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Testing the spatial and temporal framework of speciation in an ancient lake species flock: the leech genus *Dina* (Hirudinea: Erpobdellidae) in Lake Ohrid

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Abstract. Ancient Lake Ohrid on the Balkan Peninsula is considered to be the oldest ancient lake in Europe with a suggested Plio-/Pleistocene age. Its exact geological age, however, remains unknown. Therefore, molecular clock data of Lake Ohrid biota may serve as an independent constraint of available geological data, and may thus help to refine age estimates. Such evolutionary data may also help unravel potential biotic and abiotic factors that promote speciation events.

Here, mitochondrial sequencing data of one of the largest groups of endemic taxa in the Ohrid watershed, the leech genus *Dina*, is used to test whether it represents an ancient lake species flock, to study the role of potential horizontal and vertical barriers in the watershed for evolutionary events, to estimate the onset of diversification in this group based on molecular clock analyses, and to compare this data with data from other endemic species for providing an approximate time frame for the origin of Lake Ohrid.

Based on the criteria speciosity, monophyly and endemicity, it can be concluded that *Dina* spp. from the Ohrid watershed, indeed, represents an ancient lake species flock. Lineage sorting of its species, however, does not seem to be complete and/or hybridization may occur. Analyses of population structures of *Dina* spp. in the Ohrid watershed indicate a horizontal zonation of haplotypes from spring and lake populations, corroborating the role of lake-side springs, particularly the southern feeder springs, for evolutionary processes in endemic Ohrid taxa. Vertical differentiation of lake taxa, however, appears to be limited, though differences between populations from the littoral and the profundal are apparent. Molecular clock analyses indicate that the most recent common ancestor of extant species of this flock is approximately 1.99 ± 0.83 million years (Ma) old, whereas the split of the Ohrid *Dina* flock from a potential sister taxon outside the lake is estimated at 8.30 ± 3.60 Ma. Comparisons with other groups of endemic Ohrid species indicated that in all cases, diversification within the watershed started ≤ 2 Ma ago. Thus, this estimate may provide information on a minimum age for the origin of Lake Ohrid. Maximum ages are less consistent and generally less reliable. But cautiously, a maximum age of 3 Ma is suggested. Interestingly, this time frame of approximately 2–3 Ma ago for the origin of Lake Ohrid, generated based on genetic data, well fits the time frame most often used in the literature by geologists.

1 Introduction

Analyses of major diversification events in ancient lakes, that is, lakes that have continuously existed for a hundred thousand or even million years, play a major role in studies of speciation and radiation patterns (e.g. Brooks, 1950; Martens et al., 1994; Martens, 1997; Rossiter and Kawanabe, 2000). From a temporal point of view, ancient lakes constitute prime systems for studying evolutionary events over geological time scales. Due to their prolonged existence, they have accumulated thick sedimentary layers, which often provide an excellent paleoenvironmental record (e.g. Cohen, 2003). Moreover, since these lake basins typically constitute

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relatively closed systems, they are ideal for documenting the effects of environmental events on endemic lake faunas. Examples for such events acting on local and regional scales include lake-level fluctuations (e.g. Cohen, 1994; Cohen et al., 2007; Schultheiß et al., 2009), salinity changes (Cohen, 2003), and volcanic eruptions (Degens et al., 1973; Wagner et al., 2008; Sulpizio et al., 2010). On a global scale, tectonic and oceanic events, climate change, anoxia, and meteoric impact are among the most influential physical disturbances that are thought to have triggered biotic changes (e.g. Benton, 2009).

The deep drilling programs in Lake Baikal, for example, have revealed cyclicities of 20 000, 40 000, and 100 000 years in physical parameters, all of which are related to the three Milankovich orbital parameters (e.g. Kashiwa et al., 1999). In fact, continental deep drilling programs have been conducted in several ancient lakes, opening a new era of exploration of lake histories (e.g. Scholz et al., 2007).

Comparisons of drilling core and other geological data with dated molecular phylogenies of extant endemic species, however, often indicate severe discrepancies between old lakes with a long limnological history on the one hand and young endemic faunal elements on the other. This is because catastrophic environmental events may have repeatedly wiped out or at least depleted ancient lakes faunas. Examples include Lake Malawi (e.g. Sturmbauer et al., 2001; Schultheiß et al., 2009; Genner et al., 2010), Lake Kivu (e.g. Degens et al., 1973), and Lake Titicaca (e.g. Mourguiart and Montenegro, 2002). Even for the oldest lake in the world, Lake Baikal, researchers have shown that endemic faunal elements are often one order of magnitude younger than the lake itself (e.g. Sherbakov, 1999; Wilke, 2004). One of the few ancient lakes for which no such discrepancy has been reported so far, is the European ancient Lake Ohrid situated on the Balkan Peninsula (for a review of the limnology and biodiversity of Lake Ohrid see Albrecht and Wilke, 2008). Several ancient lake species flocks, that is, relatively species-rich monophyletic groups of endemic species (Greenwood, 1984), are known for Lake Ohrid that very likely evolved within the lake and that are up to 2 Ma old. At the same time, many researchers suggest a lake age of 2–3 Ma (see Discussion for details). The problem, however, is that the latter estimate is largely based on preliminary geological data that is about 100 years old (i.e. Cvijić, 1911; also see the Discussion for details). Therefore, these estimates have to be treated with caution pending further investigation. In fact, evolutionary data on Lake Ohrid biota may serve as an independent constraint to available geological data, and may thus help refining age estimates.

The second dimension of speciation events in Lake Ohrid besides time – the spatial one – might also be closely linked to geological and environmental events such as lake-level fluctuations and major earthquakes. Within the Ohrid watershed, different spatial scales of endemicity have been proposed by Albrecht and Wilke (2008):

- species endemic to Lake Ohrid and its adjacent springs,
- species endemic to the surrounding springs,
- species endemic to the lake proper, and
- species endemic to parts of the lake separated by horizontal and vertical barriers.

Previous studies of selected species flocks in Lake Ohrid (e.g. Albrecht et al., 2006; Wilke et al., 2007; Wysocka et al., 2008) have provided first insights into patterns of allopatric and parapatric speciation in Lake Ohrid. Two of the most interesting findings are that (i) adjacent springs of Lake Ohrid (particularly the southern feeder springs around Sv. Naum on the Macedonian side and Zagorican/Tushemisht on the Albanian side) harbour endemic species, some of them being regarded as sister species to lake taxa, and (ii) there are distinct vertical zones within the lake presumably promoting either parapatric speciation along geographical/ecological gradients or even "micro-allopatric" speciation due to bathymetrical barriers (reviewed in Albrecht and Wilke, 2008).

A comprehensive picture of patterns and processes of speciation in Lake Ohrid, however, is still missing and the role of vertical and horizontal differentiation is still subject to controversial discussions. This is partly due to the facts that (i) only a limited number of species flocks have been studied so far within a rigorous phylogenetic framework, (ii) most flocks are relatively small, typically comprising only a handful of species, and (iii) there are only very few groups that inhabit all key habitats in Lake Ohrid ranging from the littoral to the deepest parts of the profundal.

A group that fulfils the latter two criteria is the leeches (Hirudinea) of Lake Ohrid (Sket and Šapkarev, 1992). According to these authors, Lake Ohrid and its surrounding springs are inhabited by 26 species (12 of them being endemic), which belong to 5 families: Piscicolidae (2); Glossiphoniidae (9); Hirudinidae (1), Haemopidae (1), and Erpobdellidae (13). Sket (1989) first drew attention to a potentially endemic radiation in Lake Ohrid hirudinids, describing the intralacustrine speciation within the genus *Dina* (family Erpobdellidae). The putative species flock is characterized by a high degree of anatomical similarities among its members. However, body shape variation is considerable, making determination a difficult task (Sket, 1989). As with all Hirudinea, Lake Ohrid *Dina* spp. are hermaphrodites, reproducing by reciprocal fertilization. They are small to large sized predatory leeches (15–80 mm), typically with reduced eyes, small mouth openings and small caudal suckers (Fig. 1a, Nesemann and Neubert, 1999). Interestingly, there are pronounced seasonal changes in the distribution of *Dina* spp. and preliminary data suggests both a vertical and horizontal separation of taxa within the Ohrid watershed (e.g. Sket, 1989). According to Sket and Šapkarev (1992), some species occur in all depth zones of Lake Ohrid (which are all characterized by a sufficient oxygen content and a rich

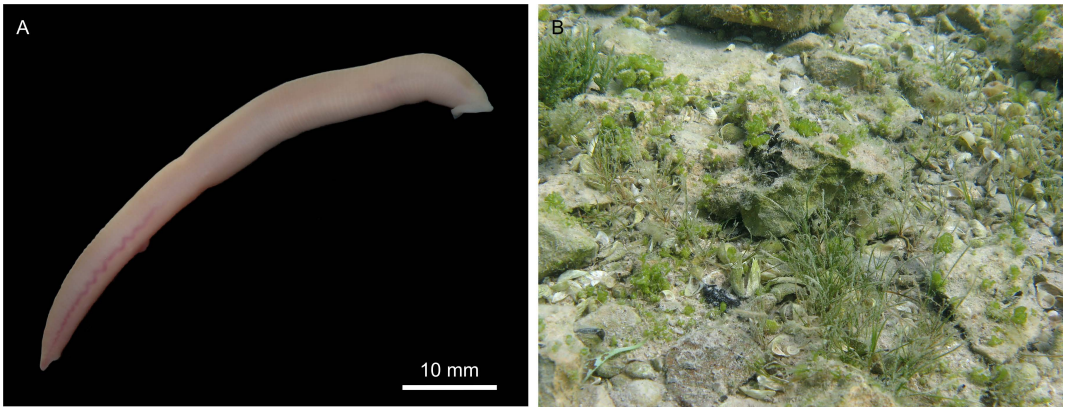

Fig. 1. (A) Live specimen of endemic *Dina lyhnida* from Lake Ohrid, a species that occurs from the lower littoral to the profundal. **(B)** Habitat photograph of the lower littoral zone of Lake Ohrid near Veli Dab. In Lake Ohrid, the lower littoral is the zone with the highest total leech density (Sket, 1992).

food supply), whereas other species are restricted to certain vertical zones (Fig. 1b). Some species also appear to show preferences for specific substrates such as stones or mud (e.g. Nesemann and Neubert, 1999). However, as the type of substratum in Lake Ohrid is strongly auto-correlated to water depth (Hauffe et al., 2010), it remains unknown which of these two abiotic factors can better explain the distribution of *Dina* spp.

Phylogenetic relationships of worldwide Hirudinea have recently been investigated (e.g. Trontelj et al., 1996; Siddall and Burreson, 1998; Apakupakul et al., 1999; Pfeiffer et al., 2004, 2005; Utevsky and Trontelj, 2004; Phillips and Siddall, 2009). Some studies also included the Erpobdellidae (Trontelj and Sket, 2000; Sket et al., 2001; Siddall, 2002; Borda and Siddall, 2004). However, no similar analyses exist as yet for the putative *Dina* flock in Lake Ohrid. This is very unfortunate because this flock is not only one of the very few presumed hirudinid ancient lake species flocks in the world, it also includes a sufficient number of species for testing patterns and processes of speciation in Lake Ohrid.

Here we used DNA sequences from the mitochondrial cytochrome *c* oxidase subunit I (COI) gene from *Dina* spp. from the Ohrid watershed in order to:

(a) test whether *Dina* spp., do indeed, represent an ancient lake species flock,

(b) study the potential role of horizontal and vertical barriers in Lake Ohrid for speciation in this group,

(c) estimate the onset of diversification in this group based on molecular clock analyses, and

(d) compare this data with data from other endemic species groups to provide an approximate time frame for the origin of Lake Ohrid solely based on evolutionary data.

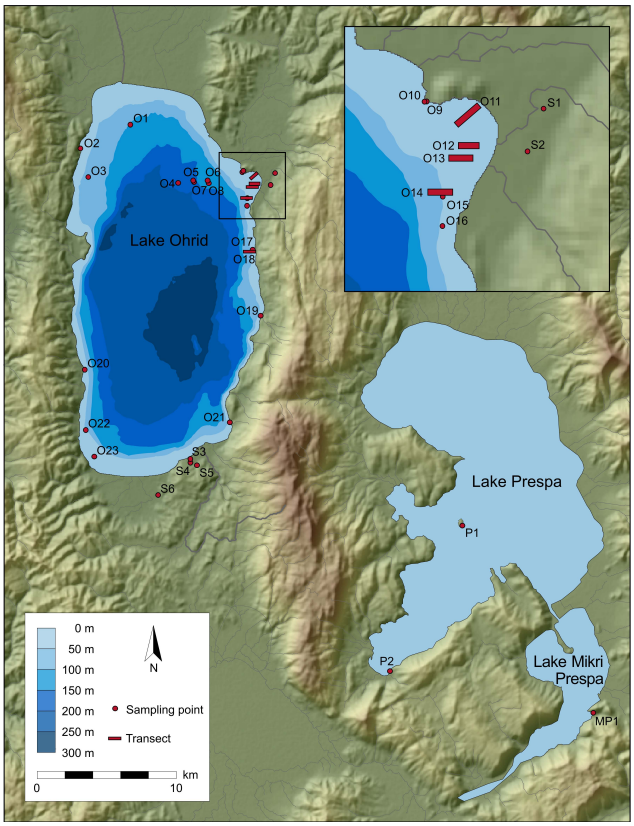

Fig. 2. Map of the Balkan lakes Ohrid, Prespa, and Mikri Prespa showing collecting sites for specimens of the family Erpobdellidae. The insert in the upper right corner shows an enlargement of the Ohrid Bay together with the respective collecting sites. Locality details are given in Table 1. Base map adapted from SRTM30 data (see http://eros.usgs.gov/#/Find_Data/Products_and_Data_Available/SRTM).

Table 1. Information on specimens of Hirudinea studied, including locality information for collecting points and transects, locality codes according to Fig. 2, and voucher numbers for specimens and DNA preps deposited at the University of Giessen Systematics and Biodiversity Collection (UGSB). Nomenclature follows Nesemann and Neubert (1999). See text for GenBank accession numbers and information on sequences obtained from GenBank.

Species	Locality (locality code)	Geographical coordinates	Specimen (DNA) voucher numbers
Dina l. lineata (O. F. Müller, 1774)	Germany, Mecklenburg-Western Pomerania, Parchim district, small pool	53.445° N, 11.910° E	UGSB1639 (5990), UGSB1640 (6800), UGSB1641 (6801)
Dina l. lacustris Sket, 1968	Macedonia, Ligemii Bogovines (type locality)	41.95069° N, 20.79578° E	UGSB4164 (9114), UGSB4165 (9115), UGSB4166 (9213)
Dina lepinja Sket & Šapkarev, 1986	Macedonia, Lake Ohrid (O14)	41.0931° N, 20.7953° E–41.0931° N, 20.7891° E	UGSB1642 (5943), UGSB1643 (5952), UGSB1644 (5996), UGSB1645 (6305)
	Macedonia, Lake Ohrid (O12)	41.1028° N, 20.8019° E–41.1028° N, 20.7967° E	UGSB1646 (5954), UGSB1647 (6001)
	Macedonia, Lake Ohrid (O16)	41.08783° N, 20.79277° E	UGSB1648 (6911)
	Macedonia, Lake Ohrid (O21)	40.93950° N, 20.77754° E	UGSB1649 (6920)
Dina cf. *svilesta* Sket, 1989	Macedonia, Lake Ohrid (O18)	41.0563° N, 20.7919° E–41.0563° N, 20.7982° E	UGSB1650 (3949)
	Macedonia, Lake Ohrid (O17)	41.05635° N, 20.79671° E	UGSB1651 (6922)
Dina lyhnida Šapkarev, 1990	Macedonia, Lake Ohrid (O4)	41.1031° N, 20.7302° E	UGSB1653 (5956), UGSB1654 (6004), UGSB1655 (6309), UGSB1656 (6799)
	Macedonia, Lake Ohrid (O5)	41.105° N, 20.744° E	UGSB1657 (5946), UGSB1658 (5961)
	Macedonia, Lake Ohrid (O6)	41.105° N, 20.757° E	UGSB1659 (6308), UGSB1660 (5962)
	Macedonia, Lake Ohrid (O14)	41.0931° N, 20.7953° E–41.0931° N, 20.7891° E	UGSB1661 (6377)
	Macedonia, Lake Ohrid (O16)	41.08783° N, 20.79277° E	UGSB1662 (6912)
	Macedonia, Lake Ohrid (O21)	40.93950° N, 20.77754° E	UGSB1663 (6918)
	Macedonia, Lake Ohrid (O3)	41.107° N, 20.649° E	UGSB1664 (3937)
	Macedonia, Lake Ohrid (O13)	41.1005° N, 20.8003° E–41.1006° N, 20.7943° E	UGSB1665 (6378)
Dina krilata Sket, 1989	Macedonia, Lake Ohrid (O1)	41.143° N, 20.687° E	UGSB1666 (3947), UGSB1667 (3948)
	Macedonia, Lake Ohrid (O12)	41.1028° N, 20.8019° E–41.1028° N, 20.7967° E	UGSB1668 (5937), UGSB1669 (5950), UGSB1670 (6307), UGSB1671 (5998)
	Macedonia, Lake Ohrid (O16)	41.08783° N, 20.79277° E	UGSB1672 (6909)
	Macedonia, Lake Ohrid (O17)	41.05635° N, 20.79671° E	UGSB1673 (6916)
	Albania, Lake Ohrid (O23)	40.91579° N, 20.65533° E	UGSB4167 (11375), UGSB4168 (11376)
	Albania, Lake Ohrid (O22)	40.93412° N, 20.6478° E	UGSB4169 (11379)

Table 1. Continued.

Dina cf. *kuzmani* Šapkarev, 1990/ *Dina* cf. *eturpshem* Sket, 1989	Macedonia, Lake Ohrid (O14)	41.0931° N, 20.7953° E–41.0931° N, 20.7891° E	UGSB1674 (5955), UGSB1675 (6304), UGSB1676 (5994)
	Macedonia, Lake Ohrid (O11)	41.1065° N, 20.7966° E–41.1100° N, 20.8026° E	UGSB1701 (3939)
	Macedonia, Lake Ohrid (O16)	41.08783° N, 20.79277° E	UGSB1677 (6913)
	Macedonia, Lake Ohrid (O11)	41.1065° N, 20.7966° E–41.1100° N, 20.8026° E	UGSB1678 (3940), UGSB1679 (3945)
	Macedonia, Lake Ohrid (O12)	41.1028° N, 20.8019° E–41.1028° N, 20.7967° E	UGSB1680 (5999), UGSB1681 (6372)
	Macedonia, Lake Ohrid (O18)	41.0563° N, 20.7919° E–41.0563° N, 20.7982° E	UGSB1682 (3955)
	Macedonia, Lake Ohrid (O17)	41.05635° N, 20.79671° E	UGSB1683 (6914)
	Macedonia, Lake Ohrid (O16)	41.08783° N, 20.79277° E	UGSB1684 (6910)
Dina cf. *profunda* Šapkarev, 1990	Macedonia, Lake Ohrid (O8)	41.1030° N, 20.7578° E	UGSB1686 (5945), UGSB1689 (5959)
	Macedonia, Lake Ohrid (O7)	41.1037° N, 20.7447° E	UGSB1687 (6798)
	Macedonia, Lake Ohrid, (O18)	41.0563° N, 20.7919° E–41.0563° N, 20.7982° E	UGSB1688 (3950)
Dina ohridana Sket, 1968	Albania, Lake Ohrid (O20)	40.97531° N, 20.64453° E	UGSB1690 (6297), UGSB1691 (6299)
	Macedonia, Lake Ohrid (O12)	41.1028° N, 20.8019° E–41.1028° N, 20.7967° E	UGSB1692 (5960), UGSB1693 (6002)
	Macedonia, Lake Ohrid (O21)	40.93950° N, 20.77754° E	UGSB1694 (6919)
	Macedonia, Lake Ohrid (O14)	41.0931° N, 20.7953° E–41.0931° N, 20.7891° E	UGSB1695 (5997)
	Macedonia, Lake Ohrid (O17)	41.05635° N, 20.79671° E	UGSB1696 (6915)
	Macedonia, Lake Ohrid (O19)	41.01262° N, 20.80513° E	UGSB1697 (6917)
	Macedonia, Lake Ohrid (O2)	41.12643° N, 20.64184° E	UGSB1698 (6298)
	Macedonia, Lake Ohrid (O2)	41.0931° N, 20.7953° E–41.0931° N, 20.7891° E	UGSB1699 (6793
	Macedonia, spring lake of Sv. Naum (S3)	40.91444° N, 20.74226° E	UGSB4170 (11363), UGSB4171 (11364), UGSB4172 (11367), UGSB4173 (11368)
	Macedonia, spring near old camping site of Sv. Naum (type locality) (S4)	40.91208° N, 20.74213° E	UGSB4174 (11381), UGSB4175 (11382), UGSB4176 (11383)
	Macedonia, spring lake near main springs of Sv. Naum (S5)	40.91019° N, 20.74793° E	UGSB4177 (11373), UGSB4178 (11374)
Dina sp.	Macedonia, Ohrid, Studeništa spring (S2)	41.102° N, 20.814° E	UGSB1652 (5991)
	Macedonia, Lake Ohrid (O1)	41.143° N, 20.687° E	UGSB1685 (3935)
	Macedonia, Lake Ohrid (O15)	41.093° N, 20.793° E	UGSB1700 (3951)
	Macedonia, Lake Ohrid (O9)	41.11084° N, 20.78839° E	UGSB1702 (6295)
	Macedonia, Lake Ohrid (O10)	41.11082° N, 20.78838° E	UGSB1703 (6296)
	Macedonia, Lake Ohrid (O12)	41.1028° N, 20.8019° E–41.1028° N, 20.7967° E	UGSB1704 (6000), UGSB1705 (6795)
	Macedonia, Lake Ohrid (O3)	41.107° N, 20.649° E	UGSB1706 (3944)

Table 1. Continued.

	Macedonia, spring Sv. Naum (S3)	40.91444° N, 20.74226° E	UGSB4179 (11365), UGSB4180 (11366), UGSB4181 (11369), UGSB4182 (11370), UGSB4183 (11372)
	Albania, Lake Ohrid (O23)	40.91579° N, 20.65533° E	UGSB4184 (11377), UGSB4185 (11378)
	Albania, Lake Ohrid (O22)	40.93412° N, 20.6478° E	UGSB4186 (11380)
	Albania, spring complex at the beginning of the village Zagorican (S6)	40.88973° N, 20.71312° E	UGSB4187 (11361), UGSB4188 (11362)
Dina latestriata Neubert & Nesemann, 1995	Albania, Lake Prespa (P2)	40.76848° N, 20.92642° E	UGSB4189 (6300)
	Greece, Lake Trichonis	38.58893° N, 21.46703° E	UGSB4190 (6363)
	Macedonia, Lake Prespa (P1)	40.86655° N, 20.98989° E	UGSB4191 (6924)
	Greece, Lake Mikri Prespa (type locality) (MP1)	40.74215° N, 21.10763° E	UGSB4192 (9206)
Dina punctata Johansson, 1927	Spain, Valencia, Province Castellon, Municipality Algimia de Almonacid, Fuente Donace	39.91800° N, 0.43760° W	UGSB2080a (9106), UGSB2080b (9107)
Erpobdella monostriata (Lindenfeld & Pietruszynski, 1890)	Germany, Mecklenburg-Western Pomerania, Ludwigslust district, Lake Neustädter See	53.39054° N, 11.55809° E	UGSB2071 (9096)
Erpobdella nigricollis (Brandes, 1900)	Germany, Mecklenburg-Western Pomerania, Ludwigslust district, Lake Neustädter See	53.39054° N, 11.55809° E	UGSB2073 (9100)
Erpobdella octoculata (Linnaeus, 1758)	Macedonia, Lake Ohrid (O14)	41.0931° N, 20.7953° E–41.0931° N, 20.7891° E	UGSB4193 (5995)
	Macedonia, Lake Prespa (P1)	40.86655° N, 20.98989° E	UGSB4194 (6923)
Erpobdella sp.	Macedonia, Ohrid, Bejbunar spring (S1)	41.110° N, 20.818° E	UGSB1720 (5933)
Erpobdella testacea (Savigny, 1822)	Germany, Mecklenburg-Western Pomerania, Ludwigslust district, Lake Neustädter See	53.39054° N, 11.55809° E	UGSB2072 (9098)
Erpobdella vilnensis (Liskiewicz, 1925)	Germany, Mecklenburg-Western Pomerania, Ludwigslust district, forest pool	53.43° N, 11.41° E	UGSB1712 (5989)
	Germany, Saxony-Anhalt, W of Osterwieck, River Ilse	51.967° N, 10.710° E	UGSB1709 (6805)
Batracobdella algira (Moquin-Tandon, 1846)	Spain, Andalusia, Province Huelva, Municipality Cortelazor, outflow of spring Fuene Lavadora	37.95559° N, 4.04210° W	UGSB2082 (9205)
Glossiphonia complanata (Linnaeus, 1758)	Germany, Mecklenburg-Western Pomerania, creek	53.81848° N, 10.92799° E	UGSB2069 (9200)

2 Materials and methods

2.1 Specimens studied

Specimens of nominal *Dina* spp. were collected from the Lake Ohrid area (lakes Ohrid, Prespa, and Mikri Prespa, including adjacent springs) between 2004 and 2009 (Fig. 2). Where possible, we included topotypical materials (Table 1). In addition, we obtained specimens of the type species of the genus *Dina*, *D. lineata*, from Germany as well as topotypes of *D. lineata lacustris* from a small glacial lake some 34 km north of Lake Ohrid. For comparison, we also studied nominal *Dina* species from Greece and Spain as well as representatives of the closely related genus *Erpobdella*. Specimens of *Glossiphonia complanata* and *Batracobdella algira* (both

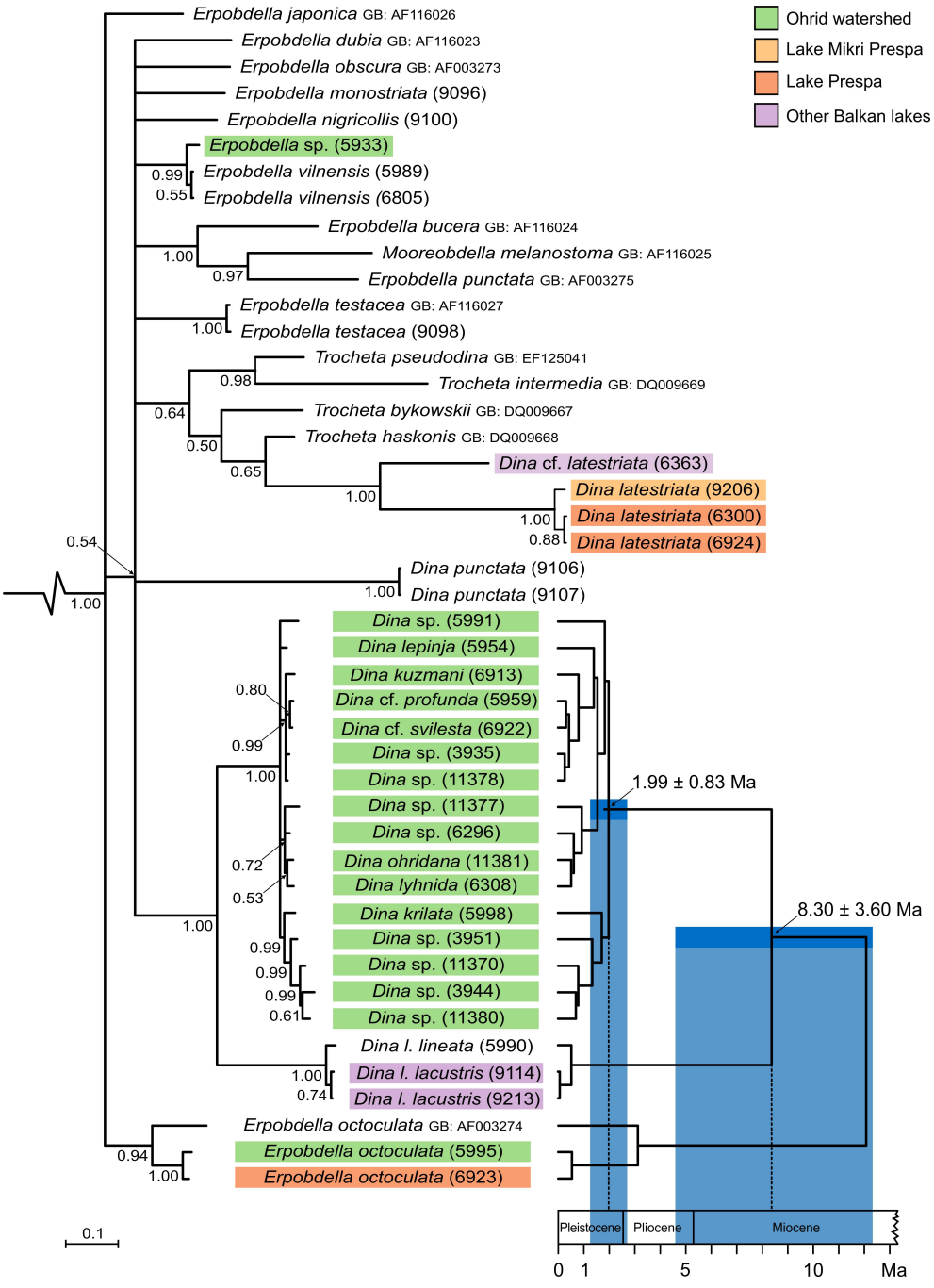

Fig. 3. Bayesian phylogeny of selected species of Erpobdellidae inferred from the mitochondrial COI gene (left). The outgroup taxa *Glossiphonia complanata* and *Batracobdella algira* were removed *a posteriori*. Bayesian posterior probabilities are given next to the nodes. The scale bar indicates the number of substitutions per site. A strict clock Bayesian tree of selected taxa from the Ohrid *Dina* flock is shown on the right. The 95% confidence intervals for the onset of diversification and for the split with taxa from outside the lake are indicated by blue bars. Specimens from the Balkans are marked by coloured bars. Number in parenthesis behind taxon names are the respective DNA voucher numbers (see Table 1 for details). Accession numbers are provided for sequences obtained from GenBank.

family Glossiphoniidae) were used for outgroup comparison. Genetic information of additional taxa (including *Erpobdella* spp., *Trocheta* spp., and *Mooreobdella melanostoma*) were obtained from GenBank (Fig. 3).

Most of the specimens, especially from the littoral, were collected using a rectangular dredge (frame dimension 400×200 mm) with a mesh size of 0.5 mm. A van Veen grab (225 cm^2) was utilized for collecting samples from the

profundal of Lake Ohrid (depths 120, 130, 180, and 200 m). Additionally, traps were prepared in Lake Ohrid using plastic bottles filled with fish bait. Specimens from springs were gathered by hand picking from stones. If possible, taxa were identified in the field by ST with a field stereomicroscope based on the morphological (e.g. size, colour, suckers) and anatomical (e.g. atrium, cornea, testes) characteristics suggested by Šapkarev (1975, 1990), Lukin (1976), Sket (1981), Sket and Šapkarev (1986), and Nesemann and Neubert (1999). After identification, specimens were preserved in 80% ethanol for DNA work.

For subsequent analyses, we labelled specimens that resemble the known morphospecies closely but which could genetically not be assigned unambiguously to the named species with the epithet *confer* (cf). Morphologically and genetically distinct specimens that could not be assigned to previously described species were referred to as "sp." (also see Table 1).

2.2 DNA isolation and sequencing

The method described in Wilke et al. (2006) was used for isolating DNA from individual leeches. The primers for amplifying a fragment of the COI gene with a target length of 658 base pairs (excluding 51 bp primer sequence) were LCO1490 and HCO2198 as described by Folmer et al. (1994). Sequences (forward and reverse) were determined using the LI-COR (Lincoln, NE) DNA sequencer Long ReadIR 4200 and the Thermo Sequenase Fluorescent Labeled Primer Cycle Sequencing kit (Amersham Pharmacia Biotech, Piscataway, NJ). The protein-coding COI sequences were unambiguously aligned in BioEdit 7.0.4.1 (Hall, 1999). The first base pairs behind the 3′ end of each primer were difficult to read. We therefore uniformly cut off these parts, leaving a 592 bp-long completely overlapping fragment for the COI gene.

The 109 newly created sequences (GenBank accession numbers HM246525−HM246633) were used for a hierarchical set of phylogenetic and phylogeographical analyses, together with 12 sequences obtained from GenBank (for GenBank accession numbers see Fig. 3).

2.3 Phylogenetic and phylogeographical analyses

2.3.1 Phylogenetic analyses

A family-level phylogenetic analysis was performed using a dataset of 47 sequences from representatives of *Dina* spp., *Erpobdella* spp., *Trocheta* spp., and *Mooreobdella* sp., with *Glossiphonia complanata* and *Batracobdella algira* as outgroup taxa. The purpose of this analysis was to infer the position of *Dina* spp. from the Ohrid watershed within the family Erpobdellidae and to test whether they represent an ancient lake species flock sensu Greenwood (1984) and Schön and Martens (2004). Based on preliminary phylogenetic analyses, we included only 16 *Dina* taxa from Lake Ohrid in this analysis in order to avoid a possible shift of the performance of the model selection, and thus the actual phylogenetic analysis, towards intraspecific relationships. These 16 taxa comprise 7 nominal species as well as 9 undetermined haplotypes.

First, we ran jModelTest 0.1.1 (Posada, 2008) in order to obtain an estimate for the general model of sequence evolution to be used in the phylogenetic analysis and to estimate the ratio of invariable sites for a test of substitutional saturation. The entropy-based test in DAMBE (Xia et al., 2003) did not reveal a substantial degree of saturation with the *Iss* for asymmetrical trees (IssAsym = 0.763) being significantly lower ($P < 0.0001$) than the *Iss.c* for symmetrical trees (IssSym = 0.798).

For phylogenetic reconstruction of our family-level dataset, we conducted a Bayesian analysis in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) with the general GTR + I + G model suggested by the Akaike criterion of jModelTest and a flat Dirichlet prior probability density. We performed two simultaneous independent runs, each starting with a different random tree; each run with one cold and three heated chains. The mixing of the MCMC chains of the two runs was monitored with Tracer 1.4.1 (Drummond and Rambaut, 2007) and the analysis was terminated after the average standard deviation of the split frequencies was smaller than 0.01. The first 25% of the sampled ∼3 million generations were discarded as burn-in.

2.3.2 Molecular clock analysis

Our molecular clock analysis aims at estimating the minimum age of onset of diversification of *Dina* spp. in the Ohrid watershed (this estimate can be regarded as a potential minimum age of the lake; see Discussion for details) and the age of the spilt with the sister group outside Lake Ohrid (which can be potentially used to establish the maximum age of the lake).

To do this, we further reduced the dataset (from hereon called molecular clock dataset) to a total of 22 specimens. They belonged to *Dina* spp. from the Ohrid watershed, the sister taxon *Dina lineata*, and the outgroup taxon *Erpobdella octoculata*.

The clock analyses were conducted closely following the flowchart provided by Wilke et al. (2009: Fig. 6). First, we ran the molecular clock dataset in jModelTest to find the best-fit model of sequence evolution based on the Akaike information criterion. Then, we proceeded to test whether the molecular clock hypothesis is accepted, that is, whether a strict molecular clock can be assumed (recall that the larger family-level dataset did not show signs of saturation). As model-choice criterion, we used the Bayes factor (see Ronquist and Deans, 2010, for a review). The Bayes factor B is the ratio of the marginal likelihoods of two given models M_0 and M_1. If the models differ by a factor of $2 \ln (B_{10}) = 6$ to

10, then there is strong evidence against model M_0 (Kass and Raftery, 1995: 777). The marginal likelihood can be approximated by the harmonic mean of the likelihood values from an MCMC sample. According to Hipsley et al. (2009), an advantage of the Bayes factor compared to likelihood ratio tests is that it automatically penalizes complex models, thus guarding against overfitting.

With harmonic means of $-\ln = 2091.07$ and $-\ln = 2095.27$ for the clock and no-clock models and a Bayes factor of 8.4, there is strong evidence against the model without clock assumption. Applying a strict-clock model therefore appears to be appropriate.

In the absence of a *Dina*-specific substitution rate, we then estimated divergence times from all clock-enforced trees of both Bayesian runs (except burn-in), utilizing the trait-specific COI Protostomia molecular clock rate of $1.24\% \pm 0.22\%\,\mathrm{Ma}^{-1}$ for the inferred HKY model suggested by Wilke et al. (2009).

Finally, we calculated 95% confidence intervals for our time estimates including both the error of the phylogenetic analyses (in terms of branch-length variation of individual trees) and the error of the clock rate (see above) by utilizing a propagation of uncertainty approach (for details see Wilke et al., 2009, 2010).

It should be noted that we did not correct our clock estimates for ancestral polymorphism since the universal Protostomia COI clock applied here is also not corrected (see Wilke et al., 2009). As the Protostomia clock, however, is based on phylogenetic events that are, on average, 3 Ma old, there might be a small bias towards the overestimation of divergence times for events younger than 3 Ma and towards underestimation for events older than that.

2.3.3 Network analysis

For phylogeographical analyses (both network analyses and tests for genetic structure) of *Dina* spp. within the Ohrid watershed, we used sequences from a total of 87 specimens (from hereon called the Ohrid dataset). First, a statistical parsimony haplotype network was constructed utilizing the program TCS 1.21 (Clement et al., 2000), with the connection limit set to 95% and gaps being treated as fifth state. Then, we ran a second analysis, reducing the connection limit to 90% in an attempt to infer connections among sub-networks.

2.3.4 Test for genetic structure

We used the Ohrid dataset to perform an exact test as suggested by Raymond and Rousset (1995) and implemented in Arlequin 3.5.1.2 (Excoffier et al., 2005) to study (a) possible genetic differentiations among taxa inhabiting different habitats, that is, springs (17 specimens), littoral (0–20 m depth; 15 specimens), sublittoral (20–50 m depth; 45 specimens) and profundal (>50 m depth; 10 specimens), and (b) the role of horizontal and vertical barriers in Lake Ohrid. We feel that this test is appropriate as lineage sorting does not seem to be complete in *Dina* spp. from the Ohrid watershed (see Results for details). The assumed null hypothesis of the test statistics is a random distribution of different haplotypes among groups (i.e. panmixia). The significance of pairwise differentiation of specimens from different habitats was evaluated by running the Markov chain for 1 000 000 steps.

3 Results

3.1 Phylogenetic analyses

The family-level phylogenetic analysis utilizing 47 sequences from representatives of *Dina* spp., *Erpobdella* spp., *Trocheta* spp. and *Mooreobdella* sp. resulted in the majority-rule consensus tree shown in Fig. 3. In this tree, basal relationships are poorly resolved, causing a large polytomy of several non-Ohrid taxa. All clades harbouring specimens of *Dina* spp., however, are extremely well resolved, all with Bayesian posterior probabilities (BPP) of 1.00. Accordingly, nominal *Dina* spp. are not monophyletic but cluster in four different clades. The first "*Dina*" clade comprises specimens of *D. latestriata* from Lake Mikri Prespa (topotypes) and Lake Prespa as well as a closely related taxon (here called *D*. cf. *latestriata*) from the Southern Balkan Lake Trichonis. The second "*Dina*" clade consists of the two specimens of *D. punctata* studied from Spain. The third clade is made up of specimens of the type species of *Dina*, *D. lineata*, comprising representatives of nominal *D. l. lineata* from Germany and topotypes of *D. l. lacustris* from a small glacial lake north of Lake Ohrid. The fourth and largest *Dina*-clade in our study contains all and only *Dina* specimens from the Ohrid watershed (incl. feeder springs). Thus, Lake Ohrid *Dina* forms a monophyletic group. This fourth clade is sister to the *Dina* clade comprising the type species of the genus. The sister-group relationship is supported by a BPP of 1.00. However, the *Dina* clade from Lake Ohrid shows a very shallow phylogenetic structure and nominal taxa are not well resolved (also see Fig. 4).

3.2 Molecular clock analyses

The molecular clock analyses (Fig. 3) indicate that the monophyletic group of endemic *Dina* species from the Lake Ohrid watershed (hence called Ohrid *Dina* flock) is relatively young. The most recent common ancestor (MRCA) of extant species of this flock is estimated to be 1.99 Ma old with a 95% confidence interval of ± 0.83 Ma.

This estimate is based on an average node depth of 0.0246 ± 0.0093 and a clock rate of $0.0124 \pm 0.0022\,\mathrm{Ma}^{-1}$ for the HKY model (see Materials and methods section).

The split of the Ohrid *Dina* flock from its presumed sister taxon outside the Ohrid watershed (i.e. *Dina lineata*) is estimated to have occurred 8.30 Ma ago with a confidence interval of ± 3.60 Ma (average node depth 0.129 ± 0.041).

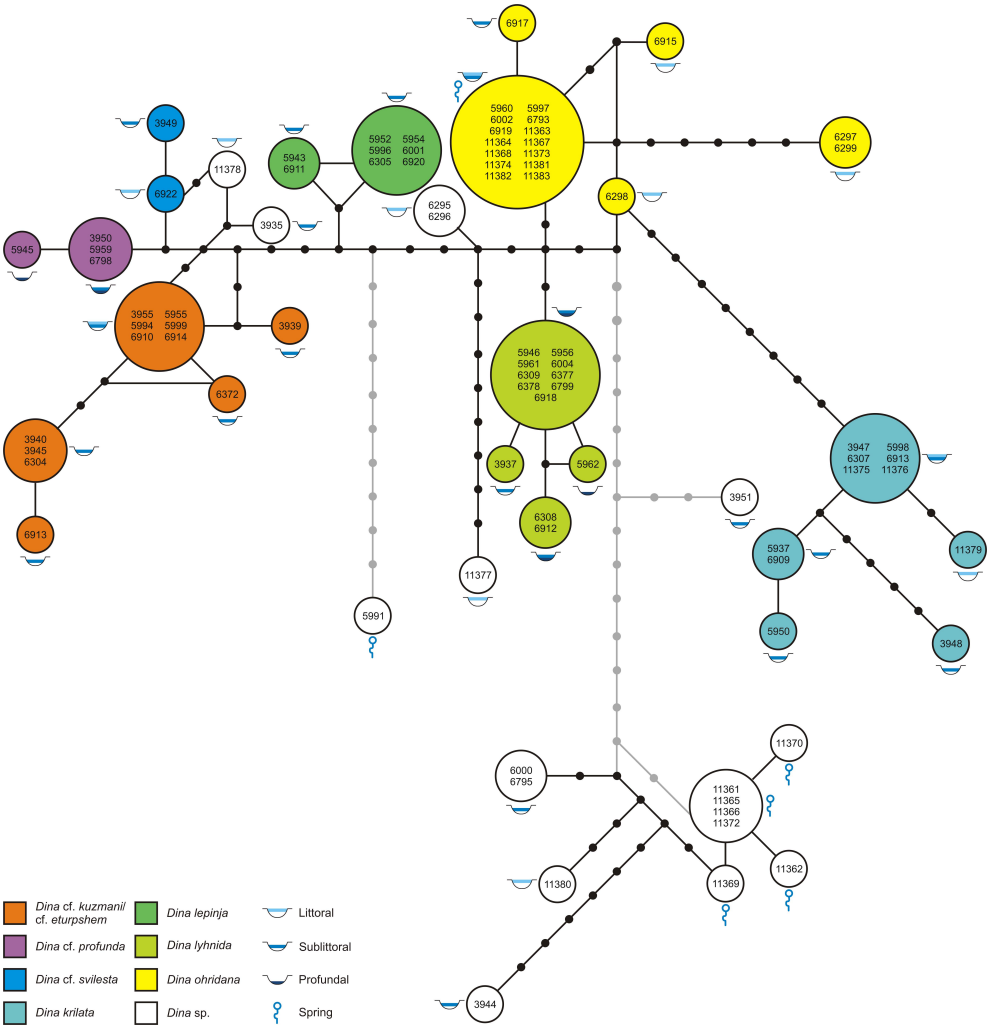

Fig. 4. Statistical parsimony haplotype network of specimens of the Ohrid *Dina* flock based on the mitochondrial COI gene (connection limit 95%). Haplotypes are colour-coded according to taxa (DNA voucher numbers are provided inside the circles; see Table 1 for details). Areas of circles representing the haplotypes found are proportional to the number of specimens sharing the respective haplotype. Missing haplotypes are indicated by black dots. The grey lines show the connections among sub-networks when reducing the TCS connection limit to 90%. Major habitat types are indicated by symbols.

3.3 Network analyses

The network analysis of the Ohrid dataset (87 specimens) applying a 95% connection limit resulted in a total of 38 haplotypes (14 of them shared) in four distinct sub-networks (Fig. 4). The first and by far the largest sub-network consists of all specimens belonging to *D*. cf. *kuzmani*/cf. *eturpshem*, *D*. cf. *profunda*, *D*. cf. *svilesta*, *D. krilata*, *D. lepinja*, *D. lyhnida*, *D. ohridana* as well as four undetermined haplotypes (*Dina* spp.). The second sub-network comprises eleven undetermined specimens with a total of seven haplotypes, whereas sub-networks three and four comprise only one undetermined haplotype. If the connection limit is lowered to 90%, all sub-networks became interconnected (see grey lines in Fig. 4).

On the one hand, a number of specimens could not be unambiguously assigned to nominal species and substitutional differences among species are generally low. On the other hand, specimens of most morphologically distinct taxa share haplotypes or cluster together. This observed pattern, together with a number of reticulations seen in the networks, indicates that lineage sorting in *Dina* spp. from the Ohrid watershed is not complete and/or that hybridization occurs.

The assignment of character states for horizontal (springs vs. lake proper) and vertical (littoral, sublittoral, profundal) distribution to haplotypes shows that haplotypes are frequently shared by specimens collected from different vertical zones such as littoral/sublittoral as well as sublittoral/profundal. There are, however, no haplotypes shared by specimens from all three vertical zones and there are no clusters of specimens from the littoral and profundal. As for

Table 2. Results of the exact test of significant pairwise genetic differences (P level 0.05) between 87 specimens of *Dina* spp. from different habitats of the Ohrid watershed. Numbers in parenthesis indicate number of specimens studied/number of haplotypes found in the respective group.

	Springs	Littoral	Sublittoral	Profundal
Springs (17/6)				
Littoral (15/14)	+			
Sublittoral (45/41)	+	–		
Profundal (10/10)	+	–	–	

the horizontal distribution, all spring haplotypes are highly distinct from lake haplotypes except for the most common haplotype in our dataset, which is shared by a total of 14 specimens from springs and the lake itself.

3.4 Test for genetic structure

The exact test of pairwise sample differences between habitats based on haplotype frequencies did not indicate significant differentiations among *Dina* specimens from the littoral, sublittoral or profundal. However, there are significant pairwise differences between haplotypes from springs and all lake habitats (Table 2).

4 Discussion

4.1 Phylogenetic position of the Ohrid *Dina* flock

In Europe, the Erpobdellidae is the predominant leech family with three species-rich nominal genera, *Erpobdella* Blainville, 1918, *Trocheta* Dutrochet, 1817, and *Dina* Blanchard, 1892. The latter genus comprises at least 24 species and subspecies (Nesemann and Neubert, 1999; Grosser et al., 2007; Grosser and Eiseler, 2008).

Systematics and phylogenetics of the family, however, are still discussed controversially. Siddall (2002), for example, has argued that the morphological and anatomical characters used to define genera of Erpobdellidae were not reliable. He also found, based on molecular data, that the genus *Dina* is not monophyletic. Therefore, he formally synonymised the genera *Dina*, *Mooreobdella*, *Nephelopsis*, and *Trocheta* under the genus *Erpobdella*. Whereas some researchers followed this suggestion (e.g. Borda and Siddall, 2004; Oceguera-Figueroa et al., 2005), other workers argued that there are morphological characters that can be used to distinguish at least some of the above genera (e.g. Hovingh, 2004) and continued to treat *Dina* and *Trocheta* as valid (e.g. Pfeiffer et al., 2005; Grosser et al., 2007; Grosser and Eiseler, 2008).

Whereas we did find European *Dina* to be paraphyletic in our dataset as previously suggested by Siddall (2002), we also found *Dina* specimens from the Ohrid watershed to form a monophyletic clade with the type species of *Dina*, *D. lineata*, as its sister taxon. Moreover, *Dina* spp. from Lake Ohrid could be unambiguously distinguished from *Erpobdella*, sympatrically occurring in the lake (see Fig. 2). Pending a comprehensive revision of the family Erpobdellidae, we therefore prefer to retain the genus name *Dina* for the clade comprising the endemic Lake Ohrid taxa and the type species of the genus.

Our phylogenetic analysis not only showed *Dina* species from the Ohrid watershed to be monophyletic, it also revealed a number of genetically (and morphologically) distinct taxa that may represent new species. Sket (1989) and Sket and Šapkarev (1992) had already suspected the existence of at least two undescribed species. The occurrence of cryptic species is not unusual in leeches as demonstrated recently for the medicinal leeches (Trontelj and Utevsky, 2005; Siddall et al., 2007). Overall, the total number of species within the Ohrid *Dina* flock remains unknown, largely because of a conflict between morphological and molecular data. Thus future studies based on detailed anatomical analyses and fast-evolving nuclear markers would have to address this problem.

Interestingly, we also found one taxon, "*Dina*" *latestriata*, which had been described from Lake Mikri Prespa, not to belong to the Ohrid group (Fig. 3), corroborating the faunal distinctness of Lake Ohrid. Such distinctness has been suggested before based on biogeographical and phylogenetical studies in diverse taxa (e.g. Albrecht and Wilke, 2008; Hauswald et al., 2008; Schultheiß et al., 2008; Albrecht et al., 2009; Wilke et al., 2010).

As to the question whether the Ohrid group, indeed, represents an ancient lake species flock, the conservative species flock definition by Greenwood (1984) is based on the following criteria: speciosity, monophyly and endemicity. All these criteria are met by the *Dina* species in the Ohrid watershed. For this reasoning, we fully support Sket (1989) in that the endemic species of Lake Ohrid represent an ancient lake species flock.

It should be noted that *D. lineata*, the presumably widespread type species, is also mentioned in the literature for Lake Ohrid (e.g. Sket and Šapkarev, 1992), though the researchers state that they are not certain as to the species identification because only juvenile specimens were studied. Our sampling indicates that *D. lineata* occurs in the vicinity of Lake Ohrid, but we were unable to find it in the actual lake.

4.2 Horizontal and vertical zonation of *Dina* spp. in Lake Ohrid

The relatively high rate of diversification within the Ohrid *Dina* flock raises questions about whether there are distinct horizontal and vertical barriers or gradients in the lake that may have promoted evolutionary events. The role of such

barriers has been discussed in the literature for more than 50 years (e.g. Hadžiše, 1956; Hubendick, 1960; Radoman, 1985). Two of the most prominently considered barriers in the Ohrid watershed are the horizontal differentiation of species occurring in springs and the lake proper and the vertical differentiation of taxa (species and subspecies) occurring above and below the "*Chara* belt" (see Albrecht and Wilke, 2008).

Whereas several cases of horizontal zonations between springs and the lake proper have been reported (e.g. Radoman, 1983; Albrecht et al., 2006, 2008; Albrecht and Wilke, 2008; Wysocka et al., 2008; also see Hauffe et al., 2010), strong vertical barriers or gradients within the lake remain mostly hypothetical (e.g. Hadžiše, 1956; Hubendick, 1960; Radoman, 1985; Albrecht and Wilke, 2008; but see Wysocka et al., 2008).

Our findings for the horizontal zonation of haplotypes from spring and lake populations corroborate the role of lakeside springs, particularly of the southern feeder springs, for evolutionary processes in endemic Lake Ohrid taxa. This, in turn, raises questions as to the role of isolation of lake and spring populations for allopatric/parapatric speciation, and why these springs, which probably were repeatedly inundated by the lake during evolutionary times (for lake-level fluctuations see Lézine et al., 2010; Lindhorst et al., 2010), continue to harbor highly distinct haplotypes. Regarding a vertical differentiation of lake taxa, population differences among the vertical zones studied appear to be small (Table 2). However, our approach tested only for differences among vertical zones but not for potential taxonomic structures (in order to avoid circularity since taxonomic identity here is partly based on the molecular findings of this study). Since both the zonation and a potential taxonomic structure may weaken each other in the test, a lack of differentiation may be due to type 2 error (false negative). Nonetheless, some structure is obvious from the vertical (littoral, sublittoral, profundal) distribution of haplotypes plotted in Fig. 4. Accordingly, specimens from the littoral and profundal never share haplotypes, whereas specimens from adjacent depth zones do. These findings support the hypotheses of Stanković (1960: 290), who suggested that the deeper parts of Lake Ohrid were gradually colonized from shallower parts, accompanying tectonic deepening and that "The differentiated profundal forms are therefore younger...".

4.3 Evolutionary evidence and the time frame for the origin of Lake Ohrid

As stated in the Introduction, the exact age of Lake Ohrid is unknown (for a review of available data see Albrecht and Wilke, 2008). Whereas there is newer geological data for the time frame for the formation of the Ohrid Graben (i.e. 5–8 Ma; Dumurdzanov et al., 2004), most information about the limnological age of the actual lake is directly or indirectly based on geological studies of Cvijić (1911: 716), who wrote that "the Ohrid valley probably originated during the Pliocene". Later, Stanković (1960) referred to Cvijić (1911) and suggested that the Ohrid Basin was formed at the end of the Pliocene. Other researchers (e.g. Spirkovski et al., 2001)

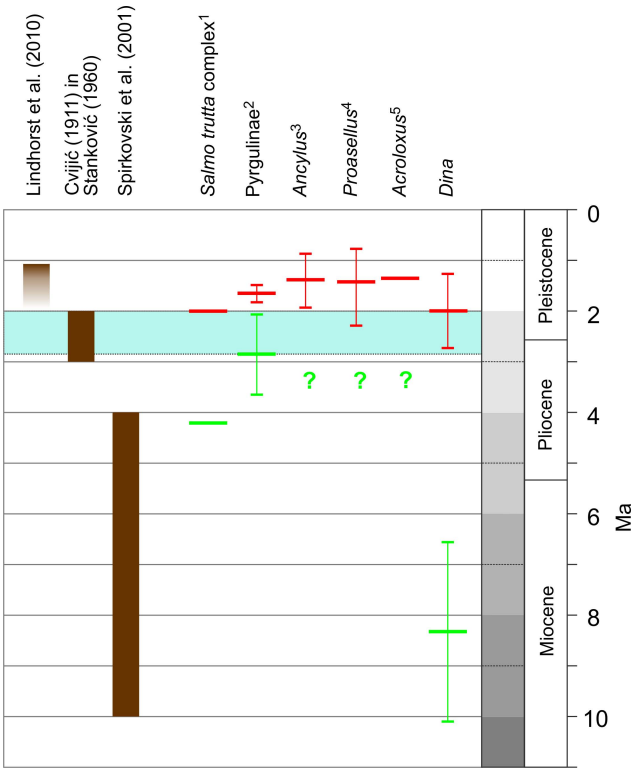

Fig. 5. Comparisons of geological and evolutionary estimates for the age of Lake Ohrid. Brown bars indicate geological estimates (note that the estimate of Lindhorst et al., 2010 represents a minimum age). The narrow red bars indicate the onset of diversification in groups of endemic Lake Ohrid taxa together with their standard deviations (not CI). The narrow green bars indicate the average timing and standard deviations of the split with their respective sister groups outside the lake. These estimates set the minimum and a potential maximum time frame for the origin of Lake Ohrid faunas (turquoise band) based on evolutionary data. See text for details.

[1] Sušnik et al., 2006.

[2] Wilke et al., 2007, 2009. Note that the authors did not include all species of the Pyrgulinae species flock in their analysis. Therefore, the onset of diversification in this group is very likely underestimated.

[3] Albrecht et al., 2006. Note that the authors regarded an endemic taxon from the feeder springs of Lake Ohrid as a non-Ohrid taxon. For reasons of consistency, this taxon is here considered to be part of the Ohrid group.

[4] Wysocka et al., 2008. Note that the authors calculated the age for the older of two *Proasellus* groups at 2.83 Ma. For their tree estimations, they used the GTR + I + G substitution model, but utilized the K2P model for subsequent molecular dating. Here we have recalculated the divergence time based on the GTR + I + G rate suggested by Wilke et al., 2009.

[5] Albrecht and Wilke, 2006.

even considered an age of Lake Ohrid of up to 10 Ma, but without stating on which evidence this information is based. There is, however, new data for the limnological age of Lake Ohrid from Lindhorst et al. (2010). Based on sediment thickness and estimated sedimentation rates, the authors suggest a minimum age of 1.1 Ma (Fig. 5).

Given the conflicting geological information on the age of Lake Ohrid, molecular clock estimates of major evolutionary events in Lake Ohrid species flocks may help to refine these dates by establishing lower and upper constraints.

The upper constraint could come from the onset of speciation in those species flocks. If we assume that speciation occurred within the basin, the first divergence event in the respective flock would constitute the minimum age of Lake Ohrid because at this time, the lake must have already existed. In contrast, if we calculate the divergence time of the MRCA of lake taxa with the presumed sister taxon from outside the lake then we would receive a potential maximum age for the lake. Of course, to do so, we would have to assume that the divergence event happened outside the lake and that the lake did not already exist at that time. Whereas data on, for example, the population structure and ecology of the taxa involved may help to provide some evidence for that, these assumptions appear to be difficult to test. Therefore, the lower constraint suggested here should be treated with caution. The molecular clock analyses conducted here for Ohrid *Dina* flock (Fig. 3) indicate that the MRCA of extant species of this flock is approximately 1.99 Ma old with a confidence interval of ± 0.83 Ma. The age of the split of the Ohrid *Dina* flock from its sister taxon outside the lake (i.e. *Dina lineata*) is estimated at 8.30 ± 3.60 Ma. The upper limit shows good concordance with the minimum ages for other endemic taxa available in the literature (Fig. 5) ranging from approximately 1.3 Ma for the limpet genus *Acroloxus* in Lake Ohrid (Albrecht and Wilke, 2006) to some 2 Ma for the endemic *Salmo trutta* complex (Sušnik et al., 2006).

Considerably less data is available for the lower constraint, which also shows a higher variability. Estimates for the split of endemic Ohrid taxa to taxa outside the lake range from approximately 2.85 ± 1.29 Ma for the pyrgulinid gastropod species flock (Wilke et al., 2007) to 8.30 ± 3.60 Ma calculated here for the *Dina* flock.

Overall, the data summarized in Fig. 5 provides good support that the minimum age of Lake Ohrid is some 2 Ma. For the reasons outlined above, support for the maximum age estimation is weaker and approximately 3 Ma is a rough estimate. Interestingly, this time frame of approximately 2–3 Myr for the origin of Lake Ohrid, generated solely based on evolutionary data, almost exactly fits the time frame most often used in the literature by geologists.

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