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Unravelling the diversity of the Crassiphialinae (Digenea: Diplostomidae) with molecular phylogeny and descriptions of five new species

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ABSTRACT

Crassiphialinae Sudarikov, 1960 is a large subfamily of the Diplostomidae Poirier, 1886 with a complex taxonomic history. It includes a diversity of species parasitic in the intestines of avian and mammalian definitive hosts worldwide. *Posthodiplostomum* Dubois, 1936 is a large and broadly distributed crassiphialine genus notorious for its association with diseases in their fish second intermediate hosts. In this study, we generated partial 28S rDNA and cytochrome *c* oxidase subunit 1 (*cox*1) mtDNA gene sequences of digeneans belonging to seven crassiphialine genera. The 28S sequences were used to study the interrelationships among crassiphialines and their placement among other major diplostomoidean lineages. Our molecular phylogenetic analysis and review of morphology does not support subfamilies currently recognized in the Diplostomidae; therefore, we abandon the current subfamily system of the Diplostomidae. Molecular phylogenetic analyses suggest the synonymy of *Posthodiplostomum*, *Ornithodiplostomum* Dubois, 1936 and *Mesoophorodiplostomum* Dubois, 1936; morphological study of our well-fixed adult specimens and review of literature revealed lack of consistent differences among the three genera. Thus, we synonymize *Ornithodiplostomum* and *Mesoophorodiplostomum* with *Posthodiplostomum*. Our phylogenetic analyses suggest an Old World origin of *Posthodiplostomum* followed by multiple dispersal events among biogeographic realms. Furthermore, our analyses indicate that the ancestors of these digeneans likely parasitized ardeid definitive hosts. Four new species of *Posthodiplostomum* collected from birds in the New World as well as one new species of *Posthodiplostomoides* Williams, 1969 from Uganda are described.

1. Introduction

Crassiphialinae Sudarikov, 1960 is a relatively large subfamily of the digenean family Diplostomidae Poirier, 1886. Its members parasitize, as adults, a variety of avian and mammalian definitive hosts worldwide. Despite the large number of studies on the Crassiphialinae, the systematics of the subfamily is complex and has always been unstable (Dubois, 1970; Shoop, 1989; Niewiadomska, 2002). Therefore, the use of DNA sequence data for phylogenetic inference and taxon differentiation within the Crassiphialinae is highly beneficial. At present, only five of the 16 genera of crassiphialines have published DNA sequences of the large ribosomal subunit (28S) from adult specimens. Previous molecular phylogenetic studies have cast doubt on the validity of the Crassiphialinae based on the position of *Crassiphiala* Van Haitsma, 1925 and *Uvulifer* Yamaguti, 1934 being separate from *Bolbophorus* Dubois, 1934, *Ornithodiplostomum* Dubois, 1936 and *Posthodiplostomum* Dubois, 1936 (e.g. Achatz et al., 2019c).

Posthodiplostomum is a large, widely distributed and often reported crassiphialine genus whose members as adults are parasitic in the

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intestine of piscivorous birds throughout the world (Dubois, 1968; Niewiadomska, 2002). This genus is well-known to fisheries biologists and wildlife disease ecologists due to the association of *Posthodiplostomum* spp. with fish diseases and a common use of these parasites as models in ecological studies (e.g. Lane et al., 2015; Boone et al., 2018). The metacercariae of *Posthodiplostomum* are known to be associated with 'black spot' disease when encysted on the skin or fins of their fish second intermediate hosts (Horák et al., 2014); these metacercariae are also commonly referred to as 'white grub' when encysting within fish tissues, often visceral organs (see Boone et al., 2018 and references therein). These 'white grub' are commonly associated with a variety of pathologies in fishes and may cause death (Hoffman, 1958; Spall and Summerfelt, 1969; Lane and Morris, 2000).

Members of the genus *Ornithodiplostomum* have attracted significant attention from researchers due to their association with disease in fishes; their metacercariae are known to encyst on the brain of their fish second intermediate hosts (e.g. Matisz et al., 2010). Another crassiphialine genus, *Mesoophorodiplostomum* Dubois, 1936, has been only reported from the Nearctic and is much less studied than some of the larger and more broadly distributed genera. A close relationship among *Posthodiplostomum*, *Ornithodiplostomum* and *Mesoophorodiplostomum* has been recently demonstrated using sequences of the ribosomal internal transcribed spacer region (ITS1 + 5.8S + ITS2) as well as the mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) gene (Blasco-Costa and Locke, 2017; López-Hernández et al., 2018).

Despite the fact that larval specimens of *Posthodiplostomum* spp. are commonly collected and studied using molecular tools (e.g. Locke et al., 2010; Blasco-Costa and Locke, 2017; Kvach et al., 2017; Stoyanov et al., 2017; Locke et al., 2018; López-Hernández et al., 2018; Cech et al., 2020), few studies which produced DNA sequences have provided species identifications based on adult morphology (e.g. Locke et al., 2018). At present, only *Posthodiplostomum centrarchi* Hoffman, 1958, *Posthodiplostomum nanum* Dubois, 1937 and *Mesoophorodiplostomum pricei* (Krull, 1934) have DNA sequence data from adult specimens (Locke et al, 2010, 2018; López-Hernández et al., 2018) while sequence data from adult *Ornithodiplostomum* are lacking.

In the present study, we generated partial 28S rDNA and *cox*1 gene sequences from 28 species/species-level lineages belonging to seven genera of crassiphialines from Africa, Europe and the New World. The newly obtained 28S sequences were used for phylogenetic inference of crassiphialine taxa to demonstrate the phylogenetic position of these taxa among other major lineages of diplostomoideans, re-evaluate their systematics and aid ecological studies and disease diagnostics. Detailed phylogenetic analyses of 28S and *cox*1 sequences were conducted for closely related *Posthodiplostomum*, *Ornithodiplostomum* and *Mesoophorodiplostomum*. Whenever possible, type-species of corresponding genera were used in our analyses. Furthermore, four new species of *Posthodiplostomum* are described from the New World as well as one new species of another crassiphialine genus, *Posthodiplostomoides* Williams, 1969, from Africa.

2. Materials and methods

2.1. Sample collection and morphological study

Adult diplostomid digeneans were obtained from the intestines of a variety of avian hosts, while larval diplostomids were collected from a variety of snail and fish species in the New World, Africa and Europe (Table 1). Live diplostomids were rinsed in saline, heat-killed with hot water and fixed in 70% ethanol. Dead digeneans were immediately fixed in 95% ethanol. Specimens for light microscopy were stained with aqueous alum carmine according to the protocol provided by Lutz et al. (2017) and studied using a DIC-equipped Olympus BX51 compound microscope (Olympus Corp., Tokyo, Japan). All measurements are provided in micrometres. Type-series and morphological vouchers were deposited in the collection of the H. W. Manter Laboratory, University of Nebraska, Lincoln, Nebraska, USA and the Museum of Southwestern Biology (MSB), University of New Mexico, Albuquerque, New Mexico, USA (Table 1). Host specimens were deposited in the Philip L. Wright Zoological Museum (UMZM), University of Montana, Missoula, Montana, USA, the MSB, and the Museum of the Universidade Federal de Mato Grosso (UFMT), Brazil.

As in several recent studies of diplostomoideans, we refer to the two distinct body parts in diplostomoideans as prosoma and opisthosoma; justification for the use of this terminology is provided in detail by Achatz et al. (2019a) and Tkach et al. (2020).

To comply with the regulations set out in Article 8.5 of the amended 2012 version of the *International Code of Zoological Nomenclature* (ICZN, 2012), details of the new species have been submitted to ZooBank. The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub:85347BC8-9AC0-498B-9DFB-FC8A0F5EBCF7. The LSIDs for the new taxa are provided in the taxonomic summaries.

2.2. Molecular study

Genomic DNA of diplostomids was isolated according to the protocol described by Tkach and Pawlowski (1999). Fragments of the nuclear ribosomal 28S rDNA and mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) genes were amplified by polymerase chain reactions (PCR). Amplifications of 28S were performed using forward primer digL2 (5′-AAG CAT ATC ACT AAG CGG-3′) and reverse primer 1500R (5′-GCT ATC CTG AGG GAA ACT TCG-3′) (Tkach et al., 2003). A fragment of the *cox*1 gene was amplified using forward primers Plat-diploCOX1F (5′-CGT TTR AAT TAT ACG GAT CC-3′), Cox1_Schist_5' (5′-TCT TTR GAT CAT AAG CG-3′), Dipl_Cox_5' (5′-ACK TTR GAW CAT AAG CG-3′) and BS_CO1_INT_F (5′-ATT AAC CCT CAC TAA ATG ATT TTT TTY TTT YTR ATG CC-3′) and reverse primers Plat-diploCOX1R (5′-AGC ATA GTA ATM GCA GCA GC-3′), acox650R (5′-CCA AAA AAC CAA AAC ATA TGC TG-3′), JB5 (5′-AGC ACC TAA ACT TAA AAC ATA ATG AAA ATG-3′), Dipl650R (5′-CCA AAR AAY CAR AAY AWR TGY TG-3′), Dipl_Cox_3' (5′-WAR TGC ATN GGA AAA AAA CA-3′) and BS_CO1_INT_R (5′-TAA TAC GAC TCA CTA TAA AAA AAA MAM AGA AGA RAA MAC MGT AGT AAT-3′) (Lockyer et al., 2003; Derycke et al., 2005; Moszczynska et al., 2009; Kudlai et al., 2015; Achatz et al., 2019a, 2021b). PCR amplifications were performed in a total volume of 25 or 50 μl using GoTaq G2 DNA Polymerase from Promega (Madison, Wisconsin, USA) or One-Taq quick load PCR mix from New England Biolabs (Ipswich, Massachusetts, USA) according to the manufacturers' instructions. An annealing temperature of 53 °C was used for ribosomal amplifications and 45 °C was used for mitochondrial amplifications.

Illustra ExoProStar PCR clean-up enzymatic kit from Cytiva (Marlborough, Massachusetts, USA) was used to purify PCR products. Purified PCR products were cycle-sequenced directly using BrightDye Terminator Cycle Sequencing Kit (MCLAB, California, USA), cleaned using a BigDye Sequencing Clean Up Kit from MCLAB and run on an ABI 3130 automated capillary sequencer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The PCR primers were used for sequencing reactions. In addition, internal forward primer DPL600F (5′-CGG AGT GGT CAC CAC GAC CG -3′) and internal reverse primer DPL700R (5′-CAG CTG ATT ACA CCC AAA G-3′) were used for sequencing of 28S amplicons (Achatz et al., 2019a). Contiguous sequences were assembled using Sequencher 4.2 software (GeneCodes Corp., Ann Arbor, Michigan, USA) and deposited in the GenBank sequence database (Table 1).

2.3. Phylogenetic analyses

Newly generated and previously published sequences were initially aligned using ClustalW as implemented in MEGA7 software (Kumar et al., 2016). All alignments were trimmed to the length of the shortest sequence included in the analyses; sites with ambiguous homology were excluded from the analyses.

Table 1
Hosts, geographical origin, GenBank IDs and Harold W. Manter Laboratory (HWML) and Museum of Southwestern Biology (MSB) accession numbers of digeneans collected in this study

Taxa	Host species	Geographical origin	Museum accession number	GenBank ID	
				28S	*cox*1
Bolbophorus cf. *confusus*	*Pelecanus onocrotalus*	Ukraine	–	MZ710936	MZ707162
Cercocotyla rhodesiensis	*Halcyon malimbica*	Uganda	HWML 216634; MSB:Para:32014	MZ710937	MZ707163
Cercocotyla sp.	*Ceryle maxima*	Uganda	–	MZ710938	MZ707164
Posthodiplostomoides kinsellae n. sp.	*Halcyon malimbica*	Uganda	HWML 216635, 216636	MZ710939	MZ707165
Posthodiplostomum cf. *anterovarium* n. comb.[a]	*Lepomis cyanellus* (liver)	Minnesota, USA	HWML 216637	MZ710940, MZ710941	MZ707166
	Lepomis gibbosus (liver)	Minnesota, USA	–	MZ710942	MZ707167
Posthodiplostomum anterovarium n. comb.[a]	*Pelecanus erythrorhynchos*[c]	New Mexico, USA	MSB:Para:32011	MZ710943, MZ710944	MZ707168
Posthodiplostomum centrarchi	*Ambloplites rupestris*	Minnesota, USA	–	MZ710945	MZ707169
	Anhinga anhinga	Mississippi, USA	HWML 216638	MZ710946, MZ710947	MZ707170, MZ707171
	Anhinga anhinga	Louisiana, USA	HWML 216639; MSB:Para:32016	MZ710948	MZ707172
	Ardea alba	Mississippi, USA	–	–	MZ707173, MZ707174
	Ardea herodias	Georgia, USA	HWML 216641; MSB:Para:32018	MZ710949, MZ710950	MZ707175, MZ707176
	Lepomis cyanellus (liver)	Minnesota, USA	HWML 216642	MZ710951, MZ710952	MZ707177, MZ707178
	Lepomis cyanellus (skin)	Minnesota, USA	HWML 216643	MZ710953	MZ707179
	Lepomis macrochirus (heart)	Minnesota, USA	–	–	MZ707180
	Lepomis macrochirus (liver)	Minnesota, USA	–	–	MZ707181
	Lepomis macrochirus (mesentery)	Minnesota, USA	–	–	MZ707182
	Lepomis macrochirus (spleen)	Minnesota, USA	–	–	MZ707183
	Megaceryle alcyon	Mississippi, USA	–	MZ710954	MZ707184
Posthodiplostomum cuticola	*Nycticorax nycticorax*	Ukraine	HWML 216644; MSB:Para:32012	MZ710955	MZ707185
Posthodiplostomum erickgreenei n. sp.	*Pandion haliaetus*[d]	Montana, USA	HWML 216645, 216646	MZ710956	MZ707186
Posthodiplostomum eurypygae n. sp.	*Eurypyga helias*[e]	Pantanal, Brazil	HWML 216647, 216648	MZ710957	MZ707187
Posthodiplostomum macrocotyle	*Busarellus nigricollis*	Pantanal, Brazil	HWML 216649	MZ710958, MZ710959	MZ707188, MZ707189
Posthodiplostomum microsicya	*Tigrisoma lineatum*	Pantanal, Brazil	HWML 216650	MZ710960	–
Posthodiplostomum minimum	*Ardea herodias*	North Dakota, USA	HWML 216651; MSB:Para:32017	MZ710961	MZ707190
	Nycticorax nycticorax	Mississippi, USA	HWML 216653	MZ710962	MZ707191
Posthodiplostomum nanum	*Ardea alba*	Mississippi, USA	HWML 216654	MZ710963	MZ707192
Posthodiplostomum orchilongum	*Ardea alba*	Mississippi, USA	HWML 216655	MZ710964	–
	Egretta caerulea	Mississippi, USA	HWML 216656; MSB:Para:32015	MZ710965, MZ710966	MZ707193
Posthodiplostomum pacificus n. sp.	*Larus californicus*	California, USA	HWML 216657	MZ710967	MZ707194
Posthodiplostomum cf. *podicipitis* n. comb.[b]	*Catostomus commersonii* (skin)	Minnesota, USA	–	MZ710968	MZ707195
	Lophodytes cucullatus	North Dakota, USA	HWML 216658	MZ710969, MZ710970	MZ707196, MZ707197
	Pimephales promelas (brain)	Minnesota, USA	–	MZ710971	MZ707198
Posthodiplostomum pricei n. comb.[a]	*Larus delawarensis*	North Dakota, USA	HWML 216659; MSB:Para:32013	MZ710972, MZ710973	MZ707199, MZ707200
Posthodiplostomum ptychocheilus n. comb.[b]	*Mergus merganser*	Minnesota, USA	HWML 216660; MSB:Para:32019	MZ710974	MZ707201
Posthodiplostomum recurvirostrae n. sp.	*Recurvirostra americana*	North Dakota, USA	HWML 216661	MZ710975	MZ707202
Posthodiplostomum sp. 11[b]	*Chrosomus eos*	Minnesota, USA	–	MZ710976	MZ707203
	Unidentified fish (eyes)	North Dakota, USA	–	MZ710977	MZ707204
Posthodiplostomum sp. 17	*Lophodytes cucullatus*	North Dakota, USA	HWML 216662	MZ710978	MZ707205
Posthodiplostomum sp. 18	*Physa gyrina*	Oregon, USA	–	MZ710979, MZ710980	MZ707206, MZ707207
Posthodiplostomum sp. 18	*Pelecanus erythrorhynchos*	Oregon, USA	HWML 216663	MZ710981	MZ707208
Posthodiplostomum sp. 19	*Physa* sp.	Minnesota, USA	–	MZ710982, MZ710983	MZ707209
Posthodiplostomum sp. 20	*Physa gyrina*	Oregon, USA	–	MZ710984	MZ707210
Posthodiplostomum sp. 20	*Physa gyrina*	Oregon, USA	–	MZ710985- MZ710988	MZ707211
Posthodiplostomum sp. 21	*Tigrisoma lineatum*	Pantanal, Brazil	–	MZ710989	MZ707212
Posthodiplostomum sp. 21	*Jabiru mycteria*	Pantanal, Brazil	–	MZ710990	MZ707213
Posthodiplostomum sp. 22	*Ardea alba*	Pantanal, Brazil	HWML 216664	MZ710991	MZ707214
Posthodiplostomum sp. 22	*Ardea cocoi*	Pantanal, Brazil	–	MZ710992	MZ707215
Posthodiplostomum sp. 22	*Tigrisoma lineatum*	Pantanal, Brazil	HWML 216665	MZ710993	MZ707216
Posthodiplostomum sp. 23	*Ardea herodias*	Georgia, USA	HWML 216666	MZ710994, MZ710995	MZ707217, MZ707218
Pulvinifer macrostomum	*Gallinago gallinago*	Minnesota, USA	HWML 216667; MSB:Para:32020	MZ710996	MZ707219

Note: The localization of metacercariae in the second intermediate host is provided, when possible, in parentheses.
[a] Previously included in *Mesoophorodiplostomum*.
[b] Previously included in *Ornithodplostomum*.
[c] Host deposited in the Museum of Southwestern Biology (NK250053; MSB:Para:19549).
[d] Host deposited in the Philip L. Wright Zoological Museum (UMZM:Bird:22149).
[e] Host deposited in the Museum of the Universidade Federal de Mato Grosso (UFMT 4865).

The phylogenetic positions of *Bolbophorus*, *Cercocotyla* Yamaguti, 1939, *Mesoophorodiplostomum*, *Ornithodiplostomum*, *Posthodiplostomoides*, *Posthodiplostomum* and *Pulvinifer* Yamaguti, 1933 within the Diplostomoidea Poirier, 1886 were determined using a 28S alignment with *Suchocyathocotyle crocodili* (Yamaguti, 1954) (Cyathocotylidae Mühling, 1896) as the outgroup based on the topology presented by Achatz et al. (2019d). This alignment included newly generated sequences of *Bolbophorus* cf. *confusus* (Krause, 1914) (type-species; $n = 1$), *Cercocotyla* spp. ($n = 2$), *M. pricei* (type-species; $n = 1$), *Ornithodiplostomum ptychocheilus ptychocheilus* (Faust, 1917) (type-species; $n = 1$), *Posthodiplostomoides kinsellae* n. sp. ($n = 1$), *Posthodiplostomum* spp. (including the type-species; $n = 6$) and *Pulvinifer macrostomum* (Jägerskiöld, 1900) (type-species; $n = 1$) and previously published sequences of other crassiphialines including *Bolbophorus* spp. ($n = 4$), *Crassiphiala* ($n = 2$), *Ornithodiplostomum* ($n = 1$), *Posthodiplostomum* ($n = 4$) and *Uvulifer* ($n = 2$). This alignment also included non-crassiphialine diplostomids ($n = 11$) as well as members of the Proterodiplostomidae Dubois, 1936 ($n = 2$) and the Strigeidae Railliet, 1919 ($n = 12$).

Based on the results of the initial, broader analysis of 28S data, two subsequent analyses based on 28S and *cox*1 of *Posthodiplostomum* +*Ornithodiplostomum*+*Mesoophorodiplostomum* were conducted. Both analyses used the unidentified genus of diplostomid sequenced by Hoogendoorn et al. (2019) as the outgroup based on the results of the initial 28S analysis. The second alignment of 28S included newly generated sequences of *Posthodiplostomum* ($n = 21$) including the type-species *Posthodiplostomum cuticola* (von Nordmann, 1832), *Ornithodiplostomum* ($n = 1$) including the type-species *O. p. ptychocheilus*, *Mesoophorodiplostomum* ($n = 3$) including the type-species *M. pricei*, and previously published sequences of *Posthodiplostomum* ($n = 8$), *Ornithodiplostomum* ($n = 1$) and previously unidentified diplostomids ($n = 4$).

The alignment of *cox*1 sequences included newly generated sequences of *Posthodiplostomum* ($n = 25$) including the type-species *Po. cuticola*, *Ornithodiplostomum* ($n = 4$) including the type-species *O. p. ptychocheilus*, *Mesoophorodiplostomum* ($n = 5$) including the type-species *M. pricei*, and previously published sequences of *Posthodiplostomum* ($n = 15$), *Ornithodiplostomum* ($n = 11$), *Mesoophorodiplostomum* ($n = 3$) and an unidentified diplostomid ($n = 1$).

Bayesian inference (BI) as implemented in MrBayes v3.2.6 software was used for the phylogenetic analyses (Ronquist and Huelsenbeck, 2003). The general time-reversible model with estimates of invariant sites and gamma-distributed among-site variation (GTR + G + I) model was identified as the best-fitting nucleotide substitution model for all alignments using MEGA7 (Kumar et al., 2016). The BI analyses for the 28S datasets were performed using MrBayes software as follows: Markov chain Monte Carlo (MCMC) chains were run for 3,000,000 generations with sample frequency set at 1,000. Log-likelihood scores were plotted and only the final 75% of trees were used to produce the consensus trees. The BI analysis for the *cox*1 dataset used similar conditions; however, the dataset was analyzed as codons and ran for 6,000,000 generations. The number of generations for each analysis was determined as sufficient because the standard deviation stabilized below 0.01. Pairwise comparisons for each locus were carried out using MEGA7.

Several genera referred to in text begin with the letter 'P'. To avoid confusion and redundancy, we refer to *Pandion* as *Pa.*, *Pelecanus* as *Pe.*, *Posthodiplostomum* as *Po.*, *Posthodiplostomoides* as *Ps.* and *Pulvinifer* as *Pu.*

3. Results and discussion

3.1. Molecular phylogenies

The initial 28S alignment was 1,092 bp long; 60 bases were excluded from the analysis due to ambiguous homology. The phylogenetic tree resulting from the BI analysis of 28S clearly demonstrated the strong non-monophyly of the Diplostomidae and Strigeidae (Fig. 1), similar to previous molecular phylogenetic analyses of the Diplostomoidea (e.g. Blasco-Costa and Locke, 2017; Hernández-Mena et al., 2017; Achatz et al., 2019b, c, d, 2020b, 2021a; Queiroz et al., 2020; Tkach et al., 2020; Locke et al., 2021). Overall, the phylogeny consisted of a large basal polytomy with multiple independent clades. Importantly, members of the subfamilies of the Diplostomidae (i.e. Crassiphialinae and Diplostominae Poirier, 1886) were non-monophyletic. Both members of the Proterodiplostomidae formed a 100% supported monophyletic clade.

Bolbophorus spp. formed two distinct clades. The first clade (unsupported) included a larval specimen of *Bolbophorus* as a sister group to a 100% supported clade of *B.* cf. *confusus* + two other unidentified *Bolbophorus* species-level lineages (Fig. 1). Interestingly, *Bolbophorus damnificus* Overstreet & Curran, 2002 was positioned in a separate clade in the basal polytomy from the other members of *Bolbophorus*. *Cercocotyla* spp. formed an independent 100% supported clade in the basal polytomy. *Uvulifer* + *Crassiphiala* + *Posthodiplostomoides* formed a 100% supported clade in the basal polytomy of the Diplostomoidea. Within this clade, *Crassiphiala* + *Posthodiplostomoides* formed a weakly supported cluster (Fig. 1). Interestingly, *Pu. macrostomum* was positioned in a strongly supported clade (97%) with non-crassiphialine diplostomids. This 97% supported clade contained two subclades of *Alaria* Schrank, 1788 + *Pulvinifer* (unsupported) and *Diplostomum* + a clade of [*Austrodiplostomum* Szidat & Nani, 1951 + *Tylodelphys* Diesing, 1850 (98% support)].

The unidentified diplostomid of Hoogendoorn et al. (2019) (GenBank: MK604826)+cluster of *Posthodiplostomum*+*Ornithodiplostomum* +*Mesoophorodiplostomum* formed a fairly well-supported monophyletic clade (92%) within the basal polytomy of the Diplostomoidea (Fig. 1). This clade of the three genera was 99% supported with *Po. cuticola* positioned as a sister group to the weakly supported clade containing the remaining taxa (Fig. 1). Phylogenetic relationships among taxa within the *Posthodiplostomum* + *Ornithodiplostomum* + *Mesoophorodiplostomum* clade are discussed in detail below.

The second 28S alignment that included only members of *Posthodiplostomum*, *Ornithodiplostomum* and *Mesoophorodiplostomum* was 1,093 bp long; 28 bases were excluded from the analysis due to ambiguous homology. The topology of the tree resulting from the phylogenetic analysis of this alignment was overall well-resolved and strongly supported (Figs. 2 and 3). In this analysis, *Po. cuticola* (type-species of *Posthodiplostomum*) was positioned as a sister group to a 100% supported clade which contained all other taxa. The four sequences from larval *Posthodiplostomum* specimens collected in Eastern Asia (Palaearctic and Indomalayan realms) formed a 100% supported clade, which was separated from the 100% supported cluster containing the remaining *Posthodiplostomum*, *Ornithodiplostomum* and *Mesoophorodiplostomum* sequences. The 100% supported cluster contained seven well-supported clades. Clades I–VI formed a weakly supported clade separated from clade VII (polytomy of *Po. nanum*+*Posthodiplostomum* sp. 23+*Posthodiplostomum* sp. of Hernández-Mena et al. (2017); 100% supported). Clades I–VI were overall positioned in a polytomy (Fig. 2).

Clades I and II clustered in a weakly supported clade within the weakly supported polytomy. Clade I (100% support) included several unidentified species-level lineages of *Posthodiplostomum* and *Ornithodiplostomum* larvae without matching sequences from adults. *Posthodiplostomum* sp. 17 appeared as a sister group to a 100% supported cluster containing the remaining members of Clade I (Fig. 2). This 100% supported cluster was mostly a polytomy that included *Posthodiplostomum* sp. 19, *Ornithodiplostomum* cf. *podicipitis* Yamaguti, 1939, *O. p. ptychocheilus* (type-species of *Ornithodiplostomum*), *Posthodiplostomum recurvirostrae* n. sp., *Ornithodiplostomum scardinii* (Shulman, 1952) and a 100% supported clade of *Posthodiplostomum* sp. 18 + (*Posthodiplostomum* sp. 20 + *Posthodiplostomum* sp. 11).

Clade II (100% support) consisted primarily of *Posthodiplostomum* taxa with morphologically identified adults (Fig. 2) and was well resolved. *Posthodiplostomum eurypygae* n. sp. was positioned as a sister group to a 100% supported clade which contained all other members of the clade. Within this clade, *Posthodiplostomum orchilongum* Noble, 1936

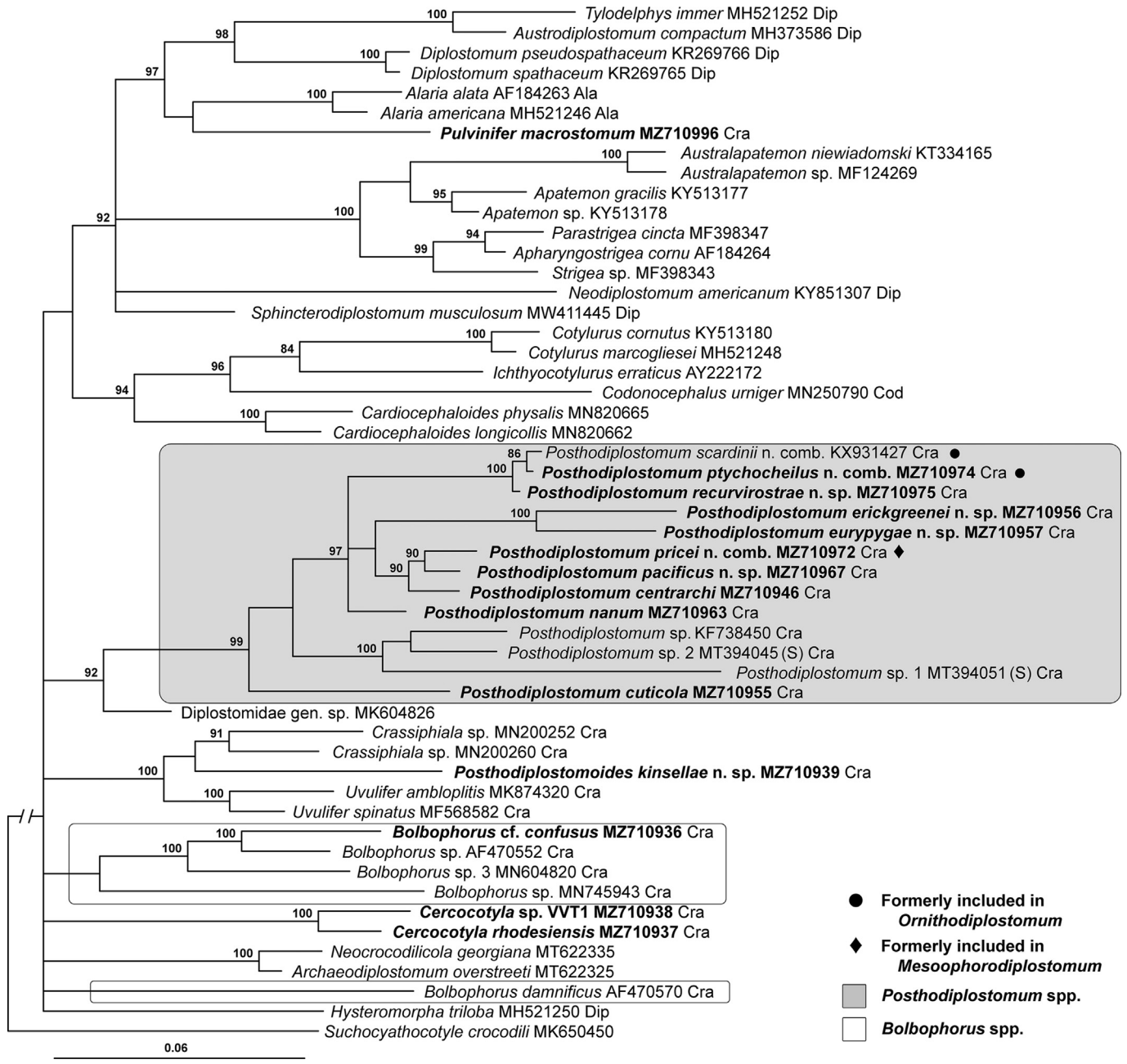

Fig. 1. Phylogenetic interrelationships among 51 diplostomoidean taxa based on Bayesian Inference (BI) analysis of partial 28S rDNA gene sequences. Bayesian inference posterior probability values lower than 80% are not shown. The new sequences generated in this study are indicated in bold. The scale-bar indicates the number of substitutions per site. Reference to the origin of species numbering/naming system of *Posthodiplostomum* spp. in the analysis is provided in parentheses after GenBank accession numbers followed by subfamilies of members of the Diplostomidae included in the analysis. Abbreviation for reference to the original designations of species-level lineages: S, Sokolov and Gordeev (2020). Abbreviations for subfamilies: Ala, Alariinae; Cod, Codonocephalinae; Cra, Crassiphialinae; Dip, Diplostominae.

formed a sister branch to a weakly supported clade containing *Posthodiplostomum erickgreenei* n. sp. + a 100% supported clade of [*Posthodiplostomum macrocotyle* Dubois, 1937 + a 99% supported clade with four other species-level lineages]. That 99% supported clade positioned *Posthodiplostomum* sp. 9 of Hoogendoorn et al. (2019) as a sister group to a 98% supported clade of [*Posthodiplostomum* sp. 21 + an 82% supported cluster of (*Posthodiplostomum* sp. 22 + *Posthodiplostomum microsicya* Dubois, 1936)].

Clades III, IV and V formed a poorly supported cluster (Fig. 2). Clade III (99% support) contained *Posthodiplostomum pacificus* n. sp. as a sister group to an unsupported polytomy of *M. pricei*, *Mesoophorodiplostomum anterovarium* Dronen, 1985 and an unidentified diplostomid (GenBank: KU221112). Clade IV (100% supported) consisted of a polytomy with *Po. centrarchi* + an unidentified diplostomid (GenBank: MK321671) + a 100% supported cluster of two unidentified diplostomids (GenBank: KY319363 and KY319364). Clade V only contained *Posthodiplostomum minimum* (MacCallum, 1921). Clade VI was positioned as an independent branch in the broader polytomy and only contained *Posthodiplostomum brevicaudatum* (von Nordmann, 1832) (Fig. 2).

The *cox*1 alignment was 363 bp long; the phylogenetic tree resulting from the analysis of the *cox*1 alignment was characterized by an overall weakly supported branch topology. Other recent molecular phylogenetic studies have repeatedly demonstrated that analyses of faster mutating genes (e.g. *cox*1; e.g. Hernández-Mena et al., 2017; López-Hernández

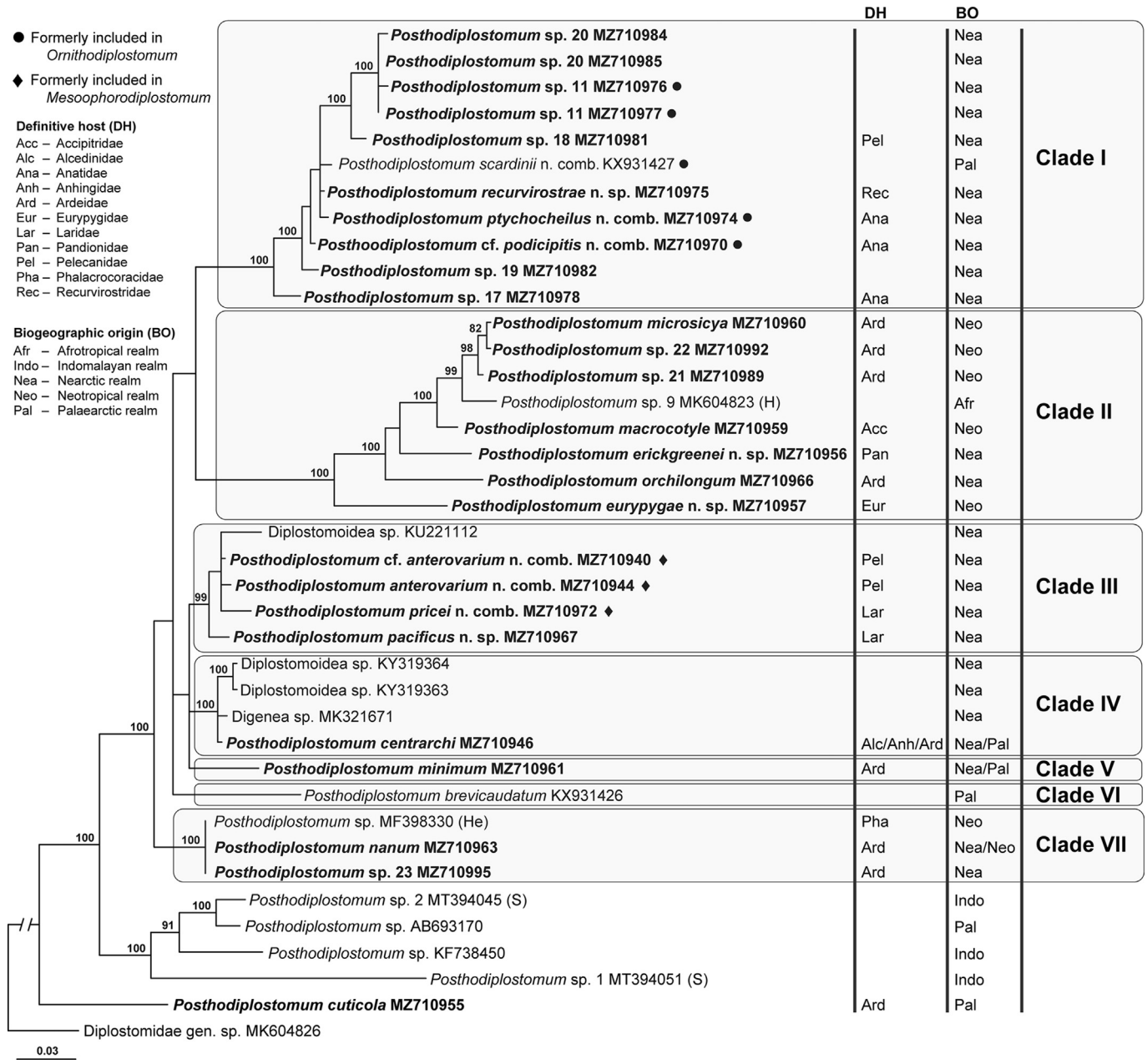

Fig. 2. Phylogenetic interrelationships among 38 taxa of *Posthodiplostomum* (syns. *Ornithodiplostomum* and *Mesoophorodiplostomum*) based on Bayesian Inference (BI) analysis of partial 28S rDNA gene sequences. Bayesian inference posterior probability values lower than 80% are not shown. The new sequences generated in this study are indicated in bold. The scale-bar indicates the number of substitutions per site. Reference to origin of species numbering/naming systems of are provided in parentheses after GenBank accession numbers. Biogeographical realm where specimens were collected and family of definitive host (for adult isolates and larvae molecularly matched to adult forms) are provided when possible. Abbreviations for references to the original designations of species-level lineages: He, Hernández-Mena et al. (2017); Ho, Hoogendoorn et al. (2019); S, Sokolov and Gordeev (2020).

et al., 2018; Hoogendoorn et al., 2019; Achatz et al., 2019a, c, 2020a; Cech et al., 2020; Tkach et al., 2020) often produce topologies which are much less resolved than those based on slower mutating genes such as 28S (e.g. Hernández-Mena et al., 2017; Hoogendoorn et al., 2019; Achatz et al., 2019a, c, 2020a; Sokolov and Gordeev, 2020; Tkach et al., 2020). Because of this, we opt to not discuss the results of this analysis in detail, although we provide the resulting tree (Supplementary Fig. S1) to allow for comparison of some of the better resolved clades. Overall, the basal clades in this phylogeny were weakly supported, while the majority of the more distal clades (containing individual species/species-level lineages) were much more strongly supported (Supplementary Fig. S1).

3.2. Non-monophyly of the Crassiphialinae

At present, the Diplostomidae contains four subfamilies: the Crassiphialinae, Diplostominae, Alariinae Hall & Wigdor, 1918 and Codonocephalinae Sudarikov, 1959. According to Niewiadomska (2002), members of the Crassiphialinae are united based on vitellarium that is typically confined to the opisthosoma, a copulatory bursa that may be protrusible and 'Neascus' type metacercariae; whereas members of the Diplostominae are united based on vitellarium located in both parts of the body, a copulatory bursa that is not protrusible and 'diplostomulum' type metacercariae. Furthermore, Niewiadomska (2002) stated that

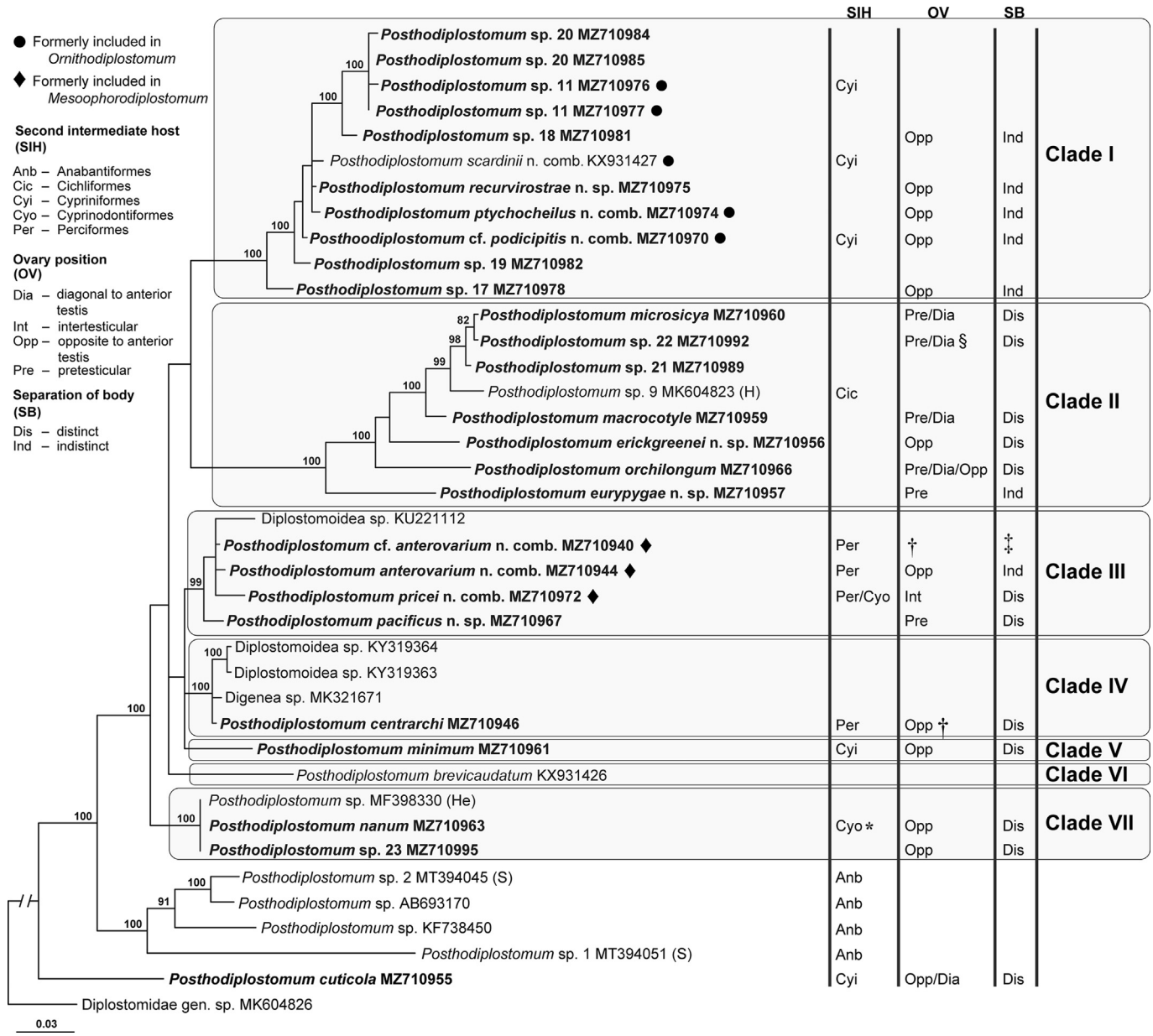

Fig. 3. Phylogenetic interrelationships among 38 taxa of *Posthodiplostomum* (syns. *Ornithodiplostomum* and *Mesoophorodiplostomum*) based on Bayesian Inference (BI) analysis of partial 28S rDNA gene sequences. Bayesian inference posterior probability values lower than 80% are not shown. The new sequences generated in this study are indicated in bold. The scale-bar indicates the number of substitutions per site. Reference to origin of species numbering/naming systems of are provided in parentheses after GenBank accession numbers. Order of second intermediate hosts (for larvae and adults molecularly matched to larval forms), position of ovary and level of distinction between prosoma and opisthosoma in adult stages provided when possible. Abbreviations for references to the original designations of species-level lineages: He, Hernández-Mena et al. (2017); Ho, Hoogendoorn et al. (2019); S, Sokolov and Gordeev (2020). * Collected from experimental infection by López-Hernández et al. (2018). § Ovary intertesticular or opposite to anterior testis in immature specimens. † Ovary intertesticular in immature specimens. ‡ Prosoma and opisthosoma distinct in immature specimens.

members of these two subfamilies only parasitize birds as adults. Members of the Alariinae also possess 'diplostomulum' type metacercariae, but often have mesocercarial stages as well. In addition, alariines parasitize mammals as adults. The only member of the Codonocephalinae, *Codonocephalus urniger* (Rudolphi, 1819), has progenetic metacercariae, an infundibular prosoma and several other unique morphological characters (Achatz et al., 2019b; Niewiadomska, 2002). Our broader analysis of 28S (Fig. 1) included multiple genera representing two out of the three diplostomid subfamilies (i.e. the Crassiphialinae and Diplostominae) which contain more than a single genus. At present, DNA sequence data are only available for a single genus from the Alariinae (i.e. *Alaria*).

Our broader analysis based on 28S sequences (Fig. 1) clearly demonstrates the non-monophyly of the Diplostomidae as well as two of its subfamilies (i.e. the Diplostominae and Crassiphialinae). Likewise, several recent molecular phylogenetic studies have demonstrated non-monophyly of these currently accepted taxa (e.g. Blasco-Costa and Locke, 2017; Hernández-Mena et al., 2017; Achatz et al., 2019b, c, d, 2020b, 2021a; Queiroz et al., 2020; Tkach et al., 2020). Prior to our study, only five genera of crassiphialines had available 28S sequence data (*Bolbophorus*, *Crassiphiala*, *Ornithodiplostomum*, *Posthodiplostomum* and *Uvulifer*). Previous studies demonstrated *Crassiphiala* and *Uvulifer* to form a clade independent from *Bolbophorus*, *Ornithodiplostomum* and *Posthodiplostomum* (e.g. Achatz et al., 2019c). Our 28S analysis included members of additional crassiphialine genera *Cercocotyla*,

Mesoophorodiplostomum, *Posthodiplostomoides* and *Pulvinifer*, as well as the type-species of *Bolbophorus* (*B.* cf. *confusus*) (Fig. 1).

The molecular phylogenetic analysis of the Diplostomoidea based on 28S (Fig. 1) did not unite the members of the Crassiphialinae or Diplostominae. Instead, members of both subfamilies formed several independent clades in the basal polytomy of the Diplostomoidea. In fact, *Alaria* (Alariinae), *Diplostomum* (Diplostominae), *Austrodiplostomum* (Diplostominae), *Tylodelphys* (Diplostominae) and *Pulvinifer* (Crassiphialinae) formed a 97% supported clade. Our analysis failed to provide any support for the currently recognized Crassiphialinae and Diplostominae.

Furthermore, morphological analysis has demonstrated the lack of any consistent morphological features in the adult stages which could be used to reliably differentiate between taxa forming the clades of the Crassiphialinae or Diplostominae (Fig. 1). The difference in distribution of vitellarium between members of the Crassiphialinae and Diplostominae is very inconsistent. Numerous crassiphialine species have vitellarium in both parts of the body (e.g. *Bolbophorus confusus* and *Posthodiplostomoides* spp.). The protrusible nature of the copulatory structures should also not be relied on for separation of subfamilies considering that only some, but not all, crassiphialines have a protrusible genital bursa (Niewiadomska, 2002). In addition, some diplostomines possess also protrusible genital bursae/cones (e.g. some species of *Dolichorchis* Dubois, 1961 and *Tylodelphys*).

Interestingly, *Codonocephalus* Diesing, 1850 was positioned within a strongly supported clade (94%) of *Cardiocephaloides* Sudarikov, 1959 and *Cotylurus* Szidat, 1928 + *Ichthyocotylurus* Odening, 1969 (Fig. 1). It is possible that familial placement of *Codoncephalus* should be re-evaluated. *Codonocephalus* shares some morphological features with both the Diplostomidae and Strigeidae (Achatz et al., 2019b; Niewiadomska, 2002).

Recently, Tkach et al. (2020) proposed discontinuing the use of subfamilies within the diplostomoidean family Proterodiplostomidae based on the non-monophyletic nature of its constituent subfamilies. The abandonment of subfamilies has also been relatively recently proposed for other large digenean families such as the Cryptogonimidae Ward, 1917, Dicrocoeliidae Looss, 1899 and Echinostomatidae Looss, 1899 (Miller and Cribb, 2008; Tkach et al., 2016, 2018). Based on our molecular phylogenetic analysis (Fig. 1), which is consistent with other recent molecular phylogenetic studies of the Diplostomidae (e.g. Hernández-Mena et al., 2017; Achatz et al., 2019b, c, d, 2020b, 2021a; Queiroz et al., 2020; Tkach et al., 2020), it is our opinion that the subfamilies of the Diplostomidae should also be abandoned. Therefore, we do not consider the four diplostomid subfamilies to be valid. It is likely that the subfamilies of the Strigeidae should also be considered invalid due to their non-monophyletic nature. However, detailed morphological study of independent clades of strigeids is necessary to determine if any morphological features may be used to erect new subfamilies (or families). Undoubtedly, a detailed re-evaluation of the system of the diplostomoidean families is required. However, such a re-evaluation is well beyond the scope of the present study.

3.3. Status of Bolbophorus

Bolbophorus spp. are associated with diseases in fishes (Markle et al., 2014, 2020). Interestingly, members of *Bolbophorus* as currently recognized formed two independent clades in our analysis of 28S (Fig. 1). The first clade was composed of four species/species-level lineages (two of which are only currently known from larvae), including the specimen tentatively identified as the type-species of the genus. The second clade only contained *B. damnificus*; the separate position of *B. damnificus* demonstrates that the species belongs to a separate genus. However, detailed morphological re-evaluation of *Bolbophorus* spp. is necessary to properly address the generic placement of *B. damnificus*.

Unfortunately, the single specimen of *B.* cf. *confusus* available in our collection was entirely used for DNA extraction. *Bolbophorus confusus* was originally described from specimens collected from Dalmatian pelican *Pelecanus crispus* Brunch from Europe by Krause (1914) and later redescribed by Dubois (1934, 1938) based on the original material and additional specimens collected from the great white pelican *Pelecanus onocrotalus* L. from Europe and the American white pelican *Pelecanus erythrorhynchos* Gmelin from Minnesota, USA. Our specimen was collected from *Pe. onocrotalus* in Ukraine. No other species of *Bolbophorus* is currently known to be distributed in Europe.

Currently there are 11 unique 28S sequences from *B. damnificus* and four unique 28S sequences of *Bolbophorus* sp. of Overstreet et al. (2002) available in GenBank. We suspect that at least some of these sequences contain errors or represent additional species, in part, due to the presence of indels limited to individual sequences (e.g. GenBank: AF470546 compared to AF470538). Furthermore, the intraspecific variation among 28S sequences of *B. damnificus* reaches 1.6% and the intraspecific variation among 28S sequences *Bolbophorus* sp. from Overstreet et al. (2002) is up to 0.4%. These levels of intraspecific variation are substantially greater than within the *Bolbophorus* sp. of Hoogendoorn et al. (2019) (0% intraspecific variation) and *Posthodiplostomum* spp. (up to 0.1% intraspecific variation) in the present study (see Section 3.7). Moreover, some *cox*1 sequences (e.g. GenBank: AF470578 compared to AF470614) generated by Overstreet et al. (2002) from isolates of these species have single-nucleotide indel sites, which is not possible in a coding gene. Sequencing of freshly collected adult specimens of *B. damnificus* and *Bolbophorus* sp. of Overstreet et al. (2002) is necessary to evaluate the status of these taxa and clarify the systematic position of *B. damnificus*.

3.4. Validity of Ornithodiplostomum and Mesoophorodiplostomum

Ornithodiplostomum and *Posthodiplostomum* are differentiated based on the presence/absence of an ejaculatory pouch (present in *Ornithodiplostomum* spp. *vs* absent in *Posthodiplostomum* spp.) as well as the level of separation between prosoma and opisthosoma (indistinct in *Ornithodiplostomum* spp. *vs* more or less distinct in *Posthodiplostomum* spp.; Fig. 4) (Dubois, 1968; Niewiadomska, 2002). *Ornithodiplostomum p. ptychocheilus*, the type-species of *Ornithodiplostomum*, was originally described as having an ejaculatory pouch; however, it was not shown on the illustrations of the adult provided by Van Haitsma (1930) and Dubois (1936, 1968). It appears that the pouch-like terminal portion of the seminal vesicle was considered an ejaculatory pouch. In our opinion, this terminal portion of the seminal vesicle is not an 'ejaculatory pouch' based on the original illustrations provided by Van Haitsma (1930) and our well-fixed adult specimens of *O. p. ptychocheilus*. Based on the original descriptions, the only *Ornithodiplostomum* species that appears to have a well-developed ejaculatory pouch is *Ornithodiplostomum garambense* (Baer, 1959), which was originally placed into the genus *Prolobodiplostomum* Baer, 1959 (Baer, 1959; Dubois, 1968). Furthermore, in our 28S analyses (Figs. 1–3) the sequence of *Po. recurvirostrae* (which clearly lacks an ejaculatory pouch) was positioned in a strongly supported clade with *O. p. ptychocheilus*.

Dubois (1944) transferred *Ornithodiplostomum podicipitis* into *Posthodiplostomum* based on the lack of an ejaculatory pouch. Later, Dubois (1968) returned it to *Ornithodiplostomum* based on the lack of clear differentiation between the prosoma and opisthosoma as well as the fact that it was not described from a member of *Ardea* L. Our specimens of *O.* cf. *podicipitis* also clearly lack an ejaculatory pouch. Similar to *Po. recurvirostrae*, this species was positioned within a clade with *O. p. ptychocheilus* (Figs. 2 and 3). The terminal portion of the seminal vesicle of some *Posthodiplostomum* spp. (e.g. *Po. minimum*, *Po. macrocotyle* also appears pouch-like) (Dubois, 1968; present material). Hence, the presence/absence of an ejaculatory pouch does not appear to be a valid feature enabling differentiation among these genera based on well-fixed adult specimens.

The adult specimens of taxa from Clade I (including *Ornithodiplostomum* spp.) in our second 28S analysis (Fig. 3) lacked a clear distinction between prosoma and opisthosoma. However, *Po. eurypygae*, which was

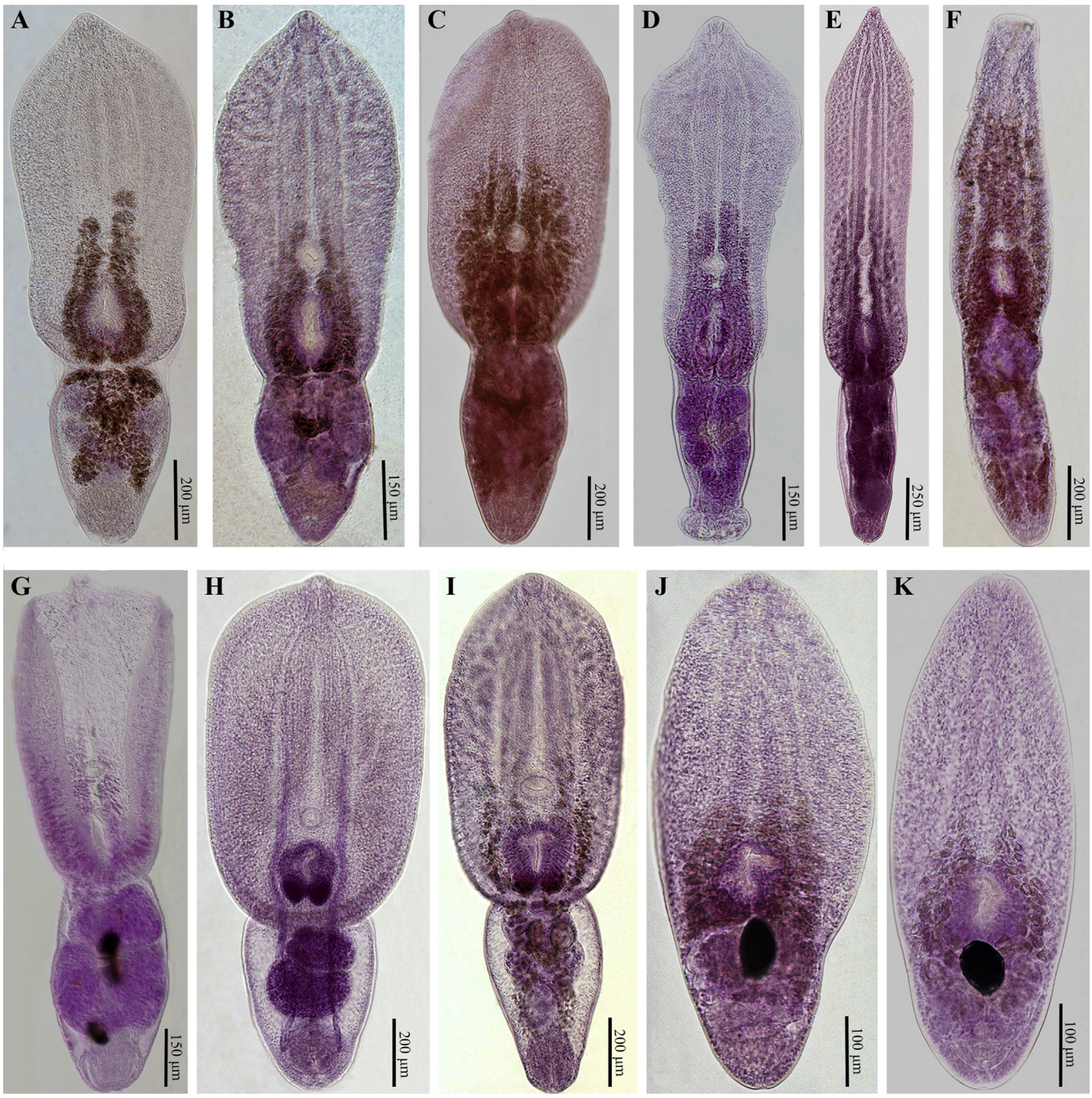

Fig. 4. Photographs of *Posthodiplostomum* spp. **A** *Po. cuticola*. **B** *Po. minimum*. **C** *Po. orchilongum*. **D** *Po. centrarchi*. **E** *Po. pricei*. **F** *Po. eurypygae* n. sp. **G** *Po. erickgreenei* n. sp. **H** *Posthodiplostomum* sp. 22. **I** *Po. macrocotyle*. **J** *Po. ptychocheilus*. **K** *Po. recurvirostrae* n. sp.

positioned as the basal branch in Clade II, also lacks a clear distinction between the prosoma and opisthosoma (Figs. 3 and 4). Other taxa with corresponding adults included in Clade II have a distinct prosoma and opisthosoma. Furthermore, *M. anterovarium*, which was positioned in Clade IV, also has a weakly separated prosoma and opisthosoma as an adult. However, *Po. pacificus* and *M. pricei*, members of Clade IV, both have a distinct prosoma and opisthosoma. Thus, the combination of molecular phylogenetic data and morphological analysis convincingly demonstrate that the lack of clear separation between prosoma and opisthosoma are not suitable for differentiation of *Ornithodiplostomum* and *Posthodiplostomum*.

The flame-cell formulae provided by Niewiadomska (2002) differ between *Ornithodiplostomum* and *Posthodiplostomum*. However, Dubois (1968) already cast doubt on the reported flame-cell formula in *O. p. ptychocheilus* (type-species of *Ornithodiplostomum*). Furthermore, a dissertation on the larvae of *O. ptychocheilus* by Hendrickson (1978) (likely *O. p. ptychocheilus*) demonstrated that the flame-cells of larval *O. ptychocheilus* are difficult to observe and the author was unable to confirm the number of flame-cells. It remains unclear if the flame-cell formula actually differs between *Ornithodiplostomum* and *Posthodiplostomum*. The flame-cell formula of *Mesoophorodiplostomum* spp. is currently unknown.

Mesoophorodiplostomum is differentiated from *Posthodiplostomum* and *Ornithodiplostomum* based on the position of the ovary (intertesticular in the type-species of *Mesoophorodiplostomum vs* pretesticular or at level of anterior testis in *Posthodiplostomum* and *Ornithodiplostomum* spp.) (Niewiadomska, 2002; López-Hernández et al., 2018; present data). However, some authors have noted that the ovary can be intertesticular in some immature specimens of *Po. centrarchi* and *Po. brevicaudatum* (see Palmieri, 1977; Stoyanov et al., 2017). The ovary of many species of *Posthodiplostomum* (e.g. *Po. recurvirostrae*, *Po. minimum*, *Posthodiplostomum obesum* (Lutz, 1928)) is positioned opposite to the anterior testis. In fact, the second known member of *Mesoophorodiplostomum* (*M. anterovarium*) has an ovary which is opposite to the anterior testis (Dronen, 1985). Dronen (1985) noted that his new species fits characteristics of both *Mesoophorodiplostomum* and *Posthodiplostomum* and only tentatively assigned its genus.

Molecular phylogenies based on 28S (Figs. 1–3) consistently positioned *Mesoophorodiplostomum* (including the type-species *M. pricei*) within clades of *Posthodiplostomum*. Interestingly, *M. pricei* and *M. anterovarium* formed a strongly supported clade with *Po. pacificus* (Figs. 2 and 3), a species with a pretesticular ovary. These results make it clear that the position of ovary is not suitable to distinguish between these three genera.

Our analyses of 28S (Figs. 1 and 2) positioned *Po. cuticola* (type-species of *Posthodiplostomum*) as a sister group to several other clades of *Posthodiplostomum*, *Ornithodiplostomum* and *Mesoophorodiplostomum*. If *Ornithodiplostomum* and *Mesoophorodiplostomum* were to be maintained as separate genera, then the several other clades of *Posthodiplostomum* would require the erection of at least four additional genera. However, morphological features in adult stages do not support the erection of these new genera. For instance, *Po. centrarchi* was originally considered a subspecies of *Po. minimum* due to its extremely similar morphology. However, the 28S phylogeny (Fig. 2) placed these taxa in only a weakly supported clade together with a clade of *Po. pacificus* + *Mesoophorodiplostomum* spp. Clade II contained another previous synonym of *Po. minimum*, namely *Po. orchilongum* (see Section 3.8), as well as several other species which closely conform to the morphological diagnosis of *Posthodiplostomum* (e.g. *Po. macrocotyle*, *Po. microsicya*). Based on the phylogenetic position of the type-species, *Po. cuticola*, and lack of consistent morphological differences in the adult stages, we consider *Ornithodiplostomum* and *Mesoophorodiplostomum* to be junior synonyms of *Posthodiplostomum*; we transfer all members of these two genera into *Posthodiplostomum*.

Considering the new synonymy, we provide updated species-level lineage numbers for the previously published *Posthodiplostomum* species-level lineages (Table 2). This increases the number of recognized *Posthodiplostomum* species-level lineages in GenBank to 23, including our data (Supplementary Table S1).

López-Hernández et al. (2018) suggested that *Posthodiplostomum* clades may potentially be separated based on the localisation of metacercariae in fishes. *Posthodiplostomum cuticola* (von Nordman, 1832) are known to encyst on the skin of fishes; it formed a sister branch to all other *Posthodiplostomum* spp. in our 28S phylogenies (Figs. 1–3). However, *Posthodiplostomum centrarchi* Hoffman, 1958 and *Posthodiplostomum* cf. *podicipitis* (Yamaguti, 1939) n. comb. were also found on the skin of fishes in the present study (Table 1), although *Po. centrarchi* was more commonly found in visceral organs (e.g. liver and spleen). Based on the currently available data, the site of infection in fishes does not seem to be suitable for separating *Posthodiplostomum* clades.

An amended diagnosis of *Posthodiplostomum* is provided below.

3.5. *Posthodiplostomum* Dubois, 1936

Diagnosis (after Niewiadomska, 2002, amended): Digenea: Diplostomidae. Body bipartite, distinctly or indistinctly; prosoma flat or concave, oval, sometimes elongate, linguiform or lanceolate; opisthosoma short or long, oval or claviform to subcylindrical. Pseudosuckers absent; holdfast organ subspherical or oval, with cavity opening *via* median slit. Oral and ventral sucker present; oral sucker often weakly developed; pharynx small. Testes two, tandem, different in size and shape; anterior testis asymmetrical or transversely-oval; posterior testis larger, bilobed, reniform or cordiform, sometimes twisted, often with indentation anteriorly. Ovary ellipsoidal or oval, pretesticular, opposite to anterior testis or intertesticular, median, lateral or diagonal to anterior testis. Vitellarium typically in prosoma and opisthosoma. Copulatory bursa eversible, with terminal or subterminal opening. Genital cone present in most species, surrounded by prepuce, encloses hermaphroditic duct, which is formed at its base by union of uterus and ejaculatory duct; ejaculatory pouch typically absent, terminal portion of seminal vesicle may appear sac-like. Typically in piscivorous birds. Cosmopolitan. Metacercariae in fishes.

Type-species: Po. cuticola (von Nordmann, 1832).

Other species: Po. anterovarium (Dronen, 1985) n. comb., *Po. australe* Dubois, 1937, *Po. bi-ellipticum* Dubois, 1958, *Po. botauri* Vidyarthi, 1938, *Po. boydae* Dubois, 1969, *Po. brevicaudatum* (von Nordmann, 1832), *Po. centrarchi* Hoffman, 1958, *Po. erickgreenei* n. sp., *Po. eurypygae* n. sp., *Po. garambense* (Baer, 1959) n. comb., *Po. giganteum* Dubois, 1988, *Po. grande* (Diesing, 1850), *Po. grayii* (Verma, 1936), *Po. ixobrychi* (Lung Tsu-pei, 1966), *Po. linguaeforme* Pearson & Dubois, 1985, *Po. macrocotyle* Dubois, 1937, *Po. mehtai* Gupta & Mishra, 1974, *Po. microsicya* Dubois, 1936, *Po. mignum* Boero, Led & Brandetti 1972, *Po. milvi* Fotedar & Bambroo, 1965, *Po. minimum* (MacCallum, 1921), *Po. nanum* Dubois, 1937, *Po. obesum* (Lutz, 1928), *Po. oblongum* Dubois, 1937, *Po. opisthosicya* Dubois, 1969, *Po. orchilongum* Noble, 1936, *Po. pacificus* n. sp., *Po. podicipitis* (Yamaguti, 1939) n. comb., *Po. pricei* (Krull, 1934) n. comb., *Po. prosostomum* Dubois & Rausch, 1948, *Po. ptychocheilus ptychocheilus* (Faust, 1917) n. comb., *Po. ptychocheilus palaearcticum* (Odening, 1963) n. comb., *Po. recurvirostrae* n. sp., *Po. scardinii* (Shulman, 1952) n. comb.

3.6. *Descriptions of new taxa*

3.6.1. *Posthodiplostomum erickgreenei* Achatz, Chermak, Cromwell & Tkach n. sp.

3.6.1.1. *Taxonomic summary*

Type-host: *Pandion haliaetus* (L.) (Aves: Pandionidae). The bird specimen in which the new digenean species was found was deposited in the Philip L. Wright Zoological Museum (UMZM), University of Montana, Missoula, Montana, USA, under accession number UMZM:Bird:22149.

Type-locality: Missoula County (46°54′40.5″N, 114°9′36.162″W), Montana, USA.

Type-material: The type-series consists of one gravid adult specimen and two non-gravid adult specimens deposited in the HWML. Holotype: HWML 216645, labeled ex *P. haliaetus*, small intestine, Missoula County, Montana, USA, 12 July 2017, coll. E. Greene. Paratypes: HWML 216646 (lot of 2 slides), labels identical to the holotype.

Site in host: Small intestine.

Representative DNA sequences: GenBank: MZ710956 (28S), MZ707186 (*cox*1).

ZooBank registration: The Life Science Identifier (LSID) for *Posthodiplostomum erickgreenei* n. sp. is urn:lsid:zoobank.org:act:58B988DD-11DB-42C9-8612-59D006A5299C.

Etymology: The species is named after Erick Greene (University of Montana) for his help with collecting the host specimens containing the new species and his contributions to our knowledge of wildlife ecology in the Rocky Mountains.

3.6.1.2. *Description.* [Based on 3 adult specimens; measurements of holotype (gravid adult) given in text; measurements of entire series given in Table 3; Fig. 5] Body 1,300 long, consisting of distinct prosoma and opisthosoma; prosoma 790 × 400, extremely concave, essentially infundibular with ventral aperture, long, truncated at anterior end,

Table 2
New and updated *Posthodiplostomum* species-level lineage numbers and their corresponding previously-accepted species-level lineage numbers

Updated species-level lineage number	Previously-accepted species-level lineage number	Representative GenBank accession number	Reference
Posthodiplostomum sp. 10	*Ornithodiplostomum* sp. 1	HM064737	Moszczynska et al. (2009)
Posthodiplostomum sp. 11	*Ornithodiplostomum* sp. 2	KT831368	Moszczynska et al. (2009)
Posthodiplostomum sp. 12	*Ornithodiplostomum* sp. 3	HM064780	Moszczynska et al. (2009)
Posthodiplostomum sp. 13	*Ornithodiplostomum* sp. 4	HM064788	Moszczynska et al. (2009)
Posthodiplostomum sp. 14	*Ornithodiplostomum* sp. 8	MH368943	Locke et al. (2010)
Posthodiplostomum sp. 15	Diplostomidae gen. sp. X	MH368849	Gordy and Hanington (2019)
Posthodiplostomum sp. 16	*Posthodiplostomum* sp. 4	MH368945	Gordy and Hanington (2019)
	Posthodiplostomum sp. UG2	LC511187	Komatsu et al. (2020)
	Posthodiplostomum sp. UG3	LC511188	Komatsu et al. (2020)
Posthodiplostomum sp. 17	–	MZ707205	Present study
Posthodiplostomum sp. 18	–	MZ707206	Present study
Posthodiplostomum sp. 19	–	MZ707209	Present study
Posthodiplostomum sp. 20	–	MZ707210	Present study
Posthodiplostomum sp. 21	–	MZ707212	Present study
Posthodiplostomum sp. 22	–	MZ707214	Present study
Posthodiplostomum sp. 23	–	MZ707217	Present study

Note: A single representative GenBank accession number is provided for each new or updated species-level lineage as well as the reference to the origin of the corresponding previously accepted species-level lineage number.

widest at level of ventral sucker; opisthosoma cylindrical, 510 × 300, somewhat narrower than prosoma. Prosoma:opisthosoma length ratio 1.5. Forebody 39% of body length. Tegument unarmed, likely due to loss of spination resulting from freezing. Oral sucker terminal, 40 × 40. Ventral sucker larger than oral sucker, 55 × 70, located near mid-length of prosoma; oral:ventral sucker width ratio 0.6. Holdfast organ posterior to ventral sucker, typically positioned in posterior-most third of prosoma, oval with ventral muscular portion, 155 × 125. Proteolytic gland dorsal to posterior part of holdfast organ. Prepharynx not observed. Pharynx oval, 45 × 35. Oesophagus 55 long, similar in length to pharynx. Caecal bifurcation in anterior-most 10% of prosoma length. Caeca slender, extending to near posterior margin of opisthosoma.

Testes 2, tandem, occupying most of opisthosoma; anterior testis entire, 150 × 210, posterior testis somewhat bilobed, 225 × 290. Seminal vesicle primarily post-testicular, portions ventral to posterior part of posterior testis, compact, continues as short ejaculatory duct. Ejaculatory duct joins metraterm dorsally to form hermaphroditic duct near proximal part of genital cone. Hermaphroditic duct opens at tip of genital cone into genital atrium; genital cone surrounded by prepuce within genital atrium (Fig. 5C). Genital cone and prepuce occupy majority of genital atrium. Genital pore subterminal, dorsal.

Ovary opposite and ventral to anterior testis, subspherical, positioned near prosoma-opisthosoma junction, 80 × 78. Oötype and Mehlis' gland not well-observed. Laurer's canal not observed. Vitellarium with anterior limits located slightly anterior to level of ventral sucker, extending posteriorly to about level of anterior margin of genital cone and prepuce. Vitelline reservoir intertesticular. Uterus ventral to gonads and seminal vesicle, contains few eggs (70–75 × 45–50).

Excretory vesicle not well-observed. Excretory pore terminal.

3.6.1.3. Remarks. *Posthodiplostomum erickgreenei* n. sp. clearly belongs to *Posthodiplostomum* based on the results of our molecular analysis of 28S (Fig. 1) as well as the presence of a prepuce that surrounds the genital cone and the lack of pseudosuckers. The new species can be distinguished from all other *Posthodiplostomum* spp., except for *Posthodiplostomum australe* Dubois, 1937, by the shape of prosoma (essentially infundibular with ventral aperture in the new species *vs* foliate or only slightly concave in all other *Posthodiplostomum* spp.).

While both *Po. erickgreenei* n. sp. and *Po. australe* have a more concave or infundibular prosoma than other *Posthodiplostomum* spp., the prosoma in the new species is more concave or infundibular-like than in *Po. australe* (Supplementary Fig. S2). The new species and *Po. australe* can be further distinguished based on the distinction between prosoma and opisthosoma (clearly distinct in the new species *vs* only a slight constriction present between prosoma and opisthosoma in *Po. australe*; Supplementary Fig. S2), posterior extent of vitellarium (almost reaches the end of opisthosoma in the new species, but only reaches near the midpoint of the opisthosoma in *Po. australe*). In addition, the two species can be separated by ovary shape and size (subspherical, 80 × 78 μm in the new species *vs* transversely oval, 45–55 × 72–100 μm in *Po. australe*) and egg length (70–75 μm in the new species *vs* 80–91 μm in *Po. australe*). The geographical distance separating the two species is also quite large (USA *vs* Australia) which may be meaningful despite the broad distribution of the avian host.

3.6.2. Posthodiplostomum eurypygae Achatz, Chermak, Bell, Fecchio & Tkach n. sp.

3.6.2.1. Taxonomic summary

Type-host: *Eurypyga helias* (Pallas) (Aves: Eurypygidae). The bird specimen in which the new digenean species was found was deposited in the Museum of the Universidade Federal de Mato Grosso, Brazil under accession number UFMT 4865.

Type-locality: Pantanal, Fazenda Retiro Novo (16°21′53″S, 56°17′31″W), Municipality of Poconé, Mato Grosso State, Brazil.

Type-material: The type-series consists of two mature specimens deposited in the HWML. Holotype: HWML 216647, labeled ex *E. helias*, small intestine, Pantanal, Fazenda Retiro Novo, Municipality of Poconé, Mato Grosso State, Brazil, 12 October 2019, coll. A. Fecchio. Paratype (Hologenophore): HWML 216648 (lot of 1 slide), label identical to the holotype.

Site in host: Small intestine.

Representative DNA sequences: GenBank: MZ710957 (28S), MZ707187 (*cox*1).

ZooBank registration: The Life Science Identifier (LSID) for *Posthodiplostomum eurypygae* n. sp. is urn:lsid:zoobank.org:act:445CE83A-CF6B-48B2-87D1-1FA5D7272FD4.

Etymology: The species is named after the genus of the definitive type-host.

3.6.2.2. Description. [Based on 2 adult specimens; measurements of holotype given in text; measurements of holotype and hologenophore given in Table 3; Fig. 6] Body 1,142 long, lanceolate, consisting of indistinct prosoma and opisthosoma; prosoma 656 × 218, slightly concave near prosoma-opisthosoma junction, widest at level of ventral sucker; opisthosoma cylindrical, 486 × 176, somewhat narrower than prosoma. Prosoma:opisthosoma length ratio 1.4. Forebody 41% of body length. Tegument armed with fine spines. Oral sucker terminal, 76 × 82. Ventral sucker smaller than oral sucker, 50 × 66, located in the posterior-

Table 3
Ranges for morphometric characters of three new *Posthodiplostomum* spp.

Feature	*Po. erickgreenei* n. sp.		*Po. eurypygae* n. sp.		*Po. recurvirostrae* n. sp.
	Holotype	Non-gravid adults ($n = 2$)	Holotype	Hologenophore (Lateral specimen)	Holotype and paratypes ($n = 3$)[a]
Body length	1,300	1,060–1,250	1,142	–	580–690 (643)
Prosoma length	790	662–700	656	–	400–521 (466)
Prosoma width	400	300–328	218	–	233–260 (243)
Opisthosoma length	510	360–588	486	425	139–213 (177)
Opisthosoma width	300	270–330	176	–	171–196 (184)
Prosoma:opisthosoma length ratio	1.5	1.1–1.9	1.4	–	2.2–3.7 (2.7)
Forebody (% of body length)	39	34–42	41	–	49–55 (52)
Oral sucker length	40	40–45	76	–	38–40 (39)
Oral sucker width	40	48–60	82	–	28–30 (29)
Ventral sucker length	55	52–60	50	–	30–35 (32)
Ventral sucker width	70	68–85	66	–	30–35 (33)
Oral sucker:ventral sucker width ratio	0.6	0.7	1.2	–	0.8–1.0 (0.9)
Holdfast organ length	155	145	90	–	100–108 (104)
Holdfast organ width	125	100	54	–	96–115 (103)
Holdfast organ position (% of prosoma length)	76	60–78	78	–	70–79 (73)
Pharynx length	45	45–52	44	–	30–34 (32)
Pharynx width	35	40	36	–	27–33 (29)
Oral sucker:pharynx length ratio	0.9	0.8–1.0	1.7	–	1.2–1.3 (1.2)
Oesophagus length	55	38–40	30	–	42–85 (69)
Anterior testis length	150	110–142	116	90	53–78 (64)
Anterior testis width	210	156–165	132	–	55–82 (68)
Posterior testis length	225	155–242	140	120	59–80 (69)
Posterior testis width	290	245–314	160	–	118–156 (136)
Ovary length	80	–	54	45	40–52 (45)
Ovary width	78	–	80	–	40–60 (48)
Number of eggs	3	0	0	0	1 (1)
Egg length	70–75	–	–	–	68–73 (70)
Egg width	45–50	–	–	–	48–56 (51)
Anterior vitellarium free zone (% of prosoma length)	51	49–54	32	–	58–62 (60)
Posterior vitellarium free zone (% of opisthosoma length)	20	10–35	14	22	50–65 (56)

[a] Mean provided for *Posthodiplostomum recurvirostrae* n. sp. in parentheses after range considering it is the only species with more than two specimens available.

most quarter of prosoma; oral:ventral sucker width ratio 1.2. Holdfast organ immediately posterior to ventral sucker, oval with ventral muscular portion, 90 × 54. Proteolytic gland not well-observed. Prepharynx not observed. Pharynx oval, 44 × 36. Oesophagus somewhat shorter than pharynx, 30 long. Caecal bifurcation in anterior-most quarter of prosoma length. Caeca slender, extending to near posterior margin of posterior testis.

Testes 2, tandem; anterior testis positioned near prosoma-opisthosoma junction, entire, 116 × 132, posterior testis somewhat bilobed, 140 × 160. Seminal vesicle primarily post-testicular, ventral to posterior testis, compact, continues as short ejaculatory duct. Ejaculatory duct joins metraterm dorsally to form hermaphroditic duct near proximal part of genital cone. Hermaphroditic duct opens at tip of genital cone into genital atrium; genital cone surrounded by prepuce within genital atrium (Fig. 6C and D). Genital cone and prepuce occupy majority of genital atrium. Genital pore subterminal, dorsal.

Ovary primarily pretesticular, posterior part of ovary ventral to anterior testis, transversely oval, positioned near prosoma-opisthosoma junction, 54 × 80. Oötype and Mehlis' gland intertesticular. Laurer's canal opens dorsally, at level of posterior margin of anterior testis. Vitellarium extending from slightly posterior to level of caecal bifurcation in prosoma to level of genital cone and prepuce in opisthosoma. Vitelline reservoir intertesticular. Uterus ventral to testes and seminal vesicle, contains no eggs.

Excretory vesicle not well-observed. Excretory pore terminal.

3.6.2.3. Remarks. Posthodiplostomum eurypygae n. sp. is a member of *Posthodiplostomum* based on the results of our molecular analyses, the presence of a prepuce that surrounds the genital cone, and the lack of pseudosuckers. This new species can be distinguished from most other *Posthodiplostomum* spp. based on the relatively indistinct separation of prosoma and opisthosoma. The only other *Posthodiplostomum* spp. which share this trait are *Posthodiplostomum anterovarium* (Dronen, 1985) n. comb., *Po. podicipitis*, *Po. ptychocheilus* (both subspecies) and another new species (*Posthodiplostomum recurvirostrae* n. sp.) which is described and differentiated below (see Section 3.6.3).

Posthodiplostomum eurypygae n. sp. can be distinguished from *Po. anterovarium* and *Po. ptychocheilus* (both subspecies) based on the position of ovary (primarily pretesticular in the new species *vs* opposite to anterior testis in the other two species). The ovary of *Po. podicipitis* is mostly opposite to the anterior testis; however, it is somewhat pretesticular as well. The vitellarium in the new species extends much farther anteriorly than in *Po. anterovarium*, *Po. podicipitis* and *Po. ptychocheilus* (both subspecies) (extends anterior to slightly posterior to the level of the caecal bifurcation in *Po. eurypygae*, while in the three other species vitellarium extends only to the level of or slightly anterior to the level of the ventral sucker). Furthermore, the body shape in the new species is completely different from *Po. ptychocheilus* (both subspecies) (lanceolate in *Po. eurypygae vs* oval in *Po. ptychocheilus*). The oral sucker of the new species is typically substantially larger than in *Po. anterovarium*, *Po. podicipitis* and *Posthodiplostomum ptychocheilus ptychocheilus* (Faust, 1917) n. comb. (76 × 82 μm in the new species *vs* 48–57 × 36–45 μm in *Po. anterovarium*, 33–36 × 26–30 μm in *Po. podicipitis* and 25–30 × 25–30 μm in *Po. p. ptychocheilus*). In addition, *Po. eurypygae* n. sp. differs from these three species by at least 5.9% in partial sequences of 28S and at least 16.5% in partial sequences of *cox*1 (Supplementary Tables S2 and S3).

3.6.3. Posthodiplostomum recurvirostrae Achatz, Chermak & Tkach n. sp.

3.6.3.1. Taxonomic summary

Type-host: *Recurvirostra americana* Gmelin (Aves: Recurvirostridae).

Type-locality: Nelson County, North Dakota, USA.

Type-material: The type-series consists of three fully mature specimens on a single slide deposited in the HWML. Holotype and paratypes: HWML

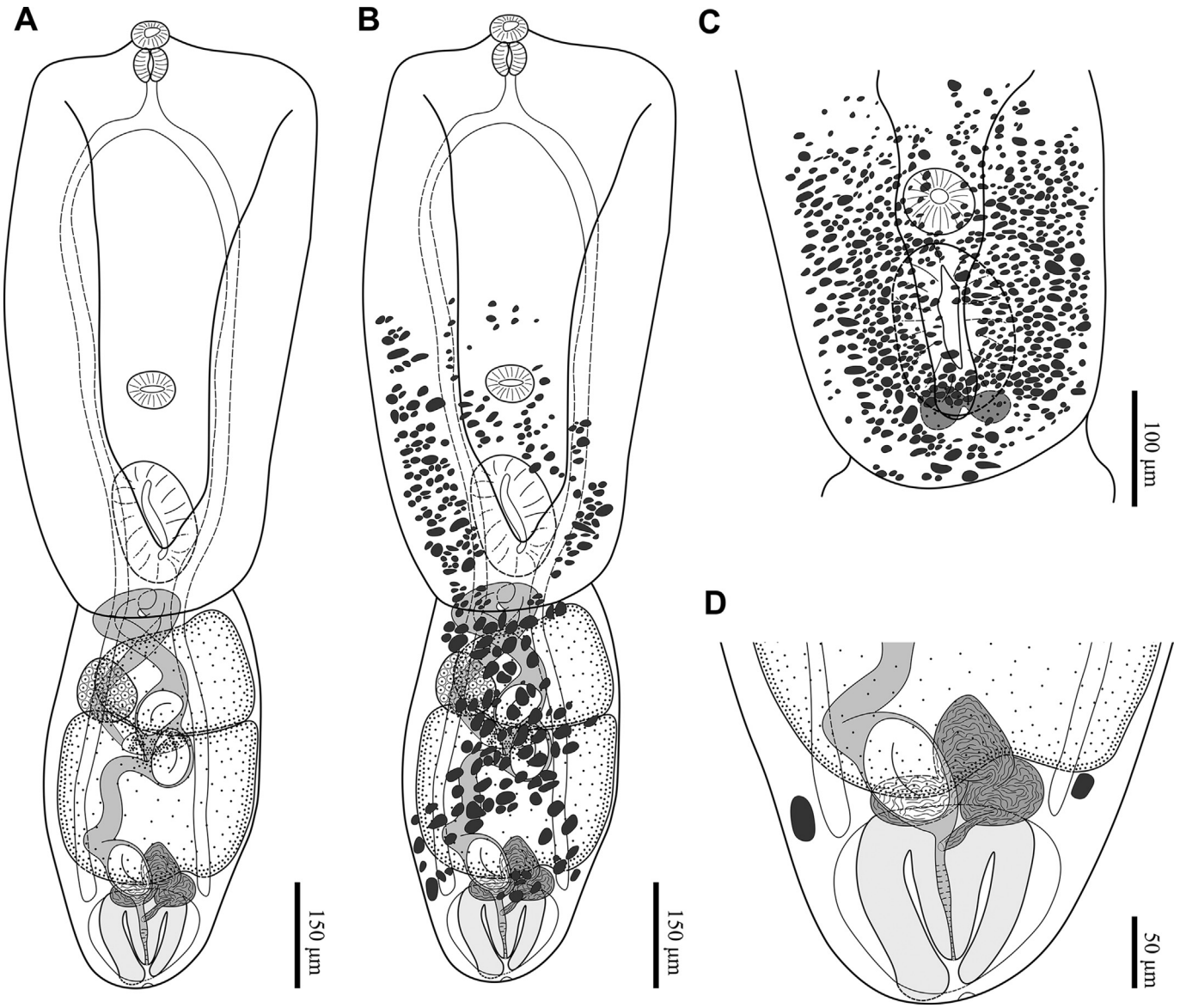

Fig. 5. *Posthodiplostomum erickgreenei* n. sp. **A** Ventral view of the holotype, vitellarium omitted. **B** Ventral view of the holotype, vitellarium shown. **C** Ventral view of hologenophore prosoma demonstrating the anterior distribution of vitellarium. **D** Posterior end of the holotype, ventral view. Posteriormost vitellarium shown.

216661, labeled ex *R. americana*, small intestine, Nelson County, North Dakota, USA, 2 September 2013, coll. V.V. Tkach.

Site in host: Small intestine.

Representative DNA sequences: GenBank: MZ710975 (28S), MZ707202 (*cox*1).

ZooBank registration: The Life Science Identifier (LSID) for *Posthodiplostomum recurvirostrae* n. sp. is urn:lsid:zoobank.org:act:85C2CAD6-F058-41D3-BFC6-A35614CE37FD.

Etymology: The species is named after the genus of the definitive type-host.

3.6.3.2. Description. [Based on 3 adult specimens; measurements of holotype given in text; measurements of entire series given in Table 3; Fig. 7] Body oval, 660 long, consisting of indistinct prosoma and opisthosoma; prosoma slightly concave, 521 × 235, widest at level of ventral sucker; opisthosoma short, rounded, 139 × 186, somewhat narrower than prosoma. Prosoma:opisthosoma length ratio 3.7. Forebody 55% of body length. Tegument armed with fine spines. Oral sucker terminal, 38 × 28. Ventral sucker similar in size to oral sucker, 30 × 33, located in posterior-most third of prosoma; oral:ventral sucker width ratio 0.85. Holdfast organ immediately posterior to ventral sucker, positioned in posterior-most quarter of prosoma, subspherical with ventral muscular portion, 108 × 98. Proteolytic gland dorsal to posterior part of holdfast organ. Prepharynx short; pharynx oval, 30 × 28. Oesophagus longer than pharynx, 81 long. Caecal bifurcation in anterior-most third of prosoma. Caeca slender, extending to near prosoma-opisthosoma junction.

Testes 2, tandem, occupying at least half of opisthosoma length; anterior testis entire, subspherical, sinistral, may be partially ventral to posterior testis, 60 × 55; posterior testis transversely-elongated, somewhat irregular, 68 × 135. Seminal vesicle primarily post-testicular, portions ventral to posterior part of posterior testis, compact, continues as extremely short ejaculatory duct. Ejaculatory duct almost immediately joins metraterm dorsally to form hermaphroditic duct near proximal part of genital cone. Hermaphroditic duct opens at tip of genital cone; genital cone surrounded by prepuce within genital atrium (Fig. 7C). Genital cone and prepuce occupy majority of genital atrium. Genital pore subterminal, dorsal.

Ovary opposite to anterior testis, spherical or subspherical, dextral, positioned near prosoma-opisthosoma junction, 40 × 40. Oötype and

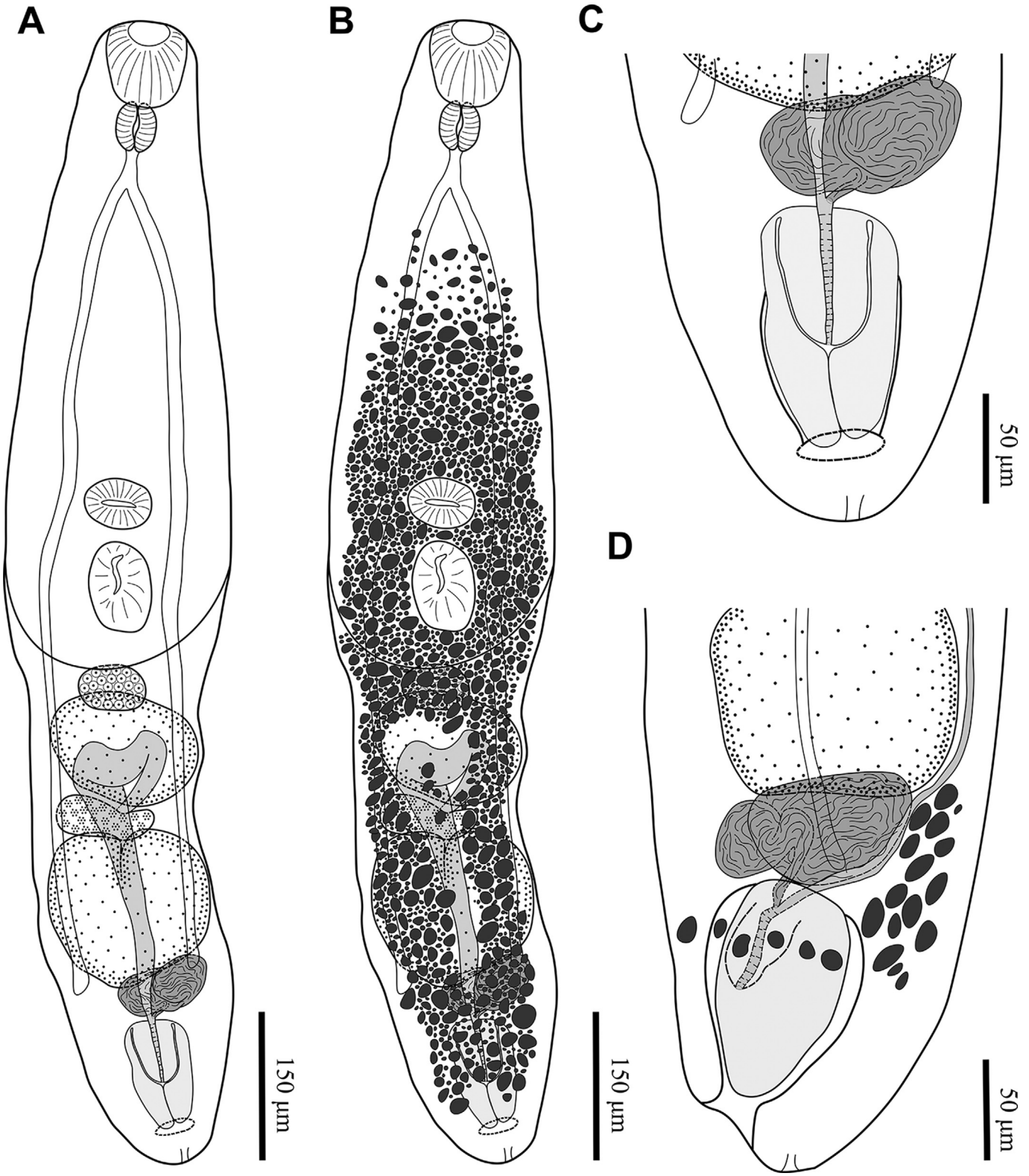

Fig. 6. *Posthodiplostomum eurypygae* n. sp. **A** Ventral view of the holotype, vitellarium omitted. **B** Ventral view of the holotype, vitellarium shown. **C** Posterior end of the holotype, ventral view, vitellarium omitted. **D** Posterior end of the paratype, lateral view. Posterior margins of vitellarium shown.

Mehlis' gland positioned between anterior testis and ovary. Laurer's canal opens dorsally at level of vitelline reservoir. Vitellarium extending from near level of ventral sucker in prosoma to about mid-level of posterior testis in opisthosoma. Vitelline reservoir positioned between testes and ovary. Uterus ventral to gonads, containing one egg (68 × 48).

Excretory vesicle not well-observed; excretory pore terminal.

3.6.3.3. Remarks. Posthodiplostomum recurvirostrae n. sp. belongs to *Posthodiplostomum* based on the results of our molecular analyses as well as the presence of a prepuce that surrounds the genital cone and the lack of pseudosuckers. The new species is most easily distinguished from all other *Posthodiplostomum* spp., except for *Po. anterovarium*, *Po. eurypygae*, *Po. podicipitis* and *Po. ptychocheilus*, based on the relatively indistinct separation of prosoma and opisthosoma.

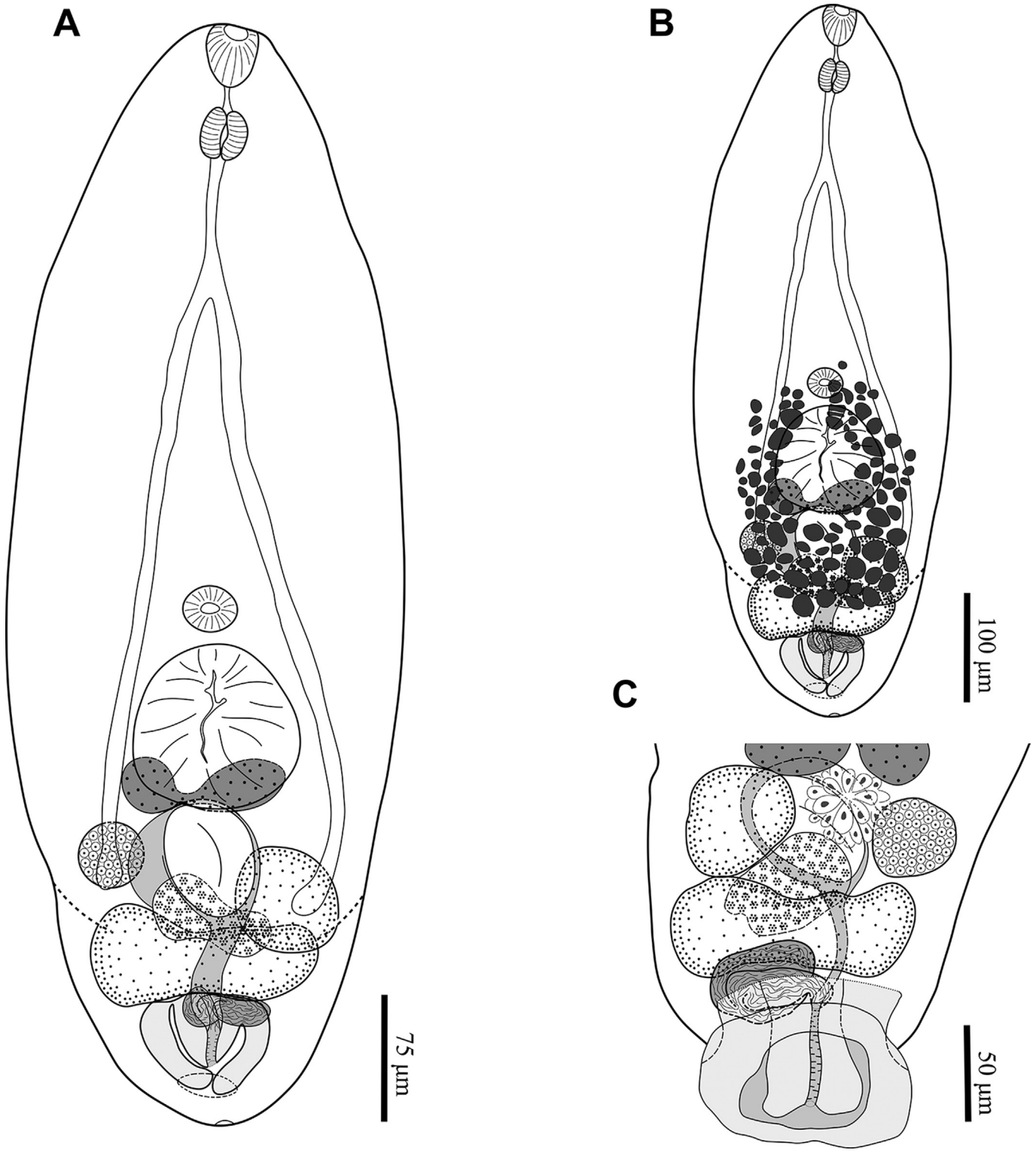

Fig. 7. *Posthodiplostomum recurvirostrae* n. sp. **A** Ventral view of the holotype, vitellarium omitted. **B** Ventral view of the holotype, vitellarium shown. **C** Posterior end of a paratype, dorsal view, vitellarium omitted.

Posthodiplostomum recurvirostrae n. sp. can be differentiated from *Po. eurypygae* based on the distribution of vitellarium (distributed between near the level of the ventral sucker to near the midlevel of the posterior testis in *Po. recurvirostrae* n. sp. *vs* distributed between slightly posterior to level of caecal bifurcation to the level of genital cone in *Po. eurypygae*). In addition, *Po. recurvirostrae* n. sp. is a substantially smaller species than *Po. eurypygae* (Table 3) and the two species differ in body shape (oval in *Po. recurvirostrae* n. sp. *vs* lanceolate in *Po. eurypygae*). These two species also differ by 6.9% in partial sequences of 28S and 18.4% in partial sequences of *cox*1.

The new species from *R. americana* can be distinguished from *Po. anterovarium* based on the smaller oral sucker:ventral sucker width ratio (0.8–1.0 in the new species *vs* 1.4 in *Po. anterovarium*), smaller ventral sucker size (30–35 × 30–35 μm in the new species *vs* 63–78 × 51–62 μm in *Po. anterovarium*), somewhat larger holdfast organ (100–108 × 96–115 μm in *Po. recurvirostrae vs* 72–114 × 54–72 μm in *Po. anterovarium*), smaller testes (e.g. anterior testis 53–78 × 55–82 μm in *Po. recurvirostrae vs* anterior testis 81–135 × 153–207 μm in *Po. anterovarium*) and smaller eggs (egg length 68–73 μm in the new species *vs* 92–95 μm in *Po. anterovarium*). Furthermore, these species differ by 2.2–2.3% in partial sequences of 28S and 16.2–17.3% in partial sequences of *cox*1 (Supplementary Tables S2 and S3).

Posthodiplostomum recurvirostrae n. sp. differs from *Po. podicipitis* in having smaller testes (e.g. anterior testis 53–78 × 55–82 μm in the new species *vs* anterior testis 75–126 × 90–180 μm in *Po. podicipitis*) and egg length (68–73 μm in the new species *vs* 90–93 μm in *Po. podicipitis*). The two species differ by 0.1% in partial sequences of 28S and 12.1% in partial sequences of *cox*1 (Supplementary Tables S2 and S3), which significantly exceeds the broadly accepted level of interspecific divergence in diplostomids.

Posthodiplostomum recurvirostrae n. sp. is morphologically closest to *Po. ptychocheilus* (both subspecies). However, the new species and *Po. p. ptychocheilus* can be differentiated based on oesophagus:pharynx length ratio (1.4–2.7, mean 2.2, in the new species *vs* less than 1 based on the original line drawings of adults by Dubois (1936) and our material) and egg length is somewhat smaller (68–73 μm in the new species *vs* 70–89 μm in *Po. p. ptychocheilus*). The new species and *Po. p. ptychocheilus* differ by 0.2% in partial sequences of 28S and 11.5% in partial sequences of *cox*1 (Supplementary Tables S2 and S3). *Posthodiplostomum recurvirostrae* n. sp. and *Posthodiplostomum ptychocheilus palaearcticum* (Odening, 1963) n. comb. most obviously differ in the body length:body width ratio (2.5–2.8 in the new species *vs* 1.3 in *Po. p. palaearcticum*) as well as the holdfast organ size (100–108 × 96–115 μm in the new species *vs* 121 × 162 μm in *Po. p. palaearcticum*).

3.6.4. *Posthodiplostomum pacificus* Achatz, Chermak, Kent & Tkach n. sp.

3.6.4.1. Taxonomic summary

Type-host: *Larus californicus* (Lawrence) (Aves: Laridae).

Type-locality: Tule Lake (41°52′45.1″N, 121°33′26.3″W), National Wildlife Refuge, California, USA.

Type-material: The-type series consists of one mature specimen deposited in the HWML. Holotype: HWML 216657, labeled ex *L. californicus*, small intestine, Tule Lake, National Wildlife Refuge, California, USA, 8 July 2013, coll. V.V. Tkach.

Site in host: Small intestine.

Representative DNA sequences: GenBank: MZ710967 (28S), MZ707194 (*cox*1).

ZooBank registration: The Life Science Identifier (LSID) for *Posthodiplostomum pacificus* n. sp. is urn:lsid:zoobank.org:act:6ED78A42-6F28-4CD6-96FB-2DD6B57ACAC2.

Etymology: The species is named after the region of the type-locality, the Pacific Coast of the USA.

3.6.4.2. Description.

[Based on one adult specimen; Fig. 8] Body 1,220 long, consisting of distinct prosoma and opisthosoma; prosoma oval, concave, 854 long, widest at mid-length, 746 wide; anterior portion of prosoma with lateral protrusions on each side of oral sucker, glandular thickening present near proximal portion of protrusions. Opisthosoma cylindrical, 366 long, much narrower than prosoma, 434 wide. Prosoma:opisthosoma length ratio 2.3. Forebody 18% of body length. Tegument unarmed likely due to loss of spination resulting from freezing. Oral sucker terminal, 70 × 76. Ventral sucker larger than oral sucker, 66 × 76, located in anterior-most third of prosoma, obscured by holdfast organ; oral:ventral sucker width ratio 1.1. Holdfast organ massive, 426 × 370, oval with muscular ventral portion, occupies approximately half of prosoma length and width, strongly protruding; protruding portion overlaps ventral sucker, positioned in central portion of prosoma. Proteolytic gland not well-observed. Prepharynx short. Pharynx large, oval, 116 × 98. Oesophagus and caeca not well-observed.

Testes 2, tandem, entire, more or less reniform, occupying most of opisthosoma; anterior testis 282 × 384, partially inside prosoma, posterior testis 208 × 382. Seminal vesicle mostly post-testicular, partly ventral to posterior part of posterior testis, compact, continues as short ejaculatory duct. Ejaculatory duct joins metraterm dorsally to form hermaphroditic duct near proximal part of genital prepuce. Genital cone absent. Hermaphroditic duct opens at midpoint of genital prepuce (Fig. 8). Genital prepuce within genital atrium. Genital pore subterminal, dorsal.

Ovary pretesticular, reniform, positioned within prosoma, dorsal to holdfast organ, 114 × 216. Oötype and Mehlis' gland not well-observed. Laurer's canal not observed. Vitellarium limited to prosoma, distributed throughout prosoma posterior to level of pharynx, vitellarium within holdfast organ. Vitelline reservoir intertesticular, positioned at prosoma-opisthosoma junction. Uterus ventral to gonads, anterior portion convoluted, without eggs.

Excretory vesicle not well-observed. Excretory pore terminal.

3.6.4.3. Remarks.

Posthodiplostomum pacificus n. sp. belongs to *Posthodiplostomum* based on the results of our molecular analyses as well as the presence of a genital prepuce and the lack of pseudosuckers. Unlike all other *Posthodiplostomum* spp., *Po. pacificus* n. sp. lacks a well-defined genital cone but still possesses a clearly defined genital prepuce. In addition, *Po. pacificus* possesses glandular thickenings near the anterior margin of the prosoma which are absent in all other members of the genus.

The vitellarium of *Po. pacificus* n. sp. is limited to the prosoma. The only other *Posthodiplostomum* spp. with vitellarium limited to the prosoma are *Posthodiplostomum mignum* Boero, Led & Brandetti, 1972 and *Po. nanum sensu* Dubois, 1937. *Posthodiplostomum pacificus* n. sp. possesses vitellarium which is distributed throughout the prosoma, while the vitellarium of *Po. mignum* is limited to the area around the ventral sucker and holdfast organ. The holdfast organ of this new species is truly massive (occupies approximately 50% of prosoma), while the holdfast organ of *Po. mignum* and *Po. nanum sensu* Dubois, 1937 have much smaller holdfast organs.

3.6.5. *Posthodiplostomoides kinsellae* Achatz, Chermak, Martens, Pulis & Tkach n. sp.

3.6.5.1. Taxonomic summary

Type-host: *Halcyon malimbica* Shaw (Aves: Alcedinidae).

Type-locality: Kibale National Park (0°21′31.4″N, 30°22′50.2″E), Manairo, Uganda.

Type-material: The type-series consists of four fully mature specimens deposited in the HWML. Holotype: HWML 216635, labeled ex *H. malimbica*, small intestine, Uganda, 20 March 2013, coll. E. Pulis.

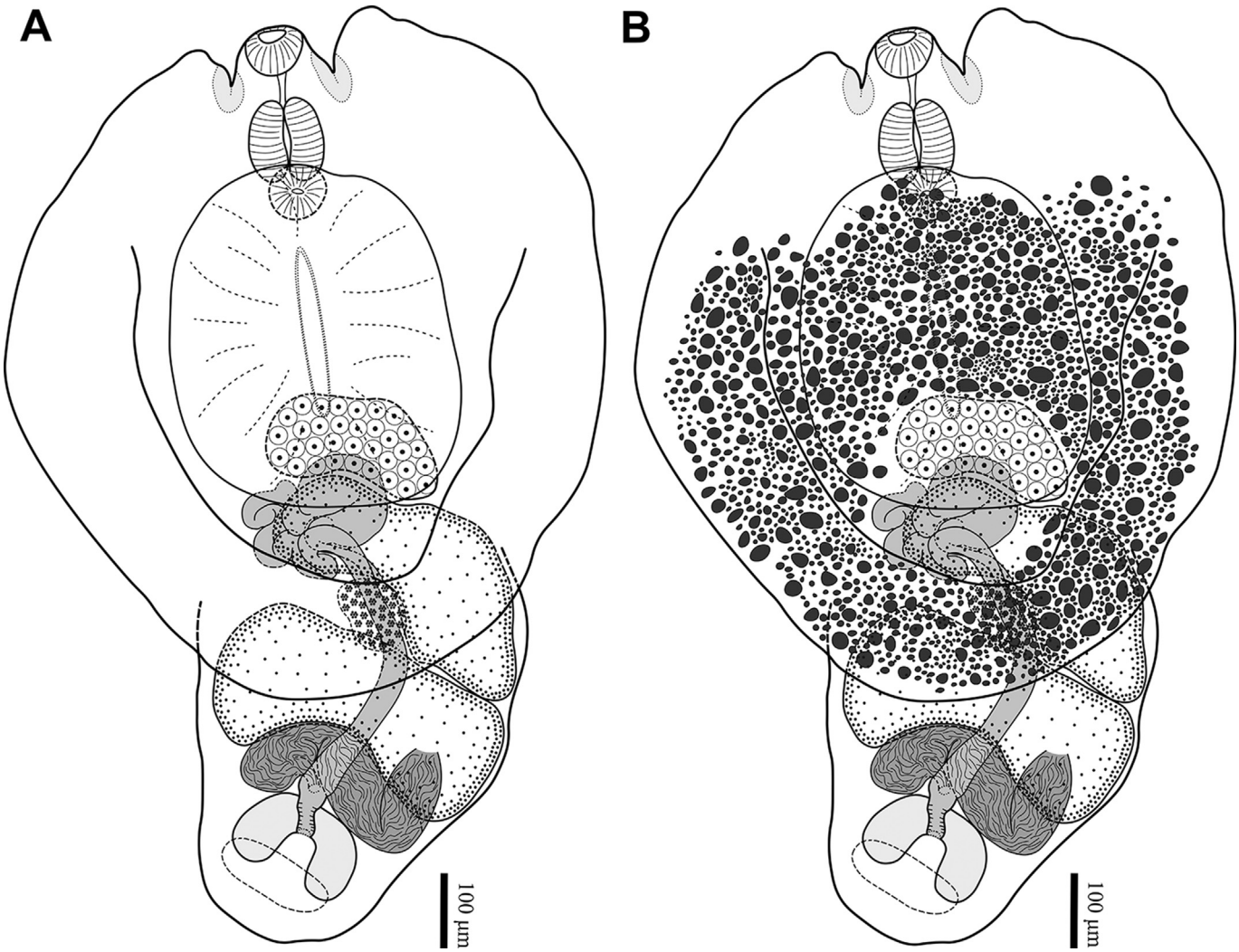

Fig. 8. *Posthodiplostomum pacificus* n. sp. **A** Ventral view of the holotype, vitellarium omitted. **B** Ventral view of the holotype, vitellarium shown.

Paratypes: HWML 216636 (lot of 2 slides), labels identical to the holotype.

Site in host: Small intestine.

Representative DNA sequences: GenBank: MZ710939 (28S), MZ707165 (*cox*1).

ZooBank registration: The Life Science Identifier (LSID) for *Posthodiplostomoides kinsellae* n. sp. is urn:lsid:zoobank.org:act:554358B0-8853-4FC4-95F3-FAF877E8DE20.

Etymology: The species is named after J. M. Kinsella for his outstanding contributions to the field of parasitology and being an incredible colleague.

3.6.5.2. Description. [Based on 4 adult specimens; measurements of holotype given in text; measurements of entire series given in Table 4; Fig. 9] Body 1,171 long, consisting of distinct prosoma and opisthosoma. Prosoma oval, widest at level of ventral sucker, 571 × 339, posterior portion somewhat concave; opisthosoma cylindrical, 580 × 206, somewhat narrower than prosoma. Prosoma:opisthosoma length ratio 1. Forebody 26% of body length. Tegument of prosoma armed with fine spines. Oral sucker subterminal, 58 × 55. Pseudosuckers present, 56–66 × 42. Ventral sucker somewhat larger than oral sucker, 59 × 73, located near mid-length of prosoma; oral:ventral sucker width ratio 0.8. Holdfast organ 151 × 127, subspherical with ventral muscular portion, posterior to ventral sucker, typically positioned in posterior-most quarter of prosoma. Proteolytic gland dorsal to posterior part of holdfast organ. Prepharynx not observed. Pharynx oval, 43 × 34. Oesophagus 29 long. Caecal bifurcation in anterior-most 25% of prosoma length. Caeca slender, extending to near posterior margin of posterior testis.

Testes 2, tandem, occupying about half of opisthosoma; anterior testis entire, subspherical or reniform, 111 × 125, posterior testis somewhat bilobed, saddle-like, 134 × 183. Seminal vesicle primarily post-testicular, portions ventral to posterior part of posterior testis, compact, was well-observed only in holotype, continues as short ejaculatory duct. Ejaculatory duct joins metraterm dorsally to form hermaphroditic duct near proximal part of genital cone. Hermaphroditic duct opens at tip of genital cone; genital cone with ventral prepuce within genital atrium. Genital cone and prepuce occupy majority of genital atrium. Genital pore terminal.

Ovary pretesticular, subspherical, 75 × 76. Oötype and Mehlis' gland not well-observed. Laurer's canal not observed. Vitellarium sparsely distributed in prosoma, extending from level of or slightly posterior to level of ventral sucker to about posterior margin of opisthosoma. Vitelline reservoir intertesticular. Uterus ventral to gonads, contains no egg in holotype, up to five eggs in paratypes (88–105 × 56–67).

Excretory vesicle and pore not observed.

3.6.5.3. Remarks. Posthodiplostomoides kinsellae n. sp. belongs to the genus based on the presence of pseudosuckers and a genital cone with genital prepuce. The new species differs from the two other known *Posthodiplostomoides* species, *Posthodiplostomoides leonensis* (Williams,

Table 4
Ranges of morphometric characters of *Posthodiplostomoides* spp.

Species	*Ps. kinsellae* n. sp.		*Ps. opisthadenicus*	*Ps. leonensis*[b]
Host	*Halcyon malimbica*		*Scopus umbretta*	*Bubulcus ibis*
Locality	Uganda		Zimbabwe	Sierra Leone
Reference	Present study		Dubois and Beverly-Burton (1971)	Williams (1967)
	Holotype and paratypes ($n = 3$)[a]	Hologenophore	($n = 9$)	(n = not provided)
Body length	1,171–1,389 (1,252)	–	Up to 1,800	950–1,100
Prosoma length	569–721 (620)	–	630–770	490–580
Prosoma width	334–360 (344)	–	250–280	320–380
Opisthosoma length	580–686 (625)	–	670–1,050	460–520
Opisthosoma width	206–246 (232)	182	200–290	240–270
Prosoma:opisthosoma length ratio	0.9–1.1 (1.0)	–	0.7[c]	1.2[c]
Forebody (% of body length)	54–58 (56)	–	66[c]	59[c]
Oral sucker length	56–58 (57)	–	47–60	50–60
Oral sucker width	55–56 (55)	–	57–68	50–80
Pseudosucker length	54–66 (59)	–	–	–
Pseudosucker width	28–43 (39)	–	–	–
Ventral sucker length	55–59 (58)	–	60–73	40–55
Ventral sucker width	67–73 (69)	–	65–78	57–75
Oral sucker:ventral sucker width ratio	0.8 (0.8)	–	0.9[c]	0.9[c]
Holdfast organ length	132–175 (153)	–	90–125	80–100
Holdfast organ width	127–167 (142)	–	90–120	80–100
Pharynx length	36–45 (41)	–	37–42	30–50
Pharynx width	34–37 (35)	–	30–37	20–30
Oral sucker:pharynx length ratio	1.2–1.6 (1.4)	–	1.23[c]	1.2[c]
Oesophagus length	29–60 (40)	–	–	–
Anterior testis length	111–127 (119)	–	85–175	80–120
Anterior testis width	125–144 (140)	–	195–270	190–260
Posterior testis length	123–141 (133)	–	160–250	120–160
Posterior testis width	183–227 (210)	–	200–270	180–240
Ovary length	75–85 (80)	72	50–68	60–100
Ovary width	76–95 (84)	85	90–105	50–70
Number of eggs	0–5	4	1	0–2
Egg length	88–97 (91)	63–67	–	73
Egg width	56–66 (61)	89–105	–	52
Anterior vitellarium free zone (% of prosoma length)	52–59 (55)	–	80[c]	46[c]
Posterior vitellarium free zone (% of opisthosoma length)	5–6 (5)	–	6[c]	16[c]

[a] Mean provided for *Posthodiplostomoides kinsellae* n. sp. in parentheses after range.
[b] Obtained from experimental infection by Williams (1967).
[c] Calculated measurements based on the line drawing in the original description.

1967) and *Posthodiplostomoides opisthadenicus* Dubois & Beverley-Burton, 1971, based on the distribution of the vitellarium (sparsely distributed in the prosoma and extending anteriorly to about the level of the ventral sucker or somewhat more posterior to it in the new species *vs* densely distributed in prosoma extending anterior to the level of the ventral sucker in *Posthodiplostomoides leonensis* and vitellarium in prosoma restricted to the area around holdfast organ in *Posthodiplostomoides opisthadenicus*), and the distinction between prosoma and opisthosoma (clearly distinct in the new species *vs* much less distinct in the two other species). This new species of *Posthodiplostomoides* can be further distinguished from the other two species in the possession of a larger holdfast organ (132–175 × 132–167 μm in *Posthodiplostomoides kinsellae* n. sp. *vs* 80–100 × 80–100 μm in *Posthodiplostomoides leonensis* and 90–125 × 90–120 μm in *Posthodiplostomoides opisthadenicus*).

3.7. Pairwise comparisons of Posthodiplostomum spp.

Many of the sequences of *Posthodiplostomum* spp. available in GenBank were obtained from larval stages; these larval stages typically cannot be reliably identified to the species based on morphology alone. Unfortunately, comparisons with the previously published sequences suggest that at least some sequences contain errors as they include numerous ambiguous sites and indels of lengths that cannot be divided by three (e.g. 1–2 nucleotides long) in the protein-coding gene *cox*1. Comparisons of DNA sequences must only utilize accurate sequences.

The interspecific divergence of 28S sequences among *Posthodiplostomum* spp. was generally low (0–9.6%; Supplementary Table S2). *Posthodiplostomum* sp. 20 *vs Posthodiplostomum* sp. 11 were the least divergent at 0%, whereas *Po. orchilongum vs Posthodiplostomum* sp. 1 of Sokolov and Gordeev (2020) (GenBank: MT394051) were the most divergent at 9.6%.

Intraspecific variation was only detected within four *Posthodiplostomum* spp. with multiple 28S sequences: *Po. anterovarium*, *Po. centrarchi*, *Posthodiplostomum* sp. 11 and *Posthodiplostomum* sp. 20. Interestingly, three out of 11 partial 28S sequences of *Po. centrarchi* contained an ambiguous site (cytosine or thymine), while the remaining eight had a thymine at the same position. *Posthodiplostomum anterovarium, Posthodiplostomum* sp. 11 and *Posthodiplostomum* sp. 20 each had a single ambiguous base.

The interspecific divergence of *cox*1 sequences among *Posthodiplostomum* spp. was much greater than among 28S sequences (4.1–22.3%; Supplementary Table S3) and overall similar to the interspecific divergence of *cox*1 sequences demonstrated within other diplostomoidean genera (3.4–19.8%) (e.g. Hernández-Mena et al., 2014; Gordy et al., 2017; Locke et al., 2018; López-Hernández et al., 2018; Achatz et al., 2020b and references therein; Tkach et al., 2020). *Posthodiplostomum minimum* (MacCallum, 1921) and *Posthodiplostomum* sp. 16 were the least divergent at 4.1%; *Posthodiplostomum cuticola* and *Posthodiplostomum brevicaudatum* were the most divergent at 22.3% (Supplementary Table S3). Despite only 0–0.1% difference between 28S sequences of *Posthodiplostomum* sp. 11 and *Posthodiplostomum* sp. 20, these two species-level lineages differed by 9.6–10.2% in *cox*1 sequences.

Due to the similarity of *cox*1 sequences among *Po. minimum* and *Posthodiplostomum* sp. 16 in the pairwise comparisons of all *Posthodiplostomum* spp., an additional alignment limited to *cox*1 sequences of Po. *minimum* and *Posthodiplostomum* sp. 16 was analyzed; this additional alignment was 72 nucleotides longer than the alignment used for general pairwise

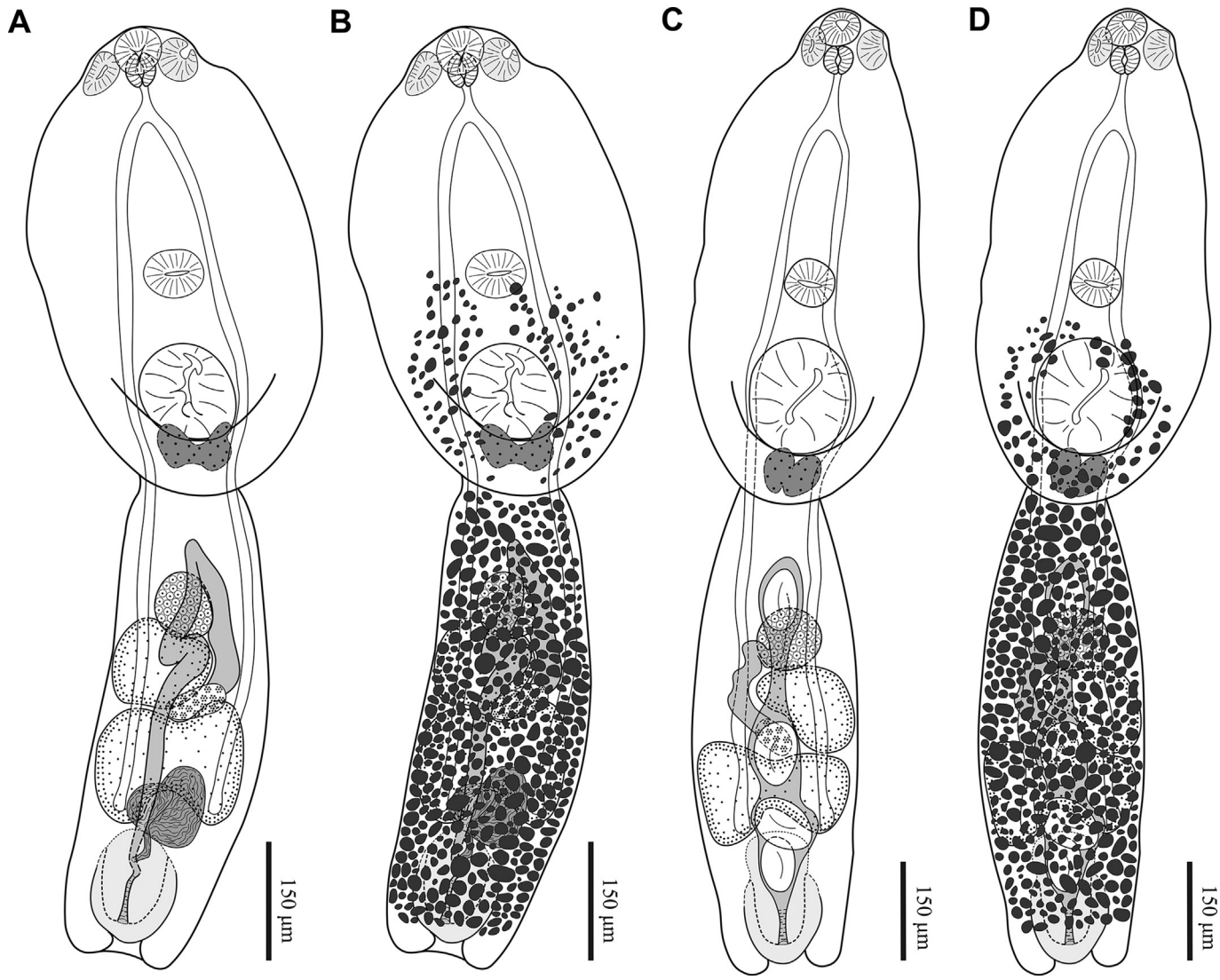

Fig. 9. *Posthodiplostomoides kinsellae* n. sp. **A** Ventral view of the holotype, vitellarium omitted. **B** Ventral view of the holotype, vitellarium shown. **C** Ventral view of a paratype, vitellarium omitted. **D** Ventral view of a paratype, vitellarium shown.

comparisons of *Posthodiplostomum* spp. (Supplementary Table S4). The pairwise comparisons based on this longer alignment demonstrated *Po. minimum vs Posthodiplostomum* sp. 16 to be 5.3–6.0% different.

The majority of *Posthodiplostomum* spp. did not demonstrate more than 2.2% intraspecific variation (Supplementary Table S3) in *cox*1 sequences. For instance, the partial *cox*1 sequences of *Po. centrarchi* (up to 1.1%), *Posthodiplostomum* sp. 11 (up to 0.5%), and *Posthodiplostomum* sp. 20 (up to 0.5%) demonstrated relatively low intraspecific variation despite having some intraspecific variations in 28S sequences (Supplementary Tables S2 and S3). Interestingly, *Po. minimum* from the Palaearctic and Nearctic only varied by up to 0.7%, and *Posthodiplostomum* sp. 16 from the Palaearctic and Nearctic varied by up to 1.8%. Exceptionally, the intraspecific variation of *Po. anterovarium* was greater than within comparisons of other species-level lineages (up to 3.6%) (Supplementary Table S3).

An additional alignment was analyzed to explore the intraspecific variation of *Po. anterovarium* (= *Posthodiplostomum* sp. 1 and sp. 2 of Moszczynska et al. (2009)). The additional alignment was 25 nucleotides longer than the alignment used for general pairwise comparisons of *Posthodiplostomum* spp. (Supplementary Table S5). The *cox*1 sequence of the adult specimen of *Po. anterovarium* (GenBank: MZ707168) was 3.0–3.5% different from the data of the larval specimens previously referred to as *Posthodiplostomum* sp. 1 and *Posthodiplostomum* sp. 2 of Moszczynska et al. (2009) as well as the sequences from our larval specimens (Supplementary Table S5); the larval specimens of the previously accepted *Posthodiplostomum* sp. 1 and sp. 2 of Moszczynska et al. (2009) differed by 2.8–3.8%. Our *cox*1 sequences from larvae and *Posthodiplostomum* sp. 2 of Moszczynska et al. (2009) varied by up to 2.5%. Importantly, the level of variation among *cox*1 sequences of the adult *Po. anterovarium* and genetically similar larvae is gradual (Supplementary Table S5). In our opinion, the differences detected among the *cox*1 sequences of these isolates do not provide enough support to consider these separate species/species-level lineages without clear morphological differences in adult specimens. As such, we consider these larvae (e.g. *Posthodiplostomum* spp. 1 and 2 of Moszczynska et al. (2009)) to be *Po.* 'cf.' *anterovarium* until matching sequences from adults will become available.

3.8. Remarks on Posthodiplostomum diversity

In the present study, we have generated new ribosomal and mitochondrial DNA sequences of the type-species of *Bolbophorus* Dubois, 1934, two species of *Cercocotyla* Yamaguti, 1939, one new species of *Posthodiplostomoides*, 23 species/species-level lineages of *Posthodiplostomum* (syns. *Mesoophorodiplostomum* and *Ornithodiplostomum*) and the type-species of *Pulvinifer*. We provided DNA sequence data from adults of 19 species/species-level lineages, 14 of which were identified to

species based on adult morphology. In addition, our DNA sequences represent 14 species/species-level lineages of *Posthodiplostomum*, which lacked previously published DNA sequence data.

Our results show that the currently known diversity of *Posthodiplostomum* is underestimated. The genus, as recognized in this study, was represented in the Nearctic by 12 nominal species. Our data, combined with previous studies, demonstrated the presence of at least 17 species-level lineages in the Nearctic. Furthermore, the morphology of our specimens of *Posthodiplostomum* sp. 21 and 22 suggests the presence of at least two additional species in the Neotropics; however, our adult specimens of these species-level lineages are not sufficient for description. We hypothesize that the diversity of *Posthodiplostomum* in other biogeographic realms has been similarly underestimated.

Our specimens of *Po. minimum* from the great blue heron *Ardea herodias* L. and black-crowned night heron *Nycticorax nycticorax* (L.) closely conform to the original description of *Po. minimum* collected from *A. herodias* in a zoo in New York, USA by MacCallum (1921) and the subsequent description of *Po. minimum* provided by Dubois and Rausch (1948) based on specimens collected from *A. herodias* and *N. nycticorax* in the Midwestern United States (e.g. Wisconsin, Michigan and Ohio). *Posthodiplostomum* sp. UG1 of Komatsu et al. (2020) (GenBank: LC511186) is clearly conspecific with our *Po. minimum* based on comparison of *cox*1 data (0–0.7% divergence in partial *cox*1 sequences; Supplementary Table S4). At the same time, *Posthodiplostomum* sp. 16 (= *Posthodiplostomum* sp. 4 of Gordy and Hanington (2019); e.g. GenBank: MH368945) and *Posthodiplostomum* sp. UG2 and UG3 of Komatsu et al. (2020) (GenBank: LC511187 and LC511188) appear to be conspecific based on comparison of *cox*1 sequences (0–1.8% divergence in partial *cox*1 sequences; Supplementary Table S4). The *cox*1 sequences of *Po. minimum* (= *Posthodiplostomum* sp. 4 of Moszczynska et al. (2009)) and *Posthodiplostomum* sp. 16 (= *Posthodiplostomum* sp. 4 of Gordy and Hanington (2019) and UG2 and UG3 of Komatsu et al. (2020)) also differ by 5.3–6.0% (Supplementary Table S4). In our opinion, this range of divergence exceeds what can be reasonably expected for intraspecific variation based on currently available data for the diplostomoideans. It is critical that adults which correspond to the genotype of *Posthodiplostomum* sp. 16 are collected for proper morphological comparison with *Po. minimum*. The presently available data demonstrate that at least three species of *Posthodiplostomum*, *Po. centrachi*, *Po. minimum* and *Posthodiplostomum* sp. 16, have Holarctic distributions.

Posthodiplostomum orchilongum is currently considered a synonym of *Po. minimum* (see Dubois, 1938, 1968). Our phylogenetic analyses (Figs. 2 and 3) clearly demonstrate that these taxa represent distinct species-level lineages. These two species are most easily distinguished based on differences in the holdfast organ (typically subspherical or transversely-oval in *Po. orchilongum vs* longitudinally-oval in *Po. minimum*) as well as the anterior extent of vitellarium (extending more anteriorly to the level of the ventral sucker in *Po. orchilongum vs* typically only reaching to the level of or slightly anterior to the level of the ventral sucker in *Po. minimum*). Based on the results of our molecular phylogenetic analyses as well as morphological differences, we restore *Po. orchilongum* as an independent species. We expect that additional differences may be found in other stages of the life-cycle.

Prior to this study, *Posthodiplostomum nanum* was known to be distributed only in the Neotropics (Dubois, 1937; López-Hernández et al., 2018). This is the first report of *Po. nanum* in the Nearctic region. However, it is important to note that *Po. nanum* studied by López-Hernández et al. (2018) has vitellarium in both the prosoma and opisthosoma, whereas the material originally described by Dubois (1937) has vitellarium only in the prosoma. Our specimens are conspecific with *Po. nanum* studied by López-Hernández et al. (2018) based on morphology as well as the comparison of *cox*1 sequences (1.4% difference). The distribution of the vitellarium has been demonstrated to be rather stable within a *Posthodiplostomum* species (Pérez-Ponce de León, 1995; present study). It is likely that the specimens currently identified as *Po. nanum* represent a novel species. Similar to the situation regarding *Po. minimum*, DNA sequences from specimens that conform to the original description of *Po. nanum* by Dubois (1937) are needed to test if the two morphotypes are conspecific.

Our specimens of *Po.* cf. *podicipitis* from a hooded merganser *Lophodytes cucullatus* (L.) are morphologically similar to the original description of specimens from the little grebe *Tachybaptus ruficollis* (Pallas) (*Podiceps ruficollis*) collected in Japan by Yamaguti (1939). It is possible that our material represents a novel species based on the difference in the order of definitive host (Anseriformes *vs* Podicipediformes) as well as the fact that the distribution range of *Ta. ruficollis* does not extend into the Nearctic, nor does the geographical range of *L. cucullatus* extend into the Palaearctic. Unfortunately, data on snail intermediate hosts of these taxa are not available. However, at this point we consider the description of our material as a novel species premature until comparable data of *Po. podicipitis* from *Ta. ruficollis* in Japan become available.

Mesoophorodiplostomum was previously considered a separate genus (Dubois, 1936; Niewiadomska, 2002), in part, based on the position of the ovary (intertesticular in *Posthodiplostomum pricei* (Krull, 1934) n. comb., the former type-species of *Mesoophorodiplostomum*). Our examination of ovary position of *Posthodiplostomum* spp. included in our 28S analysis (Fig. 3) demonstrated some clades to have relatively stable position of ovary (e.g. the ovary of members of Clade I was opposite to the anterior testis). However, other clades that include multiple species/species-level lineages (i.e. Clades II and III) had a variable position of the ovary. Importantly, previous authors have demonstrated that the position of the ovary may change during development (e.g. Stoyanov et al., 2017) or in adults (e.g. Palmieri, 1977). Our specimens of *Po. anterovarium*, *Po. centrachi* and *Posthodiplostomum* sp. 22 demonstrate variation in ovary position between the more immature and mature adult specimens (e.g. intertesticular in immature forms that transitions to pretesticular in adults of *Po. centrarchi*) (Fig. 3). Therefore, the exact position of the ovary should not be heavily relied upon for differentiation of *Posthodiplostomum* spp. except in fully mature adult specimens.

Most *Posthodiplostomum* spp. have a relatively distinct prosoma and opisthosoma. However, members of the former *Ornithodiplostomum* (Clade I; Fig. 3) as well as *Po. anterovarium* (Clade III; Fig. 3) and *Po. eurypygae* (Clade II; Fig. 3) have relatively indistinct separation between prosoma and opisthosoma. While this feature is suitable for assisting with differentiation of many *Posthodiplostomum* spp., it is clearly not suitable for supra-specific systematics. It is worth noting that among *Posthodiplostomoides* spp., only the new species described here has a clearly distinct prosoma and opisthosoma. At the same time, all other morphological features support its generic placement.

Our analyses demonstrate that Diplostomoidea sp. (GenBank: KU221112, KY319363 and KY319364), Digenean sp. (GenBank: MK321671) and Diplostomidae gen. sp. X (GenBank: MH368849) belong to *Posthodiplostomum* (Figs. 1–3). Identity of these forms will need to be established in the future by matching their sequences to sequences of properly fixed and identified adult digeneans.

3.9. Biogeography and host associations of Posthodiplostomum

Considering the ecological relevance of members of *Posthodiplostomum*, notably as major causative agents of ‘white grub’ and ‘black spot’ disease in fishes, it is critical to understand the diversity of *Posthodiplostomum* spp. worldwide as well as their host-associations throughout their life-cycles.

The 28S analysis of *Posthodiplostomum* spp. positioned *Po. cuticola* from the Palaearctic (Ukraine) as a strongly supported sister group to all other *Posthodiplostomum* spp. (Fig. 2). Likewise, four isolates of

Posthodiplostomum spp. larvae from the Indomalayan (India and Vietnam) and Palaearctic (Japan) realms were positioned in a 100% supported clade separate from the 100% supported clade containing the remaining *Posthodiplostomum* spp. The position of *Po. cuticola* and the clade from the Indomalayan and Palaearctic realms strongly suggest an Old World origin of the genus. The strong support and branch lengths of the cluster of the four *Posthodiplostomum* spp. larvae from the Indomalayan (India and Vietnam) and Palaearctic (Japan) realms suggest that members of the cluster may be endemic to Southeastern Asia and nearby regions (i.e. Japan).

Only two of the seven clades within the larger internal cluster of *Posthodiplostomum* spp. (Fig. 2) contained species from a single biogeographic realm, Nearctic in case of Clade III and Palaearctic in case of Clade VI. The remaining five clades contained representatives from more than one biogeographic realm. The branch topology within Clade II suggests a dispersal from the Neotropics into the Nearctic and Afrotropics (Fig. 2) while the branch topology in Clade I clearly suggests the dispersal of *Po. scardinii* from Nearctic to Palaearctic. Clades IV, V and VII failed to demonstrate any clear patterns of biogeography. *Posthodiplostomum centrarchi* (Clade IV; Nearctic and Palaearctic), *Po. minimum* (Clade V; Nearctic and Palaearctic) and *Po. nanum* (Clade VII; Nearctic and Neotropics) were collected in two biogeographic realms. Distribution of diplostomoideans (e.g. *Diplostomum ardeae* Dubois, 1969 and *Diplostomum huronense* (La Rue, 1927)) across multiple biogeographic realms has been previously demonstrated with DNA sequence data (e.g. Locke et al., 2020; Achatz et al., 2021c). In part, the extremely broad distribution of some *Posthodiplostomum* spp. may be facilitated by the broad geographical distribution and migratory nature of many of the avian definitive hosts; for instance, *Ardea alba* and *N. nycticorax* both have essentially worldwide distributions and are semi-migratory. The wide geographical distribution of *Posthodiplostomum* spp. is also possible due to the ubiquity of their potential snail intermediate hosts.

Based on the positions of *Po. cuticola* as well as *Po. centrarchi*, *Po. nanum* and *Posthodiplostomum* sp. 23 (Fig. 2), it would not be unreasonable to hypothesize that the ancestors of these diplostomoideans parasitized ardeid definitive hosts (e.g. herons). Additional 28S sequence data from other species of *Posthodiplostomum*, many of which parasitize ardeids, are necessary to further test this hypothesis. In addition, our phylogenetic analysis of *Posthodiplostomum* spp. based on 28S sequences (Fig. 2) revealed several secondary definitive host-switching events in the evolutionary history of *Posthodiplostomum*.

Clades I, II, III and VII (Fig. 2) included species which originate from a variety of definitive hosts. The members of Clade I included adults collected from anatids (common merganser *Mergus merganser* L. and *L. cucullatus*; three *Posthodiplostomum* species/species-level lineages), a recurvirostrid (American avocet *R. americana* Gmelin; *Po. recurvirostrae*) and a pelecanid (*Pe. erythrorhynchos*; *Posthodiplostomum* sp. 18). The position of *Posthodiplostomum* sp. 17 from *L. cucullatus* as a sister branch to the 100% supported clade which contained other members of Clade I, as well as the positions of *Po.* cf. *podicipitis* (collected from *L. cucullatus*) and *Po. ptychocheilus* (collected from a *M. merganser*) within the 100% supported clade suggest a possible host switch from merganser ducks to avocets and pelicans (Fig. 2; Table 1). However, the adult specimens of the other five species-level lineages within this clade remain to be collected and sequenced, which should clarify the picture of their host associations. Clade II demonstrates multiple transitions among lineages of avian definitive hosts (Fig. 2). For instance, *Po. eurypygae* from a eurypygid (sun bittern *E. helias* (Pallas)) was positioned as a sister group to species collected from ardeids (great egret *A. alba* L., cocoi heron *Ardea cocoi* L., little blue heron *Egretta caerulea* (L.) and rufescent tiger heron *Tigrisoma lineatum* (Boddaert); four *Posthodiplostomum* species/species-level lineages), accipitrids (black-collared hawk *Busarellus nigricollis* (Latham); *Po. macrocotyle*), a ciconiid (jabiru *Jabiru mycteria* (Lichtenstein)) and a pandionid (western osprey *P. haliaetus* (L.); *Po. erickgreenei*). Interestingly, three species/species-level lineages (*Po. microsicya*, *Posthodiplostomum* sp. 21 and 22) from *T. lineatum* formed a strongly supported clade (99%) which indicates a single transition to *T. lineatum*. Clade III included species collected from larids (California gull *L. californicus* (Lawrence) and ring-billed gull *Larus delawarensis* Ord; two *Posthodiplostomum* species/species-level lineages) and a pelecanid (*Pe. erythrorhynchos*; *Po. anterovarium*). Clade VII included two species/species-level lineages from ardeids (*A. alba* and *A. herodias*) and a single species-level lineage from a phalacrocoracid (Neotropic cormorant *Nannopterum brasilianum* (Gmelin)). More data on definitive and intermediate hosts are necessary to address the directionality of host-switching within these two clades.

Our 28S tree of *Posthodiplostomum* spp. (Fig. 3) revealed some associations between the strongly supported clusters/clades and the order of their fish second intermediate hosts. For instance, four species-level lineages from the Indomalayan and Palaearctic realms (GenBank: AB693170, KF738450, MT394045 and MT394051) were collected from fishes in the order Anabantiformes Britz, whereas three species-level lineages from Clade I (Fig. 3) were collected from fishes in the order Cypriniformes Bleeker. Although all former members of *Mesoophorodiplostomum* (Clade III; Fig. 3) were collected from perciform fishes, one species (*Po. pricei*) was found in fishes from the order Cyprinodontiformes Berg. The fish second intermediate hosts of many *Posthodiplostomum* species-level lineages are currently unknown, thus, it can be anticipated that some of these relationships may change once more data regarding the second intermediate hosts become available.

To the best of our knowledge, this is the first report of *Posthodiplostomum* spp. (or its new synonyms) from sunbitterns (Eurypygidae Selby), anhingas (Anhingidae Reichenbach) and avocets (Recurvirostridae Bonaparte). Based on our newly collected and sequenced specimens (Table 1) it is clear that *Posthodiplostomum* spp. and its new synonyms parasitize at least members of the orders Accipitriformes Vieillot (e.g. hawks and osprey), Charadriiformes Huxley (e.g. gulls, avocets), Eurypygiformes Hackett, Kimball, Reddy, Bowie, Braun, Braun, Chojnowski, Cox, Han, Harshman, Huddleston, Marks, Miglia, Moore, Sheldon, Steadman, Witt & Yuri (sunbitterns), Pelecaniformes Sharpe (e.g. pelicans, herons) and Suliformes Sharpe (e.g. anhingas, cormorants). It is worth noting that literature data (e.g. Dubois, 1968) claim that *Posthodiplostomum* spp. parasitize other orders of avian definitive hosts (e.g. Podicipediformes). It will be interesting to see how taxa collected from members of other avian orders, such as Podicipediformes (grebes), will impact the topologies of the *Posthodiplostomum* phylogenies.

Management strategies focused on the definitive hosts of *Posthodiplostomum* spp. must target a wide diversity of fish-eating birds, besides the most commonly reported ardeid hosts, as previously suggested by some authors (e.g. Lane and Morris, 2000). Our data from adult specimens expand the reference set of *Posthodiplostomum* spp. sequences which is critical for future ecological and systematic studies on agents of 'white grub' and 'black spot' disease worldwide. Our results further demonstrate that management strategies should also consider other birds that may not be commonly viewed as piscivorous, such as avocets. However, snail controlling measures may be the more realistic and efficient avenue as opposed to limiting access of avian definitive hosts to water bodies.

4. Conclusions

The results of our molecular phylogenetic analysis of 28S (Fig. 1) as well as the available data on morphology convincingly demonstrate the non-monophyly of two major subfamilies of the Diplostomidae, therefore we propose abandonment of the subfamilies in the system of the Diplostomidae. Based on the review of the morphology of *Posthodiplostomum*, *Ornithodiplostomum* and *Mesoophorodiplostomum* combined with molecular phylogenetic data (Figs. 1–3) we synonymize *Ornithodiplostomum* and *Mesoophorodiplostomum* with *Posthodiplostomum*. Newly generated sequence data for 28 species/species-level lineages of diplostomids including sequences of 19 adult forms and first sequences for species of

Cercotyla, *Posthodiplostomoides* and *Pulvinifer* significantly enhanced the current picture of the phylogenetic interrelationships within the family and expanded the reference database for future studies. Collection and sequencing of adult specimens of the numerous lineages currently known only from larval stages, as well as broader sampling from insufficiently studied hosts and geographical regions (e.g. Afrotropics and Australasia), are critical for the improvement of our understanding of the diversity and evolution of *Posthodiplosomum* as well as of the Diplostomidae as a whole.

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Ethical approval

All applicable institutional, national and international guidelines for the care and use of animals were followed. Euthanasia of animals was carried out in accordance with approved Institutional Animal Care and Use Committee (University of North Dakota IACUC protocol IACUC protocol 0610-1). Bird carcasses for parasitological examination were either obtained from hunters during regular hunting seasons, or from museum ornithological teams upon euthanasia as approved by IACUC (usually collected by firearm). Collecting of all birds other than game birds provided by hunters holding regular hunting permits, was done based on appropriate governmental permits in corresponding countries. No hosts were held in laboratory live prior to parasitological examination.

CRediT author statement

Tyler Achatz, Taylor Chermak, Jakson Martens, Vasyl Tkach: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Resources, Writing – original draft, Writing – review & editing. Eric Pulis, Alan Fecchio, Jeffrey Bell, Stephen Greiman, Kara Cromwell, Sara Brant, Michael Kent: Resources, Writing – review & editing.

Data availability

The newly generated sequences are deposited in the GenBank database under the accession numbers MZ710936-MZ710996 (28S) and MZ707162-MZ707219 (*cox*1). Type- and voucher material is deposited in the collection of the H. W. Manter Laboratory, University of Nebraska, Lincoln, Nebraska, USA and the Museum of Southwestern Biology (MSB), University of New Mexico, Albuquerque, New Mexico, USA.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crpvbd.2021.100051.

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