Taxonomy, systematics, and life cycle of digeneans (Platyhelminthes: Trematoda) infecting freshwater fishes

by

Triet Nhat Truong

A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
December 9, 2023

Keywords: Taxonomic revision, systematics, emendation, life cycle, resurrection

Approved by

Stephen A. Bullard, Chair, Professor, School of Fisheries, Aquaculture, and Aquatic Sciences
Anita M. Kelly, Extension Professor, School of Fisheries, Aquaculture, and Aquatic Sciences
Nathan V. Whelan, Asst. Res. Professor, School of Fisheries, Aquaculture, and Aquatic Sciences
Stephen S. Curran, Research Assistant, School of Fisheries, Aquaculture, and Aquatic Sciences

Abstract

This dissertation is structured in 7 chapters. In the dissertation, I described 4 new digenean species and redescribed 3 species belonging to 7 genera of 5 families, erected 1 new genus, resurrected and emended the diagnoses of 3 genera, and elucidated the life cycle of 3 species, using combined morphological and nucleotide sequence-based (internal transcribed spacer regions [ITS1 and ITS2] and 28S rDNA) evidence. The results of this dissertation are 7 papers that have been published, in press, or under reviewed in peer-reviewed scientific journals, including Journal of Parasitology, Systematic Parasitology, Comparative Parasitology, and Parasitology International.

Posthovitellinum psiloterminae n. gen., n. sp. (Lissorchiidae) infects the intestine of *Cyclocheilos enoplos* (Bleeker, 1849) (Cypriniformes: Cyprinidae), a migratory riverine carp from the Mekong River (Dong Thap province, Vietnam). *Posthovitellinum psiloterminae* is a unique asymphylodorine by having a well-developed cirrus-sac, an unarmed ejaculatory duct and metraterm, a follicular vitellarium distributing in 2 lateral fields located between the posterior margin of the ventral sucker and the mid-level of the testis, and a sinistral, submarginal genital pore.

Pseudoparamacroderoides Gupta and Agrawal, 1968 (Macroderoididae) differs from other macroderoidid genera by having the combination of a subspherical oral sucker that lacks distinctly-enlarged circumoral spines; caeca that extend posteriad beyond the testes without forming a cyclocoel; testes that are approximately ≤1/3 maximum body width in diameter; a cirrus sac that is claviform, slightly dorsal to and predominantly lateral to the ventral sucker; symmetrical vitelline fields that extend posteriad to the middle of the post-testicular space and that remain separate anteriorly and posteriorly; and an excretory vesicle that is I-shaped and

wholly post-ovarian, inter-testicular, or median to the posterior testis. *Pseudoparamacroderoides dongthapensis* n. sp. infects the intestine of a riverine catfish, *Mystus mysticetus* Roberts, (Siluriformes: Bagridae) in the Mekong River, Vietnam and differs from its congeners by having an elongate hindbody and an excretory vesicle that is approximately half as long as the body and that extends anteriad beyond the anterior testis.

Plesiocreadium Winfield, 1929 (Macroderoididae) differs from other macroderoidids by having a dorsoventrally flat forebody, ceca that extend posteriad beyond the testes and that do not form a cyclocoel, testes that are greater than one-half of maximum body width, a cirrus sac that is dorsal to the ventral sucker and arches dextrad or sinistrad, a uterine seminal receptacle, asymmetrical vitelline fields that remain separated anteriorly and posteriorly and that extend anteriad to the level of the ventral sucker, and an I-shaped excretory vesicle. *Plesiocreadium typicum* Winfield, 1929 infects the intestine of bowfins, *Amia calva* Linnaeus, 1766 (Amiiformes: Amiidae), captured in the L'Anguille River (Mississippi River Basin, Arkansas), Big Lake (Pascagoula River Basin, Mississippi), Chittenango Creek (Oneida Lake, New York), and Reelfoot Lake (Tennessee River Basin, Tennessee).

Plagioporus wataugaensis n. sp. (Opecoelidae) infects the intestine of the northern hogsucker, *Hypentelium nigricans* (Lesueur, 1817), and the white sucker, *Catostomus commersonii* (Lacepède, 1803), (both Cypriniformes: Catostomidae) in the eastern USA. *Plagioporus wataugaensis* has vitelline fields that are discontinuous at the level of the ventral sucker and follicles that surround the ceca and that span the midline dorsal to the testes and an excretory vesicle that is wholly post-testicular and short (6–9% of the body length).

Proterometra wigglewomble n. sp. (Azygiidae) asexually reproduces in the compact elimia, *Elimia showalteri* (Lea, 1860) (Cerithioidea: Pleuroceridae) and matures in the esophagus of the

blackbanded darter, *Percina nigrofasciata* (Agassiz, 1854) (Perciformes: Percidae) in Cahaba River, Alabama. Adults of the new species differ from congeners by having a small body and eggs having a wholly fimbriated surface that appears as a cilia-like brush border. Live naturally-shed cercariae of the new species differ from those of its congeners by having a strongly claviform tail stem bearing aspinose mammillae, a single furca, excretory pores that open on the posterior margin of the single furca, and few eggs in the cercarial distome. Naturally-shed cercariae of *P. wigglewomble* secrete a jelly-like adhesive that coats the surface of the furca and evidently facilitates attachment to the surface of glass, plastic, and snail shell and vigorously wiggle about once attached, as if mimicking the larva of a stream insect so as to lure the blackbanded darter to eat it.

Adults of *Leuceruthrus stephanocauda* (Faust, 1921) Womble and Bullard, 2022 (Azygiidae) infect the stomach of spotted bass, *Micropterus punctulatus* (Rafinesque, 1819), green sunfish, *Lepomis cyanellus* Rafinesque, 1819, and longear sunfish, *Lepomis megalotis* (Rafinesque, 1820) from Chewacla Creek (Tallapoosa River, Alabama) and mottled sculpin, *Cottus bairdii* Girard, 1850 (juvenile) from Raccoon Creek (Chattooga River, Georgia) and differ from its congeners by having an elongated body and an asymmetrical vitellarium. The cercaria of *L. stephanocauda* sheds from yellow elimia, *Elimia flava* (Lea, 1862) (Cerithioidea: Pleuroceridae) in Chewacla Creek and Moores Mill Creek (Tallapoosa River) and *Elimia caelatura georgiana* (Lea, 1862) in Raccoon Creek. Naturally-shed cercariae of *L. stephanocauda* are unique by having bilateral, discontinuous fields of black tail stem pigmentation posterior to the withdrawn distome and along the margins of the paired furcae, prominent subtriangular spines on the anterior end of the tail stem anterior and posterior to the distome, and broadly rounded to lanceolate furcae that are longer than wide and that bear numerous marginal and submarginal protuberances.

Transversotrema cf. *patialense* (Transversotrematidae) asexually reproduces in the red-rimmed melania, *Melanoides tuberculata* (Müller, 1774) (Cerithioidea: Thiaridae) and matures beneath the scales of the zebrafish, *Danio rerio* (Hamilton, 1822) (Cypriniformes: Danionidae) within a spring-fed earthen pond aquaculture system (a private aquaculture facility) in the vicinity of Ruskin, Florida. The adult of *T.* cf. *patialense* has a body that is 1.8–2.2× wider than long, a ventral sucker that is 1.2–1.7× wider than the pharynx, and a primarily extra-cecal follicular vitellarium extending anteromediad nearly to the level of the eyespots. Cercariae actively swam once liberated from the crushed snails and had well-defined arm-like processes each bearing an adhesive pad, an elongate tail stem, oar-shaped furcae each marginated with a membranous, pleated fin fold, well-developed male and female genitalia, and lacked vitelline follicles. The redia had a broadly rounded anterior body end and a constricted, diminutive tail process at the posterior body end.

Acknowledgments

I would like to sincerely and especially thank Dr. Stephen Bullard, my advisor and mentor, for generously providing all necessary laboratory equipment and resources used for my studies. From my very early days at the laboratory, Dr. Bullard was always willing to spent hours of his extremely busy schedule to teach me how to properly use dissecting and compound microscropes, stain and mount specimens, examine the whole mounts and identify the digenetic worms and their fish hosts using the dichotomous keys. During the 5-year program, I have learned a lot from his detailed guidance, corrections, enthusiasm, and continuous encouragement. When I started working on my first scientific paper, he sat down next to me and showed me how to write the first sentence using telegraphic description, make a line drawing of a worm using his most valuable compound microscope, and format the manuscript following the journal guidelines. As a wonderful mentor, Dr. Bullard always cared a lot about the success of his students, encouraged his students to continuously work hard to achieve their goals under a well-organized, feasible plan. One of the best thing I learned from him is to make a “to-do list”. A complicated, seemingly undoable project could be done if we split it into smaller parts, make a detailed plan for each part, and keep making and finishing daily to-do lists to achieve each of these parts. Without his guidance and help, I definitely could not finish this dissertation and my PhD program. Dr. Bullard also acted as a good friend and helped me overcome my most difficult time during my life in 2023.

My especial thank to Dr. Stephen Curran, who is also one of my friend and PhD committee members, for his strong interest, encouragement, expertise, and guidance in the taxonomy, systematics, and life cycle of digeneans. I thank Steven Ksepka, Micah Warren, Haley Dutton, Jake Shurba, John Brule, and Kamila Mora, my best-ever labmates and colleagues, who were

always willing to spend uncountable days with me in the field and in the laboratory helping collect fishes, snails and examine them for parasitic infections and identifications.

I would like to especially thank my parents, Trung V. Truong and Luy T. Nguyen, for providing unconditional financial and mental support when I need; Binh T. Quang and Mai T. Ho, my parents-in-law who fully trusted me and accepted me to marry their second oldest, beloved daughter.

Finally, I would like to honor my deeply beloved wife and life partner, Yen T. M. Quang, who has been always beside me, loved and cared about me no matter what happened in life. We have met over sixteen years ago and have been sharing life goals, responsibilities, sorrows, and happiness as well as overcoming numerous difficulties during. That time period seems not to be so long, but I will never forget your faithful, unconditional, and forever love you gave me. Our two wonderful, beloved daughters, Quynh N. Truong and Quynh T. Truong are the sweet outcome of our forever love and the wonderful legacy you left for me. You are forever present and filled up my heart.

Table of Contents

List of Tables

List of Figures

Chapter 1

CHAPTER 1: *POSTHOVITELLINUM PSILOTERMINAE* N. GEN., N. SP. (DIGENEA: LISSORCHIIDAE) INFECTING THE INTESTINE OF *CYCLOCHEILOS ENOPLOS* (CYPRINIFORMES: CYPRINIDAE) IN THE MEKONG RIVER, VIETNAM

***Published in Journal of Parasitology (Available 1 June 2021)**

Authors: Triet N. Truong, Micah B. Warren, Steven P. Ksepka, Stephen S. Curran, and Stephen A. Bullard

ABSTRACT

We herein describe a new species and propose a new genus, *Posthovitellinum psiloterminae* n. gen., n. sp. (Lissorchiidae: Asymphylodorinae), based on specimens that infect the intestine of *Cyclocheilos enoplos* (Bleeker, 1849) (Cypriniformes: Cyprinidae), a migratory riverine carp from the Mekong River (Dong Thap province, Vietnam). The new species is assigned to Lissorchiidae by having a combination of features: spinous tegument, subterminal oral sucker, pre-equatorial ventral sucker, median and pretesticular ovary, submarginal genital pore at level of the ventral sucker, follicular vitellarium distributing in 2 lateral fields, and lacking eyespot pigment in the adult. It cannot be assigned to any existing asymphylodorine genus because it has the combination of a well-developed cirrus-sac, an unarmed ejaculatory duct and metraterm, a follicular vitellarium distributing in 2 lateral fields located between the posterior margin of the ventral sucker and the mid-level of the testis, and a sinistral, submarginal genital pore. The new species has an elongate, claviform cirrus-sac, a single, large, elongate-oval testis at the posterior extremity of the body, operculate eggs, and an I-shaped excretory bladder with secondary branches at the level of the testis and extending anteriad to the level of the pharynx. Bayesian inference analysis of the partial large subunit ribosomal DNA gene (*28S rDNA*) recovered the new species sister to *Asaccotrema vietnamiense* Sokolov and Gordeev, 2019; these species

differed by 118 nucleotides (12%; 983 bp fragment). This is the first lissorchiid reported from the Mekong River; only the second from southern Vietnam; and the fourth reported from a cyprinid fish in Vietnam. The aforementioned phylogenetic analysis included previously unpublished sequences representing lissorchiids infecting the intestine of North American suckers (Cypriniformes: Catostomidae): *Lissorchis* cf. *nelsoni* from spotted sucker, *Minytrema melanops* (Rafinesque, 1820) and *Lissorchis* cf. *gullaris* (immature) from smallmouth buffalo, *Ictiobus bubalus* (Rafinesque, 1818). *Asymphylodora atherinopsidis* Annereaux, 1947 herein is treated as a species *incertae sedis*. The *28S* tree topology suggests that Lissorchiinae may comprise more than 1 lineage but additional species are needed to confidently assert this.

Monorchioidea Odhner, 1911 comprises the 3 digenean families Monorchiidae Odhner, 1911 (>290 species in 58 genera; see Madhavi, 2008), Lissorchiidae Magath, 1917 (76 species in 9 genera; see Bray, 2008; Sokolov and Gordeev, 2019), and Deropristidae Cable and Hunninen, 1942 (7 species in 3 genera; see Bray, 2005; Choudhury, 2009; Sokolov et al., 2020); which all primarily mature in fishes. Monorchiids infect the intestine of littoral marine fishes (Bray, 2008). Deropristids infect only Holarctic acipenseriform and anguilliform fishes and have freshwater, brackish, and marine life histories (Sokolov et al., 2020). Lissorchiids mature in wholly freshwater Holarctic fishes (principally intestine). A few have progenetic metacercariae that infect molluscan (Biguet et al., 1956; Stunkard, 1959; Tang, 1980; Kudlai, 2010) or piscine (Stunkard, 1959; Schell, 1973; Sokolov and Gordeev, 2019) intermediate hosts, and some species descriptions are based on progenetic metacercariae only (Sokolov and Gordeev, 2019). Lissorchiidae, the focus taxon for the present work, has 2 subfamilies: Lissorchiinae Magath, 1917 (32 species each having 2 testes) and Asymphylodorinae Szidat, 1943 (43 species each

having 1 testis). The Asymphylodorinae has 5 accepted genera: *Asymphylodora* Looss, 1899; *Brahamputrotrema* Gupta, 1955; *Wangxiyunia* Bray, 2008; *Prosovitellina* Wang, 1985; and *Asaccotrema* Sokolov and Gordeev, 2019 (Bray, 2008; Sokolov and Gordeev, 2019). *Tigrotrema* Bhaduria and Dandotia, 1984 is *incertae sedis* because its description is incomplete and includes several features that do not conform to the diagnosis of Lissorchiidae, e.g., a median and postbifurcal genital pore in the forebody, a post-testicular ovary, and an extensively follicular vitellarium in the hindbody (Bray, 2008). Although Vietnam is a hotspot of diversity for Cypriniformes (minnows and carps) (see Orsi, 1974), the primary host group for asymphylodorines, only 3 previous reports detail these Vietnamese fishes as hosts for asymphylodorine trematodes.

The Mekong River Delta (MRD) comprises >40,500 km^2 of the Mekong River and its fisheries resources together feed >60 million residents, making it the world's largest combined freshwater fishery (International Rivers, 2017). An estimated 322 fish species of 77 families range in the MRD (Tran et al., 2013), making it among the most fish biodiverse rivers (International Rivers, 2017). Few fish digeneans have been described or reported from the MRD: *Nomasanguinicola canthoensis* Truong and Bullard, 2013 (Digenea: Aporocotylidae) infecting branchial vessels of *Clarias macrocephalus* Günther, 1864 (Siluriformes: Clariidae) (see Truong and Bullard, 2013) plus 3 lissorchiids (i.e., adults of *Asymphylodora japonica* Yamaguti, 1938 and *Asymphylodora* sp. infecting the intestine of the common carp, *Cyprinus carpio* Linnaeus, 1758 [Cypriniformes: Cyprinidae] [see Ha and Duc, 2005] plus metacercariae of *Asaccotrema vietnamiense* Sokolov and Gordeev, 2019 encysted in the liver of the sidestripe rasbora, *Rasbora paviana* Tirant, 1885 [Cyprinidae] [see Sokolov and Gordeev, 2019]).

There is urgency associated with documenting fish and fish parasite diversity in the Mekong River. Seven hydropower dams have been or will be soon built on the mainstem of the Mekong River. Ten more dams will soon be constructed in China, Lao People's Democratic Republic (Lao PDR), and Cambodia; despite obvious conservation concerns for the aquatic fauna of the downstream nations of Cambodia and Vietnam (International Rivers, 2017). Dams will almost certainly reduce endemic fish (and fish parasite) biodiversity and productivity in the MRD, and migratory fishes, like the type host for the new species we describe herein, could be extirpated due to lack of river passage to critical spawning habitats (Baran, 2006; Mekong River Commission, 2015).

During a survey of fish parasites in the Mekong River during 2018, we collected numerous lissorchiid specimens infecting the intestine of a single individual of *Cyclocheilos enoplos* (Bleeker, 1849) (Cypriniformes: Cyprinidae), a highly migratory riverine fish. We herein describe these specimens as a new species and propose a new asymphylodorine genus to accommodate the new species. Additionally, we sequenced the partial *28S rDNA* gene fragment of the new species combined with several new sequences from North American lissorchiids to determine interrelationships among Monorchioidea.

MATERIALS AND METHODS

Fish were purchased alive from Cao Lanh Fish Market, Dong Thap province, Vietnam (10°27′12.8″N, 105°38′14.3″E) during September 2018. These fish were captured from the Mekong River Delta (the senior author [TNT] resided in and was raised in that area; it is common knowledge that this market handles locally captured fishes only). Fish were bagged, transported on ice to a nearby laboratory (Dong Thap Community College), euthanized by spinal severance, and dissected on the same day. Fish identification follows Tran et al. (2013). The host

for the new species was identified as *C. enoplos* by having a slender body, a pointed mouth with 4 barbels and smooth lips, and a lateral line with 35–37 scales. Fish were dissected, associated viscera excised and placed in isolated dishes filled with saline solution (0.9% NaCl), and the intestine was examined for parasites using a Wild Heerbrugg M5A (Wild Heerbrugg, Heerbrugg, Switzerland) stereodissecting microscope and fiber optic light sources in the field. From that collection, we obtained 40 live, mature trematode specimens from the intestine of an individual *C. enoplus*. In North America, we collected 4 spotted suckers, *Minytrema melanops* (Rafinesque, 1820) (Pascagoula River, Mississippi; 30°36′10″N, 88°37′42″W; 18–20 March 2012) infected with 10 adults of *Lissorchis* cf. *nelsoni*; 1 highfin carpsucker, *Carpiodes velifer* (Rafinesque, 1820) (Pascagoula River, Mississippi; 30°50′00″N, 88°44′42″W; 19 March 2012) infected with 5 adults of *Lissorchis kritskyi* Barnhart and Powell, 1979; 1 smallmouth buffalo, *Ictiobus bubalus* (Rafinesque, 1818) infected with 5 immature specimens of *Lissorchis* cf. *gullaris*, 1 black buffalo, *Ictiobus niger* (Rafinesque, 1819) and 2 quillbacks, *Carpioides cyprinus* (Lesueur, 1817) (Lake Chotard, Mississippi; 32°35′25″N, 91°01′24″W; 23 March 2012), each infected with at least 1 adult of *L*. cf. *gullaris*; and 1 smallmouth buffalo (Reelfoot Lake, Tennessee; 36°22′34″N, 89°25′56″W; 18 October 2003) infected with 1 adult of *L*. cf. *gullaris*.

Trematode specimens intended for morphology were rinsed with physiologic saline using fine artists' brushes to remove debris, flame-killed on a microscope slide using a coverslip to restrain the specimen but without putting any pressure on the specimen, and fixed in 10% neutral buffer formalin. Specimens for DNA extraction were placed directly in 95% ethanol. Fixed specimens were stained in Van Cleave's and Ehrlich's hematoxylins overnight or in Meyer's hematoxylin or Semichon's aceto-carmine for 30 min. Stained specimens were dehydrated in an ethanol (EtOH) series, cleared in clove oil, and permanently mounted on glass slides using

Canada balsam. Illustrations were made using an Olympus BX51 microscope (Olympus Corp. of the Americas, Center Valley, Pennsylvania) equipped with differential interference contrast optical components and drawing tube. Measurements were made using an ocular micrometer and reported in micrometers (μm) as the range followed by the mean ± standard deviation and sample size in parentheses.

Scientific names, including taxonomic authorities and dates, for fishes follow Eschmeyer et al. (2016). Morphological terms and nomenclature for intestinal flukes follows Bray (2008), Sokolov and Gordeev (2019), and Panyi et al. (2020).

Type specimens (1 holotype and 4 paratypes; all adults) of the new species, 3 vouchers (adults) of *L.* cf. *nelsoni*, 2 vouchers (adults) of *L. kritskyi*, and 8 vouchers (4 adults, 4 immatures) of *L.* cf. *gullaris* were deposited in the National Museum of Natural History's Invertebrate Zoology Collection (USNM, Smithsonian Institution, Washington, D. C.).

DNA was extracted from at least 2 specimens of each collected lissorchiid species (see above) using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Following extraction, DNA concentration was measured using a NanoDrop–1000 spectrophotometer (Thermo Scientific, Nanodrop Technologies, Waltham, Massachusetts), diluted to 20 ng/μl, and stored at –20 C. A fragment of the large subunit rDNA (*28S*) was amplified from the Vietnamese worms using primers U178 (5'–GCACCGCTAAYTTAAG–3') and L1642 (5'–CCAGCGCCATCCATTTTCA3') and the internal transcribed spacer 2 region (*ITS2*) using primers GA1 (5'–AGAACATCGACATCTTGAAC–3') and ITS2.2 (5'–CCTGGTTAGTTTCTTTTCCTCCGC–3') (Orélis-Ribeiro et al., 2017). PCR reactions for amplifying the *ITS2* and *28S rDNA* gene were performed with the following thermocycling parameters: initial denaturation step of 94 C for 4

min, followed by 40 cycles of 94 C for 40 sec, 60 C for 30 sec, 72 C for 2 min, with a final extension step of 72 C for 5 min. In addition to U178 and L1642, the 2 sequencing primers 300F (5'–CAAGTACCGTGAGGGAAAGTTG–3') and 1200R (5'–GCATAGTTCACCATCTTCGG–3') were used to improve sequencing coverage on the *28S* (Orélis-Ribeiro et al., 2017). Two replicates were used to identify any potential variability in the *28S* or *ITS2*. PCR product purification was conducted using the QIAquick PCR Purification kit (Qiagen). DNA sequencing was performed by ACGT Incorporated (Wheeling, Illinois). Forward and reverse sequences of the new species were aligned using MAFFT and low quality read-ends were trimmed resulting in sequences of 1,501 and 1,317 (*28S*) and 397 and 394 (*ITS2*) nucleotides (Katoh and Standley, 2013). A fragment of rDNA spanning the internal transcribed spacer regions (partial *ITS1*, complete *5.8S* gene, and complete *ITS2* region) and a partial fragment of the *28S rDNA* gene were targeted from the worms collected from Mississippi. These sequences were prepared following the methods outlined in Andres et al. (2015) and using the primers cited in Claxton et al. (2017). These resulting sequences measured at least 2,495 bp long. The obtained *5.8S*, and *ITS1* sequences were not used in the present study because there are not enough available data in GenBank for making comparison. All nucleotide sequence data from the new species and newly generated sequences of *L.* cf. *nelsoni*, *L. kritskyi*, and *L.* cf. *gullaris* were deposited in GenBank (Table I).

Taxon selection for phylogenetic analyses was based on earlier studies investigating the systematics of Monorchioidea (see Olson et al., 2003; Atopkin et al., 2017; Wee et al., 2018; Panyi et al., 2020; Petkevičiūtė et al., 2020; Sokolov et al., 2020) and included 2 sequences of the new species, 22 sequences representing the ingroup (Lepocreadioidea Odhner, 1905, Monorchioidea Odhner, 1911), and the outgroup was represented by Apocreadiidae Skrjabin,

1942 (Table I). Sequences were aligned using MAFFT (Katoh and Standley, 2013). JModelTest 2 version 2.1.10 was implemented to perform statistical selection of the best-fit models of nucleotide substitution based on Bayesian information criteria (BIC) (Darriba et al., 2012). Aligned sequences were reformatted (from .fasta to .nexus) using the web application ALTER (Glez-Peña et al., 2010) to run Bayesian inference analysis (BI). BI was performed in MrBayes version 3.2.5 (Ronquist and Huelsenbeck, 2003) using substitution model averaging (nst-mixed) and a gamma distribution to model rate-heterogeneity. Defaults were used in all other parameters. Three independent runs with four Metropolis-coupled chains were run for 5,000,000 generations, sampling the posterior distribution every 1,000 generations. Convergence was checked using Tracer v1.6.1 (Rambaut et al., 2014b) and the sump command in MrBayes. All runs appeared to reach convergence after discarding the first 25% of generation as burn-in. A majority rule consensus tree of the post burn-in posterior distribution was generated with the sumt command in MrBayes. The inferred phylogenetic tree was visualized using FigTree v1.4.3 (Rambaut et al., 2014a) and further edited for visualization purposes with Adobe Illustrator (Adobe Systems).

DESCRIPTION

Posthovitellinum Truong, Curran, and Bullard n. gen.

(Figs. 1–7)

With characters of Lissorchiidae, Asymphylodorinae after Bray (2008). Body fusiform. Tegument spinous on both sides. Oral sucker subspherical, smaller than ventral sucker. Pre-pharynx short, wider than long. Pharynx muscular, nearly spherical. Esophagus long (at least twice pharynx length), tubular, bifurcating at level of ventral sucker. Ceca broad, extending to anterior half or middle of testis. Cirrus-sac claviform, well-developed. Internal seminal vesicle

bipartite. Prostatic vesicle compact, surrounded by thick prostate gland cells. Ejaculatory duct unarmed, elongate. Genital atrium present (nearly indistinct). Genital pore sinistral, submarginal, at level of ventral sucker. Ovary subspherical, pretesticular. Uterus tubular, having coils distributing from ventral sucker to posterior body end. Metraterm prominent, muscular, aspinous. Eggs numerous and operculate. Vitelline follicles in 2 lateral fields, confined in the hindbody. Excretory bladder I-shaped. Excretory pore opens at posterior extremity. Adults infecting a cyprinid fish in Mekong River basin.

Taxonomic summary

Type and only known species: *Posthovitellinum psiloterminae* Truong, Curran, and Bullard n. sp.

ZooBank registration:

Etymology: *Posthovitellinum* is a Latin neuter (noun) for the vitellarium confined to the hindbody.

***Posthovitellinum psiloterminae* Truong, Curran, and Bullard n. sp.**

(Figs. 1–7)

Description (based on 10 stained, mounted specimens of adult worms). Body small, tapering at posterior extremity, 1,025–1,200 (1,086 ± 63; 10) long, 350–435 (382 ± 23; 10) wide, widest at level of posterior margin of ventral sucker. Forebody 210–310 (260 ± 34; 10) long or 20–28% (24 ± 3%; 10) of body length. Hindbody almost twice as long as forebody, 640–800 (682 ± 47; 10) long or 58–67% (63 ± 3%; 10) of body length. Tegument covered with spines; spines conical, distributing on ventral and dorsal body surfaces, denser near anterior end. Eyespot pigmentation absent. Oral sucker wider than long, 90–145 (116 ± 14; 10) long and 130–185 (144 ± 17; 10) wide and covered by smaller spines than those on the tegument. Ventral sucker pre-equatorial, median, subspherical, wider than long, 120–195 (154 ± 21; 10) long, 165–230 (182 ±

18; 10) wide and also covered by small spines. Oral sucker to ventral sucker width ratio 1:1.3 (1:1.1–1.4; 10) (Figs. 1, 2). Pre-pharynx 10–45 (24 ± 10; 10) long and 13–45 (26 ± 9; 10). Pharynx well-developed, 50–95 (68 ± 12; 10) long, 45–80 (53 ± 11; 10) wide. Esophagus 130–230 (181 ± 31; 10) long, 20–45 (29 ± 8; 10) wide. Ceca extend bilaterally to mid-testis (Figs. 1, 2).

Testis single, large, median, ellipsoid to elongated-ovoid, 220–290 (254 ± 22; 10) long, 120–165 (141 ± 14; 10) wide, close to posterior extremity, overlapping ovary posterodorsally. Vas deferens single, thin duct, connecting the testis and the seminal vesicle in the cirrus-sac. External seminal vesicle absent. Cirrus-sac muscular, 143–375 (284 ± 67; 10) long, 60–85 (73 ± 7; 10) wide, occasionally recurved, laying posterodorsally to ventral sucker. Internal seminal vesicle 100–145 (114 ± 13; 10) long, 38–63 (47 ± 7; 10) wide, positioning at proximal end of the cirrus-sac. Prostate gland cells surrounding the prostatic vesicle and terminal male duct. Prostatic vesicle short, filled with nucleated cells, locating at proximal end of the ejaculatory duct and connecting it with the internal seminal vesicle. Ejaculatory duct muscular, eversible, 108–265 (159 ± 43; 10) long, 14–28 (20 ± 5; 10) wide. Genital atrium small, indistinct in some specimens. Genital pore submarginal to marginal, 15–60 (29 ± 16; 10) long, 10–55 (26 ± 13; 10) wide, opening in posterior half of ventral sucker (Figs. 1, 2, 6).

Ovary single, median, 88–140 (111 ± 15; 10) long, 100–145 (114 ± 15; 10) wide, overlapping testis anteroventrally. Oviduct long, having cluster of striated-like muscle on dorsal surface. Oötype thick-walled, surrounded with Mehlis' gland. Seminal receptacle saccular, 40–45 (43 ± 4; 2) long, 15–18 (17 ± 2; 2) wide, located at about the mid-oviduct and the vitelline reservoir. Laurer's canal not observed (Figs. 1, 2, 7). Uterine seminal receptacle not observed (Figs. 1, 2). Vitelline fields spanning extracecal and intercecal zone, usually distributing from

posterior margin of ventral sucker or posterior to ventral sucker to anterior margin of testis or middle of testis; vitelline fields connecting by a pre-ovarian transverse reservoir (Figs. 1, 2). Uterus filled with eggs, ascending from oötype and forming pre-ovarian coils on right side of hindbody, then coils descending laterally on the right side of hindbody and reaching into postesticular space, then ascending back to pre-ovarian zone and crossing to the left side of hindbody, before again descending laterally to postesticular zone and finally ascending back once again on the left side and forming a well-defined metraterm; metraterm broad, opening at genital atrium (Figs. 1, 2, 6). Uterine eggs ovoid or slightly elongate, 23–35 (29 ± 3; 22) long, 10–18 (15 ± 3; 22) wide (Fig. 5).

Excretory bladder tubular, dorsal, bifurcating at level of testis; excretory ducts branching at level of ventral sucker; subdivided ducts extending to pharynx. Excretory pore subterminal (Figs. 1–4).

Taxonomic summary

Type and only reported host: Cyclocheilos enoplos (Bleeker, 1849) (Cypriniformes: Cyprinidae).

Type locality: Lower Mekong River Basin (Cao Lanh Fish Market, Dong Thap province, Vietnam, 10°27′12.8″N, 105°38′14.3″E).

Specimens deposited: Holotype (USNM 1620811) and 4 paratypes (USNM 1620812–1620815); *Lissorchis* cf. *nelsoni* (USNM 1620816–1620818), 3 vouchers; *Lissorchis kritskyi* (USNM 1620819, USNM 1620820), 2 vouchers; *Lissorchis* cf. *gullaris* (USNM 1620821–1620824, USNM 1620828), 5 vouchers from *Ictiobus bubalus*, 1 voucher from *Ictiobus niger* (USNM 1620825), 2 vouchers from *Carpioides cyprinus* (USNM 1620826, USNM 1620827).

Sequences deposited: Posthovitellinum psiloterminae identical fragments spanning *ITS2* (GenBank Nos. MT928347, MT928348) and partial *28S rDNA* gene (MT928351, MT928352);

Lissorchis kritskyi partial *ITS1*, complete *5.8S*, complete *ITS2* region, partial *28S rDNA* gene (MT928329); *Lissorchis* cf. *nelsoni* partial *ITS1*, complete *5.8S*, complete *ITS2* region, partial *28S rDNA* gene (MT928354); *Lissorchis* cf. *gullaris* partial *ITS1*, complete *5.8S*, complete *ITS2* region, partial *28S rDNA* gene (MT928353).

Site in host: Intestine.

Prevalence and intensity: One specimen of *C. enoplos* was infected with 40 adult specimens of the new species.

Zoobank registration:

Etymology: The specific epithet *psiloterminae* is a Latin noun; *psilo* (smooth) and *terminus* refers to each of the male and female terminal genital ducts, which lack armament. The ending is neuter to agree with the genus and because the term represents both sexes.

Remarks

The new species, like most of other lissorchiids, infects the intestine of a freshwater fish. It is clearly a lissorchiid by having a spinous tegument, a pretesticular ovary, a submarginal genital pore, a follicular vitellarium in 2 lateral fields, and eggs that are operculate and lack polar filaments as well as by lacking eyespot pigment in the body. We assign the new species to Asymphylodorinae since it has a single testis. In having the vitellarium confined to the hindbody, the new species differs from *Prosovitellina* spp. (vitellarium confined to forebody) and *Brahamputrotrema* spp. (vitellarium predominantly distributed in forebody). By having unarmed male and female terminal genitalia, the new species differs from *Prosovitellina* spp. (spined ejaculatory duct and metraterm). By having a sinistral submarginal genital pore, it differs from *Brahamputrotrema* spp. (dextral submarginal genital pore). *Posthovitellinum psiloterminae* differs from *Asaccotrema vietnamiense* (known only from progenetic metacercariae infecting

liver of sidestripe rasbora in Vietnam) by having a well-developed cirrus-sac and a sinistral, submarginal genital pore at level of the ventral sucker. In contrast, *Asaccotrema vietnamiense* lacks a cirrus-sac and has a dextral submarginal genital pore that opens more anteriorly at level of the esophagus. By having a subspherical oral sucker and unarmed terminal genitalia, *P. psiloterminae* differs from *Wanxiyunia cyprini* (Wang, 1982) Bray, 2008, which has a massive infundibuliform oral sucker and armed ejaculatory duct and metraterm. The new species is superficially most similar to species in *Asymphylodora* by having a claviform cirus-sac, a genital pore that opens at level of ventral sucker, a pretesticular ovary, a uterus that distributes in hindbody, and operculate eggs; however, it differs from all species of *Asymphylodora* by having an unarmed ejaculatory duct and metraterm.

The main stem of the excretory bladder of *P. psiloterminae* ascends to the level of the anterior region of the testis, nearly to the posterior margin of the ovary, in the dorsal aspect of the hindbody where it splits into 2 symmetrical collecting ducts. Each duct ascends diagonally into the extracecal body space and subdivides again, with each of the 4 remaining tubes ascending to the level of the esophagus and pharynx where they terminate (Figs. 3, 4).

Phylogenetic results

The resulting *28S rDNA* gene fragments from both specimens representing the new species were identical. After alignment with other sequences (983 bp, including gaps), the *28S* sequences of the new species were most similar to those of *Asaccotrema vietnamiense* (MK863409) but differed by 118 nucleotides (12%). The *28S* fragment from *P. psiloterminae* differed from *L. kritskyi* (EF032689), *L.* cf. *gullaris* (MT928353), and *L.* cf. *nelsoni* (MT928354) by 135 (14%), 140 (14%), and 135 (14%) nucleotides, respectively. The *28S* sequences of the new species differed from 4 sequences of *Asymphylodora progenetica* Serkova and Bykhowskii, 1940

(MT103402), *Asymphylodora perccotti* Besprozvannykh, Ermolenko and Atopkin, 2012 (FR822731), *Asymphylodora* sp. (MT153917), and *Asymphylodora* sp. (MN726955) by 157 (16%), 164 (17%), 140 (14%), and 156 (16%) nucleotides, respectively. The *28S* sequences of *P. psiloterminae* differed from *Palaeorchis incognitus* Szidat, 1943 (MT103410) by 133 (14%) nucleotides. An *ITS2* alignment was created with each of the generated sequences from *P. psiloterminae*, *L. kritskyi*, *L.* cf. *gullaris*, and *L.* cf. *nelsoni* and 3 other available lissorchiid sequences obtained from GenBank (*A. progenetica* [MT103399], *Asymphylodora* sp. [MT153914], and *P. incognitus* [MT103406]) that measured 304 bp long, including gaps. No intraspecific variation was observed within replicate sequences from each species. *Posthovitellinum psiloterminae* differed from *L. kritskyi* (MT928329), *L.* cf. *gullaris* (MT928353), and *L.* cf. *nelsoni* (MT928354) by 105 (35%), 103 (34%), and 102 (33%) nucleotides, respectively. The *ITS2* sequence of the new species differed from *A. progenetica*, *Asymphylodora* sp., and *P. incognitus* by 106 (35%), 96 (32%), and 95 (31%) nucleotides, respectively. The *28S* tree (Fig. 8) recovered the new species within a clade that included the only other Vietnamese sequence from a lissorchiid (*Asaccotrema vietnamiense*).

This analysis indicated monophyly of Lissorchiidae and Monorchiidae, which is consistent with previous phylogenetic studies (Olson et al., 2003; Atopkin et al., 2017; Sokolov and Gordeev, 2019; Panyi et al., 2020; Petkevičiūtė et al., 2020; Sokolov et al., 2020). All of the asymphylodorine sequences were monophyletic and recovered as a sister clade with the European lissorchiine *P. incognitus*. Three species of *Lissorchis* (Lissorchiinae) from North America formed a well-supported higher clade with a clade including all Palearctic *Asymphylodora* spp. + Southeast-Asian asymphylodorines (*Posthovitellinum* + *Asaccotrema*) + European *Palaeorchis* (Fig. 8).

DISCUSSION

The configuration of the uterus in *Lissorchis* spp. is important to detail in taxonomic descriptions (Barger, 2010; Gale et al., 2014). Gale et al. (2014) diagnosed species of *Lissorchis* by assessing 3 uterine configurations: uterine coils that laterally descend from and ascend back to the preovarian zone on both sides of the hindbody (only sinistral loops extend posterior to the testis) (= “type A”); as in type A but uterine loops are present in the sinistral side of the hindbody only (= “type B”); as in type A but both dextral and sinistral uterine loops extend posterior to the testis) (= “type C”). Sokolov and Gordeev (2019) considered this character to be problematic and ignored uterine configuration; however, we think that this feature is indeed an important taxonomic feature that should be described. In *P. psiloterminae*, the uterine pattern closely conforms to type A (Gale et al., 2014).

Posthovitellinum is the sixth asymphylodorine genus and 10th accepted lissorchiid genus to be proposed (Bray, 2008; Sokolov and Gordeev, 2019). The new species is the fourth lissorchiid reported from Vietnam and, whereas other lissorchiids range in northern and southern Vietnam, the first from the MRD. Little is known in general about the digeneans of the MRD: few parasitologists work in this area and no routine or sustained parasitological research is being conducted in the region by local scientists. As this river system is second only to the Amazon River in aquatic biodiversity (International Rivers, 2017), future parasitological studies promise to reveal additional new species of digeneans infecting fishes there.

Lissorchiids show a range of host specificity: some genera include species that are highly host specific to the definitive fish host and others less so. Lissorchiids principally mature in cyprinids and catostomids (Cypriniformes) ranging in North America, the Palaearctic region, India, and Southeast Asia (Bray, 2008; see Table II herein). Species of *Lissorchis* Magath, 1917

and *Neopaleorchis* Schell, 1973 (mainly infecting North American catostomids), *Prosovitellina*, *Wangxiyunia*, *Asaccotrema*, and *Palaeorchis* Szidat, 1943 (mainly infecting Palaearctic cyprinids), *Brahamputrotrema* (infecting channids in India), and *Asymphylotrema* Dvoryadkin and Besprozvannykh, 1985 (infecting cobitids and cyprinids in the Palaearctic region) exhibit a high degree of host specificity. However, species of *Asymphylodora* exhibit low host specificity (maturing in minnows and carps [Cyprinidae], snakeheads [Channidae], gobies [Gobiidae, Odontobutidae], catfishes [Bagridae, Siluridae], and pupfishes [Cyprinodontidae]) and have a global distribution (Table II). *Asymphylodora atherinopsidis* Annereaux, 1947 (described from a single specimen from *Atherinopsis californiensis* Girard, 1854 [Atheriniformes: Atherinopsidae], a marine fish in California) has a "bipartite metraterm" that we consider to represent a terminal organ, a definitive feature for monorchiids. We therefore consider it as a species *incertae sedis*.

Among the 76 accepted lissorchiid species, few are represented by nucleotide sequences in GenBank, i.e., only 7 partial *28S rDNA* sequences representing 7 lissorchiid species were available prior the present study (Petkevičiūtė et al., 2020; Table I herein). Our sequences herein comprise the eighth partial *28S rDNA* and the fourth *ITS2 rDNA* nucleotide sequences for Lissorchiidae, including the first available sequences of *ITS1* and *ITS2* regions for *L. kritskyi*, and the first available sequences spanning the *ITS1*, *5.8S*, *ITS2*, and partial *28S rDNA* gene for *L.* cf. *nelsoni* and *L.* cf. *gullaris*.

The estimated phylogenetic tree is insufficient for testing the validity of the 2 lissorchiid subfamilies since we included only 4 lissorchiine sequences and 6 asymphylodorine sequences; sequences representing additional North American and Asian taxa are needed. However, it is noteworthy that the European species of *Palaeorchis* is more closely related to all available asymphylodorine species (*Asymphylodora* + *Posthovitellinum* + *Asaccotrema*) rather than to 3

species representing North American lissorchiines (Fig. 8). This finding is consistent and further supports the phylogenetic relationships among species of Lissorchiidae reported in Petkevičiūtė et al. (2020). Their results suggested that *P. incognitus* (with 2 testes) was closely related to other asymphylodorines having 1 testis. These phylogenetic studies indicate that testes count may not be a good character for distinguishing lissorchiid subfamilies.

Of the 3 species of *Lissorchis* used in the present analysis, only the identity of *L. kritskyi* is well-supported. These vouchers conformed perfectly to the published description of this species (Barnhart and Powell, 1979), and the partial *28S rDNA* representing our specimens of *L. kritskyi* were identical to those of *L. kritskyi* in GenBank (AY222250, EF032689). Specimens of *L.* cf. *nelsoni* collected from spotted suckers in the Pascagoula River conformed to the description of *Lissorchis nelsoni* Gale, Choudhury, Bailey and Sutherland 2014 (Gale et al., 2014). This species was described from the same host species but from the northeastern portion of the Mississippi River Basin (Wisconsin), which is vastly disjunct from the Pascagoula River; which is east of the Mississippi River Basin. Because of that, the identity of the specimens of *L. nelsoni* from the Pascagoula River should remain provisional until sequence data is available from specimens from the Mississippi River Basin. We think that our immature specimens of *L.* cf. *gullaris* are *Lissorchis gullaris* Self and Campbell, 1956 but that identification is tentative for 2 reasons. First, our voucher specimens of *L.* cf. *gullaris* were collected from a single smallmouth buffalo and comprised immature specimens only. We collected 5 immature specimens of *L.* cf. *gullaris* plus adults of *L.* cf. *gullaris* from black buffalo during the same collection (same time and locality as the infected smallmouth buffalo detailed above) and also from smallmouth buffalo from Reelfoot Lake, Tennessee, as part of another collection. Second, *L. gullaris* could be a junior subjective synonym of *L. fairporti* (see Choudhury and Nelson, 1998) because both

species have an armed, protrusible cirrus, both mature in fishes of *Ictiobus* and *Carpioides*, and the 2 named species are morphologically similar. The identification of *L*. cf. *gullaris* (rather than *L*. cf. *fairporti*) is warranted herein because the description of *L. fairporti* is insufficient and the species requires redescription. The synonomy of the species should be the subject of an investigation using nucleotide evidence and well-fixed specimens for morphology.

Cyclocheilos enoplos, the type host of *P. psiloterminae*, is endemic to the Mekong River. It is highly migratory within the river and requires unobstructed river channel habitat for spawning (Mekong River Commission, 2015). Its movements likely are or will be interrupted by dams, reducing its range or perhaps extirpating it from the river entirely. This has obvious impacts on the diversity of parasites in the Mekong River and in *C. enoplos*.

ACKNOWLEDGMENTS

We thank Thuy Ha Nguyen, Rector of Dong Thap Community College, Dong Thap, Vietnam, for hosting us during the collection. This study was supported by research grants from the Auburn University Vice President for Research and Economic Development, Southeastern Cooperative Fish Parasite and Disease Project (Alabama Department of Conservation and Natural Resources), Alabama Agriculture Experiment Station, and US Department of Agriculture. Earlier exploratory aspects of this project were supported by grants from the National Science Foundation.

LITERATURE CITED

Andres, M. J., S. S. Curran, T. J. Fayton, E. E. Pulis, and R. M. Overstreet. 2015. An additional genus and two additional species of Forticulcitinae (Digenea: Haploporidae) Folia Parasitologica 62: 025. doi:10.14411/fp.2015.025.

Andres, M. J., E. E. Pulis, S. S. Curran, and R. M. Overstreet. 2018. On the systematics of some marine haploporids (Trematoda) with the description of a new species of *Megasolena* Linton, 1910. Parasitology International 67: 805–815.

Annereaux, R. F. 1947. Three new trematodes from marine fishes of California. Transactions of the American Microscopical Society 66: 249–255.

Atopkin, D. M., V. V. Besprozvannykh, H. D. Ngo, N. V. Ha, N. V. Tang, A. V. Ermolenko, and A. Y. Beloded. 2017. Morphometric and molecular data of the two digenean species *Lasiotocus lizae* Liu, 2002 (Monorchiidae) and *Paucivitellosus vietnamensis* sp. n. (Bivesiculidae) from mullet fish in Tonkin Bay, Vietnam. Journal of Helminthology 91: 346–355.

Baran, E. 2006. Fish migration triggers in the Lower Mekong Basin and other tropical freshwater systems. MRC Technical Paper No. 14, Mekong River Commission, Vientiane, 56 p.

Barger, M. A. 2010. A new species of *Lissorchis* (Trematoda: Lissorchiidae) from creek chubsuckers (*Erimyzon oblongus*) in the Big Thicket National Preserve, Texas, U.S.A. Comparative Parasitology 77: 1–5.

Barnhart, M. C., and E. C. Powell. 1979. *Lissorchis kritskyi* sp. n. (Digenea: Lissorchiidae) from the River carpsucker, *Carpiodes carpio* (Rafinesque). Proceedings of the Helminthological Society of Washington 46: 47–51.

Besprozvannykh, V. V., A. V. Ermolenko, and D. M. Atopkin. 2012. The life cycle of *Asymphylodora perccotti* sp. n. (Trematoda: Lissorchiidae) in the Russian Southern Far East. Parasitology International 61: 235–241.

Bhadauria, S., and M. R. Dandotia. 1984. Studies on the trematode parasites of freshwater fishes with special reference to Gwalior region. Part II. On one new genus and some unknown and known species. Rivista di Parasitologia 45: 341–384.

Bhadauria, S., and M. R. Dandotia. 1988. Studies on the digenetic trematodes of freshwater fishes with special reference to Gwalior region. Part IV. Description of ten new and six already known forms belonging to eight genera of trematodes. Rivista di Parasitologia 47: 353–397.

Biguet, J., S. Deblock, and A. Capron. 1956. Description d'une metacercaire progenetique du genre *Asymphylodora* Loos, 1899, decouverte chez *Bythinia leachi* Sheppard dans le Nord de la France. Annales de Parasitologie Humaine et Comparée. 31: 525–542.

Bray, R. A. 2005. Family Deropristiidae Cable and Hunninen, 1942. *In* Keys to the Trematoda. Vol. 2. Jones, A., R. A. Bray, and D. I. Gibson (eds.). CAB International and Natural History Museum, Cambridge, U.K., p. 653–656.

Bray, R. A. 2008. Family Lissorchiidae Magath, 1917. *In* Keys to the Trematoda. Vol. 3. Bray, R. A., D. I. Gibson and A. Jones (eds.). CAB International and Natural History Museum, Cambridge, U.K., p. 177–186.

Chen, P., and Z. Tang. 1984. A new species of the genus *Asymphylodora* (Trematoda: Monorchiidae). Acta Zootaxonomica Sinica 9: 12–14.

Chernyshenko, A. 1949. New helminths of fishes in the Black Sea. Trudy Odesskogo Derzhavnogo Universiteta imeni I. I. Mechnikova 57: 79–91.

Choudhury, A. 2009. A new deropristiid species (Trematoda: Deropristiidae) from the lake sturgeon *Acipenser fulvescens* in Wisconsin, and its biogeographical implications. Journal of Parasitology 95: 1159–1164.

Choudhury, A., and P. A. Nelson. 1998. *Lissorchis macropharynx* n. sp. (Digenea: Lissorchiidae) from the shorthead redhorse, *Moxostoma macrolepidotum* (Lesueur) (Oxsteichthyes: Catostomidae). Journal of Parasitology 84: 1196–1202.

Christensen, B. M., P. K. Wellner, and L. N. Gleason. 1982. Two new species of *Lissorchis* Magath (Digenea: Lissorchiidae) from the spotted sucker, *Minytrema melanops* (Rafinesque), with a key to species. Proceedings of the Helminthological Society of Washington 49: 22–27.

Claxton, A. T., A. D. Fuehring, M. J. Andres, T. D. Moncrief, and S. S. Curran. 2017. Parasites of the vermilion snapper, *Rhomboplites aurorubens* (Cuvier), from the western Atlantic Ocean. Comparative Parasitology 84: 1–14.

Cort, W. W. 1918. A new cercariaeum from North America. Journal of Parasitology 5: 86–91.

Cribb, T. H., N. Q.-X. Wee, R. A. Bray, and S. C. Cutmore. 2018. *Monorchis lewisi* n. sp. (Trematoda: Monorchiidae) from the surf bream, *Acanthopagrus australis* (Sparidae), in Moreton Bay, Australia. Journal of Helminthology 92: 100–108.

Curran, S. S., V. V. Tkach, and R. M. Overstreet. 2006. A review of *Polylekithum* Arnold, 1934 and its familial affinities using morphological and molecular data, with description of *Polylekithum catahoulensis* sp. nov. Acta Parasitologica 51: 238–248.

Dantotia, M. R., and S. Bhadauria. 1979. On a new species of the genus *Brahmputrotrema* [sic] Dayal and Gupta, 1954 (Family: Lissorchiidae Poche, 1926) from a fresh water fish, *Puntius sophore*. Rivista di Parassitologia 40: 57–61.

Darriba, D., G. L. Taboada, R. Doallo, and D. Posada. 2012. jModelTest 2: More models, new heuristics, and parallel computing. Nature Methods 9: 772. doi:10.1038/nmeth.2109.

Dvoriadkin, V. A., and V. V. Besprozvannykh. 1985. Systematic position and life cycle of *Asymphylotrema macracetabulum* comb. nov. (Trematoda, Monorchidae). Parazitologiya 19: 394–398.

Eschmeyer, W. N., R. Fricke, and R. Van der Laan. 2016. Catalog of Fishes: Genera, Species, References. Available at: http://researcharchive.calacademy.org/research/ichthyology/fishcatmain.asp. Accessed 16 April 2020.

Fayton, T. J., S. S. Curran, M. J. Andres, R. M. Overstreet, and C. T. McAllister. 2016. Two new species of *Homalometron* (Digenea: Apocreadiidae) from Nearctic freshwater fundulids, elucidation of the life cycle of *H. cupuloris*, and molecular phylogenetic analysis of some congeners. Journal of Parasitology 102: 94–104.

Fischthal, J. H. 1942. *Triganodistomum hypentelii* n. sp. (Trematoda: Lissorchiidae) from the hog sucker, *Hypentelium nigricans* (Le Sueur). Journal of Parasitology 28: 389–393.

Fischthal, J. H. 1979. *Asymphylodora fishelsoni* sp. n. (Trematoda: Monorchiidae) from a cyprinodontid fish, *Aphanius dispar richardsoni* (Boulenger), from Israel, and its allometric growth. Journal of Helminthology 53: 357–362.

Fischthal, J. H., and J. D. Thomas. 1972. Digenetic trematodes of marine fishes from Senegal. Bulletin de l'Institut Fondamental d'Afrique Noire 34: 292–322.

Gale, E. J., A. Choudhury, J. L. Bailey, and D. J. Sutherland. 2014. A new species of *Lissorchis* (Trematoda: Lissorchiidae) from the spotted sucker, *Minytrema melanops* (Actinopterygii: Catostomidae), in Wisconsin, U.S.A. Comparative Parasitology 81: 61–66.

Gibson, D. I., R. A. Bray, and E. A. Harris. 2005. Host-Parasite database of the Natural History Museum, London. Available at: http://www.nhm.ac.uk/research-curation/scientific-resources/taxonomy-systematics/host-parasites/. Accessed 19 August 2020.

Glez-Peña, D., D. Gómez-Blanco, M. Reboiro-Jato, F. Fdez-Riverola, and D. Posada. 2010. ALTER: program-oriented conversion of DNA and protein alignments. Nucleic Acids Research 38: W14–W18. doi:10.1093/nar/gkq321.

Gupta, S. P. 1955. Trematode parasites of fresh-water fishes. Indian Journal of Helminthology 5: 1–80.

Gupta, S. P., and V. Agrawal. 1967. On a trematode, *Asymphylodora ritai* n. sp. from the intestine of a freshwater fish, *Rita rita* (Ham.). Indian Journal of Helminthology 18: 50–53.

Gupta, S. P., and S. L. Verma. 1976. On some trematode parasites of freshwater fishes. Rivista di Parassitologia 37: 171–182.

Ha, N. V., and N. V. Duc. 2005. New records of endohelminth parasites in common carp (*Cyprinus carpio*) from Vietnam. Journal of Biology 27: 12–16.

Haderlie, E. C. 1950. A new species of *Triganodistomum* (Trematoda: Lissorchiidae) from the sacramento sucker, *Catostomus occidentalis* Ayres. Journal of Parasitology 36: 297–300.

Haderlie, E. C. 1953. Parasites of the fresh-water fishes of northern California. University of California Publications in Zoology 57: 303–440.

Hasnain, M., and U. Sahay. 1990. *Asymphylodora puntiusii* (Monorchiidae: Asymphylodorinae) from *Puntius puntius*. Indian Journal of Parasitology 14: 17–21.

Huston, D. C., T. L. Miller, S. C. Cutmore, and T. H. Cribb. 2019. A new genus and species of the trematode family Gyliauchenidae Fukui, 1929 from an unexpected, but plausible, host, *Kyphosus cornelii* (Perciformes: Kyphosidae). Parasitology 146: 937–946.

International Rivers. 2017. A dangerous trajectory for the Mekong River, an update on the status of Mekong mainstream dams. Available at: www.internationalrivers.org/sites/default/files/attached-files/mekong_mainstream_damsupdate_2017_english.pdf. Accessed 11 March 2020.

Issaitschikov, I. M. 1923. To the knowledge of parasitic worms of cyprinids of the river Kuban. Trudy GIEV T. II. 2.

Ito, J. 1960. Contributions to the morphology of cercariae obtained from a snail host, *Semisulcospira libertina* in Japan. Japanese Journal of Medical Science and Biology 13: 59–72.

Karimov, S. B., and G. K. Mukhamedov. 1990. A description of the new species of the genus *Asymphylotrema* (Trematoda, Monorchidae). Parazitologiya 24: 80–81.

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.

Koval, V. P. 1950. Digenetic trematodes of fishes of the lower Dnieper. Trudy Biologogruntosnavchego Fakulta Kiivs'k 5: 187–207.

Krygier, B. B., and R. W. Macy. 1969. *Lissorchis heterorchis* sp. n. (Trematoda: Lissorchiidae) from *Catostomus macrocheilus* Girard in Oregon. Proceedings of the Helminthological Society of Washington 36: 136–139.

Kudlai, O. S. 2010. The first record of *Asymphylodora progenetica* (Trematoda, Monorchiidae) in Ukraine. Vestnik Zoologii 44: e45–48.

Kulakova, A. P. 1972. A new species of the genus *Parasymphylodora* (Trematoda, Monorchidae) and its larva. Parazitologiya 6: 137–142.

Kulakovskaja, O. P. 1947. *Asymphylodora markewitschi* n. sp., trematode digénétique des poissons du fleuve Dniepr. Travaux de l'Institut Zoologique de l'Académie des Sciences de la République Ukrainienne, Réunion des Travaux de Parasitologie 1: 152–154.

Lühe, M. 1909. Parasitische Plattwürmer. I: Trematodes. Süsswasserfauna Deutschlands 17: 1–217.

Macy, R. W., and A. K. Berntzen. 1970. On the life cycle and in vitro culture of *Laterocreadium problematicum*, gen. et sp. n. (Trematoda: Allocreadiidae) from Oregon. Journal of Parasitology 56(Suppl.): 445.

Madhavi, R. 2008. Family Monorchiidae Odhner, 1911. *In* Keys to the Trematoda. Vol. 3. Bray, R. A., D. I. Gibson, and A. Jones (eds.). CAB International and Natural History Museum, Cambridge, U.K., p. 145–175.

Magath, T. B. 1917. The morphology and life history of a new trematode parasite, *Lissorchis fairporti* nov. gen., et nov. spec. from the Buffalo fish, *Ictiobus*. Journal of Parasitology 4: 58–69.

Markowski, S. 1935. Die paratischen Würmer von Gobius minutus Pall. des polnischen Balticums. Bulletin de l'Académie Polonaise des Sciences 6–7: 251–260.

Maurya, R., R. Gupta, and A. M. Saxena. 2018. A new species of *Asymphylodora* (Trematoda: Lissorchiidae) from Bata, *Labeo bata* (Cyprinidae), in the Sharda canal of India, and amendment of the diagnosis for *Asymphylodora*. Comparative Parasitology 85: 153–158.

Mekong River Commission. 2015. The council study on sustainable management and development of the Mekong River, including impacts of mainstream hydropower projects. Interim Technical Report 1: Vol.1. Specialists' report, 569 p.

Mikailov, T. K. 1975. Parazity ryb vodoemov Azerbaidzhana. (Sistematika, dinamika proiskhozhdenie). (Parasites of fish in reservoirs of Azerbaidjan. Systematics, dynamics and origin). ELM, Baku, Azerbaijan, 299 p.

Mueller, J. F., and H. J. Van Cleave. 1932. Parasites of the Oneida Lake fishes. Part II. Descriptions of new species and some general taxonomic considerations, especially concerning the trematode family Heterophyidae. Roosevelt Wild Life Annals 3: 79–137.

Mühling, P. 1898. Studien aus Ostpreussens Helminthenfauna. Vorläufige Mittheilung. Zoologischer Anzeiger 21: 16–24.

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., K. M. Halanych, B. T. Dang, M. D. Bakenhaster, C. R. Arias, and S. A. Bullard. 2017. Two new species of *Elopicola* (Digenea: Aporocotylidae) from Hawaiian ladyfish, *Elops hawaiensis* (Eastern Sea) and Atlantic tarpon, *Megalops atlanticus* (Gulf of Mexico) with a comment on monophyly of elopomorph blood flukes. Parasitology International 66: 305–318.

Orsi, J. J. 1974. A check list of marine and freshwater fishes of Vietnam. Publications of the Seto Marine Biological Laboratory 21: 153–171.

Pan, J., X. Ding, and D. An. 1987. A new species of *Palaeorchis* from freshwater fishes of China (Digenea: Monorchiidae). Annual Bulletin of the Society of Parasitology Guangdong Province 8–9: 139–140.

Panyi, A. J., S. S. Curran, and R. M. Overstreet. 2020. Phylogenetic affinity of *Genolopa* (Digenea: Monorchiidae) with descriptions of two new species. Diversity 12: 51. doi:10.3390/d12020051.

Parker, J. H., S. S. Curran, R. M. Overstreet, and V. V. Tkach. 2010. Examination of *Homalometron elongatum* Manter, 1947 and description of a new congener from *Eucinostomus currani* Zahuranec, 1980 in the Pacific Ocean off Costa Rica. Comparative Parasitology 77:154–163.

Petkevičiūtė, R., G. Stanevičiūtė, and V. Stunžėnas. 2020. Exploring species diversity of lissorchiid trematodes (Digenea: Lissorchiidae) associated with the gravel snail, *Lithoglyphus naticoides*, in European freshwaters. Journal of Helminthology 94: e152. doi:10.1017/S0022149X2000036X.

Qir, Z., and J. W. Wang. 1995. Study of the Asymphylodorinae flukes of freshwater fishes in Tianjin, China (Digenea: Lissorchiidae). *In* The Society of Parasitology of China (eds.) Proceedings of the Tenth Anniversary of the Founding of China Parasitological Society. Chinese Scientific and Technical Publishers, Beijing, China, p.113–116.

Rambaut, A., M. A. Suchard, D. Xie, and A. J. Drummond. 2014a. FigTree v1.4.4. Available at: http://tree.bio.ed.ac.uk/software/figtree. Accessed 17 April 2020.

Rambaut, A., M. A. Suchard, D. Xie, and A. J. Drummond. 2014b. Tracer v1.6, Available at: http://beast.bio.ed.ac.uk/Tracer. Accessed 17 April 2020.

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

Schell, S. C. 1973. The life history of *Neopaleorchis catostomi* gen. et sp. n. (Trematoda: Monorchiidae), an intestinal parasite of the coarsescale sucker, *Catostomus macrocheilus* Girard. Journal of Parasitology 59: 463–468.

Schwelm, J., O. Kudlai, N. J. Smit, C. Selbach, and B. Sures. 2020. High parasite diversity in a neglected host: Larval trematodes of *Bithynia tentaculata* in Central Europe. Journal of Helminthology 94: e120. doi:10.1017/S0022149X19001093.

Self, J. T., and J. W. Campbell. 1956. A study of the helminth parasites of the buffalo fishes of Lake Texoma with a description of *Lissorchis gullaris* n. sp. (Trematoda: Lissorchiidae). Transactions of the American Microscopical Society 75: 397–401.

Serkova, O. P., and B. E. Bykhovskii. 1940. *Asymphylodora progenetica* n. sp. with some information on its morphology and life cycle. Parazitologicheskii Sbornik 8: 162–175.

Shimazu, T. 2016. Digeneans Parasitic in Freshwater Fishes (Osteichthyes) of Japan VI. Lissorchiidae. Bulletin of the National Museum of Nature and Science 42: 1–22.

Simer, P. H. 1929. Fish Trematodes from the Lower Tallahatchie River. American Midland Naturalist 11: 563–588.

Singh, J. P. 1980. On a new trematode *Asymphylodora puntiussi* from a freshwater fish *Puntius sarana* (Ham. Buch.) from Kanpur, U. P. India. Proceedings of the Indian Science Congress Association 67(3 Suppl.(vii)): 146.

Singh, S. P., and D. P. Sinha. 1975. Two new trematodes from fresh water fishes of Bihar. Dr. B. S. Chauhan Commemorative Volume: 411–417.

Sokolov, S. G., and I. I. Gordeev. 2019. *Asaccotrema vietnamiense* n. gen.; n. sp. (Trematoda: Monorchioidea), a new aberrant representative of lissorchiid trematodes from the sidestripe

rasbora, *Rasbora paviana* Tirant (Actinopterygii: Cyprinidae), Vietnam. Zootaxa 4674: 451–462.

Sokolov, S., E. Voropaeva, and D. Atopkin. 2020. A new species of deropristid trematode from the starlet *Acipenser ruthenus* (Actinopterygii: Acipenseridae) and revision of superfamily affiliation of the family Deropristidae. Zoological Journal of the Linnean Society 20: zlaa015. doi:10.1093/zoolinnean/zlaa015.

Srivastava, N. N. 1951. A new trematode *Asymphylodora kedarai* n. sp. of the family Monorchiidae Odhner 1911. Indian Journal of Helminthology 3: 7–12.

Stunkard, H. W. 1959. The morphology and life-history of the digenetic trematode, *Asymphylodora amnicolae* n. sp.; the possible significance of progenesis for the phylogeny of the Digenea. Biological Bulletin 117: 562–581.

Szidat, L. 1943. Die Fischtrematoden der Gattung *Asymphylodora* Looss, 1899 und Verwandte. Zeitschrift für Parasitenkunde 13: 25–61.

Tang, Z. 1980. Progenetic development of *Asymphylodora stenothyrae* n. sp. Acta Hydrobiologica Sinica 7: 231–244.

Tkach, V. V., J. Pawlowski, J. Mariaux, and Z. Swiderski. 2001. Molecular phylogeny of the suborder Plagiorchiata and its position in the system of Digenea. *In* Interrelationships of the Platyhelminthes. D. T. J. Littlewood and R. A. Bray (eds.). Taylor and Francis, London, U.K., p 186–193.

Tran, D. D., K. Shibukawa, P. T. Nguyen, H. P. Ha, L. X. Tran, H. V. Mai, and K. Utsugi. 2013. Fishes of the Mekong Delta, Vietnam. Can Tho University Publishing House, Can Tho, Vietnam, 174 p.

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) with comments on related catfish aporocotylids. Folia Parasitologica 60: 237–247.

Wang, H. 1982. Parasitic trematodes from fishes of Poyang Lake. 1. Three new species of Monorchiidae. Acta Zootaxonomica Sinica 7: 1–5.

Wang, H. 1983. Parasitic trematodes from fishes of Poyang Lake 6. Description of four new species of Opecoelidae and other families. Acta Zootaxonomica Sinica 8: 337–344.

Wang, P., Y. Sun, Y. Zhao, W. Zhang, and Y. Wang. 1985. Notes on some digenetic trematodes of vertebrates from Wuyishan, Fujian. Wuyi Science Journal 5: 129–139.

Wee, N. Q., S. C. Cutmore, P. Sasal, and T. H. Cribb. 2020. Three new species of *Allobacciger* Hafeezullah and Siddiqi, 1970 (Digenea: Monorchiidae) from Australia and French Polynesia. Marine Biodiversity 50: 16. doi:10.1007/s12526-019-01029-8.

Wesenberg-Lund, C. J. 1934. Contributions to the development of the Trematoda Digenea. Part II. The biology of the freshwater cercariae in Danish freshwaters. Kongelige Danske Videnskabernes Selskabs Skrifter 5: 1–223.

Yamaguti, S. 1936. Studies on the helminth fauna of Japan. Part 15. Trematodes of fishes, II. Satyu Yamaguti, Kyöto: 1–6.

Yamaguti, S. 1938. Studies on the helminth fauna of Japan. Part 21. Trematodes of Fishes, IV. Kyöto: Yamaguti, S., 139 p.

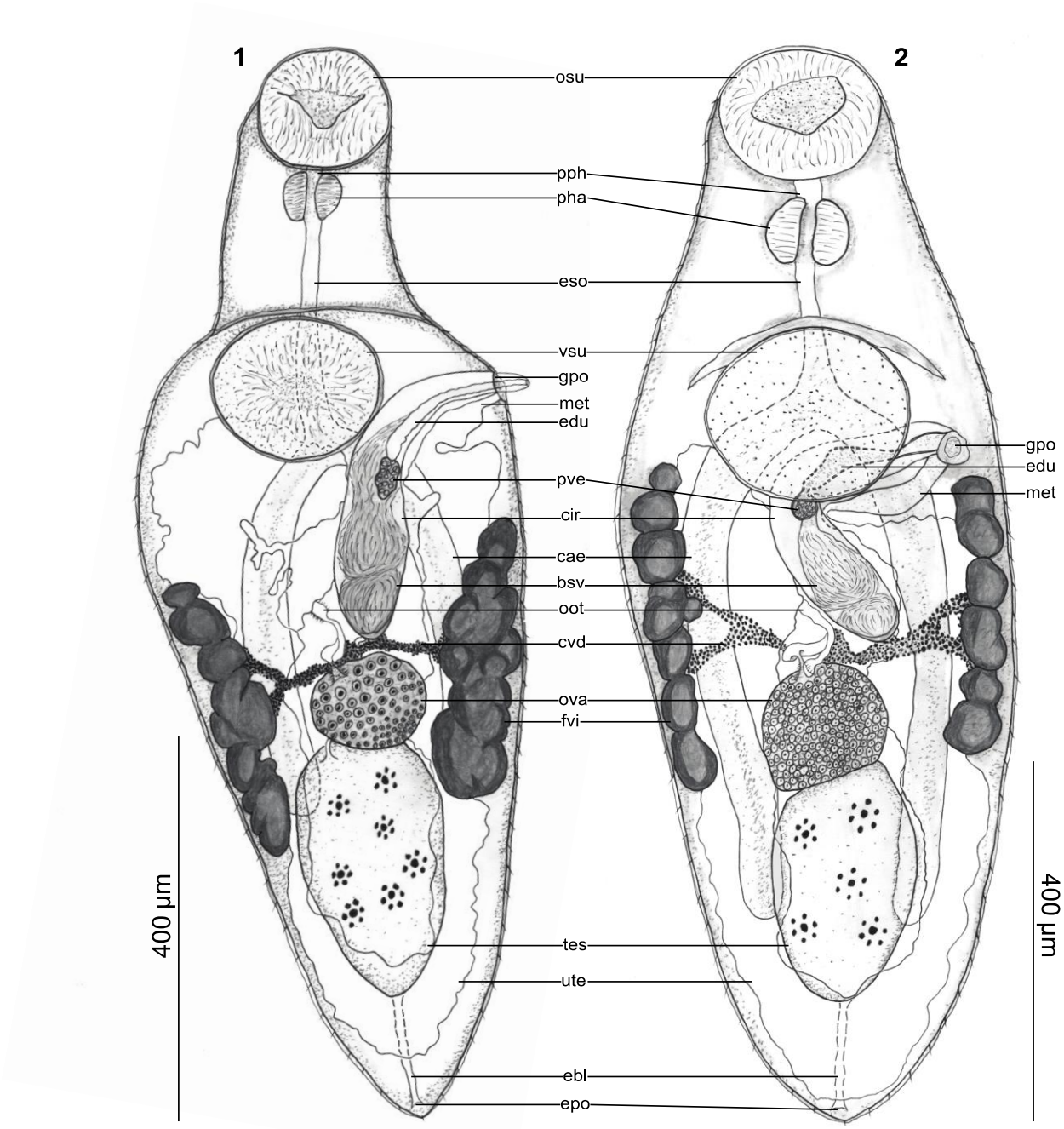
Zhang, T. 1988. Parasitic trematodes from fishes of Sichuan Province in China, 1. Two new species of Monorchiidae (Trematoda: Digenea). Acta Zootaxonomica Sinica 13: 324–328.

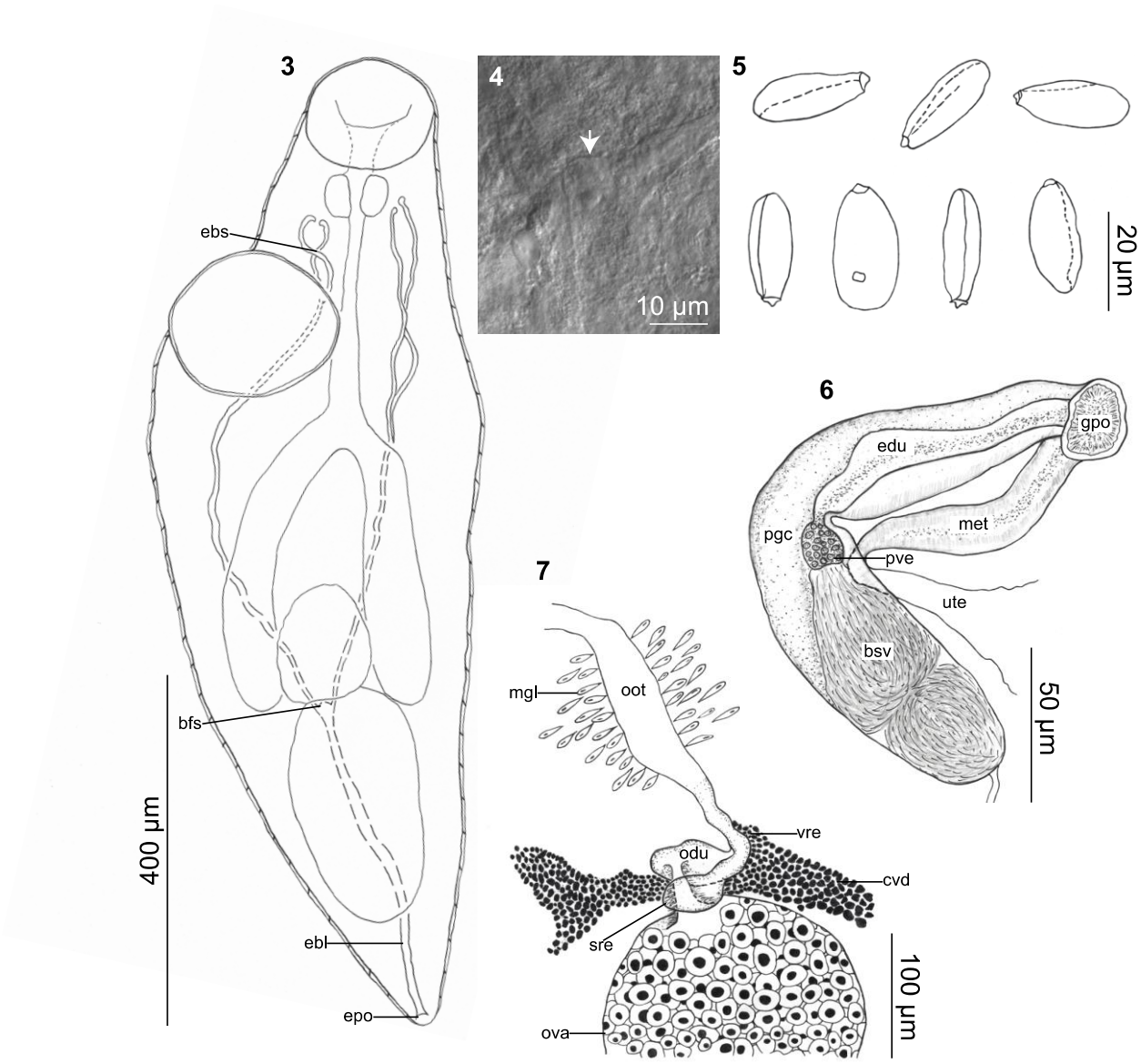
FIGURES 1, 2. *Posthovitellinum psiloterminae* n. gen., n. sp. infecting the intestine of *Cyclocheilos enoplos* (Bleeker, 1849) (Cypriniformes: Cyprinidae) in Dong Thap province, Vietnam. (**1**) Ventral view, whole-mounted of holotype (USNM 1620811), showing oral sucker (osu), pre-pharynx (pph), pharynx (pha), esophagus (eso), ventral sucker (vsu), genital pore (gpo), metraterm (met), ejaculatory duct (edu), prostatic vesicle (pve), cirrus-sac (cir), ceca (cae), bipartite seminal vesicle (bsv), ootype (oot), common vitellarium duct (cvd), ovary (ova), follicular vitellarium (fvi), testis (tes), uterus (ute), excretory bladder (ebl), excretory pore (epo). (**2**) Ventral view, paratype (USNM 1620812), showing the same features.

FIGURES 3–7. *Posthovitellinum psiloterminae* n. gen., n. sp. infecting the intestine of *Cyclocheilos enoplos* (Bleeker, 1849) (Cypriniformes: Cyprinidae) in Dong Thap province, Vietnam. (**3**) Partly lateroventral view, whole-mounted of paratype (USNM 1620813) showing the I-shaped excretory system, excretory branching site (ebs), bifurcating site (bfs), excretory bladder (ebl), excretory pore (epo). (**4**) Photo illustration of the excretory bladder, paratype (USNM 1620813), showing the terminal end (arrow). (**5**) Eggs (holotype, USNM 1620811) in different views showing operculate eggs located in different parts of the uterus. (**6**) Ventral view, paratype (USNM 1620812), terminal genitalia showing ejaculatory duct (edu), genital pore (gpo), metraterm (met), prostatic vesicle (pve), prostate gland cell (pgc), uterus (ute), bipartite seminal vesicle (bsv). (**7**) Ventral view, paratype (USNM 1620814), ovary complex showing Mehlis' gland (mgl), ootype (oot), vitellarium reservoir (vre), oviduct (ovi), common vitellarium duct (cvd), seminal receptacle (sre), ovary (ova).

FIGURE 8. Phylogenetic relationships of *Posthovitellinum psiloterminae* n. gen., n. sp. and other Monorchioidea, and Lepocreadioidea taxa based on the Bayesian inference analysis of the

28S rDNA data. Values aside nodes are posterior probability. Scale bar is in substitutions per site. Taxonomic names were followed by GenBank accession numbers in parentheses.


1
2
osu
pph
pha
eso
vsu
gpo
met
edu
pve
cir
cae
bsv
oot
cvd
ova
fvi
tes
ute
ebl
epo
gpo
edu
met
400 µm
400 µm


3
ebs
bfs
ebl
epo
400 µm
4
10 µm
5
20 µm
6
gpo
edu
met
pgc
pve
ute
bsv
50 µm
7
mgl
oot
vre
odu
cvd
sre
ova
100 µm

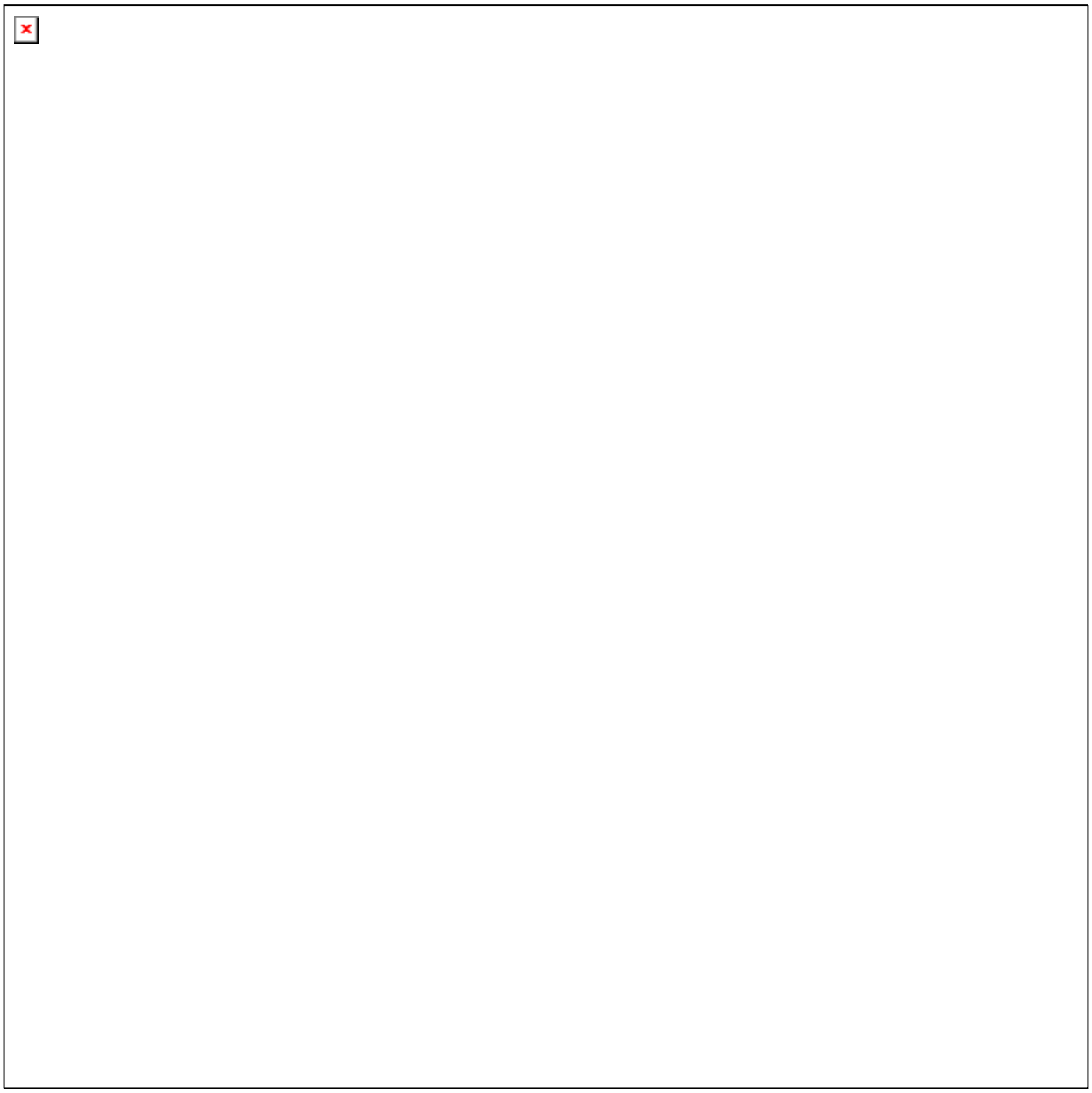


Table I. Partial *28S rDNA* sequences used in the present study.

Species	Host	Locality	GenBank Accession #s	References
Monorchioidea Odhner, 1911				
Monorchiidae Odhner, 1911				
Monorchis monorchis (Stossich, 1890) Monticelli, 1893	*Diplodus vulgaris* (Geoffroy Saint-Hilaire, 1817)	near Corsica, France	AF184257	Tkach et al., 2001
Lasiotocus glebulentus Overstreet, 1971	*Mugil curema* Valenciennes, 1836	Beaufort, North Carolina, USA	MN984476	Panyi et al., 2020
Monorchis lewisi Cribb, Wee, Bray, and Cutmore, 2018	*Acanthopagrus australis* (Günther, 1859)	Western Moreton Bay, Queensland, Australia	MF503309	Cribb et al., 2018
Genolopa ampullacea Linton, 1910	*Haemulon flavolineatum* (Desmarest, 1823)	Islamorada, Florida, USA	MN984474	Panyi et al., 2020
Postmonorchis orthopristis Hopkins, 1941	*Haemulon flavolineatum* (Desmarest, 1823)	Upper Matecumbe Key, Florida, USA	MN984475	Panyi et al., 2020
Allobacciger annulatus Wee, Cutmore, Sasal, and Cribb, 2020	*Centropyge tibicen* (Cuvier, 1831)	Heron Island, Queensland, Australia	MK955782	Wee et al., 2020
Diplomonorchis leiostomi Hopkins, 1941	*Leiostomus xanthuru* Lacepède, 1802	Gulf of Mexico, Ocean Springs, Mississippi, USA	AY222252	Olson et al., 2003
Hurleytrematoides chaetodoni (Manter, 1942) Yamaguti, 1954	*Chaetodon striatus* Linnaeus, 1758	West Florida Middle Grounds, Gulf of Mexico, Florida, USA	MH244116	Andres et al., 2018
Lissorchiidae Magath, 1917				
Posthovitellinum psiloterminae Truong, Curran, and Bullard n. gen., n. sp.	*Cyclocheilos enoplos* (Bleeker, 1849)	Cao Lanh Fish Market, Dong Thap, Vietnam	MT928351, MT928352	Present study
Asaccotrema vietnamiense Solokov and Gordeev, 2019	*Rasbora paviana* Tirant, 1885	Cat Tien National Park, Dong Nai, Vietnam	MK863409	Solokov and Gordeev, 2019
Asmphylodora sp.	*Bithynia tentaculata* (Linnaeus, 1758)	River Lippe, Germany	MN726955	Schwelm et al., 2020
Asymphylodora sp.	*Lithoglyphus naticoides* (Pfeiffer, 1828)	Danube River, Hungary	MT153917	Petkevičiūtė et al., 2020
Asmphylodora perccotti Besprozvannykh, Ermolenko, and Atopkin, 2012	*Perccottus glenii* Dybowski, 1877	Bolshaya Ussurka River Basin, Primorsky Region, Russian Southern Far East of Russia	FR822731	Besprozvannykh et al., 2012
Asymphylodora progenetica Serkova and	*Bithynia tentaculata*	Jeruzalė pond, Vilnius, Lithuania	MT103402	Petkevičiūtė et

Species	Host	Locality	GenBank Accession #s	References
Bykhowskii, 1940	(Linnaeus, 1758)			al., 2020
Lissorchis kritskyi Barnhart and Powell, 1979	*Minytrema melanops* (Rafinesque, 1820)	Pascagoula River, George County, Mississippi, USA	EF032689	Curran et al., 2006
Lissorchis cf. *nelsoni*	*Minytrema melanops* (Rafinesque, 1820)	Pascagoula River, Jackson County, Mississippi USA	MT928354	Present study
Lissorchis cf. *gullaris*	*Ictiobus bubalus* (Rafinesque, 1818)	Lake Chotard, Mississippi; River, Issaquena County, Mississippi USA	MT928353	Present study
Palaeorchis incognitus Szidat, 1943	*Lithoglyphus naticoides* (Pfeiffer, 1828)	Balaton Lake, Hungary	MT103410	Petkevičiūtė et al., 2020
Deropristidae Cable and Hunninen, 1942				
Skrjabinopsolus nudidorsalis Sokolov, Voropaeva, and Atopkin, 2020	*Acipenser ruthenus* Linnaeus, 1758	Oka River near Kletino village, Ryazan Oblast, Russia	MN700996	Sokolov et al., 2020
Lepocreadioidea Odhner, 1905				
Lepocreadiidae Odhner, 1905				
Preptetos caballeroi Pritchard, 1960	*Naso vlamingii* (Valenciennes, 1835)	Heron Island, Coral Sea, Great Barrier Reef, Queensland, Australia	AY222236	Olson et al., 2003
Gorgocephalidae Manter, 1966				
Gorgocephalus kyphosi Manter, 1966	*Kyphosus vaigiensis* (Quoy and Gaimard, 1825)	Lizard Island, Coral Sea, Great Barrier Reef, Queensland, Australia	AY222234	Olson et al., 2003
Gyliauchenidae Fukui, 1929				
Endochortophagus protoporus Huston, Miller, Cutmore, and Cribb, 2019	*Kyphosus cornelii* (Whitley, 1944)	off Point Peron, Western Australia, Australia	MK396257	Hutson et al., 2019
Enenteridae Yamaguti, 1958				
Enenterum aureum Linton, 1910	*Kyphosus vaigiensis* (Quoy and Gaimard, 1825)	Fish market, Moorea, French Polynesia, France	AY222232	Olson et al., 2003
Outgroup				
Apocreadioidea Skrjabin, 1942				
Apocreadiidae Skrjabin, 1942				
Homalometron pallidum Stafford, 1904	*Fundulus heteroclitus* (Linnaeus, 1766)	Long Island Sound, Stonington, Connecticut, USA	HM038044	Parker et al., 2010
Homalometron cupuloris (Ramsey, 1965) Cribb and Bray, 1999	*Lepomis microlophus* (Günther, 1859)	Tchoutacabouffa River, Harrison County, Mississippi, USA	KT823420	Fayton et al., 2016

Table II. Host and geographical distribution among species of Lissorchiidae. In each genus, type species is placed first, followed by other species in alphabetic order.

Parasites	Type host or commonly reported hosts	Host Family	Localities	References
Asymphylodorinae				
Asymphylodora				
A. tincae	*Tinca tinca*	Cyprinidae	Germany	Lühe, 1909
A. abdurachmanovi	*Vimba vimba*	Cyprinidae	Azerbaijan	Mikailov, 1975
A. amnicolae	*Amnicola limosus*	Amnicolidae	Massachusetts, USA	Stunkard, 1959
*A. atherinopsidis**	*Atherinopsis californiensis*	Atherinopsidae	California, USA	Annereaux, 1947
A. carassii	*Carassius auratus*	Cyprinidae	Fujian, China	Chen and Tang, 1984
A. carpiae	*Cyprinus carpio*	Cyprinidae	Syria	Szidat, 1943
A. demeli	*Pomatoschistus minutus*	Gobiidae	Polish Baltic Sea	Marchowski, 1935
A. dollfusi	*Bithynia leachii*	Bithyniidae	France	Biguet et al., 1956
A. ferruginosa	*Barbus barbus*	Cyprinidae	Germany	Lühe, 1909
A. fishelsoni	*Aphanius dispar*	Cyprinodontidae	Israel	Fischthal, 1979
A. hupehensis	*Silurus asotus* *Pseudorasbora parva*	Siluridae Cyprinidae	China	Wang and Pan, 1964
A. imitans	*Abramis brama*	Cyprinidae	Kaliningrad, Russia	Mühling, 1898
A. innominata	*Opsariichthys uncirostris* *Tribolodon hakonensis* *Hemibarbus barbus* *Phoxinus steindachneri* *Gymnogobius isaza*	Cyprinidae	Japan	Shimazu, 2016
A. japonica	*Cyprinus carpio*	Cyprinidae	Japan	Yamaguti, 1938
A. kedarai	*Puntius sophore*	Cyprinidae	Uttar Pradesh, India	Srivastava, 1951
A. kubanica	*Abramis brama* *Cyprinus carpio*	Cyprinidae	Kuban river, Russia	Issaitschikov, 1923
A. longicaeca	*Systomus sarana*	Cyprinidae	Bihar, India	Singh and Sinha, 1975
A. markewitschi	*Carassius carassius*	Cyprinidae	Ukraine	Kulakowskaja, 1947
A. megalobramae	*Megalobrama terminalis*	Cyprinidae	Fujian, China	Wang et al., 1985
A. ovaliformae	*Spinibarbus sinensis*	Cyprinidae	Fujian, China	Wang et al., 1985
A. parasquamosa	*Rutilus rutilus* *Leuciscus idus* *Blicca bjoerkna* *Abramis brama* *Abramis ballerus*	Cyprinidae	Northwest European Russia	Kulakova, 1972
A. perccotti	*Perccottus glenii*	Odontobutidae	Primorsky Region, Russian Southern Far East	Besprozvannykh et al., 2012
A. pontica	*Neogobius melanostomus*	Gobiidae	Black Sea	Chernyshenko, 1949

Parasites	Type host or commonly reported hosts	Host Family	Localities	References
A. poyangensis	*Cyprinus carpio*	Cyprinidae	Jiangxi, China	Wang, 1982
A. progenetica	*Bithynia tentaculata*	Bithyniidae	Leningrad, Russia	Sercova and Bykhovskii, 1940
A. punctatusi	*Channa punctata*	Channidae	Uttar Pradesh, India	Gupta and Verma, 1976
A. puntiusii	*Puntius puntio*	Cyprinidae	Bihar, India	Hasnain and Sahay, 1990
A. puntiussi	*Puntius sarana*	Cyprinidae	Uttar Pradesh, India	Singh, 1980
A. renale	*Elopichthys bambusa*	Cyprinidae	China	Lee et al., 1958
A. ritai	*Rita rita*	Bagridae	Uttar Pradesh, India	Gupta and Agrawal, 1967
A. sinensis	*Sarcocheilichthys sinensis* *Carassius auratus* *Cyprinus carpio*	Cyprinidae	China	Wang and Pan, 1964
A. sinica	(?)	(?)	China	Gibson et al. (2005)
A. sitapurensis	*Labeo bata*	Cyprinidae	Uttar Pradesh, India	Maurya et al., 2018
A. stenothyrae	*Stenothyra divalis*	Stenothyridae	China	Tang, 1980
Asaccotrema				
A. vietnamiense	*Rasbora paviana*	Cyprinidae	Dong Nai, Vietnam	Sokolov and Gordeev, 2019
Brahamputrotrema				
B. punctata	*Channa puncta*	Channidae	India	Guppta, 1955
B. channa	*Channa puncta*	Channidae	India	Bhadauria and Dandotia, 1988
B. gwaliorensis	*Puntius sophore*	Cyprinidae	Madhya Pradesh, India	Dantotia and Bhadauria, 1979
Prosovitellina				
P. rhinogobio	*Rhinogobio typus* *Saurogobio dabryi*	Cyprinidae	Jiangxi, China	Wang, 1982
P. elopichthydis	*Elopichthys bambusa*	Cyprinidae	Fujian, China	Wang et al., 1985
Wangxiyunia				
W. cyprini	*Cyprinus carpio*	Cyprinidae	Jiangxi, China	Wang, 1982
W. borealis	*Cyprinus carpio* *Macropodus opercularis*	Cyprinidae Osphronemidae	Tianjin, China	Qir and Wang, 1995
W. sinensis	*Cyprinus carpio*	Cyprinidae	China	Wang and Pan, 1964
Lissorchiinae				
Lissorchis				
L. fairporti	*Ictiobus cyprinellus* *Ictiobus bubalus*	Catostomidae	Iowa, USA	Magath, 1917
L. amniculensis	*Erimyzon oblongus*	Catostomidae	Texas, USA	Barger, 2010
L. attenuatus	*Catostomus commersonii*	Catostomidae	New York, USA	Mueller and Van Cleave, 1932
L. calentinei	*Minytrema melanops*	Catostomidae	Kentucky, USA	Christensen et al., 1982

Parasites	Type host or commonly reported hosts	Host Family	Localities	References
L. crassicrurum	*Catostomus rimiculus*	Catostomidae	California, USA	Haderlie, 1953
L. garricki	*Carpoides velifer*	Catostomidae	Mississippi, USA	Simer, 1929
L. gullaris	*Ictiobus bubalus*	Catostomidae	Oklahoma, USA	Self and Campbell, 1956
L. heterorchis	*Catostomus macrocheilus*	Catostomidae	Oregon, USA	Krygier and Macy, 1969
L. hypentelii	*Hypentelium nigricans*	Catostomidae	Michigan, USA	Fischthal, 1942
L. kritskyi	*Carpiodes carpio*	Catostomidae	Iowa, USA	Barnhart and Powell, 1979
L. macropharynx	*Moxostoma macrolepidotum*	Catostomidae	Manitoba, Canada	Choudhury and Nelson, 1998
L. minytremi	*Minytrema melanops*	Catostomidae	Kentucky, USA	Christensen et al., 1982
L. mutabilis	*Planorbella smithii*	Planorbidae	Michigan, USA	Cort, 1918
L. nelsoni	*Minytrema melanops*	Catostomidae	Wisconsin, USA	Gale et al., 2014
L. polylobatum	*Catostomus occidentalis*	Catostomidae	California, USA	Krygier and Macy, 1969
L. simeri	*Catostomus commersonii*	Catostomidae	New York, USA	Mueller and Van Cleave, 1932
L. translucens	*Ictiobus bubalus*	Catostomidae	Mississippi, USA	Simer, 1929
Asymphylotrema				
A. macracetabulum	*Misgurnus anguillicaudatus*	Cobitidae	Russian Southern Far East	Dvoriadkin and Besprozvannykh, 1985
A. leninabadi	*Gobio gobio*	Cyprinidae	Tadzhikistan	Karimov and Mukhamedov, 1990
A. monostyloides	*Cobitis biwae*	Cobitidae	Japan	Ito, 1960
Neopaleorchis				
N. catostomi	*Catostomus macrocheilus*	Catostomidae	Idaho, USA	Schell, 1973
Palaeorchis				
P. diplorchis	*Pseudogobio esocinus*	Cyprinidae	Japan	Yamaguti, 1936
P. crassus	*Pisidium amnicum*	Sphaeriidae	Finland	Wesenburg-Lund, 1934
P. incognitus	*Rutilus rutilus*	Cyprinidae	Kaliningrad, Russia	Szidat, 1943
P. lobiovaris	*Saurogobio dabryi*	Cyprinidae	Sichuan, China	Zhang, 1988
P. panjionghuai	*Saurogobio dabryi*	Cyprinidae	Guangdong, China	Pan et al., 1987
P. postovaris	*Pseudogyrinocheilus prochilus*	Cyprinidae	Sichuan, China	Zhang, 1988
P. problematicus	*Ptychocheilus oregonensis*	Cyprinidae	Oregon, USA	Macy and Berntzen, 1970
P. senegalensis	*Pomadasys suillus*	Haemulidae	Senegal	Fischthal and Thomas, 1972
P. sinensis	*Rhinogobio typus*	Cyprinidae	Jiangxi, China	Wang, 1983
P. skrjabini	*Padogobius bonelli*	Gobiidae	Ukraine	Koval, 1950
P. unicus	*Blicca bjoerkna*	Cyprinidae	Kaliningrad,	Szidat, 1943

Parasites	Type host or commonly reported hosts	Host Family	Localities	References
			Russia	
Tigrotrema				
*T. gwaliorense**	*Channa punctata*	Channidae	India	Bhadauria and Dandotia, 1984

*: taxonomic identity is undetermined (*incertae sedis*).

CHAPTER 2: RESURRECTION AND EMENDATION OF *PSEUDOPARAMACRODEROIDES* GUPTA & AGRAWAL, 1968 (DIGENEA: MACRODEROIDIDAE), DESCRIPTION OF A NEW SPECIES FROM VIETNAM, AND COMMENTS ON THE SYSTEMATICS OF MACRODEROIDID GENERA

***Published in Systematic Parasitology (Available 23 October 2021)**

Authors: Triet N. Truong, Stephen S. Curran, Haley R. Dutton, and Stephen A. Bullard

Abstract

We herein resurrect and emend *Pseudoparamacroderoides* Gupta & Agrawal, 1968 (Digenea: Macroderoididae) and describe a new species, *Pseudoparamacroderoides dongthapensis* n. sp., from adult specimens infecting the intestine of a riverine catfish, *Mystus mysticetus* Roberts, (Siluriformes: Bagridae) in the Mekong River, Vietnam. *Pseudoparamacroderoides* (*Pseudoparamacroderoides seenghali* Gupta & Agrawal, 1968 [type species]; *Pseudoparamacroderoides vittati* Kakaji, 1969 [= *Ps. vittatusi*]; *Pseudoparamacroderoides raychaudhurii* Agarwal & Kumar, 1983; and *Pseudoparamacroderoides keni* Agarwal & Agarwal, 1984) differs from other macroderoidid genera by having the combination of a subspherical oral sucker that lacks distinctly-enlarged circumoral spines; caeca that extend posteriad beyond the testes without forming a cyclocoel; testes that are approximately ≤1/3 maximum body width in diameter; a cirrus sac that is claviform, slightly dorsal to and predominantly lateral to the ventral sucker (cirrus sac partially dorsolateral to dextral or sinistral margin of ventral sucker); symmetrical vitelline fields that extend posteriad to the middle of the post-testicular space (not restricted to the inter-gonadal space) and that remain separate (not confluent) anteriorly and posteriorly; and an excretory vesicle that is I-shaped (with or without anterior swelling) and wholly post-ovarian, inter-testicular, or median to the posterior testis. *Pseudoparamacroderoides dongthapensis* n. sp.

differs from its congeners by having an elongate hindbody (>2× forebody length) and an excretory vesicle that is approximately half as long as the body and that extends anteriad beyond the anterior testis. This is the first record of a species of *Pseudoparamacroderoides* from beyond the Indian sub-continent, from *M. mysticetus*, and from the Mekong River or from Vietnam. A diagnostic key to macroderoidid genera and a key to *Pseudoparamacroderoides* spp. are provided.

Introduction

Gupta & Agrawal (1968) described *Pseudoparamacroderoides seenghali* Gupta & Agrawal, 1968 (type species) (Macroderoididae McMullen, 1937) based on three and eight mature and immature specimens, respectively, infecting the intestine of a giant-river catfish, *Sperata seenghala* (Sykes), (Siluriformes: Bagridae) captured from the Gomti River (also known as the Gumti River or Gomati River, Uttar Pradesh, India), a tributary of the Ganges River. They assigned *Pseudoparamacroderoides* Gupta & Agrawal, 1968 to Walliniinae Yamaguti, 1958 within Allocreadiidae Looss, 1902 (as "Allocreadijdae"). The generic diagnosis adequately differentiated the type species from all other genera (i.e., including detail of the size, shape, distribution, and extent of the spines, suckers, oesophagus, caeca, testes, cirrus sac, ovary, seminal receptacle, uterus, vitellarium, and excretory vesicle) (Gupta & Agrawal, 1968). A year later, also from the Gomti River, Kakaji (1969) described *Pseudoparamacroderoides vittati* Kakaji, 1969 (Note: "*vittatusi*," the original spelling, is a malformed suffix as per International Commission of Zoological Nomenclature Article 11.9.1.4 [ICZN, 1999]), based on a single specimen infecting the intestine of a striped dwarf catfish, *Mystus vittatus* (Bloch), (Siluriformes: Bagridae). Yamaguti (1971) made *Pseudoparamacroderoides* a subgenus of *Paramacroderoides* Venard, 1941, thereby reassigning the genus to Macroderoididae from Allocreadiidae without

providing an argument for the decision. Baffling is that Kumari et al. (1972), apparently unaware of Yamaguti (1971), synonymised *Ps. seenghali* and *Ps. vittati* (both having an I-shaped excretory vesicle) with *Astiotrema reniferum* (Looss, 1898) Looss, 1900 (which has a Y-shaped excretory vesicle), making *Pseudoparamacroderoides* a junior subjective synonym of *Astiotrema* Looss, 1900 (placed in Astiotrematinae Baer, 1924; Plagiorchiidae Lühe, 1901). Agarwal & Kumar (1983) and Agarwal & Agarwal (1984) retained *Pseudoparamacroderoides* and described *Pseudoparamacroderoides raychaudhurii* Agarwal & Kumar, 1983 (three specimens from one host) (Varuna River, Ganges River Basin, Uttar Pradesh) and *Pseudoparamacroderoides keni* Agarwal & Agarwal, 1984 (four specimens from two hosts) (Ken River, Ganges River Basin, Madhya Pradesh, India), respectively, from the intestine of the same host species as *Ps. vittati*, *My. vittatus*. Hence, all but one of the congeners were from the same host species (*My. vittatus*) and the same river basin (Ganges River).

No worker since 1984 has collected, described, and published any detail of a museum specimen or newly-collected specimen of a species of *Pseudoparamacroderoides*, and, to our knowledge, no voucher of a named congener exists. Font & Lotz (2008) accepted that *Pseudoparamacroderoides* was a junior subjective synonym of *Paramacroderoides* without comment. Tkach et al. (2010), without detailing an argument for the synonymy, transferred the type species of *Pseudoparamacroderoides* (*Ps. seenghali*) and *Ps. raychaudhurii* to *Macroderoides* Pearse, 1924 (Digenea: Macroderoididae); thereby making *Pseudoparamacroderoides* a junior subjective synonym of *Macroderoides*. Tkach et al. (2010) did not treat the status of *Ps. vittati* and *Ps. keni*; leaving them without a generic assignment. This decision contradicted Yamaguti (1971), Kumari et al. (1972), and Font & Lotz (2008). Karar et al. (2021) disagreed with Kumari et al. (1972) and considered

Pseudoparamacroderoides distinct from *Astiotrema* (see Remarks). We are aware of no other taxomomic work that has treated *Pseudoparamacroderoides*.

Herein, we discovered numerous adult macroderoidid specimens infecting the intestine of cá chốt sọc (in Vietnamese) or trey kanchos chhnoht (in Khmer), *Mystus mysticetus* Roberts, (Siluriformes: Bagridae) from the Mekong River, Dong Thap Province, Vietnam. We describe our macroderoidid specimens as a new species of *Pseudoparamacroderoides*, resurrect and emend *Pseudoparamacroderoides*, comment on the systematics of related genera and provide a key to the genera of Macroderoididae and a key to *Pseudoparamacroderoides* spp. Our generic emendation of *Pseudoparamacroderoides* herein provides additional generic features to accommodate all congeners and help better differentiate the genus with other macroderoidids.

Materials and Methods

Three specimens of the riverine catfish, *My. mysticetus* **were purchased live from a fish market** (Cao Lanh City Market, Dong Thap Province, Vietnam; 10°27'12.8"N, 105°38'14.3"E) during September 2018. The fish were caught locally but the exact location is unknown. Fish were bagged, transported to a nearby laboratory (Dong Thap Community College, Dong Thap), euthanized, and dissected such that each organ was excised and isolated in a dish of sodium citrate (7 g $Na_3C_6H_5O_7$ dissolved in 1 L of distilled water; yielding a 7 ppt solution of sodium citrate) before being examined with the aid of a Wild Heerbrugg M5A stereodissecting microscope (Wild Heerbrugg, Heerbrugg, Switzerland) and fiber optic light source. Live adult trematodes were collected from the intestine of each infected catfish, rinsed in citrated saline, flame-killed on microscope slides using coverslips to restrain the specimens but without putting pressure on the specimen, and fixed in 10% neutral buffer formalin. Fixed specimens were stained in a mixture of Van Cleave's and Ehrlich's hematoxylins (500:1) overnight, dehydrated in

an ethanol series, cleared in clove oil, and permanently mounted on glass slides using Canada balsam. Illustrations were made using an Olympus BX51 microscope (Olympus Corp. of the Americas, Center Valley, Pennsylvania, USA) equipped with differential interference contrast optical components and drawing tube. Measurements were made using an ocular micrometer and reported in micrometers as the range followed by the mean ± standard deviation and sample size in parentheses. Fish were identified as *My. mysticetus* by having a short adipose fin, three dark stripes along the body, and a black spot behind the head (Tran et al., 2013). Taxonomic authorities for fishes follow Fricke et al. (2021). Anatomical terms for *Pseudoparamacroderoides* spp. and other macroderoidids follow Font & Lotz (2008) except that "pre-pharyngeal oesophagus" and "post-pharyngeal oesophagus" are used instead of "pre-pharynx" and "oesophagus", respectively. The so-called "pre-pharynx" is the portion of the oesophagus anterior to the pharnyx; it is not a distinctive organ but part of the oesophagus proper. The pharynx is a muscular organ that wraps around the tube (oesophagus). The length of the pre-pharyngeal segment of the oesophagus is a character state related to pharynx position. "Forebody" and "hindbody" herein refers to the pre- and post-acetabular portions of the body, respectively (Manter, 1970). Types of the new species were deposited in the National Museum of Natural History's Invertebrate Zoology Collection (USNM, Smithsonian Institution, Washington, D.C., USA).

Superfamily Plagiorchioidea Lühe, 1901

Family Macroderoididae McMullen, 1937

***Pseudoparamacroderoides* Gupta & Agrawal, 1968**

Emended generic diagnosis

Body elongate, spinous, with posterior end rounded, <5.0× longer than wide; forebody not distinctly dorsoventrally-compressed; tegumental spines triangular in outline, widest at base, sharply pointed, becoming more sparse posteriad. Posterior extent of tegumental spines variable, terminating at level of ventral sucker, testes, or covering entire body surface. Eyespot pigmentation absent. Oral sucker subterminal, subspherical, lacking unique spines (distinctively-enlarged circumoral spines absent). Ventral sucker prominent, having distinct lumen (not vestigial), subspherical, approximately same diameter as oral sucker, in anterior half of body. Pharynx subpherical or ovoid. Pre-pharyngeal segment of oesophagus shorter than pharynx or indistinct. Post-pharyngeal segment of oesophagus longer than pharynx. Intestine bifurcating in mid-forebody or immediately anterior to ventral sucker; caeca extending posteriad beyond testes, terminating approximately at mid-level of post-testicular space or to near posterior body end, without forming a cyclocoel. Testes diagnonal or nearly opposite, positioning in middle of hindbody, separated (not abutting), having diameter approximately ≤1/3 maximum body width. Cirrus sac claviform, slightly dorsal to and predominantly lateral to ventral sucker (cirrus sac partially dorsolateral to dextral or sinistral margin of ventral sucker); internal seminal vesicle present, unipartite; cirrus aspinous. External seminal vesicle absent. Genital atrium prominent; pore median or submedian, immediately anterior to ventral sucker. Ovary pre-testicular, sinistral or dextral (not median), posterolateral to ventral sucker or abutting ventral sucker. Mehlis' gland and Laurer's canal present. Seminal receptacle blind-ending, dextral or sinistral, posteromedian or median to ovary. Uterus partly inter-testicular, extending posteriad to near posterior body extremity. Metraterm unarmed. Eggs ovoid, operculate. Vitellarium follicular, in two symmetrical lateral fields, extending from level of or slightly anterior to ventral sucker posteriad approximately to mid-level of post-testicular space (not restricted to inter-gonadal space),

separate (not confluent) anteriorly and posteriorly. Excretory vesicle I-shaped (with or without anterior swelling), massive, wholly post-ovarian, inter-testicular, extending anterior to or beyond anterior testis or median to posterior testis; pore terminal. Parasites of the intestine of freshwater bagrid catfishes ranging in the Indian-subcontinent and Southeast Asia.

Type-species: Pseudoparamacroderoides seenghali Gupta & Agrawal, 1968.

Other species: Pseudoparamacroderoides vittati Kakaji, 1969; *Pseudoparamacroderoides raychaudhurii* Agarwal & Kumar, 1983; *Pseudoparamacroderoides keni* Agarwal & Agarwal, 1984; *Pseudoparamacroderoides dongthapensis* n. sp.

Differential diagnosis

Body elongate, <5.0× longer than wide, with broadly rounded posterior end. Oral sucker subspherical (not funnel-shaped), lacking distinctively-enlarged circumoral spines. Ventral sucker prominent, having distinct lumen (not vestigial). Suckers approximately equal in diameter. Caeca terminating in post-testicular space near posterior body end, without forming a cyclocoel. Testes approximately ≤1/3 maximum body width in diameter. Cirrus sac slightly dorsal to and predominantly lateral to ventral sucker (partially dorsolateral to dextral or sinistral margin of ventral sucker). Vitelline fields symmetrical, separate (not confluent) anteriorly and posteriorly (not restricted to inter-gonadal space). Excretory vesicle I-shaped, with or without slight anterior swelling, massive, wholly post-ovarian, inter-testicular, extending anterior to or beyond anterior testis or median to posterior testis.

***Pseudoparamacroderoides dongthapensis* Truong, Curran, & Bullard n. sp.**

Type-host: Mystus mysticetus Roberts, (Siluriformes: Bagridae), cá chốt sọc or trey kanchos chhnoht.

Type-locality: Mekong River (Cao Lanh Fish Market, Dong Thap Province, Vietnam; 10°27'12.8"N, 105°38'14.3"E).

Type-material: Holotype (USNM 1660548); paratypes (USNM 1660549–1660553).

Site in host: Intestine.

Prevalence and intensity of infection: Two of three (67%) *My. mysticetus* were infected by two and five adult specimens of the new species.

ZooBank registration: urn:lsid:zoobank.org:act:1399C795-D5BD-447A-B358-5C5F9E911F20.

Etymology: The specific epithet is for the type locality.

Description (Figs. 1–7)

(Based on light microscopy of 7 stained, whole-mounted adult specimens [USNM 1660548–1660553]).

Body with rounded ends, 1,610–2,480 (2,085 ± 302; n = 6) long or 3.4–4.9× (4.0 ± 0.6; n = 6) longer than wide, 410–680 (528 ± 103; n = 6) wide, widest at level of mid-body; forebody 450–630 (550 ± 62; n = 6) long or 25–29% (27 ± 2; n = 6) of body length (BL); hindbody 1,010–1,620 (1,358 ± 222; n = 6) long or 62–67% (65 ± 2; n = 6) of BL or >2× forebody length (Fig. 1). Tegumental spines present on ventral and dorsal body surfaces, wholly covering forebody, extending posteriad to level of ventral sucker or testes (Fig. 1). Ventral tegumental spines at level of oral sucker 11–13 (12 ± 1; n = 10) long, 8–9 (8 ± 1; n = 10) wide (Figs. 2, 3); spines between suckers 11–12 (12 ± 1; n = 10) long, 6–7 (6 ± 0; n = 10) wide (Figs. 2, 3); spines at level of gonads 13–18 (15 ± 2; n = 10) long, 10–11 (10 ± 1; n = 10) wide. Dorsal tegumental spines at level of oral sucker 11–13 (12 ± 1; n = 10) long, 6–8 (7 ± 1; n = 10) wide; spines between suckers 11–12 (12 ± 1; n = 10) long, 6–8 (7 ± 1; n = 10) wide; spines at level of gonads 12–14 (13 ± 1; n = 10) long, 6–7 (6 ± 1; n = 10) wide. Oral sucker broad, embedded in tegument, 130–

188 (160 ± 22; n = 6) long, 148–225 (194 ± 30; n = 6) wide or 30–45% (37 ± 5; n = 6) of body width (BW) (Figs. 1, 2). Ventral sucker median, slightly wider than long, 135–213 (176 ± 29; n = 6) long, 143–225 (188 ± 28; n = 6) wide or 30–44% (36 ± 5; n = 6) of BW, 1.0–1.2× (1.0 ± 0.1; n = 6) oral sucker width (Fig. 1). Pre-pharyngeal oesophagus indistinct, 5–15 (11 ± 4; n = 6) long, 33–55 (47 ± 9; n = 6) in maximum width. Pharynx subspherical, 80–130 (113 ± 17; n = 6) long, 78–170 (132 ± 33; n = 6) wide or 19–31% (25 ± 5; n = 6) of BW. Post-pharyngeal oesophagus sinuous or straight, 113–238 (169 ± 52; n = 6) long or 6–12% (8 ± 2; n = 6) of BL, 33–65 (55 ± 11; n = 6) in maximum width (Fig. 1). Intestinal bifurcation in forebody, 380–480 (438 ± 41; n = 6) from anterior body end or 19–24% (21 ± 2; n = 6) of BL. Caeca terminating 60–120 (93 ± 25; n = 6) or 3–6% (5± 1; n = 6) of BL from posterior body end (Figs. 1, 4).

Testes subspherical, typically oblique or nearly opposite in two of seven specimens, each having a diameter 28–35% of maximum BW; anterior testis 143–208 (170 ± 21; n = 6) long, 115–228 (167 ± 41; n = 6) wide, 920–1,310 (1,117 ± 144; n = 6) from anterior end of body or 50–57% (54 ± 3; n = 6) of BL; posterior testis slightly larger than anterior testis, 153–225 (187 ± 28; n = 6) long, 145–238 (192 ± 37; n = 6) wide; post-testicular space 510–930 (710 ± 177; n = 6) or 25–40% (34 ± 5; n = 6) of BL (Figs. 1, 4). Cirrus sac elongate, 300–503 (396 ± 67; n = 6) long or 15–24% (19 ± 3; n = 6) of BL, 40–80 (64 ± 15; n = 6) in maximum width, dextral to ventral sucker, extending 60–130 (95 ± 32; n = 6) posteriad beyond ventral sucker (Figs. 1, 5). Internal seminal vesicle elongate, approximately 1/2 cirrus sac length, 158–258 (220 ± 41; n = 6) long, 38–73 (58 ± 13; n = 6) wide (Fig. 5). Pars prostatica 60–145 (103 ± 35; n = 4) long or 12–48% (28 ± 15; n = 4) cirrus sac length, 13–23 (20 ± 2; n = 4) wide; prostatic gland cells numerous, surrounding male terminal duct; cirrus muscular, bulbous, 60–103 (77 ± 18; n = 4) long, comprising 15–20% (18 ± 2; n = 4) of cirrus sac length, 35–55 (46 ± 9; n = 4) wide; cirrus

everted in one specimen (USNM 1660551), 145 long (Fig. 5). Genital atrium 28–53 (40 ± 13; n = 3) long, 15–25 (21 ± 5; n = 3) wide (Fig. 5). Genital pore median, posterior to intestinal bifurcation (Figs. 1, 5).

Ovary subspherical, submedian, pre-testicular, abutting proximal end of cirrus sac or ventral sucker, 113–163 (145 ± 21; n = 6) long, 133–190 (164 ± 20; n = 6) wide, 630–870 (753 ± 84; n = 6) from anterior end or 34–39% (36 ± 2; n = 6) of BL (Figs. 1, 5, 6). Oviduct slender, ventral to other components of female genitalia. Oötype thick-walled, connecting to surrounding Mehlis' gland cells. Seminal receptacle ovoid, dorsal to oviduct, post-ovarian, 105–225 (156 ± 57; n = 4) long, 48–90 (60 ± 20; n = 4) in maximum width; seminal receptacle connection to oviduct not observed. Laurer's canal proximal portion observed in two of seven specimens, dextral, extending posteriad lateral to seminal receptacle, dorsal to common vitelline duct (Fig. 6). Vitellarium comprising irregularly-shaped follicles; fields primarily ventral to caeca, extending 55–575 (365 ± 197; n = 6) posteriad beyond posterior testis, terminating 300–490 (353 ± 72; n = 6) from posterior body end or 12–24% (15 ± 4; n = 6) of BL, connecting medially to common vitelline reservoir via two slender ducts; vitelline reservoir post-ovarian, dorsal to uterus, connecting with oviduct approximately in middle of oviduct (Fig. 6). Uterus extensive in hindbody, filling inter-vitelline space, extending posteriad beyond caecal tips. Metraterm prominent, entering genital atrium ventral relative to cirrus sac, partly dorsal to ventral sucker (Fig. 5). Eggs filling uterus in all specimens, 28–33 (30 ± 2; n = 30) in maximum length, 10–13 (13 ± 1; n = 30) in maximum width (largest eggs nearest metraterm) (Figs. 1, 7).

Excretory vesicle dorsal to uterus, transversely inflated at distal end (anterior swelling), extending 48–205 (n = 2) anteriad beyond anterior testis; excretory vesicle length 1,030–1,270 (n = 2) or 49–55% of BL (Fig. 4).

Remarks

Species of *Pseudoparamacroderoides* resemble *Macroderoides typicus* (Winfield, 1929) Van Cleave & Mueller, 1932 (originally *Plesiocreadium typicum* Winfield, 1929, type species of *Plesiocreadium* Winfield, 1929) and *Macroderoides flavus* Van Cleave & Mueller, 1932. Van Cleave & Mueller (1932) transferred *Pl. typicum* to *Macroderoides*; thereby synonymising *Plesiocreadium* with *Macroderoides* ("*We can find no point wherein Plesiocreadium of Winfield, 1929 differs from the concept of Macroderoides*"). Although no explicit argument for that synonymy was presented by Van Cleave & Mueller (1932), subsequent authors have accepted it. *Pseudoparamacroderoides* spp. and the aforementioned species have an elongate body that is <5.0× longer than wide, suckers that are approximately equal in size, a subterminal oral sucker that is not funnel-shaped, a short or indistinct pre-pharyngeal oesophagus, testes that are typically diagonal in the middle of the hindbody, an ovary that is posterolateral to or abutting the ventral sucker, a uterus that is partly inter-testicular, and a vitellarium that is separate anteriorly and posteriorly and that extends from the level of or slightly anterior to the ventral sucker posteriad beyond the posterior testis (not to level of the caecal tips). *Pseudoparamacroderoides* spp. also resemble *Macroderoides minutus* Tkach & Kinsella, 2011 in having having an ovoid body and small testes. *Pseudoparamacroderoides* spp. differ from *Ma. typicus* and *Ma. flavus* by having a forebody that is not distinctly dorsoventrally-compressed (vs. distinctly dorsoventrally-compressed) and a broadly rounded posterior end (vs. with a strongly tapering posterior end), a cirrus sac that is slightly dorsal to and predominantly lateral to the ventral sucker (vs. dorsally overlapping the ventral sucker), a blind-ending seminal receptacle (vs. lacking; having a uterine seminal receptacle), a vitellarium that is in symmetrical fields (vs. asymmetrical), and an excretory vesicle that is I-shaped, with or without an anterior swelling, inter-testicular or median

to the posterior testis (vs. a saccate excretory vesicle that is wholly post-testicular or extending to level of the testes). An investigation on the taxonomic identity of species originally assigned to *Plesiocreadium* is forthcoming by the present authors. *Pseudoparamacroderoides* spp. differ from *Ma. minutus* by having testes that are separate (vs. abutting), an ovary that is posterolateral to or abutting the ventral sucker (vs. slightly dorsal to the ventral sucker), a seminal receptacle that is post-acetabular, posteromedian or median to the ovary (vs. dorsal to the ventral sucker and ventral to the ovary), and a uterus that is partly inter-testicular (vs. wholly ventral to the testes) (Gupta & Agarwal, 1968; Tkach & Kinsella, 2011).

Gupta & Agrawal's (1968) diagnosis of *Pseudoparamacroderoides* effectively differentiated the genus from other macroderoidid genera, and we fail to see an extant argument for why it should be regarded as a junior subjective synonym of *Paramacroderoides* or *Macroderoides*. The key we provide herein (Key 1) differentiates the resurrected genus from other accepted genera of the family. It differs from *Macroderoides* (except *Ma. typicus*, *Ma. flavus*, and *Ma. minutus*) and *Paramacroderoides* by having a more ovoid, less elongated body (~3–5× vs. 8–12× and 7–18× longer than wide, respectively), a cirrus sac that is slightly dorsal to and predominantly lateral to the ventral sucker (vs. wholly dorsal), smaller testes (diameter ~20–35% of maximum BW vs. ~50–80% and 50–90% of maximum BW, respectively), an ovary that is posterolateral to or abuts the ventral sucker (vs. an ovary that is far posterior to the ventral sucker), and an excretory vesicle that is ~30–50% of BL (vs. an excretory vesicle that is confined to the post-testicular space; 16% of BL in *Macroderoides texanus* Tkach, Strand, & Froese, 2008 and ~20% of BL in *Paramacroderoides* spp.) (Pearse, 1924; Vernard, 1941; Gupta & Agrawal, 1968; Tkach et al., 2008). *Pseudoparamacroderoides* further differs from *Paramacroderoides* by having a subterminal, subspherical oral sucker that lacks either distinctively-enlarged circumoral

spines or a constriction between the oral sucker and the forebody (vs. a terminal, funnel-shaped oral sucker with distinctively-enlarged circumoral spines and having a post-oral constriction) (Vernard, 1941; Gupta & Agrawal, 1968). All *Pseudoparamacroderoides* spp. infect species of Bagridae.

Pseudoparamacroderoides differs from *Perezitrema* Baruš & Moravec, 1967 (as emended by Moravec & Salgado-Maldonado [2002]) by having a subterminal, subspherical oral sucker (vs. a terminal, funnel-shaped oral sucker) that is approximately equal in diameter to the ventral sucker (vs. >2× wider in diameter than the ventral sucker), caeca that do not form a cyclocoel (vs. cyclocoel-ending caeca), and testes that are more anteriad in the hindbody, post-testicular space 24–32% of BL (vs. testes are more posteriad; post-testicular space 9% of BL) (Gupta & Agrawal, 1968; Moravec & Salgado-Maldonado, 2002). *Pseudoparamacroderoides* differs from monotypic *Cirkennedya* Gibson & Bray, 1979 by having a distinctly smaller body (1,160–2,480 × 410–680 vs. 6,000–9,500 × 1,500–2,250), a prominent (luminal) ventral sucker in the anterior half of the body (vs. a vestigial, without lumen ventral sucker in the anterior third of the body), testes that are widely separated and smaller (diameter ~20–35% of maximum BW) (vs. testes abutting; diameter ~45–70% of maximum BW), and a vitellarium that is separate (not confluent) anteriorly and posteriorly (vs. a vitellarium that is confluent anteriorly and posteriorly) (Gupta & Agrawal, 1968; Gibson & Bray, 1979). *Pseudoparamacroderoides* differs from monotypic *Malawitrema* Bray & Hendrix, 2007 by having intestinal caeca that terminate in the post-testicular space near the posterior body end (vs. wholly pre-testicular caeca), a cirrus sac that is slightly dorsal to and predominantly lateral to the ventral sucker (vs. dorsally overlapping), an ovary that is posterolateral to or abuts the ventral sucker (vs. dorsally overlapping), and a vitellarium that extends from level of or slightly anterior to the ventral sucker posteriad

approximately to the middle of the post-testicular space (vs. a restricted vitellarium that is limited to a region between the ovary and testes) (Gupta & Agrawal, 1968; Bray & Hendrix, 2007). *Pseudoparamacroderoides* differs from both *Rauschiella* Babero, 1951 and *Gauhatiana* Gupta, 1953 by having an I-shaped excretory vesicle with or without a slight anterior swelling (*Rauschiella* and *Gauhatiana* are the only macroderoidid genera that possess a Y-shaped excretory vesicle) (Babero, 1951; Gupta, 1953; Gupta & Agrawal, 1968). Further, *Rauschiella* differs from *Pseudoparamacroderoides* by having an oral sucker that is much larger than the ventral sucker, wholly extra-caecal vitelline follicles, and a massive uterus that fills the post-ovarian inter-caecal space and that presents as a compact, transverse portion in the post-testicular space (Babero, 1951). *Gauhatiana* further differs from *Pseudoparamacroderoides* by having a vitellarium that is discontinuous at level of the ventral sucker (Gupta, 1953). *Pseudoparamacroderoides* differs from *Glossidium* Looss, 1899 and *Astiotrema* (both regarded as *incertae sedis* in Plagiorchioidea) by having an I-shaped (not Y-shaped) excretory vesicle (Gupta & Agrawal, 1968; Pojmańska et al., 2008; Karar et al., 2021).

Pseudoparamacroderoides dongthapensis n. sp. differs from other congeners by having a more elongate hindbody, >2× longer than the forebody (2.2–2.6 vs. 1.4–1.9× longer), and a long excretory vesicle (extending anteriad beyond the anterior testis, 49–55% of BL vs. extends to mid-level of the posterior testis or anterior testis; 33–44% of BL). The new species further differs from *Ps. seenghali*, *Ps. raychaudhurii*, and *Ps. keni* by having a cirrus sac that extends posteriad to the ventral sucker (Gupta & Agrawal, 1968; Agarwal & Kumar, 1983; Agarwal & Agarwal, 1984). It further differs from *Ps. seenghali*, *Ps. vittati*, and *Ps. raychaudhurii* by having a uterus that extends posteriad to the post-caecal space near the posterior body end, post-uterine space ~1% of BL (vs. a uterus that is restricted to the pre-caecal space; post-uterine space ~13% of BL

in *Ps. seenghali*, ~10% of BL in *Ps. vittati*; ~4% of BL in *Ps. raychaudhurii*) (Gupta & Agrawal, 1968; Kakaji, 1969; Agarwal & Kumar, 1983). It further differs from *Ps. vittati* by having a shorter post-pharyngeal oesophagus that is 6–12% of BL (vs. 19% of BL) and proportionally larger testes (diameter 28–35% vs. 15–17% of maximum BW) (Kakaji, 1969). It further differs from *Ps. keni* by having a more elongate body (~3–5× vs. ~2× longer than wide), caeca that extend posteriad to near the posterior body end (vs. to mid-level of the post-testicular space), a subspherical (vs. lobed) ovary, and vitelline follicles that extend anteriad to level of the ventral sucker (vs. extending more anteriad in the forebody; to level of the pharynx) (Agarwal & Agarwal, 1984). We additionally provide a diagnostic key (Key 2) for all nominal species of *Pseudoparamacroderoides*.

The body spination of *Pseudoparamacroderoides* spp. could be taxonomically important to differentiate species, but needs further investigation. In the generic diagnosis of *Pseudoparamacroderoides* and the description of *Ps. seenghali*, Gupta & Agrawal (1968) stated, "*oral sucker with spines larger than body spines*". Since then, subsequent authors (Kakaji [1969]; Agarwal & Kumar [1983]; Agarwal & Agarwal [1984]) used that feature and the posterior extent of the tegumental spines but did not report a measurement. We emphasise here that the spines detailed by Gupta & Agrawal (1968) are like those of the new species described herein: they comprise triangular, scale-like tegumental body spines that are slightly larger anteriorly, becoming smaller posteriorly on the ventral and dorsal body surface. These spines are clearly distinct from the circumoral spines (either larger or smaller than the tegumental spines) of the macroderoidids *Ma. texanus* and species of *Paramacroderoides*.

The four species originally assigned to *Pseudoparamacroderoides*, which collectively infect the intestine of two bagrid catfish species in India, deserve attention. Three of the four Indian

species of *Pseudoparamacroderoides* infect a single fish species in the same river basin (Ganges River); none has been reported since nor has been sequenced, and the types are evidently lost or inaccessible. The illustrations for *Ps. seenghali* and *Ps. vittati* are reportedly all in dorsal view and appear to be enantiomorphic. The same is true for *Ps. raychaudhurii* and *Ps. keni*, which were illustrated in ventral view and are apparently also enantiomorphic. We are aware of no other genus of trematodes wherein the majority of species are enantiomorphic; which is a relatively rare occurrence among flatworms (Winfield, 1929; Patella & Bullard, 2013; Dutton et al., 2021; Curran et al., 2021).

Based on the published description alone, *Ps. seenghali* could be distinctive from its Indian congeners by the combination of having a proportionally short post-pharyngeal oesophagus (13–15% of BL), proportionally large testes (diameter = 20–21% of maximum BW), a uterus that restricted to the pre-caecal space (post-uterine space = 13% of BL), and an excretory vesicle that extends anteriad along the midline and terminates between the testes. The excretory vesicle of *Ps. vittati* terminates at mid-level of the posterior testis, whereas that of *Ps. seenghali* terminates between the testes. Kakaji (1969) stated, "*excretory bladder* (= vesicle) *upto middle of posterior testis instead of upto anterior end of anterior testis*," but the whole body drawing of *Ps. seenghali* in Gupta & Agrawal (1968) shows that the excretory vesicle does not reach the anterior margin of the anterior testis. Kakaji (1969) further differentiated this species from *Ps. seenghali* by "*ovary some distance behind ventral sucker instead of overlapping*" and "*receptaculum seminis* (seminal receptacle) *lateral to ovary*." The taxonomic utility of the slight separation between the ventral sucker and ovary should be assessed in more than a single specimen. We reject the assertion that the position and shape of the seminal receptacle in *Ps. vittati* are distinct from those in *Ps. seenghali*: the seminal receptacle is the same shape and in the

same position in both descriptions by Gupta & Agrawal (1968) and Kakaji (1969). Kakaji (1969) used the distribution of the vitellarium and distribution (inter-caecal or extra-caecal) of so-called "*uterine coils*" to differentiate *Ps. seenghali* and *Ps. vittati*. Regarding the vitellarium, Kakaji (1969) stated, "*extension of vitellaria from anterior end of ventral sucker* (as in *Ps. vittati*) *instead of intestinal bifurcation or a little anterior to it* (as in *Ps. seenghali*)." Problematic is that the illustrations of these taxa are identical regarding the anterior extent of the vitellarium (both have a vitellarium that extends from a level immediately posterior to the intestinal bifurcation to the posterior body end; terminating anterior to the caecal tips). Hence, either the narrative description or the illustration of *Ps. vittati* is erroneous; casting doubt on the status of this character state. Regarding the so-called "*uterine coils*" for *Ps. seenghali*, Gupta & Agrawal (1968) stated, "*Uterine coils numerous, intercaecal passing between testes*." In an apparent lapse, Kakaji (1969) differentiated *Ps. vittati* from *Ps. seenghali* by stating that *Ps. vittati* was the only congener with inter-caecal uterine coils.

Hence, in summary, the length or anterior extent of the excretory vesicle is evidently the strongest, and perhaps the only, evidence that *Ps. vittati* is distinct from *Ps. seenghali*. The use of the other features (separation between ovary and ventral sucker, seminal receptacle shape and position, anterior extent of vitellarium, and inter-caecal/extra-caecal uterine coils) is dubious and certainly not well-justified. For that reason, we think that *Ps. vittati* would benefit from a redescription; especially regarding the extent of the excretory vesicle.

Agarwal & Kumar (1983) differentiated *Ps. raychaudhurii* from *Ps. seenghali* by the presence/absence of a so-called "*pre-pharynx*" (see Materials and Methods), a separation between the ovary and ventral sucker (a dubious feature; see above), and a reportedly coiled seminal vesicle as well as by the distribution of the vitellarium. Since the forebody and

oesophagus of these flukes are distensible, we are skeptical that length of the pre-pharyngeal oesophagus is not vulnerable to fixation artifact – even in well-fixed, heat-killed specimens. Likewise, regarding the gap between the ovary and ventral sucker, we further doubt this feature as reliable since the adult depicted in Figure 1A of Agarwal & Kumar (1983) is evidently pinched in the exact location to artifactitiously push the ovary away from the ventral sucker. Regarding the "*coiled vesicula seminalis*" of *Ps. raychaudhurii*, Agarwal & Kumar (1983) drew a seminal vesicle that was medially constricted (see their Figure 1B) while their narrative description described the seminal vesicle as "coiled", which could have led Karar et al. (2021) to conclude that *Pseudoparamacroderoides* spp. have a bipartite seminal vesicle. We regard all species of *Pseudoparamacroderoides* as having a unipartite seminal vesicle until further evidence to the contrary is provided. Regarding the extension of the vitellarium, Agarwal & Kumar (1983) reported "*vitelline follicles extend from anterior end of ventral sucker to a little posterior to posterior testis.*" Aside from paraphrasing, there is no illustrated difference between the vitellarium distribution in *Ps. seenghali*, *Ps. vittati*, and *Ps. raychaudhurii*. In summary, the description of *Ps. raychaudurii* weakly differentiates it from the type species *Ps. seenghali*; these descriptions are in fact strikingly similar to each other.

Regarding the comparison between *Ps. raychaudhurii* and *Ps. vittati*, Agarwal & Kumar (1983) used the proportional size of the suckers, oesophagus length, excretory vesicle length, and seminal receptacle shape to justify the new species. First, regarding proportional size of the suckers, Gupta & Agrawal (1968), Kakaji (1969), and Agarwal & Kumar (1983) stated, "*ventral sucker smaller than oral sucker*" (for *Ps. seenghali*), "*ventral sucker nearly as large as oral sucker*" (for *Ps. vittati*), and "*ventral sucker more or less equal to oral sucker*" (for *Ps. raychaudhurii*), respectively. The published illustrations of these flukes show that the suckers are

nearly equal; there is no difference between them in that regard. Regarding the oesophagus length, we presume that these authors measured the post-pharyngeal length of the oesophagus (the distance from the posterior margin of the pharynx to the intestinal bifurcation). Comparing the illustrations of these species, that section of the oesophagus is evidently longest in *Ps. vittati* and seemingly equal in length between *Ps. seenghali* and *Ps. raychaudhurii*. The excretory vesicle of *Ps. raychaudhurii* is identical to that described for *Ps. seenghali*. Seminal receptacle shape has been described putatively as "*saccular*" (in *Ps. raychaudhurii*), "*flask shaped*" (*Ps. vittati*), and "*pear-shaped*" (*Ps. seenghali*). We do not see a demonstrable difference between these putative shape descriptors, and the published illustrations fail to differentiate them. Hence, the narrative description seems to comprise paraphrasing and the published illustrations lack sufficient detail to confirm the putatively unique shapes of the seminal receptacle described by these authors. As such, we are skeptical that *Ps. raychaudhurii* is a distinct species; it too needs a redescription.

The description of *Ps. keni* is also problematic (Agarwal & Agarwal, 1984). These authors did not treat *Ps. raychaudhurii*; which either comprises a lapse or indicates that, without comment, these authors did not accept *Ps. raychaudhurii* as distinctive from its congeners. These authors stated that *Ps. keni* differed from *Ps. seenghali* and *Ps. vittati* by "*the extension of vitelline follicles and in having short oesophagus. It further differs from Ps. seenghali in having a pre-pharynx and from Ps. vittati in ratio of suckers.*" The vitellarium of *Ps. keni* is illustrated by Agarwal & Agarwal (1984) as extending from the pharynx/intestinal bifurcation posteriad to end immediately anterior to the caecal tips. In *Ps. seenghali, Ps. vittati,* and *Ps. raychaudhurii*, the anterior extent of the vitellarium is posterior to the intestinal bifurcation. The presence of the so-called "pre-pharynx" was used as a diagnostic feature for *Ps. keni*. However, *Ps. vittati* and

Ps. raychaudhurii both were illustrated as having a gap between the pharynx and oral sucker ("pre-pharynx present"), and we suspect that this feature is present in all members of this group depending on the state of contraction upon fixation. The oesophagus of *Ps. keni* is shorter than that of *Ps. vittati* but indistinct from that of *Ps. seenghali* and *Ps. raychaudhurii*. The ratio of the ventral sucker diameter to oral sucker diameter appears to be identical in all illustrations of *Pseudoparamacroderoides* spp. We are baffled by the use of this feature as diagnostic since all of the drawings of these species seem to have an oral sucker that is approximately the same diameter as that of the ventral sucker; the ratio is ~1:1 in each species. Based on the aforementioned taxonomic features used by Agarwal & Agarwal (1984) to differentiate *Ps. keni* from two of its three congeners, we think that this species also should be recollected and redescribed. The published description indicates that *Ps. keni* may be distinct by having five or six vitelline follicles that are illustrated anterior to the intestinal bifurcation. If nucleotide data become available, we expect these species to be similar or identical in the ITS2 region and 28S rDNA gene.

Discussion

Macroderoididae is in need of systematic revision. The type genus *Macroderoides* is paraphyletic (Hernández-Mena et al., 2016), some synonymized genera appear morphologically distinct, and some species clearly seem exceptionally distinct from their congeners. Since Font & Lotz's (2008) chapter in Keys to the Trematoda, *Magnivitellinum* Kloss, 1966 and *Alloglossidium* Simer, 1929 have been reassigned from Macroderoididae to Alloglossidiidae Hernández-Mena, Mendoza-Garfias, Ornelas-García, & Pérez-Ponce de León, 2016 (see Hernández-Mena et al., 2016). This subtraction left seven macroderoidid genera that were accepted before the present study (i.e., *Macroderoides*; *Paramacroderoides*; *Rauschiella*;

Gauhatiana; *Perezitrema*; *Cirkennedya*; *Malawitrema*). The present study resurrects *Pseudoparamacroderoides*, bringing the total number of accepted genera to eight. We regard the synonymy of *Pseudoparamacroderoides* (see Yamaguti, 1971; Font & Lotz, 2008; Tkach et al., 2010) as indefensible since no argument has been articulated to justify it. We think that the genus comprises a morphologically-similar group of nominal species that collectively is distinct from related macroderoidids. Moreover, and although irrelevant from a strict taxonomic sense, *Pseudoparamacroderoides* includes species that infect an endemic catfish lineage (Bagridae) in a particular geographic area (Indian sub-continent, Southeast Asia). Despite the morphological distinctiveness of the genus and the associated host and geographic locality data, our examination of the literature shows that this genus has been essentially ignored by parasitologists outside of India. Yamaguti's (1971) decision to relegate *Pseudoparamacroderoides* as a sub-genus lacked an argument, omitted or ignored several useful features clearly diagnosed by Gupta & Agrawal (1968), and introduced at least one error. Yamaguti's (1971) over-simplified diagnosis comprised the presence of diagonal testes, a "*weakly-developed*" (thin-walled?) cirrus sac, a post-ovarian seminal receptacle, and, erroneously, a vitellarium that commences approximately at level of the ventral sucker. The last feature is a lapse by Yamaguti (1971) because *Ps. seenghali* (along with *Ps. raychaudhurii* and *Ps. keni*) has a vitellarium that extends anterior to the ventral sucker. Despite these issues, subsequent workers (Font & Lotz, 2008; Tkach et al., 2010) accepted Yamaguti's (1971) action.

The excretory vesicle and seminal receptacle are likely important diagnostic features for macroderoidids. Pearse (1924) and Font & Lotz (2008) did not mention the anterior extent (length) of the excretory vesicle in their diagnoses of *Macroderoides* but we think it is reliable to differentiate genera of Macroderoididae. This important feature has been described in only *Ma.*

texanus, which has a short, wholly post-testicular, slender I-shaped excretory vesicle (Tkach et al., 2008). Winfield (1929) and Hunter (1932) diagnosed *Ma. typicus* and *Macroderoides parvus* (Hunter, 1932) Van Cleave & Mueller, 1934, respectively as having a "bag-shaped" excretory vesicle, which does not help diagnose the shape of the structure. Regarding the seminal receptacle, Pearse (1924) and Font & Lotz (2008) did not mention the presence/absence of a seminal receptacle in their diagnoses or amendment of *Macroderoides*. At least *Ma. texanus*, *Ma. minutus*, and *Macroderoides luki* Kusy & Barger, 2017 have a seminal receptacle (Tkach et al., 2008; Tkach & Kinsella, 2011; Kusy & Barger, 2017); *Ma. typicus* and *Ma. parvus* lack a seminal receptacle (having a uterine seminal receptacle). Taylor (1978) asserted the presence of a seminal receptacle in *Macroderoides*. Consequently, seven genera (*Macroderoides*, *Paramacroderoides*, *Rauschiella*, *Gauhatiana*, *Perezitrema*, *Pseudoparamacroderoides*, and *Malawitrema*) include at least some species that have a seminal receptacle (Pearse, 1924; Venard, 1941; Babero, 1951; Gupta, 1953; Gupta & Agrawal, 1968; Moravec & Salgado-Maldonado, 2002; Bray & Hendrix, 2007).

The biodiversity of digeneans infecting fishes of the Mekong River Delta (MRD) remains vastly under-explored due to a lack of focused parasitological surveys there (Truong et al., 2021). Bagridae (the only known host group of *Pseudoparamacroderoides* spp.) is clearly under-sampled for macroderoidid infections. It currently comprises 23 genera and 189 species that range in tropical and temperate rivers and estuaries (Ferraris, 2007). Only three species (long whiskers catfish, *Mystus gulio* Hamilton; cá chốt trắng [in Vietnamese] or trey kanchos [in Khmer], *Mystus wolffii* [Bleeker], and cá chốt vàng [in Vietnamese], *Mystus velifer* Ng) reportedly inhabit estuarine and coastal habitats in the Indian sub-continent and Southeast Asia (Ng, 2012; Ng et al., 2015). Nine bagrids (seven riverine plus two estuarine) range in the MRD,

Vietnam (Tran et al., 2013). Given that only three bagrids are currently known as hosts for macroderoidids (including the new species described herein; see above), we suspect that necropsies of these bagrids could reveal new macroderoidid species. Presently, no molecular data is available for any species of *Pseudoparamacroderoides*.

Acknowledgments

We thank Thuy Ha Nguyen, former Rector of Dong Thap Community College, for hosting us during the collection in Vietnam; Steven P. Ksepka, Micah B. Warren (Auburn University, Auburn, Alabama, USA), and Carlos F. Ruiz (El Paso, Texas, USA) for helping with fish collection, necropsy and identification. This study was supported by research grants from the Auburn University Vice President for Research and Economic Development, Southeastern Cooperative Fish Parasite and Disease Project (Alabama Department of Conservation and Natural Resources), Alabama Agriculture Experiment Station, and US Department of Agriculture. Earlier exploratory aspects of this project were supported by grants from the National Science Foundation.

References

Agarwal, G. P., & Kumar, R. (1983). On a new digenetic trematode *Pseudoparamacroderoides raychaudhurii* n. sp. from the intestine of a freshwater fish *Mystus vittatu*s (Bl.) at Varanasi, India. *Rivista di Parassitologia, 44,* 313–316.

Agarwal, L. N., & Agarwal, G. P. (1984). On a new species of the genus *Pseudoparamacroderoides* Gupta & Agrawal 1968 (Trematoda: Macroderoidae) from the intestine of *Mystus vittatus* (Bl.). *Indian Journal of Helminthology, 36,* 51–54.

Babero, B. B. (1951). *Rauschiella tineri* n. g., n. sp., a trematode (Plagiorchiinae) from a frog. *Journal of Parasitology, 37,* 560–562.

Bray, R. A., & Hendrix, S. S. (2007). A new genus and species of Macroderoididae, and other digeneans from fishes of Lake Malawi, Africa. *Journal of Parasitology, 93,* 860–865.

Curran, S. S., Dutton, H. R., Warren, M. B., du Preez, L., & Bullard, S. A. (2021). Two new species of Cephalogonimidae Looss, 1899 (Digenea: Plagiorchioidea) from Africa (Mozambique and Guinea), including a new phylogenetic hypothesis for related plagiorchioids. *International Journal for Parasitology: Parasites and Wildlife, 14*, 228–240.

Dutton, H. R., du Preez, L. H., Verneau, O., Whelan, N. V., & Bullard, S. A. (2021). First record of a polystome from alligator snapping turtle, *Macrochelys temminckii* (Cryptodira: Chelydridae) or Mississippi; with comments on "*Neopolystoma orbiculare* (Stunkard, 1916)" and its junior subjective synonyms. *Journal of Parasitology, 107,* 74–88.

Ferraris, C. J. (2007). Checklist of catfishes, recent and fossil (Osteichthyes: Siluriformes), and catalogue of siluriform primary types. *Zootaxa, 1418,* 1–628.

Font, W. F., & Lotz, J. M. (2008). Family Macroderoididae McMullen, 1937. In: Bray, R. A., Gibson, D. I., & Jones, A. (Eds.), *Keys to the Trematoda. Volume 3*. CAB International & Natural History Museum, Cambridge, U.K. pp. 373–380.

Fricke, R., Eschmeyer, W. N., & Van der Laan, R. (2021). *Eschmeyer's Catalog of Fishes: Genera, Species, References*. Available at: http://researcharchive.calacademy.org/research/ichthyology/catalog/fishcatmain.asp. Accessed 5 May 2021.

Gibson, D. I., & Bray, R. A. (1979). *Cirkennedya porlockensis*, a new genus and species of digenean from the sunfish *Mola mola* (L.). *Journal of Helminthology, 53,* 245–250.

Gupta, S. P. (1953). Trematode parasites of fresh-water fishes. *Indian Journal of Helminthology, 5,* 1–80.

Gupta, S. P., & Agrawal, V. (1968). *Pseudoparamacroderoides seenghali* n. g., n. sp. (Allocreadiidae: Walliniinae) from the intestine of a fresh water fish, *Mystus seenghala* (Sykes) from Lucknow, India. *Indian Journal of Helminthology, 20,* 70–74.

Hernández-Mena, D. I., Mendoza-Garfias, B., Ornelas-García, C. P., & Pérez-Ponce de León, G. (2016). Phylogenetic position of *Magnivitellinum* Kloss, 1966 and *Perezitrema* Baruš & Moravec, 1967 (Trematoda: Plagiorchioidea: Macroderoididae) inferred from partial 28S rDNA sequences, with the establishment of Alloglossidiidae n. fam. *Systematic Parasitology, 93,* 525–538.

Hunter, G. W. (1932). A new trematode (*Plesiocreadium parvum*, sp. nov.) from fresh water fish. *Transactions of the American Microscopical Society, 51,* 16–21.

International Commission on Zoological Nomenclature. (1999). *International Code of Zoological Nomenclature* (4th ed.). International Trust for Zoological Nomenclature, London, U.K., 306 pp.

Kakaji, V. L. (1969). Studies on helminth parasites of Indian fishes. Part 3. Some trematode parasites of fresh water fishes of Uttar Pradesh. *Indian Journal of Helminthology, 21*, 49–80.

Karar, Y. F. M., Blend, C. K., Dronen, N. O., & Adel, A. (2021). Towards resolving the problematic status of the digenean genus *Astiotrema* Looss, 1900: An updated concept and revision of species composition for *Astiotrema* (*sensu stricto*). *Zootaxa, 4991*, 36–72.

Kumari, N. V., Srivastava, C. B., & Chauhan B. S. (1972). Redescription of *Astiotrema reniferum* (Looss, 1898) Looss, 1900 with comments on the status of the genus *Pseudoparamacroderoides* Gupta & Agarwal, 1968 (Trematoda: Plagiorchiidae). *Records of the Zoological Survey of India*, *67*, 315–323.

Kusy, H., & Barger, M. A. (2017). A new species of *Macroderoides* (Trematoda: Macroderoididae) from spotted gar, *Lepisosteus oculatus* (Lepisosteidae), in the Big Thicket National Preserve and surrounding areas, Texas, U.S.A. *Comparative Parasitology, 84*, 28–31.

Manter, H. W. (1970). The terminology and occurrence of certain structures of digenetic trematodes, with special reference to the Hemiuroidea. In: Singh, K. S., & Tandan, B. K (Eds.), *H. D. Srivastava Commemoration Volume*, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India. pp. 27–33.

Moravec, F., & Salgado-Maldonado, G. (2002). Redescription of *Perezitrema bychowskyi* (Caballero & Caballero, 1975) (Trematoda: Macroderoididae), with remarks on the

systematic status of *Perezitrema* Baruš & Moravec, 1996. *Systematic Parasitology*, *53*, 199–206.

Ng, H. H. (2012). *Mystus velifer*, a new species of catfish from Indochina (Teleostei: Bagridae). *Zootaxa*, *3398*, 58–68.

Ng, H. H., Hadiaty, R. K., Lundberg, J. G., & Luckenbill, K. R. (2015). A new genus and species of bagrid catfish from northern Sumatra (Siluriformes: Bagridae). *Proceedings of the Academy of Natural Sciences of Philadelphia*, *164*, 149–157.

Patella, R., & Bullard, S. A. (2013). Hexabothriids of devil rays (Mobulidae): New genus and species from gill of *Mobula hypostoma* in the Gulf of Mexico and redescription of a congener from *Mobula rochebrunei* in the eastern Atlantic Ocean. *Journal of Parasitology*, *99*, 856–867.

Pearse, A. S. (1924). Observations on parasitic worms from Wisconsin fishes. *Transactions of the Wisconsin Academy of Sciences, Arts and Letters*, *21*, 147–160.

Pojmańska, T., Tkach, V. V., & Gibson, D. I. (2008). Genera *incertae sedis*, genera *inquirenda*, *nomina nuda*, larval or collective names and recently erected genera. In: Bray, R. A., Gibson, D. I., & Jones, A. (Eds.), *Keys to the Trematoda. Volume 3*. CAB International & Natural History Museum, Cambridge, U.K. pp. 735–755.

Taylor, P. W. (1978). *Macroderoides trilobatus* sp. n. (Digenea: Maroderoididae) from the bowfin, *Amia calva*, and emendation of the genus. *Journal of Parasitology*, *64*, 393–394.

Tkach, V. V., & Kinsella, J. M. (2011). New *Macroderoides* (Digenea: Macroderoididae) from Florida gar, with molecular phylogeny of the genus. *Journal of Parasitology*, *97*, 920–923.

Tkach, V. V., Pulis, E. E., & Overstreet, R. M. (2010). A new *Paramacroderoides* species (Digenea: Macroderoididae) from two species of gar in the southeastern United States. *Journal of Parasitology, 96,* 1002–1006.

Tkach, V. V., Strand, E. J., & Froese, L. (2008). *Macroderoides texanus* n. sp. (Digenea: Macroderoididae) from alligator gar, *Atractosteus spatula* in Texas. *Parasitology Research, 104,* 27–33.

Tran, D. D., Shibukawa, K., Nguyen, P. T., Ha, H. P., Tran, L. X., Mai, H. V., & Utsugi, K. (2013). *Fishes of the Mekong Delta, Vietnam*. Can Tho University Publishing House, Can Tho, Vietnam, 174 pp.

Truong, T. N., Warren, M. B., Ksepka, S. P., Curran, S. S., & Bullard, S. A. (2021). *Posthovitellinum psiloterminae* n. gen., n. sp. (Digenea: Lissorchiidae) infecting intestine of *Cyclocheilos enoplos* (Cypriniformes: Cyprinidae) in the Mekong River, Vietnam. *Journal of Parasitology, 107,* 431–445.

Van Cleave, H. J., & Mueller, J. F. (1932). Parasites of Oneida Lake fishes. Part I. Descriptions of new genera and new species. *Roosevelt Wild Life Annals, 3,* 5–71.

Van Cleave, H. J., & Mueller, J. F. (1934). Parasites of Oneida Lake fishes. Part III. A biological survey of the worm parasites. *Roosevelt Wild Life Annals, 7,* 161–334.

Venard, C. E. (1941). Studies on parasites of Reelfoot Lake fish. III. A new genus and new species of trematode (Plagiorchioidea; Macroderoididae) from *Lepisosteus platostomus*. *Journal of the Tennessee Academy of Science, 16,* 379–383.

Winfield, G. F. (1929). *Plesiocreadium typicum,* a new trematode from *Amia calva*. *Journal of Parasitology, 16,* 81–87.

Yamaguti, S. (1971). *Synopsis of digenetic trematodes of vertebrates*. Tokyo: Keigaku Publishing Co., 1074 pp.

Figs. 1–4 *Pseudoparamacroderoides dongthapensis* n. sp. infecting the intestine of the freshwater catfish *Mystus mysticetus* Roberts, (Siluriformes: Bagridae) from the Mekong River Delta, Vietnam. 1, Whole body (ventral view) of the holotype (USNM 1660548); the uterine conformation is stylized to show its outer extension and distribution in the hindbody. 2, Anterior end (ventral view) of a paratype (USNM 1660551) showing the size and shape of the tegumental spines at level of the oral sucker and those between suckers. 3, Tegumental spines at level of the oral sucker (left, ventral view) of a paratype (USNM 1660551) and those between suckers (right, ventral view) of the same specimen. 4, Sketchy outline of the hindbody (dorsal view) of a paratype (USNM 1660552) showing anterior extent of the excretory vesicle. *Abbreviations:* os, oral sucker; pro, pre-pharyngeal oesophagus; ph, pharynx; poo, post-pharyngeal oesophagus; ce, intestinal caeca; gp, gential pore; cs, cirrus sac; vs, ventral sucker; ova, ovary; sr, seminal receptacle; ut, uterus; ate, anterior testis; pte, posterior testis; vf, vitteline follicles; exp, excretory pore; tso, tegumental spines at level of the oral sucker; tss, tegumental spines between suckers; exv, excretory vesicle.

Figs. 5–7 *Pseudoparamacroderoides dongthapensis* n. sp. infecting the intestine of the freshwater catfish *Mystus mysticetus* Roberts, (Siluriformes: Bagridae) from the Mekong River Delta, Vietnam. 5, Terminal male genitalia (dorsal view) of a paratype (USNM 1660552) showing the cirrus and the unipartite internal seminal vesicle. 6, Ovary complex (ventral view) of the holotype (USNM 1660548) showing the ovary and other components of the female genitalia. 7, Uterine eggs (in different views) of the holotype. *Abbreviations:* ga,

genital atrium; gp, gential pore; mt, metraterm; vs, ventral sucker; cir, cirrus; cs, cirrus sac; pp, pars prostatica; gc, prostate gland cells; sv, seminal vesicle; ova, ovary; put, proximal end of the utetus; oot, oötype; mg, Mehlis' gland; vr, vitelline reservoir; sr, seminal receptacle; ovi, oviduct; lc, Laurer's canal.

Key 1 Key to genera of Macroderoididae

1a. Excretory vesicle Y-shaped …….. 2

1b. Excretory vesicle I-shaped ……...……...…. 3

2a. Vitelline fields continuous, completely extra-caecal; testes in anterior half of body ……..…...
……...……...…. *Rauschiella* Babero, 1951

2b. Vitelline fields interrupted at level of ventral sucker; testes in posterior half of body ……..
.…….……...…...……. *Gauhatiana* Gupta, 1953

3a. Caeca terminating in pre-testicular space; vitelline fields restricted to inter-gonadal space …..
……..……..... *Malawitrema* Bray & Hendrix, 2007

3b. Caeca terminating in post-testicular space, extending to near posterior body end; vitelline fields not restricted to inter-gonadal space ……...…... 4

4a. Ventral sucker vestigial (without lumen); vitelline fields confluent anteriorly and posteriorly
……..... *Cirkennedya* Gibson & Bray, 1979

4b. Ventral sucker not vestigial (with typical lumen); vitelline fields separate (not confluent) anteriorly and posteriorly .....……..…….……...…… 5

5a. Oral sucker funnel-shaped, larger than ventral sucker …….....……...… 6

5b. Oral sucker subspherical, diameter approximately equal to that of ventral sucker …..... 7

6a. Oral sucker with posterior constriction, rows of distinctly-enlarged circumoral spines present; caeca blind-ending ……..……... *Paramacroderoides* Vernard, 1941

6b. Oral sucker without posterior constriction, rows of distinctly-enlarged circumoral spines absent; caeca forming cyclocoel …….…… *Perezitrema* Baruš & Moravec, 1967

7a. Cirrus sac slightly dorsal to and predominantly lateral (partially dorsolateral to dextral or sinistral margin of ventral sucker) to ventral sucker; testes approximately ≤ 1/3 max body width in diameter ..... *Pseudoparamacroderoides* Gupta & Agrawal, 1968

7b. Cirrus sac dorsal to ventral sucker; testes ≥1/2 max body width in diameter ……. ……..…….. *Macroderoides* Pearse, 1924

Key 2 Key to *Pseudoparamacroderoides* spp.

1a. Excretory vesicle extending anteriad to level of posterior testis; testes <1/5 of max body width in diameter …..……..……... *Ps. vittati*

1b. Excretory vesicle extending anteriad to level of or beyond anterior testis; testes >1/4 of max body width in diameter ……...……...…….. 2

2a. Hindbody >2× longer than forebody; excretory vesicle extending anteriad beyond anterior testis ……...…….. *Ps. dongthapensis*

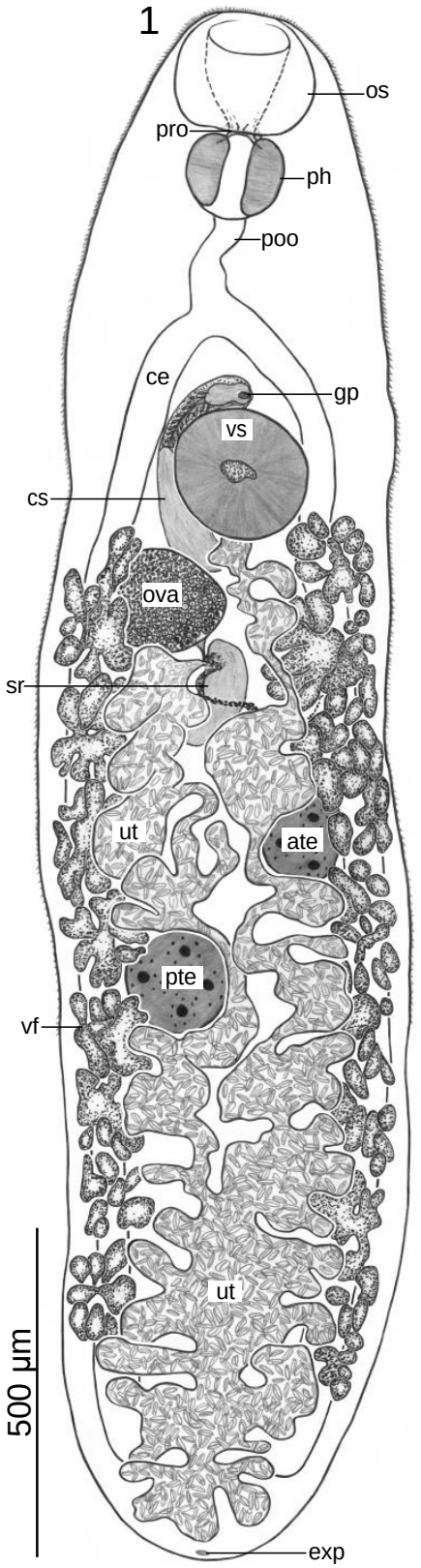
2b. Hindbody <2× longer than forebody; excretory vesicle extending anteriad to level of anterior testis ……...…….…… 3

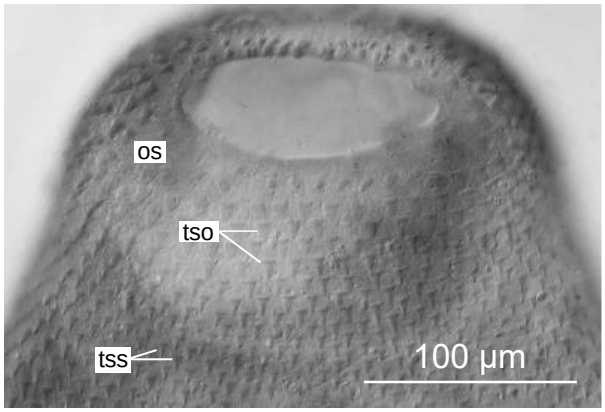
3a. Caeca terminating at middle of post-testicular space; uterus extending posteriad beyond caecal tips …….. *Ps. keni*

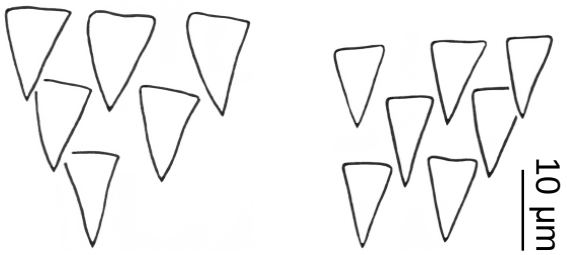
3b. Caeca terminating near posterior body end; uterus not extending posteriad beyond caecal tips ……... 4

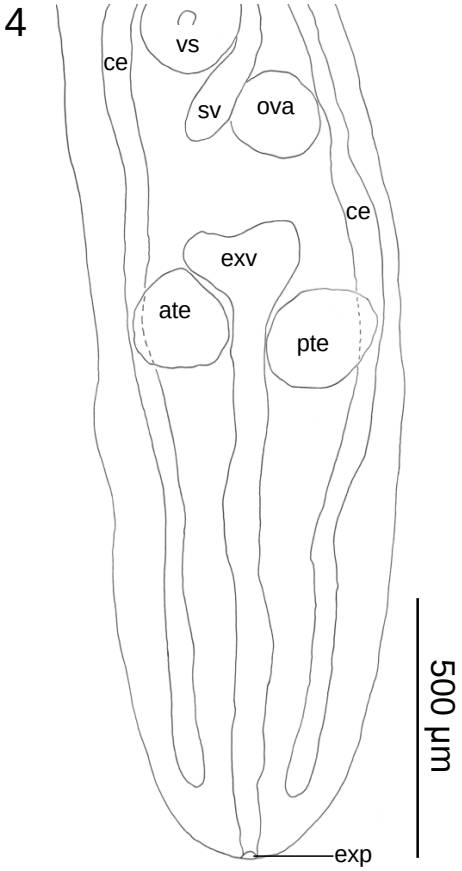
4a. Uterus extending posteriad anterior to caecal tips; post-uterine space >10% of body length … ….……...….. *Ps. seenghali*

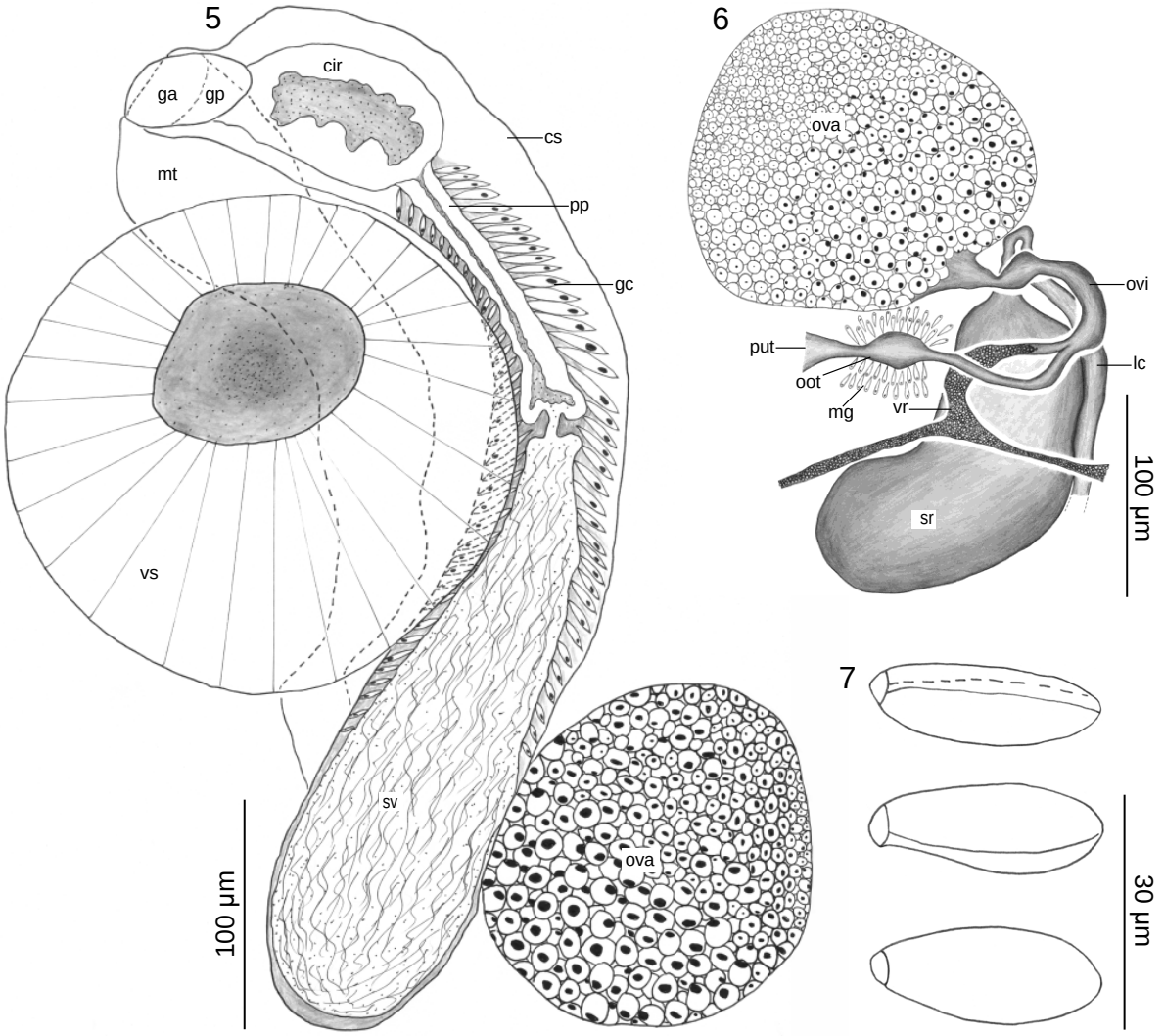
4b. Uterus extending posteriad to level of caecal tips; post-uterine space <5% of body length ….. ...……. *Ps. raychaudhurii*


1
os
pro
ph
poo
ce
gp
vs
cs
ova
sr
ut
ate
pte
vf
ut
500 µm
exp


2
os
tso
tss
100 µm


3
10 µm


4
ce
vs
sv
ova
ce
exv
ate
pte
500 µm
exp


5
cir
ga
gp
cs
mt
pp
gc
vs
sv
ova
100 µm
6
ova
ovi
put
lc
oot
mg
vr
sr
100 µm
7
30 µm

CHAPTER 3: RESURRECTION OF *PLESIOCREADIUM* WINFIELD, 1929 (DIGENEA: MACRODEROIDIDAE) WITH PHYLOGENETIC ANALYSES AND SUPPLEMENTAL OBSERVATIONS OF ITS TYPE SPECIES FROM RIVERS IN ARKANSAS, MISSISSIPPI, NEW YORK, AND TENNESSEE

***Published in Journal of Parasitology (Available 27 April 2023)**

Authors: Triet N. Truong, Stephen S. Curran, Florian B. Reyda, Matthew Horton, and Stephen A. Bullard

ABSTRACT

We herein resurrect and emend *Plesiocreadium* Winfield, 1929 (Digenea: Macroderoididae) and provide a supplemental description of its type species, *Plesiocreadium typicum* Winfield, 1929, based on adult specimens collected from the intestine of bowfins, *Amia calva* Linnaeus, 1766 (Amiiformes: Amiidae), captured in the L'Anguille River (Mississippi River Basin, Arkansas), Big Lake (Pascagoula River Basin, Mississippi), Chittenango Creek (Oneida Lake, New York), and Reelfoot Lake (Tennessee River Basin, Tennessee). *Plesiocreadium* spp. (*Pl. typicum* and *Plesiocreadium flavum* [Van Cleave and Mueller, 1932] n. comb.) differ from other macroderoidids by having a dorsoventrally flat forebody, ceca that extend posteriad beyond the testes and that do not form a cyclocoel, testes that are greater than one-half of maximum body width, a cirrus sac that is dorsal to the ventral sucker and arches dextrad or sinistrad, a uterine seminal receptacle, asymmetrical vitelline fields that remain separated anteriorly and posteriorly and that extend anteriad to the level of the ventral sucker, and an I-shaped excretory vesicle. Bayesian phylogenetic analyses (*ITS2* and *28S*) recovered monophyletic *Plesiocreadium sensu stricto* (as defined herein) sister to *Macroderoides trilobatus* Taylor, 1978 and that clade sister to the remaining macroderoidids, with sequences ascribed to species of *Macroderoides* Pearse, 1924 recovered as paraphyletic. We regard *Macroderoides parvus* (Hunter, 1932) Van Cleave

and Mueller, 1934, *M. trilobatus*, and *Rauschiella* Babero, 1951 as *incertae sedis*. Arkansas, New York, and Tennessee comprise new locality records for *Pl. typicum*.

We accept 8 genera of Macroderoididae McMullen, 1937: *Macroderoides* Pearse, 1924, *Plesiocreadium* Winfield, 1929, *Paramacroderoides* Vernard, 1941, *Gauhatiana* Gupta, 1953, *Perezitrema* Baruš and Moravec, 1967, *Pseudoparamacroderoides* Gupta and Agrawal, 1968, *Cirkennedya* Gibson and Bray, 1979, and *Malawitrema* Bray and Hendrix, 2007 (see Truong et al., 2021). *Rauschiella* Babero, 1951 and *Gauhatiana* have a Y-shaped excretory bladder, whereas the remaining genera have an I-shaped excretory vesicle (Babero, 1951; Gupta, 1953; Font and Lotz, 2008). Monotypic *Cirkennedya* has a uniquely 'vestigial' (lacking prominent lumen) ventral sucker and bilateral vitellarium extending anteriad into the forebody and spanning the midline anteriorly and posteriorly (Gibson and Bray, 1979). We provisionally accept *Gauhatiana* and *Cirkennedya* as macroderoidids (Gibson and Bray, 1979; Font and Lotz, 2008). Recent phylogenetic studies recovered Macroderoididae and its type genus *Macroderoides* as paraphyletic (Razo-Mendivil et al., 2006; Hernández-Mena et al., 2016; Dumbo et al., 2019), suggesting that those taxa need revision (Truong et al., 2021).

Winfield (1929) proposed Plesiocreadiinae Winfield, 1929 for the monotypic *Plesiocreadium* and assigned Plesiocreadiinae to Allocreadiidae Looss, 1902 (as Allocreadiidae Stossich, 1904). He described *Plesiocreadium typicum* Winfield, 1929, type species, based on specimens that infected the intestine of a bowfin, *Amia calva* Linnaeus, 1766 (Amiiformes: Amiidae), from Douglas Lake, east of Lake Michigan. Hunter (1932) described *Plesiocreadium parvum* Hunter, 1932 from adult specimens infecting the intestine of both longnose gar, *Lepisosteus osseus* (Linnaeus, 1758) (Lepisosteiformes: Lepisosteidae), and bowfin from Lake Champlain, New

York. Hunter (1932) stated that *Pl. parvum* was nearly identical to *Pl. typicum* but differed from it by a combination of features related to body size and shape, sucker sizes, esophagus and pharynx size and position, testes sizes, Laurer's canal length, Mehlis' gland cell distribution, vitellarium distribution, and tegumental spine density. He further emended *Plesiocreadium* by including the ventral sucker position, ovary position, and vitellarium extent. Van Cleave and Mueller (1932) reassigned the type species, *Pl. typicum*, to *Macroderoides* without a detailed explanation, thereby making *Plesiocreadium* a junior subjective synonym of *Macroderoides*. Because the 2 works were published at approximately the same time during January 1932, Van Cleave and Mueller (1932) were unlikely to have seen the description of *Pl. parvum* by Hunter (1932). Later, Van Cleave and Mueller (1934) transferred *Pl. parvum* to *Macroderoides* (as *Macroderoides parvus* [Hunter, 1932] Van Cleave and Mueller, 1934) without justification. Subsequent authors have accepted the systematic decisions of Van Cleave and Mueller (1932, 1934) without a doubt. McMullen (1937) proposed Macroderoididae based on *Macroderoides* (type; *Macroderoides spiniferus* Pearse, 1924, type species). Tkach et al. (2001) summarized the systematic history of *Macroderoides* since McMullen (1937).

Herein, we supplement the description of *Pl. typicum* based on specimens we collected from bowfins in the eastern United States. We also resurrect and emend *Plesiocreadium*.

MATERIALS AND METHODS

Two bowfins were gillnetted from Reelfoot Lake, Tennessee (36°23′05″N, 89°21′25″W) on 30 June 2002 and on 1 July 2002. Another bowfin was gillnetted from Big Lake, Pascagoula River, Mississippi (30°36′12″N, 88°37′36″W) on 14 April 2011. Five bowfins were electrofished from Chittenango Creek, Oneida Lake, New York from July through September 2020. Four additional bowfins were electrofished from New Lake, adjacent to Second Creek, (35°05′49.3″N,

90°57′34.2″W), L'Anguille River, Arkansas on 31 May 2022. Trematode specimens were collected from the intestine, rinsed, and cleaned in 8.5 ppt saline, heat-killed in hot water (~60 C), and fixed in 10% neutral buffered formalin. Fixed specimens were stained overnight in Van Cleave's hematoxylin mixed with several drops of Ehrlich's hematoxylin. Stained specimens were made basic at 70% ethanol (EtOH) with 2 drops of lithium carbonate (Li_2CO_3) saturated in 70% EtOH and 1 drop of butylamine ($CH_3[CH_2]_3NH_2$), dehydrated in an EtOH series, cleared in clove oil, and permanently mounted on glass slides using Canada balsam. Specimens intended for DNA extraction were placed directly in 95% EtOH without any heat treatment. Illustrations were made using an Olympus BX51 microscope (Olympus Corporation of the Americas, Center Valley, Pennsylvania) equipped with differential interference contrast optical components and a drawing tube. Measurements were made using an ocular micrometer and reported in micrometers (μm) as the range, followed by the mean ± standard deviation. Taxonomic authorities for fishes followed Fricke et al. (2022). Anatomical terms for *Plesiocreadium* spp. and other macroderoidids followed Truong et al. (2021). Vouchers of *Pl. typicum* were deposited in the National Museum of Natural History's Invertebrate Zoology Collection (NMNH, Smithsonian Institution, Washington, D.C.) (see Taxonomic summary).

Two EtOH-preserved adult specimens of *Pl. typicum* (as 2 replicates) from New Lake were used separately to extract genomic DNA using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA concentration was measured using a NanoDrop–1000 spectrophotometer (Thermo Scientific, Nanodrop Technologies, Waltham, Massachusetts), diluted to 20 ng/μl, and stored at –20 C. The partial *28S* sequence was amplified using forward primer U178 (5'–GCACCGCTAAYTTAAG–3') and reverse primer L1642 (5'–CCAGCGCCATCCATTTTCA3') (Lockyer et al., 2003); the internal transcribed spacer 2 region

(*ITS2*) was amplified using forward primer GA1 (5'–AGAACATCGACATCTTGAAC–3') and reverse primer ITS2.2 (5'–CCTGGTTAGTTTCTTTTCCTCCGC–3') (Anderson and Barker, 1998; Cribb et al., 1998). PCR reactions for amplifying the *ITS2* and *28S* rDNA gene were performed with the following thermocycling parameters: initial denaturation step of 94 C for 4 min, followed by 42 cycles of 94 C for 40 sec, 53 C for 30 sec, 72 C for 2 min, with a final extension step of 72 C for 5 min. PCR product purification was conducted using the QIAquick PCR Purification kit (Qiagen). Both PCR primers were used for DNA sequencing reactions. Additionally, 2 internal primers 300F (5'–CAAGTACCGTGAGGGAAAGTTG–3') and 1200R (5'–GCATAGTTCACCATCTTCGG–3') were used to improve sequencing coverage on the *28S* (Lockyer et al., 2003). DNA sequencing was performed by Genewiz (South Plainfield, New Jersey). All nucleotide sequences generated in the present study were deposited in the NCBI GenBank (see Taxonomic summary).

Phylogenetic analysis of the *ITS2* included the newly-generated sequence of *Pl. typicum* and all available sequences of macroderoidids (8 sequences) plus an alloglossidiid sequence as the outgroup. Taxon and outgroup selection for the *28S* phylogenetic analysis was based on the previous studies on the systematics of Macroderoididae and related families (Hernández-Mena et al., 2016; Dumbo et al., 2019). Selected *28S* sequences (31) included *Alloglossidium corti* (Lamont, 1921) Van Cleave and Mueller, 1934 (JF440783), *Alloglossidium floridense* Kasl, Fayton, Font, and Criscione, 2013 (KC812276), *Alloglossidium fonti* Tkach and Mills, 2011 (JF440763), *Alloglossidium geminum* (Mueller, 1930) Van Cleave and Mueller, 1934 (JF440771), *Alloglossidium kenti* Simer, 1929 (JF440808), *Aptorchis aequalis* Nicoll, 1914 (EF014729), *Aptorchis glandularis* Tkach and Snyder, 2008 (EU334368), *Aptorchis megacetabulus* Tkach and Snyder, 2007 (EF014730), *Auridistomum chelydrae* (Stafford, 1900)

Stafford, 1905 (AY116872), *Choledocystus hepaticus* (Lutz, 1928) Sullivan, 1977 (AY875679), *Haplometra cylindracea* (Zeder, 1800) Looss, 1899 (AF151933), *Haplometroides intercaecalis* Silva, Ferreira and Strüssmann, 2007 (MH206169), *Macroderoides minutus* Tkach and Kinsella, 2011 (HQ680850), *Macroderoides spiniferus* Pearse, 1924 (AF433674, EU850400), *Macroderoides texanus* Tkach, Strand, and Froese, 2008 (EU850398), *Macroderoides trilobatus* Taylor, 1978 (EU850406), *Pl. typicum* (AF433673, HQ680846), *Magnivitellinum saltaensis* Davies, Liquin, Lauthier, Párraga, Saravia, Davies, and Ostrowski de Núñez, 2021 (MN744313), *Magnivitellinum simplex* Kloss, 1966 (KU535683), *Paramacroderoides echinus* Venard, 1941 (MH041375), *Paramacroderoides kinsellai* Tkach, Pulis, and Overstreet, 2010 (HM137661), *Perezitrema bychowskyi* (Caballero and Caballero, 1975) Brooks, 1980 (KU535686), *Plagiorchis elegans* (Rudolphi, 1802) Braun, 1902 (KJ533392), *Plagiorchis maculosus* (Rudolphi, 1802) Braun, 1902 (MK641807), *Plagiorchis muelleri* Tkach and Sharpilo, 1990 (AF184250), *Plagiorchis vespertilionis* (Müller, 1780) Braun, 1900 (AF151931), *Plesiocreadium flavum* (Van Cleave and Mueller, 1932) n. comb. (HQ680851), *Pl. typicum* (present study), *Rauschiella poncedeleoni* (Razo-Mendivil and León-Régagnon, 2001) Razo-Mendivil, León-Régagnon, and Pérez-Ponce de León, 2006 (AY875678), *Rauschiella tineri* Babero, 1951 (AY875677). Other *28S* sequences (3) representing the outgroup included *Creptotrema astyanace* (Scholz, Aguirre-Macedo, and Choudhury, 2004) Franceschini, Aguiar, Zago, de Oliveira Fadel Yamada, Bertholdi Ebert, and da Silva, 2021 (KF631422), *Creptotrema lobata* (Hernández-Mena, Lynggaard, Mendoza-Garfias, and Pérez-Ponce de León, 2016) Franceschini, Aguiar, Zago, de Oliveira Fadel Yamada, Bertholdi Ebert, and da Silva, 2021 (KX954173), *Creptotrema tica* (Hernández Mena, Pinacho-Pinacho, García-Varela, Mendoza-

Garfias, and Pérez-Ponce de León, 2018) Franceschini, Aguiar, Zago, de Oliveira Fadel Yamada, Bertholdi Ebert, and da Silva, 2021 (MH997001).

The newly-generated *ITS2* and *28S* sequences of *Pl. typicum* were aligned and compared with the previously published macroderoidid sequences. Sequences were aligned using MAFFT (Katoh and Standley, 2013). JModelTest 2 version 2.1.10 was implemented to perform a statistical selection of the best-fit models of nucleotide substitution based on Bayesian information criteria (BIC) (Darriba et al., 2012). Aligned sequences were trimmed to the length of the shortest sequence and reformatted (from .fasta to .nexus) using the web application ALTER (Glez-Peña et al., 2010). The alignments were subjected to Bayesian inference (BI) analyses. The BI analyses were performed in MrBayes version 3.2.5 (Ronquist and Huelsenbeck, 2003) using substitution model averaging (nst-mixed) and a gamma distribution to model rate-heterogeneity. Three independent runs with 4 Metropolis-coupled chains were run for 5,000,000 generations, sampling the posterior distribution every 1,000 generations. Defaults were used in all other parameters. Convergence was checked using Tracer v1.7.1 (Rambaut et al., 2018) and the sump command in MrBayes. All runs appeared to reach convergence after discarding the first 25% of generations as burn-in. Majority-rule consensus tree of the post-burn-in posterior distribution was generated with the sumt command in MrBayes. The inferred phylogenetic trees were visualized using FigTree v1.4.4 (Rambaut et al., 2014) and further edited for visualization purposes with Adobe Illustrator 24.1.3 version (2020) (Adobe Systems, San Jose, California).

DESCRIPTION

Plesiocreadium Winfield, 1929, emended

Figs. 1–11

Diagnosis: Body elongate, spinous, tapering at posterior end; forebody dorsoventrally flat, distinct from hindbody. Tegumental spines triangular in outline, widest at base, sharply pointed, becoming more sparse posteriad, absent from posterior body end. Eyespot pigmentation absent. Suckers equally sized. Oral sucker subspherical, terminal or subterminal. Ventral sucker having prominent lumen (not vestigial), circular in outline, one-third to two-fifths of body length from anterior body end. Pharynx subspheroid, muscular. Pre-pharyngeal segment of esophagus shorter than pharynx or indistinct. Post-pharyngeal segment of esophagus longer than pharynx. Intestine bifurcating in mid-level or posterior half of forebody; ceca extending posteriad beyond testes without forming cyclocoel, terminating approximately at mid-level of post-testicular space or to near posterior body end. Testes subspheroid or ovoid, typically diagonal, occasionally nearly tandem; diameter greater than one-half of maximum body width. Cirrus sac claviform, dorsal to ventral sucker, arching dextrad or sinistrad, extending beyond posterior margin of ovary or to level of anterior testis; internal seminal vesicle bipartite, posterior portion usually longer; cirrus aspinous, sinuous, thick; prostatic gland cells numerous. External seminal vesicle absent. Genital atrium present; pore median, immediately anterior to ventral sucker. Ovary subspheriod or subtriangular, pre-testicular, immediately posterior to or overlapping posterior margin of ventral sucker, dextral or sinistral to midline. Mehlis' gland and Laurer's canal present. Seminal receptacle absent. Uterus primarily ventral to testes, partly inter-testicular; uterine coils loosely convoluted, post-ovarian, extending posteriad beyond cecal tips or restricted to pre-cecal space; uterine seminal receptacle present. Metraterm unarmed. Eggs ovoid, operculate. Vitellarium follicular, separate (not spanning midline) anteriorly and posteriorly; fields posteriorly asymmetrical (field on ovarian side shorter than field on opposite side), extending from posterior half of ventral sucker posteriad beyond posterior testis (not restricted to inter-gonadal space, not

extending posteriad to level of cecal tips). Excretory vesicle I-shaped (saccate or elongate), wholly post-testicular or extending anteriad to level of testes (not inter-testicular); pore terminal. Parasites of the intestine of North American freshwater fishes (Amiidae, Esocidae, and Lepisosteidae).

Differential diagnosis: Forebody distinctly dorsoventrally flat. Suckers equally sized. Ceca extending posteriad beyond testes, not forming cyclocoel. Cirrus sac dorsal to ventral sucker, arching dextrad or sinistrad. Seminal receptacle absent. Vitellarium posteriorly asymmetrical, separate anteriorly and posteriorly, extending anteriad to level of ventral sucker. Excretory vesicle I-shaped.

Type species: Plesiocreadium typicum Winfield, 1929.

Other species: Plesiocreadium flavum (Van Cleave and Mueller, 1932) n. comb.

Remarks on *Plesiocreadium*

Plesiocreadium resembles *Pseudoparamacroderoides* by having an elongate body, equally sized suckers, a subspherical oral sucker, a short or indistinct pre-pharyngeal esophagus, diagonal testes that are in the middle of the hindbody, an ovary that is posterolateral to or abutting the ventral sucker, a partly inter-testicular uterus, and a vitellarium that is separate (not spanning the midline) anteriorly and posteriorly and that extends posteriad beyond the posterior testis (not to the level of the cecal tips) (Truong et al., 2021). Species of *Plesiocreadium* also resemble *M. minutus* by having a body that is <6.5× longer than wide, diagonal testes that are abutting or immediately posterolateral to the ovary, a cirrus sac that extends posteriad to the level of the ovary or the anterior testis, and a vitellarium that extends from the level of the ventral sucker posteriad beyond the posterior testis. *Plesiocreadium* differs from *Pseudoparamacroderoides* by having a dorsoventrally flat forebody (vs. not dorsoventrally flat),

a cirrus sac that is dorsal to the ventral sucker (vs. predominantly lateral to the ventral sucker), a uterine seminal receptacle (vs. blind seminal receptacle), vitelline fields that are posteriorly asymmetrical (vs. symmetrical), and an excretory vesicle that is wholly post-testicular or extending to the level of the testes (vs. inter-testicular) (Truong et al., 2021). *Plesiocreadium* further differs from *Pseudoparamacroderoides* by having a bipartite seminal vesicle (vs. unipartite) and cirrus sac position relative to the ovary. Our examination of specimens of *Plesiocreadium* spp. (holotype [USNM 1320873] and 2 paratypes [USNM 1320874, 1350574] of *Pl. typicum* of Winfield [1929], 49 specimens of *Pl. typicum* herein from all 4 localities [see Figs. 1–4] plus 2 and 4 museum vouchers of *Pl. typicum* of Tkach et al. [2011] USNPC 104583 and 104584, respectively; 9 specimens of *Pl. flavum* [SSC collection; 19 May 2006] infecting the intestine of the bowfin in Kelsey's Pond, Portland, Connecticut, several syntypes and 4 vouchers of *Pl. flavum* of Van Cleave and Mueller [1932] USNM 1321309 and of Tkach et al. [2011] USNPC 104582 [preserved in 95% EtOH], respectively) shows that they have an ovary and a cirrus sac always located on the same side of the body (either primarily dextral or sinistral to the midline), whereas the cirrus sac and ovary among species of *Pseudoparamacroderoides* either positioned on the same side or opposite sides of the body. *Plesiocreadium* spp. differ from *M. minutus* by having a pharynx that overlaps or is immediately posterior to the oral sucker (the pre-pharyngeal esophagus is indistinct or remarkably shorter than the post-pharyngeal esophagus vs. approximately equal in length), a uterine seminal receptacle (vs. having a distinct blind seminal receptacle), and vitelline fields that are asymmetrical posteriorly (vs. symmetrical).

Plesiocreadium spp. differ from other macroderoidids by having a distinctly dorsoventrally flat forebody; *Pl. typicum*, *Pl. flavum*, and *M. parvus* are the only macroderoidids that reportedly have a dorsoventrally flat anterior body end. *Plesiocreadium* spp. have a body that is <6.5×

longer than wide (vs. 8–12× and 7–18× longer than wide among *Macroderoides* spp. [except *M. minutus*] and *Paramacroderoides* spp., respectively). *Plesiocreadium* spp. have oral and ventral suckers that are approximately equal in size (*Perezitrema* spp. have an oral sucker that is much larger than the ventral sucker), an oral sucker that is not funnel-shaped (vs. funnel-shaped in *Paramacroderoides* spp. and *Perezitrema* spp.), and a ventral sucker that has a typical lumen (vs. vestigial in the monotypic *Cirkennedya*). *Plesiocreadium* spp. have ceca that terminate in the post-testicular space without forming a cyclocoel (vs. terminating at the level of the testes in the monotypic *Malawitrema*; merging posteriorly to form a cyclocoel in *Perezitrema* spp.). *Plesiocreadium* spp. have testes that are typically diagonal (present in *Pseudoparamacroderoides* spp., *Gauhatiana* spp., and *M. minutus*; diagonal or symmetrical in *Rauschiella* spp.; symmetrical in the monotypic *Malawitrema*; tandem in *Paramacroderoides* spp., the monotypic *Cirkennedya*, *Perezitrema* spp., and most species of *Macroderoides* [except *M. minutus*]) and that are in the posterior half of the body (vs. anterior half of the body in *Rauschiella* spp.). *Plesiocreadium* spp. have a vitellarium that is distributed as 2 lateral asymmetrical fields that are separate (not spanning the midline) anteriorly and posteriorly and that extend from the posterior half of the ventral sucker posteriad to the level of or beyond the posterior testis (not to the level of the cecal tips) (vs. symmetrical in all other macroderoidids [except *Pa. kinsellai*]; restricted to the hindbody in *Macroderoides* spp. [except *M. minutus*] and *Paramacroderoides* spp.; confined to the inter-gonadal space in the monotypic *Malawitrema*; extending into the forebody and spanning the midline anteriorly and posteriorly in the monotypic *Cirkennedya*; extending posteriad to the level of the cecal tips in *M. parvus*). Additionally, *Plesiocreadium* spp. have an I-shaped excretory vesicle that is wholly post-testicular or extends anteriad to the level of the testes and not inter-testicular (vs. inter-testicular in

Pseudoparamacroderoides spp.; *Rauschiella* spp. and *Gauhatiana* spp. exceptionally have a Y-shaped excretory vesicle). Regarding the seminal receptacle, Winfield (1929, p. 83) asserted, "*There is no definite seminal receptacle but rather the sperms remain in a mass in the portion of the uterus* (= uterine seminal receptacle) *most proximal to the ovary*". Our thorough examinations of the present specimens of both *Pl. typicum* and *Pl. flavum* (see above) further supported observation by Winfield (1929). Hence, species of *Plesiocreadium* further differ from other macroderoidids (except the monotypic *Cirkennedya*) by having a uterine seminal receptacle (vs. having a blind seminal receptacle) (Winfield, 1929; Gibson and Bray, 1979).

Plesiocreadium flavum n. comb. was reassigned herein because it fits within our emended diagnosis of the genus; Truong et al. (2021) detailed similarities between *Pl. typicum* and *Pl. flavum*. This species has been documented from the intestine and rectum of chain pickerel, *Esox niger* Lesueur, 1818 (Esociformes: Esocidae), from the Oneida Lake (Van Cleave and Mueller, 1932), Big Bay, Lake Superior (Van Cleave and Mueller, 1934), and Connecticut River (Tkach and Kinsella, 2011; present study) as well as from the intestine of the Florida gar, *Lepisosteus platyrhincus* DeKay, 1842 (Lepisosteiformes: Lepisosteidae), in the Orange Lake (Tkach and Kinsella, 2011).

***Plesiocreadium typicum* Winfield, 1929**

(Figs. 1–11; Table I)

Based on light microscopy of 49 stained, whole-mounted adult specimens: Body broadly rounded anteriorly, widest at level of ventral sucker or in hindbody between ventral sucker and testes; hindbody longer than forebody. Tegumental spines distributing from anterior end posteriad to level of posterior testis (Figs. 1–4). Oral sucker subterminal, opening ventrally, typically less than one-half of maximum body width. Ventral sucker in anterior half of body

(Figs. 1–4). Pharynx subequal in length and width, dorsally overlapping or immediately posterior to oral sucker. Intestine bifurcating in posterior half of forebody; ceca extending approximately to mid-level of post-testicular space (Figs. 1–4).

Testes subspheriod, diagonal, abutting or separated by uterus nearly at middle of hindbody; anterior testis approximately equal in size to posterior testis (Figs. 1–4). Vasa efferentia and vas deferens not evident. Cirrus sac muscular, extending to level of anterior testis; cirrus eversible, convoluted when inverted into cirrus sac (Figs. 1–8). Genital pore median (Figs. 1–4).

Ovary subtriangular or oval, abutting or overlapping posterior margin of ventral sucker (Figs. 1–4, 9–11). Oviduct sinuous, extending sinistrad from ovary (Figs. 9–11). Oötype lateral to ovary and ventral to oviduct, surrounded by Mehlis' gland. Laurer's canal ascending or descending dorsally from oviduct; Laurer's canal pore submedian, dextral, opening dorsally between proximal end of cirrus sac and anterior testis, visible only in a few specimens (Figs. 9–11). Uterus comprising descending and ascending portions; descending portion ventrally to testes, coursing either sinistrally or dextrally around anterior testis depending on ovarian position, winding between or ventral to testes to other body side, coursing around posterior testis before coiling dextrally in post-testicular space past cecal tips; ascending portion coursing anteriorly following same course as descending portion or primarily coiling sinistrally, with coils ventral to both testes; uterine distal end ventral to cirrus sac (Figs. 1–8). Uterine seminal receptacle storing within descending portion of uterus between or lateral to testes (Figs. 1, 2). Metraterm sinistral to cirrus sac, entering genital atrium dorsal to distal end of cirrus sac (Figs. 5–8). Eggs numerous. Vitellarium irregularly follicular with variously-sized follicles, primarily ventral to ceca, extending anteriad to level of middle or posterior margin of ventral sucker; vitelline field on ovarian side extending posteriad to middle or slightly beyond posterior margin

of posterior testis; opposite field to ovarian side extending posteriad to posterior margin of posterior testis or to approximately middle of post-testicular space; fields connected through two slender transverse ducts, post-ovarian and immediately anterior to, or at level of, anterior half of anterior testis; vitelline reservoir median or submedian, entering oviduct dorsal to anterior testis (Figs. 1–4, 9–11).

Excretory vesicle wholly post-testicular, extending anteriad beyond cecal tips and dorsal to uterine coils; posterior end of excretory vesicle constricted as a diminutive duct before opening into excretory pore; length approximately less than one-fifth of body length (Figs. 2, 4).

Taxonomic summary

Type host: Bowfin, *Amia calva* Linnaeus, 1766 (Amiiformes: Amiidae) (Winfield, 1929).

Type locality: Douglas Lake, Michigan (Winfield, 1929).

Other hosts: Florida gar, *Lepisosteus platyrhincus* DeKay, 1842 (Lepisosteiformes: Lepisosteidae) (Tkach and Kinsella, 2011). A record of *Pl. typicum* (as *M. typicus*) reported from the shortnose gar, *Lepisosteus platostomus* Rafinesque, 1820 (Lepisosteiformes: Lepisosteidae) (see Tkach et al., 2001) is dubious (see Discussion).

Other localities: Orange Lake (29°28′46″N, 82°9′43″W), Alachua County, Florida (Tkach and Kinsella, 2011); Illinois and Mississippi Rivers (Van Cleave and Mueller, 1932); Big Lake, Pascagoula River (30°36′12″N, 88°37′36″W), Mississippi (present study); Reelfoot Lake (36°23′05″N, 89°21′25″W), Tennessee (present study); Chittenango Creek, Oneida Lake tributary (43°9′20″N, 75 58′21″W), New York (present study); New Lake adjacent to Second Creek (35°05′49.3″N, 90°57′34.2″W), L'Anguille River, Arkansas (present study).

Specimens examined: Plesiocreadium flavum n. comb. (as *M. flavus*) (USNM 1321309, syntypes; USNPC 104582, 4 vouchers); *M. trilobatus* (USNM 1370180, holotype; USNM

1370181, 2 paratypes); *Pl. typicum* (USNM 1320873, holotype; USNM 1320874 and 1350574, 2 paratypes); *Pl. typicum* (as *M. typicus*) (USNPC 104584, 6 vouchers) (Tkach et al. [2008; 2010] and Tkach and Kinsella [2011] erroneously stated that the types for *Pl. typicum* were lost or undesignated).

Specimens and DNA sequences deposited: 10 adult specimens, including USNM 1679386–88 (Big Lake), USNM 1679389–90 (Reelfoot Lake), USNM 1679384–85 (Chittenango Creek), USNM 1679391–92, 1679394 (New Lake); *ITS2* (OQ357794), partial *28S* rDNA (OQ357776).

Site in host: Intestine.

Prevalence and intensity: 1 of 1 (100%) (Big Lake), 2 of 2 (100%) (Reelfoot Lake), 2 of 5 (40%) (Chittenango Creek), and 2 of 4 (50%) (New Lake) *A. calva* each was infected by numerous adult specimens of *Pl. typicum*.

Remarks on *Plesiocreadium typicum* and related species

Our specimens of *Pl. typicum* matched the original description by Winfield (1929), except that some specimens from Big Lake and Reelfoot Lake had a larger body length to width ratio and some specimens from Reelfoot Lake were slightly larger in body length and width (Table I). Winfield (1929) reported a limited set of measurements for those specimens (see Table I), which makes comparing them challenging. Van Cleave and Mueller (1932) differentiated *Pl. flavum* from *Pl. typicum* by using body size and shape, pre-pharyngeal esophagus length, and egg size. *Plesiocreadium typicum* further differs from *Pl. flavum* by having an excretory vesicle that is confined to the post-testicular space (approximately less than one-fifth of body length vs. extending anteriad to the level of the testes and approximately one-fourth of body length).

The taxonomic status of *M. parvus*, *M. trilobatus*, and *M. minutus* is problematic. Although *M. parvus* was originally assigned to *Plesiocreadium*, we regard this species as *incertae sedis* because it has a series of exceptional features that makes it an outlier within *Plesiocreadium* and *Macroderoides*: a distinctly dorsoventrally flat forebody (present in *Plesiocreadium* spp. but absent in *Macroderoides* spp.); an ovary that is posteromedian to the ventral sucker, medial between the ventral sucker and testes (vs. immediately posterolateral to or overlapping the ventral sucker in *Plesiocreadium* spp. or typically closer to the testes than to the ventral sucker in *Macroderoides* spp.); and a vitellarium that is wholly post-ovarian (vs. partly pre-ovarian in *Macroderoides* spp. or extending anteriad to the level of the ventral sucker in *Plesiocreadium* spp.), that primarily fills the testicular and post-testicular inter-cecal space (vs. distributing in 2 lateral fields that are separate anteriorly and posteriorly in *Plesiocreadium* spp. and *Macroderoides* spp.), and that extends posteriad to the level of the cecal tips (vs. extending to the level of or posterior to the posterior testis, not extending to the level of the cecal tips in *Plesiocreadium* spp. and *Macroderoides* spp.).

Hunter (1932) emended *Plesiocreadium* by assigning *Pl. parvus* to the genus. Hunter's (1932) emendation, however, failed to detail several important diagnostic features useful in differentiating *Plesiocreadium* spp. from other macroderoidids. Specifically, these features include the arrangement of the testes, distribution of the vitellarium, and the shape and anterior extent of the excretory vesicle (see Remarks on *Plesiocreadium*). Like species of *Plesiocreadium*, *M. parvus* reportedly lacked a blind seminal receptacle (having a uterine seminal receptacle) (see Hunter, 1932), but its distinct morphological features do not conform to our emended diagnosis of *Plesiocreadium*. Since the original description of Hunter (1932), no other published study has reported, redescribed, or deposited a voucher specimen of *M. parvus* in

a curated museum collection, thereby no DNA sequence has been generated for this species. Thus, new collection and redescription of *M. parvus*, as well as a proposal of a new macroderoidid genus to accommodate *M. parvus*, are needed.

We regard *M. trilobatus* as a species *incertae sedis*. Although it superficially resembles other *Macroderoides* spp., *M. trilobatus* differs in several key features. *Macroderoides trilobatus* has a cirrus sac that is wholly lateral to (sinistral, but illustrated as dextral in Taylor [1978]; partly wrapping around the ventral sucker) the ventral sucker (vs. dorsal to the ventral sucker in other *Macroderoides* spp.). It has an ovary with 3 distinct lobes (vs. entire, smooth ovary; cf. *Macroderoides luki* Kusy and Barger, 2017). Additionally, *M. trilobatus* has distinctly smaller eggs (20–27μm long × 10–17μm wide vs. 36–55μm long × 17– 35μm wide in other *Macroderoides* spp.). Taylor (1978) did not detail the spine distribution, seminal receptacle, uterus, and excretory vesicle of *M. trilobatus*. The illustration of the holotype (USNM 1370180; see fig. 1 in Taylor, 1978) is further incorrect in at least 2 features. First, the tegumental spines illustrated by Taylor (1978) are distributed from the anterior body end posteriad to the level of the ovary; however, our examination of the holotype showed that the spines are restricted to the forebody. Secondly, Taylor (1978) drew the cirrus sac dextral to the ventral sucker; however, our examination revealed the cirrus sac is sinistral to the ventral sucker. Moreover, the type specimens of *M. trilobatus* were collected from the intestine of dead bowfins (the number of hosts examined was not specified) (Taylor, 1978). Perhaps, these trematode specimens had deteriorated such that we could not determine the form of the seminal receptacle, if present. Hence, a redescription based on properly-fixed specimens of *M. trilobatus* from the type host and type locality is needed to determine its generic assignment.

The status of *M. minutus* (described from a single specimen) is similar. It is also exceptional among its congeners because it has an ovoid body (2.9× vs. 8–12× longer than wide). *Macroderoides minutus* has small testes (diameter ~40% vs. ~50–80% of maximum body width) that are slightly diagonal and abutting (vs. tandem and separated by the uterus) and that are immediately posterolateral to the ventral sucker (vs. approximately in the middle of the hindbody). It has an ovary that overlaps the posterior margin of the ventral sucker and that is immediately anterolateral to and nearly abutting the anterior testis (vs. far posterior to the ventral sucker and is well-separated from the testes by the uterus). The seminal receptacle of *M. minutus* dorsally overlaps the ventral sucker and is ventral to the ovary (vs. post-ovarian either in *M. texanus* and *M. luki*; indeterminate in *M. spiniferus*). Additionally, *M. minutus* has a vitellarium that extends anteriad to the level of the ventral sucker (vs. far posterior to the ventral sucker in the remaining *Macroderoides* spp.) (Tkach et al., 2008; Tkach and Kinsella, 2011; Kusy and Barger, 2017).

Sequence comparisons and phylogenetic analyses

The *ITS2* and *28S* sequences from the 2 specimens of *Pl. typicum* from the New Lake were identical. The aligned *ITS2* and *28S* sequences of *Pl. typicum* and other macroderoidids were 266 and 1,184 bp (including gaps), respectively. Our *ITS2* (OQ357794) and *28S* (OQ357776) sequences were identical to those ascribed to *Pl. typicum* (HQ680846–49). The aligned *ITS2* sequence (OQ357794) of *Pl. typicum* differed from those of *M. trilobatus* (EU850406) and *Pl. flavum* HQ680851) by 6 (97.7% similarity) and 7 (97.3%) nucleotides, respectively (Table II). The *28S* sequence (OQ357776) of *Pl. typicum* differed from that of *Pl. flavum* (AF433674) by 20 (98.3%) nucleotides (Table III). Intriguingly, the *28S* sequence formerly identified as "*Pl. typicum*" (as *M. typicus*) (AF433673, Tkach et al. [2001]) differed from both those of *Pl. typicum*

(OQ357776, present study) and *Pl. typicum* (HQ680846, Tkach and Kinsella [2011]) by 44 (96.6%) nucleotides, and from that of *M. spiniferus* (AF433674, Tkach et al. [2001]) by only 1 (99.9%) nucleotide but was identical to that of *M. spiniferus* (EU850400, Tkach et al. [2008]) (Table III).

The *ITS2* analysis (270 bp) recovered a paraphyletic *Macroderoides* because *M. trilobatus* (EU850406) was sister to *Plesiocreadium* as revised herein. *Plesiocreadium typicum* (OQ357794) was sister to *Pl. typicum* (HQ680846) in a clade that also included *Pl. flavum* (HQ680851) in a polytomy. This clade shared a common ancestor with a clade comprising *Paramacroderoides* spp. sister to a clade of *Macroderoides* spp. (Fig. 12). The *28S* analysis (1,163 bp) recovered both Macroderoididae and *Macroderoides* as paraphyletic. The topology of the *28S* tree was similar to that of the *ITS2* tree except that the *28S* analysis recovered 2 identical sequences of *Pl. typicum* (OQ357776, HQ680846) sister to *Pl. flavum* (HQ680851). Together, these species were sisters to *M. trilobatus* (EU850406) in a clade that shared a recent common ancestor with a clade including the remaining macroderoidids. *Paramacroderoides* spp. were sister to a clade comprising *Pe. bychowskyi* (KU535686) sister to *Macroderoides* spp. The sequence of "*Pl. typicum*" (AF433673) was nested within the main *Macroderoides* clade and was sister to *M. spiniferus* (AF433674, EU850400) (Fig. 13). The *ITS2* and *28S* phylogenetic analyses supported the morphological diagnosis of *Plesiocreadium* spp. and *M. trilobatus* from *Macroderoides* spp. (see Remarks on *Plesiocreadium* and *Pl. typicum* and related species).

Two sequences of *Rauschiella* (*R. tineri* [AY875677] and *R. poncedeleoni* [AY875678]) were recovered in an earlier branching clade in the *28S* tree that was phylogenetically distant from the clade comprising all accepted macroderoidids (Fig. 13). This phylogenetic result apparently rejected the provisional assignment of *Rauschiella* to Macroderoididae by Font and

Lotz (2008) but further supported earlier phylogenetic results that species of *Rauschiella* were more closely related to plagiorchiids than to macroderoidids (see fig. 1 in Razo-Mendivil et al., 2006; fig. 1 in Hernández-Mena et al., 2016). *Rauschiella* was proposed for *Rauschiella tineri* Babero, 1951, which has an unidentified ("*a Mexican green frog, probably a species of Rana*", host from an unspecified locality in Mexico (Babero, 1951, p. 560). The genus was originally assigned to Plagiorchiinae Pratt, 1902 within Plagiorchiidae. Brooks (1977) relegated *Rauschiella* to a subgenus within *Glypthelmins* Stafford, 1905. Razo-Mendivil et al. (2006), without a specific assignment to a family, resurrected *Rauschiella*. Font and Lotz (2008) provisionally transferred *Rauschiella* to Macroderoididae but noted its systematic uncertainty because species of the genus exceptionally have testes located in the anterior body half, a massive uterus that fills the post-ovarian inter-cecal space and that presents as a compact, transverse portion in the post-testicular space, and a Y-shaped excretory vesicle. *Rauschiella* spp. further differ from other macroderoidids by having a wholly extra-cecal vitellarium. Moreover, although irrelevant from a strictly taxonomic sense, *Rauschiella* spp. represent the only macroderoidids that infect frogs and snakes, whereas other macroderoidids infect freshwater and marine fishes (Sullivan, 1977; Font and Lotz, 2008). Species of *Rauschiella*, however, share some important morphological features with plagiorchiids in having an elongate cirrus sac that contains a convoluted seminal vesicle and a Y-shaped excretory vesicle with the main stem that bifurcates at the level of, or anterior to, the testes. Further, species of *Rauschiella* superficially resemble some plagiorchiids (i.e., *Bilorchis* Mehra, 1937; *Allopharynx*, Strom, 1928) in having a wholly extra-cecal vitellarium and a compact inter-cecal and inter-testicular uterus in the hindbody. Based on the above morphological evidence and current phylogenetic knowledge, we

exclude *Rauschiella* from Macroderoididae and consider the genus *incertae sedis* in Plagiorchioidea.

DISCUSSION

We accept 8 macroderoidid genera. The present study resurrects *Plesiocreadium* and excludes *Rauschiella* from Macroderoididae, making both the family and its type genus (*Macroderoides*) monophyletic (considering *M. trilobatus* as *incertae sedis*). As such, Macroderoididae includes only digeneans infecting fishes. Collectively, species of *Macroderoides*, *Paramacroderoides*, *Plesiocreadium*, and *Perezitrema* infect the amiid, *E. niger*, lepisosteids, and ictalurids of North and Central America. *Pseudoparamacroderoides* spp. infect bagrids on the Indian subcontinent and Southeast Asia. Monotypic *Cirkennedya* infects the ocean sunfish, *Mola mola* (Linnaeus, 1758) (Tetraodontiformes: Molidae), from the Northeast Atlantic Ocean. *Gauhatiana* spp. infect the Philippine catfish, *Clarias batrachus* (Linnaeus, 1758) (Siluriformes: Clariidae), on the Indian sub-continent and an unidentified marine fish in the northeastern part of the Indian Ocean. Monotypic *Malawitrema* infects the North African catfish, *Clarias gariepinus* (Burchell, 1822) (Siluriformes: Clariidae), and the kampoyo, *Bagrus meridionalis* Günther, 1894 (Siluriformes: Bagridae), from the Lake Malawi, southeastern Africa.

Plesiocreadium and *Pseudoparamacroderoides* are the only macroderoidid genera to have species with enantiomorphic genitalia, and perhaps this possible synapomorphy could relate these genera. Since the resurrection of both *Pseudoparamacroderoides* (see Truong et al., 2021) and *Plesiocreadium* (present study) and excluding the *incertae sedis* taxa *M. parvus* and *M. trilobatus*, we accept only *M. spiniferus* (type species), *M. texanus*, *M. minutus* (see Remarks on *Pl. typicum*), and *M. luki* as members of *Macroderoides*. The only species of *Macroderoides*

having a lobed ovary is *M. luki*, which most closely resembles *M. trilobatus* but differs from it only by having a larger body (2,800–4,730 μm long × 228–415 μm wide vs. 1,680–2,232 μm long × 168–264 μm wide), a greater proportional distance between the cirrus sac and ovary (3–15% of the body length vs. overlapping or abutting to each other), and larger eggs (40–50 μm long × 22–35 μm wide vs. 20–27 μm long × 10–17 μm wide) (Kusy and Barger, 2017). All of the aforementioned species range in North America.

Current knowledge of the taxonomy and systematics of Macroderoididae strongly supports *Plesiocreadium* as a distinct genus. Our study proves that species of *Plesiocreadium* are morphologically distinct from and cannot be transferred to *Macroderoides*. The combination of the body shape, testes arrangement, vitellarium distribution, and a uterine seminal receptacle differentiates between the 2 genera (see Remarks on *Plesiocreadium*). We think the synonymies of Van Cleave and Mueller (1932, 1934) were indefensible as they provided no morphological evidence ("*We can find no point wherein Plesiocreadium of Winfield, 1929 differs from the concept of Macroderoides as delinated by Pearse, 1924, and confirmed by Simer, 1929. We therefore feel that it is necessary to consider Plesiocreadium as a synonym of Macroderoides*", in Van Cleave and Mueller [1932, p. 54]) but ignored several important morphological differences between species of *Plesiocreadium* and *Macroderoides*. Further, several previous phylogenetic studies have consistently recovered species of *Plesiocreadium* in an earlier branching clade sister to *M. trilobatus*. Three species of *Macroderoides* (*M. spiniferus*, *M. minutus*, and *M. texanus*) cladded with *Pe. bychowskyi* (see fig. 1 in Hernández-Mena et al., 2016; fig. 4 in Dumbo et al., 2019; present study Fig. 13). Based on sequence comparison (partial *18S*, complete *ITS1*, complete *5.8S*, complete *ITS2*, partial *28S*) between *M. trilobatus* with *M. spiniferus*, *M. texanus*, and *Pa. kinsella*, Tkach et al. (2010) indicated that *M. trilobatus* could

represent a new genus. As we did, Tkach and Kinsella (2011) recovered *Pl. typicum* (as *M. typicus*) and *Pl. flavum* (as *M. flavus*) as sister taxa, thereby recovering a monophyletic *Plesiocreadium* despite not commenting on the validity of the genus therein. The present phylogenetic analyses have more taxa of macroderoidids than any previous study.

The family assignment of *Gauhatiana*, which is the only macroderoidid that has a Y-shaped excretory vesicle, is uncertain. McMullen (1937) and Font and Lotz (2008) differentiated Macroderoididae from Plagiorchiidae by the shape of the excretory vesicle (I-shaped vs. Y-shaped, respectively), but Font and Lotz (2008) nevertheless provisionally transferred *Gauhatiana* to Macroderoididae. *Gauhatiana* further differs from macroderoidid genera by having a vitellarium comprising 2 lateral clusters in the forebody and hindbody that are discontinuous at the level of the ventral sucker. Liang-Sheng and Fotedar (1958), without providing a justification, synonymized *Gauhatiana* with *Astiotrema* Looss, 1900 (regarded as *incertae sedis* in Plagiorchioidea Lühe, 1901 by Pojmańska et al. [2008]). Hernández-Mena et al. (2016), without detailed morphological evidence, speculated that *G. batrachii* (misspelled therein as "*Gauthiana*" *batrachii*) belonged to Alloglossidiidae Hernández-Mena, Mendoza-Garfias, Ornelas-García, and Pérez-Ponce de León, 2016 (diagnosed as having an I-shaped excretory vesicle only) because it matures in catfishes (Siluriformes) like species of *Alloglossidium* Simer, 1929 (type genus of Alloglossidiidae). Karar et al. (2021), disagreeing with Liang-Sheng and Fotedar (1958), considered *Gauhatiana* distinct from *Astiotrema*, but synonymized *Gauhatiana fusiformis* Wang, 1981 and *Gauhatiana pseudobagri* Wang, 1983 with *Astiotrema fotedari* Dhar, 1977 and *Astiotrema reniferum* (Looss, 1898) Looss, 1900, respectively. Both species lack i) discontinuous vitelline fields at the level of the ventral sucker and ii) an ovary that is in the middle of the body, which are both diagnostic features of

Gauhatiana (see Karar et al., 2021). Hence, the only congener, *Gauhatiana lebedevi* Gupta and Miglani, 1976, was described from 8 specimens infecting the intestine of an unidentified marine fish in Fort Blair (Andaman and Nicobar Islands, Bay of Bengal). This species is likely not a macroderoidid because it has an aspinous tegument and a cirrus sac that is wholly lateral (sinistral) to the ventral sucker (Gupta and Miglani, 1976). Further investigations are needed to confirm the tegumental spination and relative position of the cirrus sac to the ventral sucker in *G. lebedevi*. No nucleotide sequence is publicly available for a species of *Gauhatiana*.

The *28S* sequence AF433673 of Tkach et al. (2001) is identical to *M. spiniferus* (EU850400) (see Table III) and is likely that species, not *Pl. typicum*. AF433673 (1,250 bp) was generated from 1 specimen collected from the shortnose gar, and no voucher specimen was deposited (Tkach et al., 2001). Four identical sequences representing *Pl. typicum* (HQ680846–49, 2,447 bp) were generated from 2 specimens infecting the Florida gar and 2 specimens infecting bowfins in Orange Lake (with 6 deposited voucher specimens USNPC 104583–84) (Tkach and Kinsella, 2011). Six sequences of *M. spiniferus* (EU850400–05; 2,514 bp, with 1 single-site polymorphism in the position '112' from the 5' end) were generated from 1 specimen infecting the alligator gar, *Atractosteus spatula* (Lacepède, 1803) (Lepisosteiformes: Lepisosteidae), in Nueces River (Texas), 2 specimens infecting the longnose gar and shortnose gar in Red Creek and Pascagoula River (both in Mississippi), respectively, and 3 specimens infecting the Florida gar in Cross Creek near Gainesville (Florida) (no voucher specimen) (Tkach et al., 2008).

ACKNOWLEDGMENTS

We thank Thomas Brooking and Tony Van de Valk, both of Cornell University Biological Field Station, for helping collect Oneida Lake fish; Haley Dutton, Steven Ksepka, Micah Warren, John Brule, and Justin Krol (Southeastern Cooperative Fish Parasite and Disease

Laboratory, Auburn University) for helping collect the other fish parasites studied herein; and Anna Phillips, Kathryn Ahlfeld, and Amanda Robinson (all United States National Museum, Washington, D.C.) for taking care of our deposited specimens and for loaning critical type materials to us. This study was supported by research grants to SAB from the Southeastern Cooperative Fish Parasite and Disease Project, Alabama Agriculture Experiment Station, Auburn University Vice President for Research and Economic Development, United States Department of Agriculture, and United States Fish and Wildlife Service. The collections in New York were supported by a research grant to FBR from State University of New York College at Oneonta Research Foundation.

LITERATURE CITED

Anderson, G. R., and S. C. Barker. 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.

Babero, B. B. 1951. *Rauschiella tineri* n. g., n. sp., a trematode (Plagiorchiinae) from a frog. Journal of Parasitology 37: 560–562.

Brooks, D. R. 1977. Evolutionary history of some plagiorchioid trematodes of anurans. Systematic Zoology 26: 277–289.

Cribb, T. H., G. R. Anderson, R. D. Adlard, and R. A. Bray. 1998. A DNA-based demonstration of a three-host life-cycle for the Bivesiculidae (Platyhelminthes: Digenea). International Journal for Parasitology 28: 1791–1795.

Darriba, D., G. L. Taboada, R. Doallo, and D. Posada. 2012. jModelTest 2: More models, new heuristics, and parallel computing. Nature Methods 9: 772. https://doi.org/10.1038/nmeth.2109.

Dumbo, J. C., Q. M. dos Santos, and A. Avenant-Oldewage. 2019. Morphological and molecular characterization of *Glossidium pedatum* Looss, 1899 and *Orientocreadium batrachoides* Tubangui, 1931 from sharptooth catfish, *Clarias gariepinus* (Burchell, 1822). African Zoology 54: 43–61.

Font, W. F., and J. M. Lotz. 2008. Family Macroderoididae McMullen, 1937. *In* Keys to the Trematoda. Vol 3., R. A. Bray, D. I. Gibson, and A. Jones (eds.). CAB International and Natural History Museum, Cambridge, U.K., p. 373–380.

Fricke, R., W. N. Eschmeyer, and R. Van der Laan. 2022. Eschmeyer's Catalog of Fishes: Genera, Species, References. Available at:

http://researcharchive.calacademy.org/research/ichthyology/catalog/fishcatmain.asp. Accessed 20 April 2022.

Gibson, D. I., and R. A. Bray. 1979. *Cirkennedya porlockensis*, a new genus and species of digenean from the sunfish *Mola mola* (L.). Journal of Helminthology 53: 245–250.

Glez-Peña, D., D. Gómez-Blanco, M. Reboiro-Jato, F. Fdez- Riverola, and D. Posada. 2010. ALTER: Program-oriented format conversion of DNA and protein alignments. Nucleic Acids Research 38: W14–W18. doi:10.1093/nar/gkq321.

Gupta, S. P. 1953. Trematode parasites of fresh-water fishes. Indian Journal of Helminthology 5: 1–80.

Gupta, N. K., and A. Miglani. 1976. Digenetic trematodes from marine food fishes and wild ducks of Port Blair (Andaman and Nicobar Islands) India. Revista Iberica de Parasitologia 36: 219–248.

Hernández-Mena, D. I., B. Mendoza-Garfias, C. P. Ornelas-García, and G. Pérez-Ponce de León. 2016. Phylogenetic position of *Magnivitellinum* Kloss, 1966 and *Perezitrema* Baruš and Moravec, 1967 (Trematoda: Plagiorchioidea: Macroderoididae) inferred from partial 28S rDNA sequences, with the establishment of Alloglossidiidae n. fam. Systematic Parasitology 93: 525–538.

Hunter, G. W. 1932. A new trematode (*Plesiocreadium parvum*, sp. nov.) from fresh water fish. Transactions of the American Microscopical Society 51: 16–21.

Karar, Y. F. M., C. K. Blend, N. O. Dronen, and A. Adel. 2021. Towards resolving the problematic status of the digenean genus *Astiotrema* Looss, 1900: An updated concept and revision of species composition for *Astiotrema* (*sensu stricto*). Zootaxa 4991: 36–72.

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.

Kusy, H., and M. A. Barger. 2017. A new species of *Macroderoides* (Trematoda: Macroderoididae) from spotted gar, *Lepisosteus oculatus* (Lepisosteidae), in the Big Thicket National Preserve and surrounding areas, Texas, U.S.A. Comparative Parasitology 84: 28–31.

Liang-Sheng, Y., and D. N. Fotedar. 1958. A review of the trematode genus *Astiotrema* in the family Plagiorchiidae. Journal of Helminthology 32: 17–32.

Lockyer, A. E., P. D. Olson, and D. T. J. Littlewood. 2003. Utility of complete large and small subunit rRNA genes in resolving the phylogeny of the Neodermata (Platyhelminthes): implications and a review of the cercomer theory. Biological Journal of the Linnean Society 78: 155–171.

McMullen, D. B. 1937. A discussion of the taxonomy of the family Plagiorchiidae Luhe, 1901, and related trematodes. Journal of Parasitology 23: 244–258.

Moravec, F., and G. Salgado-Maldonado. 2002. Redescription of *Perezitrema bychowskyi* (Caballero and Caballero, 1975) (Trematoda: Macroderoididae), with remarks on the systematic status of *Perezitrema* Baruš and Moravec, 1996. Systematic Parasitology 53: 199–206.

Pojmańska, T., V. V. Tkach, and D. I. Gibson. 2008. Genera *incertae sedis*, genera *inquirenda*, *nomina nuda*, larval or collective names and recently erected genera. *In* Keys to the Trematoda. Vol 3., R. A. Bray, D. I. Gibson, and A. Jones (eds.). CAB International and Natural History Museum, Cambridge, U.K., p. 735–755.

Rambaut, A., M. A. Suchard, D. Xie, and A. J. Drummond. 2014. FigTree v1.4.4. http://tree.bio.ed.ac.uk/software/figtree. Accessed on 20 August 2022.

Rambaut, A., A. J. Drummond, D. Xie, B. Baele, and M. A. Suchard. 2018. Posterior summarization in Bayesian phylogenetics using Tracer 1.7. Systematic Biology 67: 901–904.

Razo-Mendivil, U. J., V. León-Règagnon, and G. Pérez-Ponce de León. 2006. Monophyly and systematic position of *Glypthelmins* (Digenea), based on partial lsrDNA sequences and morphological evidence. Organisms Diversity and Evolution 6: 308–320.

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

Sullivan, J. J. 1977. Revision of the genus *Rauschiella* Babero, 1951 (Digenea: Plagiorchiidae) with a redescription of *R. palmipedis* (Lutz, 1928) n. comb. from Venezuelan frogs. Proceedings of the Helminthological Society of Washington 44: 82–86.

Taylor, P. W. 1978. *Macroderoides trilobatus* sp. n. (Digenea: Maroderoididae) from the bowfin, *Amia calva*, and emendation of the genus. Journal of Parasitology 64: 393–394.

Tkach, V. V., and J. M. Kinsella. 2011. New *Macroderoides* (Digenea: Macroderoididae) from Florida gar, with molecular phylogeny of the genus. Journal of Parasitology 97: 920–923.

Tkach,V. V., E. E. Pulis, and R. M. Overstreet. 2010. A new *Paramacroderoides* species (Digenea: Macroderoididae) from two species of gar in the southeastern United States. Journal of Parasitology 96: 1002–1006.

Tkach, V. V., S. D. Snyder, and Z. Swiderski. 2001. On the phylogenetic relationships of some members of Macroderoididae and Ochetosomatidae (Digenea, Plagiorchioidea). Acta Parasitologica 46: 267–275.

Tkach, V. V., E. J. Strand, and L. Froese. 2008. *Macroderoides texanus* n. sp. (Digenea: Macroderoididae) from alligator gar, *Atractosteus spatula* in Texas. Parasitology Research 104: 27–33.

Truong, T. N., S. S. Curran, H. R. Dutton, and S. A. Bullard. 2021. Resurrection and emendation of *Pseudoparamacroderoides* Gupta and Agrawal, 1968 (Digenea: Macroderoididae), description of a new species from Vietnam, and comments on the systematics of macroderoidid genera. Systematic Parasitology 98: 697–711.

Van Cleave, H. J., and J. F. Mueller. 1932. Parasites of Oneida Lake fishes. Part I. Descriptions of new genera and new species. Roosevelt Wild Life Annals 3: 5–71.

Van Cleave, H. J., and J. F. Mueller. 1934. Parasites of Oneida Lake fishes. Part III. A biological survey of the worm parasites. Roosevelt Wild Life Annals 7: 161–334.

Winfield, G. F. 1929. *Plesiocreadium typicum*, a new trematode from *Amia calva*. Journal of Parasitology 16: 81–87.

Figures 1–4. *Plesiocreadium typicum* infecting the intestine of the bowfin, *Amia calva* Linnaeus, 1766 (Amiiformes: Amiidae), from 4 localities in the eastern United States. (**1**) Voucher (ventral view) from Big Lake, Pascagoula River (Mississippi) (USNM 1679386). (**2**) Voucher (ventral view) from Chittenango Creek, Oneida Lake tributary (New York) (USNM 1679384). (**3**) Voucher (ventral view) from Reelfoot Lake (Tennessee) (USNM 1679389). (**4**) Voucher (ventral view) from New Lake (Arkansas) (USNM 1679391). Abbreviations: os, oral sucker; pre, pre-pharyngeal esophagus; ph, pharynx; poe, post-pharyngeal esophagus; ce, intestinal cecum; gp, genital pore; cs, cirrus sac; cir, cirrus; vs, ventral sucker; ova, ovary; vf,

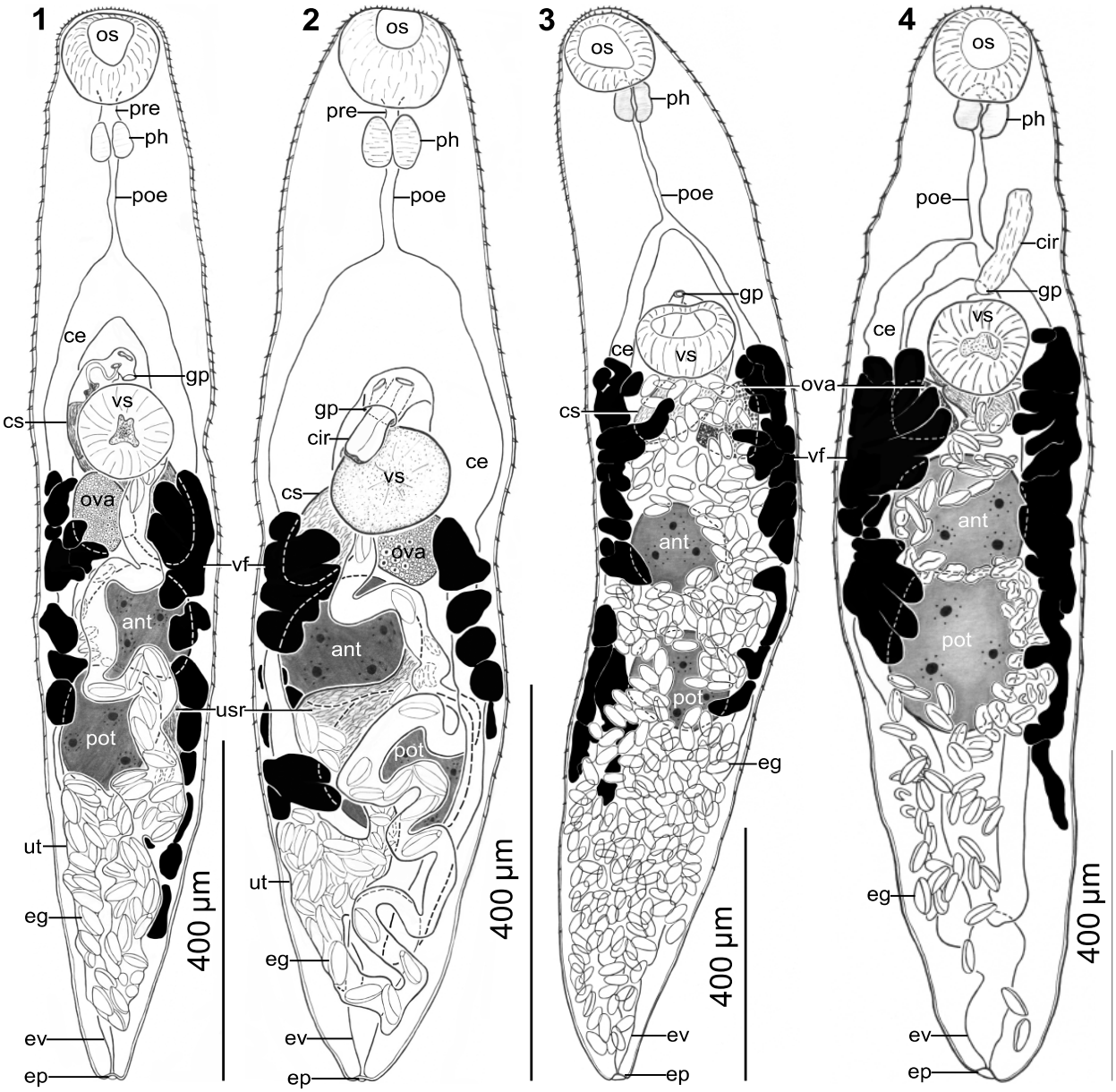
vitelline follicles; ant, anterior testis; pot, posterior testis; usr, uterine seminal receptacle; ut, uterus; eg, egg; ev, excretory vesicle; ep, excretory pore.

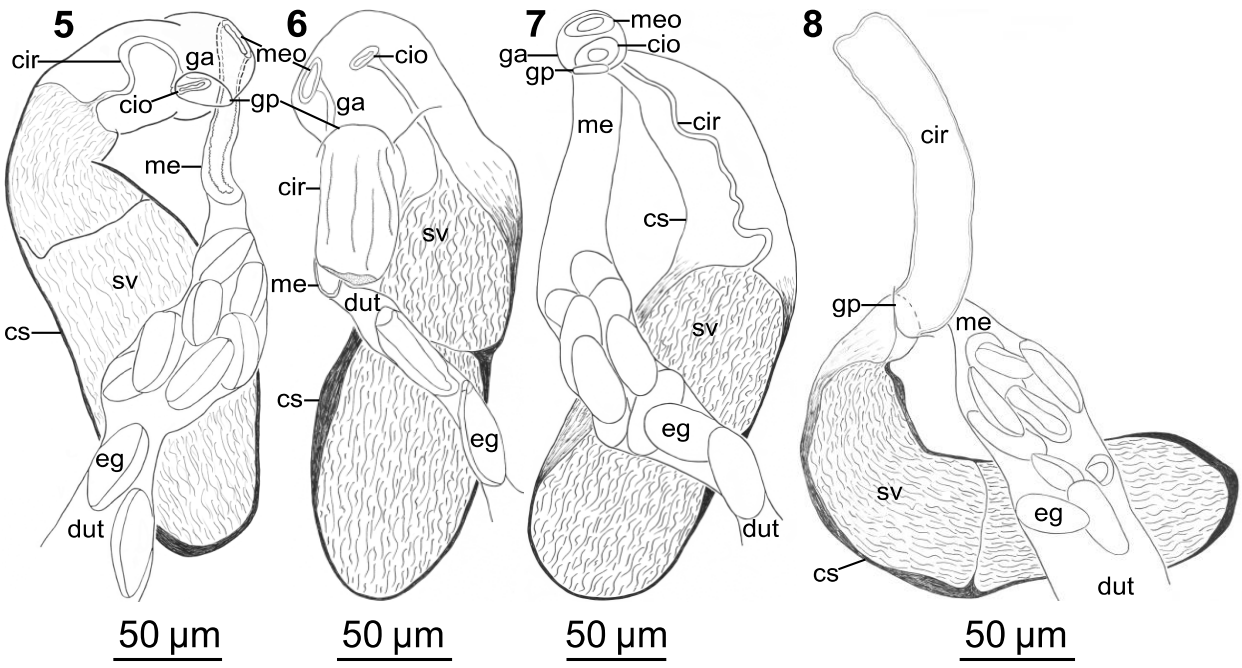
Figures 5–8. *Plesiocreadium typicum* from 4 localities in the eastern United States, showing terminal genitalia. (**5**) Voucher (ventral view) from Big Lake, Pascagoula River (Mississippi) (USNM 1679386). (**6**) Voucher (ventral view) from Chittenango Creek, Oneida Lake tributary (New York) (USNM 1679384). (**7**) Voucher (ventral view) from Reelfoot Lake (Tennessee) (USNM 1679390). (**8**) Voucher (ventral view) from New Lake (Arkansas) (USNM 1679392). Abbreviations: cir, cirrus; cs, cirrus sac; meo, metraterm opening; ga, genital atrium; cio, cirrus opening; gp, genital pore; me, metraterm; sv, seminal vesicle; eg, egg; dut, distal end of the uterus.

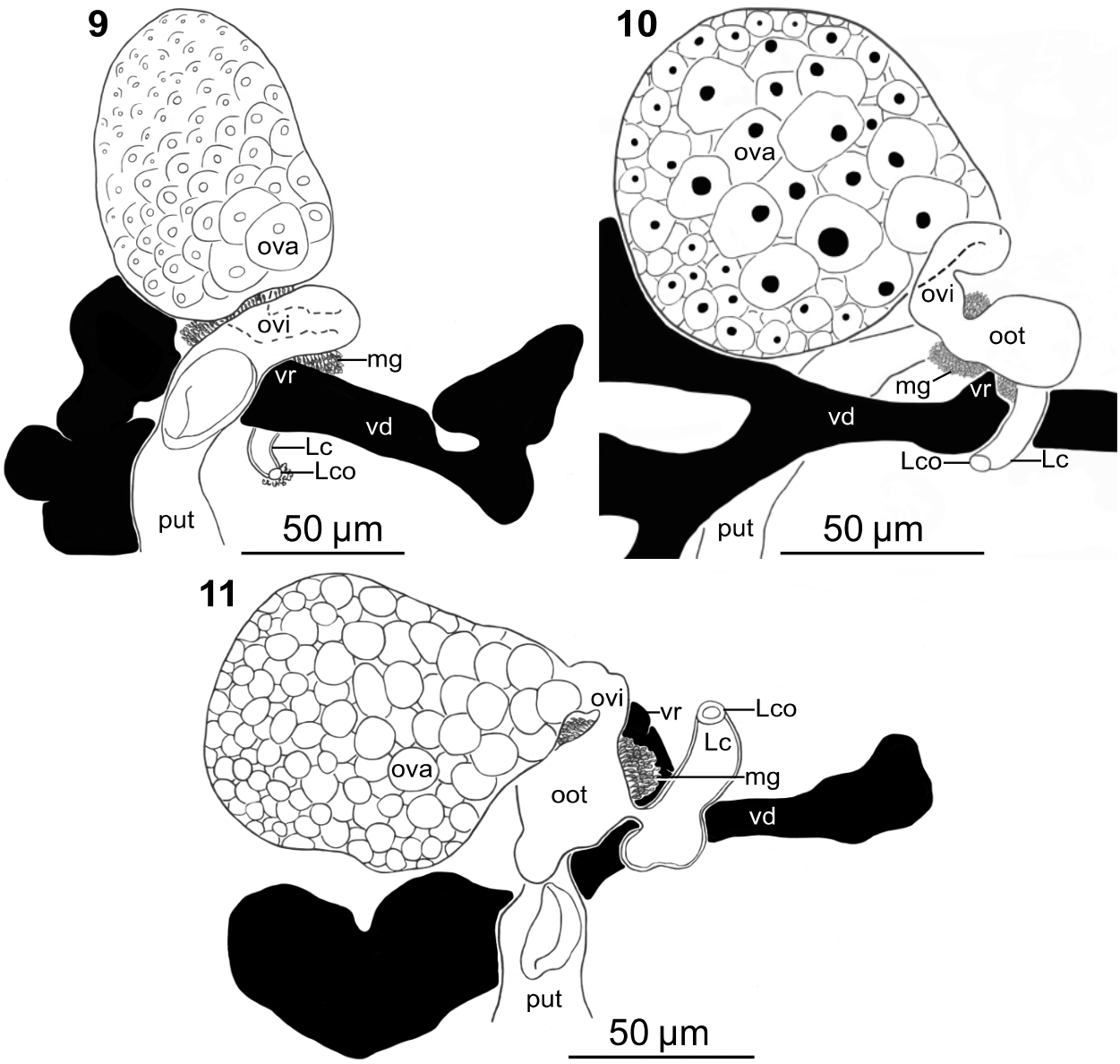
Figures 9–11. *Plesiocreadium typicum*, showing ovarian complex. (**9**) Voucher (ventral view) from Big Lake, Pascagoula River (Mississippi) (USNM 1679386). (**10**) Voucher (dorsal view) from Chittenango Creek, Oneida Lake (New York) (USNM 1679385). (**11**) Voucher (dorsal view) from New Lake (Arkansas) (USNM 1679394). Abbreviations: ova, ovary; ovi, oviduct; oot, oötype; vr, vitelline reservoir; mg, Mehlis' gland; vd, vitelline duct; Lc, Laurer's canal; Lco, Laurer's canal opening; put, proximal end of the uterus.

Figure 12. *ITS2* phylogeny for Macroderoididae. Values aside nodes are posterior probability. Scale bar is in substitutions per site.

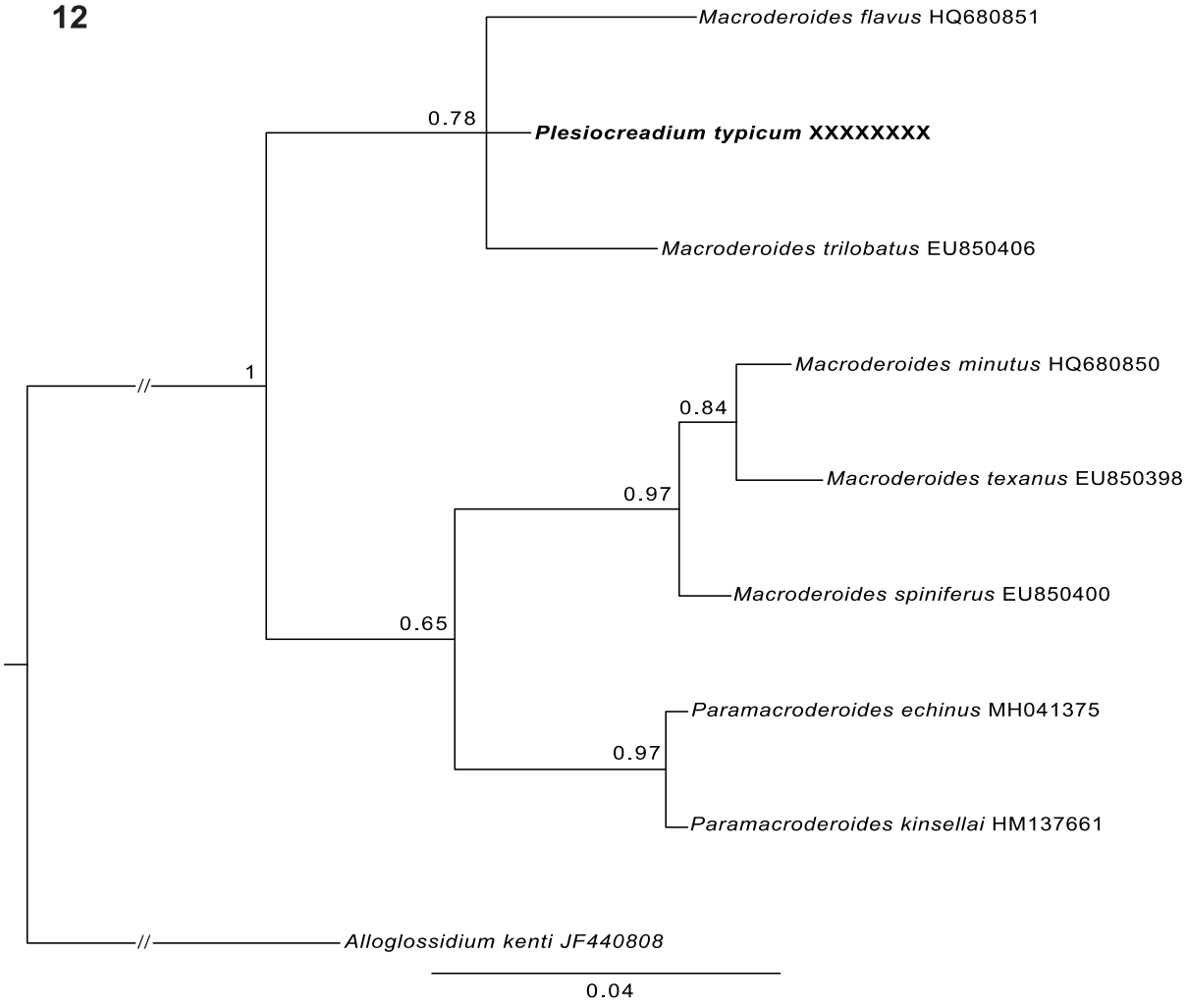
Figure 13. *28S* phylogeny for Macroderoididae. Values aside nodes are posterior probability. Scale bar is in substitutions per site.


1
os
pre
ph
poe
ce
gp
vs
cs
ova
vf
ant
usr
pot
ut
eg
ev
ep
400 µm
2
os
pre
ph
poe
gp
cir
vs
ce
cs
ova
ant
pot
ut
eg
ev
ep
400 µm
3
os
ph
poe
gp
ce
vs
ova
cs
vf
ant
pot
eg
ev
ep
400 µm
4
os
ph
poe
cir
gp
vs
ce
ant
pot
eg
ev
ep
400 µm


5
cir
ga
meo
cio
gp
me
sv
cs
eg
dut
50 µm
6
cio
ga
cir
sv
me
dut
cs
eg
50 µm
7
meo
cio
ga
gp
me
cir
cs
sv
eg
dut
50 µm
8
cir
gp
me
sv
eg
cs
dut
50 µm


9
ova
ovi
mg
vr
vd
Lc
Lco
put
50 µm
10
ova
ovi
oot
mg
vr
vd
Lco
Lc
put
50 µm
11
ovi
vr
Lco
Lc
ova
mg
oot
vd
put
50 µm

12


Macroderoides flavus HQ680851
0.78
Plesiocreadium typicum XXXXXXXX
Macroderoides trilobatus EU850406
1
Macroderoides minutus HQ680850
0.84
Macroderoides texanus EU850398
0.97
Macroderoides spiniferus EU850400
0.65
Paramacroderoides echinus MH041375
0.97
Paramacroderoides kinsellai HM137661
Alloglossidium kenti JF440808
0.04

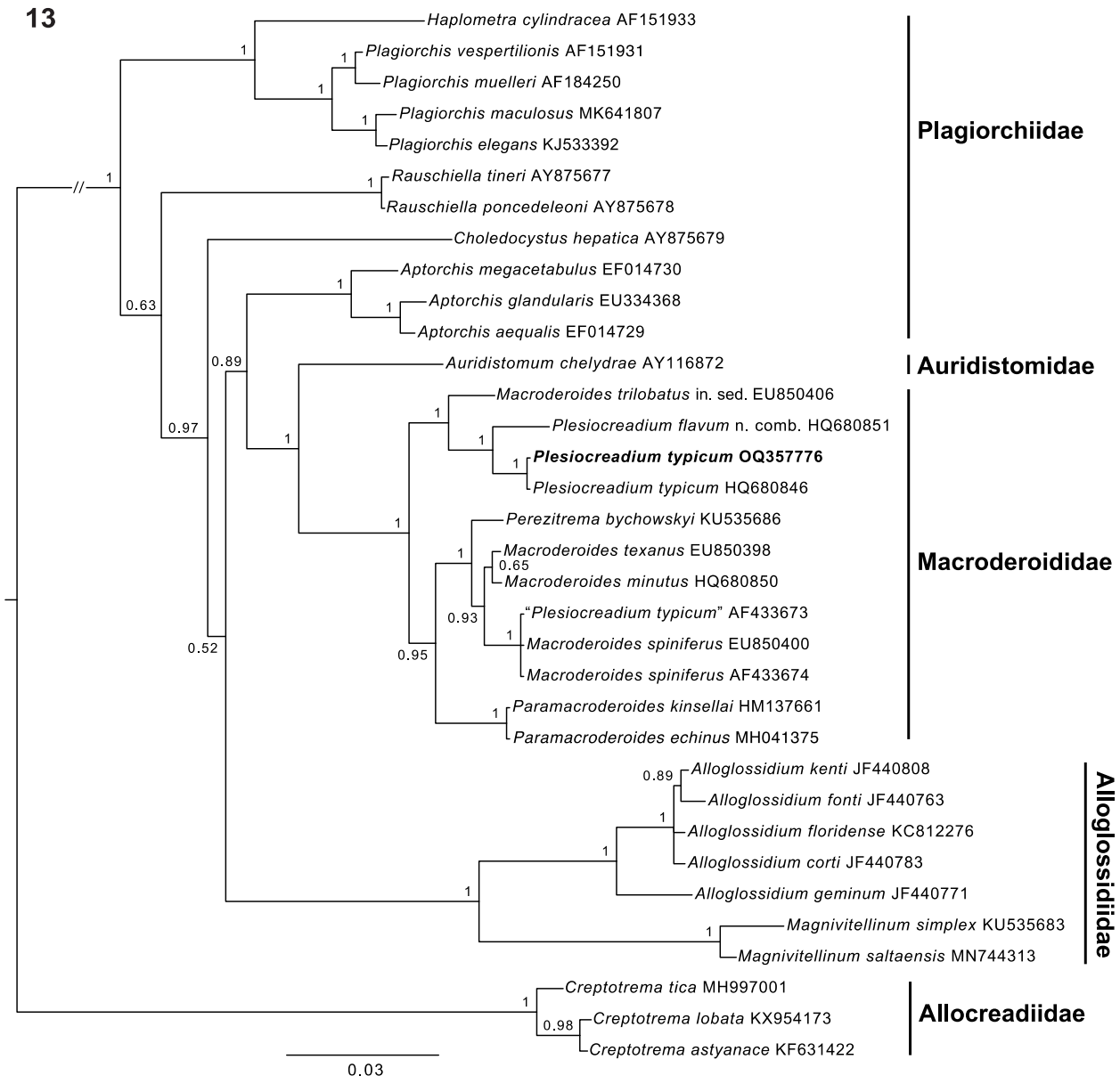

13
Haplometra cylindracea AF151933
Plagiorchis vespertilionis AF151931
Plagiorchis muelleri AF184250
Plagiorchis maculosus MK641807
Plagiorchis elegans KJ533392
Rauschiella tineri AY875677
Rauschiella poncedeleoni AY875678
Choledocystus hepatica AY875679
Aptorchis megacetabulus EF014730
Aptorchis glandularis EU334368
Aptorchis aequalis EF014729
Auridistomum chelydrae AY116872
Macroderoides trilobatus in. sed. EU850406
Plesiocreadium flavum n. comb. HQ680851
Plesiocreadium typicum OQ357776
Plesiocreadium typicum HQ680846
Perezitrema bychowskyi KU535686
Macroderoides texanus EU850398
Macroderoides minutus HQ680850
"Plesiocreadium typicum" AF433673
Macroderoides spiniferus EU850400
Macroderoides spiniferus AF433674
Paramacroderoides kinsellai HM137661
Paramacroderoides echinus MH041375
Alloglossidium kenti JF440808
Alloglossidium fonti JF440763
Alloglossidium floridense KC812276
Alloglossidium corti JF440783
Alloglossidium geminum JF440771
Magnivitellinum simplex KU535683
Magnivitellinum saltaensis MN744313
Creptotrema tica MH997001
Creptotrema lobata KX954173
Creptotrema astyanace KF631422
0.03
Plagiorchiidae
Auridistomidae
Macroderoididae
Alloglossidiidae
Allocreadiidae

Table I. Measurements of *Plesiocreadium typicum* infecting the intestine of *Amia calva* in the eastern United States.

Characters	Douglas Lake Michigan Winfield (1929) (n = 38)	Big Lake Mississippi Present study (n = 7)	Reelfoot Lake Tennessee Present study (n = 15)	New Lake Arkansas Present study (n = 17)	Chittenango Creek New York Present study (n = 10)
Body length (BL)	778–1,365 (1,083)	1,130–1,260 (1,199 ± 44)	742–1,700 (1,198 ± 284)	811–1,471 (1,035 ± 165)	890–1,080 (1,024 ± 54)
Body width (BW)	173–294 (211)	195–280 (222 ± 34)	146–325 (255 ± 51)	271–377 (321 ± 35)	205–262 (241 ± 19)
BL:BW ratio	4.5–4.6	4.0–6.3 (5.5 ± 0.9)	3.9–5.8 (4.7 ± 0.5)	2.7–4.3 (3.2 ± 0.5)	4.0–5.0 (4.3 ± 0.3)
Forebody (FB) length	–	400–445 (421 ± 17)	172–470 (340 ± 73)	251–424 (335 ± 43)	305–420 (388 ± 35)
% FB to BL	–	33–37 (35 ± 2)	23–37 (29 ± 4)	27–37 (33 ± 3)	34–41 (38 ± 2)
Hindbody (HB) length	–	610–740 (671 ± 41)	412–1,090 (751 ± 222)	459–903 (592 ± 121)	495–567 (536 ± 25)
% HB to BL	–	54–59 (56 ± 2)	53–70 (62 ± 5)	50–64 (57 ± 3)	49–56 (52 ± 2)
Oral sucker (OS) length	94–136 (114)	90–103 (96 ± 5)	71–128 (98 ± 16)	76–112 (97 ± 10)	83–95 (88 ± 4)
OS width	89–131 (116)	98–118 (109 ± 6)	73–125 (102 ± 15)	85–119 (103 ± 12)	100–110 (106 ± 4)
% OS width to BW	45–51	35–56 (50 ± 8)	34–49 (40 ± 4)	27–40 (32 ± 4)	41–50 (44 ± 3)
Ventral sucker (VS) length	96–145 (117)	98–115 (107 ± 6)	70–123 (102 ± 17)	91–137 (113 ± 13)	90–113 (102 ± 7)
VS width	89–150 (119)	103–118 (112 ± 6)	74–158 (114 ± 22)	88–144 (115 ± 15)	90–120 (108 ± 9)
%VS width to BW	51	41–59 (51 ± 6)	38–49 (45 ± 3)	30–45 (36 ± 4)	42–49 (45 ± 2)
VS to OS width ratio	–	0.9–1.2 (1.0 ± 0.1)	1.0–1.3 (1.1 ± 0.1)	1.0–1.3 (1.1 ± 0.1)	0.9–1.1 (1.0 ± 0.1)
Pharynx length	–	43–55 (51 ± 4)	35–63 (50 ± 9)	43–67 (54 ± 6)	50–58 (53 ± 3)
% pharynx length to BL	–	4–5 (4 ± 0)	3–6 (4 ± 1)	4–7 (5 ± 1)	5–6 (5 ± 0)
Pharynx width	47–70 (57)	48–53 (51 ± 2)	35–60 (48 ± 8)	45–65 (55 ± 8)	55–60 (59 ± 2)
% pharynx width to BW	24–27	19–27 (23 ± 3)	16–26 (19 ± 3)	14–22 (17 ± 2)	23–29 (25 ± 2)
Pre-pharyngeal esophagus (eso) length	–	13–53 (28 ± 13)	6–18 (10 ± 4)	0–13 (4 ± 5)	10–23 (16 ± 4)
% pre-pharyngeal eso to BL	–	1–4 (2 ± 1)	0–2 (1 ± 1)	0–1	1–2 (2 ± 0)
Post-pharyngeal eso length	–	103–133 (118 ± 10)	65–168 (99 ± 28)	61–145 (95 ± 28)	78–128 (94 ± 16)
% post-pharyngeal eso to BL	–	8–11 (10 ± 1)	6–11 (8 ± 2)	6–15 (9 ± 2)	8–13 (9 ± 1)
Intestinal bifurcation (Int bif) to anterior body end	–	265–300 (281 ± 14)	210–345 (253 ± 37)	178–290 (232 ± 34)	220–285 (247 ± 19)

Characters	Douglas Lake Michigan Winfield (1929) (n = 38)	Big Lake Mississippi Present study (n = 7)	Reelfoot Lake Tennessee Present study (n = 15)	New Lake Arkansas Present study (n = 17)	Chittenango Creek New York Present study (n = 10)
% int bif-anterior body end to BL	–	21–25 (24 ± 1)	17–30 (21 ± 3)	19–29 (22 ± 2)	22–28 (24 ± 2)
Ceca extent from anterior end	–	1,030–1,130 (1,063 ± 37)	693–1,520 (1,066 ± 278)	762–1,285 (945 ± 142)	800–990 (932 ± 56)
% ceca extent from anterior end to BL	–	86–93 (89 ± 2)	84–93 (90 ± 3)	85–97 (91 ± 3)	90–92 (91 ± 1)
Anterior testis (AT) length	–	108–148 (122 ± 14)	86–160 (118 ± 23)	111–176 (130 ± 19)	105–135 (120 ± 9)
AT width	94–159 (132)	110–138 (122 ± 13)	80–180 (135 ± 26)	110–200 (166 ± 25)	103–145 (124 ± 11)
% AT width to BW	54	53–63 (58 ± 4)	45–64 (54 ± 6)	41–67 (52 ± 7)	49–59 (52 ± 4)
Posterior testis (PT) length	–	120–145 (138 ± 9)	93–181 (134 ± 23)	91–181 (146 ± 24)	103–310 (151 ± 61)
PT width	89–164 (133)	103–155 (124 ± 18)	84–193 (147 ± 29)	143–250 (179 ± 25)	105–165 (129 ± 17)
% PT width to BW	51–56	51–64 (58 ± 5)	50–67 (58 ± 6)	46–69 (56 ± 6)	49–65 (54 ± 6)
Pre-testicular space	–	540–650 (616 ± 36)	325–770 (563 ± 128)	396–723 (528 ± 79)	455–587 (543 ± 44)
% pre-testicular space to BL	–	48–53 (51 ± 2)	26–55 (48 ± 7)	43–56 (51 ± 3)	46–56 (53 ± 3)
Post-testicular space	–	310–370 (332 ± 22)	198–732 (386 ± 154)	128–441 (247 ± 86)	215–420 (253 ± 60)
% post-testicular space to BL	–	26–30 (28 ± 2)	23–63 (32 ± 9)	14–33 (23 ± 5)	21–42 (25 ± 6)
Cirrus sac (CS) length	–	228–338 (280 ± 35)	190–355 (262 ± 48)	189–346 (243 ± 45)	150–293 (205 ± 52)
% CS length to BL	–	19–30 (23 ± 4)	17–28 (23 ± 3)	18–30 (24 ± 3)	15–27 (20 ± 4)
CS width	–	65–133 (85 ± 22)	43–91 (66 ± 13)	45–89 (63 ± 11)	40–74 (58 ± 11)
Seminal vesicle (SV) length	–	175–258 (220 ± 28)	94–175 (133 ± 30)	81–275 (157 ± 56)	133–203 (174 ± 27)
SV length to CS length	–	71–92 (79 ± 7)	36–62 (51 ± 8)	38–113 (65 ± 21)	64–97 (87 ± 12)
SV width	–	58–128 (80 ± 22)	34–82 (60 ± 14)	27–99 (55 ± 17)	35–68 (53 ± 11)
Cirrus length	–	73–120 (89 ± 19)	100–165 (130 ± 22)	70–223 (125 ± 49)	138–193 (162 ± 21)
Cirrus width	–	–	–	14–37 (30 ± 6)	35–48 (40 ± 6)
Genital atrium length	–	15–28 (22 ± 5)	11–33 (19 ± 8)	18	–
Genital atrium width	–	25	10–43 (24 ± 11)	16	–
Ovary length	–	80–115 (96 ± 10)	55–118 (94 ± 20)	61–114 (87 ± 13)	78–88 (82 ± 3)
Ovary width	65–108 (96)	55–88 (72 ± 13)	37–115 (75 ± 19)	56–101 (81 ±14)	75–100 (85 ± 9)

Characters	Douglas Lake Michigan Winfield (1929) (n = 38)	Big Lake Mississippi Present study (n = 7)	Reelfoot Lake Tennessee Present study (n = 15)	New Lake Arkansas Present study (n = 17)	Chittenango Creek New York Present study (n = 10)
% Ovary width to BW	37–38	28–37 (33 ± 3)	24–41 (29 ± 4)	17–35 (26 ± 5)	30–41 (36 ± 4)
Pre-ovarian space	–	465–540 (504 ± 25)	344–630 (465 ± 80)	330–552 (429 ± 55)	380–500 (463 ± 35)
% pre-ovarian space to BL	–	40–44 (42 ± 1)	32–46 (39 ± 4)	35–47 (42 ± 3)	43–49 (45 ± 2)
Egg length	39–49 (43)	39–45 (43 ± 2)	41–45 (43 ± 1)	40–46 (43 ± 2)	39–46 (42 ± 2)
Egg width	19–23 (20)	18–22 (20 ± 1)	18–23 (21 ± 1)	15–22 (18 ± 1)	18–23 (20 ± 2)
Pre-vitelline (vit) space	–	460–540 (492 ± 30)	275–530 (405 ± 62)	294–521 (384 ± 56)	350–510 (453 ± 46)
% Pre-vit space to BL	–	38–44 (41 ± 2)	28–41 (35 ± 4)	31–42 (37 ± 3)	39–48 (44 ± 3)
Post-vit space	–	160–370 (286 ± 72)	131–530 (295 ± 120)	92–392 (195 ± 75)	180–390 (250 ± 56)
% post-vit space to BL	–	13–32 (24 ± 6)	18–45 (24 ± 7)	11–27 (18 ± 5)	18–39 (24 ± 6)
Vit field on ovarian side	–	263–385 (327 ± 45)	210–570 (402 ± 118)	262–415 (331 ± 48)	235–295 (258 ± 18)
% vit field on ovarian side to BL	–	22–32 (27 ± 4)	24–41 (33 ± 4)	26–41 (32 ± 4)	22–30 (25 ± 2)
Vit field opposite to ovary	–	340–535 (451 ± 65)	330–700 (537 ± 130)	378–705 (497 ± 92)	290–410 (341 ± 35)
% vit field opposite to ovary to BL	–	30–43 (38 ± 5)	38–51 (45 ± 3)	39–64 (48 ± 6)	28–40 (34 ± 4)
Excretory vesicle length	–	210–235 (226 ± 12)	195–208	52–234 (147 ± 50)	93–193 (162 ± 46)
% excretory vesicle length to BL	–	18–21 (19 ± 2)	14–17	6–18 (14 ± 4)	9–18 (16 ± 4)

Table II. Pairwise comparisons of macroderoidid *ITS2* sequences (266 bp; above the diagonal: percent nucleotide similarity; below the diagonal: nucleotide differences).

Species	*P. typicum* (OQ357794)	*P. typicum* (HQ680846)	*P. flavum* n. comb.	*M. minutus*	*M. spiniferus*	*M. texanus*	*M. trilobatus*	*P. echinus*	*P. kinsellai*
Plesiocreadium typicum (OQ357794)	–	100	97.3	92.1	92.1	91.3	97.7	92.7	93.1
Plesiocreadium typicum (HQ680846)	0	–	97.3	92.1	92.1	91.3	97.7	92.7	93.1
Plesiocreadium flavum n. comb.	7	7	–	89.4	89.8	89.4	95.8	92.3	92.7
Macroderoides minutus	21	21	28	–	98.5	98.5	90.6	91.7	92.1
Macroderoides spiniferus	21	21	27	4	–	97.7	90.6	92.1	92.5
Macroderoides texanus	23	23	28	4	6	–	89.8	91.7	92.0
Macroderoides trilobatus	6	6	11	25	25	27	–	92.3	92.3
Paramacroderoides echinus	19	19	20	22	21	22	20	–	99.6
Paramacroderoides kinsellai	18	18	19	21	20	21	20	1	–

Table III. Pairwise comparisons of macroderoidid *28S* sequences (1,184 bp; above the diagonal: percent nucleotide similarity; below the diagonal: nucleotide differences).

Species	*P. typicum* (OQ357776)	*P. typicum* (HQ680846)	*"P. typicum"* (AF433673)	*P. flavum* n. comb.	*M. minutus*	*M. spiniferus* (EU850400)	*M. spiniferus* (AF433674)	*M. texanus*	*M. trilobatus*	*P. echinus*	*P. kinsellai*	*P. bychowskyi*
Plesiocreadium typicum (OQ357776)	–	100	96.3	98.3	96.6	96.3	96.2	96.5	97.7	96.3	96.3	96.8
Plesiocreadium typicum (HQ680846)	0	–	96.3	98.3	96.6	96.3	96.2	96.5	97.7	96.3	96.3	96.8
"Plesiocreadium typicum" (AF433673)	44	44	–	96.1	99.2	100	99.9	99.2	97.5	97.5	97.5	98.6
Plesiocreadium flavum n. comb.	20	20	46	–	96.3	96.1	96.0	96.2	97.5	95.9	95.9	96.3
Macroderoides minutus	40	40	9	44	–	99.2	99.2	99.7	97.4	97.8	97.8	99.1
Macroderoides spiniferus (EU850400)	44	44	0	46	9	–	99.9	99.2	97.5	97.5	97.5	98.6
Macroderoides spiniferus (AF433674)	45	45	1	47	10	1	–	99.1	97.5	97.4	97.4	98.6
Macroderoides texanus	41	41	10	45	3	10	11	–	97.3	97.9	97.9	99.2
Macroderoides trilobatus	27	27	29	29	31	29	30	32	–	96.7	96.7	97.0
Paramacroderoides echinus	44	44	30	48	26	30	31	25	39	–	100	97.6
Paramacroderoides kinsellai	44	44	30	48	26	30	31	25	39	0	–	97.6
Perezitrema bychowskyi	38	38	16	44	11	16	17	10	36	28	28	–

CHAPTER 4: *PLAGIOPORUS WATAUGAENSIS* N. SP. (DIGENEA: OPECOELIDAE) INFECTING INTESTINE OF NORTHERN HOGSUCKER, *HYPENTELIUM NIGRICANS*, AND WHITE SUCKER, *CATOSTOMUS COMMERSONII*, (CYPRINIFORMES: CATOSTOMIDAE) FROM THE EASTERN USA, INCLUDING AN EMENDED DIAGNOSIS, KEY TO NEARCTIC CONGENERS, AND PHYLOGENETIC ANALYSIS

***Published in Parasitology International (Available 29 March 2022)**

Authors: Triet N. Truong, Stephen S. Curran, Florian B. Reyda, Jacob M. Rash, and Stephen A. Bullard

ABSTRACT

We describe a new species of *Plagioporus* Stafford, 1904 infecting the intestine of two catostomids in the eastern USA. We emend *Plagioporus* to account for Nearctic congeners having ceca terminating at the level of the testes (previously diagnosed as having ceca terminating in the post-testicular space only) and testes in the posterior body extremity (a feature not previously considered as having generic importance). Of the accepted Nearctic species, *Plagioporus wataugaensis* n. sp. resembles *Plagioporus serotinus* Stafford, 1904, *Plagioporus hypentelii* Hendrix, 1973, and *Plagioporus hageli* Fayton and Andres, 2016 but differs from them by the distribution of the vitellarium and proportional length and relative extent of the excretory vesicle. *Plagioporus wataugaensis* has vitelline fields that are discontinuous at the level of the ventral sucker (vs. continuous in *P. serotinus* and *P. hypentelii*) and follicles that surround the ceca (vs. wholly ventral to the ceca in *P. hageli*) and that span the midline dorsal to the testes (vs. slightly overlapping the lateral margins of the testes). The excretory vesicle of *P. wataugaensis* is wholly post-testicular and short (6–9% of the body length) (vs. reaching the level of the posterior testis, 14–24% of the body length). Phylogenetic analyses of the 28S, ITS1, 5.8S, and ITS2 rDNA recovered *P. wataugaensis* sister to *Plagioporus sinitsini* Mueller, 1934. A

key to the Nearctic *Plagioporus* spp. is provided. We regard *Plagioporus shawi* (McIntosh, 1939) Margolis, 1970, *Plagioporus serratus* Miller, 1940, and *Plagioporus loboides* (Curran, Overstreet, and Tkach, 2007) Fayton and Andres, 2016 as *incertae sedis*.

1. Introduction

Plagioporus Stafford, 1904 is one of the most speciose genera of Opecoelidae Ozaki, 1925, comprising >60 accepted species [1–4] that collectively range in the Holarctic region [5] and the Indian sub-continent. Over 40 species formerly assigned to *Plagioporus* have been reassigned to other opecoelid genera: *Podocotyle* Dujardin, 1845, *Allopodocotyle* Pritchard, 1966, *Neolebouria* Gibson, 1976, *Gaevskajatrema* Gibson and Bray, 1982, and *Macvicaria* Gibson and Bray, 1982 (see Cribb [5]). *Plagioporus* resembles *Podocotyle*, *Allopodocotyle*, *Neolebouria*, and *Macvicaria* in that all typically having species with an ovoid or elongate body, a sinistral genital pore in mid-forebody or immediately anterior to the ventral sucker, testes that are seperated from the posterior body end, and a compact uterus that occupies space between the genital pore and the posterior testes [5]. Recent phylogenetic analyses recovered *Plagioporus* spp. as monophyletic and sister to the the clade that includes species of *Neoplagioporus* Shimazu, 1990 (paraphyletic). *Neoplagioporus ayu* (Takahashi, 1928) Shimazu, 1990 and *Neoplagioporus zacconis* (Yamaguti, 1934) Shimazu, 1990 are sister taxa sharing a common ancestor with a clade including *Neoplagioporus elongatus* (Goto and Ozaki, 1930) Shimazu, 1990 but also *Urorchis goro* Ozaki, 1927 and *Urorchis acheilognathi* Yamaguti, 1934) (see Fayton and Andres [2], Fayton, Choudhury et al. [3], Fayton, McAllister et al. [4], and Martin, Cutmore et al. [6]). Fayton, Choudhury et al. [3] and Fayton, McAllister et al. [4] accepted 19 Nearctic species of *Plagioporus*; nucleotide data (partial 18S, internal transcribed spacer regions [ITS1, 5.8S, ITS2],

and partial 28S rDNA) exist for 11 of those but only a 28S sequence is available for *Plagioporus loboides* (Curran, Overstreet, and Tkach, 2007) Fayton and Andres, 2016.

Life cycles for 5 *Plagioporus* spp. have been published; all are Nearctic [7–12]. The typical life cycle pattern comprises a molluscan first intermediate host, an arthropod second intermediate host, and a fish definitive host (*op. cit.*). Exceptionally, *Plagioporus sinitsini* Mueller, 1934 exhibits progenetic development within the first intermediate host [12,13]. *Plagioporus* spp. collectively exhibit a low degree of definitive host specificity, typically infecting the intestine and rarely the gall bladder of Nearctic freshwater and diadromous fishes (catostomids, centrarchids, a cottid, fundulids, a gasterosteid, a hiodontid, an ictalurid, leuciscids, percids, and salmonids) [4,8,10,14,15] and Eurasian cyprinids [16].

In the present study, we collected mature and immature opecoelid specimens infecting the intestine of the northern hogsucker, *Hypentelium nigricans* (Lesueur, 1817), and the white sucker, *Catostomus commersonii* (Lacepède, 1803), (both Cypriniformes: Catostomidae) in the eastern USA. We describe the specimens as a new species of *Plagioporus*, emend the generic diagnosis, provide a key to Nearctic *Plagioporus* spp., and conduct phylogenetic analyses.

2. Materials and methods

2.1. Morphological study

Ten white suckers were electrofished from Pass Run (38°42'18.0”N, 78°26'43.4”W), Hawksbill Creek, South Fork Shenandoah River, Page County, Virginia, USA on 21 May 2019. Four northern hogsuckers were also electrofished from the Watauga River (36°09'23.0”N, 81°46'11.2”W), Watauga County, North Carolina, USA on 15 October 2020. Viscera from each fish were excised and separated into dishes filled with a physiological saline (9 grams of sodium chloride [NaCl] dissolved in 1 L of distilled water; making a 9 ppt solution of NaCl). Each

intestine was examined for parasites using a Wild Heerbrugg M5A stereodissecting microscope (Wild Heerbrugg, Heerbrugg, Switzerland) and fiber optic light sources. A total of 29 (8 adults, 21 juveniles) and 20 (all adults) trematodes were collected from the intestine of 3 northern hogsuckers and 2 white suckers, respectively. Trematodes intended for morphology were rinsed with physiological saline, gently cleaned with fine artists' brushes to remove debris, heat-killed in hot water (~60 °C), and fixed in 10% neutral buffer formalin. Fixed specimens were stained overnight in a glass stender dish containing Van Cleave's hematoxylin mixed with 2 drops of Ehrlich's hematoxylin. Stained specimens were made basic at 70% ethanol (EtOH) with drops of lithium carbonate (Li_2CO_3) saturated in 70% EtOH and butylamine ($CH_3[CH_2]_3NH_2$), dehydrated in an EtOH series, cleared in clove oil, and permanently mounted on glass slides using Canada balsam. Specimens intended for DNA extraction were placed directly in 95% EtOH. Illustrations were made using an Olympus BX51 microscope (Olympus Corporation of the Americas, Center Valley, Pennsylvania, USA) equipped with differential interference contrast optical components and drawing tube. Measurements were made using an ocular micrometer and reported in micrometers (μm) as the range, followed by the mean ± standard deviation and sample size in parentheses. Length and width of reported vitelline follicles were based on measurements of a haphazard selection of small, medium, and large follicles from 6 adults [4]. Scientific names, including taxonomic authorities and dates, for fishes follow Fricke et al. [17]. Morphological and anatomical terms for *Plagioporus* spp. follow Fayton, McAllister et al. [4], except that "genital pore", "pre-pharyngeal esophagus" and "post-pharyngeal esophagus" are used instead of "gonopore", "pre-pharynx", and "esophagus", respectively. "Forebody" and "hindbody" herein refer to the portions of the body anterior and posterior to the ventral sucker, respectively [18].

Type specimens were deposited in the National Museum of Natural History's Invertebrate Zoology Collection (USNM, Smithsonian Institution, Washington, D. C., USA).

2.2. DNA extraction, sequencing, and phylogenetic analyses

Three EtOH-preserved specimens (1 adult and 1 immature from 2 northern hogsuckers; 1 adult from 1 white sucker) of the new species were used separately (as 3 replicates) to extract genomic DNA using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA concentration was measured using a NanoDrop–1000 spectrophotometer (Thermo Scientific, Nanodrop Technologies, Waltham, Massachusetts, USA), diluted to 20 ng/µl, and stored at –20 °C. PCR primers previously used for *Plagioporus* spp. (forward primer ITSF [5′–CGC CCG TCG CTA CTA CCG ATT G–3′] and reverse primer 1500R [5′–GCT ATC CTG AGG GAA ACT TCG–3′]) were adopted to amplify the ITS region and the partial 28S [2–4] with the following thermocycling parameters: initial denaturation step of 94 °C for 4 min, followed by 40 cycles of 94 °C for 40 s, 55 °C for 30 s, 72 °C for 2 min, with a final extension step of 72 °C for 5 min. PCR product purification was conducted using the QIAquick PCR Purification kit (Qiagen). In addition to ITSF and 1500R, digl2 (5′–AAG CAT ATC ACT AAG CGG–3′), digl2R (5′–CCG CTT AGT GAT ATG CTT–3′), 300F (5′–CAA GTA CCG TGA GGG AAA GTT G–3′), 300R (5′–CAA CTT TCC CTC ACG GTA CTT G–3′), 900F (5′–CCG TCT TGA AAC ACG GAC CAA G–3′), and ECD2 (5′–CTT GGT CCG TGT TTC AAG ACG GG–3′) were used for DNA sequencing. DNA sequencing was performed by Genewiz (South Plainfield, New Jersey, USA). All nucleotide sequences of the new species were deposited in NCBI GenBank (see Taxonomic summary).

The ITS2 and the partial 28S sequences of the new species were annotated using the ITS2 Ribosomal Database [19] and the length of the complete 5.8S of Nearctic *Plagioporus* spp. was

estimated according to annotated sequences in Fayton and Andres [2] and Fayton, Choudhury et al. [3], Fayton, McAllister et al. [4]. For pairwise comparisons, sequences of the new species were aligned and compared with those of the 12 Nearctic congeners. The phylogenetic analyses of the ITS and 28S markers included sequences of the new species, 17 and 16 opecoelids, and 3 and 4 outgroup taxa, respectively (Figs. 3,4): *Plagioporus boleosomi* (Pearse, 1924) Peters, 1957 (KX553953), *Plagioporus shawi* (McIntosh, 1939) Margolis, 1970 (KX553951), *Plagioporus chiliticorum* (Barger and Esch, 1999) Cribb, 2005 (KX553943), *P. loboides* (EF523477), *Plagioporus kolipinskii* Tracey, Choudhury, Cheng, and Ghosh, 2009 (KX553952), *Plagioporus hageli* Fayton and Andres, 2016 (KX553950), *P. sinitsini* (KX553944), *Plagioporus aliffi* Fayton, Choudhury, McAllister, and Robison, 2017 (KX905056), *Plagioporus limus* Fayton, Choudhury, McAllister, and Robison, 2017 (KX905055), *Plagioporus fonti* Fayton, Choudhury, McAllister, and Robison, 2017 (KX905054), *Plagioporus carolini* Fayton, McAllister, Robison, and Connior, 2018 (MG214680), *Plagioporus ictaluri* Fayton, Robison, and McAllister, 2018 (MG214679), *N. ayu* (KX553947), *N. elongatus* (KX553948), *N. zacconis* (KX553949), *U. acheilognathi* (KX553945), *U. goro* (KX553946), *Helicometra boseli* Nagaty, 1956 (KU320600), *Helicometra fasciata* (Rudolphi, 1819) Odhner, 1902 (KU320597), *Helicometra manteri* Andres, Ray, Pulis, Curran and Overstreet, 2014 (KJ701238), *Opecoeloides furcatus* (Bremser, 1819) Odhner, 1928 (AJ241790), *Poracanthium furcatum* Dollfus, 1948 (AJ241791), and *Podocotyle scorpaenae* (Rudolphi, 1919) Bartoli and Gibson, 1991 (AJ241794) (see Bray et al. [1], Fayton and Andres [2], Fayton, Choudhury et al. [3], Fayton, McAllister et al. [4], Jousson et al. [20], and Andres et al. [21]). Taxon and outgroup selection was based on Fayton and Andres [2], Fayton, Choudhury et al. [3], Fayton, McAllister et al. [4], Martin, Cutmore et al. [6], Martin, Huston et al. [22], and Sokolov et al. [23].

For the phylogenetic analyses, sequences of the new species and other selected opecoelids were aligned and trimmed to the length of the shortest sequence in each alignment using MAFFT [24]. JModelTest 2 version 2.1.10 was used to perform a statistical selection of the best-fit models of nucleotide substitution based on Bayesian information criteria [25]. Aligned sequences were reformatted (from .fasta to .nexus) using the web application ALTER [26]. Both alignments were independently subjected to Bayesian inference (BI) analyses. The BI analyses were performed in MrBayes version 3.2.5 [27] using substitution model averaging (nst-mixed) and a gamma distribution to model rate-heterogeneity. Defaults were used in all other parameters. Three independent runs with 4 Metropolis-coupled chains were run for 5,000,000 generations, sampling the posterior distribution every 1,000 generations. Convergence was checked using Tracer v1.7.1 [28] and the sump command in MrBayes. All runs appeared to reach convergence after discarding the first 25% of generations as burn-in. Majority-rule consensus trees of the post burn-in posterior distribution were generated with the sumt command in MrBayes. Trees were visualized using FigTree v1.4.4 [29] and further edited for visualization purposes with Adobe Illustrator 24.1.3 version (2020) (Adobe Systems, San Jose, California, USA).

3. Results

3.1. Plagioporus Stafford, 1904, emdended (based on adults of Nearctic species)

Body ovoid, fusiform, or elongate, with broadly rounded or tapering posterior end, widest at level of ventral sucker or in hindbody. Eyespot pigment absent. Oral sucker subspherical, ventral, subterminal or terminal. Ventral sucker subspherical, wider than oral sucker, in anterior half or middle of body. Pharynx subspherical or ovoid, abutting or nearly abutting oral sucker (i.e., pre-pharyngeal segment of esophagus indistinct or shorter than pharynx; post-pharyngeal segment of esophagus distinct, straight or sinuous, longer than pharynx). Intestine bifurcating in

mid-forebody or dorsal to anterior half of ventral sucker; ceca terminating at level of or beyond testes. Testes two, subspheroid, tandem or diagonal, separate, abutting or overlapping, in middle or posterior half of hindbody. External seminal vesicle absent. Cirrus sac claviform, oval or elongate, straight or sinuous, proximal end anterior or dorsal to ventral sucker. Internal seminal vesicle unipartite or bipartite, straight or convoluted. Pars prostatica indistinct or distinct. Cirrus aspinous, eversible. Genital atrium present or indistinct. Genital pore ventral, sinistral, lateral or submedian, in mid-forebody or immediately anterior to ventral sucker. Ovary entire or slightly lobed, immediately pre-testicular or nearly opposite or overlapping anterior testis. Mehlis' gland and Laurer's canal present; Laurer's pore dorsolateral, at level of or anterior to ovary. Seminal receptacle canalicular, dorsal to ovary. Uterine field compact, occupying space between genital pore and posterior testis. Metraterm aspinous, lateral to cirrus sac. Eggs ovoid, operculate, lacking polar filaments. Vitellarium follicular, in two lateral fields, extending into forebody or confined to hindbody, with or without follicles spanning midline anteriorly or posteriorly; follicles surrounding or wholly ventral to ceca; vitelline fields continuous or ventrally discontinuous at level of ventral sucker. Excretory vesicle I-shaped (saccular or elongate), restricted to post-testicular space or extending to level of ovary; pore terminal. Infecting intestine and gall bladder of freshwater and diadromous Holarctic fishes.

Type-species: Plagioporus serotinus Stafford, 1904.

3.1.1. Remarks on Plagioporus

Plagioporus has been emended by many authors [2–5,9,30–32]. Gibson and Bray [31] erected *Macvicaria* (type species, *Macvicaria alacris* [Looss, 1901] Gibson and Bray, 1982), for the marine species formerly of *Plagioporus* that have a "long" excretory vesicle that extends anteriad to at least beyond the posterior testis and onto the level of the ovary [5,31]. As such,

Plagioporus became restricted to freshwater species with a "short" excretory vesicle confined to the post-testicular space or extending to the level of the posterior testis [5,31,32]. However, subsequent workers [2–4,16] have challenged the use of excretory vesicle length as diagnostic because some species of *Plagioporus* have an excretory vesicle that extends farther anteriad; contradicting the diagnoses in Gibson and Bray [31] and Cribb [5]. *Plagioporus siliculus* Sinitsin, 1931, *P. limus*, and *P. ictaluri* have an excretory vesicle that reaches the anterior testis [3,4,7]; *P. shawi* and *P. carolini* have an excretory vesicle that extends to the level of the ovary [4,33]. Fayton and Andres [2] transferred *Plagiocirrus loboides* Curran, Overstreet, and Tkach, 2007 to *Plagioporus* based on its 28S nucleotide similarity with *Plagioporus* spp. and a 28S tree topology; thereby, without a formal emendation, including the following features in the diagnosis of *Plagioporus*: a lobed ovary, a uterus that distributes from the level of the genital pore to the posterior body end, and a vitellarium that is restricted between the ventral sucker and the posterior testis. Also without a formal emendation, Fayton, McAllister et al. [4] included *P. shawi* and *P. carolini* in *Plagioporus*; thereby adding the following feature to the genus diagnosis: an excretory vesicle that extends anteriad to the level of the ovary.

Emending *Plagioporus* is needed because several Nearctic plagioporines cannot be assigned to *Plagioporus sensu* Cribb [5], Fayton and Andres [2], or Fayton, McAllister et al. [4]: *Plagioporus cooperi* (Hunter and Bangham, 1932) Price, 1934, *P. sinitsini, Plagioporus serratus* Miller, 1940, *P. ictaluri*, and *P. carolini* have ceca that terminate at the level of the testes [4,30,34,35]. Cribb's [5] diagnosis of *Plagioporus* included, "*ceca blind, extend posteriorly beyond testes towards posterior end of body.*" Further, *P. sinitsini, P. serratus, P. ictaluri*, and *P. carolini* have testes located in the posterior body extremity [4,30,35]. Cribb's [5] diagnosis included, "*testes two, tandem to oblique, well separated from posterior end of body.*" Hence, we

emend *Plagioporus* to include species that have ceca that terminate at the level of the testes and testes that are in the posterior body extremity.

The taxonomic status of some Nearctic species is problematic. *Plagioporus shawi* is a morphologically bizarre congener. It has entire or lobed testes and an elongate cirrus sac that extends posteriad to the level of the ovary [33,36]. Also bizarre, *P. serratus* has a papillate tegument, which is covered by spiniform papillae [32]. The spinous tegument of *P. serratus* reported by Miller [35] is dubious (see Discussion). The taxonomic status of *P. loboides* also needs to be reconsidered. The reassignment of *Plagiocirrus loboides* to *Plagioporus* (see Fayton and Andres [2]) relied upon only the 28S sequence similarity (96.3–98.6% within 1,227 nucleotides) to 6 Nearctic *Plagioporus* spp. and a phylogenetic analysis (no morphological evidence). *Plagioporus loboides* differs from the Nearctic *Plagioporus* spp. by having the combination of an extensive uterus that extends posteriad far beyond the posterior testis and a vitellarium that is restricted between the ventral sucker and the posterior testis [37]. The high percent nucleotide similarity among plagioporines is unsurprising: 28S sequences of *Neoplagioporus* spp. are 97.1–99.1% similar to those of *Urorchis* spp. (see Fayton and Andres [2]). Regarding the 28S tree topology in Fayton and Andres [2], *P. loboides* was recovered sister to *P. hageli* (<80% support) within the clade of *Plagioporus* spp. However, it is odd to accept the assignment of *Plagiocirrus loboides* to *Plagioporus* regarding its distinct morphology to Nearctic *Plagioporus* spp. Also, *P. loboides* cannot be assigned to *Plagiocirrus* (as its original assignment) without an emendation of that genus regarding the position and shape of the ovary and the distribution of the vitellarium. Hence, until these issues are further investigated and/or more evidence is presented, we herein regard *P. shawi*, *P. serratus* and *P. loboides* as species *incertae sedis*.

3.2. Plagioporus wataugaensis Truong, Curran, and Bullard n. sp.

Description: (Figs. 1A–B; 2A–C)

Body fusiform, 810–1,600 (1,247 ± 301; n = 16) long, 3.0–4.1× (3.5 ± 0.4; n = 16) longer than wide, 245–440 (358 ± 65; n = 16) wide, widest at level of ventral sucker or mid-body; forebody 280–530 (409 ± 81; n = 16) long, 29–40% (33 ± 3; n = 16) of body length (BL); hindbody 340–860 (613 ± 195; n = 16) long, 40–54% (48 ± 3; n = 16) of BL (Figs. 1A,B; 2A). Oral sucker 85–150 (123 ± 22; n = 16) long, 90–160 (129 ± 24; n = 16) wide, 31–42% (37 ± 3; n = 16) of body width (BW) (Figs. 1A,B; 2A). Ventral sucker wider than long, 170–280 (226 ± 35; n = 16) long, 180–310 (256 ± 42; n = 16) wide, 63–83% (72 ± 5; n = 16) of BW; 1.9–2.2× (2.0 ± 0.1; n = 16) oral sucker width (Figs. 1A,B; 2A). Pre-pharyngeal esophagus 8–25 (20 ± 7; n = 15) long, 8–20 (16 ± 4; n = 15) wide; pharynx subspherical, 45–75 (60 ± 10; n = 15) long 45–75 (63 ± 12; n = 15) wide, 16–20% (18 ± 1; n = 15) of BW; post-pharyngeal esophagus 75–200 (125 ± 36; n = 16) long, 7–16% (10 ± 2; n = 16) of BL, 10–35 (24 ± 7; n = 16) wide. Intestine bifurcating in mid-forebody 200–350 (273 ± 45; n = 16) from anterior body end, 23–28% (26 ± 2; n = 4) of BL; ceca slender, extending posteriad to slightly beyond posterior testis; post-cecal space 50–260 long (134 ± 57; n = 15), 6–18% (10 ± 3; n = 15) of BL (Figs. 1A,B; 2A).

Testes subspheroid, median, abutting or slightly overlapping, in middle of hindbody (Figs. 1A,B; 2A); anterior testis slightly wider than long, 100–190 (158 ± 31; n = 16) long, 108–240 (191 ± 45; n = 16) wide, 540–960 (746 ± 142; n = 16), 55–68% (61 ± 4; n = 16) of BL from anterior body end; posterior testis dorsal to and narrower than anterior testis, 105–230 (175 ± 46; n = 16) long, 93–225 (170 ± 44; n = 16) wide, 620–1,140 (819 ± 182; n = 16), 66–80% (72 ± 4; n = 16) of BL from anterior body end; post-testicular space 65–290 long (182 ± 79; n = 16), 7–21% (14 ± 4; n = 16) of BL (Figs. 1A,B; 2A). Vasa efferentia uniting at level of posterior half of

ventral sucker, connecting proximal end of cirrus sac through short vas deferens (Fig. 2A). Cirrus sac claviform, elongate, 200–500 (347 ± 96; n = 16) long, 48–110 (84 ± 23; n = 16) at widest part, dorsally overlapping anterior half to 3/4 of ventral sucker, length accounting for 23–32% (28 ± 3; n = 16) of BL (Figs. 1A; 2A,B); internal seminal vesicle convoluted, S-shaped, 130–520 (306 ± 127; n = 16) long, 40–90 (68 ± 17; n = 16) wide, occupying 57–113% (86 ± 17; n = 16) of cirrus sac length (Figs. 1A,B; 2A,B); cirrus tubular, 70–160 (103 ± 24; n = 16) long, 16–56% (31 ± 11; n = 16) of cirrus sac length, 8–30 (16 ± 5; n = 16) wide (Figs. 1A; 2A,B). Genital atrium conspicuous, 43–60 (50 ± 6; n = 7) long, 28–48 (38 ± 6; n = 7) wide (Fig. 2B); pore submedian, slightly anterior to intestinal bifurcation, anterior extent at 200–320 (252 ± 37; n = 16), 18–25% (21 ± 3; n = 16) of BL from anterior body end (Figs. 1A; 2A,B).

Ovary entire, submedian, slightly wider than long, 45–120 (88 ± 22; n = 16) long, 50–150 (105 ± 29; n = 16) wide, 45–64% (55 ± 7; n =16) of anterior testis maximum width, ventrodextrally overlapping anterior testis by 24–51% (33 ± 7; n = 13) of its length; pre-ovarian space 510–860 long (689 ± 121; n = 16), 50–65% (56 ± 5; n = 16) of BL; (Figs. 1A,B; 2A,C). Oviduct pre-testicular, longitudinal (Fig. 2A). Mehlis' gland surrounding oötype. Seminal receptacle median, dorsal to anterior half of anterior testis (Figs. 1B; 2A,C). Laurer's canal curving dorsally; distal end opening between posterior margin of ventral sucker and ovary (indistinct except in dorsally- and laterally-mounted specimens) (Figs. 2A,C). Vitelline fields continuous or ventrally discontinuous at level of ventral sucker; follicles subglobular to irregularly-shaped, variously-sized, 13–90 (48 ± 18; n = 50) long, 13–63 (36 ± 12; n = 50) wide, extending from slightly anterior to or at level of genital pore to post-testicular space near posterior body end, surrounding ceca, spanning midline and dorsal to testes; pre-vitellarium space 175–300 long (235 ± 34; n = 16), 16–27% (19 ± 3; n = 16) of BL from anterior body end;

post-vitellarium space 750–1,520 long (1,172 ± 278; n = 16), 90–97% (94 ± 2; n = 16) of BL from anterior body end (Figs. 1A,B); vitelline ducts dorsal to and at level of ovary; vitelline reservoir median, dorsally overlapping anterior margin of anterior testis and seminal receptacle (Figs. 1B; 2C). Uterus wholly pre-testicular or ventrally overlapping anterior half of anterior testis (Figs. 1A,B; 2A). Metraterm sinistrolateral to cirrus sac, entering genital atrium dorsal to cirrus sac (Figs. 1A; 2B). Eggs few to numerous, 5–63 (28 ± 18; n = 14); ovoid or slightly elongate, 60–75 (67 ± 4; n = 27) long, 25–40 (32 ± 4; n = 27) wide (Figs. 1A,B; 2A,B).

Excretory vesicle short, saccular, wholly post-testicular, 58–145 (97 ± 27; n = 14) long, 6–9% (8 ± 1; n = 14) of BL, 8–30 (18 ± 7; n = 14) wide, extending anteriad to level of or slightly anterior to cecal tips (Figs. 1A,B; 2A).

3.2.2. Taxonomic summary

Type host: Northern hogsucker, *Hypentelium nigricans* (Lesueur, 1817), (Cypriniformes: Catostomidae).

Site of infection: Intestine.

Type-locality: Watauga River (36°09’23.0”N, 81°46’11.2”W), Watauga County, North Carolina, USA.

Other host and locality: White sucker, *Catostomus commersonii* (Lacepède, 1803), (Cypriniformes: Catostomidae); Pass Run (38°42’18.0”N, 78°26’43.4”W), Hawksbill Creek, South Fork Shenandoah River, Page County, Virginia, USA.

Type-specimens deposited: Holotype (USNM 1661969), 3 adult paratypes (USNM 1661970–72) from northern hogsucker and 5 adult paratypes (USNM 1661973–77) from white sucker.

Specimens studied: Sixteen stained, whole-mounted adult specimens studied and measured from northern hogsucker and white sucker.

Etymology: The species name *wataugaensis* refers to the type locality of the new species.

Representative DNA sequence: Partial ITS1, complete 5.8S, complete ITS2 region, and partial 28S rDNA gene, GenBank accession no ON059353.

Prevalence and intensity: Three of 4 (75%) northern hogsuckers were infected with 5 adults plus 10 juveniles of the new species, 3 adults, and 11 juveniles, respectively. Two of 10 (20%) white suckers were infected with 9 and 11 adults of the new species, respectively.

3.2.3. Remarks on Plagioporus wataugaensis

The new species resembles *P. serotinus*, *Plagioporus hypentelii* Hendrix, 1973, and *P. hageli* by having a fusiform body, a ventral sucker that is >1.5× wider than the oral sucker, an ovary that dextrally overlaps the anterior testis, ceca that terminate in the post-testicular space, and a vitellarium that extends anteriad to the forebody and that spans the midline of the post-testicular space [2,35,38,39]. It differs from these species by having vitelline fields that are ventrally discontinuous at the level of the ventral sucker (vs. continuous in *P. serotinus* and *P. hypentelii*); follicles that surround the ceca (vs. wholly ventral to the ceca in *P. hageli*; indeterminate for *P. serotinus* and *P. hypentelii*) and that span the midline dorsal to the testes (vs. do not span the midline dorsal to the testes; follicles slightly overlap the lateral margins of the testes). The new species also has a short (6–9% of BL), wholly post-testicular excretory vesicle (vs. extending anteriad to the level of the posterior testis; 16%, 14–16%, and 20–24% of BL in *P. serotinus*, *P. hypentelii*, and in *P. hageli*, respectively). The new species further differs from *P. serotinus* by having smaller eggs (60–75 × 25–40 vs. 70–90 × 50–60) [35]. It further differs from *P. hypentelii* by having a convoluted (S-shaped) seminal vesicle (vs. not convoluted; elongate seminal vesicle) and an ovary that ventrally overlaps the anterior testis (vs. dorsally) [39].

The new species differs from *P. boleosomi*, *Plagioporus lepomis* Dobrovolny, 1939, *P. chiliticorum*, *P. loboides*, *P. aliffi*, *P. fonti*, and *P. limus* by having vitelline follicles in the forebody (vs. restricted to the hindbody or not extending anteriad beyond the anterior margin of the ventral sucker) [3,9,15,37,40]. The new species can be distinguished from *P. siliculus* by having a cirrus sac that dorsally overlaps the ventral sucker (vs. wholly anterior to the ventral sucker), more elongate eggs (60–75 × 25–40 vs. 58 × 42), and a wholly post-testicular excretory vesicle (vs. extending anteriad to the level of the anterior testis) [7]. The new species differs from *P. cooperi*, *P. sinitsini*, *P. serratus*, *P. ictaluri*, and *P. carolini* by having ceca that terminate in the post-testicular space (vs. terminating at the level of the posterior testis) [4,30,34,35]. Further, the vitellarium of the new species distinguishes it from these congeners. The vitelline fields are ventrally discontinuous at the level of the ventral sucker (vs. continuous), and it has follicles spanning the midline dorsal to the testes (vs. slightly overlapping the lateral margins of the testes). In addition, the new species has post-testicular vitelline follicles (vs. follicles at the level of the posterior testis in *P. ictaluri* and *P. carolini*). *Plagioporus wataugaensis* can be easily distinguished from *P. sinitsini*, *P. shawi*, and *P. loboides* by having an ovary with relatively smooth margins (“entire”) (vs. slightly or deeply lobed). The new species differs from *Plagioporus macrouterinus* Haderlie, 1953 and *P. loboides* by having a compact uterine field that is between the genital pore and the anterior testis (vs. extending posteriad to the level of the posterior testis in *P. macrouterinus*; *P. loboides* has a uterus filling the post-testicular space) [37,41]. The new species further differs from *P. cooperi* by having a more elongate body (3.0–4.1× vs. 1.4–2.0× longer than wide), tandem testes (vs. diagonal), and an ovary that ventrodextrally overlaps the anterior testis (vs. not overlapping; the ovary is at the level of and nearly opposite to the anterior testis) [34]. The new species further differs from *P. shawi* by

having a cirrus sac that does not extend posteriad beyond the posterior margin of the ventral sucker (vs. extending posteriad beyond the ventral sucker and to the level of the ovary) and by having an excretory vesicle that is restricted to the post-testicular space (vs. extending anteriad beyond the testes and to the level of the ovary) [33,36]. It further differs from *P. serratus* by having a substantially larger body (approximately twice as large) (810–1,600 × 245–440 vs. 410–510 × 140–180), a smooth tegument (vs. a tegument having "spiniform papillae"), and an ovary that is approximately 1/2 the anterior testis width (vs. ~2.0× wider) [32,35]. *Plagioporus wataugaensis* differs from *P. kolipinskii* by having a smaller (63–83% of BW) ventral sucker (vs. equal to BW), an ovary that is approximately 1/2 the anterior testis width (vs. approximately equal), and a short (6–9% of BL), wholly post-testicular excretory vesicle (vs. 18–23% of BL, extending anteriad to the level of the posterior testis) [16]. We provide a diagnostic key for accepted Nearctic *Plagioporus* spp.

3.3. Sequence comparison and phylogenetic analyses

The nucleotide sequences from the 3 specimens of the new species (2 and 1 from the northern hogsucker and white sucker, respectively) were pairwise identical and 2,530–2,561 bp. The aligned ITS1, 5.8S, ITS2, and 28S sequences of *P. wataugaensis* and other *Plagioporus* spp. were 381, 156, 253, and 1,222 bp, respectively (including gaps). The ITS1 and ITS2 of the new species differed from that of *P. sinitsini* by 7 (98.1% similarity) and 2 (99.2%) nucleotides, respectively. The 5.8S of the new species was identical to that of *P. shawi* but differed from 10 other congeners by 1 or 2 nucleotides only. The 28S of the new species differed from *P. aliffi* and *P. sinitsini* by 18 (98.5%) and 21 (98.3%) nucleotides, respectively (Table 1).

The 28S phylogenetic analysis (1,226 bp, including gaps) recovered the new species sister to *P. sinitsini*. *Plagioporus* was monophyletic and sister to the clade comprising *Neoplagioporus*

(paraphyletic) + *Urorchis* (Fig. 3). *Plagioporus shawi* (infecting salmonids) was sister to all other Nearctic species of *Plagioporus*. *Plagioporus limus* (infecting a percid) was sister to a clade comprising the remaining analyzed Nearctic congeners. One clade included the new species + *P. sinitsini* (infecting catostomids and leuciscids, respectively) and *P. chiliticorum* + *P. carolini* + *P. ictaluri* (infecting leuciscids, a cottid, and an ictalurid, respectively). Another clade comprised *P. hageli* (infecting a salmonid), *P. loboides* (infecting fundulids and a leuciscid), and *P. kolipinskii* (infecting a gasterosteid). The third clade included *P. aliffi*, *P. boleosomi*, *P. fonti* (infecting percids) (Fig. 3).

The topologies of the ITS1 + 5.8S + ITS2 (826 bp, including gaps) and 28S trees were similar, except that the ITS1 + 5.8S + ITS2 analysis recovered *P. aliffi*, *P. boleosomi*, *P. fonti*, and *P. limus* (all infect percids) as monophyletic (weakly supported) and sister to a clade comprising seven Nearctic congeners (Figs. 3,4).

4. Discussion

Plagioporus is in need of a taxonomic review. No published taxonomic work on the genus exists that has inclusively treated non-Nearctic congeners. Bray et al. [1] estimated the total number of accepted species (55) but did not list them. Numerous non-Nearctic freshwater and marine species assigned to the genus have exceptional features: a tegument covered by "small tubercles" (spines?) (present in *Plagioporus anguillus* El-Naffar, 1991); suckers approximately equal in diameter (*Plagioporus dogieli* Pogorelzeva, 1975; *Plagioporus parathalassomatis* Wang, 1982; *Plagioporus varicorhini* Feng and Wang, 1997; and *Plagioporus pomacanthi* Shen and Li, 2000); an indistinct esophagus (*Plagioporus heterorchis* Bilqees, 1977); testes that are symmetrical (*P. pomacanthi*), transversely-elongated (*Plagioporus panchax* Vasandakumar and Janardanan, 2002), or lobed (*Plagioporus preporatus* Manter, 1954; *Plagioporus glomeratus*

Roĭtman, 1963; *Plagioporus polymixiae* Yamaguti, 1970; *P. heterorchis*; *Plagioporus sichuanensis* Wang and Jiang, 1985; and *P. varicorhini*); a cirrus sac that is lateral to the ventral sucker (*Plagioporus collichthydis* Wang, 1982 and *P. parathalassomatis*) and that extends posteriad beyond the ventral sucker (*P. heterorchis*, *Plagioporus epinepheli* Shen, 1985; and *P. panchax*); a genital pore that is marginal (*P. pomacanthi*), median (*Plagioporus pseudospari* Wang, 1982), submedian, dextral (*Plagioporus sinicus* Wang, 1977 and *P. anguillus*), or at the level of the oral sucker (*P. preporatus*); an ovary that is dextral to and is at the level of the ventral sucker (*Plagioporus kamalai* [Gupta, 1956] Martin, Cutmore, Ward, and Cribb, 2017) or transversely-elongated (*P. heterorchis*); a wholly extra-cecal vitellarium (*P. panchax*); a uterus that is partly inter-testicular and that extends posteriad beyond the testes (*P. pomacanthi*); and an excretory vesicle that is elongate and extends anteriad beyond the ovary (*P. pomacanthi*), extends to the level of the ventral sucker (*P. kamalai*), or that is Y-shaped (*P. anguillus*) (see Manter [42], Gupta [43], Roĭtman [44], Yamaguti [45], Solonchenko [46], Bilqees [47], Wang [48], Wang [49], Shen [50], Wang and Jiang [51], El-Naffar [52], Feng and Wang [53], Shen and Li [54], and Vasandakumar and Janardanan [55]). These species clearly do not fit the diagnosis of *Plagioporus*. Their appropriate taxonomic assignments need further investigations. Hence, we think that adding any of these features to the diagnosis of *Plagioporus* is dubious at this time, but we expect the number of accepted non-Nearctic congeners to be reduced. Presently, no nucleotide sequence is available for a non-Nearctic species of *Plagioporus*.

Our emendation of *Plagioporus* is based on the features of the accepted Nearctic species only. We emend *Plagioporus* by adding new features associated with the posterior extent of the ceca and testes position. This emendation enables inclusion of at least 4 Nearctic congeners that have already been assigned to the genus: *P. cooperi*, *P. sinitsini*, *P. ictaluri*, and *P. carolini* (see

Remarks on *Plagioporus*). These additions accommodate emendations by Cribb [5], Fayton and Andres [2], and Fayton, McAllister et al. [4]. Regarding *P. shawi*, *P. serratus* and *P. loboides* (all herein *incertae sedis*), if these species are to be reassigned to *Plagioporus*, the diagnosis must be further emended to include the following features: a papillate tegument (present in *P. serratus*), lobed testes (*P. shawi*), a cirrus sac that extends posteriad to the level of the ovary (*P. shawi*), a uterine seminal receptacle (*P. loboides*), a uterus that extends posteriad beyond testes (*P. loboides*), and a vitellarium that is restricted between the ventral sucker and the posterior testis (*P. loboides*).

We herein accept 17 Nearctic species of *Plagioporus* (see the Key). The new species is the 3rd species of *Plagioporus* that infects the northern hogsucker (after *P. sinitsini* and *P. hypentelii*) and is also the 3rd infecting the white sucker (after *P. serotinus* and *P. sinitsini*). The new species is the 4th congener reported from catostomids (after *P. serotinus*, *P. sinitsini*, and *P. hypentelii*) [30,35,38,39]. The new species resembles its congeners that infect catostomids (see Remarks) and resolves sister to *P. sinitsini* (Figs. 3,4; Table 1). However, if *P. serotinus* and *P. hypentelii* are sequenced, we expect nucleotide sequences of the new species to be most similar to those of these species. *Plagioporus sinitsini* infects the gall bladder of catostomids and leuciscids [8,14,30,56,57]. *Plagioporus serratus*, originally described as having a spinose body surface [35], is the only other Nearctic congener that infects the gall bladder. Gibson [32] re-examined the type specimens of *P. serratus* and reported that tegumental spines were absent ("*Having re-examined the type specimens of P. serratus, I now consider that there is no "cuticular" element to the "spines" of this species*"). He questioned its distinction from *P. sinitsini* because both species have a lobed ovary. Gibson [32] further treated the "*broad-based spines*" of Miller [35] as "*spiniform papillae*" and that *P. serratus* probably represents a junior subjective synonym of

P. sinitsini. McAllister, Choudhury et al. [57] and McAllister, Font et al. [58] asserted that the only morphological distinctions of *P. serratus* from *P. sinitsini* were the body size and host association, but Miller [35] obviously differentiated it from all *Plagioporus* spp. by having a small body and a spinose tegument. *Plagioporus sinitsini* and *P. serratus* were assigned to *Caudotestis* Issaitschikov, 1928 (as a subgenus of *Plagioporus*) by Yamaguti [59] because they have testes in the posterior body end; however, both were assigned to *Plagioporus* by Tracey et al. [16], Fayton, Choudhury et al. [3], and Fayton, McAllister et al. [4]. Newly-collected specimens and nucleotide sequences of *P. serratus* from the type host and type locality are needed to test if it is distinct from *P. sinitsini*.

The 2 accepted species of *Plagiocirrus* Van Cleave and Mueller, 1932 by Fayton and Andres [2] (*Plagiocirrus primus* Van Cleave and Mueller, 1932 [infecting a leuciscid] and *Plagiocirrus testeus* Fritts, 1959 [infecting a catostomid]) are morphologically distinct from *Plagioporus* spp. Cribb [5] differentiated species of *Plagiocirrus* from all opecoelids by having a restricted vitellarium but noted that they resembled *Plagioporus* spp. because these species have a short excretory vesicle and infect freshwater fishes. Fayton and Andres [2] questioned Van Cleave and Mueller's [60] morphological distinctions (i.e., uterus and vitellarium distribution) of *Plagiocirrus* from *Plagioporus* and suspected that *Plagiocirrus* should be synonymized with *Plagioporus*. Species of *Plagiocirrus* differs from those of *Plagioporus* by having a genital pore that is at the level of, or immediately posterior to, the pharynx (vs. at the level of, or slightly anterior to, the intestinal bifurcation), testes that are subspheroid or lobed (vs. subspheroid), an ovary that is entire and wholly pre-testicular (vs. entire or lobed, immediately pre-testicular or nearly opposite or overlapping the anterior testis), a uterus that extends posteriad far in the post-testicular space (vs. a compact uterus that occupies space between the genital pore and the

posterior testis), a restricted vitellarium that occupies the space between the ventral sucker and the anterior testis (vs. extending anteriad into the forebody or confined to the hindbody posteriad to the level of or beyond the testes), and an excretory vesicle that is saccular and post-testicular (vs. saccular or elongate and is restricted to the post-testicular space or extends anteriad to the level of the ovary). To date, no published phylogenetic study has included a sequence of either species of *Plagiocirrus*.

Acknowledgements

We thank Kinnon Hodges, Thomas Johnson, Kennith Lingerfelt, and Dylan Owensby (North Carolina Wildlife Resources Commission), Brian Mullin, Claire Curtin, and Cheyenne Pommelle (State University of New York College at Oneonta) for helping with the fish collections as well as Haley Dutton, Steven Ksepka, Micah Warren, and Justin Krol (Aquatic Parasitology Laboratory, Auburn University) for helping collect parasites and conducting fish necropsies. This study was supported by research grants from the Southeastern Cooperative Fish Parasite and Disease Project (Alabama Department of Conservation and Natural Resources), Alabama Agriculture Experiment Station, Auburn University Vice President for Research and Economic Development, North Carolina Wildlife Resources Commission, State University of New York College at Oneonta Research Foundation, and US Department of Agriculture.

References

[1] R.A. Bray, T.H. Cribb, D.T.J. Littlewood, A. Waeschenbach, 2016, The molecular phylogeny of the digenean family Opecoelidae Ozaki, 1925 and the value of morphological characters, with the erection of a new subfamily, Folia Parasitol. 63, 013. https://doi.org/10.14411/fp.2016.013.

[2] T.J. Fayton, M.J. Andres, New species of *Plagioporus* Stafford, 1904 (Digenea: Opecoelidae) from California, with an amendment of the genus and a phylogeny of freshwater plagioporines of the Holarctic, Syst. Parasitol. 93 (8) (2016) 731–748, https://doi.org/10.1007/s11230-016-9664-6.

[3] T.J. Fayton, A. Choudhury, C.T. McAllister, H.W. Robison, Three new species of *Plagioporus* Stafford, 1904 from darters (Perciformes: Percidae), with a redescription of *Plagioporus boleosomi* (Pearse, 1924) Peters, 1957, Syst. Parasitol. 94 (2) (2017) 159–182, https://doi.org/10.1007/s11230-016-9697-x.

[4] T.J. Fayton, C.T. McAllister, H.W. Robison, M.B. Connior, Two new species of *Plagioporus* (Digenea: Opecoelidae) from the Ouachita madtom, *Noturus lachneri*, and the banded sculpin, *Cottus carolinae*, from Arkansas, J. Parasitol. 104 (2) (2018) 145–156, https://doi.org/10.1645/16-114.

[5] T.H. Cribb, Family Opecoelidae Ozaki, 1925, in: A. Jones, R.A. Bray, D.I. Gibson (Eds.), Keys to the Trematoda, 2, CAB International and Natural History Museum, Cambridge, U.K., 2005, pp. 443–531.

[6] S.B. Martin, S.C. Cutmore, T.H. Cribb, Revision of *Neolebouria* Gibson, 1976 (Digenea: Opecoelidae), with *Trilobovarium* n. g., for species infecting tropical and subtropical

shallow-water fishes, Syst. Parasitol. 94 (3) (2017) 307–338, https://doi.org/10.1007/s11230-017-9707-7.

[7] D. Sinitsin, Studien über die phylogenie der trematoden. IV. The life histories of *Plagioporus siliculus* and *Plagioporus virens*, with special reference to the origin of the Digenea, Z. Wiss. Zool. 138 (1931) 409–456.

[8] C.G. Dobrovolny, Life history of *Plagioporus sinitsini* Mueller and embryology of new cotylocercous cercariae (Trematoda), Trans. Am. Micros. Soc. 58 (2) (1939) 121–155, https://doi.org/10.2307/3222930.

[9] C.G. Dobrovolny, The life history of *Plagioporus lepomis*, a new trematode from fishes, J. Parasitol. 25 (6) (1939) 461–470, https://doi.org/10.2307/3272330.

[10] S.C. Schell, The life history of *Plagioporus shawi* (McIntosh 1939) (Trematoda: Opecoelidae), an intestinal parasite of salmonid fishes, J. Parasitol. 61 (5) (1975) 899–905, https://doi.org/10.2307/3279231.

[11] S.S. Hendrix, The life history and biology of *Plagioporus hypentelii* Hendrix, 1973 (Trematoda: Opecoelidae), J. Parasitol. 64 (4) (1978) 606–612, https://doi.org/10.2307/3279943.

[12] M.A. Barger, G.W. Esch, *Plagioporus sinitsini* (Digenea: Opecoelidae): a one-host life cycle, J. Parasitol., 86 (1) (2000), 150–153, https://doi.org/10.2307/3284926.

[13] S.B. Martin, A.J. Downie, T.H. Cribb, A new subfamily for a clade of opecoelids (Trematoda: Digenea) exploiting marine fishes as second-intermediate hosts, with the first report of opecoelid metacercariae from an elasmobranch, Zool. J. Linn. Soc. 188 (2) (2020) 455–472, https://doi.org/10.1093/zoolinnean/zlz084.

[14] J.H. Fischthal, Parasites of northwest Wisconsin fishes. I. The 1944 survey, Trans. Wis. Acad. Sci. Arts Lett. 37 (1945) 157–220.

[15] M.A Barger, G.W. Esch, *Allopodocotyle chiliticorum* n. sp. (Digenea: Opecoelidae) from redlip shiners, *Notropis chiliticus*, in Basin Creek, North Carolina, J. Parasitol. 85 (5) (1999) 891–892, https://doi.org/10.2307/3285827.

[16] J.K. Tracey, A. Choudhury, J.M. Cheng, S. Ghosh, A new opecoelid species (Trematoda: Opecoelidae) from the threespine stickleback *Gasterosteus aculeatus* L. in California, J. Parasitol. 95 (5) (2009) 1177–1182, https://doi.org/10.1645/GE-1999.1.

[17] R. Fricke, W.N. Eschmeyer, R. Van der Laan (Eds.), Eschmeyer's catalog of fishes: genera, species, references, 2021. http://researcharchive.calacademy.org/research/ichthyology/catalog/fishcatmain.asp (Accessed 19 April 2021).

[18] H.W. Manter, in K.S. Singh, and B.K. Tandan (Eds.), The terminology and occurrence of certain structures of digenetic trematodes, with special reference to the Hemiuroidea, H.D. Srivastava Commen., Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India, 1970, 27–33, https://digitalcommons.unl.edu/parasitologyfacpubs/593.

[19] A. Keller, T. Schleicher, J. Schultz, T. Müller, T. Dandekar, M. Wolf, 5.8S-28S rRNA interaction and HMM-based ITS2 annotation, Gene 430 (1–2) (2009) 50–57, https://doi.org/10.1016/j.gene.2008.10.012.

[20] O. Jousson, P. Bartoli, J. Pawlowski, Molecular identification of developmental stages in Opecoelidae (Digenea), Int. J. Parasitol. 29 (11) (1999) 1853–1858, https://doi.org/10.1016/S0020-7519(99)00124-1.

[21] M.J. Andres, C.L. Ray, E.E. Pulis, S.S. Curran, R.M. Overstreet, Molecular characterization of two opecoelid trematodes from fishes in the Gulf of Mexico, with a description of a new species of *Helicometra*, Acta Parasitol. 59 (3) (2014) 405–412, https://doi.org/10.2478/s11686-014-0258-7.

[22] S.B. Martin, D.C. Huston, S.C. Cutmore, T.H. Cribb, A new classification for deep-sea opecoelid trematodes based on the phylogenetic position of some unusual taxa from shallow-water, herbivorous fishes off south-west Australia, Zool. J. Linn. Soc. 186 (2) (2019) 385–413, https://doi.org/10.1093/zoolinnean/zly081.

[23] S.G. Sokolov, S.V. Shchenkov, I.I. Gordeev, Records of opecoeline species *Pseudopecoelus* cf. *vulgaris* and *Anomalotrema koiae* Gibson & Bray, 1984 (Trematoda, Opecoelidae, Opecoelinae) from fish of the North Pacific, with notes on the phylogeny of the family Opecoelidae, J. Helminthol. 93 (4) (2019) 475–485, https://doi.org/10.1017/S0022149X18000573.

[24] K. Katoh, D.M. Standley, MAFFT Multiple sequence alignment software version 7: Improvements in performance and usability, Mol. Biol. Evol. 30 (4) (2013) 772–780, https://doi.org/10.1093/molbev/mst010.

[25] D. Darriba, G.L. Taboada, R. Doallo, D. Posada, jModelTest 2: More models, new heuristics, and parallel computing, Nat. Methods 9 (2012), 772, https://doi.org/10.1038/nmeth.2109.

[26] D. Glez-Peña, D. Gómez-Blanco, M. Reboiro-Jato, F. Fdez-Riverola, D. Posada, ALTER: program-oriented conversion of DNA and protein alignments, Nucleic Acids Res. 38 (suppl. 2) (2010) W14–W18, https://doi.org/10.1093/nar/gkq321.

[27] F. Ronquist, J.P. Huelsenbeck, MrBayes 3: Bayesian phylogenetic inference under mixed models, Bioinformation 19 (12) (2003) 1572–1574, https://doi.org/10.1093/bioinformatics/btg180.

[28] A. Rambaut, A.J. Drummond, D. Xie, G. Baele, M.A. Suchard, Posterior summarisation in Bayesian phylogenetics using Tracer 1.7, Syst. Biol. 67 (5) (2018) 901–904, https://doi.org/10.1093/sysbio/syy032.

[29] A. Rambaut, M.A. Suchard, D. Xie, A.J. Drummond, FigTree v1.4.4, 2014, http://tree.bio.ed.ac.uk/software/figtree.

[30] J.F. Mueller, Two new trematodes from Oneida Lake fishes, Trans. Am. Microsc. Soc. 53 (3) (1934) 231–236, https://doi.org/10.2307/3222099.

[31] D.I. Gibson, R.A. Bray, A study and reorganization of *Plagioporus* Stafford, 1904 (Digenea: Opecoelidae) and related genera, with special reference to forms from European Atlantic waters, J. Nat. Hist. 16 (4) (1982) 529–559, https://doi.org/10.1080/00222938200770431.

[32] D.I. Gibson, Trematoda, in: L. Margolis, Z. Kabata (Eds.), Guide to the parasites of fishes of Canada. Part IV, Can. Spec. Publ. Fish. Aquat. Sci. 124, NRC Research Press, Ottawa, Canada, 1996, 1–373.

[33] A. McIntosh, A new allocreadiid trematode *Podocotyle shawi*, n. sp., from the silver salmon, J. Wash. Acad. Sci. 29 (9) (1939) 379–381.

[34] G.W. Hunter, R.V. Bangham, Studies on fish parasites of Lake Erie. I. New trematodes (Allocreadiidae), Trans. Am. Microsc. Soc. 51 (2) (1932) 137–152, https://doi.org/10.2307/3222429.

[35] M.J. Miller, Parasites of freshwater fish. III. Further studies on the internal trematodes of fish in the central St. Lawrence watershed, Can. J. Res. 18D (12) (1940) 423–434, https://doi.org/10.1139/cjr40d-038.

[36] L. Margolis, The trematode *Plagioporus shawi* (McIntosh, 1939) n. comb. (Opecoelidae: Plagioporinae) from the North Pacific sockeye salmon, *Oncorhynchus nerka*, An. Inst. Biol. Univ. Nac. Auton. Mex., Ser. Zool. 41 (1) (1970) 99–108.

[37] S.S. Curran, R.M. Overstreet, V.V. Tkach, Phylogenetic affinities of *Plagiocirrus* Van Cleave and Mueller, 1932 with the description of a new species from the Pascagoula River, Mississippi, J. Parasitol. 93 (6) (2007) 1452–1458, https://doi.org/10.1645/GE-1207.1.

[38] J. Stafford, Trematodes from Canadian fishes, Zool. Anz. 27 (16–17) (1904) 481–495.

[39] S.S. Hendrix, *Plagioporus hypentelii* sp. n. (Trematoda: Opecoelidae) from the hogsucker, *Hypentelium nigricans* (LeSueur) (Osteichthys: Catostomidae), Proc. Helminthol. Soc. Wash. 40 (1973) 144–146.

[40] A.S. Pearse, Observations on parasitic worms from Wisconsin fishes, Trans. Wis. Acad. Sci. Arts Lett. 21 (1924) 147–160.

[41] E.C. Haderlie, Parasites of the fresh-water fishes of northern California, Univ. Calif. Publ. Zool. 57 (5) (1953) 303–440.

[42] H.W. Manter, Some digenetic trematodes from fishes of New Zealand, Trans. Roy. Soc. New Zealand 82 (1954) 475–568.

[43] S.P. Gupta, Two new trematodes of the family Allocreadiidae from the fresh-water fishes of U.P., Indian J. Helminthol. 8 (1956) 100–106.

[44] V.A. Roĭtman, New species of trematodes of fishes of the Amur basin, Tr. Gelmintol. Lab. 8 (1963) 303–312.

[45] S. Yamaguti, Digenetic trematodes of Hawaiian fishes, Keigaku Publishing, Tokyo, 1970.

[46] A.I. Solonchenko, Family Opecoelidae Ozaki, 1926. in: V.N. Greze, S.L. Delyamure, V.M. Nikolaeva (Eds.), Key to the parasites of vertebrates in the Black and Azov Seas. Parasitic invertebrates of fish, fish-eating birds and marine mammals, Naukova Dumka, Kiev., 1975, pp. 126–133.

[47] F.M. Bilqees, *Plagioporus heterorchis* sp. n. (Trematoda: Opecoelidae) from the fish *Pomadasys olivaceus* (Day) of Karachi Coast, Pak. J. Sci. Ind. Res. 20 (1977) 258–260.

[48] P. Wang, A survey of the parasites of eels from Fujian Province, J. Fujian Teachers Univ. Nat. Sci. 1977 (1), 53–63.

[49] P. Wang, Some digenetic trematodes of marine fishes from Fujian Province, China. Oceanol. Limnol. Sin. 13 (1982) 179–194.

[50] J. Shen, Digenetic trematodes of fishes from the Xisha Islands, II, Stud. Mar. Sin. 24 (1985) 167–180.

[51] P. Wang, X. Jiang, Studies on the helminth fauna of Sichuan, Part 1. Trematodes of fishes, Sichuan Dong Wu 4 (2) (1985) 1–7.

[52] M.K. El-Naffar, On two new species of trematodes from the Nile fishes at Assiut city, A, R, Egypt. Bull. Fac. Sci., Assiut Univ. 20 (2-E) (1991) 291–299.

[53] W. Feng, W. Wang, New digenetic trematodes of fishes from Wuling Mountains area, in: D. Song (Eds.), Invertebrates of Wuling Mountains Area, south-western China, Serial Publications of Biosciences Survey, Science Press, Beijing, 1997, pp. 223–234.

[54] J. Shen, X. Li, Addendum to the parasitic digenetic trematodes from marine fishes in Hainan Island, Stud. Mar. Sin. 42 (2000) 153–162.

[55] M.V. Vasandakumar, K.P. Janardanan, The life cycle of *Plagioporus panchax* sp. nov. (Digenea: Opecoelidae) from freshwater fish in Kerala, India, Riv. Parassitol. 19 (3) (2002) 197–204.

[56] D.D. Williams, M.J. Ulmer, Host and locality records for *Plagioporus sinitsini* (Trematoda: Opecoelidae), Proc. Helminthol. Soc. Wash. 47 (2) (1980) 263.

[57] C.T. McAllister, A. Choudhury, T.J. Fayton, D.G. Cloutman, C.R. Bursey, H.W. Robison, C.M. Whipps, T. Scholz, Parasites of Western Creek chubsucker *Erimyzon claviformis* (Cypriniformes: Catostomidae) from Arkansas and Oklahoma, U.S.A., Comp. Parasitol. 88 (1) (2021) 113–119, https://doi.org/10.1654/COPA-D-20-00013.

[58] C.T. McAllister, W.F. Font, M.B. Connior, H.W. Robison, T.J. Fayton, N.G. Stokes, C.D. Criscione, Trematode parasites (Digenea) of the slender madtom *Noturus exilis* and Black River madtom *Noturus maydeni* (Siluriformes: Ictaluridae) from Arkansas, U.S.A., Comp. Parasitol. 82 (1) (2015) 137–143, https://doi.org/10.1654/4714.1.

[59] S. Yamaguti, Systema helminthum, Vol. I. The digenetic trematodes of vertebrates, Interscience Publishers, Inc., New York, 1958.

[60] H.J. Van Cleave, J.F. Mueller, Parasites of Oneida Lake fishes, Part I. Descriptions of new genera and new species, Roosevelt Wild Life Ann. 3 (1) (1932) 5–71.

Figs. 1. A–B. *Plagioporus wataugaensis* n. sp. infecting the intestine of *Hypentelium nigricans* (Lesueur, 1817), (Cypriniformes: Catostomidae) in North Carolina, USA. A. Wholemount of holotype (USNM 1661969) (ventral view) showing morphological and anatomical features. B. Wholemount of paratype (USNM 1661970) (dorsal view) showing the same features. *Abbreviations*: os, oral sucker; pph, pre-pharyngeal esophagus; ph, pharynx; eso, esophagus; gp,

genital pore; met, metraterm; cir, cirrus; cs, cirrus sac; sv, S-shaped internal seminal vesicle; vf, vitelline follicles; vs, ventral sucker; ce, ceca; eg, egg; ov, ovary; ant, anterior testis; pot, posterior testis; exv, excretory vesicle; epo, excretory pore; vd, vitelline duct; vr, vitellarium reservoir; sr, seminal receptacle.

Figs. 2. A–C. *Plagioporus wataugaensis* n. sp. infecting the intestine of *Hypentelium nigricans* (Lesueur, 1817), (Cypriniformes: Catostomidae) in North Carolina, USA. A. Wholemount of a paratype (USNM 1661972) (lateral view) showing relative positions of the testes, terminal male genitalia and ovary complex, and excretory system; vitellarium is not illustrated for clarity. B. Terminal male genitalia of a paratype (USNM 1661971) (ventral view) in higher magnification. C. Ovary complex of a paratype (USNM 1661972) (lateral view). *Abbreviations*: os, oral sucker; ph, pharynx; eso, esophagus; ce, ceca; vs, ventral sucker; gp, genital pore; cir, cirrus; cs, cirrus sac; sv, S-shaped internal seminal vesicle; vde, vas deferens; vef, vasa efferentia; eg, egg; lc, Laurer's canal; sr, seminal receptacle; ov, ovary; ant, anterior testis; pot, posterior testis; exv, excretory vesicle; epo, excretory pore; ga, genital atrium; met, metraterm; ut, uterus; mg, Mehlis' gland; vd, vitelline duct; vr, vitellarium reservoir; vf, vitelline follicles.

Fig. 3. Phylogenetic relationships of *Plagioporus* spp. based on partial 28S rDNA sequences. Values aside nodes are posterior probability. Scale bar is in substitutions per site.

Fig. 4. Phylogenetic relationships of *Plagioporus* spp. based on partial ITS1, complete 5.8S and complete ITS2 sequences. Values aside nodes are posterior probability. Scale bar is in substitutions per site.

Key to Nearctic *Plagioporus* spp.

1a Vitellarium not extending into forebody …….……..... 2

1b Vitellarium extending into forebody …….……...……. 7

2a Vitelline follicles not spanning midline posteriorly ….…….....*P. chiliticorum*

2b Vitelline follicles spanning midline posteriorly ….……...……...……...…..... 3

3a Vitellarium wholly ventral to ceca ….……...……. *P. lepomis*

3b Vitellarium surrounding ceca ….……...…..... 4

4a Testes diagonal; excretory vesicle extending anteriad to level of anterior testis …….. *P. limus*

4b Testes tandem; excretory vesicle extending anteriad to level of posterior testis …….. 5

5a Body >5.0× longer than wide; testes width <45% of max BW ……...……... *P. aliffi*

5b Body 3.6–4.6× longer than wide; testes width >50% of max BW ……...…….……... 6

6a Ventral sucker subequal in length and width, width 81–88% of max BW; metraterm thickly muscular at distal end ……...…….……... *P. boleosomi*

6b Ventral sucker wider than long, width 65–76% of max BW; metraterm thinly muscular at distal end …….…….. *P. fonti*

7a Vitelline follicles not spanning midline posteriorly; testes in the posterior body end …….. 8

7b Vitelline follicles spanning midline posteriorly; testes well-separated from posterior body end ……...……...……...…..…….. 10

8a Posterior testis width <1/3 of max BW; seminal vesicle straight (not convoluted) ... *P. sinitsini*

8b Posterior testis width 46–67% of max BW; seminal vesicle convoluted (S-shaped) 9

9a Ventral sucker width 53–71% of max BW; ovary pre-testicular or overlapping anterior margin of anterior testis …….……... *P. ictaluri*

9b Ventral sucker width 80–92% of max BW; ovary at level of anterior testis …… *P. carolini*

10a Uterus extending posteriad to posterior testis ……..…… *P. macrouterinus*

10b Uterus extending posteriad not further than level of anterior testis ……..……..…. 11

11a. Ventral sucker width equal to max BW ……....…..…… *P. kolipinskii*

11b. Ventral sucker width remarkably smaller than max BW ……..…….....…….……... 12

12a. Cirrus sac pre-acetabular; excretory vesicle extending anteriad to level of anterior testis

..…..……..……..…….. *P. siliculus*

12b. Cirrus sac overlapping dorsally ventral sucker; excretory vesicle not extending anteriad further than level of posterior testis ……...……... 13

13a. Vitelline fields ventrally discontinuous at level of ventral sucker ……...……...…… 14

13b. Vitelline fields continuous at level of ventral sucker ……...…..……..... 15

14a. Vitellarium wholly ventral to ceca; excretory vesicle extending anteriad to level of posterior testis, length 20–24% of BL ……..... *P. hageli*

14b. Vitellarium surrounding ceca; excretory vesicle post-testicular, length 6–9% of BL

……..... *P. wataugaensis*

15a. Ceca terminating at level of testes; testes diagonal; ovary at level of and opposite to anterior testis …….. *P. cooperi*

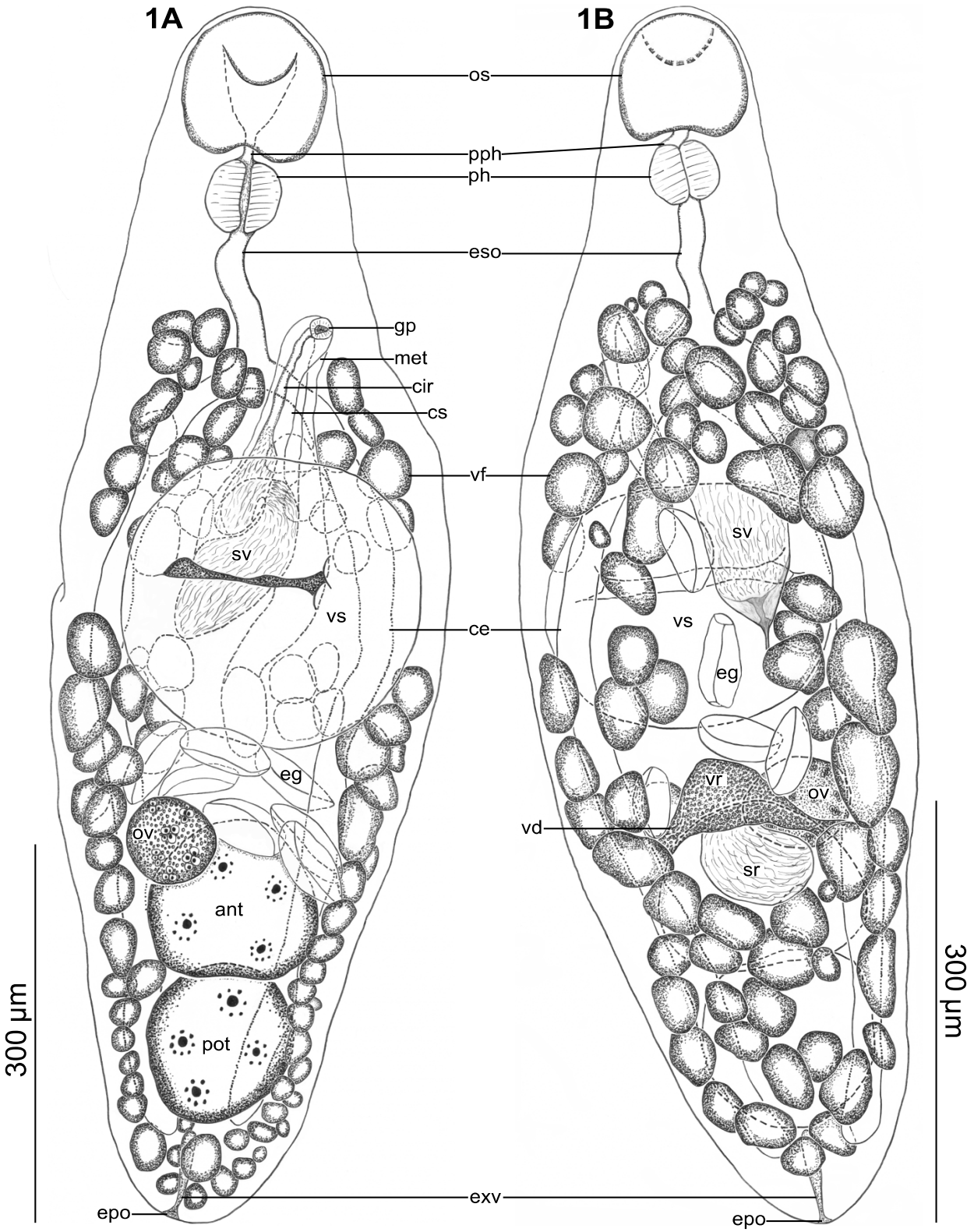
15b. Cecal terminating in post-testicular space; testes tandem; ovary pre-testicular or overlapping anterior testis ……. 16

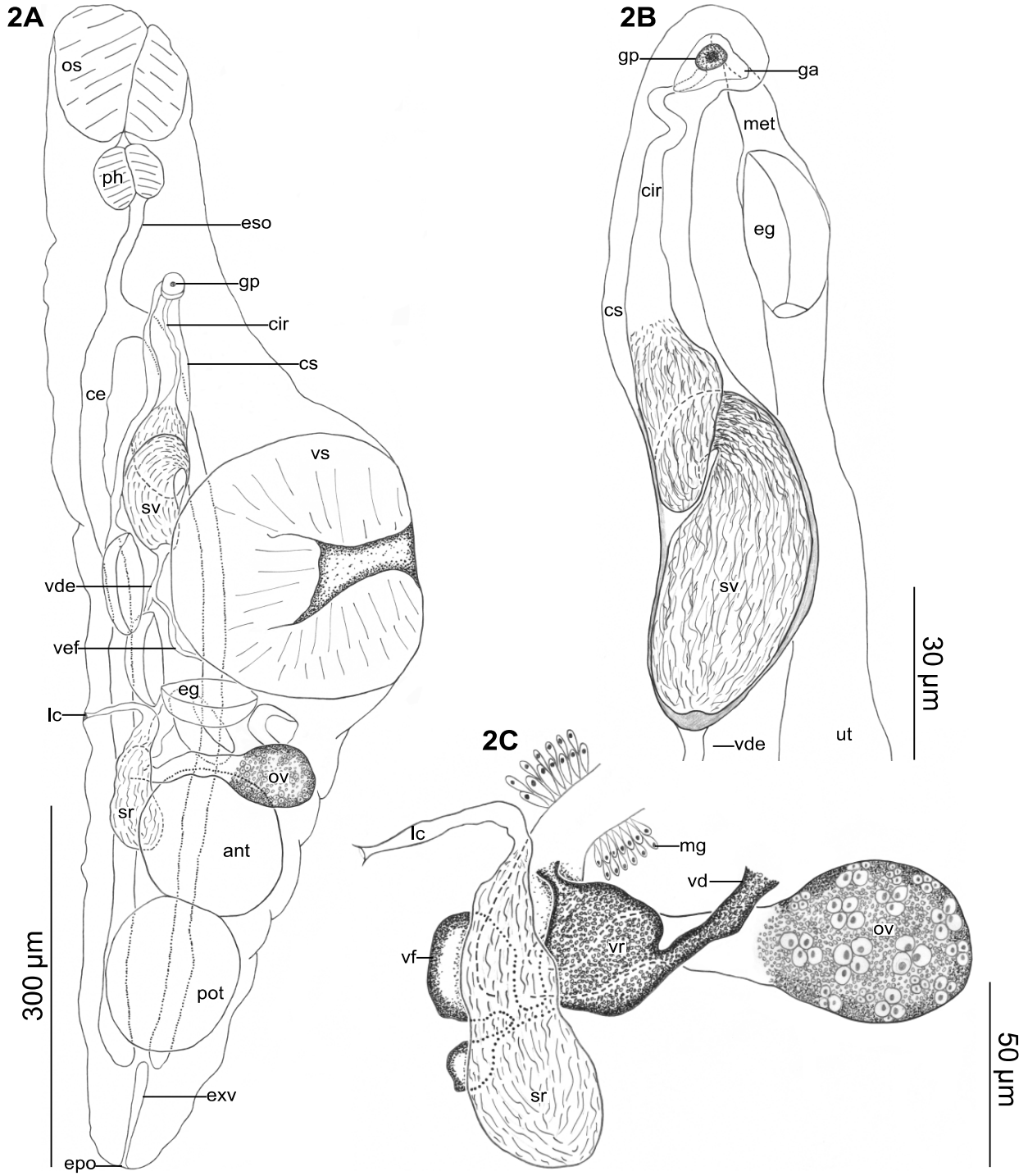
16a. Anterior testis width 63–79% of max BW; ovary width 38–45% of max BW; eggs 45–71 µm long × 26–41 µm wide ……..…..……..…… *P. hypentelii*

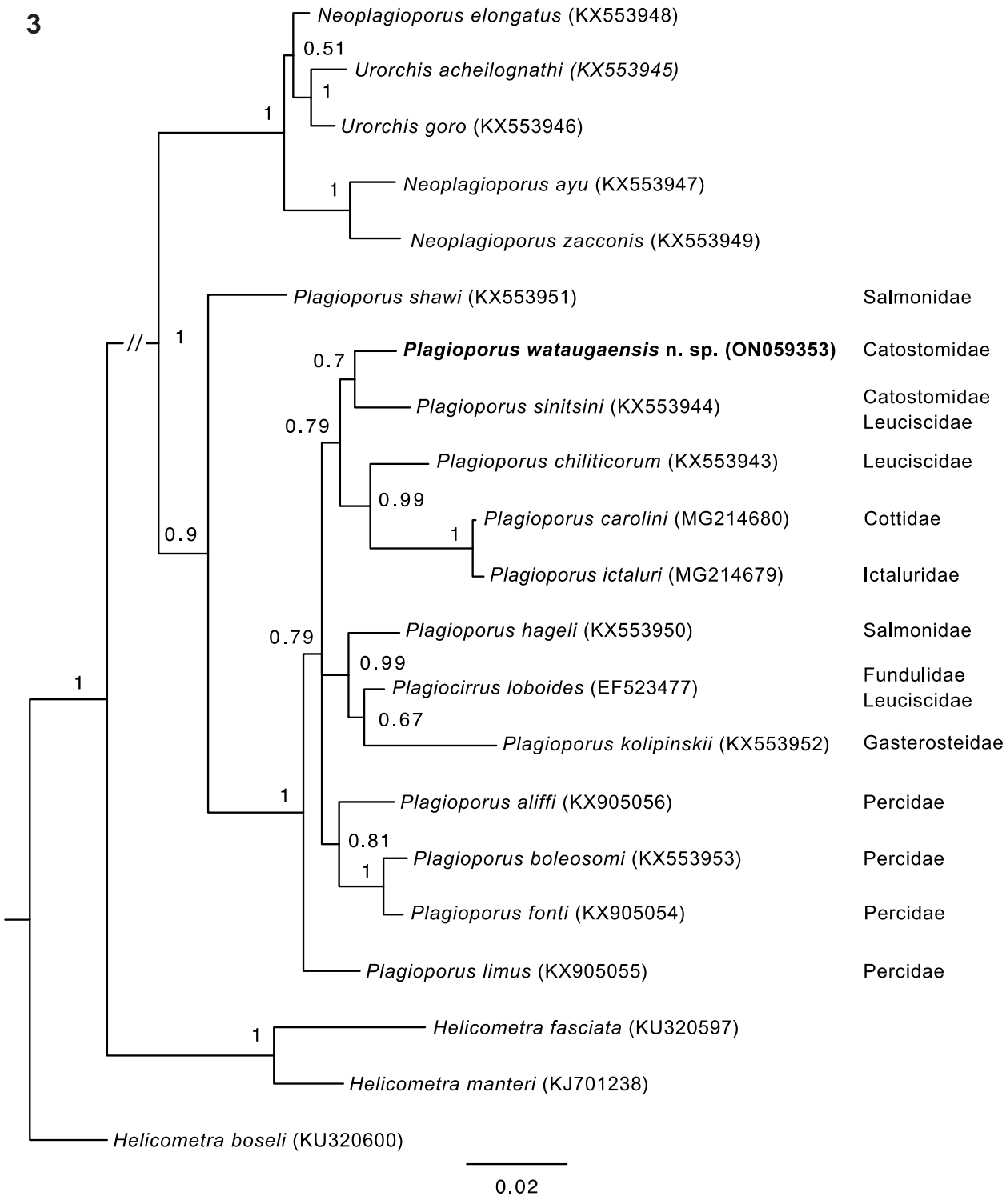
16b. Anterior testis width 52–57% of max BW; ovary width ~26% of max BW; eggs 70–90 µm long × 50–60 µm wide ……...…… *P. serotinus*

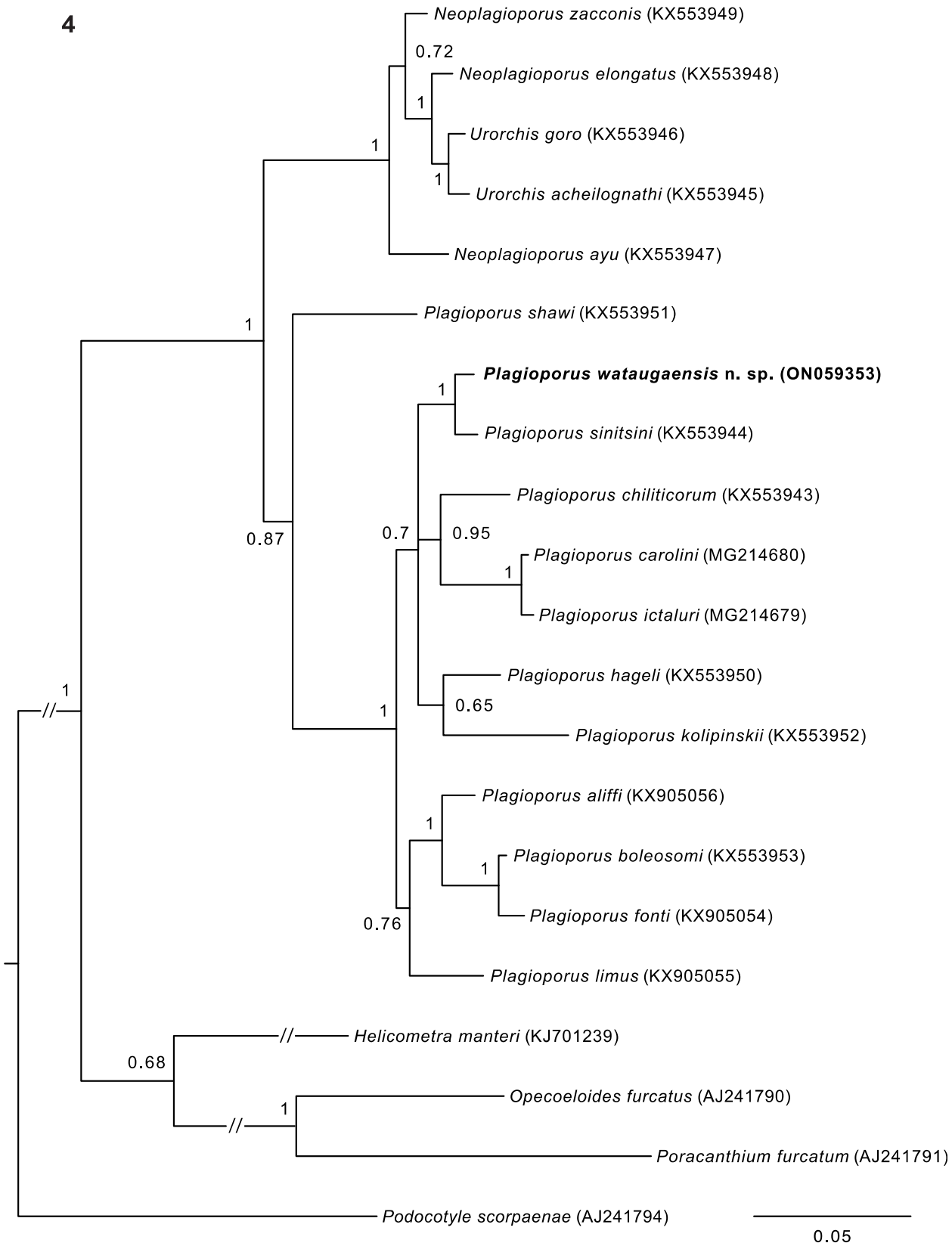
Table 1.
Pairwise comparisons of ITS1, 5.8S, ITS2, and 28S rDNA sequences of the new species, 10 Nearctic *Plagioporus* spp., *P. shawi*, and *P. loboides* (reported as percent nucleotide identity followed by the number of nucleotide differences in parentheses) from NCBI GenBank (NA: not available).

Species	*P. aliffi*	*P. boleosomi*	*P. carolini*	*P. chiliticorum*	*P. fonti*	*P. hageli*	*P. ictaluri*	*P. kolipinskii*	*P. limus*	*P. shawi*	*P. sinitsini*	*P. loboides*
ITS1												
Plagioporus	93.0	92.2	94.4	94.7	92.5	93.3	94.4	91.7	94.9	87.6	98.1	NA
wataugaensis n. sp.	(26)	(29)	(21)	(20)	(28)	(25)	(21)	(31)	(19)	(47)	(7)	
5.8S												
Plagioporus	98.7	98.7	98.7	99.4	98.7	98.7	98.7	99.4	98.7	100	99.4	NA
wataugaensis n. sp.	(2)	(2)	(2)	(1)	(2)	(2)	(2)	(1)	(2)	(0)	(1)	
ITS2												
Plagioporus	96.8	96.8	96.0	95.2	96.4	96.8	95.6	93.7	95.2	84.8	99.2	NA
wataugaensis n. sp.	(8)	(8)	(10)	(12)	(9)	(8)	(11)	(16)	(12)	(38)	(2)	
28S												
Plagioporus	98.5	97.6	96.5	97.5	97.7	97.6	96.3	95.9	97.6	96.1	98.3	97.9
wataugaensis n. sp.	(18)	(29)	(43)	(31)	(28)	(29)	(45)	(50)	(29)	(48)	(21)	(26)


1A
1B
os
pph
ph
eso
gp
met
cir
cs
vf
sv
vs
ce
eg
ov
ant
pot
epo
exv
vr
vd
sr
300 µm
300 µm


2A
os
ph
eso
gp
cir
cs
ce
vs
sv
vde
vef
eg
lc
ov
sr
ant
pot
exv
epo
300 µm
2B
gp
ga
met
cir
eg
cs
sv
vde
ut
30 µm
2C
lc
mg
vd
ov
vf
vr
sr
50 µm


3
Neoplagioporus elongatus (KX553948)
0.51
Urorchis acheilognathi (KX553945)
1
1
Urorchis goro (KX553946)
1
Neoplagioporus ayu (KX553947)
Neoplagioporus zacconis (KX553949)
Plagioporus shawi (KX553951)
Salmonidae
//
1
0.7
Plagioporus wataugaensis n. sp. (ON059353)
Catostomidae
Plagioporus sinitsini (KX553944)
Catostomidae
Leuciscidae
0.79
Plagioporus chiliticorum (KX553943)
Leuciscidae
0.99
Plagioporus carolini (MG214680)
Cottidae
0.9
1
Plagioporus ictaluri (MG214679)
Ictaluridae
0.79
Plagioporus hageli (KX553950)
Salmonidae
0.99
1
Plagiocirrus loboides (EF523477)
Fundulidae
Leuciscidae
0.67
Plagioporus kolipinskii (KX553952)
Gasterosteidae
1
Plagioporus aliffi (KX905056)
Percidae
0.81
Plagioporus boleosomi (KX553953)
Percidae
1
Plagioporus fonti (KX905054)
Percidae
Plagioporus limus (KX905055)
Percidae
1
Helicometra fasciata (KU320597)
Helicometra manteri (KJ701238)
Helicometra boseli (KU320600)
0.02


4
Neoplagioporus zacconis (KX553949)
0.72
Neoplagioporus elongatus (KX553948)
1
Urorchis goro (KX553946)
1
1
Urorchis acheilognathi (KX553945)
Neoplagioporus ayu (KX553947)
Plagioporus shawi (KX553951)
1
Plagioporus wataugaensis n. sp. (ON059353)
1
Plagioporus sinitsini (KX553944)
Plagioporus chiliticorum (KX553943)
0.87
0.7
0.95
Plagioporus carolini (MG214680)
1
Plagioporus ictaluri (MG214679)
Plagioporus hageli (KX553950)
1
//
1
0.65
Plagioporus kolipinskii (KX553952)
Plagioporus aliffi (KX905056)
1
Plagioporus boleosomi (KX553953)
1
0.76
Plagioporus fonti (KX905054)
Plagioporus limus (KX905055)
//
Helicometra manteri (KJ701239)
0.68
Opecoeloides furcatus (AJ241790)
1
//
Poracanthium furcatum (AJ241791)
Podocotyle scorpaenae (AJ241794)
0.05

CHAPTER 5: DESCRIPTION, LIFE CYCLE, AND PHYLOGENETICS OF *PROTEROMETRA WIGGLEWOMBLE* N. SP. (DIGENEA: AZYGIIDAE) FROM THE CAHABA RIVER, ALABAMA, U.S.A.

***In review, Systematic Parasitology (Submitted 17 May 2023)**

Authors: Triet N. Truong, Nathan V. Whelan, Paul D. Johnson, Michael L. Buntin, and Stephen A. Bullard

Abstract

We herein describe *Proterometra wigglewomble* n. sp. (Digenea: Azygiidae: Azygiinae) from the Cahaba River, Alabama, USA, which asexually reproduces in the compact elimia, *Elimia showalteri* (Lea, 1860) (Cerithioidea: Pleuroceridae) and matures in the oesophagus of the blackbanded darter, *Percina nigrofasciata* (Agassiz, 1854) (Perciformes: Percidae). Adults of the new species differ from congeners by having a small body and eggs having a wholly fimbriated surface that appears as a cilia-like brush border. Live naturally-shed cercariae of the new species differ from those of its congeners by having a strongly claviform tail stem bearing aspinose mammillae, a single furca, excretory pores that open on the posterior margin of the single furca, and few eggs in the cercarial distome. The behaviour of the cercaria further differentiates the new species. Naturally-shed cercariae of *P. wigglewomble* secrete a jelly-like adhesive that coats the surface of the furca and evidently facilitates attachment to the surface of glass, plastic, and snail shell. Attached cercariae vigorously wiggle and thrash about once attached, as if mimicking the larva of a stream insect so as to lure the blackbanded darter to eat it. Phylogenetic analyses recovered monophyletic Azygiidae, comprising monophyletic Leuceruthrinae Goldberger, 1911 and polyphyletic Azygiinae Lühe, 1909. The present study is the largest taxon sampling for Azygiidae and the first to include *28S* sequences of *Leuceruthrus*.

Compact elimia and blackbanded darter are new host records for *Proterometra*. The new species is the 3rd congener reported from the Cahaba River, a region renowned for its fish and snail endemic biodiversity.

Introduction

Azygiidae Lühe, 1909 comprises Azygiinae Lühe, 1909 for species with post-ovarian testes and Leuceruthrinae Goldberger, 1911 for species with pre-ovarian testes (Gibson, 2002; Womble & Bullard, 2022). Species of *Proterometra* Horsfall, 1933 (Digenea: Azygiidae: Azygiinae), the focus of the present study, are unique within the family by having gonads that cluster near the posterior body end, wholly or primarily post-ovarian testes (testes occasionally overlapping posterior margin of the ovary in some species), and a uterus and a vitellarium that extend anteriad well into the forebody (Womble et al., 2015; 2016a, b; Womble and Bullard, 2022). Womble et al. (2015; 2016a, b) accepted 12 species of *Proterometra*, all of which range in North America. All *Proterometra* spp. are generally considered as being progenetic (cercariae having eggs): ten species have been confirmed as progenetic (Womble et al., 2015, 2016b; Womble & Bullard, 2022), whereas *Proterometra melanophora* (Smith, 1932) Smith 1936 and *Proterometra hodgesiana* (Smith, 1932) Smith, 1936 are indeterminate as progenetic. Womble et al. (2015) regarded *Proterometra macrostoma* (Faust, 1918) Horsfall, 1933 (type species), *P. melanophora*, and *P. hodgesiana* as *species inquirendae*, and Womble et al. (2015, 2016b) treated the four species from China (*Proterometra guangzhouensis* Lu, 1992, *Proterometra sillagae* Wu, Lu & Zhu, 1997, *Proterometra brachyuran* Wu, Lu & Zhu, 1997, and *Proterometra lamellorchis* Wu, Lu & Zhu, 1997) as *incertae sedis*.

The life cycles of *Proterometra* spp. comprise a first molluscan intermediate host and a fish definitive host (no encysted metacercaria is known) (Womble et al., 2015; Womble et al., 2016a;

b; Womble & Bullard, 2022). *Proterometra dickermani* Anderson, 1962 is progenetic and exceptional in that no wild definitive fish host has been identified for this azygiid (Anderson & Anderson, 1963; Uglem et al., 1990). *Proterometra* spp. asexually reproduce in freshwater snails: primarily pleurocerids (*Elimia* spp., *Pleurocera* spp., and the Shawnee rocksnail, *Lithasia obovata* [Say, 1829]) but also the highland campeloma, *Campeloma subsolidum* (Anthony, 1860) (Viviparidae) (see Womble et al., 2015), which was considered as a subjective synonym of the ponderous campeloma, *Campeloma crassulum* Rafinesque, 1819 (see Wu et al., 1997 and references therein). Naturally-shed furcocystocercous cercariae of *Proterometra* spp. are macroscopic and their morphology and behaviours have been used to delimit species and classify them (Anderson & Anderson, 1967; LaBeau & Peters, 1995; Riley & Uglem, 1995; Womble et al., 2015). These cercariae have a withdrawn distome (Womble et al., 2015) that extrudes upon cercarial ingestion by the fish host; although *P. dickermani* and *Proterometra autraini* LaBeau & Peters, 1995 could comprise exceptions (Anderson, 1962; LaBeau & Peters, 1995).

The described cercariae of *Proterometra* spp. are known as active pelagic swimmers that 'flamboyantly' swim vertically upwards in the water column, cease movement, sink, and then swim again upwards (Prior & Uglem, 1979; Prior & Uglem, 1983; TNT & SAB, personal observations). Previous authors have asserted that this behaviour is adaptive for enticing (luring) the definitive fish host to swallow the cercaria, whereupon the distome everts and immediately attaches to (colonizes, infects) the buccal cavity/gastro-intestinal epithelium of the fish. At least some fishes (Centrarchidae spp.) readily pursue and swallow these theorized luring cercariae (personal observations SAB). Exceptionally, *Proterometra hodgesiana* (Smith, 1932) Smith, 1936 and *Proterometra edneyi* Uglem & Aliff, 1984 purportedly do not swim and are inactive after shedding (Smith, 1936; Uglem & Aliff, 1984).

Womble et al. (2015) listed the reported fish hosts for *Proterometra* spp., comprising primarily sunfishes and black basses (Centrarchiformes: Centrarchidae) in southern North America but also darters (Perciformes: Percidae), Mexican tetra, *Astyanax mexicanus* (De Filippi, 1853) (Characiformes: Characidae), banded sculpin, *Cottus carolinae* (Gill, 1861) (Perciformes: Cottidae), burbot, *Lota lota* (Linnaeus, 1758) (Gadiformes: Lotidae), and tadpole madtom, *Noturus gyrinus* (Mitchill, 1817) (Siluriformes: Ictaluridae). Taxonomically, the adults of *Proterometra* spp. are morphologically similar such that interspecific diagnostic characters are few and challenging. Womble et al. (2015) provided a key for adults of *Proterometra* spp. but the cercariae are much more readily differentiated at the species level using morphology than are their corresponding adults. Womble et al. (2015, 2016a, b) elucidated life cycles of some *Proterometra* spp. by matching nucleotide sequences (ribosomal internal transcribed spacer 2 region [*ITS2*]) between their cercariae and adults; including *Proterometra catenaria* Smith, 1934, *Proterometra epholkos* Womble, Orélis-Ribeiro, & Bullard, 2015 and *Proterometra ariasae* Womble, Orélis-Ribeiro, & Bullard, 2016.

We herein describe a new species of *Proterometra* (adult and cercaria), elucidate its life cycle in the Cahaba River, Alabama, USA, provide a phylogeny of all accepted azygiid genera, and comment on the systematics of azygiid subfamilies.

Materials and Methods

On 5 May 2022, 162 snails were collected by hand in the Cahaba River (33°10'25.0"N, 87°01'30.6"W; a major tributary of the Alabama River). Snails were maintained in 20 L buckets with aerated river water and transported to the Aquatic Parasitology Laboratory. In the laboratory, snails were isolated in 6-well tissue culture plates (VWR, Radnor, Pennsylvania,

USA) with river water at 20°C. The water in each well was exchanged twice daily, and each well was examined four times per day. Each infected snail shedding an azygiid cercaria was secondarily isolated in a 350 ml glass stender dish with river water and subsequently maintained as above. Naturally-shed, adhered cercariae were observed alive, photographed and videoed, detached using minutien pins, heat-killed in hot water (60°C), and fixed in 10% neutral buffered formalin (n. b. f.). Fixed specimens were stained overnight in Van Cleave's hematoxylin with several drops of Ehrlich's hematoxylin, made basic with two drops of lithium carbonate and one drop of butylamine saturated in 70% ethanol (EtOH), dehydrated in an EtOH series, cleared in clove oil, and permanently mounted on glass slides using Canada balsam. Live cercariae intended for DNA extraction were preserved directly in 95% EtOH. Collected snails were identified as compact elimia, *Elimia showalteri* (Lea, 1860) (Cerithioidea: Pleuroceridae) based on comparisons to type material and following Whelan et al. (2022) (Fig. 1). Although snail identification was based on the best available data, the taxonomy of Pleuroceridae continues to require additional revisionary work (Whelan et al., 2022). *Elimia showalteri* is closely related to *Elimia ampla* (Anthony, 1854) (Cerithioidea: Pleuroceridae) and has a similar morphology. We identified the host as *E. showalteri* based on the specimens having a less ovate aperture and inflated body whorl than *E. ampla* (Lea, 1860). Future taxonomic work on Pleuroceridae may result in taxonomic revisions that would have implications for the identity of the host snail (Johnson et al., 2013; Whelan et al., 2022).

On 29 September 2022, four blackbanded darters, *Percina nigrofasciata* (Agassiz, 1854) (Perciformes: Percidae) were electrofished from the Cahaba River (33°10'08.3"N, 87°01'19.9"W; ~1,000 m upstream of the snail collection site) with personnel from the Alabama Department of Conservation and Natural Resources, euthanized, placed on ice, transported to the Aquatic

Parasitology Laboratory, and dissected with special attention to the buccal cavity, pharyngeal sphincter, oesophagus, stomach, and intestine using a Leica Wild Heerbrugg M8 (Heerbrugg, Switzerland) stereo-dissecting microscope. Adult trematodes were removed from the host using minutien pins and fine forceps, transferred to a stender dish using a pipette, studied alive, and subsequently stained, fixed, mounted, and preserved as the cercariae above. The fish were identified as *P. nigrofasciata* by having i) a smooth preopercular rear margin, ii) five branchiostegal rays, iii) a scaled belly with prominent scutes, iv) a short snout (i.e., the distance from the tip of the snout to the junction of the gill membranes is less than the distance from the gill membranes to the base of the pelvic fin), v) joined gill membranes, vi) seven saddles on the dorsum, vii) a fleshy frenum, viii) vertically elongate blotches connecting to the dorsum saddles, ix) 12 dorsal fin spines, x) a caudal fin base lacking light-coloured blotches, xi) 12 blotches on the flank, xii) a first dorsal fin without a light border, xiii) underside of head not reddish-orange, xiv) dorsal saddles narrower than interspaces, and xv) first dorsal fin lacking a yellow orange band (Boschung & Mayden, 2004).

Illustrations were made using an Olympus BX51 microscope (Olympus Corporation of the Americas, Center Valley, Pennsylvania) equipped with differential interference contrast optical components (D. I. C.) and a drawing tube. Measurements were made using the camera software Jenoptik Gryphax® version 2.1.0.724 (Jena, Germany) and reported in micrometers (μm) as the range followed by the mean ± standard deviation. Taxonomic authorities for fishes followed Fricke et al. (2022). The use of anatomical terms for azygiids follows Womble et al. (2016b). Types of the new species (three adults; ten cercariae) as well as shell vouchers of the infected snail hosts were deposited in the National Museum of Natural History's Invertebrate Zoology Collection (NMNH, Smithsonian Institution, Washington, D.C., USA).

Genomic DNA of the new species was extracted from the EtOH-preserved specimens (four naturally-shed cercariae and two adults) using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA concentration was measured using a NanoDrop–1000 spectrophotometer (Thermo Scientific, Nanodrop Technologies, Waltham, Massachusetts, USA), diluted to 20 ng/µl, and stored at –20 °C. Forward and reverse primers and thermocycling parameters of PCR reactions followed Truong et al. (2022). PCR product purification was conducted using the QIAquick PCR Purification kit (Qiagen). Two internal primers 300F (5'–CAAGTACCGTGAGGGAAAGTTG–3') and 1200R (5'–GCATAGTTCACCATCTTCGG–3') were also used to improve *28S* sequencing coverage (Lockyer, et al., 2003). DNA sequencing was performed by Genewiz (South Plainfield, New Jersey, USA) in both directions using the same primers as used for PCR. Additional *28S* sequences of *Leuceruthrus micropteri* Marshall & Gilbert, 1905 and *Leuceruthrus* cf. *ksepkai* were generated from adult specimens both infecting the stomach of the largemouth bass, *Micropterus salmoides* [Lacepède, 1802] [Centrarchiformes: Centrarchidae] collected from Wheeler Reservoir, Tennessee River, Alabama (34°37'17.8"N, 86°49'51.47"W) on 13 August 2013 and 24 September 2013 and from Holmes Creek, Choctawhatchee River, Florida, USA (30°36'25"N, 85°44'49"W) on 25 March 2015, respectively; comprising unpublished sequences from Womble & Bullard (2022). All generated nucleotide sequences of the present study were deposited in the NCBI GenBank (see Taxonomic summary).

Taxon and outgroup selection for *28S* and *ITS2* phylogenetic analyses were based on Olson et al. (2003) and Womble et al. (2016a), respectively. The selected *28S* sequences (18 in total) included two of the new species (one cercaria, one adult), *Azygia longa* (Leidy, 1851) Manter, 1926 (KC985234), *Derogenes varicus* (Müller, 1784) Looss, 1901 (AY222189), *Dinurus*

longisinus Looss, 1907 (AY222202), *Hemipera manteri* (Crowcroft, 1947) Yamaguti, 1958 (AY222196), *Lecithochirium caesionis* Yamaguti, 1942 (AY222200), *Lecithocladium excisum* (Rudolphi, 1819) Lühe, 1901 (AY222203), *Lecithophyllum botryophoron* (Olsson, 1868) Odhner, 1905 (AY222205), *L.* cf. *ksepkai*, *L. micropteri*, *Opisthadena dimidia* Linton, 1910 (AY222198), *Otodistomum cestoides* (Van Beneden, 1870) Odhner, 1911 (AY222187), *Nematobothrium scombri* (Taschenberg, 1879) Ishii, 1935 (AY222195), *Plerurus digitatus* (Looss, 1899) Looss, 1907 (AY222201), *P. macrostoma* (MF927953), *Proterometra* sp. (KC985237), and *Bivesicula unexpecta* Cribb, Bray, & Barker, 1994 (AY222181) as the outgroup. Selected *ITS2* sequences (12) included two of the new species (one cercaria, one adult), *A. longa* (KT808319), *Leuceruthrus blaisei* Womble & Bullard, 2022 (ON877229), *Leuceruthrus* cf. *ksepkai* (ON877231), *Leuceruthrus* cf. *stephanocauda* (ON877232), *Leuceruthrus ksepkai* Womble & Bullard, 2022 (ON877233), *L. micropteri* (KT808320), *P. ariasae* (KT808317), *P. epholkos* (KM503118), *P. macrostoma* (MF927955), and *B. unexpecta* (KR092222) as the outgroup.

Sequences of the new species and publicly available sequences (Tables 1, 2) were aligned using MAFFT (Katoh & Standley, 2013) with the –auto flage and default paramterts. Comparisons among aligned sequences were made with Geneious prime version 2023.0.4 (Biomatters Inc., Boston, Massachusetts, USA). Nucleotide similarity was calculated as percentage of identical bases per total aligned bases, whereas nucleotide difference was number of unidentical bases, both including the indels (nucleotide insertions and deletions). Gene trees for *ITS2* and *28S* were inferred independently with MrBayes version 3.25, following Truong et al. (2022). Briefly, best-fit models were inferred with JModelTest 2.1.10 (Darriba et al., 2012). Tree inference for each gene used three independent runs, each with 4 Metropolis couples chains

and run for 5,000,000 generations, sampling the posterior distribution every 1,000 generations. Model averaging and a gamma distribution to model rate-heterogeneity were used for substitions models. All other parameters and priors were set to defaults. Evidence for convergence was visualized with Tracer 1.7 (Rambaut et al., 2018) and further examined with the MrBayes sump command. Convergence was assumed to have occurred when trace plots among runs overlapped and effective samples size for each parameter was at least 200. Inferred phylogenetic trees were visualized using FigTree v1.4.4 (Rambaut et al., 2014) and further edited for visualization purposes with Adobe Illustrator 24.1.3 version (2020) (Adobe Systems, San Jose, California, USA).

Superfamily Azygioidea Lühe, 1909

Family Azygiidae Lühe, 1909

Proterometra wigglewomble **Truong & Bullard n. sp.**

Type-host: Percina nigrofasciata (Agassiz, 1854) (Perciformes: Percidae), blackbanded darter.

Other host: Elimia showalteri (Lea, 1860) (Cerithioidea: Pleuroceridae), compact elimia.

Type-locality: Cahaba River (33°10'08.3"N, 87°01'19.9"W), Shelby County, Alabama (blackbanded darter).

Other locality: Cahaba River (33°10'25.0"N, 87°01'30.6"W), Shelby County, Alabama (compact elimia).

Site in host: Oesophagus (blackbanded darter); indeterminate (compact elimia).

Prevalence and intensity of infection: One of four (25%) blackbanded darters was infected by seven adult specimens of the new species; eight of 162 (4.9%) compact elimia shed cercariae of the new species.

Specimens and DNA sequences deposited: syntypes, three adult specimens (USNM 1606709–1606711), ten cercariae (USNM 1606712–1606721); intermediate host vouchers (USNM 1606722–1606728); *ITS2* (OR470687), *28S* rDNA (OR470686).

ZooBank registration: urn:lsid:zoobank.org:act:D96E7942-ECCA-4584-ABD3-226F56F9B806.

Etymology: The species name refers to the behaviour of the cercaria and honours Matthew Ryan Womble (Fairhope, Alabama, USA) for his contribution to the natural history of azygiids in North America.

Description (Figs. 1–14)

Diagnosis of adult (based on live specimens and light microscopy of three stained, whole-mounted specimens):

Body subovoid, having broadly rounded ends, having anterior end broader than posterior end, 1353–1366 long, 841–902 wide, widest in posterior half of forebody, 1.5–1.7× longer than wide (Fig. 2); body of live specimens amber-coloured. Tegument aspinous, without plications, 10–24 thick. Forebody 765–870 long (57–64% of body length); hindbody 313–380 long (23–29% of body length), forebody 2.0–2.8× longer than hindbody (Fig. 2). Oral sucker subspherical, ventrally subterminal, 407–499 long (31–37% of body length), 437–527 wide (56–58% of body width), 1.1× wider than long, 62–91 from anterior body end (5–7% of body length), 814–854 from posterior body end (60–64% of body length), 2.0–2.4× longer than ventral sucker (Fig. 2). Ventral sucker subspherical, in posterior half of body, 180–218 long (14–16% of body length), 217–221 wide (25–28% of body width), 1.1–1.2× wider than long, 281–326 from posterior margin of oral sucker (21–24% of body length) (Fig. 2). Mouth subterminal, opening ventrally, directed anteriad. Pre-pharyngeal oesophagus apparently absent. Pharynx ovoid, dorsally overlapping posterior margin of oral sucker, 84–142 long (6–10% of body length), 103–139 wide

(13–15% of body width). Oesophagus extending posteriad 21–35 from pharynx (2–3% of body length) before bifurcating; dextral branch of oesophagus 237–329 long (18–24% of body length), 64–114 in maximum width; sinistral branch of oesophagus 188–360 long (14–27% of body length), 67–138 in maximum width (Fig. 2). Dextral caecum 843–939 long (62–69% of body length), 114–172 in maximum width; sinistral caecum 846–1,050 long (63–77% of body length), 94–194 in maximum width; pre-caecal space 417–563 (131–41% of body length); post-caecal space 16–83 (1–7% of body length) (Fig. 2).

Testes two in number, ovoid, diagonal, in posterior half of hindbody, ventral to caeca, anterior to or at level of caecal tips; separated from ventral sucker by extensive uterine coils; dextral testis 82–168 long (6–12% of body length), 126–164 wide (17–21% of body width); sinistral testis 155–159 long (11–12% of body length), 119–137 wide (13–18% of body width); pre-testicular space 1038–1138 (78–84% of body length); post-testicular space 40–86 (3–6% of body length) (Fig. 2). Vasa efferentia originating from anterior half of testes as narrow ducts, extending anteriad to level of ventral sucker, connecting to prostatic sac without involving a vas deferens. Prostatic sac ovoid, thin-walled, medial, slightly overlapping anterior margin of ventral sucker, 185–268 long (14–20% of body length), 122–140 wide (16% of body width), 584–651 from anterior body end (44–47% of body length), 524–566 from posterior body end (38–43% of body length) (Figs. 2, 3). Internal seminal vesicle convoluted, within proximal portion of prostatic sac, 303–392 long, comprising anterior and posterior portions; proximal portion of internal seminal vesicle 174–209 long (53–57% of total seminal vesicle length), 32–48 in maximum width; distal portion of internal seminal vesicle 129–138 long (43–47% of seminal vesicle length), 23–27 in maximum width (56–72% width of proximal portion) (Fig. 3). Pars prostatica thick-walled, posteriorly communicating with distal portion of seminal vesicle via

short verschlussapparat, positioned within distal portion of prostatic sac, slightly curved, swollen at proximal portion, 144–205 long; proximal portion 27–32 in maximum width; distal portion 14–18 in maximum width (44–67% width of proximal portion) (Fig. 3). Prostatic gland cells numerous, surrounding pars prostatica for its entire length. Ejaculatory duct unarmed, 44–55 long (3–4% of body length), 16–23 in maximum width, merging distally with metraterm to form hermaphroditic duct (Fig. 3). Hermaphroditic duct 40 long (3% of body length), 44 in maximum width, opening distally into genital atrium via hermaphroditic pore; hermaphroditic pore anterior to ventral sucker, at level of middle of prostatic sac (Fig. 3). Sinus organ ventral to prostatic sac, directing ventrally, enclosing hermaphroditic duct. Genital atrium large, ventral to sinus sac, pre-acetabular, capable to contain several eggs or empty, 252–335 wide (32–37% of body width) (Fig. 3). Genital pore immediately pre-acetabular, 749–769 from anterior body end (56% of body length), at level of middle of prostatic sac (Figs. 2, 3).

Ovary elliptical in outline, median, dorsally overlapping testes, separated from ventral sucker by extensive uterine coils, 106–119 long (8–9% of body length), 158–191 wide (20–21% of body width); pre-ovarian space 1025–1104 (75–83% of body length); post-ovarian space 132–208 (10–15% of body length) (Figs. 2, 4). Oviduct dorsal to ovary, extending transversely towards sinistral side of body, with sphincter at proximal portion of oviduct (47 long, 40 wide) (Fig. 4). Oötype pre-ovarian. Mehlis' gland cells distributed in dense cluster from level of ovary to posterior margin of ventral sucker, surrounding oötype (Fig. 4). Laurer's canal originating from oviduct at level of oviducal sphincter, dorsal to ovary, filled with sperm, extending transversely from commissure with oviducal sphincter before looping dextrally to ovary, extending anteriad; Laurer's canal pore dextral, dorsal, immediately pre-ovarian (Fig. 4). Uterine seminal receptacle comprising proximal portion of uterus. Uterus extensively coiling, occupying space between

ovary to nearly middle of oral sucker, filling inter-caecal space between ovary and ventral sucker, sinistral to ventral sucker, partly ventral to caeca and extra-caecal anteriorly, crossing midline between pharynx and prostatic sac, connecting with terminal genitalia dorsal to ventral sucker; uterine field 849–1003 long (64–73% of body length), 853–864 wide (72–95% of body width); metraterm unarmed, extending anteriad posterolateral to prostatic sac and dorsal to ventral sucker, merging distally with ejaculatory duct, 189–257 long (14–19% of body length), 33–39 wide (Figs. 2, 3). Uterine eggs ovoid, operculate, numerous, shape and sizes depending on location within uterus; eggs in proximal portion of uterus smooth-surfaced (lacking wholly fimbriated surface), lacking developed miracidium, 54–72 long, 30–44 wide; eggs in distal portion of uterus, metraterm, and genital atrium having a wholly fimbriated surface, appearing colloquially as a cilia-like brush border (cf. Womble et al. [2015]), fully embryonated (= containing miracidium), 70–80 long, 40–54 wide (Figs. 2–6). Vitellarium follicular, wholly ventral to caeca, comprising 2 lateral fields, extending anteriad from posterior half of oral sucker posteriad to level of testes; dextral vitelline field 704–941 long (52–71% of body length, 77–103% of dextral caecum length); pre-dextral vitelline field space 287–546 (22–40% of body length); post-dextral vitelline field space 130–208 (10–15% of body length); sinistral vitelline field 713–969 long (52–73% of body length, 68–115% of sinistral caecum length); pre-sinistral vitelline field space 342–474 (26–35% of body length), post-sinistral vitelline space 85–269 (6–20% of body length); distance between anterior ends of vitelline fields 449 (58% of body width); distance between posterior ends of vitelline fields 316 (41% of body width) (Fig. 2). Transverse vitelline ducts asymmetrical, extending posteromedian before fusing to form vitelline reservoir; dextral transverse vitelline duct 209–284 long, 589–743 from anterior end of dextral vitelline field (79–84% of dextral vitelline field length); sinistral transverse vitelline duct 154–566 long,

660–684 from anterior end of sinistral vitelline field (68–94% of sinistral vitelline field length). Vitelline reservoir dorsal to ovary, posteromedian or posterolateral to ovary, 78–188 long, 24–77 wide. Excretory pore terminal (Fig. 2).

Diagnosis of cercaria (based on live cercariae and light microscopy of 27 stained, whole-mounted, naturally-shed cercariae with a withdrawn distome):

Cercaria furcocystocercous, presenting as a withdrawn distome (within a cavity) occupying anterior end of tail stem, having a single diminutive furca that secretes an adhesive and that is nearly indistinguishable from the tail stem, 2598–3330 (2988 ± 199) in total length (= tail stem + furca), 462–652 (557 ± 45) in maximum width at anterior terminal end, 4.5–6.8× (5.4 ± 0.6) longer than wide (Figs. 9–11), light orange to amber in colour in live specimens, shedding in early morning or infrequently early afternoon, adhering to substratum immediately after shedding, actively and continuously thrashing/wiggling tail stem subsequent to attachment to substratum as if mimicking the larva of a stream insect. Tail stem cylindrical, claviform, aspinous, 2273–3063 (2669 ± 197) long (86–92% [89 ± 1] of total cercarial length), widest anteriorly and narrowing posteriad, mammillate, enclosing a column of glandular cells in tail stem; tail stem mammillae largest and most numerous anteriorly, becoming smaller and less numerous posteriorly, 43–91 (59 ± 11) in maximum height, 73–135 (106 ± 16) in maximum base width, 1.1–2.8× (1.8 ± 0.4) wider than long, distributed in anterior 2/3–3/4 of tail stem only and covering an area 1894–2300 (2093 ± 120) long (62–76% [70 ± 4] of total cercarial length), terminating approximately 304–1005 (576 ± 155) from base of furca (12–30% [19 ± 4] of total cercarial length) (Figs. 9–12).

Furca single, diminutive and greatly reduced in size compared to other *Proterometra* spp., base of furca merging with posterior end of tail stem, synthesis to posterior end of tail stem not

evident, 229–452 (317 ± 48) long (8–14% [11 ± 1] of total cercarial length), 124–245 (175 ± 30) in maximum width (19–53% [32 ± 8] of maximum cercarial width); furcal tegument refractory, having a pleated appearance, thickened relative to tegument of tail stem, thicker at furcal tip, gradually becoming thinner toward base, secreting an adhesive, 11–34 (19 ± 7) thick; adhesive gelatinous, nearly transparent, coating furca for its entire length, 41–71 (49 ± 20) thick or approximately twice as thick as furcal tegument (Figs. 7, 9–11, 13, 14); column of glandular cells in tail stem median or submedian, distributed along tail stem typically posterior to distome (anterior to distome in some specimens) posteriad to level of or slightly posterior to termination of mammillae, with each cell evidently having a granular cytoplasm and well-defined spheroid nucleus, slightly eosinophilic, 963–2215 (1645 ± 488) long (40–82% [64 ± 19] of tail stem length), 59–130 (100 ± 26) wide (10–24% [18 ± 5] of maximum tail stem width) (Figs. 9, 10).

Excretory system comprising a main duct originating at tail stem cavity opening, extending posteriad before bifurcating into two primary collecting canals at level of distome; excretory collecting canals extending posteriad along tail stem, occasionally crossing (but not merging) along their courses posteriorly, opening separately at furcal tip; pores terminal (Figs. 9–11, 14).

Distome aspinose, oval or lingulate in outline, having equally broadly rounded ends, 817–1022 (908 ± 55) long (27–35% [30 ± 2] of total cercarial length), 366–521 (440 ± 41) wide (66–94% [79 ± 8] of maximum cercarial width), widest at level of prostatic sac, 1.8–2.7× (2.1 ± 0.3) longer than wide, 99–650 (281 ± 116) from anterior end of tail stem (3–23% [10 ± 4] of total cercarial length) (Figs. 9–11). Forebody 442–573 (498 ± 35) long (52–57% [55 ± 1] of distome length), 1.6–2.0× (1.8 ± 0.1) longer than hindbody; hindbody 235–320 (276 ± 23) long (28–32% [30 ± 1] of distome length) (Figs. 9–11). Oral sucker subspherical, ventrally subterminal, 271–360 (299 ± 25) long (29–36% [33 ± 2] of distome length), 279–334 (300 ± 13) wide (57–83%

[69 ± 7] of distome width), 0.9–1.2× (1.0 ± 0.1) longer than wide, 1.9–2.6× (2.2 ± 0.2) longer than ventral sucker, 1.6–1.9× (1.7 ± 0.1) wider than ventral sucker (Figs. 9–11). Ventral sucker subspherical, 128–160 (139 ± 8) long (13–17% [15 ± 1] of distome length), 151–195 (173 ± 8) wide (34–48% [40 ± 4] of distome width), 1.1–1.4× (1.2 ± 0.1) wider than long (Figs. 9–11). Pre-pharyngeal oesophagus apparently absent. Pharynx ovoid, 62–92 (76 ± 6) long (7–10% [8 ± 1] of distome length), 73–116 (91 ± 8) wide (18–24% [21 ± 2] of distome width) (Figs. 9–11). Oesophagus bifurcating into two lateral branches immediately posterior to pharynx; dextral branch of oesophagus 101–194 (136 ± 20) long (11–20% [15 ± 2] of distome length); sinistral branch of oesophagus 111–187 (149 ± 21) long (11–21% [16 ± 3] of distome length). Dextral caecum 427–594 (507 ± 48) long (50–61% [56 ± 3] of distome length); sinistral caecum 427–612 (516 ± 43) long (52–62% [57 ± 2] of distome length); pre-caecal space 293–512 (347 ± 61) (34–55% [38 ± 4] of distome length); post-caecal space 27–82 (63 ± 15) (3–10% [7 ± 2] of distome length) (Figs. 9–11).

Testes two, ovoid, diagonal, contiguous, having dextral testis either anterior or posterior to sinistral testis, ventral to caeca, at level of caecal tips and overlapping posterior margin of ventral sucker; dextral testis 140–207 (167 ± 18) long (3–10% [7 ± 2] of distome length), 84–168 (130 ± 18) wide (16–23% [18 ± 2] of distome width); sinistral testis 111–200 (161 ± 20) long (23–37% [30 ± 4] of distome length), 83–197 (127 ± 26) wide (13–22% [18 ± 2] of distome width); pre-testicular space 497–716 (603 ± 54) (61–71% [66 ± 3] of distome length); post-testicular space 40–115 (69 ± 17) (4–14% [8 ± 2] of distome length) (Figs. 9–11). Prostatic sac subspherical, dorsally overlapping anterior margin of ventral sucker and posterior margin of pharynx; vasa efferentia, vas deferens, seminal vesicle, verschlussapparat, pars prostatica, ejaculatory duct, hermaphroditic duct, and sinus organ not developed (Figs. 9, 10). Genital atrium pre-acetabular,

74–121 (94 ± 9) wide (17–26% [22 ± 2] of distome width). Genital pore immediately pre-acetabular, 428–621 (488 ± 47) from anterior end of distome (49–68% [54 ± 3] of distome length) (Figs. 9, 10).

Ovary ovoid, dorsally overlapping anterior margin of testes and posterior margin of ventral sucker, 88–159 (188 ± 15) long (10–18% [13 ± 2] of distome length), 90–145 (118 ± 14) wide (21–31% [27 ± 2] of distome width); pre-ovarian space 418–701 (550 ± 71) (49–69% [60 ± 5] of distome length). Oviduct, oötype, Mehlis' gland, Laurer's canal, uterine seminal receptacle, and metraterm not developed. Uterus pre-ovarian, containing a few eggs (0–4) (Figs. 8, 11). Uterine eggs ovoid, non-fimbriated, lacking discernible miracidium, 39–60 (50 ± 7) long, 21–40 (29 ± 6) wide (Figs. 8, 11). Vitellarium follicular, bilateral, wholly ventral to caeca and gonads; vitelline fields extending from posterior half of ventral sucker posteriad to level of testes; dextral vitelline field 422–577 (496 ± 42) long (47–61% [55 ± 4] of distome length); sinistral vitelline field 360–594 (511 ± 56) long (42–66% [56 ± 5] of distome length), pre-vitelline space 218–337 (273 ± 30) (24–35% [30 ± 3] of distome length); post-vitelline space 61–190 (138 ± 26) (7–21% [15 ± 3] of distome length). Transverse vitelline ducts extending posteromedian to form vitelline reservoir; dextral transverse vitelline duct branching at 274–486 (366 ± 59) from anterior end of dextral vitelline field (60–85% [73 ± 8] of dextral vitelline field length); sinistral transverse vitelline duct branching at 268–460 (366 ± 47) from anterior end of sinistral vitelline field (54–87% [72 ± 8] of sinistral vitelline field length). Vitelline reservoir dorsal to ovary, posteromedian or posterolateral to ovary (Figs. 9–11).

Excretory system of distome Y-shaped, main stem 76–199 (128 ± 33) long (7–23% [14 ± 4] of distome length), bifurcating at level of testes into collecting ducts; collecting ducts extending anteriad laterally to distome body; anterior extent difficult to trace; pore terminal (Figs. 9, 10).

Remarks

The new species is assigned to *Proterometra* because its adults have a subovoid body with a forebody that is longer than the hindbody, an aspinous tegument, an oral sucker that is >2× larger than the ventral sucker in length and width, gonads that cluster near the posterior body end, a primarily pre-testicular ovary, a uterus that extends into the forebody and that is partly extra-caecal anteriorly, and a vitellarium that extends well into the forebody (to the posterior half of the oral sucker). In addition, cercariae of the new species are macroscopic, progenetic, and have a primarily pre-testicular ovary.

Adults of the new species resemble those of *P. epholkos* but differ from them by having a body that is not ventrally concave (vs. ventrally concave) and that lacks tegumental projections (vs. having tegumental projections covering entire body), an ovary that dorsally overlaps the testes (vs. pre-testicular), a metraterm that extends anteriad to the genital atrium dorsal to the ventral sucker (vs. extending transversely from the left side to right side of the body and is entirely anterior to the ventral sucker), and mature eggs that have a wholly fimbriated surface, appearing colloquially as a cilia-like brush border (vs. eggs that have minute fimbriae on one pole) (Womble et al., 2015). Adults of the new species differ from those of all other species of *Proterometra* by a combination of having i) a small body (only *P. edneyi* is smaller), ii) an oral sucker that is >2× larger than the ventral sucker in length and width, iii) a vitellarium that extends anteriad to the posterior half of the oral sucker posteriad to the level of the testes, and iv) mature eggs that have a wholly fimbriated surface (vs. having smooth shell, except for *P. macrostoma* and *P. autraini*, which also have wholly fimbriated eggshell [Horsfall, 1933, 1934;

LaBeau & Peters, 1995]; *Proterometra albacauda* Anderson & Anderson, 1967 has eggs bearing minute fimbriae on one pole [Womble et al., 2015]).

We observed live naturally-shed cercariae and only measured stained, whole-mounted, naturally-shed cercariae of the new species for comparisons. Fully-developed (having an inverted distome) cercariae of the new species differ from those of its congeners by having i) a strongly claviform tail stem (vs. tail stem spindle-shaped or dorsoventrally flat) that has aspinose mammillae (vs. tail stem having spinose mammillae, except for *P. dickermani* and *P. sagittaria*, which also have aspinose mammillae [Dickerman, 1946; Anderson, 1962]), ii) a single furca (vs. paired furcae) that secretes an adhesive and that adheres the tail stem to the substratum upon shedding (the cercaria is benthic, not a pelagic swimming cercaria, and wiggles or thrashes while attached to the substratum), iii) excretory pores that open on the posterior margin of the single furca, and iv) few eggs in the cercarial distome (maximum of 4 vs. >10).

The new species is the only nominal azygiid that has a single furca. It is also the only congener having a column of glandular cells within the tail stem. The new species is also the only known azygiid that adheres to a substratum upon being shed from the snail. We do not know the function of these gland cells in the new species; however, we speculate that they are glandular and perhaps provide the adhesive that is delivered to the secretory surface of the furca.

Phylogenetic results

ITS2 and *28S* sequences generated from the four cercariae and two adults of the new species were identical to each other. Based on nucleotide similarity, the new species was most similar to *P. macrostoma* (*28S*) and *P. epholkos* (*ITS2*). The *28S* sequence of the new species differed from that of *P. macrostoma* by 40 nucleotides (96.8% similarity of 1247 bp alignment) (Table 1). The

ITS2 sequence of the new species differed from that of *P. epholkos* by three nucleotides (99.3% similarity of 368 bp) (Table 2).

The *28S* tree (1199 bp, including gaps) recovered a monophyletic Azygiidae that comprised a monophyletic Leuceruthrinae (with only two leuceruthrines included) but a polyphyletic Azygiinae (Fig. 15). *Proterometra wigglewomble* was sister to *P. macrostoma*. Monophyletic *Proterometra* was recovered sister to the clade including *Leuceruthrus* (*L. micropteri* + *L.* cf. *ksepkai*) (Leuceruthrinae) and *Azygia longa* (Azygiinae). These taxa were sisters to *O. cestoides* (Azygiinae).

The *ITS2* tree (368 bp, including gaps) recovered a monophyletic Leuceruthrinae but a polyphyletic Azygiinae and paraphyletic *Proterometra* (Fig. 16). The new species was sister to *P. epholkos*. Womble et al. (2016a, b) recovered *Proterometra* as monophyletic, and Womble & Bullard (2022) recovered it as paraphyletic; suggesting that additional taxon sampling is needed to better understand the evolutionary history of these parasites.

Discussion

The present study brings the total number of the accepted species of *Proterometra* to 13, making it the second most speciose azygiid genus (*Azygia* Looss, 1899 has 25 species [WoRMS editorial board, 2023]). Species of *Proterometra* collectively exploit a diverse group of freshwater snails as intermediate hosts and mature in freshwater fishes that are sympatrically endemic in rivers, streams, and lakes on North America, primarily east of the mainstem of the Mississippi River (Womble et al., 2015). *Proterometra wigglewomble* is the second congener that infects darters (after *P. edneyi*); both species possess a relatively small body as compared to their congeners (Uglem & Aliff, 1984), perhaps an adaptation to the small body size of their definitive fish hosts. The blackbanded darter, definitive host of the new species, is a common

benthic forager that ubiquitously distributes in streams and rivers throughout the southeastern United States. It inhabits all river drainages in Alabama except for the Tennessee River (Boschung & Mayden, 2004). We are the first to elucidate an azygiid life cycle by matching both *ITS2* and *28S* sequences between the cercariae and adults that infected wild, sympatric freshwater snail and fish hosts, respectively. Compact elimia and blackbanded darter represent new intermediate and definitive host records, respectively, of *Proterometra*. *Proterometra wigglewomble* is the third congener reported from the Cahaba River (after *P. macrostoma* and *P. melanophora*; see Horsfall [1934], Smith [1936]).

The subfamilial diagnoses for Azygiinae and Leuceruthrinae (see Gibson, 2002) need revision. Azygiinae is clearly polyphyletic, which was evidenced by our *28S* and *ITS2* analyses (Figs. 15, 16). Previous preliminary phylogenetic studies of the *ITS2* (Blair et al., 2018; Womble et al, 2015; 2016a, b; Womble & Bullard, 2022) have consistently recovered Azygiinae as paraphyletic or polyphyletic, although none of these analyses have rejected monophyly of Leuceruthrinae. However, our *28S* tree further recovered *Leuceruthrus* spp. sister to *A. longa*, representative of the type genus for Azygiinae, in a clade (with high nodal support) sister to monophyletic *Proterometra* spp. (also assigned to Azygiinae) (Fig. 15). Hence, the only diagnostic feature used by Gibson (2002) (i.e., position of the ovary relative to the testes) does not seem to differentiate azygiid subfamilies. Womble & Bullard (2022) questioned the systematic position of *Otodistomum* Stafford, 1904. It is the only marine azygiid and infects the stomach and body cavity of chondrichthyans. Unlike any other azygiid, it has an oral sucker that is smaller in length and width than the ventral sucker, gonads that are confined in the anterior half of the body, a coiled ejaculatory duct, a genital pore that is near the pharynx and anterior to the prostatic sac, and a vitellarium that is confluent posteriorly. Based on these features and the

nucleotide evidence, it should likely be reassigned to its own subfamily. Based on morphology of the accepted azygiid genera and available phylogenetic evidence, Azygiinae should include *Azygia* and *Leuceruthrus*, whereas Proterometrinae Yamaguti, 1958 should comprise *Proterometra* only. Additional *28S* sequences for all of the recognized genera (especially *Azygia lucii* [Müller, 1776] Lühe, 1909, type species) will help further test evolutionary relationships among the various azygiid lineages.

Acknowledgments We thank Micah Warren, Haley Dutton, Jacob Shurba, John Brule, Justin Krol, and Steven Ksepka (all Southeastern Cooperative Fish Parasite and Disease Laboratory, Auburn University) for helping collect the snails and fish studied herein. The findings and conclusions in this article are those of the authors and do not necessarily represent the views of the U.S. Fish and Wildlife Service. This study was supported by the Southeastern Cooperative Fish Parasite and Disease Project, the US Fish and Wildlife Service (Department of Interior), National Sea Grant (National Oceanic and Atmospheric Administration), United States Department of Agriculture (National Institute of Food and Agriculture), Federal Aid in Sport Fish Restoration (Alabama Department of Conservation and Natural Resources, Inland and Marine Resources Divisions), and the Alabama Agricultural Experiment Station (Auburn University, College of Agriculture).

References

Anderson, M. G. (1962). *Proterometra dickermani*, sp. nov. (Trematoda: Azygiidae). *Transactions of the American Microscopical Society, 81*(3), 279–282. https://doi.org/10.2307/3224052

Anderson, M. G., & Anderson, F. M. (1963). Life History of *Proterometra dickermani* Anderson, 1962. *Journal of Parasitology, 49*(2), 275–280. https://doi.org/10.2307/3275994

Anderson, M. G., & Anderson, F. M. (1967). The life histories of *Proterometra albacauda* and *Proterometra septimae*, spp. n. (Trematoda: Azygiidae) and a redescription of *Proterometra catenaria* Smith, 1934. *Journal of Parasitology*, *53*(1), 31–37. https://doi.org/10.2307/3276617

Blair, P., Franklin, H., Kelner, N., Ke-Lind, P., Paulmier, M., & Shrestha, M. (2018). Targeted rDNA sequence determination from geographically isolated populations of *Proterometra macrostoma* (Trematoda: Azygiidae). *Proceedings of the Indiana Academy of Science*, *127*(1), 82–88.

Boschung, H. T., & Mayden, R. L. (2004). *Fishes of Alabama*. Smithsonian Books, Washington D. C., USA., 960 pp.

Darriba, D., Taboada, G. L., Doallo, R., & Posada, D. (2012). jModelTest 2: More models, new heuristics, and parallel computing. *Nature Methods 9*, 772, https://doi.org/10.1038/nmeth.2109.

Dickerman, E. E. (1946). Studies on the trematode family Azygiidae. III. The morphology and life cycle of *Proterometra sagittaria* n. sp. *Transactions of the American Microscopical Society, 65*(1), 37–44. https://doi.org/10.2307/3223284

Fricke, R., Eschmeyer, W. N., & Van der Laan, R. (2022). *Eschmeyer's Catalog of Fishes: Genera, Species, References*. Retrieved December 20, 2022, from http://researcharchive.calacademy.org/research/ichthyology/catalog/fishcatmain.asp

Gibson, D. I. (2002). Superfamily Azygioidea Lühe, 1909. In: D. I. Gibson, A. Jones, & R. A. Bray (Eds.), *Keys to the Trematoda*. *Volume 1* (pp. 19–24). CABI Publishing & the Natural History Museum, London, U.K.

Horsfall, M. W. (1933). Development of *Cercaria macrostoma* (Faust) into *Proterometra* (nov. gen.) *macrostoma*. *Science, 78*(2017), 175–176. https://doi.org/10.1126/science.78.2017.175

Horsfall, M. W. (1934). Studies on the life history and morphology of the cystocercous cercariae. *Transactions of the American Microscopical Society, 53*(4), 311–347. https://doi.org/10.2307/3222382

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*(6), 247–282. https://doi.org/10.1080/03632415.2013.785396

Katoh, K., & Standley, D. M. (2013). MAFFT Multiple sequence alignment software version 7: Improvements in performance and usability. *Molecular Biology and Evolution, 30,* 772–780. https://doi.org/10.1093/molbev/mst010

LaBeau, M. R., & Peters, L. E. (1995). *Proterometra autraini* n. sp. (Digenea: Azygiidae) from Michigan's upper Peninsula and a key to species of *Proterometra*. *Journal of Parasitology, 81*(3), 442–445. https://doi.org/10.2307/3283829

Lockyer, A. E., Olson, P. D., & Littlewood, D. T. J. (2003). Utility of complete large and small subunit rRNA genes in resolving the phylogeny of the Neodermata (Platyhelminthes): implications and a review of the cercomer theory. *Biological Journal of the Linnean Society, 78,* 155–171. https://doi.org/10.1046/j.1095-8312.2003.00141.x

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). *International Journal for Parasitology, 33,* 733–755. https://doi.org/10.1016/S0020-7519(03)00049-3

Prior, D. J., & Uglem, G. L. (1979). Behavioural and physiological aspects of swimming in cercariae of the digenetic trematode, *Proterometra macrostoma*. *Journal of Experimental Biology, 83*(1), 239–247. https://doi.org/10.1242/jeb.83.1.239

Rambaut, A., Suchard, M. A., Xie, D., & Drummond, A. J. (2014). *FigTree v1.4.4.* Retrieved December 22, 2022, from http://tree.bio.ed.ac.uk/software/figtree

Rambaut, A., Drummond, A. J., Xie, D., Baele, G., & Suchard, M. A. (2018). Posterior summarization in Bayesian phylogenetics using Tracer 1.7. *Systematic Biology, 67*(5), 901–904. https://doi.org/10.1093/sysbio/syy032

Riley, M. W., & Uglem, G. L. (1995). *Proterometra macrostoma* (Digenea: Azygiidae): variations in cercarial morphology and physiology. *Parasitology, 110*(4), 429–436. https://doi.org/10.1017/S0031182000064763

Smith, S. (1936). Life-cycle studies of *Cercaria hodgesiana* and *Cercaria melanophora*. *Journal of the Alabama Academy of Science, 8,* 30–32.

Truong, T. N., Curran, S. S., Reyda, F. B., Rash, J. M., & Bullard, S. A. (2022). *Plagioporus wataugaensis* n. sp. (Digenea: Opecoelidae) infecting intestine of northern hogsucker, *Hypentelium nigricans,* and white sucker, *Catostomus commersonii,* (Cypriniformes:

Catostomidae) from the eastern USA, including an emended diagnosis, key to Nearctic congeners, and phylogenetic analysis. *Parasitology International, 89*(2022), 102580. https://doi.org/10.1016/j.parint.2022.102580

Uglem, G. L., & Aliff, J. V. (1984). *Proterometra edneyi* n. sp. (Digenea: Azygiidae): behavior and distribution of acetylcholinesterase in cercariae. *Transactions of the American Microscopical Society, 103*(4), 383–391. https://doi.org/10.2307/3226475

Uglem, G. L., Lewis, M. C., & Short, T. M. (1990). Contributions to the life history of *Proterometra dickermani* (Digenea: Azygiidae). *Journal of Parasitology, 76*(3), 447–450. https://doi.org/10.2307/3282688

Uglem, G. L., & Prior, D. J. (1983). Control of swimming in cercariae of *Proterometra macrostoma* (Digenea). *Journal of Parasitology, 69*(5), 866–870. https://doi.org/10.2307/3281048

Whelan, N. V., Johnson, P. D., Garner, J. T., Garrison, N. L., & Strong, E. E. (2022). Prodigious polyphyly in Pleuroceridae (Gastropoda: Cerithioidea). *Bulletin of the Society of Systematic Biologists, 1*(2): 8419. https://doi.org/10.18061/bssb.v1i2.8419

Womble, M. R., Orélis-Ribeiro, R., & Bullard, S. A. (2015). *Proterometra epholkos* sp. n. (Digenea: Azygiidae) from Terrapin Creek, Alabama, USA: Molecular characterization of life cycle, redescription of *Proterometra albacauda*, and updated lists of host and geographic locality records for *Proterometra* spp. in North America. *Parasitology International, 64*, 50–69. https://doi.org/10.1016/j.parint.2014.09.004

Womble, M. R., Arias, C. R., & Bullard, S. A. (2016a). Taxonomic redescription, morphological and molecular diagnosis, and life cycle of *Proterometra catenaria* Smith, 1934 (Digenea:

Azygiidae) from the Choctawhatchee River, Florida, U.S.A. *Comparative Parasitology, 83*(2), 137–148. https://doi.org/10.1654/4814i.1

Womble, M. R., & Bullard, S. A. (2022). Azygiid parasites of North American endemic pleurocerids and centrarchids: revision of *Leuceruthrus* Marshall and Gilbert, 1905 (Digenea: Azygiidae), description of two new species, and phylogenetic analysis. *Journal of Parasitology, 108*(4), 374–394. https://doi.org/10.1645/22-36

Womble, M. R., Orélis-Ribeiro, R., & Bullard, S. A. (2016b). New species of *Proterometra* (Digenea: Azygiidae) and its life cycle in the Chickasawhay River, Mississippi, USA, with supplemental observations of *Proterometra autraini*. *Parasitology International, 65*, 31–43. https://doi.org/10.1016/j.parint.2015.09.005

WoRMS Editorial Board. (2023). *World Register of Marine Species*. Retrieved February 6, 2023, from https://www.marinespecies.org/aphia.php?p=taxdetails&id=158634

Wu, S.-K., Oesch, R. D., & Gordon, M. E. (1997). *Missouri aquatic snails*. Natural History Series, No. 5. Missouri Department of Conservation: Jefferson, Missouri, USA., 97 pp.

Fig. 1. Photographs of the compact elimia, *Elimia showalteri* (Lea, 1860) (Cerithioidea: Pleuroceridae) from the Cahaba River, Alabama, USA that shed cercariae of *Proterometra wigglewomble* n. sp. A, Voucher USNM 1606722. B, Voucher USNM 1606723. C, Voucher USNM 1606724. D, Voucher USNM 1606725. E, Voucher USNM 1606726. F, Voucher USNM 1606727. G, Voucher USNM 1606728.

Figs. 2–4 Adults of *Proterometra wigglewomble* n. sp. from the oesophagus of the blackbanded darter, *Percina nigrofasciata* (Agassiz, 1854) (Perciformes: Percidae) collected from the Cahaba River, Alabama, USA. 2, Ventral view of USNM 1606709. 3, Ventrolateral view of

USNM 1606710 showing the terminal male genitalia. 4, Ventral view of USNM 1606710 showing the ovarian complex. *Abbreviations:* dc, dextral caecum; dt, dextral testis; eb, oesophageal branch; ebl, excretory bladder; ed, ejaculatory duct; eg, egg; ep, excretory pore; ga, genital atrium; gp, genital pore; hp; hermaphroditic pore; Lc, Laurer's canal; Lp, Laurer's canal opening pore; m, metraterm; od, oviduct; oot, oötype; os, oral sucker; ova, ovary; ovs, oviduct sphincter; pf, transparent projecting filaments; ph, pharynx; pp, pars prostatica; ps, prostatic sac; sc, sinistral caecum; st, sinistral testis; sv, seminal vesicle; ut, uterus; va, vas efferent; ve, verschlussapparat; vd, vitelline duct; vf, vitelline follicles; vs, ventral sucker.

Figs. 5–8 Photomicrographs of live adults and cercariae of *Proterometra wigglewomble* n. sp. 5, Eggs from the distal portion of the uterus of an adult specimen showing eggs with wholly fimbriated surface, appearing colloquially as a cilia-like brush border. 6, An embryonated egg from the distal portion of the uterus of an adult showing miracidium. 7, Ventral view of the furca of a cercaria showing the translucent adhesive substance that has been secreted from the pleat-like tegument of the furca. 8, Ventral view of the distome of a cercaria with two eggs. *Abbreviations:* di, distome; dt, dextral testis; f, furca; fim, fimbria; mi, miracidium; ova, ovary; sl, secretory layer; st, sinistral testis; tas, translucent adhesive substance; vs, ventral sucker.

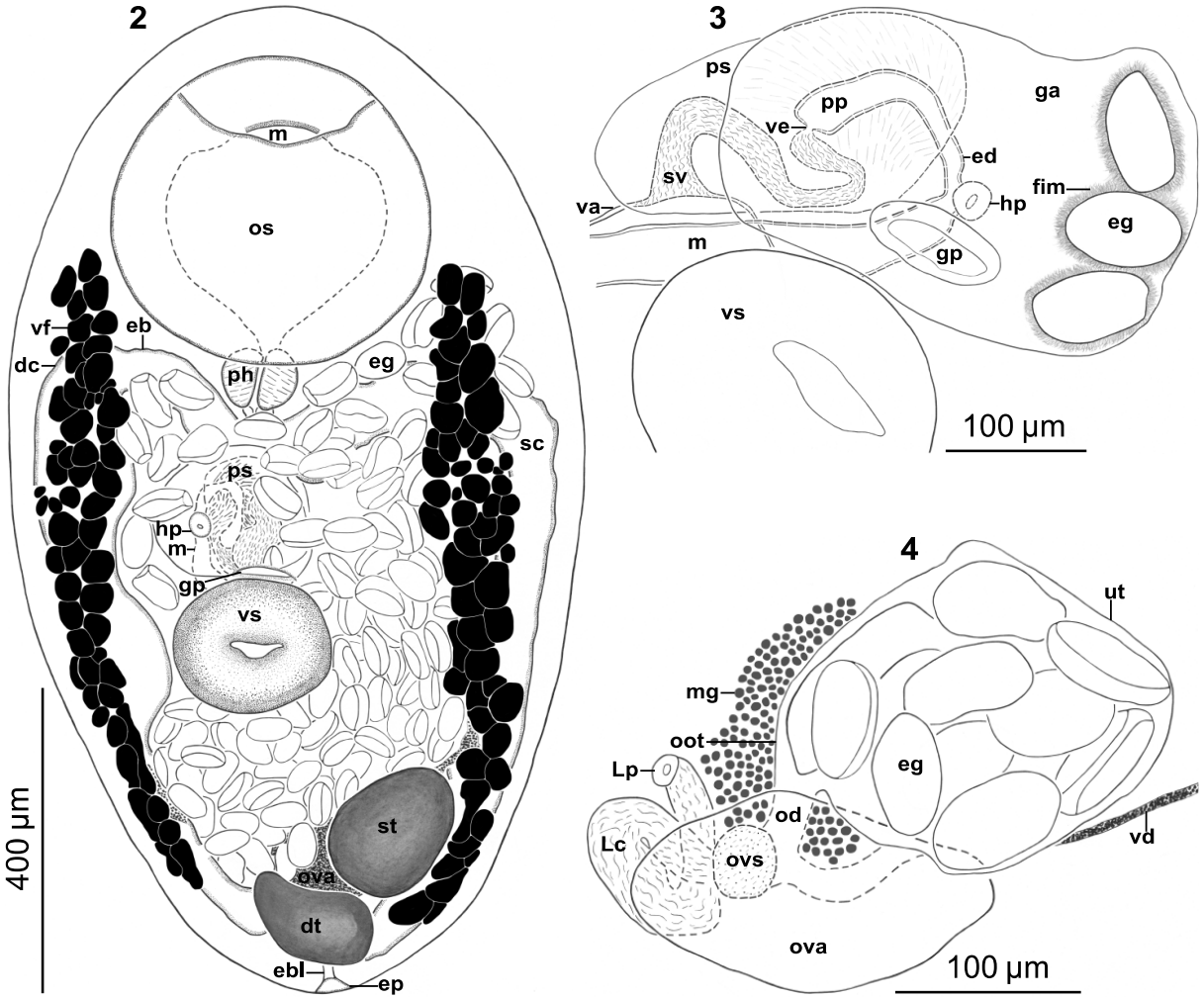
Figs. 9–14 Naturally-shed cercariae of *Proterometra wigglewomble* n. sp. from the compact elimia, *Elimia showalteri* (Lea, 1860) (Cerithioidea: Pleuroceridae) collected from the Cahaba River, Alabama, USA. 9, Ventral view of USNM 1606712 showing morphology and anatomical organ arrangement. 10, Ventral view of USNM 1606713 showing similar features to those in Fig. 9 but enantiomorphic arrangement of the testes. 11, Lateral view of USNM 1606714 showing the two terminal opening pores of the primary excretory canals (column of

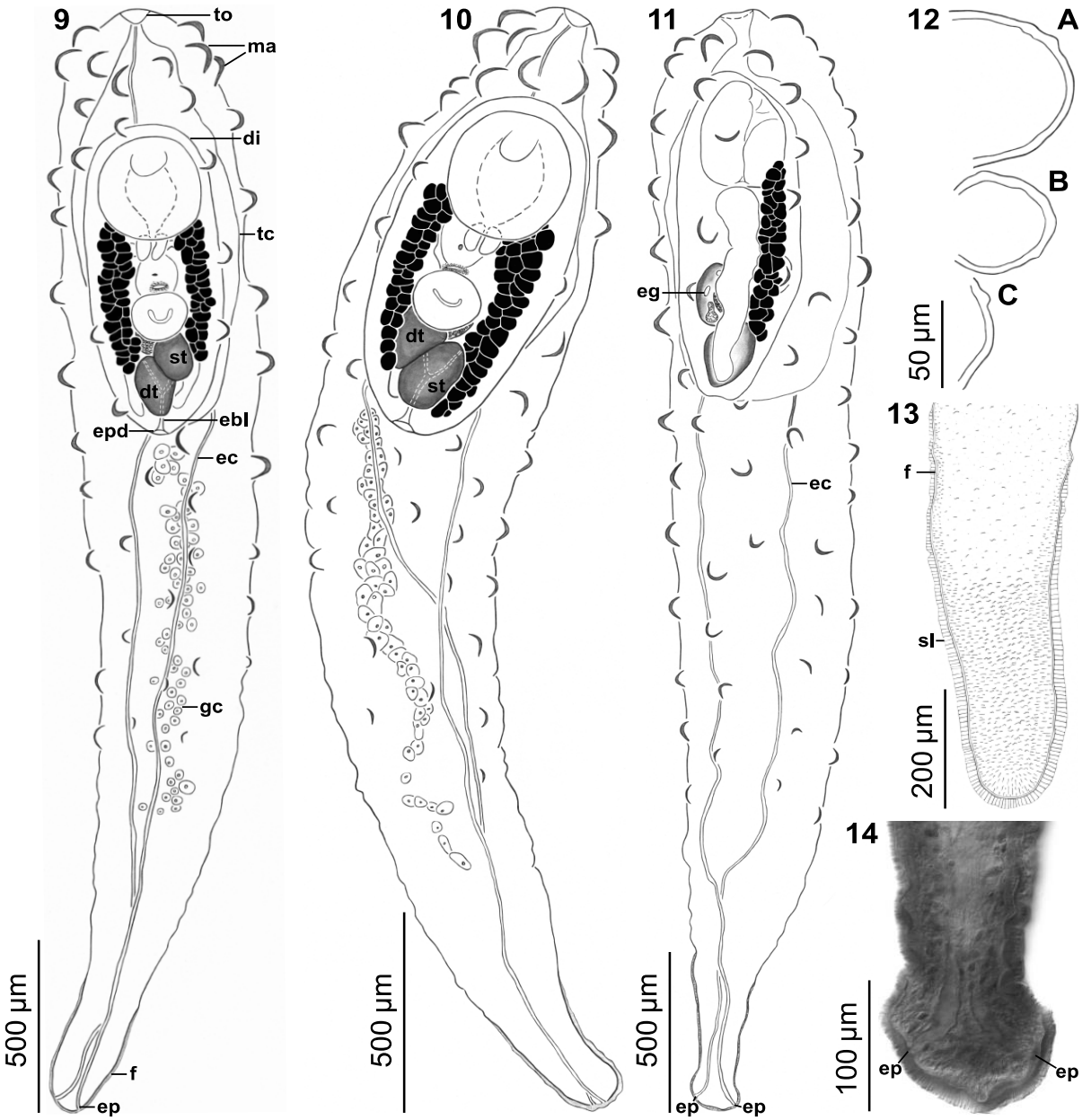
the gland cells not shown). 12, Tail stem mammillae, all with same scale bar; A, mammilla at the anterior end of the tail stem; B, mammilla immediately posterior to the distome; C, mammilla near the posterior end of the tail stem. 13, Ventral view of the furca of USNM 1606715 showing the pleat-like tegumental layer (secretory layer) that we theorize to secretes the adhesive. 14, Photomicrograph (lateral view) of the furca of USNM 1606714 showing the two terminal opening pores of the primary excretory collecting canals. *Abbreviations:* di, distome; dt, dextral testis; ebl, excretory bladder; ec, excretory collecting canal; ep, excretory pore; epd, excretory pore of distome; eg; egg; f, furca; gc, gland cells; ma, mammillae; ova, ovary; sl, secretory layer; st, sinistral testis; tc, tail stem cavity; to, tail stem cavity opening.

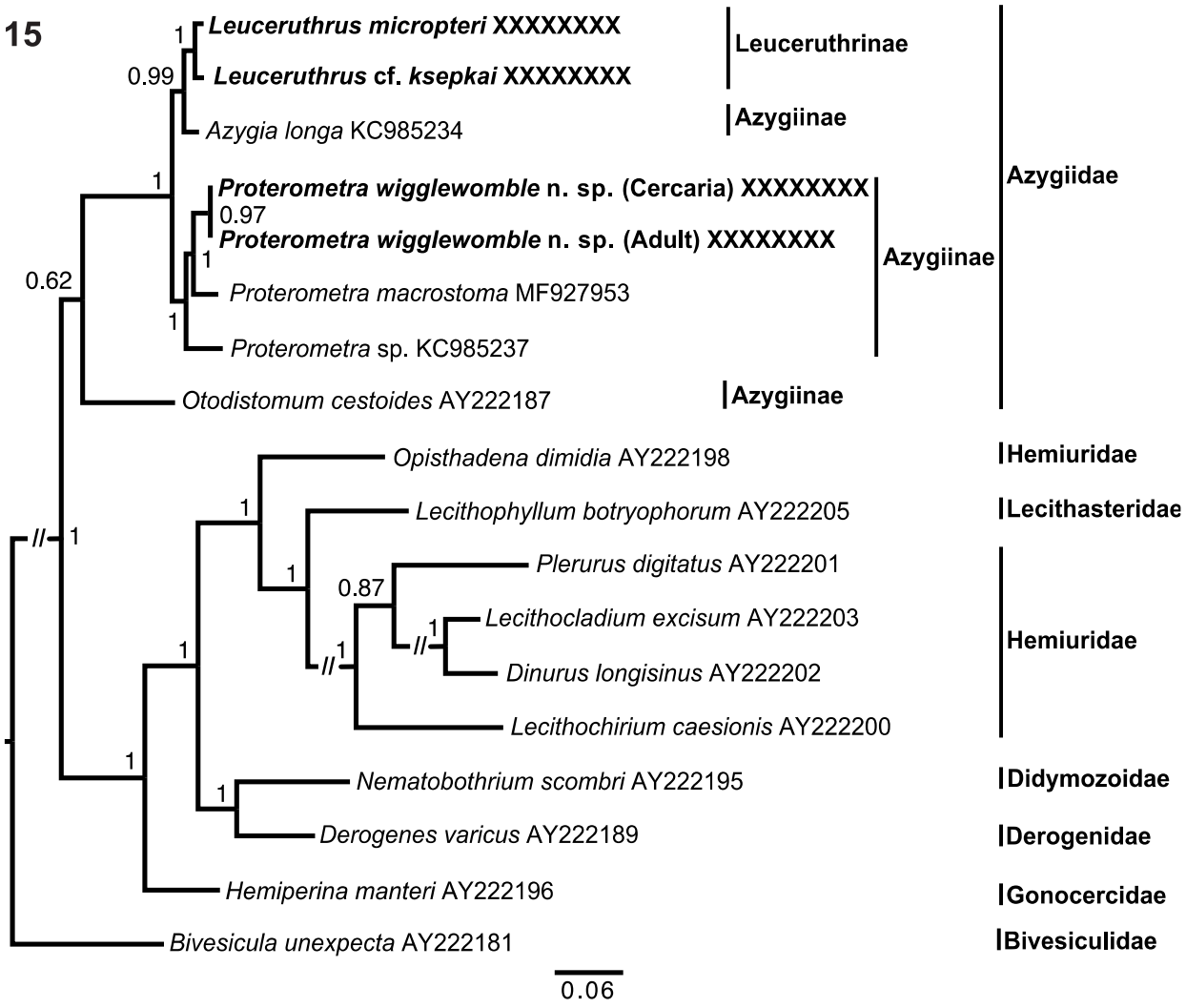
Fig. 15 *28S* phylogeny. Values aside nodes are posterior probability. Sequences in bold are those from the present study. Scale bar is in substitutions per site.

Fig. 16 *ITS2* phylogeny. Values aside nodes are posterior probability. Sequences in bold are those from the present study. Scale bar is in substitutions per site.


1
A
B
C
D
E
F
G
10 mm


2
m
os
vf
eb
dc
ph
eg
sc
ps
hp
m
gp
vs
st
ova
dt
ebl
ep
400 µm
3
ps
pp
ga
ve
ed
sv
fim
va
hp
eg
m
gp
vs
100 µm
4
ut
mg
oot
Lp
eg
od
vd
Lc
ovs
ova
100 µm


9
to
ma
di
tc
st
dt
epd
ebl
ec
gc
f
ep
500 µm
10
dt
st
500 µm
11
eg
ec
500 µm
ep
ep
12
A
B
C
50 µm
13
f
sl
200 µm
14
100 µm
ep
ep


15
Leuceruthrus micropteri XXXXXXXX
Leuceruthrus cf. ksepkai XXXXXXXX
Azygia longa KC985234
Proterometra wigglewomble n. sp. (Cercaria) XXXXXXXX
Proterometra wigglewomble n. sp. (Adult) XXXXXXXX
Proterometra macrostoma MF927953
Proterometra sp. KC985237
Otodistomum cestoides AY222187
Opisthadena dimidia AY222198
Lecithophyllum botryophorum AY222205
Plerurus digitatus AY222201
Lecithocladium excisum AY222203
Dinurus longisinus AY222202
Lecithochirium caesionis AY222200
Nematobothrium scombri AY222195
Derogenes varicus AY222189
Hemiperina manteri AY222196
Bivesicula unexpecta AY222181
Leuceruthrinae
Azygiinae
Azygiinae
Azygiinae
Azygiidae
Hemiuridae
Lecithasteridae
Hemiuridae
Didymozoidae
Derogenidae
Gonocercidae
Bivesiculidae
0.06

16

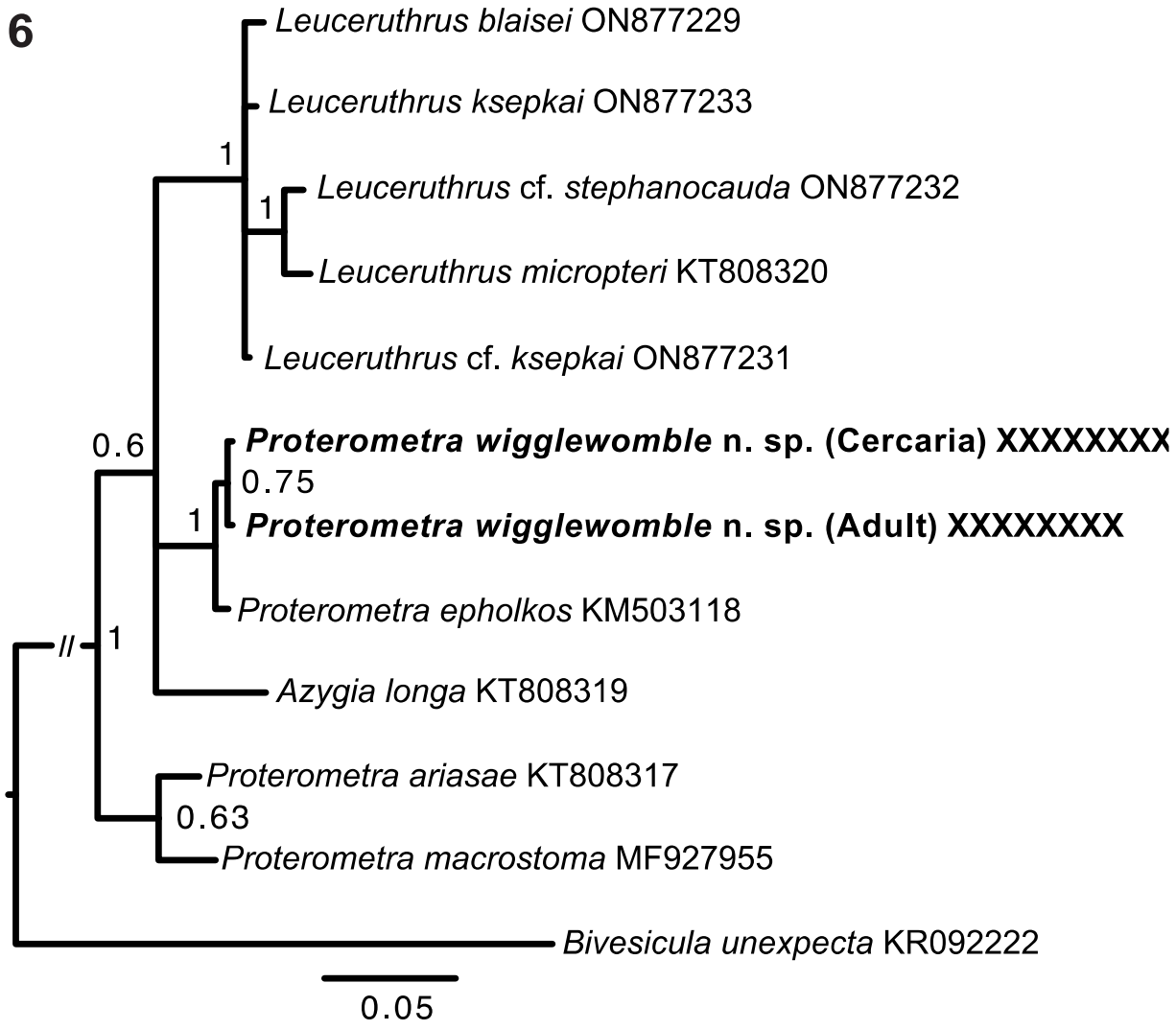

Leuceruthrus blaisei ON877229
Leuceruthrus ksepkai ON877233
1
Leuceruthrus cf. *stephanocauda* ON877232
1
Leuceruthrus micropteri KT808320
Leuceruthrus cf. *ksepkai* ON877231
0.6
***Proterometra wigglewomble* n. sp. (Cercaria) XXXXXXXX**
0.75
1
***Proterometra wigglewomble* n. sp. (Adult) XXXXXXXX**
Proterometra epholkos KM503118
//
1
Azygia longa KT808319
Proterometra ariasae KT808317
0.63
Proterometra macrostoma MF927955
Bivesicula unexpecta KR092222
0.05

Table 1 Pairwise comparisons among 28S sequences of seven azygiids and *Bivesicula unexpecta* (1247 bp; above the diagonal: percent nucleotide similarity; below the diagonal: nucleotide differences).

Species	*P. wigglewomble* n. sp.	*P. macrostoma*	*Proterometra* sp.	*L. micropteri*	*L.* cf. *ksepkai*	*A. longa*	*O. cestoides*	*B. unexpecta*
Proterometra wigglewomble n. sp.	–	96.8	95.5	95.2	95.2	95.2	86.1	78.4
Proterometra macrostoma	40	–	94.7	94.9	95.1	95.0	86.0	78.2
Proterometra sp.	56	66	–	94.2	94.1	94.7	86.3	77.8
Leuceruthrus micropteri	60	63	72	–	98.6	97.5	86.4	78.4
Leuceruthrus cf. *ksepkai*	60	61	73	17	–	97.4	86.2	78.2
Azygia longa	60	62	66	31	32	–	87.0	78.2
Otodistomum cestoides	173	174	170	169	171	162	–	77.7
Bivesicula unexpecta	269	271	277	269	271	272	278	–

Table 2 Pairwise comparisons among ITS2 sequences of ten azygiids and *Bivesicula unexpecta* (368 bp; above the diagonal: percent nucleotide similarity; below the diagonal: nucleotide differences).

Species	*P. wigglewomble* n. sp.	*P. ariasae*	*P. epholkos*	*P. macrostoma*	*L. blaisei*	*L. ksepkai*	*L. micropteri*	*L.* cf. *ksepkai*	*L.* cf. *stephanocauda*	*A. longa*	*B. unexpecta*
Proterometra wigglewomble n. sp.	–	93.4	99.3	92.6	93.7	94.0	92.6	93.7	92.8	93.1	67.4
Proterometra ariasae	23	–	93.3	96.0	92.5	92.5	91.7	92.3	91.4	91.4	68.8
Proterometra epholkos	3	24	–	93.0	93.0	93.3	92.4	93.0	92.1	93.4	67.7
Proterometra macrostoma	26	14	25	–	92.5	92.2	90.8	92.0	90.5	91.4	69.1
Leuceruthrus blaisei	22	26	25	26	–	99.1	97.1	98.9	97.4	92.3	67.6
Leuceruthrus ksepkai	21	26	24	27	3	–	97.4	99.1	97.7	92.6	67.3
Leuceruthrus micropteri	26	29	27	32	10	9	–	97.1	98.6	92.3	66.8
Leuceruthrus cf. *ksepkai*	22	27	25	28	4	3	10	–	97.4	92.3	67.2
Leuceruthrus cf. *stephanocauda*	25	30	28	33	9	8	5	9	–	92.0	66.5
Azygia longa	24	30	23	30	27	26	27	27	28	–	68.8
Bivesicula unexpecta	119	113	118	112	118	119	121	120	122	114	–

CHAPTER 6: LIFE CYCLE OF *LEUCERUTHRUS STEPHANOCAUDA* (FAUST, 1921) WOMBLE AND BULLARD, 2022 (DIGENEA: AZYGIIDAE), A NEW AZYGIID PHYLOGENY, AND COMBINED EVIDENCE OF LOW HOST SPECIFICITY TO ITS INVERTEBRATE AND VERTEBRATE HOSTS

***In review, Comparative Parasitology (Submitted 22 August 2023)**

Authors: Triet N. Truong, Nathan V. Whelan, and Stephen A. Bullard

Abstract

We elucidate the life cycle of *Leuceruthrus stephanocauda* (Faust, 1921) Womble and Bullard, 2022, provide the first description of its adult, and redescribe its cercaria based on specimens infecting centrarchiform and perciform fishes and pleurocerid snails in the southeastern United States. Based on morphological and nucleotide-based (*28S* and *ITS2*) evidence, adults of *L. stephanocauda* infect the stomach of spotted bass, *Micropterus punctulatus* (Rafinesque, 1819), green sunfish, *Lepomis cyanellus* Rafinesque, 1819, and longear sunfish, *Lepomis megalotis* (Rafinesque, 1820) from Chewacla Creek (Tallapoosa River, Alabama) and mottled sculpin, *Cottus bairdii* Girard, 1850 (juvenile) from Raccoon Creek (Chattooga River, Georgia). The adult of *L. stephanocauda* differs from its congeners by having an elongated body and an asymmetrical vitellarium. The cercaria of *L. stephanocauda* sheds from yellow elimia, *Elimia flava* (Lea, 1862) (Cerithioidea: Pleuroceridae) in Chewacla Creek and Moores Mill Creek (Tallapoosa River) and *Elimia caelatura georgiana* (Lea, 1862) in Raccoon Creek. Naturally-shed cercariae of *L. stephanocauda* are unique by having bilateral, discontinuous fields of black tail stem pigmentation posterior to the withdrawn distome and along the margins of the paired furcae, prominent subtriangular spines on the anterior end of the tail stem anterior and posterior to the distome, and broadly rounded to lanceolate furcae that are longer than wide and that bear numerous marginal and submarginal protuberances. Our *28S* phylogenetic analysis recovered *L. stephanocauda* sister to *Leuceruthrus micropteri* Marshall and Gilbert, 1905 within

monophyletic Leuceruthrinae Goldberger, 1911. No species of *Leuceruthrus* Marshall and Gilbert, 1905 was reported previously from the Tallapoosa River.

Species of *Leuceruthrus* Marshall and Gilbert, 1905 (Digenea: Azygiidae: Leuceruthrinae) have an adult with a lingulate body, an oral sucker that is larger than the ventral sucker, oblique and pre-ovarian testes, a straight ejaculatory duct, a uterus that is entirely inter-cecal, partly inter-testicular and between the ovary and the oral sucker, and a vitellarium that is primarily extra-cecal and restricted to the hindbody (Womble and Bullard, 2022). Womble and Bullard (2022) revised *Leuceruthrus*, accepted 3 previously-described species (*Leuceruthrus micropteri* Marshall and Gilbert, 1905 [type species], *Leuceruthrus ocalana* [Smith, 1935] Womble and Bullard, 2022 [*species inquirenda*], and *Leuceruthrus stephanocauda* [Faust, 1921] Womble and Bullard, 2022), and described 4 taxa (*Leuceruthrus blaisei* Womble and Bullard, 2022, *Leuceruthrus ksepkai* Womble and Bullard, 2022, *Leuceruthrus* cf. *ksepkai*, and *Leuceruthrus* cf. *stephanocauda*). *Leuceruthrus micropteri* and *L.* cf. *ksepkai* are the only congeners with a morphologically-described adult. The remaining species have been described based on their cercaria only (Faust, 1921; Horsfall, 1934; Smith, 1935; Womble and Bullard, 2022). Smith (1935) recovered adults of *L. ocalana* (as "*Cercaria stephanocauda ocalana* Smith, 1935") from natural and experimental infections but did not measure or draw the adults and (erroneously) considered them to be conspecific with *L. micropteri*. Womble and Bullard (2022) described the cercaria of *Leuceruthrus* cf. *stephanocauda*, which resembles *L. stephanocauda*, from Alabama as well as the adult of *Leuceruthrus* cf. *ksepkai* (recovered sister to *L. ksepkai* in a phylogenetic analysis of the internal transcribed spacer 2 [*ITS2*]) from Florida. *Leuceruthrus micropteri* is the only congener with a known life cycle. It asexually reproduces in several freshwater pleurocerids (*Elimia* Adams and Adams, 1854, *Leptoxis* Rafinesque, 1819, *Pleurocera*

Rafinesque, 1818) and matures in the stomach of endemic freshwater centrarchids (primarily black basses [*Micropterus* Lacepède, 1802] and sunfishes [*Lepomis* Rafinesque, 1819]) in eastern North America (Womble and Bullard, 2022). Species of *Leuceruthrus* have apparently non-progenetic (no cercaria of a species of *Leuceruthrus* reportedly has eggs) furcocystocercous cercariae that are macroscopic, flamboyantly swim, and are morphologically distinctive and can reliably diagnose species (Womble and Bullard, 2022). On the other hand, the adults of *Leuceruthrus* spp. are more challenging regarding their taxonomy because they are quite similar to each other and can be filled with eggs that obscure potentially taxonomically informative features associated with the male and female genitalia (Smith, 1935; Womble and Bullard, 2022). Nucleotide sequences of leuceruthrines are publicly available for *L. blaisei* (*ITS2*), *L.* cf. *ksepkai* (*ITS2*, partial *28S*), *L.* cf. *stephanocauda* (*ITS2*), *L. ksepkai* (*ITS2*), and *L. micropteri* (*ITS2, 28S*).

We herein use morphology and nucleotide sequences to elucidate the life cycle of *L. stephanocauda*, provide the first morphological description of the adult, supplement the morphological description of the cercaria from the southeastern United States, and provide an updated phylogenetic analysis for Azygiidae Lühe, 1909.

MATERIALS AND METHODS

Eighty *Elimia caelatura georgiana* (Lea, 1862) (Cerithioidea: Pleuroceridae) were collected by hand from Raccoon Creek (34°28′30.5″N, 85°24′40.1″W) (Chattooga River, Chattooga County, Georgia, USA) on 25 August 2021. A total of 287 yellow elimia, *Elimia flava* (Lea, 1862) (Cerithioidea: Pleuroceridae) were collected by hand from Moores Mill Creek (32°32′56.3″N, 85°28′41.2″W) and Chewacla Creek (32°32′09.8″N, 85°29′48.0″W) (both Tallapoosa River, Lee County, Alabama, USA) on 15 May 2022 and on 19 May 2022, respectively. Collected snails were kept alive in 5-gal buckets and coolers filled with aerated creek water before being transferred to Auburn University, Auburn, Alabama. In the

laboratory, snails were isolated and maintained as per Truong et al. (*in review*). Naturally-shed, free swimming cercariae from these snails were collected using a 5-ml transferred pipette, studied alive in a small stender dish, and subsequently heat-killed in hot water (~60°C), fixed in 10% neutral buffered formalin (n. b. f.), stained overnight in Van Cleave's hematoxylin mixed with several drops of Ehrlich's hematoxylin, dehydrated in an ethanol (EtOH) series, cleared in clove oil, and permanently mounted on glass slides using Canada balsam. Additional live cercariae were preserved directly in 95% EtOH for DNA extraction. The collected snails from Chewacla Creek and Moores Mill Creek were identified as *E. flava*, which is the only *Elimia* species in the lower Tallapoosa drainage (Figs. 1–5) (Whelan et al., 2022). The snails from Raccoon Creek were identified as *E. caelatura georgiana* by being from the Chattooga drainage and having an ovate aperture with pronounced lips, a moderately inflated body whorl, and vertical striae on the body whorl (Figs. 6–8). The most recent authorative taxonomic list for Pleuroceridae does not recognize subspecies, but the shells identified here as *E. caelatura georgiana* follow the subspecies concepts of Burch and Tottenham (1980). Furthermore, the shells are unique from other *E. caelatura* subspecies likely indicate a distinct species (Whelan et al., 2022).

Four mottled sculpins, *Cottus bairdii* Girard, 1850 (Perciformes: Cottidae) were collected by kick netting from Raccoon Creek (34°28′30.5″N, 85°24′40.1″W) on 25 August 2021. Three spotted basses, *Micropterus punctulatus* (Rafinesque, 1819), 2 green sunfish, *Lepomis cyanellus* Rafinesque, 1819, and 3 longear sunfish, *Lepomis megalotis* (Rafinesque, 1820) (all Centrarchiformes: Centrarchidae) were electrofished from Chewacla Creek (32°32′09.8″N, 85°29′48.0″W) on 19 May 2022. Collected fish were examined by screening the buccal cavity, esophageal sphincter, intestine, and stomach for azygiid infections using a Wild Heerbrugg M8 (Heerbrugg, Switzerland) stereo-dissecting microscope with aid of fiber optic light sources. Adult and juvenile azygiids were removed from the host using minutien

pins and fine forceps, transferred to a stender dish using a pipette, studied alive, heat-killed, and subsequently stained, fixed, mounted, and preserved the same as the aforementioned cercariae.

Fishes were identified as per Boschung and Mayden (2004). We identified our specimens as the mottled sculpin by having slightly connected dorsal fins, an incomplete lateral line, a uniformly pigmented chin, palatine teeth, relatively wide saddles, 9 infraorbital pores, 4 pelvic fin rays, and 14 pectoral fin rays. The spotted bass had an elongated body with a standard length ~3.6× longer than body depth, an upper jaw not extending beyond the posterior margin of the eye, a dark lateral band with dark blotches, an emarginated caudal fin, a shallow notch separating the soft dorsal fin and spinous dorsal fin, the longest dorsal spine ~1.6× length of the last dorsal spine, 3 anal fin spines, 72 lateral scale rows, 17 scale rows on the cheek, 23 circumpeduncular scales, and 7 and 12 scale rows above and below the lateral line, respectively. The green sunfish had a standard length ~2.3× longer than the body depth, a body depth ~ equal to the distance between the tip of the snout to the anterior margin of the dorsal fins, cheek and preopercular areas that lacked distinct bars radiating from the eye, an emarginated caudal fin, a rounded pectoral fin with 15 rays not extending beyond the anterior margin of the eye, long and slender gill rakers on the first arch, a short membranous opercular tab with a white border, a tongue without teeth, 49 lateral scale rows, and 3 anal fin spines. The longear sunfish had a standard length ~2.2× the body depth, cheek and preopercular areas lacking distinct bars radiating from the eye, an emarginated caudal fin, a rounded pectoral fin with 15 rays not extending beyond the anterior margin of the eye, short and stubby gill rakers on first arch, an elongated membranous opercular tab with a white border, a lateral line with 37 scale rows and lacking a red streak, a tongue without teeth, 5 scale rows on the cheek, and 3 anal fin spines.

Line drawings were made using an Olympus BX51 microscope (Olympus Corporation of the Americas, Center Valley, Pennsylvania, USA) equipped with differential interference contrast optical components (D. I. C.) and a drawing tube. Measurements were made using the camera software Jenoptik Gryphax® version 2.1.0.724 (Jena, Germany) and reported in micrometers (μm) as the range followed by the mean ± standard deviation. Taxonomic authorities for fishes follow Fricke et al. (2023). Anatomical terms for azygiids follow Truong et al. (*in review*). Vouchers of *L. stephanocauda* (3 adults, 2 juveniles, and 5 cercariae) and 5 and 3 shell vouchers of *E. flava* and *E. c. georgiana*, respectively, were deposited in the National Museum of Natural History's Invertebrate Zoology Collection (Smithsonian Institution, Washington, D.C., USA).

Four adult azygiids (2 from 1 spotted bass, 1 from green sunfish, and 1 from longear sunfish; all from Chewacla Creek), 4 naturally-shed cercariae from 2 *E. flava* individuals (Moores Mill Creek), and 1 naturally-shed cercaria from *E. flava* (Chewacla Creek) were used separately (as replicates) to extract genomic DNA. One juvenile azygiid from mottled sculpin and 2 naturally-shed azygiid cercariae from 2 *E. c. georgiana* (both from Raccoon Creek) were also used for DNA extraction. DNA extraction, PCR reactions, and sequencing of azygiids in the present study were performed as per Truong et al. (*in review*). Additional *28S* sequences of *Proterometra ariasae* Womble, Orélis-Ribeiro, and Bullard, 2016 were generated from 2 naturally-shed, morphologically-identified cercariae from the smooth hornsnail, *Pleurocera prasinata* (Conrad, 1834) (Cerithioidea: Pleuroceridae) collected from the Cahaba River (33°10′25.0″N, 87°01′30.6″W) on 5 May 2022.

Taxon and outgroup selection for the present *28S* phylogenetic analysis were based on Truong et al. (*in review*) (11 sequences total): *Azygia longa* (Leidy, 1851) Manter, 1926 (KC985234), *L.* cf. *ksepkai* (OR470684), *L. micropteri* (OR470685), 2 of *L. stephanocauda* (1 cercaria from Raccoon Creek, 1 adult from Chewacla Creek), *Otodistomum cestoides* (Van

Beneden, 1870) Odhner, 1911 (AY222187), *P. ariasae* (XXXXXXXX), *Proterometra macrostoma* (Faust, 1918) Horsfall, 1933 (MF927953), *Proterometra* sp. (KC985237), a new species of *Proterometra* described in Truong et al. (*in review*) (OR470686), and *Bivesicula unexpecta* Cribb, Bray, and Barker, 1994 (AY222181; outgroup).

Analyzed *28S* sequences were aligned using MAFFT (Katoh and Standley, 2013) with the –auto flage and default paramterts. Sequence comparison was made with Geneious prime version 2023.0.4 (Biomatters Inc., Boston, Massachusetts, USA). The *28S* tree was inferred using MrBayes version 3.25. The best-fit model was inferred with JModelTest 2.1.10 (Darriba et al., 2012). Tree inference used 3 independent runs, each with 4 Metropolis couples chains and run for 5,000,000 generations, sampling the posterior distribution every 1,000 generations. Model averaging and a gamma distribution to model rate-heterogeneity were used for substitions models. All other parameters and priors were set to defaults. Evidence for convergence was visualized with Tracer 1.7 (Rambaut et al., 2018) and further examined with the MrBayes sump command. Convergence was assumed to have occurred when trace plots among runs overlapped and effective samples size for each parameter was at least 200. Inferred phylogenetic tree was visualized using FigTree v1.4.4 (Rambaut et al., 2014) and further edited for visualization purposes with Adobe Illustrator 27.7 version (2023) (Adobe Systems, San Jose, California, USA).

RESULTS

Azygiidae Lühe, 1909

Leuceruthrus Marshall and Gilbert, 1905

Leuceruthrus stephanocauda [Faust, 1921] Womble and Bullard, 2022

(Figs. 9–18)

Description

Diagnosis of adult (based on live specimens and light microscopy of 5 stained, whole-mounted specimens): (Figs. 9–12).

Body lingulate in outline, having broadly rounded anterior end and tapering posterior end, typically widest in posterior half of forebody, occasionally widest in anterior half of hindbody, having numerous transverse plications throughout body, 4074–7777 (5983 ± 1610) long, 1165–2291 (1740 ± 505) wide, 3.1–3.9× (3.5 ± 0.3) longer than wide (Fig. 9); live specimens pink to milky white in color. Tegument aspinous, thickly muscular, 30–66 (45 ± 14) thick. Forebody 1565–2686 (2127 ± 476) long (33–38% [36 ± 2] of body length); hindbody 2016–4301 (3209 ± 979) long (49–55% [53 ± 2] of body length), 1.3–1.7× (1.5 ± 0.2) longer than forebody (Fig. 9). Oral sucker subspherical, ventrally subterminal, 748–1230 (982 ± 229) long (15–18% [17 ± 1] of body length), 728–1179 (956 ± 214) wide (51–63% [56 ± 4] of body width), 1.0–1.1× (1.0 ± 0.0) longer than wide, 1.4–1.5× (1.5 ± 0.0) longer than ventral sucker, 1.3–1.4× (1.4 ± 0.1) wider than ventral sucker, 152–254 (200 ± 39) from anterior body end (2–4% [3 ± 1] of body length), 3170–6381 (4775 ± 1379) from posterior body end (78–82% [79 ± 2] of body length) (Fig. 9). Ventral sucker subspherical, in anterior half of body, 492–817 (658 ± 147) long (10–13% [11 ± 1] of body length), 547–914 (706 ± 160) wide (38–47% [41 ± 4] of body width), 1.0–1.1× (1.1 ± 0.0) wider than long, 653–1340 (939 ± 285) from posterior margin of oral sucker (13–17% [16 ± 2] of body length) (Fig. 9). Mouth subterminal, opening ventrally in anterior half of oral sucker, directing anteriad. Pre-pharyngeal esophagus apparently absent. Pharynx ovoid, dorsally overlapping posterior margin of oral sucker, 209–379 (286 ± 69) long (4–5% [5 ± 1] of body length), 224–386 (307 ± 78) wide (16–19% [18 ± 1] of body width) (Fig. 9). Esophagus originating from mouth, immediately bifurcating posterior to pharynx; esophageal branches extending slightly anterolaterad from pharynx; dextral branchial esophagus 157–220 (182 ± 27) long (3–4% [3 ± 1] of body length), maximum width 86–153 (118 ± 34); sinistral branchial esophagus 134–

197 (172 ± 27) long (3–5% [3 ± 1] of body length), maximum width 83–137 (108 ± 27) (Fig. 9). Ceca extending posteriad to near posterior body end; dextral cecum 3075–6766 (4886 ± 1585) long (75–87% [81 ± 5] of body length), maximum width 120–254 (172 ± 64); sinistral cecum 3210–6586 (4963 ± 1437) long (79–85% [83 ± 2] of body length), maximum width 112–263 (171 ± 74); pre-cecal space 817–1203 (1044 ± 166) (15–20% [18 ± 2] of body length); post-cecal space 324–501 (423 ± 79) (5–9% [7 ± 1] of body length) (Fig. 9).

Testes 2 in number, ovoid, diagonal or nearly opposite, inter-cecal, pre-ovarian, wholly posterior to or dorsally overlapping ventral sucker; dextral testis either anterior or posterior to sinistral testis, 327–513 (414 ± 71) long (6–9% [7 ± 1] of body length), 273–402 (350 ± 49) wide (18–30% [21 ± 5] of body width); sinistral testis 286–493 (393 ± 87) long (5–9% [7 ± 1] of body length), 237–421 (336 ± 85) wide (18–21% [20 ± 1] of body width); pre-testicular space 1872–3357 (2538 ± 597) (36–46% [43 ± 4] of body length); post-testicular space 1647–3762 (2828 ± 960) (40–53% [47 ± 5] of body length) (Fig. 9). Vasa efferentia and vas deferens not observed. Prostatic sac ovoid, median, immediately pre-acetabular, 235–617 (426 ± 197) long (6–10% [7 ± 2] of body length), 194–494 (344 ± 163) wide (17–25% [21 ± 5] of body width), 1327–2092 (1626 ± 360) from anterior body end (27–33% [29 ± 3] of body length), 2538–5085 (3648 ± 1178) from posterior body end (62–65% [64 ± 1] of body length) (Figs. 9, 10). Internal seminal vesicle highly convoluted, swollen for entire length, 645 long, 69 wide (Figs. 9, 10). Pars prostatica thick-walled, positioned within anterior half of prostatic sac, slightly curved, swollen proximally, becoming narrower distally; proximal end communicating with distal portion of seminal vesicle via short verschlussapparat organ; distal end becoming indistinguishable from proximal end of ejaculatory duct in ventrally and dorsally whole-mounted specimens (Fig. 10). Ejaculatory duct unarmed, narrow along entire length, opening distally to genital atrium via hermaphroditic pore. Hermaphroditic duct, sinus organ, and prostatic gland cells not evident. Hermaphroditic pore anterior to ventral sucker,

at level of middle of prostatic sac. Genital atrium large, pre-acetabular, circular in outline, ventromedian to prostatic sac, capable to enlarge and contain numerous eggs, 134–657 (385 ± 215) wide (11–29% [19 ± 8] of body width). Genital pore pre-acetabular, at level of posterior half of prostatic sac, 1486–2513 (1991 ± 438) from anterior body end (32–36% [34 ± 2] of body length (Figs. 9, 10).

Ovary ovoid, median, inter-cecal, at level of middle of hindbody, separated from testes by uterine coils, 409–2020 (1090 ± 697) posterior to posterior margin of testes (10–26% [18 ± 7] of body length), 236–370 (282 ± 76) long (5–6% [6 ± 0] of body length), 228–379 (280 ± 86) wide (18–20% [19 ± 1] of body width); pre-ovarian space 2982–4744 (3619 ± 977) long (69–74% [72 ± 3] of body length); post-ovarian space 942–1407 (1178 ± 211) long (17–24% [21 ± 3] of body length) (Figs. 9, 11). Oviduct primarily dorsal to ovary, winding and extending anteriorly from oviducal sphincter. Oviducal sphincter dorsal to anterior half of ovary, connecting to the proximal end of oviduct via short, constricted duct, 71 long, 74 wide (Fig. 11). Oötype and Mehlis' gland cells not evident. Laurer's canal median, dorsal to ovary, originating from proximal portion of oviduct immediately posterior to oviducal sphincter, looping at level of middle of ovary before opening dorsally; Laurer's canal pore dorsomedian to ovary, posterolateral to oviducal sphincter (Fig. 11). Uterine seminal receptacle pre-ovarian, filling with sperm within short proximal portion of uterus anteroventral to ovary (Fig. 12). Uterus wholly inter-cecal, comprising numerous transverse loops between gonads, occupying space between ovary and ventral sucker, extending anteriad between testes before connecting with terminal genitalia dorsal to ventral sucker; uterine field 1202–3782 (2393 ± 1118) long (30–53% [38 ± 10] of body length), 271–1278 (756 ± 407) wide (22–56% [41 ± 14] of body width); metraterm unarmed, ventral to prostatic sac, extending anteriad to genital atrium dorsal to ventral sucker, merging distally with ejaculatory duct, 244–562 (442 ± 173) long (6–9% [7 ± 1] of body length), 31–36 (34 ± 3) wide (Fig. 9). Uterine eggs ovoid, having

smooth surface (fimbria apparently absent), unembryonated, numerous, slightly varying in sizes within uterus; eggs in proximal portion of uterus 56–71 (61 ± 5) long, 28–40 (33 ± 4) wide; eggs in distal portion of uterus and genital atrium 59–73 (68 ± 4) long, 29–39 (34 ± 3) wide (Fig. 9). Vitellarium follicular, comprising 2 lateral fields, wholly ventral and primarily extra-lateral to ceca, asymmetrical in length, extending anteriad from posterior half or posterior margin of oral sucker posteriad to near posterior body end, not to level of cecal tips; longer vitelline field either dextral or sinistral in body, 1523–3694 (2554 ± 937) long (37–47% [42 ± 5] of body length); shorter vitelline field 1275–3380 (2215 ± 821) long (31–43% [36 ± 4] of body length, 78–92% [87 ± 6] of longer vitelline field length); pre-vitelline space 2077–3496 (2711 ± 617) long (42–51% [46 ± 3] of body length); post-vitelline space 492–1027 (739 ± 248) long (8–16% [13 ± 3] of body length); distance between anterior ends of vitelline fields 600–1243 (921 ± 280) long (48–57% [53 ± 3] of body width); distance between posterior ends of vitelline fields 304–916 (531 ± 252) long (24–40% [29 ± 6] of body width) (Fig. 9). Transverse vitelline ducts nearly symmetrical, extending slightly posteromedian before fusing to form vitelline reservoir; dextral transverse vitelline duct 311–331 (321 ± 14) long, branching 802–1745 (1226 ± 422) from anterior end of dextral vitelline field (52–60% [55 ± 4] of dextral vitelline field length); sinistral transverse vitelline duct 197–312 (255 ± 81) long, branching 797–2127 (1284 ± 598) from anterior end of sinistral vitelline field (53–63% [57 ± 4] of sinistral vitelline field length) (Fig. 9). Vitelline reservoir median, dorsal to anterior half ovary, 119–135 (127 ± 11) long, 19–92 (56 ± 52) wide. Ovovitelline duct dorsal to ovary, slightly curved, originating sinistrally from vitelline reservoir, extending anterosinistrad before communicating to distal oviduct immediately anterior to ovary (Fig. 11).

Excretory system Y-shaped; main stem of bladder slender posteriorly for most of its length, swollen anteriorly, extending anteriad 823–1108 (958 ± 154) from pore (14–20% [17

± 3] of body length) before bifurcating in post-ovarian space; excretory bifurcation inter-cecal, 112–284 (199 ± 75) posterior to ovary (1–5% [4 ± 2] of body length); collecting ducts extending anteriad bilaterally to body, anterior extent difficult to trace; pore terminal (Fig. 9).

Diagnosis of juveniles (based on live specimens and light microscopy of 2 stained, whole-mounted specimens): (Fig. 13).

Body lingulate in outline, having broadly rounded anterior end and tapering posterior end, widest in posterior half of forebody, having numerous transverse plications throughout body, 1840–1927 long, 866–909 wide, 2.1× longer than wide; live specimens light pink in color. Tegument aspinous, thickly muscular, 24–25 thick. Forebody 767–888 long (42–46% of body length), 1.0–1.3× longer than hindbody; hindbody 674–748 long (35–41% of body length). Oral sucker subspherical, ventrally subterminal, approximately equal in length and width, 467–479 long (24–26% of body length), 474–487 wide (54–55% of body width), 1.3–1.5× longer than ventral sucker, 1.2–1.3× wider than ventral sucker, 93–110 from anterior body end (5–6% of body length), 1264–1382 from posterior body end (69–72% of body length). Ventral sucker subspherical, approximately in middle of body, 328–370 long (18–19% of body length), 371–408 wide (43–45% of body width), 1.1× wider than long, 188–342 from posterior margin of oral sucker (10–18% of body length). Mouth subterminal, opening ventrally in anterior half of oral sucker, directing anteriad. Pre-pharyngeal esophagus apparently absent. Pharynx ovoid, dorsally overlapping posterior margin of oral sucker, 188–194 long (10% of body length), 169–175 wide (19–20% of body width). Esophagus originating from mouth, immediately bifurcating posterior to pharynx; esophageal branches extending anteriad from pharynx; dextral branchial esophagus 153 long (8% of body length); sinistral branchial esophagus 204 long (11% of body length). Ceca extending posteriad to near posterior body end; dextral cecum 1212–1328 long (66–69% of body length), maximum width 146–189; sinistral cecum 1242–1290 long (64–70% of body length), maximum width

174–216; pre-cecal space 402–489 long (21–27% of body length); post-cecal space 215–263 long (11–14% of body length).

Testes 2 in number, ovoid, diagonal or nearly opposite, inter-cecal, pre-ovarian, dorsally overlapping ventral sucker; dextral testis either anterior or posterior to sinistral testis, 147–186 long (8–10% of body length), 128–155 wide (15–17% of body width); sinistral testis 186–190 long (10% of body length), 139–145 wide (16% of body width); pre-testicular space 918–1080 long (50–56% of body length); post-testicular space 525–699 long (27–38% of body length). Vasa efferentia and vas deferens not evident. Prostatic sac ovoid, median, immediately pre-acetabular, 115–120 long (6% of body length), 97–117 wide (11–13% of body width), 639–739 from anterior body end (35–38% of body length), 1070–1091 from posterior body end (56–59% of body length). Internal seminal vesicle highly convoluted, filled with sperm; its course difficult to trace. Pars prostatica, ejaculatory duct, hermaphroditic duct, hermaphroditic pore, sinus organ, and prostatic gland cells not evident. Genital atrium large, circular in outline, overlapping posterior half of prostatic sac and anterior margin of ventral sucker, 117–124 wide (13–14% of body width). Genital pore immediately pre-acetabular, 724–866 from anterior body end (39–45% of body length).

Ovary ovoid, median, inter-cecal, approximately at middle of hindbody, 43–97 from posterior margin of testes (2–5% of body length), 128–133 long (7% of body length), 81–119 wide (9–13% of body width); pre-ovarian space 1191–1509 long (65–78% of body length); post-ovarian space 307–532 long (16–29% of body length). Oviducal sphincter, oviduct, oötype, Mehlis’ gland cells, Laurer’s canal, Laurer’s canal pore, ovovitelline duct, and uterine seminal receptacle not evident. Uterus wholly inter-cecal, appearing as some loops between gonads, extending anteriad between testes before connecting with genital atrium dorsal to ventral sucker; metraterm not evident. Uterine eggs and vitellarium not developed in juveniles. Excretory system indistinct; pore terminal.

Supplemental observations of cercaria (based on live and formalin-fixed specimens; light microscopy of 20 stained, whole-mounted, naturally-shed cercariae): (Figs. 14–18).

Cercaria furcocystocercous, comprising a tail stem, a withdrawn distome occupying anterior end of tail stem, and paired furcae attaching to posterior end of tail stem, 3209–3916 (3550 ± 204) in total length (= tail stem + furca), 389–511 (445 ± 33) in maximum width at level of distome, 6.5–9.1× (8.0 ± 0.7) longer than wide (Figs. 14–16); live specimens light amber in color, shedding in early morning or occasionally in early afternoon, actively swimming immediately after shedding. Tail stem claviform in outline, apparently lacking mammillae and granular gland cells, rimmed at approximately middle of tail stem, 2718–3264 (3014 ± 158) long (82–87% [85 ± 1] of total cercarial length), 5.6–7.8× (6.8 ± 0.6) longer than wide, comprising anterior and posterior portions; anterior portion cylindrical, spinous, enclosing withdrawn distome, having tegumental plications for almost its entire length; spines subtriangular, approximately equal in sizes, 21–43 (31 ± 6) long, 21–50 (33 ± 7) wide, encircling tail stem in 1–3 irregular rows at anterior end of distome and in 1 row on rim of tail stem near posterior end of distome; posterior portion posterior to distome, dorsoventrally flat, distinct from anterior portion, aspinous, having black pigmentation (visible on live and formalin-fixed specimens, appearing absent on conventional whole-mounted specimens) and minute protuberances distributed bilaterally for almost its entire length; pigmentation distributed in discontinuous fields, 1625–1865 (1677 ± 93) long (46–51% [49 ± 3] of tail stem length, 39–44% [42 ± 2] of total cercarial length) (Figs. 14–16). Tail cavity not evident. Tail cavity terminal, opening anteromediad (Figs. 14, 15).

Furcae paired, broadly rounded to lanceolate, dorsoventrally flat, symmetrical in shape and length, bearing protuberances; margins of each furca entirely serrated and pigmented; protuberances minute, numerous, distributed on margins and submarginal regions of each furca; ventral furca 453–641 (535 ± 57) long (13–19% [15 ± 1] of total cercarial length),

310–477 (378 ± 48) in maximum width (63–106% [85 ± 13] of maximum cercarial width), 1.2–1.7× (1.4 ± 0.1) longer than wide; dorsal furca 456–622 (532 ± 55) long (13–18% [15 ± 1] of total cercarial length), 307–451 (376 ± 41) in maximum width (63–102% [85 ± 12] of maximum cercarial width), 1.2–1.6× (1.4 ± 0.1) longer than wide (Figs. 14–18).

Excretory system of cercaria comprising a main collecting duct, extending posteriad along tail stem before bifurcating into 2 secondary ducts at synthesis of furcae and tail stem; each secondary excretory duct extending along midline of each furca, opening at furcal tip; pore terminal to each furca (Figs. 14–16).

Distome aspinous, lingulate in outline, having broadly rounded ends, entirely withdrawn into anterior end of tail stem in naturally-shed cercaria, 1289–1660 (1465 ± 98) long (36–47% [41 ± 3] of total cercarial length), 345–483 (401 ± 39) wide (77–98% [90 ± 5] of maximum cercarial width), 2.7–4.2× (3.7 ± 0.4) longer than wide, 29–93 (55 ± 18) from anterior end of tail stem (1–2% [2 ± 0] of total cercarial length) (Figs. 14–16). Forebody 653–838 (759 ± 54) long (49–54% [52 ± 1] of distome length), 1.3–1.8× (1.6 ± 0.1) longer than hindbody; hindbody 413–616 (480 ± 47) long (30–37% [33 ± 2] of distome length). Oral sucker subspherical, ventrally subterminal, 274–346 (307 ± 19) long (18–23% [21 ± 1] of distome length), 261–317 (286 ± 16) wide (57–81% [72 ± 7] of distome width), 0.9–1.2× (1.1 ± 0.1) longer than wide, 1.1–1.5× (1.4 ± 0.1) longer than ventral sucker, 1.2–1.4× (1.3 ± 0.1) wider than ventral sucker, 28–62 (44 ± 9) from anterior end of distome (2–5% [3 ± 1] of body length) (Figs. 14–16). Ventral sucker subspherical, 202–254 (228 ± 16) long (14–18% [16 ± 1] of distome length), 206–237 (222 ± 9) wide (48–63% [56 ± 5] of distome width), 0.9–1.2× (1.0 ± 0.1) longer than wide, 300–468 (408 ± 46) from posterior margin of oral sucker (23–31% [28 ± 2] of body length) (Figs. 14–16). Mouth subterminal, opening ventrally at anterior half of oral sucker, directing anteriad. Pre-pharyngeal esophagus apparently absent. Pharynx ovoid, dorsally overlapping oral sucker, 106–134 (122 ± 7) long

(7–9% [8 ± 1] of distome length), 84–104 (94 ± 5) wide (20–26% [24 ± 2] of distome width). Esophagus originating from mouth, extending posteriad before immediately bifurcating posterior to pharynx into 2 anterolateral branches; dextral branch of esophagus 82–198 (159 ± 30) long (5–14% [11 ± 2] of distome length); sinistral branch of esophagus 67–210 (165 ± 40) long (5–14% [11 ± 3] of distome length). Ceca extending anteriad to level of middle of oral sucker before extending posteriad to near posterior body end; dextral cecum 973–1419 (1266 ± 108) long (75–92% [86 ± 4] of distome length), 54–101 (78 ± 12) wide; sinistral cecum 1068–1445 (1266 ± 112) long (80–93% [86 ± 3] of distome length), 57–91 (72 ± 9) wide; pre-cecal space 125–281 (184 ± 46) (8–22% [13 ± 3] of distome length); post-cecal space 50–99 (76 ± 14) (3–7% [5 ± 1] of distome length) (Figs. 14, 15).

Testes 2 in number, ovoid, diagonal or nearly symmetrical, inter-cecal, overlapping posterior margin of or wholly posterior to ventral sucker; dextral testis either anterior or posterior to sinistral testis, 42–74 (57 ± 9) long (3–5% [4 ± 1] of distome length), 34–63 (46 ± 8) wide (9–15% [12 ± 2] of distome width); sinistral testis 44–69 (58 ± 8) long (3–5% [4 ± 1] of distome length), 29–69 (47 ± 9) wide (8–16% [12 ± 2] of distome width); pre-testicular space 829–1092 (988 ± 77) (64–71% [67 ± 2] of distome length); post-testicular space 310–456 (380 ± 41) (22–29% [26 ± 2] of distome length) (Figs. 14, 15). Prostatic sac subspherical, immediately anterior to ventral sucker, 64–96 (78 ± 7) long (5–6% [5 ± 0] of distome length), 50–77 (68 ± 8) wide (12–20% [17 ± 2] of distome width), 547–763 (681 ± 54) from anterior end of distome (43–49% [46 ± 2] of distome length), 624–851 (710 ± 54) from posterior end of distome (46–51% [48 ± 1] of distome length); vasa efferentia, vas deferens, seminal vesicle, verschlussapparat organ, pars prostatica, ejaculatory duct, hermaphroditic duct, and sinus organ not evident (Figs. 14, 15). Genital atrium circular in outline, pre-acetabular, ventromedian to prostatic sac, 32–60 (46 ± 7) wide (8–15% [11 ± 2]

of distome width). Genital pore pre-acetabular, at mid-level of prostatic sac, 616–787 (710 ± 50) from anterior end of distome (46–51% [49 ± 2] of distome length) (Figs. 14, 15).

Ovary ovoid, inter-cecal, post-testicular, 35–130 (83 ± 26) posterior to posterior margin of testes (2–8% [6 ± 2] of distome length), 49–79 (68 ± 9) long (10–18% [13 ± 2] of distome length), 35–52 (45 ± 5) wide (21–31% [27 ± 2] of distome width); pre-ovarian space 996–1305 (1168 ± 88) (74–83% [80 ± 2] of distome length); post-ovarian space 181–293 (232 ± 29) (13–21% [16 ± 2] of distome length) (Figs. 14, 15). Oviduct, oötype, Mehlis' gland, Laurer's canal, ovovitelline duct, uterine seminal receptacle, and metraterm not evident. Uterus pre-ovarian, apparently lacking eggs (= cercaria not progenetic), appearing as a straight or winding thin-walled duct, partly inter-testicular, extending anteriad to level of genital atrium dorsal to ventral sucker; metraterm not evident (Figs. 14, 15). Vitellarium not developed in cercarial distome.

Excretory system of distome Y-shaped; main stem slender for its entire length, 178–284 (225 ± 24) long (12–20% [15 ± 2] of distome length), bifurcating into 2 collecting ducts approximately at level of rim of tail stem; excretory bifurcation inter-cecal, 7–34 (19 ± 8) posterior to posterior margin of ovary (1–2% [1 ± 0] of distome length); collecting ducts extending anteriad laterally, anterior extent difficult to trace; excretory pore terminal (Figs. 14, 15).

Taxonomic summary

Type host: No type host designated. Faust (1921) did not mention which snail species was infected by *L. stephanocauda*. He examined both the sharp-crest elimia, *Elimia carinifera* (Lamarck, 1822) (as *Goniobasis carinifera* Lamarck, 1822) and the crested mudalia, *Leptoxis carinata* (Bruguière, 1789) (as *Anculosa carinata* Bruguière, 1789) (both Cerithioidea: Pleuroceridae).

Site of infection: Stomach (fishes); indeterminate (snails).

Type locality: "the region of Rome, Georgia'' Faust (1921).

Other hosts and localities: Table 1, 2.

Specimens deposited: Three adults (vouchers, USNM XXXXXXX), 2 juveniles (vouchers, USNM XXXXXXX), 5 naturally-shed cercariae (vouchers, USNM XXXXXXX); intermediate host vouchers (5 *E. flava* USNM XXXXXXX and 3 *E. c. georgiana* USNM XXXXXXX).

Specimens studied: Five stained, whole-mounted adults; 2 stained, whole-mounted juveniles; and 20 stained, whole-mounted, naturally-shed cercariae studied and measured.

Representative DNA sequences: ITS2 (adult, XXXXXXXX from spotted bass, Chewacla Creek), partial *28S* rDNA (adult, XXXXXXXX from spotted bass, Chewacla Creek; cercaria, XXXXXXXX, Raccoon Creek).

Prevalence and intensity: One of 3 (33.3%) spotted basses was infected by 7 adults of *L. stephanocauda*, 1 of 2 (50%) green sunfish was infected by 1 adult, 1 of 3 (33%) longear sunfish was infected by 1 adult, 1 of 4 (25%) mottled sculpins was infected by 3 juveniles; 3 of 72 (4.2%, Moores Mill Creek) and 2 of 215 (0.9%, Chewacla Creek) of *E. flava* and 4 of 80 (5.0%, Raccoon Creek) of *E. c. georgiana* shed cercariae of *L. stephanocauda*.

Remarks

Womble and Bullard (2022) accepted *L. stephanocauda* and described a furcocystocercous cercaria that they identified as *Leuceruthrus* cf. *stephanocauda* infecting *Elimia* cf. *carinifera* and *Elimia* cf. *modesta* (both Cerithioidea: Pleuroceridae). Their description of the cercaria (Figs. 3–8 therein) was based on 2 naturally-shed, mature specimens (having a withdrawn distome). They did not observe live specimens or comment on the presence or absence of pigmentation in live cercarial specimens. They differentiated their cercaria (as *L.* cf. *stephanocauda*) based on fixed, whole-mounted specimens that

lacked cercarial pigmentation and had numerous protuberances on the tail stem; which both Faust (1921) and Horsfall (1934) could have omitted or not observed.

Our furcocystocercous cercariae from all localities were morphologically identical to each other and all had i) pre-ovarian testes, ii) black pigmentation that was distributed bilaterally to the tail stem posterior to the withdrawn distome and marginally on the paired furcae (present in both live and formalin-fixed specimens but absent in stained, whole-mounted specimens), iii) subtriangular spines that were distributed in rows on the tail stem approximately anterior and posterior to the distome, and iv) numerous marginal and submarginal protuberances on the paired furcae (Figs. 14–18). Our cercariae collectively had all morphological features to diagnose *L. stephanocauda* as per Faust (1921), Horsfall (1934), and Smith (1935) and *L.* cf. *stephanocauda* as per Womble and Bullard (2022). Hence, we identified our cercariae as *L. stephanocauda* and considered those identified as *L.* cf. *stephanocauda* by Womble and Bullard (2022) as conspecific. However, our cercaria of *L. stephanocauda* had a tail stem that was more elongated than that in Womble and Bullard (2022) (5.6–7.8× vs. 4.5–5.1× longer than wide) but less elongated than that of the mature cercariae from Oconomowoc River in Horsfall (1934) (~8.0× longer than wide). In addition, our cercarial specimens had paired furcae that were proportionally shorter than those in Womble and Bullard (2022) (1.2–1.7× vs. 1.7–2.3× longer than wide). Our specimens of *L. stephanocauda* additionally had tegumental plications on the anterior end of the tail stem along the withdrawn distome and had broadly rounded to lanceolate furcae that bear numerous marginal and submarginal protuberances (Figs. 14–18). *Leuceruthrus* cf. *stephanocauda* lacks tegumental plications and has lanceolate furcae that lack submarginal protuberances (see Figs. 3–8 in Womble and Bullard [2022]).

The cercaria of *L. stephanocauda* differs from that of its congeners by having i) black pigmentation that distributed in bilateral, discontinuous fields on the tail stem posterior to the

withdrawn distome and that along the margins of the paired furcae, ii) prominent subtriangular spines that are distributed in 1–3 rows on the tail stem approximately anterior and posterior to the distome, and iii) broadly rounded to lanceolate furcae that are longer than wide and that bear numerous marginal and submarginal protuberances. Of the accepted species of *Leuceruthrus*, only cercariae of *L. ocalana* and *L. ksepkai* have black pigmentation that is distributed along the margins of the paired furcae (observed on live specimens only); both species, however, lack bilateral fields of pigmentation on the tail stem posterior to the distome (Smith, 1935; Womble and Bullard, 2022). *Leuceruthrus ocalana* has "irregular patches" of black pigmentation on margins of the furcae (Smith, 1935). *Leuceruthrus ksepkai* has 2 prominent ridges on the tail stem anterior and posterior to the distome and additionally has minute protuberances encircling a short portion of the tail stem immediately posterior to the distome (Womble and Bullard, 2022).

Adult *L. stephanocauda* differ from that of its known congeners by having i) a more elongated body (3.1–3.9× vs. 1.7–3.0× and 2.3–3.4× longer than wide in *L.* cf. *ksepkai* and *L. micropteri*, respectively) and ii) a bilateral vitellarium that is asymmetrical, with the shorter vitelline field 78–91% [87 ± 6] of the length of the longer vitelline field (vs. having a symmetrical vitellarium as in *L.* cf. *ksepkai* and *L. micropteri*). Adult *L. stephanocauda* resemble that of *L. micropteri* but differ by having smaller eggs in the distal uterus (59–73 μm long × 29–39 μm wide vs. 70–95 μm long × 35–45 μm wide in *L. micropteri*). Adult *L. stephanocauda* further differ from that of *L.* cf. *ksepkai* by having proportionally smaller suckers, with an oral sucker length 15–18% of body length (vs. 21–27% of body length in *L.* cf. *ksepkai*) and a ventral sucker length that is 10–13% of the body length (vs. 14–20% of the body length in *L.* cf. *ksepkai*).

This is the first description of the adult of *L. stephanocauda* since its cercaria was described >100 y ago by Faust (1921). The life cycle of only 2 species of *Leuceruthrus* (*L.*

micropteri and *L. stephanocauda*) is known. The present study comprises the first report of a species of *Leuceruthrus* from the Tallapoosa River and from a sculpin (Cottidae). *Elimia flava*, *E. c. georgiana*, and green sunfish together represent new host records of *Leuceruthrus*.

Sequence comparisons and phylogenetic results

The *28S* sequences from 4 adults and 5 cercariae of *L. stephanocauda* from Moores Mill Creek and Chewacla Creek were identical to each other but differed from those (1 juvenile and 2 cercariae, all identical sequences) from Raccoon Creek by only 1 nucleotide (at alignment position '636' from the 5' end), which we regard as intraspecific variation given their morphological idenitity. Based on nucleotide similarity, the *28S* sequences of *L. stephanocauda* (GenBank number XXXXXXXX, Chewacla Creek; XXXXXXXX, Raccoon Creek) were most similar to that of *L. micropteri* (OR470685) (99.1% and 99.2% similarity, respectively; 1226 bp) but differed by 11 and 10 nucleotides, respectively (Table 3). The *ITS2* sequences of *L. stephanocauda* (XXXXXXXX) (adults, juvenile, and cercariae) from all sites were identical to each other and to the only other nucleotide sequence of *L. stephanocauda* (ON877232; as "*L.* cf. *stephanocauda*" in Womble and Bullard [2022]) from Big Canoe Creek, Alabama (see Fig. 18 in Womble and Bullard [2022] and Table 2 and Fig. 16 in Truong et al. [*in review*]).

The *28S* tree (1226 bp, including gaps) recovered *L. stephanocauda* sister to *L. micropteri*. *Proterometra* Horsfall, 1933 (4 species included) was recovered as monophyletic and sister to the clade including *Leuceruthrus* spp. and *A. longa*. *Otodistomum cestoides* was recovered sister to all other azygiids. Like in Truong et al. (*in review*), the present *28S* tree recovered a monophyletic Leuceruthrinae Goldberger, 1911 and a polyphyletic Azygiinae Lühe, 1909 (Fig. 19).

DISCUSSION

Future studies on the taxonomy of azygiids should include observations on the behaviors and morphological features of live, naturally-shed cercariae in addition to stained, whole-mounted, naturally shed cercariae. Several species of *Proterometra* have been diagnosed by cercarial morphology and behavior (Womble et al., 2015, 2016a, b; Truong et al. [*in review*]). Regarding *L. stephanocauda*, pigmentation distributed along the tail stem and furcae is clearly visible in live and formalin-fixed cercariae, but it vanishes when a specimen is cleared in clove oil.

This study is the first to combine morphology and nucleotide sequences to demonstrate low specificity to the molluscan and fish hosts by a species of *Leuceruthrus*. *Leuceruthrus micropteri* (see Table 1 in Womble and Bullard [2022]) and *L. stephanocauda* (see Table 2 herein) exhibit low definitive fish host specificity. However, only records of *L. stephanocauda* (which infects 4 fish species belonging to 2 families from 2 localities in the southeastern United States) have accompanying sequences (*28S* and *ITS2*) whereas those of *L. micropteri* (infecting fishes of 3 families ranging in the eastern United States) were identified using morphology only. In fact, many of these reported hosts of *L. micropteri* need to be confirmed in future studies (Womble and Bullard, 2022).

Little is known on the life cycles of species of *Leuceruthrus*. Our study is the first to elucidate the life cycle of a species of *Leuceruthrus* using morphology and nucleotide evidence (*ITS2* and *28S*) in a single place and time. That is, no previous study on the taxonomy and life cycles of *Leuceruthrus* has described and deposited type specimens of the adult and its cercaria within the same river (Womble and Bullard, 2022). Future work focusing on collecting and examining endemic pleurocerids (primarily *Elimia* spp. and *Pleurocera* spp.), cottids, and centrarchids (primarily *Micropterus* spp., *Lepomis* spp., and other black basses) from lakes, streams, and rivers of eastern United States could further help

elucidate life cycles of the 4 remaining species (*L. blaisei*, *L. ksepkai*, *L. ocalana*, and the innominate congener *L.* cf. *ksepkai*) as well as help discover new azygiids.

ACKNOWLEDGEMENTS

The authors thank Micah Warren, Haley Dutton, Jacob Shurba, John Brule, and Steven Ksepka (all Southeastern Cooperative Fish Parasite and Disease Laboratory, Auburn University) for helping collect the snails and fish studied herein. The findings and conclusions in this article are those of the authors and do not necessarily represent the views of the U.S. Fish and Wildlife Service. This study was funded by the Southeastern Cooperative Fish Parasite and Disease Project, the US Fish and Wildlife Service (Department of Interior), National Sea Grant (National Oceanic and Atmospheric Administration), United States Department of Agriculture (National Institute of Food and Agriculture), Federal Aid in Sport Fish Restoration (Alabama Department of Conservation and Natural Resources, Inland and Marine Resources Divisions), and the Alabama Agricultural Experiment Station (Auburn University, College of Agriculture).

LITERATURE CITED

Boschung, H. T., and R. L. Mayden. 2004. Fishes of Alabama, Smithsonian Books, Washington D. C., U.S.A. 960 pp.

Burch, J. B., and J. L. Tottenham. 1980. North American freshwater snails, IV. Species lists, ranges and illustrations. Walkerana 1:81–215.

Darriba, D., G. L. Taboada, R. Doallo, and D. Posada. 2012. jModelTest 2: More models, new heuristics, and parallel computing, Nature Methods 9:772. doi.org/10.1038/nmeth.2109.

Faust, E. C. 1921. Larval flukes from Georgia. Transactions of the American Microscopical Society 40:49–58.

Fricke, R., W. N. Eschmeyer, and R. Van der Laan (Eds.). 2023. Eschmeyer's catalog of fishes: genera, species, references. http://researcharchive.calacademy.org/research/ichthyology/catalog/fishcatmain.asp. Accessed 2023 April 4.

Horsfall, M. W. 1934. Studies on the life history and morphology of the cystocercous cercariae. Transactions of the American Microscopical Society 53:311–347.

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.

Marshall, W. S., and N. C. Gilbert. 1905. Three new trematodes found principally in black bass, Zoologische Jahrbücher Abteilung für Systematik 22:477–488.

Rambaut, A., A. J. Drummond, D. Xie, G. Baele, and M. A. Suchard. 2018. Posterior summarization in Bayesian phylogenetics using Tracer 1.7. Systematic Biology 67:901–904.

Rambaut, A., M. A. Suchard, D. Xie, and A. J. Drummond. 2014. FigTree v1.4.4, http://tree.bio.ed.ac.uk/software/figtree. Accessed 2023 June 23.

Smith, S. 1935. *Cercaria stephanocauda ocalana*, a new subspecies of cystocercous cercariae from Florida; its life-cycle and distribution. Journal of the Alabama Academy of Science 7:18–19.

Truong, T. N., N. V. Whelan, P. D. Johnson, M. L. Buntin, and S. A. Bullard. Description, life cycle, and phylogenetics of *Proterometra wigglewomble* n. sp. (Digenea: Azygiidae) from the Cahaba River, Alabama, U.S.A., Systematic Parasitology (*in review*).

Whelan, N. V., P. D. Johnson, J. T. Garner, N. L. Garrison, and E. E. Strong. 2022. Prodigious polyphyly in Pleuroceridae (Gastropoda: Cerithioidea). Bulletin of the Society of Systematic Biologists 1:8419. doi.org/10.18061/bssb.v1i2.8419.

Womble, M. R., C. R. Arias, and S. A Bullard. 2016a. Taxonomic redescription, morphological and molecular diagnosis, and life cycle of *Proterometra catenaria* Smith, 1934 (Digenea: Azygiidae) from the Choctawhatchee River, Florida, U.S.A. Comparative Parasitology 83:137–148.

Womble, M. R., R. Orélis-Ribeiro, and S. A. Bullard. 2015. *Proterometra epholkos* sp. n. (Digenea: Azygiidae) from Terrapin Creek, Alabama, USA: Molecular characterization of life cycle, redescription of *Proterometra albacauda*, and updated lists of host and geographic locality records for *Proterometra* spp. in North America. Parasitology International 64:50–69.

Womble, M. R., R. Orélis-Ribeiro, and S. A. Bullard. 2016b. New species of *Proterometra* (Digenea: Azygiidae) and its life cycle in the Chickasawhay River, Mississippi, USA, with supplemental observations of *Proterometra autraini*. Parasitology International 65:31–43.

Womble, M. R., and S. A. Bullard. 2022. Azygiid parasites of North American endemic pleurocerids and centrarchids: revision of *Leuceruthrus* Marshall and Gilbert, 1905 (Digenea: Azygiidae), description of two new species, and phylogenetic analysis. Journal of Parasitology 108:374–394.

FIGURE LEGENDS

Figures 1–8. Photographs of the yellow elimia, *Elimia flava* (Lea, 1862) (Cerithioidea: Pleuroceridae) (1–5) from the Chewacla Creek (Tallapoosa River, Alabama) and *Elimia caelatura georgiana* (Lea, 1862) (Pleuroceridae) (6–8) from Raccoon Creek (Chattooga River, Georgia); both shed cercariae of *Leuceruthrus stephanocauda*. **1.** Voucher USNM XXXXXX. **2.** Voucher USNM XXXXXX. **3.** Voucher USNM XXXXXX. **4.** Voucher USNM XXXXXX. **5.** Voucher USNM XXXXXX. **6.** Voucher USNM XXXXXX. **7.** Voucher USNM XXXXXX. **8.** Voucher USNM XXXXXX.

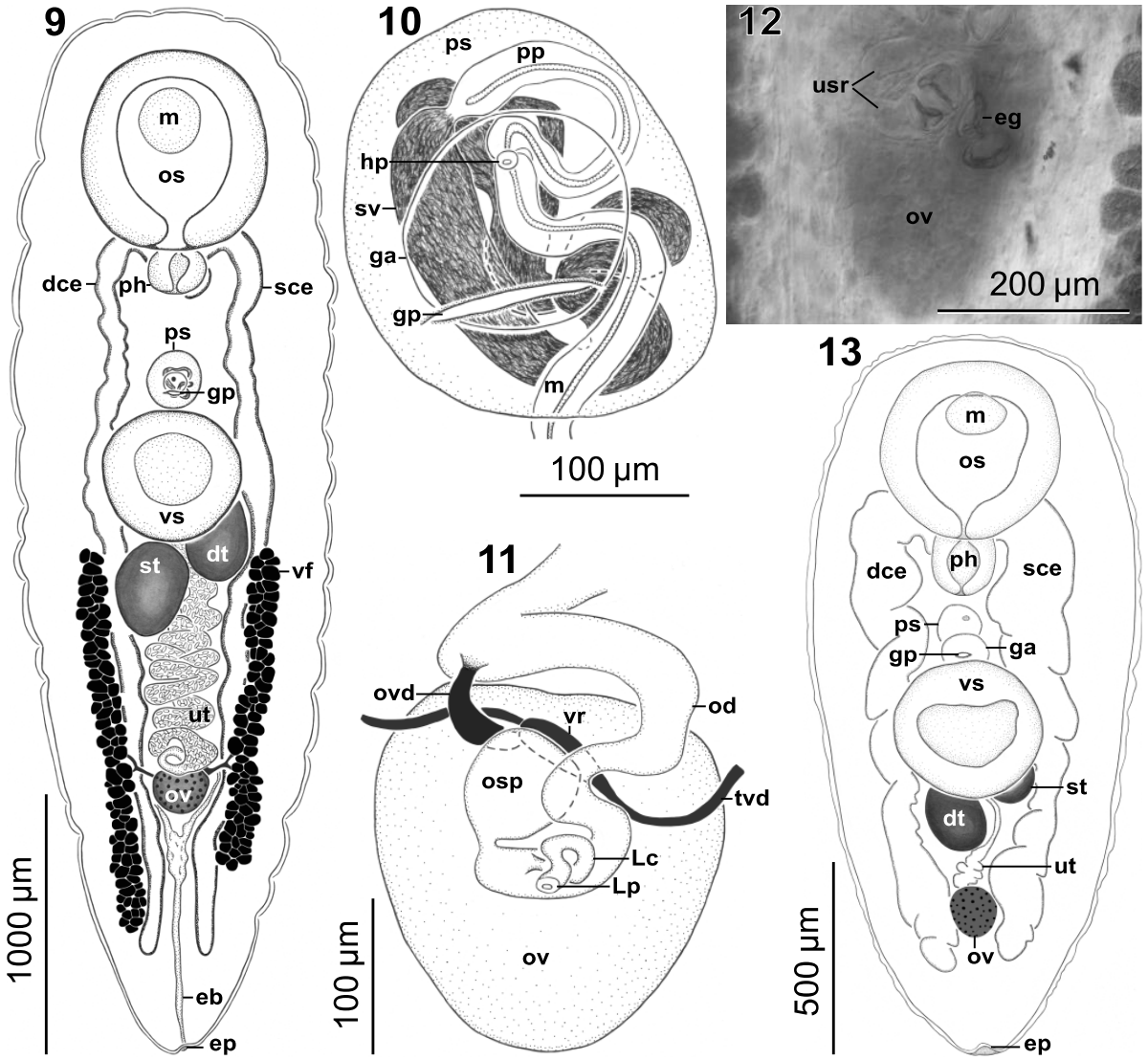
Figures 9–13. *Leuceruthrus stephanocauda* [Faust, 1921] Womble and Bullard, 2022 infecting the stomach of the spotted bass, *Micropterus punctulatus* (Rafinesque, 1819) (Centrarchiformes: Centrarchidae) (adult) from Chewacla Creek (Tallapoosa River, Alabama) and infecting the stomach of the mottled sculpins, *Cottus bairdii* Girard, 1850 (Perciformes: Cottidae) (juvenile) from Raccoon Creek (Chattooga River, Georgia). **9.** Ventral view of USNM XXXXXX (adult) showing the whole body. **10.** Ventrolateral view of USNM XXXXXX (adult) showing the terminal male genitalia. **11.** Ventral view of USNM XXXXXX (adult) showing the ovarian complex. **12.** Ventral view of USNM XXXXXX (adult) showing the uterine seminal receptacle. **13.** Ventral view of USNM XXXXXX (juvenile) showing the whole body. Abbreviations: dce, dextral cecum; dt, dextral testis; eb, excretory bladder; eg, egg; ep, excretory pore; ga, genital atrium; gp, genital pore; hp; hermaphroditic pore; Lc, Laurer’s canal; Lp, Laurer’s canal opening

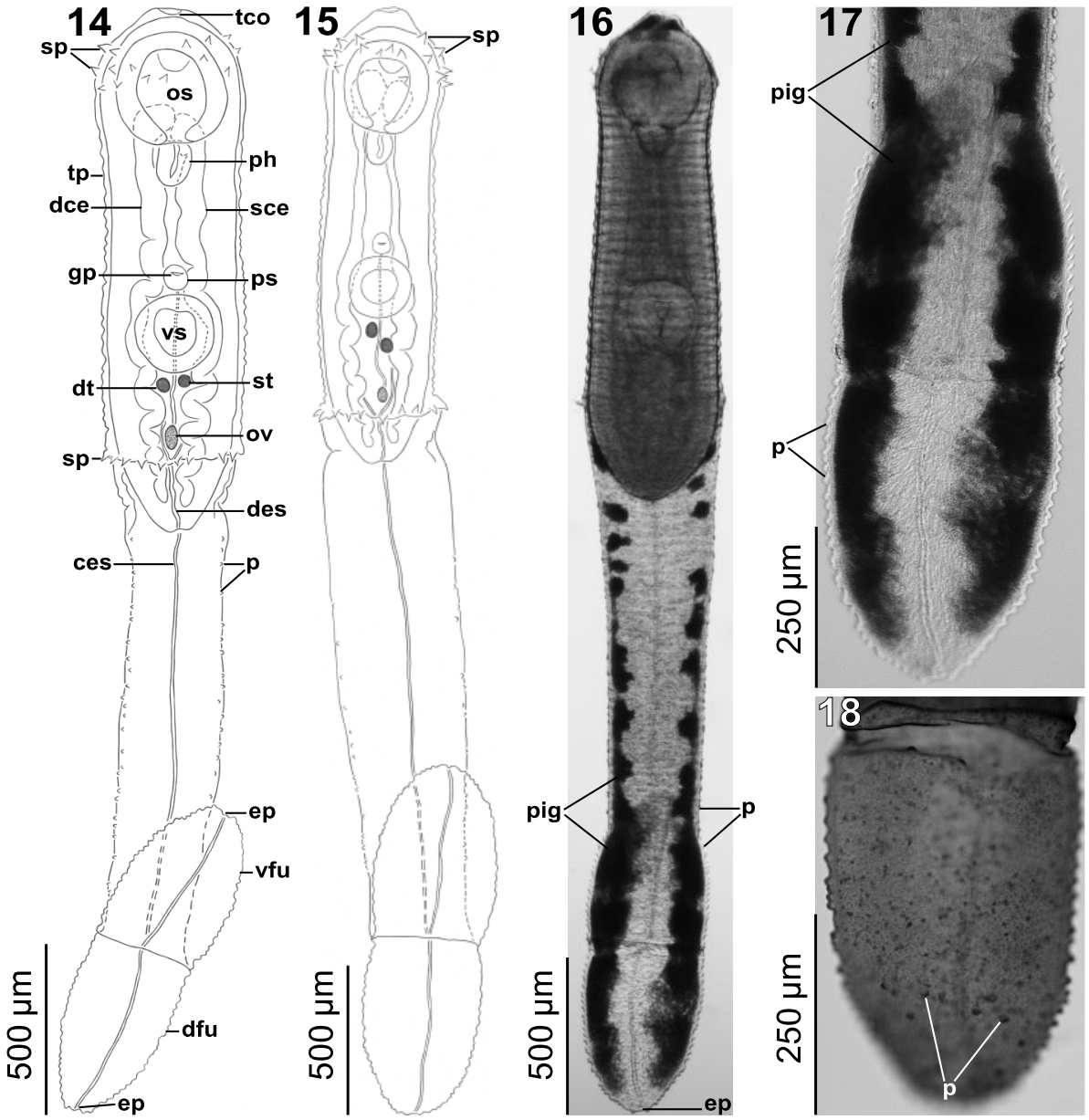
pore; m, metraterm; m, mouth; od, oviduct; os, oral sucker; osp, oviducal sphincter; ov, ovary; ovd, ovovitelline duct; ph, pharynx; pp, pars prostatica; ps, prostatic sac; sce, sinistral cecum; st, sinistral testis; sv, seminal vesicle; tvd, transverse vitelline duct; usr, uterine seminal receptacle; ut, uterus; vf, vitelline follicles; vs, ventral sucker.

Figures 14–18. Naturally-shed cercaria of *Leuceruthrus stephanocauda* [Faust, 1921] Womble and Bullard, 2022 from the yellow elimia, *Elimia flava* (Lea, 1862) (Cerithioidea: Pleuroceridae) from the Chewacla Creek (Tallapoosa River, Alabama). **14.** Ventral view of USNM XXXXXX showing anatomical organ arrangement and lanceolate furcae. **15.** Ventral view of USNM XXXXXX showing similar features to those in Fig. 14 but broadly rounded furcae. **16, 17.** Photomicrographs of a wet-mount of a formalin-fixed cercaria (ventral view); 16. Pigmentation pattern and protuberances on the tail stem and paired furcae; 17. Higher magnification of the same specimen in Fig. 16 showing the same features. **18.** Photomicrographs of USNM XXXXXX (ventral view) showing submarginal protuberances on the ventral furca. Abbreviations: ces, excretory system of cercaria; dce, dextral cecum; des, excretory system of the distome; dfu, dorsal furca; dt, dextral testis; ep, excretory pore at the tip of each furca; gp, genital pore; os, oral sucker; ov, ovary; p, protuberances; ph, pharynx; ps, prostatic sac; sce, sinistral cecum; sp, spines; st, sinistral testis; tco, tail stem cavity opening; tp, tegumental plications; ut, uterus; vfu, ventral furca; vs, ventral sucker.

Figure 19. *28S* phylogeny. Values aside nodes are posterior probability. Sequences in bold are those from the present study. Scale bar is in substitutions per site. Abbreviations: Che, Chewacla Creek; Rac, Raccoon Creek.


1
2
3
4
5
6
7
8
10 mm


9
m
os
dce
ph
sce
ps
gp
vs
st
dt
vf
ut
ov
eb
ep
1000 µm
10
ps
pp
hp
sv
ga
gp
m
100 µm
11
ovd
vr
od
osp
tvd
Lc
Lp
ov
100 µm
12
usr
eg
ov
200 µm
13
m
os
dce
ph
sce
ps
gp
ga
vs
st
dt
ut
ov
ep
500 µm


14
tco
sp
os
ph
tp
dce
sce
gp
ps
vs
dt
st
ov
sp
des
ces
p
ep
vfu
dfu
ep
500 µm
15
sp
500 µm
16
pig
p
ep
500 µm
17
pig
p
250 µm
18
p
250 µm

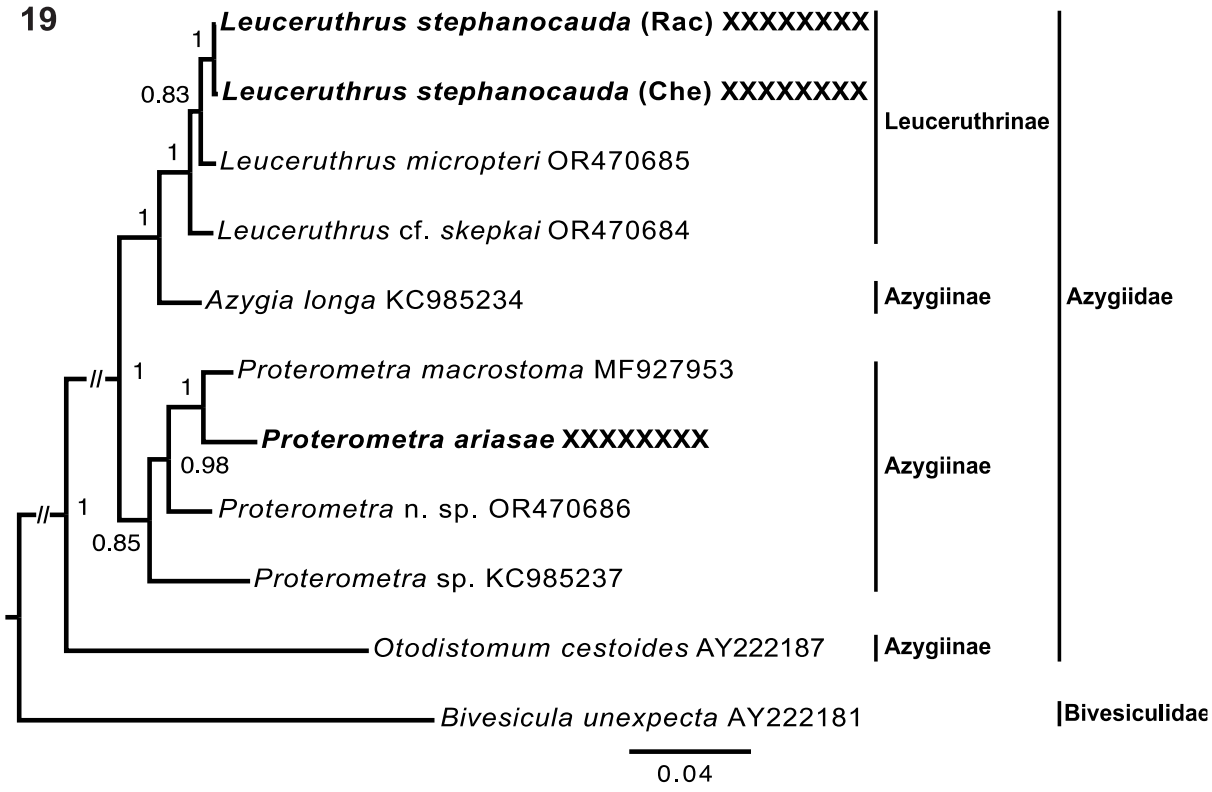

19
Leuceruthrus stephanocauda (Rac) XXXXXXXX
Leuceruthrus stephanocauda (Che) XXXXXXXX
Leuceruthrus micropteri OR470685
Leuceruthrus cf. skepkai OR470684
Azygia longa KC985234
Proterometra macrostoma MF927953
Proterometra ariasae XXXXXXXX
Proterometra n. sp. OR470686
Proterometra sp. KC985237
Otodistomum cestoides AY222187
Bivesicula unexpecta AY222181
1
0.83
1
1
1
1
0.98
1
0.85
Leuceruthrinae
Azygiinae
Azygiinae
Azygiinae
Azygiidae
Bivesiculidae
0.04

Table 1. Intermediate snail hosts of *Leuceruthrus stephanocauda* (Faust, 1921) Womble and Bullard, 2022.

Host	Locality	Reference
Leptoxis carinata (Bruguière, 1789) (Cerithioidea: Pleuroceridae)*	"the region of Rome", Georgia	Faust, 1921
Elimia carinifera (Larmarck, 1822) (Cerithioidea: Pleuroceridae)*	"the region of Rome", Georgia	Faust, 1921
Pleurocera acuta Rafinesque, 1831 (Cerithioidea: Pleuroceridae)	Oconomowoc River, Wisconsin	Horsfall, 1934
Elimia caelatura georgiana (Lea 1862) (Cerithioidea: Pleuroceridae)	Raccoon Creek, Chattooga River, Georgia (34°28'30.5"N, 85°24'40.1"W)	Present study
Elimia flava (Lea, 1862) (Cerithioidea: Pleuroceridae)	Moores Mill Creek, Tallapoosa River, Alabama (32°32'56.3"N, 85°28'41.2"W)	Present study
	Chewacla Creek, Tallapoosa River, Alabama (32°32'09.8"N, 85°29'48.0"W)	Present study
Elimia cf. *carinata* (Cerithioidea: Pleuroceridae)†	Big Canoe Creek, Coosa River, Alabama (33°48'21"N, 86°28'57"W)	Womble and Bullard, 2022
Elimia cf. *modesta* (Cerithioidea: Pleuroceridae)†	Big Canoe Creek, Coosa River, Alabama (33°48'21"N, 86°28'57"W)	Womble and Bullard, 2022

* Faust (1921) did not mention which species was the type host of *L. stephanocauda*.
† Reported as hosts of *Leuceruthrus* cf. *stephanocauda* in Womble and Bullard (2022).

Table 2. Fish hosts of *Leuceruthrus stephanocauda* (Faust, 1921) Womble and Bullard, 2022.

Host	Locality	Reference
Cottus bairdii Girard, 1850 (Perciformes: Cottidae), mottled sculpin	Raccoon Creek (34°28'30.5"N, 85°24'40.1"W), Chattooga River, Georgia	Present study
Micropterus punctulatus (Rafinesque, 1819) (Centrarchiformes: Centrarchidae), spotted bass	Chewacla Creek (32°32'09.8"N, 85°29'48.0"W), Tallapoosa River, Alabama	Present study
Lepomis cyanellus Rafinesque, 1819 (Centrarchiformes: Centrarchidae), green sunfish	Chewacla Creek (32°32'09.8"N, 85°29'48.0"W), Tallapoosa River, Alabama	Present study
Lepomis megalotis (Rafinesque, 1820) (Centrarchiformes: Centrarchidae), longear sunfish	Chewacla Creek (32°32'09.8"N, 85°29'48.0"W), Tallapoosa River, Alabama	Present study

Table 3. Pairwise comparisons among 28S sequences of 9 azygiids and *Bivesicula unexpecta* (1226 bp; above the diagonal: percent nucleotide similarity; below the diagonal: nucleotide differences).

Species	*L. stephanocauda* Chewacla Creek, AL	*L. stephanocauda* Raccoon Creek, GA	*L.* cf. *ksepkai*	*L. micropteri*	*A. longa*	*O. cestoides*	*P ariasae*	*P. macrostoma*	*Proterometra* sp.	*Proterometra* n. sp	*B. unexpecta*
Leuceruthrus stephanocauda, Chewacla Creek, AL	–	99.9	98.8	99.1	97.2	86.1	94.4	94.7	94.0	94.9	77.7
Leuceruthrus stephanocauda, Raccoon Creek, GA	1	–	98.8	99.2	97.3	86.1	94.5	94.8	94.1	95.0	77.7
Leuceruthrus cf. *ksepkai*	14	15	–	98.6	97.4	86.0	94.5	95.0	94.0	95.1	77.8
Leuceruthrus micropteri	11	10	17	–	97.5	86.1	94.5	94.8	94.1	95.1	78.0
Azygia longa	34	33	32	31	–	86.7	94.8	94.9	94.6	95.1	77.8
Otodistomum cestoides	170	169	171	169	162	–	85.5	85.7	86.1	85.8	77.3
Proterometra ariasae	68	67	67	67	63	177	–	97.4	94.2	95.9	77.9
Proterometra macrostoma	64	63	61	63	62	174	32	–	94.6	96.7	77.8
Proterometra sp.	73	72	73	72	66	170	70	66	–	95.4	77.4
Proterometra n. sp*	62	61	60	60	60	173	50	40	56	–	78.0
Bivesicula unexpecta	273	273	271	269	272	278	271	271	277	269	–

AL: Alabama; GA: Georgia. * A new species of *Proterometra* described in Truong et al. *(in review)*.

CHAPTER 7: MORPHOLOGICAL AND NUCLEOTIDE-BASED CONFIRMATION OF THE LIFE CYCLE OF *TRANSVERSOTREMA* CF. *PATIALENSE* (DIGENEA: TRANSVERSOTREMATIDAE) IN NORTH AMERICA

***In review, Journal of Parasitology (Submitted 09 October 2023)**

Authors: Triet N. Truong and Stephen A. Bullard

Abstract

We herein describe the life cycle of *Transversotrema* cf. *patialense* in North America based on morphological and nucleotide evidence. This trematode asexually reproduces in the red-rimmed melania, *Melanoides tuberculata* (Müller, 1774) (Cerithioidea: Thiaridae) and matures beneath the scales of the zebrafish, *Danio rerio* (Hamilton, 1822) (Cypriniformes: Danionidae) within a spring-fed earthen pond aquaculture system (a private aquaculture facility) in the vicinity of Ruskin, Florida. The adult of *T.* cf. *patialense* has a body that is 1.8–2.2× wider than long, a ventral sucker that is 1.2–1.7× wider than the pharynx, and a primarily extra-cecal follicular vitellarium extending anteromediad nearly to the level of the eyespots. Cercariae actively swam once liberated from the crushed snails and had well-defined arm-like processes each bearing an adhesive pad, an elongate tail stem, oar-shaped furcae each marginated with a membranous, pleated fin fold, well-developed male and female genitalia, and lacked vitelline follicles. The redia had a broadly rounded anterior body end and a constricted, diminutive tail process at the posterior body end. The large subunit ribosomal DNA (*28S*) and ribosomal internal transcribed spacer 2 (*ITS2*) sequences of *T.* cf. *patialense* from Florida were most similar to those from *T. patialense* infecting red-rimmed melania from Mayagüez, Puerto Rico and differed by 134 and 69 nucleotides, respectively. The *28S* phylogenetic analysis recovered our sequence sister to a clade comprising those of *T. patialense* plus *Transversotrema* sp. infecting an unspecified freshwater snail from Chiang Mai, Thailand, and the *ITS2* analysis recovered our

sequence sister to that of *T. patialense*. Both phylogenetic analyses recovered i) *Prototransversotrema steeri* Angel, 1969 sister to all *Transversotrema* spp. and ii) *Crusziella formosa* Cribb, Bray, and Barker, 1992 nested within a clade of *Transversotrema* spp. The latter result rejected monophyly of *Transversotrema* and suggested that *Transversotrema* requires revision or that *Crusziella* should be considered a junior subjective synonym of *Transversotrema*.

Transversotrematidae Witenberg, 1944 comprises 4 accepted genera (*Circuitiocoelium* Wang, 1981; *Crusziella* Cribb, Bray, and Barker, 1992; *Prototransversotrema* Angel, 1969; *Transversotrema* Witenberg, 1944) diagnosed by body length to width ratio, presence/absence of the oral sucker, relative position of the mouth to the body, shape of the seminal vesicle, distribution of the vietllarium, and development of eggs in the adults (Cribb, 2002). *Prototransversotrema* and *Transversotrema* have a primarily extra-cecal vitellarium and non-embryonated eggs. *Prototransversotrema* differs from *Transversotrema* by having an oral sucker (vs. lacking an oral sucker), a mouth that opens at the oral sucker (vs. at the pharynx), and a tubular seminal vesicle (vs. a bipartite seminal vesicle). *Circuitiocoelium* and *Crusziella* are both monotypic and differ from *Transversotrema* and *Prototransversotrema* by having a reduced, wholly intra-cecal vitellarium and embryonated eggs. *Circuitiocoelium* differs from *Crusziella* by having a body that is longer than wide (vs. wider than long), an oral sucker (vs. lacking an oral sucker), and a mouth that opens at the oral sucker (vs. at the pharynx) (Cribb, 2002).

Transversotrema, the focus genus of the present study, has only 2 accepted freshwater species (*Transversotrema patialense* [Soparkar, 1924] Crusz and Sathananthan, 1960 and *Transversotrema chauhani* Agrawal and Singh, 1960) and 31 marine species (see Womble et al.,

2015; Cutmore et al., 2016, 2023; WoRMS Editorial Board, 2023). Cribb et al. (1992) regarded *Transversotrema chackai* Mohandas, 1973, *Transversotrema koliense* (Olivier, 1947) Yamaguti, 1971, *Transversotrema laruei* Velasquez, 1958, and *Transversotrema soparkari* (Pandey, 1971) Pande and Shukla, 1972 as junior subjective synonyms of *T. patialense*. Cutmore et al. (2016, 2023) asserted that the taxonomy of *Transversotrema* spp. should rely on nucleotide sequences (*28S*, ribosomal internal transcribed spacer 2 [*ITS2*], and cytochrome c oxidase subunit 1 mitochondrial region [cox1]) and phylogenetic analyses as well as provided evidence that morphology of the adults differentiated few congeners. The cercaria of *Transversotrema* spp. morphologically resembles its adult and has well-developed male and female genitalia (Womble et al, 2015; Perales Macedo et al., 2022). *Transversotrema* spp. have 2-host life cycles (no encysted metacercaria nor second intermediate host has been found) (Soparkar, 1924; Cribb, 1988, 2002; Womble et al, 2015; Perales Macedo et al., 2022). The life cycles of only two transversotrematids are known: *T. patialense* and *Prototransversotrema steeri* Angel, 1969 (Soparkar, 1924; Velasquez, 1958; Crusz and Sathananthan, 1960; Cribb, 1988; Cribb et al., 1992). The life cycle of no marine transversotrematid is known.

Transversotrematid records from the Americas are scant, and the identities of various transversotrematid specimens that morphologically resemble *T. patialense* there are intriguing (Womble et al., 2015; Perales Macedo et al., 2022). At least one transversotrematid could naturally range in North America: Dechtiar and Christie (1988) documented (but did not describe nor deposit a voucher specimen of) *Prototransversotrema* sp. beneath the scales of wild-caught common shiner, *Luxilus cornutus* (Mitchill, 1817) (Cypriniformes: Leuciscidae) from the Credit River, Ontario, Canada. The other transversotrematid records since then have been attributed to *T. patialense* based on morphology of the adults and cercariae related to infections in the

exotic/introduced zebrafish, *Danio rerio* (Hamilton, 1822) (Cypriniformes: Danionidae) and other freshwater fishes. Womble et al. (2015) first reported and described adults they identified as *T. patialense* that infected the space beneath the scales of zebrafish purchased from an recirculating aquaculture system in North America. Perales Macedo et al. (2022) documented that cercariae of *T. patialense* shed from the red-rimmed melania, *Melanoides tuberculata* (Müller, 1774) (Cerithioidea: Thiaridae) in Quebrada de Oro, Mayagüez, Puerto Rico could infect a phylogenetically diverse array of native Puerto Rican fishes. This study was important because it showed that endemic fishes could be infected by a transversotrematid that first was established on invasive zebrafish. Further, *T. patialense* is vectored by the red-rimmed melania, another invasive species that has long been established in particular freshwater environments in North America (including Pacific and Atlantic drainages).

Herein, we use morphology and nucleotide sequences to elucidate the first life cycle of a transversotrematid in North America, describing the adult, cercaria, and redia of *T.* cf. *patialense*, and provide updated phylogenetic analyses for Transversotrematidae.

MATERIALS AND METHODS

Nine zebrafish were collected within a spring-fed earthen pond aquaculture system in Ruskin, Florida, bagged with half-filled water, and mailed to the Aquatic Parasitology Laboratory, Auburn University, Auburn, Alabama on 22 September 2022. Fish were euthanized in MS-222 (250 ppm) and dissected using a Leica Wild Heerbrugg M8 (Heerbrugg, Switzerland) stereodissecting microscope. Fish scales were gently detached from the skin using a pair of fine forceps to examine for the presence of adult transversotrematids. Adult trematodes were promptly transferred to a small glass stender dish using a 5ml plastic transfer pipette, and studied alive. Adult trematodes were subsequently heat-killed in hot water (~60 °C), and fixed in 10% neutral

buffered formalin. Fixed specimens were stained overnight in Van Cleave's hematoxylin mixed with several drops of Ehrlich's hematoxylin. Stained specimens were made basic with 2 drops of lithium carbonate and 1 drop of butylamine saturated in 70% ethanol (EtOH), dehydrated in an EtOH series, cleared in clove oil, and permanently mounted on glass slides using Canada balsam. Additional live, adult trematodes intended for DNA extraction were placed directly in 95% EtOH without any heat treatment.

A total of 391 red-rimmed melania were collected from the spring inlet to the earthen pond that harbored the infected zebrafish. Snails were bagged with water, and mailed overnight to the Aquatic Parasitology Laboratory on 28 September 2022. In the laboratory, snails were isolated in 6-well tissue culture plates (VWR, Radnor, Pennsylvania) with pond water at 20°C or crushed and individually examined in a small stender dish. Rediae and actively swimming cercariae from crushed snails were promptly transferred to a clean stender dish using a 5ml plastic transfer pipette, studied alive, subsequently stained, fixed, mounted, and preserved in the same ways as for the adult worms. Collected snails were identified as *M. tuberculata* based on shell morphology as per (Burch, 1989). Specifically, the shell had rounded whorls that were sculptured with spiral grooves (appearing as low costae) and weakly vertical curved ribs (Figs. 1–5).

Illustrations of the trematodes were made using an Olympus BX51 microscope (Olympus Corporation of the Americas, Center Valley, Pennsylvania) equipped with differential interference contrast optical components and a drawing tube. Measurements were made using a microscope camera software Jenoptik Gryphax® version 2.1.0.724 (Jena, Germany) and reported in micrometers (μm) as the range, followed by the mean ± standard deviation. Taxonomic authorities for fishes followed Fricke et al. (2023). Anatomical terms for *Transversotrema* spp. followed those of Womble et al. (2015) (adult), Perales Macedo et al. (2022) (cercaria; except

that “ventral sucker” and “arm-like process” were used instead of “acetabulum” and “caudal appendage”, respectively), and Soparkar (1924) (redia). Vouchers of the present transversotrematids (5 whole-mounted adults, 5 cercariae, 5 rediae) and 5 snail host shells were deposited in the National Museum of Natural History’s Invertebrate Zoology Collection (NMNH, Smithsonian Institution, Washington, D.C.) (see Taxonomic summary).

Genomic DNA (*ITS2* and partial *28S*) were separately extracted from 2 adults and 2 cercariae (all EtOH-preserved specimens) (as replicates) using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. DNA concentration was measured using a NanoDrop–1000 spectrophotometer (Thermo Scientific, Nanodrop Technologies, Waltham, Massachusetts), diluted to 20 ng/µl, and stored at –20 °C. Forward and reverse primers and thermocycling parameters of PCR reactions to amplify the *ITS2* and *28S* followed those in Truong et al. (2022). PCR product purification was conducted using the QIAquick PCR Purification kit (Qiagen). All PCR primers were used for DNA sequencing reactions. Two internal primers 300F (5’–CAAGTACCGTGAGGGAAAGTTG–3’) and 1200R (5’–GCATAGTTCACCATCTTCGG–3’) were additionally used to improve sequencing coverage on the *28S* (Lockyer, et al., 2003). DNA sequencing was performed by Genewiz (South Plainfield, New Jersey). All nucleotide sequences were deposited in the NCBI GenBank (see Taxonomic summary).

Sequences were aligned using MAFFT (Katoh and Standley, 2013). Taxon and outgroup selection for the *28S* and *ITS2* phylogenetic analyses were based on previous transversotrematid phylogenetic studies, i.e., Perales Macedo et al. (2022) and Cutmore et al. (2023). Sequence alignments and all parameter settings for the Bayesian inference (BI) analyses followed those in Truong et al. (2022). Briefly, JModelTest 2 version 2.1.10 was implemented to perform a

statistical selection of the best-fit models of nucleotide substitution (Darriba et al., 2012). The BI analyses were performed in MrBayes version 3.2.5 (Ronquist and Huelsenbeck, 2003) using substitution model averaging (nst-mixed) and a gamma distribution to model rate-heterogeneity. Three independent runs with 4 Metropolis-coupled chains were run for 5,000,000 generations, sampling the posterior distribution every 1,000 generations. Defaults were used in all other parameters. Convergence was checked using Tracer v1.7.1 (Rambaut et al., 2018) and the sump command in MrBayes. All runs appeared to reach convergence after discarding the first 25% of generations as burn-in. Convergence was assumed to have occurred when trace plots among runs overlapped and effective samples size for each parameter was at least 200. Majority-rule consensus tree of the post-burn-in posterior distribution was generated with the sumt command in MrBayes. The inferred phylogenetic trees were visualized using FigTree v1.4.4 (Rambaut et al., 2014) and further edited for visualization purposes with Adobe Illustrator 27.7 version (2023) (Adobe Systems, San Jose, California).

DESCRIPTION

Transversotrema cf. *patialense*

Diagnosis of adult (based on light microscopy of 13 stained, whole-mounted adult specimens): (Figs. 6–8)

Body distinctly dorsoventrally flat, transversely elongate, D-shaped in outline, widest at level of pharynx, slightly indented at middle of posterior body end, 329–497 (393 ± 45) long at level of midline, 327–496 (396 ± 45) long at level of dextral vitelline margin, 229–510 (391 ± 66) long at level of sinistral vitelline margin, 664–942 (790 ± 81) in maximum width, 1.8–2.2× (2.0 ± 0.2) wider than middle body length (Figs. 6, 7). Tegument spinous; spines subtriangular, denser and larger at anterior body end, becoming smaller toward posterior body end; ventral and dorsal

spines approximately equal in sizes; ventral spines 7–9 (8 ± 1) long, 6–7 (6 ± 0) wide; dorsal spines 6–10 (8 ± 1) long, 6–7 (7 ± 0) wide. Eyespots paired, prominent, positioning symmetrically to midline, at level of posterior half of pharynx, approximately equal in sizes, 15–25 (20 ± 3) in maximum width, 84–150 (109 ± 19) long from anterior body end (23–36% [28 ± 3] of middle body length); distance between eyespots 89–168 (122 ± 21) long (13–21% [15 ± 2] of maximum body width). Pharynx ovoid, 62–80 (71 ± 6) long (16–21% [18 ± 1] of middle body length), 67–90 (78 ± 7) wide (9–11% [10 ± 1] of maximum body width), 1.0–1.2× (1.1 ± 0.1) wider than long, 31–81 (53 ± 13) long from anterior body end (8–16% [13 ± 2] of middle body length) (Figs. 6, 7). Oral sucker absent. Ventral sucker circular in outline, thin, weakly muscular, stalked, ventrally overlapping posterior margin of pharynx, with ventral surface armed with spines, 85–124 (106 ± 13) long (22–30% [27 ± 3] of middle body length), 86–130 (115 ± 14) wide (11–18% [15 ± 2] of maximum body width), 0.9–1.3× (1.1 ± 0.1) wider than long, 1.2–1.7× (1.5 ± 0.1) longer than pharynx, 1.2–1.7× (1.5 ± 0.2) wider than pharynx, 78–136 (110 ± 17) long from anterior body end (21–31% [28 ± 3] of middle body length), 137–239 (179 ± 28) long from posterior body end (39–49% [45 ± 3] of middle body length) (Figs. 6, 7). Mouth subterminal, opening ventrally at middle of pharynx, directed anteriad, 26–48 (36 ± 7) long (6–13% [9 ± 2] of middle body length), 3–29 (13 ± 7) wide (0–4% [2 ± 1] of maximum body width), 44–88 (65 ± 12) long from anterior body end (12–20% [17 ± 2] of middle body length). Esophagus thin-walled, originating from mouth, extending 27–67 (49 ± 12) posteriorly from pharynx (7–18% [13 ± 3] of middle body length) before bifurcating at level of middle of ventral sucker and at 143–209 (163 ± 19) from anterior body end (38–46% [42 ± 3] of middle body length); esophageal branches extending slightly anterolaterad; dextral branchial esophagus 100–164 (133 ± 19) long (25–43% [34 ± 5] of middle body length), maximum width 9–22 (14 ± 4); sinistral branchial

esophagus 93–171 (125 ± 21) long (25–42% [32 ± 5] of middle body length), maximum width 9–25 (16 ± 5) (Figs. 6, 7). Ceca thick-walled, comprising numerous papilla-like projections along its entire length, arching approximately parallelly to body margin on each side, before extending transversely toward midline near posterior body end and fusing to form cyclocoel, 663–920 (773 ± 79) long (169–229% [199 ± 18] of middle body length), maximum width 36–58 (48 ± 7); cecal space 178–260 (210 ± 23) long (51–59% [54 ± 3] of middle body length), 376–508 (445 ± 42) wide (50–67% [57 ± 5] of maximum body width); pre-cecal space 79–149 (106 ± 19) long (21–31% [27 ± 3] of middle body length); post-cecal space 66–102 (77 ± 11) long (16–22% [20 ± 2] of middle body length) (Figs. 6, 7).

Testes 2 in number, ovoid, lobed or entire, opposite, inter-cecal, posterolateral to ventral sucker, approximately equal in sizes, occupying space 269–369 (328 ± 34) wide (37–50% [42 ± 4] of maximum body width); dextral testis 63–95 (82 ± 12) long (16–26% [21 ± 3] of middle body length), 92–151 (118 ± 17) wide (11–18% [15 ± 2] of maximum body width); sinistral testis 61–112 (92 ± 15) long (18–29% [23 ± 3] of middle body length), 105–164 (127 ± 18) wide (13–19% [16 ± 2] of maximum body width); distance between testes 49–126 (85 ± 23) long (6–16% [11 ± 3] of maximum body width); pre-testicular space 151–277 (183 ± 34) long (41–56% [46 ± 5] of middle body length); post-testicular space 76–138 (111 ± 16) long (23–32% [28 ± 3] of middle body length) (Figs. 6, 7). Vasa efferentia and vas deferens not observed. Seminal vesicle distinctly bipartite, membranous, wholly dextral to midline, filled with sperm, with 2 parts connected by thin duct ventral to dextral branch of esophagus, 246–389 (328 ± 45) long (70–94% [84 ± 7] of middle body length); proximal part ellipsoid, inter-cecal, 53–118 (83 ± 22) long (17–33% [25 ± 5] of seminal vesicle length), 19–56 (34 ± 12) wide; distal part convoluted, constricted at level of pharynx, extra-cecal, swollen proximally and becoming narrower toward distal end,

communicating with genital atrium at its distal end, 184–294 (245 ± 33) long (67–83% [75 ± 5] of seminal vesicle length), 2.0–4.9× (3.2 ± 0.9) longer than proximal part, 21–46 (33 ± 9) wide (Figs. 6, 7). Pars prostatica and ejaculatory duct absent. Genital atrium small, circular in outline, 16–28 (23 ± 4) wide. Genital pore subterminal, ventral, dextral and close to midline, 5–12 (8 ± 2) long from anterior body end (1–3% [2 ± 1] of middle body length) (Figs. 6, 7).

Ovary ovoid or ellipsoid, lobed or entire, inter-cecal, anterior and diagonal to sinistral testis, at level of ventral sucker, wider than long, 48–91 (66 ± 13) long (13–22% [17 ± 2] of middle body length), 74–143 (99 ± 19) wide (10–17% [12 ± 2] of maximum body width); pre-ovarian space 110–191 (140 ± 24) long (29–41% [35 ± 4] of middle body length); post-ovarian space 143–237 (183 ± 24) long (39–53% [47 ± 4] of middle body length) (Figs. 6–8). Oviduct originating from dextral margin of ovary, extending posteriorly short distance before curving dextrally along space between ovary and sinistral testis, 85–112 (95 ± 9) long (22–27% [25 ± 2] of middle body length). Oötype immediately anterior to sinistral testis, communicating to proximal region of oviduct. Mehlis' gland cells not evident. Laurer's canal filled with sperms; ventral and connecting to distal end of oviduct, comprising 2 distinct regions, 168–208 (186 ± 15) long (43–55% [49 ± 5] of middle body length); proximal region swollen for its entire length, originating from distal oviduct, extending anterodextrally to level of oötype, 88–122 (99 ± 13) long (45–59% [53 ± 5] of Laurer's canal length); distal region primarily dorsal to sinistral testis, extending posterosinistrally, becoming narrow for its entire length, 79–109 (87 ± 11) long (41–55% [47 ± 5] of Laurer's canal length); Laurer's canal pore inter-cecal, dorsal to anterior half of sinistral testis, 205–265 (232 ± 20) from anterior body end (57–64% [60 ± 3] of middle body length (Figs. 6, 8). Uterine seminal receptacle in proximal uterus, primarily between testicular space. Uterus ventral to dextral branchial esophagus, lacking convolutions, comprising proximal

and distal portions; proximal portion wholly inter-cecal, originating from oötype, extending transversely toward dextral cecum, coursing along anterior margins of both testes and posterior margin of vitelline reservoir, occupying space 143–221 (203 ± 22) wide (20–29% [26 ± 3] of maximum body width); distal portion wholly extra-cecal, extending anteriad dextrally along seminal vesicle, connecting to genital atrium separately from seminal vesicle (hermaphroditic duct absent), 133–208 (174 ± 23) long (34–50% [44 ± 5] of middle body length); metraterm not evident (Figs. 6–8). Eggs (in early developmental stage) present in proximal uterus of only 1 of 13 specimens. Vitellarium comprising numerous follicles primarily extra-cecal, occasionally having 1 to 2 inter-cecal follicles between cecum and testes; vitelline fields wrapping around outer surface of intestinal ceca, extending anteromediad approximately to level of eyespots, occupying space 273–439 (348 ± 50) long (75–115% [26 ± 3] of middle body length) and 143–221 (203 ± 22) wide (20–29% [26 ± 3] of maximum body width); vitelline follicles more condense anteriorly and at middle of body, absent around indented area at posterior body end; pre-vitelline space 23–65 (36 ± 12) long (6–16% [9 ± 3] of middle body length); post-vitelline space 17–48 (29 ± 8) long (4–13% [7 ± 2] of middle body length); distance between anterior vitelline margins 148–235 (188 ± 30) long (19–30% [24 ± 3] of maximum body width) (Figs. 6, 7). Ovovitelline duct anterior to sinistral testis and posterodextral to ovary, originating sinistrally from vitelline reservoir, communicating to proximal oviduct immediately posterior to ovary (Fig. 8). Transverse vitelline ducts nearly symmetrical, ventral to ceca and ovary, branching from vitelline fields approximately at level of testes, extending anteromedially and anteriorly to testes before fusing at level of ventral sucker to form vitelline reservoir. Vitelline reservoir large, median, C-shaped in outline, primarily inter-testicular, overlapping posterior half of ventral sucker, 42–86 (66 ± 13) long, 90–156 (123 ± 22) wide (Figs. 6–8).

Excretory bladder median, I-shaped, slightly swollen anteriorly, extending anteriad 45–84 (65 ± 20) from pore (12–21% [16 ± 5] of maximum body length); collecting ducts branching near excretory pore, nearly symmetrical, extending laterad some distance posteriorly to vitelline fields, ascending through vitelline fields and along ceca to level of ventral sucker before curving toward body margins and descending parallelly along body margins to some distance in post-testicular space to make a loop around vitelline follicles; pore subterminal, dorsal, median, opening approximately at middle of post-cecal space, 14–44 (33 ± 10) long from posterior body end (4–12% [8 ± 3] of middle body length) (Fig. 7).

Diagnosis of cercaria (based on light microscopy of 12 stained, whole-mounted cercariae from crushed snails): (Figs. 9, 10)

Cercaria furcocercous, delicate, comprising cercarial body, a tail stem attaching to posterior end of cercarial body, a pair of arm-like processes attaching to anterior end of tail stem, and a pair of furcae attaching to posterior end of tail stem, 893–1169 (1061 ± 82) in total length (= body + tail stem + furca), 506–659 (587 ± 44) in maximum width at level of cercarial body, 1.6–2.0× (1.8 ± 0.1) longer than wide (Figs. 9, 10). Cercarial body spinous, distinctly dorsoventrally flat, transversely elongated, D-shaped in outline, widest at level of pharynx, having relatively straight margin at anterior end and curved margin at posterior end, 279–348 (307 ± 21) long at midline (27–34% [29 ± 2] of total cercarial length), 1.7–2.1× (1.9 ± 0.1) wider than long (Figs. 9, 10). Tegument spines denser and larger at anterior body end; ventral spines slightly larger than dorsal spines; ventral spines 5–7 (6 ± 1) long, 4–6 (5 ± 0) wide; dorsal spines 4–6 (5 ± 1) long, 3–5 (4 ± 1) wide. Eyespots paired, prominent, symmetrical in position and sizes, immediately posterior to posterior margin of pharynx, 15–20 (18 ± 1) in maximum width, 85–117 (102 ± 10) long from anterior body end (28–38% [33 ± 2] of cercarial body length); distance between eyespots 68–128

(88 ± 16) long (13–19% [15 ± 2] of maximum body width) (Figs. 9, 10). Pharynx ovoid, 44–53 (48 ± 3) long (15–18% [16 ± 1] of cercarial body length), 41–59 (51 ± 5) wide (8–10% [9 ± 1] of maximum body width), 0.9–1.2× (1.0 ± 0.1) wider than long, 57–77 (63 ± 5) long from anterior body end (19–25% [20 ± 2] of cercarial body length). Oral sucker absent. Ventral sucker circular in outline, thin, weakly muscular, stalked, overlapping posterior margin of pharynx, with ventral surface armed with spines, 92–116 (107 ± 8) long (31–41% [35 ± 3] of cercarial body length), 94–116 (108 ± 7) wide (16–23% [18 ± 2] of maximum body width), 0.9–1.2× (1.0 ± 0.1) wider than long, 2.0–2.4× (2.2 ± 0.1) longer than pharynx, 1.9–2.6× (2.1 ± 0.2) wider than pharynx, 83–124 (88 ± 16) long from anterior body end (27–38% [33 ± 3] of cercarial body length), 70–122 (94 ± 13) long from posterior body end (23–35% [31 ± 3] of cercarial body length) (Figs. 9, 10). Mouth subterminal, opening ventrally within anterior half of pharynx, directed anteriad, 17–28 (22 ± 3) long (6–9% [7 ± 1] of cercarial body length), 4–10 (7 ± 2) wide (1–2% [1 ± 0] of maximum body width), 58–81 (67 ± 6) long from anterior body end (20–26% [22 ± 2] of cercarial body length). Esophagus thin-walled, originating from mouth, extending 32–60 (44 ± 9) posteriorly from pharynx (11–18% [14 ± 2] of cercarial body length) before bifurcating at level of middle of ventral sucker and at 129–165 (148 ± 13) long from anterior body end (45–53% [48 ± 3] of cercarial body length); esophageal branches extending slightly anterolaterad; dextral branchial esophagus 46–167 (92 ± 28) long (15–54% [30 ± 9] of cercarial body length), maximum width 5–19 (11 ± 4); sinistral branchial esophagus 53–102 (84 ± 13) long (19–33% [27 ± 4] of cercarial body length), maximum width 6–21 (12 ± 5) (Figs. 9, 10). Ceca relatively thick-walled, arching approximately parallelly to body margin on each side, before extending transversely toward midline near posterior body end and fusing to form cyclocoel, 447–677 (556 ± 69) long (159–215% [181 ± 16] of cercarial body length), maximum width 11–34 (19 ± 7);

cecal space 115–172 (140 ± 16) long (41–52% [46 ± 2] of cercarial body length), 240–349 (288 ± 34) wide (43–56% [49 ± 4] of maximum body width); pre-cecal space 100–145 (118 ± 13) long (34–43% [38 ± 3] of cercarial body length); post-cecal space 41–67 (57 ± 8) long (12–22% [18 ± 3] of cercarial body length) (Figs. 9, 10).

Testes 2 in number, ovoid, entire or lobed, opposite, inter-cecal, posterolateral to or slightly overlapping posterior margin of ventral sucker, approximately equal in sizes, occupying space 176–239 (210 ± 21) wide (32–40% [36 ± 2] of maximum body width); dextral testis 58–86 (70 ± 9) long (17–28% [23 ± 3] of cercarial body length), 73–106 (90 ± 10) wide (13–17% [15 ± 1] of maximum body width); sinistral testis 59–83 (70 ± 8) long (20–27% [23 ± 2] of cercarial body length), 76–102 (91 ± 8) wide (14–18% [16 ± 1] of maximum body width); distance between testes 25–63 (39 ± 10) long (4–12% [7 ± 2] of maximum body width); pre-testicular space 141–207 (165 ± 21) long (44–62% [54 ± 5] of cercarial body length); post-testicular space 57–92 (72 ± 10) long (17–30% [23 ± 4] of cercarial body length) (Fig. 9, 10). Vasa efferentia and vas deferens not observed. Seminal vesicle distinctly bipartite, membranous, dextral to midline, filled with sperms, with 2 parts connected by thin duct ventral to dextral branchial esophagus, 267–361 (307 ± 28) long (94–128% [100 ± 10] of cercarial body length); proximal part ellipsoid, inter-cecal, 53–80 (65 ± 9) long (18–24% [21 ± 2] of seminal vesicle length), 10–32 (23 ± 6) wide; distal part convoluted, extra-cecal, swollen proximally and becoming gradually narrower toward distal end, 207–296 (242 ± 24) long (76–82% [79 ± 2] of seminal vesicle length), 3.2–4.6× (3.7 ± 0.5) longer than proximal part, 11–36 (23 ± 8) wide (Figs. 9, 10). Pars prostatica and ejaculatory duct absent. Genital atrium small, circular in outline, 14–32 (22 ± 5) wide. Genital pore close to anterior body margin, dextral and close to midline, 3–12 (9 ± 2) long from anterior body end (1–4% [3 ± 1] of cercarial body length) (Figs. 9, 10).

Ovary ovoid or ellipsoid, inter-cecal, anterosinistral to sinistral testis, at level of ventral sucker, wider than long, 27–60 (42 ± 12) long (9–21% [14 ± 4] of cercarial body length), 61–115 (78 ± 17) wide (10–18% [13 ± 3] of maximum body width); pre-ovarian space 101–152 (132 ± 14) long (36–47% [43 ± 3] of cercarial body length); post-ovarian space 107–140 (124 ± 9) long (35–46% [40 ± 3] of cercarial body length) (Figs. 9, 10). Oviduct, oötype, Mehlis' gland cells, uterine seminal receptacle, ovovitelline duct, vitelline follicles, transverse vitelline ducts, and vitelline reservoir not developed. Laurer's canal posteromedian to ovary, extending posteriorly between ovary and sinistral testis, 59–74 (69 ± 5) long (19–26% [23 ± 2] of cercarial body length); Laurer's canal pore inter-cecal, immediately anterosinistral or dorsal to sinistral testis, 165–226 (195 ± 20) long from anterior body end (58–80% [63 ± 6] of cercarial body length). Uterus appearing as thin-walled duct, ventral to dextral branchial esophagus, comprising proximal and distal portions; proximal portion wholly inter-cecal, coursing transversely along anterior margins of both testes and partly inter-testicular, occupying space 134–197 (158 ± 22) wide (22–34% [27 ± 3] of maximum body width); distal portion wholly extra-cecal, extending anteriad dextrally along seminal vesicle, communicating to genital atrium separately from seminal vesicle, 144–199 (167 ± 14) long (48–70% [54 ± 5] of cercarial body length); metraterm not evident (Figs. 9, 10). Eggs not observed in cercaria.

Excretory system of cercarial body comprising a bladder and collecting ducts; bladder median, I-shaped, swollen anteriorly, extending anteriad 33–70 (46 ± 11) from pore (10–20% [15 ± 3] of cercarial body length); collecting ducts, appearing as 2 extra-cecal pairs, nearly symmetrical in length and course, extending laterad from proximal and distal ends of excretory bladder, coursing along cercarial body margins and cyclocoel to level of testes or ventral sucker, farther anterior extent difficult to trace in whole-mounted specimens; excretory pore subterminal,

median, opening dorsally in post-cecal space, 13–25 (19 ± 4) from posterior body end (4–8% [6 ± 1] of cercarial body length) (Fig. 9).

Tail stem aspinous, dorsoventrally flat, elongated, lacking pigmentation in live, fixed, and whole-mounted specimens, approximately uniform in width, dorsally attaching to indented posterior end of cercarial body, vulnerable to physical manipulations and mounting medium, easily detached from cercarial body (along with arm-like processes and furcae) in whole-mounts, having numerous granular cells randomly distributed along its length, 356–568 (460 ± 54) long (40–50% [43 ± 3] of total cercarial length), 76–117 (102 ± 12) wide (14–20% [17 ± 2] of maximum body width), 3.8–5.1× (4.5 ± 0.4) longer than wide.

Arm-like processes aspinous, lacking pigmentation and granular cells, dorsal to tail stem, approximately symmetrical in shape and length, positioning at synthesis of tail stem and cercarial body, each having an adhesive pad at distal end, appearing as an attachment organ of cercaria; dextral process 66–105 (81 ± 11) long (6–9% [8 ± 1] of total cercarial length), 23–37 (28 ± 4) in maximum width (4–6% [5 ± 1] of maximum body width), 2.1–4.2× (3.0 ± 0.6) longer than wide; sinistral process 57–97 (75 ± 12) long (5–8% [7 ± 1] of total cercarial length), 21–38 (29 ± 7) in maximum width (3–7% [5 ± 1] of maximum body width), 2.1–3.6× (2.6 ± 0.5) longer than wide (Figs. 9, 10).

Furcae aspinous, oar-shaped, cylindrical proximally, becoming dorsoventrally flat toward distal end, lacking pigmentation, symmetrical in shape and length, indented medially at distal tip, having numerous granular cells randomly distributed along its length and marginated with membranous, pleated fin fold; dextral furca 250–318 (284 ± 22) long (24–29% [27 ± 2] of total cercarial length), 78–115 (99 ± 12) in maximum width (14–19% [17 ± 2] of maximum body width), 2.5–3.3× (2.9 ± 0.3) longer than wide; sinistral furca 246–304 (285 ± 18) long (25–31%

[27 ± 2] of total cercarial length), 74–111 (93 ± 12) in maximum width (14–19% [16 ± 2] of maximum body width), 2.6–3.6× (3.1 ± 0.3) longer than wide (Figs. 9, 10).

Excretory system of tail stem and furcae comprising a main collecting duct and secondary collecting ducts; main collecting duct connecting to excretory bladder of cercarial body at proximal end of tail stem, extending posteriad along midline of tail stem, bifurcating into 2 secondary ducts at synthesis of furcae and tail stem; secondary collecting ducts extending posteriad along midline of each furca; excretory pores terminal, opening at furcal indents (Figs. 9, 10).

Diagnosis of redia (based on light microscopy of 20 stained, whole-mounted rediae from crushed snails): (Figs. 11–14)

Redia broadly rounded at anterior end and markedly tapering at posterior end, comprising a body and a constricted, diminutive tail process, 426–729 (550 ± 70) long (= body + tail process), 177–274 (226 ± 27) wide, 2.1–3.3× (2.5 ± 0.3) longer than wide. Body of redia comprising myriad granular cells, a muscular pharynx at anterior end, a digestive tract (intestine) immediately posterior to pharynx, and 4–15 (9 ± 3) germ balls at various sizes (which each developing into a cercaria) and developing cercariae (Figs. 11, 12, 14), or occasionally only a daughter redia (Fig. 13). Tail process 36–102 (63 ± 18) long (7–18% [11 ± 3] of total body length). Pharynx ovoid, 64–125 (87 ± 17) long (11–20% [16 ± 3] of total body length), 75–116 (94 ± 10) wide (31–61% [43 ± 8] of body width), 0.8–1.4× (1.1 ± 0.2) wider than long. Intestine sac-like, in anterior half of body, 106–227 (164 ± 34) long (21–38% [30 ± 6] of total body length). Excretory system not evident in whole-mounts.

Daughter redia (observed in only 1 of 20 examined specimens) ovoid, median, approximately at middle of body and posterior to intestine of mother redia, having an ovoid pharynx at anterior

body end, 186 long (32% of mother redia length), 115 wide (56% of maximum mother redia width); pharynx of daughter redia 49 long (26% of daughter redia length), 46 wide (40% of maximum daughter redia width); germ balls and intestine not developed within daughter redia (Fig. 13).

Taxonomic summary

Hosts: Zebrafish, *Danio rerio* (Hamilton, 1822) (Cypriniformes: Danionidae) (adults); red-rimmed melania, *Melanoides tuberculata* (Müller, 1774) (Cerithioidea: Thiaridae) (cercariae and rediae).

Locality: A spring-fed earthen pond aquaculture system (a private aquaculture facility) in the vicinity of Ruskin, Florida.

Specimens examined: Transversotrema patialense (MSB:Para:33302–08, adult; MSB:Para:33310–11, cercaria; MSB:Para:33309, redia).

Specimens and DNA sequences deposited: 5 adults of *T.* cf. *patialense* (USNM XXXXXXX), 5 cercariae (USNM XXXXXXX), 5 rediae (USNM XXXXXXX); 5 snail shells (USNM XXXXXXX); *ITS2* (XXXXXXXX), partial *28S* rDNA (XXXXXXXX).

Sites in host: beneath and between scales (zebrafish); indeterminate (red-rimmed melania).

Prevalence and intensity: 3 of 9 (33.3%) zebrafish were infected by 1, 1, and 15 adults of *T.* cf. *patialense*; 7 of 391 (1.8%) red-rimmed melania were infected by cercariae and rediae of *T.* cf. *patialense*.

Remarks

Our transversotrematid specimens (adult, cercaria, and redia) had all of the morphological features to diagnose *T. patialense* as per Soparkar (1924), Crusz et al. (1964), Cribb et al. (1992), and Womble et al. (2015). However, we refrain from identifying these specimens as *T. patialense*

(and thereby identified them as *T.* cf. *patialense*) because i) no extant type specimen of *T. patialense* exists (Cribb et al., 1992; Womble et al., 2015), ii) no DNA sequence of *T. patialense* from the type host (red-rimmed melania) and from the type locality (i.e., "*a shallow stream near Pinjore gardens about three miles from Kalka*", Haryana, India; see Soparkar [1924]) is available in GenBank to match with the present nucleotide sequences, and iii) many morphologically cryptic and genetically closely related species of *Transversotrema* have been recently discovered and described (Hunter et al., 2010; Hunter and Cribb, 2012; Cribb et al., 2014; Cutmore et al., 2016). In short, the identity of *T. patialense* is indeterminate, and in light of the nucleotide differences between our specimens and those previously reported from Puerto Rico, we decided to identify our specimens as *T.* cf. *patialense*.

The morphological identity of transversotrematid infections in North America and nearby is intriguing. Perales Macedo et al. (2022) identified cercariae naturally-shed from red-rimmed melania as well as adults from 6 native Peurto Rican fishes from experimental infections (the river goby, *Awaous banana* [Valenciennes, 1837] and the sirajo, *Sicydium plumieri* [Bloch, 1786] [both Gobiiformes: Gobiidae], the mountain mullet, *Dajaus monticola* [Bancroft, 1834] [Mugiliformes: Mugilidae], the bigmouth sleeper, *Gobiomorus dormitor* Lacepède, 1800 [Gobiiformes: Eleotridae], *Oreochromis* sp. [Cichliformes: Cichlidae], and the guppy, *Poecilia reticulata* Peters, 1859 [Cyprinodontiformes: Poeciliidae] and from a wild-caught mountain mullet) as *T. patialense*. Perales Macedo et al. (2022, p. 6) described their cercariae of *T. patialense* as "*lacking eggs and developed vitelline fields*" but our examination of their voucher specimens showed that these cercariae (MSB:Para:33310–11, 6 cercariae) had numerous extra-cecal vitelline follicles (Figs. 16, 17; Fig. 3A on p. 4 of Perales Macedo et al. 2022). Intriguingly, no previously-described, naturally-shed transversotrematid cercaria reportedly has vitelline

follicles in the cercarial body (see Soparkar, 1924; Olivier, 1947; Velasquez, 1961; Crusz et al., 1964; Rao and Ganapati, 1967; Nadakal et al., 1969; Pandey, 1971; Cribb, 1988). The rediae of *T. patialense* from Puerto Rico (MSB:Para:33309, 3 rediae) have a broadly rounded posterior body end and several prominent tegumental projections (Fig. 18) distributed on the posterior 2/3 of the body. Previous descriptions of *T. patialense* (and its junior synonyms) (Soparkar, 1924; Olivier, 1947; Velasquez, 1961; Crusz et al., 1964; Rao and Ganapati, 1967; Nadakal et al., 1969; Pandey, 1971) characterized its redia as having a posterior appendage/tail process and lacking tegumental projections. Interstingly, the presence of tegumental projections of the redia of *T. patialense* from Puerto Rico resembles those (as projections and pseudopods) of the redia of *P. steeri* infecting the Brazier's pebble snail, *Posticobia brazieri* (Smith, 1882) (Truncatelloidea: Tateidae) from Queensland, Australia (see Cribb et al. [1988] and Figs. 7–12 therein). Perales Macedo et al. (2022) collected these rediae from crushed red-rimmed melania and deposited them in the Southwestern Museum of Biology (University of New Mexico, Albuquerque, NM) but did not describe nor draw them. Our cercariae of *T.* cf. *patialense* from Florida lacked visible vitelline follicles (Figs. 9, 10) and the rediae had a prominent diminutive tail process at the posterior body end and lacked tegumental projections (Figs. 11–14).

Nucleotide sequence comparisons and phylogenetic analyses

The present *28S* and *ITS2* sequences from 2 adults and 2 cercariae of *T.* cf. *patialense* were identical to each other, respectively. The aligned *28S* of *T.* cf. *patialense* was most similar to that of the sequence ascribed to *T. patialense* (OP099865) from Puerto Rico (87.3% similarity of 1169 bp alignment) but differed by 134 nucleotides. It differed from *28S* sequence of the only other freshwater species of *Transversotrema* (*Transversotrema* sp., KU820963, infecting an unspecified freshwater snail in Thailand) by 157 bp (85.3% similarity) and from all analyzed

marine congeners by 216–260 nucleotides. Interestingly, untrimmed *28S* sequences of 4 marine species (*Transversotrema elegans* Hunter, Ingram, Adlard, Bray, and Cribb, 2010 [KX186730], *Transversotrema gigantica* Hunter, Ingram, Adlard, Bray, and Cribb, 2010 [KX186732], *Transversotrema haasi* Witenberg, 1944 [AY222186, type species], and *Transversotrema hyperionis* Cutmore, Corner, and Cribb, 2023 [KX186729]) differed from each other by only 1 nucleotide; *T. elegans* and *T. hyperionis* were identical. The *ITS2* sequence of *T.* cf. *patialense* was most similar to that of *T. patialense* (OP088731) (80.2% similarity of 396 bp alignment) but differed by 69 nucleotides. It differed from all marine congeners by 87–107 nucleotides. The aligned *ITS2* of *T. elegans* (OR129482) differed from that of *T. gigantica* (OR129484) by 5 nucleotides; *T. elegans* differed from *T. hyperionis* (OR129486) by only 2 nucleotides. No nucleotide sequence is publicly available for the monotypic *Circuitiocoelium*.

The *28S* tree (1169 bp, including gaps) recovered *P. steeri* (AY222184) sister to a clade including all analyzed species of *Transversotrema* (Fig. 19). *Transversotrema* was recovered as polyphyletic. Three freshwater species of *Transversotrema* (*T.* cf. *patialense*, *T. patialense*, and *Transversotrema* sp.) were monophyletic and sisters to a clade that included all marine *Transversotrema* spp. + *Crusziella formosa* Cribb, Bray, and Barker, 1992 (KX186726). *Crusziella formosa* was sister to a clade including *Transversotrema lacerta* Hunter, Ingram, Adlard, Bray, and Cribb, 2010 (OR129453) + (*T. elegans*, *T. gigantica*, *T. haasi*, *T. hyperionis*, and *Transversotrema titanis* Cutmore, Corner, and Cribb, 2023 [OR129454]) within a clade of all analyzed marine *Transversotrema* spp. (Fig. 19). The *ITS2* tree (396 bp, including gaps) had similar topology to that of the *28S* tree, except that *Transversotrema* was paraphyletic and *C. formosa* (OR129481) was sister to a clade including all marine *Transversotrema* spp. (Fig. 20).

The present *28S* and *ITS2* trees suggested that *Transversotrema* requires revision or that *Crusziella* should be considered a junior subjective synonym of *Transversotrema*.

DISCUSSION

Little is known about the biodiversity of freshwater species of *Transversotrema*. Soparkar (1924) first described the cercaria and redia of *T. patialense* (as *Cercaria patialensis*). Velasquez (1958) first described the adult of *T. patialense* (as *T. laruei*) from the barramundi, *Lates calcarifer* (Bloch, 1790) (Latidae) in Rizal, Philippines; Crusz and Sathananthan (1960) regarded *C. patialensis* as a larval form of *T. patialense* and briefly described the adult (as metacercaria) of *T. patialense* from the spiketail paradisefish, *Pseudosphromenus cupanus* [Cuvier, 1831] [Anabantiformes: Osphronemidae] in Batalagoda, Sri Lanka. Since the study of Crusz and Sathananthan (1960), *T. patialense* (and its synonyms) has been reported from numerous freshwater and euryhaline fish species over a wide geographic range (see Table 1 in Womble et al. [2015] for reported hosts and localities of *T. patialense*), but Perales Macedo et al. (2022) provided the first DNA sequences for *T. patialense*. Our adult specimens of *T.* cf. *patialense* are morphologically indistinguishable to those of *T. patialense* in Womble et al. (2015) and in Perales Macedo et al. (2022). We think our specimens are conspecific to those in Womble et al. (2015) because they have identical morphology and both infect a widely distributed, ornamental and experimental definitive fish host (zebrafish) in North America. Unfortunate in this regard is that we do not have a sequence or any genomic DNA of the specimens reported by Womble et al. (2015). Based on cercarial and redial morphology as well as the large percent differences in the *ITS2* and *28S* (see Nucleotide sequence comparisons), our specimens and those from Puerto Rico likely represent 2 species of *Transversotrema*. However, we could not confirm if our specimens of *T.* cf. *patialense* or those of *T. patialense* from Puerto Rico (or neither of them) are conspecific

with those of *T. patialense* in Soparkar (1924) *sensu stricto*. A recollection and sequencing of *T. patialense* from the type host and type locality (or nearby area) is required to resolve the identity of *T. patialense*. The only other accepted freshwater species of *Transversotrema*, *T. chauhani* infecting the gangetic leaffish, *Nandus nandus* (Hamilton, 1822) (Anabantiformes: Nandidae) in Lucknow, India, seems to be distinct from *T. patialense* by having a ventral sucker that is much more narrow than the pharynx, a vitellarium that is confluent at the anterior body end (vs. lacking within the space between the eyespots at the anterior body end), and markably smaller eggs (30–33 μm long × 25–29 μm wide) (Agrawal and Singh, 1981). Since that study, no worker has collected and published a record of this species and, to our knowledge, no type material exists for *T. chauhani*.

This study comprises the first confirmatory life cycle of a transversotrematid in North America, based on combined morphological and nucleotide sequence evidence. Presently, red-rimmed melania and zebrafish are the only known intermediate snail host and definitive fish host, respectively, for *Transversotrema* in North America. In this study, the hosts of *T.* cf. *patialense* were both sourced from the same outdoor, static earthen pond wherein other native freshwater scaled fishes also cohabited. Given that *T.* cf. *patialense* shares the low definitive fish host specificity as to *T. patialense*, we speculate that these native fishes in that pond are likely susceptible to *T.* cf. *patialense*. In addition to *T.* cf. *patialense*, we also found an intense infection of a gyrodactylid on the skin and gill of these zebrafish. These specimens potentially represent a new gyrodactylid species and a morphological description of that gyrodactylid is forthcoming.

ACKNOWLEDGEMENTS

We thank Dr. Nathan Whelan and Samantha Donohoo (School of Fisheries, Aquaculture, and Aquatic Sciences, Auburn University; Southeast Conservation Genetics Laboratory, Warm

Springs Fish Technology Center, US Fish and Wildlife Service) for helping indentify and take photos of the snails; Jacob Shurba and John Brule (Southeastern Cooperative Fish Parasite and Disease Laboratory, Auburn University) for helping examine the snails and fish in the present study; Dr. Sara Brant (Southwestern Museum of Biology, University of New Mexico) for loaning the transversotrematid specimens. This study was supported by research grants to SAB from the Southeastern Cooperative Fish Parasite and Disease Project, Alabama Agriculture Experiment Station, Auburn University Vice President for Research and Economic Development, United States Department of Agriculture, and United States Fish and Wildlife Service.

LITERATURE CITED

Agrawal, N., and H. S. Singh. 1981. On a rare trematode, *Transversotrema chauhani* n. sp., from a freshwaterfish, *Nandus nandus* (Ham.). Current Science 9: 426–427.

Burch, J. B. 1989. North American freshwater snails. Malacological Publications, Michigan, 365 p.

Cribb, T. H. 1988. Life cycle and biology of *Prototransversotrema steeri* Angel, 1969 (Digenea: Transversotrematidae). Australian Journal of Zoology 36: 111–129.

Cribb, T. H. 2002. Superfamily Transversotrematoidea Witenberg, 1944. *In* Keys to the Trematoda. Vol 1., D. I. Gibson, A. Jones, and R. A. Bray, (eds.). CAB International and Natural History Museum, Cambridge, U.K., p. 469–473.

Cribb, T. H., R. A. Bray, and S. C. Barker. 1992. A review of the family Transversotrematidae (Trematoda: Digenea) with the description of a new genus, *Crusziella*. Invertebrate Taxonomy 6: 909–935.

Crusz, H., and A. H. Sathananthan. 1960. Metacercaria of *Transversotrema patialense* in the fresh-water fish *Macropodus cupanus*. Journal of Parasitology 46: 613–614.

Crusz, H., W. E. Ratnayake, and A. H. Sathananthan. 1964. Observations on the structure and life-cycle of the digenetic fish-trematode *Transversotrema patialense* (Soparkar). Ceylon Journal of Science (Biological Sciences) 5: 8–17.

Cutmore, S. C., B. K. Diggles, and T. H. Cribb. 2016. *Transversotrema* Witenberg, 1944 (Trematoda: Transversotrematidae) from inshore fishes of Australia: description of a new species and significant range extensions for three congeners. Systematic Parasitology 93: 639–652.

Cutmore, S. C., R. D. Corner, T. H. Cribb. 2023. Morphological constraint obscures richness: a mitochondrial exploration of cryptic richness in *Transversotrema* (Trematoda: Transversotrematidae). International Journal for Parasitology 53: 595–635.

Darriba, D., G. L. Taboada, R. Doallo, and D. Posada. 2012. jModelTest 2: More models, new heuristics, and parallel computing. Nature Methods 9: 772. doi: 10.1038/nmeth.2109.

Dechtiar, A. O., and W. J. Christie. 1988. Survey of the parasite fauna of Lake Ontario fishes, 1961 to 1971. *In* Parasites of fishes in the Canadian waters of the Great Lakes. S. J. Nepszy (ed.). Great Lakes Fishery Commission Technical Report No. 51., Ontario Ministry of Natural Resources, Ottawa, Canada, p. 66–95.

Fricke, R., W. N. Eschmeyer, and R. Van der Laan (Eds.). 2023. Eschmeyer's catalog of fishes: genera, species, references. Available at: http://researcharchive.calacademy.org/research/ichthyology/catalog/fishcatmain.asp. Accessed 23 June 2023.

Hunter, J. A., E. Ingram, R. D. Adlard, R. A. Bray, and T. H. Cribb. 2010. A cryptic complex of *Transversotrema* species (Digenea: Transversotrematidae) on labroid, haemulid and lethrinid fishes in the Indo–West Pacific Region, including the description of three new species. Zootaxa 2652: 17–32.

Hunter, J. A., K. A. Hall, and T. H. Cribb. 2012. A complex of Transversotrematidae (Platyhelminthes: Digenea) associated with mullid fishes of the Indo-West Pacific Region, including the descriptions of four new species of *Transversotrema*. Zootaxa 3266: 1–22.

Hunter, J. A., and T. H. Cribb. 2012. A cryptic complex of species related to *Transversotrema licinum* Manter, 1970 from fishes of the Indo-West Pacific, including descriptions of ten new

species of *Transversotrema* Witenberg, 1944 (Digenea: Transversotrematidae). Zootaxa 3176: 1–44.

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.

Lockyer, A. E., P. D. Olson, and D. T. J. Littlewood. 2003. Utility of complete large and small subunit rRNA genes in resolving the phylogeny of the Neodermata (Platyhelminthes): implications and a review of the cercomer theory. Biological Journal of the Linnean Society 78: 155–171.

Nadakal, A. M., A. Mohandas, and V. Sunderaraman. 1969. *Cercaria chackai* sp. n. (Transversotrematidae) from Kerala, India. Journal of Parasitology: 1187–1190.

Olivier, L. 1947. *Cercaria koliensis*, a new fork-tailed cercaria from Guadalcanal. Journal of Parasitology 33: 234–240.

Pandey, K. C. 1971. On a rare cercaria, *Cercaria soparkari* n. sp., (Transversotrematidae) from Lucknow, India. Journal of Helminthology 45: 321–32.

Perales Macedo, D. M. B., S. C. Díaz Pernett, M. G. Díaz González, G. M. Torres Nieves, C. J. Santos Flores, A. M. Díaz Lameiro, and S. A. Locke. 2022. Autochthonous transmission of the Indomalayan parasite, *Transversotrema patialense*, in the Caribbean: molecular, morphological, and experimental evidence. Experimental Parasitology 242 (2022) 108368. doi: 10.1016/j.exppara.2022.108368.

Rambaut, A., A. J. Drummond, D. Xie, B. Baele, and M. A. Suchard. 2018. Posterior summarization in Bayesian phylogenetics using Tracer 1.7. Systematic Biology 67: 901–904.

Rambaut, A., M. A. Suchard, D. Xie, and A. J. Drummond. 2014. FigTree v1.4.4. Available at: http://tree.bio.ed.ac.uk/software/figtree. Accessed December 22, 2022.

Rao, K. H., and P. N. Ganapati. 1967. Observations on *Transversotrema patialensis* (Soparkar, 1924) (Trematoda) from Waltair, Andhra Pradesh (India). Parasitology 57: 661–664.

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

Soparkar, M. B. 1924. A new cercaria from northern India. *Cercaria patialensis* nov. spec. Indian Journal of Medical Research 11: 933–942.

Truong, T. N., S. S. Curran, F. B. Reyda, J. M. Rash, and S. A. Bullard. 2022. *Plagioporus wataugaensis* n. sp. (Digenea: Opecoelidae) infecting intestine of northern hogsucker, *Hypentelium nigricans,* and white sucker, *Catostomus commersonii,* (Cypriniformes: Catostomidae) from the eastern USA, including an emended diagnosis, key to Nearctic congeners, and phylogenetic analysis. Parasitology International, 89, 102580. doi: 10.1016/j.parint.2022.102580.

Velasquez, C. C. 1961. Further Studies on *Transversotrema laruei* Velasquez with observations on the life cycle (Digenea: Transversotrematidae). Journal of Parasitology 47: 65–70.

Womble, M. R., S. J. Cox-Gardiner, T. H. Cribb, and S. A. Bullard. 2015. First record of *Transversotrema* Witenberg, 1944 (Digenea) from the Americas, with comments on the taxonomy of *Transversotrema patialense* (Soparkar, 1924) Crusz and Sathananthan, 1960, and an updated list of its hosts and geographic distribution. Journal of Parasitology 101: 717–725.

WoRMS Editorial Board. (2023). World register of marine species. https://www.marinespecies.org/aphia.php?p=taxdetails&id=345925. Accessed 31 August 2023.

Figures 1–5. Photographs of the red-rimmed melania, *Melanoides tuberculata* (Müller, 1774) (Cerithioidea: Thiaridae) collected from Ruskin, Florida. **(1)** Voucher USNM XXXXXX. **(2)** Voucher USNM XXXXXX. **(3)** Voucher USNM XXXXXX. **(4)** Voucher USNM XXXXXX. **(5)** Voucher USNM XXXXXX.

Figures 6–8. Adult of *Transversotrema* cf. *patialense* (Digenea: Transversotrematidae) infecting beneath and between scales of the zebrafish, *Danio rerio* (Hamilton, 1822) (Cypriniformes: Danionidae) from Ruskin, Florida. **(6)** Ventral view of USNM XXXXXX showing anatomical organs. **(7)** Photomicrograph of USNM XXXXXX. **(8)** Ovarian complex of the female genitalia. Abbreviations: ce, cecum; cy, cyclocoel; dLc, distal Laurer's canal; dt, dextral testis; ep, excretory pore; es, eyespot; ga, genital atrium; gp, genital pore; Lp, Laurer's canal pore; m, mouth; od, oviduct; ot, oötype; ov, ovary; ovd, ovovitelline duct; ph, pharynx; pLc, proximal Laurer's canal; st, sinistral testis; sv, seminal vesicle; tvd, transverse vitelline duct; ut, uterus; vf, vitelline follicles; vr, vitelline reservoir; vs, ventral sucker.

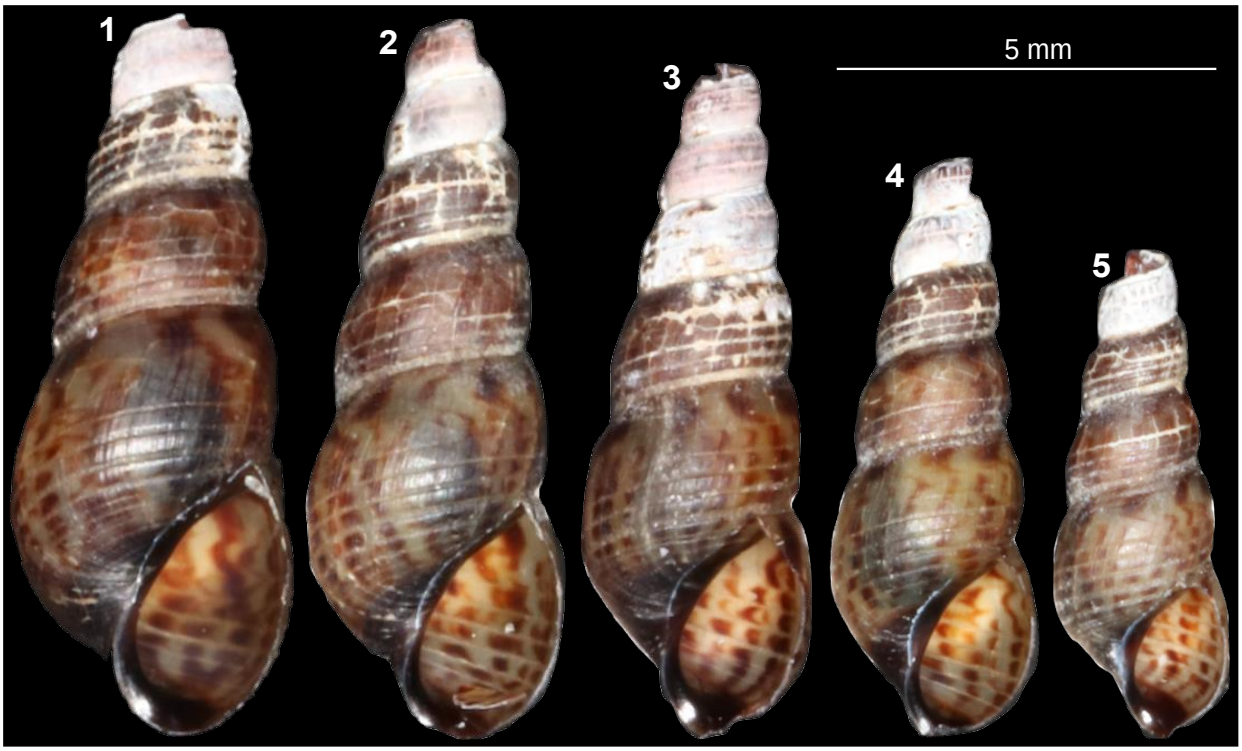
Figures 9, 10. Cercaria of *Transversotrema* cf. *patialense* (Digenea: Transversotrematidae) infecting the red-rimmed melania, *Melanoides tuberculata* (Müller, 1774) (Cerithioidea: Thiaridae) from Ruskin, Florida. **(9)** Ventral view of USNM XXXXXX showing anatomical organs of the cercarial body, tail stem, and the furcae. **(10)** Photomicrograph of USNM XXXXXX. Abbreviations: ap, adhesive pad; apr, arm-like process; ce, cecum; dt, dextral testis; eb, excretory bladder; emc, excretory main collecting duct; ep, excretory pore; esd, excretory secondary collecting duct; f, furca; ff, fin fold; ga, genital atrium; gc, granular cells; gp, genital pore; m, mouth; ov, ovary; ph, pharynx; st, sinistral testis; sv, seminal vesicle; ut, uterus; vs, ventral sucker.

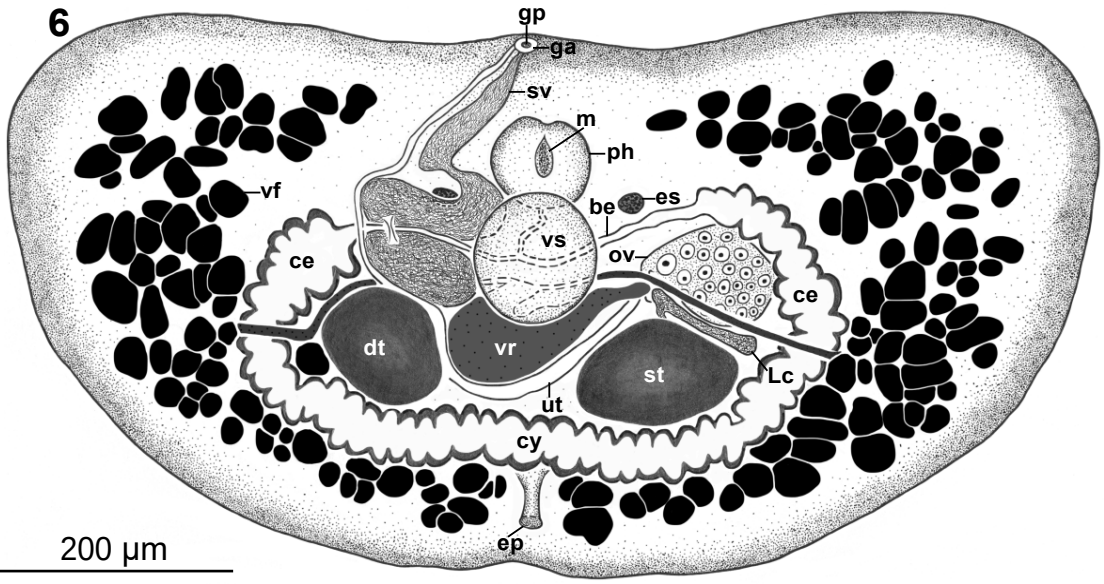
Figures 11–14. Redia of *Transversotrema* cf. *patialense* (Digenea: Transversotrematidae) infecting the red-rimmed melania, *Melanoides tuberculata* (Müller, 1774) (Cerithioidea: Thiaridae) from Ruskin, Florida. **(11)** USNM XXXXXX showing the body, tail process, germ balls, and birth pore. **(12)** USNM XXXXXX showing developing cercaria with eyespots. **(13)** USNM XXXXXX showing the daughter redia. **(14)** Photomicrograph of USNM XXXXXX. Abbreviations: bp, birth pore; dc, developing cercaria; dr, daughter redia; gb, germ ball; in, intestine; ph, pharynx; tp, tail process.

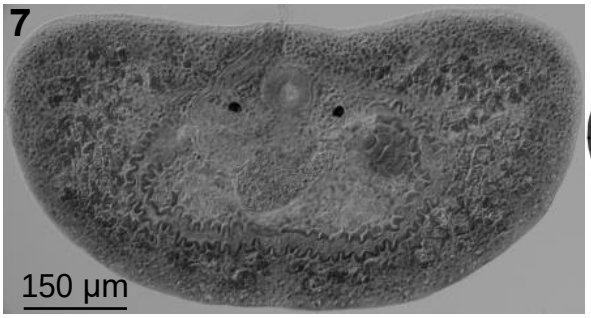
Figures 15–18. Adult, cercaria, and redia of specimens ascribed to *Transversotrema patialense* (Soparkar, 1924) Crusz and Sathananthan, 1960 infecting the red-rimmed melania, *Melanoides tuberculata* (Müller, 1774) (Cerithioidea: Thiaridae) from Puerto Rico. **(15)** A gravid adult (MSBPara33304–1, ventral view). **(16)** A naturally-shed, whole-mounted cercaria (MSBPara33310–1, ventral view). **(17)** Cercarial body of a naturally-shed, whole-mounted cercaria (MSBPara33311–1, dorsal view) showing vitelline follicles. **(18)** A redia (MSB33309) showing a broadly rounded posterior body end and numerous projections in the posterior 2/3 of body. Abbreviations: eg, egg; gb, germ ball; ph, pharynx; tp, tegumental projections; vf, vitelline follicles.

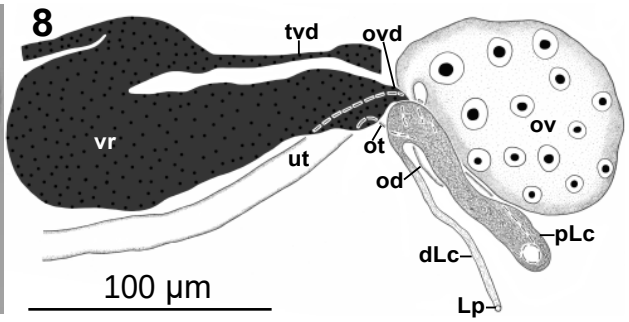
Figure 19. *28S* phylogeny. Values aside nodes are posterior probability. Sequences in bold are those from the present study. Scale bar is in substitutions per site.

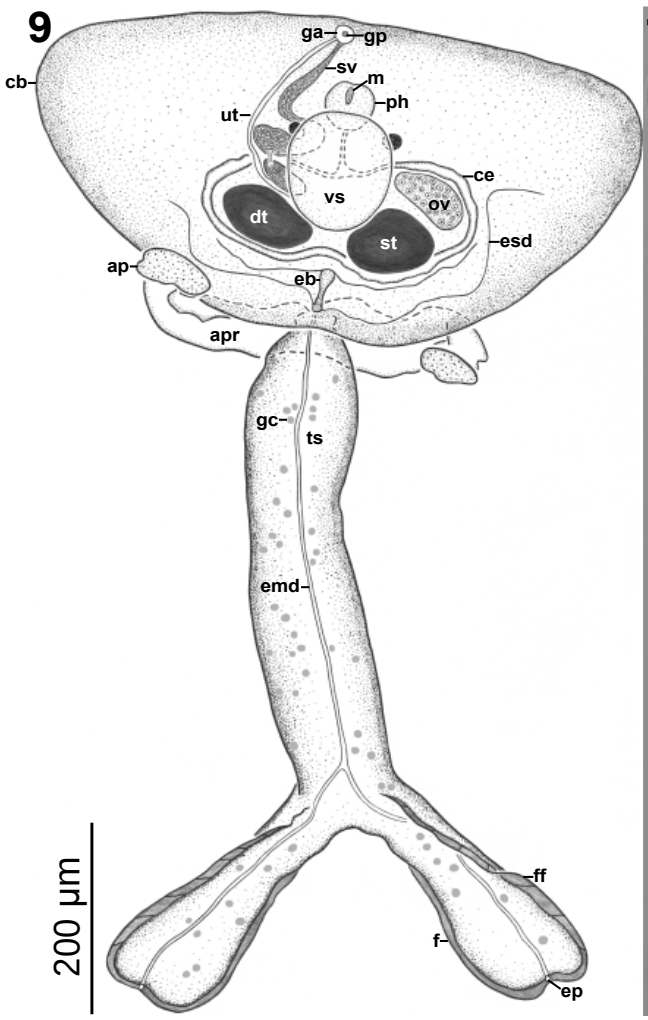
Figure 20. *ITS2* phylogeny. Values aside nodes are posterior probability. Sequences in bold are those from the present study. Scale bar is in substitutions per site.


1
2
3
4
5
5 mm

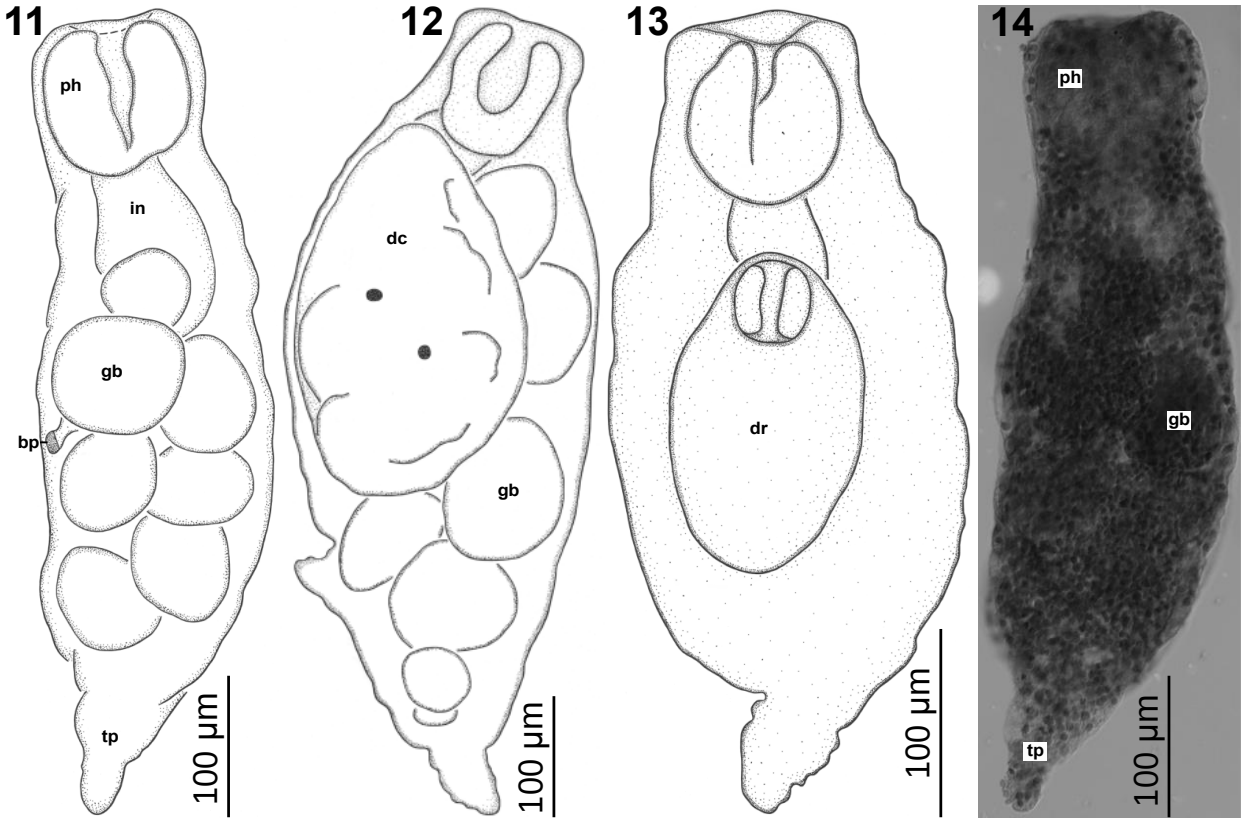

6
gp
ga
sv
m
ph
vf
es
be
vs
ov
ce
ce
dt
vr
st
Lc
ut
cy
ep
200 µm

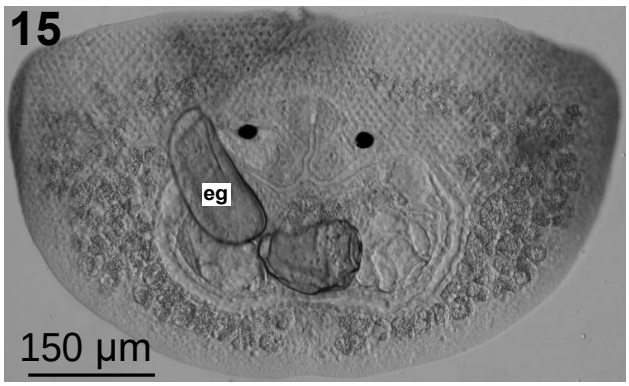

7
150 µm

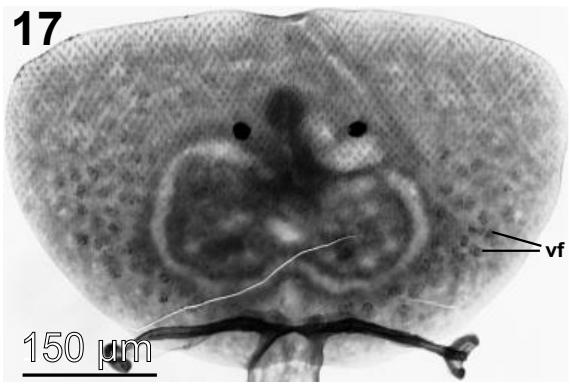

8
tvd
ovd
ov
vr
ot
ut
od
pLc
dLc
Lp
100 µm

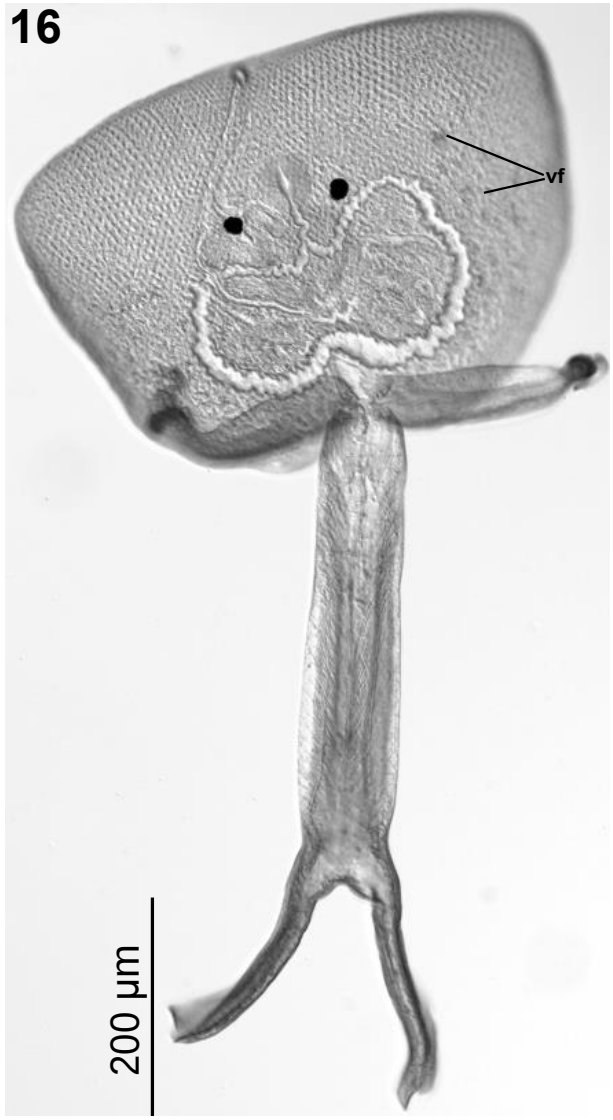

9
ga
gp
sv
m
ph
cb
ut
ce
vs
ov
dt
st
esd
ap
eb
apr
gc
ts
emd
ff
f
ep
200 µm

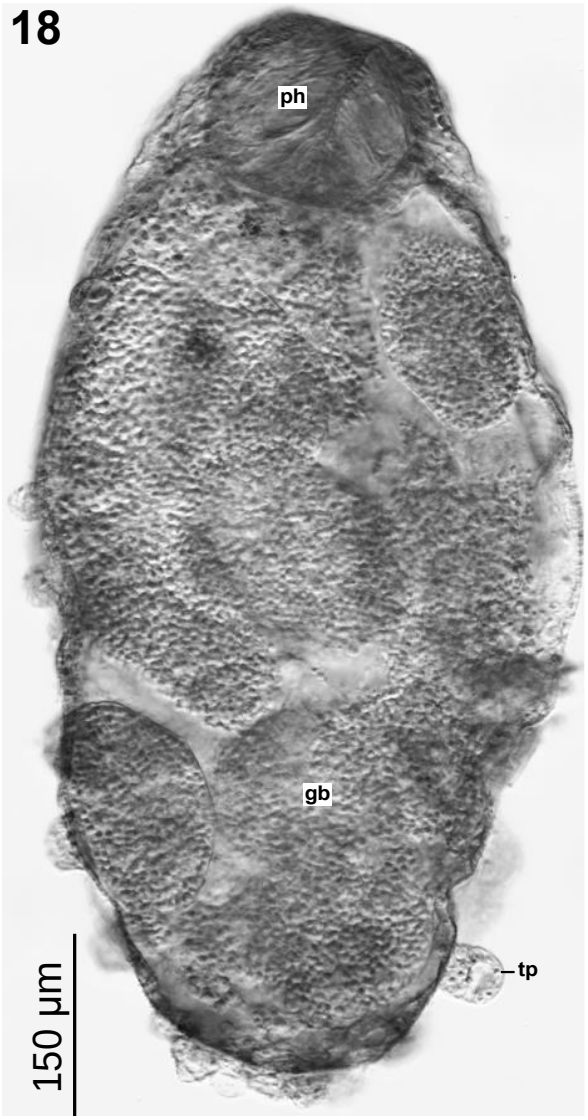

10
200 µm

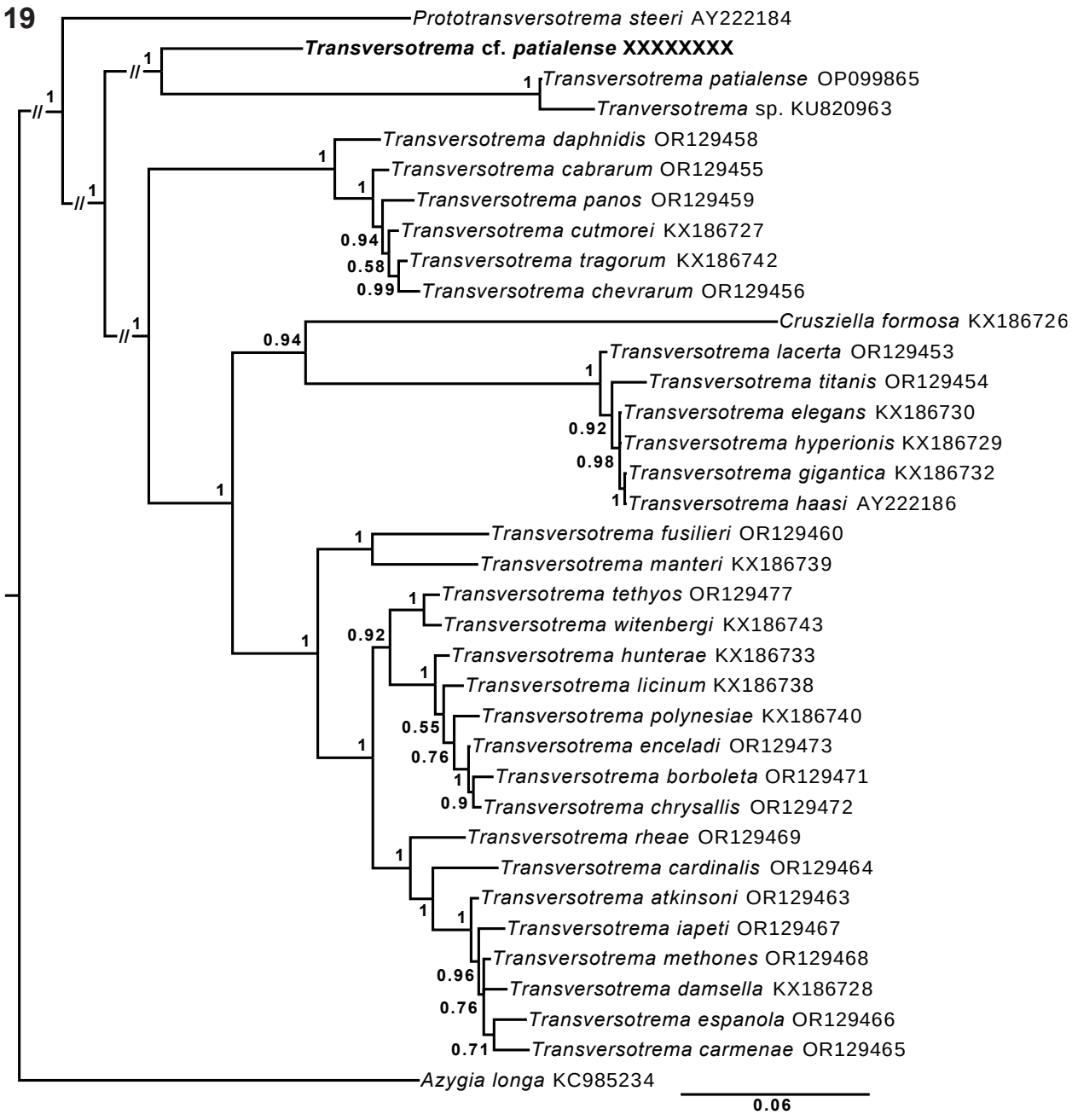

11
ph
in
gb
bp
tp
100 µm
12
dc
gb
100 µm
13
dr
100 µm
14
ph
gb
tp
100 µm

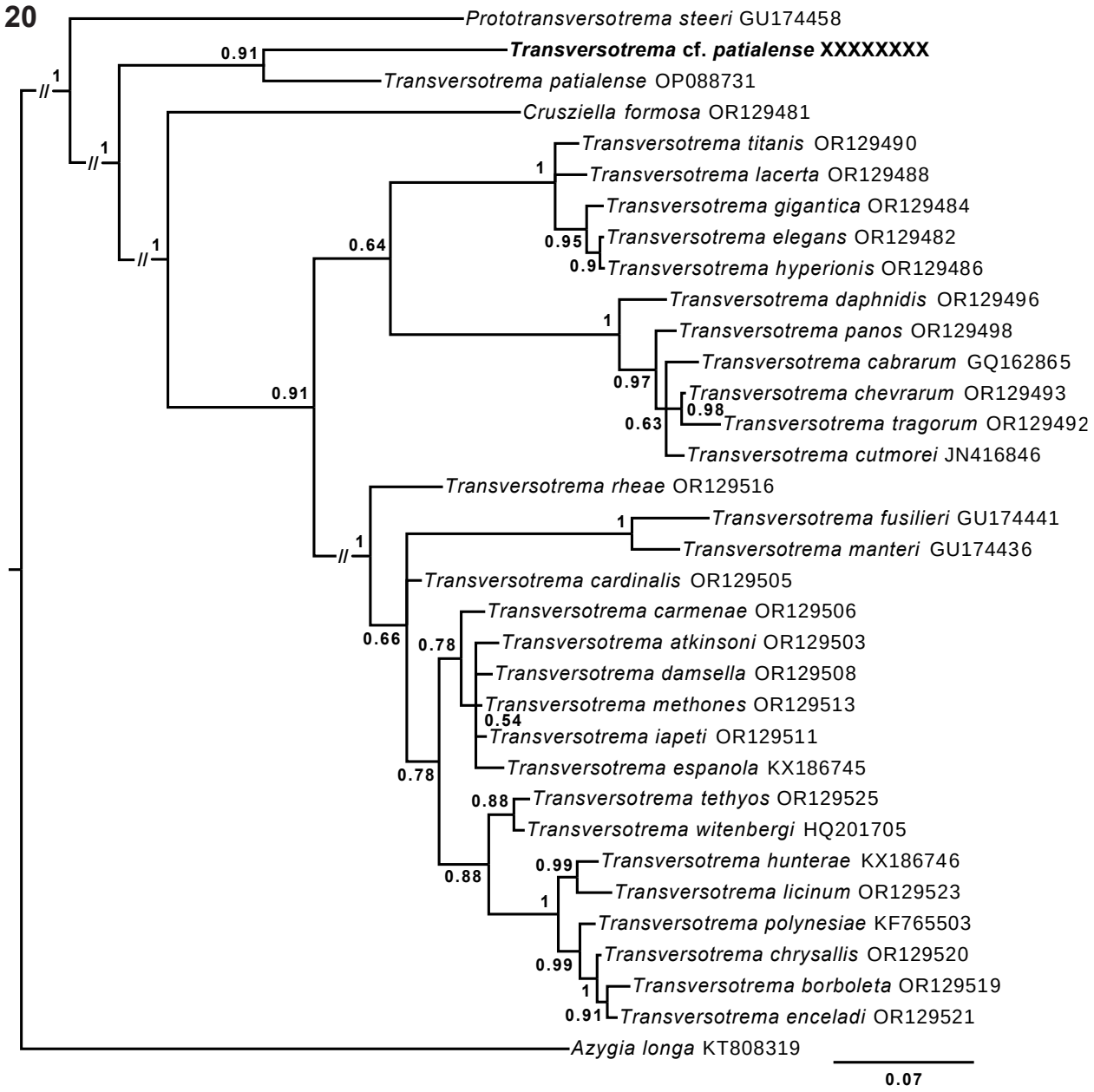

15
eg
150 µm


17
vf
150 µm


16
vf
200 µm


18
ph
gb
tp
150 µm


19
Prototransversotrema steeri AY222184
Transversotrema cf. patialense XXXXXXXX
Transversotrema patialense OP099865
Tranversotrema sp. KU820963
Transversotrema daphnidis OR129458
Transversotrema cabrarum OR129455
Transversotrema panos OR129459
Transversotrema cutmorei KX186727
Transversotrema tragorum KX186742
Transversotrema chevrarum OR129456
Crusziella formosa KX186726
Transversotrema lacerta OR129453
Transversotrema titanis OR129454
Transversotrema elegans KX186730
Transversotrema hyperionis KX186729
Transversotrema gigantica KX186732
Transversotrema haasi AY222186
Transversotrema fusilieri OR129460
Transversotrema manteri KX186739
Transversotrema tethyos OR129477
Transversotrema witenbergi KX186743
Transversotrema hunterae KX186733
Transversotrema licinum KX186738
Transversotrema polynesiae KX186740
Transversotrema enceladi OR129473
Transversotrema borboleta OR129471
Transversotrema chrysallis OR129472
Transversotrema rheae OR129469
Transversotrema cardinalis OR129464
Transversotrema atkinsoni OR129463
Transversotrema iapeti OR129467
Transversotrema methones OR129468
Transversotrema damsella KX186728
Transversotrema espanola OR129466
Transversotrema carmenae OR129465
Azygia longa KC985234
0.06


20
Prototransversotrema steeri GU174458
Transversotrema cf. patialense XXXXXXXX
Transversotrema patialense OP088731
Crusziella formosa OR129481
Transversotrema titanis OR129490
Transversotrema lacerta OR129488
Transversotrema gigantica OR129484
Transversotrema elegans OR129482
Transversotrema hyperionis OR129486
Transversotrema daphnidis OR129496
Transversotrema panos OR129498
Transversotrema cabrarum GQ162865
Transversotrema chevrarum OR129493
Transversotrema tragorum OR129492
Transversotrema cutmorei JN416846
Transversotrema rheae OR129516
Transversotrema fusilieri GU174441
Transversotrema manteri GU174436
Transversotrema cardinalis OR129505
Transversotrema carmenae OR129506
Transversotrema atkinsoni OR129503
Transversotrema damsella OR129508
Transversotrema methones OR129513
Transversotrema iapeti OR129511
Transversotrema espanola KX186745
Transversotrema tethyos OR129525
Transversotrema witenbergi HQ201705
Transversotrema hunterae KX186746
Transversotrema licinum OR129523
Transversotrema polynesiae KF765503
Transversotrema chrysallis OR129520
Transversotrema borboleta OR129519
Transversotrema enceladi OR129521
Azygia longa KT808319
0.07