Taxonomy and Systematics of Fish Blood Flukes (Digenea: Schistosomatoidea) using Morphology, Phylogenetics, and Life History

by

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ABSTRACT

The monophyletic fish blood flukes (Platyhelminthes: Digenea: Schistosomatoidea Stiles, 1898) comprise ~186 species of 46 genera infecting freshwater, marine, and estuarine fishes. They are occasional pathogens of cultured fishes and are ancestral to the turtle blood flukes and the human-pathogenic blood flukes (Schistosomatidae Stiles and Hassal, 1898). The life cycles of aporocotylids include a mollusk or polychaete intermediate host (wherein the parasite undergoes clonal asexual reproduction) and a fish definitive host (wherein the parasite matures). Blood flukes described in this dissertation were collected from 410 fish assigned to nine genera in nine families. I use alpha taxonomy, scanning electron microscopy (SEM), genetic sequence techniques (PCR; large subunit ribosomal DNA [28S], and internal transcribed spacer 2 [ITS2]), and molecular phylogenetic analysis (Bayesian inference) to characterize two new families, four new genera, and five new species as well as revise two genera. I describe *Cardicola uterohamus* n. sp. from the heart of the yellowedge grouper, *Hyporthodus flavolimbatus*, *Aetohemecus kirstenjensenae* n. gen., n. sp. and *Homestios janinecairae* n. gen., n. sp. from the heart of the banded eagle ray, *Aetomylaeus nichofii*, *Sanguinicola plehnae* n. sp. from the heart of the Northern pike, *Esox lucius*, *Pseudosanguinicola occidentalis* n. gen., n. comb. from the heart of the walleye, *Sander vitreus*, and a new genus and new species from the heart of the white mullet, *Mugil curema* as well as provide supplemental descriptions for *Cardicola cardiocola* Manter, 1954, *Nomasanguinicola dentata* Paperna, 1964, and *Sanguinicola volgensis* (Rašín, 1929) Mcintosh, 1934. I revise and diagnose the fish blood fluke families: Chimaerohemecidae Yamaguti, 1971, Acipensericolidae n. fam., Elopicolidae n. fam., Sanguinicolidae Poche, 1926, and Aporocotylidae Odhner, 1912.

This work culminates in the revision of all families of fish blood flukes as well as several genera, the first report of a chimaerohemecid host infected with more than one species of blood fluke, and the first blood fluke infecting mullet from North America. Further, I provide the first genetic sequences from species of Sanguinicolidae and the first genetic sequence from an adult sanguinicolid from Africa. Further, I include 12 nucleotide sequences belonging to nominal and

innominate chimaerohemecids and aporocotylids. This work has resulted in publications in Journal of Parasitology and International Journal for Parasitology: Parasites and Wildlife.

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ABSTRACT

Cardicola Short, 1953 is the most speciose aporocotylid genus (35 species) and includes marine and estuarine species of fish blood flukes that infect higher ray-finned fishes (Euteleostei). Several clades within *Cardicola* are recovered in phylogenetic analyses of the large subunit ribosomal DNA (*28S*) but morphological synapomorphies for those nucleotide-based clades remain elusive. The type species, *Cardicola cardiocola* (Manter, 1947) Short, 1953, has not been recollected in 73 yr and the original description was incomplete; making a genus revision challenging because of the ambiguous systematic position of its type species. Herein, we redescribe *C. cardiocola* using the holotype (USNM 1337732) and new specimens collected from the type host, jolthead porgy, *Calamus bajonado* (Sparidae), from nearby the type locality. It differs from its congeners by the combination of having a body that is 5 × longer than wide, an anterior sucker with concentric rows of spines, 2–6 tegumental body spines per row, an esophageal gland that is 22–43% of the esophageal length, a testis that is 3–5 × longer than wide and that fills the intercecal space, a vitelline duct connecting to the anterior aspect of the oötype, an ascending uterus that lacks any coil, a descending uterus yielding a single coil, an obvious cirrus sac separated by constriction from the seminal vesicle, a tegumental protrusion surrounding the terminal end of cirrus sac, and a male genital pore that is posterior to the

remainder of the genitalia. We also describe a new congener infecting the heart of yellowedge grouper, *Hyporthodus flavolimbatus* (Serranidae) from the Gulf of Mexico. It differs from its congeners by the combination of having an anterior sucker that does not extend beyond the anterior body margin, 2–5 tegumental body spines per row, posterior ceca that are 9 × length of the anterior ceca and that lack any coil, a testis that is 3 × longer than wide and that does not fill the intercecal space, an ovary that is >60% of the body width, a vitelline duct that connects to the anterior aspect of the oötype, a uterus that is >10% of the body width and that extends posterior to all genitalia, and a rounded posterior body margin. It is the first species of *Cardicola* to be described from a grouper (Serranidae). The *28S* and internal transcribed spacer 2 (*ITS2*) phylogenetic analyses recovered the new species as a distinct lineage within the clade of *Cardicola* spp.

KEY WORDS Taxonomy, Systematics, Fish Blood Fluke, Type species, Phylogenetics, Morphology, Sparidae, Large Subunit Ribosomal (*28S*), Internal Transcribed Spacer 2 (*ITS2*)

Seventy-three years have passed since Manter (1947) described the type species of *Cardicola* (Digenea: Aporocotylidae Odhner, 1912; see Bullard et al., 2009), *Cardicola cardiocola* (Manter, 1947) Short, 1953 (as *Psettarium cardiocolum*) (see Manter, 1947; Short, 1953; Smith, 1997). Since Short (1953) proposed *Cardicola* and designated Manter's species as the type, 34 congeners have been described. All but one infect 'higher ray-finned fishes' (Euteleostei): the host for *Cardicola dhangeli* Hutson, Vaughan, and Blair, 2019, which is morphologically clearly a 'fish blood fluke', is reported as dugong, *Dugong dugon* (Müller, 1776) (Sirenia: Dugongidae) off Australia (Hutson et al., 2019). Four congeners (*Cardicola ahi* Yamaguti, 1970; *Cardicola congruenta* Lebedev and Mamaev, 1968; *Cardicola kurochkini* [Parukhin, 1976] Bullard and

Overstreet, 2006; *Cardicola mugilis* Yamaguti, 1970) are considered *incertae sedis* (Bullard, 2010a).

Herein we: redescribe the type species (*C. cardiocola*) using the holotype (USNM 1337732) and newly-collected specimens infecting the type host (jolthead porgy, *Calamus bajonado* [Bloch and Schneider, 1801] Robins and Ray, 1986) from nearby the type locality; describe a new congener infecting the yellowedge grouper, *Hyporthodus flavolimbatus* (Poey, 1865) Craig and Hastings, 2007 (Perciformes: Serranidae) collected from the northern Gulf of Mexico (the first record of a species of *Cardicola* infecting a serranid); and, use the large subunit ribosomal (*28S*) and internal transcribed spacer 2 (*ITS2*) to explore relationships between the new species and congeners.

MATERIALS AND METHODS

In 2015 and 2017, the heart of 2 of 4 (50%) jolthead porgies (*Cal. bajonado*) from the northeastern Gulf of Mexico off southern Florida were infected with 7 adults of *C. cardiocola*. Jolthead porgy were captured by bottom trawl deployed from the *R/V Tommy Munro*, 2 uninfected specimens in April 2015 (off Naples, Florida) and 2 infected specimens in April 2017 (off Charlotte Harbor, Florida). At necropsy, the heart was excised intact, placed in sample bags (heart bisected), exposed to 70 C freshwater, shaken vigorously, and preserved in 5–10% neutral buffered formalin (NBF). In the laboratory, heart tissues were examined with the aid of a dissecting microscope and fiber optic light source to isolate fluke specimens for morphology. The heart was teased apart with forceps to reveal adult blood flukes (n = 7), and sediment from the heart was examined with the aid of a settling column.

In addition, the heart of 2 of 5 (40%) yellowedge groupers (*H. flavolimbatus*) from the north-central Gulf of Mexico were infected with 3 adults and several schistosomula of a new species

that is congeneric with *C. cardiocola*. Yellowedge grouper (n = 5) were captured by longline in August (East of Pascagoula, Mississippi) and September (West of Pascagoula, Mississippi) in 2017 from the Gulf of Mexico, aboard the *R/V Oregon II*. At necropsy, the heart and gill were excised intact and separated (heart bisected, gill arches separated). On the ship, all tissues were examined with the aid of a dissecting microscope and fiber optic light source to isolate fluke specimens for morphology and genetic sequencing. The heart was teased apart with forceps to reveal the single adult blood fluke, and sediment from the heart and gill was examined with the aid of a settling column. The adult fluke (n = 1) for morphology was heat-killed on a glass slide using a butane hand lighter under little or no coverslip pressure as per Bullard et al. (2019). Schistosomulum specimens (n = 3) were unmoving and presumed dead, so no attempt was made to relax them before their direct transfer to 10% NBF. Adult specimens (n = 2) collected for DNA extraction were wet mounted on glass slides and examined to confirm their identity, placed directly into 95% EtOH, and stored at −20 C until DNA was extracted (see below).

Adult flukes fixed in formalin were rinsed with distilled water, cleaned with fine brushes to remove any debris, stained overnight in Van Cleave's hematoxylin with two additional drops of Ehrlich's hematoxylin, dehydrated using an ethanol series, cleared in clove oil, permanently mounted in Canada balsam, illustrated using a Leica DM 2500 (Leica, Wetzler, Germany) microscope equipped with differential interference contrast (DIC), measured using an ocular micrometer, and illustrated using a drawing tube. Measurements are reported in micrometers (μm) as the range followed by the mean, standard deviation, and sample size in parentheses. Scientific names, including taxonomic authorities and dates, for fishes follow Eschmeyer et al. (2016). Classification and anatomical terms for fish blood flukes follow Manter (1947), Short (1953), Bullard (2010a), and Bullard et al. (2012). The holotype, *C. cardiocola* (USNM

1337732), was borrowed from the National Museum of Natural History's Invertebrate Zoology Collection (USNM, Smithsonian Institution, Washington, D. C.). Type and voucher materials of the new species and *C. cardiocola,* respectively, were deposited in the National Museum of Natural History's Invertebrate Zoology Collection (USNM, Smithsonian Institution).

Using the 2 EtOH-preserved and microscopically identified blood flukes, total genomic DNA (gDNA) was extracted from 1 specimen (the other did amplify, thus yielding negative results) using DNeasyTM Blood and Tissue Kit (Qiagen, Valencia, California) as per the manufacturer's protocol except that the proteinase-K incubation period was extended overnight and the final elution step used 100 µl of elution buffer to increase the final DNA concentration. The *28S* and the *ITS2* were amplified using primers U178-F (5'-GCACCCGCTGAAYTTAAG-3'); L1642-R (5'-CCAGCGCCATCCATTTTCA-3') and GA1-F (5'-AGAACATCGACATCTTGAAC-3'); ITS2.2-R (5'-CCTGGTTAGTTTCTTTTCCTCCGC-3'), respectively. PCR amplifications were performed with the cycling profile identified in Warren et al. (2017) except that the annealing temperature was set at 57 C for 30 sec for both the *28S* and *ITS2*. All PCR reactions were carried out in an MJ Research PTC-200 (BioRad, Hercules, California). PCR products (12 µl) were verified on a 1% agarose gel and stained with ethidium bromide. PCR products were purified by microcentrifugation with the QIAquick PCR Purification Kit (Qiagen) according to manufacturer's protocols except that the last elution step was performed with autoclaved nanopure H_2O rather than with the provided buffer. DNA sequencing was performed by ACGT, Incorporated (Wheeling, Illinois). Reactions were sequenced using BigDye terminator version 3.1, cleaned with magnetic beads (CleanSeq dye terminator removal kit), and analyzed using an ABI 3730 XL or 3730 Genetic Analyzer (ACGT, Incorporated). Sequence assembly and analysis of chromatograms were performed with Geneious version 2019.2.3 (http://www.geneious.com).

All nucleotide sequence data were deposited in GenBank (Table I).

The phylogenetic analyses included one sequence of the new species plus all sequences taken from nominal species of *Cardicola* that were available (Table I). The out-group (*28S* tree) is represented by sequences of the genus *Psettarium* Goto and Osaki, 1930 and (*ITS2* tree) *Paradeontacylix* McIntosh, 1934 (Table I) because these groups have proven consistent in determining the interrelationships in previous publications (Yong et al., 2016). Sequences were aligned with the multiple alignment tool using fast Fourier transform (MAFFT) (Katoh and Standley, 2013) and trimmed to the length of the shortest sequence (1214 [*28S*]; 461 [*ITS2*] base pairs). JModelTest 2 version 2.1.10 was implemented to perform a statistical selection of the best-fit models of nucleotide substitution based on Bayesian Information Criterion (BIC) (Darriba et al., 2012). Aligned sequences were reformatted (from .fasta to .nexus) using the web application ALTER (Glez-Peña et al., 2010) to run Bayesian inference (BI). BI was performed in MrBayes version 3.2.5 (Ronquist and Huelsenbeck, 2003) using substitution model averaging (*nst-mixed*) and a gamma distribution to model rate-heterogeneity. Defaults were used in all other parameters. Three independent runs with 4 Metropolis-coupled chains were run for 5,000,000 generations, sampling the posterior distribution every 1,000 generations. Convergence was checked using Tracer v1.6.1 (Rambaut et al., 2014) and the *sump* command in MrBayes: all runs appeared to reach convergence after discarding the first 25% of generation as burn-in. A majority-rule consensus tree of the post-burn-in posterior distribution was generated with the "sumt" command in MrBayes. The inferred phylogenetic tree was visualized using FigTree v1.4.4 (Rambaut et al., 2014) and further edited for visualization purposes with Adobe Illustrator (Adobe Systems).

DESCRIPTION

Cardicola cardiocola (Manter, 1947) Short, 1953

(Figs. 1−4)

Redescription of adult based on light microscopy of the holotype (USNM 1337732) and 7 newly collected whole-mounted adult specimens; USNM coll. nos. 1622601−1622607): Body flat, ventrally concave, oval in shape, 1,150–1,490 (1,383 ± 110, 8) long, 160–310 (274 ± 52, 8) at greatest width, 4.5–7.2 (5.1 ± 0.86, 8) × longer than wide (Figs. 1, 2). Tegumental body spines 4–5 (20) long, 1 (20) wide at base, not fused at base; tegumental spine rows extending along entire body length, with 249–286 (268 ± 26, 5) rows per side or total of 498–556 (527 ± 41, 5); each row 5–20 (5) long, with number of spines per row increasing from anterior end to middle of body and then decreasing posteriorly, generally following pattern 2, 3, 4, 5, 6, 5, 4 spines per row. Anterior sucker 13–45 (23 ± 10, 8) long, 18–50 (27 ± 10, 8) at greatest width or 6–17% of body width; anterior sucker spines small, <1 (1) long, in 5 concentric rows (1), located between distal end and slightly anterior to body margin; at limits of light microscopy. Ventrolateral nerve cord 1,112–1,245 (1,207 ± 49, 5) long, 8–13 (11 ± 3, 5) wide near mid-body at widest level, 35–58 (48 ± 8, 5) from body margin. Nerve commissure perpendicular to midline of body, connecting ventrolateral nerve-cords, 110–133 (118 ± 8, 8) or 8–10% of body length from anterior end of body, 45–63 (53 ± 6, 8) across width of worm, 13–18 (14 ± 2, 8) in breadth; secondary commissure and nerve cords not evident in whole-mounts.

Mouth 3 (3) in diameter, 3–5 (4 ± 1, 3) from terminal end of anterior sucker. Esophagus 420–595 (550 ± 55, 8) in total length or 37–42% of body length, 10–43 (32 ± 10, 8) in maximum width (at level of anterior ceca), extending sinuously posteriad along midline, curving 5–9 (8) times, widening posteriorly (Fig. 2); esophageal wall thickening from 2–3 (8) near mouth, 5–10

(8) in middle portion, and 3–5 (8) in posterior half of esophagus (Fig. 2). Esophageal gland surrounding esophagus, 125–233 (157 ± 38, 8) long or 22–43% of esophagus length, 55–78 (67 ± 9, 8) wide or 19–27% of body width. Intestine X- or H-shaped, with paired anterior and posterior ceca intersecting medially (Figs. 1, 2); Cecal intersection of anterior and posterior ceca 425–605 (562 ± 57, 8) or 37–43% of body length from anterior body end; anterior ceca 73–124 (99 ± 19, 8) long or 5–9% (9% ± 1, 2) of body length, 28–43 (36 ± 6, 8) wide, ventral to lateral nerve cord, containing granular material within lumen of some individuals (Fig. 2); posterior ceca asymmetrical, 319–450 (401 ± 47, 6) long or 28–31% (29% ± 1, 6) of body length, 17–37 (27 ± 8, 6) wide, ventral to testis (Figs. 1, 2); Post-cecal space 325–410 (366 ± 28, 6) long or 26–28% of body length.

Testis 288–415 (371 ± 42, 7) long or 25–29% of body length, 58–110 (98 ± 18, 7) wide or 32–39% of body width, 3–5 (4 ± 1, 7) × longer than wide, intercecal, filling entire intercecal space, not extending lateral to intestine (Figs. 1, 2). Post-testicular space 360–425 (401 ± 23, 7) long or 28–31% of body length. Vas deferens 163–238 (207 ± 25, 7) long, 13–23 (16 ± 5, 7) wide, emanating from posteroventral portion of testis, following midline before becoming confluent with seminal vesicle. Seminal vesicle 93–133 (108 ± 13, 7) long, 15–33 (26 ± 6, 7) wide, 3.3–6.7 × longer than wide, containing sperm in 6 of 8 specimens, extending posteriad before curving and connecting to cirrus sac, posterior-most portion of genitalia, 63–88 (77 ± 9, 7) or 5–6% of body length from posterior body end (Figs. 1–4). Cirrus sac 18–33 (27 ± 5, 7) long or comprising 18–33% (26% ± 5, 7) length of seminal vesicle, 8–13 (11 ± 2, 7) wide or 55–83% (71% ± 11, 7) of seminal vesicle width, having extremely thin wall approximately 1 (7) thick, including cirrus; everted cirrus 13 and 18 (2) long, 3 (2) wide (Figs. 3, 4).

Ovary medial, irregular in shape, not deeply lobed, 60–105 (78 ± 19, 7) in maximum length or 4–7% of body length, 80–150 (116 ± 23, 7) wide or 34–50% of body width, 1.1–2 × wider than long, immediately post-testicular, ventral to lateral nerve-cords; post-ovarian space 275–383 (326 ± 34, 7) long or 22–28% of body length (Figs. 1–4). Oviduct (including oviducal seminal receptacle) 75–138 (98 ± 22, 7) long, 5–13 (10 ± 3, 7) wide; oviducal seminal receptacle 15–68 (55 ± 19, 7) long or 20–84% of oviduct length, 10–50 (39 ± 13, 7) wide. Oötype 15–70 (41 ± 19, 7) long, 10–33 (24 ± 9, 7) wide (Figs. 2, 4). Vitellarium having follicles compacted in dense lobules, occupying space dorsal and lateral to testis and ceca, extending from nerve commissure to terminal end of posterior ceca (Fig. 3); common collecting duct 150–515 (377 ± 125, 7) long, 15–33 (25 ± 6, 7) wide. Uterus extending directly posteriad from oötype, 18–88 (42 ± 28, 7) long or 1–7% of body length, 5–30 (12 ± 8, 7) wide; ascending portion 128–198 (173 ± 23, 7) long or 11–14% of body length, 20–43 (33 ± 7, 7) wide; extending diagonally anteriad across mid-line and dorsal to seminal vesicle, containing eggs in 7 of 8 specimens (Figs. 1–4), before curving and connecting with descending portion; descending portion 125–195 (167 ± 27, 7) long or 9–14% of body length, 15–25 (20 ± 4, 7) wide, coiling once, with wall 1 (7) thick, extending posteriad before connecting with metraterm; metraterm 23–43 (36 ± 6, 7) long or 7–10% of uterine length, 8–23 (20 ± 5, 7) wide, comprising distal-most portion of female reproductive tract, (Figs. 3, 4), with wall 4 thick. Uterine eggs 8–12 (10 ± 2, 20) in diameter, dense lipid-like bodies, with thin shell (Fig. 4). Female genital pore sinistral, post-ovarian, lateral to proximal portion of seminal vesicle, 60–98 (86 ± 13, 7) from male genital pore, 125–178 (162 ± 18, 7) or 11–12% of body length from posterior body end (Figs. 1–4). Excretory bladder small, 8–10 (9 ± 1, 3) long, 5–8 (6 ± 2, 3) wide, medial.

Taxonomic summary

Type and only reported host: Jolthead porgy, *Calamus bajonado* (Bloch and Schneider 1801) Robins and Ray, 1986 (Spariformes: Sparidae).

Type locality: Dry Tortugas, Florida.

Other localities: Northeast Gulf of Mexico, (26°45′00.8″N, 82°48′23.4″W), 150 km west of Boca Grande, Florida.

Site of infection: Heart lumen.

Prevalence and intensity of infection: Two of 4 (prevalence = 50%) jolthead porgy hearts sampled on 14 April 2015 (n = 2, neither infected) and 18 April 2017 (n = 2, both infected) were infected by 7 specimens of *C. cardiocola*.

Specimens examined: Holotype of *C. cardiocola* (USNM 1337732).

Specimens deposited: Vouchers (USNM 1622601 − 1622607)

Remarks

The description of *C. cardiocola* by Manter (1947) (as *Psettarium cardiocolum*) is generalized and omits details of several morphological features that recent workers have used to differentiate species of the genus. For example, there is no mention of any detail of the anterior sucker and its spines, esophageal gland, oviducal seminal receptacle, or oötype. Further, the tegumental spines were misinterpreted as ‘fused’ (corrected by Short [1953]) and the seminal vesicle was described as ‘vas deferens swollen to function as seminal vesicle’ (Manter, 1947, p. 368). The original description is based on a single specimen (USNM 1337732) that is small, significantly de-stained, and twisted (Fig. 1). This species differs from its congeners by the combination of having a body that is 5 × longer than wide, an anterior sucker with concentric rows of spines, 2–6 tegumental body spines per row, an esophageal gland that is 22–43% of the

esophageal length, a testis that is 3–5 × longer than wide and that fills the intercecal space, a vitelline duct connecting to the anterior aspect of the oötype, an ascending uterus that lacks any coil, a descending uterus yielding a single coil, an obvious cirrus sac separated by constriction from the seminal vesicle, a tegumental protrusion surrounding terminal end of cirrus sac, and a male genital pore that is posterior to the remainder of the genitalia.

Morphologically, *C. cardiocola* is most similar to *Cardicola euzeti* (Bullard and Overstreet, 2003) Nolan and Cribb, 2006 (as *Elaphrobates*), which infects snappers (*Lutjanus campechanus* [Poey, 1860] Rivas, 1966 and *Lutjanus griseus* [Linnaeus, 1758] Matsuura in Uyeno et al., 1983) (Bullard and Overstreet, 2003), by having an anterior sucker with concentric rows of spines, a medial portion of the oviduct containing an oviducal seminal receptacle, a vitelline duct that connects to the anterior aspect of the oötype, a descending uterus with a single coil, and a male genital pore that is posterior to all other genitalia (Bullard and Overstreet, 2003). It differs from *C. euzeti* by having tegumental spines 4–5 long (vs. 9–16 long), a testis 3–5 × longer than wide (vs. 1.3–2.6 × longer than wide) and that fills the intercecal space (vs. a testis that does not fill the intercecal space), and a metraterm 7–10% of the uterine length (vs. 17–55% of the uterine length).

The phylogenetic position of *C. cardiocola* using nucleotide sequences is still undetermined, but given its morphological similarity, we predict *C. cardiocola* shares a recent common ancestor with *C. euzeti*. Host affiliation would predict that it clades with the other sparid blood flukes, *Cardicola aurata* Holzer, Montero, Repullés, Sitja-Bobadilla, Alvarez-Pellitero, Zarza, and Raga, 2008 and *Cardicola langeli* Bullard, 2013. However, morphologically, *C. aurata* and *C. langeli* are differentiated from *C. cardiocola* by having a testis that does not fill the intercecal space (vs. a testis filling the intercecal space in *C. cardiocola*) and a vitelline duct that connects

to the posterior aspect of the oötype (vs. a vitelline duct connecting to the anterior aspect of the oötype in *C. cardiocola*). Further, *C. aurata* has a male genital pore located at the terminal end of the body adjacent to the excretory vesicle (vs. a male genital pore located on the sinistral body margin in *C. cardiocola*) as well as a descending uterus that lacks a coil (vs. a single coil in *C. cardiocola*). *Cardicola langeli* differs from *C. cardiocola* by having a seminal vesicle that does not have a clear separation from the vas deferens (vs. a seminal vesicle with clear constriction separating it from the vas deferens in *C. cardiocola*), an oviducal seminal receptacle that is posterior to all of the genitalia (vs. an oviducal seminal receptacle anterior to the oötype in *C. cardiocola*), a convoluted ascending uterus (vs. an ascending uterus that lacks a coil in *C. cardiocola*), and a female genital pore that is dorsal to the seminal vesicle and oötype (vs. a female genital pore that is lateral to the seminal vesicle in *C. cardiocola*) (Bullard, 2013).

***Cardicola uterohamus* Warren and Bullard n. sp.**

(Figs. 5, 6)

Diagnosis of adult (based on 1 whole-mounted adult specimen; USNM coll. no. 1622608): Body flat, ventrally concave, oval in shape, tapering anteriorly, rounded posterior end, 1720 long, 380 at greatest width, 4.5 × longer than wide (Fig. 5). Tegumental body spines obscured by dorsal mounted specimen, <1 (20) long, 1 (20) wide at base, not fused at base. Tegumental spines rows extending to level of ovary, with 185 rows per side or total of 370; each row 4–7 (20) long, with number of spines per row increasing from anterior end to middle of body and then decreasing posteriorly, generally following pattern 2, 3, 4, 5, 6, 5, 4 spines per row. Anterior sucker 20 long, 30 at greatest width or 8% of body width. Ventrolateral nerve cord discernable anterior of ovary, 1,100 long, 15 wide near mid-body at widest level, 50 from body margin. Nerve commissure perpendicular to midline of body, connecting ventrolateral nerve-cords, 5%

of body length from anterior end of body, 53 across width of worm, 13 in breadth (Fig. 5); secondary commissure and nerve cords not evident in whole-mounts.

Mouth 3 in diameter, 8 from terminal end of anterior sucker (Fig. 5). Esophagus 600 in total length or 35% of body length, 10 in maximum width anterior to nerve commissure, 35 in maximum width (medial portion), 25 in maximum width (terminal portion of esophagus), extending sinuously posteriad along midline, curving 8 times (Fig. 5); esophageal wall thickening from 2 near mouth, 8 in middle portion, and 5 in posterior half of esophagus (Fig. 5). Esophageal gland surrounding esophagus, 500 long or 83% of esophagus length, 120 wide or 32% of body width. Intestine X- or H-shaped, with paired anterior and posterior ceca intersecting medially (Fig. 5); Cecal intersection of anterior and posterior ceca 600 or 35% of body length from anterior body end; anterior ceca 74 in average length or 4% of body length, 67 wide or 18% of body width, ventral to lateral nerve cord, containing granular material within lumen of some individuals (Fig. 5); posterior ceca asymmetrical, 650 in average length or 38% of body length, 9 × longer than anterior ceca, 53 average width or 14% of body width, ventral to testis (Fig. 5); Post-cecal space 420 long or 24% of body length.

Testis 450 long or 26% of body length, 155 wide or 41% of body width, 3 × longer than wide, intercecal, not filling entire intercecal space, not extending lateral from intestine (Fig. 5). Pre-testicular space 790 long or 46% of body length from cecal bifurcation. Post-testicular space 490 long or 28% of body length. Vas deferens 288 long, 6 at wide, emanating from postero-ventral portion of testis, following midline before turning sinistrally and expanding as rudimentary seminal vesicle, 105 long, 18 wide, 6 × longer than wide, 188 or 11% of body length from posterior body end. Cirrus sac and cirrus not evident (Fig. 6).

Ovary predominantly dextral, triangular in shape, not deeply lobed, 253 in maximum length or 15% of body length, 250 wide or 66% of body width, immediately post-testicular; post-ovarian space 285 long or 17% of body length (Figs. 5, 6). Oviduct (including oviducal seminal receptacle) 140 long, 4 wide; oviducal seminal receptacle 73 long or 52% of oviduct length, 18 wide. Oötype 43 long, 28 wide (Figs. 5, 6). Vitellarium having glandular appearance, occupying space dorsal and lateral to testis and ceca, extending from mid-esophagus to terminal end of posterior ceca. Uterus extending laterally from oötype, making complete U-turn before extending dextrally and making another U-turn back sinistrally and connecting to ascending portion; ascending portion large, hook-shaped, 488 long or 28% of body length, 58 wide or 15% of body width, posterior-most portion of genitalia, 50 from posterior end, containing eggs, abutting margin of ovary before connecting to descending convoluted portion; convoluted portion consisting of descending and ascending portions before connecting to metraterm, 435 long or 25% of body length, containing eggs; metraterm 35 long or 3% of uterine length, 15 wide, comprising distal-most portion of female reproductive tract (Figs. 5, 6), without eggs, with wall 3 thick; total uterine length with convolutions 1,110 long or 64% of body length. Uterine eggs 14–26 (18 ± 2, 20) in length or 48% of uterus width, dense lipid-like bodies, with thin shell (Fig. 6). Female genital pore sinistral, post-ovarian, at level of oviducal seminal receptacle, 73 from male genital pore, 228 or 13% of body length from posterior body end (Figs. 5, 6).

Diagnosis of schistosomulum (based on 3 stained, whole-mounted specimens): Body flat, ventrally concave, oval in shape, tapering anteriorly, 820–1,390 (1,063 ± 294, 3) long, 128–270 (179 ± 79, 3) at greatest width, 5.1–7.7 (6.2 ± 1.3, 3) × longer than wide.

Taxonomic summary

Type and only reported host: Yellowedge grouper, *Hyporthodus flavolimbatus* (Poey, 1865) Craig and Hastings, 2007 (Perciformes: Serranidae).

Type locality: Northern Gulf of Mexico, (28°37′42.0″N, 85°57′24.5″W), 170 km south of Panama City, Florida.

Site of infection: Heart lumen.

Prevalence and intensity of infection: 2 of 5 (prevalence = 40%) yellowedge grouper sampled on 19, 24 August 2017, 5 September 2017, and 13 September 2017 were infected by 6 specimens of *C. uterohamus*.

Specimens deposited: Holotype (USNM 1622608).

Zoobank registration: urn: lsid: zoobank.org:act:76B722D2-63D9-40BB-A947-5BB90F5D3CFA.

Etymology: The Latin "*uterohamus*" refers to the general shape of the uterus.

Phylogenetic results

The amplified *28S* and *ITS2* fragments representing the new species comprised 1615 and 466 nucleotides, respectively (GenBank accession no. MW147714 and MW145112). The *28S* sequence is similar to the sequences for *Cardicola forsteri* Cribb, Daintith, and Munday, 2000 (KT119353), *Cardicola opisthorchis* Ogawa, Ishimaru, Shirakashi, Takami, and Grabner, 2011 (HQ324227), and *Cardicola orientalis* Ogawa, Tanaka, Sugihara, and Takami, 2010 (AB742425) differing by 42 (3.6%), 46 (3.9%), and 51 (4.3%) nucleotides, respectively. The *ITS2* sequence is similar to the sequences for *Cardicola milleri* Nolan and Cribb, 2006 (DQ059640), *Cardicola beveridgei* Nolan, Miller, Cutmore, Cantacessi, and Cribb, 2014 (KX523189), *Cardicola parvus* Bullard, Baker, and de Buron, 2012 (KY817376), and *Cardicola*

chaetodontis Yamaguti, 1970 (DQ059633) differing by 40 (13%), 41 (14%), 41 (14%), and 42 (14%), respectively.

The phylogenetic analyses (Bayesian Analyses [Figs. 7, 8]) produced similar *28S* and *ITS2* trees both with low nodal support (<0.60 posterior probability [PP]). The recovered *ITS2* tree has multiple polytomies (Fig. 8) and differed from the *28S* tree in recovering the clade including *Cardicola jiigurru* Yong, Cutmore, Miller, Wee, and Cribb, 2016 and *Cardicola suni* Yong, Cutmore, Miller, Wee, and Cribb, 2016 in a polytomy that includes *C. langeli* (vs. sister to *C. orientalis*). Further, *C. langeli* is recovered sister to *C. aurata* and *Cardicola bullardi* Nolan, Miller, Cutmore, Cantacessi, and Cribb, 2014 forming a clade (*ITS2*) rather than included in a clade with *C. aurata* (*28S*). In addition, sequences of *Cardicola currani* Bullard and Overstreet, 2004*, Cardicola laruei* Short, 1953, *Cardicola palmeri* Bullard and Overstreet, 2004*, C. parvus,* and *Cardicola yuelao* Yong, Cutmore, and Cribb, 2018 do not align with more than 600 base pairs marking them deficient for the *28S* study (Bullard and Overstreet, 2004; Yong et al., 2018a; Siegel et al., 2018). However, the *ITS2* sequences for these taxa are included in the *ITS2* tree. Nucleotide sequences for *C. dhangeli* (*28S*; *ITS2*), *C. euzeti* (*ITS2*), *Cardicola nonamo* Bullard, 2010 (*28S*; *ITS2*), and *Cardicola whitteni* Manter, 1954 (*28S*; *ITS2*) are also not yet available for study. The new species described herein was recovered in the *28S* tree sister to a clade including *C. euzeti*, *C. chaetodontis*, and *C. beveridgei* (Fig. 7) and in the *ITS2* tree as a distinct lineage, but part a large polytomy (Fig. 8).

Remarks

Cardicola uterohamus n. sp. differs from its congeners by the combination of having an anterior sucker that does not extend beyond the anterior body margin, 2–5 tegumental body spines per row, posterior ceca 9 × longer than the anterior ceca and that lack any coil, a testis 3 ×

longer than wide and that does not fill the intercecal space, an ovary that is >60% of the body width, a vitelline duct that connects to the anterior aspect of the oötype, a uterus that is >10% of the body width and that extends posterior to all genitalia, and a rounded posterior body margin. It is the only species of *Cardicola* that infects a grouper (Serranidae).

Until this publication, *C. langeli* lacked an associated nucleotide sequence. The sister relationships among *C. langeli*, *C. aurata*, *C. bullardi, Cardicola abu* Yong, Cutmore, and Cribb, 2018, *C. forsteri*, and *C. opisthorchis* are noteworthy because these species are markedly morphologically distinctive, i.e., the testis is anterolateral to the intestine (*C. forsteri*), post-cecal (*C. opisthorchis*), or ventral to the intestine (*C. abu*) (Cribb et al., 2000; Ogawa et al., 2011; Yong et al., 2018a), and infect phylogenetically unrelated ray-finned fishes: *C. forsteri* and *C. opisthorchis* infect true tunas (*Thunnus* spp.) whereas *C. abu* infects a damselfish (Pomacentridae), and further, *C. langeli* and *C. aurata* infect porgies (Sparidae) (Cribb et al., 2000; Ogawa et al., 2011).

Several species within the genus (*C. aurata*, *C. langeli*, and the *Cardicola* spp. infecting rabbitfishes) infecting phylogenetically related hosts are recovered together in a phylogenetic analysis while others (*C. currani, C. euzeti*, *C. laruei, C. orientalis, C. palmeri, C. parvus*) are not. *Cardicola euzeti* recovered sister to a clade including *C. beveridgei* and *C. chaetodontis*, which is surprising because *C. beveridgei* also infects a snapper (*Lutjanus argentimaculatus* [Forsskål, 1775] Kyushin et al. 1977). However, *C. beveridgei* and *C. chaetodontis* are easily differentiated from *C. euzeti* by having a uterus that is posterior to all other genitalia (vs. a male genital pore that is posterior to all other genitalia). Like many previous publications, *C. orientalis*, a species that infects Pacific bluefin tuna (*Thunnus orientalis* (Temminck and Schlegel, 1844), was recovered sister to the species of *Cardicola* that infect rabbitfishes

(Siganidae) (Yong et al., 2016; Yong et al., 2018a; Yong et al., 2018b; Siegel et al., 2018). However, morphologically, *C. orientalis* is distinct by having a vitelline duct that connects to the anterior aspect of the oötype (vs. connecting to the posterior aspect of the oötype) and a uterus with 3 coils (vs. a uterus that is extensively convoluted). The ITS2 phylogenetic analysis recovered the sciaenid infecting blood flukes (*C. currani, C. laruei, C. palmeri, C. parvus*) in 2 separate clades. *Cardicola laruei* and *C. parvus* share a recent common ancestor with a clade comprising *C. beveridgei*, *C. chaetodontis*, and *C. milleri*. *Cardicola currani* and *C. palmeri* are recovered in a polytomy that includes a clade comprising *C. abu*, *C. forsteri*, and *C. opisthorchis*. Morphologically, *C. currani* and *C. palmeri* are differentiated from *C. laruei* and *C. parvus* by having an oral sucker that extends past the anterior body margin (vs. a partially invaginated minute oral sucker) and an oviducal seminal receptacle that extends across the midline (vs. extending in parallel with the midline).

DISCUSSION

Previous studies have indicated that the same definitive host species across a broad geographic area (Gulf of Mexico) can be differentially infected by blood flukes, perhaps relating to fish host population differences or the abundance or distribution of the intermediate host shedding infective cercariae (Ogawa et al., 2011). The new species appears relatively host-specific to yellowedge grouper since 21 sympatric red groupers, *Epinephelus morio* (Valenciennes, 1828) Matsuura in Uyeno et al., 1983 (Perciformes: Serranidae), were not infected by this blood fluke. Before the present study, 4 fish blood flukes of 2 genera have been identified infecting serranids (Overstreet and Køie, 1989; Nolan and Cribb, 2004; Bullard, 2012), all within the Epinephelinae. With the addition of the second genus (*Cardicola*) to the group of aporocotylids infecting groupers, we can speculate the diversity within this subfamily of fishes is

high and should be examined more thoroughly. Yellowedge groupers caught west of Pascagoula, MS, were also negative for infections, limiting the infections to only fish caught east of Mobile Bay, Alabama. Similarly, *Selachohemecus benzi* Bullard, Overstreet, and Carlson, 2006, a fish blood fluke that infects black tip sharks, *Carcharhinus limbatus* (Valenciennes 1839) Compagno 1973, has been found only east of the Mississippi River (Bullard et al., 2006). Given this information, the geographic range of *C. uterohamus* seems to be restricted to the east of Pascagoula, Mississippi. If true, it is likely linked to the overall range of the intermediate host.

ITS2 may not be ideal for inferring interspecific relationships among *Cardicola* spp. The *ITS2* is frequently used in phylogenetics of fish blood flukes (Yong et al., 2016, 2018a, 2018b; Siegal et al., 2018) as well as turtle blood flukes (Roberts et al., 2019). However, in the present study, the *ITS2* analysis did not recover a well-resolved tree with high nodal support (Fig. 8). Siegel et al. (2018) had a similar result, recovering species of *Cardicola* as paraphyletic with species of *Braya* Nolan and Cribb, 2006, which have species that are morphologically similar to species of *Cardicola*. For example, Nolan and Cribb (2006) differentiated species of *Braya* from *Cardicola* spp. by testis position relative to the intercecal field, presence or absence of a cirrus sac, the position of the male genital pore relative to the end of the body, and the female genital pore relative to the male genital pore. *Cardicola aurata* has a male genital pore that extends to the terminal end of the body; *C. opisthorchis* has a testis that is post-cecal; and *C. abu*, *C. aurata, C. bullardi*, *C. dhangeli*, *C. forsteri, C. langeli*, *C. opisthorchis*, and *C. whitteni* all lack a cirrus sac. Further, all species of *Braya* infect scarids (Scaridae), adding to the complexity of the genus. Other than Siegel et al. (2018), nucleotide sequences from *Cardicola* spp. that infect sciaenids (*C. currani*, *C. laruei*, *C. palmeri*, and *C. parvus*) have been absent from the prior studies including sequences for species of *Cardicola* (Brooks et al., 2017; Yong et al., 2018a). When

these sequences are removed from the phylogenetic analysis (*ITS2*), we recover well-resolved trees, without a polytomy. These discrepancies in evaluating the interspecific relationships using nucleotide evidence highlight the importance of quality morphological descriptions of new species as well as recollected nominal species.

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FIGURE LEGENDS

Figures 1–4. *Cardicola cardiocola* (Manter, 1947) Short, 1953 (Digenea: Aporocotylidae) infecting the heart of the jolthead porgy, *Calamus bajonado* (Bloch and Schneider, 1801) Robins and Ray, 1986 (Perciformes: Sparidae) from the northeast Gulf of Mexico, (26°45′00.8″N, 82°48′23.4″W), 150 km west of Boca Grande, Florida. Scale values aside bars. (**1**) Body of holotype (USNM No. 1337732) and (**2**) paratype (USNM No. 1622601) showing cecal bifurcation (cb), esophagus (es), esophageal gland (eg), anterior ceca (ac), vitellarium (v), posterior ceca (pc), vitelline duct (vit), testis (t), ovary (ov), and oötype (oo). (**3**) Genitalia of the holotype (USNM No. 1337732) showing testis (t), ovary (ov), vitelline duct (vit), oviduct (o), uterus (u), female genital pore (fp), seminal vesicle (sv), cirrus sac (cs), and cirrus (c). (**4**) Genitalia of paratype (USNM No. 1622601) showing testis (t), ovary (ov), oviducal seminal vesicle (osr), uterus (u), vas deferens (vd), oötype (oo), female genital pore (fp), seminal vesicle (sv), cirrus sac (cs).

Figures 5, 6. *Cardicola uterohamus* Warren and Bullard n. sp. (Digenea: Aporocotylidae) from the heart of the yellowedge grouper, *Hyporthodus Flavolimbatus* (Poey, 1865) Craig and Hastings, 2007 (Perciformes: Serranidae) from the northern Gulf of Mexico, (28°37′42.0″N, 85°57′24.5″W), 170 km south of Panama City, Florida. (**5**) Body of holotype (USNM No. 1622608) showing mouth (mo), cecal bifurcation (cb), esophagus (es), esophageal gland (eg), anterior ceca (ac), vitellarium (v), posterior ceca (pc), testis (t), ovary (ov), female genital pore (fp), Male genital pore (mp), and uterus (u). and oötype (oo). (**6**) Genitalia of holotype (USNM No. 1622608) showing testis (t), posterior ceca (pc), ovary (ov), vitelline duct (vit), metraterm (met), female genital pore (fp), oviduct (o), oötype (oo), Mehlis gland (mg), uterine egg (ue), and uterus (u).

Figure 7. Phylogenetic relationships of species within *Cardicola* reconstructed using Bayesian inference analysis using the large subunit ribosomal (*28S*) gene. The new species is shown in bold.

Figure 8. Phylogenetic relationships of species within *Cardicola* reconstructed using Bayesian inference analysis using the internal transcribed spacer 2 (*ITS2*) gene. The new species is shown in bold.

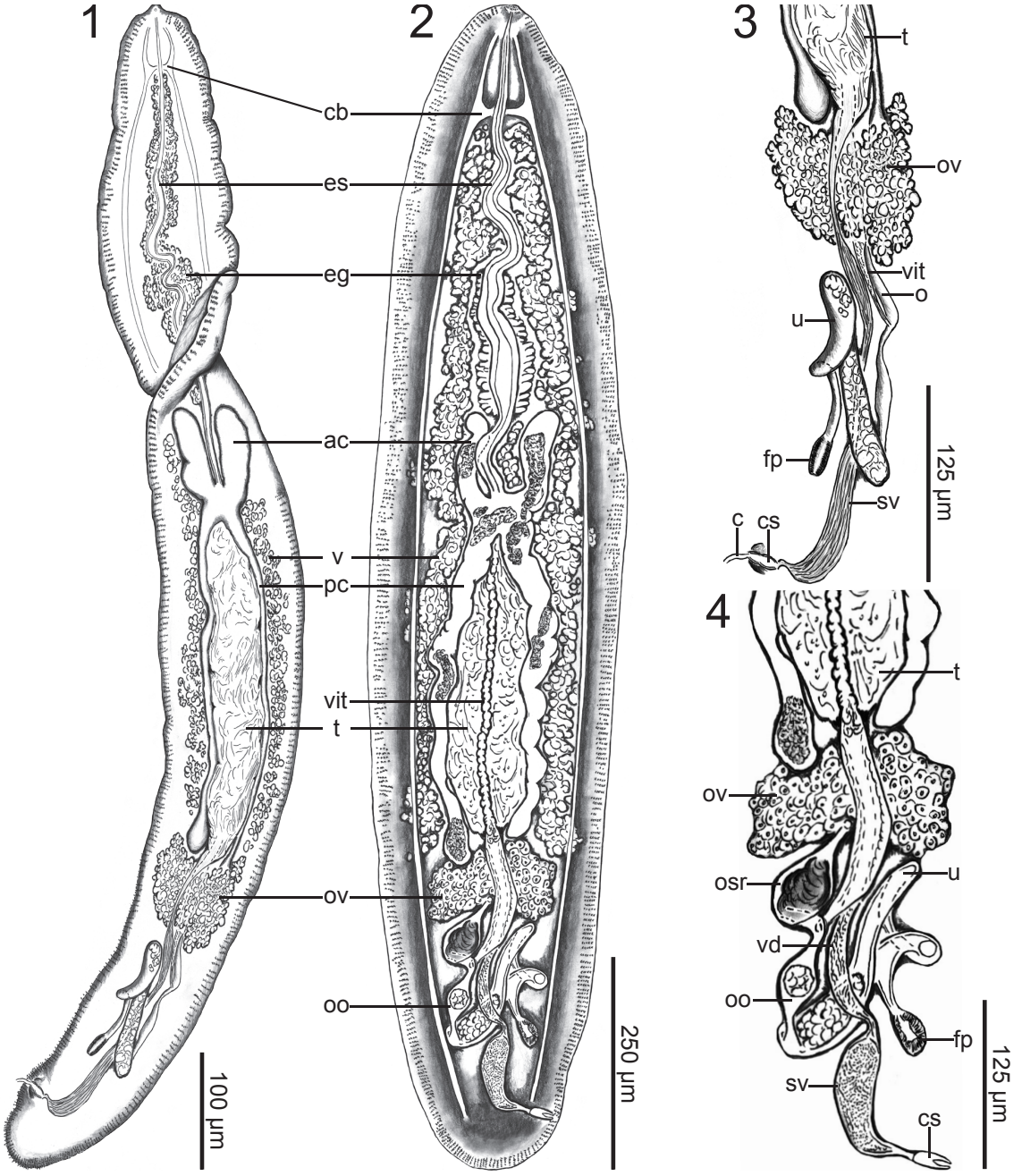
Table I. Sequences used in the present study.

Species	Host	Locality	GenBank Accession #s		Reference
			28S	ITS2	
Cardallagium anthicum (Bullard and Overstreet, 2006) Yong, Cutmore, Jones, Gauthier, and Cribb, 2018	cobia, *Rachycentron canadum* (Linnaeus 1766) Monod, 1973 (Perciformes: Rachycentridae)	Northern Gulf of Mexico off Mississippi, USA	KX840316		Warren et al. (2017)
Cardallagium cf. *anthicum* (Bullard and Overstreet, 2006) Yong, Cutmore, Jones, Gauthier, and Cribb, 2018	cobia, *Rachycentron canadum* (Linnaeus 1766) Monod, 1973 (Perciformes: Rachycentridae)	Vietnam	KX840319		Warren et al. (2017)
Cardicola abu Yong, Cutmore, and Cribb, 2018	Whitley's sergeant, *Abudefduf whitleyi* Allen and Robertson, 1974 (Percifomres: Pomacentridae)	North Wistari Reef, off Heron Island, Queensland, Australia	MH161379	MH161380	Yong et al. (2018a)
Cardicola aurata Holzer, Montero, Repulles, Nolan, Sitja-Bobadilla, Alvarez-Pellitero, Zarza, and Raga, 2008	gilt-head bream, *Sparus aurata* Linnaeus, 1758 (Perciformes: Sparidae)	Western Mediterranean, Spain, North of Valencia	AM910616	AM910617	Holzer et al. (2008)
Cardicola bartolii Nolan and Cribb, 2006	golden-lined spinefoot, *Siganus lineatus* (Valenciennes 1835) Allen and Swainston, 1988 (Perciformes: Siganidae)	Heron Island, Queensland, Australia	MF140285	DQ059631	Nolan and Cribb, (2006)
Cardicola beveridgei Nolan, Miller, Cutmore, Cantacessi, and Cribb, 2014	mangrove red snapper, *Lutjanus argentimaculatus* (Forsskål, 1775) Kyushin et al. 1977 (Perciformes: Lutjanidae)	off Lizard Island, Queensland, Australia	KX523188	KX523189	Yong et al. (2016)
Cardicola bullardi Nolan, Miller, Cutmore, Cantacessi, and Cribb, 2014	Australian spotted mackeral, *Scomberomorus munroi* Collette and Russo, 1980 (Perciformes: Scombridae)	Moreton Bay, Queensland, Australia	KX523190	KX523191	Yong et al. (2016)
Cardicola chaetodontis Yamaguti, 1970	Rainford's butterflyfish, *Chaetodon rainfordi* McCulloch, 1923 (Perciformes: Chaetodontidae)	Heron Island, Queensland, Australia	KX523192		Yong et al. (2013)
	blueblotch butterflyfish, *Chaetodon plebeius* Cuvier, 1831 (Percifomes: Chaetodontidae)	Lizard Island. Queensland, Australia		KF049003	Yong et al. (2016)
Cardicola coeptus Nolan and Cribb, 2006	goldspotted spinefoot, *Siganus punctatus* (Schneider and Forster, 1801) Allen and Swainston, 1988 (Perciformes: Siganidae)	Heron Island, Queensland, Australia	MF140284	DQ059630	Nolan and Cribb, (2006)

Cardicola covacinae Nolan and Cribb, 2006	goldspotted spinefoot, *Siganus punctatus* (Schneider and Forster, 1801) Allen and Swainston, 1988 (Perciformes: Siganidae)	Heron Island, Queensland, Australia	MF140283	DQ059634	Nolan and Cribb, (2006)
Cardicola currani Bullard and Overstreet, 2004	red drum, *Sciaenops ocellatus* (Linnaeus, 1766) Chao, 1978 (Perciformes: Sciaenidae)	Northern Gulf of Mexico, Mississippi Sound, Mississippi, USA	KJ572524	KY817380	Orélis-Ribeiro et al. (2014)
Cardicola forsteri Cribb, Daintith, and Munday, 2000	Southern bluefin tuna, *Thunnus maccoyii* (Castelnau 1872) Gibbs and Collette, 1967 (Perciformes: Scombridae)	Port Lincoln, Australia	AB742426	AB742428	Shirakashi et al. (2016)
Cardicola jigurru Yong, Cutmore, Miller, Wee, and Cribb, 2016	milkfish, *Chanos chanos* (Forsskål, 1775) Kyushin et al. 1977 (Gonorynchiformes: Chanidae)	Lizard Island, Queensland, Australia	KX463506	KX463507	Yong et al. (2016)
Cardicola lafii Nolan and Cribb, 2006	pinspotted spinefoot, *Siganus fuscescens* (Houttuyn 1782) Lindberg and Krasyukova, 1975 (Perciformes: Siganidae)	Lizard Island, Queensland, Australia	MF140282	DQ059639	Nolan and Cribb, (2006)
Cardicola langeli Bullard, 2013	sheepshead, *Archosargus probatocephalus* (Walbaum, 1792) Lee et al. 1980 (Perciformes: Sparidae)	off Horn Island, Mississippi Sound, Mississippi, USA	MW158544		present study
Cardicola laruei Short, 1953	spotted seatrout, *Cynoscion nebulosus* (Cuvier, 1830) Chao, 1978 (Perciformes: Sciaenidae)	Northwestern Atlantic Ocean, South Atlantic Bight	KY817378	KY817375	Siegel et al. (2018)
Cardicola miller Nolan and Cribb, 2006	two-spot red snapper, *Lutjanus bohar* (Forsskål, 1775) Kyushin et al. 1977 (Perciformes: Lutjanidae)	Lizard Island, Queensland, Australia		DQ059640	Nolan and Cribb, (2006)
Cardicola mogilae Brooks, Cutmore, Yong, and Cribb, 2017	pinspotted spinefoot, *Siganus fuscescens* (Houttuyn 1782) Lindberg and Krasyukova, 1975 (Perciformes: Siganidae)	Lizard Island, Queensland, Australia	MF140280	DQ059635	Nolan and Cribb, (2006)
Cardicola opisthorchis Ogawa, Ishimaru, Shirakashi, Takami, and Grabner, 2011	*Terebella* sp. Linnaeus, 1767(Terabellida: Terabellidae)	Tsushima Island, Nagasaki, Japan	AB829900	AB830082	Sugihara et al. (2014)
Cardicola orientalis Ogawa, Tanaka, Sugihara, and Takami, 2009	Southern bluefin tuna, *Thunnus maccoyii* (Castelnau 1872) Gibbs and Collette, 1967 (Perciformes: Scombridae)	Port Lincoln, Australia	AB742425	AB742427	Shirakashi et al. (2016)
Cardicola palmeri Bullard and Overstreet, 2004	black drum, *Pogonias cromis* (linnaeus, 1766) Chao, 1978 (Perciformes: Sciaenidae)	Northern Gulf of Mexico, Mississippi Sound, Mississippi, USA	KJ572525	KY817379	Orélis-Ribeiro et al. (2014)
Cardicola parvus Bullard, Baker, and de Buron, 2012	Atlantic croaker, *Micropogonias undulatus* (Linnaeus, 1766) Chao, 1978	Northwestern Atlantic Ocean, South Atlantic	KY817377	KY817376	Bullard et al. (2012)

	(Perciformes: Sciaenidae)	Bight			
Cardicola sp. Wangetti	milkfish, *Chanos chanos* (Forsskål, 1775) Kyushin et al. 1977 (Gonorynchiformes: Chanidae)	Wangetti Beach, Queensland, Australia	KX463508		Yong et al. (2016)
Cardicola suni Yong, Cutmore, Miller, Wee, and Cribb, 2016	milkfish, *Chanos chanos* (Forsskål, 1775) Kyushin et al. 1977 (Gonorynchiformes: Chanidae)	Moreton Bay, Queensland, Australia	KX463511	KX463510	Yong et al. (2016)
Cardicola tantabiddi Nolan and Cribb, 2006	pinspotted spinefoot, *Siganus fuscescens* (Houttuyn 1782) Lindberg and Krasyukova, 1975 (Perciformes: Siganidae)	Ningaloo Reef, Australia	MF140279	DQ059642	Nolan and Cribb, (2006)
Cardicola uterohamus n. sp. Warren and Bullard	yellowedge grouper, *Hyporthodus flavolimbatus* (Poey, 1865) Craig and Hastings, 2007 (Perciformes: Serranidae)	Northern Gulf of Mexico, off Florida, USA	MW147714	MW145112	present study
Cardicola yuelao Yong, Cutmore, and Cribb, 2018	flagtail triggerfish, *Sufflamen chrysopterum* (Bloch and Schneider 1801) Matsuura in Masuda et al. 1984 (Tetraodontiformes: Balistidae)	Off Lizard Island, northeast Queensland, Australia	MH161381	MH161382	Yong et al. (2018a)
Cardicola euzeti (Bullard and Overstreet, 2003) Nolan and Cribb, 2006a	red snapper, *Lutjanus campechanus* (Poey, 1860) Rivas, 1966 Perciformes: Lutjanidae)	Northern Gulf of Mexico off Mississippi, USA	KJ572526		Orélis-Ribeiro et al. (2014)
Littorellicola bilhawkinsi Bullard, 2010	Florida pompano, *Trachinotus carolinus* (Linnaeus, 1766) (Perciformes: Carangidae)	Northern Gulf of Mexico off Mississippi, USA	MW152328		present study
Paradeontacylix belearicus Repullés-Albelda, Montero, Holzer, Ogawa, Hutson, and Raga, 2008	greater amberjack, *Seriola dumerili* (Risso, 1810) Hureau and Tortonese, 1973 (Perciformes: Carangidae)	Off Mallorca, Spain	AM489594	AM489600	Repullés-Albelda et al. (2008)
Paradeontacylix buri Ogawa, Akiyama, and Grabner, 2015	Japanese amberjack, *Seriola quinqueradiata* Temminck and Schlegel, 1845 (Perciformes: Carangidae)	Miyazaki Prefecture, Japan	AB904154	AB904153	Ogawa et al. (2015)
Paradeontacylix godfreyi Hutson and Whittington, 2006	yellowtail amberjack, *Seriola lalandi* Valenciennes, 1833 (Perciformes: Carangidae)	Port Augusta, Australia	AM489597	AM489602	Repullés-Albelda et al. (2008)
Paradeontacylix grandispinus Ogawa and Egusa, 1986	greater amberjack, *Seriola dumerili* (Risso, 1810) Hureau and Tortonese, 1973 (Perciformes: Carangidae)	Ushine, Kagoshima, Japan	AM489596	AM489601	Repullés-Albelda et al. (2008)
Paradeontacylix ibericus Repullés-Albelda, Montero, Holzer, Ogawa, Hutson, and	greater amberjack, *Seriola dumerili* (Risso, 1810) Hureau and Tortonese, 1973 (Perciformes: Carangidae)	Murcia, Murcia, Spain	AM489593	AM489598	Repullés-Albelda et al. (2008)

Raga, 2008					
Paradeontacylix kampachi Ogawa and Egusa, 1986	greater amberjack, *Seriola dumerili* (Risso, 1810) Hureau and Tortonese, 1973 (Perciformes: Carangidae)	Ushine, Kagoshima, Japan	AM489595	AM489598	Repullés-Albelda et al. (2008)
Outgroup					
Psettarium hawaienses (as *Paracardicola*) (Martin, 1960) Yong, Cutmore, Jones, Gauthier, and Cribb, 2018	stars and stripes puffer, *Arothron hispidus* (Linnaeus, 1758) Kyushin et al. 1977 (Tetraodontiformes: Tetraodontidae)	Moreton Bay region, southeast Queensland, Australia	MG709035		Yong et al. (2018b)
Psettarium hustoni Yong, Cutmore, Jones, Gauthier, and Cribb, 2018	black-spotted puffer, *Arothron nigropunctatus* (Bloch and Schneider, 1801) Dor 1984 (Tetraodontiformes: Tetraodontidae)	Flora Reef, Queensland, Australia	MG709037		Yong et al. (2018b)
Psettarium martini Yong, Cutmore, Jones, Gauthier, and Cribb, 2018	starry puffer, *Arothron stellatus* (Anonymous 1798) Fricke 1999 (Tetraodontiformes: Tetraodontidae)	Gold Coast Seaway, Gold Coast, southeast Queensland, Australia	MG709040		Yong et al. (2018b)
Psettarium pandora Yong, Cutmore, Jones, Gauthier, and Cribb, 2018	yellow boxfish, *Ostracion cubicus* Linnaeus, 1758 (Tetraodontiformes: Ostraciidae)	North Wistari Reef, off Heron Island, Queensland, Australia	MG709046		Yong et al. (2018b)
Psettarium pulchelum Yong, Cutmore, Bray, Miller, Semarariana, Palm & Cribb, 2016	narrow-lined puffer, *Arothron manilensis* (Marion de Procé 1822) Matsuura in Masuda et al. 1984 (Tetraodontiformes: Tetraodontidae)	Off Peel Island, Moreton Bay, southeast Queensland, Australia	MG709049		Yong et al. (2018b)
Psettarium sinensis (as *Paradeontacylix*) (Liu, 1997) Yong, Cutmore, Jones, Gauthier, and Cribb, 2018	Japanese puffer, *Takifugu rubripes* (Temminck and Schlegel, 1850) Matsuura in Masuda et al. 1984 (Tetraodontiformes: Tetraodontidae)		EU082007		unpublished
Psettarium yoshidai Yong, Cutmore, Jones, Gauthier, and Cribb, 2018	map puffer, *Arothron mappa* (Lesson 1831) Matsuura in Masuda et al. 1984 (Tetraodontiformes: Tetraodontidae)	Gold Coast Seaway, Gold Coast, southeast Queensland, Australia	MG709051		Yong et al. (2018b)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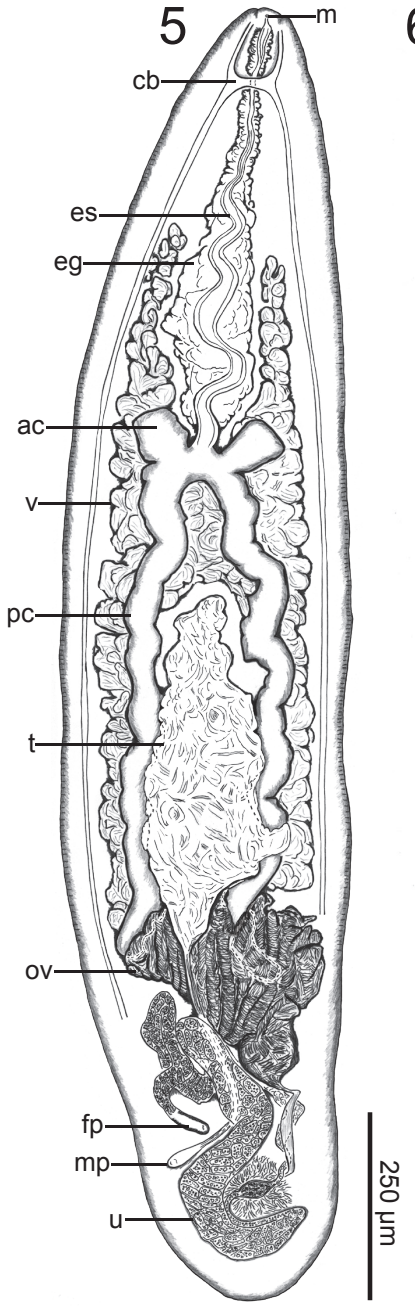
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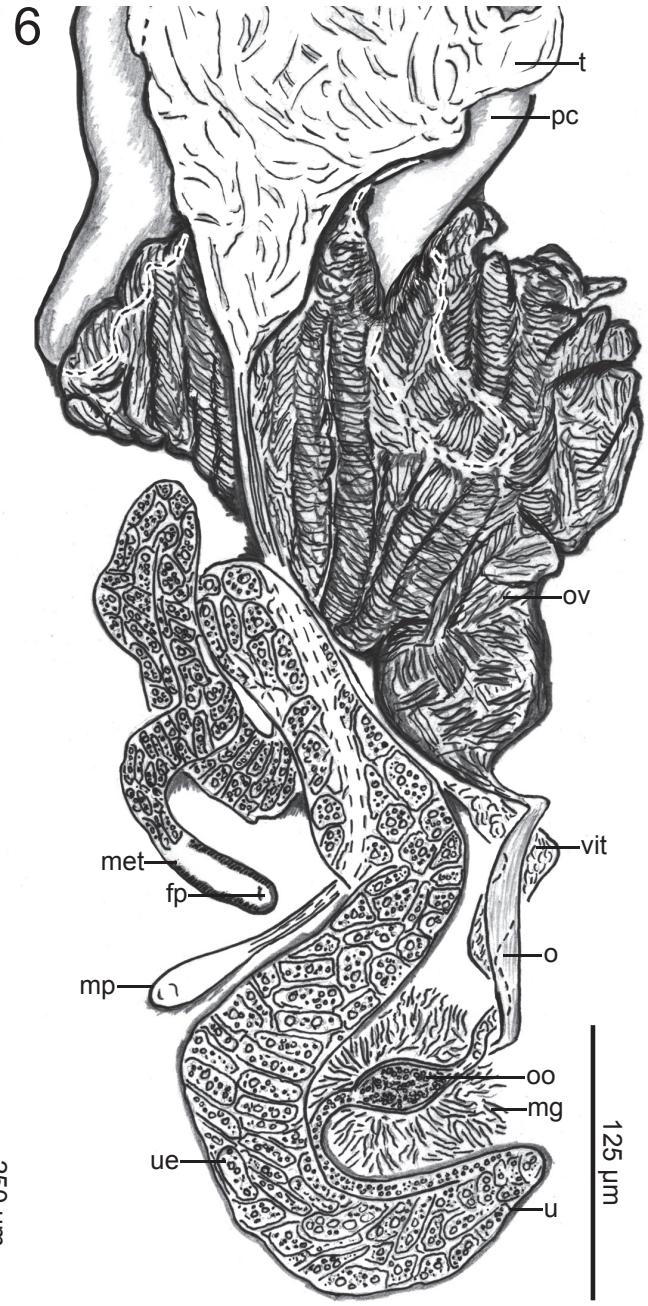
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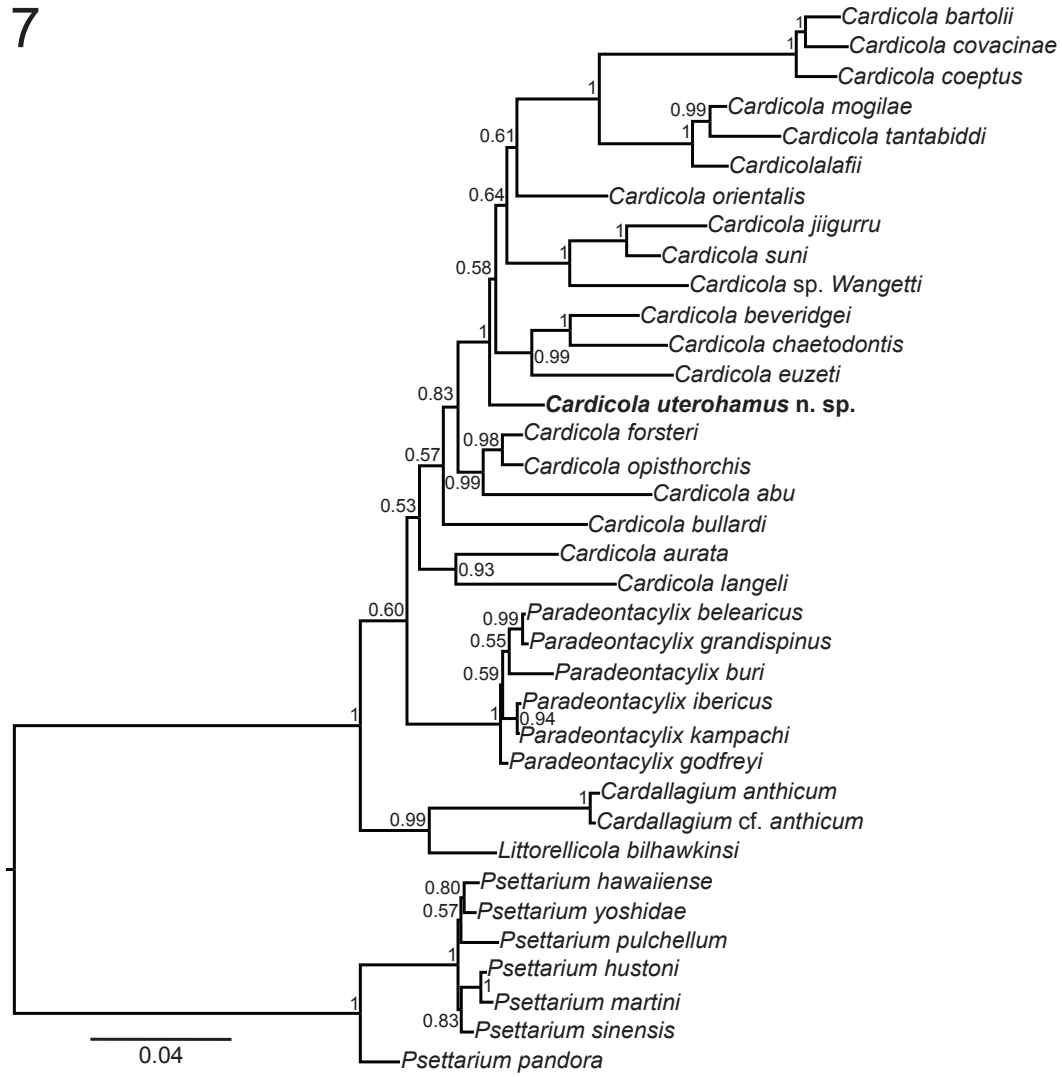
Cardicola bartolii
Cardicola covacinae
Cardicola coeptus
Cardicola mogilae
Cardicola tantabiddi
Cardicolalafii
Cardicola orientalis
Cardicola jiigurru
Cardicola suni
Cardicola sp. Wangetti
Cardicola beveridgei
Cardicola chaetodontis
Cardicola euzeti
Cardicola uterohamus n. sp.
Cardicola forsteri
Cardicola opisthorchis
Cardicola abu
Cardicola bullardi
Cardicola aurata
Cardicola langeli
Paradeontacylix belearicus
Paradeontacylix grandispinus
Paradeontacylix buri
Paradeontacylix ibericus
Paradeontacylix kampachi
Paradeontacylix godfreyi
Cardallagium anthicum
Cardallagium cf. anthicum
Littorellicola bilhawkinsi
Psettarium hawaiiense
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Psettarium pulchellum
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Psettarium sinensis
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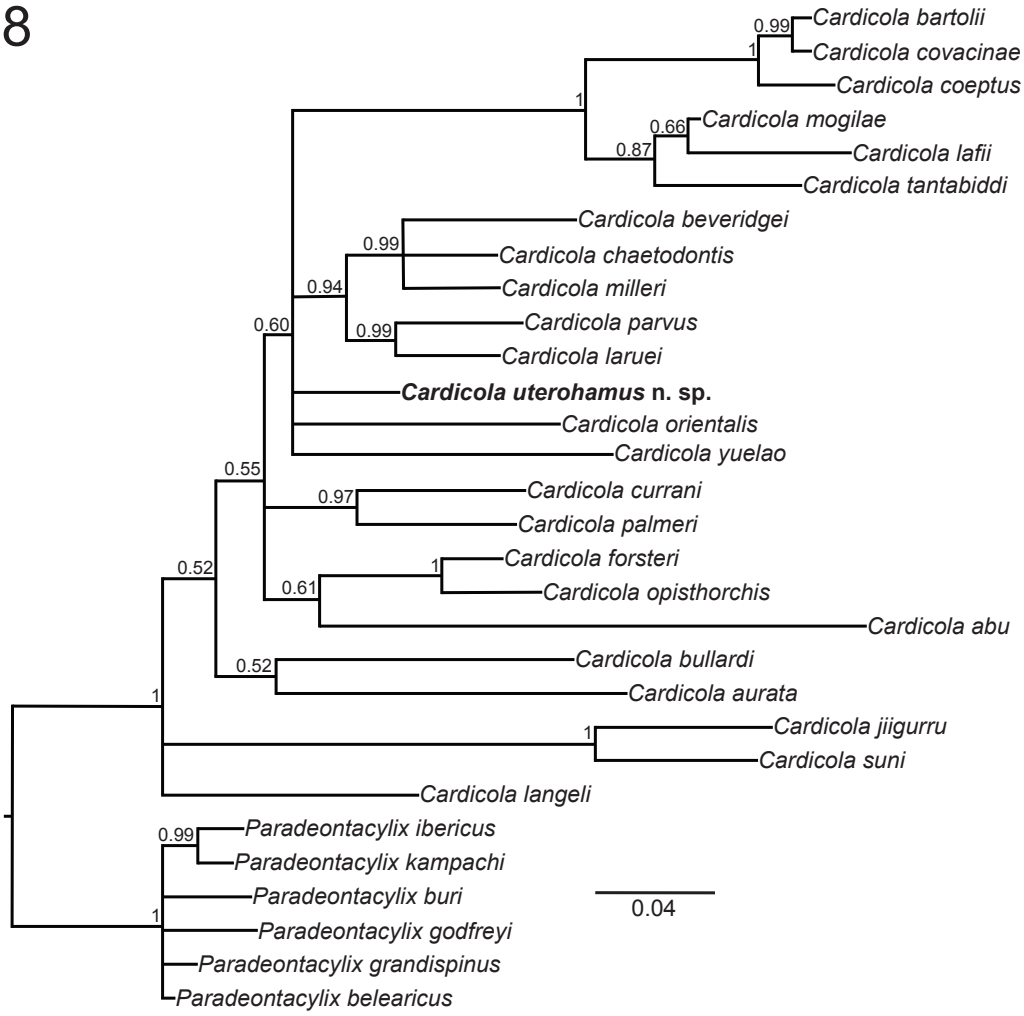
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Cardicola suni
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CHAPTER 2: FISH BLOOD FLUKES (DIGENEA: APOROCOTYLIDAE) FROM INDONESIA: TWO NEW GENERA AND SPECIES INFECTING THE BANDED EAGLE RAY, *AETOMYLAEUS NICHOFII* (BLOCH AND SCHNEIDER, 1801) CAPAPÉ AND DESOUTTER, 1979 (MYLIOBATIFORMES: MYLIOBATIDAE) FROM BORNEO

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Authors: Micah B. Warren and Stephen A. Bullard

ABSTRACT

Specimens representing two new species of blood flukes (Digenea: Aporocotylidae), each representing a new genus, were collected from the banded eagle ray, *Aetomylaeus nichofii* (Bloch and Schneider, 1801) Capapé and Desoutter, 1979, in Borneo, Indonesia. *Aetohemecus kirstenjensenae* n. sp., n. gen. infected the heart of a banded eagle ray from Manggar, East Kalimantan, Borneo, Indonesia, and differs from its congeners by having an oviducal ampullae, an oötype posterior to all genitalia, and a uterus that extends anterior to the ovary. The new species resembles *Selachohemecus* spp., which infect requiem sharks (Carcharhinidae) in the Northwestern Atlantic Ocean and Gulf of Mexico, by having a single ventrolateral row of large C-shaped tegumental spines, X- or H-shaped intestine, and a post-caecal ovary. Specimens of *Homestios janinecairae* n. sp., n. gen. infected the heart of a banded eagle ray from Takisung, South Kalimantan, Borneo, Indonesia. The new species resembles other blood flukes that infect rays (Batoidea) by having a single, curving testis and an inverse U-shaped intestine as well as by lacking tegumental spines. It differs from all aporocotylids infecting batoids that lack spines by having a uterus that extends anteriad beyond the level of the seminal vesicle. The present study comprises the first record of an aporocotylid from Indonesia or from an eagle ray (Myliobatidae). To our knowledge, these are the first trematodes reported from a species of *Aetomylaeus*. The

proposals of new genera and the description of two new species herein brings the total number of nominal chondrichthyan blood flukes to 13 species of 11 genera.

Keywords: Taxonomy, Systematics, Chondrichthyes, Elasmobranchii, Fish Blood Fluke, Batoidea

1. Introduction

Currently, there are 633 nominal ray species (Chondrichthyes: Elasmobranchii: Batoidea) ranging in both marine and freshwater habitats (Last et al., 2016). More than 55 nominal batoid species of 12 families range in Indonesia, where the elasmobranch diversity and commercial fisheries landings (~110,000 tonnes) are high (Dharmadi et al., 2009). A large proportion of elasmobranch landings are bycatch and, because these sharks and rays typically are not identified to species at landing, this proportion is likely higher (Dharmadi et al., 2009; Dharmadi et al., 2013). Given that locations with such high elasmobranch landings (tonnes of fish put on the dock) are rare, Indonesia is lucrative regarding opportune collections of parasites from high diversity of elasmobranchs (Dharmadi et al., 2009). No aporocotylid (Platyhelminthes: Digenea: Aporocotylidae Odhner, 1912) has been described from this region. Despite the fact that most of the major batoid lineages (Myliobatiformes, Rhinopristiformes, Torpediniformes) have been confirmed as aporocotylid hosts, only six batoid species (0.09% of the total batoids) have been reported as blood fluke hosts (Table 1). The only record from a skate (Rajiformes) is that of Bazikalova (1932), who reported an aporocotylid, which evidently remains innominate, infecting the lumen of the intestine of thorny skate, *Amblyraja radiata* (as *Raja*) (Donovan, 1808) Stehmann, 1973 (Rajiformes: Rajidae) (Bullard and Jensen, 2008). In comparison, of the 516 nominal shark species, only four (0.07%) have been reported as aporocotylid hosts (Table 1).

The aporocotylids that infect chondrichthyans comprise 11 spp. of nine genera (Table 1) and have large C-shaped tegumental spines (infecting sharks, a ray, and a chimaera) or lack spines (infecting batoids [except *Gymnurahemecus bulbosus* Warren, Ruiz, Whelan, Kritsky, and Bullard, 2019]) (Short, 1954; Van der Land, 1967; Madhavi and Rao, 1970; Maillard and Ktari, 1978; Bullard et al., 2006; Bullard and Jensen, 2008; Orélis-Ribeiro et al., 2013; Cutmore et al., 2018; Warren et al., 2019; Warren and Bullard, 2019; Warren et al., 2020). Further, aporocotylids infecting batoids (except *G. bulbosus*) have a curving testis (Madhavi and Rao, 1970; Bullard and Jensen, 2008; Cutmore et al., 2018; Warren and Bullard, 2019; Warren et al., 2020). The aporocotylids infecting bony fishes comprise ~155 spp. of 32 genera and differ by having transverse rows of tegumental spines or rosethorn-shaped spines (McIntosh, 1934; Bullard, 2013) or by lacking spines (Truong and Bullard, 2013, Orélis-Ribeiro and Bullard, 2015). Species of *Plehniella* infect the body cavity of catfishes, lack spines, and have a star-shaped intestine (Truong and Bullard, 2013, Orélis-Ribeiro and Bullard, 2015). The current separation of these two morphologically distinct lineages of fish blood flukes is further represented in nucleotide-based phylogenetic studies using the large subunit of ribosomal DNA (*28S rDNA*) (Warren et al., 2019; Warren and Bullard, 2019).

Herein, we describe two new species of fish blood flukes infecting the heart of two banded eagle rays, *Aetomylaeus nichofii* (Bloch and Schneider, 1801) Capapé and Desoutter, 1979 (Myliobatiformes: Myliobatidae) from Borneo, Indonesia and propose two new genera to accommodate each one. The present report comprises the first record of an aporocotylid infection from Indonesia and from an eagle ray (Myliobatidae) as well as the first record of a digenean infection for any species of *Aetomylaeus*. Further, we provide a key to all fish blood flukes that infect chondrichthyans (KEY).

2. Materials and methods

2.1 Specimen collection and preparation

One hundred and twenty specimens of 25 species of sharks and rays (21 rays, four sharks) were collected from 26 November 2006 – 4 August 2008 using gill nets and commercial trawls (Koch et al., 2012). Herein, we report the parasitological results for the banded eagle ray (*Aetomylaeus nichofii*) only. During collection, on 2 December 2006 and 3 August 2008, the hearts from two banded eagle rays (*Aetomylaeus nichofii*) in Takisung, South Kalimantan, Borneo (03°52'28.00"S, 114°36'37.00"E) and Manggar, East Kalimantan, Borneo (01°12'55.20"S, 116°58'27.50"E), Indonesia, respectively, were collected. At necropsy, the heart and spiral intestine were excised intact (heart bisected, spiral valve opened). Hearts and half of the spiral valve were placed in sampled bags and fixed with 10% neutral buffered formalin (nbf). From 2008–7 July 2020 the hearts (including the two hearts from the banded eagle rays) of all 120 shark and ray specimens were examined with the aid of a Meiji Techno RZ dissecting microscope to isolate fluke specimens for morphology. The hearts were teased apart with forceps to reveal adult aporocotylids, and sediment from the heart was examined using sedimentation method using a plastic cylinder.

Adult flukes (n=5) were transferred to vials filled with 10% nbf, rinsed with distilled water, cleaned with fine brushes to remove any host tissue or debris, stained overnight in Van Cleave's hematoxylin with several additional drops of Ehrlich's hematoxylin, dehydrated using an ethanol series, cleared in clove oil, and permanently mounted in Canada balsam. Drawings were made with Leica DM 2500 and Leica DMR (Leica, Wetzler, Germany) microscopes each equipped with differential interference contrast (DIC), measured using an ocular micrometer, and illustrated using a drawing tube. Measurements are reported in micrometres (μm) as the range

followed by the mean and sample size in parentheses. Scientific names, including taxonomic authorities and dates, for fishes follow Eschmeyer et al. (2016). Morphological terms and nomenclature for aporocotylids follows Bullard and Jensen (2008), Orélis-Ribeiro et al. (2013), Warren et al. (2019), Warren et al. (2020). Type and voucher materials of the new species were deposited in the National Museum of Natural History's Invertebrate Zoology Collection (USNM, Smithsonian Institution, Washington, D. C.).

3. Results

3.1 Aetohemecus n. gen. (Figs. 1 − 2)

3.1.1 Generic diagnosis

Body 4–6 × longer than wide, dorsoventrally flattened, ventrally concave, having anterior and posterior ends tapering equally, spinous; lateral tegumental spines C-shaped, directed ventrally, each on a muscular peduncle, distributing in a single ventrolateral column, not continuous anteriorly nor posteriorly. Rosethorn-shaped spines lacking. Nervous system comprising paired lateral nerve cords. Anterior sucker aspinous, lacking peduncle, diminutive, occupying space between anterior-most lateral tegumental spines. Mouth on mid-ventral surface of anterior sucker. Pharynx not evident. Oesophagus extending sinuously posteriad along mid-line for 1/3 of body length. Intestinal caeca X- or H-shaped, connecting to oesophagus ventrally, lacking diverticulae, posterior caeca terminating in anterior half of body. Testis single, medial, occupying middle 1/3 of body. Auxiliary external seminal vesicle lacking. Cirrus-sac present, enveloping internal seminal vesicle and cirrus. Ovary medial, ventral to ascending uterus, post-caecal, post-testicular; post-ovarian space comprising 1/6–1/4 of body length. Oviducal ampulla present. Laurer's canal absent. Oötype medial, posterior to genitalia, comprising an inconspicuous ovoid chamber. Uterus extending anterior to ovary; uterine eggs irregular, thin-

shelled. Vitellarium follicular, symmetrical posteriorly, filling space between nerve commissure to ovary; common vitelline collecting duct extending from dextral branch of vitellarium. Common genital pore dorsal, post-gonadal, anterior to level of oötype.

3.1.2 Differential diagnosis

Body 4–6 × longer than wide; lateral tegumental spines C-shaped, directed ventrally, each on a muscular peduncle, distributing in a single ventrolateral column, not continuous anteriorly nor posteriorly. Intestinal caeca X- or H-shaped, posterior caeca terminating in anterior half of body. Testis single, medial, occupying middle 1/3 of body. Ovary medial, ventral to ascending uterus, post-caecal, post-testicular; post-ovarian space comprising 1/6–1/4 of body length. Oviducal ampulla present. Laurer's canal absent. Oötype medial, posterior to genitalia, comprising an inconspicuous ovoid chamber. Uterus extending anterior to ovary. Common vitelline collecting duct extending from dextral branch of vitellarium. Common genital pore post-gonadal, anterior to level of oötype.

3.1.3 Taxonomic summary

Type-species: Aetohemecus kirstenjensenae n. sp.

Type host: Banded eagle ray, *Aetomylaeus nichofii* (Bloch and Schneider, 1801) Capapé and Desoutter, 1979 (Myliobatiformes: Myliobatidae).

Etymology: The Greek "*Aeto*" meaning eagle and "*heme*" meaning blood refers to the type species infecting the blood of an eagle ray.

3.2 Aetohemecus kirstenjensenae n. sp. (Figs. 1 − 2)

3.2.1 Diagnosis of adult specimens (based on four whole-mounted specimens; USNM coll. nos. 1642775−1642778).

Body 1000–1320 (1121 ± 152, 4) long, 235–245 (240 ± 4, 4) at greatest width, 4–6 × longer than wide (Fig. 1). Lateral tegumental spines 93–120 (106 ± 12, 3) per side of body or a total of 189–228 (210 ± 20, 3), ending 18–38 (27 ± 10, 4) or 1–3% (2 ± 0.09, 3) of body length from posterior end of body, base slightly bifurcate at posterior margin, tissue not associated with base on anterior-most lateral tegumental spines (Fig. 1), approximately equal in size throughout length of body; lateral tegumental spines in anterior region 7–8 (7.7 ± 0.5, 4) long, 1–2 (1.6 ± 0.5, 4) wide; mid-body 5–7 (6 ± 1, 4) long, 1–2 (1.2 ± 0.4, 4) wide, and posterior region 4–5 (4.5 ± 0.5, 4) long, 1 (1, 9) wide (Fig. 1); peduncles supporting lateral tegumental spines approximately equal in size; anterior peduncles 7–8 (7.7 ± 0.5, 4) long, 3–5 (4 ± 0.8, 4) wide; mid-body and posterior peduncles 5–7 (6 ± 0.5, 4) long, 5–6 (5.5 ± 0.5, 4) wide.

Ventrolateral nerve-cord 924–1120 (995 ± 109, 3) long, 10–15 (12 ± 2, 4) wide near mid-body at widest level, 50–58 (53 ± 4, 4) from body margin. Primary commissure perpendicular to mid-line of body, connecting ventrolateral nerve-cords, 95–115 (105 ± 8, 4) or 8%–10% (9% ± 1, 4) of body length from anterior end of body, 25–28 (26 ± 1.5, 4) across width of worm, 10–18 (12 ± 4, 4) in breadth; (Fig. 1); secondary commissure and nerve cords not evident in wholemounts.

Mouth 1–3 (2 ± 1.2, 3) in diameter, 1–8 (4 ± 4, 3) from terminal end of anterior sucker (Fig. 1). Oesophagus 325–425 (379 ± 43, 4) in total length or 30%–37% (34% ± 0.03, 4) of body length, 18–23 (21 ± 2, 4) in maximum width, ventral to primary nerve-commissure (Fig. 1); oesophageal wall thickening from 1–2 (1.5 ± 0.5, 4) near mouth to 5–10 (6.3 ± 2.5, 4) posteriorly. Caecal bifurcation 281−415 (355 ± 56, 4) or 21%−38% (32% ± 0.07, 4) of body length from anterior body end; anterior caeca 22−39 (31 ± 8, 4) in mean length or 2%–4% of body length, 21–27 (4) in mean width; posterior caeca 38–68 (57 ± 13, 4) in mean length or 4%–

6% of body length, 19–34 (4) in mean width (Fig. 1).

Testis 230–270 (250 ± 20, 3) long or 23%–25% (24% ± 0.01, 3) of body length, 53–85 (74 ± 15, 4) wide or 22%–35% (31% ± 0.06, 4) of body width, 3–5 (4 ± 0.9, 3) × longer than wide, post-caecal (Fig. 1). Post-testicular space 335–380 (362 ± 20, 4) long or 29%–36% (33% ± 0.03, 4) of body length. Vasa efferentia comprising interconnecting meshwork of fine ducts entwined throughout testicular tissue, 8 (1) in diameter; vas deferens 118–210 (152 ± 40, 4) long, 8–18 (13 ± 4, 4) wide, emanating from postero-ventral portion of testis, curving dextrad ventral to anterior portion of ovary before curving mediad ventral to ovary to connect with cirrus-sac (Fig. 2). Cirrus-sac 53–88 (64 ± 16, 4) long, having extremely thin wall approximately 1–2 (2 ± 0.5, 4) thick, including seminal vesicle and cirrus; seminal vesicle 45–75 (55 ± 14, 4) long, 13–15 (14 ± 1, 4) wide, filling breadth of cirrus sac, curving dextrad, narrowing and opening dorsal (Fig. 2); everted cirrus long, 61 long or 1.8 × seminal vesicle length, 4 wide (Fig. 1); internal cirrus 27 long or 37% of seminal vesicle length, 3 wide (Fig. 2). Common genital pore 178–298 (227 ± 50, 4) or 18%–23% (20% ± 2, 4) of body length from posterior end of body, 50–58 (55 ± 3, 4) from sinistral body margin, 113–130 (123 ± 7, 4) from dextral body margin (Figs. 1, 2).

Ovary medial, lobed, 90–118 (102 ± 14, 4) long or 7%–11% (9% ± 0.01, 4) of body length, 63–100 (77 ± 16, 4) wide or 26%–42% (32% ± 0.07, 4) of body width, 0.9–1.6 (1.4 ± 0.3, 4) × longer than wide, post-caecal, post-testicular; post-ovarian space 205–253 (234 ± 20, 4) long or 18%–24% (21% ± 0.02, 4) of body length (Figs. 1, 2). Oviduct and Laurer's canal not evident; oviducal ampulla 10–20 (15 ± 7, 2) long, 15 (2) wide (Fig. 2). Oötype 10–15 (13 ± 3, 3) in diameter, posterior to all genitalia (Figs. 1, 2). Vitellarium having follicles compacted in dense lobules, occupying space dorsal and lateral to oesophagus, caeca, and testis; common collecting duct 163–223 (196 ± 25, 4) long, 13–20 (17 ± 3, 4) wide.

Uterus extending anteriad from oötype, 185–223 (207 ± 18, 4) long or 17–20 (19 ± 0.01, 4) of body length, 58–93 (74 ± 16, 4) wide, with wall 1 (4) thick; ascending portion extending sinuously anteriad and dorsal to seminal vesicle, common vitelline duct, and ovary before extending anterior to ovary and connecting to descending portion, containing eggs in all (4) specimens (Figs. 1, 2); descending portion 80–105 (90 ± 11, 4) long or 36%–49% (44% ± 6, 4) of ascending uterus length, extending posteriad before connecting with metraterm; metraterm 30–58 (47 ± 14, 4) long or 11%–19% (16% ± 0.03, 4) of descending uterus, 8–15 (12 ± 3, 4) wide, comprising distal-most portion of female reproductive tract, demarcated from descending uterus by obvious constriction (Fig. 2). Uterine eggs 10–13 (12 ± 1, 4) in diameter or 40%–67% (54% ± 0.1, 4) of uterus width, containing a large spheroid body plus several smaller, dense lipid-like bodies, with thin shell (Fig. 2).

3.2.2 Taxonomic summary

Type and only reported host: Banded eagle ray, *Aetomylaeus nichofii* (Bloch and Schneider, 1801) Capapé and Desoutter, 1979 (Myliobatiformes: Myliobatidae).

Site in host: Heart lumen.

Type locality: Off Manggar, Makassar Strait, (01°12'55.20"S, 116°58'27.50"E), East Kalimantan, Borneo, Indonesia.

Prevalence and intensity of infection: 1 (prevalence = 100%) banded eagle ray sampled on 3 August 2008 was infected by 4 specimens of *A. kirstenjensenae*.

Specimens deposited: Holotype (USNM 1642775), paratypes (USNM 1642776–1642778)

Etymology: The specific epithet "*kirstenjensenae*" honors Prof. Kirsten Jensen (Senior Curator of Invertebrate Zoology for the Biodiversity Institute and Natural History Museum;

Associate Chair of Ecology and Evolutionary Biology at The University of Kansas, Lawrence, Kansas, USA) for her contributions to our knowledge of elasmobranch parasites.

3.2.3 Taxonomic remarks

Aetohemecus kirstenjensenae is most similar to *Selachohemecus benzi* Bullard, Overstreet, and Carlson, 2006, *Selachohemecus olsoni* Short, 1954, and *G. bulbosus* by the combination of having a single row of C-shaped lateral tegumental spines, posterior caeca terminating in the middle 1/3 of the body, and an ascending and descending uterus (Short, 1954; Bullard et al., 2006; Warren et al., 2019). Further, the new species is similar to *G. bulbosus* by having an oviducal ampulla and an oötype positioned posterior to the genitalia. *Aetohemecus kirstenjensenae* differs from *S. olsoni* and *S. benzi* by the combination of having an extruded cirrus that is as long as the seminal vesicle, an oviducal ampulla, a uterus that extends anterior to the ovary, and an oötype that is posterior to the genitalia. *Aetohemecus kirstenjensenae* differs from *G. bulbosus* by having a X- or H-shaped intestine, a uterus that extends anterior to the ovary, and an extruded cirrus that is as long as the seminal vesicle as well as by lacking an oesophogeal bulb (KEY) (Warren et al., 2019).

Aetohemecus kirstenjensenae further resembles several other fish blood flukes that infect chondrichthyans having large C-shaped spines (*Chimaerohemecus trondheimensis* Van der Land, 1967; *Hyperandrotrema cetorhini* Maillard and Ktari, 1978; *Hyperandrotrema walterboegeri* Orélis-Ribeiro and Bullard, 2013) (Table 1). The new species differs from *C. trondheimensis* and *Hyperandrotrema* spp. by the combination of having a single row of C-shaped lateral tegumental spines (vs. two rows or a field of spines), an X- or H-shaped intestine (vs. inverse U-shaped) that terminates in the middle 1/3 of the body (vs. the posterior end of the body), a post-caecal ovary

(vs. intercaecal), and post-caecal common genital pore (vs. intercaecal) (KEY) (Van der Land, 1967; Maillard and Ktari, 1978; Orélis-Ribeiro et al., 2013).

Further, *A. kirstenjensenae* differs from other fish blood flukes that infect chondrichthyans in that those species lack lateral tegumental spines (*Achorovermis testisinuosus* Warren and Bullard, 2020; *Electrovermis zappum* Warren and Bullard 2019; *Myliobaticola richardheardi* Bullard and Jensen, 2008; *Ogawaia glaucostegi* Cutmore, Cribb, and Yong, 2019; and *Orchispirium heterovitellatum* Madhavi and Rao, 1970) (Table 1). Until now, species of *Selachohemecus* were the only aporocotylids infecting chondrichthyans reported to have a X- or H-shaped intestine (KEY). Regarding the hosts for blood flukes, *A. kirstenjensenae* is the only nominal blood fluke reported from an eagle ray (Myliobatidae).

3.3 Homestios janinecairae n. gen. (Figs. 3 − 4)

3.3.1 Generic diagnosis

Body 9 × longer than wide, dorsoventrally flattened, muscular, aspinous. Rosethorn-shaped spines absent. Nervous system comprising paired lateral nerve cords. Anterior sucker aspinous, lacking peduncle, diminutive. Mouth subterminal. Pharynx absent. Oesophagus extending sinuously posteriad along midline for ≤ 1/4 of body length; posterior oesophageal swelling present. Intestine inverse U-shaped, asymmetrical; posterior caeca shorter than oesophagus, connecting to oesophagus ventrally, lacking diverticulae, terminating in anterior half of body. Testis single, medial, curving, lacking lobed margins. Vas deferens long, > 70% of seminal vesicle length, extending posteriad from testis. Cirrus-sac present, enveloping internal seminal vesicle and cirrus. Internal seminal vesicle distinct, longer than vas deferens. Extruded cirrus short, < 5% of seminal vesicle length. Auxiliary external seminal vesicle absent. Common genital pore dorsal, post-gonadal. Ovary medial, post-caecal, wholly anterior to uterus; post-

ovarian space comprising 1/3 of body length. Vitellarium follicular, diffuse, asymmetrical. Laurer's canal absent. Oötype indistinct. Uterus post-gonadal, not extensively convoluted, extending posteriad before curving anteriad extending anterior to posterior margin of ovary before crossing midline and extending posteriad; uterine eggs irregular. Uterine seminal receptacle absent. Excretory vesicle small, medial, with arms, visible in posterior most region of body.

3.3.2 Differential diagnosis

Body approx. 9 × longer than wide; aspinous, lacking lateral tubercles. Anterior sucker aspinous, lacking peduncle, diminutive. Pharynx absent. Medial and posterior oesophageal swelling present. Intestine inverse U-shaped, asymmetrical; posterior caeca terminating in anterior half of body, lacking diverticulae. Testis single, lacking lobed margins, curving < 40 times. Vas deferens long, > 70% of seminal vesicle length. Internal seminal vesicle distinct, longer than vas deferens, enveloped by cirrus sac. Extruded cirrus short, < 5% of seminal vesicle length. Common genital pore post-caecal, post-gonadal. Ovary medial, post-caecal, dorsal to posterior portion of testis, wholly anterior to uterus. Laurer's canal absent. Uterus post-gonadal, uterus that extends anteriad beyond the level of the seminal vesicle, not extensively convoluted.

3.3.3 Taxonomic summary

Type-species: Homestios janinecairae n. sp. (Digenea: Aporocotylidae).

Type host: Banded eagle ray, *Aetomylaeus nichofii* (Bloch and Schneider, 1801) Capapé and Desoutter, 1979 (Myliobatiformes: Myliobatidae).

Etymology: The Greek "*Homestios*" meaning 'dwelling with' refers to the same batoid species hosting the type species for both genera.

3.4 Homestios janinecairae n. sp. (Figs. 3 − 4)

3.4.1 Description of adult (based on a single whole-mounted specimen; USNM coll. no. 1642774).

Body 1305 long, 153 at greatest width, 9 × longer than wide (Fig. 3), muscular, tapering gradually until bluntly rounded. Nerve commissures not evident in whole-mount. Ventrolateral nerve-cords located 13 from lateral body margin, 5 in maximum width, becoming confluent posteriorly, 8 from posterior body margin. Anterior sucker, aspinous, centered on mouth. Mouth 3 in diameter, 3 from terminal end of body (Fig. 3). Oesophagus 408 in total length or 31% of body length, 18 in maximum width (Fig. 3). Caecal bifurcation 163 or 12% of body length from anterior body end; caeca extending posteriad in parallel, asymmetrical, dextral caecum 153 long or 11% of body length, sinistral caecum 178 long or 14% of body length, 22 in mean width or 14% of body width (Fig. 3); post-caecal space 765 long or 59% of body length (Fig. 3).

Testicular mass 363 long or 28% of body length, 103 wide, occupying 67% of body width, 4 × longer than wide, dorsal to caeca, curving 33 times (Fig. 3), narrowing, and becoming confluent with vas deferens. Post-testicular space 418 long or 32% of body length. Vas deferens 181 long or 71% of seminal vesicle length, 4 wide, emanating from postero-ventral portion of testis, meandering sinistral to ovary and posteriad between ascending and descending uterine portions before connecting to the cirrus sac (Figs. 3, 4). Cirrus-sac having thin wall 1 thick, including seminal vesicle and cirrus; seminal vesicle extending sinuously posteriad, 258 long or 20% of body length, 20 wide or 13% of body width, 13 × longer than wide, running between ascending and descending portions of the uterus, curving 6 times (Figs. 3, 4), narrowing and curving sinistrally towards body margin (Figs. 3, 4); cirrus short, 10 long or 3% of seminal vesicle length, 8 wide, cirrus pore 5 in diameter (Fig. 4). Common genital pore 120 or 9% of

body length from posterior end of the body, 18 from sinistral body margin, 75 from dextral body margin (Figs. 3, 4).

Ovary medial, small, irregular in shape, not extending lateral to nerve cords, 38 long or 2% of body length, 70 wide or 46% of body width, 2 × wider than long; post-ovarian space 380 long or 29% of body length (Figs. 3, 4). Oviduct, vitelline duct, and oötype indistinct. Laurer's canal not observed. Ascending uterus 318 long, 15 in maximum width (Fig. 4), extending anteriad arching dorsally over medial portion of vas deferens before connecting to descending portion; descending uterus extends posteriorly 238 long or 75% of ascending uterus length, 13 in maximum width; post-uterine space 75 long or 6% of body length (Fig. 3). Uterine eggs13 long, 6 wide or 46% of uterus width, containing many small dense bodies, with thin shell (Fig. 4). Excretory vesicle 8 long, < 1 wide, with arms (Fig. 3).

3.4.2 Taxonomic summary

Type and only reported host: Banded eagle ray, *Aetomylaeus nichofii* (Bloch and Schneider, 1801) Capapé and Desoutter, 1979 (Myliobatiformes: Myliobatidae).

Site in host: Heart lumen.

Type locality: Off Takisung, Java Sea, (03°52'28.00"S, 114°36'37.00"E), South Kalimantan, Borneo, Indonesia.

Prevalence and intensity of infection: 1 (prevalence = 100%) banded eagle ray sampled on 2 December 2006 was infected by 1 specimen of *H. janinecairae*

Specimens deposited: Holotype (USNM 1642774).

Etymology: The specific epithet "*janinecairae*" honors Prof. Janine N. Caira (University of Connecticut, Storrs, Connecticut USA) for her contributions to our knowledge of elasmobranch parasites.

3.2.3 Taxonomic remarks

3.4.3 Taxonomic remarks

Homestios janinecairae is most similar to *M. richardheardi* and all other nominal fish blood flukes infecting batoids (except *G. bulbosus* and *A. kirstenjensenae*) by the combination of having an aspinous, diminutive anterior sucker, an asymmetrical inverse U-shaped intestine, a curving testis, and a post-caecal common genital pore as well as by lacking any spine along the lateral tegument (Madhavi and Rao, 1970; Bullard and Jensen, 2008; Cutmore et al., 2018; Warren and Bullard, 2019; Warren et al., 2020). It differs from *M. richardheardi* by the combination of having >30 testicular curves (vs. 9–10), a vas deferens that is >70% of the seminal vesicle length, a seminal vesicle that curves 6 times (vs. 9–10), and a uterus that extends anteriad beyond the level of the seminal vesicle (KEY) (Bullard and Jensen, 2008). *Homestios janinecairae* differs from all other fish blood flukes infecting batoids (except *G. bulbosus* and *A. kirstenjensenae*) by having a body 9 × longer than wide, an oesophagus that is 1/3 of body length, >30 testicular curves, a vas deferens that is >70% of the seminal vesicle length, a sharply curved seminal vesicle that is 1/5 of body length, a cirrus that is <5% of seminal vesicle length, an ovary that is wider than long, and a uterus that extends anteriad beyond the level of the seminal vesicle (KEY). The only other species to have a uterus that extends anteriad beyond the level of the seminal vesicle is *O. heterovitellatum* and *O. glaucostegi* (Madhavi and Rao, 1970; Cutmore et al., 2018). *Orchispirium heterovitellatum* is unique by having lateral tubercles along the tegument, posterior caeca that extend into the posterior half of the body, and a testis that is intercecal and bearing lobes along the margin (Madhavi and Rao, 1970). The new species differs from *O. glaucostegi* by having a uterus that extends anteriad beyond the terminal margin of the testis (vs. remaining posterior to the ovary) before folding ventrally and continuing posterior to

the common genital pore. Further, *O. glaucostegi*, *E. zappum*, and *A. testisinuosus* differ by having a body that is > 15 × longer than wide (vs. 9 in the new species and *M. richardheardi*) (KEY) (Cutmore et al., 2019; Warren and Bullard, 2019; Warren et al., 2020).

4. Discussion

4.1 Host-switching

Host-switching is emerging as a key phenomenon to understanding the natural history of fish blood flukes. *Aetomylaeus nichofii* is infected by blood flukes of two species in two genera; one (*A. kirstenjensenae*) could be the result of a host-switching event. Schistosomes have been documented to switch intermediate hosts (Lockyer et al., 2002) but fish and turtle blood fluke host-switching are rarely discussed (Bullard et al., 2019; Warren et al., 2019). The 13 nominal species of chondrichthyan blood flukes can be split into two morphological groups: (i) those having C-shaped spines (*H. cetorhini, H. walterboegeri, C. trondheimensis, G. bulbosus, A. kirstenjensenae, S. benzi,* and *S. olsoni*) and (ii) aspinous species (*O. heterovitellatum, O. glaucostegi, A. testisinuosus, H. janinecairae, M. richardheardi,* and *E. zappum*) (Fig. 5). Not including the new taxa described herein, these two groups were recovered as monophyletic in a recent phylogenetic analysis (Warren and Bullard, 2019). We expected that batoids would host a fish blood fluke resembling *H. janinecairae* (aspinous, inverse U-shaped intestine, curving testis) but *A. kirstenjensenae* (with C-shaped spines) was unexpected (because it has a C-shaped tegumental spines, a X-shaped intestine, a single testis without curves, and an oötype posterior to the genitalia) and likely does not share a common ancestor with other batoid blood flukes, similar to *G. bulbosus* (Fig. 5).

4.2 The intestinal morphology of chondrichthyan blood flukes

The intestinal morphology of fish blood flukes could inform ancestry. *Aetohemecus kirstenjensenae* is the only batoid blood fluke that has a X-shaped intestine, similar to *S. olsoni* and *S. benzi*. (Fig. 1; 5). Because of this, the new species likely shares a recent common ancestor with *Selachohemecus* spp. (Bullard et al., 2006). However, *A. kirstenjensenae* also has an oötype that is posterior to all genitalia, like that of *G. bulbosus*, which has an inverse U-shaped intestine (Warren et al., 2019). The most recent phylogenetic analysis available places *G. bulbosus* sister to *C. trondheimensis*, which are two clearly, morphologically different lineages of fish blood flukes. We predict that *A. kirstenjensenae* will clade with *Selachohemecus* spp. and that they share a recent common ancestor with *G. bulbosus* (Fig. 5). This prediction is based on the single row of C-shaped spines, which is a shared trait by all species of these 3 genera (Short, 1954; Bullard et al, 2006; Warren et al., 2019).

Other than *Selachohemecus* spp. and *A. kirstenjensenae*, fish blood flukes infecting chondrichthyans have inverse U-shaped intestines similar to that of several fish blood flukes that infect bony fishes (*Acipensericola glacialis* Warren, Roberts, Arias, Koenigs, and Bullard, 2017; *Acipensericola petersoni* Bullard, Snyder, Jensen, and Overstreet, 2008; *Paracardicoloides yamaguti* Martin, 1974) and turtle blood flukes (*Spirorchis* spp.) (Martin, 1974, Bullard et al., 2008, Warren et al., 2017, Bullard et al., 2019). Orélis-Ribeiro et al. (2017) recovered *P. yamaguti* sister to species of *Elopicola* Bullard, 2014 forming a clade that shares a recent common ancestor with *A. petersoni* using the second internal transcribed spacer of ribosomal DNA (*ITS-2*) region (Orélis-Ribeiro et al., 2017). This is significant because in *28S rDNA* phylogenies species of *Elopicola* are recovered as sister to all other fish blood flukes that infect actinopterygians, with exception to sequences sourced from cercariae shed from freshwater snails (Cribb et al, 2017; Warren et al., 2019). Further, no other fish blood fluke that infects a

bony fish (except *Acipensericola* spp. and *P. yamagutii*) has an inverse U-shaped intestine (Martin, 1974, Bullard et al., 2008; Warren et al., 2017). Because of this, we predict that the inverse U-shaped (two posterior caeca) intestine is pleisiomorphic but has evolved independently in several lineages: *Plehniella* spp. Szidat, 1951; *Nomasanguinicola canthoensis* Truong and Bullard, 2013, *Cardicola* spp. Short, 1953 (Short, 1953; Truong and Bullard, 2013, Orélis-Ribeiro and Bullard, 2015).

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FIGURE LEGENDS

Figures 1–2 *Aetohemecus kirstenjensenae* Warren and Bullard n. gen., n. sp. (Digenea: Aporocotylidae) from the heart of the banded eagle ray, *Aetomylaeus nichofii* (Bloch and Schneider, 1801) Capapé and Desoutter, 1979 (Myliobatiformes: Myliobatidae). (**1**) Body of holotype (USNM No. 1642775), dorsal view. Bar = 250 µm. (**2**) Genitalia, paratype (USNM No. 1642776), ventral view. Bar = 100 µm. Mouth (mo), nerve commissure (nc), oesophagus (os), vitellarium (vit), intestine (i), testis (t), uterus (u), metraterm (met), ovary (o), vas deferens (v), seminal vesicle (sv), cirrus sac (cs), cirrus (c), vitelline duct (vd), common genital pore (cgp), oviducal ampullae (oa), and oötype (oo).

Figures 3–4 *Homestios janinecairae* Warren and Bullard n. gen., n. sp. (Digenea: Aporocotylidae) from the heart of the banded eagle ray, *Aetomylaeus nichofii* (Bloch and Schneider, 1801) Capapé and Desoutter, 1979 (Myliobatiformes: Myliobatidae). (**3**) Body of holotype (USNM No. 1642774), dorsal view. Bar = 250 µm. (**4**) Genitalia, paratype (USNM No. 1642774), ventral view. Bar = 100 µm. Mouth (mo), oesophagus (os), vitellarium (vit), intestine (in), testis (t), ovary (ov), vas deferens (vd), uterus, (u), ascending uterus (au), descending uterus (du), seminal vesicle (sv), cirrus (c), and common genital pore (cgp).

Figure 5 Phylogenetic relationships of chondrichthyan blood flukes based on morphological characters (tegumental spines, shape of intestines). Host affiliations are included. Dashed lines indicate species with no nucleotide sequences. Boxes indicate spine rows: blue = 2 + spine rows, green = 1 spine row, and red = no spines. Shape of the intestine (⋂) inverse U-shaped and (X) X-shaped.

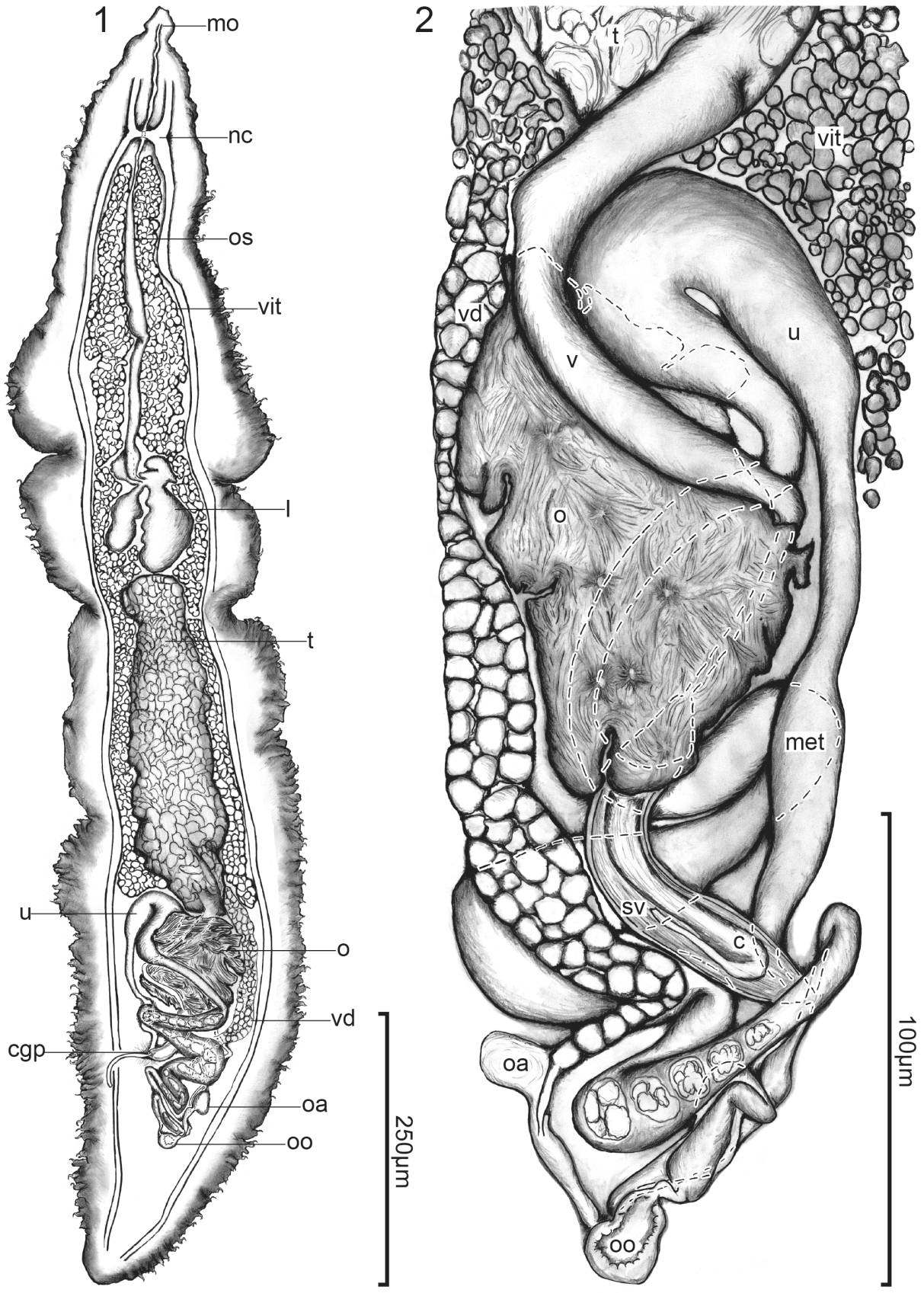
Table 1. The blood flukes (Digenea: Aporocotylidae) infecting cartilaginous fishes (Chondrichthyes).

Parasite	Host	Site of infection	Locality	Reference
Aetohemecus kirstenjensenae n. sp., n. gen.	banded eagle ray, *Aetomylaeus nichofii* (Bloch and Schneider, 1801) Capapé and Desoutter, 1979	heart	Makassar Strait, (01°12'55.20"S, 116°58'27.50"E), off Manggar, East Kalimantan, Borneo, Indonesia	present study
Achorovermis testisinuosus Warren and Bullard, 2020	smalltooth sawfish, *Pristis pectinata* Latham, 1796	heart	Eastern Gulf of Mexico, off Naples, Florida, USA	Warren et al., 2020
Chimaerohemecus trondheimensis Van der Land, 1967	rabbit fish, *Chimaera monstrosa* Linnaeus, 1758	dorsal aorta	NE Atlantic, off Bergen, Norway	Van der Land, 1967; Lockyer et al., 2003b
	spook fish, *Hydrolagus mitsukurii* (Jordan and Snyder, 1904) Nakaya, 1984	dorsal aorta and postcardinal vein around kidney	Saruga Bay, Japan	Kamegai et al., 2002
Electrovermis zappum Warren and Bullard, 2019	lesser electric ray, *Narcine bancroftii* (Griffith and Smith, 1834) Carvalho, 2001	heart	Gulf of Mexico, off Fort Morgan, Alabama, USA	Warren and Bullard, 2019
Gymnurahemecus bulbosus Warren, Ruiz, Whelan, Kritsky, and Bullard, 2019	smooth butterfly ray, *Gymnura micrura* (Bloch and Schneider, 1801) Uyeno, 1983	heart	Gulf of Mexico, off Mobile, Alabama, USA	Warren et al., 2019
Homestios janinecairae n. sp., n. gen.	banded eagle ray, *Aetomylaeus nichofii* (Bloch and Schneider, 1801) Capapé and Desoutter, 1979	heart	Java Sea, (03°52'28.00"S, 114°36'37.00"E), off Takisung, South Kalimantan, Borneo, Indonesia	present study
Hyperandrotrema cetorhini Maillard and Ktari, 1978	basking shark, *Cetorhinus maximus* (Gunnerus, 1765) Springer, 1973	circulatory system; heart	Mediterranean Sea, off Tunisia; Oslofjorden, Norway; North Sea, off	Malliard and Ktari, 1978; Smith, 1972

			Montrose, Scotland	
Hyperandrotrema walterboegeri Orélis-Ribeiro and Bullard, 2013	shortfin mako shark, *Isurus oxyrinchus* Rafinesque, 1810	luminal surface (endocardium) of heart atrium and ventricle	Viosca Knoll, northern Gulf of Mexico, 123 km south/southwest of Dauphin Island, Alabama, USA	Orélis-Ribeiro et al., 2013
Myliobaticola richardheardi Bullard and Jensen, 2008	Atlantic stingray, *Hypanus sabinus* (Lesueur, 1824) Last, Manjaji-Matsumoto, Naylor, and White, 2016	intertrabecular spaces of heart	Deer Island, Mississippi Sound, Northern Gulf of Mexico off Biloxi, Mississippi, USA	Bullard and Jensen, 2008
Ogawaia glaucostegi Cutmore, Cribb, and Yong, 2018	giant shovelnose ray, *Glaucostegus typus* (Anonymous [Bennett], 1830) Compagno, Last, Stevens, and Alava, 2005	valves of conus arteriosus; ventricle	Moreton Bay, Queensland, Australia	Cutmore et al., 2018
Orchispirium heterovitellatum Madhavi and Rao, 1970	Bengal whipray, *Brevitrygon imbricata* (Bloch and Schneider, 1801) Last, Manjaji-Matsumoto, Naylor, and White, 2016	mesenteric blood vessels	Western Bay of Bengal, waters off Waltair, India	Madhavi and Rao, 1970
Selachohemecus benzi Bullard, Overstreet, and Carlson, 2006	blacktip shark, *Carcharhinus limbatus* (Valenciennes, 1839) Compagno, 1973	heart	Apalachicola Bay, Florida, USA; Northern Gulf of Mexico, off Mississippi, USA	Bullard et al., 2006
Selachohemecus olsoni Short, 1954	Atlantic sharpnose shark, *Rhizoprionodon terraenovae* (Richardson, 1837) Springer, 1964	heart	Alligator Harbor, Florida, USA; Apalachicola Bay, Florida, USA; Mississippi Sound, Mississippi, USA	Short, 1954; Bullard et al., 2006

KEY. Key to the identification of fish blood flukes (Digenea: Aporocotylidae) infecting chondrichthyans.

1a. Body spinous, lateral tegumental spines (LTSs) C-shaped	2
1b. Body aspinous	8
2a. Intestinal caeca X-or H-shaped	3
2b. Intestinal caeca inverse U-shaped	5
3a. Oviducal ampullae present; oötype posterior to all genitalia	*Aetohemecus kirstenjensenae* n. gen, n. sp.
3b. Oviducal ampullae absent; oötype anterior to common genital pore	4
4a. Body minute (< 1.4 mm long), LTSs numbering > 170 per side of body	*Selachohemecus olsoni*
4b. Body large (≥ 1.4 mm long), LTSs < 100 per side of body	*Selachohemecus benzi*
5a. LTSs distributed in a single column, large oesophogeal bulb present	*Gymnurahemecus bulbosus*
5b. LTSs distributed in multiple columns or lateral field	6
6a. Caeca short, terminating at level of genital pores	*Chimaerohemecus trondheimensis*
6b. Caeca elongate, terminating posterior to genitalia	7
7a. Body 2 × longer than wide, mid-body LTSs < 20 μm long	*Hyperandrotrema cetorhini*
7b. Body 7–8 × longer than wide, mid-body LTSs ≥ 25 μm long	*Hyperandrotrema walterboegeri*
8a. Body margin having lateral tubercles	*Orchispirium heterovitellatum*
8b. Body margin lacking lateral tubercles	9
9a. Body minute (< 1 mm long), testis curving < 15 times	*Myliobaticola richardheardi*
9b. Body elongated, testis curving > 15 times	11
11a. Testis curving < 50 times	12
11b. Testis curving > 50 times	13
12a. Seminal vesicle > 40% of body width; uterus not extending anterior to seminal vesicle	*Electrovermis zappum*
12b. Seminal vesicle < 20% of body width; uterus extending anterior to seminal vesicle	*Homestios janinecairae*
13a. Testis curving < 80 times; uterus extending anterior to posterior margin of testis	*Ogawaia glaucostegi*
13b. Testis curving > 100 times; uterus wholly posterior to ovary	*Achorovermis testisinuosus*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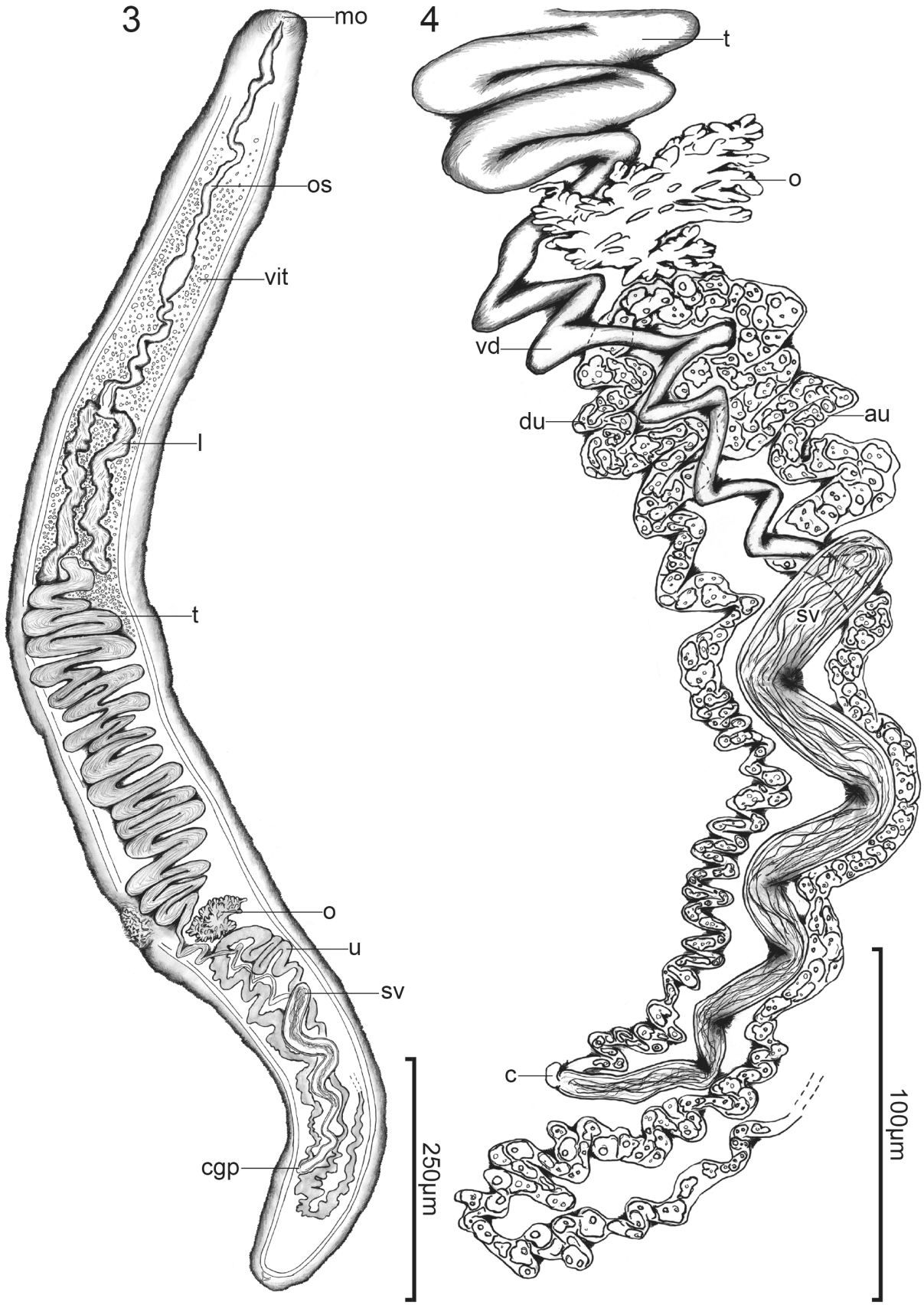
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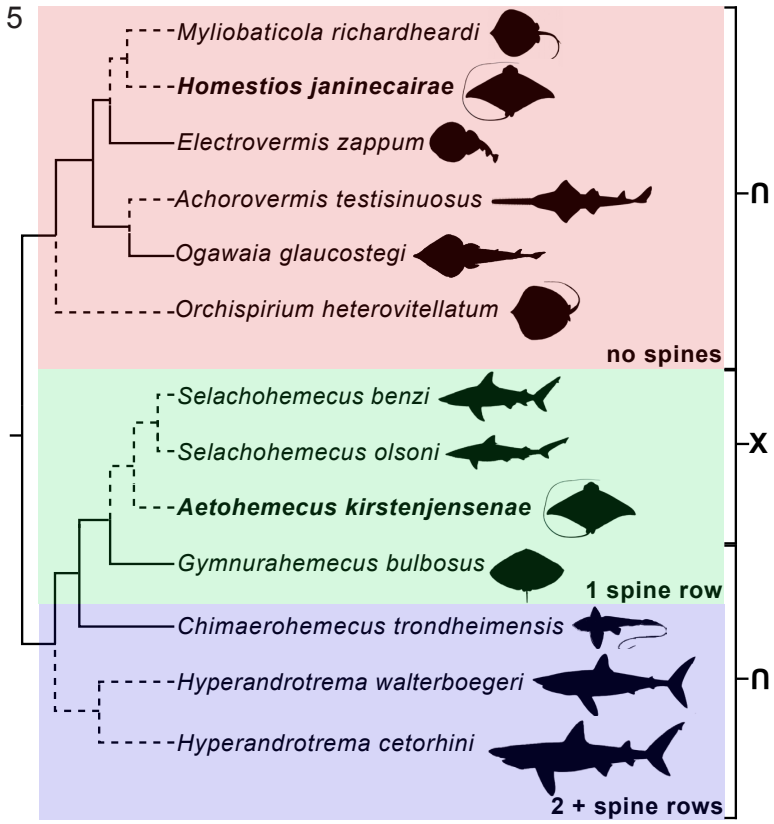
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Myliobaticola richardheardi
Homestios janinecairae
Electrovermis zappum
Achorovermis testisinuosus
Ogawaia glaucostegi
Orchispirium heterovitellatum
no spines
Selachohemecus benzi
Selachohemecus olsoni
Aetohemecus kirstenjensenae
Gymnurahemecus bulbosus
1 spine row
Chimaerohemecus trondheimensis
Hyperandrotrema walterboegeri
Hyperandrotrema cetorhini
2 + spine rows
n
x
n

CHAPTER 3: REVISION OF *SANGUINICOLA* PLEHN, 1905 WITH REDESCRIPTION OF *SANGUINICOLA VOLGENSIS* (RAŠÍN, 1929) MCINTOSH, 1934, DESCRIPTION OF A NEW SPECIES, PROPOSAL OF A NEW GENUS, AND PHYLOGENETIC ANALYSIS

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ABSTRACT

Sanguinicola Plehn, 1905 comprises 26 species that collectively infect fishes from 8 orders (Cypriniformes, Characiformes, Siluriformes, Esociformes, Salmoniformes, Labriformes, Centrarchiformes, and Perciformes). Its revision is warranted because several species assigned to the genus could represent new genera, nucleotide sequences are wanting, many species have incomplete descriptions, and types for most species are missing or of poor quality. Herein, we emend *Sanguinicola* based on morphology and the first nucleotide-based phylogenetic analysis that includes multiple sequences from morphologically identified adult specimens. We describe *Sanguinicola plehnae* Warren and Bullard n. sp. from the heart of northern pike, *Esox lucius* Linnaeus, 1758 from Russia; provide supplemental observations of *Sanguinicola volgensis* (Rašín, 1929) McIntosh, 1934 from the heart of sabrefish (type species), *Pelecus cultratus* (Linnaeus, 1758) Berg, 1949 from Russia; describe *Sanguinicola* cf. *volgensis* from the heart of ide, *Leuciscus idus* (Linnaeus, 1758) Berg, 1949 from Russia; and describe *Pseudosanguinicola occidentalis* (Van Cleave and Mueller, 1932) Warren and Bullard n. gen., n. comb. from the heart of walleye, *Sander vitreus* (Mitchill, 1818) Bailey, Latta, and Smith, 2004 from eastern North America. *Sanguinicola plehnae* differs from its congeners by having lateral tegumental spines that total 118–122, are small (3% of body width), and protrude 2–3 μm from the tegument

(lacking associated conical protrusion) as well as by having a large testis (>40% of body length). *Sanguinicola volgensis* differs from its congeners by having posteriorly-directed lateral tegumental spines encased in a tegumental conical protrusion as well as by having an ovoid egg. Specimens of *S.* cf. *volgensis* differ from those of *S. volgensis* by having a body that is 5–6 × longer than wide (vs. 2–3 × in *S. volgensis*) and <90 lateral tegumental spines (vs. >95). *Pseudosanguinicola* Warren and Bullard n. gen. differs from *Sanguinicola* by having densely transverse rows of lateral tegumental spines (vs. a single column of large spines). The phylogenetic analysis utilizing the large subunit ribosomal DNA (*28S*) failed to reject monophyly of *Sanguinicola.*

KEY WORDS

Taxonomy, Systematics, Revision, Fish blood fluke, Type species, Phylogenetics, Morphology, Large subunit ribosomal DNA (*28S*)

Sanguinicola Plehn, 1905 is a neglected genus of blood fluke that got off to a bad start (Bullard et al., 2009 and Warren and Bullard, *in press*). Plehn (1905) assigned *Sanguinicola armata* Plehn, 1905 (type species) and *Sanguinicola inermis* Plehn, 1905 (see Plehn, 1905) to the Turbellaria and then to a group of monozoic cestodes (Plehn, 1908; Bullard et al., 2009). These species were redescribed by Ejsmont (1926) with a level of thoroughness unmatched by some current blood fluke descriptions. No researcher has conducted anatomical work as detailed as that of Ejsmont (1926) on a species of *Sanguinicola* and addressed the obvious systematic issues with the genus. Plehn (1905) assigned the markedly morphologically distinct species, *S. armata* and *S. inermis,* to the same genus. Ejsmont (1926) accepted this assignment in his redescription of *S. armata* and *S. inermis*. Until 2008, all freshwater fish blood flukes were assigned to *Sanguinicola*, except for *Plehniella coelomicola* Szidat, 1951 and *Paracardicoloides yamagutii*

Martin, 1974 (see Szidat, 1951; Martin, 1974). Disregarding the obvious, stark morphological and ecological differences between the genera, Yamaguti (1958) synonymized *Plehniella* Szidat, 1951 with *Sanguinicola* (see Orélis-Ribeiro and Bullard, 2015). Subsequently, Madhavi and Hanumantha Rao (1970) and Smith (1972, 1997, 2002) accepted this synonymy. Since 2007, 5 freshwater fish blood fluke species assigned to 4 genera and from 3 continents (Asia, North America, South America) have been described (*Acipensericola petersoni* Bullard, Snyder, Jensen, and Overstreet, 2008; *Nomasanguinicola canthoensis* Truong and Bullard, 2013; *Cladocaecum tomasscholzi* Orélis-Ribeiro and Bullard, 2016; *Kritsky platyrhynchi* Orélis-Ribeiro and Bullard, 2016; *Acipensericola glacialis* Warren and Bullard, 2017). Additionally, 2 new species of *Plehniella* were described and the genus was resurrected and revised (*Plehniella sabajperezi* Orélis-Ribeiro and Bullard, 2015; *Plehniella armbrusteri* Orélis-Ribeiro and Bullard, 2016). *Sanguinicola* remains in need of revision. It comprises 26 nominal species that collectively infect fishes assigned to 8 orders (Cypriniformes, Characiformes, Siluriformes, Esociformes, Salmoniformes, Labriformes, Centrarchiformes, Perciformes) (Smith, 1997a, 1997b) (Tables I, II). It is the most speciose of the 7 freshwater fish blood fluke genera (*Sanguinicola*; *Plehniella*; *Paracardicoloides* Martin, 1974; *Acipensericola* Bullard, Snyder, Jensen, and Overstreet, 2008; *Nomasanguinicola* Truong and Bullard, 2013; *Cladocaecum* Orélis-Ribeiro and Bullard, 2016; *Kritsky* Orélis-Ribeiro and Bullard, 2016).

Herein, using newly-collected material from North America and Russia, we emend *Sanguinicola*; describe a new species infecting northern pike, *Esox lucius* Linnaeus, 1758; redescribe *Sanguinicola volgensis* (Rašín, 1929) McIntosh, 1934 (see Rašín, 1929) infecting the type-host, sabrefish, *Pelecus cultratus* (Linnaeus, 1758) Berg, 1949; describe an innominate species of *Sanguinicola* infecting ide, *Leuciscus idus* (Linnaeus, 1758) Berg, 1949; and propose a

new genus infecting walleye, *Sander vitreus* (Mitchill, 1818) Bailey, Latta, and Smith, 2004 in North America. We also use the large subunit ribosomal DNA (*28S*) to explore their relationships with the other blood fluke lineages.

MATERIALS AND METHODS

Fish blood flukes were collected during 2020–2021 from the heart of walleye (*S. vitreus*; Oneida Lake, New York and Fox River, Wisconsin), sabrefish (*P. cultratus*; Upper Volga River, Russia), ide (*L. idus*; Upper Volga River, Russia), and northern pike (*E. lucius*; Upper Volga River, Russia). The heart was excised intact, placed in a sample bag (heart bisected), exposed to 70 C freshwater, shaken vigorously, and fixed in 5–10% neutral buffered formalin (n.b.f.). In the laboratory, each heart was examined with the aid of a dissecting microscope and fiber optic light source to isolate flukes. The heart was teased apart with forceps to reveal adult blood flukes and sediment from the fixed heart was taken from a settling column and examined. Adult specimens collected for DNA extraction were wet mounted on glass slides and examined to confirm their identity, preserved in 95% ethanol (EtOH), and stored at −20 C. Adult flukes (n = 15) fixed in formalin and intended for morphology were rinsed with distilled water, cleaned with fine brushes to remove any debris, stained overnight in Van Cleave's hematoxylin with 3 additional drops of Ehrlich's hematoxylin, dehydrated using an ethanol series, cleared in clove oil, permanently mounted in Canada balsam, illustrated using an Olympus BX51 microscope (Olympus Corporation of the Americas, Center Valley, Pennsylvania) equipped with differential interference contrast (DIC), measured using an ocular micrometer, and illustrated using a drawing tube. Methods for electron microscopy are from Poddubnaya et al. (2023). Measurements are reported in micrometers (μm) as the range followed by the mean, standard deviation, and sample size in parentheses unless otherwise indicated. Scientific names, including

taxonomic authorities and dates, for fishes follow Eschmeyer et al. (2016; online version updated 2022). Classification and anatomical terms for fish blood flukes follow Bullard (2010), Bullard et al. (2012), and Warren et al. (2019, 2021). Types and vouchers were deposited in the National Museum of Natural History's Invertebrate Zoology Collection (USNM, Smithsonian Institution, Washington, D. C.).

A total of 8 EtOH-preserved and microscopically-identified fish blood flukes were used for DNA extraction and sequencing: 2 specimens from walleye, *S. vitreus* (Perciformes: Percidae), 2 from sabrefish, *P. cultratus* (Cypriniformes: Leuciscidae), 2 from ide, *L. idus* (Cypriniformes: Leuciscidae), and 2 from northern pike, *E. lucius* (Esociformes: Esocidae). Total genomic DNA (gDNA) was extracted using DNeasyTM Blood and Tissue Kit (Qiagen, Valencia, California, USA) as per the manufacturer's protocol except that the proteinase-K incubation period was extended overnight, and the final elution step used 100 microliter (µl) of elution buffer to increase the final DNA concentration. The *28S* was amplified using primers outlined in Warren et al. (2021). PCR amplifications were performed with the cycling profile identified by Warren et al. (2017b) except that the annealing temperature was set at 56 C for 30 sec. PCR reactions were carried out in a MJ Research PTC-200 (BioRad, Hercules, California). PCR products (12 µl) were verified on a 1% agarose gel and stained with ethidium bromide. PCR products were purified by microcentrifugation with the QIAquick PCR Purification Kit (Qiagen, Valencia, California) according to the manufacturer's protocols except that the last elution step was performed with autoclaved nanopure H_2O rather than with the provided buffer. DNA sequencing was performed by Genewiz, Incorporated (South Plainfield, New Jersey). Sequence assembly and analysis of chromatograms were performed with Geneious version 2022.0.2 (http://www.geneious.com). Nucleotide sequence data were deposited in GenBank (Table III).

The phylogenetic analyses included the new freshwater fish blood fluke sequences and selected sequences representing species of fish blood flukes that were available on GenBank (Table III). The out-group is represented by the turtle blood flukes *Baracktrema obamai* Roberts, Platt, and Bullard, 2016, *Spirorchis artericola* (Ward, 1921) Stunkard, 1921, and *Vasotrema robustum* for the analysis (Table III). The turtle blood fluke sequences have been recovered repeatably as a sister taxon to the fish blood flukes (Olson et al., 2003; Orélis-Ribeiro et al., 2014). Sequences were aligned with the multiple alignment tool using fast Fourier transform (MAFFT) (Katoh and Standley, 2013) and trimmed to the length of the shortest sequence presented herein (1,362 [*28S*] base pairs [bp]). JModelTest 2 version 2.1.10 (Darriba et al., 2012) was implemented to perform a statistical selection of the best-fit models of nucleotide substitution based on Bayesian Information Criterion (BIC). Aligned sequences were reformatted (from .fasta to .nexus) using the web application ALTER (Glez-Peña et al., 2010) to run Bayesian inference (BI). BI was performed in MrBayes version 3.2.5 (Ronquist and Huelsenbeck, 2003) using substitution model averaging ("nst-mixed") and a gamma distribution to model rate-heterogeneity. Defaults were used in all other parameters. Three independent runs with 4 Metropolis-coupled chains were run for 5,000,000 generations, sampling the posterior distribution every 1,000 generations. Convergence was checked using Tracer v1.6.1 (Rambaut et al., 2014) and the "sump" command in MrBayes: all runs appeared to reach convergence after discarding the first 25% of generations as burn-in. A majority rule consensus tree of the post burn-in posterior distribution was generated with the "sumt" command in MrBayes. The inferred phylogenetic tree was visualized using FigTree v1.4.4 (Rambaut et al., 2014) and further edited for visualization purposes with Adobe Illustrator (Adobe Systems).

DESCRIPTIONS

Sanguinicola Plehn, 1905, emended

(Figs. 1–17)

Diagnosis: Body of adult <9 × longer than wide, dorsoventrally flattened, lacking posterolateral protuberance, tapering equally anteriorly and posteriorly, spined; tegumental spines straight and not distally recurved, deeply rooted in tegument and only slightly protruding from it, arranging in a single column along lateral body margin, orienting laterally or slightly postero-laterally. Rosethorn-shaped spines absent. Ventrolateral nerve cords and dorsolateral nerve cords present; ventrolateral nerve cord extending nearly entire body length, appearing slightly sub-terminal, with commissure anteriorly. Anterior body extremity proboscis-like, accommodating mouth; mouth medioventral, subterminal, with associated tooth-like mouth apparatus (Figs. 4I, 12B). Pharynx present. Esophagus medial, straight, not looping, extending posteriad less than one-fourth to one-third body length, with anterior and posterior esophageal swellings, with esophageal sunken glandular cells enveloping middle and posterior portion of esophagus, connecting with ceca, anteromedially. Intestine thin-walled, medial, comprising 4–5 radial ceca that collectively appear generally as an X-shaped structure; each cecum can be dendritic. Testis single, having an array of lateral lobes, as wide or slightly wider than breadth of intestine, appearing as multiple testes distributing in 2 tandem rows flanking midline from level of ceca to posterior third or posterior quarter of body; middle testicular lumen extending along entire length of testis; sperm duct (vas deferens) single, arising from posterior middle regions of testis; spermatozoa having 9 + 0 axoneme pattern. Cirrus-sac surrounding seminal vesicle, inconspicuous in some wholemounts, prostatic glands present. Male genital pore dextral, slightly lateral to midline. Ovary single, medial, with superficially lobed margins, with lateral portions

extending anteriad and lateral to posterior portion of testicular field, appearing butterfly wing-shaped to varying degrees, as wide or wider than testicular field, occupying posterior one-third of body. Vitelline cells widely distributed throughout space from approximant level of anterior nerve commissure to seminal vesicle and mixed with other cell types comprising diffuse follicles. Oviduct a narrow duct extending directly posteriad from posteromedial surface of ovary, connecting with distal portion of vitelline reservoir to or near level of male genital pore; oviducal seminal receptacle present or absent. Ovo-vitelline duct connecting with oötype posteriorly or extending laterad and connecting to anterior aspect of oötype. Oötype compact, oblong, posterior to level of male genital pore, posterior to gonads and genital ducts. Laurer's canal absent. Uterus short, not coiled, post-ovarian; uterine seminal receptacle lacking; uterine eggs triangular or ovoid; triangular eggs bearing a midbody stub-like extension. Female genital pore anteromedial to male genital pore. Excretory vesicle appearing V-shaped or Y-shaped or diminutive; excretory pore terminal. Undergoing asexual reproduction in hydrobiid, semisulcospirid, planorbid, pleurocerid, lymnaeid, and valvatid snails (Warrren and Bullard, *in press*); maturing in blood vascular system of Cypriniformes and Esociformes (Table I).

Differential diagnosis: Body <9 × longer than wide; lateral tegumental spines straight (not recurved), deeply rooted in tegument or slightly protruding from it, arranging in a single column along lateral body margin, orienting laterally or slightly posterolaterally. Intestine thin-walled, medial, comprising 4–5 radial ceca that collectively appear generally as an X-shaped structure; each cecum can be dendritic, terminating in anterior half of the body. Testis single, having an array of lateral lobes, as wide or slightly wider than breadth of intestine, appearing as if multiple testes distributing in 2 tandem rows flanking midline from level of ceca to posterior third or posterior quarter of body. Male genital pore lateral to female genital pore, ending along midline.

Ovary butterfly wing-shaped, post-cecal, post-testicular. Oötype posterior to genitalia. Uterus short, not coiled. Eggs triangular or ovoid.

Type species: Sanguinicola armata Plehn, 1905 (ex. heart lumen of tench, *Tinca tinca* [Linnaeus, 1758] Berg, 1949 [Cypriniformes: Tinicidae] [type host] from Freising, Germany [type locality]).

Synonyms: Janickia Rašín, 1929.

Other accepted species: Sanguinicola intermedia Ejsmont, 1926, *Sanguinicola volgensis* (Rašín, 1929) McIntosh, 1934*, Sanguinicola lophophora* Erickson and Wallace, 1959, *Sanguinicola skjabini* Akhmerov, 1960, *Sanguinicola magnus* Hu, Long, and Lee, 1965, *Sanguinicola lungensis* Tang and Lin, 1975, *Sanguinicola rhodei* Wang, 1983, *Sanguinicola rutili* Simón-Martín, Rojo-Vásquez, and Simón-Vicente, 1988, *Sanguinicola hasegawai* Shimazu, 2013 (Table I).

Species incertae sedis: Sanguinicola inermis Plehn, 1905, *Sanguinicola chalmersi* Odhner, 1924, *Sanguinicola huronis* Fischthal, 1949, *Sanguinicola argentinensis* Szidat, 1951, *Sanguinicola davisi* Wales, 1958, *Sanguinicola klamathensis* Wales, 1958, *Sanguinicola alseae* (Meade and Pratt, 1965) Holmes, 1971, *Sanguinicola idahoensis* Schell, 1974, *Sanguinicola sanliense* Wang, 1982, *Sanguinicola clarias* Imam, Marzouk, Hassan, and Itman, 1984, *Sanguinicola fontinalis* Hoffman, Fried, and Harvey, 1985, *Sanguinicola maritimus* Nolan and Cribb, 2005, *Sanguinicola ugui* Shimazu, 2007 (Table II).

Nomina nuda: Sanguinicola incognita Akhmerov, 1959, *Sanguinicola shantsuensis* Lung and Chen, 1965, *Sanguinicola megalobramae* Li, 1980 (Table II).

Remarks

The revised diagnosis herein includes several additional taxonomic characters and further detail of other features used to differentiate *Sanguinicola sensu stricto* (Table I) from other fish blood fluke genera. The diagnosis has been emended several times (Ejsmont, 1926; Yamaguti, 1958; Smith, 2002) but several useful characters and details have been left out or ignored. In specific, lateral tegumental spines are present in *Sanguinicola* spp.; no accepted species of *Sanguinicola* lacks spines. *Sanguinicola* has a single column of spines and lacks transverse rows of spines; with each spine being straight and lacking a recurved tip. We detail the position and shape of the nerve cords, mouth, esophagus, intestinal ceca, testis, vasa deferens, vitelline distribution, ovary, oviducal seminal receptacle, oötype, genital pores, excretory vesicle, and uterus.

Sanguinicola sensu stricto (Table I), as revised herein, is most similar to monotypic *Nomasanguinicola*, which infects a bighead catfish, *Clarias macrocephalus* Günther, 1864 (Siluriformes: Clariidae) in the Mekong River, Vietnam, and monotypic *Parasanguinicola* Herbert and Shaharom-Harrison, 1995 (see Herbert and Shaharom-Harrison, 1995), which infects a sea bass (Perciformes: Latidae) in Malaysia. It differs from *N. canthoensis* most notably by lacking denticles flanking the mouth (not to be confused with minute concentric rows of spines). Further, *N. canthoensis* differs by having a uterus with ascending and descending portions (appearing as an inverse U-shape) as well as by lacking lateral tegumental spines (Truong and Bullard, 2013). *Parasanguinicola vastispina* Herbert and Shaharom-Harrison, 1995 (see Herbert and Shaharom-Harrison, 1995) differs by the number of spines along the tegument (<90), having lateral tegumental spines without a tegumental conical protrusion, a testis lacking lateral lobes, and (reportedly) a pre-ovarian female genital pore (Herbert and Shaharom-

Harrison, 1995). The location of the female genital pore needs confirmation. The position of the female genital pore in the illustration provided by Herbert and Shaharom-Harrison (1995) resembles the vitelline collecting duct and not an anteriorly extending uterus. We think that Herbert and Shaharom-Harrison (1995) mistook the proximal portion of the vitelline collecting duct as the distal portion of the uterus (Fig. 2, Herbert and Shaharom-Harrison, 1995). No other species of *Sanguinicola* has a pre-ovarian genital pore.

The other freshwater fish blood fluke genera (*Acipensericola, Plehniella,* monotypic *Cladocecum,* monotypic *Kritsky*) differ from *Sanguinicola* by features associated with the anterior sucker, lateral tegumental spines, intestine, and genitalia. Species of *Acipensericola*, which infect sturgeons and paddlefish (Appy and Dadswell, 1972; Bullard et al., 2008; Orélis-Ribeiro and Bullard, 2015; Warren et al., 2017), differ from *Sanguinicola* by having a large, pedunculate, bowl-shaped anterior sucker, lateral tegumental spines in transverse rows, an inverse U-shaped intestine, and a testicular column with 6 testes (including a post-genital testis) (Bullard et al., 2008; Warren et al., 2017b). *Plehniella*, monotypic *Cladocecum*, and monotypic *Krtisky* comprise species that infect catfishes only (Orélis-Ribeiro and Bullard, 2015). *Plehniella* spp. and *Kritsky platyrhynchi* (Guidelli, Isaac, and Pavanelli, 2002) Orélis-Ribeiro and Bullard, 2016 infect the body cavity but differ from *Sanguinicola* spp. by having an intestine comprising 6 radial ceca, a genital atrium, and a vas deferens that traverses anterior to the uterus (Orélis-Ribeiro and Bullard, 2015; 2016). Unique among fish blood flukes, *Cladocecum tomasscholzi* Orélis-Ribeiro and Bullard, 2016 has 1 pair of elongate anterior ceca plus a medial cecum with numerous branches extending laterad (Orélis-Ribeiro and Bullard, 2016).

We accept 10 species of *Sanguinicola*: *S. armata, S. hasegawai*, *S. intermedia*, *S. lophophora*, *S. lungensis*, *S. magnus*, *S. skjabini, S. rhodei*, *S. rutili*, and *S. volgensis* (Table I).

These species differ from the actinopterygian blood flukes by having lateral tegumental spines that are not distally recurved and that are arranged in a single column (vs. lateral transverse rows of spines) (Bullard and Overstreet, 2003), an intestine comprising radial ceca (vs. long anterior and posterior ceca) (Bullard, 2013; Warren et al., 2021), and a single testis with lateral lobes extending from a massive seminal column (vs. a large testicular mass with vasa efferentia as an interconnected meshwork of ducts or multiple testes) (Bullard et al., 2012; Warren et al., 2021). Further, all blood flukes infecting chondrichthyans differ from *Sanguinicola* spp. by having a Laurer's canal. They further differ by having C-shaped lateral tegumental spines and a non-sinusoidal testis or lacking spines and having a sinusoidal testis (Warren et al., 2019; Warren and Bullard, 2021).

A total of 16 species originally assigned to *Sanguinicola* need additional investigation regarding the lateral tegumental body spines, oral sucker spines, testis, and ovary to confidently assign them to a genus (Table II). *Sanguinicola alseae, S. davisi, S. fontinalis, S. idahoensis, S. klamathensis, S. maritimus*, *S. occidentalis,* and *S. ugui* have transverse rows or tufts (*S. fontinalis*) of spines (vs. a single column of large spike-like spines), and all are reported from North America except *S. maritimus* and *S. ugui*. *Sanguinicola maritimus* infects marine fishes in Australia (Nolan and Cribb, 2005; Shimazu, 2007). *Sanguinicola inermis, S. huronis*, and *S. argentinensis* were described as lacking lateral tegumental body spines (Ejsmont, 1926; Fischthal, 1947; Szidat, 1951). Further, *S. inermis* and *S. argentinensis* were described as having 'bristles' distributed along the lateral body surface (Ejsmont, 1926; Szidat, 1951) but we speculate that these 'bristles' could be sensory cilia, not spines (Fig. 4C–E). Moreover, *S. alseae, S. davisi, S. idahoensis*, and *S. klamathensis*, all of which infect North American salmoniforms, have an anterior 'proboscis-like' sucker with circumoral spines. This spine distribution is like

those observed in several marine lineages of fish blood flukes (*Elaphrobates euzeti* Bullard and Overstreet, 2003) (Wales, 1958; Holmes, 1971; Schell, 1974; Bullard and Overstreet, 2003). *Sanguinicola clarias* differs from *Sanguinicola* spp. by having 4 denticles (described as rose-thorn spines) that flank the mouth and a uterus that is inverse U-shaped (Imam et al., 1984). Truong and Bullard (2013) suggested that this species and *Plehniella dentata* Paperna, 1964 should be reassigned to *Nomasanguinicola* because they have 2 columns of denticles flanking the mouth and other key features of the genus (Table II) (Paperna, 1964; Truong and Bullard 2013). We herein reassign them as *Nomasanguinicola clarias* (Imam, Marzouk, Hassan, and Itman, 1984) Warren and Bullard n. comb. and *Nomasanguinicola dentata* (Paperna, 1964) Warren and Bullard n. comb. Imam et al. (1984) included supplemental observations of *S. chalmersi*, which has a column of 7 denticles on each side of the mouth (Odhner, 1924; Imam et al., 1984); perhaps suggesting that a new genus is warranted for this taxon (Truong and Bullard, 2013). Finally, we consider *S. incognita*, *S. megalobramae,* and *S. shantsuensis* as *nomina nuda* because no description or illustration for these names could be found.

We propose several reasons for the lack of new information on *Sanguinicola* spp. First, specimens of *Sanguinicola* deteriorate extremely rapidly. We have observed that within an hour of euthanizing a fish host, the blood flukes are non-motile, evidently dead, and seemingly deteriorated. Moreover, these flukes are exceptionally minute, delicate, and difficult to stain. Even with stains that work well for other blood flukes, we have observed that specimens of *Sanguinicola* appear to lose stain or never take it up to begin with. We suspect that the exceptionally poor condition of deposited types and vouchers of *Sanguinicola* spp. are at least in part explained by these methodological challenges; certainly, the extant types of *Sanguinicola* spp. are of limited value and are simply poor specimens. Additionally, whereas few

parasitologists examine freshwater fishes for parasites (Scholz and Choudhury, 2014), even fewer examine them for blood fluke infections. Hence, we think many infections go undetected. Finally, because so many nominal species descriptions are diagrammatical or incomplete, the taxonomist has little to go on when differentiating newly collected specimens.

***Sanguinicola plehnae* Warren and Bullard n. sp.**

(Figs. 1–4)

Light microscopy of adult based on 1 whole-mounted adult specimen and scanning electron microscopy of 9 adult specimens: USNM collection no. 1688209): Body flat, ventrally concave, tapering posteriorly and anteriorly, 1,200–3,000 (2,100) long, 350–550 (450) at greatest width, 4.2 × longer than wide (Figs. 1, 4B). Tegumental body spines 10–22 (16) from anterior end (Figs. 1, 4H), 5–7 (6) between spines anteriorly and 13–15 (14) posterior two-thirds of body (Figs. 2, 4G), 13 long, 2 wide at base, 6.4 × longer than wide; distal region of spines projecting 2–3 (2.5) through tegument, best observed with SEM (Figs. 2, 4A, C–E, G); area surround protruding spines flattened, smooth, tegumental layer surrounded by a gathering of ciliated sensory endings (Fig. 4C–E, L), tegumental spines extending to level of oötype in posterior half, 215 from posterior end (Fig. 1), with 120 and 122 per side or total of 242 (95–97 visible with SEM) (Fig. 1). Anterior body extremity proboscis-like, small, 10 long, 25 wide; spines absent. Mouth terminal, 1 long, 2 wide, possessing tegumental elevations into mouth cavity (Fig. 4H, I). Pharynx present. Ventrolateral nerve cord length indistinct, 11 wide near mid-body at widest level, 78 from body margin. Nerve commissure perpendicular to mid-line of body, connecting ventrolateral nerve-cords, 233 or 11% of body length from anterior end of body, 90 or 18% of body width across width of worm, 9 in breadth; secondary commissure branches 10 in maximum width. Esophagus 636 in total length or 30% of body length, 18 in maximum width (at level just

anterior to nerve commissure), extending sinuously posteriad along midline, widening slightly posteriorly (Fig. 1). Esophageal sunken glandular cells present in the middle and posterior esophagus. Intestine thin-walled, medial, comprising 4 radial ceca, X-shaped (Fig. 1); cecal intersection of anterior and posterior ceca 584 or 27% of body length from anterior body end; anterior ceca 54 long or 2.5% of body length, 23 wide, ventral to lateral nerve cord, containing granular material within lumen of some individuals (Fig. 1); posterior ceca asymmetrical, 72 long or 3% of body length, 25 wide, ventral to testis; post-cecal space 1,479 long or 69% of body length.

Testis 876 long or 41% of body length, 208 wide or 41% of body width, 4.2 × longer than wide, post-cecal; testicular lobes irregular, not paired, extending laterally, 119 in length towards body margin (Figs. 1, 3); testicular central column large, resembling a column throughout the length of the testis, 26 wide (Figs. 1, 3). Post-testicular space 562 long or 26% of body length. Vas deferens 196 long, 13 wide, emanating from middle posteroventral portion of testis, following midline before become confluent with seminal vesicle. Cirrus-sac present, having wall approximately 4 thick, including seminal vesicle, ejaculatory duct, and cirrus; seminal vesicle 184 long, 39 wide, 4.7 × longer then wide (Figs. 1, 3); everted cirrus 10 long; male genital pore towards midline, post-ovarian, sinistral to female genital pore, 204 or 10% of body length from posterior body end (Fig. 4F).

Ovary medial, double-winged in shape, appearing as loose aggregation of cells, 190 in maximum length or 9% of body length, 159 wide or 32% of body width, immediately post-testicular; post-ovarian space 399 long or 19% of body length (Figs. 1, 3). Oviduct 302 long or 14% of body length; oviducal seminal receptacle indistinct. Oötype 51 long, 49 wide; 159 long or 7% of body length from posterior end (Fig. 3). Vitellarium appearing as loose follicles,

occupying space dorsal and lateral to testis and ceca, extending from nerve commissure to ovary (Fig. 1); common collecting duct 447 long, 14 wide. Uterus short, extending directly anteriad from oötype, 53 long, 32 wide (Fig. 3). Female genital pore medial, post-ovarian, lateral to seminal vesicle, 224 or 10% of body length from posterior body end (Figs. 3, 4F); 40 from male genital pore (Fig. 3). Excretory vesicle small, 30 long, 9 wide, medial.

Transmission electron microscopy (TEM) of 3 adult specimens: The presence of irregular depressions and prominences of different size and shape were revealed on the surface of the distal tegumental cytoplasmic layer of the worms via SEM and TEM observations (Fig. 4J, K). The cytoplasmic matrix of this layer is moderately dense and contains a high concentration of rounded or ovoid dense granules 0.2–0.4 (0.3) μm in diameter and electron-lucent vesicles (Fig. 4K). Detailed description of ultrastructure features of spines, tegument, and sensory receptors of *S. plehnae* (see Poddubnaya et al., 2020). This taxon is the same species as that referred to as *S. inermis* in this above-mentioned paper.

Taxonomic summary

Type and only known host: Northern pike, *Esox lucius* Linnaeus, 1758 (Esociformes: Esocidae).

Type locality: Upper Volga River, Russia.

Site of infection: Ventral aorta and bulbus arteriosus of heart.

Prevalence and intensity of infection: 24 of 121 (19.8%) northern pike sampled in 2021 were collectively infected by 28 specimens of *S. plehnae*.

Specimens deposited: Holotype (USNM 1688209).

ZooBank registration: urn: lsid: zoobank.org:act:2720BEA5-16A6-436D-B15D-66C1625C2B06.

Etymology: The specific epithet honors Dr. Marianne Plehn (1863–1946; Bavarian Biological Experimental Institute) for her contributions to our knowledge of *Sanguinicola*, fish parasitology, and fish pathology as well as for her pioneering career as the first woman to be awarded i) a doctorate by Eidgenössische Technische Hochschule in Zürich, Switzerland, ii) a Royal Professorship by King Ludwig III in Germany and iii) doctoral status for the faculty of Veterinary Medicine at the University of Munich, Germany (Ogilvie and Harvey, 2000).

Remarks

Sanguinicola plehnae differs from its congeners by having lateral tegumental spines that total 118–122, are small (3% of body width), and protrude 2–3 μm from the tegument (lacking associated conical protrusion) as well as by having a large testis (>40% of body length) (Figs. 1–3, 4A). The new species is most similar to *S. hasegawai*, *S. rutili*, and *S. skrjabini* by having a similar body length-to-width ratio (>4) and a testis length >40% of body length (Akhmerov, 1960; Simón-Martín et al., 1988; Shimazu, 2013). However, *S. hasgawai* and *S. rutili* have <100 lateral tegumental spines per body side (vs. >115). *Sanguinicola skrjabini* differs from the new species by having a larger body length-to-width ratio (>6) and >300 tegumental spines. Only 2 other species assigned to the genus, *S. armata,* and *S. volgensis*, infect an esociform (both infecting northern pike, *E. lucius*). Given the tumultuous history of the genus, the similarities between species of *Sanguinicola*, and that it is unusual for a blood fluke to infect numerous fish hosts, we suspect that these records could be dubious and represent infections by several blood fluke species (Bikhovskaya-Pavlovskaya et al., 1964; Kirk and Lewis, 1994).

***Sanguinicola volgensis* (Rašín, 1929) Mcintosh, 1934**

(Figs. 5–13)

Light microscopy of 7 newly collected whole-mounted adult specimens and scanning electron microscopy of 8 specimens; USNM collection nos. 1688210–1688216): Body flat, ventrally concave, tapering posteriorly and anteriorly, 1,052–1,299 (1,146 ± 85, 6) long, 315–419 (358 ± 32, 7) at greatest width, 2.7–3.3 (3 ± 0.27, 6) × longer than wide (Figs. 5, 12D); surface with numerous, narrow, short surface bulges, covered with heavy concentrations of shallow knob-like outgrowths (Fig. 12A, J, K), not present on the anterior body extremity, lateral conical protrusion, and cirrus (Fig. 12A, B, H, I, F, G). Tegumental body spines 21–34 (25 ± 6, 4) from anterior end (Figs. 5, 12A), 23–33 (28 ± 4, 20) long, 3 (20) wide at base, 8.3–11 × longer than wide, completely encased in conical protrusion, 10–12 (20) wide (Figs. 6, 12D, H, I); tegumental spines extending to level of oötype (Figs. 5, 12D), 127–228 (153 ± 34, 7) from posterior end (Figs. 5, 12E), with 96–109 (102 ± 4, 5) per side or total of 196–211 (204 ± 7, 5). Anterior body extremity proboscis-like, small, 12–18 (15 ± 3, 3) long, 13–33 (25 ± 8, 4) wide; spines absent. Ventrolateral nerve cord 999–1,074 (1,035 ± 31, 4) long, 4–12 (8 ± 3, 6) wide near mid-body at widest level, 58–76 (66 ± 7, 6) from body margin. Nerve commissure perpendicular to mid-line of body, connecting ventrolateral nerve-cords, 153–192 (167 ± 13, 6) or 15% of body length from anterior end of body, 75–110 (90 ± 12, 6) across width of worm, 7–11 (10 ± 2, 6) in breadth; secondary commissure branches 5–6 (5.6 ± 0.5, 6) in maximum width. Mouth small, 3 (3) in diameter, 3–5 (4 ± 1, 5) from terminal end of anterior body extremity (Fig. 12A–C). Pharynx present; pharyngeal canal surrounded by well-developed muscle complex of circular and radial muscle fibers (Fig. 13B). Esophagus 319–406 (341 ± 32, 6) in total length or 29–32% of body length, 11–21 (16 ± 4, 6) in maximum width (at level just anterior to nerve commissure), extending sinuously posteriad along midline, widening slightly posteriorly (Figs. 5, 8–11); anterior esophageal middle portion 59–79 (69 ± 14, 2) long, 60–75 (68 ± 11, 2) wide; posterior

portion 78–109 (93 ± 16, 3) long, 54–68 (63 ± 8, 3) wide; esophageal sunken glandular cells concentrated in the middle and posterior portions. Intestine X-shaped, dendritic, with radial ceca intersecting medially, 1 specimen contained a fifth cecal lobe (Figs. 8–11); cecal intersection of anterior and posterior ceca 319–406 (341 ± 32, 6) or 29–32% of body length from anterior body end; anterior ceca 37–57 (44 ± 9, 6) long or 3–5% of body length, 14–21 (17 ± 3, 6) wide, containing granular material within lumen of some individuals (Figs. 5, 8–11); posterior ceca asymmetrical, 41–77 (59 ± 18, 6) long or 3–37% of body length, 22–46 (28 ± 12, 6) wide (Figs. 5, 8–11); post-cecal space 678–817 (740 ± 50, 6) long or 64–65% of body length.

Testis 309–500 (390 ± 59, 7) long or 29–35% of body length, 158–225 (177 ± 26, 7) wide or 37–47% of body width, 2 × longer than wide, post-cecal, testicular lobes irregular, not paired, extending laterally, 56–106 (75 ± 16, 7) in length towards body margin (Figs. 5, 7); testicular central column large, resembling a column throughout the length of the testis, 20–36 (28 ± 6, 7) wide (Fig. 5). Post-testicular space 333–550 (413 ± 67, 7) long or 31–36% of body length. Vas deferens 56–113 (75 ± 21, 7) long, 9–16 (12 ± 3, 6) wide, emanating from postero-ventral portion of testis, following midline before becoming confluent with seminal vesicle. Cirrus-sac present, wall approximately 3–14 (8 ± 3.6, 7) thick, including seminal vesicle, ejaculatory duct, and cirrus; seminal vesicle 208–282 (241 ± 28, 7) long, 26–33 (29 ± 3, 7) wide, 7–10 × longer then wide (Fig. 7). Male genital pore toward midline, post-ovarian, posterior to female genital pore, 117–185 (138 ± 25, 6) or 11–14% of body length from posterior body end (Figs. 5, 12E–G).

Ovary medial, double-winged in shape, 110–160 (136 ± 18, 7) in maximum length or 10–13% of body length, 151–207 (180 ± 16, 7) wide or 44–58% of body width, 1.3–2 × wider than long, immediately post-testicular; post-ovarian space 248–400 (291 ± 51, 7) long or 23–26% of

body length (Fig. 5). Oviduct (including oviducal seminal receptacle) 211–330 (270 ± 43, 7) long; oviducal seminal receptacle 110–252 (169 ± 51, 7) long or 54–76% of oviduct length, 12–30 (19 ± 6, 7) wide. Oötype 30–38 (34 ± 3, 7) long, 22–30 (26 ± 3, 7) wide (Fig. 7). Vitellarium follicular, compacted in dense lobules, occupying space dorsal and lateral to testis and ceca, extending from nerve commissure to terminal end of posterior ceca (Fig. 5); common collecting duct 167–250 (216 ± 33, 7) long, 8–15 (11 ± 2, 7) wide. Uterus short, extending directly anteriad from oötype, 40–52 (46 ± 6, 6) long or 4% of body length, 11–28 (16 ± 8, 6) wide; containing single egg in 1 of 7 specimens; uterine egg 19 (1) long, 11 (1) wide, with thin shell. Female genital pore central, post-ovarian, lateral to seminal vesicle, 153–238 (177 ± 32, 6) or 15–18% of body length from posterior body end (Figs. 5, 7, 12E–G). Excretory vesicle small, 13 (1) long, 7 (1) wide, medial.

Transmission electron microscopy (TEM) of 4 adult specimens: The body surface is bounded by the distal tegumental syncytial layer, which is limited by surface and basal membranes (Fig. 13F, G). Due to numerous, periodic, surface infoldings, bulges 0.7–0.8 (7.5) µm thick are present (Fig. 13F). Also, the thickness of the outer tegumental layer between the bulges is 0.35 ± 0.05 µm. A deep invagination of the basal membrane extends into the central area of each bulge (Fig. 13F, G). The tegumental cytoplasmic matrix is moderately electron-dense and finely fibrous in appearance forming beneath the surface plasma membrane a dense fibrous zone, which bears regular, knob-like outgrowths (Fig. 13F, G). Numerous rounded vesicles with a slightly fibrous content are recognizable within the cytoplasm throughout the whole length of the worm's body (Fig. 13F, G). The electron-dense spines are uniform in shape, a large proportion of spine length occurs deep beneath the level of the distal cytoplasm (Fig. 13A, D, E). In sections, diameter of the spines is 2.2–3.0 (2.6) µm; the distance between the spines varies from 4.5–6.2 (5.35) µm

(Fig. 13A, C–E). Well-developed muscle fibers flattened sarcoplasmic extensions are associated with each spine (Fig. 13C). The hemidesmosomes are located at the tapering ends of the muscle fibers and spine bodies (Fig. 13C).

Taxonomic summary

Type host: Sabrefish, *Pelecus cultratus* (Linnaeus, 1758) Berg, 1949 (Cypriniformes: Leuciscidae).

Other hosts: Zope, *Ballerus ballerus* (Linnaeus, 1758) (Cypriniformes: Leuciscidae) (as *Abramis ballerus*) (Wierzbicka, 1977); common bream, *Abramis brama* (Linnaeus, 1758) (Wierzbicka, 1977) (Leuciscidae); common bleak, *Alburnus alburnus* (Linnaeus, 1758) (Leuciscidae) (Rašín, 1929); white bream, *Blicca bjoerkna* (Linnaeus, 1758) (Leuciscidae) (Wierzbicka, 1977); chub, *Squalius cephalus* (Linnaeus, 1758) (Leuciscidae) (as *Leuciscus cephalus*) (Kirk and Lewis, 1994); ide, *Leuciscus idus* (Linnaeus, 1758) (Leuciscidae) (Bikhovskaya-Pavlovskaya et al., 1964); and common dace, *Leuciscus leuciscus* (Linnaeus, 1758) (Kirk and Lewis, 1994); Italian rudd, *Scardinius hesperidicus* Bonaparte, 1845 (Cypriniformes: Leuciscidae) (as *Scardinus erythrophthalmus* [Linnaeus, 1758]) (Ergens et al., 1975); northern pike, *Esox 82ucius* Linnaeus, 1758 (Esociformes: Esocidae) (Bikhovskaya-Pavlovskaya et al., 1964; Molnar, 1969; Kirk and Lewis, 1994).

Type locality: Upper Volga River, Russia.

Site of infection: Heart lumen.

Prevalence and intensity of infection: 12 of 82 (14.6%) sabrefish sampled in 2021 were collectively infected by 20 specimens of *S. volgensis*.

Specimens deposited: Vouchers (USNM 1688210–1688216).

Sanguinicola* cf. *volgensis

(Figs. 14–17)

Light microscopy of whole-mounted adult (1) and juvenile (1) specimen and scanning electron microscopy of 5 specimens; USNM collection nos. 1688217–1688218): Body flat, ventrally concave, oval, 1,227 long, 239 at greatest width, 5 × longer than wide, bearing longitudinal row of conical protrusions (Figs. 14; 16A, B, G, H). Tegumental surface consisting of irregular depressions and prominences (Fig. 16G, H, F); apical surface of prominences bears knob-like ornamentations (Fig. 16K). Ciliated sensory endings present, including lateral protrusions (Fig. 16H, F). Tegumental body spines 29 (10) long, 2.5 (10) wide at base, 11 × longer than wide (Fig. 16A, G, H, I), completely encased in conical protrusion (Fig. 16G), 10 (10) wide, or spine emerging 2.5 from conical protrusion (Fig. 16I); tegumental spines extending to level of oötype (Fig. 16B), with 87 and 89 spines per side or total of 176. Anterior body extremity proboscis-like, small, 14 long, 24 at greatest width; spines absent (Fig. 16A, B). Ventrolateral nerve cord indistinct posteriorly, 10 wide near mid-body at widest level, 45 from body margin. Nerve commissure perpendicular to mid-line of body, connecting ventrolateral nerve-cords, 187 or 15% of body length from anterior end of body, 45 or 19% of body width across width of worm, 10 in breadth. Mouth 2 in diameter, 7 from terminal end of anterior body extremity (Fig. 16C, D). Esophagus 358 in total length or 29% of body length, 10 in maximum width (at level just anterior to nerve commissure), extending sinuously posteriad along midline, widening slightly posteriorly (Fig. 14); esophageal wall 2 wide in posterior half of esophagus. Esophageal sunken glandular cells present. Intestine X-shaped, dendritic, with radial ceca intersecting medially (Fig. 14); cecal intersection of anterior and posterior ceca 358 or 29% of body length from anterior body end; anterior ceca 38 long or 3% of body length, 21 wide or 7% of body width, containing granular material within lumen (Fig. 14); posterior ceca asymmetrical,

54 long or 4% of body length, 14 wide or 6% of body width, anterior to testis (Fig. 14); post-cecal space 792 long or 65% of body length.

Testis 384 long or 31% of body length, 106 wide or 44% of body width, 3.6 × longer than wide, post-cecal, testicular lobes irregular, not paired, extending laterally, 39 (10) in length towards body margin (Fig. 14); testicular central column large, resembling a column throughout the length of the testis, 16 (10) wide (Fig. 14). Post-testicular space 410 long or 33% of body length. Vas deferens 84 long, 8 wide, emanating from postero-ventral portion of testis, following midline before becoming confluent with seminal vesicle. Cirrus-sac and cirrus indistinct; seminal vesicle 180 long or 15% of body length, 23 wide or 10% of body width, 15 × longer then wide, 144 or 12% of body length from posterior body end (Fig. 14). Male genital pore toward midline, post-ovarian, sinistral to female genital pore, 137 or 13% of body length from posterior body end (Figs. 14, 16E).

Ovary medial, double-winged in shape, 108 in maximum length or 8% of body length, 116 wide or 49% of body width, 1.1 × wider than long, immediately post-testicular; post-ovarian space 293 long or 24% of body length (Fig. 14). Oviduct 224 or 18% long. Oötype 33 long, 20 wide; 95 or 8% of body length from posterior end (Fig. 14). Vitellarium comprising diffuse follicles compacted in dense lobules, occupying space dorsal and lateral to testis and ceca, extending from anterior to nerve commissure to posterior to ovary (Fig. 14). Uterus short, extending directly anteriad from oötype, 34 long or 3% of body length, 3 wide. Female genital pore medio-dextral, post-ovarian, lateral to seminal vesicle, 144 or 11% of body length from posterior body end (Fig. 16E, J). Excretory vesicle indistinct.

Light microscopy of juvenile specimen: Body flat, ventrally concave, ovoid, 1,324 long, 205 at greatest width, 6 × longer than wide (Fig. 15). Tegumental body spines 27 (10) long, 3 (10)

wide at base, completely encased in tegument (Fig. 15), tegumental spines extending to level of oötype, with 92 and 94 spines per side or total of 186. Esophagus 406 in total length or 31% of body length, 22 in maximum width, extending sinuously posteriad along midline, widening slightly posteriorly. Esophageal sunken glandular cells concentrated immediately anterior to cecal bifurcation, 185 in total length or 46% of esophagus length, 47 in maximum width (Fig. 15). Intestine X-shaped, with paired anterior and posterior ceca intersecting medially; cecal intersection of anterior and posterior ceca 406 or 31% of body length from anterior body end; anterior ceca 42 long or 3% of body length, 22 wide or 11% of body width, containing granular material within lumen (Fig. 15); posterior ceca asymmetrical, 75 long or 6% of body length, 37 wide or 18% of body width, anterior to testis; post-cecal space 832 long or 63% of body length. Testis 316 long or 24% of body length, 108 wide or 53% of body width, 2.9 × longer than wide, post-cecal, testicular lobes irregular, not paired, extending laterally, 53 (10) in length towards body margin (Fig. 15). Post-testicular space 358 long or 27% of body length. Terminal genitalia and excretory vesicle not observed.

Transmission electron microscopy (TEM) of 3 adult specimens: TEM shows that distal tegumental cytoplasmic layer of the body tegument greatly varies in thickness 0.1–2.0 (1.05) µm, depending on the degree of surface irregularity, which is penetrated by various kinds of numerous, large, and deep surface invaginations, forming numerous prominences (Fig. 17B, G, H). The apical ends of these prominences are electron-dense of about 0.25 ± 0.05 µm in their length, forming knob-like ornamentation shown via SEM (Figs. 16K, 17G, H). The tegumental cytoplasmic matrix is moderately electron-dense and finely fibrous in appearance with a dense fibrous zone beneath the surface plasma membrane and contains a high concentration of small, electron-lucent vesicles and rare single electron-dense, oval granules 0.3 ± 0.01 x 0.2 ± 0.01 µm

(Fig. 17G–I). The vesicles are elongated about 0.15 ± 0.01 x 0.06 ± 0.01 µm and can be recognized by their electron-dense walls (Fig. 17I). The basal plasma membrane of the syncytial layer extends into long invaginations, penetrating the central area of the prominences (Fig. 17G, H). The conical protrusions are scattered along the lateral body margins (Fig. 17A, C, D). Each protrusion possesses 1 spine, much of the length of which occurs deep beneath the level of the distal cytoplasm (Fig. 17C, D). The diameter of the spines varies between 1.7–2.2 (1.95) µm. The distance between spines is 6.5–8.0 (7.25) µm. Spines are uniform in the shape and have an electron-dense body surrounded by muscle fibers and flattened sarcoplasmic extensions (Fig. 17B–F). Hemidesmosomes are located between the muscle fibers and the spine body (Fig. 17F).

Taxonomic summary

Type host: ide, *Leuciscus idus* (Linnaeus, 1758) Berg, 1949 (Cypriniformes: Leuciscidae).

Type locality: Upper Volga Region, Russia.

Site of infection: Heart lumen

Prevalence and intensity of infection: 22 of 67 (32.8%) ide sampled in 2021 were infected by a collective total of 47 specimens of *S*. cf. *volgensis*.

Specimens deposited: Vouchers (USNM 1688217, 1688218).

Remarks

We identified the blood fluke specimens infecting ide as *Sanguinicola* cf. *volgensis* because our specimens were morphologically indistinguishable from the published description and the newly collected material (see above) while having a larger body length: width ratio (5–6 vs. 2–3) and <90 lateral tegumental spines (vs. >95). The juvenile specimen had large lateral tegumental spines but lacked the conical protrusion associated with the spine (Fig. 15), which is noteworthy regarding their taxonomic identity.

Sanguinicola volgensis differs from its congeners by having posteriorly-directed lateral tegumental spines encased in a tegumental conical protrusion (Figs. 5, 6,12H–I, 13D). Rašín (1929) described *Sanguinicola volgensis* (as *Janickia* Rašín, 1929) based on the length of the metraterm (longer than oötype vs. shorter than oötype) and egg shape (oval vs. triangular). McIntosh (1934) synonymized *Janickia* with *Sanguinicola* stating *"Janickia is regarded by the writer as a synonym of Sanguinicola, since, according to Rašín (1929), it does not differ from the genus Sanguinicola except in shape and size of the egg, oötype, and metraterm; the species Janickia volgensis Rašín, 1929, therefore, becomes Sanguinicola volgensis (Rašín, 1929) n. comb."* (p. 465, McIntosh, 1934). McIntosh did not elaborate further. Because several species assigned to the *Sanguinicola sensu stricto* are described as having an ovoid egg, we accept the synonymy, i.e., *Janickia* herein remains a junior subjective synonym of *Sanguinicola*. Four species assigned to *Sanguinicola sensu stricto* reportedly have ovoid eggs (*S. lophophora*, *S. rhodei*, *S. hasegawai*, *S. magnus*), 4 reportedly have triangular eggs (*S. armata, S. intermedia*, *S. lungensis*, *S. rutili*), and 1 has *"approximately triangular"* eggs (*S. rhodei*) (p. 341, Wang, 1983). No description of an egg was given for *S. skrjabini* (Akhmerov, 1960). Further, we did not observe an egg in the uterus for *S. plehnae*. In 1 of 7 of our whole-mounted specimens of *S. volgensis*, we observed a single ovoid egg within the lumen of the distal uterus. Further collections are required to determine the egg shape in this species.

Pseudosanguinicola **Warren and Bullard n. gen.**

(Figs. 18–20)

Diagnosis: Body of adult <7 × longer than wide, dorsoventrally flat, lacking posterolateral protuberance, tapering equally anteriorly and posteriorly, ventrally concave, spined; tegumental spines delicate, straight, and not distally recurved, arranging in densely compacted transverse

rows along lateral body margin, appearing irregular or discordant. Rosethorn-shaped spines absent. Ventrolateral nerve cords and dorsolateral nerve cords present; ventrolateral nerve cord appearing slightly sub-terminal, with commissure anteriorly. Anterior sucker diminutive, appearing concave, accommodating mouth; mouth medioventral, subterminal, with associated tooth-like mouth apparatus (Figs. 4I, 12B). Pharynx indistinct. Esophagus medial, straight, not looping, extending posteriad less than one-fourth body length, with anterior and posterior esophageal swellings, with esophageal gland enveloping posterior portion of esophagus, connecting with ceca, anteromedially. Intestine thin-walled, medial, comprising 4 radial ceca that collectively appear generally as an X-shaped structure. Testis diffuse, an array of lateral lobes, with breadth equal or slightly greater than that of intestine, giving the appearance of multiple testes in 2 tandem rows flanking midline from level of ceca to middle third of body. Vasa efferentia prominent, appearing to occupy one-third of the testicular width, with secondary ducts extending from lateral margins of testicular lobes and coalescing ventrally along midline, narrowing medially before connecting with proximal portion of seminal vesicle. Cirrus-sac surrounding seminal vesicle present, but inconspicuous in some wholemounts. Male genital pore dextral, slightly lateral to midline. Ovary single, medial, with superficial lobed margins, with lateral portions extending anteriad and lateral to posterior portion of testicular field, appearing butterfly wing-shaped to varying degrees, as wide or wider than testicular field, occupying posterior one-third of body. Vitellarium follicular, at least occupying space from ~level of anterior nerve commissure to terminal end of ovary. Oviduct a narrow duct extending directly posteriad from posteromedial surface of ovary, connecting with distal portion of vitelline reservoir to or near level of male genital pore; oviducal seminal receptacle present or absent. Ovo-vitelline duct connecting with oötype posteriorly. Oötype large, oblong, lateral and posterior

to level of male genital pore, posterior to genitalia. Laurer's canal absent. Uterus short, straight; uterine seminal receptacle lacking. Female genital pore anteromedial to male genital pore. Excretory vesicle not observed.

Differential diagnosis: Body <7 × longer than wide; tegumental spines delicate, straight, and not distally recurved, arranging in densely compacted transverse rows along lateral body margin, appearing irregular or discordant. Intestine X-shaped, with 4 radial ceca, terminating in anterior half of the body. Testis single, an array of lateral lobes, with breadth equal or slightly greater than that of intestine, giving the appearance of multiple testes in 2 tandem rows flanking midline from level of ceca to middle one-third of body. Seminal vesicle medial, large, one-fourth to one-third of body length. Male genital pore lateral to female genital pore, terminating along midline. Ovary butterfly wing-shaped, post-cecal, post-testicular. Oötype large, posterior to genitalia. Uterus short, straight, equal in length of oötype.

Type species: Pseudosanguinicola occidentalis (Van Cleave and Mueller, 1932) Warren and Bullard n. comb.

ZooBank registration: urn: lsid: zoobank.org:act:D09CC45D-3EB2-40E0-AECD-76D745B80578.

Etymology: The Greek '*pseudo*' refers to the previous assignment of this species to *Sanguinicola*.

Remarks

Pseudosanguinicola Warren and Bullard n. gen. resembles *Sanguinicola* and *Nomasanguinicola* by having an anterior esophageal swelling, an intestine terminating in the anterior half of the body, a vitelline duct dorsal to the testis, an ovary that is butterfly wing-shaped, and male and female genital pores that open near the midline. It differs from

Nomasanguinicola by lacking an anterior sucker with 2 columns of denticles flanking the mouth (Truong and Bullard, 2013). Further, it differs from *Sanguinicola sensu stricto* by having lateral tegumental spines that are delicate, straight, and not distally recurved, distributed in densely compacted transverse rows along the lateral body margin, appearing grass-like and discordant (Figs. 18, 19). *Pseudosanguinicola* is most similar to species we regard as *incertae sedis* (*S. alseae*, *S. davisi*, *S. fontinalis*, *S. idahoensis, S. klamathensis*, *S. maritimus*, and *S. ugui*) (Table II) by having tegumental spines in transverse rows rather than a single column. Of those 7 species, *S. idahoensis* is most similar by having "*numerous spines along the lateral tegument*" (p. 562, Schell, 1974). The illustration of *S. idahoensis* does not help determine homology of the spines. Further, *S. alseae*, *S. davisi*, *S. idahoensis*, and *S. klamathensis* have a prominent proboscis-like anterior sucker with circumoral spines (Wales, 1958; Holmes, 1971; Schell, 1974). Considering these differences (transverse rows and a prominent proboscis-like anterior sucker) and that they infect species from North America, the blood flukes infecting salmoniforms may represent a new genus.

Pseudosanguinicola occidentalis **(Van Cleave and Mueller, 1932) Warren and Bullard n. comb.**

(Figs. 18–20)

Description of adults based on light microscopy of 5 newly collected whole-mounted specimens; USNM collection nos. 1688219–1688223): Body flat, ventrally concave, ovoid, 1,145–1,527 (1,324 ± 157, 5) long, 162–271 (230 ± 41, 5) at greatest width, 5–7.1 (5.7 ± 0.92, 5) × longer than wide (Fig. 18); body constricted posteriorly producing tail-like appendage, 176–223 (205 ± 20, 4) from terminal end (Fig. 18). Tegumental body spines 2–3.5 (2.8 ± 1.1, 20) long, 1–1.5 (1.3 ± 0.4, 20) wide at base, densely packed, not extending beyond tegument,

tegumental spines extending to level of female genital pore in posterior half (Fig. 19). Anterior sucker, small, 11–17 (14 ± 4, 2) long, 19–21 (20 ± 1, 2) at greatest width; spines absent. Ventrolateral nerve cord indistinct. Nerve commissure perpendicular to mid-line of body, connecting ventrolateral nerve-cords, 129–149 (139 ± 14, 2) or 11% of body length from anterior body end, 54–63 (59 ± 6.4, 2) across width of worm, 9–11 (10 ± 2, 2) in breadth; secondary commissure branches indistinct. Mouth 2.5 in diameter, 2 from anterior of body. Esophagus 291–359 (325 ± 34, 3) in total length or 24% of body length, 16–22 (19 ± 3, 3) in maximum width (at level just anterior to nerve commissure), extending sinuously posteriad along midline, widening in anterior portion 34 long and 13 wide; esophageal wall thickening from 3–7 (5 ± 1, 4) in maximum width. Esophageal gland indistinct. Intestine thin-walled, medial comprising 4 radial ceca that collectively appear generally X-shaped (Fig. 18); anterior ceca 25–32 (29 ± 5, 2) long or 2% of body length, 15 wide, containing granular material within lumen of some individuals; posterior ceca asymmetrical, 34–40 (37 ± 5, 2) long or 3% of body length, 17 wide, anterior to testis (Fig. 18); post-cecal space 879–1034 (957 ± 110, 2) long or 61–70% of body length.

Testis 389–409 (392 ± 12, 4) long or 28–34% of body length, 39–101 (31 ± 3, 4) wide or 24–30% of body width, 5–10 × longer than wide, post-cecal, testicular lobes irregular, not paired, extending laterally, 57–76 (64 ± 9, 4) in length towards body margin, (Figs. 18, 20); testicular middle column 12–59 (33 ± 17, 5) wide. Post-testicular space 367–660 (510 ± 147, 3) long or 32–46% of body length. Vas deferens 101–150 (119 ± 27, 3) long, 5–12 (7 ± 4, 3) wide, emanating from postero-ventral portion of testis, following midline before becoming confluent with seminal vesicle. Cirrus-sac present, including seminal vesicle and cirrus; seminal vesicle 239–480 (340 ± 125, 3) long, 15–38 (30 ± 11, 4) wide, 21–33% (3) of body length (Fig. 20).

Cirrus 13–37 (25 ± 17, 2) long or 5–12% of seminal vesicle length, 6–8 (7 ± 1.4, 2) wide. Male genital pore toward midline, post-ovarian, sinistral to female genital pore, 114–187 (143 ± 39, 3) or 9–12% of body length from posterior body end (Fig. 20).

Ovary medial, double-winged in shape, 120–313 (192 ± 106, 3) long or 10–21% of body length, 66–123 (99 ± 29, 3) wide or 41–45% of body width, immediately post-testicular; post-ovarian space 232–390 (326 ± 83, 3) long or 20–29% of body length (Figs. 18, 20). Oviduct (including oviducal seminal receptacle) 282–433 (365 ± 77, 3) long; oviducal seminal receptacle 85 long or 30% of oviduct length, 13 wide. Oötype 28–46 (39 ± 9, 4) long, 16–47 (33 ± 14, 4) wide, 65–120 (84 ± 31, 4) long from posterior terminal end (Figs. 18, 20). Vitellarium comprising follicles compacted in dense lobules, occupying space dorsal and lateral to testis and ceca, extending from anterior to nerve commissure to terminal end of ovary (Fig. 18); common collecting duct 375–404 (390 ± 21, 2) long, 11–19 (15 ± 3.3, 2) wide. Uterus short, extending directly anteriad from oötype, 23–50 (35 ± 11, 4) long or 2–3% of body length, 17–22 (20 ± 3, 4) wide; uterine eggs not observed. Female genital pore medial, post-ovarian, lateral to male genital pore, 133–213 (167 ± 34, 4) or 12–14% of body length from posterior body end (Figs. 18, 20). Excretory vesicle not observed.

Taxonomic summary

Type host: Walleye, *Sander vitreus* (Mitchill, 1818) Bailey et al., 2004 (Perciformes: Percidae).

Other hosts: Yellow perch, *Perca flavescens* (Mitchill 1814) Collette and Bănărescu, 1977 (Perciformes: Percidae).

Type locality: Oneida Lake, New York, USA.

Other locality: Lewis Point, Oneida Lake, Lenox, NY (Madison County): (43º10′24″N, 75º46′35″W); Wisconsin, USA.

Site of infection: Heart lumen.

Prevalence and intensity of infection: 2 of 2 (100%) walleye sampled on 7 July 2020 were infected by 1 specimen each of *P. occidentalis*; 6 of 16 (38%) walleye sampled on 20 Aug 2020 collectively were infected by 8 specimens of *P. occidentalis*.

Specimens deposited: Vouchers (USNM 1688219–1688223).

Remarks

Pseudosanguinicola occidentalis resembles the accepted *Sanguinicola* spp. (Table I) by having an intestine that is X-shaped, with 4 radial ceca terminating in the anterior half of the body, a single testis with lobes extending laterally, a double-winged or butterfly wing-shaped ovary, male and female genital pores terminating along the midline, and a short uterus. It differs from *Sanguinicola* spp. most notably by the arrangement of the lateral tegumental spines, which are densely compacted transverse lateral rows vs. single column of large spines (Figs. 2, 6, 19). *Pseudosanguinicola occidentalis* also resembles *S. rhodei* by having a lappet-like posterior end that begins at level of the genital pores and oötype (Fig. 18). Before collecting new material, we (MBW, SAB) incorrectly predicted that this was fixation artifact. Van Cleave and Mueller (1932) described 2 vitelline ducts connecting to the oötype (Van Cleave and Mueller, 1932). Our newly collected specimens have a single vitelline duct (Fig. 20), which is large and obvious. Depending on the condition of Van Cleave and Mueller's specimens, these delicate ducts could have been difficult for them to discern. They also described minute cuticular spines covering most of the body, but this is not the case in our material and likely represents sensory cilia (Ejsmont, 1926; Van Cleave and Mueller, 1932).

Results of Phylogenetic Analysis

The amplified *28S* and internal transcribed spacer 2 region (*ITS2*) fragments representing *S. volgensis*, *S.* cf. *volgensis*, and *S. plehnae* are 1,584, 1,585, and 1,574 nucleotides and 451, 461, and 452 nucleotides, respectively. The amplified *28S* fragment representing *P. occidentalis* is 1,589 nucleotides. Sequences representing *S. volgensis* and *S.* cf. *volgensis* were recovered sister to one another and only differed by a single base pair in the *28S* (Fig. 21) and 2 base pair differences in the *ITS2* (2 bp; 99.6% similar). The *28S* sequence representing *S. plehnae* differed from *S. volgensis* and *S.* cf. *volgensis* by 82 and 83 nucleotides (*28S*) and by 37 and 39 nucleotides (*ITS2*), respectively. The species was recovered sister to the clade including *S. volgensis* and *S.* cf. *volgensis*. The *28S* sequences (*S. volgensis*, *S.* cf. *volgensis*, *S. plehnae*) grouped sister to the cercarial sequence for *Sanguinicola* cf. *inermis* (AY222180) and yielded a percent similarity of 81% (218 bps) (Olson et al., 2003) (Fig. 21). It is noteworthy that this *28S* sequence (AY222180) is from a cercaria infecting a gastropod from a lake in Poland that has never been confirmed with the adult specimen (Olsen et al., 2003). That said, *S. volgensis* (like the type species, *S. armata*) differs from *S. inermis* (see Remarks) by having large spike-like tegumental spines in a single column (Fig. 6) (Plehn, 1905; Ejsmont, 1926). The large % bp difference between *S. volgensis* and *S.* cf. *inermis* (218 bp) is similar to intergeneric sequence variability rather than interspecific variation. For example, sequences representing species of *Psettarium* Goto and Ozaki, 1930 and *Cardallagium* Yong, Cutmore, Jones, Gauthier, and Cribb, 2018 (formally assigned to *Psettarium*) differ by 217 bp (Warren et al., 2017a; Yong et al., 2018).

Pseudosanguinicola occidentalis was recovered sister to all species of *Sanguinicola* and differed from *S. volgensis*, *S.* cf. *volgensis*, *S. plehnae,* and *S.* cf. *inermis* by 158, 157, 183, and

236 bp (*28S*), respectively (Fig. 21). Further, *P. occidentalis* and *Sanguinicola* spp. were monophyletic and sister to the remaining actinopterygian blood flukes assigned to *Elopicola* Bullard, 2014*, Aporocotyle* Odhner, 1900*, Plethorchis* Martin, 1975, *Neoparacardicola* Yamaguti, 1970, *Skoulekia* Alama-Bermejo, Montero, Raga, and Holzer, 2011, and *Psettarium* Goto and Ozaki, 1930.

The chondrichthyan blood flukes (*Chimaerohemecus trondheimensis* Van der Land, 1967, *Gymnurahemecus bulbosus* Warren, Ruiz, Whelan, Kritsky, and Bullard, 2019, *Ogawaia glaucostegi* Cutmore, Cribb, and Yong, 2018, *Electrovermis zappum* Warren and Bullard, 2019) and acipenseriform blood flukes (*Acipensericola* spp.) were recovered sister to all other actinopterygian blood flukes (Fig. 21; Table III). *Acipensericola petersoni* Bullard, Snyder, Jensen, and Overstreet, 2008 and *Acipensericola glacialis* Warren and Bullard, 2017 were recovered sister to the chondrichthyan blood flukes with low nodal support (0.51). *Acipensericola petersoni* and *A. glacialis* differ from *P. occidentalis* and species of *Sanguinicola* by 310 and 363 bp (79% similarity), respectively. Given the low nodal support for the clade representing the chondrichthyan blood flukes and actinopterygian blood flukes, we expect some change to the tree topology as more taxa are added and analyzed.

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FIGURE LEGENDS

Figures 1–3. *Sanguinicola plehnae* Warren and Bullard n. sp. infecting the heart of northern pike, *Esox lucius* Linnaeus, 1758 (Esociformes: Esocidae) from the upper Volga River, Russia. Scale values aside bars. Dorsal view. (**1**) Body of holotype (USNM No. 1688209). (**2**) spines of holotype. (**3**) genitalia of holotype (USNM No. 1688209). Abbreviations: esophagus (es), excretory system (e), female genital pore (fgp), intestine (in), lateral tegumental spines (s), male genital pore (mgp), mouth (mo), nerve commissure (nc), oötype (oo), ovary (o), oviduct (ov), seminal vesicle (sv), testis (t), and vitellarium (v).

Figure 4. *Sanguinicola plehnae* Warren and Bullard n. sp. infecting the heart of northern pike, *Esox lucius* Linnaeus, 1758 (Esociformes: Esocidae) from the upper Volga River, Russia. Scanning electron microscopy (**A – J**) and transmission electron microscopy (**K, L**). (**A**) Part of the ventro-lateral row of the anterior region of the worms distal tegumental cytoplasm. (**B**) Ventral view of entire worm. (**C–E**) Distal region of the spines projecting through the body surface. (**F**) Dorsal view of the posterior body. (**G**) Ventro-lateral area of the middle body. (**H**) Anterior terminal outgrowth. (**I**) Mouth with 2 tegumental elevations directed into the mouth cavity. (**J**) Ventral surface of mid-body. (**K**) Region of the distal tegumental cytoplasm. (**L**) Distal region of a lateral tegumental spine extending above distal tegumental cytoplasmic layer.

Abbreviations: anterior end (ae), anterior terminal outgrowth (ato), ciliated sensory ending (ce), dense granules (dg), distal tegumental cytoplasm (dtc), flattened syncytial cytoplasm (fc), flattened, smooth tegumental layer (ft), lateral tegumental spines (s), male genital pore (mgp), female genital pore (fgp), mouth (mo), posterior end (pe), surface depressions (sd), surface prominence (sp), tegumental elevations (te) and vesicles (v).

Figures 5–7. *Sanguinicola volgensis* (Rašín, 1929) McIntosh, 1934 from the heart of the sabrefish, *Pelecus cultratus* (Linnaeus, 1758) Berg, 1949 (Cypriniformes: Cyprinidae) from the upper Volga River, Russia. Scale values aside bars. Dorsal view. (**5**) Body of adult (USNM No. 1688210). (**6**) spines of adult (USNM No. 1688210). (**7**) genitalia of adult (USNM No. 1688211). Abbreviations: cirrus-sac (cs), esophagus (es), excretory system (e), female genital pore (fgp), intestine (in), lateral tegumental spines (s), male genital pore (mp), mouth (mo), nerve commissure (nc), oötype (oo), ovary (o), oviduct (ov), seminal vesicle (sv), testis (t), uterus (u), vas deferens (vd), vitellarium (v), and vitelline duct (vit).

Figures 8–11. Intestines of *Sanguinicola volgensis* (Rašín, 1929) McIntosh, 1934 from the heart of the sabrefish, *Pelecus cultratus* (Linnaeus, 1758) Berg, 1949 (Cypriniformes: Cyprinidae) from the upper Volga River, Russia. Scale value aside bar. (**8, 9, 10, 11**) Intestine of adults (USNM No. 1688211, 1688212, 1688213, 1688214).

Figure 12. *Sanguinicola volgensis* (Rašín, 1929) McIntosh, 1934 from the heart of the sabrefish, *Pelecus cultratus* (Linnaeus, 1758) Berg, 1949 (Cypriniformes: Cyprinidae) from the upper Volga River, Russia. Scanning electron microscopy. (**A, B**) Ventral view of anterior end. (**C**) Mouth on terminal outgrowth. (**D**) Ventral view of full body. (**E, F**) Dorsal view of posterior end. (**G**) Dorsal view of the posterior end. (**H, I**) Body surface of mid-body. (**J, K**) Body surface. Abbreviations: anterior end (ae), body surface (bs), everted cirrus (ec), female genital pore (fgp), knob-like ornamentation (ko), large specimens (I), lateral conical protrusions (lcp), lateral

conical, protrusions (lcp), lateral conical protrusions (lcp), male genital pore (mgp), mouth (mo), posterior end (pe), small (H), surface bulges (sb), and tegumental elevations (te).

Figure 13. *Sanguinicola volgensis* (Rašín, 1929) McIntosh, 1934 from the heart of the sabrefish, *Pelecus cultratus* (Linnaeus, 1758) Berg, 1949 (Cypriniformes: Leuciscidae) from the upper Volga River, Russia. Transmission electron microscopy. (**A**) Lateral body tegument. (**B**) Section of the anterior outgrowth. (**C**) Basal portion of the lateral tegumental spine. (**D, E**) Lateral conical protrusion. (**F, G**) Distal tegumental cytoplasm layer. Abbreviations: anterior esophagus (aes), ciliated sensory ending (ce), dense tegumental granules (dg), distal tegumental cytoplasmic layer (dtc), flattened sarcoplasmic extensions (fse), hemidesmosomes (hd), invagination of basal tegumental membrane (iv), knob-like ornamentation (ko), lateral tegumental spine (s), mouth (mo), muscle fibers (mf), pharynx (ph), surface bulges (sb), and vesicles (v).

Figures 14–15. *Sanguinicola* cf. *volgensis* from the heart of ide, *Leuciscus idus* (Linnaeus, 1758) Berg, 1949 (Cypriniformes: Leuciscidae) from the upper Volga River, Russia. Scale values aside bars. Dorsal view. (**14**) Body of adult (USNM No. 1688217). (**15**) Body of juvenile (USNM No. 1688218). Abbreviations: esophageal gland (eg), esophagus (es), excretory system (e), intestine (in), lateral tegumental spines (s), nerve commissure (nc), oötype (oo), ovary (o), testis (t), uterus (u), and vitellarium (v).

Figure 16. *Sanguinicola* cf. *volgensis* from the heart of ide, *Leuciscus idus* (Linnaeus, 1758) Berg, 1949 (Cypriniformes: Leuciscidae) from the upper Volga River, Russia. Scanning electron microscopy. (**A**) Ventral view of anterior end. (**B**) Full body. (**C**) Ventral view of anterior end. (**D**) Ventral view of mouth. (**E**) Dorsal body surface. (**F**) Body surface. (**G**) Lateral body surface. (**H**) Lateral body surface. (**I**) Lateral body surface. (**J**) Dorsal view of posterior end. (**K**) Ventral body surface. Abbreviations: anterior (ae), body surface (bs), ciliated receptor (cr), ciliated sensory ending (ce), everted cirrus (ec), female genital pore (fgp), knob-like ornamentation (ko), lateral conical protrusion (lcp), male genital pore (mgp), mouth (mo), posterior end (pe), spermatozoa (sz), spine (s), surface depressions (sd), surface prominences (sp), and tegumental elevations (te).

Figure 17. *Sanguinicola* cf. *volgensis* from the heart of ide, *Leuciscus idus* (Linnaeus, 1758) Berg, 1949 (Cypriniformes: Leuciscidae) from the upper Volga River, Russia. Transmission electron microscopy. (**A**) Lateral body tegument. (**B**) Section of lateral tegumental spines. (**C**, **D**) Lateral conical protrusions. (**E**) Section of individual lateral tegumental spine. (**F**) Basal portion of lateral tegumental spine. (**G**, **H**) Body surface. (**I**) Cytoplasm of the tegumental layer. Abbreviations: distal tegumental cytoplasmic layer (dtc), electron-dense granules (dg), flattened sarcoplasmic extensions (fse), hemidesmosomes (hd), invagination of basal tegumental membrane (iv), knob-like ornamentation (ko), lateral conical protrusions (lcp), lateral tegumental spine (s), muscle fibers (mf), surface prominences (sp), and vesicles (v).

Figures 18–20. *Pseudosanguinicola occidentalis* (Van Cleave and Mueller, 1932) Warren and Bullard n. comb. infecting the heart of walleye, *Sander vitreus* (Mitchill, 1818) Bailey et al., 2004 (Perciformes: Percidae) from Wisconsin and Oneida Lake, New York, USA. Scale values aside bars. Ventral view. (**18**) Body of voucher (new material) (USNM No. 1688219). (**19**) Spines of voucher (USNM No. 1688219). (**20**) Genitalia of voucher (USNM No. 1688219). Abbreviations: cirrus-sac (cs), esophagus (es), female genital pore (fgp), intestine (in), lateral

tegumental spines (s), male genital pore (mp), mouth (mo), nerve commissure (nc), oötype (oo), ovary (o), oviduct (ov), seminal vesicle (sv), testis (t), vas deferens (vd), vitellarium (v), and vitelline duct (vit).

Figure 21. Phylogenetic relationships of freshwater fish blood fluke species (Table III) reconstructed using Bayesian inference analysis using the large subunit ribosomal DNA (*28S*) gene. New sequences are shown in bold.

Table I. Species of *Sanguinicola* Plehn, 1905 accepted herein.

Species	Type Host	Locality	Reference
Sanguinicola armata Plehn, 1905 (type species)	tench, *Tinca tinca* (Linnaeus, 1758) Berg, 1949 (Cyprinininformes: Tincidae)	Europe	Plehn, 1905
Sanguinicola hasegawai Shimazu, 2013	loach, *Barbatula toni* (Dybowski, 1869) Okada, 1961 (Cypriniformes: Nemacheilidae)	Japan	Shimazu, 2013
Sanguinicola intermedia Ejsmont, 1926	Crucian carp, *Carassius carassius* (Linnaeus, 1758) Berg, 1949 (Cypriniformes: Cyprinidae)	E Europe	Ejsmont, 1926
Sanguinicola lophophora Erickson and Wallace, 1959	spottail shiner, *Hudsonus hudsonius* (Clinton, 1824) Gilbert, 1978 (Cypriniformes: Leuciscidae)	N America	Erickson and Wallace, 1959
Sanguinicola lungensis Tang and Lin, 1975	goldfish, *Carrasius auratus* (Linnaeus, 1758) Berg, 1949 (Cypriniformes: Cyprinidae)	China	Tang and Lin, 1975
Sanguinicola magnus Hu, Long, and Lee, 1965	grass carp, *Ctenopharyngodon idella* (Valenciennes, 1844) (Cypriniformes: Xenocyprididae)	Asia	Hu et al., 1965
Sanguinicola plehnae n. sp. Warren and Bullard, 2023	Northern pike, *Esox lucius* Linnaeus, 1758 (Esociformes: Esocidae)	Upper Volga River, Russia	present study
Sanguinicola rhodei Wang, 1983	giant Chinese bitterling, *Acheilognathus macropterus* (Bleeker, 1871) Chen and Li, 1989 (Cypriniformes: Acheilognathidae) (as *Acanthorhodeus taenianalis*)	Asia	Wang, 1983
Sanguinicola rutili Simón-Martín, Rojo-Vásquez, and Simón-Vicente, 1988	bermejuela, *Achondrostoma arcasii* (Steindachner, 1866) Robalo et al., 2007 (Cypriniformes: Leuciscidae) (as *Rutilus arcasi*)	Europe	Simon-Martin, 1988
Sanguinicola skrjabini Akhmerov, 1960	silver carp, *Hypophthalmichthys molitrix* (Valenciennes, 1844) Berg, 1949 (Cypriniformes: Xenocyprididae)	E Europe	Akhmerov, 1960
Sanguinicola volgensis (Rašín, 1929) McIntosh, 1934	sabrefish, *Pelecus cultratus* (Linnaeus, 1758) Berg, 1949 (Cypriniformes: Leuciscidae)	Upper Volga River, Russia	Rašín, 1929; present study

Table II. Species of *Sanguinicola* Plehn, 1905 needing additional study.

Species	Host	Locality	Reference
Sanguinicola alseae (Meade and Pratt, 1965) Holmes, 1971 *incertae sedis*	cutthroat trout, *Oncorhynchus clarkii* (Richardson, 1836) Lee et al., 1980 (Salmoniformes: Salmonidae)	North America	Meade and Pratt, 1965
Sanguinicola argentinensis Szidat, 1951 *incertae sedis*	streaked prochilod, *Prochilodus lineatus* (Valenciennes, 1837) Malabarba, 1989 (Characiformes: Prochilodontidae) (as *Prochilodus platensis)*	South America	Szidat, 1951
Sanguinicola chalmersi Odhner, 1924 *incertae sedis*	giraffe catfish, *Auchenoglanis occidentalis* (Valenciennes, 1840) Bailey and Stewart, 1984 (Siluriformes: Claroteidae)	Africa	Woodland, 1923; Odhner, 1924
Sanguinicola davisi Wales, 1958 *incertae sedis*	cutthroat trout, *Oncorhynchus clarkii* (Richardson, 1836) Lee et al., 1980 *(*Salmoniformes: Salmonidae)	North America	Wales, 1958
Sanguinicola fontinalis Hoffman, Fried, and Harvey, 1985 *incertae sedis*	brook trout, *Salvelinus fontinalis* (Mitchill, 1814) Okada, 1961 (Salmoniformes: Salmonidae)	N America	Hoffman et al., 1985
Sanguinicola huronis Fischthal, 1949 *incertae sedis*	smallmouth bass, *Micropterus dolomieu* Lacepède, 1802 (Centrarchiformes: Centrarchidae)	North America	Fischthal, 1949
Sanguinicola idahoensis Schell, 1974 *incertae sedis*	rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792) Tomelleri and Eberle, 1990 (Salmoniformes: Salmonidae)	North America	Schell, 1974
Sanguinicola incognita Akhmerov, 1959 *nomen nudum*	grass carp, *Ctenopharyngodon idella* (Valenciennes, 1844) (Cypriniformes: Xenocyprididae)	Eastern Europe	Akhmerov, 1959
Sanguinicola inermis Plehn, 1905 *incertae sedis*	Eurasion carp, *Cyprinus carpio* Linnaeus, 1758 (Cypriniformes: Cyprinidae)	Europe	Plehn, 1905
Sanguinicola klamathensis Wales, 1958 *incertae sedis*	cutthroat trout, *Oncorhynchus clarkii* (Richardson, 1836) Lee et al., 1980 *(*Salmoniformes: Salmonidae)	North America	Wales, 1958
Sanguinicola maritimus Nolan and Cribb, 2005 *incertae sedis*	brownspotted wrasse, *Notolabrus parilus* (Richardson, 1850) Russell, 1988 (Labriiformes: Labridae)	Southwest Pacific	Nolan and Cribb, 2005
Sanguinicola megalobramae Li, 1980 *nomen nudum*	Wuchang bream, *Megalobrama amblycephala* Yih, 1955 (Cypriniformes: Xenocyprididae)	Asia	Li, 1980
Sanguinicola sanliense Wang, 1982 *incertae sedis*	yellow catfish, presumably *Tachysurus sinensis* Lacepède, 1803 (Siluriformes: Bagridae) (scientific name not included in publication)	Asia	Wang, 1982

Sanguinicola shantsuensis Lung and Chen, 1965 *nomen nudum*	Crucian carp, *Carassius carassius* (Linnaeus, 1758) Berg, 1949 (Cypriniformes: Cyprinidae)	Asia	Lung and Shen, 1965 (cited by Li, 1980, Tang and Lin, 1975, Shimazu, 2007)
Sanguinicola ugui Shimazu, 2007 *incertae sedis*	big-scaled redfin, *Pseudaspius hakonensis* (Günther, 1877) Dyldin et al., 2020 (Cypriniformes: Leuciscidae) (as *Tribolodon hakonensis*)	Japan	Shimazu, 2013

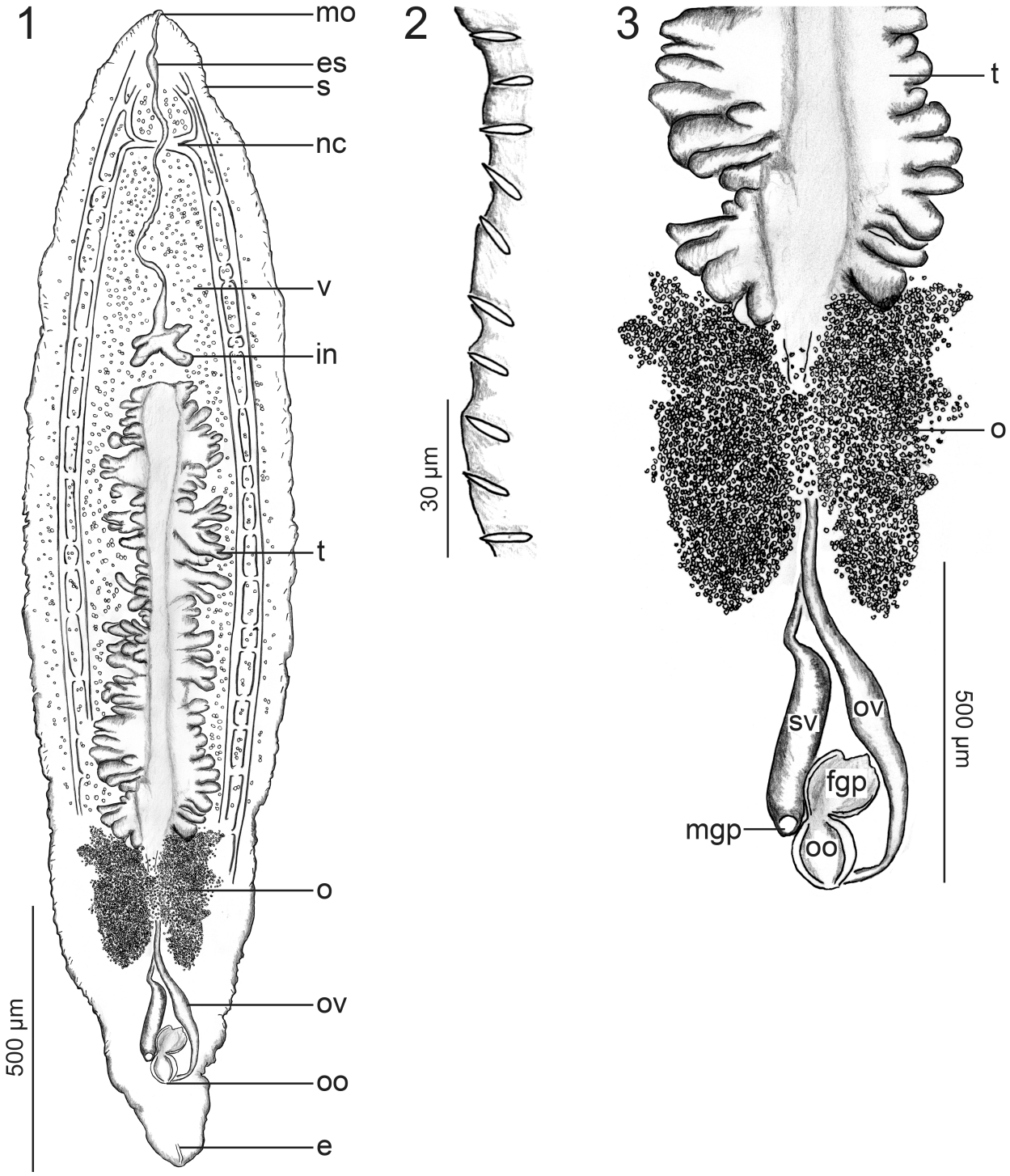
Table III. Fish blood fluke *28S* sequences used in the present study.

Taxa	Host	Locality	GenBank Accession #s	Reference
Acipensericola glacialis Warren and Bullard, 2017	lake sturgeon, *Acipenser fulvescens* (Rafinesque, 1817) (Acipenseriformes: Acipenseridae)	Lake Winnebago and Lake Butte des Morte, Wisconsin, USA	MF186849	Warren et al., 2017b
Acipensericola petersoni Bullard, Snyder, Jensen, and Overstreet, 2008	American paddlefish, *Polyodon spathula* (Walbaum, 1792) (Acipenseriformes: Polyodontidae)	Mississippi River and Tennessee River (Mississippi River Basin), USA	KY243879	Orélis-Ribeiro et al., 2017
Aporocotyle argentinensis Smith, 1969	Argentine hake, *Merluccius hubbsi* Marini, 1933 (Gadiformes: Merlucciidae)	off north Patagonia, Argentina	JX094803	Hernández-Orts et al., 2012
Aporocotyle mariachristinae Hernández-Orts, Alama-Bermejo, Carrilo, Garcia, Crespo, Rasga, and Montero, 2012	pink cusk-eel, *Genypterus blacodes* (Forster, 1801) Chong, 1985 (Ophidiiformes: Ophidiidae)	off north and central Patagonia, Argentina	JX094802	Hernández-Orts et al., 2012
Aporocotyle spinosicanalis Williams, 1958	European hake, *Merluccius merluccius* (Linnaeus, 1758) Svetovidov, 1973 (Gadiformes: Merlucciidae)	off Orkney islands, NE Atlantic Ocean (North Sea)	AY222177	Olson et al., 2003
Chimaerohemecus trondheimensis Van der Land, 1927	rabbit fish, *Chimaera monstrosa* Linnaeus, 1758 (Chimaeriformes: Chimaeridae)	NE Atlantic, off Bergen, Norway	AY157239	Lockyer et al., 2003
Electrovermis zappum Warren and Bullard, 2019	lesser electric ray, *Narcine bancroftii* Say, 1821 (Torpediniformes: Narcinidae)	Gulf of Mexico, off Fort Morgan, Alabama, USA	MN244242	Warren and Bullard, 2019
Elopicola bristowi Orélis-Ribeiro and Bullard, 2017	Hawaiian ladyfish, *Elops hawaiensis* Reagan, 1909 (Elopiformes: Elopidae)	Eastern Sea, off Nha Trang, Vietnam	KY243881	Orélis-Ribeiro et al., 2017
Elopicola franksi Orélis-Ribeiro and Bullard, 2017	tarpon, *Megalops atlanticus* Valenciennes, 1847 (Elopiformes:	Gulf of Mexico, off Florida, USA	KY243882	Orélis-Ribeiro et

	Megalopidae)			al., 2017
Elopicola nolancribbi Bullard, 2014	ladyfish, *Elops saurus* Linnaeus, 1766 (Elopiformes: Elopidae)	Gulf of Mexico, off Ship Island, Mississippi, USA	KY243880	Orélis-Ribeiro et al., 2017
Gymnurahemecus bulbosus Warren and Bullard, 2019	smooth butterfly ray, *Gymnura micrura* (Bloch and Schneider, 1801) Uyeno and Miyake, 1983 (Myliobatiformes: Gymnuridae)	Gulf of Mexico, Mobile, Alabama, USA	MH555432	Warren et al., 2019
Neoparacardicola nasonis Yamaguti, 1970	bluespine unicornfish, *Naso unicornis* (Forsskål, 1775) Lindberg and Krasyukova, 1975 (Acanthuriformes: Acanthuridae)	off Lizard Island, Australia	AY222179	Olson et al., 2003
Ogawaia glaucostegi Cutmore, Cribb, and Yong, 2018	giant shovelnose ray, *Glaucostegus typus* (Anonymous [Bennett], 1830) Compagno et al., 2005 (Rhinopristiformes: Glaucostegidae)	Moreton Bay, Queensland, Australia	MF503308	Cribb et al., 2017a
Plethorchis acanthus Martin, 1975	striped mullet, *Mugil cephalus* Linnaeus, 1758 (Mugiliformes: Mugilidae)	Brisbane River, Queensland, Australia	AY222178	Olson et al., 2003
Psettarium hustoni Yong, Cutmore, Jones, Gauthier, and Cribb, 2018	black-spotted puffer, *Arothron nigropunctatus* (Bloch and Schneider, 1801) Dor 1984 (Tetraodontiformes: Tetraodontidae)	Flora Reef, Queensland, Australia	MG709037	Yong et al., 2018
Psettarium pandora Yong, Cutmore, Jones, Gauthier, and Cribb, 2018	yellow boxfish, *Ostracion cubicus* Linnaeus, 1758 (Tetraodontiformes: Ostraciidae)	North Wistari Reef, off Heron Island, Queensland, Australia	MG709046	Yong et al., 2018
Psettarium pulchelum Yong, Cutmore, Bray, Miller, Semarariana, Palm and Cribb, 2016	narrow-lined puffer, *Arothron manilensis* (Marion de Procé 1822) Matsuura in Masuda et al. 1984 (Tetraodontiformes: Tetraodontidae)	Off Peel Island, Moreton Bay, southeast Queensland, Australia	MG709049	Yong et al., 2018
Psettarium yoshidai Yong, Cutmore, Jones, Gauthier, and Cribb, 2018	map puffer, *Arothron mappa* (Lesson 1831) Matsuura in Masuda et al. 1984 (Tetraodontiformes:	Gold Coast Seaway, Gold Coast, southeast Queensland, Australia	MG709051	Yong et al., 2018

	Tetraodontidae)			
Pseudosanguinicola occidentalis n. gen., n. comb. Warren and Bullard, 2023	walleye, *Sander vitreus* (Mitchill, 1818) Bailey et al. 2004 (Perciformes: Percidae)	Wisconsin, USA	OQ715339	present study
Pseudosanguinicola occidentalis n gen, n. comb. Warren and Bullard, 2023	walleye, *Sander vitreus* (Mitchill, 1818) Bailey et al. 2004 (Perciformes: Percidae)	Oneida Lake, New York, USA	OQ715338	present study
Sanguinicola plehnae n. sp. Warren and Bullard, 2023	Northern pike, *Esox lucius* Linnaeus, 1758 (Esociformes: Esocidae)	Upper Volga River, Russia	OQ715334	present study
Sanguinicola plehnae n. sp. Warren and Bullard, 2023	Northern pike, *Esox lucius* Linnaeus, 1758 (Esociformes: Esocidae)	Upper Volga River, Russia	OQ715335	present study
Sanguinicola cf. *inermis*	great pond snail, *Lymnaea stagnalis* (Gastropoda: Lymnaeidae)	Warminia-Mazury Region, Poland	AY222180	Olson et al., 2003
Sanguinicola cf. *volgensis*	ide, *Leuciscus idus* (Linnaeus, 1758) Berg, 1949 (Cypriniformes: Leuciscidae)	Upper Volga River, Russia	OQ715336	present study
Sanguinicola cf. *volgensis*	ide, *Leuciscus idus* (Linnaeus, 1758) Berg, 1949 (Cypriniformes: Leuciscidae)	Upper Volga River, Russia	OQ715337	present study
Sanguinicola volgensis (Rašín, 1929) McIntosh, 1934	sabrefish, *Pelecus cultratus* (Linnaeus, 1758) Berg, 1949 (Cypriniformes: Leuciscidae)	Upper Volga River, Russia	OQ715332	present study
Sanguinicola volgensis (Rašín, 1929) McIntosh, 1934	sabrefish, *Pelecus cultratus* (Linnaeus, 1758) Berg, 1949 (Cypriniformes: Leuciscidae)	Upper Volga River, Russia	OQ715333	present study
Skoulekia erythrini Palacios-Abella, Georgieva, Mele, Raga, Isbert, Kostadinova, and Montero, 2017	common pandora, *Pagellus erythrinus* (Linnaeus, 1758) Tortonese, 1973 (Perciformes: Sparidae)	Off Santa Pola, Spain	MF043944	Palacios-Abella et al., 2017
Skoulekia meningialis Alama-Bermejo, Montero, Raga, and Holzer, 2011	common two-banded sea bream, *Diplodus vulgaris* (Geoffroy St. Hilaire, 1817) Tortonese, 1973 (Perciformes: Sparidae)	off Valencia, Spain	FN652293	Alama-Bermejo et al., 2011

Out-group				
Baracktrema obamai Roberts, Platt, and Bullard, 2016	black marsh turtle, *Siebenrockiella crassicollis* (Gray, 1831) (Cryptodira: Geoemydidae)	Perak, Perak River, Malaysia	KX061500	Roberts et al., 2016
Spirorchis artericola (Ward, 1921) Stunkard, 1921	painted turtle, *Chrysemys picta* (Schneider, 1783) (Cryptodira: Emydidae)	Reelfoot Lake, Tennessee, USA	AY604704	Snyder, 2004
Vasotrema cf. *robustum*	Gulf Coast spiny softshell turtle, *Apalone spinifera aspera* (Agassiz, 1857) (Cryptodira: Trionychidae)	Round Lake, Cahaba River, Alabama, USA	MH843490	Roberts and Bullard, 2017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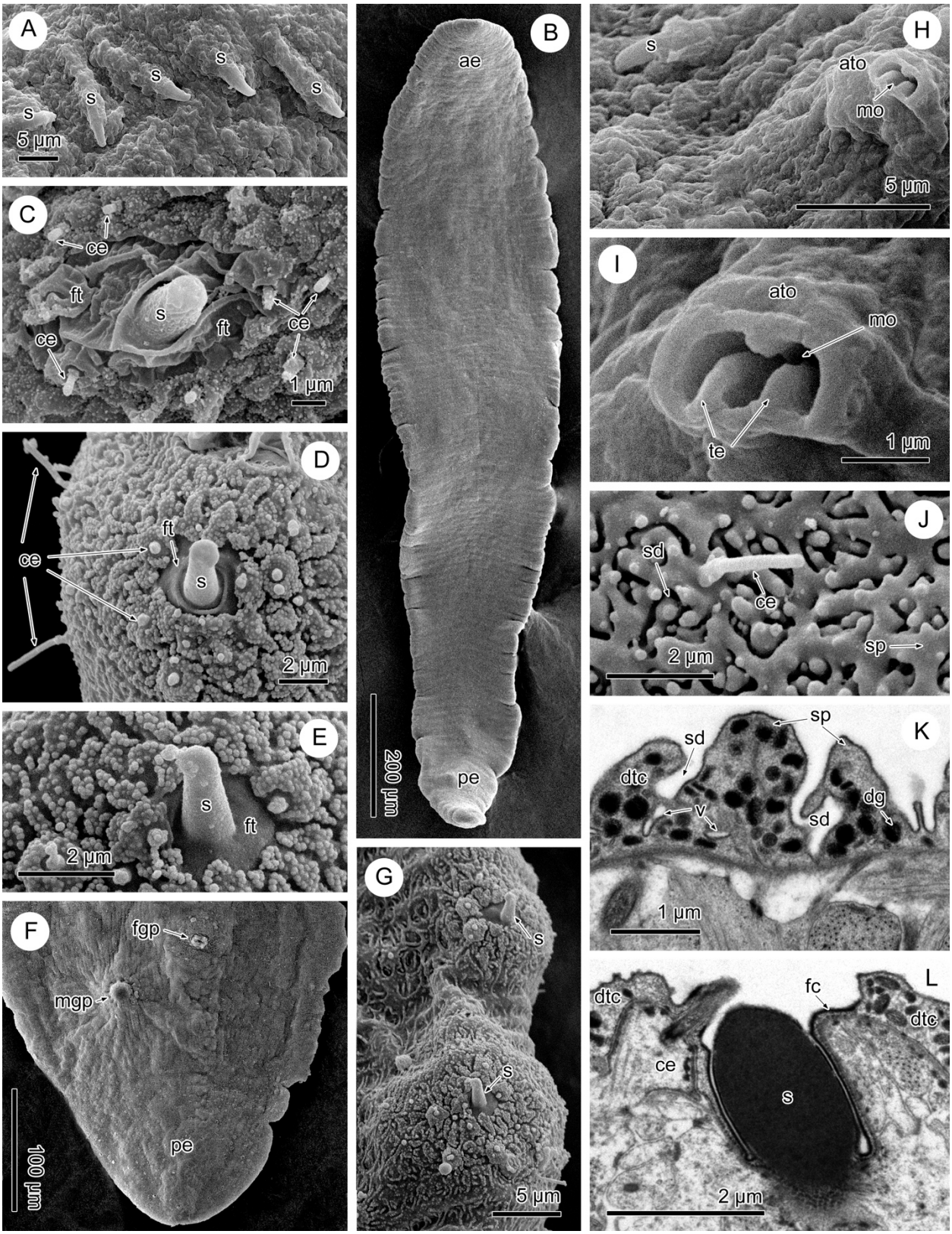
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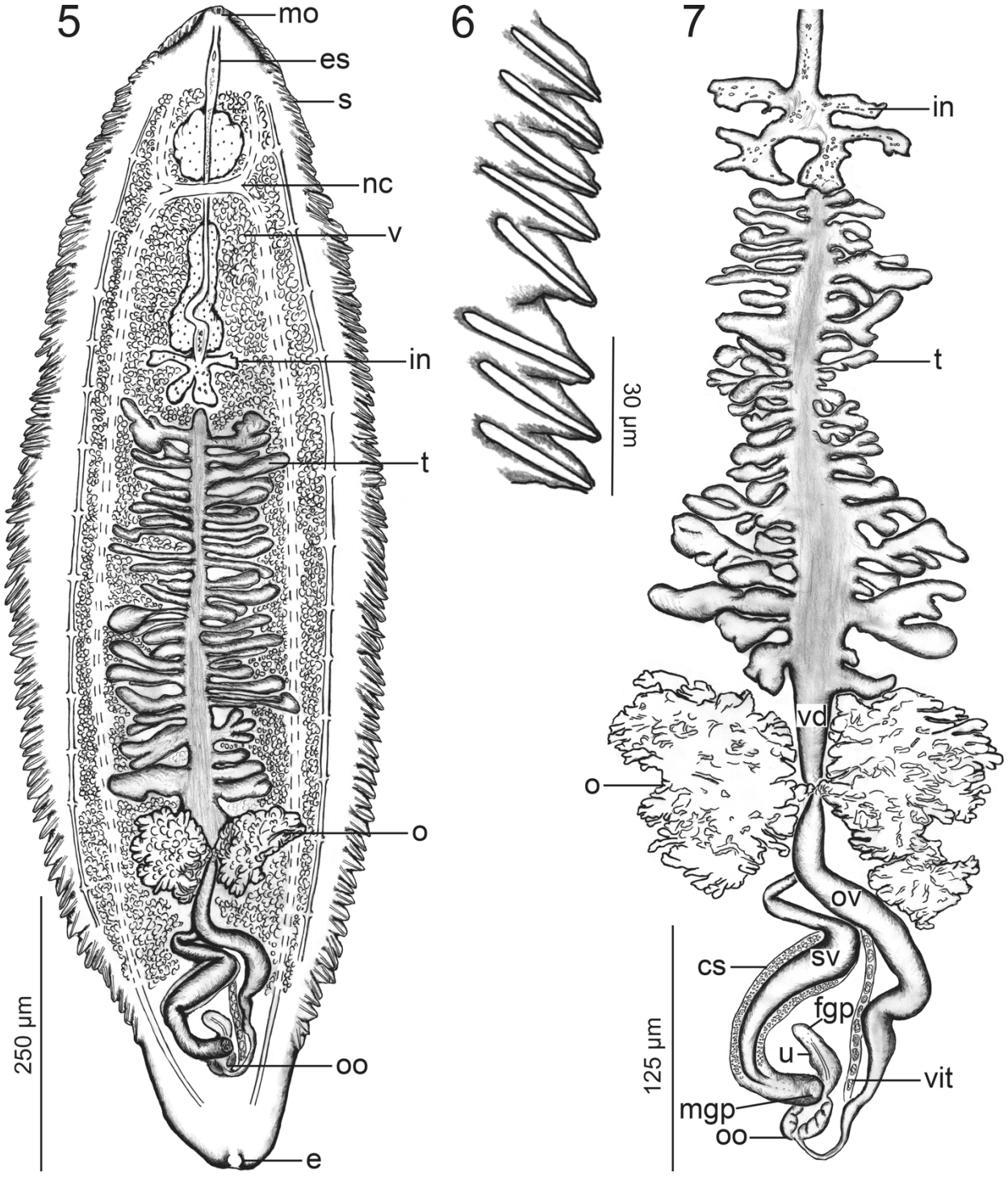
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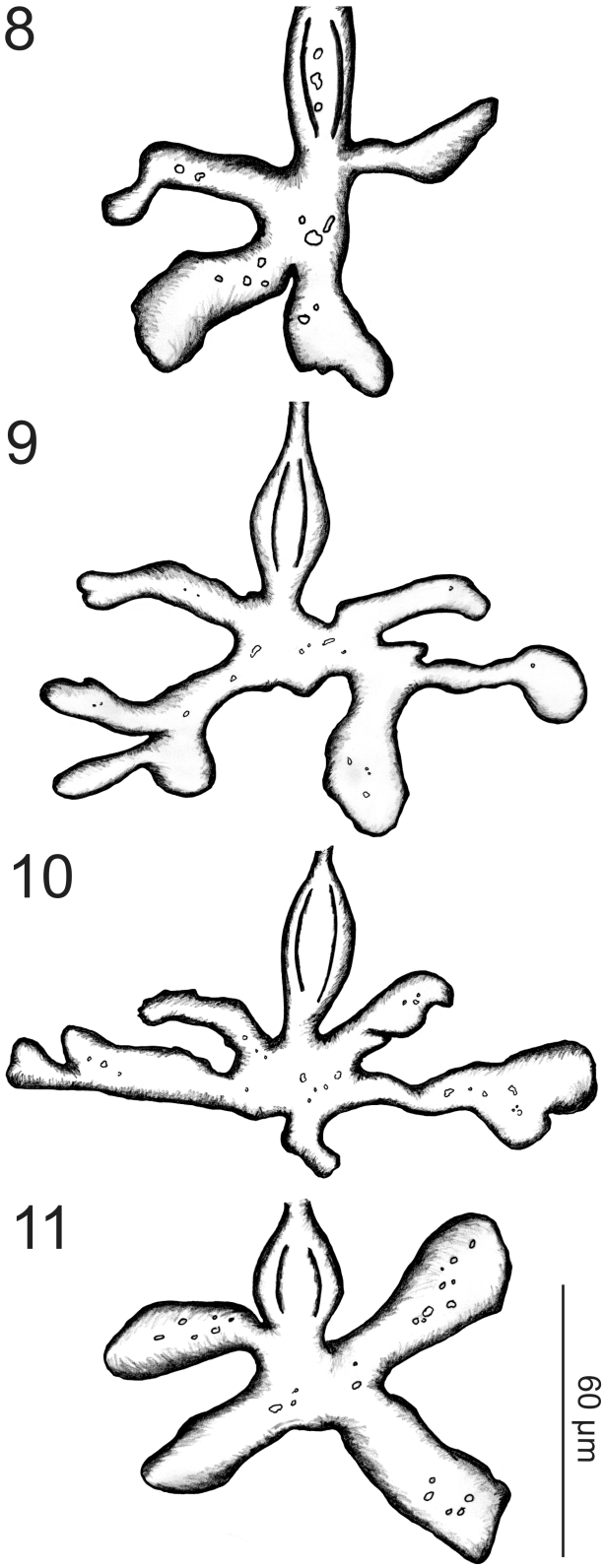
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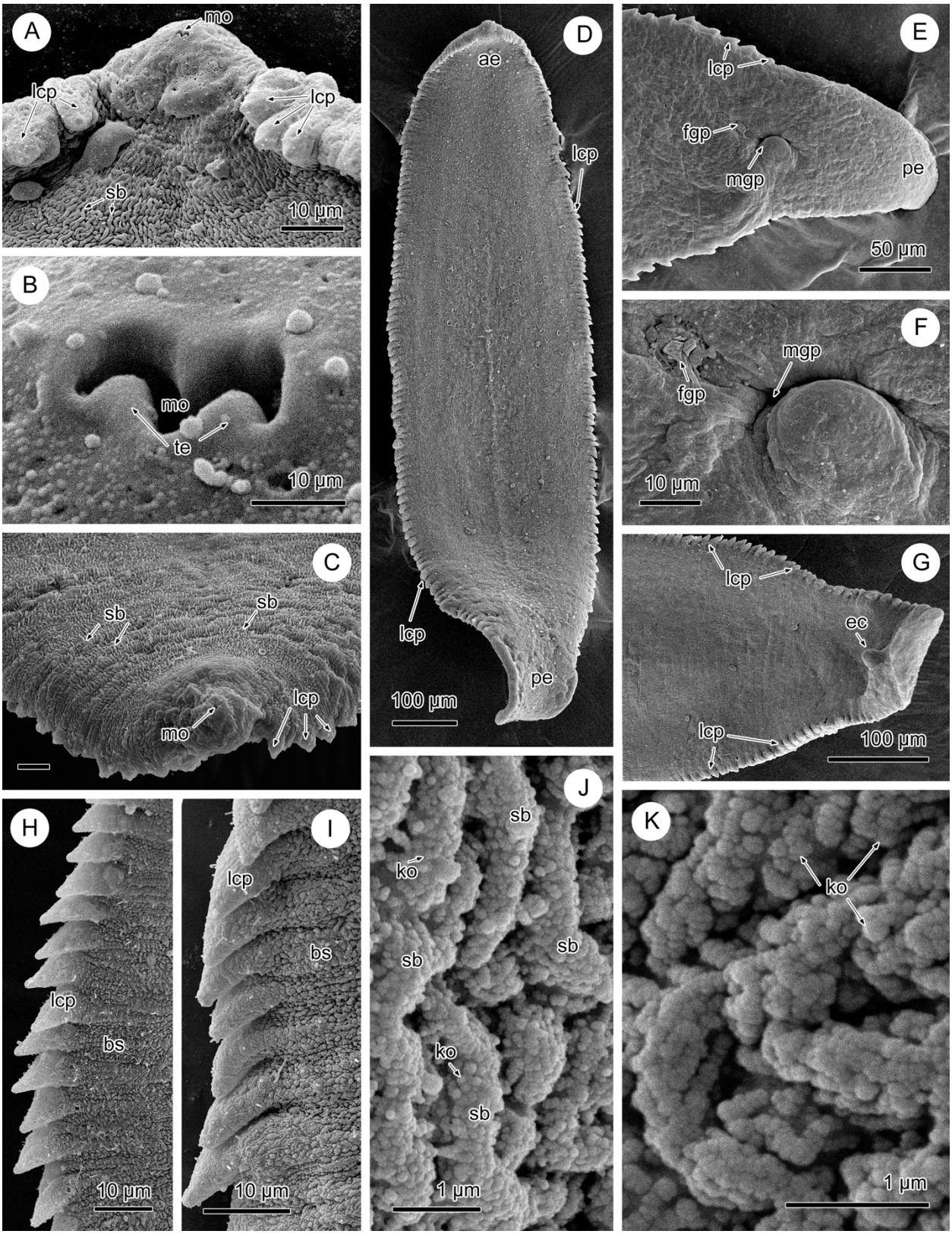
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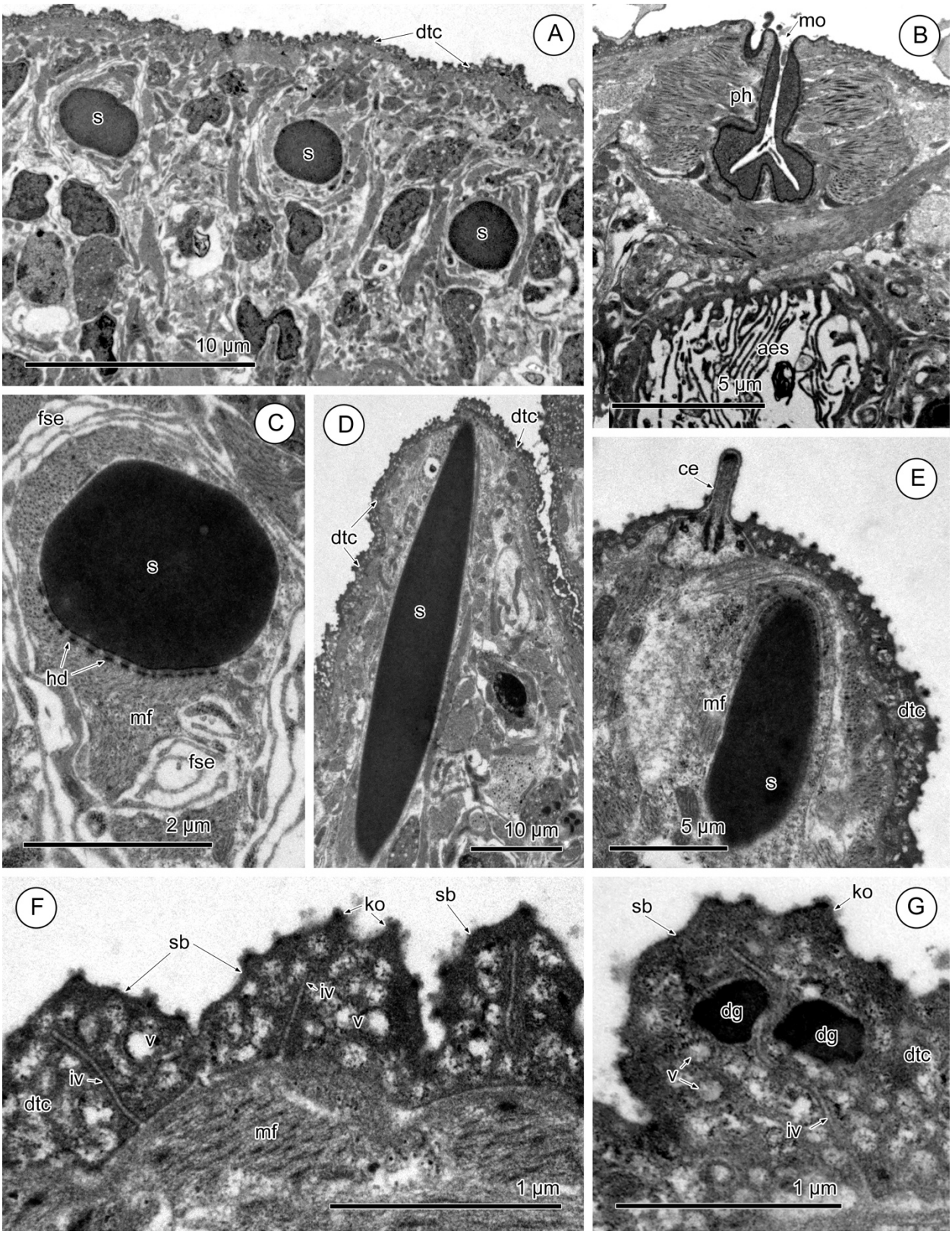
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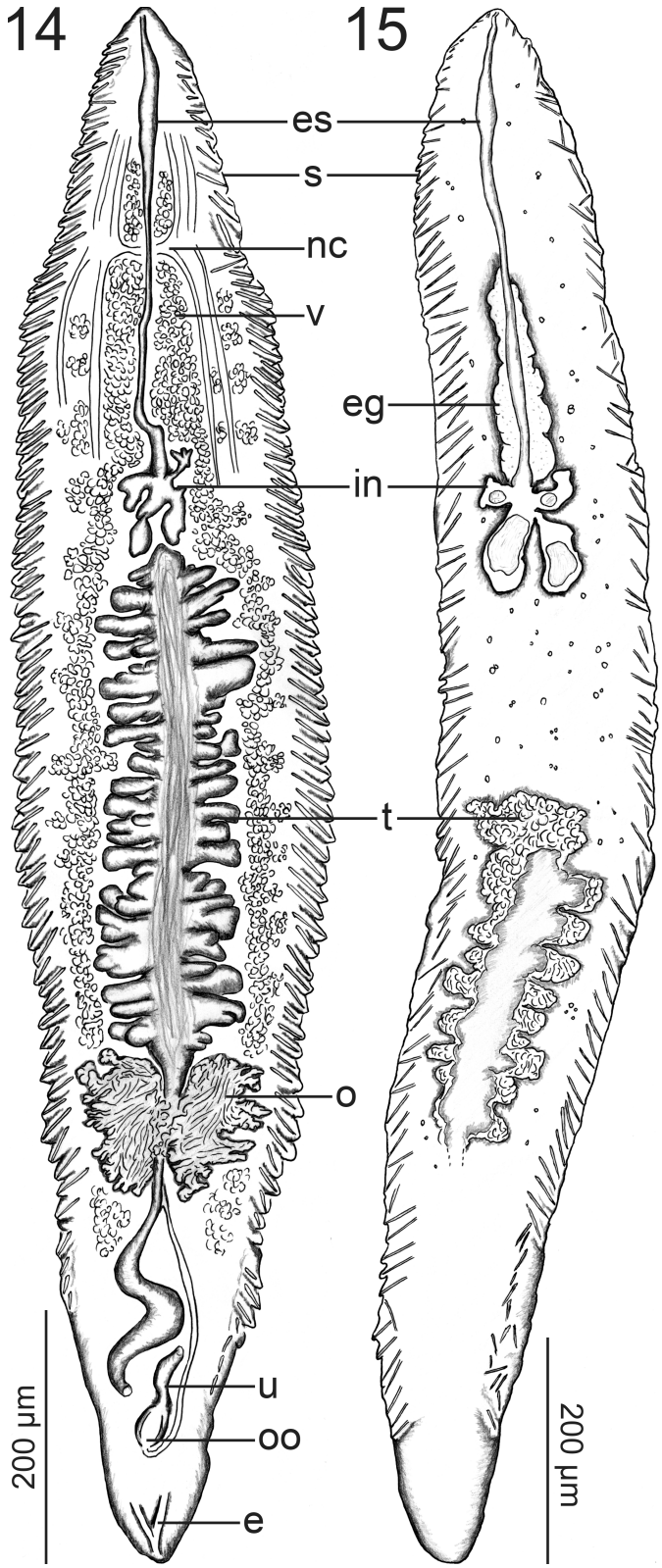
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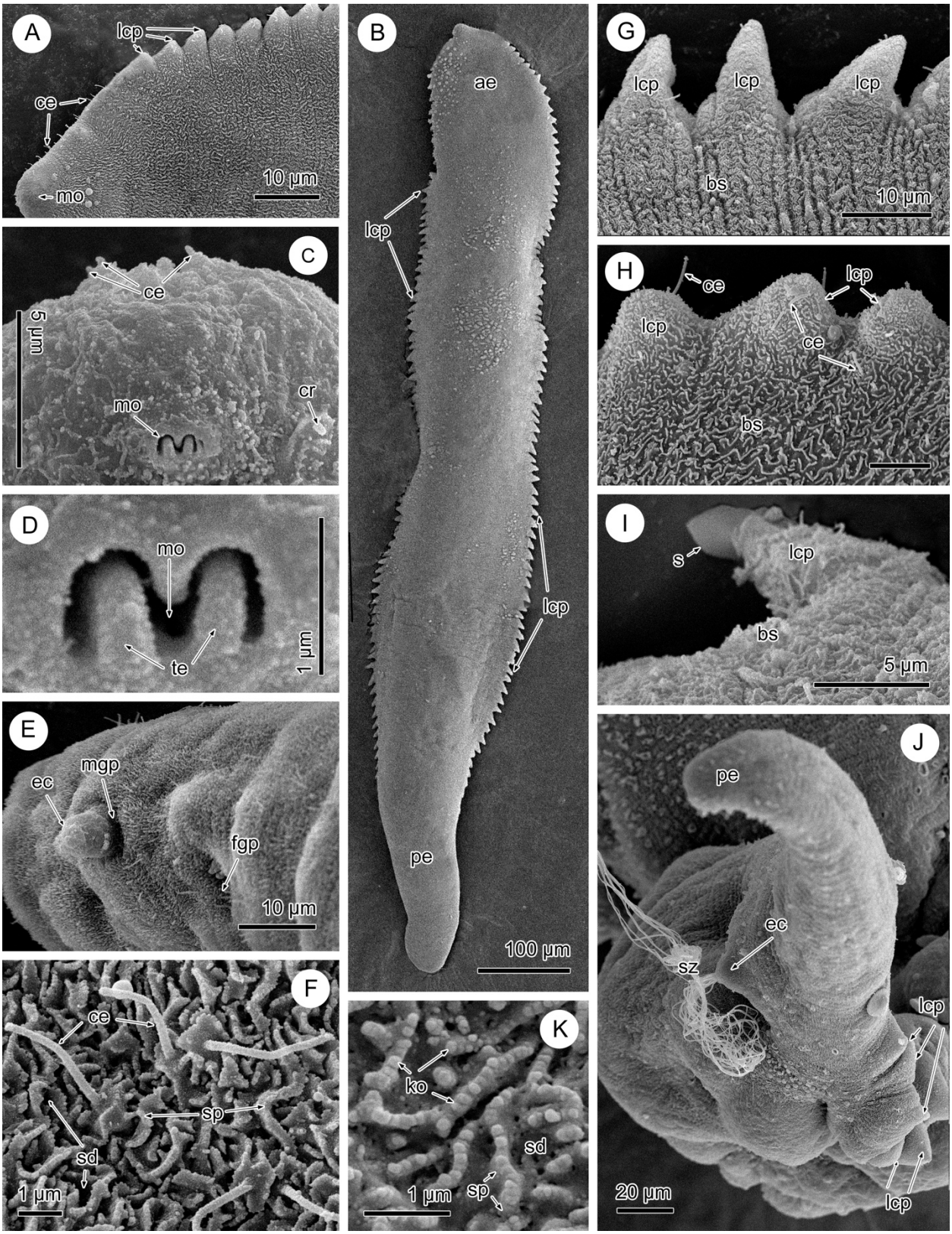
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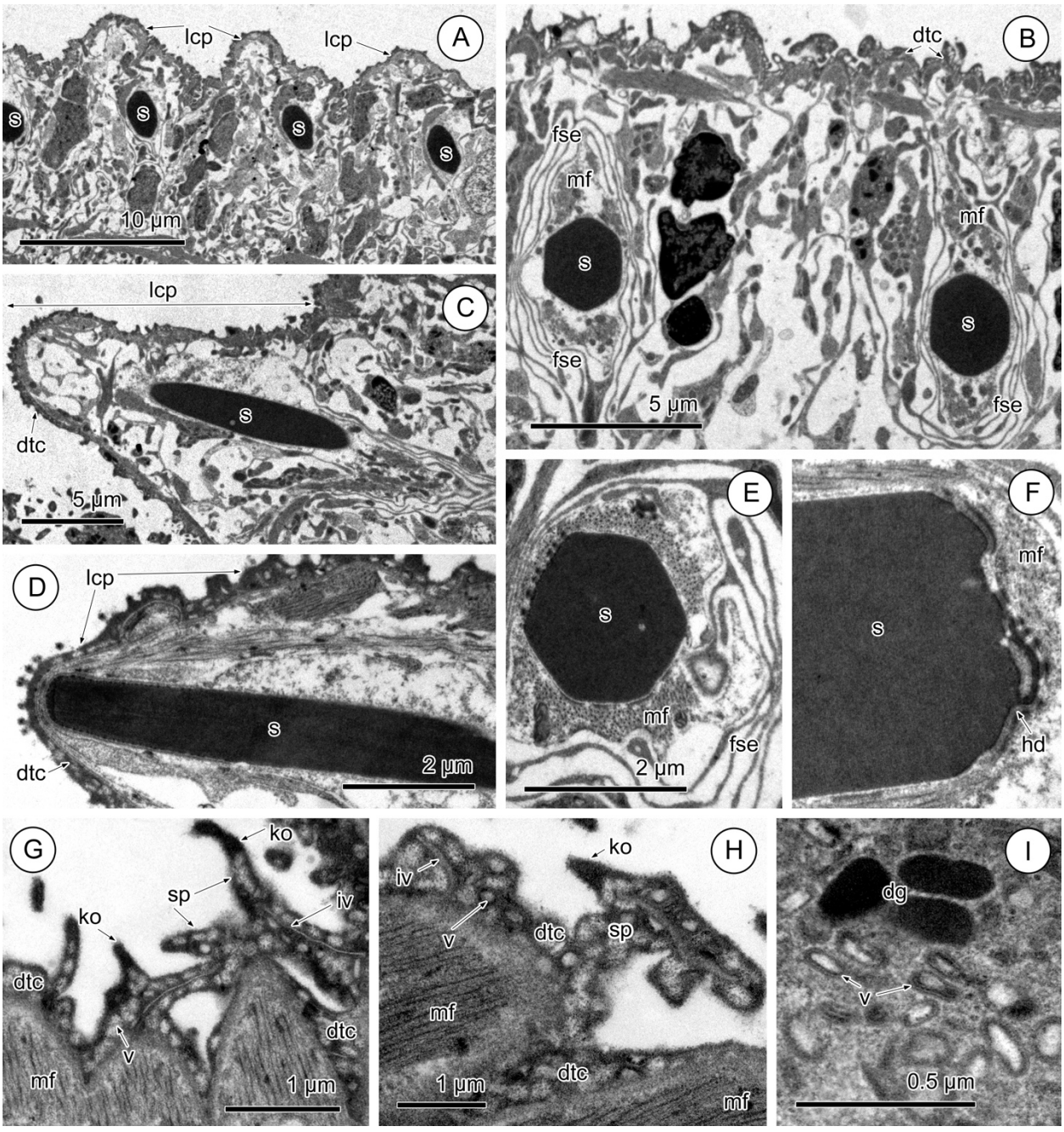
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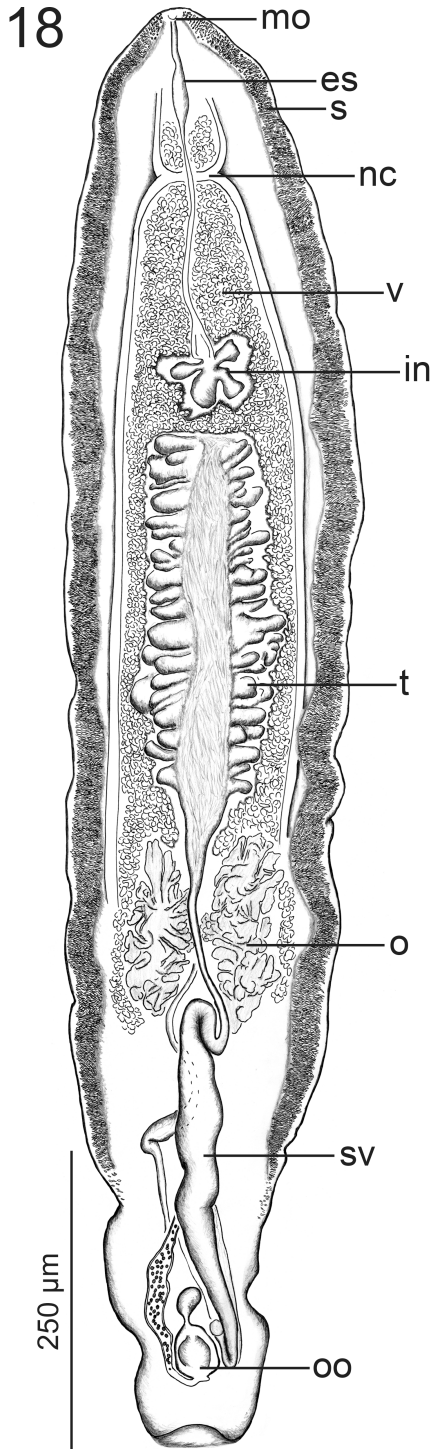
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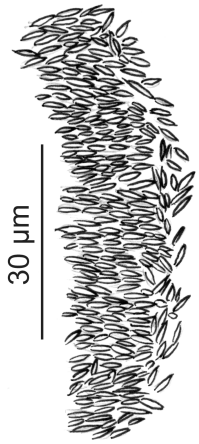
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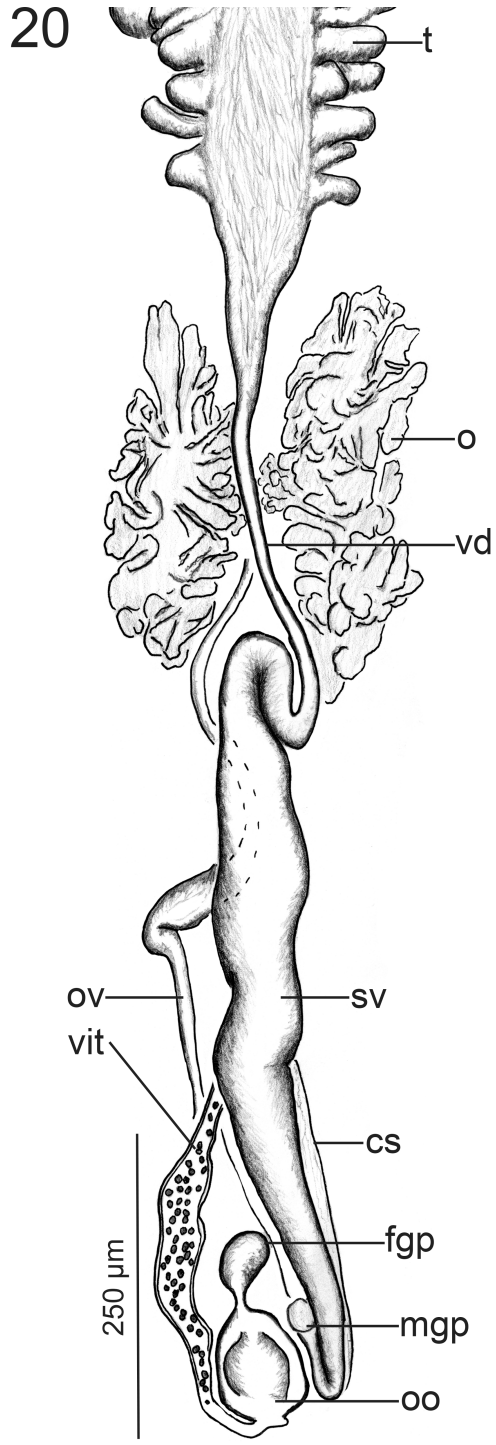
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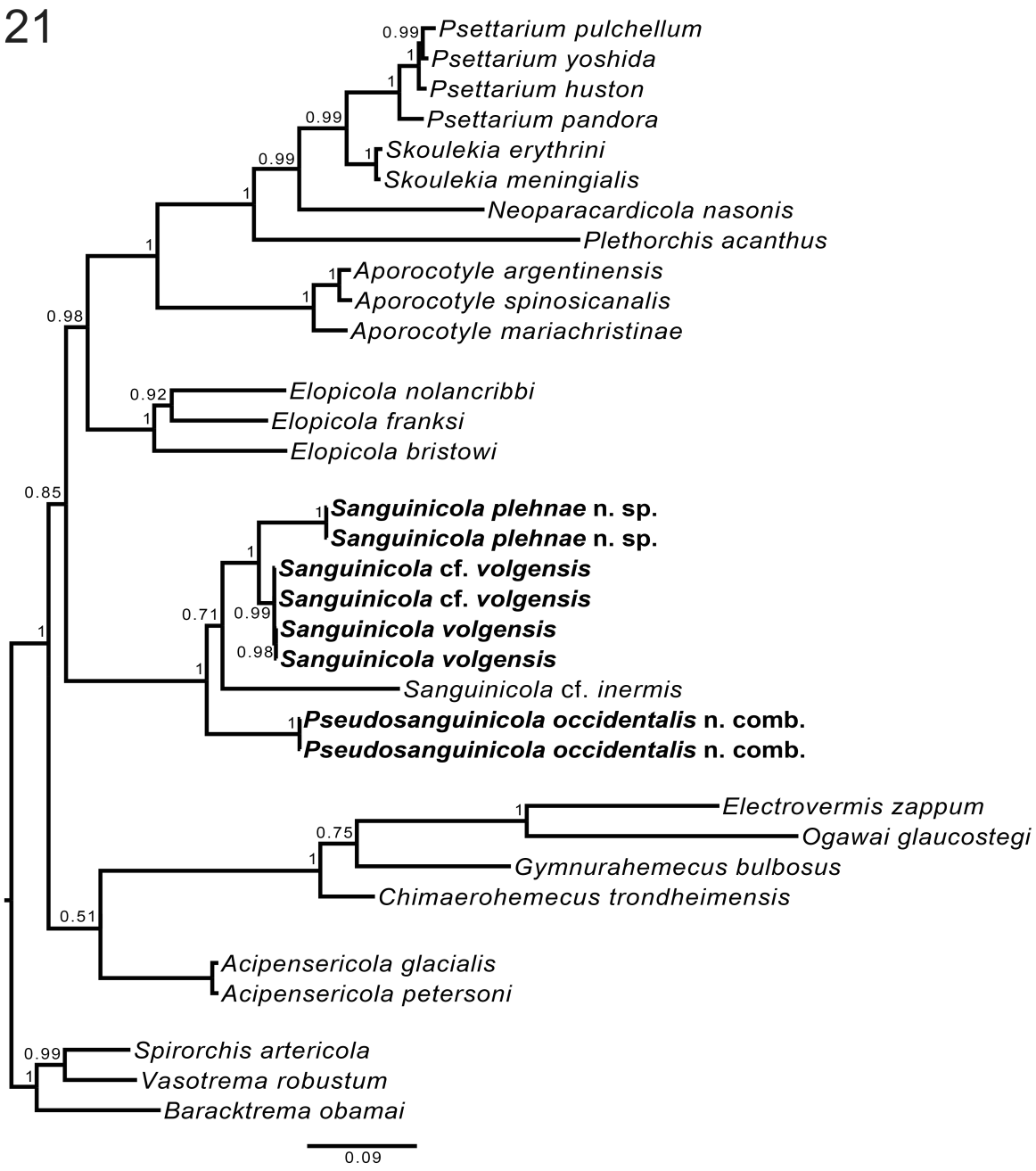
21
Psettarium pulchellum
Psettarium yoshida
Psettarium huston
Psettarium pandora
Skoulekia erythrini
Skoulekia meningialis
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Plethorchis acanthus
Aporocotyle argentinensis
Aporocotyle spinosicanalis
Aporocotyle mariachristinae
Elopicola nolancribbi
Elopicola franksi
Elopicola bristowi
Sanguinicola plehnae n. sp.
Sanguinicola plehnae n. sp.
Sanguinicola cf. volgensis
Sanguinicola cf. volgensis
Sanguinicola volgensis
Sanguinicola volgensis
Sanguinicola cf. inermis
Pseudosanguinicola occidentalis n. comb.
Pseudosanguinicola occidentalis n. comb.
Electrovermis zappum
Ogawai glaucostegi
Gymnurahemecus bulbosus
Chimaerohemecus trondheimensis
Acipensericola glacialis
Acipensericola petersoni
Spirorchis artericola
Vasotrema robustum
Baracktrema obamai
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CHAPTER 4: SYSTEMATIC REVISION OF THE FISH BLOOD FLUKES WITH DIAGNOSES OF CHIMAEROHEMECIDAE YAMAGUTI, 1971, ACIPENSERICOLIDAE N. FAM., SANGUINICOLIDAE POCHE, 1926, ELOPICOLIDAE N. FAM., AND APOROCOTYLIDAE ODHNER, 1912

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Authors: Micah B. Warren and Stephen A. Bullard

ABSTRACT

We herein morphologically diagnose the 5 natural groups of fish blood flukes and name them. Species of Chimaerohemecidae Yamaguti, 1971 infect chimeras, sharks, and rays (Chondrichthyes) and have C-shaped lateral tegumental spines and a non-sinusoidal testis or lack spines and have a sinusoidal testis. Species of Acipensericolidae n. fam. infect sturgeons and paddlefish (Acipenseriformes) and have a robust, bowl-shaped, pedunculate anterior sucker, lateral tegumental spines that are spike-like (not C-shaped), an inverse U-shaped intestine (anterior ceca absent) with posterior ceca terminating near the excretory bladder, 6 testes (inter-cecal, ovoid or oblong, lacking deep lobes; including 1 post-ovarian testis), a Laurer's canal, and a dextral common genital pore. Species of Sanguinicolidae Poche, 1926 infect primarily later-branching freshwater ray-finned fishes (Teleostei) and have a diminutive anterior sucker, a medial esophageal swelling (pouch), short, radial ceca of approximately equal length or short anterior ceca plus an elongate, dendritic posterior cecum, testis with appendix-like lateral lobes, no Laurer's canal, and separate or common genital pores. Species of Elopicolidae n. fam. infect ladyfishes, tarpons, and catadromous eels (Elopomorpha) and have a robust, bowl-shaped, pedunculate anterior sucker, lateral tegumental spines that are spike-like (can be lost in adult), short or indistinct anterior ceca, posterior ceca that terminate at level of the testis(es), a single

testis or 2 testes, a Laurer's canal present or absent, and a sinistral common genital pore and atrium. Species of Aporocotylidae Odhner, 1912 primarily infect later branching marine and estuarine ray-finned fishes (Teleostei) and have a spheroid anterior sucker with concentric rows of circumferential spines or the spheroid anterior sucker is lost in adults or adults have a diminutive anterior sucker, a sinuous esophagus lacking a pouch, an X- or H-shaped intestine having 4 ceca, long anterior ceca (or secondarily lost), smooth posterior ceca that extend posteriad in parallel with respective body margin and terminate near the posterior body end, testis(es) that lack appendix-like lateral lobes, no Laurer's canal, and a sinistral common genital pore or separate genital pores that are sinistral. Our *28S* phylogeny recovered the fish blood flukes as monophyletic and each of the morphologically-diagnosed families as monophyletic and sister to the remaining blood flukes infecting turtles and homeotherms. Acipensericolidae was recovered sister to the clade comprising Chimaerohemecidae + Sanguinicolidae and Elopicolidae + Aporocotylidae. The branching order and inter-relationships of these families remains unsettled perhaps because of low taxon sampling among non-aporocotylids and extinction of intermediate taxa.

KEY WORDS

Fish, Blood fluke, Digenea, Taxonomy, Systematics

The fish blood flukes, "Aporocotylidae Odhner, 1912" (see Bullard et al., 2009), presently comprise 5 morphologically distinct but as of yet undiagnosed lineages, 46 accepted genera, and >175 spp. Yamaguti (1958, 1971) proposed several subfamilies for the relatively few genera that were accepted at the time; however, none of those subfamilies were adopted because their diagnoses were based on erroneous character state assignments or homoplasy and because they are routinely and unequivocally recovered as paraphyletic or polyphyletic (Bullard et al., 2006,

2008, 2012; Orélis-Ribeiro et al., 2014; Orélis-Ribeiro and Bullard, 2015, 2016; Warren and Bullard, 2019, 2021; Warren et al., *in review*, 2017, 2019, 2020, 2021). Since Smith (1997a, 1997b, 2002) reviewed the 20 genera of fish blood flukes that were accepted in 2002, an additional 26 genera and >100 species have been described. This later body of work collectively included many new taxonomic characters and character states with which to diagnose, differentiate, and classify fish blood fluke genera and the 5 main clades they comprise (Bullard and Jensen, 2008; Bullard and Overstreet, 2003, 2008; Bullard, 2010a, 2010b, 2014; Bullard et al., 2006, 2008, 2012; Cutmore et al., 2022; Orélis-Ribeiro et al., 2013, 2014, 2017; Truong and Bullard, 2013; Orélis-Ribeiro and Bullard, 2015, 2016; Cribb et al., 2017; Warren et al., *in review*, 2017, 2019, 2020, 2021; Warren and Bullard, 2019, 2020). We herein diagnose these lineages using morphology, propose new families for them, and test this new classification scheme using a *28S* phylogeny.

MATERIALS AND METHODS

The new and elevated family group names herein follow the recommendations of the International Commission on Zoological Nomenclature (ICZN, 1999; Notton et al., 2011). In specific, Article 29.1., Formation of family-group names, states that "*A family-group name is formed by adding to the stem of the name [Art. 29.3] of the type genus, or to the entire name of the type genus [see Article 29.6], a suffix as specified in Article 29.2*" (Yamaguti, 1958, 1971). Article 29.3., Determination of stem in names of type genera, states that "*The stem of a family-group name is based on the name of its type genus [Art. 63]...*" Article 36, Principal of Coordination, states that "*A name established for a taxon at any rank in the family group* (which includes the subfamily, family, and superfamily) *is deemed to have been simultaneously established for nominal taxa at all other ranks in the family group; all these taxa have the same*

type genus, and their names are formed from the stem of the name of the type genus [Art. 29.3] with appropriate change of suffix [Art. 34.1]. The name has the same authorship and date at every rank." Article 64., Choice of type genus, states that "*An author who wishes to establish a new nominal family-group taxon may choose as type genus any included nominal genus the name of which he or she regards as valid [Art. 11.7.1], not necessarily that having the oldest name. The choice of type genus determines the stem of the name of the nominal family-group taxon [Art. 29.1]*."

Each family diagnosis herein includes the available information on the known/reported intermediate and definitive hosts, site of infection in the definitive host, cercarial morphology, egg shape and site of infection, schistosomulum morphology, and adult morphology. For some of the proposed families, the life cycles of none, few, or several of the species are known.

Phylogenetic methods follow that of Warren and Bullard (2019), Bullard and Dutton (2022), and Whelan et al. (2022) (Table I). Members of Liolopidae Dollfus, 1934, Diplostomoidea Poirier, 1886, and Brachylaimoidea Joyeuz and Foley, 1930 were selected as functional outgroup taxa for the Schistosomatoidea Stiles and Hassal, 1898 (see De León and Hernández-Mena, 2019; Bullard and Dutton, 2022). All taxa included in the phylogenetic analysis have been published with morphological descriptions except the following unpublished sequences of ours: Sanguinicolidae sp. from heart of *Micropterus* sp. collected in the Coosa River, Alabama; Sanguinicolidae sp. from the body wash of *Mystus* cf. *mysticetus* in the Mekong River, Vietnam; Sanguinicolidae sp. from the body cavity of *Pangasius* cf. *macronema* collected in the Mekong River, Vietnam; Chimaerohemecidae sp. from the gill epithelium of the smooth butterfly ray, *Gymnura micrura* (Bloch and Schneider, 1801) collected in Mobile Bay, Alabama; Chimaerohemecidae sp. from the heart of smalltooth sawfish, *Pristis pectinata* Latham, 1796

collected in the Gulf of Mexico; and Chimaerohemecidae sp. from the heart of *Gymnura* cf. *poecilura* collected in the South China Sea off Vietnam. The specimens that produced the sequences we labeled herein as "Sanguinicolidae sp." (several species; Fig. 1; Table I) had the diagnostic features for the family as diagnosed herein. Those of "Chimaerohemecidae sp." were diagnosed by having C-shaped lateral tegumental spines (autopomorphic for that family) with exception of that from the smooth butterfly ray. That sequence is by definition a nonugen (Roberts et al., 2018) and was sourced from blood fluke eggs in the gill. No family-level diagnostic features for blood fluke miracidia have been proposed but we conservatively identified the eggs as a species of the family based on the sequence being recovered within other morphologically identified sequences of that family.

DESCRIPTIONS

Chimaerohemecidae Yamaguti, 1971

Accepted genera: *Chimaerohemecus* Van der Land, 1967 [type]; *Selachohemecus* Short, 1954; *Orchispirium* Madhavi and Rao, 1970; *Hyperandrotrema* Maillard and Ktari, 1978; *Myliobaticola* Bullard and Jensen, 2008; *Ogawaia* Cutmore, Cribb, and Yong, 2018; *Gymnurahemecus* Warren, Ruiz, Whelan, Kritsky, and Bullard, 2019; *Electrovermis* Warren and Bullard, 2019; *Achorovermis* Warren and Bullard, 2020; *Aetohemecus* Warren and Bullard, 2021; *Homestios* Warren and Bullard, 2021.

Diagnosis: Hermaphroditic, asexual reproduction in marine bivalves (*Electrovermis*), lacking encysted metacercaria or second intermediate host (Table II). Sporocyst spheroid or ovoid, with few cercariae. Cercaria non-acetabulate, apharyngeate, non-ocellate; spinous anterior sucker present; tegumental body spines distributing as lateral transverse rows; body fin present; tail brevifurcate; furcae asymmetrical, lacking fins; discernable gonads or genitalia indistinct. Extra-

uterine eggs embedding within and undergoing considerable larval development in gill epithelium of definitive host.

Body of adult dorsoventrally flat, ovoid or elongate, spinose and having each spine mounted on a muscular peduncle (*Aetohemecus*, *Chimaerohemecus*, *Gymnurahemecus*, *Hyperandrotrema*, *Selachohemecus*) or aspinose and lacking peduncles or tubercles (*Achorovermis*, *Electrovermis*, *Homestios*, *Myliobaticola*, *Ogawaia*) or having aspinose lateral tubercles (*Orchispirium*); spine bosses, posterolateral body protuberance, head collar, copulatory bursa, acetabulum, and median esophageal pouch or plicate organ lacking. Tegumental spines (if present) robust, C-shaped, each mounted on a muscular peduncle, directed ventrally, distributing in a single lateral column (*Aetohemecus*, *Gymnurahemecus*, *Selachohemecus*) or 2 lateral columns (*Chimaerohemecus*) or a lateral field of inter-spaced spines (*Hyperandrotrema*). Rosethorn-shaped spines absent. Sensory papillae absent. Anterior sucker nearly indistinct, aspinose, diminutive, not pedunculate, not bowl-shaped; mouth subterminal, a minute pore-like opening. Nervous system obvious depending on species; dorsolateral and ventrolateral nerve cords extending for nearly entire body length, forming anterior and posterior nerve commissures; secondary lateral branches obvious or indistinct. Pharynx absent. Esophagus long, sinuous, or straight, medial, having a medial swelling (esophageal bulb) and/or posterior swelling (chamber) immediately anterior to cecal bifurcation; esophageal gland surrounding esophagus for entire length or limited to middle or posterior portion of esophagus (indistinct in *Aetohemecus*, *Homestios*, *Myliobaticola*). Intestine inverse U-shaped or (secondarily) X-shaped (*Selachohemecus*, *Aetohemecus*), bifurcating at midline, not crossing opposing cecum; posterior ceca slightly asymmetrical (*Aetohemecus*, *Chimaerohemecus*, *Gymnurahemecus*, *Homestios*, *Hyperandrotrema*, *Myliobaticola*, *Orchispirium*, *Selachohemecus*) or markedly asymmetrical (*Achorovermis*, *Electrovermis*,

Ogawaia), blind ended, paired, terminating in anterior half of body (*Achorovermis*, *Aetohemecus*, *Electrovermis*, *Gymnurahemecus*, *Homestios*, *Myliobaticola*, *Selachohemecus, Ogawaia*) or posterior body extremity (*Chimaerohemecus*, *Hyperandrotrema*, *Orchispirium*). Genitalia occupying posterior one-third of body, post-gonadal.

Testis single, having smooth borders or exceptionally having posteriorly directed lobes (*Orchispirium*), non-sinusoidal (*Aetohemecus*, *Chimaerohemecus*, *Gymnurahemecus*, *Hyperandrotrema*, *Selachohemecus*) or sinusoidal (*Achorovermis*, *Electrovermis*, *Homestios*, *Myliobaticola*, *Ogawaia*, *Orchispirium*), anterior to ovary and genitalia, post-cecal (*Achorovermis*, *Aetohemecus*, *Electrovermis*, *Gymnurahemecus*, *Homestios*, *Myliobaticola*, *Ogawaia*, *Selachohemecus*) or inter-cecal (*Chimaerohemecus*, *Hyperandrotrema*, *Orchispirium*), not extending anterior to cecal bifurcation. Vasa efferentia coalescing ventrally and in posterior portion of testis, uniting to form vas deferens in posterior margin of testis; vas deferens straight (*Achorovermis*, *Chimaerohemecus*, *Hyperandrotrema*, *Selachohemecus*, *Orchispirium*, *Ogawaia*) or curved (*Aetohemecus*, *Gymnurahemecus*, *Homestios*) or looped (*Electrovermis*). Cirrus sac present, enveloping internal seminal vesicle and cirrus, post-gonadal or lateral to ovary (*Aetohemecus*, *Orchispirium*). Auxiliary external seminal vesicle absent.

Female reproductive system comprising a single ovary, oviduct, seminal receptacle that can comprise part of oviduct (oviducal seminal receptacle) (*Myliobaticola*, *Orchispirium*, *Selachohemecus*) or oviducal ampullae (*Aetohemecus*, *Gymnurahemecus*, *Hyperandrotrema*), vitellarium and primary vitelline collecting duct, oötype, uterus, and metraterm. Ovary single, wholly inter-cecal (*Chimaerohemecus*, *Hyperandrotrema*) or wholly post-cecal (*Achorovermis*, *Aetohemecus*, *Electrovermis*, *Gymnurahemecus*, *Homestios*, *Myliobaticola*, *Ogawaia*, *Orchispirium*, *Selachohemecus*), primarily post-testicular, medial or lateral to midline

(*Gymnurahemecus, Orchispirium*, *Selachohemecus*), deeply lobed (*Aetohemecus*, *Chimaerohemecus*, *Gymnurahemecus*, *Homestios*, *Hyperandrotrema*, *Selachohemecus*) or superficially lobed (*Electrovermis*) or a loose aggregation of ova (*Achorovermis*, *Myliobaticola*, *Orchispirium*). Oviduct a thin-walled duct emanating from ovary, straight to sinuous (*Aetohemecus*, *Chimaerohemecus*, *Gymnurahemecus*, *Selachohemecus*, *Ogawaia*, *Orchispirium*) or looping (*Achorovermis*, *Myliobaticola*); *Hyperandrotrema walterboegeri* has a straight to sinuous oviduct and *H. cetorhini* has a looping oviduct. Indistinct in *Electrovermis* and *Homestios.* Vitellarium follicular, diffuse (*Achorovermis*, *Electrovermis*, *Myliobaticola*), in dense lobules (*Aetohemecus*, *Gymnurahemecus, Homestios*), or granular (*Selachohemecus*, *Ogawaia*, *Orchispirium*), symmetrical (*Aetohemecus*, *Electrovermis*, *Myliobaticola*, *Ogawaia*) or asymmetrical (*Achorovermis*, *Chimaerohemecus*, *Gymnurahemecus*, *Homestios*, *Orchispirium*, *Selachohemecus*; *H. walterboegeri* is asymmetrical and *H. cetorhini* is symmetrical), filling space from nerve commissure to proximal portion of testis (*Achorovermis*, *Homestios*), ovary (*Aetohemecus, Gymnurahemecus*, *Selachohemecus*), or genital pore (*Chimaerohemecus*, *Electrovermis*, *Hyperandrotrema*, *Myliobaticola*, *Ogawaia, Orchispirium*). Primary vitelline duct extending posteriad along dextral body margin or along midline, ventral to ovary (*Chimaerohemecus*), not forming a transverse vitelline duct or reservoir, uniting with oviduct in posterior body end proximal to and near oötype. Indistinct in *Electrovermis*, *Homestios*, and *Myliobaticola*. Laurer's canal present (*Chimaerohemecus*, *Hyperandrotrema*, *Gymnurahemecus*) or absent (*Aetohemecus*, *Achorovermis*, *Electrovermis*, *Homestios*, *Myliobaticola*, *Ogawaia*, *Orchispirium*, *Selachohemecus*). Oötype post-cecal or exceptionally inter-cecal in *Hyperandrotrema*, posterior to common genital pore or exceptionally dextral to common genital pore (*Orchispirium*) or anterior to common genital pore (*Selachohemecus*), comprising an

inconspicuous ovoid chamber; Mehlis' gland indistinct, staining poorly and difficult to visualize with light microscopy of fixed, stained specimens. Uterus post-testicular or overlapping posterior margin of testis (*Ogawaia*), post-cecal or inter-cecal (*Chimaerohemecus*, *Hyperandrotrema*), typically straight or exceptionally convoluted (*Aetohemecus*, *Chimaerohemecus*, *Hyperandrotrema*, *Ogawaia*, *Orchispirium*), having ascending and descending portions; ascending portion extending anteriad along midline from oötype (*Aetohemecus*, *Chimaerohemecus*, *Hyperandrotrema*, *Gymnurahemecus*) or extending posteriad before turning anteriad and extending along midline (*Achorovermis*, *Ogawaia*, *Selachohemecus*), typically curving posteriad at level of testis (*Aetohemecus*, *Ogawaia*, *Orchispirium*), ovary (*Achorovermis*, *Chimaerohemecus*, *Gymnurahemecus*, *Homestios*, *Hyperandrotrema*, *Myliobaticola*, *Selachohemecus*), or seminal vesicle (*Achorovermis*); descending portion extending posteriad along sinistral body margin, uterus + metraterm flanking cirrus sac, metraterm muscularized to varying degrees or indistinct; uterine eggs thin-shelled, oblong (*Aetohemecus*, *Achorovermis*, *Electrovermis, Homestios, Myliobaticola, Ogawaia Orchispirium*) or circular (*Gymnurahemecus*, *Hyperandrotrema*, *Selachohemecus*- SAB observations), lacking filaments, lacking discernible miracidium, undergoing considerable larval development in tissue of definitive host (at least 1 species has eggs that hatch while in the gill of the fish host). Common genital pore dorsal, on sinistral body margin, post-gonadal, aspinose, post-cecal or exceptionally inter-cecal in *Hyperandrotrema*; common genital atrium present in *Chimaerohemecus* and *Hyperandrotrema*, indeterminate for other genera.

Excretory vesicle minute, medial, having excretory arms or exceptionally lacking excretory arms (*Gymnurahemecus*) (indeterminate for *Aetohemecus, Electrovermis, Myliobaticola*, *Ogawaia*). Maturing in blood of sharks, rays, and chimaeras (Chondrichthyes).

Differential diagnosis: Adults having C-shaped lateral tegumental spines each mounted on muscular peduncle and having non-sinusoidal testis or lacking lateral tegumental spines and having sinusoidal testis. Maturing in blood of sharks, rays, and chimaeras (Chondrichthyes).

Remarks

Based on morphology, we diagnose 2 groups within Chimaerohemecidae. One group matures in chimaeras, sharks, and epipelagic rays (Gymnuridae, Myliobatidae) and differs from all other blood flukes by having C-shaped lateral tegumental spines and a non-sinusoidal testis (Fig. 1). The other chimaerohemecids infect benthic rays (Narcinidae, Prisitidae, Glaucostegidae, Dasyatidae) and differ from all other blood flukes by lacking spines and having a sinusoidal testis. Warren and Bullard (2021) theorized that chimaerohemecids could eventually be split into different groups (subfamilies) based on the presence or absence of lateral tegumental spines and testis shape. Noteworthy also is that the X-shaped intestine of *Selachohemecus* and *Aetohemecus* is homoplasy, not homologous to the X-shaped intestine of *Sanguinicola* spp., for example, evidently evolved from the inverse U-shaped intestine of other chimaerohemecids (Warren and Bullard, 2021; present study) (Fig. 1).

Only one study has matched cercariae and adults of a chimaerohemecid in the bivalve and chondrichthyan hosts (Warren and Bullard, 2019): *Electrovermis zappum* Warren and Bullard, 2019 infects the variable coquina clam, *Donax variabilis* Say, 1812 (Donacidae Fleming, 1828) and matures in the lesser electric ray, *Narcine bancroftii* (Griffith and Smith, 1834) (Narcinidae) in high energy open beach habitat in the northern Gulf of Mexico. Other studies have collected trematode cercariae from bivalves, sequenced them, conducted a phylogenetic analysis, inferred their taxonomic identity from the resulting tree topology, and presumed the identity of the definitive host group based on their clade assignment; which assumes no possibility of host

switching (Cribb et al., 2017; Cutmore et al., 2022). Although we do not know the definitive host for any chimaerohemecid cercarial sequence other than *E. zappum*, sequences of these innominate cercariae clade with *E. zappum* and other batoid chimaerohemecids that lack C-shape spines and have a sinusoidal testis. The cercaria for a blood fluke whose adults have C-shaped spines and lack a sinusoidal testis has not been reported.

Yamaguti (1971) proposed Chimaerohemecinae Yamaguti, 1971 for *Chimaerohemecus trondheimensis* Van der Land, 1967 (see Van der Land, 1967; Yamaguti, 1971), but his subfamily diagnosis was erroneous, including objective errors regarding the absence of lateral tegumental spines and the structure of the testis (Orélis-Ribeiro et al., 2013; Van der Land, 1967). Herein, we elevated the subfamily name to family as per the recommendations of the ICZN despite the core problems of Yamaguti's subfamily diagnosis.

Acipensericolidae n. fam.

Accepted genera: *Acipensericola* Bullard, Snyder, Jensen, and Overstreet, 2008.

ZooBank registration: urn: lsid: zoobank.org:act:3647841C-A71D-48C5-8534-DC28C48FA73A.

Etymology: Acipensericolidae refers to the definitive host group.

Diagnosis: No life cycle known. Body of adult dorsoventrally flat, strongly ventrally concave, spinose; posterolateral body protuberance, head collar, copulatory bursa, acetabulum, and median esophageal pouch or plicate organ lacking. Tegumental body spines distributing as lateral transverse rows, spike-like, lacking re-curved tip. Rosethorn-shaped spines absent. Sensory papillae abundant, occupying ventrolateral body surface between lateral nerve cord and body margin.

Anterior sucker bowl-shaped, centered on mouth, demarcated from the anterior body end by peduncle, having minute spines on inner antero-ventral surface only; mouth subterminal, a relatively large opening (cf. chimaerohemecids). Nervous system well-developed; dorso- and ventrolateral nerve cords present, extending for nearly entire body length, meeting to form anterior and posterior nerve commissures; secondary lateral branches obvious or indistinct. Pharynx between anterior sucker and nerve commissure, highly muscular, intensely basophilic. Esophagus medial, extending straight before connecting with intestine. Intestine inverse U-shaped, bifurcating at midline, with long posterior ceca only and no anterior cecum, lacking secondary rami, not crossing opposing cecum, extending posteriad to near body end.

Testes inter-cecal, 6 in number, comprising 5 pre-ovarian testes and 1 post-ovarian testis. Vasa efferentia entwining throughout testicular tissue, with anterior and posterior trunks linking testicular column and posterior-most testis; vas deferens straight. Cirrus sac dextral, post-ovarian, enveloping seminal vesicle and cirrus; cirrus everting dorsally near dextral body margin. Common genital atrium present; common genital pore dorsal, opening on dextral body margin, inter-cecal or dorsal to dextral cecum, post-ovarian, aspinose. Auxiliary external seminal vesicle absent.

Ovary single, medial, inter-cecal, separating anterior column of testes from single posterior testis, immediately posterior to testicular column, deeply lobed, located within posterior one-fourth of body. Oviduct a thin-walled duct emanating from ovary, sinistral, functioning as oviducal seminal receptacle, extending to level of posterior testis before recurving before meeting oötype. Vitellarium diffuse, extending dorsal and ventral to gonads and ceca, not extending laterally beyond ventrolateral nerve cords; primary vitelline duct extending posteriad, sinistral, single/unpaired, not forming a transverse vitelline duct or reservoir, arcing sinistral to

posterior testis in column, uniting with oviduct in posterior body end proximal to and near oötype. Laurer's canal present. Oötype spherical, inter-cecal, inter-testicular, ventral to ovary, medial to cirrus sac. Uterus long, ventral to ovary, inter-cecal, having ascending and descending portions, convoluted, extending anterior from oötype to level of posterior portion of testicular column before curving posteriad, typically containing several large eggs; uterine eggs oblong, one-third uterus maximum width, lacking filaments, lacking mature miracidium. Genitalia occupying posterior one-fourth of body, inter-testicular, lateral to ovary and oötype.

Excretory vesicle large, Y-shaped, cradling posterior testis, thin-walled; excretory pore dorsal, subterminal. Maturing in blood of sturgeons and paddlefish (Chondrostei: Acipenseriformes).

Differential diagnosis: Anterior sucker robust, bowl-shaped, pedunculate, having minute spines on inner anteroventral surface only. Lateral tegumental spines spike-like (not C-shaped, lacking recurved tip); distributing in ventrolateral transverse rows, with distal end having a sharp tip protruding slightly from tegument. Pharynx present. Intestine inverse U-shaped; anterior ceca absent; posterior ceca terminating near excretory bladder. Testes 6 in number (5 pre-ovarian testes plus 1 post-ovarian testis), inter-cecal, having shallow lobes, non-sinusoidal. Laurer's canal present. Vitellarium symmetrical; primary vitelline collecting duct sinistral. Common genital pore dextral. Maturing in blood of sturgeons and paddlefishes (Acipenseriformes)

Remarks

Acipensericolids mature in sturgeons and paddlefish (Acipenseriformes) and are readily differentiated from all other fish blood flukes by the combination of having a robust, bowl-shaped, pedunculate anterior sucker, lateral tegumental spines that are spike-like (not C-shaped), an inverse U-shaped intestine (anterior ceca absent) with posterior ceca terminating near the

excretory bladder, 6 testes (inter-cecal, ovoid or oblong, lacking deep lobes; including 1 post-ovarian testis), a Laurer's canal, and a dextral common genital pore.

Elopicolidae n. fam.

Accepted genera: *Elopicola* Bullard, 2014 (type genus) and *Paracardicoloides* Martin, 1974.

ZooBank registration: urn: lsid: zoobank.org:act:79EFE8E4-FB25-4297-AA93-8FA90CEEEC01.

Etymology: Elopicolidae refers to the definitive host group.

Diagnosis: Hermaphroditic, asexual reproduction in freshwater gastropods (*Paracardicoloides*) and marine bivalves (*Elopicola*), lacking encysted metacercaria or second intermediate host. Sporocyst oblong, harboring few cercariae. Cercaria non-acetabulate, apharyngeate, non-ocellate, having spinous anterior sucker, tegumental body spines, body fin, and symmetrical furcae with fins, lacking discernable gonads or genitalia, undergoing considerable larval development in vertebrate definitive host. Extra-uterine eggs embedding within and undergoing considerable larval development in gill epithelium of definitive host.

Body of adult dorsoventrally flat, thick-bodied, spinose (*Paracardicoloides* spp. and juveniles of *Elopicola* spp.) or aspinose (adults of *Elopicola* spp.), lacking posterolateral body protuberance, head collar, copulatory bursa, acetabulum, and median esophageal pouch or plicate organ lacking. Tegumental body spines (if present) straight, spike like, lacking recurved tip, not distributing as ventrolateral transverse rows, with tips enveloped by tegument. Rosethorn-shaped spines absent. Sensory papillae abundant, occupying ventrolateral body surface between lateral nerve cord and body margin. Anterior sucker bowl-shaped, centered on mouth, demarcated from anterior body end by peduncle, aspinous; mouth subterminal, a relatively large opening (cf. chimaerohemecids). Nervous system well-developed; dorso- and ventrolateral nerve cords

present, extending for nearly entire body length, meeting to form anterior and posterior nerve commissures; secondary lateral branches indistinct. Sensory papillae present (*Elopicola*) or absent (*Paracardicoloides*), bearing cilia (*Elopicola*) or lacking cilia (*Paracardicoloides*), not distributing in regular dorsolateral bands. Pharynx present (*Elopicola*) or absent (*Paracardicoloides*), between anterior sucker and nerve commissure, muscular, intensely basophilic. Esophagus medial, extending straight before connecting with intestine. Intestine typically inverse U-shaped or with abbreviated lobes of cecum directing anteriad, bifurcating at midline, with long posterior ceca, terminating in middle one-third of body.

Testes single (*Elopicola*) or 2 in number (*Paracardicoloides*), inter-cecal (*Elopicola*) or post-cecal (*Paracardicoloides*), medial, deeply lobed, in posterior half of body. Vasa efferentia entwining throughout testicular tissue, coalescing in antero-sinistral region of testis to form vas deferens (*Elopicola*) or comprising anterior and posterior trunks linking anterior and posterior testes (*Paracardicoloides*); vas deferens straight, not convoluted, meeting with cirrus sac. Cirrus sac sinistral, pre-ovarian, enveloping internal seminal vesicle and cirrus; cirrus everting dorsally in sinistral body half (*Paracardicoloides*) or everting dorsally near midline (*Elopicola*), post-cecal. Common genital atrium present; common genital pore dorsal, near sinistral body margin, post-cecal, pre-ovarian. Auxiliary external seminal vesicle absent.

Ovary medial, post-cecal, inter-testicular (*Paracardicoloides*) or post-testicular (*Elopicola*), deeply lobed. Oviduct a thin-walled duct emanating from ovary, medial, post-cecal, post-genital pore; oviducal seminal receptacle present. Vitellarium follicular, 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 ceca; primary vitelline duct single/unpaired, not forming a transverse

vitelline duct or reservoir, arcing along postero-dextral margin of testis/anterior testis, connecting with oötype posteriorly (*Paracardicoloides*) or laterally (*Elopicola*). Laurer's canal present (*Paracardicoloides*) or absent (*Elopicola*). Oötype oblong, pre-ovarian, anterior to common genital pore (*Paracardicoloides*) or posterior to common genital pore (*Elopicola*). Uterus comprising short ascending and descending portions, not convoluted, pre-ovarian, inter-cecal (*Paracardicoloides*) or post-cecal (*Elopicola*); uterine eggs ovoid; metraterm pre-ovarian, medial to cirrus sac.

Excretory vesicle large, Y-shaped; excretory pore dorsal, subterminal. Maturing in blood of elopomorphs (Teleostei: Elopomorpha).

Differential diagnosis: Anterior sucker robust, bowl-shaped, aspinose in adults, pedunculate. Lateral tegumental spines spike-like not C-shaped, not distributing in ventrolateral transverse rows, with distal end having a sharp tip enveloped by tegument (adults of *Elopicola* spp. are aspinose); mouth comprising a large opening. Pharynx present (*Elopicola*) or absent (*Paracardicoloides*). Intestine generally inverse U-shaped; anterior ceca short or nearly indistinct; posterior ceca terminating at level of testis (*Elopicola*)/anterior-most testis (*Paracardicoloides*). Testis(es) deeply lobed, non-sinusoidal, comprising 1 pre-ovarian and inter-cecal testis plus 1 post-ovarian and post-cecal testis (*Paracardicoloides*) or comprising 1 inter-cecal testis (*Elopicola*); common genital atrium and pore present, sinistral, pre-ovarian. Vitellarium symmetrical; primary vitelline collecting duct dextral. Laurer's canal present (*Paracardicoloides*) or absent (*Elopicola*). Egg in gill epithelium of fish host having elongate polar filaments. Maturing in blood of elopomorphs (Teleostei: Elopomorpha).

Remarks

Elopicolids mature in fishes having a leptocephalus larva: ladyfishes (*Elops* spp.), tarpons (*Megalops* spp.), and catadromous eels (*Anguilla* spp.) (all Elopomorpha). They differ from all other blood flukes by the combination of having a robust, bowl-shaped, pedunculate anterior sucker, lateral tegumental spines that are spike-like (can be lost in adult), short or nearly indistinct anterior ceca, posterior ceca that terminate at level of the testis(es), a single testis or 2 testes, a Laurer's canal present or absent, and a sinistral common genital pore and atrium.

Sanguinicolidae Poche, 1926

Accepted genera: *Sanguinicola* Plehn, 1905 (type), *Plehniella* Szidat, 1951, *Nomasanguinicola* Truong and Bullard, 2013, *Kritsky* Orélis-Ribeiro and Bullard 2016, *Pseudosanguinicola* Warren and Bullard, *in review*, and *Cladocaecum* Orélis-Ribeiro and Bullard 2016; we provisionally include *Parasanguinicola* Herbert and Shaharom-Harrison, 1995.

Diagnosis: Hermaphroditic, asexual reproduction in freshwater gastropods (*Sanguinicola*), lacking encysted metacercaria or second intermediate host, having migratory larva (schistosomulum) infecting lymphatic and blood vascular system, having adult infecting blood and body cavity of vertebrate definitive host. Sporocyst present (*Sanguinicola*) or sporocyst and redia present (*Sanguinicola*). Sporocyst spheroid (*Sanguinicola*) or ovoid (*Sanguinicola*), having few or having many cercariae (*Sanguinicola*). Cercaria non-acetabulate, apharyngeate, non-ocellate; having spinous anterior sucker; tegumental body spines present or absent; body fin present or absent; lacking discernable gonads or genitalia; tail furcae symmetrical, fins present or absent; undergoing considerable larval development in vertebrate definitive host; extra-uterine eggs embedding in fish gill epithelium and undergoing considerable larval development; miracidium can hatch and emerge from eggs embedded in fish gill epithelium.

Body of adult dorsoventrally flat, ventrally concave, aspinose or spinose (*Sanguinicola, Parasanguinicola, Pseudosanguinicola*); head collar, copulatory bursa, acetabulum, and median esophageal pouch or plicate organ lacking. Tegumental body spines straight, elongate, not distally recurved, deeply rooted in tegument (*Sanguinicola*) or only slightly protruding from tegument (*Sanguinicola*, *Parasanguinicola*) or delicate and appearing irregular or discordant (*Pseudosanguinicola*) distributing in a single column (*Sanguinicola*, *Parasanguinicola*) or distributing as densely compacted transverse rows along lateral body margin (*Pseudosanguinicola*). Rosethorn-shaped spines absent. Sensory papillae present (*Sanguinicola*, *Nomasanguinicola, Pseudosanguinicola*) or absent (*Cladocaecum, Kritsky, Plehniella*) bearing cilia (*Sanguinicola*, *Nomasanguinicola, Parasanguinicola*, *Pseudosanguinicola*) or lacking cilia (*Cladocaecum, Kritsky, Plehniella*), not distributing in regular dorsolateral bands. Anterior sucker a proboscis accommodating the mouth, not separated from body by peduncle, having denticles in 2 columns flanking mouth (*Nomasanguinicola*) or lacking denticles (*Cladocaecum*, *Kritsky*, *Parasanguinicola, Plehniella*, *Pseudosanguinicola, Sanguinicola*), having concentric spine rows in small adults (*Kritsky*, *Plehniella*) or aspinose (*Cladocaecum, Sanguinicola, Parasanguinicola, Pseudosanguinicola*); mouth medioventral, subterminal, comprising a minute pore-like opening, with associated tooth-like mouth apparatus of Ejsmount (1926) (*Pseudosanguinicola, Sanguinicola*). Nervous system well-developed; dorso- and ventrolateral nerve cords extending for nearly entire body length, meeting to form anterior and posterior nerve commissures; secondary lateral branches obvious (*Sanguinicola*) or indistinct (*Cladocaecum*, *Kritsky*, *Nomasanguinicola, Parasanguinicola, Plehniella, Pseudosanguinicola*). Pharynx present (*Cladocaecum*, *Kritsky*, *Nomasanguinicola*, *Parasanguinicola, Plehniella, Sanguinicola*) or absent (*Pseudosanguinicola*), not highly muscular, not intensely basophilic. Esophagus with

anterior and posterior esophageal swellings. Intestine comprising 4 (exceptionally 5) ceca forming an X-shaped configuration (*Parasanguinicola*, *Pseudosanguinicola*, *Sanguinicola*) or having 5 or 6 radial ceca (*Kritsky, Nomasanguinicola*, *Plehniella*) or exceptionally having paired anterior ceca plus a medial cecum with numerous branches extending laterally (*Cladocaecum*), restricted to anterior half of body (*Kritsky, Nomasanguinicola*, *Parasanguinicola, Pseudosanguinicola, Plehniella, Sanguinicola*) or extending in posterior half (*Cladocaecum*), bifurcating at midline, blind ending, not overlapping another cecum.

Testis single, anterior to ovary and genitalia, diffuse, having laterally-directed lobes or exceptionally (purportedly) not having lateral-directed lobes (*Parasanguinicola*), post-cecal (*Kritsky, Nomasanguinicola, Parasanguinicola, Pseudosanguinicola, Plehniella, Sanguinicola*) or dorsal to cecum (*Cladocaecum*), in middle one-third of body (*Kritsky, Nomasanguinicola, Parasanguinicola, Plehniella, Pseudosanguinicola, Sanguinicola*) or posterior half of body (*Cladocaecum*). Vasa efferentia gathering secondary ducts emanating from lateral margins of testicular lobes, coalescing ventrally along midline, forming large seminal column (*Pseudosanguinicola, Sanguinicola)* or not forming large seminal column (*Cladocaecum, Kritsky, Nomasanguinicola, Parasanguinicola*, *Plehniella*), narrowing medially before connecting to vas deferens; vas deferens straight (*Cladocaecum, Kritsky, Nomasanguinicola*, *Parasanguinicola, Pseudosanguinicola, Sanguinicola*) or looping (*Plehniella*), meeting with cirrus sac. Cirrus sac sinistral, post-ovarian, thin-walled; internal seminal vesicle present; cirrus everting dorsally in sinistral body half (*Plehniella*) or everting dorsally near midline (*Kritsky, Sanguinicola*) or indistinct (*Cladocaecum, Nomasanguinicola, Parasanguinicola, Pseudosanguinicola*), post-cecal; body protuberance associated with cirrus present (*Plehniella, Sanguinicola*) or absent (*Cladocaecum*, *Kritsky*, *Nomasanguinicola*, *Parasanguinicola*,

Pseudosanguinicola), directing dorsally, medial. Common genital atrium and common genital pore present (*Plehniella*) or absent (*Cladocaecum*, *Kritsky*, *Nomasanguinicola, Parasanguinicola*, *Pseudosanguinicola*, *Sanguinicola*); separate genital pores dorsal, post-cecal; female genital pore medial (*Cladocaecum*, *Kritsky*, *Nomasanguinicola, Pseudosanguinicola*, *Sanguinicola*) or exceptionally (purportedly) sinistral (*Parasanguinicola*), post-ovarian (*Cladocaecum*, *Kritsky*, *Nomasanguinicola, Pseudosanguinicola*, *Sanguinicola*) or exceptionally (purportedly) pre-ovarian (*Parasanguinicola*), aspinose. Auxiliary external seminal vesicle absent.

Ovary medial, post-cecal, post-testicular, can appear butterfly-shaped, having deep lobes (*Kritsky, Parasanguinicola, Plehniella*) or superficial lobes (*Cladocaecum*, *Nomasanguinicola, Pseudosanguinicola, Sanguinicola*), as wide as testicular field (*Cladocaecum*, *Kritsky*, *Pseudosanguinicola*, *Plehniella*, *Sanguinicola*) or wider than testicular field (*Nomasanguinicola*, *Parasanguinicola*). Oviduct a thin-walled duct emanating from ovary, medio-dextral, curving slightly sinistrally; oviducal seminal receptacle present. Vitellarium diffuse, symmetrical, follicular, mostly anterior to genital pores; primary vitelline duct single/unpaired, extending posteriad along midline, not forming a transverse vitelline duct or reservoir, connecting with oviduct in posterior body end proximal to and near oötype. Laurer's canal absent. Oötype spheroid (*Cladocaecum*, *Kritsky*, *Pseudosanguinicola*, *Sanguinicola*) or oblong (*Nomasanguinicola, Parasanguinicola, Plehniella*). Uterus short, having ascending portion only (*Parasanguinicola, Pseudosanguinicola, Sanguinicola*) or ascending and descending portions (*Cladocaecum*, *Kritsky*, *Nomasanguinicola, Plehniella*), not convoluted, not containing sperm; uterine seminal receptacle absent; uterine eggs thin-shelled, not operculate, triangular or ovoid (*Parasanguinicola*, *Sanguinicola*) or spheroid (*Cladocaecum*, *Kritsky*, *Nomasanguinicola*) or

indistinct (*Plehniella*, *Pseudosanguinicola*), lacking discernible miracidium; metraterm robust and strongly muscular (*Plehniella*) or indistinct (*Cladocaecum, Kritsky, Nomasanguinicola, Parasanguinicola, Pseudosanguinicola, Sanguinicola*).

Excretory vesicle Y-shaped or a small gland (*Parasanguinicola, Sanguinicola*) or indistinct (*Cladocaecum*, *Kritsky*, *Nomasanguinicola, Plehniella*); excretory pore terminal. Maturing in blood or body cavity of later branching ray-finned freshwater fishes (Teleostei).

Differential diagnosis: Anterior sucker a proboscis with mouth, not pedunculate, having denticles or lacking denticles or having concentric spine rows in small adults or aspinose; mouth comprising a minute pore-like opening; pharynx present or absent; medial esophageal swelling (pouch) present. Intestine comprising short radial ceca of approximate equal length or having anterior ceca plus a single medial and dendritic posterior cecum. Testis with appendix-like lateral lobes, non-sinusoidal. Genital pores common or separate. Laurer's canal absent. Vitellarium symmetrical. Maturing in blood and body cavity of later branching freshwater ray-finned fishes (Teleostei).

Remarks

Sanguinicolids primarily infect later branching freshwater ray-finned fishes (Teleostei) and differ from all other blood flukes by the combination of having a diminutive anterior sucker, a medial esophageal swelling (pouch), short, radial ceca of approximate equal length or having short anterior ceca plus an elongate, dendritic posterior cecum, testis with appendix-like lateral lobes, and no Laurer's canal. Warren et al. (*in review*) treated the taxonomic problems and systematics of *Sanguinicola*.

Sanguinicola maritimus Nolan and Cribb (2005) is the only sanguinicolid that infects a marine fish. The identity of the molluscan intermediate host is indeterminate but could comprise

a littoral euryhaline snail, for example. It has some unique morphological features highlighted by Nolan and Cribb (2005) that could warrant proposal of a new sanguinicolid genus. No nucleotide sequence for this species exists in GenBank.

The original description of *Parasanguinicola* reported that its type species lacked lateral lobes of the testis and had a pre-ovarian female genital pore lateral to the testis, but these features are dubious and need to be confirmed (Herbert and Shaharom-Harrison, 1995).

Aporocotylidae Odhner, 1912, emended

Accepted genera: *Aporocotyle* Odhner, 1900; *Deontacylix* Linton, 1910; *Psettarium* Goto and Ozaki, 1929; *Paradeontacylix* Mcintosh, 1934; *Cardicola* Short, 1953; *Psettaroides* Lebedev and Mamaev, 1968; *Neoparacardicola* Yamaguti, 1970; *Metaplehniella* Lebedev and Parukhin, 1972; *Plethorchis* Martin, 1975; *Pearsonellum* Overstreet and Køie, 1978; *Pseudocardicola* Parukhin, 1985; *Cruoricola* Herbert, Shaharom-Harrison, and Overstreet, 1994; *Elaphrobates* Bullard and Overstreet, 2003; *Ankistromeces* Nolan and Cribb, 2004; *Adelomyllos* Nolan and Cribb, 2004; *Chaulioleptos* Nolan and Cribb, 2005; *Phthinomita* Nolan and Cribb, 2006; *Braya* Nolan and Cribb, 2006; *Littorellicola* Bullard, 2010; *Skoulekia* Alama-Bermejo, Montero, Raga, and Holzer, 2011; *Rhaphidotrema* Yong and Cribb, 2011; *Primisanguis* Bullard, 2012; *Cardallagium* Yong, Cutmore, Jones, Gauthier, and Cribb, 2018; *Allocardicola* Yong, Cribb, and Cutmore, 2021; *Holocentricola* Cutmore and Cribb, 2021.

Diagnosis: Hermaphroditic, asexual reproduction in estuarine and marine polychaetes, lacking encysted metacercaria or second intermediate host, having migratory schistsomulum infecting lymphatic and blood vascular system, having adult infecting blood and body cavity of later branching ray-finned fishes (Euteleosti). Sporocyst present (*Cardicola*) or absent (*Aporocotyle*), fusiform (*Cardicola*), having few or many cercariae (*Cardicola*). Redia present

(*Aporocotyle*) or absent (*Cardicola*); fusiform, having many cercariae. Cercaria non-acetabulate, apharyngeate, non-ocellate, having spinous anterior sucker, lacking body fin and tegumental body spines discernable gonads or genitalia, tail furcae present and symmetrical with fins (*Aporocotyle*) or absent (*Cardicola*); undergoing considerable larval development in vertebrate definitive host. Extra-uterine eggs embedding in fish gill epithelium and undergoing considerable larval development; miracidium can hatch and emerge from eggs embedded in fish gill epithelium.

Body of adult strongly dorsoventrally flat (*Adelomyllos*, *Allocardicola*, *Aporocotyle, Braya, Cardallagium, Cardicola, Chauliolaptos, Crouricola, Deontacylix, Elaphrobates, Holocentricola, Littorellicola, Metaplehniella, Neoparacardicola, Paradeontacylix, Pearsonellum, Plethorchis, Primisanguis, Psettarium, Psettaroides, Pseudocardicola, Rhaphidotrema, Skoulekia*) or exceptionally cylindrical/thread-like (*Ankistromeces*, *Phthinomita*), spinous; head collar, copulatory bursa, acetabulum, and median esophageal pouch or plicate organ lacking. Tegumental body spines distributing in ventrolateral transverse rows or exceptionally having spine bosses (*Aporocotyle* only), having distally recurved tip (*Cardicola*, *Elaphrobates*, *Holocentricola*, *Littorellicola*, *Paradeontacylix*, *Pearsonellum*, *Skoulekia*) or not having distally recurved tip (*Adelomyllos*, *Allocardicola, Ankistromeces*, *Aporocotyle*, *Cardallagium, Deontacylix, Phthinomita, Psettarium, Rhaphidotrema*); rosethorn-shaped spines present in *Paradeontacylix* only. Anterior sucker diminutive, spheroid, lacking peduncle, having rows of concentric spines anterior to mouth (*Adelomyllos, Allocardicola, Ankistromeces, Aporocotyle, Cardicola, Elaphrobates, Holocentricola, Pearsonellum*, *Phthinomita, Psettarium*) or lacking spheroid anterior sucker (*Cardallagium, Littorellicola, Metaplehniella, Neoparacardicola, Paradeontacylix, Psettaroides, Rhaphidotrema*, *Skoulekia*); mouth

subterminal, comprising a minute pore-like opening. Nervous system well-developed or indistinct (*Ankistromeces, Phthinomita*); dorso- and ventrolateral nerve cords extending for nearly entire body length, forming anterior and posterior nerve commissures; secondary lateral branches indistinct. Sensory papillae present (*Cardallagium, Cardicola, Littorellicola, Psettarium, Primisanguis*) or absent (*Adelomyllos*, *Ankistromeces*, *Aporocotyle, Braya, Chaulioleptos, Cruoricola, Deontacylix, Elaphrobates, Holocentricola, Metaplehniella, Neoparacardicola, Paradeontacylix, Pearsonellum, Phthinomita, Plethorchis, Primisanguis, Psettaroides, Pseudocardicola, Rhaphidotrema*, *Skoulekia*). Pharynx absent. Esophagus enveloped by glandular region (*Adelomyllos, Aporocotyle, Braya, Cardallagium, Cardicola, Chaulioleptos, Cruoricola, Elaphrobates, Holocentricola, Littorellicola, Metaplehniella, Neoparacardicola, Paradeontacylix*, *Pearsonellum, Plethorchis, Primisanguis, Psettarium, Psettaroides, Pseudocardicola, Skoulekia*) or not (*Ankistromeces*, *Deontacylix*, *Phthinomita, Rhaphidotrema*); posterior esophageal swelling absent; having a nearly indistinct posterior swelling in (*Adelomyllos* only).

Intestine comprising 4 ceca (1 pair of anterior ceca plus 1 pair of posterior ceca) forming an X-shaped configuration (*Neoparacardicola*) or an H-shaped configuration (*Adelomyllos*, *Ankistromeces*, *Aporocotyle, Braya, Cardallagium, Cardicola, Chaulioleptos, Crouricola, Deontacylix, Elaphrobates, Holocentricola, Littorellicola, Metaplehniella, Neoparacardicola, Paradeontacylix, Pearsonellum, Phthinomita, Plethorchis, Primisanguis, Psettarium, Psettaroides, Pseudocardicola, Rhaphidotrema*, *Skoulekia*) or anterior ceca secondarily lost (*Allocardicola*), bifurcating at midline, blind ending; posterior ceca not overlapping corresponding cecum, terminating in the posterior one-third of the body (*Ankistromeces, Aporocotyle, Braya, Cardicola, Chaulioleptos, Cruoricola, Elaphrobates, Littorellicola,*

Paradeontacylix, Primisanguis, Psettarium, Psettaroides, Pseudocardicola, Rhaphidotrema, *Skoulekia*) or terminating in the middle one-third of the body (*Adelomyllos, Allocardicola, Cardallagium*, *Deontacylix, Holocentricola, Metaplehniella, Pearsonellum, Phthinomita, Plethorchis*) or terminating in the anterior one-third of the body (*Neoparacardicola*). Genitalia restricted to posterior one-fourth of body, post-cecal (*Adelomyllos*, *Allocardicola, Ankistromeces, Braya, Cardicola, Chaulioleptos, Cruoricola, Deontacylix, Elaphorbates, Holocentricola, Metaplehniella, Neoparacardicola, Phthinomita, Plethorchis, Primisanguis, Psettarium, Psettaroides, Pseudocardicola, Rhaphidotrema*, *Skoulekia*) or inter-cecal (*Aporocotyle*), post-testicular (*Ankistromeces, Allocardicola, Aporocotyle*, *Braya, Cardicola, Cruoricola, Deontacylix, Elaphorbates, Holocentricola, Metaplethniella, Pearsonellum*, *Plethorchis*, *Primisanguis, Psettaroides, Pseudocardicola, Rhaphidotrema*, *Skoulekia*) or inter-testicular (*Adelomyllos*, , *Chaulioleptos, Neoparacardicola, Phthinomita, Psettarium*), anterior to oötype (*Adelomyllos*, *Allocardicola, Ankistromeces, Aporocotyle, Cardallagium, Chaulioleptos, Cruoricola, Littorellicola, Neoparacardicola, Paradeontcylix, Pearsonellum, Phthinomita, Plethorchis, Primisanguis, Psettarium, Pseudocardicola, Rhaphidotrema, Skoulekia*) or posterior to oötype (*Cardicola*, *Elaphrobates*, *Braya*, *Deontacylix, Metaplehniella*) or female genitalia is anterior to oötype and male genitalia is posterior to oötype (*Holocentricola, Psettaroides*).

Testis(es) single (*Allocardicola*, *Ankistromeces, Braya, Cardallagium, Cardicola, Cruoricola Elaphrobates, Holocentricola, Metaplehniella, Pearsonellum, Primisanguis, Psettaroides, Skoulekia*) or multiple (*Adelomyllos, Aporocotyle*, *Chaulioleptos, Deontacylix, Littorellicola, Neoparacardicola, Paradeontacylix*, *Phthinomita, Plethorchis, Psettarium*, *Pseudocardicola*, *Rhaphidotrema*), dendritic (*Metaplehniella*, *Neoparacardicola*) or rectangular (*Adelomyllos*, *Allocardicola, Ankistromeces*, *Braya, Cardallagium, Cardicola, Chaulioleptos, Crouricola,*

Deontacylix, Elaphrobates, Holocentricola, Pearsonellum, Phthinomita, Primisanguis, Psettarium, Psettaroides, Rhaphidotrema, Skoulekia) or ovoid (*Pseudocardicola*) or cobblestone-like (*Aporocotyle*, *Paradeontacylix*, *Plethorchis*, *Littorellicola*); anterior testis(es) pre-ovarian, occupying space anterior to genitalia, inter-cecal (*Ankistromeces, Aporocotyle, Braya, Cardicola, Chaulioleptos, Cruoricola, Elaphorbates, Littorellicola, Paradeontacylix, Pearsonellum, Phthinomita, Primisanguis, Psettarium, Psettaroides, Pseudocardicola, Skoulekia*) or post-cecal (*Adelomyllos*, *Allocardicola*, *Cardallagium, Deontacylix, Holocentricola, Metaplehniella, Neoparacardicola, Plethorchis, Rhaphidotrema*); posterior testis post-ovarian, occupying space posterior to genitalia, post-cecal. Vasa efferentia coalescing in posteroventral region of testis, uniting into a vas deferens in anterior or posterior margin of testis; vas deferens straight to sinuous. Auxiliary external seminal vesicle present only in *Pearsonellum* only. Cirrus sac sinistral, pre-ovarian (*Aporocotyle, Neoparacardicola*), post-ovarian (*Adelomyllos, Allocardicola, Ankistromeces*, *Braya, Cardallagium, Cardicola, Chaulioleptos, Cruoricola, Deontacylix*, *Elaphrobates, Holocentricola, Littorellicola, Metaplehniella, Paradeontacylix*, *Pearsonellum, Phthinomita, Plethorchis, Primisanguis, Psettarium, Psettaroides, Pseudocardicola, Rhaphidotrema, Skoulekia*), thin-walled (the cirrus sack can be indistinct without differential interference contrast optical components); internal seminal vesicle present; cirrus appearing appendix-like when everted; posterolateral body protuberance associated with cirrus present (*Ankistromeces, Cardallagium, Cardicola, Holocentricola, Littorellicola, Metaplehniella, Neoparacardicola, Primisanguis, Psettarium, Psettaroides, Skoulekia*) or absent (*Adelomyllos, Allocardicola, Aporocptyle, Braya, Chaulioleptos, Cruoricola, Deontacylix, Elaphrobates, Paradeontacylix, Pearsonellum, Plethorchis, Pseudocardicola, Rhaphidotrema*), directing dorsally. Common genital atrium absent. Genital

pores separate, dorsal, sinistral, aspinose, or exceptionally having stylet with *Rhaphidotrema* only.

Ovary medial (*Adelomyllos*, *Allocardicola*, *Ankistromeces, Cardallagium*, *Cardicola, Chaulioleptos, Cruoricola, Deontacylix, Elaphrobates, Littorellicola*, *Metaplehniella, Paradeontacylix*, *Pearsonellum*, *Phthinomita, Plethorchis*, *Psettarium, Psettaroides, Pseudocardicola, Rhaphidotrema*, *Skoulekia*) or lateral to midline (*Braya, Neoparacardicola, Primisanguis*), post-testicular (*Ankistromeces, Cardallagium, Cardicola, Cruoricola*, *Deontacylix, Elaphrobates, Littorellicola, Metaplehniella, Paradeontacylix, Pearsonellum, Phthinomita, Plethorchis, Psettaroides, Pseudocardicola, Rhaphidotrema*, *Skoulekia*) or inter-testicular (*Adelomyllos, Chaulioleptos, Neoparacardicola, Phthinomita*, *Psettarium*), having smooth margins (*Allocardicola*, *Ankistromeces, Aporocotyle, Littorellicola, Pearsonellum, Phthinomita, Rhaphidotrema*, *Skoulekia*) or having lobed margins (*Adelomyllos, Braya, Cardalllagium, Cardicola, Chaulioleptos, Cruoricola, Deontacylix, Elaphorbates, Holocentricola, Neoparacardicola, Paradeontacylix, Plethorchis, Primisanguis, Psettarium, Psettaroides, Pseudocardicola*) or purportedly dendritic (*Metaplehniella*), having well-differentiated refractive acini. Oviduct a thin-walled duct emanating from ovary, medial (*Aporocotyle, Metaplehniella*) or dextral (*Adelomyllos, Allocardicola, Ankistromeces, Braya, Cardallagium, Cardicola, Chaulioleptos, Cruoricola, Deontacylix, Elaphrobates, Holocentricola, Littorellicola, Neoparacardicola, Paradeontacylix, Pearsonellum, Phthinomita, Plethorchis, Primisanguis, Psettarium, Psettaroides, Pseudocardicola, Rhaphidotrema, Skoulekia*), straight to sinuous or exceptionally looping in *Pseudocardicola* only; oviducal seminal receptacle present (*Braya, Cardicola, Deontacylix, Elaphrobates, Littorellicola, Paradeontacylix, Pearsonellum*) or absent (*Adelomyllos*, *Allocardicola*, *Ankistromeces,*

Aporocotyle, Cardallagium, Chaulioleptos, Cruoricola, Holocentricola, Metaplehniella, Neoparacardicola, Phthinomita, *Plethorchis, Primisanguis, Psettarium, Psettaroides, Pseudocardicola, Rhaphidotrema*, *Skoulekia*). Vitellarium diffuse or densely compacted, extending from cecal bifurcation to genitalia, symmetrical (*Allocardicola*, *Aporocotyle, Braya, Cardallagium, Cardicola, Cruoricola, Deontacylix, Holocentricola, Littorellicola, Metaplehniella, Neoparacardicola, Paradeontacylix, Primisanguis, Psettarium, Psettaroides, Pseudocardicola, Rhaphidotrema*) or asymmetrical (*Adelomyllos, Ankistromeces, Aporocotyle, Chauliolaptos, Elaphrobates, Pearsonellum*, *Phthinomita, Plethorchis, Skoulekia*), mostly anterior to genital pores, coalescing along midline (*Adelomyllos, Allocardicola, Braya, Cardallagium, Cardicola, Cruoricola, Deontacylix, Elaphrobates, Holocentricola, Littorellicola, Metaplehniella, Neoparacardicola, Paradeontacylix, Primisanguis, Psettarium, Psettaroides, Rhaphidotrema*, *Skoulekia*) or coalescing laterally (*Ankistromeces, Aporocotyle*, *Chaulioleptos, Pearsonellum, Phthinomita, Plethorchis*, *Pseudocardicola*); primary vitelline duct single/unpaired, extending posteriad along midline (*Adelomyllos, Braya*, *Cardicola, Cruoricola, Elaphrobates, Metaplehniella, Neoparacardicola, Psettaroides, Skoulekia*) or dextral (*Ankistromeces, Aporocotyle, Cardallagium, Chaulioleptos, Deontacylix, Holocentricola, Littorellicola, Paradeontacylix, Pearsonellum, Phthinomita, Plethorchis, Primisanguis, Psettarium, Pseudocardicola, Rhaphidotrema*), not forming a transverse vitelline duct or reservoir, uniting with oviduct in posterior body end near oötype. Laurer's canal absent. Oötype spheroid to oblong. Uterus elongate and convoluted (*Adelomyllos, Allocardicola, Aporocotyle, Braya, Cardallagium, Cardicola, Cruoricola, Deontacylix, Elaphrobates, Holocentricola, Littorellicola, Metaplehniella, Paradeontacylix, Pearsonellum, Primisanguis, Psettarium, Psettaroides, Pseudocardicola, Rhaphidotrema, Skoulekia*) or straight (*Ankistromeces,*

Chaulioleptos, *Phthinomita, Plethorchis*), initially extending anteriad from oötype (*Adelomyllos, Allocardicola, Ankistromeces, Aporocotyle, Cardallagium, Cruoricola, Deontacylix*, *Holocentricola, Littorellicola, Metaplehniella, Pearsonellum, Phthinomita, Psettarium, Psettaroides, Pseudocardicola, Rhaphidotrema, Skoulekia*) or initially extending slightly posteriad (*Braya, Cardicola, Elaphrobates, Paradeontacylix, Primisanguis*), curving anteriad along midline, curving posteriad at level of ovary (*Adelomyllos, Allocardicola, Braya, Cardallagium, Cardicola, Chaulioleptos, Cruoricola, Elaphrobates, Holocentricola, Littorellicola, Metaplehniella, Paradeontacylix, Phthinomita, Primisanguis, Psettarium, Psettaroides, Rhaphidotrema, Skoulekia*), curving posteriad at level of testis (*Ankistromeces, Aporocotyle, Deontacylix, Neoparacardicola, Pearsonellum, Plethorchis, Pseudocardicola*) or having ascending and descending portions; uterine seminal receptacle present in *Primisanguis* only; uterine eggs thin-shelled, operculate (*Pearsonellum*) or non-operculate, pliable, elongate (*Adelomyllos, Braya, Neoparacardicola, Skoulekia*) or spheroid (*Ankistromeces, Aporocotyle, Cardallagium, Cardicola, Deontacylix, Elaphorbates, Littorellicola, Pearsonellum, Phthinomita, Primisanguis, Psettarium, Rhaphidotrema*) or elliptical to ovoid (*Chaulioleptos, Cruoricola, Holocentricola, Paradeontacylix, Plethorchis*), lacking discernible miracidium; metraterm weakly muscular.

Excretory vesicle V-shaped (*Plethorchis*) or Y-shaped (*Adelomyllos*, *Aporocotyle, Cruoricola*, *Elaphrobates*, *Littorellicola*, *Primisanguis*, *Psettarium*, *Rhaphidotrema*) or a small gland (*Ankistromeces, Braya, Cardicola, Chaulioleptos, Holocentricola, Pearsonellum, Phthinomita*); excretory pore terminal. Maturing in blood of principally late branching ray-finned marine fishes (Euteleostei).

Differential diagnosis: Anterior sucker diminutive, not bowl-shaped, lacking peduncle, spheroid and having rows of concentric spines anterior to mouth or lacking spheroid anterior sucker; mouth comprising a minute pore-like opening; medial esophageal swelling (pouch) absent; pharynx absent. Lateral tegumental spines distributing in ventrolateral transverse rows or spine bosses (*Aporocotyle* only), not C-shaped. Testis(es) non-sinusoidal, lacking appendix-like lateral lobes. Intestine comprising 4 ceca (1 pair of anterior ceca plus 1 pair of posterior ceca) forming an X- or H-shaped configuration; anterior ceca long or secondarily lost (*Allocardicola* only); posterior ceca comprising a pair of elongate ceca each extending posteriad in parallel with respective body margin and terminating in middle or posterior 1/3 of body or anterior 1/3 (*Neoparacardicola* only). Genital pores sinistral or having sinistral common genital pore. Vitellarium asymmetrical or symmetrical. Laurer's canal absent. Maturing in blood of principally later branching ray-finned marine fishes (Teleostei).

Remarks

Aporocotylids primarily infect later branching marine and estuarine ray-finned fishes (Teleostei) and differ from all other blood flukes by the combination of having a spheroid anterior sucker with concentric rows of circumferential spines or the spheroid anterior sucker is lost in the adult or adults have a diminutive anterior sucker, a sinuous esophagus lacking a medial swelling, an X- or H-shaped intestine having 4 ceca, long anterior ceca, long smooth posterior ceca that extend posteriad in parallel with respective body margin and terminate near the posterior body end, testis(es) that lack appendix-like lateral lobes, no Laurer's canal, and a sinistral common genital pore or separate genital pores that are sinistral.

Three genera (*Chanicola* Yong, Cribb, and Cutmore, 2021; *Spirocaecum* Yong, Cribb, and Cutmore, 2021; *Balistidicola* Cutmore and Cribb, 2022) were recently proposed based on the

identities of their definitive hosts, a combination of morphological features common to many genera of Aporocotylidae (*sensu stricto*, as diagnosed herein), and *28S* and *ITS2* phylogenetic analyses (Yong et al., 2021; Cutmore and Cribb, 2022). Cutmore and Cribb (2022) asserted that *Balistidicola* was unique by having short anterior ceca, long posterior ceca, a lanceolate body, a single testis, a post-ovarian uterus, and an oötype that is dextral and anterior to the cirrus sac. The type species of *Cardicola* (*Cardicola cardiocola* [Manter, 1947] Short, 1953) has each of those features (Manter, 1947; Short, 1953; Warren et al., 2021). The narrative description as well as Figure 4 of Warren et al. (2021), wherein the type species of *Cardicola* is redescribed based on the Manter's holotype and newly collected specimens, clearly shows that this species has each of those features—in particular the oötype is clearly anterior and dextral to the cirrus sac. Further, *Cardicola abu* Yong, Cutmore, and Cribb, 2018, *Cardicola ambrosioi* Braicovich, Etchegoin, Timi, and Sardella, 2006, *Cardicola coridodacis* Manter, 1954, *Cardicola currani* Bullard and Overstreet, 2004, *Cardicola forsteri* Cribb, Daintith, and Munday, 2000, *Cardicola nonamo* Bullard, 2010, *Cardicola opisthorchis* Ogawa, Ishimaru, Shirakashi, Takami, and Grabner, 2011, *Cardicola orientalis* Ogawa, Tanaka, Sugihara, and Takami, 2010, *Cardicola ahi* Yamaguti, 1970 *incertae sedis* (see Warren et al., 2021), and *Cardicola kurochkini* (Parukhin, 1976) Bullard and Overstreet, 2006 *incertae sedis* (see Warren et al., 2021) have this combination of features. Because of these facts, we reassign the type species of *Balistidicola* to *Cardicola* as *Cardicola corneri* (Cutmore and Cribb, 2022) n. comb. as well as reassign *Balistidicola yuelao* Yong, Cutmore, and Cribb, 2018 as *Cardicola yuelao* Yong, Cutmore, and Cribb, 2018. The reassignment of the type species of *Balistidicola* to *Cardicola* makes the genus a junior subjective synonym of *Cardicola*.

Regarding *Chanicola* and *Spirocaecum*, Yong et al. (2021) stated that these genera were diagnosed with morphological features that do not differentiate them from *Cardicola*, i.e., the diagnoses are not diagnostic. To justify this approach, Yong et al. (2021; p. 1) advocated that, "*molecular phylogenetics has shown that emphasizing phenetics alone is unreliable.*" These authors detailed their experiential concept of what they called "*wastebasket taxonomy*" wherein the morphological features they chose failed them in predicting the evolutionary relationships they recovered by their nucleotide-based analyses. We have the antithetical perspective based on available evidence. Our experience is that nucleotide-based phylogenetic reconstruction recovers morphologically similar and diagnosable blood fluke taxa as sharing a common ancestor; correspondingly, the morphological features used by us previously and herein diagnose and differentiate the monophyletic families (KEY) and their genera. Herein, for the lack of functional diagnoses (see above), we reassign the type species of *Chanicola* and the type species of *Spirocaecum* to *Cardicola* as *Cardicola jiigurru* Yong, Cutmore, Miller, Wee, and Cribb, 2016 and *Cardicola coeptus* Nolan and Cribb, 2006, respectively. These reassignments make *Chanicola* and *Spirocaecum* junior synonyms of *Cardicola*. As a result, the additional species are reassigned to *Cardicola*: *Cardicola suni* Yong, Cutmore, Miller, Wee, and Cribb, 2016 (formerly *Chanicola*) as well as the species formerly assigned to *Spirocaecum* (*Cardicola bartolii* Nolan and Cribb, 2006; *Cardicola covacinae* Nolan and Cribb, 2006; *Cardicola lafii* Nolan and Cribb, 2006; *Cardicola tantabiddii* Nolan and Cribb, 2006; *Cardicola mogilae* Brooks, Cribb, Yong and Cutmore, 2017).

We continue to view *Cardicola* spp. and the *Cardicola*-like aporocotylids as recently-evolved and co-radiating with euteleost fishes (Bullard, 2013; Orélis-Ribeiro et al., 2014). This could explain the slight morphological differences between them. Some fish blood fluke genera

include species that are morphologically similar but have large nucleotide differences between them (Bullard, 2014; Orélis-Ribeiro et al., 2017; Yong et al., 2018; Warren et al., 2021; Cutmore et al., 2022), and, alternatively, some fish blood flukes that are morphologically easily differentiated have few nucleotide differences (Bullard and Overstreet, 2003; Warren et al., 2021; Yong et al., 2021; Cutmore et al., 2022; Cutmore and Cribb, 2022). Given that there is no direct empirical correlation between morphological similarity/ dissimilarity and the corresponding degree of nucleotide similarity for specific lineages of trematodes, we think that it is perilous to assume that tree branch length justifies new genera as suggested by Yong et al. (2021). Without a morphological differential diagnosis, the limits of a genus would be arbitrary (because the tree topology changes upon adding a sequence) and depend on ingroup taxon selection, the marker being used, the methodological details of the phylogenetic analysis, and the outgroup selection.

Key to the families of fish blood flukes based on morphological diagnoses above

1a. Having C-shaped lateral tegumental spines each mounted on a muscular peduncle and having non-sinusoidal testis or lacking lateral tegumental spines and having sinusoidal testis; vitellarium symmetrical or asymmetrical, having a dextral primary vitelline collecting duct. Maturing in blood of sharks, rays, and chimaeras (Chondrichthyes)……
…….....**Chimaerohemecidae Yamaguti, 1971**

1b. Lacking C-shaped lateral tegumental spines; lacking sinusoidal testis; vitellarium asymmetrical or symmetrical.....2

2a. Anterior sucker robust, bowl-shaped, pedunculate; mouth comprising a large opening; pharynx present or absent; lateral tegumental spines spike-like (not C-shaped, lacking

recurved tip; can be lost in adults of some species); Laurer's canal present or absent; common genital atrium and pore present.....3

2b. Anterior sucker not bowl-shaped nor pedunculate; mouth comprising a minute pore-like opening; Laurer's canal absent; genital pores separate or common.....4

3a. Anterior sucker having minute spines on inner anteroventral surface in adults; tegumental spines distributing in ventrolateral transverse rows, with distal end having a sharp tip protruding slightly from tegument; pharynx present; intestine inverse U-shaped (anterior ceca absent) with posterior ceca terminating near the excretory bladder; 6 testes (5 pre-ovarian testes plus 1 post-ovarian testis), intercecal, having shallow lobes; common genital pore dextral, post-ovarian. Maturing in blood of sturgeons and paddlefishes (Acipenseriformes) .....**Acipensericolidae n. fam.**

3b. Anterior sucker aspinose in adults; tegumental spines not distributing in ventrolateral transverse rows, with distal end having a sharp tips enveloped by tegument (adults of *Elopicola* spp. are aspinose); pharynx present or absent; intestine generally inverse U-shaped; anterior ceca short or nearly indistinct; posterior ceca terminating at level of testis (*Elopicola*) / anterior-most testis (*Paracardicoloides*); 2 deeply lobed testes (1 pre-ovarian and intercecal testis plus 1 post-ovarian and post-cecal testis) (*Paracardicoloides*) or 1 intercecal, deeply-lobed testis (*Elopicola*); common genital pore sinistral, pre-ovarian. Egg in gill epithelium of fish host having elongate polar filaments. Maturing in blood of elopomorphs (Teleostei: Elopomorpha).....**Elopicolidae n. fam.**

4a. Medial esophageal swelling (pouch) present; intestine comprising short radial ceca of approximate equal length or having anterior ceca plus a single medial and dendritic posterior cecum; testis with appendix-like lateral lobes; genital pores common or separate. Maturing in

blood and body cavity of later branching freshwater ray-finned fishes (Teleostei) ..... **Sanguinicolidae Poche, 1926**

4b. Medial esophageal swelling (pouch) absent; pharynx absent; intestine X- or H-shaped; anterior ceca long or secondarily lost (*Allocardicola*); posterior ceca comprising a pair of elongate ceca each extending posteriad in parallel with respective body margin and terminating in middle or posterior 1/3 of body or in anterior 1/3 of body (*Neoparacardicola*); testes lacking appendix-like lateral lobes; genital pores sinistral or having sinistral common genital pore. Maturing in blood or body cavity of later branching marine ray-finned fishes (Teleostei).....**Aporocotylidae Odhner, 1912**

Phylogenetic remarks

Our *28S* phylogeny recovered the fish blood flukes as monophyletic and sister to the remaining blood flukes infecting turtles and homeotherms (Fig. 1) (Bullard et al., 2019; Bullard and Dutton, 2022). Each of the morphologically-diagnosed families was recovered as monophyletic. This tree resembles most previously-published blood fluke trees and comprises the largest taxon sampling for fish blood flukes to date. Acipensericolidae was recovered as a sister to all other fish blood flukes, with two main clades comprising Chimaerohemecidae + Sanguinicolidae and Elopicolidae + Aporocotylidae.

DISCUSSION

Testing the so-called deep cophyly between blood flukes and their craniate hosts remains a key question in the natural history of blood flukes, including that for the schistosomes (Bullard and Dutton, 2022). Because the natural history of the fish blood flukes explains the origin of the schistosomes, their systematics and host-parasite relationships are of broad interest. Smith (1972) and Maillard (1982) asserted that fish blood flukes showed no evidence of strict phylogenetic

host specificity at the level of genus or species. However, our current understanding of the fish blood flukes is that i) morphologically similar, phylogenetically-related fish blood flukes mature in phylogenetically-related fish lineages (KEY), ii) the branching order (based on *28S* phylogenetic analyses) for fish blood flukes does not mirror that for craniates (i.e., the chimaerohemecids are not sister to all blood flukes) but does indicate that marine ray-finned fishes are a late branching lineage among fish blood flukes, and that iii) the phylogenetic position of these major fish blood fluke lineages (each of which is robustly monophyletic) remains unsettled (Fig. 1; Table I). This new understanding derives mainly from discoveries of fish blood flukes infecting chondrichthyans, early branching actinopterygians, and other freshwater fishes; all of which previously were under-explored for blood fluke infections (Orélis-Ribeiro et al., 2013, 2014, 2017; Orélis-Ribeiro and Bullard, 2015, 2016; Warren et al., *in review*, 2017, 2019, 2020, 2021; Warren and Bullard, 2019, 2021). Further, although few fish blood fluke life cycles are known, 11 from freshwater fishes and 8 from marine fishes (Warren and Bullard, 2019; Cutmore et al., 2022) (Table II), the fish blood flukes appear to show some level of specificity (and perhaps some level of cophyly) to/with their molluscan first intermediate hosts (Cribb et al., 2017; Warren and Bullard, 2019; Cutmore et al., 2022).

The blood flukes (Schistosomatoidea) seem to be an old lineage because they mature in collectively all major craniate lineages and exploit a large diversity of intermediate hosts. They mature in hosts belonging to the earliest branching extant jawed vertebrates (Chondrichthyes), to the early branching actinopterygians (Acipenseriformes), to late branching ray-finned fishes (Tetraodontiformes), and freshwater and marine turtles and homeotherms (birds and mammals). Further, they collectively exploit the greatest diversity of invertebrate hosts of any trematode lineage; undergoing asexual reproduction in bivalves and snails but also polychaetes (Table II).

Alternatively, but seemingly less likely is that the blood flukes recently evolved and rapidly colonized and radiated across the craniate tree of life. Extinct early branching gnathostome lineages could have harbored blood fluke infections, and those blood flukes would comprise key missing links to piece together the natural history of the blood flukes. For example, a blood fluke could have infected any number of extinct fish lineages (pteraspidomorphs, anaspidomorphs, thelodontomorphs, osteostracomorphs, placodermiomorphs) or the 47 extinct lineages (families and orders) of chondrichthyans (Nelson et al., 2016). However, unless the lateral tegumental spines, for example, are discovered in a fossil fish belonging to one of these lineages, we could never know. The most alluring craniates that remain to be explored for blood fluke infections are the extant jawless fishes (monophyletic Agnatha) and stem sarcopterygians (lungfishes [Dipnoi] and coelacanths [Coelacanthomorpha]). Despite having examined limited numbers of lampreys and lungfishes (S. A. Bullard, pers. obs.), no infection has been detected so far. Further, the extant agnathans, lungfishes, and coelacanths are each but a relic of a previously more species-rich lineage. Perhaps this theorized extinction of some fish, turtle, and tetrapod blood fluke lineages explains why robust clades of blood flukes are unsettled regarding their phylogenetic position.

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FIGURE LEGENDS

Figure 1. Maximum likelihood phylogeny based on the large subunit ribosomal DNA (*28S*). Labels in front of nodes are bootstrap support values. Values <80 not shown. New sequences generated along with GenBank accession numbers for this study in Table I. Scale bar indicates substitutions per site. Sequences for blood flukes not yet described or cercarial sequences follow associated numbering: [1]Sanguinicolidae spp., cercariae ex. *Posticobia brazieri* (Smith 1882), Brisbane River, Queensland, Australia (Cutmore et al., 2022); [2]Sanguinicolidae sp. W5003, cercaria ex. *Plotiopsis balonnensis* (Conrad, 1850), Northern Territory, Victoria River, Australia (Brant et al., 2006); [3]Sanguinicolidae sp. ex *Juga plicifera* (Lea, 1838), King, Hills, Dell, Taylor Creek; McKenzie River, Oregon, U.S.A. (Preston et al., 2020); [4]Sanguinicolidae sp. ex *Micropterus* sp. (Lacepède 1802), Coosa River, Alabama, U.S.A.; [5]Sanguinicolidae sp. W5004, cercaria ex. *Amerianna carina* (Adams, 1861), Northern Territory, Mary River Floodplain, Australia (Brant et al., 2006); [6]Sanguinicolidae sp. ex. *Mystus* cf. *mysticetus*, Mekong River, Vietnam; [7]Sanguinicolidae sp. ex. *Pangasius* cf. *macronema*, Mekong River, Vietnam; [8]Chimaerohemecidae sp., cercaria ex. *Plebidonax deltoides* (Lamarck, 1818), Stockton Beach, New South Wales, Australia (Cribb et al., 2017); [9]Chimaerohemecidae sp. H, cercaria ex. *Megapitaria squalida* (G. B. Sowerby I, 1835), Off Santa Rosalía, Gulf of California, Mexico Gulf of California, Mexico (Cutmore et al., 2022); [10]Chimaerohemecidae sp., cercaria ex. *Solen viridis* (Say, 1821), Gulf of Mexico, U.S.A. (Warren and Bullard, 2019); [11]Chimaerohemecidae sp. ex. *Gymnura micrura* (Bloch and Schneider, 1801), Mobile Bay, Alabama, U.S.A; [12]Chimaerohemecidae sp. ex. *Pristis pectinata* Latham, 1796, Gulf of Mexico, U.S.A.; [13]Chimaerohemecidae sp. ex. *Gymnura* cf. *poecilura*, Nha Trang, Vietnam; [14]Schistosomatoidea sp. W1134 ex. *Biomphalaria sudanica* (E von Martens, 1870), Queen Elizabeth National Park, Uganda (Brant et al., 2006); [15]Schistosomatoidea sp. W1284 ex. *Segmentorbis kanisaensis* (Preston, 1914), Lake Victoria, Kenya (Brant et al., 2006).

Table I. New *28S* sequences generated for the present study. Superscripts indicate specimens in Figure 1.

Species	Host	Locality	GenBank Accession #s
Chimaerohemecidae sp.[13]	longtail butterfly ray, *Gymnura* cf. *poecilura* (Shaw, 1804) Dor, 1984 (Myliobatiformes: Gymnuridae)	Eastern Sea, off Nha Trang, Vietnam	OQ709101
Chimaerohemecidae sp.[12]	smalltooth sawfish, *Pristis pectinata* Latham, 1796 (Rhinopristiformes: Pristidae)	Eastern Gulf of Mexico, off Naples, Florida, USA	OQ709102
Chimaerohemecidae sp.[11]	smooth butterfly ray, *Gymnura micrura* (Bloch and Schneider, 1801) Uyeno and Miyake, 1983 (Myliobatiformes: Gymnuridae)	northern Gulf of Mexico, Mobile Bay, Alabama, USA	OQ709103
Sanguinicolidae sp.[4]	bass, *Micropterus* sp. (Lacepède 1802) Berg, 1949 (Centrarchiformes: Centrarchidae)	Coosa River, Alabama, USA	OQ709104
Sanguinicolidae sp.[6]	*Mystus* cf. *mysticetus* Roberts, 1992 (Siluriformes: Bagridae)	Dong Thap fish market (Mekong River), Vietnam	OQ709105
Sanguinicolidae sp.[7]	*Pangasius* cf. *macronema* Bleeker, 1850 (Siluriformes: Pangasiidae)	Dong Thap fish market (Mekong River), Vietnam	OQ709106
Nomasanguinicola canthoensis Truong and Bullard, 2013	broadhead catfish, *Clarias macrocephalus* Günther 1864 (Siluriformes: Clariidae)	Can Tho fish market, (Mekong River), Vietnam	OQ709107
Hyperandrotrema walterboegeri Orélis-Ribeiro and Bullard, 2013	shortfin mako shark, *Isurus oxyrinchus* Rafinesque, 1810 (Lamniformes: Lamnidae)	northern Gulf of Mexico, off Dauphin Island, Alabama, USA	OQ709108
Myliobaticola richardheardi Bullard and Jensen, 2008	Atlantic stingray, *Hypanus sabinus* (Lesueur, 1824) Last, Manjaji-Matsumoto, Naylor, and White, 2016 (Myliobatiformes: Dasyatidae)	Mississippi Sound, northern Gulf of Mexico, off Biloxi, Mississippi, USA	OQ709109
Selachohemecus benzi Bullard, Overstreet, and Carlson, 2006	blacktip shark, *Carcharhinus limbatus* (Valenciennes, 1839) Compagno, 1973 (Carcharhiniformes: Carcharhinidae)	northern Gulf of Mexico, Mississippi, USA	OQ709110
Selachohemecus olsoni Short, 1954	Atlantic sharpnose shark, *Rhizoprionodon terraenovae* (Richardson, 1837) Springer, 1964 (Carcharhiniformes: Carcharhinidae)	Mississippi Sound, northern Gulf of Mexico, Mississippi, USA	OQ709111

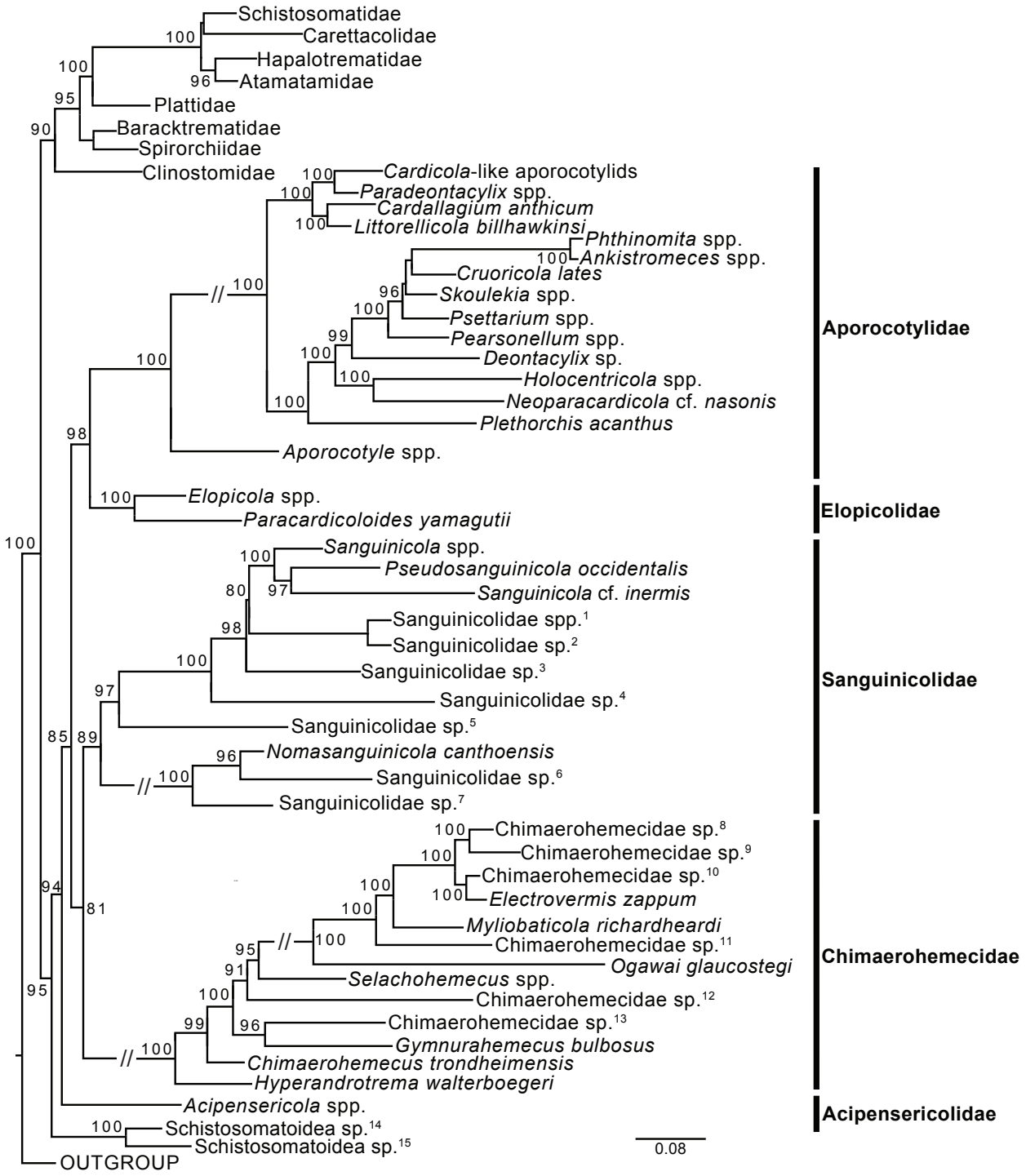
Table II. Reports of intermediate hosts and completed life cycles of fish blood flukes.

Host	Cercaria	Locality	Reference
GASTROPODA			
Amerianna carinata (H. Adams, 1861) (Hygrophila: Planorbidae); *Glyptophysa gibbose* (A. Gould, 1847) (Hygrophila: Planorbidae)	Sanguinicolidae sp.[5] (W5004)	northern Territory, Mary River Floodplain, Australia	Brant et al., 2006
Biomphalaria sudanica (E von Martens, 1870) (Hygrophila: Planorbidae)	Schistosomatoidea sp.[14] (W1134)	Queen Elizabeth National Park, Uganda	Brant et al., 2006
Campeloma decisum (Say, 1817) (Architaenioglossa: Viviparidae)	*Pseudosanguinicola occidentalis*	New York	Bacha, 1966
Fluminicola seminalis (Hinds, 1842) (Littorinimorpha: Hydrobiidae)	*Sanguinicola klamathensis*	Darrah Springs Hatchery, Tehama Co., California	Wales, 1958
Fluminicola virens (I. Lea, 1838) (Littorinimorpha: Hydrobiidae)	*Sanguinicola idahoensis*	Clearwater River, Clearwater Co., Idaho	Schell, 1974
Heleobia australis (Orbigny, 1835) (Littorinimorpha Cochliopidae)	Aporocotylidae gen sp. 1	Arroyo Cangrejo, Buenos Aires province, Argentina	Matías et al., 2014
Juga plicifera (Lea, 1838) (as *Oxytrema silicula*) (Caenogastropoda: Semisulcospiridae)	*Sanguinicola alseae*	Alsea River, Benton Co., Oregon	Meade and Pratt, 1965
Juga plicifera (Lea, 1838) (as *Oxytrema silicula*) (Caenogastropoda: Semisulcospiridae)	Sanguinicolidae sp.[3] (Aporocotylidae sp.)	King, Hills, Dell, Taylor Creek; McKenzie River	Preston et al., 2020
Ancylus fluviatilis O. F. Müller, 1774 (Hygrophila: Planorbidae)	*Sanguinicola rutili*	Salamanca, Spain	Simon-Martin et al., 1987
Leptoxis carinata (Bruguiere, 1792) "*Oxytrema circumlineata*" (Caenogastropoda: Pleuroceridae)	*Sanguinicola fontinalis*	Susquehanna River, Hariisburg, Pennsylvania	Hoffman et al., 1985
	Sanguinicola davisi	Darrah Springs Hatchery, Tehama Co., California	Wales, 1958
	Sanguinicola klamathensis	Klamath River, Oregon	Meade, 1967

Littorodinops monroensis Frauenfeld, 1863 (Littorinimorpha: Hydrobiidae)	Aporocotylidae sp. cercaria type 1	northern Gulf of Mexico, Grand Lagoon, Florida	Bullard dissertation, 2008
Lymnaea stagnalis (Linnaeus, 1758) (Hygrophila: Lymnaeidae)	*Sanguinicola* sp.	Lake Glubokoye, Russia	Nikolaeva, 1985
Peregriana peregra (O. F. Müller, 1774) (as *Lymnaea*) (Hygrophila: Lymnaeidae)	*Sanguinicola inermis*	Europe	Kirk and Lewis, 1993
Planorbella trivolvis Say, 1817) (as *Helisoma*) (Hygorphila: Planorbidae)	*Cercaria brevifurca*	vicinity of St. Louis, Missouri	McCoy, 1929
	Cercaria whitentoni	Stillwater Tourist Park, Stillwater, Oklahoma	Croft, 1933
Plotiopsis balonnensis (Conrad, 1850) (Caenogastropoda: Thiaridae) (as *Thiara balannensis*)	Sanguinicolidae sp.[2] (W5003)	northern Territory, Victoria River, Australia	Brant et al., 2006
Posticobia brazieri (Smith 1882) (Littorinimorpha: Tateidae)	*Paracardicoloides yamagutii*	Downfall Creek, Wivenhoe Pocket, Queensland, Australia	Nolan and Cribb, 2004
	Sanguinicolidae sp.[1] (Aporocotylidae sp. B)	unnamed tributary of Brisbane River, Australia; Fairnie Brook, Queensland, Australia	Cutmore et al., 2022
	Sanguinicolidae sp.[1] (Aporocotylidae sp. C)	Moggill Creek, Queensland, Australia; unnamed tributary of Brisbane River, Queensland, Australia	Cutmore et al., 2022
	Sanguinicolidae sp.[1] (Aporocotylidae sp. D)	unnamed tributary of Brisbane River, Queensland, Australia	Cutmore et al., 2022
	Sanguinicolidae sp.[1] (Aporocotylidae sp. E)	unnamed tributary of Brisbane River, Queensland, Australia	Cutmore et al., 2022
	Sanguinicolidae sp.[1] (Aporocotylidae sp. F)	Moggill Creek, Queensland, Australia	Cutmore et al., 2022
	Sanguinicolidae sp.[1] (Aporocotylidae sp. G)	Churchbank Weir, Queensland, Australia	Cutmore et al., 2022
Segmentorbis kanisaensis (Preston, 1914) (Hygrophila: Planorbidae)	Schistosomatoidea sp.[15] (W1284)	Lake Victoria, Kenya	Brant et al., 2006
Valvata tricarinata (Say, 1817)	*Sanguinicola*	Lake Francis, Isanti Co., Minnesota	Erickson and Wallace,

(Heterobranchia: Valvatidae)	*lophophora*		1959
BIVALVIA			
Anadara trapezia (Deshayes, 1839) (Arcida: Arcidae)	*Elopicola bristowi*	eastern Moreton Bay, Queensland, Australia	Cutmore et al., 2022
Argopecten irradians (Lamarck, 1819) (Pectinida: Pectinidae)	*Cercaria martini*	northwestern Atlantic Ocean, Woods Hole, Massachusetts	Linton, 1915; Stunkard, 1983
Chione cancellata (Linnaeus, 1767) (Venerida: Veneridae)	*Cercaria cristulata*	northern Gulf of Mexico, Alligator Point, Florida	Holliman, 1961
Donax variabilis Say, 1822 (Cardiida: Donacidae)	*Cercaria asymmetrica*	northern Gulf of Mexico, Alligator Point, Florida	Holliman, 1961
	Electrovermis zappum	northern Gulf of Mexico, Fort Morgan, Alabama	Warren and Bullard, 2019
Ensis macha (Molina, 1782) (Adapedonta: Pharidae)	Aporocotylidae sp. cercaria	La Tapera, San Matias Gulf, Patagonia, Argentina	Vázquez et al., 2013; Orellana and Lohrmann, 2015
Eucallista purpurata (Lamarck, 1818) (Venerida, Veneridae) (as *Amiantis purpurata*)	Aporocotylidae sp. cercaria	El Molino Beach, San Matias Gulf, Patagonia, Argentina	Gilardoni et al., 2011; Carvalho et al., 2015
Mactra isabelleana d'Orbigny, 1846 (Venerida: Mactridae)	Aporocotylidae sp. cercaria	Cassino Beach, San Matias Gulf, Patagonia, Argentina	Carvalho et al., 2015
Megapitaria squalida (G. B. Sowerby I, 1835) (Venerida, Veneridae)	Chimaerohemecidae sp.[9] (Aporocotylidae sp. H)	Off Santa Rosalía, Gulf of California, Mexico	Yee-Duarte et al., 2017; Cutmore et al., 2022
Mercenaria capechiensis (Gmelin, 1791) (Venerida: Veneridae)	*Cercaria mercenariae*	western Gulf of Mexico, Galveston Island, Texas	Wardle, 1979
Mesodesma donacium (Lamarck, 1818) (Venerida: Mesodesmatidae)	Aporocotylidae sp. cercaria	Carelmapu, Chile	López et al., 2014
Plebidonax deltoides (Lamarck, 1818) (as *Donax*) (Cardiida: Psammobiidae)	Chimaerohemecidae sp.[8] (Aporocotylidae sp. NSW1)	Stockton Beach, New South Wales, Australia	Cribb et al., 2017
Solemya velum Say, 1822 (Solemyida: Solemyidae)	*Cercaria solemyae*	northwestern Atlantic Ocean, Woods Hole, Massachusetts	Martin, 1944
Solen viridis (Say, 1821)	Chimaerohemecidae	northern Gulf of Mexico, Mississippi	Bullard dissertation,

(Adapedonta: Solenidae)	sp.[10] (Aporocotylidae sp. cercaria type 2)	Sound, Mississippi	2008; Warren and Bullard, 2019
Tagelus divisus (Spengler, 1794) (Cardiida: Solecurtidae)	Aporocotylidae sp. cercaria	northwestern Atlantic Ocean, Biscayne Bay, Florida	Fraser, 1967
ANNELIDA			
Amphicteis gunneri (Sars, 1835) (Terebellida: Ampharetidae)	*Cercaria amphicteis*	northern Gulf of Mexico, Apalachicola River, Florida	Olgesby, 1961
Amphitrite ornata (Leidy, 1855) (Terebellida: Terebellidae)	*Cardicola parvus*	Oyster landing in North Inlet, near Georgetown, SC and Charleston, SC	Siegel et al., 2018
Amphitrite sp. Müller, 1771 (Terebellida: Terebellidae)	*Cardicola forsteri*	Kushimoto, Wakayama Prefecture, Japan	Shirakashi et al., 2016
Artacama proboscidea Malmgren, 1866 (Terebellida: Terebellidae)	*Aporocotyle simplex*	Øresund, north of the island Veen, between Denmark and Sweden	Køie, 1982
Enoplobranchus sanguineus (Verrill, 1873) (Terebellida: Terebellidae)	*Cardicola parvus*	Oyster landing in North Inlet, near Georgetown, SC and Charleston, SC	Siegel et al., 2018
Hydroides dianthus Verrill, 1873 (Sabellida: Serpulidae)	*Cercaria loossi*	Northwestern Atlantic Ocean, Woods Hole, Massachusetts	Linton, 1915
Lanassa nordenskioldi Malmgren, 1866 (Terebellida: Terebellidae)	"morphologically indistinguishable" from *Aporocotyle simplex*	Seyðisfjörður, Eastern Iceland	Køie and Petersen, 1988
Lanicides vayssierei (Gravier, 1911) (Terebellidae: Terebellidae)	*Cercaria hartmanae*	northwestern Atlantic Ocean, Woods Hole, Massachusetts	Martin, 1952
Longicarpus modestus (Quatrefages, 1866) (Terebellida: Terebellidae)	*Cardicola forsteri*	off Port Lincoln, South Australia	Cribb et al., 2011
Nicolea gracilibranchis (Grube, 1878) (Terebellida: Terebellidae)	*Cardicola orientalis*	Kushimoto, Wakayama Prefecture, Japan	Shirakashi et al., 2016
Terebella lapidaria Linnaeus, 1767 (Terebellida: Terebellidae)	*Cardicola laruei*	Oyster landing in North Inlet, near Georgetown, SC and Charleston, SC	Siegel et al., 2018
Terebella sp. Linnaeus, 1767 (Terebellida: Terebellidae)	*Cardicola opisthorchis*	off Tsushima, Nagasaki, Japan	Sugihara et al., 2014


Schistosomatidae
Carettacolidae
Hapalotrematidae
Atamatamidae
Plattidae
Baracktrematidae
Spirorchiidae
Clinostomidae
Cardicola-like aporocotylids
Paradeontacylix spp.
Cardallagium anthicum
Littorellicola billhawkinsi
Phthinomita spp.
Ankistromeces spp.
Cruoricola lates
Skoulekia spp.
Psettarium spp.
Pearsonellum spp.
Deontacylix sp.
Holocentricola spp.
Neoparacardicola cf. nasonis
Plethorchis acanthus
Aporocotyle spp.
Aporocotylidae
Elopicola spp.
Paracardicoloides yamagutii
Elopicolidae
Sanguinicola spp.
Pseudosanguinicola occidentalis
Sanguinicola cf. inermis
Sanguinicolidae spp.1
Sanguinicolidae sp.2
Sanguinicolidae sp.3
Sanguinicolidae sp.4
Sanguinicolidae sp.5
Nomasanguinicola canthoensis
Sanguinicolidae sp.6
Sanguinicolidae sp.7
Sanguinicolidae
Chimaerohemecidae sp.8
Chimaerohemecidae sp.9
Chimaerohemecidae sp.10
Electrovermis zappum
Myliobaticola richardheardi
Chimaerohemecidae sp.11
Ogawai glaucostegi
Selachohemecus spp.
Chimaerohemecidae sp.12
Chimaerohemecidae sp.13
Gymnurahemecus bulbosus
Chimaerohemecus trondheimensis
Hyperandrotrema walterboegeri
Chimaerohemecidae
Acipensericola spp.
Acipensericolidae
Schistosomatoidea sp.14
Schistosomatoidea sp.15
OUTGROUP
0.08

CHAPTER 5: FIRST REPORT OF A FISH BLOOD FLUKE FROM SUB-SAHARAN AFRICA: *NOMASANGUINICOLA DENTATA* (PAPERNA, 1964) WARREN AND BULLARD, 2023 INFECTING AFRICAN SHARPTOOTH CATFISH, *CLARIAS GARIEPINUS* (BURCHELL, 1822) TEUGLES, 1982 IN THE OKAVANGO RIVER, NAMIBIA WITH COMMENTS ON NATURAL HISTORY OF CATFISH BLOOD FLUKES

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ABSTRACT

We herein provide a supplemental description of *Nomasanguinicola dentata* (Paperna, 1964) Warren and Bullard, 2023 (Digenea: Sanguinicolidae) and provide a *28S* phylogeny to test relationships among freshwater fish blood flukes. We examined the heart of three African sharptooth catfish, *Clarias gariepinus* (Burchell, 1822) Teugles, 1982 from the Okavango River (northeastern Namibia) that was infected with adults of *N. dentata*. This blood fluke differs from *N. canthoensis* by having a body 5.3–6.7 longer than wide (vs. 3.5–4.6), an anterior esophageal swelling 7–8% (vs. 14–24%) of total esophageal length, a posterior esophageal swelling 3–5% (vs. 8–10%) of total esophageal length, a pre-cecal (vs. wholly post-cecal) testis, and an ovary that does not extend laterally beyond the nerve cords. The *28S* sequence for *N. dentata* differed from that of *N. canthoensis* by 144 bp (9% difference), and the phylogenetic analysis recovered these species as sister taxa and Sanguinicolidae as monophyletic. This is the first report of a fish blood fluke from sub-Saharan Africa, and the first report of a species of *Nomasanguinicola* from Africa in ~40 yrs.

Keywords: sub-Saharan, fish blood fluke, taxonomy, systematics, Africa

1. Introduction

The Otophysa comprises four fish orders (Cypriniformes, Characiformes, Gymnotiformes, Siluriformes) and 67% of all primary division freshwater fishes (Nakatani et al. [1]; Diogo [2]). Among these are the catfishes (Siluriformes) that range on every continent except Antarctica (only fossils are reported from Antarctica) and inhabit freshwater, estuarine, and marine environments (Nakatani et al. [1]; Diogo [2]; Nelson et al. [3]). The blood flukes that mature in primary division freshwater fishes comprise 35 species assigned to three families (Sanguinicolidae Poche, 1926, Acipensericolidae Warren and Bullard, 2023, Elopicolidae Warren and Bullard, 2023) (see Warren and Bullard [4]). Among these families, six genera assigned to Sanguinicolidae infect otophysans (*Sanguinicola* Plehn, 1905; *Plehniella* Szidat, 1951; *Nomasanguinicola* Truong and Bullard, 2013; monotypic *Cladocaecum* Orélis-Ribeiro and Bullard, 2016; monotypic *Kritsky* Orélis-Ribeiro and Bullard, 2016; and monotypic *Pseudosanguinicola* Warren and Bullard, 2023). They infect carps (Cypriniformes) (Plehn [5]; Warren et al. [6]), prochilods (Characiformes) (Szidat [7]), catfishes (Siluriformes) (Szidat [7]; Guidelli et al. [8]; Truong and Bullard [9]; Orélis-Ribeiro and Bullard [10]; [11]), and pickerels (Esociformes) (Warren et al. [6]). Ten sanguinicolids infect catfishes (*Nomasanguinicola* spp. [3 spp.], *Plehniella* spp. [3 spp.], monotypic *Cladocaecum,* monotypic *Kritsky*, *Sanguinicola chalmersi* Odhner, 1924 *incertae sedis*, *Sanguinicola sanliense* Wang, 1982 *incertae sedis* [see Warren et al. [6]).

Relative to North America and Mexico, African freshwater fishes are under sampled for parasites (Scholz and Choudhury [12]) and the blood flukes described from Africa are poor and incomplete. In fact, no parasite has been reported for over 80% of the fish species in the four

largest African rivers (Niger, Nile, Congo, and Zambezi) and the African Great Lakes (Lakes Victoria, Malawi, and Tanganyika) (Smit et al. [13]). Further, the two fish blood flukes that have been reported from African catfishes (Siluriformes), both have incomplete morphological descriptions and lack any nucleotide data, and both were collected from northern Africa (*S. chalmersi* [Sudan] and *Nomasanguinicola clarias* {Imam, Marzouk, Hassan, and Itman, 1984} Warren and Bullard 2023 [Egypt]) (Woodland [14]; Odhner [15]; Imam et al. [16]; Warren et al. [6]). *Nomasanguinicola clarias* is morphologically similar to, infects the same host (African sharptooth catfish, *Clarias gariepinus* [Burchell, 1822] Teugles, 1982), and was collected in the same geographic location (Egypt vs. Israel) as *Nomasanguinicola dentata* (Paperna, 1964) Warren and Bullard, 2023 (Paperna [17], Truong and Bullard [9]). The problem is *N. dentata* also has an incomplete and seemingly erroneous morphological description and lacks a nucleotide sequence. Hence, supplemental descriptions of both *N. dentata* and *N. clarias* are needed to determine if they should be synonymized.

Herein, we provide a supplemental description for *N. dentata* and reconstruct a phylogenetic analysis using the large subunit ribosomal DNA (*28S*) to support the morphological description and test relationships and monophyly of the Sanguinicolidae. We also comment on the monophyly of catfish blood flukes.

2. Materials and methods

2.1. Fish collection, parasite specimen collection, preparation, and deposition

On 7 Dec 2021, 3 African sharptooth catfish (*C. gariepinus*) from the Okavango River (northeastern Namibia) were captured by hook and line and identified using Skelton [18] by having a mottled dorsal surface, a white ventral surface, 3 pairs of barbels (1 pair dorsal, 1 pair lateral, 1 pair vertical), palantine teeth fused and inverse V-shaped, first and second dorsal fin

rays half the length of third ray, dorsal fin rays (71), anal fin rays (53), pelvic fin rays (4), and pectoral fin rays (9) counts. The heart was excised intact, sliced longitudinally, immersed, and shaken in saline, and examined with the aid of a dissecting microscope and fiber optic light source. The heart was teased apart with forceps to reveal adult blood flukes, and sediment from the fixed heart was taken from a settling column and examined. Adult specimens intended for morphology were observed microscopically, heat-killed on glass slides using a butane hand lighter under no coverslip pressure and fixed in 10% neutral buffered formalin (n.b.f.). Flukes collected for DNA extraction were wet mounted on glass slides and examined to confirm their identity, preserved in 95% ethanol (EtOH), and stored at –20ºC. Upon returning to the laboratory morphological specimens were rinsed with distilled water, stained overnight in Van Cleave's hematoxylin with 3 additional drops of Ehrlich's hematoxylin, dehydrated using an ethanol series, cleared in clove oil, permanently mounted in Canada balsam, illustrated using a Olympus BX51 microscope (Olympus Corporation of the Americas, Center Valley, Pennsylvania, USA) equipped with differential interference contrast (DIC), measurements were obtained by using a Jenoptik Gryphax camera (Jenoptik AG, Jena, Germany), and illustrated using a drawing tube. Measurements are reported in micrometers (μm) as the range followed by the mean, standard deviation, and sample size in parentheses unless otherwise indicated. Scientific names, including taxonomic authorities and dates, for fishes follow Eschmeyer et al. [19]. Classification and anatomical terms for fish blood flukes follow Warren and Bullard [4] and Warren et al. [6]. Types and vouchers were deposited in the National Museum of Natural History's Invertebrate Zoology Collection (USNM, Smithsonian Institution, Washington, DC).

2.2. DNA extraction, amplification, sequencing, and Phylogenetic analysis

Two EtOH-preserved and microscopically identified fish blood flukes were used for DNA extraction and sequencing. DNA extraction, primers used, PCR amplification, sequencing, sequence assembly and analysis follow that of Warren et al. 6, 20, 21]. The phylogenetic analyses included the new freshwater fish blood fluke sequence and selected sequences representing species of sanguinicolids that were available on GenBank (Warren et al. [6]; Warren and Bullard [4]). The out-group comprises sequences representing the chimaerohemecids from Warren and Bullard [4] for the analysis. Sequences were aligned with the multiple alignment tool using fast Fourier transform (MAFFT) (Katoh and Standley [22]) and trimmed to the length of the shortest sequence presented herein (1,362 [*28S*] base pairs [bp]). JModelTest 2 version 2.1.10 was implemented to perform statistical selection of the best-fit models of nucleotide substitution based on Bayesian Information Criterion (BIC) (Darriba et al. [23]). Aligned sequences were reformatted (from .fasta to .nexus) using the web application ALTER (Glez-Peña et al. [24]) to run Bayesian inference (BI). BI was performed in MrBayes version 3.2.7a (Ronquist and Huelsenbeck [25]) using substitution model averaging ("nst-mixed") and a gamma distribution to model rate-heterogeneity. Defaults were used in all other parameters. Three independent runs with 4 Metropolis-coupled chains were run for 5,000,000 generations, sampling the posterior distribution every 1,000 generations. Convergence was checked using Tracer v1.6.1 (Rambaut et al. [26]) and the "sump" command in MrBayes: all runs appeared to reach convergence after discarding the first 25% of generations as burn-in. A majority rule consensus tree of the post burn-in posterior distribution was generated with the "sumt" command in MrBayes. The inferred

phylogenetic tree was visualized using FigTree v1.4.4 (Rambaut et al. [26]) and further edited for visualization purposes with Adobe Illustrator (Adobe Systems).

3. Results

3.1 Nomasanguinicola dentata (Paperna, 1964) Warren and Bullard, 2023 (Figs. 1A–C; 2A–D)

3.1.1 Light microscopy of adult based on 4 whole-mounted adult specimens: USNM collection nos. XXXXXXX–XXXXXXX)

Body elongate, ovoid in shape, 1,814–3,249 (2,229 ± 683, 4) long, 303–482 (364 ± 81, 4) in maximum width, 5.3–6.7 × longer than wide (Figs. 1A, B); lateral body margin having regularly spaced tegumental papillae, 2.3–8.6 (4.6 ± 3, 40) long, 2.1–16 (6.9 ± 6.4, 40) wide (Fig. 2D). Ventrolateral nerve cords 10–15 (12 ± 2, 4) wide near mid-body, 48–84 (59 ± 17, 4) or 14–17% of body width from body margin; secondary branches and dorsolateral nerve cords not evident; commissure of ventrolateral nerve cord 210–316 (243 ± 49, 4) or 10–12% of body length from anterior body end, 54–84 (67 ± 13, 4) across body width, 15–18 (16 ± 2, 4) in breadth, coursing dorsal to esophagus (Figs. 1A, B). Tegumental sensory papillae present, distributing across ventral and dorsal surface of body from level of anterior sucker to posterior end, approximately 4.2–6.4 (5.4 ± 0.9, 4) in diameter, cilia not observed.

Anterior sucker 38–57 (50 ± 8, 4) long, 55–72 (61 ± 8, 4) wide at base or 15–18% of body width, 1–1.9 × wider than long (Fig. 2A); terminal papillae on anterior margin of anterior sucker not observed; denticles of anterior sucker 23–24 (23.6 ± 0.3, 4) in total length, 4–4.5 (4.2 ± 0.4, 4) in maximum width, extending 4 (7) from tegument; shaft 13–17 (15 ± 2, 4) long, 4.1–4.3 (4.2 ± 0.1, 4) in maximum width; aperture 8.8–10 (9.3 ± 0.7, 4); with each flanking column 28–41 (34 ± 6, 4) long or 56–79% of anterior sucker length (Figs. 2A–C). Mouth 3–4 (3.3 ± 1, 4) in diameter, medioventral, 13–16 (14 ± 1.5, 4) or 28–29% of anterior sucker length from anterior

end, trilobate (Fig. 2A); pharynx not observed. Esophagus 600–960 (726 ± 160, 4) in total length or 30–36% of body length, beginning as narrow tube extending posteriad 94–143 (114 ± 22, 4) or 4–7% of body length before connecting with anterior esophageal swelling; anterior esophageal swelling 48–69 (56 ± 10, 4) long or 7–8% of esophagus total length, 10–14 (11 ± 2, 4) wide or 3–4% of maximum body width, at level midway between ventrolateral nerve commissure and anterior sucker; esophagus narrowing from anterior esophageal swelling and extending posteriad; posterior esophageal swelling immediately anterior to ceca 26–35 (30 ± 4, 4) long or 3–5% of esophageal total length, 20–33 (26 ± 6, 4) wide or 1–3% × maximum esophagus width (Figs. 1A, B). Esophageal gland indistinct.

Intestine X-shaped, having 4 distinct radial ceca (Figs. 1A, B); cecal intersection 608–967 (732 ± 160, 4) or 30–36% of body length from anterior body end; anterior ceca 33–54 (43 ± 11, 4) long or 2% of body length, 17–24 (19 ± 4, 4) wide, containing granular material within lumen of some individuals; posterior ceca 42–54 (47 ± 6, 4) long or 2–3% of body length, 20–24 (22 ± 2, 4) wide, ventral to testis; post-cecal space 1,161–2,253 (1465 ± 527, 4) long or 63–69% of body length.

Testis 697–1,571 (931 ± 427, 4) long or 35–48% of body length, 107–165 (123 ± 28, 4) wide or 31–36% of body width, 6–10 × longer than wide, lobed, extending anterior to cecal bifurcation (Figs. 1A, B); post-testicular space 566–1,039 (732 ± 210, 4) long or 29–36% of body length. Vasa efferentia comprising several dendritic ducts, 18–26 (21 ± 3, 4) wide. Vas deferens 122–205 (159 ± 35, 4) long, 9–17 (14 ± 3, 4) wide, emanating from postero-ventral portion of testis, curving sinistrally before sharply curving towards midline and becoming confluent with seminal vesicle. Cirrus-sac present, having wall approximately 5–9 (7 ± 1.7, 4) thick, including seminal vesicle, cirrus indistinct; seminal vesicle 158–503 (293 ± 148, 4) long,

27–51 (36 ± 11, 4) wide, 5–10 × longer than wide (Figs. 1A–C); male genital pore toward midline, 4–12 (8 ± 3, 4) in diameter, dorsal, post-ovarian, sinistral to and posterior to female genital pore, 285–377 (323 ± 40, 4) or 12–17% of body length from posterior body end.

Ovary medial, lobed, 116–299 (166 ± 89, 4) in maximum length or 6–9% of body length, 138–222 (168 ± 38, 4) wide or 42–49% of body width, immediately post-testicular; post-ovarian space 541–767 (605 ± 108, 4) long or 24–30% of body length (Figs. 1A–C). Oviduct arching in dextral half of body posterior to ovary and medial to cirrus sac, 405–616 (467 ± 100, 4) long or 19–23% of body length including oviducal seminal receptacle; oviducal seminal receptacle 207–290 (245 ± 41, 4) long or 47–62% of oviduct length, 38–79 (51 ± 19, 4) wide or 4–7 × longer than wide (Fig. 1C). Vitellarium appearing as loose follicles, occupying space dorsal and lateral to testis and ceca, extending from nerve commissure to seminal vesicle (Figs. 1A, B); collecting duct 799–1,846 (1,322 ± 740, 4) long, 27–55 (43 ± 13, 4) wide. Oötype 12–21 (17 ± 5, 4) long, 12–14 (13 ± 1, 4) wide; post-oötype space 258–321 (288 ± 32, 4) long or 10–14% of body length from posterior end (Fig. 1C). Mehlis' gland 65–94 (79 ± 14, 4) wide, surrounding oötype (Fig. 1C). Uterus inverse U-shaped, post-cecal, post-gonadal, occupying space between seminal vesicle and oviducal seminal receptacle; ascending uterus 168–373 (237 ± 93, 4) long or 8–12% of body length, 9–14 (12 ± 2, 4) in maximum width; descending uterus 152–347 (212 ± 91, 4) long or 84–93% of ascending uterus length, 16–56 (28 ± 19, 4) in maximum width (Fig. 1C). Female genital pore medial, dorsal, post-ovarian, dextral to and anterior to male genital pore. Excretory system indistinct.

3.1.2 Taxonomic summary

Type and only known host: African sharptooth catfish, *Clarias gariepinus* (Burchell, 1822) Teugles, 1982 (Siluriformes: Clariidae).

Type locality: Hule Nature Reserve, Israel.

Other localities: Okavango River, Namibia.

Prevalence and intensity of infection: 1 of 3 African sharptooth catfish were infected by 6 specimens of *N. dentata*.

Specimens deposited: Vouchers (USNM XXXXXXX, XXXXXXX, XXXXXXX, XXXXXXX).

Site of infection: heart.

3.1.3 Taxonomic remarks

We identified the newly collected specimens herein as *N*. *dentata* by having an anterior sucker with two columns of denticles flanking the mouth, a lateral body margin having regularly spaced tegumental papillae, an esophagus with an anterior swelling, an intestine with 4 radial ceca, and a testis that extends anterior to the intestine. *Nomasanguinicola dentata* differs from *N*. *canthoensis* by having a body 5.3–6.7 longer than wide (vs. 3.5–4.6), an anterior esophageal swelling 7–8% (vs. 14–24%) of total esophageal length, a posterior esophageal swelling 3–5% (vs. 8–10%) of total esophageal length, a pre-cecal (vs. wholly post-cecal) testis, and an ovary that does not extend laterally beyond the nerve cords. The denticles of *N*. *dentata* should be viewed in lateral view to accurately illustrate the morphology of the denticle as having a distinct shaft and hook (which are both mostly embedded in the tegument). If the denticle is in frontal or dorso-ventral view, the denticle outline appears as an elongate oval or pyriform (Figs. 2A, C) (Truong and Bullard [9]). Further, in the largest specimen of *N*. *dentata* examined (USNM XXXXXXX), the denticles apparently lacked a pointed tip. Specimens of *N*. *canthoensis* likely have a similar denticle morphology but confirmation is needed as this character state is likely an important synapomorphy for the sanguinicolids.

The species identities of *N. dentata* and *N. clarias* are doubtful because no type specimen could be located for either species, the illustrations are superficial and incomplete, and both species are reported to infect *C. gariepinus* in Israel and Egypt (Paperna's [17]; Imam et al.'s [16]; Imam and El-Askalany [27]). The species description of *N. clarias* includes the presence of lateral tegumental spines, but no spine was illustrated in the original description. We cannot be certain of whether this description represents a new species or if it is conspecific with *N. dentata*. Because of that, we consider *N. clarias* as *species inquirenda* until a new specimen from Egypt is collected and studied (Imam et al.'s [16]). A neotype should be designated for *N. clarias* as well as *N. dentata*.

3.2 Phylogenetic analysis

The amplified *28S* fragments representing the two specimens of *N. dentata* were identical and comprised 1,546 nucleotides. They differed from that of *N. canthoensis* by 144 bp (9% difference). The new sequences differed from species of *Sanguinicola* (*Sanguinicola volgensis* [Rašín, 1926] McIntosh, 1934, *S.* cf. *volgensis*, *Sanguinicola plehnae* Warren and Bullard, 2023) and *Pseudosanguinicola occidentalis* (Van Cleave and Mueller, 1932) Warren and Bullard, 2023 by 356 bp (22% difference), 355 bp (22% difference), 370 bp (24% difference), and 380 bp (24% difference), respectively. Further, the new sequence differed from the unidentified cercarial sequence (*Sanguinicola* cf. *inermis* [AY222180]) by 364 bp (29% difference). The phylogenetic analysis recovered *Nomasanguinicola dentata* and *N. canthoensis* as sister taxa and sister to two innominate sanguinicolids that infect two other catfishes in the Mekong River, Vietnam (GenBank numbers: OQ709105, OQ709106) as well as a monophyletic Sanguinicolidae (see Warren and Bullard [4]; Fig. 3). *Nomasanguinicola dentata* differs from the innominate species infecting *Pangasius* cf. *macronema* (Siluriformes: Pangasidae) (OQ709106) and *Mystus* cf.

mysticetus (Siluriformes: Bagridae) (OQ709105) by 168 bp (11% difference) and 204 bp (13% difference) nucleotides, respectively. This monophyletic catfish-infecting clade is recovered sister to all other species assigned to Sanguinicolidae.

4. Discussion

Presently, seven families of catfishes are known hosts for sanguinicolids (Pimelodidae Swaison, 1838; Clariidae Bonaparte, 1846; Bagridae Bleeker, 1858; Pangasidae Bleeker, 1858; Auchenipteridae Bleeker, 1862; Claroteidae Bleeker, 1862; Mochokidae Jordan, 1923). These records are from Africa, South America, and Asia (Woodland [14]; Odhner [15]; Szidat [7]; Wang [28]; Imam et al. [16]; Lunaschi [29]; Paperna [26]; Truong and Bullard [9]; Orélis-Ribeiro and Bullard [10,11]). No record of a catfish blood fluke exists from a marine catfish (Ariidae), and none has been reported from North America or Australia. Further, the sequences generated for the present study and those by Warren et al. [6] (n = 4) are the only available catfish blood fluke sequences. Given the diversity of siluriforms (4,159 species assigned to 501 genera of 40 families), there are likely several distinct freshwater fish blood fluke lineages awaiting discovery (Fricke et al. [30]). For example, the innominate sanguinicolid that infects *M.* cf. *mysticetus* from Vietnam (OQ709105) has an anterior sucker with two rows of denticles flanking the mouth and lateral tegumental spines. However, the denticles associated with this innominate species are in rows of 5 (vs 4) (Truong and Bullard [9]; present study) and lateral tegumental spines are only described from *N. clarias*, *S. chalmersi*, and *S. sanliense* (Imam et al. [16]; Wang [28]; Warren et al. [6]). The other innominate sanguinicolid infecting *P.* cf. *macronema* from Vietnam (OQ709106) is further unique by lacking spines or denticles.

All known catfish blood flukes represented by nucleotides share a common ancestor (Fig. 3), objectively suggesting a high level of phylogenetic host specificity to catfishes. This level of

phylogenetic host specificity will be tested as additional blood fluke species are discovered and described. Based on available evidence, there is no pattern of cophyly between freshwater fishes and their blood flukes (Warren and Bullard [4; 31]; Betancur et al. [32]). In the future, freshwater sanguinicolids should be the focus in Africa and elsewhere as they will likely reveal several unknown lineages that can better the systematics of the group.

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Competing interests Statement: No author has a competing interes

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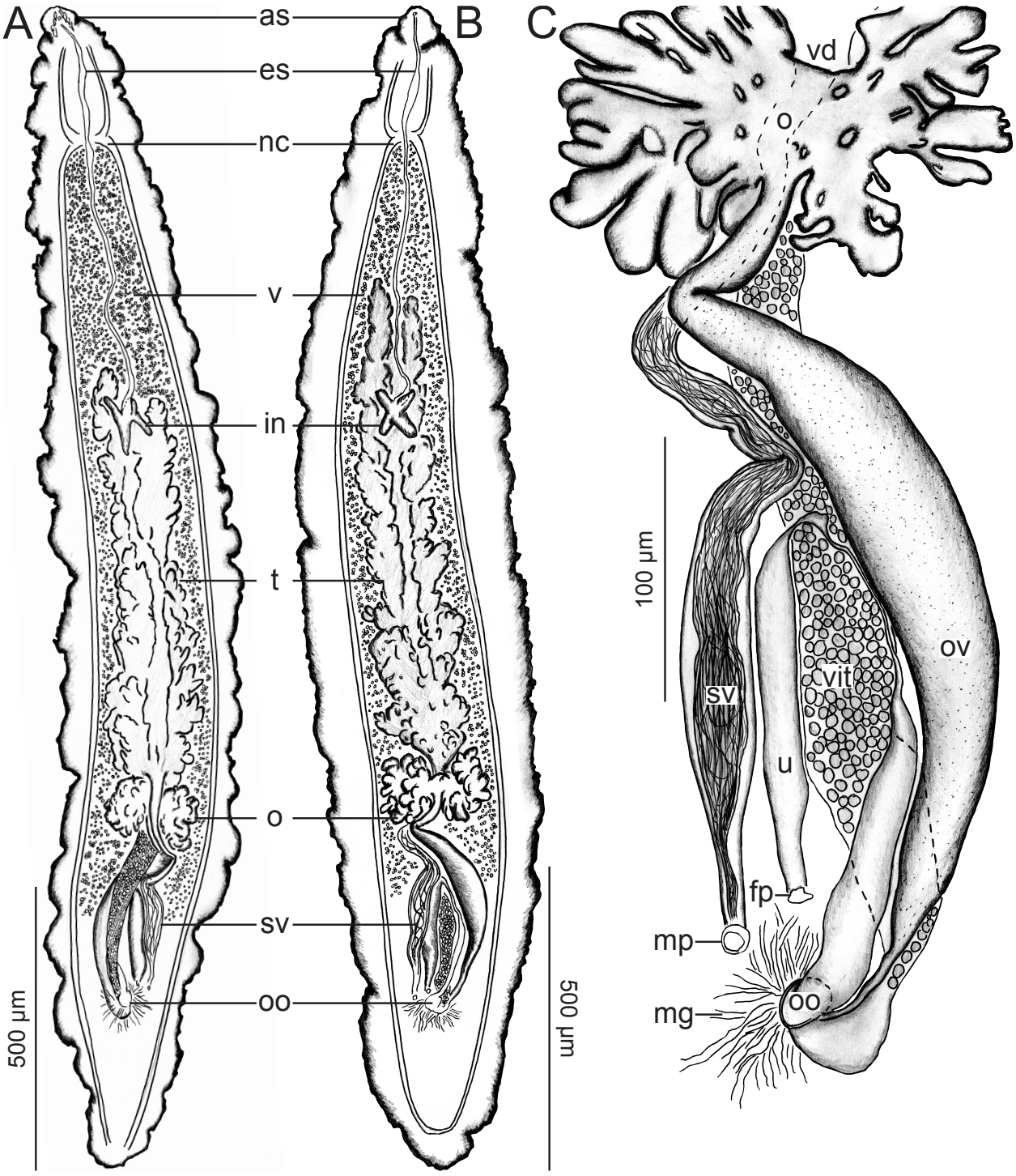
FIGURE LEGENDS

Figs. 1 *Nomasanguinicola dentata* (Paperna, 1964) Warren and Bullard, 2023 (Digenea: Sanguinicolidae Poche, 1926) infecting the heart of African sharptooth catfish, *Clarias gariepinus* (Burchell, 1822) Teugles, 1982 (Siluriformes: Clariidae) from the Okavango River (northeastern Namibia). Scale values aside bars. (**A**) Body of voucher (USNM No. XXXXXXX) showing anterior sucker (as), esophagus (es), nerve commissure (nc), vitellarium (v), intestine (in), testis (t), ovary (o), seminal vesicle (sv), and oötype (oo). Ventral view. (**B**) Body of voucher (USNM No. XXXXXXX). Dorsal View. (**C**) genitalia of voucher (USNM No. XXXXX) showing vas deferens (vd), ovary (o), oviducal seminal vesicle (ov), vitelline duct (vit), seminal vesicle (sv), uterus (u), female genital pore (fp), male genital pore (mp), oötype (oo), and Mehlis' gland (mg). Dorsal View.

Figs. 2 *Nomasanguinicola dentata* (Paperna, 1964) Warren and Bullard, 2023 (Digenea: Sanguinicolidae Poche, 1926) infecting the heart of African sharptooth catfish, *Clarias gariepinus* (Burchell, 1822) Teugles, 1982 (Siluriformes: Clariidae) from the Okavango River (northeastern Namibia). Scale values aside bars. (**A**) Anterior sucker of voucher (USNM No. XXXXXXX) showing mouth (m), denticles (d), and esophagus (es). Ventral view. (**B**) Lateral view of denticles of voucher (USNM No. XXXXXXX) showing shaft (s), crescent-shaped hook (h), and aperture (a). (**C**) Dorso-ventral view of denticles of voucher (USNM No. XXXXX). (**D**) Lateral tegumental margin of voucher (USNM No. XXXXXXX) showing lateral papillae.

Fig. 3 Phylogenetic relationships of freshwater fish blood fluke species assigned to Sanguinicolidae Poche, 1926. Reconstructed using Bayesian inference analysis using the large subunit ribosomal DNA (*28S*) gene. New sequence is shown in bold and GenBank accession number, host family, and continent locality aside taxa.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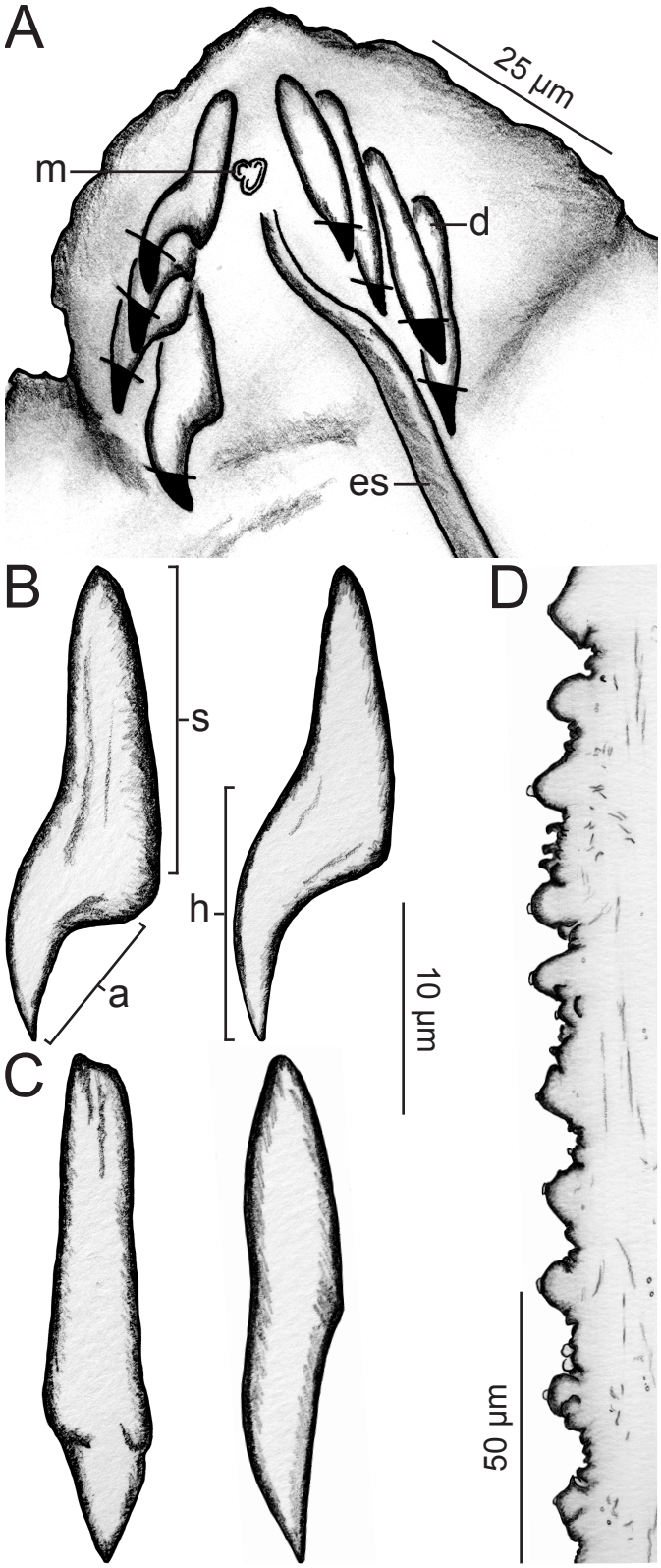
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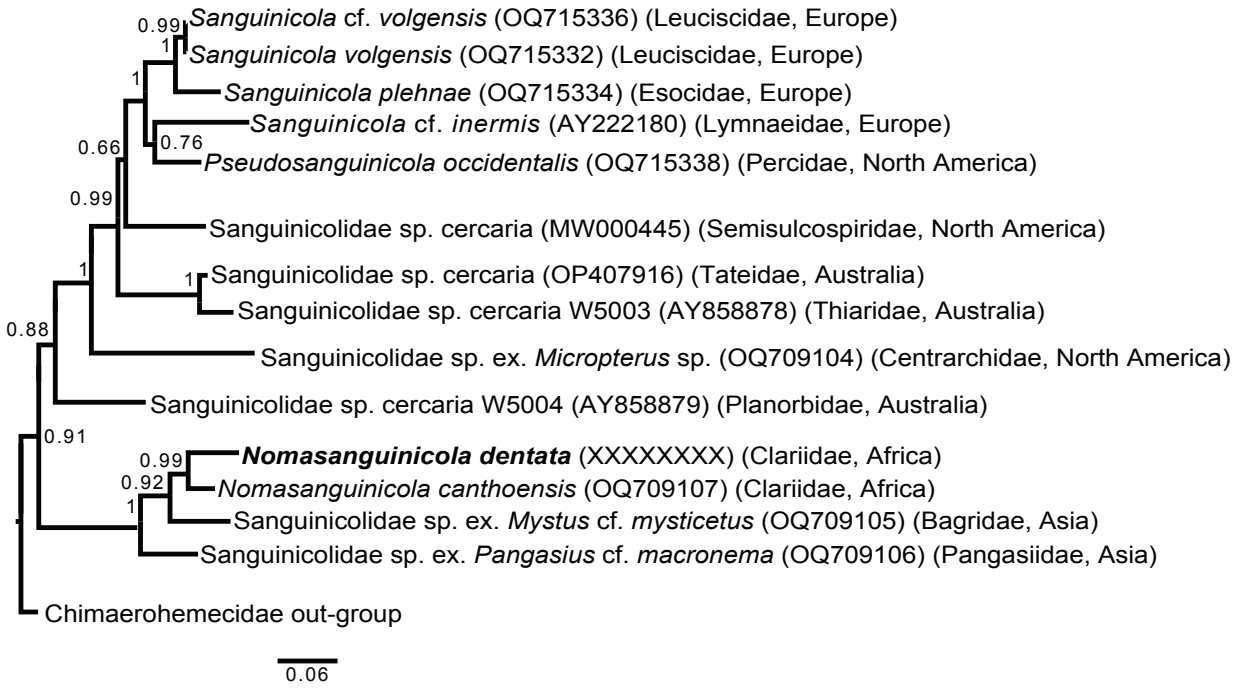
0.99
Sanguinicola cf. volgensis (OQ715336) (Leuciscidae, Europe)
1
Sanguinicola volgensis (OQ715332) (Leuciscidae, Europe)
1
Sanguinicola plehnae (OQ715334) (Esocidae, Europe)
Sanguinicola cf. inermis (AY222180) (Lymnaeidae, Europe)
0.66
0.76
Pseudosanguinicola occidentalis (OQ715338) (Percidae, North America)
0.99
Sanguinicolidae sp. cercaria (MW000445) (Semisulcospiridae, North America)
1
1
Sanguinicolidae sp. cercaria (OP407916) (Tateidae, Australia)
Sanguinicolidae sp. cercaria W5003 (AY858878) (Thiaridae, Australia)
0.88
Sanguinicolidae sp. ex. Micropterus sp. (OQ709104) (Centrarchidae, North America)
Sanguinicolidae sp. cercaria W5004 (AY858879) (Planorbidae, Australia)
0.91
0.99
Nomasanguinicola dentata (XXXXXXXX) (Clariidae, Africa)
0.92
Nomasanguinicola canthoensis (OQ709107) (Clariidae, Africa)
1
Sanguinicolidae sp. ex. Mystus cf. mysticetus (OQ709105) (Bagridae, Asia)
Sanguinicolidae sp. ex. Pangasius cf. macronema (OQ709106) (Pangasiidae, Asia)
Chimaerohemecidae out-group
0.06

CHAPTER 6: DESCRIPTION AND PATHOLOGY OF A NEW GENUS AND SPECIES OF FISH BLOOD FLUKE (DIGENEA: APOROCOTYLIDAE) FROM WHITE MULLET, *MUGIL CUREMA* VALENCIENNES, 1836 (MUGILIFORMES: MUGILIDAE) IN MOBILE BAY, ALABAMA.

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ABSTRACT

We herein describe a new genus and species of Aporocotylidae Odhner, 1912 infecting white mullet, *Mugil curema* Valenciennes, 1836 (Mugiliformes: Mugilidae) collected from the mouth of Deer River, a tributary of Mobile Bay, Alabama. The heart of 87 of 108 (81%) white mullet (*M. curema*) was infected with adults of the new species. The new species differs from the mugilid infecting aporocotylids, *Cardicola mugilis* Yamaguti, 1970 and *Cardicola brasiliensis* Knoff and Amato, 1991, by having the combination of 2 testes (vs. single testis), post-cecal testes (vs. inter-cecal testis), a uterus with straight ascending and descending portions (vs. convoluted), and a common genital pore (vs. separate genital pores). It differs from *Plethorchis acanthus* Martin, 1975, by having a post-cecal testes (vs. partially inter-cecal), 2 testes (vs. >100), a post-ovarian uterus (vs. pre-ovarian), and uterine eggs that are oval (vs. fusiform) as well as by lacking uterine egg spines or filaments. The *28S* nucleotide sequence for the new species was 21 % (215 bp) different from *P. acanthus*, and our phylogenetic analysis recovered these species as sister taxa. Monophyly of Aporocotylidae *sensu stricto* of Warren and Bullard, 2023 was not rejected. Adults of the new species infected the atrium, ventricle, and bulbus arteriosus of the heart. Variable endocardial hyperplasia was observed including a thickened endothelium with accompanying endocarditis. In heart, eggs were observed in the cardiac muscle

of the myocardium where they were encapsulated by granuloma composed of epithelioid histiocytes. In gill, eggs infected the afferent artery of gill filaments. Proliferating epithelium extended to the height of the gill lamellae, leaving the lamellae indiscernible from surrounding tissue. In one gill filament with numerous eggs, the afferent artery was disrupted and hemorrhaging into adjacent tissues was observed. This is the first aporocotylid infecting a mullet from the northwestern Atlantic Ocean, and the third description of endocarditis attributed to a fish blood fluke infection.

KEY WORDS

taxonomy, systematics, phylogenetics, morphology, large subunit ribosomal (*28S*)

The mullets, Mugilidae Jarocki, 1822 (Mugiliformes), comprises 78 species of 26 genera (Eschmeyer et al., 2016). Three fish blood flukes infect mullets: *Cardicola mugilis* Yamaguti, 1970 and *Plethorchis acanthus* Martin, 1975 infect grey mullet (*Mugil cephalus* Linnaeus, 1758) off Hawaii (Central Pacific Ocean) and in the Brisbane River (Australia), respectively and *Cardicola brasiliensis* Knoff and Amato, 1992 infects Lebranche mullet, *Mugil liza* Valenciennes, 1836 from Sepetiba Bay (Brazil, Southwestern Atlantic Ocean) (Yamaguti, 1970; Martin, 1975; Knoff and Amato, 1992). No fish blood fluke has been reported from a mullet in the Gulf of Mexico.

Recently, studies using mitochondrial genes and cytogenetic data have revealed that *M. cephalus* and *Mugil curema* Valenciennes, 1836 each comprise a complex of cryptic species (Durand and Borsa, 2015; Nirchio et al., 2017). Concerning fish blood fluke taxonomy, this is interesting because fish blood flukes are host specific with few examples of more than one distinct species infecting the same host (Ogawa and Egusa, 1986; Repullés-Albelda, et al., 2008; Ogawa et al., 2011; Shirakashi et al., 2012, 2013; Warren and Bullard, 2021). Because two

distinct species (*C. mugilis* and *P. acanthus*) infect the same host (*M. cephalus*) from different geographic localities (off Hawaii and Australia), this may provide further support that grey mullets from Hawaii and those from Australia are distinct species (Durand and Borsa, 2015; Nirchio et al., 2017). Further, undescribed mullet species could be infected by innominate aporocotylids and, conversely, knowledge of parasites of cryptic mullet species could signal the presence of new mullet species.

Aporocotylids infections are pathogenic in aquaculture when infection intensity is high and when eggs or adults occlude branchial cessels and interfere with oxygen exchange in the gill (Thulin, 1980; Ogawa and Fukudome, 1994; Colquitt et al., 2001; Bullard and Overstreet, 2002; Munday et al., 2003). Damage to the gill epithelium is also reported from eggs, exiting miracidia, or penetrating cercariae (Crespo et al., 1992; Kirk and Lewis, 1992; Herbert et al., 1995; Bullard and Overstreet, 2002, 2008). Further, adult aporocotylids infecting heart can cause a thickening of the endocardium and endocardial thrombi; which could decrease blood flow (Warren et al., 2017). There is limited information regarding pathological effects on cardiac tissue attributed to fish blood fluke infections. Because of this, and that mullets are an important resource in aquaculture and fisheries settings (primarily in Brazil and Mexico) (Avigliano et al., 2015; Pacheco-Almanzar et al., 2017; Santana et al., 2018; Lima et al., 2019), new and existing fish blood fluke histopathological investigations are warranted.

We herein describe a new genus and species of Aporocotylidae Odhner, 1912 infecting white mullet (*M. curema*) collected from the mouth of Deer River, a tributary of Mobile Bay, Alabama. We present a phylogenetic analysis using the large subunit ribosomal (*28S*) gene to test the monophyly of the family as well as explore phylogenetic relationships. Further, we provide details on the blood fluke infection using histology. This study reports the first aporocotylid

infecting a mullet from North America, and the first mugiliform blood fluke description in > 30 y.

MATERIALS AND METHODS

In 2017 and 2021, the heart of 20 of 20 (100%) and 67 of 88 (76%) white mullet (*M. curema*) from north fork Deer River, off Mobile Bay, Alabama was infected with a new fish blood fluke. Fish were captured by gill net in March 2017 (20 fish) and cast net in March 2021 (88 fish), then transferred on ice and relocated to the laboratory for examination some hours later. At necropsy in the laboratory, the heart and gill were excised intact and examined with the aid of a dissecting microscope and fiber optic light source to isolate fluke specimens and eggs for morphology and nucleotide sequencing. The heart was bisected with fine forceps and scissors to reveal adult blood flukes. Gill filaments were cut with scissors and mounted on glass slides to examine for blood fluke eggs. Adult flukes for morphology were heat-killed on a glass slide using a butane hand lighter under little or no coverslip pressure as per Bullard et al. (2019) and fixed in 10% neutral buffered formalin (n.b.f). Adult specimens intended for DNA extraction were wet mounted on glass slides and examined to confirm their identity, placed directly into 95% EtOH, and stored at −20 °C until DNA was extracted (see below).

Adult flukes fixed for morphology were rinsed with distilled water, cleaned with fine brushes to remove any debris, stained overnight in Van Cleave's hematoxylin with two additional drops of Ehrlich's hematoxylin, dehydrated using an ethanol series, cleared in clove oil, and permanently mounted in Canada balsam. Measurements were obtained by using an Olympus BX51 microscope (Olympus Corporation of the Americas, Center Valley, Pennsylvania, USA) equipped with differential interference contrast (DIC) and a Jenoptik Gryphax camera (Jenoptik AG, Jena, Germany) and illustrations were completed using a drawing tube. Measurements are

reported in micrometers (μm) as the range followed by the mean, standard deviation, and sample size in parentheses. Scientific names, including taxonomic authorities and dates, for fishes follow Eschmeyer et al. (2016). Classification and anatomical terms for fish blood flukes follow Warren et al. (2023) and Warren and Bullard (2023). Type and voucher materials were deposited in the National Museum of Natural History's Invertebrate Zoology Collection (USNM, Smithsonian Institution, Washington, DC).

For histopathology the heart, gill, and viscera from infected fish were fixed in 10% n.b.f and routinely processed. After fixation, 12 gill arches (2 each from the gill of 6 white mullet), 4 hearts, and 2 visceral masses were processed for histopathology sections. Heart and gill were left intact, and each visceral mass was transversally cut into 4, 1 cm-long pieces, yielding 24 pieces of tissue; which was then rinsed in water for 2 h, dehydrated in an ethanol series, embedded in paraffin, sectioned at 5 μm, and stained with Gill's 3 hematoxylin and eosin per Luna (1992). Gill arches were decalcified in EDTA for 1 week prior to embedding. Ten slides per portion of tissue were cut yielding > 720 sections on 240 slides. Blood fluke eggs were photographed and measured.

Using 2 of the EtOH-preserved and microscopically identified blood flukes, total genomic DNA (gDNA) was extracted using DNeasyTM Blood and Tissue Kit (Qiagen, Valencia, California, USA) as per the manufacturer's protocol except that the proteinase-K incubation period was extended overnight, and the final elution step used 100 microliter (μl) of elution buffer to increase the final DNA concentration. Primer choice, PCR amplification cycling profile is outlined in Warren et al. (2017) and Warren et al. (2021). All PCR reactions were carried out in a MJ Research PTC-200 (BioRad, Hercules, California, USA). PCR products (12 μl) were verified on a 1% agarose gel and stained with ethidium bromide. PCR products were purified by

microcentrifugation with the QIAquick PCR Purification Kit (Qiagen, Valencia, California, USA) according to manufacturer's protocols except that the last elution step was performed with autoclaved nanopure H_2O rather than with the provided buffer. DNA sequencing was performed by Genewiz, Incorporated (South Plainfield, New Jersey). Sequence assembly and analysis of chromatograms were performed with Geneious version 2021.2.2 (http://www.geneious.com). Nucleotide sequence data were deposited in GenBank.

The phylogenetic analysis included 2 nucleotide sequences of the new species plus selected aporocotylids sequences from GenBank. The out-group comprised *Elopicola* spp. (Elopicolidae Warren and Bullard, 2023) and was informed by results presented by Warren et al. (2019, 2023) and Warren and Bullard (2023). Sequence alignment, trimming (1128 [*28S*] base pairs [bp]), model selection, alignment reformatting, is outlined in Warren et al. (2023). BI was performed in MrBayes version 3.2.7a (Ronquist and Huelsenbeck, 2003) using substitution model averaging ("nst-mixed") and a gamma distribution to model rate-heterogeneity. Defaults were used in all other parameters. Three independent runs with 4 Metropolis-coupled chains were run for 5,000,000 generations, sampling the posterior distribution every 1000 generations. Convergence was checked using Tracer v1.6.1 (Rambaut et al., 2014) and the "sump" command in MrBayes: all runs appeared to reach convergence after discarding the first 25% of generation as burn-in. A majority rule consensus tree of the post burn-in posterior distribution was generated with the "sumt" command in MrBayes. The inferred phylogenetic tree was visualized using FigTree v1.4.4 (Rambaut et al., 2014) and further edited for visualization purposes with Adobe Illustrator (Adobe Systems).

DESCRIPTION

N. GEN. Warren and Bullard

(Figs. X−X)

Diagnosis: Body of adult >15 × longer than wide, threadlike, dorsoventrally flat posteriorly, lacking posterolateral protuberance, tapering equally anteriorly, bluntly rounded posteriorly, spined; tegumental spines arranging in regularly spaced transverse rows along lateral body margin. Rosethorn-shaped spines absent. Anterior sucker diminutive, appearing concave, accommodating mouth; mouth medioventral, subterminal. Pharynx not observed. Esophagus medial, straight, not looping, extending posteriad one-third body length, with posterior esophageal swelling, connecting with intestine, anteromedially. Intestine medial, H-shaped, lacking diverticula, posterior ceca terminating in middle third of body. Testes 2 in number, medial, flanking genitalia anteriorly and posteriorly, deeply lobed. Vasa efferentia coalescing at posterior margin of anterior testis and anterior margin of posterior testis to form large ducts that coalesce to form vas deferens; vas deferens forming from anterior and posterior ducts of vasa efferentia at level of ovary and proximal portion of cirrus sac, extending directly posterior and connecting with cirrus sac, ventral to ovary and dorsal to vitelline duct. Auxiliary external seminal vesicle absent. Cirrus sac medial, post-ovarian, post-cecal, inter-testicular; enclosing internal seminal vesicle and cirrus; cirrus everting dorsally in sinistral body half, post-cecal, inter-testicular. Ovary single, medial, post-cecal, inter-testicular, deeply lobed. Oviduct post-cecal; oviducal seminal receptacle present. Laurer's canal absent. Vitellarium diffuse, follicular, occupying space from anterior body end to level of ovary. Vitelline duct longitudinal, connecting with oötype posteriorly, extending anteriorly to middle third of esophagus. Oötype post-ovarian, inter-testicular. Uterus comprising ascending and descending portions, post-ovarian, inter-

testicular; uterine eggs large, wider than uterus, having ovoid body, lacking filaments. Metraterm indistinct. Male and female reproductive tracts opening into common atrium and sharing a common pore; common genital pore dorsal, sinistral, post-cecal, inter-testicular.

Differential diagnosis: Body >15 × longer than wide; tegumental spines spaced 3 × longer than spine row length, in transverse rows along lateral body margin. Intestine H-shaped, posterior ceca terminating in middle third of body. Testes 2 in number, flanking genitalia anteriorly and posteriorly. Auxiliary external seminal vesicle absent. Ovary post-cecal. Laurer's canal absent. Oötype inter-testicular. Uterine eggs ovoid, lacking filaments, > 90% of uterine width. Common genital pore present, post-cecal, inter-testicular.

Taxonomic summary

Type and only nominal species: N. GEN., n. sp. Warren and Bullard.

N. GEN., n. sp. Warren and Bullard

(Figs. 1–7)

Light microscopy of 3 complete whole-mounted adult specimens and 8 partial specimens; USNM collection nos. XXXXXXX–XXXXXXX): Body elongate, anterior body thread-like, posterior body at level of genitalia flattened, tapering anteriorly, 1,241–1,681 (1,481 ± 223, 3) long, 50–71 (60 ± 8, 10) at greatest width, 17–30 (24 ± 6, 3) × longer than wide (Fig. 1); tegumental body spines minute, 18–23 (22 ± 2, 4) from anterior end (Figs. 4–5), 2–4 (2.5 ± 0.4, 20) long, <0.2 wide at base, at limits of light microscopy, extending to posterior body end (Figs. 2–3); tegumental spine rows 3–4 (3.3 ± 0.6, 20) long, 0.4–0.5 (0.4 ± 0.03, 20) wide, regularly spaced apart 5–8 (6 ± 1.2, 10) near anterior sucker, 12–20 (16 ± 3.4, 10) in middle body near intestine, 6–12 (9 ± 2.6, 10) in posterior body at level of posterior testis, 3–5 (4 ± 1, 20) spines per row (Fig. 5), with 106–124 (115 ± 9, 2) per side or total of 217–240 (229 ± 16, 2). Anterior

sucker 12–18 (15 ± 3, 3) long, 13–33 (25 ± 8, 4) wide at base, terminal papillae on anterior margin of anterior sucker present, paired, each approximately 1 long and 1 wide; anterior sucker spines small, <1 (1) long, in 5 concentric rows (1) (Fig. 1, 4). Ventrolateral nerve cord and nerve commissure not observed. Mouth small, 3 (2) in diameter, 8 from terminal end of anterior body extremity (Fig. 4). Pharynx absent. Esophagus 466–589 (521 ± 60, 4) in total length or 31–38% of body length, 8–13 (10 ± 2, 4) in maximum width (at level just anterior intestine), extending sinuously posteriad along midline, widening posteriorly (Figs. 1, 4, 6); anterior esophageal swelling absent. Intestine H-shaped; cecal intersection of anterior and posterior ceca 757 or 61% of body length from posterior end; anterior ceca 21–65 (41 ± 22, 3) long or 1–2% of body length, 13–22 (17 ± 4, 3) wide, containing granular material within lumen of some individuals (Figs. 1, 6); posterior ceca asymmetrical, 123–234 (178 ± 56, 3) long or 10–15% of body length, 15–22 (18 ± 4, 3) wide (Figs. 1, 6); post-cecal space 602–729 (666 ± 90, 2) long or 48–49% of body length or 13–15% of body length from anterior testis.

Testes 2 in number, post-cecal; anterior testis 99–212 (155 ± 48, 8) long or 11–13% of body length, 35–59 (49 ± 11, 8) wide or 63–83% of maximum body width, 3–5 × longer than wide, terminating 249–584 (351 ± 95, 9) or 21–47% of body length from posterior body end (Figs. 1–3); posterior testis 107–139 (118 ± 18, 8) long or 7–9% of body length or 65–76% of anterior testis length, 36–63 (51 ± 14, 9) wide or 71–89% of maximum body width, 2–3 × longer than wide, terminating in posterior body extremity (Figs. 1–3). Vasa efferentia difficult to trace in fixed specimens, an interconnecting meshwork of fine ducts entwining throughout testicular tissue, approximately 7–10 (8.5 ± 1.3, 5) wide, containing sperm in all specimens, coalescing in posterior portion of anterior testis and anterior portion of posterior testis to each form larger ducts (Figs. 2–3, 7); anterior vasa efferentia duct 62–110 (82 ± 25, 4) long or 4–7% of body

length, 7–9 (8 ± 1, 4) in maximum width; posterior vasa efferentia duct 101–134 (121 ± 18, 4) long or 1.2–2.2 × longer than anterior duct of vasa efferentia or 8% of body length, 8 (4) in maximum width; vas deferens forming from anterior and posterior ducts of vasa efferentia at level of ovary and proximal portion of cirrus sac, extending directly posterior 3–5 (4 ± 1, 4) or 6–10% of seminal vesicle length, 1–3 (2 ± 1, 4) or 6–18% of cirrus sac width in maximum width, and connecting with cirrus sac, containing sperm in all specimens (Figs. 2–3, 7). Cirrus-sac present, wall approximately 1.5–3 (2.1 ± 0.5, 9) thick, including seminal vesicle, and cirrus; seminal vesicle 41–57 (50 ± 5, 9) long, 9–19 (15 ± 3, 9) wide, 3 × longer then wide (Figs. 2–3, 7); everted cirrus 17–23 (20 ± 4, 5) long or 33–47% of seminal vesicle length, 6 (5) wide, 3 × longer then wide (Fig. 3, 7). Common genital pore 164–224 (194 ± 30, 4) or 13% of body length from posterior end of the body, 11–13 (12 ± 1.4, 3) from sinistral body margin, 39–41 (40 ± 1.4, 3) from dextral body margin (Figs. 1–3, 7).

Ovary medial, irregular in shape, 93–111 (102 ± 13, 8) in maximum length or 7% of body length, 57–62 (60 ± 4, 8) wide or 80–87% of body width, 1.5–1.9 × longer than wide, immediately posterior to anterior testis; post-ovarian space 209–273 (241 ± 45, 8) long or 17–22% of body length (Fig. X). Oviduct (including oviducal seminal receptacle) 108–129 (120 ± 11, 8) long; oviducal seminal receptacle 87–104 (94 ± 9, 8) long or 38–42% of oviduct length, 14–18 (15 ± 2, 8) wide. Oötype 10 (1) in diameter. Vitellarium follicular, occupying space ventral and lateral to testis, extending from anterior sucker to ovary; vitelline collecting duct 800–1155 (977 ± 251, 2) long, 13–19 (15 ± 3, 8) wide. Uterus extending directly anteriad from oötype, 90–110 (102 ± 11, 7) long or 7% of body length, 15–17 (16 ± 1, 7) wide, with wall 3–4 (3.7 ± 0.6, 7) thick, containing eggs in all specimens (Figs. 2–3); ascending portion extending anteriad and dorsal to seminal vesicle, curving sinistral to posterior margin of ovary before

connecting with descending portion; descending portion 55–60 (57 ± 3, 7) long or 4% of body length, 13–17 (15 ± 1, 7) wide or 14% of body width, with wall 3–4 (3.3 ± 0.6, 7) thick, extending posteriorly before connecting with common genital pore (Figs. 2–3). Uterine eggs 21–28 (26 ± 3, 8) long or 47–49% of descending uterus length, 13–16 (15 ± 1.3, 8) wide or 93–94% of descending uterus width, with wall 1–2 (1.3 ± 0.6, 7) thick, containing dense lipid-like bodies (Figs. 1–3). Excretory vesicle indistinct.

Taxonomic summary

Type host: White mullet, *Mugil curema* Valenciennes, 1836 (Mugiliformes: Mugilidae).

Type locality: Deer River (30°32'02.4"N 88°06'20.3"W), Mobile Bay, Alabama.

Site of infection: Heart lumen.

Prevalence of infection: 20 of 20 (100%) white mullet sampled in 2017 and 67 of 88 (76%) white mullet sampled in 2021 were infected by specimens of the new species.

Specimens deposited: Holotype (USNM XXXXXXX), paratype (USNM XXXXXXX), and vouchers (USNM XXXXXXX, XXXXXXX, XXXXXXX, XXXXXXX, XXXXXXX, XXXXXXX, XXXXXXX, XXXXXXX).

Remarks

The new genus is most similar to *Psettarium* Goto and Ozaki, 1930, monotypic *Neoparacardicola* Yamaguti, 1970, monotypic *Adelomyllos* Nolan and Cribb, 2004b, monotypic *Chaulioleptos* Nolan and Cribb, 2005, and *Phthinomita* Nolan and Cribb, 2006b by having the combination of lateral tegumental spines in transverse rows (vs. single column of spines) (Warren et al., 2023), an H-shaped intestine with elongate ceca (vs. inverse U-shaped) (Bullard, 2014; Orélis-Ribeiro et al., 2017), and two testes (vs. a single testis or multiple [>2] testes)

(Mcintosh, 1934; Bullard et al., 2008; Santoro et al., 2015; Warren et al., 2017; Warren et al., 2021) (Figs. 1–3, 7).

The new species is most similar to *Psettarium* spp. described with two testes (*Psettarium hustoni* Yong, Cutmore, Jones, Gauthier, and Cribb, 2018, *Psettarium hawaiiensis* [Martin, 1960] Yong, Cutmore, Jones, Gauthier, and Cribb, 2018, *Psettarium yoshidae* Yong, Cutmore, Jones, Gauthier, and Cribb, 2018, *Psettarium martini* Yong, Cutmore, Jones, Gauthier, and Cribb, 2018) and differs by having an intestine that terminates in the middle third of the body (vs. terminating in posterior third), a vitelline duct that extends to middle third of esophagus (vs. post-cecal bifurcation), a uterus with straight ascending and descending portions (vs. convoluted), and a common genital pore (vs. separate genital pores). The new species differs from the other five aporocotylids that have two testes by having a common genital pore (vs. separate genital pores) (Yamaguti, 1970, Nolan and Cribb, 2004, Nolan and Cribb, 2005, Cutmore et al., 2021).

The new species differs from *C. mugilis* and *C. brasiliensis* by having 2 testes (vs. single testis), post-cecal testis (vs. inter-cecal testis), a uterus with straight ascending and descending portions (vs. convoluted), and a common genital pore (vs. separate genital pores). It differs from *P. acanthus* by having elongate anterior ceca (vs. reduced), post-cecal testes (vs. partially inter-cecal), 2 testes (vs. >100), a vitelline duct extending to middle third of esophagus (vs. post-cecal), a post-ovarian uterus (vs. extending pre-ovarian), a descending portion of uterus 14% of body width (>50% of body width), and uterine eggs that are oval (vs. fusiform) as well as by lacking uterine egg spines or filaments.

Phylogenetic results

The amplified *28S* fragments representing the new species comprised 1,515 nucleotides (GenBank accession no. XXXXXXXX and XXXXXXXX), were identical, and 21% (215 bp) different from *P. acanthus*. The new species was recovered sister to *P. acanthus*, supporting a monophyletic mullet infecting blood fluke clade (Fig. 8). This is interesting because *P. acanthus* infects the gray mullet (*M. cephalus*) from Australia and is morphologically distinct (see Remarks). The clade was recovered sister to *Psettarium* spp., monotypic *Neoparacardicola*, monotypic *Adelomyllos*, monotypic *Chaulioleptos*, and *Phthinomita* spp. (Fig. 8). This tree topology is consistent with previous studies concerning the fish blood flukes (Warren et al., 2023; Warren and Bullard, 2023).

Histology results

Adults of the new species infected the atrium, ventricle, and bulbus arteriosus of the heart and were coelozoic, occupying the luminal spaces of the heart, either loose in the lumen or within the intertrabecular spaces of the heart (Figs, 9–10). Variable endocardial hyperplasia was observed, ranging from several cells thick with no accompanying endocarditis (Fig 9) to a significantly thickened endothelium with accompanying endocarditis (Fig. 11) (Warren et al., 2017). Endocarditis was characterized by an acute granulomatous inflammatory response composed of eosinophilic granulocytes, mononuclear inflammatory infiltrates, and clusters of free granules, from degranulation of eosinophilic granulocytes, present in proliferating endothelial cells (Fig. 11). No adults were observed in the vessels of the gill or viscera.

Eggs infected the heart and gill, but live/viable eggs were observed in the gill only. In heart, eggs were observed in the cardiac muscle of the myocardium where they were encapsulated by granuloma composed of epithelioid histiocytes (Fig. 12). In gill, eggs were infecting the afferent

artery of gill filaments, and each had a miracidium. No egg was observed in a gill lamella (Figs 13–15). The distribution of eggs in gill filaments was uneven and the host response varied based on the number of eggs present. In gill filaments with few eggs, no demonstrable host response was observed, with eggs being encapsulated by a granuloma composed of a few layers of epithelioid cells (Fig. 13). In gill filaments with numerous eggs, the encapsulating granuloma was thicker than those surrounding eggs in lightly infected gill filaments and the overlying epithelium was hyperplastic. Proliferating epithelium extended to the height of the gill lamellae, leaving the lamellae indiscernible from surrounding tissue (Fig. 14). In one gill filament with numerous eggs, the afferent artery was disrupted and hemorrhaging into adjacent tissues was observed (Fig. 15). No eosinophilic granulocytes were observed associated with eggs in the heart or gill.

DISCUSSION

The intermediate host infected with the new species is currently unknown, but we expect that it may be present in the estuary or low salinity river mouth (off the esturary). Attempts to collect invertebrates from the type locality have been conducted albeit with negative results. Martin (1974) and Nolan and Cribb (2004) examined the gastropod, *Posticobia brazieri* (Smith 1882) (Littorinimorpha: Tateidae), due to its high abundance in the Brisbane River (also the type locality for *P. acanthus*), but the snails yielded no infection of the mugiliform blood fluke. However, this gastropod has been shown to be the intermediate host for *Paracardicoloides yamagutii* Martin, 1974 (Elopicolidae Warren and Bullard, 2023; see Warren and Bullard, 2023) that is reported to infect catadromous eels (*Anguilla* spp.) in rivers of Australia and New Zealand (Martin, 1974; Nolan and Cribb, 2004). Further, this gastropod is a host for several innominate sanguinicolid cercariae from the Brisbane River, Australia (Cutmore et al., 2023). Given the

evolutionary distance between these two fish host lineages and the aporocotylids that infect them, we suspect that the mugilid blood flukes likely use a distantly related intermediate host, such as a polychaete. Determining the intermediate host could be vital in predicting other unknown intermediate hosts as the mugilid blood flukes (*P. acanthus* and the new species herein) are recovered sister to a clade comprising *Deontacylix* Linton, 1910, *Psettarium* Goto and Ozaki, 1930, monotypic *Neoparacardicola* Yamaguti, 1970, *Pearsonellum* Overstreet and Køie, 1989, monotypic *Cruoricola* Herbert, Shaharom-Harrison, and Overstreet, 1994, *Ankistromeces* Nolan and Cribb, 2004, *Phthinomita* Nolan and Cribb, 2006b, *Skoulekia* Alama-Bermejo, Montero, Raga, and Holzer, 2011, and *Holocentricola* Cutmore and Cribb, 2021, which have no intermediate host information.

Published work characterizing the effects of adult blood flukes on the endocardium of fishes is scarce which is noteworthy considering that pathological lesions can affect normal functions of the heart. Warren et al. (2017) described endocarditis associated with *Cardallagium* cf. *anthicum* (as *Psettarium* cf. *anthicum*) infecting cultured cobia (*Rachycentron canadum* [Linnaeus, 1766] Monod, 1973 [Rachycentridae]) from Vietnam. The primary results of that study revealed a thickened endocardium and endocardial thrombi where the adult blood flukes were located. Loss of muscle striation in the myocardium was also noted and melanin-like pigment was observed suggesting the presence of melanomacrophage aggregates (Warren et al., 2017). McElroy et al. (2020) diagnosed the infection and pathology caused by *Cardicola laruei* Short, 1953 infecting spotted seatrout (*Cynoscion nebulosus* [Cuvier, 1830] Chao, 1978 [Sciaenidae]) from estuaries in South Carolina. The pathology reported was granulomatous myocarditis associated with blood fluke eggs as well as blister-like extrusions on the epicardium. Further, in one blister-like extrusion they observed yellow pigmented cells and suggest these

may be melanomacrophages. In contrast to Warren et al. (2017), there was no apparent cellular host reaction to the adult blood flukes (McElroy et al. 2020). The contrasting cellular host reactions to the adult is interesting. This cellular response to the adult is likely associated with *C.* cf. *anthicum* threading itself through the myocardium and remaining seemingly stationary as an adult rather than crawling freely in the lumen of the heart, like that of *C. laruei* (Warren et al., 2017, McElroy et al. 2020). Like *C.* cf. *anthicum*, the new species was found occupying the intertrabecular spaces of the heart. It may be that fish blood flukes with elongate or thread-like body morphologies that utilize the intertrabecular spaces are more likely to contribute to endocarditis, but this hypothesis awaits further histopathology studies.

Endocardial cells are second to hepatocytes in the supply of cytochrome p450, a gene superfamily coding for enzymes involved in the detoxification of blood and the metabolism of fatty acids, steroids, and vitamins (Poppe and Ferguson, 2006, Uno et al., 2012, Warren et al., 2017). Additionally, specialized endocardial cells have a high capacity for endocytosis removing waste and biological macromolecules from the blood (Poppe and Ferguson, 2006). These properties likely afford the endocardium a role in regulating systemic disease and detoxification of blood. As a result, changes to the endocardium may increase susceptibility to systemic diseases and reduce ability to phagocytize waste or detoxify pollutants from blood, in addition to any reduction in cardiac output resulting from the volume of the lumen of the heart blood flukes occupy. It is unclear if the thickening of the endocardium and the presence of eosinophilic granulocytes and free granule clusters observed associated with infections by the new species negatively impact function of the heart of white mullet. Future work on the how changes to the endocardium impact regulation of infectious disease, detoxification of blood, and metabolism would prove invaluable to better understanding the impacts of blood flukes on their hosts.

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FIGURE LEGENDS

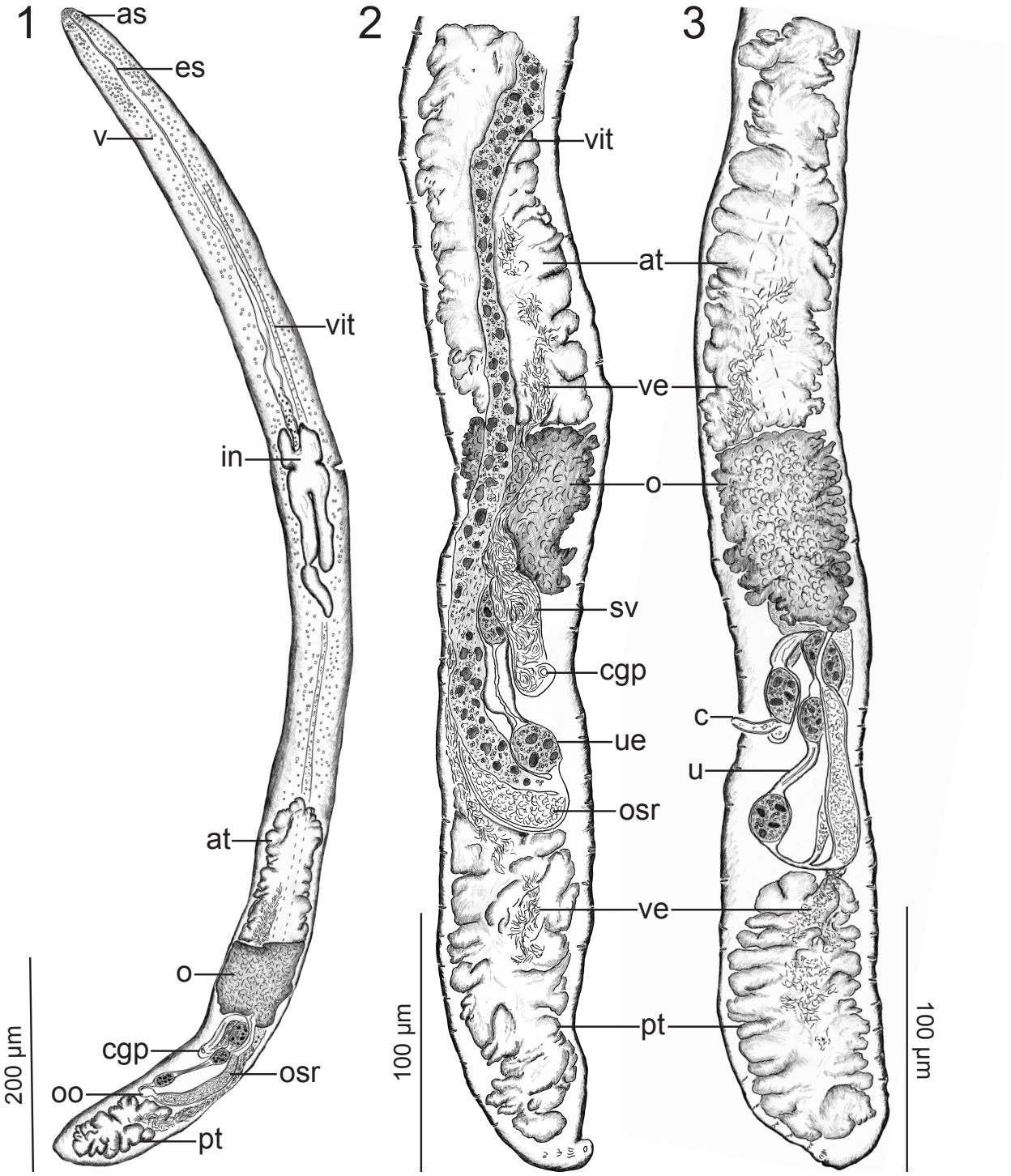
Figures 1–3. N. GEN., n. sp. Warren and Bullard (Digenea: Aporocotylidae) infecting the heart of white mullet, *Mugil curema* Valenciennes, 1836 (Mugiliformes: Mugilidae) from the mouth of Deer River, a tributary of Mobile Bay, Alabama, (30°32′ 3.401″ N, 88°6′ 21.113″ W). Scale values aside bars. (**1**) Body of holotype (USNM No. XXXXXXX), (**2**) Genitalia of paratype (USNM No. XXXXXXX, XXXXXXX), ventral, and (**3**) Genitalia of paratype (USNM No. XXXXXXX, XXXXXXX), dorsal. Abbreviations: anterior sucker (as), anterior testis (at), cirrus (c), common genital pore (cgp), esophagus (es), intestine (in), oötype (oo), ovary (o), oviducal seminal receptacle (osr), posterior testis (pt), seminal vesicle (sv), uterine egg (ue), uterus (u), vasa efferentia (ve), and vitelline duct (vit).

Figures 4–7. N. GEN., n. sp. Warren and Bullard (Digenea: Aporocotylidae) infecting the heart of white mullet, *Mugil curema* Valenciennes, 1836 (Mugiliformes: Mugilidae) from the mouth of Deer River, a tributary of Mobile Bay, Alabama, (30°32′ 3.401″ N, 88°6′ 21.113″ W). Scale values aside bars. (**4**) Anterior sucker of holotype (USNM No. XXXXXXX), dorsal, (**5**) Lateral tegumental spines of holotype (USNM No. XXXXXXX), ventral, (**6**) Intestine of voucher (USNM No. XXXXXXX), ventral, (**7**) Male genital system of voucher (USNM No. XXXXXXX) dorsal.Abbreviations: anterior testis (at), anterior vasa efferentia duct (ad), circumoral spines (cs), cirrus (c), esophagus (es), intestine (in), mouth (mo), papillae (pa), posterior testis (pt), posterior vasa efferentia duct (pd), seminal vesicle (sv), vas deferens (vd), and vasa efferentia (ve).

Figure 8. Phylogenetic relationships of species within the Aporocotylidae Odhner, 1912 reconstructed using Bayesian inference analysis using the large ribosomal subunit (*28S*) gene. Numbers aside nodes indicate posterior probability. The new genus and species is shown in bold.

Figures 9–12. Histological sections (hematoxylin and eosin) of white mullet, *Mugil curema* Valenciennes, 1836 (Mugiliformes: Mugilidae) infected by N. GEN., n. sp. Warren and Bullard (Digenea: Aporocotylidae). (**9**) Heart showing adults in the lumen of the ventricle. (**10**) Adult in the intertrabecular space of heart. (**11**) Endocardium showing hyperplasia of the endocardium and endocarditis composed of eosinophilic granulocytes (arrows), mononuclear inflammatory infiltrates (*), and free granule clusters (triangles). (**12**) Granuloma (*) encapsulating egg in cardiac muscle of the myocardium.

Figures 13–15. Histological sections (hematoxylin and eosin) of white mullet, *Mugil curema* Valenciennes, 1836 (Mugiliformes: Mugilidae) gill infected by eggs of N. GEN., n. sp. Warren and Bullard (Digenea: Aporocotylidae) (**13**) Egg in afferent artery of the gill filament encapsulated by epithelioid cells (*). (**14**) Eggs in the afferent artery of the gill showing epithelioid cells (arrows) encapsulating eggs and hyperplasia of the overlying gill epithelium (*). (**15**) Eggs in gill showing disruption of the afferent artery (*) and hemorrhaging (arrows).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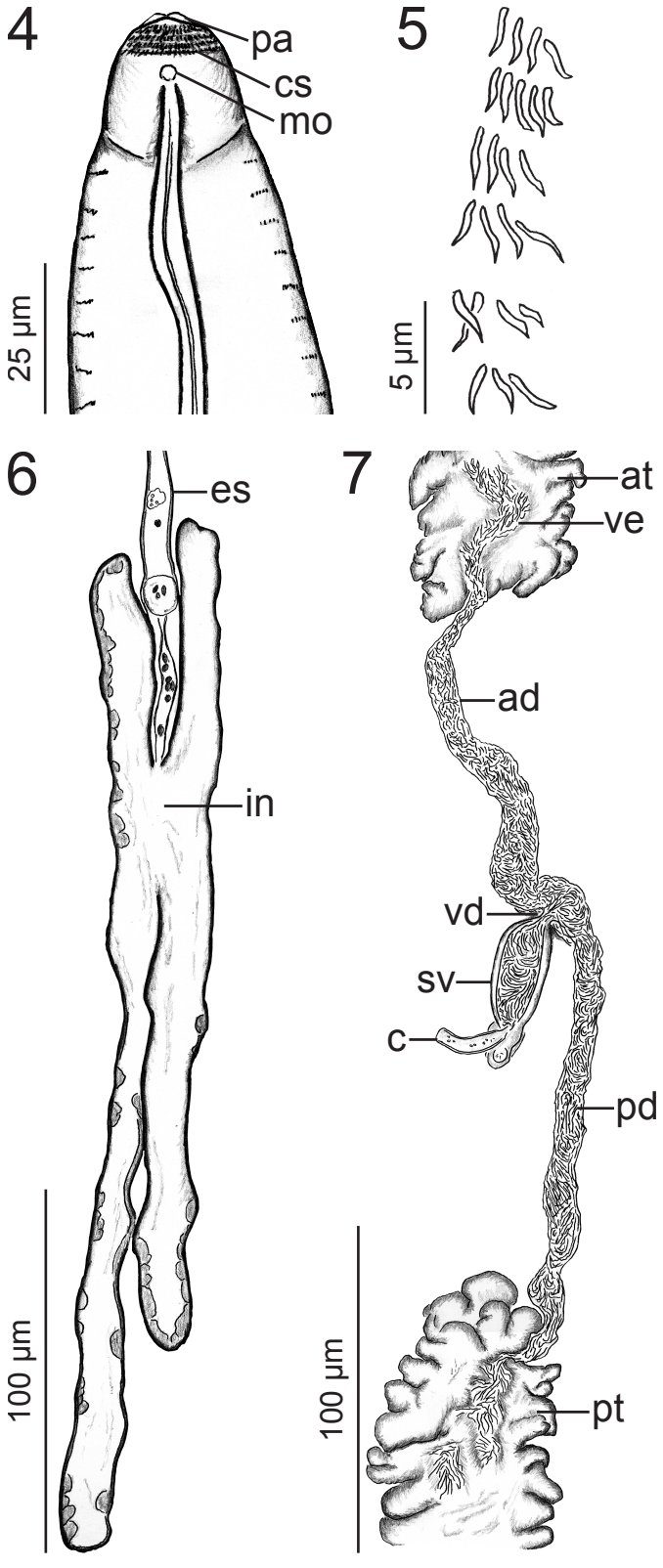
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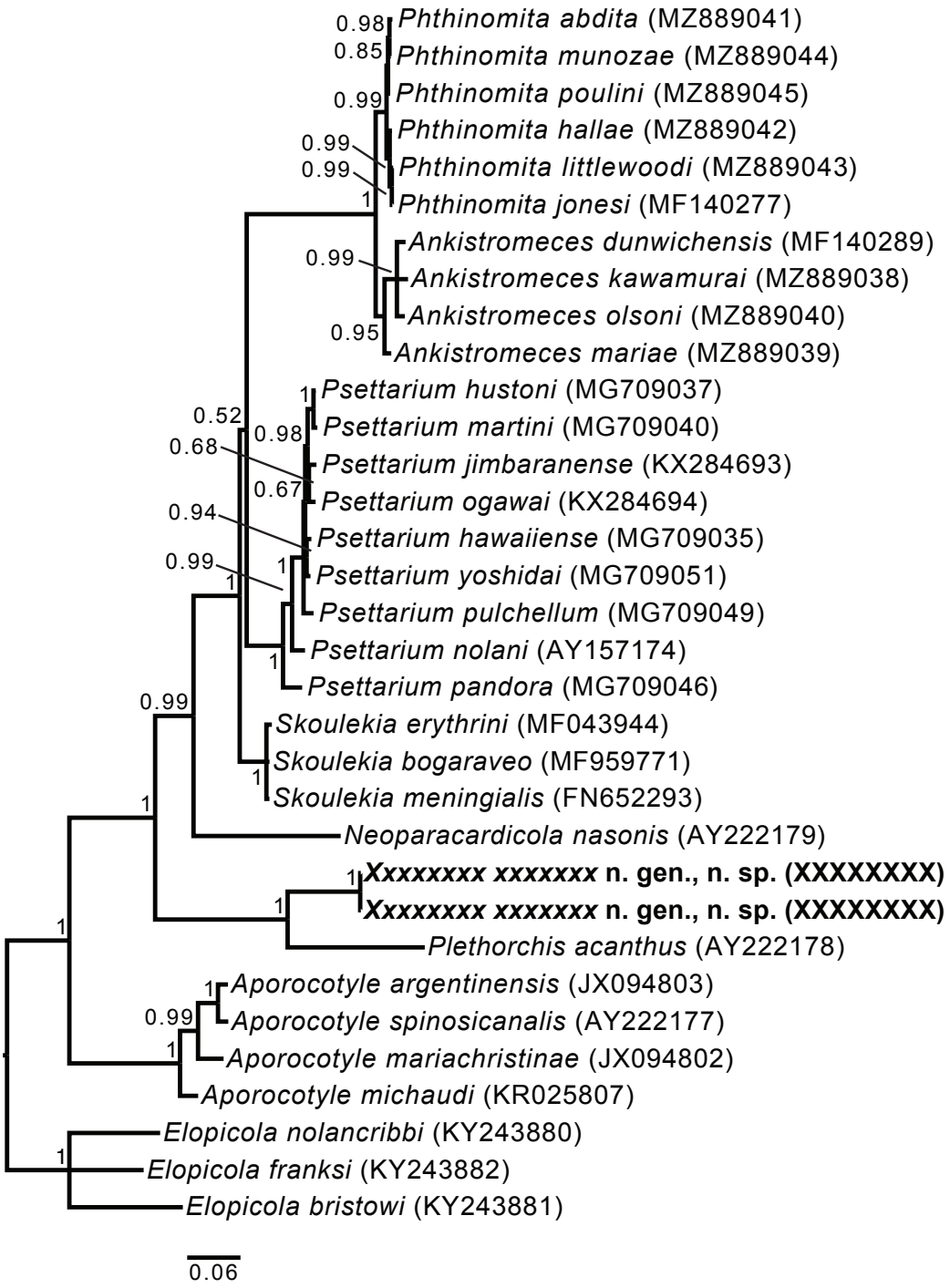
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Ankistromeces kawamurai (MZ889038)
Ankistromeces olsoni (MZ889040)
Ankistromeces mariae (MZ889039)
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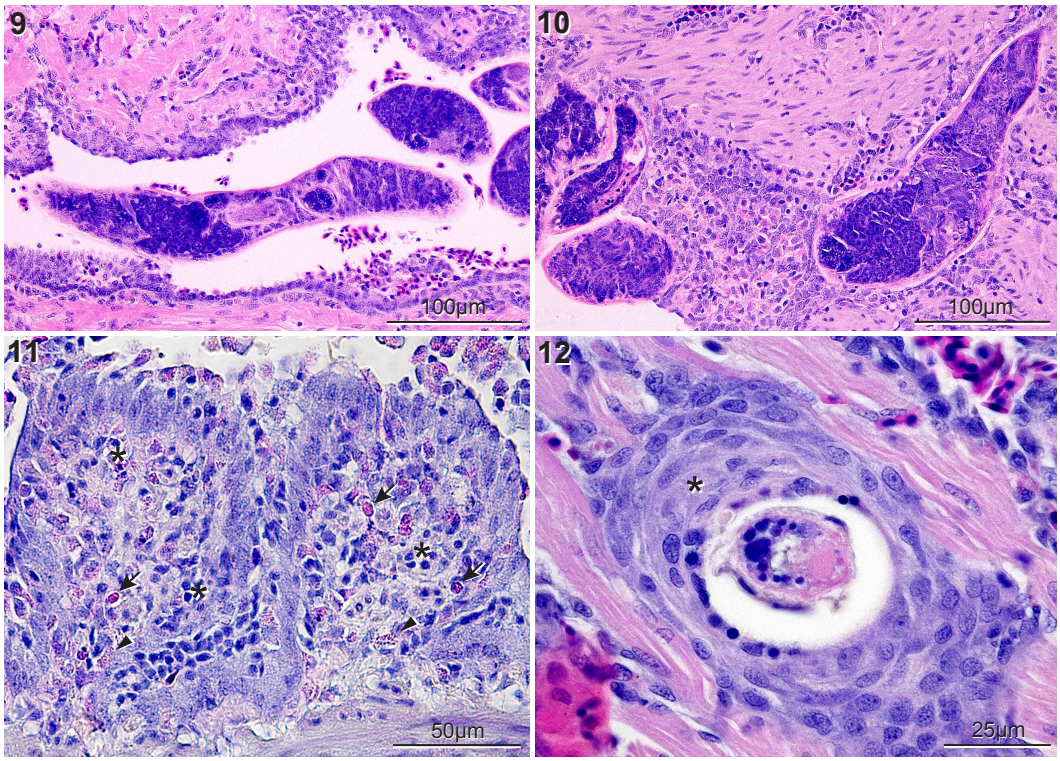
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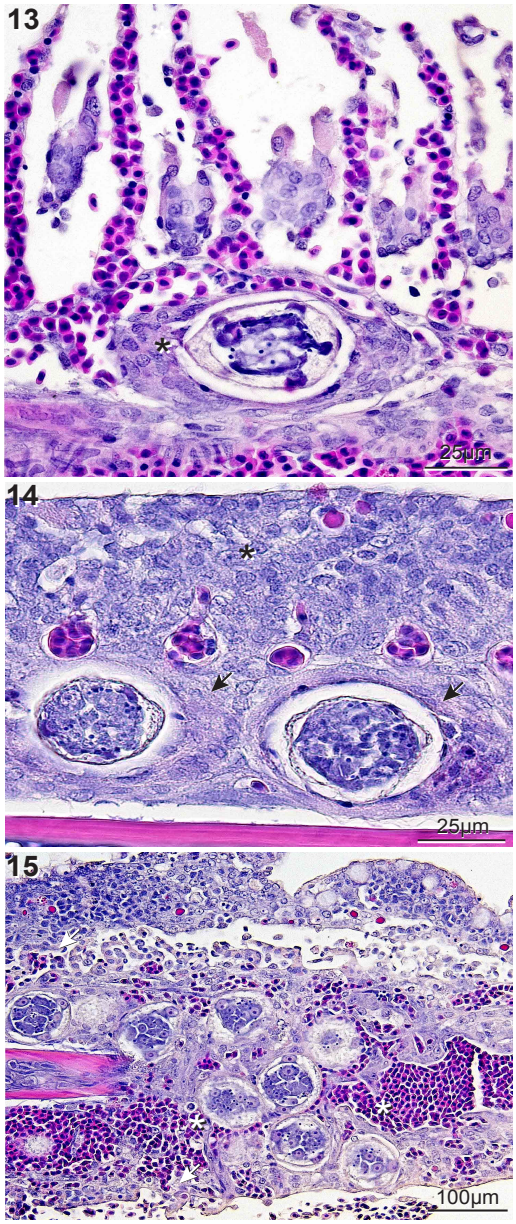
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CHAPTER 7: SUMMARY.

The foundation of this dissertation uses both taxonomic and systematic approaches to describe and revise several fish blood fluke groups now comprising ~186 species of 46 genera among five families. More specifically this work contributes to this group of parasitic helminths by revising three fish blood fluke families, the proposal of two new families, the proposal of four genera, the revision of a genus, the description of five new species, the redescription of three species, the synonymy of three genera and two species, the addition of 20 previously unpublished nucleotide sequences from new species herein as well as other fish blood fluke species, and the second documented pathology from a fish blood fluke infecting the heart. The results clearly suggest a high level of diversity among the fish blood flukes, and it hints that research with this group is still in its infancy.

Considering the trends related to fish blood fluke taxonomic publications, it becomes clear that there is a pressing need to enhance the quality of morphological descriptions. In recent years, with the rise of nucleotide sequencing, publications focused on taxonomy have often downplayed the importance of morphology in favor of phylogenetic results. Routinely, many new species descriptions are notably incomplete or rely on molecular techniques to determine whether morphological distinctions are necessary. Intriguingly, most phylogenetic analyses remain rather straightforward, primarily utilizing a single gene tree in their descriptions.

As molecular tools continue to advance, it is tempting to envision a future where the identification of new species can be accomplished solely by analyzing tissue samples. However, genetic-based databases face significant challenges, with one of the most severe being the

potential for misidentifying the organism being sequenced. This could perpetuate an error cascade, leading to confusion and a lack of clarity.

To mitigate these error cascades and ensure a comprehensive understanding of the natural world, it is crucial to have scientists well-versed in both the morphology and genetic characteristics of organisms. Taxonomy serves as a vital framework that offers insights into the natural history of organisms, and eliminating details about these organisms will not lead to a resolution of the questions that nature presents.

The future directions for research in this field will likely include a more comprehensive exploration of the diversity and distribution of fish blood flukes, improvements in morphological and genetic characterization, and a deeper understanding of the interactions between parasites and their hosts. Moreover, delving into the factors that influence the distribution patterns, such as the availability and abundance of intermediate hosts, variations in host populations, and environmental variables, will have significant implications for the conservation of fish species, particularly those of commercially valuable or threatened. A comprehensive understanding of the prevalence and effects of these parasites on fish populations can serve as a foundation for the development of informed conservation strategies.