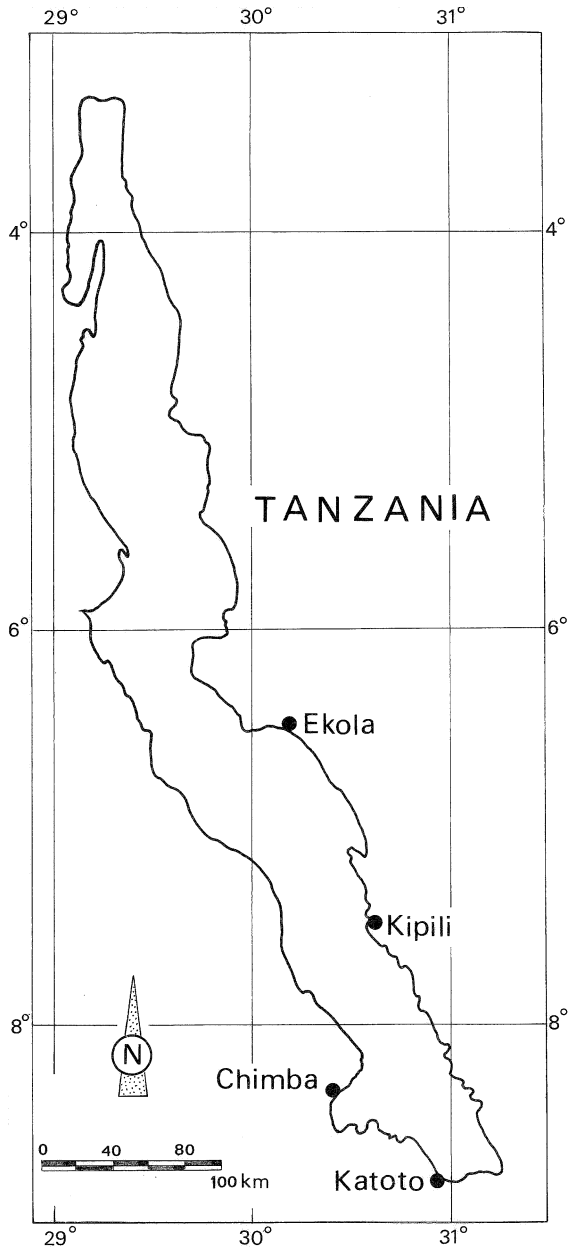

Fig. 1 Map with sampling sites in the Southern part of Lake Tanganyika

on the Tamura-Nei model; Tamura & Nei, 1993) and gamma distances.

Testing for cryptic species

We applied the K/θ method (Birky et al., 2010; Birky, 2013) to test for the presence of species-like entities from genetic data, which is based on the evolutionary species concept and has been developed for both asexual (Birky et al., 2010) as well as sexual (Birky, 2013) taxa. This method has already been successfully applied to non-marine ostracods for the detection of cryptic species (Bode et al., 2010; Martens et al., 2012; Schön et al., 2012). The K/θ method relies on the fact that populations which have been evolving independently for a sufficiently long time form clusters that are separated by gaps that are too deep to result only from a random genetic drift within a species. Such gaps can only be formed by diversifying selection or by long-term physical isolation, for example, in different lake sub-basins (see below) and represent speciation. Gaps owing to drift alone have an average depth of $2N_e$ generations, with a 95% confidence interval of $4N_e$ generations. Thus, species can be identified as sister clades separated by gaps greater than $t = 4N_e$ generations deep (for more details, see Birky et al. 2010; Birky, 2013).

Age estimates

We followed Wilke et al. (2009) for conducting relative age estimates using a molecular clock and first confirmed that our ingroup sequences had similar rates of molecular evolution by conducting Likelihood ratio tests on Maximum Likelihood trees with and without clock assumption in TreePuzzle (Schmidt et al., 2002; but see Bromham et al., 2000). We then calculated genetic distances between phylogenetic clades with parameters from the best model for molecular sequence evolution as identified by jModeltest. These distances were divided by 2 to transform into substitution rates. To the latter, the mitochondrial invertebrate clock (GTR+I+G) from Wilke et al. (2009) of 1.76 ± 0.66 myr was applied to obtain age estimates, taking the SD of the estimated substitution rates and the clock into account.

Results

Overall genetic diversity

From all four geographic populations, we could successfully obtain sequences for all three genomic regions (see Table 1). These include 61 sequences of 498 basepairs (bp) for the mitochondrial 16S region, 24 sequences with 540 bp for the nuclear ITS region,

and 12 sequences of 828 bp for the nuclear LSU region. There is a remarkable difference in the general patterns of genetic diversity between the sequenced nuclear and mitochondrial regions. While the average *p* distance among the Tanganyikan *Romecytheridea* "*ampla*" here analyzed is 9.6% ± 0.8% for the mitochondrial 16S region, there is no variation at all in the nuclear ITS and LSU regions. Because of the absence of any genetic divergences of the nuclear regions, only the genetic patterns of the mitochondrial 16S are further analyzed and described below.

Phylogenetic trees

The four geographic populations form separate clades in the phylogenetic 16S tree with high statistical support by both bootstrapping in ML and posterior probabilities with Bayesian Inference (BI). Branches leading to the different clades are relatively long, while branches within each clade are rather shallow (Fig. 2). The phylogenetic relationships were similar with both methods for tree constructions. Ostracods from Chimba and Katoto form a sister clade, which is phylogenetically the closest to ostracods from Ekola. *Romeocytheridea* sampled in Kipili are more distant from the other three populations and are furthermore split into two phylogenetic clades which are statistically well supported (Fig. 2).

Genetic diversity and structure at the population level, phylogeography

The high genetic differentiation between the various geographic populations is reflected in the structure of the 16S network (Fig. 3). The four different geographic populations form separate unconnected networks with few missing haplotypes in each network and no haplotypes being shared between populations. While the 16 ostracods from the two water depths in Katoto are connected through nine haplotypes in the same network, the Kipili population is further split into two different networks of two and 14 haplotypes. The Ekola population contains at five the lowest number of haplotypes among the eight investigated specimens, while the 19 ostracods from Chimba display at 14 the highest number of haplotypes, which are connected by up to 11 mutational steps.

The network structures fit the high estimates of genetic distances (estimated with the Maximum Composite Likelihood model) between the different geographic populations varying for 16S between 8.6 and 35.7% (see Table 2), with the lowest between Chimba and Katoto and the highest between Chimba and Kipili. This pattern is in conformity with the results of the AMOVA analyses where 79.36% of genetic variation was found among the geographically different populations ($P < 0.001$), 15.01% between two genetically different groups in Kipili (see above; $P < 0.001$), and 2.06% within each genetic group ($P > 0.05$).

The strong genetic differentiation between the different populations is also illustrated by the high F_{st} estimates of more than 0.95 for all population comparisons, which were all statistically significant, and also between the two genetic groups from Kipili (see Table 2) and the closely situated Chimba and Katoto.

Tests for cryptic species

When applying the K/θ method (Birky et al. 2010; Birky, 2013), we find that the two genetic groups from Kipili did not fulfill its statistical criteria (see Table 3), because the K/θ ratio was smaller than 4. Thus, they cannot be considered as two different evolutionary genetic species. However, all geographically different populations (Kipili with all samples, Ekola, Chimba, and Katoto) fulfill the criteria of the K/θ method (Table 3) as their K/θ ratios are larger than 4, and they should therefore be regarded as different evolutionary (cryptic) genetic species based on the mitochondrial sequence data.

Age estimates

When applying the GTR+I+G clock of Wilke et al. (2009) to the substitution rates, the populations from Southern Chimba and Katoto would have split around 2.4 myr ago (SD = 1.4–4.8 myr), while, for example, Chimba and Ekola would have been separated for 7.4 myr (SD = 4.1–14.7 myr) with Chimba and Kipili being the most distant in the phylogenetic tree (Fig. 2) for 10.1 myr (SD = 5.7–19.8 myr).

Discussion

Mitochondrial versus nuclear DNA

We observed a strong contrast in the genetic variability between the sequenced regions of the nuclear and

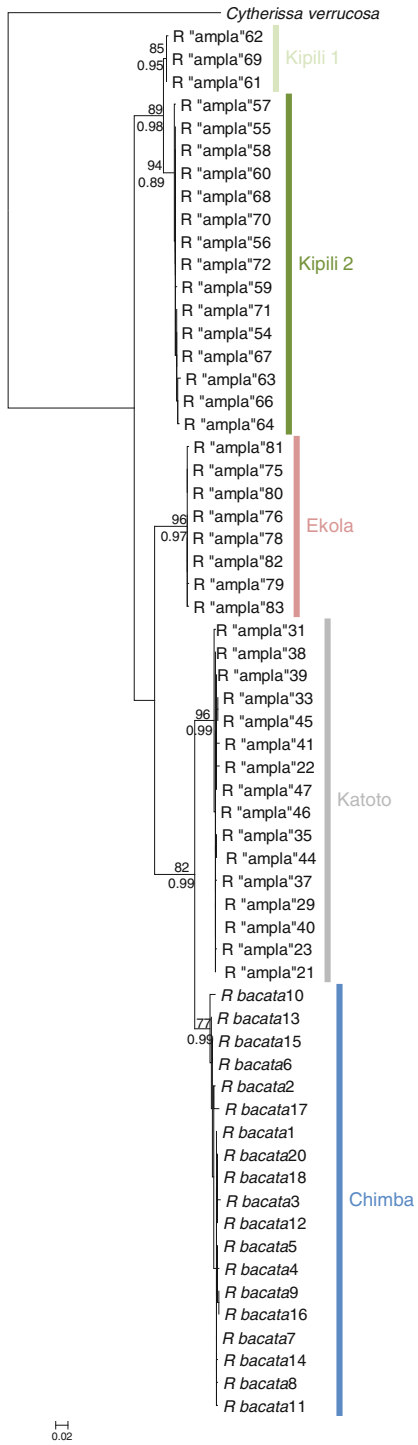

Fig. 2 16S phylogeny constructed with Maximum Likelihood (ML) methods and Bayesian Inference (BI). Numbers above branches are bootstrap values of 1,000 replicates of the ML tree, numbers below branches posterior probabilities of BI. Kipili 1 and 2 refer to genetic groups. Statistical support for branches being below 50% is not shown

mitochondrial genome. While there was no variation at all in the nuclear ribosomal ITS and LSU regions (see above) between the four geographically different populations of *Romecytheridea* "*ampla*" studied here, genetic variability of the mitochondrial 16S region reached more than 35% between populations (Table 2). Similar patterns with no genetic variability in nuclear regions but considerable variation in a mitochondrial region have also been reported from non-marine ostracods with asexual reproduction (Schön et al., 1998; Schön et al., 2010; Koenders et al., 2012), while other ostracods showed very low nuclear variability together with high mitochondrial variation (Schön & Martens, 2012; Schön et al., 2012). It remains to be further investigated whether this might be owing to strongly differing rates of molecular evolution in nuclear and mitochondrial ostracod genomes as proposed by Schön et al. (2003), highly efficient, concerted evolution in the ribosomal nuclear DNA, or other molecular mechanisms such as hybridization or introgression. The latter seems to be rather common in cichlid fish from different ancient lakes (Koblmüller et al., 2007; Nevado et al., 2009, 2011; Mims et al., 2010; Genner & Turner, 2011; Anseeuw et al., 2012; Schwarzer et al., 2012) and deserves special attention in future molecular investigations of ostracods from ancient lakes. Sex-specific differences in migration frequencies, which are known to cause different gene flows of mitochondrial and nuclear regions (Hedrick, 2007), can most likely be ruled out as an explanation for the contrasting genetic pattern described in our study here because of the small size (<1 mm) and slow, crawling movements of *Romecytheridea* ostracods.

Phylogenetic and phylogeographic patterns of genetic diversity

We found several lines of evidence supporting strong genetic segregation between geographically different *Romecytheridea* "*ampla*" ostracod populations from the South and Central parts of Lake Tanganyika: well-supported clades in phylogenetic analyses (Fig. 2), unconnected networks with unique haplotypes (Fig. 3), statistically significant high F_{st} values, and large genetic distances (Table 2). The latter are smaller for ostracods from Katoto and Chimba (Table 2) than for the other comparisons, which can most likely be explained by their closer geographic proximity of

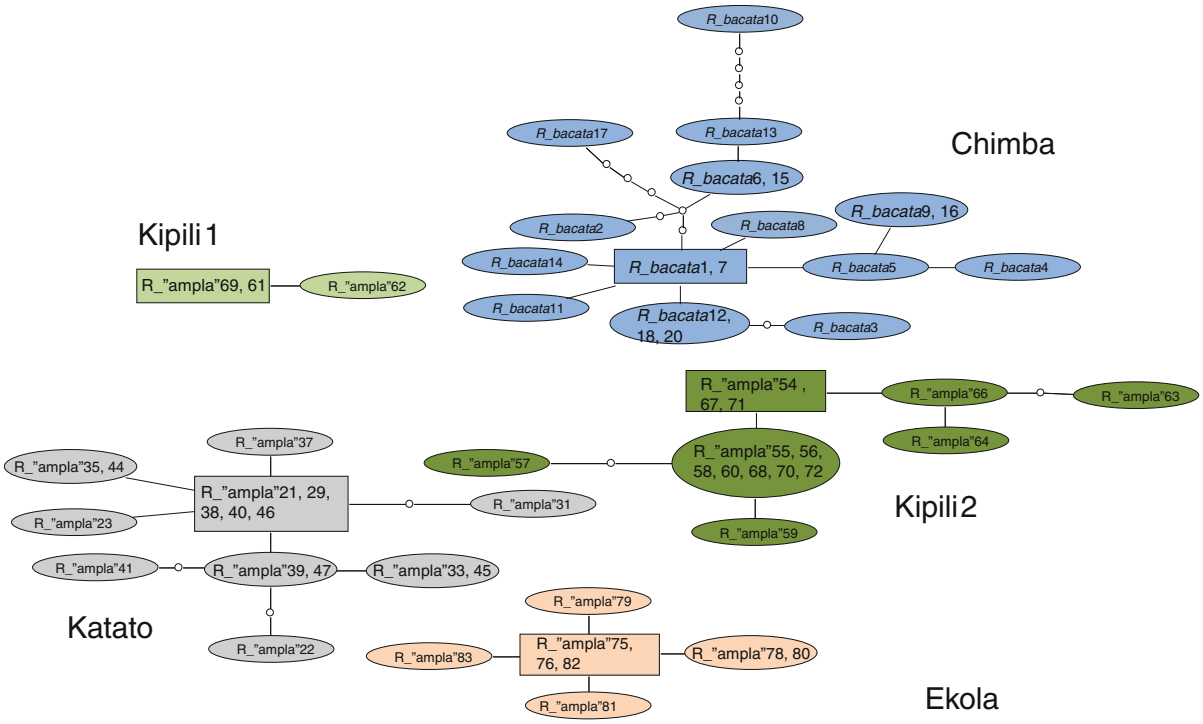

Fig. 3 Parsimonious 16S network, connected at the 95% probability limit with 8 mutational steps. *Squares* represent ancestral haplotypes, *small circles* missing haplotypes. The size of the squares or large circles is proportional to the number of individuals with that haplotype

Table 2 Genetic distances and F_{st} estimates between genetic groups based on 16S sequence data

	Ekola	Kipili1	Kipili2	Kipili	Katoto	Chimba
Ekola		0.98**	0.98***	0.94***	0.98***	0.95***
Kipili1	*0.266 ± 0.064*		0.89**		0.98***	0.97***
Kipili2	*0.296 ± 0.071*	*0.032 ± 0.010*			0.98**	0.96***
Kipili	*0.291 ± 0.068*				0.95***	0.94***
Katoto	*0.292 ± 0.072*	*0.325 ± 0.076*	*0.332 ± 0.076*	*0.331 ± 0.075*		0.91***
Chimba	*0.261 ± 0.063*	*0.333 ± 0.075*	*0.361 ± 0.081*	*0.356 ± 0.079*	*0.086 ± 0.019*	

Genetic distances were estimated with the gamma parameter and the Maximum Composite Likelihood model (left hand of the table in Italics), while F_{st} estimates are shown at the right hand of the table. ** $P < 0.01$; *** $P < 0.001$

about 100 km. These two sampling stations are furthermore located on Western shallow shores in the Southern part of Lake Tanganyika (see Fig. 1).

These different genetic *Romecytheridea* entities furthermore fulfill the statistical criteria of the K/θ method (Table 3 and Fig. 3). The K/θ method is a different approach than applying, for example, the barcoding gap (Hebert & Gregory, 2005) as it is based on population and evolution genetic theory and provides statistical means to test for species boundaries from DNA sequence data (see above and Birky et al., 2010; Schön et al., 2012 and Birky, 2013 for more details). In cases of recent speciation, the K/θ method might fail, but so would other methods for species limitation. The K/θ method can erroneously identify different species from mitochondrial data under female philopatry and male migration (Birky, 2013), but there is no evidence for such migration patterns for ostracods nor has speciation of these endemic ostracods been recent. Thus, the different

Table 3 Results of applying the K/θ method (Birky, 2013) to test for cryptic species

Population	*n*	θ within clade	Sister clade	K/θ with sister clade
Kipili 1	3	0.0024		
Kipili 2	15	0.0036	Kipili 1	1.17
Kipili	18	0.011		
Ekola	8	0.0018	Chimba	**16.2**
Ekola	8	0.0018	Katoto	**38.3**
Katoto	16	0.0039		
Chimba	19	0.0082	Katoto	**10.5**

n = number of sequences for each clade. Sister clades were identified from phylogenetic grouping (see Fig. 1). K/θ is calculated by dividing the p distances between sister clades by the highest estimate of θ the two clades. If K/θ is larger than 4, the criteria for the phylogenetic species concept are fulfilled (Birky, 2013). If this is the case for our data, D is printed in bold

Romecytheridea populations studied here should be regarded as different (cryptic) species when taking the mitochondrial DNA sequence data into account. If the nuclear data are considered, they would be one genetic species. According to Birky (2013), mitochondrial genes segregate earlier during speciation events than most nuclear genes. Wouters & Martens (2007) have meanwhile investigated samples from Chimba and found that the *Romecytheridea* ostracods there actually belong to the new species, *R. bacata* Wouters & Martens (2007) and not to *R. ampla* as originally assumed. Whether the other populations studied here indeed hold cryptic, morphologically unrecognizable species or new and identifiable, yet undescribed, species needs to be further investigated.

Linking Romecytheridea *speciation to the lake level history of Lake Tanganyika*

Schön and Martens (2012) recently dated speciation events of endemic Baikalian and Tanganyikan ostracods with mitochondrial COI sequence data (using the clock of Wilke et al., 2009) and fossil data. Although we sequenced 16S here, and not COI (another mitochondrial region), we assume that the clock of Schön and Martens (2012) could be applied to our dataset, mainly because others have found comparable patterns of genetic variability in both 16S and COI for the same ostracod dataset (Bode et al., 2010), with 16S being only slightly less variable. In this case, the different (cryptic) *R.* "*ampla*" species studied here would have been separated for a minimum of 2.4 (Chimba and Katoto) or 7.4 myr (Chimba and Ekola) up to 10 myr. The age estimates for *Romecytheridea* from Chimba and Ekola are very close to the 7 myr that Schön & Martens (2012) estimated for the *R. ampla* group in the general phylogeny from the central part of Lake Tanganyika (near Kigoma) and the age estimate of 7–8 myr for the Tanganyikan *Mastacembelus* eel flock (Brown et al., 2010). Assuming that this mitochondrial molecular clock is reliable, the different (cryptic) *Romecytheridea* "*ampla*" species would thus be younger than the geological age of Lake Tanganyika, which is 9–12 myr (Tiercelin & Mondeguer, 1991; Cohen et al., 1993). The different (cryptic) *Romecytheridea* species from Chimba and Katoto might have split more recently from each other and their age estimate appears to be similar to the Tanganyikan platythelphusid crab radiation for which an age of 2.4–3.3 myr was estimated (Marijnissen et al., 2006). During its long geological history, Lake Tanganyika underwent several severe lake level fluctuations. Geological data suggest that 1.1 myr ago when water stands might have been 650–700 m lower than today, three separate sub-basins were formed (Lezzar et al., 1996; Cohen et al., 1997). Subsequent water level fluctuations took place 390–360, 290–260, and 190–170 kyr ago, with the lowest water levels being down to 250–350 m less than today (Cohen et al., 1997). Our age estimates of the *Romecytheridea* "*ampla*" species complex clearly exceed all of these periods with large lake level fluctuations. The shallow parts of especially the Southern basin, where the two (cryptic) *Romecytheridea* "*ampla*" species were sampled for the current study, must have dried out during most of these periods when water levels were low (Cohen, 2012). This implies that the different (cryptic) *Romecytheridea* "*ampla*" species must have been formed long before water levels dropped and must have remained genetically isolated as "good species" when they came into contact with each other again during these periods of low lake level stands, especially at shallow parts of the lake. During periods of low lake levels, suitable ostracod habitats could also have been available next to the receded steep shoreline, in which case these species might have remained geographically isolated.

Alternatively, ostracods might have become extinct in Lake Tanganyika itself during these periods and recolonized the lake again when water levels increased. In that case, speciation could have taken place outside of the actual Lake Tanganyika, for example, from source populations in different refugia. However, such an evolutionary scenario outside of Lake Tanganyika during dry periods is less likely for ostracods, because it can be expected that shallow lakes, temporary pools, and river pools also dry out at a time when even Lake Tanganyika is affected by severe drops in lake level. Furthermore, related ostracod species like *Cyprideis torosa* do not occur in rivers and do not produce drought-resistant resting eggs as some other non-marine ostracods do (Martens, 1994). Salzburger et al. (2002) showed that certain cichlids from Lake Tanganyika did not originate from river systems, but that endemic fish radiations in the lake basin caused a secondary colonization of surrounding river systems. Similarly, Wilson et al. (2004) found that a group of gastropods now widespread in Africa actually evolved from taxa found in Lake Tanganyika today. In order to further resolve speciation patterns in *Romecytheridea* from Lake Tanganyika during periods of low lake level stands, the phylogenetic position and age of extralacustrine relatives to this species complex or the *Cyprideis* flock would need to be verified.

Certain cichlid fish speciated in Lake Tanganyika in periods following low lake level stands (Sturmbauer et al., 2003) or showed signals of population expansion (Koblmüller et al., 2011). Other cichlids speciated in geographic separation when separate sub-basins were formed because of low lake level stands (Rüber et al., 1999, 2001; Sturmbauer et al., 2001; Nevado et al., 2009, 2011). If the same kind of scenarios would apply to speciation of the species of *Romecytheridea* investigated here, these different (cryptic) species would be maximally 75,000–135,000 years old (last severe drop in lake level; Cohen, 2012). To achieve an estimated age of 100,000 years for the split between the different (cryptic) *Romecytheridea* "*ampla*" species, the molecular mitochondrial clock of these ostracods would have to be exceptionally fast, about 100 times faster than classic mitochondrial clocks from invertebrates (Wilke et al., 2009), which seems unlikely. In addition, Wilson et al. (2004) reported that thalassoid gastropods from Lake Tanganyika evolved 40 myr ago, long before the current lake existed, and concluded that patterns of fast diversification are not common among all ancient lake taxa, but might only be typical for cichlid fish.

Lineage segregation

The persistent lineage segregation between Ekola and Kipili from the Eastern shore of Lake Tanganyika on one hand and the Chimba/Katoto localities from the Western shore on the other can most likely be conveniently understood by simple geographic isolation. These localities are 100–200 km apart and thus even if their populations had to follow receding lake levels during arid climates, it is unlikely that they came into direct contact with each other during low lake stands. The presence of two different genetic lineages at Kipili might indicate that lineage sorting at this, more central, part of the lake, has been incomplete. More likely, they are still in the process of speciating as they split more recently (see above). The two lineages might both have formed around Kipili, as the different islands have some measure of isolation between them, but at least one of the two lineages might also have been of allochthonous origin.

However, in Chimba and Katoto, which are both in the same shallow part of the south-western part of the lake, lineage sorting appears to be complete already. During low lake levels, when these populations had to follow the receding lake, they were most likely bound to get into contact with each other and yet after several series of low lake level stands since their presumed origin several millions of years ago, there is no mix between the two cryptic species. This is paradoxical and requires further investigation. One possible avenue of exploration is that competitive exclusion (Hardin, 1960; Quenouille et al., 2011) occurred as soon as ostracods recolonized the previously inaccessible parts of the lake during reflooding, when the climate became more humid again. We know that such processes of competitive exclusion can be fast, if there is little niche overlap and if there are significant regional differences in habitats.

Outlook

Our detection of cryptic species in endemic ostracods from Lake Tanganyika significantly increases the standing biodiversity estimates of this group. Given the fact that we have only studied a limited number of

sites from the Southern and Central part of the lake and assuming that most morphospecies hold similar amounts of cryptic diversity as the *R.* "*ampla*" studied here, the *Cyprideis* flock might very well consist of more than 100 endemic ostracod species. Our results also have important more general implications for diversity estimates of non-marine ostracods as ancient lakes contribute about 25% to the currently known 2,000 non-marine ostracod morphospecies (Martens et al., 2008). Speculation aside, it is our hope that the planned deep drilling in Lake Tanganyika (Cohen, 2012) will happen soon, because ostracod fossils could then provide the urgently required real-time estimates to calibrate molecular clocks from ostracods and aid in reconstructing the evolutionary history of endemic ostracods from Lake Tanganyika and their amazing diversity.

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