TWO NEW SPECIES OF *ELOPICOLA* (DIGENEA: APOROCOTYLIDAE) FROM HAWAIIAN LADYFISH, *ELOPS HAWAIENSIS* (EASTERN SEA) AND ATLANTIC TARPON, *MEGALOPS ATLANTICUS* (GULF OF MEXICO) WITH A COMMENT ON MONOPHYLY OF ELOPOMORPH BLOOD FLUKES

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ABSTRACT

Elopicola bristowi sp. n. infects the blood vascular system of Hawaiian ladyfish, *Elops hawaiiensis*, in the Eastern Sea. It differs from the only nominal congener *Elopicola nolancribbi* by the combination of having rows of ventrolateral tegumental spines, a proportionally long oesophagus, anterior caeca, vasa efferentia coalescing ventral to the posterodextral margin of the testis, a post-testicular metraterm, a dextral common genital pore lateral to the oötype, and genitalia that are enantiomorphic relative to those of *E. nolancribbi*. *Elopicola franksi* sp. n. infects the heart and blood vascular system of Atlantic tarpon, *Megalops atlanticus*, in the Gulf of Mexico. It differs from its congeners by the combination of lacking ventrolateral tegumental spines and having an elongate body (6× longer than wide), a proportionally long oesophagus, a compact testis at level of the distal ends of the posterior caeca, and a post-testicular common genital pore at level of the oötype. Phylogenetic analyses based on the small subunit ribosomal DNA (18S), large subunit ribosomal DNA (28S), and internal transcribed spacer 2 (ITS2) genes revealed considerable genetic differences between these taxa. The 18S+28S tree showed a monophyletic *Elopicola* sister to all aporocotylids infecting fishes of Euteleostomorpha. The ITS2 tree showed *Paracardicoloides yamagutii* as the sister taxon to *Elopicola* spp.

Keywords: Taxonomy, systematics, fish blood flukes, cophyly, evolution

1. Introduction

At present, only five fish blood flukes (Aporocotylidae) reportedly infect early-branching ray-finned fishes (Actinopterygii) (Table 1). This knowledge gap hampers understanding broad scale evolutionary relationships among blood flukes (Schistosomatoidea) (Orélis-Ribeiro et al. [5]). Herein, we use morphology and molecular sequence data (18S, ITS2, and 28S rDNA) to describe and classify two new species of *Elopicola* Bullard, 2014, emend the diagnosis of that genus, and test monophyly of the blood flukes that infect tarpons and ladyfishes (Elopiformes).

2. Materials and methods

2.1. Specimen collection and preparation

A collection of Hawaiian ladyfish, *Elops hawaiiensis*, (standard length [SL] 300–750 mm) gill-netted from littoral waters of the Eastern Sea off Nha Trang, Vietnam, were purchased from the Vĩnh Hải Fish Market (12°16'44.8"N; 109°11'38.9"E) and necropsied during June 2015. To

prioritize infected vs. putatively non-infected hosts, several excised gill filaments from each fish were first examined as wet mounts and with the aid of a compound light microscope to detect aporocotylid eggs. Fish infected with aporocotylid eggs in gill epithelium were prioritized for immediate dissection to attempt collection of live or fresh-dead flukes. Heart, gill, and viscera were excised, isolated in separate dishes filled with saline, and examined with a dissecting microscope. After that initial examination, the fluid remaining in each dish was transferred to a settling column filled with fresh saline, left for 15 min, decanted, diluted again with fresh saline, allowed to settle for 15 min, and re-examined. Flukes intended as whole-mounts were transferred to a vial of 5-10% neutral buffered formalin (n.b.f.), and those for DNA analysis were preserved in 100% EtOH and stored at -20°C . Two dead Atlantic tarpon (SL 698 mm and 558 mm) were opportunistically sampled on 13 January 2010, after apparently succumbing to cold shock, in Bayboro Harbor, Florida, a boat basin of Tampa Bay ($27^{\circ} 45'41.41''\text{N}$; $82^{\circ}37'55.37''\text{W}$). A third Atlantic tarpon (total length [TL] 1,700 mm) was killed unintentionally by an angler on 19 June 2014 off North Captiva Island (near $26^{\circ}35'46''\text{N}$ $82^{\circ}13'44''\text{W}$; Gulf of Mexico), Florida, and submitted for examination to the Florida Fish and Wildlife Conservation Commission's Fish and Wildlife Research Institute (FWRI). Three additional Atlantic tarpon (SL 359–591 mm) were collected by FWRI fisheries independent monitoring field staff using a seine net on 29 July 2014 from a coastal retention pond in Tampa Bay, but no blood fluke was collected from these fish. For all examined Atlantic tarpon, hearts were excised, macerated and rinsed in saline, examined in a dish using a dissecting microscope, and processed in settling columns as indicated above for Hawaiian ladyfish. Flukes intended as whole mounts were placed in 5% n.b.f. One damaged specimen was cut in half and allocated to 95% EtOH and RNAlater.

Specimens for morphology were rinsed thoroughly with distilled water and cleaned with fine brushes to remove host tissue debris, stained overnight in Van Cleave's hematoxylin with several additional drops of Ehrlich's hematoxylin, made basic in 70% ethanol with lithium carbonate and butyl-amine, dehydrated, cleared in clove oil, and permanently mounted in Canada balsam. Illustrations of stained, whole-mounted specimens were made with the aid of a Leica DM-2500 (Leica, Wetzlar, Germany) equipped with differential interference contrast (DIC) optical components and a drawing tube. Measurements were obtained with a calibrated ocular micrometer (as straight-lines along the course of each duct) and are herein reported in micrometers (μm) followed by their mean and the number measured in parentheses. Scientific names including taxonomic authorities and dates for fishes follow Eschmeyer et al. [6]. Common names are taken from FishBase (Froese and Pauly [7]). Nomenclature for Aporocotylidae follows Bullard et al. [8]. Type and voucher materials are deposited in the United States National Museum (USNM, Washington, D.C.).

2.2. DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from two specimens of the new species from Hawaiian ladyfish and a specimen of the new species from Atlantic tarpon plus one specimen each of *A. petersoni*, *E. nolancribbi*, and the turtle blood fluke *Baracktrema obamai* Roberts, Platt, and Bullard, 2016 (Table 2) using DNeasy™ Blood and Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer-recommended protocol, except that the incubation period with proteinase-K was extended overnight and only 100 µl of elution buffer was used in the final elution step to increase the final DNA concentration in the eluate. Extracted DNA was for amplification of 18S, 28S, and ITS2 rDNA gene fragments using the set of primers described in Table 3. PCR amplifications were carried out in a total volume of 25 µl of a solution containing 2 µl of DNA template, 0.4 µM of each primer along with 1× buffer, 2.5 mM MgCl₂ (New England Biolabs, Ipswich, MA), 1 mM dNTP mixture, and 0.3 µl *Taq* polymerase (5 U/µl) (New England Biolabs, Ipswich, MA). The 18S and 28S rDNA amplification reactions were performed with a cycling profile of 4 min at 94°C for initial denaturation, followed by 40 repeating cycles of 94°C for 40 s for denaturation, 50°C for 30 s for annealing, and 72°C for 2 min for extension, followed by a final 7 min at 72°C for extension. The ITS2 rDNA amplification reactions were performed with a cycling profile of 4 min at 94°C for initial denaturation, followed by 40 repeating cycles of 94°C for 40 s for denaturation, 50°C for 30 s for annealing, and 72°C for 1 min for extension, followed by a final 5 min at 72°C for extension. All PCR reactions were performed in a Veriti Thermal Cycler (Applied Biosystems). PCR products (5 µl) were verified on a 1% agarose gel and stained with ethidium bromide. PCR amplicons were gel-excised using QIAquick™ Gel Extraction Kit (QIAGEN) following the manufacturer's protocol. Bidirectional DNA sequencing was performed by GENEWIZ with ABI Prism 3730xl DNA analyzer (GENEWIZ, Inc., South Plainfield, NJ). 18S, ITS2, and 28S genes were sequenced using PCR primers and some additional internal forward primers listed in Table 3. Sequence assembling and editing were conducted using BioNumerics version 7.0 (Applied Maths, Sint-Martens-Latem, Belgium). GenBank accession numbers are given in Table 3. A hologenophore (USNM 1422064) (sensu Pleijel et al. [21]) of the new species from Hawaiian ladyfish is deposited in the United States National Museum (USNM, Washington, D.C.).

2.3. Sequence alignments and phylogenetic analysis

Two datasets were assembled for phylogenetic analyses. To test monophyly of aporocotyliids from early-branching lineages of ray-finned fishes and their relationships with turtle blood flukes, the first dataset (hereafter referred as “18S+28S dataset”) comprised a combined matrix of

near-complete 18S and partial 28S sequences derived from the newly sequenced taxa plus publicly available data comprising a holocephalan blood fluke, a putative cypriniform blood fluke (see discussion about the identity of this sequence in Orélis-Ribeiro et al. [5]), and marine euteleost blood flukes. Outgroup selection for this dataset was based on the phylogeny of Digenea published by Olson et al. [11] and included representatives of the superfamily Diplostomoidea. The second dataset (hereafter referred to as “ITS2 dataset”), which excludes the new species from Atlantic tarpon, tested monophyly of elopomorph blood flukes and comprised complete ITS2 sequences derived from the newly sequenced taxa plus *P. yamagutii*. The outgroup for this dataset comprised publicly available sequences of fish blood flukes (Table 2).

For each dataset, sequences were aligned using MAFFT (Katoh and Toh [22]) with default settings implemented in the CIPRES Science Gateway V. 3.3 (Miller et al. [23]). The resulting alignment was refined by eye using MEGA version 5.2.2 (Tamura et al. [24]) and ends of each fragment were trimmed to match the shortest sequence. The concatenated alignment (18S–28S) was created using the web application FaBox 1.35 (Villesen [25]). Ambiguous positions in single gene alignments were identified and removed using the Gblock server (Castresana [26]) with settings for a less stringent selection. Bayesian inference (BI) was performed using the Metropolis-coupled Markov chain Monte Carlo method (MC³) in MrBayes version 3.2.6 (Huelsenbeck [27], Ronquist and Huelsenbeck [28], Huelsenbeck and Ronquist [29]) and run on CIPRES (Miller et al. [23]). Model of evolution was selected based on the Akaike Information Criterion (Posada and Buckley [30]) as implemented in the jModelTest version 2.1.4 (Darriba et al. [31]; Guindon and Gascuel [32]). The GTR + I + G (proportion of invariable sites = 0.430 and gamma distribution = 0.849) and the TVM+ G (gamma distribution = 0.743) models were inferred as the best estimator for 18S+28S dataset and ITS2 dataset, respectively. Therefore, BI for 18S+28S dataset used the following parameters: nst = 6, rates = invgamma, ngammat = 4, and default priors; and for the ITS2 dataset the following parameters: nst = 6, rates = gamma, ngammat = 4, and default priors. Analyses were run in duplicate each containing four independent chains (three heated and one cold chain) (nchains = 4) for 1.0×10^7 generations (ngen = 10,000,000) sampled at intervals of 1,000 generations (samplefreq = 1,000). Results of the first 2,500 sampled trees were discarded as burn-in based on the stationarity of the likelihood values, assessed by plotting the log-likelihood values of the sample points against generation time using Tracer version 1.5 (Rambaut and Drummond [33]). All retained trees were used to estimate posterior probability of each node. A majority rule consensus tree with average branch lengths was constructed for the remaining trees using ‘summarize the trees’ (sumt) in MrBayes. Additionally, a maximum likelihood (ML) analysis was performed on both datasets in RAxML v.7.2.6 (Stamatakis [34]) and also performed on CIPRES (Miller et al. [23]), with default

parameters. GTRGAMMA model was employed for both datasets. Bootstrap values were estimated from 1,000 replicates. Resulting phylogenetic trees were visualized using FigTree v1.4.2 (Rambaut [33]) and further edited with Adobe Illustrator CS3 (Adobe Systems). Branch supports for BI and ML analyses were considered as significant when posterior probabilities were >0.95 and bootstrap values were >70%, respectively.

3. Results

3.1. *Elopicola* Bullard, 2014, emended

3.1.1. *Diagnosis*

Body flat, oval in outline, ventrally concave, lacking posterolateral body protuberance, anterior and posterior ends tapering equally; ventrolateral tegumental body spines present or absent in adult, present in juvenile and schistosomulum, minute, straight (lacking recurved tip), distributing in ventrolateral transverse rows or not, enveloped by tegument. Rosethorn-shaped spines absent. Anterior sucker bowl-shaped (not a spinous, spheroid anterior sucker with medioventral mouth), demarcated from anterior body end by peduncle, aspinous in adult. Muscular pharynx occupying space between anterior sucker and nerve commissure. Oesophagus terminating in middle third of body. Intestine inverse U-shaped or having anterior caeca; posterior caeca lacking diverticula, terminating in middle third or posterior third of body. Testis single, medial, intercaecal, pre-ovarian. Auxiliary external seminal vesicle absent. Cirrus sac dextral or sinistral, pre-ovarian, enclosing internal seminal vesicle and cirrus; cirrus everting dorsally near midline or dextrally, post-caecal. Ovary single, medial, occupying posterior body extremity, immediately anterior to excretory vesicle. Oviduct post-caecal, extending anteriorly from ovary; oviducal seminal receptacle present. Primary vitelline duct extending posteriorly near dextral or sinistral body margin or slightly ventral to testis, curving medially before uniting with oviduct to form ovo-vitelline duct. Ovo-vitelline duct short, transverse, connecting with oötype laterally. Oötype post-testicular, pre-ovarian. Uterus comprising short ascending and descending portions, pre-ovarian, post-caecal; eggs in gill of fish host tetrahedral, with elongate polar filaments. Metraterm post-caecal, pre-ovarian, medial to cirrus sac. Male and female reproductive tracts opening into common atrium; common genital pore dorsal, dextral or sinistral, post-caecal. Excretory vesicle prominent posteriorly, Y-shaped; excretory pore dorsal, subterminal. Adults infecting fishes of Elopiformes.

3.1.2. *Differential diagnosis*

Ventrolateral tegumental body spines straight if present. Anterior sucker bowl-shaped. Pharynx present. Oesophagus terminating in middle third of body. Intestine inverse U-shaped or

having anterior caeca; posterior caeca lacking diverticula, terminating in middle third or posterior third of body. Testis single, medial, intercaecal, pre-ovarian. Ovary immediately anterior to excretory vesicle. Oviduct post-caecal, extending anteriad. Oviducal seminal receptacle present. Oötype pre-ovarian. Uterus and metraterm post-caecal, pre-ovarian, medial to cirrus sac. Common genital pore dorsal, post-caecal. Excretory vesicle prominent, Y-shaped.

Type species: Elopicola nolancribbi Bullard, 2014

3.1.3. Remarks

Elopicola Bullard 2014 resembles monotypic *Paracardicoloides* Martin, 1974 and *Acipensericola* Bullard, Jensen, Snyder, and Overstreet, 2008 by having a bowl-shaped anterior sucker and straight ventrolateral tegumental body spines (Martin [1]; Bullard et al. [2]). This combination of features differentiates these three genera from all other accepted aporocotylid genera. The excretory vesicle in species of these genera is massive and Y-shaped, which may also prove a useful synapomorphy for these genera; however, too few descriptions of the excretory system of other adult aporocotylids exist to draw a conclusion in that regard. *Elopicola* differs from *Paracardicoloides* by having a pharynx, a single testis, an ovary that is immediately anterior to the excretory vesicle (posterior to all other reproductive structures), a post-caecal uterus and metraterm, and a post-testicular oötype (Bullard [3]; present study).

Paracardicoloides lacks a pharynx and has an anterior testis and a posterior testis, which flank the ovary, a partly intercaecal and pre-testicular uterus, and a pre-testicular oötype (Bullard [3]). *Acipensericola* is most easily distinguished from *Elopicola* and *Paracardicoloides* by having a column of intercaecal testes and a Laurer's canal, among other distinguishing features (Bullard et al. [2]).

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

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

Body 1,268–1,868 (1,632; 11) long, 318–525 (419; 11) wide, 3–5× (4; 11) longer than wide, with maximum width near midbody (Figs. 1, 2, 4). Ventrolateral tegumental body spines 10 long, 3 wide or approximately 3× longer than wide, numbering 496–524 (508; 4) spines per side of body or a total of 1,012–1,020 (1,016; 2) spines, in transverse rows for entire body length (BL) from base of anterior sucker to posterior body end, not confluent posteriorly, with numerous spines seemingly detached; rows numbering 201–221 (211; 4) or a total of 410–433 (422; 2) rows, spaced 6–9 (8; 4) apart, comprising 1–2 (2; 4), 4 (4; 4), and 2 (2; 4) spines per row in anterior, middle, and posterior portions of body, respectively; row breadth 3–8 (6; 4), 25–29 (27; 4), and 6–9 (8; 4), respectively (Figs. 2, 3). Dorsolateral nerve cords difficult to trace for most of BL, 4–10 (7; 10) wide near mid-body at widest level, 45–58 (52; 10) or 10–17% (13%; 10) of

body width (BW) from body margin at mid-body, becoming confluent with paired cord 35–63 (50; 7) or 2–5% (3%; 7) of BL from posterior body end; anterior commissure 140–220 (177; 10) or 9–13% (11%; 10) of BL from anterior body end, 83–163 (102; 10) or 19–31% (24%; 10) of BW across width of worm, 3–13 (8; 10) wide, perpendicular to long axis of body, coursing dorsal to posterior end of pharynx (Figs. 1, 2, 4). Anterior sucker 53–68 (61; 11) in diameter or 10–20% (15%; 11) of BW, strongly muscular, extending anteriad 28–55 (44; 11) or 2–3% (3%; 9) of BL or 51–94% (71%; 9) of anterior sucker diameter; mouth 5–10 (7; 11) in diameter, centered on anterior sucker, 28–43 (34; 11) or 1–3% (2%; 11) of anterior sucker length from anterior end of body, surrounded by muscular rim of sucker (Figs. 1, 2, 4). Oesophagus 600–818 (707; 11) long or 41–47% (43%; 11) of BL (Figs. 1, 2, 4); pharynx not extending laterally beyond nerve cord, between level of mouth and anterior nerve commissure, 63–93 (80; 11) long or 10–15% (12%; 11) of oesophagus length or 4–6% (5%; 11) of BL, 30–55 wide (42; 11) or 7–13% (10%; 11) of BW, 1–3× (2; 11) longer than wide (Figs. 1, 2, 4); middle portion of oesophagus sinuous, thick-walled, 440–633 (536; 11) long or 65–83% (76%; 11) of oesophagus length or 27–36% (33%; 11) of BL, 28–63 (45; 11) wide, with wall 8–13 (10; 11) thick or 16–37% (23%; 11) of middle portion of oesophagus width; posterior oesophageal swelling thick-walled, separated from medial portion of oesophagus by a constriction, immediately anterior to caecal bifurcation, 50–105 (85; 11) long or 7–14% (12%; 11) of oesophagus length or 3–7% (5%; 11) of BL, 30–80 (51; 11) wide, 7–19% (12%; 11) of BW, 1–2× (2; 11) longer than wide, with wall 3–8 (5; 11) thick or 5–21% (10%; 11) of posterior oesophageal swelling width (Figs. 1, 2, 4). Oesophageal gland enveloping oesophagus from level of pharynx to caecal bifurcation, lacking birefringent rodlet-like structures, concentrating in an area surrounding posterior oesophageal swelling, 55–160 (107; 10) long or 8–21% (15%; 10) of oesophagus length, 65–123 (87; 10) wide or 1× (1; 10) wider than long. Caecal bifurcation immediately posterior to posterior oesophageal swelling, 688–850 (734; 11) or 42–55% (45%; 11) of BL from anterior body end; anterior caeca directing anteriad, 50–90 (68; 20) long or 8–13% (10%; 10) of oesophagus length, 25–83 (38; 20) wide or 6–16% (9%; 20) of BW, posterior caeca slightly sinuous, containing brownish or yellowish contents; dextral and sinistral posterior caeca asymmetrical, 380–528 (461; 11) and 353–565 (485; 11) long, respectively, extending posteriad 355–530 (461; 22) or 24–32% (28%; 22) of BL or 56–76% (65%; 22) of oesophagus length, 20–60 (40; 22) wide, ending 345–540 (456; 22) or 25–32% (28%; 22) of BL from posterior body end (Figs. 1, 2, 4).

Testis 205–368 (313; 11) long or 14–23% (19%; 11) of BL, 133–255 (191; 11) wide or 37–58% (46%; 11) of BW, 1–2× (2; 11) longer than wide, terminating 8–63 (34; 9) or 1–4% (2%; 9) of BL from distal tips of sinistral posterior caecum, 323–460 (407; 11) or 24–27% (25%; 11) of BL from posterior body end; testis lobes 25–95 (46; 36) long, 25–60 (41; 36) wide or 1–3× (1; 36) longer than wide; vasa efferentia 5–13 (8; 13) wide; vas deferens 23–75 (45; 10) long or 1–

4% (3%; 10) of BL (Figs. 1, 2, 4, 7, 8). Cirrus sac beginning ventral to posterior margin of testis 378–575 (495; 11) or 27–33% (30%; 11) of BL from posterior body end, 78–178 (145; 11) long, 28–63 (46; 10) wide or 3–5× (3; 10) longer than wide, with wall 2–3 (2; 11) thick, terminating 23–73 (55; 11) or 1–5% (4%; 11) of BL from posterior end of testis, 293–413 (356; 11) or 20–24% (22%; 11) of body length from posterior body end; internal seminal vesicle more or less filling cirrus sac depending on amount of sperm in duct, directing posteriad, 38–158 (109; 11) long or 1–6× (3; 10) vas deferens length, 25–63 (45; 11) wide; cirrus 38–63 (49; 3) long, 25–38 (31; 3) wide (Figs. 1, 2, 4, 7, 8).

Ovary appearing as a loose aggregation of ova, immediately anterior to posterior nerve confluence, 95–183 (144; 11) long or 6–11% (9%; 11) of BL, 95–188 (140; 11) wide or 22–43% (34%; 11) of BW, 1–2× (1; 11) longer than wide; post-ovarian space 93–193 (153; 11) long or 6–12% (9%; 11) of BL. Oviduct S-shaped, extending anteriad from center of ovary, 183–400 (306; 11) long; oviducal seminal receptacle 95–225 (145; 11) long or 38–56% (48%; 11) of oviduct length, 38–118 (66; 11) wide, 1–4× (2; 11) longer than wide, varying in length and width depending on amount of sperm in duct (Figs. 1, 2, 4, 7, 8). Primary vitelline collecting duct sinistral, extending 188–503 (347; 11) posteriad and coursing between testis and sinistral body margin or dorsal to sinistral margin of testis, 15–30 (22; 10) wide, curving mediad at level of posterior margin of testis, with distal portion extending in parallel with oviducal seminal receptacle before uniting with distal portion of oviduct immediately proximal to oötype (Figs. 1, 2, 4, 7, 8); oötype spheroid, 18–53 (42; 11) or 1–3% (3%; 11) of BL from posterior margin of testis, at approximate level of posterior margin of cirrus sac and oviducal seminal receptacle, 15–28 (22; 11) long, 15–25 (19; 11) wide or 1–2× (1; 11) longer than wide, 285–418 (351; 11) or 19–24% (22%; 11) of BL from posterior body end; Mehlis' gland indistinct in fixed material (Figs. 1, 2, 4, 7, 8). Uterus short relative to many other aporocotylids, C-shaped; ascending uterus 53–88 (71; 11) long, 15–25 (18; 11) wide, 38–77% (51%; 11) of oviduct seminal receptacle length, with cuboidal cells lining lumen, extending anterodextrad from oötype before curving mediad to connect with metraterm near posterior margin of testis (Figs. 1, 2, 4, 7, 8). Metraterm post-testicular, between distal portion of primary vitelline collecting duct and cirrus sac, 95–215 (138; 11) long or 6–12% (9%; 11) of BL, 15–30 (23; 11) wide or 4–9× (6; 11) longer than wide; proximal portion of metraterm transverse, crossing midline; distal portion of metraterm extending posterodextrad, with muscular wall 5–6 (5; 11) thick; metraterm eggs 12–50 (25; 25) long, 4–8 (6; 25) wide (Figs. 1, 2, 4, 7, 8). Common genital pore 20–45 (29; 11) in diameter, opening at level of oötype, 298–415 (359; 11) or 20–24% (22%; 11) of BL from posterior body end, 38–118 (77; 11) or 9–31% (19%; 11) of BL from dextral body margin, 148–308 (222; 11) or 47–59% (53%; 11) of BL from sinistral body margin (Figs. 1, 2, 4, 7, 8).

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

3.2.2. *Diagnosis of juvenile (based on 2 stained, whole-mounted specimens)*

Body 1,450–1,480 (1,465; 2) long, 205–240 (223; 2) wide, 6–7× (7; 2) longer than wide (Fig. 5). Ventrolateral tegumental body spines resembling those of adults; spines 3–5 (4; 10) in maximum length, 2 (2; 10) wide or 2–3× (2; 10) longer than wide, numbering 609–627 (618; 2) per side of body or totaling 1,236 (1,236; 1) spines; rows numbering 269–282 (276; 2) or a total of 551 (551; 1), 5 (5; 2) apart, comprising 1 (1; 2), 3 (3; 2), 2 (2; 2) spines per row in anterior, middle and posterior portions of body, respectively, 2 (2; 2), 10 (10; 2), and 4 (4; 2) in breadth in anterior, middle and posterior portions of body, respectively. Dorsolateral nerve cords difficult to trace for most of BL, 3–5 (4; 2) wide near mid-body at widest level, 43 (43; 2) or 18–21% (19%; 2) of BW from body margin at mid-body, paired, contiguous anteriorly and posteriorly, becoming confluent with paired cord 35 (35; 1) or 2% (2%; 1) of BL from posterior body end; anterior commissure 167–178 (173; 2) or 11–12% (12%; 2) of BL from anterior body end, 55–60 (58; 2) or 25–27% (26%; 2) of BW across width of worm, 5–13 (9; 2) wide (Fig. 5). Anterior sucker 48–58 (53; 2) in diameter or 23–24% (24%; 2) of BW, strongly muscular, extending anteriad 23–25 (24; 2) or 2% (2%; 2) of BL or 38% (38%; 2) of anterior sucker diameter; mouth 3–8 (6; 2) diameter, 23–25 (24; 2) or 2% (2%; 2) of anterior sucker length from anterior end of body (Fig. 5). Oesophagus 608–728 (668; 2) long or 42–49% (46%; 2) of BL, anterior portion of oesophagus comprising a muscular pharynx, 70–80 (75; 2) long or 10–13% (11; 2) of oesophagus total length or 1% (1%; 2) of BL, 38 wide (38; 2) or 16–19% (17%; 2) of BW, 2× (2; 2) longer than wide (Fig. 5); middle portion of oesophagus 458–560 (509; 2) long or 75–77% (76%; 2) of oesophagus total length or 32–38% (35%; 2) of BL, 35–38 (37; 2) wide, with wall 5 (5; 2) thick or 13–14% (14%; 2) of middle portion of oesophagus width; posterior oesophageal swelling 70–98 (84; 11) long or 12–13% (12%; 2) of oesophagus total length or 5–7% (6%; 2) of BL, 40–45 (43; 2) wide, 17–22% (19%; 2) of BW, 2× (2; 2) longer than wide, with wall 13 (13; 2) thick or 29–33% (31%; 2) of posterior oesophageal swelling width (Fig. 6). Oesophageal gland 103–113 (108; 2) long or 14–19% (16%; 2) of oesophagus length, 73–80 (77; 2) wide or 1× (1; 2) wider than long. Caecal bifurcation 390–748 (569; 2) or 27–51% (39%; 2) of BL from anterior body end; anterior caeca directing anteriad 50–73 (60; 4) long or 9% (9%; 2) of oesophagus length, 7–40 (20; 4) wide or 7–15% (11%; 2) of BW; dextral and sinistral posterior caeca asymmetrical, 355–380 (368; 2) and 408–413 (411; 2) long, respectively, extending posteriad 358–410 (386; 4) or 24–31% (27%; 4) of BL or 49–67% (59%; 4) of oesophagus length, 25–45 (35; 4) wide, ending 345–450 (401; 4) or 23–31% (28%; 4) of BL from posterior body end (Fig. 6).

Testis anlage 230–245 (238; 2) long or 16–17% (16%; 2) of BL, 85–88 (87; 2) wide or 37–41% (39%; 2) of BW, 2–3x (2; 2) longer than wide, terminating 10–60 (35; 2) or 1–4% (2%; 2) of BL from caeca or 333–350 (342; 2) or 23–24% (23%; 2) of BL from posterior body end; lobes 13–53 (34; 4) long, 13–23 (19; 4) wide, 1–3x (2; 2) longer than wide; vasa efferentia and vas deferens indistinct. Cirrus sac anlage near level of distal tips of posterior caeca, 393–418 (406; 2) or 27–29% (28%; 2) of BL from posterior body end, 98–130 (114; 2) long, 25–30 (28; 2) wide, 3–5x (4; 2) longer than wide, with wall 3 (3; 2) thick, terminating 23–30 (27; 2) or 2% (2%; 2) of BL from posterior end of testis, terminating 295–335 (315; 2) or 20–23% (22%; 2) of BL from posterior body end (Fig. 5).

Ovary anlage 75–130 (103; 2) long or 5–9% (7%; 2) of BL, 63–90 (77; 2) wide or 31–38% (34%; 2) of maximum BW, 1–2x (1; 2) longer than wide (Fig. 5); post-ovarian space 130–193 (162; 2) long or 9–13% (11%; 2) of BL. Oviduct anlage 135–170 (153; 2) long; oviducal seminal receptacle anlage 68–70 (69; 2) long or 41–50% (46%; 2) of oviduct length, 13–35 (24; 2) wide, 2–5x (4; 2) longer than wide (Fig. 5). Laurer's canal, primary vitelline collecting duct, and Mehlis' gland indistinct. Oötype anlage 15–18 (17; 2) long, 10–15 (13; 2) wide or 1–2x (1; 2) longer than wide, 23–48 (36; 2) or 2–3% (2%; 2) of BL from posterior margin of testis, 275–315 (295; 2) or 19–22% (20%; 2) of BL from posterior body end. Uterus anlage 65–78 (72; 2) long, 15–18 (17; 2) wide, 93–115% (104%; 2) of oviducal seminal receptacle length, with cuboidal cells lining lumen (Fig. 5). Metraterm filled with basophilic cells, 40 (40; 2) long or 3% (3%; 2) of BL, 13–15 (14; 2) wide, 3x (3; 2) longer than wide, with wall 3 (3; 2) thick. Common genital pore 10–20 (15; 2) in diameter, 293–323 (308; 2) or 20–22% (21%; 2) of BL from posterior body end, 40–50 (45; 2) or 17–24% (21%; 2) of BL from dextral body margin, 100–150 (125; 2) or 49–63% (56%; 2) of BL from sinistral body margin (Fig. 5).

Excretory vesicle 18–25 (22; 2) long or 1–2% (2%; 2) of BL, 28–33 (31; 2) wide; arms of excretory vesicle each 123–138 (129; 4) long, 18–30 (23; 4) wide (Fig. 5).

3.2.3. *Diagnosis of schistosomulum (based on 2 stained, whole-mounted specimens)*

Body 1,150–1,293 (1,222; 2) long, 213–220 (217; 2) wide or 5–6x (6; 2) longer than wide, aspinous (Fig. 6). Anterior sucker 48–51 (50; 2) in diameter, 23% (23%; 2) of BW, extending anteriad 28 (28; 2) or 2% (2%; 2) of BL or 55–58% (57%; 2) of anterior sucker diameter; mouth 5–8 (7; 2) diameter, 13–28 (21; 2) or 1–2% (2%; 2) of anterior sucker length from anterior end of body (Fig. 6). Oesophagus 565–593 (579; 2) long or 44–52% (48%; 2) BL; pharynx 55–78 (67; 2) long or 10–13 (11%; 2) oesophagus length or 1% (1%; 2) of BL, 30 (30; 2) wide; middle portion of oesophagus 435 (435; 2) long or 73–77% (75%; 2) of oesophagus total length or 34–38% (36%; 2) of BL, 33–35 (34; 2) wide, with wall 5 (5; 2) thick or 14–15% (15%; 2) of middle portion of oesophagus width; posterior oesophageal swelling resembling that of adult specimens, 75–80 (78; 2) long or 13% (13%; 2) of oesophagus length or 6–7% (6%; 2) of BL,

33–40 (37; 2) wide or 15–19% (17%; 2) of BW, 2× (2; 2) longer than wide, with wall 13 (13; 2) thick or 33–39% (36%; 2) of posterior oesophageal swelling width (Fig. 6). Oesophageal gland 90–103 (97; 2) long or 15–18% (17%; 2) of oesophagus length, 70–88 (79; 2) wide or 1× (1; 2) wider than long. Caecal bifurcation 610–623 (617; 2) or 47–54% (51%; 2) of BL from anterior end; anterior caeca 33–48 (41; 4) long or 6–8% (7%; 4) of oesophagus length, 25–38 (32; 4) wide or 14–16% (15%; 4) of BW; dextral and sinistral posterior caeca asymmetrical, 258–343 (301; 2) and 298–338 (318; 2) long respectively, 22–26% (25%; 4) of BL or 43–59% (53%; 4) of oesophagus length, 35–43 (40; 4) wide, ending 158–363 (289; 4) or 14–28% (24%; 4) of BL from posterior body end (Fig. 6).

Testis anlage 198–255 (227; 2) long or 17–20% (18%; 2) of BL, 25–58 (42; 2) wide or 12–26% (19%; 2) of BW, 4–8× (6; 2) longer than wide, terminating 23 (23; 1) or 2% (2%; 1) of BL from distal tips of posterior caeca, 233–338 (286; 2) or 20–26% (23%; 2) of BL from posterior body end (Fig. 6); cirrus anlage filled with basophilic cells, at level of distal tip of dextral posterior cecum, 298–363 (331; 2) or 26–28% (27%; 2) of BL from posterior body end, 70–88 (79; 2) long, 13–20 (17; 2) wide or 4–5× (5; 2) longer than wide, terminating 10–45 (28; 2) or 1–3% (2%; 2) of BL from posterior end of testis, 225–275 (250; 2) or 20–21% (20%; 2) of BL from posterior body end.

Ovarian anlage 43–45 (44; 2) long or 3–4% (4%; 2) of BL, 38–48 (43; 2) wide or 17–23% (20%; 2) of maximum BW, 1–2× (1; 11) longer than wide; post-ovarian space 165–168 (167; 2) long or 13–15% (14%; 2) of BL (Fig. 6). Oviduct, oötype, and uterus indistinct. Common genital pore post-testicular, 10–13 (12; 2) in diameter, opening at level of oötype, 228–280 (254; 2) or 20–22% (21%; 2) of BL from posterior body end, 35 (35; 2) or 16% (16%; 2) of BL from dextral body margin, 103–123 (113; 2) or 48–56% (52%; 2) of BL from sinistral body margin (Fig. 6).

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

3.2.4. Taxonomic summary

Type host: *Elops hawaiiensis* (Reagan, 1909) (Elopomorpha: Elopiformes: Elopidae), Hawaiian ladyfish.

Type locality: Eastern Sea, off Nha Trang, Vietnam.

Site in host: Blood vascular system. Adults and juveniles from wash of heart, gill, branchial sinuses, and viscera; a juvenile infected branchial sinuses and heart, respectively.

Prevalence and intensity of infection by adult and juveniles of E. bristowi: Five of 6 (83%)

Hawaiian ladyfish had 1–19 (mean intensity = 6 ± 0.5) fluke specimens. No adult fluke was collected from the remaining Hawaiian ladyfish examined, despite having aporocotylid eggs in gill epithelium.

Specimens deposited: Holotype (USNM 1422060); Paratypes (USNM 1422063; USNM 1422062; USNM 1422061).

Etymology: The specific epithet honors the late Professor Glenn A. Bristow (Department of Biology, University of Bergen, Bergen, Norway; Institute of Biotechnology and Environment, Nha Trang University, Nha Trang, Vietnam) for his invaluable contributions to parasitology in Vietnam.

3.2.5. Remarks

The new species differs from *E. nolancribbi*, the only nominal congener, by having rows of ventrolateral tegumental spines (in adults and juveniles), a proportionally long oesophagus (41–47% of BL), anterior caeca (in adults, juveniles, and schistosomula), vasa efferentia coalescing ventral to the posterodextral margin of the testis, a post-testicular metraterm, a dextral common genital pore that is lateral to the oötype, and genitalia that are enantiomorphic relative to that of *E. nolancribbi*. For example, *E. bristowi* has a cirrus sac and a primary vitelline duct that are dextral plus female terminal genitalia that are directed dextrad; whereas, *E. nolancribbi* has the opposite: a cirrus sac and a primary vitelline duct that are sinistral plus female terminal genitalia that are directed sinistrad (Figs. 6, 7; see also Figs. 1–12 from Bullard [3]). *Elopicola nolancribbi* lacks tegumental spines (in adults) and has a short oesophagus (<40% of the BL), minute or no discernible anterior caeca, vasa efferentia coalescing in the anterolateral portion of the testis, a metraterm that is medial to the testis, and a sinistral common genital pore that is anterior to the oötype. Because body spines can apparently become detached easily in poorly fixed or degraded aporocotylid specimens (Bullard [35]; McVay et al. [36]), new collections of living specimens comprising *E. nolancribbi* may reveal the presence of spines. Bullard [3] reported that spines were present in the schistosomulum of *E. nolancribbi*.

3.3. *Elopicola franksi* Oréllis-Ribeiro and Bullard sp. n. (Figs. 9–10)

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

Body 1,240–1,395 (1,318; 21) long, 208–215 (212; 2) wide, 6× (6; 2) longer than wide, with maximum width near genitalia (Fig. 9). Ventrolateral tegumental body spines not observed. Dorsolateral nerve cords indistinct. Anterior sucker 40–48 (44; 2) in diameter or 33–38% (36%; 2) of BW, strongly muscular, extending anteriad from anterior end of body 33–38 (36; 2) or 2–3% (3%; 2) of BL or 69–95% (82%; 2) of anterior sucker diameter; mouth opening within anterior sucker, 3–4 (4; 2) diameter, surrounded by muscular rim of sucker (Fig. 9). Oesophagus 608–858 (733; 2) long or 49–62% (55%; 2) of BL, including anterior (muscular pharynx), middle, and posterior (posterior oesophageal swelling) portions (Fig. 9); pharynx 63–65 (64; 2) long or 8–10% (9%; 2) of oesophagus total length or 5% (5%; 2) of BL, 30–40 wide (35; 2) or 14–19%

(17%; 2) of BW, 2× (2; 2) longer than wide (Fig. 9); middle portion of oesophagus sinuous, thick-walled, separated from pharynx by a marked constriction, 485–738 (612; 2) long or 80–86% (83%; 2) of oesophagus total length or 39–53% (46%; 2) of BL, 18 (18; 2) wide; posterior oesophageal swelling thick-walled, separated from medial portion of oesophagus by a marked constriction, immediately anterior to caecal bifurcation, 55–60 (58; 2) long or 6–10% (8%; 2) of oesophagus total length or 4–5% (4%; 2) of BL, 23–25 (24; 2) wide, 11–12% (11%; 2) of BW, 2× (2; 2) longer than wide (Fig. 9). Oesophageal gland indistinct. Caecal bifurcation immediately posterior to posterior oesophageal swelling, 633–818 (726; 2) or 51–59% (55%; 2) of BL from anterior body end; anterior caeca 23–50 (37; 2) long or 6% (6%; 1) of oesophagus length, 15 (15; 2) wide or 7% (7%; 2) of BW; dextral and sinistral posterior caeca asymmetrical, 248–250 (249; 2) and 258 (258; 2) long, respectively, extending posteriad 223–253 (243; 2) or 18–20% (19%; 2) of BL or 29–42% (34%; 2) of oesophagus length, 15–28 (21; 2) wide, ending 338–368 (352; 2) or 24–30% (27%; 2) of BL from posterior body end, poorly delineated from surrounding parenchyma (Fig. 9).

Testis at level of distal tips of posterior caeca, anterior half occupying intercaecal space, 150–153 (152; 2) long or 11–12% (12%; 2) of BL, 85–100 (93; 2) wide or 40–48% (44%; 2) of maximum BW, 2× (2; 2) longer than wide, terminating 35–75 (55; 2) or 3–5% (4%; 2) of BL from distal tips of sinistral longest posterior caecum, 295–308 (302; 2) or 22–24% (23%; 2) of BL from posterior body end; testis lobes 70–75 (72; 3) long, 33–35 (34; 3) wide, 2× (2; 3) longer than wide; vasa efferentia 5 (5; 10) wide; vas deferens extending a short distance posteriad before meeting cirrus sac and internal seminal vesicle, 78–113 (96; 2) long or 6–8% (7%; 2) of BL (Figs. 9, 10). Cirrus sac between testis and sinistral nerve cord, beginning near level of distal tips of dextral cecum, 155–190 (173; 2) long, 63–65 (64; 2) wide, 2× (2; 2) longer than wide, with wall 2 (2; 2) thick, terminating 80–118 (99; 2) or 6–10% (8%; 2) of BL from posterior margin of testis, 210–213 (212; 2) or 15–17% (16%; 2) of BL from posterior body end; cirrus 38–45 (42; 2) long, 20–25 (23; 2) wide, lacking spines (Figs. 9, 10).

Ovary a loose aggregation of ova, 80–103 (92; 2) long or 6–8% (7%; 2) of BL, 83–93 (88; 2) wide or 39–45% (42%; 2) of maximum BW (Figs. 9, 10); post-ovarian space 70–90 (80; 2) long or 6% (6%; 2) of BL. Oviduct S-shaped, extending anteriad from near center of ovary, 175–205 (190; 2) long; oviducal seminal receptacle a thin-walled sac, 93–105 (99; 2) long or 45–60 (53; 2) of oviduct length, 40–43 (42; 2) wide, 2–3× (2; 2) longer than wide, varying in length and width depending on amount of sperm in duct (Figs. 9, 10). Laurer's canal indistinct. Primary vitelline collecting duct dextral, extending 125–230 (178; 2) posteriad and coursing between testis and dextral body margin, 18–23 (21; 2) wide, curving mediad at level of posterior margin of testis, uniting with distal portion of oviduct immediately proximal to oötype (Figs. 9, 10); oötype spheroid, post-caecal, 220–240 (230; 2) or 16–19% (18%; 2) of BL from posterior margin

of testis, slightly anterior to or at level of posterior margin of cirrus sac, 16–18 (17; 2) long, 15–18 (17; 2) wide or 1× (1; 2) longer than wide, 220–240 (230; 2) or 16–19% (18%; 2) of BL from posterior body end. Mehlis' gland indistinct. Uterus relatively short; ascending uterus 40–60 (50; 2) long, 8 (8; 2) maximum width, 38–65% (51%; 2) of oviduct seminal receptacle length, extending anterosinistrad from oötype before connecting with metraterm near the posterior margin of testis (Figs. 9, 10). Metraterm post-testicular, 63–65 (64; 2) long or 5% (5%; 2) of BL, 20–25 (23; 2) wide, 3× (3; 2) longer than wide, with muscular wall 2–3 (3; 2) thick; metraterm eggs 10 (10; 5) long, 5 (5; 5) wide, lacking tendril-like filaments. Common genital pore post-testicular, 30–33 (32; 2) in diameter, opening at level of oötype, 243–280 (262; 2) or 20% (20%; 2) of BL from posterior body end (Figs. 9, 10). Excretory vesicle indistinct.

3.3.2. Taxonomic summary

Type host: *Megalops atlanticus* (Valenciennes, 1847) (Elopomorpha: Elopiformes:

Megalopidae), Atlantic tarpon.

Type locality: North Captiva Island (near 26°35'46"N 82°13'44"W), Gulf of Mexico off Peninsular Florida, USA; *Other locality:* Bayboro Harbor (27°45'41.41"N; 82°37'55.37"W), Tampa Bay, Gulf of Mexico off Peninsular Florida, USA.

Site in host: Lumen of heart.

Prevalence of infection by adults of E. franksi: Three of 5 (60%) Atlantic tarpon infected.

Specimens deposited: Holotype (USNM 1422065); Paratype (USNM 1422066).

Etymology: The specific epithet honors Mr. James "Jim" Franks (Gulf Coast Research Laboratory) for his efforts in understanding the biology of Atlantic tarpon in the Gulf of Mexico.

3.3.3. Remarks

Elopicola franksi differs from its congeners by lacking ventrolateral tegumental spines and by having an elongate body (6× longer than wide), a proportionally long oesophagus (49–62% of the BL), a compact testis at level of the distal ends of the posterior caeca, and a post-testicular common genital pore at level of the oötype.

We observed aporocotylid eggs, perhaps those of *E. franksi*, embedded beneath the gill epithelium of two young Atlantic tarpon (830 and 890 mm FL) captured from the low-salinity estuarine waters of Biloxi Bay, Mississippi (30°26'16.35"N; 89°01'37.56"W; northern Gulf of Mexico) on 13 April 2007. These eggs, each having a ciliated miracidium, were morphologically characteristic of those belonging to *Elopicola* (see Bullard [3]) by having a tetrahedral body with extremely elongate polar filaments. These observations indicated that this blood fluke may infect Atlantic tarpon in the northern Gulf of Mexico as well as the region of the type locality, i.e., eastern Gulf of Mexico off Florida. No adult fluke was recovered from these Atlantic tarpon; no molecular sequence data was sourced from an egg; and we cannot at this time specifically

diagnose eggs by morphology alone. Hence, identifying this infection in Atlantic tarpon off Mississippi is pending.

3.4. Phylogenetic analyses

Elopicola nolancribbi differed from *E. bristowi* and *E. franksi* by 87 (87/1698; 95%) and 145 (145/1698; 91%) base pairs, respectively, in the 18S and 219 (219/1280; 82%) and 197 (197/1280; 84%) base pairs, respectively, in the 28S. *Elopicola bristowi* and *E. franksi* differed by 149 (149/1698; 91%) base pairs in the 18S and 212 (212/1280; 83%) base pairs in the 28S. *Elopicola nolancribbi* differed from *E. bristowi* by 235 (235/638; 53%) base pairs in the ITS. Regarding the 18S+28S dataset (Fig. 11), the aligned sequence data had a total of 2,978 positions, of which 6% were excluded because of ambiguous positional homology according to criteria adopted in Gblocks. The final data matrix had 2,804 positions per taxon (1,616 conserved, 1,188 variable, and 861 parsimony-informative). Tree topologies recovered by BI and ML analyses were congruent; however, the former had higher nodal support values (Fig. 11). The acipenseriform blood fluke *A. petersoni* clustered with the holoccephalan blood fluke *Chimaerohemecus trondheimensis* van der Land, 1967 rather than with elopiform blood flukes, which comprised (*Elopicola bristowi* (*Elopicola franksi*, *E. nolancribbi*)). The latter clade was sister to *Sanguinicola* cf. *inermis* plus all marine aporocotylids. All aporocotylids analyzed were monophyletic and sister to all turtle blood flukes (Fig. 11). Blood flukes from *Elops* spp. were not sister taxa.

Regarding the ITS2 dataset (Fig. 12), the aligned sequence data comprised 638 positions, 46% of which were excluded because of ambiguous positional homology according to the criteria adopted in Gblocks (Fig. 12). Thus, this final data matrix consisted of 293 positions per taxon (104 conserved, 189 variable, and 142 parsimony-informative). Recovered tree topologies by BI and ML analyses were congruent with the former yielding slightly higher nodal support. The resulting tree showed that *P. yamagutii* is sister to a clade comprising *E. nolancribbi* and *E. bristowi*; hence, elopomorph (ladyfish + eel) blood flukes were monophyletic. ITS2 sequence data for *E. franksi* was not available: only low quality sequences were recovered. We attempted to further investigate monophyly of elopomorph blood flukes by adding two short 18S and 28S rDNA sequences (i.e., 316 and 131 bp, respectively) of *P. yamagutii* retrieved from GenBank (accession numbers U42569 and U42562) into the 18S+28S dataset. Although both BI and ML resulting trees were mostly poorly resolved, *P. yamagutii* was, indeed, recovered sister to all elopiform blood flukes (results not shown).

4. Discussion

4.1. Aporocotylid interrelationships

The 18S+28S topology (Fig. 11) and the ITS2 topology (Fig. 12) presented herein generally agreed with previously published hypotheses of generic interrelationships among aporocotylids (Aiken et al. [38]; Alama-Bermejo et al. [18]; Bray et al. [39]; Bullard et al. [2]; Cribb et al. [40]; Holzer et al. [41]; Nolan and Cribb [9,12]; Ogawa et al. [42]; see Orélis-Ribeiro [5]). The only previous phylogenetic hypothesis to include *A. petersoni* was Bullard et al. [2], who recovered *A. petersoni* within a clade of actinopterygian blood flukes; all of which were sister to *C. trondheimensis* (a holocephalan blood fluke). In the present 18S+28S analysis, *A. petersoni* is sister to *C. trondheimensis*; which together comprise a clade sister to that of all other actinopterygian blood flukes (Fig. 11). That blood flukes of acipenseriforms and chondrichthyans are monophyletic and sister to all other blood flukes of actinopterygians rejects the notion of ordinal cophyly between blood flukes and craniates. This result could be due to low taxon sampling, and analysis of additional sequences sourced from non-euteleosteomorph blood flukes is pending. Yet, the branching pattern for the other fish blood flukes resembled that of their fish host lineages (Orélis-Ribeiro et al. [5]; Betancur [37]; Smith [43–46]). For example, the blood flukes of fishes assigned to Teleostei (Elopomorpha + remaining members of Teleostei) are monophyletic: *Elopicola* (parasites of Elopomorpha) is monophyletic and comprises the sister taxon to the blood flukes infecting the remaining members of Teleostei, i.e., the Osteoglossocephalai. In addition, *Sanguinicola* cf. *inermis* (a parasite of Cypriniformes) is sister to all other blood flukes infecting members of Osteoglossocephalai; mirroring the sister taxa relationships hypothesized for those respective host lineages (Betancur et al. [37]). Additionally, we found it curious that *E. nolancribbi* and *E. bristowi* were not sister taxa (Fig. 11), given their morphological similarities and host affiliations (infecting species of *Elops*). On the other hand, such is not surprising considering that *E. nolancribbi* and *E. franksi* both range in the Gulf of Mexico (*E. bristowi* ranges in the western Pacific Ocean). Regarding the ITS2 topology, previous works based on comparative morphology have asserted that *Paracardicoloides*, *Acipensericola*, and/or *Elopicola* share a recent common ancestor (Bullard et al. [2]; Bullard [3]; present study), and the results presented herein do not refute those assertions: using ITS2 sequence data these taxa are monophyletic and sister to all other aporocotylids analyzed (Fig. 12).

4.2. Future directions for aporocotylid species discovery and phylogenetics

A gap in knowledge regarding fish blood fluke natural history and evolution is that we know of few aporocotylids that infect “early branching” (formerly, colloquially referred to as “basal”) fish lineages, i.e., those excluded from Euteleosteiomorphia sensu Betancur et al. [37]. These early branching fish lineages, which are predominantly primary-division freshwater fishes, comprise Cladistia (Polypteriformes [bichirs]), Chondrostei (Acipenseriformes [paddlefishes, sturgeons]), Holostei (Amiiformes [bowfin], Lepisosteiformes [gars]), Elopomorpha (Elopiformes [ladyfishes, tarpons], Anguilliformes [eels], Albuliformes [bonefishes] et al.), Osteoglossomorpha (Osteoglossiformes [bonytongues]), and Otomorpha (Clupeiformes [herrings], Alepocephaliformes [slickheads, tubeshoulders], Gonorhynchiformes [milkfishes], and Cypriniformes [carps], Gymnotiformes [knifefishes], Characiformes [characins], Siluriformes [catfishes]). Of those, only the following lineages include documented aporocotylid hosts: Acipenseriformes (*A. petersoni* [see Bullard et al. [2]], Elopomorpha (3 species of *Elopicola* [see Bullard [3]; present study]), Gonorhynchiformes (2 nominal species of *Cardicola* Short, 1953 [Yong et al. [55]]), Cypriniformes (several species of *Sanguinicola* Plehn, 1907 [Smith [44,45]]), Characiformes (*Sanguinicola argentinensis* Szidat, 1951), and Siluriformes (species *incertae sedis* as *Sanguinicola* and *Plehniella*, respectively, 4 accepted species of *Plehniella* Szidat, 1951 [see Szidat, [47]]; Oréllis-Ribeiro et al. [48]), *Nomasanguinicola canthoensis* Truong and Bullard, 2013 [see Truong and Bullard [49]; Oréllis-Ribeiro and Bullard [50]]; as well as monotypic *Cladocaecum* Oréllis-Ribeiro and Bullard, 2016 and *Kritsky* Oréllis-Ribeiro and Bullard, 2016 [see Oréllis-Ribeiro and Bullard [50]).

Another considerable gap in our knowledge comprises the lack of molecular data available from potential infections in other species of ladyfishes, tarpons, eels, and bonefishes. For example, *Paracardicoloides* is monotypic and it would seem that few examinations of other species of *Anguilla* have been conducted (Nolan and Cribb [4]; Bullard [3]). As another example, Adams et al. [51] accepted 7 species of *Elops*, of which only 2 are documented as aporocotylid hosts. The southern ladyfish, *Elops smithi* McBride, Rocha, Ruiz-Carus, and Bowen, 2010 (see McBride et al. [52]) was recently described and ranges in the northwestern Atlantic Ocean in sympatry with *E. saurus*. Given that *E. saurus* is the only known definitive host for *E. nolancribbi* (Bullard [3]), a parasitological survey targeting *E. smithi* could reveal a new species of *Elopicola* or perhaps indicate more broad definitive host specificity for *E. nolancribbi*. Interestingly, McBride and Horodysky [53] reported that *E. smithi* and *E. saurus* have different life history parameters (recruiting to coastal estuarine localities during the autumn and winter-spring, respectively), such that their association with potential estuarine snail, bivalve, or polychaete intermediate hosts may also be distinctly timed. Interesting also would be to examine the eastern Pacific Ocean species *Elops affinis* Regan, 1909, which to our knowledge has not a single parasite reported from it. Further, regarding Atlantic tarpon, populations from Florida, the

eastern Pacific Ocean off Panama, the Gulf of Guinea, and Costa Rica are genetically distinct (Ward et al. [54]). We have not examined Atlantic tarpon outside of the Gulf of Mexico, but of interest would be to compare the morphological and genetic differences among their blood flukes, if present, in light of the delineated host populations. Finally, no report of a blood fluke exists from any of the bonefishes (Albuliformes: Albulidae: *Albula* spp.) (Adams et al. [51]). Parasitological surveys of these hosts could provide further insights on the taxonomy and ancestry of elopomorph and other blood flukes.

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Figure legends

Figs. 1–3. *Elopicola bristowi* sp. n. (Digenea: Aporocotylidae) from the blood vascular system of ladyfish, *Elops hawaiiensis* Regan, 1909, (Elopiformes: Elopidae) from the Eastern Sea off Nha Trang (Vietnam). Scale values aside each bar. (1) Body of adult ([holotype] USNM No. 1422060) showing anterior sucker (as), pharynx (ph), dorsolateral nerve commissure (dc), nerve cord (nc), oesophagus (oe), vitellarium (v), posterior oesophageal swelling (pos), anterior caecum (ac), posterior caecum (pc), testis (t), vasa efferentia (ve), vas deferens (vd), cirrus sac (cs), internal seminal vesicle (isv), metraterm (met), common genital pore (cgp), uterus (u), oötype (oo), oviducal seminal receptacle (osr), oviduct (ov), ovary (o), excretory vesicle (ev). Dorsal view. (2) Body of adult ([paratype] USNM No. 1422063) showing same features as in Fig. 1. Ventral view. (3) Rows of ventrolateral tegumental spines distributing in posterior portion of body (*).

Figs. 4–6. *Elopicola bristowi* sp. n. (Digenea: Aporocotylidae) from the blood vascular system of ladyfish, *Elops hawaiiensis* Regan, 1909, (Elopiformes: Elopidae) from the Eastern Sea off Nha Trang, Vietnam. (4) Body of adult ([holotype] USNM No. 1422060) showing same features as in Fig. 1. Dorsal view. (5) Body of juvenile ([paratype] USNM No. 1422062) showing same features as in Fig. 1 plus testis anlage (ta), cirrus sac anlage (csa), basophilic cells (bc), metraterm anlage (meta), uterine anlage (ua), oötype anlage (ooa), oviducal seminal receptacle anlage (osra), oviduct anlage (ova), ovarian anlage (oa). Dorsal view. (6) Schistosomulum ([paratype] USNM No. 1422061) showing same features as in Figs. 4 and 5. Ventral view.

Figs. 7–8. *Elopicola bristowi* sp. n. (Digenea: Aporocotylidae) from the blood vascular system of ladyfish, *Elops hawaiiensis* Regan, 1909, (Elopiformes: Elopidae) from the Eastern Sea off Nha Trang, Vietnam. (7) Genitalia of adult specimen ([holotype] USNM No. 1422060) showing testis (t), distal tip of dextral caecum (ddc), distal tip of sinistral caecum (dsc), vasa efferentia (ve), vas deferens (vd), internal seminal vesicle (isv), cirrus sac (cs), common genital pore

(cgp), everted cirrus (ec), vitelline duct (vt), metraterm eggs (me), metraterm (met), uterus (u), oötype (oo), oviducal seminal receptacle (osr), oviduct (ov), ovary (o), excretory vesicle (ev). Dorsal view. (8) Body of adult ([paratype] USNM No. 1422063) showing same features as in Fig. 7. Ventral view.

Figs. 9–10. *Elopicola franksi* sp. n. (Digenea: Aporocotylidae) from the heart of Atlantic tarpon, *Megalops atlanticus* Valenciennes, 1847, (Elopiformes: Megalopidae) from the Gulf of Mexico. Scale values aside each bar. (9) Body of adult ([holotype] USNM No. 1422065) showing anterior sucker (as), pharynx (ph), oesophagus (oe), posterior oesophageal swelling (pos), anterior caecum (ac), posterior caecum (pc), testis (t), vasa efferentia (ve), vas deferens (vd), vitelline duct (vt), cirrus sac (cs), internal seminal vesicle (isv), metraterm (met), uterus (u), oötype (oo), common genital pore (cgp), oviducal seminal receptacle (osr), oviduct (ov), ovary (o). Lateral view. (10) Genitalia of adult specimen ([holotype] USNM No. 1422065) showing testis (t), distal tip of dextral caecum (ddc), distal tip of sinistral caecum (dsc), vasa efferentia (ve), vas deferens (vd), internal seminal vesicle (isv), cirrus sac (cs), everted cirrus (ec), vitelline duct (vt), metraterm eggs (me), metraterm (met), oötype (oo), oviducal seminal receptacle (osr), oviduct (ov), ovary (o). Lateral view.

Figure 11. Phylogenetic relationships of blood flukes reconstructed by Bayesian inference and based on concatenated fragments of 18S and 28S rDNA genes from 26 taxa (majority rule consensus tree). Numbers aside tree nodes indicate posterior probability (Bayesian inference, left) and bootstrap values (Maximum Likelihood, right); definitive hosts are indicated by icons.

Figure 12. Phylogenetic relationships of aporocotylids reconstructed by Bayesian inference and based on ITS2 rDNA sequences from 12 taxa (majority rule consensus tree). Numbers aside tree nodes indicate posterior probability (Bayesian inference, left) and bootstrap values (Maximum Likelihood, right); definitive hosts are indicated by icons.