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First integrative study of the diversity and specificity of metacercariae of *Posthodiplostomum* Dubois, 1936 from native and introduced fishes in the Caribbean

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Research Article

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Abstract

Metacercariae of the genus *Posthodiplostomum* are often recorded in freshwater fish hosts. While the diversity and taxonomy of this genus are receiving increasing attention in molecular phylogenetic studies, available data remain geographically biased. Most molecular studies of *Posthodiplostomum* and morphologically similar (neascus) worms originate in North America and Europe and Asia (more than 60% of DNA sequences are from USA and Canada), with few data currently available from the Neotropics, where high host diversity suggests high and under-sampled parasite diversity. In this study, we report molecular and morphological data from metacercariae of *Posthodiplostomum* in fish in Puerto Rico, where only a single species has been previously reported. Partial sequences of cytochrome *c* oxidase subunit 1 from metacercariae from *Dajaus monticola* (native to Puerto Rico) and the introduced fishes *Poecilia reticulata*, *Parachromis managuensis*, *Lepomis macrochirus* and *Micropterus salmoides* revealed 7 genetically distinct species-level lineages, of which 4 were novel. We report novel molecular life-cycle linkages in *Posthodiplostomum macrocotyle* (metacercariae in muscle of the cichlid *Pa. managuensis*), a species previously known only from adults in birds from South America; and in *Posthodiplostomum* sp. 23 (metacercariae in poeciliids), which has recently been found in *Ardea herodias* in Georgia, USA. We also report the first molecular data from *Posthodiplostomum* sp. 8 in *M. salmoides* in the Caribbean. Metacercariae of most species were morphologically distinguished and all displayed narrow specificity for fish hosts, with no indication of parasite sharing among introduced and native fishes.

Introduction

The genus *Posthodiplostomum* Dubois, 1936 (Platyhelminthes: Diplostomidae) is a large and widely distributed group of digenetic trematodes in which larval stages infect snails and fish, and adults occur in the intestine of piscivorous birds. In freshwater fish hosts, metacercariae of *Posthodiplostomum* can negatively impact growth, change feeding rates (Osorio-Sarabia *et al.*, 1986), affect response to predation (Ondračková *et al.*, 2006) or cause mortality (Lane and Morris, 2000).

Over the last decade, molecular studies have revealed novel species diversity and linked unknown larvae with identified adults in *Posthodiplostomum* (Locke *et al.*, 2010; Nguyen *et al.*, 2012; Stoyanov *et al.*, 2017; Boone *et al.*, 2018; López-Hernández *et al.*, 2018; Pérez-Ponce de León *et al.*, 2022). Several studies also demonstrated a need for revision in *Posthodiplostomum* and related genera (Blasco-Costa and Locke, 2017; López-Hernández *et al.*, 2018; Achatz *et al.*, 2019), culminating in the recent work of Achatz *et al.* (2021), who synonymized *Ornithodiplostomum* and *Mesoophorodiplostomum* with *Posthodiplostomum* and presented several new molecular life-stage linkages. These authors also found no support for the traditional subfamily system within the Diplostomidae, which rested partly on larval morphotypes shared among various genera. These include the neascus (from Greek, 'new bladder'), a type of metacercariae that Hughes (1927) named for the prominent reserve bladder that occurs in *Posthodiplostomum* and certain other diplostomid genera such as *Uvulifer* Yamaguti, 1934 and *Crassiphiala* Van Haitsma, 1925 (Hoffman, 1955; Gibson, 1996). Regardless of the status of neascus as a character within the Diplostomidae, it remains a useful concept for characterization of diplostomid diversity, as it effectively narrows down the identity of genera and species of metacercariae under consideration.

A review of molecular studies of *Posthodiplostomum* and other neascus shows strong geographic bias (Supplementary Table 1). Two-thirds (11/16) of all studies have been carried out in the Palaearctic (5) and Nearctic (6), with a sequencing effort disproportionately intense in North America (586/838 sequences). Probably as a consequence of this bias, most (at least 2/3) of the genetically distinguished *Posthodiplostomum* species come from North America (more precise estimates of regional species diversity are impeded by the use of different markers by different authors, raising the possibility of the same species delineated with different loci in different studies). Moreover, from the relatively few sequences sampled outside the Nearctic

and Palaearctic regions (238/838), most (143) originate in a single recent study by Pérez-Ponce de León *et al.* (2022), 1 of only 3 with data from the Neotropics. This unbalanced sampling effort is particularly noteworthy because the diversity of fish and bird hosts is higher in Neotropic and Afrotropic regions compared to regions further north (Balian *et al.*, 2008), which suggests that parasite diversity is also higher. Still, while the study effort given in Supplementary Table 1 shows strong regional sampling and sequencing bias, it nonetheless represents an improvement. In 2016, <3% of specimens sequenced from the superfamily Diplostomoidea were obtained from the southern hemisphere and non-temperate regions (Blasco-Costa and Locke, 2017). Overall, however, additional data from neascus-forming taxa in the Neotropical regions are still needed to characterize the distribution, diversity and host-use patterns of *Posthodiplostomum* and related parasites.

Views of the identity and host specificity of metacercariae of *Posthodiplostomum* have changed in light of molecular data. In northeastern North America, for example, metacercariae of genetically distinguished species of *Posthodiplostomum* have proven to be specific to narrow ranges of centrarchid or leuciscid fish hosts (Locke *et al.*, 2010; Boone *et al.*, 2018). Metacercariae of *Posthodiplostomum minimum*, *Posthodiplostomum centrarchi* and *Posthodiplostomum* sp. 8 have subsequently shown similar specificity in novel environments such as in Europe (Kvach *et al.*, 2017; Stoyanov *et al.*, 2017), Puerto Rico (Locke *et al.*, 2018) and Japan (Komatsu *et al.*, 2020; Achatz *et al.*, 2021). In Central America, however, the metacercariae of most lineages of *Posthodiplostomum* surveyed by Pérez-Ponce de León *et al.* (2022) were found in multiple families of fish, suggesting patterns of host specificity initially observed in temperate regions may not generalize to tropical regions. The sequences from the extensive survey of *Posthodiplostomum* in Central America by Pérez-Ponce de León *et al.* (2022) have yet to be compared with those of Achatz *et al.* (2021), who sequenced adults across the Americas, partly because both studies were published nearly simultaneously.

In the Caribbean, little work has been conducted on metacercariae of *Posthodiplostomum*. Bunkley-Williams and Williams (1994) reported metacercariae of *Po. minimum* in centrarchids and in *Poecilia reticulata*, all of which are introduced to the island. Mohammed *et al.* (2020) reported metacercariae of *Posthodiplostomum* from the guppy *Poe. reticulata* in its native range (Trinidad). Aside from a DNA sequence and report of *Po. centrarchi* in *Lepomis microlophus* in Puerto Rico (Locke *et al.*, 2018), no genetic studies have been carried out to characterize the diversity of these worms in introduced or native freshwater fishes in the Caribbean region. The general trends of diversity that emerge from molecular surveys of diplostomoid and particularly neascus metacercariae suggest that diversity may be underestimated in the Caribbean and in Central and South America.

The present work aimed to characterize the diversity and host use of *Posthodiplostomum* and similar neascus-type metacercariae in fishes from western Puerto Rico, by integrating molecular and morphological analysis. Seven genetically distinct neascus-type metacercariae were found in native and introduced freshwater fishes, including 3 novel lineages, all of which display narrow specificity for their second-intermediate hosts on the island.

Materials and methods

Sample collection

Most parasites studied herein were from fish from freshwater bodies in western Puerto Rico: Quebrada de Oro and Rio Yagüez in Mayagüez, a small pond in Centro Vacacional de Añasco, Cerrillos Reservoir near Ponce, and Rio Rosario in Maricao. Additional sequences were obtained from metacercariae from *Gambusia affinis* sampled in the Pascagoula River, Mississippi (USA), from *Lepomis macrochirus* in Lake Ontario and Lake Opinicon, Ontario (Canada), *Lepomis gibbosus* in St. Didace, Quebec (Canada), *Micropterus salmoides* in Canadarago Lake, New York (USA), and *Micropterus dolomieu* in Ottawa River, and Lake Saint Pierre, Quebec (Canada) and in Oaks Creek, New York (USA). In Puerto Rico, fish were caught using a backpack or boat-mounted electrofisher, while those from sites on the North American mainland were collected with a beach seine, cast net or hook and line. Fish collected in Puerto Rico were transported to the laboratory where they were maintained in aquaria until euthanization by immersion in clove oil solution until necropsy (Kvach *et al.*, 2017). Fish were identified based on Sterba (1967) as well as using molecular data (see below), and prior to necropsy were measured and weighed. Guppies and small fish were crushed whole between glass plates. Metacercariae were extracted from cysts manually with forceps for preservation in 95% alcohol and refrigeration at 4°C. Prevalence and mean abundance were calculated according to Bush *et al.* (1997).

At the same site of the Quebrada de Oro from which infected *Poe. reticulata* and *Dajaus monticola* were sampled, 1639 *Tarebia granifera* and 124 *Melanoides tuberculata* (Thiaridae), 747 *Physa acuta* (Physidae) and 202 Neritidae gen. sp. were collected. Ten *Marisa cornuarietis* were collected from the small pond in Añasco where infected *Parachromis managuensis* were obtained. No other snail species were observed at these localities. Most snails were housed in a small container with dechlorinated water for acclimation, placed in a multi-well culture plate and exposed to a 5 h light and dark cycle (a minority were dissected directly). Snails were examined twice for cercariae infections under a dissecting microscope. Non-shedding snails were crushed and examined for sporocysts.

Morphological study

Metacercariae were gradually rehydrated from 95% alcohol concentration to pure water, stained with Semichon's acid carmine, dehydrated in pure alcohol, cleared in clove oil and mounted on permanent slides using Permount toluene medium. Three types of morphological vouchers were deposited in the Museum of Southwestern Biology (MSB:Para:33294-301): paragenophores (from pairs of complete worms from the same individual host separated for either morphological or molecular evaluation), syngenophores (representatives from a different individual host of the same species collected from the same locality from which sequenced worms were obtained) and hologenophores (individual sub-sectioned worms from which DNA was extracted and sequenced) (Pleijel *et al.*, 2008).

Morphological characterization of metacercariae was based on features studied by Dubois (1970*b*), Athokpam and Tandon (2014) and Stoyanov *et al.* (2017) with metrical data reported in micrometres. Principal component analysis (PCA), analysis of similarities (ANOSIM) and permutational analysis of multivariate dispersion (PERMDISP) were performed using PRIMER-e software (Clarke and Gorley, 2015) to assess morphometric variation within and between genetically distinguished species, based on Euclidean morphometric distances among specimens calculated from log-transformed measurements. Line drawings were made with the aid of a camera lucida Nikon Alphaphot YS and a drawing tube.

Molecular and phylogenetic analysis

Extraction of DNA from excysted metacercariae preserved in 95% ethanol employed the protocol for invertebrates of Ivanova *et al.*

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(2006) and the Canadian Centre for DNA Barcoding protocols. The DNA barcode region of the cytochrome *c* oxidase subunit 1 (CO1) gene was initially amplified using the polymerase chain reaction (PCR), and degenerate primers Dice1F forward with the Dice11-R including D3Ar sequence reverse primer (Van Steenkiste *et al.*, 2015) and/or primers MplatCOX1dF/R of Moszczynska *et al.* (2009). In *Posthodiplostomum* and other members of the former Crassiphialinae, the barcode region of CO1 can be difficult to sequence bidirectionally because of poly-T regions (Van Steenkiste *et al.*, 2015). Nonetheless, this region of CO1 has utility because even lower quality contigs (single read, or only partially bidirectional reads) typically yield data allowing reliable discrimination of species. For example, Locke *et al.* (2010) highlighted several species in which CO1 variation was likely inflated by problems with contig assembly and electropherogram quality, but the species distinguished have generally been supported in subsequent studies of the same taxa (Boone *et al.*, 2018; Achatz *et al.*, 2021). In addition, the standardized use of this region of CO1 ensures comparability among a wide range of animals (Ondrejicka *et al.*, 2014), including *Posthodiplostomum* and related taxa (Locke *et al.*, 2010, 2015; Boone *et al.*, 2018; López-Hernández *et al.*, 2018).

Individual hosts suspected to be *Pa. managuensis* were sub-adult and did not show all diagnostic characters, and belong to Cichlidae, in which morphological variability can complicate identification (Sowersby, 2017). In poeciliids, phenotypic plasticity and sexual dimorphism, with some species-specific characters only in males, can also hamper identifications (Schories *et al.*, 2009; Mise *et al.*, 2015). Identification of *Pa. managuensis* and *Poe. reticulata* was therefore confirmed by amplification and sequencing CO1 using primers FishF1 (TCAACCAACCACAAAGACATTGGCAC) and FishR1 (TAGACTTCTGGGTGGCCAAAGAATCA) (Ward *et al.*, 2005) after extracting DNA from muscle tissue using a Qiagen DNeasy blood & tissue kit (Qiagen, Germantown, Maryland, USA) following the manufacturer's protocols. The extracts were amplified in 25 μL reactions [3 μL template, 8.5 μL H_2O, 0.5 μL of each primer, 12.5 μL Taq 2× Master Mix (New England Biolabs, Ippswich, Massachusetts, USA)] with initial denaturation at 95°C for 2 min followed by 30 cycles of 0.5 min at 94°C, 0.5 min at 54°C and 1 min at 72°C; followed by a final 10 min at 72°C. PCR products were visualized on 1.0% agarose gels and sequenced at Genewiz (Genewiz, South Plainfield, New Jersey, USA).

Forward and reverse electropherograms were assembled using Geneious Prime v. 2020.2.3 (Biomatters Ltd., New Zealand). In many sequences obtained from parasites, a manual alignment was required to assemble contigs because unidirectional amplicons overlapped only in a poly-T region, where bidirectional contigs were obtained. Alignments were then generated using MUSCLE implemented in MEGA v.10.1.8 (Kumar *et al.*, 2018) and pairwise uncorrected *P*-distances were calculated between and within clusters using the same program. Sequences from other studies used in the present analysis are provided in Supplementary Table 2. For phylogenetic reconstruction, maximum likelihood (ML) and Bayesian inference (BI) were used. The best nucleotide substitution model determined for CO1 (GTR + G + I) was selected based on the Bayesian information criterion (Kumar *et al.*, 2018). The ML analysis of non-redundant (<100% identical) CO1 sequences was carried out using RAxML v. 8 (Stamatakis, 2014) in Geneious v.11.2. Phylogenetic accuracy was estimated by bootstrapping the constructed trees with 500 replicates (Pattengale *et al.*, 2010). For BI gene tree estimation, MrBayes 3.2.6 (Huelsenbeck and Ronquist, 2001) was used with 2 runs of the Markov chain Monte Carlo for 1.1 million generations, sampled every 200 generations, a heating parameter value of 0.2, burn-in length of 100 000 and *Diplostomum spathaceum* set as outgroup. Species were delineated based on reciprocal monophyly in phylogenetic analysis and considering the magnitudes of *P*-distances within and between clades thus distinguished. Barcode index numbers (BINs), which are unique identifiers assigned in BOLD based on CO1 distance cluster quality (Ratnasingham and Hebert, 2007, 2013) were also used as a species-delineation proxy, and the geographic distributions, host spectra, infection site and morphology of specimens were also considered in determining species boundaries.

Pérez-Ponce de León *et al.* (2022) sequenced CO1 in metacercariae of *Posthodiplostomum* in a wide range of fishes across Central America and obtained results highly relevant to the present study. However, sequences of DNA from *Posthodiplostomum* in the study by Pérez-Ponce de León *et al.* (2022) were from a different region of CO1 than those obtained in the present study, and direct comparison was not possible. Some sequences from Achatz *et al.* (2021), which all include the same region of CO1 sequenced herein, extend to the region sequenced by Pérez-Ponce de León *et al.* (2022), allowing indirect comparisons of the latter data with sequences in the present study. We used Discontinuous Megablast (Wheeler *et al.*, 2003) to query CO1 sequences of Achatz *et al.* (2021) against taxonomic identifiers created for lineages I–V in Pérez-Ponce de León *et al.* (2022).

Results

Infection levels

Neascus resembling *Posthodiplostomum* spp. were found in 64 of 170 (38%) fish belonging to 4 families collected in western Puerto Rico. Prevalence was highest in *M. salmoides* (17/29 infected), followed by *Pa. managuensis* (8/14), *Poe. reticulata* (28/58) and *D. monticola* (10/54) (Table 1). A total of 144 metacercariae were recovered from the mesentery, viscera and body cavity in *Poe. reticulata*, *M. salmoides* and *L. microlophus*, and from muscle tissue of *Pa. managuensis* and *D. monticola*. No diplostomid sporocysts or cercariae were recovered from snails.

Molecular analyses

Amplification and direct sequencing of CO1 was successful in 64/88 specimens from Puerto Rico (GenBank accession numbers: OP071162–225, Supplementary Table 3). Forward and reverse chromatograms were aligned to generate bidirectional contigs when possible. In most samples, a 13 bp poly-T region common in CO1 in diplostomids (Van Steenkiste *et al.*, 2015) caused short or low-quality electropherograms in downstream sequencing and contigs were therefore based on single-direction reads assembled on either side of the poly-T span. After including previously published data, and removing identical and short sequences (<300 bp), the final alignment of 78 CO1 consensus sequences was 441 bp in length. All study sequences were between 14.5 and 22.4% divergent from those of *Posthodiplostomum brevicaudatum* (KX931418-20), which were excluded from subsequent analysis because they resulted in a shortened contiguous alignment. In ML and BI analyses, CO1 sequences from specimens collected in the present study fell into 7 well-supported and reciprocally monophyletic species-level clades (hereafter, species) (Fig. 1) with interspecific divergence of at least 6.4% and mean intraspecific mean distances of 0.9% (range 0–3.8%) (Table 2). Deeper nodes in the ML and BI phylogenies were generally weakly supported (bootstrap support <70%, posterior probability <0.9), but in both ML and BI trees sequences generated in the present study fell into a monophyletic clade consisting of data from *Posthodiplostomum* spp. Separate ML/BI analysis based on translated amino acids, or on only the first 2 codons, did not clarify

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Table 1. Origins and biometric parameters (mean ± standard deviation in parentheses) of fish collected in Puerto Rico and other localities in the present study

Host	Status in Puerto Rico	*N* infected/ examined	*N* sequenced/*N* studied morphologically	Weight (g)	Total length (cm)	Locality
Poeciliidae						
Poecilia reticulata	Introduced (native to Lesser Antilles, Venezuela, Guyana)	28/58	16/41	ND	2.3 ± 0.4	Quebrada de Oro (18.2129, −67.1429)
Gambusia affinis	Introduced (native to Southeastern USA, Mississippi drainage)	1/1	2/0	ND	ND	Pascagoula River, Mississippi, USA
Mugilidae						
Dajaus monticola	Native	10/54	11/43	38.4 ± 74.9	10.7 ± 5.5	Río Yagüez (18.2051, −67.1299), Quebrada de Oro (18.2129, −67.1429)
Cichlidae						
Parachromis managuensis	Introduced (native to Honduras, Nicaragua, Costa Rica)	8/14	15/10	46.1 ± 28.8	14.7 ± 3.2	Unnamed pond, Villas de Añasco (18.2941, −67.2009)
Centrarchidae						
Lepomis auritus	All centrarchids introduced (native to North America)	0/17		ND	17.23 ± 3.55	Cerrillos reservoir (18.0803, −66.5775)
Lepomis macrochirus		0/1		ND	17.5	Cerrillos reservoir (18.0803, −66.5775)
L. macrochirus		1/1	3/2	ND	ND	Salmon Point, Lake Ontario, Canada (43.8399, −77.221)
L. macrochirus		1/1	1/2	ND	ND	Lake Opinicon, Ontario, Canada (44.5663, −76.3074)
Lepomis microlophus		1/14	1/0	ND	12.49 ± 4.69	Cerrillos reservoir (18.0803, −66.5775)
Lepomis gibbosus		ND	3/6	ND	ND	Lac Thomas, QC, Canada (46.3832, −73.2405), Eastern Lake Ontario, ON Canada (43.8399, −77.221)
Micropterus salmoides		17/29	1/2	ND	19.46 ± 9.89	Cerrillos reservoir (18.0803, −66.5775), Rio Rosario (18.1703, −66.9871)
M. salmoides		1	1/0	ND	ND	Lake Canadarago, NY, USA (42.817, −75.0076)
Micropterus dolomieu		1	4/0	ND	ND	Lake St. Pierre, QC, Canada (46.115, −73.021)
M. dolomieu		1	1/2	ND	ND	Ottawa River, ON, Canada (45.579, −75.136)
M. dolomieu		1	1/0	ND	ND	Oaks Creek, NY, USA (42.6892, −74.9577)

ND, no data.

relationships, and therefore, only the ML tree based on all nucleotide positions is shown herein (Fig. 1). Sequences from the present study were comprised of 3 species from *Poe. reticulata* (body cavity) (*Posthodiplostomum* spp. 23, 24 and 26), 1 from *D. monticola* (muscle) (*Posthodiplostomum* sp. 25), 1 from *Pa. managuensis* (muscle) (*Posthodiplostomum macrocotyle*), 1 from *M. dolomieu* and *M. salmoides* (liver) (*Posthodiplostomum* sp. 8) and 1 from *L. macrochirus* (liver, kidney) and *L. gibbosus* (heart, liver) (*Po. centrarchi*). Most of the species we distinguished were consistent with the BINs assigned in BOLD (Ratnasingham and Hebert, 2007, 2013), except in *Posthodiplostomum* sp. 23 and 24, which each contained 2 BINs. Within *Posthodiplostomum* sp. 23, the BIN algorithm separated 2 sequences from parasites from *G. affinis* in Mississippi (BIN: AAM7098) from 7 sequences from parasites from *Poe. reticulata* in Puerto Rico in the present study, and 1 from an adult from *Ardea herodias* from Georgia, USA, from Achatz *et al.* (2021) (BIN: ADE4068), based on BOLD calculations of 2.52% CO1 distance between these BINs. Nonetheless, we provisionally place both clusters of sequences in AAM7098 and ADE4068 within *Posthodiplostomum* sp. 23 (following nomenclature of Achatz *et al.*, 2021), because this level of genetic divergence is within that recorded in other studies of *Posthodiplostomum* (e.g. Achatz *et al.*, 2021), intermediate hosts belong to the same family (Poeciliidae), the data originate from a plausible geographic scope for a single species of *Posthodiplostomum* and genetic distances based on our own alignments differed (Table 2). In what we consider to be *Posthodiplostomum* sp. 24, 2 BINs (AEF4897, $n = 5$; AEF4552, $n = 1$) differing by 3.81% in CO1 were distinguished on BOLD. Although this is an unusually high level of intraspecific variation in CO1 in *Posthodiplostomum*, in the absence of any other evidence, such as differential host use, we provisionally assign sequences in both AEF4897 and AEF4552 to a single putative species. Notably, the single specimen in AEF4552 was found in the same individual guppy as a specimen of AEF4897.

Four of the 7 species sequenced in the present study presented species-level matches with published data. Six CO1 sequences from *Po. centrarchi* in *L. macrochirus* from Ontario were 99–100% similar to those of *Po. centrarchi* from Canada, Europe and Puerto Rico (Locke *et al.*, 2010, 2018; Stoyanov *et al.*, 2017). Six CO1 sequences from metacercariae from the liver of *M. salmoides* from Cerrillos Reservoir matched (98–99.8%) *Posthodiplostomum* sp. 8 from *Micropterus* spp. from North America (Locke *et al.*, 2010; Boone *et al.*, 2018). Fifteen CO1 sequences from metacercariae from the muscle of *Pa. managuensis* showed 100% identity with adult *Po. macrocotyle* from *Busarellus nigricollis* from the Pantanal, Brazil (Achatz *et al.*, 2021). Eight CO1 sequences from metacercariae infecting *Poe. reticulata* and 2 of *G. affinis* in Mississippi matched (99.6%) an adult of *Posthodiplostomum* sp. 23 from *A. herodias* in Georgia, USA (Achatz *et al.*, 2021). The CO1 sequences of *Posthodiplostomum* sp. 23 from Achatz *et al.* (2021) were long enough for comparison with data from *Posthodiplostomum* of Pérez-Ponce de León *et al.* (2022), but no species-level match was observed for this or other species in the 2 studies (Table 3).

Preliminary morphological identifications of representative cichlid and poeciliid hosts were supported with CO1 data. A 595-bp CO1 sequence from a sub-adult cichlid (OP071160) collected in Añasco was 99.5–100% identical to 12 CO1 sequences of *Pa. managuensis* in GenBank (HQ654748-52, HQ654748, KP728467, KP728467, MG496183-6). A 596-bp CO1 sequence from a guppy (OP071161) collected in Quebrada de Oro, Mayagüez, averaged 97.66 (range 86.6–99.8% similarity) to 63 sequences from *Poe. reticulata* in GenBank. The lower range of these similarities was driven by an unpublished sequence (MT428036) attributed to *Poe. reticulata* that differed from other CO1 sequences from *Poe. reticulata* by ≥12.4%; MT428036 is likely misidentified as it has high similarity (>99%) with numerous CO1 sequences from *Poecilia gillii*, *Poecilia mexicana*, *Poecilia orri* and *Poecilia sphenops*, which form part of a species complex that does not include *Poe. reticulata* (Bagley *et al.*, 2015). Excluding MT428036, the CO1 sequence from *Poe. reticulata* in Quebrada de Oro averaged 97.83 (range 94.8–99.8% similarity) to the remaining 62 CO1 sequences from *Poe. reticulata* in GenBank. Tissue vouchers from these 2 sequenced fish hosts were deposited at the Museum of Southwestern Biology (*Poe. reticulata* MSB:Host:24820, *Pa. managuensis* MSB:Host:24821).

Morphology of parasites

A total of 147 neascus metacercariae were recovered from fish in western Puerto Rico, of which 66 were from the body cavity of *Poe. reticulata*, 37 were from muscle of *D. monticola* and 44 from muscle of *Pa. managuensis*. For morphometric analysis, 105 worms were stained and measured, including vouchered metacercariae from centrarchid hosts sampled in Canada and USA. The morphology of the neascus metacercariae was consistent with *Posthodiplostomum* spp. Worms were in voluminous cysts, exhibited a bipartite body, and lacked lateral pseudosuckers in the foliaceous prosoma (Fig. 2).

The 105 specimens studied morphometrically included 6 hologenophores from 2 *Posthodiplostomum* species from *Poe. reticulata* (*Posthodiplostomum* spp. 23 and 24; no hologenophore was obtained from *Posthodiplostomum* sp. 26). All voucher specimens, including all those from *Poe. reticulata* that likely comprise a mixture of *Posthodiplostomum* spp. 23, 24 and 26, were characterized morphometrically with accompanying statistical analysis of morphometric distances. Because only a single species of *Posthodiplostomum* distinguished genetically was recovered from each of *Pa. managuensis*, *D. monticola*, *Lepomis* spp. and *Micropterus* spp., unsequenced voucher specimens from each of these hosts were considered para- or syngenophores for purposes of morphological characterization. Consistent with this assumption, metacercariae from the same host species were more morphometrically similar than those from different host species (ANOSIM global: $R = 0.389$, $P = 0.001$, 999 permutations of Euclidean distances based on 16 log-transformed measurements). Morphometric distances between worms from all pairs of host species were significantly greater than those within host species (pairwise ANOSIM: $R = 0.219$–0.966, $P \leq 0.001$) except between worms from *Lepomis* and *Micropterus* ($R = 0.004$, $P = 0.396$). The greatest morphometric distances were between parasites from *Pa. managuensis* and those from *D. monticola* ($R = 0.902$, $P = 0.001$) and *Micropterus* spp. ($R = 0.966$, $P = 0.002$).

These differences are apparent in PCA, in which the first 2 axes explain the 75.3% of total morphometric variation (Fig. 3). Along PC1 (62.8% of total variation), worms from *Pa. managuensis* were morphometrically distinct from all others except for some originating from *Poe. reticulata*. Along PC2, 2 groups were formed, one scoring positively and comprising metacercariae from all host species, and a second scoring negatively and comprising a subset of worms from *Poe. reticulata* and *D. monticola*. Morphometric variability was highest in worms from *Poe. reticulata*, which were widely dispersed along PC1 and PC2. Morphometric dispersion (approximated in 2 dimensions by the spread of the data cloud in Fig. 3) differed among metacercariae from different host species (PERMDISP: F ratio = 4.435, $P = 0.0024$). Worms from *Poe. reticulata* presented the highest morphometric dispersion, with an average spread to median of morphometric distances of 1.360 (S.D. 0.075), followed by

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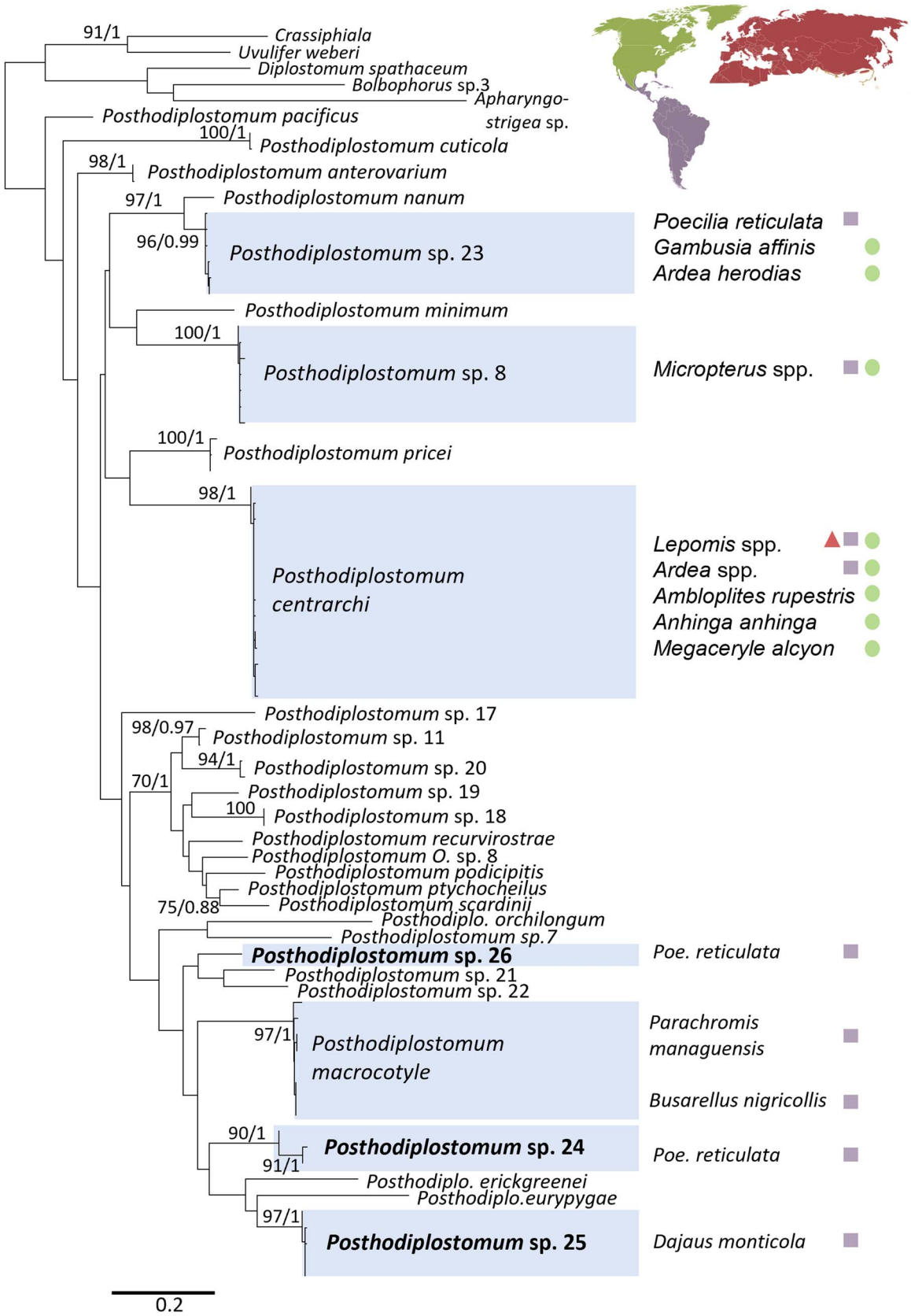

Fig. 1. Maximum Likelihood (ML) tree of 75 sequences of partial CO1 from *Posthodiplostomum* spp. and other diplostomids using the GTR + G + I model of nucleotide evolution. Nodes with ML bootstrap values above 70% in 1000 replicates are annotated with ML bootstrap support/BI posterior probability based on 8252 trees. Species present in Puerto Rico are indicated by shaded boxes and annotated with hosts and geographic realms (purple square = Neotropical; green circle = Nearctic; red triangle = Palaearctic); those newly characterized are in bold text.

parasites from *D. monticola* (average spread to median = 1.048, S.D. 0.053). The high morphometric variability of specimens from *Poe. reticulata* is consistent with the molecular results indicating the presence of 3 species (*Posthodiplostomum* spp. 23, 24 and 26) in this host. Consequently, only hologenophores were used to describe metacercariae of species from

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Table 2. Mean uncorrected *P*-distance (%) in partial CO1 among and within species of *Posthodiplostomum* from Puerto Rico (ranges in parentheses) and heterospecific species with minimal genetic distance (i.e. nearest neighbour)

Group or species name	Intraspecific	Interspecific	Nearest neighbour
Posthodiplostomum centrarchi	0.5 (0–1.2)	16.7 (13.7–21.5)	*Posthodiplostomum* sp. 8
Posthodiplostomum macrocotyle	0.8 (0–1.7)	16.4 (12.5–20.4)	*Posthodiplostomum* sp. 21
Posthodiplostomum sp. 23	0.7 (0.2–1.1)	16.4 (6.4–21.7)	*Posthodiplostomum nanum*
Posthodiplostomum sp. 24	2.5 (0.6–3.8)	16.2 (10.9–20.9)	*Posthodiplostomum* sp. 26
Posthodiplostomum sp. 25	0.3 (0–0.7)	16.4 (12.5–21.1)	*Posthodiplostomum erickgreenei*
Posthodiplostomum sp. 26	–	15.5 (10.3–20.1)	*Posthodiplostomum* sp. 24
Posthodiplostomum sp. 8	0.7 (0.3–1.4)	16.7 (13.9–23.2)	*Posthodiplostomum anterovarium*

Table 3. Summary of genetic distances (uncorrected-*P*) among sequences of CO1 from species of *Posthodiplostomum* in Achatz *et al*. (2021) and Pérez-Ponce de León *et al*. (2022), based on BLAST searches

Query sequence from Achatz *et al*. (2021)	Similarity to lineages of *Posthodiplostomum* in Pérez-Ponce de León *et al*. (2022) from Cichlidae, Poeciliidae, Goodeidae, Profundulidae and Leuciscidae in México, Guatemala, Honduras, Nicaragua and Costa Rica	Alignment length
Posthodiplostomum erickgreenei (MZ707186) from *Pandion haliaetus* (Montana, USA)	81.66–85.55% to I–V	334–338
Posthodiplostomum cf. *podicipitis* (MZ707196) from *Lophodytes cucullatus* (North Dakota, USA), *Catostomus commersonii* and *Pimephales promelas* (Minnesota, USA)	92.31–93.85% to I 81.69–88.73% to II–V	59–71
Posthodiplostomum sp. 17 (MZ707205) from *L. cucullatus* (North Dakota, USA)	80.43–89.77% to I–V	73–92
Posthodiplostomum sp. 21 (MZ707213) from *Tigrisoma lineatum*, *Jabiru mycteria* (Pantanal, Brazil)	82.04–86.45% to I–V	338–396
Posthodiplostomum sp. 22 (MZ707215) from *Ardea alba*, *Ardea cocoi*, *T. lineatum* (Pantanal, Brazil)	82.04–86.45% to I–V	332–338
Posthodiplostomum sp. 23 (MZ707217) from *Ardea herodias* (Georgia, USA)	92.20–92.77% to I 81.29–84.80% to II–V	342–346

Poe. reticulata, while other species were characterized also based on para- and syngenophores (Table 4, see sections Descriptions and Remarks).

Descriptions

Posthodiplostomum sp. 23
Host: *Poecilia reticulata*, *Gambusia affinis* (Poeciliidae).
Localities: Quebrada de Oro (18.2129, −67.1429), Mayagüez, Puerto Rico; Pascagoula River, Mississippi, USA.
Site of infection: mesentery.
Voucher specimen: MSB:Para:33301.
BINs: ADE4068, AAM7098.
GenBank: OP071188-97.
Description based on 1 hologenophore from *Poe. reticulata* from Quebrada de Oro (Fig. 2C; Table 4). Body slightly bipartite, with prosoma elongated and round, opisthosoma round. Prepharynx not observed. Oral sucker oval, well developed, slightly larger than ventral sucker. Muscular holdfast organ close to ventral sucker and to posterior margin of prosoma. Copulatory bursa and genital primordia not visible. In spacious, colourless, thin-walled cysts.

Posthodiplostomum sp. 24
Host: *Poecilia reticulata* (Poeciliidae).
Locality: Quebrada de Oro (18.2129, −67.1429), Mayagüez, Puerto Rico.
Site of infection: mesentery.
Voucher specimen: MSB:Para:33296.
BINs: AEF4897, AEF4552.
GenBank: OP071198-205.
Description based on 1 hologenophore (Fig. 2D; Table 4). Body small. Prosoma spatulated and bigger than opisthosoma, separated by marked constriction. Oral sucker terminal and oval. Ventral sucker oval, muscular and slightly larger than oral sucker. Prepharynx not observed. Oval holdfast organ well-developed located close to ventral sucker. Holdfast gland not observed. Testes and ovary in opisthosoma, poorly developed. Copulatory bursa terminal and elongate. In spacious, colourless, thin-walled cysts.

Posthodiplostomum sp. 25
Host: *Dajaus monticola* (Mugilidae).
Localities: Rio Yaguez (18.2051, −67.1299) and Quebrada de Oro (18.2129, −67.1429), Mayagüez, Puerto Rico.
Site of infection: muscle.
Voucher specimens: MSB:Para:33294-5, MSB:Para:33298.
BIN: ADE2986.
GenBank: OP071206-18.
Description based on 12 paragenophores (Fig. 2B; Table 4). Prosoma spatulated, separated by marked constriction from oval or round opisthosoma. Oral sucker small, round, at anterior extremity of prosoma. Prepharynx not observed, pharynx oval, longer than wide, observed in 4 specimens. Ventral sucker usually larger than oral sucker, located in middle of prosoma, well-separated from holdfast organ. Holdfast organ large, slightly

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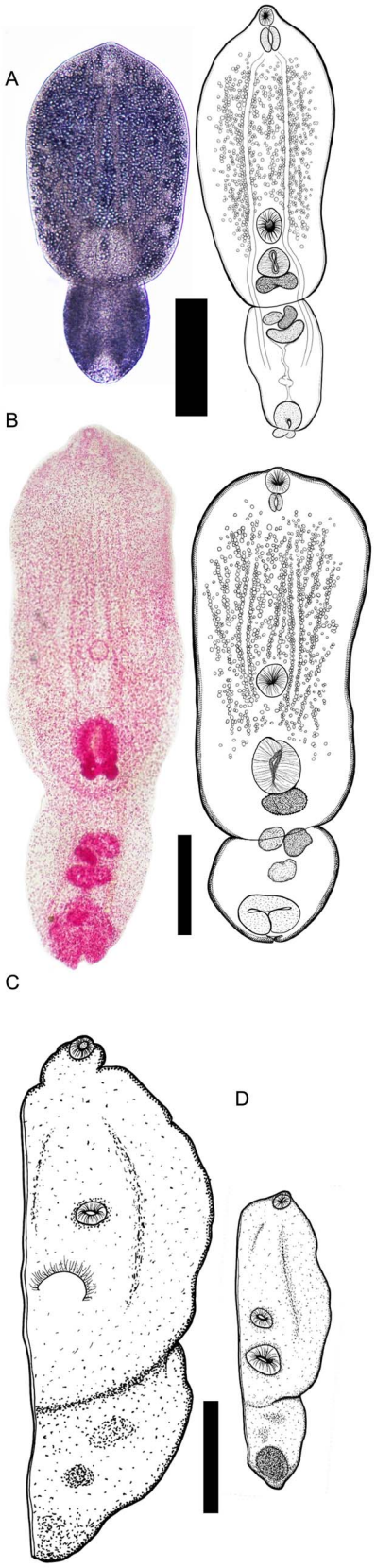

Fig. 2. Line drawings and whole mounts of metacercariae of *Posthodiplostomum* from fish collected in western Puerto Rico. (A) Paragenophores of *Posthodiplostomum macrocotyle* from the muscle of *Parachromis managuensis*. Scale bar: 100 μm. Left specimen is a temporary mount of a live, unstained specimen. (B) Paragenophores of *Posthodiplostomum* sp. 25 from the muscle of *Dajaus monticola*. Left specimen is stained and permanently mounted. (C, D) Hologenophores of metacercariae from body cavity of a single individual *Poecilia reticulata*. Scale bar: 200 μm. (C) *Posthodiplostomum* sp. 23; (D) *Posthodiplostomum* sp. 24.

oval, holdfast glands usually absent. Testis and ovary poorly differentiated, copulatory bursa at posterior of opisthosoma, rounded. In yellowish, semi-opaque, thicker-walled cysts, possibly consisting partly of encapsulating tissue of host origin.

Posthodiplostomum macrocotyle Dubois, 1937
Host: *Parachromis managuensis* (Cichlidae).
Locality: Tres Hermanos National Park (18.2941, −67.2009), Añasco, Puerto Rico.
Site of infection: muscle.
Voucher specimens: MSB:Para:33297, MSB:Para:33299-300.
BIN: AEF4898.
GenBank: OP071172-87.
Description based on 11 paragenophores (Fig. 2A; Table 4). Body small, distinctly bipartite. Prosoma spatulated, opisthosoma oval. Prepharynx not observed. Oral sucker elongated slightly from superior of prosoma, but round. Muscular oval ventral sucker located close to margin anterior to holdfast organ. Holdfast organ smaller, closest to posterior margin of prosoma. Copulatory bursa oval, contractile. In colourless, transparent, thin-walled cysts.

Remarks

The metacercariae of the 7 genetically distinguished species reported herein were assigned to the genus *Posthodiplostomum* based on morphological features such as the bipartite body divided into a foliate prosoma lacking pseudosuckers and a roughly spherical opisthoma, as well as molecular phylogenetic analysis. Morphological characterization of *Posthodiplostomum* spp. 23, 24 was limited to hologenophores (Table 4; Fig. 2) and no morphological characterization of *Posthodiplostomum* sp. 26 was possible. *Posthodiplostomum* spp. 23, 24 and 26 were all from the same host (*Poe. reticulata*), sometimes in mixed infections, making it unclear whether unsequenced specimens could be considered paragenophores of *Posthodiplostomum* sp. 23, 24 or 26. Consistent with the higher species diversity of parasites in *Poe. reticulata*, morphometric heterogeneity was significantly greater in vouchered metacercariae from guppies than in parasites from other hosts, in which only single species of *Posthodiplostomum* were detected in DNA sequences. Despite these limitations, most of the genetically distinguished species can be differentiated from each other based on straightforward characters such as total length. For example, hologenophores of *Posthodiplostomum* spp. 23 and 24 differ markedly in size (Fig. 2; Table 4) and, notably, these 2 metacercariae were collected from the same individual guppy. The metacercaria of *Posthodiplostomum* sp. 23 resembles that of *Posthodiplostomum nanum* from guppies as described by López-Hernández *et al.* (2018), but in *Posthodiplostomum* sp. 23 the oral sucker is larger (49 × 58 μm *vs* maximum of 41 × 41 μm in 20 metacercariae of *Po. nanum*, López-Hernández *et al.*, 2018), and the ventral sucker is further from the tribocytic organ (cf. Fig. 2 herein and Fig. 1D in López-Hernández *et al.*, 2018).

Metacercariae of *Po. macrocotyle* were the smallest worms, overlapping in total length only with *Posthodiplostomum* sp. 24 and smaller individuals of *Posthodiplostomum* sp. 25. These 2 smallest species, *Posthodiplostomum* sp. 24 and *Po. macrocotyle*, can be distinguished by the width of the ventral sucker and the distances between the ventral sucker and the anterior and posterior margins of the prosoma.

Metacercariae of *Posthodiplostomum* sp. 8 and *Po. centrarchi*, the 2 species infecting centrarchid hosts, were generally larger than those of other species encountered. As in Kvach *et al.* (2017), morphological distinctions were not observed between the metacercariae of these 2 species, which can be distinguished genetically and by their tendency to infect either *Lepomis* or *Micropterus* (Locke *et al.*, 2010; Boone *et al.*, 2018).

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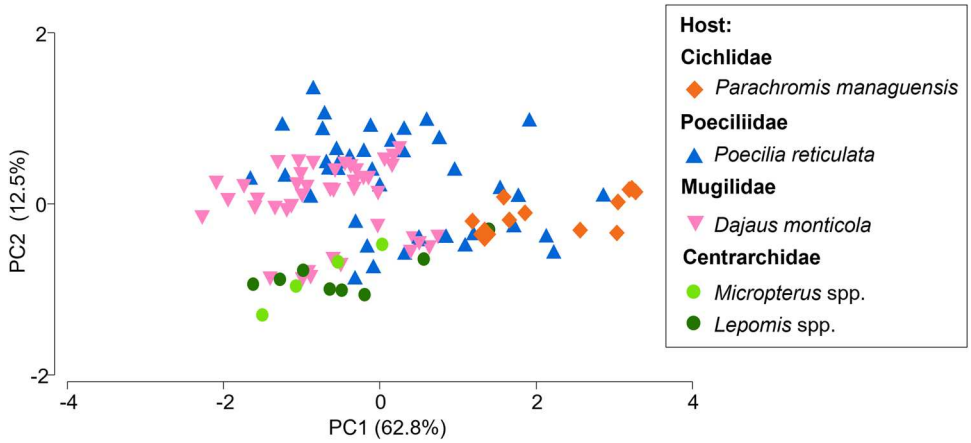

Fig. 3. Principal Component Analysis of morphometric distances among 105 metacercariae of species of *Posthodiplostomum* infecting fishes in Puerto Rico. Axes (PC1, PC2) are labelled with amount of morphometric variation explained and points with host species (see inset legend). Euclidean morphometric distances are based on 16 log-transformed measurements.

Metacercarial paragenophores of *Posthodiplostomum* sp. 25, from the muscle of *D. monticola*, were variable in size, such that some specimens were as small as the smaller species (*Posthodiplostomum* sp. 24 and *Po. macrocotyle*) and some were as large as the larger species from centrarchids. Unlike the other species studied here, metacercariae of *Posthodiplostomum* sp. 25 were usually found in a pale-yellow capsule or cyst, possibly including tissue of host origin.

In *Posthodiplostomum* spp. 8, 24, 25 and *Po. centrarchi*, 69–73% of the total body length is accounted for by the prosoma, while in *Posthodiplostomum* sp. 23 and *Po. macrocotyle*, the prosoma represented 64–66% of the total length. The length of the ventral sucker was 13% of the length of the prosoma in *Posthodiplostomum* sp. 24, while it accounted for 6–8% of the prosoma length in *Po. centrarchi* and *Posthodiplostomum* sp. 23 and 25.

Pérez-Ponce de León *et al.* (2022) recently reported 6 lineages of *Posthodiplostomum* infecting various fishes throughout Central America, but using internal transcribed spacer (ITS) rDNA and a different region of CO1 than sequenced in the present study. Consequently, morphological comparisons between lineages of *Posthodiplostomum* encountered in the same host taxa by Pérez-Ponce de León *et al.* (2022) and in the present study are of particular interest. Based on specimen drawings in Pérez-Ponce de León *et al.* (2022), the 3 lineages that these authors recovered from poeciliids have estimated total lengths of 971 (lineage I), 1045 (lineage II) and 948 (lineage IV) μm. The hologenophore of one of the species recovered from *Poe. reticulata* in Puerto Rico, *Posthodiplostomum* sp. 24, is substantially smaller (Table 4). The oral sucker of the hologenophore of *Posthodiplostomum* sp. 23 (also from *Poe. reticulata*) was larger than those of these lineages (estimated to be 14, 25 and 14 μm in diameter in lineages I, II and IV, respectively). Interestingly, both *Posthodiplostomum* sp. 23 and lineage IV in Pérez-Ponce de León *et al.* (2022) are closely related to *Po. nanum*, and other than the aforementioned difference in the size of the oral sucker, appear morphologically similar. However, CO1 differs substantially in *Posthodiplostomum* sp. 23 and lineage IV, indicating they are different species (see Remarks above on MZ707217, and Table 3).

Pérez-Ponce de León *et al.* (2022) recovered lineage III from cichlids in the amphilophine clade (*Archocentrus nigrofasciata*, *Amatitlania siquia*) collected in the native range of *Pa. managuensis*, also an amphilophine (Concheiro Pérez *et al.*, 2007). However, lineage III appears to be 1725 μm in total length, much larger than any specimens we recovered, particularly the diminutive metacercariae of *Po. macrocotyle* that originated in *Pa. managuensis* introduced in Puerto Rico.

Discussion

Unrecognized species diversity frequently emerges from molecular surveys of diplostomoids (e.g. Blasco-Costa and Locke, 2017) and was therefore expected in this survey of metacercariae in Puerto Rico. Of 7 genetically distinct species of *Posthodiplostomum* detected, 3 are probably new additions to sequence databases and the present records add host and geographic range data for the other 4. The present results suggest that substantial species diversity remains to be discovered in *Posthodiplostomum* in this region, and point to certain host taxa as deserving of further parasitological study.

In phylogenetic analysis, all CO1 sequences fell within a clade composed of species of *Posthodiplostomum*, corroborating morphological identifications at the genus level. Within most of the 7 well-supported species-level clades, CO1 varied by <2%, despite the inclusion of data from geographically distant samples, such as *Po. centrarchi* from Canada and Europe, *Posthodiplostomum* sp. 23 from Georgia, USA and *Po. macrocotyle* from the Pantanal, Brazil. In contrast, interspecific CO1 distances were at least 6.4%, which exceeds the smallest interspecific CO1 distances (5.3%) reported by Achatz *et al.* (2021), in a wide survey in which many species were distinguished with the added support of 28S and morphology from adults. When considered alongside differences in metacercarial morphology, host use and infection sites, and reciprocal monophyly in phylogenetic analysis, this gap in CO1 distances constitutes strong evidence the sympatric lineages we encountered correspond to 7 distinct species.

Molecular data show that 4 of 7 species of *Posthodiplostomum* in Puerto Rico are conspecific with those sequenced in other studies, yielding new information on host spectra and geographic distributions of these parasites. One of these 4 species-level matches was published by Locke *et al.* (2018), i.e. identical CO1 in *Po. centrarchi* from North American mainland samples (Locke *et al.*, 2010; Boone *et al.*, 2018) and from *L. microlophus* in Puerto Rico. The other 3 species-level matches are newly reported here. CO1 from metacercariae from *Poe. reticulata* in Puerto Rico and from another poeciliid, *G. affinis*, in Mississippi matches *Posthodiplostomum* sp. 23, previously known only from *A. herodias* in the southeastern USA (Achatz *et al.*, 2021).

Phylogenetic analysis indicates *Posthodiplostomum* sp. 23 is closely related to *Po. nanum* (this also emerges from analysis of 28S by Achatz *et al.*, 2021). Interestingly, similar to

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Table 4. Comparative morphometrics of metacercariae of *Posthodiplostomum* from the present study: mean (range in parentheses) in μm

Species	*Posthodiplostomum* sp. 23	*Posthodiplostomum* sp. 24	*Posthodiplostomum* sp. 25	*Posthodiplostom macrocotyle*	*Posthodiplostomum* sp. 8	*Posthodiplostomum centrarchi*
Host	*Poecilia reticulata*	*Poe. reticulata*	*Dajaus monticola*	*Parachromis managuensis*	*Micropterus salmoides*, *M. dolomieu*	*Lepomis macrochirus*, *L. gibbosus*
N	1	1	43	11	4	8
Body *L*	895	511	902 (589–1480)	470 (324–673)	1038 (725–1286)	1070 (637–1373)
Prosoma *L*	571	363	624 (400–1032)	312 (218–425)	762 (562–967)	747 (475–965)
Prosoma *W*	445	187	338 (224–528)	183 (121–367)	317 (191–385)	227 (160–390)
Opisthosoma *L*	333	146	280 (169–528)	161 (102–249)	283 (170–431)	350 (165–504)
Opisthosoma *W*	401	122	236 (137–360)	109 (55–275)	260 (205–290)	243 (144–358)
Oral sucker *L*	49	26	32 (20–50)	21 (13–38)	43 (30–56)	40 (27–54)
Oral sucker *W*	58	31	33 (18–48)	20 (12–41)	47 (33–73)	32 (22–40)
Ventral sucker *L*	48	34	50 (28–77)	31 (24–45)	67 (57–77)	49 (27–79)
Ventral sucker *W*	47	48	49 (30–72)	26 (18–43)	52 (44–56)	39 (24–62)
Pharynx *L*	ND	ND	39 (33–42)	16	31	ND
Pharynx *W*	ND	ND	24 (21–28)	11	20	ND
Holdfast organ *L*	124	44	111 (66–232)	46 (32–75)	104 (78–121)	95 (56–127)
Holdfast organ *W*	171	55	87 (54–136)	44 (23–73)	86 (79–89)	81 (51–102)
Copulatory bursa *L*	ND	63	110 (54–180)	50 (29–81)	90 (77–106)	88 (40–144)
Copulatory bursa *W*	ND	37	113 (56–176)	42 (21–95)	81 (69–91)	77 (44–114)
Ventral sucker to holdfast organ *L*	139	48	84 (32–227)	47 (29–75)	146 (59–198)	135 (58–174)
Ventral sucker to superior prosoma *L*	452	105	358 (186–568)	201 (135–288)	512 (404–652)	509 (333–628)
Ventral sucker to posterior prosoma *L*	136	261	435 (97–920)	113 (75–168)	255 (161–330)	238 (142–338)

L, length; *W*, width; ND, no data.

Posthodiplostomum sp. 23, *Po. nanum* is also known from *Poe. reticulata* (López-Hernández *et al.*, 2018) and has also recently been recovered from an ardeid (*A. herodias*) in the southeastern USA (Achatz *et al.*, 2021). Based on sequences of ITS rDNA, Pérez-Ponce de León *et al.* (2022) placed lineage IV, from *P. sphenops* and *Profundulus* spp. from Oaxaca, Mexico in a clade with *Po. nanum* (along with an unidentified species from *Tilapia sparrmanii* from South Africa, Hoogendoorn *et al.*, 2019). However, by comparison with longer sequences of *Posthodiplostomum* sp. 23 (e.g. MZ707217, 1024 bp) of Achatz *et al.* (2021), a species-level match between *Posthodiplostomum* sp. 23 in the present study with lineage IV of Pérez-Ponce de León *et al.* (2022) can be excluded. Taken together, these findings indicate that 3 closely related species of *Posthodiplostomum* share overlapping geographic distributions across the Americas, and similar hosts in their life cycles [*Po. nanum*, *Posthodiplostomum* sp. 23 of Achatz *et al.* (2021) and *Posthodiplostomum* lineage IV of Pérez-Ponce de León *et al.* (2022) all have metacercariae in *Poecilia*, and adults of *Po. nanum* and *Posthodiplostomum* sp. 23 occur in *A. herodias* in North America].

The present results also provide the first molecular links to larval forms of *Po. macrocotyle*, the first account of its metacercarial morphology, and the first report of this species outside South America. Morphology-based records of adults of *Po. macrocotyle* originate from diverse fish-eating birds [*Spheniscus magellanicus* (Sphenisciformes), *B. nigricollis* (Accipitriformes), several ardeids (Pelecaniformes) and the type host *Rynchops niger* (Charadriiformes)] in Cuba, Brazil and Argentina (Dubois, 1937, 1970*a*, 1970*b*; Dubois and Macko, 1972; Travassos *et al.*, 1969; Brandaõ *et al.*, 2013; Drago *et al.*, 2014). The CO1 obtained from metacercariae from the introduced jaguar guapote *Pa. managuensis* in Puerto Rico matches with adults from the accipitrid *B. nigricollis* in the Pantanal, Brazil, which have recently been sequenced by Achatz *et al.* (2021). Previous reports of metacercariae of *Po. macrocotyle* from South American cichlids were questionable. Azevedo *et al.* (2006, 2011) and Mesquita *et al.* (2011) identified *Po. macrocotyle* metacercariae from cichlid (*Geophagus brasiliensis*) and catfish (*Trachelyopterus striatulus*) hosts in Brazil, but provided no support other than citation of Travassos *et al.* (1969), who only reproduced the description of Dubois (1937, 1938) of the adult form, which presents ambiguous morphological relation to the metacercaria. For example, Travassos *et al.* (1969) reported the adult of *Po. macrocotyle* to be 890–1170 μm in total length, much larger than the metacercariae observed herein. Moreover, cysts of *Po. macrocotyle* were only found in muscle in the present study, while these earlier works reported this species from unusual sites such as buccal cavity and stomach (Azevedo *et al.*, 2006; Mesquita *et al.*, 2011). The parasites reported as *Po. macrocotyle* by Azevedo *et al.* (2006, 2011) and Mesquita *et al.* (2011) are therefore suspected to represent a different species.

Cichlids are one of the most species-rich and widespread families of freshwater fish, and are often reported to harbour *Posthodiplostomum* or neascus-type parasites, particularly in the Neotropics (Salgado-Maldonado *et al.*, 2004, 2020; Neves *et al.*, 2013; Tavares-Dias and Oliveira, 2017; Santos *et al.*, 2018; Salgado *et al.*, 2020). Two of 6 molecular lineages (III and VI) of *Posthodiplostomum* sampled by Pérez-Ponce de León *et al.* (2022) across North and Middle America were from cichlids, 1 lineage widely distributed between northeastern Mexico and Costa Rica, and the other recovered only in southeastern Mexico. The only other molecular data from neascus from this host family are those from *Posthodiplostomum* sp. 9 from *T. sparrmanii* from South Africa (Hoogendoorn *et al.*, 2019). Despite these efforts, this is the first report of *Posthodiplostomum* in the jaguar guapote *Pa. managuensis*, one of the largest (Conkel, 1993) and most widely introduced (Holmes *et al.*, 2020) cichlids.

The remaining 2 CO1-based matches with prior work were in species of *Posthodiplostomum* (*Po. centrarchi* and *Posthodiplostomum* sp. 8) from centrarchid fish hosts. *Posthodiplostomum centrarchi* was previously found in *L. microlophus* introduced to the island (Locke *et al.*, 2018), while the present record extends the geographic distribution of *Posthodiplostomum* sp. 8 to the Caribbean. Both these species are known mainly from North America (Locke *et al.*, 2010; Boone *et al.*, 2018; Achatz *et al.*, 2021), but have also been introduced in Europe (Kvach *et al.*, 2017). Based on ITS sequences, Pérez-Ponce de León *et al.* (2022) cast doubt on the separation of *Posthodiplostomum* sp. 8 and *Po. centrarchi*, but we found unambiguous separation of these species in analysis of available CO1 sequences, and their exclusive recovery from different centrarchid hosts in multiple studies provides strong support of their distinct status.

The 3 remaining species detected in the present study (*Posthodiplostomum* spp. 24, 25 and 26) have not been reported previously, at least not using the barcode locus of the CO1 gene. *Posthodiplostomum* sp. 25 from the muscle of *D. monticola* (Mugilidae) is new in terms of not being previously sequenced, as well as a new record for the genus *Posthodiplostomum* in this host. Parasitological surveys of *D. monticola* in Mexico (Salgado-Maldonado *et al.*, 2004, 2020) and Costa Rica (Sandlund *et al.*, 2010) have reported few digeneans. Although *D. monticola* is common and abundant in Puerto Rico (Kwak *et al.*, 2007; Cancel-Villamil and Locke, 2022), and the present results indicate infections with *Posthodiplostomum* sp. 25 are not uncommon, no neascus has been reported previously in this host. The only helminths reported from *D. monticola* in Puerto Rico are cystidicolid nematodes in the genus *Spinitectus* and metacercariae of *Echinochasmus donaldsoni* (Bunkley-Williams and Williams, 1994; Dyer *et al.*, 1998). Compared with the numerous records in the wide spectra of leuciscids and centrarchids (Hoffman, 1999), *Posthodiplostomum* spp. are infrequently reported from a small number of fishes of the Mugilidae (Domitch and Sarabeev, 2000; Özer and Yilmaz Kirca, 2015; Sarabeev *et al.*, 2017).

The 2 remaining species of *Posthodiplostomum* newly sequenced herein (spp. 24 and 26) were both from the guppy *Poe. reticulata*, a fish native to Trinidad and Venezuela that has been introduced widely (Deacon *et al.*, 2011). The poeciliids are a species-rich group of Neotropical origin, but molecular work on their parasites has just begun (López-Hernández *et al.*, 2018; Pérez-Ponce de León *et al.*, 2022). Metacercariae of *Posthodiplostomum* were recorded in 11% of 270 *Poe. reticulata* sampled in its native range in Trinidad (Mohammed *et al.*, 2020), and *Po. nanum* has been recorded in *Poe. reticulata* in Brazil (López-Hernández *et al.*, 2018). Hoffman (1999) compiled records of metacercariae of *Posthodiplostomum* (as *Po. minimum*) in *G. affinis* in Tennessee and in *Poe. mexicana* in Mexico. Four lineages of *Posthodiplostomum* were recorded from poeciliids (though not *Poe. reticulata*) and other cyprinodontiform fishes by Pérez-Ponce de León (2022) in Central America.

The present results add to an evolving picture of metacercarial host-specificity in *Posthodiplostomum*. In the more intensively sequenced species of *Posthodiplostomum*, patterns of host specificity emerging from early surveys (Locke *et al.*, 2010) have generally been maintained. These patterns are consistent with subspecies Hoffman (1958) created based largely on the compatibility of cercariae with different fishes. In molecular surveys, metacercariae of *Po. minimum* have been recovered only from leuciscid hosts (Locke *et al.*, 2010; Komatsu *et al.*, 2020; Achatz *et al.*, 2021), and those of *Po. centrarchi* nearly exclusively from *Lepomis* spp., which mirrors the specificity of cercariae in Hoffman's (1958) experiments. Notably, except for 7 specimens from 3 fish that Boone *et al.* (2018) suspected to be *M. salmoides* × *Micropterus punctulatus* hybrids, *Po. centrarchi* has not

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been recovered from species in the centrarchid genus *Micropterus* in modern sequencing surveys, and cercariae of *Po. centrarchi* were also incompatible with *M. salmoides* in Hoffman's (1958) experimental infections. In other words, what Hoffman (1958) and later authors have called the 'centrarchid line' does not infect all centrarchids. Instead, in molecular surveys, members of *Micropterus* commonly harbour *Posthodiplostomum* sp. 8. All of these nearly exclusive associations have remained consistent in sequencing surveys, despite the novel hosts that cercariae of *Po. minimum*, *Posthodiplostomum* sp. 8 and *Po. centrarchi* encounter in diverse and widespread habitats (present study; Locke *et al.*, 2010, 2018; Kvach *et al.*, 2017; Stoyanov *et al.*, 2017; Boone *et al.*, 2018; Stoyanov *et al.*, 2018; Cech *et al.*, 2020; Komatsu *et al.*, 2020). Our data from Caribbean hosts and parasites not previously surveyed are thus consistent with both these particular patterns and the host specificity that generally prevails among this group of parasites. It is relevant to note that other than what is reported herein, no similar neascus have been observed in hundreds of additional fish, including all native species to the island and over a dozen of introduced species (Locke SA, unpublished data).

The present results thus suggest a narrow second-intermediate host use in 7 lineages of *Posthodiplostomum* in Puerto Rico that is consistent with most molecular surveys to date. In contrast, Pérez-Ponce de León *et al.* (2022) recently reported 6 lineages of *Posthodiplostomum*, 4 infecting multiple families of fish throughout Central America. Unfortunately, a direct comparison of sequences with those of Pérez-Ponce de León *et al.* (2022) is impossible. One or more of the species encountered in Puerto Rico may be conspecific with lineages I–VI from Central America of Pérez-Ponce de León *et al.* (2022), particularly *Po. macrocotyle*, and *Posthodiplostomum* spp. 24, 25 and 26, some of which were found in host families (cichlids and poeciliids) also sampled by Pérez-Ponce de León *et al.* (2022). While morphological comparisons do not support conspecificity, the differences observed (see Remarks) may be to some extent related to geographic (Stoyanov *et al.*, 2017) and host-associated (Palmieri, 1975, 1977*a*, 1977*b*, 1977*c*) morphological variation within species. If the same species of *Posthodiplostomum* have indeed been studied and sequenced in our study and throughout Central America by Pérez-Ponce de León *et al.* (2022), then differences in host range in the 2 studies could be attributed to the comparatively depauperate fish fauna of Puerto Rico. For example, Pérez-Ponce de León *et al.* (2022) reported 3 lineages (I, II and IV) from families of fish [Leuciscidae (as Cyprinidae), Goodeidae, Profundulidae, Fundulidae] that are absent or uncommon in Puerto Rico, as well as in various poeciliids, which are represented in our collections by *Poe. reticulata*. In the present study, lineages I, II or IV of Pérez-Ponce de León *et al.* (2022) may have been restricted to a locally available poeciliid host (*Poe. reticulata*) because of the absence of other compatible host taxa on the island.

However, we consider that Pérez-Ponce de León *et al.* (2022) probably underestimated the number of species represented in their sequence data, and that other interpretations of host specificity in their samples are possible. For example, levels of CO1 divergence within lineages III (8%) and IV (6%) reported by Pérez-Ponce de León *et al.* (2022) exceed those known within species of *Posthodiplostomum* [maxima of intraspecific CO1 divergence of 2.26% (Locke *et al.*, 2010), 1.7% (Stoyanov *et al.*, 2017), 3.91% (Boone *et al.*, 2018) and 3.8% (Achatz *et al.*, 2021)] and other digeneans (e.g. the suggested 5% cutoff of Vilas *et al.*, 2005), including *Diplostomum* species in which hundreds of individuals were sequenced with geographically widespread sampling (maximum intraspecific CO1 K2P = 2.66% in Locke *et al.*, 2015). Pérez-Ponce de León *et al.* (2022) also delineated most species based on ITS with incomplete support from CO1. For example, while the ITS sequences of lineage I were obtained from parasites from multiple fish families, CO1 was only available from a subset from poeciliids, meaning that the wide host range of lineage I is effectively based on ITS alone. This is problematic because ITS can vary little or not at all between recently separated species (Vilas *et al.*, 2005; Locke *et al.*, 2015; Cribb *et al.*, 2022).

To some extent, the contrasting host-use patterns emerging from this study and that of Pérez-Ponce de León *et al.* (2022) will be clarified by determining which species of *Posthodiplostomum* are co-distributed in fish in both Central America and Puerto Rico (most pragmatically achievable by sequencing homologous CO1). The available data show that 3 of 7 species we found in Puerto Rico (*Po. centrarchi*, *Posthodiplostomum* spp. 8 and 23) were not encountered by Pérez-Ponce de León *et al.* (2022). However, regardless if species co-occur in both regions, we believe it to be unlikely that the host specificity of metacercariae of *Posthodiplostomum* differs fundamentally in the Caribbean and Middle America, and for this reason, we believe that 2 types of additional data are necessary to reconcile our results with those of Pérez-Ponce de León *et al.* (2022). First, as most of the species of *Posthodiplostomum* encountered herein are only known from a limited study area, CO1-based records from additional samples in novel environments will allow assessment of metacercarial host specificity against the background of a different or richer host fauna. Second, we contend that the multi-family host range of several lineages in Pérez-Ponce de León *et al.* (2022) remains to be verified, for example through sequencing CO1 in isolates of lineage I from non-poeciliid hosts. In addition, care is needed in interpreting host ranges of lineages in Pérez-Ponce de León *et al.* (2022) due to conflicts between the paper and GenBank records. For example, OK315743 and OK314903 appear to be sequences from the same individual worm (see Table 1 in Pérez-Ponce de León *et al.*, 2022), but these GenBank records list different hosts (*Poecilia latipunctata* and *Herichthys labridens*) and lineages (I and III).

Our prediction is that subsequent studies will reveal most species of *Posthodiplostomum* to have metacercariae restricted to a narrow range of hosts (e.g. a single family). This expectation is based on molecular surveys of metacercariae of *Posthodiplostomum*, which have revealed 3 generalists and 11 specialists. The generalists comprise *Posthodiplostomum pricei*, with CO1-based records of metacercariae in Fundulidae, Centrarchidae and Moronidae (Blasco-Costa and Locke, 2017); *Posthodiplostomum* cf. *podicipitis*, with CO1-based records in Leuciscidae and Catostomidae (Achatz *et al.*, 2021) and *Posthodiplostomum* lineage II, with CO1-based records in Leuciscidae and Goodeidae (Pérez-Ponce de León *et al.*, 2022). In contrast, the CO1-based species delimitations of the following 11 species reveal single-family host ranges: *Po. centrarchi*, *Po. minimum*, *Posthodiplostomum* cf. *anterovarium* and *Posthodiplostomum* sp. 8 (sampled in multiple contexts in the present study, Locke *et al.*, 2010, 2018; Kvach *et al.*, 2017; Stoyanov *et al.*, 2017; Boone *et al.*, 2018; Stoyanov *et al.*, 2018; Cech *et al.*, 2020; Komatsu *et al.*, 2020; Achatz *et al.*, 2021), and 7 species of *Posthodiplostomum* sampled in the St. Lawrence River [*Posthodiplostomum* spp. 5 and 7 in Locke *et al.* (2010) and *Posthodiplostomum* spp. 10–14 of Achatz *et al.* (2021) (=*Ornithodiplostomum* spp. 1–3, 4, 8 of Locke *et al.* (2010)].

The modern world provides species of *Posthodiplostomum* with many opportunities to colonize newly encountered host taxa, by reshuffling host assemblages through changing climate (Marcogliese, 2001) and species introductions (Rahel, 2010). In Puerto Rico, the accidental or intentional introduction of non-

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native fish species has occurred since the early 1900s (Neal *et al.*, 2004, cited in Neal *et al.*, 2009). Forty-six freshwater fishes have been reported in the island, of which only 9 species are native (Rodríguez-Barreras *et al.*, 2020). Introduction of new host species can cause a decrease in abundance of native species through co-introduction of pathogens (reviewed by Daszak *et al.*, 2000 and Lafferty *et al.*, 2005), although this has not been studied in Puerto Rico freshwater fish assemblages. Bunkley-Williams and Williams (1994) encountered varying infection levels of *Posthodiplostomum*-type neascus from freshwater fishes introduced to Puerto Rico, but found none in native fishes. These authors reported intensities of 1–2 metacercariae per host of *Po. centrarchi* (as *Po. minimum*) in *Lepomis* spp., of which 9 of 10 were infected. Only 1 of 33 *Micropterus* sp. was infected (presumably with *Posthodiplostomum* sp. 8). Few (2/8) guppies were infected, but intensity was high (8–10 metacercariae per host). In the present study, a similar infection intensity was observed in guppies, and overall infection levels were higher in introduced hosts than in the native *D. monticola*. No evidence was seen for 'spill over' of *Posthodiplostomum* between introduced and native fish hosts, nor for that matter were species of *Posthodiplostomum* shared among any host species. This stands in contrast to the recent discovery of a digenean native to the Indomalayan region, *Transversotrema patialense*, in introduced snails and both native and introduced fishes in the Quebrada de Oro, i.e. the same small stream studied here (Perales Macedo *et al.*, 2022).

Attempts to identify the first-intermediate hosts of any of the *Posthodiplostomum* species found in fish were unsuccessful, although the presence of metacercariae provides unequivocal evidence that the life cycle is completed locally. In 2722 individual snails, no diplostomid infections were observed in the single native gastropod species (Neritidae gen. sp.) nor the other species, all of which are introduced in Puerto Rico. Multiple species of *Physa*, such as *Ph. anatina*, *Ph. gyrina*, *Ph. fontinalis* and *Ph. integra,* have been reported to be suitable hosts for cercariae of *Posthodiplostomum* and *Ornithodiplostomum* species (Hoffman, 1958; Sankurathri and Holmes, 1976; Radabaugh, 1980; Hendrickson, 1986). Among the snails collected, *Physa acuta* is therefore the most likely first-intermediate host of the *Posthodiplostomum* species observed in *Poe. reticulata* and *D. monticola*, in view of prior records of *Posthodiplostomum* and similar neascus-forming species in members of the Hygrophila such as Physidae and Planorbidae (Hoffman, 1960; Ostrowski de Núñez, 1973; Velázquez-Urrieta and Pérez-Ponce de León, 2021). The absence of infections in 747 *Ph. acuta* collected from the same localities as the infected fish is consistent with reported prevalences, which are particularly low in non-native populations of *Ph. acuta* (Ebbs *et al.*, 2018). Diplostomid infections were also absent from 1763 thiarids belonging to 2 species, and a small number of *M. cornuarietis* (Ampullariidae), which is unsurprising given the absence of prior reports of *Posthodiplostomum* in these snail families. However, the recent expansion of the genus *Posthodiplostomum* by Achatz *et al.* (2021), along with the high number of species known only genetically, or in which snail hosts are unknown, suggests that new host–parasite combinations in *Posthodiplostomum* remain to be discovered, particularly for snails. Diplostomoids have been recorded from the thiarid species we sampled in their native range in Southeast Asia (Krailas *et al.*, 2014; Veeravechsukij *et al.*, 2018), but not in introduced populations. Nasir *et al.* (1969) recorded a longifurcocercous cercariae in *M. cornuarietis* in its native range (Venezuela). However, the furcocercous cercariae recorded by Krailas *et al.* (2014), Veeravechsukij *et al.* (2018) and Nasir *et al.* (1969) all possess well-developed ventral suckers and other characters that distinguish them from cercariae known from *Posthodiplostomum* (Blair, 1977; Hendrickson, 1986; Ritossa *et al.*, 2013; López-Hernández *et al.*, 2018).

Compared with previous records based on morphology (Bunkley-Williams and Williams, 1994), the present molecular results constitute a substantial increase in species diversity, particularly given that our sampling effort was not especially intense: 58 infected guppies, 54 mountain mullet, 14 jaguar guapote and 6 from *Micropterus* spp. Clearly, this indicates that further studies, particularly with greater host species coverage, are likely to reveal additional species of *Posthodiplostomum* in the Caribbean. Remarkably, most species of *Posthodiplostomum* recovered herein were from non-native hosts, which typically harbour fewer species of parasites than native hosts (Torchin *et al.*, 2003). Taken together with the findings of Pérez-Ponce de León *et al.* (2022), these results suggest a particularly fruitful area of further molecular prospecting in *Posthodiplostomum* will be in poeciliids and cichlids in their native ranges.

To our knowledge, this is the first report of *Posthodiplostomum* infecting *Pa. managuensis* and *D. monticola*. These results also suggest that non-native hosts in Puerto Rico (*Pa. managuensis*, *Poe. reticulata*, *L. macrochirus*, *L. gibbosus, M. salmoides*, *M. dolomieu*) have co-introduced diplostomid parasites into the local environment, or facilitated their establishment, although no evidence was observed for *Posthodiplostomum* species shared among native and introduced freshwater fishes on the island. The relatively small geographical area surveyed demonstrates that the diversity of this group of parasites in Puerto Rico and the Caribbean is potentially much higher.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182022001214.

Data availability. The newly generated sequences are deposited in the GenBank database under the accession numbers OP071160-225. Morphological vouchers of parasites and of host tissues were deposited in the Museum of Southwestern Biology (MSB:Para:33294-301, MSB:Host:24820-1).

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Author contributions. S. A. L. and S. C. D. P. conceived, designed and conducted the study. S. C. D. P. performed statistical and phylogenetic analyses. S. A. L., S. C. D. P. and S. V. B. wrote and revised the article.

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Conflict of interest. The authors declare there are no conflicts of interest.

Ethical standards. This study was conducted with approval of the UPRM IACUC (OLAW assurance D20-01098).

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