Taxonomy and phylogenetics of fish blood flukes using morphology, life history, and molecular markers

by

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ABSTRACT

Fish blood flukes (Digenea: Aporocotylidae) are of rapidly progressing and emerging interest to ecology and evolutionary biologists because some lineages may have co-evolved with the major lineages of non-tetrapod vertebrates (= “fishes”). They are relevant to medical researchers because they are the putative immediate ancestors to the blood flukes that cause schistosomiasis (Schistosomatidae), a disease that debilitates millions of people annually. Aquaculture industry personnel regard them as pathogens of high-value fishes in marine aquaculture operations. Taxonomists are interested in them as well because the rate of new species discovery is proportionally high relative to that of other fish trematode groups. Yet, ambiguity regarding fish blood fluke interrelationships obstructs a deeper understanding of the evolutionary origins of flatworm parasitism in craniates, including the origin of schistosomes. In order to address this issue, in my Ph.D. dissertation research, I have employed alpha taxonomy and molecular phylogenetics approaches to explore the taxonomic diversity of fish blood flukes that infect underexplored hosts and generate the most comprehensive phylogeny of Schistosomatoidea *sensu lato* to date. This work has resulted in publications in the *Journal of Parasitology*, and *Folia Parasitologica* comprising the taxonomic characterization of 6 new species of fish blood flukes of 4 genera (i.e., *Hyperandrotrema walterboegeri* Orélis-Ribeiro and Bullard, 2013 n. sp., *Plehniella sabajperezi* Orélis-Ribeiro and Bullard, 2015 n. sp.*, Plehniella armbrusteri* Orélis-Ribeiro and Bullard, 2015

n. sp*., Cladocaecum tomasscholzi* Orélis-Ribeiro and Bullard, 2016 n. gen., n. sp., *Elopicola* n. sp. 1, and *Elopicola* n. sp. 2), emended diagnosis of 3 of those genera, and re-description plus erection of a new genus (*Kritsky platyrhynchi* [Guidelli, Isaac, and Pavanelli, 2002] Orélis-Ribeiro and Bullard n. gen., n. comb.). The specimens described herein were derived from a wide taxonomic scope of fish hosts that included a lamniform shark (shortfin mako shark, *Isurus oxyrinchus* [Rafinesque]), pimelodid catfishes (long-whiskered catfishes, *Pimelodus albofasciatus* [Mees], *Pimelodus blochii* [Valenciennes], *Pimelodus grosskopfii* [Steindachner]), *Hemisorubim platyrhynchos* [Valenciennes]), an auchenipterid catfish (driftwood catfish (*Ageneiosus inermis* [Linnaeus]), and elopiform fishes (Hawaiian ladyfish, *Elops hawaiensis* [Reagan], and tarpon, *Megalops atlanticus* [Valenciennes]). Fishes were also collected from diverse sites throughout South and North America, and Asia. Additionally, my collaborators and I published in *Advances of Parasitology* a synoptic review of all published molecular studies (life history, taxonomy, phylogeny) and summarized all GenBank sequences and primer sets for the fish blood flukes. Further, I lead the analysis of new and all available sequence data for the partial D1–D2 domains of 28S rDNA from 83 blood fluke taxa, and explored the evolutionary expansion of flatworm parasitism in the blood of craniates. In the last chapter of my Ph.D. dissertation research, my collaborators and I substantially improved this previous phylogeny by targeting a denser taxon sampling among underexplored blood fluke lineages that infect chondrichthyan and actinopterygian hosts, and using a combination of 2 nuclear ribosomal genes (18S and 28S rDNA). Based on a dataset that comprises 97 blood fluke taxa, the resultant tree topologies represented the most well-resolved, large-scale phylogeny of blood flukes to

date. Although no significant novel relationships were recovered among tetrapod blood flukes (“spirorchiids” and schistosomes), our phylogenetic trees provided meaningful insights about the evolution of fish blood flukes. This is the first phylogenic reconstruction that tested and supported monophyly of chondrichthyan, elopiform, and otophysan blood flukes. The earliest-branching monophyletic group sister to the remaining fish blood flukes comprised an acipenseriform blood fluke, *Acipensericola petersoni*, plus all chondrichthyan blood flukes. This clade was recovered sister to elopiform blood flukes that, in turn, were sister to a clade comprising blood flukes that infect otophysan plus neoteleost fishes. Such branching order matches that of their hosts. In the context of Schistosomatoidea *sensu lato*, these results support the notion that blood flukes exhibit co-phyly with their craniate hosts.

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CHAPTER 1: DIVERSITY AND ANCESTRY OF FLATWORMS INFECTING BLOOD OF NONTETRAPOD CRANIATES ("FISHES")

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ABSTRACT

We herein review all published molecular studies (life history, taxonomy, phylogeny) and summarize all GenBank sequences and primer sets for the "fish blood flukes". Further, by analysing new and all available sequence data for the partial D1–D2 domains of 28S from 83 blood fluke taxa, we explore the evolutionary expansion of flatworm parasitism in the blood of craniates. Based on this analysis, the blood flukes infecting marine bony fishes (Euteleostei) are monophyletic. The clade comprising the chondrichthyan blood fluke plus the marine euteleost blood flukes is the sister group to tetrapod blood flukes (spirorchiids and schistosomes). The innominate blood fluke cercariae from freshwater gastropods were monophyletic and sister to the clade comprising spirorchiids and schistosomes, but low nodal support indicated that they may represent a distinct blood fluke lineage with phylogenetic affinities also to fish blood flukes. Blood flukes that utilize gastropod intermediate hosts were monophyletic (unidentified gastropod cercariae + tetrapod blood flukes) and those utilizing bivalves and polychaetes were monophyletic (marine fish blood flukes). Low or no taxon sampling among blood flukes of basal fish lineages and primary division freshwater fish lineages are significant data gaps needing closure. We also note that no record of an

infection exists in a hagfish (Myxiniformes), lamprey (Petromyzontiformes), or nontetrapod sarcopterygiian, i.e., coelocanth (Coelacanthimorpha) or lungfish (Dipnoi). The present phylogenetic analysis reiterated support for monophyly of Schistosomatidae and paraphyly of spirorchiids, with the blood flukes of freshwater turtles basal to those of marine turtles and schistosomes.

1. INTRODUCTION

Blood flukes (Platyhelminthes: Digenea: Schistosomatoidea) historically have been assigned to three families, each corresponding to the vertebrate definitive host lineages they infect (Amemiya et al., 2013; Nelson, 2006). Fish blood flukes (Digenea: Aporocotylidae; also as “Sanguinicolidae”; hereafter referred to as “FBFs”) (Bullard et al., 2009; Smith, 1972, 1997a, 1997b, 2002) infect nontetrapod craniates, i.e., paraphyletic fishes. Turtle blood flukes (Digenea: paraphyletic “Spirorchiidae”) principally infect marine and freshwater turtles (Chelonia) (Snyder, 2004), with recent molecular phylogenetic support for inclusion of the crocodile-infecting, dioecious blood fluke *Griphobilharzia amoena* (see Brant and Loker, 2005; Loker and Brant, 2006; Platt et al., 1991; 2013). Schistosomes (Digenea: Schistosomatidae) (see Lockyer et al., 2003b) infect birds and mammals (Cribb et al., 2001, Olson et al., 2003) and have been the most studied, making them among the best known of trematode families (Brant et al., 2006). They cause human schistosomiasis and are among the world’s most economically important metazoan parasites, with species of *Schistosoma* (principally *S. mansoni*, *S. japonicum*, and *S. haematobium*) infecting >230 million people from 28

countries and killing an estimated 280,000 people annually in sub-Saharan Africa (Rollinson et al., 2013; Van der Werf et al., 2003; World Health Organization, 2010). Most phylogenetic attention has focused on schistosomes, and abundant evidence exists that they comprise a monophyletic group. Two molecular phylogenetic studies have been published for turtle blood flukes (Snyder, 2004; Tkach et al., 2009) and nine for that of FBFs. The latter analyses typically include a limited number of taxa and markers (Tables 1–3) and treat relationships between or within genera, natural history, or life cycles (Aiken et al., 2007; Alama-Bermejo et al., 2011; Bray et al., 2012; Bullard et al., 2008; Cribb et al., 2011; Holzer et al., 2008; Nolan and Cribb, 2004a, 2006a,b; Ogawa et al., 2011).

FBFs are of rapidly emerging interest to ecology and evolutionary biology because some lineages may have coevolved with the major lineages of nontetrapod craniates (Bullard et al., 2008). They are relevant to medical researchers because they are the likely immediate ancestors to the tetrapod blood flukes (Brant et al., 2006), including those that cause schistosomiasis (Schistosomatidae) (op. cit.). They are of critical concern to aquatic animal health personnel who regard them as serious pathogens of high-value fishes kept in marine (Cribb et al., 2011; Ogawa et al., 2007) and freshwater (Kirk, 2012; Meade and Pratt, 1965; Meade, 1967; Schell, 1974) aquaculture operations (Bullard and Overstreet, 2002; 2008). Taxonomists also are interested in them because the rate of new species discovery is proportionally high relative to that of other fish trematode groups (Cribb and Bray, 2011). Yet, FBFs remain among the most poorly understood of trematode families, and ambiguity regarding their interrelationships

obstructs a nuanced understanding of the evolutionary origins of flatworm parasitism in the blood of craniates, including the origin of schistosomes.

An abundance of recent molecular phylogenetics data supports the notion that FBFs are the direct aquatic counterparts and likely ancestors of the tetrapod blood flukes (i.e., turtle blood flukes plus schistosomes). Yet, they are one of the most poorly understood of trematode families (Cribb and Bray, 2011). They require a single intermediate host, a gastropod, bivalve, or polychaete, and mature in the blood and body cavity, rarely other sites (Alama-Bermejo et al., 2011), of fishes ranging worldwide in rivers (Bullard et al., 2008; Truong and Bullard, 2013), estuaries (Bullard, 2013), coral reefs (Nolan and Cribb, 2006a,b), and offshore epipelagic waters (Ogawa et al., 2010; Orélis-Ribeiro et al., 2013). FBFs presently comprise 136 accepted species assigned to 35 genera, 20 of which are monotypic (Fig. 1). They thus outnumber both schistosomes (94 species in 15 genera)[1] and spirorchiids (85 species in 19 genera).[2] Nearly half of the recognized FBF genera (15 of 35) have been proposed since 2002 (Smith, 2002; Cribb and Bray, 2011), and many species have been described since 2002. Most recent descriptions have been conducted by workers in the western Pacific Ocean off Australia (T. Cribb and colleagues) and Japan (K. Ogawa and colleagues), the Mediterranean Sea (J.A. Raga and colleagues), and the Gulf of Mexico, Caribbean Sea, and northwestern Atlantic Ocean (S.A. Bullard and colleagues). The large number of monotypic genera (Fig. 1), the regional nature of described species, i.e., most species typically are known from single collections from a single geographic locality, and the large proportion of fishes that have yet to be critically examined for infections together indicate that many species of FBFs remain undiscovered in each of these regions and in adjacent waters.

FBFs are relevant to both basic research and applied research especially considering (i) host-parasite coevolution and (ii) health implications for aquacultured fishes. Regarding coevolution, FBFs exploit the spectrum of vertebrate lineages (Amemiya et al., 2013; Nelson, 2006), from the most primitive jawed (gnathostome) craniates (Chondrichthyes: sharks, rays, and chimaeras) (Bazikolova, 1932; Bullard and Jensen, 2008; Bullard et al., 2006; Madhavi and Rao, 1970; Orélis-Ribeiro et al., 2013; Short, 1954; van der Land, 1967) to the most highly derived of bony fishes (Pleuronectiformes and Tetraodontiformes) (Goto and Ozaki, 1929; Manter, 1940; Martin, 1960; Nolan and Cribb, 2004c; Ogawa et al., 2007; Young and Cribb, 2011) (Figs. 1–3). However, phylogenetic studies of FBFs have not kept pace with discoveries of new species and proposals of new genera. Phylogenetic inferences including each accepted genus and relevant out-group exist for Schistosomatidae and paraphyletic “Spirorchiidae” (op. cit.), but no comparable phylogeny exists for FBFs. As such, monophyly of the FBFs remains untested and, we argue, likely based on the assumption that all nontetrapod blood flukes are “fish blood flukes” and therefore members of a monophyletic Aporocotylidae. Hence, our understanding of the evolutionary origins of the blood flukes infecting terrestrial craniates remains somewhat opaque since we have not yet scrutinized deep phylogenetic interrelationships of FBFs. Available evidence from morphology (Bullard, in press; Bullard et al., 2006; Orélis-Ribeiro et al., 2013; Truong and Bullard, 2013) and molecular biology (Bullard et al., 2008; Cribb et al., 2011; present study) hints that blood flukes have coevolved with the major lineages of craniates, perhaps accompanying craniates onto land. Noteworthy is that no record of a blood fluke exists from any nontetrapod lineage of Sarcopterygii, i.e.,

coelacanths and lungfishes (Fig. 2E), which represents an obvious gap in our knowledge regarding the natural distribution and evolutionary expansion of these blood parasites in aquatic craniates (mostly fishes) and terrestrial craniates (tetrapods) (Amemiya et al., 2013; Nelson, 2006; Fig. 2E). With that suspected, long-shared evolutionary history between these flukes and their fish hosts as a backdrop, we argue that a better understanding of the evolutionary interrelationships and natural history of FBFs will underpin new approaches to further understanding those attributes in their putative descendants, the tetrapod blood flukes.

The study of FBF infections has applications to aquaculture because FBFs debilitate or kill fish within intensive culture systems (Bullard and Overstreet, 2002; 2008; Hardy-Smith et al., 2012; Ishimaru et al., 2013). Requirements to survey nearby invertebrates and fishes for infections and manage diseases in those settings, especially in offshore net pens ("sea cages"), have hastened FBF life cycle and epidemiological studies (Alama-Bermejo et al., 2011; Cribb et al., 2011; Hayward et al., 2010; Holzer et al., 2008; Ogawa et al., 2007, 2011; Shirakashi et al., 2012). In fact, the intensity of research activities focused on fish pathogenic FBFs in aquaculture has greatly increased our knowledge of their biology and life cycles. The tuna blood fluke *Cardicola forsteri*, which matures in commercially prized southern bluefin tuna (*Thunnus maccoyii*), has arguably become the most intensively studied of marine fish trematodes. Moreover, as aquaculture technologies expand to include more fish species cultured under a wider diversity of freshwater, marine, and estuarine environments, novel FBF pathogens may emerge that constrain those sectors of the aquaculture industry.

We review published molecular biological studies of FBFs in the areas of life history, taxonomy, and phylogenetics. We also provide a new phylogenetic analysis for the flatworms infecting the blood vascular system of craniates and discuss the relevance of FBFs to studies of tetrapod blood flukes (spirorchiids and Schistosomatidae).

2. LIFE HISTORY

FBF cercariae are minute, are difficult to identify morphologically, and typically have an extremely low prevalence of infection among invertebrate intermediate host populations (Cribb et al., 2011). Perhaps as a result of these attributes, the intermediate host(s) is unknown for all but a few FBF species: several species of the freshwater genus *Sanguinicola* (see Bullard and Overstreet, 2008) and the marine species *Aporocotyle simplex* (see Køie, 1982; Køie and Petersen, 1988, experimental infections; no molecular markers applied), *Paracardicoloides yamagutii* (see Nolan and Cribb, 2004a; see succeeding text), *Cardicola forsteri* (see Cribb et al., 2011; see succeeding text), and *Cardicola opisthorchis* (see Sugihara et al., 2014; see succeeding text). ITS2 rDNA sequences have been used to match morphologically indeterminate larval FBF specimens, i.e. cercariae, sporocysts, and rediae, collected from invertebrate hosts with morphologically distinctive adult specimens from sympatric fish hosts. Expeditiously and inexpensively, molecular markers enable rapid detection of infections, identification of the infective agent, by phylogenetic inference or by sequence homology to an already-sequenced taxon, and identification of intermediate hosts, thus linking life history stages of potentially pathogenic FBFs. Certainly, determining the identity of intermediate and

definitive hosts comprises a critical first step in understanding the life cycle of FBFs. Beyond diagnostics approaches, however, much remains to be learned about specific details of the host-parasite relationship among FBFs and their polychaete, gastropod, and bivalve intermediate hosts. After all, their evolutionary history may well be influenced as much, or more so, by these intermediate hosts as their definitive hosts. As such, we argue that molecular studies should not wholly supplant classical experimental studies that make direct microscopy observations of larval and adult FBFs in host tissues subsequent to exposures of naive invertebrate and definitive fish hosts (Køie, 1982).

Much of the information concerning FBF life cycles originates from aquatic animal health programs linked to commercial aquaculture: 17 of 109 (16%) of the available FBF sequences in GenBank derive from infections in marine aquaculture (Table 1). FBFs comprise one of the few trematode groups whose members can harm the definitive fish host as adults that occlude blood vessels and cause asphyxia, as eggs that damage or obstruct gill epithelia and branchial vessels, and as miracidia that hatch from eggs embedded in gill epithelium and bore out of the fish (Bullard and Overstreet, 2002; 2008). As such, their life cycles are of concern to commercial aquaculture operations because FBFs can kill or debilitate fish and cause economic losses in freshwater ponds, raceways, and offshore marine cages. Sequences sourced from nonadult FBFs infecting gastropod, bivalve, and polychaete hosts, which harbour FBF asexual reproductive stages —sporocyst, rediae, cercariae—are far less numerous (4 of 109) (Table 1) than those from adult specimens infecting fish hosts.

In the first published FBF life cycle study determined by the application of a molecular method, Nolan and Cribb (2004a) documented 2 cercarial morphotypes ("Type A" and "Type B") infecting 80 of 11,314 (0.7%) specimens of the hydrobiid gastropod *Posticobia brazieri* in tidal creeks of Queensland, Australia. ITS2 rDNA sequences from cercaria Type A aligned (100% agreement) with adult specimens of *P. yamagutii* that were collected from the blood (the dorsal aorta, atrium, ventricle, gills, kidney, and blood vessels of the intestine and swim bladder) of speckled longfin eels, *Anguilla reinhardtii*. In another study, responding to concerns about diseases associated with infections of *C. forsteri* infecting cultured southern bluefin tuna *Thunnus maccoyii* off South Australia, Cribb et al. (2011) screened 9,351 of individuals of 11 bivalve, 2 gastropod, and 24 polychaete families for FBF infections. ITS2 rDNA sequence data derived from cercaria that infected *Longicarpus modestus* (Polychaeta: Terebellidae) aligned with 100% agreement with adult specimens of *C. forsteri* from the heart of nearby southern bluefin tuna in a net pen at Port Lincoln, Australia. They also sequenced the 28S rDNA fragment (721 base pairs in D1–D2 region) from cercaria of *C. forsteri* that infected *L. modestus*. Following the approach of Cribb et al. (2011), Sugihara et al. (2014) focused on polychaetes while examining 744 invertebrates for FBF infections in a culture site of Pacific bluefin tuna, *Thunnus orientalis*, off southern Japan. Sporocysts and cercaria of FBFs were found in five individuals of a terebellid polychaete (*Terebella*) collected from within the shell of dead barnacles taken from the substratum and from ropes and floats below the sea cage. ITS2 and 28S sequences from sporocysts were identical (100%) to those of adults of *C. opisthorchis* from the heart of cultured Pacific bluefin tuna.

Shirakashi et al. (2012) studied concurrent infections of *Cardicola orientalis* and *Cardicola opisthorchis* in Pacific bluefin tuna. They used ITS2 rDNA sequence data to differentiate the crescent-shaped eggs of *C. opisthorchis* in the afferent filament artery from ovoid eggs of *C. orientalis* infecting the gill lamellae. They stated that species-specific PCR primers applied to gill tissue samples could complement histopathology and help diagnose infections before eggs and adults were numerous enough to be readily observed with light microscopy. Yong et al. (2013) used complete ITS2 rDNA sequence data to identify the FBF eggs lodged in the gill of five species of butterflyfish (Perciformes: Chaetodontidae) from the Great Barrier Reef as a single species, *Cardicola chaetodontis* (a single base-pair difference in two samples). FBF eggs are not infrequently observed in gill epithelium and branchial arterioles of fishes during routine fish necropsies (Bullard, personal observations), but in many instances, corresponding adult specimens that infect the blood of the individual fish are not recovered. Molecular techniques that effectively extract and amplify DNA from FBF eggs, which are minute, for example, eggs of *C. chaetodontis* are 40–60 μm in total length, promise to reveal the presence of unnamed FBF species and hitherto undocumented hosts if resulting sequences are placed in a phylogenetic context with sequences from named species. The same can be said for sequences derived from cercariae (see succeeding text). Using quantitative polymerase chain reaction (qPCR), Norte dos Santos et al. (2012) identified the eggs of *C. forsteri* aggregated in the gill of ranched southern bluefin tuna. Similarly, Kirchhoff et al. (2012) applied this method to diagnose eggs of *C. forsteri* infecting *T. maccoyii*. Polinski et al. (2013) took the approach a step further by developing a sensitive and accurate real-time PCR technique that can also be used for

identification of *C. forsteri*, *C. orientalis*, and *C. opisthorchis* in non-lethal samples. No cross-species or host genomic amplification was detected in either method tested, i.e., SYBR-based qPCR and a common reporter TaqMan assay; however, their combined application improved the reliability to differentiate species. These methods have confirmed concurrent infections of *C. forsteri* and *C. orientalis* in *T. maccoyii*, also indicating the higher prevalence and distribution of *C. orientalis* in the host.

Recently, the usefulness of both developed techniques was supported to assess the pathological consequences of FBF infections. Polinski et al. (2014) combined qPCR with host gene immune transcription to quantify amounts of DNA from tissues infected by *C. opisthorchis* and *C. orientalis*. They also quantified the temporal host immune response to those infections in caged Pacific bluefin tuna. Previous data documented adults and eggs of *C. orientalis* in the gill (Ogawa et al., 2010, Shirakashi et al., 2012), whereas adults and eggs of *C. opisthorchis* infected heart and afferent branchial arteries, respectively (Ogawa et al., 2011; Shirakashi et al., 2012). Polinski et al.'s (2014) results, however, revealed a correlation of IgM transcription to the high quantities of '*C. orientalis* only' infections in the gill tissues but not to DNA of *C. opisthorchis*, suggesting that such an immune response in this organ might be triggered by presence of adults rather than of eggs. Moreover, high levels of DNA of *C. orientalis* in the heart were attributed to the presence of juvenile flukes. Although such methods cannot identify FBF life history stages or whether or not the flukes are alive or dead, this quantitative approach enables conjecture about infection intensity and infection status, which is itself a promising tool for future epidemiological studies involving wild or cultured fishes.

Brant et al. (2006) used phylogenetic inference (28S, 18S, COI) in treating several unidentified putative FBF cercariae isolated from gastropods (Planorbidae and Thiaridae) in Uganda, Kenya, and Australia. They morphologically characterized the cercariae with photomicrographs and based upon presence/absence of eyespots, fin folds on the cercarial tail and body, and tail shape. Although a consistent and phylogenetically coherent, morphology-based definition (diagnosis) of FBF cercariae is lacking, these cercariae are typically distinctive: minute, forktailed, without a ventral sucker, with a distinctive penetrating organ that likely comprises a specialized anterior sucker (Truong and Bullard, 2013), a fin fold on the cercarial body, and a fin fold present or absent on the tail furcae (Cribb et al., 2011). One of these cercaria (W5004) was especially morphologically bizarre, i.e., described as apharyngeate and furcocercous but with “extravagant lateral tail membranes and a pointed body shape”, and none of these cercariae had all of the typical features of FBF cercariae (see preceding text). However, the combined 18S and 28S analysis of these cercariae clustered them with other FBF sequences (we presume that “Sanguinicolid sp.” is that of “*Sanguinicola* cf. *inermis*” [AY222180]). For that reason and because they were morphologically bizarre relative to known FBF cercariae, these authors posited that a much greater morphological diversity of FBF cercariae exists than has been recognized in the literature to date. The taxonomic identity and phylogenetic significance of these sequences are further discussed later in the text.

3. TAXONOMY

Most available FBF DNA sequence information is derived from adult FBFs infecting the heart of marine fishes (Table 1). As of 14 February 2014, GenBank contained 109 FBF nucleotide sequences derived from 22 published papers plus one sequence from an unpublished work (Tables 1 and 2). In total, these sequences represent 48 of 136 (35%) nominal FBFs assigned to 15 of 35 (43%) accepted genera and infecting 42 bony fish species and one chondrichthyan species (Tables 1 and 2). For most genera, very few or no species have been sequenced, and adults of only one freshwater FBF have been sequenced to date (Fig. 1; Bullard et al., 2008). ITS2 rDNA comprises 65 of 109 (59%) GenBank nucleotide sequences for 41 of 48 (85%) FBF species; 28S comprises 28 (26%) sequences for 20 (42%) species, 18S comprises 10 (9%) sequences for 10 (21%) species; COI comprises 6 (6%) sequences for 5 (10%) species. Noteworthy also is that 29 of 48 (61%) FBF species in GenBank are represented only by a single gene: ITS2 (27 species), 28S (1), or 18S (1). Sequence data from the combination of 28S plus ITS2 genes exist for 12 species and those of 18S plus 28S genes exist for 9 species. Few FBF species have been characterized by more than two genes: 18S, 28S, and COI sequence data are available for *Chimaerohemecus trondheimensis*, and that for 18S, 28S, and ITS2 exists for *P. yamagutii*, *Psettarium* (as *Paradeontacylix*) *sinensis*, *Plethorchis acanthus*, and *Skoulekia meningialis*. All ITS2 nucleotide sequences are represented by complete fragments (ITS2: ~390 bp), whereas the other fragments are depicted mostly by near-complete (18S: ~1800 bp, 28S: ~3700 bp, and COI: ~1100 bp) and partial sequences (28S: ~1300 bp, and COI: ~400 bp) (Table 1). In addition, several sequences representing innominate FBFs have resulted from parasitological surveys of wild (Alama-Bermejo et al., 2011; Hernández-Orts et al., 2012; Nolan and Cribb, 2004a,

2006a,b) and cultured fishes (Holzer et al., 2008; Ogawa et al., 2007, 2011; Repulles-Albelda et al., 2008; see succeeding text) (Table 1).

Nolan and Cribb (2006a) characterized a high level of genetic diversity, interpreted as FBF species richness (two new species of *Ankistromeces* and nine new species of *Phthinomita*), among morphologically similar FBF specimens infecting siganid, labrid, and mullid reef fishes off Australia and Palau. They sequenced replicates of complete ITS2 rDNA from specimens of each new species from a total of 29 host/parasite/locality combinations. The study revealed 19 distinct genotypes (having 1−41 base differences), which defined those species along with morphological characters—together representing the first published evidence of cryptic speciation among FBFs that otherwise would have been undetected or underestimated if morphology alone had been considered. This level of ITS2 sequence conservation is interesting because variation in the ITS1 is detectable within a species or an individual among other Digenea (Nolan and Cribb, 2005) as well as other fish-parasitic platyhelminths, for example, ectoparasitic capsaline monogenoids (Bullard et al., 2011).

In a case of concurrent infection, Shirakashi et al. (2013) sequenced partial 28S and complete ITS2 rDNA regions to complement morphological identification of *C. orientalis* and *C. forsteri* infecting gill and heart, respectively, of ranched southern bluefin tuna off South Australia. This was the first report of *C. orientalis* infecting *T. maccoyii*, not only expanding the known geographic distribution of this FBF but also raising new concerns to the aquaculture industry.

To our knowledge, only one study has applied molecular tools to test a biogeographical hypothesis with FBFs. Aiken et al. (2007) tested whether or not

parasites of epipelagic fishes are as geographically widespread and genetically similar as their hosts. Complete ITS2 rDNA sequences of *C. forsteri* from wild and cultured southern bluefin tuna off the Great Australian Bight as well as those from cultured Atlantic bluefin tuna, *Thunnus thynnus*, in the Mediterranean Sea off Spain matched 100%. A similar match was demonstrated among sequences of *Cardicola* sp. from cultured Pacific bluefin tuna off Mexico and cultured Atlantic bluefin tuna from Spain. Partial 28S rDNA sequences of *C. forsteri* from tunas off Australia and Spain differed by 1 base-pair (Holzer et al., 2008), perhaps explained by highly variable regions of the 28S (Olson and Tkach, 2005). Beyond basic research on biogeography, molecular characterizations and delineations of specific blood fluke strains in a given geographic area are relevant to the aquaculture industry since some of those strains may have different levels of pathogenicity to different host populations. Given how fish in the food aquaculture industry and in the ornamental pet trade are transported globally, such information could be also relevant to bio-security and conservation biology of endemic fish populations.

4. PHYLOGENY

A phylogenetic hypothesis based on morphological or molecular data and including species from the majority of accepted FBF genera has yet to be published. Early studies incorporating FBF sequence data used 18S rDNA to root phylogenies testing monophyly and interrelationships of Schistosomatidae (Lockyer et al., 2003b; Snyder and Loker, 2000), Digenea (Cribb et al., 2001; Olson et al., 2003), and Platyhelminthes

(Littlewood et al., 1999, 2001; Lockyer et al., 2003a). Later studies using FBF sequences have focused on interrelationships among genera and species within the family (including ribosomal 18S, ITS2, and 28S plus mitochondrial COI genes; Table 1 and 2). Most of these studies rely on already-deposited GenBank sequences and add one or a few novel sequences to generate a phylogeny. Nearly all of these sequences derive from adult FBFs collected from marine bony fishes (Euteleostei) (Holzer et al., 2008; Nolan and Cribb, 2006a, b). No shark or ray FBF has been sequenced to date, but the holocephalan blood fluke *C. trondheimensis* has been hypothesized as a lineage basal to all other FBFs (Bullard et al., 2008) or as a close relative of '*Sanguinicola'* cf. *inermis* with indeterminate phylogenetic affiliation to the other FBFs (Cribb et al., 2011).

Nolan and Cribb (2006a) used complete ITS2 rDNA to show genetic distance and intraspecific conservation of ITS2 sequences between and within, respectively, select species of *Phthinomita* (*P. littlewoodi*, *P. jonesi*, and *P. hallae*), making the case that ITS2 is a reliable species-level barcode for FBFs. These same authors presented an ITS2 phylogeny for *Phthinomita* spp. and *Ankistromeces* spp. and overlaid the host affiliations for each FBF species. Morphologically similar species of *Phthinomita* infecting siganids were not monophyletic nor was *Phthinomita*. The species of *Ankistromeces* were monophyletic, forming a crown group sister to "*Phthinomita* sp. C" infecting siganids and related to all species of paraphyletic *Phthinomita*. The species of *Ankistromeces* likewise infected not only siganids but also a filefish (Tetraodontiformes: Monocanthidae). Noteworthy is that ITS2 data show that sister species of *Phthinomita* and *Ankistromeces* infected bony fishes that are phylogenetically unrelated but occupy similar niches on the Great Barrier Reef. This perhaps indicates that ecological factors

(proximity, abundance, and habitat distribution of invertebrate intermediate hosts) and host switching drive FBF speciation in that coral reef system more so than definitive host ancestry. The identities of invertebrate intermediate hosts for species of *Phthinomita* and *Ankistromeces* remain indeterminate. Nolan and Cribb (2006b) used distance analysis (minimum evolution and neighbor joining) of complete ITS2 rDNA sequences to differentiate 4 clades among *Cardicola* and *Braya* on the Great Barrier Reef. These clades agreed with the infected host groups: *Cardicola* spp. infecting rabbitfishes (Siganidae), snappers (Lutjanidae), butterflyfishes (Chaetodontidae), and tunas (Scombridae) and *Braya* spp. infecting parrotfishes (Scaridae) only. Their results demonstrated paraphyly of *Cardicola*, with *Cardicola* spp. infecting tunas comprising a sister clade to that of *Braya* spp. This result was consistent with previous morphology-based studies of *Cardicola* spp. (Bullard, 2010; 2013; Bullard and Overstreet, 2003; Bullard et al., 2012) that indicated a taxonomic revision of the genus was needed concomitant with a reconsideration of *Elaphrobates*, which may not be distinct from *Cardicola*.

ITS2 rDNA sequence data and analysis have yielded inconsistent phylogenetic results. Aiken et al. (2007) used Bayesian inference for complete ITS2 rDNA sequence data for *Pearsonellum corventum* (out-group, parasite of groupers (Serranidae)), *Braya* spp., *C. forsteri*, several innominate species of *Cardicola*, and nine species of *Cardicola* described by Nolan and Cribb (2006b). Their resultant topology, like that of Nolan and Cribb (2006b) revealed that *C. milleri* is sister to all other named, non-*C. forsteri* taxa. However, unlike Nolan and Cribb (2006b), Aiken et al. (2007) reported 2 clades comprising (*C. covacinae*(*C. coeptus*(*C. bartolii*, *C. watsonensis*))) and (*C. lafii*, *C.*

parilus(*Cardicola* sp. 3(*C. tantabiddii*, *Cardicola.* sp. 2))), whereas Nolan and Cribb (2006b) showed (*C. coeptus*(*C. covacinae*(*C. bartolii*, *C. watsonensis*))). Significant also is that the tuna blood flukes *C. forsteri*, "*Cardicola* sp. 3," and "*Cardicola* sp. 4" were monophyletic and sister to the *Braya* spp. in the phylogeny of Nolan and Cribb (2006b), but they were paraphyletic in Aiken et al., (2007), with *Cardicola* spp. that infect mackerels (*Scomberomorus* spp.) ("*Cardicola* sp. 4",*"Cardicola* sp. 5") sister to tuna blood flukes, snappers (Lutjanidae), and rabbitfishes (Siganidae). Noteworthy here is that, although using the same molecular marker (ITS2) applied to most of the same in-group taxa (except *C. chaetodontis*, not included in Aiken's et al. (2007) analysis), outgroup choice seemingly influenced the resultant topology: instead of using *P. corventum* as the out-group, Nolan and Cribb (2006b) used *C. forsteri*. Although results differ, both indicate that *Cardicola* needs revision (op. cit.) and perhaps that distinct FBF genera are justified for snappers (Lutjanidae), with FBFs of spinefoots (Siganidae) harboring species of a closely related but distinct genus.

Bullard et al. (2008) compared near-complete small subunit ribosomal DNA sequence data for *C. trondheimensis*, *Acipensericola petersoni*, the putative species of *Sanguinicola* ("*Sanguinicola* cf. *inermis*"), *Aporocotyle spinosicanalis*, *P. acanthus*, and *Neoparacardicola nasonis*. The resultant topology suggested that basal gnathostomes (represented by Chondrichthyes) and basal actinopterygians (represented by Acipenseriformes) harbour lineages of FBFs that are basal to those of higher bony fishes (Euteleostei). This contradicts Smith (1997a), who argued that FBFs lack detectable phylogenetic specificity to particular host lineages. Reports from a growing number of FBF genera indicate some level of phylogenetic host specificity based on

both molecular data (Aiken et al., 2007; Holzer et al., 2008; Nolan and Cribb, 2006a, b) and morphology. Simply, closely related blood flukes seemingly infect closely related hosts, for example, blood flukes of lamniform sharks (Orélis-Ribeiro et al., 2013), sturgeon and paddlefish (Acipenseriformes) (Bullard et al., 2008), walking catfishes (Siluriformes: Clariidae) (Truong and Bullard, 2013), drums (Perciformes: Sciaenidae) (Bullard and Overstreet, 2004; Bullard et al., 2012), groupers (Serranidae) (Bullard, 2012; Nolan and Cribb, 2004a; Overstreet and Køie, 1989), and amberjacks (*Seriola* spp.) (Holzer et al., 2008).

Holzer et al. (2008) conducted a phylogenetic analysis of partial 28S rDNA sequences from several putative FBF cercaria (see Brant et al., 2006), some of which were curiously basal to *C. trondheimensis* and all remaining FBFs analysed. Their analysis showed that the blood flukes (*Paradeontacylix* spp.) of amberjacks (*Seriola* spp.) were monophyletic and formed a clade sister to *C. forsteri* and *Cardicola coeptus*. Their analysis of ITS2 sequence data yielded a phylogeny that left unresolved the relationship between *Psettarium sinensis* (as *Paradeontacylix*), *Braya* spp., and a clade including *Cardicola* spp. and *Paradeontacylix* spp. This phylogeny proved *Cardicola* to be paraphyletic and had a topology for the remaining taxa that was comparable to that of Nolan and Cribb (2006b; see preceding text for discrepancies between Aiken et al. (2007) and these works). Their ITS2 results grouped *Cardicola aurata* as the sister taxon to *Paradeontacylix* (0.54 nodal support).

Cribb et al. (2011) conducted a Bayesian analysis of partial large subunit rDNA sequences across available FBF sequences to establish the taxonomic identity of cercaria isolated from the terebellid polycheate *L. modestus*. Although not the primary

purpose of their study, this phylogeny (see Fig. 1 of Cribb et al., 2011) along with the analyses conducted by Bray et al. (2012) (see later text for more details) offers the most phylogenetic breadth previously published for blood flukes, including 52 taxa. That tree is interesting for several reasons. First, Aporocotylidae is paraphyletic, a result that is concordant with the paraphyly of “fishes”. Second, *C. trondheimensis* and an assumed species of *Sanguinicola* are in a clade that forms a polytomy with two lineages of blood flukes, including schistosomes and the paraphyletic spirorchiids plus marine FBFs. This tree topology potentially foretells the level of taxonomic revision necessary for not only FBFs but also Schistosomatoidea *sensu lato*. Cribb et al. (2011) also detailed records of known and probable FBF cercaria, their hosts, and their morphological features and included a detailed discussion of how much we do not know about the morphology of FBF cercariae. Such discussion echoed that of Brant et al. (2006), who also suggested that diagnostic morphological features of FBF cercaria have been insufficiently explored.

Alama-Bermejo et al. (2011) presented topologies based upon partial 28S rDNA and complete ITS2 rDNA. The former phylogenetic hypothesis comprised 3 distinct lineages: *P. acanthus*, a clade comprising monophyletic *Cardicola* spp. and *Paradeontacylix* spp., and a clade including *S. meningialis* (an ecologically bizarre FBF that infects the ectomeningeal veins of the brain in common two-banded seabream, *Diplodus vulgaris*), *Psettarium sinensis* (as *Paradeontacylix sinensis*), and *Sasala nolani* (as “Sanguinicolid sp. Moorea-DTJL-2002”). The latter tree showed the 2 species of *Pearsonellum* as sister to (*S. meningialis* (*Psettarium* sp. KH2007(*Psettarium* sp. Aburatsubo 3.2 EF544056))).

Ogawa et al. (2011) presented trees based on complete ITS2 rDNA and partial 28S rRNA. The ITS2 analysis (Bayesian) resulted in a topology that showed *Psettarium sinensis* (as *Paradeontacylix sinesis*) and *P. corventum* as two lineages basal to the clade comprising four distinct lineages: *Braya* spp., *Cardicola* spp. ("*Cardicola* 1"), three species of *Cardicola* and monophyletic *Paradeontacylix*, and three species of *Cardicola* that infect tunas (Scombridae). The latter topology showed a paraphyletic *Cardicola*, a monophyletic *Paradeontacylix*, and *Psettarium sinensis* and *C. orientalis* as distinct lineages.

Bray et al. (2012) conducted a Bayesian analysis assembling three datasets, i.e., 18S, 28S, and concatenated 18S+28S, to test the phylogenetic position of *Sasala nolani* with other available FBFs sequences. Due to greatest taxonomic coverage, only the 28S tree including 31 taxa was reported, showing a topology concordant with Cribb et al.'s (2011) tree regarding paraphyly of the family. However, noteworthy is that Bray et al. (2012) reported 2 clades comprising (*N. nasonis*(*P. acanthus*(*S. nolani*, *P. sinensis*(*S. meningialis*)))) and (*Paradeontacylix ibericus*(*P. grandispinus*(*P. balearicus*, *P. kampachi*(*P. godfreyi*(*C. aurata*), whereas Cribb et al. (2011) showed (*P. acanthus*(*N. nasonis*(*S. meningialis*(*S. nolani*, *P. sinensis*)))) and (*P. grandispinus*, *P. balearicus*(*P. godfreyi*(*P. kampachi*, *P. ibericus*))), with *C. aurata* in a sister clade, i.e., *Cardicola* clade, grouping with *C. coeptus*, *C. forsteri*, and cercaria from *L. modestus* and *Reterebella aloba*. Such discrepancies may be attributable to the differences in taxon sampling, resulting in differing alignment length and nucleotide substitution models estimated for the datasets. In specific, Cribb et al. (2011) studied an alignment

comprising 859 nucleotide bases and TVM+I+G was the model predicted; whereas, Bray et al. (2012) considered 693 bases and GTR+I+G was the model chosen.

5. APPROACH TO OUR PHYLOGENETIC ANALYSIS

We analyzed GenBank sequence data for the partial D1–D2 domains of 28S and reconstructed a phylogeny that included 88 taxa from Lockyer et al. (2003b), Morgan et al. (2003), Snyder (2004), Brant (2007), Brant and Loker (2009), Hanelt et al. (2009), Tkach et al. (2009), Cribb et al. (2011), and Brant et al. (2012). This phylogeny included new sequences from *Cardicola currani* (KJ272524), *Cardicola palmeri* (KJ572525), and *Elaphrobates euzeti* (KJ572526). Total genomic DNA from newly collected specimens was extracted using DNeasy™ Tissue kit (QIAGEN) according to the manufacturer's protocol. PCR was carried out using the primer combination U178+L1642 (Table 3) following the method described in Lockyer et al. (2003b). The PCR products were purified using the QIAquick Gel Extraction Kit (QIAGEN) following the manufacturer's protocols. DNA sequencing was performed by Genewiz with ABI Prism 3730xl DNA analysers (Genewiz, Inc., South Plainfield, NJ) using the same primers as used in the PCR. We thought that the inclusion of additional schistosome and FBF taxa could be helpful because dense taxon sampling can yield more accurate estimates of evolutionary relationships (Heath et al., 2008).

Regarding the out-group, initially, the 28S data set was analysed with an aspidogastrean taxon (*Rugogaster hydrolagi* (AY157176)) and three bivesiculids (*Bivesicula claviformis* (AY222182), *Bivesicula unexpecta* (AY222181), and

Bivesiculoides fusiformis (AY222183)) along with all available FBF sequences, "Spirorchiidae" and Schistosomatidae plus those of select members of Diplostomoidea (*Hysteromorpha triloba* (HM114365); *Alaria alata* (AF184263)), Strigeidae (*Ichthyocotylurus erraticus* (AY222172)), Clinostomidae (*Clinostomum* spp. (AY222175-6)), and Brachylaimidae (*Brachylaima virginianum* (DQ060330) and *Brachylaima thompsoni* [AF184262]). In that analysis, the *G. amoena*+*Hapalorhynchus gracilis* clade was the most basal tetrapod blood fluke lineage, i.e., sister to the remaining turtle blood flukes and schistosomes. Previous phylogenetic inferences position that clade sister to a group comprising marine turtle blood flukes and schistosomes, with blood flukes of freshwater turtles as basal to those lineages, for example, Brant and Loker (2005) and Loker and Brant (2006). Diplostomoidea has previously been considered the sister taxon to all blood flukes (Olson et al., 2003), and, hence, our remaining analyses were performed with Diplostomoidea as the out-group.

Regarding the in-group, previous authors have used unspecified larval clinostomes (AY222175: metacercaria from unspecified site in firetail gudgeon, *Hypseleotris galii* (Perciformes: Eleotridae) from coastal streams in Australia, and AY222176: metacercaria from unspecified site in American bullfrog, *Rana catesbeiana* from the United States) in blood fluke phylogeny (Bray et al., 2012; Cribb et al., 2011; Olson et al., 2003). The justification for the inclusion of clinostomes in resolving blood fluke phylogeny arises from similarity in sequence data and cercarial morphology. The ssrDNA+lsrDNA phylogeny of Olson et al. (2003) shows a sister group relationship between those unspecified clinostome sequences and that of some FBFs. In addition, and perhaps highly significant, clinostome cercariae have a reportedly uncanny

morphological similarity to the blood flukes of fishes and tetrapods (Dönges, 1974; Kirk and Lewis, 1993). In addition, as stated earlier in the text, several sequences sourced from unspecified cercarial infections of freshwater gastropods from Eastern Europe, Africa, and Australia similarly have been used (Brant et al., 2006; Olson et al., 2003; discussions in the preceding text). Olson et al. (2003) used sequences derived from cercariae (AY222180) to represent "Sanguinicolidae" that were identified as "*Sanguinicola* cf. *inermis*" because they infected a freshwater gastropod (*Lymnaea stagnalis*) in a water body known to harbour carps (*Cyprinus* spp.) infected by an alleged species of *Sanguinicola* (see Bullard et al., 2008; Kirk, 2012). The original identification of these specimens has drifted, with subsequent authors misrepresenting Olson et al.'s (2003) identification by reporting it as "*Sanguinicola inermis*" or by using AY222180 as a definitive representative of the genus *Sanguinicola* in FBF phylogenetic studies. Nonexistent, however, is published morphological evidence that those specimens were a species of *Sanguinicola* or *S. inermis*, and, to our knowledge, no voucher specimen exists. For this reason, we caution authors in using AY222180 as a definitive representative of *Sanguinicola*. Brant et al. (2006) used phylogenetic inference to identify their cercariae as FBFs (AY585878-81; see discussion earlier in the text), and including AY222180 as "Sanguinicolid sp." Noteworthy here is that, except for the 18S sequence data from adults of *A. petersoni* (see Bullard et al., 2008), nonexistent is sequence data sourced from a definitively identified adult FBF that infects a primary division freshwater fish host. Obviously, molecular phylogenetic inference is a powerful tool in identification of cercaria; however, it should be understood that we remain unconvinced of the species-, genus-, or family-level identities of these cercariae that

have collectively been derived from freshwater gastropods and previously treated as FBFs. This is particularly problematic regarding our understanding of the marine/freshwater origins of blood fluke lineages. Adding to the complexity of the matter, "*Sanguinicola*" traditionally has been used as a repository for any freshwater FBF, and the genus as it is broadly interpreted now likely includes several genera.

To test the effect of including 28S sequence data from species of *Clinostomum* and the freshwater gastropod cercariae of Olson et al. (2003) and Brant et al. (2006), we analyzed the dataset as +fw gastropod cercariae / –clinostomes (Fig. 2A), –fw gastropod cercariae / +clinostomes (Fig. 2B), +fw gastropod cercariae / +clinostomes (Fig. 2C), and –fw gastropod cercariae / –clinostomes (Fig. 2D). Sequences were aligned using MUSCLE version 3.7 (Edgar, 2004) with ClustalW sequence weighting and UPGMA clustering for iterations 1 and 2. Resultant alignment was refined by eye using MEGA version 5.2.2 (Tamura et al., 2011) and ends of each fragment were trimmed to match the shortest sequence. Ambiguously aligned positions were identified and removed using a Gblocks server (Castresana, 2000) with all default settings for a less stringent selection. Bayesian inference was performed using MrBayes version 3.2.2 (Huelsenbeck and Ronquist, 2005; Huelsenbeck et al., 2001; Ronquist and Huelsenbeck, 2003) run on CIPRES portal (Miller et al., 2010). The software jModelTest version 2.1.4 (Darriba et al., 2012; Guindon and Gascuel, 2003) was used to select an appropriate substitution model. The GTR+I+G (proportion of invariable sites=0.287 and gamma distribution=1.352) model was inferred as the best estimator by the Akaike Information Criterion (AIC), therefore Bayesian analyses used the following parameters: nst=6, rates=invgamma, ngammacat=4, and default priors. Analyses were run in

duplicate each containing four simultaneous Markov chain Monte Carlo (MCMC) methods (nchains=4) for 1.0×10^7 generations (ngen=10,000,000) sampled at intervals of 1000 generations (samplefreq=1000). Results of the first 3000 trees were discarded as "burn-in" based on the ‘stationarity’ of all parameters sampled by the chains and assessed using Tracer version 1.5 (Rambaut and Drummond, 2008). All retained trees were used to estimate posterior probability of each node. The resulting data matrix for the analysis discussed below (Fig. 2A; 3) comprised 647 positions per taxon (224 conserved, 423 variable, and 358 parsimony-informative), and posterior probability provided strong support to individual nodes.

6. RESULTS FROM OUR PHYLOGENETIC ANALYSIS

The phylogeny based on the inclusion of the freshwater gastropod cercariae and exclusion of clinostomes (Figs. 2A and 3) forms the foundation for the discussion of the succeeding text. However, we find it noteworthy how the topology changes by including/excluding those sequences (Figs. 2B–D). If including the freshwater gastropod cercariae only (Fig. 2A), FBFs are monophyletic (all sequences sourced from adult specimens) and the freshwater gastropod cercariae are the sister group to all other blood flukes. If including clinostomes only (Fig. 2B), nodal support for a monophyletic Aporocotylidae increases slightly (0.79–0.89) and nodal support for a monophyletic turtle blood flukes plus schistosomes decreases slightly (0.93–0.88). If both the freshwater cercariae and clinostomes are included (Fig. 2C), the freshwater gastropod cercariae, marine bony FBFs, and *C. trondheimensis* are recovered as distinct lineages

forming a polytomy, with relatively low (0.73) nodal support for the monophyly of clinostomes plus all non-FBFs.

Noteworthy here is that when clinostomes are included, they are the sister group to the blood flukes that infect tetrapods (spirorchiids and schistosomes) and they make more ambiguous the phylogenetic relationship between the freshwater gastropod cercariae and the blood flukes of fishes and tetrapods. On the face of it, some of us (SAB, ROR) initially found it difficult to accept that spirorchiids and schistosomes share a more recent common ancestor with the clinostomes than with the FBFs, i.e., clinostome membership within Schistosomatoidea. Yet, one of us (THC), who is more familiar with clinostomes, finds it not inconceivable that the clinostomid life cycle is an extension of a two-host blood fluke life cycle wherein a vertebrate definitive host consumed the “original” definitive host such that the blood fluke infection remained viable in the predator host. Indeed, it is relatively bizarre that clinostomes infect the esophagus of their vertebrate hosts, rather than the intestine as most other trematodes do. Moreover, clinostomids, spirorchiids and schistosomatids have ventral suckers; whereas, FBFs lack a ventral sucker. Hence, and taking into account the uncanny morphological similarity of their cercariae (see preceding text), although we accept that adult morphology and definitive host associations make the clinostome+blood fluke association unlikely, our minds are open to novel, future interpretations that may seem strange now.

If freshwater gastropod cercariae and clinostomes both are excluded (Fig. 2D), a clear sister group relationship between the monophyletic FBFs and all other blood flukes is recovered with high nodal support for both clades (0.90 and 0.98, respectively).

However, we think this latter scenario is oversimplified and dodges one of the most interesting aspects of blood fluke phylogeny: the hitherto unresolved phylogenetic affinities among blood flukes of basal fishes, especially those infecting primary division freshwater fishes, euteleosts, tetrapods, and non-tetrapod sarcopterygians. As such, and although the taxonomic identities of Olson et al.'s (2003) and Brant et al.'s (2006) cecariae are indeterminate, including them in the analysis is preferable to us because these cercariae (i) undoubtedly show clear molecular phylogenetic affinities to both turtle blood flukes and FBFs, (ii) were collected from freshwater localities, (iii) infected gastropod species belonging to taxonomic groups known as FBF hosts (although little is known about turtle blood fluke intermediate hosts), and (iv) may well prove to represent FBFs, possibly species of *Sanguinicola* or novel, closely-related genera. We also note the possibility, however distant, that one or several of Brant et al.'s (2006) cercariae from Africa could mature in lungfishes (Sarcopterygii: Dipnoi: Ceratodontiformes: Protopteridae), which would prove particularly exciting because lungfishes are the immediate ancestor to all terrestrial craniates, the sister lineage to Tetrapoda, and presently lack any record of an infection by a blood fluke. Indeed, the localities for these cercarial infections (Brant et al., 2006) comprise rivers harbouring populations of African lungfishes, *Protopterus* spp. (Berra, 2007). Finally, and perhaps not coincidentally, the present parasite phylogeny recovered that places those cercariae as the sister group to the tetrapod blood flukes (Figs. 2A and 3) mirrors the phylogenetic arrangement of craniates (Fig. 2E) regarding Actinopterygii as the sister group to lungfishes and terrestrial craniates (Amemiya et al., 2013; Nelson, 2006), if coelacanths are excluded.

7. SUMMARY OF PHYLOGENETIC STUDY

Considering these aspects, we primarily discuss the tree that includes the freshwater gastropod cercariae along with all other sequences sourced from adult FBFs (Fig. 3). Several main observations are worthy of note.

(1) With the lowest taxon sampling of all blood flukes in the present analysis, considering only sequences derived from adult specimens, and without sequence data sourced from taxonomically identified adults of any freshwater FBF, the monophyly of Aporocotylidae was not rejected. *Chimaerohemecus trondheimensis*, a blood fluke that matures in chimaeras (Chondrichthyes: Holocephali), clearly represents a distantly related lineage that is the sister taxon to the FBFs infecting marine bony fishes (Figs. 2A and 3). It clusters with FBFs infecting marine bony fishes with a higher posterior probability (0.90) when clinostomes and the freshwater gastropod cercariae were excluded from the analysis. The presence of C-shaped tegumental body spines, unique to species of *Chimaerohemecus*, *Selachohemecus*, and *Hyperandrotrema*, suggests that the blood flukes of chimaeras and sharks are monophyletic. Sequence data from those additional taxa are pending.

(2) The blood flukes of bony fishes (Euteleostei) were monophyletic. No 28S sequence data derived from a definitively identified adult FBF infecting a primary division freshwater fish exists to date (see discussion earlier in the text). Hence, by default, all of the FBFs that infect bony fishes and for which 28S sequence data exist are marine species. Nevertheless, the present analysis shows that those taxa are monophyletic. If the clade that includes the unidentified African and Australian cercariae

and *Sanguinicola* cf. *inermis* does in fact represent blood flukes that mature in primary division freshwater fishes, then the “monophyly” of the marine FBFs we show in the present phylogeny would become more meaningful. Indeed, it would also indicate paraphyly of the Aporocotylidae. This gap in sequence data representative of “freshwater FBFs” needs to be closed and should include data from *Sanguinicola*, *Plehniella*, *Nomasanguinicola*, *Acipensericola* as well as the other genera of blood flukes that infect primary division freshwater fish lineages. Adult specimens corresponding to those sequences should be lodged in curated helminthological museum. Until then, discussion of the marine and freshwater monophyly and/or origins of the FBFs is speculative at best. However, insights from comparative morphology have been discussed in detail in a series of systematic works for FBFs that infect primary division freshwater fishes (Bullard et al., 2008; Bullard, 2013; Bullard, in press; Orélis-Ribeiro and Bullard, unpublished observations; Truong and Bullard, 2013). These results together strongly indicate that the blood flukes of primary division freshwater fishes likely share a recent common ancestor and that they exhibit phylogenetic affinities to the turtle blood flukes, suggesting paraphyly of FBFs.

(3) Generic interrelationships among the blood flukes of marine bony fishes were consistent with previous phylogenetic studies: *Paradeontacylix* and *Cardicola* were closely related (Holzer et al., 2008); *Cardicola* was not supported as monophyletic and apparently needs taxonomic revision (Bullard, 2013); *Elaphrobates* was not supported as valid (Bullard and Overstreet, 2003; Nolan and Cribb, 2006b); and *Aporocotyle* and *Paradeontacylix* were each monophyletic (see also discussions above). No sequence data exists for the type species of *Cardicola* (*Cardicola cardiocolum*), although the

present study provides such data for the type and only nominal species of *Elaphrobates*. In fact, few type species have been sequenced, and those that have been sequenced belong to monotypic genera, i.e., *Chimaerohemecus*, *Plethorchis*, *Elaphrobates*, *Skoulekia*, *Sasala*, *Paracardicoloides*, and *Acipensericola* (Fig. 1). The only genera that are not monotypic and that have had their type species sequenced comprise *Ankistromeces*, *Braya*, *Pearsonellum*, and *Phthinomita* (Fig. 1). This represents another obvious gap in molecular taxonomic data that should be closed in order to test monophyly of these genera, objectively assess homology, and test reliability of differential morphological features used in generic diagnoses and species descriptions.

(4) Cercariae previously identified as putative FBFs based upon phylogenetic inference (Brant et al., 2006) formed a clade sister to all spirorchiids and schistosomes. However, we remain unconvinced that the cercaria from *Lymnaea stagnalis* (AY222180) is a species of *Sanguinicola*, and the present analysis, although perhaps overly conservative, does not definitively support its membership as a FBF. This sequence remains problematic because its species affiliation was never determined, no voucher material exists, and no sequence data derived from an adult specimen of a species of *Sanguinicola* exists to date (see discussion earlier in the text). If such an affiliation is confirmed, monophyly of Aporocotylidae would be rejected.

(5) All of the known life cycles for freshwater FBFs use gastropods as intermediate hosts (Hoffman et al., 1985; Meade, 1967; Meade and Pratt, 1965; Schell, 1974; Wales, 1958). Similarly, along with the putative cercariae we discussed in the preceding text, all spirorchiids and schistosomes use gastropods as intermediate hosts (Brant et al.,

2006). Marine FBFs reportedly use bivalves and polychaetes only, not gastropods (Fraser, 1967; Holliman, 1961; Køie, 1982; Linton, 1915; Martin, 1952; Oglesby, 1961; Wardle, 1979). Considering this ecological similarity, we acknowledge the possibility that the freshwater FBFs likewise have a closer phylogenetic affiliation with spirorchiids and schistosomes than with marine FBFs. We know strikingly little about the life cycles for the majority of named marine and freshwater FBFs (see text earlier) and almost nothing about any marine turtle blood fluke life cycle (see Stacy et al. (2010) for a molecular inference that suggested a limpet (*Fissurella nodosa*) intermediate host). However, the putatively profound dichotomy comprising the freshwater and marine FBFs may reflect this pattern of intermediate host natural history.

(6) Regarding the blood flukes that do not infect fishes, i.e., those of tetrapods, our analysis, which used more taxa than any previous analysis, but less sequence data, did not significantly refute the previously published tree topologies for blood flukes, i.e., monophyly of Schistosomatidae and paraphyly of Spirorchiidae (see Brant and Loker, 2005, 2013; Brant et al., 2006; Lockyer et al., 2003b; Loker and Brant, 2006; Snyder, 2004; Snyder and Loker, 2000).

(7) Regarding schistosome interrelationships, the resulting tree generally matched those recovered by previous workers (Brant and Loker, 2005; Brant et al., 2006; Lockyer et al., 2003b; Loker and Brant, 2006; Snyder, 2004; Snyder and Loker, 2000): (i) high support for clades AO (*Austrobilharzia*, *Ornithobilharzia*), BSO (*Bivitellobilharzia*, *Schistosoma, Orientobilharzia*), SH (*Schistosomatium, Heterobilharzia*), and BTGB (*Bilharziella*, *Trichobilharzia, Dendritobilharzia, Gigantobilharzia*); (ii) clade AO basal; (iii) clade SH sister to

Dendritobilharzia+*Bilharziella*+*Gigantobilharzia*+*Trichobilharzia*+*Allobilharzia*+ *Anserobilharzia*; (iv) *Bivitellobilharzia* sister to clade SO; and (v) Asian schistosomes basal (Brant and Loker, 2005, 2009; 2013; Brant et al., 2006; Lawton et al., 2011; Lockyer et al., 2003b, Loker and Brant, 2006; Snyder, 2004; Snyder and Loker, 2000; Wang et al., 2009; Webster et al., 2006; Webster and Littlewood, 2012).

(8) Although not the focus of the work presented here, we note that some schistosome interrelationships were unlike those previously recovered, perhaps because of the proportionally smaller amount of molecular sequence data that were included in our analysis: (i) *Dendritobilharzia* basal to (*Gigantobilharzia* (*Bilharziella*+*Trichobilharzia*); (ii) *Macrobilharzia*, *Bivitellobilharzia*, SO, and SH+BTGB unresolved. Moreover, the present study indicated that mammal schistosomes are monophyletic, indicating three independent colonizations of birds. Noteworthy also is that *G. amoena* was sister to the freshwater turtle blood flukes, apparently not wholly supporting the notion that schistosomes colonized modern archosaurs (birds) from ancestral archosaurs (crocodilians), i.e., *G. amoena*, although reported dioecious, is not a member of Schistosomatidae (see Brant and Loker, 2005; Brant et al., 2013; and Loker and Brant, 2006;).

8. FUTURE DIRECTIONS

The Digenea is perhaps the largest group of endoparasitic metazoan parasites and includes 150 families with 2700 nominal genera, >18,000 nominal species, and conservatively ~40,000 extant species (Cribb et al., 2001). The ubiquity of FBFs reveals

them as likely significant actors in freshwater, marine, and estuarine ecosystems (Cribb et al., 2001). However, limited taxon sampling in FBF phylogenic studies has hampered understanding of coevolution with host taxa and placement of “Aporocotylidae” within Schistosomatoidea. We think that an understanding of the evolutionary origins of flatworm parasitism in the blood of craniates will be advanced significantly by continued studies on the morphology and molecular biology of blood flukes that infect basal fishes, for example, Chondrichthyes, Acipenseriformes, Elopiformes, Siluriformes. As with the sarcopterygian fish lineages, coelacanths and lungfishes, we also emphasize the need to examine hagfishes (Myxiniformes) and lampreys (Petromyzontiformes) for the presence of blood fluke infections. To our knowledge no such examinations have been conducted, but finding and describing blood fluke specimens from these fascinating and phylogenetically unique craniates would be exciting and impactful to our understanding of the evolution of blood flukes as a whole. Hagfishes and lampreys may well harbour novel genera and/or families of blood flukes. Such increased taxon sampling for morphological features and molecular sequence data as well as the development and application of novel molecular markers should help resolve the interrelationships of the FBFs, analogous to the resolution of the paraphyletic “Spirorchiidae” that included 18S rDNA and 28S rDNA sequences from freshwater and marine representatives of eight spirorchiid genera (Snyder, 2004). Moreover, a comprehensive molecular phylogeny of blood flukes underpins testing hypotheses about the origin of diseases caused by blood flukes in craniates as well as trematode dioecity (Platt and Brooks, 1997), a condition that is unusual among flatworms and may have independently evolved several times in the Digenea.

A substantial quantity of genomic information, which exists for schistosomes, is lacking and wholly unexplored in non-human blood flukes, especially among FBFs. Massive parallel sequencing platforms, i.e., next- or second-generation sequencing, are opening new avenues to life sciences research by high-throughput technology that can inexpensively and within days produce thousands of megabases of nucleotide information. The commercially available platforms are distinguished by a combination of specific protocols that can be arranged as template preparation (clonally amplified or single-molecule templates), sequencing and imaging, and data analysis (Metzker, 2010). Such technology recently has improved the draft genome of *Schistosoma mansoni*, which is not surprising given its medical importance, and hastened completion of its genome, i.e., the first among parasitic flatworms (Berriman et al., 2009; Protasio et al., 2012; Tsai et al., 2013). The study of the evolution of complex body plans, parasite-host relationships, parasite sensory systems, disease pathogenesis, and new drugs and vaccine sites have been explored by the use of molecular tools applied to genome studies in *S. haematobium*, *S. mansoni*, and *S. japonicum* (see Berriman et al., 2009; The *Schistosoma japonicum* Genome Sequencing and Functional Analysis Consortium, 2009; Young et al., 2012). Analogous questions could be applied similarly to FBF studies. Second-generation sequencing technologies have provided high-resolution maps of temporal changes in gene expression among cercaria, schistosomula, and adult stages of *Schistosoma* spp. (Protasio et al., 2012). Similarly, Collins et al. (2013) recently used genomic resources and RNA-seq-based gene expression profiling to identify the gene responsible for the maintenance of neoblast-like cells. These data are directly relevant to a molecular understanding of the way in which these human

pathogens infect the host, and they are relevant because blocking infection means preventing disease. These processes likely originated in, and likely remain to be elucidated in, FBF-fish relationships, underscoring the need for genome studies comprising FBFs that infect the major non-tetrapod craniate lineages.

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FIGURE LEGENDS

Figure 1. Number of accepted fish blood fluke species per accepted genus (black bars) and that has been characterized with nucleotide data (white bars). Categorized by general ecological providence (marine, estuarine, and freshwater) and presented from left to right in approximate phylogenetic order (basal to derived; based on Nelson, 2006) of the definitive host group infected by the species of that genus. ***** A genus whose type species has been sequenced.

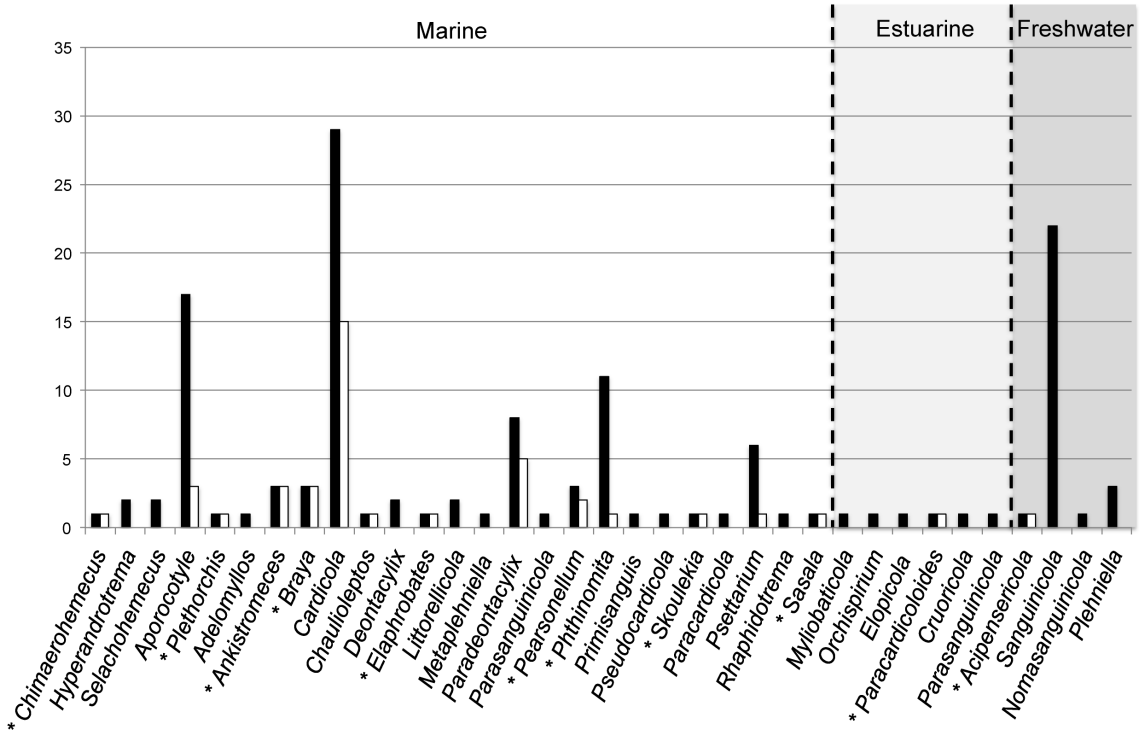
Figure 2. Simplified phylogenetic relationships of blood flukes (Bayesian inference, partial 28S sequences (see text for out-groups), and posterior probability aside each node) that (A) include the freshwater gastropod (snail) cercariae (Brant et al., 2006; Olson et al., 2003) and exclude clinostomes, (B) include clinostomes and exclude the freshwater snail cercariae, (C) include both freshwater snail cercariae and clinostomes, and (D) exclude both freshwater snail cercariae and clinostomes. (E) Simplified phylogeny for Gnathostomata (craniates other than Myxiniformes (hagfishes) + Petromyzontiformes (lampreys)) based on Nelson (2006) and Amemiya et al. (2013), showing the position of sharks, skates, rays, and chimaeras (Chondrichthyes); ray-finned fishes (Actinopterygii); and the three major lineages of Sarcopterygii: coelacanths (Coelacanthiformes), lungfishes (Ceratodontiformes), and terrestrial craniates and their descendants (Tetrapoda).

Figure 3. Phylogenetic relationships of blood flukes reconstructed by Bayesian inference and based on partial D1–D2 domains of 28S from 83 blood fluke taxa (majority rules consensus tree). Numbers aside tree nodes indicate posterior probability. *Taxa sequenced in the present study.

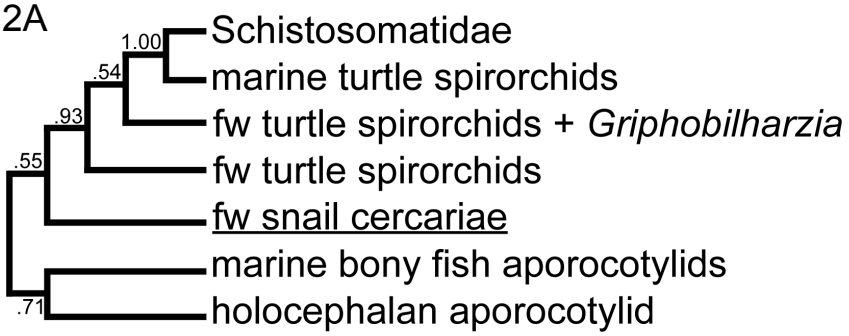
FOOTNOTES

[1]Basch (1991) counted 86 species and 13 genera; Khalil (2002) accepted *Jilinobilharzia* as valid, which includes 2 species; Müller and Kimmig (1994), Horák et al. (1998), and Kolářová et al. (2013) described 3 new species of *Trichobilharzia*; Attwood et al. (2002) and Hanelt et al., (2009) described 2 new species of *Schistosoma*; Aldhoun and Littlewood (2012) considered *Orientobilharzia* a junior subjective synonym of *Schistosoma*; Kolářová et al. (2006) and Brant et al. (2013) proposed 2 new genera and species (*Allobilharzia visceralis* and *Anserobilharzia brantae* [syn. *Trichobilharzia brantae* Farr and Blankemeyer, 1956], respectively).
[2]Smith (1997b) listed 82 species of 21 genera; Platt (2002) accepted 19 genera; Tkach et al. (2009) and Platt and Sharma (2012) described 1 and 2 new species, respectively.

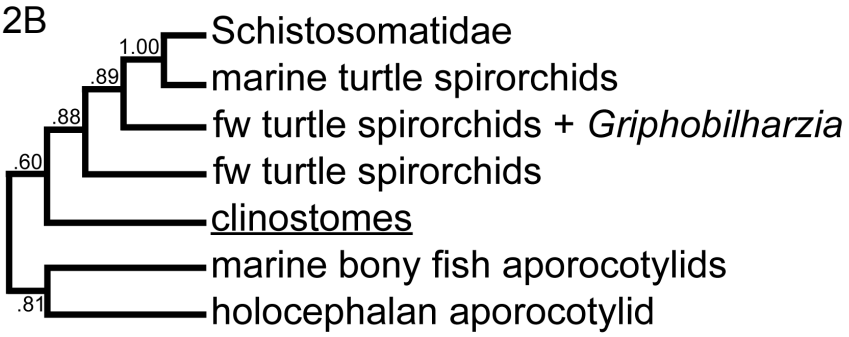
1


Marine
Estuarine
Freshwater
35
30
25
20
15
10
5
0
* Chimaerohemecus
Hyperandrotrema
Selachohemecus
Aporocotyle
* Plethorchis
Adelomyllos
* Ankistromeces
* Braya
Cardicola
Chaulioleptos
Deontacylix
* Elaphrobates
Littorellicola
Metaplehniella
Paradeontacylix
Parasanguinicola
* Pearsonellum
* Phthinomita
Primisanguis
Pseudocardicola
* Skoulekia
Paracardicola
Psettarium
Rhaphidotrema
* Sasala
Myliobaticola
Orchispirium
Elopicola
* Paracardicolides
Cruoricola
Parasanguinicola
* Acipensericola
Sanguinicola
Nomasanguinicola
Plehniella

2A


1.00
.54
.93
.55
.71
Schistosomatidae
marine turtle spirorchids
fw turtle spirorchids + Griphobilharzia
fw turtle spirorchids
fw snail cercariae
marine bony fish aporocotylids
holocephalan aporocotylid

2B


1.00
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.60
.81
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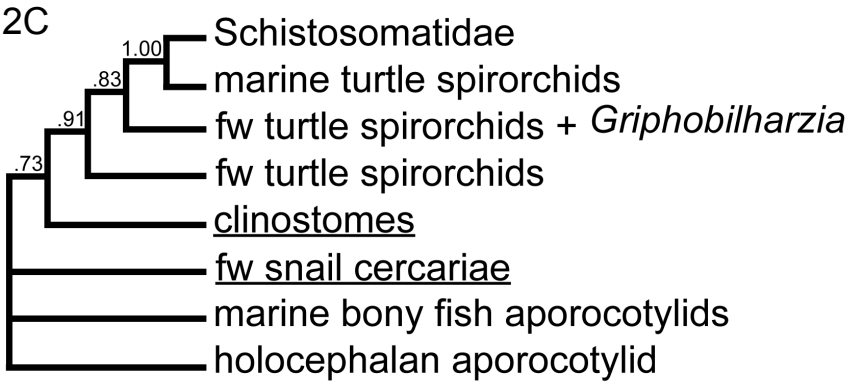
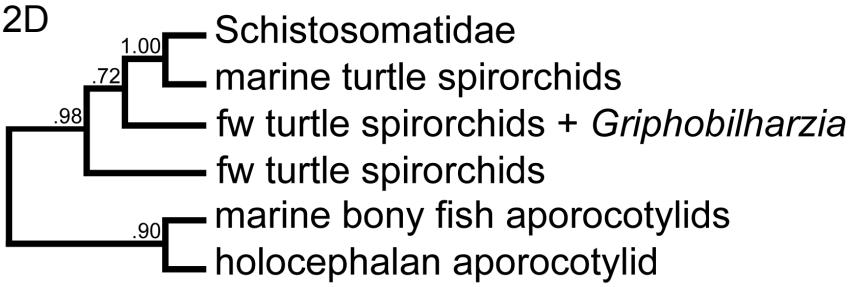
marine turtle spirorchids
fw turtle spirorchids + Griphobilharzia
fw turtle spirorchids
clinostomes
marine bony fish aporocotylids
holocephalan aporocotylid

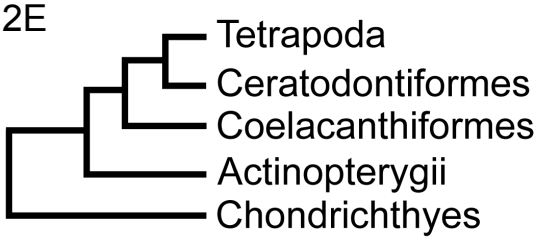
2C


1.00
.83
.91
.73
Schistosomatidae
marine turtle spirorchids
fw turtle spirorchids + Griphobilharzia
fw turtle spirorchids
clinostomes
fw snail cercariae
marine bony fish aporocotylids
holocephalan aporocotylid

2D


1.00
.72
.98
.90
Schistosomatidae
marine turtle spirorchids
fw turtle spirorchids + Griphobilharzia
fw turtle spirorchids
marine bony fish aporocotylids
holocephalan aporocotylid

2E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Ceratodontiformes
Coelacanthiformes
Actinopterygii
Chondrichthyes

3

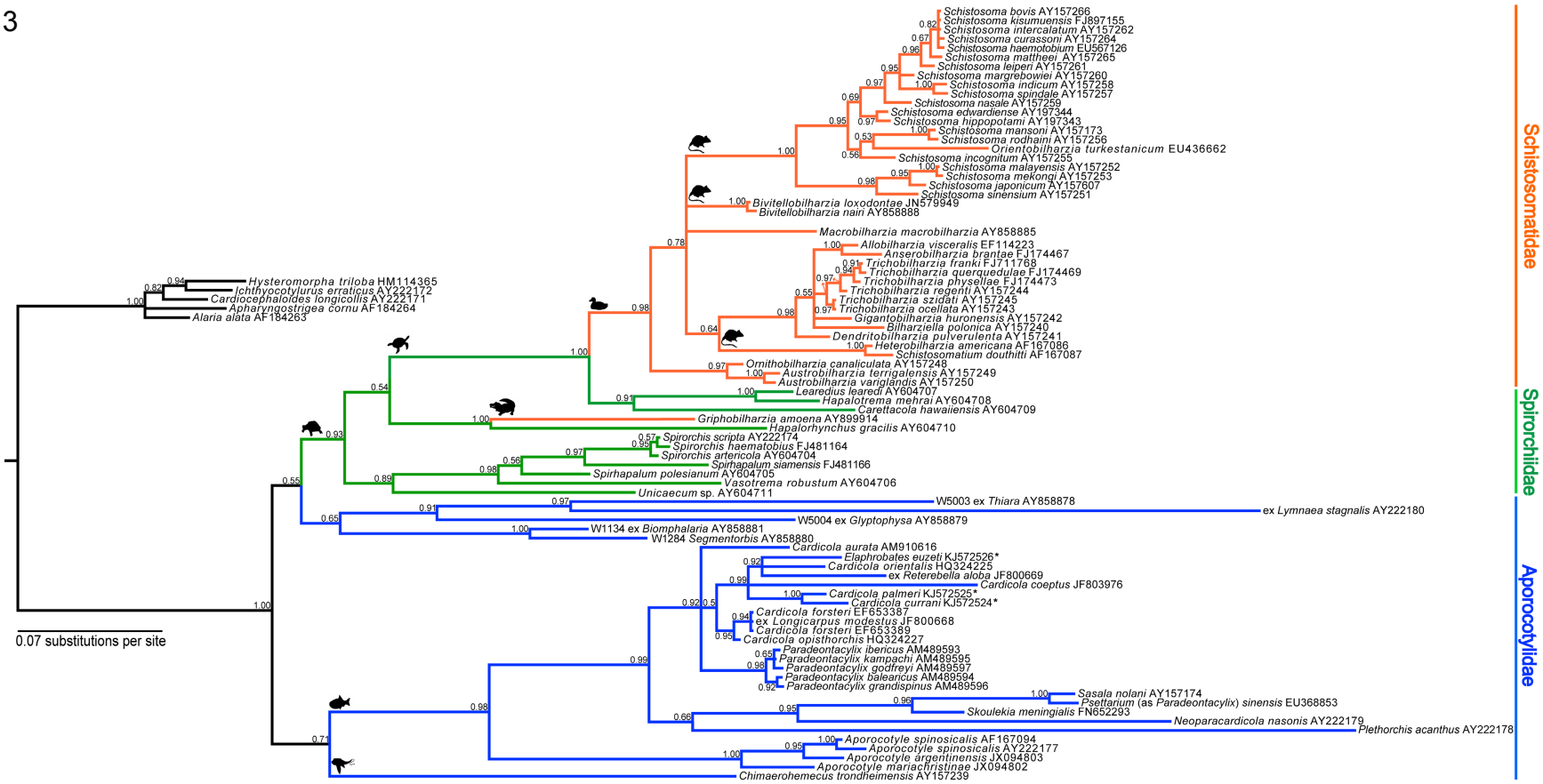
Schistosoma bovis AY157266
Schistosoma kisumuensis FJ897155
Schistosoma intercalatum AY157262
Schistosoma curassoni AY157264
Schistosoma haematobium EU567126
Schistosoma mattheei AY157265
Schistosoma leiperi AY157261
Schistosoma margrebowiei AY157260
Schistosoma indicum AY157258
Schistosoma spindale AY157257
Schistosoma nasale AY157259
Schistosoma edwardiense AY197344
Schistosoma hippopotami AY197343
Schistosoma mansoni AY157173
Schistosoma rodhaini AY157256
Orientobilharzia turkestanicum EU436662
Schistosoma incognitum AY157255
Schistosoma malayensis AY157252
Schistosoma mekongi AY157253
Schistosoma japonicum AY157607
Schistosoma sinensium AY157251
Bivitellobilharzia loxodontae JN579949
Bivitellobilharzia nairi AY858888
Macrobilharzia macrobilharzia AY858885
Allobilharzia visceralis EF114223
Anserobilharzia brantae FJ174467
Trichobilharzia franki FJ711768
Trichobilharzia querquedulae FJ174469
Trichobilharzia physellae FJ174473
Trichobilharzia regenti AY157244
Trichobilharzia szidati AY157245
Trichobilharzia ocellata AY157243
Gigantobilharzia huronensis AY157242
Bilharziella polonica AY157240
Dendritobilharzia pulverulenta AY157241
Heterobilharzia americana AF167086
Schistosomatium douthitti AF167087
Ornithobilharzia canaliculata AY157248
Austrobilharzia terrigalensis AY157249
Austrobilharzia variglandis AY157250
Learedius learedi AY604707
Hapalotrema mehrai AY604708
Carettacola hawaiiensis AY604709
Griphobilharzia amoena AY899914
Hapalorhynchus gracilis AY604710
Spirorchis scripta AY222174
Spirorchis haematobius FJ481164
Spirorchis artericola AY604704
Spirhapalum siamensis FJ481166
Spirhapalum polesianum AY604705
Vasotrema robustum AY604706
Unicaecum sp. AY604711
W5003 ex Thiara AY858878
ex Lymnaea stagnalis AY222180
W5004 ex Glyptophysa AY858879
W1134 ex Biomphalaria AY858881
W1284 Segmentorbis AY858880
Cardicola aurata AM910616
Elaphrobates euzeti KJ572526*
Cardicola orientalis HQ324225
ex Reterebella aloba JF800669
Cardicola coeptus JF803976
Cardicola palmeri KJ572525*
Cardicola currani KJ572524*
Cardicola forsteri EF653387
ex Longicarpus modestus JF800668
Cardicola forsteri EF653389
Cardicola opisthorchis HQ324227
Paradeontacylix ibericus AM489593
Paradeontacylix kampachi AM489595
Paradeontacylix godfreyi AM489597
Paradeontacylix balearicus AM489594
Paradeontacylix grandispinus AM489596
Sasala nolani AY157174
Psettarium (as Paradeontacylix) sinensis EU368853
Skoulekia meningialis FN652293
Neoparacardicola nasonis AY222179
Plethorchis acanthus AY222178
Aporocotyle spinosicalis AF167094
Aporocotyle spinosicalis AY222177
Aporocotyle argentinensis JX094803
Aporocotyle mariachristinae JX094802
Chimaerohemecus trondheimensis AY157239
Hysteromorpha triloba HM114365
Ichthyocotylurus erraticus AY222172
Cardiocephaloides longicollis AY222171
Apharyngostrigea cornu AF184264
Alaria alata AF184263
0.07 substitutions per site
Schistosomatidae
Spirorchiidae
Aporocotylidae

Table 1. GenBank DNA sequences for fish blood flukes.

Parasite	Host	Stage	Locality	Setting	GenBank Accession Numbers				Reference(s)
					18S	28S	ITS2	COI	
Acipensericola petersoni	*Polyodon spathula*	A*	Mississippi Delta, USA	wild	DQ534192§				Bullard et al. 2008
Ankistromeces dunwichensis	*Siganus fuscescens*	A	SW Pacific, off North Stradbroke Island, Australia	wild			DQ335838ϕ		Nolan and Cribb, 2006a
Ankistromeces mariae	*Meuschenia freycineti*	A	SW Pacific, off Stanley Harbour, Australia	wild			DQ335839ϕ		Nolan and Cribb, 2006a
Ankistromeces olsoni	*Siganus fuscescens*	A	SW Pacific, off Heron Island, Australia	wild			DQ335840ϕ		Nolan and Cribb, 2006a
Aporocotyle argentinensis	*Merluccius hubbsi*	A	SW Atlantic, off Patagonia, Argentina	wild		JX094803†			Hernández-Orts et al. 2012
Aporocotyle mariachristinae	*Genypterus blacodes*	A	SW Atlantic, off Patagonia, Argentina	wild	JX094801§	JX094802†			Hernández-Orts et al. 2012
Aporocotyle spinosicanalis	*Merluccius merluccius*	A	NE Atlantic	wild		AF167094†			Snyder and Loker, 2000
		A	NE Atlantic, UK	wild	AJ287477ϕ				Cribb et al. 2001
		A	NE Atlantic, off Orkney Islands, UK	wild		AY222177†			Olson et al. 2003
Braya jexi	*Scarus frenatus*	A	SW Pacific, off Heron Island, Australia	wild			DQ059624ϕ		Nolan and Cribb, 2006b
Braya psittacus	*Scarus ghobban*	A	SW Pacific, off Heron Island, Australia	wild			DQ059625ϕ		Nolan and Cribb, 2006b
Braya yantschi	*Chlorurus microrhinos*	A	SW Pacific, off Heron Island, Australia	wild			DQ059628ϕ		Nolan and Cribb, 2006b
Cardicola aurata	*Sparus aurata*	A	Mediterranean Sea, off Valencia, Spain	cultured		AM910616†	AM910617ϕ		Holzer et al. 2008
Cardicola bartolii	*Siganus lineatus*	A	SW Pacific, off Heron Island, Australia	wild			DQ059631ϕ		Nolan and Cribb, 2006b
Cardicola chaetodontis	*Chaetodon aureofasciatus*	A	SW Pacific, off Lizard Island, Australia	wild			KF049000ϕ		Yong et al. 2013
	Chaetodon baronessa	E	SW Pacific, off Lizard Island, Australia	wild			KF049004ϕ		Yong et al. 2013
	Chaetodon kleinii	E	SW Pacific, off Heron Island, Australia	wild			JN418931ϕ		Yong et al. 2013
	Chaetodon lunulatus	E	SW Pacific, off Lizard Island, Australia	wild			KF049002ϕ		Yong et al. 2013
	Chaetodon plebeius	E	SW Pacific, off Lizard Island, Australia	wild			KF049003ϕ		Yong et al. 2013

	Chaetodon rainfordi	A	SW Pacific, off Heron Island, Australia	wild		JN418932ϕ	Yong et al. 2013
	Chaetodon unimaculatus	E	SW Pacific, off Heron Island, Australia	wild		DQ059633ϕ	Nolan and Cribb, 2006b
	Chaetodon unimaculatus	E	SW Pacific, off Lizard Island, Australia	wild		KF049001ϕ	Yong et al. 2013
Cardicola coeptus	*Siganus punctatus*	A	SW Pacific, off Heron Island, Australia	wild		DQ059629ϕ	Nolan and Cribb, 2006b
		A	SW Pacific, off Heron Island, Australia	wild	JF803976†		Cribb et al. 2011
Cardicola coeptus	*Siganus vulpinus*	A	SW Pacific, off Heron Island, Australia	wild		DQ059630ϕ	Nolan and Cribb, 2006b
		A	SW Pacific, off Heron Island, Australia	wild	JF803977†		Cribb et al. 2011
Cardicola covacinae	*Siganus punctatus*	A	SW Pacific, off Heron Island, Australia	wild		DQ059634ϕ	Nolan and Cribb, 2006b
Cardicola currani	*Sciaenops ocellatus*	A	Gulf of Mexico, off Davis Bayou, USA		KJ272524†		Present study
Cardicola forsteri	*Longicarpus modestus*	C	SW Pacific, off Port Lincoln, S Australia	wild	JF800668†	JF800670ϕ	Cribb et al. 2011
Cardicola forsteri	*Thunnus maccoyii*	A	SW Pacific, off S Australia	cultured		DQ059637ϕ	Nolan and Cribb, 2006b
		A	SW Pacific, off Port Lincoln, S Australia	cultured	EF653387†	EF661575ϕ	Aiken et al. 2007
		A	SW Pacific, off Cabbage Patch, Australia	wild	EF653389†	EF653394ϕ	Aiken et al. 2007
		A	SW Pacific, off Port Lincoln, S Australia	cultured	AB742426†	AB742428ϕ	Shirakashi et al. 2013
Cardicola forsteri	*Thunnus thynnus*	A	Mediterranean Sea, off Puerto de Mazarrón, Spain	cultured	EF653388†	EF653395ϕ	Aiken et al. 2007
Cardicola lafii	*Siganus fuscescens*	A	SW Pacific, off Lizard Island, Australia	wild		DQ059639ϕ	Nolan and Cribb, 2006b
Cardicola milleri	*Lutjanus bohar*	A	SW Pacific, off Lizard Island, Australia	wild		DQ059640ϕ	Nolan and Cribb, 2006b
Cardicola opisthorchis	*Thunnus orientalis*	A	NW Pacific, Japan	cultured	HQ324227†	HQ324228ϕ	Ogawa et al. 2011
Cardicola opisthorchis	*Terebella* sp.	C	NW Pacific, off Tsushima, Japan	wild	AB829900†	AB830082ϕ	Sugihara et al. 2014
Cardicola orientalis	*Thunnus maccoyii*	A	SW Pacific, off Port Lincoln, S Australia	cultured	AB742425†	AB742427ϕ	Shirakashi et al. 2013

Cardicola orientalis	*Thunnus orientalis*	A	NW Pacific, off Kushimoto, Japan	cultured		HQ324225†	HQ324226ϕ		Ogawa et al. 2011
Cardicola palmeri	*Pogonias cromis*	A	Gulf of Mexico, off Back Bay, USA			KJ572525†			Present study
Cardicola parilus	*Siganus fuscescens*	A	Indian, off Ningaloo Reef, W Australia	wild			DQ059638ϕ		Nolan and Cribb, 2006b
Cardicola tantabiddii	*Siganus fuscescens*	A	Indian, off Ningaloo Reef, W Australia	wild			DQ059642ϕ		Nolan and Cribb, 2006b
Cardicola watsonensis	*Siganus corallinus*	A	SW Pacific, off Lizard Island, Australia	wild			DQ059643ϕ		Nolan and Cribb, 2006b
Chimaerohemecus trondheimensis	*Chimaera monstrosa*	A	NE Atlantic, off Bergen, Norway	wild	AY157213§	AY157239§		AY157185§	Lockyer et al. 2003b
Elaphrobates euzeti	*Lutjanus campechanus*	A	Gulf of Mexico, USA			KJ572526†			Present study
Neoparacardicola nasonis	*Naso unicornis*	A	SW Pacific, off Lizard Island, Australia	wild	AY222097§	AY222179†			Olson et al. 2003
Paracardicoloides yamagutii	*Anguilla reinhardtii*	A	Churchbank Weir, Australia	wild	U42569†	U42562†			Barker and Blair, 1996
		A	Brisbane River tributaries, Australia	wild			AY465872ϕ		Nolan and Cribb, 2004a
	Posticobia brazieri	C	Brisbane River tributaries, Australia	wild			AY465869ϕ		Nolan and Cribb, 2004a
Paradeontacylix balearicus	*Seriola dumerili*	A	Mediterranean Sea, off Majorca, Spain	ns		AM489594†	AM489600ϕ	AM489604†	Repullés-Albelda et al. 2008
Paradeontacylix godfreyi	*Seriola lalandi*	A	SW Pacific, off Port Lincoln, S Australia	ns		AM489597†	AM489602ϕ	AM489607†	Repullés-Albelda et al. 2008
Paradeontacylix grandispinus	*Seriola dumerili*	A	NW Pacific, off Ushine, Japan	ns		AM489596†	AM489601ϕ		Repulles-Albelda et al. 2008
Paradeontacylix ibericus	*Seriola dumerili*	A	Mediterranean Sea, off Santa Pola, Spain	ns		AM489593†	AM489598ϕ	AM489603†	Repullés-Albelda et al. 2008
Paradeontacylix kampachi	*Seriola dumerili*	A	NW Pacific, off Ushine, Japan	ns		AM489595†	AM489599ϕ	AM489605† AM489606†	Repullés-Albelda et al. 2008
Pearsonellum corventum	*Plectropomus leopardus*	A	SW Pacific, off Heron Island, Australia	wild			AY465873ϕ		Nolan and Cribb, 2004b
Pearsonellum pygmaeus	*Cromileptes altivelis*	A	SW Pacific, off Lizard Island, Australia	wild			AY465874ϕ		Nolan and Cribb, 2004b
Phthinomita adlardi	*Siganus argenteus*	A	Indian, off Ningaloo Reef, W Australia	wild			DQ335844ϕ		Nolan and Cribb, 2006a
Phthinomita	*Siganus virgatus*	A	Indian, off Ningaloo Reef,	wild			DQ335845ϕ		Nolan and Cribb,

brooksi			W Australia					2006a
Phthinomita hallae	*Siganus corallinus*	A	SW Pacific, off Heron Island, Australia	wild			DQ335846ϕ	Nolan and Cribb, 2006a
	Siganus doliatus	A	SW Pacific, off Heron Island, Australia	wild			DQ335847ϕ	Nolan and Cribb, 2006a
	Siganus vulpinus	A	SW Pacific, off Heron Island, Australia	wild			DQ335848ϕ	Nolan and Cribb, 2006a
Phthinomita ingramae	*Siganus punctatus*	A	SW Pacific, off Lizard Island, Australia	wild			DQ335849ϕ	Nolan and Cribb, 2006a
Phthinomita jonesi	*Siganus argenteus*	A	SW Pacific, off Lizard Island, Australia	wild			DQ335850ϕ	Nolan and Cribb, 2006a
	Siganus doliatus	A	SW Pacific, off Lizard Island, Australia	wild			DQ335851ϕ	Nolan and Cribb, 2006a
	Siganus lineatus	A	SW Pacific, off Lizard Island, Australia	wild			DQ335852ϕ	Nolan and Cribb, 2006a
	Siganus vulpinus	A	SW Pacific, off Lizard Island, Australia	wild			DQ335853ϕ	Nolan and Cribb, 2006a
Phthinomita littlewoodi	*Siganus corallinus*	A	SW Pacific, off Lizard Island, Australia	wild			DQ335854ϕ	Nolan and Cribb, 2006a
	Siganus lineatus	A	SW Pacific, off Heron Island, Australia	wild			DQ335855ϕ	Nolan and Cribb, 2006a
Phthinomita munozae	*Choerodon venustus*	A	SW Pacific, off Heron Island, Australia	wild			DQ335856ϕ	Nolan and Cribb, 2006a
Phthinomita poulini	*Parupeneus barberinus*	A	SW Pacific, off Lizard Island, Australia	wild			DQ335857ϕ	Nolan and Cribb, 2006a
	Parupeneus bifasciatus	A	SW Pacific, off Lizard Island, Australia	wild			DQ335858ϕ	Nolan and Cribb, 2006a
	Parupeneus cyclostomus	A	SW Pacific, off Lizard Island, Australia	wild			DQ335859ϕ	Nolan and Cribb, 2006a
Phthinomita robertsthomsoni	*Siganus argenteus*	A	SW Pacific, off Lizard Island, Australia	wild			DQ335860ϕ	Nolan and Cribb, 2006a
Phthinomita sasali	*Siganus doliatus*	A	Indo-West Pacific, Palau	wild			DQ335861ϕ	Nolan and Cribb, 2006a
Phthinomita symplocos	*Siganus lineatus*	A	SW Pacific, off Lizard Island, Australia	wild			DQ335867ϕ	Nolan and Cribb, 2006a
Plethorchis acanthus	*Mugil cephalus*	A	Brisbane River, Australia	wild	AY222096§	AY222178†		Olson et al. 2003
		A	SW Pacific, off Heron Island, Australia	wild			AY465875ϕ	Nolan and Cribb, 2006a
Psettarium (*as*	*Takifugu rubripes*	A	Fuzhou City, China	cultured	EU081899§		EU082007ϕ	Chen et al. 2008

Paradeontacylix) *sinensis*		A	ns	ns	EU368853†			Unpublished[1]
Sasala nolani	*Arothron meleagris*	A	S Pacific off Moorea, French Polynesia	wild	AY157184§	AY157174§		Lockyer et al. 2003a
Skoulekia meningialis	*Diplodus vulgaris*	A	Mediterranean Sea, off Valencia, Spain	wild	FN652294§	FN652293†	FN652292ϕ	Alama-Bermejo et al. 2011

Ns, not specified;
* A, adult; C, cercaria; E, egg;
ϕ Complete nucleotide sequence, § Near-complete nucleotide sequence; † Partial nucleotide sequence;
[1] Chen, C.M., Wang, Y.Y. & Wen, J.X. (Submitted to GenBank in 04 Aug 2007, unpublished data)

Table 2. Molecular studies of fish blood flukes; deep phylogeny (DP); life cycles (LC); species differentiation (SD).

Reference	Approach	Taxonomic level	18S	ITS2	28S	COI	Sets of PCR primers*	
							PCR+Sequencing	Additional sequencing
Barker and Blair (1996)	DP	Inter-family	✓		✓		3, 4, 59, 60	133–135
Snyder and Loker (2000)	DP	Inter-family			✓		57, 58	137–139
Cribb *et al*. (2001)	DP	Inter-family	✓				1, 2, 5–8	None
Littlewood and Olson (2001)	DP	Inter-family	✓				6, 7, 9–43	None
Olson *et al*. (2003)	DP	Inter-family	✓		✓		10, 42, 57, 61	146, 148, 149†, 150
Lockyer *et al*. (2003*a*)	DP	Inter-family	✓		✓		1, 2, 63–68	146, 147, 149–163
Lockyer *et al*. (2003*b*)	DP	Inter-family	✓		✓	✓	1, 2, 63–68, 121, 122	146, 147, 149–169
Nolan and Cribb (2004*a*)	LC/SD	Intra-family		✓			93†–95	None
Nolan and Cribb (2004*b*)	SD	Intra-family		✓			93†–95	None
Snyder (2004)	DP	Inter-family	✓		✓		9, 42, 63, 64	140–147, 149†, 150
Brant *et al*. (2006)	LC/SD	Intra-family	✓		✓	✓	9, 42, 44–54, 58, 63, 64, 69–92, 123–130	140–147, 149†, 150
Nolan and Cribb (2006*a*)	SD	Intra-family		✓			94, 95	None
Nolan and Cribb (2006*b*)	SD	Intra-family		✓			94, 95	None
Aiken *et al*. (2007)	SD	Intra-family		✓	✓		57, 62, 93†–95	None
Ogawa *et al*. (2007)	SD	Intra-family		✓			94, 95	None
Bullard *et al*. (2008)	DP	Intra-family	✓				9, 42	140–143
Chen *et al*. (2008)	SD	Intra-family	✓	✓			55, 56, 94, 95	None
Holzer *et al*. (2008)	SD	Intra-family		✓	✓		63, 64, 93†, 94	None
Repulles-Albelda *et al*. (2008)	SD	Intra-family		✓	✓	✓	63, 64, 93†, 94, 131, 132	152
Cribb *et al*. (2011)	DP/LC	Intra-family		✓	✓		57, 62†, 94, 95	None
Ogawa *et al*. (2011)	SD	Intra-family		✓	✓		63, 64, 93†, 94	None
Alama-Bermejo *et al*. (2011)	SD	Intra-family	✓	✓	✓		10, 42, 63, 64, 94, 95	136, 152
Bray *et al*. (2012)	DP/SD	Intra-family	✓		✓		1, 2, 63–68	146, 147, 149–163
Hernández-Orts *et al*. (2012)	SD	Intra-family	✓		✓		10, 42, 63, 64	136, 152
Kirchhoff *et al*. (2012)[1]	SD	Intra-family		✓				
Norte dos Santos *et al*. (2012)[1]	SD	Intra-family		✓				
Shirakashi *et al*. (2012)	SD	Intra-family		✓			96–101	None
Shirakashi *et al*. (2013)	SD	Intra-family		✓	✓		63, 64, 93†, 94	152
Yong *et al*. (2013)	SD	Intra-family		✓			94, 95	None
Polinski *et al*. (2013)	SD	Intra-family		✓			102–120‡	None
Polinski *et al*. (2014)	SD	Intra-family		✓			108, 109, 113–115, 120	None
Sugihara *et al*. (2014)	LC/SD	Intra-family		✓	✓		93†, 94, 63, 64	

[1]Authors did not report primers used. (*) See Supplementary Table S1 for details about primer IDs in the present article. (†) Primer modified from original reference (see Supplementary Table S1). (‡) Primers and probes for real-time qPCR detection and restriction free cloning.

Table 3. Oligonucleotide primers for fish blood flukes (Digenea: Aporocotylidae).

Primer ID (direction)		Sequence 5' to 3'	Reference
Original	In article		
Amplification and Sequencing			
18S rDNA			
A (F)	1	AMCTGGTGGATCCTGCCG	Medlin *et al*. 1988
B (R)	2	TGATCCATCTGCAGGTTCACCT	Medlin *et al*. 1988
SB8	3	GGGTGGA TTTATTAGAACAG	Barker and Blair, 1996
PB	4	CCGTCAATTCMTTTRAGTTT	Barker and Blair, 1996
400F (F)	5	TCCGGAGAGGGAGCCTGA	Littlewood *et al*. 2000
600R (R)	6	ACCGCGGCKGCTGGCACC	Littlewood *et al*. 2000
1270F (F)	7	ACTTAAAGGAATTGACGG	Littlewood *et al*. 2000
1630R (R)	8	TAAGGGCATCACAGACCTG	Littlewood *et al*. 2000
18SE (alias 18S-A) (F)	9	CCGAATTCGTCGACAACCTGGTTGATC CTGCCAGT	Littlewood and Olson, 2001
Worm A (F)	10	GCGAATGGCTCATTAAATCAG	Littlewood and Olson, 2001
18S-7 (F)	11	GCCCTATCAATTTGTTGGTA	Littlewood and Olson, 2001
18S-10 (R)	12	TACCATCGACAGTTGATAGGGC	Littlewood and Olson, 2001
300F (F)	13	AGGGTTCGATTCCGGAG	Littlewood and Olson, 2001
400R (alias 300R) (R)	14	TCAGGCTCCCTCTCCGGA	Littlewood and Olson, 2001
Cestode-1 (alias CEST1R) (R)[3]	15	TTTTTCGTCACTACCTCCCC	Littlewood and Olson, 2001
600F (F)	16	GGTGCCAGCMGCCGCGGT	Littlewood and Olson, 2001
18S-8 (F)	17	GCAGCCGCGGTAACTCCAGC	Littlewood and Olson, 2001
Pace-A (F)	18	GAGTTACCGCGGCTGCTG	Littlewood and Olson, 2001
18S-9 (F)	19	TTTGAGTGCTCAAAGCAG	Littlewood and Olson, 2001
930F (F)	20	GCATGGAATAATGAAATAGG	Littlewood and Olson, 2001
18S-A27 (R)	21	CCATACAAATGCCCCCGTCTG	Littlewood and Olson, 2001
Ael-5 (F)	22	TGTTTTCATTGATCAGGAGC	Littlewood and Olson, 2001
1100F (F) [1]	23	CAGAGTTTCGAAGACGATC	Littlewood and Olson, 2001
1100R (R)	24	GATCGTCTTCGAACCTCTG	Littlewood and Olson, 2001
Ael-3 (R)	25	GTATCTGATCGTCTTCGAAA	Littlewood and Olson, 2001
Pace-B (R)	26	CCGTCAATTCCTTTAAGTTT	Littlewood and Olson, 2001
1270R (R)	27	CCGTCAATTCCTTTAAGT	Littlewood and Olson, 2001
Pace-BF (F)	28	AAACTTAAAGGAATTGACGG	Littlewood and Olson, 2001
18S-11F (F)	29	AACGGCCATGCACCACCACCC	Littlewood and Olson, 2001
1262R (alias 1055R) (R)	30	CGGCCATGCACCACC	Littlewood and Olson, 2001
18S-11F (F)	31	GGGTGGTGGTGCATGGCCGTT	Littlewood and Olson, 2001
1262F (alias 1055F) (F)	32	GGTGGTGCATGGCCG	Littlewood and Olson, 2001
18S-2 (F)	33	ATAACAGGTCTGTGATGCCCTTAGA	Littlewood and Olson, 2001
1200F (F)	34	CAGGTCTGTGATGCCC	Littlewood and Olson, 2001
18S-3 (R)	35	TCTAAGGGCATCACAGACCTGTTAT	Littlewood and Olson, 2001
1200R (R)	36	GGGCATCACAGACCTG	Littlewood and Olson, 2001
18S-5 (F)	37	CCCTTTGTACACACCGCCCGTCGCT	Littlewood and Olson, 2001
1400F (F)	38	TGYACACACCGCCCGTC	Littlewood and Olson, 2001
18S-4 (R)	39	AGCGACGGGCGGTGTGTAC	Littlewood and Olson, 2001
1400R (R)	40	ACGGGCGGTGTGTAC	Littlewood and Olson, 2001
Cestode-6 (R)	41	ACGGAAACCTTGTTACGACT	Littlewood and Olson, 2001
Worm B (R)	42	CTTGTTACGACTTTTACTTCC	Littlewood and Olson, 2001
18S-F (alias 18S-	43	CCAGCTTGATCCTTCTGCAGGTTCACC	Littlewood and Olson, 2001

B) (F)		TAC	
JB1 (F)	44	CCAACCTGGTTGATCCTGCCAGT	Morgan *et al*. 2003
18SA (F)	45	AACCTGGTTGATCCTGCCAGT	Morgan *et al*. 2003
18SF2.1 (F)	46	ATCTAAGGAAGGCAGCAGGCG	Morgan *et al*. 2003
18SF1 (F)	47	CGGGACTCAATTGAGGCTCCGT	Morgan *et al*. 2003
18SF2 (F)	48	ACTTTGAACAAATTTGAGTGCTCA	Morgan *et al*. 2003
18H (F)	49	GCTGAAACTTAAAGGAATTGA	Morgan *et al*. 2003
18SR0 (R)	50	CGCGGCTGCTGGCACCAGACTTGCC	Morgan *et al*. 2003
18SR1(R)	51	CAGTGTCCGGGCCGGGTGAG	Morgan *et al*. 2003
18J (R)	52	GGGCATCACAGACCTGTTATTG	Morgan *et al.* 2003
R18A (R)	53	GATCCTTCCGCAGGTTCACCTACG	Morgan *et al*. 2003
18SB (R)	54	TGATCCTTCTGCAGGTTCACCTAC	Morgan *et al*. 2003
F2 (F)	55	GCCATGCATGTCCAAGTACATAC	Chen *et al*. 2008
R2 (R)	56	TCGCTAAACCATTCAATCGGTAG	Chen *et al.* 2008
28S rDNA			
LSU5 (F)	57	TAGGTCGACCCGCTGAAYTTAAGCA	Littlewood, 1994
LSU3 (R)	58	TAGAAGCTTCCTGAGGGAAACTTCGG	Littlewood, 1994
Ns[2]	59	GATTACCCGCTGAACTTAAGCATAT	Barker and Blair, 1996
Ns[3]	60	GCTGCATTCACAAACACCCCGACTC	Barker and Blair, 1996
1500R (R)	61	GCTATCCTGAGGGAAACTTCG	Olson *et al*. 2003
EC-D2 (alias ECD2, ECD-2) (R)[1]§	62	CCTTGGTCCGTGTTTCAAGACGGG	Littlewood *et al*. 1997
U178 (F)	63	GCACCCGCTGAAYTTAAG	Lockyer et al. 2003*a*
L1642 (R)	64	CCAGCGCCATCCATTTTCA	Lockyer et al. 2003*a*
U1148 (F)	65	GACCCGAAAGATGGTGAA	Lockyer et al. 2003*a*
L2450 (R)	66	GCTTTGTTTTAATTAGACAGTCGGA	Lockyer et al. 2003*a*
U1846 (F)	67	AGGCCGAAGTGGAGAAGG	Lockyer et al. 2003*a*
L3449 (R)	68	ATTCTGACTTAGAGGCGTTCA	Lockyer et al. 2003*a*
28SF6 (F)	69	GCACCCGCTGAAYTTAAG	Morgan *et al*. 2003
C1 (F)	70	ACCCGCTGAATTTAAGCAT	Morgan *et al*. 2003
ITS2.2F (F)	71	GCGGAGGAAAAGAAACTAAC	Morgan *et al*. 2003
28SF4 (F)	72	AGTACCGTGAGGGAAAGTTG	Morgan *et al*. 2003
28SF3 (F)	73	CGAAACCCAAAGGCGCAGTGA	Morgan *et al*. 2003
28SF7 (F)	74	CCCGAAAGATGGTGAACTATGCTT	Morgan *et al*. 2003
28SF9 (F)	75	GTATAGGGGCGAAAGACTAATCG	Morgan *et al*. 2003
28SF10 (F)	76	AGCAGGACGGTGGCCATGGAAG	Morgan *et al*. 2003
28SF8 (F)	77	AGGCCGAGGTGGAGAAGGGTTC	Morgan *et al*. 2003
28SF11 (F)	78	TACCCATATCCGCAGCAGGTCTC	Morgan *et al*. 2003
28SF12 (F)	79	AAACGGCGGGAGTAACTATGA	Morgan *et al*. 2003
28SF13 (F)	80	ATGGATGTAGTATAGGTGGGAGC	Morgan *et al*. 2003
28SF14 (F)	81	AAGAGGTGTCAGAAAAGTTACC	Morgan *et al*. 2003
D2 (R)	82	TGGTCCGTGTTTCAAGAC	Morgan *et al*. 2003
28SR3 (R)	83	CTCAGGCATAGTTCACCATC	Morgan *et al*. 2003
28SR1 (R)	84	AGCGCCATCCATTTTCAGGG	Morgan *et al*. 2003
28SR6 (R)	85	GACCAAGTGCAGCTTGCCCTC	Morgan *et al*. 2003
28SR9 (R)	86	AGACCTGCTGCGGATATGGGT	Morgan *et al*. 2003
28SR7 (R)	87	GCTTTGTTTTAATTAGACAGTCGGATTC	Morgan *et al*. 2003
28SR10 (R)	88	GGGAATCTCGTTAATCCATTCA	Morgan *et al*. 2003
28SR11 (R)	89	TCACCATAGGACACCCGCGT	Morgan *et al*. 2003
28SR12 (R)	90	TGAACCTGCGGTTCCTCTCGTA	Morgan *et al*. 2003
28SR13 (R)	91	ACTTAGAGGCGTTCAGTCTTAA	Morgan et al. 2003
28SR8 (R)	92	ATTCTGACTTAGAGGCGTTCA	Morgan *et al*. 2003
ITS2 rDNA			
3S (F)*	93	GGTACCGGTGGATCACTCGGCTCGTG	Bowles *et al*. 1993
ITS2.2 (R)	94	CCTGGTTAGTTTCTTTTCCTCCGC	Cribb *et al*. 1998

GA1 (F)	95	AGAACATCGACATCTTGAAC	Anderson and Barker, 1998
CO-F (F)	96	GCTATTCCTAGATGTTTACG	Shirakashi *et al*. 2012
CO-R (R)	97	GCAAAGAAACATTGCATCG	Shirakashi *et al*. 2012
CH-F (F)	98	TTTTCCTAAATGTGTGTGCA	Shirakashi *et al*. 2012
CH-R (R)	99	AGGCAACAAGTATCAAAACA	Shirakashi *et al*. 2012
BF-F (F)	100	GGAAATTGTGCYACCTGGCA	Shirakashi *et al*. 2012
BF-R (R)	101	AGCACAAGCCGCTACCA	Shirakashi *et al.* 2012
Cfor_F	102	TGATTGCTTGCTTTTTCTCGAT	Polinski *et al*. 2013
LCfor_F	103	TGCACAATTCACGACTCACGATCCACA CGGTCTCGCACTGGCACGGGTGATTG CTTGCTTTTTCTCGAT	Polinski *et al*. 2013
Cfor_R	104	TATCAAAACATCAATCGACATC	Polinski *et al*. 2013
RF_Cfor_F[4]	105	CGACTCACTATAGGGCAGATCTTCGAA TGATTGCTTGCTTTTTCTCGATATG	Polinski *et al*. 2013
RF_Cfor_R[4]	106	GGCCTTGACTAGAGGGTACCAGATATC AAAACATCAATCGACATCTCA	Polinski *et al*. 2013
Cori_F	107	TGCTTGCTATTCCTAGATGTTTAC	Polinski *et al*. 2013
L_Cori_F	108	TGCACAATTCACGACTCACGATCATCC GCTCCGACGACACGAACGGGTGCTTG CTATTCCTAGATGTTTAC	Polinski *et al*. 2013
Cori_R	109	AACAACTATACTAAGCCACAA	Polinski *et al*. 2013
RF_Cori_F[4]	110	CGACTCACTATAGGGCAGATCTTCGAA TGCTTGCTATTCCTAGATGTTTACG	Polinski *et al*. 2013
RF_Cori_R[4]	111	GGCCTTGACTAGAGGGTACCAGAAACA ACTATACTAAGCCACAACCT	Polinski *et al*. 2013
Copt_F	112	TTCCTAAATGTGTGTGCA	Polinski *et al*. 2013
L_Copt_F	113	TGCACAATTCACGACTCACGATCATCC GCTCCGACGACACGAACGGGTTCCTAA ATGTGTGTGCA	Polinski *et al*. 2013
Copt_R	114	TCAAAACATCAATCGACACT	Polinski *et al*. 2013
RF_Copt_F[4]	115	CGACTCACTATAGGGCAGATCTTCGAA TTCCTAAATGTGTGTGCATTTGTG	Polinski *et al*. 2013
RF_Copt_R[4]	116	GGCCTTGACTAGAGGGTACCAGATCAA AACATCAATCGACACTTCAC	Polinski *et al*. 2013
L_UP	117	GCACAATTCACGACTCACGA	Polinski *et al*. 2013
L_FAM_1[5]	118	FAM-CCACACGGTCTCGCACTGGC-BHQ1	Polinski *et al*. 2013
L_HEX_1[5]	119	HEX-CCACACGGTCTCGCACTGGC-BHQ1	Polinski *et al*. 2013
L_HEX_2[5]	120	HEX-CATCCGCTCCGACGACACGA-BHQ1	Polinski *et al*. 2013
CO1 mtDNA			
Cox1_schist_5k (F)	121	TCTTTRGATCATAAGCG	Lockyer *et al*. 2003b
Cox1_schist_3k (R)	122	TAATGCATMGGAAAAAAACA	Lockyer *et al*. 2003b
CO1F5 (F)	123	TTGRTTTGTYTCTTTRGATC	Morgan *et al*. 2003
CO1F6 (F)	124	TTTGTYTCTTTRGATCATAAGCG	Morgan *et al*. 2003
CO1F4 (F)	125	ATTTGGWACTGCTTTTTTTGAGCC	Morgan *et al*. 2003
CO1F3 (F)	126	CATTTATTTTGGTTTTTTGGTCA	Morgan *et al*. 2003
CO1R9 (R)	127	TTDTTHCTTADABTCATACA	Morgan *et al*. 2003
CO1R8 (R)	128	CCAAYCATRAACATATGATG	Morgan *et al*. 2003
CO1R4 (R)	129	ACCTAAATAATGCATAGGAAA	Morgan *et al*. 2003
CO1R5 (R)	130	GATCATARCAWCTWACACGACG	Morgan *et al*. 2003
JB3 (F)	131	TTTTTTGGGCATCCTGAGGTTTAT	Bowles *et al*., 1993
JB4.5 (R)	132	TAAAGAAAGAACATAATGAAAATG	Bowles *et al*., 1993

Additional sequencing[6]			
18S rDNA			
SB9	133	TTTCACCTCTAACACCGC	Barker and Blair, 1996
SB3	134	GGAGGGCAAGUCUGGUGC	Barker and Blair, 1996
A27	135	CCATACAAATGCCCCCGTCTG	Barker and Blair, 1996
Lin 3 (F)	136	GCGGTAATTCCAGCTCCA	Lin *et al*. 1999
D2F (F)	137	CTTTGAAGAGAGAGTTC	Littlewood, 1994
D3RM (R)	138	GCATAGTTCACCATCTTTC	Littlewood and Johnston, 1995
D4AR (R)	139	CCGTGTTTCAAGACGGG	Littlewood and Johnston, 1995
388F (F)	140	AGG GTT CGA TTC CGG AG	Littlewood and Olson, 2001
1100F (F)[1]	141	CAGAGTTTCGAAGACGATC	Littlewood and Olson, 2001
CEST1R (R)[1]	142	TTTTTCGTCACTACCTCCCC	Littlewood and Olson, 2001
1270R (R)	143	CCGTCAATTCCTTTAAGT	Littlewood and Olson, 2001
28S rDNA			
Dig12 (F)	144	AAGCATATCACTAAGCGG	Tkach *et al*. 1999
LSU1500R (R)	145	GCTATCCTGAGGGAAACTTCG	Tkach *et al*. 1999
300F (F)	146	CAAGTACCGTGAGGGAAAGTTG	Lockyer *et al*. 2003a
300R (R)	147	CAACTTTCCCTCACGGTACTTG	Lockyer *et al*. 2003a
400R (R)	148	GCA GCT TGA CTA CAC CCG	Olson *et al*. 2003
EC-D2 (alias ECD2, ECD-2) (R)[1]	149	CCTTGGTCCGTGTTTCAAGACGGG	Littlewood *et al*. 1997
900F (F)	150	CCGTCTTGAAACACGGACCAAG	Lockyer *et al*. 2003a
1200F (F)	151	CCCGAAAGATGGTGAACTATGC	Lockyer *et al*. 2003a
1200R (alias LSU1200R) (R)	152	GCATAGTTCACCATCTTTCGG	Lockyer *et al*. 2003a
1600F (F)	153	AGCAGGACGGTGGCCATGGAAG	Lockyer *et al*. 2003a
U2229 (F)	154	TACCCATATCCGCAGCAGGTCT	Lockyer *et al*. 2003a
L2230 (R)	155	AGACCTGCTGCGGATATGGGT	Lockyer *et al*. 2003a
U2562 (F)	156	AAACGGCGGGAGTAACTATGA	Lockyer *et al*. 2003a
L2630 (R)	157	GGGAATCTCGTTAATCCATTCA	Lockyer *et al*. 2003a
U2771 (F)	158	AGAGGTGTAGGATARGTGGGA	Lockyer *et al*. 2003a
L2984 (R)	159	CTGAGCTCGCCTTAGGACACCT	Lockyer *et al*. 2003a
U3119 (F)	160	TTAAGCAAGAGGTGTCAGAAAAGT	Lockyer *et al*. 2003a
U3139 (F)	161	AAGTTACCACAGGGATAACTGGCT	Lockyer *et al*. 2003a
LSU3_4160 (R)	162	GGTCTAAACCCAGCTCACGTTCCC	Lockyer *et al*. 2003a
L3358 (R)	163	AACCTGCGGTTCCTCTCGTACT	Lockyer *et al*. 2003a
CO1 mtDNA			
CO1560Fa (F)	164	TTTGATCGTAAATTTGGTAC	Lockyer *et al*. 2003b
CO1560Fb (F)	165	TTTGATCGGAATTTTGGTAC	Lockyer *et al*. 2003b
CO1560R (R)	166	GCAGTACCAAATTTACGATC	Lockyer *et al*. 2003b
CO1800F (F)	167	CATCATATGTTTATGGTTGG	Lockyer *et al*. 2003b
CO1800Ra (R)	168	CCAACCATAAACATATGATG	Lockyer *et al*. 2003b
CO1800Rb (R)	169	CCAACCATAAACATGTGATG	Lockyer *et al*. 2003b

Ns, not specified.

[1]Primers presented in "Amplification and Sequencing" as well as "Additional sequencing" category in order to better characterize their distinctive role in different articles (see Table 2).

[2]Primer only identified by its 3' starting position in *S. mansoni* sequence (i.e., position 45) (see Barker and Blair, 1996).

[3]Primer only identified by its 3' starting position in *S. mansoni* sequence (i.e., position 278) (see Barker and Blair, 1996).

[4]Restriction-free bridging primers.

[5]Real-time PCR probe.

[6]Primers were classified as "additional sequencing" just when this was specified in the original reference.

*Nolan and Cribb (2004*a*), Nolan and Cribb (2004*b*), Holzer *et al*. (2008), Ogawa *et al*. (2011) and Shirakashi *et al*. (2013) use an oligonucleotide sequence modified from original reference (i.e., 5' - GGTACCGGTGGATCAC**GT**GGCT**A**GTG - 3').
§ Snyder *et al*. (2004), Cribb *et al*. (2011) employ an oligonucleotide sequence modified from original reference with the subtraction of a cytosine in 5' end.

CHAPTER 2: BLOOD FLUKES (DIGENEA: APOROCOTYLIDAE) OF EPIPELAGIC LAMNIFORMS: *HYPERANDROTREMA CETORHINI* FROM BASKING SHARK (*CETORHINUS MAXIMUS*) AND A NEW CONGENER FROM SHORTFIN MAKO SHARK (*ISURUS OXYRINCHUS*) OFF ALABAMA

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ABSTRACT

We redescribe the type species *Hyperandrotrema cetorhini* Maillard and Ktari, 1978 (Digenea: Aporocotylidae Odhner, 1912) based on the holotype and 2 paratypes collected from the heart of basking shark (*Cetorhinus maximus*), emend the original generic diagnosis for *Hyperandrotrema* Maillard and Ktari, 1978, and describe *Hyperandrotrema walterboegeri* Orélis-Ribeiro and Bullard n. sp. based on 6 adult specimens collected from the heart of a shortfin mako shark (*Isurus oxyrinchus* Rafinesque, 1810) captured from Viosca Knoll (N29°11.70'; W88°33.32'; 123 km southwest of Dauphin Island, Alabama), northern Gulf of Mexico. *Hyperandrotrema* spp. infect lamniforms and differ from all other nominal aporocotylids at least by having a ventrolateral field of robust C-shaped spines (rather than transverse rows of minute, shaft-like spines), an inverse U-shaped intestine with extremely elongate ceca terminating near the level of the excretory bladder, and a common genital pore that comprises the dorsal opening of a common genital atrium. Specimens of *H. walterboegeri* n. sp. exceed 12 mm in total length, making it the largest named blood fluke on Earth. The new species further differs from *H. cetorhini* by the combination of having an adult body that is 7–8 × longer than wide, tegumental spines at level of the

testis measuring 25–38 μm long × 10–15 μm wide, a long vas deferens 4–5% of the body length that connects with the cirrus sac and internal seminal vesicle well posterior to the union of the uterus and metraterm, a testis 9–11 × longer than wide, and a large ootype 105–150 μm long × 85–105 μm wide. This is the first report of *Hyperandrotrema* from the Gulf of Mexico and the second aporocotylid species reported from an epipelagic elasmobranch. Our results demonstrate that ecologically-related (epipelagic, marine) and phylogenetically-related (Lamniformes) definitive hosts are infected by morphologically similar (congeneric) fish blood flukes.

INTRODUCTION

The fish blood flukes (Digenea: Aporocotylidae Odhner, 1912) that infect epipelagic marine fishes are underexplored regarding their taxonomic diversity. At present, 17 of ~130 (12%) nominal species of Aporocotylidae assigned to 7 genera are known to mature in 17 epipelagic fishes of 13 genera: *Aporocotyle simplex* Odhner, 1900 from roundnose grenadier, *Coryphaenoides rupestris* Gunnerus, 1765; *Aporocotyle pacifica* Yamaguti, 1970 from oilfish, *Ruvettus pretiosus* Cocco, 1833; *Paradeontacylix sanguinicoloides* McIntosh, 1934 from yellowtail amberjack, *Seriola lalandi* Valenciennes, 1833 and Samson fish, *Seriola hippos* Günther, 1876; *Paradeontacylix kampachi* Ogawa and Egusa, 1986, *Paradeontacylix grandispinus* Ogawa and Egusa, 1986, *Paradeontacylix baleariicus* Repullés-Albeida, Montero, Holzer, Ogawa, Hutson, and Raga, 2008, and *Paradeontacylix ibericus* Repullés-Albeida, Montero, Holzer, Ogawa, Hutson, and Raga, 2008 all from greater amberjack, *Seriola dumerili* (Risso,

1810); *Paradeontacylix godfreyi* Hudson and Whittington, 2006 from yellowtail amberjack, *Seriola lalandi* Valenciennes, 1833; *Psettarium* cf. *grandis* (Lebedev and Mamaev, 1968) Bullard and Overstreet, 2006 from indo-Pacific sailfish, *Istiophorus platypterus* (Shaw, 1792), Indo-Pacific blue marlin, *Makaira mazara* (Jordan and Snyder, 1901), and black marlin, *Istiompax indica* (Cuvier, 1832); *Cardicola congruenta* Lebedev and Mamaev, 1968 from kawakawa, *Euthynnus affinis* (Cantor, 1849); *Cardicola ahi* Yamaguti, 1970 from yellowfin tuna, *Thunnus albacares* (Bonnaterre, 1788); *Cardicola forsteri* Cribb, Daintith, and Munday, 2000 from southern bluefin tuna, *Thunnus maccoyii* (Castelnau, 1872) and northern bluefin tuna, *Thunnus thynnus* (Linnaeus, 1758); *Cardicola opisthorchis* Ogawa et al., 2011 and *Cardicola orientalis* from pacific bluefin tuna, *Thunnus orientalis* (Temminck and Schlegel, 1844); *Chimaerohemecus trondheimensis* van der Land, 1967 from rabbit fish, *Chimaera monstrosa* Linnaeus, 1758 and spookfish, *Hydrolagus mitsukurii* (Jordan and Snyder, 1904); *Hyperandrotrema cetorhini* Maillard and Ktari, 1978 from basking shark, *Cetorhinus maximus* (Gunnerus, 1765); and *Paracardicoloides yamagutii* Hine, 1978 from short-finned eel *Anguilla australis* Richardson, 1841. Of the 6 aporocotylid species of 5 genera that infect chondrichthyans (Table 1), only *C. trondheimensis* and *H. cetorhini* reportedly infect fishes that are regular residents of the epipelagic realm (Van der Land, 1967; Maillard and Ktari, 1978; Kamegai et al., 2002; Karlsbakk et al., 2002; Bullard et al., 2006; Bullard and Jensen, 2009; Castro, 2011).

From a parasitological perspective, these fish-aporocotylid relationships make for a fascinating study system because the completion of these life cycles seems intuitively improbable in the vast, open ocean. Yet, the taxonomic diversity of aporocotylids that

infect these fishes indicates that epipelagic hosts are not rare acquisitions. Herein, we throw light on aporocotylid biodiversity in the epipelagic zone by describing a new species that infected a shortfin mako shark, *Isurus oxyrinchus* Rafinesque, 1810 (Lamniformes: Lamnidae), in the Gulf of Mexico off Alabama. This is the first report of an aporocotylid from a pelagic shark in this ocean basin and only the second aporocotylid species reported from an epipelagic shark.

MATERIALS AND METHODS

The infected shortfin mako shark (female, 340 cm total length, 250 kg total weight) was identified by the diagnostic characters of Castro (2011) (= length of pectoral fins 16% of total length; ventral side of snout and mouth white, lacking blue or black coloration). It was captured by baited hook and line by recreational fishermen Tim King and Kevin Higgenbotham aboard the F/V *Finatic* on 8 January 2012 from the Horse Shoe Rigs at Viosca Knoll (N29°11.70'; W88°33.32', northern Gulf of Mexico, 123 km south/southwest of Dauphin Island, Alabama), killed at sea, iced, returned to the dock, and opportunistically necropsied on 9 January 2012. There, the heart was removed, bisected, and examined for the presence of fish blood flukes with the aid of a stereo dissecting microscope. Resulting aporocotylid specimens (6 total= 3 whole specimens, 3 partial specimens) intended as stained, whole mounted specimens were temporarily mounted on a slide, killed with heat from an EtOH burner flame, transferred to and held in a vial of 5% neutral buffered formalin, rinsed thoroughly with distilled water and cleaned with fine brushes to remove any debris, stained overnight in Van Cleave's

hematoxylin with several additional drops of Ehrlich's hematoxylin, made basic at 70% ethanol with lithium carbonate and butyl-amine, dehydrated, cleared in clove oil, and permanently mounted on glass slides using Canada balsam (Bullard, 2010a; b). Illustrations of stained, whole-mounted specimens were made with the aid of a Leica DM-2500 equipped with differential interference contrast optical components and a drawing tube. Measurements from these 6 specimens were obtained by using a calibrated ocular micrometer and are herein reported in micrometers (μm) following the number of measurements in parentheses. Subsequently, the partial specimens were demounted by immersion in xylene overnight, rinsed several times in xylene to remove Canada balsam, rinsed in 100% EtOH overnight, and prepared for scanning electron microscopy (SEM) by dehydrating them, immersion in hexamethyldisilazane for 30 min, air drying for 45 min, and sputter-coating with 15 nm gold palladium (Bullard, *in press*).

Scientific names including taxonomic authorities and dates for fishes follows Eschmeyer (2012). Higher level fish classification and nomenclature follows Nelson (2006) and Castro (2011). Nomenclature for Aporocotylidae follows Bullard et al. (2009). Brown (1956) was used to help construct the genus name and specific epithet. Specimens of related aporocotylids were borrowed from the United States National Parasite Collection (Beltsville, Maryland, USA; USNPC) courtesy of Eric Hoberg and Patricia Pilitt, and the holotype and 2 paratypes of the new species were deposited there.

DESCRIPTIONS

***Hyperandrotrema* Maillard & Ktari, 1978, emended**

(Figs. 1–20)

Diagnosis: Body of adult ovoid or elongate, 2–8× longer than wide, dorsoventrally flattened, lacking posterolateral protuberance, lacking distinctive anterior sucker demarcated from body, spined; field of tegumental body spines ventrolateral, having 1–7 spines across breadth of field depending on location on body, not comprising spines evenly and clearly distributing in transverse rows, discontinuous posteriorly (=posterior area of body aspinous at level of excretory vesicle); tegumental body spines C-shaped (base and hook shaft resembling a log hook), smallest at body ends, largest at midbody; base of tegumental body spines broad, crenulated or straight shaft robust, strongly recurved and perpendicular to hook base, with sharp-pointed tip; tegument supporting spines pedunculate, having longitudinal muscle extending from proximal base of spine to body proper, directing ventrally. Rosethorn-shaped spines absent. Nerve cords confluent posteriorly at level of excretory bladder; ventrolateral nerve commissure perpendicular to long axis of body, dorsal to esophagus. Mouth medial, subterminal. Pharynx absent. Buccal cavity absent. Esophagus medial, sinuous, extending posteriad <15% of body length, including posterior esophageal swelling; posterior esophageal swelling thick-walled, occupying space between cecal bifurcation and ventrolateral nerve commissure; esophageal gland surrounding posterior esophageal swelling (seemingly best visualized in heat-killed, formalin-fixed, and hematoxylin-stained specimens observed with differential interference contrast microscopy). Intestine inverse U-shaped (lacking anterior ceca), straight or slightly sinuous (not convoluted or looping extensively), lacking diverticula or secondary rami, comprising cecal bifurcation

and paired ceca, terminating <3% of body length from posterior body; sinistral cecum coursing dorsal to cirrus sac and forming a distinctive arch at level of genital atrium. Testis single, 2–11 × longer than wide, intercecal and occupying space between cecal bifurcation and ovary; vasa efferentia comprising interconnecting meshwork of fine ducts dispersed throughout testicular field, coalescing ventrally in posterior margin of testicular field and forming single vas deferens; vas deferens emerging from medioventral aspect of posterior margin of testis, extending posteriad before connecting with proximal portion of cirrus sac. Cirrus sac enveloping internal seminal vesicle, cirrus, and prostatic gland cells, thin-walled, weakly muscular, enclosing whispy non-staining contents surrounding internal seminal vesicle, sinistral, post-gonadal; internal seminal vesicle a laterally-expanded distal portion of vas deferens within proximal portion of cirrus sac; cirrus massive, appendix-like, everting laterally; prostatic gland cells encircling non-everted cirrus in distal portion of ejaculatory duct. Auxiliary external seminal vesicle absent. Opening of male genital tract connecting with a common atrium; opening of common genital atrium dorsal, at level of arch of sinistral cecum. Ovary single, medial, intercecal, post-testicular, middle portion narrow and comprising dorsal and ventral cords spanning midline, bearing deep-notched lateral lobes and appearing dendritic, having hourglass- or butterfly-shaped outline laterally, occupying posterior 1/4 of body. Vitellarium follicular, coextensive with ceca and principally lateral to testicular field from level of anterior nerve commissure to genital atrium, with symmetrical or asymmetrical posterior extremities. Oviduct extending from dorsal surface of ovarian cord, including oviducal ampulla; oviducal ampulla comprising a laterally-expanded chamber in extreme distal portion of oviduct, proximal to ootype. Oviducal seminal

receptacle of proximal oviduct absent. Laurer's canal extending dorsomediad from oviduct approximately at level of oviducal ampulla, surrounded by intensely basophilic glandular cells, opening dorsally at level of cirrus sac. Primary vitelline duct dextral, connecting with oviduct on anterior or lateral surface of ootype. Ootype spheroid, surrounded by extensive Mehlis' gland, posterior to genital ducts and gonads, intercecal. Uterus extensively convoluted for entire length, occupying space between ovary and ootype as well as between cirrus sac and dextral cecum, lacking descending uterus; uterine eggs thin shelled, minute, spheroid, lacking discernible miracidium. Metraterm straight, sinistral. Opening of female genital tract connecting with a common atrium, anterior to corresponding connection with male genital tract. Excretory vesicle medial, Y-shaped including arms; excretory pore dorsal, subterminal. In heart of epipelagic elasmobranchs (Elasmobranchii: Lamniformes).

Differential diagnosis: Body of adult lacking posterolateral protuberance; field of tegumental body spines ventrolateral, discontinuous posteriorly, comprising robust C-shaped spines. Esophagus extending posteriad <15% of body length. Intestine inverse U-shaped, terminating <3% of body length from posterior body, with sinistral cecum arching at level of genital atrium. Testis approximately 2–11× longer than wide; vasa efferentia coalescing ventrally in posterior margin of testicular field; vas deferens emerging from medioventral aspect of posterior margin of testis. Cirrus sac enveloping internal seminal vesicle, cirrus, and prostatic gland cells; internal seminal vesicle a laterally-expanded distal portion of vas deferens within confines of cirrus sac; cirrus massive, appendix-like. Auxiliary external seminal vesicle absent. Ovary middle portion comprising dorsal and ventral cords spanning midline, dendritic, occupying posterior 1/4

of body. Oviducal ampulla present. Oviducal seminal receptacle absent. Laurer's canal present. Primary vitelline duct dextral. Ootype posterior to genital ducts and gonads, intercecal. Uterus extensively coiled, occupying space between cirrus sac and dextral cecum, lacking descending uterus; uterine eggs minute, spheroid. Genital tracts opening into common atrium; common atrium opening on dorsal surface.

Type species: Hyperandrotrema cetorhini Maillard and Ktari, 1978.

Remarks

Hyperandrotrema Maillard & Ktari, 1978 (monotypic), *Chimaerohemecus* Van der Land, 1967 (monotypic), and *Selachohemecus* Short, 1954 (2 species) (Table 1) include all nominal species of Aporocotylidae that infect sharks and chimaeras (Maillard and Ktari, 1978; Van der Land, 1967; Short, 1954; Kamegai et al., 2002; Karlsbakk et al., 2002; Bullard et al., 2006; present study). Species of these genera are unique among all other named aporocotylids in having robust C-shaped tegumental body spines that are each associated with a muscular peduncle. These distinctive spines are distributed in a single lateral column (*Selachohemecus*), 2–4 lateral columns (*Chimaerohemecus*), or a ventrolateral field (*Hyperandrotrema*). The majority of aporocotylids that infect true bony fishes (Euteleosti) typically have myriad ventrolateral, transverse rows of tegumental body spines, with each spine having a slightly recurved distal tip (see Bullard and Overstreet, 2003; 2004; Bullard, 2010a; 2012; Bullard et al., 2012; McVay et al., 2011). The lateral tegumental body spines of species of *Hyperandrotrema*, *Chimaerohemecus*, and *Selachohemecus* are discontinuous posteriorly, i.e., the posterior most spines of each body margin terminate well anterior to the posterior body end. In addition, all species of these genera have an ovary that is

immediately post-testicular, intercecal, and markedly dendritic, with the lateral projections of the ovary giving the organ an hourglass- or butterfly-shaped outline. Also, the common vitelline duct of species of these genera originates in the posterior region of the body at level of the uterus or genital pore. Euteleost blood flukes typically have a prominent, medial common vitelline duct that originates at level of the cecal intersection and extends posteriad to join the oviduct and ootype.

There are significant morphological differences among the genera of chondrichthyan aporocotylids. Clearly *Selachohemecus* significantly differs from the other genera by having an intestine that is X-shaped (consisting of short anterior and posterior caeca of approximate equal length), a single ventrolateral column of tegumental body spines, a post-cecal ovary, an ootype that is anterior to a portion of the oviduct, and a common genital pore as well as by lacking a Laurer's canal and oviducal ampulla. These are major differences that seem to indicate a more distant relationship between species of this genus and those of *Hyperandrotrema* and *Chimaerohemecus*. Further, *Orchispirium* Madhavi and Rao, 1971 and *Myliobaticola* Bullard and Jensen, 2009, both monotypic presently, include species infecting stingrays. However, and despite having an inverse U-shaped intestine, they are not obviously morphologically allied with other chondrichthyan aporocotylids (Madhavi and Rao, 1970; Bullard and Jensen, 2009).

Hyperandrotrema is morphologically most similar to *Chimaerohemecus*, both having similarly-shaped spines (log hook-shaped, "C-shaped"), an inverse U-shaped intestine, intercecal gonads and genitalia, a Laurer's canal, an oviducal ampulla (= "receptaculum seminis" of Van der Land [1967]), an ootype that is posterior to the genitalia, a massive cirrus and associated cirrus sac that encloses the internal seminal vesicle, and an

extensively coiled uterus that occupies the space between the cirrus sac and dextral cecum. No other named aporocotylid reportedly possess an oviducal ampulla or this combination of morphological features. *Hyperandrotrema* can be most easily differentiated from *Chimaerohemecus*, and all other proposed fish blood fluke genera, by having a field of lateral tegumental body spines, posterior ceca that terminate in the posterior body extremity at level of the excretory bladder, and a common genital pore (comprising the dorsal opening of a common genital atrium). *Chimaerohemecus* has 2 ventrolateral columns of tegumental body spines and ceca that terminate at level of the cirrus sac. Maillard and Ktari (1978) used (i) esophagus length:body length (esophagus proportionally shorter in *Hyperandrotrema* than in *Chimaerohemecus*) and the posterior extent of the ceca (nearly terminal in *Hyperandrotrema*) to justify the proposal of a new genus to accommodate *H. cetorhini*. We concur that those are strong generic features differentiating these genera, and provide additional ones herein (see emended diagnosis above).

The literature holds confusion regarding the presence/absence of a common genital pore or separate genital pores in species of *Hyperandrotrema* and *Chimaerohemecus*. Because this feature is of generic significance among aporocotylids, it is worthy of clarification herein. Van der Land (1967) and Maillard and Ktari (1978) reported that the genital pores were separate in *C. trondheimensis* and *H. cetorhini*, respectively. Kamegai et al. (2002) reported a common genital pore in *Chimaerohemecus* sp. The openings of the genital tracts in the holotype and paratypes of *H. cetorhini* we studied are indeed separate. They open into a common atrium before that atrium opens dorsally at level of arch of the sinistral cecum (Figs. 3, 4). Likewise, voucher specimens of *C.*

tronheimensis (230895-10 [2 specimens]; 230196-16 [1 specimen]) we studied from the dorsal aorta of the type host from near the type locality in the northeastern Atlantic Ocean off Norway had a similar arrangement; with both genital tracts opening into a shallow, common atrium that then opens on the dorsal body surface. Although a minute difference and bordering on semantics, these observations confirm the presence of a common genital opening in all aporocotylids that infect sharks (Short, 1954; Maillard and Ktari, 1978; Bullard et al., 2006; present study) and chimaeras (Van der Land, 1967; Kamegai et al., 2002; Karlsbakk et al., 2002).

***Hyperandrotrema cetorhini* Maillard and Ktari, 1978**

(Figs. 1–4)

Diagnosis of adult based on light microscopy of the whole-mounted holotype (68PE-TJ-19) and paratypes (1 slide with 2 specimens, both 68- PE-TJ-20): With characters of *Hyperandrotrema* Maillard and Ktari, 1978 as emended. Body of adult ovoid, appearing equally rounded anteriorly and posteriorly, 6,600–8,120 (3) long, 3,020–3,640 (3) wide, 2 × longer than wide, with body margin having variously placed lateral folds resulting from contractions and disposition of specimen when fixed, with 1 paratype having contracted sinistral body margin at level of the opening of the genital atrium (which should not be misinterpreted as a posterolateral body protuberance) (Fig. 1). Field of tegumental body spines comprising hundreds of spines, approximately 41–48 (2) lateral-most spines per side of body between mouth and cecal bifurcation, with many spines seemingly detached in type specimens, comprising 1–2 spines abreast anteriorly and posteriorly and typically 6–7 abreast in midbody, with breadth of field varying accordingly (from approximately 10–25 [2] in breadth at level of mouth to approximately

83–143 [2] in breadth at midbody depending on contraction of lateral body margin), ending 68–75 (2) or 1% of body length from posterior end of body (Figs 1, 2); spines near mouth 10–13 (20) long, 8 (20) wide at base; spines at level of cecal bifurcation 10–13 (20) long, 8–10 (20) wide at base; spines at level of testis 10–15 (20) long, 8–10 (20) wide at base; spines at level of ootype 10–13 (20) long, 8 (20) wide at base; spines posterior to level of ootype 10–13 (20) long, 8 (20) wide at base; tegumental peduncles supporting spines 13 (3) long × 10–13 (3) wide at level of esophagus, 8–10 (4) long × 10–13 (4) wide in midbody and posteriorly. Ventrolateral nerve-cords evident only in anterior body region, obscured by ceca and vitellarium for most of body length, with secondary branches not evident but likely obscured by overlying vitellarium; ventrolateral nerve commissure 268–343 (3) or 4–5% (3) body length from anterior end of body, 453–663 (3) across width of worm or 14–18% (3) body width, 13–25 (3) in maximum diameter; dorsolateral nerve commissure not evident. Mouth 25–33 (2) in diameter, distance from anterior body end not measurable due to specimen folding (Fig. 1). Esophagus 688–720 (3) long or 9–11% of body length, 275–428 (2) in maximum width or 3 × esophagus width at level of ventrolateral nerve commissure, containing yellowish granular material (Fig. 1); musculo-glandular region surrounding anterior portion of esophagus 178–273 (3) long, 128–353 (3) wide, 0.7–1.4 × longer than wide (Fig. 1); posterior esophageal swelling 345–373 (3) long, 275–428 (2) wide, 0.7–1.3 × longer than wide, surrounded by gland cells likely comprising an esophageal gland, occupying space between ventrolateral nerve commissure and cecal bifurcation (Fig. 1); esophagus wall 3–5 (3) thick near mouth, 25–75 (3) thick near cecal bifurcation; esophageal gland not enveloping esophagus for entire length, conspicuous at level of

esophageal swellings (best visualized with stereoscope because of improved depth of field), concentrating in an area 375–468 (3) long × 338–575 (3) wide (Fig. 1). Ceca bifurcating 563–760 (3) or 8–12% of body length from anterior body end, 5,765–7,319 (3) long or 87–91% of body length, terminating 38–75 (3) or approximately 1% of body length from posterior body end, 158–165 (3) wide or 4–5% maximum body width at level of midbody, containing granular, brownish material in lumen of all specimens (Fig. 1).

Testis oblong, approximately 4,095–5,472 (3) long or 62–69% of body length, 2,350–3,005 (3) wide or 78–84% of body width, 1.6–1.8 × longer than wide (Fig. 1). Post-testicular space 1,505–1,910 (3) long or 22–26% of body length. Vasa efferentia 13–25 (3) in diameter; vas deferens 43–60 (2) in maximum width at level of ovary or 2% of maximum testis width, extending posteriad approximately 138–145 (2) before connecting with cirrus sac (Figs. 1, 3, 4). Cirrus-sac 835–1,140 (3) long or 12–15% of body length, 348–538 (3) in maximum width before cecal arch, with wall 5–8 (3) thick, S-shaped in lateral profile, having a proximal kink and a distal and laterally-curved portion at level of opening of genital atrium; internal seminal vesicle S-shaped within cirrus sac, convoluted, with wall approximately 3–5 (3) thick, 188–700 (3) long or 6–19% of cirrus sac length, 75–103 (3) in maximum width before ceca kink or 18–24% of maximum cirrus sac width; sperm duct and inverted cirrus opening 775–1,048 (3) or 10–16% of body length from posterior end of body, opening 238–353 (3) or 7–12% of maximum body width from sinistral body margin, 350–525 (2) long or 32–63% of cirrus sac length, 65–73 (3) in maximum width or 63–97% of seminal vesicle width, with wall 10–13 (3) thick and having minute external crenulations (Figs. 1, 3, 4).

Ovary 1,050–1,275 (3) long or 16–36% of body length, 2,100–2,400 (3) wide or 66–

70% of body width; ovarian lobes 975–1,075 (10) long or 41–45% of ovary maximum width, 28–40 (10) in maximum width or 2–3% of ovary maximum length, 26–37 × longer than wide; secondary and tertiary branches 90–133 (10) long or 4–6% of ovary maximum width, 28–50 (10) in maximum width or 2–4% of ovary maximum length, 2–4 × longer than wide (Figs. 1, 3, 4). Vitellarium having asymmetrical posterior branches in paratype (dextral portion longest and extending to level of oviducal ampulla), having symmetrical posterior branches in holotype, longest posterior branch of vitellarium terminating 1,008–1,125 (2) or 14–16% of body length from posterior end (Fig. 1). Oviduct extending dextrad from mid-dorsal surface of ovarian cord before curving posteriad 1,647–1,745(2) or 22–25% of body length, 28–35 (3) in maximum width at level of ovary, running posteriorly 603-770 (2) before contorting extensively (3–4 loops) at level of cirrus sac, continuing 318-425 (2) posteriad approximately in parallel with dextral body margin before expanding laterally and meeting with oviducal ampulla and base of Laurer's canal posterior to level of cirrus sac (Figs. 3, 4); oviducal ampulla comprising anterodorsal and posteroventral compartments, 275–315 (3) in total length or 22–29% of oviduct total length, 108–148 (3) in maximum width or 3.1–5.3 × oviduct minimum width, 638–838 (2) or 10% of body length from posterior end of body, containing ova only (no sperm or vitelline material) in holotype and paratypes; Laurer's canal 150–183 (3) long or 12–17% of oviduct total length, 75–98 (3) in maximum width or 2.5–3.5 × oviduct minimum width, extending anteriad from origin of oviducal ampulla, having glandular wall 3–5 (3) thick, containing ova only (no sperm or vitelline material) in holotype and paratypes, with 1 paratype showing ova being ejected from the dorsal pore of the canal (Figs. 3, 4). Oviduct distal to oviducal ampulla extending mediad 153–

273 (2), crossing midline before arching posteriad and connecting with anterodorsal surface of ootype (Figs. 3, 4). Common vitelline duct not presenting as a robust medial duct (cf. primary vitelline duct of *Cardicola* spp.), originating from medial aspect of dextral vitelline field at level of uterus, extending mediad and connecting with oviduct on anterodorsal surface of ootype (Figs. 3, 4). Ootype 50–75 (3) long, 48–63 (3) wide or 2 × oviduct width, 6–8% of body length from posterior body end; Mehlis gland comprising a dense field of refractive, non-staining processes extending perpendicular to long axis of body (Figs. 3, 4). Uterus extending anteriorly from ootype and immediately becoming extensively convoluted, 118–138 (3) in maximum width at level of seminal vesicle; uterine mass 890–1,308 (3) in maximum length or 14–16% of body length, 580–635 (3) in maximum width or 17–19% of body width, lacking obvious aggregations of sperm (lacking uterine seminal receptacle) (Figs. 3, 4); uterine eggs 5–10 (20) in diameter; metraterm comprising a narrow, straight distal portion of uterus, with wall 8–10 (3) thick, 75–100 (3) wide or 54–85% of maximum uterus width, extending posteriad 1,025 (1) lateral to cirrus sac before passing ventral to arch of sinistral cecum and opening into common genital atrium (Figs. 3, 4).

Excretory bladder 43 (1) long, 83 (1) wide or 1.9 × longer than wide (Fig. 1); arms of excretory bladder each 13–25 (2) wide; excretory pore 5–10 (2) wide.

Taxonomic summary

Type and only known host: *Cetorhinus maximus* (Gunnerus, 1765) (Lamniformes: Cetorhinidae), the basking shark.

Site: Adults in blood vascular system and heart.

Type locality: Eastern Atlantic Ocean off Tunisia.

Specimen examined: Holotype (68PE-TJ-19) and paratype (68PE-TJ-20) (both Muséum National d'histoire Naturelle, Paris [MNHNP]).

Remarks

Maillard and Ktari's (1978) description of *H. cetorhini* was based on 35 stained, whole-mounted specimens plus 6 serially-sectioned specimens (no observation of a living specimen was provided). The holotype and paratypes we studied were strongly flattened, with some folding, and apparently had lost many lateral tegumental spines. However, these specimens were well-stained and matched the published description of *H. cetorhini* in most regards. The original description of *H. cetorhini* was excellent and comprised a general account of the anatomy of the species; however, it had few measurements of the internal anatomy (body length, body width, 'oral cavity' diameter, testis length, testis width, esophagus length, lateral tegumental spine length). The present redescription provides morphometric data on the body, gut, genitalia, and excretory system, which were required to delineate it from the new species described below. After studying the holotype and paratype of *H. cetorhini*, and with the benefit of having specimens of an additional species of the genus, we detected a few features that needed further description and some that required correction regarding the anatomy of *H. cetorhini*.

Body depression. Maillard and Ktari (1978) described a slight depression on the sinistral side of the body about the opening of the cirrus sac. While we could not discern this feature in the type materials we studied, it is likely this feature, if present, was visualized with histology and living specimens. We did not, however, see any evidence in the holotype or paratypes that the posterior end of the body had a posterolateral

protuberance.

Lateral tegumental body spines. These authors originally reported that the ventrolateral field of tegumental spines comprised 3 or 4 spines abreast (and later in the differential diagnosis as 2 abreast); however, the holotype and paratype, while not showing this distribution clearly, did have regions of the spine field that had at least 7 spines abreast.

Digestive tract. Scant detail of the oral region and esophagus of *H. cetorhini* was provided by Maillard and Ktari (1978). The circumoral muscles we observed in the type specimens are pronounced, but do not comprise a distinct, well-delineated sucker that is separated from the anterior end of the body. The posterior portion of the esophagus is markedly thick-walled and likely comprises a functional chamber associated with digestion. This region is enveloped by the most easily discernible region of the esophageal gland, which in the type specimens is weakly staining but associated with dorsoventral acini. In addition, although ceca were correctly described in the description section, Maillard and Ktari (1978) may have mistook the vitellarium for the cecum in the Discussion section, wherein they state that the ceca terminate at level of or slightly posterior to the ovary. Whether or not the vitellarium of blood fluke species is symmetrical (extending posteriad equally) or not is likely an important generic feature.

Vitellarium symmetry and common duct. Maillard and Ktari (1978) reported that the vitellarium was slightly asymmetrical, with the dextral portion of the vitellarium extending slightly more posteriad than the sinistral portion. The holotype of *H. cetorhini* has a symmetrical vitellarium and the paratypes have an asymmetrical vitellarium, so the feature apparently represents intraspecific variation. However, that the vitellarium is

approximately symmetrical in *H. cetorhini* may represent a reliable specific feature differentiating it from closely related species infecting sharks. For example, *S. benzi* has a markedly asymmetrical vitellarium (Bullard et al., 2006). Maillard and Ktari (1978) depicted the common vitelline duct as connecting with the posterior portion of the oviducal ampulla; however, we observed in the holotype and paratypes that the duct connects on the anterodorsal aspect of the ootype. Van der Land (1967) reported that the common vitelline duct of *C. trondheimensis* was medial and originated "somewhere ventral of the ovary and leads directly to the ootype." We examined the voucher specimens of *C. trondheimensis* and the duct connects to the ootype anterodorsally.

***Hyperandrotrema walterboegeri* Orélis-Ribeiro and Bullard n. sp.**

(Figs. 5–20)

Diagnosis of adult based on light microscopy of 6 heat-killed, whole- mounted specimens plus subsequent SEM observations of 3 demounted, sputter-coated specimens: Body of adult elongate, appearing more rounded anteriorly than posteriorly, 11,220–12,520 (3) long, 1,500–1,800 (5) wide, 7–8 times longer than wide, with body margin strongly ventrally concave and reflected inward in some regions of some specimens depending on state of contraction when fixed (Figs. 5, 9). Dorsal body surface having honeycomb-like tegumental ridges (Fig. 11) covering surface except tegument immediately dorsal to mouth (Fig. 10). Ventral body surface lacking tegumental ridges (Fig. 12). Field of tegumental body spines (Figs. 5, 6, 9, 13–16) comprising hundreds of spines, approximately 48–51 (2) lateral-most spines per side of body between mouth and cecal bifurcation, comprising 2–7 spines abreast anteriorly and posteriorly and typically 7 abreast in midbody, with breadth of field varying

accordingly (from approximately 30–175 [4] anteriorly at level of mouth and esophagus; 175– 225 [4] in midbody at level of testis; 88–140 [4] at level of ootype), ending 120–193 (3) or 1–2% of body length from posterior end of body (Fig. 5); spines near mouth 13–20 (20) long, 8–10 (20) wide at base (Figs. 9, 15); spines at level of cecal bifurcation 25–38 (20) long, 10–12 (20) wide at base (Figs. 9, 16); spines at level of testis 25–38 (20) long, 10–12 (20) wide at base (Fig. 6); spines at level of ootype 18–25 (20) long, 8–10 (20) wide at base; spines posterior to level of ootype 13–20 (20) long, 8–10 (20) wide at base; tegumental peduncles supporting spines 13–30 (20) long 3 13–20 (20) wide at level of esophagus, 15–33 (20) long 3 15–28 (20) wide in midbody and posteriorly (Figs. 13, 15, 16). Ventrolateral nerve-cords indistinct except in anterior body end and midbody, 20–28 (4) in maximum width at midbody, 250–375 (4) or 15–21% of body width from body margin at midbody; with secondary branches indistinct; ventrolateral nerve com- missure 570–770 (4) or 6% of body length from anterior end of body, 238–300 (4) across width of worm or 13–19% of body width, 13–15 (4) in maximum diameter (Fig. 5); dorsolateral nerve commissure not evident. Mouth subterminal, 20–25 (2) in diameter (Figs. 5, 9). Esophagus 870–1,205 (4) long or 7–10% of body length, 140–165 (4) in maximum width or 2–4 times esophagus width at level of ventrolateral nerve commissure; anterior esophageal swelling 325–405 (4) long, 135–153 (4) wide, 2.3–2.7 times longer than wide, thin-walled, occupying space anterior to ventrolateral nerve commissure; posterior esophageal swelling 313–400 long, 75–168 (4) wide, 2–5 times longer than wide, surrounded by gland cells likely comprising an esophageal gland, occupying space between ventrolateral nerve commissure and cecal bifurcation; esophageal wall 3–8 (5) thick near mouth, 5–38 (4) thick near cecal bifurcation;

esophageal gland resembling that of H. cetorhini, concentrating in an area 325–550 (4) long 3 335–363 (4) wide (Fig. 5). Ceca bifurcating 955–1,300 (3) or 8% of body length from anterior body end, 8,720–10,250 (3) long or 70–89% of body length, terminating 103–285 (3) or 1–3% of body length from posterior body end, 50–140 (4) wide or 3–9% maximum body width, containing similar granular material in lumen of all specimens (Fig. 5).

Testis elongate, rectangular, approximately 6,825–7,450 (4) long or 60– 63% of body length, 630–835 (4) wide or 41–49% of body width, 9–11 times longer than wide (Fig. 5). Post-testicular space approximately 3,145– 3,760 (3) long or 28–31% of body length (Fig. 5). Vasa efferentia 15–33 (4) in diameter; vas deferens 70–125 (3) in maximum width at level of ovary or 10–15% of maximum testis width, extending slightly diagonally sinistrad for 460–590 (3) or 4–5% of body length before connecting with cirrus sac posterior to union of uterus and metraterm (Figs. 5, 7, 8). Cirrus-sac 1,056–1,290 (3) long or 9–11% of body length, 230–426 (3) in maximum width before ceca arch, with wall 5–10 (4) thick, L-shaped in lateral profile, expanded distally (Figs. 5, 7, 8); internal seminal vesicle straight or slightly sinuous, with wall approximately 5–8 (4) thick, 675–800 (3) long or 54–76% of cirrus sac length, 70–88 (3) in maximum width at level of middle of cirrus sac or 21–30% of maximum cirrus sac width (Figs. 5, 7, 8); sperm duct and inverted cirrus opening 1,990 (1) or 17% of body length from posterior end of body, opening 170–280 (3) or 9–17% of maximum body width from sinistral body margin, 423–650 (3) long or 41–52% of cirrus sac length, 93–113 (3) in maximum width at level of posterior portion of cirrus sac or 27–49% of maximum cirrus sac width, with wall 10–13 (3) thick and having minute crenulations (Fig. 7); everted cirrus (Fig. 18)

opening 1,175–2,200 (2) or 15–18% of body length from posterior end of body, opening 165–295 (3) 9–17% of maximum body width from sinistral body margin (Fig. 17), 170–575 (3) long or 19–72% of seminal vesicle length, 210–285 (3) in maximum width or 3–4 times seminal vesicle width, with wall 10–13 (3) thick and having minute external crenulations or ridges (Figs. 8, 18, 19).

Ovary 875–1,205 (6) long or 9–11% of body length, 950–1,200 (5) wide or 56–73% of body width (Figs. 5, 7, 8); ovarian lobes resembling those of H. cetorhini, having primary branches 405–550 (10) long or 34–46% of ovary maximum width, 20–40 (10) in maximum width or 2–3% of ovary maximum length, 14–20 times longer than wide, having secondary and tertiary branches 50–248 (10) long or 4–21% of ovary maximum width, 23–30 (10) in maximum width or 2–3% of ovary maximum length, 2–8 times longer than wide (Figs. 5, 7, 8). Vitellarium having asymmetrical posterior branches, with dextral portion longest and extending to level of distal portion of cirrus sac and terminating 1,775–2,240 (3) or 16–18% of body length from posterior end (Fig. 5). Oviduct extending dextrad from mid-dorsal surface of ovarian cord before curving posteriad 2,015–2,423 (3) long or 16–19% of body length, 23–25 (4) in maximum width at level of ovary, running posteriorly 1,520-1,875 (3) before joining oviducal ampulla posterior to level of cirrus sac (Figs. 5, 7, 8); oviducal ampulla 275–358 (3) long or 14–16% of oviduct total length, 95–143 (3) in maximum width or 4.1–7.2 times oviduct minimum width (narrowest portion of oviduct), 1,275–1,745 (3) or 11–14% of body length from posterior end of body, containing ova, vitelline material, and sperm in all specimens (Figs. 5, 7, 8); Laurer's canal 373–525(3) long or 17–23% of oviduct total length, 78– 80 (3) in maximum width or 3–4 times oviduct minimum width, extending

anteriad from anterior aspect of oviducal ampulla, having glandular wall 8–10 (3) thick (Figs. 5, 7, 8), containing ova and sperm in all specimens; dorsal pore (Fig. 20) of canal in 4 of 6 specimens showing sperm and ova being ejected. Oviduct distal to oviducal ampulla extending mediad 190– 248 (3), curving and extending directly posteriad before connecting with ootype (Figs. 7, 8). Common vitelline duct 950–1,200 (4) long, 20–23 (4) wide at the level of oviducal ampulla, connecting with vitellarium in dextral half of body near posterior margin of testis, extending posteriad in parallel with dextral ventrolateral nerve-cord before curving mediad and connecting with anterodorsal surface of ootype (Figs. 7, 8). Ootype 105– 150 (5) long, 85–105 (5) wide or 4–5 times oviduct width, 9–12% of body length from posterior body end (Figs. 7, 8). Mehlis' gland comprising a dense field of refractive, non-staining processes radiating from ootype (Figs. 7, 8). Uterus following similar general course as in H. cetorhini, 103– 123 (3) in maximum width; uterine mass 1,550–1,910 (3) in maximum length or 14–16% of body length, 385–475 (3) in maximum width or 25– 28% of body width, lacking obvious aggregations of sperm (lacking uterine seminal receptacle) (Figs. 5, 7, 8); uterine eggs 5–13 (20) in diameter, with one specimen ejecting eggs 10–13 (20) in diameter; metraterm similar to that of H. cetorhini, with wall 8–13 (3) thick, 50– 75 (4) wide or 34–73% of maximum uterus width, extending posteriad 400–995 (4) and following similar course as in H. cetorhini (Figs. 7, 8).

Excretory bladder 128–213 (4) long, 45–68 (4) wide or 2.3–3.8 times longer than wide (Fig. 5); arms of excretory bladder each 15–25 (5) wide; excretory pore 5–8 (3) wide.

Taxonomic summary

Type and only known host: Isurus oxyrinchus Rafinesque, 1810 (Lamniformes: Lamnidae), the shortfin mako shark.

Site: Adults attached to luminal surface (endocardium) of heart atrium and ventricle.

Type locality: Viosca Knoll (N29°11.70'; W88°33.32'), northern Gulf of Mexico, 123 km south/southwest of Dauphin Island, Alabama.

Specimens deposited: Holotype USNPC 107005. Paratype USNPC 107006.

Prevalence of infection: One shortfin mako shark was infected with 6 specimens of *H. walterboegeri*.

Etymology: The specific name *walterboegeri* honors ROR's former MSc thesis advisor, Prof. Walter Antonio Pereira Boeger (Federal University of Paraná, Curitiba, Brazil), and also is in reference to the exceptionally large body size of adults of this aporocotylid.

Remarks

The new species is the largest known blood fluke. It further differs from *H. cetorhini*, the only known congener, by the combination of having an adult body that is 7–8 × longer than wide, tegumental spines at level of the testis measuring 25–38 μm long × 10–15 μm wide, a long vas deferens 4–5% of the body length that connects with the cirrus sac and internal seminal vesicle well posterior to the union of the uterus and metraterm, a testis 9–11 × longer than wide, and a large ootype 105–150 μm long × 85–105 μm wide. *Hyperandrotrema cetorhini* has a smaller (6–8 mm in total length, albeit still relatively large among other fish blood flukes) adult body that is 2 × longer than wide, spines at level of the testis measuring 10–15 long × 8–10 wide, a proportionally shorter vas deferens 1–2% of the body length and that connects with the cirrus sac and

internal seminal vesicle at level of or immediately posterior to the union of the uterus and metraterm, a testis that is 2 × longer than wide, and a smaller ootype 50–75 (3) long × 48–63 (3) wide.

The functional significance of the oviducal ampulla and its homology with the various forms of the so-called ‘oviducal seminal receptacle’ of other aporocotylid genera is indeterminate, but it may function as a seminal receptacle or fertilization chamber (La Rue and Barone, 1932). We observed ova in the oviducal ampulla of the holotype and paratypes of *H. cetorhini* (see Description) as well as ova and sperm within the oviducal ampulla of the new species. However, given the morphological distinctiveness of the ampulla in species of *Hyperandrotrema* and *Chimaerohemecus*, we are hesitant to label it an ‘oviducal seminal receptacle’ and thereby assume homology with that feature in euteleost blood flukes.

DISCUSSION

Hyperandrotrema spp. infect geographically wide-ranging sharks, and certainly too few records of these flukes infecting these charismatic fishes exist to speculate on regional differences in fluke prevalence or taxonomic diversity. Basking sharks are the second-largest fish in the world (Castro, 2011). They can and do cross ocean basins, visit nearshore localities or remain far offshore, and have the ability to remain at great depths for prolonged periods (Gore et al., 2008; Skomal et al., 2009). Records of basking sharks in the Gulf of Mexico exist (Hoffmayer et al., 2011) but sighting a basking shark there is generally regarded as a rare occurrence, especially in the

northern Gulf of Mexico. As summarized by Castro (2011), basking sharks likely migrate across ocean basins as they track seasonal plankton blooms. Shortfin mako sharks are open ocean apex predators, which are seldom observed near land other than oceanic islands. They eat other sharks, swordfish (*Xiphias gladius* Linnaeus, 1758), and bluefish (*Pomatomus saltatrix* [Linnaeus, 1766]) (see Castro, 2011) and move great distances, with great speed, in the northwestern Atlantic Ocean. For example, Casey and Kohler (1992) recorded a tagged shortfin mako shark that traveled an average of 107 km/day. Some information is known about the movements of shortfin mako sharks in the Gulf of Mexico (Castro, 2011). Their occurrence seemingly coincides with that of yellowfin tunas and blackfin tunas (*Thunnus atlanticus* [Lesson, 1831]) during mid-October until March, which are also the periods of coolest water temperatures. These sharks comprise the only known hosts for species of Hyperandrotrema, but we predict that other epipelagic lamniforms like the great white shark, *Carcharodon carcharias* (Linnaeus, 1758), (Lamnidae) are likely susceptible to infections. At least we are particularly excited about the prospect of discovering such an infection. Additional examinations of basking sharks, shortfin mako sharks, and other epipelagic sharks are certainly needed to elucidate the biodiversity and geographic distributions of lamniform blood flukes. However, as previously mentioned, basking sharks and shortfin mako sharks indeed are sympatric in the Gulf of Mexico, and perhaps their blood flukes are as well.

Our results demonstrate that epipelagic, phylogenetically related (Lamniformes) definitive fish hosts are infected by morphologically similar (congeneric) fish blood flukes, perhaps suggestive of parasite-host co-evolution in the epipelagic zone. That

epipelagic fishes with markedly different feeding ecologies, i.e., filter feeding in basking shark and piscivory in shortfin mako shark, are infected by congeneric aporocotylids is not surprising given that no evidence exists to support the notion that any aporocotylid life cycle is mediated directly by trophic interactions among its hosts (Køie, 1982; Cribb et al., 2011).

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FIGURE LEGENDS

Figures 1–2. *Hyperandrotrema cetorhini* Maillard and Ktari, 1978 (Digenea: Aporocotylidae) from the heart of the basking shark, *Cetorhinus maximus* (Gunnerus, 1765) (Lamniformes: Cetorhinidae). Paratype, the smaller of the 2 specimens on slide 68-PE-TJ-20. Scale values aside each bar. **(1)** Body of adult showing the mouth (m), musculo-glandular area (mga) surrounding esophagus, ventrolateral nerve commissure (vnc), posterior esophageal swelling (pes), esophageal gland, (eg), cecal bifurcation (cb), testis (t), vasa efferentia (ve), ceca (c), ovary (o), gut looping (l) over terminal genitalia, uterus (ut), vitelline follicles (v) near termination of vitellarium, common genital pore (cgp), oviducal ampulla (oa), ootype (oo), cecal termination (ct), and excretory bladder (eb) and pore. Dorsal view. **(2)** Ventrolateral tegumental body spines and their associated tegumental peduncles distributing in anterior portion of body (*) at level of cecal bifurcation.

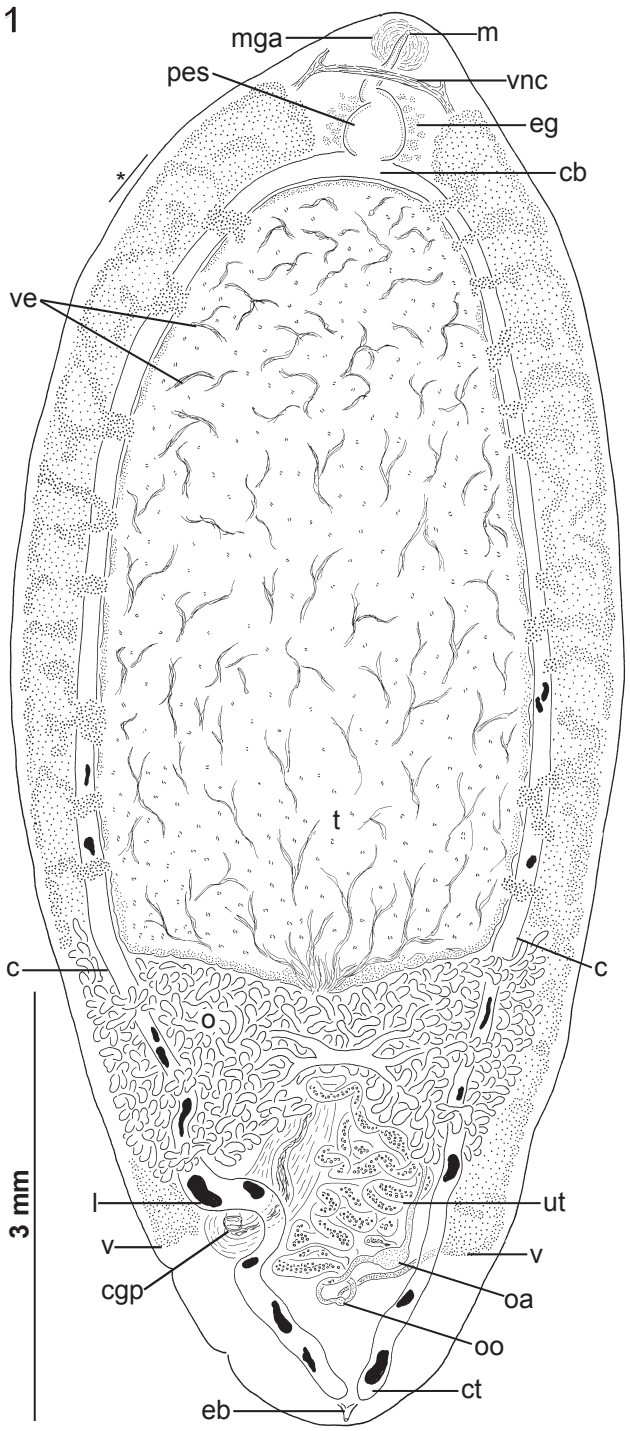
Figures 3–4. *Hyperandrotrema cetorhini* Maillard and Ktari, 1978 (Digenea: Aporocotylidae) from the heart of the basking shark, *Cetorhinus maximus* (Gunnerus, 1765) (Lamniformes: Cetorhinidae). Scale values aside each bar. **(3)** Genitalia of holotype (68PE-TJ-19) showing location of vas deferens (vd), cirrus sac (cs), internal seminal vesicle (isv), inverted cirrus (ic), gut looping (l) over terminal genitalia, portion of cirrus sac, gland cells (gc) of male terminal genitalia, male genital opening (mgo) into common atrium and pore (*), proximal cord-like portion of ovarian lobes (ol), origin of oviduct (ovo), oviduct (ov), Laurer's canal (Lc), oviducal ampulla (oa), common vitelline duct (cvd), ootype (oo), Mehlis gland (mg), uterus (u), metraterm (met), and female genital opening (fgo) into common atrium. Dorsal view. **(4)** Genitalia of paratype (68-PE-TJ-20, see Fig. 1) showing comparable features as Fig. 3.

Figures 5–6. *Hyperandrotrema walterboegeri* Orélis-Ribeiro and Bullard n. sp. (Digenea: Aporocotylidae) from the heart of the shortfin mako shark, *Isurus oxyrinchus* Rafinesque, 1810 (Lamniformes: Lamnidae). Scale values aside each bar. **(5)** Body of holotype (USNPC No. 107005) as anterior (left) and posterior (right) portions showing mouth (m), anterior esophageal swelling (aes), ventrolateral nerve cord (vnc), posterior esophageal swelling (pes), esophageal gland (eg), cecal bifurcation (cb), vasa efferentia (ve), vitellarium (v), vas deferens (vd), proximal cord-like portion of ovarian lobes (ol), uterus (ut), common genital pore (cgp), gut looping (l) over terminal genitalia, oviducal ampulla (oa), ootype (oo), cecal termination (ct), and excretory bladder (eb). Ventral view. **(6)** Ventrolateral tegumental body spines of holotype (USNPC No. 107005) and their associated tegumental peduncles distributing in anterior portion of body (*) at level of cecal bifurcation. Note that these spines are illustrated at the same scales as those of the type species *Hyperandrotrema cetorhini* (cf. Fig. 2).

Figures 7–8. *Hyperandrotrema walterboegeri* Orélis-Ribeiro and Bullard n. sp. (Digenea: Aporocotylidae) from the heart of the shortfin mako shark, *Isurus oxyrinchus* Rafinesque, 1810 (Lamniformes: Lamnidae). Scale value aside bar, both same scale. **(7)** Genitalia of holotype (USNPC No. 107005) showing location of vas deferens (vd), ovarian lobes (ol), origin of oviduct (ovo), cirrus sac (cs), internal seminal vesicle (isv),

metraterm (met), female genital opening (fgo) into common atrium, male genital opening (mgo) into common atrium and pore (*), vitelliarium (v), inverted cirrus (ic), gut looping (l) over terminal genitalia portion of cirrus sac, ootype (oo), Mehlis gland (mg), common vitelline duct (cvd), oviducal ampulla (oa), Laurer's canal (Lc), oviduct (ov), and uterus (u). Ventral view. **(8)** Genitalia of paratype (USNPC No. 107006) showing comparable features as in Fig. 7 as well as everted cirrus (ec). Dorsal view.

Figures 9–20. *Hyperandrotrema walterboegeri* Orélis-Ribeiro and Bullard n. sp. (Digenea:Aporocotylidae) from the heart of the short fin mako shark, *Isurus oxyrinchus* Rafinesque, 1810 (Lamniformes: Lamnidae). Scanning electron micrographs. Scale value aside each bar. (9) Anterodextral portion of body showing crimped body margin and field of lateral tegumental spines (sf) and its associated muscular peduncles, ventral body surface (*), and location of mouth (arrow). Ventral view. (10) Apex of anterior body end showing tegument (*) immediately dorsal to mouth. This region lacks spines, honeycomb-like tegumental ridges, or any discernible form of sucker demarcated from the body proper. Dorsal view. (11) Dorsal body surface showing honeycomb-like system of ridges that covers most of the body surface. Dorsal view. (12) Ventral body surface showing lack of honeycomb-like ridges (cf. dorsal body surface, Fig. 11, at same scale). The flecks of material on this surface are likely host material. Ventral view. (13) Field of lateral tegumental spine rows approximately at level of cecal bifurcation. Ventral view. (14) Dorsal body surface showing rippled lateral body margins. The lateral most spines of the ventrolateral spine field are evident (arrows). Dorsal view. (15) Ventrolateral field of tegumental spines (spines strongly retracted) near mouth showing muscular peduncles supporting spines. Note that the lateral most peduncles (*) are spaced such that the spines medial to them are nestled between them; giving the distribution of spines within the field an alternating ‘‘offset’’ appearance. Distal tips (arrows) of retracted spines are exposed despite most of spine shaft being obscured by neighboring peduncles. Ventral view. (16) Ventrolateral tegumental spines approximately at level of cecal bifurcation showing C-shaped spine shaft (s), laterally expanded base of spine (*), and muscular peduncle (p). Ventral view. (17) Opening of common genital atrium (arrow) and Laurer's canal (*). Note that opening of genital atrium has a tegumental rim. Body margin at top of micrograph. Dorsal view. (18) Everted cirrus (ec) and probable female genital pore opening at base of everted cirrus. Dorsal view. (19) Higher magnification view of everted cirrus showing fine detail of surface striations. Dorsal view. (20) Higher magnification view of opening of Laurer's canal (Fig. 17) and the well-developed tegumental rim that encircles the pore. Dorsal view.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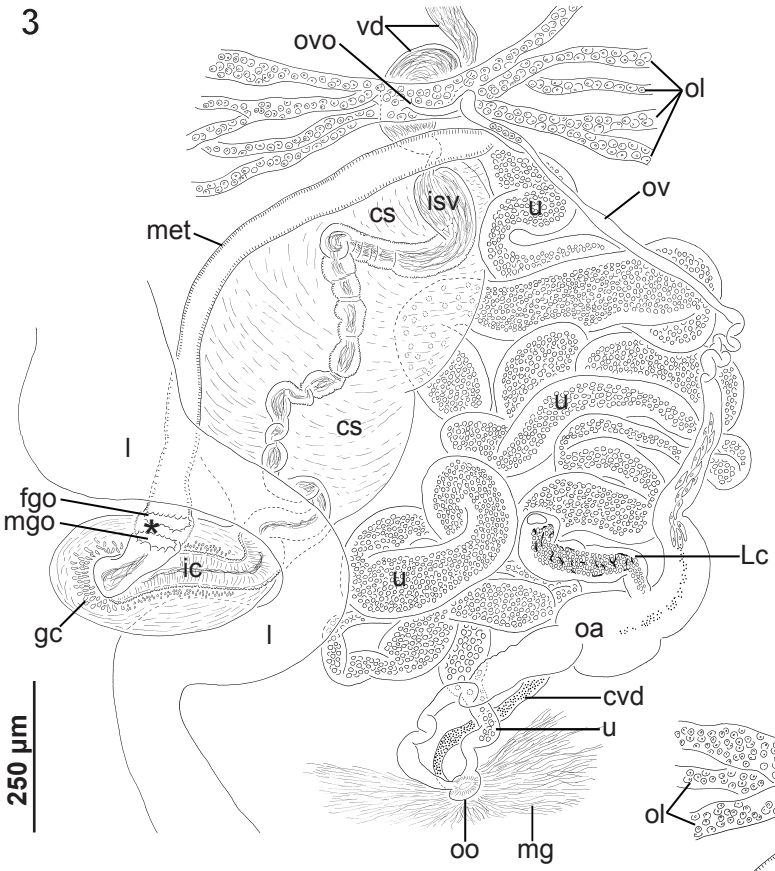
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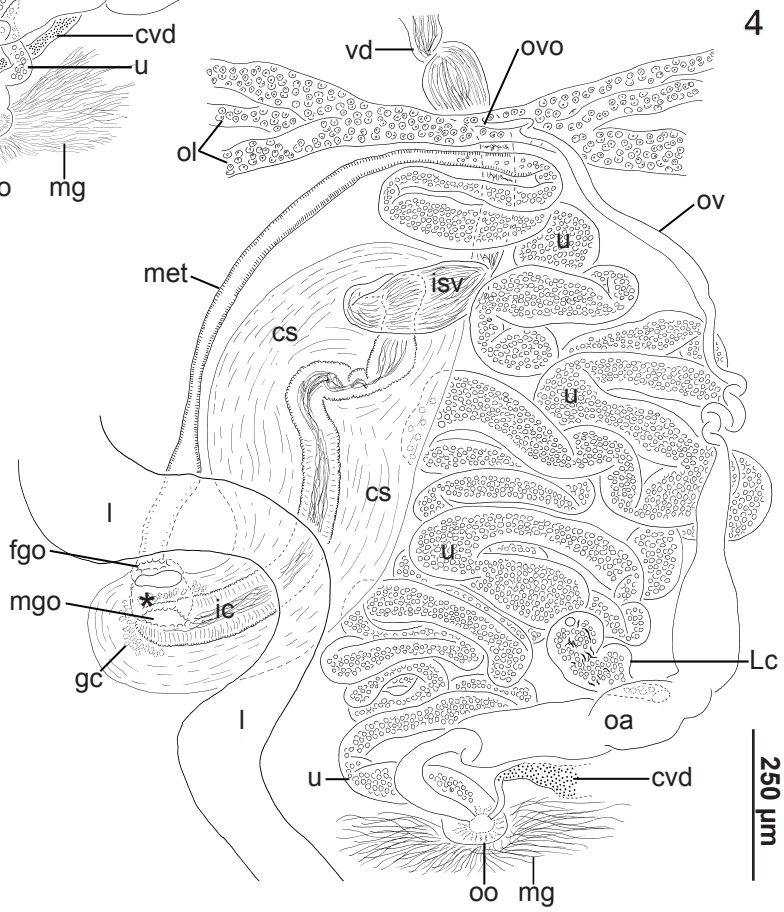
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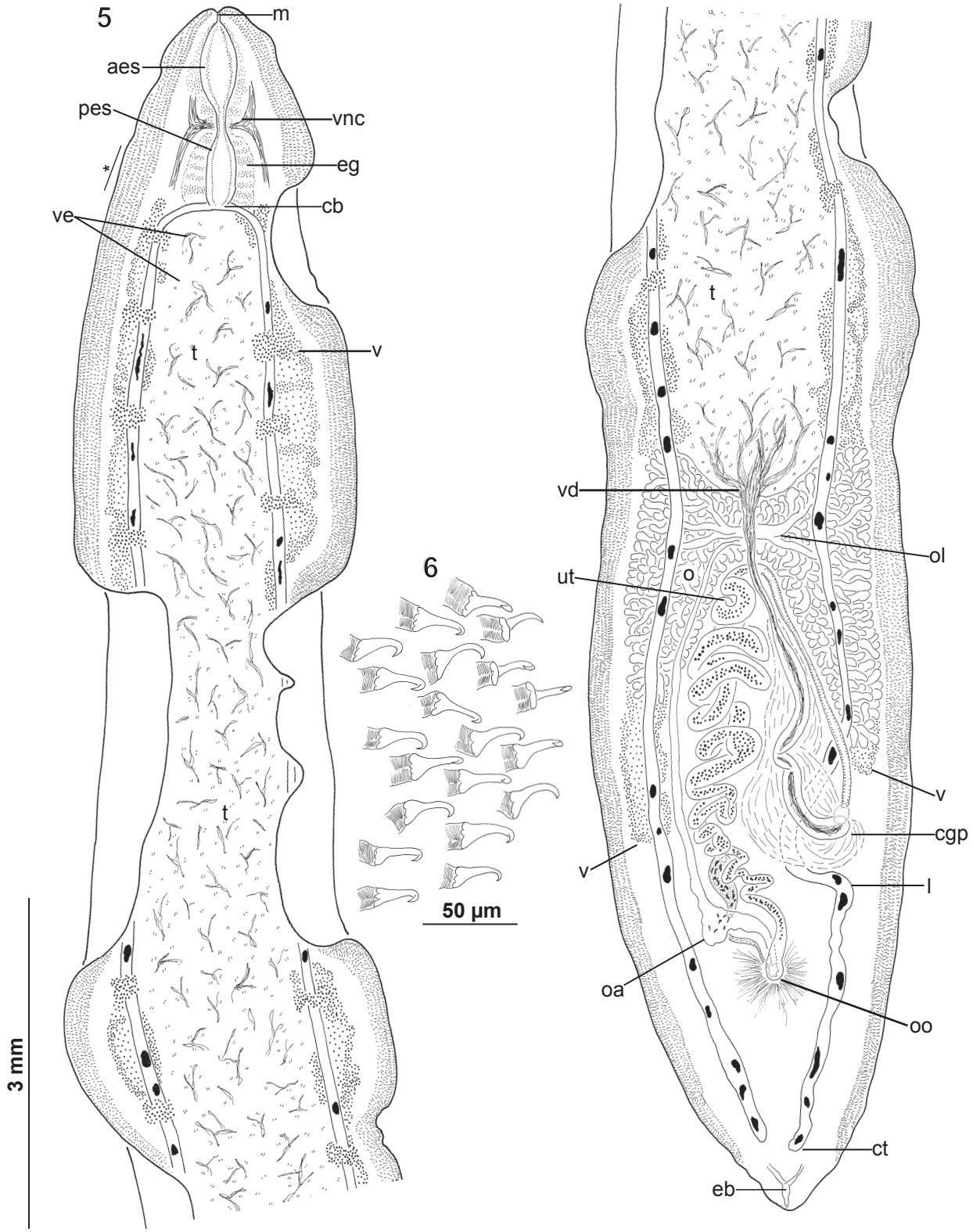
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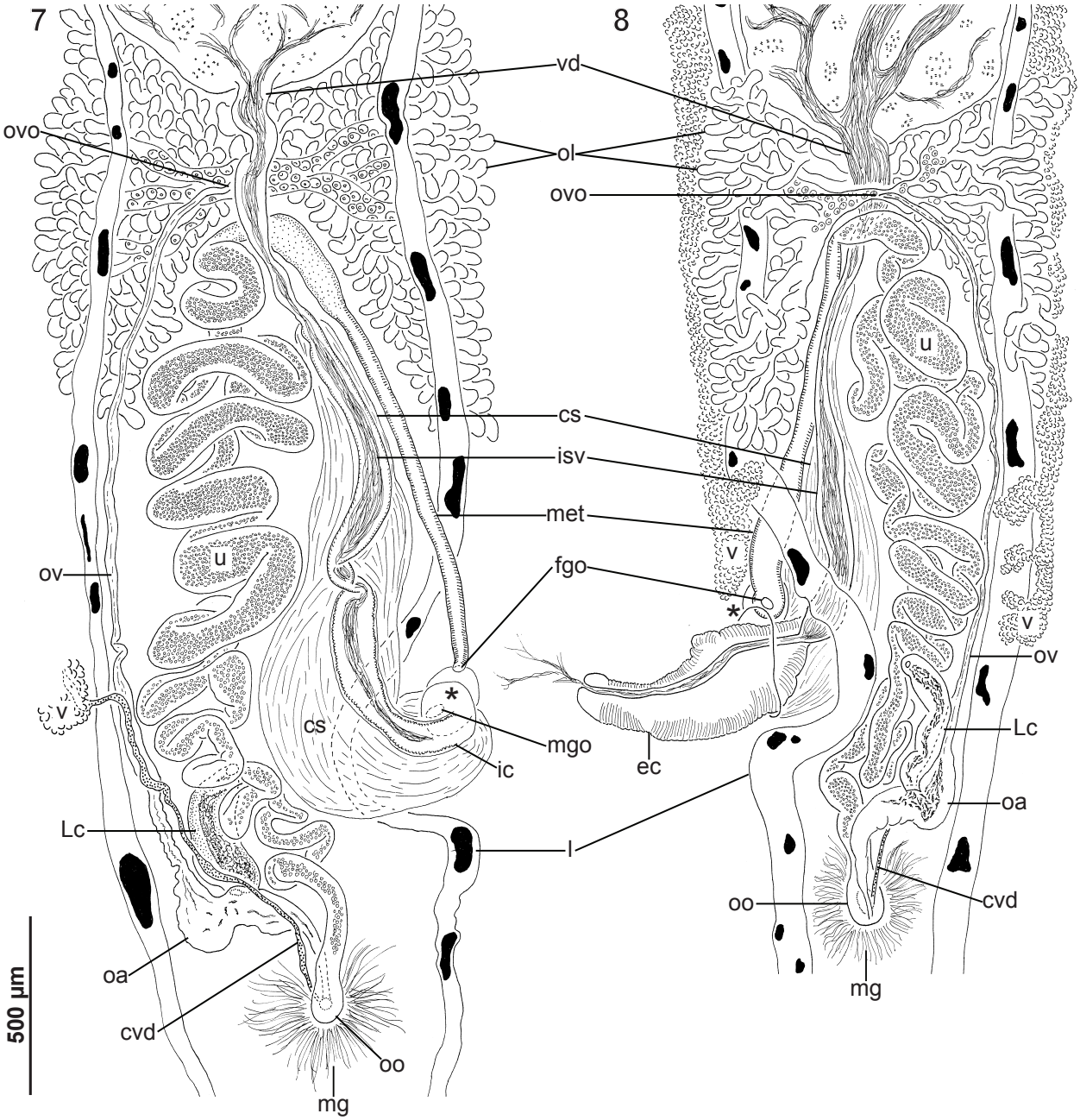
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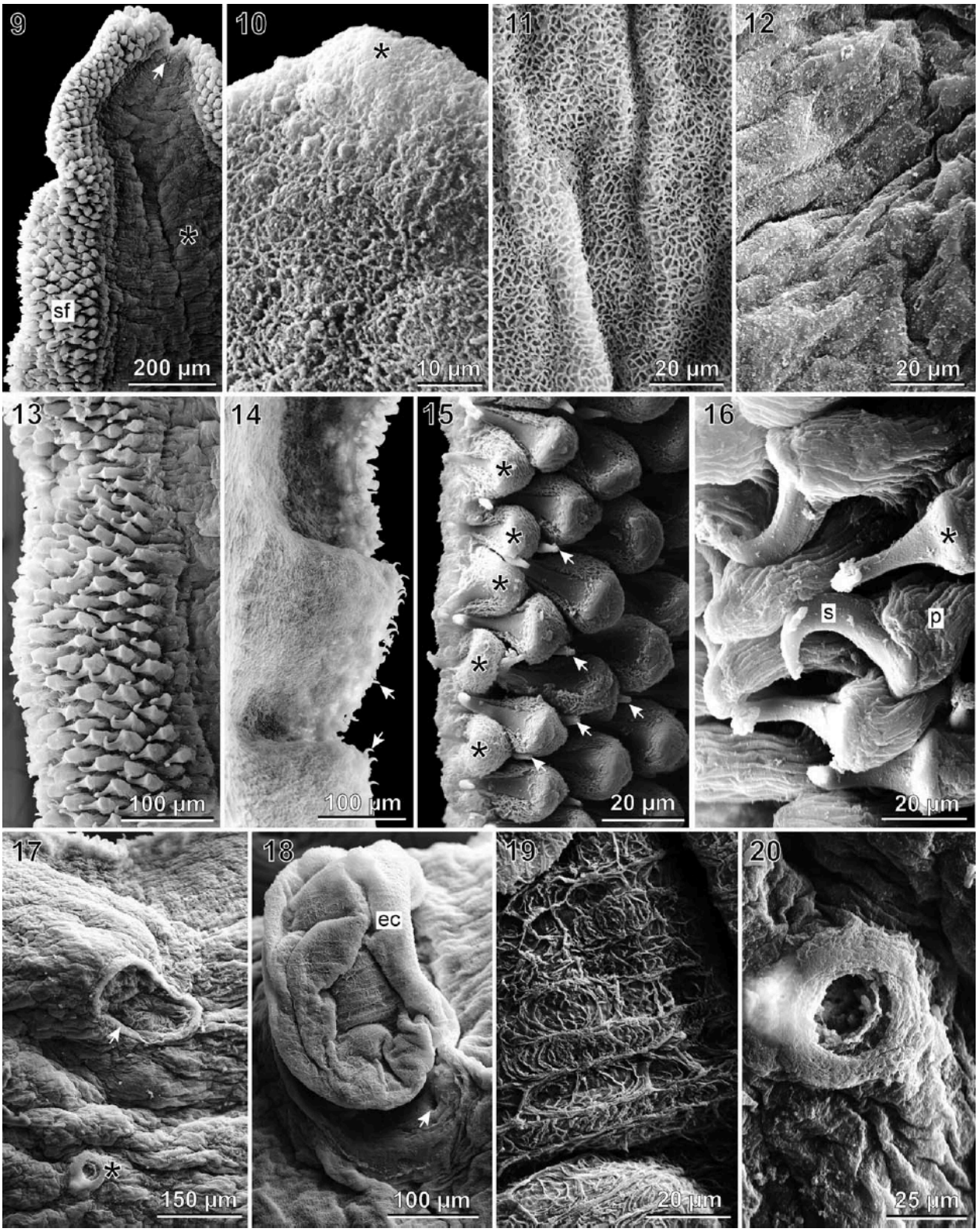
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Table 1. Fish blood flukes (Digenea: Aporocotylidae) reported from chondrichthyans.*

Aporocotylid	Host	Site	Geographic locality	Reference
Selachohemecus olsoni Short, 1954	*Rhizoprionodon terranovae* Richardson, 1836, Atlantic sharpnose shark	heart	Gulf of Mexico, Alligator Harbor; Gulf of Mexico, Apalachicola Bay; Gulf of Mexico, Mississippi Sound	Short, 1954; Bullard et al., 2006; Bullard et al., 2006
Selachohemecus benzi Bullard, Overstreet, and Carlson, 2006	*Carcharhinus limbatus* Müller & Henle, 1839, blacktip shark	heart, kidney	Gulf of Mexico, Apalachicola Bay; Gulf of Mexico, Mississippi Sound; Gulf of Mexico, Tampa Bay	Bullard et al., 2006; Bullard et al., 2006; Bullard et al., 2006
Chimaerohemecus trondheimensis Van der Land, 1967	*Chimaera monstrosa* Linnaeus, 1758, rabbit fish	dorsal aorta	Trondheim Fjord (Norway)	Van der Land, 1967
	Hydrolagus mitsukurii Jordan & Snyder, 1904, spookfish	dorsal aorta, kidney	Suruga Bay (Japan)	Kamegai et al., 2002
Chimaerohemecus sp.	*Hydrolagus affinis* Brito Capello, 1868, smalleyed rabbitfish	heart	North Atlantic Ocean (Greenland)	Karlsbakk et al., 2002
Orchispirium heterovitellatum Madhavi & Rao, 1970	*Himantura imbricata* Bloch & Schneider, 1801, scaly whipray	mesenteric vessels	Bay of Bengal (India)	Madhavi & Rao, 1970
Hyperandrotrema cetorhini Maillard and Ktari, 1978	*Cetorhinus maximus* Gunnerus, 1765, basking shark	heart	Mediterranean Sea (Tunisia); Oslo Fjord, (Norway); North Sea (Scotland)	Maillard & Ktari, 1978 ; Smith, 1972; Smith, 1972
Hyperandrotrema walterboegeri. n. sp.	*Isurus oxyrinchus* Rafinesque, 1810, shortfin mako shark	heart	Gulf of Mexico, Viosca Knoll	Present study
Myliobaticola richardheardi Bullard and Jensen, 2008	*Dasyatis sabina* Lesueur, 1824, Atlantic stingray	heart	Gulf of Mexico, Mississippi Sound	Bullard & Jensen, 2008

* Bazikalova (1932) reported ‘‘Aporocotylidae () gen. sp.’’ from the intestinal lumen of a thorny skate, Amblyraja radiata (Donovan, 1808), and this record is discussed in Smith (1972) as well as Bullard and Jensen (2008).

CHAPTER 3: BLOOD FLUKES (DIGENEA: APOROCOTYLIDAE) INFECTING BODY CAVITY OF SOUTH AMERICAN CATFISHES (SILURIFORMES: PIMELODIDAE): TWO NEW SPECIES FROM RIVERS IN BOLIVIA, GUYANA, AND PERU WITH A RE-ASSESSMENT OF *PLEHNIELLA* SZIDAT, 1951

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ABSTRACT

Plehniella Szidat, 1951 is emended based on new collections from South American long-whiskered catfishes. It is clearly differentiated from *Sanguinicola* Plehn, 1905 by lacking lateral tegumental body spines and by having 6 asymmetrical caeca. *Plehniella sabajperezi* sp. n. infects body cavity of *Pimelodus albofasciatus* (Mees) from the Demerara and Rupununi Rivers (Guyana) and *Pimelodus blochii* (Valenciennes) from Lake Tumi Chucua (Bolivia) and Napo River (Peru). It differs from *Plehniella coelomicola* Szidat, 1951 (type species) by having a thin-walled vas deferens that greatly exceeds the length of cirrus-sac and that joins the cirrus-sac at level of ovovitelline duct and ootype, an internal seminal vesicle that is absent or diminutive, and a cirrus-sac that is spheroid, nearly marginal, and envelops the laterally-directed distal portion of the male genitalia. *Plehniella armbrusteri* sp. n. infects body cavity of *P. blochii* from Lake Tumi Chucua (Bolivia). It differs from *P. coelomicola* and *P. sabajperezi* by having a relatively ovoid body, a massive intestine comprising caeca that are deeply-lobed to diverticulate and terminate in the posterior half of the body, a testis that flanks the distal tips of the posteriorly-directed caeca, and a proximal portion of the vas deferens that loops ventral to the testis. Small adults

(Plehniella sp.) collected from body cavity of *Pimelodus grosskopfii* (Steindachner) from Cienega de Jobo and Canal del Dique (Colombia) differ from congeners by having a posteriorly-constricted body region, an anterior sucker with concentric rows of minute spines, an elongate anterior oesophageal swelling, short and wide caeca, and a male genital pore that opens proportionally more anteriad. This study nearly doubles the number of aporocotylids documented from South America Rivers and comprises the first record of a fish blood fluke from *P. blochii, P. albofasciatus* and *P. grosskopfii* as well as from Bolivia, Colombia, Guyana or Peru.

INTRODUCTION

A dearth of information exists regarding the taxonomic diversity, hosts and geographic distributions of 'fish blood flukes' (Digenea: Aporocotylidae) in South American rivers. This significant portion of the Neotropical region arguably harbours the most diverse freshwater ichthyofauna on the Earth, estimated to exceed 4000 species (Reis 2013). However, only three of 136 (2%) aporocotylid species assigned to two genera reportedly infect five South American freshwater fishes from four genera: *Sanguinicola argentinensis* Szidat, 1951 infects heart and vessels leading to the gills of the streaked prochilodid, *Prochilodus platensis* (Holmberg) = *Prochilodus lineatus* (Valenciennes) (Characiformes: Prochilodontidae); *Plehniella coelomico- la* Szidat, 1951 infects the body cavity of the long-whiskered catfishes (Siluriformes: Pimelodidae) *Iheringichthys labrosus* (Lütken), *Pimelodus albicans* (Valenciennes) and *Pimelodus clarias maculatus* (Bloch) = *Pimelodus maculatus* (Lacépède); and

Plehniella platyrhynchi (Guidelli, Isaac et Pavanelli, 2002) (originally as *Sanguinicola platyrhynchi* Guidelli, Isaac et Pavanelli, 2002) infects body cavity of porthole shovelnose catfish, *Hemisorubim platyrhynchos* (Valenciennes) (Pimelodidae) (Table 1).

Pimelodidae includes 109 accepted species assigned to approximately 30 genera (Lundberg et al. 2010, Eschmeyer and Fong 2015) that range primarily in South America but also north to the central Panamanian Isthmus and into Mexico (Nelson 2006). Among catfishes as hosts, Pimelodidae has the highest number of records for aporocotylid infections (Truong and Bullard 2013) (Table 1), and it seems likely that additional species of Aporocotylidae remain unnamed in pimelodids ranging in South America. Nearly all of the known blood flukes that infect freshwater fishes mature in blood but those infecting pimelodids mature in body cavity (Szidat 1951, Truong and Bullard 2013). However, seemingly few workers search the body cavity of fishes for infections, and perhaps this unusual site of infection explains why so few species of *Plehniella* are known to date. The taxonomy of nominal aporocotylids infecting pimelodids also needs revision. As reflected in the taxonomic literature since the 1950's, *Plehniella* has largely been ignored, and no taxonomic assessment of the genus based upon a study of specimens has been published since Lunaschi (1985).

Herein, we describe two new species of *Plehniella* and emend the diagnosis of the genus. We also provide several new host and geographic locality records for *Plehniella* (Table 1), including the first records of a fish blood fluke from Bolivia, Colombia, Guyana, and Peru.

MATERIALS AND METHODS

Specimens of *P. blochii* were captured by seine and cast net from Napo River (3°29'S; 73°5'W) (Peru) and killed by spinal severance at necropsy. Living flukes intended as whole-mounts were killed with heat from an ethanol-burner flame under slight coverslip pressure and transferred to a vial of 5% neutral buffered formalin (n.b.f.). Three species of pimelodid catfish from the Auburn University Natural History Museum Fish Collection (AUM) were examined for the presence of aporocotylid infections. The number, collection date, geographic locality, and AUM catalogue numbers of examined fishes is as follows: for *P. albofasciatus*, three fish specimens collected 17–18 October 1998 from Demerara River (5.93333°N; -58.30611°W), Atlantic Ocean Basin, Guyana, AUM 27947; five fish specimens collected 15 November 2007 from Rupununi River (3.91798°N; -059.10053°W), Essequibo River Basin, Guyana, AUM 49616; for *P. blochii*, 14 specimens collected 5–10 November 1981 from Lake Tumi Chucua, tributary to Beni River (10°07'S; 66°11'W), Madeira River Basin, Bolivia, AUM 23544; and for *P. grosskopfii***,** nine specimens collected 7 September 1978 from Cienega de Jobo and Canal del Dique (10.35°S; -74.96667°W), Magdalena River Basin, Colombia, AUM 35398. These fish were formalin-fixed in the field (immersed and abdominally injected) and subsequently transferred to, and held in, 70% EtOH. The body cavity of each fish was slit by a ventrolongitudinal incision by scalpel and rinsed with distilled water over a Petri dish such that the contents could then be examined for the presence of fish blood flukes with the aid of a stereo dissecting microscope. Discovered aporocotylid specimens then were transferred to

and held in a vial of 5% n.b.f.

All resulting aporocotylid specimens intended as whole mounted specimens were removed from n.b.f., rinsed thoroughly with distilled water and cleaned with fine brushes to remove any debris, stained overnight in Van Cleave's hematoxylin with several additional drops of Ehrlich's hematoxylin, made basic at 70% ethanol with lithium carbonate and butylamine, dehydrated, cleared in clove oil, and permanently mounted on glass slides using Canada balsam. Illustrations of stained, whole-mounted specimens were made with the aid of a Leica DM-2500 equipped with differential interference contrast optical components and a drawing tube. Measurements were made using a calibrated ocular micrometer (as straight-lines along the course of the ducts) and are herein reported in micrometres (μm) as a range followed by, in parentheses, the mean and number of measurements taken. Scientific names including taxonomic authorities and dates for fishes follow Eschmeyer (2015). Common names are taken from FishBase (Froese and Pauly 2015). Higher level fish classification and nomenclature follows Nelson (2006). Nomenclature for the Aporocotylidae follows Bullard et al. (2009). Brown (1956) was used to help construct the genus name and specific epithet. Stephen S. Curran (Gulf Coast Research Laboratory, Ocean Springs, Mississippi) collected the aporocotylid specimen from a Bloch's catfish that was captured in the Napo River (Peru) on 6 August 2001.

RESULTS

Plehniella Szidat, 1951 emended (Figs. 1–16)

Diagnosis. Body elongate or oblong in outline, with slight sinistral indentation at level of genitalia or not, approximately 1.5–4.0× longer than wide, dorsoventrally flattened, ventrally concave, anterior and posterior ends tapering equally or having a broadly rounded posterior end, lacking tegumental body spines, rod-like, or bristle-like structures in adult. Ventrolateral nerve cords indistinct; dorsolateral nerve cords present. Mouth medioventral. Anterior sucker with concentric spine rows present in small adult specimens, demarcated from body by posterior constriction of tegument, an obvious proboscis with concentric spine rows. Pharynx diminutive, apparently muscular, with triradiate lumen, in anterior portion of oesophagus. Oesophagus medial, moderately sinuous, extending posteriad approximately 1/3–1/2 of body length, including anterior and posterior oesophageal swellings enveloped by glands. Intestine having glandular wall; comprising 6 caeca; caeca asymmetrical, smooth or diverticulate. Testis single, diffuse, having many laterally-directed lobes. Vasa efferentia extensive, having secondary ducts extending from lateral margins of testicular lobes and coalescing ventrally along midline and forming a single large vas deferens; vas deferens transverse, crossing midline, with proximal portion extending straight or looping ventral to testis. Cirrus-sac present. Ovary single, medial or slightly dextral, post-testicular, occupying posterior 1/3 of body, a loose aggregation of spheroid ova bound by a thin membrane, strongly diverticulate, having deep lobes with middle portion narrow and comprising dorsal and ventral cords spanning midline of body, hourglass- or butterfly-shaped in outline, dorsal to vas deferens. Vitellarium follicular, occupying space from mid-oesophagus to testis or middle of ovary. Oviduct emanating from posteromedial dorsal surface of ovary, expanding in proximal portion

to form an oviducal seminal receptacle, connecting with distal portion of vitelline duct before joining with ootype. Ootype massive, post-ceacal, post-gonadal. Laurer's canal absent. Uterus a relatively abbreviated duct connecting ootype and metraterm, straight (lacking convolutions or coils), post-caecal, post-gonadal; uterine seminal receptacle not evident; uterine egg not observed. Metraterm present, sinistral. Male and female reproductive tracts sharing common atrium and pore; dorsal, post-gonadal, sinistral, submarginal. In body cavity of South American pimelodid catfishes.

Differential diagnosis. Ventrolateral tegumental body spines absent. Anterior sucker absent in large adult specimens; small adult specimens having spinous anterior sucker with concentric spine rows. Pharynx present. Oesophagus 1/3–1/2 of body length, with anterior and posterior oesophageal swellings enveloped by oesophageal glands. Intestine comprising 6 asymmetrical and sac-like caeca. Testis single, diffuse, having many laterally-directed lobes. Vas deferens transverse, crossing midline, with proximal portion extending straight or comprising anterior loops ventral to testis. Cirrus-sac present. Ovary having deep-lobes. Ootype massive. Uterus a relatively abbreviated duct connecting ootype and metraterm, straight (lacking convolutions or coils). Common genital atrium and pore present.

Type species: *Plehniella coelomicola* Szidat, 1951

Remarks. Szidat (1951) proposed *Plehniella* for *P. coelomicola,* specimens of which he excised from the body cavity of several formalin-fixed specimens of *Iheringichthys labrosus* (Lütken) originally collected from the filters of a water treatment plant in Buenos Aires. He also reported immature specimens of *Plehniella* sp. from the body cavity of similarly-sourced specimens of *Pimelodus maculatus*

(Lacépède) (as *P. clarias* [Bloch]). He posited that these small adult specimens infecting *P. maculatus* could have represented a species distinct from *P. coelomicola* that simply was not adapted for infecting this host and, as a result, did not develop further.

Regarding the nomenclature of the type species, some publications have reported the specific epithet as "*coelomica*" (Avendaño de Mac Intosh and Ostrowski de Núñez 1998, Brasil-Sato and Pavanelli 2004, Takemoto et al. 2009, Brasil-Sato 2003) rather than "*coelomicola*" as per Szidat (1951). We suspect that after Yamaguti (1958) re-assigned *P. coelomicola* to *Sanguinicola*, it was considered necessary to have agreement in gender between the specific-group name and the generic name with which it is combined. However, articles 31.2.1 and 34.2.1 of the ICZN state that "*if the species-group name is a noun*," its ending does not need to agree in gender with the generic name with which it is combined. Hence, we accept only *P. coelomicola* and consider "*Plehniella coelomica*" (op. cit.) an invalid nomenclatural act.

Szidat (1951) and Lunaschi (1985) diagnosed *Plehniella*, and the character states detailed in each diagnosis are worthy of comparison with those of the emended diagnosis herein. Szidat's (1951) original diagnosis was relatively non-restrictive but included the presence of 6 caeca, which is a key feature that differentiates the genus from all other aporocotylid genera. An additional feature that readily differentiates adult specimens of *Plehniella* from those of *Sanguinicola* and many other aporocotylid genera is the absence of lateral tegumental body spines. Szidat's (1951) diagnosis (translated from German) stated, "*Aporocotylidae with wide oval, leaf-shaped body that dwells in body cavity of their hosts (so far freshwater fishes of South America).*

Inner organization of gut apparatus and of reproductive glands resembles Sanguinicola *Plehn, 1905; however, the cirrus-sac is transformed into a large half-circle shaped organ because of the strongly developed prostatic part. Uterus short, containing one egg. Always six gut diverticula.*"

Among the accepted genera of Aporocotylidae, *Plehniella* is probably most similar to "*Sanguinicola*"; however, the intestine of *Plehniella* spp. is markedly distinct from that of *Sanguinicola armata* Plehn, 1905 (type species) in that it has 6 caeca (present study, Szidat 1951) rather than 4 (or 5) as in *Sanguinicola* (Plehn 1905, Ejsmont 1926). Similarly, the genitalia typical of species in these genera are markedly distinct: in *Plehniella* the vas deferens is strongly sinuous and runs transverse to the midline of body, the terminal male genitalia includes a pronounced cirrus-sac, and the female genitalia includes an ootype and metraterm that together form an arch at level of the cirrus-sac. In *S. armata*, the vas deferens extends approximately directly posteriad, the cirrus-sac is indeterminate but diminutive if present, and the uterus is truncated, comprising a short duct that extends anteriad from the ootype (Plehn 1905, Ejsmont 1926). We were unable to confirm an egg in any of our specimens, but it is noteworthy that Szidat's mention of "*uterus containing one egg*" is similar to that reported by Ejsmont (1926) for *S. armata*.

Further, *Sanguinicola* spp. seemingly have a lanceolate body with tegumental spines arranged in a single lateral column, testicular lobes perpendicular to the midline, and an ovary margin that has a deep median notch anteriorly and posteriorly (giving the ovary an outline resembling an hourglass or butterfly wings). Plehn's (1905) original descriptions of *Sanguinicola armata* Plehn, 1905 (type species) and

Sanguinicola inermis Plehn, 1905 are depicted as having 4 or 5 caeca, respectively, but that latter configuration needs confirmation. We have not seen it in any specimen of any species of *Sanguinicola* (personal observations SAB) and no description of any species of *Sanguinicola* since Plehn (1905) has included a 5th caecum. Finally, although spine shape and distribution need confirmation for several species of *Sanguinicola*, we regard the genus as having a single column of lateral tegumental body spines (as exhibited by the type species of the genus); whereas, large adult specimens of *Plehniella* spp. lack tegumental spines altogether.

Expanding on those features of *Plehniella* 34 years later, Lunaschi's (1985) diagnosis (translated from Spanish) stated, *"Body leaf-shaped; cuticle smooth* [lacking spines]*. Mouth subterminal with tri-radiate lumen, internally marked by three structures or muscular processes; pharynx absent; oesophagus long; intestine comprising six short diverticula* [caeca] *that reach equatorial region of body. Common genital pore dorsal, subterminal and submedian. Cirrus-sac not defined. Testis diffuse, medial, in posterior part of middle third of body, between intestinal diverticula and ovary; prostatic region large. Ovary with a strong central constriction, forming two large symmetrical lobes, each lobe multilobed, oviduct large; ootype ample; uterus routing in parallel to the prostatic duct, opening into a small genital atrium. Vitelline glands comprising very small follicles distributing from posterior part of oesophagus to ovary; common vitelline duct passing parallel to oviduct to which it is united in its entrance to ootype. Parasites of coelomic cavity of freshwater fishes."*

We confirmed the presence of nearly all of these features in our specimens and concur that some reliably circumscribe *Plehniella*. However, in disagreement with

Lunaschi, we regard species of *Plehniella* as having a pharynx, an intestine that extends posteriad and that may or may not reach the midbody region, a prominent cirrus-sac, and an ovary with asymmetrical lobes (see emended diagnosis above).

Plehniella spp. differ from the chondrichthyan blood flukes by lacking robust, C-shaped lateral tegumental body spines and a Laurer's canal. Features associated with the anterior sucker, lateral tegumental spines, and genitalia differentiate *Plehniella* from the freshwater fish blood flukes assigned to *Acipensericola* Bullard, Snyder, Jensen et Overstreet, 2008 and *Nomasanguinicola* Truong et Bullard, 2013. *Acipensericola* differs from *Plehniella* by having a bowl-shaped anterior sucker, robust peg-like lateral tegumental spines, a column of testes, and a Laurer's canal; among other diagnostic features (Bullard et al. 2008). *Nomasanguinicola* differs from *Plehniella* mostly notably by the presence of an anterior sucker with denticles directing posteroventrally, forming a column per each side of mouth (Truong and Bullard 2013).

We are baffled by Yamaguti's (1958) decision to synonymize *Plehniella* with *Sanguinicola* and by the acceptance of this decision by subsequent authorities working with this group (Smith 2002, Nolan and Cribb 2005), especially considering the aforementioned, well-documented morphological and ecological differences between species of these genera. Regarding the obvious problems with Yamaguti's (1958) diagnosis of *Sanguinicola*, as written it does not accommodate *Plehniella* as diagnosed and clearly-illustrated by Szidat (1951). In specific, several features listed by Yamaguti (1958) as diagnostic for *Sanguinicola* are, beyond all doubt, lacking in *Plehniella*: "*body lanceolate*," "*with fine marginal striations or denticulations*," "*intestine X-shaped, occasionally divided into 5 branches*," "*testes in two rows in median field*

between ovary and intestine," "*uterus very short, containing only one egg, opening beside or anterior to male pore*," and "*parasitic in circulatory system of freshwater fishes*."

Szidat (1951) reported that the genital pores in *P. coelomicola* were separate; whereas, Lunaschi (1985) interpreted them as opening at a common pore. However, Szidat (1951) misinterpreted the shape and position of the uterus, which likely ultimately led to a misinterpretation of the genital opening. Szidat (1951) illustrated the uterus as a short inverted J-shaped feature connecting with a structure he labelled as the female genital pore. However, in our specimens the uterus arcs sinistrad before connecting to a metraterm. Szidat (1951) also likely misinterpreted the metraterm as an excretory bladder because he stated, "*Of the excretory system one can only see the stem of the slightly sinuous collecting vessel or bladder which opens posterior to the male genital opening*;" clearly describing the feature labeled as 'Ex.' ('Exkretionsblase') in his figures 4 and 5.

Plehniella platyrhynchi, the other aporocotylid that has 6 caeca and infects the body cavity of a pimelodid catfish in South America (i.e., *H. platyrhynchos*), which was provisionally reassigned to *Plehniella* from *Sanguinicola* (Truong and Bullard 2013), appears to have adjacent but separate genital pores, among other diagnostic features, and its taxonomic status will be treated in greater detail elsewhere.

***Plehniella sabajperezi* sp. n.** Figs. 1–5

ZooBank number for species: **urn:lsid:zoobank.org:act:03FF9D2A-11E3-4E8D-BD8B-C9474EBB22B1**

Diagnosis (based on light microscopy of 10 whole-mounted large adult

specimens): Body of adult 651–1352 (918; 9) long, 210–445 (294; 9) wide or 2.6–3.5 (3.1; 9) × longer than wide; anterior body end more tapered than posterior end, notched on sinistral side at level of terminal genitalia, posterior end broadly rounded (Figs 1, 2). Ventrolateral or lateral tegumental body spines absent in large adult specimens. Ventral and dorsal sensory papillae not evident with light microscopy. Ventrolateral nerve cords indistinct, secondary branches indistinct, dorsolateral nerve cords difficult to trace for most of body length; commissure of dorsolateral nerve cord 104–202 (150; 6) or 11–24% (17%; 6) of body length from anterior body end, 78–118 (96; 5) across width of the worm or 18–56% (29%; 5) of maximum body width, 3–11 (6; 4) in diameter, perpendicular to long axis of body, coursing dorsal to posterior end of oesophageal anterior swelling (Fig. 1).

Anterior sucker not evident as a clearly delineated sucker separate from anterior region of body in large adult specimens; rows of spines on anterior end of body not evident; terminal papillae on anterior margin not present; denticles not present (Fig. 1, 2). Mouth 2–4 (3; 10) in diameter, 1% (1%; 10) of maximum body width, 7–14 (10; 10) long or 1–2% (1%; 10) of body length from anterior body end (Fig. 1, 2); pharynx immediately posterior to mouth, ovoid in outline, minute, 6–10 (8; 9) long, 6–8 (7; 10) wide, 3–4 (3; 10) thick (Fig. 2).

Oesophagus 289–452 (351; 10) long or 32–44% (38%; 9) of body length, typically (8 out 10 specimens) dilating to 7–11 (9; 8) immediately posterior to pharynx, extending sinuously posteriad for 12–35 (20; 8) or 4–9% (6%; 8) of oesophagus total length before gradually narrowing to 3–8 (5; 8) and extending 17–67 (31; 10) or 4–18% (9%; 10) of oesophagus total length before connecting with anterior oesophageal

swelling; anterior oesophageal swelling 47–87 (70; 10) long or 6–9% (8%; 10) of oesophagus total length, 12–27 (19; 10) wide or 5–8% (7%; 9) of maximum body width, with wall 4–10 (7; 10) thick, 46–88 (62; 10) or 5–8% (7%; 9) of body length from anterior body end, delineated posteriorly by marked recurving of oesophagus ventrally. Oesophagus narrowing to 7–12 (9; 10) posterior to anterior oesophageal swelling and extending sinuously posteriad 168–269 (197; 10) before connecting with posterior oesophageal swelling (Fig. 1). Posterior oesophageal swelling with elongate anterior portion and bulb-like posterior portion; anterior portion delineated anteriorly from narrow region of oesophagus by sharp bend of oesophagus; posterior portion immediately anterior to caecal ramification, 27–51 (36; 10) long or 9–12% (10; 10) of oesophagus length, 20–33 (24; 10) wide or 0.9–1.7 (1.3; 10) × maximum oesophagus width, with wall 4–7 (5; 7) thick, 215–392 (291; 10) or 29–33% (31%; 9) of body length from anterior body end, oblong, oriented diagonally (not parallel with longitudinal body axis), connecting with intestine anteromedially (Figs. 1, 3). Anterior oesophageal gland 140–162 (152; 10) long, 86–132 (100; 10) wide or 4.3–7.9 (5.3; 10) × width of anterior oesophageal swelling (Fig. 1); posterior oesophageal gland 107–247 (146) long or 34–55% (41%; 10) of oesophagus length, 37–62 (46; 10) wide or 1.5–2.8 (2; 10) × width of posterior oesophageal swelling, a loose aggregation of large gland-like cells bound by a thin and lightly-staining membrane (Figs. 1, 3).

Intestine 240–377 (310; 10) or 23–37% (34%, 9) of body length from anterior body end, with 6 clearly-differentiated caeca in all specimens examined; caeca (clockwise in ventral view from oesophagus-intestine connection) 25–65 (39; 9), 30–63 (45; 9), 30–65 (51; 9), 50–81 (65; 9), 42–80 (60; 9), 28–60 (45; 9) long or approximately 4–7%

of body length and 10–17% of oesophagus length, 15–24 (20; 9), 18–40 (28; 9), 20–47 (35; 9), 25–61 (43; 9), 26–51 (40; 9), and 20–56 (31; 9) wide or approximately 10–16% of maximum body width and 1.1–1.8 × maximum oesophagus width, smooth (lacking diverticula), wall glandular (not illustrated), containing refractive content (not illustrated); caecal field 106–210 (142; 9) long or 16–18% (16%; 8) of body length and 26–50% (41%; 9) of oesophagus length, 72–146 (116; 10) wide or 31–53% (42%, 9) of maximum body width; post-caecal distance 320–563 (439; 10) or 38–56% (48%; 9) of body length from anterior body end, 324–771 (499; 10) or 49–60% (53%; 9) of body length from posterior body end (Figs. 1, 3).

Testis 154–456 (246; 10) long or 20–35% (25%; 9) of body length, 111–248 (165; 10) wide or 41–71% (54%; 9) of body width or 1.1–1.9 (1.5; 10) × longer than wide, containing dense field of vasa efferentia intertwining among densely-packed testicular cells; testicular cells circular, each measuring 1–5 (3; 25) in diameter; post-testicular space 179–342 (281; 10) long or 25–38% (30%; 9) of body length (Fig. 1). Vasa efferentia secondary ducts 2–25 (8; 29) wide, extending from lateral margins of testicular lobes (Fig. 1). Vas deferens a thin-walled duct, including a proximal portion ventral to testis and a post-testicular portion; proximal portion robust, 11–50 (31; 10) wide, extending posteriad along midline; post-testicular portion 250–526 (407; 10) long or 35–51% (44%; 9) of body length, 14–50 (35; 10) wide at level of posterior margin of testis, extending posteriad 30–107 (72; 10) or 3–12% (8%; 9) of body length before curving and extending 27–92 (52; 9) toward dextral body margin, 9–45 (28; 8) wide at level of ovary, curving ventromedially and crossing midline before narrowing to 5–22 (14; 10) or 2–6.3% (4.6; 9) of maximum body width, continuing posteriad

approximately in parallel with sinistral body margin before curving medially and connecting to ventral aspect of cirrus-sac (Figs. 1, 4, 5).

Cirrus-sac spheroid, nearly marginal, 46–112 (74; 9) long or 4–14% (8%; 8) of body length, 47–104 (79; 9) wide or 14–44% (26%; 8) of maximum body width, 0.8–1.1 (1; 9) × longer that wide, having wall approximately 1 (1; 10) thick; internal seminal vesicle indistinct (Figs. 1, 4, 5). Inverted cirrus not observed. Everted cirrus and sperm duct laterally-directed, 36–61 (49; 2) long or 41–76% (58%; 2) of cirrus-sac length, base 42–53 (48; 2) wide, expanding to 53 (1 of 2 specimens), narrowing in the distal portion to 36–39 (38; 2), 53 (2) in maximum width or 0.5 (2) times cirrus-sac width, having minute external crenulations or ridges (not illustrated), with sperm tube coursing near ventral surface, and extending from common genital pore to beyond posterior body margin (Figs. 1, 4, 5).

Ovary having 3–7 (5; 7) dextral and 2–4 (3; 7) sinistral branches each 5–18 (8; 45) wide, dextral and sinistral halves of ovary measuring 30–123 (78; 9) and 25–90 (52; 9) in maximum length or approximately 4–14% (8; 8) and 3–8% (5%; 8) of body length, 70–207 (145; 8) in maximum width or 33–60% (46%; 7) of body width, 1–3 (2; 8) × wider than long; post-ovarian space 165–233 (202; 9) long or 17–24% (21%; 9) of body length (Figs. 1, 5). Oviduct curving sinistrally immediately posterior to ovary and lateral to vas deferens, 186–334 (255; 10) long or 24–32% (27%; 9) of body length, including an abbreviated proximal duct, a dilated portion (= oviducal seminal receptacle), and a narrow distal portion; proximal duct emanating from posteroventral surface of ovary extending sinistrally 15–37 (29; 10), 4–12 (8; 10) in maximum width, curving posteromediad to connect with oviducal seminal receptacle; oviducal seminal

receptacle filled with sperm and ova in all specimens, 97–246 (167; 10) long or 51–74% (63%; 10) of total oviduct length, 13–45 (26; 10) wide or 3–10 (6; 10) × longer than wide, occupying space between vas deferens and sinistral body margin, crossing vas deferens dorsally, post-ovarian; distal portion of oviduct 5–11 (8; 10) or 21–54% (33%, 10) of oviducal seminal receptacle width, continuing posteriad approximately in parallel with dextral body margin before uniting with vitelline duct (Figs. 1, 5). Ootype 42–107 (77; 9) long, 16–48 (33; 9) wide, 1.8–4 (2.5; 9) × longer than wide, connecting with vitelline duct and oviduct posteriorly, slightly dextral, orienting parallel to long axis of body; post-ootype distance 55–96 (69; 9) or 6–8% (7%; 8) (Figs. 1, 5).

Uterus occupying space between ootype and vas deferens, extending 31–65 (43.5; 8) or 4–6% (5%; 7) of body length, 5–14 (8; 8) in maximum width, curved or straight before connecting with metraterm (Figs. 1, 5). Metraterm 62–116 (96; 9) long or 1.5–3.2 (2.3; 8) × uterus length, 9–12% (10%; 8) of body length, 12–38 (25; 9) in maximum width, with wall 4–10 (5.8; 8) thick, connecting with common genital atrium 15–46 (27; 9) or 2–5% (3%; 9) from posterior body end (Figs. 1, 4, 5). Common genital pore 20–40 (29; 8) in diameter, opening 5–10 (7; 3) or 1–2% (1%; 3) of body length from posterior body end (Figs. 1, 4, 5). Uterine eggs not observed.

Excretory system not observed.

Type host: *Pimelodus albofasciatus* (Mees, 1974) (Siluriformes: Pimelodidae).

Other hosts: Bloch's catfish, *Pimelodus blochii* (Valenciennes, 1840).

Type locality: Demerara River (5.93333°N; -58.30611°W), Atlantic Ocean Drainage, Guyana.

Other localities: Specimens of *P. albofasciatus* captured from Rupununi River,

Kwatamang Landing (3.91798°N; -059.10053°W), Essequibo River Drainage, Guyana; Bloch's catfish captured from Lake Tumi Chucua, tributary to Beni River, Dept. Beni, Prov. Vaca Diez (10°07'S; 66°11'W), Madeira River Basin, Bolivia; and Napo River (3°29'S; 73°5'W), a tributary of the Amazon River, Peru.

Site in host: Body cavity.

Prevalence and intensity of infection: Two of 3 (66.7%) *P. albofasciatus* collected from Demerara River had 2–3 (mean intensity= 2.5 ±0.5) specimens; one of 5 (20%) *P. albofasciatus* collected from Rupununi River had 2 specimens; eight of 14 (57.1%) Bloch's catfish collected from Lake Tumi Chucua had 1–13 (mean intensity= 3.6 ±0.5) specimens.

Specimen deposited: Holotype and two paratypes at the United States National Museum (USNM Coll. No. 1283479, 1283480 and 1283481); one paratype at the Institute of Parasitology, Academy of Sciences of the Czech Republic, České Budějovice (IPCAS D-717).

Etymology: The specific epithet *'sabajperezi'* honors Dr. Mark Henry Sabaj Pérez (Department of Ichthyology, The Academy of Natural Sciences, Philadelphia) for his contributions to our knowledge of fish biodiversity in South America.

Remarks. Our specimens of *P. sabajperezi* resemble Szidat's (1951) original description of *P. coelomicola* by each having an ovoid, extremely flattened body, a minute pharynx with a triradiate lumen, an oesophagus <1/2 body length, a thick-walled, glandular anterior and posterior oesophageal swelling, an intestine comprising 6 short caeca, a testis that is extensively branched, a cirrus-sac, an ovary in the posterior 1/3 of body and having deep-lobed lateral lobes, and a prominent ootype

that is slightly dextral. Both species also lack an anterior sucker in large adult specimens as well as lateral tegumental body spines or spination about the anterior body end and mouth.

These taxa can be most easily differentiated based upon the morphology of the terminal male genitalia. In *P. coelomicola*, and as described by Szidat (1951), the vas deferens crosses the ovary medioventrally and curves dextrad before extending laterad and crossing the midline where it becomes confluent with the massive, strongly musculo-glandular internal seminal vesicle enveloped by the cirrus-sac. That is, the connection between the thin-walled vas deferens and musculo-glandular internal seminal vesicle in *P. coelomicola* is located between the level of the ovary and ootype. Szidat (1951) detailed the internal seminal vesicle as a "*narrow duct surrounded by an immense layer of gland cells which contain a very fine glandular secretion*." The internal seminal vesicle (and cirrus-sac) of *P. coelomicola* comprises over half of the length of the herein so-called 'afferent sperm duct' (comprising the vas deferens and internal seminal vesicle) and extends well anterior to the metraterm and ootype. As already stated, Szidat's (1951) figure 5 likely misinterprets the metraterm and female genital pore as an excretory duct/bladder extending posteriad along the midline, i.e., in figure 5 of Szidat (1951) the structure labeled "Ex." is the metraterm, not an excretory duct.

In contrast, the new species has a vas deferens that extends sinuously posteriad between the metraterm and sinistral dorsolateral nerve cord before connecting with the medial surface of the cirrus-sac in the posterior body extremity at level of the ovo-vitelline duct (Figs. 1, 5). We could not discern an internal seminal vesicle in our

whole-mounted specimens; however, we suspect that one may be present, although likely diminutive if indeed present, as indicated by the presence of putatively secretory or glandular cells that are sporadically distributed at level of the cirrus-sac (Fig. 4). The cirrus-sac itself of the new species is spheroid, occupies the space posterior to the metraterm, and envelops the distal extent only of the terminal male genitalia. That is, the vas deferens of the new species is thin-walled for nearly its entire length, not enveloped by a sac and not associated with a thickened glandular or muscular wall. Features associated with the wall of the duct should not be confused with the amount of sperm in the duct; which could modulate the diameter of the vas deferens and internal seminal vesicle. In addition, in the new species the cirrus everts laterad (Figs. 1, 4), not mediad (cf. Figs. 6, 9, 10 plus Szidat's [1951] figure 5), and the cirrus-sac itself is marginal in the sinistral side of the body.

In summary, the new species can be most easily differentiated from *P. coelomicola* by having a thin-walled vas deferens that greatly exceeds the length of cirrus-sac and that joins the cirrus-sac at level of ovo-vitelline duct and ootype, an internal seminal vesicle that is absent or diminutive, and a cirrus-sac that is spheroid, nearly marginal, and envelops the laterally-directed distal portion of the male genitalia only.

***Plehniella armbrusteri* sp. n.** Figs. 6–10

ZooBank number for species: **urn:lsid:zoobank.org:act:C75B9533-A380-42DC-A8EC-F6BF9870A55C**

Diagnosis (based on light microscopy of 7 whole-mounted large adult specimens): Body of adult 1078–1438 (1270; 7) long, 514–716 (602; 7) wide or 1.6–2.4 (2.1; 7) × longer than wide, with ends tapering equally (Figs. 6, 7). Ventrolateral or lateral

tegumental body spines absent in large adult specimens. Ventral and dorsal sensory papillae not evident with light microscopy. Ventrolateral nerve cords indistinct, secondary branches indistinct, dorsolateral nerve cords difficult to trace for most of length in all specimens; commissure of dorsolateral nerve cord 177–198 (190; 4) or 14–16% (15%; 4) of body length from anterior body end, 116–192 (144; 3) across width of the worm or 19–30% (23; 3) of maximum body width, 5–10 (6; 3) in diameter, perpendicular to long axis of body, coursing dorsal to posterior end of oesophageal anterior swelling.

Anterior sucker not evident as a clearly delineated sucker separate from anterior region of body in large adult specimens; rows of spines on anterior end of body not evident; terminal papillae on anterior margin not present; denticles not present (Figs. 6, 7). Mouth 8–13 (11; 7) in diameter, 1–2% (2%; 7) of maximum body width, 18–23 (20; 7) long or 1–2% (2%; 7) of body length from anterior body end (Figs. 6, 7); pharynx immediately posterior to mouth, ovoid in outline, minute, 13–15 (14; 7) wide, 5–7 (6; 7) thick (Fig. 7).

Oesophagus 456–560 (512; 7) long or 36–44% (41%; 7) of body length, typically (6 of 7 specimens) dilating to 14–23 (18; 6) immediately posterior to pharynx, extending sinuously posteriad for 18–38 (29; 6) or 3–12% (7%; 6) of oesophagus total length, gradually narrowing to 4–11 (9; 6) and dilating again to 12–24 (19; 6), extending 20–52 (38; 6) or 4–10% (7%; 6) of oesophagus total length, narrowing to 7–13 (10, 7) before connecting with anterior oesophageal swelling (Figs. 6); anterior oesophageal swelling 98–121 (110; 7) long or 8–10% (9%; 7) of oesophagus total length, 30–35 (33; 7) wide or 5–7% (6%; 7) of maximum body width, with wall 9–13

(11; 7) thick, 80–99 (90; 7) or 7–8% (7%; 7) of body length from anterior body end, delineated posteriorly by marked recurving of oesophagus ventrally. Oesophagus narrowing to 17–22 (20; 7) posterior to anterior oesophageal swelling and extending sinuously posteriad 230–328 (264; 7) before connecting posterior oesophageal swelling (Figs. 6). Posterior oesophageal swelling with elongate anterior portion and bulb-like posterior portion, anterior portion delineated anteriorly from narrow region of oesophagus by sharp bend of oesophagus; posterior portion immediately anterior to caecal ramification, 62–75 (69; 7) long or 12–15% (14%; 7) of oesophagus length, 38–56 (48; 7) wide or 1.1–1.7 (1.5; 7) × maximum oesophagus width, with wall 6–10 (8.7; 7) thick, ovoid in outline, oriented diagonally (not parallel with longitudinal body axis), connecting with intestine anteromedially (Figs. 6, 8). Anterior oesophageal gland 167–257 (209; 7) long, 143–235 (179; 7) wide or 4.8–6.7 (5.5; 7) × width of anterior oesophageal swelling; posterior oesophageal gland 225–345 (267; 7) long or 42–62% (52%; 7) of oesophagus length, 61–95 (74; 7) wide or 1.3–1.9 (1.5; 7) × width of posterior oesophageal swelling, a loose aggregation of large gland-like cells bound by a thin and lightly-staining membrane.

Intestine 394–505 (455; 5) or 32–38% (35%, 5) of body length from anterior body end, with 6 clearly-differentiated caeca in all specimens examined, caeca (clockwise in ventral view from oesophagus-intestine connection) 105–130 (114; 6), 145–240 (191; 4), 205–270 (245; 4), 225–393 (302; 4), 160–363 (252; 4), and 135–235 (171; 5) long or approximately 15–19% of body length and 33–48% of oesophagus length, 57–130 (92; 6), 92–152 (121; 4), 65–148 (103; 4), 122–287 (166; 4), 95–204 (148; 4), and 54–160 (99; 5) wide or approximately 16–25% of maximum body width and 5.7–12.6 ×

maximum oesophagus width, deep-lobed to diverticulate, wall glandular (not illustrated), containing refractive content; caecal field 305–541 (444; 5) or 28–38% (34%; 5) of body length and 67–102% (87%; 5) of oesophagus length, 243–425 (360; 4) wide or 37–70% (60%, 4) of maximum body width; post-caecal distance 660–930 (812; 5) or 61–65% (62%;5) of body length from anterior body end, 395–521 (460; 6) or 34–39% (37%; 6) of body length from posterior body end (Figs. 6, 8).

Testis 198–305 (249; 6) long or 15–25% (20%; 6) of body length, 325–445 (388; 6) wide or 56–75% (66%; 4) of body width or 0.5–0.8 (0.6; 5) × longer than wide, containing dense field of vasa efferentia intertwining among densely-packed testicular cells; testicular cells circular, each measuring 3–5 (4; 7) in diameter; anteriorly flanking the distal tips of the 3 posteriorly-directed caeca; post-testicular space 290–446 (361; 6) long or 26–39% (29%; 6) of body length (Fig. 6). Vasa efferentia secondary ducts 2–30 (9; 16) wide extending from lateral margins of testicular lobes (Fig. 6). Vas deferens a thin-walled duct, including a proximal portion ventral to testis and a post-testicular portion; proximal portion robust, 16–23 (19; 4) wide looping anteriorly before posterior end of testis. Post-testicular portion 193–245 (215; 6) long, 16–20 (18; 3) wide at level of posterior margin of testis, extending posteriad 33–111 (75; 6) or 3–9% (6%; 6) of body length before curving and extending 47–170 (99; 6) toward dextral body margin, 16–29 (22; 6) wide at level of ovary, curving ventromedially and extending 19–51 (41; 6), narrowing to 10–18 (14; 7) or 2–3% (2; 7) of maximum body width before connect with cirrus-sac and internal seminal vesicle, ventral to ovary, containing sperm in all specimens (Figs. 6, 9, 10).

Cirrus-sac C-shaped in outline, appendix-like, 403–522 (480.9; 7) long or 31.6–

42.5 (38%; 7) of body length, 32–48 (40.1; 7) in maximum width or 5–9.3% (6.8%; 7) of body width, 9.6–15.4 (12.2; 7) × longer that wide, with wall 3–4 (3.4; 7) thick, wall glandular (Figs. 6, 9, 10); internal seminal vesicle occupying breadth and length of cirrus-sac to varying degrees depending on amount of sperm present in duct, 330–446 (393; 7) long, 5–25 (12; 7) wide or 17–84 (41; 7) × longer that wide, extending toward sinistral margin before continuing posteriad approximately in parallel with sinistral body margin (Figs. 6, 9, 10); inverted cirrus 72–100 (88; 7) long or 14–22% (18%; 7) of cirrus-sac length; opening 24–29 (26; 7) or 2–3% (2%; 7) of body length from posterior body end, opening 65–145 (102; 7) or 10–28% (17%; 7) of maximum body width from sinistral body margin, opening 80–170 (120; 7) or 15–24 % (20%; 7) of maximum body width from dextral body margin; slightly posteriad to female genital pore, slightly anteriad or coinciding with common genital pore opening (Figs. 6, 9, 10).

Ovary having 3–6 (5; 5) dextral and 2–3 (2.5; 2) sinistral branches each 3–18 (7.5; 23) wide, dextral and sinistral halves of ovary measuring 85–133 (101; 6) and 158–182 (170; 2) in maximum length or approximately 7–12% (8; 6) and 2–3% (3%; 2) of body length, sinistral half of ovary difficult to trace in most of the specimens examined (5 of 7 specimens), 185 in maximum width or 28% of body width, 1.7 × wider than long; post-ovarian space 192–285 (253; 6) long or 18–22% (20%; 6) of body length (Figs. 6, 9, 10). Oviduct curving sinistrally immediately posterior to ovary and lateral to vas deferens, 368–483 (421.6; 7) long or 29–39% (33%; 7) of body length, including an abbreviated proximal duct, a dilated portion (= oviducal seminal receptacle), and a narrow distal portion; proximal duct emanating from posteroventral surface of ovary extending sinistrally 37–98 (67.4; 7), 9–13 (10; 6) in maximum width, curving

posteromediad to connect with oviducal seminal receptacle; oviducal seminal receptacle filled with sperm and ova in all specimens, 149–340 (239.1; 7) long or 35–73% (54%; 6) of total oviduct length, 25–45 (31; 7) wide or 2.8–5 (4.1; 7) × longer than wide, occupying space between vas deferens and sinistral body margin, crossing vas deferens dorsally, post-ovarian, distal portion of oviduct 8–12 (10; 7) or 27–44% (32%, 7) of oviducal seminal vesicle width, continuing posteriad approximately in parallel with dextral body margin before uniting with vitelline duct (Figs. 6, 9, 10). Ootype 75–109 (92; 7) long, 32–52 (44; 6) wide, 1.7–2.5 (2.1; 7) × longer than wide, connecting with vitelline duct and oviduct posteriorly, slightly dextral, orienting parallel to long axis of body; post-ootype distance 62–106 (74; 7) or 5–8% (6%; 7) (Figs. 6, 9, 10).

Uterus occupying space between ootype and cirrus; extending 62–90 (75; 6) or 5–7% (6%; 6) of body length, 11–14 (13; 6) in maximum width, orienting diagonally anterosinistrad before connecting with metraterm (Figs. 6, 9, 10). Metraterm 156–256 (206; 6) long or 2–4 (2.9; 4) × longer than uterus, 12–20% (16%; 6) of body length, 21–42 (30; 6) in maximum width, with wall 5–10 (6; 6) thick; connecting with common genital atrium 40–51 (44; 7) or 3–4% (4%; 7) from posterior body end (Figs. 6, 9, 10). Common genital pore 16–20 (18; 7) in diameter, opening 19–26 (22; 7) or 2% (2%; 7) of body length from posterior body end (Figs. 6, 9, 10). Uterine eggs not observed.

Excretory system not observed.

Type and only known host: Bloch’s catfish, *Pimelodus blochii* (Valenciennes, 1840) (Siluriformes: Pimelodidae).

Type locality: Lake Tumi Chucua, tributary to Beni River, 26Km, Dept. Beni, Prov. Vaca Diez (10°07S; 66°11'W), Madeira River Basin, Bolivia.

Site in host: Body cavity.

Prevalence and intensity of infection: Two of 14 (14.3%) Bloch's catfish had 1–8 (mean intensity= 4.5 ±0.5).

Specimen deposited: Holotype and one paratype at the United States National Museum (USNM Coll. No. 1283477 and 1283478); one paratype at the Institute of Parasitology, Academy of Sciences of the Czech Republic, České Budějovice (IPCAS D-716).

Etymology: The specific name *armbrusteri* honors Dr. Jonathan W. Armbruster (Department of Biological Sciences, Auburn University Museum of Natural History) for his important contributions to the taxonomy of Neotropical catfishes.

Remarks. *Plehniella armbrusteri* is most easily distinguished from its congeners by the combination of having a relatively ovoid body 1.6–2.4 times longer than wide, a massive intestine (1/3 and 2/3 of body length and width, respectively) comprising caeca that are deeply-lobed to diverticulate and that terminate in the posterior half of the body, a testis that flanks the distal tips of the posteriorly-directed caeca, and a proximal portion of the vas deferens that loops anteriad and ventral to the testis. As indicated previously, *P. coelomicola* has a smooth, non-diverticulate intestine that is proportionally smaller (1/20 and 1/7 of body length and width, respectively) and terminates in the anterior half of the body. This species differs from *P. armbrusteri* also in that the testis does not extend anteriad to flank the posterior caeca, and the vas deferens does not loop ventral to the testis. Also as indicated previously, *P. sabajperezi* differs from *P. armbrusteri* by the combination of having a relatively elongate body that is 2.6–3.5 times longer than wide and a posterior body end that is

markedly more rounded than the anterior end, a relatively short intestine that terminates in the anterior body end and lacks diverticula, an anterior portion of the testicular field that surrounds only the most posterior caecum, an anterior portion of vas deferens that extends straight towards the end of testicular field without looping, an indistinct seminal vesicle, and a spheroid cirrus-sac.

***Plehniella* sp.** Figs. 11–16

Diagnosis (based on light microscopy of 10 whole-mounted small adult specimens): Body of small adults 365–500 (426; 10) long, 104–150 (135; 7) wide or 2.7–3.6 (3.2; 10) × longer than wide, markedly recessed 42–87 (64; 10) or 10–19% (15%; 10) of body length from posterior end of body, ends tapering equally (Figs. 11). Ventrolateral or lateral tegumental body spines absent in small adult specimens. Ventral and dorsal sensory papillae not evident with light microscopy. Ventrolateral nerve cords indistinct, secondary branches indistinct, dorsolateral nerve cords difficult to trace for most of body length; commissure of dorsolateral nerve cord difficult to trace for most specimens.

Anterior sucker base 35–42 (39; 10) width along constriction of tegument or 26–36% (29%; 10) of body width, 21–26 (23; 10) long or 1.5–1.9 (1.7; 10) × wider than long; about 3 concentric rows of < 1 long spines associated with anterior end, spines barely discernible with light microscope; terminal papillae on anterior margin not present; denticles not evident (Figs. 11, 12, 13). Mouth 3 (10) in diameter, 2–3% (2%; 10) of maximum body width, 4–15 (9; 10) or 1–3% (2%; 10) of body length from anterior body end (Figs. 11, 12, 13); pharynx immediately posterior to mouth, ovoid in outline, minute, 5–8 (6.3; 9), 4–7 (6; 10) wide, 2–3 (3; 10) thick (Figs. 11, 12, 13).

Oesophagus 167–228 (197; 10) long or 41–53% (46%; 10) of body length, beginning as a narrow tube 2–6 (3.4; 10), extending slightly straight 23–39 (30; 10) or 12–18% (15%; 10) of oesophagus total length before connecting with anterior oesophageal swelling (Figs. 11); anterior oesophageal swelling 29–39 (34; 10) long or 14–20% (17%; 10) of oesophagus total length, 9–12 (10; 10) wide or 7–10% (8%; 10) of maximum body width, with wall 4–5 (4; 10) thick, 33–47 (41; 10) or 9–12% (10%; 10) of body length from anterior body end, delineated posteriorly by marked recurving of oesophagus ventrally. Oesophagus narrowing to 3–6 (4.6; 10) posterior to anterior oesophageal swelling and extending sinuously posteriad 91–131 (115.3; 10) before connecting with posterior oesophageal swelling. Posterior oesophageal swelling with elongate anterior portion and a bulb-like posterior portion, anterior portion delineated anteriorly from narrow region of oesophagus by sharp bend of oesophagus; posterior portion immediately anterior to caecal ramification, 3–26 (18; 10) long or 3–5% (4; 10) of oesophagus length, 8–12 (10; 10) wide or 0.7–1.2 (1; 10) × maximum oesophagus width, with wall 1–2 (2; 10) thick, ovoid in outline, oriented diagonally (not parallel with longitudinal body axis), connecting with intestine anteromedially (Figs. 11, 14). Anterior oesophageal gland 45–53 (48; 3) long, 36–67 (55; 9) wide or 3.6–6.7 (5.3; 9) × width of anterior oesophageal swelling; posterior oesophageal gland 65–109 (90; 10) long or 37–55% (46%; 10) of oesophagus length, 18–32 (24; 10) wide or 1.6–4 (2.4; 10) × width of posterior oesophageal swelling, a loose aggregation of large gland-like cells bound by a thin and lightly-staining membrane as in *P. sabajperezi* and *P. armbrusteri*.

Intestine 149–201 (174; 9) or 38–47% (41%, 4) of body length from anterior body

end; with 6 clearly-differentiated caeca in all specimens examined, caeca (clockwise in ventral view from oesophagus-intestine connection) 32–41 (38; 10), 36–56 (45; 10), 40–62 (48; 10), 35–63 (49; 10), 38–63 (48; 10), and 33–56 (44; 10) long or approximately 9–12% of body length and 16–26% of oesophagus length, 25–33 (30; 10), 24–45 (37; 10), 24–47 (36; 10), 23–61 (42; 10), 20–43 (30; 10), and 12–35 (28; 10) wide or approximately 21–29% (25%; 10) of maximum body width and 6–10.8 (8.3; 10) × maximum oesophagus width, extending directly out from caecal ramification, deep-lobed to diverticulate, some caeca branching near extremity, wall glandular (not illustrated), containing refractive content (not illustrated); caecal field 69–120 (94; 10) or 19–26% (22%; 10) of body length and 35–62% (48%; 10) of oesophagus length, 81–111 (101; 10) wide or 71–82% (75%, 10) of maximum body width; post-caecal distance 201–283 (234; 9) or 50–59% (55%; 9) of body length from anterior body end, post-caecal distance 152–243 (188; 9) or 41–49% (45%; 9) of body length from posterior body end (Figs. 11, 14).

Testicular anlage 49–101 (69; 10) long or 12–20% (16%; 10) of body length, 51–110 (85; 10) wide or 49–73% (63%; 10) of body width or 0.6–1 (0.8; 10) × longer than wide, containing dense field of vasa efferentia intertwining among densely-packed testicular cells; testicular cells circular, each measuring 1 in diameter; post-testicular space 110–163 (133; 10) long or 27–36% (31%; 10) of body length (Figs. 11). Vasa efferentia secondary ducts 11–35 (21; 10) in maximum width (Figs. 11). Vas deferens a thin-walled duct, including a proximal portion ventral to testicular anlage and a post-testicular portion; proximal portion robust, comprising 1 trunk, 11–35 (21; 10) in maximum width, extending from lateral margins of testicular lobes, typically orienting

diagonally posterosinistral (6 of 10 specimens), and occasionally orienting diagonally posterodextral (2 of 10 specimens) or along midline (2 of 10 specimens); post-testicular field portion 66–157 (110; 10) long, 6–22 (13; 10) wide at the level of posterior margin of testicular anlage, typically extending diagonally sinistrad 12–38 (26; 9) or 3–9% (5%; 9) of body length before curving toward dextral body margin dorsally (9 specimens vs. 1 ventrally) and extending 15–45 (33; 10), 5–15 (11; 9) wide at level of ovary, curving ventromedially (9 specimens vs. 1 dorsally) and crossing midline for 32–80 (54; 10), 9–17 (14; 10) wide or 9–13% (11; 10) of maximum body width before connect with cirrus-sac and internal seminal vesicle, ventral to ovary (Figs. 11, 15, 16).

Cirrus-sac oblong, 45–65 (53; 10) long or 9–14% (13%; 10), 11–19 (16; 10) in maximum width or 11–14% (12%; 10) of body width, having wall approximately 1 (1; 10) thick, thin-walled; internal seminal vesicle robust, 32–65 (52; 10) long, 5–11 (7; 10) wide or 5.6–9.8 (7.6; 10) × longer that wide, curving posteriad and orienting diagonally posteromedial (Figs. 11, 15, 16). Male genital pore circular, 2–3 (3; 10) wide, slightly sinistral, opening 24–41 (34; 10) or 20–29% (25%; 10) of maximum body width from sinistral body margin, opening 35–51 (45; 10) or 27–41 % (33%; 10) of maximum body width from dextral body margin, 48–80 (62; 10) or 13–18% (14%; 10) of body length from posterior body end (Figs. 11, 15, 16).

Ovarian anlage hard to delineate in most of the specimens fixed, having approximately 3 (3; 1) dextral and 2 (2; 1) sinistral branches each 3(3; 5) wide, branches difficult to delineate in most specimens; post-ovarian space 100–103 (102; 2) long or 25–26% (25%; 2) of body length (Figs. 11, 15, 16). Oviduct curving

sinistrally immediately posterior to ovary and lateral to vas deferens, 105–158 (126; 6) long or 25–32% (29%; 6), including an abbreviated proximal duct, a dilated portion (= oviducal seminal receptacle), and a narrow distal portion; proximal duct emanating from posteroventral surface of ovary extending sinistrally 10–20 (13; 6), 2–3 (2; 6) in maximum width, curving posteromediad to connect with oviducal seminal receptacle; oviducal seminal receptacle 40–80 (59; 6) long or 37–52% (47%; 5) of total oviduct length, 5–16 (9; 6) wide or 5–11 (8; 6) × longer than wide, occupying space between vas deferens and sinistral body margin, crossing vas deferens dorsally, post-ovarian, Distal portion of oviduct 2–3 (2; 10) or 13–60% (35%, 6) of oviducal seminal receptacle width, continuing posteriad approximately in parallel with dextral body margin before uniting with vitelline duct (Figs. 11, 15, 16). Ootype 10–18 (16; 10) long, 7–11 (9; 10) wide, 1.4–2.1 (1.7; 10) × longer than wide, slightly dextral to midline, orienting parallel to long axis of body; post-ootype distance 35–65 (50; 10) or 10–14% (12%; 10) (Figs. 11, 15, 16). Uterine anlage occupying space between cirrus-sac and distal portion of oviduct; 16–30 (24; 9) long or 4–8% (6%; 9) of body length, first portion of ascending uterus difficult to trace in most specimens (7 of 10 specimens), approximately 5–9 (7; 7), becoming refractive and curving sinistrad 13–22 (18; 7) before connecting metraterm; metraterm dilating 5–8 (7; 9) and extending 6–11 (8; 9), narrowing to 2 (2; 9) and extending about 4–6 (5; 9) (Figs. 11, 15, 16). Female genital pore not observed.

Excretory system not observed.

Type and only host: *Pimelodus grosskopfii* (Steindachner, 1879) (Siluriformes: Pimelodidae).

Type and only locality: Cienega de Jobo and Canal del Dique (10.35°S; -74.96667°W), Magdalena River Drainage, Colombia.

Site in host: Body cavity.

Prevalence and intensity of infection: Three of 9 (33.3%) *P. grosskopfii* collected at Cienega de Jobo and Canal del Dique, Colombia had 7–26 (mean intensity= 14 ±0.5) specimens.

Specimens deposited: Two vouchers at the United States National Museum (USNM Coll. No. 1283482 and 1283483); one voucher at the Institute of Parasitology, Academy of Sciences of the Czech Republic, České Budějovice (IPCAS D-718).

Remarks. We identified these specimens as *Plehniella* sp. based on the presence of the following characteristics: body ovoid, lateral tegumental spines lacking; pharynx lumen triradiate, minute; anterior and posterior oesophageal swellings robust, thick-walled; intestine comprising 6 caeca; testis lobed; cirrus-sac present; ootype prominent; uterus short; metraterm present. The specimens of *Plehniella* sp. differ from their congeners by the combination of having a posteriorly-constricted body region, an anterior sucker with concentric rows of minute spines, an elongate anterior oesophageal swelling (1/6 oesophagus length), short and wide caeca (1/10 body length; >1/2 body width), and a male genital pore that opens proportionally more anteriad (1/7 body length from posterior body end). As described above, *P. coelomicola* and *P. sabajperezi* differ from the specimens of *Plehniella* sp. by having a relatively elongate body and relatively short caeca (terminating in anterior body half). Our specimens of *P. armbrusteri* can be differentiated most easily from those of *Plehniella* sp. by having a relatively oblong-ovoid body and long caeca (nearly 1/2

body length). None of the aforementioned nominal species has a posterior body constriction, an anterior sucker, or spines associated with the body; however, all have a male genital pore that opens near the posterior body extremity.

We speculate that the specimens of *Plehniella* sp. were small adult specimens based on the proportionally large size of the genitalia (nearly 1/2 body length). Because of this, we posit that some of the differences between these putatively younger specimens and our other specimens (those of *P. sabajperezi* and *P. armbrusteri*) are likely associated with fluke development, e.g., presence/absence of anterior sucker and associated spination, location of genital pore. However, noteworthy is that other workers have used body size to differentiate congeneric adult fish blood flukes (e.g., Nolan and Cribb 2006a,b, Bullard et al. 2012). Hence, we included a detailed description of these specimens herein so that a future worker(s) could explore this host for infections, perhaps revealing the presence of another new species of *Plehniella*.

It is noteworthy that the small, putatively young, adult specimens of *Plehniella* sp. have a spheroid anterior sucker with concentric spine rows, whereas the large specimens of the new species lack a demarcated anterior sucker or associated spines. Although the specimens of *Plehniella* sp. were collected from body cavity, we speculate that perhaps they had recently arrived in that site and that the sucker and spines are lost during subsequent development. Whether or not any species of *Plehniella* keeps a spinous anterior sucker throughout life, or whether younger specimens of the new species have a spinous anterior sucker, is indeterminate.

DISCUSSION

A major portion of the results presented herein are based on specimens collected from formalin-fixed fishes (collected between 1978 and 2007) that reside within the Auburn University Museum of Natural History. From a parasitological perspective, fish collections offer a great opportunity to sample a wide geographic, ecological, and phylogenetic diversity of fishes without having to expend resources to support a field expedition. This approach seems particularly promising in filling geographic and taxonomic gaps for aporocotylids that infect the body cavity. As evidenced by the present study, taxonomically useful, although not ideal, specimens can be readily obtained from museum-curated fish specimens without causing significant damage to the fish specimen (our parasite materials were obtained by making a slit in abdomen and rinsing the body cavity). Moreover, assessments of museum fish specimens for parasite infections could represent a critical component in “before-after” baseline comparisons, once those collections offer a unique opportunity to obtain samples from time points before a man-made disturbance takes place. Currently, those comparisons are available for studies of human parasites, where those data are recorded during a disease outbreak but little information is known for comparisons in fish parasite populations. For example, the construction of dams have been implicated as the major factor leading to increased prevalence and transmission of schistosomiasis, a disease caused by blood flukes that are close relatives of aporocotylids (Gryseels et al. 1994, N’Goran et al. 1997, Orélis-Ribeiro et al. 2014).

Herein, we report a concurrent infection of aporocotylids (i.e., *P. sabajperezi* and

P. armbrusteri) in a South American pimelodid (i.e. Bloch's catfish, *P. blochii*). Few hosts have been reportedly concurrently infected by congeneric fish blood flukes: *Cardicola forsteri* Cribb, Daintith et Munday, 2000, and *Cardicola orientalis* Ogawa, Tanaka, Sugihara et Takami, 2010 from southern bluefin tuna, *Thunnus maccoyii* Castelnau, 1872 (Shirakashi et al. 2013)*; Cardicola orientalis* and *Cardicola opisthorchis* from pacific bluefin tuna, *Thunnus orientalis* (see Ogawa et al. 2011, Shirakashi et al. 2012); *Paradeontacylix grandispinus* Ogawa et Egusa, 1986 and *Paradeontacylix kampachi* Ogawa et Egusa, 1986 from greater amberjack, *Seriola dumerili* Risso, 1810 (Ogawa and Egusa 1986, Repullés-Albelda et al. 2008). We mention this here simply because it may be ecologically significant regarding host-parasite immuno-compatibility and, clearly, few examples of concurrent infections by blood-dwelling flatworms exist in the primary literature.

Several morphology-based studies have supported the view that phylogenetically-related definitive hosts are infected by morphologically similar (congeneric) fish blood flukes (Bullard et al. 2008, Bullard and Overstreet 2004, Bullard and Overstreet 2006, Orélis-Ribeiro et al. 2013, Bullard 2014). Within a host family or genus, that phenomenon is harder to discern. For example, *Pimelodus* is a paraphyletic assemblage that requires revision (Lundberg and Littmann 2003, Lundberg et al. 2011). Yet, the available evidence from morphological and molecular data on the interrelationships of South American pimelodids suggest that related species of *Pimelodus* have morphologically similar species of *Plehniella*. For example, according to Lundberg et al. (1991) and Lundberg and Parisi (2002), the presence of a trigeminofacial nerve-complex is shared by 4 catfishes that are infected by *Plehniella*

spp.; i.e., *P. maculatus* (type species), *P. blochii*, *P. grosskopfii*, and *Iheringichthys labrosus*. Noteworthy also is that the topology of the latest molecular phylogeny on South American pimelodids indicated a *P. blochii* species complex (Lundberg et al. 2011). As such, we believe that the concurrent infection of *P. armbrusteri* and *P. sabajperezi* in *P. blochii* may prove to be a further evidence of this cryptic speciation.

Approximately 17% (19 of 109) of the accepted species of Pimelodidae have been described since 2005 (Eschmeyer and Fong 2015), and likely many species remain unnamed (Armbruster 2011). As such, it seems plausible to also predict an increasing number of overlooked infections of *Plehniella* spp. Beyond the cryptic diversity, it seems clear that much remains to be documented regarding the definitive host range, geographic distribution, general biology, and taxonomy of blood flukes that infect pimelodid species. Adding complexity to this scenario, no intermediate host is known for any species of South American aporocotylid, although no shortage of candidates exist. Alda and Matorelli (2014) recently described a cercaria that infects gonad and digestive gland of the intertidal mud snail *Heleobia australis* (Rissooidea: Cochliopidae) in Argentina. It had most of the key morphological features of aporocotylid cercariae: minute body with an anterior sucker armed with concentric spines, forked tail with fin fold present on the tail furcae, and no ventral sucker (Orélis-Ribeiro et al. 2014).

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FIGURE LEGENDS

Figs. 1–4. *Plehniella sabajperezi* sp. n. (Digenea: Aporocotylidae), from body cavity of *Pimelodus albofasciatus* (Mees, 1974) (Siluriformes: Pimelodidae), from Demerara River, Guyana. **Fig. 1.** Holotype (USNM Coll. No. 1283479), ventral view. **Fig. 2.** Anterior end of holotype (USNM Coll. No. 1283479), ventral view. **Fig. 3.** High magnification view of caeca of paratype (IPCAS D-717), ventral view. **Fig. 4.** Higher magnification view of terminal genitalia of paratype (IPCAS D-717), ventral view. *Abbreviations*: aog – anterior oesoph- ageal gland; aos – anterior oesophageal swelling; bc – basophilic cells; c – caecum; C1–C6 – sac-like caeca; cgp – common genital pore; cs – cirrus-sac; dc – anterior commissure of dorsolateral nerve cord; ec – everted cirrus; m – mouth; me – metraterm; mga – me- traterm connection with common genital atrium; mgp – male genital pore; nc – nerve cord; o – ovary; oe – oesophagus; oo – ootype; osr – oviducal seminal receptacle; p – pharynx; pog – posterior oesophageal gland; pos – posterior oesophageal swelling; st – sperm tube; t – testis; u – uterus; v – vitellarium; vd – vas deferens; ve – vasa efferentia; vt – vitelline duct (proximal extent not illustrated).

Fig. 5. Genitalia of *Plehniella sabajperezi* sp. n. (Digenea: Aporocotylidae), from body cavity of *Pimelodus albofasciatus* (Mees, 1974) (Siluriformes: Pimelodidae), from Demerara River, Guyana. Terminal genitalia of paratype (IPCAS D-717), ventral view. *Abbreviations*: bi – body indentation; cs – cirrus-sac; ec – everted cirrus; mgp – male genital pore; me – metraterm; mga – metraterm connection with common genital atrium; oo – ootype; o – ovary; osr – oviducal seminal receptacle, st – sperm tube; t – testis; u – uterus; ve – vasa efferentia; vd – vas deferens (vd); v – vitellarium, vt – vitelline duct (vt).

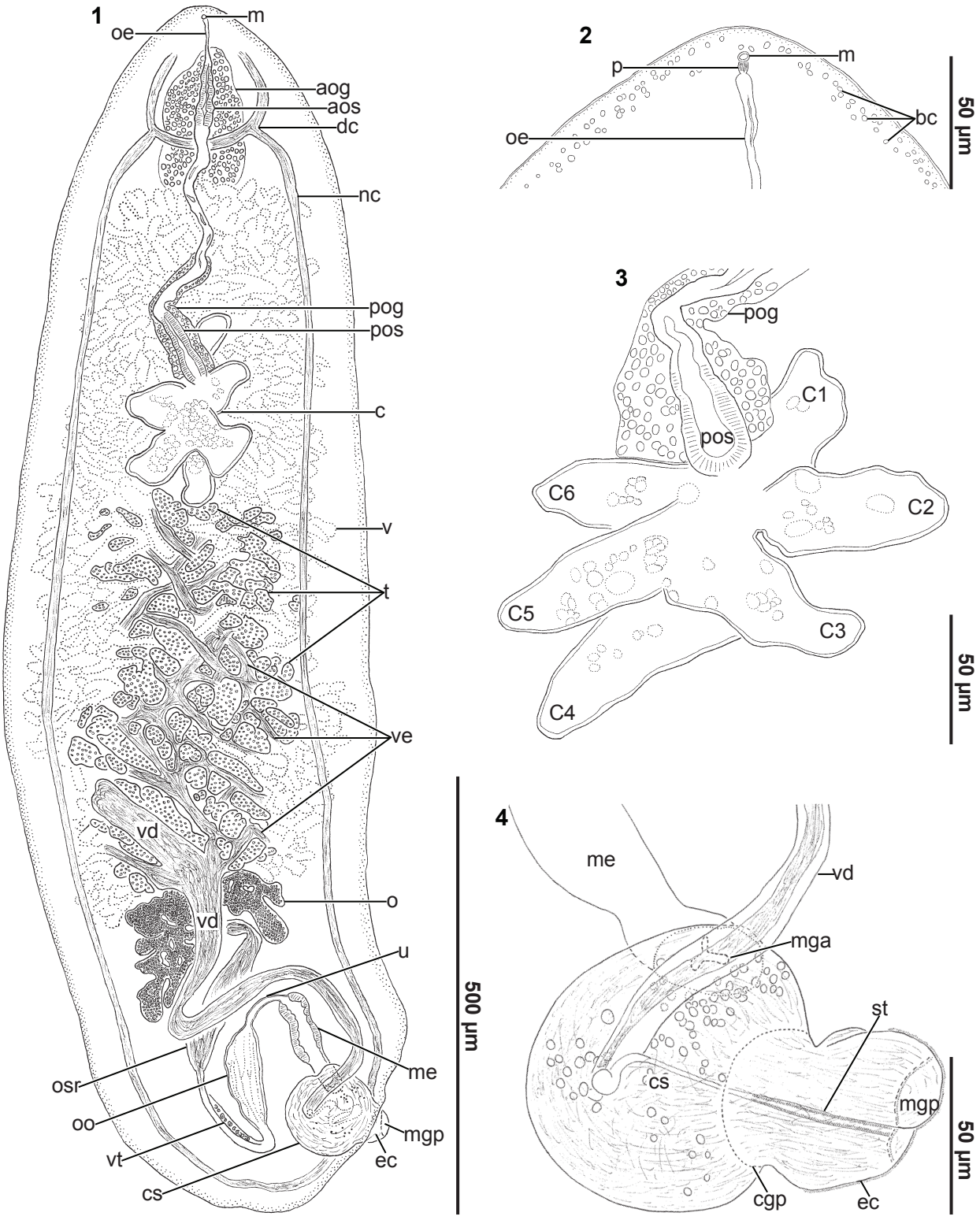
Figs. 6–8. *Plehniella armbrusteri* sp. n. from body cavity of Bloch's catfish, *Pimelodus blochii* (Valenciennes, 1840) (Siluriformes: Pimelodidae), from Lake Tumi Chucua, Bolivia. **Fig. 6.** Holotype (USNM Coll. No. 1283477), ventral view. **Fig. 7.** Anterior end of holotype (USNM Coll. No. 1283477), ventral view. **Fig. 8.** High magnification view of caeca of paratype (USNM Coll. No. 1283477), ventral view. *Abbreviations:* aog – anterior oesophageal gland; aos – anterior oesophageal swelling; bc – basophilic cells; c – caecum; C1–C6 – sac-like caeca; cs – cirrus-sac; cgp – common genital pore; dc – anterior commissure of dorsolateral nerve cord; m – mouth; me – metraterm; nc – nerve cord; o – ovary; oe – oesophagus; oo – ootype; osr – oviducal seminal receptacle; p – pharynx; pog – pos- terior oesophageal gland; pos –posterior oesophageal swelling; t – testis; u – uterus; v – vitellarium; vd – vas deferens; ve – vasa efferentia; vt – vitelline duct.

Figs. 9–10. Genitalia of *Plehniella armbrusteri* sp. n. from body cavity of Bloch's catfish, *Pimelodus blochii* (Valenciennes, 1840) (Siluriformes: Pimelodidae), from Lake Tumi Chucua, Bolivia. **Fig. 9.** Terminal genitalia of holotype (USNM Coll. No. 1283477), ventral view. **Fig. 10.** Terminal genitalia of paratype (IPCAS D-716), dorsal view. *Abbreviations*: cgp – common genital pore; cs – cir- rus-sac; ic – inverted cirrus; isv – internal seminal vesicle; me – metraterm; mga – metraterm connection with common genital atrium; mgp – male genital pore; o – ovary; oo – ootype; osr –

oviducal seminal receptacle; ov – ova; t – testis; u – uterus; vd – vas deferens; ve – vasa efferentia; vt – vitelline duct.

Figs. 11–14. *Plehniella* sp. from body cavity of *Pimelodus grosskopfii* (Steindachner, 1879) (Siluriformes: Pimelodidae), from Cienega de Jobo and Canal del Dique, Colombia. **Fig. 11.** Voucher (USNM Coll. No. 1283482), ventral view. **Fig. 12.** Anterior end of voucher (IPCAS D-718), ventral view. **Fig. 13.** Anterior end of voucher (USNM Coll. No. 1283482), ventral view. **Fig. 14.** High magnification view of caeca of voucher (IPCAS D-718). *Abbreviations*: aog – anterior oesophageal gland; aos – anterior oesophageal swelling; as – anterior sucker; bc – basophilic cells; c – caecum; C1–C6 – sac-like caeca; cs – cirrus-sac; dc – anterior commissure of dorsolateral nerve cord; isv – internal seminal vesicle; me – metraterm; mgp – male genital pore; nc – nerve cord; o – ovary; oe – oe- sophagus; oo – ootype; osr – oviducal seminal receptacle; p – pharynx; pog – posterior oesophageal gland; pos – posterior oesophageal swelling; s – row of spines; t – testis; u – uterus; ve – vasa efferentia; vd – vas deferens; vt – vitelline duct.

Figs. 15–16. Genitalia of *Plehniella* sp. from body cavity of *Pimelodus grosskopfii* (Steindachner, 1879) (Siluriformes: Pimelodidae), from Cienega de Jobo and Canal del Dique, Colombia. **Fig. 15.** Terminal genitalia of voucher (IPCAS D-718), ventral view. **Fig. 16.** Terminal genitalia voucher (USNM Coll. No.1283483), dorsal view. *Abbreviations*: bc – basophilic cells; cs – cirrus-sac; isv – internal sem- inal vesicle; me – metraterm; mgp – male genital pore; o – ovary; oo – ootype; osr – oviducal seminal receptacle; t – testis; u – uterus; vd – vas deferens; vt – vitelline duct.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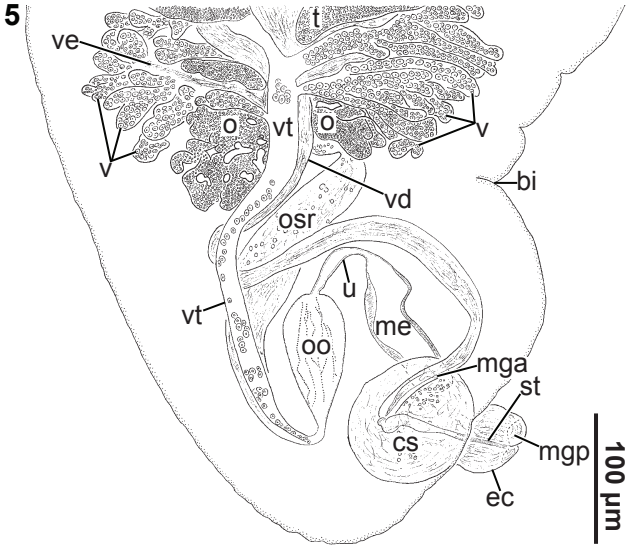

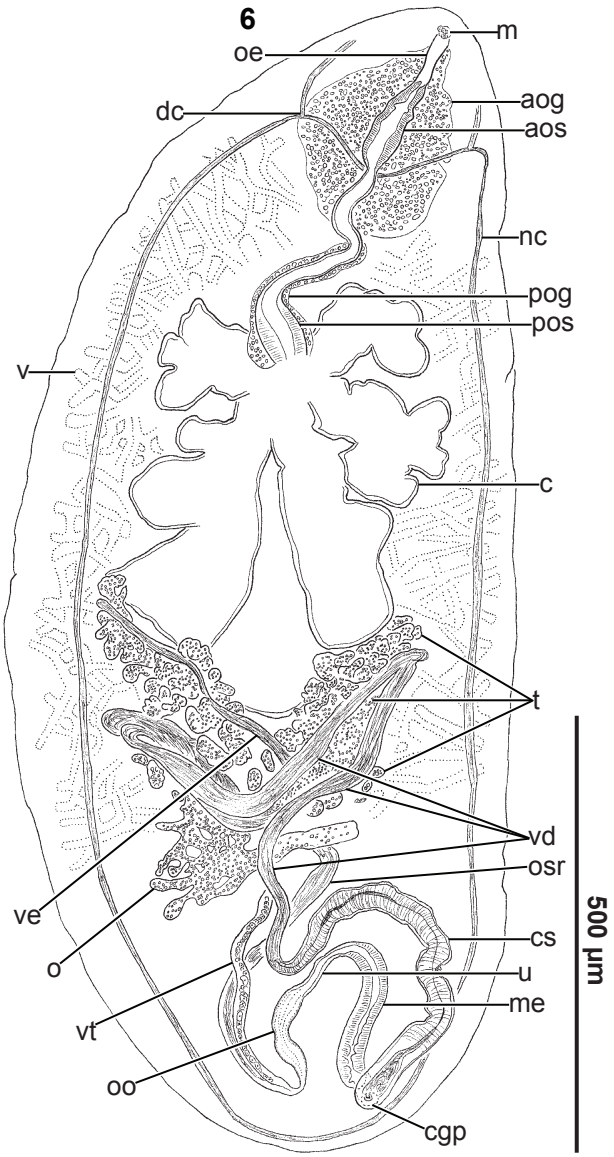
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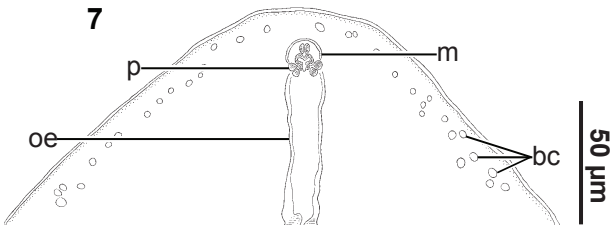
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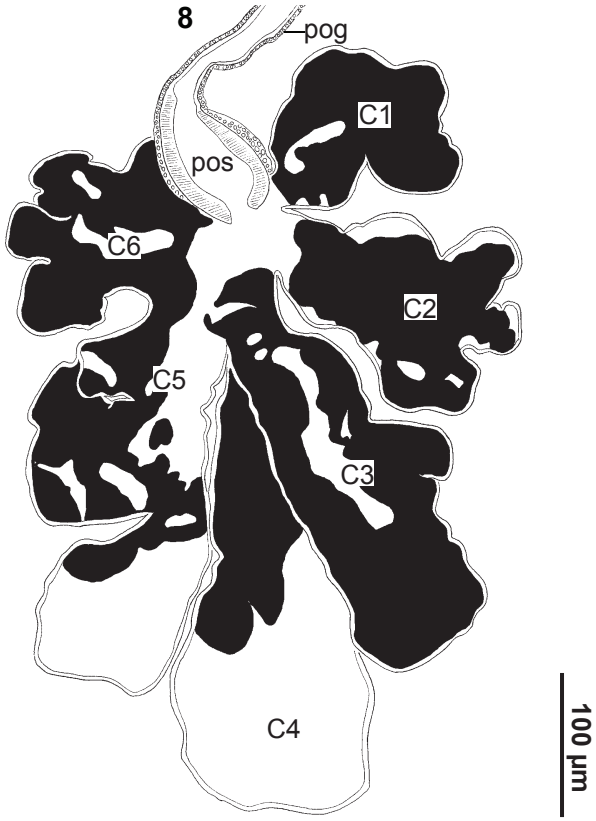
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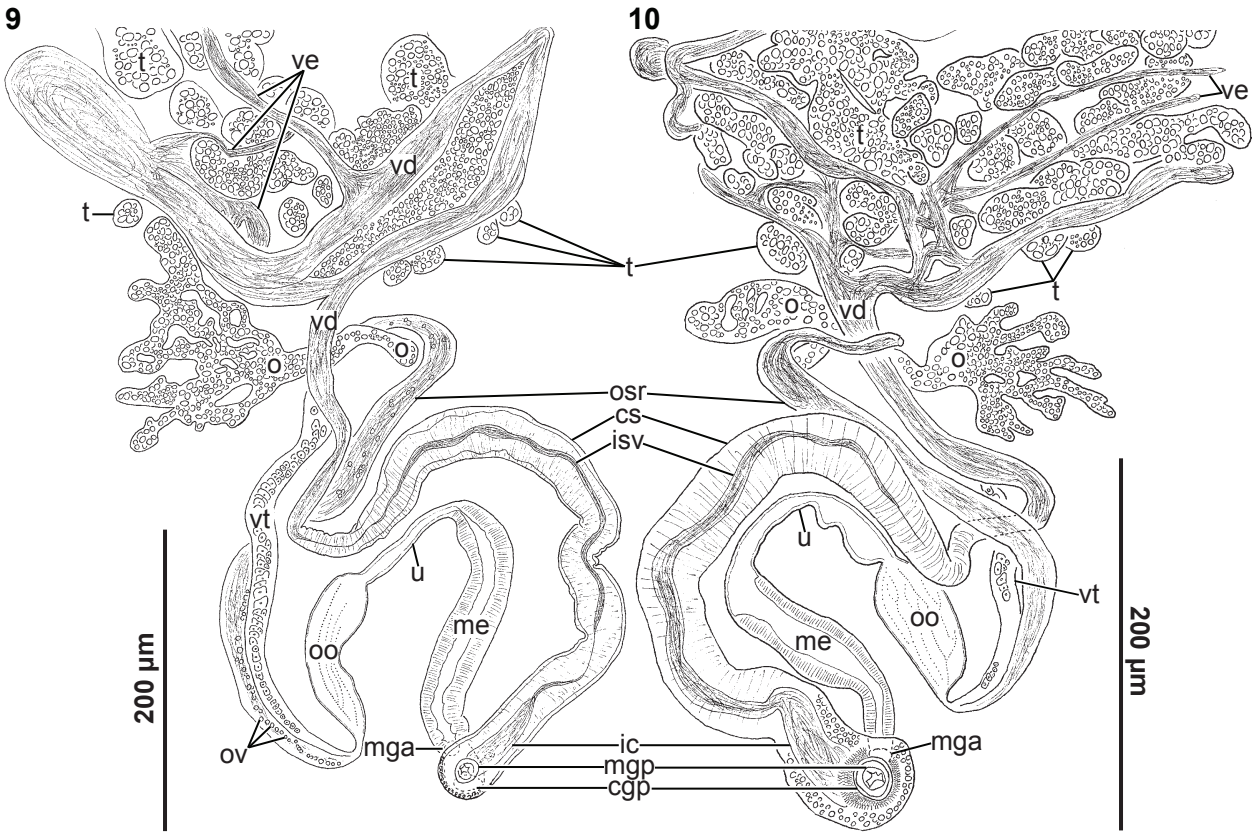
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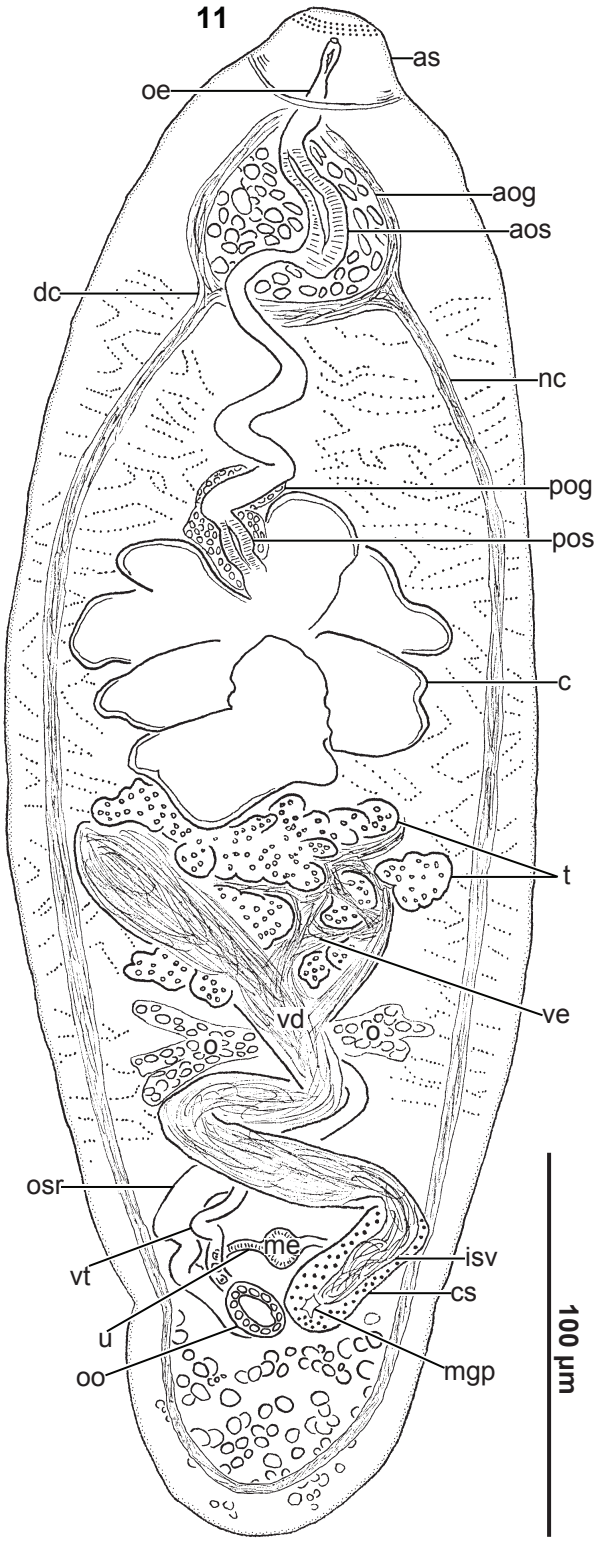
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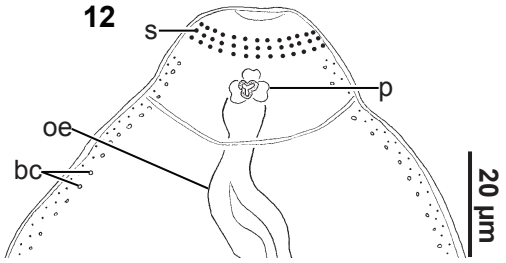
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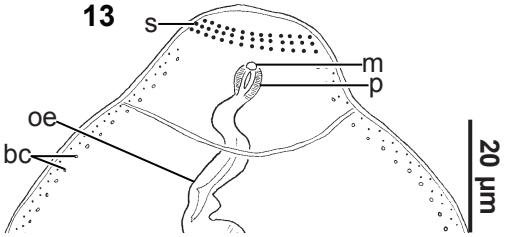
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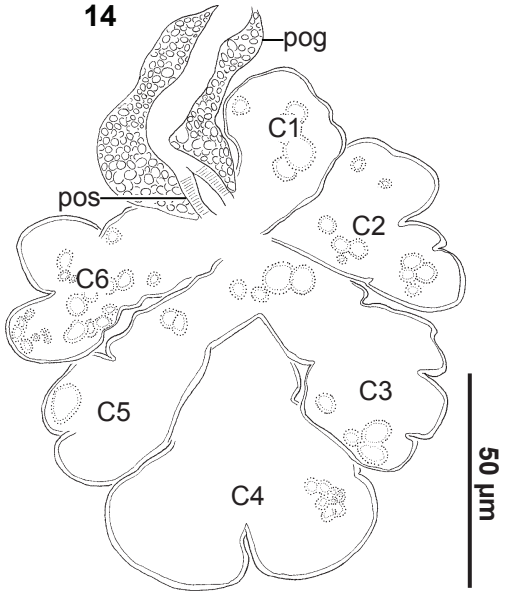
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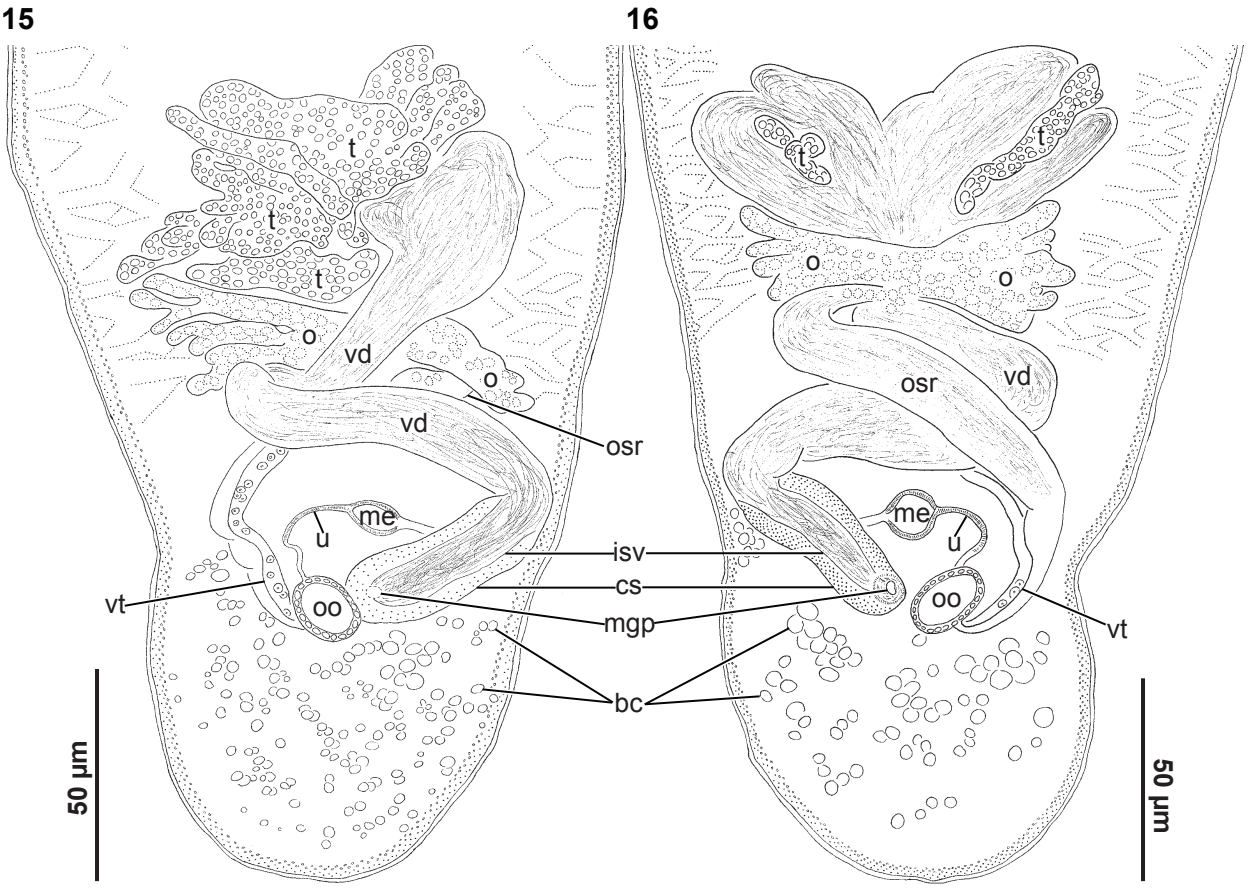
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Table 1. Blood flukes (Digenea: Aporocotylidae) of catfishes (Siluriformes).

Aporocotilydae	Host	Site	Locality	Reference(s)
Nomasanguinicola canthoensis Truong et Bullard, 2013	*Clarias macrocephalus* (Günther) (Clariidae)	branchial vessels	Can Tho fish market, Vietnam (Mekong River)	Truong and Bullard (2013)
***Plehniella armbrusteri* sp. n.**	*Pimelodus blochii* (Valenciennes) (Pimelodidae)	body cavity	Lake Tumi Chucua, Bolivia	present study
Plehniella coelomicola Szidat, 1951	*Iheringichthys labrosus* (Lütken) (Pimelodidae)	body cavity	La Plata River, Argentina	Szidat (1951)
	Pimelodus albicans (Valenciennes) (Pimelodidae)	body cavity	La Plata River, Argentina	Lunaschi (1985), Avendaño de Mac Intosh and Ostrowski de Núñez (1998)
	Pimelodus maculatus (Lacépède) (Pimelodidae) (as *P. clarias maculatus* [Bloch])	body cavity	La Plata River, Argentina	Lunaschi (1985)
	Pimelodus maculatus (Lacépède) (Pimelodidae)	body cavity	La Plata River, Argentina	Avendaño de Mac Intosh and Ostrowski de Núñez (1998)
	Pimelodus maculatus	body cavity	Paraná River Basin, Brazil	Brasil-Sato and Pavanelli (2004), Takemoto et al. (2009)
	Pimelodus maculatus	body cavity	São Francisco River Basin, Brazil	Brasil-Sato (2003), Brasil-Sato and Pavanelli (2004)
Plehniella dentata Paperna, 1964 *incertae sedis*	*Clarias gariepinus* (Burchell) (Clariidae) (as *C. lazera* [Valenciennes])	"intestine" (probably mesenteric vessels)	Lake Tiberia and Hule Nature Reserve, Israel	Paperna (1964)
Plehniella platyrhynchi (Guidelli, Isaac et Pavanelli, 2002) n. comb. (originally as *Sanguinicola*)	*Hemisorubim platyrhynchos* (Valenciennes) (Pimelodidae)	body cavity	Paraná River, Brazil	Guidelli et al. (2002), Truong and Bullard (2013)
***Plehniella sabajperezi* sp. n.**	*Pimelodus albofasciatus* (Mees) (Pimelodidae)	body cavity	Demerara River, Guyana	present study
	Pimelodus albofasciatus	body cavity	Rupununi River, Guyana	present study

	Pimelodus blochii (Valenciennes) (Pimelodidae)	body cavity	Lago Tumi Chucua, Bolivia	present study
	Pimelodus blochii	body cavity	Napo River, Peru	present study
Plehniella sp.	*Pimelodus grosskopfii* (Steindachner) (Pimelodidae)	body cavity	Cienega de Jobo and Canal del Dique, Colombia	present study
Sanguinicola chalmersi Odhner, 1924	*Auchenoglanis occidentalis* (Valenciennes) (Claroteidae)	blood, heart	Sudan, Africa	Woodland (1923), Odhner (1924), Khalil (1969, 1971), Paperna (1996)
	Synodontis schall (Block and Schneider) (Mochoidae)	mesenteric and branchial blood vessels	Cairo and Giza fish markets, Egypt	Woodland (1923), Imam et al. (1984)
Sanguinicola clarias Imam, Marzouk, Hassan et Itman, 1984 *incertae sedis*	*Clarias gariepinus* (Burchell) (Clariidae) (as *C. lazera*)	"mesenteric and other blood vessels"	Cairo and Giza fish markets, Egypt	Imam et al. (1984)
		not specified	Beni-Suef fish market, Egypt	Imam and El-Askalany (1990)

CHAPTER 4: TWO NEW GENERA OF FISH BLOOD FLUKES (DIGENEA: APOROCOTYLIDAE) FROM CATFISHES IN THE PERUVIAN AMAZON

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Authors: Raphael Orélis-Ribeiro and Stephen A. Bullard

ABSTRACT

Cladocaecum tomasscholzi n. gen., n. sp. infects the heart (lumen of ventricle) of driftwood catfish, *Ageneiosus inermis* Linnaeus, 1766 (Siluriformes: Auchenipteridae) from the Nanay River (Amazon River Basin, near Iquitos, Peru). It differs from all other aporocotylid genera by having a highly branched intestine comprising a central cecum that terminates immediately anterior to the ovary and that has numerous laterally-directed diverticula. *Kritsky platyrhynchi* (Guidelli, Isaac, and Pavanelli, 2002) n. gen., n. comb. (= *Plehniella p.*) is redescribed based on paratypes plus new specimens collected from the body cavity of the type host (porthole shovelnose catfish, *Hemisorubim platyrhynchos* Valenciennes, 1840) (Pimelodidae) from the nearby Itaya River. *Kritsky* differs from *Sanguinicola* Plehn, 1905*, Plehniella* Szidat, 1951, *Nomasanguinicola* Truong and Bullard, 2013, and *Cladocaecum* by the combination of having a spinous anterior sucker, an intestine comprising 6 asymmetrical ceca, a lanceolate body, a straight vas deferens, an ovary with finger-like lateral projections, a small and spheroid oötype, numerous, minute, spheroid uterine eggs, and separate genital pores. An updated list of hosts, tissues infected, and geographic localities for the catfish blood flukes (9 spp.; 5 genera) is provided. This is the first report of a fish blood fluke infecting a member of Auchenipteridae and first proposal of a new genus

of blood fluke (Schistosomatoidea) from South America in 64 yr. It brings the total number of Amazonian fish blood flukes to a mere 4 species.

INTRODUCTION

Catfishes (Siluriformes), comprising 3,695 species of 39 families, are among the most species rich vertebrate taxa, totaling 1 in 9 fishes and 1 in 18 vertebrate species (Chapman, 2009; Eschmeyer and Fong, 2015). South America is a catfish biodiversity focus (Reis et al., 2003), including species that are highly-prized by the aquarium pet trade (Mitchell and Thomas, 2008), commercial and recreational fishing industries (Welcomme et al., 2014), and production aquaculture sector (Queiroz et al., 2002). Despite the regional economic importance of catfishes, as well as their ecological and phylogenetic diversity, few studies have explored their metazoan parasites; especially their fish blood flukes (Digenea: Aporocotylidae) (Szidat, 1951; Guidelli et al., 2002; Orélis-Ribeiro and Bullard, 2015). Catfishes worldwide host 8 aporocotylids (of 138 accepted aporocotylid species, 6%) currently assigned to *Plehniella* Szidat, 1951, *Nomasanguinicola* Truong and Bullard, 2013, and "*Sanguinicola* Plehn, 1905" (see Truong and Bullard, 2013; Orélis-Ribeiro and Bullard, 2015). Catfish blood flukes mature in the circulatory system (Woodland, 1923, 1924; Odhner, 1924; Paperna, 1964, 1996; Truong and Bullard, 2013) and the body cavity (Szidat, 1951; Guidelli et al., 2002; Orélis-Ribeiro and Bullard, 2015) of 10 catfishes of 6 genera and 4 families (Table I).

Herein, we propose 2 new genera, describe a new species, and redescribe a

nominal species of Aporocotylidae based on specimens that infect the heart and body cavity of catfishes in the Peruvian Amazon. This is the first proposal of a new genus of blood fluke (Schistosomatoidea) from South America since Szidat's (1951) proposal of *Plehniella*.

MATERIALS AND METHODS

Hosts were captured by seine or cast net near Iquitos, Peru (from 2005 to 2006) and examined with a dissecting microscope immediately after euthanasia. Flukes were pipetted onto a glass slide and heat-killed with an EtOH burner flame before being transferred to a vial of 5% neutral buffered formalin. Later, whole specimens were rinsed thoroughly with distilled water and cleaned with fine brushes to remove any debris, stained overnight in Van Cleave's hematoxylin with several additional drops of Ehrlich's hematoxylin, made basic in 70% ethanol with lithium carbonate and butyl-amine, dehydrated, cleared in clove oil, and permanently mounted in Canada balsam. Illustrations of stained, whole-mounted specimens were made with the aid of a Leica DM-2500 (Leica, Wetzler, Germany) equipped with differential interference contrast (DIC) optical components and a drawing tube. Measurements were obtained by using a calibrated ocular micrometer (as straight-lines along the course of each duct) and are herein reported in micrometers (μm) followed by their mean and the number measured in parentheses. Morphometric data for the borrowed paratypes (Coleção Helmintológica do Instituto Oswaldo Cruz [CHIOC] collection nos. 34361a and 34361b) are reported in brackets following the measurements of the newly-

collected voucher specimens. Scientific names including taxonomic authorities and dates for fishes follow Eschmeyer (2015). Common names are taken from FishBase (Froese and Pauly, 2015). Higher level fish classification and nomenclature follows Nelson (2006). Nomenclature for Aporocotylidae follows Bullard et al. (2009). Brown (1956) was used to help construct the genus name and specific epithet. Type and voucher materials are deposited in the United States National Museum (USNM, Washington, D.C.) and Coleção Helmintológica do Instituto Oswaldo Cruz (CHIOC, Rio de Janeiro, Brazil).

DESCRIPTIONS

Cladocaecum n. gen.

(Figs. 1–4)

Diagnosis: Body of adult elongate, < 6× longer than wide, strongly dorsoventrally flattened, ventrally concave, lacking posterolateral protuberance, anterior body end less tapered than posterior end, lacking tegumental body spines (‘Marginalstacheln’), rods and bristles (‘Stäbchen-Börstchen’), and rosethorn-shaped spines. Ventrolateral nerve cords indistinct. Dorsolateral nerve cords extending nearly entire body length (hereafter referred to as BL), with commissures anteriorly and posteriorly. Anterior sucker indistinct. Mouth medioventral. Pharynx a diminutive zone of muscle encircling anterior end of the esophagus immediately posterior to mouth. Esophagus medial, extending directly posteriad (not convoluted) approximately 1/3 of BL, connecting with ceca anteromedially, including subtle anterior and posterior esophageal swellings

immediately anterior to nerve commissure and cecal ramification (respectively); anterior esophageal gland enveloping anterior esophageal swelling, between mouth and anterior nerve commissure; posterior esophageal gland enveloping posterior esophageal swelling, occupying space anterior to cecal ramification. Intestine comprising paired anterior ceca plus a medial cecum with numerous branches extending laterad, asymmetrical, lacking clearly discernible paired posterior ceca, penetrating into posterior body half. Testis single, medial, immediately anterior to ovary. Cirrus sac indistinct. Male genital pore slightly sinistral, post-gonadal, post-cecal, opening approximately 1/12 of BL from posterior body end. Ovary medial, post-cecal, immediately post-testicular, as wide as testis, occupying posterior 1/4 of body. Oviduct extending posteriad from posteromedial surface of ovary, connecting with vitelline duct near level of male genital pore; oviducal seminal receptacle present, immediately post-ovarian. Ovo-vitelline duct short, connecting with oötype dextrally; oötype spheroid, thick-walled, enveloping large gland cells, post-cecal, post-gonadal, posterior to genital pores. Laurer's canal absent. Uterus post-cecal, primarily post-gonadal, with straight (not convoluted) ascending and descending segments; uterine seminal receptacle lacking; uterine eggs spheroid, lacking appendages or filaments. Metraterm indistinct. Female genital pore dorsal, anteromedial to male genital pore. Excretory vesicle indistinct. Adults in the heart of ‘driftwood catfishes’ (Siluriformes: Auchenipteridae).

Differential diagnosis: Ventrolateral tegumental body spines absent. Anterior sucker indistinct. Pharynx present. Esophagus approximately 1/3 of BL, with anterior and posterior esophageal swellings enveloped by respective esophageal gland.

Intestine comprising paired anterior ceca plus a medial cecum with numerous branches extending laterad, asymmetrical, lacking clearly discernible paired posterior ceca, penetrating into posterior body half. Testis single, pre-ovarian. Proximal portion of oviduct comprising an oviducal seminal receptacle. Oötype spheroid, thick-walled, enveloping large gland cells. Laurer's canal absent. Uterus with straight ascending and descending segments. Metraterm indistinct. Genital pores separate.

Type and only known species: Cladocaecum tomasscholzi n. sp.

Etymology: The genus name is for the highly branched (clados) gut of the type species.

Remarks

Cladocaecum differs from all other aporocotylid genera by having a highly branched intestine comprising a central cecum that terminates immediately anterior to the ovary and that has numerous laterally-directed diverticula. It is most similar to several other aporocotylid genera (i.e., *Sanguinicola* Plehn, 1905; *Plehniella* Szidat, 1951; *Nomasanguinicola* Truong and Bullard, 2013) accommodating blood flukes that mature in primary division freshwater fishes.

Sanguinicola Plehn, 1905, the aporocotylid genus that has historically accommodated the vast majority of freshwater fish blood flukes, needs systematic revision. No type materials exist for the type species *Sanguinicola armata* Plehn, 1905 but Ejsmont's (1926) treatment of that species is most taxonomically informative. Because *Sanguinicola* includes several species of doubtful identity and uncertain systematic position, we base our generic comparisons on Ejsmont's (1926) description of *S. armata*. The new genus resembles *Sanguinicola* by having an

esophagus that is 1/3 of BL, a pre-ovarian testis, an oötype that is posterior to all other segments of the male and female genitalia, a post-ovarian uterus, and separate genital pores as well as by lacking a Laurer's canal. The oötype of these genera is thick-walled and highly-glandular, so much so that it may appear muscular in some whole mounts (Schell, 1974). This type of oötype differs from that of *Cardicola* spp. (Bullard and Overstreet, 2004), which have an ovoid, thin-walled oötype surrounded by an obvious Mehlis' gland. The new genus is most easily differentiated from *Plehniella* also by the shape of the intestine. *Cladocaecum* resembles *Nomasanguinicola*, the other genus of catfish blood fluke, by lacking ventrolateral tegumental spines, rods, and bristles and a Laurer's canal and by having a post-testicular ovary, a post-ovarian uterus with ascending and descending segments, and separate genital pores. The new genus is most easily distinguished from the closely-related *Sanguinicola* and *Nomasanguinicola* by lacking lateral tegumental body spines and by having a highly branched intestine that extends to the ovary. *Sanguinicola* and *Nomasanguinicola* have a compact, multi-lobed intestine that does not extend into the posterior half of the body. In addition to that feature, *Sanguinicola* has a column of lateral tegumental body spines; whereas, *Nomasanguinicola* has an anterior sucker with denticles forming a column per each side of mouth.

***Cladocaecum tomasscholzi* n. sp.**

(Figs. 1–4)

Diagnosis of adult (measurements and illustrations based on light microscopy of 1 whole-mounted specimen): Body 2,925 long, 553 wide, maximum width at level of ovary, 5.4× longer than wide (Fig. 1). Ventral and dorsal sensory papillae not evident

with light microscopy. Dorsolateral nerve cords difficult to trace for most of BL; 10 wide near mid-body at widest level; 110 or 20% of body width from body margin at mid-body, paired, contiguous anteriorly and posteriorly, becoming confluent with paired cord 40 or 1% of BL from posterior body end; commissure 375 or 13% of BL from anterior body end, 125 or 23% of body width across width of worm, 13 wide, perpendicular to long axis of body, coursing dorsal to posterior end of esophageal anterior swelling (Fig. 1). Mouth 5 in diameter, 13 long or 0.4% of BL from anterior body end (Figs. 1, 2); pharynx ovoid, 25 or 3% of esophagus length, 20 wide or 1.3× longer than wide, with muscular wall 8 thick. Esophagus 943 long or 34% of BL, including a (i) distal portion, an (ii) anterior esophageal swelling portion, an (iii) elongated narrow portion, and a (iv) posterior swelling portion; distal portion dilating to 25 after pharynx and extending directly posteriad for 133 or 14% of esophagus total length, with wall 3 thick, narrowing to 10 wide before connecting with anterior esophageal swelling; anterior esophageal swelling 175 long or 19% of esophagus total length, 30 wide or 5% of maximum body width, with wall 13 thick, 171 or 6% of BL from anterior body end; elongated narrow portion 15 wide, with wall 5 thick, extending directly posteriad 435 before connecting with posterior esophageal swelling; posterior esophageal swelling delineated anteriorly from narrow region of esophagus by slight bend, 200 long or 21% of esophagus length, 43 wide or 1.4× maximum esophagus width, with wall 13 thick, posterior portion immediately anterior to cecal ramification, connecting with intestine anteromedially (Figs. 1, 2). Anterior esophageal gland 200 long or 21% of esophagus length, 170 wide or 5.7× maximum width of anterior esophageal swelling (Figs. 1, 2); posterior esophageal gland 593 long or 63%

of esophagus length or 3× length of anterior portion of esophageal gland, 150 in maximum width or 3.5× maximum width of posterior esophageal swelling (Fig. 1). Intestine thick-walled, with crenulated luminal surface (Fig. 3); ceca bifurcation 980 or 34% of BL from anterior body end; anterior ceca extending approximately in parallel with body margin and esophagus, not extending laterad beyond dorsolateral nerve cord; anterodextral cecum 318 long or 11% of BL, 80 wide; anterosinistral cecum 286 long or 10% of BL, 32 wide; medial branch of posterior cecum extending directly posteriad along midline 1,303 or 45% of BL, approximately 4× length of either anterior cecum, 80 wide or approximately as wide as either anterior cecum; secondary cecal branches 159–318 (n = 11) long, approximately 80 wide, not extending laterad far beyond dorsolateral nerve cord (Fig. 1).

Testis 675 long or 14% of BL, 500 wide or 93% of body width, rectangular, poorly delineated from surrounding parenchyma (Fig. 1); post-testicular space 783 or 27% of BL. Vas deferens extending slightly diagonally toward sinistral body margin before connecting with seminal vesicle; seminal vesicle S-shaped, 239 long, containing sperm for entire length (Figs. 1, 4). Male genital pore 225 or 8% of BL from posterior body end, 100 from sinistral body margin, 200 from dextral body margin (Fig. 4).

Ovary 330 long or 11% of BL, 350 wide or 63% of body width, dorsal to vas deferens, having an irregular margin (Figs. 1, 4); post-ovarian space 300 long or 10% of BL. Oviduct curving sinistrad immediately posterior to ovary and lateral to vas deferens, 408 long, 68 in maximum width, comprising (i) abbreviated proximal duct, (ii) dilated portion (= oviducal seminal receptacle), and (iii) distal portion; proximal duct extending 75 toward sinistral body margin, 8 wide, curving posteromediad to connect

with oviducal seminal receptacle; oviducal seminal receptacle filled with sperm, 215 long or 53% of total oviduct length, 68 wide or 6× longer than wide; distal portion of oviduct narrow, 10 wide or 15% of oviducal seminal vesicle maximum width, continuing posteriad approximately in parallel with dextral body margin before uniting with vitelline duct (Fig. 4). Primary vitelline collecting duct indistinct in region anterior to ovary, passing ventral to ovary before extending ventrally 335 along oviducal seminal receptacle to unite with distal portion of oviduct immediately proximal to oötype. Oötype 50 in diameter, 125 or 4% of BL from posterior body end; oötype wall 20 thick, enveloping clearly delineated spheroid cells (Figs. 1, 4). Mehlis' gland indistinct but perhaps an amorphous glandular region primarily concentrating immediately posterior to oötype (Fig. 4). Uterus medial, flanked by primary vitelline duct and seminal vesicle; ascending portion of uterus extending 238 anteriad from oötype, curving dorsally immediately posterior to posterior margin of ovary, 78 in maximum width; descending uterus extending 140 posteriad from posterior margin of ovary, 0.6× length of ascending uterus, 38 in maximum width or approximately 1/2 width of ascending uterus, lacking muscular wall; uterine eggs thin-shelled, spheroid, 15 (15; 63) in diameter. Female pore 10 in diameter.

Excretory system indistinct.

Type and only known host: Driftwood catfish, *Ageneiosus inermis* Linnaeus, 1766 (Siluriformes: Auchenipteridae).

Site in host: Heart, ventricle lumen.

Type locality and collection date: Nanay River (3°43'12"S, 73°16'50"W), Amazon River Basin, near Iquitos, Peru, August 2005.

Specimen deposited: Holotype USNM Coll. No. 1254657.

Prevalence of infection: 1 of 5 (20%) *A. inermis* was infected by one fluke.

Etymology: The specific name *tomasscholzi* honors Professor Tomáš Scholz (Institute of Parasitology Biology Centre, ASCR, Helminthology, Czech Republic) in recognition of his significant contributions to fish parasitology.

***Kritsky* n. gen.**

(Figs. 5–9)

Diagnosis: Body of adult lanceolate, 6× longer than wide, strongly dorsoventrally flattened, ventrally concave, lacking posterolateral protuberance, more rounded posteriorly than anteriorly, lacking tegumental body spines (“Marginalstacheln”), rods and bristles (“Stäbchen-Börstchen”), and rosethorn-shaped spines. Ventrolateral nerve cords indistinct. Dorsolateral nerve cords present, extending nearly entire BL, with commissures anteriorly and posteriorly. Anterior sucker with concentric spine rows anterior to mouth and ornamenting anterior aspect of sucker, demarcated from body by posterior constriction of tegument, comprising an obvious proboscis accommodating mouth, lacking lateral denticles. Mouth medioventral. Pharynx diminutive, with tri-radiate lumen, in anterior portion of esophagus. Esophagus medial, extending directly posteriad (not convoluted) >1/4 of BL, connecting with ceca anteromedially, including anterior and posterior esophageal swellings enveloped by respective esophageal glands. Intestine restricted to anterior half of body, having glandular wall, comprising 6 ceca; ceca asymmetrical, saccular, each approximately 1/9 of esophagus length, smooth (lacking diverticula). Testis single, medial, post-cecal, pre-ovarian, >1/3 of BL, 4× longer than wide, diffuse, extensively lobed,

extending laterad beyond nerve cords. Vasa efferentia extensive, having secondary ducts extending from lateral margins of testicular lobes and coalescing ventrally along midline; vas deferens predominantly straight, lacking sinistral or dextral loops. Cirrus sac present, sinistral, post-gonadal, enclosing seminal vesicle and cirrus; inverted cirrus having a transverse pore; everted cirrus having a proximal base circular, everting dorsally near midline; male genital pore post-gonadal, post-cecal, 1/12 of BL from posterior body end. Ovary medial or slightly dextral, post-testicular, occupying posterior 1/4 of body, a loose aggregation of spheroid ova bound by a thin membrane, with finger-like laterally directed lobes and narrow middle portion (hourglass- or butterfly-shaped), dorsal to vas deferens, extending lateral to dorsolateral nerve cords, as wide as testis. Vitellarium follicular, occupying space from middle of esophagus to posterior end of testis. Oviduct emanating from posteromedial dorsal surface of ovary, connecting with vitelline duct near level of male genital pore; oviducal seminal receptacle present, immediately post-ovarian. Ovo-vitelline duct short, connecting with oötype dextrally; oötype longer than wide, post-cecal, post-gonadal, at level of or posterior to genital pores. Laurer's canal absent. Uterus inverse J-shaped, post-cecal, post-gonadal; ascending portion longer than descending portion; ascending portion straight or slightly convoluted; uterine seminal receptacle lacking; uterine eggs spheroid, lacking appendages or filaments. Metraterm indistinct. Female genital pore transverse, anteromedial to male genital pore. Excretory vesicle indistinct. Adults in body cavity of South American pimelodids.

Differential diagnosis: Ventrolateral tegumental body spines absent. Spinous anterior sucker with concentric spine rows present in adult. Pharynx present.

Esophagus > 1/4 of BL, with anterior and posterior esophageal swellings enveloped by respective esophageal glands. Intestine comprising 6 asymmetrical, saccular ceca. Testis single, > 1/3 of BL, diffuse, with many lobes extending laterad beyond nerve cords. Vas deferens straight, lacking sinistral or dextral loops. Cirrus sac present; inverted cirrus having a transverse pore. Male genital pore post-gonadal, post-cecal, 1/12 of BL from posterior body end. Ovary with finger-like laterally directed lobes and narrow middle portion. Proximal portion of oviduct comprising an oviducal seminal receptacle. Oötype occupying space at level of or posterior to genital pores. Laurer's canal absent. Uterus inverse J-shaped. Genital pores separate, transverse.

Type and only known species: *Kritsky platyrhynchi* (Guidelli, Isaac, and Pavanelli, 2002) n. gen., n. comb.

Etymology: The generic epithet honors Professor Delane Kritsky (Idaho State University, Pocatello) for his many contributions to the study of helminth biodiversity in South American freshwater fishes.

Remarks

Kritsky n. gen., *Nomasanguinicola* Truong and Bullard, 2013, *Plehniella* Szidat, 1951, and *Sanguinicola* Plehn, 1905 are the only aporocotylid genera that lack a Laurer’s canal and have the combination of an esophagus that extends at least 1/3 of the BL, an extensively lobed testis that occupies the space between the ceca and ovary, an approximately butterfly wing-shaped ovary, an oötype post-gonadal, and a post-ovarian uterus (Plehn, 1905; Ejsmont, 1926; Szidat, 1951; Truong and Bullard, 2013; Orélis-Ribeiro and Bullard, 2015; present study). The new genus resembles *Plehniella* by having that combination of features plus a body that lacks lateral

tegumental spines as well as having a minute pharynx, anterior and posterior esophageal glands corresponding to esophageal swellings, an intestine comprising 6 asymmetrical and saccular ceca, and a prominent cirrus sac. Members of both genera mature in the body cavity of pimelodid catfishes from South America (Szidat, 1951; Orélis-Ribeiro and Bullard, 2015; present study). *Nomasanguinicola* can be easily differentiated from these genera by the presence of an anterior sucker with denticles directing posteroventrally, forming a column per each side of mouth (Truong and Bullard, 2013). *Sanguinicola armata,* and therefore *Sanguinicola sensu lato*, differs from these genera by the combination of having lateral tegumental body spines arranged in a single lateral column, 4–5 but not 6 ceca, and a large, tetrahedral egg in the oötype (Ejsmont, 1926; Kirk and Lewis, 1993).

Monotypic *Kritsky* differs from *Plehniella* by a combination of morphological features associated with body shape, anterior sucker, male genitalia, female genitalia, genital pores, and eggs. The adult body in the new genus is lanceolate, approximately 6× longer than wide, and more rounded posteriorly than anteriorly (Fig. 5). In *Plehniella*, the adult body is elongate or oblong (approximately 2–4× longer than wide) with a slight sinistral indentation at level of genitalia or not and anterior and posterior ends tapering equally or having a broadly rounded posterior end (Szidat, 1951; Orélis-Ribeiro and Bullard, 2015). Regarding the anterior sucker, *Kritsky* has a demarcated anterior sucker with concentric spine rows in large adult specimens, whereas a spinous anterior sucker has only been reported from small, putatively young, adult specimens of *Plehniella* spp. but never from large adult specimens (Orélis-Ribeiro and Bullard, 2015). *Kritsky* has a vas deferens that is predominantly straight, lacking

sinistral or dextral loops (Fig. 5). *Plehniella* has a vas deferens that is strongly sinuous, forming obvious sinistral and dextral loops (Szidat, 1951; Orélis-Ribeiro and Bullard, 2015). The ovary of *Kritsky* has finger-like lateral extensions; whereas, that of *Plehniella* comprises an extensively lobed mass (Szidat, 1951; Orélis-Ribeiro and Bullard, 2015). The oötype of the new genus is a small, spheroid chamber (Figs. 5, 8, 9); whereas, that of *Plehniella* is an elongate chamber that exceeds the length of the ascending uterus (i.e., the ascending uterus is >3× oötype length in *K. platyrhynchi* vs. 1–2× oötype length in *Plehniella* spp.) (Szidat, 1951; Orélis-Ribeiro and Bullard, 2015). The new genus has numerous, minute, spheroid uterine eggs (Fig. 8); whereas, *Plehniella* spp. typically have a single, large, ovoid uterine egg (Szidat, 1951; Orélis-Ribeiro and Bullard, 2015). *Kritsky* has separate, relatively anteromedial genital pores (Figs. 5, 8, and 9); whereas, *Plehniella* has a common atrium and pore that is circular, lateral, and opens near the posterior body extremity, appearing almost terminal (Szidat, 1951; Orélis-Ribeiro and Bullard, 2015).

The new genus differs from the blood flukes that infect chondrichthyans by lacking robust, C-shaped lateral tegumental spines and a Laurer's canal (see Short, 1954; Van der Land, 1967; Maillard and Ktari, 1978; Bullard et al., 2006; Orélis-Ribeiro et al., 2013). *Kritsky* can be most easily differentiated from the blood fluke genera including species that infect basal fish lineages, i.e., *Acipensericola* Bullard, Snyder, Jensen, and Overstreet, 2008, *Elopicola* Bullard, 2014, and *Paracardicoloides* Martin, 1974, by lacking a bowl-shaped anterior sucker, lateral tegumental spines, U-shaped intestine, Laurer's canal, inter-gonadal oötype, and common genital atrium and pore (Martin, 1974; Bullard et al., 2008; Bullard, 2014). The new genus differs from aporocotylid

genera accommodating species that infect higher bony fishes (Euteleostei) by the combination of lacking lateral tegumental body spines and an intestine comprising paired anterior and posterior ceca (Bullard and Overstreet, 2003, 2004; Bullard, 2010, 2012, 2013; Bullard et al., 2012; McVay et al., 2011).

***Kritsky platyrhynchi* (Guidelli, Isaac, and Pavanelli, 2002) n. gen., n. comb.**

(Figs. 5–9)

Diagnosis of adult (measurements and illustrations based on 2 paratypes of P. platyrhynchi *[CHIOC 34361a and 34361b] plus 4 stained, whole-mounted specimens):* Body 1,955–2,210 (2,075; 3) [1,868, 3,358] long, 272–518 (390; 4) [300, 600] wide or 5.4–7.2 (6.1; 3) [6.2, 5.6]× longer than wide (Fig. 5). Ventral and dorsal sensory papillae not evident with light microscopy. Dorsolateral nerve cords 6–12 (9; 4) [5, 8] wide near mid-body at widest level; 53–77 (67; 4) [65, 82] or 12–20% (18%; 4) [22%, 14%] of body width from body margin at mid-body, paired, contiguous anteriorly and posteriorly, becoming confluent with paired cord 35–55 (45; 3) [25, 67] or 2–3% (2%, 3) [1%, 2%] of BL from posterior body end; commissure of dorsolateral nerve cord 143–151 (148; 4) [148, 240] or 7% (7%; 3) [8%, 7%] of BL from anterior body end, 52–77 (65; 4) [70, 130] across width of the worm or 15–19% (17%; 4) [23%, 22%] of maximum body width, 4–7 (5; 4) [7, 10] in diameter, perpendicular to long axis of body, coursing dorsal to mid-portion of esophageal anterior swelling (Fig. 5). Anterior sucker base width 50–70 (58; 4) [52, 72] or 13–18% (15%; 4) [17%, 12%] of body width, 34–41 (38; 4) [34, 51] long or 1.3–1.7 (1.5; 4) [1.5, 1.4]× wider than long; 4 concentric rows of approximately 1 long 1 wide spines associated with anterior end; terminal papillae on anterior margin not present; denticles not present (Figs. 5, 6).

Mouth 2–4 (3; 4) [3, 3] in diameter, 9–14 (12; 4) [10, 16] or 27–34% (30%; 4) [29%, 31%] of anterior sucker length from anterior end (Figs. 5, 6); pharynx immediately posterior to mouth, ovoid, 7–9 (8; 4) [9, 10] long or 1–2% (1%; 4) [2%, 1%] of esophagus length, 7–10 (9; 4) [8, 9] wide or 1 (1; 4) [1, 1]× longer than wide, with muscular wall 3–4 (4; 4) [4, 4] thick, not extending posteriad along esophagus beyond posterior margin of anterior sucker (Fig. 6). Esophagus 540–624 (570; 4) [588, 949] long or 26–28% (27%; 3) [32%, 28%] of BL, including a (i) distal portion, an (ii) anterior esophageal swelling portion, an (iii) elongated narrow portion, and a (iv) posterior swelling portion; distal portion typically (5 of 6 specimens) dilating to 9–15 (12; 4) [10, 7 - not dilating] immediately after pharynx and extending straight posteriad for 18–25 (20; 4) [35, not dilating] or 3–4% (4%; 4) [6%, not dilating] of esophagus total length, narrowing to 5–8 (7; 4) [8, 10] and extending 30–44 (37; 4) [30, 84 - not narrowing] or 5–9% (7%; 4) of esophagus total length before connecting with anterior esophageal swelling; anterior esophageal swelling 101–143 (124; 4) [110, 197] long or 19–23% (22%; 4) [19%, 21%] of esophagus total length, 19–26 (23; 4) wide or 5–7% (6%; 4) [7%, 4%] of maximum body width, with wall 7–8 (8; 4) [6, 6] thick, 66–83 (77; 4) [85, 106] or 3–4% (4%; 3) [5%, 3%] of BL from anterior body end; elongated narrow portion 11–15 (13; 4) [16, 22] wide, with wall 3–5 (4; 4) [3, 3] thick, extending directly posteriad 223–297 (265; 4) [273, 471] before connecting with posterior esophageal swelling; posterior esophageal swelling with elongate anterior portion and bulb-like posterior portion, anterior portion delineated anteriorly from narrow region of esophagus by slight bend of esophagus; posterior portion immediately anterior to cecal ramification 71–169 (111; 4) [70, 197] long or 13–27% (19%; 4) [12%, 21%] of

esophagus length, 24–33 (30; 4) [21, 52] wide or 1.2–1.4 (1.3; 4) [1.1, 2]× maximum esophagus width, with wall 5–7 (6; 4) [6, 12] thick, ovoid, medial (Figs. 5, 7). Anterior esophageal gland 132–175 (149; 4) [indistinct, 235] long, 43–85 (62; 4) [indistinct, 135] wide or 2.3–3.3 (2.7; 4) [indistinct, 5.2]× width of anterior esophageal swelling (Fig. 5); posterior esophageal gland 154–207 (175) [140, 290] long or 27–38% (31%; 4) [24%, 31%] of esophagus length, 55–85 (70; 4) [58, 130] wide or 2.1–2.6 (2.4; 4) [2.8, 2.5]× width of posterior esophageal swelling, a loose aggregation of large gland-like cells bound by a thin and lightly-staining membrane (Figs. 5, 7). Intestine 532–623 (576; 4) [528, 963] or 27–28% (27%, 3) [28%, 29%] of BL from anterior body end; with six clearly-differentiated ceca in all specimens examined, ceca (clockwise in ventral view from esophagus-intestine connection) 32–49 (43; 4) [63, 100], 46–79 (63; 4) [94, 145], 65–87 (74; 4) [75, 303], 61–111 (87; 4) [118, 224], 48–73 (61; 4) [90, 184], and 33–58 (46; 4) [75, 90] long or approximately 3–4% [5, 5] of BL and 10–14% [15, 18] of esophagus length, 21–47 (36; 4) [55, 106], 31–67 (55; 4) [47, 137], 37–75 (50; 4) [50, 94], 29–42 (36; 4) [63, 90], 40–56 (50; 4) [63, 90], and 34–47 (39; 4) [53, 72] wide or approximately 11–12% [18%, 16%] of maximum body width and 1.4–1.6 [2.6, 1.9]× maximum esophagus width, smooth (lacking diverticula), containing refractive content (not illustrated), cecal field 165–214 (191; 4) [215, 493] long or 9–10% (10%; 4) [12%, 15%] of BL and 26–39% (34%; 4) [37%, 52%] of esophagus length, 125–170 (140; 4) [221, 292] wide or 33–46% (37%, 4) [74%, 49%] of maximum body width; post-cecal distance 1,211–1,460 (1,330; 3) [1,135, 2,025] or 62–66% (64%; 3) [61%, 60%] of BL from posterior body end (Figs. 5, 7).

Testis 718–1054 (871; 4) [595, 1,238] long or 37–42% (39%; 3) [32%, 37%] of BL, 183–302 (239; 4) [220, 480] wide or 58–67% (62%; 4) [73%, 80%] of body width or 3.5–3.9 (3.7; 4) [2.7, 2.6]× longer than wide, containing dense field of vasa efferentia intertwining among densely-packed testicular cells; testicular cells circular, each measuring 2 (2; 20) [2, 2] in diameter; post-testicular space 498–588 (539; 3) [430, 857] long or 26–27% (26%; 3) [23%, 26%] of BL. Vasa efferentia secondary ducts 2–13 (8; 15) [6{3}, 21{5}] wide, extending from lateral margins of testicular lobes (Fig. 5). Vas deferens a thin-walled duct, including a proximal portion ventral to testis and a post-testicular portion; proximal portion robust, comprising approximately 3–6 (5; 4) [3, 7] swollen trunks 9–40 (26; 17) [12{3}, 30{7}] wide, extending from lateral margins of testicular lobes before uniting in post-testicular portion; post-testicular portion 236–297 (259; 3) [172, 353] long or 11–13% (13%; 3) [9%, 11%] of BL, 44–65 (53; 4) [14, 95] wide at the level of posterior margin of testis, narrowing to 11–12 (11; 3) [12, 45] at level of ovary before curving sinistrad to connect with cirrus sac and internal seminal vesicle, ventral to ovary, containing sperm in all specimens (Figs. 5, 8, 9). Cirrus sac appendix-like, 159–178 (167; 3) [224, 343] long or 8% (8%; 4) [12%, 10%] of BL, 21–31 (26; 3) [27, 32] in maximum width or 7–8% (8%; 3) [9%, 5%] of body width, with glandular wall 1 (1, 3) [1, 1] thick (Figs. 5, 8, 9); internal seminal vesicle robust, occupying breadth and length of cirrus sac to varying degrees depending on amount of sperm present in duct, 15–21 (18; 3) [27, 14] in maximum width or 9.6–10.6 (8.5; 3) [8.3, 24.5]× longer that wide, extending toward sinistral margin before continuing posteriad approximately in parallel with sinistral body margin (Figs. 5, 8, 9); inverted cirrus opening in ventral view obliquely angled at ~70° from midline, 31–41 (35; 3) [not

inverted, 52] wide, 4–10 (7; 3) [not inverted, 5] height, opening 57–88 (76; 3) [not inverted, 102] or 21–23% (22%; 3) [not inverted, 17%] of maximum body width from sinistral body margin, 85–143 (117; 3) [not inverted, 145] or 31–37% (33%; 3) [not inverted, 24%] of maximum body width from dextral body margin, posterolateral to female genital pore; 162–192 (173; 3) [not inverted, 260] or 8–9% (8%; 3) [not inverted, 8%] of BL from posterior body end; everted cirrus proximal base [25, not everted] wide, distal portion [8, not everted] wide, opening [54, not everted] or [18%, not everted] of maximum body width from sinistral body margin, [87, not everted] or [29%, not everted] of maximum body width from dextral body margin, posterolateral to female genital pore; [124, not everted] or [7%, not everted] of BL from posterior body end.

Ovary divided into dextral and sinistral fields of dendritic branches, having approximately 7 (7; 2) [indistinct, 7] dextral and 4 (4; 2) [indistinct, 5] sinistral narrow branches each 2–3 (3; 33) [indistinct, 7 {10}] wide, branches may coalesce, dextral and sinistral fields measuring 133–155 (142; 2) [indistinct, 212] and 70–113 (92; 2) [indistinct, 141] in maximum length or approximately 7% (7; 2) [indistinct, 6%] and 4–6% (5%; 2) [indistinct, 4%] of BL, 165–253 (209; 2) [indistinct, 354] in maximum width or 60–67% (64%; 2) [indistinct, 59%] of body width, 1.6–1.9 (1.8; 2)× wider than long; post-ovarian space 379–387 (383; 3) [indistinct, 640] long or 19% (19%; 2) [indistinct, 19%] of BL. Oviduct curving sinistrally immediately posterior to ovary and lateral to vas deferens, 325–368 (367; 3) [326, 675] long or 17–18% (18%; 3) [17%, 20%] of BL, including an (i) abbreviated proximal duct, a (ii) dilated portion (= oviducal seminal receptacle), and a (iii) narrow distal portion; proximal duct emanating from

posteroventral surface of ovary extending sinistrally 27–38 (31; 3) [33, 75] with 8–12 (10; 3) [5, 8] maximum width, curving posteromediad to connect with oviducal seminal receptacle; oviducal seminal receptacle filled with sperm and ova in all specimens, 154–203 (182; 3) [153, 367] long or 47–51% (49%; 3) [47%, 54%] of total oviduct length, 23–79 (57; 3) [49, 134] wide or 5.2–14.1 (8.2; 3) [6.7, 5]× longer than wide, occupying space between vas deferens and sinistral body margin, crossing vas deferens dorsally, post-ovarian, distal portion of oviduct 7–8 (8; 3) [6, 11] or 9–35% (19%, 3) [12%, 8%] of oviducal seminal vesicle width, continuing posteriad approximately in parallel with dextral body margin before uniting with vitelline duct. Oötype 25–40 (31; 3) [23, 58] long, 12–26 (17; 3) [10, 23] wide, 1.5–2.1 (1.9; 3) [2.3, 2.5]× longer than wide, connecting with vitelline duct and oviduct dorso-posteriorly, slightly dextral, orienting diagonally (not parallel with longitudinal body axis); post-oötype distance 135–147 (140; 3) [95, 204] or 7% (7%; 3) [5%, 6%] of BL from posterior body end (Figs. 5, 8, 9). Uterus occupying space between oötype and cirrus sac; 179–230 (209; 3) [214, 280] long or 9–11% (10; 3) [11%, 8%], 17–21 (19; 3) [21, 36] in maximum width, beginning with an ascending uterus 89–127 (112; 3) [136, 172] long or 5–7% (6; 3) [7%, 6%] of BL, typically (3 of 5 specimens) convoluted dorsally along ascending segment length, descending uterus 71–81 (77; 3) [77, 107] long or 62–80% (70%; 3) [57%, 62%] ascending uterus length; uterine eggs thin-shelled, ovoid, 4–10 (6; 22) [no eggs, 15 {5}] long, 3–8 (5; 22) [no eggs, 11 {5}] wide. Female genital pore in ventral view obliquely angled at ~45° from midline, 37–55 (48; 3) [45, 71] wide, 2–5 (3; 3) [3, 5] height, 67–88 (81; 3) [70, 115] or 23–25% (23%; 3) [23%, 19%] of BL from sinistral body margin, 79–114 (98; 3) [79, 160] or 27–29% (28%; 3)

[26%, 27%] of BL from dextral body margin, 182–192 (188; 3) [120, 311] or 9–10% (9%; 3) [6%, 9%] of BL from posterior body end.

Excretory system indistinct.

Type and only host: Porthole shovelnose catfish, *Hemisorubim platyrhynchos* Valenciennes, 1840 (Siluriformes: Pimelodidae).

Type locality: Baía River, floodplain of upper Paraná River Basin, Brazil.

Other localities: Itaya River, Amazon River Basin, Iquitos, Peru.

Site in host: Body cavity.

Intensity of infection: 10 specimens infected 1 porthole shovelnose catfish.

Specimens examined: CHIOC paratypes 34361a and 34361b plus 4 stained, whole-mounted adult specimens from body cavity of porthole shovelnose catfish (field number PI 432a; 15 cm total length; collection date: 13 September 2006) in the Itaya River (03°45'60"S, 73°14'44"W), Amazon River Basin, near Iquitos, Peru.

Specimens deposited: USNM Coll. Nos. 1254658 and 1254659 (2 vouchers); CHIOC No. 38217 (1 voucher).

Remarks

Our observations of the paratypes and newly-collected specimens of *Kritsky platyrhynchi* n. gen., n. comb. differed from the original description in several regards. Guidelli et al. (2002) did not report observations of an anterior sucker but one is clearly present, albeit comprising the typically diminutive anterior sucker of fish blood flukes. They described the mouth as “apical and very small, surrounded by 4 rows of denticles” (see Guidelli et al. [2002], fig. 2d). They related these sclerites with those of *Plehniella dentata* Paperna, 1964 (very likely a species of *Nomasanguinicola*, see

Truong and Bullard [2013]). Truong and Bullard (2013) argued that all clariid aporocotylids (Siluriformes: Clariidae) (i.e., *Nomasanguinicola canthoensis* Truong and Bullard, 2013, *P. dentata*, and *Sanguinicola clarias* Imam, El-Askalany, Hassan, and Itman, 1984) are congeners and likely have 2 columns of 4 denticles each that flank the mouth. These denticles should not be confused with the minute, straight spines arranged in 4 concentric rows described herein (see Truong and Bullard [2013] for a discussion on homology of spines). We regard the presence of this spinous anterior sucker as an important generic feature differentiating *K. platyrhynchi* from members of *Nomasanguinicola*, *Plehniella*, and *Sanguinicola*. Guidelli et al. (2002) described the diminutive pharynx as a “muscular organ” posterior to the mouth and summarized published descriptions of the pharynx in *S. inermis* Plehn, 1905, *S. argentinensis* Szidat, 1951, and *P. coelomicola* Szidat, 1951. The pharynx of the *K. platyrhynchi* resembles that of *N. canthoensis* and other species of *Plehniella* (Truong and Bullard, 2013; Orélis-Ribeiro and Bullard, 2015). Guidelli et al. (2002) indicated the presence of the posterior esophageal swelling as “enlarged and surrounded by numerous cells.” We confirmed that the anterior esophageal swelling and its associated gland is present in all of the specimens we studied. Guidelli et al. (2002) described the vas deferens as “directed toward the posterior extremity of the body parallel and to the left of the uterus.” The paratypes and newly-collected specimens clearly show that the vas deferens is dextral to the oviducal seminal receptacle (Figs. 5, 8, 9). In addition, Guidelli et al. (2002) described the vas deferens as, “bending to the right and again to the medial region of the body.” The vas deferens curves sinistrad once before connecting with a cirrus sac and an internal seminal vesicle

(Figs. 5, 8, 9). Guidelli et al. (2002) described the female genital pore as dorsal to the ovary (see Guidelli et al.'s [2002] figs. 2a, c) but in all specimens we studied the pore is clearly lateral to the male genital pore (Figs. 5, 8, 9). Perhaps related to this error, Guidelli et al. (2002) described the oviducal seminal receptacle as a distal portion of the uterus; the vitelline duct as the oviduct; and the uterus as a "prolonged and ascending seminal receptacle."

DISCUSSION

The species and genus-level taxonomic diversity of catfish blood flukes (Table I) and the large proportion of catfishes that have yet to be thoroughly examined for infections together underscore the potential for aporocotylid species discovery. Including the present study, 9 species of blood flukes in 4 genera infect 11 catfishes of 7 genera among Auchenipteridae, Clariidae, Claroteidae, Mochoidae, and Pimelodidae (Table I). Yet, no data are available for blood fluke infections in some species-rich catfish families. For example, the Neotropical family Loricariidae (907 species; Eschmeyer and Fong, 2015) is the fifth most diverse vertebrate family (Lujan et al., 2015) but lacks a single blood fluke record. This comprises a markedly under-sampled fish lineage for blood fluke infections.

Like in other fish blood fluke lineages (Bullard et al., 2008; Bullard and Overstreet, 2004; Bullard and Overstreet, 2006; Orélis-Ribeiro et al., 2013; Bullard, 2014), morphologically-similar blood flukes seemingly infect phylogenetically-related catfishes (Orélis-Ribeiro and Bullard, 2015). The present study revealed some

uncanny similarities between *K. platyrhynchi* and *Plehniella* spp., all of which infect body cavity of South American pimelodid catfishes (Remarks; Table I); for example, Truong and Bullard (2013) reported that congeneric aporocotylids infect walking catfishes (Clariidae) (see Table I).

Although redescriptions of *P. dentata* and *S. clarias* are needed, the presence of 2 columns of 4 denticles flanking either side of the mouth of those blood flukes and *N. canthoensis*, likely indicate that they are congeners. Moreover, recollection and redescription of new specimens of *Sanguinicola chalmersi* Odhner, 1924 sourced from the type host and type locality in Africa may result in its assignment to a new genus. If so, the blood flukes of African catfishes (a multifamily clade informally named by Sullivan et al. [2006] as “Big Africa”) may also comprise a taxonomically diverse and closely related group. Likewise, the relationship between catfish ancestry and the evolution of their blood flukes will be greatly advanced by examining additional South American catfishes for infections.

Because catfishes range or have ranged on all continents, including Antarctica (Grande and Eastman, 1986; Armbruster, 2011), focused collections targeting body cavity and vascular system of extant species for the presence of blood fluke infections could provide data to test biogeographic hypothesis regarding intra- and inter-continental relationships among the catfish blood flukes. The South American catfish blood flukes *C. tomasscholzi* and *K. platyrhynchi* and the African-Asian catfish blood flukes *N. canthoensis*, *P. dentata*, and *S. clarias* have a combination of a minute pharynx, an esophagus with anterior and posterior esophageal swellings enveloped by esophageal glands, a single testis that is pre-ovarian, a cirrus sac, a butterfly wing-

shaped ovary, a post-ovarian uterus having ascending and descending segments, and separate genital pores as well as lacking a Laurer's canal. Thus, available morphological evidence suggests that the ancestor of those catfish blood flukes infected a Gondwanaland catfish.

On a continental scale, we report the first locality record of *K. platyrhynchi* from the upper Amazon Basin (Itaya River, Peru), approximately 3,000 km away from its type locality in the upper Paraná Basin (Baía River, Brazil). This distribution is consistent with the existence of a dispersal route between the Amazon and Paraná basins (Hubert and Renno, 2006; Hubert et al., 2007). Given that no catfish blood fluke life cycle has been demonstrated, the identity of the molluscan (or polychaete) intermediate host represents an obvious gap in our knowledge that should be closed in order to test hypotheses concerning their biogeography: a given blood fluke's distribution may just as well be explained by the geographic distribution of its intermediate host(s).

Most blood flukes infect a variety of sites within the blood vascular system; many fewer infect body cavity. In fact, these are the only blood flukes (Schistosomatoidea) that mature outside of the circulatory system. *Kritsky platyrhynchi* and *Plehniella* spp. represent 2 lineages of freshwater fish blood flukes that reportedly infect the body cavity of South American pimelodids (see Table I). *Deontacylix* spp., which are morphologically unlike the aforementioned genera in many ways, are the only lineage of marine fish blood flukes that infect the body cavity of sea chubs: *Deontacylix ovalis* Linton, 1910 (type species), from yellow sea chub, *Kyphosus incisor* Cuvier, 1831; Bermuda sea chub, *K. sectatrix* Linnaeus, 1758, and Cortez sea chub, *K. elegans*

Peters, 1869 (Linton, 1910; Manter, 1947; Léon-Régagnon et al., 1997); and *D. kyphosi* Yamaguti, 1970, from blue sea chub, *K. cinerascens* Forsskål, 1775 (Yamaguti, 1970). Such a departure from infecting circulatory system likely has a physiological basis and associated advantages and disadvantages that would be exciting to explore. For example, perhaps body cavity flukes avoid the host immune response but have access to less host resources, given that they are no longer associated with blood. Such studies would require that we learn what fish blood flukes in both sites eat. Noteworthy also is that no information exist regarding the sites of egg deposition from any of these lineages. Szidat (1951) pointed out that, although he had thoroughly searched for eggs of *P. coelomicola* in the kidney and gill of fishes infected by adult flukes, he observed undeveloped eggs in the body cavity only; however, he did not detail the disposition or location of undeveloped eggs in the body cavity. Perhaps there are significant advantages to infecting body cavity with respect to egress of eggs through the host's intestine, which would apparently represent a significant evolutionary departure from that of other fish blood flukes wherein miracidia are assumed to hatch from eggs embedded in gill epithelium and bore out of the fish (references in Bullard and Overstreet, 2008; Orélis-Ribeiro et al., 2014).

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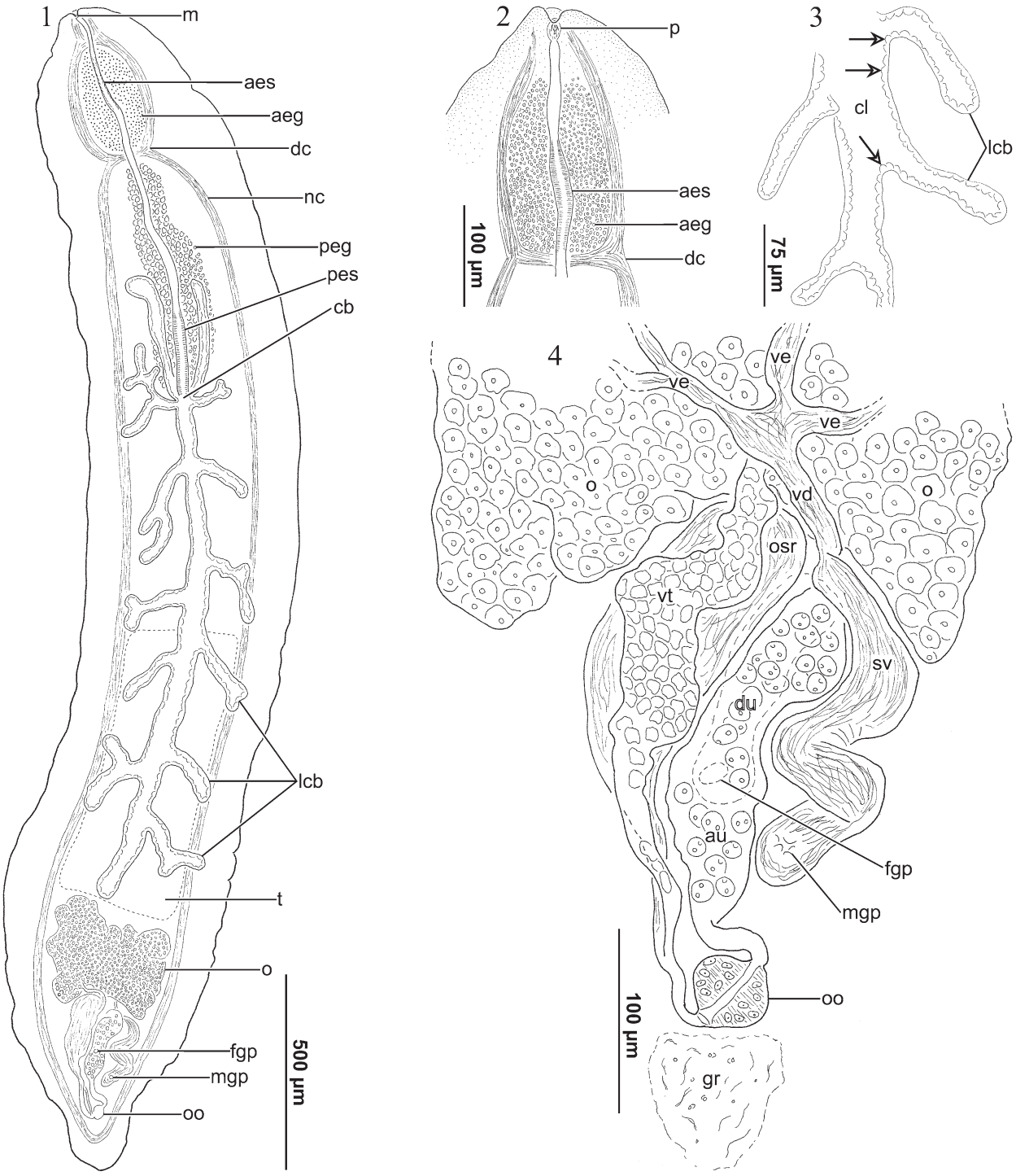
FIGURE LEGENDS

Figures 1–4. *Cladocaecum tomasscholzi* n. gen., n. sp. (Digenea: Aporocotylidae) from the heart of the driftwood catfish *Ageneiosus inermis* Linnaeus, 1766 (Siluriformes: Auchenipteridae), from the Nanay River, Peru. Ventral view. Holotype USNM Coll. No. 1254657. Scale values aside each bar. **(1)** Body of adult showing mouth (m), anterior esophageal swelling (aes), anterior esophageal gland (aeg), anterior commissure of dorsolateral nerve cord (dc), dorsolateral nerve cord (nc), posterior esophageal gland (peg), posterior esophageal swelling (pes), esophagus (e), cecal bifurcation (cb), lateral cecal branches (lcb), putative testis (t, dashed), ovary (o), female genital pore (fgp), male genital pore (mgp), oötype (oo) **(2)** Anterior end showing mouth residing within anterior concavity and minute, weakly-muscular pharynx (p) enveloping extreme distal end of esophagus. **(3)** Laterally extending cecal branches (lcb) showing mononucleate cells (arrows) lining the inner walls of the cecal lumen (cl). **(4)** Genitalia showing vasa efferentia (ve), seminal vesicle (sv), male genital pore (mgp), ovary (o), vitelline duct (vt), oviducal seminal receptacle (osr), oötype (oo), glandular region (gr), ascending uterus (au), descending uterus (du, dashed) and female genital pore (fgp).

Figures 5–7. *Kritsky platyrhynchi* n. gen., n. sp. (Digenea: Aporocotylidae) from the body cavity of porthole shovelnose catfish, *Hemisorubim platyrhynchos* Valenciennes, 1840 (Siluriformes: Pimelodidae), from the Itaya River, Peru. Scale values aside each bar. **(5)** Body of adult specimen (USNM Coll. No. 1254658) showing location of anterior sucker (as), esophagus (e), anterior esophageal gland (aeg), anterior esophageal swelling (aes), anterior commissure of dorsolateral nerve cord (dc), dorsolateral nerve cord (nc), posterior esophageal gland (peg), posterior esophageal swelling (pes), vittelarium (v), ceca (c), testis (t), vasa efferentia (ve), vas deferens (vd), ovary (o), oviducal seminal receptacle (osr), cirrus sac (cs), internal seminal vesicle (isv), male genital pore (mgp), uterus (u), oötype (oo), vitelline duct (vt), female genital pore (fgp); ventral view. **(6)** Anterior end of voucher specimen (USNM Coll. No. 1254659) showing row of spines (s), mouth (m), pharynx (p), esophagus (e); dorsal view. **(7)** High magnification view of ceca of voucher specimen (USNM Coll. No. 1254659) showing posterior esophageal gland (peg), posterior esophageal swelling (pes), ceca (C1–C6); dorsal view.

Figures 8–9. Genitalia of *Kritsky platyrhynchi* n. gen., n. sp. (Digenea: Aporocotylidae) from the body cavity of porthole shovelnose catfish, *Hemisorubim platyrhynchos* Valenciennes,1840 (Siluriformes: Pimelodidae), dorsal views. Scale values aside each bar. Oviducal seminal receptacle (osr), vitelline duct (vt), oötype (oo), ascending uterus (au), descending uterus (du), female genital pore (fgp), vas deferens (vd), internal seminal vesicle (isv), cirrus sac (cs), male genital pore (mgp), everted cirrus (ec), opening of sperm tube (ost). **(8)** Voucher specimen (USNM Coll. No. 1254659) from Itaya River, Peru. **(9)** Paratype (CHIOC 34361a) from Baía River, Brazil.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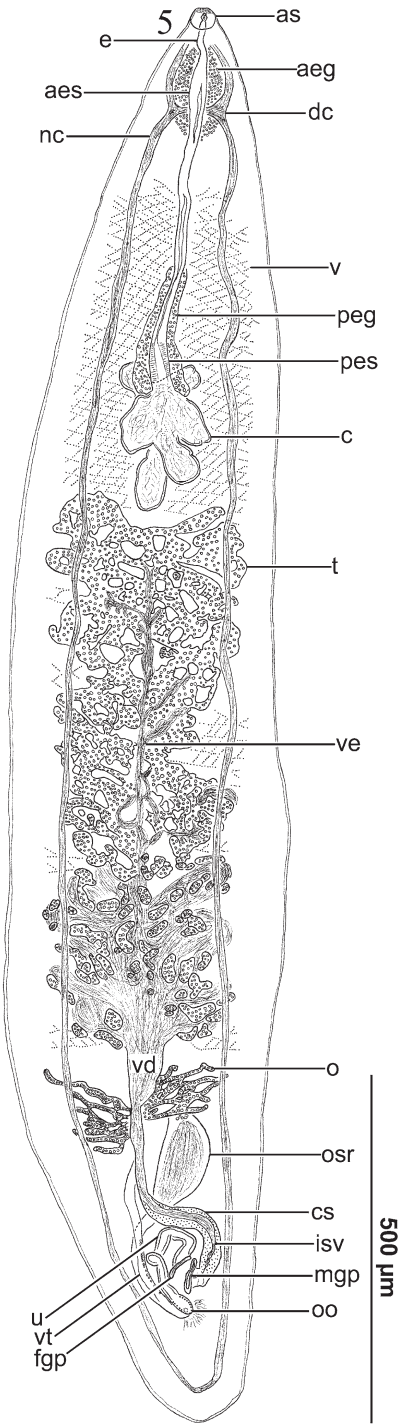
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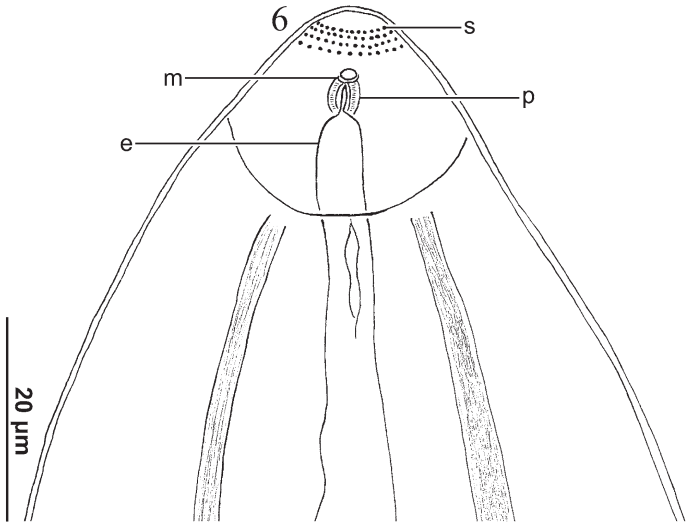
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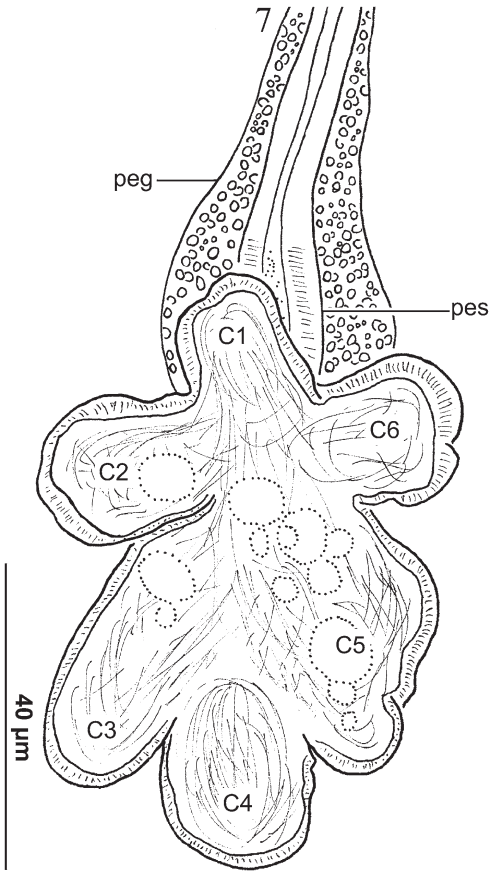
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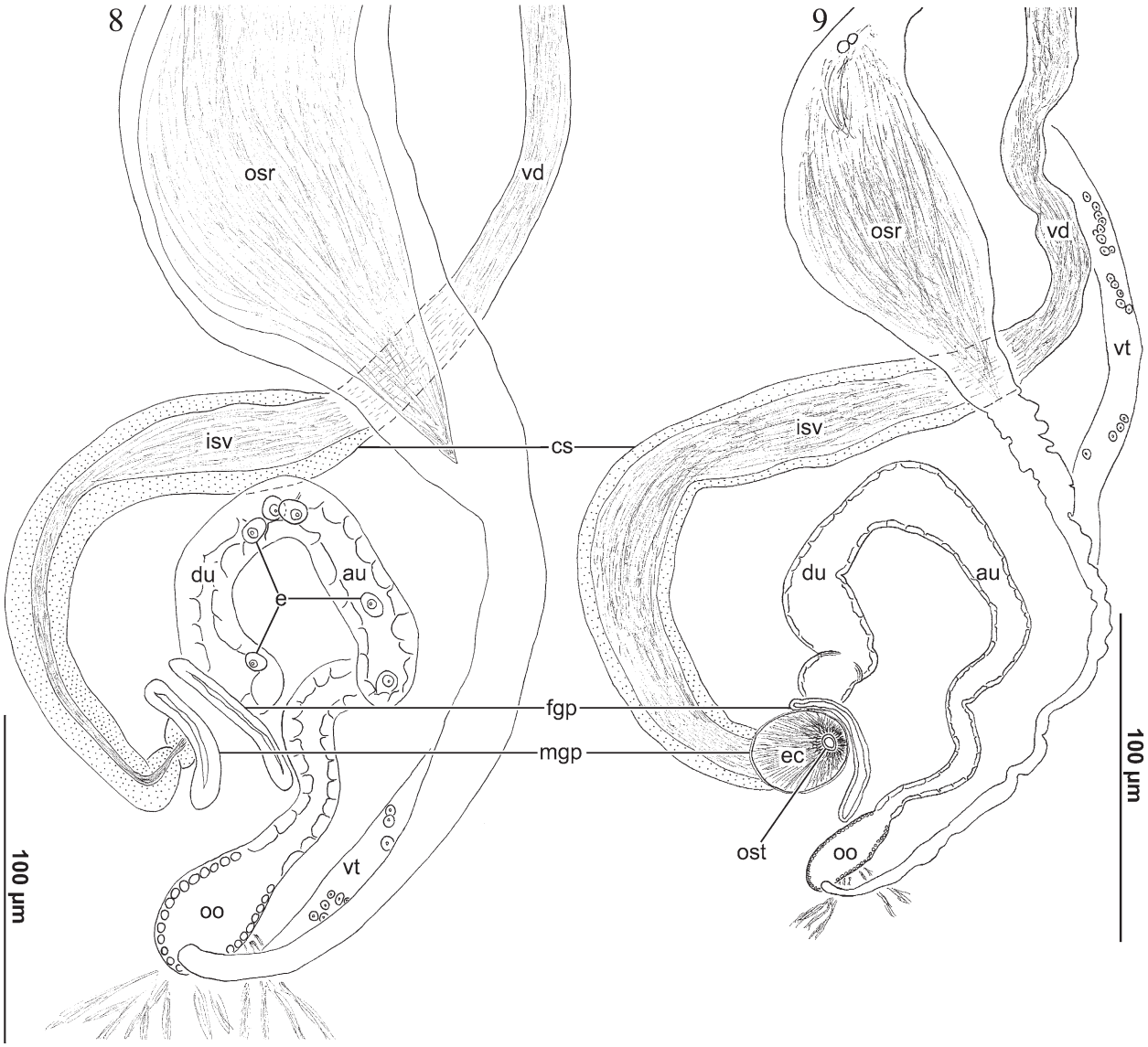
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Table 1. Blood flukes (Digenea: Aporocotylidae) of catfishes (Siluriformes).

Aporocotylid	Host	Site	Locality	Reference(s)
Cladocaecum tomasscholzi n. gen., n. sp.	*Ageneiosus inermis* Linnaeus, 1766 (Auchenipteridae)	Ventricle in heart	Nanay River, Peru	present study
Kritsky platyrhynchi (Guidelli, Isaac, and Pavanelli, 2002) n. gen., n. comb. (originally as *Sanguinicola*)	*Hemisorubim platyrhynchos* Valenciennes, 1840 (Pimelodidae)	body cavity	Baía River, Brazil	Guidelli et al., 2002; 2003 (as *Plehniella* in Truong and Bullard, 2013)
		body cavity	Itaya River, Peru	present study
Nomasanguinicola canthoensis Truong and Bullard, 2013	*Clarias macrocephalus* Günther, 1864 (Clariidae)	branchial vessels	Can Tho fish market, Vietnam (Mekong River)	Truong and Bullard, 2013
Plehniella armbrusteri Orélis-Ribeiro and Bullard, 2015	*Pimelodus blochii* Valenciennes, 1840 (Pimelodidae)	body cavity	Lake Tumi Chucua, Bolivia	Orélis-Ribeiro and Bullard, 2015
Plehniella coelomicola Szidat, 1951	*Iheringichthys labrosus* Lütken, 1874 (Pimelodidae)	body cavity	La Plata River, Argentina	Szidat, 1951
	Pimelodus albicans Valenciennes, 1840 (Pimelodidae)	body cavity	La Plata River, Argentina	Lunaschi, 1985; Avendaño de Mac Intosh and Ostrowski de Núñez, 1998
	Pimelodus maculatus Lacépède, 1803 (Pimelodidae) (as *P. clarias*)	body cavity	La Plata River, Argentina	Lunaschi, 1985
		body cavity	La Plata River, Argentina	Avendaño de Mac Intosh and Ostrowski de Núñez, 1998
		body cavity	Paraná River Basin, Brazil	Brasil-Sato and Pavanelli, 2004; Takemoto et al., 2009
		body cavity	São Francisco River Basin, Brazil	Brasil-Sato, 2003; Brasil-Sato and Pavanelli, 2004
Plehniella dentata Paperna, 1964 *incertae sedis* (likely a species of *Nomasanguinicola*)	*Clarias gariepinus* Burchell, 1822 (Clariidae) (as *C. lazera*)	"intestine" (probably mesenteric vessels)	Lake Tiberias and Hule Nature Reserve, Israel	Paperna, 1964; Truong and Bullard, 2013

Plehniella sabajperezi Orélis-Ribeiro and Bullard, 2015	*Pimelodus albofasciatus* Mees, 1974 (Pimelodidae)	body cavity	Demerara River, Guyana	Orélis-Ribeiro and Bullard, 2015
		body cavity	Rupununi River, Guyana	Orélis-Ribeiro and Bullard, 2015
	Pimelodus blochii Valenciennes, 1840 (Pimelodidae)	body cavity	Lago Tumi Chucua, Bolivia	Orélis-Ribeiro and Bullard, 2015
		body cavity	Napo River, Peru	Orélis-Ribeiro and Bullard, 2015
Plehniella sp.	*Pimelodus grosskopfii* Steindachner, 1879 (Pimelodidae)	body cavity	Cienega de Jobo and Canal del Dique, Colombia	Orélis-Ribeiro and Bullard, 2015
Sanguinicola chalmersi Odhner, 1924	*Auchenoglanis occidentalis* Valenciennes, 1840 (Claroteidae)	blood, heart	Sudan, Africa	Woodland, 1923; Odhner, 1924; Khalil, 1969, 1971; Paperna, 1996
	Synodontis schall Block and Schneider, 1801 (Mochoidae)	mesenteric and branchial blood vessels	Cairo and Giza fish markets, Egypt	Woodland, 1923; Imam et al., 1984
Sanguinicola clarias Imam, Marzouk, Hassan, and Itman, 1984 *incertae sedis* (likely a species of *Nomasanguinicola*)	*Clarias gariepinus* Burchell, 1822 (Clariidae) (as *C. lazera*)	"mesenteric and other blood vessels"	Cairo and Giza fish markets, Egypt	Imam et al., 1984; Truong and Bullard, 2013
		not specified	Beni-Suef fish market, Egypt	Imam and El-Askalany, 1990

CHAPTER 5: BLOOD FLUKES (DIGENEA: APOROCOTYLIDAE) OF ELOPIFORMES: TWO NEW SPECIES OF ELOPICOLA SPP. FROM HAWAIIAN LADYFISH, *ELOPS HAWAIENSIS* AND TARPON, *MEGALOPS ATLANTICUS* AND THEIR PHYLOGENETIC RELATIONSHIPS WITH OTHER BLOOD FLUKES (SCHISTOSOMATOIDEA)

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ABSTRACT

We emend the original generic diagnosis for *Elopicola* Bullard, 2014, based on a collection from elopiform fishes. *Elopicola* n. sp. 1 infects the Hawaiian ladyfish, *Elops hawaiensis* (Elopiformes: Elopidae), in the South China Sea off Nha Trang, Vietnam. The new species is most easily differentiated from *Elopicola nolancribbi*, the only nominal congener, by the presence of an enantiomorphic terminal genitalia. *Elopicola* n. sp. 1 further differs from *E. nolancribbi* by the combination of having rows of tegumental body spines in adults and juveniles, pharynx width that is 7–13% of body width, oesophagus that is 41–47% of body length, short lobes of caeca directing anteriad present in adults, large juveniles, and schistosomula, testis that occupies most of the intercaecal space, vasa efferentia coalescing in postero-dextral region of testis to form vas deferens, ootype that is located well posterior to the testis, and common genital pore post-testicular, and at the level of ootype. *Elopicola* n. sp. 2 infects the tarpon, *Megalops atlanticus* (Elopiformes: Megalopidae), in the Gulf of Mexico off Tampa Bay, Florida, USA. It differs from *Elopicola* n. sp. 1 and *E. nolancribbi* by having a body 6× longer than wide, an anterior sucker that is 33–38% of body width, an oesophagus that

is 49–62% of total body length, a testis that has its anterior half occupying intercaecal space and a size that is 11–12% of body length, and a common genital pore post-testicular, at the level of ootype. Phylogenetic analyses based on three ribosomal genes (18S, ITS, and 28S rDNA) support blood flukes infecting elopomorph fishes as a monophyletic, early-diverging lineage that is sister to the blood flukes infecting marine bony fishes. This report brings the total number of nominal Gulf of Mexico and South China Sea aporocotylids to 15 and 3, respectively. In the light of our results, we discuss the relevance of exploring the role of enantiomorphism in the evolution of elopiform blood flukes as well as the potential of *Elopicola* spp. as biological tags.

1. INTRODUCTION

At present, only 3 of ~142 nominal species of fish blood fluke (Digenea: Aporocotylidae) assigned to 3 genera reportedly mature in early-branching lineages of ray-finned fishes (Chordata: Actinopterygii). Specifically, infections of aporocotylids have been described from one early-branching actinopterygian fish (Chondrostei: Acipenseriformes) plus two early-branching teleost fishes (Teleostei: Elopomorpha) of three genera (Table 1). Most of these fish lineages have recreational and commercial value. A remarkable example is the tarpon, *Megalops atlanticus* (Valenciennes, 1847), one of the first saltwater species to be declared a game fish (IGFA 2012). As such, sport fishing of this highly sought-after species have generated an economic impact of $6 billion USD and supported approximately 100,000 jobs in Gulf of Mexico and southeastern U.S. (Ault and Luo 2013). Noteworthy is that, despite the research

attention and conservation concerns (Levesque 2011) directed to this species, no information exists on their blood parasites.

In an evolutionary biology perspective, this particular knowledge gap in the taxonomic diversity of fish blood flukes has hampered our understanding of the evolutionary relationships within Schistosomatoidea (Bullard et al. 2008, Orélis-Ribeiro et al. 2014). Morphological evidence not only suggests that the blood flukes infecting all those early-diverging host lineages share a common ancestor, but also indicates that they might have a close association with tetrapod blood flukes (paraphyletic "spirorchiids" plus schistosomes) (Bullard et al. 2008). However, no previous morphology or molecular-based phylogeny has ever attempted to infer those relationships (Orélis-Ribeiro et al. 2014).

Herein we describe two new species of *Elopicola* – one from Hawaiian ladyfish, *Elops hawaiensis* and the other from the tarpon, *Megalops atlanticus*; and emend the diagnosis of the genus. Phylogenetic analyses based on three nuclear ribosomal genes (18S, ITS, and 28S) were used to support the taxonomic identity and phylogenetic placement of the new taxa. The present study comprises the second and third species of fish blood fluke reported from elopiforms. In addition, the new species from Hawaiian ladyfish is the third record of an aporocotylid from the South China Sea, whereas the new species from tarpon is the fifteenth report of fish blood flukes from the Gulf of Mexico.

2. MATERIALS AND METHODS

2.1 Specimen collection and preparation

Six specimens of Hawaiian ladyfish were captured from the South China Sea before being purchased fresh from Chợ Vĩnh Hải fish market (12°16'44.8"N; 109°11'38.9"E) in Nha Trang, Vietnam and necropsied between 1–16 June 2015. To optimize the duration of dissection efforts, infected individual Hawaiian ladyfish were first identified by searching for aporocotylid eggs lodged in the gill epithelium, which were examined as wet-mounts with the aid of a compound microscope at ×200–400 magnification. Heart, gill arches and associated branchial vessels were each removed and examined separately in petri dishes for the presence of adult and juvenile flukes before the remaining contents of each dish were transferred to pilsner glasses, allowed to settle for 15 min, decanted, and re-examined. In addition, sediment derived from rinsing macerated head, trunk, and body cavity were also examined. Flukes intended as whole-mounts were transferred to a vial of 10% neutral buffered formalin (n.b.f.). Specimens intended for DNA analysis were preserved in 100% EtOH and stored stored at −20 °C.

Three specimens of tarpon were opportunely sampled from the Gulf of Mexico off Tampa Bay (Florida, USA) during investigations conducted by the Florida Fish and Wildlife Conservation Commission's (FWC) Fish and Wildlife Research Institute (FWRI). Flukes were collected exclusively from the heart and fixed as described above.

Morphological specimens were rinsed thoroughly with distilled water and cleaned with fine brushes to remove any debris, stained overnight in Van Cleave's hematoxylin with several additional drops of Ehrlich's hematoxylin, made basic in 70% ethanol with lithium carbonate and butyl-amine, dehydrated, cleared in clove oil, and permanently mounted in Canada balsam. Illustrations of stained, whole-mounted specimens were

made with the aid of a Leica DM-2500 (Leica, Wetzler, Germany) equipped with differential interference contrast (DIC) optical components and a drawing tube. Measurements were obtained by using a calibrated ocular micrometer (as straight-lines along the course of each duct) and are herein reported in micrometers (μm) followed by their mean and the number measured in parentheses. Scientific names including taxonomic authorities and dates for fishes follow Eschmeyer et al. (2016). Common names are taken from FishBase (Froese and Pauly 2016). Higher level fish classification and nomenclature follows Nelson (2006). Nomenclature for Aporocotylidae follows Bullard et al. (2009). Brown (1956) was used to help construct the genus name and specific epithet. Type and voucher materials are deposited in the United States National Museum (USNM, Washington, D.C.).

2.2 DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from individual adult flukes sourced from two specimens of the new species from Hawaiian ladyfish and one specimen from the new species from tarpon plus one specimen of the aporocotylid *Acipensericola petersoni*, *Elopicola nolancribbi*, and 1 specimen of the turtle blood fluke *Baracktrema obamai* (Table 2) using DNeasy^{TM} Blood and Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer-recommended protocol, except for the incubation period with proteinase-K that was extended to overnight and the final elution step wherein only 100 μl of elution buffer was used, in order to increase the final DNA concentration in the eluate. Extraction products served as templates for the amplification of the 18S, ITS2, and 28S rDNA genes using the set of primers described in Table 3. PCR amplifications were

carried out in a total volume of 25 µl containing approximately 2 µl of DNA template, 0.4 µM of each primer along with 1× buffer, 2.5 mM $MgCl_2$ (New England Biolabs, Ipswich, MA), 1 mM dNTP mixture, and 0.3 µl *Taq* polymerase (5 U/µl) (New England Biolabs, Ipswich, MA). The 18S and 28S rDNA amplification reactions were performed with a cycling profile of 4 min at 94°C for initial denaturation, followed by 40 repeating cycles of 94°C for 40 s for denaturation, 50°C for 30 s for annealing, and 72°C for 2 min for extension, followed by a final 7 min at 72°C for extension. The ITS2 rDNA amplification reactions were performed with a cycling profile of 4 min at 94°C for initial denaturation, followed by 40 repeating cycles of 94°C for 40 s for denaturation, 50°C for 30 s for annealing, and 72°C for 1 min for extension, followed by a final 5 min at 72°C for extension. All PCR reactions were performed in a Veriti Thermal Cycler (Applied Biosystems). PCR products (5 µl) were verified on a 1 % agarose gel and stained with ethidium bromide. PCR amplicons were gel-excised using QIAquick™ Gel Extraction Kit (QIAGEN) following the manufacturer's protocol. DNA sequencing was performed by GENEWIZ with ABI Prism 3730xl DNA analyzer (GENEWIZ, Inc., South Plainfield, NJ). 18S, ITS2, and 28S genes were sequenced using PCR primers and some additional internal forward primers listed in Table 3. Analysis of chromatograms of the forward and reverse DNA strands, as well as sequence assembling and editing were conducted using BioNumerics version 7.0 (Applied Maths, Sint-Martens-Latem, Belgium). New sequences generated by this work were submitted to GenBank (Table 3). A hologenophore (*sensu* Pleijel et al. 2008) of the new species from Hawaiian ladyfish is deposited in the United States National Museum (USNM, Washington, D.C.).

2.3 Sequence alignments and phylogenetic analyses

Two datasets were assembled for the phylogenetic analyses. To test the monophyly of aporocotylids from early-branching lineages of ray-finned fishes and their relationships with turtle blood flukes, the first dataset (hereafter referred as 18S+28S dataset) comprised a combined matrix of near-complete18S and partial 28S sequences derived from the newly sequenced taxa plus publicly available data comprising a holocephalan blood fluke, a putative cypriniform blood fluke (i.e., *Sanguinicola* cf. *inermis*; see discussion on Orélis-Ribeiro et al. 2014), and marine euteleost blood flukes. Outgroup selection for this dataset was based on the phylogeny of the Digenea published by Olson et al. (2003) and included representatives of the superfamily Diplostomoidea. The second dataset (ITS2 dataset) aimed to test the monophyly of the elopomorph blood flukes and comprised complete ITS2 sequences derived from the newly sequenced taxa plus an elopomorph blood fluke, *Paracardicoloides yamagutii*. The outgroup for this dataset comprised publicly available sequences of marine euteleost blood flukes (Table 2).

For each dataset, sequences were aligned using MAFFT (Katoh and Toh 2010) with default settings implemented in the CIPRES Science Gateway V. 3.3 (Miller et al. 2010). The resulting alignment was refined by eye using MEGA version 5.2.2 (Tamura et al. 2011) and ends of each fragment were trimmed to match the shortest sequence. The concatenated alignment (18S–28S) was created using the web application FaBox 1.35 (Villesen 2007). Ambiguous positions in the single gene alignments were identified and removed using the Gblocks server (Castresana 2000) with settings for a less stringent selection. Bayesian inference (BI) was performed using the Metropolis-coupled Markov

chain Monte Carlo method (MC^3) in MrBayes version 3.2.6 (Huelsenbeck et al. 2001, Ronquist and Huelsenbeck 2003, Huelsenbeck and Ronquist 2005) and run on CIPRES (Miller et al. 2010). Model of evolution was selected based on the Akaike Information Criterion (Posada and Buckley, 2004) as implemented in the jModelTest version 2.1.4 (Darriba et al. 2012; Guindon and Gascuel, 2003). The GTR + I + G (proportion of invariable sites = 0.430 and gamma distribution = 0.849) and the TVM+ G (gamma distribution = 0.743) models were inferred as the best estimator for 18S+28S dataset and ITS2 dataset, respectively; therefore, BI used for 18S+28S dataset the following parameters: nst = 6, rates = invgamma, ngammacat = 4, and default priors; and for the ITS2 dataset the following parameters: nst = 6, rates = gamma, ngammacat = 4, and default priors. Analyses were run in duplicate each containing four independent chains (three heated and one cold chain) (nchains = 4) for 1.0×10^7generations (ngen = 10,000,000) sampled at intervals of 1000 generations (samplefreq = 1000). Results of the first 2500 sampled trees were discarded as burn-in based on the stationarity of the likelihood values, assessed by plotting the log-likelihood values of the sample points against generation time using Tracer version 1.5 (Rambaut and Drummond 2009). All retained trees were used to estimate posterior probability of each node. A majority rule consensus tree with average branch lengths was constructed for the remaining trees using ‘summarize the trees’ (sumt) in MrBayes. Additionally, a maximum likelihood (ML) analysis was performed on both datasets in RAxML v.7.2.6 (Stamatakis 2006) and also performed on CIPRES (Miller et al. 2010), with default parameters. GTRGAMMA model was employed for both datasets. Bootstrap values were estimated from 1,000 replicates. Resulting phylogenetic trees were visualized using FigTree v1.4.2 (Rambaut

2009) and further edited with Adobe Illustrator CS3 (Adobe Systems). Branch supports for BI and ML analyses were considered as significant when posterior probabilities were >0.95 and bootstrap values were >70%, respectively.

3. RESULTS

3.1 Elopicola Bullard, 2014, emended

3.1.1 Diagnosis

Body flat, oval, ventrally concave, lacking posterolateral body protuberance, anterior and posterior ends tapering approximately equally; tegumental body spines in adult and juvenile specimens minute, straight, delicate and barely discernable with light microscopy in juveniles, lacking recurved tip, distributing in ventrolateral transverse rows or not, enveloped by tegument. Rose thorn-shaped spines absent. Nervous system with dorsolateral nerve cords and commissure; dorsolateral nerve cord paired, contiguous anteriorly and posteriorly. Anterior sucker bowl-shaped, not comprising a spheroid anterior sucker with a medio-ventral mouth, demarcated from anterior body end by peduncle, aspinous in adult. Muscular pharynx occupying space between anterior sucker and nerve commissure. Oesophagus sinuous, ventral to anterior nerve commissure, extending sinuously posteriad approximately 1/3–1/2 of body length, comprising morphologically distinctive anterior, middle, and posterior regions; anterior and posterior portions of oesophagus comprising distinctive swellings. Intestine inverse U-shaped or with abbreviated lobes of cecum directing anteriad in adult and juvenile specimens, with long posterior caeca, lacking diverticula or secondary rami, extending

sinuously posteriad but lacking loops or coils, terminating in middle third of body. Testis single, medial, intercaecal, pre-ovarian, deeply lobed; vasa efferentia coalescing in antero-sinistral or postero-dextral region of testis to form vas deferens; vas deferens extending posteriad before narrowing distally and connecting to cirrus sac. Auxiliary external seminal vesicle absent. Cirrus sac dextral or sinistral, pre-ovarian, enclosing internal seminal vesicle and cirrus; cirrus everting dorsally near midline or dextrally, post-caecal. Ovary single, medial, post-caecal, post-testicular, occupying posterior body extremity. Oviduct transverse, post-caecal; oviducal seminal receptacle present. Vitellarium an extensive network of narrow, interconnecting, branching bands having granular vitelline material, extending laterad beyond ventrolateral nerve cords, occupying space from anterior nerve commissure posteriad to level of distal tips of posterior caeca; primary vitelline duct extending posteriad near dextral or sinistral body margin or slightly ventral to testis, curving mediad before uniting with oviduct to form ovo-vitelline duct. Ovo-vitelline duct short, transverse, connecting with ootype laterally. Ootype pre-ovarian. Uterus comprising short ascending and descending portions, pre-ovarian, post-caecal; uterine eggs having tetrahedral body, with elongate polar filaments. Metraterm pre-ovarian, medial to cirrus sac. Male and female reproductive tracts opening into common atrium and sharing a common pore; common genital pore dorsal, dextral or sinistral, post-caecal. Excretory vesicle prominent posteriorly, Y-shaped; excretory pore dorsal, subterminal. Adults infecting members of Elopiformes.

3.1.2 Differential diagnosis

Body lacking posterolateral body protuberance. Anterior sucker bowl-shaped, demarcated from anterior body end by peduncle, aspinous. Muscular pharynx occupying space between anterior sucker and nerve commissure. Oesophagus having anterior and posterior swellings. Intestine inverse U-shaped or with abbreviated lobes of cecum directing anteriad. Testis single, pre-ovarian, deeply lobed; vasa efferentia coalescing in antero-sinistral or postero-dextral region of testis to form vas deferens; vas deferens extending posteriad before narrowing distally and connecting to cirrus sac. Cirrus sac present. Ovary post-caecal, post-testicular, occupying posterior body extremity. Oviducal seminal receptacle present. Ovo-vitelline duct short, transverse, connecting with ootype laterally. Ootype pre-ovarian. Uterus pre-ovarian, post-caecal; uterine eggs having tetrahedral body, with elongate polar filaments. Male and female reproductive tracts opening into common atrium and sharing a common pore.

Type species: *Elopicola nolancribbi*

3.1.3 Remarks

Elopicola is most easily differentiated from all other nominal aporocotylid genera by a combination of morphological features associated with the anterior sucker, tegumental body spines, intestine, and gonads. The anterior sucker of *Elopicola* is bowl-shaped. *Elopicola* has tegumental body spines (when present, in juveniles of *E. nolancribbi,* and adults plus juveniles of *Elopicola* n. sp. 1) that lack a recurved tip, and may be distributed in ventrolateral rows. The intestine is inverse U-shaped or with abbreviated lobes of cecum directing anteriad (when present, in adults of *E. nolancribbi* and *Elopicola* n. sp. 3*,* and adults plus juveniles of *Elopicola* n. sp. 1). The testis is

intercaecal and the ovary is single, medial, post-caecal, post-testicular, occupying posterior body extremity. Considering these features, *Elopicola* is most similar to monotypic *Paracardicoloides* Martin, 1974 by having a bowl-shaped anterior sucker, tegumental body spines that lack a recurved tip, and inverse U-shaped caeca. However, *Elopicola* can be easily differentiated from *Paracardicoloides* by having a single testis rather than having an anterior and posterior testes. *Elopicola* further differs from *Paracardicoloides* by having a muscular pharynx, a post-testicular ovary that occupies the posterior body extremity, and a post-cecal uterus that is lateral to the testis. *Paracardicoloides* lacks a muscular pharynx, and has an ovary that is intercaecal, as well as a partly pre-testicular uterus that is also intercaecal.

3.2 Elopicola n. sp. 1 Orélis-Ribeiro and Bullard (Figs. 1–8)

3.2.1 Diagnosis of adult (based on light microscopy of 11 stained, whole-mounted specimens)

Body 1,268–1,868 (1,632; 11) long, 318–525 (419; 11) wide, approximately 3–5× (4; 11) longer than wide, with maximum width near midbody (Figs. 1, 2, 4). Tegumental body spines straight, lacking recurved distal tips, directing laterally, medially, perpendicularly, or orienting at 45º angle posteriad, minute, each spine 10 in maximum length, each spine 3 in maximum width or approximately 3× longer than wide, numbering approximately 496–524 (508; 4) spines per side of body or totaling 1,012–1,020 (1,016; 2) spines, in transverse rows (Figs. 2, 3). Tegumental spine rows distributing along ventrolateral body surface for entire body length from near the base of the oral sucker to the extreme posterior body end, not confluent posteriorly, with many

spines seemingly detached in type specimens, distributing in approximately 201–221 (211; 4) rows each spaced 6–9 (8; 4) apart per side of body or a total of 410–433 (638; 2) rows, comprising 1–2 (2; 4), 4 (4; 4), 2 (2; 4) spines per row in anterior, middle and posterior portions of body, respectively, with breadth of field varying accordingly, approximately 3–8 (6; 4), 25–29 (27; 4), and 6–9 (11; 4) in breadth in anterior, middle and posterior portions of body, respectively (Figs. 2, 3). Dorsolateral nerve cords difficult to trace for most of body length (BL); 4–10 (7; 10) wide near mid-body at widest level; 45–58 (52; 10) or 10–17% (13%; 10) of body width from body margin at mid-body, paired, contiguous anteriorly and posteriorly, becoming confluent with paired cord 35–63 (50; 7) or 2–5% (3%; 7) of BL from posterior body end; anterior commissure 140–220 (177; 10) or 9–13% (11%; 10) of BL from anterior body end, 83–163 (102; 10) or 19–31% (24%; 10) of body width across width of worm, 3–13 (8; 10) wide, perpendicular to long axis of body, coursing dorsal to posterior end of oesophageal anterior swelling (Figs. 1, 2, 4).

Anterior sucker 53–68 (61; 11) in diameter or 10–20% (15%; 11) of body width, strongly muscular, extending anteriad from anterior end of body approximately 28–55 (44; 11) or 2–3% (3%; 9) of body length or 51–94% (71%; 9) of anterior sucker diameter; mouth opening within anterior sucker, 5–10 (7; 11) diameter, 28–43 (34; 11) or 1–3% (2%; 11) of anterior sucker length from anterior end of body, surrounded by muscular rim of sucker (Figs. 1, 2, 4). Oesophagus ventral to nerve commissure, 600–818 (707; 11) long or 41–47% (43%; 11) of body length, including an anterior (muscular pharynx), middle, and posterior (posterior oesophageal swelling) portions (Figs. 1, 2, 4); anterior portion of oesophagus comprising a muscular pharynx, not extending laterally

beyond nerve cord, between level of mouth and anterior nerve commissure, 63–93 (80; 11) long or 10–15% (12%; 11) of oesophagus total length or approximately 4–6% (5%; 11) of body length, 30–55 wide (42; 11) or approximately 7–13% (10%; 11) of body width, 1–3 (2; 11) longer than wide, markedly thick-walled and muscular, with muscular wall of approximately equal thickness to muscular rim of anterior sucker (Figs. 1, 2, 4); middle portion of oesophagus sinuous, thick- walled, separated from muscular pharynx by a marked constriction, 440–633 (536; 11) long or 74–92% (78%; 11) of oesophagus total length or approximately 27–36% (33%; 11) of body length, 28–63 (45; 11) wide, with wall 8–13 (10; 11) thick or 16–37% (23%; 11) of middle portion of oesophagus width; posterior oesophageal swelling bulbous, thick-walled, separated from medial portion of oesophagus by a marked constriction, immediately anterior to caecal bifurcation, 50–105 (85; 11) long or 8–14% (12%; 11) of oesophagus total length or approximately 3–7% (5%; 11) of body length, 30–80 (51; 11) wide, approximately 7–19% (12%; 11) of body width, 1–2× (2; 11) longer than wide, with wall approximately 3–8 (5; 11) thick or 5–21% (10%; 11) of posterior oesophageal swelling width (Figs. 1, 2, 4). Oesophageal gland enveloping oesophagus from level of pharynx to caecal bifurcation, lacking birefringent rodlet-like structures, concentrating in an area surrounding posterior oesophageal swelling 55–160 (107; 10) long or 9–21% (16%; 10) of oesophagus length, 65–123 (87; 10) wide or 1× (1; 10) wider than long. Caecal bifurcation immediately posterior to posterior oesophageal swelling, 688– 850 (734; 11) or 42–55% (45%; 11) of body length from anterior body end; abbreviated lobes of caecum directing anteriad discernable in all examined specimens, short, 50–90 (68; 20) long or 8–13% (10%; 10) of oesophagus length, 25–83 (38; 20) wide or 6–16% (9%; 20)

of body width, posterior caeca slightly sinuous, arching posterolaterad from caecal bifurcation and extending posteriad approximately in parallel with lateral body margin, containing brown or yellow-colored contents in lumen; dextral and sinistral posterior caeca asymmetrical, 380–528 (461; 11) and 353–565 (485; 11) long respectively, extending posteriad approximately 355–530 (461; 22) or 24–32% (28%; 22) of body length or 56–86% (65%; 22) of oesophagus length, 20–60 (40; 22) in maximum width, ending approximately 345–540 (456; 22) or 25–32% (28%; 22) of body length from posterior body end (Figs. 1, 2, 4).

Testis occupying most of the intercaecal space, 205–368 (321; 11) long or 14–23% (19%; 11) of body length, 133–255 (191; 11) wide or 37–58% (46%; 11) of maximum body width, 1–2× (2; 11) longer than wide, terminating at same level or 8–63 (34; 9) or 1–4% (2%; 9) of body length from distal tips of the sinistral, longest posterior caecum, 323–460 (407; 11) or 24–27% (25%; 11) of body length from posterior body end; testis lobes 25–95 (46; 36) long, 25–60 (41; 36) wide, 1–3× (1; 36) longer than wide; vasa efferentia difficult to trace in most of the specimens examined, an interconnecting meshwork of fine ducts entwining throughout testicular tissue, 5–13 (8; 13) wide, containing sperm in all specimens; vas deferens extending a short distance posteriad before meeting cirrus sac and internal seminal vesicle, 23–75 (45; 10) long or 1–4% (3%; 10) of body length, containing sperm in all specimens (Figs. 1, 2, 4, 7, 8). Cirrus sac located between posterior end of testis and dextral nerve cord, initiating near the level of the distal tips of the dextral, shortest posterior caecum, 378–575 (495; 11) or 27–33% (30%; 11) of body length from posterior body end, 78–178 (145; 11) long, 28–63 (46; 10) wide, 3–5× (3; 10) longer than wide, with wall 2–3 (2; 11) thick, terminating

23–73 (55; 11) or 1–5% (4%; 11) of body length from posterior testis end, 293–413 (356; 11) or 20–24% (22%; 11) of body length from posterior body end, enveloping well-delineated internal seminal vesicle; internal seminal vesicle occupying breadth and length of cirrus sac to varying degrees depending on amount of sperm present in duct, directing posteriad and orienting parallel with long axis of body, 38–158 (109; 11) long or 1–6× (3; 10) vas deferens length, 25–63 (45; 11) in maximum width; cirrus everting dorsally, slightly dextral, 25–38 (45; 3) long and 28–33 (31; 3), directing dextrad and not extending from common genital pore to beyond lateral body margin in the specimens examined, lacking spines (Figs. 1, 2, 4, 7, 8).

Ovary a loose aggregation of probable ova, immediately anterior to posterior nerve confluence, 95–183 (144; 11) long or 6–11% (9%; 11) of body length, 95–188 (140; 11) wide or 22–43% (34%; 11) of maximum body width, 1–2× (1; 11) longer than wide; post-ovarian space 93–193 (153; 11) long or 6–12% (10%; 11) of body length. Oviduct S-shaped, extending anteriad from near the center of ovary, 183–400 (306; 11) long; oviducal seminal receptacle a thin-walled sac containing sperm in all specimens, 95–225 (145; 11) long or 38–56% (48%; 11) of oviduct length, 38–118 (66; 11) wide, 1–4× (2; 11) longer than wide, varying in length and width depending on amount of sperm in duct (Figs. 1, 2, 4, 7, 8). Laurer's canal not observed in any of the specimens studied. Primary vitelline collecting duct sinistral, extending 188–503 (347; 11) posteriad and coursing between testis and sinistral body margin or dorsal to sinistral margin of testis, 15–30 (22; 11) in maximum width, curving mediad at level of posterior margin of testis, with distal portion extending in parallel with oviducal seminal receptacle before unite with distal portion of oviduct immediately proximal to ootype (Figs. 1, 2, 4, 7, 8); ootype

difficult to trace from surrounding tissue in most specimens, approximately spheroid, post-caecal, 18–53 (42; 11) or 1–3% (3%; 11) of body length from posterior margin of testis, residing at the level of the posterior margin of cirrus sac, immediately anterior or dorsal to anterior margin of oviducal seminal receptacle midpoint, 15–28 (22; 11) long, 15–25 (19; 11) wide, or 1–2× (1; 11) longer than wide, 285–418 (351; 11) or 19–24% (22%; 11) of body length from posterior body end; Mehlis' gland indistinct in fixed material (Figs. 1, 2, 4, 7, 8). Uterus short relative to many other aporocotylids, C-shaped; ascending uterus 53–88 (71; 11) long, 15–25 (18;11) maximum width, 38–77% (51%, 11) of oviduct seminal receptacle length, with cuboidal cells lining lumen, extending anterodextrad from ootype before curving mediad to connect with metraterm near the posterior margin of testis (Figs. 1, 2, 4, 7, 8). Metraterm post-testicular, between distal portion of primary vitelline collecting duct and cirrus sac, 95–215 (138; 11) long or 6–12% (9%; 11) of body length, 15–30 (23; 11) maximum width, 4–9× (6; 11) longer than wide, proximal portion transverse, crossing midline, distal portion extending posterodextrad, with muscular wall 5–6 (5; 11) thick; metraterm eggs capsular, straight, C-shaped, or S-shaped, having body 12–50 (25; 25) long, 4–8 (6; 25) wide, tendril-like filaments not observed in the specimens studied (Figs. 1, 2, 4, 7, 8). Common genital pore post-testicular, 20–45 (28; 11) in diameter, opening at level of ootype, 298–415 (359; 11) or 20–24% (22%; 11) of body length from posterior body end, 38–118 (77; 11) or 9–31% (18%; 11) of body length from dextral body margin, 148–308 (222; 11) or 47–59% (53%; 11) of body length from sinistral body margin (Figs. 1, 2, 4, 7, 8).

Excretory vesicle 25–53 (35; 10) long or 1–3% (2%; 10) of body length, 13–88 (48; 10) wide; excretory arms each 95–175 (125; 20) long, 15–58 (23; 20) wide (Figs. 1, 2, 4, 7, 8).

3.2.2 Diagnosis of schistosomulum (based on 2 stained, whole-mounted specimens)

Body 1,250–1,293 (1,222; 2) long, 213–220 (217; 2) wide or 5–6× (6; 2) longer than wide, spines not observed in the specimens studied (Fig. 6). Nervous system not evident.

Anterior sucker 48–51 (50; 2) in diameter, 23% (23%; 2) of body width, extending anteriad from anterior end of body approximately 28 (28; 2) or 2% (2%; 2) of body length or 55–58% (57%; 2) of anterior sucker diameter; mouth opening within anterior sucker, 5–8 (7; 2) diameter, 13–28 (21; 2) or 1–2% (2%; 2) of anterior sucker length from anterior end of body (Fig. 6). Oesophagus 565–593 (579; 2) long or 44–52% (48%, 2) body length; anterior portion of oesophagus comprising a muscular pharynx, 55–78 (67; 2) long or 10–13 (11%; 2) oesophagus length or approximately 1% (1%; 2) of body length, 30 (30; 2) wide; middle portion of oesophagus resembling that of adult specimens, 435 (435; 2) long or 73–77% (75%; 2) of oesophagus total length or approximately 34–38% (36%; 2) of body length, 33–35 (34; 2) wide, with wall 5 (5; 2) thick or 14–15% (15%; 2) of middle portion of oesophagus width; posterior oesophageal swelling resembling that of adult specimens, 75–80 (78; 2) long or 13% (13%; 2) of oesophagus total length or approximately 6–7% (6%; 2) of body length, 33–40 (37; 2) wide, 15–19% (17%; 2) of maximum body width, 2× (2; 2) longer than wide, with wall approximately 13 (13; 2) thick or 33–39% (36%; 2) of posterior oesophageal swelling

width (Fig. 6). Oesophageal gland 90–103 (97; 2) long or 15–18% (17%; 2) of oesophagus length, 70–88 (79; 2) wide or 1× (1; 2) wider than long. Caecal bifurcation 610–623 (617, 2) or 47–54% (51%; 2) of body length from anterior end; abbreviated lobes of caecum directing anteriad resembling those of adults, 33–48 (41; 4) long or 6–8% (7%; 4) of oesophagus length, 25–38 (32; 4) wide or 14–16% (15%; 4) of body width; dextral and sinistral posterior caeca asymmetrical, 258–343 (301; 2) and 298–338 (318; 2) long respectively, extending posteriad approximately 255–335 (301; 4) or 22–26% (25%; 4) of body length or 43–59% (53%; 4) of oesophagus length, 35–43 (40; 4) in maximum width, ending approximately 158–363 (289; 4) or 14–28% (24%; 4) of body length from posterior body end (Fig. 6).

Testicular anlage 198–255 (227; 2) long or 17–20% (18%; 2) of body length, 25–58 (42; 2) wide or 12–26% (19%; 2) of body width, 4–8× (6; 2) longer than wide, terminating at same level or 23 (23; 1) or 2% (2%; 1) of body length from distal tips of the sinistral, longest posterior caecum, 233–338 (286; 2) or 20–26% (23%; 2) of body length from posterior body end (Fig. 6); probable cirrus anlage filled with basophilic cells, initiating near the level of the distal tips of the dextral, shortest posterior caecum, 298–363 (331; 2) or 26–28% (27%; 2) of body length from posterior body end, 70–88 (79; 2) long, 13–20 (17; 2) wide or 4–5× (5; 2) longer than wide, wall difficult to delineate, terminating 10–45 (28; 2) or 1–3% (2%; 2) of body length from posterior testis end, 225–275 (250; 2) or 20–21% (20%; 2) of body length from posterior body end.

Ovarian anlage 43–45 (44; 2) long or 3–4% (4%; 2) of body length, 38–48 (43; 2) wide or 17–23% (20%; 2) of maximum body width, 1–2× (1; 11) longer than wide); post-ovarian space 165–168 (167; 2) long or 13–15% (14%; 2) of body length (Fig. 6).

Female terminal genitalia anlage filled with basophilic cells, oviduct, ootype, and uterus not morphologically distinctive. Common genital pore post-testicular, 10–13 (12; 2) in diameter, opening at level of ootype, 228–280 (254; 2) or 20–22% (21%; 2) of body length from posterior body end, 35 (35; 2) or 16% (16%; 2) of body length from dextral body margin, 103–123 (113; 2) or 48–56% (52%; 2) of body length from sinistral body margin (Fig. 6).

Excretory vesicle 13–30 (22; 2) long or 1–2% (2%; 2) of body length, 25–58 (42; 2) wide; excretory arms each 78–88 (82; 4) long, 13–35 (29; 4) wide (Fig. 6).

3.2.3 Diagnosis of large juvenile (based on 2 stained, whole-mounted specimens)

Body 1,450–1,480 (1,465; 2) long, 205–240 (223; 2) wide, approximately 6–7× (7; 2) longer than wide (Fig. 5). Tegumental body spines resembling those of large adults, each spine 3–5 (4; 10) in maximum length, each spine 2 (2; 10) in maximum width or approximately 2–3× (2; 10) longer than wide, numbering approximately 609–627 (618; 2) spines per side of body or totaling 1,236 (1,236; 1) spines, distributing in a ventrolateral field (Fig. 6). Tegumental spine rows distributing in approximately 269–282 (276; 2) rows each spaced 5 (5; 2) apart per side of body or a total of 551 (551; 1) rows, comprising 1 (1; 2), 3 (3; 2), 2 (2; 2) spines per row in anterior, middle and posterior portions of body, respectively, with breadth of field varying accordingly, approximately 2 (2; 2), 10 (10; 2), and 4 (4; 2) in breadth in anterior, middle and posterior portions of body, respectively. Dorsolateral nerve cords difficult to trace for most of body length (BL); 3–5 (4; 2) wide near mid-body at widest level; 43 (43; 2) or 18–21% (19%; 2) of body width from body margin at mid-body, paired, contiguous anteriorly and posteriorly,

becoming confluent with paired cord 35 (35; 1) or 2% (2%; 1) of BL from posterior body end; anterior commissure 167–178 (173; 2) or 11–12% (12%; 2) of BL from anterior body end, 55–60 (58; 2) or 25–27% (26%; 2) of body width across width of worm, 5–13 (9; 2) wide (Fig. 6).

Anterior sucker 48–58 (53; 2) in diameter or 23–24% (24%; 2) of body width, strongly muscular, extending anteriad from anterior end of body approximately 23–25 (24; 2) or 2% (2%; 2) of body length or 38% (38%; 2) of anterior sucker diameter; mouth opening within anterior sucker, 3–8 (6; 2) diameter, 23–25 (24; 2) or 2% (2%; 2) of anterior sucker length from anterior end of body (Fig. 6). Oesophagus similar to large adults, 608–728 (668; 2) long or 42–49% (46%; 2) of body length, anterior portion of oesophagus comprising a muscular pharynx, 70–80 (75; 2) long or 10–13% (11; 2) of oesophagus total length or approximately 1% (1%; 2) of body length, 38 wide (38; 2) or approximately 16–19% (17%; 2) of body width, 2 (2; 2) longer than wide (Fig. 6); middle portion of oesophagus, 458–560 (509; 2) long or 75–77% (76%; 2) of oesophagus total length or approximately 32–38% (35%; 2) of body length, 35–38 (37; 2) wide, with wall 5 (5; 2) thick or 13–14% (14%; 2) of middle portion of oesophagus width; posterior oesophageal swelling 70–98 (84; 11) long or 12–13% (12%; 2) of oesophagus total length or approximately 5–7% (6%; 2) of body length, 40–45 (43; 2) wide, approximately 17–22% (19%; 2) of body width, 2× (2; 2) longer than wide, with wall approximately 13 (13; 2) thick or 29–33% (31%; 2) of posterior oesophageal swelling width (Fig. 6). Oesophageal gland103–113 (108; 2) long or 14–19% (16%; 2) of oesophagus length, 73–80 (77; 2) wide or 1× (1; 2) wider than long. Caecal bifurcation 390–748 (569; 2) or 27–51% (39%; 2) of body length from anterior body end; abbreviated lobes of caecum

directing anteriad 50–73 (60; 4) long or 9% (9%; 2) of oesophagus length, 7–40 (20; 4) wide or 7–15% (11%; 2) of body width, posterior caeca with dextral and sinistral posterior caeca asymmetrical, 355–380 (368; 2) and 408–413 (411; 2) long respectively, extending posteriad approximately 358–410 (386; 4) or 24–31% (27%; 4) of body length or 49–67% (59%; 4) of oesophagus length, 25–45 (35; 4) in maximum width, ending approximately 345–450 (401; 4) or 23–31% (28%; 4) of body length from posterior body end (Fig. 6).

Testicular anlage resembling that of large adults, 230–245 (238; 2) long or 16–17% (16%; 2) of body length, 85–88 (87; 2) wide or 37–41% (39%; 2) of maximum body width, 2–3× (2; 2) longer than wide, terminating 10–60 (35; 2) or 1–4% (2%; 2) of body length from distal tips of the sinistral, longest posterior caecum, 333–350 (342; 2) or 23–24% (23%; 2) of body length from posterior body end; testis lobes 13–53 (34; 4) long, 13–23 (19; 4) wide, 1–3× (2; 2) longer than wide; vasa efferentia not evident; vas deferens not evident. Cirrus sac anlage containing few basophilic cells, initiating near the level of the distal tips of the dextral, shortest posterior caecum, 393–418 (406; 2) or 27–29% (28%; 2) of body length from posterior body end, 98–130 (114; 2) long, 25–30 (28; 2) wide, 3–5× (4; 2) longer than wide, with wall 3 (3; 2) thick, terminating 23–30 (27; 2) or 2% (2%; 2) of body length from posterior testis end, 295–335 (315; 2) or 20–23% (22%; 2) of body length from posterior body end (Fig. 6).

Ovarian anlage 75–130 (103; 2) long or 5–9% (7%; 2) of body length, 63–90 (77; 2) wide or 31–38% (34%; 2) of maximum body width, 1–2× (1; 2) longer than wide (Fig. 6); post-ovarian space 130–193 (162; 2) long or 9–13% (11%; 2) of body length. Oviduct anlage 135–170 (153; 2) long; oviducal seminal receptacle anlage 68–70 (69; 2) long or

41–50% (46%; 2) of oviduct length, 13–35 (24; 2) wide, 2–5× (4; 2) longer than wide (Fig. 6). Laurer's canal not observed in any of the specimens studied. Primary vitelline collecting duct not observed; ootype anlage difficult to trace from surrounding tissue, 23–48 (36; 2) or 2–3% (2%; 2) of body length from posterior margin of testis, 15–18 (17; 2) long, 10–15 (13; 2) wide, or 1–2× (1; 2) longer than wide, 275–315 (295; 2) or 19–22% (20%; 2) of body length from posterior body end; Mehlis' gland indistinct in fixed material. Uterine anlage filled with basophilic cells, 65–78 (72; 2) long, 15–18 (17; 2) maximum width, 93–115% (104%, 2) of oviduct seminal receptacle length, with cuboidal cells lining lumen (Fig. 6). Metraterm filled with basophilic cells, 40 (40; 2) long or 3% (3%; 2) of body length, 13–15 (14; 2) maximum width, 3× (3; 2) longer than wide, with wall 3 (3; 2) thick. Common genital pore 10–20 (15; 2) in diameter, 293–323 (308; 2) or 20–22% (21%; 2) of body length from posterior body end, 40–50 (45; 2) or 17–24% (21%; 2) of body length from dextral body margin, 150–100 (125; 2) or 49–63% (56%; 2) of body length from sinistral body margin (Fig. 6).

Excretory vesicle 18–25 (22; 2) long or 1–2% (2%; 2) of body length, 28–33 (31; 2) wide; excretory arms each 123–138 (129; 4) long, 18–30 (23; 4) wide (Fig. 6).

3.2.4 Taxonomic summary

Type host: Elops hawaiensis (Reagan, 1909)

Type locality: South China Sea, off Nha Trang, Vietnam.

Site in host: Indeterminate. Most adults and juveniles in sediment derived from rinsing macerated head, trunk, and body cavity; 1 large juvenile in branchial sinuses and 1 large juvenile in heart.

Prevalence and intensity of infection: Five of 6 (83%) Hawaiian ladyfish collected had 1–19 (mean intensity = 6 ± 0.5) specimens.

Specimens deposited: One holotype (large adult, specimen #1 USNM XXXXXX); three paratypes (adult, specimen #2 USNM XXXXXX; specimen #3 large juvenile, USNM XXXXXX; schistosomulum, specimen #4 USNM XXXXXX).

3.2.5 Remarks

The new species is most easily differentiated from *E. nolancribbi*, the only nominal congener, by the presence of an enantiomorphic terminal genitalia. Following Palmer's (2005) terminology, enantiomorphs are the two mirror images of an asymmetric (chiral) form. Specifically, *Elopicola* n. sp. 1 has a cirrus sac and a primary vitelline duct that is dextral plus a female terminal genitalia orienting dextrad, whereas *E. nolancribbi* has the opposite, a cirrus sac and a primary vitelline duct that is sinistral plus a female terminal genitalia orienting sinistrad (see Figs. 6, 7, and 1–12 from Bullard, 2014). *Elopicola* n. sp. 1 further differs from *E. nolancribbi* by the combination of having (i) rows of tegumental body spines in adults and juveniles, (ii) pharynx width that is 7–13% of body width, (iii) oesophagus that is 41–47% of body length, (iv) short lobes of caeca directing anteriad present in adults, large juveniles, and schistosomula, (v) testis that occupies most of the intercaecal space, (vi) vasa efferentia coalescing in postero-dextral region of testis to form vas deferens, (vii) ootype that is located well posterior to the testis, and (viii) common genital pore post-testicular, and at the level of ootype. *Elopicola nolancribbi* has (i) body spines only observed in juveniles, (ii) pharynx width that is 1–3% of body width, (iii) oesophagus that is 23–39% of body length, (iv) short

lobes of caeca directing anteriad observed only in large adults, (v) testis midpoint at level of distal tips of posterior ceca (i.e., just anterior half occupying intercaecal space), (vi) vasa efferentia coalescing in antero-sinistral region of testis to form vas deferens, (vii) ootype at the level of posterior margin of testis, and (viii) common genital pore sinistral to the posterior half of testis and well anterior to the ootype. Because body spines can be easily displaced in poorly fixed or degrading specimens (Bullard, 2010; McVay et al., 2011), new collections of specimens may reveal the presence of this feature in adults of *Elopicola nolancribbi*. Noteworthy also is that, although the pharynx is assumedly pliable in nature based on its striated, muscular wall, we decided to include it as a diagnostic feature because no granular material resulting from ingestion of blood where observed filling the pharynx of any of the specimens of *Elopicola* n. sp. 1 studied.

To the best of our knowledge, the literature holds no specific designation for an intermediary stage of blood flukes between the immature, migratory schistosomulum and the fully-developed, adult fluke. However, herein we provide the diagnosis of large juveniles based on specimens characterized by proportionally narrower genitalia (nearly 1/2 width of genitalia from fully-developed adults), and a cirrus sac and oviducal seminal receptacle anlage filled with relatively little amount of sperm and ova. While the schistosomulum also has extremely reduced genitalia, its male and female terminal genitalia are filled with basophilic cells that preclude the delineation of their different portions.

3.3 Elopicola n. sp. 2 Orélis-Ribeiro and Bullard (Figs. 9–10)

3.3.1 Diagnosis of adult (based on light microscopy of 2 stained, whole-mounted specimens)

Body 1,240–1,395 (1,318; 21) long, 208–215 (212; 2) wide, approximately 6× (6; 2) longer than wide, with maximum width near genitalia (Fig. 9). Tegumental body spines not observed. Dorsolateral nerve cords indistinct in the fixed material.

Anterior sucker 40–48 (44; 2) in diameter or 33–38% (36%; 2) of body width, strongly muscular, extending anteriad from anterior end of body approximately 33–38 (36; 2) or 2–3% (3%; 2) of body length or 69–95% (82%; 2) of anterior sucker diameter; mouth opening within anterior sucker, 3–4 (4; 2) diameter, surrounded by muscular rim of sucker (Fig. 9). Oesophagus 608–858 (733; 2) long or 49–62% (55%; 2) of body length, including an anterior (muscular pharynx), middle, and posterior (posterior oesophageal swelling) portions (Fig. 9); muscular pharynx, 63–65 (64; 2) long or 8–10% (9%; 2) of oesophagus total length or approximately 5% (5%; 2) of body length, 30–40 wide (35; 2) or approximately 14–19% (17%; 2) of body width, 2 (2; 2) longer than wide, wall indistinct in the fixed specimens (Fig. 9); middle portion of oesophagus sinuous, thick-walled, separated from muscular pharynx by a marked constriction, 485–738 (612; 2) long or 80–86% (83%; 2) of oesophagus total length or approximately 39–53% (46%; 2) of body length, 18 (18; 2) wide; posterior oesophageal swelling bulbous, thick-walled, separated from medial portion of oesophagus by a marked constriction, immediately anterior to caecal bifurcation, 55–60 (58; 2) long or 6–10% (8%; 2) of oesophagus total length or approximately 4–5% (4%; 2) of body length, 23–25 (24; 2) wide, approximately 11–12% (11%; 2) of body width, 2× (2; 2) longer than wide (Fig. 9). Oesophageal gland indistinct. Caecal bifurcation immediately posterior to posterior oesophageal swelling,

633– 818 (726; 2) or 51–59% (55%; 2) of body length from anterior body end; abbreviated lobes of caecum directing anteriad, short, 23–50 (37; 2) long or 6% (6%; 1) of oesophagus length, 15 (15; 2) wide or 7% (7%; 2) of body width, posterior caeca slightly sinuous, arching posterolaterad from caecal bifurcation and extending posteriad approximately in parallel with lateral body margin; dextral and sinistral posterior caeca asymmetrical, 248–250 (249; 2) and 258 (258; 2) long respectively, extending posteriad approximately 223–253 (243; 2) or 18–20% (19%; 2) of body length or 29–42% (34%; 2) of oesophagus length, 15–28 (21; 2) in maximum width, ending approximately 338–368 (352; 2) or 24–30% (27%; 2) of body length from posterior body end, poorly delineated from surrounding parenchyma (Fig. 9).

Testis midpoint approximately at level of distal tips of posterior, anterior half occupying intercaecal space, 150–153 (152; 2) long or 11–12% (12%; 2) of body length, 85–100 (93; 2) wide or 40–48% (44%; 2) of maximum body width, 2× (2; 2) longer than wide, terminating 35–75 (55; 2) or 3–5% (4%; 2) of body length from distal tips of the sinistral, longest posterior caecum, 295–308 (302; 2) or 22–24% (23%; 2) of body length from posterior body end; testis lobes difficult to delineate in most of the specimens examined, 70–75 (72; 3) long, 33–35 (34; 3) wide, 2× (2; 3) longer than wide; vasa efferentia difficult to trace in most of the specimens examined, an interconnecting meshwork of fine ducts entwining throughout testicular tissue, 5 (5; 10) wide, containing sperm in all specimens; vas deferens extending a short distance posteriad before meeting cirrus sac and internal seminal vesicle, 78–113 (96; 2) long or 6–8% (7%; 2) of body length, containing sperm in all specimens (Figs. 9, 10). Cirrus sac located between testis and sinistral nerve cord, initiating near the level of the distal tips

of the dextral, shortest posterior caecum, 155–190 (173; 2) long, 63–65 (64; 2) wide, 2× (2; 2) longer than wide, with wall 2 (2; 2) thick, terminating 80–118 (99; 2) or 6–10% (8%; 2) of body length from posterior testis end, 210–213 (212; 2) or 15–17% (16%; 2) of body length from posterior body end, enveloping well-delineated internal seminal vesicle; internal seminal vesicle occupying breadth and length of cirrus sac to varying degrees depending on amount of sperm present in duct (Figs. 9, 10); cirrus everting dorsally, 38–45 (42; 2) long and 20–25 (23; 2), lacking spines (Figs. 9, 10).

Ovary a loose aggregation of probable ova, 80–103 (92; 2) long or 6–8% (7%; 2) of body length, 83–93 (88; 2) wide or 39–45% (42%; 2) of maximum body width, 1× (1; 2) longer than wide (Figs. 9, 10); post-ovarian space 70–90 (80; 2) long or 6% (6%; 2) of body length. Oviduct S-shaped, extending anteriad from near the center of ovary, 175–205 (190; 2) long; oviducal seminal receptacle a thin-walled sac containing sperm in all specimens, 93–105 (99; 2) long or 45–60 (53; 2) of oviduct length, 40–43 (42; 2) wide, 2–3× (2; 2) longer than wide, varying in length and width depending on amount of sperm in duct (Figs. 9, 10). Laurer's canal not observed in any of the specimens studied. Primary vitelline collecting duct dextral, extending 125–230 (178; 2) posteriad and coursing between testis and dextral body margin, 18–23 (21; 2) in maximum width, curving mediad at level of posterior margin of testis, uniting with distal portion of oviduct immediately proximal to ootype (Figs. 9, 10); ootype difficult to trace from surrounding tissue in most specimens, approximately spheroid, post-caecal, 220–240 (230; 2) or 16–19% (18%; 2) of body length from posterior margin of testis, residing slightly anterior or at the level of the posterior margin of cirrus sac, 16–18 (17; 2) long, 15–18 (17; 2) wide, or 1× (1; 2) longer than wide, 220–240 (230; 2) or 16–19% (18%; 2) of body

length from posterior body end; Mehlis' gland indistinct in fixed material. Uterus difficult to trace in the specimens studied, short relative to many other aporocotylids, ascending uterus 40–60 (50; 2) long, 8 (8;2) maximum width, 38–65% (51%, 2) of oviduct seminal receptacle length, extending anterosinistrad from ootype before connecting with metraterm near the posterior margin of testis (Figs. 9, 10). Metraterm post-testicular, 63–65 (64; 2) long or 5% (5%; 2) of body length, 20–25 (23; 2) maximum width, 3× (3; 2) longer than wide, with muscular wall 2–3 (3; 2) thick; metraterm eggs capsular, straight, or C-shaped, having body 10 (10; 5) long, 5 (5; 5) wide, tendril-like filaments not observed in the specimens studied. Common genital pore post-testicular, 30–33 (32; 2) in diameter, opening at level of ootype, 243–280 (262; 2) or 20% (20%; 2) of body length from posterior body end (Figs. 9, 10).

Excretory vesicle indistinct.

3.3.2 *Taxonomic summary*

Type host: Megalops atlanticus (Valenciennes, 1847)

Type locality: Gulf of Mexico off Tampa Bay, Florida, USA.

Site in host: Heart.

Specimens deposited: One holotype (adult, specimen #1 USNM XXXXXX); one paratype (adult, specimen #2 USNM XXXXXX)

3.3.3 Remarks

The new species differs from *E. nolancribbi* and *Elopicola* n. sp. 1 by the combination of having a body 6× (3–4× in *E. nolancribbi*; 3–5× in *Elopicola* n. sp. 1)

longer than wide, an anterior sucker that is 33–38% (8–24% in *E. nolancribbi*; 10–20% in *Elopicola* n. sp. 1) of body width, an oesophagus that is 49–62% (23–39% in *E. nolancribbi*; 36–47% in *Elopicola* n. sp. 1) of total body length, a testis that has its anterior half occupying intercaecal space (testis anterior half occupying intercaecal space in *E. nolancribbi*; approximately the entire testis occupying the intercaecal space in *Elopicola* n. sp. 1) and a size that is 11–12% (19–33% in *E. nolancribbi*; 14–23% in *Elopicola* n. sp. 1) of body length, and a common genital pore post-testicular, at the level of ootype.

3.4 Phylogenetic analyses

Regarding the 18S+28S dataset, the aligned sequence data comprised a total of 2978 positions, 6% of which were excluded because of ambiguous positional homology according to the criteria adopted in Gblock. Thus, this final data matrix consisted of 2804 positions per taxon (1616 conserved, 1188 variable, and 861 parsimony-informative). Tree topologies recovered by either BI and ML analyses were completely congruent, however, the former yielded relatively higher nodal support values (Fig. 11). In both analyses, blood flukes that mature in early-divergent lineages of ray-finned fishes were rendered polyphyletic, with the acipenseriform blood fluke *Acipensericola petersoni* clustering with the holocephalan blood fluke *Chimaerohemecus trondheimensis*, rather than with the elopiform blood flukes, that formed a clade comprising (*Elopicola* n. sp. 1*(Elopicola* n. sp. 2, *E. nolancribbi)*. The latter clade formed a monophyletic group that is sister to *Sanguinicola* cf. *inermis* plus all marine euteleost

blood flukes. Furthermore, all members of Aporocolitylidae formed a clade that is sister to a clade comprising all turtle blood flukes (Fig. 11).

Regarding the ITS2 dataset, the aligned sequence data rendered a total of 638 positions, 46% of which were excluded because of ambiguous positional homology according to the criteria adopted in Gblock (Fig. 12). Thus, this final data matrix consisted of 293 positions per taxon (104 conserved, 189 variable, and 142 parsimony-informative). Recovered tree topologies by either BI and ML analyses were also completely congruent, and the former yielded a slightly higher nodal support. Resulting tree showed that the eel blood fluke *Paracardicoloides yamagutii* is sister to a clade formed by the ladyfish blood fluke *Elopicola nolancribbi* and the new species described from *Elops hawaiensis* (Fig. 12). Hence, elopomorph blood flukes were recovered monophyletic. Unfortunately, no ITS2 sequence data were generated from the specimen of *Elopicola* n. sp. 2. We suspect that the sequencing process in the 3'–5' direction was precluded by a mutation in the annealing site of the corresponding oligonucleotide primer (i.e., ITS2.2), because only poor quality sequences were recovered. While the design of a new set of primers is pending, we attempted to further support the monophyly of elopomorph blood flukes by adding two short 18S and 28S rDNA sequences (i.e., 316 and 131 bp, respectively) of *P. yamagutii* retrieved from GenBank (accession numbers U42569 and U42562) into the 18S+28S dataset. Although both BI and ML resulting trees were mostly poorly resolved, *P. yamagutii* was, indeed, recovered sister to all elopiform blood flukes (data not shown).

4. DISCUSSION

4.1 Phylogenetic analyses

Our phylogenetic analyses did not significantly refute the only previously published tree topology that includes a blood fluke that infects an early-branching lineage of ray-finned fish (i.e., *Acipensericola petersoni*). Bullard et al.'s (2008) tree recovered the chondrichthyan blood fluke *Chimaerohemecus trondheimensis* as sister taxon to a clade formed by *A. petersoni* + (*Sanguinicola* cf. *inermis*(*Aporocotyle spinosicalis*(*Plethrochis acanthus*, *Neoparacardicola nasonis*))). Although in our tree topologies *A. petersoni* was also sister to all other actinopterygian blood flukes, this species was recovered in a clade comprising *C. trondheimensis*. At first glance, considering insights from comparative morphology, it seems odd that *A. petersoni* is more closely related to a chondrichthyan blood fluke than to an elopiform blood fluke (*Elopicola* spp.; see *Elopicola* emended diagnosis above). Yet, given that most of these morphological features are also shared with turtle blood flukes (Bullard et al. 2008), the placement of *A. petersoni* as one of the most earlier-branching taxa seems plausible. Although addressing co-phyly is beyond the scope of the present study and will be treated in greater detail elsewhere, it is worth noting that the branching pattern observed in the topology presented herein is approximately congruent with the phylogenetic 'tree of life' of fishes published by Betancur et al. (2013). While the blood fluke infecting an holocephalan was recovered forming a clade with the acipenseriform blood fluke, rather than forming a sister relationship with the actinopterygian plus sarcopterygian blood flukes, congruence exists with the branching order of elopiform and otophysan fishes. However, as remarked by Orélis-Ribeiro et al. (2014), the sequence identified on

Genbank as *Sanguinicola* cf. *inermis* should be used cautiously as a definitive representative of an otophysan blood fluke. Regarding the relationship between *Elopicola* spp., noteworthy is that the topologies based on the 18S+28S dataset pointed out that their evolution is seemingly driven by ecological aspects instead of host ancestry. Aporocotylids infecting species of *Elops* were recovered paraphyletic. Indeed, the elopiform aporocotylids sympatric in the Gulf of Mexico (i.e., ladyfish aporocotylid *Elopicola nolancribbi* and tarpon aporocotylid *Elopicola* n. sp. 2) were more closely related to each other than to the Hawaian ladysh aporocotylid *Elopicola* n. sp. 1. However, these relationships may be reflecting an insufficient taxon sampling within the genus.

4.2 Enantiomorphism

Although genital asymmetry is an evolutionary trend well known throughout a wide variety of metazoan phyla (Schilthuizen, 2013), the present study reports the first record of interspecific enantiomorphism in blood flukes (Schistosomatoidea). While it seems reasonable to accept how this modification would play a central role at a prezigotic isolation and subsequent speciation, the relative rarity of this phenomena among Platyhelminthes is puzzling (Ogawa and Egusa 1981, Kritsky et al. 2011, Patella and Bullard 2013, Schilthuizen 2013). Noteworthy is that most of the concepts used by studies of genital asymmetry comprise frequency of distinctive genitalia configurations within a population (see ‘Definitions and terminology’ in Schilthuizen [2013]). We mention this here because a more statistically representative sampling size of *Elopicola* spp. will likely expand our understanding on the role of this feature in the evolution of

elopiform blood flukes.

4.3 Use of elopiform blood flukes as biological tags

Tarpon and ladyfish blood flukes may comprise an accurate biological tag that help to inform stock differentiation among elopiform fishes. Fish stock assessments, i.e., “intraspecific group of randomly mating individuals with temporal and spatial integrity” (Ihssen et al. 1981) or “units below species that are naturally occurring” (Waldman 2004), are imperative for establishing appropriate management regulations in fisheries and could be particularly impactful for species with a wide geographical distribution, wherein multiple stocks are under different exploitation pressure throughout its range (Waldman 2004). Genetic studies on tarpon, *Megalops atlanticus*, pointed out that populations from Florida, the Pacific of Panama, Gulf of Guinea, and Costa Rica, were genetically differentiated (Ward *et al.* 2008). To the best of our knowledge, Florida is the only locality, among those populations, where tarpon fishing is regulated. Moreover, because of the estimated decline in tarpon landings (Adams et al. 2013), we believe that blood flukes could represent a fruitful model to further assess the structure of those stocks and perhaps provide valuable information to substantiate their conservation and management.

Another potential application of elopiform blood flukes as biological tags could be supporting the diagnosis of host species that are sympatric and difficult to differentiate morphologically. McBride et al. (2010) found just one diagnostic feature that distinguishes the new species *E. smithi* from its sympatric congener *E. saurus*, i.e. myomere (larvae) or vertebrae (adults) counts. Given that *E. saurus* is known as the

only host for *E. nolancribbi* (Bullard 2014), a parasitological survey targeting *E. smithi* may reveal the reliability of the use of blood flukes as biological tags. Noteworthy also is that in the regions of sympatry, McBride and Horodysky (2004) reported that the recruitment of *E. smithi* and *E. saurus* occurs in the autumn and winter-spring, respectively. Parasitological surveys during those periods could not only potentially provide further insights on the biology of those fish species, but also will likely shed light on the life cycle of elopiform blood flukes. Based on the presence of a juvenile specimen of *E. nolancribbi* infecting a juvenile *E. saurus*, Bullard (2014) inferred that the life cycle of this aporocotylid occurs near shore or in low salinity waters, perhaps within a nearby river. The present study supports those observations, given that all specimens, representing different stages (i.e., eggs, schistosomulum, large juveniles, and adults) of Elopicola n. sp. 1 were collected from juvenile *E. hawaiensis* (FL 370mm [6]).

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FIGURE LEGENDS

Figs. 1–3. *Elopicola* n. sp. 1 (Digenea: Aporocotylidae) from the blood vascular system and viscera of ladyfish, *Elops hawaiensis* Regan, 1909, (Elopiformes: Elopidae) from the South China Sea off Nha Trang (Vietnam) and adjacent waters. Scale values aside each bar. (1) Body of adult (specimen #1 [holotype] USNM No. XXXXXX) showing location of muscular anterior sucker (as), pharynx (ph), dorsolateral nerve commissure (dc), oesophagus (oe), nerve cord (nc), vitelline follicles (v), posterior oesophageal swelling (pos), anterior caecum (ac), posterior caecum (pc), testicular field (tf), vasa efferentia (ve), vas deferens (vd), cirrus sac (cs), internal seminal vesicle (isv), metraterm (met), common genital pore (cgp), uterus (u), ootype (oo), oviducal seminal receptacle (osr), oviduct (ov), ovary (o), excretory arms (ea), excretory vesicle (ev). Dorsal view. (2) Body of adult (specimen #2 [paratype] USNM No. XXXXXX) showing same features as in Fig. 1. Ventral view. (3) Rows of ventrolateral tegumental body spines distributing in posterior portion of body (*) at level of ovary.

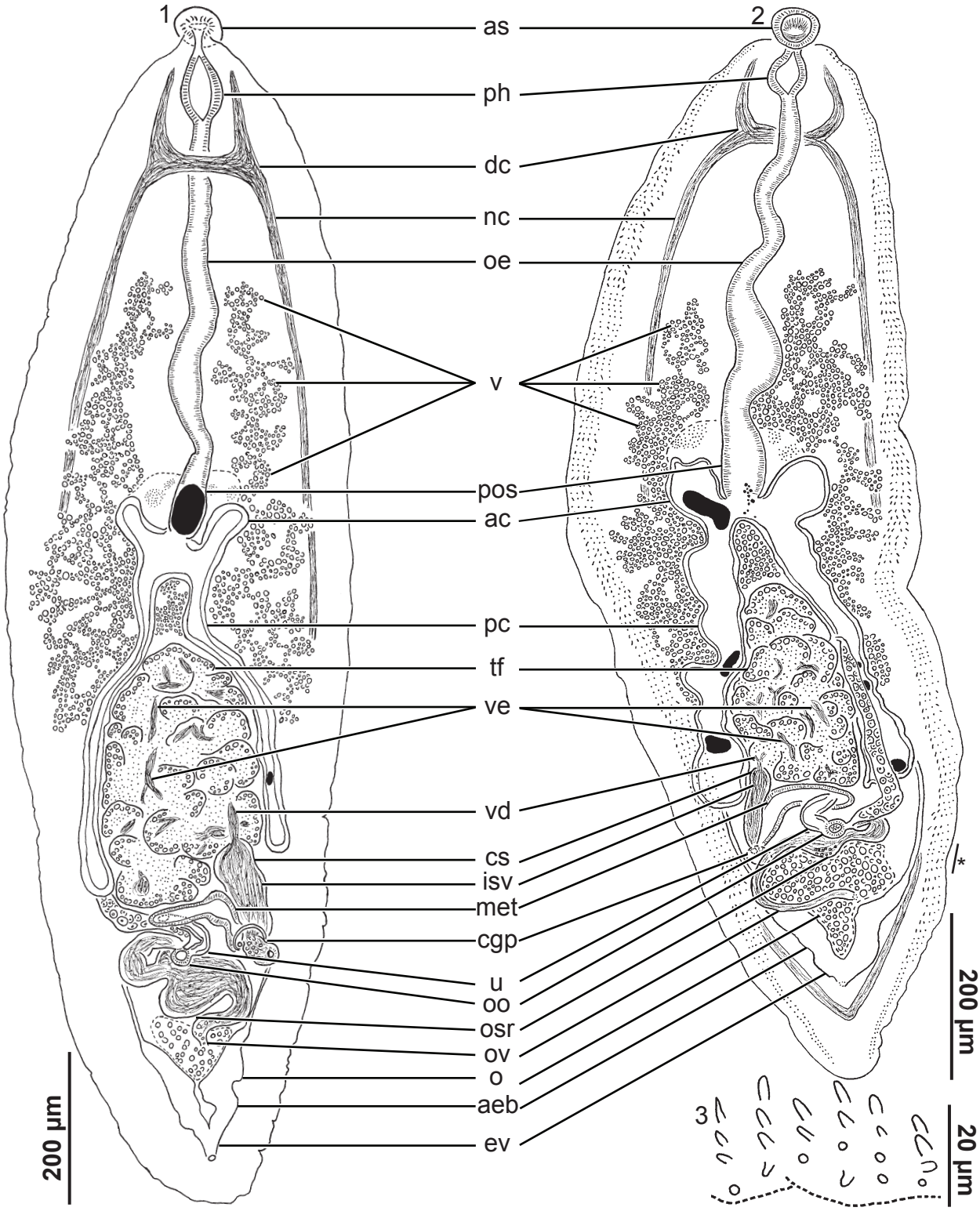
Figs. 4–6. *Elopicola* n. sp. 1 (Digenea: Aporocotylidae) from the blood vascular system and viscera of ladyfish, *Elops hawaiensis* Regan, 1909, (Elopiformes: Elopidae) from the South China Sea off Nha Trang (Vietnam) and adjacent waters. (4) Body of adult (specimen #1 [holotype] USNM No. XXXXXX) showing same features as in Fig. 1. Dorsal view. (5) Body of large juvenile (specimen #3 [paratype] USNM No. XXXXXX) showing location of muscular anterior sucker (as), pharynx (ph), dorsolateral nerve commissure (dc), oesophagus (oe), nerve cord (nc), vitelline follicles (v), posterior oesophageal swelling (pes), anterior caecum (ac), posterior caecum (pc), testicular anlage (ta), cirrus sac anlage (csa), basophilic cells (bc), common genital pore (cgp), metraterm anlage (meta), uterine anlage (ua), ootype anlage (ooa), oviducal seminal receptacle anlage (osra), oviduct anlage (ova), ovarian anlage (oa), excretory arms (ea), excretory vesicle (ev). Dorsal view. (6) Schistosomulum (specimen #4 [paratype] USNM No. XXXXXX) showing location of muscular anterior sucker (as), pharynx (ph), dorsolateral nerve commissure (dc), oesophagus (oe), nerve cord (nc), vitelline follicles (v), posterior oesophageal swelling (pes), anterior caecum (ac), posterior caecum (pc), testicular anlage (ta), basophilic cells (bc), common genital pore (cgp), ovarian anlage (oa), excretory arms (ea), excretory vesicle (ev). Ventral view.

Figs. 7–8. *Elopicola* n. sp. 1 (Digenea: Aporocotylidae) from the blood vascular system and viscera of ladyfish, *Elops hawaiensis* Regan, 1909, (Elopiformes: Elopidae) from the South China Sea off Nha Trang (Vietnam) and adjacent waters. (7) Genitalia of adult specimen (specimen #1 [holotype] USNM No. XXXXXX) showing testis (t), distal tip of dextral caecum (ddc), distal tip of sinistral caecum (dsc), vasa efferentia (ve), vas deferens (vd), internal seminal vesicle (isv), cirrus sac (cs), common genital pore (cgp), everted cirrus (ec), vitelline duct (vt), metraterm eggs (me), metraterm (met), uterus (u), ootype (oo), oviducal seminal receptacle (osr), oviduct (ov), ovary (o), excretory arms (ea), excretory vesicle (ev). Dorsal view. (8) Body of adult (specimen #2 [paratype] USNM No. XXXXXX) showing same features as in Fig. 7. Ventral view.

Figs. 9–10. *Elopicola* n. sp. 2 (Digenea: Aporocotylidae) from the heart of tarpon, *Megalops atlanticus* Valenciennes, 1847, (Elopiformes: Megalopidae) from the Northern Gulf of Mexico off Tampa Bay and adjacent waters. Scale values aside each bar. (9) Body of adult (specimen #1 [holotype] USNM No. XXXXXX) showing location of muscular anterior sucker (as), pharynx (ph), oesophagus (oe), posterior esophageal swelling (pes), anterior caecum (ac), putative posterior caecum (ppc), testicular field (tf), vasa efferentia (ve), vas deferens (vd), cirrus sac (cs), internal seminal vesicle (isv), metraterm (met), common genital pore (cgp), uterus (u), ootype (oo), oviducal seminal receptacle (osr), oviduct (ov), ovary. Lateral view. (10) Genitalia of adult specimen (specimen #1 [holotype] USNM No. XXXXXX) showing testis (t), putative distal tip of dextral caecum (ddc), putative distal tip of sinistral caecum (dsc), vasa efferentia (ve), vas deferens (vd), internal seminal vesicle (isv), cirrus sac (cs), common genital pore (cgp), everted cirrus (ec), sperm (s), vitelline duct (vt), metraterm eggs (me), metraterm (met), uterus (u), ootype (oo), oviducal seminal receptacle (osr), oviduct (ov), ovary (o). Lateral view.

Figure 11. Phylogenetic relationships of blood flukes reconstructed by Bayesian inference and based on concatenated fragments of 18S and 28S rDNA genes from 26 taxa (majority rule consensus tree). Numbers aside tree nodes indicate posterior probability (Bayesian inference, left) and bootstrap values (Maximum Likelihood, right). Definitive hosts are indicated by icons aside tree nodes. Newly generated sequences are highlighted in bold. Shaded area indicates *Elopicola* spp.

Figure 12. Phylogenetic relationships of fish blood flukes reconstructed by Bayesian inference and based on ITS2 rDNA sequences from 12 taxa (majority rule consensus tree). Numbers aside tree nodes indicate posterior probability (Bayesian inference, left) and bootstrap values (Maximum Likelihood, right). Definitive hosts are indicated by icons aside tree nodes. Newly generated sequences are highlighted in bold. Shaded area indicates elopomorph blood flukes.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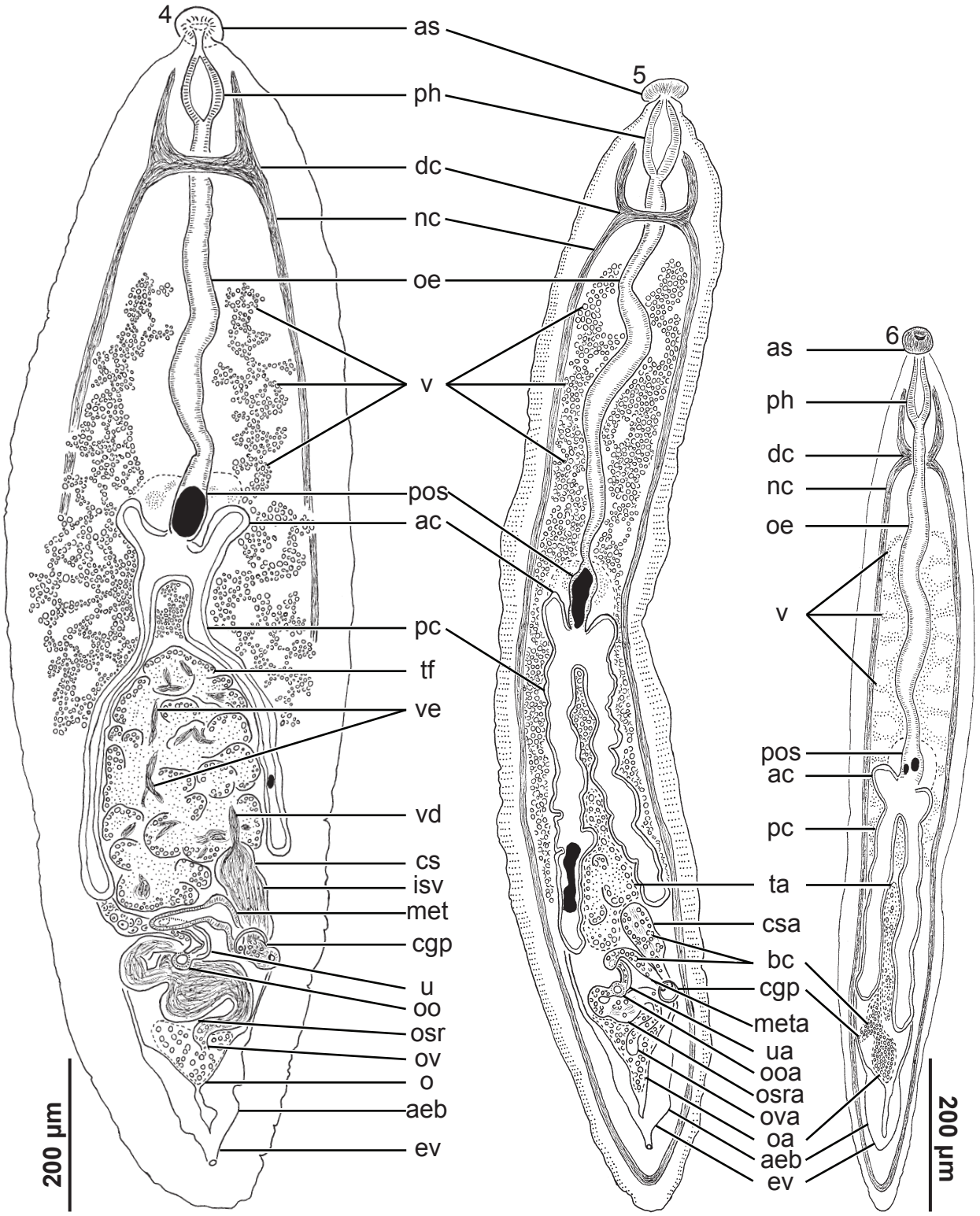
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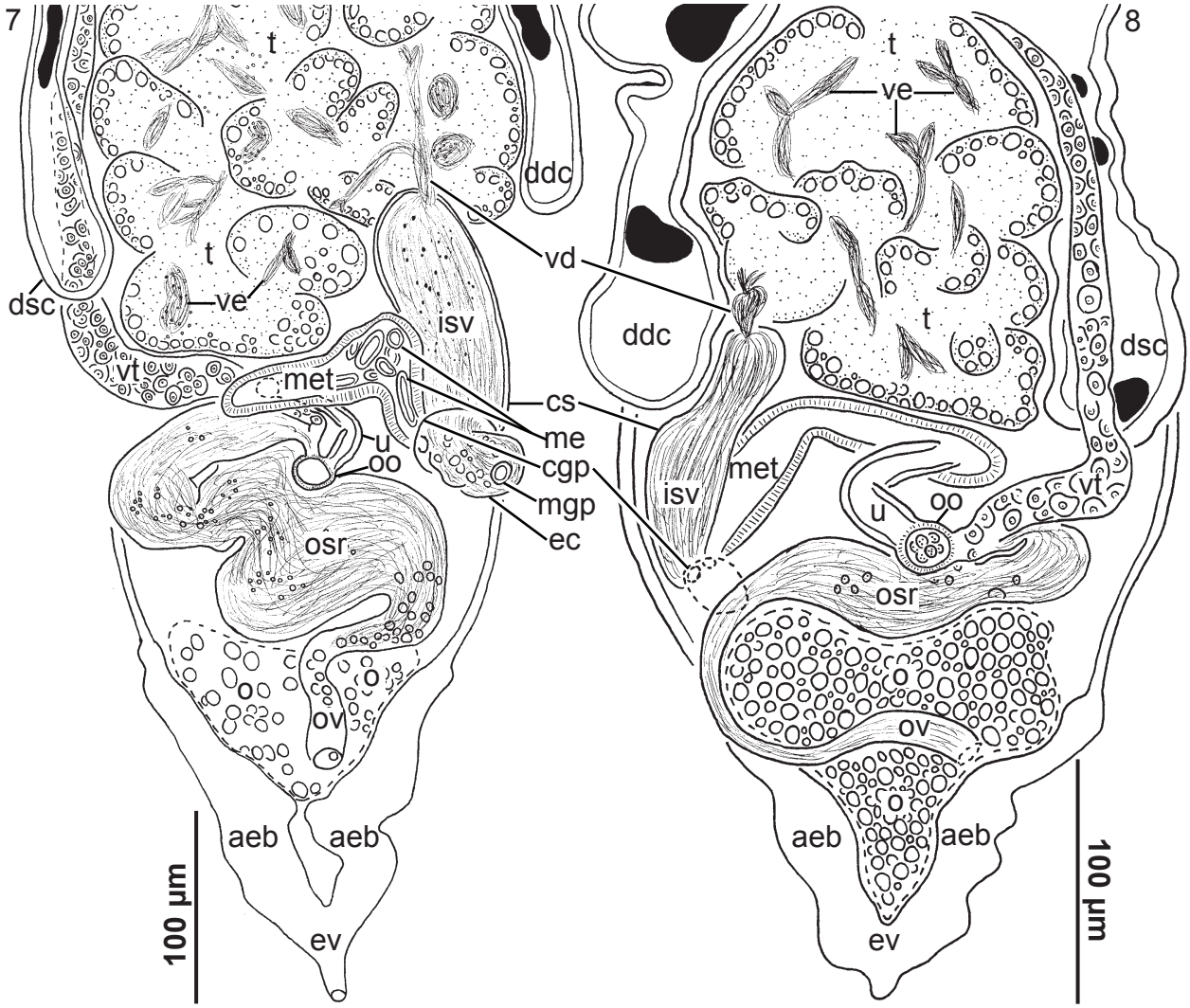
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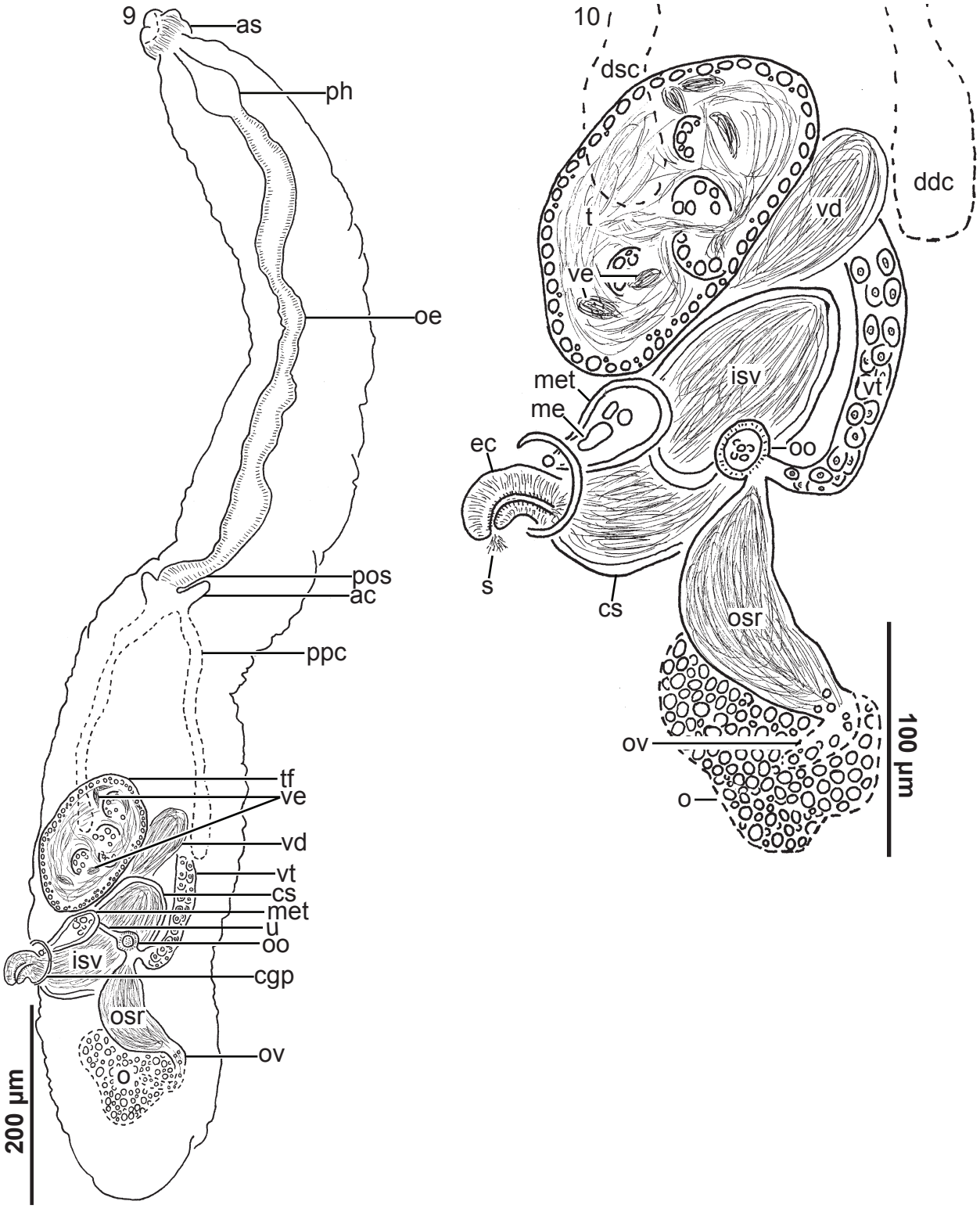
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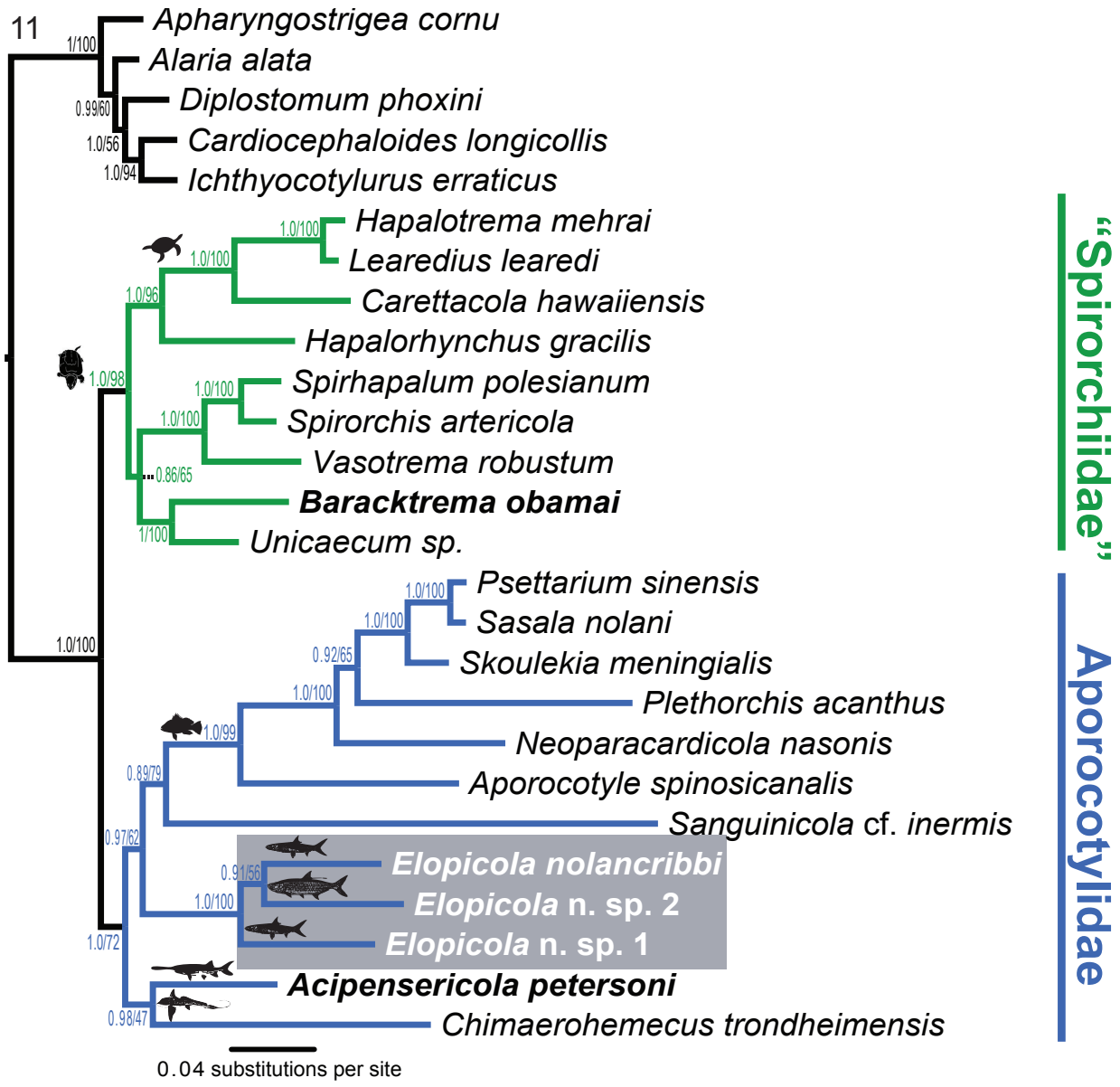
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1.0/56
1.0/94
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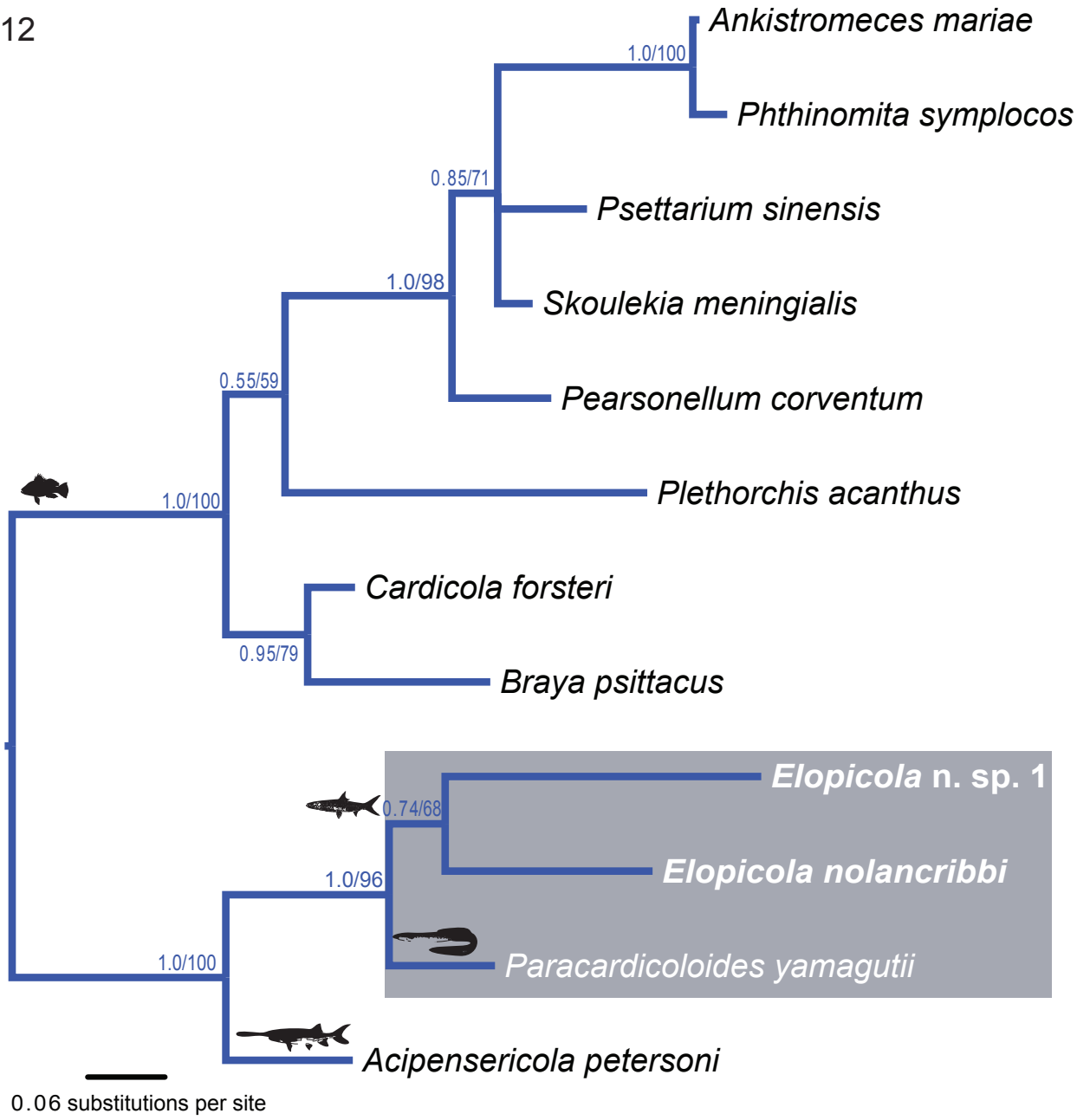
Alaria alata
Diplostomum phoxini
Cardiocephalaides longicollis
Ichthyocotylurus erraticus
1.0/100
1.0/100
1.0/96
1.0/98
1.0/100
1.0/100
0.86/65
1/100
Hapalotrema mehrai
Learedius learedi
Carettacola hawaiiensis
Hapalorhynchus gracilis
Spirhapalum polesianum
Spirorchis artericola
Vasotrema robustum
Baracktrema obamai
Unicaecum sp.
1.0/100
1.0/100
0.92/65
1.0/100
1.0/99
0.89/79
0.97/62
0.91/56
1.0/100
1.0/72
0.98/47
Psettarium sinensis
Sasala nolani
Skoulekia meningialis
Plethorchis acanthus
Neoparacardicola nasonis
Aporocotyle spinosicanalis
Sanguinicola cf. inermis
Elopicola nolancribbi
Elopicola n. sp. 2
Elopicola n. sp. 1
Acipensericola petersoni
Chimaerohemecus trondheimensis
0.04 substitutions per site
"Spirorchiidae"
Aporocotylidae

12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Pearsonellum corventum
Plethorchis acanthus
1.0/100
Cardicola forsteri
0.95/79
Braya psittacus
Elopicola n. sp. 1
0.74/68
Elopicola nolancribbi
1.0/96
Paracardicoloides yamagutii
1.0/100
Acipensericola petersoni
0.06 substitutions per site

Table 1. Blood flukes (Digenea: Aporocotylidae) of members of the early-branching actinopterygians (Chondrostei: Acipenseriformes) and teleosts (Teleostei: Elopomorpha).

Aporocotylid	Host	Site	Locality	Reference(s)
Acipensericola petersoni Bullard, Jensen, Snyder, and Overstreet, 2008	*Polyodon spathula* (Walbaum, 1792) (Acipenseriformes: Polyodontidae)	atrium, ventricle, and bulbus arteriosus of heart	Six Mile Lake, MS, USA	Bullard et al. (2008)
Elopicola nolancribbi Bullard, 2014	*Elops saurus* (Linnaeus, 1766) (Elopiformes: Elopidae)	indeterminate	Ship Island, off MS, Gulf of Mexico	Bullard (2014)
Elopicola n. sp. 1	*Elops hawaiensis* (Reagan, 1909) (Elopiformes: Elopidae)	indeterminate	Chợ Vĩnh Hải fish market, Nha Trang, Vietnam (South China Sea)	present study
Elopicola n. sp. 2	*Megalops atlanticus* (Valenciennes, 1847) (Elopiformes: Megalopidae)	heart	Off Florida, Gulf of Mexico	present study
Paracardicoloides yamagutii Martin, 1974	*Anguilla reinhardtii* Steindachner, 1867 (Elopomorpha: Anguilliformes)	Blood vessels, dorsal aorta	Brisbane River and tributaries, Australia	Martin 1974, Nolan and Cribb 2004

Table 2. DNA sequences used in the present study.

Digenean taxon	Host species	Locality	GenBank Accession Numbers			Reference(s)
			18S	28S	ITS2	
Superfamily Schistosomatoidea						
Family Aporocotylidae						
Acipensericola petersoni	*Polyodon spathula*	Tennessee River, USA	XXXXXXX	XXXXXXX	XXXXXXX	Present study
Ankistromeces mariae	*Meuschenia freycineti*	SW Pacific, off Stanley Harbour, Australia			DQ335839	Nolan and Cribb (2006a)
Aporocotyle spinosicanalis	*Merluccius merluccius*	NE Atlantic	AJ287477	AY222177		Cribb et al. (2001), Olson et al. (2003)
Braya psittacus	*Scarus ghobban*	SW Pacific, off Heron Island, Australia			DQ059625	Nolan and Cribb (2006b)
Cardicola forsteri	*Thunnus maccoyii*	SW Pacific, off Port Lincoln, S Australia			AB742428	Shirakashi et al. (2013)
Chimaerohemecus trondheimensis	*Chimaera monstrosa*	NE Atlantic, off Bergen, Norway	AY157213	AY157239		Lockyer et al. (2003b)
Elopicola nolancribbi	*Elops saurus*	Gulf of Mexico, off Ship Island, USA	XXXXXXX	XXXXXXX	XXXXXXX	Present study
Elopicola n. sp. 1	*Elops hawaiensis*	W Pacific, off Nha Trang, Vietnam	XXXXXXX	XXXXXXX	XXXXXXX	Present study
Elopicola n. sp. 2	*Megalops atlanticus*	Gulf of Mexico, off FL, USA	XXXXXXX	XXXXXXX	XXXXXXX	Present study
Neoparacardicola nasonis	*Naso unicornis*	SW Pacific, off Lizard Island, Australia	AY222097	AY222179		Olson et al. (2003)
Paracardicoloides yamagutii	*Anguilla reinhardtii*	Brisbane River tributaries, Australia			AY465872	Nolan and Cribb (2004a)
Pearsonellum corventum	*Plectropomus leopardus*	SW Pacific, off Heron Island, Australia			AY465873	Nolan and Cribb (2004b)
Phthinomita symplocos	*Siganus lineatus*	SW Pacific, off Lizard Island, Australia			DQ335867	Nolan and Cribb (2006a)
Plethorchis acanthus	*Mugil cephalus*	Australia	AY222096	AY222178	AY465875	Olson et al. (2003), Nolan and Cribb (2006a)
Psettarium (*as Paradeontacylix*) *sinensis*	*Takifugu rubripes*	Fuzhou City, China	EU081899	EU368853	EU082007	Chen et al. (2008)
Sanguinicola cf. *inermis*	*Lymnaea stagnalis*	Warminia-Mazury Region, Poland	AY222098	AY222180		Olson et al. (2003)
Sasala nolani	*Arothron meleagris*	S Pacific off Moorea, French Polynesia	AY157184	AY157174		Lockyer et al. (2003a)

Skoulekia meningialis	*Diplodus vulgaris*	Mediterranean Sea, off Valencia, Spain	FN652294	FN652293	FN652292	Alama-Bermejo et al. (2011)
Family Spirorchiidae						
Baracktrema obamai	*Siebenrockiella crassicolli*	Malaysia	XXXXXXX	XXXXXXX	XXXXXXX	Present study
Carettacola hawaiiensis	*Chelonia mydas*	Pacific Ocean, HI, USA	AY604717	AY604709		Snyder (2004)
Hapalorhynchus gracilis	*Chelydra serpentina*	Reelfoot Lake, TN, USA	AY604718	AY604710		Snyder (2004)
Hapalotrema mehrai	*Chelonia mydas*	Pacific Ocean, HI, USA	AY604716	AY604708		Snyder (2004)
Learedius learedi	*Chelonia mydas*	Pacific Ocean, HI, USA	AY604715	AY604707		Snyder (2004)
Spirhapalum polesianum	*Emys orbicularis*	Lesniki, Ukraine	AY604713	AY604705		Snyder (2004)
Spirorchis artericola	*Chrysemys picta*	Reelfoot Lake, TN, USA	AY604712	AY604704		Snyder (2004)
Unicaecum sp.	*Trachemys scripta*	Reelfoot Lake, TN, USA	AY604719	AY604711		Snyder (2004)
Vasotrema robustum	*Apalone spinifera*	Nishnabotna River, IA, USA	AY604714	AY604706		Snyder (2004)
Superfamily Diplostomoidea						
Family Diplostomidae						
Alaria alata	*Nyctereutes procyonoides*	Kherson Region, Ukraine	AY222091	AF184263		Olson et al. (2003)
Diplostomum phoxini	*Phoxinus phoxinus*	Aberystwyth, Wales	AY222090	AY222173		Olson et al. (2003)
Family Strigeidae						
Apharyngostrigea cornu	*Ardea cinerea*	Kherson Region, Ukraine	AY222092	AF184264		Olson et al. (2003)
Cardiocephaloides longicollis	*Larus ridibundus*	Kherson Region, Ukraine	AY222089	AY222171		Olson et al. (2003)
Ichthyocotylurus erraticus	*Coregonus autumnalis*	Lough Neagh, UK	AJ287526	AY222172		Olson et al. (2003)

Table 3. Oligonucleotide primers used in the present study.

Primer ID (direction)	Sequence 5' to 3'	Reference
Amplification and Sequencing		
18S rDNA		
18SE (alias 18S-A) (F)	CCGAATTCGTCGACAACCTGGTT GATCCTGCCAGT	Littlewood and Olson (2001)
Worm B (R)	CTTGTTACGACTTTTACTTCC	Littlewood and Olson (2001)
28S rDNA		
U178 (F)	GCACCCGCTGAAYTTAAG	Lockyer et al. (2003a)
L1642 (R)	CCAGCGCCATCCATTTTCA	Lockyer et al. (2003a)
ITS2 rDNA		
GA1 (F)	AGAACATCGACATCTTGAAC	Anderson and Barker (1998)
ITS2.2 (R)	CCTGGTTAGTTTCTTTTCCTCCGC	Cribb et al. (1998)
Additional sequencing		
18S rDNA		
388F (F)	AGG GTT CGA TTC CGG AG	Littlewood and Olson (2001)
1100F (F)	CAGAGTTTCGAAGACGATC	Littlewood and Olson (2001)
CEST1R (R)	TTTTTCGTCACTACCTCCCC	Littlewood and Olson (2001)
1270R (R)	CCGTCAATTCCTTTAAGT	Littlewood and Olson (2001)
28S rDNA		
300F (F)	CAAGTACCGTGAGGGAAAGTTG	Lockyer et al. (2003a)
900F (F)	CCGTCTTGAAACACGGACCAAG	Lockyer et al. (2003a)
1200R (alias LSU1200R) (R)	GCATAGTTCACCATCTTTCGG	Lockyer et al. (2003a)

CHAPTER 6: PHYLOGENY OF CRANIATE BLOOD FLUKES (PLATYHELMINTHES: DIGENEA: SCHISTOSOMATOIDEA), WITH EMPHASIS ON "FISH BLOOD FLUKES" (APOROCOTYLIDAE)

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ABSTRACT

Blood flukes (Digenea: Schistosomatoidea) comprise a diverse group of hematozoic digeneans that mature in the vascular system (rarely other sites) of a wide spectrum of craniate hosts. Because of the veterinary and medical importance of schistosomes, previous molecular systematics studies have focused on interrelationships among tetrapod blood flukes. However, uncertainty stemming from fish blood fluke (Digenea: Aporocotylidae) interrelationships obstructs a deeper understanding of the evolutionary origins of flatworm parasitism in craniates, including the origin of schistosomes. Herein, we combine 2 nuclear ribosomal genes (18S and 28S rDNA) to provide an updated phylogeny for the blood flukes. Although no significant novel relationships have been recovered among tetrapod blood flukes ("spirorchiids" and schistosomes), our phylogenetic trees provided meaningful insights about the evolution of fish blood flukes. This is the first phylogenic reconstruction that tested and supported monophyly of chondrichthyan, elopiform, and otophysan blood flukes. The earliest-branching monophyletic clade sister to the remaining fish blood flukes comprised the acipenseriform blood fluke *Acipensericola petersoni* plus all chondrichthyan blood flukes. This clade was recovered sister to elopiform blood flukes that, in turn, were sister

to a clade comprising blood flukes that infect otophysan plus neoteleosteans. Such internal node order is congruent to that of their hosts. Although chondrichthyan blood flukes were not sister to Sarcopterygii and Actinopterygii, resulting tree topology broadly support the notion that blood flukes exhibit co-phyly with their tetrapod and bony fish hosts. Phylogenomic and taxonomic studies investigating blood flukes that potentially infect hagfish, lamprey, coelacanth, and lungfish could improve our current understanding of blood fluke-craniate co-phyly.

1. INTRODUCTION

Blood flukes (Digenea: Schistosomatoidea) are hematozoic digeneans that comprise 321 species assigned to 74 genera and infect a wide spectrum of definitive hosts, including sharks, rays, chimaeras, bony fishes, turtles, birds and mammals, and use gastropods, bivalves, and polychaetes as intermediate hosts (Smith 1997 a, b, Khalil 2002; Platt 2002; Orélis-Ribeiro et al. 2014). Collectively, these hosts are associated with marine, estuarine, and freshwater habitats and their distribution covers a wide band of latitude (Smith 1997 a, b, Khalil 2002, Platt 2002, Orélis-Ribeiro et al. 2014). While most blood flukes mature in blood, some infect body cavity (Linton 1910, Szidat 1951, Orélis-Ribeiro et al. 2014, Orélis-Ribeiro and Bullard 2015) and the ectomeningeal veins of the brain (Alama-Bermejo et al. 2011). Because of the severity of some of these infections, several species of blood flukes are considered of medical and veterinary importance, with some members emerging as pathogens in aquaculture settings

(Meade and Pratt, 1965; Meade 1967; Schell 1974; Bullard and Overstreet 2002, 2008; Ogawa et al. 2007; Cribb et al. 2011; Kirk 2012)

Historically, the systematics of blood flukes is not based on a phylogenetic hypothesis, but on the vertebrate definitive hosts they infect: the fish blood flukes (Aporocotylidae; hereafter “FBFs”) infect chondrichthyans and bony fishes; turtle blood flukes (paraphyletic “Spirorchiidae”; hereafter “TBFs”) infect freshwater and marine turtles, and the schistosomes (Schistosomatidae) infect a crocodilian, birds, and mammals (Orélis-Ribeiro et al. 2014). Given that schistosomes are perhaps the most devastating human parasitic platyhelminth, with estimated mortalities of 280,000 people per year in sub-Saharan Africa (Van Der Werf et al. 2003), most of the early molecular systematics works have seemingly neglected members of other blood fluke families and prioritized a dense taxon sampling within Schistosomatidae (Lockyer et al. 2003b, Olson et al. 2003, Snyder 2004, Cribb et al. 2011, Orélis-Ribeiro et al. 2014). The most comprehensive phylogeny of the Digenea (Olson et al. 2003) was the first study that included molecular data from all families of blood flukes. However, Olson’s et al. (2003) trees comprised only 19% of the generic diversity of Schistosomatoidea and a bias towards schistosomes (53%) relative to TBFs and FBF (5% and 13%, respectively). In a subsequent phylogenetic hypothesis, Snyder’s (2004) analyses supported paraphyly of Spirorchiidae by including 38% and 67% of TBFs and schistosomes genera (respectively); whereas, only 5% of FBF genera known at that time were included. Although maintaining approximately the same generic coverage for TBFs and schistosomes (38% and 73%, respectively), Cribb et al.’s (2011) analysis significantly increased the taxonomic coverage of FBFs, including 24% of the accepted genera.

Recently, Orélis-Ribeiro et al. (2014) published the most comprehensive phylogeny of Schistosomatoidea to date, including 43% of the generic diversity of the group. Despite the higher generic coverage of schistosomes (87%), TBFs (43%), and FBFs (26%) on the latter study, the taxononomic coverage of TBFs and FBFs still represents an obvious gap in our knowledge regarding evolutionary relationships of blood flukes.

Some unique atributes underpin the importance of FBFs to our understanding of the evolution of blood flukes. They are the most diverse family of blood flukes, outnumbering TBFs and schistosomes regarding the number of species and genera (Orélis-Ribeiro et al., 2014). Regarding evolutionary divergence of hosts (Amemiya et al., 2013; Nelson, 2006; Betancur et al. 2013), FBFs are the only members of Schistosomatoidea that exploit a spectrum of definitive hosts that ranges from the most early-branching jawed craniates (Bazikalova 1932, Short 1954, Van der Land 1967, Madhavi and Rao 1970, Bullard et al. 2006, Bullard and Jensen 2008, Orélis-Ribeiro et al. 2013) to the most highly-derived bony fishes (Pleuronectiformes and Tetraodontiformes) (Goto and Ozaki 1929, Manter 1940, Martin 1960, Nolan and Cribb 2004c, Ogawa et al. 2007, Yong and Cribb 2011). In addition, FBFs are likely ancestors of tetrapod blood flukes (TBFs plus schistosomes) and could contribute to the understanding of the origins of virulence and pathogenicity within Schistosomatoidea (Orélis-Ribeiro et al., 2014).

We herein present the most complete superfamily-level phylogeny of blood flukes including 97 species covering 58% (43 of 74 genera) of the accepted generic diversity of the group. Our goals were to (i) elucidate the interrelationships among all blood fluke families, (ii) test FBF monophyly, and (iii) test tetrapod blood fluke monophyly. We

herein extend previous molecular work by exploring two sets of nuclear ribosomal gene sequences (18S and 28S rDNA) derived from a denser taxon sampling of FBFs, including sequence data from FBF lineages that infect elasmobranchs (sharks and rays), early-branching actinopterygians (Polyodontidae, Elopidae, Megalopidae, Pangasiidae, and Clariidae) plus derived actinopterygians (members of the families Rachycentridae and Kyphosidae). We also expand taxon sampling of TBFs, generating new sequence data for five of the 21 accepted genera, including 13 sequences from innominate taxa.

2. MATERIALS AND METHODS

2.1 Taxonomic sampling

FBFs and TBFs were collected between 2012–2015 from several fishes and turtles from Asia and North America. Newly collected specimens from 38 species were included in our analyses. Of these species, 25 are FBFs of 15 genera and 12 are TBFs of seven genera. Noteworthy is that because some of these collections comprised surveys of novel geographical localities and/or hosts, one FBF and 10 TBFs are innominate. Our dataset was supplemented with molecular sequence data derived from 67 taxa published by Lockyer et al. (2003b), Morgan et al. (2003), Snyder (2004), Brant (2007), Chen et al. (2008), Brant and Loker (2009), Hanelt et al. (2009), Tkach et al. (2009), Cribb et al. (2011), Brant et al. (2012), and Hernández-Orts et al. (2012). With the exception of *Sanguinicola* cf. *inermis*, which represents a sequence sourced from an unidentified cercaria, all sequences are derived from morphologically identified adult

flukes or, in the case of some schistosomes, cercariae sourced from laboratory-reared strains. Although no morphological evidence exists that those specimens were a species of *Sanguinicola* or *S. inermis*, we use this sequence in the present study based on their molecular phylogenetic affiliation to other FBFs (Olson et al. 2003, Snyder 2004, Cribb et al. 2011). Where available, voucher material (hologenophores and paragenophores, *sensu* Pleijel et al. 2008) have been deposited at United States National Museum (USNM, Washington, D.C.). Table 1 provides the list of species used in this study with their hosts, geographical localities, museum voucher numbers, and GenBank accession numbers.

2.2 DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from newly collected specimens fixed and stored in 100% EtOH using DNeasy^{TM} Blood and Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer protocol, except for the incubation period with proteinase-K that was extended to overnight and the final elution step wherein only 100 µl of elution buffer was used, in order to increase the final DNA concentration in the eluate. Extraction products served as templates for the amplification of the 18S and 28S rDNA genes using the set of primers described in Table 2. PCR amplifications were carried out in a total volume of 25 µl containing approximately 2 µl of DNA template, 0.4 µM of each primer along with 1× buffer, 2.5 mM $MgCl_2$ (New England Biolabs, Ipswich, MA), 1 mM dNTP mixture, and 0.3 µl *Taq* polymerase (5 U/µl) (New England Biolabs, Ipswich, MA). Amplification reactions were performed with a cycling profile of 4 min at 94 °C for initial denaturation, followed by 40 repeating cycles of 94 °C for 40 s for denaturation, 50 °C

for 30 s for annealing, and 72 °C for 2 min for extension, followed by a final 7 min at 72 °C for extension. All PCR reactions were performed in a Veriti Thermal Cycler (Applied Biosystems). PCR products (5 µl) were verified on a 1 % agarose gel and stained with ethidium bromide. PCR amplicons were gel-excised using QIAquick™ Gel Extraction Kit (QIAGEN) following the manufacturer's protocol. DNA sequencing was performed by GENEWIZ with ABI Prism 3730xl DNA analyzer (GENEWIZ, Inc., South Plainfield, NJ). Primers used in sequencing of 18S and 28S rDNA genes included the PCR primers and some additional internal primers described in Table 2. Inspection, editing, and assembling of chromatograms of the forward and reverse DNA strands were performed on BioNumerics version 7.0 (Applied Maths, Sint-Martens-Latem, Belgium). New sequences generated by this work were submitted to GenBank (Table 1).

2.3. Sequence alignments and phylogenetic analyses

Three datasets were generated: (i) 18S (105 taxa; 1690 positions); 28S (105 taxa; 1114 positions); 18S+28S (2804 taxa; 105 positions). Outgroup selection for these dataset was based on the phylogeny of the Digenea published by Olson et al. (2003) and included representatives of the superfamily Diplostomoidea (Table 1).

For each dataset, sequences were aligned using MAFFT (Katoh and Toh, 2010) with default settings implemented in the CIPRES Science Gateway V. 3.3 (Miller et al., 2010). The resulting alignment was refined by eye using MEGA version 5.2.2 (Tamura et al., 2011) and ends of each fragment were trimmed to match the shortest sequence. The concatenated alignment (18S+28S) was created using the web application FaBox

1.35 (Villesen, 2007). Ambiguous positions in the single gene alignments were identified and removed using the Gblocks server (Castresana, 2000) with settings for a less stringent selection. Bayesian inference (BI) was performed using the Metropolis-coupled Markov chain Monte Carlo method (MC^3) in MrBayes version 3.2.6 (Huelsenbeck et al. 2001, Ronquist and Huelsenbeck 2003, Huelsenbeck and Ronquist 2005) and run on CIPRES (Miller et al. 2010). Model of evolution was selected based on the Akaike Information Criterion (Posada and Buckley, 2004) as implemented in the jModelTest version 2.1.4 (Darriba et al. 2012, Guindon and Gascuel 2003). The GTR + I + G model was inferred as the best estimator for all datasets, respectively; therefore, BI used the following parameters: nst = 6, rates = invgamma, ngammacat = 4, and default priors. Analyses were run in duplicate each containing four independent chains (three heated and one cold chain) (nchains = 4) for 1.0×10^7generations (ngen = 10,000,000) sampled at intervals of 1000 generations (samplefreq = 1000). Results of the first 2500 sampled trees were discarded as “burn-in” based on the stationarity of the likelihood values, assessed by plotting the log-likelihood values of the sample points against generation time using Tracer version 1.5 (Rambaut and Drummond, 2009). All retained trees were used to estimate posterior probability of each node. A majority rule consensus tree with average branch lengths was constructed for the remaining trees using ‘summarize the trees’ (sumt) in MrBayes. Additionally, a maximum likelihood (ML) analysis was performed on all datasets in RAxML v.7.2.6 (Stamatakis, 2006) and also performed on CIPRES (Miller et al. 2010), with default parameters. GTRGAMMA model was employed for all datasets. Bootstrap values were estimated from 1,000 replicates. Resulting phylogenetic trees were visualized using FigTree v1.4.2 (Rambaut, 2009) and

further edited with Adobe Illustrator CS3 (Adobe Systems). Branch supports for BI and ML analyses were considered as significant when posterior probabilities were >0.95 and bootstrap values were >70%, respectively. Accession numbers for each gene sequenced are provided in Table 1.

3. RESULTS

3.1 Taxonomic coverage

A total of 38 near-complete 18S rDNA and partial 28S rDNA sequences were produced, including newly generated data for 25 FBFs, 12 TBFs, and 1 clinostomatid (Table 1). The type species of 12 FBF genera, 4 TBF genera, and 10 schistosome genera are represented in the dataset, including 7 and 1 newly generated sequences for type species of FBFs and TBFs, respectively. This is the first molecular phylogenetic analyses to simultaneously include FBF species that infect sharks (2 species, 2 genera), stingrays (2 species, 1 genus), early-branching actinopterygians of Polyodontidae (1 species, 1 genus), Elopidae (2 species, 1 genus), Megalopidae (1 species, 1 genus), Pangasiidae (1 species, 1 genus), and Clariidae (1 species, 1 genus) plus actinopterygians of Rachycentridae (2 putative species, 1 genus) and Kyphosidae (1 species, 1 genus). This data, in combination with the publicly available sequence data, included 52%, 43%, and 93% of the generic diversity of FBFs, TBFs, and schistosomes.

3.2 Molecular phylogeny

BI and ML analyses of the individual loci recovered tree topologies showing a poor resolution in the relationships among the deepest nodes. Conversely, BI and ML analyses of the concatenated dataset yielded well-resolved topologies that will form the foundation of the results presented below (Fig. 1). Also, because most of the novel relationships were recovered within Aporocotylidae, greater detail will be provided to these outcomes in the following discussion.

The concatenated dataset yielded a final alignment comprising 2,804 sites (1,690 and 1,114 sites from 18S and 28S rDNA, respectively), 14% of these sites comprised uncertain homologous positions that were excluded by Gblock. Thus, this final data matrix consisted of 2,498 (1,569 and 929 derived from 18S and 28S rDNA fragments, respectively) positions per taxon (1,221 conserved, 1,277 variable, and 1,067 parsimony-informative). Tree topologies recovered by either BI and ML analyses ere largely congruent, however, the former often recovered higher support values.

Regarding the interrelationships among the blood fluke families, our resulting topologies rendered Aporocotylidae monophyletic and sister to clinostomatids plus tetrapod blood flukes (TBFs and schistosomes), with TBFs paraphelytic and schistosomes monophyletic (Fig. 1). Within Aporocotylidae, the earliest branching monophyletic group sister to the remaining taxa comprised an acipenseriform blood fluke, *Acipensericola petersoni*, plus all chondrichthyan blood flukes (FBFs of the genera *Chimaerohemecus*, *Hyperandrotrema*, *Selachohemecus*, and *Myliobaticola*). This clade was recovered sister to elopiform blood flukes that, in turn, were sister to a clade comprising blood flukes that infect otophysan plus neoteleostean fishes. Regarding the generic-level interrelationships within the latter crown clade, tree

topologies yielded *Aporocotyle* spp. (blood flukes of gadiform and ophidiiform fishes) as sister to two major clades comprising (*Plethorchis acanthus* (*Neoparacardicola nasonis* (*Deontacylix* sp. (*Phthinomita* spp. (*Skoulekia meningialis* (*Sasala nolani,Psettarium sinensis*) and (*Littorellicola billhawkinsi* (*Psettarium* spp. (*Paradeontacylix* sp. (*Cardicola* spp.)))). Species of *Cardicola* that infect sciaenids were rendered paraphyletic in our analyses, with *Cardicola laruei*, the blood fluke that matures in *Cynoscion arenarius* (Perciformes: Sciaenidae), and *Elaphrobates euzeti*, the FBF that infects *Lutjanus campechanus* (Lutjanidae) showing closer phylogenetic affinities than the two species infecting the sciaenids *Sciaenops ocellatus* and *Pogonias cromis* (*Cardicola currani* and *Cardicola palmeri*, respectively).

FBFs that mature in body cavity were paraphyletic. Both *Deontacylix* sp. and an unidentified aporocotylid (putative new genus) that mature in the body cavity of *Kyphosus* cf. *vaigiensis* (Perciformes: Kyphosidae) and *Pangaseus macronema* (Siluriformes: Pangasiidae), respectively, were recovered in different positions in the trees. While *Deontacylix* sp. clustered with neoteleostean blood flukes as mentioned before, the pangasiid blood fluke formed a clade with another siluriform blood fluke, *Nomasanguinicola canthoensis*. Noteworthy also is that, with the only exception of *Psettarium* spp., all genera represented by more than one species were recovered monophyletic (i.e., *Selachohemecus* spp., *Myliobaticola* spp., *Elopicola* spp., *Aporocotyle* spp., and *Cardicola* spp.) (Fig. 1).

Regarding the relationships among the paraphyletic “Spirorchiidae”, two major clades were recovered with strong nodal support: (*Baracktrema obamai*, *Unicaecum* sp.) plus (*Vasotrema* spp. (*Spirhapalum* spp.(*Spirorchis* spp., unidentified spirorchiid n.

sp. 1, n. gen. 2), and (*Griphobilharzia amoena* (*Hapalorhynchus* spp. (*Coeuritrema platti*, unidentified spirorchiid n. sp. 1, n. gen. 1))) + (*Carettacola hawaiiensis* (*Hapalotrema mehrai* (*Learedius learedi*))) plus schistosomes. While the former clade was comprised exclusively of blood flukes that infect freshwater turtles, the latter also included blood flukes that infect a freshwater crocodile, marine freshwater turtles, birds, and mammals.

Within Schistosomatidae, tree topologies retrieved the bird schistosomes *Macrobilharzia macrobilharzia* as the earliest divergent taxon sister to two major clades. One comprising the mammalian schistosomes (*Heterobilharzia americana*+ *Schistosomatium douthitti*) as sister group to bird schistosomes of the genera *Bilharziella*, *Gigantobilharzia*, *Dendritobilharzia*, *Trichobilharzia*, *Anserobilharzia*, and *Allobilharzia*. The other major clade rendered the bird schistosomes (*Ornithobilharzia canaliculata*+*Austrobilharzia* spp.) as sister group to the elephant schistosome *Bivitellobilharzia nairi* plus *Schistosoma* spp.

4. DISCUSSION

4.1 Selection of molecular markers

By increasing the taxonomic coverage within FBFs and using a combination of sequences from 2 nuclear ribosomal genes, we presented herein the most comprehensive and robust phylogeny of Schistosomatoidea. The addition of 18S rDNA has substantially improved previous phylogenetic inferences based on 28S rDNA alone (Cribb et al., 2011; Orélis-Ribeiro et al. 2014), and supported other flatworm studies

where the combination of these 2 genes also has added stability and resolution to the resulting tree topologies (Olson et al. 2001, Olson and Littlewood 2002, Olson et al. 2003).

4.2 Interrelationships within Schistosomatoidea

Despite using a relatively wider taxonomic scope and large sequence data, our analyses did not refute the previously published family-level relationships among blood flukes, i.e., the monophyletic Aporocotylidae sister to the paraphyletic “Spirorchiidae” plus monophyletic Schistosomatidae (see Olson, et al. 2003; Snyder, 2004; Brant et al., 2006; Orélis-Ribeiro, et al. 2014). Noteworthy also is that members of the family Clinostomatidae were recovered sister to the clade comprising the tetrapod blood flukes, this is consistent with the topologies published by Snyder (2004) and Orélis-Ribeiro et al. (2014) (see discussion in Orélis-Ribeiro et al., 2014).

4.3 Monophyly of Aporocotylidae

Given the inclusion in our analyses of sequences derived from FBFs maturing in early-branching lineages of jawed craniates (Chondricthyes) and ray-finned fishes (Actinopterygii), perhaps the most striking result of the present study is that monophyly of Aporocotylidae was not rejected. Indeed, as mentioned previously, FBFs that infect elasmosbranchs, elopiforms, siluriforms, and neoteleosts each formed strongly supported monophyletic sister groups in the tree topologies. Noteworthy is that the branching pattern within Aporocotylidae largely reflects the topology recovered for bony fishes (see Fig. 1 in Betancur et al. 2013). Specifically, although chondrichthyan blood

flukes were not sister to Sarcopterygii and Actinopterygii, the internal node order observed for the acipenseriform, elopiform, otophysan, and neoteleost blood flukes matches that of their hosts (Fig. 1).

Regarding the tree topologies recovered in previous molecular phylogenetic studies, with due consideration to their restricted taxonomic range, our analyses rendered relatively similar branching pattern. With exception of Cribb et al. (2011), no study rejected monophyly of Aporocotylidae. Cribb et al.'s (2011) tree recovered *C. trondheimensis* clustered with the sequence derived from a cercariae that were identified as "*Sanguinicola* cf*. inermis*", whereas all other euteleostean blood flukes grouped in a separate clade. Our analyses supported *Sanguinicola* cf*. inermis* clustered together with *N. canthoensis*, which suggests that this species has a phylogenetic affiliation with the otophysan blood fluke. Yet, no sequence derived from a morphologically identified adult of *Sanguinicola* exist, and thus, we caution authors in using this sequence as a definitive representative of this genus. Noteworthy also is that Cribb et al.'s (2011) results may have been influenced by the addition of sequences derived from an unidentified freshwater gastropod cercariae reported by Brant et al. (2006). We mention this because the inclusion of those cercariae by Orélis-Ribeiro et al. (2014) have also seemed to negatively affect topological resolutions and support values. While no evidence is provided that these cercariae have any relationship with FBFs, we also recommend caution on the use of their molecular data for phylogenetic purposes.

Generic interrelationships among euteleostean blood flukes matched earlier phylogenetic studies: *Aporocotyle* was monophyletic (Orélis-Ribeiro et al. 2014),

Ankistromeces and *Phthinomita* were closely related (Nolan and Cribb 2006a), *Paradeontacylix* and *Cardicola* clustered together (Holzer et al. 2008, Orélis-Ribeiro et al. 2014), *Cardicola* was supported as monophyletic (Alama-Bermejo et al. 2011), *Elaphrobates* clustered among species presently assigned to *Cardicola* (Bullard and Overstreet 2003, Nolan and Cribb 2006b, Orélis-Ribeiro et al. 2014).

We sequenced specimens comprising 12 FBF type species, most belonging to monotypic genera (92%). This low taxon sampling within FBFs genera precludes the use of our tree topologies to inform and stabilize the taxonomy of those genera, however, some remarks are worthy of note. The neoteleostean blood flukes *Littorellicola billhawkinsi* and *Psettarium anthicum* were recovered monophyletic. Despite infecting phylogenetically distantly-related hosts (Carangidae and Rachycentridae) and differing in the number of testis (8 vs. 1), their elongated slender body having a posterolateral protuberance may represent synapomorphies for this clade. Noteworthy is that another species of *Psettarium* clustered elsewhere in the trees. *Psettarium sinensis* (Liu, 1977) Ogawa, Nagano, Akai, Sugita, and Hall, 2007, originally described as a member of *Paradeontacylix*, was tentatively reassigned to *Psettarium*. This issue could be addressed by a morphological re-description of newly collected specimens of the type species of *Psettarium* (*Psettarium japonicum* [Goto and Ozaki, 1929] Goto and Ozaki, 1930), supplemented by molecular data. As stated by Bullard (2012), the same approach could render a substantial change in the number of species assigned to the genus *Cardicola*.

While comparative morphological evidence has supported most of the interrelationships retrieved within this family, some unexpected results have been

observed. Specifically, although *Acipensericola* and *Elopicola*, both blood fluke lineages that mature in early-branching ray-finned fishes, share a suit of unique features (i.e., bowl-shaped anterior sucker, straight lateral tegumental body spines, inverse U-shaped intestine, an intertesticular ovary, and a Laurer's canal) (Bullard et al. 2008, Bullard 2014, Orélis-Ribeiro et al. manuscript in preparation) that distinguish them from other FBFs, their monophyly was rejected. Noteworthy also is that these blood flukes share some resemblance with TBFs (i.e., shape and relative positions of spines, oral sucker, and ceca), but their placement in the tree topologies were distinct as well. However, the closer association rendered between *A. petersoni* and all chondrichthyan blood flukes was not well supported (PP= 0.92, BS=32, Fig. 1), suggesting that a denser taxon sampling among other blood flukes that infect early-branching ray-finned fishes may substantially influence the branching pattern in this portion of the tree. Nonetheless, monophyly of chondrichthyan blood flukes was strongly supported. This is the first molecular phylogenetic study that tested and confirmed a tight, distinct clade including species of *Chimaerohemecus*, *Hyperandrotrema*, *Myliobaticola*, and *Selachohemecus*. Perhaps the most unexpected result of the present study is the presence of stingray blood flukes within this clade. The holocephalan and shark blood flukes (*C. trondheimensi*s, *Hyperandrotrema* spp., and *Selachohemecus* spp.) are markedly distinct from all other blood flukes by having robust C-shaped tegumental body spines, however, the stingray blood flukes, *Orchispirium heterovitellatum* and *Myliobaticola richardheardi*, have an aspinous body. This suggests that the C-shaped spines may constitute a synapomorphy of this clade that was lost on the blood flukes of stingrays. Noteworthy also is that some blood flukes within this clade retrieved the longest branch

lengths in the trees, which support the large divergence among those species and all remaining blood flukes (Fig. 1).

4.4 Interrelationships within paraphyletic "Spirorchidae"

Regarding generic relationships among TBFs, our analyses were consistent with previous published tree topologies by Snyder (2004) and Orélis-Ribeiro et al. (2014), however, the addition of new taxa provided new insights in the two major clades recovered. Comparative morphological evidence support the clade formed by *Baracktrema obamai*, which matures in the geoemydid turtles *Siebenrockiella crassicollis* (type host) and *Cuora amboinensis* (Roberts et al., *in press*), and *Unicaecum* sp., which matures in *Trachemys scripta* (Snyder, 2004). These are the only accepted TBF genera that have a combination of a single cecum, single testis, oviducal seminal receptacle, and uterine pouch (Stunkard, 1925; Roberts et al., *in press*). Our analyses also supported resurrection of *Coeuritrema*, which was sister to *Hapalorhynchus* (see Roberts et al., *in press*). Interestingly, *Coeuritrema* clustered with a putative new species and genus (unidentified spirorchiid n. sp. 1, n. gen. 2) collected from an unidentified turtle host in Vietnam. The relatively long branch length of this new taxa support their status herein as a putative new genus. Noteworthy also is that *Coeuritrema platti*, *Hapalorhynchus* spp., and the unidentified spirorchiid n. sp. 1, n. gen. 2 together formed a clade that was sister to the crocodilian blood fluke *G. amoena*. While previous phylogenetic studies support this placement for the molecular data attributed to this taxa (Brant and Loker, 2005; Brant et al., 2006; Loker and Brant, 2006; Orélis-Ribeiro et al. 2014), evidences from comparative morphology are strikingly

discordant. *Griphobilharzia* resembles *Coeuritrema platti*, *Hapalorhynchus* spp., and unidentified spirorchiid n. sp. 1, n. gen. 2 by having a ventral sucker. However, *Griphobilharzia* differs from these TBFs by having a single testis, and from all known TBFs by dioecity. Indeed, *G. amoena* resembles the schistosomes more than any known TBF. *Griphobilharzia* has a well-developed gynaecophoric canal, resembling several genera of Schistosomatinae Stiles and Hassall, 1898: *Schistosoma* Weinland, 1858, *Ornithobilharzia* Odhner, 1912, *Austrobilharzia* Johnston, 1917, *Macrobilharzia* Travassos, 1922, *Schistosomatium* Tanabe, 1923, *Heterobilharzia* Price1929, *Bivitellobilharzia* Vogel and Minning, 1940, and *Orientobilharzia* Dutt and Srivastava, 1955 (see Khalil, 2002). Taken these evidences together, we urge the need for additional sequence data derived from specimens identified morphologically as *G. amoena*.

Regarding monophyly of TBFs genera, our analyses were consistent with previously published tree topologies, recovering *Spirhapalum* as paraphyletic (Tkach et al., 2009; Cribb et al., 2011; Orélis-Ribeiro et al. 2014), and *Spirorchis* as monophyletic (Tkach et al., 2009; Orélis-Ribeiro et al. 2014). Moreover, our analyses are the first molecular phylogenetic study to support monophyly of *Vasotrema* and *Hapalorhynchus*.

4.5 Interrelationships within monophyletic Schistosomatidae

With the exception of the bird schistosome *Macrobilharzia macrobilharzia* that was retrieved as the earliest branching taxon within Schistosomatoidea, intergeneric relationships within this family largely corresponded those rendered by previous study (Snyder and Loker, 2000; Lockyer et al., 2003b; Snyder, 2004; Brant and Loker, 2005,

2013; Brant et al., 2006; Loker and Brant, 2006; Orélis-Ribeiro et al., 2014). Specifically, clades AO (*Austrobilharzia*, *Ornithobilharzia*), BS (*Bivitellobilharzia*, *Schistosoma*), SH (*Schistosomatium*, *Heterobilharzia*), and BTGD (*Bilharziella*, *Trichobilharzia*, *Dendritobilharzia, Gigantobilharzia*) were recovered with relatively high nodal support. In addition, our trees rendered the clade SH sister to *Dendritobilharzia* + *Bilharziella* + *Gigantobilharzia* + *Trichobilharzia* + *Allobilharzia* + *Anserobilharzia*. *Bivitellobilharzia* was yielded sister to *Schistosomes*. Within the latter genus, Asian schistosomes were retrieved as the earliest-branching lineage. Historically, Asian schistosomes were recognized as a distinct group within *Schistosoma* based on egg morphology, intermediate host specificity, and geographical distribution (Lockyer, et al., 2003b). That, coupled with the results from all molecular phylogenetic studies, warrants a taxonomic revision of this genus.

5. CONCLUSION

Contradicting the notion that some families of blood flukes lack a conspicuous phylogenetic specificity to certain host lineages (Smith 1997a), our results suggest that members of Schistosomatoidea exhibit co-phyly with major lineages of craniates. By focusing on the collection of strategically-targeted FBFs, we were able to recover unprecedented relationships within Aporocotylidae that further support these cophylogenetic patterns. No blood fluke is known to infect the key craniate lineages hagfish, lamprey, coelacanth, and lungfish, and discovery of infections in those hosts would be critical. Moreover, exploration using a phylogenomic approach will likely

elucidate a more comprehensive picture of the evolutionary relationships of blood flukes and their craniate hosts.

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FIGURE LEGENDS

Figure 1. Phylogenetic relationships of blood flukes reconstructed by Bayesian inference and based on concatenated fragments of 18S and 28S rDNA genes from 105 taxa (majority rule consensus tree). Numbers aside tree nodes indicate posterior probability (Bayesian inference, left) and bootstrap values (Maximum Likelihood, right); incongruent nodes between the two analyses are indicated by a dash (-). Type species are indicated by an asterisk (*); newly generated sequences are highlighted in bold. Definitive hosts are indicated by icons aside tree nodes.

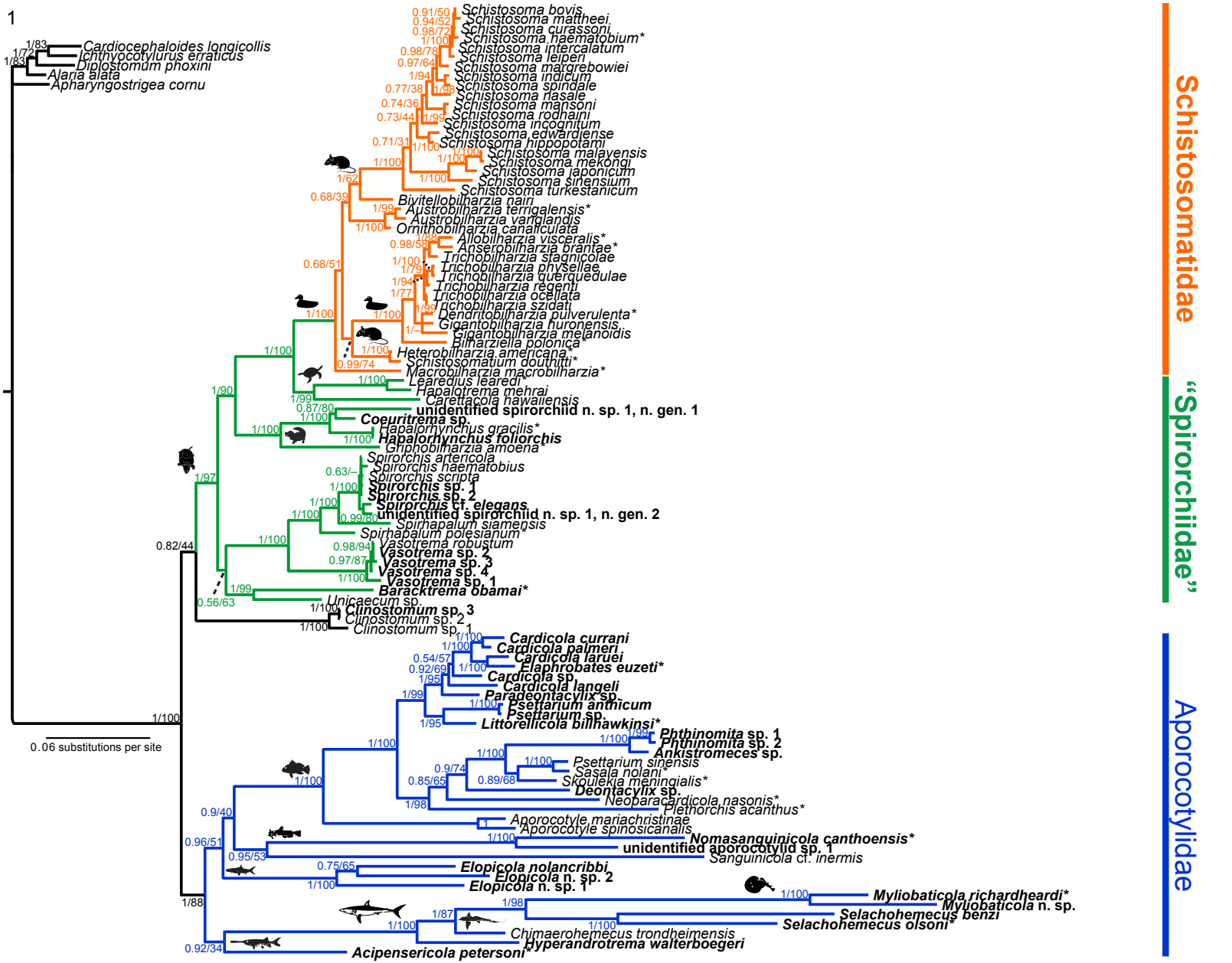
Schistosomatidae
"Spirorchiidae"
Aporocotylidae
Schistosoma bovis
Schistosoma mattheei
Schistosoma curassoni
Schistosoma haematobium*
Schistosoma intercalatum
Schistosoma leiperi
Schistosoma margrebowiei
Schistosoma indicum
Schistosoma spindale
Schistosoma nasale
Schistosoma mansoni
Schistosoma rodhaini
Schistosoma incognitum
Schistosoma edwardiense
Schistosoma hippopotami
Schistosoma malayensis
Schistosoma mekongi
Schistosoma japonicum
Schistosoma sinensium
Schistosoma turkestanicum
Bivitellobilharzia nairi
Austrobilharzia terrigalensis*
Austrobilharzia variglandis
Ornithobilharzia canaliculata
Allobilharzia visceralis*
Anserobilharzia brantae*
Trichobilharzia stagnicolae
Trichobilharzia physellae
Trichobilharzia querquedulae
Trichobilharzia regenti
Trichobilharzia ocellata
Trichobilharzia szidati
Dendritobilharzia pulverulenta*
Gigantobilharzia huronensis
Gigantobilharzia melanoidis
Bilharziella polonica*
Heterobilharzia americana*
Schistosomatium douthitti*
Macrobilharzia macrobilharzia*
Leared ius learedi*
Hapalotrema mehrai
Carettacola hawaiiensis
unidentified spirorchiid n. sp. 1, n. gen. 1
Coeuritrema sp.
Hapalorhynchus gracilis*
Hapalorhynchus foliorchis
Griphobilharzia amoena*
Spirorchis arteticola
Spirorchis haematobius
Spirorchis scripta
Spirorchis sp. 1
Spirorchis sp. 2
Spirorchis cf. elegans
unidentified spirorchiid n. sp. 1, n. gen. 2
Spirhapalum siamensis
Spirhapalum polesianum
Vasotrema robustum
Vasotrema sp. 2
Vasotrema sp. 3
Vasotrema sp. 4
Vasotrema sp. 1
Baracktrema obamai*
Unicaecum sp.
Clinostomum sp. 3
Clinostomum sp. 2
Clinostomum sp. 1
Cardicola currani
Cardicola palmeri
Cardicola laruei
Elaphrobates euzeti*
Cardicola sp.
Cardicola langeli
Paradeontacylix sp.
Psettarium anticum
Psettarium sp.
Littorellicola billhawkinsi*
Phthinomita sp. 1
Phthinomita sp. 2
Ankistromeces sp.
Psettarium sinensis
Sasala nolani*
Skoulekia meningialis*
Deontacylix sp.
Neoparacardicola nasonis*
Plethorchis acanthus*
Aporocotyle mariachristinae
Aporocotyle spinosicanalis
Nomasanguinicola canthoensis*
unidentified aporocotylid sp. 1
Sanguinicola cf. inermis
Elopicola nolancribbi
Elopicola n. sp. 2
Elopicola n. sp. 1
Myliobaticola richardheardi*
Myliobaticola n. sp.
Selachohemecus benzi
Selachohemecus olsoni*
Chimaerohemecus trondheimensis
Hyperandrotrema walterboegeri
Acipensericola petersoni*
Cardiocephaloides longicollis
Ichthyocotylurus erraticus
Diplostomum phoxini
Alaria alata
Apharyngostrigea cornu
0.06 substitutions per site

Table 1. Taxon sampling and sequences used in the present study.

Digenean taxon	Stage	Host species (common names)	Geographical origin	Museum Nº	GenBank Accession Numbers		Source
					18S	28S	
Superfamily Schistosomatoidea							
Family Aporocotylidae							
*Acipensericola petersoni**	A	*Polyodon spathula* (American paddlefish)	Tennessee River, USA	USNMXXXXXX	XXXXXXXX	XXXXXXXX	Present study
Ankistromeces sp.	A	*Siganus* sp. (spinefoot)	W Pacific, off Nha Trang, Vietnam		XXXXXXXX	XXXXXXXX	Present study
Aporocotyle mariachristinae	A	*Genypterus blacodes* (pink cusk-eel)	SW Atlantic, off Wild Patagonia, Argentina		JX094801	JX094802	Hernández-Orts et al. (2012)
Aporocotyle spinosicanalis	A	*Merluccius merluccius* (European hake)	NE Atlantic, UK		AJ287477	AY222177	Olson et al. (2003)
Cardicola currani	A	*Sciaenops ocellatus* (red drum)	Gulf of Mexico, off Davis Bayou, USA	USNMXXXXXX	XXXXXXXX	XXXXXXXX	Present study
Cardicola langeli	A	*Archosargus probatocephalus* (sheepshead)	Gulf of Mexico, USA		XXXXXXXX	XXXXXXXX	Present study
Cardicola laruei	A	*Cynoscion arenarius* (sand weakfish)	Gulf of Mexico, off Tampa Bay, USA		XXXXXXXX	XXXXXXXX	Present study
Cardicola palmeri	A	*Pogonias cromis* (black drum)	Gulf of Mexico, off Back Bay, USA	USNMXXXXXX	XXXXXXXX	XXXXXXXX	Present study
Cardicola sp.	A	*Scomberomorus* cf. *commerson* (spanish mackerel)	W Pacific, off Nha Trang, Vietnam	USNMXXXXXX	XXXXXXXX	XXXXXXXX	Present study
*Chimaerohemecus trondheimensis**	A	*Chimaera monstrosa* (chimaera)	NE Atlantic, Norway		AY157213	AY157239	Lockyer et al. (2003b)
Deontacylix sp.	A	*Kyphosus cf. vaigiensis* (brassy chub)	W Pacific, off Nha Trang, Vietnam	USNMXXXXXX	XXXXXXXX	XXXXXXXX	Present study
*Elaphrobates euzeti**	A	*Lutjanus campechanus* (red snapper)	Gulf of Mexico, USA		XXXXXXXX	XXXXXXXX	Present study
*Elopicola nolancribbi**	A	*Elops saurus* (ladyfish)	Gulf of Mexico, off Ship Island, USA		XXXXXXXX	XXXXXXXX	Present study
Elopicola sp. 1	A	*Elops hawaiensis* (Hawaiian ladyfish)	W Pacific, off Nha Trang, Vietnam	USNMXXXXXX	XXXXXXXX	XXXXXXXX	Present study

Elopicola sp. 2	A	*Megalops atlanticus* (Atlantic tarpon)	Gulf of Mexico, off FL, USA		XXXXXXXX	XXXXXXXX	Present study
Hyperandrotrema walterboegeri	A	*Isurus oxyrinchus* (mako shark)	Gulf of Mexico, off Viosca Knoll, USA		XXXXXXXX	XXXXXXXX	Present study
*Littorellicola billhawkinsi**	A	*Trachinotus carolinus* (Florida pompano)	Gulf of Mexico, Off Horn Island, USA		XXXXXXXX	XXXXXXXX	Present study
*Myliobaticola richardheardi**	A	*Dasyatis sabina* (Atlantic stingray)	Gulf of Mexico, USA			XXXXXXXX	Present study
Myliobaticola sp. n. 1	A	*Narcine bancroftii* (Caribbean electric ray)	Gulf of Mexico, off Orange Beach, USA		XXXXXXXX	XXXXXXXX	Present study
*Neoparacardicola nasonis**	A	*Naso unicornis* (bluespine unicornfish)	Australia		AY222097	AY222179	Olson et al. (2003)
*Nomasanguinicola canthoensis**	A	*Clarias macrocephalus* (bighead catfish)	Can Tho, Vietnam	USNMXXXXXX	XXXXXXXX	XXXXXXXX	Present study
Paradeontacylix sp.	A	*Seriola dumerili* (greater amberjack)	Gulf of Mexico, USA		XXXXXXXX	XXXXXXXX	Present study
Phthinomita sp.1	A	*Siganus virgatus* (barhead spinefoot)	W Pacific, off Nha Trang, Vietnam	USNMXXXXXX	XXXXXXXX	XXXXXXXX	Present study
Phthinomita sp.2	A	*Siganus cf. guttatus* (goldlined spinefoot)	W Pacific, off Nha Trang, Vietnam	USNMXXXXXX	XXXXXXXX	XXXXXXXX	Present study
*Plethorchis acanthus**	A	*Mugil cephalus* (flathead grey mullet)	Brisbane River, Australia		AY222096	AY222178	Olson et al. (2003)
Psettarium anthicum	A	*Rachycentron canadum* (cobia)	Gulf of Mexico, off South of Chandeleur, USA		XXXXXXXX	XXXXXXXX	Present study
Psettarium sp.	A	*Rachycentron canadum* (cobia)	Nha Trang, Vietnam	USNMXXXXXX	XXXXXXXX	XXXXXXXX	Present study
Psettarium sinensis (as *Paradeontacylix sinensis*)	A	*Takifugu rubripes* (Japanese pufferfish)	Fuzhou, China		EU081899	EU368853	Chen et al. (2008)
Sanguinicola cf. *inermis*	C	*Lymnaea stagnalis* (gastropod)	Warminia-Mazury Region, Poland		AY222098	AY222180	Olson et al. (2003)
*Sasala nolani**	A	*Arothron meleagris* (guineafowl puffer)	S Pacific, off Moorea, French Polynesia		AY157184	AY157174	Lockyer et al. (2003a)
Selachohemecus benzi	A	*Carcharhinus limbatus* (blacktip shark)	Gulf of Mexico, USA		XXXXXXXX	XXXXXXXX	Present study
*Selachohemecus olsoni**	A	*Rhizoprionodon terraenovae* (Atlantic sharpnose shark)	Gulf of Mexico, USA		XXXXXXXX	XXXXXXXX	Present study
*Skoulekia meningialis**	A	*Diplodus vulgaris* (two-banded seabream)	Mediterranean Sea, off Valencia, Spain		FN652294	FN652293	Alama-Bermejo et al. (2011)

Unidentified aporocotylid sp. 1	A	*Pangaseus macronema* (shark catfish)	Can Tho, Vietnam	USNMXXXXXX	XXXXXXXX	XXXXXXXX	Present study
Family Schistosomatidae							
*Allobilharzia visceralis**	A	*Cygnus columbianus* (tundra swan)	USA		EF114221	EF114222	Brant (2007) ϕ
*Anserobilharzia brantae**	C	*Gyraulus parvus* (gastropod)	USA		FJ174450	FJ174466	Brant and Loker (2009)
*Austrobilharzia terrigalensis**	C	*Batillaria australis* (marine gastropod)	Sydney Harbour, Australia		AY157223	AY157249	Lockyer et al. (2003b)
Austrobilharzia variglandis	A	*Larus delawarensis* (ring-billed gull)	Delaware, USA		AY157224	AY157250	Lockyer et al. (2003b)
*Bilharziella polonica**	A	*Anas platyrhynchos* (mallard duck)	Kherson Oblast, Ukraine		AY157214	AY157240	Lockyer et al. (2003b)
Bivitellobilharzia nairi	M	*Elephas maximus* (Indian elephant)	Rambukkana, Sri Lanka		AY829261	AY858888	Brant et al. (2006)
*Dendritobilharzia pulverulenta**	A	*Gallus gallus* (chicken)	laboratory infection, New Mexico, USA.		AY157215	AY157241	Lockyer et al. (2003b)
Gigantobilharzia huronensis	A	*Agelaius phoeniceus* (red-winged blackbird)	Winnebago County, Wisconsin, USA.		AY157216	AY157242	Lockyer et al. (2003b)
Gigantobilharzia melanoidis	C	*Melanoides tuberculata* (gastropod)	United Arab Emirates		JX875067	JX875068	Schuster et al. (2014)
*Griphobilharzia amoena**	A	*Crocodylus johnstoni* (Australian freshwater crocodile)	Australia		AY899915	AY899914	Brant and Loker (2005)
*Heterobilharzia americana**	A	*Mesocricetus auratus* (golden hamster)	laboratory infection, USA		AY157220	AY157246	Lockyer et al. (2003b)
*Macrobilharzia macrobilharzia**	A	*Anhinga anhinga* (anhinga)	Louisiana, USA		AY829260	AY858885	Brant et al. (2006)
Ornithobilharzia canaliculata	A	*Larus delawarensis* (ring-billed gull)	Texas, USA		AY157222	AY157248	Lockyer et al. (2003b)
*Schistosomatium douthitti**	A	*Mesocricetus auratus* (golden hamster)	laboratory infection, Indiana, USA		AY157221	AY157247	Lockyer et al. (2003b)
Schistosoma bovis	A	*Mus musculus* (mouse)	laboratory infection, originally isolated from Tanzania		AY157238	AY157266	Lockyer et al. (2003b)
Schistosoma curassoni	A	*Mesocricetus auratus* (golden hamster)	laboratory infection, originally isolated from Senegal		AY157236	AY157264	Lockyer et al. (2003b)
Schistosoma	C	*Biomphalaria sudanica*	Lake Edward,		AY197344	AY197344	Morgan et al.

edwardiense		(gastropod) (gastropod)	Uganda			(2003)
*Schistosoma haematobium**	A	*Mesocricetus auratus* (golden hamster)	Lake Edward, Mali	Z11976	AY157263	Lockyer et al. (2003b)
Schistosoma hippopotami	C	*Bulinus truncatus* (gastropod)	Uganda: Lake Edward	AY197343	AY197343	Morgan et al. (2003)
Schistosoma incognitum	A	*Bandicota indica* (greater bandicoot rat)	Phitsanulok, Thailand	AY157229	AY157255	Lockyer et al. (2003b)
Schistosoma indicum	A	*Bos taurus* (cow)	Mymensingh, Bangladesh	AY157231	AY157258	Lockyer et al. (2003b)
Schistosoma intercalatum	A	*Mus musculus* (mouse)	laboratory infection, São Tomé	AY157235	AY157262	Lockyer et al. (2003b)
Schistosoma japonicum	A	*Mus musculus* (mouse)	laboratory infection, originally isolated from the Philippines	AY157226	AY157607	Lockyer et al. (2003b)
Schistosoma leiperi	A	*Mesocricetus auratus* (golden hamster)	laboratory infection, originally isolated from South Africa	AY157234	AY157261	Lockyer et al. (2003b)
Schistosoma malayensis	A	*Mus musculus* (mouse)	laboratory infection, originally isolated from Malaysia	AY157227	AY157252	Lockyer et al. (2003b)
Schistosoma mansoni	ns	ns	Egyptian strain	U65657	AY157173	Hanelt et al. (1996)ϕ
Schistosoma margrebowiei	A	*Mus musculus* (mouse)	laboratory infection, originally isolated from Zambia.	AY157233	AY157260	Lockyer et al. (2003b)
Schistosoma mattheei	A	*Mus musculus* (mouse)	laboratory infection, originally isolated from Zambia	AY157237	AY157265	Lockyer et al. (2003b)
Schistosoma mekongi	A	*Mus musculus* (mouse)	laboratory infection, originally isolated from Laos	AY157228	AY157253	Lockyer et al. (2003b)
Schistosoma nasale	A	*Capra hircus* (domestic goat)	Sri Lanka	AY157232	AY157259	Lockyer et al. (2003b)
Schistosoma rodhaini	A	*Mus musculus* (mouse)	laboratory infection	AY157230	AY157256	Lockyer et al. (2003b)
Schistosoma sinensium	A	*Mus musculus* (mouse)	laboratory infection, originally isolated from China	AY157225	AY157251	Lockyer et al. (2003b)
Schistosoma spindale	C	*Mus musculus* (mouse)	laboratory infection, originally isolated	Z11979	AY157257	Lockyer et al. (2003b)

			from Sri Lanka				
Schistosoma turkestanicum (as *Orientobilharzia turkestanicum*)	A	*Ovis aries* (sheep)	Iran		AF442499	AY157254	Lockyer et al. (2003b)
Trichobilharzia ocellata	C	*Lymnaea stagnalis* (gastropod)	Germany		AY157217	AY157243	Lockyer et al. (2003b)
Trichobilharzia physellae	A	*Aythya affinis* (lesser scaup)	New Mexico		FJ174457	FJ174473	Lockyer et al. (2003b)
Trichobilharzia querquedulae	A	Anas discors (blue-winged teal)	Florida		FJ174453	FJ174469	Brant and Loker (2009)
Trichobilharzia regenti	C	*Radix peregra* (gastropod)	laboratory infection, Horak Lab., Prague, Czech Rep.		AY157218	AY157244	Brant and Loker (2009)
Trichobilharzia stagnicolae	A	*Mergus merganser* (common merganser)	Michigan		FJ174462	FJ174478	Lockyer et al. (2003b)
Trichobilharzia szidati	C	*Lymnaea stagnalis* (gastropod)	laboratory infection, Horak Lab., Prague, Czech Republic		AY157219	AY157245	Brant and Loker (2009)
Family "Spirorchiidae"							
*Baracktrema obamai**	A	*Siebenrockiella crassicollis* (black marsh turtle)	Malaysia		XXXXXXXX	XXXXXXXX	Present study
Carettacola hawaiiensis	A	*Chelonia mydas* (green sea turtle)	Pacific Ocean, HI, USA		AY604717	AY604709	Snyder (2004)
Coeuritrema platti	A	*Pelodiscus sinensis* (Chinese softshell turtle)	W Pacific, off Nha Trang, Vietnam	USNMXXXXXX	XXXXXXXX	XXXXXXXX	Roberts et al. (*in press*)
*Hapalorhynchus gracilis**	A	*Chelydra serpentina* (common snapping turtle)	Reelfoot Lake, TN, USA		AY604718	AY604710	Snyder (2004)
Hapalorhynchus foliorchis	A	*Chelydra serpentina* (common snapping turtle)	E.W. Shell Ponds, Auburn, AL, USA	USNMXXXXXX	XXXXXXXX	XXXXXXXX	Present study
Hapalotrema mehrai	A	*Chelonia mydas* (green sea turtle)	Pacific Ocean, HI, USA		AY604716	AY604708	Snyder (2004)
*Learedius learedi**	A	*Chelonia mydas* (green sea turtle)	Pacific Ocean, HI, USA		AY604715	AY604707	Snyder (2004)
*Spirhapalum polesianum**	A	*Emys orbicularis* (european pond turtle)	Lesniki, Ukraine		AY604713	AY604705	Snyder (2004)
Spirhapalum siamensis	A	Cuora amboinensis (Amboina box turtle)	Mae Sot, Thailand		FJ481165	FJ481166	Tkach et al. (2009)
Spirorchis artericola	A	*Chrysemys picta* (painted	Reelfoot Lake, TN,		AY604712	AY604704	Snyder (2004)

		turtle)	USA				
Spirorchis cf. *elegans*	A	*Trachemys scripta* (pond slider)	E.W. Shell Ponds, Auburn, AL, USA		XXXXXXXX	XXXXXXXX	Present study
Spirorchis haematobius	A	*Chelydra serpentina* (common snapping turtle)	Fremont County, IA, USA		FJ481163	FJ481164	Tkach et al. (2009)
Spirorchis scripta	A	*Trachemys scripta scripta* (yellow-bellied slider)	Van Cleave, MS, USA		AY222093	AY222174	Olson et al. (2003)
Spirorchis sp. 1	A	*Pseudemys concinna* (river cooter)	Pascagoula River, MS, USA	USNMXXXXXX	XXXXXXXX	XXXXXXXX	Present study
Spirorchis sp. 2	A	*Graptemys pulchra* (Alabama map turtle)	Yellow River, Givens Bridge, AL, USA	USNMXXXXXX	XXXXXXXX	XXXXXXXX	Present study
Unicaecum sp.	A	*Trachemys scripta* (pond slider)	Reelfoot Lake, TN, USA		AY604719	AY604711	Snyder (2004)
unidentified spirorchiid n.sp.1, n.gen.1	A	bush meat	Can Tho, Vietnam	USNMXXXXXX	XXXXXXXX	XXXXXXXX	Present study
unidentified spirorchiid n.sp.1, n.gen.2	A	*Deirochelys reticularia* (chicken turtle)	Salt Pond, Conecuh National Forest, AL, USA	USNMXXXXXX	XXXXXXXX	XXXXXXXX	Present study
Vasotrema robustum	A	*Apalone spinifera* (spiny softshell turtle)	Nishnabotna River, USA		AY604714	AY604706	Snyder (2004)
Vasotrema sp. 1	A	*Apalone spinifera* (spiny softshell turtle)	Dean's Pond, Springville, AL, USA	USNMXXXXXX	XXXXXXXX	XXXXXXXX	Present study
Vasotrema sp. 2	A	*Apalone spinifera* (spiny softshell turtle)	Perry Lakes State Park, Marion, AL, USA	USNMXXXXXX	XXXXXXXX	XXXXXXXX	Present study
Vasotrema sp. 3	A	*Apalone spinifera* (spiny softshell turtle)	Dean's Pond, Springville, AL, USA	USNMXXXXXX	XXXXXXXX	XXXXXXXX	Present study
Vasotrema sp. 4	A	*Apalone spinifera* (spiny softshell turtle)	Dean's Pond, Springville, AL, USA	USNMXXXXXX	XXXXXXXX	XXXXXXXX	Present study
Superfamily Clinostomoidea Family Clinostomidae							
Clinostomum sp.1	M	*Hypseleotris galii* (firetailed gudgeon)	Moggil Creek, Australia		AY222094	AY222175	Olson et al. (2003)
Clinostomum sp.2	M	*Rana catesbeiana* (north american bullfrog)	Reelfoot Lake, TN, USA		AY222095	AY222176	Olson et al. (2003)
Clinostomum sp.3	M	*Micropterus coosae* (redeye bass)	USA		XXXXXXXX	XXXXXXXX	Present study

Superfamily Diplostomoidea Family Diplostomidae						
Alaria alata	A	*Nyctereutes procyonoides* (Racoon dog)	Kherson Region, Ukraine	AY222091	AF184263	Olson et al. (2003)
Diplostomum phoxini	M	*Phoxinus phoxinus* (common minnow)	Aberystwyth, Wales	AY222090	AY222173	Olson et al. (2003)
Family Strigeidae						
Apharyngostrigea cornu	A	*Ardea cinerea* (grey heron)	Kherson Region, Ukraine	AY222092	AF184264	Olson et al. (2003)
Cardiocephaloides longicollis	A	*Larus ridibundus* (black-headed gull)	Kherson Region, Ukraine	AY222089	AY222171	Olson et al. (2003)
Ichthyocotylurus erraticus	A	*Coregonus autumnalis* (arctic cisco)	Lough Neagh, UK	AJ287526	AY222172	Olson et al. (2003)

ns, not specified
* type species
A, adult; C, cercaria; E, egg
ϕ Unpublished

Table 2. Oligonucleotide primers used in the present study.

Primer ID (direction)	Sequence 5' to 3'	Reference
Amplification and Sequencing		
18S rDNA		
18SE (alias 18S-A) (F)	CCGAATTCGTCGACAACCTGGTTGAT CCTGCCAGT	Littlewood and Olson (2001)
Worm B (R)	CTTGTTACGACTTTTACTTCC	Littlewood and Olson (2001)
28S rDNA		
U178 (F)	GCACCCGCTGAAYTTAAG	Lockyer et al. (2003a)
L1642 (R)	CCAGCGCCATCCATTTTCA	Lockyer et al. (2003a)
Additional sequencing		
18S rDNA		
388F (F)	AGG GTT CGA TTC CGG AG	Littlewood and Olson (2001)
1100F (F)	CAGAGTTTCGAAGACGATC	Littlewood and Olson (2001)
CEST1R (R)	TTTTTCGTCACTACCTCCCC	Littlewood and Olson (2001)
1270R (R)	CCGTCAATTCCTTTAAGT	Littlewood and Olson (2001)
28S rDNA		
300F (F)	CAAGTACCGTGAGGGAAAGTTG	Lockyer et al. (2003a)
900F (F)	CCGTCTTGAAACACGGACCAAG	Lockyer et al. (2003a)
1200R (alias LSU1200R) (R)	GCATAGTTCACCATCTTTCGG	Lockyer et al. (2003a)