



Gymnurahemecus bulbosus gen. et sp. nov. (Digenea: Aporocotylidae) infecting smooth butterfly rays, *Gymnura micrura* (Myliobatiformes: Gymnuridae) in the northern Gulf of Mexico, with a taxonomic key and further evidence for monophyly of chondrichthyan blood flukes

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Abstract

Gymnurahemecus bulbosus gen. et sp. nov. infects the heart of smooth butterfly rays, *Gymnura micrura* in the Gulf of Mexico. *Gymnurahemecus* differs from all other accepted aporocotylid genera by having one column of C-shaped lateral tegumental spines, a medial oesophageal bulb anterior to a diverticulate region of the oesophagus, inverse U-shaped intestinal caeca, a non-looped testis, an oviducal ampulla, a Laurer's canal, and a post-caecal common genital pore. The new species, the shark blood flukes (*Selachohemecus* spp. and *Hyperandrotrema* spp.), and the chimaera blood fluke *Chimaerohemecus trondheimensis* are unique by having C-shaped lateral tegumental spines. *Selachohemecus* spp. and the new species have a single column of lateral tegumental spines, whereas *Hyperandrotrema* spp. and *C. trondheimensis* have 2–7 columns of lateral tegumental spines. The new species differs from *Selachohemecus* spp. most notably by having an inverse U-shaped intestine. The other ray blood flukes (*Orchispirium heterovitellatum*, *Myliobaticola richardheardi*, and *Ogawaia glaucostegi*) differ from the new species by lacking lateral tegumental spines, a medial oesophageal bulb, and a Laurer's canal and by having a looped testis. Phylogenetic analysis using large subunit ribosomal DNA (28S) indicated that the new species is sister to the clade that includes the other sequenced adult blood fluke (*O. glaucostegi*), which infects a ray in Australia. These results agree with and extend previous morphology- and nucleotide-based phylogenetic assertions that the blood flukes of early-branching jawed craniates (Chondrichthyes) are monophyletic and phylogenetically separated from the blood flukes of later-branching ray-finned fishes (Actinopterygii: Euteleostei).

Keywords Blood fluke · New genus · Chondrichthyes · Taxonomy · Phylogeny

Introduction

The fish blood flukes (Digenea: Aporocotylidae Odhner, 1912; see Bullard et al. 2009) that infect sharks, skates, and

rays (Chondrichthyes: Elasmobranchii) plus chimaeras (Chondrichthyes: Holocephali) presently comprise eight nominal species assigned to six genera: *Selachohemecus olsoni* Short, 1954 from the Atlantic sharpnose shark, *Rhizoprionodon terraenovae* (Richardson) (Short 1954; Bullard et al. 2006); *Selachohemecus benzi* Bullard et al., 2006 from the blacktip shark, *Carcharhinus limbatus* (Valenciennes) (Bullard et al. 2006); *Chimaerohemecus trondheimensis* van der Land, 1967 from rabbit fish, *Chimaera monstrosa* Linnaeus and spookfish, *Hydrolagus mitsukurii* Jordan and Snyder (van der Land 1967; Kamegai et al. 2002); *Orchispirium heterovitellatum* Madhavi and Rao, 1970 from the Bengal whipray, *Brevitrygon imbricata* (Bloch and Schneider) (Madhavi and Hanumantha Rao 1970; Bullard and Jensen 2008); *Myliobaticola richardheardi* Bullard and Jensen, 2008 from the Atlantic stingray, *Hypanus sabinus*

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(Lesueur) (Bullard and Jensen 2008); *Hyperandrotrema cetorhini* Malliard and Ktari, 1978 from the basking shark, *Cetorhinus maximus* (Gunnerus) (Maillard and Ktari 1978; Orélis-Ribeiro et al. 2013); *Hyperandrotrema walterboegeri* Orélis-Ribeiro et al., 2013 from shortfin mako shark, *Isurus oxyrinchus* Rafinesque (Orélis-Ribeiro et al. 2013); and *Ogawaia glaucostegi* Cutmore et al., 2018 from the giant shovelnose ray, *Glaucostegus typus* (Anonymous [Bennett]) (Cutmore et al. 2018). They collectively infect the heart, kidney, mesenteric blood vessels, and gill epithelium of four sharks and three rays in the northwestern Atlantic Ocean (Gulf of Mexico) (Short 1954; Bullard et al. 2006; Bullard and Jensen 2008; Orélis-Ribeiro et al. 2013) and Indian Ocean (Madhavi and Hanumantha Rao 1970; Maillard and Ktari 1978). The single blood fluke named from chimaeras has been reported from off Norway (van der Land 1967) and Japan (Kamegai et al. 2002), but another congener may exist off Greenland (Karlsbakk et al. 2002). A new species of blood fluke infecting the heart of smalltooth sawfish, *Pristis pectinata* Latham in the eastern Gulf of Mexico off Florida, USA, is being described by us (MBW and SAB) and will comprise the ninth named blood fluke infecting a chondrichthyan. Compared to the taxonomic diversity of blood flukes reported from ray-finned fishes (Actinopterygii), those infecting early-branching fish lineages, especially chondrichthyans, remain vastly underexplored.

Herein, we propose a new genus and describe a new species of blood fluke from the heart of smooth butterfly rays, *Gymnura micrura* (Bloch and Schneider), (Myliobatiformes: Gymnuridae) captured in the northern Gulf of Mexico. This is the fourth species described and fourth genus proposed for a blood fluke infecting a ray.

Materials and methods

A total of 74 smooth butterfly rays were captured using a 10-m otter trawl during summer 2016 ($n = 54$) and 2017 ($n = 20$) from the mouth of Mobile Bay (30°13'22.61"N, 88°3'18.57"W) (northern Gulf of Mexico). Each smooth butterfly ray was identified using the dichotomous key of McEachran and de Carvalho (2002) and by having a tail without serrated spines and no tentacle on the posterior margin of the spiracle. Live smooth butterfly rays were removed from the trawl and placed in a flow-through seawater tank prior to necropsy. At necropsy, each smooth butterfly ray was killed by pithing before the heart and all gill arches were excised intact, placed in separate sample bags (heart was bisected; gill arches separated), exposed to 60 °C freshwater, shaken vigorously, and preserved in 5–10% neutral buffered formalin. Gill arches, gill filaments, and corresponding sediment from the sample bags were examined using a stereo-dissecting microscope for the presence of adults and eggs of the new species. Several gill filaments

from each set of gill arches were excised, wet-mounted on a glass slide, and examined with the aforementioned compound microscopes to detect blood fluke eggs in the branchial arterioles and gill epithelium (observations of eggs will be reported elsewhere). Twenty live smooth butterfly rays captured in 2017 were pithed, placed on ice, and examined for the purposes of extracting blood flukes for DNA extraction: one adult blood fluke was isolated from the heart of two smooth butterfly rays, wet-mounted on a glass slide to confirm their identity, placed directly into 95% EtOH, and stored at -20 °C until DNA was extracted (see below). All bagged, formalin-fixed tissues were examined with the aid of a stereo-dissecting microscope and fiber optic light source to isolate blood flukes for morphology. The heart was teased apart with fine forceps to reveal adult blood flukes, and sediment from each sample bag was examined for blood flukes with aid of a settling column.

Specimens for morphology were rinsed with distilled water, cleaned with fine brushes to remove any 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, permanently mounted in Canada balsam, illustrated using Leica DM 2500 and Leica DMR microscopes each equipped with differential interference contrast (DIC) optical components, measured using an ocular micrometer, and illustrated using a drawing tube. Blood fluke measurements are reported in micrometers (μm) as the range followed by the mean, \pm standard deviation, and sample size in parentheses. Scientific names, including taxonomic authorities and dates for fishes, follow Eschmeyer et al. (2016). Morphological terms and nomenclature for blood flukes follows Bullard et al. (2006, 2009), Bullard and Jensen (2008) and Orélis-Ribeiro et al. (2013). Type and voucher materials are deposited in the National Museum of Natural History's Invertebrate Zoology Collection (USNM, Smithsonian Institution, Washington, D.C.) and the Auburn University Museum of Natural History (AUMNH, Auburn, AL).

Using the two EtOH-preserved and microscopically identified blood flukes, total genomic DNA (gDNA) was extracted using DNeasy™ Blood and Tissue Kit (Qiagen, Valencia, CA, USA) as per the manufacturer's protocol with one exception: 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. Amplification and sequencing of the large subunit ribosomal DNA (28S) used the set of primers described in Orélis-Ribeiro et al. (2017). PCR amplifications were performed according to Warren et al. (2017) with one exception: the annealing temperature was 61 °C for 30 s. DNA sequencing was performed by ACGT, Incorporated (Wheeling, IL, USA). Sequence assembly and analysis of chromatograms were performed with Geneious version 11.0.5 (<http://www.geneious.com>; Kearse et al. 2012). All nucleotide sequence data were deposited in GenBank (Table 1).

The phylogenetic analysis included two sequences of the new species plus the taxa included in Cribb et al. (2017) (Table 1). The outgroup comprised the three turtle blood fluke taxa *Hapalorhynchus gracilis* Stunkard, 1922, *Spirorchis artericola* (Ward, 1921), and *Vasotrema robustum* Stunkard, 1928. Sequences were aligned using MAFFT (Katoh and Standley 2013). JModelTest 2 version 2.1.10 was implemented to perform statistical selection of the best-fit models of nucleotide substitution based on Bayesian information criteria (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 four 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 v1.4.3 (Rambaut et al. 2014) and further edited for visualization purposes with Adobe Illustrator (Adobe Systems).

Results

Gymnurahemecus gen. nov. (Figs. 1–5, 6–8, and 9–10)

Diagnosis: Body 6–10× 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 1/4–1/3 of body length, with middle portion having an oesophageal bulb; oesophageal bulb delimited anteriorly and posteriorly by marked constrictions. Intestinal caeca inverse U-shaped, connecting to oesophagus ventrally, lacking diverticulae, 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 sinistral, post-caecal, post-testicular; post-ovarian space comprising 1/3 of body length. Oviducal ampulla present. Laurer’s canal present, opening on

dorsal surface. Oötype medial, posterior to genitalia, comprising an inconspicuous ovoid chamber. Uterus post-gonadal, not extensively convoluted, extending anteriad from oötype before crossing mid-line and extending posteriad; uterine eggs spheroid, thin-shelled. Vitellarium follicular, asymmetrical 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. Excretory vesicle small, medial, visible in posterior most region of specimen body.

Differential diagnosis: Body 6–10× longer than wide; lateral tegumental spines C-shaped, distributing in 1 column. Oesophageal bulb present. Intestinal caeca inverse U-shaped, terminating in anterior half of body. Testis single. Ovary sinistral, post-testicular. Oviducal ampulla and Laurer’s canal present. Oötype posterior to genitalia. Common vitelline collecting duct extending from dextral branch of vitellarium. Common genital pore post-gonadal, anterior to level of oötype.

Taxonomic summary

Type and only nominal species: *Gymnurahemecus bulbosus* sp. nov.

Etymology: *Gymnurahemecus* refers to the type species infecting the blood of a gymnurid.

***Gymnurahemecus bulbosus* sp. nov.** (Figs. 1–5, 6–8, and 9–10)

Diagnosis of adult specimens (based on 10 whole-mounted specimens and 2 SEM prepared specimens: body 830–1130 (986 ± 101 , 9) long, 88–155 (135 ± 22 , 9) at greatest width, 6–10× longer than wide (Figs. 1–3). Lateral tegumental spines 83–97 (91 ± 4 , 8) per side of body or a total of 168–193 (183 ± 7.4 , 8), ending 13–25 (19 ± 4.2 , 8) or 1–3% (2 ± 0.05 , 8) 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. 4), approximately equal in size throughout length of body; lateral tegumental spines in anterior region 5–8 (6.2 ± 1.2 , 9) long, 1–2 (1.6 ± 0.5 , 9) wide; lateral tegumental spines in mid-body and posterior region 4–8 (5 ± 1.3 , 9) long, 1–2 (1.2 ± 0.4 , 9) wide (Fig. 5); peduncles supporting lateral tegumental spines approximately equal in size throughout length of body; peduncles in anterior region of body 5–8 (7.1 ± 1.1 , 9) long, 3–5 (3.9 ± 0.8 , 9) wide; peduncles in mid-body and posterior region of body 5–7 (6 ± 0.5 , 9) long, 2–6 (3.4 ± 0.8 , 9) wide. Ventrolateral nerve cord 760–970 (851 ± 107 , 3) long, 8–10 (9 ± 1 , 3) wide near mid-body at widest level, 13–18 (15 ± 2.5 , 3) from body margin. Primary commissure perpendicular to mid-line of body, connecting ventrolateral nerve-cords, 78–125 (109 ± 19 , 5) or 6–14% ($11\% \pm 0.2$, 5) of body length from anterior end of body, 20–30 (25 ± 5 , 5) across width of worm, 8–10 (9.2 ± 1.1 , 5) in breadth; (Figs. 1–3, 6, 7); secondary commissure and nerve cords not evident in wholemounts.

Table 1 DNA sequences used in the present study

Blood fluke	Host	Locality	GenBank 28S Accession #	Reference
Aporocotylid cercaria W5003 Brant et al., 2006	<i>Plotiopsis balonnensis</i> (Conrad)	Victoria River, Northern Territory, Australia	AY858878	Brant et al. 2006
Aporocotylid cercaria W5004 Brant et al., 2006	<i>Glyptophysa gibbosa</i> (Gould)	Mary River, Northern Territory, Australia	AY858879	Brant et al. 2006
Aporocotylid cercaria NSW1 Cribb et al., 2017	<i>Plebidonax deltoides</i> (Lamarck)	Stockton beach, New South Wales, Australia	MF503307	Cribb et al. 2017
<i>Aporocotyle argentinensis</i> Smith, 1969	<i>Merluccius hubbsi</i> Marini	Off north Patagonia, Argentina	JX094803	Hernández-Orts et al. 2012
<i>Aporocotyle mariachristinae</i> Hernández-Orts et al., 2012	<i>Genypterus blacodes</i> (Forster)	Off north and central Patagonia, Argentina	JX094802	Hernández-Orts et al. 2012
<i>Aporocotyle spinosicanalis</i> Williams, 1958	<i>Merluccius merluccius</i> (Linnaeus)	Off Orkney islands, NE Atlantic Ocean	AY222177	Olson et al. 2003
<i>Cardicola beveridgei</i> Yong et al., 2016b	<i>Lutjanus argentimaculatus</i> (Forsskål)	Off Lizard Island, Australia	KX523188	Yong et al. 2016b
<i>Cardicola bullardi</i> Yong et al., 2016b	<i>Scomberomorus munroi</i> Collette and Russo	Moreton Bay, Queensland, Australia	KX523190	Yong et al. 2016b
<i>Cardicola forsteri</i> Shirakashi et al., 2016	<i>Thunnus orientalis</i> (Temminck and Schlegel)	Off Wakayama Prefecture, Japan	KT119353	Shirakashi et al. 2016
<i>Cardicola opisthorchis</i> Ogawa et al., 2011	<i>Thunnus orientalis</i> (Temminck and Schlegel)	Off Wakayama Prefecture, Japan	HQ324227	Ogawa et al. 2011
<i>Cardicola suni</i> Yong et al., 2016b	<i>Chanos chanos</i> (Forsskål)	Moreton Bay, Queensland, Australia	KX463511	Yong et al. 2016b
<i>Chimaerohemecus trondheimensis</i> van der Land, 1927	<i>Chimaera monstrosa</i> Linnaeus	NE Atlantic, off Bergen, Norway	AY157239	Lockyer et al. 2003b
<i>Elopicola nolancribbi</i> Bullard, 2014	<i>Elops saurus</i> Linnaeus	Gulf of Mexico, off Ship Island, MS	KY243880	Orélis-Ribeiro et al. 2017
<i>Elopicola bristowi</i> Orélis-Ribeiro et al., 2017	<i>Elops hawaiiensis</i> Reagan	Eastern Sea, off Nha Trang, Vietnam	KY243881	Orélis-Ribeiro et al. 2017
<i>Elopicola franksi</i> Orélis-Ribeiro et al., 2017	<i>Megalops atlanticus</i> Valenciennes	Gulf of Mexico, off FL	KY243882	Orélis-Ribeiro et al. 2017
<i>Gymnurahemecus bulbosus</i> gen. et sp. nov.	<i>Gymnura micrura</i> (Bloch and Schneider)	Gulf of Mexico, mouth of Mobile Bay, AL	MH555432	Present study
<i>Gymnurahemecus bulbosus</i> gen. et sp. nov.	<i>Gymnura micrura</i> (Bloch and Schneider)	Gulf of Mexico, mouth of Mobile Bay, AL	MH555433	Present study
<i>Neoparacardicola nasonis</i> Yamaguti, 1970	<i>Naso unicornis</i> (Forsskål)	Off Lizard Island, Australia	AY222179	Olson et al. 2003
<i>Ogawaia glaucostegi</i> Cutmore et al., 2018	<i>Glaucostegus typus</i> (Anonymous [Bennett])	Moreton Bay, Queensland, Australia	MF503308	Cribb et al. 2017
<i>Paradeontacylix buri</i> Ogawa et al., 2015	<i>Seriola quinqueradiata</i> Temminck and Schlegel	Off Miyazaki Prefecture, Japan	AB904154	Ogawa et al. 2015
<i>Paradeontacylix grandispinus</i> Repullés-Albelda et al., 2008	<i>Seriola dumerili</i> (Risso)	Off Kagoshima Prefecture, Japan	AM489596	Repullés-Albelda et al. 2008
<i>Paradeontacylix ibericus</i> Repullés-Albelda et al., 2008	<i>Seriola dumerili</i> (Risso)	Off Murcia, Spain	AM489593	Repullés-Albelda et al. 2008
<i>Plethorchis acanthus</i> Martin, 1975	<i>Mugil cephalus</i> Linnaeus	Brisbane River, Queensland, Australia	AY222178	Olson et al. 2003
<i>Psettarium jimbaranense</i> Yong et al., 2016a	<i>Arothron reticularis</i> (Bloch and Schneider)	Off Bali, Indonesia	KX284693	Yong et al. 2016a
<i>Psettarium nolani</i> (Bray et al. 2012) Yong et al., 2016	<i>Arothron meleagris</i> (Anonymous)	Off Moorea, French Polynesia	AY157174	Lockyer et al. 2003a
<i>Psettarium sinense</i> (Liu, 1977) Orélis-Ribeiro et al., 2014	<i>Takifugu rubripes</i> (Temminck and Schlegel)	Fuzhou, China	EU368853	
<i>Sanguinicola</i> cf. <i>inermis</i> Plehn, 1905	<i>Lymnaea stagnalis</i> (Linnaeus)	Warminia-Mazury Region, Poland	AY222180	Olson et al. 2003
<i>Skoulekia meningialis</i> Alama-Bermejo et al., 2011	<i>Diplodus vulgaris</i> (Geoffroy St. Hilaire)	Off Valencia, Spain	FN652293	Alama-Bermejo et al. 2011

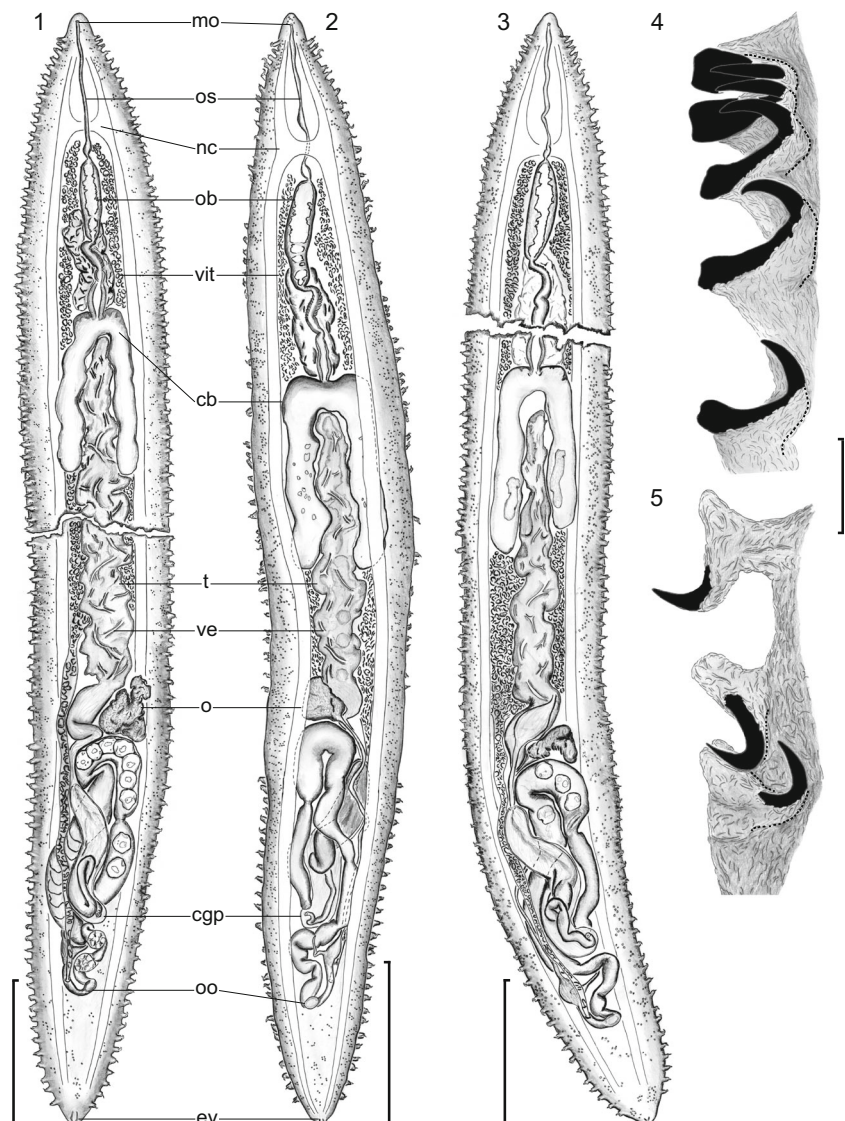
Table 1 (continued)

Blood fluke	Host	Locality	GenBank 28S Accession #	Reference
Turtle blood flukes				
<i>Haplorhynchus gracilis</i> Stunkard, 1922	<i>Chelydra serpentina</i> (Linnaeus)	Reelfoot Lake, TN	AY604710	Snyder 2004
<i>Spirorchis artericola</i> (Ward, 1921)	<i>Chrysemys picta</i> (Schneider)	Reelfoot Lake, TN	AY604704	Snyder 2004
<i>Vasotrema robustum</i> Stunkard, 1928	<i>Apalone spinifera</i> (LeSueur)	Nishnabotna River, IA	AY604706	Snyder 2004

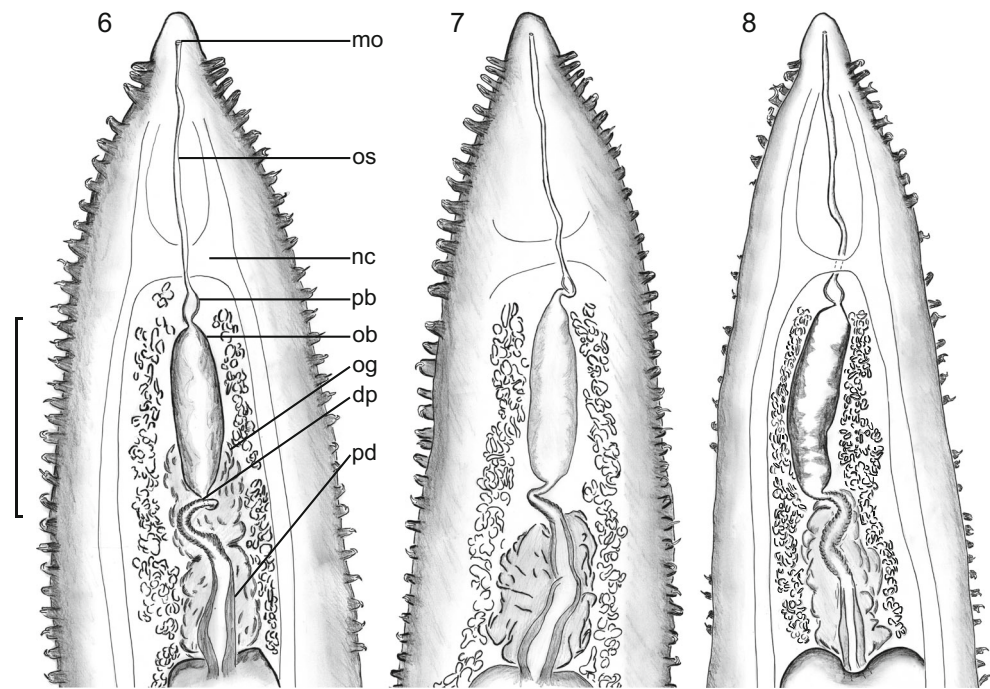
Mouth 2–3 (2.4 ± 0.5 , 7) in diameter, 6–9 (7 ± 1 , 7) from terminal end of anterior sucker (Figs. 1–3). Oesophagus 238–325 (288 ± 31 , 7) in total length or 25–35% ($29\% \pm 0.03$, 7) of body length, 13–30 (24 ± 4 , 7) in maximum width (at level of oesophageal bulb), ventral to primary nerve-commissure, comprising several distinct segments (anterior portion, pre-

oesophageal bulb dilation, medial oesophageal bulb, diverticulate portion, and pre-caecal dilation); anterior portion a narrow duct extending directly or slightly sinuously posteriad, 105–145 (128 ± 13 , 7) long or 41–48% ($44\% \pm 0.02$, 7) of oesophagus length, 1–3 (2.5 ± 0.75 , 8) wide or 1–2% ($1.8\% \pm 0.003$, 8) of body width; medial oesophageal bulb

Figs. 1–5 *Gymnura hemecus bulbosus* Warren and Bullard gen. et sp. nov. (Digenea: Aporocotylidae) from the heart of the smooth butterfly ray, *Gymnura micrura* (Bloch and Schneider) (Myliobatiformes: Gymnuridae). (1) Body of holotype, ventral view. (2) Body of paratype 1, dorsal view. (3) Body of paratype 2, ventral view. Mouth (mo), oesophagus (os), nerve commissure (nc), oesophageal bulb (ob), vitellarium (vit), caecal bifurcation (cb), testis (t), vasa efferentia (ve), ovary (o), common genital pore (cgp), oötype (oo), and excretory vesicle (ev). Bars = 150 μ m. (4) Anterior-most ventrolateral tegumental spines and supporting peduncles. Dorsal view. (5) Mid-body ventrolateral tegumental spines and supporting peduncles. Black shading indicates exposed portion of lateral tegumental spine. Bar = 4 μ m



Figs. 6–8 *Gymnurahemecus bulbosus* Warren and Bullard gen. et sp. nov. (Digenea: Aporocotylidae) from the heart of the smooth butterfly ray, *Gymnura micrura* (Bloch and Schneider) (Myliobatiformes: Gymnuridae). Oesophagus of adults demonstrating morphological consistency. All specimens show mouth (mo), oesophagus (os), nerve commissure (nc), pre-oesophageal bulb dilation (pb), oesophageal bulb (ob), oesophageal gland (og), diverticulated portion (dp), and pre-cecal dilation (pd). Bar = 100 μ m. (Oesophageal gland surrounds oesophagus but its enveloping portion is not illustrated so as not to obscure the oesophagus)



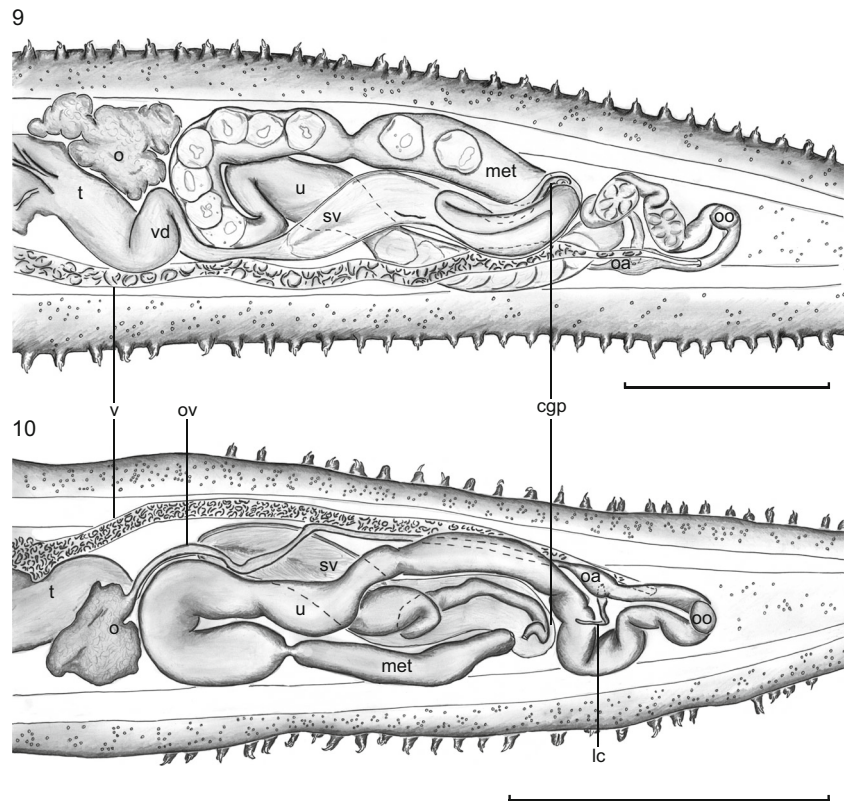
a markedly laterally expanded chamber occupying approximate middle portion of oesophagus, 70–98 (90 ± 11 , 6) long or 23–33% ($30\% \pm 4$, 6) of oesophagus length, 18–30 (24 ± 4 , 6) wide or 15–20% ($18\% \pm 2$, 6) of body width, immediately following a slightly expanded portion of oesophagus, having wall 2–3 (2.3 ± 0.5 , 6) thick; diverticulate portion having a wall comprising diverticulae-like structures, 32–53 (39 ± 8 , 6) long or 10–18% ($13\% \pm 0.03$, 6) of oesophagus length, 8–20 (12 ± 4 , 6) wide or 6–14% ($8\% \pm 0.02$, 6) of body width, immediately following medial oesophageal bulb, having wall 2–3 (2.5 ± 0.5 , 6) thick. Oesophageal gland enveloping oesophagus pre-caecal dilation, diverticulate portion, and posterior portion of oesophageal bulb, 63–135 (103 ± 23 , 8) long or 21–52% ($36\% \pm 8$, 8) of oesophagus length, 28–48 (39 ± 7 , 8) wide or 26–33% ($29\% \pm 2$, 8) of body width. Caecal bifurcation 175–360 (280 ± 69 , 8) or 17–38% ($29\% \pm 7$, 8) of body length from anterior body end; caeca extending posteriad in parallel, 127–192 (162 ± 23 , 8) long or 15–19% ($16\% \pm 1$, 8) of body length, 14–31 (25 ± 5.5 , 8) wide, ventral to lateral nerve cord, containing granular material within lumen of some individuals (Figs. 1–3).

Testis 203–345 (286 ± 45 , 8) long or 24–32% ($29\% \pm 2$, 8) of body length, 18–55 (36 ± 13 , 8) wide or 15–36% ($27\% \pm 7$, 8) of body width, 6–15 (9 ± 3.4 , 8) \times longer than wide, intercaecal (Figs. 1–3), processes extending dorsoventrally. Post-testicular space 210–455 (349 ± 73 , 8) long or 20–41% ($36\% \pm 7$, 8) of body length. Vasa efferentia comprising interconnecting meshwork of fine ducts entwined throughout testicular tissue, 10–13 (2) in diameter, extending primarily dorsoventrally and along ventral surface of testis, coalescing in posterior region of testis; vas deferens 30–75 (46 ± 16 , 6)

long, 3–8 (4 ± 2 , 6) wide, emanating from postero-ventral portion of testis, extending dextrad lateral to ovary before curving mediad and posteriad and extending posteriad aside ascending uterus for a short distance before becoming confluent with cirrus sac. Cirrus sac 103–168 (151 ± 22 , 7) long or comprising 71–81% ($77\% \pm 0.04$, 7) length of male terminal genitalia, 15–33 (25 ± 5 , 7) wide or 16–22% ($18\% \pm 0.02$, 7) of body width, having extremely thin wall approximately 1–2 (2 ± 0.5 , 8) thick, including seminal vesicle and cirrus; seminal vesicle 98–180 (150 ± 19 , 7) long, 18–30 (26 ± 4.3 , 7) wide, anterior half filling breadth of cirrus sac, posterior half filling 39–60% ($51\% \pm 0.06$, 7) breadth of cirrus sac, containing sperm in 6 of 10 specimens, extending sinuously posteriad before narrowing and curving anterodorsally. Common genital pore 155–220 (184 ± 20 , 8) or 17–20% ($18\% \pm 1$, 8) of body length from posterior end of body, 28–50 (38 ± 7 , 8) from sinistral body margin, 50–85 (65 ± 12 , 8) from dextral body margin (Figs. 1–3, 9, 10).

Ovary sinistral to testis, slightly lobed, 25–53 (41 ± 10 , 8) long or 3–5% ($4\% \pm 0.08$, 8) of body length, 18–63 (40 ± 15 , 8) wide or 20–47% ($28\% \pm 9$, 8) of body width, 0.7–1.8 (1 ± 0.4 , 8) \times wider than long, immediately post-testicular, ventral to lateral nerve-cords; post-ovarian space 275–405 (344 ± 43 , 7) long or 33–36% ($34\% \pm 4$, 7) of body length (Figs. 9, 10). Oviduct (including oviducal ampulla) 153–288 (243 ± 43 , 8) long, 6–9 (7 ± 1.1 , 6) wide; oviducal ampulla 13–30 (21 ± 6 , 6) long or 6–12% ($8\% \pm 2$, 6) of oviduct length, 10–28 (17 ± 6.3 , 6) wide. Laurer's canal 10–33 (22 ± 9 , 6) long, 3–5 (5 ± 0.7 , 6) wide, sinistral to oviducal seminal ampulla (Figs. 9, 10). Oötype 13–28 (18 ± 5.2 , 7) long, 8–15 (13 ± 2.3 , 7) wide, posterior to all other genitalia (Figs. 9, 10). Vitellarium having

Figs. 9–10 *Gymnurahebecus bulbosus* Warren and Bullard gen. et sp. nov. (Digenea: Aporocotylidae) from the heart of the smooth butterfly ray, *Gymnura micrura* (Bloch and Schneider) (Myliobatiformes: Gymnuridae). **(9)** Genitalia of holotype, ventral view. **(10)** Genitalia of paratype, dorsal view. Testis (t), ovary (o), vitelline duct (v), vas deferens (vd), oviduct (ov), ascending and descending portions of the uterus (u), common genital pore (cgp), seminal vesicle (sv), metraterm (met), Laurer's canal (lc), oviducal ampullae (oa), and oötype (oo). Bars = 100 μ m. Note that nerve cords are drawn dorsal so as not to obscure the genitalia



follicles compacted in dense lobules, occupying space dorsal and lateral to testis and caeca; common collecting duct 178–385 (281 ± 68 , 7) long, 8–13 (10 ± 1.5 , 7) wide. Uterus extending directly anterior from oötype, 155–298 (251 ± 45 , 8) long or 18–32 (25 ± 0.04 , 8) of body length, 13–30 (22 ± 5 , 8) wide, with wall 2 (8) thick; ascending portion extending sinuously anterior and dorsal to seminal vesicle before coursing diagonally across mid-line, containing eggs in 5 of 10 specimens (Figs. 1 and 9), containing sperm in 3 of 10 specimens (superficially resembling seminal vesicle), curves dextrally before immediately curving anteriorly and sinistral to posterior margin of ovary before connecting with descending portion; descending portion 28–65 (52 ± 12 , 8) long or 17–28% (20 ± 4 , 8) of ascending uterus length, 15–28 (21 ± 4 , 8) wide, with wall 1 (8) thick, containing eggs in 5 of 10 specimens (Figs. 1, 9), extending posteriad before connecting with metraterm; metraterm 73–110 (92 ± 10 , 8) long or 1.4–2.6 (1.8 ± 0.4 , 8) \times longer than the descending uterus, 15–28 (21 ± 4 , 8) wide, comprising distal-most portion of female reproductive tract, demarcated from descending uterus by obvious constriction (Figs. 9, 10), with eggs in 3 of 10 specimens, with wall 2 (8) thick. Uterine eggs 18–25 (21 ± 2.8 , 7) in diameter or 51–82% (71 ± 14 , 6) of uterus width, containing a large spheroid body plus several smaller, dense lipid-like bodies, with thin shell (Fig. 9). Excretory bladder small, 4–10 (7.5 ± 2.3 , 7) long, 2–7 (4.2 ± 1.5 , 7) wide, medial (Figs. 1–3).

Taxonomic summary

Type and only reported host: smooth butterfly ray, *Gymnura micrura* (Bloch and Schneider) (Myliobatiformes: Gymnuridae).

Site in host: Heart lumen.

Type locality: Mobile Bay ($30^{\circ} 13' 22.61''$ N, $88^{\circ} 3' 18.57''$ W), North central Gulf of Mexico.

Prevalence and intensity of infection: Seven of 54 (prevalence = 13%) smooth butterfly rays sampled in 2016 were infected by 1, 1, 1, 1, 2, 2, and 4 specimens of *G. bulbosus* (mean intensity = 1.7). Three of 20 (15%) smooth butterfly rays sampled in 2017 were infected by 1 specimen of *G. bulbosus* each (mean intensity = 1.0).

Specimens deposited: Holotype GM17-1, paratypes GM17-2, GM17-3, GM17-4, GenBank Nos. (28S: MH555432 and MH555433).

Etymology: The specific epithet “*bulbosus*” refers to the distinct oesophageal bulb of the new species.

Sequence comparison and phylogenetic results

The amplified 28S fragment from the two specimens of the new species comprised 1622 nucleotides (MH55432 and MH555433-GenBank). These were identical (100% similar) to each other; differed from the 28S sequence of *C. trondheimensis* (AY157239) by 256 nucleotides (15%); and differed from those of *O. glaucostegi* and *Aporocotylidae* sp. NSW1 by 371 (32%) and 349 (30%)

nucleotides, respectively. The recovered 28S phylogeny (Fig. 11) placed the new species within a clade that included sequences from two adult blood flukes (*C. trondheimensis* and *O. glaucostegi*) infecting chondrichthyans as well as that of a cercaria extracted from a bivalve. Expectedly, the BI tree recovered four clades comprising the blood flukes of marine actinopterygians, euryhaline elopomorphs, freshwater cercariae, and marine chondrichthyans.

Key for identification of fish blood flukes (Aporocotylidae) infecting chondrichthyans

1. Body spinous, lateral tegumental spines (LTSs) C-shaped, each on a muscular peduncle.....2
- Body aspinous.....3
2. Intestinal caeca X-shaped, oviducal ampulla absent.....5
- Intestinal caeca inverse U-shaped, oviducal ampulla present.....6
3. Adults infecting mesenteric vessels; body margin having lateral tubercles.....*Orchispirium heterovitellatum*
- Adults infecting heart; body lacking lateral tubercles.....4
4. Body minute (< 1 mm long), testis curving < 15 times.....*Myliobaticola richardheardi*

- Body extremely elongated (≥ 4 mm long), testis curving > 15 times.....*Ogawaia glaucostegi*
5. Body minute (< 1.4 mm long), LTSs numbering > 170 per side of body.....*Selachohemecus olsoni*
- Body large (≥ 1.4 mm long), LTSs < 100 per side of body.....*Selachohemecus benzi*
6. LTSs distributed in a single column.....*Gymnurahemecus bulbosus*
- LTSs distributed in multiple columns or lateral field.....7
7. Caeca short, terminating at level of genital pores.....*Chimaerohemecus trondheimensis*
- Caeca elongate, extending to near posterior body end, terminating posterior to genitalia.....8
8. Body 2 \times longer than wide, mid-body LTSs < 20 μ m long.....*Hyperandrotrema cetorhini*
- Body 7–8 \times longer than wide, mid-body LTSs ≥ 25 μ m long.....*Hyperandrotrema walterboegeri*

Taxonomic remarks

The new genus is most similar to *Hyperandrotrema* (two species, both infecting lamniform sharks) and *Chimaerohemecus* (monotypic, infecting chimaeras) by the combination of having a minute, diminutive, aspinous anterior sucker that is not demarcated from the body, C-shaped lateral

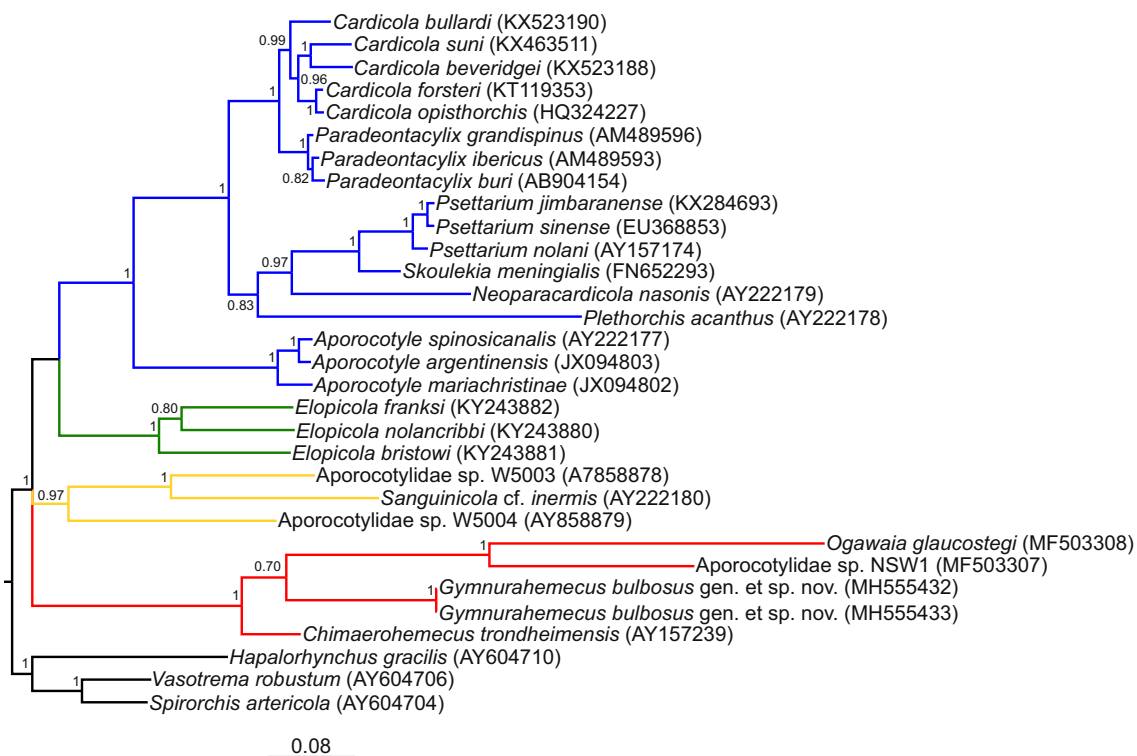


Fig. 11 Phylogenetic relationships of blood flukes reconstructed using Bayesian inference with the large subunit ribosomal DNA (28S) gene. Clades are indicated by colors: blue = marine actinopterygians, green = euryhaline elopomorphs, yellow = freshwater cercariae, red = marine

chondrichthyans, and black = turtle blood fluke outgroup. Numbers aside tree nodes indicate posterior probability. Scale bar is in substitutions per site

tegumental spines each associated with a muscular peduncle, intestinal caeca that are inverse U-shaped (lacking anterior caeca) and that lack diverticulae, an internal seminal vesicle and cirrus sac, an oviducal ampulla, a Laurer's canal, an oötype that is posterior to the remainder of the genitalia, a dextral vitelline duct (posteriorly asymmetrical vitellarium), and a common genital pore (van der Land 1967; Maillard and Ktari 1978; Oréllis-Ribeiro et al. 2014). The new genus can be easily differentiated from *Chimaerohemecus* and *Hyperandrotrema* by the combination of having a single column of lateral tegumental spines, a medial oesophageal bulb anterior to a diverticulate region of the oesophagus, intestinal caeca that terminate in the middle 1/3 of the body, a sinistral, post-caecal, post-testicular ovary, and a post-caecal common genital pore. This combination of features differentiates the new genus, *Chimaerohemecus*, and *Hyperandrotrema* from the remaining chondrichthyan blood fluke genera, i.e., *Selachohemecus* (comprised of two species, both infecting carcharhiniform sharks) (Short 1954; Bullard et al. 2006) plus *Orchispirium*, *Myliobaticola*, and *Ogawaia* (each monotypic, infecting rays) (Madhavi and Hanumantha Rao 1970; Bullard and Jensen 2008; Cutmore et al. 2018). Further, these features distinguish the new genus and its relatives infecting chondrichthyans from all genera of blood flukes that infect ray-finned fishes (Actinopterygii). *Selachohemecus* is unique among chondrichthyan blood fluke genera by having X-shaped intestinal caeca and an oötype at level of the seminal vesicle as well as by lacking an oviducal ampulla (the oviducal seminal receptacle in *S. olsoni* and *S. benzi* comprises a swelling of the proximal portion of the oviduct) and Laurer's canal. *Orchispirium*, *Myliobaticola*, and *Ogawaia* are distinct by having a looped testis and a post-caecal common genital pore as well as lacking lateral tegumental spines, an oviducal ampulla (*Orchispirium* and *Myliobaticola* have an oviducal seminal receptacle comprising a swelling of the proximal portion of the oviduct), and a Laurer's canal.

Discussion

Because chondrichthyans are the earliest branching, extant lineage of jawed vertebrate (Craniata: Gnathostomata), their blood flukes comprise an obvious and requisite group with which to test hypotheses concerning fish blood fluke monophyly and blood fluke-craniate cophyly. This area of research has progressed considerably since the late 1990s, when it was accepted that the blood flukes of fishes exhibited no detectable level of phylogenetic host specificity (Smith 1997a). The present study, comprising only the 2nd and 3rd published elasmobranch and chondrichthyan blood fluke 28S sequences (Cribb et al. 2017), respectively, provides further evidence of a monophyletic chondrichthyan blood fluke lineage. This result agrees with and extends previous morphology- and/or

nucleotide-based phylogenetic assertions that the blood flukes of early-branching jawed craniates are monophyletic and phylogenetically distant from the blood flukes of ray-finned fishes (Actinopterygii: Euteleostei) (Bullard et al. 2006, 2008; Bullard and Jensen 2008; Cribb et al. 2017; Oréllis-Ribeiro et al. 2013, 2014, 2017). Clearly, the chondrichthyan blood flukes share morphological features that set them apart as unique and differentiate them from other blood flukes infecting ray-finned fishes, turtles, crocodiles, birds, and mammals (present study; Bullard et al. 2006; Oréllis-Ribeiro et al. 2013). However, relatively low taxon sampling for chondrichthyan blood flukes used in molecular phylogenetic studies limits our ability to thoroughly test these patterns. Further, no life cycle for a chondrichthyan blood fluke is known. *Aporocotylidae* sp. NSW1 is a cercarial sequence, and the definitive host for that taxon is indeterminate; however, Cribb et al. (2017) assumed that it matured in an elasmobranch because it claded with *O. glaucostegi*, and that clade is sister to *C. trondheimensis* (Cutmore et al. 2018).

Although it has been extensively demonstrated that morphologically similar fish blood flukes mature in phylogenetically related craniates (Bullard et al. 2006, 2008; Bullard and Jensen 2008; Oréllis-Ribeiro et al. 2013, 2014, 2017), no molecular phylogenetic evidence definitively supports a strict cophyly hypothesis for the blood flukes of craniates (Oréllis-Ribeiro et al. 2017). For example, existing blood fluke phylogenies, including ours herein, fail to recover a topology reflective of the current deep classification system for the subphylum Craniata (see Nelson et al. 2016), i.e., the blood flukes of cartilaginous fishes are monophyletic and sister to all remaining craniate blood flukes. Instead, the recovered phylogenies generally show a monophyletic *Aporocotylidae*, which is sister to the paraphyletic turtle blood flukes ("Spirorchhiidae") and monophyletic Schistosomatidae (Fig. 11) (Oréllis-Ribeiro et al. 2014; Cribb et al. 2017). Again, low taxon sampling among the blood flukes of early-branching craniates is a contributing factor to this conundrum, and this lack of information underscores the importance of robust taxonomic descriptions coupled with molecular sequence data from chondrichthyan blood flukes. Sequences from blood flukes infecting chondrichthyans (especially sharks), early-branching actinopterygians (Acipenseriformes [sturgeons and paddlefishes], Polypteriformes [bichirs], Amiiformes [bowfin], Lepisosteiformes [gars]), and early-branching members of Teleostomorpha (see Nelson et al. 2016) could result in a topology that mirrors that of craniates (Chondrichthyes sister to Actinopterygii + Sarcopterygii). Obviously, obtaining a blood fluke from a hagfish (Myxiniiformes) or a lamprey (Petromyzontiformes) would be a breakthrough in this regard.

The recovered 28S phylogeny (Fig. 11) indicates four fish blood fluke lineages corresponding to the phylogenetic and ecological affiliation of their definitive hosts: marine

actinopterygians, euryhaline elopomorphs, freshwater cercariae, and marine chondrichthyans. Cribb et al. (2017) recovered the same clades and discussed them; however, they recovered the blood flukes infecting rays and chimaeras as sister to those infecting freshwater actinopterygians. Based on only a few life cycles from marine and freshwater fish blood flukes, Oréllis-Ribeiro et al. (2014) suggested a dichotomy between marine and freshwater fish blood flukes that infect polychaetes and bivalves vs. those that infect snails, respectively. Doubtless, previous speculation on the evolutionary history of the blood flukes is wanting for information about their life cycles. Although several workers have described cercariae morphologically ascribed to the fish blood flukes (Smith 1997b), *Aporocotylidae* sp. NSW1 (MF503307) is the only published cercarial sequence that clades with adult blood flukes infecting chondrichthyans (Cribb et al. 2017). Although no life cycle for a chondrichthyan aporocotylid is known and despite not knowing the identity of the definitive host for the aforementioned cercaria, Cribb et al. (2017) speculated that all elasmobranch blood flukes utilize a bivalve intermediate host rather than a gastropod or polychaete (this assumes also that all blood flukes of marine ray-finned fishes utilize polychaetes as exemplified so far by *Aporocotyle simplex* Odhner, 1900 [see Køie 1982]; *Cardicola forsteri* Cribb et al., 2000 [see Cribb et al. 2011]; *Cardicola parvus* Bullard et al., 2012 [see Bullard et al. 2012; Siegal et al. 2018]; and *Cardicola laruei* Short, 1953 [see McVay et al. 2011; Siegal et al. 2018] only). They predicted that this could explain the phylogenetic separation between the blood flukes that mature in chondrichthyans and those of actinopterygians.

Regarding hosts and blood fluke ancestry, within the marine actinopterygian clade (Fig. 11), it is noteworthy that *Elopicola* spp. are sister to all of the blood flukes infecting the remaining marine actinopterygians. The hosts for *Elopicola* spp. comprise euryhaline fishes, ladyfishes (*Elops* spp.: Elopidae), and tarpons (*Megalops* spp.: Megalopidae) (Oréllis-Ribeiro et al. 2017) that spend considerable periods of their life in freshwater or low salinity estuarine waters (Zale and Merrifield 1989; Adams and Cooke 2015). Bullard (2014) documented eggs and schistosomula larvae of *Elopicola nolancribbi* Bullard, 2014 in the gill epithelium and blood of juvenile ladyfish (*Elops saurus* Linnaeus) in a northern Gulf of Mexico estuary; suggesting that the life cycle was being completed in that estuary or a nearby river(s). Species of *Elopicola* Bullard, 2014 and *Paracardicoloides* Martin, 1974 infect fishes with a leptocephalus larva (Euteleostei: Elopomorpha), are morphologically similar, and are sister taxa based on ITS2 sequence data (Bullard 2014; Oréllis-Ribeiro et al. 2017); all of which strongly suggest cophyly among elopomorphs and their blood flukes. Nolan and Cribb (2004) demonstrated the life cycle of

P. yamagutii, which infects a riverine freshwater snail (*Posticobia brazieri* [Smith] [Littorinimorpha: Tateidae]) and matures in the Australian long-finned eel, *Anguilla reinhardtii* Steindachner. The assertion that related blood flukes infect related intermediate hosts predicts that *Elopicola* spp. (like the related *P. yamagutii*) use gastropods rather than bivalves or polychaetes. Cercariae ascribed to the fish blood flukes by molecular barcoding and phylogenetic inference (i.e., *Aporocotylidae* sp. W5003, *Sanguincola* cf. *inermis*, and *Aporocotylidae* sp. W5004; see Cribb et al. 2017) also infect gastropods but are distantly related to *Elopicola* spp. Hence, Cribb et al.'s (2017) tree and ours herein (if *Elopicola* spp. have a gastropod intermediate host) suggest that gastropod blood flukes are paraphyletic.

We do not know the intermediate host for *G. bulbosus*, but based upon Cribb et al.'s (2017) prediction, it should be an estuarine or littoral bivalve. Scant information is available on the life histories of the smooth butterfly rays throughout their range; however, this fish is considered to be a relatively non-vagile resident of estuaries, with females producing offspring year-round (Yokota and Lessa 2006; Yokota et al. 2012). Given the residency of this elasmobranch, the host is likely littoral or estuarine. Examinations of estuarine invertebrates in the northern Gulf of Mexico continue (MBW) in an attempt to isolate infected intermediate hosts, especially bivalves.

Phylogenetic analyses involving the chimaera blood fluke *C. trondheimensis* and the ray blood flukes consistently produce long branches (Bullard et al. 2008; Bray et al. 2012; Oréllis-Ribeiro et al. 2013, 2014, 2017; Santoro et al. 2015; Yong et al. 2016a, b, 2018; Cribb et al. 2017; Siegal et al. 2018). We speculate that the genetic differences that produce these long branches are indicative of a long phylogenetic separation from other blood flukes and that morphological distinctness is reflected by genetic distinctness. The inferred long branches could also be a result of limitations to taxon sampling associated with extinction in chimaera and ray blood flukes (and their hosts). Furthermore, unresolved, deep relationships among chondrichthyan and ray-finned fish blood flukes (Fig. 11) are likely a result of long branch artifacts. Greater taxon and character sampling will ultimately be needed to resolve relationships among these major lineages.

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Compliance with ethical standards

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

Conflict of interest The authors declare that they have no conflict of interest.

Disclaimer The findings and conclusions in this article are those of the authors and do not necessarily represent the views of the US Fish and Wildlife Service.

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