

## Rapid evolution of an ancient lake species flock: Freshwater limpets (Gastropoda: Ancyliidae) in the Balkan Lake Ohrid

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### Abstract

Ancient lakes have long been recognized as evolutionary theatres and hot spots of endemism; the evolution of their morphologically often highly diverse species flocks has received much attention. However, as each ancient lake has its own geological and evolutionary history, modes of speciation may differ from system to system. Ancient lakes can act as evolutionary reservoirs that assure the survival of relict species, but at the same time extant species may evolve through intralacustrine speciation. Other aspects of interest are the actual rates of immigration, diversification or extinction as well as the temporal framework of morphological change. Many of these questions have been addressed in the African (e.g. Lake Tanganyika) and Asian (e.g. Lake Baikal) ancient lakes. For an European ancient lakes (e.g. Lakes Ohrid and Prespa), such studies are largely missing. In the present paper, extraordinarily shaped endemic freshwater limpets of the genus *Ancylus* from the Balkan Lake Ohrid are used in a phylogeographic and phylogenetic context to test whether they represent an ancient lake species flock, to study the mode of speciation, and to assess the timing of morphological change. Based on DNA data from two mitochondrial genes (COI, LSU rDNA), it has been found that the Lake Ohrid *Ancylus* species form an endemic monophyletic group. In addition, the lake's feeder springs are inhabited by another, undescribed *Ancylus* species. All other studied waterbodies within the watershed do not support their own *Ancylus* lineages but are inhabited by a widespread Mediterranean taxon. The split between the species endemic to the lake and its sister taxon is dated to  $1.4 \pm 0.6$  million years ago. The study presents the first genetic confirmation for the existence of a species flock in a European ancient lake. Contrary to the prevailing opinion it shows that, concerning *Ancylus*, Lake Ohrid represents a site of intralacustrine speciation rather than an evolutionary reservoir. Moreover, it provides the first evidence for rapid morphological change in an European ancient lake species flock.

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### Introduction

Only few lakes in the world have existed continuously for more than 100,000 years or even as long as 20

million years (Gorthner 1994). In contrast to short-lived, late- or post-Pleistocene lakes, which are no more than approximately 18,000 years old, those lakes are called 'long-lived lakes' (Gorthner 1994), 'extant ancient lakes' (e.g. Martens 1997), or simply 'ancient lakes' (Brooks 1950). Ancient lakes have long been recognized as evolutionary theatres and hot spots of endemism (e.g. Brooks 1950; Martens et al. 1994; Martens 1997). As ancient lake faunas may live in the cradle in which they originated (Brooks 1950), these lakes provide prime models for studying in situ evolution as they allow for quasi-experimental approaches (Martens 1997; Rossiter and Kawanabe 2000; Schön and Martens 2004).

Rapid intralacustrine evolution of morphologically often highly diverse ancient lake species flocks has received considerable attention. However, as pointed out by Martens (1997), the faunas of ancient lakes can show various convergent developments, many of which remain ill understood. For example, it has been shown only recently (Wilson et al. 2004) that the impressive conchological diversity of gastropods presently found within Lake Tanganyika has two origins that largely differ in time and space. There are lineages that predate lake formation by as much as 40 million years; on the other hand, a widespread group of African gastropods appears to have evolved from taxa presently found in the lake.

Originally, the definition of 'species flocks' referred to speciosity, monophyly and endemism (Greenwood 1984). Today, the term is applied to groups of as few as 3–4 closely related endemic species, with the immediate ancestor not necessarily endemic to the lake (see Schön and Martens (2004) for a review of termini and definitions of species flocks).

Given the nature of ancient lake species flocks, it is generally acknowledged that their evolution should be studied in the context of the geological and evolutionary history of the respective ancient lake. Although ancient lakes *per se* are considered to be geologically old, they need not have remained constant over time in terms of watershed and isolation, water level, chemistry, biota and other characteristics (Martens et al. 1994; Coulter 1994). Naturally, a lake's history must be reflected in the taxa that have lived or continue to live in these settings. Two contrasting hypotheses are involved in studies on ancient lake species flock origins. Ancient lakes themselves can act as evolutionary reservoirs that assure the survival of glacial, marine or other types of relict species, but at the same time extant species may evolve through intralacustrine speciation (Martens 1997). Therefore, the actual cause for such patterns needs to be tested in a rigorous way for each lake system.

Another issue not yet adequately addressed in many ancient lake studies are the actual rates of immigration, speciation and extinction. Is there an increased evolutionary rate with high morphological change? Or have

changes just accumulated gradually over a long period of time? Rapid change, for example, was found in haplochromatid cichlids (Seehausen 2002), whereas thalassoid gastropods from Lake Tanganyika do not seem to show an increased rate (Wilson et al. 2004). Different modes of speciation have been suggested for different lakes and taxa (Schön and Martens 2004).

Much of our knowledge of faunal evolution in ancient lakes is based on studies in African ancient lakes (e.g. Fryer 2000) and in the Asian Lake Baikal (e.g. Sherbakov 1999). Comparatively little is known about the European ancient lakes. In fact, no European ancient lake is covered, e.g., in the latest textbook on the biology, ecology and evolution of ancient lakes (Rossiter and Kawanabe 2000).

Part of the problem is that it is still unclear which European lakes qualify as ancient lakes.

Undisputedly ancient is the oligotrophic and karstic Lake Ohrid, a steep-sided graben with rift formation origin, situated at 695 m a.s.l. in the Ohrid Valley (South Adriatic-Ionian biogeographic region). The lake is fed by springs and drained by the River Crni Drim, which belongs to the Adriatic drainage system. Lake Ohrid has a maximum length of 30.4 km and a maximum width of 14.8 km; its surface area is 370 km<sup>2</sup> (Stankovic 1960). Depth averages 164 m, with a maximum at 289 m (Spirkovski et al. 2001). It is the oldest lake in Europe; age estimates range from 2 or 3 million years (Stankovic 1960) to 10 million years (Spirkovski et al. 2001).

For Lake Ohrid, Watzin et al. (2002) estimated the degree of endemism to between 47% and 86% for various benthic animal taxa, with the highest value found for the Gastropoda. Although it is clear that Lake Ohrid is home to a disproportionately large number of gastropod endemics, the evolutionary history and processes leading to these unique faunas are largely unknown. Moreover, gastropod endemism and evolution in Lake Ohrid primarily have been studied in non-pulmonate gastropod groups like the rissoidae (summarized in Radoman 1983, 1985), whereas pulmonates and other gastropod groups are only poorly studied. This is very unfortunate, because the major gastropod groups differ considerably in their life-history strategies, and comparative studies would help to unravel effects of life-history properties on patterns and processes of evolution (Boss 1978; Michel 1994; Wesselingh and Michel 2001). Though our knowledge of pulmonate gastropods in Lake Ohrid is very limited, it should be mentioned that Hubendick (1960a) utilized the endemic representatives of the basommatophoran genus *Ancylus* to establish first hypotheses about intralacustrine speciation in Lake Ohrid.

The genus *Ancylus* is more or less continuously distributed mainly in the western Palaearctic, in northern Africa (Hubendick 1970, 1972), and in the northeast

to the Caspian Sea and the Transcaucasian region (Soldatenko and Starobogatov 2004). Outside the continuous core range, there are few isolated occurrences, e.g. in Egypt, southern Arabia and Turkestan. While Hubendick (1970) synonymized more than one hundred nominal taxa into his 'form-group *Ancylus fluviatilis*', recent studies reported four highly divergent lineages within *Ancylus* from Europe (Bugert 2001; Pfenninger et al. 2003). These clades can be differentiated only by means of genetic and morphometric data. Therefore, it was concluded that these *Ancylus* lineages are cryptic and that ecological speciation was involved in the diversification process in the species that mainly thrive in lentic environments. In contrast to the widespread *Ancylus* taxa from most parts of Europe, the putatively endemic freshwater ancylids of Lake Ohrid are characterized by their peculiar shell shape and structure (Hubendick 1960a,b). Initially, two species were recognized from Lake Ohrid: *Ancylus tapirulus* Polinski, 1929 is characterized by a sharp apex, whereas *A. scalariformis* Stankovic & Radoman, 1953 has a very characteristic structural shell feature, namely a number of concentric eaves-like projections. Both species reportedly live in the so-called shell zone of dead or live zebra mussel, *Dreissena stankovici*, as part of the sublittoral between depths of 18 and 35 m. A third species, *Ancylus lapicidus* Hubendick, 1960, resembles *A. scalariformis* in possessing comarginal ribs. As far as known, it is restricted to the littoral zone of the central part of the eastern shore of Lake Ohrid. The endemic ancylids of Lake Ohrid were regarded as palaeoendemics or relict species (Polinski 1932; Hadžišće 1956; Stankovic 1932, 1960), but traditional age estimations were not possible up to now because fossils are completely lacking.

Radoman (1955, 1985) stated that insufficient knowledge of recent malacofaunas from the southern Balkan lakes hindered inferences on the origin of the Lake Ohrid endemic gastropod fauna. However, intralacustrine speciation has been suggested for many polytypic genera in Lake Ohrid (Radoman 1961, 1985).

Here, we use a multi-level approach to study the origin and evolution of the putative species flock of *Ancylus* spp. in Lake Ohrid. Within a phylogenetic framework, all species described from the lake, as well as many *Ancylus* populations in the immediate surroundings, are studied genetically. On a more regional scale, the whole Ohrid basin is included, as are the southern Balkans and southeastern Mediterranean region. Finally, a comprehensive analysis covering most of the total distribution area of the genus *Ancylus* was possible, using our own data as well as previously published sequences (Bugert 2001; Pfenninger et al. 2003).

We utilize sequence data from the mitochondrial cytochrome oxidase c subunit I (COI) and large-subunit rDNA (LSU rDNA) genes for the three endemic species,

*A. scalariformis*, *A. lapicidus* and *A. tapirulus* as well as for several non-endemic European taxa, in order to:

- (1) determine the phylogenetic and putatively endemic status of the known Ohrid species in the genus *Ancylus* within the framework of other European species;
- (2) test whether the putative Ohrid endemics represent an ancient lake species flock;
- (3) study the putative intralacustrine mode of diversification of the Ohrid endemics;
- (4) investigate the evolution of shell shape in these limpets.

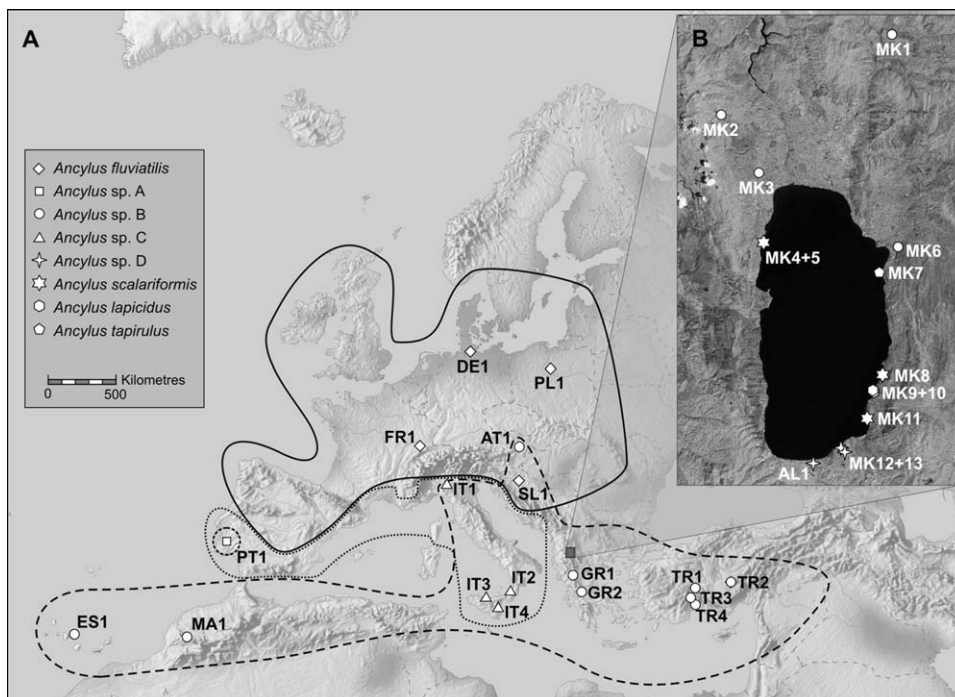
## Material and methods

### Specimens studied

Material from Lake Ohrid and surrounding areas was obtained mainly during two field trips in May 2003 and September 2004. Individual specimens were collected by hand from hard substrates in shallow waters, or from stones and rocks lifted from depths down to 5 m by snorkelling. Deeper parts of the littoral and sublittoral down to 60 m were sampled using a dredge, either from a small boat or from the research vessel of the Hydrobiological Institute (HBI) in Ohrid. While most of the collecting was done in the Republic of Macedonia, fresh material was obtained also from Greece, Albania, and Turkey (Fig. 1; for colour version see the accompanying Organisms Diversity and Evolution Electronic Supplement at <http://www.senckenberg.de/odes/06-12.htm>). For all locality data, collecting details, voucher and GenBank accession numbers, etc., see the Appendix in the Electronic Supplement. Shell shapes and structures are exemplarily demonstrated in Fig. 2 (for colour version see the Electronic Supplement).

### DNA isolation, PCR, and sequencing

DNA from ethanol-preserved snails was extracted utilizing the protocol of Winnepenninckx et al. (1993). For amplifying a fragment of the COI gene, the universal primers of Folmer et al. (1994) were used with slight modifications (for details see Albrecht et al. 2004). A fragment of the LSU rDNA gene was amplified using the primers of Schwenk et al. (1998). The quality of LSU rDNA and COI fragments was checked on a 1.4% agarose gel. PCR products were purified using the E.Z.N.A. Cycle-Pure Kit (Peqlab) or QIAquick Gel Extraction Kit (Quiagen) after cutting out corresponding bands. A total of 50–100 ng DNA were subjected to cycle sequencing using the ABI Prism Big Dye terminator kit (Perkin-Elmer). Sequences for both forward and reverse strands of each gene fragment were



**Fig. 1.** European distribution of *Ancyclus* spp. and populations studied from Lake Ohrid and surroundings; see Appendix for data on individual localities and samples. (A) Solid line = included European populations and inferred general distribution range of *Ancyclus fluviatilis* (Clade 1 of Pfenninger et al. 2003); short-dashed line (locality PT1) = *Ancyclus* sp. A (Clade 2 of Pfenninger et al. 2003); long-dashed line = *Ancyclus* sp. B (Clade 3 of Pfenninger et al. 2003); dotted line = *Ancyclus* sp. C (Clade 4 of Pfenninger et al. 2003). (B) Locations of *Ancyclus* populations studied in Lake Ohrid.

obtained with an ABI 377 automated DNA sequencer at the Scientific Research Lab, Frankfurt/Main.

The protein-coding COI sequences (638 bp long) were aligned unambiguously by eye using BioEdit 5.09 (Hall 1999). There were no insertions or deletions, except for a 15 bp long (five amino acids, positions 94–109), known insert in the outgroup *Planorbis planorbis*. The initial alignment of the LSU rDNA sequences, using default settings in ClustalW (Higgins et al. 1996), was slightly refined manually and resulted in a 482 bp long data set. There are a total of eight gaps within the ingroup taxa, with sizes ranging from 1 to 6 bp.

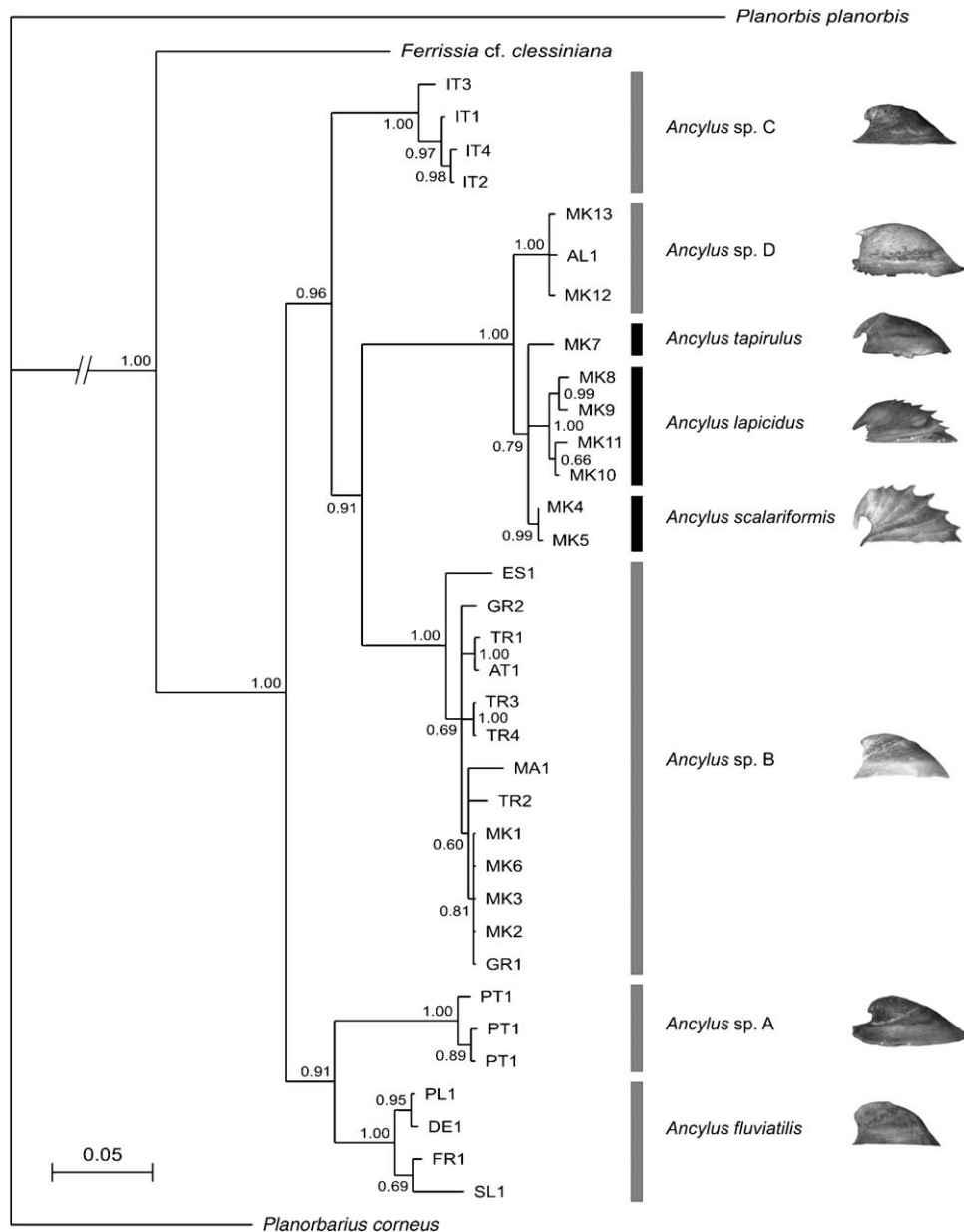
### Phylogenetic analyses

Prior to the phylogenetic analyses, the computer program Modeltest 3.6 (Posada and Crandall 1998) was used in order to find the optimal model of DNA substitution for each of the two partitions, based on the Akaike information criterion. For the COI data set, the TrN + I +  $\Gamma$  model was selected, with base frequencies of  $A = 0.3030$ ,  $C = 0.1192$ ,  $G = 0.1404$ ,  $T = 0.4373$ ; a rate matrix of  $[A-C] = 1.0000$ ,  $[A-G] = 20.7676$ ,  $[A-T] = 1.0000$ ,  $[C-G] = 1.0000$ ,  $[C-T] = 13.3154$ ,  $[G-T] = 1.000$ ; a proportion of invariable sites of 0.3670; and a gamma distribution shape parameter of  $\alpha = 0.2499$ . The

LSU rDNA partition was best described by a TVM +  $\Gamma$  model with base frequencies of  $A = 0.3632$ ,  $C = 0.1209$ ,  $G = 0.1481$ ,  $T = 0.3677$ ; a rate matrix of  $[A-C] = 2.1751$ ,  $[A-G] = 5.5159$ ,  $[A-T] = 1.6129$ ,  $[C-G] = 0.0000$ ,  $[C-T] = 5.5159$ ,  $[G-T] = 1.000$ ; and a gamma distribution shape parameter of  $\alpha = 0.2074$ .

The relative performance of different phylogenetic methods depends on numerous factors such as degree of heterogeneity, sample size, and model of sequence evolution (e.g. Wiens and Servedio 1998; Felsenstein 2004; Kolaczkowski and Thornton 2004). Therefore, we used both maximum parsimony (MP) and Bayesian inference (BI) methods in PAUP\* 4.0b10 (Swofford 2002) and MrBayes 3.0b4 (Huelsenbeck and Ronquist 2001), respectively. For a general understanding of phylogenetic relationships within the genus *Ancyclus*, BI analyses of 34 ingroup and three outgroup specimens were performed based on the best-fit model for each partition. Note that only one randomly selected individual per ingroup population was used. During a preliminary run, the log-likelihoods started at around  $-8600$  and quickly converged on a stable value of about  $-4750$  after approximately 15,000 generations. The final run was then carried out with four chains (one cold, three heated) and 1,000,000 sampled generations, with the current tree saved at intervals of ten generations. A 50% majority rule tree was constructed from all





**Fig. 2.** BI phylogram for European *Ancyclus* species based on 1120 nucleotide positions of concatenated COI and 16S DNA sequences. Tree rooted with the outgroups *Planorbarius corneus*, *Planorbis planorbis*, and *Ferrissia cf. clessiniana*. Scale bar indicates expected number of substitutions per site according to the model of sequence evolution applied. Bayesian posterior probabilities provided above branches. Along right-side margin, shells representative of the sequenced populations are displayed. For explanations of species assignments see text. New species-level clades consecutively lettered for convenience. Endemic Lake Ohrid species indicated by black bars.

sampled trees, with the first 2000 trees (= 20,000 generations) ignored as burn-in.

In order to obtain more detailed insight into the phylogenetic relationships of *Ancyclus* spp. from Lake Ohrid and their sister taxon outside the lake, a MP analysis using only individuals of the Ohrid endemics and from a feeder spring at Tušemišta (Albania) was conducted (heuristic search, 1000 random-addition-sequence replications, TBR branch swapping).

In a third step, we utilized a statistical parsimony (SP) network analysis to demonstrate preliminary phylogeographic relationships among the putatively closely related endemic *Ancyclus* species from Lake Ohrid. The SP network was derived with TCS 1.18 (Clement et al. 2000). The network approach was chosen, because traditional tree-building approaches may perform poorly in phylogeographic analyses within or between closely related species (Posada and Crandall 2001).

## Molecular clock approach

The “vagaries” of the molecular clock (Ayala 1997) remain one of the most debated issues in phylogenetics. Problems of molecular clock estimations involve, among others, the reliability of clock calibration points (e.g. Hillis et al. 1996), the occurrence of deviant lineages and/or rate heterogeneity (e.g. Sanderson, 1997; Huelssenbeck et al. 2000; Kishino et al. 2001), and the calculation of meaningful estimates for confidence limits of the clock estimations (Hillis et al. 1996; Wilke 2004). Moreover, problems tend to become more severe in clock analyses involving only distantly related taxa and/or time frames during which the respective genes become saturated, i.e. in a global molecular clock approach (e.g. Brown et al. 1979; Avise 2000).

Unfortunately, no local clock rate estimates for the COI or LSU rDNA genes are available for representatives of the genus *Ancylus*. In fact, no such information seems to exist for any basommatophoran taxon. We therefore employed the approaches previously used by Wilke (2003, 2004) for estimating COI-based divergence times in rissoidian gastropods to date the split between the endemic *Ancylus* taxa in Lake Ohrid and their closest relative outside the lake (*Ancylus* sp. D from Sv. Naum springs). Note that the relationship among these taxa is not affected by saturation.

First, we performed log-likelihood ratio tests (LRT's; Huelssenbeck and Rannala 1997) to test whether the molecular clock null hypothesis is rejected for a reduced COI data set consisting of nine haplotypes of the three Ohrid endemics and two haplotypes of *Ancylus* sp. D.

The clock hypothesis was not rejected, neither under the respective best-fit HKY+I model of sequence evolution ( $\log L_0 = -1376.78$ ,  $\log L_1 = -1368.28$ ,  $-2 \log A = 17.00$ ,  $DF = 12$ ,  $P = 0.150$ ) nor under a simpler K2P model ( $\log L_0 = -1417.52$ ,  $\log L_1 = -1411.11$ ,  $-2 \log A = 12.82$ ,  $DF = 12$ ,  $P = 0.382$ ). Therefore, it can be assumed that the COI fragment is suitable for estimating divergence times among these taxa.

In order to calculate the nucleotide divergence and meaningful confidence limits for the split between the endemic taxa from Lake Ohrid and their sister taxon from Sv. Naum springs, we used actual branch lengths from 100 clock-constrained bootstrap trees generated in PAUP under the maximum-likelihood criterion and the K2P model (note that the K2P model was chosen in order to be able to calibrate the divergences with known local COI clock rates from several Protostomia lineages; see Wilke 2003 for details). This highly conservative approach of calculating divergences and confidence limits from clock-constrained bootstrap trees might account for most, if not all, variation in our clock estimates (see also Wilke 2004).

Finally, we estimated the time of divergence for the split of the Lake Ohrid endemic *Ancylus* species and

their closest relative outside the lake. We compiled information for local COI clocks in Protostomia lineages that were previously calibrated using major geological events (e.g. Isthmus of Panama, Mediterranean Salinity Crisis). As some of these rates are based on uncorrected distances whereas others used K2P distances, we recalculated all divergences used in those studies based on the respective data available from GenBank. The recalculated rates in % K2P divergence per 1 million years are shown in Table 1 and plotted in Fig. 5.

A comparison of five local COI clock estimates for different Protostomia lineages with roughly the same body size and lifespan indicates relatively congruent rates ranging from 1.86% K2P/million years for the data set of Farrell (2001) to 2.40% K2P/million years for the Hellberg and Vacquier (1999) data set, with an average clock rate of  $2.23 \pm 0.22\%$  K2P/million years. Note that most of the data sets in GenBank did not allow for a correction for ancestral polymorphism. We therefore refrained from correcting our *Ancylus* data set for ancestral polymorphism to avoid a possible underestimation of divergent times (see Edwards and Beerli 2000 for a discussion of ancestral polymorphism).

## Distribution data and ecological characterization

Distributional data were mapped onto a Landsat7 image in ArcGIS 9 (ESRI Inc., Redland, CA). For the genetically studied populations, the habitat types occupied by the species (bathymetric range of occurrence and substrate type) were treated as unordered characters and most parsimoniously mapped (MPR) on the inferred phylogeny using MacClade 4.0 (Maddison and Maddison 2000) by linear reconstruction of ancestral states with full ranges. All most parsimonious states were represented at each node. Alternatively, mapping of characters by means of likelihood was performed with the Ancestral State Reconstruction Package for Mesquite 1.0 (Maddison and Maddison 2003) under the assumption of the Markov k-state 1 parameter model (Mkv; Lewis 2001).

## Results

### Phylogeny

The concatenated COI/LSU rDNA data set of 1120 nucleotide positions comprised 362 variable sites of which 242 were parsimony-informative. The MP and BI trees showed identical overall branching patterns; the BI tree is presented in Fig. 2.

Within Lake Ohrid, three clades and lineages are found that correspond to the three endemic *Ancylus* species

**Table 1.** Range of molecular clock rate estimates (in percentage sequence divergence per my) proposed for COI genes in Protostomia lineages separated by fewer than 10 million years

Classification	Taxon	Original molecular clock rate	Recalculated clock rate based on K2P divergences
Vetigastropoda	<i>Tegula verrucosa</i> ssp.	2.40 (Hellberg and Vacquier 1999)	2.40
Coleoptera	<i>Tetraopes</i> spp.	0.87–3.19 (Farrell 2001)	1.86
Decapoda	<i>Sesarma</i> spp.	2.33 (Schubart et al. 1998)	2.33
Caenogastropoda	<i>Salenthydrobia/Peringia</i>	1.83 (Wilke 2003)	2.195
Decapoda	<i>Alpheus</i> spp.	1.47 <sup>a</sup> (Knowlton and Weigt 1998)	2.35 <sup>a</sup>

<sup>a</sup>Knowlton and Weigt (1998) argued in their clock calibration that the youngest of the 15 Atlantic/Pacific species pairs studied should be used to calibrate the clock based on the closure of the Isthmus of Panama. However, as their youngest pair showed highly unequal branch lengths, we used the average divergence of six additional closely related pairs of species from their study to recalculate their clock rate.

previously recognized: *A. scalariformis*, *A. lapicidus*, and *A. tapirulus*.

These endemic taxa form a monophyletic group, i.e. are derived from a common ancestor. However, the relationship between the three species was not resolved fully in the BI analysis, resulting in a trifurcation.

The sister-group to this clade is a well supported monophylum (Bayesian posterior probability [BPP] of 1.00) comprising *Ancylus* sp. D specimens from springs near the southeastern edge of the lake (Sveti Naum in Macedonia; near Tušemišta in Albania).

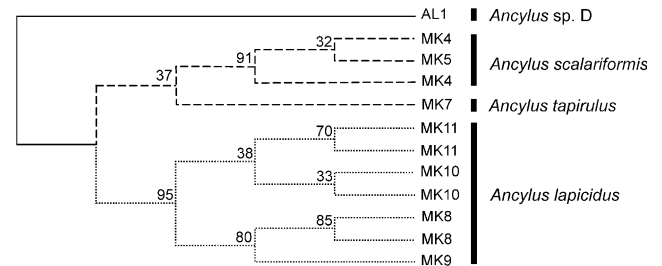
The closest relative to the above four species from Lake Ohrid and its southern feeder springs is the southern and eastern Mediterranean *Ancylus* sp. B. Sister to this group is *Ancylus* sp. C, a western and central Mediterranean species. Of all *Ancylus* taxa studied in the present paper, *A. fluviatilis* and *Ancylus* sp. A appear to be the most distantly related to the species from Lake Ohrid.

A MP analysis of Lake Ohrid *Ancylus* specimens with *Ancylus* sp. D as outgroup (Fig. 3) found *A. tapirulus* to be the sister species of *A. scalariformis*. However, as in the BI analysis, this branching was weakly supported (bootstrap value 39%).

The SP network of eleven specimens of the three Ohrid endemic species (Fig. 4) shows good separation between *A. tapirulus*, *A. scalariformis* and *A. lapicidus*. Acknowledging that only one specimen of *A. tapirulus* was available for genetic studies, *A. scalariformis* appears to be its sister taxon. This is concordant with the MP analysis (see above). The SP network suggested one haplotype of *A. scalariformis* (MK 4/2) as having the highest probability of being the ancestral haplotype in the analysis.

### Tempo and age of evolutionary history

The genetic distance (based on actual branch lengths of 100 clock-enforced ML bootstrap trees under the K2P model) for the split between the three endemic Lake Ohrid species and their sister species



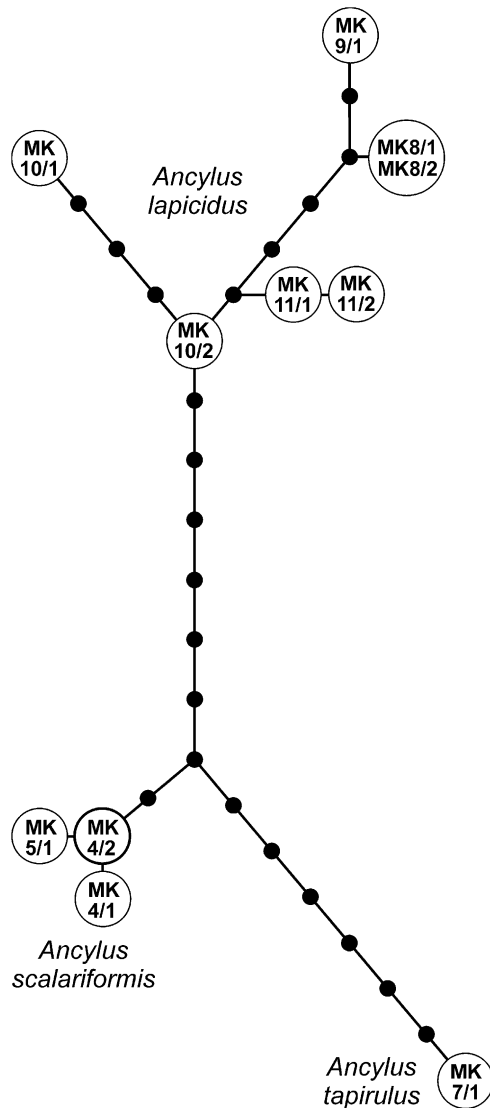
**Fig. 3.** MP cladogram for *Ancylus* species from Lake Ohrid based on 1120 nucleotide positions of concatenated COI and 16S DNA sequences. Tree rooted with outgroup *Ancylus* sp. D. MP bootstrap support (1000 replicates) provided at each node. A parsimony optimization mapping of habitat types on the phylogeny inferred is given. Dotted line indicates occurrence in rocky littoral at 0–15 m depth, dashed line refers to population living in sublittoral shell zone at 18–30 m depth.

(*Ancylus* sp. D) was calculated to  $0.0303 \pm 0.0126$  ( $3.03 \pm 1.26\%$ ). Based on the average local COI molecular clock rates for Protostomia lineages of  $2.23 \pm 0.22\%$  K2P distance per million years, the split between the Ohrid and non-Ohrid species can be estimated to have occurred approximately  $1.4 \pm 0.6$  million years ago (see Fig. 5).

### Distribution and ecology

Occurrence of the endemic Lake Ohrid *Ancylus* species is patchy and restricted mainly to the littoral and sublittoral of the karstic and rocky eastern section of the lake. Nonetheless, there are interspecific differences. While *A. scalariformis* and *A. tapirulus* occur in the sublittoral (mainly on shells of the zebra mussel *Dreissena stankovici*) of the northern and northwestern parts of the lake, *A. lapicidus* has been found only on the eastern side (on rocky substrate).

While *A. lapicidus* is restricted to the littoral from 0 to 5 m depth, *A. tapirulus* and *A. scalariformis* were found mainly between 15 and 30 m (Fig. 3).



**Fig. 4.** TCS parsimonious network of Lake Ohrid endemic *Ancylus* species based on 638 bp of COI. Maximum number of steps parsimoniously connecting two haplotypes is indicated. Haplotype with highest outgroup probability shown in a square.

## Discussion

### Phylogenetic status of the Lake Ohrid endemic species of the genus *Ancylus*

The Lake Ohrid *Ancylus* species, *A. tapirulus*, *A. scalariformis* and *A. lapicidus*, are endemic. No other *Ancylus* taxon appears to live in Lake Ohrid. The Macedonian and Albanian feeder springs of the lake are inhabited by yet another endemic taxon, *Ancylus* sp. D. Despite extensive searching, we have been unable to find this taxon in the lake, even though water flow from the springs to the lake has the character of a midsize stream.

The three Ohrid endemics are monophyletic in both the BI and MP analyses. It can therefore be assumed that these extant species share a single common ancestor.

The Lake Ohrid species and *Ancylus* sp. D from the feeder springs form a monophyletic group as well (see Fig. 2) that likely has evolved in the Ohrid Valley. The group is genetically very distinct; its closest relative is the southern European taxon *Ancylus* sp. B (Figs. 1 and 2). The overall phylogenetic relationships found for the non-Ohrid species of *Ancylus* are compatible with the inferences of Pfenninger et al. (2003). Only the relationship between *Ancylus* sp. B and *Ancylus* sp. C changes due to the inclusion of the Ohrid Valley endemics.

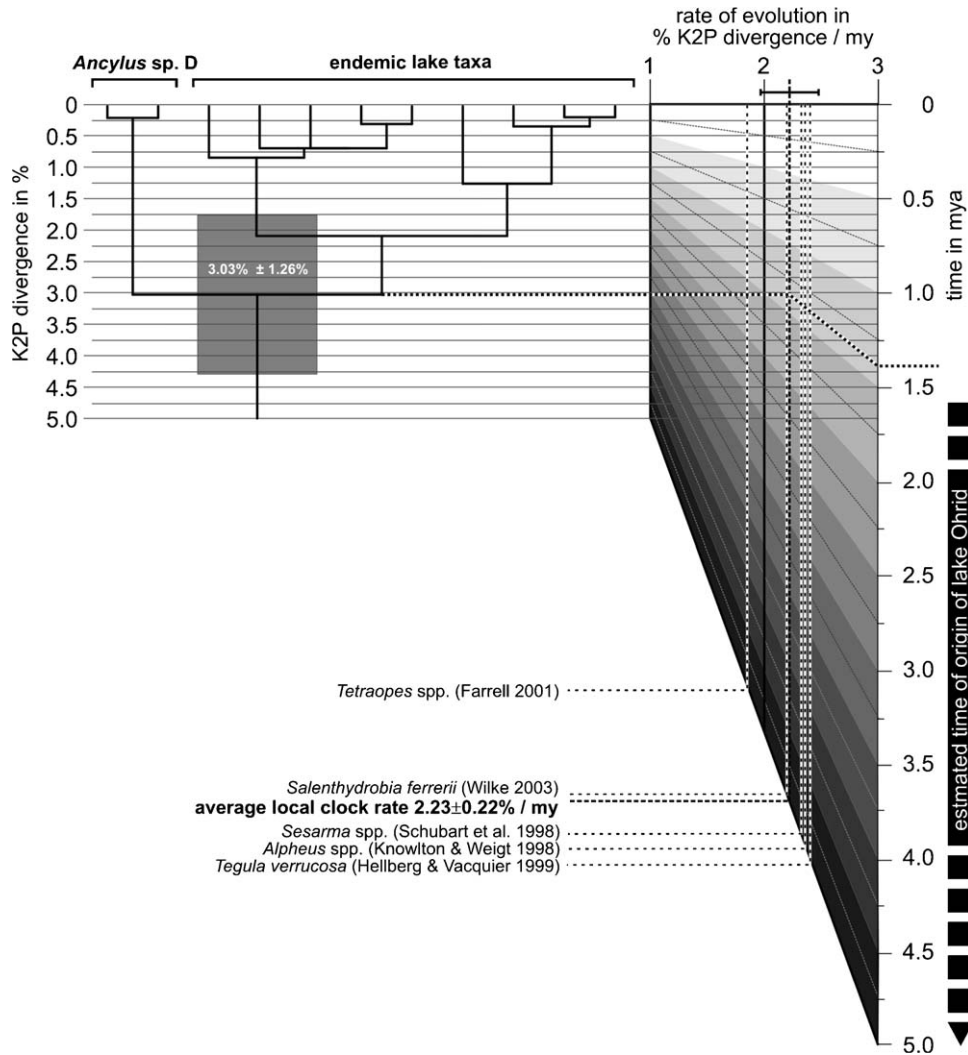
*Ancylus* sp. D and the endemics from Lake Ohrid differ from all other known *Ancylus* lineages in shell shape and structure. The validity of the ancestral position of the sublittoral *A. scalariformis*, suggested by our preliminary MP network analysis (Fig. 4), must be tested by further fine-scale populational studies. However, both the MP and network analyses hint to a sister-species relationship between *A. tapirulus* and *A. scalariformis*. Previously, *A. lapicidus* and *A. scalariformis* were thought to be closely related, due to their shell appearance with ribs, whereas *A. tapirulus* was regarded as the sister species to *A. fluviatilis* (Hubendick 1960a). This is not supported by our data. Radoman (1985) speculated that *A. tapirulus*, which he called “aberrant” (p. 127), is the most ancestral of the endemics and originated while Lake Ohrid was still connected with lacustrine basins. However, that was based on an assumed sister-species relationship between *A. fluviatilis* and *A. tapirulus*.

A genus-wide phylogeny of *Ancylus* is beyond the scope of the present paper. Additional studies involving more taxa in the *A. fluviatilis* form-group, from Mediterranean islands like Sardinia, the Balearic Islands or Crete as well as northern African species like *A. regularis* and *A. ashagiensis* (Brown 1994) and Caucasian taxa (Soldatenko and Starobogatov 2004), are necessary to further clarify the phylogeography of the European species. Some systematic remarks can be found in the Appendix in the Electronic Supplement.

### Do the Ohrid endemics represent an ancient lake species flock?

The conservative species flock definition by Greenwood (1984) is based on the following criteria: speciosity, monophyly and endemism (see the above Introduction). The latter two criteria are met by the *Ancylus* species in Lake Ohrid: the three taxa are monophyletic and do not occur outside the lake. The speciosity criterion is very subjective and hard to test. It is clear from our data that the *Ancylus* radiation in Lake





**Fig. 5.** Time scale for beginning of radiation in Lake Ohrid endemic *Ancyclus* species. Outgroups of molecular clock tree removed a posteriori. Bar represents population divergence ( $\pm$ SE) for split between lake taxa and spring taxa in southeastern part of recent lake. Rates of evolution (1–3% population divergence per million years), some recent molecular clock estimates for gastropods, and estimated age of Lake Ohrid are given. Endemic lake taxa refer to *Ancyclus scalariformis*, *A. lapicidus*, and *A. tapirulus*, whereas *Ancyclus* sp. D represents specimens from springs Sv. Naum and Tušemišta.

Ohrid is a species-poor radiation. However, as pointed out by Schön and Martens (2004), even a group of only 3–4 species may qualify as a species flock. We therefore consider the endemic species of Lake Ohrid as representing an ancient lake species flock.

Interestingly, other nearby springs on the northern and northwestern edges of the lake do not support their own *Ancyclus* lineages. Instead, all studied *Ancyclus* populations in Ohrid valley springs belong to the widespread Mediterranean taxon *Ancyclus* sp. B. Our data suggest that this taxon is the only widespread *Ancyclus* species in the eastern Mediterranean region where *Ancyclus*, in general, is rather rare (Hubendick 1970; Schütt 1982; Kinzelbach 1986).

### Mode of diversification of the Ohrid endemics relative to the origin of the lake

Inferring the actual mode of diversification is one of the most challenging parts in speciation studies (e.g. Schluter 2000, 2001) and involves knowledge of evolutionary processes in time and space. Particularly in ancient lake studies, the timing of evolutionary events (i.e. the question whether radiation started before the respective lake was formed, with the genesis of the lake or after its formation) is of great significance to an understanding of diversification modes.

Unfortunately, the precise age of Lake Ohrid is unknown. Usually it is given as 2–5 million years (e.g.

Stankovic 1960); sometimes even 10 million years are suggested (Sell and Spirkovski 2004). However, most recent studies (e.g. Salemaa 1994) assume an age no higher than approx. 2 million years.

Based on our conservative local clock estimates, the age of the split between the Ohrid and non-Ohrid species is estimated to  $1.4 \pm 0.6$  million years (see Fig. 5). This suggests that the three Ohrid endemics evolved after the establishment of Lake Ohrid or its precursor lake, i.e. we assume intralacustrine evolution. As the sister taxon of the Lake Ohrid endemics still lives in the feeder springs south of the lake, one possible scenario would be that the Lake Ohrid *Ancylus* species flock originated from specimens that invaded the young lake from these feeder springs.

However, given the relatively high standard deviation for our clock estimate and the uncertainty of the lake's age, we cannot completely rule out a second possibility, i.e. that the evolution of the Lake Ohrid *Ancylus* species flock coincided with lake genesis. The lake probably originated from damming of the upper Drim River which was fed by the same springs that nowadays feed the lake. It is possible that the formation of the lake resulted in a vicariance event separating *Ancylus* in the part of the Drim River that became the lake from those in the springs. Whereas the population in the springs did not radiate, radiation in the newly established lake took place due to the availability of new niches. It should be noted that Cvijić (1911, 1924) and Milojević (1957) suggested that the springs of Sv. Naum and Tušemišta once were sublacustrine springs. However, Stankovic (1960) and Stankovic et al. (1971) mentioned periodic lake level fluctuations during the glacial ages, and postglacial lake level reductions; also, Radoman (1985) believed in significantly earlier disconnections of the springs from the lake. Nevertheless, a distinct lineage exists in the two springs close to the southeastern edge of lake. These examples show that the interface between the springs and the lake would provide an ideal system to study ongoing speciation. Common origin of the recent Lake Ohrid endemics and *Ancylus* sp. D is not only supported by their genetic similarities but also by the shared possession of a very characteristic radula with solid teeth without cusplets (C. Albrecht, unpublished data). It should be noted that *Ancylus* is not the only gastropod taxon with putative sister-taxon relations between the feeder springs and in the actual lake. Other examples include *Carinogyraulus*, *Ohridohauffenia* and *Pyrgohydrobia* (Radoman 1985) as well as *Radix* (Burch and Hadžišće 1974).

It has been shown recently that speciation in invertebrates can be very rapid (e.g. Mendelson and Shaw 2005). In ancient lakes, the situation is equivocal. For Lake Baikal, distinct categories of species flocks have been proposed. For example, the local (endemic) gammarid species and the planorbid genus *Choanom-*

*phalus* were shown to be old species flocks (Papusheva et al. 2003). In contrast, sculpin fish and gastropods of the endemic family Baicaliidae are considered to be young flocks with estimated ages between 2.5 and 3 million years (Zubakov et al. 1997). The Ohrid *Ancylus* flock seems to be even younger than the youngest Baikalian species flocks, with an estimated start of its radiation in the early Pleistocene. Recently, Lake Ohrid has been interpreted as a glacial refuge for distinct lineages of trout fish (Sell and Spirkovski 2004). However, given the age of the Lake Ohrid *Ancylus* species flock as well as the characteristic distribution of *Ancylus* species in and particularly around the lake, the latter very likely did not constitute an evolutionary reservoir for *Ancylus* spp.

No matter whether the evolution of the Lake Ohrid *Ancylus* species flock started after the lake had formed or with the genesis of the lake, it is a major finding of the present study that evolution was intralacustrine. Although intralacustrine speciation was suggested before for several invertebrate groups in Lake Ohrid, the present study appears to be the first to support the assumption with results of molecular analyses.

Taking into account the generally low speciation rates in freshwater pulmonates as compared to non-pulmonate Gastropoda (Boss 1978), it is astonishing that three species have evolved already in Lake Ohrid in a relatively short period of time. The generally patchy distribution of ancylids in Lake Ohrid and assumed low dispersal capacities should both enhance conditions for speciation.

Radoman (1961, p. 113) found that “Intralacustrine speciation in Lake Ohrid is mainly allopatric, in mentioned cases microgeographical.” According to our preliminary data, the divergence process involves both sympatric and (micro-)allopatric components. *Ancylus tapirulus* and *A. scalariformis* both live in the shell zone (18–30 m deep), mainly on the zebra mussel *Dreissena stankovici*. This species pair seems to be allopatrically separated from *A. lapicidus* which is restricted to the littoral and lives attached to rocks or in the Ohrid-specific so-called littoral interlithon habitat (0–15 m deep). *Ancylus lapicidus* seems to be separated from the other two endemic *Ancylus* species bathymetrically and due the existence of a barrier, the *Chara* spp. algal belt (Trajanovska 2002). This barrier was already discussed by Hubendick (1960a) and is reflected in the phylogenetic results of our study. There are several characteristics of the *Chara* that cause isolation of benthic invertebrates. Migration is complicated because these macrophytic algae form a dense net, which would increase distance to be travelled by individual snails. Sediment surfaces are almost sealed by the plant cover. Interstitial water eventually becomes anoxic or even toxic at the base of that cover (Berger and Schagerl 2003). Future geneflow studies will need to evaluate how

effective this barrier is. It should be noted that there exists another pair of closely related basommatophoran limpets in Lake Ohrid: whereas *Acroloxus improvisus* lives in the shell zone, *A. macedonicus* seems to be restricted to the upper littoral (Hubendick 1960a).

### Shell shape evolution

The relatively young age of the Lake Ohrid *Ancylus* species flock indicates that shell shape and structure of Ohrid species have evolved in a very short time. Gorthner (1992) discusses non-adaptive explanations for the development of complex shell structures in ancient lakes. Shell sculpturing is very rare among freshwater pulmonates and apparently restricted to few ancient lake taxa (Albrecht and Glaubrecht 2006). However, the existence of unique ribbing in *A. scalariformis* and *A. lapicidus* is difficult to explain. Von Rintelen et al. (2004) have demonstrated the role of habitat and trophic specialisation in a caenogastropod radiation in ancient lakes on Sulawesi with additional allopatric speciation. Escalation was involved in these lakes, too, where molluscivorous crabs occur. A well known example of coevolutionary adaptation is the crab-gastropod interaction in Lake Tanganyika, with observed modifications of the prey's shell and the predator's chelae (e.g. Vermeij and Covich 1978; West and Cohen 1996). If such processes were causal to the development of the enigmatic rib structure in *A. scalariformis* and *A. lapicidus*, one would need to identify actual predators. Freshwater crabs (*Telphusa fluviatilis*) do not occur in the eastern part of Lake Ohrid (Stankovic 1960). Certain fish species are known to prey on molluscs in the Balkan Lake Scutari/Skadar (Stein et al. 1975). Gorthner (1992) attributed no importance to Lake Ohrid fish species regarding molluscivorous behaviour. Hirudinea are potential predators on freshwater snails (e.g. Streit 1985). Contrary to Gorthner (1992), who stated that leeches were not frequent in Lake Ohrid and would therefore be of no significance as predators, we would like to raise a cautionary note here. Leeches are common in the habitats where *Ancylus* occurs, and there are candidates for molluscivory, e.g. endemic species belonging to the genus *Dina*. We have observed mature leeches attaching their cocoons to the shells of limpets in Lake Ohrid. Thus it seems conceivable that juvenile leeches use ancyliids to feed during the first stages of development. Several species co-occur with ancyliids in both the littoral and sublittoral. In the lower littoral, the non-endemic *Herpobdella octoculata* occurs, which might act as predator. Gorthner (1992) argued that sculptural elements on the surface of the shells would be meaningless as defence mechanisms. However, for *Ancylus* enhanced attachment to the substrate combined with sharp ribs on the

shell could possibly boost survival. On the other hand, it is not obvious why *A. tapirulus* possesses no ribbing. Judging from the phylogenetic reconstruction, ribbing was likely lost secondarily. The absence of ribbing in *A. tapirulus* weakens the argument for a defence function of the unusual shell structuring in Lake Ohrid limpets.

### Concluding remarks

In its group of endemic *Ancylus* species, the ancient Lake Ohrid exhibits an interesting case of diversification in a generally species-poor group of freshwater gastropods.

A combination of spatial isolation, patchiness and low mobility of ancyliid populations could potentially have promoted the evolutionary pattern observed. The present study is the first molecular analysis in a European ancient lake that (1) confirms an ancient lake species flock, (2) demonstrates intralacustrine speciation, and (3) shows rapid morphological evolution. Future studies should focus on the selective regimes acting in Lake Ohrid. Small-scale ecological differences, predation and even sexual selection could be involved. To address these questions, Balkan limpets are currently studied in the Giessen lab using AFLP fingerprinting.

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