Taxonomy and systematics of freshwater turtle blood flukes (Digenea: Schistosomatoidea)

by

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ABSTRACT

Blood flukes (Platyhelminthes: Trematoda: Digenea: Schistosomatoidea) currently comprise three families: Schistosomatidae Stiles and Hassall, 1898 (species infecting mammals and birds and one crocodilian), Aporocotylidae Odhner, 1912 (species infecting elasmobranchs and fishes), and the paraphyletic "Spirorchiidae Stunkard, 1921" (species infecting marine and freshwater turtles; TBFs hereafter). Before this thesis, the turtle blood flukes comprised 84 species belonging to 19 genera, with much of the previous work focusing on TBFs infecting marine turtles (30 spp. [36%] of 10 genera described from three marine turtle species). Herein, I used morphology and molecular gene sequence data (large subunit rDNA [28S], small subunit rDNA [18S], internal transcribed region 2 [ITS2]) to explore alpha taxonomy and to revise the current systematics of freshwater turtle blood flukes domestic and abroad. Blood flukes examined herein were collected from turtles comprising 16 species of five families (Cryptodira: Chelydridae, Emydidae, Geoemydidae, Kinosternidae, Trionychidae) and from river drainages in Mississippi, Alabama, Florida (U.S.A.), Malaysia, and Vietnam. Collectively from these hosts, I identified 21 TBFs comprising six genera (*Spirorchis* MacCallum, 1918, *Hapalorhynchus* Stunkard, 1922, *Vasotrema* Stunkard, 1926, *Coeuritrema* Mehra, 1933 [resurrected herein], *Baracktrema* Roberts, Platt, and Bullard, 2016, and *Platt* Roberts and Bullard n. gen.). Newly collected specimens were compared to museum material of 22 TBF species comprising holotypes, paratypes, cotypes, syntypes, and vouchers (107 slides) from the United States National Museum, Smithsonian (USNM), American Museum of Natural History (AMNH), and the Harold W. Manter Laboratory Collection (HWML). From the newly collected 21 TBFs, I proposed two new genera (*Baracktrema*, *Platt*), resurrected one genus (*Coeuritrema*), revised one genus (*Vasotrema*), emended three genera (*Spirorchis*, *Hapalorhynchus*, *Unicaecum*), reassigned nine species, described eight new species, and reported 13 additional turtle blood fluke taxa (either previously described or innominate at this time). This thesis comprises the first records of any turtle blood fluke infecting turtles from Alabama river drainages.

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CHAPTER 1: NEW GENUS OF BLOOD FLUKE (DIGENEA: SCHISTOSOMATOIDEA) FROM MALAYSIAN FRESHWATER TURTLES (GEOEMYDIDAE) AND ITS PHYLOGENETIC POSITION WITHIN SCHISTOSOMATOIDEA

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ABSTRACT

Baracktrema obamai n. gen., n. sp. infects the lung of geoemydid turtles (black marsh turtle, *Siebenrockiella crassicollis* [type host] and southeast Asian box turtle, *Cuora amboinensis*) in the Malaysian states of Perak, Perlis, and Selangor. *Baracktrema* and *Unicaecum* Stunkard, 1925 are the only accepted turtle blood fluke genera having the combination of a single cecum, single testis, oviducal seminal receptacle, and uterine pouch. *Baracktrema* differs from *Unicaecum* by having a thread-like body approximately 30−50× longer than wide and post-cecal terminal genitalia. *Unicaecum* has a body approximately 8−12× longer than wide and terminal genitalia that are anterior to the distal end of the cecum. The new genus further differs from all other accepted turtle blood fluke genera by having a cecum that is highly convoluted for its entire length, a spindle-shaped ovary between the cirrus sac and testis, a uterine pouch that loops around the primary vitelline collecting duct, a Laurer's canal, and a dorsal common genital pore. Phylogenetic analysis of the D1–D3 domains of the nuclear large subunit ribosomal DNA (28S) revealed, with high nodal support and as predicted by morphology, that *Baracktrema* and *Unicaecum* share a recent common ancestor and form a clade sister to the freshwater turtle blood flukes of *Spirorchis*, paraphyletic *Spirhapalum*, and *Vasotrema* and that, collectively, these flukes were sister

to all other tetrapod blood flukes (*Hapalorhynchus* + *Griphobilharzia* plus the marine turtle blood flukes and schistosomes). Pending a forthcoming emended morphological diagnosis of the family, the clade including *Spirorchis* spp., paraphyletic *Spirhapalum*, *Vasotrema*, *Baracktrema*, and *Unicaecum* is a likely placeholder for "Spirorchiidae Stunkard, 1921" (type genus *Spirorchis* MacCallum, 1918; type species *Spirorchis innominatus* Ward, 1921). The present study comprises the 17th blood fluke known to infect geoemydid turtles and the first proposal of a new genus of turtle blood fluke in 21 yr.

INTRODUCTION

Blood flukes (Schistosomatoidea; see Orélis-Ribeiro et al., 2014) that mature in turtles ("turtle blood flukes", hereafter TBFs) have historically been included in paraphyletic "Spirorchiidae" (see Platt, 2002; Snyder, 2004; Orélis-Ribeiro et al., 2014). They total 84 accepted species (54 freshwater; 30 marine) assigned to 19 genera, and infect 40 turtle species (37 freshwater; 3 marine; Smith 1997a, 1997b; Platt, 1993, 2002; Tkach et al., 2009; Platt and Sharma, 2012). Nearly all (320 of 327; 98%) extant turtle species occur in freshwater but a disproportionate number (36%) of TBFs have been described from only 3 of the 7 (43%) extant marine turtles, i.e., *Caretta caretta* (Linnaeus, 1758), *Chelonia mydas* (Linnaeus, 1758), and *Eretmochelys imbricata* (Linnaeus, 1766). Because (i) only 40 of 327 (12%) turtle species are documented as TBF hosts, (ii) there are more than twice as many accepted TBF species (84) as documented turtle hosts, and (iii) only 7 of 327 (2%) turtle species are marine (Smith, 1997a, 1997b; Platt, 2002; Snyder, 2004; van Dijk et al., 2014), we suspect that a large proportion of TBF biodiversity remains to be discovered, especially among freshwater turtles.

The iconic status and conservation rearing of marine turtles perhaps has hastened the study of their parasites and diseases (e.g., Witham, 1973; Glazebrook and Campbell, 1990a, 1990b) but freshwater turtles are less studied, despite a growing freshwater turtle aquaculture industry (Haitao et al., 2008). Like their marine counterparts, however, freshwater turtles are gaining protection and obtaining higher conservation status (IUCN, 2015) such that examination of these hosts for parasites may become increasingly prohibited (Platt and Sharma, 2012). As such, efforts toward TBF species discovery will likely focus on wide-ranging turtle species of low conservation concern or on aquacultured turtles. Yet, as with marine turtles, an understanding of host-parasite relationships, life cycles, immunology, and pathogenesis will become increasingly relevant due to the need for monitoring, management, and conservation of imperiled freshwater turtle populations (Benz and Collins, 1997). Specific details about how freshwater turtles are cultured are lacking (Haitao et al., 2008) but turtles confined in high densities and kept in extensive systems (e.g., earthen ponds) could develop intense TBF infections (e.g., Holliman and Fisher, 1968) in the presence of a susceptible snail host. This is analogous to sporadic aquaculture disease issues associated with fish blood flukes in cultured marine and freshwater fishes (Bullard and Overstreet, 2002, 2008). Moreover, in the face of scarcity of clean water and expansion of hydroelectric development, freshwater turtle habitats will be increasingly subjected to anthropogenic change globally; possibly leading to deleterious changes in the host-parasite relationship or local extinction of some blood flukes (depending on the biology of the intermediate host). Observations of marine TBF infections indicate that some TBFs can be pathogenic but less is known about those aspects of the adult TBF-freshwater turtle relationship (Holliman and Fisher, 1968; Holliman et al., 1971); although previous work has focused on host cellular response to TBF eggs (Goodchild and Dennis, 1967).

Extant turtles are classified in the suborders Pleurodira (Chelidae + Pelomedusidae + Podocnemididae) and Cryptodira (11 families, including Geoemydidae) (van Dijk et al., 2014; Crawford et al., 2015). Eight of those 14 turtle families host TBFs: Geoemydidae (10 of 69; Table I), Chelidae (2 of 56 species infected), Cheloniidae (3 of 6), Chelydridae (1 of 4), Emydidae (10 of 53), Kinosternidae (3 of 26), Pelomedusidae (2 of 18), and Trionychidae (9 of 31). Over half of the species in each of these turtle families have not been examined for TBF infections. Regarding geoemydids, the host family of the present study, 10 of 69 (14%) species ranging from central Europe to southeast Asia host 16 TBFs assigned to 5 genera (Mehra, 1934, 1940; Sinha, 1934; Takeuti, 1942; Simha, 1958; Dwivedi, 1967; Rohde et al., 1968; Mehrotra, 1973a, 1973b; Tandon and Gupta, 1982, 1985; Tkach et al., 2009; Platt and Sharma, 2012; van Dijk et al., 2014). That 10 geoemydids are infected by species assigned to 5 TBF genera indicates a proportionally high level of generic, and perhaps species-level, TBF diversity among geoemydids.

Herein, we propose a new TBF genus based on specimens collected from 2 previously reported geoemydid hosts, i.e., the black marsh turtle, *Siebenrockiella crassicollis* (Gray, 1830) and the southeast Asian box turtle, *Cuora amboinensis* (Riche in Daudin, 1801) as well as conduct a phylogenetic analysis including the new genus and based on sequence data for the partial D1–D3 domains of 28S rDNA. This is the first proposal of a new TBF genus in 21 yr (Platt and Pichelin, 1994), i.e., since *Uterotrema* Platt and Pichelin, 1994.

MATERIALS AND METHODS

Turtles were purchased from commercial turtle trappers during January and February 2008. Turtles were injected with a lethal dose of sodium pentabarbitol and necropsied by TRP using standard procedures (Platt, 1988, 1998) after the animal failed to respond with an eye blink when touched with a camel-hair paint brush. TBFs were killed by placing them in a small vial with enough saline to prevent desiccation, followed by the addition of steaming 5% neutral buffered formalin before the vial was capped and gently shaken. TBFs were stored in formalin, rinsed in water to remove formalin before staining, stained with Ehrlich’s hematoxylin, cleared in methyl salicylate, prepared as whole mounts in Canada balsam, and studied using a compound microscope with differential interference contrast (DIC) optics. Illustrations of stained, whole-mounted specimens were made with the aid of a Leica DM-2500 microscope (Leica Microsystems, Wetzlar, Hesse, Germany) equipped with DIC and a drawing tube. Measurements are herein reported in micrometers (µm), followed by the number measured in parentheses. Turtle scientific names and taxonomic authorities follow van Dijk et al. (2014). Higher-level turtle classification and nomenclature follows Guillon et al. (2012) and Crawford et al. (2015), and anatomical terms for turtles follow Ashley (1974).

Classification and anatomical terms for TBFs follow Byrd (1939: uterine pouch), Luhman (1935: genital sucker), Platt (1998, 2002: genital spines, plicate organ, and most other terms; 1993: median esophageal pouch), and Yamaguti (1971: Manter's organ).

Specimens for molecular analyses were preserved in 95% EtOH and processed for sequencing the D1–D3 domains of the nuclear large subunit ribosomal DNA (28S). Total genomic DNA was extracted using DNeasy^{TM} Blood and Tissue Kit (Qiagen, Valencia, California). Polymerase chain reaction (PCR) was carried out using the forward primer "U178"(5′-GCA CCC GCT GAA YTT AAG) (where Y is C or T) and the reverse primer "L1642"(5′-CCA GCG CCATCC ATT TTC A) (Lockyer et al., 2003). PCR amplifications were performed in a total volume of 25 μl, consisting of approximately 25–40 ng of template of gDNA, 0.2 μM of each primer along with 2.5 μl of 10× buffer with $MgCl_2$, 2μl of dNTP mixture and 0.6 units of *Taq* polymerase as provided in the Takara Ex Taq kit (Takara Biomedicals, Otsu, Japan). The cycling profile consisted of an initial 4 min at 94 C for denaturation, followed by 40 repeating cycles of 94 C for 30 sec for denaturation, 50–56 C for 30 sec for annealing, and 72 C for 2 min for extension, followed by a final 5 min at 72 C for extension. All PCR reactions were carried out in a Perkin Elmer GeneAmp 2400 thermocycler (Perkin Elmer, Waltham, Massachusetts). PCR products were purified using the PCR Product Pre-Sequencing Kit (USB Corporation, Cleveland, Ohio) following the manufacturer's protocols. Sequences were determined directly from PCR templates using a Beckman/Coulter CEQ2000XL DNA sequencer and dye-terminator chemistry. Primers used in sequencing reactions included a forward primer on the 5' end of the fragment, Dig12 (5'-AAG CAT ATC ACT AAG CGG) and a reverse primer on the 3' end of the fragment, LSU1500R (5'-GCT

ATC CTG AGG GAA ACT TCG) (Tkach et al., 1999, 2000). Internal primers included the forward primers 300F (5’-CAA GTA CCG TGA GGG AAA GTT G) and 900F (5’-CCG TCT TGA AAC ACG GAC CAA G) and the reverse primers 300R (5’-CAA CTT TCC CTC ACG GTACTT G) (Lockyer et al., 2003) and ECD2 (5’-CTT GGT CCGTGT TTC AAG ACG GG) (Olson et al., 2003).

Sequences obtained were aligned with those for blood flukes available in GenBank. Homologous sequences from representatives of Diplostomoidea were used as outgroups (Orélis-Ribeiro et al., 2014). The ingroup consisted of the sequence data from the new species (GenBank No. XXXXXXXX) plus 73 taxa from Orélis-Ribeiro et al. (2014) (GenBank accession numbers reported therein), excluding short sequences from *Aporocotyle spinosicalis* (AF167094) and *Cardicola coeptus* (JF803976) and blood fluke cercariae. Sequences were aligned using MAFFT (Katoh and Toh, 2010) with default settings implemented in the CIPRES Science Gateway V. 3.3 (Miller et al., 2010). The alignment was refined by eye using MEGA version 5.2.2 (Tamura et al., 2011) and ends of each fragment were trimmed to match the shortest sequence. Ambiguous positions were identified and removed using the Gblocks server (Castresana, 2000) with settings for a less stringent selection and the following parameters: minimum number of sequences for a conserved position = 40; minimum number of sequences for a flanking position = 40; maximum number of contiguous non-conserved positions = 8; minimum length of a block = 5; allowed gap positions = with half. Bayesian inference (BI) was performed using the Metropolis-coupled Markov chain Monte Carlo method (MC^3) in MrBayes version 3.2.6 (Huelsenbeck et al., 2001; Ronquist and Huelsenbeck, 2003; Huelsenbeck and Ronquist, 2005) and run on CIPRES (Miller et al., 2010). Model of evolution was selected based on the Akaike Information Criterion (Posada and Buckley, 2004) as implemented in the jModelTest version 2.1.4 (Darriba et al., 2012; Guindon and Gascuel, 2003). The GTR + I + G (proportion of invariable sites = 0.288 and gamma distribution = 1.352) model was inferred as the best estimator; therefore, BI used the following parameters: nst = 6, rates = invgamma, ngammacat = 4, and default priors. Analyses were run in duplicate each containing 4 independent chains (3 heated and 1 cold chain) (nchains = 4) for 1.0×10^7generations (ngen = 10,000,000) sampled at intervals of 1,000 generations (samplefreq = 1,000). Results of the first 2,500 sampled trees were discarded as "burn-in" based on the stationarity of the likelihood values, assessed by plotting the log-likelihood values of the sample points against generation time using Tracer version 1.5 (Rambaut and Drummond, 2009). All retained trees were used to estimate posterior probability of each node. A majority rule consensus tree with average branch lengths was constructed for the remaining trees using 'summarize the trees' (sumt) in MrBayes. Resulting phylogenetic trees were visualized using FigTree v1.3.1 (Rambaut, 2009). Branch supports were considered as significant when posterior probabilities were >0.95.

DESCRIPTION

Baracktrema **n. gen. Roberts, Platt, and Bullard**

(Figs. 1−10)

Diagnosis: Body cylindrical (not flattened or ventrally concave), thread-like, extremely elongate (approximately 30−50× longer than wide), with posterior end tapering more sharply than anterior end, lacking tegumental spines, body protuberances, and ventral sucker. Anterior sucker terminal, comprising an apparently protrusive and eversible hemisphere directing anteriad. Pharynx present, enveloping anterior extremity of esophagus, centered on mouth, aspinous. Esophagus short (approximately 5% of body length), ventral to anterior nerve commissure, lacking diverticula, straight anteriorly and slightly sinuous posteriorly, expanding laterally to form esophageal swelling immediately anterior to esophageal-intestinal junction; esophageal gland surrounding esophagus from level of nerve commissure to esophageal-intestinal junction, strongly basophilic anterior to esophageal-intestinal junction. Plicate organ and median esophageal pouch absent. Intestine comprising a single cecum, smooth (lacking diverticula or secondary rami), extremely elongate (up to 90% of body length), terminating posterior to gonads, extensively convoluted along entire length. Testis dorsal or lateral to cecum, occupying middle to posterior portion of body, exceeding 1/4−1/3 of body length. Cirrus sac present. Genital sucker and genital spines absent. Ovary single, longer than wide, spindle-shaped, lacking lobes, dextral, dorsal to cecum, anterior to distal tip of cecum, post-testicular. Oviduct extending posteriad from ovary, medial; middle portion of oviduct functioning as oviducal seminal receptacle. Laurer's canal post-cecal, post-gonadal, opening dorsally near level of oötype. Vitellarium follicular, filling space surrounding cecum between esophageal-intestinal junction and ovary. Oötype a strongly glandular and thick-walled spheroid chamber, post-cecal, post-gonadal, anterior to common genital pore. Uterus post-gonadal, post-cecal, extensively convoluted, with associated uterine pouch; uterine pouch ventral to uterus and all other genitalia, post-cecal, post-gonadal, inverse U-shaped, comprising distinctive ascending (proximal) and descending (distal) portions; ascending portion extending dorsoanteriad from uterus, ventral to vas deferens, curving mediad before connecting with descending portion of uterine pouch; descending portion of uterine pouch laterally expanded, massive, extensively convoluted, extending posteriad before ending blindly. Common genital pore dorsal, sinistral. Excretory vesicle y-shaped; excretory pore terminal. Manter's organ absent.

Differential diagnosis: Body cylindrical, extremely elongate (approximately 30−50× longer than wide), lacking spines and ventral sucker. Anterior sucker a protrusive and eversible hemisphere directing anteriad. Esophagus short (approximately 5% of body length). Intestine comprising a single cecum, extremely elongate, terminating posterior to gonads, extensively convoluted. Testis exceeding 1/4−1/3 of body length. Ovary spindle-shaped, anterior to distal tip of cecum, post-testicular, occupying posterior 1/5 of body. Laterally-expanded medial portion of oviduct an oviducal seminal receptacle. Laurer's canal post-cecal, post-gonadal, opening dorsally near oötype. Oötype a glandular and thick-walled spheroid chamber, post-cecal, post-gonadal, anterior to common genital pore. Uterus post-gonadal, post-cecal, convoluted along entire length; uterine pouch post-cecal, post-gonadal, inverse U-shaped, with ascending and descending portions. Common genital pore dorsal, sinistral. Manter's organ absent.
Type species: Baracktrema obamai n. sp.

Baracktrema obamai n. sp. Roberts, Platt, and Bullard

(Figs. 1−9)

Description of adult (based on 17 individuals comprising 5 and 12 intact and partial specimens, respectively; number in parentheses indicates number of specimens *measured):* Body 3,550−6,530 (5) long, 58−130 (11) wide or 1−2% (4) body length at level of esophageal-intestinal junction, 68−158 (13) wide or 2−3% (4) body length at level of testis (typically maximum width of specimen), 43−75 (11) wide or 1−2% (5) body length at level of genital pore (Figs. 1−7). Anterior sucker 10−23 (10) long or <1% (4) body length, 23−40 (10) wide or 21−28% (5) maximum body width (Fig. 2). Pharynx 25−45 (11) long or 13−20% (10) esophagus length, 23−43 (11) wide or 2.7−4.6 (10) × maximum esophagus width (Fig. 2). Nerve commissure 48−113 (11) or 1−2% (5) of body length from anterior body end (Fig. 2). Esophagus 148−340 (10) long or 3−5% (4) of body length, 5−15 (11) wide anteriorly, with wall 3−5 (11) thick anteriorly (Figs. 1, 2); posterior esophageal swelling 25−55 (12) long or 11−23% (10) of esophagus total length, 15−30 (12) wide or 1.7−3 (11) × wider than anterior portion of esophagus, with wall 7−25 (12) thick; esophageal gland 100−175 (6) long or 2% (2) of body length, 45−113 (6) wide or 48−92% (6) of body width and 2−4.5 (6) × width of esophagus at swelling. Intestine 3,150−6,170 (7) long or 80−89% (5) of body length, 20−110 (10) wide or 29−95% (9) of body width at esophageal-intestinal junction, 15−83 (14) wide or 16−58% (13) of body width at level of testis; post-cecal distance 278−850 (10) or 9−15% (5) of body length (Figs. 1−7).

Testis 1,275−3,078 (6) long or 26−42% (3) of body length, 40−113 (11) in maximum width or 58−95% (8) of body width at level of testis; post-testicular space 400−1,150 (8) long or 11−21% (4) of body length (Figs. 1, 4−6). Vas deferens 450−875 (7) long or 12−16% (4) of body length, narrow, extending posteriad from testis in parallel with body margin, sinistral, 10−25 (8) in maximum width at level of testis, narrowing posteriorly to 3−8 (10) before joining seminal vesicle (Figs. 7−9). Seminal vesicle 138−333 (10) long, 23−38 (10) wide or 6−14 × longer than wide, nearly filling cirrus-sac lumen (Figs. 8, 9). Cirrus 25−125 (10) long, 5−30 (10) wide; cirrus sac a membranous, thin-walled sac surrounding terminal male genitalia, directing posteriad, straight or slightly sinuous, 208−408 (10) long or 4−8% (4) of body length, 28−40 (10) wide or 21−36% (9) of body width at level of cirrus sac (Figs. 8, 9).

Ovary parallel with long axis of body, with anterior portion slightly overlapping posterior margin of testis, 110−305 (11) long or 2−7% (5) of body length, 33−90 (11) wide or 37−74% (9) of maximum body width, 1.6−6.8 × longer than wide; post-ovarian distance 294−1,215 (11) or 8−19% (5) of body length (Figs. 7−9). Oviduct extending 188−375 (8) or 5−7% (3) of body length posteriad from posterior end of ovary, 10−15 (9) in maximum width, becoming sinuous posteriorly before expanding laterally to form oviducal seminal receptacle; oviducal seminal receptacle posterior to distal end of cecum, U-shaped, extending sinistrad slightly before sharply curving mediad, extending anteriad aside posteriorly-directed proximal portion, curving dorsally and mediad before extending again anteriad and connecting with oötype, 28−63 (9) in maximum width or 34−54% (8) of body width at oviducal seminal receptacle (Figs. 7−9). Laurer's canal a narrow duct extending posteriad from 40 (1) before oötype, dextral to oötype, opening dorsal to oviducal seminal receptacle, 50 (1) long, 5 (1) wide (Figs. 7−9). Vitellarium comprising a series of interconnected spheroid masses of large follicles, beginning 310−485 (8) or 7−8% (3) of body length from anterior end (near esophageal-intestinal junction), lateral to cecum and testis in anterior body region, dorsal to cecum and testis in posterior body region, terminating 715−1,160 (8) or 13−23% (3) of body length from posterior body end (lateral to ovary); primary vitelline collecting duct J-shaped, emerging from posterior extent of vitellarium in sinistral half of body lateral to ovary, extending posteriad and parallel to body margin for 448−663 (7) or 7−13% (3) of body length before turning mediad for 5−65 (7) well posterior to level of distal tip of cecum before turning and extending anteriad 80−258 (7) or 2−4% (3) of body length and connecting with distal portion of oviduct to form ovo-vitelline duct; ovo-vitelline duct a short duct connecting vitellarium and oviduct with oötype, 8−13 (7) long or <1% (3) body length, 8−13 (7) wide or 6−12% (7) of body width at level of ovo-vitelline duct. Oötype immediately posterior to distal end of cecum and immediately anterior to oviducal seminal receptacle, medial, dorsal to primary vitelline collecting duct (Figs. 7−9). Mehlis' gland 38−63 (8) long, 33−55 (8) wide. Uterus a large duct extending sinuously posteriad from oötype and approximately parallel with body margin medial to male reproductive tract, passing dorsal to oviducal seminal receptacle and vitelline duct anteriorly, ventral to terminal male genitalia, 13−23 (10) in width at level of vitelline loop. Uterine pouch (Byrd, 1939) overlaying uterus and obscuring its path and shape in most specimens, highly convoluted; ascending portion of uterine pouch emanating from middle portion of uterus 163−408 (9) or 4−6% (4) of body length from posterior body end near cirrus sac origin, extending anteriad 118−260 (9) long or 2−6% (4) of body length in sinistral half of body before curving mediad and looping around primary vitelline collecting duct; descending portion of uterine pouch extending posteriad from vitelline duct loop 158−370 (10) or 4−7% (4) of body length in dextral half of body and curving at least 6−9 (9) times before ending blindly 165−283 (10) or 3−6% (4) of body length from posterior body end, including 1−11 (5) eggs 8−15 (5) long (Figs. 8, 9). Common genital pore opening 13−53 (11) or 1% (5) of body length from posterior end. Excretory bladder difficult to discern extent of structure, 5−8 (5) wide near genital pore.

Taxonomic summary

Type host: Siebenrockiella crassicollis (Gray, 1830) (Testudines: Geoemydidae), black marsh turtle; *other host: Cuora amboinensis* (Riche in Daudin, 1801) (Geoemydidae), southeast Asian box turtle.

Site of infection: Lung.

Prevalence and intensity: S. crassicollis – 6/24 (25%), 3.5 (1–7); *C. amboinensis* – 2/25 (8%), 1.5 (1–2).

Type locality: Perak (probably Perak River), Malaysia; *Other localities:* Perlis (probably Perlis River) and Selangor (probably Selangor River Drainage), Malaysia.

Specimens deposited: Holotype USNM XXXXXX; paratype USNM XXXXXXX.

Materials examined: Unicaecum dissimilis – USNM 9228, holotype (mislabeled as cotype), 2 slides comprising 1 sectioned specimen (as 370 sections) *ex. Trachemys scripta troostii* (as *Pseudemys troostii*) from Reelfoot Lake, Tennessee (Byrd, 1939); *Unicaecum ruszkowskii* – AMNH 1272-5 (holotype) and AMNH 1272-1–4 (paratypes), 5 slides comprising 5 whole-mounted specimens *ex. T. scripta* (as *Pseudemys s.*) from Raleigh, North Carolina (Stunkard, 1925); AMNH 1273-1–9 (slides nos. 1, 3, 5–8 are cracked), paratypes (mislabeled as “1929”), 1 whole-mounted specimen plus 8 slides comprising 3 sectioned specimens *ex. T. scripta* (as *Pseudemys s.*) from New Orleans, Louisiana (Stunkard, 1927); USNM 80654, vouchers, 19 slides comprising 20 whole-mounted specimens (small adults) *ex.T. scripta troostii* (as *Pseudemys t.*) from Reelfoot Lake (Byrd, 1939).

Etymology: The new genus and species is named in honor of Barack Obama, 44th president of the United States of America, and 5th cousin, twice removed, of TRP.

Remarks

Baracktrema and *Unicaecum* Stunkard, 1925 are the only accepted TBF genera reportedly having the combination of a single cecum, single testis, oviducal seminal receptacle, and a uterine pouch. In addition, as in *Baracktrema*, we confirmed the presence of a pharynx in the type and voucher specimens of *Unicaecum* spp., and its generic diagnosis should be emended accordingly. As in the new genus, the anterior position of the pharynx makes it easily mistaken for the distal portion of the anterior sucker. *Baracktrema* is most easily differentiated from *Unicaecum* by the combination of having a thread-like body that is approximately 30−50× longer than wide and post-cecal terminal genitalia, i.e., the gonads are anterior to the termination of the cecum, which does not extend to the extreme posterior end of the body. *Unicaecum*, comprising 2 nominal species (*U. ruszkowskii* and *U. dissimilis*), has a body approximately 8−12× longer than wide and terminal genitalia that are anterior to the distal end of the cecum, which reaches to near the posterior body extremity. The new genus further differs from all other accepted TBF genera by having a cecum that is highly convoluted for its entire length, a spindle-shaped ovary between the cirrus sac and testis, a uterine pouch that loops around the primary vitelline collecting duct, a Laurer's canal, and a dorsal common genital pore.

Clearly, *Unicaecum* is morphologically similar and closely related to the new genus; however, several morphological features of the female genitalia of *Unicaecum* remain unresolved. We mention them here in case a future worker encounters specimens of these apparently rare blood flukes. *Unicaecum* is sparsely treated in the taxonomic literature (i.e., phylogenetic analyses [Platt, 1992; Snyder, 2004; Tkach et al., 2009; Orélis-Ribeiro et al., 2014; Pinto et al., 2015], surveys [Hughes et al., 1942; Brooks, 1979; Platt, 1990], historical review [Wall, 1951], compared with *Carettacola bipora* [see Manter and Larson, 1950], and included in keys to genera [Schell, 1985; Platt, 2002]). The remarks below derive from observations of voucher specimens plus all available type specimens as well as all published descriptions. Stunkard's types of *U. ruszkowskii* were originally deposited in the "Laboratory of Parasitology of the University of Paris" and ultimately accessioned at the AMNH (AMNH 1272, 1273). To our knowledge, no other extant type material of this species exists. The holotype of *U. dissimilis* (USNM 9228) was serially sectioned, and no other type or voucher specimen exists, including within Byrd's personal collection deposited by TRP in the USNM (Platt, 1990).

The oviducal seminal receptacle is obvious in the new species and reportedly present in *Unicaecum* spp. Stunkard (1925, 1926, 1927) provided contradictory assessments of the seminal receptacle in *U. ruszkowskii*. He initially (1925, 1926) stated that it was absent, but later he (1927; see figure 3, page 124) illustrated a spheroid chamber comprising that portion of the oviduct between the ovary and Mehlis' gland. He did not report spermatozoa within the spheroid chamber. We assessed figure 3 of Stunkard (1927) by studying his holotype (AMNH 1272−5). The "spheroid chamber" (= oviducal seminal receptacle) is clearly laterally-expanded and proximal to an oötype, which is marked by a well-developed Mehlis' gland and contains an ovum. Apparently, Stunkard (1927) misinterpreted the oviducal seminal receptacle for an oötype. He specifically stated that ova would be required to move toward the ovary (‘upstream’) within the oviduct to reach the spheroid chamber (oviducal seminal receptacle) that he thought was an oötype. Based on vouchers of *U. ruszkowskii* (USNM 80654), Byrd (1939) emended the diagnosis for *Unicaecum* to include seminal receptacle present: “*we have been able to distinguish a definitely dilated area in the oviduct adjacent to the posterior end of the ovary*.” However, he immediately contradicted that diagnosis by reporting that *U. dissimilis* lacked a seminal receptacle. Byrd’s vouchers of *U. ruszkowskii* (AMNH 80654 M1615−25, M1616−2) have an oviduct that is laterally-expanded proximal to the oötype, indicating an oviducal seminal receptacle. We failed to locate the oviducal seminal receptacle in the serial sections comprising Byrd’s holotype of *U. dissimilis*, perhaps because many sections were missing: his specimens reportedly were approximately 3,030 µm long yet only 370 sections were mounted (if each section was 4 µm thick, >800 sections should have been mounted on the slides comprising the holotype).

Stunkard (1925, 1926, 1927) reported that *U. ruszkowskii* lacked a Laurer’s canal. This duct is, however, not easily visualized in blood flukes and can be missed without studying live specimens or exceptionally well-stained whole-mounts. Interestingly, Stunkard’s (1927) figure 3 shows, and his description further details, the confluence of 2 ducts (“*deux vitelloductes et les conduits des glandes de Mehlis*”) about a constricted segment of the oviduct. The only structures familiar to us that could be confluent with the oviduct at this location are the primary vitelline collecting duct, Laurer’s canal, and the minute, wispy ducts that extend from the Mehlis’ gland to the oviduct. We confirmed that the holotype of *U. ruszkowskii* (AMNH 1272−5) has a Laurer's canal extending 195 μm dorsosinistrad from the oviduct. Likewise, for *U. dissimilis*, Byrd (1939) illustrated and described a Laurer's canal that connects to the oviduct in exactly the aforementioned location; however, he reported that the canal was absent in *U. ruszkowskii*. We confirmed that Byrd's vouchers of *U. ruszkowskii* (USNM 80654 M1615−19, M1615−21) also have a Laurer's canal.

Stunkard (1925, 1926, 1927) did not report the presence of a uterine pouch in *U. ruszkowskii*. Byrd's (1939) generic diagnosis included, "*Uterus with spherical dilation or posterior tubular extension*," the latter likely referring to the uterine pouch. He reported that his specimens of *U. ruszkowskii* had a simple uterus that extended directly from the putative oötype to the genital pore. For *U. dissimilis*, he reported, "*Uterus with large median pouch that extends posteriorly to very near end of caecum.*" No mention of this feature is included in his discussion of that species but his illustration (figure 12, page 161) shows a longitudinal, appendix-like out-pocketing of the uterus. This configuration differs markedly from that described herein for *Baracktrema*. The types and vouchers of *U. ruszkowskii* we examined did not exhibit a clear uterine pouch, and the disposition of the holotype of *U. dissimilis* precludes a definitive assessment of that feature (many sections are missing from the series of sections comprising the holotype). However, our study of the holotype did reveal a laterally-expanded duct (probable uterine pouch) that was medial to the cecum, contained ova and vitelline material, and extended from near the genital pore posteriad. These observations make it probable that this feature is indeed a uterine pouch but newly-collected, stained and whole-mounted specimens are required to confirm it.

Regarding the phylogenetic results of the present study (Fig. 10), the data matrix used for Bayesian inference (BI) analyses comprised 982 positions per taxon (359 conserved, 623 variable, and 539 parsimony informative), and the resulting tree was well resolved with 80% (58/73) of nodes having posterior probability values >0.95. With high nodal support and as predicted by morphology, *Baracktrema* and *Unicaecum* share a recent common ancestor and form a clade sister to the freshwater turtle blood flukes of *Spirorchis*, paraphyletic *Spirhapalum*, and *Vasotrema* and that, collectively, these flukes were sister to all other tetrapod blood flukes (*Hapalorhynchus* + *Griphobilharzia* plus the marine turtle blood flukes and schistosomes). The sequences for *Baracktrema* and *Unicaecum* produced relatively long branch lengths (comparable to those of the freshwater TBF *Hapalorhynchus* and of the crocodilian blood fluke *Griphobilharzia*), perhaps reflective of the marked morphological differences between these taxa; further justifying their assignment to distinct genera. The present phylogenetic analysis also reiterated support for monophyly of Schistosomatidae and paraphyly of TBFs (as well as *Spirhapalum*), with the blood flukes of freshwater turtles sister to all other tetrapod blood flukes (TBFs of marine turtles and schistosomes). We noted that some schistosome interrelationships differed from those previously reported (Orélis-Ribeiro et al., 2014), e.g., *Bivitellobilharzia* spp. were sister to the clade comprising all non-*Schistosoma* schistosomatids. Interrelationships of the fish blood flukes (Aporocotylidae Odhner, 1912) echoed that of Orélis-Ribeiro et al. (2014) wherein Aporocotylidae was monophyletic and sister to all tetrapod blood flukes (TBFs + schistosomes), and the euteleostean aporocotylids were monophyletic and sister to the chondrichthyan blood fluke *Chimaerohemecus trondheimensis* van der Land, 1967.

DISCUSSION

The life cycle of *B. obamai* should resemble that of other TBFs and schistosomes, requiring a snail first intermediate host and having a furcocercous cercariae that directly penetrates the vertebrate definitive host. Adults of *B. obamai* infect the lung arterioles, where clusters of tens to hundreds of eggs infected lung alveoli of specimens of both turtle host species. The mechanism involved for the transit of the eggs from the circulatory system to the alveoli could rely on the immune system of the host, as demonstrated for some schistosomes (Damian, 1987; Pearce, 2005). Alternatively, or in addition, eggs of *B. obamai* may be deposited directly into the luminal space of the alveoli by the fluke. How eggs travel from the lung to the external environment is not known to us; however, we speculate that some combination of active expulsion (coughing) and the bronchial escalator are involved.

The life cycles of TBFs and schistosomes differ regarding egg development: schistosome eggs are fully-developed before they are released from the definitive host and hatch shortly after contact with water (Tanabe, 1923; Stunkard and Hinchliffe, 1952; Wu, 1953; Najim, 1956; Sinha and Srivastava, 1960; Greene, 1962; Lee and Wykoff, 1966; Yamaguti, 1975); however, TBF eggs apparently require a period of development in the external environment before hatching (Goodchild and Kirk, 1960; Holliman and Fisher, 1968; Wall, 1941a, 1941b, 1951). Examination of the eggs of *B. obamai* in saline at the time of necropsy by TRP revealed larvated eggs each having a fully-developed, ciliated miracidium occupying 1/3−1/2 of the egg lumen. Miracidia swam actively inside the egg, immediately emerged upon the addition of freshwater and swam in the observation dish. Although the function of the uterine pouch in the new species is unknown, it is possible that the transit (or placement) of the eggs from the circulatory system into the lung occurs too quickly to allow for completion of larval development. Fertilized eggs could be held in the uterine pouch until development is complete such that eggs would be capable of hatching upon contact with environmental water. This scenario is highly speculative, and we did not observe a mass of eggs in the uterine pouch of any live specimen; however, the notion is intuitive based upon available facts regarding the life cycle of this and related species.

The morphological and molecular phylogenetic affinities between *Baracktrema* (1 species infecting Geoemydidae in the Indomalayan region only) and *Unicaecum* (2 species infecting Emydidae in North America only) (see Remarks) are intriguing regarding co-diversification of blood flukes and their turtle hosts. *Baracktrema obamai* infects *C. amboinensis* and *S. crassicollis*; *U. ruszkowskii* infects *G. pseudogeographica* and *T. scripta*; and *U. dissimilis* infects *Graptemys geographica* (Le Sueur, 1817) and *T. scripta*. These turtle families as well as Testudinidae plus monotypic *Platysternon* (Gray, 1831) comprise superfamily Testudinoidea, which has an extensive fossil record in Asia; where most extant species range (Crawford et al., 2015). Crawford et al. (2015) hypothesized that the Emysternia (Emydidae + *Platysternon*) entered the nearctic via high-latitude dispersal routes during the Paleogene (65−1.5 mya; a warming period) (see also Estes and Hutchison, 1980; Holroyd et al., 2001; Zachos et al., 2001; Eberle et al., 2010; Hutchison, 2013). In light of hypothesized Paleogene dispersal, the close morphological and molecular similarities among *Baracktrema* and *Unicaecum* are noteworthy. This also potentially applies to other TBF genera of the Indo-Malayan, Palearctic, and Nearctic regions (Orélis-Ribeiro et al., 2014; Crawford et al., 2015). *Platysternon megacephalum* (Gray, 1831), the monotypic extant member of Emysternia (Crawford et al., 2015), and other unsampled geoemydids are critical hosts to examine for testing such biogeographic hypotheses.

Molecular phylogenetics of turtle and fish blood flukes remains, unsurprisingly, in a state of flux. Tree topologies differ, sometimes markedly, among the various laboratories studying them; perhaps related to outgroup choice, taxon sampling, fragment length, analysis method, alignment parameters, treatment of gaps, and model of evolution used. Pending a revised morphological diagnosis, we considered only existing 28S sequence data (see Orélis-Ribeiro et al. [2014] for a complete list) from adult specimens with confirmed identities (i.e., excluding sequences derived from cercariae, which we think are not defensibly morphologically identifiable, as of yet, to the level of species, genus, or blood fluke family). As a result, we view the clade including *Spirorchis* spp., paraphyletic *Spirhapalum*, *Vasotrema*, *Baracktrema*, and *Unicaecum* as a likely placeholder for “Spirorchiidae Stunkard, 1921” (type genus *Spirorchis* MacCallum, 1918; type species *Spirorchis innominatus* Ward, 1921; see MacCallum, 1918; Stunkard, 1921; Ward, 1921; Platt, 2002) (Fig. 10). Accepting the current definition of monophyletic Schistosomatidae (crown group within Schistosomatoidea [see Orélis-Ribeiro et al., 2014]), this system would leave 2 TBF clades (i.e., the *Hapalorhynchus* and *Griphobilharzia* clade plus the marine TBF clade comprising *Carettacola*, *Learedius*, and *Hapalotrema*) in ‘systematic limbo,’ without a family assignment. Along with generating additional sequence data from other TBF species and genera, morphological diagnoses of these lineages/clades are in progress, which will help alleviate the nomenclatural ambiguity resulting from Snyder's (2004) indication of TBF paraphyly. Alternatively, perhaps additional TBF sequence data from 28S and other loci plus discovery of TBF species representing novel genera may demonstrate TBF monophyly.

The "*Spirorchis* clade" (Fig. 10), excluding *H. gracilis*, comprises all TBFs that infect freshwater cryptodirans (sensu Bickham and Carr [1983]; Guillon et al. [2012]; for sub-order Cryptodira, i.e., the "hidden neck turtles," freshwater turtles that retract their neck vertically [Bonin et al., 2006]) and for which 28S sequences are publically available. Although many more TBFs (50 species) infect cryptodirans, molecular data for those species currently is lacking. All extant marine turtles are assigned to Cryptodira but their blood flukes comprise the sister lineage to schistosomatids not those of freshwater cryptodirans (Fig. 10). Further in regards to the lack of basic knowledge of TBF biodiversity and biogeography, the present phylogenetic analysis further highlights the need for increased molecular taxonomic coverage among freshwater TBFs in general: only 9 of 55 (16%) freshwater TBF species and 6 of 10 (60%) freshwater TBF genera are associated with published 28S sequence data (Fig. 10). This predicament is even worse among the fish blood flukes, which are almost exclusively represented by marine taxa (Orélis-Ribeiro et al., 2014). The present phylogenetic analysis clustered as sister taxa 2 genera that occur on opposite sides of the planet: *Unicaecum* spp. reportedly range in North America only (Stunkard, 1925, 1927; Byrd, 1939; Brooks, 1979) and *B. obamai* reportedly ranges in the Indomalayan region only (present study); further suggestive of the fact that unnamed TBF lineages remain to be studied, especially among freshwater turtles outside of North America.

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LITERATURE CITED

Ashley, L. M. 1974. Laboratory anatomy of the turtle, 1st edition. W. M. C. Brown Company Publishers, Dubuque, Iowa, 48 p.

Benz, G. W., and D. E. Collins (eds.). 1997. Aquatic fauna in peril: The southeastern perspective. Special Publication 1, Southeast Aquatic Research Institute, Lenz Design and Communications, Decatur, Georgia, 554 p.

Bickham, J. W., and J. L. Carr. 1983. Taxonomy and phylogeny of the higher categories of cryptodiran turtles based on a cladistic analysis of chromosomal data. Copeia **4:** 918−932.

Bonin, F., B. Devaux, and A. Dupré. 2006. Turtles of the world. The Johns Hopkins University Press, Baltimore, Maryland, 416 p.

Brooks, D. R. 1979. New records for amphibians and reptile trematodes. Proceedings of the Helminthological Society of Washington **46:** 286–289.

Brooks, D. R., and J. R. Palmieri. 1979. *Neopronocephalus orientalis* sp. n. (Digenea: Pronocephalidae) and *Spirhapalum elongatum* Rohde, Lee, and Lim, 1968 (Digenea: Spirorchiidae) from *Cuora amboinensis* (Daudin) in Malaysia. Proceedings of the Helminthological Society of Washington **46:** 55−57.

Bullard, S. A., and R. M. Overstreet. 2002. Potential pathological effects of blood flukes (Digenea: Sanguinicolidae) on pen-reared marine fishes. Proceedings of the 53rd Gulf and Caribbean Fisheries Institute **53:** 10−25.

Bullard, S. A., and R. M. Overstreet. 2008. Digeneans as enemies of fishes. *In* Fish Diseases. J. Eiras, H. Segner, T. Wahil, B. G. Kapoor (eds.). Science Publishers, Enfield, New Hampshire, p. 817−976.

Byrd, E. E. 1939. Studies on the blood flukes of the family Spirorchidae. Part II. Revision of the family and description of new species. Journal of the Tennessee Academy of Science **14:** 116−161.

Castresana, J. 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Molecular Biology and Evolution **17:** 540–552.

Crawford, N. G., J. F. Parham, A. B. Sellas, B. C. Faircloth, T. C. Glenn, T. J. Papenfuss, J. B. Henderson, M. D. Hansen, and W. B. Simison. 2015. A phylogenomic analysis of turtles. Molecular Phylogenetics and Evolution **83:** 250−257.

Damian, R. T. 1987. The exploitation of host immune responses by parasites. Journal of Parasitology **73:** 3-13.

Darriba, D., G. L. Taboada, R. Doallo, and D. Posada. 2012. jModelTest 2: more models, new heuristics and parallel computing. Nature Methods **9:** 772.

Dwivedi, M. P. 1967. Contribution to the family Spirorchiidae Stunkard, 1921 (Digenea: Trematoda). Indian Journal of Helminthology **19:** 1−14.

Eberle, J. J., H. C. Fricke, J. D. Humphrey, L. Hackett, M. G. Newbrey, and J. H. Hutchison. 2010. Seasonal variability in Arctic temperatures during early Eocene time. Earth and Planetary Science Letters **296:** 481−486.

Estes, R., and J. H. Hutchison. 1980. Eocene lower vertebrates from Ellesmere Island, Canadian Arctic Archipelago. Palaeogeography, Palaeoclimatology, Palaeoecology **30:** 325−347.

Glazebrook, J. S., and R. S. F. Campbell. 1990a. A survey of the diseases of marine turtles in northern Australia. I. Farmed turtles. Diseases of Aquatic Organisms **9:** 83−95.

Glazebrook, J. S., and R. S. F. Campbell. 1990b. A survey of the diseases of marine turtles in northern Australia. II. Oceanarium-reared and wild turtles. Diseases of Aquatic Organisms **9:** 97−104.

Goodchild, C. G., and E. S. Dennis. 1967. Comparative egg counts and histopathology in turtles infected with *Spirorchis* (Trematoda: Spirorchiidae). Journal of Parasitology **53:** 38−45.

Goodchild, C. G., and D. E. Kirk. 1960. The life history of *Spirorchis elegans* Stunkard, 1923 (Trematoda: Spirorchiidae) from the painted turtle. Journal of Parasitology **46:** 219−229.

Greene, N. D. 1962. The life history of *Heterobilharzia americana* Price, 1929 (Trematoda: Schistosomatidae). Journal of Parasitology **48:** 48−49.

Guillon, J. M., L. Guéry, V. Hulin, and M. Girondot. 2012. A large phylogeny of turtles. Contributions to Zoology **81:** 147−158.

Guindon, S., and O. Gascuel. 2003. A simple, fast and accurate method to estimate large phylogenies by maximum-likelihood. Systematic Biology **52:** 696–704.

Gupta, N. K., and V. Mehrotra. 1975. On three blood flukes (Spirorchiidae: Spirorchiinae) from freshwater chelonians and discussion on the synonymy of *Plasmiorchis pellucidus* Mehra, 1934. Rivista di Parassitologia **36:** 165−170.

Haitao, S., J. F. Parham, F. Zhiyong, H. Meiling, and Y. Feng. 2008. Evidence for the massive scale of turtle farming in China. Oryx **42:** 147−150.

Holliman, R. B., and J. E. Fisher. 1968. Life cycle and pathology of *Spirorchis scripa* Stunkard, 1923 (Digenea: Spirochiidae) in *Chrysemys picta picta*. Journal of Parasitology **54:** 310−318.

Holliman, R. B., J. E. Fisher, and J. C. Parker. 1971. Studies on *Spirorchis parvus* (Stunkard, 1923) and its pathological effects on *Chrysemys picta picta*. Journal of Parasitology **57:** 71−77.

Holroyd, P. A., J. H. Hutchison, and S. G. Strait. 2001. Changes in turtle diversity and abundance through the earliest Eocene Willwood Formation: preliminary results. University of Michigan Papers in Paleontology **33:** 97−107.

Huelsenbeck, J. P., and F. Ronquist. 2005. Bayesian analysis of molecular evolution using MrBayes. *In* Statistical methods in molecular evolution. R. Nielsen (ed.). Springer Verlag, New York, New York, p. 183–232.

Huelsenbeck, J. P., F. Ronquist, R. Nielsen, and J. P. Bollback. 2001. Bayesian inference of phylogeny and its impact on evolutionary biology. Science **294:** 2310–2314.

Hughes, R. C., J. W. Higginbotham, and J. W. Clary. 1942. The trematodes of reptiles, part I, systematic section. The American Midland Naturalist **27:** 109−134.

Hutchison, J. H. 2013. New turtles from the Paleogene of North America. *In* Morphology and evolution of turtles. D. B. Brinkman, P. A. Holroyd, and J. D. Gardner (eds.), p. 477−497.

IUCN (International Union for Conservation of Nature). 2015. Available at http://www.iucnredlist.org. Accessed 20 August 2015.

Katoh, K., and H. Toh. 2010. Parallelization of the MAFFT multiple sequence alignment pro- gram, Bioinformatics **26:** 1899–1900.

Lee, H., and D. E. Wykoff. 1966. Schistosomes from wild rats in Thailand. Journal of Parasitology **52:** 323−326.

Lockyer, A. E., P. D. Olson, P. Ostergaard, D. Rollinson, D. A. Johnston, S. W. Attwood, V. R. Southgate, P. Horak, S. D. Snyder, T. H. Le, et al. 2003. The phylogeny of the Schistosomatidae based on three genes with emphasis on the interrelationships of *Schistosoma* (Weinland, 1858). Parasitology **126:** 203–224.

Luhman, M. 1935. Two new trematodes from the loggerhead turtle (*Caretta caretta*). Journal of Parasitology **21:** 274−276.

MacCallum, G. A. 1918. Notes on the genus *Telorchis* and other trematodes. Zoopathologica **1:** 77−98.

Manter, H. W., and M. I. Larson. 1950. Two new blood flukes from a marine turtle, *Caretta caretta*. Journal of Parasitology **36:** 595−599.

Mehra, H. R. 1934. New blood flukes of the family Spirorchidae Stunkard from Indian fresh-water tortoises with discussion on the synonymy of certain genera and the relationships of the families of blood flukes. Part II. Bulletin of the Academy of Sciences of the United Provinces of Agra and Oudh **3:** 169−196.

Mehra, H. R. 1940. A new distome *Enterohaematotrema* n. g. and a new blood fluke *Hemiorchis bengalensis* n. sp. belonging to the family Spirorchidae Stunkard, and a new species of the genus *Dendritobilharzia* Skrjabin and Zakharow belonging to the family Schistosomatidae Poche, with remarks on the evolution of the blood flukes. Proceedings of the National Academy of Sciences of India, Section B, Biological Sciences **10:** 100−118.

Mehrotra, V. 1973a. Digenea from some reptile hosts in India. Part I. Proceedings of the Sixtieth Indian Science Congress. Part IV, 46 p.

Mehrotra, V. 1973b. Digenea from some reptile hosts in India. Part II. Proceedings of the Sixtieth Indian Science Congress. Part IV, p. 46−47.

Miller, M. A., W. Pfeiffer, and T. Schwartz. 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. Proceedings of the Gateway Computing Environments Workshop (GCE), New Orleans, Louisiana, p. 1–8.

Najim, A. T. 1956. Life history of *Gigantobilharzia huronensis* Najim, 1950. A dermatitis-producing bird blood-fluke (Trematoda-Schistosomatidae). Parasitology **46**: 443−469.

Olson, P. D., T. H. Cribb, V. V. Tkach, R. A. Bray, and D. T. J. Littlewood. 2003. Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda). International Journal for Parasitology **33:** 733–755.

Orélis-Ribeiro, R., C. R. Arias, K. M. Halanych, T. H. Cribb, and S. A. Bullard. 2014. Diversity and ancestry of flatworms infecting blood of nontetrapod craniates "fishes." Advances in Parasitology **85:** 1−64.

Ozaki, Y. 1932. A new trematode , *Leurosoma orientale*, gen. et sp. n., from the turtle, *Ocadia sinensis* Gray. Annals and Magazine of Natural History **10:** 42−45

Ozaki, Y. 1939. [A new blood-fluke, *Hapalorhynchus yoshidai*.] Volumen Jubilare pro Professore Sadao Yoshida **1:** 29−37. (In Japanese.)

Pearce, E. J. 2005. Priming of the immune response by schistosome eggs. Parasite Immunology **27:** 265−270.

Pinto, H. A., A. L. de Melo, and S. V. Brant. 2015. Where are the South American turtle blood flukes (Trematoda: Spirorchiidae)? The first morphological and molecular analysis of spirorchiid cercariae from freshwater snails in Brazil. Parasitology International **64:** 553−558.

Platt, T. R. 1988. *Hapalorhychus brooksi* sp. n. (Trematoda: Spirorchiidae) from the snapping turtle (*Chelydra serpentina*), with notes on *H. gracilis* and *H. stunkardi*. Proceedings of the Helminthological Society of Washington **55:** 317−323.

Platt, T. R. 1990. Deposition of type and voucher material from the helminthological collection of Elon E. Byrd. Systematic Parasitology **16:** 27−34.

Platt, T. R. 1992. A phylogenetic and biogeographic analysis of the genera of Spirorchinae (Digenea: Spirorchidae) parasitic in freshwater turtles. Journal of Parasitology **78:** 616−629.

Platt, T. R. 1993. Taxonomic revision of *Spirorchis* MacCallum, 1919 (Digenea: Spirorchidae). Journal of Parasitology **79:** 337−346.

Platt, T. R. 1998. Redescription of *Hapalotrema mistroides* (Monticelli, 1896) and *Hapalotrema synorchis* Luhman, 1935 (Digenea: Spirorchidae), with comments on other species in the genus. Journal of Parasitology **84:** 594−600.

Platt, T. R. 2002. Family Spirorchiidae Stunkard, 1921. Pages 453-468 *in* D. I. Gibson, A. J. Jones, and R. A. Bray, eds. Keys to the Trematoda, Vol. 1. CABI Publishing, Wallingford, Oxford, U.K. 521 p.

Platt, T. R. and S. Pichelin. 1994. *Uterotrema australispinosa* n. gen., n. sp. (Digenea: Spirorchidae), a parasite of a freshwater turtle *Emydura macquarii* from southern Queensland, Australia. Journal of Parasitology **80:** 1008−1011.

Platt, T. R. and R. S. K. Sharma. 2012. Two new species of *Hapalorhynchus* (Digenea: Spirorchiidae) from freshwater turtles (Testudines: Geoemydidae) in Malaysia. Comparative Parasitology **79:** 202−207.

Posada, D., and T. R. Buckley. 2004. Model selection and model averaging in phylogenetics: advantages of Akaike Information Criterion and Bayesian approaches over likelihood ratio tests. Systematic Biology **53:** 793–808.

Rambaut, A. 2009. FigTree v1.2.3, Institute of Evolutionary Biology, Univ. of Edinburgh. Available at: http://tree.bio.ed.ac.uk/software/figtree.

Rambaut, A., and A. J. Drummond. 2009. Tracer Version 1.5. Program. Available at:

http://beast.bio.ed.ac.uk/Tracer/.

Rohde, K., S. K. Lee, and H. W. Lim. 1968. Ueber drei malayische Trematoden. Annales de Parasitologie Humaine et Comparée **43:** 33−43.

Ronquist, F., and J. P. Huelsenbeck. 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics **19:** 1572–1574.

Schell, S. C. 1985. Trematodes of North America north of Mexico. University Press of Idaho, University Station, Moscow, Idaho, 263 p.

Simha, S. S. 1958. Studies on the trematode parasites of reptiles found in Hyderabad State. Zeitschrift für Parasitenkunde **18:** 161−218.

Simha, S. S. and D. R. Chattopadhyaya. 1980. Studies on the trematode parasite of reptiles found in India. Contribution to the knowledge of blood flukes from marine turtles, from the Gulf of Manar, South India. Journal of the Zoological Society of India **30:** 69−78.

Singh, R. N. 1952. The cercaria of *Plasmiorchis orientalis* Mehra (1934). Proceedings of the National Academy of Sciences, India, Section B **22:** 61−71.

Sinha, B. B. 1934. A new genus of blood flukes of the family Spirorchidae, from the tortoise, *Hardella thurgi* (Gray). Records of the Indian Museum **36:** 147−151.

Sinha, P. K., and H. D. Srivastava. 1960. Studies on *Schistosoma incognitum* Chandler, 1926 II. On the life history of the blood fluke. Journal of Parasitology **46:** 629−641.

Smith, J. W. 1997a. The blood flukes (Digenea: Sanguinicolidae and Spirorchidae) of cold-blooded vertebrates: Part I. A Review of the literature published since 1971, and bibliography. Helminthological Abstracts **66:** 255−294.

Smith, J. W. 1997b. The blood flukes (Digenea: Sanguinicolidae and Spirorchidae) of cold-blooded vertebrates: Part II. Appendix I: Comprehensive parasite-host list; Appendix II: Comprehensive host-parasite list. Helminthological Abstracts **66:** 329−344.

Snyder, S. D. 2004. Phylogeny and paraphyly among tetrapod blood flukes (Digenea: Schistosomatidae and Spirorchiidae). International Journal for Parasitology **34:** 1385−1392.

Stunkard, H. W. 1921. Notes on North American blood flukes. American Museum Novitates 12: 1−5.

Stunkard, H. W. 1925. A new blood fluke, *Unicaecum ruszkowskii* n.g., n.sp.; a contribution to the relationship of the blood-infesting trematodes (published abstract). Anatomical Record **31:** 317.

Stunkard, H. W. 1926. A new blood fluke, *Unicaecum ruszkowskii* n. g. n. sp.; a contribution to the relationship of the blood infesting trematodes (published abstract). Journal of Parasitology **12:** 158−176.

Stunkard, H. W. 1927. Sur l'*Unicaecum ruszkowskii*, Trématode sanguicole des tortues d'eau douce de l'Amérique du Nord. Annales de Parasitologie Humaine et Comparée **5:** 117−126.

Stunkard, H. W., and M. C. Hinchliffe. 1952. The morphology and life-history of *Microbilharzia variglandis* (Miller and Northrup, 1926) Stunkard and Hinchliffe, 1951, avian blood-flukes whose larvae cause "swimmer's itch" of ocean beaches. Journal of Parasitology **38:** 248−270.

Takeuti, E. 1942. New blood flukes of the family Spirorchidae from Japanese fresh-water tortoise and marine turtles. Japanese Journal of Medical Sciences, VI, Bacteriology and Parasitology **2:**161−174.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution **28:** 2731–2739.

Tanabe, B. 1923. The life history of a new schistosome, *Schistosomatium pathlocopticum* Tanabe, found in experimentally infected mice. Journal of Parasitology **9:** 183−198.

Tandon, V., and N. K. Gupta. 1982. On some blood flukes (Spirorchiidae: Coeuritrematinae) from freshwater chelonians in India. Proceedings of the Indian Academy of Sciences, Animal Science **91:** 275−282.

Tandon, V., and N. K. Gupta. 1985. On the blood fluke, *Cardiotrema roparensis* Mehrotra, 1973 and validity of *C. longivesticulata* Dwivedi, 1967 (Spirorchiidae: Coeuritrematinae). Research Bulletin (Science) of the Panjab University **36:** 331−334.

Tkach, V. V., B. Grabda-Kazubska, J. Pawlowski, and Z. Swiderski. 1999. Molecular and morphological evidences for close phylogenetic affinities of the genera *Macrodera*, *Leptophallus*, *Metaleptophallus* and *Paralepoderma* (Digenea, Plagiorchioidea). Acta Parasitologica **44:** 170–179.

Tkach, V., J. Pawlowski, and J. Mariaux. 2000. Phylogenetic analysis of the suborder Plagiorchiata (Platyhelminthes, Digenea) based on partial lsrDNA sequences. International Journal for Parasitology **30:** 83–93.

Tkach, V. V., S. D. Snyder, and J. A. Vaughan. 2009. A new species of blood fluke (Digenea: Spirorchiidae) from the Malayan box turtle, *Cuora amboinensis* (Cryptodira: Geoemydidae) in Thailand. Journal of Parasitology **95:** 743−746.

van Dijk, P. P., J. B. Iverson, A. G. J. Rhodin, H. B. Shaffer, and R. Bour. 2014. Turtles of the world, 7th Edition: Annotated checklist of taxonomy, synonymy, distribution with maps, and conservation status. Chelonian Research Monographs **5:** 329−479.

Wall, L. D. 1941a. Life history of *Spirorchis elephantis* (Cort, 1917), a new blood fluke from *Chrysemys picta*. American Midland Naturalist **25:** 402−412.

Wall, L. D. 1941b. *Spirorchis parvus* (Stunkard), its life history and development of its excretory system (Trematoda: Spirorchiidae). Transactions of the American Microscopical Society **60:** 221−260.

Wall, L. D. 1951. The life history of *Vasotrema robustum* (Stunkard, 1928), Trematoda: Spirorchiidae. Transactions of the American Microscopical Society **70:** 173−184.

Ward, H. B. 1921. A new blood fluke from turtles. Journal of Parasitology **7:** 114−128.

Witham, R. 1973. A bacterial disease of hatchling loggerhead sea turtles. Quarterly Journal of the Florida Academy of Science **36:** 226−228.

Wu, L. 1953. A study of the life history of *Trichobilharzia cameroni* sp. nov. (Family Schistosomatidae). Canadian Journal of Zoology **31:** 351−373.

Yamaguti, S. 1971. Synopsis of digenetic trematodes of vertebrates, Vol. I. Keigaku Publishing Company, Tokyo, 1074 p.

Yamaguti, S. 1975. A synoptical review of life histories of digenetic trematodes of vertebrates. Keigaku Publishing Company, Tokyo, Japan, 590 p.

Zachos, J., M. Pagani, L. Sloan, E. Thomas, and K. Billups. 2001. Trends, rhythms, and aberrations in global climate 65 Ma to present. Science **292:** 686–693.

FIGURE LEGENDS

Figure 1. Body of holotype (**USNM Coll. No. XXXXXXX**) of *Baracktrema obamai* n. gen., n. sp. from black marsh turtle, *Siebenrockiella crassicollis* (Gray, 1831), (Testudines: Geoemydidae) from Perak, Perlis, and Selangor states of Malaysia. Scale value aside bar. Anterior sucker (as), esophagus (es), esophageal-cecal junction (ecj), anterior extent of testis (at), posterior extent of testis (pt), ovary (ov), cecal termination (ct), and common genital pore (cgp). Roman numerals and dashed lines indicate body segments that are illustrated at higher magnification (see Figs. 2−7). Principally ventral view; anterior portion is ventrolateral view.

Figures 2−7. Holotype (**USNM Coll. No. XXXXXXX**) of *Baracktrema obamai* n. gen., n. sp. from black marsh turtle, *Siebenrockiella crassicollis* (Gray, 1831), (Testudines: Geoemydidae) from Perak, Perlis, and Selangor states of Malaysia. Scale value aside bar, all figures are the same scale. (**2**) Anterior body segment (II) showing anterior sucker (as), pharynx (ph), nerve commissure (nc), esophagus (es), esophageal gland (eg), esophageal swelling (sw), cecum (ce), and vitellarium (v). Ventrolateral view. (**3**) Body segment (III). Ventral view. (**4**) Body segment (IV). Ventral view. (**5**) Body segment (V). Ventral view. (**6**) Body segment (VI). Ventral view. (**7**) Body segment (VII) showing ovary (ov), junction of vitellarium and primary vitelline collecting duct (jvc), vas deferens (vd), junction of ovary and oviduct (joo), vitelline duct (vt), cecal termination (ct), oötype (oo), oviducal seminal receptacle (osr), ascending portion of uterine pouch (aup), junction of uterine pouch with uterus (jup), descending portion of uterine pouch (dup), seminal vesicle (sv), cirrus (ci), distal portion of uterus (diu), common genital pore (cgp), and excretory pore (ep). Ventral view.

Figures 8−9. *Baracktrema obamai* n. gen., n. sp. (**8**) Genitalia of holotype (**USNM Coll. No. XXXXXXX**) from lung of black marsh turtle, *Siebenrockiella crassicollis* (Gray, 1831), (Testudines: Geoemydidae) from Perak, Perlis, and Selangor states of Malaysia. Junction of vitelline duct with oviduct (jvo), oviduct (od), Laurer's canal (Lc), Laurer's canal pore (Lcp), vitelline duct (vt), junction of uterine pouch with uterus (jup), descending portion of uterine pouch (dup), distal portion of uterus (diu), vas deferens (vd), oötype (oo), uterus (u), oviducal seminal receptacle (osr), ascending portion of uterine pouch (aup), seminal vesicle (sv), cirrus (ci), common genital pore (cgp), and excretory pore (ep). Ventral view. (**9**) Genitalia of paratype (**USNM Coll. No. XXXXXXX**) from lung of Southeast Asian box turtle, *Cuora amboinensis* (Daudin, 1801), Geoemydidae, from the Selangor state of Malaysia, showing comparable features as in Figure 8. Dorsal view.

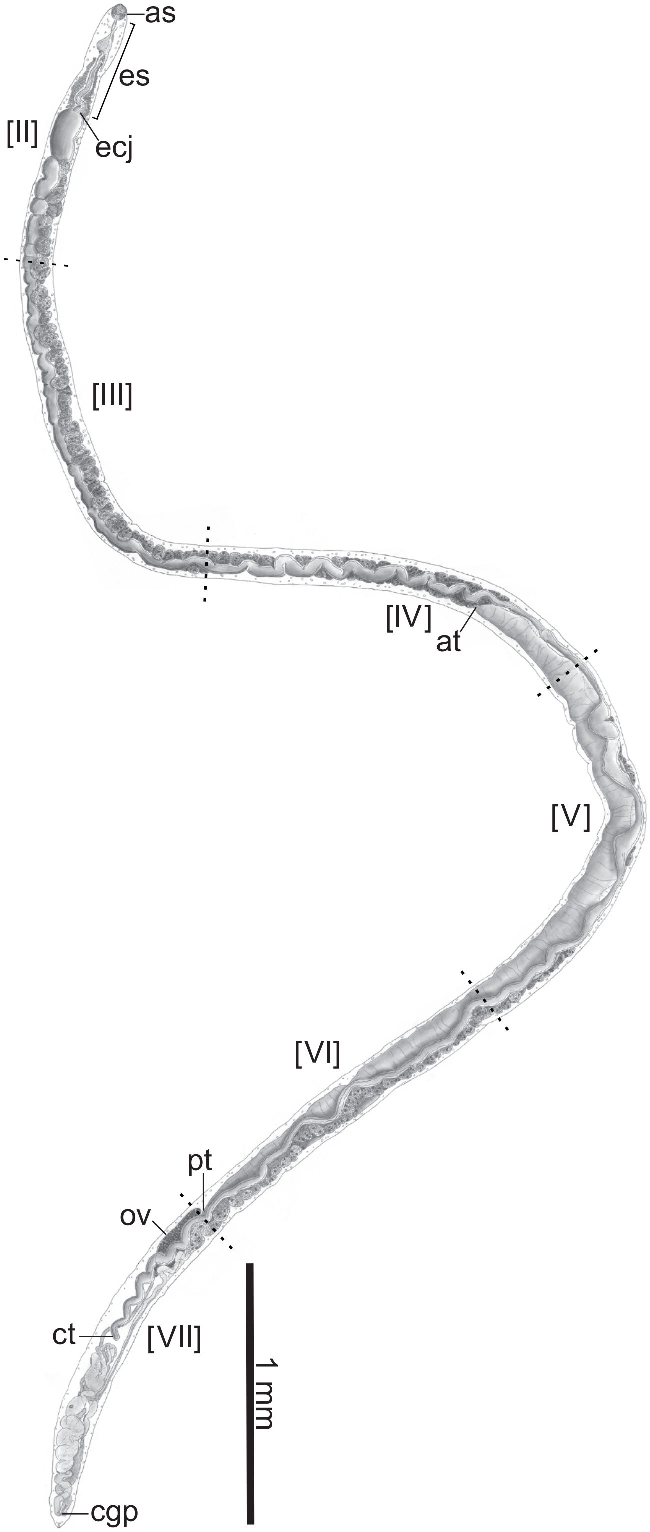
Figure 10. Phylogenetic relationships of blood flukes reconstructed by Bayesian inference and based on partial D1–D3 domains of 28S from 79 taxa (majority rules consensus tree). Numbers aside tree nodes indicate posterior probability. Definitive hosts are indicated by icons aside tree nodes.

Table 1. Blood flukes infecting turtles of Geoemydidae

Turtle host	Blood fluke	Site in host	Locality	Museum Nos.	Reference
Batagur dhongoka	*Cardiotrema vaidya*	ventricle of heart (adult)	Jabalpur, Hiren River, India	Not reported	Dwivedi (1967)
	Plasmiorchis hardelli	ventricle of heart (adult)	Allahabad, Ganga River, India	Not reported	Mehra (1934)
	Plasmiorchis orientalis	ventricle of heart (adult)	Allahabad, Ganga River, India	Not reported	Mehra (1934)
Batagur kachuga	*Cardiotrema vaidya*	ventricle of heart (adult), intestinal and body cavity wash (adult), liver (adult)	Jabalpur, Gokalpur Tank, India	Not reported	Dwivedi (1967)
	Enterohaematotrema hepaticum	liver (adult)	Hyderabad, Godavari River, India	Osmania University Zoology Museum: Accession number not reported	Simha (1958)
		liver (adult), kidney (adult)	Hyderabad, Godavari River, India	Not reported	Simha and Chattopadhyaya (1980)
Cuora amboinensis	*Spirhapalum elongatum*	mesenteric arteries (adult)	Selangor, Selangor River, Malaysia	University of Malaysia, Kuala Lumpur Zoology Department; Coll. Nos. 779−785	Rohde et al. (1968)
		Not specified	Selangor, Selangor River, Malaysia	USNM Coll. Nos. 73055	Brooks and Palmieri (1979)
	Spirhapalum siamensis	heart between atria and ventricle (adult)	Mae Sot, Moei River, Thailand	Harold W. Manter Laboratory of	Tkach et al. (2009)

	***Baracktrema obamai* n. gen., n. sp.**	**lung (egg, adult)**	**Selangor, Selangor River, Malaysia**	Parasitology Coll. No. 49027 **USNM Coll. No. XXXXXXX**	**Present study**
Geoclemys hamiltonii	*Cardiotrema roparensis*	body wash (adult), liver (adult)	Karnal, Gangoh River, India	Not reported	Tandon and Gupta (1985)
Hardella thurjii	*Plasmiorchis bengalensis*	ventricle of heart (adult)	Ranaghat, Hooghly River, India	Not reported	Mehra (1940)
	Plasmiorchis hardelli	ventricle of heart (adult), aortic arch (adult)	Allahabad, Ganga River, India	Not reported	Mehra (1934)
		ventricle of heart (adult), aortic arch (adult)	Allahabad, Ganga River, India	Not reported	Mehra (1940)
		ventricle of heart (adult)	Allahabad, U.P. Government Fish Nursery, India	Not reported	Singh (1952)
	Plasmiorchis sanguina	large blood vessels (adult)	Lucknow, Gomti River, India	Not reported	Sinha (1934)
Malayemys subtrijuga	*Hapalorhynchus snyderi*	body wash (adult)	Perlis, Perlis River, Malaysia	USNM 105194	Platt & Sharma (2012)
Mauremys sinensis	*Hapalorhynchus ocadiae*	heart (adult)	Zhuoshui River, Taiwan	Not reported	Takeuti (1942)
	Hapalorhynchus sp.	lung (egg, adult)	Shanghai, Yangtse River, China	Not reported	Ozaki (1932)
	Hapalorhynchus yoshidai	Not specified	Shanghai, Yangtse River, China	Not reported	Ozaki (1939)
Pangshura sylhetensis	*Cardiotrema roparensis*	Not specified	Rupnagar, Sutlej River, India	Not reported	Mehrotra (1973)b
		body wash (adult), liver (adult)	Rupnagar, Sutlej River, India	Not reported	Tandon and Gupta (1985)
	Hapalorhynchus sutlejensis	Not specified	Rupnagar, Sutlej River, India	Not reported	Mehrotra (1973)b

		heart (adult)	Rupnagar, Sutlej River, India	Not reported	Tandon and Gupta (1982)
	Plasmiorchis orientalis	heart (adult)	Rupnagar, Sutlej River, India	Not reported	Gupta and Mehrotra (1975)
	Plasmiorchis stunkardi	Not specified	Rupnagar, Sutlej River, India	Not reported	Mehrotra (1973)a
		heart (adult)	Rupnagar, Sutlej River, India	Personal collection of V. Mehrotra, Punjab University, India	Gupta and Mehrotra (1975)
Pangshura tecta	*Hapalorhynchus sheilae*	Not specified	Rupnagar, Sutlej River, India	Not reported	Mehrotra (1973)b
		heart (adult), liver (adult)	Rupnagar, Sutlej River, India	Not reported	Tandon and Gupta (1982)
Siebenrockiella crassicollis	*Hapalorhynchus tkachi*	body wash (adult)	Perak, Perak River, Malaysia	USNM 105196	Platt and Sharma (2012)
	***Baracktrema obamai* n. gen., n. sp.**	**lung (egg, adult)**	**Perak, Perak River, Malaysia**	**USNM XXXXX**	**Present study**
		lung (egg, adult)	**Perlis, Perlis River, Malaysia**	**USNM XXXXX**	**present study**
		lung (egg, adult)	**Selangor, Selangor River, Malaysia**	**USNM XXXXX**	**present study**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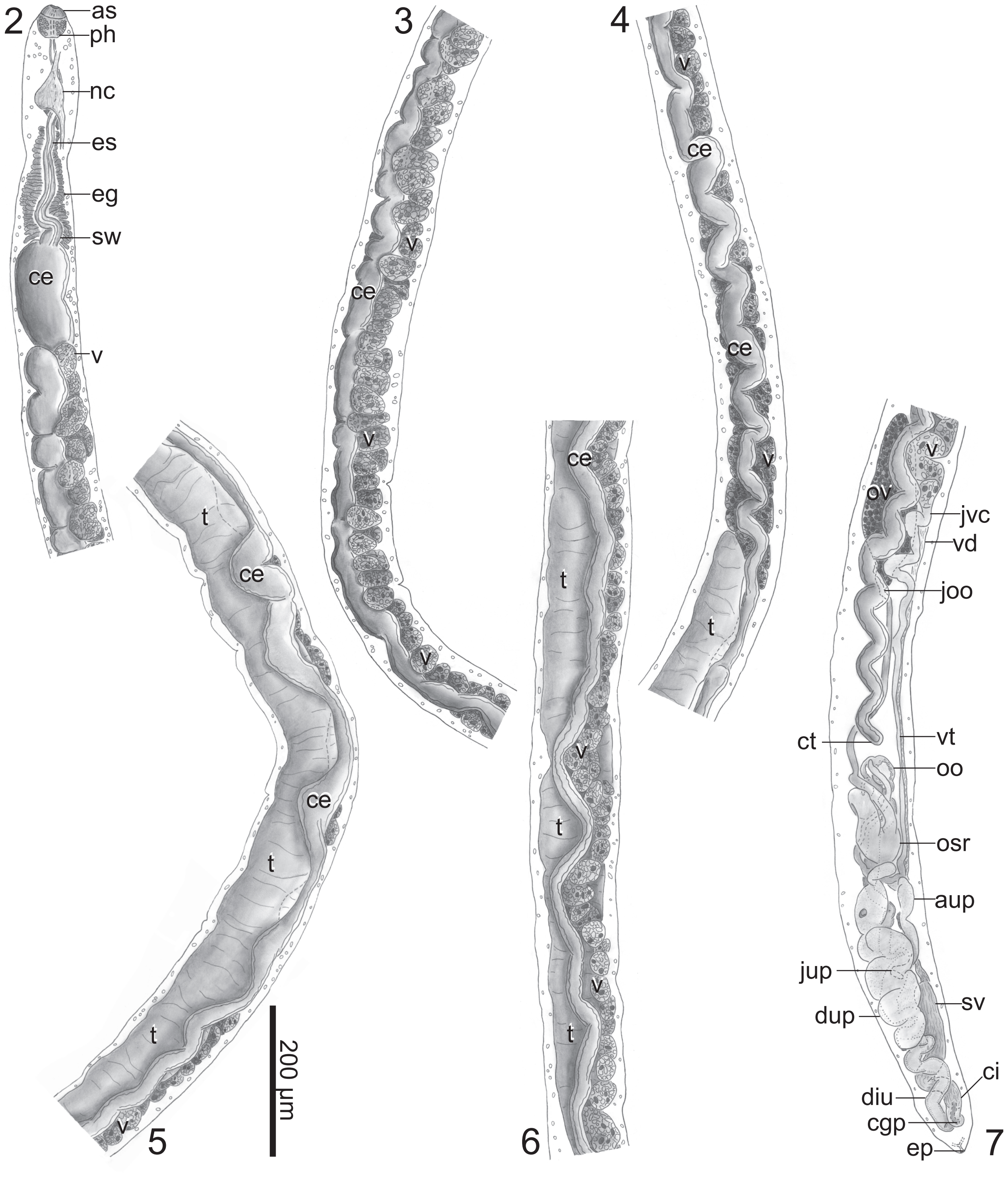
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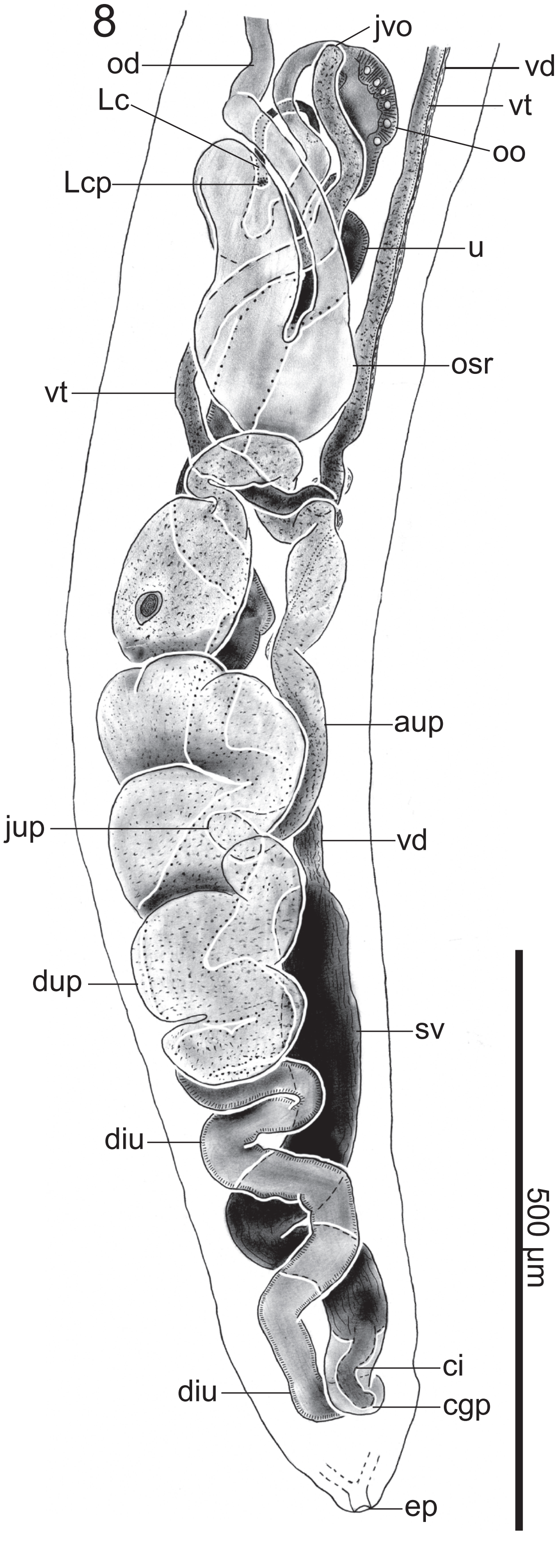
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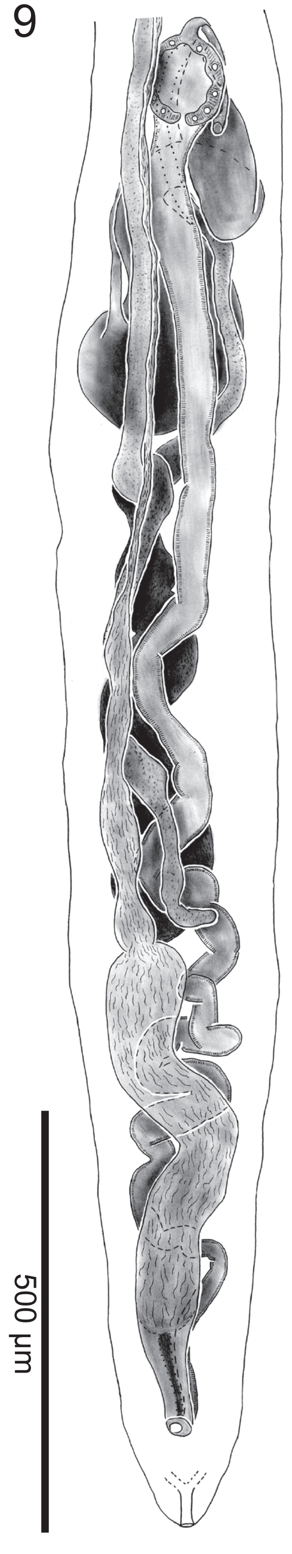
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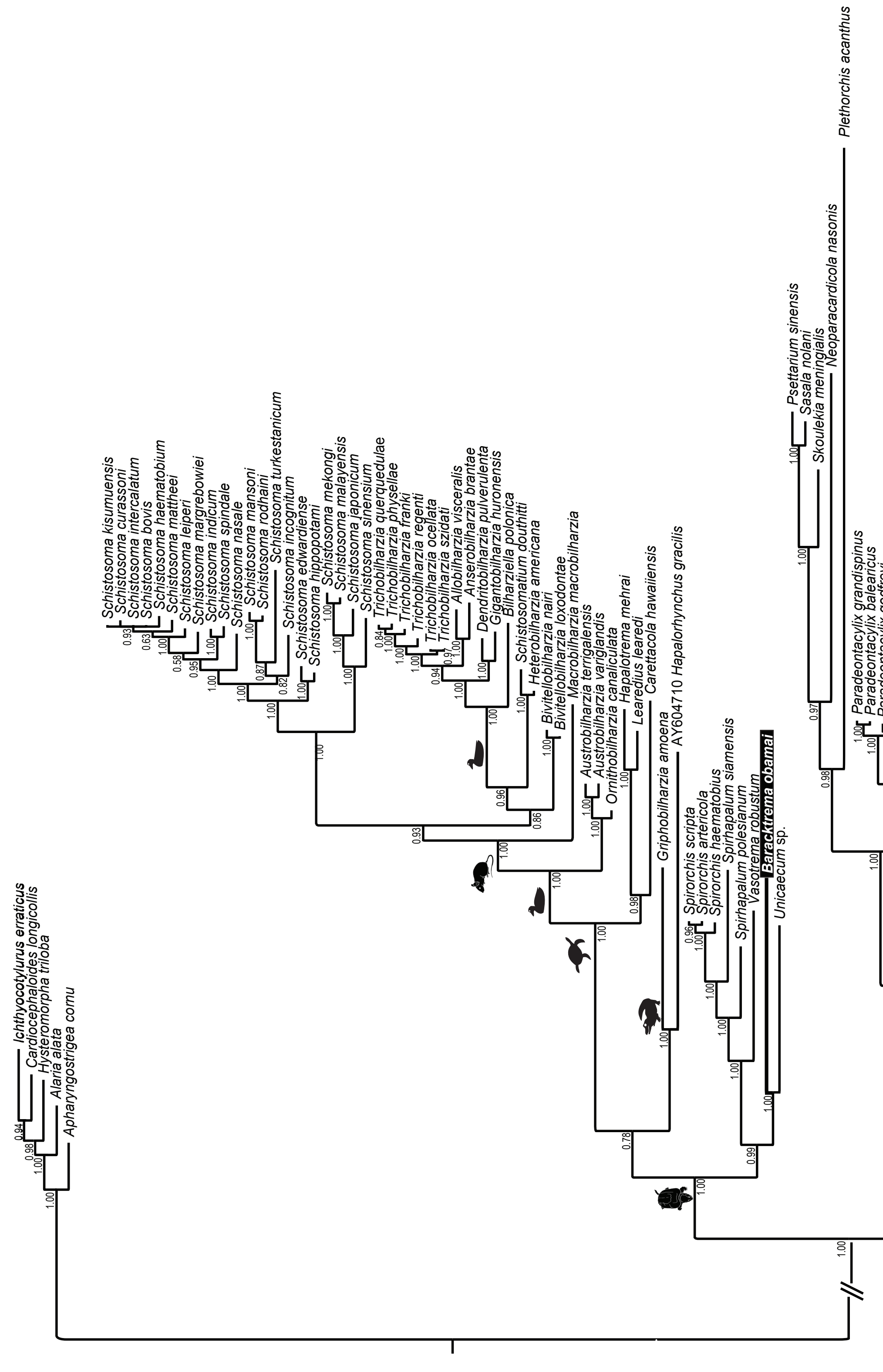
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Anserobilharzia brantae
Dendritobilharzia pulverulenta
Gigantobilharzia huronensis
Bilharziella polonica
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Bivitellobilharzia nairi
Bivitellobilharzia loxodontae
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Cardicola palmeri
Elaphrobates euzeti
Cardicola orientalis
Cardicola forsteri
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CHAPTER 2: BLOOD FLUKES OF ASIATIC SOFTSHELL TURTLES: REVISION OF *COEURITREMA* MEHRA, 1933 (DIGENEA: SCHISTOSOMATOIDEA) AND A NEW SPECIES INFECTING CHINESE SOFTSHELL TURTLES, *PELODISCUS SINENSIS*, (TRIONYCHIDAE) FROM THE DA RANG RIVER, VIETNAM

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ABSTRACT

Coeuritrema Mehra, 1933, previously regarded as a junior subjective synonym of *Hapalorhynchus* Stunkard, 1922, herein is revised to include *Coeuritrema lyssimus* Mehra, 1933 (type species), *Coeuritrema rugatus* (Brooks et Sullivan, 1981) comb. n., and *Coeuritrema platti* Roberts et Bullard sp. n. These genera are morphologically similar by having a ventral sucker, non-fused caeca, two testes, a pre-testicular cirrus sac, an intertesticular ovary, and a common genital pore that opens dorsally and in the sinistral half of the body. Phylogenetic analysis of the D1–D3 domains of the nuclear large subunit ribosomal DNA (28S) suggested that *Coeuritrema* and *Hapalorhynchus* share a recent common ancestor. *Coeuritrema* is morphologically most easily differentiated from *Hapalorhynchus* by having ventrolateral tegumental papillae and a definitive metraterm that is approximately 3−7× the uterus length. *Coeuritrema* comprises species that reportedly infect Asiatic softshell turtles (Testudines: Trionychidae) only; whereas, *Hapalorhynchus* (as currently defined) comprises blood flukes that reportedly infect those hosts plus North American musk turtles (*Sternotherus*) and mud turtles (*Kinosternon*), both Kinosternidae, North American snapping turtles (Chelydridae), Asiatic hard-shelled turtles (Geoemydidae), and African pleurodirans (Pelomedusidae). *Coeuritrema platti* infects the blood of Chinese softshell turtles, *Pelodiscus sinensis* (Wiegmann, 1835), cultured in the Da Rang River Basin (Phu Yen Province, Vietnam). It differs from *C. lyssimus* by having a narrow hindbody (<1.6× forebody width), ventrolateral tegumental papillae restricted to the hindbody, a short cirrus sac (<10% of corresponding body length), a transverse ovary buttressing the caeca, a short, wholly pre-ovarian metraterm (~10% of corresponding body length), and a submarginal genital pore. It differs from *C. rugatus* by having small ventrolateral tegumental papillae, testes without deep lobes, and a Laurer's canal pore that opens posterior to vitelline reservoir and dorsal to the oviducal seminal receptacle. The new species is only the second turtle blood fluke reported from Vietnam.

INTRODUCTION

The turtle blood flukes (TBFs; paraphyletic "Spirorchiidae") comprise 84 accepted species (54 freshwater; 30 marine) assigned to 20 genera and that infect 37 freshwater and three marine turtle species (Smith 1997a,b, Platt 1993, 2002, Tkach et al. 2009, Platt and Sharma 2012, Orélis-Ribeiro et al. 2014, Roberts et al. 2016). Seventeen TBFs of five genera (*Hapalorhynchus* Stunkard, 1922, *Vasotrema* Stunkard, 1926, *Coeuritrema* Mehra, 1933, *Enterohaematotrema* Mehra, 1940, *Cardiotrema* Dwivedi, 1967) infect softshell turtles (Testudines: Trionychidae) (Table 1), but only nine of 31 (29%) trionychids host a TBF; suggesting that perhaps numerous innominate TBFs infect this turtle lineage. Softshell turtles of *Pelodiscus* (*P. axenaria* [Zhou, Zhang et Fang, 1991], *P. maackii* [Brandt, 1857], *P. parviformis* Tang, 1997, *P. sinensis* [Wiegmann, 1835]) are commercially prized and cultured for food and magic throughout

Asia (Alves et al. 2008, Fritz et al. 2010, Stuckas and Fritz 2011). A survey of Chinese aquaculture facilities indicated that >300 million Chinese softshell turtles are in captivity now and that ~125 million individuals are available for sale now in China alone (Haitao et al. 2008). Even though they are abundant in southeast Asian aquaculture, several of these species have become scarce in the wild (van Dijk et al. 2000). Despite their commercial importance, the taxonomy of their blood parasites remains as underexplored as the infectious diseases that afflict these turtles in aquaculture.

During a recent parasitological expedition to Vietnam, we encountered a new TBF species infecting Chinese softshell turtles, which led to a consideration of all TBFs infecting softshell turtles. Herein, we revise *Coeuritrema*, emend its diagnosis, redescribe *Coeuritrema rugatus* (Brooks et Sullivan 1981) comb. n. (formerly *Hapalorhynchus*) based on museum specimens, describe a new species, and provide an updated phylogeny for the TBFs inclusive of the new taxon. This is the second TBF species reported from Vietnam.

MATERIAL AND METHODS

Turtles were purchased from commercial turtle trappers in Nha Trang, Vietnam and from a turtle farm in Phu Yen Province during 1−16 June 2015. Turtles were killed by decapitation. Necropsies were performed using 7.0 g/L sodium citrate saline solution. Living flukes intended as whole-mounts were killed with a butane hand lighter under little or no coverslip pressure and transferred to a vial of 5% neutral buffered formalin (n.b.f.). TBFs were maintained in 5% n.b.f. until staining. After washing with distilled water, specimens intended as whole mounts were stained in Van Cleave's hematoxylin with several additional drops of Ehrlich's hematoxylin, dehydrated using an ethanol series, cleared in clove oil, and permanently mounted in Canada balsam.

Whole mounts were examined using a Leica DM 2500 microscope with differential interference contrast optical components. Parasite measurements are reported in micrometers (µm) followed by the mean and number of specimens measured in parentheses. Turtle scientific names and taxonomic authorities follow van Dijk et al. (2014). Classification and anatomical terms for TBFs follow Luhman (1935: genital sucker), Byrd (1939; uterine pouch [see also Roberts et al. 2016]), Yamaguti (1971: Manter's organ), and Platt (1993: median oesophageal pouch; 1998, 2002: genital spines, plicate organ, and most other terms).

Specimens for molecular analyses were handled with camel-hair brushes, pipettes, or fine forceps, and immediately preserved in a vial of absolute EtOH and stored at −20 °C. Total genomic DNA (gDNA) was extracted using DNeasyTM Blood and Tissue Kit (Qiagen, Valencia, California, USA) according to the manufacturer's protocol, except for the incubation period with proteinase-K that was extended to overnight, and the final elution step wherein only 100 µl of elution buffer was used to increase the final DNA concentration in the eluate. Partial 28S rDNA (domains D1–D3; ~1,400 bp) was amplified using the forward primer "U178" (5′-GCA CCC GCT GAA YTT AAG-3′) and the reverse primer "L1642" (5′-CCA GCG CCATCC ATT TTC A-3′) (Lockyer et al. 2003). PCR amplifications were performed using a total volume of 25 µl containing approximately 2 µl of DNA template, 0.4 µM of each primer along with 1× buffer, 2.5 mM $MgCl_2$ (New England Biolabs, Ipswich, Massachusetts, USA), 1 mM dNTP mixture, and

0.3 µl Taq polymerase (5 U/µl) (New England Biolabs, Ipswich, Massachusetts, USA). Thermocycling profile consisted of an initial 4 min at 94°C for denaturation, followed by 40 repeating cycles of 94°C for 30 s for denaturation, 50°C for 30 s for annealing, and 72°C for 2 min for extension, followed by a final 5 min at 72°C for extension. All PCR reactions were carried out in a Veriti Thermal Cycler (Applied Biosystems, Waltham, Massachusetts, USA). PCR products (5 µl) were verified on a 1% agarose gel and stained with ethidium bromide. PCR amplicons were gel-excised using QIAquickTM Gel Extraction Kit (Qiagen, Valencia, California, USA) following the manufacturer's protocol. DNA sequencing was performed by GENEWIZ with ABI Prism 3730xl DNA analyzer (GENEWIZ, Inc., South Plainfield, New Jersey, USA). Primers used in sequencing of 28S rDNA included the PCR primers and the internal forward primers 300F (5′-CAA GTACCGTGAGGGAAAGTTG-3′) and 900F (5′-CCGTCTTGAAACACGGACCAAG-3′) and reverse primer 1200R (5′-GCATAGTTCACCATCTTTCGG-3′) (Lockyer et al. 2003). Sequence assembling and analysis of chromatograms were conducted using BioNumerics version 7.0 (Applied Maths, Sint-Martens-Latem, Belgium).

The sequence data for nuclear 28S rDNA generated during this study was aligned with those for selected blood flukes (Schistosomatoidea) available on GenBank. Outgroups were selected from representative fish blood flukes, Aporocotylidae Odhner, 1912 (see Bullard et al. 2009). The ingroup comprised newly-generated sequence data from the new species (XXXXXXXX) and *Hapalorhynchus foliorchis* Brooks et Mayes, 1975 (XXXXXXXX) plus publicly available sequences from *Baracktrema obamai* Roberts, Platt et Bullard, 2016 (KX061500), *Griphobilharzia amoena* Platt et Blair, 1991 (AY899914), and ten other TBFs (Orélis-Ribeiro et al. 2014). The specimens of *H.*

foliorchis were collected in Alabama from *Chelydra serpentina* (Linnaeus, 1758) during October, 2014 (Roberts et al. *in press*). Sequences were aligned using MAFFT (Katoh and Toh 2010) with default settings implemented in the CIPRES Science Gateway V.3.3 (Miller et al. 2010). The resulting alignment was refined by eye using MEGA version 5.2.2 (Tamura et al. 2011) and the ends of each fragment were trimmed to match the shortest sequence. Ambiguous positions were identified and removed using the Gblocks server (Castresana 2000) with settings for a less stringent selection. Bayesian inference (BI) was performed using the Metropolis-coupled Markov chain Monte Carlo method (MC3) in MrBayes version 3.2.6 (Huelsenbeck and Ronquist 2005, Huelsenbeck et al. 2001, Ronquist and Huelsenbeck 2003) and run on CIPRES (Miller et al. 2010). The model of nucleotide substitution was selected based on the Akaike Information Criterion (Posada and Buckley 2004) as implemented in the jModelTest version 2.1.4 (Darriba et al. 2012, Guindon and Gascuel 2003). The GTR + I + G (proportion of invariable sites = 0.385 and gamma distribution = 1.393) model was inferred as the best estimator; therefore, BI used the following parameters: nst = 6, rates = invgamma, ngammacat = 4, and default priors. Analyses were run in duplicate each containing 4 independent chains (three heated and one cold chain) (nchains = 4) for 1.0 × 107 generations (ngen = 10,000,000) sampled at intervals of 1000 generations (samplefreq = 1000). Results of the first 2,500 sampled trees were discarded as “burn-in” based on the stationarity of the likelihood values, assessed by plotting the log-likelihood values of the sample points against generation time using Tracer version 1.5 (Rambaut and Drummond 2009). All retained trees were used to estimate posterior probability of each node. A majority rule consensus tree with average branch lengths was constructed for the remaining trees using ‘summarize the trees’ (sumt) in MrBayes. Resulting phylogenetic trees were visualized using FigTree v1.3.1 (Rambaut 2009) and further edited with Adobe Illustrator CS3 (Adobe Systems, San Jose, California, USA). Branch supports were considered as significant when posterior probabilities were >0.95.

RESULTS

Coeuritrema Mehra, 1933 emended (Figs. 1−7)

Diagnosis. Body dorsoventrally flattened (not cylindrical), 3−7× longer than wide, constricted at level of ventral sucker, having hindbody 1−3× longer than forebody, aspinous, papillate; ventrolateral tegumental papillae present, distributing from oral sucker posteriad or from ventral sucker posteriad. Oral sucker robust, aspinous, demarcated from body by posterior constriction. Ventral sucker present, aspinous. Dorsolateral and ventrolateral nerve chords present. Pharynx present, enveloping anterior extremity of oesophagus. Oesophagus extending posteriad <1/4 of body length, ventral to anterior nerve commissure, lacking diverticula, plicate organ, or median oesophageal pouch, straight or slightly sinuous; oesophageal gland surrounding oesophagus from posterior margin of pharynx to oesophageal-intestinal junction, strongly basophilic, widest at level of caecal bifurcation. Intestine comprising non-fused caeca, inverse U-shaped, smooth (lacking diverticula or secondary rami), extending 1/2−3/4 of body length directly posteriad, not extensively convoluted, bifurcating anterior to ventral sucker, terminating in posterior body extremity. Testes comprising one anterior testis and one posterior testis, in posterior half of body, intercaecal, having deep lobes or slightly irregular margins. Vas deferens extending directly anteriad and ventral to cirrus sac before turning posteriad and expanding to form external seminal vesicle; external seminal vesicle posterior to ventral sucker, intercaecal, abutting anterodextral margin of cirrus sac, at level of common genital pore. Cirrus sac robust, pre-testicular, containing variously-sized secretory cells and large *pars prostatica*. Ovary single, wider than long, triangular, lacking lobes, intercaecal, intertesticular. Oviduct emerging from dextral margin of ovary, directing posteriad; oviducal seminal receptacle comprising middle portion of oviduct between ovary and posterior testis. Laurer's canal intercaecal, intertesticular, post-ovarian, extending anteriad or posteriad from oviduct at level of vitelline reservoir, opening dorsally. Vitellarium follicular, occupying space from caecal bifurcation to caecal termination; vitelline reservoir intercaecal, intertesticular, ventral to ovary. Oötype a weakly glandular and thin-walled chamber, intercaecal, occupying space between anterior testis and ovary, posterior to cirrus sac. Uterus intercaecal, intertesticular, straight (not coiled); uterine pouch absent. Egg single, occupying female reproductive tract proximal to metraterm. Metraterm massive, approximately 3−7× uterus length, longitudinal (extending anteriad in parallel with body margin), between ventral sucker and vitelline reservoir, sinistral to anterior testis, having an obviously muscular thick wall, apparently not storing egg(s). Common genital pore dorsal, sinistral, posterior to ventral sucker, predominantly anterior to genitalia, aspinous, lacking suckers. Excretory vesicle globular, extensively lobed, extending posteriad from distal ends of caeca to posterior body end. Excretory pore terminal. Manter's organ absent. In blood of Asiatic trionychids.

Differential diagnosis. Body 3−7× longer than wide, aspinous; ventrolateral tegumental papillae present. Ventral sucker present. Oesophagus <1/4 of body length, lacking diverticula, plicate organ, or median oesophageal pouch. Intestine comprising non-fused caeca. Testes comprising one anterior and one posterior testis. External seminal vesicle abutting anterodextral margin of cirrus sac and at level of common genital pore. Ovary intertesticular. Oviducal seminal receptacle comprising middle portion of oviduct between ovary and posterior testis. Laurer's canal intercaecal, intertesticular, post-ovarian. Vitelline reservoir intertesticular. Uterine pouch absent. Egg single, occupying female reproductive tract proximal to metraterm. Metraterm massive, approximately 3−7× uterus length, apparently not storing egg(s). Common genital pore dorsal, sinistral, predominantly anterior to genitalia.
Type species: *Coeuritrema lyssimus* Mehra, 1933 from heart ventricle of Indian flapshell turtle, *Lissemys punctata* (Bonnaterre, 1789), (Testudines: Trionychidae) in the Ganges River, India.

Remarks. Mehra (1933) proposed *Coeuritrema* for *C. lyssimus* and *Hapalorhynchus odhnerensis* (Mehra, 1933) Byrd, 1939. That diagnosis included “body wall with or without small papillae” (= ventrolateral tegumental papillae) such that *H. odhnerensis*, which lacks ventrolateral tegumental papillae, could be included. As emended herein, however, *Coeuritrema* includes papillate TBFs only. This genus has long been regarded as a junior subjective synonym of *Hapalorhynchus* (Byrd, 1939, Hughes et al. 1942, Brooks and Mayes 1976, Bourgat 1990, Platt 1988, 2002), which lacks ventrolateral tegumental papillae. *Coeuritrema* further differs from *Hapalorhynchus* by having an external seminal vesicle that abuts the anterodextral margin of the cirrus sac and a definitive metraterm that is approximately 3−7× the uterus length.

The oötype and uterus are both relatively short and together, rather than the metraterm, may store the egg in species of *Coeuritrema*. If a large egg, presumably one that is developed and ready for passage to the metraterm and/or for ejection, is present in the proximal portion of the female reproductive tract, the egg occupies the luminal spaces of both the oötype and uterus (Fig. 4). In that case, the oötype and uterus are difficult to differentiate (Fig. 4). Without an egg, the clear distinction between the oötype and the uterus is evident: the oötype comprises the laterally-expanded chamber immediately distal to the ovo-vitelline duct and the uterus is the narrow, short duct connecting it with the metraterm (Fig. 6). If our understanding is correct, it may be useful to consider the oötype and uterus functioning together as an ‘egg chamber.’ Noteworthy along these lines also is that we did not observe an egg in the metraterm of any specimen, indicating that perhaps the metraterm of *Coeuritrema* spp. does not retain the egg. These observations match those of previous workers handling specimens of the type species and *C. rugatus.* Mehra (1933) explicitly reported that he never observed more than a single egg (“ovum”) in the female reproductive tract of *C. lyssimus* (type species) and that each egg resided “in the proximal portion of the metraterm,” which, based on his illustrations (see plate 1, fig. 1 of Mehra 1933) could be interpreted as the putative egg chamber. Brooks and Sullivan (1981) likewise reported that only a single egg occurred in the uterus of ovigerous specimens. Perhaps this is related to the gestation time for the large egg. The metraterm of other whole-mounted blood flukes does hold eggs (Bullard and Overstreet 2006; Bullard et al. 2006; Bullard and Jensen 2008); however, eggs in those species are smaller, much more numerous in the uterus and metraterm, and seemingly less developed. Perhaps this is because they must infect minute branchial vessels before transiting to the gill epithelium and hatching (Bullard and Overstreet 2008). The extent to which the metraterm serves as a storage organ for TBF eggs is indeterminate, but if particular genera have or lack such an ‘egg chamber’ (=oötype + uterus), it is likely a significant differential diagnostic feature relating genera. Resin-based ultramicrotomy of the wall of the proximal portion of the female reproductive tract could reveal cellular changes to the putative egg chamber wall in the presence/absence of the large egg.

Coeuritrema and *Hapalorhynchus* are similar morphologically by having a ventral sucker, non-fused caeca, two testes, a pre-testicular cirrus sac, an intertesticular ovary, and a common genital pore that opens dorsally and in the sinistral half of the body. Phylogenetic analysis of the D1–D3 domains of the nuclear large subunit ribosomal DNA (28S) suggests that *Coeuritrema* and *Hapalorhynchus* indeed share a recent, common ancestor (Fig. 7; see below). We concur with Platt (2002) in that several apapillate turtle blood fluke species originally assigned to *Coeuritrema* should be assigned to *Hapalorhynchus*: *H. odhnerensis*, *Hapalorhynchus ocadiae* (Takeuti, 1942) Bourgat, 1990, *Hapalorhynchus oschmarini* (Belous, 1963) Bourgat, 1990, *Hapalorhynchus macrotesticularis* (Rohde, Lee et Lim, 1968) Brooks et Mayes, 1976, *Hapalorhynchus mica* (Oschmarin, 1971) Bourgat, 1990, *Hapalorhynchus sheilae* (Mehrotra, 1973) Bourgat, 1990, and *Hapalorhynchus sutlejensis* (Mehrotra, 1973) Bourgat, 1990 (see Takeuti 1942, Rohde et al. 1968, Oschmarin 1971, Mehrotra 1973).

This brings the total number of accepted species of *Coeuritrema* and *Hapalorhynchus* to 3 and 19, respectively.

Yamaguti (1958) considered *Coeuritrema* Mehra, 1933 a junior synonym of *Tremarhynchus* Thapar, 1933; however, the diagnosis of *Coeuritrema* was published in May 1933 and that of *Tremarhynchus* was published immediately thereafter in June. Hence, the former genus has taxonomic priority (Dwivedi, 1967). Byrd (1939) and Platt (2002) considered *Tremarhynchus* (and *Coeuritrema*) a junior subjective synonym of *Hapalorhynchus*. No author has treated *Tremarhynchus* since 2002.

Tremarhynchinae Yamaguti, 1958 included *Enterohaematotrema* Mehra, 1940 and *Tremarhynchus* (type genus) (see Yamaguti, 1958). Dwivedi (1967) commented that the sub-family should be renamed Coeuritrematinae Dwivedi, 1967 (type genus *Coeuritrema*) to correct Yamaguti’s (1958) name for the subfamily based on his erroneous acceptance of *Tremarhynchus* as a senior synonym of *Coeuritrema* by taxonomic priority. This decision was probably based upon the International Code of Zoological Nomenclature’s Article 61.1.2. (under “Statement of the Principle of Typification”), which states that “a nominal family-group taxon is the nominal genus on which its name is based.” (ICZN 2000). Hence, because post-1967 *Coeuritrema* was correctly understood to have taxonomic priority over *Tremarhynchus*, the sub-family name had to be changed. Yamaguti (1971) accepted Coeuritrematinae Dwivedi, 1967 (as “1968”) and included *Coeuritrema*, *Enterohaematotrema*, and *Cardiotrema* based on the presence of a ventral sucker, paired caeca not united posteriorly, two testes, a cirrus sac “more or less strongly developed” between ventral sucker and anterior testis, an intertesticular ovary, a vitellarium as long as caeca, and a genital pore between the ventral sucker and testes. These genera plus *Hapalorhynchus* and *Vasotrema* include species infecting softshell turtles (Trionychidae) (Table 1). No molecular sequence data exists for any species of *Enterohaematotrema* or *Cardiotrema*, and *Vasotrema* does not group with the *Coeuritrema* + *Hapalorhynchus* clade (Fig. 7). Hence, presently, no support exists for monophyly of softshell TBFs; suggesting that TBFs have repeatedly, independently colonized softshell turtles.

Coeuritrema differs from *Enterohaematotrema* by having a dorsal, sinistral genital pore (rather than a ventral, medial genital pore); from *Cardiotrema* by having an intertesticular ovary (rather than a sinistral ovary) and a large ventral sucker >1/2 body width (rather than <1/5 body width for *Cardiotrema*); and from *Vasotrema* by having two testes and a dorsal genital pore (rather than one testis posterior to the ovary and a ventral genital pore). Mehra (1934) provided a key to TBF genera and differentiated *Coeuritrema* and *Hapalorhynchus* by the presence or absence of a cirrus sac and cirrus. These features have been the focus of intense study because of their stated value as differential features. Our observations herein confirmed the presence of an obvious cirrus sac among the accepted species of *Coeuritrema,* but this feature may or may not be of generic importance in other TBF genera. For example, within the closely-related *Hapalorhynchus* the presence or absence of the cirrus sac and cirrus have been accepted as generic features by some (Mehra 1939, Mehra 1940, Yamaguti 1958, Yamaguti 1971) and rejected by others (Byrd 1939, Brooks and Mayes 1976, Platt 1988, Bourgat 1990, Platt 2002). Brooks and Mayes (1976) provided supplemental observations of the male genitalia of *Hapalorhynchus stunkardi* Byrd, 1939 and emended *Hapalorhynchus* to include “well-developed or poorly-developed cirrus.” Platt

(1988) observed, in histological sections, prostate cells not bound by a limiting membrane but scattered in the surrounding parenchyma in *Hapalorhynchus gracilis* Stunkard, 1922, *H. foliorchis*, and *H. stunkardi*. He also observed that the prostatic complex of *Hapalorhynchus albertoi* Lamothe-Argumedo, 1978 and *Hapalorhynchus brooksi* Platt, 1988 was membrane-bound (= cirrus sac) and that the distal portion of the ejaculatory duct (= cirrus) was eversible (Lamothe-Argumedo 1978, Platt 1988). As a result, he emended the diagnosis as “cirrus sac present or absent.” As additional species of *Coeuritrema* are documented, special care should be taken to assess the cirrus sac and cirrus.

Coeuritrema rugatus Brooks et Sullivan, 1981 comb. n. (Figs. 1, 2)

Diagnosis (based on light microscopy of eight whole-mounted adult specimens, including paratypes): Body 1250−1540 (1423; 7) long or 5.3−7.2× (6.5; 7) longer than wide, 150−200 (177; 8) wide or 12−13% (12%; 7) of body length at level of caecal bifurcation, 200−265 (219; 8) wide or 14−19% (15%; 7) of body length at ovary (typically maximum width of specimen), 150−205 (174; 8) wide or 11−15% (12%; 7) of body length at caecal termination; forebody (middle of ventral sucker to anterior end) 340−470 (439; 7) long or 24−34% (31%; 7) of body length, lacking ventrolateral tegumental papillae; hindbody (middle of ventral sucker to posterior end) 840−1090 (968; 8) long or 66−76% (69%; 7) of body length, having ventrolateral tegumental papillae (Fig. 1). Ventrolateral tegumental papillae 17−20 (19; 8) along dextral body margin, 18−20 (19; 8) along sinistral body margin, 35−39 (38; 8) total (Fig. 1); anterior papillae 8−35 (20; 8 [3 papillae measured per specimen]) long, with base 15−38 (24; 8) wide, 0.6−2.5× (1.3; 8) wider than long; middle papillae 25−43 (32; 8) long, with base 35−53 (43; 8) wide, 1.0−1.8× (1.3; 8) wider than long; posterior papillae 8−38 (23; 8) long, base 13−45 (29; 8) wide, 0.7−2.9× (1.3; 8) wider than long. Oral sucker 60−75 (67; 7) long or 4−5% (5%; 7) of body length, 78−100 (93; 7) wide or 38−50% (43%; 7) of maximum body width (Fig. 1). Ventral sucker 103−125 (114; 8) long or 7−9% (8%; 7) of body length, 103−133 (120; 8) wide or 57−75% (68%; 8) of body width at caecal bifurcation, 1.3−1.5× (1.3; 7) wider than oral sucker (Fig. 1). Nerve commissure 193−225 (202; 7) or 13−16% (14%; 7) of body length from anterior body end. Pharynx 53−68 (59; 7) long or 20−25% (23%; 7) of oesophagus length, 60−83 (77; 7) wide or 0.8−1.5× (1.1; 7) maximum oesophagus width (Fig. 1). Oesophagus 225−295 (261; 7) long or 17−21% (18%; 7) of body length, 15−25 (19; 7) wide posterior to pharynx, with wall 10−18 (14; 7) thick, 53−95 (73; 8) maximum width or 29−53% (41%; 8) of body width at caecal bifurcation, with wall 10−50 (24; 8) thick (Fig. 1), narrowing to 13−25 (20; 8) width or 7−14% (11%; 8) of body width at caecal bifurcation, with wall 10−20 (15; 8) thick; oesophageal gland 205−245 (223; 8) long or 14−17% (16%; 7) of body length and 81−93% (86%; 7) of oesophagus length, 100−135 (121; 8) wide or 56−84% (70%; 8) of body width at caecal bifurcation. Intestine bifurcating 262−330 (285; 7) or 17−23% (20%; 7) of body length from anterior end; sinistral caecum 850−1050 (962; 8) long or 67−72% (69%; 7) of body length, 73−100 (87; 7) wide at bifurcation or 46−54% (49%; 7) of body width, 20−30 (26; 8) wide at ovary or 9−15% (12%; 8) of body width, 23−45 (31; 8) wide at termination or 13−24% (18%; 8) of body width; dextral caecum 820−1100 (953; 8) long or 65−73% (68%; 7) of body length, 65−90 (77%; 8) wide at bifurcation or

38−50% (43%; 8) of body width, 15−30 (24; 8) wide at ovary or 7−13% (11%; 8) of body width, 25−45 (32; 8) wide at termination or 15−24% (18%; 8) of body width; caecal termination 115−145 (130; 8) or 8−10% (9%; 7) from posterior end.

Anterior testis lobed, intensely follicular marginally, 118−188 (143; 8) long or 9−13% (10%; 7) of body length, 70−115 (87; 8) wide or 35−44% (40%; 8) of body width at gonads; intertesticular space 63−90 (83; 8) or 4−7% (6%; 7) of body length (Figs. 1, 2). Posterior testis as anterior testis, 138−208 (167; 8) long or 10−15% (12%; 7) of body length, 70−135 (91; 8) wide or 35−51% (41%; 7) of body width at gonads; post-testicular space 340−430 (391; 8) or 26−30% (28%; 7) of body length from posterior end. Anterior trunk of vasa efferentia emanating from ventral surface of anterior testis, extending anteriad 20−28 (22; 8) or <1% (7) of body length from testis, 5 (8) wide or 2−3% (2%; 8) of body width (Fig. 2); posterior trunk of vasa efferentia emanating from ventral surface of posterior testis, extending anteriad 200−273 (234; 8) or 16−19% (17; 7) of body length, 3−5 (4; 8) wide or 1−3% (2%; 8) of body width, coalescing with anterior trunk of vasa efferentia ventral to anterior portion of anterior testis (Fig. 2). Vas deferens 88−153 (111; 8) long or 6−10% (8%; 7) of body length, 5−8 (5; 8) in maximum width, extending anteroventrally. External seminal vesicle 40−80 (60; 8) long or 3−6% (4%; 7) of body length, 25−115 (46; 8) wide, 0.7−2.0× (1.5; 8) longer than wide, narrowing posteriorly and proximal to internal seminal vesicle (Figs. 1, 2); internal seminal vesicle 75−88 (80; 8) long or 1.0−2.1× (1.4; 8) external seminal vesicle length, 25−35 (31; 8) wide or 2.2−3.1× (2.6; 8) longer than wide or 22−132% (81%; 8) of external seminal vesicle width (Figs. 1, 2). *Pars prostatica* surrounding distal portion of internal seminal vesicle, 48−58 (52; 8) long, 30−35 (32; 8) wide or 1.5−1.9× (1.6; 8) longer than wide (Fig. 2).

Cirrus 5−10 (8; 8) long, 3−5 (4; 8) wide; cirrus sac enveloping most of internal seminal vesicle and entirety of *pars prostatica*, 88−160 (119; 8) long or 6−11% (8%; 7) of body length, 55−95 (67; 8) wide or 24−44% (31%; 8) of maximum body width, containing large putative secretory cells (Figs. 1, 2).

Ovary triangular, with broadest portion in sinistral half of body, 260−355 (310; 8) or 21−25% (22%; 7) of body length from middle of ventral sucker buttressing caeca, 58−100 (77; 8) long or 5−6% (6%; 7) of body length, 93−140 (112; 8) wide or 44−58% (51%; 7) of body width, 1.3−1.8× (1.5; 8) wider than long; post-ovarian space 495−640 (581; 8) or 40−45% (41%; 7) of body length (Fig. 1); oocytes largest and most basophobic near oviduct (Fig. 2). Oviduct extending posteriad, turning dorsally, proceeding counterclockwise for 48−65 (54; 8) or 3−5% (4%; 7) of body length, 5−10 (8; 8) in maximum width, expanding laterally to form oviducal seminal receptacle; oviducal seminal receptacle extending sinistrad for 53−80 (63; 8) or 58−112% (84%; 8) of ovary width, 25−33 (28; 8) in maximum width at origin or 9−16% (13%; 8) of maximum body width, narrowing before turning dorsal, extending anterodextrad 58−95 (82; 8) or 5−7% (6%; 7) of body length, 10−13 (12; 8) in maximum width or 5−7% (5%; 8) of body width (Fig. 2). Laurer's canal a narrow duct extending 23−43 (31; 8) anterosinistrad from middle portion of oviduct, 8−13 (11; 8) wide, opening dorsally at level of middle portion of ovary (Fig. 2). Vitellarium comprising a series of interconnected spheroid masses of follicles, distributing from level of caecal bifurcation to distal ends of caeca, lateral collecting ducts coalescing at level of posterior margin of ovary to form transverse vitelline duct; transverse vitelline duct ventral to ovary, 315−415 (369; 8) or 25−29% (27%; 7) of body length from middle of ventral sucker (Figs. 1, 2); vitelline reservoir sac-like, ventral to oviducal seminal receptacle; vitelline duct extending anterodextrad and dorsal 55−85 (64; 8) or 4−6% (5%; 7) of body length before connecting with oviduct at oötype (Fig. 2). Oötype difficult to discern in gravid specimens, 30−43 (36; 8) long, 28−43 (37; 8) wide, dorsal to anterior margin of ovary (Fig. 2). Uterus comprising proximal portion and metraterm (Fig. 2), with a single egg in seven of eight specimens; proximal portion of uterus extending anterosinsitrad from oötype, 28−50 (39; 8) long or 2−4% (3%; 7) of body length, 18−30 (27; 8) wide or 9−15% (12%; 8) of maximum body width; metraterm extending anterosinistrad, 148−218 (175; 8) long or 11−15% (12%; 7) of body length, maximum width of 25−45 (34; 8) or 12−21% (16%; 8) of maximum body width, 3.2−6.6× (4.7; 8) proximal uterus length. Uterine egg ovoid, 63−78 (72; 7) long or 5% (6) of body length, 23−33 (27; 7) wide or 9−16% (12%; 7) of maximum body width, 1.9−3.3× (2.7; 7) longer than wide (Fig. 2). Common genital pore 95−150 (123; 8) or 8−10% (9%; 7) of body length posterior to middle of ventral sucker (Fig. 1).

Excretory vesicle 130−165 (144; 8) long or 10−12% (10%; 7) of body length, 78−100 (87; 8) wide or 45−55% (50%; 8) of body width at caecal termination; wall 8−13 (10; 8) thick (Fig. 1).

Type and only known host: Asiatic softshell turtle, *Amyda cartilaginea* (Boddaert, 1770) (Testudines: Trionychidae).

Type locality: Sungei Jempol, Ulu Jempol, State of Negeri Sembilan, Malaysia.

Site in host: Mesenteric blood vessels.

Specimens examined: *Hapalorhynchus rugatus* – Harold W. Manter Laboratory (HWML) of Parasitology Coll. No. 21339, paratypes, eight slides comprising eight whole-mounted specimens, seven of the eight slides labeled as *Hapalorhynchus rugosus*, *ex.*

Amyda cartilaginea (as *Trionyx cartilageneus*) from Sungei Jempol, Ulu Jempol, State of Negeri Sembilan, Malaysia (Brooks and Sullivan 1981).

Remarks. Our interpretation of some features associated with type materials (see above) of *C. rugatus* contradicted those of Brooks and Sullivan (1981), who did not detail the vasa efferentia, vas deferens, oötype, or Laurer's canal. They reported that *C. rugatus* lacked a pharynx; however, the paratypes have a large pharynx surrounding the anterior extremity of the oesophagus immediately posterior to the mouth (Fig. 1). *Coeuritrema* and *Baracktrema* Roberts, Platt et Bullard, 2016 are the only accepted TBF genera described as having a pharynx, and the pharynx is immediately posterior to the mouth and muscular oral sucker. Several species of *Hapalorhynchus*, *Spirorchis* MacCallum, 1918, and *Vasotrema* also have this configuration and will be treated in later taxonomic works (personal observations JRR, SAB).

Regarding the oviduct, Brooks and Sullivan (1981) illustrated it as originating from the posteromedial aspect of the ovary and turning anterodorsally, but we confirmed that it originates from the dextral side of the ovary. Regarding the oviducal seminal receptacle, they illustrated a sac-like structure branching from the oviduct and filled with sperm proximal to the uterus (fig. 3, pg. 1336). No paratype had such a structure. They stated that the vitelline reservoir (Fig. 2) was immediately postovarian and ventral to the ovary, but we observed that the reservoir coalesces sinistro-dorsal to the ovary (Fig. 2).

Coeuritrema platti sp. n. (Figs. 3−7)

Diagnosis (based on light microscopy of seven whole-mounted adult specimens): Body 1035−1410 (1218; 6) long or 5.5−6.9× (6.0; 6) longer than wide, 110−170 (147; 6) wide or 11−15% (12%; 6) of body length at level of caecal bifurcation, 150−225 (203; 6) wide or 14−18% (17%; 6) of body length at ovary (typically maximum width of specimen), 120−180 (165; 6) wide or 12−16% (14%; 6) of body length at caecal termination; forebody 390−519 (444; 6) long or 32−39% (36%; 6) of body length, hindbody 660−930 (783; 6) long or 61−68% (64%; 6) of body length, having ventrolateral tegumental papillae (Figs. 3, 5). Ventrolateral tegumental papillae extending posteriad from middle of ventral sucker to posterior end 18−19 (18; 5) along dextral body margin, 17−20 (18; 5) along sinistral body margin, 35−39 (36; 5) total (Figs. 3, 5); anterior papillae 8−25 (17; 3) long, with base 10−33 (21; 5) wide, 0.7−2.5× (1.2; 3) wider than long; middle papillae 10−25 (17; 3) long, with base 18−35 (26; 5) wide, 1.0−2.8× (1.7; 3) wider than long; posterior papillae 5−25 (14; 3) long, with base 8−28 (17; 5) wide, 0.6−2.0× (1.3; 3) wider than long. Oral sucker 45−70 (58; 4) long or 4−6% (5%; 4) of body length, 50−100 (80; 6) wide or 33−45% (39%; 6) of maximum body width (Figs. 3, 5). Ventral sucker 75−138 (115; 6) long or 7−13% (10%; 6) of body length, 65−138 (114; 5) wide or 59−95% (77%; 5) of body width at caecal bifurcation, 1.3−1.6× (1.4; 5) wider than oral sucker (Figs. 3, 5). Nerve commissure 150−238 (188; 6) or 13−17% (15%; 6) of body length from anterior body end. Pharynx 45−63 (58; 6) long or 20−25% (23%; 6) of oesophagus length, 45−83 (65; 6) wide or 1.6−2.2× (1.7; 6) maximum oesophagus width (Figs. 3, 5). Oesophagus straight, 229−301 (259; 6) long or 18−23% (21%; 6) of body length, 10−15 (13; 6) wide posterior to pharynx, with wall 7−10 (9; 6) thick, 25−50 (39; 6) maximum width or (26%; 6) of body width at caecal bifurcation, with wall 10−27 (20; 6) thick, constricting to 13−18 (16; 6) width or 8−16% (11%; 6) of body width at caecal bifurcation, with wall 10−15 (12; 6) thick; oesophageal gland 198−288 (254; 6) long or 17−24% (21%; 6) of body length and 0.9−1.1× (1.0; 6) oesophagus length, 75−125 (104; 6) wide or 68−74% (71%; 6) of body width at caecal bifurcation (Figs. 3, 5). Intestine bifurcating 235−365 (285; 6) or 17−27% (24%; 6) of body length from anterior end; sinistral caecum 585−940 (754; 6) long or 51−68% (62%; 6) of body length, 35−45 (39; 6) wide or 24−32% (27%; 6) of body width at bifurcation, 13−30 (20; 6) wide or 6−15% (10%; 6) of body width at ovary, 20−35 (27; 6) wide or 12−19% (16%; 6) of body width at termination; dextral caecum 585−940 (743; 6) long or 52−67% (61%; 6) of body length, 38−45 (41; 6) wide or 24−36% (29%; 6) of body width at bifurcation, 18−35 (25; 6) wide or 10−18% (12%; 6) of body width at ovary, 18−35 (27; 6) wide or 11−20% (16%; 6) of body width at termination; caecal termination 92−140 (120; 6) or 8−14% (10%; 6) from posterior end.

Anterior testis follicular throughout, lacking lobes, 60−143 (106; 6) long or 5−11% (9%; 6) of body length, 50−95 (71; 6) wide or 25−43% (35%; 6) of body width at gonads; intertesticular space 30−103 (74; 6) or 3−7% (6%; 6) of body length (Figs. 3−6). Posterior testis as anterior testis, 90−158 (130; 6) long or 8−15% (11%; 6) of body length, 55−125 (89; 6) wide or 28−57% (44%; 6) of body width at gonads; post-testicular space 293−335 (310; 6) or 24−28% (27%; 6) of body length from posterior end. Anterior trunk of vasa efferentia emanating from ventral surface of anterior testis, extending anteriad 10−35 (26; 5) or 1−3% (2%; 5) of body length from testis, 3 (5) wide or 1−2% (1%; 5) of body width (Figs. 4−6); posterior trunk of vasa efferentia emanating from ventral surface of posterior testis, extending anteriad 188−255 (224; 5) or 17−21%

(18%; 5) of body length, 3−5 (4; 5) wide or 1−3% (2%; 5) of body width, coalescing with anterior trunk of vasa efferentia ventral to anterior portion of anterior testis (Figs. 4−6). Vas deferens 80−138 (101; 5) long or 6−10% (8%; 5) of body length, 3−5 (4; 3) in maximum width, extending anteroventrally. External seminal vesicle 63−88 (75; 5) long or 6% (5) of body length, 25−38 (33; 5) wide, 1.9−2.5× (2.3; 5) longer than wide, constricting posteriorly prior to internal seminal vesicle (Figs. 3−6); internal seminal vesicle 88−118 (100; 5) long or 1.1−1.6× (1.4; 5) external seminal vesicle length, 13−35 (23; 6) wide or 3.4−6.8× (4.4; 5) longer than wide or 52−92% (73%; 5) of external seminal vesicle width (Figs. 3−6). *Pars prostatica* surrounding distal portion of internal seminal vesicle (Figs. 4, 6), 53−55 (54; 3) long, 20−30 (25; 3) wide or 1.8−2.7× (2.2; 3) longer than wide. Cirrus 5−8 (6; 3) long, 3 (3) wide; cirrus sac enveloping most of internal seminal vesicle and entirety of *pars prostatica*, 78−128 (100; 6) long or 7−9% (8%; 6) of body length, 45−65 (52; 6) wide or 22−30% (26%; 6) of maximum body width, containing large putative secretory cells (Figs. 3−6).

Ovary triangular, with broadest portion in sinistral half of body, 160−320 (246; 6) or 15−23% (20%; 6) of body length from middle of ventral sucker, buttressing caeca, 35−115 (84; 6) long or 3−9% (7%; 6) of body length, 65−153 (111; 6) wide or 42−70% (54%; 6) of body width, 1.1−1.9× (1.4; 6) wider than long; post-ovarian space 425−510 (458; 6) or 35−45% (28%; 6) of body length (Figs. 3−6); oocytes uniform in size and basophilic throughout ovary (Figs. 4, 6). Oviduct extending posteriad, turning dorsally, proceeding sinistrad, turning dorsal again before extending posteriad for 35−105 (61; 6) or 3−7% (5%; 6) of body length, 5−10 (8; 6) in maximum width, expanding laterally to form oviducal seminal receptacle; oviducal seminal receptacle extending sinistrad for

65−80 (71; 3) or 63−78% (73%; 3) of ovary width, 25−40 (34; 5) in maximum width at origin or 11−20% (16%; 5) of maximum body width, narrowing before turning dorsal, extending anterodextrad 78−80 (79; 3) or 6−7% (7%; 3) of body length, 10−13 (11; 3) in maximum width or 5−6% (5%; 3) of body width (Figs. 3−6). Laurer's canal a narrow duct extending 15−25 (21; 3) anterosinistrad from middle portion of oviduct, 5−8 (6; 3) wide, opening dorsal and over proximal portion of oviducal seminal receptacle (Figs. 4, 6). Vitellarium comprising a series of interconnected spheroid masses of follicles, distributing from level of caecal bifurcation to distal ends of caeca, lateral collecting ducts coalescing at level of posterior margin of ovary to form transverse vitelline duct; transverse vitelline duct ventral to ovary, 245−385 (297; 3) or 21−27% (24%; 3) of body length from middle of ventral sucker, lobed dorsally (Figs. 3−6); vitelline reservoir sac-like, ventral to oviducal seminal receptacle; vitelline duct extending anterodextrad and dorsal 30−58 (47; 5) or 2−5% (4%; 5) of body length before connecting with oviduct at oötype (Figs. 3−6). Oötype difficult to discern in gravid specimens, 25−40 (32; 3) long, 33−43 (38; 3) wide, dorsal to dextral half of ovary (Fig. 6). Uterus comprising proximal portion and metraterm (Figs. 3−6), with a single egg in four of seven specimens; proximal portion of uterus extending anterosinistrad from oötype, 25−45 (36; 3) long or 2−4% (3% 3) of body length, 23−38 (29; 3) wide or 12−18% (14%; 3) of maximum body width; metraterm extending anterosinistrad, 128−163 (147; 5) long or 11−13% (12%; 5) of body length, 45−63 (53; 3) wide or 13−32% (23%; 3) of maximum body width, 2.8−5.8× (4.3; 3) proximal uterus length. Uterine egg ovoid, 80−95 (86; 4) long or 6−8% (7%; 4) of body length, 30−38 (35; 4) wide or 14−17% (16%; 4) of maximum body width, 2.3−2.7× (2.5; 4) longer than wide (Fig. 4). Common genital pore 63−138 (103; 6) or 6−11% (8%; 6) of body length posterior to middle of ventral sucker.

Excretory vesicle 120−180 (165; 6) long or 12−17% (14%; 6) of body length, 38−90 (74; 6) wide or 32−53% (44%; 6) of body width at caecal termination, with wall 5−15 (9; 4) thick (Figs. 3, 5).

Type and only known host: Chinese softshell turtle, *Pelodiscus sinensis* (Wiegmann, 1835) (Testudines: Trionychidae).

Type locality: Da Rang River Basin, Phu Yen Province, Vietnam.

Site in host: Heart, mesentery, lung.

Prevalence and intensity of infection: None of two *P. sinensis* collected from the Cai River Basin in Nha Trang yielded specimens; two (100%) *P. sinensis* from an aquaculture facility in the Da Rang River Basin had four and five specimens of *C. platti*.

Specimens/materials deposited: Holotype, United States National Museum (USNM) Coll. No. **XXXXX**; one paratype, United States National Museum (USNM) Coll. No. **XXXXX**; one paratype (Institute of Parasitology, Academy of Sciences of the Czech Republic, České Budějovice (IPCAS) Coll. No. **XXXXX**; GenBank Accession No. **XXXXXX**.

Material examined: *Hapalorhynchus gracilis* – American Museum of Natural History (AMNH) Coll. No. 125, holotype, one slide comprising one whole-mounted specimen, *ex. Chelydra serpentina* from North Judson, Indiana (Stunkard 1922); AMNH 1269, paratypes, four slides (three of the four labeled *Hapalorhychus gracilis*) comprising four whole-mounted specimens, *ex. Chelydra serpentina* from North Judson, Indiana (Stunkard 1922); *Vasotrema attenuatum* − AMNH 806, syntype, 17 slides comprising

25 whole-mounted specimens, *ex. Apalone ferox* and *A. spinifera* from Florida and Indiana respectively (Stunkard 1928). *Hapalorhynchus rugatus* − HWML 21339.

Etymology: The specific epithet *platti* honors our friend and colleague Professor Thomas R. Platt (Professor Emeritus, Saint Mary's College, Notre Dame, Indiana) for his extensive, sustained contributions to the taxonomy and systematics of turtle blood flukes.

Remarks. *Coeuritrema platti* is most similar to *C. rugatus* by the combination of having ventrolateral tegumental papillae restricted to the hindbody as well as a hindbody <1.6× forebody width. The new species is most easily distinguished from *C. rugatus* by having small ventrolateral tegumental papillae (35 μm maximum base width), testes without deep lobes, and a Laurer's canal pore opening posterior to the vitelline reservoir and dorsal to the oviducal seminal receptacle. *Coeuritrema rugatus* has large ventrolateral tegumental papillae (53 μm maximum base width), deeply lobed testes, and a Laurer's canal pore opening anterior to the vitelline reservoir and oviducal seminal receptacle. *Coeuritrema platti* differs from *C. lyssimus* by having a narrow hindbody (1.1−1.4× forebody width), ventrolateral tegumental papillae restricted to the hindbody, a short cirrus sac extending 7−9% of body length, a transverse ovary buttressing the caeca, a short, wholly pre-ovarian metraterm (11-13% of body length), and a submarginal genital pore. *Coeuritrema lyssimus* has a wide hindbody (1.7× forebody width), ventrolateral tegumental papillae distributing from the oral sucker to the excretory pore, a long cirrus sac extending 10−13% of body length, a sinistral ovary that does not buttress the caeca, a metraterm that begins lateral to the ovary and extends anteriad 17−18% of the body length, and a marginal genital pore.

Molecular phylogenetic results (Fig. 7)

The results of our phylogenetic analysis of the D1–D3 domains of the nuclear large subunit ribosomal DNA (28S) produced three clades: the chondrichthyan blood fluke *Chimaerohemecus trondheimensis* van der Land, 1967, the blood flukes of bony fishes (*Aporocotyle spinosicanalis* Williams, 1958, *Plethorchis acanthus* Martin, 1975, and *Neoparacardicola nasonis* Yamaguti, 1970), and the TBFs; including the enigmatic crocodilian blood fluke *Griphobilharzia amoena* (Fig. 7). Within TBFs, and as already reported elsewhere (Snyder 2004; Orélis-Ribeiro et al. 2014; Roberts et al. *in press*), the marine (*Carettacola* Manter et Larson, 1950 clade) and freshwater TBFs clustered separately and with high nodal support. Within the freshwater TBFs, we recovered separate *Hapalorhynchus* and *Spirorchis* clades. The new species was sister to *Hapalorhynchus* spp., and that clade was sister to *G. amoena*. The phylogenetic distance (branch lengths) within the *Hapalorhynchus* clade supported our conclusions from morphology in that *Coeuritrema* is distinctive from other accepted TBF genera. Additional sequences from *Enterohaematotrema* and *Cardiotrema* are required to test monophyly of Coeuritrematinae (see above).

DISCUSSION

Our morphological descriptions and comparisons coupled with our molecular phylogenetic results indicated that *Coeuritrema* shares a recent common ancestor with

Hapalorhynchus (Fig. 7). However, definitive host ecology, phylogenetic affiliation, and geographic distribution do not explain the observed sister-taxa relationships. In specific, both *H. gracilis* (type species) and *H. foliorchis* infect the common snapping turtle, *Chelydra serpentina*, (Testudines: Chelydridae); whereas, *C. platti* infects *P. sinensis* (Trionychidae). *Chelydra serpentina* and *P. sinensis* are assigned to different families, and those families are not phylogenetically closely related (Guillon et al. 2012, Crawford et al. 2015). Moreover, none of these TBFs nor their turtle hosts have overlapping geographic distributions: *H. gracilis* and *H. foliorchis* range in North America only; whereas, *C. platti* ranges in Vietnam only (Table 1). Excluding definitive host ancestry and biogeography, we predict that knowledge of the life cycles of species of *Hapalorhynchus*, *Coeuritrema,* and *Griphobilharzia* could help explain the observed topology. Unfortunately, no life cycle is known for any species of the *Hapalorhynchus* clade, precluding a deeper discussion of this matter herein. Orélis-Ribeiro et al. (2014) concluded that blood fluke clades can be identified by their molluscan intermediate hosts, with marine flukes infecting bivalves plus freshwater and estuarine flukes infecting snails. Additional morphological, life history, and sequence data sourced from the other 17 accepted species of *Hapalorhynchus* and additional species of *Coeuritrema* are required to further test the phylogenetic pattern we recovered herein (Fig. 7).

Especially noteworthy herein is the sister relationship between *G. amoena* and the *Coeuritrema* + *Hapalorhynchus* clade. Morphologically, *G. amoena* appears as a schistosome (although having a single testis and lacking fused caeca), with markedly distinctive morphological features that do not intuitively align it with *Coeuritrema* nor

Hapalorhynchus or other TBFs for that matter. *Griphobilharzia* resembles schistosomatids by dioecity and having a ventral sucker (Platt et al. 1991, Platt et al. 2013). Further, by having a well-developed gynaecophoric canal (see Khalil 2002), it resembles several genera of Schistosomatinae Stiles et Hassall, 1898: *Schistosoma* Weinland, 1858, *Ornithobilharzia* Odhner, 1912, *Austrobilharzia* Johnston, 1917, *Macrobilharzia* Travassos, 1922, *Schistosomatium* Tanabe, 1923, *Heterobilharzia* Price1929, *Bivitellobilharzia* Vogel et Minning, 1940, and *Orientobilharzia* Dutt et Srivastava, 1955. The stark discordance between comparative morphology and gene sequence analysis reiterate the need for additional molecular gene sequence data from specimens identified as *G. amoena*. *Griphobilharzia amoena* differs from all other TBFs by being dioecious and by maturing in the freshwater crocodile, *Crocodylus johnstoni* Krefft, 1873. *Griphobilharzia* resembles all TBFs, except *Baracktrema* (single caecum), *Neospirorchis* Price, 1934 (fused caeca), and *Unicaecum* Stunkard, 1925 (single caecum), by having two non-fused caeca. *Griphobilharzia* resembles *Baracktrema*, *Neospirorchis*, *Unicaecum*, *Uterotrema* Platt et Pichelin, 1994, and *Vasotrema* by having a single testis.

Regarding the systematics of blood flukes *sensu lato*, for now, this clade as well as the marine TBF clade (*Carettacola*, *Hapalotrema* Looss, 1899, and *Learedius* Price, 1934), must continue to remain in systematic limbo, without a familial assignment (Looss 1899, Price 1934, Manter and Larson 1950, Orélis-Ribeiro et al. 2014, Roberts et al. *in press*).

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LITERATURE CITED

Alves R.R.N., Vieira W.L.S., Santana G.G. 2008: Reptiles used in traditional folk medicine: conservation implications. Biodivers. Conserv. 17: 2037−2049.

Belous E.V. 1963: [Helminth fauna of water turtles, *Amyda sinensis*, of the Far East. Helminthologia.] 4: 79−99. (In Russian)

Bourgat R. 1990: Extension taxonomique et biogeographique du genre *Hapalorhynchus* (Trematoda, Spirorchiidae). Bull. Soc. Fran. Parasitol. 8: 289−294.

Brooks D.R., Mayes M.A. 1975: Platyhelminths of Nebraska turtles with descriptions of two new species of spirorchiids (Trematoda: Spirorchiidae). J. Parasitol. 61: 403−406.

Brooks D.R., Mayes M.A. 1976: *Telorchis gutturosi* sp. n. (Trematoda: Telorchiidae) from *Graptemys pseudogeographica* Gray in Nebraska, with reports of additional species of trematodes from Nebraska turtles. J. Parasitol. 62: 901−905.

Brooks D.R., Sullivan J.J. 1981. *Hapalorhynchus rugatus* sp. nov. (Digenea: Spirorchidae) from a Malaysian freshwater turtle. Can. J. Zool. 59: 1335−1338.

Bullard S.A., Jensen K., Overstreet R.M. 2009. Historical account of the two family-group names in use for the single accepted family comprising the “fish blood flukes.” Acta Parasit. 54(1): 78−84.

Bullard S.A., Overstreet R.M. 2006. *Psettarium anthicum* sp. n. (Digenea: Sanguinicolidae) from the heart of cobia *Rachycentron canadum* (Rachycentridae) in the northern Gulf of Mexico. Folia Parasit. 53: 117−124.

Bullard S.A., Overstreet R.M. 2008. Chapter 14: Digeneans as enemies of fishes. In: J. Eiras, H. Segner, T. Wahil, B.G. Kapoor (Eds.), Fish Diseases. Science Publishers, New Hamphsire, USA, 817-976 pp.

Bullard S.A., Overstreet R.M., Carlson, J.K. 2006. *Selachohemecus benzi* n. sp. (Digenea: Sanguinicolidae) from the blacktip shark *Carcharhinus limbatus* in the northern Gulf of Mexico. Syst. Parasitol. 63: 143−154.

Bullard S.A., Jensen K. 2008. Blood flukes (Digenea: Aporocotylidae) of stingrays (Myliobatiformes: Dasyatidae): *Orchispirium heterovitellatum* from *Himantura imbricata* in the Bay of Bengal and a new genus and species from *Dasyatis sabina* in the Northern Gulf of Mexico. J. Parasitol. 94: 1,311−1,321.

Byrd E.E. 1939: Studies on the blood flukes of the family Spirorchidae. Part II. Revision of the family and description of new species. J. Tenn. Acad. Sci. 14: 116−161.

Castresana J. 2000: Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol. Biol. Evol. 17: 540−552.

Crawford N.G., Parham J.F., Sellas A.B., Faircloth B.C., Glenn T.C., Papenfuss T.J., Henderson J.B., Hansen M.H., Simison W.B. 2015: A phylogenomic analysis of turtles. Mol. Phylogenet. Evol. 83: 250−257.

Darriba D., Taboada G.L., Doallo R., Posada D. 2012: jModelTest 2: more models, new heuristics and parallel computing. Nat. Methods. 9: 772.

Dwivedi M.P. 1967: Contribution to the family Spirorchiidae Stunkard, 1921 (Digenea: Trematoda). Ind. J. Helminthol. 19: 1−14.

Fritz U., Gong S., Auer M., Kuchling G., Schneeweif, Hundsdörfer A. K. 2010: The world's economically most important chelonians represent a species complex (Testudines: Trionychidae: *Pelodiscus*). Org. Divers. Evol. 10: 227−242.

Guillon J., Guéry L., Hulin V., Girondot M. 2012: A large phylogeny of turtles (Testudines) using molecular data. Contr. Zool. 81: 147−158.

Guindon S., Gascuel O. 2003: A simple, fast and accurate method to estimate large phylogenies by maximum-likelihood. Syst. Biol. 52: 696−704.

Haitao S., Parham J.F., Zhiyong F., Meiling H., Feng Y. 2008. Evidence for the massive scale of turtle farming in China. Oryx. 42: 147−150.

Huelsenbeck J.P., Ronquist F. 2005: Bayesian analysis of molecular evolution using MrBayes. In: R. Nielsen (Ed.), Statistical methods in molecular evolution. Springer Verlag, New York, USA, 183−232 pp.

Huelsenbeck J.P., Ronquist F., Nielsen R., Bollback J.P. 2001: Bayesian inference of phylogeny and its impact on evolutionary biology. Science. 294: 2310−2314.

Hughes R.C., Higginbotham J.W., Clary J.W. 1942: The trematodes of reptiles, Part I. Am. Mid. Nat. 27: 109−134.

International Commission on Zoological Nomenclature (ICZN). 2000: International Code of Zoological Nomenclature, 4th Ed., 306 pp.

Katoh K., Toh. H. 2010: Parallelization of the MAFFT multiple sequence alignment program. Bioinformatics. 26: 1899−1900.

Khalil L.F. 2002: Family Schistosomatidae Stiles & Hassall, 1898. In: D. I. Gibson, A. J. Jones, and R. A. Bray (Eds.), Keys to the Trematoda, Vol. 1. CABI, Wallingford, 419−432 pp.

Lamothe-Argumedo R. 1978: Trematodes de reptiles 1. description de una especie nueve de la familia Spirorchidae, parásita de *Kinosternon* leucostomum de Villahermosa, Tabasco, México. An. Inst. Biol. Univ. Nal. Autón. 49: 19−24.

Lockyer A.E., Olson P.D., Ostergaard P., Rollinson D., Johnston D.A., Attwood S.W., Southgate V.R., Horak P., Snyder S.D., Le T. H., Agatsuma T., McManus D.P., Carmichael A.C., Naem S., Littlewood D.T.J. 2003: The phylogeny of the Schistosomatidae based on three genes with emphasis on the interrelationships of *Schistosoma* Weinland, 1858. Parasitology. 126: 203−224.

Loftin H. 1960: An annotated check-list of trematodes and cestodes and their vertebrate hosts from northwest Florida. Q. J. Fla. Acad. Sci. 23: 302−314.

Looss A. 1899: Weitere beiträge zur kenntnis der Trematoden-Fauna Aegyptens, zugleich versuch einer natürlichen Gleiderung des genus *Distomum* Retzius. Zool. Jahrb. 12: 521−784.

Luhman M. 1935: Two new trematodes from the loggerhead turtle (*Caretta caretta*). J. Parasitol. 21: 274−276.

Manter H.W., Larson M.I. 1950: Two new blood flukes from a marine turtle, *Caretta caretta*. J. Parasitol. 36: 595−599.

Mehra H.R. 1933: New blood flukes of the family Spirorchidae Stunkard from Indian fresh-water tortoises with discussion on the synonymy of certain genera and the relationships of the families of blood flukes. Part I. Bull. Acad. Sci. United Prov. Agra Oudh, India. 2: 203−225.

Mehra H.R. 1934: New blood flukes of the family Spirorchidae Stunkard from Indian fresh-water tortoises with discussion on the synonymy of certain genera and the relationships of the families of blood flukes. Part II. Bull. Acad. Sci. United Prov. Agra Oudh, India. 3: 169−196.

Mehra H.R. 1939: New blood flukes of the family Spirorchidae Stunkard (Trematoda) from the marine turtle *Chelone mydas* of the Arabian sea with observations on the synonymity of certain genera and classification of the family. Proc. Nat. Acad. Sci. India. 9: 155−167.

Mehra H.R. 1940: A new distome *Enterohaematotrema* n. g. and a new blood fluke *Hemiorchis bengalensis* n. sp. belonging to the family Spirorchidae Stunkard, and a new species of the genus *Dendritobilharzia* Skrjabin and Zakharow belonging to the family Schistosomatidae Poche, with remarks on the evolution of the blood flukes. Proc. Nat. Acad. Sci. India. Section B, Biol Sci. 10: 100−118.

Mehrotra V. 1973: Digenea from some reptile hosts in India, Part II (in continuation with Abstract No. 286 in Part III of the Proceedings). Proc. Sixtieth Ind. Sci. Cong. 4: 46−47.

Miller M.A., Pfeiffer W., Schwartz T. 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. Proceedings of the Gateway Computing Environments Workshop (GCE). New Orleans, Louisiana, 8 pp.

Orélis-Ribeiro R., Arias C.R., Halanych K.M., Cribb T.H., Bullard S.A. 2014: Diversity and ancestry of flatworms infecting blood of nontetrapod craniates "fishes." Adv. Parasitol. 85: 1−64.

Oschmarin P.G. 1971: [A new species of trematode from the blood vessels of Chelonia.] In: Parazity Zhivotnykh I Rasternii Dal'nego Vostoka, Vladivostok, USSR, Dal'nevostochnoe Knizhnoe Izdatel'stvo, 142−143 pp. (In Russian)

Platt T.R. 1988: *Hapalorhychus brooksi* sp. n. (Trematoda: Spirorchiidae) from the snapping turtle (*Chelydra serpentina*), with notes on *H. gracilis* and *H. stunkardi*. Proc. Helminthol. Soc. Wash. 55: 317−323.

Platt T.R. 1993: Taxonomic revision of *Spirorchis* MacCallum, 1919 (Digenea: Spirorchidae). J. Parasitol. 79: 337−346.

Platt T.R. 1998: Redescription of *Hapalotrema mistroides* (Monticelli, 1896) and *Hapalotrema synorchis* Luhman, 1935 (Digenea: Spirorchidae), with comments on other species in the genus. J. Parasitol. 84: 594−600.
Platt T.R. 2002: Family Spirorchiidae Stunkard, 1921. In: D. I. Gibson, A. J. Jones, and R. A. Bray (Eds.), Keys to the Trematoda, Vol. 1. CABI, Wallingford, 453−467 pp.

Platt T.R., Blair D., Purdie J., Melville L. 1991: *Griphobilharzia amoena* n. gen., n. sp. (Digenea: Schistosomatidae), a parasite of the freshwater crocodile *Crocodylus johnstoni* (Reptilia: Crocodylia) from Australia, with the erection of a new subfamily, Griphobilharzinae. J. Parasitol. 77: 65−68.

Platt T.R., Hoberg E.P., Chisholm L.A. 2013: On the morphology and taxonomy of *Griphobilharzia amoena* Platt and Blair, 1991 (Schistosomatoidea), a dioecious digenetic trematode parasite of the freshwater crocodile, *Crocodylus johnstoni*, in Australia. J. Parasitol. 99: 888−891.

Platt T.R., Prestwood A.K. 1990: Deposition of type and voucher material from the helminthological collection of Elon E. Byrd. Syst. Parasitol. 16: 27−34.

Platt T.R., Snyder S.D. 2007: Redescription of *Hapalorhynchus reelfooti* Byrd, 1939 (Digenea: Spirorchiidae) from *Sternotherus odoratus* (Latreille, 1801). Comp. Parasitol. 74: 31−34.

Platt T.R., Sharma R.S.K. 2012: Two new species of *Hapalorhynchus* (Digenea: Spirorchiidae) from freshwater turtles (Testudines: Geoemydidae) in Malaysia. Comp. Parasitol. 79: 202−207.

Posada D., Buckley T.R. 2004: Model selection and model averaging in phylogenetics: advantages of Akaike Information Criterion and Bayesian approaches over likelihood ratio tests. Syst. Biol. 53: 793−808.

Price E.W. 1934: New genera and species of blood flukes from a marine turtle, with a key to the genera of the family Spirorchidae. J. Wash. Acad. Sci. 24: 132−141.

Rambaut A. 2009: FigTree v1.2.3, Institute of Evolutionary Biology, Univ. of Edinburgh. Available at: http://tree.bio.ed.ac.uk/software/figtree.

Rambaut A., Drummond A.J. 2009: Tracer Version 1.5. Program. Available at: http://beast.bio.ed.ac.uk/Tracer/.

Roberts J.R., Platt T.R., Orélis-Ribeiro R., Bullard S.A. 2016: New genus of blood fluke (Digenea: Schistosomatoidea) from Malaysian freshwater turtles (Geoemydidae) and its phylogenetic position within Schistosomatoidea. J. Parasitol. 102: (in press).

Rohde K., Lee S. K., Lim H.W. 1968: Ueber drei malayische Trematoden. Ann. Parasitol. Hum. Comp. 43: 33−43.

Ronquist F., Huelsenbeck J.P. 2003: MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics. 19: 1572−1574.

Shrivastava P.S. 1959: Cercaria of *Enterohaematotrema palaeorticum* Mehra, 1940. Proc. First All-India Cong, Zool. 2: 460−465.

Smith J.W. 1997a: The blood flukes (Digenea: Sanguinicolidae and Spirorchidae) of cold-blooded vertebrates: Part I. A Review of the literature published since 1971, and bibliography. Helminthol. Abstr. 66: 255−294.

Smith J.W. 1997b: The blood flukes (Digenea: Sanguinicolidae and Spirorchidae) of cold-blooded vertebrates: Part II. Appendix I: Comprehensive parasite-host list; Appendix II: Comprehensive host-parasite list. Helminthol. Abstr. 66: 329−344.

Snyder S.D. 2004. Phylogeny and paraphyly among tetrapod blood flukes (Digenea: Schistosomatidae and Spirorchiidae). Int. J. Parasitol. 34: 1385−1392.

Stuckas H., Fritz U. 2011: Identity of *Pelodiscus sinensis* revealed by DNA sequences of an approximately 180-year-old type specimen and a taxonomic reappraisal of *Pelodiscus* species (Testudines: Trionychidae). J. Zool. Syst. Evol. Res. 49: 335−339.

Stunkard H.W. 1922: Two new genera of North American blood flukes. Am. Mus. Novit. 39: 1−8.

Stunkard H.W. 1926: A new trematode *Vasotrema amydae* n. g., n. sp., from the vascular system of the soft-shelled turtle, *Amyda*. Anat. Rec. 34: 165.

Stunkard H.W. 1928: Observations nouvelles sur les trématodes sanguicoles du genre *Vasotrema* (Spirorchidae) avec description des deux espèces nouvelles. Ann. Parasitol. Hum. Comp. 6: 303−320.

Takeuti E. 1942. New blood flukes of the family Spirorchidae from Japanese fresh-water tortoise and marine turtles. Japanese Journal of Medical Sciences, VI, Bact. Parasitol. 2: 161−174.

Tamura K.D., Peterson D., Peterson N., Stecher G., Nei M., Kumar S. 2011: MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28: 2731−2739.

Tandon V., Gupta N.K. 1982: On some blood flukes (Spirorchiidae: Coeuritrematinae) from freshwater chelonians in India. Proc. Ind. Acad. Sci. An. Sci. 91: 275−282.

Tandon V., Gupta, N.K. 1985: On the blood fluke, *Cardiotrema roparensis* Mehrotra, 1973 and validity of *C. longivesticulata* Dwivedi, 1967 (Spirorchiidae: Coeuritrematinae). Res. Bull. (Sci.) Panjab Univ. 36: 331−334.

Thapar G. S. 1933. A new blood fluke from an Indian tortoise, *Trionyx gangeticus*. J. Helminthol. 11: 163−168.

Tkach V., Snyder S.D., Vaughan J.A. 2009: A new species of blood fluke (Digenea: Spirorchiidae) from the Malayan box turtle, *Cuora amboinensis* (Cryptodira: Geoemydidae) in Thailand. J. Parasitol. 95: 743−746.

van Dijk P.P., Stuart B.L., Rhodin A.G.L. 2000: Asian Turtle Trade: Proceedings of a Workshop on Conservation and Trade of Freshwater Turtles and Tortoises in Asia--Phnom Penh, Cambodia, 1-4 December 1999. Chelonian Research Foundation, Lunenburg, Massachusetts, USA, 164 pp.

van Dijk P.P., Iverson J.B., Rhodin A.G. J., Shaffer H.B., Bour R. 2014: Turtles of the world, 7th Edition: Annotated checklist of taxonomy, synonymy, distribution with maps, and conservation status. Chel. Res. Mono. 5: 329−479.

Wall L.D. 1951: The life history of *Vasotrema robustum* (Stunkard, 1928), Trematoda: Spirorchiidae. Trans. Am. Microsc. Soc. 70: 173−184.

Yamaguti S. 1958: Systema helminihum. Volume 1, The digenetic trematodes of vertebrates, Part I. Interscience Publishers, New York, USA, 979 pp.

Yamaguti S. 1971: Synopsis of digenetic trematodes of vertebrates, Vol. I. Keigaku Publishing Company, Tokyo, Japan, 1,074 pp.

FIGURE LEGENDS

Figures 1−2. *Coeuritrema rugatus* (Brooks et Sullivan, 1981) comb. n. (Digenea: Schistosomatoidea) from the mesenteric vessels of *Amyda cartilaginea* (Boddaert) (Testudines: Trionychidae) from Sungei Jempol, Ulu Jempol, State of Negeri Sembilan, Malaysia. **Fig. 1.** Paratype (HWML Coll. No. 21339), ventral view. **Fig. 2.** Genitalia of paratype (HWML Coll. No. 21339), ventral view. *Abbreviations*: at − anterior testis; ave − anterior trunk of vasa efferentia; cb − caecal bifurcation; cgp − common genital pore; cs − cirrus sac; dc − dextral caecum; dln − dorsolateral nerve chord; ec − eversible cirrus; egg − egg in utero; ep − excretory pore; esv − external seminal vesicle; ev − excretory vesicle; isv − internal seminal vesicle; Lc − Laurer's canal; lvd − lateral vitelline collecting duct; mt − metraterm; nc − nerve commissure; od − oviduct; oe − oesophagus; og − oesophageal gland; os − oral sucker; oo − oötype; osr − oviducal seminal receptacle; ov − ovary; ph − pharynx; pp − *pars prostatica*; pt − posterior testis; pve − posterior trunk of vasa efferentia; sc − sinistral caecum; tvd − transverse vitelline duct; ut − uterus; vd − vas deferens; vlp − ventrolateral tegumental papillae; vr − vitellarium; vrs − vitelline reservoir; vs − ventral sucker; vt − vitelline duct.

Figures 3−4. *Coeuritrema platti* sp. n. (Digenea: Schistosomatoidea) from viscera of *Pelodiscus sinensis* (Wiegmann) (Testudines: Trionychidae) from Da Rang River Basin, Vietnam. **Fig. 3.** Holotype (USNM Coll. No. **XXXXXX**), dorsal view. **Fig. 4.** Genitalia of holotype (USNM Coll. No. XXXXXX), dorsal view. *Abbreviations*: at − anterior testis; ave − anterior trunk of vasa efferentia; cb − caecal bifurcation; cgp − common genital pore; cs − cirrus sac; dc − dextral caecum; ec − eversible cirrus; egg − egg in utero; ep − excretory pore; esv − external seminal vesicle; ev − excretory vesicle; isv − internal seminal vesicle; Lc − Laurer's canal; lvd − lateral vitelline collecting duct; mt − metraterm; nc − nerve commissure; od − oviduct; oe − oesophagus; og − oesophageal gland; os − oral sucker; osr − oviducal seminal receptacle; ov − ovary; ph − pharynx; pp − *pars prostatica*; pt − posterior testis; pve − posterior trunk of vasa efferentia; sc − sinistral caecum; tvd − transverse vitelline duct; vd − vas deferens; vln − ventrolateral nerve chord; vlp − ventrolateral tegumental papillae; vr − vitellarium; vrs − vitelline reservoir; vs − ventral sucker; vt − vitelline duct.

Figures 5−6. *Coeuritrema platti* sp. n. (Digenea: Schistosomatoidea) from viscera of *Pelodiscus sinensis* (Wiegmann) (Testudines: Trionychidae) from Da Rang River Basin, Vietnam. **Fig. 5.** Paratype (USNM **XXXXXX**), ventral view. **Fig. 6.** Genitalia of paratype (USNM **XXXXXX**), ventral view. *Abbreviations*: at − anterior testis; ave − anterior trunk of vasa efferentia; cb − caecal bifurcation; cgp − common genital pore; cs − cirrus sac; dc − dextral caecum; ec − eversible cirrus; ep − excretory pore; esv − external seminal vesicle; ev − excretory vesicle; isv − internal seminal vesicle; Lc − Laurer's canal; lvd − lateral vitelline collecting duct; mt − metraterm; nc − nerve commissure; od − oviduct; oe − oesophagus; og − oesophageal gland; os − oral sucker; oo − oötype; osr − oviducal seminal receptacle; ov − ovary; ph − pharynx; pp − *pars prostatica*; pt − posterior testis; pve − posterior trunk of vasa efferentia; sc − sinistral caecum; tvd − transverse vitelline duct; ut − uterus; vd − vas deferens; vln − ventrolateral nerve chord; vlp − ventrolateral tegumental papillae; vr − vitellarium; vrs − vitelline reservoir; vs − ventral sucker; vt − vitelline duct.

Figure 7. Phylogenetic relationships of blood flukes reconstructed by Bayesian inference and based on partial D1−D3 domains of 28S from 18 taxa (13 TBFs, one crocodilian blood fluke, four fish blood flukes). Numbers aside tree nodes indicate posterior probability. Definitive hosts are indicated by icons aside tree nodes.

Table 1. Blood flukes infecting softshell turtles (Testudines: Trionychidae)

Turtle	Fluke	Site in host	Locality	Museum nos.	Reference
Amyda cartilaginea, Asiatic softshell	***Coeuritrema rugatus* comb. n.**	mesenteric blood vessels	Sungei Jempol, Ulu Jempol, State of Negeri Sembilan, West Malaysia	HWML 21339	Brooks and Sullivan (1981)
Apalone ferox, Florida softshell	*Vasotrema amydae* (type species)	blood	probably a Gulf of Mexico river drainage, Florida, USA	AMNH 791	Stunkard (1926)
		blood	probably a Gulf of Mexico river drainage, Florida, USA	AMNH 791	Stunkard (1928)
	Vasotrema attenuatum	blood	probably a Gulf of Mexico river drainage, Florida, USA	AMNH 806, 807	Stunkard (1928)
	Vasotrema sp.	not specified	Apalachicola River, Franklin County, Florida, USA	not specified	Loftin (1960)
Apalone mutica, smooth softshell	*Vasotrema attenuatum*	blood	Missouri River Drainage, Nebraska, USA	not specified	Brooks and Mayes (1975)
	Vasotrema brevitestis	blood	Missouri River, 2.4 km south of Blair, Nebraska, USA	USNM 73817−73819; HWML 20077	Brooks and Mayes (1975)
	Vasotrema robustum	blood	Missouri River Drainage, Nebraska, USA	not specified	Brooks and Mayes (1975)
Apalone spinifera, spiny softshell	*Vasotrema amydae* (type species)	blood	Ohio River Drainage, Indiana, USA	AMNH 791	Stunkard (1926)
		blood	Ohio River Drainage, Indiana, USA	AMNH 791	Stunkard (1928)
	Vasotrema attenuatum	blood	Ohio River Drainage, Indiana, USA	AMNH 806, 807	Stunkard (1928)
		mesenteric blood vessels	Reelfoot Lake, Tennessee, USA	HWML9227	Byrd (1939) (see also Platt and Snyder

					2007)
		not specified	Missouri River Drainage, Nebraska, USA	not specified	Brooks and Mayes (1975)
	Vasotrema brevitestis	not specified	Atkinson Lake, Atkinson Recreation Area, 0.8 km west of Atkinson, Nebraska, USA	USNM 73817−73819; HWML 20076	Brooks and Mayes (1975)
	Vasotrema longitestis	arterial circulation	Reelfoot Lake, Tennessee, USA	USNM 9229, 80656-8; HWML 31121-8 (see Platt and Prestwood 1990)	Byrd (1939)
	Vasotrema robustum	blood	Ohio River Drainage, Indiana, USA	AMNH 808, 809	Stunkard (1928)
		arterial system	Reelfoot Lake, Tennessee, USA	USNM 80657-2; HWML 31122-2 (see Platt and Prestwood 1990)	Byrd (1939)
		heart, large blood vessels	Huron River, Washtenaw County, Michigan, USA	USNM 37306	Wall (1951)
		not specified	Missouri River Drainage, Nebraska, USA	HWML 20075	Brooks and Mayes (1975)
		not specified	Nishnabotna River, Floyd County, Iowa, USA	HWML 45795	Snyder (2004)
Dogania subplana, Malayan softshell turtle	*Hapalorhynchus macrotesticularis*	heart, arteries near heart	unknown drainage, Kuala Lumpur (purchased from Chinese merchant), Malaysia	Helminthological Coll. No. R. 769, Zoology Dept., University of Malaysia, Kuala Lumpur	Rohde et al. (1968)
Lissemys punctata, Indian flapshell	*Cardiotrema roparensis*	not specified	Sutlej River, Ropar, Punjab State, India	not specified	Mehrotra (1973)
		body wash, hepatic blood vessels	Sutlej River, Ropar, Punjab State; Yamuna River, Karnal, Haryana State, India	not specified	Tandon and Gupta (1985)
	Enterohaematotrema palaeorticum (type	small intestine	Ganges River, Allahabad, Uttar Pradesh	not specified	Mehra (1940)

species)	(probably mesenteric blood vessels)	State, India		
Coeuritrema lyssimus **(type species)**	ventricle of the heart (adult)	Ganges River, Allahabad, Uttar Pradesh State, India	not specified	Mehra (1933)
	heart (adult)	Ganges River Drainage, Rudrapur, Uttarakhand State, India	not specified	Tandon and Gupta (1982)
Hapalorhynchus odhnerensis	ventricle of heart	Ganges River, Allahabad, Uttar Pradesh State, India	not specified	Mehra (1933)
Hapalorhynchus sheilae	not specified	Patiala River (Ghaggar River Drainage), Patiala, Punjab State; Sutlej River, Ropar, Punjab State; Ghaggar River, Sangrur, Punjab State; Ganges River Drainage, Rudrapur, Uttarakhand State, India	not specified	Mehrotra (1973)
	heart, blood, liver	Ganges River Drainage, Rudrapur, Uttarakhand State; Patiala River (Ghaggar River Drainage), Patiala, Punjab State; Ghaggar River, Sangrur, Punjab State, India	not specified	Tandon and Gupta (1982)
Hapalorhynchus sutlejensis	not specified	Sutlej River, Ropar, Punjab State; Ghaggar River, Sangrur, Punjab State, India	not specified	Mehrotra (1973)
	ventricle of heart	Gomti River, Lucknow, Uttar Pradesh State;	not specified	Tandon and Gupta (1982)

			Ghaggar River, Sangrur, Punjab State, India		
Nilssonia gangeticus, Indian softshell	*Hapalorhynchus indicus*	heart, large blood vessels	Gomti River, Lucknow, Uttar Pradesh State, India	not specified	Thapar (1933)
Nilssonia hurum, Indian peacock softshell	*Enterohaematotrema palaeorticum* (type species)	intestine (probably mesenteric blood vessels)	pond in Kotah, Raipur, Madhya Pradesh State, India	not specified	Shrivastava (1959)
Pelodiscus sinensis, Chinese softshell	***Coeuritrema platti* sp. n.**	heart, mesentery, lung	Da Rang River, Phuyen Province, Vietnam	USNM XXXX	Present study
	Hapalorhynchus mica	hepatic blood vessels	Duong River, Hai Phong, Vietnam	not specified	Oschmarin (1971)
	Hapalorhynchus oschmarini	hepatic blood vessels	Lake Khanka, Russia	not specified	Belous (1963)

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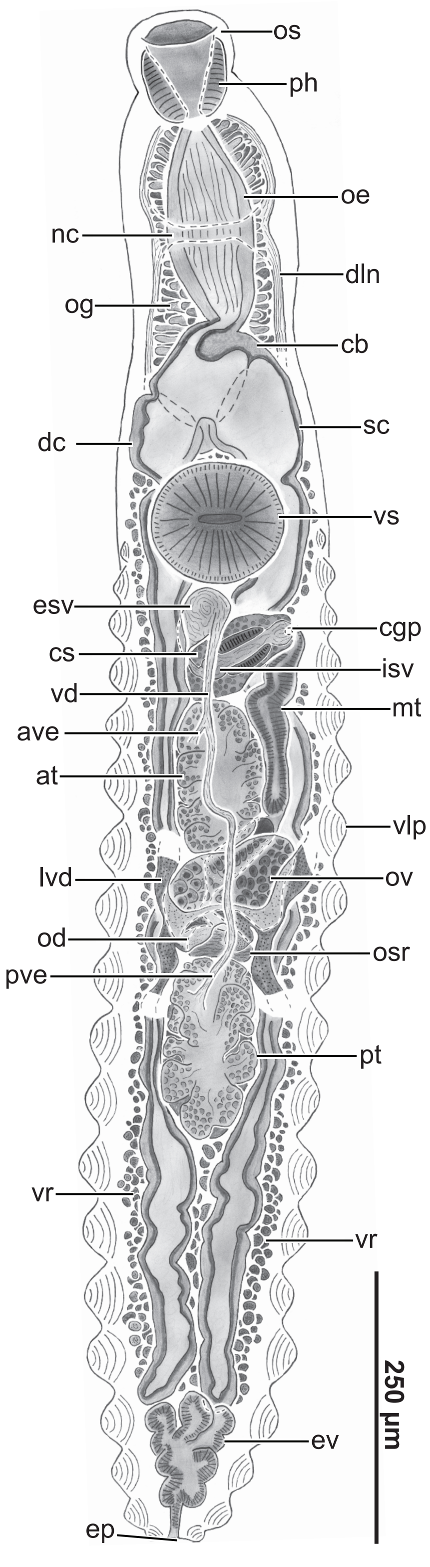
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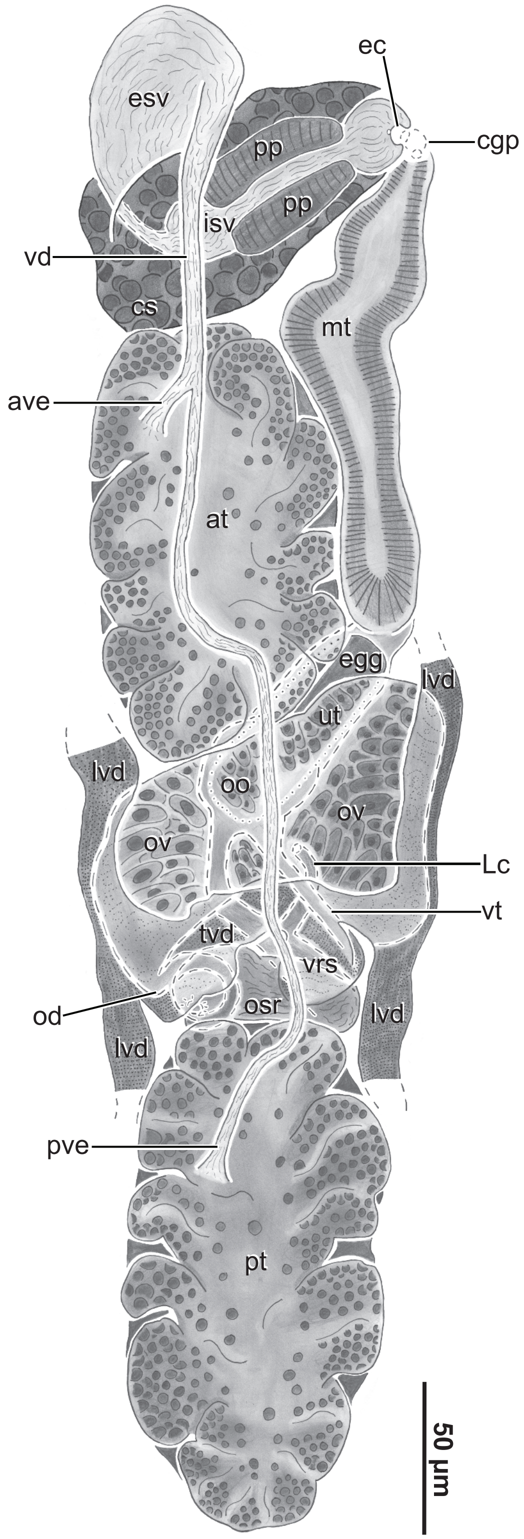
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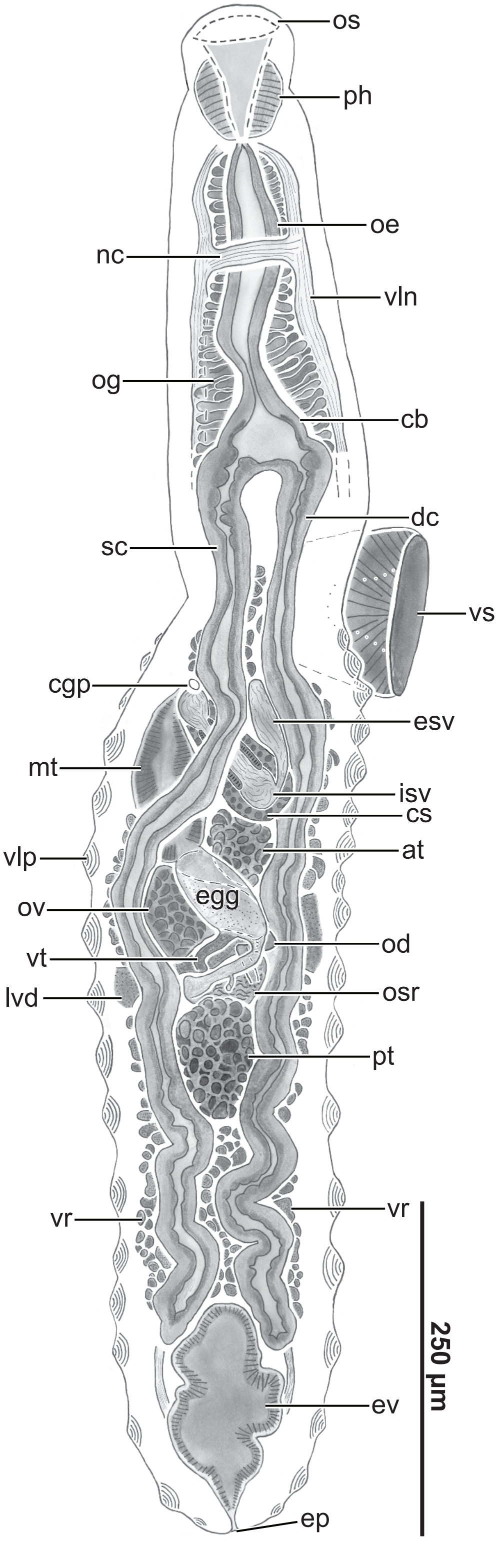
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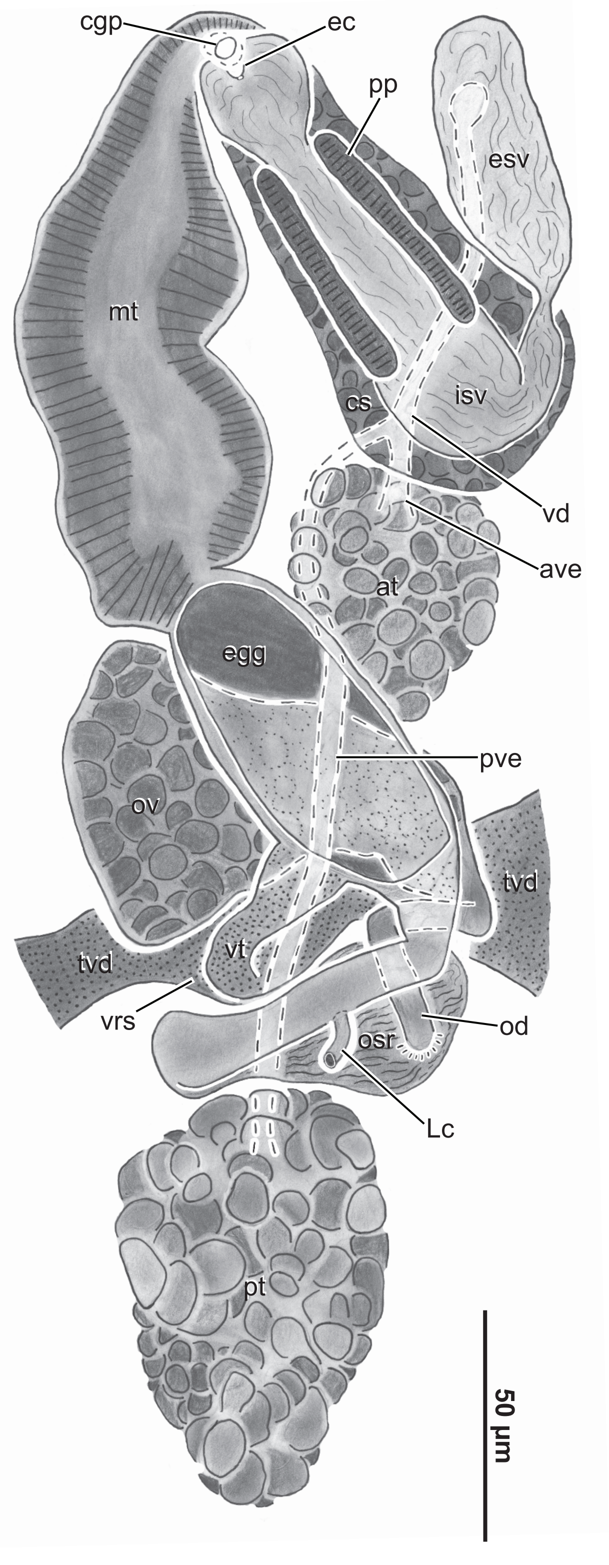
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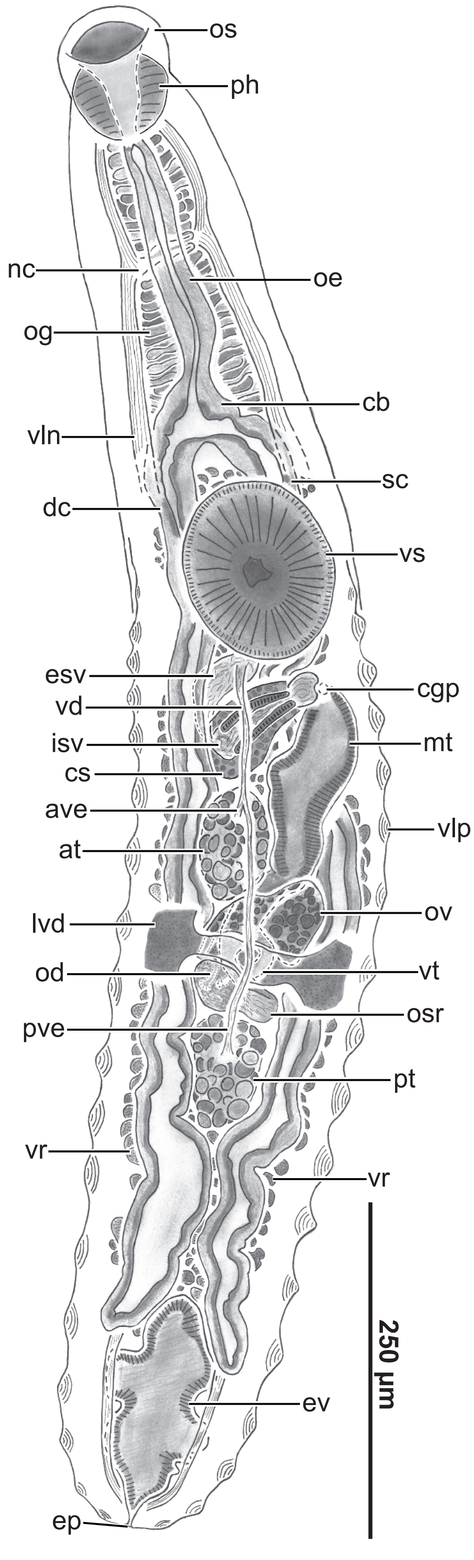

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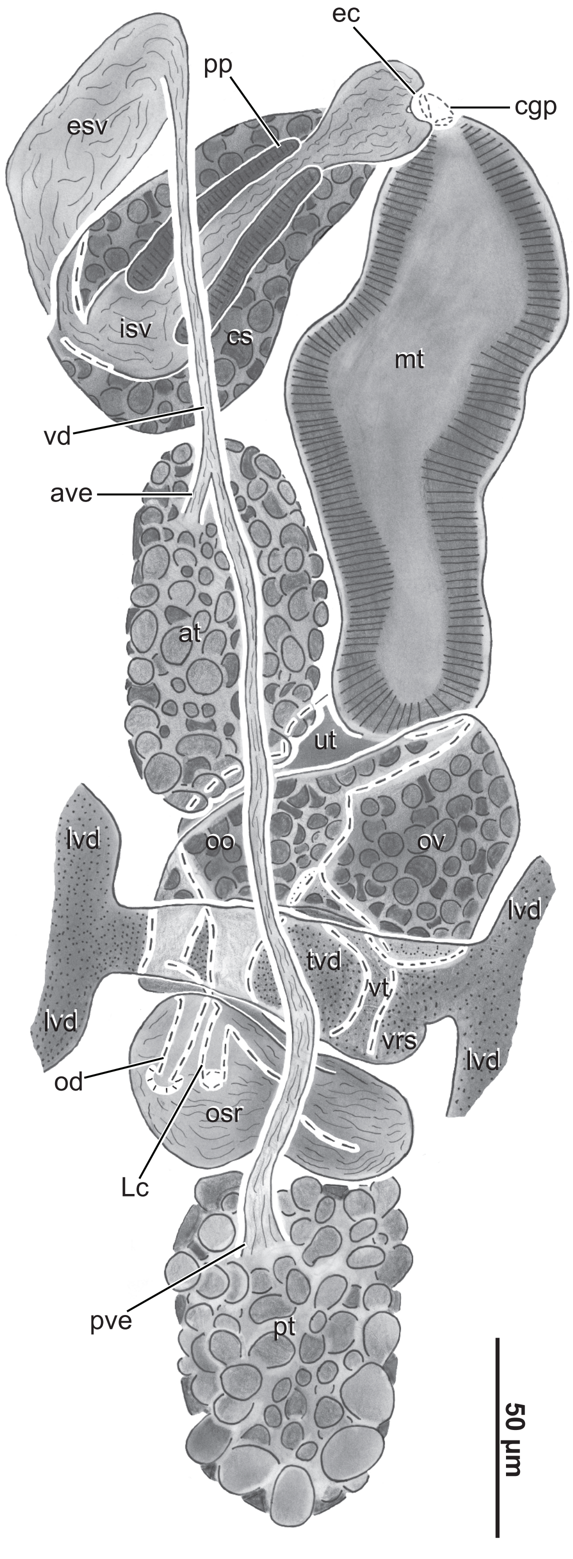

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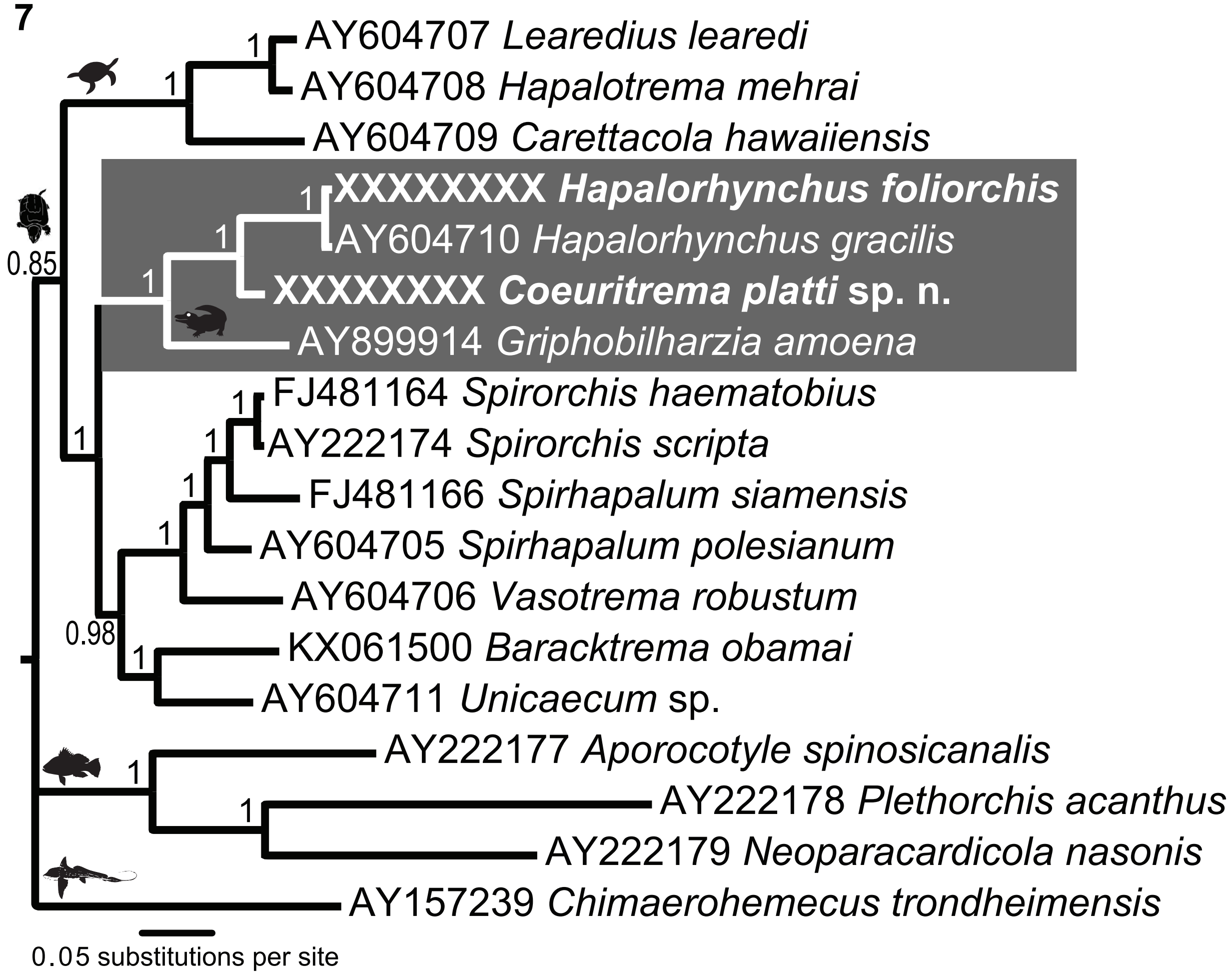
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XXXXXXXXX *Coeuritrema platti* sp. n.
AY899914 *Griphobilharzia amoena*
FJ481164 *Spirorchis haematobius*
AY222174 *Spirorchis scripta*
FJ481166 *Spirhapalum siamensis*
AY604705 *Spirhapalum polesianum*
AY604706 *Vasotrema robustum*
KX061500 *Baracktrema obamai*
AY604711 *Unicaecum* sp.
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CHAPTER 3: A NEW SPECIES OF *SPIRORCHIS* MACCALLUM, 1918 (DIGENEA: SCHISTOSOMATOIDEA) AND *SPIRORCHIS* CF. *SCRIPTA* FROM CHICKEN TURTLE, *DEIROCHELYS RETICULARIA*, (EMYDIDAE), WITH AN EMENDATION AND MOLECULAR PHYLOGENY OF *SPIRORCHIS*

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ABSTRACT

Chicken turtles, *Deirochelys reticularia* (Latreille *in* Sonnini and Latreille), (Testudines: Emydidae) from Salt Pond (31°10'13.88"N, 86°32'17.49"W; Yellow River, Alabama, USA) and Big Beaver Pond (32°25'44.03"N, 85°38'44.87"W; Tallapoosa River, Alabama, USA) were infected by *Spirorchis collinsi* Roberts et Bullard sp. n. and *Spirorchis* cf. *scripta*. The new species is most easily differentiated from its congeners by the combination of having caeca that extend far beyond the genitalia, intercaecal genitalia positioned in the middle portion of the body, a testicular column that nearly abuts the caecal bifurcation, a cirrus sac positioned between the testes and ovary, a massive Mehlis' gland, an elongate, longitudinal metraterm that extends anteriad beyond the level of the ovary, a pre-ovarian genital pore, and a prominent, intercaecal Manter's organ. The specimens of *S.* cf. *scripta* differed from the holotype and published descriptions of *Spirorchis scripta* Stunkard, 1923 by several subtle morphological features, perhaps comprising intraspecific variation, but collectively warranted a detailed description herein. Based on examinations of the aforementioned specimens plus the holotype, paratypes, and vouchers of morphologically-similar congeners, *Spirorchis* MacCallum, 1918 is emended to include the presence of oral sucker spines, a pharynx, lateral oesophageal diverticula (aka "plicate organ"), and a

median oesophageal diverticulum (aka “oeseophageal pouch”). Phylogenetic analysis of the nuclear large subunit rDNA (28S) recovered *S. collinsi* sister to *Spirorchis picta* Stunkard, 1923, >99% similarity between *S.* cf. *scripta* and *S. scripta*, and a monophyletic *Spirorchis* MacCallum, 1918. No blood fluke infection has been reported previously from these drainages, Alabama, or this turtle species. This is the first new species of *Spirorchis* to be described from North America in 26 years.

INTRODUCTION

The 22 turtle blood fluke (TBF) species ranging in North America are assigned to *Spirorchis* MacCallum, 1918, *Hapalorhynchus* Stunkard, 1922, *Unicaecum* Stunkard, 1925, and *Vasotrema* Stunkard, 1928. Collectively, they infect 16 freshwater turtle species: four infect snapping turtles (Chelydridae), ten infect pond turtles (Emydidae), three infect mud/musk turtles (Kinosternidae), and five infect softshell turtles (Trionychidae) (see Platt 1993, Smith 1997a, b, Platt 2002, Orélis-Ribeiro et al. 2014). One third (29 of 86; 34%) of the freshwater turtle species in North America range in rivers and lakes of Alabama (van Dijk et al. 2014, Guyer et al. 2015), and three of the 13 (23%) species of planorbid snails that range in Alabama (Johnson et al. 2013) are known to shed cercariae of *Spirorchis* spp. elsewhere (Wall 1939, 1940, 1941a, b, Pieper 1953, Goodchild and Kirk 1960, Holliman and Fisher 1968, Holliman et al. 1971). Yet, no TBF infection has been reported from a turtle or snail in Alabama, and few (11 of 29; 38%) turtles that occur there are known as TBF hosts elsewhere (Smith 1997a, b).

Parasitological examinations of two chicken turtles, *Deirochelys reticularia* (Latreille *in* Sonnini and Latreille) from Alabama rivers revealed infections by a new species of *Spirorchis* and *Spirorchis* cf. *scripta*. Herein, we describe these specimens and compare them to type materials of *Spirorchis innominatus* Ward, 1921 (type species), *Spirorchis artericola* (Ward, 1921) Stunkard, 1921, *Spirorchis haematobius* (Stunkard, 1922), Price, 1934, *Spirorchis elegans* Stunkard, 1923, *Spirorchis parvus* (Stunkard, 1923) Price, 1934, *Spirorchis picta* Stunkard, 1923, *Spirorchis scripta* Stunkard, 1923, *Spirorchis minutus* Byrd, 1939, and *Spirorchis kirki* (Platt, 1990) Platt, 1992. We also emend the diagnosis for *Spirorchis* and provide an updated phylogeny based on all available sequences representing members of the genus. This is the first report of a TBF from Alabama and from this turtle species as well as the first new species of TBF described from North America in 26 years, since *Spirorchis kirki* (Platt, 1990) Platt, 1992.

MATERIAL AND METHODS

Two chicken turtles (one juvenile [carapace 53.6 mm x 44.1 mm, plastron 47.1 mm x 34.0 mm]; one adult female [carapace 199.8 mm x 143.2 mm, plastron 193.0 mm x 116.9 mm]) were captured by hand from Salt Pond (31°10'13.88"N, 86°32'17.49"W; Yellow River, Alabama, USA) and by seine from Big Beaver Pond (32°25'44.03"N, 85°38'44.87"W; Tallapoosa River, Alabama, USA) on 5 and 17 March 2016, respectively. Seven pond sliders, *Trachemys scripta* (Thunberg *in* Schoepff), were captured by hand from earthen ponds for fish aquaculture at the EW Shell Fisheries Center (32°38'52.78"N, 85°29'7.20"W; Tallapoosa River drainage, Auburn University, Auburn, Alabama, USA) on 16 January 2015. Turtles were transported alive to the laboratory in a cooler with a small portion of pond water (carried within an air conditioned vehicle cab), decapitated immediately before necropsy, and examined with the aid of 7.0 g/L sodium citrate saline solution and a stereo-dissection microscope. Each host organ (brain, eye, heart, lung, spleen, liver, intestine, mesentery, kidney, rectum) was isolated in a glass container filled with saline. Portions of each organ then were excised and macerated in a petri dish while viewing under high magnification with the 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 or fallen from the excised organ/tissue. Living flukes were pipetted from saline dishes, concentrated in a clean glass dish with saline, rinsed in saline, pipetted onto glass slides, cover-slipped (only to keep the flukes from curling; no pressure exerted on specimen by coverslip), and killed with a 2-sec exposure to heat emitted from a butane hand lighter. After heat killing, a few drops of saline were applied to the edge of the coverslip before the coverslip was lifted carefully with fine forceps and the fluke was washed from the slide and into a clean dish of 5% neutral buffered formalin (n.b.f.). These flukes, intended for morphology, were held in 5% n.b.f. overnight, rinsed with distilled water, stained in Van Cleave's hematoxylin with several drops of Ehrlich's hematoxylin, dehydrated with a graded ethanol 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 in Canada balsam. Drawings were made with Leica DM2500 (Leica, Wetzler, Germany) and Leica DMR compound microscopes, each equipped with differential interference contrast optical components and a drawing tube. Measurements were obtained with a calibrated ocular micrometer (as straight-lines along the course of each duct) and are herein reported in micrometres (μm) followed by their mean and number measured in parentheses. For convenient comparison, measurements of the holotype (American Museum of Natural History [AMNH] Cat. No. 128) and paratype (AMNH 130) of *Spirorchis scripta* are indicated in brackets (and included in the treatment of *S.* cf. *scripta*), e.g., “[holotype measurement, paratype measurement],” immediately following the parentheses (“n/a” indicates not available due to poor specimen quality or a damaged specimen). Turtle scientific names and taxonomic authorities follow van Dijk et al. (2014). Classification and anatomical terms for TBFs follow Roberts et al. (2016a).

Specimens intended for molecular biology comprised the anterior end of one specimen of *Spirorchis* cf. *scripta* and the posterior end of one specimen of the new species. The remainder of each of specimen (posterior end for *S*. cf. *scripta*, anterior end for new species) was deposited as a voucher (hologenophore) in the United States National Museum (USNM Cat. Nos. **XXXXXX** and **XXXXXX**, respectively). Samples for molecular analyses were stored in a vial of absolute ethanol at -20°C. Total genomic DNA (gDNA) was extracted using the DNeasyTM Blood and Tissue Kit (Qiagen, Valencia, California, USA), except for the incubation period with proteinase-K that was extended to overnight and the final elution step wherein only 100 μl of elution buffer was used to increase the final DNA concentration in the eluate. Partial 28S rDNA (domains D1– D3; ~1,400 bp) was amplified using the forward primer “U178” (5'-GCA CCC GCT GAA YTT AAG-3') and the reverse primer “L1642” (5'-CCA GCG CCATCC ATT TTC A-3') (Lockyer et al. 2003). PCR amplifications were performed using a total volume of 25 µl containing approximately 2 µl of DNA template, 0.4 µM of each primer along with 1× buffer, 2.5 mM $MgCl_2$ (New England Biolabs, Ipswich, Massachusetts, USA), 1 mM dNTP mixture, and 0.3 µl Taq polymerase (5 U/µl) (New England Biolabs, Ipswich, Massachusetts, USA). Thermocycling profile consisted of an initial five min at 95°C for denaturation, followed by 40 repeating cycles of 94°C for 30 s for denaturation, 50°C for 30 s for annealing, and 72°C for two min for extension, followed by a final five min at 72°C for extension. All PCR reactions were carried out in a Veriti Thermal Cycler (Applied Biosystems, Waltham, Massachusetts, USA). PCR products (5 µl) were verified on a 1% agarose gel and stained with ethidium bromide. DNA Sanger sequencing was performed by Lucigen Corporation (Madison, Wisconsin, USA) using the same PCR primers. Sequence assembling and analysis of chromatograms were conducted using BioNumerics version 7.0 (Applied Maths, Sint-Martens-Latem, Belgium). Sequences were aligned with those of other blood flukes available on GenBank. The outgroup comprised *Baracktrema obamai* Roberts, Platt et Bullard, 2016 (KX061500) and *Unicaecum* sp. (AY604711). The ingroup comprised newly-generated sequence data from *Spirorchis* cf. *scripta* (**XXXXX**), the new species (**XXXXXX**) and *Spirorchis picta* (**XXXXX**) plus six other TBFs in GenBank (Table 1). Methods for sequence alignment and phylogenetic tree construction follow Roberts et al. (2016b).

The holotype and paratypes of the new species and vouchers of the redescribed species were deposited in the United States National Museum (USNM, Washington, D.C.) and the Institute of Parasitology (IPCAS, Academy of Sciences, České Budějovice, Czech Republic).

RESULTS

Spirorchis MacCallum, 1918, emended (Figs. 1−8)

Diagnosis: Body dorsoventrally flattened (not cylindrical), 3−10× longer than wide, aspinous, apapillate. Oral sucker spheroid, spinous or aspinous. Ventral sucker absent. Dorsolateral and ventrolateral nerve chords present. Pharynx present, enveloping anterior extremity of oesophagus. Oesophagus sinuous, extending posteriad <1/3 of body length, ventral to anterior nerve commissure; lateral oesophageal diverticula increasing in size and number posteriad; median oesophageal diverticulum present or absent; oesophageal gland surrounding oesophagus from posterior margin of pharynx to caecal bifurcation, strongly basophilic, widest at level immediately anterior to median oesophageal diverticulum or caecal bifurcation (Figs. 5, 6). Intestine inverse U-shaped, comprising paired caeca extending 2/3−9/10 of body length directly posteriad and in parallel with body margin, terminating in posterior end of body, smooth (lacking diverticula). Testes 4−11 in number, oblong, lobes present or absent, distributing in an intercaecal or postcaecal column, having abutting or overlapping anterior and posterior margins or relatively far separated from each other. Vas deferens extending posteriad ventral to testicular column, receiving laterally directed vasa efferentia at midline, extending posteriad from anterior testis; external seminal vesicle post-testicular, abutting posterior testis, intercaecal; internal seminal vesicle constricted medially, comprising proximal and distal swellings. Cirrus sac present, post-testicular; cirrus present. Ovary lobed or not, intercaecal or postcaecal, post-testicular. Oviduct emerging from posterior margin of ovary, extending posteriad; oviducal seminal receptacle comprising middle portion of oviduct, at level of or posterior to genital pore. Laurer's canal intercaecal or postcaecal, post-testicular, post-ovarian, extending posteriad from oviduct at level of vitelline reservoir, opening dorsally. Vitellarium follicular, occupying space from middle region of oesophagus to distal ends of caeca; transverse vitelline duct intercaecal, post-gonadal. Oötype diminutive, post-gonadal, intercaecal, anterior to transverse vitelline duct. Mehlis' gland present or indistinct in adults. Uterus partly or wholly comprising metraterm; metraterm post-gonadal or lateral to ovary. Uterine pouch absent. Uterine egg typically observed as a single egg occupying the oötype and uterus proximal to metraterm. Common genital pore ventral, opening ventral to sinistral caecum, lacking suckers. Excretory vesicle y-shaped; Manter's organ present or absent; excretory pore dorsal, subterminal or nearly terminal. In blood of North American chelydrids and emydids.

Differential diagnosis: Body 3−10× longer than wide, aspinous, apapillate. Ventral sucker absent. Pharynx present. Oesophagus <1/3 of body length; lateral oesophageal diverticula present; median oesophageal diverticulum present or absent. Intestine inverse U-shaped. Testes 4-11 in number, distributing in an intercaecal column. External seminal vesicle abutting posterior testis, intercaecal or postcaecal. Internal seminal vesicle constricted medially. Cirrus sac present, post-testicular. Ovary post-testicular. Oviducal seminal receptacle at level of or posterior to genital pore. Laurer's canal post-testicular, post-ovarian. Common genital pore ventral, opening ventral to sinistral caecum. Manter's organ present or absent.

Type species: *Spirorchis innominatus* Ward, 1921

Remarks

The correct date of publication for MacCallum's proposal of *Spirorchis* is 1918 (see Anonymous [1919], which confirms that MacCallum's work was printed in 1918). Previous authors (Platt 1992, 1993, 2002) have indicated that the date was 1919, but this is incorrect (Thomas R. Platt, personal communication).

We accept nine species of *Spirorchis*: *S. innominatus*, *S. artericola*, *S. haematobius*, *S. elegans*, *S. parvus*, *S. picta*, *S. scripta*, *S. minutus*, and *S. kirki*. These species can be coarsely grouped based upon features of the caeca, gonads, and genitalia: *S. scripta* and *S. elegans* have a testicular column that nearly abuts the caecal bifurcation and caeca that extend posteriad far beyond the genitalia; *S. innominatus* and *S. artericola* have a testicular column that nearly abuts the caecal bifurcation and caeca that extend posteriad only slightly beyond the genitalia; and the remaining species have a testicular column that is markedly posterior to the caecal bifurcation (*S. haematobius*, *S. picta*, *S. parvus*, *S. minutus*) or that is wholly post-caecal (*S. kirki*) plus caeca that do not extend posteriad far beyond the genitalia (Ward 1921, Stunkard 1922, 1923, Price 1934, Byrd 1939, Platt 1990, 1992).

Spirorchis most closely resembles *Spirhapalum* Ejsmont, 1927, *Plasmiorchis* Mehra, 1934, and *Carretacola* Manter et Larson, 1950 by having multiple testes typically distributing in an intercaecal column. *Spirorchis*, *Spirhapalum*, and *Plasmiorchis* are further similar to each other and unique among other TBFs by having lateral oesophageal diverticula and a median oesophageal diverticulum (Ward 1921, Stunkard 1923, Ejsmont 1927, Mehra 1934, Sinha 1934, Mehra 1939, Gupta and Mehrotra 1975, Platt 1993, 2002, Tkach et al. 2009). *Spirorchis* differs from *Carretacola* by lacking a ventral sucker and by having post-testicular female genitalia and a post-testicular genital pore. *Carretacola* has a ventral sucker, pre-testicular ovary, and pre-gonadal common genital pore. *Spirorchis* differs from *Plasmiorchis* and *Spirhapalum* by lacking a ventral sucker. In addition, it differs from *Spirhapalum* by lacking a post-ovarian testis and from *Plasmiorchis* by having more-or-less straight caeca that do not turn anteriad.

The diagnosis for *Spirorchis* herein is emended to include the presence of oral sucker spines, a pharynx, lateral oesophageal diverticula (aka "plicate organ"), and a median oesophageal diverticulum (aka "oeseophageal pouch"). Regarding the spines, previous authors have treated *Spirorchis* as comprising species that are aspinous (MacCallum 1918, Ward 1921, Stunkard 1922, Stunkard 1923, Platt 1993, 2002). We confirmed the lack of tegumental body spines in museum materials and newly collected specimens of various *Spirorchis* spp., but in some well-prepared specimens, i.e., *Spirorchis scripta* and *Spirorchis* sp. (Roberts and Bullard; unpubl. data), we observed minute (< 5 μm in total length), putative spines on the surface of the oral sucker. Although spinous anterior suckers are well-documented among fish blood flukes (Aporocotylidae) (Bullard et al. 2008, McVay et al. 2011, Bullard 2012, Bullard 2013, Truong and Bullard 2014), this is the first report of spines associated with the oral sucker of a TBF. Although materials at our disposal herein precluded an ultrastructural study of these minute spines, a follow-up study treating them is planned once additional specimens have been collected and prepared. Regarding the pharynx, like *Baracktrema*, *Unicaecum*, and *Coeuritrema* Mehra, 1933 (see Roberts et al. 2016a, b), a pharynx is present in *Spirorchis* spp. MacCallum (1918) diagnosed *Spirorchis* as having a pharynx but mislabeled it in the description of *S. innominatus* as the nerve commissure (Stunkard 1923). Further, we speculate that the pharynx was previously interpreted by some workers as part of the oral sucker, as it is dorsal to the mouth. Regarding the lateral oesophageal diverticula, species of *Spirorchis*, *Spirhapalum*, and *Plasmiorchis* (see Platt 2002) have an oesophagus with lateral diverticula (oesophageal lumen extending into each diverticulum) that become longer, more numerous, and more dense posteriad (Figs. 5, 6). These diverticula have been referred to variously but most commonly as comprising a plicate organ because they appear pleated (Ward 1921, Mehra 1934, Platt 1992, 1993, 2002). The association of the oesophageal gland and lateral oesophageal diverticula has perhaps caused some confusion. Based on the museum specimens we examined (Table 2), it seems likely that Stunkard (1923) mistook the "beaded appearance" of the lateral oesophageal diverticula for an oesophageal gland, and, likewise, we suspect that Mehra's (1934) "plicated lumen" refers to the lateral oesophageal diverticula. Apparently, Rohde et al. (1968) mistook the lateral oesophageal diverticula as latitudinal muscle striation. Regarding the median oesophageal diverticulum, Ward (1921) first described this oesophageal outpocketing as a "median diverticulum." Noteworthy also is that the same structure has been referred to as "median pocket" (Stunkard 1923), "petite poche" (Ejsmont 1927), "oesophageal vesicle" (Mehra 1934, Gupta and Mehrotra 1975), "pocket-like structure" (Sinha 1934), and "median oesophageal pouch" (Platt 1992, 1993, 2002). We suggest the term "median oesophageal diverticulum" because Ward's (1921) description was the first to identify and name the structure and because we suspect that this feature may be homologous with the lateral oesophageal diverticula.

Interpreting the distal portion of the female genitalia, i.e., differentiating the oötype, thin-walled proximal portion of the uterine tract, and distal metraterm, is challenging in gravid specimens because the presence of the large egg blurs definition of these structures in whole-mounted specimens viewed with light microscopy. This is taxonomically problematic because gravid adults of *Spirorchis* spp. are common (Roberts pers. obs.). MacCallum (1918) did not discern an oötype or uterus in the gravid holotype of the type species, *S. innominatus*. Ward (1921) studied gravid specimens of *S. artericola* and stated that, “the egg lies really in the ootype and a true uterus is lacking.” Later, Stunkard (1923), Byrd (1939), and Ulmer (1959) histologically differentiated the proximal portion of the uterus from the metraterm in species of *Spirorchis*. These structures probably do comprise a functional “egg chamber” (Roberts et al. 2016b) as in *S. elegans* and *S. scripta*; however, *S. picta* and the new species described herein have a uterus comprised wholly of a metraterm immediately distal to the oötype.

***Spirorchis* cf. *scripta* (Figs. 1−5, 9)**

Description of adult (based on light microscopy of five adult specimens): Body 1,260−2,090 (1,724; 5) [n/a, 1,150] long or 3.3−4.4× (3.8; 5) [n/a, 4.8] longer than wide, 360−500 (404; 5) [255, 225] wide or 19−30% (24%; 5) [n/a, 20%] of body length at level of caecal bifurcation, 380−560 (448; 5) [280, 240] wide or 23−30% (26%; 5) [n/a, 21%] of body length at level of middle testis (typically maximum body width), 200−230 (214; 5) [150, 110] wide or 10−17% (13%; 5) [n/a, 10%] of body length at level of caecal terminus (Figs. 1, 3). Oral sucker 25−45 (39; 5) [n/a, 30] long or 2−3% (2%; 5) [n/a, 3%] of body length, 50−65 (56; 5) [n/a, 60] wide or 10−18% (14%; 4) [n/a, 27%] of body width at level of caecal bifurcation (Figs. 1, 3). Nerve commissure 140−188 (161; 4) [n/a, 120] or 7−12% (9%; 4) [n/a, 10%] of body length from anterior body end. Pharynx 60−90 (77; 4) [n/a, 53] long or 15−20% (16%; 4) [n/a, 18%] of oesophagus length, 55−70 (61; 5) [n/a, 48] wide or 2.8−4.8× (3.7; 5) [n/a, 3.7] oesophagus width immediately posterior to pharynx, 1.0−1.4× (1.2; 4) [n/a, 1.1] longer than wide (Figs. 1, 3). Oesophagus extending posteriad 250−565 (427; 5) [n/a, 301] long or 20−30% (25%; 5) [n/a, 26%] of body length from mouth to posterior margin of median oesophageal diverticulum, 13−25 (17; 5) [n/a, 13] wide posterior to pharynx, with wall 10−15 (12; 5) [n/a, 10] thick, 35−50 (40; 5) [n/a, 15] wide at oesophagus median or 8−13% (10%; 5) [n/a, 7%] of body width at level of caecal bifurcation, with wall 15−25 (19; 5) [n/a, 12] thick, 45−65 (55; 5) [n/a, 35] wide anterior to median oesophageal diverticulum or 12−15% (14%; 5) [n/a, 7%] of body width at level of caecal bifurcation, with wall 35−45 (40; 5) [n/a, 30] thick; median oesophageal diverticulum 200−475 (344; 5) [n/a, 250] or 16−25% (20%; 5) [n/a, 22%] of body length from anterior body end, 75−130 (109; 5) [100, 68] long or 21−30% (26%; 5) [n/a, 23%] of oesophagus length, 58−90 (78; 5) [35, 50] wide or 13−23% (20%; 5) [14%, 22%] of body width at level of caecal bifurcation; oesophageal gland 173−410 (271; 5) [n/a, 195] long or 12−22% (15%; 5) [n/a, 17%] of body length, 100−135 (117; 5) [n/a, 105] wide or 26−35% (29%; 5) [n/a, 47%] of body width at level of caecal bifurcation (Figs. 1, 3). Intestinal bifurcation 230−525 (389; 5) [n/a, 280] from anterior body end or 18−28% (22%; 5) [n/a, 24%] of body length, dorsal to median oesophageal diverticulum (Figs. 1, 3); sinistral caecum 850−1,530 (1,180; 5) [1,095, 770] long or 60−73% (68%; 5) [n/a, 67%] of body length, 30−53 (43; 5) [25, 25] wide or 8−15% (11%; 5) [10%, 11%] of body width at level of caecal bifurcation, 28−50 (34; 5) [20, 18] wide or 7−9% (8%; 5) [7%, 8%] of body width at level of middle testis, 18−40 (30; 3) [20, 18] wide or 9−20% (14%; 5) [13%, 16%] of body width at level of caecal terminus; dextral caecum 850−1,470 (1,138; 5) [1,095, 770] long or 60−72% (66%; 5) [n/a, 67%] of body length, 25−45 (36; 5) [25, 25] wide or 7−13% (9%; 5) [10%, 11%] of body width at level of caecal bifurcation, 28−45 (35; 5) [20, 20] wide or 7−8% (8%; 5) [7%, 8%] of body width at level of middle testis, 25−35 (30; 5) [20, 18] wide or 12−18% (14%; 5) [13%, 16%] of body width at level of caecal terminus; post-caecal distance 115−235 (185; 5) [145, 125] or 9−12% (11%; 5) [n/a, 11%] of body length.

Testes 10 (5) [10, 10] in number, having abutting or overlapping anterior and posterior margins; testicular column 23−90 (46; 5) [35, 8] or 1−5% (3%; 5) [n/a, 1%] of body length from caecal bifurcation, 428−880 (632; 5) [630, 425] long or 31−42% (36%; 5) [n/a, 37%] of body length; anterior testis (t1) 48−135 (83; 5) [85, 48] long or 4−6% (5%; 5) [n/a, 4%] of body length, 88−135 (113; 5) [85, 65] wide or 20−30% (25%; 5) [30%, 27%] of body width at level of middle testis, 1.0−2.0× (1.5; 5) [1.0, 1.4] wider than long, 8−35 (25; 5) [15, 5] from sinistral caecum, 8−50 (32; 5) [15, 8] from dextral caecum; middle testis (t5) 45−85 (63; 5) [60, 50] long or 3−4% (4%; 5) [n/a, 4%] of body length, 88−200 (138; 5) [120, 88] wide or 23−36% (30%; 5) [43%, 37%] of body width at level of middle testis, 1.7−3.3× (2.2; 5) [2.0, 1.8] wider than long, 8−30 (20; 5) [abutting, abutting] from sinistral caecum, 5−35 (19; 5) [abutting, abutting] from dextral caecum; posterior-most testis 325−745 (577; 5) [565, 375] or 17−43% (34%; 5) [n/a, 33%] of body length from posterior body end, 34−120 (79; 5) [75, 53] long or 3−6% (4%; 5) [n/a, 5%] of body length, 68−170 (129; 5) [100, 100] wide or 18−36% (29%; 5) [36%, 42%] of body width at level of middle testis, 1.4−2.0× (1.7; 5) [1.3, 1.9] wider than long, abutting or 13−15 (14; 2) [abutting, abutting] from sinistral caecum, abutting or 18−35 (28; 3) [abutting, abutting] from dextral caecum (Figs. 1, 3). Vas deferens 525−750 (605; 3) [540, 413] long or 28−37% (33%; 3) [n/a, 36%] of body length, 10−13 (11; 4) [13, 13] wide, ventral to testicular column, laterally expanding before joining external seminal vesicle posterior to posterior testis (Figs. 2, 4); external seminal vesicle 88−115 (101; 5) [103, 83] long or 4−8% (6%; 5) [n/a, 7%] of body length, 65−133 (106; 5) [78, 93] wide or 15−28% (24%; 5) [28%, 39%] of body width at level of middle testis, 0.7−1.4× (1.0; 5) [1.3, 0.9] longer than wide (Figs. 2, 4); internal seminal vesicle 50−80 (69; 5) [60, 43] long or 3−5% (4%; 5) [n/a, 4%] of body length, 20−28 (24; 5) [18, 15] maximum width or 80−100% (91%; 5) [60%, 43%] of cirrus sac width, 1.8−3.5× (2.9; 5) [3.3, 2.9] longer than wide (Figs. 2, 4); proximal portion of internal seminal vesicle 18−30 (25; 5) [20, 20] long or 28−41% (36%; 5) [33%, 47%] of total internal seminal vesicle length, 20−28 (23; 5) [18, 15] wide; distal portion of internal seminal vesicle 30−52 (44; 5) [40, 23] long or 59−72% (64%; 5) [67%, 53%] of total internal seminal vesicle length, 20−28 (24; 5) [13, 13] wide. Cirrus sac 75−115 (95; 5) [85, 68] long or 5−6% (6%; 5) [n/a, 6%] of body length, 25−30 (27; 5) [30, 35] wide or 4−7% (6%; 5) [11%, 15%] of body width at level of middle testis; cirrus straight, 20−35 (28; 5) [n/a, 20] long or 1−2% (2%; 5) [n/a, 2%] of body length, 5−13 (9; 5) [n/a, 8] wide or 1−3% (2%; 5) [n/a, 3%] of maximum body width.

Ovary lobed, 3−6 lobes (5; 5) [4, 6], intercaecal, posterior to external seminal vesicle, lateral to cirrus sac, 98−120 (106; 5) [115, 100] long or 5−8% (6%; 5) [n/a, 9%] of body length, 93−180 (126; 5) [93, 88] wide or 24−32% (28%; 5) [33%, 37%] of body width at level of middle testis, 0.9−1.5× (1.2; 5) [0.8, 0.9] wider than long (Figs. 1−4); post-ovarian distance 280−530 (442; 5) [410, 300] or 13−36% (27%; 5) [n/a, 26%] of body length (Figs. 1, 3). Oviduct extending posteriad 20−38 (30; 5) [38, 20] total length or 2%

(5) [n/a, 2%] of body length, 8−13 (12; 5) [10, 8] wide; oviducal seminal receptacle 50−63 (55; 5) [38, 35] long or 3−4% (3%; 5) [n/a, 3%] of body length, 35−45 (42; 5) [30, 30] wide or 8−12% (9%; 5) [11%, 13%] of maximum body width, oviduct continuing posterosinistrad 20−145 (93; 5) [55, 30] or 2−8% (5%; 5) [n/a, 3%] of body length, 10−20 (15; 5) [10, 10] or 2−5% (3%; 5) [n/a, 4%] of maximum body width before connecting with oötype (Figs. 2, 4). Laurer's canal a narrow duct originating 30−43 (35; 5) [25, 18] or 29−44% (33%; 4) [22%, 18%] of ovary length from distal margin of seminal receptacle, extending 20−50 (32; 5) [45, 28] or 1−3% (2; 5) [n/a, 2%] of body length posterosinistrad, 8−20 (13; 5) [8, 13] wide or 2−4% (3%; 5) [n/a, 5%] of maximum body width. Vitellarium comprising a series of interconnected spheroid masses of small follicles, distributing from mid-level of oesophagus to caecal terminus, ventrolateral to testes and caeca, terminating 50−190 (118; 5) [130, 108] or 3−10% (7%; 5) [n/a, 9%] of body length from posterior body end, coalescing into lateral vitelline collection ducts posterior to oviduct (Figs. 2, 4); lateral vitelline collecting ducts 15−20 (18; 4) [20, 18] in maximum width, coalescing in intercaecal space posterior to ovary and joining at midline to form transverse vitelline duct; transverse vitelline duct 88−130 (108; 4) [70, 58] in breadth or 20−29% (23%; 4) [25%, 24%] of body width at level of middle testis, 15−30 (20; 4) [15, 13] in maximum width, 225−505 (398; 5) [350, 275] or 18−26% (23%; 3) [n/a, 24%] of body length from posterior body end; primary vitelline duct a narrow duct 13−23 (19; 4) [25, 8] wide, extending posteriad 43−58 (50; 4) [45, 28] before curving ventrally, extending 38−53 (46; 4) [58, 25] anteriad or 2−3% (3%; 4) [n/a, 2%] of body length before merging with female genitalia at oviduct-oötype junction (Figs. 2, 4). Oötype indiscernible from uterus in gravid specimens. Mehlis’ gland not observed.

Uterus proximal portion reduced, forming egg chamber with oötype in gravid individuals; egg chamber (oötype + proximal portion of uterus) 42−65 (53; 5) [62, 40] long or 40−66% (50%; 5) [54%, 40%] of ovary length, 35−40 (36; 5) [38, 33] wide or 19−43% (30%; 5) [41%, 38%] of ovary width; metraterm 15−18 (17; 5) [18, 13] long or 23−43% (33%; 5) [30%, 33%] of egg chamber length, 13−20 (16; 5) [18, 10] wide or 33−57% (46%; 5) [47%, 30%] of egg chamber width. Uterine egg 55−60 (57; 5) [75, 45] long or 3−5% (3%; 5) [n/a, 4%] of body length, 25−30 (27; 5) [35, 20] wide or 11−14% (12%; 5) [n/a, 18%] of body width at level of middle testis, 1.8−2.4× (2.2; 5) [2.1, 2.3] longer than wide. Common genital pore opening ventral, sinistral (Figs. 1−4), 370−525 (455; 5) [405, 295] or 25−29% (27%; 59) [n/a, 26%] of body length from posterior body end.

Excretory vesicle difficult to discern, 25 (1) [18, 18] long or 1% (1) [n/a, 2%] of body length, 5−8 (7; 2) [5, 5] wide or 2−4% (3%; 2) [3%, 5%] of body width at caecal terminus; Manter’s organ coiled, turning 7−10 (8; 3) [10, 3] times, extending between caeca, 128−348 (226; 5) [250, 178] long or 7−17% (13%; 5) [n/a, 15%] of body length, 20−30 (25; 3) [25, 13] wide or 9−14% (12%; 5) [17%, 12%] of body width at caecal terminus, joining excretory vesicle near excretory pore (Figs. 1, 3); excretory pore dorsal, 13−15 (14; 3) [13, 13] or 1% (3) [1%, 1%] from posterior body end (Figs. 1, 3).

Taxonomic Summary

Type host: *Trachemys scripta* (Thunberg *in* Schoepff), (holotype and paratype slides labeled as *Pseudemys scripta*, Stunkard 1923).

New host: *Deirochelys reticularia* (Latreille *in* Sonnini and Latreille) (Testudines: Emydidae).

New locality: Big Beaver Pond (32°25'44.03"N, 85°38'44.87"W) near Uphapee Creek, Tallapoosa River, near Tuskegee, Alabama, USA.

Site in host: Lung, kidney, body wash

Intensity: Eight specimens *S.* cf. *scripta* infected a single chicken turtle.

Specimens deposited: Vouchers USNM Cat. No. **XXXXXX**, IPCAS Cat. No. **XXXXXX**, GenBank Accession No. **XXXXXX**.

Materials examined: See Table 2.

Remarks

We identified our specimens as *Spirorchis* cf. *scripta* because they were morphologically similar to museum specimens (holotype, AMNH 128; paratype AMNH 130) and published descriptions of *S. scripta* (Stunkard 1923, Byrd 1939, Goodchild and Martin 1969, Platt 1993). Noteworthy, however, is that we detected several morphological differences, which, although subtle (i.e., likely comprising intraspecific variation), were seemingly in accord with the slight molecular differences (>99% similarity, 2 of 1263 bases differed) between our specimens and those attributed to *S. scripta* in GenBank (Fig. 9). Our specimens of *S.* cf. *scripta* had a proportionally smaller oral sucker (10−18% of body width vs. 27% in paratype), wider oesophagus (12−15% of body width vs. 7% in paratype), more narrow oesophageal gland (26−35% of body width vs. 47% in paratype), compressed middle testis that did not abut the caeca (23−36% of body width vs. 43% and 37%, and abutting caeca, in holotype and paratype), more narrow cirrus sac (4−7% of body width vs. 11% and 15% in holotype and paratype), longer oviduct (29−44% of ovary length vs. 22% and 18% in holotype and paratype), and more narrow uterine eggs (11−14% of body width; 18% in paratype).

After examining the holotype and paratypes as well as newly collected vouchers of *S. scripta* and *S. picta* from Alabama (Table 1), we accept *S. picta* and differentiate it from *S. scripta* by the combination of having rounded anterior and posterior body ends, a testicular column not abutting the median oesophageal diverticulum, testes that are relatively far separated (5−30 apart, with anterior testis separated from second testis [30, 20]), and a spherical ovary that is much larger (3.0× testis width) than any testis. *Spirorchis scripta* has sharply tapered anterior and posterior body ends, a testicular column abutting the caecal bifurcation, testes having abutting or overlapping anterior and posterior margins, and a smaller, lobed ovary (0.7−1.4× testis width). Byrd (1939) considered *S. picta* a junior subjective synonym of *S. elegans* based on similar body size, gonad and genital pore positions, vitellarium distribution, and testes number. Goodchild and Martin (1969) accepted this synonymy, but also considered *S. elegans* a junior subjective synonym of *S. scripta* because they interpreted testes shape and intercaecal position as ontogenetic and, therefore, unreliable as diagnostic. Platt (1993) rejected that, accepting *S. elegans* as distinct and *S. picta* as a junior subjective synonym of *S. scripta*.

***Spirorchis collinsi* Roberts et Bullard sp. n. (Figs. 6−9)**

Description of adult (based on light microscopy of four adult specimens): Body 2,660−3,820 (3,313; 3) long or 7.2−7.6× (7.5; 3) longer than wide, 220−400 (328; 4) wide or 10−11% (11%; 3) of body length at level of caecal bifurcation, 335−460 (394; 4) wide or 12−13% (12%; 3) of body length at level of middle testis, 350−500 (430; 4) wide or 13−14% (13%; 3) of body length at level of Mehlis' gland (typically maximum body width), 245−400 (328; 3) wide or 9−12% (10%; 3) of body length at level of caecal terminus. Oral sucker 20−33 (27; 4) long or 1% (1%; 3) of body length, 58−75 (67; 4) wide or 15−30% (22%; 4) of body width at level of caecal bifurcation (Figs. 6, 7). Nerve commissure 155−200 (174; 4) or 5−6% (5%; 4) of body length from anterior body end. Pharynx 75−85 (81; 4) long or 10−18% (15%; 4) of oesophagus length, 60−68 (64; 4) wide or 3.0−5.2× (4.4; 4) oesophagus width immediately posterior to pharynx (Figs. 6, 7), 1.2−1.3× (1.3; 4) longer than wide. Oesophagus extending posteriad 471−780 (559; 4) long or 15−20% (18%; 3) of body length from mouth to posterior margin of median oesophageal diverticulum, 13−20 (15; 4) wide posterior to pharynx, with wall 8−12 (10; 4) thick, 35−50 (44; 4) wide at oesophagus median or 9−20% (14%; 4) of body width at level of caecal bifurcation, with wall 25−40 (33; 4) thick, 65−90 (78; 4) wide anterior to median oesophageal diverticulum or 17−34% (25%; 4) of body width at level of caecal bifurcation, with wall 55−70 (65; 4) thick; median oesophageal diverticulum 385−660 (486; 4) or 13−17% (16%; 3) of body length from anterior body end, 58−115 (82; 4) long or 12−16% (15%; 4) of oesophagus length, 63−80 (70; 4) wide or 18−31% (22%; 4) of body width at level of caecal bifurcation; oesophageal gland 280−605 (408; 4) long or 11−16% (14%; 3) of body length, 105−145 (118; 4) wide or 28−51% (38%; 4) of body width at level of caecal bifurcation (Figs. 6, 7). Intestinal bifurcation 205−730 (474; 4) from anterior body end or 14−19% (17%; 3) of body length, dorsal to median oesophageal diverticulum; sinistral caecum 2,000−3,070 (2,533; 3) long or 73−80% (76%; 3) of body length, 30−55 (40; 4) wide or 10−14% (12%; 4) of body width at level of caecal bifurcation, 13−40 (28; 4) wide or 3−9% (7%; 4) of body width at level of Mehlis' gland, 35−63 (48; 3) wide or 13−16% (14%; 3) of body width at level of caecal terminus; dextral caecum 1,930−2,880 (2,450; 3) long or 73−75% (74%; 3) of body length, 38−45 (43; 4) wide or 11−20% (14%; 4) of body width at level of caecal bifurcation, 15−40 (28; 4) wide or 3−10% (7%; 4) of body width at level of Mehlis' gland, 35−73 (49; 3) wide or 12−18% (15%; 3) of body width at level of caecal terminus; post-caecal distance 210−370 (277; 3) or 7−11% (8%; 3) of body length.

Testes 9−10 (10; 4) in number, having abutting or overlapping anterior and posterior margins; testicular column abutting caecal bifurcation or 15−75 (38; 3) or <1−2% (1%; 3) of body length from caecal bifurcation, 465−870 (684; 4) long or 20−25% (23%; 3) of body length; anterior testis (t1) 75−110 (91; 4) long or 2−3% (3%; 3) of body length, 100−163 (123; 4) wide or 24−38% (31%; 4) of body width at level of middle testis, 1.3−1.5× (1.3; 4) wider than long, 5−35 (19; 4) from sinistral caecum, abutting or 13−50 (28; 3) from dextral caecum; middle testis (t5) 70−110 (90; 4) long or 3% (3) of body length, 113−220 (155; 4) wide or 32−51% (39%; 4) of body width at level of middle testis, 1.4−2.0× (1.7; 4) wider than long, abutting or 3−25 (17; 3) from sinistral caecum, 8−43 (32; 4) from dextral caecum; posterior-most testis 1,520−2,380 (1,980; 3) or 57−62% (59%; 3) of body length from posterior body end, 50−103 (81; 4) long or 2−3% (3%; 3) of body length, 130−240 (171; 4) wide or 37−56% (43%; 4) of body width at level of middle testis, 1.8−2.6× (2.2; 4) wider than long, abutting or 5−10 (7; 3) from sinistral caecum, 5−30 (18; 4) from dextral caecum (Fig. 7). Vas deferens 375−730 (523; 4) long or 11−21% (18%; 3) of body length, 8−15 (12; 4) wide, ventral to testicular column, laterally expanding before joining external seminal vesicle posterior to testes

(Figs. 7, 8); external seminal vesicle 75−190 (121; 4) long or 3−5% (4%; 3) of body length, 140−195 (160; 4) wide or 32−40% (37%; 4) of body width at level of Mehlis' gland, 0.5−1.2× (0.8; 4) longer than wide (Figs. 7, 8); internal seminal vesicle 155−243 (179; 4) long or 4−9% (6%; 3) of body length, 38−50 (44; 4) maximum width or 36−84% (62%; 4) of cirrus sac width, 3.2−6.4× (4.2; 4) longer than wide (Fig. 8); proximal portion of internal seminal vesicle 33−50 (39; 4) long or 14−31% (23%; 4) of total internal seminal vesicle length, 25−50 (38; 4) wide; distal portion of internal seminal vesicle 113−210 (141; 4) long or 69−86% (77%; 4) of total internal seminal vesicle length, 38−48 (42; 4) wide. Cirrus sac 188−250 (210; 4) long or 5−7% (6%; 3) of body length, 45−135 (79; 4) wide or 12−27% (18%; 4) of body width at level of Mehlis' gland; cirrus massive, sinuous, 148−333 (233; 4) long or 6−9% (7%; 3) of body length, 15−55 (35; 4) wide or 4−11% (8%; 4) of maximum body width.

Ovary spheroid, lacking lobes, intercaecal, posterior to cirrus sac, 70−165 (113; 4) long or 3−4% (4%; 3) of body length, 103−130 (118; 4) wide or 26−29% (28%; 4) of body width, 0.8−1.6× (1.1; 4) wider than long (Figs. 7, 8); post-ovarian distance 1,290−1,900 (1,643; 3) or 48−50% (50%; 3) of body length (Fig. 7). Oviduct extending posteriad 25−35 (30; 4) or 1% (3) of body length, 13−20 (15; 4) wide; oviducal seminal receptacle 45−60 (53; 4) long or 1−2% (2%; 3) of body length, 30−45 (38; 4) wide or 8−11% (9%; 4) of maximum body width, oviduct continuing posterosinistrad 83−150 (116; 4) or 1−4% (2%; 3) of body length, 10−20 (17; 4) wide or 3−5% (4%; 4) of maximum body width before connecting with oötype (Fig. 8). Laurer's canal a narrow duct originating 25−75 (46; 4) or 34−46% (40%; 4) of ovary length from distal margin of seminal receptacle, extending 60−80 (69; 4) or 2% (3) of body length posterosinistrad, 13−35 (19; 4) wide or 3−7% (4%; 4) of maximum body width. Vitellarium comprising a series of interconnected spheroid masses of small follicles (Fig. 7), distributing from caecal bifurcation to caecal terminus, ventral to caeca and testis anteriorly and flanking caeca posteriorly, terminating 155−350 (237; 3) or 5−10% (7%; 3) of body length from posterior body end, coalescing into lateral vitelline collection ducts posterior to oviduct; lateral vitelline collecting ducts 13−25 (20; 4) in maximum width, coalescing in intercaecal space posterior to ovary and joining at midline to form transverse vitelline duct; transverse vitelline duct 113−225 (155; 4) in breadth or 29−45% (36%; 4) of body width at level of Mehlis' gland, 13−25 (19; 4) in maximum width, 1,170−1,740 (1,513; 3) or 44−47% (46%; 3) of body length from posterior body end (Figs. 7, 8); primary vitelline duct a narrow duct 23−55 (42; 4) wide, extending posteriad 25−75 (49; 4) before curving ventrally, extending 25−100 (58; 4) anteriad or 1−3% (2%; 3) of body length before merging with female genitalia at oviduct-oötype junction (Fig. 8). Oötype 38−75 (53; 4) long or 41−54% (48%; 4) of ovary length, 13−40 (22; 4) wide or 12−31% (18%; 4) of ovary width (Fig. 8). Mehlis' gland obvious, 125−320 (199; 4) long or 4−8% (7%; 3) of body length, 170−270 (199; 4) wide or 35−54% (46%; 4) of body width (Figs. 7, 8). Uterus comprising metraterm only (Fig. 8); metraterm apparently comprising the entire length of the uterus from oötype extending anteriad, 218−375 (280; 4) long or 7−10% (8%; 3) of body length, 25−60 (36; 4) wide or 6−12% (8%; 4) of maximum body width, wall 20−22 (21; 4) wide, dorsal to cirrus (Fig. 8). Uterine egg absent. Common genital pore 1,460−2,160 (1,847; 3) or 55−57% (56%; 3) of body length from posterior body end (Fig. 7).

Excretory vesicle 30−45 (35; 3) long or 1% (3) of body length, 10−15 (13; 3) wide or 3−6% (4%; 3) of body width at caecal terminus; Manter's organ turning 3−10 (7; 3) times proximally, sinuous distally, intercaecal, 385−500 (425; 3) long or 11−14% (13%; 3) of body length, 35−60 (45; 3) wide or 12−15% (14%; 3) of body width at level of caecal terminus, joining excretory vesicle at excretory pore (Fig. 7); excretory pore dorsal, 25−45 (35; 3) or 1−2% (1%; 3) from posterior body end (Fig. 7).

Taxonomic Summary

Type and only known host: Chicken turtle, *Deirochelys reticularia* (Latreille *in* Sonnini and Latreille) (Testudines: Emydidae).

Type locality: Big Beaver Pond (32°25'44.03"N, 85°38'44.87"W) near Uphapee Creek, Tallapoosa River, near Tuskegee, Alabama, USA.

Other locality: Salt Pond (31°10'13.88"N, 86°32'17.49"W), Yellow River, near Andalusia, Alabama, USA.

Site in host: Kidney blood vessels and body wash.

Prevalence and intensity of infection: Two (100%) chicken turtles were infected by four and two specimens of *S. collinsi*, respectively.

Specimens/materials deposited: Holotype (USNM Cat. No. **XXXXXX**), paratype (USNM Cat. No. **XXXXXX**), paratype (IPCAS Cat. No. **XXXXXX**)**,** GenBank Accession No. **XXXXXX**.

Etymology: The specific epithet *collinsi* is in gratitude to Dr. Michael D. Collins (Department of Biological Sciences, Rhodes College, Memphis, Tennessee, USA) for mentoring JRR during his Bachelor's degree program.

Remarks

The new species is most easily differentiated from its congeners by the combination of having caeca that extend far beyond the genitalia, intercaecal genitalia positioned in the middle portion of the body, a testicular column that nearly abuts the caecal bifurcation, a cirrus sac positioned between the testes and ovary, a massive Mehlis' gland, an elongate, longitudinal metraterm that extends anteriad beyond the level of the ovary, a pre-ovarian genital pore, and a prominent, intercaecal Manter's organ.

The new species resembles *S. scripta*, *S. elegans*, *S. innominatus*, and *S. artericola* by having a testicular column that is positioned such that its anterior-most testis is close to the caecal bifurcation; however, the most obvious feature that differentiates the new species from these and other congeners is the mid-body position of the genitalia. Further, *S. haematobius* has a post-ovarian genital pore; *S. kirki* has four or five testes, a post-ovarian genital pore, indiscernible Mehlis' gland, and no Manter's organ; *S. minutus* has a testicular column that does not abut the caecal bifurcation, genitalia in the posterior 1/5 of body, and an ovary that is lateral to the cirrus sac and genital pore; *S. parvus* has four or five testes, a post-ovarian genital pore, an indiscernible Mehlis' gland, and a postcaecal Manter's organ; and *S. picta* has an ovary that abuts the testis.

The phylogenetic analysis of the 28S sequence data indicated that *S. collinsi* and *S. picta* were sister taxa within a clade that included *S. haematobius*, >99% (2 of 1263 bases differed) similarity between *S.* cf. *scripta* and *S. scripta*, and a monophyletic *Spirorchis* MacCallum, 1918 (Fig. 9). Members of the *S. haematobius* clade have a prominent median oesophageal diverticulum, ten testes, a cirrus sac positioned between the testes and ovary, a spheroid, non-lobed ovary, an obvious Mehlis' gland,

and an intercaecal Manter's organ. *Spirorchis collinsi* and *S*. *picta* have a Mehlis' gland that fills the intercaecal space from the level of the ovary to the anterior margin of the transverse vitelline duct (Fig. 8). Stunkard (1923) did not detail a Mehlis' gland in *S. picta*, and the holotype and paratype are poorly stained (perhaps destained); however, our specimens have an obvious Mehlis' gland similar to that of *S. collinsi*. Some of the museum specimens of *S*. *haematobius* we examined appeared to show intraspecific variation in ovary shape: being weakly lobed to spheroid. Ulmer (1959) suspected that the lobes resulted from excessive coverslip pressure (see Ulmer's [1959] figures 2−10, p. 83).

DISCUSSION

In addition to being a never-before-reported TBF host, the chicken turtle was a high priority target for TBF sampling because of its phylogenetic position within Emydidae (van Dijk et al. 2014, Spinks et al. 2016) and because of its overwintering behavior (Buhlmann 2005, Guyer et al. 2015). *Deirochelys* Agassiz is monotypic, comprises an early branching lineage within Deirochelyinae Agassiz (*Chrysemys* Gray, *Deirochelys*, *Graptemys* Agassiz, *Malaclemys* Gray, *Pseudemys* Gray, *Trachemys* Agassiz), and is the sister taxon to all other deirochelyines. Hence, we anticipated that this phylogenetically distinct turtle lineage would harbor new TBF taxa. Regarding overwintering behavior, of all reported turtle hosts for *Spirorchis* spp., *D. reticularia* and the western pond turtle, *Actinemys marmorata* Baird and Girard (see Thatcher 1954), are the only species that reportedly primarily overwinter terrestrially (Reese and Welsh 1997, Rathbun et al. 2002, Buhlmann 2005, Ernst and Lovich 2009, Guyer et al. 2015).

Such extended, annual, terrestrial forays suggest that perhaps these TBFs have increased adult longevity to maximize egg release when the turtles return to water in Spring. Terrestrial aestivation during colder months by chicken turtles differs from that of pond sliders, *Trachemys scripta* (the type host for *S. scripta*), which overwinter in aquatic shelters, e.g., submerged logs, scour habitat, and river or pond banks (Bodie and Semlitsch 2000, Ryan et al. 2008, Ernst and Lovich 2009). Perhaps these host behaviors are reflected by divergent parasite life history strategies, which may have resulted in genetic isolation and subtle morphological differences among these TBFs. Intensive, seasonal sampling of these ecologically different turtle species for TBF infections, genetic comparisons among the resulting TBFs, and identification of the TBF intermediate hosts could help test the hypothesis that turtle host ecological differences result in TBF genetic isolation and morphological divergence. We suspect that the TBFs of chicken turtles and pond sliders may comprise a good model system with which to test such a hypothesis. Noteworthy regarding TBF-turtle cophyly, or rather the apparent lack thereof, although *Spirorchis haematobius*, *S. collinsi*, and *Vasotrema* spp. reportedly exhibit a high degree of host specificity, the TBF clade (Roberts et al. 2016a) and the *Spirorchis* clade (Fig. 9) are not concordant with recent turtle phylogenies (Guillon et al. 2014, Spinks et al. 2016). Yet, few TBFs have been included in molecular phylogenies and additional TBF taxon sampling will help further test TBF-turtle cophyly.

Tkach et al. (2009), Orélis-Ribeiro et al. (2014), and Roberts et al. (2016a, b) provided molecular phylogenetic analyses that recovered a paraphyletic *Spirhapalum*, with *Spirhapalum polesianum* Ejsmont, 1927 sister to *Spirorchis* spp.+ *Spirhapalum siamensis* Tkach, Snyder et Vaughn, 2009. Tkach et al. (2009) considered proposing a new genus for *S. siamensis* but lacked morphological details to justify it. *Spirhapalum siamensis* and *Spirhapalum elongatum* Rohde, Lee et Lim, 1968 infect *Cuora amboinensis* (Riche *in* Daudin), an Asian geoemydid; whereas, *S. polesianum* was reported from *Emys orbicularis* (Linnaeus), an early branching emydid (Rohde et al. 1968, Brooks and Palmieri 1979, Snyder 2004, Tkach et al. 2009, Spinks et al. 2016). The phenomenon of congeneric TBFs infecting turtles of multiple families is exemplified by *Cardiotrema* spp. infecting geoemydids and trionychids, *Spirorchis* spp. infecting chelydrids and emydids, and *Hapalorhynchus* spp. infecting chelydrids, kinosternids, geoemydids, and pelomedusids. Collectively, this suggests either low host specificity of the TBFs comprising these genera or the presence of new TBF genera. However, inaccessibility (or nonexistence) of museum material for all TBFs from the Indian subcontinent (see Platt 2002) remains a major block to exploring the taxonomy and systematics of this group of blood flukes.

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LITERATURE CITED

Anonymous. 1919: Twenty-third annual report of the New York Zoological Society. Clark and Fritts Printers, New York, USA, 204 pp.

Bodie J.R., Semlitsch R.D. 2000: Spatial and temporal use of floodplain habitats by lentic and lotic species of aquatic turtles. Oecologia. 122: 138−146.

Brooks D.R., Palmieri J.R. 1979: *Neopronocephalus orientalis* sp. n. (Digenea: Pronocephalidae) and *Spirhapalum elongatum* Rohde, Lee, and Lim, 1968 (Digenea: Spirorchiidae) from *Cuora amboinensis* (Daudin) in Malaysia. Proc. Helminthol. Soc. Wash. 46: 55−57.

Byrd E.E. 1939: Studies on the blood flukes of the family Spirorchidae. Part II. Revision of the family and description of new species. J. Tenn. Acad. Sci. 14: 116−161.

Buhlmann K.A. 2005: Habitat use, terrestrial movements, and conservation of the turtle, *Deirochelys reticularia* in Virginia. J. Herpetol. 29: 173−181.

Bullard S.A. 2012: A new aporocotylid (Digenea) species from blood vascular system of gag grouper, *Mycteroperca microlepis* (Perciformes: Serranidae), off Alabama, with an emendation of *Pearsonellum* Overstreet & Køie, 1989. J. Parasitol. 98:323−327.

Bullard S.A. 2013: *Cardicola langeli* n. sp. (Digenea: Aporocotylidae) from heart of sheepshead, *Archosargus probatocephalus*, (Actinopterygii: Sparidae) in the Gulf of Mexico, with an updated list of hosts, infection sites and localities for *Cardicola* spp. Folia Parasitol. 60:17−27.

Bullard S.A, Snyder S.D., Jensen K., Overstreet R.M. 2008: New genus and species of Aporocotylidae (Digenea) from a lower actinopterygian, the American paddlefish, *Polyodon spathula*, (Polyodontidae) from the Mississippi Delta. J. Parasitol. 94: 487−495.

Ejsmont L. 1927: *Spirhapalum polesianum* n. g., n. sp. trématode du sang d'*Emys orbicularis* L. Ann. Parasitol. Hum. Comp. 5: 220−235.

Ernst C.H., Lovich J.E. 2009: Turtle of the United States and Canada, 2nd Ed., The Johns Hopkins University Press, Baltimore, Maryland, USA, 827 pp.

Goodchild C.G., Kirk D.E. 1960: The life history of *Spirorchis elegans* Stunkard, 1923 (Trematoda: Spirorchiidae) from the painted turtle. J. Parasitol. 46: 219−229.

Goodchild C.G., Martin V.L. 1969: Speciation in *Spirorchis* (Trematoda: Spirorchiidae) infecting the painted turtle, *Chrysemys picta*. J. Parasitol. 55: 1169−1173.

Guillon J., Guéry L., Hulin V., Girondot M. 2012: A large phylogeny of turtles (Testudines) using molecular data. Contr. Zool. 81: 147−158.

Gupta N.K., Mehrotra V. 1975: On three blood flukes (Spirorchiidae: Spirorchiinae) from freshwater chelonians and discussion on the synonymy of *Plasmiorchis pellucidus* Mehra, 1934. Riv. Parassitol. 36: 165−170.

Guyer C., Bailey M.A., Mount R.H. 2015: Turtles of Alabama, The University of Alabama Press, Tuscaloosa, Alabama, USA, 266 pp.

Holliman R.B., Fisher J.E. 1968: Life cycle and pathology of *Spirorchis scripa* Stunkard, 1923 (Digenea: Spirochiidae) in *Chrysemys picta picta*. J. Parasitol. 54: 310−318.

Holliman R.B., Fisher J.E., Parker J.C. 1971: Studies on *Spirorchis parvus* (Stunkard, 1923) and its pathological effects on *Chrysemys picta picta.* J. Parasitol. 57: 71−77.

Johnson P.D., Bogan A.E., Brown K.M., Burkhead N.M., Cordeiro J.R., Garner J.T., Hartfield P.D., Lepitzki D.A.W., Mackie G.L., Pip E., Tarpley T.A., Tiemann J.S., Whelan N.V., Strong E.E. 2013: Conservation status of freshwater gastropods of Canada and the United States. Fisheries. 38: 247−282.

Lockyer A.E., Olson P.D., Ostergaard P., Rollinson D., Johnston D.A., Attwood S.W., Southgate V.R., Horak P., Snyder S.D., Le T. H., Agatsuma T., McManus D.P., Carmichael A.C., Naem S., Littlewood D.T.J. 2003: The phylogeny of the Schistosomatidae based on three genes with emphasis on the interrelationships of *Schistosoma* Weinland, 1858. Parasitology. 126: 203−224.

MacCallum G.A. 1918: Notes on the genus *Telorchis* and other trematodes. Zoopathologica. 1: 77−98.

MacCallum G.A. 1926: Revue du genre *Spirorchis* MacCallum. Ann. Parasitol. hum. comp. 4: 97−103.

McVay M.J., Bakenhaster M.D., Bullard S.A. 2011: *Cardicola laruei* Short, 1953 (Digenea: Aporocotylidae) from heart of seatrouts, *Cynoscion* spp., (Perciformes: Sciaenidae) in the Gulf of Mexico and Atlantic Ocean: taxonomic redescription, first observations of egg and miracidium, and comments on geographic distribution and host specificity. Comp. Parasitol. 78:291−305.

Mehra H.R. 1934: New blood flukes of the family Spirorchidae Stunkard from Indian fresh-water tortoises with discussion on the synonymy of certain genera and the relationships of the families of blood flukes. Part II. Bull. Acad. Sci. United Prov. Agra Oudh, India. 3: 169−196.

Mehra H.R. 1939: New blood flukes of the family Spirorchidae Stunkard (Trematoda) from the marine turtle *Chelone mydas* of the Arabian Sea with observations on the synonymity of certain genera and classification of the family. Proc. Nat. Acad. Sci. India. 9: 155−167.

Olson P.D., Cribb T.H., Tkach V.V., Bray R.A., Littlewood D.T.J. 2003. Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda). Int. J. Parasitol. 33: 733−755.

Orélis-Ribeiro R., Arias C.R., Halanych K.M., Cribb T.H., Bullard S.A. 2014: Diversity and ancestry of flatworms infecting blood of nontetrapod craniates "fishes." Adv. Parasitol. 85: 1−64.

Pieper M.B. 1953: The life history and germ cell cycle of *Spirorchis artericola* (Ward, 1921). J. Parasitol. 39: 310−325.

Platt T.R. 1990: *Aphanospirorchis kirki* n. gen., n. sp. (Digenea: Spirorchidae), a parasite of the midland painted turtle, *Chrysemys picta marginata*, from northwestern Indiana, with comments on the proper spelling of the family name. J. Parasitol. 76: 650−652.

Platt T.R. 1992: A phylogenetic and biogeographic analysis of the genera of Spirorchinae (Digenea: Spirorchidae) parasitic in freshwater turtles. 78: 616−629.

Platt T.R. 1993: Taxonomic revision of *Spirorchis* MacCallum, 1919 (Digenea: Spirorchidae). J. Parasitol. 79: 337−346.

Platt T.R. 2002: Family Spirorchiidae Stunkard, 1921. In: D. I. Gibson, A. J. Jones, and R. A. Bray (Eds.), Keys to the Trematoda, Vol. 1. CABI, Wallingford, 453−467 pp.

Price E.W. 1934: New genera and species of blood flukes from a marine turtle, with a key to the genera of the family Spirorchidae. J. Wash. Acad. Sci. 24: 132−141.

Rathbun G.B., Scott Jr. N.J., Murphey T.G. 2002: Terrestrial habitat use by Pacific pond turtles in a Mediterranean climate. Southwest. Nat. 47: 225−235.

Reese D.A., Welsh H.H. 1997: Use of terrestrial habitat by western pond turtles, *Clemmys marmorata*: implications for management. In: Proceedings: Conservation, restoration, and Management of Tortoises and Turtles−An International Conference. New York Turtle and Tort. Soc. New York City, New York, USA, pp. 352−357.

Roberts J.R., Platt T.R., Orélis-Ribeiro R., Bullard S.A. 2016a: New genus of blood fluke (Digenea: Schistosomatoidea) from Malaysian freshwater turtles (Geoemydidae) and its phylogenetic position within Schistosomatoidea. J. Parasitol. 102: 451−462.

Roberts J.R., Orélis-Ribeiro R., Dang B.T., Halanych K. M., Bullard S.A. 2016b: Blood flukes of Asiatic softshell turtles: revision of *Coeuritrema* Mehra, 1933 (Digenea: Schistosomatoidea) and a new species infecting Chinese softshell turtles, *Pelodiscus sinensis*, (Trionychidae) from the Da Rang River, Vietnam. Folia Parasitol. 63: 031 doi.10.14411/fp.2016.031.

Rohde K., Lee S. K., Lim H.W. 1968: Ueber drei malayische Trematoden. Ann. Parasitol. Hum. Comp. 43: 33−43.

Ryan T.J, Conner C.A., Douthitt B.A., Sterrett S.C., Salsbury C.M. 2008: Movement and habitat use of two aquatic turtles (*Graptemys geographica* and *Trachemys scripta*) in an urban landscape. Urban Ecosyst. 11: 213−225.

Sinha B.B. 1934: A new genus of blood flukes of the family Spirorchidae, from the tortoise, *Hardella thurgi* (Gray). Rec. Ind. Mus. 36: 147−151.

Smith J.W. 1997a: The blood flukes (Digenea: Sanguinicolidae and Spirorchidae) of cold-blooded vertebrates: Part I. A Review of the literature published since 1971, and bibliography. Helminthol. Abstr. 66: 255−294.

Smith J.W. 1997b: The blood flukes (Digenea: Sanguinicolidae and Spirorchidae) of cold-blooded vertebrates: Part II. Appendix I: Comprehensive parasite-host list; Appendix II: Comprehensive host-parasite list. Helminthol. Abstr. 66: 329−344.

Snyder S.D. 2004. Phylogeny and paraphyly among tetrapod blood flukes (Digenea: Schistosomatidae and Spirorchiidae). Int. J. Parasitol. 34: 1385−1392.

Spinks P.Q., Thomson R.C., McCartney-Melstad E., Shaffer H.B. 2016: Phylogeny and temporal diversification of the New World pond turtles (Emydidae). Mol. Phylogenet. Evol. 103: 85−97.

Stunkard H.W. 1922: Two new genera of North American blood flukes. Am. Mus. Novit. 39: 1−8.

Stunkard H.W. 1923: Studies on North American blood flukes. Bull. Am. Mus. Nat. Hist. 48: 165−221.

Thatcher V.E. 1954: Some helminthes parasitic in *Clemmys marmorata*. J. Parasitol. 40: 481−482.

Tkach V., Snyder S.D., Vaughan J.A. 2009: A new species of blood fluke (Digenea: Spirorchiidae) from the Malayan box turtle, *Cuora amboinensis* (Cryptodira: Geoemydidae) in Thailand. J. Parasitol. 95: 743−746.

Truong T.N., Bullard S.A. 2013: Blood flukes (Digenea: Aporocotylidae) of walking catfishes (Siluriformes: Clariidae): new genus and species from the Mekong River (Vietnam) and a note on catfish aporocotylids. Folia Parasitol. 60:237−247.

Ulmer M.J. 1959: Studies on *Spirorchis haematobium* (Stunkard, 1922) Price, 1934 (Trematoda: Spirorchiidae) in the definitive host. Trans. Am. Microsc. Soc. 78: 81−89.

van Dijk P.P., Iverson J.B., Rhodin A.G. J., Shaffer H.B., Bour R. 2014: Turtles of the world, 7th Edition: Annotated checklist of taxonomy, synonymy, distribution with maps, and conservation status. Chel. Res. Mono. 5: 329−479.

Wall L.D. 1939: Life history of *Spirorchis* sp. (Trematoda: Spirorchiidae). J. Parasitol. 25: 28.

Wall L.D. 1940: Life history of *Spirorchis parvus* (Stunkard) Trematoda: Spirorchiidae. Science. 92: 362−363.

Wall L.D. 1941a: Life history of *Spirorchis elephantis* (Cort, 1917), a new blood fluke from *Chrysemys picta*. Am. Midl. Nat. 25: 402−412.

Wall L.D. 1941b: *Spirorchis parvus* (Stunkard) its life history and the development of its excretory system (Trematoda: Spirorchiidae). Trans. Am. Microsc. Soc. 60: 221−260.

Ward H.B. 1921: A new blood fluke from turtles. J. Parasitol. 7: 114−128.

FIGURE LEGENDS

Figures 1−2. *Spirorchis* cf. *scripta* (Digenea: Schistosomatoidea) from the lung of *Deirochelys reticularia* (Latreille *in* Sonnini and Latreille) (Testudines: Emydidae) from Big Beaver Pond (32°25'44.03"N, 85°38'44.87"W), Tallapoosa River, near Tuskegee, Alabama, USA. **Fig. 1.** Body of voucher (USNM Cat. No. XXXXX), dorsal view. **Fig. 2.** Genitalia of voucher (USNM Cat. No. XXXXXX), dorsal view. *Abbreviations*: cb − caecal bifurcation; cgp − common genital pore; ci − cirrus; cs − cirrus sac; dc − dextral caecum; egg − uterine egg; ep − excretory pore; esv − external seminal vesicle; ev − excretory vesicle; isv − internal seminal vesicle; Lc − Laurer's canal; lvd − lateral vitelline collection duct; Mo − Manter's organ; mod − median oesophageal diverticulum; nc − nerve commissure; od − oviduct; oe − oesophagus; og − oesophageal gland; osr − oviducal seminal receptacle; ov − ovary; ph − pharynx; sc − sinistral caecum; t1− anterior testis; t8 − testis no. 8; t9 − testis no. 9; t10 − posterior-most testis; tvd − transverse vitelline collection duct; vd − vas deferens; ve − vasa efferentia; vln − ventrolateral nerve chords; vr − vitellarium; vt − vitelline duct.

Figures 3−4. *Spirorchis* cf. *scripta* (Digenea: Schistosomatoidea) from the lung of *Deirochelys reticularia* (Latreille *in* Sonnini and Latreille) (Testudines: Emydidae) from Big Beaver Pond (32°25'44.03"N, 85°38'44.87"W), Tallapoosa River, near Tuskegee, Alabama, USA. **Fig. 3.** Voucher (USNM Cat. No. XXXXX), ventral view. **Fig. 4.** Genitalia of voucher (USNM Cat. No. XXXXXX), ventral view. *Abbreviations*: cgp − common genital pore; ci − cirrus; cs − cirrus sac; ct − caecal terminus; dc − dextral caecum; egg − uterine egg; ep − excretory pore; esv − external seminal vesicle; ev − excretory vesicle; isv − internal seminal vesicle; Lc − Laurer's canal; lvd − lateral vitelline collection duct; Mo − Manter's organ; mod − median oesophageal diverticulum; nc − nerve commissure; od − oviduct; oe − oesophagus; og − oesophageal gland; os − oral sucker; osr − oviducal seminal receptacle; ov − ovary; ph − pharynx; sc − sinistral caecum; t1− anterior testis; t8 − testis no. 8; t9 − testis no. 9; t10 − posterior-most testis; tvd − transverse vitelline collection duct; vd − vas deferens; ve − vasa efferentia; vln − ventrolateral nerve chords; vr − vitellarium; vt − vitelline duct.

Figures 5−6. Oesophagus, lateral oesophageal diverticula, and oesophageal gland characteristic of *Spirorchis* MacCallum, 1918. **Fig. 5.** Oesophagus of paratype (AMNH 130), *Spirorchis scripta* Stunkard, 1923 (Digenea: Schistosomatoidea) from mesenteric vessels of *Trachemys scripta* (Thunberg *in* Schoepff) (Testudines: Emydidae) from Neuse River, Raleigh, North Carolina, USA, dorsal view. **Fig. 6.** Oesophagus of holotype (USNM Cat. No. XXXXX), *Spirorchis collinsi* Roberts et Bullard, sp. n. (Digenea: Schistosomatoidea) from the kidney blood vessels of *Deirochelys reticularia* (Latreille *in* Sonnini and Latreille, 1801) (Testudines: Emydidae) from Big Beaver Pond (32°25'44.03"N, 85°38'44.87"W), Tallapoosa River, near Tuskegee, Alabama, USA, dorsal view. *Abbreviations*: cb − caecal bifurcation; mo − mouth; mod − median oesophageal diverticulum; oe − oesophagus; og − oesophageal gland; os − oral sucker; nc − nerve commissure; ph − pharynx.

Figures 7−8. *Spirorchis collinsi* Roberts et Bullard, sp. n. (Digenea: Schistosomatoidea) from the kidney blood vessels of *Deirochelys reticularia* (Latreille *in* Sonnini and Latreille) (Testudines: Emydidae) from Big Beaver Pond (32°25'44.03"N, 85°38'44.87"W), Tallapoosa River, near Tuskegee, Alabama, USA. **Fig. 7.** Body of holotype (USNM Cat. No. XXXXX), dorsal view. **Fig. 8.** Genitalia of holotype (USNM Cat. No. XXXXXX), dorsal view. *Abbreviations*: cb − caecal bifurcation; cgp − common genital pore; ci − cirrus; cs − cirrus sac; dc − dextral caecum; ep − excretory pore; esv − external seminal vesicle; ev − excretory vesicle; isv − internal seminal vesicle; Lc − Laurer's canal; Mg − Mehlis' gland; Mo − Manter's organ; mod − median oesophageal diverticulum; mt − metraterm; nc − nerve commissure; od − oviduct; oe − oesophagus; og − oesophageal gland; os − oral sucker; oo − oötype; osr − oviducal seminal receptacle; ov − ovary; ph − pharynx; sc − sinistral caecum; t1− anterior testis; t8 − testis no. 8; t9 − testis no. 9; t10 − posterior-most testis; tvd − transverse vitelline collection duct; vd − vas deferens; ve − vasa efferentia; vln − ventrolateral nerve chords; vr − vitellarium; vt − vitelline duct.

Figure 9. Phylogenetic relationships of blood flukes reconstructed by Bayesian inference and based on partial D1−D3 domains of 28S from 11 TBF taxa. Numbers aside tree nodes indicate posterior probability.

Table 1. Turtle blood fluke sequences used in the present study.

Turtle blood flukes	Host species	Locality	GenBank Accession Numbers 28S	Reference(s)
Baracktrema obamai Roberts, Platt et Bullard, 2016	*Siebenrockiella crassicollis* (Gray)	Perak (probably Perak River), Malaysia	KX061500	Roberts et al. (2016a)
Spirhapalum polesianum Ejsmont, 1927	*Emys orbicularis* (Linnaeus)	Lesniki, Ukraine	AY604705	Snyder (2004)
Spirhapalum siamensis Tkach, Snyder et Vaughn, 2009	*Cuora amboinensis* (Riche *in* Daudin)	16°42'N, 98°34'E, Mae Sot, Thailand	FJ481166	Tkach et al. (2009)
Spirorchis artericola (Ward, 1921) Stunkard, 1921	*Chrysemys picta* (Schneider)	Reelfoot Lake, Tennessee, USA	AY604704	Snyder (2004)
***Spirorchis collinsi* sp. n.**	*Deirochelys reticularia* (Latreille *in* Sonnini and Latreille)	32°25'44.03"N, 85°38'44.87"W, Big Beaver Pond, near Uphapee Creek, Tallapoosa River, Tuskegee, Alabama, USA	**XXXXXX**	Present study
Spirorchis haematobius (Stunkard, 1922) Price, 1934	*Chelydra serpentina* (Linnaeus)	Missouri River, Fremont County, Iowa, USA	FJ481164	Tkach et al. (2009)
***Spirorchis picta* Stunkard, 1923**	*Trachemys scripta* (Thunberg *in* Schoepff)	32°38'52.78"N, 85°29'7.20"W, E.W. Shell Fisheries Station, Tallapoosa River drainage, Auburn University, Auburn, Alabama, USA	**XXXXXX**	Present study
Spirorchis scripta Stunkard, 1923	*Trachemys scripta*	Moungers Creek, Pascagoula Bay drainage, Vancleave, Mississippi, USA	AY222174	Olson et al. (2003)
Spirorchis* cf. *scripta	*Deirochelys reticularia*	32°25'44.03"N, 85°38'44.87"W, Big Beaver Pond, near Uphapee Creek, Tallapoosa River, Tuskegee, Alabama, USA	**XXXXXX**	Present study
Unicaecum sp.	*Trachemys scripta*	Reelfoot Lake, Tennessee, USA	AY604711	Snyder (2004)
Vasotrema robustum	*Apalone spinifera*	Nishnabotna River, Floyd	AY604706	Snyder (2004)

Stunkard, 1928	(LeSueur)	County, Iowa, USA

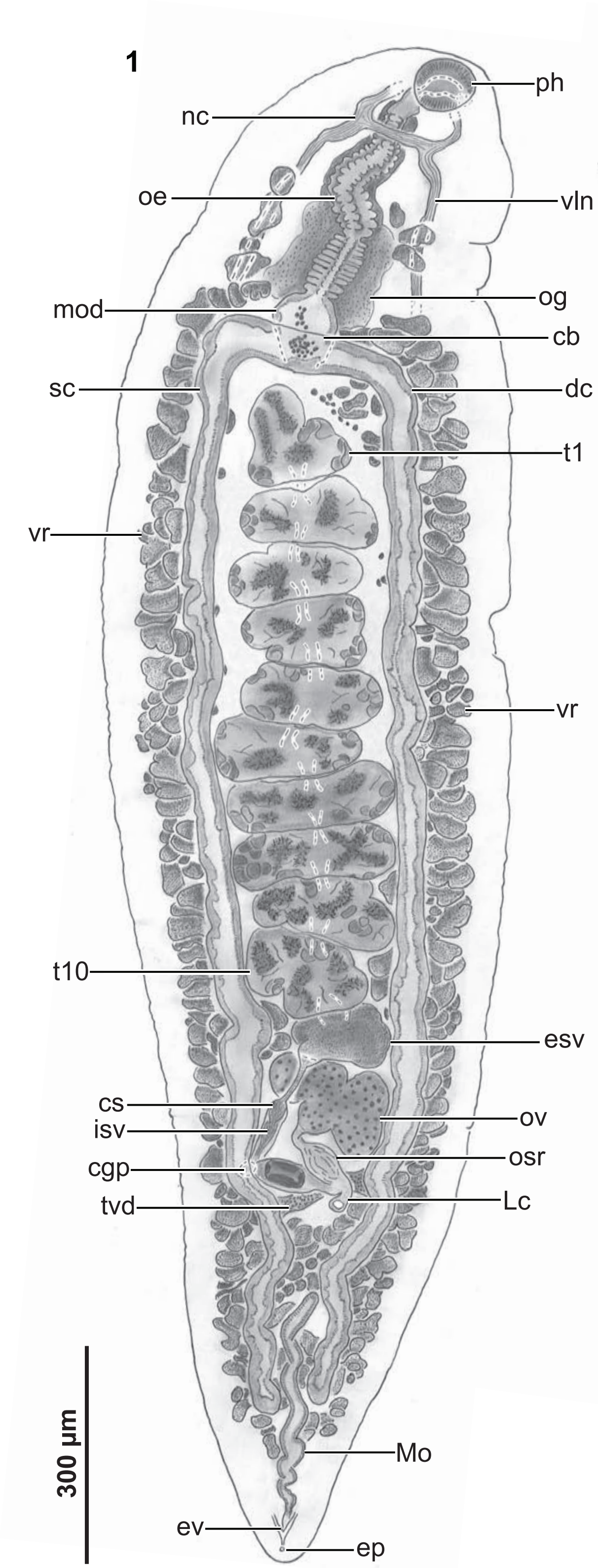
Table 2. Turtle blood fluke specimens examined in the present study.

Turtle blood fluke	Slide label	Accession No.	No. slides	Specimen	Host	Locality	Notes	Reference(s)
Spirorchis artericola (Ward, 1921) Stunkard, 1921	*Proparorchis artericola*	USNM 1350610	5	holotype; paratypes (4)	*Trachemys scripta* (Thunberg *in* Schoepff) (labeled *Pseudemys scripta*)	Illinois River, Havana, Illinois, USA		Ward (1921)
Spirorchis innominatus Ward, 1921 (type species)	*Spirorchis eustreptos*	USNM 1337308	1	holotype; paratypes (2); *Telorchis* sp. (3)	*Glyptemys insculpta* (Le Conte) (labeled as *Chelopus insculptus*)	origin not specified, New York Aquarium, New York, USA	holotype circled, labeled as "TYPE"	MacCallum (1918), (1926); see also Ward (1921)
Spirorchis haematobius (Stunkard, 1922) Price, 1934	*Henotosoma haematobium*	AMNH 126	1	holotype	*Chelydra serpentina* (Linnaeus)	Kankakee River, North Judson, Indiana, USA	misspelled as *Henostoma* in AMNH database	Stunkard (1922)
		AMNH 1264	10	paratypes (9); tissue serially sectioned		Neuse River, Raleigh, North Carolina, USA	1 intact specimen, 8 partial specimens	
Spirorchis elegans Stunkard, 1923	*Spirorchis elegans*	AMNH 134	1	holotype	*Trachemys scripta* (labeled *Pseudemys elegans*)	Illinois River, Havana, Illinois, USA		Stunkard (1923)
		AMNH 1516	1	paratype				
Spirorchis parvus (Stunkard, 1923) Price, 1934	*Haematotrema parvum*	AMNH 133	1	holotype	*Chrysemys picta* (Schneider)	Hudson River, Cold Spring, New York, USA		Stunkard (1923)
Spirorchis picta Stunkard, 1923	*Spirorchis picta*	AMNH 131	1	holotype	*Chrysemys picta*	New York City, New York, USA		Stunkard (1923)
		AMNH 1515	1	paratype				
Spirorchis scripta Stunkard, 1923	*Spirorchis scripta*	AMNH 128	1	holotype	*Trachemys scripta* (labeled	Neuse River, Raleigh, North	specimen damaged	Stunkard (1923)

					Pseudemys scripta)	Carolina, USA		
		AMNH 130	1	paratype				
Spirorchis minutus Byrd, 1939	*Spirorchis minutum*	USNM 1321965	1	holotype	*Chelydra serpentina*	Reelfoot Lake, Tennessee, USA	specimen damaged	Byrd (1939)
Spirorchis kirki (Platt, 1990) Platt, 1992	*Aphanospirorchis kirki*	Dr. Thomas R. Platt's collection	4	paratypes (4)	*Chrysemys picta marginata* (Agassiz)	St. Joseph River, Center Township, St. Joseph County, Indiana, USA	Specimens part of *TRP's personal collection, (donated to SAB)	Platt (1990)

* Dr. Thomas R. Platt, Professor Emeritus, Saint Mary's College, Notre Dame, Indiana, USA

1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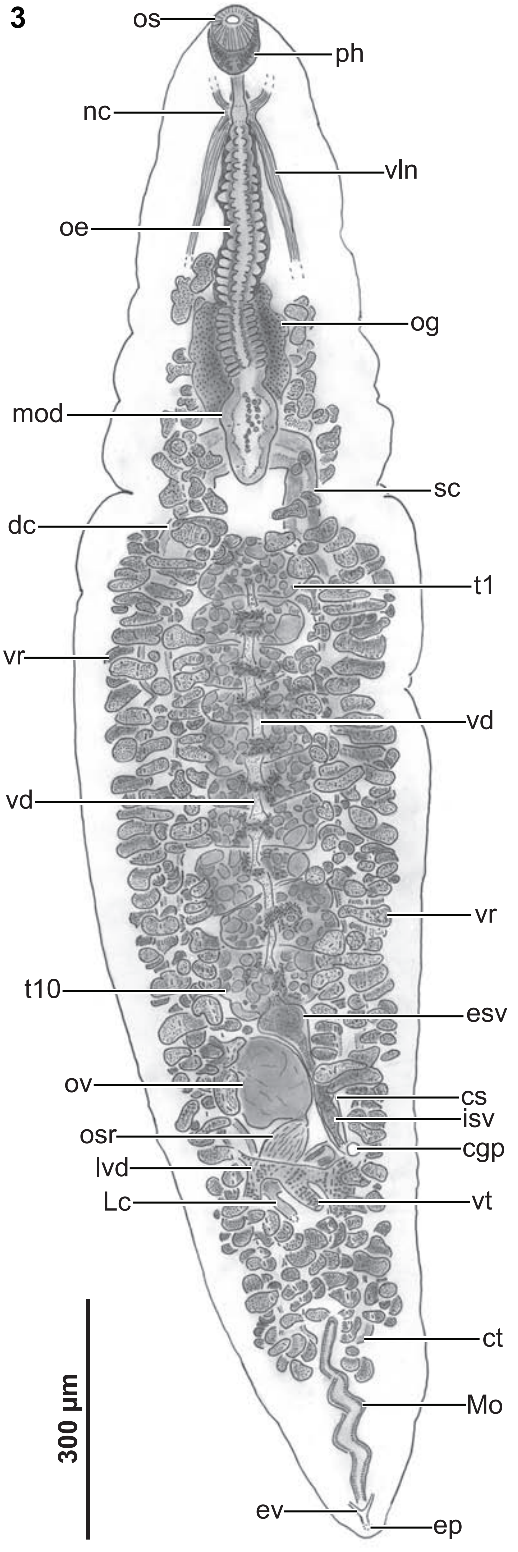
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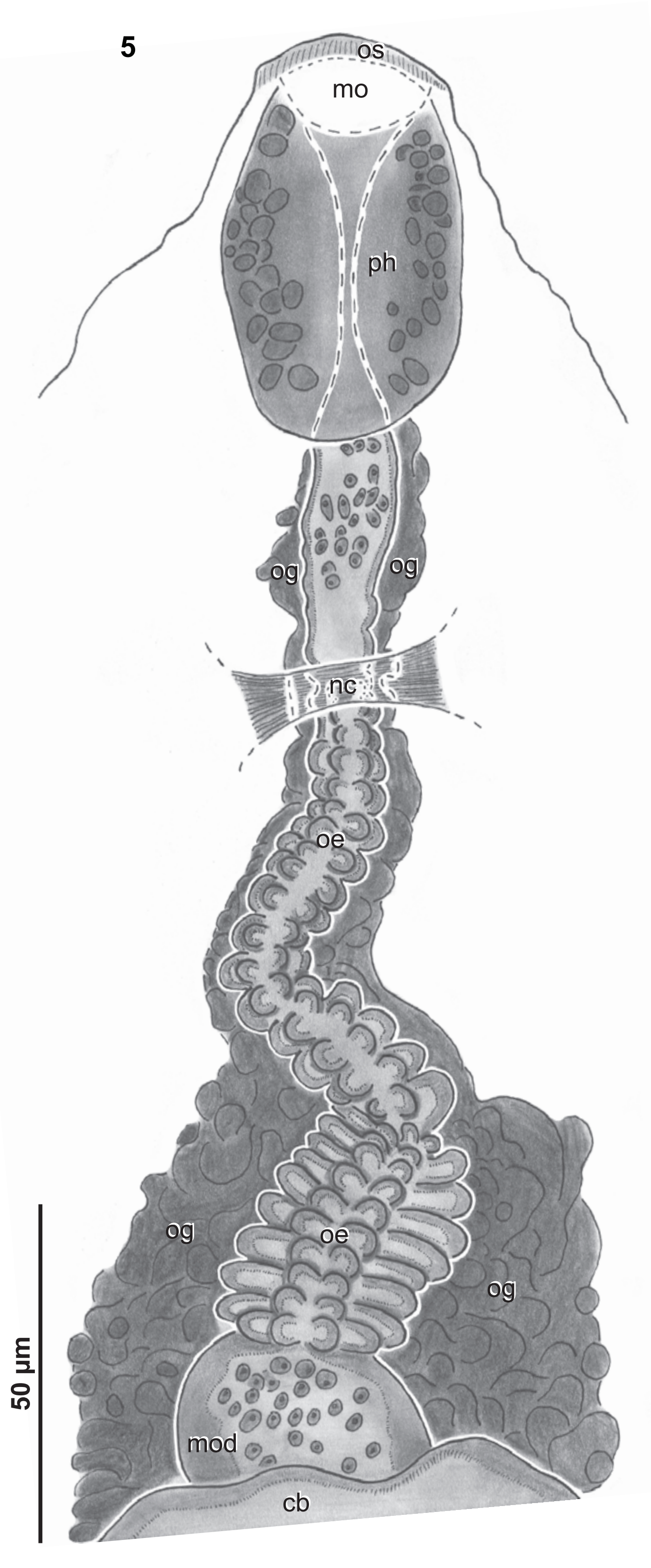
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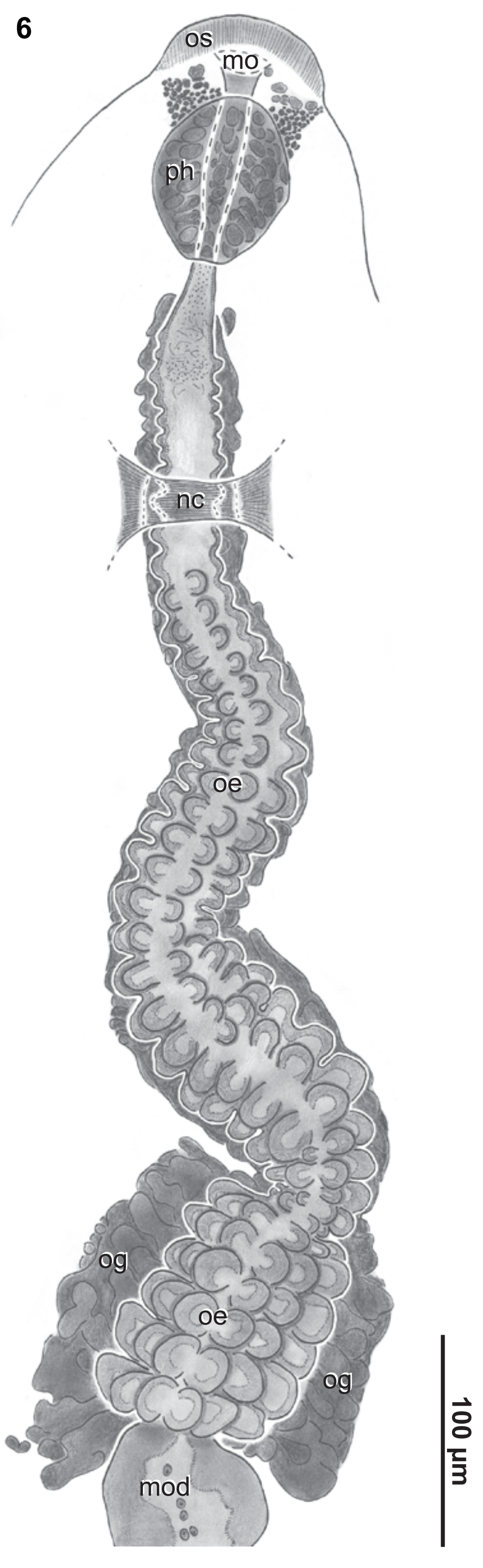
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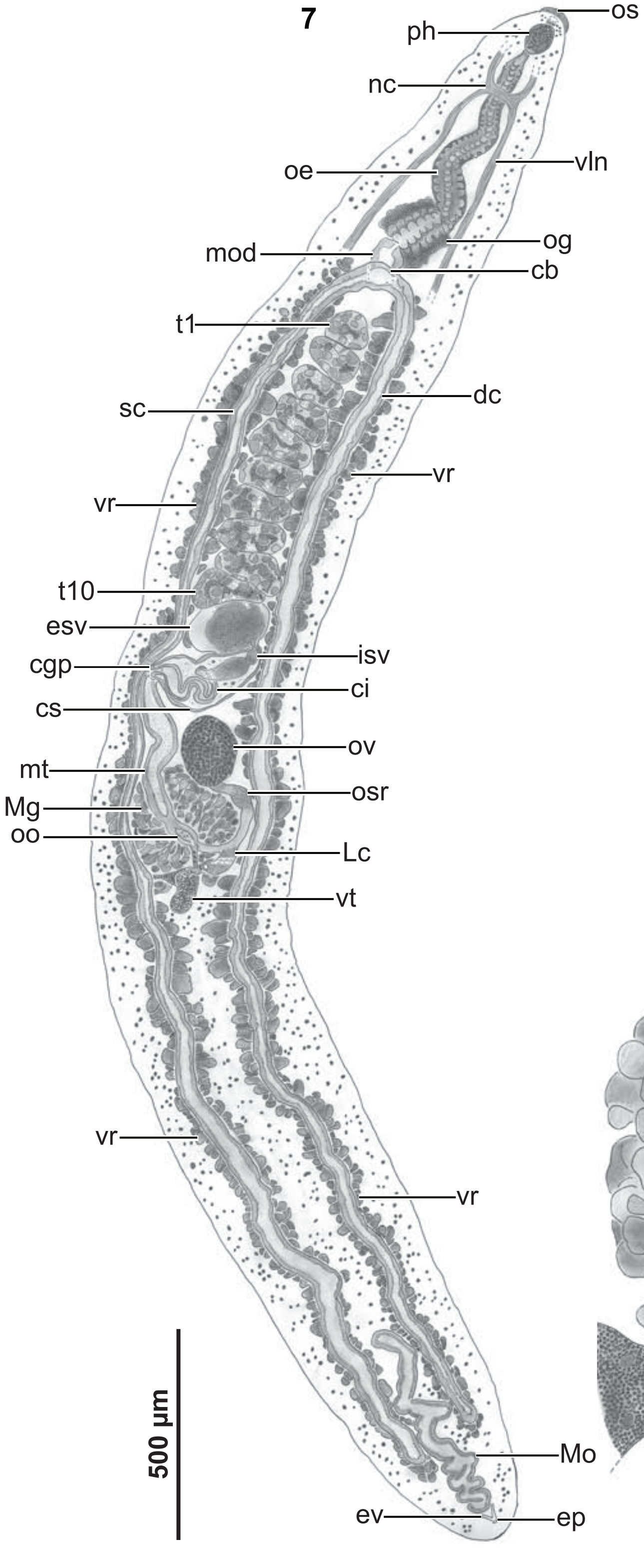
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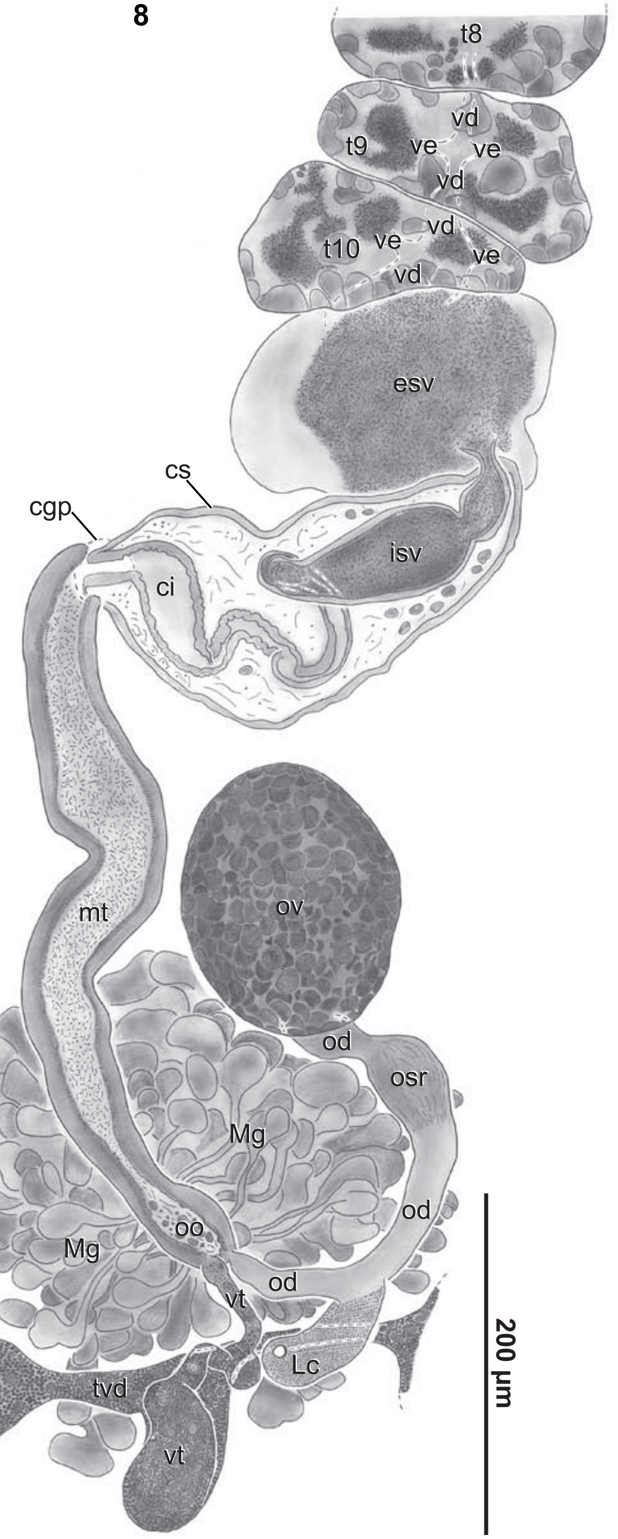
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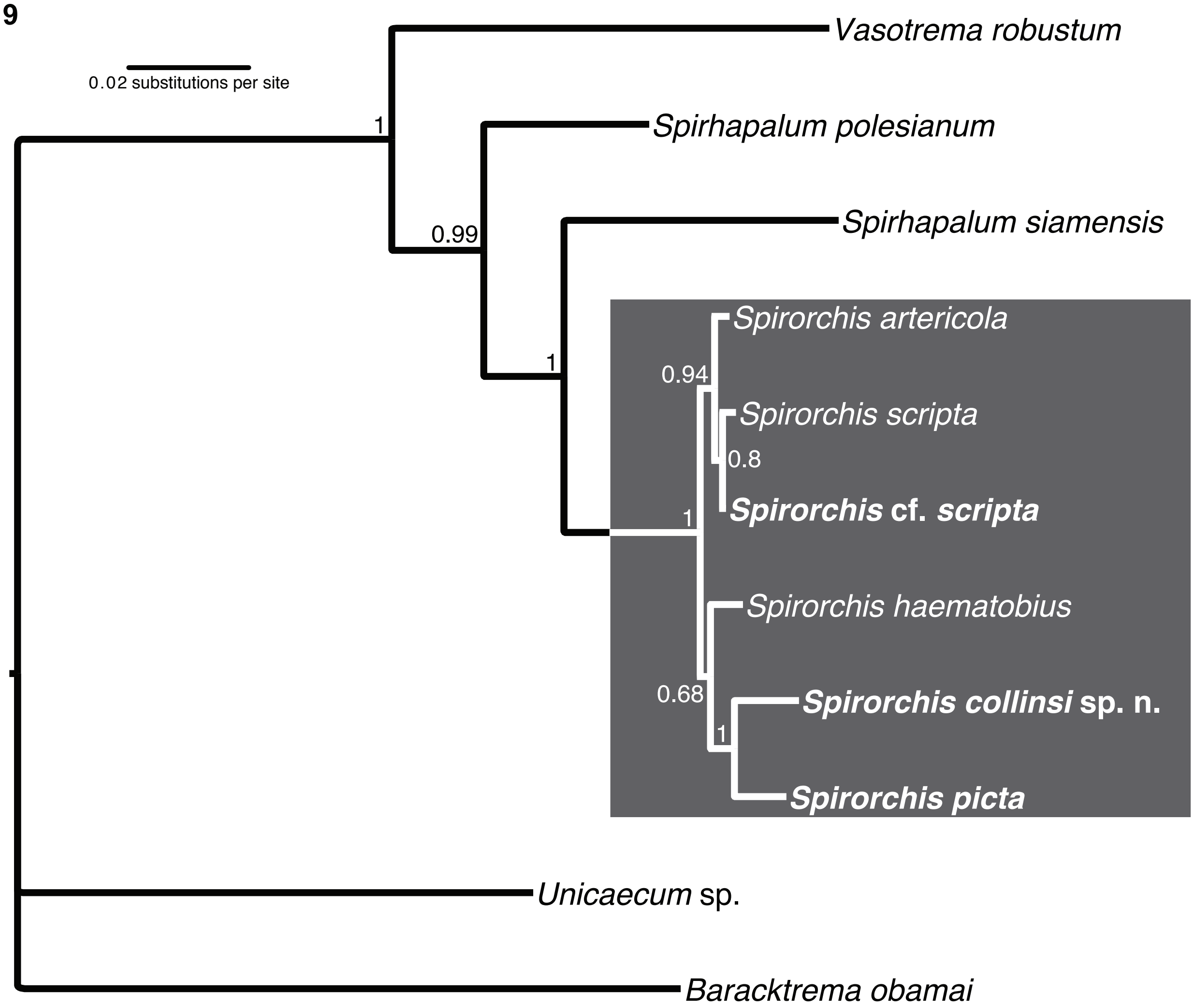
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CHAPTER 4: REVISION AND NEW SPECIES OF *VASOTREMA* STUNKARD, 1926 (DIGENEA: SCHISTOSOMATOIDEA): TURTLE BLOOD FLUKES OF NORTH AMERICAN SOFTSHELL TURTLES (TESTUDINES: TRIONYCHIDAE: *APALONE* SPP.)

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Authors: Jackson R. Roberts and Stephen A. Bullard

ABSTRACT

Gulf Coast spiny softshell turtles, *Apalone spinifera aspera* (Agassiz, 1857), (Testudines: Trionychidae) from Canoe Lake (33°47'56.16"N, 86°29'25.02"W; Springville, Alabama) and Round Lake (32°41'50.91"N, 87°14'30.39"W; Perry Lakes State Park, Marion, Alabama), were infected by *Vasotrema robustum* Stunkard, 1928, *Vasotrema longitestis* Byrd, 1939, and *Vasotrema rileyae* n. sp. The new species differs from its congeners by having papillate suckers, a short testis, an ovary dextral to the oviduct, and a pre-ovarian genital pore that is lateral to the ventral sucker. We studied the newly collected specimens and museum specimens of all congeners to revise the diagnosis of *Vasotrema* Stunkard, 1926 and redescribe and provide an updated dichotomous key to all species of the genus.

INTRODUCTION

Extant softshell turtles (Testudines: Trionychidae) comprise 13 genera and 32 nominal species that range in the Nearctic, Ethiopian, and Indomalayan realms (van Dijk et al., 2014; Guyer et al., 2015). Softshell turtles are ‘good hosts’ for turtle blood flukes (Digenea: Schistosomatoidea; paraphyletic "Spirorchiidae"; TBFs hereafter): 9 of

32 (28%) are infected by 18 TBFs assigned to 5 genera (*Coeuritrema* Mehra, 1933, *Enterohaematotrema* Mehra, 1940, *Cardiotrema* Dwivedi, 1967, *Hapalorhynchus* Stunkard, 1922, and *Vasotrema* Stunkard, 1926). These TBFs range in the Nearctic and Indomalayan realms (Smith, 1997a, 1997b; Roberts et al., 2016a, 2016b). There are 4 extant Nearctic trionychids: Gulf Coast smooth softshell turtle, *Apalone calvata* (Webb, 1959), Florida softshell turtle, *Apalone ferox* (Schneider, 1783), midland softshell turtle, *Apalone mutica* (LeSueur, 1827), and spiny softshell turtle, *Apalone spinifera* (LeSueur, 1827).

The 5 nominal species of *Vasotrema* (i.e., *Vasotrema amydae* Stunkard, 1926 [type species], *Vasotrema attenuatum* Stunkard, 1928, *Vasotrema robustum* Stunkard, 1928, *Vasotrema longitestis* Byrd, 1939, and *Vasotrema brevitestis* Brooks and Mayes, 1975) infect softshell turtles only. No record of a TBF infection exists from Gulf Coast smooth softshell turtle. Despite these being relatively commonly-encountered parasites of North American trionychids (Table I), few of these TBFs have been detailed since their original description, type materials are generally poor, and species- and genus-level anatomical details are indeterminate for most of the taxa. In some instances, the original species descriptions were sketchy and incomplete.

As part of an ongoing TBF survey in the Southeastern United States, we collected 3 TBF species from a few Gulf Coast spiny softshell turtles, *Apalone spinifera aspera* (Agassiz, 1857), in Alabama: *V. robustum*, *V. longitestis*, and a new species resembling *V. amydae*. In addition to describing the new species, we used these newly collected specimens plus examinations of all extant type materials for all species of *Vasotrema* to revise the genus, redescribe its nominal species, and produce an updated dichotomous key for species of the genus.

MATERIALS AND METHODS

Four Gulf Coast spiny softshell turtles were collected using hoop-nets baited with store-bought chicken liver and fish on 25 June 2015 and 23 July 2015 from Canoe Lake (33°47'56.16"N, 86°29'25.02"W), Springville, Alabama, and Round Lake (32°41'50.91"N, 87°14'30.39"W), Perry Lakes State Park, Marion, Alabama, respectively. Turtles were transported alive to the laboratory in a cooler with pond water within an air conditioned vehicle cab, decapitated before necropsy, and examined with the aid of 7.0 g/L sodium citrate saline solution and a stereo-dissection microscope. Each host organ (brain, eye, heart, lung, spleen, liver, intestine, mesentery, kidney, rectum) was isolated in a glass container filled with saline. Portions of each organ then were excised and macerated in a petri dish while viewing under high magnification with a Meiji Techno RZ (Meiji, Saitama, Japan) 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 or fallen from the excised organ/tissue. Living flukes were pipetted from saline dishes, concentrated in a clean glass dish with saline, rinsed in saline, pipetted onto glass slides, cover-slipped (only to ensure the flukes remained flat; no pressure exerted on specimen by coverslip), and killed with a 2-sec exposure to heat emitted from a butane hand lighter. After heat killing, a few drops of saline were applied to the edge of the coverslip before the coverslip was lifted carefully with fine forceps and the fluke was washed from the slide into a clean dish of 5% neutral buffered formalin (n.b.f.). These flukes, intended for morphology, were held in 5% n.b.f. until staining. Upon staining, specimens were rinsed with distilled water, stained in Van Cleave's hematoxylin with several drops of Ehrlich's hematoxylin, dehydrated with a graded ethanol series, dehydrated in absolute EtOH and xylene, cleared with clove oil, and permanently mounted in Canada balsam.

Whole mounts were examined using both a Leica DM 2500 (Leica, Wetzlar, Germany) and Leica DMR microscopes, both equipped with differential interference contrast (DIC). Illustrations were made using both scopes equipped with drawing tubes. Measurements were obtained with a calibrated ocular micrometer (as straight-lines along the course of each duct) and are herein reported in micrometers (µm) followed by their mean and number measured in parentheses. For analyses including newly collected vouchers and type materials (*V. robustum* and *V. longitestis*), the voucher measurements are presented first, with the type specimen measurements immediately following in brackets ("n/a" indicates not available due to poor specimen quality or absence of feature). Turtle scientific names and taxonomic authorities follow van Dijk et al. (2014) and Guyer et al. (2015). Classification and anatomical terms for TBFs follow Roberts et al. (2016a, 2016b, 2016c), and Yong et al. (2016; paired terminal papillae).

Examined museum specimens were borrowed from the United States National Museum Parasite Collection, Smithsonian Institution (USNM), the American Museum of Natural History (AMNH), and the Harold W. Manter Laboratory Collection (HWML). Holotype and paratypes of the new species and newly collected voucher specimens were deposited in the USNM. Detailed list of examined specimens is provided in Table II.

***Vasotrema* Stunkard, 1926 emended**

(Figs. 1−20)

Diagnosis: Body dorsoventrally flattened (not cylindrical), 3−20× longer than wide, aspinous; ventral body surface papillate. Oral sucker spheroid, papillate or apapillate, spinous or aspinous; paired terminal papillae present or absent; paired internal mouth papillae present or absent. Ventral sucker papillate or apapillate. Pharynx present, enveloping anterior extremity of esophagus. Esophagus sinuous, extending posteriad approximately 1/10−1/4 of body length, having numerous lateral esophageal diverticula and a single median esophageal diverticulum and associated esophageal gland; lateral esophageal diverticula surrounding esophagus for entire length, becoming larger and more numerous posteriorly; median esophageal diverticulum dorsal to intestinal bifurcation; esophageal gland surrounding esophagus from posterior margin of pharynx to cecal bifurcation, strongly basophilic, widest surrounding medial esophageal diverticulum (Figs. 5, 6). Intestine inverse U-shaped, comprising paired ceca; each cecum extending 1/2−3/4 of body length directly posteriad and in parallel with body margin, terminating in posterior end of body, smooth (lacking diverticula). Testis coiled or not, intercecal. Vas deferens extending anteriad from and ventral to anterior half of testis. External seminal vesicle present or indistinct, pre-testicular, intercecal. Internal seminal vesicle present. Cirrus sac pre-testicular. Cirrus straight. Ovary lobed or not, intercecal, pre-testicular, between cirrus sac and testis. Oviduct emerging from sinistral, dextral, or posterior margin of ovary, extending posteriad or sinistrad. Oviducal seminal receptacle comprising middle portion of oviduct, at level of or posterior to ovary. Laurer's canal intercecal, pre-testicular, pre-ovarian or post-ovarian, extending anteriad or posteriad from oviduct, opening dorsally. Vitellarium follicular, distributing from cecal bifurcation to Manter's organ. Oötype diminutive, pre-testicular, intercecal, anterior to putative transverse vitelline duct. Mehlis gland indistinct. Uterus short, accommodating a single large egg. Metraterm short, at level of ovary or pre-gonadal. Uterine pouch absent. Common genital pore opening on ventral body surface at level of sinistral cecum, lacking suckers. Excretory vesicle y-shaped; pore terminal. Manter's organ present. Infecting blood of North American trionychids.

Differential diagnosis: Body dorsoventrally flattened (not cylindrical), aspinous. Oral sucker and pharynx present. Ventral sucker present. Lateral esophageal diverticula surrounding esophagus for entire length, becoming larger and more numerous posteriorly; median esophageal diverticulum dorsal to intestinal bifurcation. Testis intercecal. Ovary intercecal, pre-testicular, between cirrus sac and testis. Vitellarium distributing from cecal bifurcation to Manter's organ. Oötype intercecal, anterior to testis and putative transverse vitelline duct. Uterus short, accommodating a single, large egg. Metraterm short, at level of ovary or pre-gonadal. Uterine pouch absent. Common genital pore opening on ventral body surface ventral to sinistral cecum, lacking suckers. Manter's organ present.

Type species: Vasotrema amydae Stunkard, 1926

Remarks

Stunkard (1926) proposed *Vasotrema* (misspelled therein as “*Vasatrema,*” which thereby is junior synonym of *Vasotrema* [ICZN Article 19.2]) to accommodate a new TBF infecting Florida softshell turtles and spiny softshell turtles. Stunkard (1928) later named 2 additional congeners and corrected the genus spelling, an error that he attributed to “transcription error.” The 5 nominal species of *Vasotrema* (*V. amydae*, *V. attenuatum*, *V. robustum*, *V. longitestis*, *V. brevitestis*) infect softshell turtles (Trionychidae) only, and we accept all of them as distinct taxa (see dichotomous key).

Recent molecular analyses indicate that *Vasotrema* is most closely related to *Spirorchis* MacCallum, 1918 and *Spirhapalum* Ejsmont, 1927 (Orélis-Ribeiro et al., 2014; Roberts et al., 2016a, 2016c). *Vasotrema* resembles these TBFs by having an oral sucker, lateral esophageal diverticula, U-shaped ceca terminating in the posterior body end, a putative transverse vitelline duct, and a ventral and sinistral common genital pore (Roberts et al., 2016c). *Vasotrema* can be differentiated from *Spirorchis* by having a ventral sucker, a median esophageal diverticulum dorsal to the cecal bifurcation, a single testis, and a pre-testicular ovary. *Spirorchis* lacks a ventral sucker and has a median esophageal diverticulum ventral to the cecal bifurcation (when present), a testicular column comprising 4−11 testes, and a post-testicular ovary. *Vasotrema* differs from *Spirhapalum* by having a single testis and a pre-testicular ovary. *Spirhapalum* has a pre-ovarian testicular column comprising 4−12 testes with a post-ovarian testis as well. Further comparisons of *Vasotrema* and *Spirhapalum* are impossible without obtaining type materials because their original descriptions are incomplete (Ejsmont, 1927; Rohde et al., 1968).

We herein revise the generic diagnosis for *Vasotrema* to include features associated with spines, papillae, pharynx, esophageal diverticula, vitellarium, and uterus. Some of these features were misinterpreted or omitted from previous concepts of the genus and descriptions of its species. Regarding spines, Wall (1951) and Brooks and Mayes (1975) reported that tegumental body spines were present on *V. robustum* and *V. brevitestis*, respectively; however, we found no evidence of a tegumental spine on any species of *Vasotrema*. Regarding sucker papillae, none had previously been detailed in any species of TBF (Platt, 2002) but we describe several types: marginal papillae of the oral sucker and ventral sucker (Figs. 1, 7, 9, 10, 13, 19), paired terminal papillae dorsal to the mouth (Figs. 1, 9, 10, 19), and paired papillae within the mouth cavity (Fig. 10). Voucher specimens of *Hapalotrema mehrai* Rao, 1976, from Thomas R. Platt's collection indicate that *Hapalotrema* Looss, 1899 may have a papillate ventral sucker also (J. R. Roberts and S. A. Bullard, unpubl. data). These specimens have a ventral sucker with large spinose crenulations similar to the marginal papillae described herein**.** Morphologically-similar papillae are present in some genera of fish blood flukes (Digenea: Aporocotylidae; Bullard and Overstreet, 2004; Truong and Bullard, 2013; Yong et al., 2016). As these structures are present in phylogenetically unrelated taxa (Orélis-Ribeiro et al., 2014), they likely evolved independently. Perhaps these papillae function to allow the fluke to sense their location within the turtle’s vascular system or to sense the presence of other flukes.

Regarding the pharynx, Stunkard (1926, 1928) and others (Byrd, 1939; Wall, 1951; Brooks and Mayes, 1975; Platt, 2002) diagnosed *Vasotrema* as lacking a pharynx. As in species of *Spirorchis*, *Unicaecum* Stunkard, 1925, *Coeuritrema*, and *Baracktrema*

Roberts, Platt, and Bullard, 2016, we suspect that previous workers misinterpreted the pharynx as a component of the oral sucker because it is immediately dorsal to the oral sucker (Roberts et al., 2016a, 2016b, 2016c). Regarding the esophageal diverticula, comprising numerous lateral diverticula plus a median diverticulum dorsal to the cecal bifurcation, previous descriptions detailed the posterior-most diverticula only. The diverticula emanating laterally from the anterior portion of the esophagus are considerably smaller and more difficult to delineate from the esophageal gland; which envelops the esophagus and its diverticula. Similar to *Spirorchis*, the diverticula surround the esophagus for its entire length and become larger and more numerous posteriorly (Figs. 5, 6; Roberts et al., 2016c). Regarding the median esophageal diverticulum, Stunkard (1928) emphasized that he did not observe a "poche [pouch]" at the esophageal-cecal junction as he did in *Spirorchis* spp. (Stunkard, 1923). Whereas the median esophageal diverticulum identified in *Vasotrema* spp. is similar to, and probably homologous to, that described in *Spirorchis* spp. (Roberts et al., 2016c), it differs by being dorsal to, rather than ventral to, the cecal bifurcation. Our results herein confirmed that the median esophageal diverticulum is present in all species of *Vasotrema*; however, it is evidently lacking in some species of *Spirorchis* (i.e., *Spirorchis elegans* Stunkard, 1923; see Stunkard, 1923; Platt, 1993; Roberts et al., 2016c).

Regarding the vitellarium, Stunkard (1928) diagnosed it as distributing from the ovary posteriad to the termination of the ceca (even though he described the vitellarium of *V. robustum* as extending to ventral sucker). The newly collected voucher specimen of *V. robustum* had a vitellarium that extended anteriad to the cecal bifurcation.

Regarding the uterus, Stunkard (1928) defined the oötype as the duct proximal to the metraterm. We observed that in gravid individuals the egg occupied the lumen of the female reproductive tract proximal to the metraterm, making the oötype and uterus difficult to differentiate even in exceptionally well-stained specimens. That these features are difficult to discern in gravid specimens led us to refer to these combined features as an egg chamber. This is similar to the condition in species of *Coeuritrema* (see Roberts et al., 2016b) and *Spirorchis* (see Roberts et al., 2016c).

How vitelline material is collected is a perplexing anatomical problem with species of *Vasotrema*. This problem likely requires the study of serially-sectioned specimens, not wholemounts. In our newly collected materials and museum specimens, a large duct passes ventral to the cecum, extends mediad, curves slightly posteriad, and expands (= primary vitelline duct; Stunkard [1928]; Wall [1951]) before extending anteriad along the midline to join the oviduct (Figs. 2, 6, 8, 11, 14, 16, 18, 20). We did not locate a collecting duct serving both the dextral and sinistral vitelline fields, i.e., a transverse vitelline duct was absent. Like in *Spirorchis* and *Spirhapalum*, which both have a transverse vitelline duct, *Vasotrema* has a bilaterally symmetrical vitellarium, with dextral and sinistral fields (Ejsmont, 1927; Rohde et al., 1968; Tkach et al., 2009; Roberts et al., 2016c). The opposite vitelline collecting duct may be diminutive or absent. If absent, vitelline material must be routed through intertwined collecting ducts before coalescing into the primary vitelline duct. Fish blood flukes lack a transverse vitelline duct, and vitelline material is gathered and routed to the primary vitelline duct as such (Bullard and Overstreet, 2004; Bullard et al., 2006, 2008; Bullard and Jensen, 2008; Truong and Bullard, 2013; Orélis-Ribeiro and Bullard, 2015).

Vasotrema amydae **Stunkard, 1926 (type species)**

(Figs. 1, 2)

Description of adult (based on light microscopy of a cotype [AMNH 791]): Body 1,310 long or 10.1× longer than wide, 105 wide or 8% of body length at level of cecal bifurcation, 120 wide or 9% of body length at level of ventral sucker, 130 wide or 10% of body length at level of ovary, 130 wide or 10% of body length at level of testis, 100 wide or 8% of body length at level of cecal terminus; forebody (middle of ventral sucker to anterior body end) 340 long or 26% of body length; hindbody (middle of ventral sucker to posterior body end) 970 long or 74% of body length, 2.9× longer than forebody (Fig. 1). Oral sucker putatively spinous, papillate, 38 long or 3% of body length, 45 wide or 43% of body width at level of cecal bifurcation; paired terminal papillae 2 in number, dorsal to mouth, together 13 long by 28 wide (Fig. 1); paired internal mouth papillae not observed. Ventral sucker papillate, 45 long or 3% of body length, 50 wide or 42% of body width. Nerve commissure 120 or 9% of body length from anterior body end. Pharynx 35 long or 12% of esophagus length, 50 wide or 10.0× esophagus width immediately posterior to pharynx, 1.4× wider than long. Esophagus extending posteriad 295 long or 23% of body length from mouth to posterior margin of median esophageal diverticulum, 5 wide posterior to pharynx, with wall 3 thick, 10 wide at esophagus median or 10% of body width at level of cecal bifurcation, with wall 5 thick; posterior-most lateral esophageal diverticula radially expanded, 100 long or 8% of body length or 34% of total esophagus length, 70 wide or 67% of body width at level of cecal bifurcation, 15 or 14% of body width from dextral body margin, 13 or 12% of body width from sinistral body margin; median esophageal diverticulum 38 long or 13% of esophagus length, 20 wide or 19% of body width at level of cecal bifurcation, with wall 12 thick; esophageal gland 245 long or 19% of body length, 100 wide or 95% of body width at level of cecal bifurcation (Fig. 1). Intestinal bifurcation 295 from anterior body end or 23% of body length; sinistral cecum 830 long or 63% of body length, 13 wide or 12% of body width at level of cecal bifurcation, 13 wide or 10% of body width at level of ovary, 13 wide or 13% of body width at level of cecal terminus; dextral cecum 810 long or 62% of body length, 18 wide or 17% of body width at level of cecal bifurcation, 15 wide or 12% of body width at level of ovary, 13 wide or 13% body width at level of cecal terminus; post-cecal distance 210 or 16% of body length (Fig 1).

Testis turning 11 times, 365 long or 28% of body length, 25 wide or 19% of body width at level of testis, 14.6× longer than wide, 195 or 15% of body length to cecal terminus, 375 or 29% of body length from posterior body end (Fig. 1). Vas deferens not observed, putatively ventral to gonads; external seminal vesicle discernible from vas deferens, intercecal, 123 long or 9% of body length, 43 wide or 33% of body width at level of testis, 2.9× longer than wide; internal seminal vesicle 40 long or 3% of body length, 15 wide or 12% of body width, 2.7× longer than wide (Figs. 1, 2). Cirrus sac 75 long or 6% of body length, 20 wide or 15% of body width at level of testis, 1.3× wider than internal seminal vesicle; cirrus straight, 28 long or 2% of body length, 8 wide or 6% of body width (Figs. 1, 2).

Ovary spheroid or weakly lobate, intercecal, nearly abutting sinistral cecum, abutting posterior margin of external seminal vesicle, 190 or 15% of body length posterior to middle of ventral sucker, 38 long or 2% of body length, 25 wide or 19% of body width, 0.7× wider than long (Fig. 1); post-ovarian distance 765 or 58% of body length (Figs. 1, 2). Oviduct extending dextrad 10 long or 1% of body length, 13 wide; oviducal seminal receptacle 20 long or 2% of body length, 23 wide or 18% of body width at level of ovary, oviduct continuing anteromediad and dextrla to ovary 105 or 8% of body length along midline of external seminal vesicle, 13 wide or 10% of body width before connecting with oötype (Fig. 2). Laurer's canal a narrow duct extending 18 or 1% of body length sinsitrad near distal margin of seminal receptacle, 8 wide (Fig. 2). Vitellarium difficult to discern, comprising a series of interconnected spheroid masses of small follicles, 400 or 31% of body length from anterior body end, distributing from common genital pore to Manter's organ, ventrolateral to testis and ceca, terminating 95 or 7% of body length from posterior body end, coalescing into primary vitelline duct anterior to anterior margin of testis (Fig. 2); primary vitelline duct difficult to discern, total length not observed, 10 wide; ovi-vitelline duct difficult to discern, 85 long or 6% of body length or 81% of oviduct length from seminal receptacle to oötype, 10 wide or 8% of body width at level of ovary. Oötype difficult to discern, 15 long or 1% of body length, 18 wide or 14% of body width. Mehlis gland not observed. Uterus 85 long or 6% of body length, 28 wide or 22% of maximum body width; egg chamber not observed; metraterm straight, 28 long or 2% of body length, 15 wide or 12% of body width; Uterine egg not observed. Common genital pore opening ventral, sinistral, 43 or 3% of body length posterior to middle of ventral sucker (Fig. 1).

Excretory vesicle difficult to discern, 30 long or 2% of body length, 5 wide or 5% of body width at cecal terminus; Manter's organ sinuous, turning 3 times, extending to cecal terminus, 130 long or 10% of body length, 23 wide or 23% of body width at cecal terminus, joining excretory vesicle (Fig. 1); excretory pore terminal.

Taxonomic summary

Type host: Not specified; Stunkard (1926) reported infections in both spiny softshell turtles, *Apalone spinifera* (LeSueur, 1827), and Florida softshell turtles, *Apalone ferox* (Schneider, 1783).

Other hosts: None.

Sites: Adults in blood.

Type locality: Not specified; Stunkard (1928) reported infected Florida softshell turtles and spiny softshell turtles from unspecified rivers in Florida and Indiana, respectively;

Other localities: Table I; Reelfoot Lake (36°21'12.23"N, 89°25'21.50"W), Tennessee (Byrd, 1939); Huron River, Washtenaw County, Michigan (Wall, 1951).

Specimens examined: Cotype (AMNH 791) (Stunkard, 1926; 1928).

Remarks

Vasotrema amydae differs from its congeners by having papillate suckers, a genital pore opening ventrally and anterior to the ovary, and a short testis that does not extend to the level of the tips of the posterior ceca.

The cotype of *Vasotrema amydae* (Figs. 1, 2; AMNH 791) is, to our knowledge, the only extant specimen that remains from the type series. Stunkard (1928) considered the oötype to comprise the entirety of what we consider the ‘egg chamber’ (= uterus and oötype). Like in specimens of *Coeuritrema* and *Spirorchis* (see Roberts et al., 2016b, 2016c), we identified a distinct oötype, uterus, and metraterm in *V. amydae*. In an apparent lapse, Stunkard (1928) described the oviduct as extending anteriad dorsal to the external seminal vesicle (as we did), but illustrated it as ventral to the seminal vesicle (Fig. 1; p. 306). The type locality and type host for *V. amydae* are indeterminate, and Stunkard's (1926, 1928) slide labels do not help resolve the matter; which likely makes it impossible to ever confirm the origin of the only existing type material for *V. amydae*. He necropsied spiny and Florida softshell turtles taken from rivers in Indiana and Florida but failed to indicate where the turtles were captured and which TBF specimens came from which turtle species. Further potentially complicating matters is that a second unidentified TBF specimen shares a slide with the cotype. The specimen is poorly stained and not worth much attention but we identified it as *Vasotrema* sp. by having diagnostic features of the genus but also having elongate esophageal diverticula and a testis that extends posteriad to near the tips of the ceca.

Specimens identified as *Hapalorhynchus evaginatus* Byrd, 1939 infect spiny softshell turtles from Reelfoot Lake, Tennessee. Byrd (1939) comprises the only record of a TBF infection in a species of *Apalone* that is not a species of *Vasotrema.* Aware of this, Platt and Snyder (2007) borrowed Byrd's holotype (USNM 9227) and considered it to be *V. attenuatum*. In examining Byrd's holotype, Platt and Snyder (2007) stated that the specimen resembled *Vasotrema* spp. by having large posterior-most esophageal diverticula, a single testis, and a sinistral and dorsal genital pore. We agree with Platt and Snyder (2007) that the illustration resembles *Vasotrema* rather than *Hapalorhynchus*, but we think the authors incorrectly reported the position of the genital pore as dorsal rather than ventral, since the genital pore is dorsal in *Hapalorhynchus* spp. and ventral in *Vasotrema* spp. Byrd's (1939) original description does not resemble *V. attenuatum* but certainly likens the specimens to *V. amydae*: the body is elongate and the posterior-most lateral esophageal diverticula surround nearly half of the esophagus length. Based on that, we suspect that *H. evaginatus* is a junior subjective synonym of *V. amydae*.

Vasotrema attenuatum **Stunkard, 1928**

(Figs. 3−6)

Description of adult (based on light microscopy of 10 adult specimens comprising paratypes [AMNH 806]): Body 1,430−1,900 (1,620; 10) long or 12.0−20.1× (16.6; 10) longer than wide, 45−75 (57; 10) wide or 2−5% (4%; 10) of body length at level of cecal bifurcation, 45−65 (56; 10) wide or 3−5% (4%; 10) of body length at level of ventral sucker, 60−115 (82; 10) wide or 4−8% (5%; 10) of body length at level of ovary, 85−120 (99; 10) wide or 5−8% (6%; 10) of body length at level of testis (typically maximum width), 50−85 (67; 10) wide or 3−6% (4%; 10) of body length at level of cecal terminus; forebody (middle of ventral sucker to anterior body end) 295−395 (339; 10) long or 20−22% (21%; 10) of body length; hindbody (middle of ventral sucker to posterior body end) 1,110−1,510 (1,281; 10) long or 78−80% (79%; 10) of body length, 3.5−4.0× (3.8; 10) longer than forebody (Figs. 3−5). Oral sucker spines not observed, apapillate, 18−30 (24; 10) long or 1−2% (1%; 10) of body length, 25−33 (28; 10) wide or 33−62% (50%; 10) of body width at level of cecal bifurcation (Figs. 3, 4). Ventral sucker papillae not observed, 30−48 (39; 10) long or 2−3% (2%; 10) of body length, 30−50 (38; 9) wide or 58−91% (69%; 9) of body width. Nerve commissure 100−135 (108; 9) or 6−7% (7%; 9) of body length from anterior body end. Pharynx 30−38 (34; 10) long or 11−18% (13%; 10) of esophagus length, 18−30 (23; 10) wide or 2.3−4.6× (3.3; 10) esophagus width immediately posterior to pharynx, 1.1−2.1× (1.5; 10) longer than wide. Esophagus extending posteriad 213−325 (263; 10) long or 14−19% (16%; 10) of body length from mouth to posterior margin of median esophageal diverticulum, 5−10 (7; 10) wide immediately posterior to pharynx, with wall 3−5 (3; 10) thick, 8−18 (13; 10) wide at esophagus median or 15−30% (23%; 10) of body width at level of cecal bifurcation, with wall 5−15 (6; 10) thick; posterior-most lateral esophageal diverticula laterally expanded, 43−60 (50; 10) long or 3−4% (3%; 10) of body length or 15−22% (19%; 10) of total esophagus length, 20−40 (29; 10) wide or 40−62% (52%; 10) of body width at level of cecal bifurcation, 10−20 (14; 8) from dextral body margin, 10−20 (14; 8) from sinistral body margin (Figs. 5, 6); median esophageal diverticulum 18−38 (28; 10) long or 7−14% (11%; 10) of esophagus length, 13−28 (20; 10) wide or 24−51% (35%; 10) of body width at level of cecal bifurcation, with wall 3−12 (7; 8) thick (Figs. 5, 6); esophageal gland 203−270 (222; 7) long or 13−15% (14%; 7) of body length, 30−45 (38; 7) wide or 60−75% (68%; 7) of body width at level of cecal bifurcation (Figs. 3, 4). Intestinal bifurcation 240−333 (273; 10) from anterior body end or 15−18% (17%; 10) of body length (Figs. 5, 6); sinistral cecum 1,020−1,390 (1,186; 10) long or 69−81% (73%; 10) of body length, 8−25 (16; 10) wide or 18−44% (28%; 10) of body width at level of cecal bifurcation, 8−18 (11; 7) wide or 7−16% (11%; 7) of body width at level of ovary, 18−40 (29; 10) wide or 30−70% (44%; 10) of body width at level of cecal terminus; dextral cecum 1,035−1,340 (1,186; 10) long or 70−80% (73%; 10) of body length, 8−25 (16; 10) wide or 18−42% (28%; 10) of body width at level of cecal bifurcation, 5−10 (7; 7) wide or 6−12% (9%; 7) of body width at level of ovary, 23−38 (29; 10) wide or 35−52% (44%; 10) of body width at level of cecal terminus; post-cecal distance 140−215 (176; 10) or 10−12% (11%; 10) of body length (Figs. 3, 4).

Testis straight, lacking coils, abutting both ceca, 488−660 (578; 9) long or 31−42% (37%; 9) of body length, 45−90 (65; 10) wide or 39−82% (66%; 10) of body width at level of testis, 5.6−14.3× (9.3; 10) longer than wide, 25−150 (65; 9) or 2−8% (4%; 9) from cecal terminus, 175−358 (226; 9) or 11−20% (14%; 9) of body length from posterior body end (Figs. 3−5). Vas deferens 275−450 (371; 10) long or 19−25% (23%; 10) of body length, 40−63 (47; 10) wide or 38−56% (48%; 10) of body width (Figs. 4, 6), ventrolateral to gonads; external seminal vesicle indiscernible from vas deferens due to lack of constriction; internal seminal vesicle 65−168 (119; 10) long or 13−26% (20%; 9) of testis length, 20−28 (24; 10) wide or 25−56% (39%; 10) of testis width, 3.2−7.3× (5.1; 10) longer than wide (Figs. 4, 6). Cirrus sac 155−238 (194; 10) long or 10−14% (12%; 10) of body length, 53−88 (68; 10) wide or 50−84% (68%; 10) of body width at level of testis, 1.9−3.8× (2.9; 10) wider than internal seminal vesicle; cirrus straight, 33−75 (50; 10) long or 2−5% (3%; 10) of body length, 10−28 (19; 10) wide or 11−33% (19%; 10) of body width at level of testis (Fig. 6).

Ovary ovoid, intercecal, abutting dextral cecum, dorsal to vas deferens, 190−290 (238; 10) or 13−16% (15%; 10) of body length posterior to middle of ventral sucker, 45−73 (58; 10) long or 3−4% (4%; 10) of body length, 25−68 (42; 10) wide or 29−91% (53%; 10) of body width, 0.9−1.9× (1.5; 10) longer than wide (Figs. 4, 6); post-ovarian distance 875−1,150 (1,003; 10) or 60−67% (62%; 10) of body length. Oviduct extending posteriad 13−45 (23; 8) or 1−3% (1%; 8) of body length, 10−13 (12; 8) wide; oviducal seminal receptacle 20−50 (32; 9) long or 1−3% (2%; 9) of body length or 37−68% (55%; 9) of ovary length, 18−28 (24; 9) wide or 21−38% (29%; 9) of body width at level of ovary, oviduct continuing anterosinistrad 63−115 (82; 5) or 4−8% (5%; 5) of body length before connecting with oötype, 10−18 (13; 5) wide or 12−23% (15%; 5) of body width (Fig. 6). Laurer's canal difficult to discern, a narrow duct extending posteriad 25−75 (49; 5) or 2−4% (3%; 5) of body length near distal margin of seminal receptacle, 15−23 (18; 5) wide or 17−31% (22%; 5) of body width at level of ovary (Figs. 4, 6). Vitellarium difficult to discern, comprising a series of interconnected spheroid masses of small follicles, 350−700 (586; 8) or 22−42% (36%; 8) of body length from anterior body end, distributing from ovary to Manter's organ, ventrolateral to ceca, terminating 85−175 (130; 9) or 6−10% (8%; 9) of body length from posterior body end, coalescing into primary vitelline duct near anterior margin of testis (Figs. 3−5); primary vitelline duct difficult to discern, 173−175 (174; 3) long or 11−12% (11%; 3) of body length, 8−55 (30; 7) wide or 11−73% (37%; 7) of body width at level of ovary; ovi-vitelline duct difficult to discern, 33−65 (46; 4) long or 2−4% (3%; 4) of body length, 10−18 (13; 4) wide or 12−23% (16%; 4) of body width. Oötype difficult to discern, 13−30 (23; 5) long or 1−2% (1%; 5) of body length, 13−25 (19; 5) wide or 15−31% (22%; 5) of body width at level of ovary. Mehlis gland not observed. Uterus 33−90 (70; 5) long or 2−5% (4%; 5) of body length, 13−28 (21; 5) wide or 15−29% (24%; 5) of body width at level of ovary; metraterm straight, 25−58 (38; 8) long or 2−4% (2%; 8) of body length, 13−20 (15; 8) wide or 13−27% (19%; 8) of body width; Uterine egg not observed. Common genital pore opening ventral, sinistral, 55−100 (75; 9) or 3−5% (5%; 9) of body length posterior to middle of ventral sucker (Figs. 3, 4).

Excretory vesicle difficult to discern, 28−50 (44; 8) long or 2−3% (3%; 8) of body length, 5−20 (9; 8) wide or 6−25% (13%; 8) of body width at cecal terminus; Manter's organ sinuous, spiraled, turning 5−8 (6; 9) times, extending to or past cecal terminus, 80−158 (132; 10) long or 5−10% (8%; 10) of body length, 18−28 (22; 10) wide or 24−47% (35%; 10) of body width at cecal terminus, joining excretory vesicle (Figs. 3, 5); excretory pore terminal.

Taxonomic summary

Type host: Not specified; Stunkard (1928) reported infections in both spiny softshell turtles, *Apalone spinifera* (LeSueur, 1827), and Florida softshell turtles, *Apalone ferox* (Schneider, 1783).

Other hosts: *Apalone* sp. (Wall, 1951); midland softshell turtle, *Apalone mutica* (LeSueur, 1827) (see Brooks and Mayes, 1975).

Sites: Adults in blood (Stunkard, 1928).

Type locality: Not specified; Stunkard (1928) reported infected Florida softshell turtles and spiny softshell turtles from unspecified rivers in Florida and Indiana, respectively.

Other localities: Table I; Not specified, USA (Wall, 1951); Not specified, Nebraska (Brooks and Mayes, 1975).

Specimens examined: Cotype (AMNH 806, 17 slides with 25 whole mounted specimens).

Remarks

Vastotrema attenuatum differs from its congeners by having a genital pore that opens ventrally and anterior to the ovary and a long, non-coiled testis that extends posteriad to the tips of the ceca.

As with the cotype of *V. amydae* and for the same reasons, we cannot confirm the type host or type locality for *V. attenuatum*. Of the extant whole-mounted specimens comprising the type series, 13 of 17 slides have no host information and 4 slides (AMNH 806-1, 2, 12, 16) indicate the host as spiny softshell turtle (as *Amyda spinifera*). Likewise, Wall’s (1951) records of *V. attenuatum*, *V. robustum*, and *V. amydae* in softshell turtles from the Huron River (Michigan) and Cumberland River (Tennessee) are equally ambiguous (Table I).

Stunkard's (1928) generally incomplete description omitted several taxonomically important features for *V. attenuatum*; however, our observations of his specimens confirmed most of the features that he did happen to describe. A significant exception is the distal portion of the female genitalia. He illustrated the uterus and metraterm as ventral to the cirrus sac (fig. 4; p. 310) but both are dorsal to the cirrus sac. Stunkard (1928) also failed to differentiate the oötype, uterus, and thick-walled metraterm; misinterpreting these structures as together comprising the oötype. We agree that these structures function as a sort of egg chamber; however, we confirmed distinct portions of the female reproductive tract corresponding to the oötype, uterus, and metraterm in non-gravid specimens. In fact, we regard the presence of these separate structures as well as the fact that the large egg of *Vasotrema* comes to occupy their luminal spaces as a generic feature for *Vasotrema*.

Vasotrema robustum **Stunkard, 1928**

(Figs. 7−11)

Description of adult (based on light microscopy of a syntype [AMNH 808] and 1 newly collected voucher [USNM XXXXXX]): Body 1,680 [2,120] long or 4.8× [5.9×] longer than wide, 290 [250] wide or 17% [12%] of body length at level of cecal bifurcation, 310 [290] wide or 18% [14%] of body length at level of ventral sucker, 350 [360] wide or 21% [17%] of body length at level of ovary (typically maximum width), 340 [325] wide or 20% [15%] of body length at level of testis, 200 [160] wide or 12% [8%] of body length at level of cecal terminus; forebody (middle of ventral sucker to anterior body end) 510 [620] or 30% [29%] of body length; hindbody (middle of ventral sucker to posterior body end) 1,170 [1,500] or 70% [71%] of body length, 2.3× [2.4×] longer than forebody (Figs. 7, 9). Oral sucker spinous, papillate [papillae not observed], 65 [60] long or 4% [3%] of body length, 98 [95] wide or 34% [38%] of body width at level of cecal bifurcation; paired terminal papillae 2 [n/a] in number, dorsal to mouth, together 10 [n/a] long by 38 [n/a] wide (Fig. 10); marginal spines 3 [n/a] in diameter (Fig. 10); paired interior mouth papillae 2 [n/a] in number, 5 [n/a] in diameter (Fig. 10); interior mouth spines 3 [3] long, terminating at level of interior mouth papillae (Fig. 10). Ventral sucker papillate, 143 [200] long or 9% [9%] of body length, 174 [223] wide or 60% [90%] of body width (Figs. 9, 11). Nerve commissure 105 [200] or 6% [9%] of body length from anterior body end. Pharynx 85 [70] long or 26% [16%] of esophagus length, 90 [90] wide or 1.6× [3.0×] esophagus width immediately posterior to pharynx, 1.1× [1.3×] wider than long. Esophagus extending posteriad 323 [450] long or 19% [21%] of body length from mouth to posterior margin of median esophageal diverticulum, 18 [18] wide posterior to pharynx, with wall 13 [13] thick, 55 [30] wide at esophagus median, with wall 30 [17] thick; posterior-most lateral esophageal diverticula radially expanded, 98 [100] long or 6% [5%] of body length and 30% [22%] of total esophagus length, 113 [103] wide or 39% [41%] of body width at level of cecal bifurcation, 55 [50] or 19% [20%] of body width from dextral body margin, 63 [43] or 22% [17%] of body width from sinistral body margin (Figs. 7, 9); median esophageal diverticulum 58 [70] long or 18% [16%] of esophagus length, 88 [75] wide or 30% [30%] of body width at level of cecal bifurcation, with wall 63 [62] thick (Figs. 7, 9); esophageal gland 250 [350] long or 15% [17%] of body length, 138 [155] wide or 48% [62%] of body width at level of cecal bifurcation (Figs. 7, 9). Intestinal bifurcation 325 [470] from anterior body end or 19% [22%] of body length; sinistral cecum 1,140 [1,510] long or 68% [71%] of body length, 50 [50] wide or 17% [20%] of body width at level of cecal bifurcation, 45 [40] wide or 13% [11%] of body width at level of ovary, 30 [45] wide or 15% [28%] of body width at level of cecal terminus; dextral cecum 1,170 [1,510] long or 70% [71%] of body length, 55 [70] wide or 19% [28%] of body width at level of cecal bifurcation, 55 [50] wide or 16% [14%] of body width at level of ovary, 33 [50] wide or 17% [31%] of body width at level of cecal terminus; post-cecal distance 150 [130] or 9% [6%] of body length (Figs. 7, 9).

Testis 270 [475] long or 16% [22%] of body length, 115 [185] wide or 34% [57%] of body width at level of testis, 2.3× [2.6×] longer than wide, 215 [250] or 13% [12%] from cecal terminus, 395 [385] or 24% [18%] of body length from posterior body end (Figs. 7, 9). Vas deferens 130 [200] long or 8% [9%] of body length, 8 [10] wide, ventral to testicular column, laterally expanding before joining external seminal vesicle; external seminal vesicle discernible from vas deferens, intercecal, 158 [270] long or 9% [13%] of body length, 125 [160] long or 7% [8%] of body length running anteriad, 33 [110] long or 2% [5%] of body length running posteriad, 80 [120] wide or 23% [33%] of body width at level of ovary, 2.0× [2.3] longer than wide; internal seminal vesicle 63 [85] long or 5% [4%] of body length, 35 [43] wide or 10% [12%] of body width, 1.8× [2.0×] longer than wide (Figs. 7−9, 11). Cirrus sac 103 [150] long or 6% [7%] of body length, 40 [63] wide or 11% [18%] of body width at level of ovary, 1.1× [1.5×] wider than internal seminal vesicle; cirrus straight, 35 [33] long or 2% [2%] of body length, 20 [13] wide or 6% [4%] of body width (Figs. 7−9, 11).

Ovary lobate, 3 [3] lobes, intercecal, abutting and slightly dorsal to external seminal vesicle, dextrolateral to cirrus sac, 375 [500] or 22% [24%] of body length posterior to middle of ventral sucker, 55 [83] long or 3% [4%] of body length, 80 [120] wide or 23% [33%] of body width, 1.5× [1.4×] wider than long (Figs. 9, 11); post-ovarian distance 740 [910] or 44% [43%] of body length (Figs. 7−9, 11). Oviduct extending posteriad 30 [63] long or 2% [3%] of body length, 15 [18] wide; oviducal seminal receptacle 48 [43] long or 3% [2%] of body length, 45 [48] wide or 13% [13%] of maximum body width, oviduct continuing anterosinistrad 88 [88] or 5% [4%] of body length, 25 [20] or 7% [6%] of body width before connecting with oötype (Figs. 8, 11). Laurer's canal a narrow duct extending 75 [88] or 4% [4%] of body length sinistrad near distal margin of seminal receptacle, 20 [13] wide or 6% [4%] of maximum body width (Figs. 8, 11). Vitellarium comprising a series of interconnected spheroid masses of small follicles, 350 [415] or 21% [20%] of body length, distributing from cecal bifurcation [ventral sucker] to Manter's organ, ventrolateral to testes and ceca (not fully illustrated to highlight paths of ceca), terminating 70 [95] or 4% [4%] of body length from posterior body end, coalescing from dextral body margin into primary vitelline duct posterior to oviduct (Figs. 7, 9); primary vitelline duct coalescing and extending posteriad 60 [80] before turning and extending anteriad 88 [103], 148 [183] long or 9% [9%] of body length, 25 [40] wide or 7% [11%] of body width, inserting at proximal margin of oötype. Oötype difficult to discern, 25 [50] long or 1% [2%] of body length, 38 [33] wide or 11% [9%] of body width (Figs. 8, 11).

Mehlis gland not observed. Uterus 50 [35] long or 3% [2%] of body length, 38 [23] wide or 11% [6%] of body width; metraterm straight, 50 [45] long or 3% [2%] of body length, 45 [45] wide or 13% [13%] of body width (Figs. 8, 11). Uterine egg (not observed in voucher) 35 long or 2% of body length, 20 wide or 6% of body width at level of ovary, 1.8× longer than wide (Fig. 8). Common genital pore opening ventral, sinistral (Figs. 7−9, 11), 375 [580] or 22% [27%] of body length posterior to middle of ventral sucker.

Excretory vesicle difficult to discern, 63 [n/a] long or 4% [n/a] of body length, 10 [n/a] wide or 5% [n/a] of body width at cecal terminus; Manter's organ coiled, turning 4 [2] times, extending past cecal terminus, 200 [200] long or 12% [9%] of body length, 35 [38] wide or 18% [24%] of body width at cecal terminus, joining excretory vesicle (Figs. 7, 9); excretory pore terminal.

Taxonomic summary

Type host: Spiny softshell turtle, *Apalone spinifera* (LeSueur, 1827).

Other hosts: Florida softshell turtle, *Apalone ferox* (Schneider, 1783) (Wall, 1951; Foster et al., 1998); midland smooth softshell, *Apalone mutica* (LeSueur, 1827) (Wall, 1951; Brooks and Mayes, 1975); Gulf coast spiny softshell, *Apalone spinifera aspera* (Agassiz, 1857) (present study).

Sites: Adults in blood (Stunkard, 1928), heart (Byrd, 1939; present study), mesenteric blood vessels (present study), and other large blood vessels (Table I).

Type locality: Not specified; Stunkard (1928) reported infected spiny softshell turtles from an unspecified river in Indiana.

Other localities: Table I; Reelfoot Lake (36°21'12.23"N, 89°25'21.50"W), Tennessee (Byrd, 1939); Huron River, Washtenaw County, Michigan (Wall, 1951); Cumberland

River, Davidson County, Tennessee (Wall, 1951); None specified, Nebraska (Brooks and Mayes, 1975); Lake Okeechobee, Palm Beach County, Florida (Foster et al., 1998); Nishnabotna River, Floyd County, Iowa (Snyder, 2004); Canoe Lake (33°47'56.16"N, 86°29'25.02"W), Coosa River, Springville, Alabama (present study); Round Lake (32°41'50.91"N, 87°14'30.39"W), Cahaba River, Perry Lakes State Park, Marion, Alabama (present study).

Specimens examined: Syntype (AMNH 808) (Stunkard, 1928).

Specimens deposited: Voucher, USNM XXXXX (Canoe Lake [33°47'56.16"N, 86°29'25.02"W], Coosa River, Springville, Alabama).

Prevalence and intensity of infection (present study): One of 3 (33%) and 1 of 1 (100%) Gulf Coast spiny softshell turtles were infected each by 1 adult of *V. robustum* from Canoe Lake and Round Lake, respectively.

Remarks

Vasotrema robustum is unique among its congeners by having a genital pore that opens ventrally at level of the ovary (Figs. 7−9, 11), far posterior to the level of the ventral sucker, and an external seminal vesicle that is anterior to the cirrus sac (Figs. 7−9, 11). The body of this species is distinctive too in that it is markedly more ovoid (Figs. 7, 9), lacking the elongate body confirmation of its congeners (Figs. 1, 3, 13, 15, 17, 19). Further, the oral sucker of *V. robustum* seems unique by having the combination of paired terminal papillae, circumoral papillae and spines, and oral spines and papillae in the mouth cavity (Fig. 10). No congener has this combination of features, although *V. longitestis* is papillate, which may indicate that *V. robustum* could ultimately be reassigned to a new genus.

Some of our observations contradict previous treatments of this species. Stunkard (1928) described the posterior-most esophageal diverticula only and compared them to those of *Spirorchis* spp. (Roberts et al., 2016c). The syntype and our newly collected specimens have lateral esophageal diverticula that are distributed over the entire length of the esophagus. These specimens also have a median esophageal diverticulum (Roberts et al., 2016c) dorsal to the cecal bifurcation. Stunkard (1928) and Wall (1951) did not describe a median esophageal diverticulum. In addition, Stunkard (1928) described the vitellarium of *V. robustum* as not extending anteriad past the ventral sucker (Figs. 9, 11) but the newly collected specimen (**USNM XXXXXX**) has a vitellarium that extends anteriad beyond the ventral sucker to the cecal bifurcation.

The type host and locality for *V. robustum* is unclear. Stunkard (1928) did not specify a host but indicated that at least 8 specimens of *V. robustum* were recovered from spiny softshell turtles. Wall (1951) stated that the description (Stunkard, 1928) was based on 13 specimens from that host in Indiana, but it is unclear where that information came from. The AMNH database lists spiny softshell turtle as the type host for *V. robustum* (syntypes 808 and 809) and Indiana as the type locality, but published host and locality records and slide labels for the extant specimens do not unambiguously confirm that.

Vasotrema longitestis **Byrd, 1939**

(Figs. 12−14)

Description of adult (based on light microscopy of the holotype [USNM 1321971] and ten newly collected vouchers [USNM XXXXXX-XXXXXX]): Body 730 −1,420 (1,144; 10) [635] long or 3.6−7.2× (5.8; 10) [2.1×] longer than wide, 100−145 (115; 10) [160] wide or 8−19% (10%; 10) [25%] of body length at level of cecal bifurcation, 100−155 (121; 10) [180] wide or 9−17% (11%; 10) [28%] of body length at level of ventral sucker, 150−285 (177; 10) [285] wide or 13−22% (17%; 10) [45%] of body length at level of ovary, 155−340 (203; 10) [300] wide or 14−28% (18%; 10) [47%] of body width at level of testis (typically maximum width), 130−250 (156; 9) [160] wide or 11−22% (14%; 10) [25%] of body length at level of cecal terminus; forebody (middle of ventral sucker to anterior body end) 195−370 (254; 10) [140] long or 18−29% (22%; 10) [22%] of body length; hindbody (middle of ventral sucker to posterior body end) 517−1,050 (890; 10) [495] long or 71−82% (78%; 10) [78%] of body length, 2.4−4.7× (3.6; 10) [3.5×] longer than wide (Figs. 12, 13). Oral sucker spines not observed, papillate [papillate], 20−35 (29; 10) [25] long or 2−5% (3%; 10) [4%] of body length, 33−48 (41; 10) [33] wide or 29−48% (36%; 10) [21%] of body width at level of cecal bifurcation (Figs 12, 13); paired terminal papillae not observed; paired internal mouth papillae not observed. Ventral sucker papillate [not observed], 50−70 (59; 10) [53] long or 5−7% (5%; 10) [8%] of body length, 48−70 (59; 10) [70] wide or 40−60% (51%; 10) [44%] of body width (Figs. 12, 13). Nerve commissure 80−140 (97; 9) [90] or 7−12% (9%; 9) [14%] of body length from anterior body end. Pharynx 30−55 (39; 10) [28] long or 16−25% (19%; 10) [n/a] of esophagus length, 30−43 (36; 10) [35] wide or 3.5−6.6× (4.7; 8) [n/a] esophagus width immediately posterior to pharynx, 0.9−1.3× (1.1; 9) [0.8×] longer than wide. Esophagus extending posteriad 155−298 (205; 10) [n/a] long or 13−25% (18%; 10) [n/a] of body length from mouth to posterior margin of median esophageal diverticulum, 5−10 (8; 9) [n/a] wide posterior to pharynx, with wall 2−7 (5; 9) [n/a] thick, 10−20 (13; 10) [n/a] wide at esophagus median or 7−18% (12%; 10) [n/a] of body width at level of cecal bifurcation, with wall 5−10 (7; 10) [n/a] thick; posterior-most lateral esophageal diverticula radially expanded, 25−60 (40; 10) [n/a] long or 2−4% (3%; 10) [n/a] of body length and 16−22% (19%; 10) [n/a] of total esophagus length, 30−55 (39; 10) [n/a] wide or 29−41% (33%; 10) [n/a] of body width at level of cecal bifurcation; 23−38 (32; 10) [n/a] or 21−38% (28%; 10) [n/a] of body width from dextral body margin, 25−38 (30; 10) [n/a] or 25−29% (26%; 10) [n/a] of body width from sinistral body margin; median esophageal diverticulum 15−35 (24; 10) [n/a] long or 10−16% (12%; 10) [n/a] of esophagus length, 20−30 (25; 10) [n/a] wide or 18−27% (22%; 10) [n/a] of body width at level of cecal bifurcation, with wall 2−15 (7; 10) [n/a] thick; esophageal gland 125−255 (159; 10) [n/a] long or 3−11% (5%; 10) [n/a] of body length, 40−78 (57; 10) [n/a] wide or 36−68% (50%; 10) [n/a] of body width at level of cecal bifurcation (Fig 13). Intestinal bifurcation 163−310 (207; 10) [n/a] from anterior body end or 15−24% (18%; 10) [n/a] of body length; sinistral cecum 435-1,050 (759; 10) [n/a] long or 59−77% (66%; 10) [n/a] of body length, 10−20 (14; 10) [n/a] wide or 8−17% (13%; 10) [n/a] of body width at level of cecal bifurcation, 5−15 (11; 10) [n/a] wide or 3−8% (6%; 10) [n/a] of body width at level of ovary, 13−28 (18; 10) [n/a] wide or 6−22% (12%; 10) [n/a] of body width at level of cecal terminus; dextral cecum 450−1,065 (764; 10) [n/a] long or 61−75% (66%; 10) [n/a] of body length, 8−18 (14; 10) [n/a] wide or 6−16% (12%; 10) [n/a] of body width at level of cecal bifurcation, 8−15 (11; 10) [n/a] wide or 4−9% (6%; 10) [n/a] of body width at level of ovary, 10−33 (21; 10) [n/a] wide or 6−23% (14%; 10) [n/a] of body width at level of cecal terminus; post-cecal distance 105−220 (177; 10) [n/a] or 11−17% (16%; 10) [n/a] of body length (Fig. 13).

Testis turning 12−17 (15; 10) [17] times, 233−710 (443; 10) [283] long or 32−50% (38%; 10) [45%] of body length, 25−65 (39; 10) [30] wide or 12−24% (19%; 10) [10%] of body width at level of testis, 9.3−15.3× (11.4; 10) [9.4×] longer than wide, 15−100 (45; 10) [15] or 2−9% (4%; 10) [2%] of body length from cecal terminus, 120−263 (214; 10) [95] or 14−22% (19%; 10) [15%] of body length from posterior body end (Figs. 12, 13). Vas deferens 100−313 (185; 10) [n/a] long or 13−22% (16%; 10) [n/a] of body length, 13−45 (26; 10) [n/a] wide, ventral to testis; external seminal vesicle comprised entirely of vas deferens; internal seminal vesicle 40−75 (53; 10) [88] long or 3−8% (5%; 10) [14%] of body length, 13−20 (16; 9) [15] wide or 5−12% (8%; 10) [5%] of body width at level of testis, 2.0−4.2× (3.4; 10) [5.9×] longer than wide (Figs. 12, 13). Cirrus sac 105−190 (139; 10) [150] long or 9−19% (12%; 10) [24%] of body length, 23−33 (27; 10) [25] wide or 8−18% (14%; 10) [8%] of body width at level of testis, 1.5−1.9× (1.7; 10) [1.7×] wider than internal seminal vesicle; cirrus straight, 40−105 (75; 10) [53] long or 4−9% (7%; 10) [8%] of body length, 5−15 (9; 10) [8] wide or 2−10% (5%; 10) [3%] of body width at level of testis (Figs. 12−14).

Ovary spheroid or weakly lobate, 2 (5) [0] lobes when present, intercecal, abutting dextral cecum, 118−255 (165; 10) [78] or 12−18% (14%; 10) [12%] of body length posterior to middle of ventral sucker, 33−55 (43; 10) [20] long or 3−5% (4%; 10) [3%] of body length, 18−68 (43; 10) [60] wide or 11−38% (24%; 10) [21%] of body width, 0.4−1.8× (1.0; 10) [3.0×] wider than long (Figs. 12−14); post-ovarian distance 380−950 (694; 10) [400] or 52−67% (60%; 10) [63%] of body length (Figs. 12−14). Oviduct extending posteriad 8−18 (13; 10) [n/a] long or 1−2% (1%; 10) [n/a] of body length, 10−15 (12; 10) [n/a] wide; oviducal seminal receptacle 18−33 (27; 10) [n/a] long or 2−3% (2%; 10) [n/a] of body length or 40−91% (64%; 10) [n/a] of ovary length, 18−30 (25; 10) [n/a] wide or 11−19% (16%; 10) [n/a] of body width at level of ovary, oviduct continuing anteriad 8−55 (27; 10) [n/a] or 1−4% (2%; 10) [n/a] of body length, 8−15 (12; 10) [n/a] wide or 5−9% (7%; 10) [n/a] of body width before connecting with egg chamber (Fig. 14). Laurer's canal a narrow duct extending 18−43 (28; 7) [n/a] or 2−3% (2%; 7) [n/a] of body length anteriad, 8−23 (13; 9) [n/a] wide (Fig. 14). Vitellarium comprising a series of interconnected large spheroid masses of small follicles, 295−500 (405; 10) [n/a] or 27−44% (36%; 10) [n/a] of body length from anterior body end, distributing from ovary to posterior end of Manter's organ, ventrolateral to testis and ceca, terminating 35−130 (92; 10) [n/a] or 5−11% (8%; 10) [n/a] of body length from posterior body end, coalescing into primary vitelline duct posterior to oviduct and immediately anterior to testis (Figs. 13, 14); primary vitelline duct a narrow and winding duct 113−245 (159; 10) [n/a] long or 9−23% (14%; 10) [n/a] of body length, 20−75 (40; 10) [n/a] wide, merging with female genitalia at proximal margin of egg chamber (Fig. 14). Oötype difficult to discern in gravid specimens, forming proximal portion of egg chamber. Mehlis gland not observed. Uterus difficult to discern in gravid specimens, forming distal portion of egg chamber; egg chamber 98−150 (129; 10) [123] long or 10−13% (11%; 10) [19%] of body length, 40−55 (44; 10) [45] wide or 19−29% (25%; 10) [16%] of body width at level of ovary; metraterm 73−108 (97; 10) [93] long or 7−15% (9%; 10) [15%] of body length, proceeding posteriad for 8−50 (28; 10) [38] or 11−49% (28%; 10) [41%] of total metraterm length, bowing and extending anteriad for 53−88 (69; 10) [55] or 51−89%

(72%; 10) [59%] of total metraterm length, 8−13 (12; 10) [13] wide or 5−9% (7%; 10) [5%] of body width at level of ovary. Uterine egg 90−138 (121; 10) [113] long or 10−12% (11%; 10) [18%] of body length, 33−48 (36; 10) [38] wide 17−25% (21%; 10) [13%] of body width. Common genital pore opening ventral, sinistral (Figs. 12−14), at level of (1) ventral sucker median or 3−20 (10; 9) [30] [or ≤1% (1%; 8) [5%] of body length anterior to middle of ventral sucker (Figs. 12−14).

Excretory vesicle difficult to discern, 23−38 (30; 10) [n/a] long or 2−3% (3%; 10) [n/a] of body length, 5−8 (7; 10) [n/a] wide or 3−6% (4%; 10) [n/a] of body width at cecal terminus; Manter's organ sinuous, turning 4−10 (6; 10) [n/a] times, extending to cecal terminus, 80−170 (134; 10) [n/a] long or 9−15% (12%; 10) [n/a] of body length, 18−30 (25; 10) [n/a] wide or 12−22% (16%; 10) [n/a] of body width at cecal terminus, joining excretory vesicle (Fig. 13); excretory pore terminal.

Taxonomic summary

Type host: Spiny softshell turtle, *Apalone spinifera* (LeSueur, 1827).

Other hosts: Gulf Coast spiny softshell turtle, *Apalone spinifera aspera* (Agassiz, 1857) (present study).

Sites: Adults in arteries (Byrd, 1939), mesenteric blood vessels (present study) (Table I).

Type locality: Reelfoot Lake (36°21'12.23"N, 89°25'21.50"W), Tennessee (Byrd, 1939).

Other localities: Table I; Canoe Lake (33°47'56.16"N, 86°29'25.02"W), Coosa River, Springville, Alabama (present study); Round Lake (32°41'50.91"N, 87°14'30.39"W), Cahaba River, Perry Lakes State Park, Marion, Alabama (present study).

Specimens examined: Holotype (USNM 1321971) (Byrd, 1939).

Specimens deposited: Voucher, USNM XXXXX (Canoe Lake [33°47'56.16"N, 86°29'25.02"W], Coosa River, Springville, Alabama); voucher, USNM XXXXX (Round Lake [32°41'50.91"N, 87°14'30.39"W], Cahaba River, Perry Lakes State Park, Marion, Alabama).

Prevalence of infection (present study): Three of 3 (100%) and 1 of 1 (100%) spiny softshell turtles were infected by *V. longitestis* from Canoe Lake and Round Lake, respectively.

Remarks

Vasotrema longitestis differs from its congeners by having a genital pore that opens ventrally and anterior to the ovary and a long, coiled testis that extends posteriad to the tips of the ceca.

The holotype of *V. longitestis* (USNM 1321971; fig. 9, p. 157; Byrd, 1939) is the only extant specimen that is unambiguously from the type series (see discussion presented in Platt and Prestwood, [1990] regarding additional specimens that may belong to the type series). However, the holotype is strongly contracted, poorly stained (perhaps it destained), and apparently strongly flattened (Fig. 12). An outline sketch of that specimen is provided herein mostly for general comparative purposes; however, some additional fine structures were evident in that specimen that had not been previously characterized. For example, we observed papillae around the mouth of the holotype and our specimens as well as papillae associated with the ventral sucker of our specimens (Fig. 13). Also, the 2 posterior esophageal dilations indicated by Byrd (1939) appear to comprise lateral esophageal diverticula. The common genital pore is immediately anterior to the ventral sucker in the holotype and our specimens (Figs. 12, 13), not at level of the posterior margin of the ventral sucker as indicated by Byrd (1939). Although Byrd (1939) described the metraterm as “short,” it is in fact proportionally the longest of any accepted species of *Vasotrema*: extending 7−15% of body length vs. 1−3% (*V. amydae*), 2−4% (*V. attenuatum*), 2−3% (*V. robustum*), and 2−3% (*V. brevitestis*). The path of the metraterm in *V. longitestis* is unique among congeners: extending posteriad before curving anteriad towards the genital pore (Figs. 12, 14).

***Vasotrema brevitestis* Brooks and Mayes, 1975**

(Figs. 15−18)

Description of adult (based on light microscopy of 5 paratypes [HWML 20076, 20077]): Body 1,130−1,330 (1,232; 5) long or 8.3−11.0× (10.0; 5) longer than wide, 110−148 (126; 5) wide or 9−11% (10%; 5) of body length at level of cecal bifurcation, 100−148 (120; 5) wide or 9−11% (10%; 5) of body length at level of ventral sucker, 95−155 (118; 5) wide or 8−12% (10%; 5) of body length at level of ovary, 105−160 (125; 5) wide or 9−12% (10%; 5) of body width at level of testis, 80−100 (88; 5) wide or 6−8% (7%; 5) of body length at level of cecal terminus; forebody (middle of ventral sucker to anterior body end) 300−430 (360; 5) long or 27−32% (29%; 5) of body length, hindbody (middle of ventral sucker to posterior body end) 830−930 (872; 5) long or 68−73% (71%; 5) of body length, 2.1−2.8× (2.5; 5) longer than forebody (Figs. 15, 17). Oral sucker spinous, apapillate, 30−38 (34; 5) long or 3% (3%; 5) of body length, 38−48 (43; 5) wide or 28−44% (35%; 5) of body width at level of cecal bifurcation (Figs. 15, 17). Ventral sucker apapillate, 45−55 (52; 5) long or 4−5% (4%; 5) of body length, 30−63

(48; 5) wide or 30−46% (39%; 5) of body width. Nerve commissure 93−125 (107; 5) or 8−10% (9%; 5) of body length from anterior body end. Pharynx 48−53 (51; 5) long or 16−21% (18%; 5) of esophagus length, 28−50 (40; 5) wide or 2.8−5.0× (3.7; 5) esophagus width immediately posterior to pharynx, 1.0−1.9× (1.3; 5) longer than wide. Esophagus extending posteriad 250−325 (279; 5) long or 20−26% (23%; 5) of body length from mouth to posterior margin of median esophageal diverticulum, 8−13 (10; 4) wide posterior to pharynx, with wall 2−10 (5; 4) thick, 10−25 (18; 4) wide at esophagus median before largest diverticula, with wall 5−22 (14; 4) thick; posterior-most lateral esophageal diverticula radially expanded, 53−113 (79; 4) long or 5−9% (6%; 4) of body length and 21−35% (29%; 4) of esophagus length, 68−75 (71; 4) wide or 56−64% (59%; 4) of body width at level of cecal bifurcation, 5−15 (12; 4) or 5−13% (10%; 4) of body width from dextral body margin, 10−13 (12; 4) or 9−11% (10%; 4) of body width from sinistral body margin; median esophageal diverticulum 25−38 (33; 4) long or 10−15% (12%; 4) of esophagus length, 25−28 (26; 4) wide or 19−23% (22%; 4) of body width at level of cecal bifurcation, with wall 10−15 (13; 4) thick; esophageal gland 168−250 (197; 4) long or 15−20% (16%; 4) of body length, 75−83 (77; 4) wide or 61−68% (64%; 4) of body width at level of cecal bifurcation (Figs. 15, 17). Intestinal bifurcation 243−315 (268; 5) from anterior body end or 19−25% (22%; 5) of body length; sinistral cecum 710-825 (779; 4) long or 57−73% (65%; 4) of body length, 25−50 (38; 4) wide or 22−42% (31%; 4) of body width at level of cecal bifurcation, 13−15 (15; 4) wide or 13−14% (13%; 4) of body width at level of ovary, 13−23 (17; 4) wide or 15−31% (20%; 4) of body width at level of cecal terminus; dextral cecum 660−825 (805; 4) long or 53−75% (67%; 4) of body length, 25−50 (38; 4) wide or 22−38% (31%; 4) of body width at level of cecal bifurcation, 13−20 (15; 4) wide or 11−17% (14%; 4) of body width at level of ovary, 13−25 (17; 4) wide or 15−31% (20%; 4) of body width at level of cecal terminus; post-cecal distance 160−220 (178; 4) or 13−18% (15%; 4) of body length (Figs. 15, 17).

Testis straight or slightly sinuous, 1 or 3 turns (2) when sinuous, 188−300 (263; 5) long or 14−24% (21%; 5) of body length, 63−138 (85; 5) wide or 54−86% (67%; 5) of body width at level of testis, 1.4−4.2× (3.4; 5) longer than wide, 150−243 (195; 4) or 13−22% (16%; 4) of body length from cecal terminus, 375−438 (405; 5) or 30−36% (33%; 5) of body length from posterior body end (Figs. 15, 17). Vas deferens 105−113 (110; 3) long or 8−10% (9%; 3) of body length, 8−13 (10; 3) wide, ventral to gonads, laterally expanding before joining external seminal vesicle; external seminal vesicle discernible from vas deferens, intercecal, 70−113 (83; 4) long or 6−9% (7%; 4) of body length, 25−30 (28; 4) wide or 25−27% (26%; 4) of body width at level of ovary, 2.5−3.8× (2.9; 4) longer than wide; internal seminal vesicle 30−43 (38; 4) long or 2−4% (3%; 4) of body length, 13−18 (15; 4) wide or 11−17% (13%; 4) of body width, 2.2−2.9× (2.5; 4) longer than wide (Figs. 15−18). Cirrus sac 58−83 (68; 4) long or 5−7% (6%; 4) of body length, 25 (4) wide or 20−23% (22%; 4) of body width at level of testis, 1.4−1.9× (1.7; 4) wider than internal seminal vesicle; cirrus straight, 23−28 (25; 4) long or 2% (4) of body length, 5−8 (6; 4) wide or 4−8% (5%; 4) of body width at level of testis (Figs. 15, 17).

Ovary spheroid, intercecal, abutting dextral cecum and posterior margin of external seminal vesicle, 30−38 (34; 5) long or 2−3% (3%; 5) of body length, 43−55 (47; 5) wide or 32−48% (41%; 5) of body width, 1.2−1.5× (1.4; 5) wider than long (Figs. 15−18); post-ovarian distance 630−740 (687; 5) or 48−64% (56%; 5) of body length. Oviduct extending posteriad 18−33 (24; 4) long or 2−3% (2%; 4) of body length, 13−15 (14; 4) wide; oviducal seminal receptacle 18−25 (22; 4) long or 2% (4) of body length, 15−30 (21; 4) wide or 13−26% (19%; 4) of body width at level of ovary, oviduct continuing anteriad 55−75 (63; 3) or 5−6% (5%; 3) of body length, 13−18 (15; 3) wide or 12−16% (14%; 3) of body width before connecting with oötype (Figs. 16, 18); ovi-vitelline duct difficult to discern, 33−58 (46; 2) long or 3−5% (4%; 2) of body length, 13−15 (14; 2) wide or 11−14% (13%; 2) of body width at level of ovary (Figs. 16, 18). Laurer's canal a narrow duct extending 18−50 (31; 3) or 2−4% (3%; 3) of body length posteriad, 8−13 (10; 3) wide (Figs. 16, 18). Vitellarium difficult to discern, comprising a series of sparse interconnected large spheroid masses of small follicles, 360−575 (460; 5) or 32−43% (37%; 5) of body length from anterior body end, distributing from common genital pore to testis terminus, dorso- and ventrolateral to testis and ceca, terminating 250−438 (358; 5) or 20−37% (29%; 5) of body length from posterior body end, coalescing into primary vitelline duct posterior to oviduct and anterior to anterior margin of testis (Figs. 16, 18); primary vitelline duct difficult to discern, a narrow duct 88−113 (101; 2) long or 7−9% (8%; 2) of body length, 18−20 (19; 2) wide, merging with female genitalia before oötype (Figs. 16, 18). Oötype difficult to discern, 23−30 (27; 4) long or 2−3% (2%; 4) of body length, 20−30 (26; 4) wide or 21−26% (24%; 4) of body width. Mehlis gland not observed. Uterus 30−40 (37; 4) long or 2−4% (3%; 4) of body length, 23−33 (28; 4) wide or 22−29% (25%; 4) of body width; metraterm straight, 20−38 (28; 4) long or 2−3% (2%; 4) of body length, 10−13 (12; 4) wide or 8−14% (11%; 4) of body width at level of ovary; Uterine egg not observed. Common genital pore opening ventral, sinistral (Figs. 15−18), 33−58 (40; 4) or 3−5% (3%; 5) of body length posterior to middle of ventral sucker (Figs. 15, 17).

Excretory vesicle difficult to discern, 28−48 (35; 4) long or 2−4% (3%; 4) of body length, 3−20 (11; 4) wide or 4−22% (12%; 4) of body width at cecal terminus; Manter's organ sinuous, turning 4−6 (5; 3) times, extending to cecal terminus, 125−130 (127; 3) long or 10−12% (11%; 3) of body length, 13−20 (16; 3) wide or 15−23% (18%; 3) of body width at cecal terminus, joining excretory vesicle (Figs. 15, 17); excretory pore terminal.

Taxonomic summary

Type host: Midland smooth softshell turtle, *Apalone mutica* (LeSueur, 1827).

Other hosts: Spiny softshell turtle, *Apalone spinifera* (LeSueur, 1827) (see Brooks and Mayes, 1975).

Sites: Adults in blood (Brooks and Mayes, 1975).

Type locality: Missouri River, a site (41°31'22.34"N, 96° 8'7.30"W) approximately 2.4 km south of Blair, Nebraska.

Other locality: Table I; Atkinson Lake (42°32'20.36"N, 99° 0'3.04"W), 0.8 km west of Atkinson, Nebraska (Brooks and Mayes, 1975).

Specimens examined: Paratypes (HWML 20076, 20077) (Brooks and Mayes, 1975).

Remarks

Vasotrema brevitestis differs from its congeners by having apapillate suckers, a genital pore opening ventrally and anterior to the ovary, and a short testis that does not extend to the level of the tips of the posterior ceca.

We herein correct several key features of this species that were misinterpreted by Brooks and Mayes (1975): tegumental spines lacking, vas deferens ventral (not dorsal) to the ovary (Figs. 16, 18), and pars prostatica lacking (perhaps confused with the internal seminal vesicle by Brooks and Mayes [1975]) (Brooks and Sullivan, 1981; Roberts et al., 2016b). Brooks and Mayes (1975) described the oviducal seminal receptacle as occupying the intercecal space between the gonads; however, the seminal receptacle is immediately post-ovarian and immediately anterior to the primary vitelline duct (Figs. 16, 18).

Vasotrema rileyae **Roberts and Bullard n. sp.**

(Figs. 19, 20)

Description of adult (based on light microscopy of ten adult specimens [USNM XXXX-XXXXXX]): Body 1,000−1,390 (1,230; 10) long or 5.3−10.0× (7.8; 10) longer than wide, 100−145 (124; 10) wide or 7−15% (10%; 10) of body length at level of cecal bifurcation, 105−165 (127; 10) wide or 8−17% (10%; 10) of body length at level of ventral sucker, 130−185 (155; 10) wide or 10−19% (13%; 10) of body length at level of ovary, 120−150 (132; 10) wide or 10−19% (13%; 10) of body length at level of testis (typically maximum width), 120−150 (132; 10) wide or 9−15% (11%; 10) of body length at level of cecal terminus; forebody (middle of ventral sucker to anterior body end) 275−375 (325; 10) long or 25−29% (27%; 10) of body length; hindbody (middle of ventral sucker to posterior body end) 725−1,045 (905; 10) long or 71−75% (73%; 10) of body length, 2.5−3.0× (2.8; 10) longer than forebody (Fig. 19). Oral sucker putatively spinous, papillate, 25−40 (35; 10) long or 2−4% (3%; 10) of body length, 25−53 (45; 10) wide or 17−45% (37%; 10) of body width at level of cecal bifurcation; paired terminal papillae 2 in number, dorsal to mouth, together 5−23 (10; 10) long by 8−30 (19; 10) wide (Fig. 19); paired internal mouth papillae not observed. Ventral sucker papillate, 45−53 (50; 10) long or 3−5% (4%; 10) of body length, 38−63 (46; 10) wide or 30−42% (36%; 10) of body width. Nerve commissure 95−120 (108; 10) or 8−10% (9%; 10) of body length from anterior body end. Pharynx 38−58 (46; 10) long or 15−23% (18%; 10) of esophagus length, 33−53 (41; 10) wide or 2.9−8.6× (5.1; 10) esophagus width immediately posterior to pharynx, 0.9−1.8× (1.1; 10) wider than long. Esophagus extending posteriad 218−305 (260; 10) long or 19−23% (21%; 10) of body length from mouth to posterior margin of median esophageal diverticulum, 5−13 (9; 10) wide posterior to pharynx, with wall 3 (10) thick, 13−18 (15; 10) wide at esophagus median or 10−15% (12%; 10) of body width at level of cecal bifurcation, with wall 8−15 (11; 10) thick; posterior-most lateral esophageal diverticula radially expanded, 75−150 (101; 10) long or 6−11% (8%; 10) of body length or 31−52% (39%; 10) of total esophagus length, 60−85 (74; 10) wide or 48−70% (59%; 10) of body width at level of cecal bifurcation, 15−30 (21; 10) or 13−24% (17%; 10) of body width from dextral body margin, 13−30 (21; 10) or 11−24% (17%; 10) of body width from sinistral body margin; median esophageal diverticulum 28−45 (35; 10) long or 11−16% (14%; 10) of esophagus length, 30−40 (36; 10) wide or 25−38% (29%; 10) of body width at level of cecal bifurcation, with wall 2−10 (5; 10) thick; esophageal gland 163−250 (214; 10) long or 16−21% (17%; 10) of body length, 50−78 (59; 10) wide or 16−21% (17%; 10) of body width at level of cecal bifurcation (Fig. 19). Intestinal bifurcation 235−315 (272; 10) from anterior body end or 20−24% (22%; 10) of body length; sinistral cecum 505-840 (692; 10) long or 51−62% (56%; 10) of body length, 8−18 (15; 10) wide or 6−18% (12%; 10) of body width at level of cecal bifurcation, 8− 20 (13; 10) wide or 5−13% (9%; 10) of body width at level of ovary, 8−25 (17; 10) wide or 7−21% (13%; 10) of body width at level of cecal terminus; dextral cecum 540−820 (702; 10) long or 54−59% (57%; 10) of body length, 13−15 (14; 10) wide or 10−13% (11%; 10) of body width at level of cecal bifurcation, 8−20 (13; 10) wide or 3−10% (7%; 10) of body width at level of ovary, 13−25 (18; 10) wide or 10−19% (14%; 10) body width at level of cecal terminus; post-cecal distance 195−275 (240; 10) or 17−22% (20%; 10) of body length (Fig. 19).

Testis turning 4−11 (6; 10) times, 225−335 (279; 10) long or 21−26% (23%; 10) of body length, 43−75 (63; 10) wide or 28−48% (39%; 10) of body width at level of testis, 3.2−6.5× (4.5; 10) longer than wide, 75−205 (143; 10) or 6−15% (12%; 10) of body length to cecal terminus, 295−490 (394; 10) or 25−36% (32%; 10) of body length from posterior body end (Fig. 19). Vas deferens 73−133 (97; 10) long or 6−10% (8%; 10) of body length, 5−15 (10; 10) wide, ventral to gonads, laterally expanding before joining external seminal vesicle; external seminal vesicle discernible from vas deferens, intercecal, 48−100 (79; 10) long or 4−8% (6%; 10) of body length, 25−73 (52; 10) wide or 15−46% (33%; 10) of body width at level of testis, 1.0−2.3× (1.6; 10) longer than wide; internal seminal vesicle 45−63 (56; 10) long or 4−6% (5%; 10) of body length, 10−23 (16; 10) wide or 6−15% (10%; 10) of body width, 2.5−6.3× (3.9; 10) longer than wide (Figs. 19, 20). Cirrus sac 88−115 (102; 10) long or 7−9% (8%; 10) of body length, 18−38 (26; 10) wide or 10−23% (16%; 10) of body width at level of testis, 1.2−2.5× (1.7; 10) wider than internal seminal vesicle; cirrus straight, 30−50 (41; 10) long or 2−4% (3%; 10) of body length, 8−13 (10; 10) wide or 5−8% (6%; 10) of body width (Figs. 19, 20).

Ovary spheroid or weakly lobate, intercecal, abutting dextral cecum, abutting posterior margin of external seminal vesicle, 95−175 (144; 10) or 10−14% (12%; 10) of body length posterior to middle of ventral sucker, 23−55 (31; 10) long or 2−5% (3%; 10) of body length, 45−70 (55; 10) wide or 29−45% (35%; 10) of body width, 0.8−2.5× (1.9; 10) wider than long (Fig. 20); post-ovarian distance 600−840 (727; 10) or 55−62% (59%; 10) of body length (Figs. 19, 20). Oviduct extending posteriad 13−23 (18; 10) long or 1−2% (1%; 10) of body length, 10−18 (13; 10) wide; oviducal seminal receptacle 28−45 (33; 10) long or 2−4% (3%; 10) of body length, 18−35 (28; 10) wide or 12−25% (18%; 10) of body width at level of ovary, oviduct continuing anterosinistrad 63−100 (83; 10) or 5−9% (7%; 10) of body length, sinistrolateral to external seminal vesicle, 10−18 (14; 10) wide or 6−12% (9%; 10) of body width before connecting with oötype (Figs. 19, 20). Laurer's canal a narrow duct extending 13−40 (30; 9) or 1−4% (3%; 9) of body length posterodextrad near distal margin of seminal receptacle, 5−13 (11; 10) wide (Fig. 20). Vitellarium difficult to discern, comprising a series of interconnected spheroid masses of small follicles, 303−400 (349; 10) or 22−34% (29%; 10) of body length from anterior body end, distributing from ventral sucker to Manter's organ, ventrolateral to testis and ceca, terminating 63−125 (103; 10) or 5−10% (8%: 10) of body length from posterior body end, coalescing into primary vitelline duct anterior to anterior margin of testis (Fig. 20); primary vitelline duct difficult to discern, 163−245 (194; 4) long or 12−18% (15%; 4) of body length, 20−50 (32; 9) wide; ovi-vitelline duct difficult to discern, 50−65 (55; 4) long or 4−5% (4%; 4) of body length or 57−81% (67%; 4) of oviduct length from seminal receptacle to oötype, 13−15 (14; 4) wide or 8−12% (9%; 4) of body width at level of ovary. Oötype difficult to discern, 20−38 (28; 8) long or 1−3% (2%; 8) of body length, 18−30 (24; 8) wide or 11−19% (15%; 8) of body width. Mehlis gland not observed. Uterus 30−73 (45; 8) long or 2−5% (4%; 8) of body length, 15−35 (21; 8) wide or 10−18% (13%; 8) of maximum body width; egg chamber (in gravid specimens) 75 and 88 (2) long or 6% and 7% of body length, 50 and 60 (2) wide or 29% and 44% of maximum body width; metraterm straight, 20−40 (29; 10) long or 1−3% (2%; 10) of body length, 13−25 (18; 10) wide or 8−17% (11%; 10) of body width; Uterine egg 70 and 83 (2) long or 60% and 61% (2) of body length, 40 and 48 (2) wide or 31% and 36% (2) of maximum body width, 1.7× and 1.8× (2) longer than wide. Common genital pore opening ventral, sinistral, 8−18 (10; 10) or 1−2% (1%; 10) of body length posterior to middle of ventral sucker (Figs. 19, 20).

Excretory vesicle difficult to discern, 13−38 (29; 10) long or 1−3% (2%; 10) of body length, 3−10 (6; 10) wide or 3−7% (4%; 10) of body width at cecal terminus; Manter's organ sinuous, turning 4−8 (6, 10) times, extending to cecal terminus, 150−225 (179; 10) long or 13−18% (15%; 10) of body length, 15−38 (22; 10) wide or 11−25% (16%; 10) of body width at cecal terminus, joining excretory vesicle (Fig. 19); excretory pore terminal.

Taxonomic summary

Type host: Gulf Coast spiny softshell turtle, *Apalone spinifera aspera* (Agassiz, 1857).

Sites: Mesenteric blood vessels.

Type locality: Round Lake (32°41'50.91"N, 87°14'30.39"W), Cahaba River, Perry Lakes State Park, Marion, Alabama.

Other locality: Canoe Lake (33°47'56.16"N, 86°29'25.02"W), Coosa River, Springville, Alabama.

Specimens deposited: Holotype (USNM XXXXX), paratypes (USNM XXXXX).

Prevalence of infection (present study): Three of 3 (100%) and 1 of 1 (100%) spiny softshell turtles were infected by *V. rileyae* from Canoe Lake and Round Lake, respectively.

Etymology: Specific epithet *rileyae* honors JRR's wife, Megan Riley Roberts.

Remarks

Vasotrema rileyae differs from its congeners by having papillate suckers, a genital pore opening ventrally and anterior to the ovary, a short testis that does not extend to the level of the tips of the posterior ceca, and a dextral ovary.

Vasotrema rileyae most closely resembles *V. amydae* by having papillate suckers, paired terminal papillae dorsal to the mouth, a genital pore opening ventrally and anterior to the ovary, and a short testis that does not extend posteriad to the level of the tips of the posterior ceca. *Vasotrema rileyae* can be further differentiated from *V. amydae* by the combination of having a genital pore at level of ventral sucker, a wider and shorter testis (3.2−6.5× longer than wide), an ovary dextral to the oviduct, and a Laurer's canal extending posteriad and dextrad. *Vasotrema amydae* has a genital pore posterior to the ventral sucker, a more elongate testis (14.6× longer than wide), an ovary sinistral to the oviduct, and a Laurer's canal extending directly sinistrad from the oviduct (Figs. 2, 20).

DISCUSSION

Some new species of *Vasotrema* may infect other North American softshell turtles and their subspecies. The Eastern spiny softshell turtle, *Apalone spinifera spinifera* (LeSueur, 1827) and Gulf Coast spiny softshell turtle (see Weisrock and Janzen, 2000; Guyer et al., 2015) range in the Mississippi River (north of Louisiana) and Coastal Plain river drainages (east of the Mississippi River), respectively. Herein, we sampled the latter subspecies, describing a new TBF species from it, but all previous records source from the other subspecies. Likewise, the Gulf Coast smooth softshell turtle, *Apalone calvata*, which is endemic to lower Coastal Plain rivers and ranges from southeastern Louisiana to eastern Alabama and the Florida panhandle, has yet to be sampled for infections by *Vasotrema* spp. This turtle species was recently accepted but long considered a subspecies of the midland softshell turtle, *A. mutica* (see Weisrock and Janzen, 2000; Guyer et al., 2015). Three TBFs (*V. attenuatum*, *V. brevitestis*, *V. robustum*) infect its close relative *A. mutica* in Nebraska (Brooks and Mayes, 1975) (Table I). Also noteworthy regarding the discovery of innominate species of *Vasotrema* is that turtle diet and behavior coupled with the gear used to trap them could also leave some softshell turtles relatively undersampled. For example, that *A. spinifera* is the most intensively sampled softshell turtle in North America (Table I) could be related to sampling bias associated with baited hoop-net trapping. Smooth softshell turtles (*A. calvata* and *A. mutica*) are more difficult to sample because they must be caught by hand; whereas, spiny softshell turtles can be captured with baited traps, perhaps because they are a dietary generalist (Guyer et al., 2015).

Concurrent infections by *Vasotrema* spp., including congeners infecting the same tissue of the same host individual, are not uncommon. This underscores the importance of morphological vouchers that accompany published molecular sequence data. The only infection records reporting a single-species infection by a species of *Vasotrema* are the parasitological surveys of Loftin (1960) and Foster et al. (1998) as well as the molecular phylogenetics study of Snyder (2004) (Table I). To ensure correct taxonomic assignments of molecular sequences, a hologenophore is ideal and can be obtained, if specimen size permits, by cutting the posterior 1/3 of the body (posterior to the testis) and preserving it in 95% EtOH for molecular biology while saving the remaining portion of the specimen for staining and whole-mounting. If the specimens are too small for that, specimens should be wet-mounted and photographed, ideally with DIC, before preserving or fixing them in EtOH and n.b.f. for molecular biology and morphology, respectively.

Key to species of *Vasotrema*:

1.	Genital pore at level of ovary	*V. robustum*
	Genital pore anterior to ovary	2
2.	Testis long, extending posteriad to tips of ceca	3
	Testis short, not extending to posteriad to tips of ceca	4
3.	Testis not coiled	*V. attenuatum*
	Testis coiled	*V. longitestis*
4.	Suckers papillate	5
	Suckers apapillate	*V. brevitestis*
5.	Ovary sinistral to oviduct	*V. amydae*
	Ovary dextral to oviduct	*V. rileyae*

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LITERATURE CITED

Brooks, D. R., and M. A. Mayes. 1975. Platyhelminths of Nebraska turtles with descriptions of two new species of spirorchiids (Trematoda: Spirorchiidae). Journal of Parasitology **61:** 403−406.

Brooks, D. R., and J. J. Sullivan. 1981. *Hapalorhynchus rugatus* sp. nov. (Digenea: Spirorchiidae) from a Malaysian freshwater turtle. Canadian Journal of Zoology **59:** 1335−1338.

Bullard, S. A., and R. M. Overstreet. 2004. Two new species of *Cardicola* (Digenea: Sanguinicolidae) in drums (Sciaenidae) from Mississippi and Louisiana. Journal of Parasitology **90:** 128-136.

Bullard, S. A., R. M. Overstreet, and J. K. Carlson. 2006. *Selachohemecus benzi* n. sp. (Digenea: Sanguinicolidae) from the blacktip shark *Carcharhinus limbatus* in the Northern Gulf of Mexico. Systematic Parasitology **63:** 143-154.

Bullard, S. A., and K. Jensen. 2008. Blood flukes (Digenea: Aporocotylidae) of stingrays (Myliobatiformes: Dasyatidae): *Orchispirium heterovitellatum* from *Himantura imbricata* in the Bay of Bengal and a new genus and species from *Dasyatis sabina* in the Northern Gulf of Mexico. Journal of Parasitology **94:** 1311-1321.

Bullard, S. A., S. D. Snyder, K. Jensen, and R. M. Overstreet. 2008. New genus and species of Aporocotylidae (Digenea) from a lower actinopterygian, the American paddlefish, *Polyodon spathula*, (Polyodontidae) from the MS Delta. Journal of Parasitology **94:** 487-495.

Byrd, E. E. 1939. Studies on the blood flukes of the family Spirorchidae. Part II. Revision of the family and description of new species. Journal of the Tennessee Academy of Science **14:** 116−161.

Ejsmont, L. 1927. *Spirhapalum polesianum* n. g., n. sp. trématode du sang d'*Emys orbicularis* L. Annales de Parasitologie **3:** 220–235.

Foster, G. W., J. M. Kinsella, P. E. Moler, L. M. Johnson, and D. J. Forrester. 1998. Parasites of Florida softshell turtles (*Apalone ferox*) from southeastern Florida. Journal of the Helminthological Society of Washington **65:** 62–64.

Guyer C., M. A. Bailey, and R. H. Mount. 2015. Turtles of Alabama, 1st ed. The University of Alabama Press, Tuscaloosa, Alabama, 266 p.

International Commission on Zoological Nomenclature (ICZN). 2000. International Code of Zoological Nomenclature, 4th ed. The Natural History Museum, London, U.K., 306 p.

Loftin, H. 1960. An annotated check-list of trematodes and cestodes and their vertebrate hosts from northwest Florida. Quarterly Journal of the Florida Academy of Sciences **23:** 302−314.

Orélis-Ribeiro, R., C. R. Arias, K. M. Halanych, T. H. Cribb, and S. A. Bullard. 2014. Diversity and ancestry of flatworms infecting blood of nontetrapod craniates "fishes." Advances in Parasitology **85:** 1−64.

Orélis-Ribeiro, R. and S. A. Bullard. 2015. Blood flukes (Digenea: Aporocotylidae) infecting body cavity of South American catfishes (Siluriformes: Pimelodidae): Two new species from rivers in Bolivia, Guyana, and Peru with a re-assessment of *Plehniella* Szidat, 1951. Folia Parasitologica **62:** 1-17.

Platt, T. R. 1993. Taxonomic revision of *Spirorchis* MacCallum, 1919 (Digenea: Spirorchidae). Journal of Parasitology **79:** 337−346.

Platt, T. R. 2002. Family Spirorchiidae Stunkard, 1921. *In* Keys to the Trematoda, Vol. 1, D. I. Gibson, A. J. Jones, and R. A. Bray (eds.). CABI Publishing, Wallingford, Oxford, U.K., p. 453-468.

Platt, T. R., and A. K. Prestwood. 1990. Deposition of type and voucher material from the helminthological collection of Elon E. Byrd. Systematic Parasitology **16:** 27−34.

Platt, T. R., and S. D. Snyder. 2007. Redescription of *Hapalorhynchus reelfooti* Byrd, 1939 (Digenea: Spirorchiidae) from *Sternotherus odoratus* (Latreille, 1801). Comparative Parasitology **74:** 31−34.

Roberts, J. R., R. Orélis-Ribeiro, B. T. Dang, K. M. Halanych, and S. A. Bullard. 2016b. Blood flukes of Asiatic softshell turtles: revision of *Coeuritrema* Mehra, 1933 (Digenea: Schistosomatoidea) and a new species infecting Chinese softshell turtles, *Pelodiscus sinensis*, (Trionychidae) from the Da Rang River, Vietnam. Folia Parasitologica **63:** 031. doi:10.14411/fp.2016.031.

Roberts, J. R., R. Orélis-Ribeiro, K. M. Halanych, C. R. Arias, and S. A. Bullard. 2016c. A new species of *Spirorchis* MacCallum, 1918 (Digenea: Schistosomatoidea) and *Spirorchis* cf. *scripta* from chicken turtle, *Deirochelys reticularia*, (Emydidae), with an emendation and molecular phylogeny of *Spirorchis*. Folia Parasitologica **63**: 041. doi:10.14411/fp.2016.041

Roberts, J. R., T. R. Platt, R. Orélis-Ribeiro, and S. A. Bullard. 2016a. New genus of blood fluke (Digenea: Schistosomatoidea) from Malaysian freshwater turtles (Geoemydidae) and its phylogenetic position within Schistosomatoidea. Journal of Parasitology **102:** 451−462.

Rohde, K., S. K. Lee, and H. W. Lim. 1968. Ueber drei malayische Trematoden. Annales de Parasitologie Humaine et Comparée **43:** 33−43.

Smith, J. W. 1997a. The blood flukes (Digenea: Sanguinicolidae and Spirorchidae) of cold-blooded vertebrates: Part I. A Review of the literature published since 1971, and bibliography. Helminthological Abstracts **66:** 255−294.

Smith, J. W. 1997b. The blood flukes (Digenea: Sanguinicolidae and Spirorchidae) of cold-blooded vertebrates: Part II. Appendix I: Comprehensive parasite-host list; Appendix II: Comprehensive host-parasite list. Helminthological Abstracts **66:** 329−344.

Snyder, S. D. 2004. Phylogeny and paraphyly among tetrapod blood flukes (Digenea: Schistosomatidae and Spirorchiidae). International Journal of Parasitology **34:** 1385−1392.

Stunkard, H. W. 1923. Studies on North American blood flukes. Bulletin of the American Museum of Natural History **48:** 165−221.

Stunkard, H. W. 1926. A new trematode *Vasotrema amydae* n. g., n. sp., from the vascular system of the soft-shelled turtle, *Amyda*. Anatomical Record **34:** 165.

Stunkard, H. W. 1928. Observations nouvelles sur les trématodes sanguicoles du genre *Vasotrema* (Spirorchidae) avec description des deux espèces nouvelles. Annales de Parasitologie Humaine et Comparee **6:** 303−320.

Tkach, V., S. D. Snyder, and J. A. Vaughan. 2009. A new species of blood fluke (Digenea: Spirorchiidae) from the Malayan box turtle, *Cuora amboinensis* (Cryptodira: Geoemydidae) in Thailand. Journal of Parasitology **95:** 743−746.

Truong, T. N., and S. A. Bullard. 2013. Blood flukes (Digenea: Aporocotylidae) of walking catfishes (Siluriformes: Clariidae): New genus and species from the Mekong River (Vietnam) and a note on catfish aporocotylids. Folia Parasitologica **60:** 237−247.

van Dijk, P. P., J. B. Iverson, A. G. J. Rhodin, H. B. Shaffer, and R. Bour. 2014. Turtles of the world, 7th Edition: Annotated checklist of taxonomy, synonymy, distribution with maps, and conservation status. Chelonian Research Monographs **5:** 329−479.

Wall, L. D. 1951. The life history of *Vasotrema robustum* (Stunkard, 1928), Trematoda: Spirorchiidae. Transactions of the American Microscopical Society **70:** 173−184.

Weisrock, D. W., and F. J. Janzen. 2000. Comparative molecular phylogeography of North American softshell turtles (*Apalone*): Implications for regional and wide-scale historical evolutionary forces. Molecular Phylogenetics and Evolution **14:** 152−164.

Yong, R. Q-Y., S. C. Cutmore, R. A. Bray, T. L. Miller, I. W. Y. Semarariana, H. W. Palm, and T. H. Cribb. 2016. Three new species of blood flukes (Digenea: Aporocotylidae) infecting pufferfishes (Teleostei: Tetraodontidae) from off Bali, Indonesia. Parasitology International **65**: 432−443.

FIGURE LEGENDS

Figures 1, 2. *Vasotrema amydae* Stunkard, 1926 (cotype, AMNH Coll. No. 791) from spiny softshell turtle, *Apalone spinifera* (LeSueur, 1827), (Testudines: Trionychidae) or Florida softshell turtle, *Apalone ferox* (LeSueur, 1827), from a river in Indiana or Florida. Scale values aside bars. (**1**) Body (dorsal view) showing oral sucker (os), paired terminal papillae (tp), pharynx (ph), nerve commissure (nc), esophagus (es), esophageal gland (eg), median esophageal diverticulum (med), sinistral cecum (sc), ventral sucker (vs), dextral cecum (dc), common genital pore (cgp), cirrus sac (cs), uterus (ut), external seminal vesicle (esv), ovary (ov), vitellarium (vr), testis (ts), cecal terminus (ct), Manter's organ (Mo), excretory vesicle (ev), and excretory pore (ep). (**2**) Genitalia (dorsal view) showing cirrus (ci), metraterm (mt), internal seminal vesicle (isv), oötype (oo), ovi-vitelline duct (ovt), oviduct (od), oviducal seminal receptacle (osr), Laurer's canal (Lc), primary vitelline duct (vt), and putative vas deferens (pv).

Figure 3−6. *Vasotrema attenuatum* Stunkard, 1928 (syntype, AMNH Coll. No. 806-4) from spiny softshell turtle, *Apalone spinifera* (LeSueur, 1827), (Testudines: Trionychidae) or Florida softshell turtle, *Apalone ferox* (LeSueur, 1827), from a river in Indiana or Florida. (**3**) Body (dorsal view) showing oral sucker (os), ventral sucker (vs), common genital pore (cgp), ovary (ov), testis (ts), and cecal terminus (ct). Scale value aside bar; dashed line indicates body segments illustrated at higher magnification in Figures 4 and 5. (**4**) Anterior portion of body (dorsal view) showing pharynx (ph), esophagus (es), nerve commissure (nc), esophageal gland (eg), median esophageal diverticulum (med), sinistral cecum (sc), dextral cecum (dc), ventral sucker (vs), cirrus sac (cs), common genital pore (cgp), metraterm (mt), cirrus (ci), uterus (ut), internal seminal vesicle (isv), ovary (ov), Laurer's canal (Lc), oviduct (od), oviducal seminal receptacle (osr), vas deferens (vd), vitelline duct (vt), and testis (ts). (**5**) Posterior portion of body (dorsal view) vitellarium (vr), cecal terminus (ct), Manter's organ (Mo), excretory vesicle (ev), and excretory pore (ep). (**6**) Genitalia (dorsal view) showing oötype (oo) and ovi-vitelline duct (ovt).

Figures 7, 8. *Vasotrema robustum* Stunkard, 1928 (syntype, AMNH Coll. No. 808) from spiny softshell turtle, *Apalone spinifera* (LeSueur, 1827), (Testudines: Trionychidae) from a river in Indiana, USA. Scale values aside bars. (**7**) Body (ventral view) showing oral sucker (os), pharynx (ph), nerve commissure (nc), esophagus (es), esophageal gland (eg), median esophageal diverticulum (med), dextral cecum (dc), sinistral cecum (sc), ventral sucker (vs), vitellarium (vr), external seminal vesicle (esv), internal seminal vesicle (isv), ovary (ov), common genital pore (cgp), oviducal seminal receptacle (osr), primary vitelline duct (vt), vas deferens (vd), testis (ts), cecal terminus (ct), Manter's organ (Mo), excretory vesicle (ev), and excretory pore (ep). (**8**) Genitalia (ventral view) showing cirrus sac (cs), cirrus (ci), oviduct (od), metraterm (mt), uterine egg (egg), uterus (ut), and oötype (oo).

Figures 9−11. *Vasotrema robustum* Stunkard, 1928 (voucher, USNM **XXX**) from Gulf Coast spiny softshell turtle, *Apalone spinifera aspera* (Agassiz, 1857), (Testudines: Trionychidae) from Canoe Lake (33°47'56.16"N, 86°29'25.02"W), Coosa River, Springville, Alabama. Scale values aside bars. (**9**) Body (ventral view) showing paired terminal papillae (tp), oral sucker (os), pharynx (ph), esophagus (es), nerve commissure (nc), esophageal gland (eg), median esophageal diverticulum (med), dextral cecum (dc), sinistral cecum (sc), ventral sucker (vs), vitellarium (vr), internal seminal vesicle (isv), external seminal vesicle (esv), common genital pore (cgp), ovary (ov), uterus (ut), oviducal seminal receptacle (osr), primary vitelline duct (vt), testis (ts), cecal terminus (ct), Manter's organ (Mo), excretory vesicle (ev), and excretory pore (ep). (**10**) Oral sucker (ventral view) showing marginal spines (ms), internal mouth spines (ims), and paired internal mouth papillae (ipa). (**11**) Genitalia (ventral view) showing cirrus sac (cs), cirrus (ci), metraterm (mt), oviduct (od), oötype (oo), Laurer's canal (Lc), and vas deferens (vd).

Figures 12−14. *Vasotrema longitestis* Byrd, 1939 from spiny softshell turtle, *Apalone spinifera* (LeSueur, 1827), (Testudines: Trionychidae) (**12**) and Gulf Coast spiny softshell, *Apalone spinifera aspera* (Agassiz, 1857), (Testudines: Trionychidae) (**13, 14**). Scale values aside bars. (12) Body (holotype, USNM 1321971, from Reelfoot Lake [36°21'12.23"N, 89°25'21.50"W], Tennessee) (ventral view) showing oral sucker (os), pharynx (ph), esophagus (es), common genital pore (cgp), ventral sucker (vs), metraterm (mt), cirrus sac (cs), uterine egg (egg), ovary (ov), testis (ts), and excretory vesicle (ev). (13) Body (voucher, USNM **XXX**, from Canoe Lake [33°47'56.16"N, 86°29'25.02"W], Coosa River, Springville, Alabama) (ventral view) showing nerve commissure (nc), median esophageal diverticulum (med), esophageal gland (eg), dextral cecum (dc), sinistral cecum (sc), vas deferens (vd), primary vitelline duct (vt), vitellarium (vr), cecal terminus (ct), Manter's organ (Mo), and excretory pore (ep). (14) Genitalia (voucher, USNM **XXX**, from Canoe Lake [33°47'56.16"N, 86°29'25.02"W], Coosa River, Springville, Alabama) (ventral view) showing cirrus (ci), metraterm (mt), internal seminal vesicle (isv), egg chamber (ec), Laurer's canal (Lc), oviduct (od), and oviducal seminal receptacle (osr).

Figures 15−18. *Vasotrema brevitestis* Brooks and Mayes, 1975 (paratype, HWML Coll. No. 20077) from midland smooth softshell turtle, *Apalone mutica* (LeSueur, 1827), (Testudines: Trionychidae) from the Missouri River, Nebraska. Scale values aside bars. (**15**) Body (paratype, HWML 20077-1) (ventral view) showing oral sucker (os), pharynx (ph), nerve commissure (nc), esophagus (es), esophageal gland (eg), median esophageal diverticulum (med), dextral cecum (dc), sinistral cecum (sc), ventral sucker (vs), common genital pore (cgp), cirrus sac (cs), uterus (ut), external seminal vesicle (esv), ovary (ov), Laurer's canal (Lc), vitellarium (vr), testis (ts), cecal terminus (ct), Manter's organ (Mo), excretory vesicle (ev), and excretory pore (ep). (**16**) Genitalia (paratype, HWML 20077-1) (ventral view) showing cirrus (ci), metraterm (mt), internal seminal vesicle (isv), oötype (oo), ovi-vitelline duct (ovt), oviduct (od), oviducal seminal receptacle (osr), primary vitelline duct (vt), and vas deferens (vd). (**17**) Body (paratype, HWML 20077-3) (dorsal view). (**18**) Genitalia (paratype, HWML 20077-3) (dorsal view).

Figures 19, 20. *Vasotrema rileyae* n. sp. (Holotype, USNM **XXX**) from Gulf Coast spiny softshell turtle, *Apalone spinifera aspera* (Agassiz, 1857), (Testudines: Trionychidae) from Round Lake (32°41'50.91"N, 87°14'30.39"W), Cahaba River, Marion, Alabama.

Scale values aside bars. (**19**) Body (dorsal view) showing oral sucker (os), paired terminal papillae (tp), pharynx (ph), esophagus (es), nerve commissure (nc), esophageal gland (eg), median esophageal diverticulum (med), sinistral cecum (sc), ventral sucker (vs), dextral cecum (dc), uterus (ut), cirrus sac (cs), external seminal vesicle (esv), ovary (ov), vitellarium (vr), testis (ts), cecal terminus (ct), Manter's organ (Mo), excretory vesicle (ev), and excretory pore (ep). (**20**) Genitalia (dorsal view) showing common genital pore (cgp), cirrus (ci), metraterm (mt), internal seminal vesicle (isv), uterus (ut), oötype (oo), ovi-vitelline duct (ovt), Laurer's canal (Lc), oviduct (od), oviducal seminal receptacle (osr), vas deferens (vd), and primary vitelline duct (vt).

Table I. Host and geographic locality records and museum specimens of *Vasotrema* spp.

Turtle host	*Vasotrema* sp.	Site in host	Riverine locality	Accession no.	Reference
Apalone ferox (Schneider, 1783)	*Vasotrema amydae* Stunkard, 1926 (type species)	blood (adult)	None specified, Florida, USA	*AMNH 791	Stunkard (1926; 1928)
	Vasotrema attenuatum Stunkard, 1928	blood (adult)	None specified, Florida, USA	†AMNH 806, 807	Stunkard (1928)
	Vasotrema robustum Stunkard, 1928	None specified (adult)	None specified, probably Fort Myers, Florida, USA	‡USNM 37306	Wall (1951)
		lumen of heart, blood vessels of liver, lung, intestine (adult)	Lake Okeechobee, Palm Beach County, Florida, USA	HWML 39326	Foster et al. (1998)
	V. sp.	None specified (adult)	Ochlockonee River, Leon County, Florida, USA	None specified	Loftin (1960)
Apalone mutica (LeSueur, 1827)	*V. attenuatum*	blood (adult)	None specified, Nebraska, USA	None specified	Brooks and Mayes (1975)
	Vasotrema brevitestis Brooks and Mayes, 1975	blood (adult)	Missouri River, site (41°31'22.34"N, 96° 8'7.30"W) 2.4 km south of Blair, Nebraska, USA	USNM 73817, 73818; HWML 20077	Brooks and Mayes (1975)
	V. robustum	None specified (adult)	None specified (possibly Cumberland River, Davidson County, Tennessee, USA)	‡USNM 37306	Wall (1951)
		circulatory system (adult)	None specified, Nebraska, USA	None specified	Brooks and Mayes (1975)
Apalone spinifera (LeSueur, 1827)	*V. amydae* (type species)	blood (adult)	None specified, Indiana, USA	*AMNH 791	Stunkard (1926; 1928)
		mesenteric blood	Reelfoot Lake	USNM 9227	Byrd (1939); Platt

	vessels (adult)	(36°21'12.23"N, 89°25'21.50"W), Tennessee, USA		and Snyder (2007); present study
	blood (adult)	Huron River, Washtenaw County, Michigan, USA	None specified	Wall (1951)
V. attenuatum	blood (adult)	None specified, Indiana, USA	†AMNH 806, 807	Stunkard (1928)
	blood (adult)	None specified, Nebraska, USA	None reported	Brooks and Mayes (1975)
V. brevitestis	blood (adult)	Atkinson Lake (42°32'20.36"N, 99° 0'3.04"W), 0.8 km west of Atkinson, Nebraska, USA	USNM 73819; HWML 20076	Brooks and Mayes (1975)
Vasotrema longitestis Byrd, 1939	arterial circulation (adult)	Reelfoot Lake (36°21'12.23"N, 89°25'21.50"W), Tennessee, USA	USNM 1321971; HWML 31121	Byrd (1939); Platt and Prestwood (1994)
V. robustum	blood	None specified, Indiana, USA	AMNH 808, 809	Stunkard (1928)
	ventricle of heart (adult)	Reelfoot Lake (36°21'12.23"N, 89°25'21.50"W), Tennessee, USA	None specified	Byrd (1939)
	lumen of heart and other large blood vessels (adult)	Huron River, Washtenaw County, Michigan, USA	‡USNM 37306	Wall (1951)
		Cumberland River, Davidson County, Tennessee, USA		
	blood (adult)	None specified, Nebraska, USA	HWML 20075	Brooks and Mayes (1975)
	None specified	Nishnabotna River,	HWML 45795	Snyder (2004)

		(adult)	Floyd County, Iowa, USA		
Apalone spinifera aspera (Agassiz, 1857)	***V. longitestis***	**mesenteric blood vessels (adult)**	**Canoe Lake (33°47'56.16"N, 86°29'25.02"W), Coosa River, Springville, Alabama, USA**	**USNM XXXX**	**Present study**
		mesenteric blood vessels (adult)	**Round Lake (32°41'50.91"N, 87°14'30.39"W), Cahaba River, Perry Lakes State Park, Marion, Alabama, USA**	**USNM XXXX**	**Present study**
	***V. rileyae* Roberts and Bullard, n. sp.**	**mesenteric blood vessels (adult)**	**Canoe Lake (33°47'56.16"N, 86°29'25.02"W), Coosa River, Springville, Alabama, USA**	**USNM XXXX**	**Present study**
		mesenteric blood vessels (adult)	**Round Lake (32°41'50.91"N, 87°14'30.39"W), Cahaba River, Perry Lakes State Park, Marion, Alabama, USA**	**USNM XXXX**	**Present study**
	V. robustum	**mesenteric blood vessels (adult)**	**Canoe Lake (33°47'56.16"N, 86°29'25.02"W), Coosa River, Springville, Alabama, USA**	**USNM XXXX**	**Present study**
		mesenteric blood vessels	**Round Lake (32°41'50.91"N,**	**Specimen destroyed**	**Present study**

		(adult)	**87°14'30.39"W), Cahaba River, Perry Lakes State Park, Marion, Alabama, USA**		
§ *Apalone* sp.	*V. attenuatum*	None specified (adult)	None specified	None specified	Wall (1951)

* Stunkard (1926; 1928) did not specify from which turtle host species this specimen came.
† Stunkard (1928) only specified on some slides the host species from which the specimens came.
‡ Wall (1951) reported infections of *V. robustum* from *A. ferox*, *A. mutica*, and *A. spinifera*. He deposited all life history stages (USNM 37306), but did not specify from which host the mounted vouchers came.
§ Wall (1951) reported an infection of *V. attenuatum* but did not specify the infected tissue, host species, or locality and did not deposit a voucher specimen.

Table II. Turtle blood fluke specimens examined in the present study.

***Vasotrema* sp.**	**Slide label**	**Accession No.**	**No. slides**	**Specimen**	**Host**	**Locality**	**Notes**	**Reference(s)**
Vasotrema amydae Stunkard, 1926	*Vasotrema amydae*	AMNH 791	1	cotype	Not specified (either *Apalone ferox* [Schneider, 1783)] or *Apalone spinifera* [LeSueur, 1827]	Not specified (either Florida or Indiana river drainage), USA	Two specimens on slide; specimen mounted center is cotype	Stunkard (1926; 1928)
Vasotrema attenuatum Stunkard, 1928	*Vasotrema ~~amydae~~ attenuatum*	AMNH 806	17	syntypes	Not specified (either *A. ferox* or *A. spinifera*); *A. spinifera* for slides 806-1, 2, 12, 16	Not specified (either Florida or Indiana river drainage), USA; Indiana, USA for slides 806-1, 2, 12, 16	slides 806-1, 2, 12, 16 have *Apalone spinifera* (labeled as *Amyda spinifera*)	Stunkard (1928)
Vasotrema robustum Stunkard, 1928	original label: *Vasotrema ~~amydae~~*; new label: *Vasotrema robustum*	AMNH 808	1	syntype	Not specified (*A. spinifera*; see Stunkard, 1928)	Not specified, Indiana, USA	*V. amydae* is written, with *amydae* scratched out	Stunkard (1928)
Vasotrema longitestis Byrd, 1939	*Vasotrema longitestis*	USNM 1321971	1	holotype	*A. spinifera* (labeled as *Amyda spinifera*)	Reelfoot Lake (36°21'12.23" N, 89°25'21.50" W), Tennessee, USA		Byrd (1939)

Vasotrema brevitestis Brooks and Mayes, 1975	*Vasotrema brevitestis*	HWML 20076	4 (loaned 2)	paratypes	*A. spinifera* (labeled as *Trionyx spiniferus*)	Atkinson Lake (42°32'20.36" N, 99° 0'3.04"W), 0.8 km west of Atkinson, Nebraska, USA,		Brooks and Mayes (1975)
		HWML 20077	6 (loaned 3)	paratypes	*Apalone mutica* (LeSueur, 1827) (labeled as *Trionyx muticus*)	Missouri River, site (41°31'22.34" N, 96° 8'7.30"W) 2.4 km south of Blair, Nebraska, USA		Brooks and Mayes (1975)

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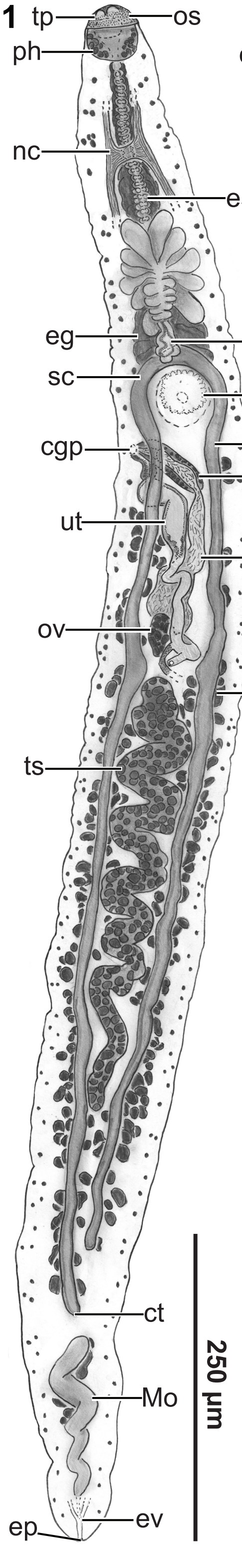
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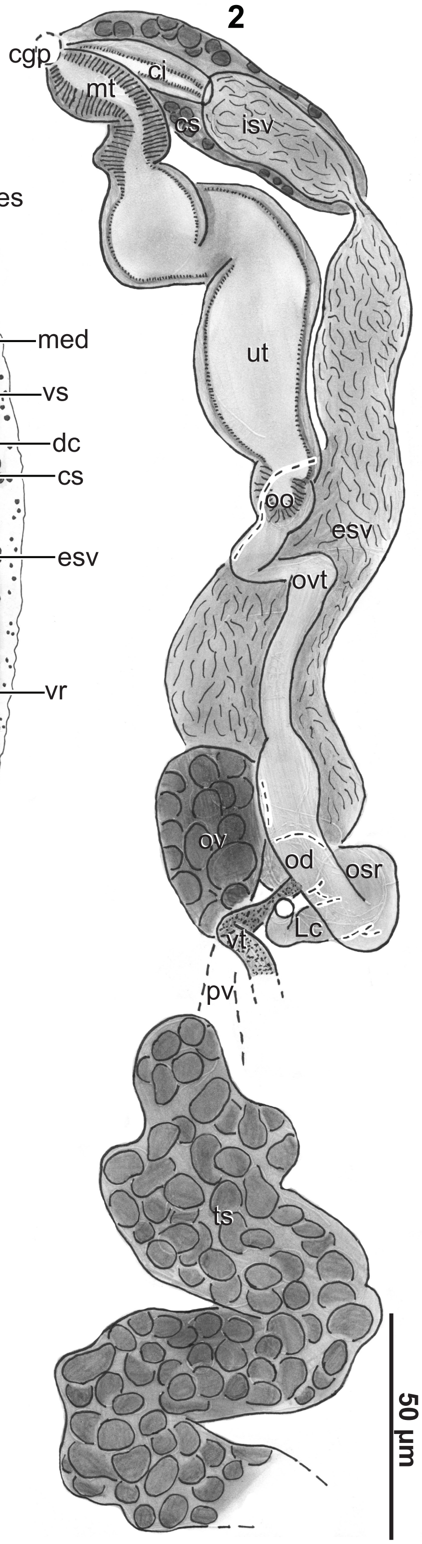
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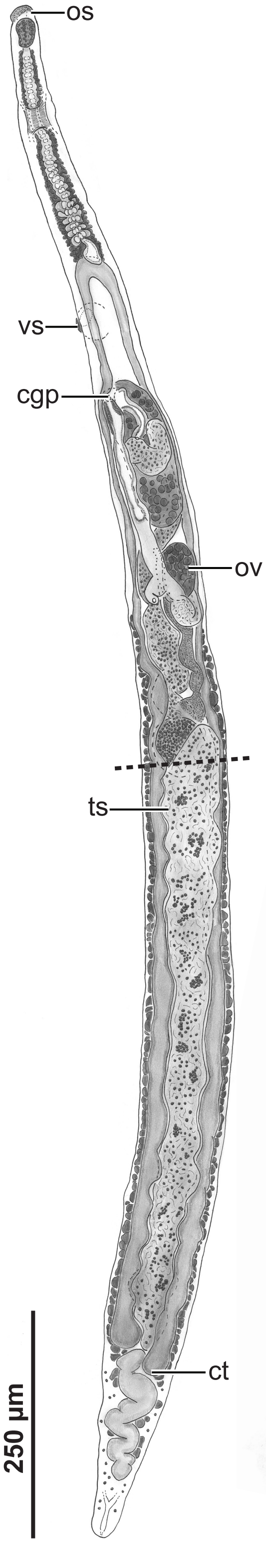

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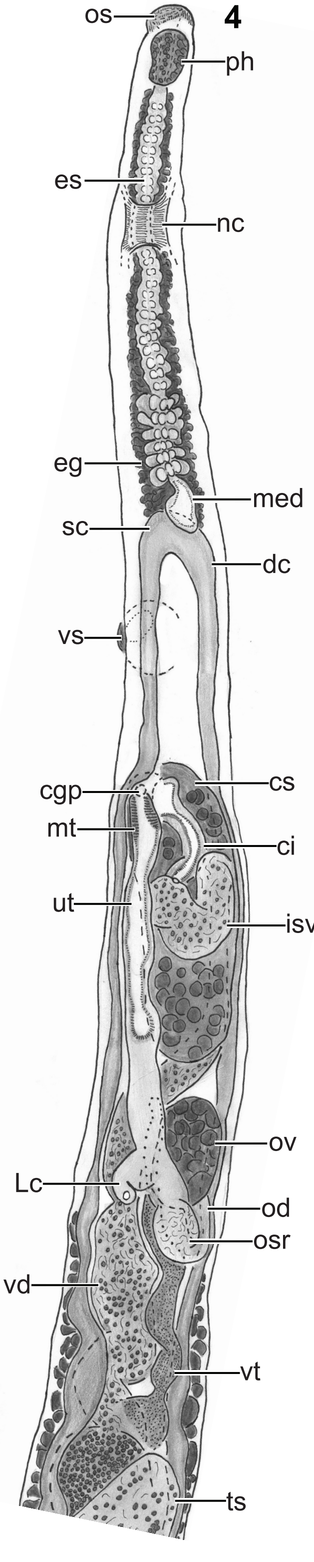

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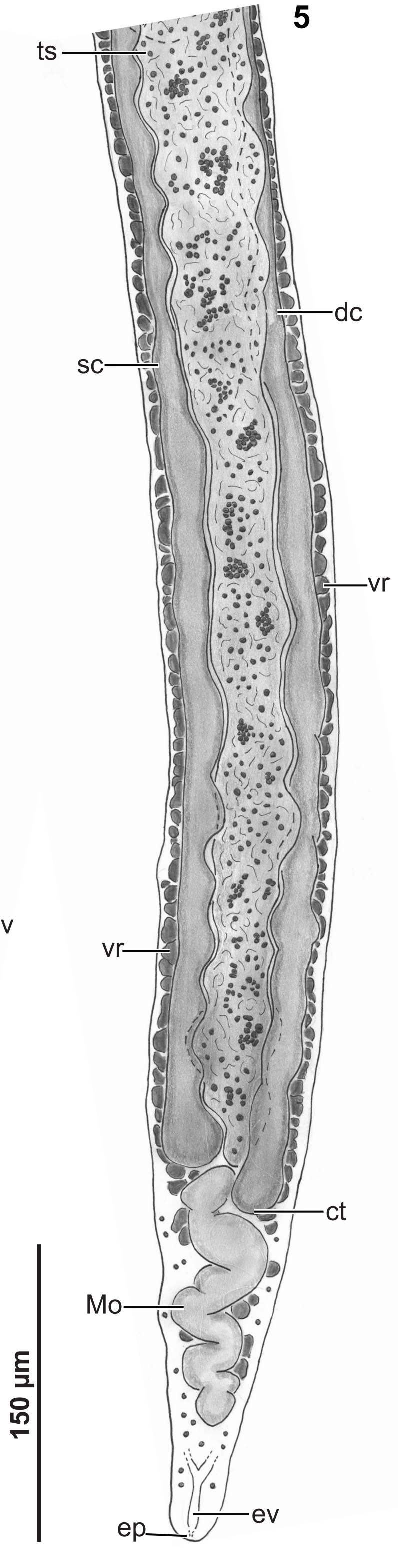

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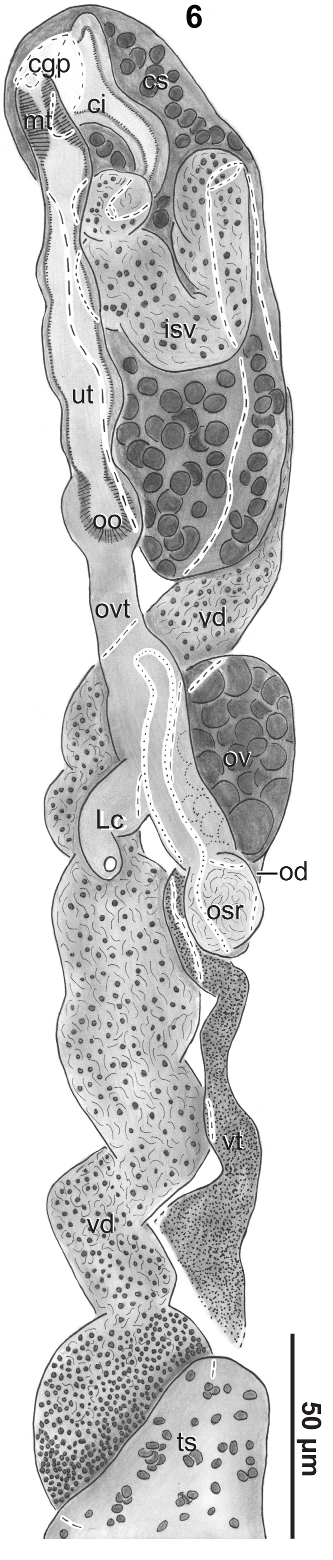

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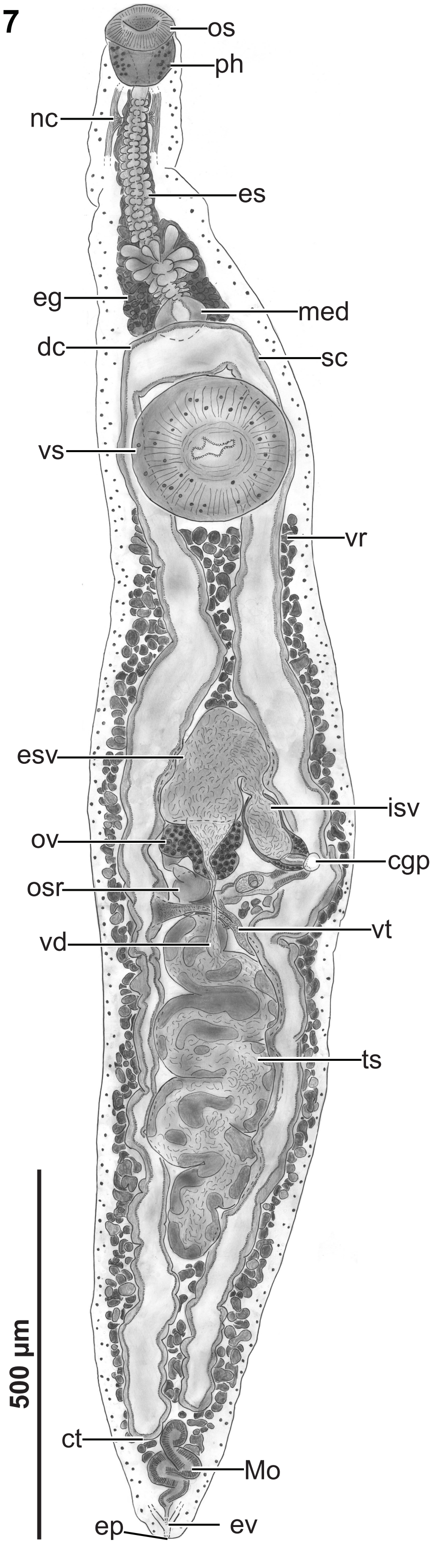
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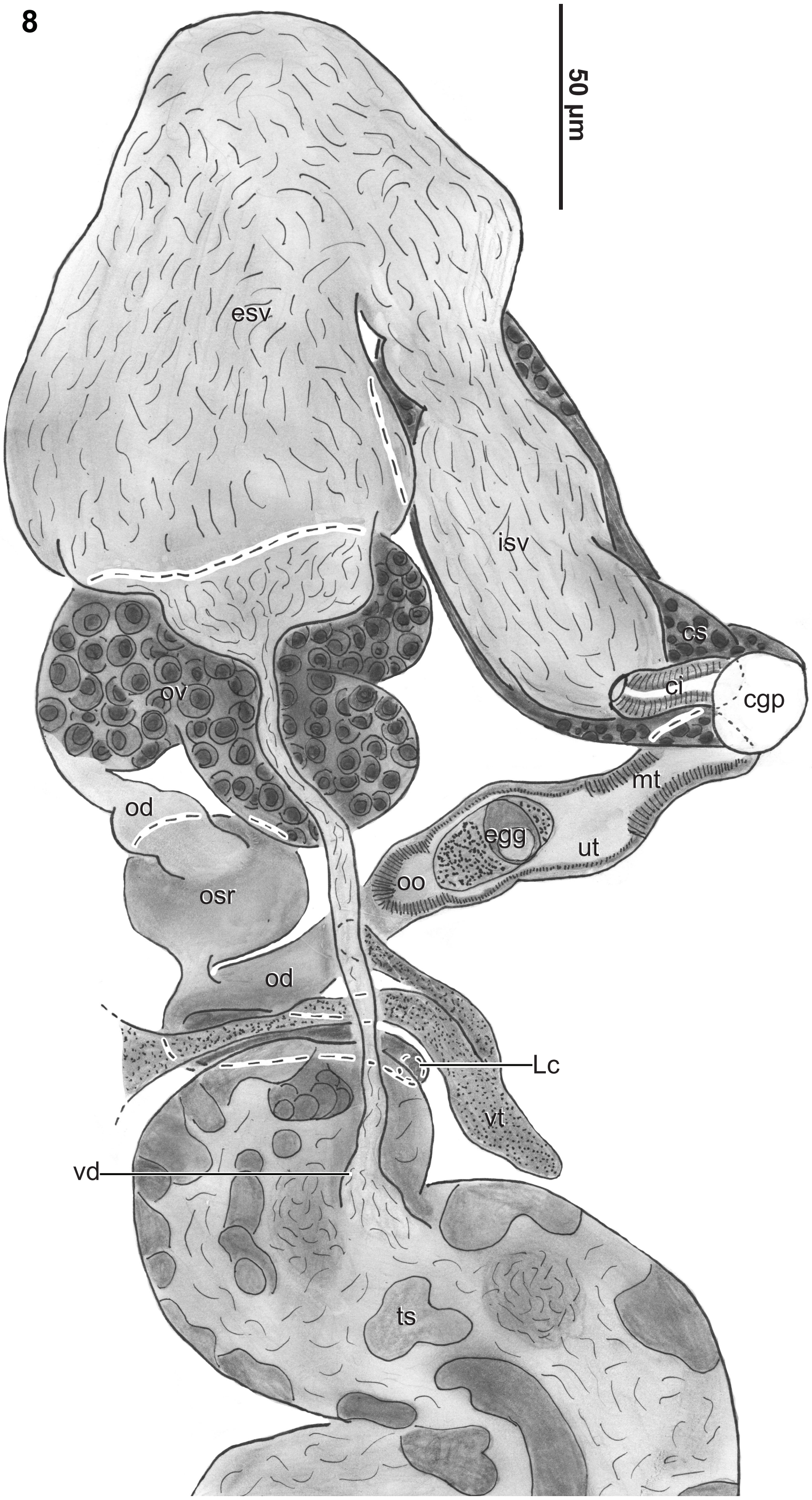
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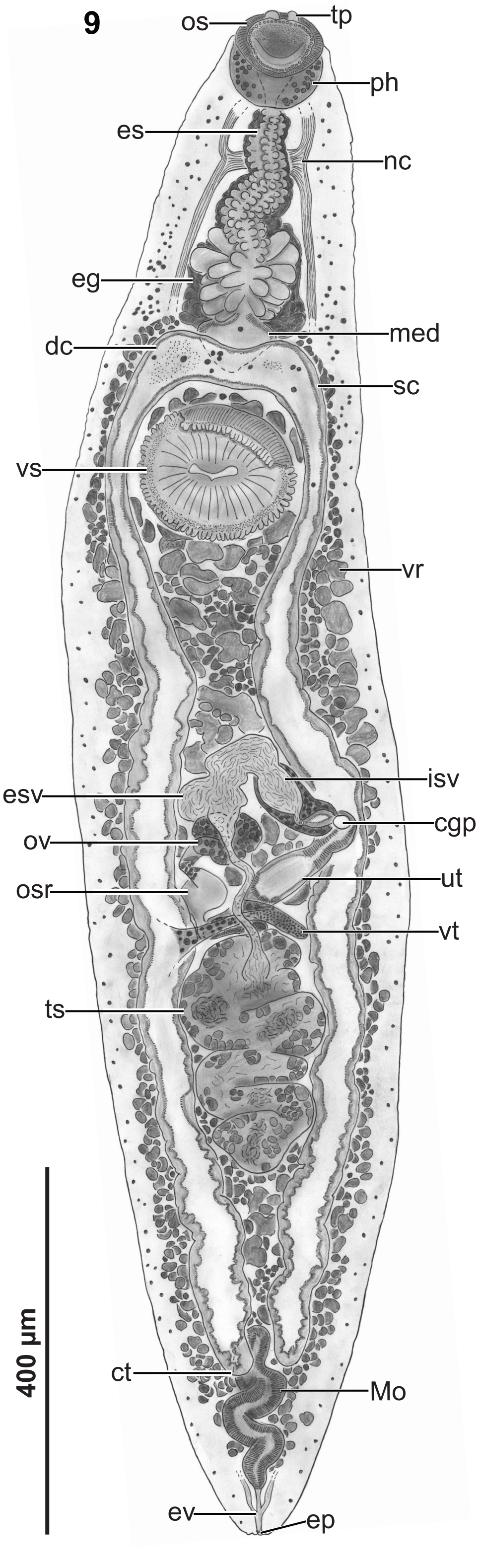
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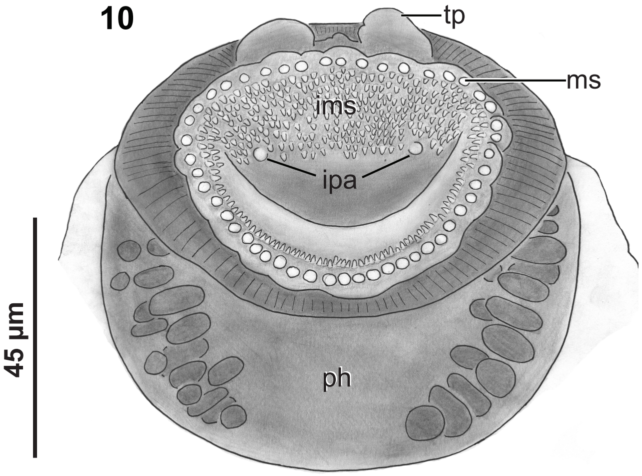
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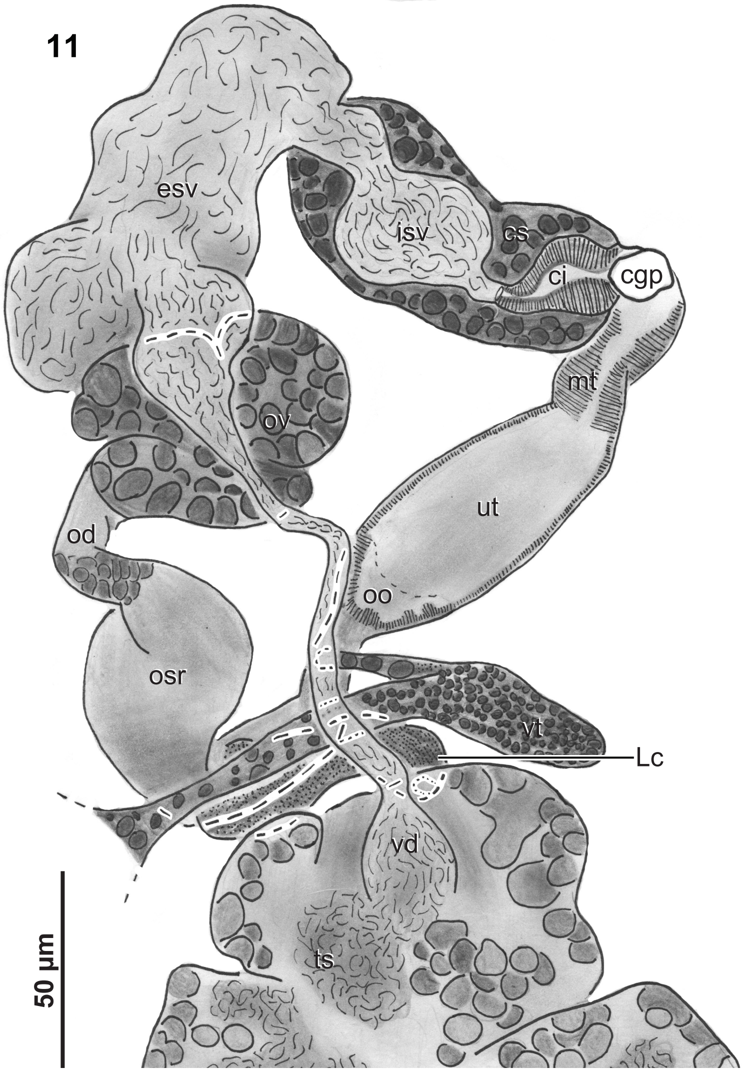
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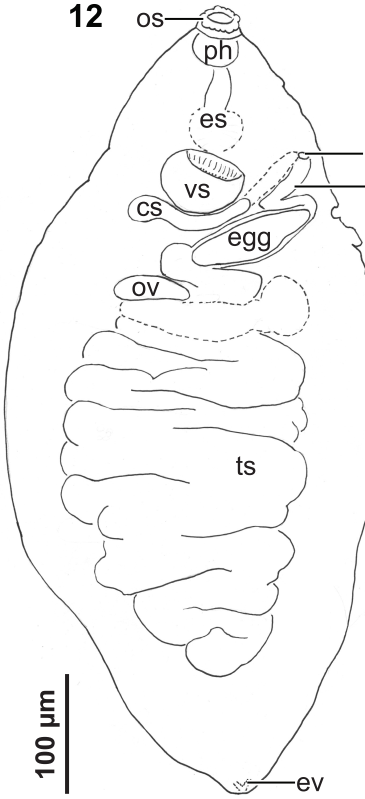
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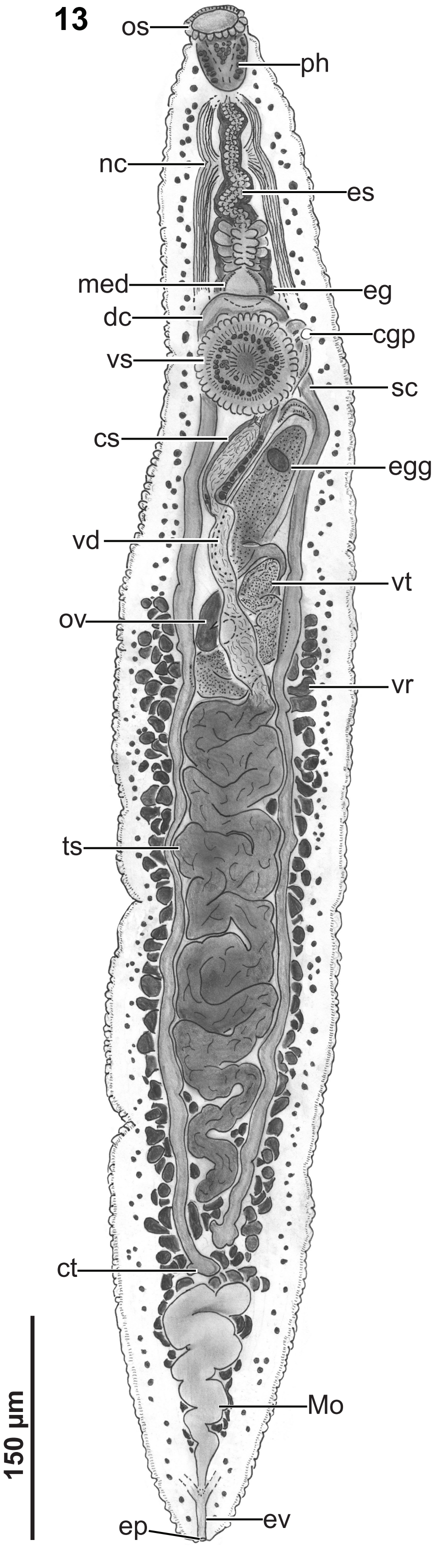
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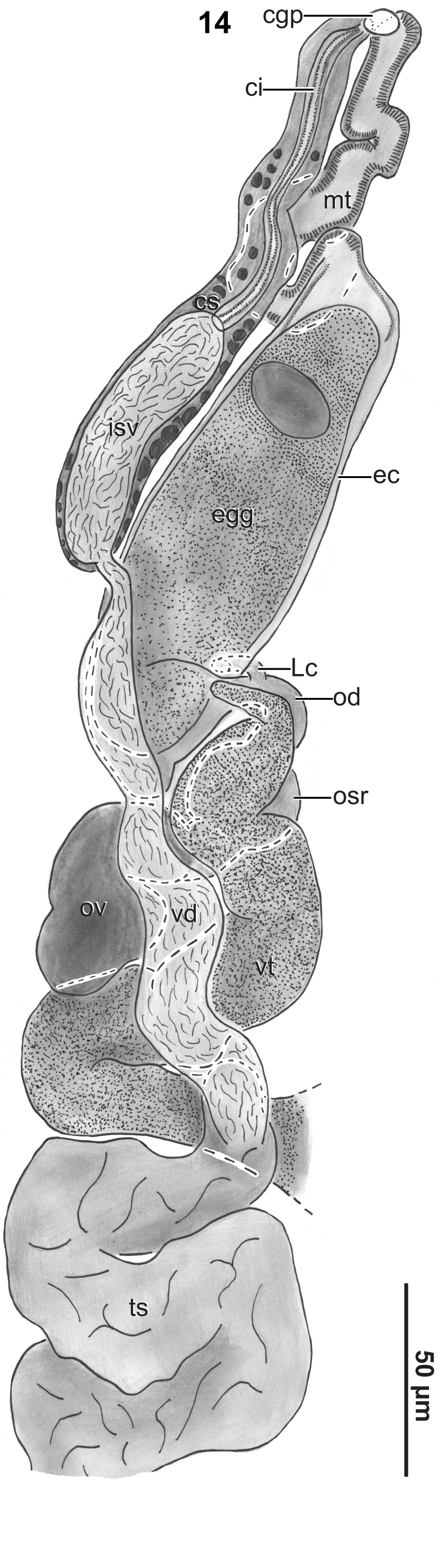
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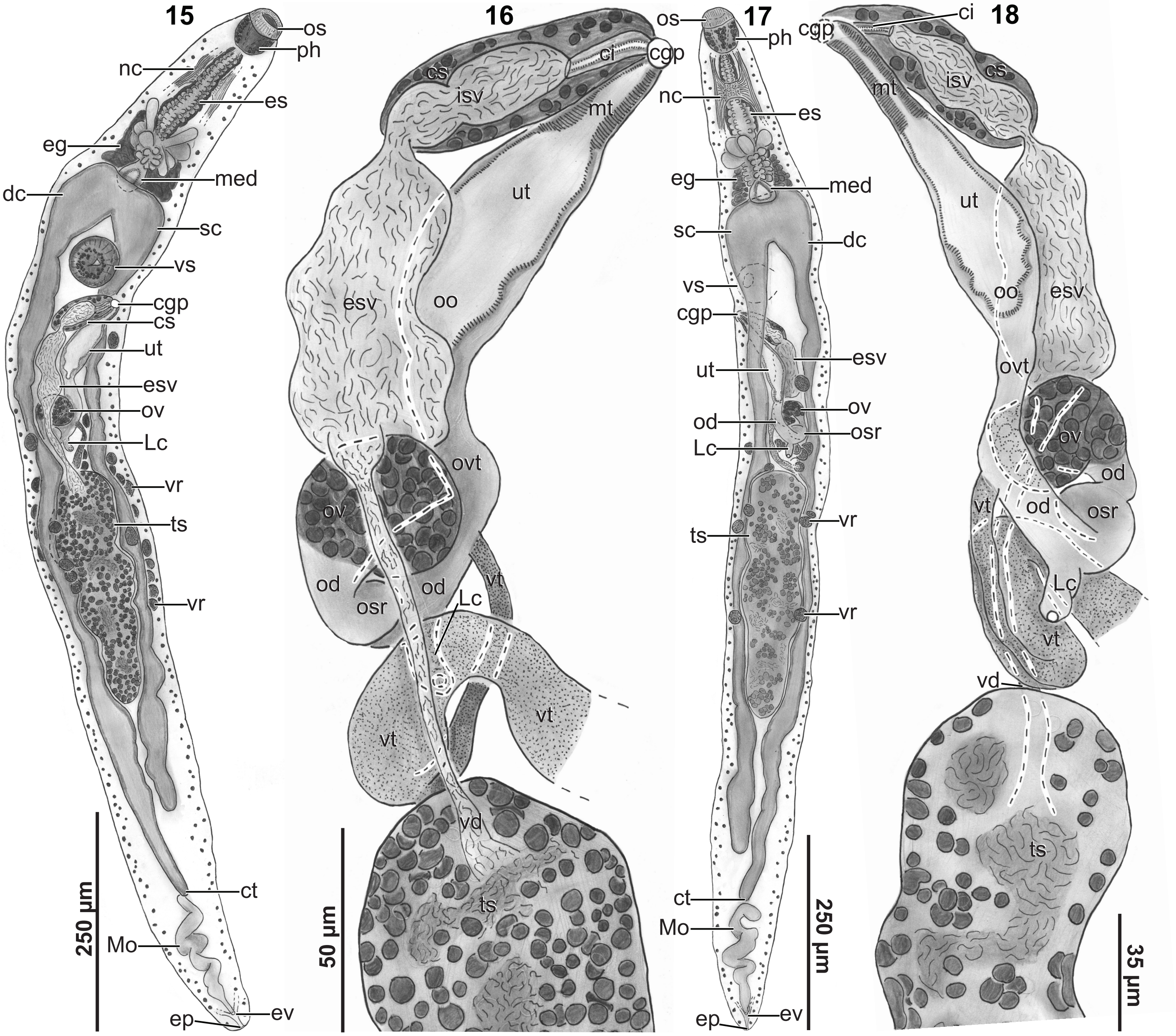
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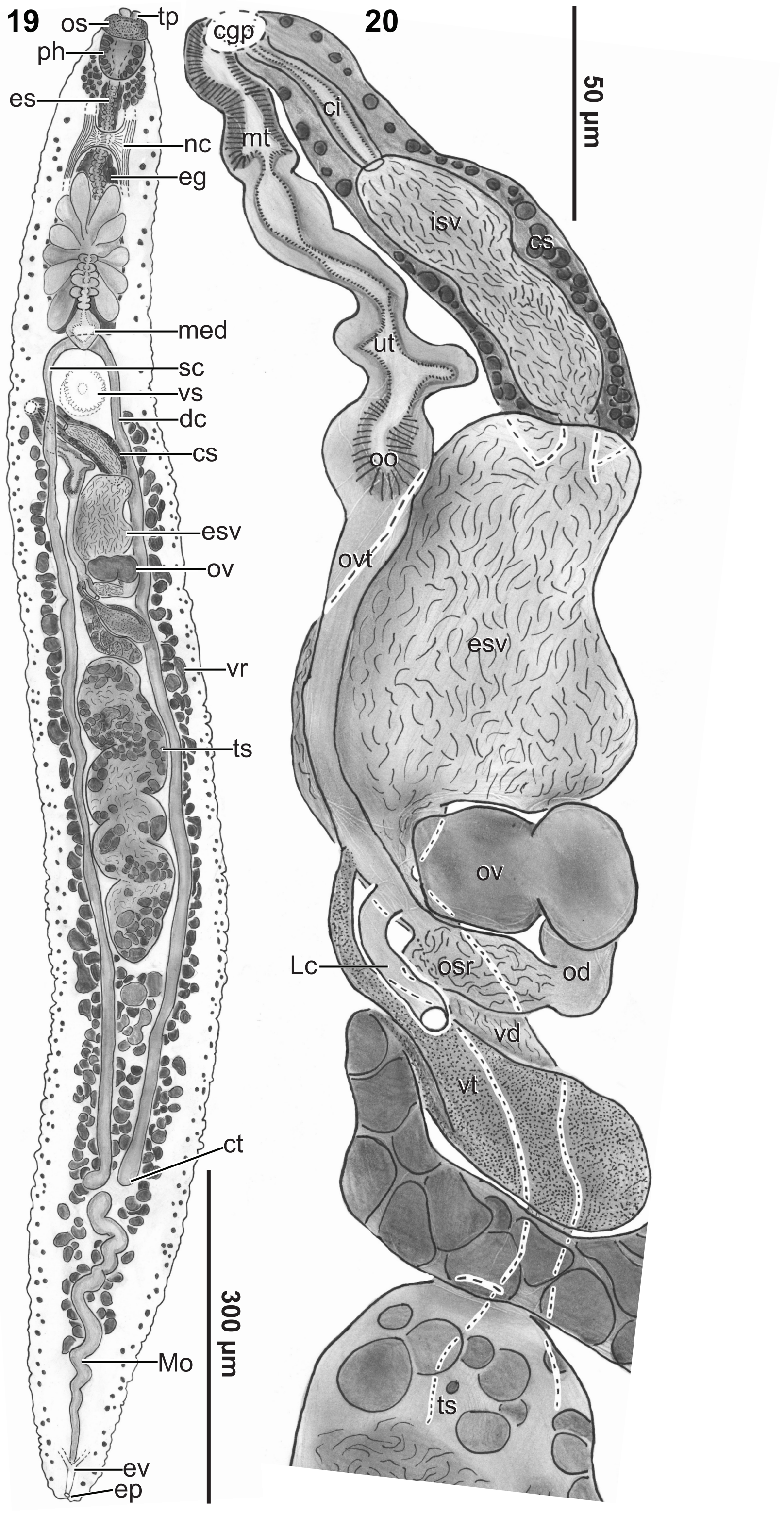
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CHAPTER 5: EMENDATION AND NEW SPECIES OF *HAPALORHYNCHUS* STUNKARD, 1922 (DIGENEA: SCHISTOSOMATOIDEA) FROM MUSK TURTLES (KINOSTERNIDAE: *STERNOTHERUS*) IN ALABAMA AND FLORIDA RIVERS

***Submitted to Parasitology International (8 May 2017)**

Authors: Jackson R. Roberts, Kenneth M. Halanych, Cova R. Arias, Brian Folt, Jeffrey M. Goessling, and Stephen A. Bullard

ABSTRACT

Hapalorhynchus Stunkard, 1922 is emended based on morphological study of existing museum specimens (type and voucher specimens) and newly-collected specimens infecting musk turtles (Testudines: Kinosternidae: *Sternotherus* spp.) from rivers in Alabama and Florida (USA). *Hapalorhynchus conecuhensis* n. sp. is described from an innominate musk turtle, *Sternotherus* cf. *minor*, (type host) from Blue Spring (31°5'27.64"N, 86°30'53.21"W; Pensacola Bay Basin, Alabama) and the loggerhead musk turtle, *Sternotherus minor* (Agassiz, 1857) from the Wacissa River (30°20'24.73"N, 83°59'27.56"W; Apalachee Bay Basin, Florida). It differs from congeners by lacking a body constriction at level of the ventral sucker, paired anterior caeca, and a transverse ovary as well as by having a small ventral sucker, proportionally short posterior caeca, nearly equally-sized anterior and posterior testes, a small cirrus sac, and a uterus extending dorsal to the ovary and the anterior testis. Specimens of *Hapalorhynchus reelfooti* Byrd, 1939 infected loggerhead musk turtles, stripe-necked musk turtles (*Sternotherus peltifer* Smith and Glass, 1947), Eastern musk turtles (*Sternotherus odoratus* [Latreille *in* Sonnini and Latreille, 1801]), and *S.* cf. *minor*. Those of *Hapalorhynchus* cf. *stunkardi* infected *S. minor* and *S. odoratus*. *Sternothorus minor, S. peltifer*, and *S.* cf. *minor* plus *S. minor* and *S. odoratus* are new host records for *H.*

reelfooti and *H.* cf. *stunkardi*, respectively. This is the first report of an infected musk turtle from the Coosa and Tallapoosa Rivers (Mobile-Tensaw River Basin), Pensacola Bay Basin, or Apalachee Bay Basin. Sequence analysis of the large subunit rDNA (28S) showed a strongly-supported clade for *Hapalorhynchus*.

1. Introduction

Hapalorhynchus Stunkard, 1922 includes 19 accepted species that infect both snapping turtles (Testudines: Chelydridae) as well as musk and mud turtles (Kinosternidae) from the Nearctic realm, side-necked turtles (Pelomedusidae) from the Ethiopian realm, and both pond turtles (Geoemydidae) and softshell turtles (Trionychidae) from the Indomalayan realm (Smith [1, 2]; Platt and Snyder [3]; Platt and Sharma [4]; Roberts et al. [5]). Three of the six species of *Hapalorhynchus* reported from North America infect kinosternids: *Hapalorhynchus reelfooti* Byrd, 1939 from the Eastern musk turtle (*Sternotherus odoratus* [Latreille *in* Sonnini and Latreille, 1801]), *Hapalorhynchus stunkardi* Byrd, 1939 from the razor-backed musk turtle (*Sternotherus carinatus* [Gray, 1856]), and *Hapalorhynchus albertoi* Lamothe-Argumedo, 1978 from the white-lipped mud turtle (*Kinosternon leucostomum* Duméril and Bibron *in* Duméril and Duméril, 1851). The present work focuses on musk turtles (*Sternotherus* spp.) in Alabama and Florida because only two of the six North American musk turtles had previously been reported as blood fluke hosts and no infected musk turtle had been reported from Alabama or Florida. Herein, loggerhead musk turtles (*Sternotherus minor* [Agassiz, 1857]), stripe-necked musk turtles (*Sternotherus peltifer* Smith and Glass, 1947), Eastern musk turtles, and specimens of an innominate loggerhead musk turtle

(*Sternotherus* cf. *minor*) were examined for blood fluke infections. *Hapalorhynchus reelfooti* Byrd, 1939, *Hapalorhynchus* cf. *stunkardi*, and a new species of *Hapalorhynchus* were identified. Based on these new collections and borrowed type materials of related blood flukes, we emend *Hapalorhynchus*, describe the new congener (the first new species of the genus described from North America in 29 years; Platt [6]), report the new host and geographic locality records for *H. reelfooti*, and provide a large subunit ribosomal DNA (28S) phylogeny for these taxa.

2. Materials and Methods

2.1. Specimen collection and preparation

Turtles were collected from the Coosa River (Canoe Lake), Tallapoosa River (Odom Creek, Parkerson Mill Creek, Chewacla Creek), and Yellow River (Blue Spring) in Alabama as well as the Aucilla River (Wacissa River) in Florida (Table 1). Two loggerhead musk turtles were captured by hand from two sites (30°20'24.73"N, 83°59'27.56"W; 30°19'39.80"N, 83°59'6.13"W, respectively) in the Wacissa River on 23 April 2016. One Eastern musk turtle was captured with a monitored, baited hoop-net from Canoe Lake (33°47'56.16"N, 86°29'25.02"W), one was captured by hand from a pond off Parkerson Mill Creek (32°35'20.04"N, 85°29'41.51"W), and two were captured by hand from a pond off Odom Creek (32°30'9.58"N, 85°26'6.07"W) on 24 June 2015, 7 September 2015, and 22−24 September 2015, respectively. Two specimens of the innominate loggerhead musk turtle were captured by hand from Blue Spring (31°5'27.64"N, 86°30'53.21"W) on 19 May 2016. One stripe-necked musk turtle was captured from Chewacla Creek (32°31'58.64"N, 85°29'53.09"W) on 15 July 2015. All turtles were decapitated immediately upon returning to the laboratory. Necropsies were performed with aid of 7.0 g/L sodium citrate saline solution to prevent blood coagulation. Live turtle blood flukes (TBFs) intended as whole mounts for morphology were heat-killed on a glass slide with coverslip (no coverslip pressure) using a butane hand lighter. Resulting specimens were transferred and maintained in 5% neutral buffered formalin for morphology or placed in 95% non-denatured ethanol (EtOH) for molecular biology (see below). Specimens for morphology were rinsed in distilled water, stained in Van Cleave's hematoxylin with several drops of Ehrlich's hematoxylin, dehydrated through an ethanol series, made basic at 70% EtOH with lithium carbonate and butly-amine, cleared in clove oil, and permanently mounted in Canada balsam. The resulting whole mounts were examined and illustrated with Leica DM 2500 and Leica DMR (Leica, Wetzler, Germany) microscopes each equipped with differential interference contrast (DIC) optical components, an ocular micrometer, and a drawing tube. Measurements of the TBF specimens are reported in micrometers (μm) followed by the mean and number of specimens measured in parentheses. Measurements for the new species are reported for the holotype and paratype, respectively. Those for *Hapalorhynchus* cf. *stunkardi* are reported separately for each voucher specimen (National Museum of National History, Smithsonian Department of Invertebrate Zoology [USNM] XXXX and XXXX, respectively), and those for the holotype of *H. stunkardi* (USNM 1321967) immediately follow in brackets ("n/a" indicates not available due to poor specimen quality). All of the newly-collected specimens were deposited in the USNM. Turtle scientific and common names follow Spinks et al. [9], van Dijk et al. [10], and Guyer et al. [11]. Classification and anatomical terms for TBFs follow Roberts et al. [5, 12, 13] and Platt [6] except that ‘anterolateral diverticula’ are herein referred to as ‘anterior caeca’.

2.2. DNA extraction, amplification, and sequencing

Specimens for molecular analyses were handled with camel hair brushes, fine-tipped forceps, or syringe tips and immediately preserved in a vial of 95% EtOH and stored at −20°C. Total genomic DNA (gDNA) was extracted using DNeasyTM Blood and Tissue Kit (Qiagen, Valencia, California, USA) according to the manufacturer’s protocol except that the incubation period with proteinase-K was extended to overnight and that the final elution step was performed using only 100 µL of elution buffer to increase the final DNA concentration. The partial 28S rDNA (domains D1–D3; ~1,400 bp) was amplified using the forward primer “U178” (5’-GCA CCC GCT GAA YTT AAG-3’) and the reverse primer “L1642” (5’-CCA GCG CCATCC ATT TTC A-3’) (Lockyer et al. [14]). PCR amplifications were performed using a total volume of 50 µl with 2 µl of DNA template, 0.4 µM of each primer along with 1× buffer, 2.5 mM $MgCl_2$, 1 mM dNTP mixture, and 0.3 µl Taq polymerase (5 U/µl) (Promega, Madison, Wisconsin, USA). The thermocycling profile comprised an initial 5 min at 95°C for denaturation, followed by 40 repeating cycles of 94°C for 30 s for denaturation, 50°C for 30 s for annealing, and 72°C for 2 min for extension, followed by a final five min at 72°C for extension. All PCR reactions were carried out in a MJ Research PTC-200 (BioRad, Hercules, California, USA). PCR products (10 µl) were verified on a 1% agarose gel and stained with ethidium bromide. PCR products were purified by microcentrifuge with the QIAquick PCR Purification Kit (Qiagen, Valencia, California, USA) according to manufacturer's protocol, except that the last elution step was performed with autoclaved nanopure H_2O rather than the provided buffer. DNA sequencing was performed by ACGT, Incorporated (Wheeling, Illinois, USA). Reactions were sequenced using BigDye terminator version 3.1, cleaned-up with magnetic beads (CleanSeq dye terminator removal kit), and analyzed using ABI 3730 XL or 3730 Genetic Analyzer. Primers used in sequencing of 28S rDNA included the PCR primers and the reverse primer 1200R (5'-GCATAGTTCACCATCTTTCGG-3') (Lockyer et al. [14]). Sequence assembly and analysis of chromatograms were performed with BioNumerics version 7.0 (Applied Maths, Saint-Martens-Latem, Belgium).

2.3. Sequence alignments and phylogenetic analyses

Assembled sequences (Table 2) were aligned with MAFFT 7.310 (Katoh and Standley [17]) and subsequently corrected by eye in Mesquite 3.2 (Maddison and Maddison [18]). Regions that could not be unambiguously aligned were excluded from further analyses. The final alignment was deposited in TreeBase (Acc. No. XXXX). Bayesian Inference was performed with MrBayes 3.5.3 (Ronquist et al. [19]). Using a GTR + Gamma model, 4 runs of 4 chains each were conducted for 1,000,000 generations. Priors were set to default values and burnin was set to 25% of generations (or 250,000). Chains were run until the average standard deviation of split frequencies was below 0.01. The resulting phylogenetic tree was viewed using FigTree v1.4.3 (Rambaut [20]) and then edited in Adobe Illustrator CC 2015.3 (Adobe Systems).

3. Results

3.1. *Hapalorhynchus* Stunkard, 1922, emended (Figs. 1−7)

3.1.1. Diagnosis

Body dorsoventrally flat (not cylindrical), 3.7−17.0× longer than wide; body constriction at level of ventral sucker present or absent; hindbody 1.4−3.3× longer than forebody, aspinous; small ventral body papillae present or absent; massive, ventrolateral tegumental papillae *sensu* Roberts et al., (2016b) absent. Oral sucker robust, demarcated from body by constriction; oral sucker spines present or absent; papillae present or absent. Ventral sucker present, aspinous. Pharynx present, enveloping anterior extremity of oesophagus. Oesophagus extending posteriad 1/10−1/4 of body length, ventral to anterior nerve commissure; lateral oesophageal diverticula present or absent; median oesophageal diverticulum absent; oesophageal gland surrounding oesophagus from pharynx to caecal bifurcation. Intestine comprising non-fused posterior caeca bifurcating anterior to ventral sucker; paired anterior caeca present (in *Hapalorhynchus brooksi* Platt, 1988) or absent; posterior caeca inverse U-shaped, extending 1/2−3/4 of body length directly posteriad, not extensively convoluted, terminating in posterior body extremity. Testes comprising one anterior and one posterior testis, in posterior 2/3 of body, intercaecal, smooth or lobed. Vas deferens extending anteriad and ventral to gonads before laterally expanding to form external seminal vesicle; external seminal vesicle posterior to ventral sucker, intercaecal, anterior or dextral to genital pore; internal seminal vesicle present (when cirrus sac present); pars prostatica difficult to discern, enveloping distal extremity of internal seminal vesicle when present. Cirrus sac absent or pre-testicular if present. Ovary ovoid or triangular in outline, intercaecal, intertesticular, transverse or sinistral. Oviduct emerging from dextral margin of ovary, extending directly posteriad or sinistrad; oviducal seminal receptacle comprising middle portion of oviduct between ovary and posterior testis. Laurer's canal intercaecal, intertesticular; Laurer’s canal pore dorsal. Vitellarium follicular, ventrolateral to caeca and gonads, distributing from oesophagus to excretory vesicle or from caecal bifurcation to ends of posterior caeca; transverse vitelline duct intertesticular, ventral to ovary, comprising lateral collecting ducts ventral to caeca. Oötype with longitudinal axis parallel to that of body, intercecal. Mehlis’ gland not observed. Uterus intercaecal, intertesticular, straight (not coiled), difficult to discern from oötype in gravid specimens, extending anteriad, dorsal or ventral to ovary; metraterm narrow, anterior to ovary, sinistral to anterior testis. Uterine pouch absent. Uterine egg single, tricornate or ovoid (with or without filaments), occupying oötype and uterus proximal to metraterm. Common genital pore sinistral, dorsal. Excretory vesicle distinctly Y-shaped or sinuous (lacking multiple laterally-directed lobes). Manter's organ absent. Excretory pore terminal or dorsal.

Type species: *Hapalorhynchus gracilis* Stunkard, 1922

3.1.2. Differential diagnosis

Body dorsoventrally flat (not cylindrical), aspinous; ventrolateral tegumental papillae *sensu* Roberts et al. [5] absent. Oral sucker robust, spinous or aspinous. Pharynx present. Median oesophageal diverticulum absent. Testes comprising one anterior and one posterior testis, intercaecal. Ovary ovoid or triangular in outline, intertesticular, transverse or sinistral. Uterus extending anteriad dorsal or ventral to ovary; metraterm narrow. Common genital pore sinistral, dorsal. Excretory vesicle distinctly Y-shaped or sinuous (lacking multiple laterally-directed lobes). Manter's organ absent.

3.1.3. Remarks

Our emended diagnosis of *Hapalorhynchus* adds or further details numerous features that help differentiate it from that of other TBF genera: details of the body surface and oral sucker, pharynx, anterior caeca, oesophageal gland, vas deferens, internal seminal vesicle, pars prostatica, oviduct, transverse vitelline duct, oötype, metraterm, and excretory pore. Yet, the genus likely needs additional revisionary work: its 19 accepted species are unusual among TBF genera in that their collective geographic distributions include North America, Asia, and Africa (Platt and Sharma [4]). The North American species of *Hapalorhynchus* infect snapping turtles (Chelydridae, infecting one of four species belonging to one of two genera) as well as the mud and musk turtles (Kinosternidae, infecting three of 26 species belonging to both genera; Table 1): *Hapalorhynchus gracilis* Stunkard, 1922 (type species), *H. reelfooti*, *H. stunkardi*, *Hapalorhynchus foliorchis* Brooks and Mayes, 1975, *H. albertoi*, and *H. brooksi*.

The Asian species of *Hapalorhynchus* infect pond turtles (Geoemydidae, infecting five of 69 species belonging to four of 19 genera) and soft-shelled turtles (Trionychidae, infecting four of 31 species belonging to four of 13 genera; all previously *Coeuritrema* unless otherwise noted): *Hapalorhynchus odhnerensis* (Mehra, 1933) Byrd, 1939, *Hapalorhynchus indicus* (Thapar, 1933) Price, 1934 (as *Tremarhynchus* Thapar, 1933), *Hapalorhynchus yoshidai* Ozaki, 1939, *Hapalorhynchus ocadiae* (Takeuti, 1942) Platt, 2002, *Hapalorhynchus oschmarini* (Belous, 1963) Platt, 2002, *Hapalorhynchus macrotesticularis* (Rohde, Lee and Lim, 1968), Brooks and Sullivan, 1981, *Hapalorhynchus mica* (Oshmarin, 1971) Platt, 2002, *Hapalorhynchus sheilae* (Mehrotra, 1973) Platt, 2002, *Hapalorhynchus sutlejensis* (Mehrotra, 1973) Platt, 2002, *Hapalorhynchus snyderi* Platt and Sharma, 2012, and *Hapalorhynchus tkachi* Platt and Sharma, 2012. All of these species except *H. snyderi* and *H. tkachi* lack complete taxonomic descriptions and their type specimens either do not exist or reside in museums or research collections that cannot or choose not to loan materials outside of the Indian subcontinent (Brooks and Sullivan [21]; Platt [22]; T. Platt, pers. comm.). The uniqueness of these taxa needs to be reassessed. *Hapalorhynchus snyderi* and *H. tkachi* (for which we examined type material) differ from North American species of *Hapalorhynchus* by having a papillate ventral sucker (similar to *Vasotrema* Stunkard, 1926; see Roberts and Bullard [23]), an external seminal vesicle abutting the anterior margin of the cirrus sac, and a cirrus sac posterior to the genital pore and directed anteriad).

The African species of *Hapalorhynchus* infect African side-necked turtles (Pelomedusidae, infecting two of 18 species belonging to one of two genera) and comprise *Hapalorhynchus beadlei* Goodman, 1987 and *Hapalorhynchus tchalimi* Bourgat and Kulo, 1987. These infections were reported only once for both *H. beadlei* and *H. tchalimi* (Goodman [24]; Bourgat and Kulo [25]). Goodman [24] described *H. beadlei* based on specimens collected from the Albert Nile mud turtle, *Pelusios williamsi lutescens* Laurent, 1965, but lost all of the original type materials before the work was accepted for publication. Goodman returned to the type locality and sampled the type host; however, according to Platt [26, 27], these newer specimens (represented by voucher USNM 79375) were not conspecific with those Goodman originally illustrated, measured, and named but rather were conspecific with those of *H. tchalimi* (Royal Museum for Central Africa, Tervuren; see Bourgat and Kulo [1987]). This matter was resolved in a petition (case no. 2653) by Platt to the International Commission on Zoological Nomenclature (Platt [26]). Several reported features distinguish *H. beadlei* and *H. tchalimi* from congeners: lateral oesophageal diverticula present, cirrus sac absent in *H. beadlei* (see Goodman [24]), and Laurer's canal originating near the proximal margin of the seminal receptacle (fig. 2, p. 438; Bourgat and Kulo [25]). Further, given the uniqueness of these taxa regarding their geographic origin and their hosts' ancestry, additional specimens of these taxa should be collected and studied so that their membership in *Hapalorhynchus* can be confirmed.

Hapalorhynchus resembles *Coeuritrema* Mehra, 1933, *Enterohaematotrema* Mehra, 1940, and *Cardiotrema* Dwivedi, 1967 by having a ventral sucker, inverse U-shaped posterior caeca, two testes, and pre-gonadal male terminal genitalia. *Hapalorhynchus* differs from *Coeuritrema* by lacking ventrolateral tegumental papillae and by having a diminutive metraterm that is difficult to discern from the uterus. *Coeuritrema* has massive, mound-like, ventrolateral tegumental papillae (*sensu* Roberts et al. [5]) and a robust metraterm (1/10-1/7 of body length) that is easily differentiated from the uterus, even in gravid specimens. *Hapalorhynchus* can be differentiated from *Enterohaematotrema* by having a moderately-sized cirrus sac (1/10-1/7 of body length when present), a diminutive metraterm, and a dorsosinistral genital pore. *Enterohaematotrema* has a massive cirrus sac (1/3 of body length), a massive metraterm (1/3 of body length), and a ventromedial genital pore posterior to the ventral sucker. Lastly, *Hapalorhynchus* differs from *Cardiotrema* by having a longer oesophagus (1/10-1/4 of body length), a larger ventral sucker (1/4−1/2 of body width), and an intertesticular ovary. *Cardiotrema* has a short oesophagus (<1/10 of body length), a small ventral sucker (<1/5 of body width), and an ovary that is sinistral to the anterior sucker.

No species of *Hapalorhynchus* had previously been described as having papillae on the body surface (Figs. 3, 4). Vouchers (USNM 1393855, 1393857) and newly collected specimens of *H. reelfooti* (USNM XXXXX, XXXXX), newly collected specimens of *H.* cf. *stunkardi* (USNM XXXXX, XXXXX), and specimens of the new species (USNM XXXXX, XXXXX) have papillae on the body surface. Papillae were indistinct in the holotype of *H. gracilis*, *H. stunkardi*, and *H. brooksi* (AMNH 125; USNM 1321967, 1375720, respectively) as well as vouchers of *H. foliorchis* (USNM XXXX). We suspect these papillae are present in other species of *Hapalorhynchus*; however, they are quite difficult to see without exceptionally well-stained material. Regarding the pharynx, like *Spirorchis* MacCallum, 1918, *Unicaecum* Stunkard, 1925, *Vasotrema*, *Coeuritrema*, and *Baracktrema* Roberts, Platt, and Bullard, 2016, species of *Hapalorhynchus* have a pharynx located in the extreme anterior end of the oesophagus such that it appears in fixed, whole-mounted specimens as immediately dorsal to the mouth. This position makes it easily missed, shrouded by the muscular oral sucker, or mistaken for a component of the oral sucker itself (Figs. 1, 5, 6; Stunkard [28]; Platt [22]; Roberts et al. [5, 12, 13]; Roberts and Bullard [23]). Regarding the oral sucker spines, no species of *Hapalorhynchus* has been described as having spines associated with the oral sucker

(Stunkard [28]; Platt [22]) but the holotype (USNM XXXX) of the new species has spines on the dorsal rim of the mouth (Fig. 3). Similar spines have been described on the surface of the ventral concavity of the oral sucker of *Vasotrema robustum* Stunkard, 1928 (see Roberts and Bullard [23]). No spine was observed in a specimen of any other species examined herein. Oral sucker papillae were herein observed in the new species and *Hapalorhynchus* cf. *stunkardi* (Figs. 3, 4). Such papillae have not been reported previously for any species of *Hapalorhynchus*; however, they have been observed in species of *Vasotrema* (see Roberts and Bullard [23]) and some fish blood flukes (Aporocotylidae) (Bullard and Overstreet [29]; Truong and Bullard [30]; Yong et al. [31]). Because these papillae are present in species assigned to evidently unrelated genera (Orélis-Ribeiro et al. [31]; Roberts et al. [12]), they likely evolved independently; perhaps as a result of strong ‘selection pressure’ for the fluke being able to detect its position in the host or to locate conspecifics.

Regarding the cirrus sac, we accept it as being present or absent among members of the genus (Platt [6, 22]): *H. gracilis*, *H. foliorchis*, and *H. beadlei* lack a cirrus sac. The holotype of *H. gracilis* (American Museum of Natural History [AMNH] Coll. No. 125) and vouchers of *H. foliorchis* (USNM XXXXX) lack a cirrus sac. As stated above, type materials of *H. beadlei* were lost, with Goodman's [24] description designated as the lectotype (Platt, 1988b; 1991). Examination of newly-collected specimens from the type host (*Pelusios* sp.) and type locality (Kampala, Uganda) are needed to assess this feature in *H. beadlei* (Platt [26, 27]). *Hapalorhynchus stunkardi* was originally described as having a “thin-walled and non-muscular (*sic*)” cirrus sac. Platt [6] reported that the prostatic cells of *H. stunkardi* were irregularly scattered about the cirrus; however, our observations of that region in the holotype of *H. stunkardi* (USNM 1321967) agree with Byrd's [8] description, i.e., the cirrus sac is present, thin-walled, and non-muscular.

3.2. Hapalorhynchus conecuhensis Roberts and Bullard, n. sp. (Figs. 1−4, 7)

3.2.1. Diagnosis of adult (based on light microscopy of two intact specimens [holotype, USNM XXXX; paratype, USNM XXXX] and two partial specimens [hologenophore, USNM XXXX; paratype, USNM XXXX]): Body 1390 and 1340 long or 4.3× (2) longer than wide, 250 and 230 wide or 18% and 17% of body length at level of caecal bifurcation, 223−275 (253; 3) wide or 19% and 21% of body length at level of ventral sucker, 245−320 (288; 4) wide or 23% and 24% of body length at level of external seminal vesicle, 260−335 (295; 4) wide or 22% and 25% of body length at level of ovary, 200−250 (224; 4) wide or 17% and 15% of body length at level of caecal terminus; forebody (middle of ventral sucker to anterior body end) 470 (2) long or 34% and 35% of body length; hindbody (middle of ventral sucker to posterior body end) 920 and 870 long or 66% and 65% of body length or 2.0× and 1.9× longer than forebody; small ventral body papillae present (Figs. 3, 4). Oral sucker 58 and 73 long or 4% and 5% of body length, 65 and 60 wide or 26% (2) of body width at level of caecal bifurcation; oral sucker spines present; papillae present (Figs. 3, 4). Ventral sucker 75 and 95 long or 5% and 7% of body length or 1.3× (2) longer than oral sucker, 88 and 100 wide or 34% and 26% of body width at level of ventral sucker or 1.4× and 1.7× wider than oral sucker. Nerve commissure 190 and 165 long or 14% and 12% of body length from anterior body end. Pharynx 40 and 50 long or 13% and 16% of oesophagus length, 63 and 58 wide or 1.9× and 1.8× wider than maximum oesophagus width. Oesophagus 310 and 308 long or 22% and 23% of body length, 20 and 13 wide posterior to pharynx with wall 10 and 8 thick, 33 (2) wide or 13% and 14% of body width at mid-oesophagus, with wall 15 and 20 thick, 65 (2) wide or 26% and 28% of body width at caecal bifurcation, with wall 55 and 47 thick; anterior oesophageal gland 130 and 114 long or 42% and 37% of oesophagus length, 75 and 57 wide or 30% and 25% of body width at level of caecal bifurcation; posterior oesophageal gland 125 and 107 long or 40% and 35% of oesophagus length, 77 and 80 wide or 31% and 35% of body width at level of caecal bifurcation. Intestine bifurcating 335 and 325 or 24% (2) of body length from anterior body end; sinistral posterior caecum 830−920 (870; 3) long or 66% and 64% of body length, 50−63 (56; 3) wide or 22% and 27% of body width at level of caecal bifurcation, 25−43 (33; 4) wide or 10−13% (11%; 4) of body width at level of ovary, 38−63 (51; 4) wide or 17−30% (23%; 4) of body width at terminus; dextral posterior caecum 775−890 (852; 3) long or 64% and 66% of body length, 38−63 (55; 3) wide or 25% and 27% of body width at level of caecal bifurcation, 25−45 (39; 4) wide or 10−15% (13%; 4) of body width at level of ovary, 38−58 (48; 4) wide or 18−25% (21%; 4) of body width at terminus; caecal terminus 140 (2) or 10% (2) of body length from posterior body end.

Anterior testis lobed, follicular, 100−178 (150; 4) long or 12% and 13% of body length or 88−95% (92%; 4) of posterior testis length, 83−150 (127; 4) wide or 32−52% (43%; 4) of body width at level of ovary or 98−106% (101%; 4) of posterior testis width; intertesticular space 28−73 (45; 4) or 3% (2) of body length. Posterior testis lobed, follicular, 113−188 (161; 4) long or 12% and 14% of body length, 78−153 (128; 4) wide or 30−52% (43%; 4) of body width at level of gonads, 243−313 (282; 3) or 23% and 22% of body length from posterior body end. Anterior trunk of vasa efferentia emanating from ventral surface of anterior testis, extending 13−50 (26; 3) or 1% (2) of body length anteriad, 5−8 (6; 3) wide; posterior trunk of vasa efferentia emanating from ventral surface of posterior testis, ventral to gonads, extending 150−255 (206; 3) or 11% and 16% of body length anteriad, 5 (4) wide, meeting anterior trunk of vasa efferentia ventral and posterior to anterior testis to form vas deferens. Vas deferens ventral, extending 133−195 (156; 3) or 14% and 10% (2) of body length anteriad, 5−10 (8; 4) wide, expanding to form external seminal vesicle. External seminal vesicle 63−113 (94; 4) long or 8% and 7% of body length, 103−258 (189; 4) wide or 42−81% (64%; 4) of body width or 1.6−2.3× (2.0; 4) wider than long, dorsal to caeca (Figs. 1, 2); internal seminal vesicle 102−150 (118; 4) long or 70−80% (74%; 4) of cirrus sac length, 25−43 (35; 4) wide or 2.4−4.1× (3.4; 4) longer than wide. Pars prostatica surrounding distal portion of internal seminal vesicle, 60−100 (72; 4) long or 52−67% (61%; 4) of internal seminal vesicle length, 45−63 (50; 4) wide or 1.3−1.6× (1.4; 4) longer than wide (Fig. 2). Cirrus sac obvious, enveloping entirety of internal seminal vesicle and cirrus, 128−205 (159; 4) long or 11% and 15% of body length, 75−113 (101; 4) wide or 31−39% (35%; 4) body width at level of external seminal vesicle; cirrus 18−28 (24; 4) long, 18−50 (34; 4) wide (Fig. 2).

Ovary triangular in outline, sinistral, 305−335 (316; 3) or 24% and 23% of body length posterior to middle of ventral sucker, 25−55 (45; 4) or 4% and 3% of body length posterior to genital pore, 100−138 (118; 4) long or 10% and 8% of body length, 80−110 (92; 4) wide or 26−34% (31%; 4) of body width; post-ovarian space 460 and 475 or 33% and 35% of body length. Oviduct turning dorsad and extending posteriad 30−50 (44; 4) or 4% and 3% (2) of body length, 8−15 (12; 4) in maximum width, laterally expanding to form seminal receptacle; oviducal seminal receptacle extending sinistrad for 50−73 (63; 4) or 57−85% (69%; 4) of ovary width, 20−35 (30; 4) wide or 7−12% (10%; 4) of body width, constricting and turning dorsad, extending anteriad for 68−100 (87; 3) or 7% (2) of body length before joining oötype, 13−18 (15; 3) wide or 4−7% (7%; 3) of body width (Figs. 1, 2). Laurer's canal originating immediately distal to seminal receptacle terminus, extending posteriad 25−35 (32; 3) or 1% (2) of body length, 8 (3) wide, opening dorsally over posterior testis (Fig. 2). Vitellarium comprising a series of interconnected spheroid masses of follicles (Fig. 1), ventro-lateral to caeca (illustrated as lateral to emphasize course of caeca), distributing from level of oesophagus median or 285 and 205 or 21% and 15% of body length from anterior body end to excretory vesicle or 55 and 88 or 4% and 7% of body length from posterior body end; lateral collecting ducts 8−20 (15; 16 [4 ducts per specimen]) wide, ventral to caeca, coalescing at level of posterior margin of ovary to form transverse vitelline duct; transverse vitelline duct ventral to ovary, 370−390 (378; 3) or 28% (2) of body length from middle of ventral sucker; primary vitelline collecting duct extending anterodextrad and dorsal 26−53 (35; 4) or 3% and 2% of body length before connecting with oviduct (Fig. 2), 9−14 (11; 4) wide or 3−5% (4%; 4) of body width. Oötype difficult to discern in gravid individuals, forming egg chamber with proximal portion of uterus, 33 and 28 long, 18 and 20 wide, dorsal to ovary and anterior testis. Uterus originating 300 or 22% (1) of body length posterior from middle of ventral sucker. In gravid individuals (2 of 4), egg chamber originating 305 and 335 or 22% (1) of body length posterior from middle of ventral sucker, 98 and 100 long or 7% (1) of body length, 55 and 50 wide or 18% (2) of body width; metraterm extending anterosinistrad, 35−52 (41; 4) long or 3% and 4% of body length, 11−30 (18; 4) wide or

3−10% (6%; 4) of body width, 1.1× and 0.9× longer than uterus. Uterine egg ovoid, 73 and 90 long or 5% (1) of body length, 45 and 43 wide or 15% and 16% of body width. Common genital pore 225−250 (238; 3) or 18% and 17% of body length posterior to middle of ventral sucker, 30−40 (35; 4) in diameter.

Excretory vesicle 25−65 (48; 3) wide or 23% and 13% of body width at level of caecal terminus; wall 10−17 (14; 3) thick (Fig. 1); excretory pore dorsal, subterminal, 13 and 18 or 1% (2) of body length from posterior body margin.

3.2.2. Taxonomic Summary

Type host: Sternotherus cf. *minor* (Testudines: Kinosternidae), innominate musk turtle; *other host*: *Sternotherus minor*, loggerhead musk turtle.

Type locality: Blue Spring (31°5'27.64"N, 86°30'53.21"W; Yellow River, Alabama, USA; *S.* cf. *minor*); *other locality*: Wacissa River (30°20'24.73"N, 83°59'27.56"W; tributary of Aucilla River, Florida, USA; *S. minor*).

Site in host: Heart, viscera wash.

Prevalence and intensity of infection by adults: Two innominate musk turtles from Blue Spring had two (whole-mounted specimens) and four (BF120 [hologenophore], BF131, three whole-mounted specimens) specimens of *H. conecuhensis*. One of two specimens of loggerhead musk turtle from the Wacissa River had one specimen of *H. conecuhensis* (BF128).

Specimens deposited: Holotype (USNM XXXX); Paratypes (USNM XXXX; XXXX); Hologenophore (USNM XXXX); GenBank (Nos. XXXX; XXXX; XXXX).

Specimens examined: Holotype of *H. gracilis* (AMNH 125); Vouchers of *H. foliorchis* (USNM XXXX, XXXX, XXX); Holotype of *H. stunkardi* (USNM 1321967); Holotype of *H. reelfooti* (USNM 1321968); Vouchers of *H. reelfooti* (USNM 1393855, 1393857); Holotype of *H. brooksi* (USNM 1375720).

Etymology: The specific epithet *conecuhensis* is for the type locality, Conecuh National Forest, Andalusia, Alabama.

3.2.3. Remarks

The new species resembles *H. brooksi* and *H. stunkardi* by having (in addition to all diagnostic characters of the genus) proportionally long posterior caeca (post-caecal space <14% of body length), a transverse external seminal vesicle (abutting posterior caeca), and lobed testes that are >40% of body width. It most closely resembles *H. stunkardi* by lacking a marked body constriction at level of the ventral sucker, paired anterior caeca, and a transverse ovary as well as by having a vitellarium distributing from the oesophagus to the excretory vesicle and an ovoid uterine egg that lacks polar filaments. *Hapalorhynchus brooksi* has a marked body constriction at level of the ventral sucker, paired anterior caeca, a transverse ovary, a vitellarium distributing from the caecal bifurcation to the caecal terminus, and an egg with polar filaments. The new species differs from *H. stunkardi* by the combination of having a relatively small ventral sucker (34−36% of body width), short posterior caeca (64−66% of body length), nearly equal-sized anterior and posterior testes (anterior testis is 88−95% and 98−106% of posterior testis length and width, respectively), a small cirrus sac (31−39% of body width), and a uterus that extends dorsal to the ovary and the anterior testis (Fig. 2).

Hapalorhynchus stunkardi has a large ventral sucker (43% of body width), long posterior caeca (72% and 74% of body length), a markedly smaller anterior testis relative to the posterior testis (<80% of posterior testis length and width), a large cirrus sac (47% of body width), and a uterus that extends ventrolateral to the anterior testis and anterior margin of ovary.

The type host for *H. conecuhensis*, *S.* cf. *minor* (see Guyer et al. [11]; pp. 21, 22), is an innominate species of musk turtle, and its taxonomic description is forthcoming (P. Scott [University of California, Los Angeles [UCLA]; Los Angeles, California, USA], personal communication to JRR).

3.3. Hapalorhynchus reelfooti Byrd, 1939 (Figs. 5, 7)

3.3.1. Diagnosis of adult (based on light microscopy of 13 intact specimens [vouchers USNM XXXX]): With features of *H. reelfooti* (see Byrd, [8] and Platt and Snyder, [3]) except for the following. Body 40−82 (62; 13) wide or 5−11% (8%; 13) of body width at level of caecal bifurcation, 33−75 (57; 13) wide or 5−10% (7%; 13) of body width at level of ventral sucker, 43−111 (78; 13) wide or 7−14% (9%; 13) of body length at level of ovary, 35−98 (66; 13) wide or 6−11% (8%; 13) at level of caecal terminus; small ventral body papillae present. Oral sucker 21−36 (26; 13) long or 2−5% (3%; 13) of body length, 23−34 (29; 13) wide or 33−63% (48%; 13) of body width at level of caecal bifurcation. Pharynx 13−36 (24; 13) long or 7−18% (13%; 13) of body length, 20−33 (26; 13) wide or 0.7−1.4× (1.2; 13) wider than maximum oesophagus width. Oesophagus 5−14 (8; 13) wide posterior to pharynx with wall 3−8 (5; 13) thick, 11−25 (18; 13) wide or 16−49% (30%; 13) of body width at mid-esophagus, with wall 7−16 (11; 13) thick, 16−32 (21; 13) wide or 23−45% (35%; 13) of body width at caecal bifurcation, with wall 8−24 (15; 13) thick; oesophageal gland 96−175 (133; 10) long or 12−18% (15%; 10) of body length, 26−44 (36; 10) wide or 39−71% (54%; 10) of body width at level of caecal bifurcation. Intestine bifurcating 153−245 (195; 13) or 19−27% (24%; 13) of body length from anterior body end; sinistral posterior caecum 297−786 (497; 13) long or 44−89% (60%; 13) of body length, 8−23 (16; 13) wide or 18−35% (26%; 13) of body width at level of caecal bifurcation, 5−14 (8; 13) wide or 5−21% (11%; 13) of body width at level of ovary, 8−14 (11; 13) wide or 11−28% (17%; 13) of body width at terminus; dextral posterior caecum 292−761 (495; 13) long or 44−89% (60%; 13) of body length, 8−23 (16; 13) wide or 14−35% (26%; 13) of body width at level of caecal bifurcation, 3−11 (8; 13) wide or 5−21% (11%; 13) of body width at level of ovary, 3−16 (11; 13) wide or 9−28% (17%; 13) of body width at terminus.

Intertesticular space 43−84 (60; 13) or 5−9% (7%; 12) of body length; posterior testis 190−311 (240; 12) or 21−32% (29%; 12) of body length from posterior body end. Anterior trunk of vasa efferentia emanating from ventral surface of anterior testis, extending 5 and 9 (2) or 1% (2) of body length anteriad, 2 and 5 (2) wide; posterior trunk of vasa efferentia emanating from ventral surface of posterior testis, ventral to gonads, extending 118 and 141 (2) or 15% and 16% (2) of body length anteriad, 2 and 5 (2) wide, meeting anterior trunk of vasa efferentia ventral and posterior to anterior testis to form vas deferens. Vas deferens ventral, extending 80 and 95 (2) or 10% and 11% (2) of body length anteriad, 2 and 5 (2) wide, expanding to form external seminal vesicle. Internal seminal vesicle 42−54 (50; 10) long or 61−84% (71%; 10) of cirrus sac length, 16−28 (21; 10) wide or 1.8−3.3× (2.4; 10) longer than wide. Pars prostatica surrounding distal portion of internal seminal vesicle, 18−26 (21; 7) long or 33−49% (42%; 7) of internal seminal vesicle length, 16−25 (19; 7) wide or 0.8−1.4× (1.1; 7) longer than wide. Cirrus sac transverse, abutting both caeca, enveloping entirety of internal seminal vesicle and cirrus, 40−81 (66; 12) long or 6−10% (8%; 12) of body length, 20−53 (40; 12) wide or 45−63% (54%; 12) body width at level of external seminal vesicle; cirrus 11−16 (13; 10) long, 4−11 (7; 10) wide.

Ovary ovoid in outline, transverse, 137−223 (171; 10) or 16−24% (20%; 10) of body length posterior to middle of ventral sucker, 28−55 (42; 12) or 4−7% (5%; 12) of body length posterior to genital pore; post-ovarian space 238−400 (326; 12) or 30−43% (39%; 12) of body length. Oviduct extending posteriad 15−44 (33; 11) or 2−6% (4%; 11) of body length, 5−14 (8; 11) in maximum width, laterally expanding to form seminal receptacle; oviducal seminal receptacle extending sinistrad for 25−41 (33; 11) or 57−85% (69%; 4) of 32−46% (39%; 11) of body width, 11−23 (19; 11) wide or 17−36% (23%; 11) of body width, constricting and turning dorsad, extending anteriad for 26−65 (43; 10) or 3−7% (5%; 10) of body length before joining oötype, 7−12 (10; 10) wide or 7−18% (11%; 9) of body width (Fig. 5). Laurer's canal originating immediately distal to seminal receptacle terminus, extending posteriad 9−30 (19; 10) or 1−3% (2; 10) of body length, 5− 11 (8; 10) wide, opening dorsally over posterior testis. Vitellarium comprising a series of interconnected spheroid masses of follicles (Fig. 5), ventro-lateral to caeca (illustrated as lateral to emphasize course of caeca), distributing from level of oesophagus median or 109−189 (141; 11) or 14−19% (16%; 11) of body length from anterior body end to excretory vesicle or 48−107 (68; 11) or 5−12% (8%; 11) of body length from posterior body end; lateral collecting ducts 5−23 (11; 40 [4 ducts per specimen]) wide, ventral to caeca, coalescing at level of posterior margin of ovary to form transverse vitelline duct; transverse vitelline duct ventral to ovary, 158−234 (195; 11) or 19−25% (23%; 11) of body length from middle of ventral sucker; primary vitelline collecting duct extending anteriad and dorsal 12 and 16 (2) or 1% and 2% (2) of body length before connecting with oviduct, 4 and 5 (2) wide or 5% and 6% (2) of body width. Oötype difficult to discern, 9−18 (12; 10) long, 9−16 (12; 10) wide, dorsal to ovary. Uterus originating 149−195 (176; 9) posteriad from middle of ventral sucker, 19−56 (32; 10) long or 2−7% (4%; 10) of body length, 11−28 (18; 10) wide or 10−35% (21%; 10) of body width; metraterm extending anterosinistrad, 14−35 (25; 10) long or 2−4% (3%; 10) of body length, 5−11 (8; 10) wide or 6−13% (9%; 10) of body width, 0.3−1.6× (0.8; 10) of uterus length. Common genital pore 66−136 (111; 13) or 11−16% (13%; 13) of body length posterior to middle of ventral sucker, 4−10 (6; 13) in diameter.

Excretory vesicle 14−40 (25; 13) wide or 19−71% (39%; 13) of body width at level of caecal terminus; wall 4−12 (8; 13) thick (Fig. 5); excretory pore dorsal, subterminal, 8−16 (11; 13) or 1−2% (1%; 13) of body length from posterior body margin.

3.3.2. Taxonomic summary

Host(s): Sternotherus minor (Agassiz, 1857) (Testudines: Kinosternidae), loggerhead musk turtle; *Sternotherus odoratus* (Latreille *in* Sonnini and Latreille, 1801) (Kinosternidae), Eastern musk turtle; *Sternotherus peltifer* Smith and Glass, 1947 (Kinosternidae), stripe-necked musk turtle; *Sternotherus* cf. *minor* (Kinosternidae), innominate musk turtle.

Locality(ies): Wacissa River (30°19'39.80"N, 83°59'6.13"W; tributary of Aucilla River, Florida, USA); Wacissa River (30°20'24.73"N, 83°59'27.56"W); Canoe Lake (33°47'56.16"N, 86°29'25.02"W; Coosa River, Alabama, USA); Pond off Odom Creek (32°30'9.58"N, 85°26'6.07"W; Tallapoosa River, Alabama); Chewacla Creek (32°31'58.64"N, 85°29'53.09"W; Tallapoosa River, Alabama); Blue Spring (31°5'27.64"N, 86°30'53.21"W; Yellow River, Alabama).

Site in host: Mesenteric blood vessels, heart, lung, viscera wash

Prevalence and intensity of infection by adults: Two of two (100%) *S. minor* were infected with four and 42 individuals; two of three (67%) *S. odoratus* individuals were infected both with two specimens; one *S. peltifer* individual was infected with five specimens; two of two (100%) *S.* cf. *minor* individuals were infected with 10 and 26 specimens.

Specimens deposited: Vouchers (USNM XXXX, XXXX, XXXX, XXXX); GenBank (Nos. XXXX, XXXX, XXXX, XXXX, XXXX, XXXX).

Specimens examined: Holotype of *H. reelfooti* (USNM 1321968); Vouchers of *H. reelfooti* (USNM 1393855; 1393857).

3.3.3. Remarks

This report comprises the first record of *H. reelfooti* from a river in Alabama or Florida, and its previous geographic distribution was limited to Reelfoot Lake (Mississippi River, Tennessee) as well as rivers in Virginia and Indiana (Table 1). It is the third species of *Hapalorhynchus* reported from Alabama, along with *H. conecuhensis* and *H. foliorchis* (see Roberts et al. [5]). It is the first species of

Hapalorhynchus reported from Florida. Additionally, all but *S. odoratus* comprise new host records for *H. reelfooti*. This is the first record of both *S. minor* and *S. peltifer* as TBF hosts.

Because the holotype of *H. reelfooti* (USNM 1321968) is in poor condition, apparently destained and strongly contracted (Platt and Snyder [3]), we compared our newly collected specimens to Platt and Snyder's [3] vouchers (USNM 1393855, 1393857) rather than relying primarily on the holotype. All of these specimens have minute, spine-like projections in the forebody and hindbody. These projections are distributed over the nearly the entire body surface and appear to be the distal tips of minute sensory papillae. *Hapalorhynchus conecuhensis*, *H. reelfooti*, and *H.* cf. *stunkardi* (below) are the only species of *Hapalorhynchus* confirmed to have them.

3.4. Hapalorhynchus cf. stunkardi (Figs. 6, 7)

3.4.1. Diagnosis of adult (based on light microscopy of 2 specimens [vouchers USNM XXXX]): With features of *H. stunkardi* (see Byrd [8]; Platt [6]) except for the following. Forebody (middle of ventral sucker to anterior body end) 415 and 368 [460] or 42% and 49% [32%] of body length; hindbody (middle of ventral sucker to posterior body end) 575 and 382 [980] or 58% and 51% [68%] of body length or 1.4× and 1.0× [2.1×] longer than forebody. Oral sucker 60 and 48 [70] long or 6% (2) [5%] of body length, 60 and 58 [93] wide or 38% and 37% [29%] of body width at level of caecal bifurcation. Ventral sucker 113 and 95 [136] long or 11% and 13% [9%] of body length or 1.9× and 2.0× [1.9×] longer than oral sucker, 88 and 93 [141] wide or 49% and 52% [43%] of body width at level of ventral sucker or 1.5× and 1.6× [1.5×] wider than oral sucker. Nerve commissure 183 and 160 [168] long or 18% and 21% [12%] of body length from anterior body end. Pharynx 38 (2) [39] long or 14% and 15% [15%] of oesophagus length, 58 and 60 [91] wide or 2.1× and 3.3× [1.6×] wider than maximum oesophagus width. Oesophagus 13 (2) [20] wide posterior to pharynx with wall 8 and 10 [15] thick, 28 and 18 [57] wide or 18% and 11% [18%] of body width at mid-esophagus with wall 18 and 15 [34] thick, 38 and 43 [64] or 24% and 27% [20%] of body width at caecal bifurcation, with wall 23 and 35 [50] thick; anterior oesophageal gland 115 and 75 [73] long or 80% and 29% [27%] of oesophagus length, 58 and 45 [141] wide or 36% and 28% [43%] of body width at level of caecal bifurcation; posterior oesophageal gland 125 and 100 [123] long or 47% and 39% [46%] of oesophagus length, 75 and 50 [143] wide or 47% and 32% [44%] of body width at level of caecal bifurcation. Intestine bifurcating 320 and 238 [297] or 32% (2) [21%] of body length from anterior body end; sinistral posterior caecum 535 and 450 [1042] long or 54% and 60% [72%] of body length, 48 and 28 [68] wide or 30% and 18% [21%] of body width at level of caecal bifurcation, 20 and 15 [41] wide or 10% (2) [14%] of body width at level of ovary, 28 and 18 [48] wide or 19% and 13% [18%] of body width at terminus; dextral posterior caecum 570 and 435 [1069] long or 58% (2) [74%] of body length, 43 and 30 [80] wide or 27% and 19% [25%] of body width at level of caecal bifurcation, 30 and 20 [18] wide or 15% and 13% [6%] of body width at level of ovary, 40 and 20 [57] wide or 27% and 14% [21%] of body width at terminus.

Anterior testis 45 and 38 [127] wide or 23% and 25% [43%] of body width at level of ovary or 100% and 76% [76%] of posterior testis width; intertesticular space 58 and 40 [86] or 6% and 5% [6%] of body length. Posterior testis 45 and 50 [168] wide or 23% and 33% [56%] of body width at level of ovary. Anterior trunk of vasa efferentia emanating from ventral surface of anterior testis, extending 18 and 21 [n/a] or 2% and 1% [n/a] of body length anteriad, 7 and 4 [n/a] wide; posterior trunk of vasa efferentia emanating from ventral surface of posterior testis, ventral to gonads, extending 126 and 123 [n/a] or 13% and 16% [n/a] of body length anteriad, 7 and 4 [n/a] wide, meeting anterior trunk of vasa efferentia ventral and posterior to anterior testis to form vas deferens. Vas deferens ventral, extending 72 and 39 [n/a] long or 7% and 5% [n/a] of body length anteriad, 5 and 7 [n/a] wide, expanding to form external seminal vesicle. External seminal vesicle 53 and 28 [57] long or 5% and 4% [4%] of body length, 33 and 13 [202] wide or 17% and 7% [64%] of body width or 1.6× and 2.2× [0.3×] longer than wide, intercaecal (Fig. 6); internal seminal vesicle 60 and 47 [136] long or 68% and 75% [59%] of anterior testis length, 28 and 12 [43] wide or 2.1× and 3.9× [3.2×] longer than wide. Pars prostatica surrounding distal portion of internal seminal vesicle, 28 and 20 [91] long or 47% and 43% [67%] of internal seminal vesicle length, 25 and 13 [59] wide or 1.1× and 1.5× [1.5×] longer than wide. Cirrus sac obvious, abutting both caeca or medial, enveloping entirety of internal seminal vesicle and cirrus, 88 and 63 [232] long or 9% and 8% [16%] of body length, 45 and 35 [150] wide or 23% and 20% [47%] of body width at level of external seminal vesicle; cirrus 20 and 9 [27] long, 25 and 8 [30] wide.

Ovary triangular in outline, sinistral, 193 and 165 [382] or 19% and 22% [27%] of body length posterior to middle of ventral sucker, 38 (2) [70] or 4% and 5% [5%] of body length posterior to genital pore; post-ovarian space 325 and 235 [473] or 33% and 31% [33%] of body length. Oviduct extending posteriad 38 and 48 [111] or 4% and 6% [8%] of body length, 8 and 13 [16] in maximum width, laterally expanding to form seminal receptacle; oviducal seminal receptacle extending sinistrad for 35 and 38 [68] or 18% and 25% [23%] of ovary width, 20 and 18 [39] wide or 10% and 12% [13%] of body width, constricting and turning dorsad, extending anteriad for 63 and 50 [125] or 6% and 7% [9%] of body length before joining oötype, 8 and 10 [25] wide or 4% and 7% [8%] of body width (Fig. 6). Laurer's canal originating immediately distal to seminal receptacle terminus, extending posteriad 30 and 47 [45] or 3% and 6% [3%] of body length, 5 and 11 [25], opening dorsally over posterior testis. Vitellarium weakly developed in specimens (Fig. 6), distributing from level of oesophagus median or 214 and 125 [184] or 22% and 17% [13%] of body length from anterior body end to excretory vesicle or 73 and 43 [45] or 7% and 6% [3%] of body length from posterior body end; lateral collecting ducts difficult to discern [n/a]; transverse vitelline duct ventral to ovary, 227 and 184 [491] or 23% and 25% [34%] of body length from middle of ventral sucker; primary vitelline collecting duct not observed [n/a]. Oötype 23 (2) [34] long, 18 and 13 wide [27], ventrolateral to ovary and anterior testis. Uterus originating 180 and 150 [386] or 18% and 20% [27%] of body length posteriad from middle of ventral sucker; metraterm extending anterosinistrad, 20 (2) [45] long or 2% and 3% [3%] of body length, 13 and 10 [23] wide or 7% (2) [8%] of body width, 80% and 71% [49%] of uterus length. Uterine egg not observed [ovoid], [86] long by [50] wide. Common genital pore 150 and 125 [309] or 15% and 17% [21%] of body length posterior to middle of ventral sucker, 10 and 8 [11] in diameter.

Excretory vesicle 43 and 40 [109] wide or 29% (2) [40%] of body width at level of caecal terminus; excretory pore dorsal, subterminal, 15 and 8 [7] or 2% and 1% [<1%] of body length from posterior body margin (Fig. 6).

3.4.2. Taxonomic summary

Host(s): Sternotherus minor (Agassiz, 1857) (Testudines: Kinosternidae), loggerhead musk turtle; *Sternotherus odoratus* (Latreille *in* Sonnini and Latreille, 1801) (Kinosternidae), Eastern musk turtle.

Locality(ies): Wacissa River (30°20'24.73"N, 83°59'27.56"W; Florida, USA); Pond off Parkerson Mill Creek (32°35'20.04"N, 85°29'41.51"W; Tallapoosa River, Alabama, USA).

Site in host: Heart, viscera wash.

Prevalence and intensity of infection by adults: One loggerhead musk turtle had one specimen and one of three (33%) Eastern musk turtles had one specimen.

Specimens deposited: Vouchers (USNM XXXXX, XXXXXX).

Specimens examined: Holotype of *H. gracilis* (AMNH 125); Vouchers of *H. foliorchis* (USNM XXXX, XXXX, XXX); Holotype of *H. stunkardi* (USNM 1321967); Holotype of *H. reelfooti* (USNM 1321968); Vouchers of *H. reelfooti* (USNM 1393855, 1393857); Holotype of *H. brooksi* (USNM 1375720).

3.4.3. Remarks

This is the first record of *H.* cf. *stunkardi* from a river in Alabama or Florida, and this species was previously known only from an aquatic locality probably within the North Oconee River (near Athens, Georgia, USA). It is the fourth species of *Hapalorhynchus* reported from an Alabama river. Both of the reported hosts comprise new host records for *H.* cf. *stunkardi*.

Our specimens of *H.* cf. *stunkardi* resembled the holotype (USNM 1321967) and published descriptions (Byrd [8]; Platt [6]) of *H. stunkardi* by having a similar ventral sucker/anterior sucker ratio (1.9× and 2.0× anterior sucker length vs. 2.4× for *H. stunkardi*), an obvious cirrus sac, a vitellarium extending from the oesophagus to the excretory vesicle, and a uterus that is lateral and slightly ventral to the anterior testis and ovary. Specimens of *H.* cf. *stunkardi* differed from those of *H. stunkardi* in numerous subtle ways: having a longer forebody (42% and 49% vs. 32% of body length), shorter caeca (52−60% of body length vs. 72−74%), smaller testes (ant. testis: 23% and 25% of body width vs. 43%; post. testis: 23% and 33% of body width vs. 56%), a narrower external seminal vesicle (1.6× and 2.2× longer than wide vs. 0.3×), a smaller cirrus sac (9% and 8% of body length vs. 16%; 23% and 20% of body width vs. 47%), and a longer metraterm (80% and 71% of uterus length vs. 49%). Despite these morphological differences, and because Byrd's [8] original description is incomplete and we lack molecular sequence data from specimens of *H. stunkardi* collected from the type host and type locality, we do not think that the specimens of *H.* cf. *stunkardi* warrant a new species description at this time. Moreover, the two specimens comprising the description above appear to be young adults with developing gonads and genitalia (Fig. 6). Larger adults from these hosts and localities are needed to address intra- or interspecific variation among these closely related species of *Hapalorhynchus*. Future workers should rely upon Platt [6] and the description herein to identify newly-collected TBFs that resemble *H. stunkardi*.

Recent taxonomic revisions and geographic range studies of species of *Sternotherus* suggest that the type host for *H. stunkardi* must be reconsidered. Byrd's

[8] description and the holotype slide label indicated that the host was a razor-backed musk turtle (*S. carinatus*) from Athens (see above; Table 1). This turtle ranges throughout the Gulf Coastal Plain from southeastern Oklahoma, central Arkansas, and into Mississippi from the Chickasawhay River south to the Gulf of Mexico (Ernst and Lovich [33]). Blankenship et al. [34] extended the range eastward to include the Escatawpa River, Mobile County, Alabama. If Byrd [8] necropsied a wild-caught turtle near Athens (North Oconee River), that host was likely the loggerhead musk turtle; not the razor-backed musk turtle as he reported. The loggerhead musk turtle was once considered to be a subspecies of the razor-backed musk turtle and ranges from east central Georgia and east central and southeastern Alabama south to central Florida (Iverson [35]; Lindeman [36]; Ernst and Lovich [33]; van Dijk et al. [10]). Unpublished records of loggerhead musk turtles from Athens exist (G. Brown, personal communication JRR).

3.5. Phylogenetic analysis

The reconstructed 28S rDNA phylogeny (Fig. 7) did not refute our morphology-based conclusions regarding respective conspecificity of our specimens of *H. conecuhensis* and *H. reelfooti*. The clade including the blood flukes (*H. foliorchis* and *H. gracilis*) that infect the common snapping turtle, *Chelydra serpentina* (Linnaeus, 1758), and those (*H. conecuhensis* and *H. reelfooti*) infecting musk turtles was strongly supported (posterior probably, pp = 1.00). Isolates of *H. reelfooti* were identical, and an isolate of *H. conecuhensis* (BF131) differed by 1 base pair (an inserted T at alignment position 1592; Treebase Acc. No. XXXX) from the otherwise identical 28S sequences for the other two conspecific specimens of *H. conecuhensis* (BF128, BF120). An updated, broad-scale molecular phylogeny that treats generic interrelationships of TBFs is in preparation, but that *Coeuritrema* and *Hapalorhynchus* share a recent common ancestor has been proposed previously (Orélis-Ribeiro et al. [31]).

4. DISCUSSION

4.1. Future directions for kinosternid TBF discovery

This is the first report of an infected musk turtle from the Coosa and Tallapoosa rivers (Mobile-Tensaw River Basin), Yellow River (Pensacola Bay Basin), or Wacissa River (Apalachee Bay Basin). Despite the broad sampling of species of *Sternotherus* for the present study, kinosternids as a whole remain largely unsampled for TBF infections, with only five of the 26 species (19%) reported as hosts. We report four of the six species of *Sternotherus* as TBF hosts, with the razor-backed musk turtle (*S. carinatus*) and the flattened musk turtle (*S. depressus*) still unsampled for blood flukes. The flattened musk turtle is a threatened species that can only be found in permanent streams of the Black Warrior River system above the Fall Line in Alabama (Guyer et al. [11]). Future sampling of razor-backed musk turtles is likely, but sampling of flattened musk turtles for TBFs is highly unlikely due to their federal and state listing (Guyer et al. [11]). The TBFs infecting mud turtles (*Kinosternon* Spix, 1824) are largely still unknown, with only one of 19 species reported as a TBF host (*H. albertoi* ex. white-lipped mud turtle [*K. leucostomum*]). *Hapalorhynchus albertoi* is a unique congener not only because it is the only TBF known from a mud turtle, but also because it is the only species of *Hapalorhynchus* reported from a kinosternid with large radially extending lateral oesophageal diverticula, resembling those of some species of *Vasotrema* (Lamothe-Argumedo [7]; Roberts and Bullard [23]). Of the 19 species of *Kinosternon*, only two are reported as ranging in the Southeastern United States: *Kinosternon baurii* Garman, 1891 and *Kinosternon subrubrum* (Bonnaterre, 1789), with the rest (17 species) ranging in the Southwestern United States down through to South America (van Dijk et al. [10]). Continued parasitological surveying of previously unsampled mud turtle species will help us better understand the true diversity of kinosternid blood flukes and their phylogenetic position within *Hapalorhynchus*.

4.2. Interrelationships of Hapalorhynchus spp.

Gene sequence data analysis suggested that the species of *Hapalorhynchus* infecting musk turtles are closely related to *H. gracilis* (type species) and *H. foliorchis* (Fig. 7), i.e., the only other congeners for which 28S sequence data are published. As the node grouping *H. gracilis* and *H. foliorchis* with *H. conecuhensis* is weakly supported (pp = 0.51), testing monophyly of the musk turtle TBFs is not possible without additional data. Despite some morphological similarities between this genus and *Coeuritrema*, these molecular data do not reject our morphology-based acceptance of *H. reelfooti* and *H. conecuhensis* as congeneric with *H. gracilis* and *H. foliorchis*. Our molecular analysis included four of the seven North American species of *Hapalorhynchus* (Fig. 7). *Hapalorhynchus conecuhensis* and *H. reelfooti* (blood flukes of *Sternotherus* spp. [Kinosternidae]) differ from *H. gracilis* and *H. foliorchis* (blood flukes of common snapping turtles [Stunkard [28]; Brooks and Mayes [37]) by having an obvious (well-developed) cirrus sac. Sequence data from *H. brooksi*, which infects common snapping turtles not musk turtles, would be a valuable addition to future analyses because *H. brooksi* is the only congener that reportedly has paired anterior caeca in addition to an obvious cirrus sac. Whereas we only have gene sequence data coverage for just over half of the known North American species of *Hapalorhynchus*, we lack a gene sequence for an Asian or African species of *Hapalorhynchus*. Future discovery and new collections of species of *Hapalorhynchus* from Asian and African turtles is imperative to better understand the taxonomy and systematics of this geographically wide-ranging TBF genus.

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LITERATURE CITED

[1] J.W. Smith, The blood flukes (Digenea: Sanguinicolidae and Spirorchidae) of cold-blooded vertebrates: Part I. A Review of the literature published since 1971, and bibliography. Helminth. Ab. 66 (1997a) 255−294.

[2] J.W. Smith, The blood flukes (Digenea: Sanguinicolidae and Spirorchidae) of cold-blooded vertebrates: Part II. Appendix I: Comprehensive parasite-host list; Appendix II: Comprehensive host-parasite list. Helminth. Ab. 66 (1997b) 329−344.

[3] T.R. Platt, S.D. Snyder, Redescription of *Hapalorhynchus reelfooti* Byrd, 1939 (Digenea: Spirorchiidae) from *Sternotherus odoratus* (Latreille, 1801). Comp. Parasitol. 74 (2007) 31−34.

[4] T.R. Platt, R.S.K. Sharma, Two new species of *Hapalorhynchus* (Digenea: Spirorchiidae) from freshwater turtles (Testudines: Geoemydidae) in Malaysia. Comp. Parasitol. 79 (2012) 202−207.

[5] J.R. Roberts, R. Orélis-Ribeiro, B.T. Dang, K.M. Halanych, S.A. Bullard, Blood flukes of Asiatic softshell turtles: revision of *Coeuritrema* Mehra, 1933 (Digenea: Schistosomatoidea) and a new species infecting Chinese softshell turtles, *Pelodiscus sinensis*, (Trionychidae) from the Da Rang River, Vietnam, Folia Parasitol. 63 (2016b) 031 doi:10.14411/fp.2016.031.

[6] T.R. Platt, *Hapalorhychus brooksi* sp. n. (Trematoda: Spirorchiidae) from the snapping turtle (*Chelydra serpentina*), with notes on *H. gracilis* and *H. stunkardi*. Proc. Helm. Soc. Wash. 55 (1988a) 317−323.

[7] R. Lamothe-Argumedo, Tremátodos de reptiles 1. Descripción de una especie nueva de la familia Spirorchidae, parásita de *Kinosternon leucostomum* de Villahermosa, Tabasco, Mexico, An. Inst. Biol. Zool. 49 (1978) 19−24.

[8] E.E. Byrd, Studies on the blood flukes of the family Spirorchidae. Part II. Revision of the family and description of new species, J. Tenn. Acad. Sci. 14 (1939) 116−161.

[9] P.Q. Spinks, R.C. Thomson, M. Gidiş, H.B. Shaffer, Multilocus phylogeny of the New-World mud turtles (Kinosternidae) supports the traditional classification of the group, Mol. Phylogenet. Evol. 76 (2014) 254–260.

[10] P.P. van Dijk, J.B. Iverson, A.G.J. Rhodin, H.B. Shaffer, R. Bour, Turtles of the world, 7th Edition: Annotated checklist of taxonomy, synonymy, distribution with maps, and conservation status, Chelon. Res. Monogr. 5 (2014) 329−479.

[11] C. Guyer, M.A. Bailey, R.H. Mount, Turtles of Alabama, 1st ed. The University of Alabama Press, Tuscaloosa, AL 2015, pp. 266.

[12] J.R. Roberts, T.R. Platt, R. Orélis-Ribeiro, S.A. Bullard, New genus of blood fluke (Digenea: Schistosomatoidea) from Malaysian freshwater turtles (Geoemydidae) and its phylogenetic position within Schistosomatoidea, J. Parasitol. 102 (2016a) 451−462.

[13] J.R. Roberts, R. Orélis-Ribeiro, K.M. Halanych, C.R. Arias, S.A. Bullard, A new species of *Spirorchis* MacCallum, 1918 (Digenea: Schistosomatoidea) and *Spirorchis* cf. *scripta* from chicken turtle, *Deirochelys reticularia*, (Emydidae), with an emendation and molecular phylogeny of *Spirorchis*, Folia Parasitol. 63 (2016c) 041 doi:10.14411/fp.2016.041.

[14] A.E. Lockyer, P.D. Olson, P. Ostergaard, D. Rollinson, D.A. Johnston, S.W. Attwood, et al., The phylogeny of the Schistosomatidae based on three genes with emphasis on the interrelationships of *Schistosoma* Weinland, 1858, Parasitology. 126 (2003) 203−224.

[15] S.D. Snyder, Phylogeny and paraphyly among tetrapod blood flukes (Digenea: Schistosomatidae and Spirorchiidae). Int. J. Parasitol. 34 (2004) 1385−1392.

[16] P.D. Olson, T.H. Cribb, V.V. Tkach, R.A. Bray, D.T.J. Littlewood, Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda), Int. J. Parasitol. 33 (2003) 733–755.

[17] K. Katoh, D. M. Standley, MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability, Mol. Biol. Evol. 30 (2013) 772-780.

[18] W.P. Maddison, D.R. Maddison, Mesquite: a modular system for evolutionary analysis. (2017) Version 3.2. http://mesquiteproject.org.

[19] F. Ronquist, M. Teslenko, P. van der Mark, D. Ayres, A. Darling, S. Höhna, et al., MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space, Syst. Biol. 61 (2011) 539–542.

[20] A. Rambaut, FigTree v1.2.3, Institute of Evolutionary Biology, Univ. of Edinburgh 2009, available at: http://tree.bio.ed.ac.uk/software/figtree.

[21] D.R. Brooks, J.J. Sullivan, *Hapalorhynchus rugatus* sp. nov. (Digenea: Spirorchidae) from a Malaysian freshwater turtle, Can. J. Zool. 59 (1981) 1335−1338.

[22] T.R. Platt, Family Spirorchiidae Stunkard, 1921, in: D.I. Gibson, A.J. Jones, R.A. Bray (Eds.), Keys to the Trematoda, Vol. 1, CABI Publishing, Wallingford, Oxford 2002, pp. 453-468.

[23] J.R. Roberts, S. A. Bullard, Revision and new species of *Vasotrema* Stunkard, 1926 (Digenea: Schistosomatoidea): turtle blood flukes of North American softshell turtles (Testudines: Trionychidae: *Apalone* spp.). J. Parasitol. XX (2017) in review.

[24] J.D. Goodman, A new blood fluke, *Hapalorhynchus beadlei* n. sp. (Spirorchiidae), and a note on *Allossostomoides* (Paramphistomidae), in *Pelusios williamsi lutescens* from Uganda, T. Am. Microsc. Soc. 106 (1987) 80–84.

[25] R. Bougat, S-D. Kulo, *Hapalorhynchus tchalimi* n. sp. (Digenea) premier Spirorchiidae de tortue d'eau douce en Afrique, Rev. Zool. Afr. 100 (1987) 435−441.

[26] T.R. Platt, *Hapalorhynchus beadlei* Goodman, 1987 (Trematoda, Digenea): proposed replacement of the holotype by a lectotype. Bull. Zool. Nomen. 45 (1988b) 258−259.

[27] T.R. Platt, Notes on the genus *Hapalorhynchus* (Digenea: Spirorchidae) from African turtles. T. Am. Microsc. Soc. 110 (1991) 182−184.

[28] H. Stunkard, Two new genera of North American blood flukes, Am. Mus. Nov. 39 (1922) 1−8.

[29] S.A. Bullard, R.M. Overstreet, Two new species of *Cardicola* (Digenea: Sanguinicolidae) in drums (Sciaenidae) from Mississippi and Louisiana, J. Parasitol. 90 (2004) 128−136.

[30] T.N. Truong, S.A. Bullard, Blood flukes (Digenea: Aporocotylidae) of walking catfishes (Siluriformes: Clariidae): new genus and species from the Mekong River (Vietnam) and a note on catfish aporocotylids, Folia Parasitol. 60 (2013) 237−247.

[31] R.Q-Y Yong, S.C. Cutmore, R.A. Bray, T.L. Miller, I.W.Y. Semarariana, H.W. Palm, T.H. Cribb, Three new species of blood flukes (Digenea: Aporocotylidae) infecting pufferfishes (Teleostei: Tetraodontidae) from off Bali, Indonesia, Parasitol. Int. 65 (2016) 432−443.

[32] R. Orélis-Ribeiro, C.R. Arias, K.M. Halanych, T.H. Cribb, S.A. Bullard, Diversity and ancestry of flatworms infecting blood of nontetrapod craniates "fishes," Adv. Parasitol. 85 (2014) 1−64.

[33] C.H. Ernst, J. E. Lovich, Turtle of the United States and Canada, 2nd ed, The Johns Hopkins University Press, Baltimore, MD 2009, pp. 827.

[34] E.L. Blankenship, M.A. Bailey, K. Schnuelle, B. Hauge, *Sternotherus carinatus*, Herp. Rev. 26 (1995) 106−107.

[35] J.B. Iverson *Sternotherus minor* (Agassiz), loggerhead musk turtle. Cat. Am. Amph. Rept. 195 (1977) 1−2.

[36] P.V. Lindeman, *Sternotherus carinatus* (Gray, 1856) − razorbacked musk turtle, razor-backed musk turtle, in: A.G.J. Rhodin, P.C.H. Pritchard, P.P. van Dijk, R.A. Saumure, K.A. Buhlmann, J.B. Iverson (Eds.) Conservation Biology of Freshwater Turtles and Tortoises: A compilation Project of the IUCN/SSC Tortoise and Freshwater Turtle Specialist Group. Chelonian Research Monographs No. 5 (2008) 012.1−012.6.

[37] D.R. Brooks, M.A. Mayes, Platyhelminths of Nebraska turtles with descriptions of two new species of spirorchiids (Trematoda: Spirorchiidae), J. Parasitol. 61 (1975) 403−406.

FIGURE LEGENDS

Figures 1, 2. Adult of *Hapalorhynchus conecuhensis* n. sp. from *Sternotherus* cf. *minor*, from Blue Spring (31°5'27.64"N, 86°30'53.21"W; Yellow River, Alabama, USA). Scale values aside each bar. **1.** Holotype (National Museum of National History, Smithsonian Department of Invertebrate Zoology [USNM] XXXX) of *H. conecuhensis* showing oral sucker (os), pharynx (ph), oesophagus (es), anterior oesophageal gland (aog), nerve commissure (nc), ventrolateral nerve chords (vln), posterior oesophageal gland (pog), vitellarium (vr), caecal bifurcation (cb), sinistral posterior caecum (sc), dextral posterior caecum (dc), ventral sucker (vs), external seminal vesicle (esv), cirrus sac (cs), vas deferens (vd), common genital pore (cgp), anterior testis (at), ovary (ov), lateral vitelline collecting duct (lvd), transverse vitelline collecting duct (tvd), oviducal seminal receptacle (osr), posterior testis (pt), caecal terminus (ct), excretory vesicle (ev), and excretory pore (ep). Ventral view. **2.** Genital complex of holotype (USNM XXXX) showing vas deferens (vd), external seminal vesicle (esv), cirrus (ci), cirrus sac (cs), pars prostatica (pp), internal seminal vesicle (isv), common genital pore (cgp), metraterm (mt), egg chamber (ec), uterine egg (egg), anterior trunk of vasa efferentia (ave), anterior testis (at), lateral vitelline collecting duct (lvd), ovary (ov), transverse vitelline collecting duct (tvd), oviducal seminal receptacle (osr), primary vitelline collecting duct (vt), posterior trunk of vasa efferentia (pve), Laurer's canal (Lc), and posterior testis (pt). Ventral view.

Figures 3, 4. Oral sucker of *Hapalorhynchus conecuhensis* n. sp. from *Sternotherus* cf. *minor* collected from Blue Spring (31°5'27.64"N, 86°30'53.21"W; Yellow River, Alabama, USA). Scale values aside each bar. **3.** Holotype (National Museum of National History, Smithsonian Department of Invertebrate Zoology [USNM] XXXX) showing oral sucker papillae (op), oral sucker spines (ss), mouth (mo), and ventral body papillae (vbp). Ventral view. **4.** Paratype (USNM XXXX) showing oral sucker papillae (op) and ventral body papillae (vbp). Dorsal view.

Figures 5, 6. *Hapalorhynchus* spp. from loggerhead musk turtles, *Sternotherus minor*, collected from the Wacissa River (30°20'24.73"N, 83°59'27.56"W; tributary of Aucilla River, Florida, USA). Scale values aside each bar. **5.** Adult of *Hapalorhynchus reelfooti* Stunkard, 1922 (National Museum of National History, Smithsonian Department of Invertebrate Zoology [USNM] XXXX) showing oral sucker (os), pharynx (ph), oesophageal gland (og), oesophagus (es), nerve commissure (nc), vitellarium (vr), caecal bifurcation (cb), dextral posterior caecum (dc), sinistral posterior caecum (sc), ventral sucker (vs), external seminal vesicle (esv), vas deferens (vd), cirrus sac (cs), internal seminal vesicle (isv), common genital pore (cgp), anterior testis (at), uterus (ut), ovary (ov), transverse vitelline collecting duct (tvd), oviducal seminal receptacle (osr), posterior trunk of vasa efferentia (pve), posterior testis (pt), caecal terminus (ct), excretory vesicle (ev), and excretory pore (ep). Ventral view. **6.** Adult of *Hapalorhynchus* cf. *stunkardi* (USNM XXXX) showing oral sucker (os), pharynx (ph), anterior oesophageal gland (aog), oesophagus (es), nerve commissure (nc), vitellarium (vr), ventrolateral nerve chords (vln), posterior oesophageal gland (pog), caecal bifurcation (cb), dextral posterior caecum (dc), sinistral caecum (sc), ventral sucker (vs), external seminal vesicle (esv), vas deferens (vd), cirrus sac (cs), internal seminal vesicle (isv), common genital pore (cgp), uterus (ut), anterior testis (at), ovary (ov), transverse vitelline collecting duct (tvd), oviducal seminal receptacle (osr), posterior trunk of vasa efferentia (pve), posterior testis (pt), caecal terminus (ct), excretory vesicle (ev), and excretory pore (ep). Ventral view.

Fig. 7. Phylogenetic relationships of blood flukes reconstructed by Bayesian inference and based on partial D1−D3 domains of the large subunit ribosomal DNA (28S) sequences from selected blood flukes (15 TBFs, three schistosomes). Numbers aside tree nodes indicate posterior probability.

Table 1
Hapalorhynchus spp. infecting mud and musk turtles (Kinosternidae)

Turtle host	Blood fluke	Site in host	Riverine locality	Accession nos.	Reference
Kinosternon leucostomum Duméril and Bibron *in* Duméril and Duméril, 1851	*H. albertoi* Lamothe-Argumedo, 1978	blood (adult)	Grijalva River, Villahermosa, Tabasco, Mexico	Colección Helmintológica del Instituto de Biología de la UNAM 229-19, 229-20	Lamothe-Argumedo [7]
Sternotherus carinatus (Gray, 1856) (probably *S. minor*; see 3.4.3. Remarks)	*H. stunkardi* Byrd, 1939	pulmonary blood vessels (adult)	North Oconee River, Athens, Georgia, USA	USNM 1321967	Byrd [8]
***Sternotherus minor* (Agassiz, 1857)**	***H. reelfooti* Byrd, 1939**	**mesenteric blood vessels, viscera wash (adult)**	**Wacissa River (tributary of Aucilla River) (30°19'39.80"N, 83°59'6.13"W), Florida, USA**	**USNM XXXX**	**Present study**
			Wacissa River (30°20'24.73"N, 83°59'27.56"W)	**USNM XXXX**	**Present study**
	H.* cf. *stunkardi	**heart (adult)**	**Wacissa River (30°20'24.73"N, 83°59'27.56"W)**	**USNM XXXX**	**Present study**
	***H. conecuhensis* n. sp.**	**heart (adult)**	**Wacissa River (30°20'24.73"N, 83°59'27.56"W)**	**GenBank XXXX**	**Present study**
Sternotherus* cf. *minor	***H. reelfooti***	**mesenteric blood vessels, viscera wash,**	**Blue Spring (Yellow River) (31°5'27.64"N, 86°30'53.21"W),**	**USNM XXXX**	**Present study**

		heart (adult)	**Alabama, USA**		
	H. conecuhensis	**heart, viscera wash (adult)**	**Blue Spring (31°5'27.64"N, 86°30'53.21"W)**	**USNM XXXX**	**Present study**
Sternotherus odoratus (Latreille *in* Sonnini and Latreille, 1801)	*H. reelfooti*	blood (adult)	Reelfoot Lake (Mississippi River) (36°21'12.23"N, 89°25'21.50"W), Tennessee, USA	USNM 1321968	Byrd [8]
		viscera wash (adult)	North Chain Lake (Kankakee River) (41°41'57.03"N, 86°22'16.51"W), Indiana, USA	USNM 1393855	Platt and Snyder [3]
		viscera wash (adult)	Westhampton Lake (James River) (37°34'38.01"N, 77°32'26.90"W), Richmond, Virginia, USA	USNM 1393857	Platt and Snyder [3]
		viscera wash (adult)	**Canoe Lake (Coosa River) (33°47'56.16"N, 86°29'25.02"W), Alabama**	**USNM XXXX**	**Present study**
		mesenteric blood vessels (adult)	**Pond off Odom Creek (Tallapoosa River) (32°30'9.58"N, 85°26'6.07"W), Alabama**	**USNM XXXX**	**Present study**
	H.* cf. *stunkardi	**heart (adult)**	**Pond off Parkerson Mill**	**USNM XXXX**	**Present study**

			Creek (Tallapoosa River) (32°35'20.04"N, 85°29'41.51"W), Alabama		
***Sternotherus peltifer* (Smith and Glass, 1947)**	***H. reelfooti***	**mesenteric blood vessels, lung (adult)**	**Chewacla Creek (Tallapoosa River) (32°31'58.64"N, 85°29'53.09"W), Alabama**	**USNM XXXX**	**Present study**

Table 2
Large subunit ribosomal DNA (28S) sequences used herein

Turtle blood fluke	Turtle host	Locality	GenBank Accession No.	Reference
Coeuritrema platti Roberts and Bullard, 2016	*Pelodiscus sinensis* (Wiegmann, 1835)	Da Rang River Basin, Phu Yen Province, Vietnam	KX712243	Roberts et al. [5]
Hapalorhynchus gracilis Stunkard, 1922	*Chelydra serpentina* Linnaeus, 1758	Reelfoot Lake (Mississippi River) (36°21'12.23"N, 89°25'21.50"W), Tennessee, USA	AY604710	Snyder [15]
Hapalorhynchus foliorchis Brooks and Mayes, 1975	*C. serpentina*	Pond off Saugahatchee Creek (Tallapoosa River) (32°39'1.36"N, 85°29'4.70"W), Alabama, USA	KX712242	Roberts et al. [5]
***Hapalorhynchus reelfooti* Byrd, 1939**	***Sternotherus minor* (Agassiz, 1857)**	**Wacissa River (tributary of Aucilla River) (30°20'24.73"N, 83°59'27.56"W), Florida, USA**	**XXXX**	**Present study**
			XXXX	**Present study**
	Sternotherus* cf. *minor	**Blue Spring (Yellow River) (31°5'27.64"N, 86°30'53.21"W), Alabama, USA**	**XXXX**	**Present study**
			XXXX	**Present study**
	Sternotherus	**Canoe Lake (Coosa**	**XXXX**	**Present**

	***odoratus* (Latreille *in* Sonnini and Latreille, 1801)**	**River) (33°47'56.16"N, 86°29'25.02"W), Alabama, USA**		**study**
		Pond off Chewacla Creek (Tallapoosa River), (32°30'9.58"N, 85°26'6.07"W), Alabama, USA	**XXXX**	**Present study**
***Hapalorhynchus conecuhensis* n. sp.**	***Sternotherus* cf. *minor***	**Blue Spring (31°5'27.64"N, 86°30'53.21"W)**	**XXXX hologenophore**	**Present study**
			XXXX	**Present study**
	S. minor	**Wacissa River (30°20'24.73"N, 83°59'27.56"W)**	**XXXX**	**Present study**
Schistosoma japonicum (Katsurada, 1904)	*Mus musculus* Linnaeus, 1758	experimental infection; original isolate from Phillipines	Z46504 AY157607	Lockyer et al. [14]; Olson et al. [16]
Schistosoma mansoni Sambon, 1907	*M. musculus*	experimental infection	AY157173	Lockyer et al. [14]
Schistosoma sinensium Pao, 1959	*M. musculus*	experimental infection; original isolate from Mianzhu, Sichuan, China	AY157251	Lockyer et al. [14]

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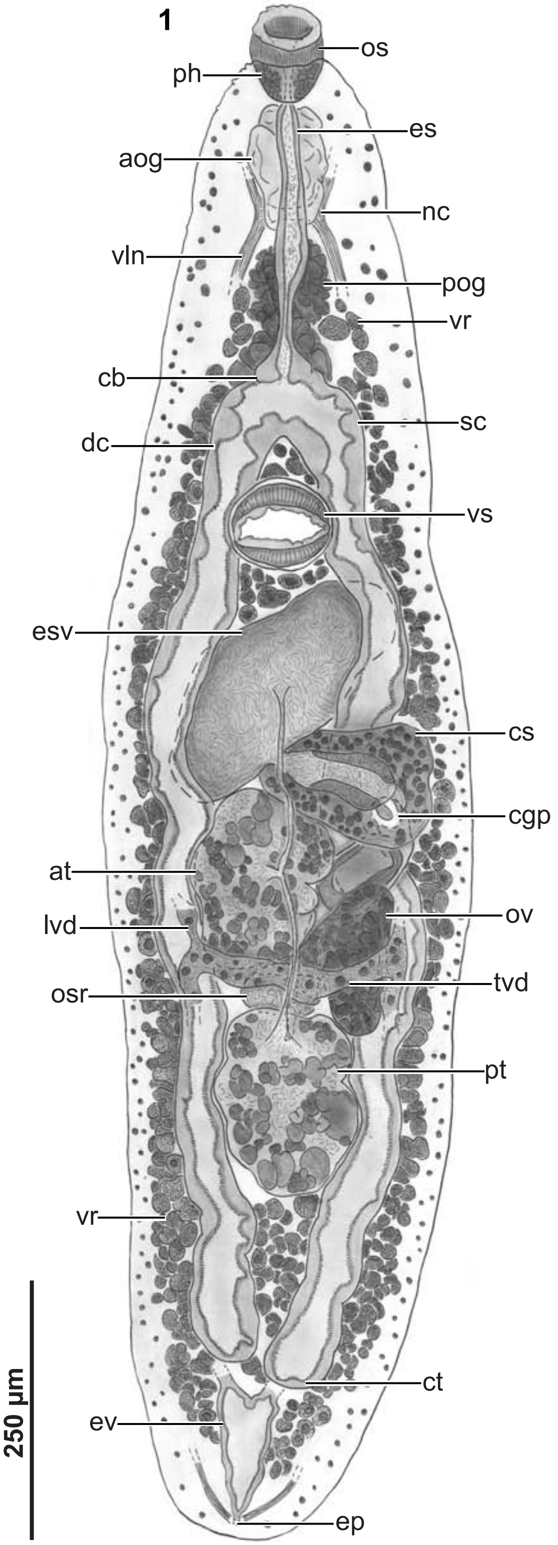
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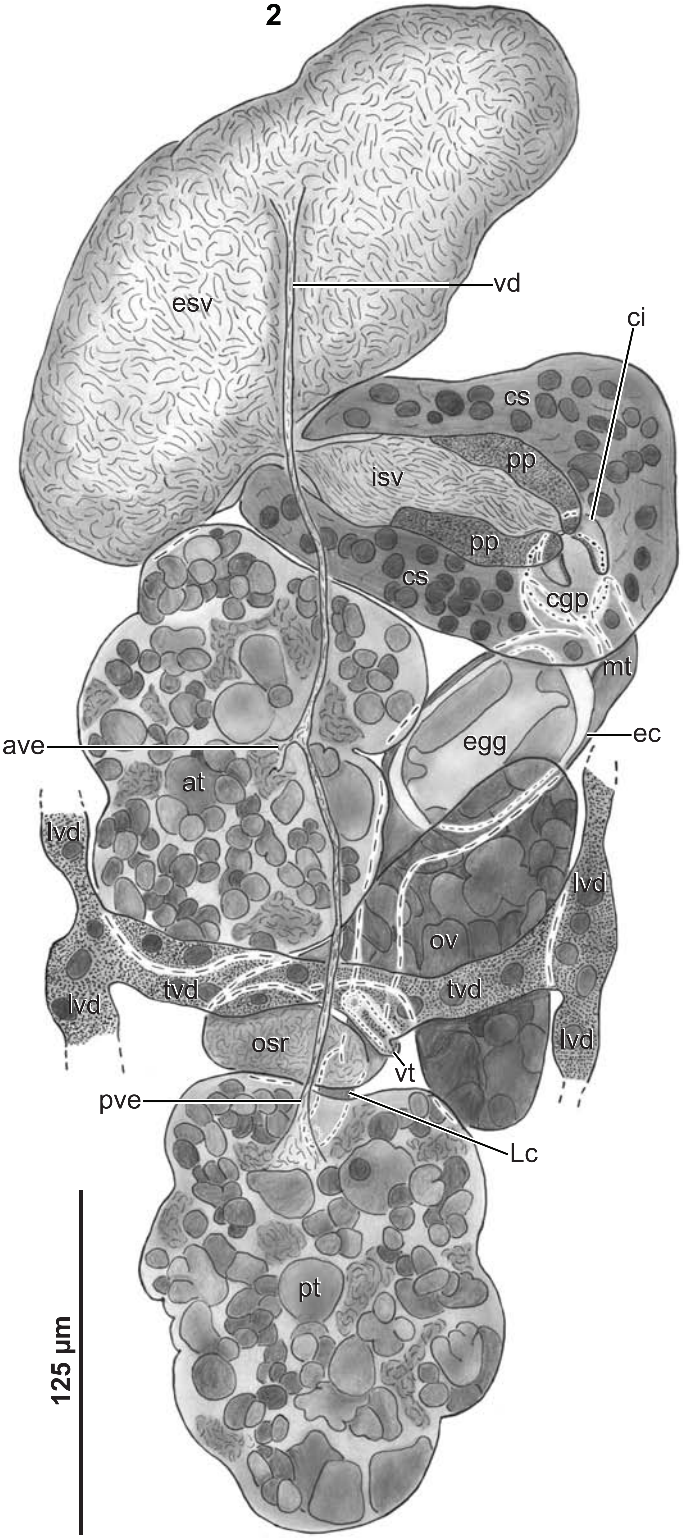
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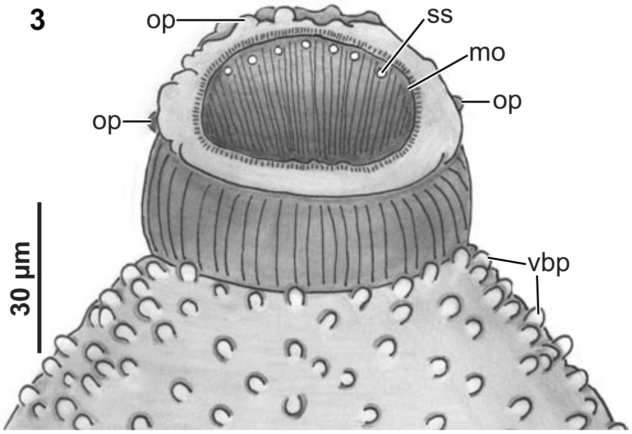

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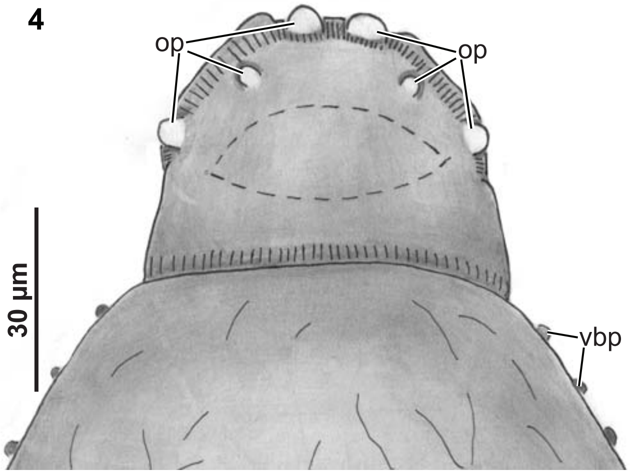

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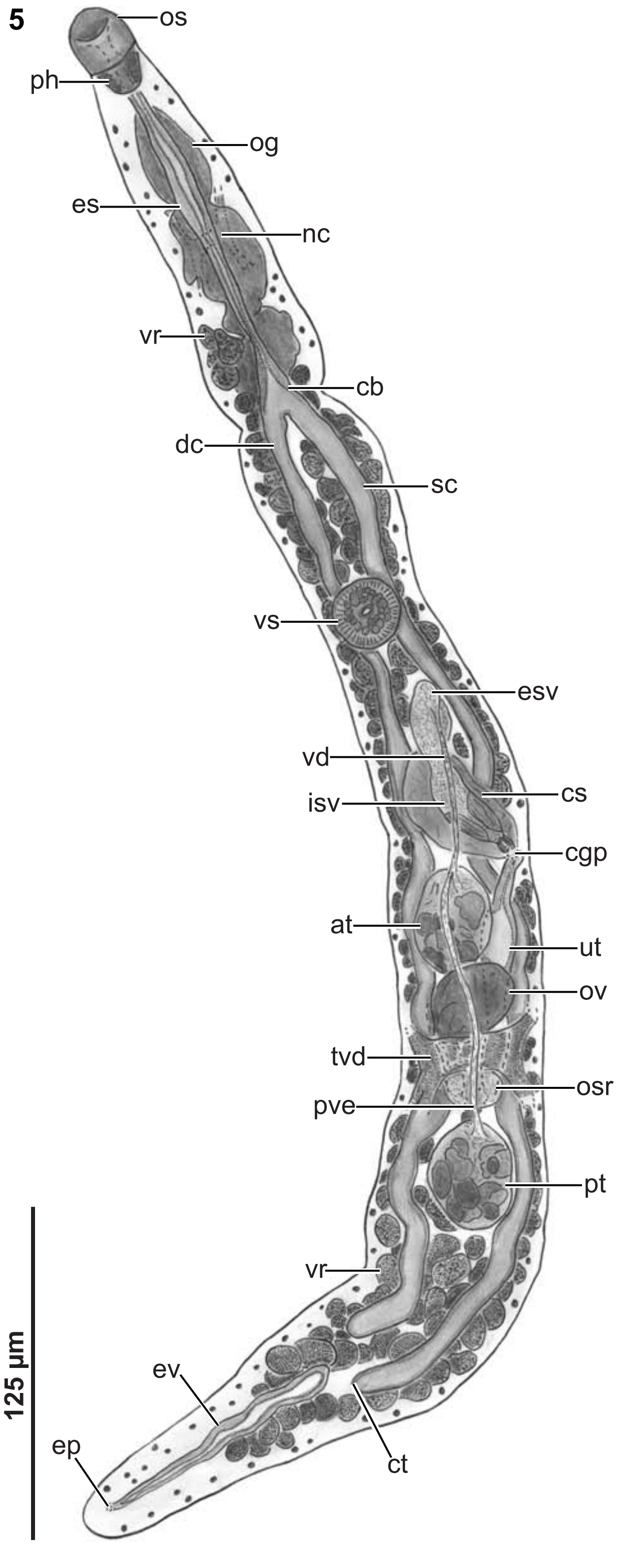
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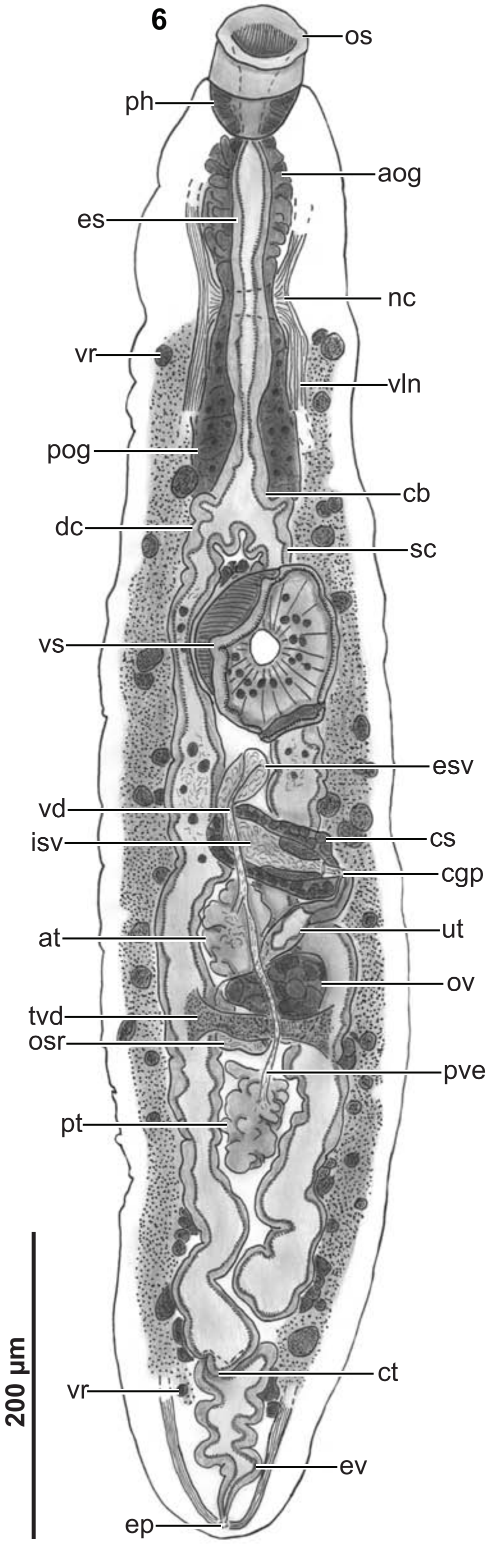
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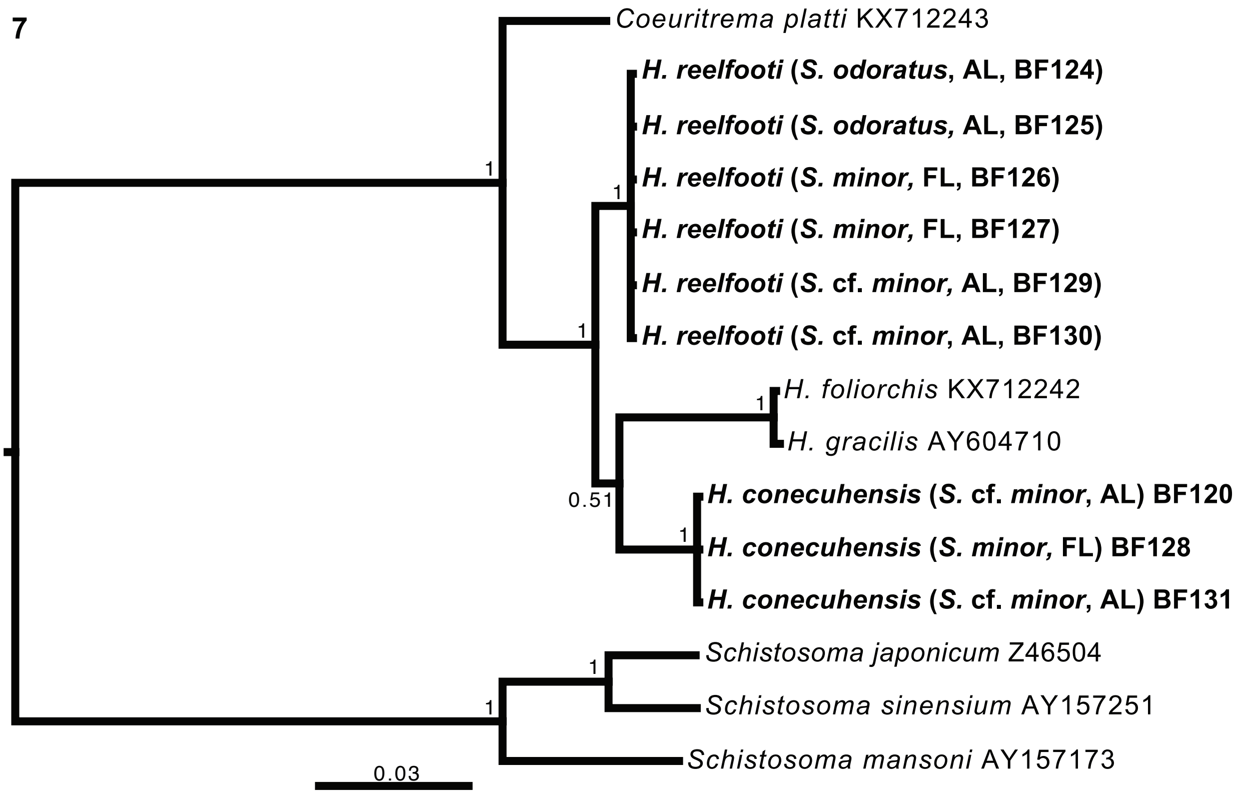

Coeuritrema platti KX712243
***H. reelfooti* (*S. odoratus*, AL, BF124)**
***H. reelfooti* (*S. odoratus,* AL, BF125)**
***H. reelfooti* (*S. minor,* FL, BF126)**
***H. reelfooti* (*S. minor,* FL, BF127)**
***H. reelfooti* (*S.* cf. *minor,* AL, BF129)**
***H. reelfooti* (*S.* cf. *minor*, AL, BF130)**
H. foliorchis KX712242
H. gracilis AY604710
***H. conecuhensis* (*S.* cf. *minor*, AL) BF120**
***H. conecuhensis* (*S. minor,* FL) BF128**
***H. conecuhensis* (*S.* cf. *minor*, AL) BF131**
Schistosoma japonicum Z46504
Schistosoma sinensium AY157251
Schistosoma mansoni AY157173
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CHAPTER 6: A NEW SPECIES OF *SPIRORCHIS* MACCALLUM, 1918 (DIGENEA: SCHISTOSOMATOIDEA) AND *SPIRORCHIS SCRIPTA* STUNKARD, 1923 INFECTING RIVER COOTER, *PSEUDEMYS CONCINNA* (LE CONTE, 1830), (TESTUDINES: EMYDIDAE) IN THE PASCAGOULA RIVER, MISSISSIPPI, U.S.A.; INCLUDING AN UPDATED PHYLOGENY FOR *SPIRORCHIS* SPP.

***Submitted to Comparative Parastiology (30 May 2017)**

Authors: Jackson R. Roberts, Kenneth M. Halanych, Cova R. Arias, Stephen S. Curran, and Stephen A. Bullard

ABSTRACT

River cooters (*Pseudemys concinna* [Le Conte, 1830]) from the Pascagoula River (30°37'07.67"N, 88°36'44.53"W; Mississippi, U.S.A.) were infected by *Spirorchis scripta* Stunkard, 1923 and *Spirorchis testiplexus* n. sp. The new species resembles *Spirorchis artericola* (Ward, 1921) Stunkard, 1921 and *Spirorchis innominatus* Ward, 1921 (type species) by having a median esophageal diverticulum, ceca that extend slightly posterior to the genitalia, 10 testes, and a testicular column that extends anteriad to near the cecal bifurcation. Based on a comparative morphological study of existing type materials, vouchers, and published descriptions of similar congeners, *S. testiplexus* is unique by the combination of having a proportionally wider body, laterally-directed cecal diverticula, deeply lobed testes, a testicular column that fills the intercecal space, and an external seminal vesicle that occupies the space between the posterior-most testis and the ovary and that passes dorsal or dorsolateral to the ovary. The large subunit rDNA (28S) Bayesian inference phylogenetic analysis (including 16 turtle blood fluke species of 7 genera) showed a strongly supported monophyletic *Spirorchis* MacCallum, 1918 sister to *Spirhapalum siamensis* Tkach, Snyder, and Vaughn, 2009.

INTRODUCTION

Cooters (Testudines: Emydidae: *Pseudemys* spp.) comprise 8 turtle species that range in Atlantic and Gulf of Mexico river drainages (Ernst and Lovich, 2009; van Dijk et al., 2014). Despite this relatively high intrageneric diversity, only the river cooter, *Pseudemys concinna* (Le Conte, 1830), has been reported as a host for blood flukes. Prior to the present study, 4 species of *Spirorchis* MacCallum, 1918 have been reported to infect this turtle: *Spirorchis innominatus* Ward, 1921 (type species), *Spirorchis artericola* (Ward, 1921) Stunkard, 1921, *Spirorchis elegans* Stunkard, 1923, and *Spirorchis scripta* Stunkard, 1923 (Table 1).

Herein, we report the presence of *S. scripta* and describe a new species in the genus based on the results of parasitological examinations of 2 river cooters opportunistically sampled from the Pascagoula River, Mississippi, U.S.A. We also contribute molecular sequence data for the new species towards an updated phylogeny for *Spirorchis* spp.

MATERIALS AND METHODS

Two river cooters were sampled by seine on 26 May 2015 from the Pascagoula River (30°37'07.67"N, 88°36'44.53"W), Mississippi, U.S.A. Turtles were killed by decapitation, and necropsies were performed using 7.0 g/L sodium citrate saline solution following Roberts et al. (2016c). Live turtle blood flukes for morphology were killed, fixed, stained, and mounted following Roberts et al. (2016c). Turtle scientific and common names follow van Dijk et al. (2014) and Guyer et al. (2015). Classification and anatomical terms for turtle blood flukes follow Roberts et al. (2016a, b, c; 2017).

Specimens intended for molecular analyses (a specimen of the new species, a specimen of *Spirorchis haematobius* (Stunkard, 1922) Price, 1934, and a specimen of *Vasotrema* cf. *robustum* Stunkard, 1928) were placed directly into absolute EtOH. The hologenophore (molecular voucher) of the new species was cut in half: the anterior end was used for DNA extraction and sequencing (GenBank XXXX [28S], XXXX [18S]) and the posterior end was stained and mounted as indicated above. Measurements are reported in micrometers (μm) followed by the mean and number of specimens measured for that feature in parentheses. Holotype, paratype, and voucher specimens of turtle blood flukes were borrowed from the American Museum of Natural History (AMNH, New York, New York), Harold W. Manter Laboratory Collection (HWML; Lincoln, Nebraska), National Museum of National History (USNM, Smithsonian Institute, Washington, D.C.), and Thomas Platt’s personal research collection (TPRC), which Platt kindly donated to SAB (see Taxonomic Summary; Materials Examined).

DNA extraction, amplification, and sequencing were performed following Roberts et al., (2017). Assembled sequences (Table 2) were aligned with MAFFT 7.310 (Katoh and Standley, 2013) and subsequently corrected by eye in Mesquite 3.2 (Maddison and Maddison, 2017). Regions that could not be unambiguously aligned were excluded from further analyses, and the final alignment was deposited in TreeBase (Acc. No. XXXX). MrBayes 3.5.3 (Ronquist et al., 2012) was used to reconstruct a Bayesian inference phylogeny. Using a GTR + Gamma model, 4 runs of 4 chains each were conducted for 1,000,000 generations. Priors were set to default values and burn-in was set to 25% of generations (or 250,000). Outgroups were selected from representative blood fluke genera informed by the phylogenetic analyses of Orélis-Ribeiro et al. (2014) and

Roberts et al. (2016a; b). The ingroup comprised all available 28S sequences for species of *Spirorchis* as well as freshwater turtle blood flukes likewise selected based on the above studies (op cit.) (Table 2). Chains were run until the average standard deviation of split frequencies was below 0.01. The resulting phylogenetic tree was viewed using FigTree v1.4.3 (Rambaut, 2009) and subsequently edited in Adobe Illustrator CC 2015.3 (Adobe Systems).

RESULTS

Spirorchis testiplexus **Roberts, Curran, and Bullard n. sp. (Figs. 1−4)**

Description

Based on light microscopy of 10 whole-mounted specimens. Body 2,650−3,850 (3,366; 10) long or 2.9−4.7× (3.7; 10) longer than wide, 660−850 (738; 10) wide or 19−26% (22%; 10) of body length at level of cecal bifurcation, 720−1,010 (922; 10) wide or 21−34% (28%; 10) of body length at level of middle testis (typically maximum width), 370−500 (421; 10) wide or 11−15% (13%; 10) (Figs. 1, 3). Oral sucker 41−86 (57; 10) long or 1−3% (2%; 10) of body length, 61−75 (70; 10) wide or 8−11% (10%; 10) of body width at level of cecal bifurcation, papillae present, spines present. Nerve commissure 193−272 (238; 10) or 6−8% (7%; 10) of body length from anterior body end. Pharynx 59−89 (70; 10) long or 9−16% (13%; 10) of esophagus length, 61−77 (70; 10) wide or 1.6−4.6× (2.5; 10) esophagus width immediately posterior to pharynx, 0.8−1.3× (1.0; 10) longer than wide (Figs. 1, 3). Esophagus extending posteriad 430−645 (550; 10) long or 15−18% (16%; 10) of body length from mouth to anterior margin of median esophageal diverticulum, 16−43 (31; 10) wide posterior to pharynx, with wall 2−20 (8; 10) thick, 27−116 (83; 10) wide at esophagus median or 4−16% (11%; 10) of body width at level of cecal bifurcation, with wall 18−72 (49; 10) thick, 111−150 (129; 10) wide anterior to median esophageal diverticulum or 15−20% (18%; 10) of body width at level of cecal bifurcation, with wall 18−80 (42; 10) thick; median esophageal diverticulum 481−710 (601; 10) or 16−19% (18%; 10) of body length from anterior body end, 105−200 (153; 10) long or 18−36% (28%; 10) of esophagus length, 30−84 (50; 10) wide or 4−12% (7%; 10) of body width at level of cecal bifurcation; esophageal gland 422−655 (546; 10) long or 15−17% (16%; 10) of body length, 250−364 (308; 10) wide or 38−49% (42%; 10) of body width at level of cecal bifurcation. Intestinal bifurcation 499−737 (623; 10) from anterior body end or 17−20% (19%; 10) of body length, dorsal to median esophageal diverticulum; sinistral cecum 1,990−3,150 (2,586; 10) long or 74−82% (77%; 10) of body length, 23−80 (49; 10) wide or 3−12% (7%; 10) of body width at level of cecal bifurcation, 68−102 (83; 10) wide or 7−10% (9%; 10) of body width at level of middle testis, 36−98 (66; 10) wide or 10−23% (16%; 10) of body width at level of cecal termini; dextral cecum 2,050−3,190 (2,643; 10) long or 75−85% (78%; 10) of body length, 34−75 (48; 10) wide or 5−11% (7%; 10) of body width at level of cecal bifurcation, 65−95 (79; 10) wide or 6−11% (9%; 10) of body width at level of middle testis, 34−107 (65; 10) wide or 9−25% (15%; 10) of body width at level of cecal termini; post-cecal distance 148−245 (182; 10) or 4−7% (5%; 10) of body length (Figs. 1, 3).

Testes 9−10 (10; 10) in number, deeply lobed, having abutting anterior and posterior margins; testicular column 18−177 (72; 10) or 1−6% (2%; 10) of body length from cecal bifurcation, abutting or 14−139 (50; 5) from median esophageal diverticulum, 1,310−2,290 (1854; 10) long or 49−61% (55%; 10) of body length; anterior testis (t1) abutting or 5−23 (13; 7) and 2−55 (26; 6) from sinistral and dextral ceca respectively, 205−525 (350; 10) long or 6−15% (10%; 10) of body length, 164−465 (297; 10) wide or 19−47% (32%; 10) of body width at level of middle testis, 0.4−1.8× (0.9; 10) wider than long; middle testis (t5), abutting both ceca or 16 from sinistral cecum (1), 150−261 (204; 10) long or 5−7% (6%; 10) of body length, 329−599 (448; 10) wide or 39−60% (48%; 10) of body width at level of middle testis, 1.3−3.1× (2.3; 10) wider than long; posterior-most testis 708−969 (797; 10) or 20−28% (24%; 10) of body length from posterior body end, abutting both ceca, 254−395 (307; 10) long or 7−11% (9%; 10) of body length, 272−499 (383; 10) wide or 34−50% (41%; 10) of body width at level of middle testis, 0.8−1.8× (1.3; 10) wider than long (Figs. 1, 3). Vas deferens 1,050−1,885 (1,533; 10) long or 37−49% (45%; 10) of body length, 13−25 (15; 10) wide, ventral to testicular column, laterally expanding before joining external seminal vesicle posterior to testes (Figs. 2, 4); external seminal vesicle abutting posterior margin of posterior-most testis, 170−295 (229; 10) long or 5−9% (7%; 10) of body length, 114−214 (169; 10) wide or 13−29% (19%; 10) of body width, 0.9−2.1× (1.4; 10) longer than wide, extending posteriad dorsal or dorsolateral to ovary (Figs. 2, 4); internal seminal vesicle 105−155 (130; 10) long or 3−5% (4%; 10) of body length, 27−43 (35; 10) maximum width or 50−75% (63%; 10) of cirrus sac width, 3.1−4.8× (3.8; 10) longer than wide (Figs. 2, 4); proximal portion of internal seminal vesicle 32−61 (47; 10) long or 21−48% (36%; 10) of total internal seminal vesicle length, 27−43 (35; 10) wide; distal portion of internal seminal vesicle 55−123 (83; 10) long or 52−79% (64%; 10) of total internal seminal vesicle length, 25−36 (30; 10) wide. Cirrus sac 139−250 (204; 10) long or 5−7% (6%; 10) of body length, 48−68 (56; 10) wide or 5−7% (6%; 10) of maximum body width;

cirrus slightly sinuous, 45−123 (77; 10) long or 1−4% (2%; 10) of body length, 23−45 (26; 10) wide or 2−5% (3%; 10) of maximum body width.

Ovary deeply lobed, intercecal, lateral to cirrus sac, ventral or ventrolateral to external seminal vesicle, 193−254 (224; 10) long or 6−7% (7%; 10) of body length,130−277 (200; 10) wide or 15−29% (22%; 10) of body width, 0.7−1.4× (0.9; 10) wider than long (Figs. 1−4); post-ovarian distance 409−591 (515; 10) or 14−18% (15%; 10) of body length (Figs. 1, 3). Oviduct extending posteriad and sinistrad 35−80 (51; 10) or 1−2% (2%; 10) of body length, 7−18 (12; 10) wide; oviducal seminal receptacle 88−130 (107; 10) long or 2−4% (3%; 10) of body length, 30−45 (35; 10) wide or 3−6% (4%; 10) of maximum body width, oviduct continuing sinistrad 83−163 (117; 10) or 2−5% (3%; 10) of body length before connecting with oötype, 14−25 (20; 10) wide or 2−3% (2%; 10) of maximum body width. Laurer's canal a narrow duct originating 23−75 (46; 10) or 11−33% (20%; 10) of ovary length from distal margin of seminal receptacle, extending 68−134 (96; 10) or 2−4% (3%; 10) of body length posterosinistrad, 14−39 (25; 10) wide or 3−8% (6%; 10) of body width at cecal termini (Figs. 3, 4). Vitellarium comprising a series of interconnected spheroid masses of small follicles (Figs. 3, 4), distributing from cecal bifurcation to cecal termini, originating 295−610 (486; 10) or 10−17% (14%; 10) of body length from anterior body end, ventrolateral to ceca and testes anteriorly and flanking ceca posteriorly (illustrated as only lateral to show course of posterior ceca, Figs. 1, 3), terminating 68−118 (92; 10) or 2−3% (3%; 10) of body length from posterior body end, coalescing into lateral vitelline collection ducts posterior to ovary; transverse vitelline duct 18−52 (32; 10) in maximum width, 190−300 (245) or 6−9% (7%; 10) of body length from cecal termini, 345−473 (427; 10) or 12−15% (13%;

10) of body length from posterior body end (Figs. 1, 3); primary vitelline duct extending posteriad 98−141 (117; 10) or 3−4% (3%; 10) of body length before turning ventrad, extending 75−113 (100; 10) anteriad or 2−4% (3%; 10) of body length before merging with female genitalia at oviduct-oötype junction, 36−64 (46; 10) wide (Figs. 2, 4). Oötype and uterus difficult to discern in gravid specimens (9 of 10 gravid). Oötype 28 long by 28 wide (1). Uterus 50 long by 25 wide (1); egg chamber 84−107 (99; 9) long or 67−85% (79%; 9) of ovary length, 45−55 (50; 9) wide or 20−40% (27%; 9) of ovary width; metraterm 41−80 (66; 10) long or 43−77% (65%; 9) or egg chamber length or 1.6× (1) longer than uterus, 16−28 (23; 9) wide or 29−60% (46%; 9) of egg chamber width or 1.1× (1) wider than uterus. Common genital pore 409−532 (481; 10) or 12−16% (14%; 10) of body length from posterior body end (Figs. 1, 3).

Excretory vesicle 16−39 (28; 10) wide or 4−9% (7%; 10) of body width at level of cecal termini; Manter's organ turning 2−5 (3; 10) times proximally, intercecal or extending to cecal termini, 139−223 (179; 10) long or 5−7% (5%; 10) of body length, 23−64 (34; 10) wide or 5−14% (8%; 10) of body width at level of cecal termini, joining excretory vesicle at excretory pore; excretory pore dorsal, 16−39 (30; 10) or 1% (10) from posterior body end (Figs. 1, 3).

Taxonomic Summary

Type host: Pseudemys concinna (Le Conte, 1830) (Testudines: Emydidae), river cooter; *other host: Chrysemys picta* (Schneider, 1783) (Testudines: Emydidae), painted turtle (Snyder, 2004).

Type locality: Pascagoula River (30°37'07.67"N, 88°36'44.53"W; Mississippi, U.S.A.); *other locality:* Reelfoot Lake (36°21'12.23"N, 89°25'21.50"W; Mississippi River Basin, Tennessee, U.S.A.; Snyder [2004]).

Site of infection in host: Mesenteric blood vessels.

Prevalence and intensity of infection by adults: One of 2 individuals of *P. concinna* from the Pascagoula River had 12 adult specimens of *S. testiplexus*.

Specimens and sequence deposited: Holotype (USNM XXXX); Paratypes (USNM XXXX; XXXX); Hologenophore (USNM XXXX); GenBank (Nos. XXXX [28S], XXXX [18S]).

Specimens examined: AMNH: holotype and paratype of *S. elegans* (AMNH 134 and AMNH 1516, respectively), holotype of *S. haematobius* (AMNH 126), holotype of *Spirorchis parvus* (Stunkard, 1923) Price, 1934 (AMNH 133), holotype of *Spirorchis picta* Stunkard, 1923 (AMNH 131), and holotype and paratype of *S. scripta* (AMNH 128 [damaged specimen] and AMNH 130). HWML: voucher of the new species described herein (HWML 45793). USNM: holotype and paratype of *S. artericola* (USNM 1350610, 2 slides comprising 2 whole-mounted specimens), holotype and paratype of *S. innominatus* (USNM 1337308, 1 slide comprising 2 whole-mounted specimens), and holotype of *Spirorchis minutus* Byrd, 1939 (USNM 1321965). TPRC: paratypes of *Spirorchis kirki* (Platt, 1990) Platt, 1992.

Etymology: The specific epithet *testiplexus* is from the Latin “plexus” (interwoven) and refers to the strongly lobed testes of the new species that resemble interlocked puzzle pieces.

Molecular results

Available 28S sequences for *S. scripta* (AY222174.1) and *S.* cf. *scripta* (KY091666; Roberts et al. 2016c) were 99.4% (8 nucleotide differences) and 99.5% (8 nucleotide differences) similar, respectively, to the new species. The available 18S sequence for *S. scripta* (AY222093) was 99.7% (5 nucleotide differences) similar to the new species. The 28S and 18S sequences of the new species were 99.8% similar (3 nucleotide differences) and identical to AY604704 and AY604712.1, respectively; both of which were previously identified as *Spirorchis artericola* (see Snyder, 2004). We borrowed Snyder's (2004) voucher specimen (HWML 45793) that was linked with those GenBank sequences and found the voucher to have a proportionally wide body, laterally-directed cecal diverticula, deeply lobed testes, a testicular column that nearly fills the intercecal space, and an external seminal vesicle that occupies the space between the posterior-most testis and the ovary and that passes dorsolateral to the ovary. This voucher specimen could be differentiated from the holotype and paratype of *S. artericola* (USNM 1350610) by the features of the testes and external seminal vesicle as well, indicating that the voucher was not conspecific with Ward's (1921) types. Therefore, and see Remarks below, we regard HWML 45793, AY6047704, and AY604712.1 as conspecific with *S. testiplexus* (Table 2).

The 28S phylogenetic analysis showed a strongly supported (posterior probability = 1.0) monophyletic *Spirorchis* sister to *Spirhapalum siamensis* Tkach, Snyder, and Vaughn, 2009. As previously reported (Tkach et al., 2009; Orélis-Ribeiro et al., 2014; Roberts et al., 2016a; c), *Spirhapalum* Ejsmont, 1927 is paraphyletic, with *Spirhapalum polesianum* Ejsmont, 1927 sister to the *Spirorchis* spp. + *S. siamensis* clade.

Remarks

The new species resembles *S. artericola* and *S. innominatus* (type species) by having a median esophageal diverticulum, ceca that extend slightly posterior to the genitalia (to level of the excretory bladder), typically 10 testes, and a testicular column that extends anteriad to near the cecal bifurcation. It further resembles *S. artericola* by having a body that is approximately 3−5× longer than wide and posterior ceca with lateral diverticula along their entire length (Figs. 1, 3). Based on a comparative morphological study of existing type materials and vouchers (see Taxonomic Summary; Specimens Examined) plus published descriptions of similar congeners, *S. testiplexus* is unique by the combination of having a proportionally wider body, laterally-directed cecal diverticula (like *S. haematobius*, *S. elegans*, *S. picta*, *S. minutus*, and *S. kirki*), deeply lobed testes, a testicular column that fills the intercecal space, and an external seminal vesicle that occupies the space between the posterior-most testis and the ovary and that passes dorsal or dorsolateral to the ovary. *Spirorchis artericola* has spheroid or weakly-lobed testes, a testicular column that does not fill the intercecal space, and an external seminal vesicle that is ventral to the ovary. *Spirorchis innominatus* has an elongate body (>6.0× longer than wide) with smooth posterior ceca. Regarding the external seminal vesicle of the new species, it is markedly laterally expanded between the posterior most testis and ovary such that it forms an apparent reservoir that is delimited anteriorly and posteriorly by a testis and the ovary, respectively (Figs. 2, 4). The position of the external seminal vesicle, which perhaps on the outset seems as if a minor feature prone to intraspecific variation, does reliably and consistently differentiate morphologically-similar species of *Spirorchis*. It is either entirely pre-ovarian (*S. minutus*, *Spirorchis collinsi* Roberts and Bullard, 2016), ventral or ventrolateral to the ovary (*S.*

innominatus, *S. artericola*, *S. haematobius*, *S. elegans*, *S. parvus*, *S. picta*, *S. kirki*), or dorsal or dorsolateral to the ovary (*S. scripta*, *S. testiplexus*). Indeed, the taxonomy of *Spirorchis* spp. relies upon subtle morphological differences among species: they are difficult to differentiate. The orientation of the external seminal vesicle, when combined with the presence or absence of other features (i.e., median esophageal diverticulum, diverticulate posterior ceca, testicular column position), reliably differentiates species of *Spirorchis*.

Specimens of *S. scripta* were collected from the same infected river cooter. We identified these turtle blood flukes as *S. scripta* based on the presence of a median esophageal diverticulum, ceca that extend far past the genitalia, testicular column that nearly abuts the cecal bifurcation, 10 spheroid and oblong testes, and an external seminal vesicle dorsal to the ovary. These specimens matched the previous taxonomic descriptions of this taxon (Stunkard, 1923; Platt, 1993) as well as borrowed museum specimens (AMNH 128, 130). In addition to the genetic differences between this taxon and the new species (see above), the latter is distinctive by the combination of having lateral cecal diverticula (vs. smooth ceca in *S. scripta*), ceca that extend slightly posteriad beyond the transverse vitelline duct (vs. extending far posteriad, approximately 13% [paratype, AMNH 130] of body length, beyond transverse vitelline duct), deeply lobed testes (vs. testes with shallow lobes), and vitelline follicles that extend anteriad near level of proximal margin of the median esophageal diverticulum (vs. extending to near the level of the nerve commissure).

This represents the second report of *S. scripta* from the Pascagoula River (Olson et al., 2003), the second report of *S. scripta* from a river cooter (Byrd, 1939), and the first report of *S. scripta* infecting a river cooter from the Pascagoula River (Tables 1, 2).

Previous authors have described the posterior ceca of *Spirorchis* spp. as smooth, simple, or sinuous (Stunkard, 1922; 1923; Platt, 1990; 2002; Roberts et al., 2016c). Based on the results of the present study, we suggest that the diagnosis of *Spirorchis* should include ‘posterior ceca smooth or having lateral diverticula.’

DISCUSSION

A given turtle species may host multiple turtle blood fluke species (Stunkard, 1922; 1928; Byrd, 1939; Ulmer, 1959; Brooks and Mayes, 1975; Roberts et al., 2016c; Roberts and Bullard, 2017) and concurrent infections are not uncommon among turtles (JRR and SAB, personal observations). This includes simultaneous infection by congeneric turtle blood flukes (Ulmer, 1959; Roberts et al., 2016c; Roberts and Bullard, 2017) or turtle blood flukes of different genera (Stunkard, 1922; Brooks and Mayes, 1975). Concurrent infection can complicate correct taxonomic identification and molecular biology. If a given specimen is large enough, a relatively common, convenient, and simple practice is to rely upon a molecular voucher (hologenophore; a specimen that cut into parts such that the same specimen can be used for morphology and molecular biology). If the specimen is minute and cannot be cut in half, we (and others) recommend wet mounting the ethanol-preserved specimen to discern the species-specific differential characters. This is not always straight forward because taxonomists may not be certain of the identity of specimens (i.e., the diagnostic characters of the taxon are indeterminate) upon dissecting the host. If the specimen is destroyed for molecular biology before it can be diagnosed, the identity of the specimen is dubious. Error cascades resulting from the use of GenBank sequences that assume taxonomic identity based upon the host identity, previously published records, gross appearance of the fluke, or the assumption of a non-concurrent infection should be avoided. As demonstrated by the subtly distinctive *Spirorchis* spp., even light microscopic study of a non-stained specimen as a temporary wet-mount may be insufficient to reliably identify the specimen to species. Related to this, the present study serves as an example of a cryptic taxon in the GenBank database and the crucial importance of molecular vouchers. A stand-alone molecular analysis (unaccompanied by morphological comparisons to physical specimens) would have barcoded the new taxon as *S. artericola* and overestimated the level of intraspecific molecular variation for a member of *Spirorchis*. Along those lines, *nomina nuda* GenBank sequences (nonugens), i.e., GenBank sequences that are unaccompanied by robust morphological evidence or a museum-curated voucher specimen that underpins their taxonomic identity, should at minimum be treated with caution or perhaps not used at all in taxonomic and phylogenetic analyses. Authors using GenBank sequences should confirm how specimens were morphologically identified and cite the original work wherein those morphological characters were diagnosed.

The Pascagoula River is the largest undammed river in the continental United States (Dynesius and Nilsson, 1994; Benke and Cushing, 2005). A high diversity of aquatic and semi-aquatic turtles live there (11 genera, 16 species; van Dijk et al., 2014; Powell et al., 2016), including some that are regarded as highly endemic. For example, the Pascagoula map turtle (*Graptemys gibbonsi* Lovich and McCoy, 1992) and the yellowblotched map turtle (*Graptemys flavimaculata* Cagle, 1954) are endemic to the Pascagoula River and its tributaries (Mississippi Natural Heritage Program, 2005; Lindeman, 2013; Selman and Qualls, 2009). Half of the Pascagoula River's turtles are reported as turtle blood fluke hosts elsewhere, and 2 have been reported as hosts from the Pascagoula River: *S. haematobius* infecting the common snapping turtle, *Chelydra serpentina* (Linnaeus, 1758), (see Brooks, 1979) and *S. scripta* infecting the pond slider, *Trachemys scripta* (Thunberg *in* Schoepff, 1792), (see Olson et al., 2003). The Pascagoula River's fishes and amphibians likely harbor relatively pristine parasite assemblages; however, this hopeful condition may not remain as such indefinitely.

The construction of 2 dams is proposed on the lower Pascagoula River that would create 2 large lakes in George County, Mississippi. This massive project is pending public comment and an environmental impact statement from the Corps of Engineers. Dams in the southeastern U.S.A. have caused mass extinctions of particular gastropod snail species that serve as intermediate hosts for digeneans (e.g., Coosa River, northeastern Alabama; Bogan et al. [1995]; Neves et al. [1997]; Johnson et al. [2013]), which obviously have the potential to also extirpate parasite species that require those snails to complete their life cycles. No study has provided before and after data on the effects of such dams on any parasite component community in a Coastal Plain river system. Given the diversity of parasite species that exploit mollusks and the extraordinary diversity of freshwater mollusks that range in the southeastern United States (op cit.), such changes have almost certainly occurred despite our inability to document them a posteriori. The perceived pristine ecological status of the Pascagoula River should be reflected by a correspondingly rich parasite community, perhaps including some endemic parasite species at risk of being extirpated if conditions there change over time due to human influence. This includes endemic host species and their presumptively unique parasite component communities as well as more geographically wide ranging but non-vagile host species that may have parasite component communities unique to the Pascagoula River. Intensive surveys of the parasites of this river system are ongoing (Curran et al., 2007; 2013; Curran and Overstreet, 2009; Tkach et al., 2013; Curran, unpublished data). In fact, parasitological exploration of the Pascagoula River's fauna comprises a large proportion of the published natural history research being conducted there, e.g., a Web of Science search on keyword "Pascagoula River" returned 10 of 73 (14%) total records that comprise parasitology. Parasitological surveys of this ecosystem are important because parasites can indicate the status of ecosystems. It is seemingly inevitable that ecological parameters there will not remain as they are now.

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LITERATURE CITED

Benke, A. C., and C. E. Cushing. 2005. Rivers of North America. Elsevier Academic Press, Burlington, Massachusetts, U.S.A.

Bogan, A. E., J. M. Pierson, and P. Hartfield. 1995. Decline in the freshwater gastropod fauna in the Mobile Bay Basin. Pages 249–252 *in* C. F. Sturm, T. A. Pearce, and A. Valdés, eds. Our living resources, a report to the Nation on the distribution, abundance and health of U.S. plants, animals and ecosystems. U.S. Department of Interior, National Biological Survey, Washington, D.C. 530 p.

Brooks, D. R. 1979. New records for amphibians and reptile trematodes. Proceedings of the Helminthological Society of Washington 46:286–289.

Brooks, D. R., and M. A. Mayes. 1975. Platyhelminths of Nebraska turtles with descriptions of two new species of spirorchiids (Trematoda: Spirorchiidae). Journal of Parasitology 61:403−406.

Byrd, E. E. 1938. Studies on blood flukes of the family Spirorchidae. I. Preliminary Report. Journal of the Tennessee Academy of Science 13:133−136.

Byrd, E. E. 1939. Studies on the blood flukes of the family Spirorchidae. Part II. Revision of the family and description of new species. Journal of the Tennessee Academy of Science 14:116−161.

Curran, S. S. and R. M. Overstreet. 2009. *Caecincola longiscens* n. sp. (Digenea: Cryptogonimidae) from the white crappie, *Pomoxis annularis,* in Mississippi, USA. Comparative Parasitology 76(1): 19-23.

Curran, S. S., R. M. Overstreet, and V. V. Tkach. 2007. Phylogenetic affinities of *Plagiocirrus* van Cleave and Mueller, 1932 with the description of a new species from the Pascagoula River, Mississippi. Journal of Parasitology 93(6): 1,452-1,458.

Curran, S. S., V. V. Tkach, R. M. Overstreet. 2013. Molecular evidence of two cryptic species of *Homalometron* (Digenea: Apocreadiidae) in freshwater fishes of the southeastern United States. Comparative Parasitology 80(2): 186-195.

Dynesius, M., and C. Nilsson. 1994. Fragmentation and flow regulation of river systems in the northern third of the world. Science 266:753−762.

Ernst, E. M., and C. H. Ernst. 1975. New hosts and localities for turtle helminths. Proceedings of the Helminthological Society of Washington 42:176−178.

Ernst, C. H., and J. E. Lovich. 2009. Turtle of the United States and Canada, 2nd ed. The Johns Hopkins University Press, Baltimore, Maryland, U.S.A. 827 pp.

Guyer, C., M. A. Bailey, and R. H. Mount. 2015. Turtles of Alabama, 1st ed. The University of Alabama Press, Tuscaloosa, Alabama, U.S.A. 266 pp.

Harwood, P. D. 1931. Parasites of Oklahoma turtles. Journal of Parasitology 18:98−101.

Johnson P. D., A. E. Bogan, K. M. Brown, N. M. Burkhead, J. R. Cordeiro, J. T. Garner, P. D. Hartfield, D. A. W. Lepitzki, G. L. Mackie, E. Pip, T. A. Tarpley, J. S. Tiemann, N. V. Whelan, and E. E. Strong. 2013. Conservation status of freshwater gastropods of Canada and the United States. Fisheries 38:247−282.

Katoh, K., and D. M. Standley. 2013. MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability. Molecular Biology and Evolution 30:772−780.

Lindeman, P. V. 2013. The map turtle and sawback atlas: ecology, evolution, distribution, and conservation. The University of Oklahoma Press, Norman, Oklahoma, U.S.A.460 p.

Maddison, W. P., and D.R. Maddison. 2017. Mesquite: a modular system for evolutionary analysis. Version 3.2. http://mesquiteproject.org.

Mississippi Natural Heritage Program. 2015. Listed species of Mississippi. Museum of Natural Science, Mississippi Department of Wildlife, Fisheries, and Parks, Jackson, Mississippi, U.S.A. 3 p.

Neves, R. J., A. E. Bogan, J. D. Williams, S. A. Ahlstedt, and P. D. Hartfield. 1997. Status of aquatic mollusks in the southeastern United States: a downward spiral of diversity. Pages 43−85 *in* G. W. Benz, and D. E. Collins, eds. Aquatic fauna in peril: the southeastern perspective. Southeast Aquatic Research Institute, Special Publication 1, Chattanooga, Tennessee, U.S.A., 554 p.

Olson, P. D., T. H. Cribb, V. V. Tkach, R. A. Bray, and D. T. J. Littlewood. 2003. Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda). International Journal for Parasitology 33:733–755.

Orélis-Ribeiro, R, C. R. Arias, K. M. Halanych, T. H. Cribb, and S. A. Bullard. 2014. Diversity and ancestry of flatworms infecting blood of nontetrapod craniates "fishes." Advances in Parasitology 85:1−64.

Platt, T. R. 1990. *Aphanospirorchis kirki* n. gen., n. sp. (Digenea: Spirorchidae), a parasite of the midland painted turtle, *Chrysemys picta marginata*, from northwestern Indiana, with comments on the proper spelling of the family name. Journal of Parasitology 76:650−652.

Platt, T. R. 1993. Taxonomic revision of *Spirorchis* MacCallum, 1919 (Digenea: Spirorchidae). Journal of Parasitology 79:337−346.

Platt, T. R. 2002. Family Spirorchiidae Stunkard, 1921. Pages 453-468 *in* D. I. Gibson, A. J. Jones, and R. A. Bray, eds. Keys to the Trematoda, Vol. 1. CABI Publishing, Wallingford, Oxford, U.K., 521 p.

Platt, T. R., and A. K. Prestwood. 1990. Deposition of type and voucher material from the helminthological collection of Elon E. Byrd. Systematic Parasitology 16:27−34.

Powell, R., R. Conant, and J. T. Collins. 2016. Peterson field guide to reptiles and amphibians of Eastern and Central North America, fourth edition. Houghton Mifflin Harcourt Publishing Company, New York, New York, U.S.A., 494 p.

Rambaut, A. 2009. FigTree v1.2.3, Institute of Evolutionary Biology, Univ. of Edinburgh, available at: http://tree.bio.ed.ac.uk/software/figtree.

Roberts, J. R., and S. A. Bullard. 2017. Revision and new species of *Vasotrema* Stunkard, 1926 (Digenea: Schistosomatoidea): turtle blood flukes of North American softshell turtles (Testudines: Trionychidae: *Apalone* spp.). Journal of Parasitology XX:XX−XX.

Roberts, J. R., T. R. Platt, R. Orélis-Ribeiro, and S. A. Bullard. 2016a. New genus of blood fluke (Digenea: Schistosomatoidea) from Malaysian freshwater turtles (Geoemydidae) and its phylogenetic position within Schistosomatoidea. Journal of Parasitology 102:451−462.

Roberts, J. R., R. Orélis-Ribeiro, B. T. Dang, K. M. Halanych, and S. A. Bullard. 2016b. Blood flukes of Asiatic softshell turtles: revision of *Coeuritrema* Mehra, 1933 (Digenea: Schistosomatoidea) and a new species infecting Chinese softshell turtles, *Pelodiscus sinensis*, (Trionychidae) from the Da Rang River, Vietnam. Folia Parasitologica 63:031 doi:10.14411/fp.2016.031.

Roberts, J. R., R. Orélis-Ribeiro, K. M. Halanych, C. R. Arias, and S. A. Bullard. 2016c. A new species of *Spirorchis* MacCallum, 1918 (Digenea: Schistosomatoidea) and *Spirorchis* cf. *scripta* from chicken turtle, *Deirochelys reticularia*, (Emydidae), with an emendation and molecular phylogeny of *Spirorchis*. Folia Parasitologica 63:041 doi:10.14411/fp.2016.041.

Roberts, J. R., K. M. Halanych, C. R. Arias, B. Folt, J. M. Goessling, and S. A. Bullard. 2017. Emendation and new species of *Hapalorhynchus* Stunkard, 1922 (Digenea: Schistosomatoidea) from musk turtles (Kinosternidae: *Sternotherus*) in Alabama and Florida rivers. Parasitology International *in review*.

Ronquist, F., M. Teslenko, P. van der Mark, D. Ayres, A. Darling, S. Höhna, B. Larget, L. Liu, M. A. Suchard, and J. P. Huelsenbeck. 2011. MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61:539–542.

Selman, W., and C. Qualls. 2009. Distribution and abundance of two imperiled *Graptemys* species of the Pascagoula River system. Herpetological Conservation and Biology 4:171−184.

Snyder, S. D. 2004. Phylogeny and paraphyly among tetrapod blood flukes (Digenea: Schistosomatidae and Spirorchiidae). International Journal for Parasitology 34:1385−1392.

Stunkard, H. W. 1922. Two new genera of North American blood flukes. American Museum Novitates 39:1−8.

Stunkard, H. W. 1923. Studies on North American blood flukes. Bulletin of the American Museum of Natural History 48:165−221.

Stunkard, H. W. 1928. Observations nouvelles sur les trématodes sanguicoles du genre *Vasotrema* (Spirorchidae) avec description des deux espèces nouvelles. Annales de Parasitologie Humaine et Comparee 6:303−320.

Teehan, W. H., and R. B. Short. 1989. Mitotic chromosomes of a species of *Spirorchis* (Trematoda: Spirorchiidae). Journal of Parasitology 75:474−476.

Tkach, V. V., S. S. Curran, J. A. Bell, and R. M. Overstreet. 2013. A new species of *Crepidostomum* (Digenea: Allocreadiidae) from *Hiodon tergisus* in Mississippi and molecular comparison with three congeners. Journal of Parasitology 99(6):1114-1121.

Tkach V. V., S. D. Snyder, and J. A. Vaughan. 2009. A new species of blood fluke (Digenea: Spirorchiidae) from the Malayan box turtle, *Cuora amboinensis* (Cryptodira: Geoemydidae) in Thailand. Journal of Parasitology 95:743−746.

Ulmer, M. J. 1959. Studies on *Spirorchis haematobium* (Stunkard, 1922) Price, 1934 (Trematoda: Spirorchiidae) in the definitive host. Transactions of the American Microscopical Society 78:81−89.

van Dijk, P. P., J. B. Iverson, A. G. J. Rhodin, H. B. Shaffer, and R. Bour. 2014. Turtles of the world, 7th Edition: Annotated checklist of taxonomy, synonymy, distribution with maps, and conservation status. Chelonian Research Monographs 5:329−479.

FIGURE LEGENDS

Figures 1, 2. Ventral view of adult of *Spirorchis testiplexus* n. sp. from river cooter, *Pseudemys concinna* (Le Conte, 1830), from the Pascagoula River (30°37'07.67"N, 88°36'44.53"W; Mississippi, U.S.A.). Scale values aside each bar. **1.** Holotype (National Museum of National History, Smithsonian Department of Invertebrate Zoology [USNM] XXXX) showing oral sucker (os), pharynx (ph), nerve commissure (nc), ventrolateral nerve chords (vln), esophagus (es), esophageal gland (eg), vitellarium (vr), median esophageal diverticulum (med), sinistral posterior cecum (sc), dextral posterior cecum (dc), anterior testis (at), vas deferens (vd), middle testis (t5), posterior-most testis (pt), external seminal vesicle (esv), ovary (ov), common genital pore (cgp), transverse vitelline duct (tvd), cecal termini (ct), Manter's organ (Mo), excretory vesicle (ev), and excretory pore (ep). **2.** Genitalia of holotype (USNM XXXX) showing ninth testis (t9), vas deferens (vd), posterior-most testis (pt), external seminal vesicle (esv), ovary (ov), internal seminal vesicle (isv), cirrus (ci), common genital pore (cgp), cirrus sac (cs), oviduct (od), oviducal seminal receptacle (osr), uterine egg (egg), metraterm (mt), egg chamber (ec), transverse vitelline duct (tvd), primary vitelline duct (vt), and Laurer's canal (Lc).

Figures 3, 4. Dorsal view of adult of *Spirorchis testiplexus* n. sp. from river cooter, *Pseudemys concinna* (Le Conte, 1830), from the Pascagoula River (30°37'07.67"N, 88°36'44.53"W; Mississippi, U.S.A.). Scale values aside each bar. **3.** Paratype (National Museum of National History, Smithsonian Department of Invertebrate Zoology [USNM] XXXX) showing oral sucker (os), pharynx (ph), nerve commissure (nc), ventrolateral nerve chords (vln), esophagus (es), esophageal gland (eg), vitellarium (vr), dextral posterior cecum (dc), median esophageal diverticulum (med), sinistral posterior cecum (sc), anterior testis (at), middle testis (t5), posterior-most testis (pt), external seminal vesicle (esv), ovary (ov), common genital pore (cgp), transverse vitelline duct (tvd), cecal termini (ct), Manter's organ (Mo), excretory vesicle (ev), and excretory pore (ep). **4.** Genitalia of paratype (USNM XXXX) showing ninth testis (t9), vas deferens (vd), posterior-most testis (pt), external seminal vesicle (esv), ovary (ov), internal seminal vesicle (isv), cirrus sac (cs), cirrus (ci), oviduct (od), oviducal seminal receptacle (osr), uterine egg (egg), common genital pore (cgp), metraterm (mt), egg chamber (ec), transverse vitelline duct (tvd), Laurer's canal (Lc), and primary vitelline duct (vt).

Figure 5. Phylogenetic relationships of selected blood flukes reconstructed by Bayesian inference and based on partial D1−D3 domains of the large subunit ribosomal DNA (28S) sequences (16 turtle blood fluke species). Numbers aside tree nodes indicate posterior probability (only values >0.95 shown).

Table 1. *Spirorchis* spp. infecting river cooters, *Pseudemys concinna* (Le Conte, 1830) (Emydidae)

***Spirorchis* sp.**		**Site in host**	**Riverine locality**	**Accession nos.**	**Reference**
Spirorchis artericola Ward, 1921	as *Pseudemys hieroglyphica* Holbrook, 1836	blood (adult)	Reelfoot Lake (Mississippi River) (36°21'12.23"N, 89°25'21.50"W), Tennessee, U.S.A.	USNM 80643, 80644; HWML 31115	Byrd (1938; 1939)
Spirorchis elegans Stunkard, 1923	as *Pseudemys elonae* (Brimley, 1928)	blood (adult)	Either the Neuse or Cape Fear River, Durham, North Carolina, U.S.A.	University of Maryland, Department of Zoology No. 790	Ernst and Ernst (1975)
***Spirorchis testiplexus*, n. sp.**	***P. concinna***	**mesentery (adult)**	**Pascagoula River (30°37'07.67"N, 88°36'44.53"W), Mississippi, U.S.A.**	**USNM XXXX**	**Present study**
Spirorchis innominatus Ward, 1921 (type species)	as *P. hieroglyphica*	blood (adult); intestine (eggs)	Lake Talawanda (Canadian River) (34°58'44.57"N, 95°47'36.96"W), McAlester, Oklahoma, U.S.A.	USNM 30867	Harwood (1931)
	as *Pseudemys concinna suwanniensis* Carr, 1937	blood (adult)	Natural Bridge (St. Mark's River) (30°17'3.74"N, 84° 9'4.45"W), Florida, U.S.A.	Not specified	Teehan and Short (1989)
Spirorchis scripta Stunkard, 1923	as **P. hieroglyphica*	blood (adult)	Reelfoot Lake (36°21'12.23"N, 89°25'21.50"W), Tennessee.	USNM 80649; HWML 31115	Byrd (1938; 1939); Platt (1993); Platt and Prestwood

					(1990)
	P. concinna	**heart (adult)**	**Pascagoula River (30°37'07.67"N, 88°36'44.53"W), Mississippi.**	**USNM XXXX**	**Present study**

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*Byrd (1939) reported *S. scripta* and described *Spirorchis blandingoides* Byrd, 1939, from *P. concinna* (as *P. hieroglyphica*). We accept *S. blandingoides* as a junior subjective synonym of *S. scripta* (see Platt, 1993).

Table 2. Turtle blood fluke sequences (28S and 18S) used in the present study.

Turtle blood flukes	Host species	Locality	GenBank Accession Numbers		Reference
			28S	18S	
Baracktrema obamai Roberts, Platt and Bullard, 2016	*Siebenrockiella crassicollis* (Gray, 1830), black marsh turtle	Perak (probably Perak River), Malaysia	KX061500		Roberts et al. (2016a)
Spirhapalum polesianum Ejsmont, 1927	*Emys orbicularis* (Linnaeus, 1758), European pond turtle	Lesniki, Ukraine	AY604705		Snyder (2004)
Spirhapalum siamensis Tkach, Snyder and Vaughn, 2009	*Cuora amboinensis* (Riche *in* Daudin, 1801), Southeast Asian pond turtle	Moei River (16°42'N, 98°34'E), Mae Sot, Thailand	FJ481166		Tkach et al. (2009)
***Spirorchis testiplexus*, n. sp. (as *Spirorchis artericola* [Ward, 1921] Stunkard, 1921)**	***Chrysemys picta* (Schneider, 1783), painted turtle**	**Reelfoot Lake (Mississippi River) (36°21'12.23"N, 89°25'21.50"W), Tennessee, U.S.A**	**AY604704**	**AY604712.1**	**Snyder (2004)**
S. testiplexus	***Pseudemys concinna* (Le Conte, 1830), river cooter**	**Pascagoula River (30°37'07.67"N, 88°36'44.53"W), Mississippi, U.S.A.**	**XXXXX**	**XXXXX**	**Present study**
Spirorchis collinsi	*Deirochelys reticularia* (Latreille *in* Sonnini and Latreille, 1801), chicken turtle	Big Beaver Pond (Tallapoosa River) (32°25'44.03"N, 85°38'44.87"W), Alabama, U.S.A.	KY091664		Roberts et al. (2016c)
Spirorchis haematobius (Stunkard, 1922) Price, 1934	*Chelydra serpentina* (Linnaeus, 1758), common snapping	Missouri River, Iowa, U.S.A.	FJ481164		Tkach et al. (2009)

	turtle			
S. haematobius	***C. serpentina***	**Pascagoula River (30°37'07.67"N, 88°36'44.53"W), Mississippi.**	**XXXXX**	**Present study**
Spirorchis picta Stunkard, 1923	*Trachemys scripta* (Thunberg *in* Schoepff, 1792), pond slider	Pond off Saugahatchee Creek (Tallapoosa River) (32°38'52.78"N, 85°29'7.20"W), Alabama.	KY091665	Roberts et al. (2016c)
Spirorchis scripta Stunkard, 1923	*T. scripta*	Moungers Creek (Pascagoula River), Mississippi.	AY222174	Olson et al. (2003)
Spirorchis cf. *scripta*	*D. reticularia*	Big Beaver Pond (Tallapoosa River) (32°25'44.03"N, 85°38'44.87"W), Alabama.	KY091666	Roberts et al. (2016c)
Unicaecum sp.	*T. scripta*	Reelfoot Lake (Mississippi River) (36°21'12.23"N, 89°25'21.50"W), Tennessee.	AY604711	Snyder (2004)
Vasotrema robustum Stunkard, 1928	*Apalone spinifera* (LeSueur, 1827), spiny softshell turtle	Nishnabotna River, Iowa.	AY604706	Snyder (2004)
Vasotrema* cf. *robustum	***Apalone spinifera aspera* (Agassiz, 1857), Gulf Coast spiny softshell turtle**	**Round Lake (Cahaba River) (32°41'50.91"N, 87°14'30.39"W), Cahaba River, Alabama.**	**XXXXX**	**Present study**

*Only 28S sequences used in the phylogenetic analysis

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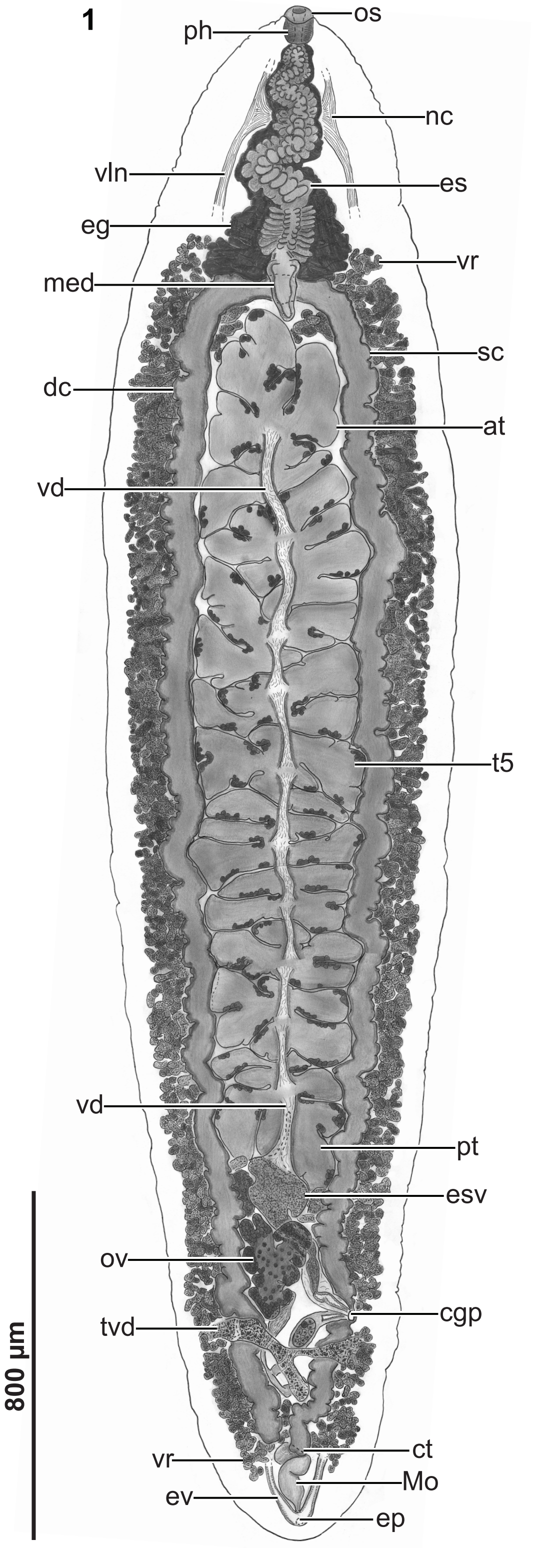
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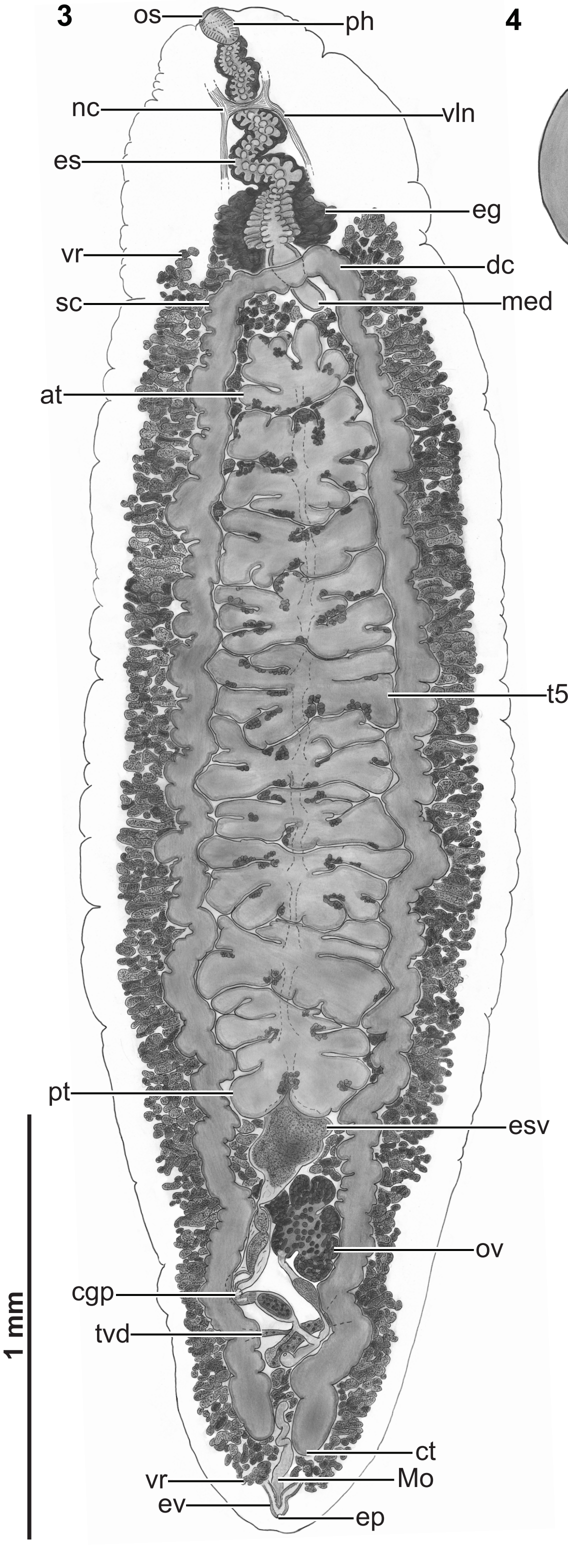
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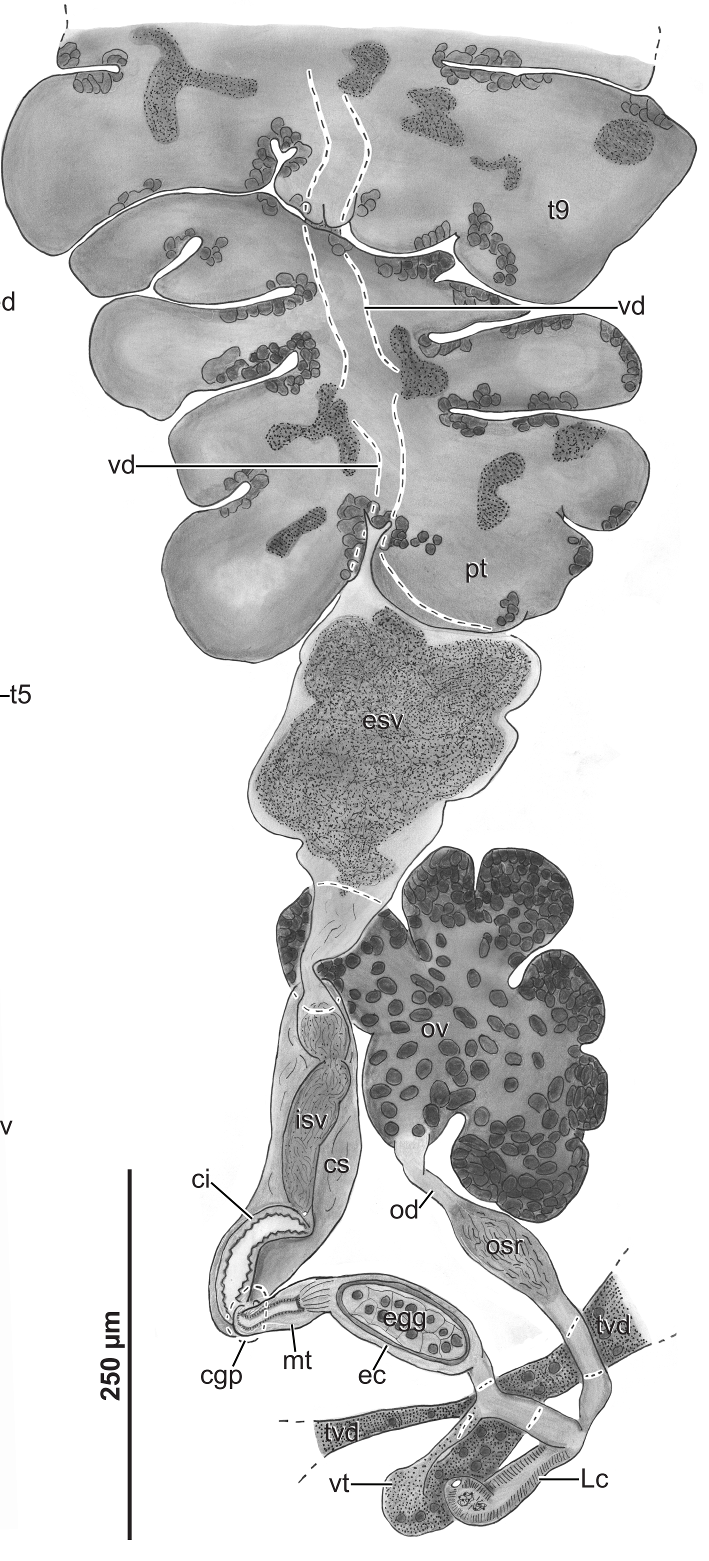
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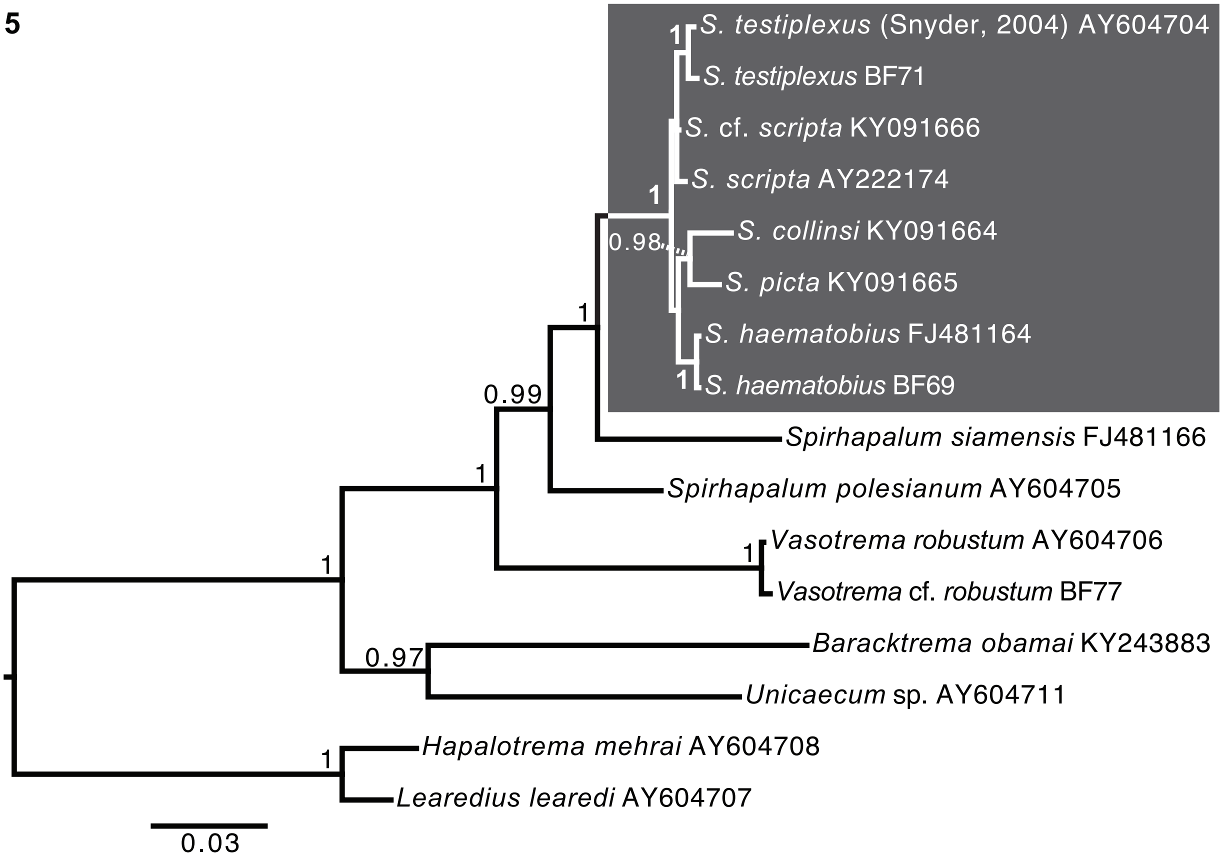

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S. testiplexus BF71
S. cf. scripta KY091666
S. scripta AY222174
S. collinsi KY091664
S. picta KY091665
S. haematobius FJ481164
S. haematobius BF69
Spirhapalum siamensis FJ481166
Spirhapalum polesianum AY604705
Vasotrema robustum AY604706
Vasotrema cf. robustum BF77
Baracktrema obamai KY243883
Unicaecum sp. AY604711
Hapalotrema mehrai AY604708
Learedius learedi AY604707
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CHAPTER 7: NEW GENUS AND SPECIES OF TURTLE BLOOD FLUKE (DIGENEA: SCHISTOSOMATOIDEA) FROM THE MEKONG SNAIL-EATING TURTLE, *MALAYEMYS SUBTRIJUGA* (SCHLEGEL & MÜLLER), (TESTUDINES: GEOEMYDIDAE) FROM VIETNAM, WITH A REASSESSMENT OF RELATED ASIATIC TURTLE BLOOD FLUKES AND MOLECULAR PHYLOGENY

***Formatted for submission to Systematic Parasitology**

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ABSTRACT

Platt sinuosus Roberts & Bullard n. g., n. sp. (type species) infects kidney and mesenteric blood vessels of Mekong snail-eating turtles, *Malayemys subtrijuga* (Schlegel & Müller), in the Mekong River Basin. *Platt* is unique by the combination of having a papillate ventral sucker, vasa efferentia that are dorsal to the gonads, an obvious, massive cirrus sac that is directed anteriad or laterad, and a vitellarium that surrounds the intestinal caeca. The new species resembles *P. ocadiae* but differs from it by having an external seminal vesicle that overlaps or is immediately posterior to the ventral sucker. Seven species of *Hapalorhynchus* herein are reassigned to *Platt* (*P. odhnerensis* [Mehra, 1933] n. comb., *P. yoshidai* [Ozaki, 1939] n. comb., *P. ocadiae* [Takeuti, 1942] n. comb., *P. oschmarini* [Belous, 1963] n. comb., *P. sutlejensis* [Mehrotra, 1973] n. comb., *P. synderi* [Platt & Sharma, 2012] n. comb., and *P. tkachi* [Platt & Sharma, 2012] n. comb.), and a dichotomous key to *Platt* spp. is provided. *Hapalorhynchus sheilae* (Mehrotra, 1973) Bourgat, 1990 and *Hapalorhynchus mica* (Oshmarin, 1971) Bourgat, 1990 are *species inquirendae*, and *Hapalorhynchus indicus* (Thapar, 1933) Price, 1934 and *Hapalorhynchus macrotesticularis* (Rohde, Lee, & Lim, 1968) Brooks & Sullivan, 1981 are *incertae sedis*. Sequence analysis of the large

subunit rDNA (28S) showed a strongly supported monophyletic Hapalorhynchus Stunkard, 1922 and monophyletic *Platt*; however, interrelationships between *Coeuritrema*, *Hapalorhynchus*, and *Platt* are still unresolved, and more sampling is still needed from this undiagnosed blood fluke lineage.

INTRODUCTION

Hapalorhynchus Stunkard, 1922 (Digenea: Schistosomatoidea) currently includes 20 nominal species that infect freshwater turtles of North America (Cryptodira: Chelydridae, Kinosternidae), Asia (Trionychidae, Geoemydidae), and Africa (Pleurodira: Pelomedusidae). As such, it is the only genus of freshwater turtle blood flukes (TBFs hereafter) that includes species infecting turtles on more than one continent (Smith, 1997a; b; Platt, 2002; Platt & Sharma 2012; Roberts et al., 2016b; 2017). Eleven of those 20 species were described from Asiatic softshell turtles (Trionychidae) or pond turtles (Geoemydidae) but none have been reported since their original description. An opportunistic examination of two butchered Mekong snail-eating turtles, *Malayemys subtrijuga* (Schlegel & Müller), (Testudines: Geoemydidae) from a market in Can Tho, Vietnam, revealed infections by two turtle blood flukes: *Hapalorhynchus snyderi* Platt & Sharma, 2012 and a new species. Herein, we propose a new genus to accommodate the new species and seven species formerly of *Hapalorhynchus*, provide a dichotomous key to all species of the new genus, and provide a phylogenetic reconstruction (based on the large subunit ribosomal DNA [28S] and internal transcribed region 2 [ITS2]) for species of *Hapalorhynchus* and *Coeuritrema platti* Roberts & Bullard, 2016.

MATERIALS AND METHODS

In November 2015, during a parasitological survey of aquatic vertebrates in the Mekong River Basin, two butchered individuals of the Mekong snail-eating turtle were opportunistically sampled from a market in Can Tho, Vietnam (10°01’42.15”N, 105°47’14.15”E). Flukes intended for morphology were killed, stained, and whole-mounted as per Roberts et al. (2017) and illustrated with the aid of Leica DM 2500 and Leica DMR (Leica, Wetzler, Germany) microscopes each equipped with differential interference contrast (DIC) optical components, an ocular micrometer, and a drawing tube. Measurements of TBF specimens are reported in micrometres (μm) followed by the mean and number of specimens measured in parentheses. Turtle scientific and common names follow van Dijk et al. (2014). Classification and anatomical terms for TBFs follow Roberts et al. (2016a; b; c) and Roberts et al., (2017) except that “ventrolateral tegumental papillae” of Roberts et al. (2016b) is replaced by “ventrolateral tegumental mamillae.”

Specimens intended for molecular biology were placed directly in 95% non-denatured ethanol (EtOH). Total genomic DNA (gDNA) was extracted using DNeasyTM Blood and Tissue Kit (Qiagen, Valencia, California, USA) according to the manufacturer’s protocol except that the incubation period with proteinase-K was extended to overnight and that the final elution step was performed using only 100 μL of elution buffer to increase the final DNA concentration. The partial 28S rDNA (domains D1–D3; ~1,400 bp) was amplified using the forward primer “U178” (5’-GCA CCC GCT

GAA YTT AAG-3’) and the reverse primer “L1642” (5’-CCA GCG CCA TCC ATT TTC A-3’) (Lockyer et al., 2003). The internal transcribed region 2 (ITS2 rDNA) was amplified using the forward primer “GA1” (5’-AGA ACA TCG ACA TCT TGA AC-3’; Anderson & Barker, 1998) and the reverse primer "ITS2.2" (5’-CCT GGT TAG TTT CTT TTC CTC CGC-3’; Cribb et al., 1998). The PCR amplifications were performed using a total volume of 50 µl with 2 µl of DNA template, 0.4 µM of each primer along with 1× buffer, 2.5 mM $MgCl_2$, 1 mM dNTP mixture, and 0.3 µl Taq polymerase (5 U/µl) (Promega, Madison, Wisconsin, USA). The thermocycling profile comprised an initial 5 min at 95°C for denaturation, followed by 40 repeating cycles of 94°C for 30 s for denaturation, 63°C for 30 s for annealing, and 72°C for 2 min for extension, followed by a final five min at 72°C for extension. All PCR reactions were carried out in a MJ Research PTC-200 (BioRad, Hercules, California, USA). PCR products (10 µl) were verified on a 1% agarose gel and stained with ethidium bromide. PCR products were purified by microcentrifuge with the QIAquick PCR Purification Kit (Qiagen, Valencia, California, USA) according to manufacturer's protocol, except that the last elution step was performed with autoclaved nanopure H_2O rather than the provided buffer. DNA sequencing was performed by ACGT, Incorporated (Wheeling, Illinois, USA). Reactions were sequenced using BigDye terminator version 3.1, cleaned-up with magnetic beads (CleanSeq dye terminator removal kit), and analyzed using ABI 3730 XL or 3730 Genetic Analyzer. Primers used in sequencing of 28S rDNA included the PCR primers and the reverse primer 1200R (5’-GCA TAG TTC ACC ATC TTT CGG-3’) (Lockyer et al., 2003). Sequence assembly and analysis of chromatograms were performed with BioNumerics version 7.0 (Applied Maths, Saint-Martens-Latem, Belgium). Sequence alignment and phylogenetic analyses follow Roberts et al. (2017). Sequences incorporated in phylogenetic analyses are presented in Table 3.

Superfamily Schistosomatoidea Stiles & Hassall, 1898

***Platt* Roberts & Bullard n. g.**

Diagnosis

Schistosomatoidea. Body dorsoventrally flat (not cylindrical), 3.7−12.0× longer than wide; body constriction at level of ventral sucker present or absent; hindbody 1.6−3.3× longer than forebody, aspinous; ventral body papillae not observed; ventrolateral tegumental mamillae absent. Oral sucker robust, demarcated from body by constriction; oral sucker spines absent; oral sucker papillae absent. Ventral sucker papillate, 1/4−1/2 of body width in diameter, aspinous. Pharynx present, enveloping anterior extremity of oesophagus. Oesophagus long, extending posteriad 1/6−1/4 of body length, ventral to anterior nerve commissure; lateral oesophageal diverticula absent; median oesophageal diverticulum absent; oesophageal gland surrounding oesophagus from pharynx to caecal bifurcation. Intestine comprising non-fused posterior caeca bifurcating immediately anterior to ventral sucker, smooth, lacking diverticula; anterior caeca absent; posterior caeca inverse U-shaped, extending 1/2−3/4 of body length directly posteriad, not extensively convoluted, terminating in posterior body extremity. Testes comprising one anterior and one posterior testis in posterior 2/3 of body, intercaecal, smooth or lobed. Male terminal genitalia pre-gonadal. Vas deferens ventral to cirrus sac; anterior and posterior trunks of vasa efferentia dorsal to gonads; external seminal vesicle posterior to ventral sucker, intercaecal, extending anteriad to level of genital pore or level of ventral sucker; internal seminal vesicle present; pars prostatica difficult to discern, enveloping distal extremity of internal seminal vesicle when present. Cirrus sac robust, extending anteriad (at 45° angle to or parallel with body margin) or laterad (perpendicular to body margin). Ovary sinistral, intercaecal, intertesticular. Oviduct emerging from dextral margin of ovary, extending directly posteriad or sinistrad; oviducal seminal receptacle comprising middle portion of oviduct between ovary and posterior testis. Laurer's canal intercaecal, intertesticular; Laurer’s canal pore dorsal. Vitellarium comprising large follicles, surrounding caeca (dorsal, ventral, and lateral), distributing between oesophagus and excretory vesicle; transverse vitelline duct intertesticular, ventral or dorsal to gonads, comprising lateral collecting ducts ventral or dorsal to caeca. Oötype longitudinal, intercaecal. Mehlis’ gland not observed. Uterus intercaecal, straight, lacking coils or extensive convolutions, difficult to discern from oötype, extending anteriad, dorsal to ovary; metraterm difficult to discern from uterus, anterior to ovary, sinistral to anterior testis. Uterine pouch absent. Uterine egg single, tricornute or ovoid (with or without filaments), occupying oötype and uterus proximal to metraterm. Common genital pore sinistral, dorsal, between sinistral cecum and body margin (lateral, extracaecal). Excretory vesicle distinctly Y-shaped or sinuous (lacking multiple laterally-directed lobes). Manter's organ absent. Excretory pore terminal. Blood and tissue parasites of Asiatic softshells (Trionychidae) and pond turtles (Geoemydidae).

Differential Diagnosis: Body dorsoventrally flat (not cylindrical), 3.7−12.0× longer than wide. Ventral sucker papillate, 1/4−1/2 of body width in diameter. Oesophagus long, extending posteriad 1/6−1/4 of body length. Posterior caeca inverse U-shaped. Testes comprising a single anterior testis and single posterior testis in posterior 2/3 of body. Male terminal genitalia pre-gonadal. Vas deferens ventral to cirrus sac; anterior and posterior trunks of vasa efferentia dorsal to gonads. Cirrus sac robust, extending anteriad (at 45° angle to or parallel with body margin) or laterad (perpendicular to body margin). Vitellarium surrounding caeca (dorsal, ventral, and lateral). Common genital pore sinistral, dorsal.

Type-species: P. sinuosus Roberts & Bullard. *Other species*: *Platt odhnerensis* (Mehra, 1933) n. comb., *P. yoshidai* (Ozaki, 1939) n. comb., *P. ocadiae* (Takeuti, 1942) n. comb., *P. oschmarini* (Belous, 1963) n. comb., *P. sutlejensis* (Mehrotra, 1973) n. comb., *P. snyderi* (Platt & Sharma, 2012) n. comb., and *P. tkachi* (Plat & Sharma, 2012) n. comb.

Etymology: Platt honors Dr. Thomas R. Platt (Professor Emeritus, St. Mary's College, Indiana, USA) for his contributions to turtle blood fluke taxonomy and systematics.

Platt sinuosus **Roberts & Bullard n. sp.**

Type-host: Malayemys subtrijuga (Schlegel & Müller), Mekong snail-eating turtle.

Type-locality: Mekong River (a market in Can Tho, Vietnam; 10°01’42.15”N, 105°47’14.15”E).

Type-material and sequences deposited: Holotype (USNM XXXX); paratypes (USNM XXXX, XXXX); 28S sequence (GenBank No. XXXXX); ITS2 sequence (GenBank No. XXXXX).

Type-materials examined: Holotype of *Hapalorhynchus gracilis* Stunkard, 1922 (AMNH 125); holotype of *Hapalorhynchus stunkardi* Byrd, 1939 (USNM 1321967); holotype of *Hapalorhynchus reelfooti* Byrd, 1939 (USNM 1321968); vouchers of *H. reelfooti* (USNM 1393855, 1393857); vouchers of *Hapalorhynchus foliorchis* Brooks and Mayes, 1975 (USNM XXXX, XXXX, XXX); holotype of *Hapalorhynchus brooksi* Platt, 1988 (USNM 1375720); holotype of *P*. *snyderi* (as *Hapalorhynchus snyderi*; USNM 105194); holotype of *P*. *tkachi* (as *Hapalorhynchus tkachi*; USNM 105196); holotype of *Hapalorhynchus conecuhensis* Roberts and Bullard, 2017 (USNM XXXX); paratypes of *H. conecuhensis* (USNM XXXX).

Site in host: Kidney and mesenteric blood vessels.

Prevalence and intensity: Two Mekong snail eating turtles were infected with 4 and 13 specimens of the new species.

Etymology: The Latin specific epithet *sinuosus* refers to the elongate, S-shaped cirrus sac.

Description (Figs. 1A, 1B)

Adult [Based on 10 whole-mounted specimens; USNM coll. nos. XXXX and XXXX.] Body 1,130−1,420 (1,250; 10) long or 9.2−11.4× (10.1; 10) longer than wide, 75−107 (93; 10) wide or 6−8% (8%; 10) of body length at level of caecal bifurcation, 89−114 (102; 10) wide or 7−9% (8%; 10) of body length at level of ventral sucker, 95−136 (115; 10) wide or 8−11% (9%; 10) of body length at level of genital pore, 105−139 (123; 10) wide or 9−11% (10%; 10) of body length at level of ovary, 86−109 (99; 10) wide or

7−9% (8%; 10) of body length at level of caecal termini; forebody (middle of ventral sucker to anterior body end) 322−438 (369; 10) long or 26−31% (29%; 10) of body length; hindbody (middle of ventral sucker to posterior body end) 798−982 (882; 10) long or 69−74% (71%; 10) of body length or 2.2−2.8× (2.4; 10) longer than forebody; small ventral body papillae not observed. Oral sucker 43−68 (52; 10) long or 4−5% (4%; 10) of body length, 43−64 (50; 10) wide or 45−70% (53%; 10) of body width at level of caecal bifurcation; oral sucker spines not observed; papillae not observed. Ventral sucker 34−68 (58; 10) long or 3−6% (5%; 10) of body length or 0.7−1.4× (1.1; 10) longer than oral sucker, 52−77 (62; 10) wide or 53−75% (61%; 10) of body width at level of ventral sucker or 1.1−1.5× (1.3; 10) wider than oral sucker (Fig. 1A). Nerve commissure 155−202 (172; 4) or 12−16% (13%; 4) of body length from anterior body end. Pharynx 34−52 (42; 10) or 14−20% (17%; 10) of oesophagus length, 25−45 (36; 10) wide or 0.9−1.7× (1.4; 10) wider than maximum oesophagus width. Oesophagus 216−272 (245; 10) long or 18−22% (20%; 10) of body length, 5−27 (15; 9) wide posterior to pharynx, with wall 4 (1) thick, 18−34 (26; 10) wide or 19−37% (28%; 10) of body width at mid-esophagus, with wall 9−19 (12; 8) thick, 18−34 (26; 10) wide or 19−35% (28%; 10) of body width at caecal bifurcation, with wall 22 and 27 (2) thick; oesophageal gland 193 and 198 (2) long or 15% and 18% (2) of body length, 55 and 75 (2) wide or 58% and 70% (2) of body width at level of caecal bifurcation. Intestine bifurcating 236−307 (267; 10) or 19−24% (21%; 10) of body length from anterior body end; sinistral posterior caecum 685−1,070 (792; 10) long or 58−75% (63%; 10) of body length, 32−45 (39; 8) wide or 34−60% (43%; 8) of body width at level of caecal bifurcation, 16−20 (17; 5) wide or 12−16% (14%; 5) of body width at level of ovary, 11−25 (19; 10) wide or 13−23% (19%; 10) of body width at terminus; dextral posterior cecum 705−1,105 (809; 10) long or 57−78% (65%; 10) of body length, 32−45 (38; 8) wide or 34−57% (41%; 8) of body width at level of caecal bifurcation, 16−23 (18; 6) wide or 12−19% (15%; 6) of body width at level of ovary, 11−35 (19; 10) wide or 10−41% (19%; 10) of body width at terminus; caecal termini 173−236 (207; 10) or 14−19% (17%; 10) of body length from posterior body end.

Anterior testis smooth, lacking lobes, 86−118 (96; 10) long or 7−10% (8%; 10) of body length or 42−66% (56%; 10) of posterior testis length, 55−84 (67; 10) wide or 41−69% (55%; 10) of body width at level of ovary or 63−120% (89%; 10) of posterior testis width; intertesticular space 41−91 (58; 10) or 3−6% (5%; 10) of body length (Figs. 1A, 1B). Posterior testis lobed, follicular, 148−236 (172; 10) long or 12−17% (14%; 10) of body length, 64−91 (77; 10) wide or 55−67% (62%; 10) of body width at level of ovary, 318−441 (366; 10) or 25−31% (29%; 10) of body length from posterior body end (Figs. 1A, 1B). Anterior trunk of vasa efferentia emanating from dorsal surface of anterior testis, extending anteriad 58 (1), 9 wide (Fig. 1B); posterior trunk of vasa efferentia emanating from the dorsal surface of the posterior testis, extending anteriad 236 (2) or 19% (1) of body length, 5 (1) wide, meeting anterior trunk 104 (1) or 8% (1) of body length posterior to genital pore to form vas deferens; vas deferens extending anteriad 193 (1) or 15% (1) of body length, 9 (1) wide before laterally expanding and turning dorsad to form external seminal vesicle (Fig. 1B). External seminal vesicle 139−250 (175; 10) long or 12−18% (14%; 10) of body length, 34−59 (40; 10) wide or 28−49% (35%; 10) of body width or 3.1−7.4× (4.5; 10) longer than wide, overlapping (8) or 4 and 20 (2) or <1% and 2% (2) of body length from posterior margin of ventral sucker, ventral to caeca (Figs. 1A, 1B); internal seminal vesicle 68−125 (105; 10) long or 6−10% (8%; 10) of body length, 18−52 (26; 10) wide or 2.3−6.9× (4.4; 10) longer than wide. Pars prostatica difficult to discern, surrounding distal portion of internal seminal vesicle, 45−100 (70; 5) long or 39−80% (63%; 5) of internal seminal vesicle length, 20−30 (25; 5) wide or 2.1−4.3× (2.8; 5) longer than wide (Fig. 1B). Cirrus sac obvious, 150−223 (189; 10) long or 12−18% (15%; 10) of body length, 50−95 (69; 10) wide or 42−94% (60%; 10) body width at level of genital pore; cirrus extending anterosinistrad 39−57 (44; 10) or 20−30% (24%; 10) of cirrus sac, 9−23 (14; 10) wide (Fig. 1B).

Ovary wedge-shaped in outline, sinistral, 232−391 (273; 10) or 19−28% (22%; 10) of body length posterior to middle of ventral sucker, 120−238 (165; 10) or 10−17% (13%; 10) of body length posterior to genital pore, 68−100 (80; 9) long or 5−8% (6%; 8) of body length, 36−45 (40; 9) wide or 28−39% (32%; 9) of body width or 39−62% (51%; 9) of ovary length; post-ovarian space 468−738 (579; 9) or 40−52% (46%; 9) of body length. Oviduct turning dorsad and extending posteriad 25−55 (42; 4) or 2−4% (3%; 4) of body length, 7−18 (15; 4) in maximum width, laterally expanding to form seminal receptacle; oviducal seminal receptacle extending sinistrad for 39−48 (43; 4) or 40−60% (51%; 4) of ovary length, 18−23 (20; 4) wide or 13−19% (16%; 4) of body width, constricting and turning dorsad, extending anteriad for 114 and 116 (2) or 9% (2) of body length before joining oötype, 9 and 16 (2) wide or 6% and 13% (2) of body width (Figs. 1A, 1B). Laurer's canal originating at distal margin of seminal receptacle, extending posterosinistrad 19 and 34 (2) or 3% (2) of body length, 5 and 8 (2) wide, opening dorsally sinistral to posterior testis (Fig. 1B). Vitellarium comprising a series of interconnected large spheroid masses of follicles (Fig. 1A), dorsal and ventral to caeca (illustrated as dorsolateral to emphasize course of caeca; Fig. 1A), distributing from level of caecal bifurcation or 250 (1) or 20% (1) of body length from anterior body end to excretory vesicle or 82 (1) or 6% (1) of body length from posterior body end; transverse vitelline duct ventral to gonads (dashed in Fig. 1B to illustrate course of oviduct and primary vitelline duct), between ovary and posterior testis, 295−454 (360; 5) or 24−32% (28%; 5) of body length from middle of ventral sucker, 18−43 (34; 5) wide or 16−33% (26%; 5) of body width; primary vitelline collecting duct turning dorsad to transverse vitelline duct, extending sinistrad 63 (1) or 5% (1) of body length before connecting with oviduct, 20 (1) wide or 14% (1) of body width. Ovi-vitelline duct extending anteriad 96 (1) or 8% of body length, 12 (1) wide (Fig. 1B). Oötype difficult to discern, 23 (2) long, 23 and 25 (2) wide, dorsolateral to anterior testis (Figs. 1, 2). Uterus originating 186 or 227 (2) or 15% and 18% (2) of body length posterior from middle of ventral sucker, 57 and 68 (2) long or 5% (2) of body length, 20 and 23 (2) wide or 19% (2) of body width; metraterm extending anterosinistrad, 39−68 (51; 9) long or 3−6% (4%; 9) of body length or 60% and 68% (2) longer than uterus, 14−23 (19; 9) wide or 12−20% (17%; 9) of body width. Uterine egg not observed. Common genital pore 68−136 (100; 10) or 5−10% (8%; 10) of body length posterior to middle of ventral sucker (Fig. 1A).

Excretory vesicle 157−211 (178; 10) long or 12−17% (14%; 10) of body length, 36−64 (49; 10) wide or 36−61% (49%; 10) of body width at level of caecal termini; wall 9−21 (16; 10) thick (Fig. 1A); excretory pore terminal.

Molecular Results (Fig. 2)

Analysis of the large subunit rDNA (28S) for all morphologically similar TBFs (with available sequence data) to *Platt*, i.e., *Hapalorhynchus* spp. + *Coeuritrema platti* Roberts & Bullard, 2016 + *Platt* spp., showed strong nodal support (posterior probability = 1) for a monophyletic *Hapalorhynchus* and a monophyletic *Platt* (Fig. 2). *Coeuritrema* currently comprises three accepted species (*C. lyssimus* Mehra, 1933 [type species], *C. rugatus* [Brooks & Sullivan, 1981], *C. platti*); however, sequence data is only available for *C. platti*, prohibiting comments on monophyly of *Coeuritrema* at this time. The phylogeny herein shows that the phylogenetic relationship between these genera is still unresolved, i.e., they form a polytomy. This unresolved relationship is more than likely due to the lack of sampling coverage across this undiagnosed lineage of freshwater TBFs (Orélis-Ribeiro et al., 2014; Roberts et al., 2016b).

In addition to 28S sequence data, we also generated ITS2 sequences for the TBFs herein as well (Table 2), hoping to construct an additional phylogeny. However, we were unable to generate a tree due to the vast base pair differences between the genera. Inability to construct a tree with the generated ITS2 data further suggests the drastic need for continued sampling of Asiatic softshells and pond turtles for known and new TBFs of this undiagnosed lineage (*Coeuritrema* spp., *Platt* spp., [and probably] *Cardiotrema* and *Enterohaematotrema*).

DISCUSSION

Platt resembles *Hapalorhynchus* Stunkard, 1922, *Coeuritrema* Mehra, 1933, *Enterohaematotrema* Mehra, 1940, and *Cardiotrema* Dwivedi, 1967 by having a ventral sucker, inverse U-shaped posterior caeca, an anterior testis and a posterior testis, and pre-gonadal male terminal genitalia. It differs from *Hapalorhynchus* by having a papillate ventral sucker, vasa efferentia that are dorsal to the gonads, an obvious, massive cirrus sac that is directed anteriad or laterad, and a vitellarium that surrounds the intestinal caeca, i.e., distributing dorsal, ventral, and lateral to the caeca. *Hapalorhynchus* has an apapillate ventral sucker, vasa efferentia that are exclusively ventral to the gonads, a diminutive cirrus sac that is directed posteriad (if present), and a vitellarium that is exclusively ventral to the intestinal caeca. The new genus is easily differentiated from *Coeuritrema* by having a smooth lateral body margin that lacks ventrolateral tegumental mamillae and by having a diminutive metraterm that is morphologically indistinct from the uterus; whereas, as emended by Roberts et al. (2016b), *Coeuritrema* has massive, mound-like ventrolateral tegumental mamillae and a massive metraterm (1/10-1/7 of body length) that is markedly distinct from the uterus. *Platt* differs from *Enterohaematotrema* by having a dorsosinistral genital pore; whereas, *Enterohaematotrema* has a ventromedial genital pore. The new genus differs from *Cardiotrema* by having a relatively large ventral sucker (1/4−1/2 of body width) that is at level immediately posterior to the caecal bifurcation, an elongate oesophagus (1/6−1/4 of body length), and an always intertesticular ovary; whereas, *Cardiotrema* has a small (rudimentary) ventral sucker (<1/5 of body width) that is far posterior to the cecal bifurcation, a short oesophagus (<1/10 of body length), and an ovary that is lateral to the anterior testis or intertesticular. Platt (2002) and Roberts et al. (2017) accepted that the ovary of *Cardiotrema* was sinistral to the anterior testis, not intertesticular; however, the ovary is reportedly sinistral to the anterior testis in *Cardiotrema vaidya* Dwivedi, 1967 and intertesticular in *Cardiotrema roparensis* Mehrotra, 1973 (see Tandon & Gupta, 1985). As a consequence, and pending an examination of found type materials or newly collected vouchers, the generic diagnosis for *Cardiotrema* (*sensu* Platt [2002]) should be emended to include ovary sinistral to anterior testis or intertesticular (Dwivedi, 1967; Mehrotra, 1973; Tandon & Gupta, 1985).

Platt snyderi (USNM XXXX; Figs. 1C, 1D) and the new species infected kidney and mesenteric blood vessels (Figs. 3−6) of both Mekong snail-eating turtles examined herein. The specimens of *P*. *snyderi* were diagnosed by having the combination of the morphological features specified in Table 1 as well as by having an elongate body (8.1−12.4× longer than wide), a short forebody (approximately 1/3 of body length), and a sinuous external seminal vesicle nearly reaching the level of the posterior margin of the ventral sucker. These specimens differed from the holotype of *P. snyderi* (USNM 105194) and its published description (Platt & Sharma, 2012) by having a vitellarium that extended to the level of the excretory vesicle (Fig. 1C) but in all other details matched the published description of this species by Platt & Sharma (2012). This is the first report of *P. snyderi* infecting a turtle from Vietnam (Table 2).

The new species resembles *P*. *snyderi*, *P*. *tkachi*, and *P*. *ocadiae* by having the combination of a sinistral caecum that is indented at level of the genital pore, a cirrus sac directed anteriad, a transverse vitelline duct that is ventral to the gonads, an intertesticular ovary that is wedge-shaped, and a genital pore that is posterior to the ventral sucker (Table 1). The new species can be readily distinguished from these species by the combination of having an external seminal vesicle that overlaps with or is immediately posterior to the level of the ventral sucker as well as a cirrus sac that is massive (extending anteriad >1/2 distance between the anterior testis and ventral sucker) and that is oriented parallel to the long axis of the body. *Platt snyderi* and *P. tkachi* have a moderately sized cirrus sac that is closest to the anterior testis and that is oriented at a 45° angle to the long body axis, and *P. ocadiae* has an external seminal vesicle at level of the genital pore.

Several Asiatic species of *Hapalorhynchus* need to be reassessed. *Hapalorhynchus sheilae* (Mehrotra, 1973) is herein considered a *species inquirenda* because its original description (Mehrotra, 1973) and redescription (Tandon & Gupta, 1982) are incomplete, no type material is extant, and it has not been treated again since its incomplete redescription. We deduce that the collection treated by Mehrotra (1973) is the same as that treated by Tandon & Gupta (1982) because the type-host and type-locality information for both *P. sutlejensis* and *H. sheilae* (*species inquirenda*) are identical, respectively, between these published works. Components of the description of *H. sheilae* are especially dubious. For example, Tandon & Gupta (1982) described the genital pore of *P. sheilae* as, “may be inter or extracaecal (since inward bending of the intestinal caeca has been found to be variable, depending upon the flattened state of the fluke)”. If the genital pore is dorsal and if the intestinal caecum is dorsal to the terminal genitalia (i.e., the terminal genitalia loop around the caecum), and in the absence of intraspecific variation, then the only way the genital pore could be intercaecal is if the specimen was severely damaged or partially destroyed when being mounted. *Hapalorhynchus mica* (Oshmarin, 1971) may be a junior subjective synonym of *P. oschmarini* (Belous, 1963). We regard it herein as *species inquirenda* until specimens of *P. mica* can be recollected and examined. The published descriptions of these taxa are both incomplete, no type or voucher material exists, and Oshmarin (1971) did not specify a characteristic that differentiated *P. mica* from *P. oschmarini*. *Hapalorhynchus indicus* (Thapar, 1933) Price, 1934 and *Hapalorhynchus macrotesticularis* (Rohde, Lee, & Lim, 1968) Brooks & Sullivan, 1981 are herein considered *incertae sedis* because they possess morphological features that differentiate them from all accepted species of *Hapalorhynchus* (see Roberts et al., 2017) and *Platt* (present study). For example, *H. indicus* has a cirrus sac that is reportedly anterior to the external seminal vesicle, and *H. macrotesticularis* has diverticulate posterior caeca and, reportedly, a blind-ending seminal receptacle rather than an oviducal seminal receptacle (Thapar, 1933; Rohde et al., 1968; Platt, 2002). No other species of *Hapalorhynchus* or *Platt* reportedly has a seminal receptacle that does not comprise an oviducal seminal receptacle (Figs. 2, 4; Platt [2002]; Roberts et al. [2017]).

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LITERATURE CITED

Anderson, G. R., & Barker, S. C. (1998). Inference of phylogeny and taxonomy within the Didymozoidae (Digenea) from the second internal transcribed spacer (ITS2) of ribosomal DNA. *Systematic Parasitology*, *41*, 87−94.

Brant, S. V., & Loker, E. S. (2005). Can specialized pathogens colonize distantly related hosts? Schistosome evolution as a case study. *PLoS Pathogens*, *1*, e38.

Belous, E.V. 1963: [Helminth fauna of water turtles, *Amyda sinensis*, of the Far East.] *Helminthologia*, *4*, 79−99 (in Russian).

Chapman, P. A., Cribb, T. H., Blair, D., Traub, R. J., Kyaw-Tanner, M. T., Flint, M., et al. (2015). Molecular analysis of the genera *Hapalotrema* Looss, 1899 and *Learedius* Price, 1934 (Digenea: Spirorchiidae) reveals potential cryptic species, with comments on the validity of the genus *Learedius*. *Systematic Parasitology*, *90*, 67−79. doi:10.1007/s11230-014-9535-y

Cribb, T. H., Anderson, G. R., Adlard, R. D., & Bray, R. A. (1998). A DNA-based demonstration of a three-host life-cycle for the Bivesiculidae (Platyhelminthes: Digenea). *International Journal for Parasitology*, *28*, 1,791−1,795.

Dwivedi, M. P. (1967). Contribution to the family Spirorchiidae Stunkard, 1921 (Digenea: Trematoda). *Indian Journal of Helminthology*, *19*, 1−14.

Lockyer, A. E., Olson, P. D., Ostergaard, P., Rollinson, D., Johnston, D. A., Attwood, S. W., et al. (2003). The phylogeny of the Schistosomatidae based on three genes with emphasis on the interrelationships of *Schistosoma* Weinland, 1858. *Parasitology*, *126*, 203−224.

Mehra, H. R. (1933). New blood flukes of the family Spirorchidae Stunkard from Indian fresh-water tortoises with discussion on the synonymy of certain genera and the relationships of the families of blood flukes. *Part I. Bulletin of the Academy of Sciences of the United Provinces of Agra and Oudh, India*, *2*, 203−225.

Mehrotra, V. (1973). Digenea from some reptile hosts in India, Part II (in continuation with Abstract No. 286 in Part III of the Proceedings). *Proceedings of the Sixtieth Indian Science Congress*, *4*, 46−47.

Oshmarin, P. G. (1971). [A new species of trematode from the blood vessels of Chelonia.] In: *Parazity Zhivotnykh I Rasternii Dal'nego Vostoka*. Vladivostok, USSR: Dal'nevostochnoe Knizhnoe Izdatel'stvo, pp. 142−143 (in Russian).

Ozaki, Y. (1939). [A new blood-fluke *Hapalorhynchus yoshidai*.] *Volumen Jubilare pro Professore Sadao Yoshida, Osaka*, *1*, 29−37 (in Japanese; English summary pp. 34−35).

Platt, T. R. (2002). Family Spirorchiidae Stunkard, 1921. In: Gibson, D. I., A.J. Jones, A. & Bray, R. A. (Eds.), *Keys to the Trematoda*, *Volume 1.* Wallingford: CABI Publishing, pp. 453-468.

Platt, T. R., & Sharma, R. S. K. (2012) Two new species of *Hapalorhynchus* (Digenea: Spirorchiidae) from freshwater turtles (Testudines: Geoemydidae) in Malaysia. *Comparative Parasitology*, *79*, 202−207.

Roberts, J. R., Platt, T. R., Orélis-Ribeiro, R., & Bullard, S. A. (2016a). New genus of blood fluke (Digenea: Schistosomatoidea) from Malaysian freshwater turtles (Geoemydidae) and its phylogenetic position within Schistosomatoidea. *Journal of Parasitology*, *102*, 451−462.

Roberts, J. R., Orélis-Ribeiro, R., Dang, B. T., Halanych, K. M., & Bullard, S. A. (2016b). Blood flukes of Asiatic softshell turtles: revision of *Coeuritrema* Mehra, 1933 (Digenea: Schistosomatoidea) and a new species infecting Chinese softshell turtles, *Pelodiscus sinensis*, (Trionychidae) from the Da Rang River, Vietnam. *Folia Parasitologica*, *63*, 031 doi:10.14411/fp.2016.031.

Roberts, J. R., Orélis-Ribeiro, R., Halanych, K. M., Arias, C. R., & Bullard, S. A. (2016c). A new species of *Spirorchis* MacCallum, 1918 (Digenea: Schistosomatoidea) and *Spirorchis* cf. *scripta* from chicken turtle, *Deirochelys reticularia*, (Emydidae), with an emendation and molecular phylogeny of *Spirorchis*. *Folia Parasitologica*, *63*, 041 doi:10.14411/fp.2016.041.

Roberts, J. R., Halanych, K. M., Arias, C. R., Folt, B., Goessling, J. M., & Bullard, S. A. (2017). Emendation and new species of *Hapalorhynchus* Stunkard, 1922 (Digenea: Schistosomatoidea) from musk turtles (Kinosternidae: *Sternotherus*) in Alabama and Florida rivers. *Parasitology International*, *in review*.

Rohde, K., Lee, S. K., & Lim, H. W. (1968). Ueber drei malayische Trematoden. *Annales de Parasitologie Humaine et Comparée*, *43*, 33−43.

Smith, J. W. (1997a). The blood flukes (Digenea: Sanguinicolidae and Spirorchidae) of cold-blooded vertebrates: Part I. A Review of the literature published since 1971, and bibliography. *Helminthological Abstracts*, *66*, 255−294.

Smith, J. W. (1997b). The blood flukes (Digenea: Sanguinicolidae and Spirorchidae) of cold-blooded vertebrates: Part II. Appendix I: Comprehensive parasite-host list; Appendix II: Comprehensive host-parasite list. *Helminthological Abstracts*, *66*, 329−344.

Snyder, S. D. (2004). Phylogeny and paraphyly among tetrapod blood flukes (Digenea: Schistosomatidae and Spirorchiidae). *International Journal Parasitology*, *34*, 1,385−1,392.

Takeuti, E. (1942). New blood flukes of the family Spirorchidae from Japanese fresh-water tortoise and marine turtles. *Japanese Journal of Medical Sciences, VI, Bacteriology and Parasitology*, *2*, 161−174.

Tandon V., & Gupta, N. K. (1982). On some blood flukes (Spirorchiidae: Coeuritrematinae) from freshwater chelonians in India. *Proceedings of the Indian Academy of Sciences (Animal Science)*, *91*, 275−282.

Tandon V., & Gupta, N. K. (1985) On the blood fluke, *Cardiotrema roparensis* Mehrotra, 1973 and validity of *C. longivesticulata* Dwivedi, 1967 (Spirorchiidae: Coeuritrematinae). *Research Bulletin (Science) of the Panjab University*, *36*, 331−334.

Thapar, G. S. (1933). A new blood fluke from an Indian tortoise, *Trionyx gangeticus*. *Journal of Helminthology*, *11*, 163−168.

van Dijk, P. P., Iverson, J. B., Rhodin, A. G. J., Shaffer, H. B., & Bour, R. (2014). Turtles of the world, 7th Edition: Annotated checklist of taxonomy, synonymy, distribution with maps, and conservation status. *Chelonian Research Monographs*, *5*, 329−479.

FIGURE LEGENDS

Fig. 1 Blood flukes ex. kidney and mesenteric blood vessels of *Malayemys subtrijuga*. A, Body of *Platt sinuosus* n. g., n. sp. (holotype, USNM XXXX), ventral view. B, Genitalia of *Platt sinuosus* n. g., n. sp. (holotype, USNM XXXX), ventral view. C, Genitalia of *Platt snyderi* n. comb. (voucher, USNM XXXX), dorsal view. D, Body of *Platt snyderi* n. comb (voucher, USNM XXXX), dorsal view. *Abbreviations*: os, oral sucker; ph, pharynx; oe, oesophagus; nc, nerve commissure; og, oesophageal gland; cb, caecal bifurcation; dc, dextral caecum; sc, sinistral caecum; vr, vitellarium; vs, ventral sucker; esv, external seminal vesicle; cs, cirrus sac; cgp, common genital pore; mt, metraterm; ut, uterus; isv, internal seminal vesicle; oo, oötype; at, anterior testis; ov, ovary; tvd, transverse vitelline duct; pt, posterior testis; ct, caecal termini; ev, excretory vesicle; ep, excretory pore; vd, vas deferens; ci, cirrus; pp, pars prostatica; ave, anterior trunk of vasa efferentia; ovd, ovi-vitelline duct; lvd, lateral collecting vitelline duct; od, oviduct; osr, oviducal seminal receptacle; Lc, Laurer's canal; pve, posterior trunk of vasa efferentia; vt, primary vitelline duct.

Fig. 2 Phylogenetic relationships of blood flukes reconstructed by Bayesian inference. Numbers aside tree nodes indicate posterior probability. Phylogeny reconstructed based sequences from the partial D1−D3 domains of the large subunit ribosomal DNA (28S).

Table 1. Key to *Platt* spp.

1.	Transverse vitelline duct dorsal to gonads	***P. sutlejensis*** (Mehrotra, 1973) n. comb.
	Transverse vitelline duct ventral to gonads	2
2.	Cirrus sac directed laterad (perpendicular to body margin)	***P. yoshidai*** (Ozaki, 1939) n. comb.
	Cirrus sac directed anteriad (at 45° angle to or parallel with body margin)	3
3.	Genital pore at level of or immediately posterior to level of ventral sucker	***P. oschmarini*** (Belous, 1963) n. comb.
	Genital pore posterior to level of ventral sucker	4
4.	Ovary ovoid; both caeca indented at level of genital pore	***P. odhnerensis*** (Mehra, 1933) n. comb.
	Ovary wedge-shaped; sinistral caecum indented at level of genital pore	5
5.	Cirrus sac short, closest to anterior testis, orienting at 45° angle to body margin	6
	Cirrus sac long, extending anteriad >1/2 distance between anterior testis and ventral sucker, orienting parallel to body margin	7
6.	Testes lobed; posterior testis far anterior to caecal termini	***P. snyderi*** (Platt & Sharma, 2012) n. comb.
	Testes smooth; posterior testis immediately anterior to caecal termini	***P. tkachi*** (Platt & Sharma, 2012) n. comb.
7.	External seminal vesicle at level of genital pore	***P. ocadiae*** (Takeuti, 1942) n. comb.
	External seminal vesicle overlapping or immediately posterior to ventral sucker	***P. sinuosus*** n. sp.

Table 2. Species of *Platt* n. g. and related Asiatic turtle blood flukes[a]

Blood fluke	Turtle host	Site	Locality	Accession nos.	Reference
Hapalorhynchus mica (Oshmarin, 1971) Bourgat, 1990[b] (*species inquirenda*; perhaps a jr. subjective synonym of *P. oschmarini*)	*Pelodiscus sinensis* (Wiegmann), Chinese softshell turtle (Geoemydidae)	liver	Not specified (purchased from a market), Haiphong, Vietnam	Not reported	Oshmarin (1971)
Platt ocadiae (Takeuti, 1942) n. comb.	*Mauremys sinensis* (Gray), Chinese stripe-necked turtle (Geoemydidae)	blood	Not specified (shipped to laboratory in Japan from Taiwan) (as Formosa)	Not reported	Takeuti (1942)
Platt odhnerensis (Mehra, 1933) n. comb.	*Lissemys punctata* (Bonnaterre), Indiana flapshell turtle (Trionychidae)	heart	Ganges River, Allahabad, Uttar Pradesh State, India	Not reported	Mehra (1933)
Platt oschmarini (Belous, 1963) n. comb.	*Pelodiscus maackii* (Brandt), Northern Chinese softshell turtle (Trionychidae)	liver	Lake Khanka (Songacha River) (44°59'45.62"N, 132°23'28.78"E), Russia	Not reported	Belous (1963)
Hapalorhynchus sheilae (Mehrotra, 1973) Bourgat, 1990[b] (*species inquirenda*)	*Pangshura tecta* (Gray), Indian roofed turtle (Geoemydidae)	heart, liver, blood vessels	Sutlej River, Ropar, Punjab State, India	Not reported	Mehrotra (1973); Tandon & Gupta (1982)
	L. p. punctata (Bonnaterre), Southern Indiana flapshell turtle (Trionychidae)	heart, liver, blood vessels	Ganges River Basin, Rudrapur, Uttarakhand State; Patiala River (Ghaggar River Basin), Patiala, Punjab State;	Not reported	

			Ghaggar River, Sangrur, Punjab State, India		
***Platt sinuosus* n. sp. (type species)**	***Malayamys subtrijuga* (Schlegel & Müller), Mekong snail-eating turtle (Geoemydidae)**	**kidney, mesenteric blood vessels**	**Mekong River (10°01’42.15”N; 105°47’14.15”E), Can Tho, Vietnam**	**USNM XXXX, XXXX**	**Present study**
***Platt snyderi* (Platt and & Sharma, 2012) n. comb.**	*M. subtrijuga* (Geoemydidae)	viscera wash	Perlis River Basin, Kangar, Perlis, Malaysia	USNM 105194, 105195[c]	Platt & Sharma (2012)
		kidney, mesenteric blood vessels	**Mekong River (10°01’42.15”N; 105°47’14.15”E) Can Tho, Vietnam**	**USNM XXXX**	**Present study**
Platt sutlejensis (Mehrotra, 1973) n. comb.	*Pangshura sylhetensis* Jerdon, Assam roofed turtle (Geoemydidae)	heart	Sutlej River, Ropar, Punjab State, India	Not reported	Mehrotra (1973); Tandon & Gupta (1982)
	L. p. punctata (Trionychidae)	heart	Gomti River, Lucknow, Uttar Pradesh State; Ghaggar River, Sangrur, Punjab State, India	Not reported	
Platt tkachi (Platt and & Sharma, 2012) n. comb.	*Siebenrockiella crassicollis* (Gray), black marsh turtle (Geoemydidae)	viscera wash	Kinta River Basin, Ipoh, Perak, Malaysia	USNM 105196, 105197[c]	Platt & Sharma (2012)
Platt yoshidai (Ozaki, 1939) n. comb.	*M. sinensis* (Geoemydidae)	blood	Yangtze River Basin, Shanghai, China	Not reported	Ozaki (1939)

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[a] *Hapalorhynchus indicus* (Thapar, 1933) Price, 1934 and *Hapalorhynchus macrotesticularis* (Rohde, Lee, & Lim, 1968) Brooks & Sullivan, 1981 are *incertae sedis* (see text).

[b] *species inquirendae* (see text).

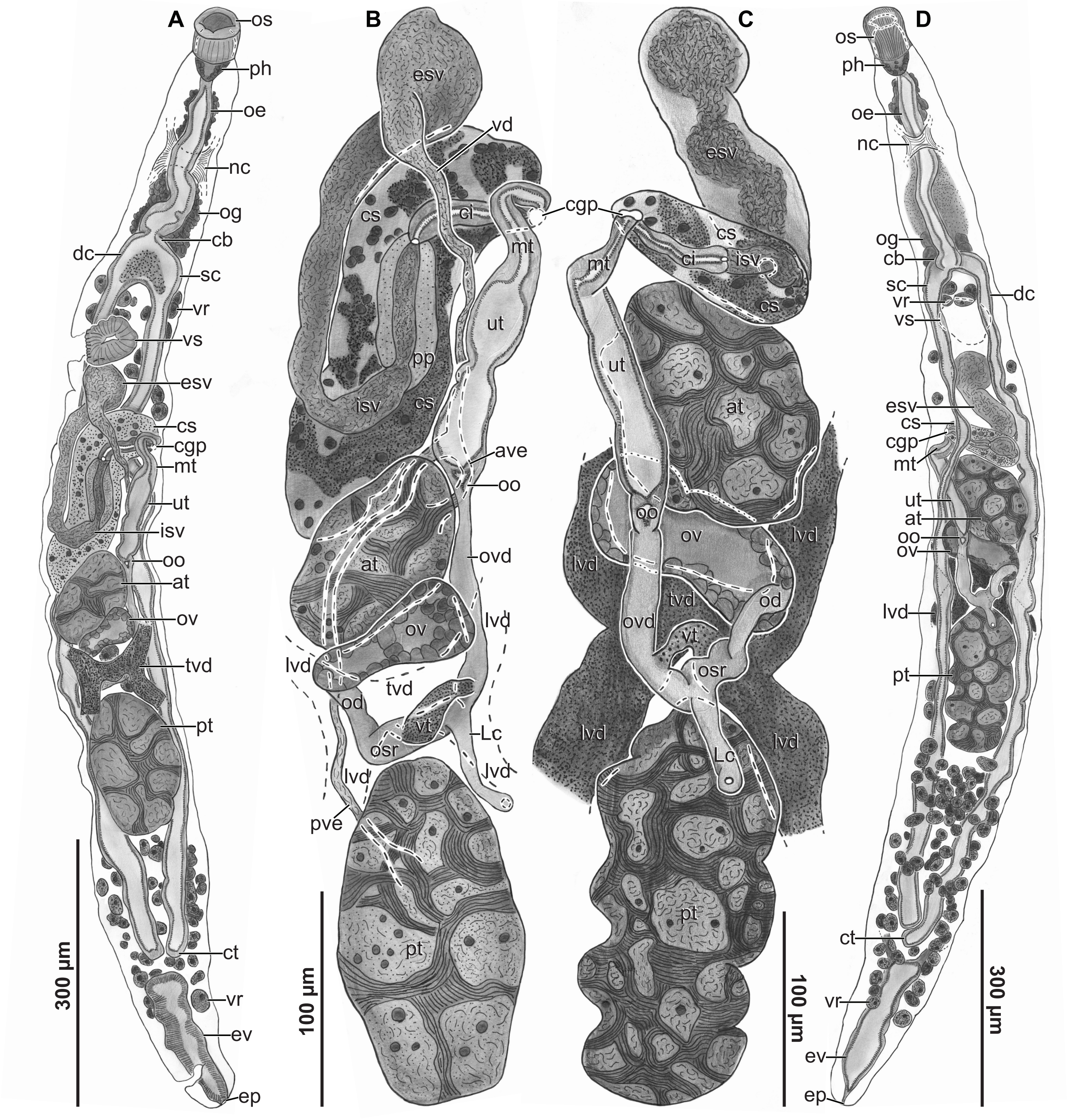
[c] Platt & Sharma (2012) incorrectly reported these accession numbers as 104195 and 104197, respectively.

Table 3. Turtle blood fluke sequences used in the present study.

Blood fluke	Turtle host	Locality	GenBank Accession Numbers		Reference(s)
			28S	**ITS2**	
***Coeuritrema platti* Roberts & Bullard, 2016**	*Pelodiscus sinensis* (Wiegmann), Chinese softhell turtle	Da Rang River Basin, Phu Yen Province, Vietnam	KX712243	**BF61**	Roberts et al. (2016b); **present study**
Griphobilharzia amoena Platt & Blair, 1991	*Crocodylus johnstoni* Krefft, freshwater crocodile	Darwin (probably Adelaide River Basin), Australia	AY899914	N/A	Brant & Loker (2005)
***Hapalorhynchus conecuhensis* Roberts & Bullard, 2017**	*Sternotherus* cf. *minor*, loggerhead musk turtle	Blue Spring (31°5'27.64"N, 86°30'53.21"W), Yellow River, Alabama, U.S.A.	BF131	**BF131**	Roberts et al. (2017); **present study**
***Hapalorhynchus foliorchis* Brooks & Mayes, 1975**	*Chelydra serpentina* (Linnaeus), common snapping turtle	Pond off Saugahatchee Creek (Tallapoosa River) (32°39'1.36"N, 85°29'4.70"W), Alabama, USA	KX712242	**BF74**	Roberts et al. (2016b); **present study**
Hapalorhynchus gracilis Stunkard, 1922	*C. serpentina*	Reelfoot Lake (Mississippi River) (36°21'12.23"N, 89°25'21.50"W), Tennessee, USA	AY604710	N/A	Snyder (2004)
***Hapalorhynchus reelfooti* Byrd, 1939**	*Sternotherus minor* (Agassiz), loggerhead musk turtle	Wacissa River (tributary of Aucilla River) (30°20'24.73"N, 83°59'27.56"W), Florida, USA	BF127	**BF127**	Roberts et al. (2017); **present study**
***Platt sinuosus* n. g., n. sp.**	***Malayamys subtrijuga* (Schlegel & Müller), Mekong snail-eating turtle**	**Mekong River (10°01'42.15"N; 105°47'14.15"E), Can Tho, Vietnam**	**BF85**	**BF85**	**Present study**
***Platt snyderi* (Platt & Sharma, 2012), n. comb.**	***M. subtrijuga***	**Mekong River (10°01'42.15"N;**	**BF132**	**BF132**	**Present study**

		105°47’14.15”E), Can Tho, Vietnam.			
Hapalotrema pambanensis Mehrotra, 1973	*Chelonia mydas* (Linnaeus), green sea turtle	indeterminate[a]	-	KM652626[a]	Chapman et al. (2015)
Learedius learedi	*C. mydas*	Coolum, Queensland, Australia	-	KM652619	Chapman et al. (2015)

[a] Mislabeled as a 28S sequence for *Learedius learedi* in Chapman et al. (2015).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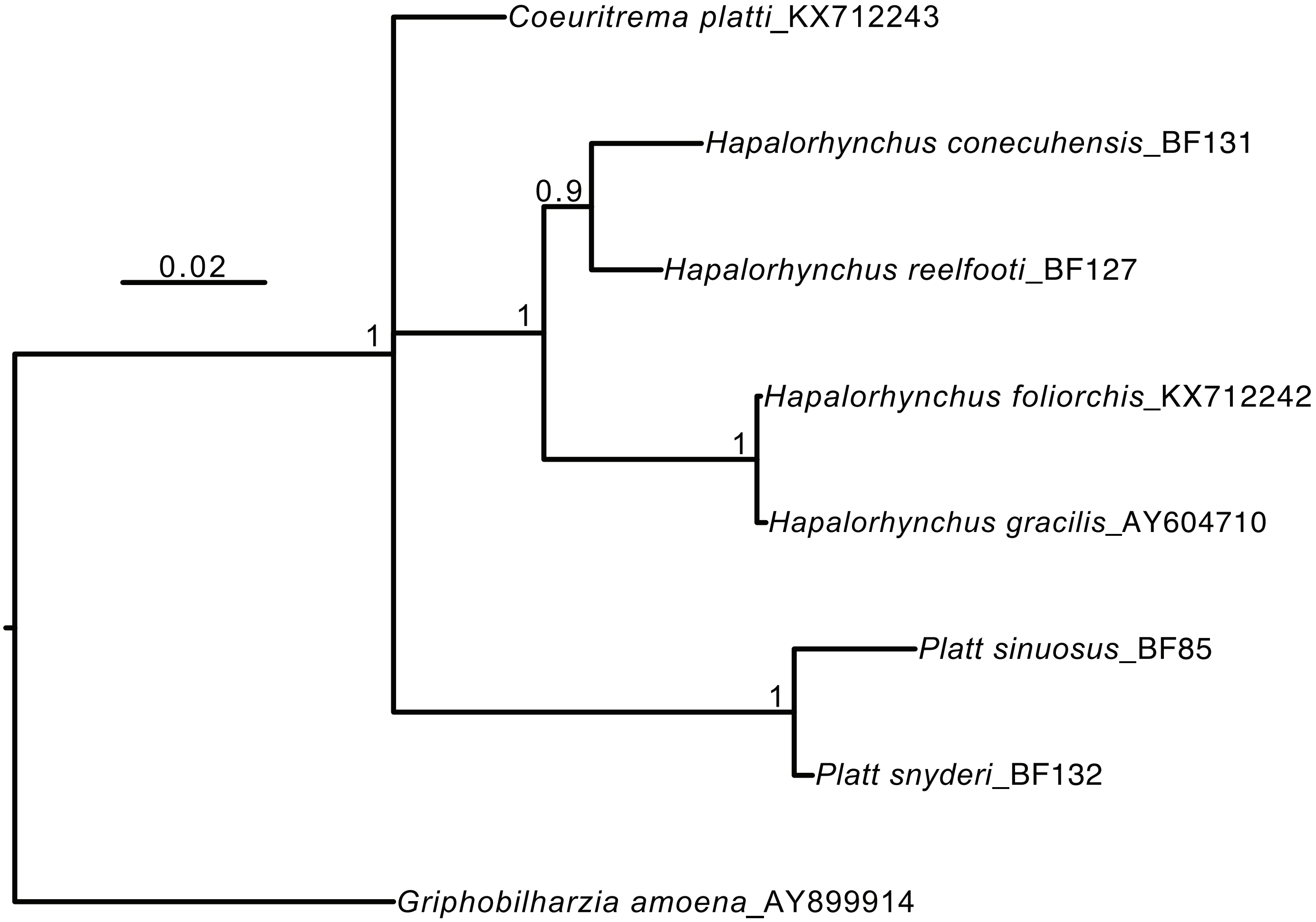
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Hapalorhynchus conecuhensis_BF131
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Hapalorhynchus foliorchis_KX712242
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Hapalorhynchus gracilis_AY604710
Platt sinuosus_BF85
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Platt snyderi_BF132
Griphobilharzia amoena_AY899914

CHAPTER 8: BLOOD FLUKES INFECTING MAP TURTLES (CRYPTODIRA: EMYDIDAE: *GRAPTEMYS* SPP.) FROM ALABAMA: INCLUDING A NEW SPECIES, MOLECULAR PHYLOGENY, AND UPDATED KEY FOR SPECIES OF *SPIRORCHIS* MACCALLUM, 1918

***Formatted for submission to Journal of Parasitology**

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ABSTRACT

Black-knobbed map turtles (*Graptemys nigrinoda* Cagle, 1954) and Alabama map turtles (*Graptemys pulchra* Baur, 1893) from the Alabama River (231°32'49.28"N, 87°30'57.82"W ; Alabama, U.S.A) and Coosa River (32°32'19.68"N, 86°12'22.69"W; Alabama), respectively, were infected by *Spirorchis paraminutus* n. sp. The new species closely resembles *Spirorchis haematobius* (Stunkard, 1922) Price, 1934, *Spirorchis minutus* Byrd, 1939, and *Spirorchis collinsi* Roberts and Bullard, 2016 by having a short testicular column (<1/4 of body length) with typically 10 testes. Of these, the new species more closely resembles *S. haematobius* and *S. minutus* by having gonads, terminal genitalia, and a common genital pore in the posterior half of the body. *Spirorchis paraminutus* can be differentiated from both *S. haematobius* and *S. minutus* by having an elongate body (12−24× longer than wide vs. 6−10× for *S. haematobius* and 8−10× for *S. minutus*) and more anterior terminal genitalia, i.e., having a genital pore 26−29% of body length anterior to posterior body end (vs. 10−11% and 12% of body length from posterior body end). Black-knobbed map turtles, Alabama map turtles, and one Escambia map turtle (*Graptemys ernsti* Lovich and McCoy, 1992) were also infected with *Spirorchis elegans* Stunkard, 1923, *Spirorchis scripta* Stunkard, 1923, and two innominate species of *Spirorchis.* All three of these map turtles have never-before

been reported as blood fluke hosts. Sequence analysis of the internal transcribed region (ITS2) showed a strongly supported monophyletic *Spirorchis*, and the new species forming a clade with *Spirorchis collinsi*, with a 99.3% simiarity with 3 differing base pairs.

Map turtles (Cryptodira: Emydidae: *Graptemys* spp.) comprise 14 species that range exclusively in Gulf of Mexico and Great Lakes river basins of the United States and Canada (Lindeman, 2013; van Dijk et al., 2014). Of these 14 accepted species, only two (14%) have previously been reported as blood fluke hosts (see Table I; Ward, 1921; Stunkard, 1923; Byrd, 1939; Rausch, 1947; Brooks, 1979).

Of all known map turtles, six species (43%) range in Alabama rivers. Of these six, only one (*Graptemys geographica* [LeSueur, 1817], common map turtle) has previously been reported as a blood fluke host. As part of a larger turtle sampling project, we sampled individuals of three map turtle species from Alabama rivers never-before-reported as blood fluke hosts: Escambia map turtle (*Graptemys ernsti* Lovich and McCoy, 1992), black-knobbed map turtle (*Graptemys nigrinoda* Cagle, 1954), and Alabama map turtle (*Graptemys pulchra* Baur, 1893). Necropsies showed infection in these hosts by five TBFs, all species of *Spirorchis* MacCallum, 1918: *S. elegans* Stunkard, 1923, *S. scripta* Stunkard, 1923, two innominate species of *Spirorchis*, and a new species. Herein, we describe the new species, provide new host and geographic locality records for species of *Spirorchis*, provide a phylogenetic analysis of the internal transcribed region (ITS2), and provide an updated key to the species of *Spirorchis* (see Platt, 1993).

MATERIALS AND METHODS

As part of a larger blood fluke survey, individuals of three never-before-sampled turtles were collected from Alabama rivers: two Escambia map turtles from the Yellow River on 3−4 August 2015 (Watkins Bridge: 31°5'44.55"N, 86°26'8.37"W; Givens Bridge: 31°0'40.93"N, 86°32'15.15"W), four black-knobbed map turtles from the Coosa River on 21 May 2016 (2 individuals; 32°32'19.68"N, 86°12'22.69"W) and the Alabama River on 14 July 2016 (2 individuals; 31°32'49.28"N, 87°30'57.82"W), and three Alabama map turtles from the Tallapoosa River (1 individual; 32°28'39.99"N, 85°41'43.25"W) and the Coosa River (2 individuals; 32°32'19.68"N, 86°12'22.69"W) on 15 April 2015 and 21 May 2016, respectively. Turtles were killed by decapitation, and necropsies were performed using 7.0 g/L sodium citrate saline solution following Roberts et al. (2016c). Live turtle blood flukes for morphology were killed, fixed, stained, and mounted following Roberts et al. (2016c). Turtle scientific and common names follow van Dijk et al. (2014) and Guyer et al. (2015). Classification and anatomical terms for turtle blood flukes follow Roberts et al. (2016a, b, c; 2017a). Blood fluke specimens intended for molecular analysis were placed directly into absolute EtOH. Holotype, paratype, and voucher specimens of turtle blood flukes were borrowed from the American Museum of Natural History (AMNH, New York, New York), National Museum of National History (USNM, Smithsonian Institute, Washington, D.C.), and Thomas Platt’s personal research collection (TPRC).

DNA extraction, amplification, and sequencing were performed following Roberts et al. (2017a). Assembled sequences (Table II) were aligned with MAFFT 7.310 (Katoh and Standley, 2013) and subsequently corrected by eye in Mesquite 3.2 (Maddison and

Maddison, 2017). Alignment herein comprised 466 base pairs. Regions that could not be unambiguously aligned were excluded from further analyses. MrBayes 3.5.3 (Ronquist et al., 2012) was used to reconstruct a Bayesian inference phylogeny. Using a GTR + Gamma model, 4 runs of 4 chains each were conducted for 1,000,000 generations. Priors were set to default values and burn-in was set to 25% of generations (or 250,000). Outgroup, i.e., *Baracktrema obamai* Roberts, Platt, and Bullard, 2016, were selected from representative blood fluke genera informed by the phylogenetic analyses of Roberts et al. (2016a; c). The ingroup comprised newly generated sequences for the internal transcribed region (ITS2) for species of *Spirorchis* (Table II). Chains were run until the average standard deviation of split frequencies was below 0.01. The resulting phylogenetic tree was viewed using FigTree v1.4.3 (Rambaut, 2009) and subsequently edited in Adobe Illustrator CC 2015.3 (Adobe Systems).

RESULTS

Spirorchis paraminutus Roberts and Bullard n. sp. (Figs. 1−7)

Description

Based on light microscopy of 7 whole-mounted specimens. Body elongate, threadlike, 1,219−2,239 (1,618; 7) long or 12.2−23.6× (18.1; 7) longer than wide, 41−100 (73; 7) wide or 3−6% (5%; 7) of body length at level of cecal bifurcation, 52−134 (91; 7) wide or 4−8% (6%; 7) of body length at level of middle testis (typically maximum body width), 39−116 (86; 7) wide or 3−7% (5%; 7) of body length at genital pore, 41−84 (64; 7) wide or 3−5% (4%; 7) of body length at level of cecal termini (Figs. 1, 3−5). Oral sucker 14−32 (22; 7) long or 1−3% (1%; 7) of body length, 23−41 (28; 7) wide or

30−56% (41%; 7) of body width at level of cecal bifurcation, papillae not observed, spines not observed. Nerve commissure 114−185 (146; 5) or 8−9% (9%; 5) of body length from anterior body end. Pharynx 34−60 (50; 7) long or 12−19% (16%; 7) of esophagus length, 18−39 (29; 7) wide or 1.0−1.8× (1.4; 6) esophagus width (Figs. 1, 3). Esophagus extending posteriad 261−432 (329; 7) long or 18−23% (20%; 7) of body length from mouth to anterior margin of median esophageal diverticulum, 5−14 (10; 7) wide posterior to pharynx, with wall 2−9 (6; 7) thick, 14−27 (21; 7) wide at esophagus median or 20−54% (31%; 7) of body width at level of cecal bifurcation, 14−27 (21; 6) wide anterior to median esophageal diverticulum or 15−46% (32%; 6) of body width at level of cecal bifurcation; median esophageal diverticulum 41−55 (46; 7) long or 11−16% (14%; 7) of esophagus length, 25−41 (33; 7) wide or 33−68% (48%; 7) of body width at level of cecal bifurcation; esophageal gland 209−409 (299; 7) long or 17−21% (18%; 7) of body length, 30−52 (41; 7) wide or 47−73% (58%; 7) of body width at level of cecal bifurcation. Intestinal bifurcation 275−477 (362; 7) from anterior body end or 21−25% (22%; 7) of body length, dorsal to median esophageal diverticulum; sinistral cecum 955−1,675 (1,266; 5) long or 65−75% (71%; 5) of body length, 11−30 (17; 6) wide or 12−32% (22%; 6) of body width at level of cecal bifurcation, 7−11 (10; 6) wide or 8−14% (10%; 6) of body width at level of middle testis, 7−16 (10; 6) wide or 7−14% (10%; 6) of body width at level of ovary, 9−27 (15; 5) wide or 13−44% (23%; 5) of body width at level of cecal termini; dextral cecum 960−1,705 (1,271; 5) long or 65−76% (71%; 5) of body length, 11−30 (17; 6) wide or 12−36% (23%; 6) of body width at level of cecal bifurcation, 7−16 (10; 6) wide or 9−12% (10%; 6) of body width at level of middle testis, 9−16 (11; 6) wide or 11−15% (12%; 6) of body width at level of ovary, 9−23 (15; 5) wide or 13−38% (22%; 5) of body width at level of cecal termini; post-cecal distance 60−159 (103; 6) or 3−11% (7%; 6) of body length (Figs. 1, 3−5).

Testes 10 (7) in number, spheroid or weakly lobed; testicular column 257−475 (353; 7) or 21−27% (22%; 7) of body length from cecal bifurcation, 257−465 (352; 7) or 21−26% (22%; 7) from median esophageal diverticulum, 212−540 (344; 7) long or 17−24% (21%; 7) of body length; anterior testis (t1) abutting or 7 (2) and 5 (1) from sinistral and dextral ceca respectively, 19−50 (35; 7) long or 2−3% (2%; 7) of body length, 18−64 (34; 7) wide or 30−48% (37%; 7) of body width at level of middle testis; middle testis (t5), abutting both ceca or 2 and 5 (2) and 4 and 7 (2) from sinistral and dextral ceca respectively, 14−54 (31; 7) long or 1−2% (2%; 7) of body length, 19−73 (36; 7) wide or 34−54% (39%; 7) of body width at level of middle testis; posterior-most testis 357−753 (558; 7) or 28−37% (34%; 7) of body length from posterior body end, abutting both ceca or 2 (1) from sinistral cecum, 21−82 (44; 7) long or 2−4% (3%; 7) of body length, 26−70 (42; 7) wide or 41−52% (46%; 7) of body width at level of middle testis (Figs. 1, 3−5). Vas deferens difficult to discern, 238−520 (324; 5) long or 16−23% (20%; 5) of body length, 7−15 (9; 5) wide, ventral to testicular column, laterally expanding before joining external seminal vesicle posterior to testes (Figs. 2, 5, 6); external seminal vesicle abutting posterior margin of posterior-most testis, 39−85 (55; 7) long or 2−5% (3%; 7) of body length, 27−71 (42; 7) wide or 29−71% (51%; 7) of body width, extending posteriad dorsal before reaching cirrus sac (Figs. 2, 5, 6); internal seminal vesicle 27−66 (44; 6) long or 2−3% (3%; 6) of body length, 16−26 (20; 6) maximum width or 53−100% (78%; 6) of cirrus sac width, 1.5−2.9× (2.2; 6) longer than wide (Figs. 2, 6). Cirrus sac 48−93 (80; 7) long or 4−7% (5%; 7) of body length, 18−34

(26; 7) wide or 21−46% (31%; 6) of body width; cirrus slightly sinuous, 27−57 (41; 7) long or 2−4% (3%; 7) of body length, 9−20 (14; 7) wide or 9−41% (18%; 7) of body width.

Ovary spheroid or weakly lobed, intercecal, ventrolateral to cirrus sac, 40−77 (66; 6) long or 3−5% (4%; 6) of body length, 25−82 (45; 6) wide or 29−71% (47%; 6) of body width, 0.3−1.1× (0.7; 6) wider than long (Figs. 1, 2, 5, 6); post-ovarian distance 272−584 (453; 6) or 22−32% (27%; 6) of body length (Figs. 1, 3, 4, 5). Oviduct extending posteriad 18−27 (21; 5) or 1−2% (1%; 5) of body length, 5−9 (7; 5) wide; oviducal seminal receptacle 68−125 (91; 5) long or 4−10% (6%; 5) of body length, 18−25 (23; 6) wide or 21−31% (24%; 6) of body width, oviduct continuing anteriad 23−46 (33; 5) or 2−3% (2%; 5) of body length before connecting with oötype, 9−14 (11; 5) wide or 10−16% (12%; 5) of body width. Laurer's canal a narrow duct originating at distal margin of seminal receptacle, extending posterosinistrad 23−80 (52; 6) or 1−5% (3%; 6) of body length, 8−23 (14; 6) wide (Figs. 2, 6). Vitellarium comprising a series of interconnected spheroid masses of small follicles (Figs. 1, 3−5), distributing from cecal bifurcation to cecal termini, originating 272−477 (370; 6) or 20−24% (22%; 6) of body length from anterior body end, ventrolateral to ceca and testes anteriorly and flanking ceca posteriorly (illustrated as only lateral to show course of posterior ceca, Figs. 1, 3−5), terminating 75−150 (106; 4) or 4−10% (7%; 4) of body length from posterior body end, coalescing into a transverse vitelline collection duct posterior to ovary; transverse vitelline duct 203−497 (382; 6) or 16−26% (22%; 6) of body length from posterior body end (Figs. 1, 5); primary vitelline duct extending posteriad from transverse vitelline duct 49−133 (79; 4) or 3−6% (4%; 4) of body length before turning dorsad, extending 34−113

(100; 10) anteriad or 2−4% (3%; 10) of body length before merging with female genitalia at oviduct-oötype junction, 36−64 (46; 10) wide (Figs. 2, 6). Oötype 26−37 (31; 6) long by 12−23 (18; 6) wide. Uterus comprised solely by muscular metraterm; metraterm 34−57 (42; 5) long or 2−3% (2%; 5) of body length, 12−23 (18; 5) wide or 14−27% (20%; 4) of body width. Common genital pore 420−584 (503; 4) or 26−29% (28%; 4) of body length from posterior body end (Figs. 1, 5).

Excretory vesicle 2−9 (7; 4) wide or 3−15% (10%; 4) of body width at level of cecal termini; Manter's organ straight or turning 2 or 4 (2) times proximally, intercecal or extending to cecal termini, 77−93 (87; 3) long or 3−6% (5%; 3) of body length, 9−16 (12; 3) wide or 15−19% (17%; 3) of body width at level of cecal termini, joining excretory vesicle at excretory pore; excretory pore dorsal, 7−14 (11; 5) or <1−1% (1%; 5) from posterior body end (Figs. 1, 5).

Taxonomic summary

Type host: Alabama map turtle, *Graptemys pulchra* Baur, 1893. *Other host:* Black-knobbed map turtle, *Graptemys nigrinoda* Cagle, 1954.

Sites: Mesenteric blood vessels.

Type locality: Coosa River (32°32'19.68"N, 86°12'22.69"W), Wetumpka, Alabama.

Other locality: Alabama River (31°32'49.28"N, 87°30'57.82"W), Monroeville, Alabama.

Specimens examined: AMNH: holotype and paratype of *Spirorchis elegans* Stunkard, 1923 (AMNH 134 and AMNH 1516, respectively), holotype of *Spirorchis haematobius* (Stunkard, 1922) Price, 1934 (AMNH 126), holotype of *Spirorchis parvus* (Stunkard, 1923) Price, 1934 (AMNH 133), holotype of *Spirorchis picta* Stunkard, 1923 (AMNH 131), and holotype and paratype of *Spirorchis scripta* Stunkard, 1923 (AMNH 128 [damaged specimen] and AMNH 130). USNM: holotype and paratype of *Spirorchis artericola* (Ward, 1921) Stunkard, 1921 (USNM 1350610, 2 slides comprising 2 whole-mounted specimens), holotype and paratype of *Spirorchis innominatus* Ward, 1921 (USNM 1337308, 1 slide comprising 2 whole-mounted specimens), holotype of *Spirorchis minutus* Byrd, 1939 (USNM 1321965), holotype of *Spirorchis collinsi* Roberts and Bullard, 2016 (USNM XXXX), and holotype of *Spirorchis testiplexus* Roberts and Bullard, 2017 (USNM XXXX). TPRC: paratypes of *Spirorchis kirki* (Platt, 1990) Platt, 1992.

Specimens deposited: Holotype (USNM XXXXX), paratypes (USNM XXXXX).

Prevalence and intensity of infection (present study): One of 3 (33%) Alabama map turtles and 1 of 4 (25%) black-knobbed map turtles were infected by 7 and 4 specimens of *S. paraminutus* from the Coosa and Alabama Rivers, respectively.

Etymology: Specific epithet *paraminutus* refers to the morphological resemblance of the new species to *Spirorchis minutus* Byrd, 1939.

DISCUSSION

The new species resembles *Spirorchis haematobius* (Stunkard, 1922) Price, 1934, *Spirorchis minutus* Byrd, 1939, and *Spirorchis collinsi* Roberts and Bullard, 2016 by having a short testicular column (<1/4 of body length) with typically 10 testes. Of these, the new species more closely resembles *S. haematobius* and *S. minutus* by having gonads, terminal genitalia, and a common genital pore in the posterior half of the body. *Spirorchis collinsi* has a testicular column abutting the cecal bifurcation and a genital pore positioned in the middle of the body (Roberts et al., 2016c). The new species can be differentiated from both *S. haematobius* and *S. minutus* by having an elongate body (12−24× longer than wide vs. 6−10× for *S. haematobius* and 8−10× for *S. minutus*) and more anterior terminal genitalia, i.e., having a genital pore 26−29% of body length anterior to posterior body end (vs. 10−11% and 12% of body length from posterior body end).

In addition to the new species, we collected specimens of *Spirorchis elegans* Stunkard, 1923, *Spirorchis scripta* Stunkard, 1923, and two innominate *Spirorchis* species (Table I). Newly collected vouchers (USNM XXX) were identified as *S. elegans* by having ceca that do not extend far past the terminal genitalia, ten testes, and by lacking a median esophageal diverticulum. Newly collected vouchers (USNM XXXX) were identified as *S. scripta* by having a large median esophageal diverticulum, ceca that extend posterior far past the terminal genitalia, and a testicular column comprised of ten testes that almost abuts the cecal bifurcation. This study comprises both new host and new locality records for these TBFs. This is the first report of TBFs infecting Escambia map turtles, black-knobbed map turtles, and Alabama map turtles. This is also the first report of these two species from the Coosa River system in Alabama (Table I).

The two innominate species of *Spirorchis* were collected from an Escambia map turtle (*Spirorchis* sp. 1) and an Alabama map turtle (*Spirorchis* sp. 2; see Table I). Two specimens of *Spirorchis* sp. 1 were collected from a one of two Escambia map turtles from the Yellow River (see Table I). One of the specimens was used for DNA extraction. The one whole-mounted specimen (AUMNH XXXX) resembles *S. artericola* by having a median esophageal pouch, ceca with lateral diverticula and that do not extend far past the genitalia, a testicular column not abutting and posterior to the cecal bifurcation, and an external seminal vesicle ventral to the ovary. The specimen differed from *S. artericola* by having small testes that do not abut either cecum. Two specimens of *Spirorchis* sp. 2 were collected from one Alabama map turtle from the Tallapoosa River (See Table I). Similar to *Spirorchis* sp. 1, one of the two specimens was used in DNA extract, leaving only one specimen for whole-mounting. Morphological analysis showed this one whole-mounted specimen (AUMNH XXXX) to be a young adult with underdeveloped gonads and terminal genitalia, making species identification impossible. More material is needed for morphological analysis before we can assign species names to these specimens.

No previous study has used solely ITS2 sequence data to test phylogenetic relationships among species of *Spirorchis*. Previous workers have shown a strongly supported monophyletic *Spirirochis* using sequences of the large subunit rDNA (28S) (Orélis-Ribeiro et al., 2014; Roberts et al., 2016c; Roberts et al., 2017b). As in these previously published phylogenies, the tree herein (Figure 7) shows a strongly supported (posterior probability = 1) monophyletic *Spirorchis*. Both isolates of the new species (BF119, BF134) were identical. The tree suggests that *S. paraminutus* is most closely related to and forms a clade with *S. collinsi*. This clade is sister to *S. haematobius* and *Spirorchis picta*. Unfortunately, *S. minutus* has never been collected after its original description (Byrd, 1939), and no sequence data is available for comparison in this study.

The two sequences of *S. paraminutus* are 99.31% (BF119) and 99.32% (BF134) similar to *S. collinsi* (both with 3 base pairs difference). This slight difference in similarity is due to sequence BF134 being longer and sharing more base pairs with *S. collinsi* than sequence BF119. Because of this high percent similarity and the difference in sequence length, the two sequences of *S. paraminutus* did not clade together each of the 1,000,000 generations, resulting in a posterior probability <1 (0.93; see Figure 7).

Key to species of *Spirorchis* (emended and updated from Platt, 1993)

1.	Testes <10 in number (typically 4−6)	2
	Testes typically 10 (never <7)	3
2.	Ceca terminate at level of anterior testis	*S. kirki*
	Ceca terminate posterior to genital complex	*S. parvus*
3.	Testicular column <1/3 body length	4
	Testicular column >1/3 body length	8
4.	Anterior testis abutting or just posterior to cecal bifurcation	*S. collinsi*
	Anterior testis in posterior body half, genital pore	5
5.	Body elongate (>12× longer than wide), genital pore 1/4 of body length from posterior body end	*S. paraminutus*
	Body robust (≤10× longer than wide), genital pore 1/10 of body length from posterior body	6
7.	Mature adults massive, >4 mm	*S. haematobius*
	Mature adults small, ≤1 mm	*S. minutus*
8.	Median esophageal pouch absent	*S. elegans*
	Median esophageal pouch present	9

9. Ceca extending far posteriad past terminal genitalia, Manter's organ extending anteriad between ceca to near level of vitelline duct *S. scripta*

Ceca extending just posterior to terminal genitalia, Manter's organ extending anteriad to cecal termini 10

10. Ceca smooth, lacking lateral diverticula *S. innominatus*

Ceca having lateral diverticula 11

11. External seminal vesicle ventral to ovary *S. artericola*

External seminal vesicle between posterior testis and ovary, dorsal or dorsolateral to ovary *S. testiplexus*

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LITERATURE CITED

Brooks, D. R. 1979. New records for amphibians and reptile trematodes. Proceedings of the Helminthological Society of Washington **46:** 286–289.

Byrd, E. E. 1939. Studies on the blood flukes of the family Spirorchidae. Part II. Revision of the family and description of new species. Journal of the Tennessee Academy of Science **14:** 116−161.

Katoh, K., and D. M. Standley. 2013. MAFFT Multiple Sequence Alignment Software Version 7: improvements in performance and usability. Molecular Biology and Evolution **30:** 772−780.

Lindeman, P. V. 2013. The map turtle and sawback atlas: ecology, evolution, distribution, and conservation. The University of Oklahoma Press, Normal, Oklahoma, U.S.A. 460 p.

Maddison, W. P., and D.R. Maddison. 2017. Mesquite: a modular system for evolutionary analysis. Version 3.2. http://mesquiteproject.org.

Orélis-Ribeiro, R, C. R. Arias, K. M. Halanych, T. H. Cribb, and S. A. Bullard. 2014. Diversity and ancestry of flatworms infecting blood of nontetrapod craniates "fishes." Advances in Parasitology **85:** 1−64.

Platt, T. R. 1993. Taxonomic revision of *Spirorchis* MacCallum, 1919 (Digenea: Spirorchidae). Journal of Parasitology **79:** 337−346.

Rambaut, A. 2009. FigTree v1.2.3, Institute of Evolutionary Biology, Univ. of Edinburgh, available at: http://tree.bio.ed.ac.uk/software/figtree.

Rausch, R. L. 1947. Observations on some helminths parasitic in Ohio turtles. American Midland Naturalist **38:** 434−442.

Roberts, J. R., and S. A. Bullard. 2017. Revision and new species of *Vasotrema* Stunkard, 1926 (Digenea: Schistosomatoidea): turtle blood flukes of North American softshell turtles (Testudines: Trionychidae: *Apalone* spp.). Journal of Parasitology **XX:** XX−XX.

Roberts, J. R., T. R. Platt, R. Orélis-Ribeiro, and S. A. Bullard. 2016a. New genus of blood fluke (Digenea: Schistosomatoidea) from Malaysian freshwater turtles (Geoemydidae) and its phylogenetic position within Schistosomatoidea. Journal of Parasitology **102:** 451−462.

Roberts, J. R., R. Orélis-Ribeiro, B. T. Dang, K. M. Halanych, and S. A. Bullard. 2016b. Blood flukes of Asiatic softshell turtles: revision of *Coeuritrema* Mehra, 1933

(Digenea: Schistosomatoidea) and a new species infecting Chinese softshell turtles, *Pelodiscus sinensis*, (Trionychidae) from the Da Rang River, Vietnam. Folia Parasitologica **63:** 031 doi:10.14411/fp.2016.031.

Roberts, J. R., R. Orélis-Ribeiro, K. M. Halanych, C. R. Arias, and S. A. Bullard. 2016c. A new species of *Spirorchis* MacCallum, 1918 (Digenea: Schistosomatoidea) and *Spirorchis* cf. *scripta* from chicken turtle, *Deirochelys reticularia*, (Emydidae), with an emendation and molecular phylogeny of *Spirorchis*. Folia Parasitologica **63:** 041 doi:10.14411/fp.2016.041.

Roberts, J. R., K. M. Halanych, C. R. Arias, B. Folt, J. M. Goessling, and S. A. Bullard. 2017a. Emendation and new species of *Hapalorhynchus* Stunkard, 1922 (Digenea: Schistosomatoidea) from musk turtles (Kinosternidae: *Sternotherus*) in Alabama and Florida rivers. Parasitology International *in review*.

Ronquist, F., M. Teslenko, P. van der Mark, D. Ayres, A. Darling, S. Höhna, B. Larget, L. Liu, M. A. Suchard, and J. P. Huelsenbeck. 2011. MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology **61:** 539–542.

Stunkard, H. W. 1923. Studies on North American blood flukes. Bulletin of the American Museum of Natural History **48:** 165−221.

van Dijk, P. P., J. B. Iverson, A. G. J. Rhodin, H. B. Shaffer, and R. Bour. 2014. Turtles of the world, 7th Edition: Annotated checklist of taxonomy, synonymy, distribution with maps, and conservation status. Chelonian Research Monographs **5:** 329−479.

Ward, H. B. 1921. A new blood fluke from turtles. Journal of Parasitology **7:** 114−128.

FIGURE LEGENDS

Figures 1−5. *Spirorchis paraminutus* n. sp. from Alabama map turtle, *Graptemys pulchra* Baur, 1893, and black-knobbed map turte, *Graptemys nigrinoda* Cagle, 1954. All scale values are aside bar. (**1**) Body (dorsal view) of holotype (USNM Coll. No. XXXX) of *S. paraminutus* from Alabama map turtle from the Coosa River (32°32'19.68"N, 86°12'22.69"W) showing oral sucker (os), pharynx (ph), esophagus (es), nerve commissure (nc), esophageal gland (eg), median esophageal diverticulum (med), cecal bifurcation (cb), sinistral posterior cecum (sc), dextral posterior cecum (dc), vitellarium (vr), anterior testis (at), middle testis (t5), posterior-most testis (pt), external seminal vesicle (esv), ovary (ov), common genital pore (cgp), oötype (oo), cecal termini (ct), Manter's organ (Mo), and excretory pore (ep). (**2**) Genitalia of holotype (USNM Coll. No. XXXX) of *Spirorchis paraminutus* showing ninth testis (t9), vas deferens (vd), internal seminal vesicle (isv), cirrus sac (cs), cirrus (ci), oviduct (od), metraterm (mt), oviducal seminal receptacle (osr), transverse vitelline duct (tvd), descending tract of primary vitelline duct (dvt), ascending tract of primary vitelline duct (avt), and Laurer's canal (Lc). (**3**) Anterior fragment (dorsal view) of paratype (USNM Coll. No. XXXX) of *S. paraminutus* from black-knobbed map turtle from the Alabama River (31°32'49.28"N, 87°30'57.82"W). (**4**) Middle fragment (ventral view) of paratype (USNM Coll. No. XXXX) of *S. paraminutus* showing the second testis (t2). (**5**) Posterior fragment (ventral view) of paratype (USNM Coll. No. XXXX) of *S. paraminutus* showing the third testis (t3), everted cirrus (ec), and excretory vesicle (ev).

Figure 6. *Spirorchis paraminutus* n. sp. from black-knobbed map turte, *Graptemys nigrinoda* Cagle, 1954 from the Alabama River (31°32'49.28"N, 87°30'57.82"W). Scale value aside bar. (**6**) Genitalia (ventral view) of paratype (USNM Coll. No. XXXX) of *S. paraminutus* showing ninth testis (t9), vas deferens (vd), posterior testis (pt), external seminal vesicle (esv), cirrus sac (cs), internal seminal vesicle (isv), ovary (ov), everted cirrus (ec), common genital pore (cgp), oviduct (od), metraterm (mt), oviducal seminal receptacle (osr), oötype (oo), transverse vitelline duct (tvd), Laurer's canal (Lc), ascending tract of primary vitelline duct (avt), and descending tract of primary vitelline duct (dvt).

Figure 7. Phylogenetic relationships of selected blood flukes reconstructed by Bayesian inference and based on internal transcribed region (ITS2) sequences (11 turtle blood fluke taxa, 13 ITS2 sequences). Numbers aside tree nodes indicate posterior probability (only values >0.90 shown).

Table I. Geographic locality records and museum specimens of blood flukes infecting map turtles (*Graptemys* spp.)

Turtle host	Blood fluke	Site in host	Riverine locality	Accession no.	Reference
***Graptemys ernsti* Lovich and McCoy, 1992, Escambia map turtle**	***Spirorchis* sp. 1**	**mesenteric blood vessels (adult)**	**Yellow River (31° 5'44.55"N, 86°26'8.37"W), Alabama, U.S.A.**	**AUMNH XXXX**	**Present study**
Graptemys geographica (LeSueur, 1817), common map turtle	*Spirorchis innominatus* Ward, 1921	heart (adult)	Union County, south of Marysville (probably Three Mile Creek [Scioto River]), Ohio, U.S.A.	None specified	Rausch (1947)
	Unicaecum dissimilis Byrd, 1939	none specified (adult)	16 km north of Yazoo City (probably Bee Lake, Mississippi River), Mississippi, U.S.A.	None specified	Brooks (1979)
***Graptemys nigrinoda* (Cagle, 1954), black-knobbed map turtle**	***Spirorchis elegans* Stunkard, 1923**	**heart, esophagus (adult)**	**Coosa River (32°32'19.68"N, 86°12'22.69"W), Wetumpka, Alabama**	**USNM XXXX**	**Present study**
	***Spirorchis scripta* Stunkard, 1923**	**lumen of heart, blood vessels of liver, lung, intestine (adult)**	**Coosa River (32°32'19.68"N, 86°12'22.69"W), Wetumpka, Alabama**	**USNM XXXX**	**Present study**
	***Spirorchis paraminutus* n. sp.**	**mesenteric blood vessels, blood vessels of bladder (adult)**	**Alabama River (31°32'49.28"N, 87°30'57.82"W), Monroeville, Alabama**	**USNM XXXX**	**Present study**
Graptemys pseudogeographica (Gray, 1831), false map turtle	*Spirorchis artericola* (Ward, 1921) Stunkard, 1921	none specified (adult)	Ochlockonee River, Leon County, Florida, U.S.A.	None specified	Ward (1921)
	S. scripta	trachea (adult)	Newton (probably	None specified	Stunkard (1923)

			Sabine River), Texas		
	Unicaecum ruszkowskii Stunkard, 1927	heart (adult)	Reelfoot Lake (36°21'12.23"N, 89°25'21.50"W), Mississippi River, Tennessee, U.S.A.	None specified	Byrd (1939)
***Graptemys pulchra* (Baur, 1893), Alabama map turtle**	***Spirorchis* sp. 2**	**heart (adult)**	**Uphapee Creek (32°28'39.99"N, 85°41'43.25"W), Tallapoosa River, Tuskegee, Alabama**	**USNM XXXX**	**Present study**
	S. elegans	**esophagus (adult)**	**Coosa River (32°32'19.68"N, 86°12'22.69"W), Wetumpka, Alabama**	**USNM XXXX**	**Present study**
	S. scripta	**heart (adult)**	**Coosa River (32°32'19.68"N, 86°12'22.69"W), Wetumpka, Alabama**	**USNM XXXX**	**Present study**
	S. paraminutus	**mesenteric blood vessels (adult)**	**Coosa River (32°32'19.68"N, 86°12'22.69"W), Wetumpka, Alabama**	**USNM XXXX**	**Present study**

Table II. Internal transcribed region (ITS2) sequences generated for molecular analysis

Turtle blood flukes	Host species	Locality	GenBank Accession Numbers
			ITS2
Baracktrema obamai Roberts, Platt and Bullard, 2016	*Siebenrockiella crassicollis* (Gray, 1830), black marsh turtle	Perak (probably Perak River), Malaysia	XXXX
Spirorchis collinsi Roberts and Bullard, 2016	*Deirochelys reticularia* (Latreille *in* Sonnini and Latreille, 1801), chicken turtle	Big Beaver Pond (Tallapoosa River) (32°25'44.03"N, 85°38'44.87"W), Alabama, U.S.A.	XXXX
Spirorchis haematobius (Stunkard, 1922) Price, 1934	*Chelydra serpentina* (Linnaeus, 1758), common snapping turtle	Pascagoula River (30°37'07.67"N, 88°36'44.53"W), Mississippi, U.S.A.	XXXX
Spirorchis paraminutus n. sp.	*Graptemys pulchra* (Baur, 1893), Alabama map turtle	Coosa River (32°32'19.68"N, 86°12'22.69"W), Wetumpka, Alabama	XXXX
	Graptemys nigrinoda (Cagle, 1954), black-knobbed map turtle	Alabama River (31°32'49.28"N, 87°30'57.82"W), Monroeville, Alabama	XXXX
Spirorchis picta Stunkard, 1923	*Trachemys scripta* (Thunberg *in* Schoepff, 1792), pond slider	Pond off Saugahatchee Creek (Tallapoosa River) (32°38'52.78"N, 85°29'7.20"W), Alabama.	XXXX
Spirorchis scripta Stunkard, 1923	*G. pulchra*	Coosa River (32°32'19.68"N, 86°12'22.69"W), Wetumpka, Alabama	XXXX
			XXXX
Spirorchis cf. *scripta*	*D. reticularia*	Big Beaver Pond (Tallapoosa River) (32°25'44.03"N, 85°38'44.87"W), Alabama.	XXXX
Spirorchis sp. 1	*Graptemys ernsti* Lovich and McCoy, 1992, Escambia map turtle	Yellow River (31° 5'44.55"N, 86°26'8.37"W), Alabama, U.S.A.	XXXX
Spirorchis sp. 2	*Graptemys pulchra* (Baur, 1893), Alabama map turtle	Uphapee Creek (32°28'39.99"N, 85°41'43.25"W), Tallapoosa River, Tuskegee, Alabama	XXXX
S. testiplexus	*Pseudemys concinna* (Le Conte, 1830), river cooter	Pascagoula River (30°37'07.67"N, 88°36'44.53"W), Mississippi, U.S.A.	XXXX
Vasotrema cf. *robustum*	*Apalone spinifera aspera* (Agassiz, 1857), Gulf Coast spiny softshell turtle	Round Lake (Cahaba River) (32°41'50.91"N, 87°14'30.39"W), Cahaba River, Alabama.	XXXX

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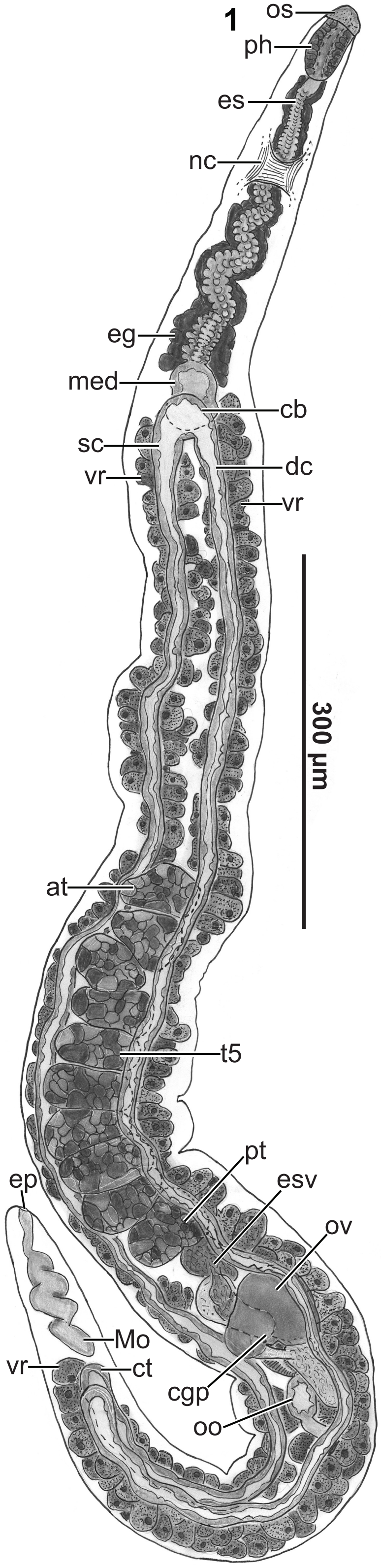
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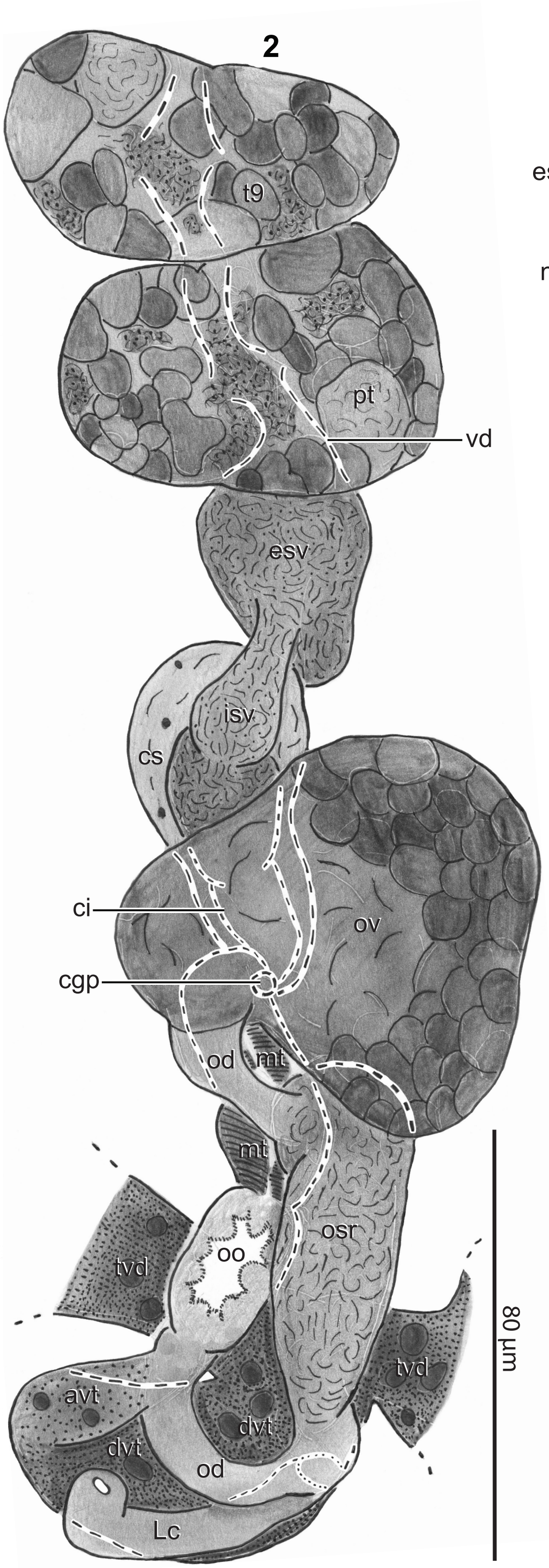
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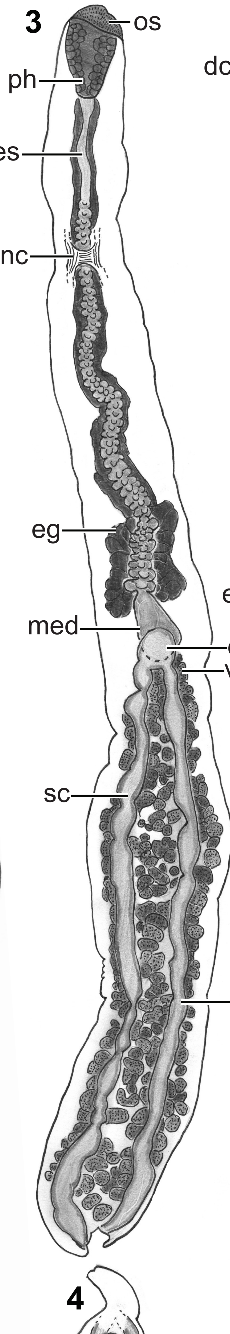
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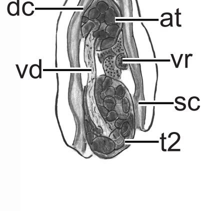
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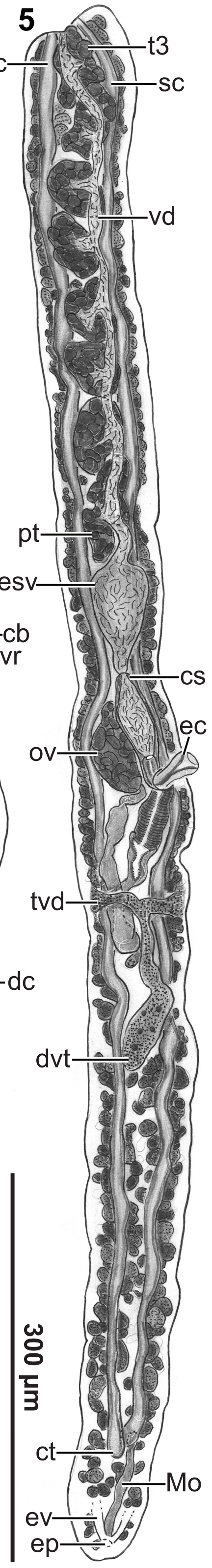
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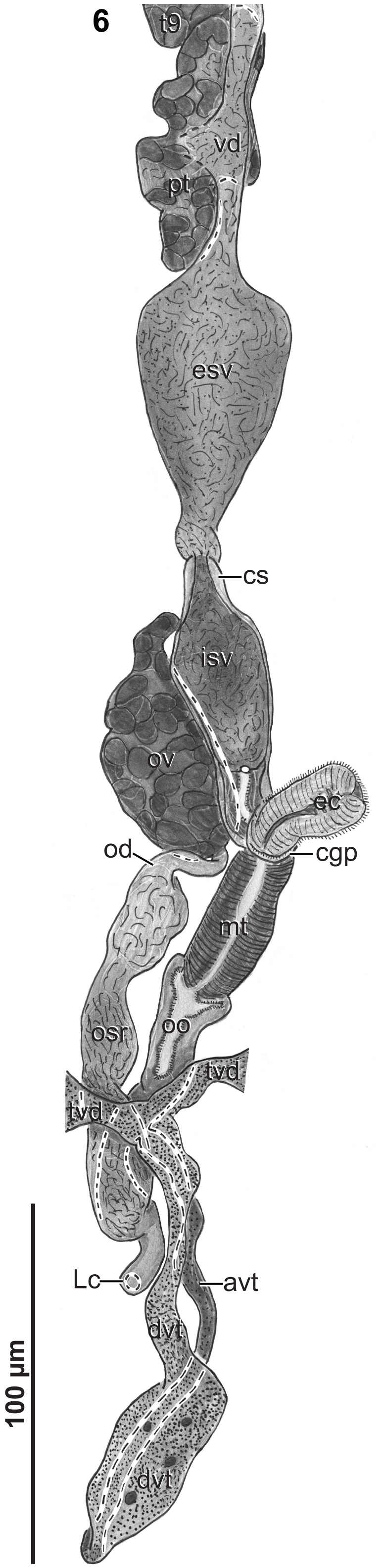
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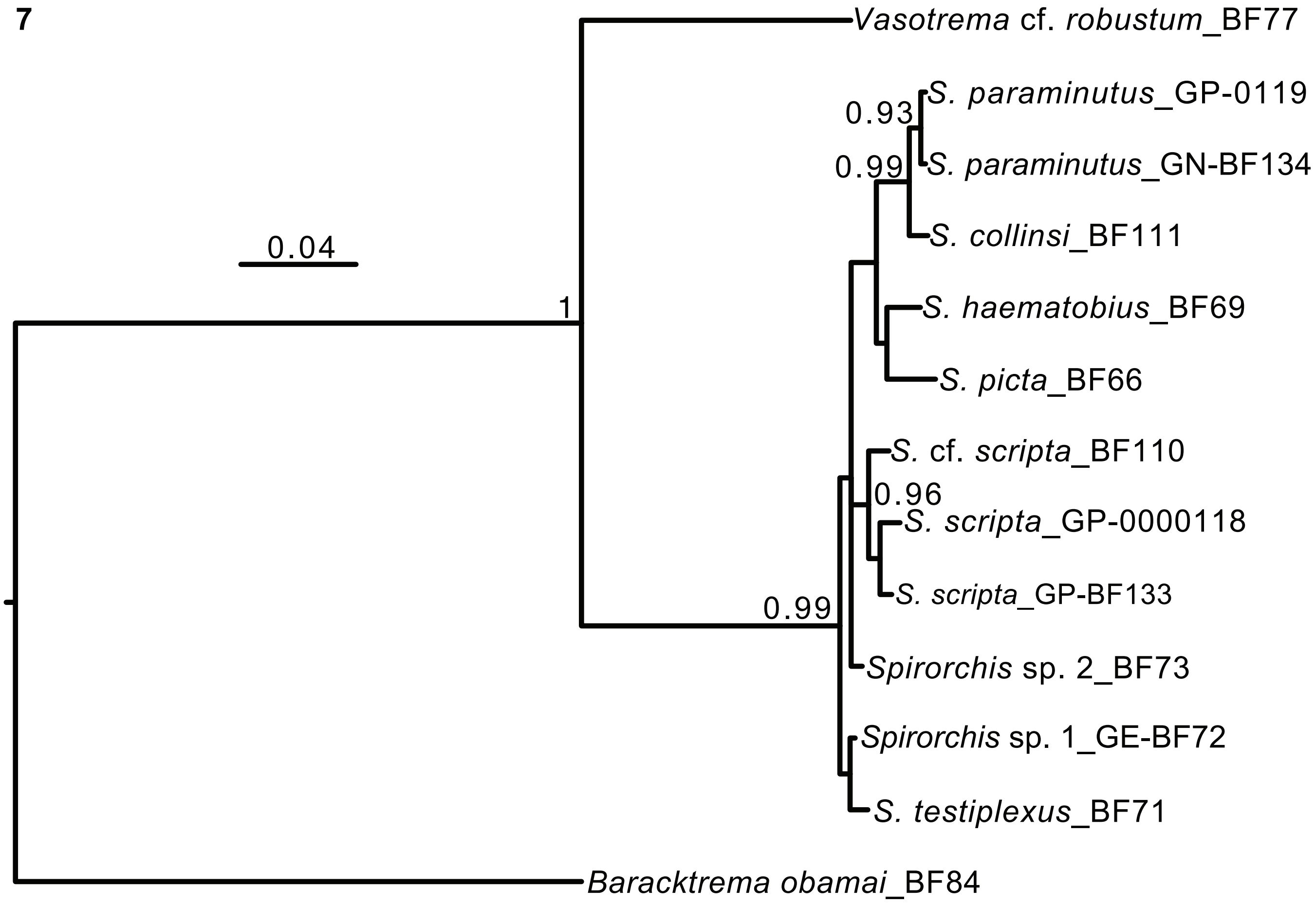

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