

1 **Constant diversification rates of endemic gastropods in ancient Lake Ohrid:**

2 **Ecosystem resilience likely buffers environmental fluctuations**

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11

12 **Abstract**

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

23 Based on phylogenetic, molecular-clock, lineage-through-time plot and diversification-rate
24 analyses we found that this potentially monophyletic group is comparatively old and that it most likely
25 evolved with a constant diversification rate. Preliminary data of the SCOPSCO deep-drilling program
26 do indicate signatures of severe environmental/climatic perturbations in Lake Ohrid. However, so far
27 there is no evidence for the occurrence of catastrophic environmental events. We therefore propose
28 that the constant diversification rate observed in endemic gastropods has been caused by two factors:
29 i) a potential lack of catastrophic environmental events in Lake Ohrid and/or ii) a probably high
30 ecosystem resilience, buffering environmental changes. Parameters potentially contributing to the
31 lake's high ecosystem resilience are its distinct bathymetry, ongoing tectonic activities, and karst
32 hydrology.

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

41

42 **1 Introduction**

43 Ancient lakes represent key ecosystems for the world's endemic freshwater biodiversity (Brooks,
44 1950; Martens et al., 1994; Martens, 1997; Rossiter and Kawanabe, 2000). Two hypotheses have
45 been suggested for the underlying processes generating their often high levels of species richness.
46 Originally, ancient lakes were considered to be evolutionary refugia that accumulate immigrating
47 elements from extralimital areas during periods of environmental changes ('reservoir function').
48 Accordingly, distantly related 'relic' species may have colonized the lake at different times and
49 possibly from different geographic areas (e.g., Hauswald et al., 2008; Wilson et al., 2004). However,
50 with the advance of molecular techniques, several researchers noted that many endemic species are
51 considerably younger than the lake they inhabit. Hence, they suggested that the high endemic
52 biodiversity in ancient lakes is predominantly a result of intra-lacustrine diversification ('cradle
53 function') (e.g., Martens, 1997; Salzburger et al., 2005; Sherbakov, 1999).

54 Though the principle role of the cradle function is hardly disputed today, rates of
55 diversification in ancient lakes remain little understood (e.g., Cristescu et al., 2010; Martens et al.,
56 1994). As ancient lakes are considered to be comparatively stable systems (Martens, 1997), originally
57 diversification rates (i.e., speciation minus extinction rates) have been assumed to be constant over
58 time. However, in the past decades, several factors, typically related to environment change, have
59 been proposed to alter the tempo of diversification in species flocks. The most renowned theory,
60 punctuated equilibrium, suggests little net evolutionary change during periods of environmental
61 stability (Gould and Eldredge, 1977; but see e.g., Pennell et al., 2014; Van Bocxlaer et al., 2008). This
62 equilibrium might be 'punctuated' during phases of rapid environmental change. Another theory
63 suggests that diversification rates can be higher in the initial phase of diversification (particularly in
64 groups that diversify through an adaptive radiation) and may decline once niche spaces becomes
65 successively occupied (e.g., Purvis et al., 2009; Schluter, 2000). This may happen after a lake first
66 came into existence or after the occurrence of major environmental events such as volcanic ash
67 deposits, severe lake-level drops, and desiccation or salinization events (Cristescu et al., 2010; Kroll
68 et al., 2012; Salzburger et al., 2014). A forth theory proposes the opposite, i.e., the existence of a
69 pronounced lag phase between colonization and onset of diversification (e.g., Cristescu et al., 2010).

70 However, these scenarios have rarely been tested in ancient lake environments due to the
71 lack of appropriate candidate lakes and suitable model taxa. Criteria for a candidate lake would be a

72 long and continuous existence, providing sufficient time for repetitive cladogenesis, and a good
73 knowledge of its palaeo-limnological history, enabling a link between geological and biotic evolution.
74 The model taxon, in turn, should be monophyletic, permitting unbiased calculations of diversification
75 rates; species rich, thus providing sufficient power for evolutionary analyses; and reasonably old,
76 allowing for studying the effect of environmental changes on speciation rates over an extended period
77 of time.

78 Of the few ancient lakes in the world, even less fulfil the above criteria. Some lakes, though
79 being old, went through a series of major environmental events, and the respective endemic species
80 are often comparable young, as observed in Lake Malawi (e.g., Schultheiß et al., 2009, 2011) and
81 Lake Titicaca (Kroll et al., 2012). Other lakes such as Lake Baikal (e.g., Ivanov et al., 2013) and Lake
82 Tanganyika (e.g., Salzburger et al., 2014; Scholz et al., 2007) might be sufficiently old but lack a
83 continuous palaeo-limnological record. In fact, one of the very few ancient lakes enabling a link
84 between geological and biotic evolution throughout its existence, is the Balkan Lake Ohrid (Wagner et
85 al., 2014). It is the oldest freshwater lake in Europe and perhaps the most speciose in the world when
86 considering lake size (Albrecht and Wilke, 2008). Though the exact age of the lake remains
87 controversially discussed, biological data suggest an age of no older than 2–3 million years (Ma) (e.g.,
88 Albrecht et al., 2006; Trajanovski et al., 2010; Wysocka et al., 2013). Newer sedimentological and
89 seismological data obtained during the recently conducted SCOPSCO deep-drilling project in Lake
90 Ohrid revealed a minimum lake age (deep-water conditions) of c. 1.2 Ma (Wagner et al., 2014), and an
91 age of its oldest sediments of approximately 2.0 Ma (Lindhorst et al., 2015). This timeframe of 1.2–2.0
92 Ma for the origin of extant Lake Ohrid is also considered in the current study.

93 Besides its relatively well characterized limnological history, Lake Ohrid also harbors a high
94 number of endemic animal species. So far, at least 185 taxa have been described (Albrecht and
95 Wilke, 2008; Pešić, 2015; Stocchino et al., 2013; Wysocka et al., 2013). In addition, there is a rich
96 protist flora. Diatoms alone account for 789 taxa with 117 of them being endemic to the lake (Levkov
97 and Williams, 2012). Accordingly, a number of more than 300 endemic eukaryotic species for ancient
98 Lake Ohrid is conceivable. The majority of the animal groups form relatively old species flocks in
99 several higher taxa including gammarids (Wysocka et al., 2008, 2013, 2014), leeches (Trajanovski et
100 al., 2010), and gastropods (Albrecht et al., 2006; Wilke et al., 2007, 2009). In fact, gastropods
101 represent the most speciose animal group in Lake Ohrid with 74 species described, 56 of which are

102 endemic to the lake and its catchment (Albrecht and Wilke, 2008; Albrecht et al., 2009, 2014; Hauffe
103 et al., 2011; Radoman, 1985). The largest share of this diversity is held by snails of the family
104 Hydrobiidae (Caenogastropoda: Truncatelloidea), including 13 pyrgulinid and 27 other endemic
105 species (Radoman, 1983). The latter group comprises the nominal genera *Dolapia*, *Gocea*, *Lyhndia*,
106 *Ohrigocea*, *Ohridohauffenia*, *Ohridohoratia*, *Polinskiola*, *Pseudohoratia*, *Strugia*, and *Zaumia* (see Fig.
107 1). Pending a formal classification of this potentially monophyletic taxon, it is from hereon called the
108 'non-pyrgulinid Hydrobiidae'. Given that this group probably represents the largest species flock in
109 Lake Ohrid (see also Radoman, 1983), it appears to be a suitable candidate taxon to study speciation
110 processes in this ancient lake.

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

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

116 ii) Utilizing lineage-through-time (LTT) plot and diversification-rate analyses, hereinafter we assess
117 changes in diversification rates over time.

118 iii) If deviations from a constant diversification model are inferred, we finally attempt to link
119 environmental and climatic fluctuations derived from the SCOPSCO program to these biotic
120 changes.

121

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

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

130

131 **2 Material and methods**

132 **2.1 Sampling**

133 Hydrobiid gastropods were collected during fieldtrips to Lake Ohrid and other waterbodies in the
134 Balkan region between 2003 and 2011 (Fig. 2; see Table 1 for details). The collection methods
135 followed those described in Schreiber et al. (2012) and included hand collecting, snorkeling, sieving
136 and dredging from small boats or the research vessel of the Hydrobiological Institute Ohrid. Samples
137 were preserved in 80% ethanol and determined in the laboratory to species level based on Radoman
138 (1983).

139

140 **2.2 DNA isolation, PCR amplification and DNA sequencing**

141 Genomic DNA was isolated from whole specimens using the CTAB protocol described in Wilke et al.
142 (2006). Voucher specimens and digital images were deposited in the Systematics and Biodiversity
143 Collection of the University of Giessen (UGSB). Fragments of the mitochondrial genes for cytochrome
144 oxidase c subunit I (COI) and large subunit rRNA (LSU rRNA or 16S rRNA) were amplified using the
145 universal primers LCO 1490 (Folmer et al., 1994) and COR722b (Wilke and Davis, 2000) as well as
146 16Sar-L and 16Sbr-H (Palumbi et al., 1991), respectively (for PCR conditions see Schreiber et al.,
147 2012). Subsequent Sanger sequencing was conducted either on a Long Read IR2 4200 sequencer
148 (LI-COR, Lincoln, NE, USA) using the Thermo Sequenase fluorescent labelled primer cycle
149 sequencing Kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) or on a ABI 3730 XL sequencer
150 (Life Technologies, Carlsbad, CA, USA) using the Big Dye Terminator Kit (Life Technologies,
151 Carlsbad, CA, USA). In total, we sequenced 65 specimens of 17 nominal species of the endemic non-
152 pyrgulinid Hydrobiidae. For comparison, we also analyzed 20 specimens of 15 closely-related species
153 occurring in lakes Prespa and Mikri Prespa as well as in surrounding waterbodies (Fig. 2; see Table 1
154 for locality details, UGSB collection numbers and GenBank accession numbers).

155

156 **2.3 Preliminary genetic analyses**

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

162

163 **2.4 Phylogenetic inference and molecular clock analyses**

164 Prior to the phylogenetic analyses, our dataset of non-pyrgulinid Hydrobiidae was supplemented with
165 sequences of closely related species. We first performed searches with BLASTN 2.2.32 (Zhang et al.,
166 2000) against the NCBI nucleotide database as well as against the nucleotide database of the Wilke
167 lab, which contains DNA information for more than 300 hydrobiid species. A preliminary phylogenetic
168 analysis was conducted in order to identify the hydrobiid groups that are most closely related to our
169 endemic non-pyrgulinid Hydrobiidae. These preliminary analyses (details not shown) indicated that
170 endemic species from the sister Lake Prespa (Fig. 2) as well as from other Balkan waterbodies are the
171 closest relatives to the Ohrid taxa. These taxa were included in our final dataset (see Table 1).

172 The main phylogenetic analyses using Bayesian inference were performed in BEAST v.
173 1.8.0 (Drummond and Rambaut, 2007). Best-fit substitution models were estimated using jModelTest
174 0.1.1 (Posada, 2008) based on the Akaike information criterion. The models suggested for the COI
175 and 16S rRNA fragments were GTR+I+ Γ and GTR+I, respectively. We tested the COI dataset for
176 substitutional saturation using the test by Xia and Xie (2001) as implemented in DAMBE 5.0.23 (Xia
177 and Xie, 2001). The value for the proportion of invariant sites ($P_{inv} = 0.46$) was obtained from the
178 jModelTest output. The observed saturation was significantly lower than the critical values ($p < 0.001$),
179 suggesting that this partition can be used for further (molecular-clock) analyses. Note that we did not
180 test for saturation in the 16S dataset as, within the family Hydrobiidae, the 16S gene is more
181 conservative than the COI gene (Wilke et al., 2001, 2013).

182 We first ran two unconstrained analyses in BEAST, one under the strict-clock and one under
183 the relaxed-clock model using relative rates for both partitions. These initial runs, however, revealed
184 an extremely low effective sample size for the prior and posterior distributions suggesting that the runs
185 might have been over-parameterized due to the complex GTR+I+ Γ model (see e.g., Grummer et al.,
186 2014; Slager et al., 2014). Hence, the less complex HKY+I+ Γ and HKY+I models were used for the
187 final analyses for the COI and 16S rRNA partition, respectively.

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

192 The final analyses (two strict-clock and two relaxed-clock runs) with a total of 85 sequences
193 were run for 100,000,000 generations each, sampling every 5,000 generations. The resulting log and
194 tree files for each strict-clock and relaxed-clock run were combined using LogCombiner v. 1.8.0
195 (BEAST) with a 50% burn-in. The maximum clade credibility (MCC) tree was identified based on the
196 posterior distribution (20,000 trees). Information from the post-burnin posterior distribution including
197 mean node ages and 95% highest posterior densities (HPD) was summarized using TreeAnnotator v.
198 1.8.0 (BEAST; no additional burn-in). A Bayes factor (BF) analysis of the likelihoods of both runs
199 (strict-clock vs. relaxed-clock model) was performed in Tracer 1.5 (Rambaut and Drummond, 2007);
200 1,000 bootstrap replicates) in order to determine the best-fitting clock model (see Newton and Raftery,
201 1994; Suchard et al., 2001).

202 The BF analysis, which compares the likelihoods of both BEAST runs (strict vs. relaxed clock
203 model), showed a decisive support for the relaxed-clock model with a BF of 45.61 ($\ln P_{\text{relaxed}} = -$
204 $5,213.40$ vs. $\ln P_{\text{strict}} = -5,318.42$).

205

206 **2.6 Lineage-through-time plot and diversification-rate analyses**

207 In a first explorative analysis, LTT plot analyses were conducted using the packages ape v. 3.3
208 (Paradis et al., 2004) and phytools v. 0.4-56 (Revell, 2012) for the R statistical environment 3.2.1 (R
209 Core Team, 2015) in order to examine whether major deviations from a constant diversification rate
210 can be directly observed through time. Thereby, all but the endemic non-pyrgulinid Hydrobiidae from
211 Lake Ohrid were pruned from the posterior distribution (20,000 trees) and the BEAST MCC tree. In a
212 second step, a LTT plot was generated for the MCC tree plus a 95% confidence interval based on the
213 posterior distribution.

214 However, generating LTT plots and detecting changes in the slope is an explorative
215 approach and might lead to misinterpretations (see Stadler, 2011). Therefore, potential shifts in
216 diversification rates through time were analyzed using the R package TreePar v. 3.3 (Stadler, 2015)
217 by testing a maximum of three shifts for ten trees randomly sampled from the posterior distribution.
218 This package implements a birth-death shift model (Stadler, 2011), which allows changes in speciation
219 and extinction rates along a phylogeny for a given timeframe and for pre-defined time intervals. Shifts
220 were analyzed along the pruned tree with default settings and a time interval of 0.1 Ma. The sampling
221 fraction was set to $17/27 = 0.63$ according to the actual number of species included divided by the

222 number of nominal species described. Results (log likelihoods of different runs, i.e., constant
223 diversification rate vs. 1 shift, 1 shift vs. 2 shifts, and 2 shifts vs. 3 shifts) were compared by applying
224 likelihood ratio tests in order to examine whether shifts in rates explain the tree significantly better than
225 a constant diversification rate (indicated by p values > 0.95; see Stadler, 2011, 2015).

226

227 **3 Results**

228 **3.1 Phylogenetic inference and molecular clock analyses**

229 The relaxed-clock MCC tree (Fig. 3) shows that the endemic non-pyrgulinid Hydrobiidae from Lake
230 Ohrid likely form a monophyletic group (Bayesian posterior probability (BPP) = 0.87). Potential sister
231 to the Lake Ohrid group is a monophyletic group containing endemic non-pyrgulinid Hydrobiidae
232 species from its sister lakes, lakes Prespa and Mikri Prespa and their catchments. The split from a
233 MRCA for these two groups is supported by a BPP of 1.0. Closest relatives to the Ohrid/(Mikri) Prespa
234 group are other Balkan hydrobiids (BPP = 1.0) previously classified into the nominal subfamilies
235 Belgrandiellinae, Belgrandiinae, and Horatiinae (see Wilke et al., 2013).

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

240

241 **3.2 Diversification-rate analysis**

242 The 95% LTT plot does not show major deviations from a constant diversification rate (Fig. 4A). This
243 finding is supported by the TreePar (birth-death shift) diversification-rate analysis. Accordingly, the
244 likelihood ratio tests suggest for eight out of ten random trees a constant rate through time. Only in two
245 of the random trees a single shift at an age of 0.1 Ma is detected (Fig. 4B and Table 2).

246

247 **4 Discussion**

248 **4.1 The Ohrid non-pyrgulinid Hydrobiidae: an old species flock**

249 Our phylogenetic analyses indicate that the non-pyrgulinid Hydrobiidae form a potential monophyletic,
250 speciose and endemic clade, and thus, by definition, represent a species flock (Greenwood et al.,

251 1984; Schön and Martens, 2004). Therefore, *in situ* diversification appears to be a main process in this
252 group. This conclusion even holds under the assumption of a non-monophyly of the Ohrid taxa.

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

265

266 **4.2 The Ohrid non-pyrgulinid Hydrobiidae: constant rate of diversification over time**

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

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

277 Interestingly, our LTT plot showed that the species flock most likely evolved with a constant
278 diversification rate. This finding is supported by our diversification-rate analysis, indicating a single
279 recent shift (drop of rates) at 0.1 Ma for two out of ten random trees (i.e., a timeframe in which
280 incomplete lineage sorting may play a role; Fig. 4B and Table 2). Therefore, our initial working

281 hypothesis – a constant diversification rate – cannot be rejected. However, we cannot rule out a type II
282 error here due to poorly resolved phylogenetic trees or insufficient sampling size. As for the quality of
283 the phylogenetic tree used for the LTT plot and the subsequent diversification-rate analyses, our
284 analyses, indeed, revealed some poorly supported (especially recent) nodes (Fig. 3). However,
285 timetree studies are relatively robust against phylogenetic uncertainties (see e.g., Morvan et al., 2013)
286 as a poorly supported topology does not necessarily affect the timing of rapid speciation events (e.g.,
287 Pagel, 1999). We are therefore confident that the low BPP support of some nodes in our tree had no
288 significant influence on the outcome of our hypothesis testing.

289 Moreover, we also think that our conclusions are not affected by a non-monophyly of the
290 Ohrid group (see random tree #2) as the split between Prespa and Ohrid taxa is, in any event, older
291 than 2 Ma and thus does not affect intra-lacustrine diversification rates within the Lake Ohrid clade.

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

301

302 **4.3 Ecosystem resilience of Lake Ohrid**

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

306 However, an important question is whether a non-constant diversification rate could not be
307 demonstrated because Lake Ohrid never experienced massive environmental and/or climatic changes
308 or whether the lake has a high ecosystem resilience that might buffer such perturbations. Preliminary
309 data of the SCOPSCO deep-drilling program based on core catcher data for the last 1.2 Ma and high-
310 resolution data for the last 640 thousand years (ka) so far do not indicate the occurrence of

311 catastrophic environmental events (Francke et al., 2015; Wagner et al., 2014), i.e., events that lead to
312 sudden drastic regime shifts (*sensu* Scheffer and Carpenter, 2003; Scheffer et al., 2001) and thus
313 potentially to mass extinction. By comparison, such events have been observed in other ancient lakes
314 including Lake Titicaca (Kroll et al., 2012; Lavenu, 1992) and Lake Malawi (Cohen et al., 2007; Scholz
315 et al., 2007; Schultheiß et al., 2009, 2011). We do, however, see signatures of severe
316 environmental/climatic perturbations in Lake Ohrid including significant lake-level drops (Lindhorst et
317 al., 2010), volcanic ash deposits (Sulpizio et al., 2010; Wagner et al., 2014), and glacial/interglacial
318 cycles (Lézine et al., 2010; Reed et al., 2010; Wagner et al., 2014).

319 Therefore, we think that the lack of changes in tempo of diversification of Lake Ohrid's non-
320 pyrgulinid Hydrobiidae might be potentially a result of two factors: i) either Lake Ohrid never
321 experienced catastrophic environmental events that resulted in the extinction of all or most of its
322 endemic taxa and thus caused a 'reset' of diversification processes; or ii) Lake Ohrid possibly has a
323 high ecosystem resilience that buffers environmental changes and potentially mitigates extinction
324 events. Note that the two factors might not be mutually exclusive as it has been shown that a loss of
325 resilience may set the scene for a catastrophic event-induced switch to an alternative state (reviewed
326 in Scheffer et al., 2001).

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

333 The suggested high ecosystem resilience of Lake Ohrid might be sustained by several
334 factors including the lake's bathymetry (deep lake with steep flanks, allowing habitats to move
335 vertically with lake-level changes; Lindhorst et al., 2010), ongoing moderate tectonic activities
336 (compensating sedimentation; Hinderer and Einsele, 2001), and its peculiar limnology (the lake is fed
337 by numerous karstic sublacustrine springs, locally buffering environmental changes; Matzinger et al.,
338 2006).

339 Although we think that the patterns inferred from mtDNA sequencing data of extant taxa are
340 highly informative, future analyses utilizing additional (nuclear) markers may help better resolving

341 some of the basal relationships of Ohrid taxa. Moreover, the temporal resolution is still limited, the
342 error rate for time estimates is relatively high, and some important processes such as extinction
343 events are difficult to infer from extant organisms (see also Rabosky, 2010 for a discussion).
344 Therefore, we encourage future palaeontological studies on endemic species using the sediment
345 cores gained during the SCOPSCO deep-drilling campaign. This concerns, for example, diatoms and
346 ostracods. Moreover, besides the main core 'DEEP', obtained from the deepest part of Lake Ohrid,
347 several other cores were retrieved in shallower parts of Lake Ohrid. The latter appears to be relatively
348 rich in mollusk fossils (see Wagner et al., 2014), which could potentially be used to directly study
349 extinction and speciation events (for a proof of principle see Albrecht et al., 2010).

350

351 **5 Conclusions**

352 Our molecular-clock analyses indicate that the non-pyrgulinid Hydrobiidae of ancient Lake Ohrid
353 represent an old endemic group, which is characterized by a constant rate of diversification. We
354 propose that this constant rate has been caused by two factors: i) a possible lack of catastrophic
355 environmental events in Lake Ohrid and/or ii) a high ecosystem resilience, buffering environmental
356 changes. Parameters potentially contributing to Lake Ohrid's ecosystem resilience are its distinct
357 bathymetry, ongoing tectonic activities, and karst hydrology. These findings are not only of interest for
358 one of the overall goals of the SCOPSCO deep-drilling program – inferring the driving forces for biotic
359 evolution in Lake Ohrid. They might also enhance our understanding of how ecosystem resilience, in
360 general, may promote a relative constant diversification in highly isolated ecosystems.

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

366

367 **Author contribution**

368 T.W. and C.A. conceived the study. K.F. and T.H. collected and identified specimens. K.F. performed
369 lab work. K.F., and B.S. performed the analyses. The manuscript was written by B.S., T.W., and K.F.,
370 with contributions from all co-authors. All authors gave final approval for publication.

371

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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	LSU rRNA	Reference
<i>Albaniana</i>	<i>albanica</i>	Albania, Ventrroku, Cave spring	40.67295	20.97403	UGSB 10730	12073	KU170805	KU170882	This study
<i>Belgrandia</i>	<i>mariafheresia</i>	Italy, Fonte di S. Cassiano	-	-	UGSB 17130	2332	KU170806	-	This study
<i>Daphniola</i>	<i>exigua</i>	Greece, large spring at Agia Paraskevi, Tembi Valley, N of Larisa	-	-	N/A	N/A	JF916470	-	Falniowski and Szarowska (2011)
	<i>graeca</i>	Greece, Dafne Spring	39.891083	22.607222	UGSB 17132	4238	KU170807	-	This study
	<i>loulisi</i>	Greece, Athens, spring at Kessariani	37.960729	23.798555	UGSB 17133	4239	KU170808	-	This study
<i>Gocea</i>	<i>ohridana 1</i>	Macedonia, Lake Ohrid, lake bank at Veli Dab	40.97324	20.78668	UGSB 10710	10493	KU170809	-	This study
	<i>ohridana 2</i>	Macedonia, Lake Ohrid, lake bank at Veli Dab	40.97324	20.78668	UGSB 10709	10332	KU170810	KU170883	This study
	<i>ohridana 3</i>	Macedonia, Lake Ohrid, Veli Dab	40.97097	20.78604	UGSB 10681	4299	KU170811	-	This study
<i>Grossuana</i>	<i>codreanui</i>	Bulgaria, Jasenovo	-	-	N/A	N/A	EF061920	-	Szarowska et al. (2007)
	<i>delphica</i>	Greece, Delphi, Kastalia Spring	38.483056	22.505278	N/A	N/A	EF061922	-	Szarowska et al. (2007)
	<i>serbica</i>	Serbia, Raska River, Spring at Sopocani Monastery	43.115833	20.370833	N/A	N/A	EF061921	-	Szarowska et al. (2007)
	sp.	Greece, E of Volos, Oros Pilon, spring E of Anilion	-	-	N/A	N/A	KC011768	-	Falniowski et al. (2012)
	<i>vurlana</i>	Greece, spring of Louras River	-	-	N/A	N/A	EF061923	-	Szarowska et al. (2007)
<i>Islamia</i>	<i>hadei</i>	Greece, Peloponnese, N Taigetos Mts., W of Sparta, spring at Dhiaseio	-	-	N/A	N/A	JF916473	-	Falniowski and Szarowska (2011)
<i>Lychnidia</i>	<i>giorgejvici 1</i>	Macedonia, feeder springs of Sveti Naum	40.91208	20.74213	UGSB 10746	12499	KU170812	-	This study
	<i>giorgejvici 2</i>	Macedonia, feeder springs of Sveti Naum	40.91208	20.74213	UGSB 10747	12500	KU170813	-	This study
	<i>giorgejvici 3</i>	Macedonia, Sveti Naum, small lake with springs	40.91029	20.74791	UGSB 10695	10286	KU170814	KU170884	This study
	<i>giorgejvici 4</i>	Macedonia, Sveti Naum, small lake with springs	40.91029	20.74791	UGSB 10696	10287	KU170815	KU170885	This study
	<i>stankovici</i>	Macedonia, Lake Ohrid, Trepica	40.95883	20.76396	UGSB 10697	10291	KU170816	KU170886	This study
<i>Malaprespia</i>	<i>albanica</i>	Albania, Lake Mikri Prespa, spring in the lake	40.67258	20.9892	UGSB 10731	12075	KU170817	KU170887	This study
<i>Ohridohauffenia</i>	<i>depressa 1</i>	Macedonia, Lake Ohrid, in front of Hotel Desaret, village Pestani	41.00927	20.80544	UGSB 10716	10507	KU170818	KU170888	This study
	<i>depressa 2</i>	Macedonia, Lake Ohrid, beach at Gorica Hill	41.08105	20.7974	UGSB 10711	10495	KU170819	KU170889	This study
	<i>minuta 1</i>	Macedonia, spring Studenicišta	41.10251	20.81491	UGSB 10712	10497	KU170820	KU170890	This study
	<i>minuta 2</i>	Macedonia, spring Bej Bunar	41.11085	20.81905	UGSB 10714	10501	KU170821	KU170891	This study
	<i>minuta 3</i>	Macedonia, spring Bej Bunar	41.11085	20.81905	UGSB 10715	10502	KU170822	KU170892	This study
	<i>rotonda</i>	Macedonia, Lake Ohrid, at camping site "Livadište"	41.12643	20.64184	UGSB 10713	10500	KU170823	KU170893	This study
	<i>sancinaumi 1</i>	Macedonia, feeder springs of Sveti Naum	40.91414	20.74147	UGSB 10738	12358	KU170824	-	This study
	<i>sancinaumi 2</i>	Macedonia, feeder springs of Sveti Naum	40.91061	20.74748	UGSB 10717	10510	KU170825	KU170894	This study
<i>Ohridohoratia</i>	<i>carinata 1</i>	Macedonia, Lake Ohrid, Veli Dab	40.97439	20.78746	UGSB 10698	10299	KU170826	KU170895	This study
	<i>carinata 2</i>	Macedonia, Lake Ohrid, Trepica	40.95868	20.77802	UGSB 10699	10305	KU170827	KU170896	This study
	<i>pygmaea 1</i>	Albania, feeder springs of Tusemisht	40.89874	20.71325	UGSB 10749	12508	KU170828	-	This study
	<i>pygmaea 2</i>	Albania, feeder springs of Tusemisht	40.89868	20.7127	UGSB 10748	12507	KU170829	-	This study
	<i>pygmaea 3</i>	Macedonia, feeder springs of Sveti Naum	40.91019	20.74793	UGSB 10750	12510	KU170830	-	This study
	<i>pygmaea 4</i>	Macedonia, Sveti Naum, spring lake at south end of island with restaurant	40.91393	20.74226	UGSB 10739	12359	KU170831	-	This study
	<i>pygmaea 5</i>	Macedonia, Sveti Naum, spring lake at south end of island with restaurant	40.91393	20.74226	UGSB 10740	12360	KU170832	-	This study
	<i>pygmaea 6</i>	Macedonia, Sveti Naum, springs at pumping station, near chapel	40.9113	20.74744	UGSB 10741	12362	KU170833	-	This study
	<i>pygmaea 7</i>	Albania, feeder springs of Tusemisht	40.89885	20.7136	UGSB 10755	12863	KU170834	-	This study
	<i>pygmaea 8</i>	Albania, Lake Ohrid, south of Lin peninsula	41.06055	20.65186	UGSB 10756	12865	KU170835	-	This study
	<i>pygmaea 9</i>	Macedonia, Lake Ohrid, in bay south of Gorica Hill	41.07304	20.79991	UGSB 10758	12867	KU170836	-	This study
	<i>pygmaea 10</i>	Macedonia, Lake Ohrid, in bay south of Gorica Hill	41.07304	20.79991	UGSB 10757	12866	KU170837	-	This study
	<i>pygmaea 11</i>	Macedonia, Lake Ohrid, northwestern bay, near Podmolje	41.16183	20.7451	UGSB 10742	12439	KU170838	KU170897	This study

continued

	<i>pygmaea</i> 12	Macedonia, Lake Ohrid, Veli Dab	40.97439	20.78747	UGSB 10759	12868	KU170839	-	This study
	<i>pygmaea</i> 13	Macedonia, Lake Ohrid, northwestern bay, near Podmolje	41.16235	20.74395	UGSB 10718	10689	KU170840	KU170898	This study
	<i>pygmaea</i> 14	Macedonia, Lake Ohrid, northwestern bay, near Podmolje	41.16183	20.7451	UGSB 10743	12440	KU170841	KU170899	This study
	<i>pygmaea</i> 15	Macedonia, Lake Ohrid, northwestern bay, near Podmolje	41.16183	20.7451	UGSB 10744	12442	KU170842	KU170900	This study
	<i>pygmaea</i> 16	Macedonia, Lake Ohrid, northwestern bay, near Podmolje	41.16429	20.7396	UGSB 10745	12444	KU170843	KU170901	This study
Ohrigocea	<i>sarmuili</i> 1	Macedonia, feeder springs of Sveti Naum	40.91287	20.74483	UGSB 10752	12514	KU170844	-	This study
	<i>sarmuili</i> 2	Macedonia, Lake Ohrid, south of Pestina	41.09239	20.63361	UGSB 10687	10153	KU170845	KU170902	This study
	<i>sarmuili</i> 3	Macedonia, Lake Ohrid, Veli Dab	40.97439	20.78747	UGSB 10688	10154	KU170846	KU170903	This study
	<i>sarmuili</i> 4	Macedonia, Lake Ohrid, bay south of Gradishte	40.99323	20.79958	UGSB 10689	10156	KU170847	KU170904	This study
	<i>sarmuili</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 Tusemisht	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
Polinskiola	<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, southeast end 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>sturanyi</i> 1	Macedonia, Lake Ohrid, bay south of Gradishte	40.99323	20.79958	UGSB 10722	10727	KU170861	KU170915	This study
	<i>sturanyi</i> 2	Macedonia, Lake Ohrid, in bay south of Gorica Hill	41.07304	20.79991	UGSB 10728	10737	KU170862	KU170916	This study
	<i>sturanyi</i> 3	Macedonia, Lake Ohrid, bay south of Gradishte	40.99323	20.79958	UGSB 10723	10728	KU170863	KU170917	This study
	<i>malaprespensis</i>	Greece, Lake Mikri Prespa, at the former Hydrobiological Institute Mikrolimni	40.74215	21.10763	UGSB 10737	12089	KU170864	KU170918	This study
	<i>valvataeformis</i> 1	Macedonia, Lake Prespa, southern shore of Golem Grad	40.86655	20.98989	UGSB 10736	12088	KU170865	KU170919	This study
	<i>valvataeformis</i> 2	Macedonia, Lake Prespa, southern shore of Golem Grad	40.86655	20.98989	UGSB 10735	12087	KU170866	KU170920	This study
	<i>valvataeformis</i> 3	Macedonia, Lake Prespa, southern shore of Golem Grad	40.86655	20.98989	UGSB 10734	12086	KU170867	KU170921	This study
<i>valvataeformis</i> 4	Greece, Lake Prespa, bay on cliff-like coast	40.82032	21.01939	UGSB 10733	12085	KU170868	KU170922	This study	
<i>valvataeformis</i> 5	Greece, Lake Prespa, bay on cliff-like coast	40.82032	21.01939	UGSB 10732	12084	KU170869	KU170923	This study	
<i>valvataeformis</i> 6	Greece, Lake Prespa, bay on cliff-like coast	40.82032	21.01939	UGSB 10686	10118	KU170870	KU170924	This study	
Pseudohoratia	<i>brusinae</i>	Macedonia, Lake Ohrid, in front of Ohrid Bay	41.10156	20.78236	UGSB 10700	10313	KU170871	KU170925	This study
	<i>lacustris</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>lacustris</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>ohridana</i> 4	Macedonia, Lake Ohrid, southeast end 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	

continued

<i>Strugia</i>	<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>kephalovrissonia</i>	Greece, spring at Thermo	-	-	N/A	N/A	EF070619	-	Szarowska (2006)

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

Random tree #	Max. age (Ma)	Rate shifts	-Log-likelihood	<i>P</i> value	Shift (Ma ago)
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	

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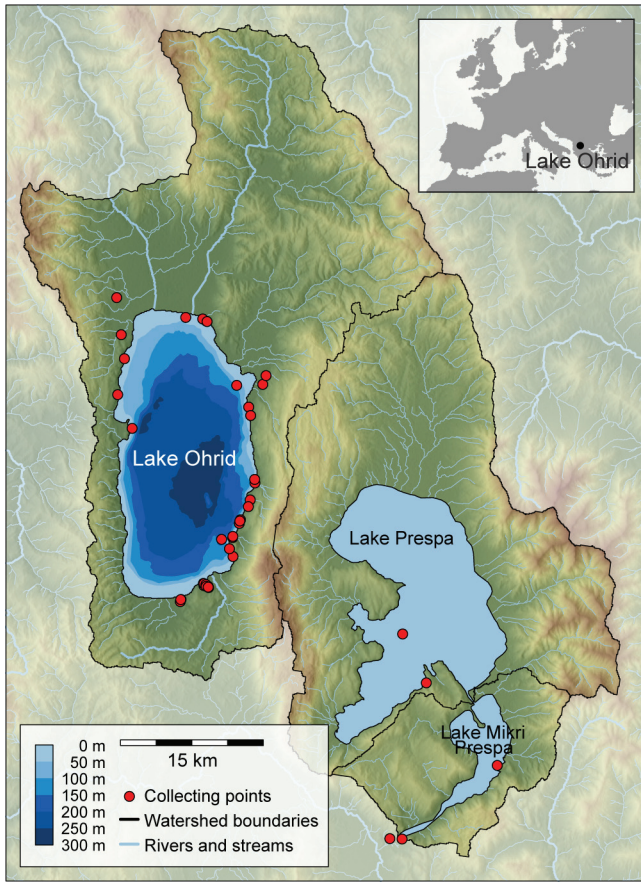
631 **Figure captions**



633 **Fig. 1.** Selected representatives of genera belonging to the endemic non-pyrgulinid Hydrobiidae from
634 Lake Ohrid. Scale bar = 1 mm.

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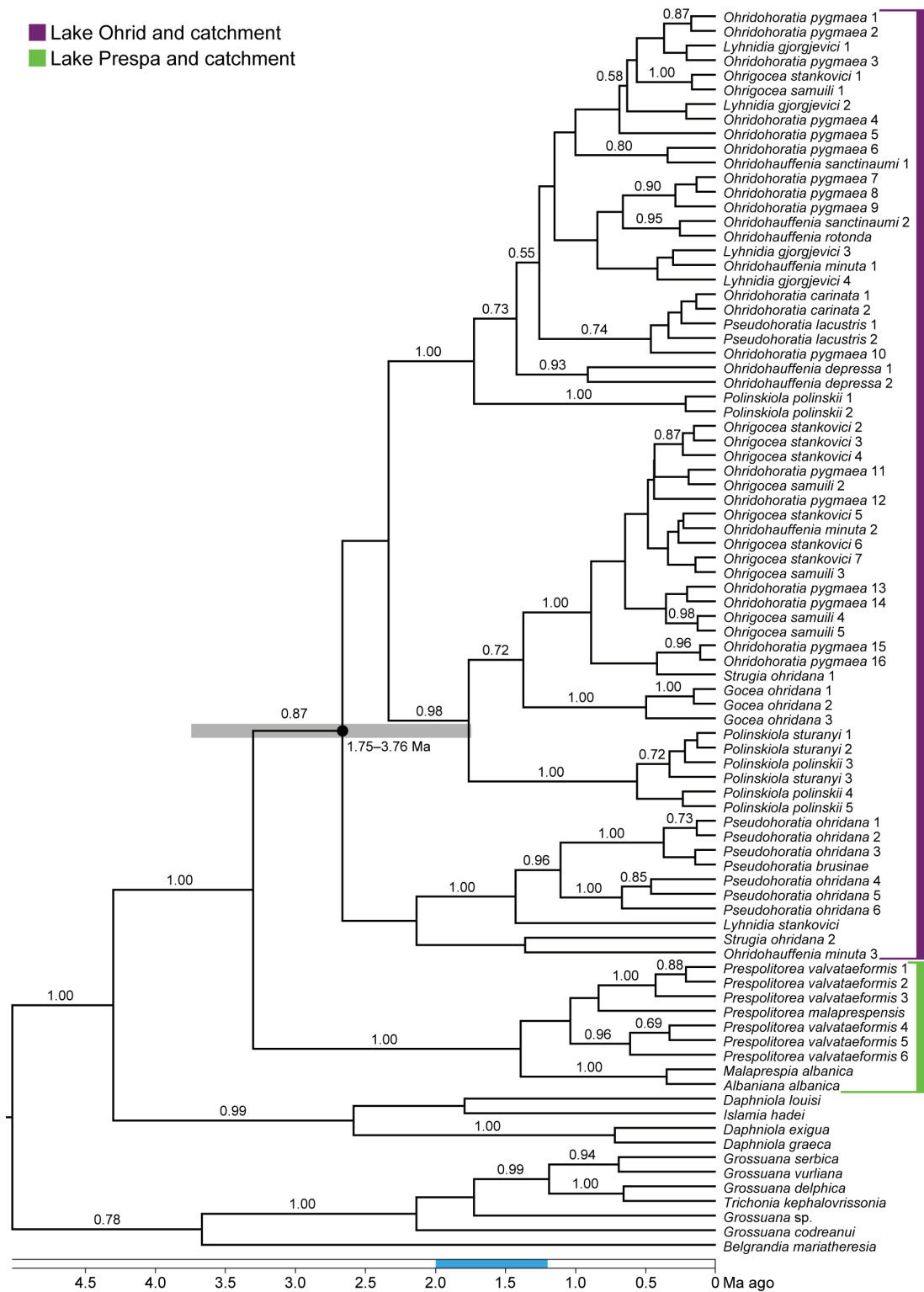


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638 **Fig. 2.** Sampling sites for non-pyrgulinid Hydrobiidae in lakes Ohrid, Prespa and Mikri Prespa, and
 639 their watersheds.

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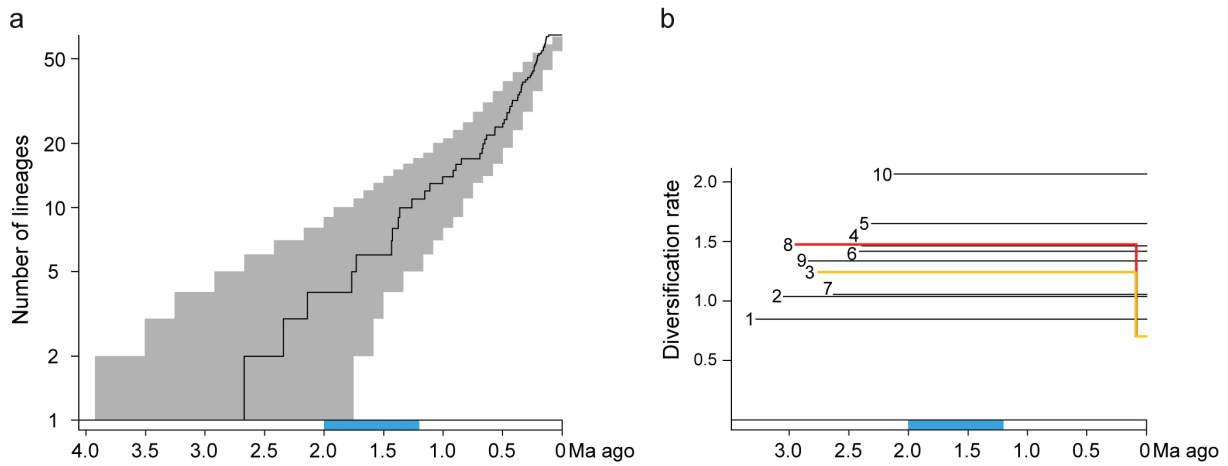
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Fig. 3. MCC tree based on a relaxed-clock BEAST analysis for non-pyrgulinid Hydrobiidae from the Balkans. Posterior probabilities >0.5 are shown at the respective branches. The grey bar indicates the 95% HPD for the age of the MRCA of the Ohrid endemics.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



648

649 **Fig. 4.** (A) LTT plot for the endemic non-pyrgulinid Hydrobiidae in Lake Ohrid. The plot is based on a
 650 relaxed-clock BEAST analysis with the black line showing the BEAST MCC and the shaded area
 651 indicating the 95% confidence interval based on 20,000 post-burnin BEAST trees. The blue bar
 652 ranging from 1.2–2.0 Ma ago shows the assumed age of Lake Ohrid. (B) Diversification rates obtained
 653 from the diversification rate-analyses (TreePar); trees with a single shift in diversification rates at 0.1
 654 Ma (trees #3 and #8) are highlighted in red and yellow.