

# 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.

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**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 anteriorly 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, grouping sister to *Spirhapalum siamensis* Tkach, Snyder, and Vaughn, 2009.

**KEY WORDS:** *Spirorchis*, Emydidae, *Pseudemys concinna*, taxonomy, Pascagoula River, Reelfoot Lake, Coastal Plain, 28S, 18S.

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 se-

quence data for the new species toward 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/liter sodium citrate saline solution following Roberts, Oréllis-Ribeiro, Halanych et al. (2016). Live turtle blood flukes for morphology were killed, fixed, stained, and mounted following Roberts, Oréllis-Ribeiro, Halanych et al. (2016). 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, Oréllis-Ribeiro, Dang et al. (2016); Roberts, Oréllis-Ribeiro, Halanych et al. (2016); Roberts, Platt et al. (2016); and Roberts et al. (2017). Specimens intended for molecular analyses (a specimen of the newly

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**Table 1. *Spirorchis* spp. infecting river cooters, *Pseudemys concinna* (Le Conte, 1830) (Emydidae). (Bold terms are infections reported herein).**

<i>Spirorchis</i> sp.	Reported host	Site in host	Locality	Accession nos.*	Reference
<i>Spirorchis arcticola</i> Ward, 1921	as <i>Pseudemys hieroglyphica</i> 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)
<i>Spirorchis elegans</i> Stunkard, 1923	as <i>Pseudemys elonae</i> (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)
<b><i>Spirorchis</i> <i>testiflexus</i>, n. sp.</b>	<b><i>P. concinna</i></b>	<b>mesentery (adult)</b>	<b>Pascagoula River (30°37'07.67"N; 88°36'44.53"W), Mississippi, U.S.A.</b>	<b>USNM 1437615, 1437616, 1437617, 1437618, 1437619</b>	<b>Present study</b>
<i>Spirorchis</i> <i>innominatus</i> Ward, 1921 (type species)	as <i>P. hieroglyphica</i>	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 <i>Pseudemys concinna</i> <i>savannensis</i> 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)
<i>Spirorchis scripta</i> Stunkard, 1923	as <i>P. hieroglyphica</i> †	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)
	<b><i>P. concinna</i></b>	<b>heart (adult)</b>	<b>Pascagoula River (30°37'07.67"N; 88°36'44.53"W), Mississippi</b>	<b>USNM 1437620</b>	<b>Present study</b>

\* USNM, National Museum of Natural History; HWML, Harold W. Manter Laboratory Collection.

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

identified species, a specimen of *Spirorchis haematobius* [Stunkard, 1922] Price, 1934, and a specimen of *Vasotrema* cf. *robustum* Stunkard, 1928) were placed directly into absolute ethanol. The hologenophore (molecular voucher) of the new species was cut in half: the anterior end was used for DNA extraction and sequencing (GenBank MH002459 [28S], MH023418 [18S]) and the posterior end was stained and mounted as indicated above. Measurements are reported in micrometers ( $\mu\text{m}$ ) as the range 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 Natural History (USNM; Smithsonian Institute, Washington, D.C.), and Thomas Platt's personal research collection (TPRC), which Professor Platt has kindly donated to S.A.B. (see Taxonomic Summary; Materials Examined).

DNA extraction, amplification, and sequencing were performed following Oréllis-Ribeiro et al. (2014). 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 (1,272 nucleotide positions with 43 excluded) was deposited in Tree-Base (<http://purl.org/phylo/treebase/phyloids/study/TB2:S22396?format=html>). MrBayes 3.5.3 (Ronquist et al., 2012) was used to reconstruct a Bayesian inference phylogeny based on the 28S rDNA fragment. Using a General Time Reversal (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). Out-groups were selected from representative blood fluke genera informed by the phylogenetic analyses of Oréllis-Ribeiro et al. (2014); Roberts, Oréllis-Ribeiro, Dang et al. (2016); and Roberts, Platt et al. (2016). The in-group comprised all available 28S sequences for species of *Spirorchis* as well as freshwater turtle blood flukes likewise selected based on the above studies (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

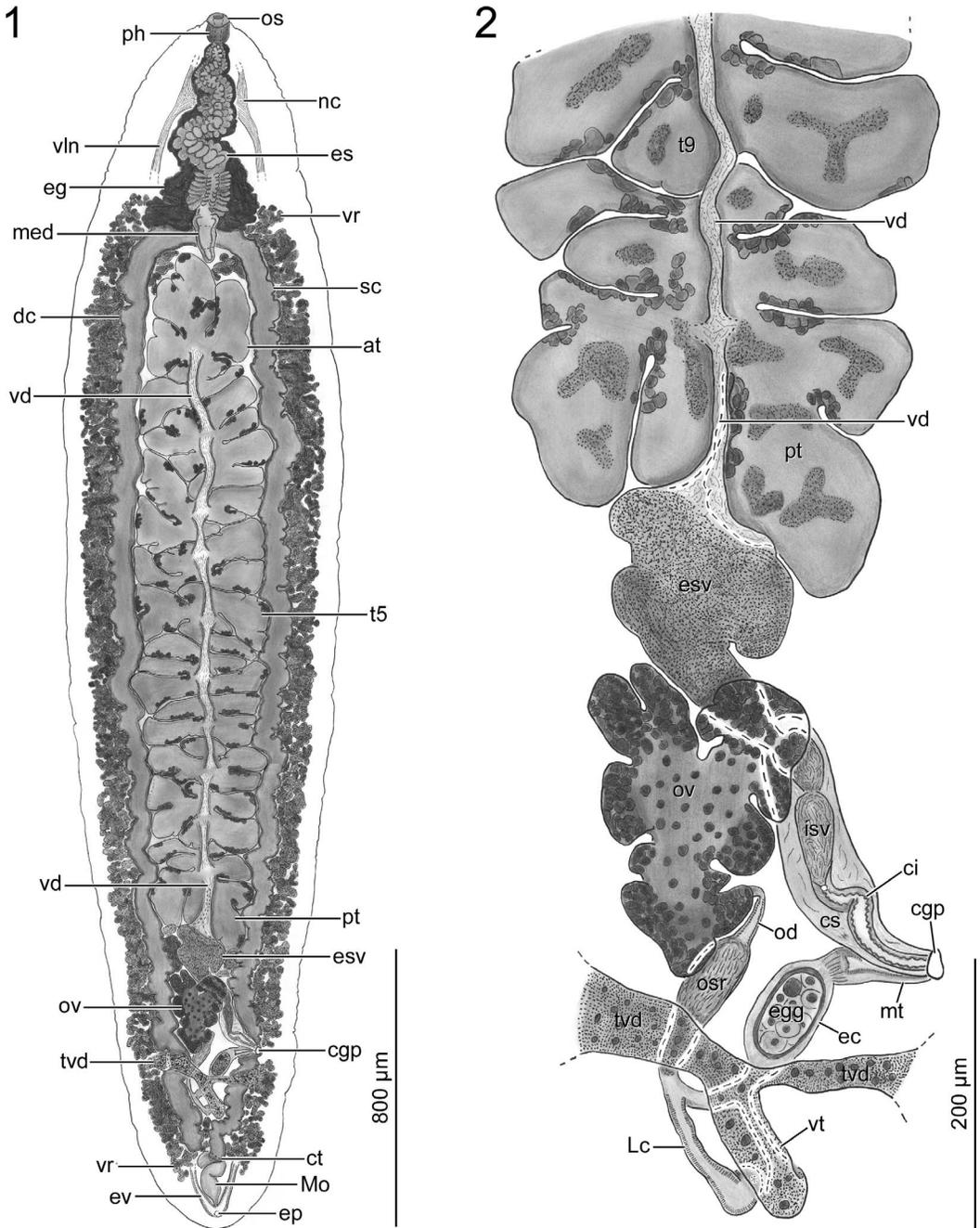
### *Spirorchis testiplexus* Roberts, Curran, and Bullard n. sp. (Figs. 1–4)

#### Description

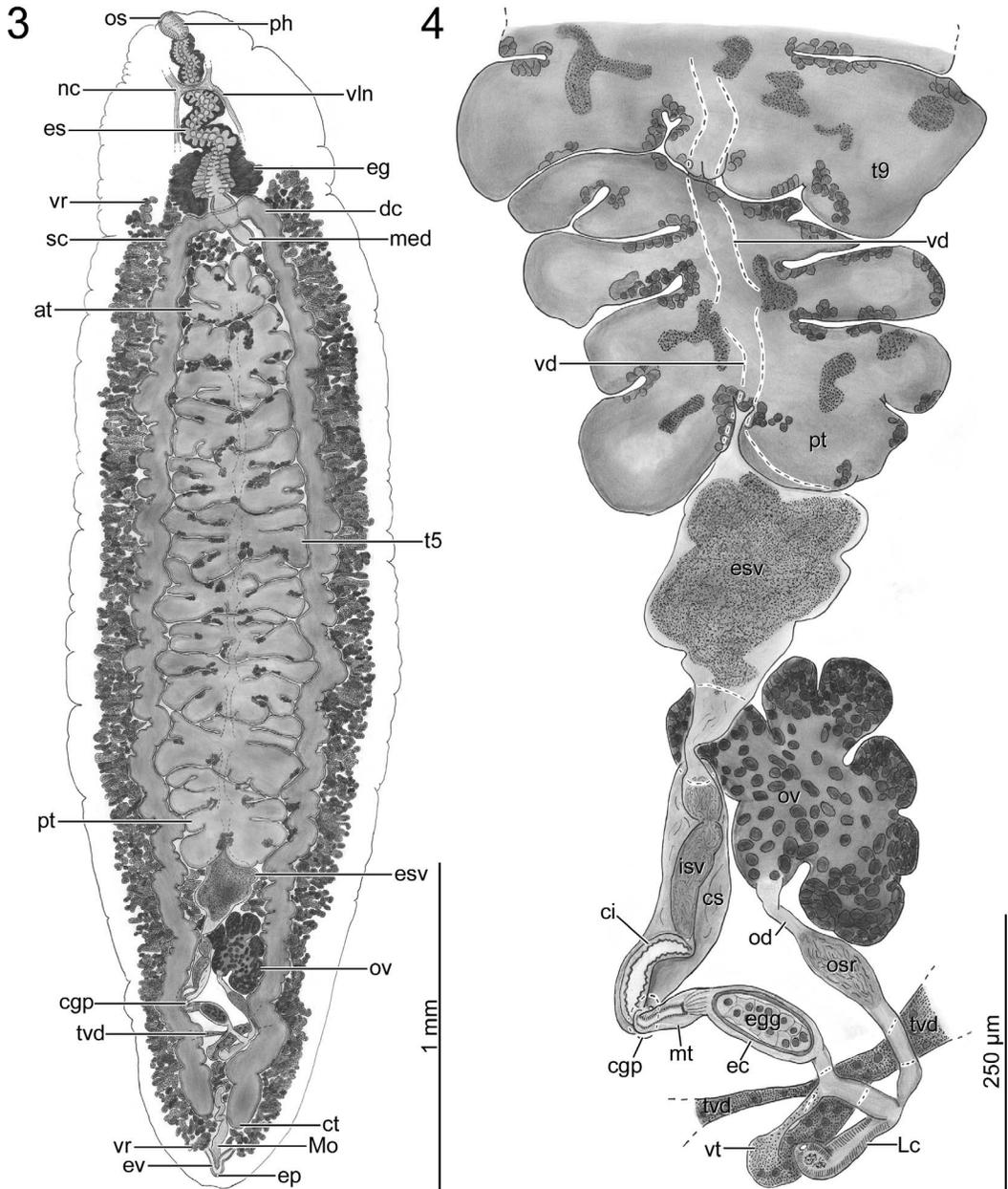
Based on light microscopy of 10 whole-mounted specimens. Body 2,650–3,850 (3,366; 10) long or 2.9–4.7 $\times$  (3.7; 10) longer than wide, 660–850 (738; 10) wide or 19–26% (22%; 10) of body length at level of cecal bifurcation, 720–1,010 (922; 10) wide or 21–34% (28%; 10) of body length at level of 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 $\times$  (2.5; 10) esophagus width immediately posterior to pharynx, 0.8–1.3 $\times$  (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

Table 2. Turtle blood fluke sequences (28S and 18S) used in the present study. (Bold sequences were either generated by or treated by authors herein.)

Turtle blood flukes	Host species	Locality	GenBank accession numbers		Reference
			28S	18S	
<i>Baracktrema obamai</i> Roberts, Platt and Bullard, 2016	<i>Siebenrockiella crassicolis</i> (Gray, 1830), black marsh turtle	Perak (probably Perak River), Malaysia	KX061500	—	Roberts, Platt et al. (2016)
<i>Spirhapalum polesianum</i> Ejsmont, 1927	<i>Enys orbicularis</i> (Linnaeus, 1758), European pond turtle	Lesniki, Ukraine	AY604705	—	Snyder (2004)
<i>Spirhapalum siamensis</i> Tkach, Snyder and Vaughn, 2009	<i>Cuora amboinensis</i> (Riche in Daudin, 1801), Southeast Asian pond turtle	Moei River (16°42'N; 98°34'E), Mae Sot, Thailand	FJ481166	—	Tkach et al. (2009)
<b><i>Spirorchis testiflexus</i>, n. sp.</b> (as <i>Spirorchis arcticola</i> [Ward, 1921] Stunkard, 1921)	<b><i>Chrysemys picta</i> (Schneider, 1783), painted turtle</b>	<b>Reelfoot Lake (Mississippi River) (36°21'12.23"N; 89°25'21.50"W), Tennessee, U.S.A</b>	<b>AY604704</b>	<b>AY604712.1</b>	<b>Snyder (2004)</b>
<i>S. testiflexus</i>	<i>Pseudemys concinna</i> (Le Conte, 1830), river cooter	<b>Pascagoula River (30°37'07.67"N; 88°36'44.53"W), Mississippi, U.S.A.</b>	<b>MH002459</b>	<b>MH023418</b>	<b>Present study</b>
<i>Spirorchis collinsi</i>	<i>Deirochelys reticularia</i> (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, Oréllis-Ribeiro, Halanych et al. (2016)
<i>Spirorchis haematobius</i> (Stunkard, 1922) Price, 1934	<i>Chelydra serpentina</i> (Linnaeus, 1758), common snapping turtle	Missouri River, Iowa, U.S.A.	FJ481164	—	Tkach et al. (2009)
<b><i>S. haematobius</i></b>	<b><i>C. serpentina</i></b>	<b>Pascagoula River (30°37'07.67"N; 88°36'44.53"W), Mississippi</b>	<b>MH002458</b>	—	<b>Present study</b>
<i>Spirorchis picta</i> Stunkard, 1923	<i>Trachemys scripta</i> (Thunberg in Schoepff, 1792), pond slider	Pond off Saughatchee Creek (Tallapoosa River) (32°38'52.78"N; 85°29'7.20"W), Alabama	KY091665	—	Roberts, Oréllis-Ribeiro, Halanych et al. (2016)
<i>Spirorchis scripta</i> Stunkard, 1923	<i>T. scripta</i>	Moungers Creek (Pascagoula River), Mississippi	AY222174	AY222093	Olson et al. (2003)
<i>Spirorchis</i> cf. <i>scripta</i>	<i>D. reticularia</i>	Big Beaver Pond (Tallapoosa River) (32°25'44.03"N; 85°38'44.87"W), Alabama	KY091666	—	Roberts, Oréllis-Ribeiro, Halanych et al. (2016)
<i>Unicaecum</i> sp.	<i>T. scripta</i>	Reelfoot Lake (Mississippi River) (36°21'12.23"N; 89°25'21.50"W), Tennessee	AY604711	—	Snyder (2004)
<i>Vasotrema robustum</i> Stunkard, 1928	<i>Apatone spinifera</i> (LeSueur, 1827), spiny softshell turtle	Nishnabotna River, Iowa	AY604706	—	Snyder (2004)
<b><i>Vasotrema</i> cf. <i>robustum</i></b>	<b><i>Apatone spinifera aspera</i> (Agassiz, 1857), Gulf Coast spiny softshell turtle</b>	<b>Round Lake (Cahaba River) (32°41'50.91"N; 87°14'30.39"W), Cahaba River, Alabama</b>	<b>MH002457</b>	—	<b>Present study</b>



**Figures 1, 2.** Line drawings of *Spirorchis testiplexus* n. sp. (holotype; USNM 1437615) from river cooter, *Pseudemys concinna* (Le Conte, 1830); ventral view of adult. **1.** Whole worm. **2.** Genitalia. Abbreviations: anterior testis (at), cecal termini (ct), cirrus (ci), cirrus sac (cs), common genital pore (cgp), dextral posterior cecum (dc), egg chamber (ec), esophageal gland (eg), esophagus (es), excretory pore (ep), excretory vesicle (ev), external seminal vesicle (esv), internal seminal vesicle (isv), Laurer's canal (Lc), Manter's organ (Mo), median esophageal diverticulum (med), metraterm (mt), middle testis (t5), nerve commissure (nc), ninth testis (t9), oral sucker (os), ovary (ov), oviducal seminal receptacle (osr), oviduct (od), pharynx (ph), posterior-most testis (pt), primary vitelline duct (vt), sinistral posterior cecum (sc), transverse vitelline duct (tvd), uterine egg (egg), vas deferens (vd), ventrolateral nerve chords (vln), vitellarium (vr).



**Figures 3, 4.** Line drawings of adult of *Spirorchis testiplexus* n. sp. from river cooter, *Pseudemys concinna* (Le Conte, 1830); dorsal view. **3.** Whole worm; paratype (USNM 1437616). **4.** Genitalia of paratype (USNM 1437616). Abbreviations: anterior testis (at), cecal termini (ct), cirrus (ci), cirrus sac (cs), common genital pore (cgp), dextral posterior cecum (dc), egg chamber (ec), esophageal gland (eg), esophagus (es), excretory pore (ep), excretory vesicle (ev), external seminal vesicle (esv), internal seminal vesicle (isv), Laurer's canal (Lc), Manter's organ (Mo), median esophageal diverticulum (med), metraterm (mt), middle testis (t5), nerve commissure (nc), ninth testis (t9), oral sucker (os), ovary (ov), oviducal seminal receptacle (osr), oviduct (od), pharynx (ph), posterior-most testis (pt), primary vitelline duct (vt), sinistral posterior cecum (sc), transverse vitelline duct (tvd), uterine egg (egg), vas deferens (vd), ventrolateral nerve chords (vln), vitellarium (vr).

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; postcecal 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 (1,854; 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,  $0.4\text{--}1.8\times$  (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,  $1.3\text{--}3.1\times$  (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,  $0.8\text{--}1.8\times$  (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,  $0.9\text{--}2.1\times$  (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,  $3.1\text{--}4.8\times$  (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,  $0.7\text{--}1.4\times$  (0.9; 10) wider than long (Figs. 1–4); postovarian 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 1437615); paratypes (USNM 1437616, 1437617, 1437618); hologenophore (USNM 1437619); GenBank (MH002459 [28S], MH023418 [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.

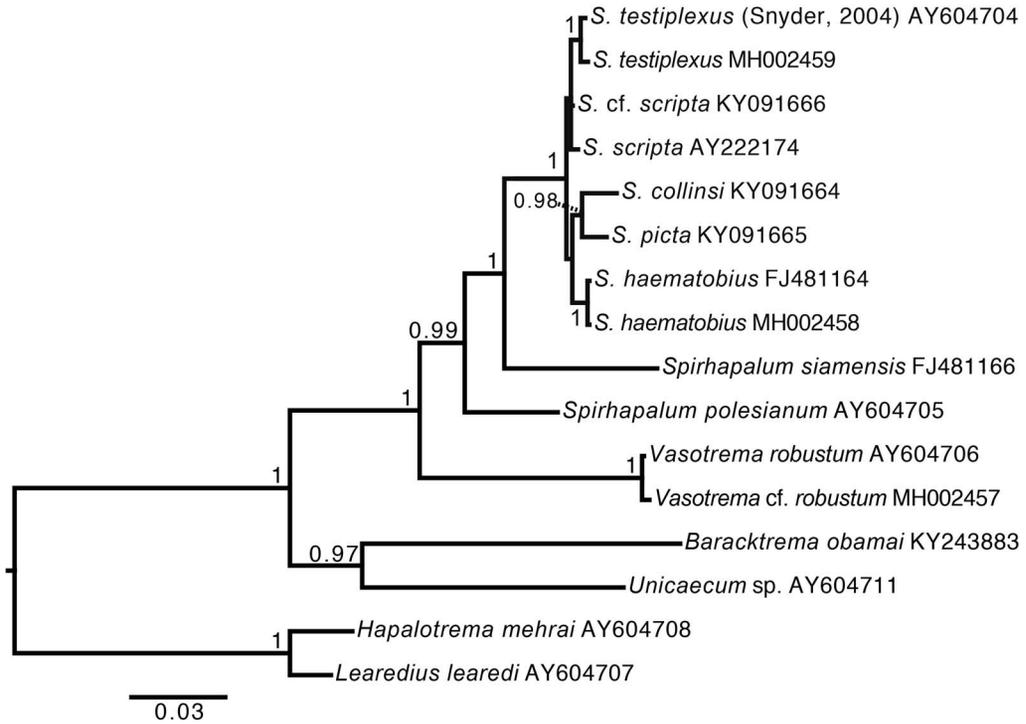
*Etymology:* The specific epithet *testiplexus* is from the Latin “plexus” (interwoven) and refers to the strongly lobed testes of the new species that resemble interlocked puzzle pieces.

### Molecular results

Available 28S sequences for *S. scripta* (AY222174.1) and *S. cf. scripta* (KY091666; Roberts, Oréris-Ribeiro, Halanych et al. 2016) were 99.4% (8 nucleotide differences) and 99.5% (8 nucleotide differences) similar, respectively, to the new species based on pairwise comparisons of the full-length sequences. 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; alignment positions 27, 721, and 817 in the 28S) 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* as sister to *Spirhapalum siamensis* Tkach, Snyder, and Vaughn, 2009 (Fig. 5). As previously reported (Tkach et al., 2009; Oréris-Ribeiro et al., 2014; Roberts, Oréris-Ribeiro, Halanych et al., 2016; Roberts, Platt et al., 2016), *Spirhapalum* Ejsmont, 1927, is paraphyletic, with *Spirhapalum polesianum* Ejsmont, 1927, sister to the *Spirorchis* spp. + *S. siamensis* clade.

## 5



**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).

### 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 anteriorly 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 differentiate morphologically similar species of *Spirorchis*. It is entirely preovarian (*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. However, the orientation of the external

seminal vesicle combined with the presence/absence of the median esophageal diverticulum and diverticulate posterior ceca, as well as testicular column position, comprise helpful differential characters for members of the genus.

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, a 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 previous taxonomic descriptions of this species (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, 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 anteriorly to near the level of the proximal margin of the median esophageal diverticulum (vs. extending to near the level of the nerve commissure). Previous authors have described the posterior ceca of *Spirorchis* spp. as smooth, simple, or sinuous (Stunkard, 1922, 1923; Platt, 1990, 2002; Roberts, Oréllis-Ribeiro, Halanych et al., 2016). Based on the results of the present study, we suggest that the diagnosis of *Spirorchis* should include “posterior ceca smooth or having lateral diverticula.”

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).

## DISCUSSION

A given turtle species may host multiple turtle blood fluke species (Stunkard, 1922, 1928; Byrd, 1939; Ulmer, 1959; Brooks and Mayes, 1975; Roberts, Oréllis-Ribeiro, Halanych et al., 2016; Roberts and Bullard, 2017) and concurrent infections are not uncommon among turtles (Roberts and Bullard, personal observations). This includes simultaneous infection by congeneric turtle blood flukes (Ulmer, 1959; Roberts, Oréllis-Ribeiro, Halanych et al., 2016; 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 is 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 straightforward because taxonomists may not be, or perhaps rarely are, 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 host identity, previously published records, gross appearance of the fluke, or the assumption of a nonconcurrent infection should be avoided. As demonstrated by the subtly distinctive *Spirorchis* spp. herein, even light microscopic study of an unstained 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 semiaquatic 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 yellow-blotched 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 Schoeff, 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 United States 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. To our knowledge, 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 high diversity of freshwater mollusks that range in the southeastern United States (Bogan et al., 1995; Neves et al., 1997; Johnson et al., 2013), 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 nonvagile 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 ex-

ploration 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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