



Extensive sampling sheds light on species-level diversity in Palearctic *Placobdella* (Annelida: Clitellata: Glossiphoniiformes)

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Abstract The bloodfeeding leech genus *Placobdella* is dominated by North American diversity, with only a single nominal species known from Central America and one from the Palearctic region. This is likely due to considerable underestimation of Palearctic biodiversity, but investigations into potential hidden diversity are lacking. To shed light on this, the present study introduces new data for specimens initially identified as *Placobdella costata* from Ukraine (close to the type locality), Italy, Germany,

Latvia, Montenegro, Bulgaria, Slovenia, Turkey, Azerbaijan, Tunisia, and Algeria, and uses both nuclear (Internal Transcribed Spacer [ITS] region) and mitochondrial (cytochrome *c* oxidase subunit I [COI]) sequence data in phylogenetic and DNA barcoding frameworks, in order to better understand species-level diversity. Seven independent lineages are present in the trees, five of which show adequate separation at the COI locus to suggest their unique species-level status (COI distances between these clades range from 4.86 to 8.10%). However, the ITS data suggest that speciation is recent or incipient in these clades, and that not enough time has passed for clear separation at this locus. We discuss the evolutionary and taxonomic implications of our findings and speculate on dispersal events that may have contributed to shaping this pattern of geographic distribution.

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Sebastian Kvist, Serge Utevsky and Federico Marrone have contributed equally to this work.

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Introduction

The genus *Placobdella* (Annelida: Clitellata: Glossiphoniiformes) currently includes 24 nominal species and all but one of these are distributed across North and Central America (Siddall et al., 2005; Bielecki et al., 2012; Oceguera-Figueroa & Pacheco-Chaves, 2012; Marrone et al., 2016; de Carle et al., 2017). The remaining species, *Placobdella costata* (Fr. Müller, 1846), is the type species for the genus and the only member with a Palearctic distribution (e.g., Siddall et al., 2005; Mabrouki et al., 2019); however, at least one record of human-mediated introduction of a North American *Placobdella* species into Europe exists (Soors et al., 2015). Members of *Placobdella* are recognized by their possession of two pairs of eyespots (the first pair coalesced), a single pair of caecate bacteriomes (bacteria-bearing organs) attached to the esophagus and bilobed ovaries (Siddall et al., 2005); these characters jointly serve as synapomorphies for members of the genus. Like most glossiphoniiform leeches, members of *Placobdella* display some degree

of parental care (they carry their offspring attached to the ventral surface) and are dorsoventrally flattened, often only slightly longer than wide, and can be found attached to the underside of rocks or submerged debris in freshwater settings, as well as attached to their hosts. All species of *Placobdella* are bloodfeeding and feed mainly on turtles, although some species will also readily feed on other amphibians and aquatic reptiles, as well as fish, birds, and mammals (including humans) (Jones & Woo, 1990; Siddall & Gaffney, 2004; Siddall & Bowerman, 2006; Vamberger & Trontelj, 2007; Moser et al., 2010; Oceguera-Figueroa et al., 2010; Schulz et al., 2011; Cichocka et al., 2021). Their feeding mode and overall high abundance in most freshwater bodies is suggestive of their high impact on ecosystems. This is further evident by virtue of members being known to vector blood-borne parasites, such as hemogregarines and trypanosomes, to their hosts but incidence and effects on fitness remain unknown (Siddall & Dessler, 1990; 1991, 2001; Arizza et al., 2016; Scardino et al., 2022).

Until recently, the phylogenetic relationships within *Placobdella* were largely unexplored, mainly due to the lack of comprehensive analyses covering a broad swath of the diversity. Beyond constraining our general knowledge of the evolutionary histories of the species, the lack of a robust phylogenetic hypothesis

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has also altered our understanding of the dispersal events between continents. In an attempt at remedying this, de Carle et al. (2017) reconstructed the phylogenetic relationships between the known members of the genus. The study revealed a large amount of undescribed North American diversity and also evinced a North American origin for the genus with a relatively late, single dispersal event to the Palearctic (de Carle et al., 2017). This might, in part, explain the overall paucity of Palearctic species diversity, a phenomenon that is mimicked in other glossiphoniiform taxa, such as members of the genera *Theromyzon* and *Helobdella* (Oosthuizen & Davies, 1993; Sağlam et al., 2018; Iwama et al., 2019); both of these genera include only few European representatives and are otherwise outweighed by North American diversity. It is still likely, however, that Palearctic diversity within *Placobdella* is grossly underestimated, and that historical and contemporary investigations have applied the name *P. costata* to any Palearctic specimen without assessing levels of morphological, genetic, and behavioral variation that might be indicative of separation between species.

The limits of the geographical distribution of *P. costata* also remain unknown, and the native status of the species in some regions has recently been questioned (e.g., Vecchioni et al. 2021). Regardless, the species has been recorded from across Scandinavia, the Baltic region, the British Isles, along the European Mediterranean coast and in most central European countries (Bielecki et al., 2012 and references therein). Indeed, records of the species also expand into several North African countries, including Morocco, Algeria, and Tunisia as well as Palestine, Syria, Iran, and the northern Caucasus and Transcaucasia in Central Asia (Pawłowski, 1936, 1968; Bielecki et al., 2012; Ben Ahmed et al., 2015; Bashirichelkasari & Yadollahvandmiandoab, 2017; Laghzaoui & Abbad, 2020; Solgi et al., 2021). The type locality for the species lies on the Crimean Peninsula (see Ben Ahmed et al., 2015), a region of recent unrest, which exacerbates any attempt at gathering fresh material. In order to assess the species-level diversity of Palearctic *Placobdella*, we herein produce new sequence data for material recently collected in Ukraine (including the Kherson Region of southern Ukraine, in proximity to the type locality), Italy, Germany, Latvia, Montenegro, Bulgaria, Slovenia, Turkey, Azerbaijan, Tunisia, and Algeria, and combine these data with previously

published information in both phylogenetic and genetic variation analyses.

Material and methods

Specimen collection

Between 2015 and 2019, several specimens of *Placobdella costata* were collected from Ukraine, Italy, Germany, Latvia, Montenegro, Bulgaria, Slovenia, Turkey, Azerbaijan, Tunisia, and Algeria (see Fig. 1 and Table 1). All specimens were initially identified using specialized literature (e.g., Mann & Watson, 1954; Sawyer, 1986; Sağlam, 2001) and the identities were later corroborated by DNA barcoding using the COI locus. Specimens were variably collected from their host, or from rocks and debris. The leeches were either relaxed by the addition of a small amount of ethanol to pond water and thereafter fixed in 95% ethanol, or fixed directly in 95% ethanol. Voucher specimens are lodged in both private and public repositories, and are available from the first authors upon request.

DNA extraction, amplification, and sequencing

To avoid contamination by bloodmeals inside of the leeches, a small piece of tissue was cut from the caudal sucker of each specimen. DNA extraction was accomplished via a DNeasy Tissue Kit (Qiagen, Valencia, CA, USA), an InnuPrep DNA Mini Kit (Analytik Jena, Jena, Germany), or a BIORON tissue kit (BIORON, Römerberg, Germany) following the manufacturers' protocols. Thereafter, partial sequences for the mitochondrial cytochrome *c* oxidase subunit I (COI) gene was amplified using the universal primers LCO1490 and HCO2198 (Folmer et al., 1994), and portions of the nuclear internal transcribed spacer (ITS) were amplified using a combination of the primers ITS3, ITS4 and ITS5 (White et al., 1990). Polymerase chain reactions (PCRs) were carried out in 25 µl reactions consisting of 16.39 µl ddH₂O; 2.5 µl buffer; 2.5 µl MgCl₂; 1 µl of each primer; 0.56 µl dNTPs at 10 mM concentration; 0.05 µl Platinum Taq polymerase (Invitrogen, Carlsbad, CA); and 1 µl total genomic DNA. PCR amplification used the following protocols for COI: 94°C (5 min) followed by 30 cycles of 94°C (45 s), 40°C (45 s), 72°C (45 s), and final

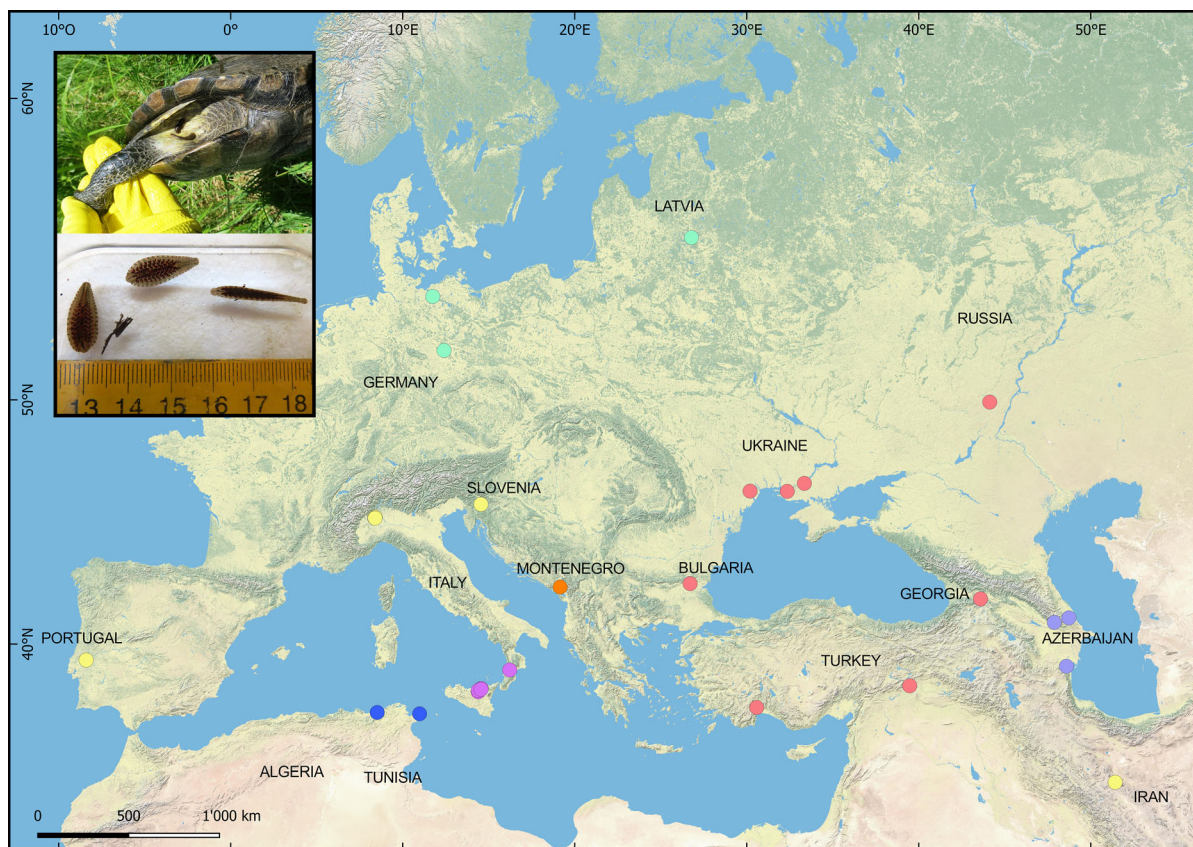


Fig. 1 Map showing the sampling sites for the included specimens of *Placobdella costata*. Note that the sites for the Iranian, Portuguese, Russian, and Georgian specimens are placed in the center of the respective countries because no

coordinates were available for those specimens. The inset in the upper left corner shows the Latvian specimens in habitus. The different colors for the sites represent the different clades found in the phylogenetic trees

extension at 72°C (7 min). For ITS, the thermocycler protocol was as follows: 95°C (5 min) followed by 35–40 cycles of 95°C (30–60 s), 48°C or 51°C (30–60 s), 72°C (30–90 s), and final extension at 72°C (8–10 min). PCR products were checked on a 1% agarose gel and amplicons were purified using either ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) or a Silica Bead DNA gel extraction kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturers' protocols. Cycle sequencing reactions were performed in 10 µl reactions, consisting of 0.5 µl ABI Big Dye Terminator ver. 3.1, 0.5 µl Big Dye 5 × sequencing buffer, 2 µl primer at 10 µM concentration, 2.5 µl purified PCR product, and 4.5 µl ddH₂O. Reaction mixtures were heated to 96°C for 1 min followed by 30 cycles of 96°C (10 s), 50°C (5 s), and 60°C (4 min). The samples were then sequenced on an ABI PRISM

3130 or 3730 (Applied Biosystems, Carlsbad, CA) at one of the following institutions: the Royal Ontario Museum (Toronto, Canada), LGC Genomics (Berlin, Germany), or Macrogen SPAIN (Madrid, Spain).

Phylogenetic analyses and genetic distances

The COI dataset for the newly generated sequences was augmented by several sequences downloaded from GenBank, including samples for all known species of *Placobdella*, several of which were derived from specimens collected at the respective type localities; the final COI dataset included 108 terminals, 22 of which are newly sequenced. The resulting COI gene tree was used to guide the choice of specimens for ITS sequencing. The ITS dataset included 48 (15 of these are newly derived) terminals representing each major clade in the COI tree. For both

Table 1 Metadata for the newly collected specimens, as well as the included GenBank sequences

Species	Collection code	COI clade	Locality	Coordinates	Host	COI accession number	ITS accession number	Reference
<i>Placobdella costata</i>	n.a	C1	Natural Park of the Lame del Sesia, Piedmont, Italy	45.42193 N, 8.405365 E	n.a	AY962461	–	Siddall et al. (2005)
<i>Placobdella costata</i>	909	C1	Cavo Regina, Piedmont, Italy	45.38125 N, 8.37511 E	<i>Emys orbicularis hellenica</i>	MW935939	–	Vecchioni et al. (2021)
<i>Placobdella costata</i>	888	C1	Madonna delle Vigne, Piedmont, Italy	45.37490 N, 8.39044 E	<i>Emys orbicularis hellenica</i>	MW935934	–	Vecchioni et al. (2021)
<i>Placobdella costata</i>	908	C1	Cavo Regina, Piedmont, Italy	45.38125 N, 8.37511 E	<i>Emys orbicularis hellenica</i>	MW935938	OK356881	Vecchioni et al. (2021); present study
<i>Placobdella costata</i>	n.a	C1	Iran	n.a	<i>Emys orbicularis persica</i>	MT260059	–	Solgi et al. (2021)
<i>Placobdella costata</i>	n.a	C1	Unknown	n.a	n.a	MF067142	MF067088	de Carle et al. (2017)
<i>Placobdella costata</i>	n.a	C1	Portugal	n.a	<i>Emys orbicularis occidentalis</i>	MF067143	MF067089	de Carle et al. (2017)
<i>Placobdella costata</i>	HA052	C1*	Draga pri Igu, Ig. Slovenia	45.9377 N, 14.5497 E	<i>Emys orbicularis</i>	OK350344	–	Present study
<i>Placobdella costata</i>	885	C2	San Fratello, Sicily, Italy	37.98346 N, 14.56649 E	<i>Emys trinacris</i>	MW935932	OK356877	Vecchioni et al. (2021); present study
<i>Placobdella costata</i>	756	C2	Lake Sambughetti Campanito, Sicily, Italy	37.83184 N, 14.39019 E	<i>Emys trinacris</i>	MW935929	–	Vecchioni et al. (2021)
<i>Placobdella costata</i>	757	C2	Lake Gianferraro, Sicily, Italy	37.95124 N, 14.49755 E	<i>Emys trinacris</i>	MW935931	–	Vecchioni et al. (2021)
<i>Placobdella costata</i>	755	C2	Lake Urio Quattroocchi, Sicily, Italy	37.90103 N, 14.39542 E	<i>Emys trinacris</i>	MW935927	–	Vecchioni et al. (2021)
<i>Placobdella costata</i>	n.a	C2	Pantana, Sicily, Italy	37.94931 N, 14.55136 E	<i>Emys trinacris</i>	KT382531	–	Marrone et al. (2016)
<i>Placobdella costata</i>	n.a	C2	Imbuttillo, Calabria, Italy	38.83116 N, 16.22326 E	<i>Emys orbicularis galloitalica</i>	KT382534	–	Marrone et al. (2016)
<i>Placobdella costata</i>	n.a	C2	Pantana, Sicily, Italy	37.94931 N, 14.55136 E	<i>Emys trinacris</i>	KT382524	–	Marrone et al. (2016)

Table 1 continued

Species	Collection code	COI clade	Locality	Coordinates	Host	COI accession number	ITS accession number	Reference
<i>Placobdella costata</i>	n.a	C2	Pantana, Sicily, Italy	37.94931 N, 14.55136 E	<i>Emys trinaeris</i>	KT382523	–	Marrone et al. (2016)
<i>Placobdella costata</i>	n.a	C2	Pantana, Sicily, Italy	37.94931 N, 14.55136 E	<i>Emys trinaeris</i>	KT382532	–	Marrone et al. (2016)
<i>Placobdella costata</i>	n.a	C2	Pantana, Sicily, Italy	37.94931 N, 14.55136 E	<i>Emys trinaeris</i>	KT382521	–	Marrone et al. (2016)
<i>Placobdella costata</i>	n.a	C2	Pantana, Sicily, Italy	37.94931 N, 14.55136 E	<i>Emys trinaeris</i>	KT382529	–	Marrone et al. (2016)
<i>Placobdella costata</i>	n.a	C2	Pantana, Sicily, Italy	37.94931 N, 14.55136 E	<i>Emys trinaeris</i>	KT382533	–	Marrone et al. (2016)
<i>Placobdella costata</i>	n.a	C2	Pantana, Sicily, Italy	37.94931 N, 14.55136 E	<i>Emys trinaeris</i>	KT382526	–	Marrone et al. (2016)
<i>Placobdella costata</i>	n.a	C2	Pantana, Sicily, Italy	37.94931 N, 14.55136 E	<i>Emys trinaeris</i>	KT383866	–	Marrone et al. (2016)
<i>Placobdella costata</i>	n.a	C2	Pantana, Sicily, Italy	37.94931 N, 14.55136 E	<i>Emys trinaeris</i>	KT382522	–	Marrone et al. (2016)
<i>Placobdella costata</i>	n.a	C2	Pantana, Sicily, Italy	37.94931 N, 14.55136 E	<i>Emys trinaeris</i>	KT382527	–	Marrone et al. (2016)
<i>Placobdella costata</i>	n.a	C2	Pantana, Sicily, Italy	37.94931 N, 14.55136 E	<i>Emys trinaeris</i>	KT382525	–	Marrone et al. (2016)
<i>Placobdella costata</i>	n.a	C2	Imbutillo, Calabria, Italy	38.83116 N, 16.22326 E	<i>Emys orbicularis hellenica</i>	KT363867	–	Marrone et al. (2016)
<i>Placobdella costata</i>	n.a	C2	Pantana, Sicily, Italy	37.94931 N, 14.55136 E	<i>Emys trinaeris</i>	KT382525	–	Marrone et al. (2016)
<i>Placobdella costata</i>	n.a	C2	Pantana, Sicily, Italy	37.94931 N, 14.55136 E	<i>Emys trinaeris</i>	KT382530	–	Marrone et al. (2016)
<i>Placobdella costata</i>	889	C3	Nohur Lake, Azerbaijan	40.95982 N, 47.88332 E	n.a	OK350335	–	Present study
<i>Placobdella costata</i>	893	C3	Akzibir Lake (Dicichi Liman), Azerbaijan	41.15838 N, 48.73478 E	n.a	OK350337	–	Present study
<i>Placobdella costata</i>	890	C3	Vitesh River, Azerbaijan	39.00154 N, 48.59039 E	n.a	OK350338	OK356883	Present study
<i>Placobdella costata</i>	640	C4	Warnow River, Mecklenburg, Germany	53.71911 N, 11.74945 E	Free living	OK350345	OK356871	Present study

Table 1 continued

Species	Collection code	COI clade	Locality	Coordinates	Host	COI accession number	ITS accession number	Reference
<i>Placobdella costata</i>	695	C4	Kapengraben, stream near Dessau, Saxony-Anhalt, Germany	51.81488 N, 12.40539 E	Free living	OK350346	OK356872	Present study
<i>Placobdella costata</i>	696	C4	Kapengraben, stream near Dessau, Saxony-Anhalt, Germany	51.81488 N, 12.40539 E	Free living	OK350347	OK356873	Present study
<i>Placobdella costata</i>	1	C4	Kaskade ponds, Silene Nature Park, Latvia	55.69078 N, 26.78860 E	<i>Emys orbicularis orbicularis</i>	OK350348	OK356874	Present study
<i>Placobdella costata</i>	2	C4	Kaskade ponds, Silene Nature Park, Latvia	55.69078 N, 26.78860 E	<i>Emys orbicularis orbicularis</i>	OK350349	OK356875	Present study
<i>Placobdella costata</i>	3	C4	Kaskade ponds, Silene Nature Park, Latvia	55.69078 N, 26.78860 E	<i>Emys orbicularis orbicularis</i>	OK350350	OK356876	Present study
<i>Placobdella costata</i>	1154	C5	Lake Tonga, Algeria	36.87881 N, 8.517920 E	<i>Emys orbicularis ssp.1</i>	OK350332	OK356884	Present study
<i>Placobdella costata</i>	1155	C5	Lake Tonga, Algeria	36.87881 N, 8.517920 E	<i>Emys orbicularis ssp.1</i>	OK350333	–	Present study
<i>Placobdella costata</i>	1	C5	Mallabi Dam, Tunisia	36.81666 N, 10.98333 E	Free living	OK350331	OK356870	Present study
<i>Placobdella costata</i>	ROMIZI12945	C6	Kozatske, Dnieper River, Ukraine	46.781827 N, 33.351710 E	Free living	OK350351	OK356879	Present study
<i>Placobdella costata</i>	ROMIZI12946	C6	Bilyayivka, Turunchuk River (west branch of the Dniester River), Ukraine	46.471184 N, 30.195211 E	Free living	OK350352	OK356878	Present study
<i>Placobdella costata</i>	891	C6	Dnieper Delta, Ukraine	46.46219 N, 32.35754 E	Free living	OK350336	–	Present study
<i>Placobdella costata</i>	912	C6	Dnieper Delta, Ukraine	46.46219 N, 32.35754 E	Free living	OK350339	OK356880	Present study
<i>Placobdella costata</i>	1099	C6	Kalecik village, Cermik, Turkey	38.11117 N, 39.48000 E	Free living	OK350334	OK356882	Present study

Table 1 continued

Species	Collection code	COI clade	Locality	Coordinates	Host	COI accession number	ITS accession number	Reference
<i>Placobdella costata</i>	n.a	C6	Strandza region, Marash River near bridge to Lozenets village, Bulgaria	42.629 N, 26.7098 E	Free living	OK350342	–	Present study
<i>Placobdella costata</i>	n.a	C6	Strandza region, Marash River near bridge to Lozenets village, Bulgaria	42.629 N, 26.7098 E	Free living	OK350343	–	Present study
<i>Placobdella costata</i>	n.a	C6	Abkhazia, Georgia	n.a	n.a	MN393268	–	Unpublished
<i>Placobdella costata</i>	n.a	C6	Rostov Oblast, Russia	n.a	n.a	MN295392	–	Unpublished
<i>Placobdella costata</i>	n.a	C6	Volgograd Oblast, Russia	n.a	n.a	MN393261	–	Unpublished
<i>Placobdella costata</i>	n.a	C6	Kirkgoz, Antalya, Turkey	37.10989 N, 30.58071 E	<i>Emys orbicularis orbicularis</i>	MK185711	–	Unpublished
<i>Placobdella costata</i>	MN4_1	C7	Danilovgrad municipality, spring “Oraska Jama”, Montenegro	42.5309 N, 19.0921 E	Free living	OK350340	–	Present study
<i>Placobdella costata</i>	MN9_1	C7	Podgorica, spring “Cmo Oko” in Bantići village, Montenegro	42.4844 N, 19.1542 E	Free living	OK350341	–	Present study
<i>Placobdella rugosa</i>	n.a		Pipestone Creek, Moosomin Regional Park, Saskatchewan, Canada	n.a	n.a	MF067101	–	de Carle et al. (2017)
<i>Placobdella rugosa</i>	n.a		Belcourt Lake, North Dakota, USA	48.88233 N, 99.75352 W	<i>Chrysemys picta bellii</i>	JX412986	–	Moser et al. (2012a)
<i>Placobdella rugosa</i>	ROMIZ10089		Mijinemungshing Lake, Ontario, Canada	n.a	Free living	MF067106	MF067057	de Carle et al. (2017)
<i>Placobdella nuchalis</i>	ROMIZ12981		Mohonk Camp, New York, USA	n.a	Free living	MF067128	MF067075	de Carle et al. (2017)
<i>Placobdella nuchalis</i>	ROMIZ12983		John Allen Pond, New York, USA	n.a	Free living	MF067126	MF067073	de Carle et al. (2017)
<i>Placobdella biannulata</i>	n.a		North Carolina, USA	n.a	Free living	AF116021	–	
<i>Placobdella picta</i>	ROMIZ110111		Kenny Lake, Ontario, Canada	n.a	Free living	MF067113	MF067062	de Carle et al. (2017)

Table 1 continued

Species	Collection code	COI clade	Locality	Coordinates	Host	COI accession number	ITS accession number	Reference
<i>Placobdella picta</i>	ROMOZI11395		Moose Mountain Provincial Park, Saskatchewan, Canada	n.a	n.a	MF067109	–	de Carle et al. (2017)
<i>Placobdella sophieae</i>	ROMIZI12994		Washington, USA	n.a	n.a	MF067097	MF067050	de Carle et al. (2017)
<i>Placobdella sophieae</i>	ROMIZI12995		Washington, USA	n.a	n.a	MF067096	MF067049	de Carle et al. (2017)
<i>Placobdella pediculata</i>	ROMIZI12987		n.a	n.a	n.a	MF067120	MF067067	de Carle et al. (2017)
<i>Placobdella pediculata</i>	n.a		Lake Pepin, Minnesota, USA	n.a	<i>Aplodinotus grunniens</i>	MN043903	–	Moser et al. (2019)
<i>Placobdella pediculata</i>	ROMIZI12986		n.a	n.a	n.a	MF067121	MF067068	de Carle et al. (2017)
<i>Placobdella mexicana</i>	ROMIZI12977		El Vergel, Mexico	n.a	n.a	MF067132	MF067078	de Carle et al. (2017)
<i>Placobdella mexicana</i>	ROMIZI12976		Mexico	n.a	n.a	MF067133	MF067079	de Carle et al. (2017)
<i>Placobdella siddalli</i>	n.a		Davis Eddy, Mississippi, USA	n.a	n.a	KY780962	–	Richardson et al. (2017)
<i>Placobdella phalera</i>	ROMIZI12990		Tennessee, USA	n.a	Free living	MF067115	–	de Carle et al. (2017)
<i>Placobdella translucens</i>	n.a		Michigan, USA	n.a	n.a	AY047328	–	Light & Siddall (1999)
<i>Placobdella phalera</i>	ROMIZI12989		n.a	n.a	Free living	MF067116	MF067063	de Carle et al. (2017)
<i>Placobdella cryptobranchii</i>	ROMIZI12988		Canopus Ramp, New York, USA	n.a	Free living	MF067117	MF067064	de Carle et al. (2017)
<i>Placobdella cryptobranchii</i>	n.a		Missouri, USA	n.a	n.a	KF601755	–	Moser et al. (2013a, b, c)
<i>Placobdella ornata</i>	n.a		Missouri, USA	n.a	n.a	KF601761	–	Moser et al. (2013a, b, c)
<i>Placobdella ornata</i>	n.a		Shivericks Pond, Massachusetts, USA	n.a	n.a	JQ812135	–	Moser et al. (2012b)
<i>Placobdella ornata</i>	n.a		The Donkmeer, East Flanders Province, Belgium	n.a	n.a	KP176597	–	Soors et al. (2015)

Table 1 continued

Species	Collection code	COI clade	Locality	Coordinates	Host	COI accession number	ITS accession number	Reference
<i>Placobdella parasitica</i>	ROMIZ110340		Pearkes Lake, Ontario, Canada	n.a	Free living	MF067122	MF067069	de Carle et al. (2017)
<i>Placobdella parasitica</i>	ROMIZ110311		North of Algonquin Park, Ontario, Canada	n.a	Free living	MF067123	MF067070	de Carle et al. (2017)
<i>Placobdella parasitica</i>	n.a		Minnesota, USA	n.a	n.a	KF058895	–	Moser et al. (2013b)
<i>Placobdella</i> sp. ²	ROMIZ112997		n.a	n.a	n.a	MF067092	MF067045	de Carle et al. (2017)
<i>Placobdella</i> sp. ²	ROMIZ112996		Canopus Ramp, New York, USA	n.a	Free living	MF067093	MF067046	de Carle et al. (2017)
<i>Placobdella papillifera</i>	ROMIZ112984		John Allen Pond, New York, USA	n.a	Free living	MF067125	MF067072	de Carle et al. (2017)
<i>Placobdella papillifera</i>	ROMIZ112985		John Allen Pond, New York, USA	n.a	Free living	MF067124	MF067071	de Carle et al. (2017)
<i>Placobdella papillifera</i>	n.a		West River, Connecticut, USA	n.a	n.a	KC505241	–	Moser et al. (2013c)
<i>Placobdella kwetlumye</i>	ROMIZ112971		Squires Lake, Washington, USA	n.a	Free living	MF067138	MF067084	de Carle et al. (2017)
<i>Placobdella kwetlumye</i>	ROMIZ112970		Idaho, USA	n.a	Free living	MF067139	MF067085	de Carle et al. (2017)
<i>Placobdella</i> sp. ¹	ROMIZ111277		Half Moon Lake, Alberta, Canada	n.a	Free living	MF067095	MF067048	de Carle et al. (2017)
<i>Placobdella</i> sp. ¹	ROMIZ111494		Adams Lake, Saskatchewan, Canada	n.a	Free living	MF067094	MF067047	de Carle et al. (2017)
<i>Placobdella burresonae</i>	ROMIZ112967		USA	n.a	Free living	MF067144	–	de Carle et al. (2017)
<i>Placobdella michiganensis</i>	n.a		n.a	n.a	n.a	MT579302	–	Moser et al. (2020)
<i>Placobdella multilineata</i>	n.a		Maurepas Swamp, Louisiana, USA	n.a	n.a	AY962464	–	Siddall et al. (2005)
<i>Placobdella multilineata</i>	n.a		Lukfata Creek, Oklahoma, USA	n.a	n.a	KM396760	–	Moser et al. (2014a)
<i>Placobdella</i> sp. ³	ROMIZ112998		Maryland, USA	n.a	n.a	MF067091	–	de Carle et al. (2017)
<i>Placobdella ali</i>	ROMIZ112966		New York, USA	n.a	Free living	MF067145	–	de Carle et al. (2017)

Table 1 continued

Species	Collection code	COI clade	Locality	Coordinates	Host	COI accession number	ITS accession number	Reference
<i>Placobdella ali</i>	ROMIZ112965		New York, USA	n.a	Free living	MF067146	–	de Carle et al. (2017)
<i>Placobdella hollensis</i>	ROMIZ110341		Frontenac Provincial Park, Ontario, Canada	n.a	Free living	MF067141	MF067087	de Carle et al. (2017)
<i>Placobdella hollensis</i>	ROMIZ110356		Frontenac Provincial Park, Ontario, Canada	n.a	Free living	MF067140	MF067086	de Carle et al. (2017)
<i>Placobdella hollensis</i>	n.a		Coonansett Pond, Massachusetts, USA	n.a	n.a	KF771652	–	Moser et al. (2014b)
<i>Placobdella lamothei</i>	ROMIZ112972		Nuevo Urecho, Michoacan, Mexico	n.a	n.a	MF067137	MF067083	de Carle et al. (2017)
<i>Placobdella lamothei</i>	ROMIZ112973		Tonatico, Estado de México, Mexico	n.a	n.a	MF067136	MF067082	de Carle et al. (2017)
<i>Placobdella</i> sp. 4	ROMIZ112999		n.a	n.a	n.a	MF067090	MF067044	de Carle et al. (2017)
<i>Placobdella ringueleti</i>	n.a		Catemaco, Veracruz, Mexico	n.a	n.a	MF067107	–	de Carle et al. (2017)
<i>Placobdella ringueleti</i>	ROMIZ112992		Chiapas, Mexico	n.a	n.a	MF067108	MF067058	de Carle et al. (2017)
<i>Placobdella nuchalis</i>	n.a		n.a	n.a	n.a	MF535240	–	Moser et al. (2017)
<i>Placobdella montifera</i>	ROMIZ112980		n.a	n.a	Free living	MF067129	MF067076	de Carle et al. (2017)
<i>Placobdella montifera</i>	ROMIZ112979		Friends Landing, Washington, USA	n.a	Free living	MF067130	MF067077	de Carle et al. (2017)
<i>Haementeria ghilianii</i>	n.a		BioPharm, French Guiana	n.a	Free living	AF329035	JN850932	Siddall & Borda (2003) and Ocegüera-Figueroa (2012)

Bold font denotes specimens for which new sequence data were produced for the present study

COI and ITS, sequences were aligned using the online version of MAFFT (Kato et al., 2019) applying automatic alignment strategy with a gap opening penalty of 5.0. Mesquite ver. 3.11 (Maddison & Maddison, 2016) and ALTER (Glez-Peña et al., 2010) were used to reformat and concatenate the datasets for phylogenetic analyses and the COI alignment was cut to cover only the “Folmer region”. Phylogenetic analyses used both maximum likelihood (ML) and parsimony approaches and the trees were rooted at *Haementeria ghilianii* de Filippi, 1849 following the previous phylogenetic hypotheses (de Carle et al., 2017).

Prior to maximum likelihood (ML) analyses, PartitionFinder ver. 1.1.1 (Lanfear et al., 2012) was used to simultaneously estimate the optimal partitioning scheme and the best fitting model of nucleotide evolution. For this purpose, each locus was assessed separately, and so was each codon position for the protein-coding COI locus. Phylogenetic analyses were performed for each of the following datasets: (i) COI only, (ii) ITS only, and (iii) COI and ITS combined. The ML analyses were carried out using RAxML ver. 8 (Stamatakis, 2014) on the CIPRES Science Gateway platform (Miller et al., 2010). Tree searches consisted of 1000 replicates with 25 initial GAMMA rate categories and final optimization using four GAMMA shape categories; nodal support values were calculated using 1000 pseudoreplicates using the rapid bootstrap algorithm applying default settings.

In addition, parsimony analyses were performed in TNT ver. 1.5 (Goloboff & Catalano, 2016) for each of the three datasets. New Technology searches were performed using 1000 iterations, each consisting of five rounds of ratcheting, without drifting or tree fusing and stipulating that the search be terminated if the optimal score was hit 10 times (“hits 10”). The results of the search were thereafter subjected to branch swapping using the “bbreak” command and bootstrap support was estimated through 1000 pseudoreplicates using default settings.

Following previous DNA barcoding approaches, MEGA ver. X (Kumar et al., 2018) was used to calculate genetic distances using uncorrected *p*-distances with uniform rates among sites and pairwise deletion of missing data. Standard errors were calculated via 500 bootstrap replicates with default settings.

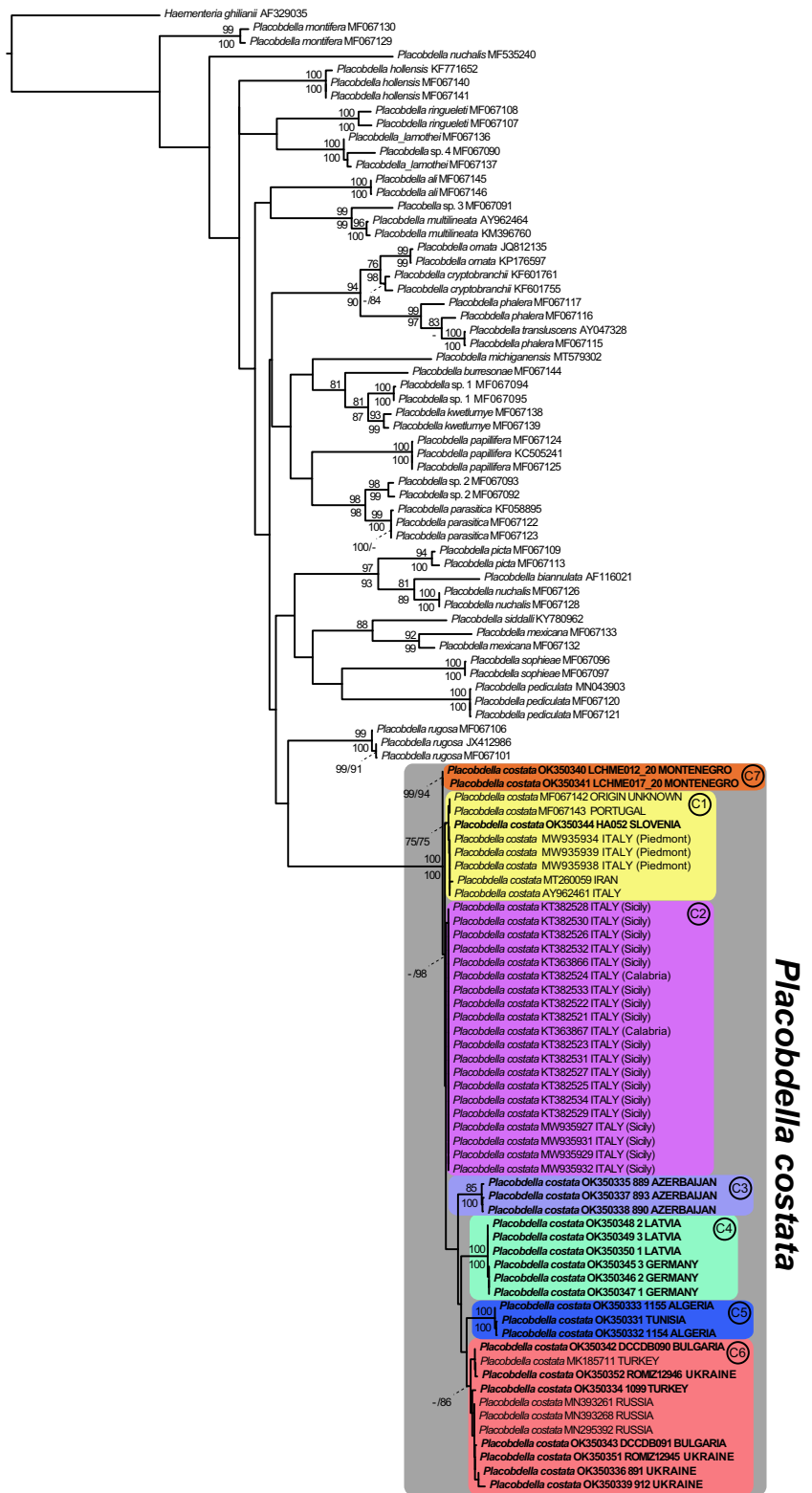
Results

Phylogeny

All newly derived sequences are deposited in GenBank under accession numbers OK350331–OK350352 (COI) and OK356870–OK356884 (ITS) (see Table 1). The final COI dataset consisted of 658 aligned sites covering the “Folmer region” and the ITS dataset consisted of 976 aligned sites; accordingly, the combined dataset consisted of 1634 aligned sites. The resulting ML trees from the analyses using the (i) COI, (ii) ITS, and (iii) combined datasets are presented in Figs. 2, 3 and 4, respectively; the corresponding parsimony trees are presented in Supplementary Figs. 1–3.

Insofar as the phylogenetic relationships of *Placobdella* have been discussed elsewhere (de Carle et al., 2017), the current study focuses only on *P. costata* and its closest constituents. Whereas each of the parsimony trees are largely unresolved for species-level relationships, the ML trees show more resolved topologies. The sister group to *Placobdella costata* differs between the trees and this position receives only very low support. In the COI tree, *Placobdella rugosa* Moore, 1901 places as the sister taxon to *P. costata* (likelihood bootstrap support [LBS] < 75%; parsimony bootstrap support [PBS] < 75%), whereas this position is occupied by larger clades in the ITS tree [composed of *P. rugosa*, *Placobdella papillifera* (Verrill, 1872), *Placobdella ringueleti* López-Jiménez & Oceguera-Figueroa, 2009, *Placobdella* sp. 4, *Placobdella lamothei* Oceguera-Figueroa & Siddall, 2008, *Placobdella* sp. 2, *Placobdella parasitica* (Say, 1824), *Placobdella mexicana* Moore, 1898, *Placobdella hollensis* (Whitman, 1892), *Placobdella pediculata* Hemingway, 1908, *Placobdella phalera* (Graf, 1899), and *Placobdella montifera* Moore, 1906; LBS < 75%; PBS < 75%] and in the combined tree [composed of *P. montifera*, *P. pediculata*, *Placobdella ornata* (Verrill, 1872), *Placobdella cryptobranchii* (Johnson & Klemm, 1977), *P. phalera*, *Placobdella translucens* (Sawyer & Shelley, 1976), *Placobdella michiganensis* (Sawyer, 1972), *Placobdella burresonae* Siddall & Bowerman, 2006, *Placobdella kwetlumye* Oceguera-Figueroa et al., 2010, *Placobdella* sp. 1, *Placobdella nuchalis* Sawyer & Shelley, 1976, *Placobdella sophieae* Oceguera-Figueroa et al., 2010, *Placobdella picta* (Verrill, 1872), *Placobdella*

Fig. 2 Highest scoring maximum likelihood tree resulting from the analysis of the COI locus ($\ln L = -8303.773428$). Likelihood bootstrap support above 75% is shown above each node and parsimony bootstrap support above 75% is shown below each node. Bold font denotes terminals for which new sequence data were produced for the present study, and GenBank accession numbers follow the taxon names. Clades C1–C7 are color-coded and are further discussed in the text



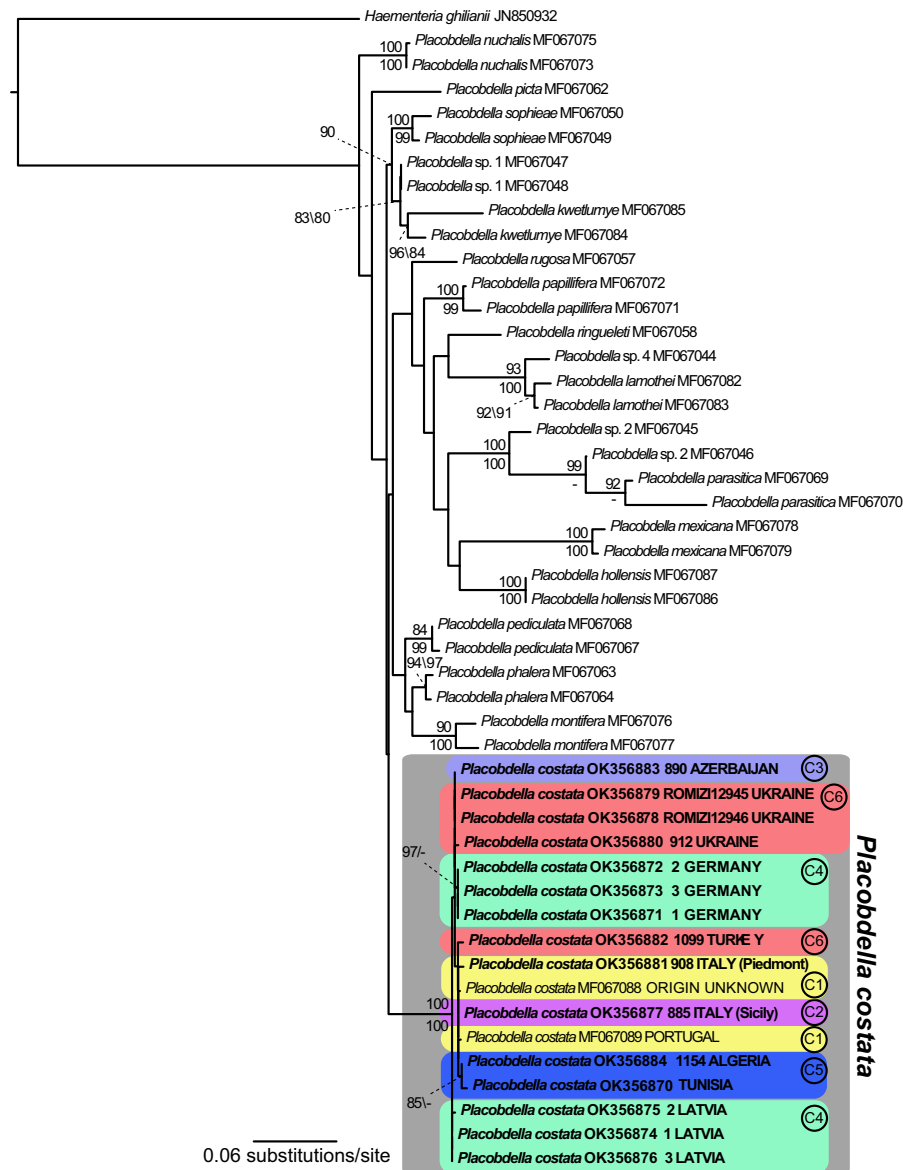


Fig. 3 Highest scoring maximum likelihood tree resulting from the analysis of the ITS locus ($\ln L = -5842.070410$). Likelihood bootstrap support above 75% is shown above each node and parsimony bootstrap support above 75% is shown below

biannulata (Moore, 1900), and *P. nuchalis*; [LBS < 75%; PBS < 75%]. In all trees, specimens labeled *P. costata* form a monophyletic group (LBS = 100%; PBS = 100% for all trees) and both the COI and combined trees recover seven lineages within *P. costata*. The first (C1) includes specimens collected in Portugal, Iran, Slovenia, and the Piedmont region of continental Italy (LBS = 75–81%; PBS = 75–77%);

each node. Newly sequenced specimens are denoted in bold font and GenBank accession numbers follow the taxon names. Clades C1–C6 are color-coded and are further discussed in the text

the second (C2) includes specimens collected on the Italian island of Sicily and in Calabria (southern peninsular Italy) (LBS = 87%; PBS = < 75%); the third (C3) includes specimens collected in Azerbaijan (LBS = 79–85%; PBS = 100%); the fourth (C4) includes specimens collected in Latvia and Germany (LBS = 97–100%; PBS = 99–100%); the fifth (C5) includes specimens collected in Tunisia and Algeria

Table 2 Average COI variation between and within the different *Placobdella costata* lineages recovered in the COI and combined trees

	C1	C2	C3	C4	C5	C6	C7
C1	0.41% ± 0.15	<i>0.49</i>	<i>0.94</i>	<i>0.92</i>	<i>0.95</i>	<i>0.85</i>	<i>0.44</i>
C2	1.62%	0.00%	<i>0.96</i>	<i>1.02</i>	<i>0.98</i>	<i>0.83</i>	<i>0.38</i>
C3	5.81%	5.60%	0.68% ± 0.28	<i>1.03</i>	<i>1.06</i>	<i>0.94</i>	<i>0.96</i>
C4	6.55%	6.66%	7.24%	0.18% ± 0.13	<i>0.97</i>	<i>0.85</i>	<i>0.95</i>
C5	6.82%	6.11%	8.10%	7.31%	0.42% ± 0.20	<i>0.83</i>	<i>0.94</i>
C6	5.46%	4.86%	6.32%	5.68%	5.80%	1.54% ± 0.29	<i>0.81</i>
C7	1.55%	0.83%	6.18%	6.84%	6.63%	5.35%	0.00%

The lower left triangle shows average interlineage distances with standard errors in the upper right triangle (italicized). The diagonal (bold font) shows average intralinear variation and standard errors

Table 3 Average ITS variation between and within the different *Placobdella costata* lineages recovered in the COI and combined trees

	C1	C2	C3	C4	C5	C6
C1	0.51% ± 0.24	<i>0.10</i>	<i>0.22</i>	<i>0.38</i>	<i>0.18</i>	<i>0.21</i>
C2	0.22%	n.a	<i>0.17</i>	<i>0.34</i>	<i>0.21</i>	<i>0.16</i>
C3	0.51%	0.25%	n.a	<i>0.16</i>	<i>0.25</i>	<i>0.08</i>
C4	1.02%	0.71%	0.27%	0.35% ± 0.22	<i>0.37</i>	<i>0.20</i>
C5	0.54%	0.43%	0.68%	0.85%	0.37% ± 0.23	<i>0.25</i>
C6	0.56%	0.34%	0.19%	0.45%	0.78%	0.37% ± 0.15

The lower left triangle shows average interlineage distances with standard errors in the upper right triangle (italicized). The diagonal (bold font) shows average intralinear variation and standard errors

(LBS = 99–100%; PBS = 99–100%); the sixth (C6) includes specimens collected in Ukraine, Russia, Georgia, Bulgaria, and Turkey (LBS < 75%; PBS 81–86%); and the seventh (C7) includes specimens collected in Montenegro (LBS = 99–100%; PBS = 94–95%). We suggest that C6 represents *Placobdella costata* sensu stricto as it includes specimens collected in close proximity to the type locality of the species. The relationships between these clades are relatively conserved between the trees; the only difference is that C1 places as the sister group to C7 in the combined tree, whereas it places as the sister group to C2 in the COI tree.

The ITS tree includes fewer terminals for the *P. costata* clade and does not recover the same seven clades, although support values are largely negligible throughout the *P. costata* clade in the ITS tree (Fig. 3). Whereas the members of C1, C4, and C6 are recovered as non-monophyletic (LBS < 75%; PBS < 75%), C5

(Algeria and Tunisia) is recovered as monophyletic (LBS = 85%; PBS < 75%); note that only a single individual was included for each of C2 and C3 and no specimens were included for C7.

Genetic variation

Genetic distances within and between the different lineages of *P. costata* are presented in Table 2 (for COI) and Table 3 (for ITS). The average COI distances within lineages ranged between 0% (in C2) and 1.54% ± 0.29 (in C6), whereas the average COI distance between lineages ranged between 1.55% ± 0.44 (between C1 and C7) and 8.10% ± 1.06 (between C3 and C5). Notably, the average variation between lineages was typically an order of magnitude larger than that within lineages, suggesting the presence of an adequately sized DNA barcoding gap for each of the comparisons. These

results lend further strength to the hypothesis that five species-level taxa are present among our “*P. costata*” sequences (C1, C2, and C7 are separated by less than 1.62% COI distance and are considered the same species by the present study); for clarity, the putative species are represented by (i) C1 + C2 + C7, (ii) C3, (iii) C4, (iv) C5, and (v) C6.

The average ITS distances between lineages were much lower than those for COI. These values ranged between $0.22\% \pm 0.10$ (between C1 and C2) and $1.02\% \pm 0.38$ (between C1 and C4), whereas the ITS distance within lineages ranged between $0.35\% \pm 0.22$ (within C4) and $0.51\% \pm 0.24$ (within C1).

Discussion

As opposed to its North American counterparts, and despite its broad geographic distribution in Europe, North Africa, and Asia, *P. costata* is a relatively rare species compared to other members of the genus (e.g., van Haaren et al., 2004; Spyra & Krodziewska, 2013). In part, its scattered distribution is likely due to heavy deforestation and the resulting decrease of woodland ponds acting as suitable habitat for the species and its hosts (Spyra & Krodziewska, 2013). Beyond abundance, the noticeable lack of species-level diversity in Palearctic members of *Placobdella* could be the result of various phenomena. Prior to the analyses performed herein, it was still unknown whether or not Palearctic populations were panmictic, which would be evident if only a single species was present across the continents; the lack of any newly described Palearctic species would certainly suggest that this is the case. Investigations into the genetic diversity of Palearctic members of *Placobdella* are virtually non-existent (but see Marrone et al., 2016; Vecchioni et al., 2021) and collection efforts have instead largely focused on North American diversity (e.g., Ocegüera-Figueroa et al., 2010; Moser et al., 2012a, b, 2014a, b; Ocegüera-Figueroa & Léon-Régagnon, 2014; de Carle et al., 2017; Mack et al., 2019). Through largely expanded geographic sampling, our results show that the COI variation between members of “*Placobdella costata*” are in line with the interspecific divergences shown for other, well-differentiated annelid taxa (e.g., Trontelj et al., 1996; Kvist, 2016; de Carle et al., 2017), which indicates that at least five separate species-level

taxa are present in the Palearctic. It seems reasonable to suggest that *Placobdella costata* sensu stricto is represented by the well-supported clade C6, seeing as specimens within that clade were collected close to the type locality in southern Ukraine. It should be noted, of course, that COI variation alone does not provide an adequate view of species delimitation. Ecological, morphological, and behavioral data should be leveraged to inform on species boundaries for our proposed taxa.

Notwithstanding the results from the COI variation analysis, the ITS locus does not support several of the separations between clades. It has been shown that ITS evolves at a much slower rate than COI, holds less power in sorting lineages, and that phylogenetic analyses using this locus often produce unresolved topologies (e.g., Martinsson et al., 2017; de Carle et al., 2017). Judging from the relatively large COI distances, we hypothesize that speciation is still ongoing (or is very recent) in *P. costata* s. l. and that isolation between populations will lead to increased variation in ITS given the appropriate amount of time. Although genetic distances are low in ITS, we still see a certain degree of lineage sorting within this locus. In particular, this relates to C5 (including specimens from Algeria and Tunisia), which is present as a distinct clade also in the ITS tree (Fig. 3). Because only a single individual sequence was included for each of C2 (including specimens from Sicily and southern Peninsular Italy) and C3 (including specimens from Azerbaijan), support for these “clades” could not be ascertained from the ITS tree.

Despite the fact that only a single species of *Placobdella* is currently recognized in the Palearctic, historical taxonomic accounts of European placobdelloid taxa might have bearing on the present study insofar as they have suggested species names that are open to accommodate the newly discovered diversity. Moquin-Tandon (1846) described *Glossiphonia catenigera* (Moquin-Tandon, 1846) (= *Placobdella costata*) from the surrounding areas of Toulouse, France and provided both figures and a short description of its morphology, showing a striking resemblance to that of *P. costata*. Although *G. catenigera* has since been subsumed by *P. costata* via a series of taxonomic transfers, we do not exclude the possibility that the species is present among our data and should perhaps be restored to species-level status within the genus *Placobdella*. The same possibility exists for

Placobdella carinata (Diesing, 1858) (= *Placobdella costata*), which Autrum (1936) discussed as being present in Asia Minor, Syria, and Astrachan (Russia) and for *Placobdella roszkowskii* Oka, 1932) (= *Placobdella costata*) from Tbilisi (Georgia) (see Autrum, 1936). Although robust morphological investigations of our newly collected taxa are needed, these three cases represent scenarios in which subsumed species might be re-erected on the basis of increased taxon sampling and species delimitation studies.

Interestingly, our phylogenetic analyses support the hypothesis that Palearctic diversity of *Placobdella* is the result of a single dispersal event from North America to Europe, insofar as the specimens of “*Placobdella costata*” form a monophyletic group (Figs. 2, 3, and 4); this result corroborates previous studies (Siddall et al., 2005; Bielecki et al., 2012). The local dispersal capability of *P. costata* is likely limited, seeing as members of *Placobdella* are typically poor swimmers and seem to preferentially disperse attached to their host (e.g., Bielecki et al., 2012). Lukin (1976) suggested that *P. costata* is the only European leech species capable of feeding on reptilian blood, although the host diversity of the species is still not fully explored. The known hosts for the species include the European pond turtle [*Emys orbicularis* (Linnaeus, 1758)], the Sicilian pond turtle (*Emys trinacris* Fritz, Fattizzo, Guicking, Tripepi, Pennisi, Lenk, Joger & Wink, 2005), the Spanish pond turtle [*Mauremys leprosa* (Schweigger, 1812)], the Balkan pond turtle [*Mauremys rivulata* (Valenciennes, 1833)], and the Caspian turtle [*Mauremys caspica* (Gmelin, 1774)] (Bielecki et al., 2012; Romero et al., 2014; Arizza et al., 2016; Bashirichelkasari & Yadollahvandmiandoab, 2017; Fediras et al., 2017; Laghzaoui et al., 2020), and few of these species are known to disperse over great distances (but see Mantziou et al. 2004; Vecchioni et al., 2020). Sapkarev (1963) recorded *P. costata* from both frogs and aquatic birds, possibly indicating that dispersal with an avian host is possible (note that the identity of the parasitized bird was not provided in that account). This could explain the expansion of its distribution into North Africa and the Middle East, but it is possible that human-mediated dispersal can account for crossing of the strait of Gibraltar. In terms of the dispersal between North America and Europe, fossil evidence suggests that *Emys orbicularis* was present in Europe in the early Miocene (Fritz et al., 1998; Lenk et al., 1999) and may

have brought the leeches attached to their bodies (Bielecki et al., 2012). It is still unknown, however, how the freshwater hosts made the transition from North America to Europe. The *P. costata*/*E. orbicularis* system provides a prime opportunity for the study of co-evolutionary patterns between the species. However, despite concerted efforts, contemporary studies have failed to recover signs of co-evolution or co-speciation between these taxa (Marrone et al., 2016; Vecchioni et al., 2021). It therefore seems unlikely that the species diversity and speciation patterns uncovered herein for *P. costata* are driven by speciation, isolation, or dispersal of the hosts.

The present study is the first to comprehensively address the genetic variation within Palearctic members of *Placobdella* and our results suggest that species-level diversity may be greater than previously thought. Given the lack of both extensive sampling and investigations into the genetic structure within the species, this is not surprising, but still provides fodder for future studies into the population dynamics of Palearctic bloodfeeding leeches and their hosts. It seems likely that more unknown biodiversity will be unveiled through approaches similar to the one used in this study.

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Data availability All DNA sequences used in the present study are deposited in GenBank under Accession Numbers OK350331-OK350352 (COI) and OK356870-OK356884 (ITS).

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that no conflict of interest exists.

Ethical approval Not applicable.

Consent to participate All authors consent.

Consent for publication All authors consent.

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