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Phylogenetic position of *Sphincterodiplostomum* Dubois, 1936 (Digenea: Diplostomoidea) with description of a second species from Pantanal, Brazil

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Abstract

Sphincterodiplostomum is a monotypic genus of diplostomid digeneans that parasitize fish-eating birds in the neotropics. The type species *Sphincterodiplostomum musculosum* has a unique, dorsal, tubular invagination in the opisthosoma with a muscular sphincter. Whereas larvae of *S. musculosum* are relatively commonly reported in Neotropical fish helminth surveys, adult specimens from birds are rarely collected. Prior to our study, no DNA sequence data for *S. musculosum* were available. Our molecular and morphological study of mature and immature adult *Sphincterodiplostomum* specimens from three species of birds and one species of crocodilian revealed the presence of at least two species of *Sphincterodiplostomum* in the neotropics. We provide the first molecular phylogeny of the Diplostomoidea that includes *Sphincterodiplostomum*. In addition, this is the first record of *S. musculosum* from caimans, along with the first record of fully mature adult *S. musculosum* from green kingfisher *Chloroceryle americana*. The new species of *Sphincterodiplostomum* (*Sphincterodiplostomum joaopinhoi* n. sp.) can be morphologically distinguished from *S. musculosum* based on the anterior extent of vitelline follicles, narrower prosoma, substantially smaller holdfast organ and structure of tegumental spines. Our data revealed 0.7% interspecific divergence in 28S and 10.6–11.7% divergence in *cox*1 sequences between the two *Sphincterodiplostomum* species.

Introduction

Sphincterodiplostomum Dubois, 1936 is a monotypic genus of diplostomoidean digeneans (Diplostomidae Poirier, 1886; Diplostominae Poirier, 1886), which parasitize the intestines of their avian definitive hosts in the neotropics (Niewiadomska, 2002; Lunaschi & Drago, 2006). The type species *Sphincterodiplostomum musculosum* Dubois, 1936 was originally described by Dubois (1936, 1938) based on immature specimens from agami heron *Agamia agami* (Gmelin) collected in Brazil. Lunaschi & Drago (2006) have described fully mature adult specimens of the species from great egret *Ardea alba* Linnaeus in Argentina. *Sphincterodiplostomum musculosum* is most easily differentiated from other members of the Diplostomidae based on the presence of a well-developed, dorsal, tubular invagination in the opisthosoma with a muscular sphincter (Niewiadomska, 2002; Lunaschi & Drago, 2006).

The complete life cycle of *S. musculosum* has not been demonstrated; however, *S. musculosum* is known to utilize a wide diversity of fish as second intermediate hosts, and has been previously collected from avian definitive hosts (e.g. Dubois, 1936; Lunaschi & Drago, 2006; Rocha *et al.*, 2015; Delgado *et al.*, 2017). Adult *S. musculosum* have been rarely collected (e.g. Lunaschi & Drago, 2006), whereas metacercariae have been reported in several studies of Neotropical fish helminths (e.g. Szidat, 1969; Zago *et al.*, 2013; Rocha *et al.*, 2015; Delgado *et al.*, 2017). To date, no DNA sequence data have been published for *S. musculosum*. Herein, we provide partial 28S ribosomal RNA (rRNA) and cytochrome *c* oxidase 1 (*cox*1) mitochondrial DNA gene sequences of *S. musculosum* and a new *Sphincterodiplostomum* species collected from avian and crocodilian hosts. The 28S DNA sequence data were used to infer the phylogenetic position of *Sphincterodiplostomum* spp. among other major diplostomoidean lineages. The sequences of *cox*1 were used for reliable *Sphincterodiplostomum* species differentiation.

Materials and methods

Vertebrate hosts were collected in Pantanal, Fazenda Retiro Novo, Municipality of Poconé, Mato Grosso State, Brazil. Mature and immature adult specimens of *S. musculosum* were obtained from cocoi heron *Ardea cocoi* Linnaeus, 1776, black-collared hawk *Busarellus*

Table 1. List of *Sphincterodiplostomum* isolates sequenced in this study, their hosts and GenBank accession numbers.

Digenean taxa	Host species	Museum number	Accession numbers	
			28S	*cox*1
Sphincterodiplostomum joaopinhoi n. sp.	*Busarellus nigricollis*	HWML-216379, 216380	MW411441, MW411442	MW410851, MW410852
Sphincterodiplostomum musculosum	*Ardea cocoi*	HWML-216381	MW411443	MW410853
S. musculosum	*Chloroceryle americana*	HWML-216382	–	MW410854
S. musculosum	*B. nigricollis*	HWML-216383	MW411444	MW410855
S. musculosum	*Caiman yacare*	HWML-216384	MW411445	MW410856

HWML, Harold W. Manter Laboratory, Lincoln, Nebraska, USA. All specimens were collected at Fazenda Retiro Novo, Municipality of Poconé, Mato Grosso State, Brazil.

nigricollis (Latham, 1790), green kingfisher *Chloroceryle americana* (Gmelin, 1788) and yacare caiman *Caiman yacare* (Daudin, 1802). In addition, mature adult specimens of the new *Sphincterodiplostomum* species were collected from *B. nigricollis* (table 1). Specimens for morphological study were stained with an aqueous alum carmine and permanently mounted according to Lutz *et al.* (2017). All measurements given in the text are in micrometres. Type and voucher specimens are deposited in the collection of the Harold W. Manter Laboratory (HWML), University of Nebraska State Museum, Lincoln, Nebraska, USA. As noted in recent publications on diplostomoideans (e.g. Achatz *et al.*, 2019a, b, c; Tkach *et al.*, 2020), we use the terms prosoma and opisthosoma to refer to the distinct anterior and posterior regions of the body.

Specimens of *Sphincterodiplostomum* spp. observed under scanning electron microscope (SEM) were dehydrated in a series of ethanol of ascending concentrations and dried with hexamethyldisilazane (Ted Pella Inc., Redding, California, USA) as a transition fluid. *Sphincterodiplostomum* spp. specimens were mounted on aluminium stubs using conductive double-sided tape, coated with gold–palladium and examined with the use of a Hitachi 4700 SEM (Hitachi USA, Mountain View, California, USA) at an accelerating voltage of 5 kV.

Genomic DNA was extracted from a part of a specimen or a whole single specimen of *Sphincterodiplostomum* spp. following the protocol described by Tkach & Pawlowski (1999) or using a ZR Genomic DNA Tissue Micro Prep kit (Zymo Research, Irvine, California, USA) following the manufacturer's instructions. Amplification and sequencing of 28S and *cox*1 fragments was carried out as described in Achatz *et al.* (2019d). Newly obtained sequences are deposited in GenBank (table 1).

Newly obtained and previously published sequences were initially aligned using ClustalW implemented in MEGA7 software (Kumar *et al.*, 2016). The position of *Sphincterodiplostomum* spp. among major diplostomoidean lineages was studied using an alignment of 28S, which included newly generated sequences of both *Sphincterodiplostomum* species and previously published sequences of 16 members of the Diplostomidae, two members of the Proterodiplostomidae Dubois, 1936 and 12 members of the Strigeidae Railliet, 1919. *Suchocyathocotyle crocodili* (Yamaguti, 1954) was selected as the outgroup based on the topology presented by Achatz *et al.* (2019d). The phylogenetic analysis was conducted using Bayesian inference (BI) as implemented in MrBayes version 3.2.6 software (Ronquist & Huelsenbeck, 2003). The best-fitting nucleotide substitution model identified by MEGA7 was the general time-reversible model with estimates of invariant sites and gamma-distributed among-site variation (GTR + I + G). The BI analysis was performed using MrBayes software as follows: Markov chain Monte Carlo (MCMC) chains were run for 3,000,000 generations with sample frequency set at 1000. Log-likelihood scores were plotted and only the final 75% of trees were used to produce the consensus trees. The number of generations for each analysis was considered sufficient as the standard deviation stabilized below 0.01. The pairwise comparisons of *Sphincterodiplostomum* isolates were performed with assistance of MEGA7 software.

Results

Systematics

Diplostomidae Poirier, 1886
Sphincterodiplostomum Dubois, 1936

Sphincterodiplostomum joaopinhoi n. sp.

Taxonomic summary

Type host. Busarellus nigricollis (Accipitriformes: Accipitridae).

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

Type material. The type series consists of two mature and four immature adult specimens deposited in the HWML. Holotype: HWML-216379, labelled ex. *B. nigricollis*, small intestine, Pantanal, Fazenda Retiro Novo, Municipality of Poconé, Mato Grosso State, Brazil, 9 June 2017, coll. A. Fecchio. Paratypes: HWML-216380 (lot of four), labels identical to the holotype.

Site in host: Small intestine.

ZooBank registration. The Life Science Identifier (LSID) for *Sphincterodiplostomum joaopinhoi* n. sp. is urn:lsid:zoobank.org:act:DB466D7B-EED6-4959-9EB9-E3C34A3D4885.

Etymology. The species is named after Dr Joao B. Pinho (Laboratório de Ecologia de Aves, Federal University of Mato Grosso, Cuiabá, Brazil) in recognition of his contributions into the knowledge of avifauna of Pantanal and his invaluable assistance with collecting specimens reported in this work.

Description

Based on two adult specimens (see figs 1, 2a–e and 3a–e). Body 978–1259 long, consisting of distinct prosoma and opisthosoma; prosoma elliptical, 580–766 long, with maximum width at level of holdfast organ, 428–460; opisthosoma cylindrical, 398–493 long, 226–241 wide. Prosoma: opisthosoma length ratio 1.5–1.6; opisthosoma width ratio 1.8–2. Forebody 347–451 long, 35–36%

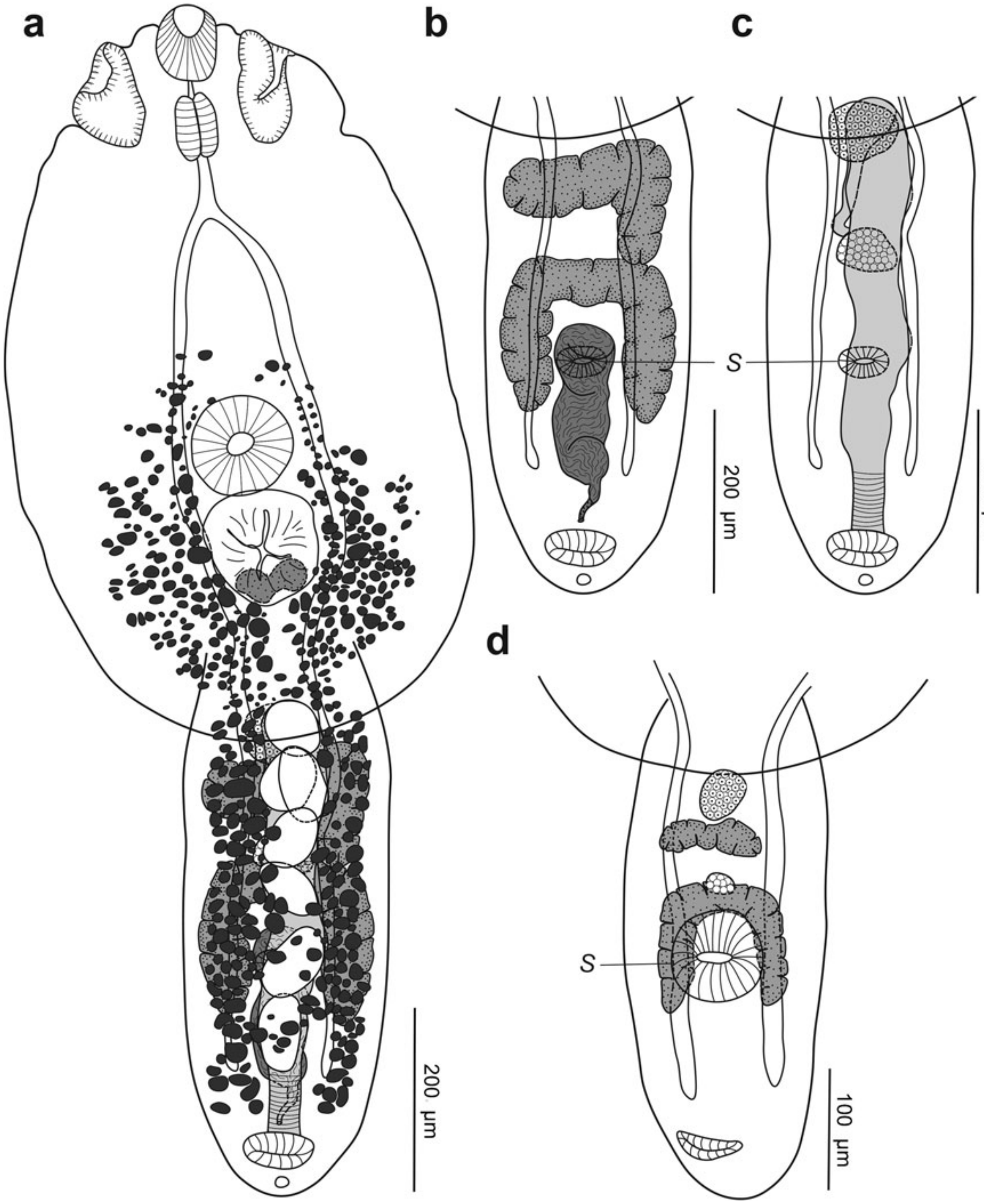

Fig. 1. Line drawings of *Sphincterodiplostomum joaopinhoi* n. sp.: (a) holotype, ventral view; (b) male reproductive system of holotype, ventral view of opisthosoma; (c) female reproductive system of holotype, ventral view of opisthosoma, vitellarium and eggs omitted; (d) paratype, immature specimen, dorsal view. Abbreviation: S, dorsal muscular sphincter associated with the tubular invagination of the opisthosoma.

of body length. Minuscule tegumental spines covering most of prosoma, absent between anterior margin of oral sucker and posterior margin of pseudosuckers; spines scale-like with several small digitiform projections at posterior edge (fig. 3d). Opisthosoma with a tubular invagination with muscular sphincter at level of posterior testis. Oral sucker terminal, oval, 64–72 × 49–56. Pseudosuckers 75–82 × 66–78. Ventral sucker with minute spines covering its base, 93–98 × 98–108, located near 60% of prosoma length; oral: ventral sucker width ratio 0.5. Holdfast organ immediately posterior to ventral sucker; subspherical or oval with ventral muscular portion, 118–128 × 110–168. Proteolytic gland at base of holdfast organ, bilobed, 43 × 79. Prepharynx 24 long. Pharynx oval, 76 × 46. Oesophagus 54–80 long. Caecal bifurcation in anterior third of prosoma. Ceca slender, extending to near posterior end of opisthosoma.

Testes 2, in tandem, lobate; anterior testis asymmetrical, 68–112 × 156–203; posterior testis symmetrical, horseshoe-shaped with anterior isthmus, 152–171 × 165–209. Seminal vesicle folded, posterior to isthmus of posterior testis; terminal efferent duct of seminal vesicle joins dorsal side of metraterm to form short hermaphroditic duct.

Ovary pretesticular, near prosoma–opisthosoma junction, subspherical or slightly transversely oval 55–60 × 64–75. Oötype

Fig. 2. Specimens of *Sphincterodiplostomum* species/species-level lineages from Pantanal, Brazil: (a) holotype of mature adult *Sphincterodiplostomum joaopinhoi* n. sp., ventral view; (b, c) paratypes of immature *S. joaopinhoi* n. sp. at different stages of development, ventral views; (d, e) opisthosoma of *S. joaopinhoi* n. sp., dorsal views; (f) mature adult *Sphincterodiplostomum musculosum* from *Chloroceryle americana*, ventral view, hologenophore; (g) immature *S. musculosum* from *Ardea cocoi*, dorsal view; (h) immature *S. musculosum* from *Caiman yacare*, dorsal view. Abbreviation: S, sphincter surrounding tubular invagination characteristic of *Sphincterodiplostomum* species.

and Mehlis' gland inter-testicular. Laurer's canal not observed. Vitelline follicles distributed as two lateral bands extending posteriorly from approximately the level of the ventral sucker to near the posterior end of the body, lateral bands sporadically confluent. Vitelline follicles absent in the first 47–68% of prosoma and last 15–20% of opisthosoma. Vitelline reservoir inter-testicular. Uterus ventral to gonads, extending anteriorly to near junction of prosoma and opisthosoma before turning and

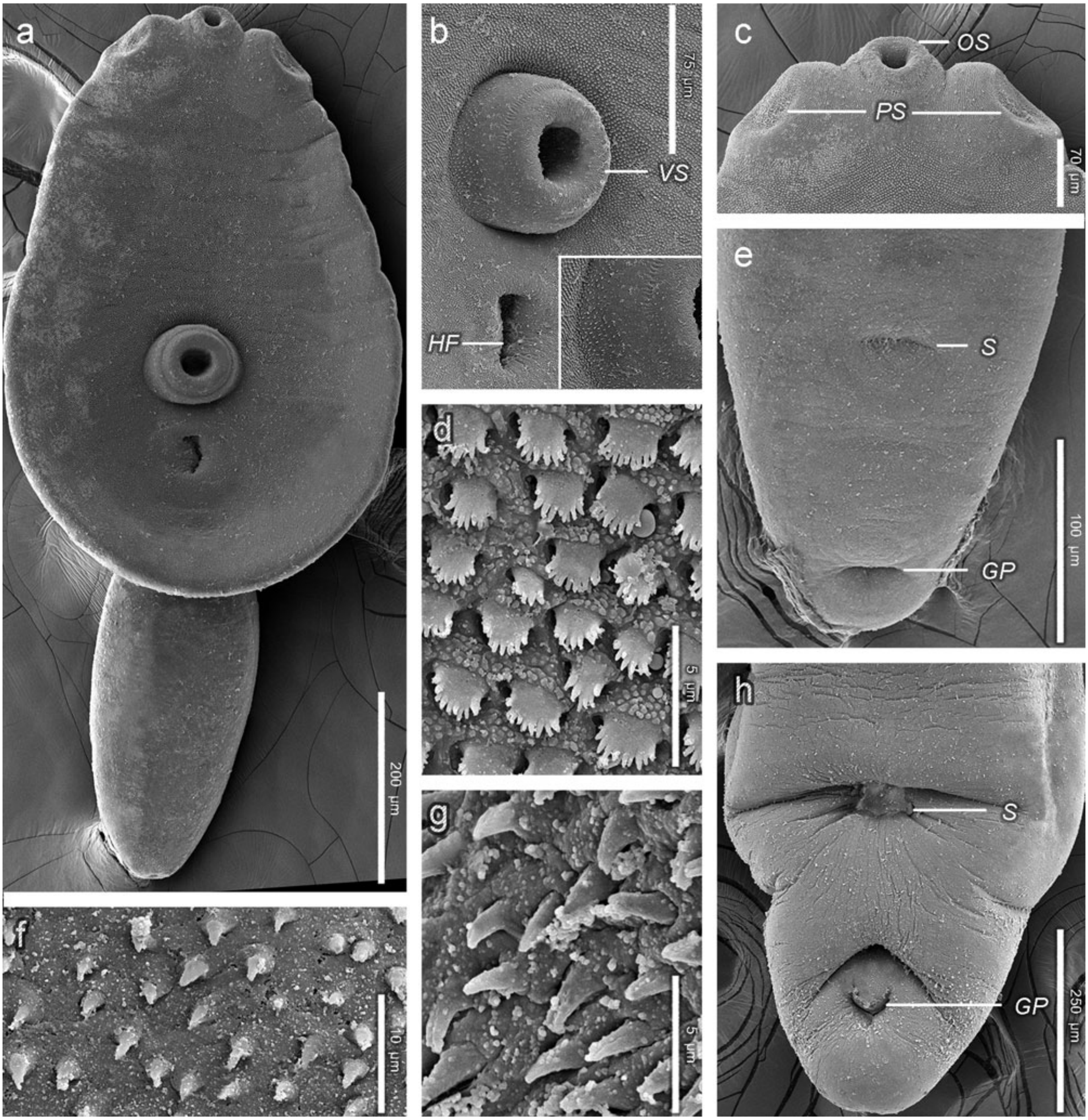

Fig. 3. Scanning electron micrographs of *Sphincterodiplostomum* spp.: (a) entire specimen of *Sphincterodiplostomum joaopinhoi* n. sp., ventral view; (b) ventral sucker and holdfast organ of *S. joaopinhoi* n. sp., ventral view; inset shows minute spines at the base of the ventral sucker; (c) anterior end of prosoma of *S. joaopinhoi* n. sp., ventral view; (d) tegumental spines with digitiform projections of *S. joaopinhoi* n. sp.; (e) posterior end of opisthosoma of *S. joaopinhoi* n. sp., dorsal view, note the sphincter surrounding tubular invagination characteristic of *Sphincterodiplostomum* species; (f, g) tegumental spines of *Sphincterodiplostomum musculosum*; (h) posterior end of opisthosoma of *S. musculosum*, dorsal view. Abbreviations: GP, genital pore; HF, holdfast organ; OS, oral sucker; PS, pseudosucker; S, sphincter surrounding tubular invagination; VS, ventral sucker.

extending posteriorly. Uterus contains up to eight eggs (74–83 × 42–53). Genital pore subterminal, on dorsal side, muscular. Excretory vesicle not well-observed. Excretory pore subterminal, on dorsal side.

Remarks

The new species clearly belongs to *Sphincterodiplostomum* based on the presence of a well-developed dorsal tubular invagination in the opisthosoma with a muscular sphincter, along with the results of our molecular phylogenetic analysis (fig. 4). The differential diagnosis below compares the new species with the description of adult *S. musculosum* by Lunaschi & Drago (2006) as they were the first to describe mature adult specimens. It is worth noting that specimens described by Lunaschi & Drago (2006) were contracted as stated by the authors and evident based on their illustrations. As we had only a single fully mature ovigerous specimen, we do not provide a description of *S. musculosum*. For the same reason, we do not provide a differential diagnosis based on

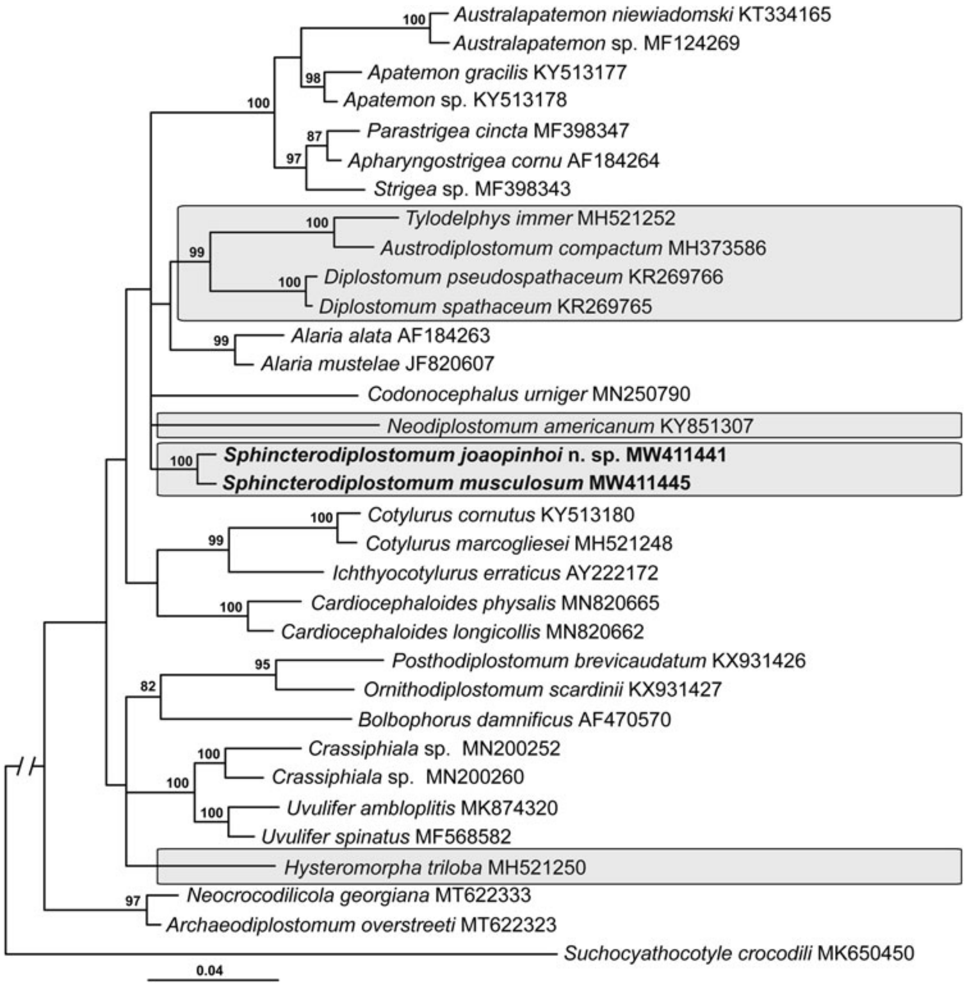

Fig. 4. Phylogenetic interrelationships among 33 diplostomoidean taxa including *Sphincterodiplostomum* spp. based on Bayesian Inference (BI) analysis of partial 28S rRNA gene sequences. Members of the sub-family Diplostominae as currently recognized are indicated by the shaded rectangles. BI posterior probability values lower than 80% are not shown. The new sequences obtained in this study are in bold. Scale bar indicates number of substitutions per site. GenBank accession numbers are provided after the names of all species.

our material, except for the tegumental spine structure and body size, which is skewed in the description by Lunaschi & Drago (2006) due to contraction.

Sphincterodiplostomum joaopinhoi n. sp. can be easily distinguished from *S. musculosum* based on the anterior extent of vitelline follicles (limited to near level of ventral sucker in the new species versus reaching near the level of the caecal bifurcation in *S. musculosum*) (figs 1 and 2).

Sphincterodiplostomum joaopinhoi n. sp. is smaller than heat-killed, properly fixed adult specimen of *S. musculosum* in our material (body length 978–1259 in the new species vs body length 1821 in *S. musculosum*). Even immature heat-killed specimens of *S. musculosum* in our material are substantially larger (body length 2145–2593) than the new species (body length 978–1259). *Sphincterodiplostomum joaopinhoi* n. sp. differs from *S. musculosum* described by Lunaschi & Drago (2006) by a much smaller prosoma width (428–460 in *S. joaopinhoi* n. sp. vs 754–1115 in *S. musculosum*), smaller oral sucker (64–72 × 49–56 in the new species vs 92–108 × 63–106 in *S. musculosum*), shorter pseudosuckers (75–82 in *S. joaopinhoi* n. sp. vs 101–150 in *S. musculosum*), smaller holdfast organ (118–128 × 110–168 in the new species vs 143–314 × 217–580 in *S. musculosum*), shorter pharynx (76 in *S. joaopinhoi* n. sp. vs 111–140 in *S. musculosum*) and wider anterior testis (156–203 in the new species vs 95–113 in *S. musculosum*).

Our SEM study demonstrated that the structure of tegumental spines of *S. joaopinhoi* n. sp. (fig. 3d) also differs from that in *S. musculosum* (fig. 3f, g). The spines of *S. joaopinhoi* n. sp. are scale-like and have several digitiform projections at the posterior edge of each spine (fig. 3d), whereas spines of *S. musculosum* are not scale-like and lack such projections (fig. 3f, g). *Sphincterodiplostomum joaopinhoi* n. sp. differs from *S. musculosum* by 0.7% (eight bases out of 1193) in the partial sequences of 28S gene, and by 10.6–11.7% (58–64 bases out of 545) in the partial sequences of *cox*1 gene.

Molecular phylogeny

After trimming to the length of the shortest sequence, the alignment of 28S was 1117 bases long; two sites were excluded due to indels. The topology of the Diplostomidae and Strigeidae in the phylogeny resulting from our analysis of 28S (fig. 4) was similar to other recent molecular phylogenetic analyses of the group (e.g. Blasco-Costa & Locke, 2017; Hernández-Mena *et al.*, 2017; Locke *et al.*, 2018;

Achatz *et al.*, 2019b, c, d, 2020; Pérez-Ponce de León & Hernández-Mena, 2019; Queiroz *et al.*, 2020; Tkach *et al.*, 2020). Importantly, both the Diplostomidae and Strigeidae were non-monophyletic. Both included representatives of the Proterodiplostomidae formed a strongly supported (97%) clade. Both members of *Sphincterodiplostomum* formed a strongly supported (100%) clade within a polytomy. This polytomy included three other clades of diplostomids and one well-supported clade of strigeids (fig. 4). The three other clades of diplostomids included (1) an unsupported clade of *Alaria* Schrank, 1788 + a 99% supported clade of [*Diplostomum* von Nordmann, 1832 + *Tylodelphys* Diesing, 1850 + *Austrodiplostomum* Szidat et Nani, 1951]; (2) *Codonocephalus* Diesing, 1850; and (3) *Neodiplostomum* Railliet, 1919. *Hysteromorpha triloba* (Rudolphi, 1819) was part of a separate, unsupported and unresolved polytomy (fig. 4).

Genetic variation

Pairwise nucleotide comparisons of partial 28S sequences revealed 0.7% difference (eight bases out of 1193) between the *Sphincterodiplostomum* species/species-level lineages. No intraspecific variation was detected among the partial 28S sequences of either species.

The pairwise comparisons of partial *cox*1 sequences demonstrated 10.6–11.7% difference (58–64 bases out of 545) between the two *Sphincterodiplostomum* species. In contrast with the 28S sequences, the *cox*1 sequences demonstrated 1.3–2.6% (7–14 bases out of 545) intraspecific variation in *S. musculosum* and 0.6% (three bases out of 545) intraspecific variation in *S. joaopinhoi* n. sp.

Discussion

This study adds a second species to the previously monotypic *Sphincterodiplostomum*. According to Niewiadomska (2002), *Sphincterodiplostomum* belongs to the sub-family Diplostominae, which has been since demonstrated to be clearly non-monophyletic (fig. 4; e.g. Blasco-Costa & Locke, 2017; Locke *et al.*, 2018; Achatz *et al.*, 2019b, c, d; Achatz *et al.*, 2020; Queiroz *et al.*, 2020; Tkach *et al.*, 2020). Both *Sphincterodiplostomum* species possess a well-developed, dorsal tubular invagination in the opisthosoma with a muscular sphincter, which is absent in other members of the Diplostominae. This fact, along with the molecular phylogenetic analysis placing the genus in its own clade with no evidence of close relationships with any other group within a polytomy, demonstrates that *Sphincterodiplostomum* represents a unique evolutionary lineage that likely evolved in South America. Whereas this evidence may be sufficient to erect a new sub-family (or family) for *Sphincterodiplostomum*, we feel that such an action would be premature until a detailed re-evaluation of all non-monophyletic members of the Diplostominae is undertaken. With the results of this study, 28S DNA sequences are only available for six of the 14 genera within the Diplostominae. Thus, it is not known how inclusion of DNA sequences of the remaining Diplostominae genera may affect the resulting topology and our understanding of the relationships among all members of the sub-family.

The intrageneric pairwise nucleotide comparisons of partial 28S (0.7%) and *cox*1 (10.6–11.7%) sequences of *Sphincterodiplostomum* spp. are similar to the levels of intrageneric variation demonstrated within other diplostomoidean genera (28S: 0–4.4%; *cox*1: 3.4–19.8%; see Achatz *et al.*, 2020 and references therein; Tkach *et al.*, 2020).

Our mature and immature adult specimens of *S. musculosum* (fig. 2f–h) conform closely to the original description of *S. musculosum* from *Ag. agami* by Dubois (1936, 1938) and redescription based on specimens from *Ar. alba* by Lunaschi & Drago (2006). Both immature and mature specimens of *S. musculosum* in our material were more similar to immature specimens described by Dubois (1936, 1938), and were substantially longer than the contracted specimens described by Lunaschi & Drago (2006). The body length of our specimens of *S. musculosum* ranged between 1821 and 2593, despite most of them being immature, whereas Dubois (1936, 1938) described his immature specimens to be up to 2900 long. In contrast, the body length of the contracted specimens described by Lunaschi & Drago (2006) ranged between 919 and 1329. This provides additional evidence that *S. musculosum* is a substantially larger digenean than *S. joaopinhoi* n. sp. Lunaschi & Drago (2006) described the tegument of *S. musculosum* as smooth. However, the tegument on the prosoma of our specimens is armed with spines (fig. 3f, g). The contradiction is explained by the extremely small size of the tegumental spines, which are difficult to observe under a light microscope.

This is the first report of *S. musculosum* from *A. cocoi*, *B. nigricollis* (or any raptor), *Ch. americana* (or any kingfisher) and *Ca. yacare* (or any crocodilian). We assume that the infection of *Ca. yacare* was accidental based on the presence of only immature specimens and the lack of any previous reports of *S. musculosum* in crocodilians. Caimans share both habitat and diet with fish-eating birds, thus making accidental infection possible. The fact that the specimens were collected during an extremely hot time of the year from a caiman in a small, shallow water body likely explains why these digeneans, normally parasitic in birds, underwent some growth and development in a cold-blooded vertebrate.

Sphincterodiplostomum joaopinhoi n. sp. is the second member of the genus and the first *Sphincterodiplostomum* species to be reported or described from *B. nigricollis*. While we did find *S. musculosum* in studied *B. nigricollis*, we did not find any fully mature specimens. It cannot be excluded that some previous reports of metacercariae of *S. musculosum* and unidentified *Sphincterodiplostomum* sp. from a variety of Neotropical fish may actually be *S. joaopinhoi* n. sp. The larvae of the two species are likely morphologically similar as larvae, as is the case for many other diplostomoideans; therefore, molecular identification of *Sphincterodiplostomum* metacercariae is recommended in the future. We hypothesize that the genus *Sphincterodiplostomum* contains additional not-yet-described species as has been recently demonstrated for several other diplostomoidean genera, such as *Crassiphiala* Van Haitsma, 1925, *Hysteromorpha* Lutz, 1931 and *Uvulifer* Yamaguti, 1934 (e.g. Locke *et al.*, 2018; López-Jiménez *et al.*, 2018; Achatz *et al.*, 2019a, c).

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Conflicts of interest. None.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of animals.

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