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Genomic characterization of a new species of *Pseudoparacreptotrema* (Digenea: Allocreadiidae) from Puerto Rico, with comments on the biogeography of the genus

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Research Paper

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Abstract

In this study, we describe a new species of *Pseudoparacreptotrema* (Allocreadiidae) from the mugilid *Dajaus monticola* collected in western Puerto Rico, where no allocreadiid has previously been reported, bringing the number of species in this genus to seven (five in *D. monticola*, two in *Profundulus* spp.). The new parasite species is distinguished from congeners by its overall size, oral-to-ventral sucker size ratio, pharynx size, cirrus sac, and oral lobe morphology, and by 0.64%–3.45% divergence in a 1019-bp alignment of 28S. We build on prior suggestions that the current concept of *P. agonostomi* likely includes multiple species and provide the first mitochondrial data (whole mitochondrial genome) as well as the complete nuclear rDNA array from *Pseudoparacreptotrema* to facilitate future phylogenetic work. Within the Allocreadiidae, phylogenetic analysis of mitochondrial genomes and 28S provides conflicting topologies for the placement of *Pseudoparacreptotrema* and *Allocreadium*. The 28S phylogeny of six species of *Pseudoparacreptotrema* resembles that of four lineages of *D. monticola* in that in both host and parasite, Pacific coastal lineages branch earliest, and a Caribbean lineage is more recently evolved.

Introduction

The Allocreadiidae Looss, 1902 are primarily found in the Nearctic, Neotropical, and Palearctic realms (Caira & Bogéa, 2005). Members of this family have freshwater life cycles with bivalves as first intermediate hosts, crustaceans and aquatic insects as second intermediate hosts, and teleosts, reptiles, and amphibians as final hosts (Caira & Bogéa, 2005).

Pseudoparacreptotrema was erected for two species found in *Profundulus* spp. (Pérez-Ponce de León et al., 2016) and later expanded to accommodate three species in *Dajaus monticola* (Pérez-Ponce de León et al., 2020). *Pseudoparacreptotrema* is distinguished from other allocreadiids by symmetrical or oblique testes (vs. tandem), a large oral sucker (relative to ventral sucker), the extent of the vitelline fields, and the genus is also the only allocreadiid known from profundulids and *D. monticola* (Pérez-Ponce de León et al., 2016; 2020; Pinacho-Pinacho et al., 2015). Among allocreadiids, *Pseudoparacreptotrema* is unusual in that muscular lobes adorn the oral sucker in some species but are absent in others; in other allocreadiid genera, all members either possess or lack these structures. The six species of *Pseudoparacreptotrema* (*P. profundulusi*, *P. macroacetabula*, *P. pacificum*, *P. axtlaensis*, *P. falciformis*, *P. agonostomi*) have only been recorded in Middle America (Salgado-Maldonado et al., 1998; Pérez-Ponce de León et al., 2016, 2020). Life cycles are unknown, but larval stages of *Pseudoparacreptotrema* spp. probably infect bivalves and arthropods, as in other allocreadiids maturing in fish (e.g., *Bunodera* spp., *Allocreadium* spp., among others, Petkevičiūtė et al., 2010; 2023; Caira & Bogéa, 2005).

As the type and only known host of four of the six species of *Pseudoparacreptotrema*, the mountain mullet, *D. monticola*, likely plays a significant role in the diversity and distribution of these parasites and is also the focal host of the present study. *Dajaus monticola* is an omnivorous, amphidromous species that spends most of its life in high-gradient rivers (Matamoros et al., 2009; Smith & Kwak, 2014). The range of *D. monticola* encompasses rivers draining into the Pacific and Atlantic oceans across Middle America, and adjacent coasts of North and South America, as well as rivers of larger Caribbean islands (Kubicek et al., 2019). Four phylogenetically distinct lineages of *D. monticola* occur: a Gulf Coast clade, a Caribbean clade, and two clades with partly overlapping distributions along the Pacific coast (McMahan et al., 2013). In Puerto Rico, where the present study takes place, *D. monticola* is among the most abundant freshwater fishes (Cancel-Villamil & Locke, 2022) and six helminths, but no allocreadiids, have been reported from this host on the island (*Ancyrocephalus* sp., *Echinochasmus donaldsoni*, *Spinitectus*

agonostomi, *Posthodiplostomum* sp. 25, and *Dulcitransversotrema patialense*) (Bunkley-Williams & Williams, 1994; Dyer et al., 1998; Díaz-Pernett et al., 2022; Perales-Macedo et al., 2022).

To date, most of the extensive molecular work on the Allocreadiidae has employed nuclear ribosomal markers, particularly 28S (e.g., Petkevičiūtė et al., 2010; Atopkin et al., 2018, 2020; Pérez-Ponce de León et al., 2016, 2020; Mendoza-Garfias et al., 2022). A recent analysis of whole mitochondrial genomes of three allocreadiids by Solórzano-García et al. (2024) provided valuable new resources for phylogenetic analysis in this group. These authors found that the mitochondrial genome of *Allocreadium* was the earliest diverging lineage in a clade also containing *Creptotrematina* and *Wallinia*, which is the same branching order seen in 28S phylogenies (Atopkin et al., 2020; Pérez-Ponce de León et al., 2016, 2020; Mendoza-Garfias et al., 2022). Here, we build on this work with the first whole mitochondrial genome sequence from *Pseudoparacreptotrema*. If recent 28S phylogenies continue to be predictive of mt genome phylogenies, then the mt genome of *Allocreadium* should remain the earliest diverging member of a clade containing *Pseudoparacreptotrema*, *Creptotrematina*, and *Wallinia*, with the latter two genera paired as more recent and closely related lineages.

Materials and methods

Sample collection

Dajaus monticola were caught using a backpack electrofisher (Halltech Aquatic Research Inc., Guelph, ON, Canada) from the Quebrada de Oro, Río Culebrinas and Río Yagüez in the Mayagüez district of western Puerto Rico. Fish were placed in aquaria until euthanasia by immersion in a solution of water and clove oil (Underwood & Anthony, 2020). Fish stomachs and intestines were extracted and screened for parasites under a dissection microscope. Adult worms, some of which were heat-killed in hot water, were placed in 95% ethanol and stored at –20 °C until morphological and molecular analysis.

Morphological analysis

Specimens were gradually rehydrated and stained in alcohol-free water with dilute acetocarmine for several minutes. Specimens were then dehydrated to pure ethanol, cleared in clove oil, and mounted on slides with Permount (Fisher Scientific, Atlanta, Georgia, USA). Mounted specimens were measured using a Nikon Eclipse TS100 and NIS-Elements Microscope Imaging Software, version 4.51 (Nikon Metrology Inc., Texas, USA). Line drawings were made with PENUP (version 3.9.13.37, Samsung, San Jose, California, USA) based on the images produced with NIS-Elements.

Molecular analysis

DNA was extracted from three whole worms from two *D. monticola*, one from Quebrada de Oro and one from Río Culebrinas, using the manufacturer's instructions for the NucleoSpin Tissue XS kit (Macherey-Nagel, Allentown, Pennsylvania, USA). In two specimens, the partial ribosomal 28S region was amplified using primers *LSU5* (5'-TAGGTCGACCCGCTGAAYTTAAGCA-3') (Littlewood, 1994) and *1500R* (5'-GCTATCCTGAGGGAAACTTCG-3') (Snyder & Tkach, 2001) with 30 cycles of the following conditions: 95 °C for 30 s, 56 °C for 45 s, followed by an extension at 68 °C for 1 minute, followed by a final incubation period at 68 °C for 5 minutes. The reaction mixtures (25 µL) comprised 3 µL of template DNA, 8.5 µL of H_2O, 0.5 µL of each primer, and 12.5 µL of Taq 2X Master Mix (New England Biolabs, Ipswich, Massachusetts, USA). Amplicons were visualized through electrophoresis on a 1% agarose gel stained with Midori Green Advance (Nippon Genetics Europe GmgH, Düren, Germany) in 1.0× TBE. Amplicons were purified and subjected to Sanger sequencing at Azenta (New Jersey, USA). In one specimen, DNA was shotgun-sequenced using an Illumina Hi Seq 4000 at Azenta, in a tenth of a lane. Nextera adapters were used for the construction of 150-bp paired-end reads.

Phylogenetic analysis

The two sequences of partial 28S generated using Sanger sequencing, along with 28S from the rDNA operon (see the following section), were compared with sequences obtained from allocreadiids on GenBank in an alignment generated using MUSCLE (Edgar, 2004) implemented in Geneious Prime (Biomatters Inc, Auckland, NZ). Phylogenetic trees were constructed using Maximum Likelihood (ML; RAxML, Stamatakis, 2014) and Bayesian Inference (BI; MrBayes, Huelsenbeck & Ronquist, 2001) using Geneious Prime using the GTR+G+I nucleotide substitution model, which was selected based on the Bayesian Information Criterion reported in MEGA 11 (Tamura et al., 2021). The ML tree included 1000 bootstrap replicates, and the BI tree was based on two Markov chain Monte Carlo simulations of 1,100,000 generations with a sampling frequency of every 200 generations, four heated chains with a heated parameter value of 0.2, and a 100,000 burn-in length. In BI analysis of 28S, *Dicrocoelium dendriticum* was set as outgroup; in BI analysis of mt genomes, *Fasciola hepatica* was set as outgroup.

Illumina reads from a specimen of *Pseudoparacreptotrema* were trimmed with BBDuk and mapped with BBmap (Bushnell, 2014) to the mitochondrial genome of *Allocreadium lobatum* (OR987847) (Solórzano-García et al., 2024) with high sensitivity. Portions of the resulting consensus with good coverage were extended in separate Geneious map-to-reference assemblies until the entire molecule was assembled with deep and even read coverage. Annotations to the final assembly initially made with MITOS2 (Bernt et al., 2013) were adjusted based on alignment with allocreadiid mt genomes of Solórzano-García et al. (2024). Both nucleotide and translated amino acid sequences of the new mt genome were aligned with other mt genomes of digeneans available in GenBank and sites with gaps were eliminated. Substitution models and phylogenetic reconstructions were performed using the same methods used for the 28S alignment. The rDNA operon was assembled from the BBDuk-trimmed Illumina reads using a similar approach to that taken with the mitochondrial genome, beginning with an iterative extension of the 18S sequence from *Allocreadium neotenicum* (JX983204).

Results

Description

Taxonomic summary

Phylum: Platyhelminthes Claus, 1887
Class: Trematoda Rudolphi, 1808
Subclass: Digenea Carus, 1863
Family: Allocreadiidae Looss, 1902
Genus: *Pseudoparacreptotrema* Pérez-Ponce de León, Pinacho-Pinacho, Mendoza-Garfias, Choudhury & García-Varela, 2016
Pseudoparacreptotrema yaguezani n. sp.

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Type host: *Dajaus monticola* (Bancroft), mountain mullet (Perciformes: Mugilidae).

Site of infection: intestine and stomach.

Type locality: Quebrada de Oro (18.214, -67.141), Mayagüez, Puerto Rico.

Other localities: Río Yagüez (18.208, -67.122), Mayagüez; Río Culebrinas (18.3931, -67.1511), Aguada, Puerto Rico.

Etymology: The specific epithet is taken from the history of the Mayagüez district, which includes the localities where the new species was collected.

Specimens are deposited in the Museum of Southwestern Biology: Holotype: MSB: 50062; paratypes MSB: 50063-5 (paragenophores and syngenophores of DNA sequences).

DNA sequence GenBank accessions: PP545471-PP545472 (partial 28S), PP548224 (rDNA operon), PP577106 (mitochondrial genome).

The name *Pseudoparacreptotrema yaguezani* n. sp. has been registered in ZooBank with the Life Science Identifier of urn:lsid:zoobank.org:act:5B303422-1309-4887-8DB0-C45F84B31DB1

Diagnosis

Body of adult large, oval in shape, widest at midbody, with bluntly rounded anterior and slightly tapered posterior extremity (Table 1, Fig. 1). Body tegument aspinose, smooth, thick. Oral sucker adorned with pair of small lanceolate lobes. Oral sucker smaller than ventral sucker. Eyespots between pharynx and anterior margin of oral sucker. Pharynx large, muscular. Esophagus absent. Caeca bifurcating posterior to pharynx. Genital pore posterior to caecal bifurcation. Cirrus sac large, dextrolateral to ventral sucker. S-shaped seminal vesicle inside cirrus sac. Ventral sucker large,

Table 1. Morphometric comparison of species of *Pseudoparacreptotrema* reported from *Dajaus monticola* and *Ictalurus balsanus* (range followed by mean in parenthesis, in μm)

Source	Present study	Salgado-Maldonado et al. 1998	Salgado-Maldonado et al. 1998	Salgado-Maldonado et al. 1998	Pérez-Ponce de León et al. 2020	Pérez-Ponce de León et al. 2020
Locality	PR	Río Cuitzmala, Jalisco, MX	Río La Palma, Veracruz, MX	Río Balsas, Guerrero, MX	Pacific Slope, Middle America	Río Axtla, San Luis Potosí, MX
Parasite	*Pseudoparacreptotrema yaguezani* n. sp.	*P. agonostomi ss**	*Pseudoparacreptotrema. sp.**	*Pseudoparacreptotrema* sp.*	*P. pacificum*	*P. axtlaensis*
Host	*Dajaus monticola*	*D. monticola*	*D. monticola*	*Ictalurus balsanus*	*D. monticola*	*D. monticola*
N. measured	8	25	27	8	26	25
BL	723–1605 (1009)	507–1464 (910)	592–1474 (956)	800–1937 (1310)	479–1052 (755)	492–1005 (645)
BW	285–701 (421)	213–595 (370)	265–777 (480)	312–662 (526)	177–564 (333)	197–359 (244)
OSL	72–175 (111)	57–145 (102)	88–153 (126)	99–168 (145)	74–130 (100)	60–117 (85)
OSW	81–214 (133)	63–182 (122)	104–200 (146)	120–207 (162)	71–138 (103)	86–146 (109)
VSL	203–407 (264)	135–327 (203)	130–293 (222)	186–336 (275)	118–248 (181)	137–231 (165)
VSW	155–360 (221)	129–283 (197)	153–299 (210)	162–333 (261)	115–259 (172)	112–218 (141)
VSL/OSL	2.3–2.8 (2.4)	1.3–2.6 (2.0)	1.2–2.2 (1.8)	1.6–2.1 (1.9)	1.3–2.3 (1.8)	1.6–2.3 (1.9)
VSW/OSW	1.7–1.9 (1.7)	1.3–2.3 (1.6)	1.0–1.7 (1.4)	1.3–1.8 (1.6)	1.15–2.13 (1.7)	1.08–1.55 (1.3)
PhL	49–106 (72)	15–59 (35)	28–83 (51)	39–69 (56)	31–74 (47)	33–44 (38)
PhW	49–92 (70)	21–67 (48)	39–98 (60)	57–81 (65)	32–70 (51)	31–46 (39)
CSL	88–540 (279)	176 – 390 (272)	246–384 (306)	225–402 (330)	110–256 (177)	130–230 (161)
CSW	58–135 (99)	57–130 (88)	84–165 (125)	87–150 (124)	52–124 (81)	57–95 (74)
LTL	128–314 (184)	81–234 (155)	93–395 (201)	135–330 (208)	92–189 (140)	85–178 (118)
LTW	65–211 (115)	69–161 (102)	80–252 (148)	75–192 (145)	61–159 (109)	64–104 (84)
RTL	117–300 (201)	75–267 (160)	114–338 (212)	126–300 (237)	92–184 (135)	89–170 (127)
RTW	59–218 (110)	54–195 (112)	70–234 (143)	84–189 (146)	53–156 (104)	64–110 (83)
OvL	62–132 (90)	45–137 (94)	65–192 (127)	96–165 (141)	55–116 (85)	56–121 (83)
OvW	60–156 (82)	51–179 (91)	78–200 (128)	105–231 (145)	60–123 (88)	49–113 (76)
Egg L	38–60 (47)	41–52 (46)	46–52 (49)	52–57 (54)	38–61 (52)	36–43 (40)
Egg W	22–41 (31)	23–31 (27)	23–33 (26)	20–31 (26)	28–39 (34)	23–32 (27)
Oral lobe	Present, well-developed	Present	Present	Present	Absent	Present, well-developed

Abbreviations: B, body; CS, cirrus sac; L, length; LT, left testes; MX, México; OS, oral sucker; Ov, ovary; Ph, pharynx; PR, Puerto Rico; RT, right testes; VS, ventral sucker; W, width.*Originally as *Creptotrema agonostomi* (Salgado-Maldonado et al., 1998).

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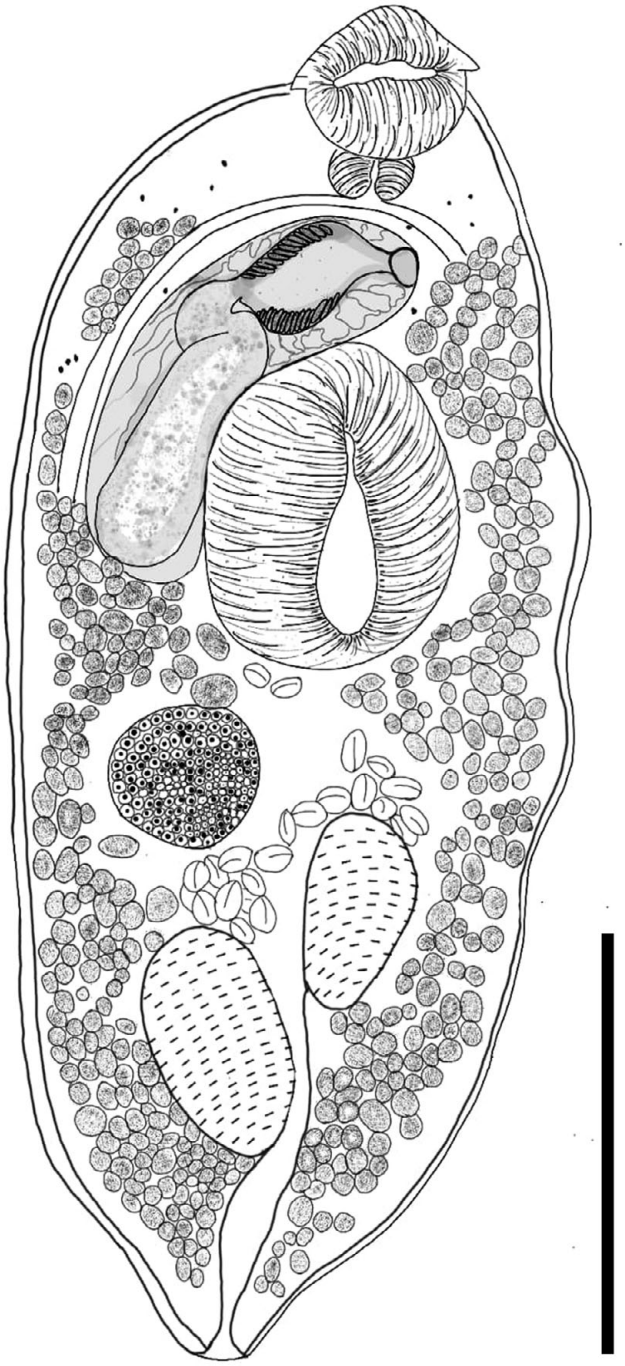

Figure 1. *Pseudoparacreptotrema yaguezani* n. sp. from *Dajaus monticola* in rivers of western Puerto Rico. Composite line drawing, scale 500 μm.

aspinose, near midbody, with narrow longitudinal opening. Relatively small ovary in midbody. Testes two, oblique, slightly tapered. Left testes below ventral sucker, slightly smaller. Right testes larger, posterior to ovary. Eggs small, oval in shape, numerous (>10), scattered in small uterus. Vitellarium in marginal fields extending from intestinal bifurcation to posterior extremity of body, not confluent. Excretory pore small, in ventral posterior extremity of body.

Phylogenetic analysis: partial 28S

Three identical sequences of 28S from *P. yaguezani* n. sp. formed a monophyletic clade within the genus *Pseudoparacreptotrema* (Fig. 2). The monophyly of this genus, the six species within it, and the relationships among these species all had strong nodal support. Species of *Pseudoparacreptotrema* with muscular lobes on the oral sucker formed a monophyletic, derived clade, nested among species lacking such lobes. The 28S sequence of *P. yaguezani* n. sp. differed from that of *P. axtlaensis* by 9–10 bp in a 1,115-bp alignment (0.64%) (Table 2). Interspecific p-distance in 28S between *P. yaguezani* n. sp. and other *Pseudoparacreptotrema* species (excluding *P. axtlaensis*) was mean 2.41 (range 1.37–3.45)%.

Among the four allocreadiid genera from which mitochondrial genomes were analyzed (see the following section), the 28S phylogeny revealed *Allocreadium* to be the earliest branching, followed

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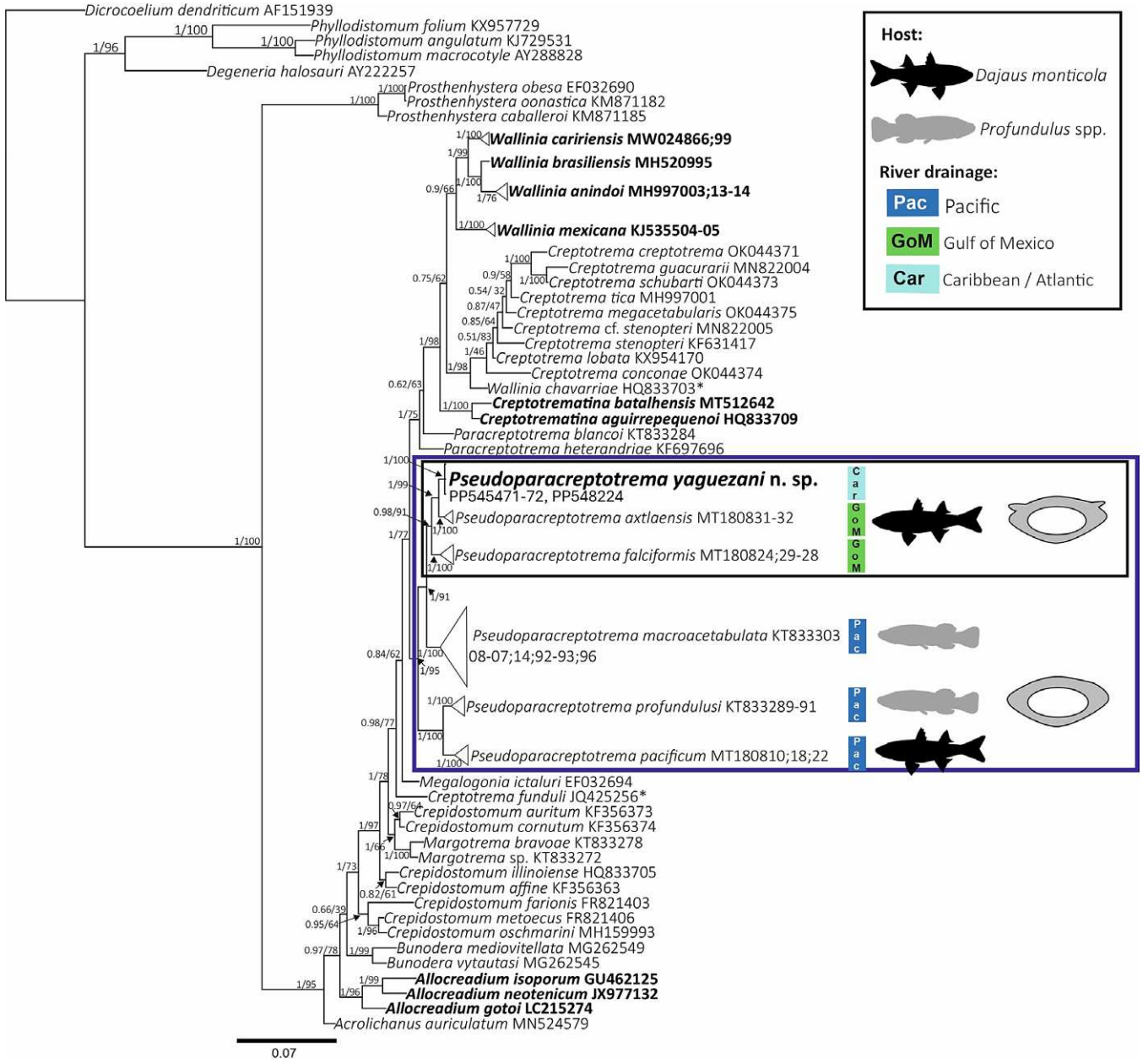

Figure 2. Phylogenetic reconstruction of partial 28S sequence evolution in the Allocreadiidae (1,115-bp alignment). Nodes in the Bayesian Inference (BI) tree presented are annotated with posterior probability and bootstrap values from separate Maximum Likelihood analysis. In *Pseudoparacreptotrema* (blue rectangle), branch tips are annotated with symbols indicating geographic origin, host (see inset legend), and presence or absence of paired muscular lobes on the oral sucker. Sequences in bold represent allocreadiid species and genera also present in mitochondrial genome phylogeny (Fig. 3). An asterisk indicates sequences from species that did not fall in monophyletic clades with congeners. *Dicrocoelium dendriticum*, *Degeneria halosauri*, *Phyllodistomum* spp. and *Prosthenhystera* spp. were used as outgroups. The scale bar indicates the number of substitutions per site.

by *Pseudoparacreptotrema*, with *Wallinia* and *Creptotrematina* branching latest (Fig. 2). The 28S phylogeny also showed non-monophyly of both *Wallinia* (due to *W. chavariae*) and *Creptotrema* (due to *C. funduli*).

Phylogenetic analysis: mitochondrial genome

After removing low-quality and low-complexity sequences, and trimming low-quality ends using BBduk, the Illumina read pool from *P. yaguezani* n. sp. was reduced from 51,771,054 sequences, all 150-bp long, to 51,755,324 sequences with mean 149.8, standard deviation 3.8, range 92–150 bp in length. The mitochondrial genome assembly of *P. yaguezani* n. sp. was 14,489-bp long with a mean of 1138.1 (range 964–1331) reads per site from the 5′ end of cox3 to the 3′ end of nad5 (i.e., excluding the difficult-to-assemble, non-coding, repetitive region between cox3 and nad5 that artifactually increases read depth). The final assembly comprised 12 protein-coding genes, two ribosomal genes, and 22 tRNA genes. The order of the 12 protein-coding genes was the same as in three other allocreadiid mitochondrial genomes (Supplementary Table S1, Supplementary Fig. S1). The nucleotide contents of the mitochondrial genome were as follows: A: 3,262 (22.5%), C: 1,807 (12.5%), G: 3,235 (22.3%), and T: 6,185 (42.7%). The frequency of AT nucleotide pairs was 34.8%, that of GC pairs, 65.2%.

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Table 2. Uncorrected p distances in 28S rDNA (ranges in percent) among species of *Pseudoparacreptotrema*

Species of *Pseudoparacreptotrema*	1	2	3	4	5	6
1. *P. yaguezani* n. sp. (3, Car)	0	0.64	1.37–1.56	1.94–2.22	3.26	3.35–3.45
2. *P. axtlaensis* (2, GoM)		0	1.28–1.47	1.84–2.12	2.97	2.97–3.06
3. *P. falciformis* (3, GoM)			0–0.27	1.66–2.13	3.16–3.25	3.25–3.44
4. *P. macroacetabulata* (7, Pac)				0–0.36	3.16–3.45	3.26–3.64
5. *P. profundulusi* (3, Pac)					0	1.19–1.28
6. *P. pacificum* (3, Pac)						0–0.09

Number of sequences and geographic origins in parentheses.
Abbreviations: Car, Caribbean; GoM, Gulf of México; Pac, Pacific.

In both ML and BI trees based on both nucleotides and translated amino acids, the mitochondrial genome of *P. yaguezani* n. sp. fell in a highly supported, monophyletic clade including other Allocreadiidae (Fig. 3): *Creptotrematina aguirrepequenoi*, *Wallinia mexicana*, and *Allocreadium lobatum*. Unlike in the *28S* phylogeny (Fig. 2), the mitochondrial genome of *Pseudoparacreptotrema* branched earlier than *Creptotrematina, Wallinia* and *Allocreadium*, and the branching order of the latter three varied in trees based on mitochondrial nucleotides (Fig 2A) or translated amino acids (Fig 2B). Relationships among nearly all major clades differed in analysis of mt genome nucleotides and translated amino acids (Fig 2, Supplementary Fig. S2). For example, in the mt nucleotide tree, the Allocreadiidae and Dicrocoeliidae (*Dicrocoelium*, *Lyperosomum*, *Eurytrema*) were together in a well-supported clade, which in turn was in a larger clade containing Prosthogonimidae (*Prosthogonimus*), Eucotylidae (*Tamerlania*), and Plagiorchioidae (*Plagiorchis*, *Orientocreadium*, *Glypthelmins*, *Haematoloechus*). However, in the amino acid topology, the Allocreadiidae was separate, diverging early from a clade containing all these latter taxa. Uncorrected nucleotide p distances between the mitochondrial genome of *P. yaguezani* n. sp. and the other three allocreadiids were 19.1%–21.1%; uncorrected amino acid p distances between

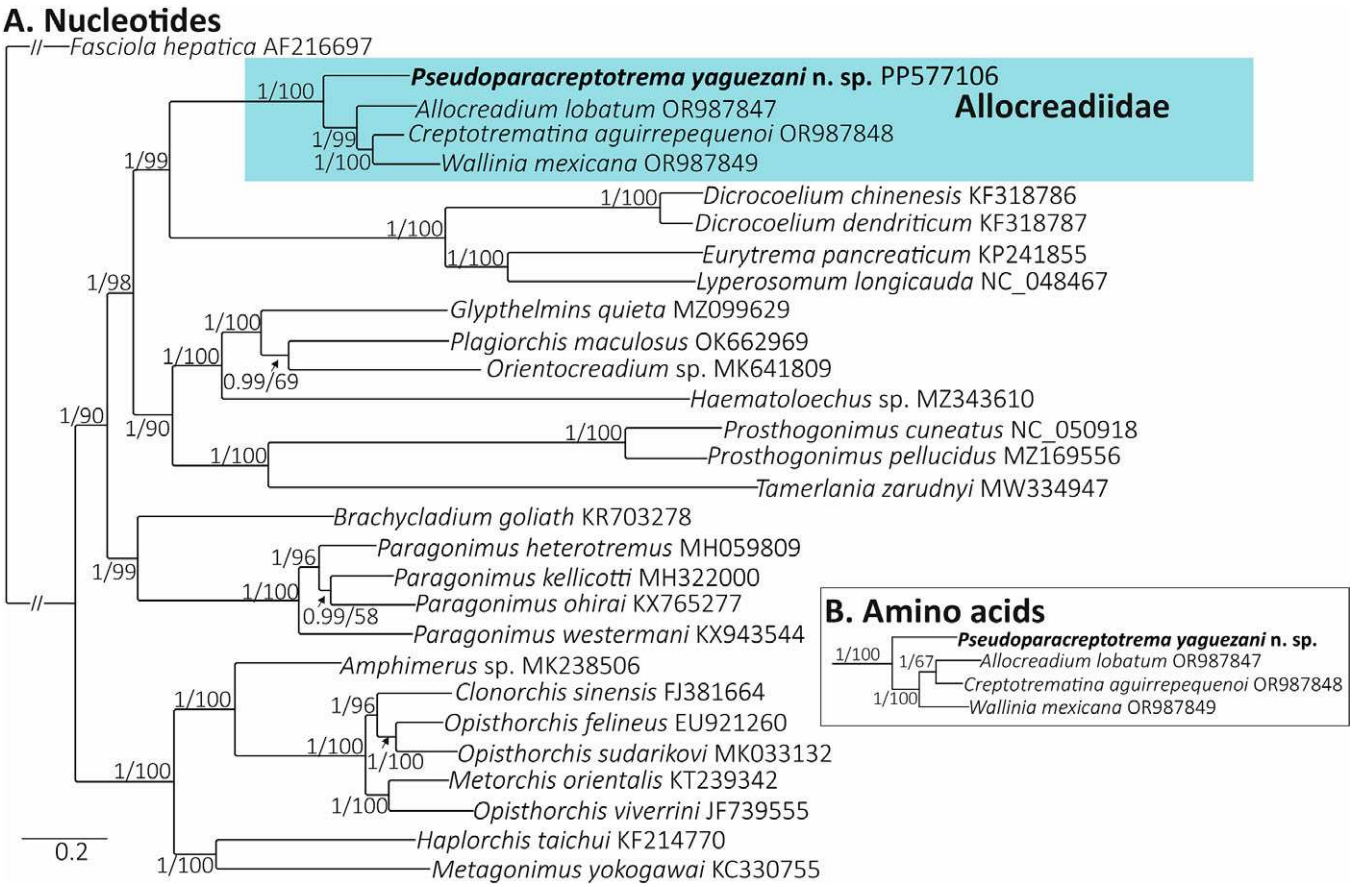

Figure 3. Phylogenetic reconstruction of mitochondrial genome evolution in the Allocreadiidae and other digeneans. A) The tree represented was obtained by Bayesian Inference (BI) analysis of an 11,814-bp nucleotide alignment; nodal support is provided by posterior probability and bootstrap values in separate Maximum Likelihood (ML) analysis (BI/ML). The Allocreadiidae are within the blue rectangle, with the sequence from the present study in bold. *Fasciola hepatica* (AF216697) was used as an outgroup. The scale bar indicates the number of substitutions per site. Phylogenetic analysis of translated amino acids is in Supplementary Figure S2; B shows the mitochondrial amino acid topology within the allocreadiid subtree.

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the mitochondrial genome of *P. yaguezani* n. sp. and the other three allocreadiids were 24.33-26.84%.

rDNA operon

Of 51,755,324 trimmed Illumina reads obtained from *P. yaguezani* n. sp., 143,195 were assembled to an rDNA operon contig 7,293-bp long, with a mean coverage of 2838 reads per site (range 842–3366) over subunit and internal transcribed spacer sites. Annotations yielded an 18S rRNA gene (1989 bp), ITS1 rDNA (698 bp), 5.8S rRNA gene (157 bp), ITS2 rDNA (279 bp), 28S rRNA gene (4170 bp), and nucleotide composition was A: 20.1%, T(U): 33.0%, G: 25.1% and C: 21.9%.

Remarks

In *Pseudoparacreptotrema yaguezani* n. sp., the cirrus sac lies wholly to the right of the ventral sucker, but this structure is partly or largely dorsal to the ventral sucker in *P. profundulusi*, *P. macroacetabula*, *P. pacificum*, *P. axtlaensis*, and *P. falciformis.* The oral lobes of *P. yaguezani* n. sp. further distinguish this species from *P. macroacetabulata* and *P. profundulusi*, which lack these structures; the latter species are also found in *Profundulus* spp., rather than *D. monticola. Pseudoparacreptotrema yaguezani* n. sp. can be differentiated from *P. pacificum* by the presence of oral lobes (vs. absent), as well as the posterior separation of vitelline fields (vs. confluent), the oblique position of the testes (vs. paired), and its larger body size. *Pseudoparacreptotrema yaguezani* n. sp. can be differentiated from *P. falciformis* by the outward extending lanceolate lobes of the oral sucker (vs. sickle-shaped). *Pseudoparacreptotrema yaguezani* n. sp. is larger and has greater oral sucker-to-ventral sucker size ratios than *P. axtlaensis*, and vitelline fields are not confluent in the posterior part of the body of *P. yaguezani* n. sp., unlike in *P. axtlaensis*, in which vitelline fields converge posteriorly.

Morphologically, *P. yaguezani* n. sp. is most similar to a species originally described by Salgado-Maldonado et al. (1998) as *Creptotrema agonostomi* from *D. monticola* collected in a Pacific drainage in Jalisco, México. These authors also reported this species in *D. monticola* in an Atlantic drainage in Veracruz and in a Pacific drainage in *I. balsanus.* After examining the specimens of Salgado-Maldonado et al. (1998), Pérez-Ponce de León et al. (2020) transferred the species to *Pseudoparacreptotrema*, and concluded that at least two species were grouped under the name *P. agonostomi* by Salgado-Maldonado et al. (1998): *P. agonostomi* s.s. from *D. monticola* in Río Cuitzmala, Jalisco, with lobes on the oral sucker, as well another species of *Pseudoparacreptotrema* from the same host and river lacking lobes on the oral sucker, *P. pacificum.* Both *P. yaguezani* n. sp. and *P. agonostomi* s.s. from Jalisco are unique among species of *Pseudoparacreptotrema* in possessing a cirrus sac passing wholly to the right of the ventral sucker. However, compared to *P. agonostomi* s.s., the new species *P. yaguezani* has a larger and more muscular pharynx, smaller, more lanceolate oral lobes, and a greater oral-sucker to ventral-sucker size ratio.

Along with the studies of Pérez-Ponce de León et al. (2016, 2020), our work shows that species of *Pseudoparacreptotrema* with molecular support are limited to narrow geographic distributions, show high specificity at the host genus level, and either have or lack lobes on the oral sucker. Given this, other species besides *P. pacificum* are likely to have been grouped under the name *P.* (*C.*) *agonostomi* by Salgado-Maldonado et al. (1998). Specimens from *I. balsanus* are probably a different species than *P. pacificum* and *P. agonostomi* s.s. as, in addition to being in a distantly related host, specimens from *I. balsanus* are larger and differ markedly in the disposition of the cirrus sac and ventral sucker opening (oval rather than slit-like) compared with those from *D. monticola* in Jalisco (Table 1, Salgado-Maldonado et al., 1998). Pérez-Ponce de León et al. (2020) also reported that some specimens that Salgado-Maldonado et al. (1998) obtained from *D. monticola* in Veracruz had oral lobes, whereas others did not. This indicates a total of five species may have been grouped under the name *P.* (*C.*) *agonostomi* by Salgado-Maldonado et al. (1998): *P. agonostomi* s.s., with lobes on oral sucker, from *D. monticola* in Río Cuitzmala, Jalisco; *P. pacificum*, without oral lobes, from *D. monticola* in Río Cuitzmala; an unnamed species, with oral lobes, from *I. balsanus* from Río Chontalcoatlan, Guerrero (Pacific drainage); an unnamed species with oral lobes from *D. monticola* from Río Máquinas, Veracruz (Atlantic drainage); and an unnamed species lacking oral lobes from *D. monticola* from Río Máquinas.

Although detailed records were not kept, we estimate the prevalence of infection of *P. yaguezani* n. sp. in *D. monticola* in the streams of western Puerto Rico sampled to be less than 10%. Most infected fish had between one and two worms (maximum six), with worms more common in larger fish.

Discussion

Pseudoparacreptotrema yaguezani n. sp. is the first allocreadiid recorded in Puerto Rico and the first member of *Pseudoparacreptotrema* outside of Middle America. Molecular studies have revealed geographically limited distributions both in species of *Pseudoparacreptotrema* (Pérez-Ponce de León et al., 2020) and in lineages of its host, *D. monticola* (McMahan et al., 2013), but the absence of molecular data from a Caribbean sample of *Pseudoparacreptotrema* has limited further comparisons until now. Our results suggest that biogeographic processes may have co-structured major divergence events in both this parasite and its host.

Nominally, the definitive host of *P. yaguezani* n. sp., *D. monticola*, is widely distributed (Matamoros et al., 2009; Kubicek et al., 2019), but two studies show multiple species are grouped under this name. Durand et al. (2012) found three lineages of *D. monticola* in Middle America using the ribosomal 16S and mitochondrial CO1 and cytochrome b (cytb) genes. Using the nuclear ribosomal S7-1 and the mitochondrial cytb genes, four lineages were recovered by McMahan et al. (2013), with estimated divergences 14.7–7.0 million years ago. These two surveys differed in markers employed, sampling localities, and particularly in the number of specimens sequenced (nine in Durand et al. 2012, 94 in McMahan et al. 2013), but both found two Pacific lineages and that a less common, more northern Pacific lineage is the most ancient. The phylogenetic analysis of McMahan et al. (2013) indicated that the subsequent lineages of *D. monticola* arose in the Gulf of México, then diverged into two lineages, one occurring widely across the western coast of México (Pacific A) and the other found throughout the Caribbean (Fig. 4, Supplementary Fig. S3). Morphometric differences among the four lineages of McMahan et al. (2013) include head shape and dorsal and anal fin insertion sites (Díaz-Murillo et al., 2017). McMahan et al. (2013) recovered the Caribbean clade of *D. monticola* in Florida, the Dominican Republic, Puerto Rico, and Jamaica, where Bancroft (1834) described this species; thus, the name *D. monticola* should be reserved for the Caribbean clade.

Incorporating a Caribbean species of *Pseudoparacreptotrema* in phylogenetic analysis reveals suggestive commonalities in the evolution of both the parasite and lineages of *D. monticola s.l.* (Figs. 2, 4). In

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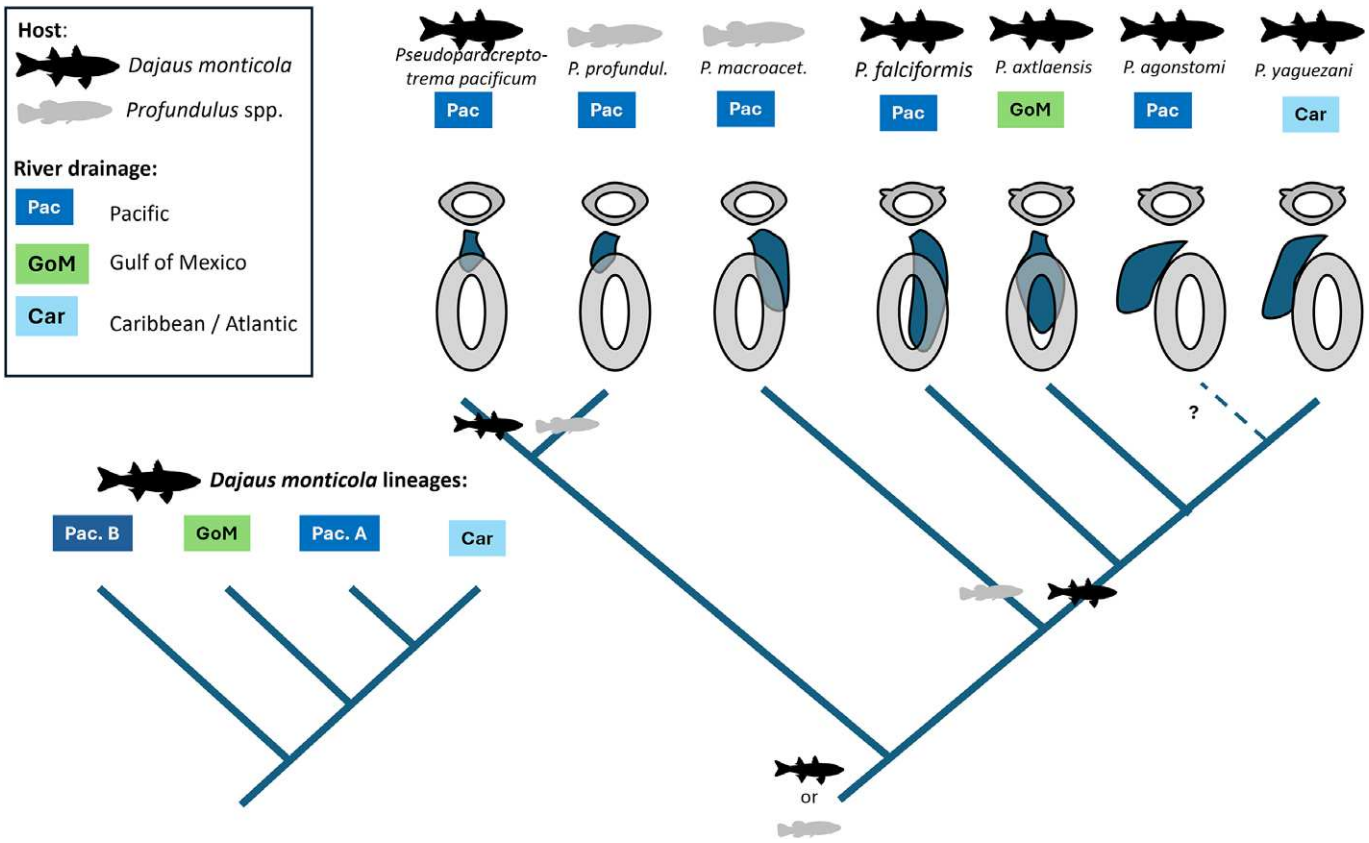

Figure 4. Schematic cladograms showing the branching order of lineages within *Dajaus monticola* (lower left, adapted from McMahan et al. 2013) and *Pseudoparacreptotrema* spp. (upper right, based on the present study). Tips of parasite cladogram are annotated with symbols indicating fish host, geographic origin, and illustration of presence/absence of paired muscular lobes on oral sucker and cirrus sac disposition with respect to ventral sucker. Branches of parasite cladogram are annotated with possible ancestral host and subsequent host-switch events. The placement of *P. agonostomi* (dashed line) is hypothesized based on morphology; the remaining topology in *Pseudoparacreptotrema* is based on phylogenetic analysis of 28S (Fig. 2). For a map with geographic localities, see Supplementary Fig. S3.

both host and parasite, Pacific coastal lineages branch earliest, and a Caribbean lineage is most recent. The greater divergence of the ancestral Pacific lineages of *Pseudoparacreptotrema* (as indicated by branch lengths in Fig. 2, see also Table 2) is consistent with the greater divergence of the ancestral Pacific lineage of *D. monticola* (Pacific B, see McMahan et al., 2013). The 28S phylogeny of *Pseudoparacreptotrema* shows that Gulf of México and Caribbean lineages arose from ancestral Pacific lineages, which contrasts with *D. monticola*, in which a Caribbean lineage is most closely related to a lineage that secondarily recolonized the Pacific (Pacific A). However, available information from *P. agonostomi*, still unsequenced, suggests it may resolve this inconsistency. The oral lobes of *P. agonostomi* suggest a close relationship with *P. falciformis* and *P. axtlaensis*, and the dextral cirrus sac further suggests a close relationship with *P. yaguezani* n. sp. A cladogram incorporating *P. agonostomi* based on these characters (Fig. 4) has the same geographic sequence as in *D. monticola s.l.*: ancestral Pacific lineages colonizing the Gulf of México, expanding to the Caribbean and back to the Pacific. In a temporally explicit phylogeny of *D. monticola*, McMahan et al. (2013) discuss the geological events that may have contributed to this sequence of diversification, which include movement of the Chortís block (Honduras, El Salvador, parts of Nicaragua and Guatemala), formation of the Yucatán Peninsula (a barrier lacking suitable river habitat, isolating *D. monticola* along the Gulf of México), and the still-open Central American Seaway permitting dispersal from the Caribbean to the Pacific. The presence of some species of *Pseudoparacreptotrema* in *Profundulus* and the higher number of lineages of parasites than hosts may reflect parasite speciation within biogeographic regions, because of host switching, including intermediate host switching. Assuming the ancestral definitive host of *Pseudoparacreptotrema* to be either *D. monticola s.l.* or *Profundulus* produces equally parsimonious scenarios (two definitive host switches). However, because of the incompleteness of the overall picture, the scenario in Figure 4 is best characterized as a prediction that highlights several research questions, including: Which of the Pacific lineages of McMahan et al. (2013) of *D. monticola* (A or B) are infected with *P. pacificum, P. macroacetabulata*, and *P. agonostomi*? What is the phylogenetic affiliation of *P. agonostomi* and the unidentified species of *Pseudoparacreptotrema* formerly grouped under this name? Is the phylogeny of *Pseudoparacreptotrema* temporally consistent with that of *D. monticola*?

Pseudoparacreptotrema yaguezani n. sp. can be distinguished from congeners both morphologically (see remarks) and based on 0.64-3.45% divergence in 28S. The geographic origins of *P. yaguezani* n. sp. in the Caribbean also indicate it is unlikely to be conspecific with any species from Middle America. The relatively small divergence in 28S (0.64%) between *P. yaguezani* n. sp. and *P. axtlaensis* falls within the lower range of interspecific distances in other Allocreadiidae (e.g., 0.58-0.82% divergence in 28S between *Bunodera* spp., Petkevičiūtė et al., 2010; 0.4%–5.0% among *Creptotrema* spp., Franceschini et al. 2021) and in other digeneans, distinct species may share identical 28S sequences (e.g., *Transversotrema*, Cutmore et al., 2023). Mitochondrial data provided here will allow more reliable species delineations within this genus.

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The results of phylogenetic analyses in the present study differed from prior work in several respects. For example, in the 28S phylogeny of Pérez-Ponce de León et al. (2020), *P. macroacetabulata* was nested between *P. falciformis* and *P. axtlaensis*, but with weak support. In our analysis (Fig. 2), the strong support for *P. macroacetabulata* diverging earlier from *P. falciformis* and *P. axtlaensis* suggests that data from *P. yaguezani* n. sp. has clarified these relationships. Another distinct aspect of our 28S topology was the paraphyly of *Wallinia*, with *W. chavarriae* emerging from the base of a *Creptotrema* clade rather than with other members of *Wallinia.* In other recent analyses of 28S, *W. chavarriae* falls within a monophyletic *Wallinia* clade (Hernández-Mena et al., 2019; Da Silva et al., 2021; Pérez-Ponce de León et al., 2020; Mendoza-Garfias et al., 2022). However, few or no *Creptotrema* sequences were included in these analyses, except that of Mendoza-Garfias et al. (2022), in which support for the monophyly of *Wallinia*, including *W. chavarriae*, was slightly weaker than herein (81% bootstrap replicates in ML, and 1.0 posterior probability in BI). The distant placement of 28S from *Creptotrema funduli* (*species inquirenda*) from other *Creptotrema* species observed here (Fig. 2) was highlighted by Franceschini et al. (2021), who discussed the need for taxonomic reassessment of this species; even prior to any molecular analysis, Manter (1962) argued *C. funduli* was not a member of *Creptotrema.*

Phylogenetic analysis of mitochondrial genomes produced variable results, which also differed from prior studies. The numerous differences between topologies based on nucleotides and amino acid translations suggest a need for cautious interpretation. One result that was consistent and different from prior work was the early branching of the mitochondrial genome of *Pseudoparacreptotrema* among allocreadiids, ancestral to *Allocreadium*, *Creptotrematina*, and *Wallinia.* In the 28S phylogeny (Fig. 2), in contrast, *Allocreadium* diverges first, a branching order also observed by Atopkin et al. (2020), Pérez-Ponce de León et al. (2016, 2020), and Mendoza-Garfias et al. (2022). The 28S tree is based on just 1,115 nucleotide characters but builds on decades of studies using Sanger sequencing (e.g., Choudhury & Règagnon, 2005) that collectively provide data from 41 allocreadiid species. The mt genome tree is based on 10 times more nucleotides but 10 times fewer allocreadiids, its poorer taxonomic representation resulting from the comparatively recent adoption of next-generation sequencing in parasite systematics, epitomized by the single prior study of Allocreadiidae of Solórzano-García et al. (2024). Because tree reliability depends more on taxonomic representation than the number of characters analyzed (e.g., Hedtke et al., 2006), the earlier branching position of *Allocreadium* to *Pseudoparacreptotrema* indicated by 28S is probably a more reliable estimate of relationships among these genera. However, several deeper nodes in the 28S tree, including those associated with the position of *Allocreadium*, are not strongly supported both in the present and other studies (Atopkin et al., 2020; Pérez-Ponce de León et al., 2016, 2020; Mendoza-Garfias et al., 2022). As mt genome sequencing proceeds in the Allocreadiidae, further disagreements with intergeneric relationships based on 28S may emerge, but topologies will probably converge on a single solution with minor variations.

The discovery and phylogenetic position of *P. yaguezani* n. sp. in Puerto Rico highlights fruitful areas for research on the biogeography of *Pseudoparacreptotrema* and its evolutionary affinities among allocreadiids. As mentioned by Pérez-Ponce de León et al. (2020), work on *Pseudoparacreptotrema* in *D. monticola* could serve as a model for work on the nematode *Spinitectus agonostomi*, which has a similar geographic distribution and specificity for *D. monticola.*

Supplementary material. The supplementary material for this article can be found at http://doi.org/10.1017/S0022149X24000567.

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