Open Access

Ancient Endemic or Recent Invader? Phylogenetic Position and the Probable Origin of the Ccladoceran *Diaphanosoma macedonicum* (Diplostraca, Sididae) from the Ancient Lakes in the Balkans

Charo López-Blanco^{1,2,}∗₪, Ohrideja Tasevska³₪, Goce Kostoski³, Eduardo Vicente⁴₪, Laura S. Epp⁵₪, and Antonio García-Alix²₪

¹Justus Liebig University Giessen, Department of Animal Ecology and Systematics, Heinrich-Buff-Ring 26 (IFZ). D-35392 Giessen, Germany. *Correspondence: E-mail: Charo.Lopez-Blanco@allzool.bio.uni-giessen.de (López-Blanco)

²Department of Stratigraphy and Paleontology, University of Granada, Avda, Fuentenueva s/n, 18071, Granada, Spain. E-mail: agalix@ugr.es (García-Alix)

³Department of Zooplankton, Hydrobiological Institute, Naum Ohridski 50, 6000 Ohrid, Northern Macedonia. E-mail: orhidejat@hio.edu.mk (Tasevska); gocekos@hio.edu.mk (Kostoski)

⁴Department of Microbiology and Ecology, University of Valencia, Dr. Moliner 50, 46100 Burjassot, Spain. E-mail: eduardo.vicente@uv.es (Vicente) ⁵Limnological Institute, University of Konstanz, Mainaustraße 252 78464 Konstanz / Egg, Germany. E-mail: laura.epp@uni-konstanz.de (Epp)

Received 24 July 2023 / Accepted 13 January 2024 / Published 3 May 2024 Communicated by Ka Hou Chu

Ancient lakes contain unique and very vulnerable fauna. Determining and understanding the origin of such biodiversity is a key factor in promoting conservation and management actions in some of the most singular ecosystems on the planet. Lake Ohrid in the Balkans is known as a natural laboratory for speciation, containing a high number of endemic species. However, the identity and origin of the planktonic cladoceran *Diaphanosoma* is uncertain. Representatives of the genus were long considered to have invaded the lake, but recent morphological studies have suggested that they belonged to the endemic taxon in the Balkans, *D. macedonicum*. Here, phylogenetic methods based on two mitochondrial gene fragments (*COI* and 16S) were used to identify *Diaphanosoma* specimens from the ancient Lake Ohrid and Lake Prespa in the Balkans and compare them with other species in Europe, including those living in nearby water bodies. Molecular evidence showed that *D. macedonicum* was constrained to the ancient lakes Ohrid, Prespa, and Mikri Prespa, which suggests reproductive isolation within the lakes. Phylogenetic analyses supported previous morphological assessments and situated *D. macedonicum* within the *D. mongolianum* species group, which contains three sibling species (*D. mongolianum*, *D. lacustris*, and *D. macedonicum*). Nuclear markers are needed to study intraspecific gene flow in these organisms and discard a potential formation of hybrids.

Key words: Ancient lakes, DNA barcoding, Lake Ohrid, Lake Prespa, Endemicity

BACKGROUND

Ancient lakes are some of the most singular ecosystems of the planet, as they comprise extant

environments that have been carrying water since before the last glacial period or even before the Pleistocene (Gorthner 1994; Martens 1997). As they have persisted during multiple climatic cycles and environmental

Citation: López-Blanco C, Tasevska O, Kostoski G, Vicente E, Epp LS, García-Alix A. 2024. Ancient endemic or recent invader? Phylogenetic position and the probable origin of the cladoceran *Diaphanosoma macedonicum* (Diplostraca, Sididae) from the ancient lakes in the Balkans. Zool Stud **63:**09. doi:10.6620/ZS.2024.63-09.

conditions, they contain continuous archives of the most recent history of the planet, and thus they have been the focus of many palaeoecological investigations (e.g., Cohen 2012; Fritz et al. 2012; Karabanov et al. 2004; Stone et al. 2011). They have also fascinated taxonomists and evolutionary biologists, since as a result their internal speciation processes, they contain high-endemic species and some of the rarest taxa on Earth (Cristescu et al. 2010). By way of illustration, the ancient Lake Ohrid in the Balkans is considered the most biodiverse ancient lake in the world in terms of the number of species per surface area (Albrecht and Wilke 2008). The rate of endemicity was estimated at 36% for all taxa and 34% for Animalia (Albrecht and Wilke 2008), but it has probably increased in recent years with the discovery of new endemic taxa, such as the cladoceran Coronatella begoniae (Sinev and López-Blanco 2018).

Regarding the Cladocera, 26 species have been cited in this lake (Stankovic 1960; Kostoski et al. 2004), two of them - Coronatella begoniae (Sinev and López-Blanco 2018) and Phreatalona smirnovi (Van Damme et al. 2009) — being endemic, which situates the endemicity rate at ca. 7.4%. The low number of endemic cladocerans encountered in this lake might either be ascribed to (i) a strong dispersal ability that could explain more cosmopolitan distributions or (ii) failure to estimate real biodiversity by conventional taxonomy based on morphology. This draws attention to the case of *Diaphanosoma* Fischer, 1850, in the ancient Lake Ohrid, because data obtained in the last few decades depicts contradictory theories about its identity, origin, and status in this ecosystem. Some information indicates that this species might be a recent invader of the lake, while further morphological analyses have suggested it to be an ancient endemism. Representatives of the genus Diaphanosoma were not known in this lake until the 1990s, when new invaders D. birgei lacustris Kořinek and Leptodora kindtii (Focke, 1844) were recorded (Guseska et al. 2019). Analysis of cladoceran subfossils in a sedimentary record in Lake Ohrid only showed sub-fossils from Diaphanosoma in the uppermost sample (mean age 1975) (López-Blanco et al. 2020). However, the sub-fossil analysis did not allow identification at the species level due to the poor taxonomical information provided by the postabdominal claws. Identification of Diaphanosoma in this lake has been unclear, pointing either to D. birgei lacustris (Gušeska et al. 2005 2014 2019) or to D. brachyurum (Kostoski et al. 2010; Tasevska et al. 2017). Recently, Korovchinsky (2022) presented detailed morphological descriptions indicating that Diaphanosoma in Lake Ohrid belong to the endemic Balkan taxon D. macedonicum. Nevertheless, this morphological

analysis was only based on parthenogenetic females, while males and gamogenetic females are needed to confirm the species identity and improve the formal species description.

In this study, we provide new genetic data from mitochondrial loci (16S and the *COI* barcoding regions) originating from specimens collected mainly in the Balkan Peninsula with two main aims: 1) to confirm the identity of the *Diaphanosoma* species in the ancient Lake Ohrid and Lake Prespa and assess its current status in these ecosystems, and 2) to help determine the phylogenetic position of the *D. macedonicum* within the phylogeny of the genus. The data provided in our study will contribute to a better understanding of ancient lake biodiversity in a poorly studied area like the Balkans and provide a baseline for management actions for some of the most precious ecosystems of the world.

MATERIALS AND METHODS

Diaphanosoma Fischer, 1850, currently includes 40 valid euplanktonic species, most of which inhabit relatively warm water bodies all over the world (Korovchinsky 2018). Five of them, D. brachyurum (Liévin, 1848), D. lacustris Korinek, 1981, D. mongolianum Ueno, 1938, D. orghidani Negrea, 1982, and D. macedonicum Korovchinsky et Petrovski 2014, occur in European inland waters (Błędzki and Rybak 2016; Korovchinsky 2018). All of them except D. macedonicum have a relatively large distribution covering several countries and biogeographical areas (Fig. 1). Only D. macedonicum has shown a very limited distribution in the Balkan Peninsula, since it was only cited in the pelagic zones of the lakes Dojran and Prespa (Korovchinsky and Petkovski 2014) and recently was also found in Mikri Prespa (Alexiou et al. 2021).

Field collection and morphological analyses

The *Diaphanosoma* specimens studied here were collected from Lake Ohrid, Lake Prespa, and geographically close water bodies in North Macedonia and Greece. In addition, material from outside the Balkan Peninsula belonging to *D. mongolianum, D. lacustris* and *D. brachyurum* was used for phylogenetic comparison (Table 1). Samples were collected by pulling a 90 μ m plankton net in the pelagic zone of the lakes/reservoirs cited in table 1. The material was preserved in a 95% ethanol solution and stored at 4°C for further analyses. Samples were sorted in the lab and identified at the species level using specific keys for European zooplankton (Alonso 1996; Błędzki and Rybak 2016; Margaritora 1983). Specimens from Lake Ohrid identified as *D. macedonicum* (see Korovchinsky 2022) were morphologically compared with described conspecifics from lakes Prespa and Dojran (Korovchinsky and Petkovski 2014).

DNA Sequencing

Total genomic DNA was extracted from single individuals following the DNeasy Blood & Tissue (Qiagen) protocol, but using half of the recommended volume for each step. Once in the lysis buffer, each specimen was energetically crushed with sterile and disposable needles to ensure that the carapace and tissues broke down. Following DNA extraction, DNA was quantified in a Nanodrop spectrophotometer, and $4-5 \mu$ L of DNA was added to a PCR, following the procedure published in López-Blanco et al. (2024) (final volume of 20 µL consisting of 2 µL of 10x Thermopol Buffer, 1.4 µL of MgCl, 1.4 µL of dNTP, 6.6 µL of dH₂O, 0.2 µL of TMAC, 1.2 µL of BSA, 1.4 µL of forward and reverse primers, and 0.4 of Taq Polymerase [New England Biolabs, 5000 U/mL]). Fragments of the mitochondrial cytochrome c oxidase subunit I (COI) were amplified and sequenced using either the universal primers for arthropods (Folmer et al. 1994) or specific primers designed for zooplankton, Zplank-F and Zplank-R (Prosser et al. 2013). The ribosomal 16S rRNA (16S) fragment was amplified and sequenced using the primer pair 16Sch-a and 16Sbr (Sacherová and Hebert 2003). The polymerase chain reaction amplification conditions followed the methods of Prosser et al. (2013): five cycles of (94°C for 40s, 45°C for 40s, 72°C for 1 min), then 35 cycles of (94°C for 40s, 51°C for 40s and 72°C for 1 min), and then a final extension of 72°C for 5 min. PCR products were visualized on a 1% agarose gel, and visually positive PCR products were selected for sequencing. The amplified PCR products were sequenced in an ABI 3730 XL sequencer (Life Technologies, Carlsbad, CA, USA) using a Big Dye Terminator kit ver. 3.1 (Life Technologies). The chromatograms for each DNA sequence were checked using the software SEQUENCHER 5.4.6 (Gene Codes, Ann Arbor, MI). The primer sequences were removed before any further analysis.

Phylogenetic analyses

The authenticity of the sequences was verified



Fig. 1. (A) Map showing the distribution of the genus *Diaphanosoma* in Europe (modified after Błędzki and Rybak 2016) and (B) close-up on the Balkan Peninsula, where *D. macedonicum* was cited. Note that *D. brachyurum* is widely distributed in Europe, and it is also present in the distribution area of *D. orghidani*.

using BLASTN 2.10 (Zhang et al. 2000) against the National Center for Biotechnology Information (NCBI) nucleotide database. These sequences, together with more than 200 sequences retrieved from GenBank (Table 2), were employed in our phylogenetic reconstructions using *Moina* cf. *micrura* (Accession number: MH708070), *Simocephalus* cf. *serrulatus* (Accession number: KC617159), and *Daphnia* cf. *pulicaria* (Accession number: EU152322) as suitable outgroup. The outgroup selection was made using the same taxa as those used in a recent *COI*-based phylogeny of *Diaphanosoma* s. l., published in Dumont et al. (2021),

in order to make the results comparable. Protein-coding *COI* sequences were aligned in Bioedit (Hall 1999) by leaving default settings, and then the alignments were inspected visually and adjusted if gaps were detected. The software jModelTest 2.1.7 (Darriba et al. 2012) of the Cyber Infrastructure for the Phylogenetic Research project (CIPRES; www.phylo.org) was used to select the best-fit model of sequence substitution (*COI*: HKY+G) under the corrected Akaike Information Criterion (AICc) (Cavanaugh 1997). Uncorrected and K2P genetic distances (550 replicates) were calculated in MEGA for the major *COI* clades inferred from the

Table 1. List of specimens sequenced in this study, location of the sampling sites, and GenBank accession numbers for COI gene

Sample ID	Species	Site	Latitude	Longitude	Elevation (m asl)	GenBank accession number	
						COI	16S
DiLa_27079	D. lacustris	Reservoir La Serena, Spain	38°54'43.17"N	5°25'55.74"W	319	OR050432	
DiLa_27080	D. lacustris	Reservoir La Serena, Spain	38°54'43.17"N	5°25'55.74"W	319	OR050433	OR039415
DiLa_27081	D. lacustris	Reservoir La Serena, Spain	38°54'43.17"N	5°25'55.74"W	319	OR050434	OR039416
DiLa_27082	D. lacustris	Reservoir La Serena, Spain	38°54'43.17"N	5°25'55.74"W	319	OR050435	OR039417
DiMa_25980	D. macedonicum	Lake Ohrid, Northern Macedonia	41°05'41.29"N	20°47'14.89"E	690	OR050417	
DiMa_25981	D. macedonicum	Lake Ohrid, Northern Macedonia	41°05'41.29"N	20°47'14.89"E	690	OR050418	
DiMa_25982	D. macedonicum	Lake Ohrid, Northern Macedonia	41°05'41.29"N	20°47'14.89"E	690	OR050419	OR039407
Dima_25985	D. macedonicum	Lake Ohrid, Northern Macedonia	41°05'41.29"N	20°47'14.89"E	690	OR050422	OR039408
DiMa_25983	D. macedonicum	Lake Ohrid, Northern Macedonia	41°05'41.29"N	20°47'14.89"E	690	OR050420	
DiMa_25984	D. macedonicum	Lake Ohrid, Northern Macedonia	41°05'41.29"N	20°47'14.89"E	690	OR050421	
DiMa_26646	D. macedonicum	Lake Prespa, Northern Macedonia	40°52'26.91"N	20°58'27.56"E	842	OR050428	OR039412
DiMo_27098	D. mongolianum	Lake Vegoritida, Greece	40°44'31.75"N	21°48'22.93"E	510	OR050436	OR039418
DiMo 27099	D. mongolianum	Lake Vegoritida, Greece	40°44'31.75"N	21°48'22.93"E	510	OR050455	OR039419
DiSp_26589	Diaphanosoma sp.	Kastoria, Greece	40°30'39.52"N	21'18'53.07"E	624	OR050423	
DiSp 26590	Diaphanosoma sp.	Kastoria, Greece	40°30'39.52"N	21'18'53.07"E	624	OR050424	
DiSp_26591	Diaphanosoma sp.	Lake Vegoritida, Greece	40°44'31.75"N	21°48'22.93"E	510	OR050425	OR039409
DiSp 26592	Diaphanosoma sp.	Lake Dojran, Northern Macedonia	41°12'36.78"N	22°44'40.65"E	140	OR050426	OR039410
DiSp_26648	Diaphanosoma sp.	Lake Kastoria, Greece	40°30'39.52"N	21'18'53.07"E	624	OR050429	OR039413
DiSp_26649	Diaphanosoma sp.	Lake Kastoria, Greece	40°30'39.52"N	21'18'53.07"E	624	OR050430	OR039414
DiSp_26650	Diaphanosoma sp.	Lake Vegoritida, Greece	40°44'31.75"N	21°48'22.93"E	510	OR050431	
DiSp_27125	D. mongolianum	Albufera de Valencia, Spain	39°20'11.41"N	0°21'03.91"W	0	OR050437	OR039420
DiSp_27126	D. mongolianum	Albufera de Valencia, Spain	39°20'11.41"N	0°21'03.91"W	0	OR050438	OR039421
DiSp_27127	D. mongolianum	Albufera de Valencia, Spain	39°20'11.41"N	0°21'03.91"W	0	OR050439	OR039422
DiSp_E132	Diaphanosoma sp.	Reservoir La Serena, Spain	38°50'30.27"N	5°06'20.40"W	350	OR050454	
DiSp_E187	Diaphanosoma sp.	Reservoir Sotonera, Spain	42°07'35.62"N	0°38'22.75"W	428	OR050440	
DiSp_E188	Diaphanosoma sp.	Reservoir Sotonera, Spain	42°07'35.62"N	0°38'22.75"W	428	OR050441	
DiMo_E192	D. mongolianum	Reservoir Moneva, Spain	41°11'00.81"N	0°49'29.41"W	632	OR050442	
DiMo_E193	D. mongolianum	Reservoir Moneva, Spain	41°11'00.81"N	0°49'29.41"W	632	OR050443	
DiMo_E194	Diaphanosoma sp.	Reservoir Moneva, Spain	41°11'00.81"N	0°49'29.41"W	632	OR050444	
DiBra_E201	D. brachyurum	Reservoir Ebro, Spain	42°58'27.69"N	4°01'11.67"W	835	OR050445	DQ470593
DiBra_E202	D. brachyurum	Reservoir Ebro, Spain	42°58'27.69"N	4°01'11.67"W	835	OR050446	
DiBra_E203	D. brachyurum	Reservoir Ebro, Spain	42°58'27.69"N	4°01'11.67"W	835	OR050447	
DiSp_E204	Diaphanosoma sp.	Reservoir Vicarias, Spain	41°22'24.25"N	2°11'01.19"W	805	OR050448	
DiSp_E206	Diaphanosoma sp.	Reservoir Vicarias, Spain	41°22'24.25"N	2°11'01.19"W	805	OR050449	
DiSp_E217	Diaphanosoma sp.	Reservoir Mequinenza, Spain	41°22'58.55"N	0°07'29.64"E	153	OR050450	
DiSp_E218	Diaphanosoma sp.	Reservoir Mequinenza, Spain	41°22'58.55"N	0°07'29.64"E	153	OR050451	
DiSp_E222	Diaphanosoma sp.	Reservoir Ortigosa, Spain	42°11'12.53"N	2°40'35.03"W	1013	OR050452	
DiSp_E223	Diaphanosoma sp.	Reservoir Ortigosa, Spain	42°11'12.53"N	2°40'35.03"W	1013	OR050453	
SiCry_26602	Sida crystallina	Lake Ohrid, Northern Macedonia	41°05'41.29"N	20°47'14.89"E	690	OR050427	OR039411

phylogenetic analyses.

Phylogenetic analyses were performed on the COI dataset using the Maximum likelihood (ML) and Bayesian Inference (BI) methods. ML analysis was conducted using the web server offering RAxML-NG available at https://raxml-ng.vital-it.ch/#/ by applying the HKY+G model. Bayesian phylogenetic analyses were performed in MRBAYES 3.2.2 (Ronquist et al. 2012) with the substitution models selected by jModelTest. The BI analysis was conducted using Markov chain Monte Carlo (MCMC) sampling in MrBayes (Ronquist et al. 2012) under the HKY+G substitution model. Markov chain Monte Carlo (MCMC) methods used random starting trees and employed four independent runs, each with one cold chain and three incrementally heated chains. Trees were sampled every 100 generations for one million generations, and the first 25% of all the trees sampled before convergence were discarded as burn-in. The 50% majority-rule consensus tree was generated from the remaining trees. Node support of the inferred trees was evaluated by bootstrapping for ML and by Bayesian posterior probabilities (BPPs) for BI. Species clusters and supports were visualized in FIGTREE 1.4.3 (Rambaut 2010).

Given the restricted phylogenetic information provided for *COI* within the *D. mongolianum* group and in order to clarify the position of the *D. macedonicum* clade, 16S sequences were added to *COI* sequences (Table 1) and two datasets were compiled (one per gene partition). The 16S fragments were automatically aligned using the MAFFT algorithm (Katoh 2002) with default options. Concatenated *COI* plus 16S alignments were performed using CONCATENATOR (Pina-Martins and Paulo 2008). The Sididae species *Sida crystallina* (Table 1) and *Penilia avirostris* (Accession numbers *COI*: KT208814 and 16S: DQ470595) were included as suitable outgroup in phylogenetic tree reconstruction based on concatenated data. jModelTest was run again and the phylogenetic analysis was performed

Table 2. List of *COI* sequences downloaded from GenBank, indicating the species name, the phylogenetic position in our *COI* tree, the region of collection and the accession number. Sequences in the table were downloaded from the following publications: (Alexiou et al. 2021; Elías-Gutiérrez et al. 2008; Lakatos et al. 2015; Liu et al. 2018; Prosser et al. 2013; Richter et al. 2007)

Species name in GenBank	Phylogenetic group	Site	GenBank accession number	Publication	
Diaphanosoma heberti					
Diaphanosoma cf. heberti	D. heberti	Mexico	KC617624	Prosser et al. (2013)	
Diaphanosoma cf. heberti	D. heberti	Mexico	KC617623	Prosser et al. (2013)	
Diaphanosoma cf. heberti	D. heberti	Mexico	KC617622	Prosser et al. (2013)	
Diaphanosoma cf. heberti	D. heberti	Mexico	KC617621	Prosser et al. (2013)	
Diaphanosoma cf. heberti	D. heberti	Mexico	KC617620	Prosser et al. (2013)	
Diaphanosoma cf. heberti	D. heberti	Mexico	KC617619	Prosser et al. (2013)	
Diaphanosoma cf. heberti	D. heberti	Mexico	KC617618	Prosser et al. (2013)	
Diaphanosoma cf. heberti	D. heberti	Mexico	KC617617	Prosser et al. (2013)	
Diaphanosoma cf. heberti	D. heberti	USA	HM884025	Direct Submission	
Diaphanosoma cf. heberti	D. heberti	USA	HM884019	Direct Submission	
Diaphanosoma cf. heberti	D. heberti	USA	HM884018	Direct Submission	
Diaphanosoma cf. heberti	D. heberti	Mexico	EU702164	Elías-Gutiérrez et al. (2008)	
Diaphanosoma cf. heberti	D. heberti	Mexico	EU702163	Elías-Gutiérrez et al. (2008)	
Diaphanosoma cf. heberti	D. heberti	Mexico	EU702162	Elías-Gutiérrez et al. (2008)	
Diaphanosoma cf. heberti	D. heberti	Mexico	EU702161	Elías-Gutiérrez et al. (2008)	
Diaphanosoma cf. heberti	D. heberti	Mexico	EU702160	Elías-Gutiérrez et al. (2008)	
Diaphanosoma cf. heberti	D. heberti	Mexico	EU702159	Elías-Gutiérrez et al. (2008)	
Diaphanosoma cf. heberti	D. heberti	Mexico	EU702158	Elías-Gutiérrez et al. (2008)	
Diaphanosoma cf. heberti	D. heberti	Mexico	EU702157	Elías-Gutiérrez et al. (2008)	
Diaphanosoma cf. heberti	D. heberti	Mexico	EU702156	Elías-Gutiérrez et al. (2008)	
Diaphanosoma cf. heberti	D. heberti	Mexico	EU702155	Elías-Gutiérrez et al. (2008)	
Diaphanosoma cf. heberti	D. heberti	Mexico	EU702154	Elías-Gutiérrez et al. (2008)	
Diaphanosoma cf. heberti	D. heberti	Mexico	EU702153	Elías-Gutiérrez et al. (2008)	
Diaphanosoma cf. heberti	D. heberti	Mexico	EU702152	Elías-Gutiérrez et al. (2008)	
Diaphanosoma cf. heberti	D. heberti	Mexico	EU702151	Elías-Gutiérrez et al. (2008)	
Diaphanosoma cf. heberti	D. heberti	Mexico	EU702150	Elías-Gutiérrez et al. (2008)	

Table 2. (Continued)

Species name in GenBank	Phylogenetic group	Site	GenBank accession number	Publication
Diaphanosoma brevireme				
Diaphanosoma brevireme	D. brevireme	Mexico	KC617616	Prosser et al. (2013)
Diaphanosoma brevireme	D. brevireme	Mexico	KC617615	Prosser et al. (2013)
Diaphanosoma brevireme	D. brevireme	Mexico	KC616986	Prosser et al. (2013)
Diaphanosoma brevireme	D. brevireme	Mexico	KC616984	Prosser et al. (2013)
Diaphanosoma brevireme	D. brevireme	Mexico	KC616983	Prosser et al. (2013)
Diaphanosoma brevireme	D. brevireme	Brazil	KY659309	Domingos et al. (2017)
Diaphanosoma brevireme	D. brevireme	Mexico	EU702149	Elías-Gutiérrez et al. (2008)
Diaphanosoma brevireme	D. brevireme	Mexico	EU702148	Elías-Gutiérrez et al. (2008)
Diaphanosoma brevireme	D. brevireme	Mexico	EU702147	Elías-Gutiérrez et al. (2008)
Diaphanosoma brevireme	D. brevireme	Mexico	EU702146	Elías-Gutiérrez et al. (2008)
Diaphanosoma brevireme	D. brevireme	Mexico	EU702145	Elías-Gutiérrez et al. (2008)
Diaphanosoma spinulosum				
Diaphanosoma spinulosum	D. spinulosum	Mexico	KC617336	Prosser et al. (2013)
Diaphanosoma spinulosum	D. spinulosum	Mexico	KC617335	Prosser et al. (2013)
Diaphanosoma spinulosum	D. spinulosum	Mexico	KC617334	Prosser et al. (2013)
Diaphanosoma spinulosum	D. spinulosum	Mexico	KC617333	Prosser et al. (2013)
Diaphanosoma spinulosum	D. spinulosum	Mexico	KC617332	Prosser et al. (2013)
Diaphanosoma spinulosum	D. spinulosum	Mexico	KC617331	Prosser et al. (2013)
Diaphanosoma spinulosum	D. spinulosum	Mexico	KC617330	Prosser et al. (2013)
Diaphanosoma orientalis				
Diaphanosoma orientalis	D. orientalis	Japan	LC060059	Lakatos et al. (2015)
Diaphanosoma orientalis	D. orientalis	Japan	LC060058	Lakatos et al. (2015)
Diaphanosoma orientalis	D. orientalis	Japan	LC060057	Lakatos et al. (2015)
Diaphanosoma macrophthalma				
Diaphanosoma cf. macrophthalma	D. orientalis	Japan	LC060056	Lakatos et al. (2015)
Diaphanosoma cf. macrophthalma	D. macrophthalma	Japan	LC060055	Lakatos et al. (2015)
Diaphanosoma cf. macrophthalma	D. macrophthalma	Japan	LC060054	Lakatos et al. (2015)
Diaphanosoma cf. macrophthalma	D. macrophthalma	Japan	LC060053	Lakatos et al. (2015)
Diaphanosoma macrophthalma	D. macrophthalma	China	KU720105	Direct Submission
Diaphanosoma dubium				
Diaphanosoma cf. dubium	D. dubium	Japan	LC060052	Lakatos et al. (2015)
Diaphanosoma cf. dubium	D. dubium	Japan	LC060051	Lakatos et al. (2015)
Diaphanosoma cf. dubium	D. dubium	Japan	LC060050	Lakatos et al. (2015)
Diaphanosoma cf. dubium	D. dubium	Japan	LC060049	Lakatos et al. (2015)
Diaphanosoma cf. dubium	D. dubium	Japan	LC060048	Lakatos et al. (2015)
Diaphanosoma dubium	D. dubium	China	KY788978	Liu et al. (2017)
Diaphanosoma dubium	D. dubium	China	KY788977	Liu et al. (2017)
Diaphanosoma dubium	D. dubium	China	KY788974	Liu et al. (2017)
Diaphanosoma dubium	D. dubium	China	KY788973	Liu et al. (2017)
Diaphanosoma dubium	D. dubium	China	KY788947	Liu et al. (2017)
Diaphanosoma dubium	D. dubium	China	KY788946	Liu et al. (2017)
Diaphanosoma dubium	D. dubium	China	KY788945	Liu et al. (2017)
Diaphanosoma dubium	D. dubium	China	KY788944	Liu et al. (2017)
Diaphanosoma dubium	D. dubium	China	KY788941	Liu et al. (2017)
Diaphanosoma dubium	D. dubium	China	KY788940	Liu et al. (2017)
Diaphanosoma dubium	D. dubium	China	KY788939	Liu et al. (2017)
Diaphanosoma dubium	D. dubium	China	KY788938	Liu et al. (2017)
Diaphanosoma dubium	D. dubium	China	KY788933	Liu et al. (2017)
Diaphanosoma dubium	D. dubium	China	KY788932	Liu et al. (2017)
Diaphanosoma dubium	D. dubium	China	KY788931	Liu et al. (2017)
Diaphanosoma dubium	D. dubium	China	KY788930	Liu et al. (2017)
Diaphanosoma dubium	D. dubium	China	KU720106	Direct Submission
Diaphanosoma dubium	D. dubium	Taiwan	AB549201	Direct Submission

Table 2. (Continued)

Species name in GenBank	Phylogenetic group	Site	GenBank accession number	Publication
Diaphanosoma amurensis				
Diaphanosoma cf. amurensis	D. amurensis	Japan	LC060047	Lakatos et al. (2015)
Diaphanosoma cf. amurensis	D. amurensis	Japan	LC060046	Lakatos et al. (2015)
Diaphanosoma cf. amurensis	D. amurensis	Japan	LC060045	Lakatos et al. (2015)
Diaphanosoma cf. amurensis	D. amurensis	Japan	LC060044	Lakatos et al. (2015)
Diaphanosoma cf. amurensis	D. amurensis	Japan	LC060043	Lakatos et al. (2015)
Diaphanosoma birgei				
Diaphanosoma cf. birgei	D. heberti	Brazil	KU315486	Direct Submission
Diaphanosoma birgei	D. birgei	Mexico	EU702144	Elías-Gutiérrez et al. (2008)
Diaphanosoma birgei	D. birgei	Mexico	EU702143	Elías-Gutiérrez et al. (2008)
Diaphanosoma birgei	D. birgei	Mexico	EU702142	Elías-Gutiérrez et al. (2008)
Diaphanosoma birgei	D. birgei	Canada	EU702141	Elías-Gutiérrez et al. (2008)
Diaphanosoma excisum				
Diaphanosoma excisum	D. excisum	China	KY788972	Liu et al. (2017)
Diaphanosoma excisum	D. excisum	China	KY788971	Liu et al. (2017)
Diaphanosoma excisum	D. excisum	China	KY788970	Liu et al. (2017)
Diaphanosoma excisum	D. excisum	China	KY788969	Liu et al. (2017)
Diaphanosoma excisum	D. excisum	China	KY788968	Liu et al. (2017)
Diaphanosoma excisum	D. excisum	China	KY788967	Liu et al. (2017)
Diaphanosoma excisum	D. excisum	China	KY788966	Liu et al. (2017)
Diaphanosoma excisum	D. excisum	China	KY788965	Liu et al. (2017)
Diaphanosoma excisum	D. excisum	China	KY788964	Liu et al. (2017)
Diaphanosoma excisum	D. excisum	China	KY788963	Liu et al. (2017)
Diaphanosoma excisum	D. excisum	China	KY788962	Liu et al. (2017)
Diaphanosoma excisum	D. excisum	China	KY788961	Liu et al. (2017)
Diaphanosoma excisum	D. excisum	China	KY /88960	Liu et al. (2017)
Diaphanosoma excisum	D. excisum	China	KY /88959	Liu et al. (2017)
Diaphanosoma excisum	D. excisum	China	KY /88958	Liu et al. (2017)
Diaphanosoma excisum	D. excisum	China	KY /8895/	Liu et al. (2017)
Diaphanosoma excisum	D. excisum	China	K I /88930	Liu et al. (2017)
Diaphanosoma excisum	D. excisum	China	K I /00933	Liu et al. (2017)
Diaphanosoma excisum	D. excisum	China	K 1 / 88934	Liu et al. (2017)
Diaphanosoma excisum	D. excisum	China	K 1 / 88955 K V 788052	Liu et al. (2017)
Diaphanosoma excisum	D. excisum	China	KV788951	Lin et al. (2017)
Diaphanosoma excisum	D. excisum	China	KV788950	Lin et al. (2017)
Diaphanosoma excisum	D. excisum	China	KV788949	Liu et al. (2017)
Diaphanosoma excisum	D. excisum	China	KY788948	Liu et al. (2017)
Diaphanosoma excisum	D. excisum	China	KY788923	Lin et al. (2017)
Diaphanosoma orghidani	Dienensum		111,00720	2
Diaphanosoma cf. orghidani	D. macrophthalma	China	KY788942	Liu et al. (2017)
Diaphanosoma cf. orghidani	D. orghidani	China	KY788883	Liu et al. (2017)
Diaphanosoma cf. orghidani	D. orghidani	China	KY788870	Liu et al. (2017)
Diaphanosoma cf. orghidani	D. orghidani	China	KY788869	Liu et al. (2017)
Diaphanosoma cf. orghidani	D. orghidani	China	KY788868	Liu et al. (2017)
Diaphanosoma cf. orghidani	D. orghidani	China	KY788867	Liu et al. (2017)
Diaphanosoma cf. orghidani	D. orghidani	China	KY788866	Liu et al. (2017)
Diaphanosoma cf. orghidani	D. orghidani	China	KY788865	Liu et al. (2017)
Diaphanosoma cf. orghidani	D. orghidani	China	KY788852	Liu et al. (2017)
Diaphanosoma cf. orghidani	D. orghidani	China	KY788851	Liu et al. (2017)
Diaphanosoma cf orghidani	D. macrophthalma	China	KY788841	Liu et al. (2017)
Diaphanosoma cf. orghidani	D. macrophthalma	China	KY788840	Liu et al. (2017)
Diaphanosoma cf. orghidani	D. macrophthalma	China	KY788839	Liu et al. (2017)
Diaphanosoma orghidani	D. orghidani	China	KY788829	Liu et al. (2017)
Diaphanosoma orghidani	D. orghidani	China	KY788828	Liu et al. (2017)

Table 2. (Continued)

Species name in GenBank	Phylogenetic group Site		GenBank accession number	Publication	
Diaphanosoma orghidani	D. orghidani	China	KY788827	Liu et al. (2017)	
Diaphanosoma orghidani	D. orghidani	China	KY788786	Liu et al. (2017)	
Diaphanosoma orghidani	D. orghidani	China	KY788785	Liu et al. (2017)	
Diaphanosoma orghidani	D. orghidani	China	KY788784	Liu et al. (2017)	
Diaphanosoma orghidani	D. orghidani	China	KY788783	Liu et al. (2017)	
Diaphanosoma orghidani	D. orghidani	China	KY788782	Liu et al. (2017)	
Diaphanosoma orghidani	D. orghidani	China	KY788781	Liu et al. (2017)	
Diaphanosoma orghidani	D. orghidani	Greece	MW259043	Alexiou et al. (2021)	
Diaphanosoma orghidani	D. orghidani	Greece	MW259042	Alexiou et al. (2021)	
Diaphanosoma orghidani	D. orghidani	Greece	MW259038	Alexiou et al. (2021)	
Diaphanosoma orghidani	D. orghidani	Greece	MW259011	Alexiou et al. (2021)	
Diaphanosoma mongolianum					
Diaphanosoma mongolianum	D. mongolianum	China	KY788836	Liu et al. (2017)	
Diaphanosoma mongolianum	D mongolianum	China	KY788835	Liu et al. (2017)	
Diaphanosoma mongolianum	D mongolianum	China	KY788834	Lin et al. (2017)	
Diaphanosoma mongolianum	D. mongolianum	China	KV788837	Liu et al. (2017)	
Diaphanosoma mongolianum	D. mongolianum	Greece	MW259036	Alexion et al. (2017)	
Diaphanosoma mongolianum	D. mongolianum	Greece	MW259035	Alexiou et al. (2021)	
Diaphanosoma mongolianum	D. mongolianum	Greece	MW259034	Alaxiou et al. (2021)	
Diaphanosoma mongolianum	D. mongolianum	Greece	MW259034	Alexiou et al. (2021)	
Diaphanosoma mongolianum	D. mongolianum	Greece	MW259033	Alexiou et al. (2021)	
Diaphanosoma mongolianum	D. mongolianum	Greece	MW259032	Alexiou et al. (2021)	
Diapnanosoma mongolianum	D. mongolianum	Greece	MW259031	Alexiou et al. (2021)	
Diaphanosoma mongolianum	D. mongolianum	Greece	MW259030	Alexiou et al. (2021)	
Diaphanosoma mongolianum	D. mongolianum	Greece	MW259025	Alexiou et al. (2021)	
Diaphanosoma mongolianum	D. mongolianum	Greece	MW259027	Alexiou et al. (2021)	
Diaphanosoma mongolianum	D. mongolianum	Greece	MW259026	Alexiou et al. (2021)	
Diaphanosoma mongolianum	D. mongolianum	Greece	MW259025	Alexiou et al. (2021)	
Diaphanosoma mongolianum	D. mongolianum	Greece	MW259023	Alexiou et al. (2021)	
Diaphanosoma mongolianum	D. mongolianum	Greece	MW259022	Alexiou et al. (2021)	
Diaphanosoma mongolianum	D. mongolianum	Greece	MW259021	Alexiou et al. (2021)	
Diaphanosoma mongolianum	D. mongolianum	Greece	MW259020	Alexiou et al. (2021)	
Diaphanosoma mongolianum	D. mongolianum	Greece	MW259019	Alexiou et al. (2021)	
Diaphanosoma mongolianum	D. mongolianum	Greece	MW259018	Alexiou et al. (2021)	
Diaphanosoma mongolianum	D. mongolianum	Greece	MW259017	Alexiou et al. (2021)	
Diaphanosoma mongolianum	D. mongolianum	Greece	MW259016	Alexiou et al. (2021)	
Diaphanosoma mongolianum	D. mongolianum	Greece	MW259015	Alexiou et al. (2021)	
Diaphanosoma mongolianum	D. mongolianum	Greece	MW259014	Alexiou et al. (2021)	
Diaphanosoma mongolianum	D. mongolianum	Greece	MW259013	Alexiou et al. (2021)	
Diaphanosoma mongolianum	D. mongolianum	Greece	MW259012	Alexiou et al. (2021)	
Diaphanosoma mongolianum	D. mongolianum	Greece	MW259010	Alexiou et al. (2021)	
Diaphanosoma brachyurum					
Diaphanosoma brachyurum	D. brachyurum	China	KY788792	Liu et al. (2017)	
Diaphanosoma brachyurum	D. brachyurum	China	KY788791	Liu et al. (2017)	
Diaphanosoma brachyurum	D. brachyurum	Italy	MH321371	Unpublished	
Diaphanosoma brachyurum	D. brachyurum	Italy	MH321370	Direct Submission	
Diaphanosoma brachyurum	D. brachyurum	Italy	MH321369	Direct Submission	
Diaphanosoma brachyurum	D. brachyurum	Italy	MH321368	Direct Submission	
Diaphanosoma brachyurum	D. brachyurum	Italy	MH321367	Direct Submission	
Diaphanosoma brachyurum	D. brachyurum	Italy	MH321366	Direct Submission	
Diaphanosoma brachvurum	D. brachyurum	Italy	MH321365	Direct Submission	
Diaphanosoma brachvurum	D. brachvurum	Italv	MH321364	Direct Submission	
Diaphanosoma brachvurum	D. brachvurum	Germanv	EF189666	Richter et al. (2007)	
Diaphanosoma macedonicum		<i>j</i>		()	
Diaphanosoma macedonicum	D. macedonicum	Greece	MW259047	Alexiou et al. (2021)	
Dianhanosoma macedonicum	D macedonicum	Greece	MW259046	Alexiou et al. (2021)	
Diaphanosoma macedonicum	D macedonicum	Greece	MW259045	Alexion et al. (2021)	
	D. maccuonicum		11111237043	/ fiexiou et al. (2021)	

as indicated before, but now on the combined dataset. The best-fit model of sequence substitution (TIM1 + G) under the AICc was selected for the rest of the phylogenetic analysis performed using the concatenated matrix, repeating the same procedure as indicated before for the *COI* dataset. Alignments for the *COI* and concatenated datasets are provided in supplementary data 1 and 2.

RESULTS

Morphological comparison

Morphologically, the specimens of *D.* macedonicum found in Lake Ohrid were quite similar to those from the two previously investigated lakes, Prespa and Dojran (see Korovchinsky and Petkovski 2014), differing only in minor features (Fig. 2). The former were smaller, and their integument lacked reinforced chitinization; denticles of posteroventral margin were on average more abundant. Morphological characteristics (body length, head length, diameter of the eye, swimming antennae length, and upper antennal length) of the specimens from Lake Ohrid were closer to those of Lake Prespa than to the specimens of Lake Dojran (Fig. 2).

Phylogenetic and coalescence analysis

Specimens retrieved from the Iberian and Balkan Peninsulas cover all the *Diaphanosoma* spp. cited in Europe. *D. lacustris*, *D. mongolianum*, *D. brachyurum* and *D. orghidani* were detected in the Iberian Peninsula, while *D. orghidani*, *D. macedonicum* and *D. mongolianum* were found in the Balkan Peninsula.

Sequences of *Diaphanosoma* specimens recovered from the ancient lakes Ohrid and Prespa were grouped together with the only available sequences of *D. macedonicum* from Mikri Prespa (Alexiou et al. 2021). Molecular data from these three locations together formed the *D. macedonicum* clade in our phylogenetic tree (Fig. 3). In Lake Dojran, although cited in the scientific literature (Alexiou et al. 2021; Korovchinsky and Petkovski 2014), we have not found specimens of *D. macedonicum* but instead of *D. mongolianum*. Mean ingroup K2P genetic distances for *D. macedonicum* are 2.36 \pm 0.31%, while the K2P distances between the closest groups, *i.e.*, *D. lacustris* and *D. mongolianum*, reached 6.9 \pm 0.11% and 8.4 \pm 0.15%, respectively (Table 3).

The phylogenetic analysis presented in figure 3 showed that *D. macedonicum*, together with *D. lacustris* and *D. mongolianum*, is part of the mongolianum

group. The term mongolianum group is used hereinafter to describe the assemblage formed by three species: D. lacustris, D. mongolianum, and D. macedonicum. However, the phylogenetic position of D. macedonicum within this group was not well resolved and/or denotes discrepancies between the Bayesian and maximum likelihood methods when using only COI markers (Fig. 3). These differences vanished when using the concatenated dataset, as shown in figure 4. Here, there are no discrepancies between the two methods, and the level of support is higher in both the Bayesian (MrBayes posterior probability; pp = 0.99 for *D. macedonicum*; pp = 1.00 for the mongolianum group) and maximum likelihood methods (ML bootstrap values, bs = 86% for D. macedonicum and bs = 99% for the mongolianum group). The distinction between the species of interest and others within the genus is further corroborated by the genetic distances presented in table 3.

DISCUSSION

Identification and phylogenetic position

The molecular results presented here indicate that the Diaphanosoma collected in the ancient lakes Ohrid and Prespa belong to the endemic species of the Balkan peninsula, D. macedonicum. This is confirmed by short genetic distances (COI: K2P < 3%) in comparison to the only available sequences of this taxon (Alexiou et al. 2021) and by the larger genetic differentiation (COI: K2P > 6%) with sister groups, such as D. lacustris and D. mongolianum. These small K2P distances are expected for the genus since Dumont et al. (2021) also found relatively small intraspecific genetic distances, mostly less than 2%, within temperate climate species of Diaphanosoma. Besides the identity of Diaphanosoma in Ohrid and Prespa, our phylogenetic analysis (Figs. 3, 4) supported previous morphological evidence (Korovchinsky and Petkovski 2014) that considered D. macedonicum as a member of the D. mongolianum species group, comprising three sibling species (D. mongolianum, D. lacustris, and D. macedonicum).

Molecular results indicate that *D. macedonicum* is restricted to the pelagic zones of lakes Ohrid, Prespa, and Mikri Prespa (Fig. 4). The sampled populations in lower-altitude lakes in the Balkans, including Lake Dojran, belong to *D. mongolianum* (Fig. 3). However, *D. macedonicum* was reported from Lake Dojran (Alexiou et al. 2021; Korovchinsky and Petkovski 2014) based on morphological identification. Figure 2 shows morphological differences (body length, head length, diameter of the eye, swimming antennae length and upper antennal length) between the Ohrid,



Fig. 2. Measurements of *Diaphanosoma* populations in the Balkan Peninsula (Lakes Ohrid, Prespa, and Dojran). Measurements of specimens from Lake Ohrid were retrieved from Korovchinsky (2022) from specimens collected in this study, while measurements from Lake Prespa and Lake Dojran were retrieved from Korovchinsky and Petkovski (2014).

Prespa and the Dojran specimens. In most cases, Ohrid and Prespa characteristics overlapped, while Dojran characteristics differed in some way (e.g., see head height vs. body length in Fig. 2). This could be ascribed to phenotypic plasticity within the species under different environmental conditions (e.g., Tollrian and Leese 2010), but requires confirmation by molecular data. The presence of several Diaphanosoma species in the same water body should also be considered as an explanatory cause for this apparently contradictory data. On the other hand, Diaphanosoma spp. are challenging to identify due to their small size, high degree of polymorphism, and conservative outer morphology. Morphological assignments are based on details of internal anatomy and complex limb arrangements that require high dissection ability and level of expertise. These factors, together with the presence of cryptic taxa, have led to incorrect morphological identification

of many zooplankton species (*e.g.*, Alonso et al. 2021; Sinev and López-Blanco 2018) and justify the use of molecular tools. For instance, *D. brachyurum* was traditionally listed in many Greek lakes but later assigned to different *Diaphanosoma* spp. (Alexiou et al. 2021; Koussouris et al. 1991; Moustaka-Gouni et al. 2006).

Origin and distribution

Regarding the distribution, molecular data suggest that populations of the three described species in the Balkans, *D. orghidani*, *D. macedonicum* and *D. mongolianum*, are geographically separated in two principal groups of lakes (Fig. 4): (i) the Dessaretes lake group (Ohrid, Prespa, Mikri Prespa, and Maliq), belonging to the Adriatic Sea basin, and (ii) the Aegean lake group (Kastoria, Vegoritida, Dojran, Volvi, Kerkini

Table 3. Genetic distances (%) of the main clades based on the *COI* dataset. Mean estimated Kimura 2-parameter (K2P) distances are shown below the diagonal and the standard deviation (SD) is shown above the diagonal

	D. lacustris	D. macedonicum	D. mongolianum	D. orghidani	D. brachyurum	D. heberti	D. brevireme
D. lacustris		1.10	0.92	2.28	2.35	2.08	2.74
D. macedonicum	6.90		1.15	2.32	2.41	2.12	2.69
D. mongolianum	5.55	8.42		2.39	2.44	2.10	2.74
D. orghidani	23.73	24.48	25.59		2.45	2.22	2.46
D. brachyurum	21.77	23.42	24.31	24.97		1.91	2.94
D. heberti	20.69	21.22	21.70	22.00	18.02		2.69
D. brevireme	31.63	31.00	31.02	27.08	32.00	32.11	
D. spinulosum	31.16	30.41	32.93	27.15	31.06	31.29	19.29
D. orientalis	25.75	26.32	26.19	16.40	24.53	24.89	30.37
D. macrophtalma	26.04	28.07	27.21	18.99	23.59	22.52	29.97
D. dubium	24.23	24.18	25.82	21.14	22.42	21.26	29.67
D. amurensis	23.08	24.51	24.19	22.65	20.39	19.26	29.90
D. birgei	23.87	23.11	24.45	25.48	20.67	16.57	30.61
D. excisum	30.08	29.30	30.15	25.45	30.93	27.69	20.19

	D. spinulosum	D. orientalis	D. macrophthalma	D. dubium	D. amurensis	D. birgei	D. excisum
D. lacustris	2.94	2.20	2.33	2.38	2.29	2.37	2.56
D. macedonicum	2.88	2.24	2.53	2.31	2.37	2.30	2.45
D. mongolianum	2.97	2.18	2.39	2.46	2.36	2.38	2.51
D. orghidani	2.58	1.60	2.05	2.15	2.18	2.42	2.30
D. brachyurum	2.95	2.19	2.20	2.27	2.09	2.08	2.76
D. heberti	2.96	2.20	2.10	2.06	2.02	1.76	2.63
D. brevireme	2.04	2.48	2.69	2.62	2.73	2.74	1.91
D. spinulosum		2.59	2.84	2.87	3.05	2.72	2.27
D. orientalis	30.58		1.84	2.38	2.00	2.35	2.65
D. macrophthalma	30.45	19.42		2.38	2.25	2.27	2.82
D. dubium	30.72	27.84	26.30		2.33	2.38	2.53
D. amurensis	32.17	22.43	23.63	24.65		2.34	2.65
D. birgei	28.65	27.62	25.17	24.95	23.15		2.51
D. excisum	23.72	31.79	32.16	28.67	30.25	28.87	



Fig. 3. Bayesian tree of the *COI* data showing the phylogenetic relationship among *Diaphanosoma* spp. in the world. Numbers next to each node posterior probabilities and bootstrap support values, respectively. *denotes the discrepancy between Bayesian and maximum likelihood methods.



Fig. 4. Top panel (A): Bayesian inference based on concatenated *COI* and 16S sequences. Bayesian posterior probabilities (top) and bootstrap support values (bottom) are provided next to each node. The scale bar indicates substitutions per site according to the applied models of sequence evolution. Letters after the species name indicate the geographical location of the sequences used in our analyses (GR: Greece; MC: Northern Macedonia; SP: Spain). Bottom panel (B): Map of the distribution of *Diaphanosoma* spp. in water bodies in the Balkan Peninsula based on recent morphological and molecular studies (Alexiou et al. 2021; Korovchinsky 2022; Korovchinsky and Petkovski 2014; this study). Each species is designated by one color in the phylogeny and the map to aid interpretation.

and Paralimni), situated eastward and draining into the Aegean Sea (Šapkarev 1970). DNA barcoding data indicate that the endemic D. macedonicum is present in the first group, while other Diaphanosoma spp. with larger geographical distribution inhabit more southeastern lakes (Fig. 4). According to Cvijic (1911), both groups of lakes have always been isolated from each other, which corresponds with the idea of the existence of an endemic species constrained only to ancient lakes. Gradual reproductive isolation in these ancient lakes might explain both the molecular results and the current geographical distribution of D. macedonicum. However, hybridization is known to occur within different species of Diaphanosoma, which can cause mitonuclear discordances in the phylogenies (Liu et al. 2018). Potential mechanisms facilitating the formation of hybrids are not known for *Diaphanosoma*, but they only affected a small proportion (6%) of the analyzed samples by Liu et al. (2018). Although the isolation of ancient lakes is thought to withstand invasion from adjacent water bodies yielding stable environments (Martens 1997), molecular markers are needed to confirm the hypothesis exposed here and to be certain that different species did not interbreed.

Interestingly, Diaphanosoma was considered to have invaded Lake Ohrid in the last few decades (Gušeska et al. 2005 2014; Kostoski et al. 2010; Tasevska et al. 2017). Was it then possible that an ancient endemic species was neglected for so long in limnological surveys? The historical and recent fossil record is concordant with the detection of Diaphanosoma in recent times in Ohrid. The first survey of the pelagic fauna in the great lakes of the Balkan Peninsula (September-October 1801) (Richard 1892) mentioned Daphnella brachyura in lakes Dojran, Ostrovo (currently Vegoritida), Bézik (currently Volvi; Sapkarev 1970) and Vendrok (probably Kastoria nowadays) (Sturany 1894), but Diaphanosoma was not found in Lake Ohrid. In the second survey in 1904 (Georgevitch 1907), conducted between April and May, which is not the optimal period for Diaphanosoma development, this taxon was found in Lake Dojran, Tachinos (at present drained) (Dill 1990), Ajvassil (at present Koronia) (Šapkarev 1970), and Ostrovo, but again it was not recorded in Ohrid. In the fossil record, Diaphanosoma postabdomens were only found in the upper sample (mean age 1975) of a sedimentary record covering the last 6650 years in Lake Ohrid (López-Blanco et al. 2020). However, cladoceran sub-fossils from planktonic species are very soft and labile; therefore, they may not truly represent the real fauna (López-Blanco et al. 2013 2016). Moreover, preserved subfossils of Diaphanosoma did not allow identifications at the species level (López-Blanco et al.

2020). Factors such as the decline of population density over time due to environmental drivers should not be ruled out as explanatory factors, since Diaphanosoma populations have shown to be affected by temperature (Herzig 1984), food (Geller and Müller 1981), competition (Matveev 1991), and/or invertebrate and vertebrate predators (Herzig and Auer 1990; Lunte and Luecke 1990; Keller and Conlon 1994). For instance, long-term surveys in Lake Constance (Germany) showed the disappearance of Diaphanosoma from the zooplankton community from 1961 to 2001 (Stich 2004). Ecological factors that might have triggered changes in Diaphanosoma density in Lake Ohrid, together with a higher sampling effort in the last few decades, might explain why its presence has been unnoticed for such a long time.

CONCLUSIONS

This study provides initial evidence of the distribution and origin of *D. macedonicum* in the Balkan Peninsula, supporting the hypothesis of an ancient endemic taxon. Molecular data indicate that this species inhabits planktonic environments of the ancient lakes Ohrid, Prespa, and Mikri Prespa, which suggests a gradual reproductive isolation within these ecosystems. Our study contributes to a better estimation of the endemic biodiversity in these ancient lakes, which is crucial to guide management and to conduct further research in some of the most unique ecosystems on the planet.

Acknowledgments: We thank Tom Wilke for hosting CLB during her Humboldt postdoctoral fellowship at the JLUG and for providing all the scientific and logistic support for conducting this research. We highly appreciate the collaboration and help of Sasho Trajanovski with the bureaucracy and sampling permits. We gratefully acknowledge the valuable help of Sergei Sereda during CLB molecular training and the invaluable suggestions and scientific inputs of Diana Delicado during the lab work and preparation of this manuscript. We also thank Silvia Nachtigal for her kind assistance in the lab at the JLUG and Maria Sahuquillo for the specimens from La Albufera. This work was funded by the Alexander von Humboldt Foundation under the project "The deep drilling program in ancient Lake Ohrid: Using fossil water flea assemblages to infer drivers of biodiversity and top-down regulators" to CLB (3.3 - 7121 - ESP/1188322). CLB also acknowledges the European Union for her Marie Sklodowska-Curie grant agreement number 892487 under Horizon 2020 funds and the research group RNM-190 of the Junta de Andalucía that supported her while writing this manuscript. Three anonymous reviewers and the editor provided constructive and useful comments on an earlier version of the manuscript.

Authors' contributions: Charo López-Blanco contributed to the research idea, sample collection, laboratory, data analysis and manuscript editing. Ohrideja Tasevska and Goce Kostoski contributed to the sample collection, logistics and permits. Eduardo Vicente contributed to the sample collection. Laura S. Epp and Antonio García-Alix together with all the authors previously mentioned contributed to the manuscript editing.

Competing interests: The authors declare that they have no competing interest.

Availability of data and materials: Sequences generated here were deposited in the publicly available repository GenBank (Accession number OR050417–OR050455 and OR039407–OR039422).

Consent for publication: Not applicable.

Ethics approval to participate: Not applicable.

REFERENCES

- Albrecht C, Wilke T. 2008. Ancient Lake Ohrid: Biodiversity and Evolution. Hydrobiologia 615:103–140. doi:10.1007/s10750-008-9558-y.
- Alexiou R, Stamou G, Minoudi S, Tourli F, Tsartsianidou V, Triantafyllidis A, Michaloudi E. 2021. The genus *Diaphanosoma* (Diplostraca: Sididae) in Greece: morphological and molecular assessment. Zootaxa **5082**:572–582. doi:10.11646/zootaxa.5082. 6.4.
- Alonso M. 1996. Crustacea Branchiopoda. Volumen 7. Fauna Ibérica. Consejo Superior de Investigaciones Científicas, Madrid, Spain.
- Alonso M, Neretina AN, Ventura M. 2021. Ceriodaphnia smirnovi (Crustacea: Cladocera), a new species from the Mediterranean Region, and a phylogenetic analysis of the commonest species. Zootaxa 4974:146. doi:10.11646/zootaxa.4974.1.1.
- Błędzki LA, Rybak JI. 2016. Freshwater crustacean zooplankton of Europe. Cladocera & Copepoda (Calanoida, Cyclopoida) Key to species identification, with notes on ecology, distribution, methods and introduction to data analysis. Springer Cham, Switzerland. doi:10.1007/978-3-319-29871-9.
- Cavanaugh JE. 1997. Unifying the derivations for the Akaike and corrected Akaike information criteria. Stat Probab Lett 33:201– 208. doi:10.1016/s0167-7152(96)00128-9.
- Cohen AS. 2012. Scientific drilling and biological evolution in ancient lakes: Lessons learned and recommendations for the future. Hydrobiologia **682:**3–25. doi:10.1007/s10750-010-0546-7.
- Cristescu ME, Adamowicz SJ, Vaillant JJ, Haffner DG. 2010. Ancient lakes revisited: From the ecology to the genetics of speciation. Mol Ecol 19:4837–4851. doi:10.1111/j.1365-294X.2010.04832.x.

Cvijic J. 1911. Fundamentals of geography and geology of Macedonia

and Old Serbia. Book III, Serbian Academy of Sciences, Special Edition, Beograd. (in Serbian)

- Darriba D, Taboada GL, Doallo R, Posada D. 2012. jModelTest 2: more models, new heuristics and parallel computing. Nature Methods **9:**772. doi:10.1038/nmeth.2109.
- Dill W. 1990. Inland fisheries of Europe. EIFAC Tech Paper 52. Food and Agriculture Organization of the United Nations, Rome, Italy.
- Dumont HJ, Fei BH, Guo FF, Chen H, Cheng D, Liu P, Xu L, Sanoamuang LO, Rietzler AC, Xu S, Vierstraete A, Elías-Gutierrez M. 2021. Toward a phylogeny and biogeography of *Diaphanosoma* (Crustacea: Cladocera). Aquat Ecol 55:1207– 1222. doi:10.1007/s10452-020-09819-0.
- Elías-Gutiérrez M, Martínez Jerónimo F, Ivanova NV, Valdez-Moreno M. 2008. DNA barcodes for Cladocera and Copepoda from Mexico and Guatemala, highlights and new discoveries. Zootaxa 1839:1–42. doi:10.11646/zootaxa.1839.1.1.
- Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. 1994. DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol **3(5)**:294–299. doi:10.1071/ZO9660275.
- Fritz SC, Baker PA, Tapia P, Spanbauer T, Westover K. 2012. Evolution of the Lake Titicaca basin and its diatom flora over the last ~370,000 years. Palaeogeogr Palaeoclimatol Palaeoecol 317–318:93–103. doi:10.1016/j.palaeo.2011.12.013.
- Geller W, Müller H. 1981. The filtration apparatus of Cladocera: Filter mesh-sizes and their implications on food selectivity. Oecologia 49:316–321. doi:10.1007/BF00347591.
- Georgevitch J. 1907. Les organisms du plankton des grands lacs de la peninsula balkanique. Mémoires de la Société de France 20:5–19.
- Gorthner A. 1994. What is an ancient lake? In: Martens K, Goddeeris B, Coulter G (eds) Speciation in ancient lakes. Archiv für Hydrobiologie 44:97–100.
- Gušeska D, Kostoski G, Tasevska O. 2005. Spatial and temporal distribution of Cladocera in Lake Ohrid pelagic zone. Nat Montenegrina **4:**33–40.
- Gušeska D, Tasevska O, Kostoski G, Guseski D. 2014. Zooplankton abundance and diversity in Lake Ohrid, Macedonia. Int J Ecosyst Ecol Sci **4:3**33–340.
- Gušeska D, Tasevska O, Kostoski G, Gušeski D. 2019. Biomass dynamics of pelagic Crustacea in Lake Ohrid, Republic of Macedonia, in the period 2000–2009. Acta Zool Bulg 13:63–67.
- Hall TA. 1999. BioEdit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT. Nucleic Acids Symp Ser **41**:95–98.
- Herzig A. 1984. Temperature and life cycle strategies of *Diaphanosoma* brachyurum: An experimental study on development, growth, and survival. Arch fur Hydrobiol **101**:143–178.
- Herzig A, Auer B. 1990. The feeding behaviour of *Leptodora kindti* and its impact on the zooplankton community of Neusiedler See (Austria). Hydrobiologia **198:**107–117. doi:10.1007/ BF00048627.
- Karabanov E, Williams D, Kuzmin M, Sideleva V, Khursevich G, Prokopenko A, Solotchina E, Tkachenko L, Fedenya S, Kerber E, Gvozdkov A, Khlustov O, Bezrukova E, Letunova P, Krapivina S. 2004. Ecological collapse of Lake Baikal and Lake Hovsgol ecosystems during the Last Glacial and consequences for aquatic species diversity. Palaeogeogr Palaeoclimatol Palaeoecol 209:227–243. doi:10.1016/j.palaeo.2004.02.017.
- Katoh K. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res 30:3059–3066. doi:10.1093/nar/gkf436.
- Keller W, Conlon M. 1994. Crustacean zooplankton communities and lake morphometry in Precambrian shield lakes. Can J Fish Aquat Sci 51:2424–2434. doi:10.1139/f94-242.
- Korovchinsky NM. 2022. Diaphanosoma macedonicum Korovchinsky

et Petkovski, 2014 (Crustacea: Cladocera: Sididae) in Lake Ohrid. Arthropoda Sel **31:**179–182. doi:10.15298/arthsel.31.2.06.

- Korovchinsky NM. 2018. Cladocera: Ctenopoda, Families Sididae, Holopediidae & Pseudopenilidae (Branchiopoda: Cladocera). *In*: Identification guides to the plankton and benthos of inland waters. Backhuys Publ. & Margraf Publ., The Netherlands.
- Korovchinsky NM, Petkovski KT. 2014. The ancient Balkan lakes harbor a new endemic species of *Diaphanosoma* Fischer, 1850 (Crustacea: Branchiopoda: Cladocera). Zootaxa **3784:**539–549. doi:10.11646/zootaxa.3784.5.3.
- Kostoski G, Albrecht C, Trajanovski S, Wilke T. 2010. A freshwater biodiversity hotspot under pressure - Assessing threats and identifying conservation needs for ancient Lake Ohrid. Biogeosciences 7:3999–4015. doi:10.5194/bg-7-3999-2010.
- Kostoski G, Guseska D, Tasevska O. 2004. A Day-Night and seasonal periodicity in the distribution of the zooplankton from Lake Ohrid at Water Observation and Information System for Balkan countries (BALWOIS), Ohrid, Northern Macedonia, 25–29 May 2004.
- Koussouris TS, Diapoulis AC, Photis GD. 1991. Evaluating the trophic status of a shallow polluted Lake, Lake Ioannina, Greece. Toxicol Environ Chem 31:303–313. doi:10.1080/02772249109357702.
- Lakatos C, Urabe J, Makino W. 2015. Cryptic diversity of Japanese *Diaphanosoma* (Crustacea: Cladocera) revealed by morphological and molecular assessments. Inland Waters 5:253– 262. doi:10.5268/IW-5.3.847.
- Liu P, Xu L, Xu SL, Martínez A, Chen H, Cheng D, Dumont HJ, Han BP, Fontaneto D. 2018. Species and hybrids in the genus *Diaphanosoma* Fischer, 1850 (Crustacea: Branchiopoda: Cladocera). Mol Phylogenet Evol **118:**369–378. doi:10.1016/ j.ympev.2017.10.016.
- López-Blanco C, Sahuquillo M, Vicente E, García-Alix A, Epp LS. 2024. Notes on the ecology and distribution of a water flea complex (Anomopoda, Daphniidae) revealed by new DNA barcodes in the Iberian Peninsula. Zool Scr 53:98–112. doi:10.1111/zsc.12630.
- López-Blanco C, Tasevska O, Kostoski G, Wagner B, Wilke T. 2020. Ancient civilizations already had an impact on cladoceran assemblages in Europe's oldest lake. Palaeogeogr Palaeoclimatol Palaeoecol 552:109734. doi:10.1016/j.palaeo.2020.109734.
- López-Blanco C, Miracle MR, Vicente E. 2013. Is there a bias between contemporary and subfossil cladoceran assemblages? Limnetica 32(2):201–214. doi:10.23818/limn.32.17.
- López-Blanco C, Sinev AY. 2016. Cladocera biodiversity in La Tembladera Lake (Ecuador): a palaeolimnological approach. Crustaceana 89:1611–1637. doi:10.1163/15685403-00003605.
- Lunte CC, Luecke C. 1990. Trophic interactions of *Leptodora* in Lake Mendota. Limnol Oceanogr 35:1091–1100. doi:10.4319/ lo.1990.35.5.1091.
- Margaritora F. 1983. Cladoceri (Crustacea: Cladocera). Consiglio Nazionale delle Richerche Aq/1/197, Rome, Italy.
- Martens K. 1997. Speciation in ancient lakes. Trends Ecol Evol **12:**177–182. doi:10.1016/s0169-5347(97)01039-2.
- Matveev VF. 1991. Self-maintaining plankton: pelagic Cladocera in small microcosms with lake water. Hydrobiologia **225:**301–307. doi:10.1007/BF00028408.
- Moustaka-Gouni M, Vardaka E, Michaloudi E, Konstantinos AK, Tryfon E, Mihalatou H, Spyros G, Lanaras T. 2006. Plankton food web structure in a eutrophic polymictic lake with a history of toxic cyanobacterial blooms. Limnol Oceanogr 51:715–727. doi:10.4319/lo.2006.51.1_part_2.0715.
- Pina-Martins F, Paulo OS. 2008. Concatenator: Sequence data matrices handling made easy. Mol Ecol Resour 8:1254–1255. doi:10.1111/j.1755-0998.2008.02164.x.

- primers for COI amplification from freshwater microcrustaceans. Mol Ecol Resour 13:1151–1155. doi:10.1111/1755-0998.12132.
- Rambaut A. 2010. FigTree. Available at: http://tree.bio.ed.ac.uk/ software/f.
- Richard J. 1892. Animaux inferieurs, notamment Entomostraces, recueillis par M. le Prof. Steindachner dans les lacs de la Macedoine. *In*: Annalen Naturhistorichen Hofmuseums Wien, Serie B 7:151–153.
- Richter S, Olesen J, Wheeler WC. 2007. Phylogeny of Branchiopoda (Crustacea) based on a combined analysis of morphological data and six molecular loci. Cladistics 23:301–336. doi:10.1111/ j.1096-0031.2007.00148.x.
- Ronquist F, Teslenko M, Van Der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard A, Huelsenbeck JP. 2012. MrBayes 3.2: Efficient bayesian phylogenetic inference and model choice across a large model space. Syst Biol 61:539–542. doi:10.1093/sysbio/sys029.
- Sacherová V, Hebert PDN. 2003. The evolutionary history of the Chydoridae (Crustacea: Cladocera). Biol J Linn Soc **79:6**29– 643. doi:10.1046/j.1095-8312.2003.00216.x.
- Šapkarev JA. 1970. The Fauna of Hirudinea of Macedonia. The Taxonomy and Distribution of Leeches of Aegean Lakes. Int Rev der gesamten Hydrobiol und Hydrogr 55:317–324. doi:10.1002/ iroh.19700550303.
- Sinev AY, López-Blanco C. 2018. A new species of *Alona* Baird, 1843 (Cladocera: Chydoridae) from the ancient Lake Ohrid. Zootaxa **4526**:434–446. doi:10.11646/zootaxa.4526.4.2.
- Stankovic S. 1960. The Balkan Lake Ohrid and its living world. Monographiae Biologicae IX, Den Haag, University of Michigan, USA.
- Stich HB. 2004. Back again: The reappearance of *Diaphanosoma brachyurum* in Lake Constance. Arch fur Hydrobiol 159:423–431. doi:10.1127/0003-9136/2004/0159-0423.
- Stone JR, Westover KS, Cohen AS. 2011. Late Pleistocene paleohydrography and diatom paleoecology of the central basin of Lake Malawi, Africa. Palaeogeogr Palaeoclimatol Palaeoecol 303:51–70. doi:10.1016/j.palaeo.2010.01.012.
- Sturany R. 1894. Zur Molluskenfauna der europäischen Türkei. Annalen des Naturhistorischen Museums in Wien **9:**369–394.
- Tasevska O, Špoljar M, Gušeska D, Kostoski G, Patcheva S, Sarafiloska EV. 2017. Zooplankton in ancient and oligotrophic Lake Ohrid (Europe) in association with environmental variables. Croat J Fish 75:95–103. doi:10.1515/cjf-2017-0013.
- Tollrian R, Leese F. 2010. Ecological genomics: steps towards unraveling the genetic basis of inducible defenses in *Daphnia*. BMC Biology **8:**51. doi:10.1186/1741-7007-8-51.
- Van Damme K, Brancelj A, Dumont HJ. 2009. Adaptations to the hyporheic in Aloninae (Crustacea: Cladocera): Allocation of *Alona protzi* Hartwig, 1900 and related species to *Phreatalona* gen. nov. Hydrobiologia **618**:1–34. doi:10.1007/s10750-008-9607-6.
- Zhang Z, Schwartz S, Wagner L, Webb M. 2000. A greedy algorithm for aligning DNA sequences. J Comput Biol **7:1**–2. doi: 10.1089/10665270050081478.

Supplementary materials

Supplementary data 1. Alignment *COI* dataset. (download)

Supplementary data 2. Alignment concatenated dataset. (download)