

Thank you for the detailed comments and reviews on our manuscript. We address all of the points raised by the Referees below.

Referee 1 (A. Weigand)

Specific comments:

1) *In the discussion section, the authors state that "[t]he findings suggest that the non-pyrgulinid Hydrobiidae form a monophyletic, speciose and endemic clade and thus, by definition, represent a species flock". However, and since Bayesian posterior probability values have a strong tendency towards high values (see e.g. Cummings et al. 2003 - Comparing bootstrap and posterior probability values in the four-taxon case), a support value of 0.87 cannot be seen as a decisive support nor as an argument for the monophyly of this organism group. Thus, hypothesis 1 must be questioned. Furthermore, and because this hypothesis is the authors self-defined requirement to perform all subsequent diversification analyses, the monophyly of this group has to be unambiguously demonstrated first. I would suggest to additionally implement Maximum Likelihood analyses complementing the BI data and perhaps an even more dedicated partitioning scheme with different partitions based on the three CO1 codon positions and different partitions for the stem and loop regions of the 16S rDNA fragment. A nuclear marker would be great, but I see that this will be hard to achieve for all the specimens.*

Response: We are thankful for this comment and would like to clarify this point. The support value of 0.87 is comparatively low for a Bayesian posterior probability and we should be cautious in drawing the final conclusion that the flock is monophyletic.

As suggested by the referee, we performed additional maximum likelihood (RAxML) analyses using a default partitioned dataset (16S, COI; ML bootstrap: 30) and a partitioned dataset, in which the COI fragment was additionally partitioned by codon position (ML bootstrap: 61). Though in both analyses, the Prespa clade is monophyletic (BPP = 1.0), there is no significant support for a monophyletic Ohrid clade.

However, we think that a potential non-monophyly of the Ohrid clade will not have significant implications for the analyses and interpretations in our manuscript. First, our LTT-analyses are strictly based on Ohrid taxa, and a single colonization event into Lake Prespa would add only a single lineage to our analysis. Second, and more importantly, the only random tree (#2), suggesting a non-monophyly of the Ohrid clade, indicates that the split between Prespa and Ohrid taxa occurred c. 2.38 Ma ago and thus before Lake Ohrid came into existence. In other words, it does not affect intra-lacustrine diversification rates.

To make this clearer to the reader, we plan to rephrase the respected section in our manuscript: "Our findings indicate that this group is comparatively old and most likely monophyletic. However, due to the comparatively low support of 0.87, a non-monophyly of the Ohrid group cannot be excluded. Nonetheless, even under the unlikely assumption that the Prespa group renders the Ohrid group paraphyletic (as in random tree #2), the molecular-clock analysis suggests that the split between Prespa and Ohrid is older than 2 Ma and thus does not affect intra-lacustrine diversification rates within the Lake Ohrid clade."

2) *Again in the discussion, the authors argue that "[o]ur LTT-plot (Fig. 4a) shows that several lineages have already existed when the lake reached deep-water conditions or even before it came into existence (see Trajanovski et al., 2010 for a potentially similar pattern in the Dina leach flock). Therefore, the term "cradle" may not only be used for monophyletic species evolving within the lake (intra-acustrine speciation) but also for a group of monophyletic species that started to evolve within the (palaeo-)basin." Important here and addressed are the two oldest nodes of the non-pyrgulid Hydrobiidae (> 2mya) and their four corresponding lineages. However, both assumed to be monophyletic groupings within the non-pyrgulinid Hydrobiidae (i.e. the resulting 4 lineages) have no posterior probability support at all (= below 0.5 according to the authors), must be collapsed and hence cannot be regarded as being monophyletic. The argument of several lineages existing prior to lake Ohrid formation thus does not hold true. Same is true for the monophyly of the mentioned species groups.*

Response: Thanks for raising these important points. In our manuscript, we wanted to point out that the term 'cradle' may not only be used for a monophyletic group of extant species whose ancestors started to evolve within the lake (intra-lacustrine speciation), but also for lineages/species that started

to evolve within the (palaeo-)basin. However, we agree that the above sentence starting with "Therefore, the term 'cradle' [...]" might be misleading and thus we will remove it from the revised manuscript.

Regarding the second last sentence of the referee, we disagree with this statement. Both, our time-dated phylogenetic tree (Fig. 3) and the LTT plot (Fig. 4) indicate that at least some lineages may have existed before 2.0 Ma and thus prior to the lake formation. However, collapsing lineages with low support will result in a polytomy with exactly the same age. This assumption is also supported by Referee 2, see point 2).

3) *Based on the inferred constant rate of diversification the authors conclude that "[the] initial working hypothesis – rate homogeneity – cannot be rejected. [...] 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 iv)." The diversification rate is defined as the net sum of speciation rate - extinction rate. Rate homogeneity thereby refers to the following (see Ricklefs 2007 - Estimating diversification rates from phylogenetic information): "The simplest diversification process presupposes that rates of speciation and extinction are the same for all lineages and do not vary over time. This is the assumption of rate homogeneity." Thus, rate homogeneity is mostly used as a model and cannot be inferred from a constant rate of diversification as it refers to constant rates of speciation and extinction. Ricklefs further states that "different combinations of speciation and extinction rates can produce the same expected clade size". This means, that different combinations in speciation and extinction rates may lead to similar inferred net diversification rates. As an example: potential environmental/climatic fluctuations (as proposed by some studies for Lake Ohrid) may have lead to extinction events and subsequent adaptive radiations within relative short periods of time. This is known for other taxa including hydrobiids from other regions of the world. The outcome would be a temporally increased rate of extinction and subsequent increased rate of speciation. However, and for the observed 0.1 my intervals, the "phylogenetic window" may be too broad to see those changes in extinction and speciation rates leading to a similar net diversification rate (i.e. birth and death of lineages) as expected under rate homogeneity. Thus, rate homogeneity as defined as constant speciation and extinction rates cannot be inferred nor supported by the data presented as only a constant rate of diversification is observed. Finally Ricklefs (2007) states that "it is unlikely that rate homogeneity can be unambiguously supported for any clade." However, the inference drawn by the authors that "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" can be partially drawn as old lineages can be observed. However, lineages may have even survived those events. If this point is still dealt with in the discussion, it should be re-formulated according to what is supported by the data. I would be really careful in drawing the final conclusion that a high ecosystem reliance can be supported by the "rate homogeneity" of the investigated gastropod taxon. The opposite may be true: If a significant rate shift is observed and can be temporarily linked to a given environmental event, this may be seen as support for the influence of this event on the diversification process. Related to this discussion, see also point 4.*

Response: We thank you for pointing us to the difference between rate homogeneity as defined by Ricklefs (2007) and constant diversification rates, which is the parameter that we actually measured. We will replace the term "rate homogeneity" by "constant diversification rate" in the revised manuscript. The definition of rate homogeneity is a very strict theoretical concept and, as correctly identified by the referee, Ricklefs acknowledges that *"it is unlikely that rate homogeneity can be unambiguously supported for any clade"*. The test of rate homogeneity is moreover challenging because there is still an ongoing debate whether extinctions rates can be reliable estimated based on molecular phylogenies (Beaulieu and O'Meara, 2015; Morlon et al., 2011; Rabosky, 2010, 2014). Our used TreePar approach, for instance, is known to overestimate the extinction/speciation ratio but identifies shifts in net diversification rate correctly (Laurent et al., 2015; Stadler, 2011).

We are still confident that the window frame (grid) of 0.1 Ma is sufficient for the TreePar analysis, because 1) this is a reasonable timeframe as changes in diversification rates will probably not occur within smaller time slots in both genetic markers, and 2) we had enough power to detect shifts in two out of ten random trees at the time of 0.1 Ma. We furthermore think that the grid used is less important than the magnitude of changes in the diversification rate from one time slot to the next. Unfortunately, only two studies analysed the power of the TreePar approach under various scenarios of tree size and extinction fractions (Laurent et al., 2015; Stadler, 2011) but none of them tested explicitly the influence of grid size. We however re-analyzed random tree #1 using a grid of 0.01 which resulted in a

significantly better log-likelihood by including a single shift at 0.07 Ma (const: 45.882 vs. 1 shift: 35.959 → $p = 1.0$). Note that with a window of 0.1 Ma, a single rate shift in this tree almost reached the critical significance level ($p = 0.947$; see Table 2). This very recent single shift, again, represents a timeframe in which incomplete lineage sorting may play a crucial role.

As for the third point, that a high ecosystem resilience may not be supported by a lack of diversification rate shifts, we only partly agree. Operational criteria for resilience are lack of regime shift and lack of extinction events (for details see the discussion paper of Jovanovska et al. in the same special issue) and we agree that the latter is very difficult to apply to molecular phylogenies (see also above). In molecular phylogenetic studies, the impact of catastrophic events has been inferred from molecular-clock analyses and/or particular topological patterns (e.g., founder flush, recent re-colonizations; see e.g., Schultheiß et al. 2009). If such a pattern is not observed in a molecular phylogeny, more complex methods are needed such as diversification-rate analyses. We think that this methodological approach is powerful even for such recent and comparatively small datasets (we could detect rate shifts in two out of ten random trees at 0.1 Ma). Our analyses did not detect shifts in diversification rates in the pre-lake phase or early phase of the lake. We therefore argue that the lack in diversification rate shifts is related to the ecosystem resilience of Lake Ohrid against particular environmental perturbations such as the observed glacial/interglacial cycles.

4) *The authors refer to the species coverage, an important factor when calculating diversification rates based on phylogenetic tree hypotheses, by saying: "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 cryptic group." It is correct, that there is a high probability that the authors cover all older/major lineages. However, at the same time, many more recent species may have been missed. This seems very plausible since a high amount of cryptic species is observed in Lake Ohrid, e.g. see Pseudohoratia ohridana. If more recent nodes accumulate, net diversification rate may show a shift in more recent times, e.g. during glacial cycles (Lindhorst et al. 2015). The oldest node referring to the taxon P. ohridana is even at 1 my of age. This taxonomic coverage is problematic and its influence on the diversification rate estimates has to be addressed more thoroughly in the discussion. However, distinguishing such a pattern from a "normal" pull-of-the-present effect due to speciation only processes will get challenging.*

Response: We will expand the discussion regarding taxonomic coverage: "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 morphotypes 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."

Discussing changes in diversification rates for recent diversification events, however, remains problematic given potential biases resulting from effects such as incomplete lineage sorting. In fact, for assessing such young events, coalescent methods might be more appropriate for phylogenetic inference but are beyond the scope of the present study. For instance, Morlon et al. (2010) developed coalescent-based methods to estimate gradual changes in diversification rate. However, no tests for distinct shifts in diversification rates are available in this framework but a pattern of sudden rate change is expected by catastrophic environmental events. We therefore refrained in our manuscript from including coalescent methods and extensively discussing young events of rate shifts. Moreover, we would be happy to include a cautionary note in our revised version pointing out some of the problems associated with such events.

5) *The authors state that "we did not test for substitutional saturations as both genes have been suggested to be not saturated within the family Hydrobiidae (Wilke et al., 2001, 2013)". However substitutional saturation is dataset- and sampling-dependant and a general statement cannot be made here. Please test your dataset for substitutional saturation as this is easily done but at the same time may heavily bias the reconstruction of the phylogenetic tree hypothesis by lowering the phylogenetic information content of the data.*

Response: We performed saturation tests for the COI gene fragment using DAMBE. Only little saturation is observed suggesting that this partition can be used for further (molecular-clock) analyses (see below). We did not perform a saturation test for the 16S dataset because 16S is known to be more conservative than COI (see also Wilke et al., 2001, 2013).

Therefore, we will rephrase this sentence in the revised manuscript: “We tested the COI dataset for nucleotide substitution 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 for the preferred GTR+I+ Γ model. 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 dataset as, within the family Hydrobiidae, the 16S gene is more conservative than the COI gene (Wilke et al., 2001, 2013).”

DAMBE output for the XIA saturation test for COI using the PINV value (0.46) obtained from jModelTest for the preferred substitution model GTR+I+G.

NumOTU	Iss	Iss.cSym	T	DF	P	Iss.cAsym	T	DF	P
4	0.117	0.793	32.297	246	0.0000	0.759	30.650	246	0.0000
8	0.132	0.748	26.935	246	0.0000	0.636	22.023	246	0.0000
16	0.135	0.715	25.575	246	0.0000	0.505	16.320	246	0.0000
32	0.138	0.698	24.915	246	0.0000	0.371	10.361	246	0.0000

6) Briefly explain the methodological procedure of the diversification rate analyses performed in TreePar in more detail, as it is used as an additional argument besides the LTT-plots. The analytical difference should become more clear.

Response: We expanded the Methods section: “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 through time were analyzed using the R package TreePar v. 3.3 (Stadler, 2015) by testing a maximum of three shifts for ten trees randomly sampled from the posterior distribution. This package implements a birth-death shift model (Stadler, 2011) that allows changes in speciation and extinction rates along a phylogeny for a given timeframe and for pre-defined time intervals. Shifts were analyzed along the pruned tree with default settings and a time interval of 0.1 Ma. 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 accounting for incomplete sampling. 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 allowed) 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).”

7) In the discussion, the authors argue that “the TreePar analysis used does account for incomplete sampling and we did infer two single rate shifts in the present study.” More precisely, a single rate shift each in two independent trees has been identified. Or do you have the assumption of two rate shifts during the evolution of non-pyrgulinid Hydrobiidae in Lake Ohrid? Would it be possible to show even more trees. I am not familiar with the standard procedure, but showing ten trees only seems rather too less.

Response: Thanks for highlighting this point. A single rate shift was found in two out of the ten random trees. We rephrased this sentence: “the TreePar 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.”

Ideally, the entire posterior distribution with 20,000 trees could be tested for changes in diversification rates. However, testing multiple random trees as done here is more robust compared to analyzing a single consensus (maximum clade credibility tree) as often performed in different other publications for both diversification rate-analyses and LTT plots.

Referee 2 (T. von Rintelen)

Specific comments:

1) *A few issues should still be addressed, though, in order to further improve the MS. As the first reviewer, A. Weigand, has already pointed out, it would be interesting to see whether the apparent support for the monophyly of the non-pyrgulinid Hydrobiidae in Lake Ohrid by a BPP of 0.87 is also found when using a ML analysis, or, as I would also suggest, a BI analysis without constraints. In contrast to the authors' statement that they ran (initially) unconstrained analyses, I would suggest that any molecular clock analysis, whether relative or calibrated, is constrained in a way by the requirement of ultrametry. Given that BEAST enforces bifurcations, a, say, MrBayes analysis would show whether the topology and support for it are stable.*

Response: We hope that the issue of monophyly vs. non-monophyly of the Lake Ohrid species flock has been sufficiently addressed in the above sections of Referee 1, point 1). We did perform further non-clock analyses using a different and faster phylogenetic method (maximum likelihood; RAxML).

2) *Based on the present tree, I cannot quite agree with the first referee on his second point, as collapsing basal (unsupported) splits would not contradict the onset of diversification in non-pyrgulinid hydrobiids before deep-water conditions set in at Lake Ohrid.*

Response: We agree. See comments above, Referee 1, point 2).

3) *Regarding the inference of rate homogeneity and LTT plots (also discussed by A. Weigand), a brief perusal of Ricklefs 2007 suggests to me that this point certainly warrants some more discussion by the authors, acknowledging potential limitations and alternative hypotheses.*

Response: We will replace the term "rate homogeneity" by "constant diversification rate" and commented on this issue above. See comments above, Referee 1, point 3).

4) *I also concur with the other points raised by the first referee. Anyhow, I am convinced that the concerns raised above can be dealt with either by providing evidence for them being unfounded and/or by providing a more balanced discussion - either way, the data are worthy of publication in this journal.*

Response: We agree and will provide a more balanced discussion highlighting the above issues and limitations in the revised manuscript.

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

2 **Ecosystem resilience likely buffers environmental fluctuations**

3

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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 monophyletic group is comparatively old and that it most likely evolved
25 with a constant diversification rate. Preliminary data of the SCOPSCO deep-drilling program do
26 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

Gelöscht: rate homogeneity

Gelöscht: probable

44 **1 Introduction**

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

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

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

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

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

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

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

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

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

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

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

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

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

132
133 **2 Material and methods**

Gelöscht: of rate heterogeneity

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Gelöscht: i) In a preliminary analysis, we first use DNA sequencing data and phylogenetic analyses for testing the monophyly of our model taxon, thus assuring that it fulfils one of the criteria for being a species flock besides endemism and speciosity.¶
i

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146 **2.1 Sampling**

147 Hydrobiid gastropods were collected during fieldtrips to Lake Ohrid and other waterbodies in the
148 Balkan region between 2003 and 2011 (Fig. 2; see Table 1 for details). The collection methods
149 followed those described in Schreiber et al. (2012) and included hand collecting, snorkeling, sieving
150 and dredging from small boats or the research vessel of the Hydrobiological Institute Ohrid. Samples
151 were preserved in 80% ethanol and determined in the laboratory to species level based on Radoman
152 (1983).

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153

154 **2.2 DNA isolation, PCR amplification and DNA sequencing**

155 Genomic DNA was isolated from whole specimens using the CTAB protocol described in Wilke et al.
156 (2006). Voucher specimens and digital images were deposited in the Systematics and Biodiversity
157 Collection of the University of Giessen (UGSB). Fragments of the mitochondrial genes for cytochrome
158 oxidase c subunit I (COI) and large subunit rRNA (LSU rRNA or 16S rRNA) were amplified using the
159 universal primers LCO 1490 (Folmer et al., 1994) and COR722b (Wilke and Davis, 2000) as well as
160 16Sar-L and 16Sbr-H (Palumbi et al., 1991), respectively (for PCR conditions see Schreiber et al.,
161 2012). Subsequent Sanger sequencing was conducted either on a Long Read IR2 4200 sequencer
162 (LI-COR, Lincoln, NE, USA) using the Thermo Sequenase fluorescent labelled primer cycle
163 sequencing Kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) or on a ABI 3730 XL sequencer
164 (Life Technologies, Carlsbad, CA, USA) using the Big Dye Terminator Kit (Life Technologies,
165 Carlsbad, CA, USA). In total, we sequenced 65 specimens of 17 nominal species of the endemic non-
166 pyrgulinid Hydrobiidae. For comparison, we also analyzed 20 specimens of 15 closely-related species
167 occurring in lakes Prespa and Mikri Prespa as well as in surrounding waterbodies (Fig. 2; see Table 1
168 for locality details, UGSB collection numbers and GenBank accession numbers).

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170 **2.3 Preliminary genetic analyses**

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

179

180 2.4 Phylogenetic inference and molecular clock analyses

181 Prior to the phylogenetic analyses, our dataset of non-pyrgulinid Hydrobiidae was supplemented with
182 sequences of closely related species. We first performed searches with BLASTN 2.2.32 (Zhang et al.,
183 2000) against the NCBI nucleotide database as well as against the nucleotide database of the Wilke
184 lab, which contains DNA information for more than 300 hydrobiid species. A preliminary phylogenetic
185 analysis was conducted in order to identify the hydrobiid groups that are most closely related to our
186 endemic non-pyrgulinid Hydrobiidae. These preliminary analyses (details not shown) indicated that
187 endemic species from the sister Lake Prespa (Fig. 2) as well as from other Balkan waterbodies are the
188 closest relatives to the Ohrid taxa. These taxa were included in our final dataset (see Table 1).

189 The main phylogenetic analyses using Bayesian inference were performed in BEAST v.
190 1.8.0 (Drummond and Rambaut, 2007). Best-fit substitution models were estimated using jModelTest
191 0.1.1 (Posada, 2008) based on the Akaike information criterion. The models suggested for the COI
192 and 16S rRNA fragments were GTR+I+ Γ and GTR+I, respectively. We tested the COI dataset for
193 substitutional saturation using the test by Xia and Xie (2001) as implemented in DAMBE 5.0.23 (Xia
194 and Xie, 2001). The value for the proportion of invariant sites (Pinv = 0.46) was obtained from the
195 jModelTest output. The observed saturation was significantly lower than the critical values ($p < 0.001$),
196 suggesting that this partition can be used for further (molecular-clock) analyses. Note that we did not
197 test for saturation in the 16S dataset as, within the family Hydrobiidae, the 16S gene is more
198 conservative than the COI gene (Wilke et al., 2001, 2013).

Gelöscht: nucleotide

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model

Gelöscht: Note that we did not test for
substitutional saturations as both genes
have been suggested to be not
saturated within the family Hydrobiidae

199 We first ran two unconstrained analyses in BEAST, one under the strict-clock and one under
200 the relaxed-clock model using relative rates for both partitions. These initial runs, however, revealed
201 an extremely low effective sample size for the prior and posterior distributions suggesting that the runs
202 might have been over-parameterized due to the complex GTR+I+ Γ model (see e.g., Grummer et al.,
203 2014; Slager et al., 2014). Hence, the less complex HKY+I+ Γ and HKY+I models were used for the
204 final analyses for the COI and 16S rRNA partition, respectively.

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

216 The final analyses (two strict-clock and two relaxed-clock runs) with a total of 85 sequences
217 were run for 100,000,000 generations each, sampling every 5,000 generations. The resulting log and
218 tree files for each strict-clock and relaxed-clock run were combined using LogCombiner v. 1.8.0
219 (BEAST) with a 50% burn-in. The maximum clade credibility (MCC) tree was identified based on the
220 posterior distribution (20,000 trees). Information from the post-burnin posterior distribution including
221 mean node ages and 95% highest posterior densities (HPD) was summarized using TreeAnnotator v.
222 1.8.0 (BEAST; no additional burn-in). A Bayes factor (BF) analysis of the likelihoods of both runs
223 (strict-clock vs. relaxed-clock model) was performed in Tracer 1.5 (Rambaut and Drummond, 2007);
224 1,000 bootstrap replicates) in order to determine the best-fitting clock model (see Newton and Raftery,
225 1994; Suchard et al., 2001).

226 The BF analysis, which compares the likelihoods of both BEAST runs (strict vs. relaxed clock
227 model), showed a decisive support for the relaxed-clock model with a BF of 45.61 ($\ln P_{\text{relaxed}} = -$
228 $5,213.40$ vs. $\ln P_{\text{strict}} = -5,318.42$).

229

230 2.6 Lineage-through-time plot and diversification-rate analyses

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

238 However, generating LTT plots and detecting changes in the slope is an explorative
239 approach and might lead to misinterpretations (see Stadler, 2011). Therefore, potential shifts in
240 diversification rates through time were analyzed using the R package TreePar v. 3.3 (Stadler, 2015)
241 by testing a maximum of three shifts for ten trees randomly sampled from the posterior distribution.

242 This package implements a birth-death shift model (Stadler, 2011), which allows changes in speciation
243 and extinction rates along a phylogeny for a given timeframe and for pre-defined time intervals. Shifts
244 were analyzed along the pruned tree with default settings and a time interval of 0.1 Ma. The sampling
245 fraction was set to $17/27 = 0.63$ according to the actual number of species included divided by the

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

253

254 3 Results

255 3.1 Phylogenetic inference and molecular clock analyses

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

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

267

268 3.2 Diversification-rate analysis

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

273

274 4 Discussion

275 4.1 The Ohrid non-pyrgulinid Hydrobiidae: an old species flock

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

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Gelöscht: The nodes of interest (see above) are very well supported and the initial diversifications within Ohrid and Prespa non-pyrgulinid Hydrobiidae received support values of 0.87 and 1.0, respectively. However, many shallower nodes are only poorly supported. ¶

Gelöscht: flock

Gelöscht: The main goal of this study was to test for changes in diversification rates over time in the largest group of endemic animals in ancient Lake Ohrid – the non-pyrgulinid Hydrobiidae. Our findings indicate that this group is, indeed, comparatively old and most likely monophyletic. However, due to the comparatively low support of 0.87, a non-monophyly of the Ohrid group cannot be excluded. Nonetheless, even under the unlikely assumption that the Prespa group renders the Ohrid group paraphyletic (as in random tree #2), the molecular-clock analysis suggests that the split between Prespa and Ohrid is older than 2 Ma and thus does not affect intra-lacustrine diversification rates within the Lake Ohrid clade. ¶ With an age of 1.75–3.76 (1.75–2.68) Ma, the onset of diversification within this flock may even predate the origin of extant Lake Ohrid c. 1.2–2.0 Ma ago. However, the most significant finding of this study is that both the LTT plot and the diversification-rate analyses suggest a rate homogeneity constant diversification rate over time. These findings are discussed in the following sections in respect to the specific goals of the current study. ¶

Gelöscht: The phylogenetic and molecular-clock analyses were primarily done to assure that the study taxon is monophyletic, and thus preventing a potential bias in our divergence-time analysis (see specific goal i). The former analyses were also performed in order to obtain temporal baseline... [1]

Gelöscht: The findings suggest

1984; Schön and Martens, 2004). Therefore, *in situ* diversification appears to be a main process in this group. This conclusion even holds under the assumption of a non-monophyly of the Ohrid taxa. The age estimates obtained from the molecular-clock analyses revealed that the Lake Ohrid flock potentially started to diversify before extant Lake Ohrid came into existence (i.e., in a pre-lake/palaeo-lake phase or in other waterbodies of the Ohrid Graben system such as rivers or springs; see Fig. 3 and Fig. 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 and 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.

Gelöscht: ('cradle function')

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Gelöscht: taxa because the split between Lake Prespa and Lake Ohrid occurred well before the origin on set of Lake Ohrid origination.

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

Gelöscht: Nonetheless, there are some important conceptual implications of our investigations, calling the strict use of 'cradle' into question relative to *in situ* diversification. Our LTT plot (Fig. 4A) shows that several lineages have already existed when the lake reached deep-water conditions or even before it came into existence (see Trajanovski et al., 2010) for a potentially similar pattern in the *Dina* leach flock). Therefore, the term 'cradle' may not only be used for monophyletic species evolving within the lake (intra-lacustrine speciation) but also for a group of monophyletic species that started to evolve within the (palaeo-)basin.¶

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 aims at estimating 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.

Gelöscht: third

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

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

Gelöscht: rate homogeneity

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

Gelöscht: believe

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

Gelöscht: morphotypes

Gelöscht: 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 cryptic group. Moreover, the TreePar analysis used does account for incomplete sampling and we did infer two rate shifts in the present study. Thus the method used seems to have enough power to detect rate heterogeneity in our data set

430 4.3 Ecosystem resilience of Lake Ohrid

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

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

Gelöscht: iv

Gelöscht: rate heterogeneity

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

Gelöscht: to be included in this special issue

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

Gelöscht: or

Gelöscht: potentially

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

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480 The suggested high ecosystem resilience of Lake Ohrid might be sustained by several
481 factors including the lake's bathymetry (deep lake with steep flanks, allowing habitats to move
482 vertically with lake-level changes; Lindhorst et al., 2010), ongoing moderate tectonic activities
483 (compensating sedimentation; Hinderer and Einsele, 2001), and its peculiar limnology (the lake is fed
484 by numerous karstic sublacustrine springs, locally buffering environmental changes; Matzinger et al.,
485 2006).

Gelöscht: (

Gelöscht: and robust

Gelöscht: the monophyly of the Ohrid taxa could not be shown unambiguously. Further (nuclear) markers should be included in

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

Gelöscht: in order to support our proposed hypothesis of a monophyletic flock

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

Gelöscht: recent

519

520 | **5 Conclusions**

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

Gelöscht: monophyletic and

Gelöscht: the

Gelöscht: rate homogeneity

Gelöscht: diversification

Gelöscht: observed

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

Gelöscht: though we think that the diversification patterns inferred in the present study are robust,

535

545 **Author contribution**

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

549

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560

Gelöscht: 12

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802

803 **Figure captions**

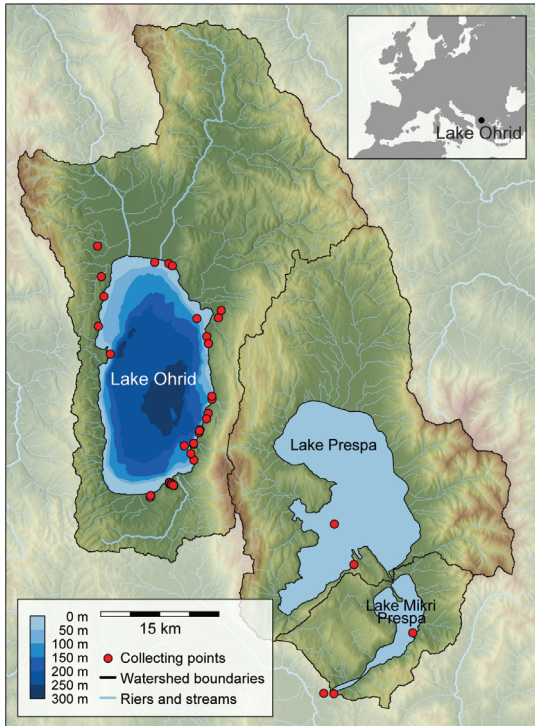
804 **Fig. 1.** Selected representatives of genera belonging to the endemic non-pyrgulinid Hydrobiidae from
805 Lake Ohrid. Scale bar = 1 mm.



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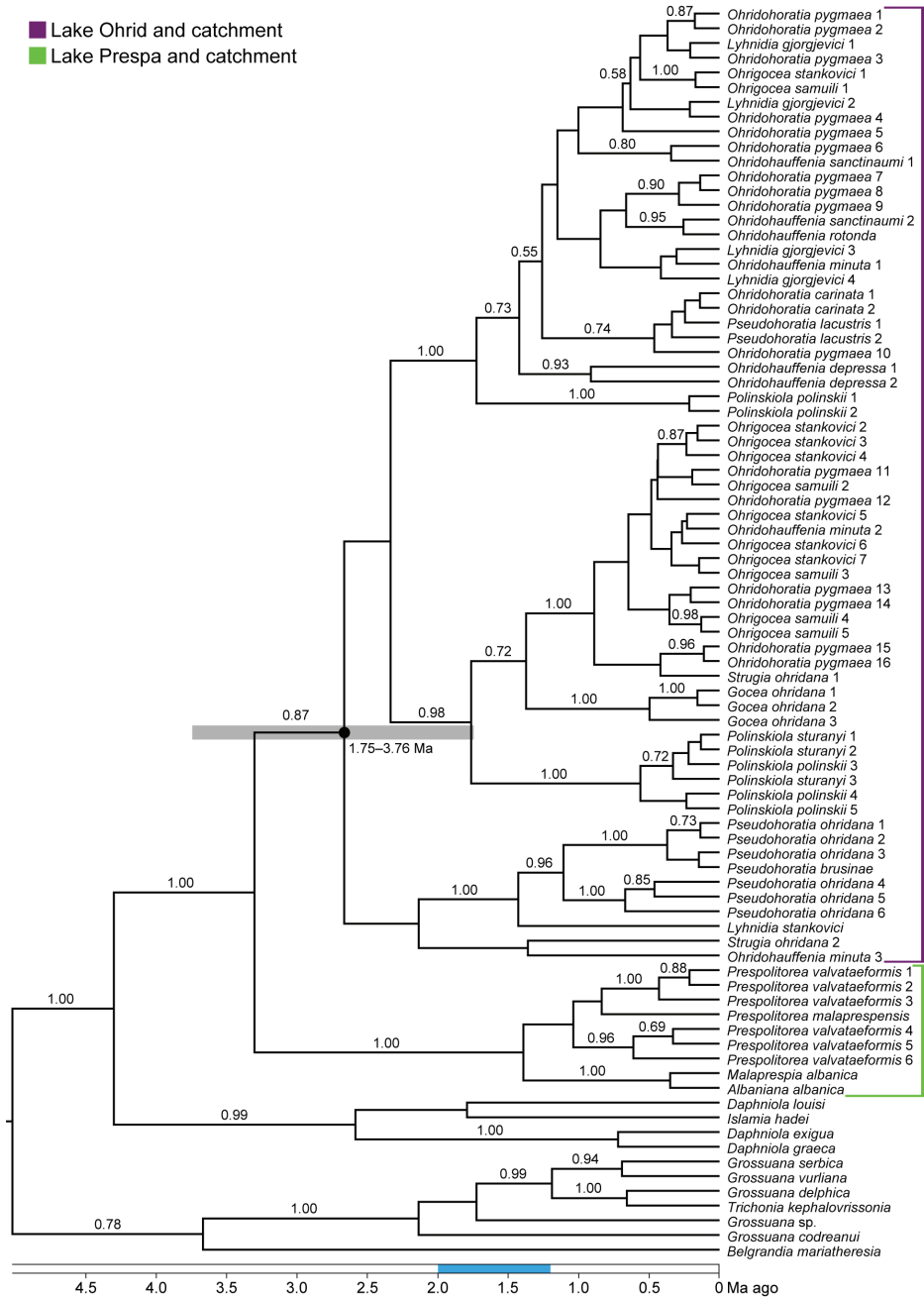
808 **Fig. 2.** Sampling sites for non-pyrgulinid Hydrobiidae in lakes Ohrid, Prespa and Mikri Prespa, and
809 their watersheds.



810

811

812 **Fig. 3.** MCC tree based on a relaxed-clock BEAST analysis for non-pyrgulinid Hydrobiidae from the
 813 Balkans. Posterior probabilities >0.5 are shown at the respective branches. The grey bar indicates the
 814 95% HPD for the age of the MRCA of the Ohrid endemics. The blue bar in the timescale ranging from
 815 1.2–2.0 Ma ago shows the assumed age of Lake Ohrid.



816

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

Gelöscht: green

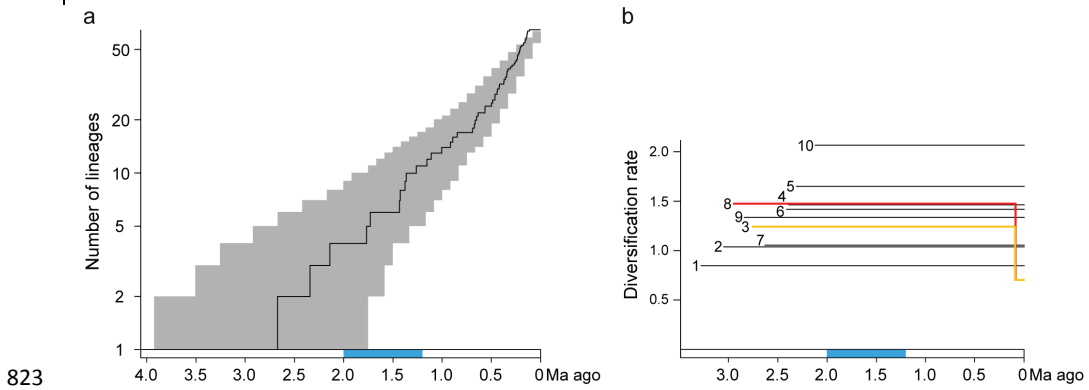


Table 1. Taxa studied including locality information, collection and DNA voucher details, and NCBI GenBank accession numbers.

Genus	Species	Locality	Latitude	Longitude	UGSB voucher no.	DNA voucher no.	GenBank accession no. LSU rRNA	Reference
<i>Albaniana</i>	<i>albanica</i>	Albania, Ventrroku, Cave spring	40.67295	20.97403	UGSB 10730	12073	x	This study
<i>Belgrandia</i>	<i>maritimesia</i>	Italy, Fonte di S. Cassiano	-	-	UGSB 17130	2332	x	This study
<i>Daphniola</i>	<i>exigua</i>	Greece, large spring at Agia Paraskevi, Tembi Valley, N of Larisa	-	-	N/A	N/A	JF916470	Fainiowski and Szarowska (2011)
	<i>graeca</i>	Greece, Dafne Spring	39.891083	22.607222	UGSB 17132	4238	x	This study
	<i>lousi</i>	Greece, Athens, spring at Kessariani	-	-	N/A	N/A	EF070618	Szarowska (2006)
<i>Gocea</i>	<i>ohridana 1</i>	Macedonia, Lake Ohrid, lake bank at Veli Dab	40.97324	20.78668	UGSB 10710	10493	x	This study
	<i>ohridana 2</i>	Macedonia, Lake Ohrid, lake bank at Veli Dab	40.97324	20.78668	UGSB 10709	10332	x	This study
	<i>ohridana 3</i>	Macedonia, Lake Ohrid, Veli Dab	40.97097	20.78604	UGSB 10681	4299	x	This study
<i>Grossuana</i>	<i>codreanui</i>	Bulgaria, Jasenov	-	-	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 Anilon	-	-	N/A	N/A	KC011768	Fainiowski et al. (2012)
	<i>vurizana</i>	Greece, spring of Louros River	-	-	N/A	N/A	EF061923	Szarowska et al. (2007)
<i>Islamia</i>	<i>hadai</i>	Greece, Peloponnese, N Taigetos Mts., W of Sparta, spring at Dhiaseleo	-	-	N/A	N/A	JF916473	Fainiowski and Szarowska (2011)
<i>Lychnidia</i>	<i>gorgjevic 1</i>	Macedonia, Lake Ohrid, in front of Hotel Desaret, village Pestani	40.91208	20.74213	UGSB 10746	12499	x	This study
	<i>gorgjevic 2</i>	Macedonia, feeder springs of Sveti Naum	40.91208	20.74213	UGSB 10746	12499	x	This study
	<i>gorgjevic 3</i>	Macedonia, feeder springs of Sveti Naum	40.91208	20.74213	UGSB 10747	12500	x	This study
	<i>gorgjevic 4</i>	Macedonia, Sveti Naum, small lake with springs	40.91029	20.74791	UGSB 10695	10286	x	This study
	<i>stankovici</i>	Macedonia, Sveti Naum, small lake with springs	40.91029	20.74791	UGSB 10696	10287	x	This study
	<i>stankovici</i>	Macedonia, Lake Ohrid, Trpica	40.95583	20.76396	UGSB 10697	10291	x	This study
<i>Malaprespia</i>	<i>albanica</i>	Albania, Lake Mikri Prespa, spring in the lake	40.67258	20.9892	UGSB 10731	12075	x	This study
<i>Ohridohauferia</i>	<i>depressa 1</i>	Macedonia, Lake Ohrid, in front of Hotel Desaret, village Pestani	41.00927	20.80544	UGSB 10716	10507	x	This study
	<i>depressa 2</i>	Macedonia, Lake Ohrid, beach at Gorica Hill	41.08105	20.7974	UGSB 10711	10495	x	This study
	<i>minuta 1</i>	Macedonia, spring Studentičista	41.10251	20.81491	UGSB 10712	10497	x	This study
	<i>minuta 2</i>	Macedonia, spring Bej Bunar	41.11085	20.81905	UGSB 10714	10501	x	This study
	<i>minuta 3</i>	Macedonia, spring Bej Bunar	41.11085	20.81905	UGSB 10715	10502	x	This study
	<i>rotunda</i>	Macedonia, Lake Ohrid, at camping site "Livadište"	41.12643	20.64184	UGSB 10713	10500	x	This study
	<i>sancitnaumi 1</i>	Macedonia, feeder springs of Sveti Naum	40.91414	20.74147	UGSB 10738	12358	x	This study
	<i>sancitnaumi 2</i>	Macedonia, feeder springs of Sveti Naum	40.91061	20.74748	UGSB 10717	10510	x	This study
	<i>carinata 1</i>	Macedonia, Lake Ohrid, Veli Dab	40.97439	20.78746	UGSB 10698	10299	x	This study
	<i>carinata 2</i>	Macedonia, Lake Ohrid, Trpica	40.95868	20.77802	UGSB 10699	10305	x	This study
	<i>pygmaea 1</i>	Albania, feeder springs of Tusemisht	40.89874	20.71325	UGSB 10749	12508	x	This study
	<i>pygmaea 2</i>	Albania, feeder springs of Tusemisht	40.89886	20.7127	UGSB 10748	12507	x	This study
	<i>pygmaea 3</i>	Macedonia, feeder springs of Sveti Naum	40.91019	20.74793	UGSB 10750	12510	x	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	x	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	x	This study
	<i>pygmaea 6</i>	Macedonia, Sveti Naum, springs at pumping station, near chapel	40.9113	20.74744	UGSB 10741	12362	x	This study
	<i>pygmaea 7</i>	Albania, feeder springs of Tusemisht	40.89885	20.7136	UGSB 10755	12863	x	This study
	<i>pygmaea 8</i>	Albania, Lake Ohrid, south of Lin peninsula	41.06055	20.65186	UGSB 10756	12865	x	This study
	<i>pygmaea 9</i>	Macedonia, Lake Ohrid, in bay south of Gorica Hill	41.07304	20.79991	UGSB 10758	12867	x	This study
	<i>pygmaea 10</i>	Macedonia, Lake Ohrid, in bay south of Gorica Hill	41.07304	20.79991	UGSB 10757	12866	x	This study
	<i>pygmaea 11</i>	Macedonia, Lake Ohrid, northwestern bay, near Podmolje	41.16183	20.7451	UGSB 10742	12439	x	This study

<i>continued</i>										
	<i>pygmaea</i> 12	Macedonia, Lake Ohrid, Veli Dab	40.97439	20.78747	UGSB 10759	12868	x	-		This study
	<i>pygmaea</i> 13	Macedonia, Lake Ohrid, northwestern bay, near Podmolje	41.16235	20.74395	UGSB 10718	10689	x	x		This study
	<i>pygmaea</i> 14	Macedonia, Lake Ohrid, northwestern bay, near Podmolje	41.16183	20.7451	UGSB 10743	12440	x	x		This study
	<i>pygmaea</i> 15	Macedonia, Lake Ohrid, northwestern bay, near Podmolje	41.16183	20.7451	UGSB 10744	12442	x	x		This study
	<i>pygmaea</i> 16	Macedonia, Lake Ohrid, northwestern bay, near Podmolje	41.16429	20.7396	UGSB 10745	12444	x	x		This study
Ohrigocea	<i>sarullii</i> 1	Macedonia, feeder springs of Sveti Naum	40.91287	20.74483	UGSB 10752	12514	x	-		This study
	<i>sarullii</i> 2	Macedonia, Lake Ohrid, south of Pestina	41.09239	20.63361	UGSB 10687	10153	x	x		This study
	<i>sarullii</i> 3	Macedonia, Lake Ohrid, Veli Dab	40.97439	20.78747	UGSB 10688	10154	x	x		This study
	<i>sarullii</i> 4	Macedonia, Lake Ohrid, bay south of Gradishte	40.99323	20.79958	UGSB 10689	10156	x	x		This study
	<i>sarullii</i> 5	Macedonia, Lake Ohrid, lake bank at Veli Dab	40.97324	20.78668	UGSB 10692	10170	x	x		This study
	<i>stankovici</i> 1	Macedonia, feeder springs of Sveti Naum	40.91029	20.74791	UGSB 10751	12512	x	-		This study
	<i>stankovici</i> 2	Albania, feeder springs of Tusemisht	40.89874	20.71325	UGSB 10753	12517	x	-		This study
	<i>stankovici</i> 3	Macedonia, feeder springs of Sveti Naum	40.91298	20.74455	UGSB 10754	12518	x	-		This study
	<i>stankovici</i> 4	Macedonia, spring at Sveti Naum	40.91135	20.745	UGSB 10690	10164	x	x		This study
	<i>stankovici</i> 5	Macedonia, Lake Ohrid, Sveti Stefan	41.07306	20.79985	UGSB 10691	10166	x	x		This study
	<i>stankovici</i> 6	Macedonia, Lake Ohrid, lake bank at Veli Dab	40.97324	20.78668	UGSB 10694	10173	x	x		This study
	<i>stankovici</i> 7	Macedonia, Lake Ohrid, lake bank at Veli Dab	40.97324	20.78668	UGSB 10693	10172	x	x		This study
Polinskiola	<i>polinski</i> 1	Macedonia, feeder springs of Sveti Naum	40.91061	20.74748	UGSB 10725	10730	x	x		This study
	<i>polinski</i> 2	Macedonia, feeder springs of Sveti Naum	40.91061	20.74748	UGSB 10724	10729	x	x		This study
	<i>polinski</i> 3	Macedonia, Lake Ohrid, Trpejca	40.95868	20.77802	UGSB 10726	10734	x	x		This study
	<i>polinski</i> 4	Macedonia, Lake Ohrid, southeast end of the lake	40.9396	20.7783	UGSB 10729	10739	x	x		This study
	<i>polinski</i> 5	Macedonia, Lake Ohrid, Trpejca	40.95752	20.77771	UGSB 10727	10735	x	x		This study
	<i>sturanjvi</i> 1	Macedonia, Lake Ohrid, bay south of Gradishte	40.99323	20.79958	UGSB 10722	10727	x	x		This study
	<i>sturanjvi</i> 2	Macedonia, Lake Ohrid, in bay south of Gorica Hill	41.07304	20.79991	UGSB 10728	10727	x	x		This study
	<i>sturanjvi</i> 3	Macedonia, Lake Ohrid, bay south of Gradishte	40.99323	20.79958	UGSB 10723	10728	x	x		This study
Prespilitorea	<i>malaprespensis</i>	Greece, Lake Mikri Prespa, at the former Hydrobiological Institute Mikrolimni	40.74215	21.10763	UGSB 10737	12089	x	x		This study
	<i>valvataeformis</i> 1	Macedonia, Lake Prespa, southern shore of Golem Grad	40.86655	20.98989	UGSB 10736	12088	x	x		This study
	<i>valvataeformis</i> 2	Macedonia, Lake Prespa, southern shore of Golem Grad	40.86655	20.98989	UGSB 10735	12087	x	x		This study
	<i>valvataeformis</i> 3	Macedonia, Lake Prespa, southern shore of Golem Grad	40.86655	20.98989	UGSB 10734	12086	x	x		This study
	<i>valvataeformis</i> 4	Greece, Lake Prespa, bay on cliff-like coast	40.82032	21.01939	UGSB 10733	12085	x	x		This study
	<i>valvataeformis</i> 5	Greece, Lake Prespa, bay on cliff-like coast	40.82032	21.01939	UGSB 10732	12084	x	x		This study
	<i>valvataeformis</i> 6	Greece, Lake Prespa, bay on cliff-like coast	40.82032	21.01939	UGSB 10686	10118	x	x		This study
Pseudohoratia	<i>brusinae</i>	Macedonia, Lake Ohrid, in front of Ohrid Bay	41.10156	20.78236	UGSB 10700	10313	x	x		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	x	x		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	x	x		This study
	<i>ohridana</i> 1	Macedonia, Lake Ohrid, south of Gradishte	40.98687	20.79729	UGSB 10707	10330	x	x		This study
	<i>ohridana</i> 2	Macedonia, Lake Ohrid, in front of Ohrid Bay	41.10156	20.78236	UGSB 10701	10314	x	x		This study
	<i>ohridana</i> 3	Macedonia, Lake Ohrid, in front of Hotel Desaret, village Pestani	41.01262	20.80513	UGSB 10705	10327	x	x		This study
	<i>ohridana</i> 4	Macedonia, Lake Ohrid, southeast end of the lake	40.94696	20.77385	UGSB 10706	10328	x	x		This study
	<i>ohridana</i> 5	Macedonia, Lake Ohrid, south of Gradishte	40.98687	20.79729	UGSB 10708	10331	x	x		This study
	<i>ohridana</i> 6	Macedonia, Lake Ohrid, in front of Hotel Desaret, village Pestani	41.01262	20.80513	UGSB 10704	10326	x	x		This study

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

Gelöscht: s

Gelöscht: rate homogeneity

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	

831