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Phylogeny and systematics of the Proterodiplostomidae Dubois, 1936 (Digenea: Diplostomoidea) reflect the complex evolutionary history of the ancient digenean group

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Abstract The Proterodiplostomidae Dubois, 1936 is a relatively small family of diplostomoidean digeneans parasitising the intestines of reptilian hosts associated with freshwater environments in tropical and subtropical regions. The greatest diversity of proterodiplostomids is found in crocodilians, although some parasitise snakes and turtles. According to the most recent revision, the Proterodiplostomidae included 17 genera within 5 subfamilies. Despite the complex taxonomic structure of the family, availability of testable morphology-based phylogenetic hypotheses and ancient hosts, molecular phylogenetic analyses of the group were practically lacking. Herein, we use novel DNA sequence data of the nuclear lsrRNA gene and mitochondrial *cox*1 gene from a broad range of proterodiplostomid taxa obtained from crocodilian, fish, and snake hosts on four continents to test the monophyly of the family and evaluate the present morphology-based classification system of the Proterodiplostomidae in comparison with the

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molecular phylogeny. This first detailed phylogeny for the Proterodiplostomidae challenges the current systematic framework. Combination of molecular phylogenetic data with examination of freshly collected quality specimens and re-evaluation of morphological criteria resulted in a number of systematic and nomenclatural changes along with a new phylogeny-based classification of the Proterodiplostomidae. As the result of our molecular and morphological analyses: (i) the current subfamily structure of the Proterodiplostomidae is abolished; (ii) three new genera, *Paraproterodiplostomum* n. g., *Neocrocodilicola* n. g. and *Proteroduboisia* n. g., are described and *Pseudoneodiplostomoides* Yamaguti, 1954 is restored and elevated from subgenus to genus level; (iii) two new species, *Paraproterodiplostomum currani* n. g., n. sp. and *Archaeodiplostomum overstreeti* n. sp., are described from the American alligator in Mississippi, USA. Comparison of the structure of terminal ducts of the reproductive system in all proterodiplostomid genera did not support the use of these structures for differentiation among subfamilies (or major clades) within the family, although they proved to be useful for distinguishing among genera and species. Our study includes the first report of proterodiplostomids from Australia and the first evidence of a snake acting as a paratenic host for a proterodiplostomid. A key to proterodiplostomid genera is provided. Questions of proterodiplostomid-host associations parasitic in crocodilians are discussed in connection with their historical biogeography. Our molecular phylogeny of the Proterodiplostomidae closely matches the current molecular phylogeny of crocodilians. Directions for future studies of the Proterodiplostomidae are outlined.

Introduction

The Proterodiplostomidae Dubois, 1936 is a relatively small family of diplostomoidean digeneans parasitising the intestines of reptilian hosts associated with freshwater environments, mostly in the tropical and subtropical regions of the world. The greatest diversity of proterodiplostomids is found in crocodilians, although some parasitise snakes and turtles (Dubois, 1979; Niewiadomska, 2002). Members of the Proterodiplostomidae are characterised by the presence of a thin- or thick-walled tubule or pouch surrounded by glandular cells associated with the terminal ducts of their reproductive system called a paraprostate (Niewiadomska, 2002).

Dubois (1936) established the Proterodiplostomidae for diplostomids from reptiles which possessed a paraprostate. The early systems of the family proposed by Dubois (1936, 1951) were based on host associations and a wide range of morphological characters including size of the holdfast organ, presence or absence of papillae on the margins of the holdfast organ, distribution of vitelline follicles, and arrangement of terminal reproductive ducts. Dubois (1953) revisited the systematics of the family and separated the Proterodiplostomidae into two "super-subfamilies" based on host associations (crocodilians and chelonians *vs* snakes). Byrd & Reiber (1942) and later Brooks et al. (1992) proposed systematic revisions of the Proterodiplostomidae with a stronger emphasis on the organization of the terminal ducts of the reproductive system. However, Niewiadomska (2002) in her most recent revision of the Proterodiplostomidae viewed the revision by Brooks et al. (1992) as too preliminary to be broadly adopted as a basis for the current system of the family. According to Niewiadomska (2002), the Proterodiplostomidae includes 17 genera within five subfamilies: Massoprostatinae Yamaguti, 1958 (1 genus), Ophiodiplostominae Dubois, 1936 (2 genera), Polycotylinae Monticelli, 1888 (8 genera), Proalarioidinae Sudarikov, 1960 (1 genus) and Proterodiplostominae Dubois, 1936 (5 genera).

Members of the family are distributed on different continents and occur in some of the most ancient groups of amniotic tetrapods, thus representing an extremely interesting model for phylogenetic and phylogeographic studies. However, the current systematics and taxonomy of the Proterodiplostomidae as well as all existing phylogenetic reconstructions of the group (e.g. Brooks, 1979; Brooks & O'Grady, 1989) are morphology-based. The lack of a molecular phylogenetic assessment of the group has prevented us from addressing such intriguing questions as the patterns of their current and past geographical distribution, host-associations, or the monophyly of recognised taxa. Likewise, the true interrelationships among the genera within the Proterodiplostomidae remain completely unknown. In fact, the position of the Proterodiplostomidae among other digeneans was tested based only on DNA sequences obtained from metacercariae of only two species belonging to two of

the 17 currently accepted genera, with only weak support (Hernández-Mena et al., 2017; Queiroz et al., 2020). Molecular data are also important as an independent set of characters that may help to assess the relative taxonomic value of morphological characters traditionally used to outline and differentiate among proterodiplostomid taxa including peculiarities of organization of the reproductive system and structure of the holdfast organ.

While significant progress has been recently achieved in the molecular phylogenetics and systematics of the Diplostomoidea Poirier, 1886 and its major constituent lineages (e.g. Blasco-Costa & Locke, 2017; Hernández-Mena et al., 2017; Locke et al., 2018; Achatz et al., 2019b, c, d; Queiroz et al., 2020), the Proterodiplostomidae remains one of the only diplostomoidean families to receive very little attention in molecular phylogenetic studies. This can be partly explained by the logistic challenges of obtaining fresh material from hosts that are often protected and difficult to collect.

This study is focused on the proterodiplostomids of crocodilians. Based on the available descriptions, taxonomic revisions, and checklists (Dubois, 1979; Catto & Amato, 1994; Tellez, 2014), there are five named species of proterodiplostomids belonging to four genera reported from crocodilians in the Nearctic: *Archaeodiplostomum acetabulata* (Byrd & Reiber, 1942); *Crocodilicola pseudostoma* Willemoes-Suhm, 1870; *Polycotyle ornata* Willemoes-Suhm, 1870; *Pseudocrocodilicola americaniense* Byrd & Reiber, 1942; and *Pseudocrocodilicola georgiana* Byrd & Reiber, 1942. There are 11 species of proterodiplostomids belonging to seven genera known from crocodilians in the Neotropics: *Cr. pseudostoma*; *Cystodiplostomum hollyi* Dubois, 1936; *Herpetodiplostomum caimancola* (Dollfus, 1935); *Mesodiplostomum gladiolum* Dubois, 1936; *Paradiplostomum abbreviatum* (Brandes, 1888); *Prolecithodiplostomum constrictum* Dubois, 1936; *Proterodiplostomum breve* Catto & Amato, 1994; *Proterodiplostomum globulare* Catto & Amato, 1994; *Proterodiplostomum longum* (Brandes, 1888); *Proterodiplostomum medusae* (Dubois, 1936); *Proterodiplostomum tumidulum* Dubois, 1936; and *Pseudoneodiplostomum groschafti* Moravec, 2001. In the Afrotropics, there are only two species of proterodiplostomids belonging to a single genus that parasitise crocodilians: *Pseudoneodiplostomum bifurcatum* (Wedl, 1861) and *Pseudoneodiplostomum thomasi* (Dollfus, 1935). A further three species of proterodiplostomids parasitise crocodilians in the Indomalayan region, each belonging to a separate genus: *Capsulodiplostomum crocodilinum* Dwivedi, 1966; *Herpetodiplostomum gavialis* (Narain, 1930); and *Pseudoneodiplostomum siamense* (Poirier, 1886). No proterodiplostomids have been previously reported from crocodilians in Australia.

In this study, we collected numerous specimens of multiple proterodiplostomid species from four species of crocodilian hosts in Australia, Brazil, South Africa, and the USA, in addition to specimens of *Heterodiplostomum lanceolatum* Dubois, 1936 from a frog and a snake from Brazil. We use partial sequences of the nuclear large ribosomal subunit RNA gene (28S) and the mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) gene to analyse the phylogenetic position of the Proterodiplostomidae, test its monophyly, and examine the interrelationships among its constituent taxa. In addition, we erect three new genera and describe two new species of proterodiplostomids from the American alligator *Alligator mississippiensis* (Daudin), re-evaluate some current proterodiplostomid genera, and provide an updated key for the identification of proterodiplostomids to genus level.

Materials and methods

Several of the genera discussed in the present work have very similar spellings, which prevents the standard use of the first or first and second letters for abbreviation. As such, we use the following abbreviations to refer to genera: *Al.*, *Alligator* Cuvier; *Ar.*, *Archaeodiplostomum* Dubois, 1944; *Co.*, *Crocodylus* Laurenti; *Cr.*, *Crocodilicola* Poche, 1926; *Cy.*, *Cystodiplostomum* Dubois, 1936; *He.*, *Heterodiplostomum* Dubois, 1936; *Me.*, *Mesodiplostomum* Dubois, 1936; *Ne.*, *Neocrocodilicola* n. g.; *Pa.*, *Paradiplostomum* La Rue, 1926; *Pe.*, *Pseudoneodiplostomum* Dubois, 1936; *Po.*, *Polycotyle* Willemoes-Suhm, 1870; *Pp.*, *Paraproterodiplostomum* n. g.; *Pr.*, *Proterodiplostomum* Dubois, 1936; *Ps.*, *Pseudocrocodilicola* Byrd & Reiber, 1942; *Pt.*, *Proteroduboisia* n. g.; *Pu.*, *Pseudoneodiplostomoides* Yamaguti, 1954.

Morphological data

Adult or immature specimens belonging to the Proterodiplostomidae were collected from the intestines of the following hosts: *Al. mississippiensis* from the Pascagoula Wildlife Management Area, Jackson Co., Mississippi, USA (30°37′07.2″N, 88°37′08.9″W), between 2004 and 2015; yacare caiman *Caiman yacare* Daudin, yellow-bellied liophis snake *Erythrolamprus poecilogyrus* (Wied-Neuwied) and Cei's white-lipped frog *Leptodactylus chaquensis* Cei from Fazenda Retiro Novo, Pantanal, Municipality of Nossa Senhora do Livramento, Mato Grosso State, Brazil, in 2016 and 2019; spectacled caiman *Caiman crocodilus* Linnaeus from the vicinity near Iquitos, Peru in 2016 (kindly provided by Dr Stephen Bullard, Auburn University); Australian freshwater crocodile *Crocodylus johnstoni* Krefft from Daly River near Oolloo Crossing, Northern Territory, Australia (14°00.31′S, 131°14.46′E) in 2006; and Nile crocodile *Crocodylus niloticus* Laurenti from the Olifants River, Limpopo Province (24°3′S, 31°13′E) and Crocodile River, Mpumalanga Province, South Africa (25°27′S, 31°58′E) in 2010 and Flag Boshielo Dam, Marble Hall, Limpopo Province (24°51′00.5″S, 29°22′55.8″E), South Africa in 2016. In addition, a proterodiplostomid metacercaria was collected from the mesenteries of the Mississippi green water snake *Nerodia cyclopion* (Duméril, Bibron & Duméril) and an immature proterodiplostomid was obtained from the intestine of the banded water snake *Nerodia fasciata* (Linnaeus) from the Pascagoula Wildlife Management Area, Jackson Co., Mississippi, USA (30°38′16.5″N, 88°36′35.9″W) in 2011–2012 (Table 1). In most cases, live digeneans removed from the hosts were briefly rinsed in saline, killed with hot water, and fixed in 80% ethanol. Live digeneans from *Co. niloticus* were killed with hot saline, fixed in 10% formalin, and transferred to 70% ethanol. Dead digeneans from the frozen carcass of the Nile crocodile from Flag Boshielo Dam were immediately fixed in 80% ethanol. Specimens for light microscopy were stained with aqueous alum carmine according to Lutz et al. (2017). Specimens were identified and measured using an Olympus BX51 compound microscope (Tokyo, Japan), equipped with differential interference contrast optics, a digital camera and Rincon measurement software (Imaging Planet, Goleta, California, USA). Drawings were made under a Leica DMC 4500 microscope (Buffalo Grove, Illinois, USA) with the aid of a drawing tube. All measurements given in the text and tables are in micrometres.

Different authors referred to the two distinct body parts in diplostomoideans as prosoma/opisthosoma, or forebody/hindbody, or anterior/posterior segments. The latest revision by Niewiadomska (2002) in the "Keys to the Trematoda" used the terms forebody and hindbody for these body parts whereas a different meaning was given to the same terms in chapters on all other distome digeneans, which was somewhat confusing. To avoid confusion, we use the terms prosoma and opisthosoma (e.g. Achatz et al., 2019a, c) to reflect the fact that these parts of the body in diplostomoideans are not segments (e.g. unlike segments or proglottides in cestodes) and the terms forebody and hindbody are universally used to designate the parts of body posterior and anterior to the ventral sucker in distome digeneans. Our use of this terminology is also consistent with its use in similar situations among other invertebrates, e.g. arachnids.

Historically, the muscular structure surrounding one or more terminal parts of the reproductive system (e.g. the paraprostate, ejaculatory duct, hermaphroditic duct, metraterm or a combination of the above) in some proterodiplostomids was called a secondary muscular pouch, a muscular sac, a muscular bulb or a capsule. These terms were used without a proper definition or distinct separation between them. Since all these terms refer to structures with a somewhat similar organisation and topology, differing only in size or their level of development, we use the unified term "muscular pouch" for these structures.

Type- and voucher specimens are deposited in the collection of the Harold W. Manter Laboratory (HWML), University of Nebraska State Museum, Lincoln, NE, USA, or the Museu Paraense Emílio Goeldi (MPEG), Belém, Pará State, Brazil. For comparative purposes we examined specimens of *Cr. pseudostoma* from *Crocodylus moreletii* Duméril & Bibron collected in Mexico and deposited by Vernon Thatcher in the HWML (accession number 21420).

Molecular data

Genomic DNA was extracted from single specimens of worms according to the protocol described by

Table 1 List of proterodiplostomid species used in our phylogenetic analyses including their host species, geographical origin of material, morphological voucher numbers and GenBank accession numbers

Digenean taxa	Host species	Geographical origin	Life-cycle stage	Museum No.	GenBank ID		Reference
					28S	*cox*1	
Archaeodiplostomum overstreeti n. sp.	*Alligator mississippiensis*	USA[a]	Adult	HWML 216298; HWML 216299	MT622323	MT603590	Present study
Archaeodiplostomum overstreeti n. sp.	*Nerodia fasciata*	USA[b]	Immature	–	MT622324	MT603591	Present study
Archaeodiplostomum overstreeti n. sp.	*Nerodia cyclopion*	USA[b]	Metacercaria	–	MT622325	MT603592	Present study
Crocodillicola pseudostoma	*Rhamdia guatemalensis*	Mexico[c]	Metacercaria	CNHE 10423	MF398328	MF398317	Hernández-Mena et al. (2017)
Cystodiplostomum hollyi	*Caiman yacare*	Brazil[d]	Adult	HWML 216300; MPEG 00251-00253	MT622326-MT622329	MT603593-MT603595	Present study
Cystodiplostomum sp.	*Caiman yacare*	Brazil[d]	Adult	–	MT622330	MT603596	Present study
Heterodiplostomum lanceolatum	*Leptodactylus chaquensis*	Brazil[d]	Metacercaria	–	MT622331	MT603597	Present study
Heterodiplostomum lanceolatum	*Erythrolamprus poecilogyrus*	Brazil[d]	Adult	HWML 216301	–	MT603598	Present study
Heterodiplostomum lanceolatum	*Leptodactylus podicipinus*	Brazil[e]	Metacercaria	UFMG-TRE 114; AMP 07454-7479	MN149353	–	Queiroz et al. (2020)
Mesodiplostomum gladiolum	*Caiman yacare*	Brazil[d]	Adult	HWML 216302	MT622332	–	Present study
Neocrocodilicola georgiana[†]	*Alligator mississippiensis*	USA[a]	Adult	HWML 216303; HWML 216304	MT622333-MT622336	MT603599-MT603602	Present study
Paradiplostomum abbreviatum	*Caiman yacare*	Brazil[d]	Adult	HWML 216305; MPEG 00254-00255	MT622337	MT603603	Present study
Polycotyle ornata	*Alligator mississippiensis*	USA[a]	Adult	HWML 216306-216308	MT622338-MT622340	MT603604-MT603606	Present study
Proterodiplostomum longum	*Caiman crocodilus*	Peru[f]	Adult	HWML 216309	MT622341	MT603607	Present study
Proterodiplostomum medusae	*Caiman yacare*	Brazil[d]	Adult	HWML 216310; HWML 216311; MPEG 00258-00260	MT622342-MT622344	MT603608-MT603610	Present study
Proterodiplostomum sp.	*Caiman yacare*	Brazil[d]	Adult	HWML 216312	MT622345	MT603611	Present study
Proteroduboisia globulare[‡]	*Caiman yacare*	Brazil[d]	Adult	HWML 216313; MPEG 00256-00257	MT622346-MT622353	MT603612-MT603617	Present study
Pseudocrocodilicola americaniense	*Alligator mississippiensis*	USA[a]	Adult	HWML 216314	MT622354-MT622356	MT603618-MT603620	Present study

Table 1 continued

Digenean taxa	Host species	Geographical origin	Life-cycle stage	Museum No.	GenBank ID		Reference
					28S	*cox*1	
Pseudoneodiplostomoides crocodilarum	*Crocodylus johnstoni*	Australia[g]	Adult	HWML 216315	MT622357	MT603621	Present study
Pseudoneodiplostomum bifurcatum	*Crocodylus niloticus*	South Africa[h,i,j]	Adult	HWML 216316; HWML 216317	MT622358-MT622362	MT603622	Present study
Pseudoneodiplostomum gabonicum	*Crocodylus niloticus*	South Africa[h,j]	Adult	HWML 216318; HWML 216319	MT622363-MT622364	–	Present study
Pseudoneodiplostomum cf. *siamense*	*Crocodylus johnstoni*	Australia[g]	Adult	–	MT622365	MT603623	Present study
Pseudoneodiplostomum thomasi	*Crocodylus niloticus*	South Africa[h]	Adult	HWML 216320	MT622366-MT622367	MT603624-MT603625	Present study
Paraproterodiplostomum currani n. g., n. sp.	*Alligator mississippiensis*	USA[a]	Adult	HWML 216321-216323	MT622368-MT622369	MT603626	Present study

[†]Previously *Pseudocrocodilicola*; [‡]Previously *Proterodiplostomum*

[a] Pascagoula Wildlife Management Area, Jackson Co., Mississippi, USA (30°37′07.2″N, 88°37′08.9″W)

[b] Pascagoula Wildlife Management Area, Jackson Co., Mississippi, USA (30°38′16.5″N, 88°36′35.9″W)

[c] Catemaco Lake, Veracruz, Mexico (18°24′46.7″N, 95°6′33.8″W)

[d] Fazenda Retiro Novo, Pantanal, Municipality of Nossa Senhora do Livramento, Mato Grosso State, Brazil (16°21′53″S, 56°17′31″W)

[e] Vicinities near a riparian forest in Véstia Stream, Municipality of Selvíria, state of Mato Grosso do Sul, Brazil (20°23′43.57″S, 51°23′39.28″W)

[f] Vicinities near Iquitos, Peru

[g] Daly River near Oolloo Crossing, Northern Territory, Australia (14°00.31′S, 131°14.46′E)

[h] Crocodile River, Mpumalanga province, South Africa (25°27′S, 31°58′E)

[i] Olifants River, Limpopo province, South Africa (24°3′S, 31°13′E)

[j] Flag Boshielo Dam, Marble Hall, Limpopo Province, South Africa (24°51′00.5″S, 29°22′55.8″E)

Abbreviations: AMP, Coleção Zoológica (ZUFMS), Universidade Federal de Mato Grosso do Sul, Campo Grande, Mato Grosso do Sul, Brazil; HWML, Harold W. Manter Laboratory, Lincoln, Nebraska, USA; CNHE, Colección Nacional de Helmintos, Instituto de Biología, Universidad Nacional Autónoma de México, México City, Mexico; MPEG, Museu Paraense Emílio Goeldi, Belém, Brazil; UFMG: Museum of the Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

Tkach & Pawlowski (1999) or using a ZR Genomic DNA™ Tissue Micro Prep kit (Zymo Research, Irvine, California, USA) following the manufacturer's protocol. An approximately 1,300-bp long fragment at the 5′-end of the 28S gene was amplified by polymerase chain reactions (PCR) on a T100™ thermal cycler (Bio-Rad, Hercules, California, USA) using the forward primer digL2 (5′-AAG CAT ATC ACT AAG CGG-3′) and the reverse primer 1500R (5′-GCT ATC CTG AGG GAA ACT TCG-3′) (Tkach et al., 2003). A fragment of the *cox*1 gene was amplified using the forward primers Plat-diploCOX1F (5′-CGT TTR AAT TAT ACG GAT CC-3′) and Cox1_Schist_5′ (5′-TCT TTR GAT CAT AAG CG-3′) and the reverse primers Plat-diploCOX1R (5′-AGC ATA GTA ATM GCA GCA GC-3′), acox650R (5′-CCA AAA AAC CAA AAC ATA TGC TG-3′) and JB5 (5′-AGC ACC TAA ACT TAA AAC ATA ATG AAA ATG-3′) (Lockyer et al., 2003; Derycke et al., 2005; Moszczynska et al., 2009; Kudlai et al., 2015). PCRs were performed in a total volume of 25 µl using One-Taq quick load PCR mix from New England Biolabs (Ipswich, Massachusetts, USA) or 50 µl using GoTaq G2 DNA Polymerase from Promega (Madison, Wisconsin, USA) according to the manufacturer's instructions and using an annealing temperature of 53 °C for nuclear rRNA amplifications and 45 °C for *cox*1 amplifications.

PCR products were purified using ExoSAP-IT PCR clean-up enzymatic kit from Affymetrix (Santa Clara, California, USA) following the manufacturer's protocol. PCR products were cycle-sequenced directly using MCLab BrightDye® terminator chemistry (Molecular Cloning Laboratories, San Francisco, California, USA), cleaned using MCLab BigDye magnetic beads and run on an ABI 3130 automated capillary sequencer (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

PCR primers along with the 28S internal forward primer DPL600F (5′-CGG AGT GGT CAC CAC GAC CG-3′) and reverse primer DPL700R (5′-CAG CTG ATT ACA CCC AAA G-3′) and *cox*1 internal forward primer BS_CO1_IntF (5′-ATT AAC CCT CAC TAA ATG ATT TTT TTY TTT YTR ATG CC-3′) and reverse primer (5′-TAA TAC GAC TCA CTA TAA AAA AAA MAM AGA AGA RAA MAC MGT AGT AAT-3′) were used for sequencing reactions (Achatz et al., 2019a, d). Contiguous sequences were assembled using Sequencher version 4.2 software (GeneCodes Corp., Ann Arbor, Michigan, USA). Newly obtained sequences are deposited in the GenBank database (Table 1).

Sequences were initially aligned using ClustalW implemented in MEGA7 software (Kumar et al., 2016). The position of proterodiplostomid genera among other diplostomoidean families was studied using an alignment that included newly obtained 28S sequences of 12 proterodiplostomid taxa, previously published sequences of *Cr. pseudostoma*, *He. lanceolatum*, 17 representatives of the Diplostomidae Poirier, 1886, and 13 taxa of the Strigeidae Railliet, 1919. *Suchocyathocotyle crocodili* (Yamaguti, 1954) was used as an outgroup based on the phylogeny published by Achatz et al. (2019d).

Interrelationships within the Proterodiplostomidae were studied using a second alignment of 28S sequences along with an alignment of *cox*1 sequences. *Alaria mustelae* Bosma, 1931 was as the outgroup in both alignments based on the previously published phylogenies and the results of our phylogeny based on the first 28S alignment (see above). The second alignment of the Proterodiplostomidae included newly obtained sequences of 19 proterodiplostomid species and previously published sequences of *Cr. pseudostoma* and *He. lanceolatum*. The *cox*1 alignment included newly obtained sequences of 18 proterodiplostomid species and a single previously published sequence of *Cr. pseudostoma*. Despite all our efforts, we were unable to successfully amplify and sequence *cox*1 for *Me. gladiolum* and *Pseudoneodiplostomum gabonicum* Dubois, 1948.

Phylogenetic analyses were conducted using Bayesian inference (BI) as implemented in MrBayes Ver. 3.2.6 software (Ronquist & Huelsenbeck, 2003). The general time-reversible model with estimates of invariant sites and gamma-distributed among-site variation (GTR + I + G) was identified as the best-fitting nucleotide substitution model for all datasets using MEGA7. Bayesian inference analysis for both 28S datasets were performed using MrBayes software as follows: Markov chain Monte Carlo (MCMC) chains were run for 6,000,000 generations with sample frequency set at 1,000. Bayesian inference analysis for the *cox*1 dataset 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 1,000. Log-likelihood scores were plotted and only the final 75% of trees

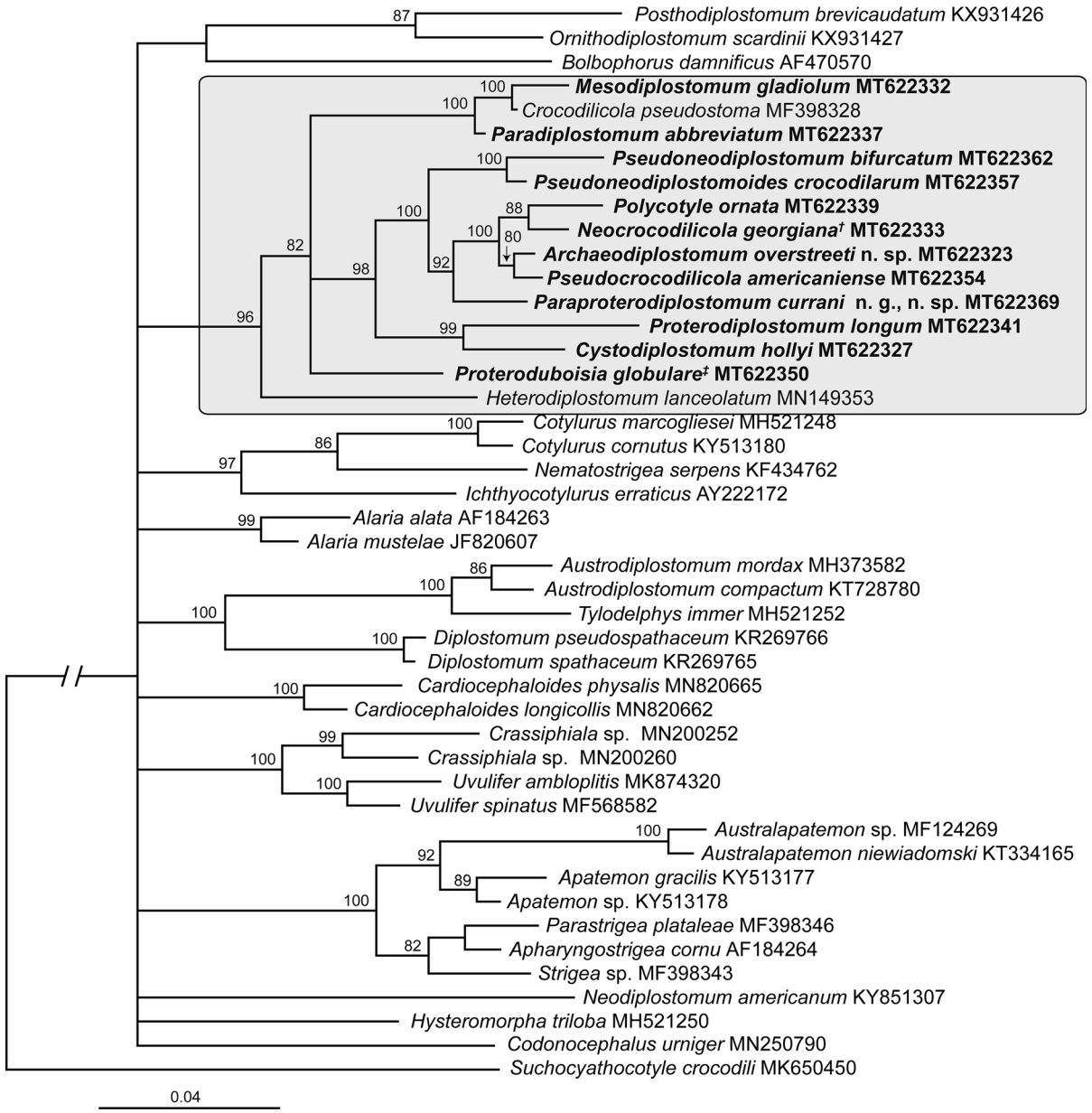

Fig. 1 Phylogenetic relationships between the taxa of the Diplostomoidea resulting from Bayesian inference (BI) analysis based on the partial sequences of the nuclear 28S rRNA gene. Bayesian inference posterior probability values are shown above branches; support values lower than 0.80 (80%) are not shown. GenBank accession numbers are provided after the names of species. The scale-bar indicates the number of substitutions per site. Newly generated sequences are highlighted in bold; shaded rectangle indicates the taxa belonging to the Proterodiplostomidae. † Previously *Pseudocrocodilicola*; ‡ Previously *Proterodiplostomum*

were used to produce the consensus trees. The number of generations for each analysis was considered sufficient as the standard deviation stabilised below 0.01 in all analyses. Pairwise sequence comparisons were done for sequences included in both 28S and *cox*1 analyses with assistance of MEGA7 software.

To comply with the regulations set out in Article 8.5 of the amended 2012 version of the *International Code of Zoological Nomenclature* (ICZN, 2012), details of all new taxa have been submitted to ZooBank. For each new taxon, the Life Science Identifier (LSID) is reported in the taxonomic summary.

Results

Molecular phylogeny

Upon trimming to the length of the shortest sequence obtained from GenBank, the first 28S alignment, which included proterodiplostomids along with members of other diplostomoidean families, was 1,104 bp long; 19 nucleotide positions were excluded due to ambiguous homology. In the phylogenetic tree resulting from the BI analysis, all members of the Proterodiplostomidae formed a strongly supported (96%) monophyletic clade (Fig. 1). This clade was overall very well resolved with high support for almost all topologies. *Heterodiplostomum lanceolatum* formed a sister branch to all other members of the Proterodiplostomidae, although the latter cluster had a somewhat low support (82%). A more detailed analysis of the interrelationships within the Proterodiplostomidae is provided below. Similar to other recent molecular phylogenies of the Diplostomoidea, the currently accepted Diplostomidae and Strigeidae were non-monophyletic. This was demonstrated and discussed in several recent studies (e. g. Blasco-Costa & Locke, 2017; Hernández-Mena et al., 2017; Locke et al., 2018; Achatz et al., 2019b, c, d, 2020; Queiroz et al., 2019); therefore, we do not describe details here.

The second 28S alignment containing only proterodiplostomids was 1,102 bp long after trimming to the length of the shortest sequence; 15 nucleotide positions were excluded due to ambiguous homology and indels. The phylogenetic tree resulting from the BI analysis of the second 28S alignment was well-resolved, except for a basal polytomy which included four strongly supported clades (Fig. 2).

The highly supported (99%) clade I contained the majority of proterodiplostomid taxa and was divided into two major sub-clades. The first major sub-clade of clade I included all Nearctic species collected from

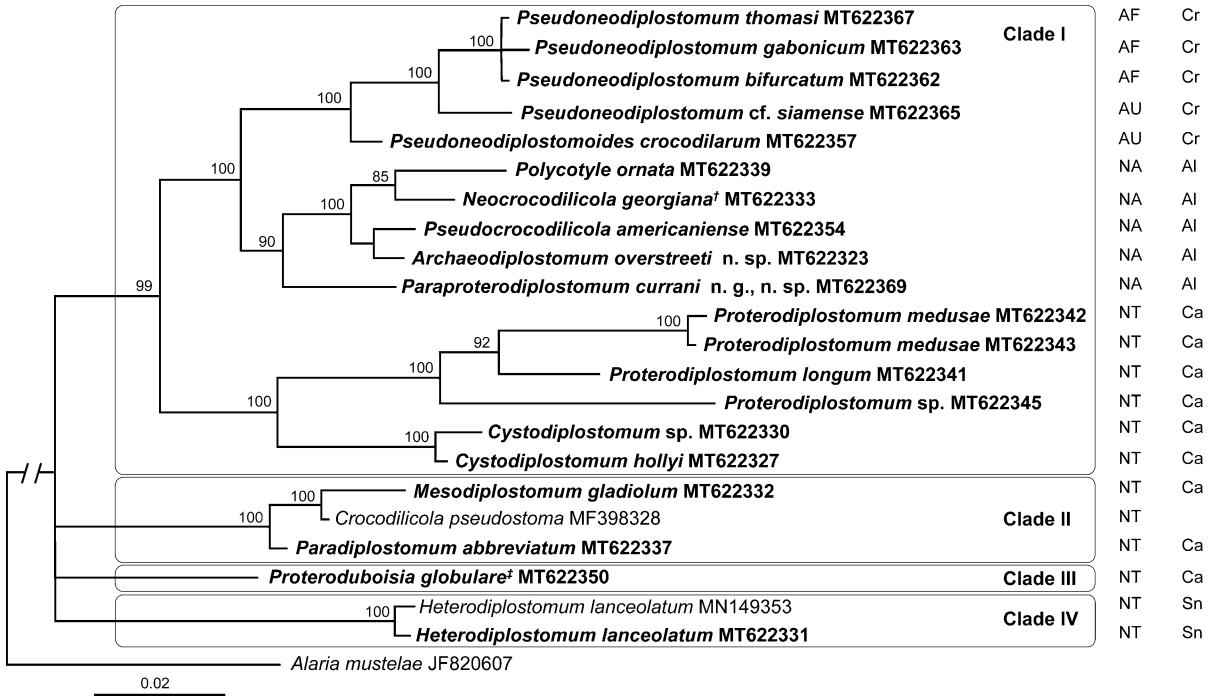

Fig. 2 Phylogenetic relationships between the taxa of the Proterodiplostomidae resulting from Bayesian inference (BI) analysis based on the partial sequences of the nuclear 28S rRNA gene. Bayesian inference posterior probability values are shown above branches; support values lower than 0.80 (80%) are not shown. The scale-bar indicates the number of substitutions per site. Newly generated sequences are highlighted in bold; rectangles indicate the four major monophyletic clades. GenBank accession numbers are provided after the names of species. Biogeographical realms and definitive host groups are indicated in two columns on the right. Abbreviations for biogeographical realms: AF, Afrotropical realm; AU, Australasian realm; NA, Nearctic realm; NT, Neotropical realm. Abbreviations for definitive host groups: Cr, true crocodiles (*Crocodylus*), Al, alligators, Ca, caimans, Sn, snakes. † Previously *Pseudocrocodilicola*; ‡ Previously *Proterodiplostomum*

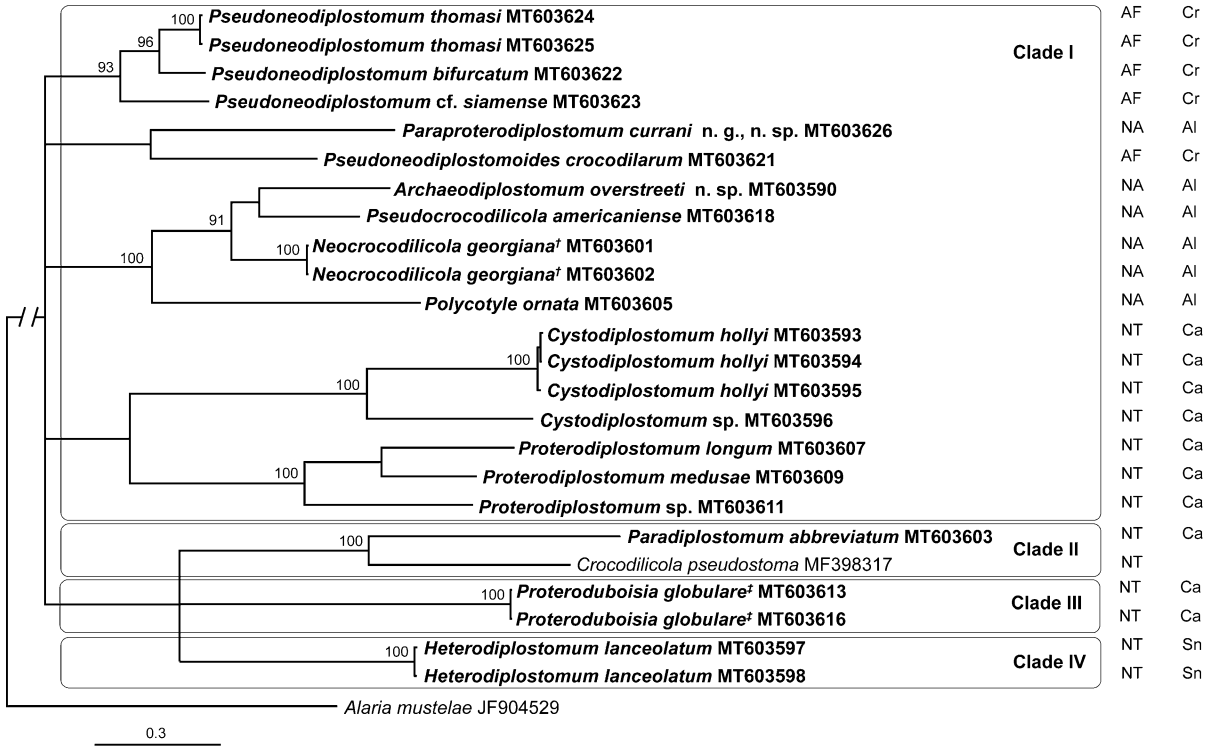

Fig. 3 Phylogenetic relationships between the taxa of the Proterodiplostomidae resulting from Bayesian inference (BI) analysis based on the partial sequences of the mitochondrial *cox*1 gene. Bayesian inference posterior probability values are shown above branches; support values lower than 0.80 (80%) are not shown. The scale-bar indicates the number of substitutions per site. Newly generated sequences are highlighted in bold; rectangles indicate the taxa belonging to 4 major monophyletic clades in the 28S tree. GenBank accession numbers are provided after the names of species. Biogeographical realms and definitive host groups are indicated in two columns on the right. Abbreviations for biogeographical realms: AF, Afrotropical realm; AU, Australasian realm; NA, Nearctic realm; NT, Neotropical realm. Abbreviations for definitive host groups: Cr, true crocodiles (*Crocodylus*), Al, alligators, Ca, caimans, Sn, snakes. † Previously *Pseudocrocodilicola*; ‡ Previously *Proterodiplostomum*

American alligators in Mississippi (91% support) and species of a clade of *Pseudoneodiplostomoides* and *Pseudoneodiplostomum* from crocodiles in Africa and Australia. Within the clade of proterodiplostomids from alligators, *Paraproterodiplostomum currani* n. g., n. sp. formed a sister branch to a 100% supported clade comprising the remaining taxa (Fig. 2). Among those, *Po. ornata* + *Neocrocodilicola georgiana* n. comb. (previously in *Pseudocrocodilicola*; see discussion below) formed a rather weakly supported clade (85%), whereas *Ps. americaniense* and *Archaeodiplostomum overstreeti* n. sp. formed a clade without meaningful support.

Pseudoneodiplostomoides crocodilarum (Tubangui & Masiluñgan, 1936) collected from Australian freshwater crocodiles formed a sister branch (100% support) to *Pseudoneodiplostomum* spp. in the clade of proterodiplostomids collected from *Co. johnstoni* and *Co. niloticus*, correspondingly. *Pseudoneodiplostomum* cf. *siamense* collected from Australian freshwater crocodiles formed a sister branch (100% support) to a strongly supported clade (100%) including the three species of *Pseudoneodiplostomum* from Nile crocodiles in South Africa.

The second major sub-clade of clade I (100% support) included members of *Cystodiplostomum* and *Proterodiplostomum* from caimans in the Neotropics. Members of each of the two genera formed corresponding 100% supported clades. Within the *Proterodiplostomum* clade, the sequence of an unidentified, immature *Proterodiplostomum* sp. formed a sister branch to the 92% supported clade of *Pr. longum* + *Pr. medusae*.

The 100% supported clade II included *Pa. abbreviatum* that appeared basal to the 100% supported group of *Cr. pseudostoma* + *Me. gladiolum*. Clade III

included only *Proteroduboisia globulare* n. comb. (previously in *Proterodiplostomum*; see discussion below) from a caiman collected in Pantanal, Brazil, while the strongly supported (100%) small clade IV comprised two species of *Heterodiplostomum* from a frog and snake in Brazil (Fig. 2).

Upon trimming to the length of the shortest sequence, the *cox*1 mtDNA alignment was 520 bp long; no sites were excluded from the analysis. In the phylogenetic tree resulting from the BI analysis (Fig. 3), the topology of the Proterodiplostomidae was much less resolved and differed slightly from the topology in the 28S analyses. Clades II, III and IV remained the same as in the 28S tree, but clade I split into 6 independent (if low support values are ignored) clades in a polytomy in the *cox*1 tree. The majority, but not all, of the well-supported clades in the *cox*1 tree represented individual proterodiplostomid genera, namely: (i) two *Cystodiplostomum* species (100%); (ii) three *Proterodiplostomum* spp. (100%); (iii) *Pt. globulare* n. comb.; (iv) *He. lanceolatum*; (v) *Pa. abbreviatum* + *Cr. pseudostoma* (100%); (vi) *Po. ornata* + *Ne. georgiana* n. comb. + *Ps. americaniense* + *Ar. overstreeti* n. sp. (100%); (vii) *Pseudoneodiplostomum* spp. (93%); (viii) *Pp. currani* n. g., n. sp.; and (ix) *Pu. crocodilarum* (Fig. 3).

It is worth noting that *Ps. americaniense* and *Ne. georgiana* n. comb. (previously in *Pseudocrocodilicola*; see discussion below) formed a 91% supported clade with *Ar. overstreeti* n. sp.; however, the internal topology within this clade was unresolved.

The 3 sequences of *Cy. hollyi* along with the 2 sequences each of *Pt. globulare* n. comb., *He. lanceolatum*, *Ne. georgiana* n. comb. and *Pe. thomasi* formed their own respective 100% supported clades (Fig. 3).

Genetic variation

The pairwise nucleotide comparison of proterodiplostomid sequences of 28S (Supplementary Table S1) showed an overall low divergence among genera (0.5–6.6% or 5–73 bases out of 1,106). The pairs *Ar. overstreeti* n. sp./*Ps. americaniense* and *Cr. pseudostoma/Pa. abbreviatum* had the lowest intergeneric divergence difference in the 28S sequences (0.5% or 5–6 bases). The greatest intergeneric divergence in the 28S sequences (6.6%) was found in the pairs *Pr. medusae* (GenBank: MT622342)/*He. lanceolatum* (MN149353), *Me. gladiolum/Pr. longum* and *Me. gladiolum/Pr. medusae* (GenBank: MT622342).

The interspecific genetic divergence among congeneric species in the 28S sequences varied greatly across different genera. Our two *Cystodiplostomum* species showed only 0.4% (4 bases) difference in their 28S sequences and *Pseudoneodiplostomum* species demonstrated the lowest interspecific divergence in the 28S sequences among congeners at 0–1% or 0–11 bases (Supplementary Table S1). At the same time, members of *Proterodiplostomum* as currently accepted, differed by 2.3–4.3% (25–48 bases) of their 28S sequences (Supplementary Table S1).

We did not detect any intraspecific variation in 28S sequences in the majority of species with multiple sequenced specimens, namely *Ar. overstreeti* n. sp. (n = 3), *Po. ornata* (n = 3), *Pp. currani* n. g., n. sp. (n = 2), *Pt. globulare* n. comb. (n = 5), *Ps. americaniense* (n = 3), *Ne. georgiana* n. comb. (n = 4), *Pe. thomasi* (n = 2), and *Pe. bifurcatum* (n = 5). Only one specimen of *Pr. medusae* (GenBank: MT622342) had a single unambiguous base pair difference compared to GenBank: MT622343 and MT622344. It is worth noting our new 28S sequence of *He. lanceolatum* (GenBank: MT622331) and the previously published sequence of *He. lanceolatum* (GenBank: MN149353) differ by 0.2% (2 bases).

In contrast, *cox*1 sequences demonstrated much greater intergeneric variation ranging from 10.4% (54 bases) between *Ps. americaniense* and *Ne. georgiana* n. comb. to 24.8% (129 bases) between *He. lanceolatum* and *Cystodiplostomum* sp. The intrageneric divergence in *cox*1 sequences ranged from 6.7% (35 bases) between *Pe. thomasi* and *Pe. bifurcatum* to 16.5% (86 bases) between *Proterodiplostomum* sp. and *Pr. longum* (Supplementary Table S2).

No intraspecific variation was detected among *cox*1 sequences of *Ar. overstreeti* n. sp., *Cr. pseudostoma*, *Po. ornata*, *Pr. medusae*, *Pt. globulare* n. comb. and *Ps. americaniense*. In species that demonstrated intraspecific variation in *cox*1, it was dramatically lower than the lowest levels of interspecific divergence and varied between 0.2% and 0.6% (1–3 bases) in *Cy. hollyi*, *He. lanceolatum* and *Ne. georgiana* n. comb. (Supplementary Table S2).

Descriptions of new taxa

Results of our molecular phylogenetic analysis and morphological examination of freshly collected high-quality specimens of proterodiplostomids have revealed the presence of two new species and a new genus in our material from American alligators. Their descriptions are provided below.

Paraproterodiplostomum Tkach, Achatz & Pulis n. g.

Diagnosis
Body bipartite; prosoma elliptical; opisthosoma elongate, cylindrical. Oral and ventral suckers well-developed; pseudosuckers absent; holdfast organ large, elliptical, protruding from prosoma. Pharynx moderately developed; caeca extending to near posterior end of opisthosoma. Testes 2, tandem, similar in size, mostly located in last third of opisthosoma. Paraprostate well-developed, claviform; ejaculatory duct joins paraprostate near its distal end to form common male efferent duct that opens into genital atrium. Ovary pretesticular. Vitellarium extends from approximately level of ventral sucker to past posterior testis. Metraterm opens separately from common male efferent duct into genital atrium. Genital atrium opening subterminal on dorsal side. Excretory pore terminal. Nearctic. In *Alligator mississippiensis.*
Type- and only species: Paraproterodiplostomum currani n. g., n. sp.
ZooBank registration: The Life Science Identifier (LSID) for *Paraproterodiplostomum* n. g. is urn:lsid:zoobank.org:act:07E1D2C7-D8EB-43A1-89CD-826B9678E6B2.
Etymology: The name of the new genus reflects its morphological similarity to *Proterodiplostomum*.

Paraproterodiplostomum currani Tkach, Achatz & Pulis n. sp.

Type-host: *Alligator mississippiensis* (Daudin) (Crocodilia: Alligatoridae).
Type-locality: Pascagoula Wildlife Management Area (30°37′07.2″N, 88°37′08.9″W), Jackson Co., Mississippi, USA.
Type-material: The type-series consists of 9 fully mature specimens deposited in the HWML. Holotype: HWML 216321, labelled ex *Alligator mississippiensis*, small intestine, Pascagoula wildlife management area, Jackson Co., Mississippi, USA, 10.vii.2015, coll. V. Tkach. Paratypes: HWML 216322, 216323 (lot of 8 slides), labels identical to the holotype.
Site in host: Small intestine.
ZooBank registration: The Life Science Identifier (LSID) for *Paraproterodiplostomum currani* n. sp. is urn:lsid:zoobank.org:act:7B9637A3-C820-48EF-8F22-9CB98C66E4E7.
Etymology: The species is named after Dr Stephen Curran in recognition of his contributions to trematodology, particularly to our knowledge of the trematodes in the Gulf of Mexico and the Gulf Coast, and his invaluable help and camaraderie in numerous collecting trips in the region and beyond.

Description

[Based on 9 adult specimens; measurements of the holotype are given in text; measurements of the entire series are given in Table 2; see Fig. 4.] Body 6,208 long, consisting of distinct prosoma and opisthosoma; prosoma elliptical, 2,031 long, with maximum width at level of holdfast organ, 965; opisthosoma elongate, cylindrical, 4,177 × 421. Prosoma:opisthosoma length ratio 0.49. Minuscule scale-like tegumental spines covering anterior part of prosoma almost to level of anterior margin of holdfast organ. Oral sucker subterminal, 111 × 119. Pseudosuckers absent. Ventral sucker slightly larger than oral sucker, 131 × 139, located near mid-length of prosoma; oral:ventral sucker width ratio 1:1.17. Holdfast organ posterior to ventral sucker, protruding from prosoma; subspherical or oval with ventral muscular portion, highly variable in shape, occupying almost entire width and of prosoma, 941 × 961. Holdfast organ equal to 46% of prosoma length. Proteolytic gland extensive, located at base of holdfast organ. Prepharynx not observed. Pharynx oval, 100 × 80. Oesophagus slightly longer than pharynx. Caecal bifurcation in anterior third of prosoma; caeca slender, extending to near posterior end of opisthosoma.

Testes 2, tandem, entire, mostly located in posterior third of opisthosoma; anterior testis 395 × 324, posterior testis 459 × 312. Seminal vesicle post-testicular, compact, coiled, ventral to posterior testis, continuing as ejaculatory duct before connecting to base of paraprostate to form common male efferent duct. Paraprostate well-developed, claviform, 415 × 128, with proximal end reaching close to posterior

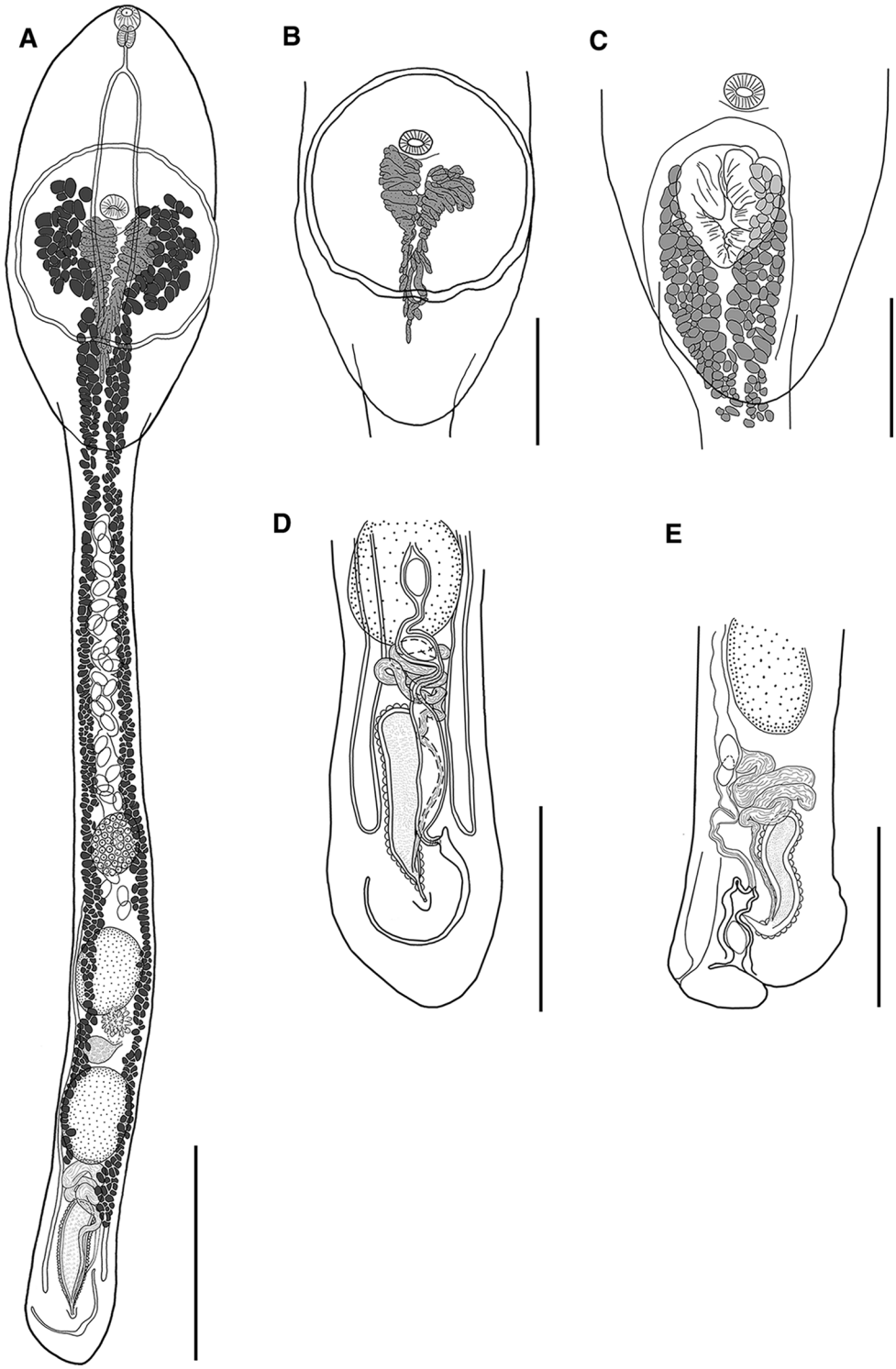

Fig. 4 *Paraproterodiplostomum currani* n. sp. A, Ventral view of the holotype; B, Proteolytic gland in the holotype; C, Proteolytic gland in a paratype; D, Posterior end of a paratype showing terminal ducts of the reproductive system, ventral view; E, Posterior end of a paratype showing terminal ducts of the reproductive system, lateral view. *Scale-bars*: A, 1 mm; B–E, 500 μm

Table 2 Metric characters of the two new proterodiplostomid species from Mississippi

Character	*Paraproterodiplostomum currani* n. sp. (n = 9)			*Archaeodiplostomum overstreeti* n. sp. (n = 4)		
	Mean ± SD	Range	CV	Mean ± SD	Range	CV
Total body length	5,779 ± 430	5,210–6,466	7.4	6,753 ± 776	6,109–7,706	11.5
Prosoma length	2,139 ± 148	1,947–2,362	6.9	3,260 ± 163	3,063–3,418	5.0
Prosoma width	924 ± 97	833–1,108	10.5	929 ± 28	905–959	3.0
Opisthosoma length	3,736 ± 328	3,368–4,198	8.8	3,561 ± 581	2,915–4,288	16.3
Opisthosoma width	401 ± 37	354–451	9.2	349 ± 37	318–390	10.6
Prosoma:opisthosoma length	0.58 ± 0.1	0.49–0.65	10.0	0.93 ± 0.12	0.8–1.1	13.3
Forebody length	1,015 ± 135	857–1,308	13.3	1,371 ± 64	1,293–1,430	4.7
Forebody:body length	0.18 ± 0.03	0.15–0.25	18.9	0.2 ± 0.02	0.18–0.22	7.6
Oral sucker length	101 ± 7	90–111	7.0	145 ± 15	128–163	10.5
Oral sucker width	111 ± 6	105–120	5.7	149 ± 7	142–156	4.7
Ventral sucker length	116 ± 14	99–132	11.6	442 ± 46	409–508	10.5
Ventral sucker width	136 ± 17	120–164	12.2	444 ± 39	412–488	8.8
Oral sucker:ventral sucker width ratio	1:1.23 ± 0.16	1:1.04–1.50	13.1	1:2.85 ± 0.42	1:2.44-3.44	14.6
Holdfast organ length	716 ± 158	540–941	22.0	522 ± 51	493–598	9.7
Holdfast organ width	695 ± 180	533–961	25.9	416 ± 43	387–465	10.3
Holdfast organ:prosoma length	0.34 ± 0.08	0.24–0.46	23.82	0.16 ± 0.01	0.14–0.18	8.8
Anterior margin of holdfast positioned at (% of prosoma length)	0.45 ± 0.11	0.31–0.56	25.0	0.7 ± 0.04	0.65–0.74	10.3
Distance between ventral sucker and holdfast organ:prosoma length	0.04 ± 0.05	0–0.12	128.3	0.28 ± 0.04	0.23–0.31	13.8
Pharynx length	105 ± 7	98–118	6.7	94 ± 14	79–106	14.6
Pharynx width	83 ± 4	78–89	4.8	88 ± 4	85–93	4.7
Esophagus length	114 ± 31	67–162	27.2	132 ± 28	107–163	21.4
Anterior testis length	304 ± 50	221–395	16.5	215 ± 28	176–243	13.1
Anterior testis width	252 ± 35	218–324	13.8	220 ± 19	199–236	8.6
Posterior testis length	350 ± 51	287–459	14.7	227 ± 30	193–248	13.0
Posterior testis width	259 ± 32	221–312	12.5	224 ± 26	199–250	11.4
Distance between posterior margin of posterior testis and end of body:opisthosoma length	0.23 ± 0.03	0.20–0.27	11.3	0.52 ± 0.05	0.46–0.57	9.0
Seminal vesicle length	880 ± 83	776–978	9.4	1,279 ± 472	822–1,874	36.9
Paraprostate length	398 ± 41	344–479	10.2	235 ± 19	218–255	7.9
Paraprostate width	125 ± 15	102–143	11.7	108 ± 6	101–112	5.9
Ovary length	272 ± 32	215–324	11.8	114 ± 15	99–129	13.6
Ovary width	194 ± 19	162–228	10.0	112 ± 14	96–129	12.1
Metraterm length	262 ± 48	228–296	18.4	321 ± 34	285–352	10.5
Egg number	20.6 ± 13.1	1–36	63.7	60 ± 23	29–84	38.3
Egg length	92 ± 3	85–96	3.1	89 ± 6	78–97	6.5
Egg width	55 ± 4	46–59	7.1	51 ± 3	47–55	6.4
Anterior vitellarium-free zone:prosoma length	0.54 ± 0.13	0.41–0.77	24.8	0.52 ± 0.04	0.48–0.56	7
Posterior vitellarium-free zone:opisthosoma length	0.22 ± 0.05	0.15–0.29	22.9	0.17 ± 0.02	0.15–0.19	10.8

Abbreviations: CV, coefficient of variation; SD, standard deviation

testis, surrounded by gland cells. Common male efferent duct opening into genital atrium separately from female opening.

Ovary pretesticular, oval or subspherical 287 × 228. Oötype, Mehlis' gland and uterine seminal receptacle inter-testicular. Vitelline follicles located around holdfast organ in prosoma and extending posteriorly to about level of paraprostate, ventral and lateral to gonads. Vitelline reservoir intertesticular. Uterus ventral to gonads, extending anteriorly from ovary to near junction of prosoma and opisthosoma before turning and extending posteriorly. Metraterm opening into genital atrium separately from common male efferent duct; genital atrium opening subterminal on dorsal side. Uterus contains numerous eggs (85–96 × 46–59). Genital atrium subterminal, on dorsal side.

Excretory vesicle not well-observed. Excretory pore terminal.

Remarks

The new genus can be differentiated from all other known proterodiplostomid genera based on a range of morphological characters. *Paraproterodiplostomum currani* n. g., n. sp. differs from *Heterodiplostomum* by the lack of a muscular pouch surrounding the paraprostate (Figs. 4, 5G, S); additionally, *Pp. currani* n. g., n. sp. differs from *He. lanceolatum* by 4.2% (36 bases) in the 28S sequence nucleotide positions and up to 20.8% (107 bases) in *cox*1 sequences (Supplementary Tables S1, S2). The new genus can be readily differentiated from *Capsulodiplostomum* Dwivedi, 1966 due to the lack of the muscular pouch enclosing the paraprostate, ejaculatory duct and metraterm. Unlike the members of *Mesodiplostomum* and *Proalarioides* Yamaguti, 1933 which lack a visible paraprostate, the new genus has a well-developed paraprostate (Figs. 4, 5G, T, U). The new genus and *Me. gladiolum* differ by 3.8% (42 bases) in the 28S sequences (Supplementary Table S1). *Paraproterodiplostomum currani* n. g., n. sp. is readily distinguishable from *Ophiodiplostomum* Dubois, 1936 species based on the relative size of the holdfast organ. In *Pp. currani* n. g., n. sp. the holdfast organ occupies on average 34% (24–46%) of the prosoma, while the holdfast organ of *Ophiodiplostomum* species is relatively larger and occupies approximately half of the prosoma length.

The terminal ducts of the male and female reproductive systems in *Pp. currani* n. g., n. sp. open separately into the genital atrium. In contrast, the metraterm of *Archaeodiplostomum*, *Crocodilicola*, *Polycotyle,* and *Pseudocrocodilicola* species joins the common male efferent duct prior to reaching the genital atrium (Fig. 5A–C, E–G). Whereas the ejaculatory duct in the new genus joins the paraprostate, in *Cheloniodiplostomum* Sudarikov, 1960, *Cystodiplostomum*, *Herpetodiplostomum* Dubois, 1936, *Massoprostatum* Caballero, 1948, *Paradiplostomum*, and *Prolecithodiplostomum* Dubois, 1936 the paraprostate opens separately from the ejaculatory duct and metraterm (Figs. 4, 5G, M–O, Q, R). The new genus differs from *Archaeodiplostomum*, *Crocodilicola*, *Polycotyle*, *Pseudocrocodilicola*, *Cystodiplostomum* and *Paradiplostomum* spp. by 1.5–3.9% (16–43 bases) in the 28S sequences and 17.3–22.3 % (90–116 bases) in the *cox*1 sequences (Supplementary Tables S1, S2).

Paraproterodiplostomum currani n. g., n. sp. clearly differs from *Proterodiplostomum* species by the absence of the sucker-like structure in the genital atrium. Furthermore, the ejaculatory duct of *Paraproterodiplostomum* n. g. joins the paraprostate at its base, whereas the ejaculatory duct of *Proterodiplostomum* does not join the paraprostate. However, the ejaculatory duct of *Proterodiplostomum* may later join the efferent duct of the paraprostate (Figs. 4, 5G, J, K). In addition, the sequences of the new genus demonstrate significant differences from *Proterodiplostomum* species in both the 28S (4.8–5.4% or 53–60 bases) and *cox*1 (19–21.3% or 99–116 bases) genes (Supplementary Tables S1, S2).

The new genus has a well-developed paraprostate compared to the relatively small and weaker developed paraprostate in *Pseudoneodiplostomum.* The two genera can be further differentiated based on the position of the ejaculatory duct and paraprostate juncture. In *Pp. currani* n. g., n. sp. the ejaculatory duct joins the paraprostate at its base, whereas in members of *Pseudoneodiplostomum* the ejaculatory duct joins the paraprostate between its midlength and proximal (anterior) end (Figs. 4, 5G, H). In addition, the new genus differs from members of *Pseudoneodiplostomum* by 2.6–2.7% (29–30 bases) in the 28S sequences and by 16.3–18.1% (85–94 bases) in the *cox*1 sequences (Supplementary Tables S1, S2).

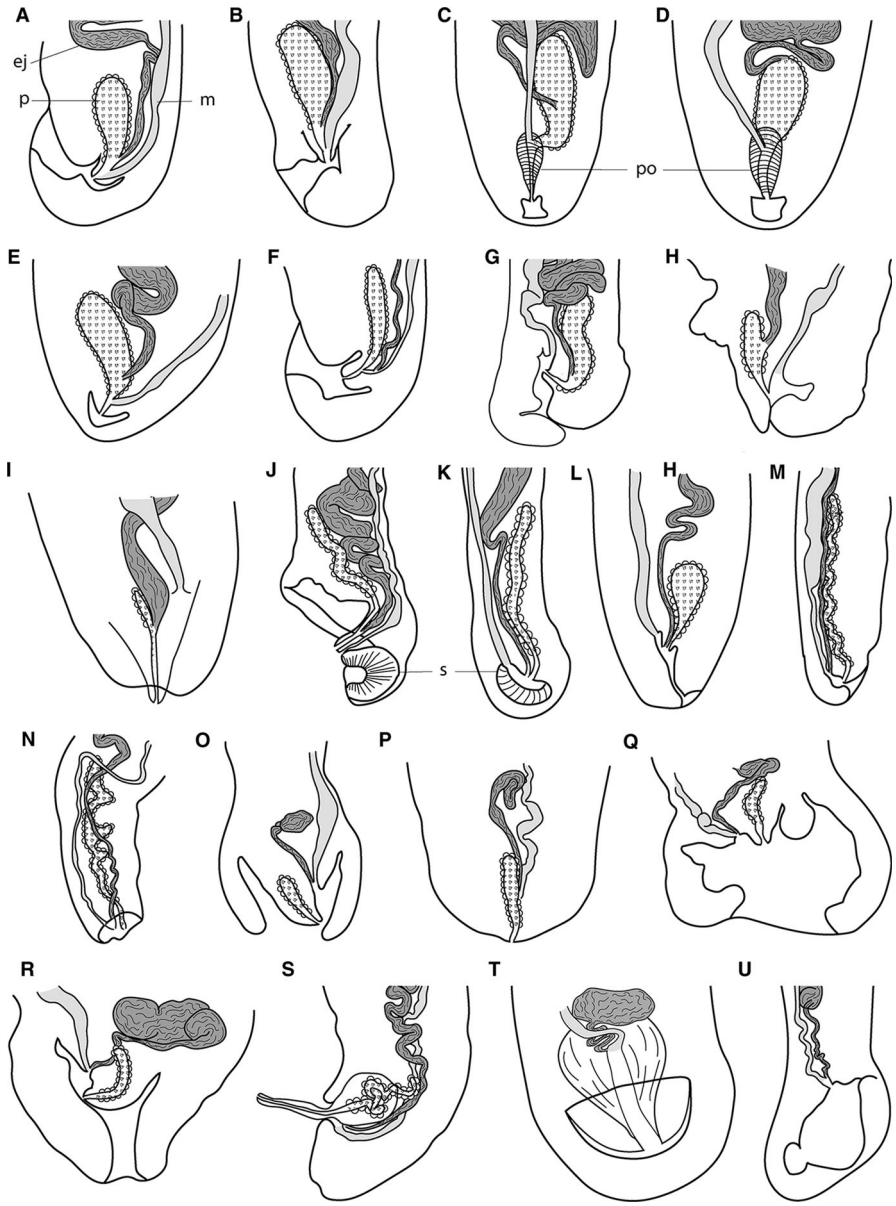

Fig. 5 Topologies of terminal reproductive ducts of representative species of all currently accepted proterodiplostomid genera with the exception of *Capsulodiplostomum* and *Cystodiplostomum*. *Capsulodiplostomum* was omitted due to a lack of any previously published, quality illustrations of the terminal ducts. *Cystodiplostomum* was not drawn separately as the topology of its terminal reproductive ducts is identical to *Prolecithodiplostomum constrictum*. A, *Archaeodiplostomum acetabulatum*, lateral view; B, *Archaeodiplostomum overstreeti* n. sp., lateral view; C, *Pseudocrocodilicola americaniense*, ventral view; D, *Neocrocodilicola georgiana* n. comb., ventral view; E, *Crocodilicola pseudostoma*, ventral view; F, *Polycotyle ornata*, lateral view; G, *Paraproterodiplostomum currani* n. g., .n. sp., lateral view; H, *Pseudoneodiplostomum gabonicum*, lateral view; I, *Pseudoneodiplostomoides crocodilarum*, dorsal view; J, *Proterodiplostomum longum*, lateral view; K, *Proterodiplostomum medusae*, lateral view; L, *Proteroduboisia globulare* n. comb., lateral view; M, *Prolecithodiplostomum constrictum*, lateral view; N, *Massoprostatum longum*, ventral view; O, *Paradiplostomum abbreviatum*, lateral view; P, *Ophiodiplostomum spectabile*, dorsal view; Q, *Cheloniodiplostomum testudinis*, lateral view; R, *Herpetodiplostomum caimancola*, lateral view; S, *Heterodiplostomum lanceolatum*, lateral view; T, *Proalarioides serpentis*, ventral view; U, *Mesodiplostomum gladiolum*, lateral view. A, C–F, after Byrd & Reiber (1942); H, after Dubois (1948); I, after Yamaguti (1954); J, M, P–S, U, after Dubois (1936); K, L, O, after Catto & Amato (1994); N, T, after Sudarikov (1960). *Abbreviations*: b, muscular bulb; ej, ejaculatory duct; m, metraterm; p, paraprostate; s, muscular sucker-like structure

***Archaeodiplostomum overstreeti* Tkach, Achatz & Pulis n. sp.**

Type-host: *Alligator mississippiensis* (Daudin) (Crocodilia: Alligatoridae).
Type-locality: Pascagoula Wildlife Management Area (30°37′07.2″N, 88°37′08.9″W), Jackson Co., Mississippi, USA.
Type-material: The type-series consists of 4 fully mature specimens deposited in the HWML. Holotype: HWML 216298, labelled ex *A. mississippiensis*, small intestine, Pascagoula wildlife management area, Jackson Co., Mississippi, USA, 17.viii.2010, coll. V. Tkach. Paratypes: HWML 216299 (lot of 3 slides), labels identical to the holotype.
Site in host: Small intestine.
ZooBank registration: The Life Science Identifier (LSID) for *Archaeodiplostomum overstreeti* n. sp. is urn:lsid:zoobank.org:act:990F2528-BE34-46A7-8810-1062D7787C70.
Etymology: The species is named after Dr Robin Overstreet in recognition of his numerous contributions to helminthology including helminths of crocodilians, and his invaluable help with collection of specimens in Mississippi.

Description

[Based on 4 adult specimens; measurements of the holotype are given in text; measurements of the entire series are given in Table 2; see Fig. 6.] Body 6,109 long, consisting of prosoma and opisthosoma; prosoma elongate, 3,194 long, much wider than opisthosoma, with maximum width at level of holdfast organ, 959; opisthosoma elongate, cylindrical, 2,915 × 340, similar in length to prosoma; prosoma:opisthosoma length ratio 1.1. Minuscule scale-like tegumental spines covering anterior part of prosoma and reaching level of posterior margin of ventral sucker. Oral sucker subterminal, 138 × 142. Pseudosuckers absent. Prepharynx not observed. Pharynx oval, 79 × 93. Oesophagus approximately twice as long as pharynx. Caecal bifurcation in anterior third of prosoma; caeca slender, blind, extending to near posterior end of opisthosoma. Ventral sucker 508 × 488, much larger than oral sucker, typically located somewhat anterior to mid-length of prosoma. Oral sucker:ventral sucker width ratio 1:3.4. Holdfast organ 496 × 387, posterior to ventral sucker, located in last third of prosoma, oval with ventral muscular portion. Holdfast organ equal to 16% of prosoma length. Proteolytic gland at base of holdfast organ.

Testes 2, tandem, smooth, mostly located in middle third of opisthosoma; anterior testis 176 × 225, posterior testis 193 × 222. Seminal vesicle post-testicular, elongated, sinuous, continuing as sinuous ejaculatory duct prior to joining base of paraprostate to form common male efferent duct. Paraprostate well-developed, claviform, 220 × 101, surrounded by gland cells. Common male efferent duct and metraterm join to form a common duct almost immediately prior to opening into genital atrium.

Ovary immediately pretesticular, subspherical, 99 × 96. Oötype and Mehlis' gland intertesticular. Seminal receptacle subspherical, immediately dorsal to oötype, smaller than ovary. Vitelline follicles distributed from level immediately posterior to ventral sucker to immediately anterior to paraprostate, ventral and lateral to gonads. Vitelline reservoir intertesticular. Uterus ventral to gonads, extending anteriorly from ovary to about level of prosoma and opisthosoma before turning and extending posteriorly and eventually transitioning into metraterm. Uterus contains numerous eggs (78–97 × 47–55). Genital atrium subterminal, on dorsal side.

Excretory vesicle not observed. Excretory pore terminal.

Remarks

The new species clearly belongs to *Archaeodiplostomum* based on the large ventral sucker, a well-developed claviform paraprostate, and an ejaculatory duct that joins the base of the paraprostate to form a common male efferent duct that subsequently merges with the metraterm to form a common duct. At present, *Archaeodiplostomum* includes a single species *Ar. acetabulata.*

Archaeodiplostomum overstreeti n. sp. differs from *Ar. acetabulata* by having a more elongated prosoma compared to the pyriform-shaped prosoma in *Ar. acetabulata* (Fig. 6; Byrd & Reiber, 1942). The new species can also be differentiated from *Ar. acetabulata* by having a longer body (6,109–7,706 μm in the new species *vs* 4,800–5,960 μm in *Ar. acetabulata*) and a typically smaller holdfast organ (493–598 × 387–465 μm in the new species *vs* 570–840 × 500–740 μm in *Ar. acetabulata*). In addition, *Ar. overstreeti* n. sp. has

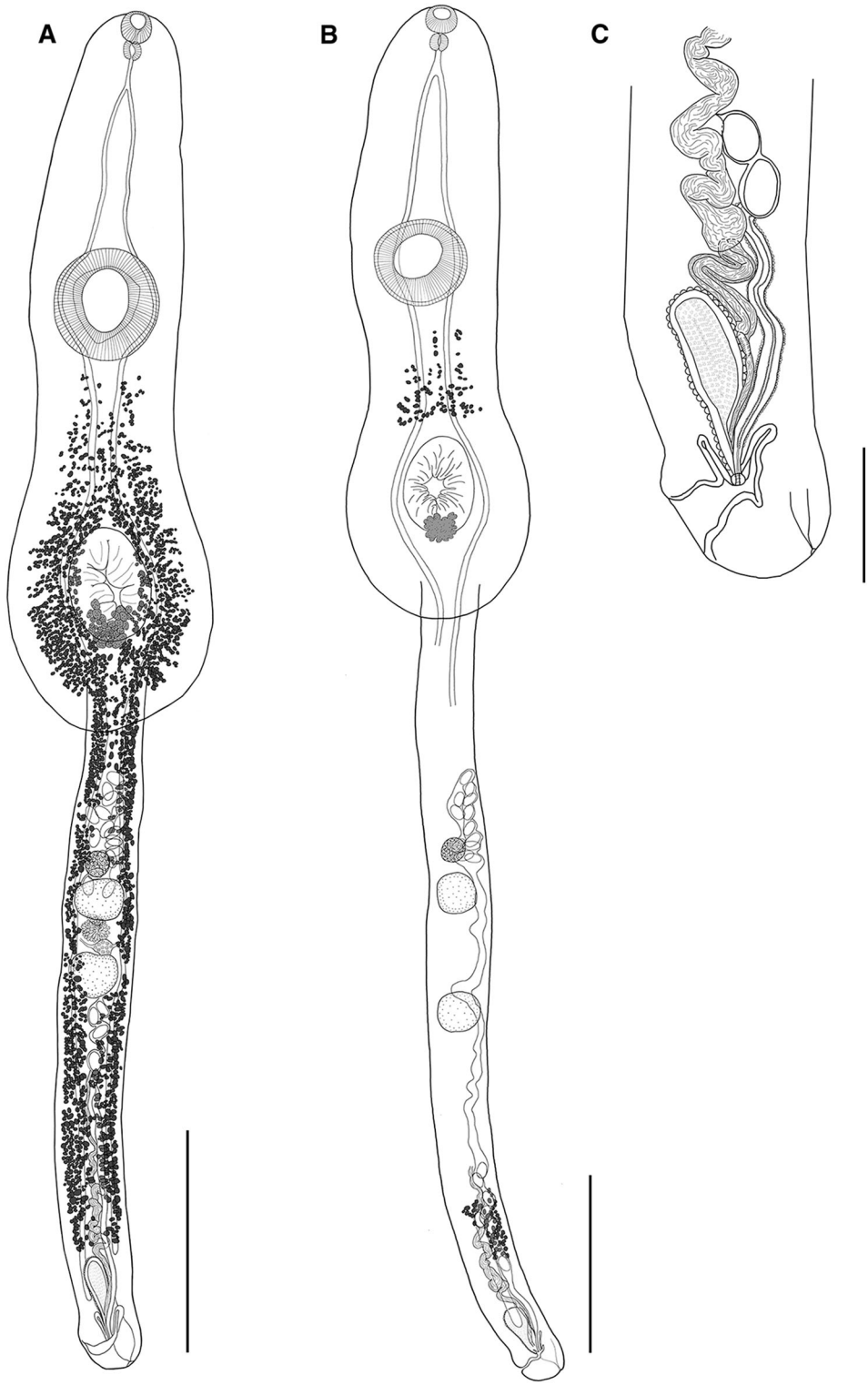

Fig. 6 *Archaeodiplostomum overstreeti* n. sp. A, Ventral view of the holotype; B, Ventral view of a paratype, anteriormost and posteriormost vitelline follicles are shown for clarity; C, Posterior end of a paratype showing terminal ducts of the reproductive system, lateral view. *Scale-bars*: A, B, 1 mm; C, 200 μm

substantially smaller paraprostate (218–255 × 101–112 μm in the new species *vs* 310–450 × 120–160 μm in *Ar. acetabulata*), ovary and testes.

Metacercariae of *Ar. overstreeti* n. sp. were recovered and sequenced from *N. fasciata* and *N. cyclopion* in Mississippi. These snakes are common in the areas where the alligators were captured. The specimen sequenced from the *N. fasciata* was found excysted in the stomach, but was not sexually mature. Gut contents of the snake contained unidentifiable fish remnants. The specimen sequenced from the *N. cyclopion* was found in the mesenteries, thus providing the first evidence that snakes can likely act as paratenic hosts for these digeneans, which are parasitic as adults in alligators.

Discussion

Abandonment of the subfamily-based system of the Proterodiplostomidae

Our molecular phylogenetic analysis of a broad diversity of proterodiplostomids from a variety of hosts from four continents strongly supports the monophyly of the Proterodiplostomidae. At the same time, our results do not support the most recent (or any of the previous) systematic arrangement of some of the taxa, particularly the current subfamily structure within the family as presented by Niewiadomska (2002). Although, this is true for both 28S and *cox*1-based phylogenies, we primarily rely on the 28S data in our subsequent considerations due to the much higher resolution at the suprageneric level provided by this gene. As has been previously suggested, the analyses based on *cox*1 data produce low resolution and numerous polytomies most likely resulting from the mutation saturation effect. Considering that the Proterodiplostomidae clearly is an ancient group of digeneans, combined with the fact that crocodilians live in warm climates where parasite life-cycles continue throughout the year, these parasites evolved over a great span of evolutionary time in terms of the number of generations. This most likely resulted in a greater mutation accumulation in fast mutating mitochondrial genes leading to lower resolution in the *cox*1 trees compared to those produced by the analyses of the slower mutating 28S gene. As noted by previous authors (e.g. Locke et al., 2018; Queiroz et al., 2020), the usefulness of commonly sequenced nuclear ribosomal and mitochondrial genes for phylogenetic inference at different taxonomic levels varies and necessitates careful assessment.

While the early systematics of the Proterodiplostomidae were based on a variety of characters traditionally used in digenean taxonomy, Brooks et al. (1992) proposed a revised system of the family with an emphasis on the structure of the terminal parts of the reproductive system. These authors split the Proterodiplostomidae based on the following four conditions: (i) paraprostate fused with ejaculatory duct and metraterm (referred to as uterus by Brooks et al. [1992]) opening separately; (ii) paraprostate and ejaculatory duct fused, then metraterm fused with common male efferent duct; (iii) paraprostate fused first with metraterm and then with ejaculatory duct; and (iv) paraprostate opening separately. This led Brooks et al. (1992) to propose two subfamilies, the Heterodiplostominae Dubois, 1936 *incertae sedis*, *sedis mutabilis* (*Heterodiplostomum* and *Ophiodiplostomum*) and the Proterodiplostominae with the latter divided into three tribes: (i) Pseudoneodiplostomini Dubois, 1936 *sedis mutabilis* (*Neelydiplostomum* Gupta, 1958, currently considered a synonym of *Herpetodiplostomum* and *Pseudoneodiplostomum*); (ii) Pseudocrocodilicolini Byrd & Reiber, 1942 *sedis mutabilis* (*Archaeodiplostomum*, *Crocodilicola*, *Pseudocrocodilicola* and *Polycotyle*); and (iii) Proterodiplostomini Dubois, 1936 *sedis mutabilis* (*Cystodiplostomum*, *Herpetodiplostomum*, *Massoprostatum*, *Mesodiplostomum*, *Paradiplostomum*, *Prolecithodiplostomum* and *Proterodiplostomum*).

Our analyses supported neither the subfamilies nor the tribes Pseudocrocodilicolini (with a caveat that the *Crocodilicola* sequence in GenBank originated from a metacercaria) and Proterodiplostomini of Brooks et al. (1992).

In the most recent revision of the Proterodiplostomidae, Niewiadomska (2002) did not accept the system proposed by Brooks et al. (1992). Her system included five subfamilies: Massoprostatinae, Ophiodiplostominae, Polycotylinae, Proalarioidinae, and Proterodiplostominae. Our molecular phylogenetic analyses included five genera of the Polycotylinae (*Crocodilicola*, *Cystodiplostomum*, *Paradiplostomum*, *Pseudocrocodilicola* and *Polycotyle*), four genera of the Proterodiplostominae (*Archaeodiplostomum*, *Mesodiplostomum*,

Pseudoneodiplostomum and *Proterodiplostomum*) and one genus of the Ophiodiplostominae (*Heterodiplostomum*). Our analyses revealed the Polycotylinae and Proterodiplostominae to be clearly paraphyletic (Figs. 1–3).

Our molecular phylogenetic results did not support any of the previously proposed systems of the Proterodiplostomidae including the most recent system proposed by Niewiadomska (2002). The use of the organisation of the terminal parts of the reproductive system as the main basis for the systematic arrangement of the proterodiplostomids was also not supported, although these characters are certainly useful for differentiation among genera and species. Our analyses do not show a clear association between well-supported proterodiplostomid clades and the structure of their terminal reproductive ducts (Figs. 2, 3, 5). For instance, in both 28S analyses (Figs. 1, 2) the representative of *Paraproterodiplostomum* n. g., which has a uterus that opens into the genital atrium separately from a common male efferent duct, formed a clade with *Archaeodiplostomum*, *Neocrocodilicola* n. comb., *Polycotyle* and *Pseudocrocodilicola*, which all have the ejaculatory duct, paraprostate and metraterm form a common duct. Likewise, members of *Cystodiplostomum*, which have a paraprostate that opens separate from the ejaculatory duct and metraterm, formed a clade with the *Proterodiplostomum* spp. (excluding *Pt. globulare* formerly included in *Proterodiplostomum*; see discussion below) possessing a metraterm that opens separately from the ejaculatory duct and the paraprostate. Although, the level of paraprostate development and the arrangement of terminal ducts of the reproductive system do not seem to be useful for identifying subfamilies of proterodiplostomids, these features are definitely suitable for differentiation among genera. We therefore provide schematic diagrams of almost all proterodiplostomid genera based on the original illustrations (Fig. 5).

Based on all previously available and new molecular as well as morphological data, we abandon the subfamily structure of the Proterodiplostomidae. This decision is reminiscent of other large digenean families that traditionally had a complex taxonomic structure, e.g. the Cryptogonimidae Ward, 1917, the Echinostomatidae Looss, 1899, and the Dicrocoeliidae Looss, 1899. In all those cases, the increasing amount of phylogenetic data indicated lack of support for existing subfamilies, which resulted in the abandonment of the subfamilies in all three families (Miller & Cribb, 2008; Tkach et al., 2016, 2018). This allows us to look at the evolution and taxonomy of this group unobstructed by the systematic schemes based on ambiguous characters with unclear evolutionary history and relative weight. We believe this will accelerate the development of a natural classification system of the Proterodiplostomidae, based on a combination of molecular phylogenetic data and better understood morphological criteria.

Revision and additional systematic changes at genus level

The status of *Pseudocrocodilicola*

Until now the genus *Pseudocrocodilicola* contained two species: *Ps. americaniense* (type-species) and *Ps. georgiana* (see Byrd & Reiber, 1942; Dubois, 1979). These species did not form a monophyletic clade in any of our analyses. *Pseudocrocodilicola georgiana* clustered with *Po. ornata* (85% support) in the 28S analysis of the Proterodiplostomidae and appeared as a separate branch in the *cox*1 tree (Figs. 2, 3), whereas *Ps. americaniense* consistently formed a poorly supported clade with *Ar. overstreeti* n. sp. in both 28S and *cox*1 analyses (Figs. 2, 3).

Besides the low branch support in the phylogenetic analyses, species of *Archaeodiplostomum* and *Pseudocrocodilicola* have very significant morphological differences, definitely warranting their placement into separate genera. *Archaeodiplostomum* spp. are characterised by having a very large ventral sucker, a prosoma and opisthosoma of similar length, vitellarium distributed in both the prosoma, and the opisthosoma and an ejaculatory duct that joins the paraprostate at its base. In contrast, members of *Pseudocrocodilicola* have a prosoma that is typically much longer than the opisthosoma, vitellarium primarily limited to the prosoma, and a muscular pouch surrounding the common duct. In addition, *Ps. americaniense* has an ejaculatory duct that joins the paraprostate at its middle and *Ps. georgiana* has an ejaculatory duct that joins the paraprostate at its proximal (anterior) end (Byrd & Reiber, 1942; Fig. 5).

The two species of *Pseudocrocodilicola* also have significant morphological differences beyond the

position of the junction of the ejaculatory duct with the paraprostate. The vitellarium of *Ps. americaniense* does not extend anteriorly to the level of the ventral sucker, whereas the vitellarium of *Ps. georgiana* always extends anteriorly beyond the level of the ventral sucker. Additionally, the metraterm of *Ps. americaniense* joins the common male efferent duct some distance after it exits the paraprostate to form the common duct (similar to that in *Archaeodiplostomum* spp.), whereas the metraterm of *Ps. georgiana* joins the distal (posterior) end of the paraprostate to form the common duct (Fig. 5C, D) (Byrd & Reiber, 1942).

Based on the phylogenetic position, genetic distances and the above morphological differences between the two *Pseudocrocodilicola* species, we believe *Ps. georgiana* needs to be transferred to a new genus. Therefore, we establish *Neocrocodilicola* n. g. with *Neocrocodilicola georgiana* n. comb. as the type- and only species. An amended diagnosis of *Pseudocrocodilicola* and diagnosis of *Neocrocodilicola* n. g. are provided below.

Pseudocrocodilicola Byrd & Reiber, 1942

Diagnosis

[After Niewiadomska (2002), amended.] Body distinctly bipartite; prosoma flattened, lanceolate, longer than cylindrical opisthosoma. Oral sucker smaller than ventral sucker. Pseudosuckers absent. Ventral sucker situated in middle or anterior to middle of prosoma; holdfast organ rather small, oval, with median slit bordered by papillae. Pharynx similar in size to oral sucker; caeca reaching level of paraprostate. Gonads occupying most of opisthosoma. Testes 2, tandem; paraprostate small, muscular, ellipsoidal, surrounded by relatively few, large gland-cells. Ejaculatory duct joins paraprostate near its midpoint. Ovary pretesticular; oötype intertesticular. Vitellarium distributed throughout posterior two thirds of prosoma, anterior extent at level of or before ventral sucker. Metraterm joins common male efferent duct to form common duct surrounded by thick-walled muscular pouch and opening into genital atrium. Excretory pore terminal. In *Alligator mississippiensis*. Nearctic.

Type-species: *Pseudocrocodilicola americaniense* Byrd & Reiber, 1942.

Neocrocodilicola Tkach, Achatz & Pulis n. g.

Diagnosis

Body distinctly bipartite; prosoma flattened, lanceolate, longer than cylindrical opisthosoma. Oral sucker smaller than ventral sucker. Pseudosuckers absent. Ventral sucker situated in middle or anterior to middle of prosoma; holdfast organ rather small, oval, with median slit bordered by papillae. Pharynx similar in size to oral sucker; caeca reaching level of paraprostate. Gonads occupying most of opisthosoma. Testes 2, tandem; paraprostate small, muscular, ellipsoidal, surrounded by relatively few large gland cells. Ejaculatory duct joins proximal end of paraprostate. Ovary pretesticular; oötype intertesticular. Vitellarium distributed throughout posterior two thirds of prosoma, always extending anteriorly beyond ventral sucker, sometimes slightly extending into opisthosoma. Metraterm joins common male efferent duct to form common duct surrounded by thick-walled muscular pouch and opening into genital atrium. Excretory pore terminal. In *Alligator mississippiensis*. Nearctic.

Type-species: *Neocrocodilicola georgiana* (Byrd & Reiber, 1942) Tkach, Achatz & Pulis n. comb.

ZooBank registration: The Life Science Identifier (LSID) for *Neocrocodilicola* n. g. is urn:lsid:zoobank.org:act:AE93B5BB-20F6-4FE5-8FFA-E09BF724541F

Etymology: The generic name reflects the parasitism in crocodilians and the fact that the name *Crocodilicola* is already preoccupied.

Remarks

Neocrocodilicola n. g. differs by 1.9–5.3% of nucleotide positions in 28S sequences and 10.4–21.7% of nucleotide positions in *cox*1sequences from all other genera with available DNA sequence data (Supplementary Tables S1, S2).

Neocrocodilicola n. g. can be differentiated from *Heterodiplostomum* by the lack of a muscular pouch surrounding the paraprostate (Fig. 5D, S). *Neocrocodilicola* n. g. also lacks a muscular pouch enclosing the paraprostate, ejaculatory duct and metraterm found in *Capsulodiplostomum*. Unlike *Mesodiplostomum* and *Proalarioides, Neocrocodilicola* n. g. has a defined paraprostate (Fig. 5D, T, U). *Neocrocodilicola* n. g. has a relatively much smaller

holdfast organ compared to *Ophiodiplostomum*, in which it occupies approximately half of the prosoma.

The metraterm of *Neocrocodilicola* n. g. joins the common male efferent duct to form the common duct. In contrast, *Proterodiplostomum*, *Pseudoneodiplostomum* and *Paraproterodiplostomum* n. g. possess a metraterm which opens separately from the male ducts (Fig. 5D, G, H, J, K). The ejaculatory duct of *Neocrocodilicola* n. g. joins the proximal half of the paraprostate (Fig. 5D), while in *Cystodiplostomum*, *Herpetodiplostomum*, *Massoprostatum*, *Paradiplostomum* and *Prolecithodiplostomum* the paraprostate, the ejaculatory duct and the metraterm open separately into the genital atrium (Fig. 5M, N, O, R).

Neocrocodilicola n. g. can be easily differentiated from *Polycotyle*, *Crocodilicola* and *Archaeodiplostomum* by the presence of a muscular pouch surrounding the common duct. Furthermore, *Neocrocodilicola* n. g. does not possess small suckers along the opisthosoma, which are characteristic of *Polycotyle*. The vitellarium in *Neocrocodilicola* n. g. is not limited to the area around the holdfast organ as in *Crocodilicola*.

The ejaculatory duct in *Neocrocodilicola* n. g. joins the near the proximal end of the paraprostate, whereas in *Archaeodiplostomum* and *Pseudocrocodilicola* it joins either the common efferent male duct or the distal end of the paraprostate, respectively (Fig. 5B–D).

The status of *Proterodiplostomum*

Proterodiplostomum at present includes six species and is the most speciose genus of proterodiplostomids in the Neotropics. All known species have an ejaculatory duct and paraprostate that open side by side or with a common pore (without a common male efferent duct) and a metraterm which opens separately from the male ducts (Dubois, 1979; Catto & Amato, 1994). Two *Proterodiplostomum* species from caimans (the type-species *Pr. longum* and *Pr. tumidulum*) were described with a sucker-like muscular structure in the genital atrium, whereas *Pr. medusae* is known to have muscular bundles which are almost sucker-like in the wall of the genital atrium (Dubois, 1936; Catto & Amato, 1994) (Fig. 5J, K). At the same time, *Pr. breve* and *Pr. globulare* (Fig. 5L) from caimans, and *Proterodiplostomum ophidum* Thatcher, 1963 from a snake were described without any sucker-like or muscular structures near the genital atrium (Thatcher, 1963; Catto & Amato, 1994).

In this study, we collected *Pr. longum*, *Pr. globulare*, *Pr. medusae* and an immature *Proterodiplostomum* species. Our specimens of *Pr. longum* have a well-defined sucker-like muscular structure in the genital atrium, whereas our specimens of *Pr. medusae* and the immature *Proterodiplostomum* sp. had well-pronounced muscle bundles in the wall of the genital atrium, which were almost sucker-like. In contrast, our specimens of *Pr. globulare* lacked any sucker-like or muscular structure in the wall of the genital atrium.

Our phylogenetic analyses revealed *Proterodiplostomum* to be non-monophyletic. *Proterodiplostomum longum* (type-species), *Pr. medusae* and the immature *Proterodiplostomum* sp. formed a strongly supported clade in our analyses (Figs. 1–3). These three species have a sucker-like structure or well-defined muscle bundles in the wall of the genital atrium. In contrast, *Pr. globulare,* which lacks the sucker-like structure in the genital atrium, formed one of the branches in the basal polytomy within the Proterodiplostomidae in all our analyses (Figs. 1–3). *Proterodiplostomum globulare* also showed 5.4–6.1% (59–67 bases) divergence in 28S sequences and significant 22.1–23.7% (116–122 bases) divergence in *cox*1 sequences from other *Proterodiplostomum* species in our study (Supplementary Tables S1, S2).

Based on the absence of a sucker-like structure in the genital atrium of *Pr. globulare* along with the strong phylogenetic evidence, we erect the genus *Proteroduboisia* n. g. for *Pr. globulare*.

Proteroduboisia Tkach, Achatz & Melo n. g.

Diagnosis

Body bipartite; prosoma elliptic, folicaeous; opisthosoma elongate, cylindrical. Oral and ventral suckers moderately developed; holdfast organ elliptical or subspherical, with papillae on margin of median slit. Pseudosuckers absent. Pharynx moderately developed; caeca reaching near level of genital atrium. Testes 2, tandem; paraprostate relatively small; ejaculatory duct and efferent duct of paraprostate open together at apex of genital cone. Ovary pretesticular. Vitellarium extends from below or at level of ventral sucker to posterior margin of anterior testis or near posterior end of body. Metraterm opens separately from male ducts into genital atrium. Genital atrium

subterminal with dorsal opening. In caimans. Neotropics.

Type-species: *Proteroduboisia globulare* (Catto & Amato, 1994) Tkach, Achatz & Melo n. comb. *Other species*: *Proteroduboisia breve* (Catto & Amato, 1994) Tkach, Achatz & Melo n. comb., *Proteroduboisia ophidum* (Thatcher, 1963) Tkach, Achatz & Melo n. comb.

ZooBank registration: The Life Science Identifier (LSID) for *Proteroduboisia* n. g. is urn:lsid:zoobank.org:act:1BD01B4D-12B0-433C-A51A-CD46E147F84F.

Etymology: The genus is named after Dr. Georges Dubois in recognition of his fundamental contributions to trematodology and particularly to our knowledge of the Proterodiplostomidae and other diplostomoideans.

Remarks

Proteroduboisia n. g. differs by 3.2–6.1% of nucleotide positions in 28S sequences and 20.2–22.3% of nucleotide positions in *cox*1sequences from all other genera with available DNA sequence data (Supplementary Tables S1, S2).

Proteroduboisia n. g. can be easily morphologically differentiated from *Heterodiplostomum* and *Capsulodiplostomum* by the lack of a muscular pouch surrounding the paraprostate in *Heterodiplostomum* (Fig. 5L, S) or the paraprostate, ejaculatory duct and metraterm in *Capsulodiplostomum* (not shown in Fig. 5 due to the very poor quality of the illustration in the original description). Although relatively small, the paraprostate of *Proteroduboisia* n. g. is well-defined compared to the apparent lack of the paraprostate in *Mesodiplostomum* and *Proalarioides* (Fig. 5L, T, U). The holdfast organ in *Proteroduboisia* n. g. occupies approximately a quarter or less of the prosoma length, whereas in *Ophiodiplostomum* the holdfast organ occupies approximately half of the prosoma length. *Proteroduboisia* n. g. can be differentiated from most other proterodiplostomid genera based on the topology of the terminal reproductive ducts. The ejaculatory duct and efferent duct of the paraprostate open side by side in *Proteroduboisia* n. g. without forming a common male efferent duct, while the metraterm opens separately. The ejaculatory duct, paraprostate and metraterm unite in different ways to form a common duct in *Archaeodiplostomum*, *Crocodilicola*, *Neocrocodilicola*, *Polycotyle* and *Pseudocrocodilicola*, whereas the paraprostate of *Cheloniodiplostomum*, *Cystodiplostomum*, *Herpetodiplostomum*, *Paradiplostomum* and *Prolecithodiplostomum* opens distinctly separately from the ejaculatory duct. In *Pseudoneodiplostomum* and *Paraproterodiplostomum* n. g. the ejaculatory duct joins the paraprostate (Fig. 5G, H). Morphological differences between *Proteroduboisia* n. g. and *Proterodiplostomum* are already discussed above.

Due to the erection of *Proteroduboisia* n. g. and transfer of three species into the new genus we provide an amended diagnosis of *Proterodiplostomum*.

Proterodiplostomum Dubois, 1936

Diagnosis

[After Niewiadomska (2002), amended.] Body distinctly bipartite; prosoma flattened, spatulate, typically much shorter than cylindrical opisthosoma. Oral sucker and ventral sucker moderately developed; holdfast organ elliptical, elongate, with papillae on margin of median slit. Pseudosuckers absent. Pharynx small or moderately developed; caeca reaching near level of genital atrium. Testes 2, tandem; anterior testis near middle of opisthosoma. Paraprostate well-developed, tubular, reaching close to posterior testis. Ejaculatory duct and efferent duct of the paraprostate open together at apex of genital cone. Ovary pretesticular; oötype intertesticular. Vitellarium distributed throughout prosoma and opisthosoma, anterior extent before or after ventral sucker, posterior extent reaching about level of paraprostate. Metraterm opens separately from male ducts near base of genital cone. Muscular sucker-like structure or denser musculature present in wall of genital atrium. Genital atrium with subterminal opening, on dorsal side. Excretory pore terminal. In crocodilians. Neotropics.

Type-species: *Proterodiplostomum longum* (Brandes, 1888).

Other species: *Proterodiplostomum tumidulum* Dubois, 1936, *Proterodiplostomum medusae* (Dubois, 1936).

Status of *Pseudoneodiplostomoides*

Prior to this study, no members of the Proterodiplostomidae had been reported from Australian crocodilians. Two members of *Pseudoneodiplostomoides*, a previously accepted subgenus of *Pseudoneodiplostomum*, were described by Tubangui & Masiluñgan (1936) and Yamaguti (1954) from saltwater crocodile *Crocodylus porosus* Schneider from the Philippines and Indonesia, respectively. Tubangui & Masiluñgan (1936) originally placed their species (*Pu. crocodilarum*) from the Philippines within the genus *Neodiplostomum* Railliet, 1919. Yamaguti (1954) later established the subgenus *Pseudoneodiplostomoides* for his newly described *Pseudoneodiplostomum* (*Pseudoneodiplostomoides*) *crocodili* Yamaguti, 1954 and *Pu. crocodilarum,* in part based on the presence of two muscular pits in the genital atrium. Dubois (1979) listed both of these species as synonyms of *Pe. siamense*. We disagree with Dubois'synonymisation because of the lack of the characteristic "pits" or concave invaginations of the genital atrium wall in *Pe. siamense*, but their presence in the members of Yamaguti's subgenus *Pseudoneodiplostomoides*. Our molecular data support this notion with 1.4 % (15 bases) divergence between *Pe. siamense* and *Pu. crocodilarum* in the 28S gene and 17.7% (92 bases) divergence in *cox*1. Considering the substantial level of sequence divergence (Supplelmentary Tables S1, S2), the results of our phylogenetic analyses (Figs. 1–3) and the lack of the characteristic invaginations in the genital atrium of all other *Pseudoneodiplostomum* species, including our specimens representing four species, we restore *Pseudoneodiplostomoides* and elevate it to genus level. Since the only character Yamaguti (1954) used to separate *Pu. crocodili* and *Pu. crocodilarum* was the width of the eggs, we consider *Pu. crocodili* a junior synonym of *Pu. crocodilarum* (Tubangui & Masiluñgan, 1936) n. comb. which becomes the type-species of *Pseudoneodiplostomoides*. Yamaguti (1954) provided an adequate diagnosis of *Pseudoneodiplostomoides*, therefore we do not include an amended diagnosis here.

Content of *Pseudoneodiplostomum*

Pseudoneodiplostomum includes four currently accepted species: *Pe. thomasi* (type-species) and *Pe. bifurcatum* from Africa, *Pe. siamense* from Southeast Asia, and *Pe. groschafti* from Cuba (Dubois, 1979; Moravec, 2001). *Pseudoneodiplostomum thomasi* was originally described by Dollfus (1935) from the intestine of the dwarf crocodile *Osteolaemus tetraspis* Cope collected in the French Congo. Dubois (1948) examined specimens collected from the intestine of the West African slender-snouted crocodile *Mecistops cataphractus* (Cuvier) collected in Gabon that were previously identified as *Pe. thomasi*. Based on these specimens, Dubois (1948) established the subspecies *Pe. thomasi gabonicum* Dubois, 1948, which differed from the nominal subspecies *Pe. thomasi thomasi* by the greater opisthosoma:prosoma ratio, narrower body, as well as the smaller ventral sucker, holdfast organ, ovary and testes.

Our specimens of *Pe. thomasi thomasi* and *Pe. thomasi gabonicum* differ by 0.2% of 28S sequences. For comparison, 28S sequences of *Pe. bifurcatum* and *Pe. thomasi* were identical despite the two species having very distinct morphologies. Based on the morphological and genetic differences we elevate *Pe. thomasi gabonicum* to species level as *Pe. gabonicum* Dubois, 1948 n. nom.

Notes on other genera

Crocodilicola pseudostoma was originally described from *Al. mississippiensis* collected in South Carolina, USA (Willemoes-Suhm, 1870) and later reported from the same host in several locations in the USA, as well as from Moreletś crocodile *Co. moreletii* in Mexico (Tellez, 2014). Our analyses of 28S and *cox*1 included sequences of *Cr. pseudostoma* from GenBank published by Hernández-Mena et al. (2017). These sequences came from a metacercaria collected from fish in Catemaco Lake, Veracruz, southern Mexico, thousands of kilometers from the type-territory of *Cr. pseudostoma* or the nearest current area populated by alligators. In our phylogenetic analyses, these sequences formed strongly supported clades with proterodiplostomids from caimans collected in Brazil (Figs. 1–3). The distribution of *Co. moreletii* overlaps with that of caimans, but not with the range of the American alligator. All proterodiplostomids from *Al. mississippiensis* included in our analyses, formed a strongly supported monophyletic group and all other genera of proterodiplostomids parasitising crocodilians showed close association with a single genus of their definitive hosts (Fig. 2).

The combination of the definitive host distribution patterns and the phylogenetic placement of *Cr. pseudostoma* sequences from GenBank suggests that the identification of these metacercariae should be considered with caution. Although *Cr. pseudostoma* was reported from *Co. moreletii* in Mexico (Dubois, 1953; Thatcher, 1964), we believe these reports were a result of misidentification due to the poor condition of the specimens. We examined specimens of *Cr. pseudostoma* from *Co. moreletii* in Mexico deposited in the HWML (see Materials and methods). Despite the very poor state of the specimens on slides it was evident that they do not belong to *Crocodilicola*. The ejaculatory duct in these specimens joins the paraprostate near its proximal end and the metraterm clearly does not join the merged ejaculatory duct and paraprostate. In *Cr. pseudostoma* the metraterm, ejaculatory duct and paraprostate merge to form a common duct. Most likely, these specimens represent a new species; however, their state does not allow for a quality description. Sequencing of an adult stage of *Cr. pseudostoma* from alligators as well as of proterodiplostomids from *Co. moreletii* in the future will eventually support or reject the identification of the metacercariae in Hernández-Mena et al. (2017). We anticipate that it will turn out to be a new genus, possibly shared between caimans and crocodiles in the Neotropics.

Cystodiplostomum hollyi is the type-species of the monotypic genus *Cystodiplostomum*. Our unidentified *Cystodiplostomum* sp. formed a 100% supported clade with *Cy. hollyi* in both 28S and *cox*1 analyses. It most likely represents a second member of the genus, but our only specimen was used for DNA extraction. Due to the relatively high level of genetic divergence, it is also possible that our specimen represents another genus not available for our analysis, such as *Prolecithodiplostomum*, in which the topology of terminal reproductive ducts is identical to that of *Cystodiplostomum* (Fig. 5M).

Unlike other proterodiplostomid taxa included in our study, adult *Heterodiplostomum* are parasites of the intestines of snakes in the Neotropics and are known to use amphibians as second intermediate hosts (Niewiadomska, 2002; Queiroz et al., 2020). At present, *Heterodiplostomum* includes two species: *He. lanceolatum* and *Heterodiplostomum helicopsis* Mañé-Garzón & Alonso, 1976. Ribosomal sequences from metacercaria of *He. lanceolatum* collected from pointed belly frogs *Leptodactylus podicipinus* (Cope) in Brazil were recently published (Queiroz et al., 2020) and differ from our sequences of *Heterodiplostomum lanceolatum* by 0.2% (2 nucleotides). No *cox*1 sequences are available for the previously published *He. lanceolatum* isolate. *Heterodiplostomum lanceolatum* has been described with caeca that terminate anterior to the copulatory bursa and vitellarium that do not extend anterior passed the holdfast organ; whereas the caeca of *He. helicopsis* terminate near the distal extremity of the opisthosoma and the vitellarium extend anteriorly to near the level of the caecal fork (Dubois, 1936; Mañé-Garzón & Alonso, 1976). Whereas our specimens of *Heterodiplostomum* from *L. chaquensis* and *E. poecilogyrus* were immature, their morphology corresponded to the description of *He. lanceolatum* (i.e. caeca terminate immediately anterior to the copulatory bursa and vitellarium does not pass the level of the holdfast organ). We suspect that the difference in the 28S gene sequences may be indicative of the presence of a cryptic species. None of the remaining proterodiplostomids included in this study had more than a single variable nucleotide site within a species and *Pe. bifurcatum* and *Pe. thomasi* had no differences in their 28S sequences (Supplementary Table S1). However, future studies will have to include sequences from adult *Heterodiplostomum* specimens along with sequences of faster mutating genes (e.g. *cox*1) to properly test for the presence of cryptic species within this genus.

As a result of the present revision of several proterodiplostomid taxa and abandonment of the subfamilies within the Proterodiplostomidae, the family now includes 21 genera. We expect additional changes in the system of this family as our knowledge of proterodiplostomid diversity and morphology, as well as greater sequencing coverage, will continue to improve with further studies. Nevertheless, we consider it useful to provide a key to the identification of the currently recognised proterodiplostomid genera. Although we do not believe that hosts or geographical distribution should be used as characters in the identification, we provide this information in the key for convenience.

Key to the genera of the Proterodiplostomidae

1a Paraprostate absent ……..… 2
1b Paraprostate present …… 3
2a Ejaculatory duct and metraterm merge to form hermaphroditic duct near apex of genital cone. Hermaphroditic duct not enclosed in a muscular pouch. Pseudosuckers absent. In crocodilians. Neotropics ……. *Mesodiplostomum*
2b Ejaculatory duct and metraterm merge to form hermaphroditic duct enclosed in a muscular pouch. Pseudosuckers present. In snakes. Palaeartic and Orient …… …… *Proalarioides*
3a Paraprostate surrounded by muscular pouch. Paraprostate duct eversible. Ejaculatory duct and metraterm open side by side. In snakes. Neotropics …… *Heterodiplostomum*
3b Paraprostate not surrounded by muscular pouch or all terminal ducts of reproductive system enclosed in single muscular pouch …… 4
4a Entire paraprostate, ejaculatory duct, and metraterm enclosed in muscular pouch. Ejaculatory duct and metraterm open separately. In crocodilians. India …… ……..*Capsulodiplostomum*
4b Paraprostate, ejaculatory duct, and metraterm not enclosed in muscular pouch. Ejaculatory duct and metraterm open separately or have a common opening …… 5
5a Vitellarium confined to opisthosoma. In crocodilians. Neotropics …… …….*Massoprostatum*
5b Vitellarium distributed differently …….. 6
6a Holdfast organ relatively massive, typically occupying approximately half of prosoma … ……. 7
6b Holdfast organ not as massive, typically occupying approximately 25–30% of prosoma ……..… 8
7a Ejaculatory duct joins distal part of paraprostate. Two muscular pits, occasionally sucker-like, present in wall of genital atrium. In crocodilians. Australasia …… …… *Pseudoneodiplostomoides*
7b Ejaculatory duct and paraprostate do not join/unite. Muscular pits in wall of genital atrium absent. In snakes. Neotropics …… …… *Ophiodiplostomum*
8a Metraterm, ejaculatory duct and paraprostate join to form a common duct or all three share common opening …… 9
8b Metraterm, ejaculatory duct and paraprostate do not form common duct. Ejaculatory duct and paraprostate or ejaculatory duct and metraterm may join or share common opening …… 13
9a Opisthosoma with longitudinal row of sucker-like structures on dorsal side. In crocodilians. Nearctic …… *Polycotyle*
9b Opisthosoma without dorsal sucker-like structures ……... 10
10a Terminal part of paraprostate, ejaculatory duct, and metraterm enclosed in muscular pouch ……11
10b Paraprostate, ejaculatory duct, and metraterm not enclosed in muscular pouch …… …12
11a Ejaculatory duct typically joins paraprostate near its midpoint. In crocodilians. Nearctic …….. *Pseudocrocodilicola*
11b Ejaculatory duct joins paraprostate near its proximal end. In crocodilians. Nearctic. …… *Neocrocodilicola* n. g.
12a Vitelline follicles confined to area around holdfast organ. Separation between prosoma and opisthosoma indistinct. In crocodilians. Nearctic and Neotropics …… …… *Crocodilicola*
12b Vitelline follicles distributed in both prosoma and opisthosoma, extending well beyond area around holdfast organ. Separation between prosoma and opisthosoma distinct. In crocodilians. Nearctic …… *Archaeodiplostomum*
13a Paraprostate opens separately from ejaculatory duct and metraterm. Ejaculatory duct and metraterm may join or share common opening ……... 14
13b Metraterm opens separately from ejaculatory duct and paraprostate. Ejaculatory duct and paraprostate may join or share common opening ……... 18
14a Genital cone present …… 15
14b Genital cone absent ……... 17
15a Genital cone massive, equal to about 1/4 of total body length. In crocodilians. Neotropics …… …… *Paradiplostomum*
15b Genital cone much smaller, not more than 1/8 of total body length …… 16

16a Holdfast organ with papillae. In crocodilians. Neotropics ……... *Herpetodiplostomum*
16b Holdfast organ without papillae. In chelonians. Neotropics ……. *Cheloniodiplostomum*
17a Thick-walled, sucker-like dorsal invagination of body present near midpoint of opisthosoma or slightly more posterior. In crocodilians. Neotropics ……... *Cystodiplostomum*
17b No thick-walled, sucker-like dorsal invagination of body present. In crocodilians. Neotropics …….. *Prolecithodiplostomum*
18a Sucker-like muscular structure (well-developed or concentrated muscle bundles) in the wall of the genital atrium present. In crocodilians. Neotropics ……..... *Proterodiplostomum*
18b Genital atrium without sucker-like structure ……... 19
19a Ejaculatory duct does not join paraprostate. Ejaculatory duct and paraprostate share common opening. In crocodilians and snakes. Neotropics …….. *Proteroduboisia* n. g.
19b Ejaculatory duct joins paraprostate …….. 20
20a Ejaculatory duct joins paraprostate near its distal end. Paraprostate well-developed. In crocodilians. Nearctic …… …… *Paraproterodiplostomum* n. g.
20b Ejaculatory duct joins proximal half of paraprostate. Paraprostate weakly developed. In crocodilians. Africa, Australasia and Neotropics ……. *Pseudoneodiplostomum*

Host and geographical associations

The Proterodiplostomidae clearly is a very old evolutionary lineage of digeneans parasitising an ancient group of hosts that already existed and strongly radiated before the separation and subsequent drift of the current continents. In a series of works, Brooks and co-authors (Brooks, 1979; Brooks & O'Grady, 1989; Brooks et al., 1992) presented morphology-based phylogenies of the Proterodiplostomidae (along with some other digenean groups parasitic in crocodilians) and analysed their historical biogeography as well as host associations. These authors emphasised that the history of proterodiplostomid associations with their crocodilian hosts extended deep into the geological and evolutionary past and was affected by major global geological events such as tectonic plate movement and accompanying radiation among and within the crocodilian lineages.

Our phylogenetic analyses supported some of the conclusions drawn in these publications, e.g. regarding the monophyly of the proterodiplostomids parasitising alligators. The arrangement of the remaining taxa showed, however, a substantial disagreement. Although our 28S tree was not fully resolved (Fig. 2) it provides some new insights into the historical biogeography and host associations of the Proterodiplostomidae. This is particularly interesting considering the recent advances in the phylogenetics of crocodilians and discovery of cryptic species based on both morphological and molecular criteria (e.g. Brochu, 1997, 2003; Bittencourt et al., 2019; Brochu & Sumrall, 2020; Roberto et al., 2020). Molecular data also suggested a relatively recent radiation and active speciation of the true crocodiles (Oaks, 2011).

Although morphology based phylogenetic hypotheses incorporating both the extant and extinct species suggested that gharials (Gavialidae Adams) represent the most basal lineage of extant crocodilians (Brochu, 1997, 2003), the molecular phylogenies (Oaks, 2011) strongly suggested that the Alligatoridae Gray is the basal extant group. Although our 28S phylogeny was not completely resolved, the interrelationships between proterodiplostomids correspond well to the phylogenetic affinities among crocodilians (Figs. 2, 7).

According to our data, the proterodiplostomids of alligatorids are not monophyletic (but those parasitic in American alligators are) because the genera associated with caimans are found in three different clades. This tree topology allows us to hypothesise that the proterodiplostomids parasitic in crocodilians have first evolved and radiated into several lineages in the ancestors of modern caimans yet in Pangea. Some of these lineages were either inherited by alligators and then true crocodiles in the process of crocodilian radiation or passed as a result of subsequent host switching events. This hypothesis corresponds with at least some of the previously suggested schemes of the biogeographic relationships among crocodilians (Sill, 1968; Brooks, 1979). The evidence of at least one genus (*Pseudoneodiplostomum*) shared between members of the genus *Crocodylus* in Africa and Australia (and Asia according to published morphology-only data) fits well the hypothesis of relatively

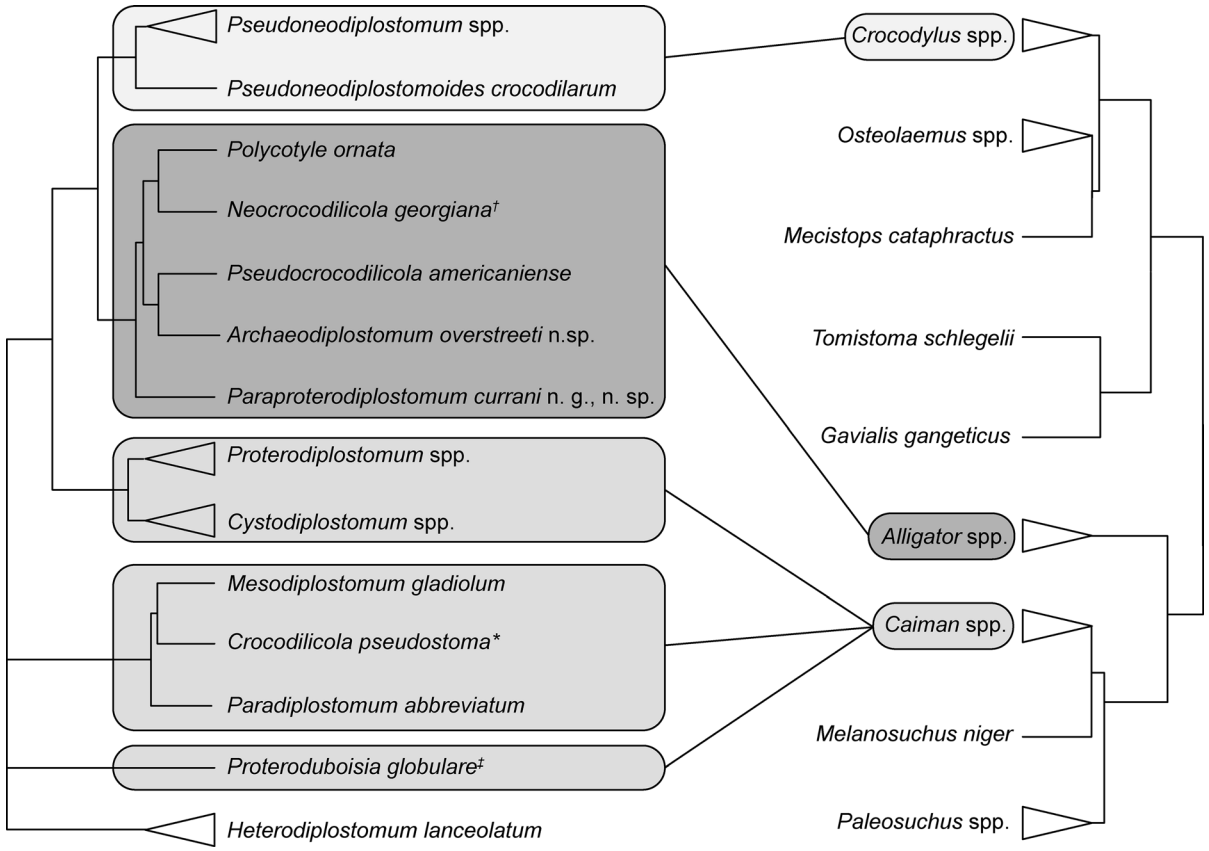

Fig. 7 Phylogenetic tree of the Proterodiplostomidae from the present study and the molecular phylogenetic tree of the Crocodilia (modified from Oaks, 2011) showing host associations between currently sequenced proterodiplostomids and extant crocodilian linages. Phylogenetic trees are presented as rectangular cladograms for convenience. Connecting lines and identical shades of grey color indicate host associations

recent active radiation of *Crocodylus* (see Oaks, 2011; Figs. 2, 7). The close relationships between clades uniting parasites of *Alligator* and *Crocodylus* suggest that as true crocodiles radiated they likely received their original proterodiplostomids from ancestors of modern alligators. It is difficult to speculate, however, where this could have happened geographically due to the broad distribution of both crocodilian lineages in the past.

Despite the paraphyly shown by the proterodiplostomids parasitic in caimans, all sub-clades in our tree (Figs. 2, 7) demonstrated strong associations between genera of proterodiplostomids and crocodilians. Proterodiplostomids from *Alligator* and *Crocodylus* formed well-supported monophyletic clades despite the high level of morphological diversification among members of each clade. The only deviation from the strict specificity to host genera in monophyletic clades in the 28S tree is the position of *Cr. pseudostoma* (GenBank: MF398328) together with *Paradiplostomum* and *Mesodiplostomum*, parasites of caimans (Fig. 2). As explained above, we believe that the sequences deposited in GenBank as *Cr. pseudostoma* were obtained from erroneously identified metacercariae.

Missing taxa and future prospects

Despite our extensive sampling effort and the broad representation of proterodiplostomid taxa in the resulting dataset, some critically important taxa and sequences from them are still missing. There are several crocodilian species in Asia, Africa, South and

Central America that have not been examined for proterodiplostomids at all. Some of them are endemic to a single island or a limited geographical area and therefore may have endemic parasite faunas. On the other hand, some crocodilians, including different species of caimans, are known as hosts of a diverse proterodiplostomid fauna, which has not been a subject of molecular systematic and phylogenetic analyses.

Some of the gaps are, however, more glaring than others. Probably the biggest gap in the available data is the lack of sequences from any proterodiplostomids parasitising gharials, which were repeatedly considered the basal group of extant crocodilians in morphology-based analyses. In addition, the distribution area of gharials lies within the overall distribution of the genus *Crocodylus* and overlaps with the current or recent historical distribution of the mugger crocodile *Crocodylus palustris* (Lesson) and *Co. porosus*. Equally missing and extremely interesting are proterodiplostomids from the Chinese alligator *Alligator sinensis* Fauvel, now critically endangered and on the brink of extinction. Therefore, only fixed museum specimens may potentially be a source of parasite samples. Other crucial hosts are the American crocodile *Crocodylus acutus* Cuvier, Orinoco crocodile *Crocodylus intermedius* Graves, and *Co. moreletii* whose geographic ranges overlap with the distribution areas of the American alligator and several species of caimans. Obtaining sequence data from proterodiplostomids parasitic in these hosts may potentially answer a variety of enticing questions regarding their evolutionary origin as well as the extent of physiological *vs* ecological specificity to their hosts. In addition to proterodiplostomids from crocodilian hosts, several known taxa of these digeneans parasitic in other hosts, such as snakes and turtles, are also awaiting sequencing and inclusion in future phylogenetic analyses.

Nevertheless, despite the lack of some important proterodiplostomids taxa in our analysis we believe that the views on their interrelationships and systematics presented here are well supported. Denser taxonomic sampling from a greater diversity of hosts and additional geographic areas should provide a solid background for a better understanding of the Proterodiplostomidae and their evolution and address the remaining unanswered questions presented in this study.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflicts of interest.

Ethical approval All applicable institutional, national and international guidelines for the care and use of animals were followed.

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