



Phylogenetic analysis of *Placobdella* (Hirudinea: Rhynchobdellida: Glossiphoniidae) with consideration of COI variation



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ABSTRACT

Placobdella is a genus of blood-feeding leeches in the family Glossiphoniidae. Historically, species of *Placobdella* have posed difficulty for systematists owing to a lack of informative morphological characters and the preponderance of inadequate or incomplete species descriptions. Here, we conduct a phylogenetic analysis of 55 individuals representing 20 of the 24 currently recognized nominal taxa using COI, ND1, 12S rDNA and ITS sequences under parsimony, maximum likelihood and Bayesian inference. We also examine the isolated COI phylogeny for the genus using an expanded dataset encompassing three additional species not included in the concatenated dataset. Finally, we assess genetic variation at the COI locus to validate initial specimen identifications and estimate how COI variation may reflect species boundaries. We conclude that *Placobdella* is a monophyletic group that places as the sister group to a clade formed by the genera *Haementeria* and *Helobdella*. We discuss the evolutionary implications of several internal relationships that are robustly resolved by all three optimality criteria, paying particular attention to the apparent fluidity of morphological characters exhibited by members of *Placobdella*. We also find preliminary evidence for the presence of cryptic and undescribed diversity within the genus.

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1. Introduction

Placobdella Blanchard, 1893 is a genus of sanguivorous, rhynchobdellid (proboscis-bearing) leeches in the family Glossiphoniidae. The genus is almost entirely North American in distribution with one species known from Europe, and one known from Central America (Bielecki et al., 2012; Marrone et al., 2016; Oceguera-Figueroa and Pacheco-Chaves, 2012; Siddall et al., 2005). Members of *Placobdella* are primarily ectoparasitic on turtles, with some specializing on amphibians or aquatic reptiles, though many species will feed opportunistically on other vertebrates including birds, fish and humans (Jones and Woo, 1990; Moser et al., 2010; Siddall and Bowerman, 2006; Siddall and Gaffney, 2004). For some hosts, members of *Placobdella* are vectors of hemogregarine and trypanosome blood parasites (Barta and Desser, 1989; Siddall and Desser, 1990, 1991, 2001).

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At present, the genus includes 24 nominal taxa, but this is likely an underestimate of the true diversity (Oceguera-Figueroa and Siddall, 2008; de Carle, unpublished data); see Figs. 1 and 2 for select representative taxa. Many original descriptions of *Placobdella* species were incomplete, lacking, for example, specific type localities (see Moser et al., 2014) or complete morphological descriptions (see Moser et al., 2013b). Species have also been revised without examination of type material, and several taxa have therefore been erroneously synonymized (see Moser et al., 2013c). For several species, type material has been lost, or was never deposited (see Moser et al., 2014, 2013a). Several of these species have been re-described in recent years (Jones and Woo, 1990; Moser et al., 2012a, 2012b, 2013a, 2013b, 2013c, 2014), highlighting the uncertain identities of several members of the genus, and the pervasiveness of inadequate descriptions. In the past decade, six new species of *Placobdella* have been described: *Placobdella ali Hughes and Siddall, 2007*; *Placobdella lamothei Oceguera-Figueroa and Siddall, 2008*; *Placobdella ringueleti López-Jiménez and Oceguera-Figueroa, 2009*; *Placobdella sophiae Oceguera-Figueroa et al., 2010*; *Placobdella kwetlumye Oceguera-Figueroa et al., 2010*; and *Placobdella siddalli Richardson*

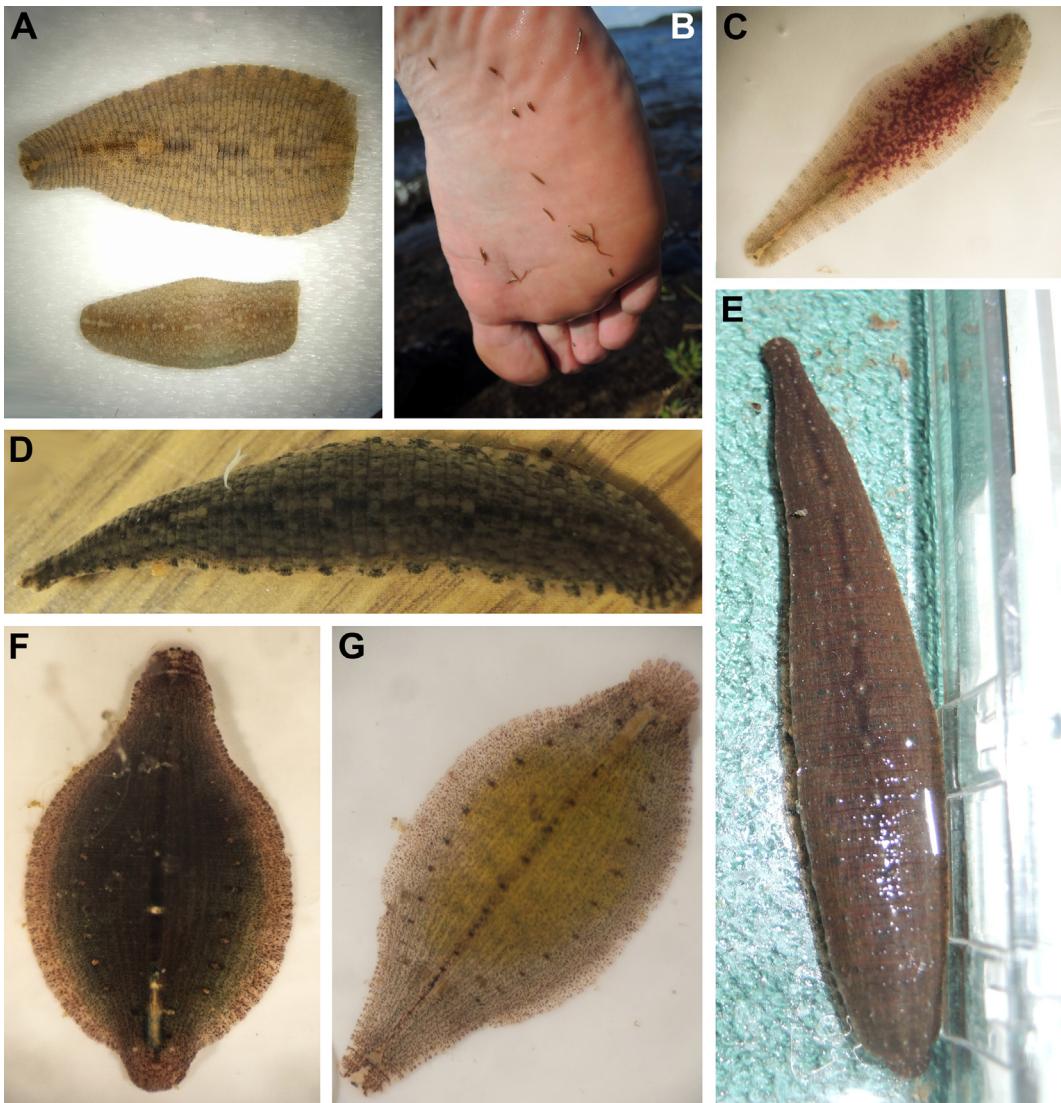


Fig. 1. Selected specimens of *Placobdella*. Photos by D. de Carle or S. Kvist except where specified. (A) Two preserved specimens of *Placobdella rugosa* showing disparate patterns of dorsal pigmentation (Manitoba, Canada; ROMIZ I11553 [top] and ROMIZ I11587 [bottom]); (B) Live juvenile specimens of *Placobdella rugosa* during collecting (Minnesota, USA; photo by A. Oceguera-Figueroa); (C) Live, recently fed juvenile *Placobdella rugosa* with crop caecae visualized by the bloodmeal (Ontario, Canada; ROMIZ I10254); (D) Live adult *Placobdella rugosa* with spermatophore attached to caudal surface (Ontario, Canada; ROMIZ I10476); (E) Live adult *Placobdella* sp. 1 (Saskatchewan, Canada; ROMIZ I11464), this specimen represents the same species as specimens ROMIZ I11277 and ROMIZ I11494 (see Discussion); (F) Live *Placobdella phalera* (Ontario, Canada; ROMIZ I10190); (G) Live *Placobdella picta* with eggs on ventral surface giving the specimen a yellow tinge (Ontario, Canada; ROMIZ I10257).

and Moser, 2017 (Hughes and Siddall, 2007; López-Jiménez and Oceguera-Figueroa, 2009; Oceguera-Figueroa et al., 2010; Richardson et al., 2017).

There is a great deal of uncertainty surrounding the taxonomy of *Placobdella*, stemming largely from a lack of phylogenetically informative morphological characters. Sawyer (1986) described *Placobdella* as including species that possess triannulate mid-body somites, two pairs of coalesced eyespots, two pairs of compact salivary glands, and one pair of bacteriomes – structures that house bacterial endosymbionts – inserting into the oesophagus. Despite these seemingly clear synapomorphies, phylogenetic evaluation of Glossiphoniidae by Light and Siddall (1999) recovered *Placobdella* as paraphyletic due to the inclusion of the genera *Oligobdella* Moore, 1918 and *Desserobdella* Barta and Sawyer, 1990 which exhibit biannulate mid-body somites and diffuse salivary glands, respectively. In a subsequent study, these genera were designated junior synonyms and subsumed within *Placobdella* (Siddall et al., 2005). In addition, constituent species have been

variously assigned to the genera *Haementeria* de Filippi, 1849, *Actinobdella* Moore, 1901; *Clepsine* Savigny, 1822 and *Batracobdella* Viguier, 1879 (Barta and Sawyer, 1990; Moser et al., 2012b; Oceguera-Figueroa, 2012). Furthermore, Auturum (1936) considered *Placobdella* a junior synonym of *Haementeria*, resulting in some nomenclatural complications. As currently defined, the genus *Placobdella* encompasses all glossiphoniid species bearing one pair of cecate bacteriomes, bilobate ovaries, and two coalesced pairs of eyespots (Siddall et al., 2005).

Recently, the unique microbiomes and secretions of *Placobdella* have been investigated. The endosymbiotic bacteria housed in bacteriomes of *Placobdella* species were shown to represent a lineage of alphaproteobacteria (*Reichenowia* Siddall, Perkins and Desser, 2004) related to Rhizobiaceae (Siddall et al., 2004). Although many other glossiphoniids house bacterial endosymbionts in similar structures, *Reichenowia* species are known only from *Placobdella*, and the two taxa likely share close co-evolutionary histories (Perkins et al., 2005; Kvist et al., 2011). Additionally, the peptides

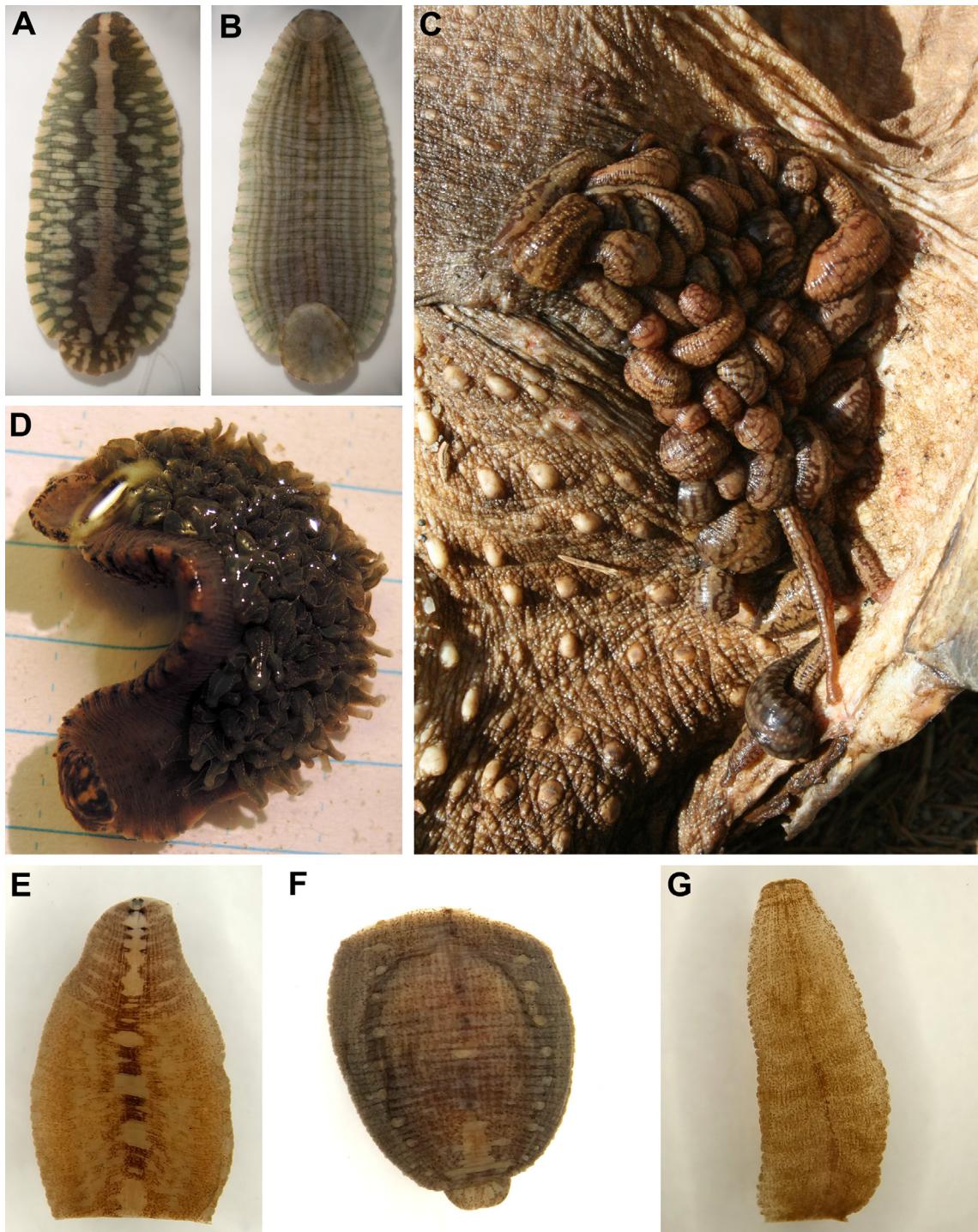


Fig. 2. Selected specimens of *Placobdella*. Photos by D. de Carle or S. Kvist except where specified. (A) Dorsal surface of preserved specimen of *Placobdella parasitica* (Ontario, Canada; ROMIZ I10340); (B) Ventral surface of preserved specimen of *Placobdella parasitica* (Ontario, Canada; ROMIZ I10340); (C) Live specimens of *Placobdella parasitica* feeding on the hind leg of a common snapping turtle (*Chelydra serpentina*) (Ontario, Canada; photo by P. Moldowan); (D) Live *Placobdella rugosa* with juveniles attached to ventral surface (Ontario, Canada; ROM IZ I10091); (E) Preserved *Placobdella hollensis* with posterior portion cut for DNA sequencing (Ontario, Canada; ROMIZ I10341); (F) Preserved *Placobdella translucens* with anterior portion cut for DNA sequencing (Michigan, USA; see Discussion); (G) Preserved *Placobdella multilineata* with posterior portion cut for DNA sequencing (Louisiana, USA).

present in leech saliva are renowned for their unparalleled anticoagulant and antimetastatic properties (e.g. Holt et al., 1989; Markwardt, 2002; Mumcuoglu, 2014). One such compound, ornatin, was first isolated from *Placobdella ornata* (Verrill, 1872), and several other anticoagulants have also been recovered from members of the genus (Mazur et al., 1991; Siddall et al., 2016).

Notwithstanding the importance of members of the genus as ectoparasites and sources of medically important compounds, the

phylogenetic relationships within the genus remain largely unknown and the phyletic status of *Placobdella* has only been tested with a limited subset of ingroup and outgroup taxa (Light and Siddall, 1999; Oceguera-Figueroa et al., 2016; Siddall et al., 2005; Siddall and Burreson, 1998). Knowing the phylogenetic associations within the genus may benefit studies into bacterial symbioses, anticoagulant evolution, and co-speciation, among other things. To address these ambiguities and facilitate future research

Table 1

Locality information and GenBank accession numbers for leech sequences used in phylogenetic analyses of *Placobdella*. Bold fonts indicate sequences that were downloaded from GenBank.

Taxon	Specimen code	Locality	GenBank accession numbers			
			COI	12S	ND1	ITS
Ingroup						
<i>Placobdella ali</i>	ROMIZ I12965	New York, USA	MF067146	–	–	–
<i>Placobdella ali</i>	ROMIZ I12966	New York, USA	MF067145	MF067200	MF067202	–
<i>Placobdella biannulata</i>		North Carolina, USA	AF116021	AY425435	AY047343	–
<i>Placobdella burresonae</i>	ROMIZ I12967	USA	MF067144	MF067199	MF067203	–
<i>Placobdella costata</i>	ROMIZ I12968	Portugal	MF067143	MF067198	MF067204	MF067089
<i>Placobdella costata</i>	ROMIZ I12969		MF067142	MF067197	MF067205	MF067088
<i>Placobdella cryptobranchii</i>		Missouri, USA	KF601755	–	–	–
<i>Placobdella cryptobranchii</i>		Missouri, USA	KF601761	–	–	–
<i>Placobdella hollensis</i>	ROMIZ I10341	Frontenac Provincial Park, Ontario, Canada	MF067141	MF067196	MF067206	MF067087
<i>Placobdella hollensis</i>	ROMIZ I10356	Frontenac Provincial Park, Ontario, Canada	MF067140	MF067195	MF067207	MF067086
<i>Placobdella kwetlumye</i>	ROMIZ I12970	Idaho, USA	MF067139	MF067194	MF067208	MF067085
<i>Placobdella kwetlumye</i>	ROMIZ I12971	Squires Lake, Washington, USA	MF067138	MF067193	MF067209	MF067084
<i>Placobdella lamothei</i>	ROMIZ I12972	Nuevo Urecho, Michoacan, Mexico	MF067137	MF067192	–	MF067083
<i>Placobdella lamothei</i>	ROMIZ I12973	Tonatino, Estado de México, Mexico	MF067136	MF067191	LT159849	MF067082
<i>Placobdella lamothei</i>	ROMIZ I12974	Tonatino, Estado de México, Mexico	MF067135	MF067190	–	MF067081
<i>Placobdella lamothei</i>	ROMIZ I12975	Mexico	MF067134	MF067189	–	MF067080
<i>Placobdella mexicana</i>	ROMIZ I12976	Mexico	MF067133	MF067188	MF067210	MF067079
<i>Placobdella mexicana</i>	ROMIZ I12977	El Vergel, Mexico	MF067132	MF067187	MF067211	MF067078
<i>Placobdella mexicana</i>	ROMIZ I12978	Mexico	MF067131	–	–	–
<i>Placobdella montifera</i>	ROMIZ I12979	Friends Landing, Washington, USA	MF067130	MF067186	MF067212	MF067077
<i>Placobdella montifera</i>	ROMIZ I12980		MF067129	MF067185	MF067213	MF067076
<i>Placobdella multilineata</i>		Lukfata Creek, Oklahoma, USA	KM396760	–	–	–
<i>Placobdella multilineata</i>		Lukfata Creek, Oklahoma, USA	KM396761	–	–	–
<i>Placobdella multilineata</i>		Maurepas Swamp, Louisiana, USA	AY962464	–	AY962451	–
<i>Placobdella nuchalis</i>	ROMIZ I12981	Mohonk Camp, New York, USA	MF067128	MF067184	MF067214	MF067075
<i>Placobdella nuchalis</i>	ROMIZ I12982	Mohonk Cove, New York, USA	MF067127	MF067183	MF067215	MF067074
<i>Placobdella nuchalis</i>	ROMIZ I12983	John Allen Pond, New York, USA	MF067126	MF067182	MF067216	MF067073
<i>Placobdella ornata</i>		The Donkmeer, East Flanders Province, Belgium	KP176597	–	–	–
<i>Placobdella ornata</i>		Shivericks Pond, Massachusetts, USA	JQ812136	–	–	–
<i>Placobdella ornata</i>		Shivericks Pond, Massachusetts, USA	JQ812135	–	–	–
<i>Placobdella papillifera</i>	ROMIZ I12984	John Allen Pond, New York, USA	MF067125	MF067181	MF067217	MF067072
<i>Placobdella papillifera</i>	ROMIZ I12985	John Allen Pond, New York, USA	MF067124	MF067180	MF067218	MF067071
<i>Placobdella papillifera</i>		West River, Connecticut, USA	KC505241	–	–	–
<i>Placobdella papillifera</i>		West River, Connecticut, USA	KC505242	–	–	–
<i>Placobdella parasitica</i>	ROMIZ I10311	North of Algonquin Provincial Park, Ontario, Canada	MF067123	MF067179	MF067219	MF067070
<i>Placobdella parasitica</i>	ROMIZ I10340	Pearkes Lake, Ontario, Canada	MF067122	MF067178	MF067220	MF067069
<i>Placobdella pediculata</i>	ROMIZ I12986		MF067121	MF067177	MF067221	MF067068
<i>Placobdella pediculata</i>	ROMIZ I12987		MF067120	MF067176	MF067222	MF067067
<i>Placobdella phalera</i>	ROMIZ I10190	Clear Lake, Ontario, Canada	MF067119	MF067175	MF067223	MF067066
<i>Placobdella phalera</i>	ROMIZ I10284	Pacaud Lake, Ontario, Canada	MF067118	MF067174	MF067224	MF067065
<i>Placobdella phalera</i>	ROMIZ I12988	Canopus Ramp, New York, USA	MF067117	MF067173	MF067225	MF067064
<i>Placobdella phalera</i>	ROMIZ I12989		MF067116	MF067172	MF067226	MF067063
<i>Placobdella phalera</i>	ROMIZ I12990	Tennessee, USA	MF067115	–	–	–
<i>Placobdella phalera</i>	ROMIZ I12991	Virginia, USA	MF067114	–	–	–
<i>Placobdella picta</i>	ROMIZ I10111	Kenny Lake, Ontario, Canada	MF067113	MF067171	MF067227	MF067062
<i>Placobdella picta</i>	ROMIZ I10235	Restoule Provincial Park, Ontario, Canada	MF067112	MF067170	MF067228	MF067061
<i>Placobdella picta</i>	ROMIZ I10257	Moore Lake, Ontario, Canada	MF067111	MF067169	MF067229	MF067060
<i>Placobdella picta</i>	ROMIZ I11395	Moose Mountain Provincial Park, Saskatchewan, Canada	MF067109	–	–	–
<i>Placobdella picta</i>	ROMIZ I10515	Nebraska, USA	MF067110	MF067168	MF067230	MF067059
<i>Placobdella ringueleti</i>	ROMIZ I12992	Chiapas, Mexico	MF067108	MF067167	–	MF067058
<i>Placobdella ringueleti</i>	ROMIZ I12993	Catemaco, Veracruz, Mexico	MF067107	MF067166	–	–
<i>Placobdella rugosa</i>	ROMIZ I10089	Mijinemungshing Lake, Ontario, Canada	MF067106	MF067165	MF067231	MF067057
<i>Placobdella rugosa</i>	ROMIZ I10100	Mijinemungshing Lake, Ontario, Canada	MF067105	MF067164	MF067232	MF067056
<i>Placobdella rugosa</i>	ROMIZ I10101	Mijinemungshing Lake, Ontario, Canada	MF067104	MF067163	MF067233	MF067055
<i>Placobdella rugosa</i>	ROMIZ I10119	Paquette Lake, Ontario, Canada	MF067103	MF067162	MF067234	MF067054
<i>Placobdella rugosa</i>	ROMIZ I10565	Nebraska, USA	MF067102	MF067161	MF067235	MF067053
<i>Placobdella rugosa</i>	ROMIZ I11411	Pipestone Creek, Moosomin Regional Park, Saskatchewan, Canada	MF067101	MF067160	MF067236	–
<i>Placobdella rugosa</i>	ROMIZ I11553	Adam Lake, Manitoba, Canada	MF067100	MF067159	MF067237	MF067052
<i>Placobdella rugosa</i>	ROMIZ I11587	Singuiish Lake, Duck Mountain Provincial Park, Manitoba, Canada	MF067099	MF067158	MF067238	MF067051
<i>Placobdella rugosa</i>	ROMIZ I11598	Unnamed pond, Duck Mountain Provincial Park, Manitoba, Canada	MF067098	MF067157	MF067239	–
<i>Placobdella rugosa</i>		Belcourt Lake, North Dakota, USA	JX412988	–	–	–
<i>Placobdella rugosa</i>		Belcourt Lake, North Dakota, USA	JX412989	–	–	–
<i>Placobdella siddalli</i>		Davis Eddy, George County, Mississippi, USA	KY780962	–	–	–
<i>Placobdella sophieae</i>	ROMIZ I12994	Washington, USA	MF067097	MF067156	MF067240	MF067050
<i>Placobdella sophieae</i>	ROMIZ I12995	Washington, USA	MF067096	MF067155	MF067241	MF067049
<i>Placobdella sp. 1</i>	ROMIZ I11277	Half Moon Lake, Alberta, Canada	MF067095	MF067154	MF067242	MF067048
<i>Placobdella sp. 1</i>	ROMIZ I11494	Adams Lake, Saskatchewan, Canada	MF067094	MF067153	MF067243	MF067047
<i>Placobdella sp. 2</i>	ROMIZ I12996	Canopus Ramp, New York, USA	MF067093	MF067152	MF067244	MF067046
<i>Placobdella sp. 2</i>	ROMIZ I12997		MF067092	MF067151	MF067245	MF067045

(continued on next page)

Table 1 (continued)

Taxon	Specimen code	Locality	GenBank accession numbers			
			COI	12S	ND1	ITS
<i>Placobdella</i> sp. 3	ROMIZ I12998	Maryland, USA	MF067091	MF067150	MF067246	–
<i>Placobdella</i> sp. 4	ROMIZ I12999		MF067090	MF067149	MF067247	MF067044
<i>Placobdella translucens</i>		Michigan, USA	AY047328	–	AY047354	
Outgroup						
<i>Americobdella valdiviana</i>		Valdivia, Chile	AY425443	AY425407	–	–
<i>Erbobdella octoculata</i>			HQ336344	AF099954	–	JX885698
<i>Alboglossiphonia heteroclita</i>		Michigan, USA	AF116016	AF099955	AY047339	–
<i>Alboglossiphonia weberi</i>		Kalihi Stream, Hawaii, USA	AY962453	–	AY962440	–
<i>Glossiphonia baicalensis</i>			AY047329	–	AY047355	–
<i>Glossiphonia complanata</i>		UK	AY047321	AY425414	AY047344	–
<i>Glossiphonia elegans</i>		West River, Connecticut, USA	JQ073866	–	JQ073889	–
<i>Haementeria acuecuyetzin</i>		Oaxaca, Mexico	JN850909	JN850871	JN850939	JN850931
<i>Haementeria depressa</i>		Tajamar en Maldonado, Uruguay	JN850902	JN850864	JN850943	JN850924
<i>Haementeria ghilianii</i>		BioPharm, French Guiana	AF329035	AY425417	AF329058	JN850932
<i>Haementeria gracilis</i>		Arroyo Aspinas, Uruguay	AF329034	AY425418	AF329057	–
<i>Haementeria lopezi</i>		Colima, Mexico	JN850898	JN850861	JN850940	JN850920
<i>Haementeria officinalis</i>		Michoacan, Mexico	JN850906	JN850868	JN850952	JN850928
<i>Haementeria paraguayensis</i>		Chaco, Argentina	JN850908	JN850870	–	JN850930
<i>Haementeria tuberculifera</i>		Iquitos, Peru	JN850910	–	JN850937	JN850933
<i>Helobdella elongata</i>		Silver Lake, Michigan, USA	AF329045	JN850882	AF329068	–
<i>Helobdella fusca</i>		Wild Goose Lake, Michigan, USA	AF329038	–	AF329061	–
<i>Helobdella lineata</i>		Gloucester, Virginia, USA	AF329039	–	AF329062	–
<i>Helobdella papillata</i>		Gloucester, Virginia, USA	AF329046	–	AF329069	–
<i>Helobdella paranensis</i>		Arroyo Aspinas, Uruguay	AF329037	AY425412	AF329060	–
<i>Helobdella robusta</i>			MF067148	–	MF067201	–
<i>Helobdella stagnalis</i>			MF067147	AY425424	AF329064	–
<i>Helobdella transversa</i>		Cheboygan State Park, Michigan, USA	AF329044	–	AF329067	–
<i>Helobdella triserialis</i>		Laguna Volcan, Bolivia	AF329054	JN850883	AF329077	–
<i>Hemiclepsis marginata</i>		Étang de la Musse, France	AF003259	AY425425	AY047336	–
<i>Marsupiobdella africana</i>		South Africa	AF116015	AY425433	AY047347	–
<i>Placobdelloides jaegerskioeldi</i>		Nelspruit, South Africa	AY962463	–	AY962450	–
<i>Placobdelloides siamensis</i>		Wat Bovorn, Thailand	AY962462	–	AY962449	–
<i>Theromyzon bifarium</i>			AY047330	–	AY047356	–
<i>Theromyzon tessulatum</i>			AY047318	AF099957	AY047338	–
<i>Hirudo medicinalis</i>			EF446712	JN118994	–	JN119038
<i>Ozobranchus margo</i>		Virginia Beach, Virginia, USA	AF003268	–	AY047331	–
<i>Calliobdella vivida</i>			AF003260	AY425409	AY047333	–
<i>Heptacyclus virgatus</i>			DQ414319	AY336048	DQ414363	–
<i>Oceanobdella sexoculata</i>		New Brunswick, Canada	DQ414332	–	DQ414377	–
<i>Oxytonostoma typica</i>		Barents Sea, Finnmarken Bank	EF405596	EF405565	EF405555	–
<i>Piscicola geometra</i>			AF003280	AY425437	AY047334	–
<i>Pontobdella muricata</i>			AY336029	AF099958	EF405545	–
<i>Pontobdella mactothela</i>			DQ414342	AY425440	DQ414387	–

on members of the genus, we here conduct a comprehensive phylogenetic analysis of *Placobdella*, encompassing more than twice the number of species used in prior studies. The main goals of this study are to determine the position of *Placobdella* within Glossiphoniidae, to test the status of the genus as a monophyletic assemblage, and to resolve internal relationships. Using a subset of the data generated for this purpose, in combination with additional data available from GenBank, we also assess COI variation within and between selected taxa to confirm species boundaries and investigate whether taxonomic labels correspond to operational taxonomic units.

2. Materials and methods

2.1. Material examined

Specimens were collected during recent Royal Ontario Museum (ROM) field expeditions across several Canadian provinces and Nebraska, USA. Leeches were collected from the undersides of rocks, wood and debris, from exposed skin, and from traps baited with beef liver. These collections were then supplemented by material from existing collections at the American Museum of Natural History (AMNH). Newly collected leeches were relaxed in ~20% ethanol

and fixed by gradually increasing the concentration of 95% ethanol. To avoid contamination by the ingesta of the leeches, tissue was cut from the caudal suckers of specimens. Tissues were stored at –20 °C prior to DNA extraction. In cases where specimens – rather than only DNA isolates – were available, the full bodies of those leeches were permanently stored in the ROM's Department of Invertebrate Zoology to serve as vouchers for identifications (Table 1). Of 24 species currently recognized within the genus, 23 – including the type species *Placobdella costata* (Müller, 1846) – were included in different constellations throughout the datasets. All but four species [*Placobdella burresonae* Siddall and Bowerman, 2006; *Placobdella biannulata* (Moore, 1900); *Placobdella ali*; and *Placobdella translucens* Sawyer and Shelley, 1976] are represented by at least two individuals affording a safeguard against incorrect specimen identifications. In the cases of *Placobdella rugosa* Moore, 1901; *Placobdella phalera* (Graff, 1899); *Placobdella picta* (Verrill, 1872) (Fig. 1G); and *Placobdella lamothei*, a high degree of intraspecific variation was evident – either morphological or genetic – and additional specimens were analysed to ensure that this diversity was represented. Outgroup sequences for other glossiphoniids, as well as representatives of Hirudinidae, Piscicolidae, Erpobdellidae, and Americobdellidae, were obtained from GenBank. Following previous phylogenetic hypotheses (Apakupakul et al., 1999; Borda and Siddall, 2004), all

Table 2

Primers used for gene amplification and sequencing for phylogenetic analyses of *Placobdella*.

Gene	Primer name	Primer sequence	Reference
<i>Nuclear</i>			
ITS	ITS5	5'-GGAAGTAAAAGTCATAACAAGG-3'	White et al. (1990)
	ITS4	5'-TCCTCCGCTTATTGATATGC-3'	White et al. (1990)
	5.8mussF	5'-CGCAGCCAGCTGCGTGAATTATGT-3'	Källersjö et al. (2005)
	5.8mussR	5'-GATGTCGATGTTCAATGTCGTCG-3'	Källersjö et al. (2005)
<i>Mitochondrial</i>			
COI	LCO Plac	5'-AYTCAACTAACATCAYAAAGAYATTGG-3'	Designed for this study
	HCO Plac	5'-TADACTTCWGGRTGACCAAAAAATCA-3'	Designed for this study
12S	12S _a	5'-AACIIGGATTAGATAACCC-3'	Simon et al. (1994)
	12S _b	5'-GAGAGTGACGGGGATGTGT-3'	Simon et al. (1994)
ND1	LND300	5'-TGGCAGAGTAGTCGATTAGG-3'	Light and Siddall (1999)
	HND1932	5'-CCTCAGCAAATCAAATGG-3'	Light and Siddall (1999)

trees resulting from the concatenated dataset were rooted at *Hirudo medicinalis* Linnaeus, 1758.

2.2. DNA extraction and sequencing

DNA from freshly collected material was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA). Mitochondrial cytochrome c oxidase subunit I (COI), NADH dehydrogenase subunit I (ND1), 12S ribosomal DNA and nuclear Internal Transcribed Spacers 1 and 2 flanking the 5.8S ribosomal gene (ITS) were amplified for each sample. Polymerase Chain Reactions (PCRs) were carried out in 25 µl volumes consisting of 16.39 µl ddH₂O; 2.5 µl buffer; 2.5 µl MgCl₂; 1 µl of each primer (Table 2; note that the primer pair for COI was designed for this study) at 10 µM concentration; 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 extension at 72 °C (7 min); for ND1, 94 °C (5 min) followed by 35 cycles of 94 °C (30 s), 46 °C (30 s), 72 °C (45 s) and final extension at 72 °C (7 min); for 12S, 94 °C (2 min) followed by 35 cycles of 94 °C (30 s), 48 °C (30 s), 72 °C (45 s) and final extension at 72 °C (7 min); for ITS, 95 °C (5 min) followed by 35 cycles of 95 °C (30 s), 48 °C (30 s), 72 °C (90 s) and final extension at 72 °C (8 min). In cases where the initial PCRs failed to amplify the DNA, annealing temperatures or volumes of DNA and/or Taq polymerase were adjusted to achieve successful amplifications.

All PCR products were run on a 1% agarose gel, and successful amplifications were subsequently purified with ExoSAP-IT (Affymetrix, Santa Clara, CA) according to the manufacturers protocol. Cycle sequencing reactions (10 µl total volume) were carried out using 0.5 µl each of ABI Big Dye Terminator V 3.1 and Big Dye 5x Sequencing Buffer (Applied Biosystems, Carlsbad, CA); 2 µl primer at 10 µM concentration (Table 2); and 2.5 µl purified PCR product. 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 3730 (Applied Biosystems, Carlsbad, CA). All DNA sequences generated by the present study are deposited in GenBank under accession numbers MF067044–MF067247 (Table 1).

2.3. Alignment and phylogenetic analysis

Sequences were assembled and edited using Geneious 9.0.4 (Kearse et al., 2012). All sequences were BLASTed (using BLASTn) against the non-redundant (nr) sequence database on GenBank in order to screen for potential contamination, and COI sequences for each sample were BLASTed to affirm specimen identifications. Sequence data for each locus were then aligned separately using the online version of MAFFT ver. 7 (Katoh and Standley, 2013), with default settings applied and choice of search strategy set to automatic. To account for small internal stretches of missing sequence data in ITS for some taxa, resulting from low quality base calls at the beginning and end of reads, the wildcard N setting was used. Resulting alignments were concatenated for phylogenetic analysis using Mesquite ver. 3.11 (Maddison and Maddison, 2016). Gaps were treated as missing data. Analyses were carried out on a combined dataset of all four loci and an expanded COI-only alignment, which included GenBank sequences for some species that were unrepresented in the concatenated dataset. The COI-only alignment contained a subset of the outgroup taxa used in the total data analysis. Tissues and DNA isolates for *Placobdella cryptobranchii* (Johnson and Klemm, 1977), *Placobdella siddalli* and *Placobdella ornata* were not available to us, but published COI sequences were retrieved from GenBank and included in the COI gene tree analysis. Due to difficulties with amplification, the ND1 sequence used for *P. lamothei* (ROMIZ I12973) was taken from a previously published mitochondrial genome of the species (GenBank accession number LT159849), which had a COI sequence that was identical to our specimen. A total of 39 outgroup taxa were used for phylogenetic analyses; these sequences were downloaded from GenBank. Taxa were included if data for at least two of the four loci were available. The ITS locus has only been sequenced for one previous study of leeches (Oceguera-Figueroa, 2012); therefore, relatively few comparative data for this locus are present on GenBank.

Maximum parsimony (MP) analysis was conducted in TNT ver. 1.5 (Goloboff et al., 2008) using a New Technology search for 1000 replications with five rounds of ratcheting and five rounds of tree fusing after the initial Wagner tree builds. Support values were estimated by 1000 rounds of standard bootstrapping, applying the same settings as above.

Prior to Bayesian inference (BI) and maximum likelihood (ML) analyses, PartitionFinder ver. 1.1.1 (Lanfear et al., 2012) was used to determine the best-fitting models of nucleotide evolution under the “greedy” search algorithm and according to the Akaike Information Criterion (AIC). All loci were separately assessed and protein-coding sequences (COI and ND1) were also analysed according to codon position.

MrBayes ver. 3.2.6 (Huelsenbeck and Ronquist, 2001) was run on the CIPRES Science Gateway (Miller et al., 2010) for 10 million generations with trees sampled every 1000 generations. PartitionFinder suggested a general time reversible (GTR) model and GAMMA distribution of nucleotide rates for all partitions, and an estimated proportion of invariable sites for all partitions except COI 3rd codon position, ND1 3rd codon position and ITS. Burn-in was set to 25% and Tracer ver. 1.6 (Bouckaert et al., 2014) was used to confirm that the MCMCMC had reached stationarity.

Maximum likelihood analyses were carried out using RaxML ver. 8 (Stamatakis, 2014) on the CIPRES platform. Tree searches consisted of 1000 replicates with 25 initial GAMMA rate categories and final optimization using four GAMMA shape categories; bootstrap values were calculated using 1000 pseudoreplicates of the rapid bootstrap algorithm, with the same settings as above.

Initial results for MP analyses showed low support for the monophyly of *Placobdella*. Translating the COI nucleotide sequences into amino acids increased support values, but also

drastically reduced the level of resolution in the tree. We therefore used [Xia et al.'s \(2003\)](#) method of inferring nucleotide substitution saturation as implemented in DAMBE ver. 6.4.2 ([Xia, 2013](#)) to determine whether this might be a confounding factor in our analysis. The analysis was performed on the COI partition of the concatenated dataset, and again on the third codon position in isolation. DAMBE analyses coded unresolved bases as question marks, considered all sequences – not only unique ones – and examined all sites for 180 jackknife replicates assuming 13% invariant sites (estimated by the software) for the entire dataset, and 1% invariant sites for third codon positions.

2.4. Species delimitation and pairwise distances

To objectively infer the number of species within the dataset, in comparison to the number of unique taxonomic labels, we used the Bayesian implementation of the PTP model for species delimitation ([Zhang et al., 2013](#)) via the bPTP webserver at <http://species.h-its.org/ptp/>. An ML tree inferred by RaxML from an alignment of COI sequences for the ingroup alone was used as input. To avoid errors associated with uneven taxon sampling (see [Zhang et al., 2013](#)), the

dataset was restricted to a maximum of two representatives per species. When more than two representatives of a species were available, the analysis considered only the individuals that were phylogenetically most distant on the trees inferred from the total dataset. Representatives of *Placobdella nuchalis* [Sawyer and Shelley, 1976](#); *Placobdella hollensis* ([Whitman, 1892](#)); *Placobdella parasitica* ([Say, 1824](#)); and *P. costata* had identical COI sequences, and only one sequence for each of the four species was included in the analysis, as presence of many short branches in the input tree can cause inaccurate results. Because the PTP method requires a correctly rooted tree, we selected *Placobdella montifera* Moore, 1906 as the root in accordance with the ML COI analysis (not shown). The bPTP analysis was run for 500,000 generations, with thinning set to 100 and burn-in set to 0.25.

Uncorrected-p distances between COI sequences for all taxa were calculated in MEGA ver. 7.0.21 ([Kumar et al., 2016](#)) with missing data ignored for each sequence pair, under the objective function of minimum evolution and equal rates for variable sites. Sequences were grouped according to taxonomic labels, and average interspecific and intraspecific distances were calculated. Because the morphology of an unidentified species, *Placobdella*

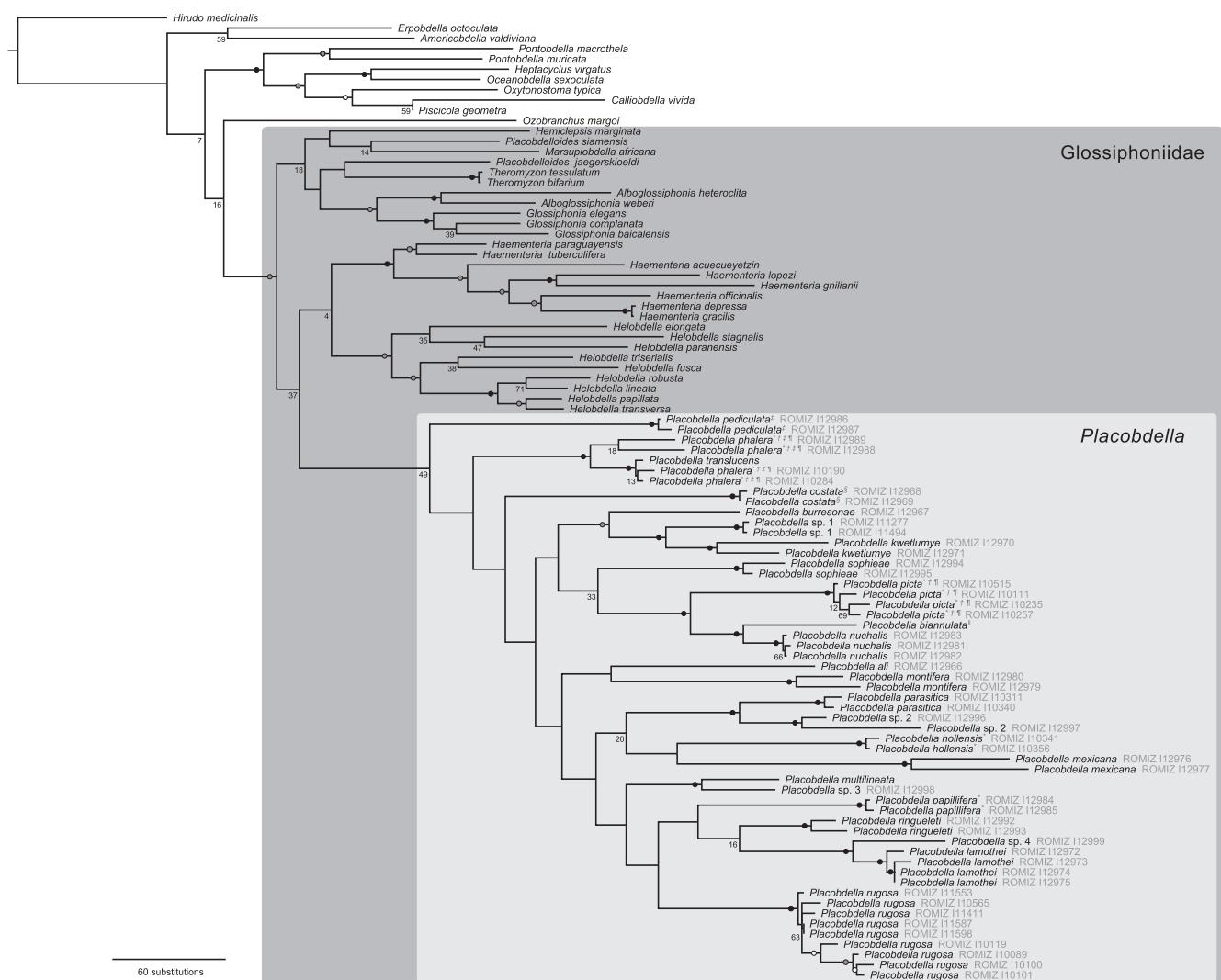


Fig. 3. Strict consensus of two most parsimonious trees depicting the phylogenetic relationships of the leech genus *Placobdella* based on combined data from COI, ND1, 12S rDNA and ITS (length = 10470 steps; Consistency Index = 0.309; Retention Index = 0.627). Black circles denote nodes with 100% bootstrap support; grey circles denote bootstrap support values of 85–99%; white circles denote 75–84% bootstrap support; bootstrap support values below 75% are indicated below nodes. Royal Ontario Museum voucher numbers or specimen codes for ingroup taxa are listed in grey. Superscript symbols indicate species that have previously been classified in other genera: ^{*}*Clepsine*; [†]*Batracobdella*; [‡]*Actinobdella*; [§]*Haementeria*; ^{||}*Oligobdella*; ^{*}*Desserobdella*.

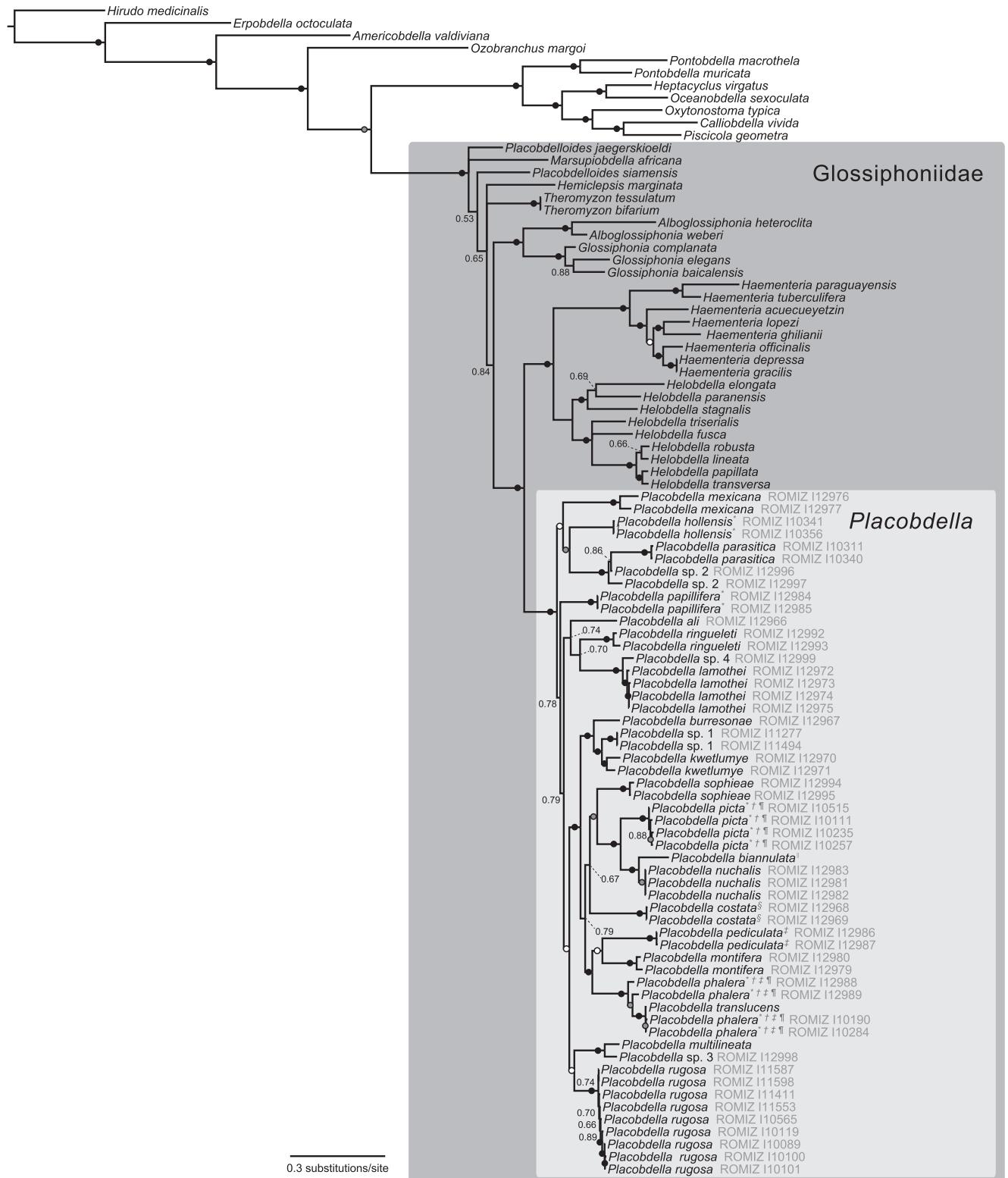


Fig. 4. Bayesian Inference tree based on combined data from COI, ND1, 12S rDNA and ITS. Black circles denote nodes with posterior probability of 1.00; grey circles denote posterior probability of 0.95–0.99; white circles denote 0.90–0.94 posterior probability; posterior probabilities below 0.90 are indicated below nodes. Royal Ontario Museum voucher numbers or specimen codes for ingroup taxa are listed in grey. Superscript symbols indicate species that have previously been classified in other genera: **Clepsine*; †*Batracobdella*; ‡*Actinobdella*; §*Haementeria*; ||*Oligobdella*; **Desserobdella*.

sp. 1 (represented by ROMIZ I11277 and ROMIZ I11494), does not conform to any available species description within the genus (see Fig. 1E), distances for this species and its sister taxon, *P. kwetlumye*,

were calculated for each locus and averaged to represent distance between the taxa, in order to securely infer the separation of these two species.

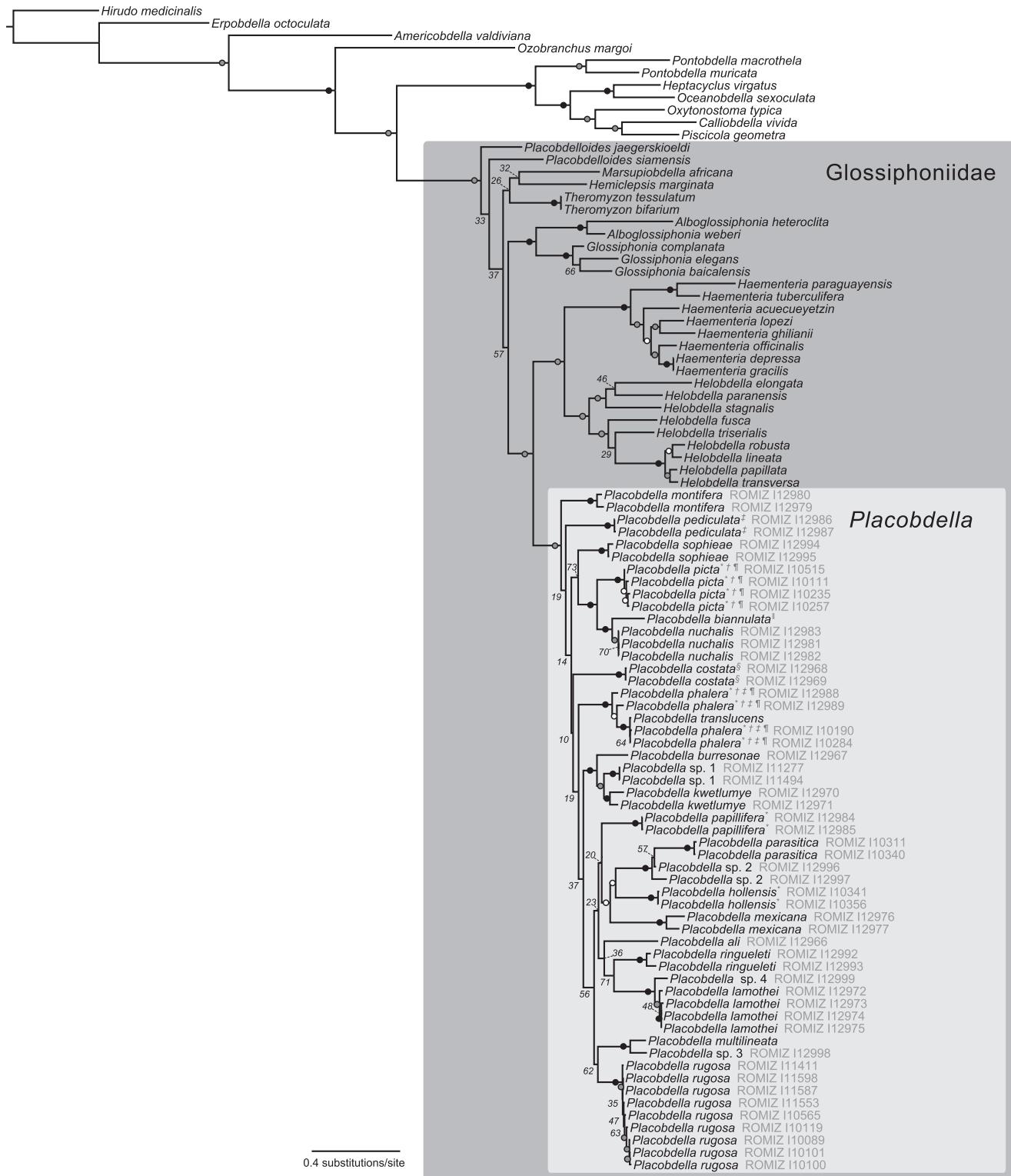


Fig. 5. Maximum likelihood tree topology based on combined data from COI, ND1, 12S rDNA and ITS (log likelihood = -47145.474664). Black circles denote nodes with 100% bootstrap support; grey circles denote bootstrap support values of 85–99%; white circles denote 75–84% bootstrap support; bootstrap support values below 75% are indicated below nodes. Royal Ontario Museum voucher numbers or specimen codes for ingroup taxa are listed in grey. Superscript symbols indicate species that have previously been classified in other genera: ^{*}Clepsine; [†]Batracobdella; [‡]Actinobdella; [§]Haementeria; ^{||}Oligobdella; [¶]Desserobdella.

3. Results

3.1. Phylogenetic analyses

Some clades were consistently recovered with high support across all analyses; however, support for several ingroup relation-

ships, especially those at deeper nodes, was low. All of our phylogenetic trees recover *Placobdella* as sister group to a clade formed by the genera *Haementeria* and *Helobdella* Blanchard, 1896. In the MP tree, this node is not well supported (parsimony bootstrap support [PBS] = 37%) (Fig. 3), but the support value is substantially increased in the BI (Fig. 4) and ML (Fig. 5) analyses (posterior prob-

ability [PP] = 1.00; ML bootstrap support [LBS] = 86%). All three analyses support the hypothesis that *Placobdella* forms a monophyletic group, but only BI and ML resolve this node with high support (PBS = 49%; LBS = 98%; PP = 1.00).

With few exceptions, the MP analysis generated a tree with low support (PBS < 80%) for nearly all ingroup nodes deeper than those joining conspecific individuals. Robust sister-group relationships between *Placobdella nuchalis* and *Placobdella biannulata*, and between that sister pair and *Placobdella picta*, are recovered (PBS = 100% for all nodes). A clade formed by *P. kwetlumye*, *Placobdella* sp. 1 (ROMIZ I11277 and ROMIZ I11449) (monophyletic with PBS = 100%) and *P. burresonae* is also well-supported (PBS = 99%), as is a sister group relationship between *Placobdella parasitica* and a separate unidentified species, *Placobdella* sp. 2 (ROMIZ I12996 and ROMIZ I12997) (PBS = 100%).

All highly supported nodes in the MP tree are also present with high support (LBS ≥ 99%; PP ≥ 0.99) in the BI and ML trees, with one exception: the unidentified taxa represented by ROMIZ I12996 and ROMIZ I12997 (see *Placobdella* sp. 2 in the parsimony analysis) do not form a clade in either the ML or BI analyses. Instead, ROMIZ I12996 groups with *P. parasitica* (LBS = 57%) and this clade, in turn, is placed as sister group to ROMIZ I12997 (LBS = 100%). Both the ML and BI analyses recover support for the aforementioned clade as sister group to *Placobdella mexicana* Moore, 1898 (LBS = 80%; PP = 0.94). Furthermore, *Placobdella multilineata* Moore, 1953 forms a sister group relationship with *P. rugosa* in both trees, but the node is only well supported by BI (LBS = 62%; PP = 0.91). Bayesian inference supports a clade formed by *Placobdella pediculata* Hemmingway, 1908 and *P. montifera* (PP = 0.91), which places as the sister taxon to *P. phalera* (PP = 1.00). The BI analysis also supports *Placobdella sophieae* as the sister group to the clade formed by *P. nuchalis*, *P. biannulata*, and *P. picta* (PP = 0.99); this node is present in all trees, but support is lower in the MP and ML analyses (PBS = 33%; LBS = 73%).

The COI-only tree recovered all hypothesized species groupings as monophyletic – i.e., all representatives for each species placed together – with high support (LBS ≥ 91%), except for *Placobdella cryptobranchii*, *Placobdella lamothei* and *Placobdella phalera*. The two individuals of *P. cryptobranchii*, grouped together, but with low support (LBS = 44%). All individuals identified as *P. lamothei* grouped together, but the clade also included the unidentified specimen ROMIZ I12999. *Placobdella phalera* was found to be paraphyletic, with *P. transluscens* nesting within the cluster. *Placobdella ornata* and *P. cryptobranchii* formed a sister group relationship (LBS = 85%). Sequences for *P. ornata*, *P. siddalli*, and *P. cryptobranchii* did not nest within any other species, suggesting that none of the previously unidentified samples belonged to any of these taxa. A key motivation for including these taxa and performing a COI-only analysis was to address the taxonomic confusion of *P. ornata* and *P. rugosa* (see Discussion).

The nucleotide substitution saturation analysis indicates that “substantial saturation” is present in a dataset if $I_{SS} > I_{SS.c}$. There was no evidence of significant saturation in the entire COI alignment (for 32 operational taxonomic units [OTUs], $I_{SS} = 0.302$ and $I_{SS.c}$ for an asymmetrical tree – i.e. ladderized – topology = 0.392). The same analysis did show substantial saturation when performed on the alignment of the third codon position only (for 32 OTUs, $I_{SS} = 0.663$ and $I_{SS.c}$ for an asymmetrical topology = 0.366). The COI third codon position was then excluded from the concatenated dataset and phylogenetic analyses were repeated for all optimality criteria, using the same settings as mentioned in the Material and Methods. The resulting trees were, however, much less resolved when third codon positions were excluded, indicating that there is valuable phylogenetic signal present in the third codon positions (the trees generated from the datasets with COI 3rd codon positions removed are presented in Supplementary

Figs. 1–3). In light of this, we chose to show the trees generated by the full dataset with all COI codon positions included.

3.2. Species delimitation and pairwise distances

The bPTP analysis indicated that the dataset contained between 22 and 35 species, and the maximum posterior probability solution identified 30 distinct terminal taxa, where we had identified 24 species (22 of which were nominal taxa). The highest PP solution lists each of the two individuals of *Placobdella ringueleti* (PP = 0.57), *P. multilineata* (PP = 0.52), and *P. lamothei* (PP = 0.52) as separate species, but these partitions are not well supported. The two different individuals for each of *P. phalera* and *P. mexicana* are also recovered as separate species, this time with high support (PP = 1.00 in all cases). The discrete taxa *Placobdella* sp. 3 (represented by specimen ROMIZ I12998) and *Placobdella* sp. 4 (represented by specimen ROMIZ I12999) do not belong to the partitions containing *P. multilineata* and *P. lamothei*, respectively, as suggested by the phylogenetic analyses. Instead, each forms its own partition (PP = 1.00 and 0.48), suggesting that they may be distinct species, separate from *P. multilineata* or *P. lamothei*. All other hypothesized species form exclusive and relatively robust partitions (PP ≥ 0.81).

The uncorrected pairwise distances for the entire ingroup COI dataset are presented in Supplementary Table 1. Overall average interspecific pairwise distance was $16.06\% \pm 1.99$; average intraspecific variation for those taxa that were split into different partitions by the bPTP analysis are as follows: *P. mexicana*, 5.99%; *P. phalera*, 4.25%; *P. ringueleti*, 5.41%; *P. multilineata*, 0.86%. The unidentified specimen ROMIZ I12999 and *P. lamothei* show a COI distance of 6.15%. Unidentified specimen ROMIZ I12998 and *P. multilineata* show a distance of 7.67%. The average uncorrected-p distances between *P. kwetlumye* and *Placobdella* sp. 1 (ROMIZ I11277 and ROMIZ I11449) for each locus are as follows: 12S rDNA ($5.70\% \pm 1.49$), COI ($7.60\% \pm 0.63$), ND1 ($7.57\% \pm 0.30$), ITS ($3.63\% \pm 2.43$). The standard deviation for ITS is driven up by the odd disparity in nucleotide sequences between the specimens of *P. kwetlumye* (6.29%).

4. Discussion

Based on molecular data from four loci, our phylogenetic analyses reveal several robust relationships within the genus *Placobdella*. We find species of *Placobdella*, as defined by Siddall et al. (2005), to be a monophyletic sister group to a clade formed by *Helobdella* and *Haementeria*. Although molecular evidence supporting monophyly of a lineage including all three genera is strong, there are no known morphological synapomorphies for this clade (Oceguera-Figueroa, 2012). The tree resulting from the concatenated dataset places all but four of the currently recognized species of *Placobdella* in a phylogenetic context. In addition, the analysis of COI-only suggests placements for three of the four remaining taxa (*P. ornata*, *P. cryptobranchii* and *P. siddalli*), which were not included in the concatenated matrix due to the dearth of available comparative data. Only *Placobdella michiganensis* (Sawyer, 1972) is not considered herein, as neither specimens nor DNA sequences were available. This is thus the most comprehensive systematic analysis of the genus to date, incorporating nuclear DNA sequence data for the first time, and more than doubling the taxonomic sampling of previous studies (Light and Siddall, 1999; Siddall et al., 2005; Siddall and Burreson, 1998). Eleven of the nominal species included in this analysis have never been considered in a phylogenetic context, and at least two other taxa may represent previously undescribed species. While our phylogeny provides robust support for several groupings within *Placobdella*, few of these are corroborated by the additional loci.

rated by synapomorphic morphological characters; selected taxa are discussed below, in this regard.

Given the lack of morphological characters supporting groups above the species level, the muddled taxonomic history of *Placobdella* is unsurprising. Each of the two members of the synonymized genus *Oligobdella* – originally recognized to include leeches with biannulate mid-body somites – are now assigned to different genera: *O. biannulata* is now included in *Placobdella* and *O. brasiliensis* is now included in the genus *Haementeria* (Siddall et al., 2005; Oceguera-Figueroa, 2012). The three species with pediculate caudal suckers, *P. sophiaeae*, *P. nuchalis* and *P. pediculata*, do not form a clade. Although *P. sophiaeae* and *P. nuchalis* are nested together in a larger clade including *P. picta* and *P. biannulata*, *P. nuchalis* shares more recent ancestry with leeches that do not exhibit this condition. At the time of its description, *Placobdella nuchalis* was thought to be a close relative of *Placobdella montifera*, as both species possess a discoid head separated from the rest of the body by a “nuchal constriction” (Sawyer and Shelley, 1976). On the basis of this uncommon feature, the authors concluded that they were “so unlike the other *Placobdella* that they [would] probably be separated from this genus in the future”. Nonetheless, a sister group relationship between these taxa is not found in any of our phylogenetic hypotheses, suggesting that a discoid head may have evolved independently in each lineage.

The genus *Desserobdella* was established in 1990 to accommodate leeches with a single pair of diffuse salivary glands (Barta and Sawyer, 1990). This genus, as originally conceived by its authors, included only *P. phalera*, *P. cryptobranchii*, *P. michiganensis*, and *P. picta*; however, *P. pediculata*, *P. sophiaeae*, *P. biannulata*, *P. nuchalis* and *P. ornata* also possess diffuse salivary tissues. In the present study, the COI-only analysis supports *P. phalera* and *P. cryptobranchii* together in a clade with *P. ornata* (Fig. 6). *Placobdella picta*, *P. sophiaeae*, *P. biannulata* and *P. nuchalis* form a clade; in the COI-only analysis, this grouping also contains *P. mexicana* and *P. siddalli*, which possess compact salivary glands (Fig. 6). These clades, furthermore, are only distantly related to one another, and *P. pediculata* does not group with either lineage. Additionally, *Placobdella translucens*, which nests within *P. phalera* possesses a pair of compact salivary glands. Both *P. burresonae* and *P. multilineata* possess salivary glands that are only weakly developed, but the species are only distantly related in all analyses. Interestingly, the taxon represented by ROMIZ I11277 and ROMIZ I11494 (*Placobdella* sp. 1) exhibits one pair of compact salivary glands in combination with diffuse salivary tissue and always places as sister taxon to *P. kwetlumye* (see below), which exhibits a single pair of compact salivary glands. Both the ML and BI trees support the hypothesis that possession of compact salivary tissues is plesiomorphic for *Placobdella*, and that diffuse salivary glands have evolved independently several times within the genus. Although the parsimony tree supports the opposite conclusion, it is worth noting that, within Glossiphoniidae, compact salivary glands are also present in all members of *Haementeria* and some species of *Helobdella* (Siddall et al., 2005). It may be that compact salivary glands were present in the common ancestor of all three genera, and that this character, though not present in all modern species, is plesiomorphic for the entire clade.

When possible, we included at least two specimens of each species as a means of teasing out potential cryptic diversity and validating our initial specimen identifications. This was especially important for species exhibiting a high degree of morphological variation such as *P. rugosa*, *P. phalera*, and *P. picta*. The only species for which the two individuals did not form a clade was *Placobdella cryptobranchii*—this is despite the fact that the two sequences were derived from specimens collected at or near the type locality as part of a recent redescription by Moser et al. (2013a). Although both specimens included in the analysis were determined to be

one species by the bPTP analysis, pairwise distance between the two sequences was 2.2%, which is only slightly greater than the average intraspecific distance across all species (1.50%). Taken together with the greater-than-expected genetic diversity within *P. cryptobranchii* recovered by Moser et al. (2013a) (up to 3.3% pairwise distance), these results indicate that this taxon warrants further investigation. We also find evidence for cryptic diversity in *P. mexicana* and *P. ringueleti*: both species exhibit intraspecific COI variation well above the average diversity for other species of *Placobdella* (*P. mexicana* = 5.99%; *P. ringueleti* = 5.41%).

Prior to our analyses, five specimens were unidentified: ROMIZ I12998, ROMIZ I12999, ROMIZ I12996, ROMIZ I12997, ROMIZ I11277 and ROMIZ I11494. The specimen ROMIZ I12998 groups with *P. multilineata*, but the bPTP species delimitation analysis and large COI pairwise distance (7.67%) indicate that the taxa may not be conspecific. Similarly, ROMIZ I12999 falls within the clade containing *P. lamothei*, but pairwise COI distance is high, at 6.15%. Specimens ROMIZ I12996 and ROMIZ I12997 remain unidentified. The two samples form a clade in the bPTP analysis as well as the MP and COI-only trees, but this clade is not recovered in the ML or BI trees. Vouchered specimens for these four samples were not available for study, so detailed morphological comparison is not possible at this time. Specimens ROMIZ I11277 and ROMIZ I11494 do not conform to any available species description, and were therefore hypothesized to represent undiscovered diversity. This conclusion is supported by all phylogenetic analyses and bPTP, as is a sister group relationship between this taxon and *P. kwetlumye*. Pairwise distances between the species, however, did not unequivocally support the separation of these species. For COI, intraspecific distances within each species (*Placobdella* sp. 1 = 0.45%; *P. kwetlumye* = 2.63%) were markedly lower than the interspecific distance (7.60%); however, intraspecific and interspecific distances for the nuclear locus ITS showed less divergence (average intraspecific distance for *Placobdella* sp. 1 = 0%; average intraspecific distance for *P. kwetlumye* = 6.29%; average interspecific distance = 3.63%; see Supplementary Table 1). This is largely due to one specimen of *P. kwetlumye* (ROMIZ I12971) which exhibited an ITS sequence more similar to those of *Placobdella* sp. 1 than to the other representative of *P. kwetlumye* (distances = 1.70% and 6.29% respectively). It is possible that the multicopy-nature of ITS is a confounding factor in our analyses, but this remains to be rigorously tested. Both *Placobdella* sp. 1 and *Placobdella kwetlumye* possess a single pair of compact salivary glands, but *Placobdella* sp. 1 also possesses diffuse salivary tissue. This combination of diffuse and compact salivary tissue is synapomorphic for the species. During a ROM field expedition in 2016, *Placobdella* sp. 1 was frequently collected across a broad geographic range within Canada, ranging from Alberta to Manitoba. In combination with our preliminary indications of cryptic and unidentified diversity, the presence of such a common, yet undescribed species highlights the need for continuing taxonomic work within *Placobdella*.

Sequences from three specimens of *Placobdella ornata* were included in the COI-only analysis. Two of these (JQ812135 and JQ812136) represent individuals collected from the type locality of that species, and had previously been determined to be conspecific with *P. ornata sensu stricto* following comparison with the syntype series (Moser et al., 2012b). The remaining sequence (KP176597) was obtained from a specimen collected in East Flanders, Belgium (Soors et al., 2015). The COI-only tree (Fig. 6) confirms that none of our unidentified specimens nests closely with *Placobdella ornata*, suggesting that this species is not otherwise represented in our dataset. By extension, this result corroborates the conclusion that *P. ornata* and *P. rugosa* constitute separate species (Moser et al., 2012a). The tree also confirms, for the first time in a phylogenetic context, that the specimen collected from Belgium is indeed *Placobdella ornata sensu stricto*. The specimen thus

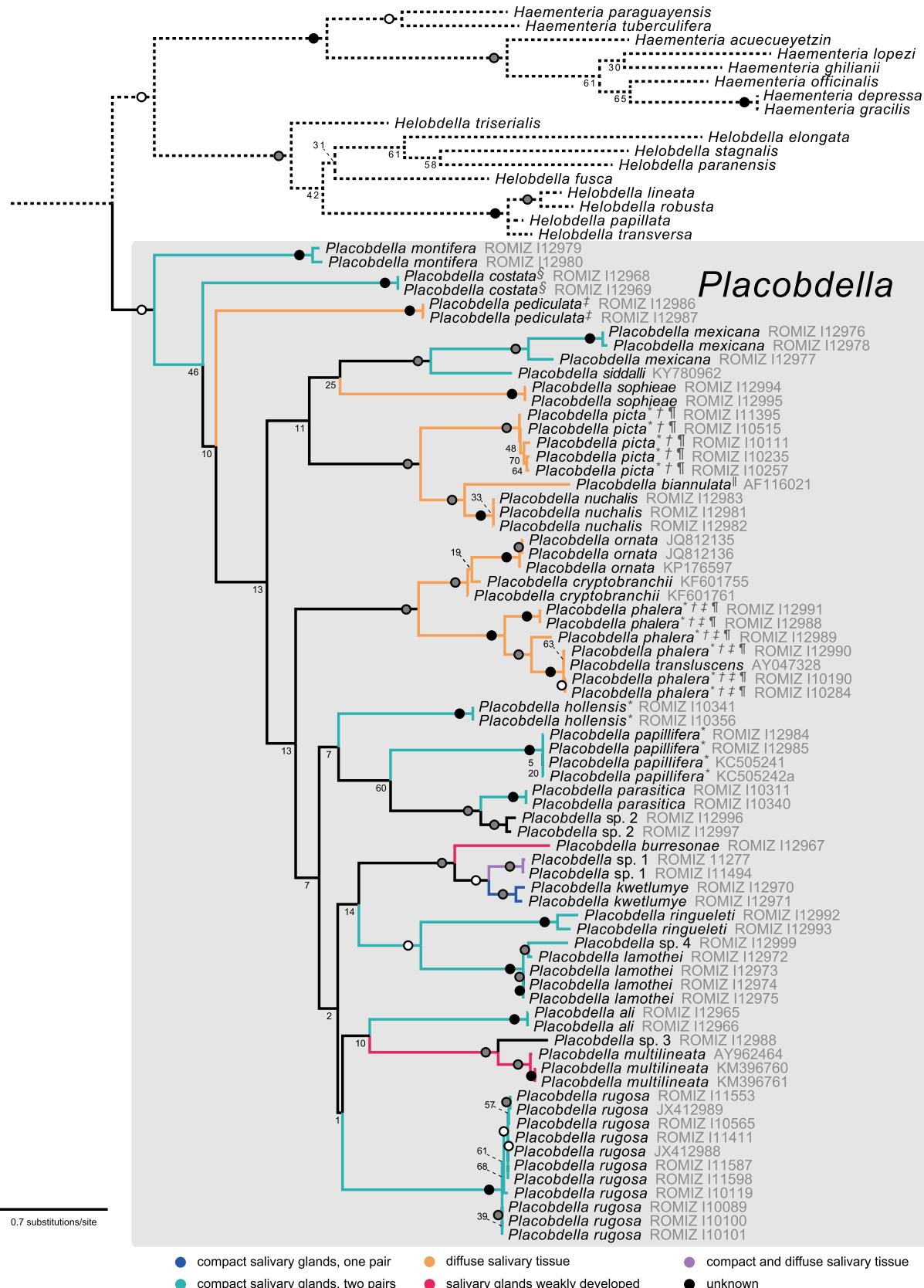


Fig. 6. Maximum likelihood tree topology based on COI sequence data (log likelihood = -9961.613401), with salivary tissue states indicated by colored branches. Black circles denote nodes with 100% bootstrap support; grey circles denote bootstrap support values of 85–99%; white circles denote 75–84% bootstrap support; bootstrap support values below 75% are indicated below nodes. Royal Ontario Museum voucher numbers or specimen codes for ingroup taxa are listed in grey. Superscript symbols indicate species that have previously been classified in other genera: *Clepsine; †Batracobdella; ‡Actinobdella; §Haementeria; ¶Oligobdella; *Desserobdella. (For interpretation of the references to color in the figure, please refer to the online version of this article.)

represents the first record of *P. ornata* outside northeastern United States, and is only the second species of *Placobdella* collected in Europe, as detailed by Soors et al. (2015). At present, *P. ornata* is not known from any other European locality, and was possibly introduced to Belgium along with its host. Although detailed information regarding the host affinities of these particular specimens are unknown, *P. ornata* is known to feed on turtles in North American and European localities alike (Siddall and Gaffney, 2004; Soors et al., 2015).

Placobdella rugosa has historically been the subject of much taxonomic confusion: it was originally described as a subspecies of *Placobdella ornata*, elevated to full species status, reassigned to *P. ornata* in the 1950s (Moore, 1952) and resurrected once again in 2012 (Moser et al., 2012a and references therein). The complex taxonomic histories of these two species underscores past confusion about their identity. Nonetheless, the two species do not group closely in the COI-only tree (the only analysis in which *P. ornata* is included; Fig. 6). Due to remarkable variation in external papillation and pigmentation – two characters commonly used to distinguish species of *Placobdella* – nine samples of *P. rugosa* were analysed to ensure that this diversity was represented (Figs. 1A–D and 2D), in addition to two COI sequences from material used in a recent redescription (Moser et al., 2012a) (Fig. 6). Unexpectedly, despite phenotypic diversity, all nine samples nest together with short branch lengths. The specimens that form this clade were collected from a broad geographic area, bounded by mid-Ontario and Nebraska to the north and south, and New York state and Saskatchewan to the east and west. While it is currently unknown how populations of leeches are connected across landscapes, it was expected that populations of *P. rugosa* would exhibit strong biogeographic signal owing to the fact that they are relatively sedentary, are not found in running waters, and are not believed to remain long on their vertebrate hosts after feeding (personal observation). Nonetheless, no clear structure emerges in the COI or total data analyses.

Moser et al.'s (2012b) redescription of *Placobdella ornata* also determined the species to be conspecific with *P. phalera* on the basis of morphological similarity and short (less than 0.5%) uncorrected-*p* distance. As a result, *P. phalera* was designated a junior synonym of *P. ornata*. Contrary to this finding, we show that individuals matching the description of *P. phalera* form a unique clade (LBS = 100%), separate from *P. ornata* (Fig. 6). However, the clade consisting of *P. ornata* + *P. cryptobranchii* (LBS = 85%) is the sister group to *P. phalera* (LBS = 98%) (Fig. 6). Additionally, *P. transluscens* nests within *P. phalera* in all trees. The two species are known to be morphologically similar, differing externally only in the degree of papillation and pigmentation (Figs. 1F and 2F) (Klemm, 1982). Both the MP and COI-only trees show two distinct, monophyletic clades within *P. phalera*, and the bPTP analysis suggests that specimens ROMIZ I12988 and ROMIZ I10284 represent separate species (PP = 1.00). The same groupings are not reiterated in the ML and BI trees but, in all cases, *P. transluscens* forms a well-supported clade with ROMIZ I10190 and ROMIZ I10284 (Figs. 3–6), which is separated from specimens ROMIZ I12988 and ROMIZ I12989 by relatively long branches. These results echo the findings of Light and Siddall (1999) and Siddall et al. (2005) who also found evidence for a close relationship between *P. phalera* and *P. transluscens*. Light and Siddall (1999) also noted morphological differences between the two clades recovered in the present study: Dphalera TN (ROMIZ I12990) was small, with a translucent body and unpigmented neck ring; DphaleraVA (ROMIZ I12991) was larger and more robust, with an opaque body and no neck ring. Both of these sequences are included in our COI-only tree (Fig. 6). The dorsal pigmentation of ROMIZ I10284 and ROMIZ I10190 more closely resembles that of ROMIZ I12989 than *P. transluscens*. The specimen identified as *P. transluscens* also has conspicuous paramedial

papillae, which are at odds with the species description (Sawyer and Shelley, 1976; Fig. 2). It is possible that ROMIZ I10190 and ROMIZ I10284 were misidentified, and in fact belong to *P. transluscens*. Although, it is equally possible that the two specimens would be more appropriately assigned to *P. phalera*, or that the two names are, in fact, synonymous. As mentioned, *P. phalera* is characterized by diffuse salivary tissue whereas *P. transluscens* possesses two pairs of compact salivary glands. Unfortunately, the anterior portion, including the head and neck region, of the only specimen of *Placobdella transluscens* available to us was used for DNA extraction (as opposed to the posterior portion; Fig. 2). We therefore cannot rely on this trait to affirm the identity of this clade. Regardless, it is clear that this taxon exhibits a great deal of genetic diversity and warrants further scrutiny, on both a molecular and morphological basis.

It bears noting that our data generated phylogenetic hypotheses that did not robustly support several of the deeper nodes, notwithstanding our use of a relatively large dataset and dense taxon sampling. Poorly resolved internal nodes are, however, not uncommon in leech phylogenetics: several previous studies, including phylogenetic assessments of the family Glossiphoniidae, have encountered similar problems (e.g. Borda and Siddall, 2004; Oceguera-Figueroa et al., 2011; Siddall, 2002a,b; Siddall and Borda, 2003; Siddall et al., 2005). This trend toward poor support may be due, in part, to the overwhelming A-T bias inherent in leech mitochondrial DNA (indeed, the G-C content for the sequences analysed in the present study was 39% for the ingroup). Whatever the reason, it is clear that future research into the systematics of *Placobdella* would prove fruitful (Siddall, 2002a,b).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ympev.2017.06.017>.

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