

Biodiversity and Phylogenetics of Metazoan Parasites Infecting Freshwater Turtles

by

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ABSTRACT

There are 356 extant turtle species (Testudines), including 60 polytypic taxa that add 122 subspecies. Among the more diverse groups of metazoan parasites that infect turtles are the turtle blood flukes (TBFs) (Digenea: Schistosomatoidea; 106 species; 24 genera). Polystomes (Monogenoidea: Polystomatidae; 61 species; 5 genera) are another underexplored group of parasites infecting turtles. They belong to a much larger group of flatworms that are, nearly without exception, fish ectoparasites and are further intriguing by infecting unusual sites (conjunctival sac, buccal cavity, urinary bladder) of turtles and frogs. Both of these taxonomic groups are understudied in North America. TBFs, especially of freshwater turtles, are largely ignored outside of North America. TBFs and polystomes infect members of both major turtle lineages, sub-order Pleurodira Cope, 1864 (side-necked turtles, withdraw neck by bending in horizontal plane; 93 species) and sub-order Cryptodira Cope, 1868 (hidden-necked turtles, withdraw head by bending neck in vertical plane; 263 species). Extant cryptodires range in the northern and southern hemispheres, whereas extant pleurodires range in southern hemisphere freshwater habitats only. Herein, I necropsied 86 turtles of 24 species (14 genera: 7 families; including cryptodires and pleurodires) from 3 continents (North America, Africa, Asia). In addition, I published a taxonomic description from TBFs collected previously by the Bullard Lab in South America (Peru). I used alpha taxonomy, light microscopy, nucleotide sequencing techniques (PCR; large subunit ribosomal DNA [28S], small subunit ribosomal DNA [18S], and Cytochrome c oxidase subunit I [CO1]), and molecular phylogenetic analyses (Bayesian inferences) to (i) redescribe a species long-regarded as a species complex, (ii) emend a genus that lacked extant type material and described a new species of the genus, (iii) proposed two new genera and described two new species, and (iv) shed light on a particular taxonomically-troubling polystome; including the first record of this lineage of parasite in the alligator snapping turtle or from Mississippi. The turtle blood fluke work is published in *The Journal of Parasitology*, *Folia Parasitologica*, and *Systematic Parasitology*. The final chapter detailing the polystome was in review at the time of the defense of this thesis.

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**CHAPTER 1: NEW GENUS AND SPECIES OF TURTLE BLOOD FLUKE
(PLATYHELMINTHES: DIGENEA: SCHISTOSOMATOIDEA) INFECTING SIX-
TUBERCLED AMAZON RIVER TURTLES, *PODOCNEMIS SEXTUBERCULATA*
(PLEURODIRA: PODOCNEMIDIDAE) FROM THE AMAZON RIVER BASIN (PERU)**

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ABSTRACT

Herein we describe a new species of turtle blood fluke (TBF) and propose a new genus to accommodate it, *Pitiutrema revelae* n. gen., n. sp. This blood fluke infects the heart of six-tubercled Amazon River turtles (*Podocnemis sextuberculata* [Cornalia, 1849] [Pleurodira: Podocnemididae]) in the headwaters of the Amazon River near Iquitos, Peru. It resembles the other 2 described species of South American freshwater TBFs (*Atamatam amazoniensis* Bullard and Roberts, 2019 and *Paratamatam iquitosiensis* Bullard and Roberts, 2019) by having a dorsoventrally flattened and ovoid body, an oral sucker with anteroventral spines, 2 inter-cecal testes arranged in a column, inter-gonadal terminal genitalia, an inter-cecal and post-ovarian Laurer's canal pore, and a Y-shaped excretory bladder. It differs from all other nominal TBFs by having the combination of an aspinose body that lacks mammillae, a tapered (not broadly rounded) posterior body end, a ventral sucker, slightly M-shaped or inverse U-shaped ceca, a deeply-lobed (dendritic) ovary, a transverse uterus, and a dispersed vitellarium. The new genus is further unique among TBF genera by having an anterior to posterior sequence of ventral sucker, anterior testis, ovary, cirrus sac (lateral to posterior half of ovary), and posterior testis. The phylogenetic results and placement of the new taxon i) were both predicted by our morphological diagnosis and comparisons with related taxa, ii) further indicated monophyly of the nominal South American freshwater TBFs, iii) reaffirmed the marine derived lineage identity of the

nominal South American freshwater TBFs, and iv) highlighted that the single cercarial sequence (TBF sp. W-810) from an ampullariid in Brazil does not share a recent common ancestor with any of the nominal South American freshwater TBFs. The new species is the eighth TBF reported from a side-necked turtle (Pleurodira), the first TBF from a member of Podocnemididae, and the third freshwater TBF from South America.

INTRODUCTION

Extant turtles comprise 2 suborders: the hidden-necked turtles (Cryptodira; 263 spp.; distributed in the northern and southern hemisphere) and the side-necked turtles (Pleurodira; 93 spp.; southern hemisphere only) (Pereira et al., 2017). Six species of *Podocnemis* Wagler, 1830 (Pleurodira: Podocnemididae) range in South America. The red-headed Amazon River turtle, *Podocnemis erythrocephala* Spix, 1824, the giant South American river turtle, *Podocnemis expansa* Scheweigger, 1812, the Magdalena River turtle, *Podocnemis lewyana* Dumeril, 1852, the six-tubercled Amazon River turtle, *Podocnemis sextuberculata* Cornalia, 1849, and the yellow-spotted river turtle, *Podocnemis unifilis* Cornalia, 1849 range in the Amazon River Basin, whereas the savanna side-neck turtle, *Podocnemis volgi* Muller, 1935, is endemic to the Orinoco River Basin.

Turtle blood flukes (“TBFs”; Platyhelminthes: Digenea: Schistosomatoidea) infect the blood vascular system of both cryptodires and pleurodires, but only 5 pleurodires reportedly are infected by 7 TBF species (*Uterotrema krefftii* Platt and Blair, 1996, *Uterotrema burnsi* Platt and Blair, 1996, *Uterotrema australispinosa* Platt and Pichelin, 1994, *Hapalorhynchus beadlei* Goodman, 1987, *Hapalorhynchus tchalimi* Bourgat and Kulo, 1987, *Atamatam amazoniensis* Bullard and Roberts, 2019, and *Paratamatam iquitosiensis* Bullard and Roberts, 2019). Hence,

the TBFs of pleurodires and those of South American freshwater turtles are under-explored for infections.

Herein, we document a new species of TBF infecting the six-tubercled Amazon River turtle, or “pitiu,” from the Peruvian Amazon and propose a new genus to accommodate it; bringing the number of accepted TBF genera to 25.

MATERIALS AND METHODS

During July 2016, the heart from each of 5, six-tubercled Amazon River turtles (*P. sextuberculata*) from the Belén Market (3°45'32.08"S, 73°14'53.15"W), Iquitos, Peru (Amazon River) was opportunistically sampled for TBF infection following previously published methods (Bullard et al., 2019). TBF specimens intended for morphology as whole-mounts were observed microscopically, heat-killed on glass slides using a butane hand lighter under little or no coverslip pressure, fixed in 10% neutral buffered formalin (NBF), rinsed with water, stained in Van Cleave's hematoxylin with several drops of Ehrlich's hematoxylin, dehydrated with a graded EtOH series, made basic at 70% EtOH with lithium carbonate and butyl-amine, dehydrated in absolute EtOH and xylene, cleared with clove oil, and permanently mounted on glass slides using Canada balsam. Specimens intended for DNA extraction and sequencing were preserved alive in 95% non-denatured ethanol (EtOH). The resulting whole mounts were examined and illustrated with the aid of compound microscopes (Leica DM2500 and Leica DMR [Leica, Wetzler, Germany]) equipped with differential interference contrast (DIC) optical components and drawing tubes. Measurements were obtained with a calibrated ocular micrometer (as straight-lines along the course of each duct) and are reported in micrometers (μm) as the range followed by the mean, \pm standard deviation, and sample size in parentheses.

Type specimens of the new species were deposited in the National Museum of Natural History's Invertebrate Zoology Collection (Smithsonian Institution, USNM Collection Nos. 1578590–1578593). Classification and anatomical terms for TBFs follow Roberts et al. (2016a, 2016b, 2016c; 2017) and Bullard et al. (2019). Turtle scientific and common names follow van Dijk et al. (2014), and higher classification of turtles follows Pereira et al. (2017).

Total genomic DNA (gDNA) was extracted from 3 EtOH-preserved and microscopically-identified blood flukes using DNeasy™ Blood and Tissue Kit (Qiagen, Valencia, California) as per the manufacturer's protocol; however, the proteinase-K incubation period was extended overnight and we used 100 µL of elution buffer to increase the final DNA concentration. Amplification and sequencing of the D1–D3 domains of the large subunit ribosomal DNA (28S) used the primer set of Oréllis-Ribeiro et al. (2017). PCR amplifications were performed according to Bullard et al. (2019). DNA sequencing was performed by ACGT, Incorporated (Wheeling, Illinois). Reactions were sequenced using BigDye terminator version 3.1, cleaned with magnetic beads (CleanSeq dye terminator removal kit; Beckman Coulter, Brea, California), and analyzed using an ABI 3730 XL or 3730 Genetic Analyzer (Applied Biosystems, Waltham, Massachusetts). Sequence assembly and analysis of chromatograms were completed with Geneious version 11.0.5 (<http://www.geneious.com>; Kearse et al., 2012). All nucleotide sequence data were deposited in GenBank (MN244241).

The phylogenetic analysis included the sequence of the new species plus all of the taxa included in Bullard et al. (2019). The outgroup comprised 3 fish blood fluke taxa: *Elopicola franksi* Oréllis-Ribeiro and Bullard, 2017 (Oréllis-Ribeiro et al., 2017), *Elopicola nolancribbi* Bullard, 2014 (Bullard, 2014), and *Elopicola bristowi* Oréllis-Ribeiro and Bullard, 2017; all sourced from Oréllis-Ribeiro et al. (2017). As per Warren et al. (2019), sequences were aligned

using MAFFT (Kato 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 4 Metropolis-coupled chains were run for 5,000,000 generations, sampling the posterior distribution every 1,000 generations. Convergence was checked using Tracer v1.6.1 (Rambaut et al., 2014) and the “sump” command in MrBayes: all runs appeared to reach convergence after discarding the first 25% of generation as burn-in. A majority rule consensus tree of the post burn-in posterior distribution was generated with the “sumt” command in MrBayes. The inferred phylogenetic tree was visualized using FigTree v1.4.3 (Rambaut et al., 2014) and further edited for visualization purposes with Adobe Illustrator (Adobe Systems). Nucleotide sequence data reported in this paper is available in the GenBank database under the submission ID number MN244241.

DESCRIPTION

***Pitiutrema* n. gen. Dutton and Bullard**

(Figs. 1–9)

Body dorsoventrally flat (not cylindrical), ventrally concave, ovoid (not thread-like), 5–6× longer than wide, having inverse U-shaped anterior end, and tapered posterior end; mammillae absent. Oral sucker robust, demarcated from body by constriction, papillate, spinous; minute oral sucker spines distributing in an ovoid patch on anteroventral surface of mouth.

Ventral sucker, crenulate, at level of body constriction (forebody more narrow than hind-body), diameter proportionate to that of the body at the constriction. Pharynx present, enveloping anterior extremity of esophagus. Esophagus sinuous, terminating in anterior one-fourth of body, dorsal to anterior nerve commissure, lateral esophageal diverticula and median esophageal diverticulum absent; esophageal gland surrounding entire length of esophagus. Intestinal ceca slightly M-shaped or inverse U-shaped, comprising non-fused ceca bifurcating in anterior one-fourth of body, cecal diverticulae absent, extending posteriad, terminating at the level of the posterior testis, slightly asymmetrical. Testes 2 in number, arranged in a column, comprising a pre-ovarian (anterior) testis and a post-ovarian (posterior) testis, inter-cecal, flanking terminal genitalia, elongate (longer than wide). Anterior and posterior trunks of vasa efferentia present, ventral to gonads, connecting to vas deferens; anterior vas efferens medial; posterior vas efferens medial; vas deferens comprising a compact external seminal vesicle; external seminal vesicle abutting anterior testis, ovary, and cirrus sac. Cirrus sac enclosing internal seminal vesicle and cirrus, extending posteromedial to ovary. Pars prostatica absent. Ovary slightly dextral, transverse, extending from 1 cecum to another, inter-testicular, closely flanked by anterior testis, dendritic (deeply lobed). Oviduct approximately perpendicular to ovary, convoluted extending posteriad before connecting to oviducal seminal receptacle; oviducal seminal receptacle at level of transverse vitelline duct. Laurer's canal inter-cecal, inter-testicular, with dorsal pore. Vitellarium symmetrical, comprising a series of dispersed spheroid masses of vitelline follicles, surrounding ceca, distributing throughout the body; transverse vitelline duct inter-testicular. Uterus transverse; metraterm strongly muscular, inter-gonadal. Common genital pore sinistral, posterior to ovary, ventral, inter-cecal, inter-testicular. Excretory vesicle Y-shaped; excretory pore dorsal, subterminal. Manter's organ absent.

Differential diagnosis: Body flat, 5–6× longer than wide, aspinous, having inverse U-shaped anterior end, and tapered posterior end. Oral sucker robust, papillate, spinous; distributing in an ovoid patch on anteroventral surface of mouth. Ventral sucker, crenulate. Esophagus dorsal to anterior nerve commissure. Intestinal ceca slightly M-shaped or inverse U-shaped, terminating at the level of the posterior testis. Testes 2, column absent, inter-cecal, flanking and abutting terminal genitalia. Anterior and posterior vas efferens medial. External seminal vesicle abutting anterior testis, ovary, and cirrus sac. Cirrus sac posteromedial to ovary. Ovary slightly dextral, transverse, flanked by anterior testis, non-inter-cecal, dendritic (deeply lobed). Oviduct approximately perpendicular to ovary, convoluted extending posteriad before connecting to oviducal seminal receptacle; oviducal seminal receptacle at level of transverse vitelline duct. Laurer's canal inter-testicular. Vitellarium symmetrical, comprising a series of dispersed spheroid masses of vitelline follicles, distributing throughout the body; transverse vitelline duct between ovary and posterior testis. Common genital pore sinistral, posterior to ovary, ventral. Manter's organ absent.

Taxonomic summary

Type and only known species: *Pitiutrema revelae* n. sp.

ZooBank registration: urn:lsid:zoobank.org:act:7C934150-42EA-4FCD-8858-0D91510D4B05

Etymology: *Pitiutrema* is from the local common name for the turtle host (“pitiu”), the six-tubercled Amazon River turtle (the type host), and *-trema* as a traditional suffix used for flukes.

***Pitiutrema revelae* n. sp. Dutton and Bullard**

(Figs. 1–9)

Description of adult (based on 4 whole-mounted specimens): Body 1,040–1,560 (1,220 ± 234; 4) long, 217–325 (277 ± 46; 4) in maximum width at level of anterior testis, 5–6× (5 ± 0; 4)

longer than wide (Figs. 1, 2); ventrolateral tegumental mammillae absent. Body surface having asymmetrically arranged tegumental projections (approximately 1–2 long, 1 wide) ventrally and dorsally.

Oral sucker 55–70 (64 ± 8 ; 4) long or 4–6% ($5\% \pm 1$; 4) of body length, 48–54 (51 ± 3 ; 4) wide or 15–23% ($19\% \pm 3$; 4) of maximum body width, (Figs. 1–5) triangle shaped; oral sucker spines projecting from tegument approximately less than 1 long (Figs. 4, 5); dorsal surface of oral sucker papillate, asymmetrically arranged. Ventral sucker 110–125 (117 ± 8 ; 3) long or 10–11% ($11\% \pm 1$; 3) of body length, 100–120 (110 ± 10 ; 3) wide or 62–83% ($75\% \pm 12$; 3) of maximum body width (Figs. 1, 3). Nerve commissure 158–195 (170 ± 17 ; 4) or 13–16% ($14\% \pm 1$; 4) of body length from anterior body end. Pharynx 55–70 (64 ± 8 ; 4) long or 20–25% ($22\% \pm 2$; 4) of esophagus length, 48–54 (51 ± 3 ; 4) wide or $2 \times (2 \pm 0$; 4) maximum esophagus width. Esophagus 263–340 (295 ± 33 ; 4) long or 22–29% ($25\% \pm 3$; 4) of body length, 6–10 (9 ± 2 ; 4) wide immediately posterior to pharynx and with wall 2–3 (3 ± 1 ; 4) thick, 15–20 (17 ± 2 ; 4) wide or 13–18% ($14\% \pm 2$; 4) of body width at mid-esophagus and with wall 4–6 (5 ± 6 ; 4) thick, 30–50 (40 ± 8 ; 4) wide or 20–33% ($26\% \pm 6$; 4) of body width at cecal bifurcation and with wall 13–25 (18 ± 6 ; 4) thick; esophageal gland 213–250 (237 ± 18 ; 4) long or 16–22% ($20\% \pm 3$; 4) of body length, 63–83 (70 ± 9 ; 4) wide or 22–31% ($26\% \pm 5$; 4) of body width. Intestine bifurcating 283–355 (312 ± 35 ; 4) or 23–26% ($25\% \pm 1$; 4) of body length from anterior body end; anterior ceca extending anteriorly in parallel with lateral body margin; sinistral anterior cecum 17–30 (21 ± 8 ; 3) long or 1–2% ($2\% \pm 0$; 3) of body length, 13–20 (15 ± 4 ; 3) wide or 6–7% ($6\% \pm 1$; 3) of body width at level of cecal bifurcation, dextral anterior cecum 12–20 (16 ± 4 ; 3) long or 6–7% ($6\% \pm 1$; 3) of body length, 10–15 (13 ± 3 ; 3) wide or 5–6% ($5\% \pm 0$; 3) of body width at level of cecal bifurcation (Figs. 3, 4); posterior ceca extending

posteriad approximately in parallel with lateral body margin, slightly sinuous, having numerous secretory cells in the lumen; sinistral posterior cecum 575–930 (758 ± 148 ; 4) long or 52–77% ($63\% \pm 10$; 4) of body length, 20–43 (28 ± 11 ; 4) wide or 14–24% ($18\% \pm 4$; 4) of body width at level of cecal bifurcation, 20–42 (29 ± 9 ; 4) wide or 8–16% ($11\% \pm 3$; 4) of body width at level of ovary, 20–35 (27 ± 6 ; 4) wide or 10–13% ($12\% \pm 1$; 3) of body width at ends of cecum, ceca terminating 220–255 (232 ± 20 ; 3) or 19–21% ($20\% \pm 1$; 3) of body length from posterior body end; dextral posterior cecum 625–700 (667 ± 38 ; 3) long or 57–67% ($60\% \pm 6$; 3) of body length, 20–38 (28 ± 9 ; 4) wide or 14–21% ($18\% \pm 3$; 4) of body width at level of cecal bifurcation, 25–40 (29 ± 7 ; 4) wide or 8–15% ($12\% \pm 3$; 4) of body width at level of ovary, 20–30 (26 ± 5 ; 3) wide or 10–14% ($12\% \pm 2$; 3) of body width at level of ends of cecum, ceca terminating 187–230 (211 ± 22 ; 3) or 17–18% ($18\% \pm 0$; 3) of body length from posterior body end (Figs. 1, 2).

Anterior testis 175–355 (273 ± 77 ; 4) long or 16–29% ($22\% \pm 6$; 4) of body length or $0.8\text{--}1.0\times$ (1.0 ± 0.1 ; 4) posterior testis length, 125–238 (181 ± 46 ; 4) wide or 58–73% ($65\% \pm 7$; 4) of body width or 82–85% ($84\% \pm 1$; 3) of posterior testis width; inter-testicular space 135–163 (144 ± 16 ; 3) long or 12–14% ($13\% \pm 1$; 3) of body length (Figs. 1, 2, 6, 9). Posterior testis 223–340 (270 ± 51 ; 4) long or 20–26% ($22\% \pm 3$; 4) of body length, 105–195 (153 ± 40 ; 4) wide or 48–60% ($55\% \pm 6$; 4) of body width, 55–90 (77 ± 19 ; 3) or 5–8% ($7\% \pm 1$; 3) of body length from posterior body end. Vasa efferentia coalescing anteriorly and posteriorly and connecting to each testis, each appearing to connect directly to external seminal vesicle ventrally (vas deferens sensu stricto is extremely short, if present); anterior vas efferens emanating from ventromedial surface of anterior testis, extending posterosinistrad 25–50 (35 ± 13 ; 3), 4–5 (5 ± 1 ; 3) wide; posterior vas efferens emanating from the dorsal surface of the posterior testis,

extending anterosinistrad 170–200 (185 ± 21 ; 2) or 15–19% ($17\% \pm 3$; 2) of body length, 3–5 (4 ± 1 ; 2) wide, lateral to or ventral to ovary (Figs. 6, 9). External seminal vesicle transverse (crossing midline), directed sinistrad, 60–88 (71 ± 15 ; 3) long or 6–7% ($6\% \pm 1$; 3) of body length, 40–65 (52 ± 13 ; 3) wide, 1.4–1.5 \times (1.4 ± 0 ; 3) wider than long, immediately posterior to anterior testis; internal seminal vesicle longitudinal, 70–85 (79 ± 8 ; 3) long or 6–8% ($7\% \pm 1$; 3) of body length, 35–49 ($41 \pm 7\%$; 3) wide, 1.7–2.0 \times (1.9 ± 0 ; 3) longer than wide (Figs. 6, 9). Cirrus sac ovoid, 95–100 (97 ± 3 ; 3) long or 8–9% ($9\% \pm 1$; 3) of body length, 55–60 (58 ± 3 ; 3) wide or 18–31% ($23\% \pm 6$; 3) body width at level of genital pore; cirrus extending posterolaterad 40–70 (56 ± 15 ; 3) or 40–72% ($58\% \pm 16$; 3) of cirrus sac length, 15–30 (21 ± 8 ; 3) wide (Figs. 6, 9). Pars prostatica absent (present in the genera *Cardiotrema* Dwivedi, 1967, *Coeuritrema* Mehra, 1933, *Hapalorhynchus* Stunkard, 1922, *Hapalotrema* Price, 1934, *Learedius* Price, 1934, and *Platt* Roberts and Bullard, 2018), prostatic glands present, surround posterior portion of cirrus sac (Fig. 6).

Ovary dendritic (having deep lobes), 135–243 (172 ± 48 ; 4) long or 10–23% ($15\% \pm 6$; 4) of body length, 90–120 (101 ± 13 ; 4) wide or 29–46% ($38\% \pm 9$; 4) of body width; post-ovarian space 375–403 (384 ± 16 ; 3) or 32–39% ($35\% \pm 4$; 3) of body length (Figs. 7, 8). Oviduct 35–49 (41 ± 7 ; 3) long or 3% ($3\% \pm 0$; 3) of body length, 20–23 (21 ± 27 ; 3) wide proximally, laterally expanding to form oviducal seminal receptacle; oviducal seminal receptacle 50–60 (55 ± 5 ; 3) long or 4–5% ($5\% \pm 1$; 3) of body length, 48–50 (49 ± 1 ; 3) wide or 15–23% ($19\% \pm 4$; 3) of body width, between ovary and posterior testis. Laurer's canal extending posterior from descending portion of uterus 55–60 (58 ± 4 ; 2), and 10 (10 ± 4 ; 2) in maximum width, with pore and dextral to uterus and dorsal to anterior margin of posterior testis (Figs. 7, 8). Vitellarium terminating at posterior body end; lateral collecting ducts dorsal to gonads, 15–75 (36 ± 27 ; 4)

wide (Figs. 1, 2), sinistral vitelline collecting duct between cirrus sac and posterior testis, dextral vitelline collecting duct between oviduct and oviducal seminal receptacle, leading to primary collecting duct that is sinistrally posteriolaterad to oviducal seminal receptacle, dorsal to gonads, transverse vitelline duct 100–175 (140 ± 34 ; 4) in breadth 25–45 (32 ± 9 ; 4) wide; primary vitelline collecting duct dorsal to transverse vitelline duct, extending posterosinistrad 18–20 (19 ± 1 ; 3) before connecting with oviduct, 9–10 (10 ± 0 ; 3) wide. Oötype not observed. Uterus 85–120 (98 ± 19 ; 3) long or 7–12% ($9\% \pm 2$; 3) of body length, 12–20 (16 ± 4 ; 3) wide, ventral to transverse vitelline duct, extending posteriorly sinistral before curving anteriorly sinistral, inter-cecal, lacking uterine pouch, with distal end comprising metraterm; metraterm 42–75 (57 ± 17 ; 3) long or 4–5% (5 ± 0 ; 3) of body length, 10–15 (13 ± 3 ; 3) wide or 5–6% ($5\% \pm 1$; 3) of body width; uterine egg not observed. Common genital pore 155–170 (162 ± 8 ; 3) or 13–14% ($14\% \pm 1$; 3) of body length from posterior body end, 6–12 (10 ± 3 ; 4) in diameter (Figs. 7, 8).

Excretory pore dorsal, subterminal; excretory vesicle 15–35 (25 ± 14 ; 3) wide or 10–16% ($13\% \pm 4$; 3) of body width at level of cecal termini; excretory pore 25–30 (28 ± 4 ; 3) or 2–3% ($2\% \pm 0$; 3) of body length from posterior body margin (Figs. 1, 2).

Taxonomic summary

Type and only reported host: *Podocnemis sextuberculata* (Cornalia, 1849) (Pleurodira:

Podocnemididae), the six-tubercled Amazon River turtle.

Type locality: Upper Amazon River Basin (Belén Market, Iquitos, Peru; [$3^{\circ}45'32.08''S$, $73^{\circ}14'53.15''W$]).

Specimens and sequences deposited: Holotype (USNM 1578590); paratype (USNM 1578591–1578593); 28S sequence (GenBank No. MN244241).

Site in host: Heart.

Prevalence and intensity: Three of 5 (prevalence = 0.6, mean intensity = 1.8) six-tubercled Amazon River turtles were infected with 7 specimens of *P. revelae*.

ZooBank registration: [urn:lsid:zoobank.org:act:88F041FB-CCCF-4561-BA11-5C0C00F2D773](https://zoobank.org/urn:lsid:zoobank.org:act:88F041FB-CCCF-4561-BA11-5C0C00F2D773)

Etymology: The specific epithet “revelae” honors Ms. Revel Weyer, HRD’s niece, whose individuality and untamed curiosity will help her succeed in anything she puts her mind to.

Molecular results

The amplified 28S fragment of the new species comprised 1,593 nucleotides (MN244241). The Bayesian analysis, using all available TBF 28S sequences (Table I) recovered the new species sister to the clade that included the other South American freshwater TBFs (*A. amazoniensis* and *P. iquitosiensis*) and 2 innominate cercarial sequences from Pinto et al. (2015). The recovered phylogeny and the placement of the new taxon i) were predicted by our morphological diagnosis and comparisons with related taxa (see Remarks, below), ii) further indicated monophyly of the nominal South American freshwater TBFs, iii) reaffirmed the marine derived lineage (MDL) identity of the nominal South American freshwater TBFs, and iv) highlighted that the single cercarial sequence (TBF sp. W-810) from an ampullariid in Brazil does not share a recent common ancestor with any other known South American freshwater TBF (Pinto et al., 2015) (see Discussion).

The topology of the recovered 28S tree resembled that of other published trees (Oréris - Ribeiro et al., 2014, de Buron et al., 2018, Roberts et al., 2018b, Bullard et al., 2019) with slight differences regarding species of *Coeritrema* Mehra, 1933 and *Spirorchis* MacCallum, 1918. Herein, *Coeritrema platti* Roberts and Bullard, 2016 claded with *Platt sinuosus* Roberts and Bullard, 2016 and *Platt snyderi* Platt and Sharma, 2012 (low support, 0.60) (Fig. 10). Bullard et al. (2019) recovered *C. platti* sister to species of *Hapalorhynchus* Stunkard, 1922 (low support

nodal, 0.48). *Coeuritrema* had long been regarded a junior subjective synonym of *Hapalorhynchus* until Roberts et al. (2016b) used presence of marginal papillae (or mammillae), ESV position (abutting anterodextral margin of cirrus sac), and presence of an obvious and muscular metraterm (3–7× uterus length) to reassign several species. *Coeuritrema* and *Platt* have a distinctive ESV position, abutting the anterodextral margin of cirrus sac; however, *Coeuritrema* has a dispersed vitellarium like that *Hapalorhynchus*. Species of *Spirorchis* are morphologically and molecularly similar but have greater inter-specific nucleotide differences in the 28S than in the ITS2 (Roberts et al., 2019). Herein, *Spirorchis haematobius* Stunkard, 1922 shares a recent common ancestor with *Spirorchis picta* Stunkard, 1923 and *Spirorchis collinsi* Roberts and Bullard, 2016 (low support, 0.55) (Fig. 10). Bullard et al. (2019) indicated that *S. haematobius* shares a recent, common ancestor with *S. scripta* (with a higher posterior probability of 0.93) rather than with *S. collinsi*, which is supported by testis morphology (Platt, 1993).

Remarks

Our previous systematic treatment of TBFs (Bullard et al., 2019) assigned all accepted TBF genera into 6 morphologically-diagnosed groups, and, by that classification scheme, the new genus cannot be assigned to any of those groups. The new genus is the only accepted TBF genus to have an anterior to posterior sequence of ventral sucker, anterior testis, ovary, cirrus sac (lateral to posterior half of ovary), and posterior testis. The new genus resembles *Amphiorchis* Price, 1934, *Hapalotrema*, *Learedius*, *Atamatam*, and *Paratamatam* (all from Groups 5 and 6 of Bullard et al. [2019]) by having a well-developed external (pre-ovarian) and internal seminal vesicle, a ventral common genital pore, an oviducal seminal receptacle that flanks the ovary anteriorly and posteriorly (respectively), an inter-cecal and post-ovarian Laurer's canal pore, and

a Y-shaped excretory bladder. It differs from the Group 5 marine TBFs by the combination of having a spinous oral sucker (Figs. 4, 5), a pharynx, 2 testes in a column, a post-ovarian testis, a convoluted oviduct, an inter-testicular Laurer's canal, a strongly muscular metraterm, and intergonadal terminal genitalia. The new genus is most similar to *Atamatam* Bullard and Roberts, 2019 by having the combination of spines on the anteroventral surface of the oral sucker, tegumental projections distributed across the ventral and dorsal body surface, inter-cecal testes, a sinistral common genital pore that is lateral to the ovary, an oviducal seminal receptacle at level of the transverse vitelline duct, and a vitellarium that extends posteriad beyond the tips of the ceca. The new genus differs from *Atamatam* by having an aspinous body (vs. spinous in *Atamatam*), smooth body margins (vs. mammillae present), an inverse U-shaped anterior end, and a crenulate ventral sucker (vs. absent) (Figs. 1, 3). The new genus further differs by having a ventromedial posterior vas efferens and mediodorsal anterior vas efferens, a transverse (vs. sinistral) dendritic (vs. smooth) ovary, a perpendicular (vs. transverse) oviduct that extends posteriad and does not cross the midline (vs. crosses midline before extending anteriorly), and a post-ovarian common genital pore (vs. lateral).

DISCUSSION

Our results demonstrate that another pleurodire lineage (Podocnemididae) was infected by a marine TBF or a TBF with a marine ancestor (Fig. 10) and that South American freshwater TBFs are monophyletic and comprise an MDL including at least 3 monotypic genera (Bullard et al., 2019; present study). Our results herein cannot reject the notion that the TBFs infecting South American pleurodires share a recent common ancestor with nearly all known marine TBFs (species of *Amphiorchis*, *Cheloneotrema* Simha and Chattopadhyaya, 1980, *Hapalotrema*, *Learedius*, *Neocaballerotrema*, *Satyanarayanotrema* Simha and Chattopadhyaya, 1980, and

Shobanotrema; Group 5 *sensu* Bullard et al., 2019), all of which infect cryptodires of Cheloniidae. Yet, because a single turtle species can harbor a high diversity of TBFs, it is intriguing to consider the possibility that there could be distinct TBF lineages that infect these turtles and whose natural history is best explained by plate tectonics and turtle natural history rather than by marine incursions and MDLs. Podocnemidids and chelids, for example, have distinct evolutionary histories determined by plate tectonics, which could affect the natural history of their blood parasites. The new species herein infects a podocnemidid in South America, whereas the other 2 TBFs named on that continent (Bullard et al., 2019) infect snake-necked turtles (or Austro-South American side-necked turtles; Chelidae). Pleurodire fossil evidence suggests that chelids (Chelidae) and the remaining side-necked turtles (pelomedusoides: Podocnemididae [South America] and Pelomedusidae [Africa]) originated and diversified in the southern and northern portions of Gondwana, respectively. During the early Cretaceous, chelids split into South American and Australian clades and pelomedusoides split into South American (Podocnemididae) and African (Pelomedusidae) clades. Both events occurring during rifting of Africa and South America (137 million years ago [Ma]) (Pereira et al., 2017). After many more TBF taxa are collected, described, and subjected to phylogenetic analyses, it will be interesting to test hypotheses concerning monophyly and sister taxa relationships among the TBFs of chelids, podocnemidids, and pelomedusids in Africa and South America. We predict that some of their TBFs will clade according to side-necked turtle biogeography and that these TBFs will not share a recent common ancestor with the MDL TBFs.

There could be extant examples of TBF lineages that have recently host-switched from marine to freshwater turtles. Intuitively, estuarine turtles could carry infections from both marine and freshwater TBFs because they are exposed (potentially) to infected snails and polychaetes

across a range of salinities. Taxonomically, this could manifest in an estuarine turtle hosting TBFs that resemble (morphologically and genetically) TBFs that infect marine or freshwater turtles. However, little information is available on the parasites of estuarine turtles, and in general it is difficult to categorize a turtle by salinity. Within Alabama, some primarily freshwater turtles are known to enter tidal/moderate salinity areas of the Mobile Bay (a large estuarine area), and at least some estuarine visits by these turtles may be extended or semi-permanent as evidenced by some individual turtles having barnacles on their shell (Guyer, 2015). At least 1 turtle in this region is considered an obligate estuarine resident: the Mississippi diamond-backed terrapin, *Malaclemys terrapin pileate* Wied, 1865 (Cryptodira: Emydidae). No TBF has been reported from this turtle, and few parasites in general have been reported from this host (Leidy, 1888; Werner, 2003). In South America, Palumbo et al. (2019) documented several adults of *Amphiorchis* sp. infecting the estuarine turtle, *Hydromedusa tectifera* Cope, 1870 (Pleurodira: Chelidae) in Río de la Plata, Brazil (Quintela et al. 2011, Jannello et al. 2016). These specimens lacked a ventral sucker (all nominal species of *Amphirochis* have a ventral sucker) and had a vitellarium distributing for the entire body length. These morphological differences suggest that these specimens may comprise a species related to but distinct from *Amphiorchis* spp. (no nucleotide information is available for this TBF because the host was preserved whole in formalin).

The phylogenetic results herein suggest that the ventral sucker of TBFs has evolved independently numerous times across the various lineages and/or, less likely, that there has been repeated, independent loss of the ventral sucker (Fig. 10). A total of 63 (of 95 nominal species) TBFs assigned to 15 (of 25 accepted genera) genera have a ventral sucker (Groups 2, 4, and 5 of Bullard et al. [2019]). In this regard, TBFs are unique among other blood flukes by being both

paraphyletic and including lineages that possess and lack a ventral sucker. The fish blood flukes are evidently monophyletic (Oréelis -Ribeiro et al., 2014), and none have a ventral sucker, whereas the schistosomes also are monophyletic (Brant and Loker, 2009), and all but 2 genera (*Gigantobilharziella* Odhner, 1910 and *Dendritobilharzia* Skrjabin and Zakharow, 1920) have a ventral sucker. *Gigantobilharziella* and *Dendritobilharzia* are sister taxa, indicating a loss of a ventral sucker. This discussion excludes the dioecious crocodilian blood fluke *Griphobilharzia amoena* Platt and Blair, 1991 as per Bullard et al. (2019). Species of *Atamatum* and *Paratamatum* are the only TBFs that lack a ventral sucker within the clade including all related marine TBFs, schistosomes, and the new species herein. This suggests that these TBFs lost the ventral sucker since both *Carrettacola* and the other related marine TBFs have a ventral sucker, and the latter lineage is sister to the South American freshwater TBFs. In addition, species of *Coeritrema*, Platt, and *Hapalorhynchus* (as well as the enigmatic *G. amoena*) all have a ventral sucker and are monophyletic; suggesting that the ventral sucker of this group comprises a robust synapomorphy. Species of *Uterotrema* and *Vasotrema* all have a ventral sucker and share a common ancestor with *Spirhapalum* and *Spirorchis*. Neither of the latter genera includes a species that has a ventral sucker, suggesting that the ventral sucker was lost in this lineage. Noteworthy also is that *Neospororchis* includes species that infect marine turtles only. Perhaps supporting the distant phylogenetic position of this marine TBF from other marine TBFs, it also is the only marine TBF genus having species that lack a ventral sucker. These observations underscore the importance of comparative anatomical study of these ventral suckers.

The presence or absence of a ventral sucker in TBFs is evidently not related to the site of infection, i.e., TBFs having a ventral sucker reportedly infect many sites within their hosts; not exclusively voluminous blood vessels. Collectively, TBFs having a ventral sucker infect heart,

mesenteric and cranial blood vessels, liver, lung, spleen, and kidney; with some records indicating only “blood” or “blood vessels.” None of those TBFs clade by tissue site of infection. In fact, few genera comprise species that infect a single organ or tissue: *Cheloneotrema* (mesenteric capillaries), *Hapalotrema* (heart), *Monticellius* Mehra, 1939 (heart), *Neocaballerotrema* Simha, 1977 (intestinal blood vessels), *Plasmiorchis* Mehra, 1934 (heart), *Satyanarayanotrema* (“blood vessels”), *Shobanatrema* Simha and Chattopadhyaya, 1980 (mesenteric capillaries of large intestine).

Few side-necked turtles (Pleurodira) have been examined for blood fluke infections, and probably many new species of TBFs infect this turtle lineage. Including this study, only 6% (6 of 92) of the named species of side-necked turtles are identified as hosts: 3 chelids (*Myuchelys latisternum*, *Emydura macquarrii*, *Chelus fimbriata*) in Australia and South America, 2 pelomedusids in Africa (*Pelusios williamsi* and *Pelusios castaneus*), and 1 podocnemidid in South America (*P. sextuberculata*) (Bourgat and Kulo, 1987; Goodman, 1987; Platt and Blair, 1996, Bullard et al., 2019; and present study). *Pitiutrema revelae* is only the third TBF described from a freshwater turtle in South America and the eighth TBF from a pleurodire worldwide. This exemplifies how vastly under represented these Southern Hemisphere TBFs are in the taxonomic literature, c.f. 27 spp. in North America and 30 spp. in Asia (Smith, 1997; Platt, 2002; Roberts and Bullard, 2017; Roberts et al., 2016a, 2017).

Key to genera of turtle blood flukes

1a. Cirrus sac and uterus/metraterm between ventral sucker and ovary (ovary pre-testicular).....	2
1b. Male and female genitalia not as 1a.....	3
2a. Testes numerous.....	<i>Caretta</i>
2b. Testis single	<i>Vasotrema</i>
3a. Testes column pre-ovarian; ESV and ovary between uterus and testicular column.....	4
3b. Testes column absent; terminal genitalia not as in 3a.....	7
4a. Anterior portion of ceca at bifurcation U-shaped; Manter's organ absent.....	<i>Monticellius</i>
4b. Anterior portion of ceca at bifurcation M-shaped; Manter's organ present	5
5a. Ventral sucker absent	<i>Spirorchis</i>
5b. Ventral sucker present.....	6
6a. Transverse vitelline duct inter-testicular.....	<i>Spirhapalum</i>
6b. Transverse vitelline duct post-testicular	<i>Plasmiorchis</i>
7a. Uterus/metraterm massive, occupying large portion of intercecal space.....	8
7b. Uterus/metraterm not massive, not occupying large portion of intercecal space	9
8a. Ventral sucker and body aspinose, ovary inter-testicular; 2 testes	<i>Enterohaematotrema</i>
8b. Ventral sucker and body spinose, ovary pre-testicular; 1 testis.....	<i>Uterotrema</i>
9a. Ovary post-testicular	10
9b. Ovary inter-testicular	12
10a. Cyclocoel present, ceca fused posteriorly.....	<i>Neospororchis</i>
10b. Cyclocoel absent, a single cecum present.....	11
11a. Terminal genitalia post-cecal	<i>Baracktrema</i>

11b. Terminal genitalia at level anterior to posterior ends of ceca.....	<i>Unicaecum</i>
12a. Cirrus sac and external seminal vesicle pre-testicular	13
12b. Cirrus sac and external seminal vesicle inter-testicular.....	16
13a. Ventral sucker papillate; cirrus sac massive, directed anteriorly or laterad	<i>Platt</i>
13b. Ventral sucker apapillate.....	14
14a. Ventrolateral tegumental mammillae present	<i>Coeuritrema</i>
14b. Ventrolateral tegumental mammillae absent	15
15a. Ventral sucker minute, far posterior to cecal bifurcation; esophagus short.....	<i>Cardiotrema</i>
15b. Ventral sucker large, typically immediately post-cecal bifurcation; esophagus long	
.....	<i>Hapalorhynchus</i>
16a. Testes >2 in number	17
16b. Testes 2 in number	18
17a. Testes divided into pre- and post-ovarian fields	<i>Hapalotrema</i>
17b. Testes pre-ovarian.....	<i>Learedius</i>
18a. Body aspinose	19
18b. Body spinose.....	21
19a. Ventral sucker present at body constriction	<i>Pitiutrema</i> , n. gen.
19b. Ventral sucker present with no body constriction	20
20a. Body slender; ceca smooth; vitellarium anterior to cecal bifurcation.....	<i>Amphiorchis</i>
20b. Body broad; ceca diverticulate anteriorly; vitellarium post-ventral sucker.....	
.....	<i>Satyanarayanotrema</i>
21a. Cyclocoel present.....	<i>Shobanotrema</i>
21b. Cyclocoel absent.....	22

22a. Ventral sucker present, oral sucker aspinose23

22b. Ventral sucker absent, oral sucker spinose24

23a. Genital pore anterior to anterior testis..... *Neocaballerotrema*

23b. Genital pore post-testicular (posterior to posterior testis).....*Chelonotrema*

24a. Body ovoid; ESR abutting anterior testis; OSR at level of TVD *Atamatam*

24b. Body thread-like; ESR not abutting anterior testis; OSR posterior to TVD.....
.....*Paramatam*

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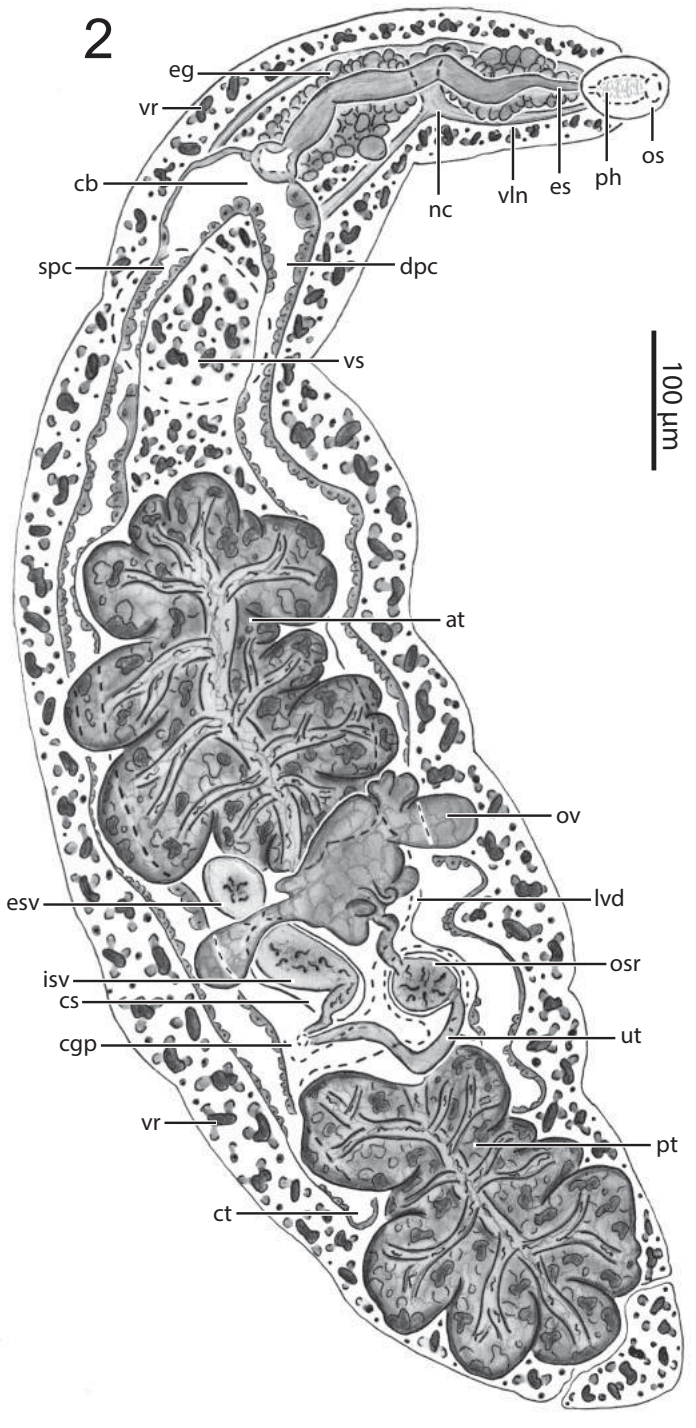
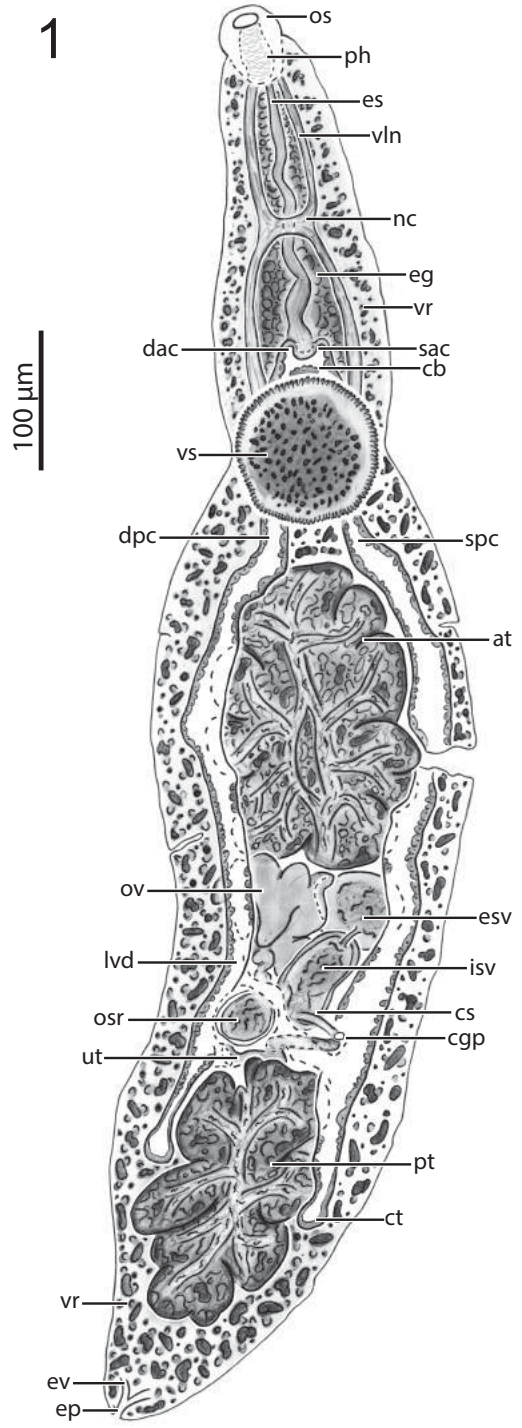
FIGURE LEGENDS

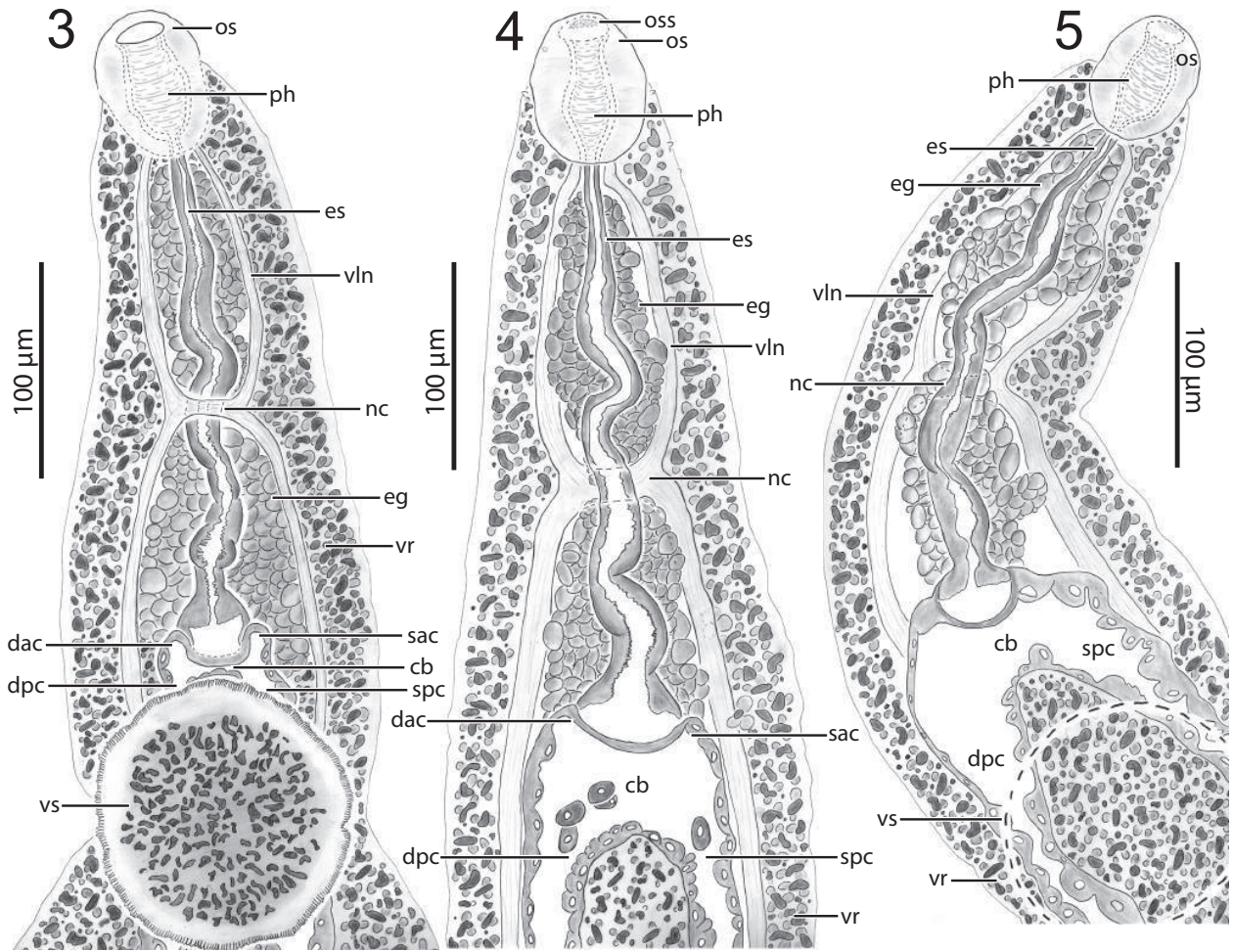
Figures 1–2. *Pitiutrema revelae* n. gen., n. sp. from six-tubercled Amazon River turtle, *Podocnemis sextuberculata* (Pleurodira: Podocnemididae) from the Amazon River in Iquitos, Peru. Scale value aside bars. **(1)** Body of holotype (USNM 1578590) showing oral sucker (os), pharynx (ph), esophagus (es), ventrolateral nerve chords (vln), nerve commissure (nc), esophageal gland (eg) illustrated behind esophagus for clarity, vitellarium (vr), sinistral anterior cecum (sac), dextral anterior cecum (dac), cecal bifurcation (cb), ventral sucker (vs), sinistral posterior cecum (spc), dextral posterior cecum (dpc), anterior testis (at), ovary (ov), external seminal vesicle (esv), internal seminal vesicle (isv), lateral vitelline collecting ducts (lvd), cirrus sac (cs), oviducal seminal receptacle (osr), common genital pore (cgp), uterus (ut), posterior testis (pt), cecal terminus (ct), excretory vesicle (ev), and excretory pore (ep). Ventral view. **(2)** Body of paratype (USNM 1578591), showing features labeled in Fig. 1. Dorsal view.

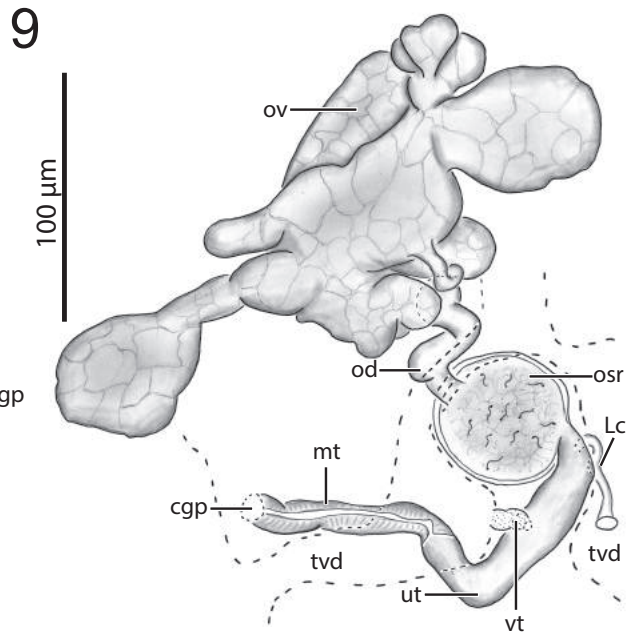
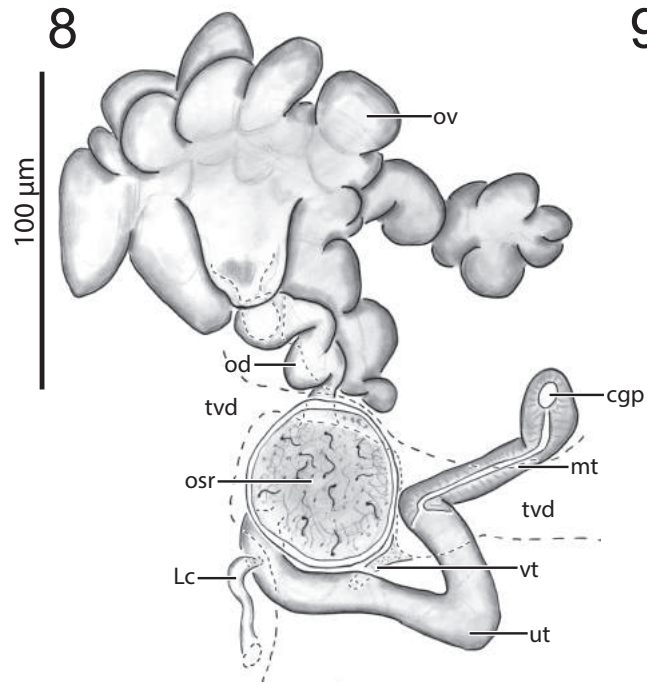
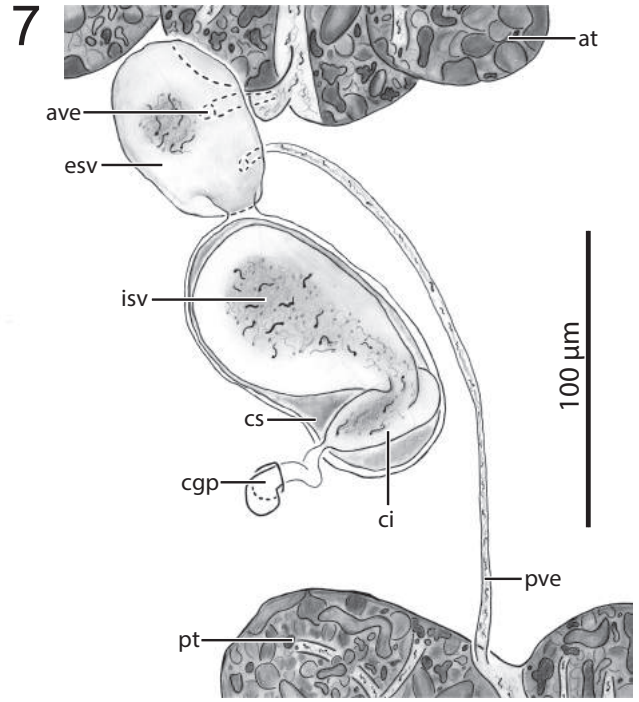
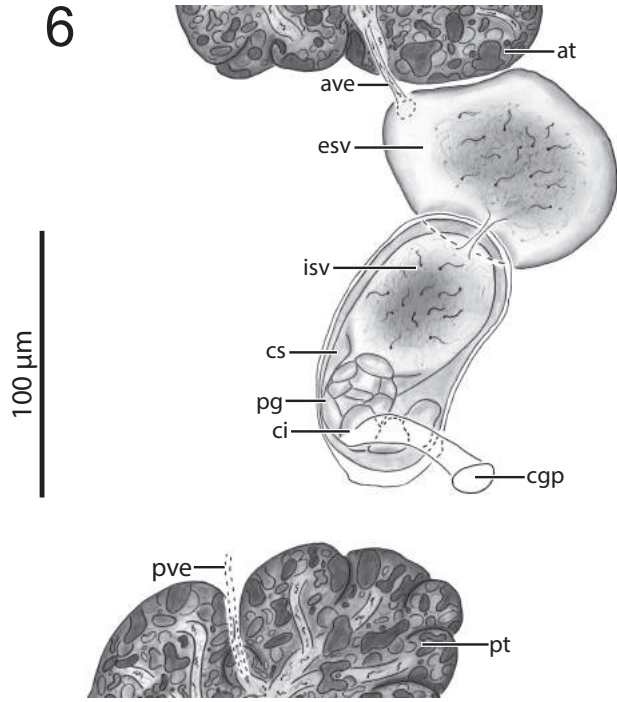
Figures 3–5. *Pitiutrema revelae* n. gen., n. sp. from six-tubercled Amazon River turtle, *Podocnemis sextuberculata* (Pleurodira: Podocnemididae) from the Amazon River in Iquitos, Peru. Scale value aside bars. **(3)** Anterior end of holotype (USNM 1578590) showing oral sucker (os), pharynx (ph), esophagus (es), ventrolateral nerve chords (vln), nerve commissure (nc), esophageal gland (eg), vitellarium (vr), sinistral anterior cecum (sac), dextral anterior cecum (dac), cecal bifurcation (cb), sinistral posterior cecum (spc), dextral posterior cecum (dpc), ventral sucker (vs). Ventral view. **(4)** Anterior end of paratype (USNM 1578593), showing features labeled in Fig. 3 in addition to oral sucker spines (oss) and with the exception of the ventral sucker (vs) that was destroyed. Dorsal view. **(5)** Anterior end of paratype (USNM 1578591), showing features labeled in Fig. 3 in addition to oral sucker spines (oss). Dorsal view.

Figures 6–9. *Pitiutrema revelae* n. gen., n. sp. from six-tubercled Amazon River turtle, *Podocnemis sextuberculata* (Pleurodira: Podocnemididae) from the Amazon River in Iquitos, Peru. Scale value aside bars. **(6)** Male genitalia of holotype (USNM 1578590) showing anterior testis (at), anterior vas efferens (ave), external seminal vesicle (esv), internal seminal vesicle (isv), cirrus sac (cs), prostate glands (pg), cirrus (ci), common genital pore (cgp), posterior vas efferens (pve), and posterior testis (pt). Ventral view. **(7)** Male genitalia of paratype (USNM 1578591), showing features labeled in Fig. 6 with the exception of the prostate glands (pg). Dorsal view. **(8)** Female genitalia of holotype (USNM 1578590) showing ovary (ov), oviduct (od), transverse vitelline duct (tvd – dashed), oviducal seminal receptacle (osr), Laurer’s canal (Lc), uterus (ut), primary vitelline duct (vt), metraterm (mt), and common genital pore (cgp). Ventral view. **(9)** Female genitalia of paratype (USNM 1578591), showing features labeled in Fig. 8. Dorsal view.

Figure 10. Bayesian phylogeny based on the large subunit ribosomal DNA (28S). South American turtle blood flukes indicated by shaded box; life history stage indicated in parentheses; ventral sucker present (dot). Values aside nodes are posterior probability. Scale bar is in substitutions per site.







10

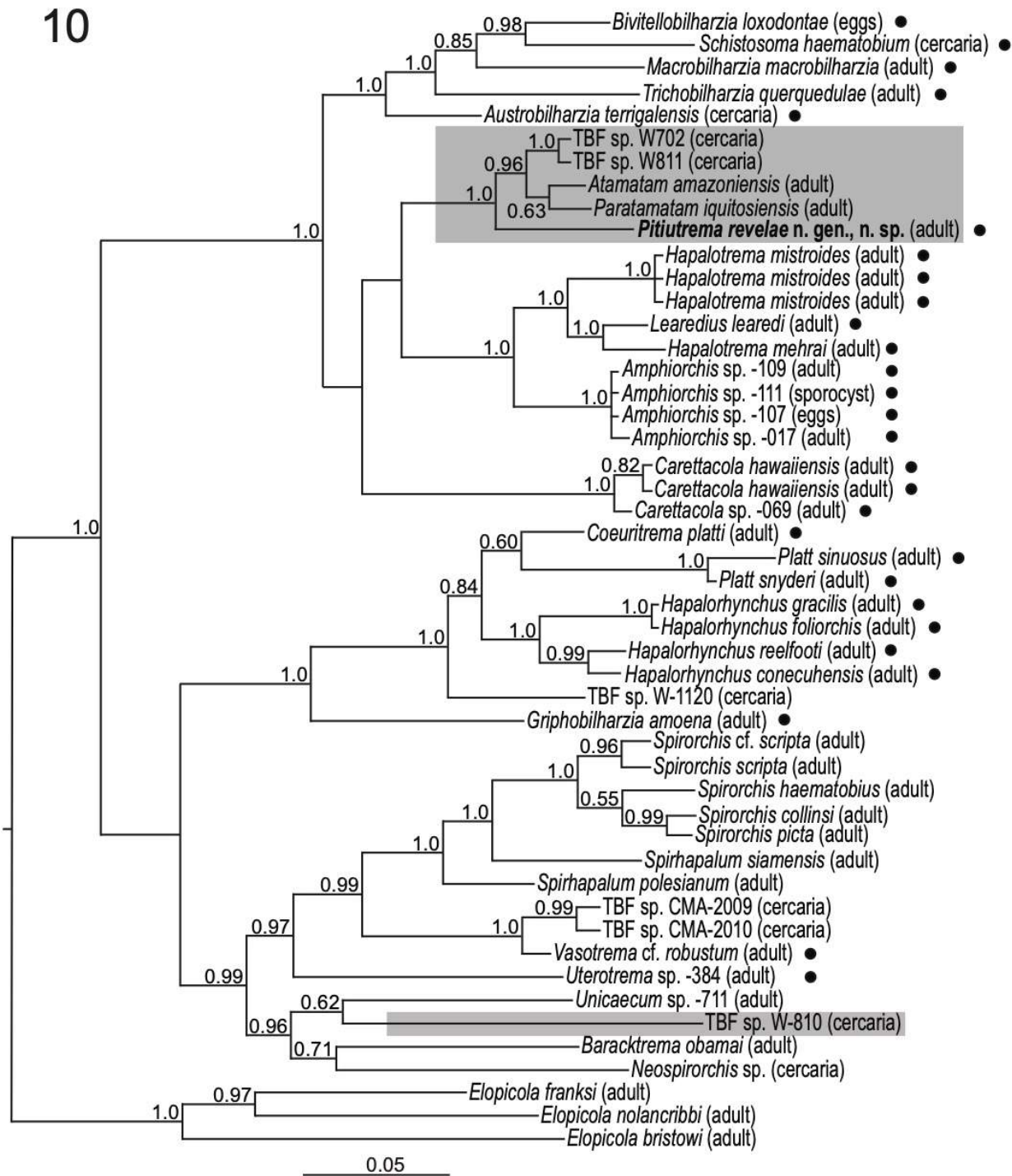


Table I. 28S sequences used herein.

Blood fluke	Host	Locality	GenBank Nos.	Reference	Source of specimens (life history stage)
			28S		
Turtle blood flukes					
<i>Amphiorchis</i> sp.	<i>Caretta caretta</i> (Linnaeus 1766), loggerhead sea turtle	Oceanographic Aquarium, Valencia, Spain	KX987107	Cribb et al., 2017	Serosa (eggs)
<i>Amphiorchis</i> sp.	<i>Caretta caretta</i> (Linnaeus 1766), loggerhead sea turtle	Florida	KX987109	Cribb et al., 2017	Not specified (adult)
<i>Amphiorchis</i> sp.	<i>Thylaeodus</i> cf. <i>rugulosus</i> , vermetid gastropod	Oceanographic Aquarium, Valencia, Spain	KX987111	Cribb et al., 2017	Not specified (sporocyst)
<i>Amphiorchis</i> sp.	<i>Chelonia mydas</i> (Linnaeus, 1758), green sea turtle	University of Florida, Florida	KU892017	Chapman et al., 2016	Not specified (adult)
<i>Atamatam amazoniensis</i> Bullard and Roberts, 2019	<i>Chelus fimbriata</i> (Schneider, 1783), matamata	Upper Amazon River Basin (3°45'32.08"S, 73°14'53.15"W), Belén Market, Iquitos, Peru	MK775718	Bullard et al., 2019	Blood vessels of kidney and mesentery (adult)
<i>Baracktrema obamai</i> Roberts, Platt, and Bullard, 2016	<i>Siebenrockiella crassicollis</i> (Gray, 1831), black marsh turtle	Perak, Perak River, Malaysia	KX061500	Roberts et al., 2016b	Lung (adult)
<i>Carettacola hawaiiensis</i> Dailey, Fast, and Balazs, 1991	<i>Chelonia mydas</i> (Linnaeus, 1758), green sea turtle	Pacific Ocean, Hawaii	AY604709	Snyder, 2004	Not specified (adult)
<i>Carettacola hawaiiensis</i> Dailey, Fast, and Balazs, 1991	<i>Chelonia mydas</i> (Linnaeus, 1758), green sea turtle	Queensland, Australia	KU600068	Chapman et al., 2016	Liver, spleen, brain, mesentery (adult)

<i>Carettacola</i> sp.	<i>Chelonia mydas</i> (Linnaeus, 1758), green sea turtle	Queensland, Australia	KU600069	Chapman et al., 2016	Brain (adult)
<i>Coeuritrema platti</i> Roberts and Bullard, 2016	<i>Pelodiscus sinensis</i> (Wiegmann, 1835), Chinese softshell turtle	Da Rang River, Phuyen Province, Vietnam	KX712243	Roberts et al., 2016b	Heart, mesentery, lung (adult)
<i>Hapalorhynchus conecuhensis</i> Roberts and Bullard, 2017	<i>Sternotherus minor</i> (Agassiz, 1857), loggerhead musk turtle	Blue Spring, Yellow River drainage, (31°5'27.64"N, 86°30'53.21"W), Alabama	MF568036	Roberts et al., 2017	Heart (adult)
<i>Hapalorhynchus foliorchis</i> Brooks and Mayes, 1975	<i>Chelydra serpentina</i> (Linnaeus, 1758), common snapping turtle	Pond off Saugahatchee Creek (Tallapoosa River) (32°39'1.36"N, 85°29'4.70"W), Alabama	KX712242	Roberts et al., 2017	Mesenteric blood vessels (adult)
<i>Hapalorhynchus gracilis</i> Stunkard, 1922	<i>Chelydra serpentina</i> (Linnaeus, 1758), common snapping turtle	Reelfoot Lake (Mississippi River) (36°21'12.23"N, 89°25'21.50"W), Tennessee	AY604710	Snyder, 2004	Not specified (adult)
<i>Hapalorhynchus reelfooti</i> Byrd, 1939	<i>Sternotherus odoratus</i> Latreille in Sonnini and Latreille, (1801), common musk turtle	Pond off Odom Creek (Tallapoosa River), (32°30'9.58"N, 85°26'6.07"W), Alabama	MF568030	Roberts et al., 2017	Mesenteric blood vessels and blood wash
<i>Hapalotrema mehrai</i> Rao, 1976	<i>Chelonia mydas</i> (Linnaeus, 1758), green sea turtle	Pacific Ocean, Hawaii	AY604708	Snyder, 2004	Not specified (adult)
<i>Hapalotrema mistroides</i> (Monticelli, 1896) Stiles and Hassall, 1908	<i>Caretta caretta</i> (Linnaeus, 1766), loggerhead sea turtle	Latium, Italy	KY499799	Santoro et al., 2017	Spleen (adult)
<i>Hapalotrema mistroides</i> (Monticelli, 1896) Stiles and Hassall, 1908	<i>Caretta caretta</i> (Linnaeus, 1766), loggerhead sea turtle	Florida	KU892016	Chapman et al., 2016	Gallbladder, liver, lung, spleen, thyroid (adult)

<i>Hapalotrema mistroides</i> (Monticelli, 1896) Stiles and Hassall, 1909	<i>Caretta caretta</i> (Linnaeus 1766), loggerhead sea turtle	Northern Adriatic coast, Italy	LT882715	Marchiori et al., 2017	Heart (adult)
<i>Learedius learedi</i> Price, 1934	<i>Chelonia mydas</i> (Linnaeus, 1758), green sea turtle	Pacific Ocean, Hawaii	AY604707	Snyder, 2004	Not specified (adult)
<i>Neospororchis</i> sp. CT-2017	<i>Caretta caretta</i> (Linnaeus 1766), loggerhead sea turtle	Northern Adriatic coast, Italy	LT882716	Marchiori et al., 2017	Mesentery and feces (eggs)
<i>Paratamata iquitosiensis</i> Bullard and Roberts, 2019	<i>Chelus fimbriata</i> (Schneider, 1783), matamata	Upper Amazon River Basin (3°45'32.08"S, 73°14'53.15"W), Belén Market, Iquitos, Peru	MK775719 MK775720	Bullard et al., 2019	Blood vessels of kidney and mesentery (adult)
<i>Pitiutrema revelae</i> n. gen. n. sp.	<i>Podocnemis sextuberculata</i> (Cornalia, 1849), six-tubercled Amazon River turtle	Upper Amazon River Basin (3°45'32.08"S, 73°14'53.15"W), Belén Market, Iquitos, Peru	MN244241	Present study	Heart (adult)
<i>Platt sinuosus</i> Roberts and Bullard, 2018	<i>Malayemys subtrijuga</i> (Schlegel and Müller, 1845), Mekong snail-eating turtle	Can Tho, Mekong River drainage (10°01'42.15"N, 105°47'14.15"E), Vietnam	MG579527	Roberts et al., 2018a	Blood vessels of kidney and mesentery (adult)
<i>Platt snyderi</i> (Platt and Sharma, 2012) Roberts and Bullard, 2018	<i>Malayemys subtrijuga</i> (Schlegel and Müller, 1845), Mekong snail-eating turtle	Can Tho, Mekong River drainage (10°01'42.15"N, 105°47'14.15"E), Vietnam	MF579528	Roberts et al., 2018a	Viscera wash (adult)
<i>Spirhupalum polesianum</i> Ejsmont, 1927	<i>Emys orbicularis</i> (Linnaeus, 1758), European pond slider	Lesniki, Kyiv Region, Ukraine	AY604705	Snyder, 2004	Not specified (adult)
<i>Spirhupalum siamensis</i> Tkach, Snyder, and Vaughan, 2009	<i>Cuora amboinensis</i> (Daudin, 1802), Malayan box turtle	Mae Sot, Moei River (16°42'N; 98°34'W), Thailand	FJ481166	Tkach et al., 2009	Heart (adult)

<i>Spirorchis artericola</i> (Ward, 1921) Stunkard, 1921	<i>Chrysemys picta</i> (Schneider, 1783), painted turtle	Reelfoot Lake (36°21'43.03"N; 89°25'44.32"W), Tennessee	AY604704	Snyder, 2004	Not specified (adult)
<i>Spirorchis collinsi</i> Roberts and Bullard, 2016	<i>Deirochelys reticularia</i> (Latreille <i>in</i> Sonnini and Latreille, [1801]), chicken turtle	Big Beaver Pond (Tallapoosa River) (32°25'44.03"N; 85°38'44.87"W), Tuskegee, Alabama	KY091664	Roberts et al., 2016c	Blood vessels of kidney and blood wash (adult)
<i>Spirorchis haematobius</i> (Stunkard, 1922) Price, 1934	<i>Chelydra serpentina</i> (Linnaeus, 1758), common snapping turtle	Pascagoula River (30°37'07.67"N; 88°36'44.53"W), Mississippi	FJ481164	Tkach et al., 2009	Not specified (adult)
<i>Spirorchis picta</i> Stunkard, 1923	<i>Trachemys scripta</i> (Thunberg <i>in</i> Schoepff, [1792]), pond slider	E.W. Shell Fisheries Station, Tallapoosa River drainage (32°38'53"N; 85°29'07"W), Auburn, Alabama	KY091665	Roberts et al., 2016c	Mesenteric blood vessels (adult)
<i>Spirorchis scripta</i> Stunkard, 1923	<i>Trachemys scripta</i> (Thunberg <i>in</i> Schoepff, [1792]), pond slider	Moungers Creek, Pascagoula Bay drainage, Vancleave, Mississippi	AY222174	Olson et al., 2003	Not specified (adult)
<i>Spirorchis cf. scripta</i>	<i>D. reticularia</i> (Latreille <i>in</i> Sonnini and Latreille, [1801]), chicken turtle	Big Beaver Pond (Tallapoosa River) (32°25'44.03"N; 85°38'44.87"W), Tuskegee, Alabama	KY091666	Roberts et al., 2016c	Lung, kidney, body wash (adult)
<i>Unicaecum</i> sp.	<i>Trachemys scripta</i> (Thunberg <i>in</i> Schoepff, 1792), pond slider	Reelfoot Lake, Tennessee	AY604711	Snyder, 2004	Not specified (adult)
<i>Uterotrema</i> sp.	Not specified	Australia	MK878384	Bullard et al., 2019	Not specified (adult)
<i>Vasotrema cf. robustum</i>	<i>Apalone spinifera aspera</i> (Agassiz,	Round Lake (Cahaba River) (32°41'50.91"N; 87°14'30.39"W), Cahaba River, Alabama	MH843490	Roberts et al., 2019	Mesenteric blood vessels (adult)

TBF sp. W-702	1857), Gulf Coast spiny softshell turtle <i>Biomphalaria</i> sp. Preston, 1910, a planorbid gastropod	Pampulha Reservoir, Belo Horizonte, state of Minas Gerais (19°51'77"S; 43°58'54"W), Brazil	KT235799	Pinto et al., 2015	Not specified (cercariae)
TBF sp. W-810	<i>Pomacea</i> sp. Perry, 1810, an ampullariid gastropod	Pampulha Reservoir, Belo Horizonte, state of Minas Gerais (19°51'77"S; 43°58'54"W), Brazil	KT235798	Pinto et al., 2015	Not specified (cercariae)
TBF sp. W-811	<i>Biomphalaria</i> sp. Preston, 1910, a planorbid gastropod	Pampulha Reservoir, Belo Horizonte, state of Minas Gerais (19°51'77"S; 43°58'54"W), Brazil	KT235800	Pinto et al., 2015	Not specified (cercariae)
TBF sp. W-1120	<i>Biomphalaria sudanica</i> (Martens, 1870), a planorbid gastropod	Kazinga Channel, Jetty no. 2 (00°11'31"S; 29°53'53"E), Mwea Safari Lodge, Queen Elizabeth National Park, Uganda	AY858884	Brant et al., 2006	Not specified (cercariae)
TBF sp. CMA-2009	<i>Physa acuta</i> Draparnaud, 1805, a physid gastropod	(35.05"N, 106.41"W) New Mexico	FJ550134	Kraus et al., 2014	Not specified (cercariae)
TBF sp. CMA-2010	<i>Physa acuta</i> Draparnaud, 1805, a physid gastropod	Bosque del Apache, New Mexico	GU270101	Pinto et al., 2015	Not specified (cercariae)
Schistosomatidae					
<i>Austrobilharzia terrigalensis</i> Johnson, 1916	<i>Batillaria australis</i> (Quoy and Gaimard, 1834), a batillariid gastropod	Rodd Point, Iron Cove, Sydney Harbor, NSW, Australia	AY157249	Lockyer et al., 2003	Not specified (cercariae)
<i>Bivitellobilharzia loxodontae</i> Vogel and Minning, 1940	<i>Loxodonta cyclotis</i> (Matschie, 1900), African forest elephant	Bai Hokou field station (2°51'34"N, 16°28'03"E), Central African Republic	JN579949	Brant and Loker, 2013	Feces (eggs)
<i>Griphobilharzia amoena</i> Platt and Blair, 1991	<i>Crocodylus johnstoni</i> Krefft, 1873, freshwater crocodile	Darwin (probably Adelaide River Basin), Australia	AY899914	Brant and Loker, 2005	Kidney, lung, liver, spleen, mesentery (adult)

<i>Macrobilharzia macrobilharzia</i> Travassos, 1922	<i>Anhinga anhinga</i> (Linnaeus, 1766), anhinga	Ascension Parish, Sorrento Timberton Hunt Club, Louisiana	AY858885	Brant et al., 2006	Mesenteric blood vessels (adult)
<i>Schistosoma haematobium</i> (Bilharz, 1852) Weinland, 1858	<i>Bulinus nyassanus</i> (E. A. Smith, 1877), a planorbid gastropod	Cape Maclear (Nankumba Peninsula), Lake Malawi-Chembe, Malawi	EU567126	Marchiori et al., 2017	Not specified (cercariae)
<i>Trichobilharzia querquedulae</i> McLeod, 1937	<i>Anas discors</i> (Linnaeus, 1766), blue-winged teal	Florida	FJ174469	Brant and Loker, 2009	Not specified (adult)
Aporocotylidae					
<i>Elopicola bristowi</i> Orélis-Ribeiro and Bullard, 2017	<i>Elops hawaiiensis</i> Reagan, 1909, Hawaiian ladyfish	Vinh Hải fish market, Nha Trang (Eastern Sea), Vietnam	KY243881	Orélis-Ribeiro et al., 2017	Blood vascular system (adult)
<i>Elopicola franksi</i> Orélis- Ribeiro and Bullard, 2017	<i>Megalops atlanticus</i> Valenciennes, 1847, Atlantic tarpon	Gulf of Mexico, North Captiva Island and Bayboro Harbor, off Florida	KY243882	Orélis-Ribeiro et al., 2017	Heart (adult)
<i>Elopicola nolancribbi</i> Bullard, 2014	<i>Elops saurus</i> Linnaeus, 1766, ladyfish	Gulf of Mexico, off Ship Island, Mississippi	KY243880	Orélis-Ribeiro et al., 2017	Blood wash (adult)

CHAPTER 2: *RUAVERMIS MIKEBARGERI* GEN. ET SP. NOV. (DIGENEA: SCHISTOSOMATOIDEA) INFECTING THE YELLOW-HEADED TEMPLE TURTLE, *HEOSEMYS ANNANDALII* (CRYPTODIRA: GEOEMYDIDAE) IN VIETNAM, INCLUDING AN UPDATED PHYLOGENY FOR THE TURTLE BLOOD FLUKES.

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ABSTRACT

Ruavermis mikebargerii Dutton and Bullard n. g., n. sp. infects the yellow-headed temple turtles (*Heosemys annandalii* [Boulenger, 1903]) in the Mekong River Basin. It resembles *Platt Roberts* and Bullard, 2018 and *Coeuritrema* Mehra, 1933 by having the anterior to posterior anatomical sequence of a ventral sucker, external seminal vesicle, cirrus sac, anterior testis, ovary, transverse vitelline duct, and posterior testis. These genera are further similar by having the combination of an elongate/ovoid aspinous body, a ventral sucker at the level of the body constriction, an oesophagus that terminates in the anterior 1/5 of the body and that is ventral to the anterior nerve commissure, intestinal caeca that bifurcate in the anterior 1/3 of the body (not immediately anterior to ventral sucker), a sinistral caecum that bends toward the midline at level of the cirrus and common genital pore, an external seminal vesicle that abuts the anterodextral margin of the cirrus sac, an oviduct that emerges from the dextral margin of the ovary, and an oviducal seminal receptacle that comprises the middle portion of the oviduct. These genera lack lateral oesophageal diverticulae and a median oesophageal diverticulum. The new genus is unique by having a papillate ventral body surface, an external seminal vesicle lateral to the cirrus sac, vasa efferentia that are ventral to the gonads, an oviduct that is convoluted, a Laurer's canal pore that is pre-ovarian, a Laurer's canal that extends anterolaterad, and an excretory vesicle that

is Y-shaped. The 28S phylogenetic analysis recovered the new species sister to *Coeuritrema platti* Roberts and Bullard, 2016, with that clade sister to *Hapalorhynchus* spp. and *Platt* spp. The new turtle blood fluke is the 4th from Vietnam, 2nd from a Vietnam geomydid, and 1st from *Heosemys* Stejneger, 1902 as well as the 1st endohelminth from the yellow-headed temple turtle.

INTRODUCTION

The yellow-headed temple turtle, *Heosemys annandalii* (Boulenger, 1903), (Cryptodira: Geoemydidae), is a large pond- and river-dwelling turtle native to Southeast Asia, inhabiting rivers and freshwater marshes as well as estuaries in the lowland areas of southern Vietnam (van Dijk et al., 2000). Although the population status of this turtle has not been assessed, van Dijk and Palasuwan (2000) asserted that this species is probably vulnerable to habitat loss and likely being overharvested for food, cultural traditions related to magic or natural therapies, and the pet trade. In China, the demand for turtles is high such that adjacent countries may be affected (Ly et al., 2011), contributing to the so-called ‘Asian turtle crisis.’ Many Asian turtle species are threatened with extinction in the wild, and an estimated 13,000 metric tons of live turtles (equal to millions of individuals) are annually exported from southeast Asia to China to meet this demand (Ly et al., 2011). At present, the only parasites that have been documented from the yellow-headed temple turtle comprise an ectoparasitic leech, *Placobdelloides siamensis* (Oka, 1917) Sawyer, 1986, (Euhirudinea: Glossiphoniidae), and a blood-dwelling haemogregarine, *Hemogregarine* sp. (Apicomplexa: Adeleina: Haemogregarinidae) (Assawawongkasem and Chansue, 2008; Chiangkul et al., 2018).

Turtle blood flukes (TBFs; Platyhelminthes: Digenea: Schistosomatoidea) infect the cardiovascular system of turtles (Bullard et al., 2019, Dutton et al., 2019). Three of the 10 nominal TBFs that range in Southeast Asia (excludes India and China) are from Vietnam: 1

(*Platt sinuosus* Roberts and Bullard, 2018) infects a geomydid and 2 (*Coeluritrema platti* Roberts and Bullard, 2016 and *Hapalorhynchus mica* Oshmarin, 1971) infect softshelled turtles (Cryptodira: Trionychidae) (Roberts et al., 2016; 2018). Eight geomydid genera include turtle species that host TBFs (*Batagur* Gray 1856, *Cuora* Gray 1856, *Geoclemys* Gray 1856, *Hardella* Gray 1870, *Malayemys* Lindholm 1931, *Mauremys* Gray 1869, *Pangshura* Gray 1856, *Siebenrockiella* Lindholm 1929). The new species described herein is the first TBF from a species of *Heosemys*, the first endohelminth from *H. annandalii*, and we provide a phylogenetic analysis to corroborate our results using morphology.

MATERIALS AND METHODS

During a parasitological expedition to Vietnam in September 2018, we encountered specimens of a new TBF species infecting the heart of a yellow-headed temple turtle that was opportunistically sampled for TBF infections from the Cao Lanh Farmer's Market (10°27'13.32"N, 105°38'14.97"E) Cao Lanh, Đồng Tháp Province, Vietnam (Mekong River). TBF specimens intended for morphology as whole-mounts were observed microscopically, heat-killed on glass slides using a butane hand lighter under little or no coverslip pressure, fixed in 10% neutral buffered formalin (nbf), rinsed with water, stained in Van Cleave's hematoxylin with several drops of Ehrlich's hematoxylin, dehydrated through a graded series of EtOHs, made basic at 70% EtOH with lithium carbonate and butyl-amine, dehydrated in absolute EtOH and xylene, cleared with clove oil, and permanently mounted on glass slides using Canada balsam (Dutton et al., 2019). The resulting whole mounts were examined and illustrated with the aid of compound microscopes (Leica DM2500 and Leica DMR [Leica, Wetzlar, Germany]) equipped with differential interference contrast (DIC) optical components and drawing tubes. Measurements were obtained with a calibrated ocular micrometer (as straight-lines along the

course of each duct) and are reported in micrometers (μm) as the range followed by the mean, \pm standard deviation, and sample size in parentheses.

Type specimens of the new species were deposited in the National Museum of Natural History's Invertebrate Zoology Collection (Smithsonian Institution, USNM Collection Nos. 1613430–1613432). Classification and anatomical terms for TBFs follow Dutton et al. (2019). Turtle scientific and common names follow Rhodin et al. (2017), and higher classification of turtles follows Pereira et al. (2017).

Total genomic DNA (gDNA) was extracted from 3 EtOH-preserved and microscopically-identified blood flukes using DNeasy™ Blood and Tissue Kit (Qiagen, Valencia, California) as per the manufacturer's protocol except that the proteinase-K incubation period was extended overnight, and 100 μL of elution buffer was used to increase the final DNA concentration. Amplification and sequencing of the D1–D3 domains of the large subunit ribosomal DNA (28S) used the primer set of Orélis-Ribeiro et al. (2017). PCR amplifications were performed according to Dutton et al. (2019). DNA sequencing was performed by ACGT, Incorporated (Wheeling, Illinois). Reactions were sequenced using BigDye terminator version 3.1, cleaned with magnetic beads (CleanSeq dye terminator removal kit; Beckman Coulter, Brea, California), and analyzed using an ABI 3730 XL or 3730 Genetic Analyzer (Applied Biosystems, Waltham, Massachusetts). Sequence assembly and analysis of chromatograms were completed with Geneious version 11.0.5 (<http://www.geneious.com>; Kearse et al., 2012). All nucleotide sequence data were deposited in GenBank (MT103553).

The phylogenetic analysis included the taxa included in Dutton et al. (2019) as well as the new taxon. As per Dutton et al. (2019), sequences were aligned using MAFFT (Kato 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 4 Metropolis-coupled chains were run for 5,000,000 generations, sampling the posterior distribution every 1,000 generations. Convergence was checked using Tracer v1.6.1 (Rambaut et al., 2014) and the “sump” command in MrBayes: all runs appeared to reach convergence after discarding the first 25% of generation as burn-in. A majority rule consensus tree of the post burn-in posterior distribution was generated with the “sumt” command in MrBayes. The inferred phylogenetic tree was visualized using FigTree v1.4.3 (Rambaut et al., 2014) and further edited for visualization purposes with Adobe Illustrator (Adobe Systems).

RESULTS

***Ruavermis* Dutton and Bullard n. gen.** (Figs. 1–3)

Generic diagnosis of adult (based on three whole-mounted specimens; USNM coll. nos. 1613430–1613432): Body dorsoventrally flat (not cylindrical), ventrally concave, ovoid (not thread-like), 4–5× longer than wide, lanceolate; mammillae absent. Oral sucker robust, demarcated from body by constriction, apapillate, aspinous. Ventral sucker present, crenulate (having papillate rim), at level of body constriction (forebody more narrow than hind-body). Pharynx present, enveloping anterior extremity of oesophagus. Oesophagus straight or slightly sinuous, terminating in anterior 1/5 of body, ventral to anterior nerve commissure, lateral oesophageal diverticula and median oesophageal diverticulum absent; oesophageal gland

surrounding oesophagus from posterior margin of pharynx to caecal bifurcation, widest at level of caecal bifurcation. Intestinal caeca inverse U-shaped, comprising non-fused caeca bifurcating in anterior 1/3 of body (not immediately anterior to ventral sucker), lacking diverticulae, not extensively convoluted, sinistral caecum bending mediad at level of cirrus and around common genital pore, extending approximately 3/4 of body length posteriad, terminating near posterior body extremity, slightly asymmetrical. Testes 2 in number, comprising a pre-ovarian (anterior) testis and a post-ovarian (posterior) testis, inter-caecal, having deep lobes or slightly irregular margins, ovoid (slightly longer than wide). Anterior and posterior trunks of vasa efferentia present, ventral to gonads, connecting to vas deferens; vas deferens extending directly anteriad ventral to cirrus sac, expanding to form external seminal vesicle; external seminal vesicle posterior to ventral sucker, lateral to the cirrus sac, inter-caecal, extending anteromediad. Cirrus sac robust, directed anterosinistrad, enclosing internal seminal vesicle and cirrus, containing variously sized gland-like cells. Pars prostatica indeterminate. Ovary primarily sinistral, slightly transverse, non inter-caecal, inter-testicular, flanked by testes anteriorly and posteriorly, having smooth margins. Proximal portion of oviduct emerging from dextral margin of ovary, directed posteriad, convoluted before connecting to oviducal seminal receptacle; oviducal seminal receptacle comprising middle portion of oviduct, posterior to transverse vitelline duct; distal portion of oviduct straight (not coiled), inter-caecal, extending anteriad, sinuous, dorsal to ovary, with distal end comprising metraterm. Laurer's canal inter-caecal, inter-testicular, extending anteriad to posterior margin of anterior testis from oviduct at anterior margin of ovary, with dorsal pore. Vitellarium distribution symmetrical, comprising a series of dispersed large asymmetrical spheroid masses of vitelline follicles, surrounding caeca, distributing from caecal bifurcation to caecal tips (surrounding caeca); transverse vitelline duct inter-testicular,

approximately at level of ovary, comprising lateral collecting ducts ventral to caeca, ventral to gonads. Metraterm anterior to ovary, longitudinal (extending anteriorly in parallel with body margin), sinistral to anterior testis, inter-gonadal, having obvious muscular thick walls; uterine pouch absent; egg not observed. Common genital pore dorsal, sinistral, marginal (extra-caecal or near body margin), between sinistral caecum and body margin, posterior to ventral sucker, predominately anterior to genitalia. Excretory vesicle Y-shaped, extending to tips of caecae; excretory pore subterminal. Manter's organ absent.

Differential Diagnosis: Body flat, 4–5× longer than wide, mammillae absent, papillate. Oral sucker aspinous. Ventral sucker crenulate, at level of body constriction. Lateral oesophageal diverticula and median oesophageal diverticulum absent. Intestinal caeca inverse U-shaped, bifurcating in anterior 1/3 of body, sinistral caecum bending toward midline at level of cirrus around common genital pore. Testes 2, inter-caecal. Male terminal genitalia pre-gonadal. Vas deferens ventral to cirrus sac; anterior and posterior trunks of vasa efferentia ventral to gonads. External seminal vesicle lateral to cirrus sac. Cirrus sac directed anterosinistral. Ovary inter-testicular. Proximal portion of oviduct emerging from dextral margin of ovary, convoluted before connecting to oviducal seminal receptacle; oviducal seminal receptacle comprising middle portion of oviduct, posterior to transverse vitelline duct. Laurer's canal inter-testicular; pore pre-ovarian, extends anterolaterad. Transverse vitelline duct inter-testicular, ventral to gonads. Metraterm anterior to ovary, sinistral to anterior testis. Common genital pore sinistral, dorsal, between sinistral caecum and body margin. Excretory vesicle Y-shaped, extending to tips of caeca; Manter's organ absent.

Taxonomic summary

Type-species: Ruavermis mikebarger Dutton and Bullard n. sp. (Digenea: Schistosomatoidea)

Type host: The yellow-headed temple, *Heosemys annandaliii* (Boulenger, 1903) (Cryptodira: Geoemydidae).

Etymology: “*Rua*” is the Vietnamese word for hard shelled turtle, and “*vermis*” is for worm.

***Ruavermis mikebarger* Dutton and Bullard n. sp.** (Figs. 1–3)

Diagnosis of adult (based on three whole-mounted specimens; USNM coll. nos. 1613430–1613432):

Body 1,800–1,952 ($1,881 \pm 76$; 3) long, 400–469 (439 ± 35 ; 3) in maximum width at level of caecal bifurcation, 4–5 (4 ± 0 ; 3) longer than wide; ventrolateral tegumental mammillae absent. Body surface having asymmetrically arranged tegumental projections (approximately 1–2 long, 1 wide) ventrally and dorsally on the anterior end.

Oral sucker 109–114 (112 ± 3 ; 3) long or 6% ($6\% \pm 0\%$; 3) of body length, 64–77 (68 ± 8 ; 3) wide or 14–17% ($16\% \pm 2\%$; 3) of maximum body width, oral sucker spines absent. Ventral sucker 191–222 (210 ± 17 ; 3) long or 10–12% ($11\% \pm 1\%$; 3) of body length, 164–182 (176 ± 10 ; 3) wide or 62–68% ($66\% \pm 4\%$; 3) of maximum body width. Nerve commissure 227–236 (233 ± 5 ; 3) or 12–13% ($12\% \pm 1\%$; 3) of body length from anterior body end. Pharynx 82–91 (85 ± 5 ; 3) or 24–27% ($25\% \pm 2\%$; 3) of oesophagus length, 100–111 (105 ± 6 ; 3) wide or 1.8–2.3 \times (2.1 ± 0.3 ; 3) wider than maximum oesophagus width. Oesophagus 336–345 (341 ± 5 ; 3) long or 18–19% ($18\% \pm 1\%$; 3) of body length, 23 (23 ± 0 ; 3) wide immediately posterior to pharynx and with wall 9 (9 ± 0 ; 3) thick, 45–52 (47 ± 4 ; 3) wide or 19–27% ($22\% \pm 4\%$; 3) of body width at mid-oesophagus and with wall 16–18 (17 ± 1 ; 3) thick, 25–45 (35 ± 10 ; 3) wide or 8–19% ($13\% \pm 5\%$; 3) of body width at caecal bifurcation and with wall 11–16 (14 ± 3 ; 3) thick;

oesophageal gland 252–295 (281 ± 25 ; 3) long or 13–16% ($15\% \pm 3\%$; 3) of body length, 157–170 (162 ± 7 ; 3) wide or 33–40% ($37\% \pm 3\%$; 3) of body width. Intestine bifurcating 102–123 (111 ± 11 ; 3) or 6–7% ($6\% \pm 0\%$; 3) of body length from anterior body end, anterior caeca absent; posterior caeca extending posteriad approximately in parallel with lateral body margin, slightly sinuous; sinistral posterior caecum 1,219–1,333 ($1,273 \pm 57$; 3) long or 65–70% ($68\% \pm 3\%$; 3) of body length, 45–57 (51 ± 6 ; 3) wide or 15–24% ($19\% \pm 4\%$; 3) of body width at level of caecal bifurcation, 45–66 (53 ± 11 ; 3) wide or 11–14% ($12\% \pm 2\%$; 3) of body width at level of ovary, 32–57 (43 ± 13 ; 3) wide or 13–23% ($17\% \pm 5\%$; 3) of body width at ends of caeca, caeca terminating 184–238 (216 ± 29 ; 3) or 11–13% ($12\% \pm 1\%$; 3) of body length from posterior body end; dextral posterior caecum 1,238–1,285 ($1,260 \pm 24$; 3) long or 65–70% ($67\% \pm 2\%$; 3) of body length, 43–50 (46 ± 4 ; 3) wide or 16–19% ($17\% \pm 1\%$; 3) of body width at level of caecal bifurcation, 48–61 (56 ± 7 ; 3) wide or 11–15% ($13\% \pm 2\%$; 3) of body width at level of ovary, 27–43 (34 ± 8 ; 3) wide or 11–17% ($14\% \pm 3\%$; 3) of body width at level of ends of caeca; caeca terminating 193–279 (237 ± 43 ; 3) or 11–15% ($13\% \pm 2\%$; 3) of body length from posterior body end.

Anterior testis 214–232 (224 ± 9 ; 3) long or 11–13% ($12\% \pm 1\%$; 3) of body length or $1.0\text{--}1.1 \times$ (1.1 ± 0.1 ; 3) posterior testis length, 136–159 (151 ± 13 ; 3) wide or 29–40% ($35\% \pm 5\%$; 3) of body width at level of ovary or 75–85% ($81\% \pm 6\%$; 3) of posterior testis width; inter-testicular space 80–95 (86 ± 8 ; 3) long or 4–5% ($5\% \pm 0\%$; 3) of body length. Posterior testis 202–207 (205 ± 3 ; 3) long or 11% ($11\% \pm 0\%$; 3) of body length, 182–193 (187 ± 6 ; 3) wide or 39–47% ($43\% \pm 4\%$; 3) of body width at level of ovary, 227–546 (358 ± 167 ; 3) or 13–28% ($19\% \pm 8\%$; 3) of body length from posterior body end (Fig. 3).

Anterior trunk of vasa efferentia emanating from ventral surface of anterior testis, extending anteriad 20–25 (23 ± 3 ; 3), 5 (5 ± 0 ; 3); posterior trunk of vasa efferentia emanating from the ventral surface of the posterior testis, extending anteriad 216–250 (239 ± 20 ; 3), or 11–14% ($13\% \pm 1\%$; 3), of body length, 4–5 (5 ± 1 ; 3) wide, ventral to ovary, meeting anterior trunk posterior to genital pore to form vas deferens; vas deferens extending anteriad 170–193 (184 ± 12 ; 3) of body length, 5 (5 ± 0 ; 3) wide before laterally expanding and turning dorsally to form external seminal vesicle (Fig. 3). External seminal vesicle longitudinal (not crossing the midline), directed anterosinistrad 107–140 (122 ± 17 ; 3) long or 6–7% ($6\% \pm 1\%$; 3) of body length, 45–67 (56 ± 11 ; 3) wide, 2–3 \times (2 ± 1 ; 3) wider than long, immediately posterior to anterior testis; internal seminal vesicle transverse, 159–170 (163 ± 6 ; 3) long or 8–9% ($9\% \pm 0\%$; 3) of body length, 52–64 (60 ± 7 ; 3) wide, 2–3 \times (3 ± 0 ; 3) longer than wide. Cirrus sac ovoid, 270–295 (287 ± 14 ; 3) long or 14–16% ($15\% \pm 1\%$; 3) of body length, 111–114 (113 ± 2 ; 3) wide or 24–29% ($26\% \pm 2\%$; 3) body width at level of genital pore; cirrus extending posterolaterad 227–272 (246 ± 23 ; 3) or 81–92% ($86\% \pm 6\%$; 3) of cirrus sac length, 23–53 (40 ± 16 ; 3) wide.

Ovary smooth and lobed, 177–205 (192 ± 14 ; 3) long or 9–11% ($10\% \pm 1\%$; 3) of body length, 120–134 (127 ± 7 ; 3) wide or 28–30% ($29\% \pm 1\%$; 3) of body width; post-ovarian space 614–791 (718 ± 93 ; 3) or 34–42% ($38\% \pm 4\%$; 3) of body length. Proximal oviduct 107–114 (112 ± 4 ; 3) long or 6% ($6\% \pm 0\%$; 3) of body length, 16–18 (17 ± 1 ; 3) wide proximally, laterally expanding to form oviducal seminal receptacle; oviducal seminal receptacle 70–100 (86 ± 15 ; 3) long or 4–5% ($5\% \pm 1\%$; 3) of body length, 45–57 (50 ± 6 ; 3) wide or 10–13% ($11\% \pm 1\%$; 3) of body width, between ovary and posterior testis. Laurer's canal extending anterior from

ascending portion of distal oviduct 45–50 (47 ± 3 ; 3), and 7–9 (8 ± 1 ; 3) in maximum width, with pore dextral to oviduct and ventral to posterior margin of anterior testis.

Vitellarium comprising a series of interconnected large spheroid masses of follicles, distributing from level of caecal bifurcation to excretory vesicle 148–205 (186 ± 33 ; 3) or 8–11% ($10\% \pm 1\%$; 3) from posterior body end; lateral collecting ducts ventral to gonads, 23–25 (24 ± 1 ; 3) wide; transverse vitelline duct 34–57 (47 ± 12 ; 3) in breadth 227–250 (235 ± 13 ; 3) wide; primary vitelline collecting duct not observed. Oötype not observed. Distal sinuous oviduct 243–284 (262 ± 21 ; 3) long or 12–15% ($14\% \pm 1\%$; 3) of body length, 20–30 (25 ± 5 ; 3) wide, dorsal to transverse vitelline duct; metraterm 141–152 (147 ± 6 ; 3) long or 7–8 % ($8\% \pm 1\%$; 3) of body length, 57–89 (78 ± 18 ; 3) wide or 14–20% ($18\% \pm 3\%$; 3) of body width; uterine egg not observed. Common genital pore 873–1085 (991 ± 108 ; 3) or 51–59% ($54\% \pm 4\%$; 3) of body length from posterior body end, 41–52 (46 ± 6 ; 3) in diameter. Excretory pore dorsal, subterminal; excretory vesicle 102–136 (117 ± 17 ; 3) wide or 41–54% ($47\% \pm 7\%$; 3) of body width at level of caecal termini; excretory vesicle 238–300 (270 ± 31 ; 3) or 14–16% ($15\% \pm 1\%$; 3) of body length from posterior body margin.

Taxonomic summary

Type and only reported host: Yellow-headed temple turtle, *Heosemys annandalii* (Boulenger, 1903), (Cryptodira: Geoemydidae).

Site in host: Heart.

Type locality: Mekong River (Cao Lãnh Farmers Market, Cao Lãnh, Đồng Tháp Province, Vietnam; $10^{\circ}27'13.32''\text{N}$, $105^{\circ}38'14.97''\text{E}$).

Prevalence and intensity of infection: Two of four (50%) yellow-headed temple turtles were infected with 26 specimens of *R. mikebarger*.

Specimens deposited: Holotype (USNM 1613430), paratypes (USNM 1613431, 1613432).

Etymology: The specific epithet “*mikebarger*” honors Dr. Michael A. Barger (School of Arts and Sciences, Peru State College, Peru, Nebraska, USA) for his contributions to helminthology, and it is in gratitude for introducing HRD to parasitology.

Phylogenetic results (Fig. 4)

The amplified 28S fragment representing the new species comprised 1,454 nucleotides and was included in an alignment comprising 968 nucleotides. The 28S sequences from 2 specimens of *R. mikebarger* were identical (100% similar) to each other and differed from *C. platti* 50 (4.1%), *H. reelfooti* 77 (6.2%), *H. conecuhensis* 82 (6.6%), *H. foliorchis* and *H. gracilis* 96 (7.8%), *P. snyderi* 94 (7.6%), and *P. sinuosus* 104 (8.4%) nucleotides, respectively. The Bayesian analysis herein recovered the new species sister to *C. platti*, with that clade sharing a recent common ancestor with species of *Hapalorhynchus* (albeit with low nodal support) (Roberts et al., 2018; Dutton et al., 2019; Bullard et al., 2019). Bullard et al. (2019; maximum likelihood) and Dutton et al. (2019; Bayesian analysis) recovered *Coeuritrema* and *Platt* as sister taxa; however, the present study added a new lineage that clades with *Coeuritrema* and recovered species of *Platt* sister to all other TBFs within that clade.

DISCUSSION

Bullard et al. (2019) assigned all accepted TBF genera to 6 morphologically-diagnosed groups, and, by that classification scheme, the new genus belongs to Group 4. This is confirmed by the phylogenetic results in that the new taxon clades with Group 4 taxa. It resembles *Cardiotrema* Dwivedi, 1967, *Coeuritrema*, *Hapalorhynchus*, and *Platt* by having the anatomical sequence (anterior to posterior) of a ventral sucker, external seminal vesicle, cirrus sac (lateral to metraterm), anterior testis, ovary, transverse vitelline duct (intertesticular), and posterior testis. In

addition to this combination of features, these genera also have an inflated oesophagus, U-shaped caeca, 2 testes (which are ovoid), a pars prostatica, a transverse cirrus sac (crosses midline, directed laterad), a sinistral, dorsal common genital pore, an intertesticular ovary and Laurer's canal, an oviducal seminal receptacle (ambiguous in *Cardiotrema*), a straight uterus directed anteriorly only, a strongly muscular metraterm, pre-gonadal terminal genitalia, and a massive, globular excretory vesicle (Manter's organ absent) (Bullard et al., 2019).

The new genus is most similar to *Platt* and *Coeuritrema* by having the combination of an elongate/ovoid aspinous body, a ventral sucker at the level of the body constriction, an oesophagus that terminates in the anterior 1/5 of the body and that is ventral to the anterior nerve commissure, intestinal caeca that bifurcate in the anterior 1/3 of the body (not immediately anterior to ventral sucker), a sinistral caecum that bends toward the midline at level of the cirrus and common genital pore, an external seminal vesicle that abuts the anterodextral margin of the cirrus sac, an oviduct that emerges from the dextral margin of the ovary, and an oviducal seminal receptacle that comprises the middle portion of the oviduct. These genera lack lateral oesophageal diverticulae and a median oesophageal diverticulum.

The new genus differs from *Coeuritrema* and *Platt* by having a papillate (vs. apapillate) ventral body surface, a proximal portion of the external seminal vesicle that is lateral to (vs. anterior to) the cirrus sac, an oviduct that is convoluted (vs. straight), a Laurer's canal origin that is pre-ovarian (vs. post-ovarian), a Laurer's canal that extends anterolaterad (vs. posterolaterad), and an excretory bladder that is Y-shaped (vs. globular/sinuuous, I-shaped, or branched). The new genus further differs from *Platt* by having a cirrus that is ventral (vs. dorsal) to the sinistral caecum, a Laurer's canal that is demarcated from the oviducal seminal receptacle by the sinuous distal portion of the oviduct (vs. immediately distal to oviducal seminal

receptacle), an excretory bladder that extends anterior to the tips of the caeca, vasa efferentia that are ventral (vs. dorsal) to the gonads, anterior and posterior vasa efferentia that unite dextral (vs. dorsal) to the metraterm, an oviducal seminal receptacle that is posterior to (vs. at level of) the transverse vitelline duct, and a vitellarium that is dispersed (vs. in clusters). The new genus further differs from *Coeuritrema* by having a tegument without mammillae (vs. mammillae present; cuticle with verrucae), a ventral sucker that is crenulate (papillate) (vs. smooth), caeca that bifurcate proportionately farther anterior to the ventral sucker (vs. immediately anterior to the ventral sucker), a common genital pore between the anterior testis and the ventral sucker (vs. common genital pore immediately posterior to ventral sucker), and a vitellarium that extends posteriad beyond the caeca (vs. from the caecal bifurcation to the caecal tips; surrounding caeca).

Coeuritrema was long regarded as a junior subjective synonym of *Hapalorhynchus* until Roberts et al. (2016) reassigned several species using the presence of mammillae, external seminal vesicle position (abutting anterodextral margin of cirrus sac), and presence of an obvious and muscular metraterm (3–7× uterus length). *Coeuritrema* and *Platt* have a uniquely positioned external seminal vesicle, abutting the anterodextral margin of the cirrus sac; however, *Coeuritrema* has a dispersed vitellarium like *Hapalorhynchus* (see Dutton et al., 2019).

Vietnam is the 9th most diverse country for turtle biodiversity, including 32 accepted turtle species. Habitat loss and overharvest of turtles in Southeast Asia probably threaten most hard shelled turtle populations there but population-level data are lacking for nearly all species (van Dijk et al., 2000, Ly et al., 2011). Only 4 TBFs are known from 3 turtle species in Vietnam, indicating that the diversity of TBFs (along with other parasites from these turtles) there is vastly underestimated.

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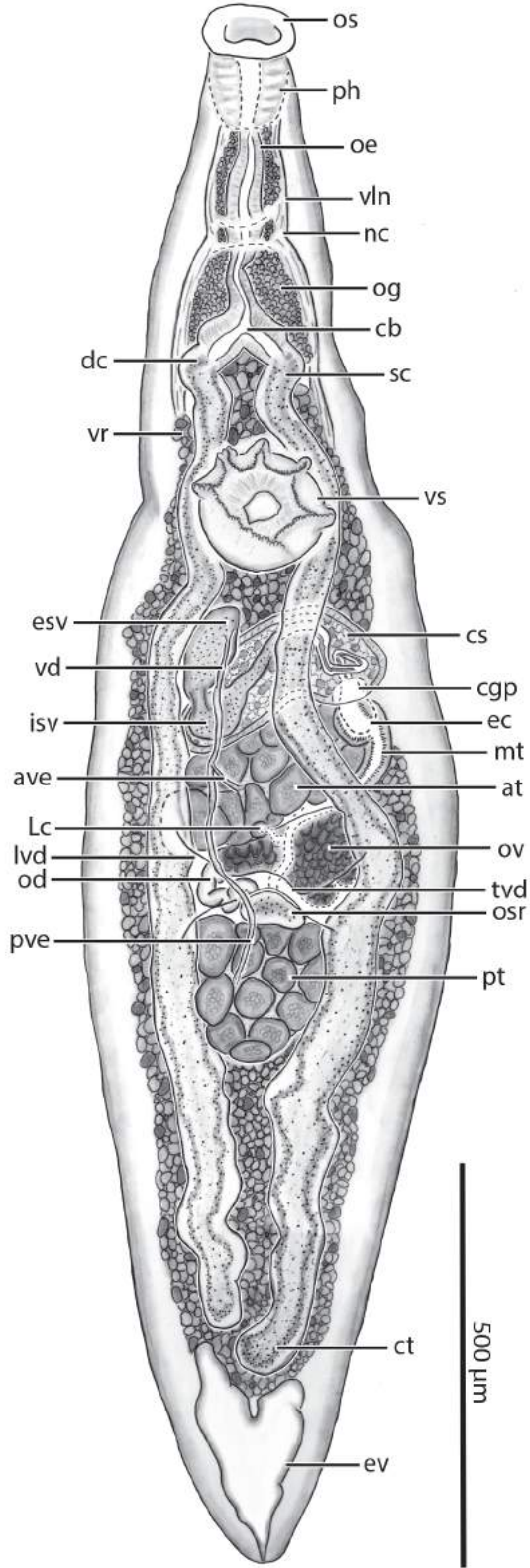
FIGURE LEGENDS

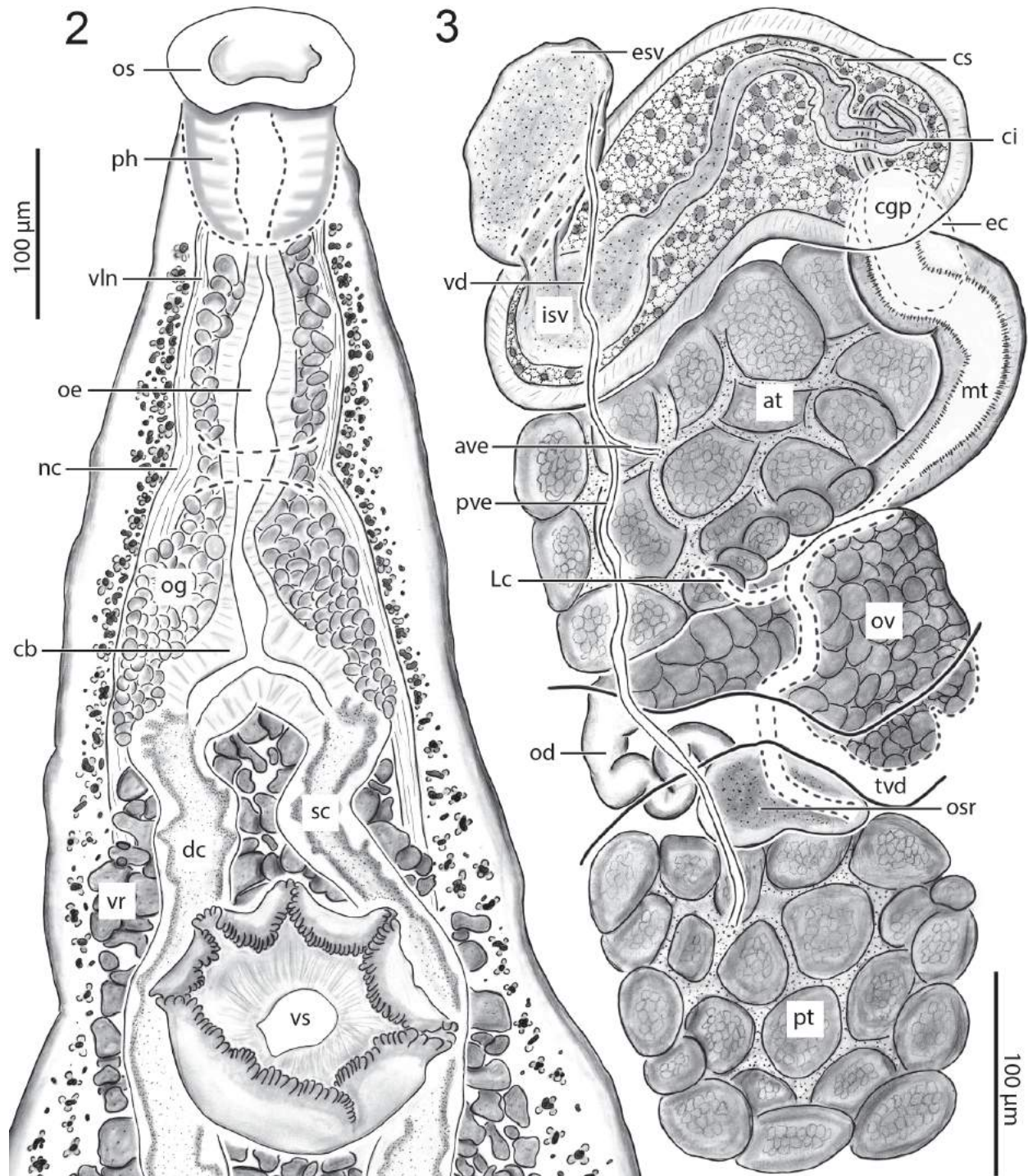
Figure 1 *Ruavermis mikebarger* Dutton and Bullard n. gen., n. sp. (Digenea: Schistosomatoidea) from the heart of the yellow-headed temple turtle, *Heosemys annandaliii* (Boulenger, 1903) (Cryptodira: Geoemydidae). **Fig. 1.** Body of holotype (USNM No. 1613430), ventral view. Bar = 500 μ m. *Abbreviations:* at – anterior testis; ave – anterior trunk of vasa efferentia; cb – caecal bifurcation; cgp – common genital pore; cs – cirrus sac; ct – caecal terminus; dc – dextral caecum; ec – eversible cirrus; esv – external seminal vesicle; ev – excretory vesicle; isv – internal seminal vesicle; Lc – Laurer’s canal; lvd – lateral vitelline collecting ducts; mt – metraterm; nc – nerve commissure; od – oviduct; oe – oesophagus; og – oesophageal gland; os – oral sucker; osr – oviducal seminal receptacle; ov – ovary; ph – pharynx; pt – posterior testis; pve – posterior trunk of vasa efferentia; sc – sinistral caecum; tvd – transverse vitelline duct; vd – vas deferens; vln – ventrolateral nerve chords; vr – vitellarium; and vs – ventral sucker.

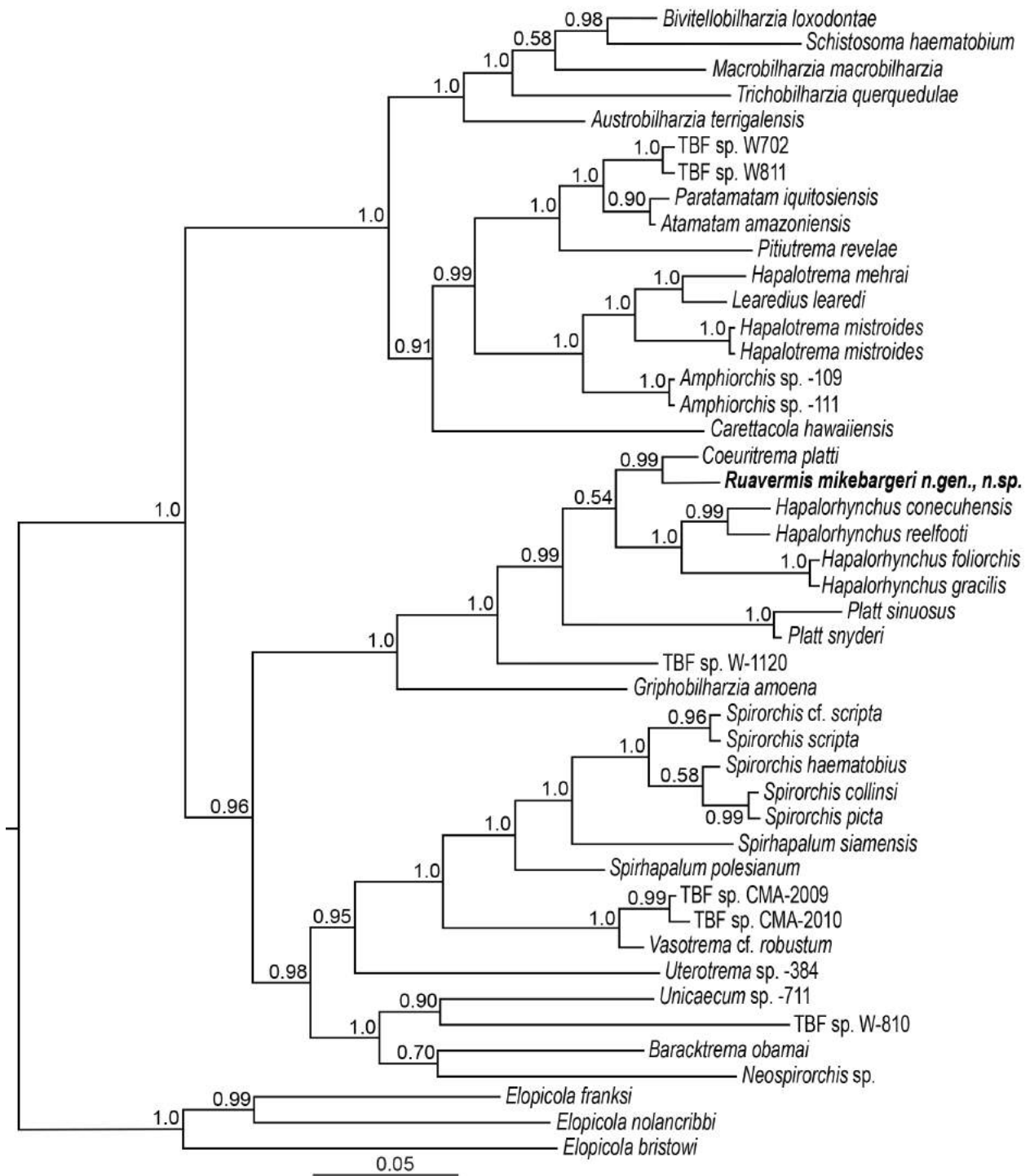
Figures 2–3 *Ruavermis mikebarger* Dutton and Bullard n. gen., n. sp. (Digenea: Schistosomatoidea) from the heart of the yellow-headed temple turtle, *Heosemys annandaliii* (Boulenger, 1903) (Cryptodira: Geoemydidae). **Fig. 2.** Anterior end of holotype (USNM No. 1613430), ventral view. **Fig. 3.** Genitalia of holotype (USNM No. 1613430), ventral view. Bar = 100 μ m. *Abbreviations:* at – anterior testis; ave – anterior trunk of vasa efferentia; cb – caecal bifurcation; cgp – common genital pore; ci – cirrus; cs – cirrus sac; dc – dextral caecum; ec – eversible cirrus; esv – external seminal vesicle; isv – internal seminal vesicle; Lc – Laurer’s canal; mt – metraterm; nc – nerve commissure; od – oviduct; oe – oesophagus; og – oesophageal gland; os – oral sucker; osr – oviducal seminal receptacle; ov – ovary; ph – pharynx; pt – posterior testis; pve – posterior trunk of vasa efferentia; sc – sinistral caecum; tvd – transverse vitelline duct; vd – vas deferens; vln – ventrolateral nerve chords; vr – vitellarium; and vs – ventral sucker.

Figure 4 Bayesian phylogeny based on the large subunit ribosomal DNA (28S). Values aside nodes are posterior probability. Scale bar is in substitutions per site.

1







**CHAPTER 3: A NEW SPECIES AND EMENDATION OF THE SELDOM REPORTED
ENTEROHAEMATOTREMA MEHRA, 1940 (DIGENEA: SCHISTOSOMATOIDEA),
INCLUDING A REVISED PHYLOGENETIC HYPOTHESIS FOR TURTLE BLOOD
FLUKES**

***Submitted to Systematic Parasitology (*in press*)**

Authors: Haley R. Dutton and Stephen A. Bullard

Abstract

Enterohaematotrema Mehra, 1940 is emended herein based upon a review of the literature and a description of a new species (*Enterohaematotrema triettruongi* n. sp.) infecting yellow-headed temple turtles, *Heosemys annandalii* (Boulenger) (Cryptodira: Geoemydidae), in the Mekong River, Vietnam. The new species differs from the published descriptions of its congeners *Enterohaematotrema palaeorticum* Mehra, 1940 and *Enterohaematotrema hepaticum* (Simha, 1958) Simha & Chattopadhyaya, 1980 by having two distinctive oesophageal glands, a short and eversible cirrus (*vs* protrusive with 3 distinct processes), a dorsal common genital pore that is sinistral (*vs* ventral and medial), a transverse (*vs* longitudinal) external seminal vesicle, an oviducal seminal receptacle that is sinistral (*vs* dextral), and a vitellarium distributing from the caecal bifurcation (anterior to the ventral sucker) to the caecal tips (*vs* vitellarium not extending anteriorly beyond ventral sucker in *E. palaeorticum* or vitellarium wholly posterior to the terminal genitalia in *E. hepaticum*). A phylogenetic analysis of the D1-D3 domains of the nuclear large subunit ribosomal DNA (28S) recovered *Enterohaematotrema* and *Platt* Roberts & Bullard, 2016 as sister taxa that share a recent common ancestor with the clade comprising *Ruavermis* Dutton & Bullard, *in press* and *Coeuritrema* Mehra, 1933. These flukes collectively comprise a monophyletic group of southeast Asian turtle blood flukes. This analysis also indicated that the

massive, longitudinal metraterm of species of *Enterohaematotrema* and *Uterotrema* Platt & Pichelin, 1994 represents homoplasy (convergent evolution). The present study comprises the first morphological study of original specimens of any species of *Enterohaematotrema* in more than 50 years and is the first molecular phylogenetic placement of the genus among the various turtle blood fluke lineages.

Introduction

The turtle blood flukes (TBFs) of *Enterohaematotrema* Mehra, 1940 comprise two accepted species that infect softshell turtles (Trionychidae) (Mehra, 1940; Shrivastava, 1959) and a pond turtle (Geomydidae) (Simha, 1958), respectively, both from India. *Enterohaematotrema palaeorticum* Mehra, 1940 (type-species) infects the softshell turtles *Lissemys punctata* Bonnaterre and *Nilssonina hurum* Gray, whereas *Enterohaematotrema hepaticum* (Simha, 1958) Simha & Chattopadhyaya, 1980 infects the pond turtle, *Batagur kachuga* Gray. The life-cycle of *E. palaeorticum*, although overlooked in the current TBF life-cycle literature (e.g. de Buron et al., 2018; Cribb et al., 2017), includes the gastropod *Indoplanorbis exustus* (Deshayes) (Planorbidae) from a pond in Kotah, India, and is currently the only Asian TBF life-cycle known (Shrivastava, 1959).

Noteworthy is that a large diversity of TBFs (6 species of 5 genera) infect *L. punctata* in southeast Asia: the only accepted TBF genus that lacks species infecting that host in that region is *Spirhpalum* Ejsmont, 1927. This emphasises the importance of examining conspecific turtles across the host's geographical distribution because a host species can collectively support a high generic and species diversity of TBFs. It also underscores the potential biodiversity of TBFs that infect turtles in this region, given that most of the turtles ranging there have yet to be examined for infections. Dutton & Bullard (*in press*) described a new species and proposed a new genus of

TBF based on specimens infecting yellow-headed temple turtles, *Heosemys annandalii* (Boulenger) (Cryptodira: Geoemydidae) in Vietnam.

The present study reports additional specimens representing a new species of *Enterohaematotrema* collected from those yellow headed temple turtles. In addition to discovering a new species, this collection of TBF specimens is important because no accessible type-materials for any species of *Enterohaematotrema* exist and no molecular data exist for any species of this genus, i.e. no phylogeny has been published that includes a species of *Enterohaematotrema*.

Herein, we emend *Enterohaematotrema*, describe a new congener (the first new species of the genus described in more than 50 years and the first to be described from Vietnam) and provide a large subunit ribosomal DNA (28S) phylogeny that includes the new species.

Materials and Methods

During a parasitological expedition to Vietnam in September 2018, we encountered specimens of a new species of *Enterohaematotrema* infecting the mesenteric vessels of 4 yellow-headed temple turtles. These turtles were opportunistically sampled for TBF infections from the Cao Lãnh Farmer's Market (10°27'13.32"N, 105°38'14.97"E), Cao Lãnh, Đồng Tháp Province, Vietnam (Mekong River). The brain, eye, heart, lung, spleen, liver, gall-bladder, kidney, bladder, and rectum from each turtle was excised, isolated in an individual container, and immersed in saline immediately after the host was killed. Portions of each organ were then excised and macerated in a Petri dish while viewing under high magnification with a stereo-dissection microscope until the entire organ had been examined. The sediment from each Petri dish and holding container was then examined to gather TBFs that had crawled free from or separated

from the infected tissue. The intestine was removed with the mesenteric vessels intact and placed under the stereo-dissection scope such that blood flukes could be observed *in vivo* and *in situ*.

TBF specimens intended for morphology as whole-mounts were observed microscopically, heat-killed on glass slides using a butane hand lighter under little or no coverslip pressure, fixed in 10% neutral buffered formalin (nbf), rinsed with water, stained in Van Cleave's hematoxylin with several drops of Ehrlich's hematoxylin, dehydrated through a graded ethanol series, made basic at 70% ethanol with lithium carbonate and butyl-amine, dehydrated in absolute ethanol and xylene, cleared with clove oil, and permanently mounted on glass slides using Canada balsam (Dutton et al., 2019). The resulting whole mounts were examined and illustrated with the aid of compound microscopes (Leica DM2500 and Leica DMR, Leica, Wetzlar, Germany) equipped with differential interference contrast (DIC) optical components and drawing tubes. Measurements were obtained with a calibrated ocular micrometer (as straight-lines along the course of each feature) and are reported in micrometres as the range followed by the mean and sample size in parentheses. Classification and anatomical terms for TBFs follow Dutton et al. (2019). Mehra (1940) referred to the distal portion of the female reproductive tract as a metraterm, whereas Platt & Pichelin (1994) and Platt & Blair (1996) referred to it as a uterus. In the new species, the distal portion of the uterus is muscular, i.e. it is a metraterm. Turtle scientific and common names follow Rhodin et al. (2017), and higher classification of turtles follows Pereira et al. (2017).

Total genomic DNA (gDNA) was extracted from 2 ethanol-preserved and microscopically-identified blood flukes using DNeasy™ Blood and Tissue Kit (Qiagen, Valencia, California) as per the manufacturer's protocol except that the proteinase-K incubation period was extended overnight, and 100 µl of elution buffer was used to increase the final DNA

concentration. Amplification and sequencing of the D1-D3 domains of the large subunit ribosomal DNA (28S) used the primer set of Orélis-Ribeiro et al. (2017). PCR amplifications were performed according to Dutton et al. (2019). DNA sequencing was performed by ACGT, Inc. (Wheeling, Illinois). Reactions were sequenced using BigDye terminator version 3.1, cleaned with magnetic beads (CleanSeq dye terminator removal kit; Beckman Coulter, Brea, California), and analysed using an ABI 3730 XL or 3730 Genetic Analyser (Applied Biosystems, Waltham, Massachusetts). Sequence assembly and analysis of chromatograms were completed with Geneious version 11.0.3 (<http://www.geneious.com>; Kearse et al., 2012).

The phylogenetic analysis included the taxa included in Dutton & Bullard (*in press*) as well as the new taxon. As per Dutton et al. (2019), sequences were aligned using MAFFT (Katoh & 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 format) 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 & Huelsenbeck, 2003) using substitution model averaging (“nst-mixed”) and a gamma distribution to model rate-heterogeneity. Defaults were used in all other parameters. Three independent runs with 4 Metropolis-coupled chains were run for 5,000,000 generations, sampling the posterior distribution every 1,000 generations. Convergence was checked using Tracer v1.6.1 (Rambaut et al., 2014) and the “sump” command in MrBayes: all runs appeared to reach convergence after discarding the first 25% of generation as ‘burn-in’. A majority rule consensus tree of the post ‘burn-in’ posterior distribution was generated with the “sumt” command in MrBayes. The inferred phylogenetic tree was visualised

using FigTree v1.4.3 (Rambaut et al., 2014) and further edited for visualization purposes with Adobe Illustrator (Adobe Systems).

Superfamily Schistosomatoidea Stiles & Hassall, 1898

Family “Spirorchiidae Stunkard, 1921” (*sensu lato*)

***Enterohaematotrema* Mehra, 1940**

Emended generic diagnosis

Body aspinose, dorsoventrally flat (not cylindrical), elongate (not thread-like), with anterior and posterior extremities equally rounded; mammillae absent. Oral sucker robust, demarcated from body by constriction, cup-shaped; oral sucker spines absent. Ventral sucker present, at 1/3–1/4 of body length from anterior extremity. Oesophagus straight or slightly sinuous, bifurcates in anterior 1/4–1/5 of body length; lateral oesophageal diverticula and median oesophageal diverticulum absent; oesophageal gland present. Intestinal caeca inverse U-shaped, terminating posterior to testes near 1/6–1/8 of body length. Testes 2, ovoid, (slightly longer than wide), smooth or lobed, anterior testis pre-ovarian or lateral to ovary, posterior testis post-ovarian testis; anterior testis immediately posterior to cirrus. External seminal vesicle posterior to ventral sucker, abutting posterodextral margin of cirrus-sac. Cirrus-sac large, robust, elongate, straight or slightly curved, thin-walled, slightly muscular, directed anteriorly, dextral to common genital pore, enclosing internal seminal vesicle and cirrus. Pars prostatica present. Ovary principally sinistral, transverse, intertesticular or lateral to anterior testis. Oviducal seminal receptacle pyriform,

dextral or sinistral, anterior to posterior testis. Laurer's canal not observed. Vitellarium well developed, surrounding caeca; transverse vitelline duct intertesticular, approximately at level of ovary and proximal end of metraterm. Metraterm massive, $>1/3-1/4$ of body length, longitudinal (extending anteriorly in parallel with body margin), sinistral, lateral to cirrus, strongly muscular (having obvious muscular thick walls), apparently not storing egg(s). Common genital pore ventral or dorsal, medial or sinistral, immediately posterior to ventral sucker, anterior to genitalia.

Type-species: Enterohaematotrema palaeorticum Mehra, 1940.

Other species: Enterohaematotrema hepaticum (Simha, 1958) Simha & Chattopadhyaya, 1980;

Enterohaematotrema triettruongi n. sp.

Differential diagnosis

Body aspinose, elongate (not thread-like); mammillae absent. Oral sucker robust. Ventral sucker $1/3-1/4$ body length from anterior end. Oesophagus terminating in anterior $1/4$ of body. Intestinal caeca inverse U-shaped. Testes 2, comprising a single anterior testis and single posterior testis. Male terminal genitalia pre-gonadal. External seminal vesicle abutting posterodextral margin of cirrus sac. Cirrus sac robust, straight or slightly curved, directed anteriorly. Ovary principally sinistral, transverse, inter-testicular or lateral to anterior testis. Metraterm massive, $>1/3-1/4$ body length, longitudinal, sinistral, lateral to cirrus, having obvious muscular thick walls. Common

genital pore ventral or dorsal, medial or sinistral, immediately posterior to ventral sucker, anterior to genitalia.

***Enterohaematotrema triettruongi* n. sp.**

Type-host: *Heosemys annandalii* (Boulenger) (Cryptodira: Geoemydidae), yellow-headed temple turtle.

Type-locality: Mekong River (Cao Lãnh Farmers Market, Cao Lãnh (10°27'17.72"N, 105°78'14.97"E), Đồng Tháp Province, Vietnam.

Type-material: Holotype (USNM XXXXXXXX), paratypes (USNM XXXXXXXX-XXXXXXXXXX).

Site in host: Mesenteric blood vessels.

Prevalence and intensity of infection: Four of four (100%) yellow-headed temple turtles were infected with 116, 32, 5 and 3 (for a total of 156) specimens of *E. triettruongi* n. sp.

Representative DNA sequences: GenBank MT103552.

ZooBank registration: To comply with the regulations set out in Article 8.5 of the emended 2012 version of the *International Code for Zoological Nomenclature* (ICZN, 2012), details of the new species have been submitted to ZooBank. The Life Science Identifier (LSID) for *Enterohaematotrema triettruongi* n. sp. is urn:lsid:zoobank.org:act:768EA4F5-1AE1-4B2B-B451-5E3B72A2C750.

Etymology: The specific epithet *triettruongi* honors Mr Triet Nhat Truong (Aquatic Parasitology Laboratory, Auburn University) for his expertise in coordinating the collection of fluke specimens in Vietnam.

Description (Figs. 1–3)

[Based on light microscopy of 7 whole-mounted specimens (USNM XXXXXXXX-XXXXXXX).]

Body 1,001–1,397 (1,162; n = 7) long, 173–245 (207; n = 7) in maximum width at level of ovary, 6 (6; n = 7) longer than wide (Fig. 1). Oral sucker 68–100 (86; n = 7) long or 5–10% (8%; n = 7) of body length, 59–68 (64; n = 7) wide or 24–39% (31%; n = 7) of maximum body width. Ventral sucker 77–98 (87; n = 7) long or 6–9% (8%; n = 7) of body length, 57–82 (73; n = 7) wide or 23–42% (36%; n = 7) of maximum body width. Nerve commissure 150–198 (170; n = 7) or 13–16% (15%; n = 7) of body length from anterior body extremity (Fig. 1).

Pharynx 45–68 (54; n = 6) or 21–31% (26%; n = 6) of oesophagus length, 18–30 (25; n = 7) wide or 0.6–0.8× (0.6×; n = 7) wider than maximum oesophagus width. Oesophagus 150–295 (207; n = 7) long or 13–22% (18%; n = 7) of body length, 9–30 (18; n = 7) wide immediately posterior to pharynx and with wall 3–11 (6; n = 7) thick, 20–45 (30; n = 7) wide or 14–34% (23%; n = 7) of body width at mid-oesophagus and with wall 9–11 (10; n = 7) thick, 32–55 (40; n = 7) wide or 23–37% (27%; n = 7) of body width at caecal bifurcation and with wall 11–20 (15; n = 7) thick; anterior oesophageal gland smooth or deeply lobed, 95–193 (127; n = 7) long or 7–14% (11%; n = 7) of body length, 68–114 (92; n = 7) wide or 33–56% (44%; n = 7) of body width; posterior oesophageal gland follicular, 91–114 (100; n = 7) long or 7–10% (9%; n = 7) of body length, 68–107 (85; n = 7) wide or 33–47% (41%; n = 7) of body width. Intestinal bifurcation 195–341 (247; n = 7) or 18–27% (22%; n = 7) of body length from anterior body extremity; anterior caeca absent; posterior caeca extending posteriad approximately in parallel with lateral body margin, slightly sinuous; sinistral posterior caecum 635–844 (685; n = 7) long or 50–64% (59%; n = 7) of body length, 16–23 (19; n = 7) wide or 11–17% (14%; n = 7) of body

width at level of caecal bifurcation, 11–23 (19; n = 7) wide or 6–14% (9%; n = 7) of body width at level of ovary, 18–34 (24; n = 7) wide or 11–21% (16%; n = 7) of body width at ends of caeca; caeca terminating 125–238 (178; n = 7) or 13–18% (16%; n = 7) of body length from posterior body end; dextral posterior caecum 614–796 (677; n = 7) long or 48–62% (59%; n = 7) of body length, 18–27 (23; n = 7) wide or 14–18% (16%; n = 7) of body width at level of caecal bifurcation, 11–23 (18; n = 7) wide or 6–14% (9%; n = 7) of body width at level of ovary, 11–27 (20; n = 7) wide or 9–20% (14%; n = 7) of body width at level of ends of caeca; caeca terminating 130–261 (190; n = 7) or 14–23% (17%; n = 7) of body length from posterior body end (Fig. 1).

Anterior testis position, pre-ovarian or lateral to ovary, 91–125 (112; n = 6) long or 8–12% (10%; n = 6) of body length or 0.8–0.9× (0.8×; n = 6) posterior testis length, 80–116 (102; n = 6) wide or 43–59% (50%; n = 6) of body width at level of ovary or 75–112% (92%; n = 6) of posterior testis width; inter-testicular space 18–57 (29; n = 6) long or 1–5% (3%; n = 6) of body length (Figs. 1–3). Posterior testis, post-ovarian, 114–186 (143; n = 7) long or 10–15% (12%; n = 7) of body length, 91–130 (112; n = 7) wide or 47–63% (54%; n = 7) of body width at level of ovary, 205–352 (289; n = 7) or 20–27% (25%; n = 7) of body length from posterior body extremity (Fig. 1). Anterior trunk of vasa efferentia emanating from ventral surface of anterior testis, extending anteriad 27–45 (35; n = 5) or 3–4% (3%; n = 5), of body length, 5–7 (6; n = 5) wide; posterior trunk of vasa efferentia not observed, meeting anterior trunk posterior to genital pore at the level of the anterior testis to form vas deferens; vas deferens extending anteriad 57–68 (64; n = 5) of body length, 5–11 (6; n = 5) wide before laterally expanding and turning dorsally to form external seminal vesicle (Fig. 3). External seminal vesicle transverse (not crossing the midline), directed mediad, lateral to cirrus-sac, 64–80 (74; n = 7) long or 6–7% (6%; n = 7) of

body length, 34–68 (53; n = 7) wide or 17–33% (25%; n = 7) of body width, 1–2× (1; n = 7) wider than long, immediately anterior to anterior testis; internal seminal vesicle transverse, 23–68 (36; n = 7) long or 2–5% (3%; n = 7) of body length, 14–45 (23; n = 7) wide, 1–2× (2×; n = 7) longer than wide. Cirrus-sac 125–272 (191; n = 7) long or 11–20% (16%; n = 7) of body length, 80–102 (91; n = 7) wide or 38–56% (49%; n = 7) body width at level of genital pore; cirrus extending anteromediad 114–216 (152; n = 7) or 67–95% (82%; n = 7) of cirrus-sac length, 27–61 (39; n = 7) wide.

Ovary smooth and lobed, 57–139 (90; n = 6) long or 6–12% (8%; n = 6) of body length, 48–75 (61; n = 6) wide or 23–33% (29%; n = 6) of body width; post-ovarian space 345–556 (429; n = 7) or 33–40% (37%; n = 7) of body length (Figs. 1–3). Oviduct 14–27 (20; n = 6) long or 1–2% (2%; n = 6) of body length, 7–9 (8; n = 6) wide proximally, ventral to transverse vitelline duct, laterally expanding to form oviducal seminal receptacle; oviducal seminal receptacle 57–91 (68; n = 6) long or 5–7% (6%; n = 6) of body length, 23–34 (26; n = 6) wide or 10–17% (13%; n = 6) of body width, between ovary and posterior testis. Laurer's canal extending laterally from proximal portion of ascending oviduct 34–45 (39; n = 6), 7–11 (9; n = 6) in maximum width; pore dextral to oviduct, inter-testicular. Vitellarium comprising a series of interconnected large irregular shaped follicular masses, distributing from level of caecal bifurcation to excretory vesicle 125–227 (154; n = 7) or 13–17% (14%; n = 7) from posterior body end; lateral collecting ducts ventral to gonads, 18–45 (28; n = 7) wide; transverse vitelline duct 80–136 (108; n = 7) in breadth 11–41 (27; n = 7) wide; primary vitelline collecting duct 14–20 (17; n = 6) long, 5–7 (6; n = 6) wide. Oötype indistinct. Metraterm 227–318 (282; n = 7) long or 21–28% (24%; n = 7) of body length, 25–45 (34; n = 7) wide or 10–22% (16%; n = 7) of body

width; uterine egg not observed. Common genital pore 578–910 (695; n = 7) or 54–67% (63%; n = 7) of body length from posterior body extremity, 9–14 (12; n = 7) in diameter.

Excretory pore dorsal, subterminal; excretory vesicle 45–91 (58; n = 7) wide or 28–57% (39%; n = 7) of body width at level of caecal termini; excretory vesicle 127–243 (162; n = 7) or 13–18% (15%; n = 7) of body length from posterior body margin (Fig. 1).

Molecular results

The amplified 28S rDNA fragment of the new species comprised 1,614 nucleotides, and the 28S rDNA dataset comprised 991 aligned nucleotide positions. The Bayesian analysis recovered the new species as sharing a recent common ancestor with *Platt sinuosus* Roberts & Bullard, 2016 and *Platt snyderi* Platt & Sharma, 2012 (Fig. 4). Species of *Hapalorhynchus* Stunkard, 1922 were recovered as monophyletic, sharing a recent common ancestor with some TBFs that infect southeast Asian turtles: *Coeritrema* Mehra, 1933 + *Ruavermis* Dutton & Bullard, *in press* and *Enterohaematotrema* + *Platt*. The low nodal support value for the southeast Asian TBF clade (0.55) indicates that this relationship is tenuous, as reported elsewhere (Bullard et al., 2019; Dutton et al., 2019; Dutton & Bullard, *in press*).

Remarks

The new species resembles the other species of *Enterohaematotrema* by having a massive oral sucker demarcated from anterior body extremity, an aspinous tegument, a caecal bifurcation anterior to the ventral sucker, a massive, curved cirrus enveloping a strongly developed pars prostatica, an external seminal vesicle between the cirrus and the anterior testis, a smooth ovary (lacking deep lobes), a massive metraterm (comprising the entire length of the uterus) that is

1/3–1/4 of body length, a metraterm and cirrus that are both between the ventral sucker and the anterior testis, and a Y-shaped excretory vesicle (only documented in *E. hepaticum*). The new species differs from the published descriptions of the only other known congeners *E. palaeorticum* and *E. hepaticum* by having two distinctive oesophageal glands, a short and eversible cirrus (*vs* protrusive with 3 distinct processes), a dorsal common genital pore that is sinistral (*vs* ventral and medial), a transverse (*vs* longitudinal) external seminal vesicle, an oviducal seminal receptacle that is sinistral (*vs* dextral), and a vitellarium distributing from the caecal bifurcation (anterior to the ventral sucker) to the caecal tips (*vs* vitellarium not extending anteriorly beyond ventral sucker in *E. palaeorticum* or vitellarium wholly posterior to the terminal genitalia in *E. hepaticum*).

Mehra's (1940) description of the type-species (*E. palaeorticum*) is incomplete, and several taxonomically important features were omitted: fine detail, shape, course, and position of the ventral sucker, caeca, Laurer's canal, vasa efferentia, vas deferens, oviduct, excretory vesicle, and excretory pore. It also included some features that are likely erroneous: pharynx absent and presence of a reservoir that is located "[on] the left side immediately behind left transverse vitelline duct". Regarding the pharynx, the pharynx of numerous TBFs comprises a muscular bulb that surrounds the anterior portion of the oesophagus such that the pharynx and oral sucker are functionally and morphologically integrated (Fig. 2). Platt (1992) listed "pharynx absent" in the diagnosis of Spirorchiidae (*sensu lato*) but subsequent descriptions of particular taxa (Bullard et al., 2019; Dutton et al., 2019; Dutton & Bullard, *in press*; Roberts et al., 2016a, b, c; Roberts et al., 2017; Roberts & Bullard, 2017; Roberts et al., 2018a, b; Roberts et al., 2019) assert the presence of a pharynx as defined above. Albeit, the pharynx is difficult to distinguish with light microscopy of whole-mounted adult specimens. Regarding the reservoir "[on] the left side

immediately behind left transverse vitelline duct”, no other TBF has such a reservoir, and it should be confirmed as present in newly collected specimens. These deficits underscore the importance of a redescription of this taxon, especially since it is the type-species for this seldom studied genus. Also noteworthy regarding dubious character states previously reported for species of *Enterohaematotrema*, Simha (1958) included presence of “cirrus ... armed with three distinct processes” in the generic diagnosis of *Hepatohaematotrema* Simha, 1958 (junior subjective synonym of *Enterohaematotrema*; see Simha & Chattopadhyaya (1980)) but we are skeptical that this feature does not comprise fixation or preparation artifact. This character state for the cirrus should be confirmed in the type materials or newly collected materials.

Discussion

Including the present study, phylogenetic analyses of blood flukes are revealing surprising examples of homoplasy. Based on morphological similarity (presence of a massive, inter-caecal metraterm that is longitudinal and located between the ventral sucker and testes) and without nucleotide data for *Enterohaematotrema*, we (Bullard et al., 2019) predicted that *Enterohaematotrema* would clade with *Uterotrema* Platt & Pichelin, 1994. The phylogenetic analysis herein resoundingly rejects this assertion, supports the phylogenetic affinity of *Enterohaematotrema* with *Hapalorhynchus*, *Cardiotrema* and *Coeluritrema* (see Yamaguti, 1971; Platt, 1992; Bullard et al., 2019) and, moreover, indicates that the metraterm of species of *Enterohaematotrema* and *Uterotrema* comprises homoplasy (convergent evolution). Similarly, Dutton et al. (2019) demonstrated that the ventral sucker of TBFs comprised homoplasy or that the sucker has been ‘lost’ repeatedly across several lineages. *Uterotrema* and *Enterohaematotrema* are indeed similar by having a common genital pore that is far anterior in

the body (immediately posterior to the ventral sucker, pre-testicular). However, these genera are distinctive in several obvious ways. *Enterohaematotrema* has an aspinous body and ventral sucker (*vs* a spinous ventral sucker and hindbody in *Uterotrema*), 2 testes (*vs* single testis), and an external seminal vesicle immediately anterior to the anterior testis (*vs* between the ventral sucker and genital pore). There is also the possibility that the sequence identified as representing *Uterotrema* sp. was misidentified; no morphological voucher is extant to accompany this sequence (see comments in Bullard et al. (2019) regarding the identity of this sequence). Regarding their geographical distributions, the three species of *Uterotrema* are known from Australian turtles only, whereas *Enterohaematotrema* comprises three species that collectively infect turtles on the Indian subcontinent and southeast Asia (Vietnam) only.

Mehra (1940) reported that *E. palaeorticum* (type-species) infected the lumen of the gut of the turtle host, which inspired him to assume that it was a true intestinal parasite and that it was ancestral to the TBFs that infect blood. No TBF has been confirmed from the intestinal lumen of a turtle, and we are skeptical that the actual site of infection for *E. palaeorticum* is the intestinal lumen. Mehra (1940) reported that he performed the necropsy and first observed the specimens of *E. palaeorticum* “more or less attached to intestinal wall and took 1/2 an hour to come out when intestine was opened in salt solution”. This leaves open the likely possibility that specimens of *E. palaeorticum* spilled from the mesenteric vessels during excision, mixed in the dish with the excised and opened intestine, and attached to the intestinal mucosa before being observed by Mehra (1940). Regardless of our speculation, the site of infection needs to be confirmed; however, our specimens, which clearly are congeneric with *E. palaeorticum* were unambiguously observed within the lumen of the mesenteric blood vessels before being removed and fixed (for morphology) or preserved (for DNA extraction).

Although not all southeast Asian TBF genera have species with available nucleotide sequence data (i.e. no species of *Plasmiorchis* Mehra, 1934 and *Cardiotrema* Dwivedi, 1967 has been sequenced to date), noteworthy is that present study further provides evidence that geomydid TBFs are paraphyletic, i.e. geomydids have been colonised as definitive hosts repeatedly. Currently, seven TBF genera include species that infect geomydids (i.e. *Baracktrema* Roberts & Bullard 2015, *Cardiotrema*, *Enterohaematotrema*, *Hapalorhynchus*, *Plasmiorchis*, *Platt*, *Ruavermis* and *Spirhapalum*) but only *Plasmiorchis* and monotypic *Baracktrema* include species that infect geomydids only.

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Compliance with ethical standards

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

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

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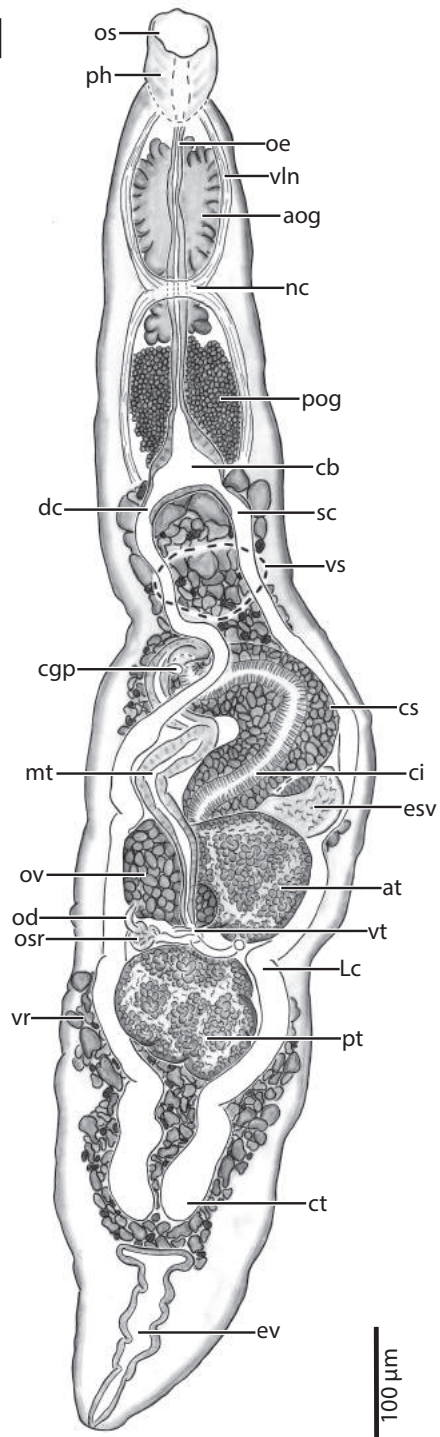
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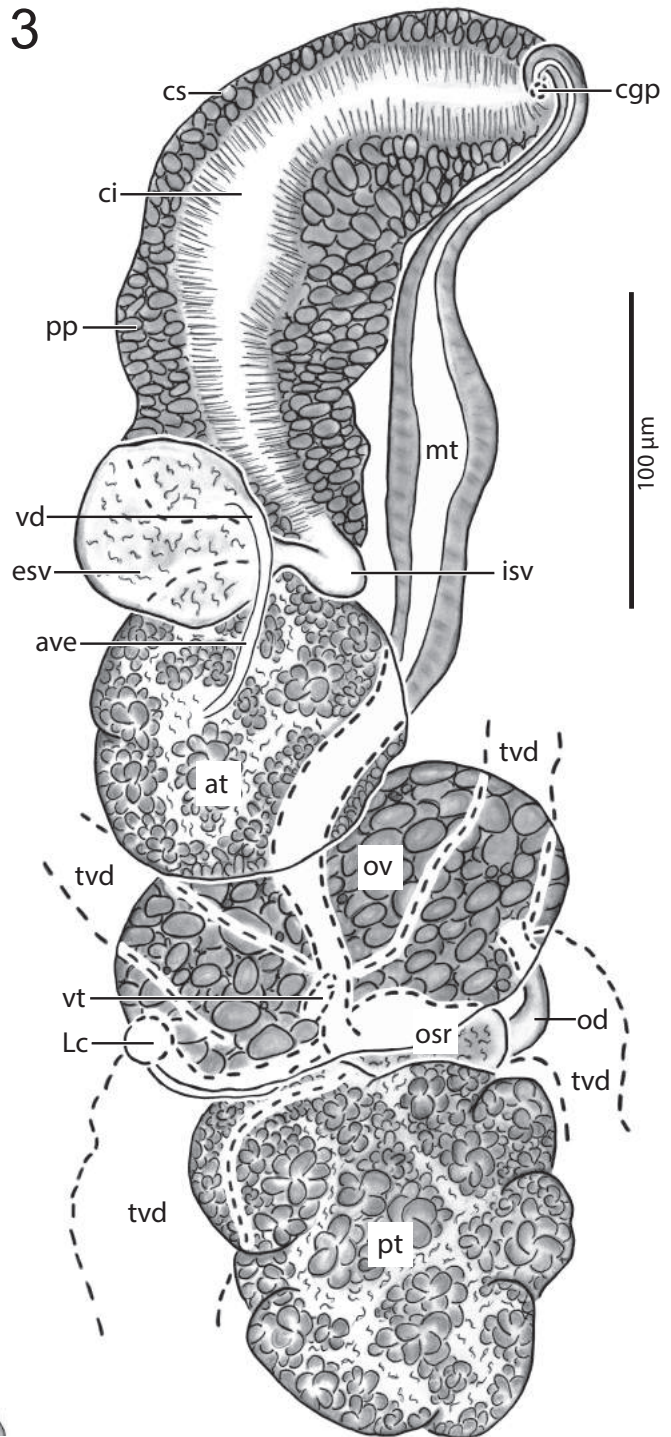
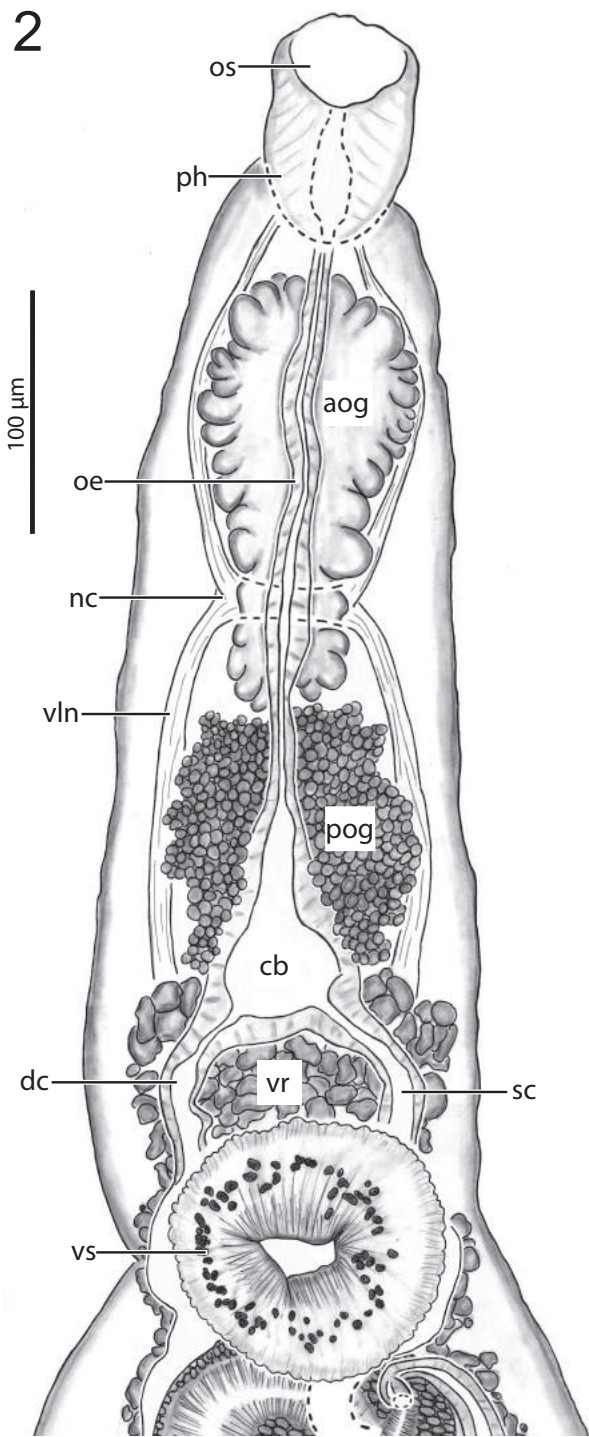
Figure 1 *Enterohaematotrema triettruongi* n. sp. **Fig. 1.** Body (dorsal view) of the holotype (USNM Coll. No. XXXXXX). Abbreviations: os, oral sucker; ph, pharynx; oe, oesophagus; vln, ventrolateral nerve chords; aog, anterior oesophageal gland; nc, nerve commissure; pog, posterior oesophageal gland; cb, caecal bifurcation; sc, sinistral posterior caecum; dc, dextral posterior caecum; vs, ventral sucker; cgp, common genital pore; ci, cirrus; mt, metraterm; esv, external seminal vesicle; ov, ovary; at, anterior testis; od, oviduct; vt, primary vitelline duct; osr, oviducal seminal receptacle; Lc, Laurer's canal; vr, vitellarium; pt, posterior testis; ct, caecal terminus; and ev, excretory vesicle.

Figures. 2, 3 *Enterohaematotrema triettruongi* n. sp. **Fig. 2.** Anterior end (ventral view) of the paratype (USNM Coll. No. XXXXX). Abbreviations: os, oral sucker; ph, pharynx; aog, anterior oesophageal gland; oe, oesophagus; nc, nerve commissure; vln, ventrolateral nerve chords; pog, posterior oesophageal gland; cb, caecal bifurcation; dc, dextral posterior caecum; sc, sinistral posterior caecum; vr, vitellarium; vs, ventral sucker; **Fig. 3.** Genitalia (ventral view) of the paratype (USNM Coll. No. XXXXXXX). Abbreviations: cgp, common genital pore; cs, cirrus-sac; ci, cirrus; pp, pars prostatica; mt, metraterm; vd, vas deferens; esv, external seminal vesicle; isv, internal seminal vesicle; ave, anterior trunk of vasa efferentia ; at, anterior testis; tvd, transverse vitelline duct; ov, ovary; vt, primary vitelline duct; Lc, Laurer's canal; od, oviduct; osr, oviducal seminal receptacle; and pt, posterior testis.

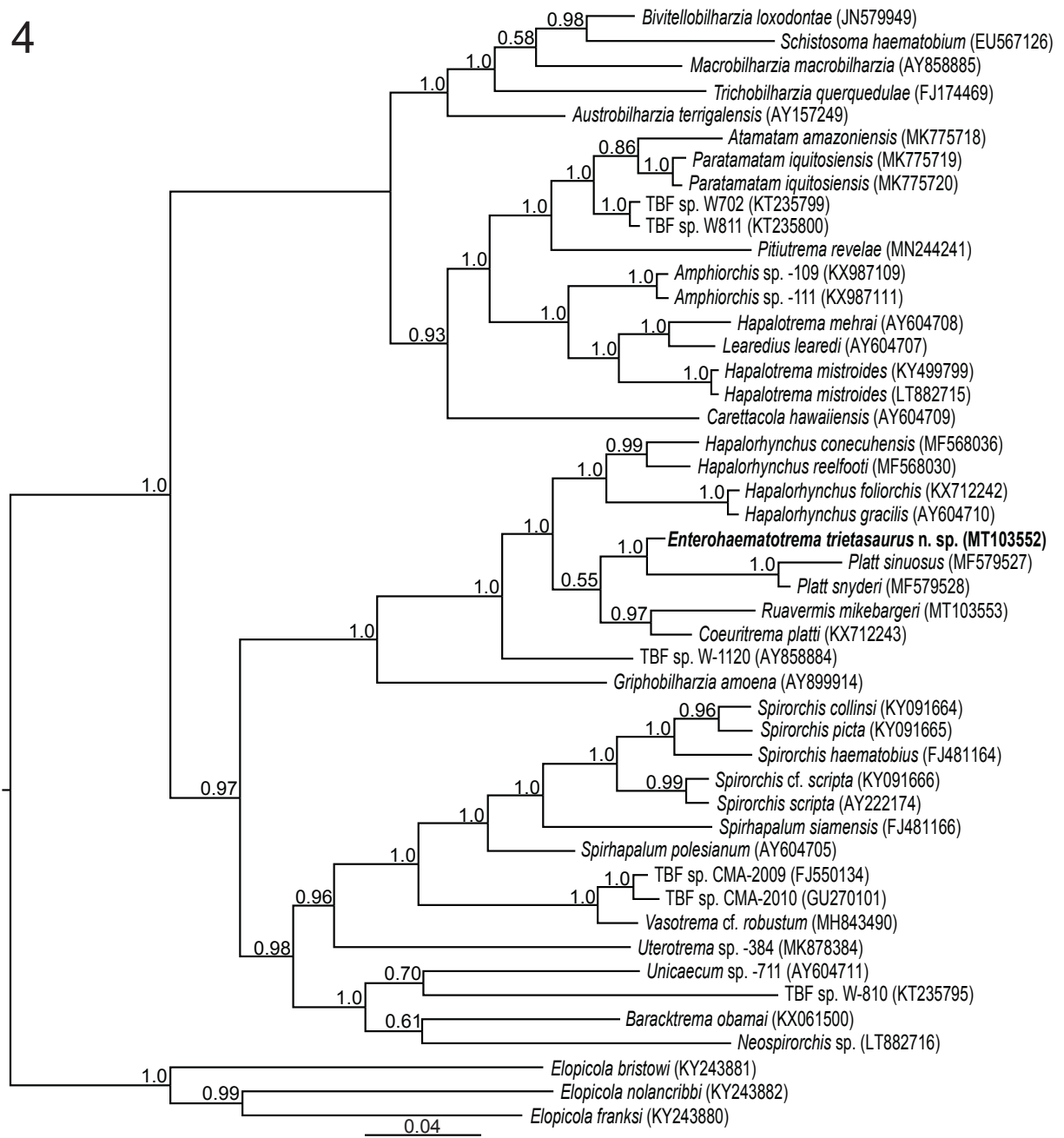
Figure 4 Phylogenetic relationships of turtle blood flukes reconstructed by Bayesian inference and based on the large subunit ribosomal DNA (28S). Numbers at the nodes indicate posterior probability. The scale-bar indicates the number of substitutions per site. The GenBank accession numbers for each taxon are indicated in parenthesis.

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CHAPTER 4: First record of a polystome (*Neopolystoma* cf. *orbiculare*) from the alligator snapping turtle, *Macrochelys temminckii* (Cryptodira: Chelydridae) or Mississippi; with comments on morphological differences among specimens ascribed to *Neopolystoma orbiculare* (Stunkard, 1916) and its subjective synonyms

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ABSTRACT

The concept of *Neopolystoma orbiculare* (Stunkard, 1916) includes records from 10 turtle species from 8 states in North America plus a European record from an introduced turtle as well as the junior subjective synonyms *N. aspidonectis*, *N. elegans*, *N. inerme*, *N. spinulosum*, *N. troosti*, and *N. floridanum*. To date, no nucleotide sequence in GenBank is tethered to a morphological description of *N. orbiculare*, and published descriptions of these species appear comparatively polymorphic. Herein, we describe a set of newly-collected specimens of *Neopolystoma* cf. *orbiculare* from the urinary bladder of 2 alligator snapping turtles, *Macrochelys temminckii* (Troost in Harland, 1835) (Cryptodira: Chelydridae Gray, 1831) from Comet Lake (30°35'46.94"N; 88°36'3.12"W), Pascagoula River, Mississippi. Our specimens differed from all previous descriptions of *N. orbiculare* and its subjective synonyms by the combination of having intestinal ceca that are diverticulate and that terminate dorsal to the haptor, distinctive hooklets each having a shank and a thumb of approximate equal length and having a much longer and curved shaft, 16 genital coronet spines that each possess 1–2 flanges per spine, pre-testicular vaginal pores, and vaginal ducts that are anterior to the junction of the oviduct and genito-intestinal canal. Nucleotide sequences (28S, 18S, COI) for our specimens were most similar to the GenBank sequences ascribed to *N. orbiculare*. The molecular

phylogenetic analyses confirmed that *Neopolystoma* is polyphyletic and that our isolates share a recent common ancestor with *N. orbiculare*. These results suggest that additional taxonomic work coupling morphology and phylogenetic analyses are needed to revise *Neopolystoma* and reconcile the identity of the polystomes presently assigned to *N. orbiculare*. This is the first record of a polystomatid from Mississippi, from the Pascagoula River, and from the alligator snapping turtle (and only the 3rd polystome species reported from any snapping turtle).

Key words: Monogenoidea, Polystomatidae, Chelonia, southeastern United States, Mississippi

“Polystomes” (Monogenoidea: Polystomatoinea Lebedev, 1986: Polystomatidae Gamble, 1896 [see Bychowsky [1937]; Lebedev [1986]; Sinappah et al., [2001]; 26 genera and ~200 accepted species) are unique among other monogenoids in that they infect tetrapods (Sarcopterygia: Tetrapoda) rather than non-tetrapod vertebrates (paraphyletic “fishes”) (Héritier et al., 2015; du Preez et al., 2017; DuPreez & Domingues, 2019). No polystome has been named from a sea turtle (Cheloniidae Oppel, 1811) but the freshwater turtles collectively host 61 nominal polystome species assigned to 3 grades (provisional genera) (Héritier et al., 2015; du Preez et al., 2017) and 2 genera (monotypic *Uteropolystomoides* Tinsley, 2017 and monophyletic *Uropolystomoides* Tinsley and Tinsley, 2016 with 13 species). The polyphyletic genera comprise *Polystomoidella* Price, 1939 (diagnosed as having a single pair of hamuli; infecting bladder only); *Polystomoides* Ward, 1917 (2 pairs of hamuli; eye, bladder, pharyngeal cavity); and *Neopolystoma* Price, 1939 (lacking hamuli; conjunctival sac, bladder, pharyngeal cavity). Problematic is that the vast majority of turtle polystomes are presently assigned to the 3 polyphyletic genera (du Preez et al., 2017) and several species assigned to those genera lack

publicly-available nucleotide sequences (18S, 28S, COI, 12S). As such, additional records, descriptions, specimens, and nucleotide sequences of polystomes are needed, including those from North America (du Preez and Van Rooyen, 2015), to help cast light on the biological diversity and evolutionary history of these parasites.

To date, and despite the high diversity of freshwater turtles in North America (Buhlmann and Gibbons, 1997; Buhlmann et al., 2008), a total of only 14 turtle-infecting polystomes have been described there: 2 species of *Polystomoidella*, 7 of *Neopolystoma*, 4 of *Polystomoides*, and 1 species of *Uteropolystomoides* (du Preez and Van Rooyen, 2015, Tinsley 2017). Because of the low number of polystome records from the southeastern United States coupled with the high endemic diversity of turtles (du Preez and Morrison, 2011; du Preez et al., 2017; Rhodin et al., 2017), we suspect that many innominate turtle polystomes range in North America; especially within the Southeastern US as a region of exceptionally high aquatic biodiversity (Benz and Collins, 1997). Especially striking regarding knowledge gaps in polystome-turtle relationships and diversity is that no record of a polystome exists from Mississippi nor Alabama. Both of these areas contain rivers that harbor high levels of aquatic faunal endemism and diversity (Benz and Collins, 1997), e.g., Pascagoula River (type locality of the new species described herein, Mississippi) and Mobile-Tensaw Basin (Alabama), including among turtles (Buhlmann and Gibbons, 1997). Accordingly, nucleotide sequences of North American polystomes are rare and few are included in phylogenetic studies. For example, regarding molecular phylogenetic studies, the most comprehensive taxon sampling for the polystomes to date is Héritier et al. (2015), who included only 2 North American polystomes (*Polystomoides oris* and *Neopolystoma orbiculare*) because no others were available at that time.

Relative to intestinal parasites of turtles, polystomes are undersampled because few parasitologist specifically look for them. Also true is that relatively few parasitologists nowadays focus on North American turtles. Whereas many turtle species in this region remain to be explored for polystome infections (du Preez and Morrison, 2011; du Preez et al., 2017; Rhodin et al., 2017), it was surprising to us that one particularly charismatic, commercially- and culturally-important turtle species had not been previously identified as a polystome host.

Herein, we describe the first polystomes documented from the alligator snapping turtle (urinary bladder), *Macrochelys temminckii* (Troost in Harland, 1835), (Cryptodira: Chelydridae). These infected turtles were taken from a small oxbow lake (Comet Lake) in the Pascagoula River, Mississippi; a state that lacks a polystome record. *Neopolystoma* includes 24 accepted species, which collectively infect the urinary bladder, ocular cavity, nasal cavity, mouth, pharynx, and cloaca of turtles that range in North America (US), Central America (Mexico, Costa Rica, Panama), Australia, Japan, Malaysia, Papua New Guinea, Africa (Tunisia), and China (Caballero, 1938; Caballero et al., 1956; Combes and Ktari, 1976; du Preez and Lim, 2000; du Preez et al., 2017; Fairfax, 1990; Harwood, 1932; Lamothe-Argumedo, 1972; Morrison and du Preez, 2011; Ozaki, 1935; Pichelin, 1995; Platt 2000a, b; Platt et al., 2011; Price, 1939; Rohde and Pearson, 1980; Rohde, 1984; Strelkov, 1950; Stunkard, 1917). Although the alligator snapping turtle is the largest extant freshwater turtle in North America, no previous record of any polystome exists from this turtle host (du Preez and Morrison, 2011).

MATERIALS AND METHODS

During June 2018 and September 2019, we collected two infected alligator snapping turtles from Comet Lake, Pascagoula River (Mississippi) and one uninfected alligator snapping turtle from the Conecuh River (Alabama). Limb lines with baited hooks were used to capture the

turtles in Mississippi, and a baited hoop net was used to capture the turtle in Alabama. Turtles were placed in coolers and immediately transported to the laboratory for examination. After decapitation, the brain, eye, heart, lung, spleen, liver, gall bladder, kidney, urinary bladder, and rectum from each turtle was excised, isolated in an individual container, and immersed in saline. Other parasites were collected from these turtles but specimens of *Neopolystoma cf. orbiculare* were observed within the lumen of the urinary bladder before being removed alive, heat-killed on a glass slides using a butane hand lighter under little or no coverslip pressure, fixed in 10% neutral buffered formalin (NBF), rinsed with water, stained in Van Cleave's hematoxylin, dehydrated with a graded EtOH series, made basic at 70% EtOH with lithium carbonate and butyl-amine, dehydrated in absolute EtOH and xylene, cleared with clove oil, and permanently mounted on glass slides using Canada balsam. Specimens intended for DNA extraction and sequencing were preserved alive in 95% non-denatured ethanol (EtOH): one large adult specimen was digested whole for DNA extraction and sequencing (GenBank Nos. XXXX-XXXX) and a small adult specimen was cut such that the haptor was stained and whole-mounted as a voucher (USNM Coll. No. XXXXXXXX) while the body was digested for DNA extraction and sequencing.

Whole mounts were examined and illustrated with the aid of a compound microscope (Olympus BX51 [Olympus, Shinjuku, Tokyo, Japan]) 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 reported in micrometers (μm) as the range followed by the mean, \pm standard deviation, and sample size in parentheses. Vouchers were deposited in the National Museum of Natural History's Invertebrate Zoology Collection (Smithsonian Institution, USNM Collection Nos. XXXXXX-XXXXXX).

Classification and anatomical terms for polystomes follow Morrison and du Preez (2011). Turtle scientific and common names generally follows Rhodin et al. (2017) with exception to the taxonomic status of the congener of *M. temminckii*, and higher classification of turtles follows Pereira et al. (2017).

Total genomic DNA (gDNA) was extracted from 2 EtOH-preserved and microscopically-identified polystomes (1 large adult, 1 small adult) using DNeasy™ Blood and Tissue Kit (Qiagen, Valencia, California, USA) as per the manufacturer's protocol; however, the proteinase-K incubation period was extended overnight and we used 100 µL of elution buffer to increase the final DNA concentration. Amplification and sequencing of three genetic markers were investigated for genetic analysis. We used the primer set of Littlewood et al. (1997) to sequence the large subunit ribosomal DNA (28S), 18S rRNA, and CO1. The combined 18S and ITS1 was amplified for a length of 2,556 bp, with 3 sets of primers for the 18S, the 1st set being the forward primer F18 (5'-ACC TGG TTG ATC CTG CCA GTA G-3') and reverse inner primer 18RG (5'-CTC TCT TAA CCA TTA CTT CGG-3'); and the other set containing the inner forward primer 18F3 (5'-GGA CGG CAT GTT TAC TTT GA-3') and reverse IR5 (5'-TAC GGA AAC CTT GTT ACG AC-3'). We used the primer set of Sinnappah et al. (2001) and Verneau et al. (1997) to obtain partial 18S rDNA plus the entire ITS1 region, using the 1st forward primer L7 (5'-TGA TTT GTC TGG TCC GAT-3'), reverse primer H7 (5'-GCT GCG TTC TTC ATC GAT ACT CG-3'), and a 2nd forward primer, S1 (5'-ATT CCG ATA ACG AAC GAG ACT-3'). The 28S was amplified in 2 overlapping fragments for a length of 1,068 bp, with 2 sets of primers. The 1st set being the forward primer LSU5' (5'-TAG GTC GAC CCG CTG AAY TTA AGC A-3') and the reverse inner primer IR16 (5'-ATT CAC ACC CAT TGA CTC GCG-3'); and the other set containing the inner forward primer IF15 (5'-GTC TGT GGC GTA GTG GTA

GAC-3') and the reverse primer LSU3' (5'-TAG AAG CTT CCT GAG GGA AAC TTC GG-3'). The CO1 region was amplified for a length of 445 bp, with the forward primer L-CO1p (5'- TTT TTT GGG CAT CCT GAG GTT TAT -3') and reverse primer H-Cox1p2 (5'-TAA AGA AAG AAC ATA ATG AAA ATG -3'). PCRs for all 18S+ITS1, 28S and CO1 fragments were conducted following the same amplification procedure: 2 min denaturation at 94°C; 35 cycles of 30 sec at 94°C, 30 sec at 50°C and 1 min at 72°C; followed by a final 10 min extension at 72°C. Success of PCR was checked in 1% agarose gels that were stained with ethidium bromide. DNA sequencing was performed by ACGT, Incorporated (Wheeling, Illinois, USA). Reactions were sequenced using BigDye terminator version 3.1, cleaned with magnetic beads (CleanSeq dye terminator removal kit), and analyzed using an ABI 3730 XL or 3730 Genetic Analyzer. Sequence assembly and analysis of chromatograms were completed with Geneious version 11.0.5 (<http://www.geneious.com>; Kearse et al. 2012). All nucleotide sequence data were deposited in GenBank (XXXXXXX-XXXXXXX).

The phylogenetic analysis included all available sequences for “*Neopolystoma orbiculare*.” Sequences were aligned using MAFFT (Kato 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). Nucleotide sequence data reported in this paper is available in the GenBank database under submission ID numbers **XXXXXX-XXXXXX**.

DESCRIPTION

Neopolystoma cf. orbiculare (Figs. 1–7)

Description of adult (based on 4 whole-mounted specimens and 1 haptor of an adult hologeneophore): Body elongate, 2,400–2,750 ($2,600 \pm 146$; 4) long, 700–950 (823 ± 117 ; 4) in maximum width at level posterior to genitalia and anterior to sucker, 650–910 (790 ± 121 ; 4) wide at level of vaginal ducts, $3 \times (3 \pm 1; 4)$ longer than wide (Figs. 1, 2). Haptor 630–910 (748 ± 132 ; 5) long or 26–33% ($30\% \pm 4\%$; 4) of body length, 780–1,100 (371 ± 198 ; 5) wide; haptoral suckers 215–710 (371 ± 198 ; 5) in diameter; hamuli and hooklets not observed; cuticular loops 20 (1) long, 5 (1) wide (Figs. 1–2). Mouth terminal, directed slightly ventral. False oral sucker 245–300 (273 ± 29 ; 4) long, 350–455 (404 ± 54 ; 4) wide. Pharynx 175–270 (220 ± 41 ; 4) long, 235–315 (266 ± 37 ; 4) wide (Figs. 1–2). Intestine comprising 2 inverse U-shaped caeca, bifurcating immediately posterior to pharynx 390–560 (478 ± 79 ; 4) from anterior body end, each 1,525–2,125 (1863 ± 269 ; 4) long or 64–77% ($71\% \pm 7\%$; 4) of body length, extending sinuously posteriad approximately in parallel with lateral body margin, terminating in body dorsal to haptor approximately at level of anterior pair of haptoral suckers, diverticulate; cecal tips 190–375 (285 ± 82 ; 4) or 12–18% ($15\% \pm 2\%$; 4) of total cecal length from anterior margin

of haptor, turning slightly mediad, not touching; post-cecal distance 50–75 (56 ± 13 ; 4) or 2–3% ($2\% \pm 0\%$; 4) of body length (Figs. 1–2).

Testis 290–390 (360 ± 47 ; 4) long, 215–275 (253 ± 27 ; 4) wide, with overall smooth appearance but having wavy, irregular margins in some specimens, not deeply lobed, occupying space 910–1,130 ($1,005 \pm 112$; 4) or 35–41% ($39\% \pm 3\%$; 4) of body length from anterior body end (Figs. 1–4). Vas deferens medial, extending directly anteriad for 255–310 (283 ± 39 ; 2), 25–30 (28 ± 4 ; 2) wide, dorsal to terminal female genitalia, coalescing anteriorly to form a seminal vesicle (Figs. 1–4). Seminal vesicle prominent, 135–140 (138 ± 4 ; 2) long or 5% (2) of body length, 115 (2) wide or 12–13% ($13\% \pm 1\%$; 2) of body width (Figs. 1–4).

Ovary convoluted, 75–115 (85 ± 20 ; 4) long, 60–85 (69 ± 11 ; 4) wide, 720–910 (813 ± 95 ; 3) or 3–5% ($3\% \pm 1\%$; 4) of body length from anterior body end (Figs. 1–4). Vitellarium indistinct or loosely dispersed throughout space surrounding caecae. Oviduct 215–300 (258 ± 60 ; 2) long, 70–75 (73 ± 4 ; 2) wide (Figs. 1–4). Genito-intestinal canal 110–125 (118 ± 11 ; 2) long, 25–50 (38 ± 18 ; 2) wide, extending postero-mediad from sinistral intestinal cecum dorsal to sinistral vaginal duct, turning slightly anteriad and expanding to become more convoluted at midline, joining intestinal cecum to oviduct anteriorly (Figs. 1–4). Vaginae 2, paired, comprising a narrow proximal duct and a thick-walled, dilated distal portion that communicates to vaginal pore, converging medially at level of the oviduct and genito-intestinal canal (920–980 [950 ± 42 ; 2] or 35% (2) of body length from anterior body end); proximal portions of vaginae 225–300 (263 ± 53 ; 2) long or 25–32% ($28\% \pm 4\%$; 2) of body width, 23–30 (27 ± 5 ; 2) wide, with wall 5–8 (7 ± 2 ; 2) thick; distal portions of vaginae comprising a relatively voluminous and strongly muscular chamber that evidently functions as a sphincter or genital sucker, 125–188 (161 ± 27 ; 4) long or 18–21% ($20\% \pm 2\%$; 4) of body width, 108–140 (124 ± 13 ; 4) wide, with wall 33–63

(47 ± 13 ; 4) thick, enveloped by strongly basophilic glandular-appearing cells absent from the parenchyma surrounding the proximal portion of the vaginae; vaginal pores marginal or slightly submarginal, directed ventrally, opening 760–970 (845 ± 96 ; 4) or 30–35% ($32\% \pm 2\%$; 4) of body length from anterior body end, 50–200 (106 ± 66 ; 4) from anterior margin of testis (Figs. 1–4). Oötype not observed. Uterus 225–260 (243 ± 25 ; 2) long, 35–60 (48 ± 18 ; 2) wide, ventral to vasa efferentia, extending anteriorly before curving mediad, containing a single egg (Figs. 1–4). Egg ovoid, operculate, 60–290 (196 ± 99 ; 4) long, 30–233 (170 ± 96 ; 4) wide (Figs. 1–4). Genital bulb 158–200 (174 ± 19 ; 4) long, 150–190 (173 ± 17 ; 4) wide, medial, posterior to cecal bifurcation, spined (having genital coronet); genital coronet comprising 16 spines, 570–710 (631 ± 72 ; 4) or 22–26% ($24\% \pm 2\%$; 4) of body length from anterior body end; genital coronet spines 45–58 (52 ± 6 ; 4) long, having 1 or 2 flanges, distally hooked (Fig. 8).

Excretory pores dorsal, subterminal, anterior to genital spines (Figs. 1, 2).

Description of small adults (based on 3 whole-mounted specimens): Identical to large adult specimens with the following supplemental observations. Body 1,375–1,550 ($1,475 \pm 90$; 3) long, 480–500 (490 ± 10 ; 3) in maximum width, 440–470 (457 ± 15 ; 3) wide at level of vaginae, $3 \times (3 \pm 0; 4)$ longer than wide. Haptor 320–360 (340 ± 28 ; 2) long or 23–24% ($24\% \pm 1\%$; 2) of body length 550–640 (590 ± 46 ; 3) wide; haptor sucker 150–180 (163 ± 15 ; 3) in diameter (Fig. 5). Marginal hooklets each having handle and guard of approximate equal length, having a much longer, curved blade, 25–25 (25 ± 0 ; 3) in total length, 13 (3) in maximum breadth of curved blade; blade 10–12 (12 ± 1 ; 3) long; guard 7–8 (8; 3) long; handle 7–10 (8 ± 1 ; 3); make some ratios of shaft length/shank length; cuticular loops 20 (3) long, 5 (3) wide (Figs. 5–7). False oral sucker 160–180 (172 ± 10 ; 3) long, 245–260 (253 ± 8 ; 3) wide. Pharynx 125–135 (132 ± 6 ; 3) long, 160–175 (167 ± 8 ; 3) wide. Intestine bifurcating 325–350 (335 ± 13 ; 3) from anterior

body end, each 1,000–1,125 ($1,050 \pm 66$; 3) long or 68–73% ($71\% \pm 2\%$; 3) of body length; cecal tips 10–50 (30 ± 28 ; 2) or 1–5% ($3\% \pm 3\%$; 2) of total cecal length from anterior margin of haptor; post cecal distance 50–75 (63 ± 18 ; 2) or 3–5% ($4\% \pm 1\%$; 2) of body length. Testis 165–225 (197 ± 30 ; 3) long, 115–125 (120 ± 5 ; 3) wide, occupying space 640–700 (680 ± 35 ; 3) or 45–47% ($46\% \pm 1\%$; 3) of body length from anterior body end. Vas deferens extending anteriorly for 150–180 (164 ± 15 ; 3), 10–13 (12 ± 2 ; 3) wide. Seminal vesicle 50 (3) long or 3–4% ($3\% \pm 0\%$; 3) of body length, 28–43 (34 ± 8 ; 3) wide or 6–9% ($7\% \pm 2\%$; 3) of body width.

Ovary 48–55 (51 ± 4 ; 3) long, 43–48 (46 ± 3 ; 3) wide, 500–560 (540 ± 35 ; 3) or approximately 3% of body length from anterior body end. Oviduct 38–55 (48 ± 9 ; 3) long, 25 (3) wide. Genito-intestinal canal 50–75 (63 ± 13 ; 3) long, 20–23 (21 ± 2 ; 3) wide. Vaginae 560–610 (593 ± 29 ; 3) or 39–41% ($40\% \pm 1\%$; 3) of body length from anterior body end; proximal portions of vaginae weakly developed, 130–188 (152 ± 31 ; 3) long or 27–38% ($31\% \pm 6\%$; 3) of body width, 13–15 (14 ± 1 ; 3) wide; distal portions of vaginae weakly developed, 50–80 (61 ± 17 ; 3) long or 10–16% ($12\% \pm 3\%$; 3) of body width, 45–60 (52 ± 8 ; 3) wide; vaginal pores nearly marginal, directed ventrally, opening 550–610 (587 ± 32 ; 3) or 39–41% ($40\% \pm 1\%$; 3) of body length anterior body end, 65–75 (70 ± 5 ; 3) from anterior margin of testis. Uterus 118–150 (134 ± 16 ; 3) long, 20 (3) wide. Egg not observed. Genital bulb 63–70 (66 ± 4 ; 3) long, 75–80 (78 ± 3 ; 3) wide, 450–480 (467 ± 15 ; 3) or 30–33% ($32\% \pm 1\%$; 3) of body length from anterior body end; genital coronet spines 40–45 (42 ± 3 ; 3) long, having 1 flange, distally hooked.

Taxonomic summary

Host: Macrochelys temminckii (Troost in Harlan 1835) (Cryptodira: Chelydridae), the alligator snapping turtle.

Locality: Comet Lake (30°35'46.94"N; 88°36'3.12"W), Pascagoula River, Mississippi.

Specimens and sequences deposited: Holotype (USNM XXXX); paratypes (USNM XXXX–XXXX); 28S sequences (GenBank Nos. XXXXXX–XXXXXX), 18S sequences (GenBank Nos. XXXXXX–XXXXXX), CO1 sequences (GenBank Nos. XXXXXX–XXXXXX), and COI sequences (GenBank Nos. XXXXXX–XXXXXX).

Site in host: urinary bladder.

Prevalence and intensity: The 2 alligator snapping turtles from Comet Lake (Mississippi) were infected with 6 (all large adult specimens) and 4 (all small adults) specimens of *Neopolystoma cf. orbiculare*, respectively. The single alligator snapping turtle from the Conecuh River (Alabama) was not infected.

ZooBank registration: XXXXXXXXXXXXXXX

Phylogenetic results

The amplified 28S fragment (1,068 bp) of *Neopolystoma cf. orbiculare* was 99.89% similar to sequences ascribed to *N. orbiculare* in GenBank, and it was 98.66% similar (12 nucleotide differences) to that of *N. cayenensis*, its sister taxon in the recovered 28S phylogeny (Fig. 9). That of the 18S (2,556 bp) of *N. cf. orbiculare* was 99.75% similar to corresponding *N. orbiculare* sequences and was 99.69% similar (6 nucleotide differences) to *N. cayenensis* (Fig. 10). Both analyses (28S and 18S) recovered *N. cf. orbiculare*, *N. orbiculare*, and *N. cayensis* as a clade sister to *Polystomoides oris*. The amplified CO1 fragment (445 bp) of *N. cf. orbiculare* was 96.85–98.74% similar (4–10 nucleotide differences) to corresponding *N. orbiculare* sequences and was 84.86% similar (48 nucleotide differences) to that of *N. cayensis* (Fig. 11). All phylogenetic trees recovered as polyphyletic *Neopolystoma* and *Polystomoides*. The 28S and 18S are highly conserved, and the CO1 is the most variable, but none of these markers rejected polyphyly of these 3 polystome genera.

Remarks

Our specimens differed from published descriptions of species of *Neopolystoma* by the combination of having intestinal ceca that are diverticulate and that terminate dorsal to the haptor, distinctive marginal hooklets each having a handle and a guard of approximate equal length and having a much longer and curved shaft, 16 genital coronet spines that each possess 1–2 flanges per spine, pre-testicular vaginal pores, and vaginal ducts that are anterior to the junction of the oviduct and genito-intestinal canal. Our smaller adult specimens were identical to the large adult specimens in all regards except that the vaginal pores are slightly more lateral (such that they appear to be nearly marginal) and the genital coronet spines have a single flange.

The number of coronet spines (which encircle the terminal male genitalia) is a common taxonomic feature to differentiate species of *Neopolystoma* (see Morrison and du Preez, 2011). Although some species apparently exhibit a wide range for the number of coronet spines, others evidently have a single number of spines. Our specimens had 16 genital coronet spines (Fig. 8), likening them to 7 congeners (*N. exhamatum*, *N. orbiculare*, *N. cayenensis*, *N. chelodinae*, *N. cyclovitellum*, *N. mackleyi*, *N. palpabrae*). In addition to this feature, all of these species (except *N. palpabrae*) have vaginal pores that are pre-testicular (*N. palpabrae* has vaginal pores at level of the testis) (Figs. X–X). Further, *N. cayenensis*, *N. chelodinae*, *N. cyclovitellum*, and *N. mackleyi* have cecae that terminate anterior to the haptor; whereas, our specimens, *N. exhamatum*, and *N. orbiculare* have cecae that terminate dorsal to the haptor (Figs. 1–2). Of those three species, *N. exhamatum* and *N. orbiculare* have genital coronet spines that each have a single flange; whereas, our large adult specimens have genital coronet spines that possess a proximal and distal flange. Strictly speaking, the number of flanges does not clearly differentiate our specimens because our small adult specimens had a single flange per genital coronet spine.

However, our specimens are further distinct from those species by having vaginal ducts that are anterior to the junction of the oviduct and genito-intestinal canal (hereafter referred to as “the junction”) (Figs. 1–4). The other two species have vaginal ducts that are at level of the junction. In addition to this combination of diagnostic features that warrant identification of our specimens as *Neopolystoma cf. orbiculare*, they clearly have cecal diverticula. Cecal diverticulae evidently can become indistinct if specimens are flattened (Platt et al., 2011); however, our specimens were heat-killed and subjected to no coverslip pressure such that the morphology of the ceca is reflective of the live specimens.

DISCUSSION

Our phylogenetic results confirm again that 3 of the 5 accepted chelonian polystome genera are polyphyletic and need taxonomic revision. Such a taxonomic revision should search for morphological similarities among the respective clades. This should include study of the type material for the genera and develop diagnostic taxonomic features associated with the male and female genitalia, in addition to hamuli characteristics.

Additional alligator snapping turtles should be necropsied for polystome infections across this turtle’s geographic distribution, within Gulf of Mexico river drainages (Southeastern US): distinct host populations or cryptic species may exist that harbor innominate polystomes. At present, the alligator snapping turtle is accepted as a single species that evidently exhibits infrequent or limited dispersal between rivers in the Southeastern US, with at least 6 evolutionarily significant units (ESUs) diagnosed with mtDNA and microsatellite DNA evidence (Echelle et al., 2010). The most distinctive ESU is alleged to be represented by alligator snapping turtles in the Suwannee River, Florida, and some authors regard these alligator snapping turtles as comprising a distinct species, *Macrochelys suwanniensis* Thomas, Grantaosky, Bourque,

Krysko, Moler, Gamble, Suarez, Leone, Enge, and Roman, 2014) (see Roman et al. [1999]; Echelle et al. [2010]; Murray et al. [2014]; Thomas et al. [2014]; Folt and Guyer [2015]). Given the high degree of host specificity exhibited by polystomes that infect turtles, it is of course possible that this putatively distinct alligator snapping turtle in the Suwannee River hosts a species of *Neopolystoma* that is not conspecific with our specimens.

Regarding the other snapping turtles (Chelydridae) as hosts, the only other species that is confirmed as a polystome host is the common snapping turtle, *Chelydra serpentina* (Linnaeus, 1758) (urinary bladder). Caballero (1938) and Lamothe-Argumedo (1972) described specimens of *N. domitalae* from Tabasco, Mexico. From common snapping turtles in Texas, Harwood (1932) and Price (1939) reported *Polystomoidella oblongum* (Wright, 1879) Price, 1939 and McAllister et al. (2008) reported *Polystomoidella whartoni*.

Regarding the record of *N. domitalae* from the snapping turtle in Tabasco, this host may have been misidentified because *C. serpentina* is now understood as ranging in the US portion of North America east of the Rocky Mountains only (Rhodin et al., 2017); whereas, the Central American snapping turtle, *Chelydra rossignonii* (Bocourt, 1868) has a delimited geographic distribution that includes the type locality of *N. domitalae*. That is, we suspect that the host of *N. domitalae* could have been *C. rossignonii*. The other nominal species of *Chelydra* Schweigger, 1812, *Chelydra acutirostris* Peters, 1862, is limited to the southern portion of Central America and the northwestern portion of Columbia (Rhodin et al., 2017). All of these chelydrids deserve attention because only 1 has been reported as a polystome host.

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FIGURE LEGENDS

Figures 1–2. *Neopolystoma cf. orbiculare* from alligator snapping turtle, *Macrochelys temminckii* (Cryptodira: Chelydridae) from the Pascagoula River in Mississippi, USA. Scale value aside bars. **(1)** Body of holotype (USNM XXXXXX) showing false oral sucker (os), pharynx (ph), esophagus (es), cecal bifurcation (cb), excretory pores (ep), genital bulb (gb), genital spines (gs), uterus (ut), seminal vesicle (sv), egg (eg), ovary (ov), vaginae (v), vas deferens (vd), testis (t), sinistral cecum (sc), dextral cecum (dc), vitellarium (vr), cecal terminus (ct), haptor (h), and haptoral sucker (hs). Ventral view. **(2)** Body of paratype (USNM XXXXXX), showing features labeled in Fig. 1. Ventral view.

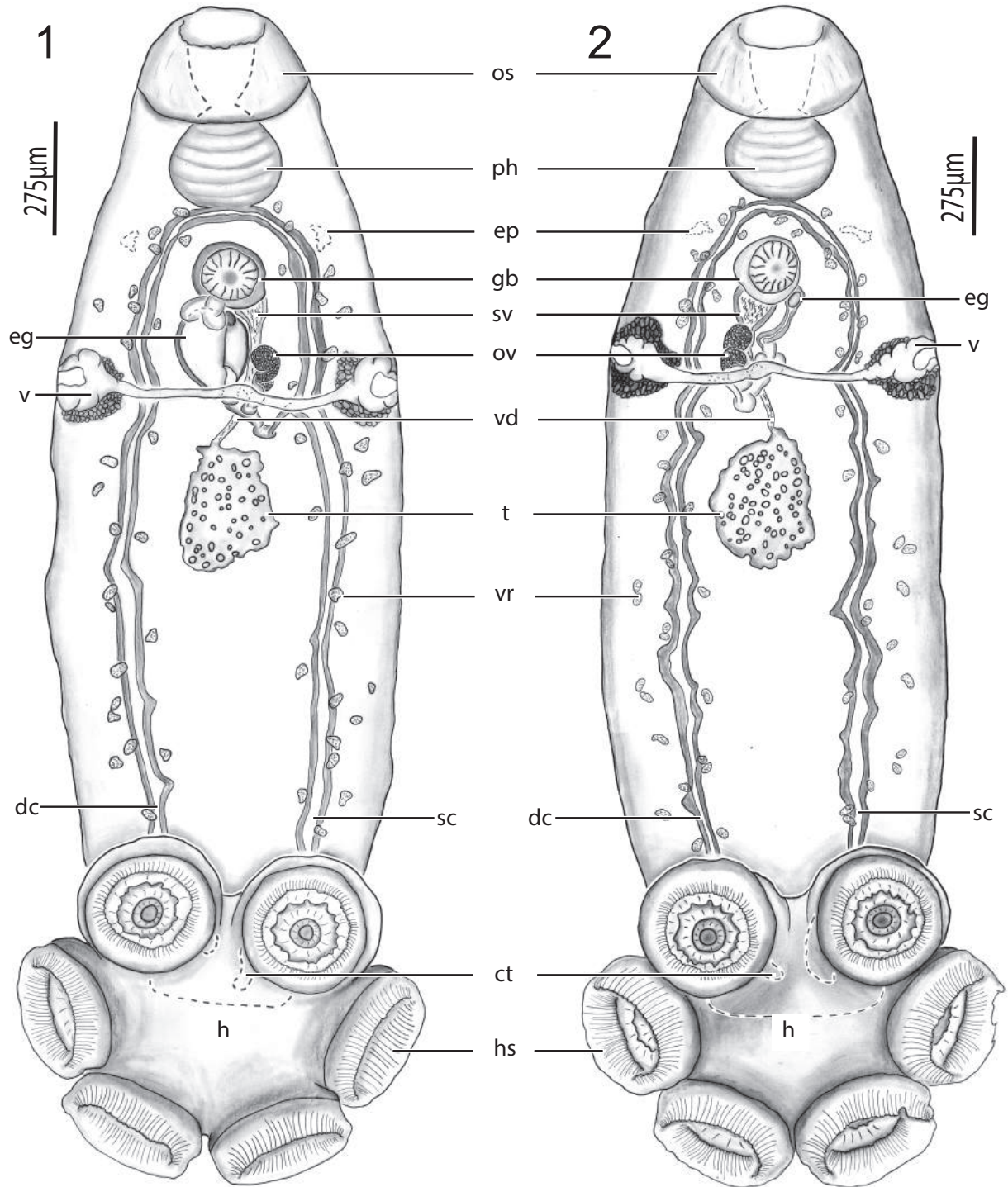
Figures 3–5. *Neopolystoma cf. orbiculare* from alligator snapping turtle, *Macrochelys temminckii* (Cryptodira: Chelydridae) from the Pascagoula River in Mississippi, USA. Scale value aside bars. **(3)** Genitalia of holotype (USNM XXXXXX) showing genital bulb (gb), genital spines (gs), genital pore (gp), egg (eg), uterus (ut), seminal vesicle (sv), ovary (ov), oviduct (od), vaginae (v), genito-intestinal canal (gc), vas deferens (vd), sinistral cecum (sc), dextral cecum (dc), and testis (t). Ventral view. **(4)** Genitalia of paratype (USNM XXXXXX), showing features labeled in Fig. 3. Ventral view.

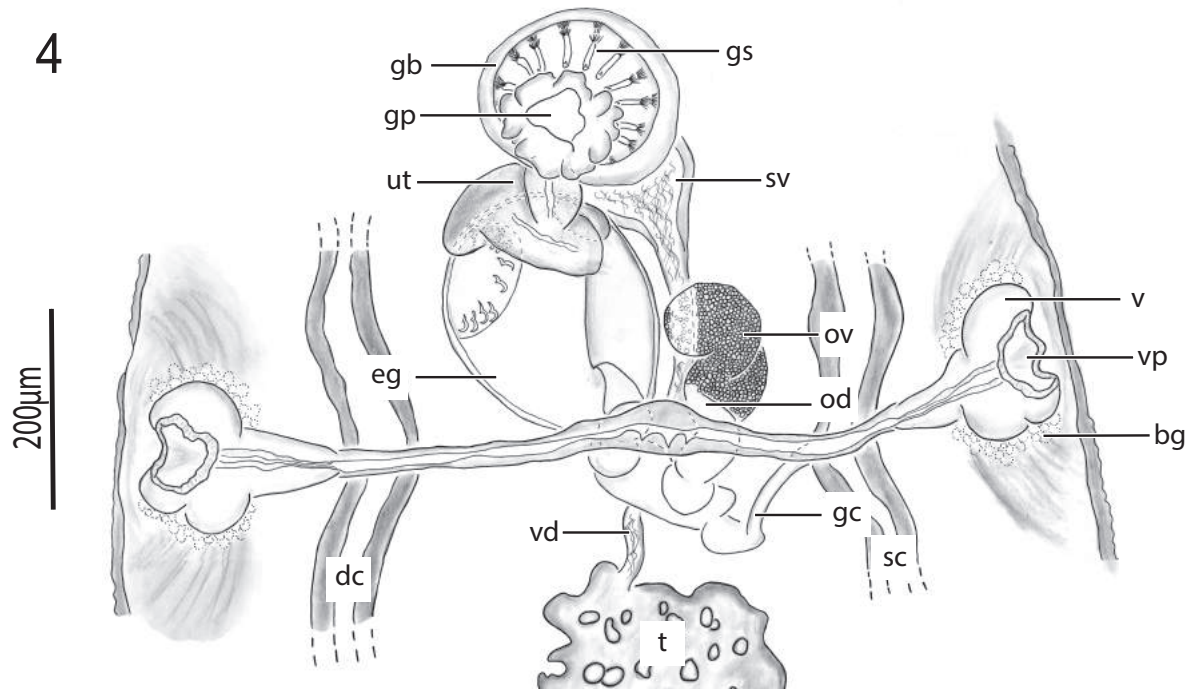
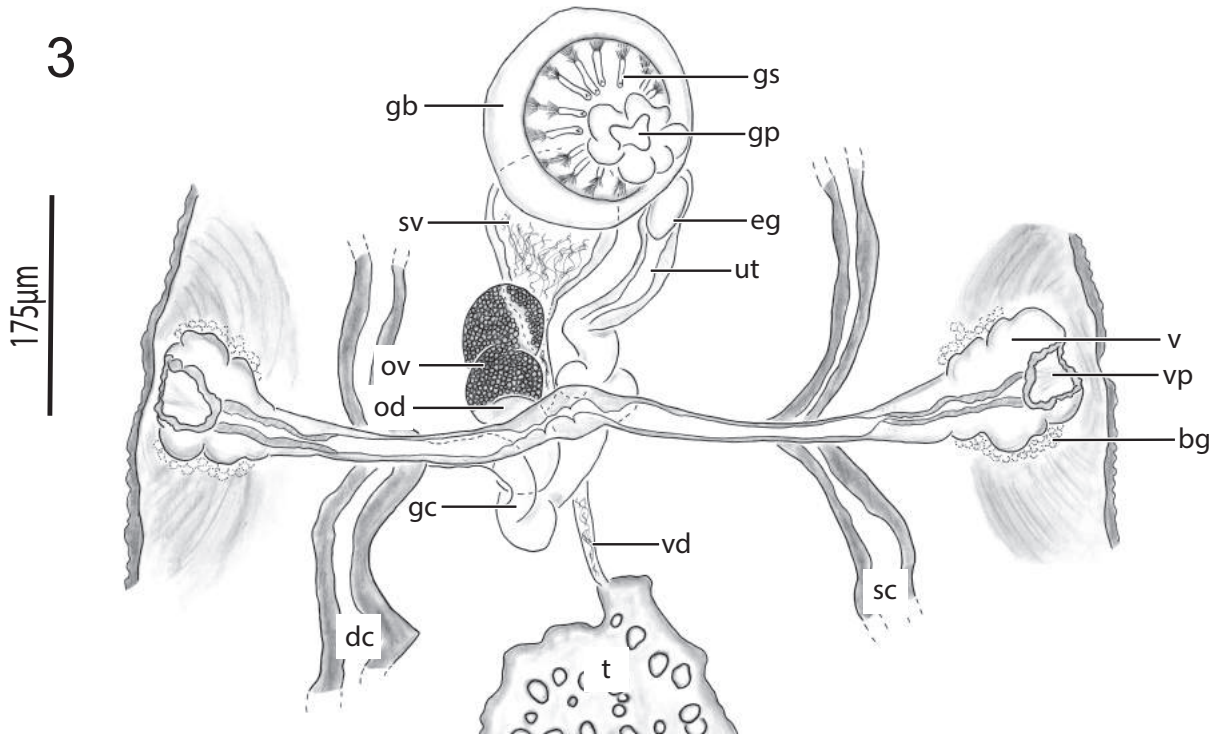
Figures 5–8. *Neopolystoma cf. orbiculare* from alligator snapping turtle, *Macrochelys temminckii* (Cryptodira: Chelydridae) from the Pascagoula River in Mississippi, USA. Scale value aside bars. **(5)** Haptor of paratype (USNM XXXXXX) showing haptoral sucker (hs), marginal hooklets (mh), and cuticular loops (cl). Ventral view. **(6)** Marginal hooklet of paratype (USNM XXXXXX). Ventral view. **(7)** Cuticular loops of paratype (USNM XXXXXX). Ventral view. **(8)** Genital coronet of holotype (USNM XXXXXX), showing genital spines (gs) and flanges (f). Ventral view.

Figure 9. 28S bayesian phylogeny. Values aside nodes are posterior probability. Scale bar is in substitutions per site.

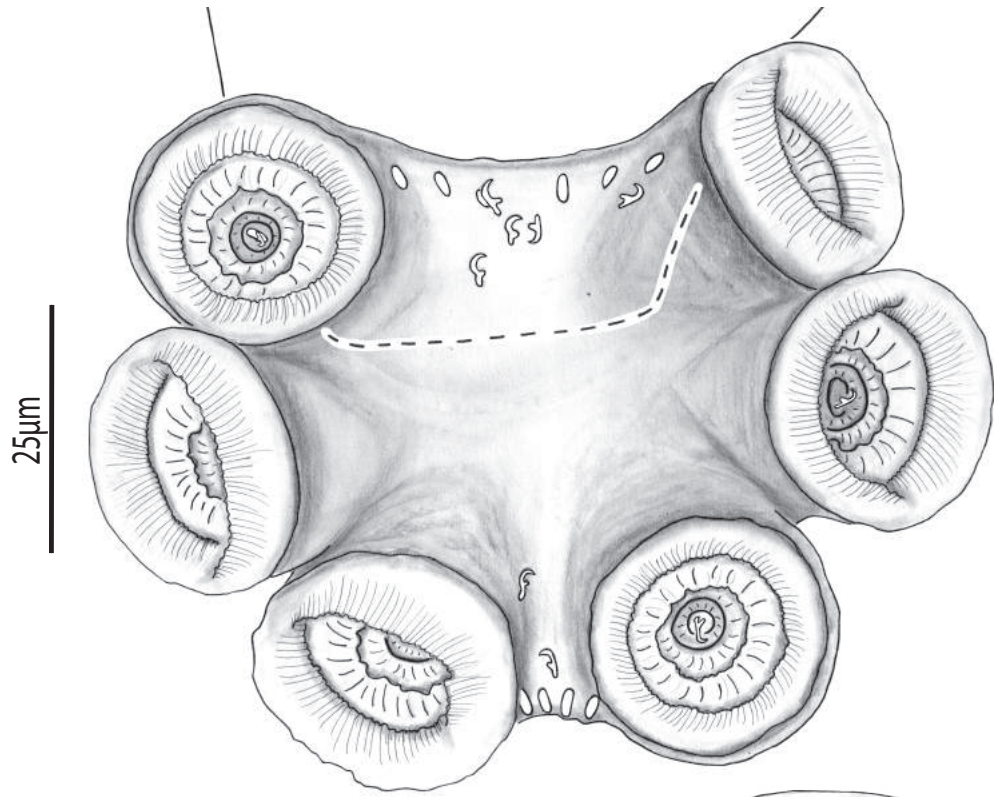
Figure 10. 18S bayesian phylogeny. Values aside nodes are posterior probability. Scale bar is in substitutions per site.

Figure 11. CO1 bayesian phylogeny. Values aside nodes are posterior probability. Scale bar is in substitutions per site.





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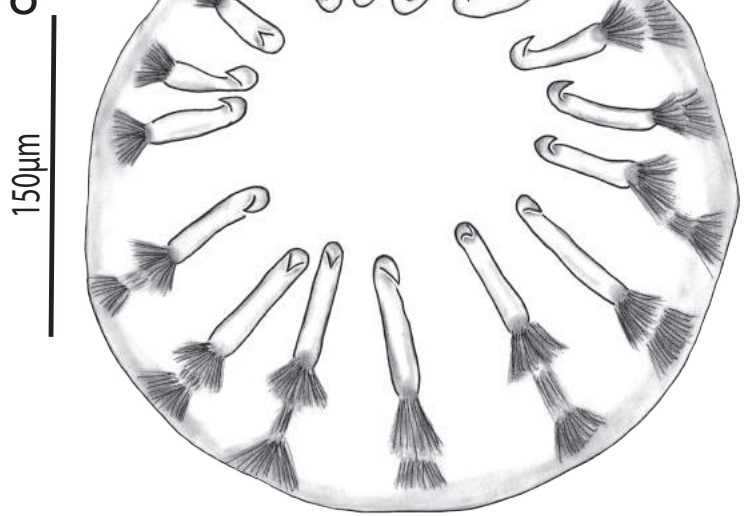
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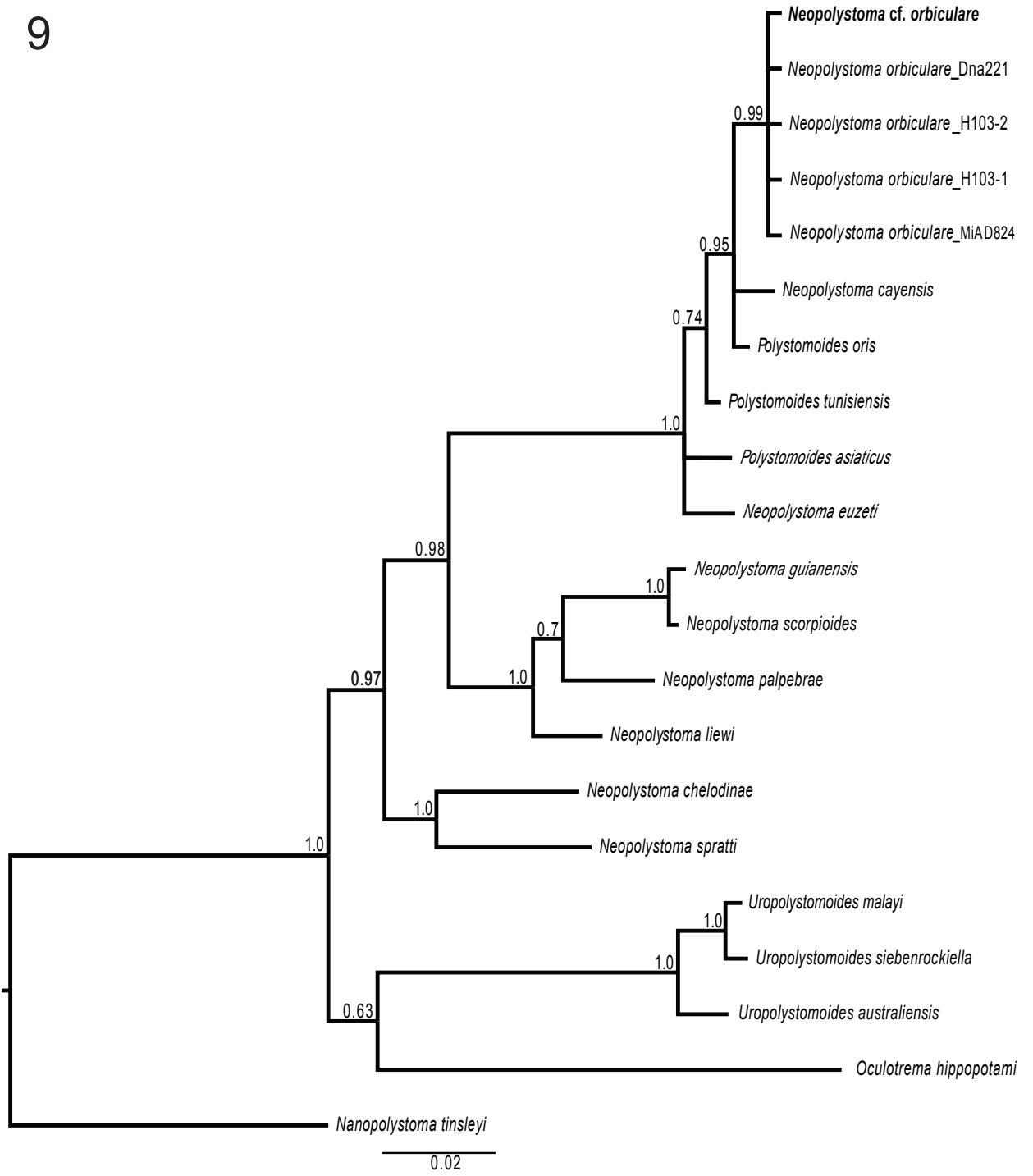


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