



Constant diversification rates of endemic gastropods in ancient Lake Ohrid: ecosystem resilience likely buffers environmental fluctuations

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Abstract. Ancient lakes represent key ecosystems for endemic freshwater species. This high endemic biodiversity has been shown to be mainly the result of intra-lacustrine diversification. Whereas the principle role of this mode of diversification is generally acknowledged, actual diversification rates in ancient lakes remain little understood. At least four types are conceivable. Diversification rates may be constant over time, they may fluctuate, rates may be higher in the initial phase of diversification, or there may be a pronounced lag phase between colonization and subsequent diversification. As understanding the tempo of diversification in ancient lake environments may help reveal the underlying processes that drive speciation and extinction, we here use the Balkan Lake Ohrid as a model system and the largest species flock in the lake, the non-pyrgulinid Hydrobiidae, as a model taxon to study changes in diversification rates over time together with the respective drivers.

Based on phylogenetic, molecular-clock, lineage-through-time plot, and diversification-rate analyses we found that this potentially monophyletic group is comparatively old and that it most likely evolved with a constant diversification rate. Preliminary data of the SCOPSCO (Scientific Collaboration On Past Speciation Conditions in Lake Ohrid) deep-drilling program do indicate signatures of severe environmental/climatic perturbations in Lake Ohrid. However, so far there is no evidence for the occurrence of catastrophic environmental events. We therefore propose that the constant diversification rate observed in endemic gastropods has been caused by two factors: (i) a potential lack of catastrophic environmental events in Lake Ohrid and/or (ii) a probably high ecosystem resilience, buffering environmental changes. Pa-

rameters potentially contributing to the lake's high ecosystem resilience are its distinct bathymetry, ongoing tectonic activities, and karst hydrology.

The current study not only contributes to one of the overall goals of the SCOPSCO deep-drilling program – inferring the driving forces for biotic evolution in Lake Ohrid. It might also enhance our understanding of how ecosystem resilience, in general, may promote relatively constant diversification rates in isolated ecosystems. However, we encourage future studies testing hypotheses about the lack of catastrophic events in Lake Ohrid. These studies should be based on high-resolution data for the entire geological history of the lake, and they should potentially involve information from the sediment fossil record, not only for gastropods but also for other groups with a high share of endemic taxa.

1 Introduction

Ancient lakes represent key ecosystems for the world's endemic freshwater biodiversity (Brooks, 1950; Martens et al., 1994; Martens, 1997; Rossiter and Kawanabe, 2000). Two hypotheses have been suggested for the underlying processes generating their often high levels of species richness. Originally, ancient lakes were considered to be evolutionary refugia that accumulate immigrating elements from extralimital areas during periods of environmental changes (“reservoir function”). Accordingly, distantly related “relic” species may have colonized a lake at different times and possibly from different geographic areas (e.g., Hauswald et al., 2008; Wilson et al., 2004). However, with the advance of

molecular techniques, several researchers noted that many endemic species are considerably younger than the lake they inhabit. Hence, they suggested that the high endemic biodiversity in ancient lakes is predominantly a result of intralacustrine diversification (“cradle function”) (e.g., Martens, 1997; Salzburger et al., 2005; Sherbakov, 1999).

Though the principle role of the cradle function is hardly disputed today, rates of diversification in ancient lakes remain little understood (e.g., Cristescu et al., 2010; Martens et al., 1994). As ancient lakes are considered to be comparatively stable systems (Martens, 1997), originally diversification rates (i.e., speciation minus extinction rates) were assumed to be constant over time. However, in the past decades, several factors, typically related to environmental change, have been proposed to alter the tempo of diversification in species flocks. The most renowned theory, punctuated equilibrium, suggests little net evolutionary change during periods of environmental stability (Gould and Eldredge, 1977; but see, e.g., Pennell et al., 2014; Van Bocxlaer et al., 2008). This equilibrium might be “punctuated” during phases of rapid environmental change. Another theory suggests that diversification rates can be higher in the initial phase of diversification (particularly in groups that diversify through an adaptive radiation) and may decline once niche spaces become successively occupied (e.g., Purvis et al., 2009; Schluter, 2000). This may happen after a lake first came into existence or after the occurrence of major environmental events such as volcanic ash deposits, severe lake-level drops, and desiccation or salinization events (Cristescu et al., 2010; Kroll et al., 2012; Salzburger et al., 2014). A fourth theory proposes the opposite, i.e., the existence of a pronounced lag phase between colonization and onset of diversification (e.g., Cristescu et al., 2010).

However, these scenarios have rarely been tested in ancient lake environments due to the lack of appropriate candidate lakes and suitable model taxa. Criteria for a candidate lake would be a long and continuous existence, providing sufficient time for repetitive cladogenesis, as well as a good knowledge of its paleo-limnological history, enabling a link between geological and biotic evolution. The model taxon, in turn, should be monophyletic, permitting unbiased calculations of diversification rates; species rich, thus providing sufficient power for evolutionary analyses; and reasonably old, allowing for studying the effect of environmental changes on speciation rates over an extended period of time.

Of the few ancient lakes in the world, even fewer fulfil the above criteria. Some lakes, despite being old, have gone through a series of major environmental events, and the respective endemic species are often comparable young, as observed in Lake Malawi (e.g., Schultheiß et al., 2009, 2011) and Lake Titicaca (Kroll et al., 2012). Other lakes such as Lake Baikal (e.g., Ivanov et al., 2013) and Lake Tanganyika (e.g., Salzburger et al., 2014; Scholz et al., 2007) might be sufficiently old but lack a continuous paleo-limnological record. In fact, one of the very few ancient lakes enabling a

link between geological and biotic evolution throughout its existence is the Balkan Lake Ohrid (Wagner et al., 2014). It is the oldest freshwater lake in Europe and perhaps the most speciose in the world when considering lake size (Albrecht and Wilke, 2008). Though the exact age of the lake remains controversially discussed, biological data suggest an age of no older than 2–3 million years (My) (e.g., Albrecht et al., 2006; Trajanovski et al., 2010; Wysocka et al., 2013). Newer sedimentological and seismological data obtained during the recently conducted SCOPSCO (Scientific Collaboration On Past Speciation Conditions in Lake Ohrid) deep-drilling project in Lake Ohrid revealed a minimum lake age (deep-water conditions) of ca. 1.2 My (Wagner et al., 2014), and an age of its oldest sediments of approximately 2.0 My (Lindhorst et al., 2015). This time frame of 1.2–2.0 My for the origin of extant Lake Ohrid is also considered in the current study.

Besides its relatively well characterized limnological history, Lake Ohrid also harbors a high number of endemic animal species. So far, at least 185 taxa have been described (Albrecht and Wilke, 2008; Pešić, 2015; Stocchino et al., 2013; Wysocka et al., 2013). In addition, there is a rich protist flora. Diatoms alone account for 789 taxa, with 117 of them being endemic to the lake (Levkov and Williams, 2012). Accordingly, a number of more than 300 endemic eukaryotic species for ancient Lake Ohrid is conceivable. The majority of the animal groups form relatively old species flocks in several higher taxa, including crustaceans (Wysocka et al., 2008, 2013, 2014), leeches (Trajanovski et al., 2010), and gastropods (Albrecht et al., 2006; Wilke et al., 2007, 2009). In fact, gastropods represent the most speciose animal group in Lake Ohrid, with 74 species described, 56 of which are endemic to the lake and its catchment (Albrecht and Wilke, 2008; Albrecht et al., 2009, 2014; Hauffe et al., 2011; Radoman, 1985). The largest share of this diversity is held by snails of the family Hydrobiidae (Caenogastropoda: Truncatelloidea), including 13 pyrgulinid and 27 other endemic species (Radoman, 1983). The latter group comprises the nominal genera *Dolapia*, *Gocea*, *Lynhnia*, *Ohrigocea*, *Ohridohaffenia*, *Ohridohoratia*, *Polinskiola*, *Pseudohoratia*, *Strugia*, and *Zaumia* (see Fig. 1). Pending a formal classification of this potentially monophyletic taxon, it is henceforth called the “non-pyrgulinid Hydrobiidae”. Given that this group probably represents the largest species flock in Lake Ohrid (see also Radoman, 1983), it appears to be a suitable candidate taxon to study speciation processes in this ancient lake.

Therefore, the major goal of the present study is to test for changes in diversification rates over time and to assess the underlying drivers. In order to achieve this objective, three specific goals are addressed.

- i. Based on molecular-clock analyses, we estimate the age of the most recent common ancestor (MRCA) of this group as a baseline for our temporal studies.



Figure 1. Selected representatives of genera belonging to the endemic non-pyruginid Hydrobiidae from Lake Ohrid. Scale bar: 1 mm.

- ii. Utilizing lineage-through-time (LTT) plot and diversification-rate analyses, hereinafter we assess changes in diversification rates over time.
- iii. If deviations from a constant diversification model are inferred, we finally attempt to link environmental and climatic fluctuations derived from the SCOPSCO program to these biotic changes.

Given that Lake Ohrid has long been considered to be a relatively stable system with considerable ecosystem resilience (*sensu* Stankovic, 1960), our working hypothesis is that there are no significant changes in diversification rates over time in the lake's non-pyruginid Hydrobiidae.

The current study will complement paleontological evidence of evolutionary processes obtained from the SCOPSCO high-resolution sediment record and thus contribute to one of the overall goals of the deep-drilling program – inferring the driving forces for biotic evolution in this fascinating ancient lake. It might also enhance our general understanding of how environmental change alters the tempo of diversification in isolated ecosystems and how ecosystem stability may buffer such changes.

2 Material and methods

2.1 Sampling

Hydrobiid gastropods were collected during field trips to Lake Ohrid and other waterbodies in the Balkan region between 2003 and 2011 (Fig. 2; see Table 1 for details). The collection methods followed those described in Schreiber et al. (2012) and included hand collecting, snorkeling, sieving, and dredging from small boats or the research vessel of the Hydrobiological Institute Ohrid. Samples were preserved in

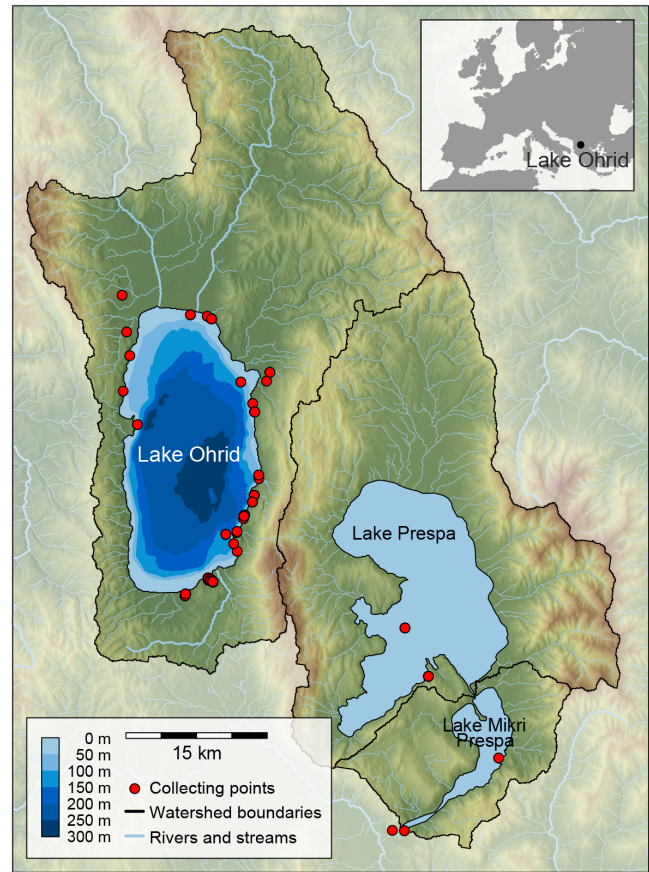


Figure 2. Sampling sites for non-pyruginid Hydrobiidae in lakes Ohrid, Prespa, and Mikri Prespa, and their watersheds.

80 % ethanol and determined in the laboratory to species level based on Radoman (1983).

2.2 DNA isolation, PCR amplification, and DNA sequencing

Genomic DNA was isolated from whole specimens using the CTAB protocol described in Wilke et al. (2006). Voucher specimens and digital images were deposited in the University of Giessen Systematics and Biodiversity collection (UGSB). Fragments of the mitochondrial genes for cytochrome oxidase c subunit I (COI) and large subunit rRNA (LSU rRNA or 16S rRNA) were amplified using the universal primers LCO1490 (Folmer et al., 1994) and COR722b (Wilke and Davis, 2000) as well as 16Sar-L and 16Sbr-H (Palumbi et al., 1991), respectively (for PCR conditions see Schreiber et al., 2012). Subsequent Sanger sequencing was conducted either on a Long Read IR2 4200 sequencer (LICOR, Lincoln, NE, USA) using the Thermo Sequenase fluorescent labelled primer cycle sequencing kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) or on a ABI 3730 XL sequencer (Life Technologies, Carlsbad, CA, USA) using the Big Dye Terminator Kit (Life Technologies, Carls-

Table 1. Taxa studied including locality information, collection and DNA voucher details, and NCBI GenBank accession numbers.

Genus	Species	Locality	Latitude	Longitude	UGSB voucher no.	DNA voucher no.	GenBank accession no. COI	16S rRNA	Reference
<i>Albaniana</i>	<i>albanica</i>	Albania, Ventroku, cave spring	40.67295	20.97403	UGSB 10730	12073	KUI70805	KUI70882	This study
<i>Belgerandia</i>	<i>marathresia</i>	Italy, Fome di S. Cassiano	–	–	UGSB 17130	2332	KUI70806	–	This study
<i>Dapimnola</i>	<i>exigua</i>	Greece, large spring at Agia Paraskevi, Tenhi Valley, N of Larisa	–	–	–	–	JF916470	–	Falhorski and Szarowska (2011)
	<i>graeca</i>	Greece, Dalne Spring	39.891083	22.607222	UGSB 17132	4238	KUI70807	–	This study
	<i>louisi</i>	Greece, Athens, spring at Kessariani	37.960729	23.798555	UGSB 17133	4239	KUI70808	–	This study
<i>Gocca</i>	<i>ohridana</i> 1	Macedonia, Lake Ohrid, lake bank at Veli Dab	40.97324	20.78668	UGSB 10710	10493	KUI70809	–	This study
	<i>ohridana</i> 2	Macedonia, Lake Ohrid, lake bank at Veli Dab	40.97324	20.78668	UGSB 10709	10332	KUI70810	KUI70883	This study
	<i>ohridana</i> 3	Macedonia, Lake Ohrid, Veli Dab	40.97097	20.78604	UGSB 10681	4299	KUI70811	–	This study
<i>Grossiana</i>	<i>coleremi</i>	Bulgaria, Jasenov	–	–	–	–	EF061920	–	Szarowska et al. (2007)
	<i>delphica</i>	Greece, Delphi, Kasatala Spring	38.483056	22.505278	–	–	EF061922	–	Szarowska et al. (2007)
	<i>serbica</i>	Serbia, Raska River Spring at Sopocan Monastery	43.115833	20.370833	–	–	EF061921	–	Szarowska et al. (2007)
	sp.	Greece, E of Volos, Oros Pilon, spring E of Anifon	–	–	–	–	KCO11768	–	Falhorski et al. (2012)
	<i>variana</i>	Greece, spring of Louros River	–	–	–	–	EF061923	–	Szarowska et al. (2007)
<i>Islamia</i>	<i>hadai</i>	Greece, Peloponnese, N Targeos Mts., W of Sparta, spring at Dhiaseho	–	–	–	–	JF916473	–	Falhorski and Szarowska (2011)
<i>Lymnida</i>	<i>glogjevici</i> 1	Macedonia, feeder springs of Svet Naum	40.91208	20.74213	UGSB 10746	12499	KUI70812	–	This study
	<i>glogjevici</i> 2	Macedonia, feeder springs of Svet Naum	40.91208	20.74213	UGSB 10747	12500	KUI70813	–	This study
	<i>glogjevici</i> 3	Macedonia, Svet Naum, small lake with springs	40.91029	20.74791	UGSB 10695	10286	KUI70814	KUI70884	This study
	<i>glogjevici</i> 4	Macedonia, Svet Naum, small lake with springs	40.91029	20.74791	UGSB 10696	10287	KUI70815	KUI70885	This study
	<i>stankovci</i>	Macedonia, Lake Ohrid, Tipelca	40.95583	20.76396	UGSB 10697	10291	KUI70816	KUI70886	This study
<i>Malaprepia</i>	<i>albanica</i>	Albania, Lake Mliri Prepsa, spring in the lake	40.67288	20.9892	UGSB 10731	12075	KUI70817	KUI70887	This study
<i>Ohridanuffenia</i>	<i>depressa</i> 1	Macedonia, Lake Ohrid, in front of Hotel Desaret, village Pestani	41.00927	20.80544	UGSB 10716	10507	KUI70818	KUI70888	This study
	<i>depressa</i> 2	Macedonia, Lake Ohrid, beach at Gorca Hill	41.08105	20.7974	UGSB 10711	10495	KUI70819	KUI70889	This study
	<i>minuta</i> 1	Macedonia, spring Studentickita	41.10251	20.81491	UGSB 10712	10497	KUI70820	–	This study
	<i>minuta</i> 2	Macedonia, spring Beg Bunar	41.11085	20.81905	UGSB 10714	10501	KUI70821	KUI70891	This study
	<i>minuta</i> 3	Macedonia, spring Beg Bunar	41.11085	20.81905	UGSB 10715	10502	KUI70822	KUI70892	This study
	<i>notonda</i>	Macedonia, Lake Ohrid, at camping site "Ljavidkite"	41.12643	20.64184	UGSB 10713	10500	KUI70823	KUI70893	This study
	<i>santrinaumi</i> 1	Macedonia, feeder springs of Svet Naum	40.91414	20.74147	UGSB 10738	12358	KUI70824	–	This study
	<i>santrinaumi</i> 2	Macedonia, feeder springs of Svet Naum	40.91061	20.74748	UGSB 10717	10510	KUI70825	KUI70894	This study
<i>Ohridohorvatia</i>	<i>carinata</i> 1	Macedonia, Lake Ohrid, Veli Dab	40.97439	20.78746	UGSB 10698	10299	KUI70826	KUI70895	This study
	<i>carinata</i> 2	Macedonia, Lake Ohrid, Tipelca	40.95868	20.77802	UGSB 10699	10305	KUI70827	KUI70896	This study
	<i>pygmaea</i> 1	Albania, feeder springs of Tushemishi	40.89874	20.71325	UGSB 10749	12508	KUI70828	–	This study
	<i>pygmaea</i> 2	Albania, feeder springs of Tushemishi	40.89686	20.7127	UGSB 10748	12507	KUI70829	–	This study
	<i>pygmaea</i> 3	Macedonia, feeder springs of Svet Naum	40.91019	20.74793	UGSB 10750	12510	KUI70830	–	This study
	<i>pygmaea</i> 4	Macedonia, Svet Naum, spring lake at island with restaurant	40.91393	20.74226	UGSB 10739	12359	KUI70831	–	This study
	<i>pygmaea</i> 5	Macedonia, Svet Naum, spring lake at island with restaurant	40.91393	20.74226	UGSB 10740	12360	KUI70832	–	This study
	<i>pygmaea</i> 6	Macedonia, Svet Naum, springs at pumping station, near chapel	40.9113	20.74744	UGSB 10741	12362	KUI70833	–	This study
	<i>pygmaea</i> 7	Albania, feeder springs of Tushemishi	40.89885	20.7136	UGSB 10755	12863	KUI70834	–	This study
	<i>pygmaea</i> 8	Albania, Lake Ohrid, south of Lin peninsula	41.06055	20.65186	UGSB 10756	12865	KUI70835	–	This study
	<i>pygmaea</i> 9	Macedonia, Lake Ohrid, in bay south of Gorca Hill	41.07304	20.79991	UGSB 10758	12867	KUI70836	–	This study
	<i>pygmaea</i> 10	Macedonia, Lake Ohrid, in bay south of Gorca Hill	41.07304	20.79991	UGSB 10757	12866	KUI70837	–	This study
	<i>pygmaea</i> 11	Macedonia, Lake Ohrid, northwest bay, near Podmolje	41.16183	20.7451	UGSB 10742	12439	KUI70838	–	This study
	<i>pygmaea</i> 12	Macedonia, Lake Ohrid, Veli Dab	40.97439	20.78747	UGSB 10759	12868	KUI70839	–	This study
	<i>pygmaea</i> 13	Macedonia, Lake Ohrid, northwestern bay, near Podmolje	41.16233	20.74395	UGSB 10718	10689	KUI70840	KUI70898	This study
	<i>pygmaea</i> 14	Macedonia, Lake Ohrid, northwestern bay, near Podmolje	41.16183	20.7451	UGSB 10743	12440	KUI70841	KUI70899	This study
	<i>pygmaea</i> 15	Macedonia, Lake Ohrid, northwestern bay, near Podmolje	41.16183	20.7451	UGSB 10744	12442	KUI70842	KUI70900	This study
	<i>pygmaea</i> 16	Macedonia, Lake Ohrid, northwestern bay, near Podmolje	41.16429	20.7396	UGSB 10745	12444	KUI70843	KUI70901	This study
<i>Ohridogocca</i>	<i>sanmili</i> 1	Macedonia, feeder springs of Svet Naum	40.91287	20.74483	UGSB 10752	12514	KUI70844	–	This study
	<i>sanmili</i> 2	Macedonia, Lake Ohrid, south of Pestani	41.09239	20.63361	UGSB 10687	10153	KUI70845	KUI70902	This study
	<i>sanmili</i> 3	Macedonia, Lake Ohrid, Veli Dab	40.97439	20.78747	UGSB 10688	10154	KUI70846	KUI70903	This study
	<i>sanmili</i> 4	Macedonia, Lake Ohrid, bay south of Gradishke	40.99323	20.79958	UGSB 10689	10156	KUI70847	KUI70904	This study

Table 1. Continued.

Genus	Species	Locality	Latitude	Longitude	UGSB voucher no.	DNA voucher no.	GenBank accession no. COI	LSU rRNA	Reference
	<i>sanniti</i> 5	Macedonia, Lake Ohrid, lake bank at Veli Dab	40.97324	20.78668	UGSB 10692	10170	KU170848	KU170905	This study
	<i>stankovici</i> 1	Macedonia, feeder springs of Sveti Naum	40.91029	20.74791	UGSB 10751	12512	KU170849	–	This study
	<i>stankovici</i> 2	Albania, feeder springs of Tushemisht	40.89874	20.71325	UGSB 10753	12517	KU170850	–	This study
	<i>stankovici</i> 3	Macedonia, feeder springs of Sveti Naum	40.91298	20.74455	UGSB 10754	12518	KU170851	–	This study
	<i>stankovici</i> 4	Macedonia, spring at Sveti Naum	40.91135	20.745	UGSB 10690	10164	KU170852	KU170906	This study
	<i>stankovici</i> 5	Macedonia, Lake Ohrid, Sveti Stefan	41.07306	20.79985	UGSB 10691	10166	KU170853	KU170907	This study
	<i>stankovici</i> 6	Macedonia, Lake Ohrid, lake bank at Veli Dab	40.97324	20.78668	UGSB 10694	10173	KU170854	KU170908	This study
	<i>stankovici</i> 7	Macedonia, Lake Ohrid, lake bank at Veli Dab	40.97324	20.78668	UGSB 10693	10172	KU170855	KU170909	This study
<i>Polinskola</i>	<i>polinski</i> 1	Macedonia, feeder springs of Sveti Naum	40.91061	20.74748	UGSB 10725	10730	KU170856	KU170910	This study
	<i>polinski</i> 2	Macedonia, feeder springs of Sveti Naum	40.91061	20.74748	UGSB 10724	10729	KU170857	KU170911	This study
	<i>polinski</i> 3	Macedonia, Lake Ohrid, Trpejca	40.95868	20.77802	UGSB 10726	10734	KU170858	KU170912	This study
	<i>polinski</i> 4	Macedonia, Lake Ohrid, southeastern part of the lake	40.9396	20.7783	UGSB 10729	10739	KU170859	KU170913	This study
	<i>polinski</i> 5	Macedonia, Lake Ohrid, Trpejca	40.95752	20.77771	UGSB 10727	10735	KU170860	KU170914	This study
	<i>sturanj</i> 1	Macedonia, Lake Ohrid, bay south of Gradishte	40.99323	20.79958	UGSB 10722	10727	KU170861	KU170915	This study
	<i>sturanj</i> 2	Macedonia, Lake Ohrid, in bay south of Gorica Hill	41.07304	20.79991	UGSB 10728	10737	KU170862	KU170916	This study
	<i>sturanj</i> 3	Macedonia, Lake Ohrid, bay south of Gradishte	40.99323	20.79958	UGSB 10723	10728	KU170863	KU170917	This study
<i>Prespolitorea</i>	<i>malaprensensis</i>	Greece, Lake Mikri Prespa, at the former Hydrobiological Institute Mikrolimni	40.74215	21.10763	UGSB 10737	12089	KU170864	KU170918	This study
	<i>valvataiformis</i> 1	Macedonia, Lake Prespa, southern shore of Golem Grad	40.86655	20.98989	UGSB 10736	12088	KU170865	KU170919	This study
	<i>valvataiformis</i> 2	Macedonia, Lake Prespa, southern shore of Golem Grad	40.86655	20.98989	UGSB 10735	12087	KU170866	KU170920	This study
	<i>valvataiformis</i> 3	Macedonia, Lake Prespa, southern shore of Golem Grad	40.86655	20.98989	UGSB 10734	12086	KU170867	KU170921	This study
	<i>valvataiformis</i> 4	Greece, Lake Prespa, bay on cliff-like coast	40.82032	21.01939	UGSB 10733	12085	KU170868	KU170922	This study
	<i>valvataiformis</i> 5	Greece, Lake Prespa, bay on cliff-like coast	40.82032	21.01939	UGSB 10732	12084	KU170869	KU170923	This study
	<i>valvataiformis</i> 6	Greece, Lake Prespa, bay on cliff-like coast	40.82032	21.01939	UGSB 10686	10118	KU170870	KU170924	This study
<i>Pseudohoratia</i>	<i>brusinae</i>	Macedonia, Lake Ohrid, in front of Ohrid Bay	41.10156	20.78236	UGSB 10700	10313	KU170871	KU170925	This study
	<i>lucustris</i> 1	Macedonia, Lake Ohrid, 2–5 m depth at northern part of the lake	41.16569	20.71836	UGSB 10702	10316	KU170872	KU170926	This study
	<i>lucustris</i> 2	Macedonia, Lake Ohrid, 2–5 m depth at northern part of the lake	41.16569	20.71836	UGSB 10703	10319	KU170873	KU170927	This study
	<i>ohridana</i> 1	Macedonia, Lake Ohrid, south of Gradishte	40.98687	20.79729	UGSB 10707	10330	KU170874	KU170928	This study
	<i>ohridana</i> 2	Macedonia, Lake Ohrid, in front of Ohrid Bay	41.10156	20.78236	UGSB 10701	10314	KU170875	KU170929	This study
	<i>ohridana</i> 3	Macedonia, Lake Ohrid, in front of Hotel Desaret, village Pestani	41.01262	20.80513	UGSB 10705	10327	KU170876	KU170930	This study
<i>Strugia</i>	<i>ohridana</i> 4	Macedonia, Lake Ohrid, southeastern part of the lake	40.94696	20.77385	UGSB 10706	10328	KU170877	KU170931	This study
	<i>ohridana</i> 5	Macedonia, Lake Ohrid, south of Gradishte	40.98687	20.79729	UGSB 10708	10331	KU170878	KU170932	This study
	<i>ohridana</i> 6	Macedonia, Lake Ohrid, in front of Hotel Desaret, village Pestani	41.01262	20.80513	UGSB 10704	10326	KU170879	KU170933	This study
	<i>ohridana</i> 1	Macedonia, small spring near Sum Spring	41.18417	20.63181	UGSB 10761	12923	KU170880	–	This study
	<i>ohridana</i> 2	Macedonia, spring near road to Kalishta	41.14912	20.63773	UGSB 10760	12922	KU170881	–	This study
<i>Trichonia</i>	<i>kephavorissonia</i>	Greece, spring at Thermo	–	–	–	–	EF070619	–	Szarowska (2006)

bad, CA, USA). In total, we sequenced 65 specimens of 17 nominal species of the endemic non-pyrgulinid Hydrobiidae. For comparison, we also analyzed 20 specimens of 15 closely related species occurring in lakes Prespa and Mikri Prespa as well as in surrounding waterbodies (Fig. 2; see Table 1 for locality details, UGSB collection numbers and GenBank accession numbers).

2.3 Preliminary genetic analyses

The protein-coding COI sequences were unambiguously aligned in BioEdit 7.0.9.0 (Hall, 1999), resulting in a final alignment of 638 base pairs (bp). The 16S rRNA sequences were aligned using the secondary structure model for the Hydrobiidae suggested by Wilke et al. (2013), resulting in a final alignment of 462 bp including gaps (an internal fragment of 45 bp was removed since no reliable alignment could be achieved for this region).

Prior to the phylogenetic analyses, our data set of non-pyrgulinid Hydrobiidae was supplemented with sequences of closely related species. We first performed searches with BLASTN 2.2.32 (Zhang et al., 2000) against the National Center for Biotechnology Information (NCBI) nucleotide database as well as against the nucleotide database of the Wilke lab, which contains DNA information for more than 300 hydrobiid species. A preliminary phylogenetic analysis was conducted in order to identify the hydrobiid groups that are most closely related to our endemic non-pyrgulinid Hydrobiidae. These preliminary analyses (details not shown) indicated that endemic species from the sister lakes Prespa and Mikri Prespa (Fig. 2) as well as from other Balkan waterbodies are the closest relatives to the Ohrid taxa. These taxa were included in our final data set (see Table 1).

2.4 Phylogenetic inference and molecular-clock analyses

The main phylogenetic analyses using Bayesian inference were performed in BEAST v. 1.8.0 (Drummond and Rambaut, 2007). Best-fit substitution models were estimated using jModelTest 0.1.1 (Posada, 2008) based on the Akaike information criterion. The models suggested for the COI and 16S rRNA fragments were GTR+I+ Γ and GTR+I, respectively. We tested the COI data set for substitutional saturation using the test by Xia and Xie (2001) as implemented in DAMBE 5.0.23 (Xia and Xie, 2001). The value for the proportion of invariant sites ($P_{inv} = 0.46$) was obtained from the jModelTest output. The observed saturation was significantly lower than the critical values ($p < 0.001$), suggesting that this partition can be used for further (molecular-clock) analyses. Note that we did not test for saturation in the 16S data set as, within the family Hydrobiidae, the 16S gene is more conservative than the COI gene (Wilke et al., 2001, 2013).

We first ran two unconstrained analyses in BEAST, one under the strict-clock and one under the relaxed-clock model, using relative rates for both partitions. These initial runs, however, revealed an extremely low effectivity sample size for the prior and posterior distributions, suggesting that the runs might have been over-parameterized due to the complex GTR+I+ Γ model (see, e.g., Grummer et al., 2014; Slager et al., 2014). Hence, the less complex HKY+I+ Γ and HKY+I models were used for the final analyses for the COI and 16S rRNA partitions, respectively.

For calibrating the molecular clock, an external trait-specific clock rate of $1.57 \pm 0.45 \text{ My}^{-1}$ for the HKY+I+ Γ model was utilized for the COI portion of our data set (Wilke et al., 2009). This rate was established for small, dioecious, subtropical or tropical Protostomia with a generation time of approximately 1 year – all of these criteria apply to our non-pyrgulinid Hydrobiidae.

The final analyses (two strict-clock and two relaxed-clock runs) with a total of 85 sequences were run for 100 000 000 generations each, sampling every 5000 generations. The resulting log and tree files for each strict-clock and relaxed-clock run were combined using LogCombiner v. 1.8.0 (BEAST) with a 50 % burn-in. The maximum clade credibility (MCC) tree was identified based on the posterior distribution (20 000 trees). Information from the post-burn-in posterior distribution including mean node ages and 95 % highest posterior densities (HPDs) was summarized using TreeAnnotator v. 1.8.0 (BEAST; no additional burn-in). A Bayes factor (BF) analysis of the likelihoods of both runs (strict-clock vs. relaxed-clock model) was performed in Tracer 1.5 (Rambaut and Drummond, 2007; 1000 bootstrap replicates) in order to determine the best-fitting clock model (see Newton and Raftery, 1994; Suchard et al., 2001).

The BF analysis, which compares the likelihoods of both BEAST runs (strict- vs. relaxed-clock model), showed a decisive support for the relaxed-clock model with a BF of 45.61 ($\ln P_{relaxed} = -5213.40$ vs. $\ln P_{strict} = -5318.42$).

2.5 Lineage-through-time plot and diversification-rate analyses

In a first explorative analysis, LTT plot analyses were conducted using the packages ape v. 3.3 (Paradis et al., 2004) and phytools v. 0.4-56 (Revell, 2012) for the R statistical environment 3.2.1 (R Core Team, 2015) in order to examine whether major deviations from a constant diversification rate can be directly observed over time. In this way, all but the endemic non-pyrgulinid Hydrobiidae from Lake Ohrid were pruned. In a second step, a LTT plot was generated for the MCC tree plus a 95 % confidence interval based on the posterior distribution.

However, generating LTT plots and detecting changes in the slope is an explorative approach and might lead to misinterpretations (see Stadler, 2011). Therefore, potential shifts in diversification rates over time were analyzed using the R

package TreePar v. 3.3 (Stadler, 2015) by testing a maximum of three shifts for 10 trees randomly sampled from the posterior distribution. This package implements a birth–death–shift model (Stadler, 2011), which allows changes in speciation and extinction rates along a phylogeny for a given time frame and for pre-defined time intervals. Shifts were analyzed along the pruned tree with default settings and a time interval of 0.1 My. The sampling fraction was set to $17/27 = 0.63$ according to the actual number of species included divided by the number of nominal species described. Results (log likelihoods of different runs, i.e., constant diversification rate vs. 1 shift, 1 shift vs. 2 shifts, and 2 shifts vs. 3 shifts) were compared by applying likelihood ratio tests in order to examine whether shifts in rates explain the tree significantly better than a constant diversification rate (indicated by p values > 0.95 ; see Stadler, 2011, 2015).

3 Results

3.1 Phylogenetic inference and molecular-clock analyses

The relaxed-clock MCC tree (Fig. 3) shows that the endemic non-pyrgulinid Hydrobiidae from Lake Ohrid likely form a monophyletic group (Bayesian posterior probability (BPP) = 0.87). The potential sister to the Lake Ohrid group is a monophyletic group containing endemic non-pyrgulinid Hydrobiidae species from its sister lakes, lakes Prespa and Mikri Prespa, and their catchments. The split from a MRCA for these two groups is supported by a BPP of 1.0. The closest relatives to the Ohrid/(Mikri) Prespa group are other Balkan hydrobiids (BPP = 1.0) previously classified into the nominal subfamilies Belgrandiellinae, Belgrandiinae, and Horatiinae (see Wilke et al., 2014).

The molecular-clock analyses indicates an age for the MRCA of the endemic non-pyrgulinid Hydrobiidae from Lake Ohrid (i.e., the onset of diversification within this group) under the favored relaxed-clock model of 1.75–3.76 My (95 % HPD; see Fig. 3). Under the inferior strict-clock model, the upper value is slightly lower at 1.75–2.68 My.

3.2 Diversification-rate analysis

The 95 % LTT plot does not suggest major deviations from a constant diversification rate (Fig. 4a). This finding is supported by the TreePar (birth–death–shift) diversification-rate analysis. Accordingly, the likelihood ratio tests suggest for 8 out of 10 random trees a constant rate over time. Only in two of the random trees is a single shift at an age of 0.1 My detected (Fig. 4b and Table 2).

4 Discussion

4.1 The Ohrid non-pyrgulinid Hydrobiidae: an old species flock

Our phylogenetic analyses indicate that the non-pyrgulinid Hydrobiidae form a potential monophyletic, speciose, and endemic clade, and thus, by definition, represent a species flock (Greenwood et al., 1984; Schön and Martens, 2004). Therefore, in situ diversification appears to be a main process in this group. The latter conclusion even holds under the assumption of a non-monophyly of the Ohrid taxa.

The age estimates obtained from the molecular-clock analysis revealed that the Lake Ohrid flock potentially started to diversify before extant Lake Ohrid came into existence (i.e., in a pre-lake/paleo-lake phase or in other waterbodies of the Ohrid Graben system such as rivers or springs; see Figs. 3 and 4a). In fact, karst springs have previously been proposed as potential ancestral habitats for other invertebrate flocks inhabiting Lake Ohrid such as the pulmonate snail genus *Ancylus* (Albrecht et al., 2006), leeches of the genus *Dina* (Trajanovski et al., 2010), and the isopod genus *Proasellus* (Kilikowska et al., 2013). This may also be the case for the species flock studied here, given that karst springs are the dominant habitat of its closest relatives outside lakes Ohrid, Prespa, and Mikri Prespa. However, testing this hypothesis is beyond the scope of the current study and probably would require a denser sampling, more precise limnological information about the early stage of Lake Ohrid, and more specific approaches such as the reconstruction of ancestral waterbodies as well as better calibration points for molecular-clock analyses.

4.2 The Ohrid non-pyrgulinid Hydrobiidae: constant rate of diversification over time

As mentioned in the Introduction, at least four modes of tempo of speciation in ancient lake species flocks are conceivable. Diversification rates may (1) be constant over time, (2) fluctuate (“punctuated equilibrium”), (3) be higher in the initial phase of diversification, or (4) show a pronounced lag phase between colonization and subsequent diversification.

As understanding the temporal frame of speciation in Lake Ohrid is of considerable importance for reaching one of the main goals of the Ohrid scientific deep-drilling program – inferring the driving forces for biotic evolution – the second specific goal of the current study is to estimate diversification rates over time. Given that Lake Ohrid has long been regarded as a relatively stable system (Stankovic, 1960), our working hypothesis assumes no significant changes in diversification rates over time in the lake’s non-pyrgulinid Hydrobiidae.

Interestingly, our LTT plot suggests that the species flock most likely evolved with a constant diversification rate. This finding is supported by our diversification-rate analysis, in-

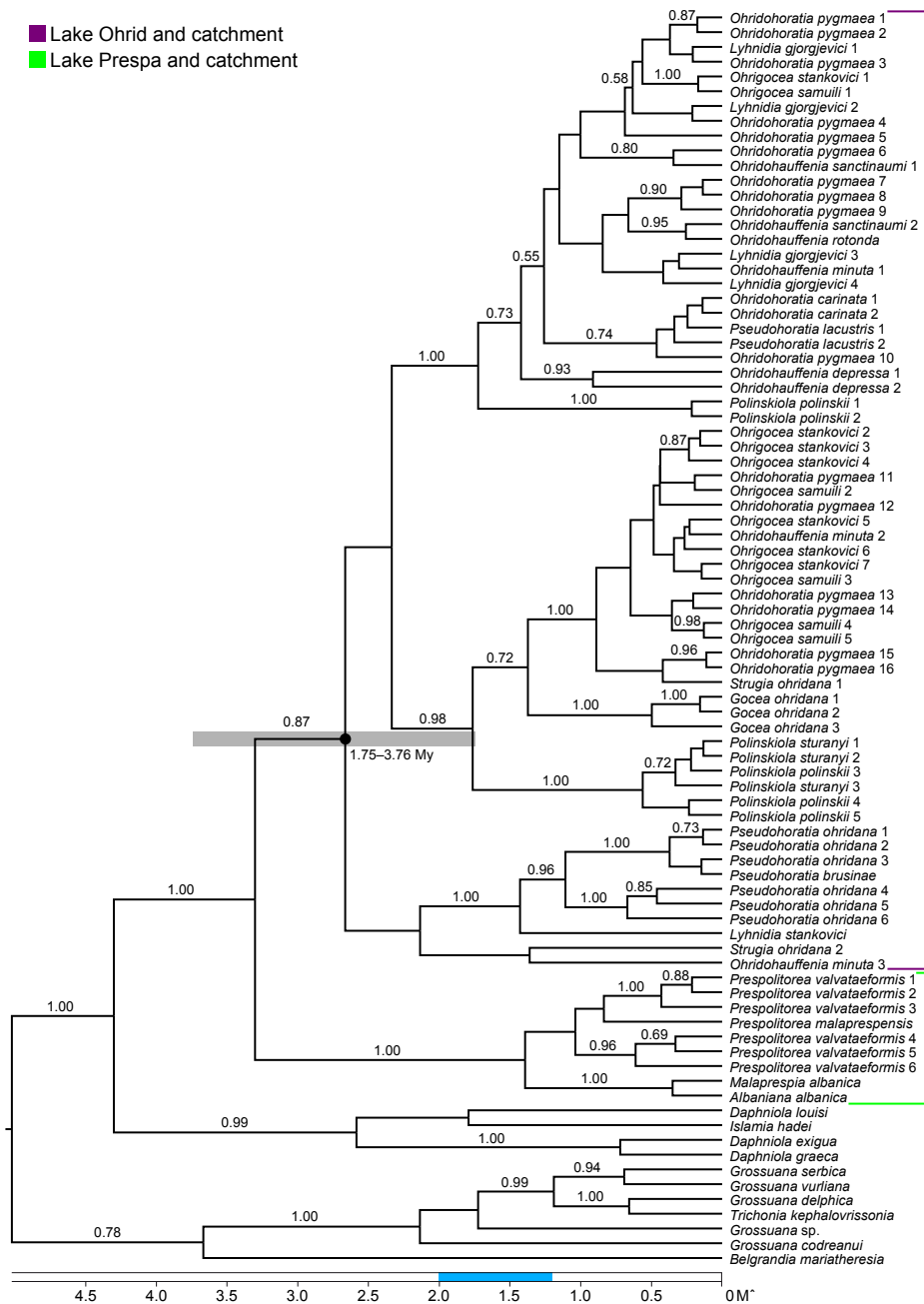


Figure 3. MCC tree based on a relaxed-clock BEAST analysis for non-pyrgulinid Hydrobiidae from the Balkans. Posterior probabilities > 0.5 are shown at the respective branches. The grey bar indicates the 95 % HPD for the age of the MRCA of the Ohrid endemics. The blue bar in the timescale ranging from 1.2 to 2.0 My shows the assumed age of Lake Ohrid.

indicating a single recent shift (drop of rates) at 0.1 Ma for 2 out of 10 random trees (i.e., a time frame in which incomplete lineage sorting may play a role; Fig. 4b and Table 2). Therefore, our initial working hypothesis – a constant diversification rate – cannot be rejected. However, we cannot rule out a type II error here due to poorly resolved phylogenetic trees or insufficient sampling size. As for the quality of the phylogenetic tree used for the LTT plot and the subsequent

diversification-rate analyses, our analyses, indeed, revealed some poorly supported (especially recent) nodes (Fig. 3). However, timetree studies are relatively robust against phylogenetic uncertainties (see, e.g., Morvan et al., 2013) as a poorly supported topology does not necessarily affect the timing of rapid speciation events (e.g., Pagel, 1999). We are therefore confident that the low BPP support of some nodes

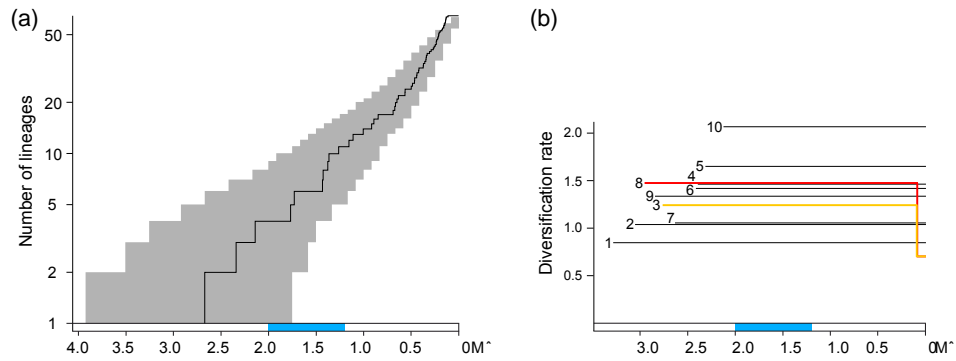


Figure 4. (a) LTT plot for the endemic non-pyrgulinid Hydrobiidae in Lake Ohrid. The plot is based on a relaxed-clock BEAST analysis, with the black line showing the BEAST MCC and the shaded area indicating the 95 % confidence interval based on 20 000 post-burn-in BEAST trees. The blue bar ranging from 1.2 to 2.0 My shows the assumed age of Lake Ohrid. (b) Diversification rates obtained from the diversification-rate analyses (TreePar); trees with a single shift in diversification rates at 0.1 My (trees nos. 3 and 8) are highlighted in red and yellow.

Table 2. Reduced output of the diversification-rate analyses (TreePar). *P* values > 0.95 indicate that a single shift in rates explains the tree significantly better than constant diversification rates, that two shifts explain the tree significantly better than a single shift, or that three shifts explain the tree significantly better than two shifts (see main text for details).

Random tree no.	Max. age (My)	Rate shifts	Log likelihood	<i>P</i> value	Shift (Ma)
1	3.29	0 vs. 1	45.882 vs. 42.043	0.947	
		1 vs. 2	42.043 vs. 38.749	0.914	
		2 vs. 3	38.749 vs. 37.060	0.663	
2	3.06	0 vs. 1	46.292 vs. 45.073	0.514	
		1 vs. 2	45.073 vs. 43.192	0.712	
		2 vs. 3	43.192 vs. 42.478	0.301	
3	2.77	0 vs. 1	36.879 vs. 32.362	0.971	1 shift: 0.1
		1 vs. 2	32.362 vs. 30.577	0.688	
		2 vs. 3	30.577 vs. 28.505	0.754	
4	2.40	0 vs. 1	28.083 vs. 26.171	0.712	
		1 vs. 2	26.171 vs. 25.507	0.278	
		2 vs. 3	25.507 vs. 23.337	0.773	
5	2.32	0 vs. 1	18.364 vs. 14.942	0.923	
		1 vs. 2	14.942 vs. 12.978	0.731	
		2 vs. 3	12.978 vs. 10.751	0.784	
6	2.42	0 vs. 1	31.850 vs. 29.342	0.829	
		1 vs. 2	29.342 vs. 27.932	0.580	
		2 vs. 3	27.932 vs. 26.219	0.670	
7	2.64	0 vs. 1	31.770 vs. 29.237	0.833	
		1 vs. 2	29.237 vs. 28.266	0.415	
		2 vs. 3	28.266 vs. 27.571	0.292	
8	2.96	0 vs. 1	34.733 vs. 30.793	0.951	1 shift: 0.1
		1 vs. 2	30.793 vs. 28.986	0.694	
		2 vs. 3	28.986 vs. 27.431	0.625	
9	2.85	0 vs. 1	34.152 vs. 31.644	0.829	
		1 vs. 2	31.644 vs. 29.433	0.781	
		2 vs. 3	29.433 vs. 27.109	0.801	
10	2.13	0 vs. 1	31.492 vs. 28.169	0.916	
		1 vs. 2	28.169 vs. 25.885	0.794	
		2 vs. 3	25.885 vs. 23.883	0.739	

in our tree had no significant influence on the outcome of our hypothesis testing.

Moreover, we also think that our conclusions are not affected by a non-monophyly of the Ohrid group (see random tree no. 2) as the split between Prespa and Ohrid taxa is, in any event, older than 2 My and thus does not affect intralacustrine diversification rates within the Lake Ohrid clade.

As for the sampling size (i.e., 17 out of 27 nominal species studied), the high diversity of evolutionary lineages found in our phylogenetic analyses indicates that our sampling design likely recovered most major evolutionary lineages within this group. Our sampling includes the majority of genera described except for *Dolapia* (which some authors included in the genus *Ohrigocea*) and *Zaumia*. We also included a variety of specimens collected at various types of habitats and type localities across the lake and its surroundings (see Fig. 2). Moreover, the diversification-rate analysis does account for incomplete sampling, and we did infer a single rate shift in two of the random trees tested in the present study. Thus, the method used seems to have enough power to detect deviations from a constant diversification rate in our data set.

4.3 Ecosystem resilience of Lake Ohrid

If we assume that the rate of diversification in the non-pyrgulinid Hydrobiidae from Lake Ohrid is constant, linking environmental/climatic fluctuations to changes in tempo of diversification becomes impossible (see specific goal iii).

However, an important question is whether a non-constant diversification rate could not be demonstrated because Lake Ohrid never experienced massive environmental and/or climatic changes or whether the lake has a high ecosystem resilience that might buffer such perturbations. Preliminary data of the SCOPSCO deep-drilling program based on core catcher data for the last 1.2 My and high-resolution data for the last 640 thousand years (ky) so far do not indicate the occurrence of catastrophic environmental events (Francke et al., 2015; Wagner et al., 2014), i.e., events that lead to sudden drastic regime shifts (sensu Scheffer and Carpenter, 2003; Scheffer et al., 2001) and thus potentially to mass extinction. By comparison, such events have been observed in other ancient lakes, including Lake Titicaca (Kroll et al., 2012; Lavenu, 1992) and Lake Malawi (Cohen et al., 2007; Scholz et al., 2007; Schultheiß et al., 2009, 2011). We do, however, see signatures of severe environmental/climatic perturbations in Lake Ohrid, including significant lake-level drops (Lindhorst et al., 2010), volcanic ash deposits (Sulpizio et al., 2010; Wagner et al., 2014), and glacial–interglacial cycles (Lézine et al., 2010; Reed et al., 2010; Wagner et al., 2014).

Therefore, we think that the lack of changes in tempo of diversification of Lake Ohrid's non-pyrgulinid Hydrobiidae might potentially be a result of two factors: (i) either Lake Ohrid never experienced catastrophic environmental events that resulted in the extinction of all or most of its endemic

taxa and thus caused a “reset” of diversification processes or (ii) Lake Ohrid possibly has a high ecosystem resilience that buffers environmental changes and potentially mitigates extinction events. Note that the two factors might not be mutually exclusive as it has been shown that a loss of resilience may set the scene for a catastrophic-event-induced switch to an alternative state (reviewed in Scheffer et al., 2001).

The second scenario is supported by the mollusk (Albrecht et al., 2010) and diatom fossil records (Cvetkovska et al., 2015; Jovanovska et al., 2015) for the past ~ 100 ky. For instance, the Campanian Ignimbrite Y5 tephra influx 39.6 ky ago (see Leicher et al., 2015) altered the water chemistry of Lake Ohrid and increased the content of silica, which in turn amplified diatom growth rates. However, it did not cause severe changes in diatom community structures or even extinction events (Jovanovska et al., 2015).

The suggested high ecosystem resilience of Lake Ohrid might be sustained by several factors, including the lake's bathymetry (deep lake with steep flanks, allowing habitats to move vertically with lake-level changes; Lindhorst et al., 2010), ongoing moderate tectonic activities (compensating sedimentation; Hinderer and Einsele, 2001), and its peculiar limnology (the lake is fed by numerous karstic sublacustrine springs, locally buffering environmental changes; Matzinger et al., 2006).

Although we think that the patterns inferred from mtDNA sequencing data of extant taxa are highly informative, future analyses utilizing additional (nuclear) markers may help better resolve some of the basal relationships of Ohrid taxa. Moreover, the temporal resolution is still limited, the error rate for time estimates is relatively high, and some important processes such as extinction events are difficult to infer from extant organisms (see also Rabosky, 2010, for a discussion). Therefore, we encourage future paleontological studies on endemic species using the sediment cores gained during the SCOPSCO deep-drilling campaign. This concerns, for example, diatoms and ostracods. Moreover, besides the main core “DEEP”, obtained from the deepest part of Lake Ohrid, several other cores were retrieved in shallower parts of Lake Ohrid. The latter appears to be relatively rich in mollusk fossils (see Wagner et al., 2014), which could potentially be used to directly study extinction and speciation events (for a proof of principle see Albrecht et al., 2010).

5 Conclusions

Our molecular-clock analyses indicate that the non-pyrgulinid Hydrobiidae of ancient Lake Ohrid represent an old endemic group, which is characterized by a constant rate of diversification. We propose that this constant rate has been caused by two factors: (i) a possible lack of catastrophic environmental events in Lake Ohrid and/or (ii) a high ecosystem resilience, buffering environmental changes. Parameters potentially contributing to Lake Ohrid's ecosystem resilience

are its distinct bathymetry, ongoing tectonic activities, and karst hydrology. These findings are not only of interest for one of the overall goals of the SCOPSCO deep-drilling program – inferring the driving forces for biotic evolution in Lake Ohrid. They might also enhance our understanding of how ecosystem resilience, in general, may promote a relatively constant diversification in highly isolated ecosystems.

However, high-resolution sedimentological data are currently only available for the last 640 ky. Therefore, we encourage future studies specifically testing hypotheses about the lack of catastrophic events in Lake Ohrid based on high-resolution data for the entire geological history of the lake, and potentially involving information from the sediment fossil record not only for gastropods but also for other groups with a high share of endemic taxa.

Author contributions. T. Wilke and C. Albrecht conceived the study. K. Föller and T. Hauffe collected and identified specimens. K. Föller performed lab work. K. Föller and B. Stelbrink performed the analyses. The manuscript was written by B. Stelbrink, T. Wilke, and K. Föller, with contributions from all co-authors. All authors gave final approval for publication.

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