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SYNONYMIZATION OF *PLACOBDELLA PICTA* (VERRILL, 1872) (HIRUDINEA: GLOSSIPHONIIDAE) WITH DESCRIPTIONS OF TWO NEW SPECIES REVEALED BY MOLECULAR SPECIES DELIMITATION

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GMYC
PTP

ABSTRACT

Species of *Placobdella* have been the frequent subject of revisionary and alpha-taxonomy in the past 2 decades. Recent molecular analyses introduced uncertainty about the taxonomic status of several broadly distributed and morphologically variable *Placobdella* species, including *Placobdella picta* (Verrill 1872), compounded by incomplete original descriptions reliant upon characters that are no longer unique in comparison to modern congeners. We assessed specimens of *P. picta* to identify any distinct phylogenetic entities that align with our morphological observations of the type series and novel topotype specimens. Using mitochondrial *COI* and *ND1* and nuclear *18S* rDNA with Maximum Likelihood and Bayesian Inference, we evaluated species boundaries using species delimitation analyses (ABGD, mPTP, bPTP, and GMYC) and molecular phylogenetics. Our analyses revealed 2 species entities equivalent to 2 non-reciprocal monophyletic clades. Morphological examinations revealed the lectotype was determined to be *Placobdella ornata* (Verrill 1872), and paralectotypes are other leech species or were poorly preserved and unidentifiable. Due to the problems with the type series, *P. picta* is now considered a junior synonym of *P. ornata*. Based on our results, we describe 2 new species: *Placobdella unimaculata* n. sp. from Connecticut and *Placobdella desseri* n. sp. from Algonquin Provincial Park, Ontario, Canada.

Leeches of the family Glossiphoniidae are well-known members of freshwater ecosystems. Many of these leeches are opportunistic ectoparasites on aquatic vertebrates, mostly turtles, amphibians, fish, and waterfowl, and some species have been shown to vector blood parasites (Sawyer, 1986; Siddall and Desser, 1992). Glossiphoniid species have the potential to be introduced to sensitive ecosystems and pose new threats to host species already facing conservation concerns (Soors et al., 2015; Reilly et al., 2023). Being able to quickly and accurately identify glossiphoniids and having baseline data on their geographic distributions is becoming a pressing concern for ecosystem monitoring and conservation. The concepts of several North American glossiphoniid species originally described in the 19th century and earlier were vague, and the diagnostic characteristics of the species have become heterogeneous over time through formal synonymy and informal usage of the names. This resulted in the species names of some of the most well-recognized glossiphoniid species in North America, such as *Placobdella picta* (Verrill 1872), being applied widely and coming to be recognized by characters that no longer distinguish the species from its congeners.

Placobdella Blanchard 1893 includes 26 species, and there is a high potential for additional unrecognized species diversity to be described (Oceguera-Figueroa and Siddall, 2008; de Carle et al., 2017; Fan et al., 2022; Ben Ahmed et al., 2023). Through efforts

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to establish accurate species boundaries among North American members of *Placobdella*, significant species-level taxonomic refinements have been undertaken in the last 2 decades based on the reexamination of type series and the collection of topotypes for morphological and molecular comparison (for example, Moser et al., 2012a, 2012b, 2013, 2014, 2016b, 2017). These revisions have been substantiated in recent molecular phylogenies (de Carle et al., 2017). Species delimitation analyses of DNA sequence data have been used to investigate the species boundaries of only a few widely distributed *Placobdella* species (de Carle et al., 2017; Mack et al., 2019), although the status of *P. picta* has not been investigated until now.

Placobdella picta was originally described by Verrill (1872) as *Clepsine picta* Verrill 1872 based on specimens collected from Whitneyville Lake and the West River, New Haven County, Connecticut, where Verrill indicated the leeches to be common on submerged or floating wood and beneath dead bark. Unfortunately, the original description of *C. picta* by Verrill is vague, and the characters used are no longer descriptive of the species, rendering contemporary specimens difficult to identify with any degree of certainty. Moore (1952) mentioned *C. picta* as a "troublesome species" because the description had multiple color variants without establishing a fundamental pattern. Moore (1952) concluded that the description of *C. picta* was likely based on multiple species, which was later reaffirmed by Barta and Sawyer (1990). To resolve these issues, Moore (1952) applied the name *picta* to specimens "agreeing with the first description but having the mouth well behind the sucker rim in [somite] III." Since then, the genus assignment of the species has seen frequent changes (for a complete list of synonyms see Autrumn, 1936; Sawyer, 1972; Klemm, 1982, 1985; Barta and Sawyer, 1990), although the transference of the name *picta* by Moore (1952) began the modern concept of *Placobdella picta sensu* Moore 1952 that has been widely recognized and applied. Barta and Sawyer (1990) redescribed the species as the type species of *Desserobdella* Barta and Sawyer 1990 (later synonymized with *Placobdella* by Siddall et al., 2005) based on novel specimens collected from Algonquin Provincial Park in Ontario, Canada, and examination of Verrill's Connecticut specimens from the Yale Peabody Museum (Table I). At present, *P. picta sensu* Moore 1952 is recognized as having the characteristics consistent with members of *Placobdella* with a brownish-green dorsum variegated with orange, a thin dark dorsal medial line, 6 to 7 rows of white-tipped papillae, and the presence of a nuchal band, and is 13–25 mm in length and is considered to be distributed east of the Rocky Mountains (Klemm, 1985; Moser et al., 2016a).

Placobdella picta has been included in several molecular phylogenies, usually represented by a single individual from Ontario (Apakupakul et al., 1999; Light and Siddall, 1999). The most recent phylogeny of *Placobdella* by de Carle et al. (2017) included several additional sequences of specimens identified as *P. picta* from Nebraska, and Ontario and Saskatchewan, Canada, and found them to nest within a strongly supported monophyletic clade sister to the clade *Placobdella biannulata* (Moore, 1898)–*Placobdella nuchalis* Sawyer and Shelley, 1976. Their analysis found the sequences of *P. picta* had short branch lengths and lacked phylogeographic structure, although the specimens exhibited a high degree of morphological variation.

Given the complex taxonomic history of *P. picta*, problems with the type series, the high degree of morphological variation, the wide geographic distribution, and the limited molecular data

Table I. Syntype specimens of *Clepsine picta* Verrill, 1872, at the Yale Peabody Museum of Natural History and subsequent determinations. Specimen lot YPM IZ 000285.AN contains 4 specimens that are labeled with letters A–D. Dash (—) indicates no notes for that specimen.

Catalog no.	Barta and Sawyer (1990) determination	Determination from this study	Locality, Collection date	Notes
YPM IZ 000251.AN	Paralectotype, *Helobdella* sp.	*Helobdella* sp.	West River, New Haven, Connecticut, 6 June 1871	Brooding eggs
YPM IZ 000285.AN (A)	Paralectotype	*Placobdella parasitica*	Whitneyville Lake, Connecticut, 4 October 1871	—
YPM IZ 000285.AN (B)	Paralectotype, *Placobdella* sp.	*Placobdella ornata*	Whitneyville Lake, Connecticut, 4 October 1871	Smaller specimen
YPM IZ 000285.AN (C)	Paralectotype, *Placobdella* sp.	*Placobdella ornata*	Whitneyville Lake, Connecticut, 4 October 1871	Larger specimen
YPM IZ 000285.AN (D)	Paralectotype, *Placobdella* sp.	*Placobdella* sp.	Whitneyville Lake, Connecticut, 4 October 1871	Poorly preserved
YPM IZ 000286.AN	Lectotype, *Placobdella picta*	*Placobdella ornata*	Whitneyville Lake, Connecticut, 4 October 1871	Designated a lectotype by Barta and Sawyer (1990)
YPM IZ 000287.AN	Paralectotype	*Placobdella* sp.	Whitneyville Lake, Connecticut, 4 October 1871	Specimen dried out previously

available, a reevaluation of the species is warranted. The main goals of this study were to evaluate species boundaries of *P. picta* with specimens from across its distribution using a combined molecular approach, to morphologically characterize the resulting phylogenetic entities, and to refine the taxonomy to accurately reflect the evolutionary lineages.

MATERIALS AND METHODS

Specimen collection

During a survey of the leech fauna of south-central Connecticut, leeches were collected by hand from submerged substrate from the Audubon Center Marsh Pond (Fairfield County, Connecticut), and Sturges Pond (Larsen Sanctuary, Fairfield County, Connecticut) on 13 August 2001, 26 March 2004, 6 May 2014, 22 October 2016, and 24 July 2022 (Table II). Specimens were relaxed, examined, and fixed as described by Moser et al. (2006). Several specimens were pressed, stained with Semichon's acetocarmine, and mounted in Canada Balsam for examination by light microscopy according to techniques outlined by Richardson (2006) and those modified by Richardson and Barger (2006). Terminology for plane shapes follows Clopton (2004). Specimens were examined using an Olympus SZX16 dissecting microscope (Olympus, Tokyo, Japan) and were photographed with a Zeiss Stemi 2000-CS microscope (Zeiss, Jena, Germany) fitted with a Q-Capture 5.0 RTV Micropublisher camera (QImaging, Surrey, Canada). Images were acquired at different focal levels, and the resulting stacks were rendered with Helicon Focus 7 Pro (Helicon Soft Ltd, Kharkiv, Ukraine) to make an extended focus image. Post-processing was done using Adobe Photoshop CC 2015 (Adobe Systems, San Jose, California). Representative specimens were deposited in the Yale Peabody Museum (YPM), Yale University, New Haven, Connecticut, and the National Museum of Natural History (USNM), Smithsonian Institution, Washington, D.C.

DNA analyses

Molecular analyses were conducted on newly collected material according to Richardson et al. (2010) as follows: DNA was isolated from the caudal suckers of individual leeches with the DNeasy Blood & Tissue Kit (Qiagen, Inc., Valencia, California), following the protocol given for the purification of total DNA from animal tissues (spin-column). For the proteinase K treatment step, tissue samples were lysed overnight at 56 C. DNA was eluted from the spin columns with 150 µl of buffer.

Partial sequences of the mitochondrial cytochrome c oxidase subunit I (*COI*) as specified by Folmer et al. (1994) and Light and Siddall (1999) as well as nicotinamide adenine dinucleotide dehydrogenase subunit I (*ND1*) by de Carle et al. (2017) were obtained to investigate phylogenetic relationships. Novel sequences in this study were generated at either Yale University or the Laboratories of Analytical Biology (L.A.B.) at the NMNH, Smithsonian Institution. At Yale University, polymerase chain reactions (PCRs) were prepared using the Illustra PuRe Taq Ready-To-Go PCR beads (GE HealthCare, Chicago, Illinois). The final volume of PCRs was 25 µl with 2 µl of genomic DNA added per reaction. DNA was amplified under the following conditions: 94 C for 5 min; 35 cycles of 94 C (30 sec), 50 C (30 sec), 72 C (45 sec); and final extension at 72 C for 7 min. Following PCR, samples were cleaned using a QIAquick PCR purification kit (Qiagen). Purified PCR products were sequenced by the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University. In the L.A.B. at the NMNH, PCRs were performed in 10 µl reactions with 1 µl of genomic DNA and final concentrations of 3 µmol of each primer, 500 µM dNTPs, 3 mM MgCl, 0.25 mg/µl of BSA (bovine serum albumin), and 0.05 U/µl of Biolase™ DNA polymerase (Bioline, Inc., Memphis, Tennessee) with buffers provided by the manufacturer. PCR reactions were performed in an Applied Biosystems 2720 Thermal Cycler under the following thermal profiles: for *COI*, 95 C for 5 min, followed by 35 cycles of 95 C (30 sec), 49 C (30 sec), 72 C (30 sec) and final extension at 72 C for 7 min; for *18S* rDNA, 95 C for (5 min), followed by 35 cycles of 95 C (30 sec), 52 C (30 sec), 72 C (30 sec), and final extension at 72 C (7 min). ExoSAP-IT (Affymetrix, Santa Clara, California) was used to purify the PCR products, and cycle sequencing was performed with BigDye Terminator v3.1 (Applied Biosystems, Foster City, California). Cycle-sequenced products were purified using Sephadex™ G-50 Fine (GE HealthCare), and DNA sequencing was performed on an ABI 3730 sequencer at the L.A.B. at the NMNH.

Chromatographs were edited and assembled with Geneious Prime, Version 2022.2.2 (https://www.geneious.com). Newly generated sequences were deposited in GenBank (Benson, 2018; Table II). Additional sequences of *COI*, *ND1*, and the nuclear ribosomal DNA of the small subunit (*18S* rDNA) were obtained from GenBank for inclusion in molecular comparisons (Apakupakul et al., 1999; Light and Siddall, 1999; Moser et al., 2011, 2013, 2017; Hopkins et al., 2014; de Carle et al., 2017; Richardson et al., 2017; Fan et al., 2022; Table II).

Outgroup representatives included *Helobdella modesta* (Verrill 1872), *Helobdella bowermani* Moser, Fend, Richardson, Hammond, Lazo-Wasem, Govedich, and Gullo 2013, and *Helobdella octatestisaca* Lai and Chang 2009. Outgroup selection was based on sister group relationships from the most recently published phylogeny of *Placobdella* (de Carle et al., 2017).

Data set assembly

Sequences were aligned using the MAFFT online platform (Katoh et al., 2005; Katoh and Standley, 2013). Alignments of *COI* and *ND1* were checked manually for gaps and then translated into amino acids as an independent assessment of sequence quality. Protein coding sequences were subsequently checked for stop codons before phylogenetic analyses using Mesquite v.3.70 (Maddison and Maddison, 2021). Individual gene data sets were concatenated using Sequence Matrix (Vaidya et al., 2011).

For species delimitation of *P. picta*, identical sequences were identified using pairwise distances in MEGA 11: Molecular Evolutionary Genetics Analysis version 11 (Tamura et al., 2021) and subsequently removed to avoid the inclusion of redundant information.

Phylogenetic analyses

Phylogenetic reconstructions were performed on individual gene data sets, as well as the concatenated data set, using Maximum Likelihood (ML) and Bayesian Inference (BI) methods.

Estimation of substitution models was done using ModelFinder within IQTREE (Kalyaanamoorthy et al., 2017), resulting in models indicated as best fit under the Bayesian information

Table II. Species, collection locality, museum catalog number, and GenBank accession information for specimens included in this study. Specimens collected from type localities indicated with an asterisk (*). New sequences generated as a part of this study are in bold. Dash (—) indicates sequence data for that gene are unavailable for that specimen. Capital letters distinguish different specimens of the same species and are repeated in Figures 1 and 2.

Species	Country	Location	Catalog no./citation	*CO1*	*ND1*	*18S*
Helobdella modesta	United States	New Haven County, Connecticut*	Moser et al., 2011	JF319988	JF326523	—
Helobdella bowermani	United States	Klamath County, Oregon*	Moser et al., 2013	KF683194	—	—
Helobdella octatestisaca	United States	Texas	Richardson et al., 2017	KY498613	—	—
Placobdella akahkway	Canada	Half Moon Lake, Alberta	ROMIZ 1277/Fan et al., 2022	MF067095	MF067242	—
Placobdella akahkway	Canada	Cypress Hills Provincial Park, Saskatchewan*	ROMIZ 1494/Fan et al., 2022	MF067094	MF067243	—
Placobdella appalachiensis (A)	United States	Smyth County, Virginia*	Hopkins et al., 2014	KF990590	—	—
Placobdella appalachiensis (B)	United States	Smyth County, Virginia*	Hopkins et al., 2014	KF990591	—	—
Placobdella appalachiensis (C)	United States	Smyth County, Virginia*	Hopkins et al., 2014	KF990592	—	—
Placobdella appalachiensis (D)	United States	Smyth County, Virginia*	Hopkins et al., 2014; this study	KF990593	**PQ557593**	—
Placobdella biannulata (A)	United States	North Carolina	Apakupakul et al., 1999; Light & Siddall, 1999	AF116021	AY047343	AF115989
Placobdella biannulata (B)	United States	Watagua County, North Carolina	USNM 1740777; this study	**PQ559250**	**PQ557594**	—
Placobdella nuchalis	United States	Gates County, North Carolina	Moser et al., 2017	MF535241	—	—
Placobdella nuchalis	United States	Orangeburg County, South Carolina*	Moser et al., 2017	MF535240	**PQ557595**	—
Placobdella desseri (A)	Canada	Algonquin Provincial Park, Ontario*	Apakupakul et al., 1999; Light & Siddall, 1999	AF116020	AY047342	AF115988
Placobdella desseri (B)	Canada	Restoule Provincial Park, Ontario	ROMIZ 10235/de Carle et al., 2017	MF067112	MF067228	—
Placobdella desseri (C)	Canada	Moore Lake, Ontario	ROMIZ 10257/de Carle et al., 2017	MF067111	MF067229	—
Placobdella desseri (D)	Canada	Kenny Lake, Ontario	ROMIZ 10111/de Carle et al., 2017	MF067113	MF067227	—
Placobdella desseri (E)	Canada	Moose Mountain Provincial Park, Saskatchewan	ROMIZ 11395/de Carle et al., 2017	MF067109	—	—
Placobdella desseri (F)	United States	Madison County, Arkansas	USNM 1740779; this study	**PQ559251**	**PQ557596**	**PQ559295**
Placobdella desseri (G)	United States	Madison County, Arkansas	USNM 1740780; this study	**PQ559252**	**PQ557597**	**PQ559296**
Placobdella desseri (H)	United States	Missouri	YPM IZ 111860; this study	**PQ559253**	—	—
Placobdella desseri (I)	United States	Nebraska	ROMIZ 10515/de Carle et al., 2017	MF067110	MF067230	—
Placobdella sophieae (A)	United States	Squires Lake, Washington*	ROMIZ 12994/de Carle et al., 2017	MF067097	MF067240	—
Placobdella sophieae (B)	United States	Squires Lake, Washington*	ROMIZ 12995/de Carle et al., 2017	MF067096	MF067241	—
Placobdella sophieae (C)	United States	Oregon	Hopkins et al., 2014	KF990594	**PQ565814**	—
Placobdella sophieae (D)	United States	Oregon	Hopkins et al., 2014	KF990595	—	—
Placobdella unimaculata (A)	United States	Fairfield, Connecticut*	This study; YPM IZ 070781	**PQ559254**	**PQ557598**	—
Placobdella unimaculata (B)	United States	Middlesex, Connecticut	This study; USNM 1740784	**PQ559255**	**PQ557599**	**PQ559300**
Placobdella unimaculata (C)	United States	Mohonk Cove, New York	ROMIZ 12982/de Carle et al., 2017	MF067127	MF067215	—
Placobdella unimaculata (D)	United States	John Allen Pond, New York	ROMIZ 12983/de Carle et al., 2017	MF067126	MF067216	—
Placobdella unimaculata (E)	United States	Mohonk Camp, New York	ROMIZ 12981/de Carle et al., 2017	MF067128	MF067214	—
Placobdella unimaculata (F)	United States	Bear Run Road, West Virginia	This study; USNM 1740783	**PQ559258**	**PQ557601**	**PQ559299**
Placobdella unimaculata (G)	United States	Summit Lake, West Virginia	This study; USNM 1740781	**PQ559256**	**PQ557600**	**PQ559298**
Placobdella unimaculata (H)	United States	Summit Lake, West Virginia	This study; USNM 1740782	**PQ559257**	—	**PQ559297**

Table III. Species delimiting results for the *Placobdella picta* clade using GMYC, PTP (m- and bPTP) calculated in BEAST, and ABGD methods for individual and combined gene data sets. To test the strength of the mPTP and bPTP results, these analyses were also run in IQTREE (values after bars). Abbreviations: ML, maximum likelihood; CI, confidence interval; Est. ent., estimated entities; dash (—), not applicable.

Analysis methods	GMYC			mPTP	bPTP		ABGD
	ML entities (CI)	Likelihood ratio	*P**	Est. ent.	Est. ent.	Mean	Est. ent.
Gene(s)							
COI	3 (1–8)	3.1842	0.2035	2|2	2–8|2–10	2.36|4.3	2
ND1	5 (2–7)	6.6436	0.0360*	2|2	2–7|2–7	2.12|4.25	2
COI+ND1	7 (2–7)	5.4022	0.0671	2|6	2–13|11–11	3.97|11.0	—
COI+18S	6 (1–14)	2.7209	0.2565	2|3	2–9|2–3	2.41|2.99	—
18S+ND1	5 (2–7)	4.6712	0.0967	2|2	2–8|10–10	2.19|10	—
COI+18S+ND1	7 (2–8)	4.0592	0.1314	2|2	2–13|2–4	2.30|2.03	—

* $P \leq 0.05$.

criterion (BIC): *COI* = GTR + I, *ND1* = HKY + G4, and *18S* rRNA = JC. A ML analysis was performed using IQTREE multicore version 2.1.3 (Nguyen et al., 2015), using the models suggested for each unlinked partition, the *-spp* option that allowed each partition to have an individual evolutionary rate, and 1,000 non-parametric bootstrap replicates (MLB; Felsenstein, 1985) on "Hydra," the Smithsonian Institution High Performance Cluster (SI/HPC).

Bayesian Inference analyses were performed using MrBayes v3.2.6 on the CIPRES Science Gateway (Miller et al., 2010). All data sets were submitted with 2 independent runs using 4 chains (3 heated, 1 cold). Each chain was allowed to run for 30 million generations, with sampling set for every 1,000 generations. The first 10 million generations were discarded as burn-in. A 50% majority-rule consensus tree with posterior probabilities (BPP) was constructed using the remaining trees after burn-in. The convergence of all MCMC runs was verified using TRACER v.1.7.2 (Rambaut et al., 2018).

Species delimitation

Using an integrative taxonomic approach, we used morphological and molecular data to determine if *P. picta* samples represented a single species or if separate, independently evolving entities exist. Three methods were used to assess species delineation, including the Automated Barcode Gap Discovery (ABGD), the Poisson tree process (PTP) including multi-rate (mPTP) and Bayesian implementation (bPTP), and the generalized mixed Yule-coalescent (GMYC). Outgroups were removed before the implementation of these methods.

The methods of mPTP, bPTP, and GMYC utilized ultrametric trees generated using Bayesian Inference in BEAST v1.10.4 as implemented on the CIPRES Science Gateway (Miller et al., 2010; Suchard et al., 2018). BEAUTi (Bayesian Evolutionary Analysis Utility) v1.10.4 generated .xml files for all BEAST runs. Independent BEAST runs were created for individual as well as combined and partitioned data sets. Tree priors for all analyses were selected under a Coalescent Process with constant population size. Nucleotide substitution models were estimated by the corrected Bayesian information criteria (BICc) using jModelTest (Posada, 2008): *COI* = GTR + G, *ND1* = HKY + G, and *18S* = JC. All data sets had independent MCMC analyses with 10^8 generations, and trees were sampled every 10,000 generations. TRACER v1.7.2 (Rambaut et al., 2018) was used to verify the convergence of all MCMC runs. A maximum clade credibility (MCC) consensus tree was obtained for each BEAST data set in Tree Annotator v1.10.4 (Suchard et al., 2018) after annotating the remaining 9,001 trees after burn-in.

To test the strength of our mPTP and bPTP delineations, these analyses were also run with trees generated with ML analyses in IQTREE. Analyses using GMYC were performed in R v.3.5.1 (R Core Team, 2021) using the package SPLITS v.1.0-19 (Ezard et al., 2017) on phylogenies obtained from individual and combined data sets. PTP analyses were carried out on the mPTP online server (http://mptp.h-its.org) and the bPTP online server (http://species.h-its.org). No changeable settings are present on the mPTP online server; however, bPTP analyses were run using 10^4 MCMC generations with a burn-in of 0.1. Individual *COI* and *ND1* data sets were uploaded on the ABGD online platform and were analyzed using preset parameters (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html).

RESULTS

Species delimitation

Delineation results are based on comparisons of mitochondrial and ribosomal data from 17 specimens distributed across central and eastern North America and western Canada. Results from the different delineation methods employed for identifying phylogenetic entities using single gene and concatenated gene data sets (i.e., *18S* rRNA, *ND1*, and *COI*) are listed in Table III and illustrated in Figure 1. We derived our findings based on phylogenetic entities using the most conservative delineation estimates in common among the genetic markers and the employed methods. Our molecular investigation into the problematic taxon *P. picta* showed that it minimally consists of 2 separate phylogenetic entities (Fig. 1; Table III). One entity, referred to as *Placobdella unimaculata* n. sp., is restricted to the eastern states of Connecticut, New York, and West Virginia. The other entity, herein referred to as *Placobdella desseri* n. sp., shows a broader distribution between Saskatchewan and Ontario, Canada, and Arkansas, Missouri, and Nebraska. GMYC analyses indicated the same 2 minimum entity estimates in all gene tree combinations except 2 (Table III). GMYC found no support when analyzing phylogenies from *COI* or *COI* + *18S* rRNA. Only the GMYC analyses of *ND1* were statistically significant ($P \leq 0.05$). Analyses using PTP methods (mPTP and bPTP) on ultrametric trees generated by BEAST estimated 2 phylogenetic entities across all data sets tested (Table III). Comparative PTP analyses using trees

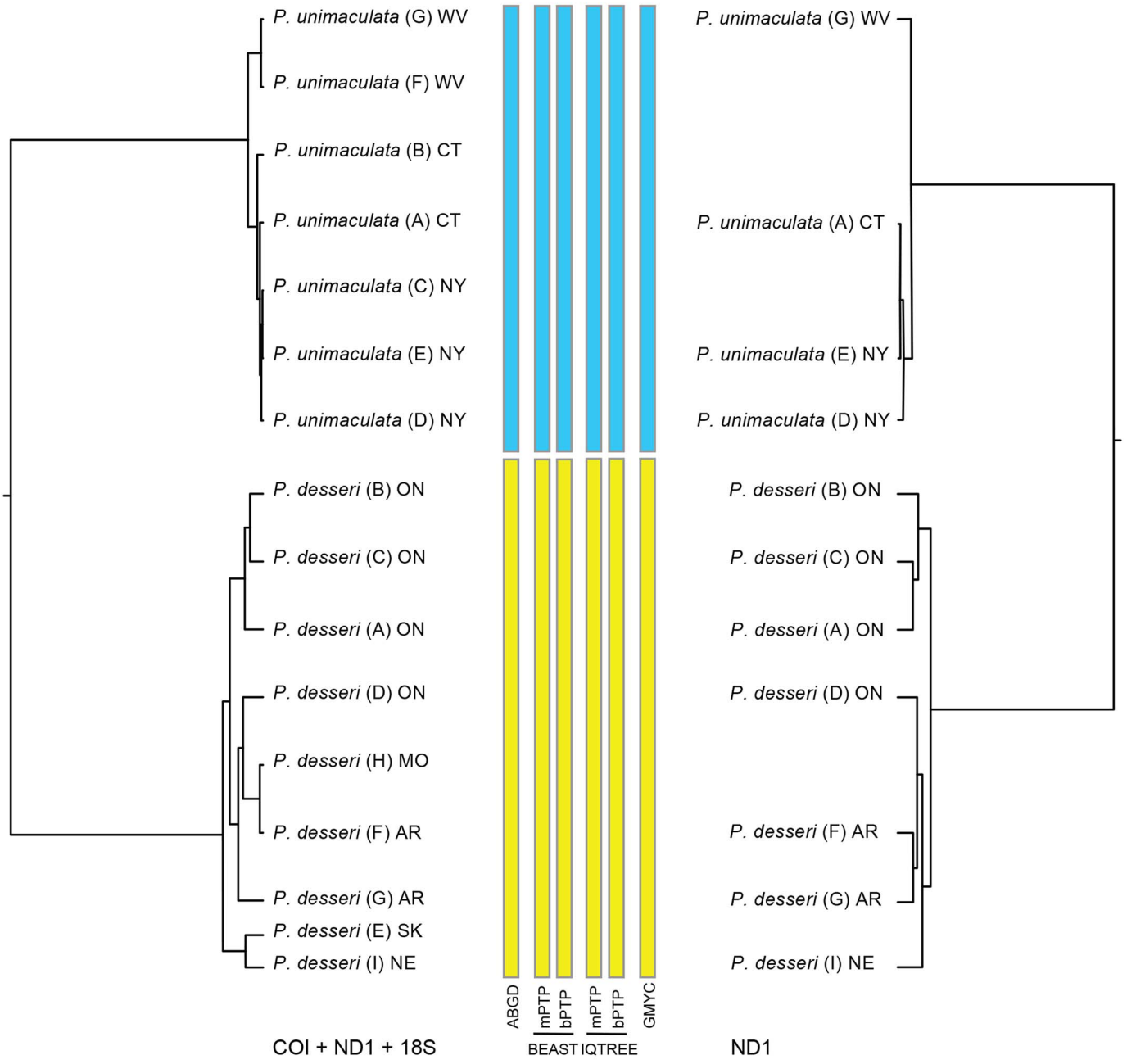

Figure 1. Ultrametric trees generated in BEAST using combined 3-gene data set (left) and *ND1* gene–only data set (right). Results of species delimitation for both trees are indicated by vertical bars above each delimitation method used. Breaks in the vertical bars indicate phylogenetic entity based on conservative estimates with that method. Differences in the number of taxa between trees are due to removal of identical sequences before phylogenetic tree generation for delimitation analyses. Abbreviations: ABGD, Automatic Barcode Gap Discovery; mPTP, multi-rate Poisson tree process; bPTP, Bayesian implementation of the Poisson tree process; GMYC, generalized mixed Yule-coalescent. Color version available online.

generated in IQTREE indicated the same 2 entities in all data sets except for 4 instances, where estimates suggested more than 2 identifiable entities (see Table III). Differences in minimum entities recovered are likely due to tree-building algorithms or how missing data are treated. ABGD analyses of *COI* and *ND1* recovered 2 distinct phylogenetic entities (Table III).

While all conclusions are based on the aforementioned delimitation analyses, we also calculated genetic distances using MEGA v11.0 (Tamura et al., 2021) using the uncorrected *p*-distance model, with uniform rates among sites, and pairwise deletion of missing data. The number of base substitutions per site between sequences was highly similar between the sequenced *COI* and *ND1* fragments. *COI* distances among sequences of *P. unimaculata* n. sp. were 0–0.9%, while the *COI* distances were between 0.18% and 2.59% in *P. desseri* n. sp. (Suppl. Table S1). *COI* distances between these 2 entities were between 10.42% and 12.89%.

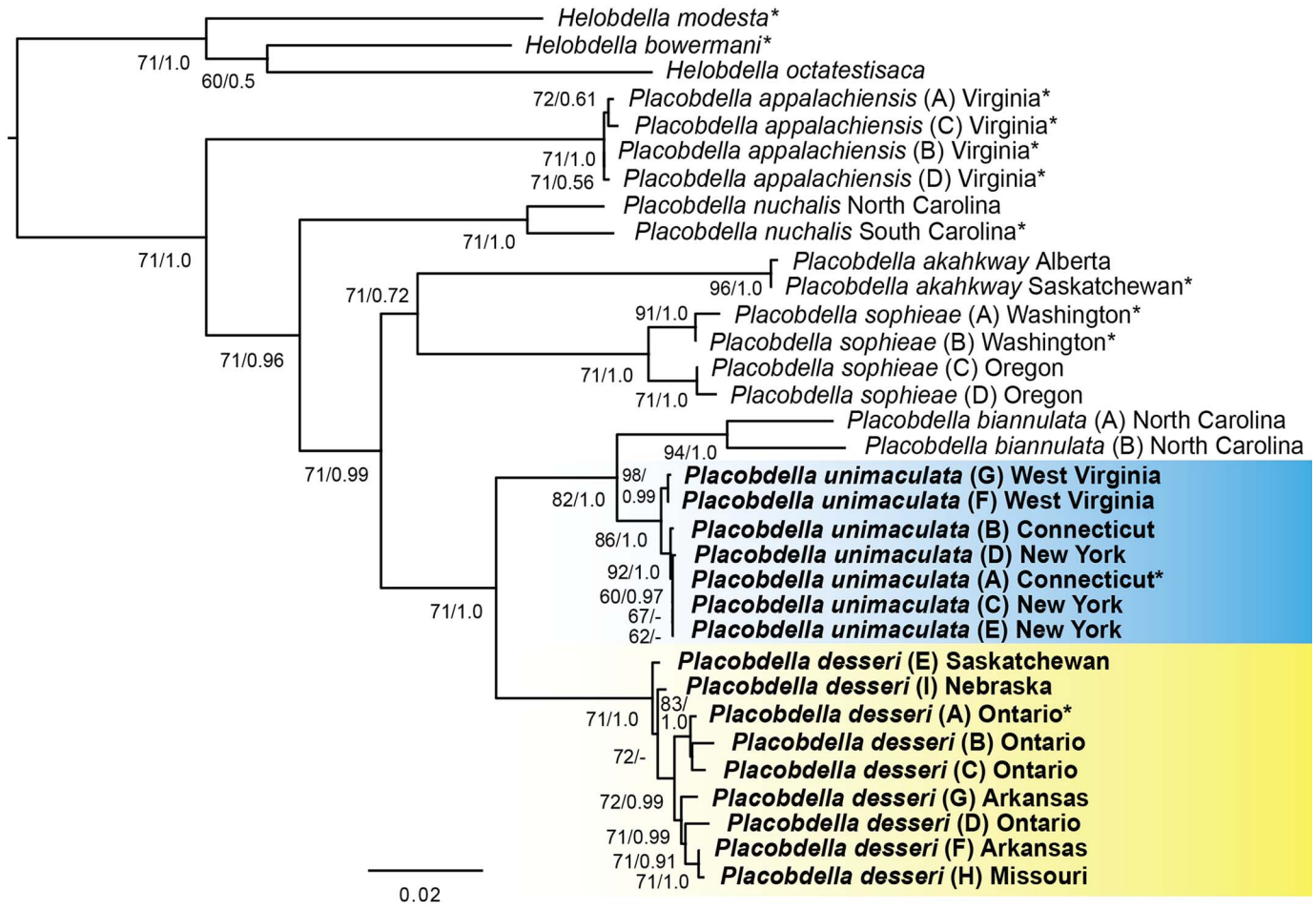

Figure 2. Phylogenetic relationships of *Placobdella* using combined gene analyses (*18S* rRNA, *COI*, *ND1*). Tree topology is based on the maximum likelihood analyses of the combined gene analyses done in IQTREE. Nodal support is indicated with both maximum likelihood bootstrapping (MLB) and Bayesian posterior probabilities (BPP). Only nodal support above MLB = 50 or BPP = 0.5 are indicated; those falling below this threshold are represented by a dash (—). An asterisk (*) indicates the sample was collected from the type locality of the species. Color version available online.

ND1 distances ranged from 0% to 0.96% in *P. unimaculata* n. sp., and 0.96% to 2.28% in *P. desseri* n. sp. but were lower when comparing between the 2 entities (10.5–11.78%) (Table S2).

Phylogenetic analyses

Our phylogenetic investigation was conducted using both Maximum Likelihood and Bayesian Inference probabilities for all single-gene data sets (not shown) as well as our final concatenated 3-gene data set (*18S* rRNA, *COI*, *ND1*). All analyses recovered *Placobdella* as monophyletic (MLB = 71; BPP = 1.0), and with several subclades corresponding to different *Placobdella* species (Fig. 2). *Placobdella unimaculata* n. sp. was recovered in a monophyletic clade comprising specimens from Connecticut, West Virginia, and New York (MLB = 86, BPP = 1.0) and was sister to *P. biannulata* (MLB = 94, BPP = 1.0). The clade *P. biannulata–P. unimaculata* n. sp. (MLB = 82, BPP = 1.0) was sister to the *P. desseri* n. sp. clade (MLB = 71, BPP = 1.0), which included representatives from Arkansas, Missouri, and Nebraska, and Ontario and Saskatchewan, Canada.

Placobdella akahkway Fan, de Carle, and Kvist 2022 (MLB = 96, BPP = 1.0) formed a clade with *Placobdella sophieae* Oceguera-Figueroa, Kvist, Watson, Sankar, Overstreet, and Siddall 2010 (MLB = 71, BPP = 0.72), which consisted of reciprocally monophyletic subclades based on collection locality (Fig. 2). Together, this clade was sister to *P. biannulata–P. desseri* n. sp. (MLB = 71, BPP = 0.99). *Placobdella nuchalis* (MLB = 71, BPP = 1.0) was recovered sister to the clade *P. akahkway–P. desseri* n. sp. (MLB = 71, BPP = 0.99). Representatives of *P. appalachiensis* Moser and Hopkins 2014 from the type locality in Virginia formed a monophyletic clade (MLB = 71, BPP = 1.0) sister to all other ingroup taxa (MLB = 71, BPP = 1.0).

Type series observations

Verrill (1872) did not indicate type material in the description and the original labels are all missing. The YPM Annelida Ledger indicates 4 specimen lots collected by and received from A. E. Verrill in 1871 from the West River, New Haven (YPM IZ 000251.AN), and Whitneyville Lake, Connecticut (YPM IZ 000285.AN–YPM IZ 000287.AN). According to the International Code of Zoological Nomenclature, these 4 lots of *C. picta* (YPM IZ 000251.AN, YPM IZ 000285.AN–YPM IZ 000287.AN) are Syntypes (Table I). In the

YPM Annelida Ledger, YPM IZ 000285.AN (4 specimens) was also mentioned as "Type" in the Remarks section.

During the redescription of *P. picta* by Barta and Sawyer (1990), YPM IZ 000286.AN was considered to be the lectotype, and the other 3 specimen lots of *C. picta* at the YPM were considered paralectotypes. Our examinations of these specimens found that YPM IZ 000286.AN is consistent with *Placobdella ornata* (Verrill, 1872) as redescribed by Moser et al. (2012b) in its possession of 3 rows of dorsal papillae and 5 pairs of pre-anal papillae. Of the 4 original specimens marked as Type in the YPM ledger from the specimen lot YPM IZ 000285.AN, 1 specimen (YPM IZ 000285.AN [A]) was determined to be *Placobdella parasitica* (Say 1824) in its possession of a smooth dorsal surface, and 2 specimens (YPM IZ 000285.AN[B, C]) were similarly consistent with *Placobdella ornata* (Verrill, 1872) as redescribed by Moser et al. (2012b). The remaining specimen (YPM IZ 000285.AN[D]) had a proboscis pore on the lip of the anterior sucker yet is poorly preserved, making diagnostic characters difficult to observe other than those identifying it as a species of *Placobdella*. As mentioned by Barta and Sawyer (1990), YPM IZ 000251.AN is an undetermined species of the genus *Helobdella*. Barta and Sawyer (1990) mentioned that YPM IZ 000287.AN was small and "could not be assigned to any currently recognized species or groups of species." Our examinations revealed that this specimen likely dried out at one time and could not be identified other than as a species of *Placobdella*. In conclusion, none of the specimens from Verrill's description can serve as a lectotype for the species *P. picta* (Table I). Barta and Sawyer (1990) subsequently based their anatomical redescription on novel specimens from Algonquin Provincial Park, Ontario, Canada.

Revision of classification

The scientific names *Clepsine picta* and *Clepsine ornata* were published simultaneously in the same publication (Verrill, 1872). Under article 24.2 and recommendation 24A of the International Code of Zoological Nomenclature (International Commission of Zoological Nomenclature, 1999), we are acting as first revisers and designating the priority of *Placobdella ornata* (Verrill, 1872; page 130) over *Placobdella picta* (Verrill, 1872; page 128) as it will best serve the stability and universality of nomenclature. The species *P. ornata* was redescribed and stabilized by Moser et al. (2012b), whereas *P. picta* has not been stabilized and comes with challenges in the description and with the type series. Given the vague and incomplete description of *C. picta* provided by Verrill (1872) in conjunction with the lectotype specimen (YPM IZ 000286.AN) and paralectotype specimens (YPM IZ 000285.AN [B, C]) designated by Barta and Sawyer (1990) to be *Placobdella ornata* (Verrill, 1872) Moser et al. 2012, *Clepsine picta* Verrill, 1872, is hereby considered a junior synonym of *P. ornata sensu* Moser et al. (2012b) along with the subsequent combinations: *Batrachobdella picta* (Verrill, 1872) Richardson, 1949, *Batracobdella picta* (Verrill, 1872) Meyer and Moore, 1954, *Desserobdella picta* (Verrill, 1872) Barta and Sawyer, 1990, and *Placobdella picta* (Verrill, 1872) Moore, 1906.

Both live and preserved specimens were examined using light microscopy and dissection techniques (Figs. 3–7). The specimens were found to have characteristics consistent with other members of *Placobdella*: 2 pairs of eyes, male and female gonopores separated by 2 annuli, a papillated body, 7 pairs of crop caeca, and bifurcated ovaries. The following morphological observations along with the molecular phylogenetic and species delimitation analyses warrant the description of 2 new species.

DESCRIPTION

Placobdella unimaculata n. sp. Moser, Richardson, and Phillips

(Figs. 3–6)

Diagnosis: Dorsum olive-green to brownish with 6 rows of unpigmented small papillae (pair of paramedial and 2 pairs of paralateral) and a very thin dorso-medial line. Cephalic region unpigmented with 2 pairs of eyespots and a large white spot posteriad of the eyespots; no nuchal band. Proboscis pore on the rim of the oral sucker and diffuse salivary cells scattered in the anterior third of the body.

External morphology: Body narrowly to very deeply ovoid to deltoid and dorso-ventrally flattened (thin); length of preserved specimens 8.3 mm ± 2.3 mm. Dorsum pigmented by scattered chromatophores that are olive green (HEX#948A4D) to brownish (HEX#4B3F3A) (Fig. 3; see hex color codes, http://www.colorhexa.com) with 6 rows of unpigmented (whitish) small papillae (pair of paramedial and 2 pairs of paralateral) on the sensory annulus (Fig. 4A). Scattered dark green to dark brown chromatophores form a very thin dorso-medial line that can be obscured by the contents of the crop ceca. Three pairs of pre-anal papillae and 6 rows of papillae start 1 annulus anteriad of pre-anal papillae (Fig. 4B). Apical cephalic region unpigmented with 2 pairs of eyespots (1 pair much larger than the other). Large white spot just below the eyespots, and there is no nuchal band (Fig. 3). Caudal sucker pigmented by scattered chromatophores and with 1 row of small unpigmented papillae near the edge of the caudal sucker. Ventrum with similar coloration as the dorsum and pigmented by scattered chromatophores. Male and female gonopores unpigmented, in furrows, and separated by 2 annuli.

Digestive system: The proboscis pore is on the rim/lip of the oral sucker. Blunt-tipped, uniformly cylindrical proboscis in membranous sheath with retractor muscles and salivary ductule bundles attaching on either side of the base of the proboscis. Diffuse salivary glands where the salivary cells are scattered in the anterior third of the body of the leech and more abundant around the base of the proboscis (Fig. 5A). Flaccid esophagus extends from the base of the proboscis with 1 pair of sac-like mycetomes. Seven pairs of diverticulated crop ceca with the last pair extending posteriad into 4 diverticulated sections (Fig. 6). Four pairs of narrowly, elliptoid intestinal ceca. Rectum opening to anus that is located 1 annulus anteriad of the caudal sucker.

Reproductive system: Male and female gonopores in furrows and separated by 2 annuli. (Male) Male gonopore slightly raised. Short, narrowly elliptoid artrial cornua that extends laterally from male gonopore into robust ejaculatory ducts that abruptly narrows at the point of attachment with the atrial cornu (Fig. 5B). Ejaculatory ducts recurve posteriorly to seminal vesicles and narrow vas deferentia connecting to testisacs. Six pairs of testisacs and each pair located in the space between a pair of crop ceca (Fig. 6). (Female) Female gonopore simple and opening to pair of bifurcated ovisacs (Fig. 5A). Anterior extensions of the

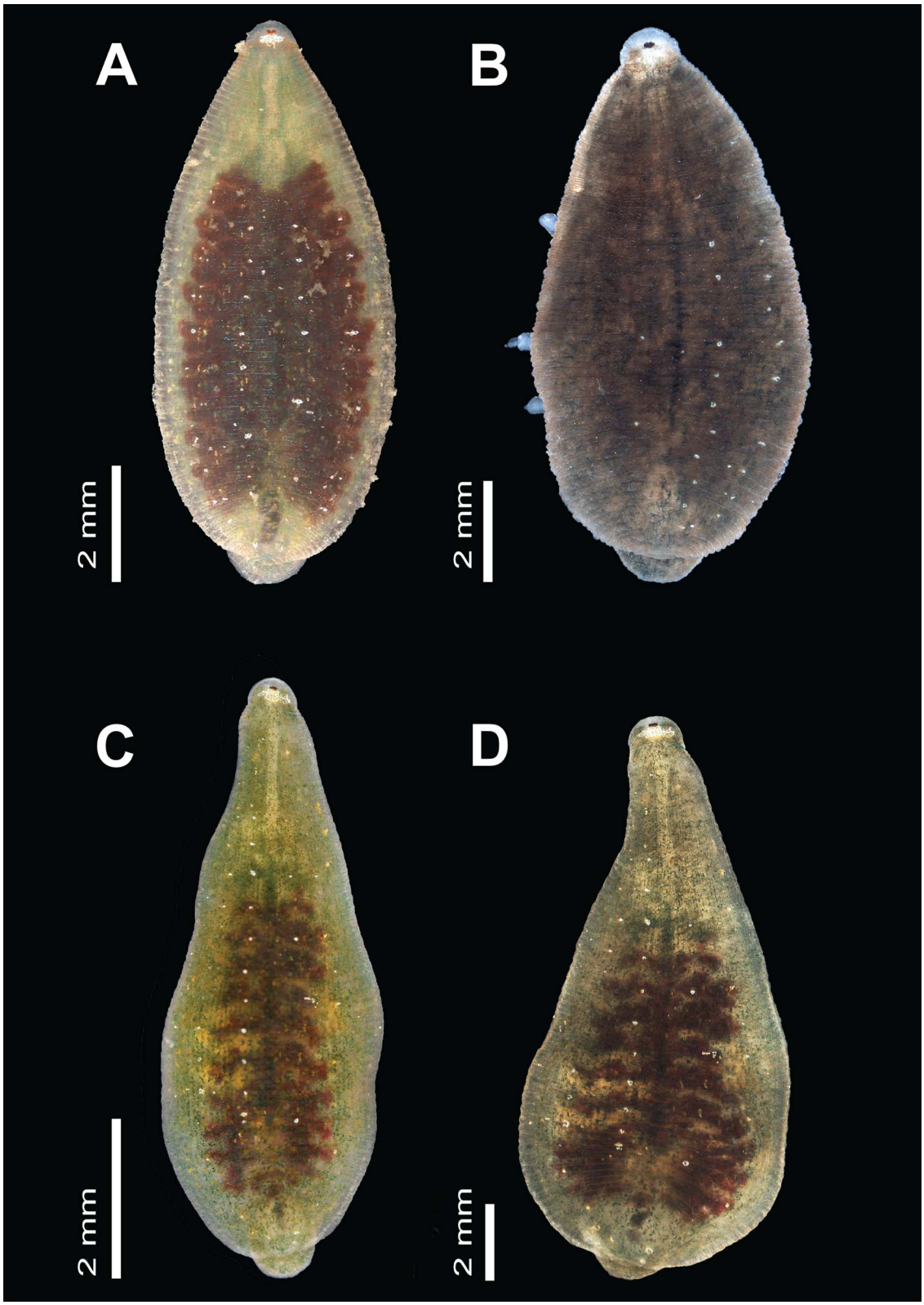

Figure 3. Images of living specimens of *Placobdella unimaculata* n. sp. from the type locality, illustrating its color variation. (**A**) YPM IZ 107059; (**B**) YPM IZ 070779; (**C**) YPM IZ 070778; (**D**) YPM IZ 070778.

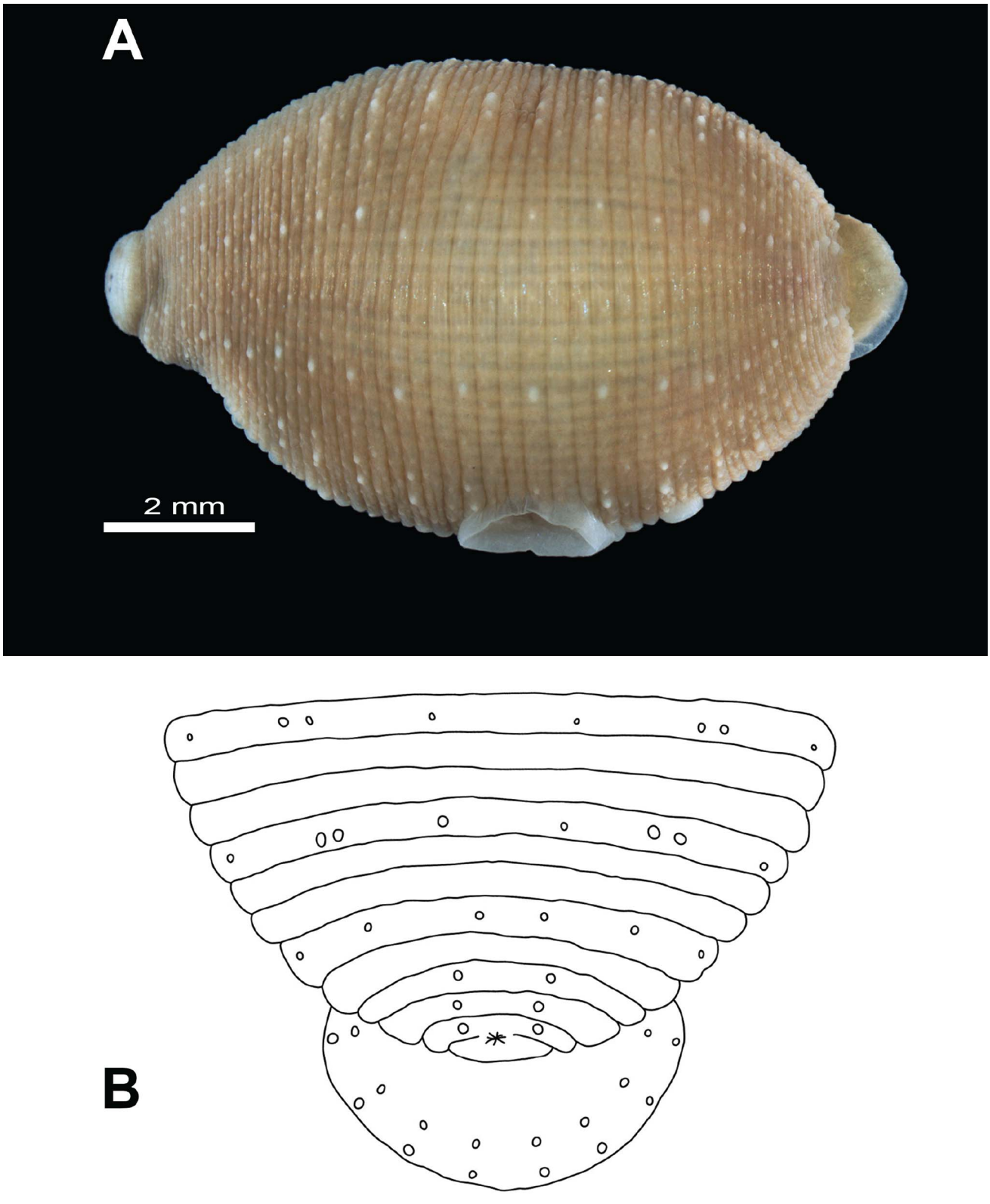

Figure 4. Dorsal papillar pattern in *Placobdella unimaculata* n. sp. (**A**) YPM IZ 070778. (**B**) Drawing of the posterior end (modified from Whitman, unpublished). Color version available online.

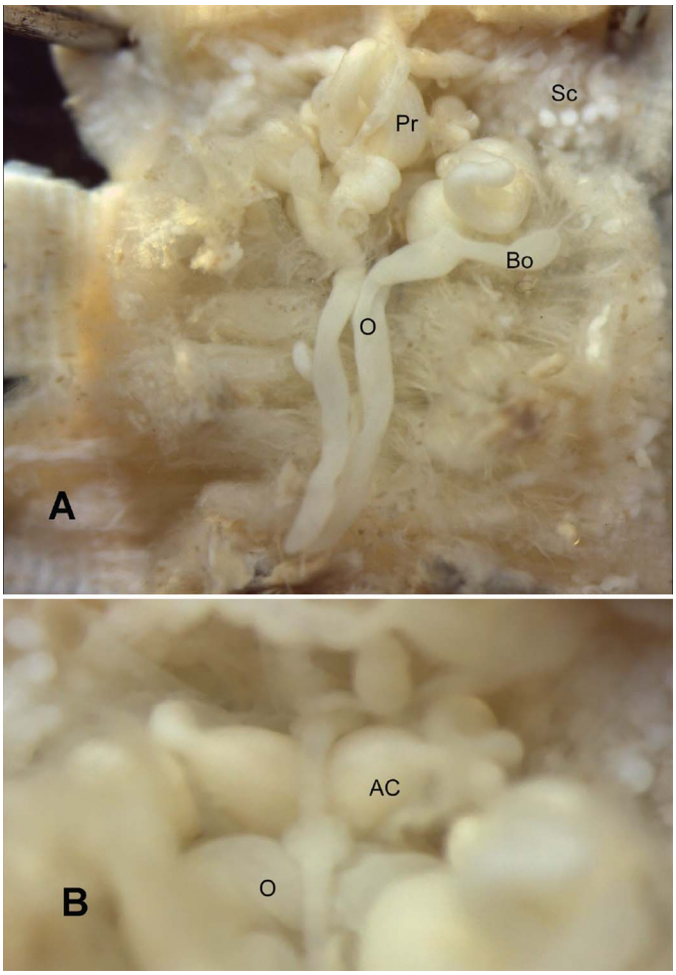

Figure 5. Dissected specimen of *Placobdella unimaculata* n. sp. (YPM IZ 070778) showing the alimentary and reproductive system. Abbreviations: AC, atrial cornua; Bo, bifurcated ovisac; O, ovisac; Pr, proboscis; Sc, salivary cells. Color version available online.

ovisacs are much smaller than the posterior section. Ovisac length depends on the reproduction condition of the leech, and ovisacs extend posteriorly for several somites.

Taxonomic summary

Material examined: Holotype: YPM IZ 070778. Paratypes: YPM IZ 070779, YPM IZ 070781, YPM IZ 111842–111843, USNM 1740770–1740774. Other material: YPM IZ 029269, YPM IZ 058077, YPM IZ 111844, USNM 1740776, USNM 1740781–1740784.

Type locality: Sturges Pond, The Roy and Margot Larsen Wildlife Sanctuary, Connecticut Audubon Society, Fairfield County, Connecticut (41°11′50.4996″N, −73°18′1.3998″W).

ZooBank registration: urn:lsid:zoobank.org:act:74D3AB7A-2BDE-4DA7-9018-1234F5434D0F.

Etymology: The specific epithet "*unimaculata*" is derived from the Latin *unus* (one) and *maculatus* (spot) referring to the single white spot that is located posterior to the eyespots.

Ecology: Individuals of *P. unimaculata* n. sp. co-occur with individuals of *P. papillifera* with the latter attached to the underside of sticks and the former attached to leaves that are suspended in the water column. *Placobdella unimaculata* n. sp. likely blood-feeds on amphibians with *Lithobates clamitans* (Latreille 1801) (green frogs), *Pseudacris crucifer* (Wied-Neuwied 1838) (spring peepers), and *Lithobates palustris* (Le Conte 1825) (pickerel frogs) being seasonally abundant in the type locality.

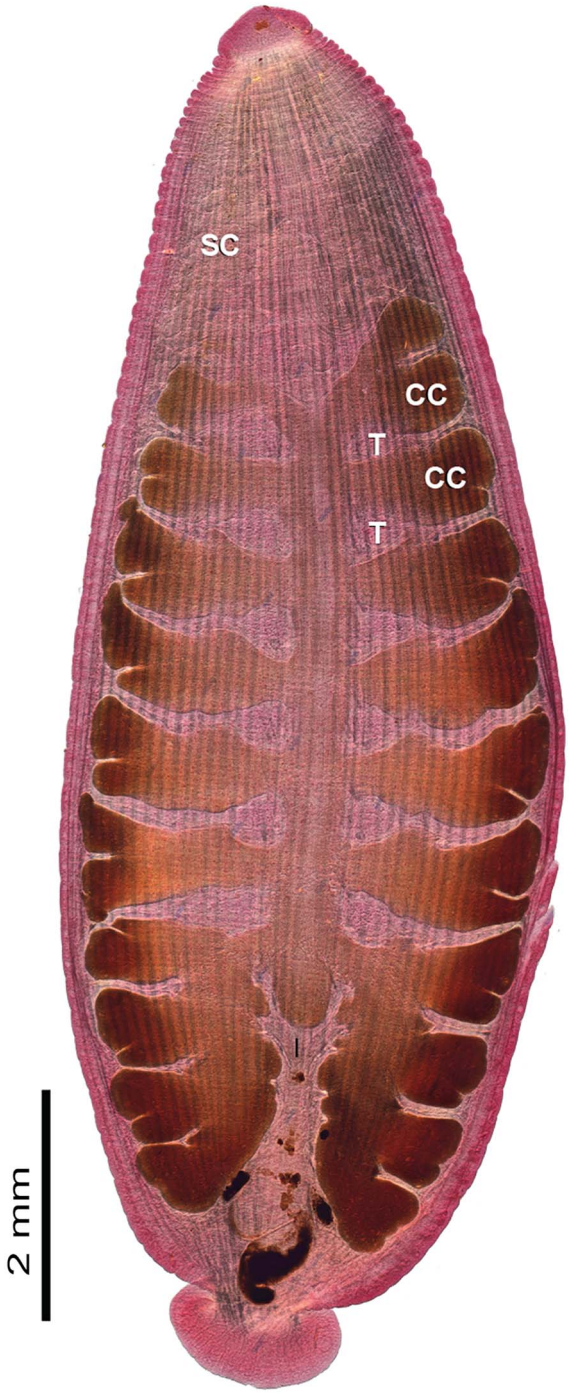

Figure 6. Internal morphology of *Placobdella unimaculata* n. sp. (YPM IZ 107059) from a cleared and stained specimen. Abbreviations: CC, crop ceca; Sc, salivary cells; T, testisac. Color version available online.

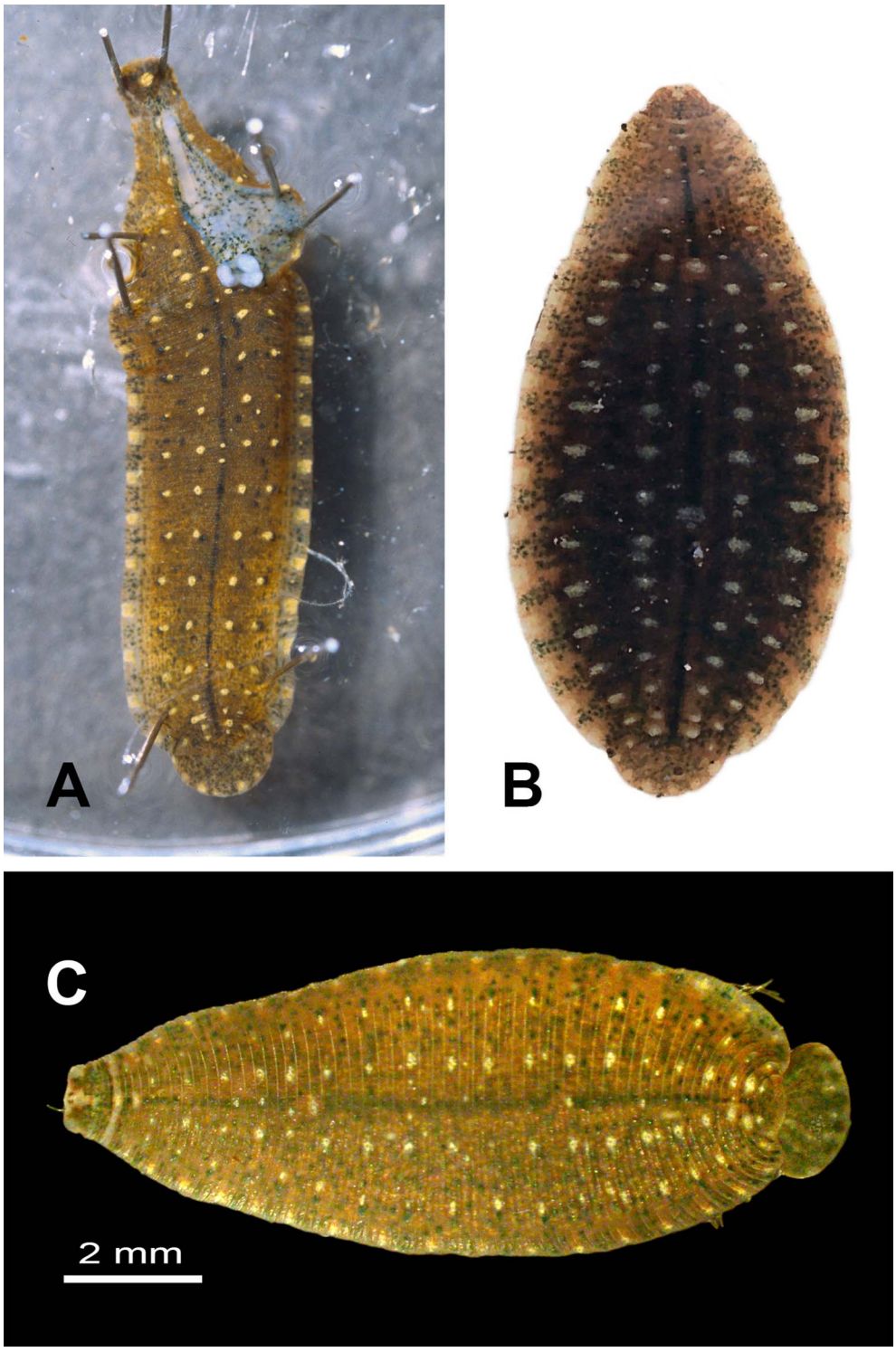

Figure 7. Dorsal surface of specimens of *Placobdella desseri* n. sp. (**A**) Dissected specimen from Algonquin Provincial Park, Ontario, Canada. (**B**) Specimen from Iowa (photographed by mwittman17). (**C**) Specimen from Arkansas.

Remarks

Placobdella unimaculata n. sp. is differentiated from *Placobdella costata* (Müller, 1846) and *Placobdella nabeulensis* Ben Ahmed, Gajda, Utevsky, Kvist, and Świątek 2023 by possessing bifurcated rather than simple ovaries. *Placobdella unimaculata* n. sp. possesses 6 rows of dorsal papillae rather than 2 rows as in *Placobdella cryptobranchi* (Johnson and Klemm, 1977), 3 rows as in *P. ornata*, *Placobdella hollensis* (Whitman 1892), and *Placobdella montifera* Moore 1906, 5 rows as in *Placobdella rugosa* (Verrill 1874), *Placobdella burresonae* Siddall and Bowerman 2006, *Placobdella michiganensis* (Sawyer 1972), *Placobdella siddalli* Richardson, Moser, Hammond, Lazo-Wasem, McAllister, and Pulis 2017, and *P. akahkway*, 7 rows as in *Placobdella kwetlumye* Oceguera-Figueroa, Kvist, Watson, Sankar, Overstreet, and

Siddall 2010 and *P. nabeulensis*, or a smooth dorsum without papillae as in *Placobdella translucens* Sawyer and Shelley 1976, *Placobdella pediculata* Hemingway 1908, *P. nuchalis*, *P. biannulata*, and *P. parasitica*. The new species can be distinguished based on its possession of a thin dorso-medial line and large white spot posterior to the eyespots on the dorsum rather than a near-transparent green dorsum without lines, spots, or patches as in *P. sophieae*. *Placobdella unimaculata* is differentiated by possessing 3 pairs of pre-anal papillae rather than no pre-anal papillae as in *P. appalachiensis*. The new species possesses a venter without stripes rather than a venter with stripes as in *Placobdella ali* Hughes and Siddall 2007, *Placobdella papillifera* (Verrill 1872), *Placobdella mexicana* (Moore 1898), *Placobdella multilineata* Moore 1953, and *Placobdella lamothei* Oceguera-Figueroa and Siddall 2008.

DESCRIPTION

Placobdella desseri n. sp. Moser, Richardson, and Phillips
(Fig. 7)

Diagnosis: Dorsum green-brown coloration with fine patches of orange pigment; with dark mid-dorsal line, pair of paralateral and paramedial rows of white papillae, and a few white papillae aligned with the mid-dorsal line. An unpigmented nuchal band is present in many specimens; unpigmented region anterior and posterior of the 2 pairs of coalesced eye spots; small mouth pore below the rim of the oral sucker and diffuse salivary cell scattered in the anterior third of the body.

Barta and Sawyer (1990) provided a thorough and complete description of the species under the name *Desserobdella picta* (Verrill, 1872) based on specimens used in their redescription (CMN 1988-0271 and CMN 1988-0272).

Taxonomic summary

Material examined: Holotype: CMN 1988-0271, Paratypes: CMN 1988-0272 and CMN 1990-0011. Other material: USNM 1740779, 1740780, and YPM IZ 111860.

Type locality: Lake of Two Rivers; Algonquin Provincial Park, Ontario, Canada (45°34′30″N, −78°29′46.9998″W).

ZooBank registration: urn:lsid:zoobank.org:act:C880BA6E-D111-4BCB-BE9C-E2ECA8F6C54E

Etymology: The specific epithet "*desseri*" is named in recognition of University of Toronto Professor Emeritus Dr. Sherwin S. Desser, who contributed much to our knowledge of this leech species.

Ecology: Habitat and life history of *P. desseri* n. sp. is as reported in previous studies by Klemm (1982, 1985) and Sawyer (1972) under the name *Batracobdella picta* (Verrill, 1872).

Remarks

Placobdella desseri is unique among members of *Placobdella* by possessing 4 rows of dorsal papillae. The morphology of the new species is most similar to *P. unimaculata*. Morphological characters in common with *P. unimaculata* are olive-green to brownish coloration, the cephalic region is mostly unpigmented, 2 coalesced eye spots, and diffuse salivary cells scattered in the anterior third of the body. However, several characters are different: *P. desseri* has a dark mid-dorsal line rather than a very thin dorso-medial line in *P. unimaculata; P. desseri* has a pair of paralateral and paramedial rows of dorsal papillae (4 rows total), whereas *P. unimaculata* has a pair of paramedial and 2 pairs of paralateral papillae for a total of 6 rows of dorsal papillae; an unpigmented nuchal band is observed in many specimens of *P. desseri*, while specimens of *P. unimaculata* have a large white spot posterior to the eyespots and no nuchal band; and the proboscis pore is located below the oral sucker rim in specimens of *P. desseri* rather than on the rim of the oral sucker as in specimens of *P. unimaculata*.

DISCUSSION

Our analyses of molecular phylogenetic and species delimitation analyses and morphological examinations support the description of 2 new species, *P. unimaculata* for the individuals in the clade collected from Fairfield, Connecticut and *P. desseri* for the clade that includes individuals from Algonquin Provincial Park, Ontario, Canada (Figs. 1, 2; Table III). Examination of the type series of *P. picta* found the morphology of the type specimens to be consistent with other species of *Placobdella* and *Helobdella* and supported the synonymization of this species name with *P. ornata*.

Using the most conservative estimates, the species delimitation analyses of single and combined gene data sets identified 2 distinct groups that correspond with *P. unimaculata* and *P. desseri* (Figs. 1, 2; Table III). In GMYC analyses, only the results of the *ND1* single-gene data set were significant. The sequences of *ND1* in our analysis include more informative sites than the *COI* sequences, indicating that *ND1* could be more informative for species-level questions of glossiphoniid leeches than other genes. It has been well documented that *COI* is saturated in leeches although the phylogenetic signal of that gene reflects the signal of other, less saturated mitochondrial loci (ex. *ND1*, *COIII*; Nakano et al., 2012). Genetic distances of *COI* have been used to distinguish leech species (reliably in most cases), although *COI* variation alone is not sufficient for assessing species boundaries and should be paired with data of additional loci and morphological examination, as well as ecological and behavioral data (Oceguera-Figueroa et al., 2011; Kvist et al., 2022). Our findings suggest that the genetic distances of *ND1* sequences in addition to those of *COI*, the traditional DNA barcoding locus, are also informative for recognizing distinct species-level entities within leeches, especially the glossiphoniids. In the future, broader approaches using larger DNA fragments, such as mitochondrial genomes, or additional mitochondrial genes will provide insight into detecting unrecognized diversity and assessing the position of new diversity in a phylogenetic context.

Species delimitation analyses supported including the specimens identified as *P. picta* (ROMIZ 10235, 10257, 10111, and 11395) by de Carle et al. (2017) within the *P. desseri* species entity, a finding that was additionally confirmed by our molecular phylogeny (Figs. 1, 2). In turn, sequences of specimens from New York identified as *P. nuchalis* by de Carle et al. (2017), and subsequently used by Fan et al. (2022), were included within the *P. unimaculata* species entity. In the molecular phylogeny, these sequences (*P. unimaculata* C, D, and E) are placed within the *P. unimaculata* clade and not close to the sequences of *P. nuchalis* from the type and paratype localities. Additionally, these sequences are all 100%

identical to the *COI* sequence of *P. unimaculata* from the type locality (YPM IZ O70781Y) and only 1.1–1.3% different using *ND1* sequences (Tables S1, S2). Based on these analyses, these specimens (ROMIZ 12981–12983) were misidentified and are consistent with *P. unimaculata*. Thus, the geographic distribution of *P. unimaculata* includes southern Connecticut, southeastern New York, and West Virginia, while *P. desseri* is known from Arkansas, Missouri, and Nebraska, and Ontario and Saskatchewan, Canada. As has been observed in other North American *Placobdella* species, the Appalachian Mountain range could be a geographic barrier between *P. unimaculata* and *P. desseri*, but further sampling efforts are needed to determine the extent of the geographic distributions of each of the new species (Richardson et al., 2020).

The findings of published research that included the species name *P. picta sensu* Moore, 1952 need to be interpreted carefully in light of the synonymization with *P. ornata*. Moser and Desser (1995) characterized the salivary glands and proboscis morphology of *P. ornata*, *P. parasitica*, and *P. picta* with light and electron microscopy. Specimens of *P. picta* in their study were from Algonquin Provincial Park, Ontario, the type locality of *P. desseri*, and their findings can be applied to our understanding of the morphological characteristics of this new species. McCallum et al. (2011) reported infestation rates of *P. picta* on amphibians in northeastern Arkansas, which falls within the distribution of *P. desseri* and geographically close to the collection localities of *P. desseri* specimens in our analyses from northwestern Arkansas. Their findings about infestation rates and life history also can be applied to our knowledge of *P. desseri*.

Additional *Placobdella* species could benefit from being assessed with species delimitation methods. De Carle et al. (2017) remarked that species entities identified in their analyses as *P. picta*, *P. rugosa*, and *Placobdella phalera* (Graf 1899), the last synonymized with *P. ornata* by Moser et al. (2012b), are widespread species that exhibit a high degree of morphological variation. Our findings suggest that *P. rugosa* and *P. phalera* have the potential to also have hidden diversity. *Placobdella rugosa* was redescribed with specimens from the type locality by Moser et al. (2012a), and Mack et al. (2019) found surprisingly low genetic variation in *COI* sequences of specimens collected throughout a large portion of the geographic distribution in Canada and the midwestern United States. The species entity identified as *P. phalera* by de Carle et al. (2017) needs to be assessed and recharacterized. This species was synonymized with *P. ornata* following examination of the type series and topotypes (Moser et al., 2012a), yet the species name has remained in use. Both of these species could benefit from the application of species delimitation methods now that the concept of these species has been anchored with a reexamination of the type series and molecular data derived from topotype material.

CONCLUSION

Placobdella picta has had a convoluted taxonomic history. Moore (1952) grafted the name *picta* to a species that he mentioned would later be fully described. In their redescription of *P. picta*, Barta and Sawyer (1990) used specimens from Ontario for their redescription and affixed the lectotype to a specimen of *P. ornata* from Connecticut (the type locality of *P. picta*). Here *Placobdella picta* is treated as a junior synonym of *P. ornata* based on a reexamination of the type series. Species delimitation analyses support novel sequences of specimens and sequences from GenBank segregating into 2 species entities that were also recovered in the molecular phylogeny. The clade that includes specimens from Connecticut is named *Placobdella unimaculata* and the clade that includes specimens from Ontario, Canada, is named *Placobdella desseri*.

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