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 (cox1) 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 diplodomoidean lineages. Our molecular phylogenetic analysis and review of morphology do 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 Posthodipllostomoides 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; Snoop, 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; Nie-
wiadowska, 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 met-
acercariae of Posthodiplostomum are known to be associated with ‘black
spot’ disease when encysted on the skin or fins of their fish second in-
termediate 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, Mesopocharodiplostomum Dubois, 1936, has been only reported
from the Neartic and is much less studied than some of the larger and
more broadly distributed genera. A close relationship among Post-
ho-diplostomum, Ornithodiplostomum and Mesopocharodiplostomum has been
recently demonstrated using sequences of the ribosomal internal tran-
scribed spacer region (ITS1 + 5.8S + ITS2) as well as the mitochondrial
cytochrome c oxidase subunit 1 (cox1) 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 spe-
cies identifications based on adult morphology (e.g. Locke et al., 2018).
At present, only Posthodiplostomum centrarchi Hoffman, 1958, Post-
ho-diplostomum nasum Dubois, 1937 and Mesopocharodiplostomum 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 cox1 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
craspiahialine taxa to demonstrate the phylogenetic position of these taxa
among other major lineages of diplodistoideans, re-evaluate their sys-
tematics and aid ecological studies and disease diagnostics. Detailed
phylogenetic analyses of 28S and cox1 sequences were conducted for
closely related Posthodiplostomum, Ornithodiplostomum and Mesopo-
charodiplostomum. Whenever possible, type-species of corresponding genera
were used in our analyses. Furthermore, four new species of Post-
ho-diplostomum 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 diplodomitid digeneans were obtained from the intestines of a
variety of avian hosts, while larval diplodomitids were collected from a
variety of snails 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 Biological (MSB), University
of New Mexico, Albuquerque, New Mexico, USA (Table 1). Host spec-
mens 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 diplodistoideans, we refer to the two
distinct body parts in diplodistoideans 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 diplodistomoids 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
(cox1) genes were amplified by polymerase chain reactions (PCR).
Amplifications of 28S were performed using forward primer dipl2
(5’-AAG CAT ATC ACT AAG CGG-3’) and reverse primer 1500R
(5’-CTC ATG AGG AAG ACT CGG-3’) (Tkach et al., 2003). A fragment of the
cox1 gene was amplified using forward primers Plat-diploCOX1F
(5’-CTT TTR AAT TAT ACG GAT GTG CC-3), Cox1_Schist_5’
(5’-TCT TTR GAT CAT AAG CGG-3’), Dipl_Cox_5’
(5’-ACK TTR GAW CAT AAG CGG-3’) and BS_C01_INT_F
(5’-ATT AIC CCT CAC TAA ATG ATT TTT TTY YTR ATG CC-3’) and
reverse primers Plat-diploCOX1R (5’-AGC ATA GTA GMA GCA
GG-3’), acox650R (5’-CCA AAA AAC CAA AAC AAT TGC TG-3’), BS5’
(5’-AGC ACC TAA ACT AAT AAC ATG AAA ATG-3’), Dipl560R
(5’-CCA AAR AAY CAR AAY AWR TGY TG-3’), Dipl5cox3’
(5’-WAR TGC ATN GGA AAA AAA CA-3’) and BS_C01_INT_R
(5’-TAA TAC GAC TCA CTA TAA AAA MAM AGA AGA RAC MAC MGT AGT
AAT-3’) (Lockyer et al., 2003; Derycke et al., 2005; Moszczynska et al.,
2009; Kudlai et al., 2014; Achatz et al., 2019a, 2021b). PCR ampliti-
fications were performed in a total volume of 25 or 50 μl using GoTaq
gDNA 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 ExproStar PCR clean-up enzymatic kit from Cytiva (Marl-
borough, Massachusetts, USA) was used to purify PCR products. Purified
PCR products were cycle-sequenced directly using BrightDye Termina-
ator Cycle Sequencing Kit (MCLAB, California, USA), cleaned using a BigDye
Sequencing Clean Up Kit from MCLAB and run on an ABI 3130 auto-
mated capillary sequencer (Thermo Fisher Scientific, Waltham, Massa-
chusetts, USA). The PCR primers were used for sequencing reactions.
In addition, internal forward primer DPL600F (5’-CCG AGT GAG CAC
GAC CG-3’) and internal reverse primer DPL700R (5’-CAG ATC ATT AGA
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 MEGAT 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   |
|-------------------------------------------|-----------------------|---------------------|-------------------------|--------------|
| **Bolbophorus cf. confusus**              | Pelecanus onocrotalus | Ukraine             | –                       | MZ701936     |
| Cercophis rhodesiensis                    | Halcyon malimbica     | Uganda              | –                       | MZ701937     |
| Cercophis sp.                             | Cerele maximia        | Ukraine             | –                       | MZ701938     |
| Posthodiplomostomidae kinselli n. sp.    | Halcyon malimbica     | Uganda              | –                       | MZ701939     |
| Posthodiplomostomum cf. antevorans n. comb. | Lepomis cyanellus (liver) | Minnesota, USA     | HWML 216637             | MZ701940, MZ701941 |
| Posthodiplomostomum antevorans n. comb.   | Lepomis Gibbonius (liver) | Minnesota, USA     | –                       | MZ701942     |
| Posthodiplomostomum centrarchi            | Amblydipsalus rapiatis | Minnesota, USA     | –                       | MZ701945     |
| Posthodiplomostomum centrarchi            | Anhinga aninga        | Mississippi, USA    | HWML 216638             | MZ701946, MZ701947 |
| Posthodiplomostomum centrarchi            | Anhinga aninga        | Louisiana, USA      | HWML 216639             | MZ701948     |
| Posthodiplomostomum centrarchi            | Ardea alba            | Mississippi, USA    | –                       | MZ701713, MZ701714 |
| Posthodiplomostomum centrarchi            | Ardea herodias        | Georgia, USA        | HWML 216641             | MZ701949, MZ701950 |
| Posthodiplomostomum centrarchi            | Lepomis cyanellus (liver) | Minnesota, USA     | HWML 216642             | MZ701951, MZ701952 |
| Posthodiplomostomum centrarchi            | Lepomis cyanellus (skin) | Minnesota, USA    | HWML 216643             | MZ701953     |
| Posthodiplomostomum centrarchi            | Lepomis macrourchis (heart) | Minnesota, USA   | –                       | MZ701980     |
| Posthodiplomostomum centrarchi            | Lepomis macrourchis (liver) | Minnesota, USA  | –                       | MZ701981     |
| Posthodiplomostomum centrarchi            | Lepomis macrourchis (mesentry) | Minnesota, USA | –                       | MZ701982     |
| Posthodiplomostomum centrarchi            | Megacotyle alicyon    | Mississippi, USA    | –                       | MZ701784     |
| Posthodiplomostomum caticola              | Nycticorax nycticoura | Ukraine             | HWML 216644             | MZ701955     |
| Posthodiplomostomum tricerygmoon n. sp.   | Pandion halliaetus    | Montana, USA        | HWML 216645             | MZ701956     |
| Posthodiplomostomum euryygrea n. sp.      | Euryzysa helia        | Pantanal, Brazil    | HWML 216647             | MZ701957     |
| Posthodiplomostomum macrocycyle           | Busarellus nigricollis | Pantanal, Brazil    | HWML 216649             | MZ701958, MZ701959 |
| Posthodiplomostomum microsycylia          | Tigrisoma lineatum    | Pantanal, Brazil    | HWML 216650             | MZ701960     |
| Posthodiplomostomum minimum               | Ardea herodias        | North Dakota, USA   | HWML 216651             | MZ701961     |
| Posthodiplomostomum nanum                 | Ardea alba            | Mississippi, USA    | HWML 216653             | MZ701962     |
| Posthodiplomostomum orchestra               | Ardea alba            | Mississippi, USA    | HWML 216654             | MZ701963     |
| Posthodiplomostomum orchestra              | Eretta caerulea       | Mississippi, USA    | HWML 216655             | MZ701964     |
| Posthodiplomostomum pacificus n. sp.      | Larus californicus    | California, USA     | HWML 216657             | MZ701967     |
| Posthodiplomostomum podicipites n. comb.   | Catostomus commersoni (skin) | Minnesota, USA  | –                       | MZ701968     |
| Posthodiplomostomum pacificus n. sp.      | Lophodytes cucullatus | North Dakota, USA   | HWML 216658             | MZ701969, MZ701970 |
| Posthodiplomostomum pelvicanus            | Penephalus promelas (brain) | Minnesota, USA | –                       | MZ701971     |
| Posthodiplomostomum pelvicanus            | Larus delawarensis    | North Dakota, USA   | HWML 216659             | MZ701972, MZ701973 |
| Posthodiplomostomum psycocinclus n. comb. | Mergus merganser      | Minnesota, USA      | HWML 216660             | MZ701974     |
| Posthodiplomostomum recurvarstra n. sp.   | Recurvirostra americana | North Dakota, USA  | HWML 216661             | MZ701975     |
| Posthodiplomostomum recurvarstra n. sp.   | Chlidonous ox         | Minnesota, USA      | –                       | MZ701976     |
| Posthodiplomostomum recurvarstra n. sp.   | Unidentified fish (eyes) | North Dakota, USA  | –                       | MZ701977     |
| Posthodiplomostomum recurvarstra n. sp.   | Lophodytes cucullatus | North Dakota, USA   | HWML 216662             | MZ701978     |
| Posthodiplomostomum recurvarstra n. sp.   | Physa gyrina          | Oregon, USA         | HWML 216663             | MZ701979, MZ701980 |
| Posthodiplomostomum recurvarstra n. sp.   | Physa sp.             | Minnesota, USA      | –                       | MZ701982, MZ701983 |
| Posthodiplomostomum recurvarstra n. sp.   | Physa gyrina          | Oregon, USA         | –                       | MZ701984     |
| Posthodiplomostomum recurvarstra n. sp.   | Physa gyrina          | Oregon, USA         | –                       | MZ701985- MZ701988 |
| Posthodiplomostomum recurvarstra n. sp.   | Physa gyrina          | Oregon, USA         | –                       | MZ701990     |
| Posthodiplomostomum recurvarstra n. sp.   | Physa gyrina          | Oregon, USA         | –                       | MZ701991     |
| Posthodiplomostomum recurvarstra n. sp.   | Ardea alba            | Pantanal, Brazil    | HWML 216664             | MZ701992     |
| Posthodiplomostomum recurvarstra n. sp.   | Ardea cocci           | Pantanal, Brazil    | –                       | MZ701993     |
| Posthodiplomostomum recurvarstra n. sp.   | Tigrisoma lineatum    | Pantanal, Brazil    | HWML 216665             | MZ701994, MZ701995 |
| Posthodiplomostomum recurvarstra n. sp.   | Ardea herodias        | Georgia, USA        | HWML 216666             | MZ701996     |
| Posthodiplomostomum recurvarstra n. sp.   | Gallinago gallinago   | Minnesota, USA      | HWML 216667             | MZ701997     |

Note: The localization of metacercariae in the second intermediate host is provided, when possible, in parentheses.

a Previously included in *Mesoophidiplomostomum*.
b Previously included in *Ornithophidiplomostomum*.
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 Universidad Federal de Mato Grosso (UFMT 4865).
The phylogenetic positions of Bolbophorus, Cercocotyla Yamaguti, 1939, Mesoophorodiplostomum, Ornithodiplostomum, Posthodiplostomum and Pulvinifer Yamaguti, 1933 within the Diplostomoidea Poirier, 1886 were determined using a 28S alignment with Suchyothacotyle crocodilii (Yamaguti, 1954) (Cathocotyliidae 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 Psychochelus psychochelis psychicchelis (Faust, 1917) (type-species; n = 1), Posthodiplostomoides kinselae 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 Pulvinifer (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 cox1 of Posthodiplostomum + Ornithodiplostomum + Mesoophoridiplostomum 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. psychochelis, Mesoophoridiplostomum (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 cox1 sequences included newly generated sequences of Posthodiplostomum (n = 25) including the type-species Po. cuticola, Ornithodiplostomum (n = 4) including the type-species O. p. psychochelis, Mesoophoridiplostomum (n = 5) including the type-species M. pricei, and previously published sequences of Posthodiplostomum (n = 15), Ornithodiplostomum (n = 11), Mesoophoridiplostomum (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 cox1 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 Po., Pelacanum 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 Diplostomoidea 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 Diplostomoidea (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 damnicus 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. Pulvinifer + 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) (Gen-Bank: MK604826) – cluster of Posthodiplostomum + Ornithodiplostomum + Mesoophoridiplostomum 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 + Mesoophoridiplostomum clade are discussed in detail below.

The second 28S alignment that included only members of Posthodiplostomum, Ornithodiplostomum and Mesoophoridiplostomum 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 Mesoophoridiplostomum sequences. The 100% supported cluster contained seven well-supported clades. Clades I–VI formed a weakly supported clade separated from clade VII (polymy 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. psychochelis (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 eurygygae 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 archilongum Noble, 1936...
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 [Posthodiplomastomum sp. 21 + an 82% supported cluster of (Posthodiplomastomum sp. 22 + Posthodiplomastomum microsicya Dubois, 1936)].

Clades III, IV and V formed a poorly supported cluster (Fig. 2). Clade III (99% support) contained Posthodiplomastomum pacificus n. sp. as a sister group to an unsupported polytomy of M. pricei, Mesoophorodiplomastomum 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 Posthodiplomastomum minimum (MacCallum, 1921). Clade VI was positioned as an independent branch in the broader polytomy and only contained Posthodiplomastomum brevicaudatum (von Nordmann, 1832) (Fig. 2).

The cox1 alignment was 363 bp long; the phylogenetic tree resulting from the analysis of the cox1 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. cox1; e.g. Hernández-Mena et al., 2017; López-Hernández...
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 Codonoccephalinae 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. 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).
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, 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.
Mesophorodiplostomum, 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 Crassiphilinae or Diplostominae. Instead, members of both subfamilies formed several independent clades in the basal polytomy of the Diplostomoidea. In fact, Alaria (Alarinae), Diplodiplostomum (Diplostominae), Austrodiplostomum (Diplostominae), Tylodelphys (Diplostominae) and Pulvinifer (Crassiphilinae) formed a 97% supported clade. Our analysis failed to provide any support for the currently recognized Crassiphilinae 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 Crassiphilinae or Diplostominae (Fig. 1). The difference in distribution of vitellaria between members of the Crassiphilinae and Diplostominae is very inconsistent. Numerous crassiphilian species have vitellaria 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, crassiphilines 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 Sudarkiv, 1959 and Corylurus Szidat, 1928 + Ichthyocotylurus Ondening, 1969 (Fig. 1). It is possible that familial placement of Codonocephalus 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 diplostomoid family Proterodiplostomidae based on the non-monophyletic nature of its constituent subfamilies. The abandonment of subfamilies has also been relatively recently proposed based on the non-monophyletic nature of its constituent subfamilies. The subfamilies within the diplostomidean family Proterodiplostomidae (Alariinae), Dicrocoeliidae Looss, 1899 and Echinostomatidae Looss, 1899 currently recognized for the type-species of Pulvinifer. On the other hand, it was not described from a member of Posthodiplostomoides sp. of Overstreet et al. (2002) is necessary to evaluate the status of these taxa and clarify the systematic position of B. damni.
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.
Mesophorodiplostomum is differentiated from Posthodiplostomum and Ornithodiplostomum based on the position of the ovary (intertesticular in the type-species of Mesophorodiplostomum vs pretesticular or at level of anterior testis in Posthodiplostomum and Ornithodiplostomum spp.). (Niewiadowska, 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 Mesophorodiplostomum (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 Mesophorodiplostomum and Posthodiplostomum and only tentatively assigned its genus.

Molecular phylogenies based on 28S (Figs. 1–3) consistently positioned Mesophorodiplostomum (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 Mesophorodiplostomum. If Ornithodiplostomum and Mesophorodiplostomum 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 + Mesophorodiplostomum spp. Clade II contained another previous synonym of Po. minimum, namely Po. orchilugum (see Section 3.8), as well as several other species which closely conform to the morphological diagnosis of Posthodiplostomum (e.g. Po. macroctylo, Po. microcylo). 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 Mesophorodiplostomum 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 Niewiadowska, 2002, amended): Digenea: Diplostomidae. Body biparite, 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. Ovaly ellipsoidal or oval, pretesticular, opposite to anterior testis or intertesticular, median, lateral or diagonal to anterior testis. Vitellarium typically in prosoma and opisthosome. 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. australis Dubois, 1937, Po. bi-ellipticum Dubois, 1958, Po. bothri Vidyarthi, 1938, Po. boydae Dubois, 1969, Po. brevicaudatum (von Nordmann, 1832), Po. centrarchi Hoffman, 1958, Po. erickgrenei n. sp., Po. euryppage n. sp., Po. garambense (Baer, 1959) n. comb., Po. giganteum Dubois, 1988, Po. grande (Diesing, 1850), Po. grayii (Verma, 1936), Po. izobrychi (Lang Tsu-pei, 1966), Po. linguaeformae Pearson & Dubois, 1985, Po. macrocytole Dubois, 1937, Po. mehtai Gupta & Mishra, 1974, Po. microsycia Dubois, 1936, Po. mingum Boero, Led & Brandetti 1972, Po. milvi Fotedar & Bambroo, 1965, Po. minimum (MacCallum, 1921), Po. nanum Dubois, 1937, Po. obsenum (Lutz, 1928), Po. oblongum Dubois, 1937, Po. opisthosycia Dubois, 1969, Po. orichlongum Noble, 1936, Po. pacificus n. sp., Po. podicipitis (Yamaguti, 1939) n. comb., Po. pricei (Krull, 1934) n. comb., Po. prosostomum Dubois & Rausch, 1948, Po. psychocheilus psychocheilus (Faust, 1917) n. comb., Po. psychocheilus 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. Paraatypes: HWML 216646 (lot of 2 slides), labels identical to the holotype.

**Site in host:** Small intestine.

**Representative DNA sequences:** GenBank: MZ710956 (28S), MZ707186 (cox1). ZooBank registration: The Life Science Identifier (LSID) for Posthodiplostomum erickgrenei n. sp. is urn:lsid:zoobank.org:act:5B9988D0-11DB-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 opisthosome; prosoma 790 × 400, extremely concave, essentially infundibular with ventral aperture, long, truncated at anterior end,
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. Segmentation unarmoured, 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 μm 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. Ootype and Mehlis’ gland not well-observed. Laurer’s canal not observed. Vitellaria 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 australis 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. australis 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. australis (Supplementary Fig. S2). The new species and Po. australis 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. australis; 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. australis). 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. australis) and egg length (70–75 μm in the new species vs 80–91 μm in Po. australis). 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: Eurypygus 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 (Holoparasite): HWML 216648 (lot of 1 slide), label identical to the holotype.

Site in host: Small intestine.

Representative DNA sequences: GenBank: MZ710957 (28S), MZ707187 (cox1).

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.
most quarter of prosoma; oral:ventral sucker width ratio 1.2. Holdfast organs immediately posterior to ventral sucker, oval with ventral muscular portion, 90 × 54. Proetendyl gland not well-observed. Pre-pharynx 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 bi-lobed, 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. Ootype and Mehls’ 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 euryygae 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 (Drönen, 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 euryygae 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 anterior to posterior 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. ptychocheilus and Po. anterovarium (both subspecies) (extends anterior to slightly posterior to the level of the caecal bifurcation in Po. euryygae, 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. euryygae 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 (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. euryygae 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 cox1 (Supplementary Tables S2 and S3).
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 (cox1).

*ZooBank registration:* The Life Science Identifier (LSID) for *Posthodiplostomum recurvostrae* 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. Ootype and

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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.
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 x 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. antervarum*, *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 cox1.

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 cox1 (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 cox1 (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 oesophagusopharynx 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 cox1 (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, Chermark, 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 (cox1).
ZooBank registration: The Life Science Identifier (LSID) for Posthodiplostomum pacificus n. sp. is urn:lsid:zoobank.org:act:6ED78A42-6F28-4CD6-96FB-2DB6B57ACA2.
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. Proso- ma:opisthosoma length ratio 2.3. Forebody 18% of body length. Tegu- ment unremarked 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. Pro- teolytic 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, poster- ior 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 her- maphroditic 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 pros- oma 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, Chermark, 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), Mbanza, 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.
Paratypes: HWML 216636 (lot of 2 slides), labels identical to the holotype.

Site in host: Small intestine.

Representative DNA sequences: GenBank: MZ710939 (28S), MZ707165 (cox1).

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. Vitellariam 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,

Fig. 8. Posthodiplostomum pacificus n. sp. A Ventral view of the holotype, vitellariam omitted. B Ventral view of the holotype, vitellariam shown.
The interspecific divergence of cox1 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 cox1 sequences demonstrated within other diplostomoid 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 cox1 sequences.

Due to the similarity of cox1 sequences among Po. minimum and Posthodiplostomum sp. 16 in the pairwise comparisons of all Posthodiplostomum spp., an additional alignment limited to cox1 sequences of Po. minimum and Posthodiplostomum sp. 16 was analyzed; this additional alignment was 72 nucleotides longer than the alignment used for general pairwise comparisons.

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

| Species                  | *Ps. kinselis* n. sp. | *Ps. opisthadenicus* | *Ps. leonensis*^a^ |
|--------------------------|-----------------------|----------------------|-------------------|
| Host                     | *Halcyon malinicia*   | *Scopus umbretta*    | *Bubulcus ibis*   |
| Locality                 | Uganda                | Zimbabwe             | Sierra Leone      |
| Reference                | Present study         | Dubois and Beverly-Burton (1971) | Williams (1967) |
|                          | (n = 3)               | (n = 9)              | (n = not provided) |
| Body length              | 1,171–1,389 (1,252)   | –                    | 950–1,100         |
| Prosoma length           | 569–721 (620)         | –                    | 490–580           |
| Prosoma width            | 334–360 (344)         | –                    | 320–380           |
| Ovipositor length        | 580–686 (625)         | –                    | 460–520           |
| Ovary length             | 206–246 (232)         | 182                  | 240–270           |
| Ovary width              | 80–111 (110)          | – 0.7^b^             | 1.2^b^            |
| Forebody (% of body length) | 54–58 (56)          | –                    | 59^c^             |
| Oral sucker length       | 56–58 (57)            | –                    | 50–60              |
| Oral sucker width        | 55–56 (55)            | –                    | 50–80              |
| Pseudosucker length      | 54–66 (59)            | –                    | –                  |
| Pseudosucker width       | 28–43 (39)            | –                    | –                  |
| Ventral sucker length    | 55–59 (58)            | –                    | 40–55              |
| Ventral sucker width     | 67–73 (69)            | –                    | 57–75              |
| Oral sucker:ventral sucker length ratio | 0.8 (0.8) | – 0.9^c^             | 0.9^c^             |
| Holdfast organ length    | 132–175 (153)         | –                    | 80–100             |
| Holdfast organ width     | 127–167 (142)         | –                    | 80–100             |
| Pharynx length           | 36–45 (41)            | –                    | 30–50              |
| Pharynx width            | 34–37 (35)            | –                    | 20–30              |
| Oral sucker:pharynx length ratio | 1.2–1.6 (1.4) | – 1.2^c^             | 1.2^c^             |
| Oesophagus length        | 29–60 (40)            | –                    | –                  |
| Anterior testis length   | 111–127 (119)         | –                    | 80–120             |
| Anterior testis width    | 125–144 (140)         | –                    | 190–260            |
| Posterior testis length  | 123–141 (133)         | –                    | 120–160            |
| Posterior testis width   | 183–227 (210)         | –                    | 180–240            |
| Ovary length             | 75–85 (80)            | 72                   | 60–100             |
| Ovary width              | 76–95 (84)            | 85                   | 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                 |
| Posterior vitellarium free zone (% of prosoma length) | 52–59 (55) | – 80^e^             | 46^e^              |
| Posterior vitellarium free zone (% of opisthosoma length) | 5–6 (5) | – 6^e^             | 16^e^              |

^a^ Mean provided for *Posthodiplostomoides kinselis* 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 kinselis* 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 cox1. 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. ochilorum* 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. antennorvarium*, *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 antennorvarium*, *Posthodiplostomum* sp. 11 and *Posthodiplostomum* sp. 20 each had a single ambiguous base.

The interspecific divergence of cox1 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 cox1 sequences demonstrated within other diplostomoid 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 cox1 sequences.
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 cox1 sequences. For instance, the partial cox1 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 S5). Importantly, the level of variation among cox1 sequences of the adult *Po. anterovarium* and genetically similar larvae is gradual (Supplementary Table S5). In our opinion, the differences detected among the cox1 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 (1946) 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 cox1 data (0–0.7% divergence in partial cox1 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 cox1 sequences (0–1.8% divergence in partial cox1 sequences; Supplementary Table S4). The cox1 sequences of Po. minimum (= Posthodiplostomum sp. 4 of Moszynska 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 diplometoideans. 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. anterovarium, Po. nanum 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 on the basis of 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 cox1 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 T. 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 T. ruficollis in Japan become available.

Mesophorodiplostomum 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 Mesophorodiplostomum). 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. centrachi) (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 Diplometoidea sp. (GenBank: KU221112, KY319363 and KY319364), Digenean sp. (GenBank: MK321671) and Diplometoidea sp. (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 Indomalayan and Palaearctic realms strongly suggest an Old World origin.

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 Afrotopics (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 diplodiplostomideans (e.g. Diplodiplostomum ardeae Dubois, 1969 and Diplodiplostomum horunense (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 Nycticorax nycticorax both have essentially worldwide distributions and are semi-migratory. The widespread 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 diplodiplostomideans parapatized 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. cullatius; 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. cullatius 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. cullatius) and Po. ptychochelius (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. eurypygaea from a eurypygids (AV) avocet (P. avocetta) was positioned as a sister group to species collected from ardeids (great egret A. alba L., cocco heron Ardea cocoi L., little blue heron Egretta caerulea (L.) and rufescent tiger heron Tigrisoma lineatum (Boddart)); four Posthodiplostomum species/species-level lineages), accipitrids (black-collared hawk Buceoletus nigriceps (Latham); Po. macrocytile), a ciconid (jabiru Jabiru mycteria (Lichtenstein)) and a pandionid (western osprey P. haliaetus (L.); Po. erickgreeni). Interestingly, three species/species-level lineages (Po. microsycya, 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 Nanopterum brasiliaternion (Gmelin)). More data on definitive and intermediate hosts are necessary to address the directionality of host-switching within these two clades.

Our 2BS 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, KF738480, 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 Mesoaphrodisiophilodiplostomum (Clade III; Fig. 3) were collected from perciform fishes, one species (Po. pricei) was found in fishes in 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 sunbittins (Euryptyaetya Selby), anhingas (Anhingidae Reichenbach) and avocets (Recuvirotridae 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), Euryptyaetya Hackett, Kimball, Reddy, Bowie, Braun, Braun, Chojnowski, Cox, Han, Harshman, Huddleston, Marks, Miglia, Moore, Sheldon, Steadman, Witt & Yuri (sunbittins), 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 Diplodiplostomidae, therefore we propose abandonment of the subfamilies in the system of the Diplodiplostomidae. Based on the review of the morphology of Posthodiplostomum, Ornithodiplostomum and Mesosporodiplostomum combined with molecular phylogenetic data (Figs. 1–3) we synonymize Ornithodiplostomum and Mesosporodiplostomum with Posthodiplostomum. Newly generated sequence data for 28 species/species-level lineages of diplodiplostomids 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 Posthodiplostomum 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
TJJA, Tyler 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 (cox1). 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.crparvbd.2021.100051.

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