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***Clinostomum poteae* n. sp. (Digenea: Clinostomidae), in the trachea of a double-crested cormorant *Phalacrocorax auritus* Lesson, 1831 and molecular data linking the life- cycle stages of *Clinostomum album* Rosser, Alberson, Woodyard, Cunningham, Pote & Griffin, 2017 in Mississippi, USA**

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Abstract *Clinostomum* spp. (Digenea: Clinostomidae) are a group of trematodes commonly found in the buccal cavity and oesophagus of a variety of piscivorous birds. The metacercariae, colloquially known as “yellow grubs,” have been reported from a diverse group of freshwater fishes worldwide. In the catfish farming region of the southeastern USA, piscivorous

birds present a continuous challenge for aquaculturists in the form of fish depredation and the introduction of trematodes into these static, earthen pond systems. *Clinostomum* spp. are commonly encountered in farm-raised catfish. While generally considered pests of minimal importance, heavy infections can result in unmarketable fillets. Of the piscivorous birds that frequent catfish aquaculture operations in the southeastern US, the double-crested cormorant (*Phalacrocorax auritus* Lesson) is one of the most damaging, although reports of *Clinostomum* spp. from *P. auritus* are limited. In this study, adult trematodes morphologically consistent with *Clinostomum* sp. were found in the trachea of a double-crested cormorant captured in Lowndes Co., Mississippi, USA. These specimens differed from other recognised *Clinostomum* spp. in several key morphological characters. Moreover, sequence data of mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox1*), nicotinamide adenine

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dinucleotide dehydrogenase subunit 1 gene (*nad1*) and ribosomal internal transcribed spacer (ITS) regions did not match any known *Clinostomum* sp. for which sequence data are available. While genetically similar to *C. marginatum* and *C. album* Rosser, Alberson, Woodyard, Cunningham, Pote & Griffin, 2017 reported from the great egret *Ardea alba* L. in Mississippi, these adult clinostomids were larger in size and limited to the trachea, whereas both *C. marginatum* Rudolphi, 1819 and *C. album* are found in the oral cavity and esophagus. Given these distinct morphological and molecular characters we propose a new member of the genus, known hereafter as *Clinostomum poteae* n. sp. Additionally, larval stages in the life-cycle of *C. album* are morphologically and molecularly identified for the first time from ramshorn snails *Planorbella trivolvis* Say and fathead minnows *Pimephales promelas* Rafinesque.

Introduction

The Clinostomidae Lühe, 1901 is a diverse family of trematodes found primarily in the buccal cavity and oesophagus of birds, reptiles, and mammals (Ukoli et al., 1966). Of the clinostomids, *Clinostomum* spp. Leidy, 1856 have been well documented in the literature and are recognised globally as parasites of economic and ecologic importance in freshwater fish and amphibians. The metacercaria stage is often referred to by aquaculturists, fish health professionals and parasitologists by the colloquial name “yellow grub”. Freshwater snails, usually of the family Planorbidae Rafinesque, serve as the first intermediate host (Krull, 1934; Pinto et al., 2015).

Currently, experimental life-cycle studies using natural definitive hosts are rarely performed as they are labor intensive and in many cases logistically unfeasible due to availability of hosts, regulatory hurdles and other interfering factors. In the absence of experimental challenges, putative life histories and the other aspects of parasite ecology can be determined by linking molecular sequence data obtained from morphologically ambiguous larval stages to gene sequences collected from adult specimens identified by morphologically distinct characters (Criscione et al., 2005). Studies investigating the global and regional diversity of *Clinostomum* species have demonstrated an unexpected number of putative

species identified by molecular and phylogenetic analyses of nuclear and mitochondrial genes (namely the internal transcribed spacer regions and cytochrome *c* oxidase subunit 1 gene) (Locke et al., 2015; Pérez-Ponce de León et al., 2016). In North America, six distinct *Clinostomum* spp. are recognised based on discrete morphological characters, all of which have molecular sequence data available for reference (Bravo-Hollis, 1947; Hutton & Sogandares-Bernal, 1960; Sereno-Urbe et al., 2013; Locke et al., 2015; Rosser et al., 2017; Briosio-Anguilar et al., 2018). At least nine additional *Clinostomum* spp. are proposed, based solely on mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox1*) and ribosomal internal transcribed spacer regions (ITS) sequences obtained from larval (Locke et al., 2015) and some adult stages (Pérez-Ponce de León et al., 2016), although corresponding morphological data for these putative species are limited. Further efforts to elucidate the species boundaries within the genus *Clinostomum* should require detailed morphometric analysis, in combination with molecular analyses of multiple gene targets, and when available, the inclusion of ecological data. Ecological data should include primary host range, tissue tropism, geographical locality and other aspects as recommended by Blasco-Costa et al. (2016).

In the southeastern USA, production of channel *Ictalurus punctatus* Rafinesque and hybrid catfish (♀ channel catfish x ♂ blue catfish *Ictalurus furcatus* Valenciennes) is hindered by losses due to depredation by piscivorous birds and the trematodes they introduce into these systems. Double-crested cormorants *Phalacrocorax auritus* Lesson are one of the most damaging on account of their high prevalence and consumption rates (Glahn & King, 2004). A recent parasitological survey of cormorants from the catfish farming region of northwestern Mississippi revealed a diversity of trematodes (O’Hear et al., 2014), several of which have since been identified as parasites of catfish (Griffin et al., 2012; Rosser et al., 2016a, b). *Clinostomum* spp. have not been reported from double-crested cormorants in Mississippi but have been reported in the great egret *Ardea alba* L. (see Rosser et al., 2017) and other ardeid hosts foraging on or near catfish operations (Overstreet & Curran 2004).

Clinostomum marginatum Rudolphi, 1819 is reported from channel catfish, but infections are considered of limited importance as no significant

pathological changes have been associated with infections and their impacts on production are negligible (Hawke & Khoo, 2004). However, in heavy infections, stunted growth may occur and the presence of numerous metacercariae in the musculature of the fish may result in rejection at processing (Hawke & Khoo, 2004). In addition to *C. marginatum*, great egrets in Mississippi are also infected with a second species, *Clinostomum album* Rosser, Alberson, Woodyard, Cunningham, Pote & Griffin, 2017. In Florida, USA, Hutton & Sogandares-Bernal (1960) noted *Clinostomum attenuatum* Cort, 1913 from the trachea of the Florida cormorant *Phalacrocorax auritus floridanus* Audubon. Similarly, Threlfall (1982) reports *C. marginatum* from the trachea and lungs of double-crested cormorant, *P. auritus*, in Florida, USA. A *Clinostomum* sp., reported as *Clinostomum complanatum* (Rudolphi, 1814), was also reported from the oral cavity and oesophagus of the Neotropical cormorant *Phalacrocorax brasilianus* Gmelin, the freshwater mollusc *Biomphalaria peregrina* d'Orbigny, four siluriform and one characiform fish in Brazil (Dias et al., 2003). However, recent morphological and molecular studies suggest the species designated as *C. complanatum* in the Americas may be misclassified (Caffara et al., 2011; Sereno-Uribe et al., 2013). Retrospective examination of specimens identified as *C. complanatum* from Central America were actually considered *C. marginatum* or the more recently described *Clinostomum tataxumui* Sereno-Uribe, Pinacho-Pinacho, García-Varela & Pérez-Ponce de León, 2013. The current literature suggests *C. complanatum* is not presently found in the New World, but further morphological and molecular studies are needed to verify these claims.

Herein we present a *Clinostomum* sp. described from the trachea of a single double-crested cormorant. Morphological and molecular data suggest this is a previously uncharacterised species, hereafter known as *Clinostomum poteae* n. sp. Additionally, we provide molecular data linking the larval stages of *C. album* in the snail and fish host. Histopathological characterisation of infections in the snail host, and novel molecular data of the nicotinamide adenine dinucleotide dehydrogenase subunit 1 gene (*nad1*) for three *Clinostomum* species are discussed.

Materials and methods

Bird trapping and necropsy

In September 2016, a single double-crested cormorant *Phalacrocorax auritus*, collected from Lowndes Co., Mississippi, USA, was euthanised using CO₂ and examined for helminth infections. The entire gastrointestinal tract was removed before being separated into four compartments: oesophagus, proventriculus and gizzard, and intestine (beginning posterior to the gizzard and terminating at the cloaca). The trachea and lower respiratory tract were also removed. All organs were opened longitudinally and examined grossly for the presence of helminths. The mucosal lining of the partitioned sections of the gastrointestinal tract were scraped manually and the contents washed with dechlorinated water into a 38- μ m aperture brass sieve. The screened contents were rinsed into a container with 0.09% saline and the contents were examined in a lined Petri dish with the aid of a dissecting stereomicroscope (Olympus SZ60, Olympus Optical Co. Ltd., Tokyo, Japan). Adult helminths were collected and placed in 0.09% saline. *Clinostomum* sp. adults found attached to the lining of the trachea were gently detached with featherweight forceps (BioQuip Products, Rancho Dominguez, California, USA) and placed in 0.09% saline. All helminths were relaxed in nearly boiling saline and fixed in 70% ethanol. Adult trematodes morphologically consistent with *Clinostomum* sp. were limited to the trachea and are the focus of this work.

Snail host and cercariae collection

Ramshorn snails *Planorbella trivolvis* Say were collected (September 2016) from vegetation along the banks of a catfish production pond in Leflore County, Mississippi, USA, using a 1.5-mm mesh aquatic dip net. Snails were placed into 18.9 l buckets containing c. 10 l of pond water and transported to the Thad Cochran National Warmwater Aquaculture Center. Snails were rinsed with well water, placed individually into plastic dilution vials (Fisher Scientific, Pittsburgh, Pennsylvania, USA) and covered with c. 10 ml of autoclaved spring water (Ozarka[®], Wilkes Barre, Pennsylvania, USA). Every 24 hours the water column in each dilution vial was examined using a dissecting microscope (Olympus SZ60, Olympus Optical Co. Ltd., Tokyo, Japan) for cercariae. When cercariae were observed, a 1-ml aliquot of cercaria

laden water was frozen at -20°C for molecular analysis from each infected snail.

Metacercariae collection

As part of a routine diagnostic investigation of fish health in a commercial catfish production pond in Lowndes County, Mississippi, USA, a single fathead minnow (6.6 cm) *Pimephales promelas* Rafinesque was examined in March 2017. Two encysted *Clinostomum* sp. metacercariae were collected from the coelomic cavity, excysted manually with metal pins, relaxed in nearly boiling 0.09% saline, and fixed in 70% ethanol for morphological and molecular analysis. An ethanol-archived specimen identified as *C. marginatum* from a blue gill *Lepomis macrochirus* Rafinesque was also trimmed for molecular analysis and included in this study.

Trematode staining and morphological analysis

A subsample ($n = 10$) of *Clinostomum* specimens from the trachea of the double-crested cormorant was transitioned from 70% ethanol to distilled water through a decreasing gradient of 1 hour ethanol washes (50%, 30% and 10%). Specimens were stained using Van Cleave's hematoxylin and mounted in Canada balsam. For a single specimen the entire worm was cleared in lactophenol and examined for body armature. A single specimen had a small section of the posterior extremity, not including reproductive organs, excised with a sterile scalpel blade and placed into a 1.5 ml tube for molecular analysis. This hologenophore was stained with Mayer's carmalum and mounted as described above.

Cercariae were studied live and vitally stained with 0.5% Jensen's neutral red [1 drop ($c.30\ \mu\text{l}$) of neutral red per 50 ml of cercariae in autoclaved spring water]. A portion were stained with Semichon's acetocarmine, destained in acidic ethanol, dehydrated in an increasing ethanol series, cleared in Hemo-De and mounted in Canada balsam as museum vouchers. Rediae were teased from the hepatopancreas of a single infected snail using insect pins, killed with near-boiling 0.09% saline and fixed in 70% ethanol. Saline wet mounts of rediae were examined for morphological characterisation. Voucher specimens of ethanol fixed rediae and cercariae were stained in Semichon's acetocarmine, destained in acidic ethanol, dehydrated in an increasing series of ethanol washes, cleared in Hemo-De, and mounted in Canada balsam. A second

infected snail was relaxed in water with menthol crystals, briefly submerged in near boiling water, the body was gently removed with feather weight forceps, and fixed in Railliet-Henry's fixative (6 g sodium chloride dissolved in 930 ml distilled water, 50 ml formaldehyde (37%, w/v), and 20 ml glacial acetic acid) for histopathological examination.

Type- and voucher specimens were submitted to the Smithsonian Institution, National Museum of Natural History, Washington, DC, USA. Line drawings were made using a camera lucida and digitised using Adobe Illustrator CC 2017 (Adobe, San Jose, California, USA). Morphological characters are reported in micrometres and measurements are presented as the range, with mean (\pm standard deviation where applicable) in parentheses.

Fish experimental challenges

Clinostomid cercariae collected from infected snails were pooled into a 500-ml beaker, the water was gently agitated with a magnetic stir bar, and the average number of cercariae per milliliter was determined from ten 0.1 ml aliquots according to previously established protocols (Griffin et al., 2012, 2014; Rosser et al., 2016b; Alberson et al., 2017). Channel catfish fingerlings ($c.2\text{--}4$ cm) reared indoors for infectious disease research at the Thad Cochran National Warmwater Aquaculture Center in Stoneville, MS, were used in infectivity studies. Fish were placed in 1 gallon containers (7 containers, 3 fish/container) containing 3.8 l of water with supplemental aeration and exposed to either 600 ($n = 9$) or 1,200 ($n = 9$) clinostomid cercariae per fish. One group of three fish were not exposed to cercariae and served as negative controls. Exposures lasted for four hours. Fish from each exposure group were placed into discrete 115-l aquaria, containing $c.80$ liters of well water ($c.26\text{--}27^{\circ}\text{C}$) supplied at 1.9 l/min, with supplemental aeration. Fish were fed ad libitum and monitored twice daily for morbidity and mortality. Once weekly, fish were netted and examined grossly for the presence of metacercariae. Fish were sacrificed at 60 days post-exposure, examined grossly for the presence of metacercariae and a subsample of fish from the 1,200 cercariae/fish exposure group ($n = 8$) were submitted for histopathological analysis.

Histopathological analysis

The single snail used in histopathological analysis was kept in Railliet-Henry's fixative for at least 24 hours,

hemisectioned, and embedded in paraffin wax for standard light microscopy slide preparation. Sections were stained with hematoxylin and eosin (HE) or periodic acid-Schiff methods for examination.

Eight channel catfish fingerlings (*c.* 5 cm long) exposed to 1,200 cercariae/fish were euthanised 60 days post-infection with an overdose of tricaine methanesulfonate, abdomens opened by incision and fixed in 10% neutral buffered formalin for a minimum of 72 hours. Fish were decalcified in Kristensen's solution for 12 hours, soaked in sodium bicarbonate rich tap water to neutralise tissue pH, and fish were trimmed in *c.* 3-mm cross-sections starting rostrally and moving caudally, with small adjustments to thickness to ensure all major organs were sampled. Tissues were processed routinely, embedded in paraffin, sectioned at approximately 4 µm, stained with hematoxylin and eosin (HE), and examined using light microscopy.

Molecular analysis

Genomic DNA was extracted from three whole adult *Clinostomum* specimens from the trachea of the cormorant and the section of the hologenophore using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, California, USA). Fragments (*c.* 500 bp) of the cytochrome *c* oxidase subunit 1 (*cox1*) and mitochondrial nicotinamide adenine dinucleotide dehydrogenase subunit 1 (*nad1*) genes were amplified by PCR and sequenced from all specimens. An *c.* 4,800-bp region of ribosomal DNA spanning the 18S rRNA gene, internal transcribed spacer (ITS) 1 region, 5.8S rRNA gene, ITS2 region, and partial 28S rRNA gene was sequenced from a single whole adult specimen, while only the ITS1, 5.8S rRNA gene, and ITS2 region was sequenced from the remaining three adult specimens. Primers used in amplifying each region are listed in Supplementary Table 1. Briefly, each 20-µl reaction consisted of 7 µl of nuclease-free water, 10 µl of Phusion Green Hot Start II High-Fidelity PCR Master Mix, 10 µM of each primer, and 1 µl of genomic DNA (*c.* 10 ng/µl). Cycling parameters for the 1F/5R, BD1/BD2, LSU5/1500R, and NDJ11/NDJ2a primer sets were 98°C for 3 min, 39 cycles at 98°C for 10 s, 48°C for 30 s, and 72 °C for 1 min, and a final extension at 72°C for 10 min. The Barker3/Barker4 primer set differed from the others by an annealing temperature of 53°C. Similarly, the Diplo1795F/Diplo2549R, Diplo2617F/Diplo3170R, 28S3431F/

28S4779R, and 28S4759F/28S5699R primer sets differed by an annealing temperature of 58°C.

Frozen 1-ml aliquots of water containing *Clinostomum* sp. cercariae released by *P. trivolvus* were thawed, contents were pelleted by centrifugation (10,000× g for 10 min), and gDNA was extracted using the DNeasy PowerSoil Kit (Qiagen Inc.) according to the manufacturer's protocol. Total gDNA from the portion of tissue excised from the each metacercaria was extracted as described for the adult *Clinostomum* sp. For all cercariae and metacercariae gDNA extracts, three regions were amplified as described previously using the BD1/BD2 primers for the ribosomal ITS regions, *cox1_schist5'/acox650r* primers for the *cox1* gene, and NDJ11/NDJ2a primers for the *nad1* gene. Additionally, the *nad1* gene was amplified from archived gDNA of an adult of *Clinostomum marginatum* from a great egret (*n* = 1) (Rosser et al., 2017), metacercaria of *C. marginatum* from an inland silverside (*n* = 1) (Rosser et al., 2016b), and an adult of *Clinostomum album* collected from a great egret (*n* = 1) (Rosser et al., 2017).

PCR products were electrophoresed through 0.8% agarose gels in the presence of ethidium bromide (0.5 µg/ml) and visualised under ultraviolet light. Each gel was run with a concurrent molecular weight ladder (HyperLadder™ 50 bp, Bionline, London, UK) to confirm presence of appropriate sized bands. Amplicons were excised and purified for sequencing using the QIAquick Gel Extraction Kit (Qiagen Inc.). Products were commercially sequenced bidirectionally with the forward and reverse primer used to generate each representative amplicon (Eurofins MWG Operon LLC, Huntsville, Alabama, USA). Electropherograms for the ribosomal and mitochondrial regions were aligned and annotated using Geneious® 10.1.3 (Biomatters Ltd., Auckland, New Zealand). The contiguous rRNA, *cox1*, and *nad1* gene sequences were compared to other *Clinostomum* species available in the National Center for Biotechnology Information non-redundant nucleotide database (NCBI nr/nt) by a BLASTN search (Altschul et al., 1990).

Phylogenetic analyses

Archived sequences of representative species of the family Clinostomidae Lühe, 1901, were downloaded from the NCBI nr/nt database and in some cases obtained from supplementary files from original works (i.e. Locke et al., 2015) (Supplementary Table 2). Downloaded sequences were limited to specimens

with data available for both the ITS regions and *cox1* gene. Additionally, two members of the Diplostomidae Poirier, 1886 (*Alaria mustelae* Bosma, 1931 and *Diplostomum baeri* Dubois, 1937) were included in the alignments as outgroups. For the ribosomal region dataset, the ITS1, 5.8S rRNA gene, and ITS2 region were extracted using ITSx v. 1.0.11 (Bengtsson-Palme et al., 2013). The *cox1* and extracted ITS1, 5.8S rRNA, and ITS2 regions were aligned with MAFFT 7.222 in Geneious® 10.1.3 (Kato et al., 2002; Kato & Standley, 2013). Positions in each alignment containing at least one gap were eliminated and the regions were concatenated for a final alignment containing 1,127 positions. The Bayesian Information Criterion was used to select the nucleotide substitution models that best described the data for each region (Nei & Kumar, 2000; Kumar et al., 2016): *cox1* codon position 1 (TN93 + I; 164 positions), *cox1* codon position 2 (HKY; 164 positions), *cox1* codon position 3 (TN93 + G; 164 positions), ITS1 region (K2 + G; 399 positions), 5.8S rRNA gene (JC; 157 positions), and ITS2 region (JC + G; 70 positions). Bayesian inference analysis was performed in MrBayes 3.2.6 using Markov chain Monte Carlo searches of two simultaneous runs of four chains with sampling of every 100th tree until convergence, when the value of the standard deviation of split frequencies was < 0.01. At this point the first 25% were discarded as ‘burn-in’ and posterior probabilities were calculated from the remaining trees. Maximum likelihood analysis was performed using IQ-Tree (Nguyen et al., 2015) on the IQ-Tree web server (Trifinopoulos et al., 2016) with the concatenated alignment and partition scheme described previously. Branch support was tested using ultrafast bootstrap support (Minh et al., 2013) with 1,000 pseudoreplicates. Dendrograms were viewed in FigTree 1.4.2 (Rambaut, 2014) and annotated in Adobe Illustrator CC 2017 (Adobe, San Jose, California, USA). Pairwise distances based on the *cox1* sequences used in the alignment for phylogenetic analysis and *nad1* sequences were calculated using MEGA7 (Kumar et al., 2016).

Family Clinostomidae Lühe, 1901
Genus *Clinostomum* Leidy, 1856

***Clinostomum poteae* n. sp.**

Type-host: *Phalacrocorax auritus* (Lesson) (Aves: Phalacrocoracidae), double-crested cormorant.

Type-locality: Lowndes County, Mississippi, USA.

Site in host: Trachea.

Type-material: Holotype USNM 1488288; 3 paratypes USNM 1488289–1488291; 1 hologenophore USNM 1488292.

Prevalence and intensity of infection: 1 of 1 *P. auritus*; 22 worms.

Representative DNA sequences: GenBank MH282567–MH282570 (ribosomal regions); MH282550–MH282553 (*cox1* gene); MH282520–MH282522 (*nad1* gene).

Zoobank registration: To comply with the regulations set out in article 8.5 of the amended 2012 version of the International Code of Zoological Nomenclature (ICZN, 2012), details of the new species have been submitted to ZooBank. The Life Science Identifier (LSID). The LSID for *Clinostomum poteae* n. sp. is urn:lsid:zoobank.org:act:25DA863A-F83C-4540-B95F-9299B24835CA.

Etymology: The species epithet is reference to and in memory of parasitologist Dr Linda Marie Wayland Pote in recognition of her contributions to the field of aquatic animal and avian parasitology, and her enthusiasm for training a new generation of parasitologists.

Description (Figs. 1, 2)

[Based on 10 ovigerous adults, Van Cleave’s hematoxylin stained and mounted in Canada balsam, see Table 1.] Body, aspinose, dorso-ventrally flattened, linguiform, widest at level of gonads, slightly constricted at ventral sucker level, 7,256–9,144 × 1,628–2,210 (8,178 × 1,930); body length to body width ratio 3.8–4.7 (4.2). Oral collar-like fold typical of *Clinostomum* spp., 652–910 × 940–1,153 (757 × 1,064) surrounding oral sucker. Oral sucker spherical, 311–365 × 337–400 (331 × 370). Pharynx not observed, branching of intestinal caeca occurs just posterior to oral collar. Paired intestinal caeca laterally extend almost entire length of body with broad diverticular foldings beginning at level of posterior border of ventral sucker. Ventral sucker large, 797–922 × 834–978 (875 × 909), located in lower to middle portion of anterior third of body. Distance between oral and ventral suckers 669–936 (769).

Testes 2, tandem, located in upper third of hindbody. Anterior testis variably lobed, 441–665 × 594–928 (550 × 792). Posterior testis



Fig. 1 Photomicrographs of Van Cleave's hematoxylin-stained *Clinostomum poteae* n. sp. from *Phalacrocorax auritus* collected in Lowndes County, Mississippi, USA. A, Holotype; B–C, Paratypes. Scale-bar: 1 mm

triangular, lobed, $463\text{--}699 \times 528\text{--}799$ (601×715). Distance between testes $472\text{--}590$ (524). Cirrus-sac, ovoid, $503\text{--}662 \times 294\text{--}445$ (553×382), located at level of and overlapping right margin of anterior testis.

Ovary dextral, intertesticular, ovoid, $328\text{--}428 \times 225\text{--}307$ (396×268). Oötype complex, intertesticular, located at midline of body. Mehlis' gland prominent, finer details of Laurer's canal obscured by presence of eggs. Uterine duct intracaecal, extending anteriorly around left margin of anterior testis before opening into uterine sac. Uterine sac tubular, extends anteriorly in midline of body to level of posterior margin of ventral sucker. Genital pore

opening into genital atrium near midline or right margin of the body at level of anterior testis. Anterior limits of vitelline fields at midpoint of ventral sucker, posterior limits at posterior end of caeca. Eggs yellowish, operculate, $105\text{--}119 \times 63\text{--}75$ (112×71).

Remarks

Clinostomum poteae n. sp. is morphologically consistent with other members of the genus and the ranges for many morphological characters overlap with other *Clinostomum* spp. from the Americas (Table 1). *Clinostomum intermedialis* Lamont, 1920 described

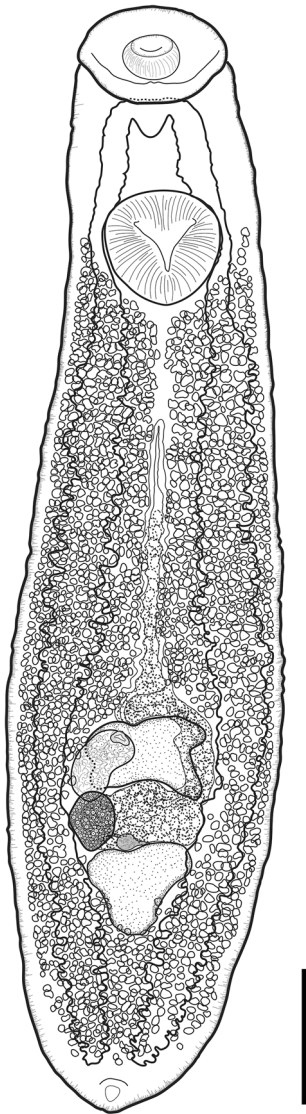


Fig. 2 Line drawing of the holotype of *Clinostomum poteae* n. sp. from *Phalacrocorax auritus* collected in Lowndes County, Mississippi, USA. Scale-bar: 1 mm

from *Phalacrocorax vigua* Vieillot differs from *C. poteae* n. sp. in the placement of the anterior testis and its size. The anterior testis of *C. intermedius* is distinctly ovoid in mature specimens and located in the left margin of the body compared to the typically lobed anterior testis of other *Clinostomum* spp. and placement in the midline of the body. Based on the most recent description by Bravo-Hollis (1947) both testes of *C. intermedius* are considerably larger in size compared to the other *Clinostomum* spp. from the Americas except for *Clinostomum heluans* Braun,

1899 in which the gonads are located posteriorly in the body. Lunaschi & Drago (2009) considered *C. intermedius* a member of the genus *Clinostomatopsis* Dollfus, 1932 based on the placement of the genital pore. Although similar in morphology to *C. attenuatum* studied by Hutton & Sogandares-Bernal (1960) from the trachea of cormorants, morphological variations exist between *C. attenuatum* and *C. poteae* n. sp. Hutton & Sogandares-Bernal (1960) provided measurements for the seven adult specimens they encountered. Using these data, additional morphometric parameters used in previous studies of *Clinostomum* spp. were evaluated. These include the ratios body length/body width, oral sucker width/body width, ventral sucker width/oral sucker width, ventral sucker width/body width, anterior testis width/anterior testis length, posterior testis width/posterior testis length, and ovary width/ovary length (Table 1). *Clinostomum attenuatum* is notably wider than *C. poteae* n. sp. in regard to body width and has a smaller ratio of body length to body width, with *C. poteae* being consistently longer. The testes size for *C. attenuatum* compared with *C. poteae* n. sp. are notably wider, although ranges for length and width slightly overlap. Hutton & Sogandares-Bernal (1960) noted the testes of *C. attenuatum* were crescent shape in most specimens with few being slightly lobed. This is in contrast to the distinctly lobed nature of the testes of *C. poteae* n. sp., especially in regard to the anterior testis. *Clinostomum marginatum* has also been reported from the trachea of double-crested cormorants in Florida; however, the placement of the gonads and size of the ovary of *C. poteae* n. sp. differs from *C. marginatum* described from ardeid birds (Caffara et al., 2011; Rosser et al., 2017).

In addition to morphological variation, *C. poteae* n. sp. is molecularly distinct from any *Clinostomum* sp. with *cox1* sequence data to date, including *C. album*, *C. attenuatum*, *C. marginatum*, and *C. tataxumui*, two of which have been reported from double-crested cormorants.

***Clinostomum album* Rosser, Alberson, Woodyard, Cunningham, Pote & Griffin, 2017**

First intermediate host: *Planorbella trivolvis* (Say) (Gastropoda: Planorbidae).

Second intermediate host: Fathead minnow *Pimephales promelas* (Rafinesque) (Cypriniformes: Cyprinidae).

Site in host: Hepatopancreas (ex *P. trivolvis*); coelomic cavity (ex *P. promelas*).

Table 1 Morphological data of *Clinostomum* spp. from birds in North America

Parasite	<i>Clinostomum album</i>	<i>Clinostomum attenuatum floridanus</i> Lesson	<i>Clinostomum golvani</i>	<i>Clinostomum helvans</i>	<i>Clinostomum marginatum</i>	<i>Clinostomum marginatum</i>	<i>Clinostomum poteae</i> n. sp.	<i>Clinostomum tataxumui</i>
Host	<i>Ardea alba</i> L.	<i>Phalacrocorax auritus</i>	<i>Butorides virescens</i>	<i>Ardea herodias</i> L.	<i>A. herodias</i> L., <i>Bubulcus ibis</i> L.	<i>Ardea alba</i> L.	<i>Phalacrocorax auritus</i>	<i>A. alba</i> L., <i>A. herodias</i> L., <i>Tigrisoma mexicanum</i> Swainson Mexico
Locality	Mississippi, USA	Florida, USA	Guadeloupe	Mexico	Canada; Florida & Texas, USA	Mississippi, USA	Mississippi, USA	
Reference	Rosser et al. (2017)	Hutton & Sogandares-Bernal (1960)	Nassi & Bayssade-Dufour (1980)	Bravo-Hollis (1947)	Caffara et al. (2011)	Rosser et al. (2017)	This study	Sereno-Uribe et al. (2013)
BL	4,402–5,929 (5,269)	6,900–7,358 (7,058)	4,020–7,460	20,706–26,145	5,900–8,200 (7,000)	5,132–6,210 (5,697)	7,256–9,144 (8,178)	3,360–9,800 (4,500)
BW	969–1,108 (1,047)	2,080–2,650 (2,429)	2,330–3,440	2,640–3,444	1,300–2,800 (2,000)	1,350–1,820 (1,562)	1,628–2,210 (1,930)	800–3,000 (1,200)
BL/BW	4.2–6.1 (5.0)	2.6–3.4 (2.9)	–	–	–	3.2–4.0 (3.7)	3.8–4.7 (4.2)	–
OCL	357–507 (428)	–	–	840–1,000	–	432–793 (561)	652–910 (757)	330–990 (460)
OCW	526–690 (617)	–	–	1,000–1,440	–	742–963 (823)	940–1,153 (1,064)	570–1,570 (760)
OSL	207–307 (256)	310–380 (331)	290–400	640–800	171–394 (311)	246–299 (268)	311–365 (331)	170–350 (206)
OSW	234–344 (263)	–	220–300	700–580	252–501 (399)	237–318 (267)	337–400 (370)	170–300 (208)
OSW/BW	0.2–0.4 (0.3)	0.12–0.15 (0.14)	–	–	–	0.15–0.19 (0.2)	0.18–0.21 (0.2)	–
VSL	480–648 (578)	790–864 (828)	600–850	880–1100	601–918 (764)	550–694 (612)	797–922 (875)	420–850 (536)
VSW	485–610 (560)	–	–	880–1100	583–966 (756)	589–677 (611)	834–978 (909)	420–777 (524)
VSW/OSW	1.5–2.4 (2.2)	2.3–2.7 (2.5)	–	–	–	2.0–2.5 (2.3)	2.3–2.6 (2.5)	–
VSW/BW	0.5–0.6 (0.5)	0.3–0.4 (0.3)	–	–	–	0.37–0.44 (0.4)	0.4–0.5 (0.5)	–

Table 1 continued

Parasite	<i>Clinostomum album</i>	<i>Clinostomum attenuatum</i>	<i>Clinostomum golvani</i>	<i>Clinostomum helvans</i>	<i>Clinostomum marginatum</i>	<i>Clinostomum marginatum</i>	<i>Clinostomum poteae</i> n. sp.	<i>Clinostomum tataxumui</i>
Host	<i>Ardea alba</i> L.	<i>Phalacrocorax auritus floridanus</i> Lesson	<i>Butorides virescens</i>	<i>Ardea herodias</i> L.	<i>A. herodias</i> L., <i>Bubulcus ibis</i> L.	<i>Ardea alba</i> L.	<i>Phalacrocorax auritus</i>	<i>A. alba</i> L., <i>A. herodias</i> L., <i>Tigrisoma mexicanum</i> Swanson Mexico
Locality	Mississippi, USA	Florida, USA	Guadeloupe	Mexico	Canada; Florida & Texas, USA	Mississippi, USA	Mississippi, USA	Mexico
Reference	Rosser et al. (2017)	Hutton & Sogandares-Bernal (1960)	Nassi & Bayssade-Dufour (1980)	Bravo-Hollis (1947)	Caffara et al. (2011)	Rosser et al. (2017)	This study	Sereno-Uribe et al. (2013)
DBS	360–827 (656)	–	–	–	640–928 (770)	640–928 (770)	669–936 (769)	–
ATL	266–469 (375)	436–500 (479)	210–360	860–900	290–743 (519)	373–526 (453)	441–665 (550)	220–650 (341)
ATW	449–535 (488)	900–1,000 (967)	550–650	1700–1840	569–1,141 (802)	534–737 (667)	594–928 (792)	370–1,005 (506)
ATW/ATL	1.1–1.7 (1.3)	1.8–2.3 (2.0)	–	–	–	1.2–1.8 (1.5)	1.1–1.8 (1.5)	–
PTL	313–473 (425)	400–518	260–400	640–840	166–587 (461)	319–589 (424)	463–699 (601)	240–420 (346)
PTW	416–571 (501)	774–980	780–800	1,440–1,740	379–1,414 (790)	569–826 (700)	528–799 (715)	450–1,220 (599)
PTW/PTL	1.0–1.3 (1.2)	1.7–2.2 (2.0)	–	–	–	1.4–1.9 (1.7)	0.9–1.4 (1.2)	–
DBT	264–354 (298)	–	–	–	–	252–378 (312)	472–590 (524)	–
OVL	201–281 (235)	273–300	280–480	640–840	118–306 (175)	187–261 (236)	328–428 (396)	160–420 (218)
OVW	180–254 (199)	346–465	–	360–714	101–267 (186)	176–256 (217)	225–307 (268)	150–360 (196)
OVL/OVW	0.8–0.9 (0.8)	0.6–0.8 (0.7)	–	–	–	0.8–1.04 (0.9)	0.5–0.8 (0.7)	–
CSL	140–307	–	–	–	151–795	316–544	503–662	250–520

Table 1 continued

Parasite	<i>Clinostomum album</i>	<i>Clinostomum attenuatum</i>	<i>Clinostomum golvani</i>	<i>Clinostomum heluans</i>	<i>Clinostomum marginatum</i>	<i>Clinostomum marginatum</i>	<i>Clinostomum poteae</i> n. sp.	<i>Clinostomum tataxamui</i>
Host	<i>Ardea alba</i> L.	<i>Phalacrocorax auritus floridanus</i> Lesson	<i>Butorides virescens</i>	<i>Ardea herodias</i> L.	<i>A. herodias</i> L., <i>Bubulcus ibis</i> L.	<i>Ardea alba</i> L.	<i>Phalacrocorax auritus</i>	<i>A. alba</i> L., <i>A. herodias</i> L., <i>Tigrisoma mexicanum</i> Swainson Mexico
Locality	Mississippi, USA	Florida, USA	Guadeloupe	Mexico	Canada; Florida & Texas, USA	Mississippi, USA	Mississippi, USA	Mexico
Reference	Rosser et al. (2017) (222)	Hutton & Sogandares-Bernal (1960)	Nassi & Bayssade-Dufour (1980)	Bravo-Hollis (1947)	Caffara et al. (2011)	Rosser et al. (2017) (418)	This study (553)	Sereno-Uribe et al. (2013) (350)
CSW	117–162 (146)	–	–	–	166–741 (497)	154–298 (220)	294–445 (382)	130–300 (172)
CSL/BL	0.02–0.1 (0.04)	–	–	–	–	0.06–0.09 (0.1)	0.1–0.1 (0.1)	–
EGGL	90–108 (100)	109–115	111–120 (116)	114–136	101–109 (105)	94–105 (101)	104–119 (112)	–
EGGW	53–67 (61)	65–73	65–71 (67)	70–80	63–79 (69)	63–72 (68)	63–75 (71)	–
EGG#	70–82 (75)	–	–	–	–	–	–	–

Abbreviations: BL, body length; BW, body width; OCL, oral collar length; OCW, oral collar width; OSL, oral sucker length; OSW, oral sucker width; VSL, ventral sucker length; VSW, ventral sucker width; DBS, distance between suckers; ATL, anterior testis length; ATW, anterior testis width; PTL, posterior testis length; PTW, posterior testis width; DBT, distance between testis; OVL, ovary length; OVW, ovary width; CSL, cirrus-sac length; CSW, cirrus-sac width; EGG, egg length; EGGW, egg width; EGG#, number of eggs

Localities: Catfish pond, Leflore County, Mississippi, USA (ex *P. trivolvis*); catfish pond, Lowndes County, Mississippi, USA (ex *P. promelas*).

Infection parameters: Prevalence 4.3% (in 10 out of 230 snails) (ex *P. trivolvis*); prevalence 100% (in 1 out of 1 fish) (ex *P. promelas*); intensity: 2 metacercariae (ex *P. promelas*).

Voucher material: 2 voucher slides of Semichon's acetocarmine-stained cercariae USNM 1488293–1488294, 3 voucher slides of Semichon's acetocarmine-stained rediae USNM 1488295–1488297, 2 voucher slides of hematoxylin and eosin stained rediae and cercariae *in situ* USNM 1488298–1488299, 1 metacercaria hologenophore USNM 1488300.

Representative DNA sequences: GenBank MH282556–MH282566 (ribosomal regions); MH282539–MH282549 (*cox1* gene); MH282526–MH282537 (*nad1* gene).

Description (Figs. 3–6)

Redia

[Based on whole mounts of 34 heat killed and ethanol-fixed rediae from a single snail host; see Supplementary Table 3.] Rediae yellow-tan in color, numerous in

the hepatopancreas of *P. trivolvis*, globular to elongate, 271–819 (455 ± 111) \times 97–235 (150 ± 31) (Figs. 3A, B, 4C). Body constricted near anterior in some specimens, usually just posterior to pharynx. Pharynx muscular, subterminal, 30–57 (47 ± 8) \times 31–58 (46 ± 7). Anterior region of body spinose, with 7–8 rows of transverse spines, extending to region at posterior margin of pharynx (Fig. 3C). Intestine tubular, elongate, 162–523 (291 ± 76) \times 28–59 (41 ± 10), length representing 44–85 (65 ± 11)% of body length. Germinal cells observed in mid-posterior to posterior portion of body. Birth pore inconspicuous, laterally posterior to pharynx. Cercariae variable in number, but present in all specimens examined, many at various stages of maturation.

Remarks

The redia of *C. album* examined in this study was morphologically consistent with descriptions of the redia of *C. marginatum* studied by Krull (1934) from *Planorbella anceps* Menke in Maryland, USA. The morphometric data presented by Krull (1934) were from the largest redia observed but fit the description of the redia of *C. album*, including the transverse rows of spines at the anterior portion of the body. Other

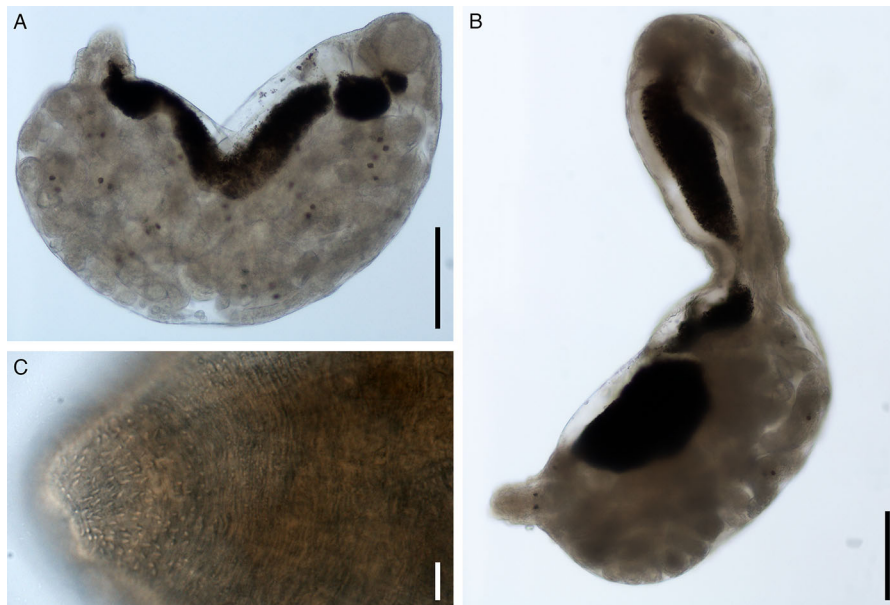


Fig. 3 Photomicrographs of unstained *Clinostomum album* rediae from *Planorbella trivolvis* collected from a catfish pond in Leflore County, Mississippi, USA. A–B, Whole rediae with numerous cercariae within; C, Anterior region showing transverse rows of spines. Scale-bars: A–B, 100 μ m; C, 10 μ m

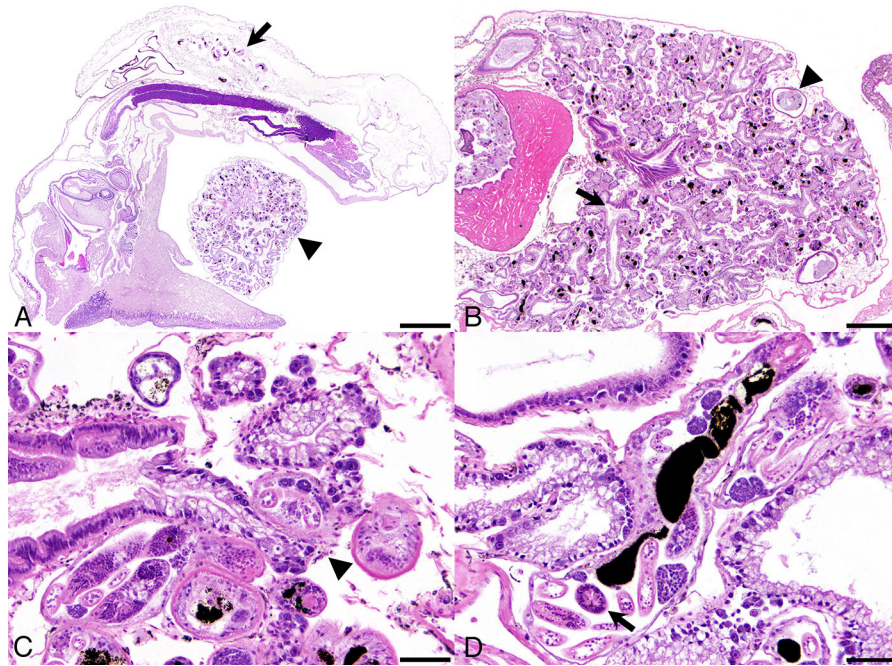


Fig. 4 Photomicrographs of *Planorbella trivolvis* infected with *Clinostomum album* collected from a catfish pond in Leflore County, Mississippi, USA. A, Subgross parasagittal view (PAS stain). Arrowhead denotes the atrophied digestive gland filled with rediae, arrow denotes rediae within the connective tissues of the lung; B, Digestive gland atrophy and heavy infection with rediae (black spots are pigment granules in rediae intestines) (HE stain). Arrow denotes digest gland tubule, arrowhead denotes intestine; C, Digestive gland tubule atrophy and compression by rediae, loss of interlobular connective tissue and fibrosis (HE stain). Arrowhead denotes a redia surrounded by fibrous tissue that blends into the basal aspect of the digestive gland tubule; D, A longitudinal profile of a redia in the interstitium of the digestive gland. Black granular pigment fills the intestine (HE stain). Arrow denotes a developing cercaria, admixed with clusters of germinal cells. Scale-bars: A, 1 mm; B, 500 μ m; C–D, 50 μ m

descriptions of the redia of *Clinostomum* spp. (Nassi & Bayssade-Dufour, 1980; Pinto et al., 2015) indicate the latter is much larger in terms of body length compared with the descriptions of redia in this study and the material examined by Krull (1934). However, each redia examined in this study and noted by Krull (1934) contained developed cercariae.

Larval parasites (rediae containing developing cercariae) were predominantly present in the interstitium of the digestive gland (hepatopancreas), and within the blood sinuses/connective tissues of the lung (Fig. 4A). Rediae contained many clusters of germinal cells admixed with developing cercariae, producing mature rediae that contained many developmental stages. Abundant fine black granular pigment filled redial intestines. The digestive gland exhibited marked atrophy of the lobules and loss of connective tissue, with concomitant expansion of the blood spaces where the parasites resided; parasite profiles comprised 50% or more of the tissue sections. Parasites within the

digestive gland stroma were surrounded by a delicate layer of fibrous tissue containing small numbers of amoebocytes. Rediae were often attached to digestive tubule walls, but they were not seen to disrupt tubular epithelium or infiltrate digestive gland lumina. In some areas, marked fibrosis of the ducts with complete loss of lobules was seen. Cercariae were commonly present in the lumen of the gizzard and intestine as well. Rediae also occurred in the reproductive tissues and ovotestes were atrophied.

Cercaria

[Based on saline wet-mounts of 30 heat-killed cercariae from 3 separate snail hosts. Flame cells studied in live specimens stained with vital stains; see Table 2.] Cercaria lophocercous, brevifurcous, pharyngeate, typical of the genus *Clinostomum*. Body elongate, ovoid, shorter than tail stem region, 106–127 (118 ± 6) \times 28–38 (33 ± 2) (Fig. 5). Eye-spots 2, present in the middle of cercarial body, 5–6 (6 ± 0.6)

Table 2 Morphological data of *Clinostomum* spp. and brevifurcous cercariae from snails in the Americas

Species	<i>Clinostomum album</i>	<i>Clinostomum marginatum</i>	<i>Cercaria ocellifera</i>		<i>Clinostomum</i> sp.
Host	<i>Planorbella trivolvis</i>	<i>Planorbella anceps</i>	<i>Biomphalaria tenagophila</i>		<i>Biomphalaria glabrata</i> , <i>B. straminea</i> , <i>B. tenagophila</i>
Locality	Mississippi, USA	Maryland, USA	Brazil		Brazil
Reference	This study	Krull (1934)	Ruiz (1953)	Moraes et al. (2009)	Pinto et al. (2015)
BL	106–127 (118)	120–138 (130)	172–185	126–132	98–128 (110)
BW	28–38 (33)	30–32 (31)	–	32–42	32–42 (35)
TSL	198–239 (223)	250–285 (268)	339–390	255–315	273–307 (288)
TSW	19–27 (23)	–	30–37	–	22–34 (27)
FL	85–108 (95)	75–98 (88)	123–195	70–120	86–106 (95)
FW	11–17 (14)	–	–	–	7–17 (12)
AOL	36–53 (43)	37–40 (39)	53	0–32	33–47 (38)
AOW	18–25 (21)	–	27	–	20–27 (24)

Abbreviations: BL, body length; BW, body width; TSL, tail stem length; TSW, tail stem width; FL, furcae length; FW, furcae width; AOL, anterior organ length; AOW, anterior organ width

× 5–6 (6 ± 0.4). Anterior organ elongate, ovoid, in anterior third of cercarial body, 36–53 (43 ± 4) × 18–25 (21 ± 2). Ventral sucker not observed. Dorsal fin-fold not observed. Tail stem long, slender, 198–239 (223 ± 10) × 19–27 (23 ± 2). Tail stem bifurcates into 2 short, tapering furcae with fin-folds, 85–108 (95 ± 6) × 11–17 (14 ± 1). Flame-cell formula 2 [(1 + 1) + (1 + 1) + [1]] = 10.

Body armature: Approximately 4–5 rows of minute pre-oral spines encircling the anterior region of cercarial body at level of anterior organ.

Remarks

The cercaria of *C. album* shares overlapping morphometric data with other clinostomid and brevifurcous cercariae described from freshwater snails in the Americas (Table 2). As with the redia, the cercaria of *C. album* closely resembles the cercaria of *C. marginatum* as described by Krull (1934) from *P. anceps*. Additionally, these also are consistent with the

cercaria of a *Clinostomum* sp. described from three *Biomphalaria* spp. in Brazil, although the rediae were not morphologically consistent with *C. album* and molecular data support these as distinct species (Pinto et al., 2015).

Metacercaria

[Based on two Mayer's carmalum stained and mounted metacercaria, one designated as a hologenophore; see Table 3.] Body slender, linguiform, 4,081–4,938 (4509) × 737–874 (805), with slight constriction at level of ventral sucker (Fig. 6). Oral collar present. Oral sucker small, ovoid, 227–267 (247) × 268–297 (283). Ventral sucker large, 593–666 (629) × 548–673 (610), located in lower anterior third of body. Pharynx indistinct; prominent oesophageal bulb present just posterior to oral sucker. Intestinal caeca prominent, bifurcating just posterior to oral sucker, extending to posterior extremity of body, diverticulate broadly in regions posterior to ventral sucker.



Fig. 5 Photomicrograph of a *Clinostomum album* cercaria shed from *Planorbella trivolvis* collected from a catfish pond in Leflore County, Mississippi, USA. Scale-bar: 100 μ m

Gonads located in anterior portion of posterior third of body. Testes 2, located in body midline. Anterior testis lobed, 270–294 (282) \times 333–334 (333). Posterior testis lobed, 273–364 (318) \times 287–301 (294). Cirrus-sac located at level of anterior testis, overlapping its right anterior lobe, extends from right margin of body toward midline.

Ovary ovoid, intertesticular, 144–148 (146) \times 125–132 (128). Genital pore visible, opening at body midline just anterior to anterior testis.

Remarks

Metacercariae of *C. album* are morphologically ambiguous and ranges for most morphological characters used to describe metacercaria overlap with *C. marginatum*, which is known to occur in cultured channel catfish (Lorio, 1989) and other forage fish in

catfish production systems in Mississippi (Rosser et al., 2016b). Catfish experimentally challenged with *C. album* cercariae and controls contained no developing metacercariae suggesting that catfish are not a host for *C. album*.

Molecular data of *Clinostomum poteae* n. sp. and larval stages of *Clinostomum album*

Ribosomal regions (ITS1, 5.8S rRNA, and ITS2 region) for all four *C. poteae* specimens were identical. The top results of a Blastn search for the entire ribosomal region (partial 18S rRNA-ITS1-5.8S rRNA-ITS2-partial 28S rRNA) sequenced from a single specimen, were *Clinostomum marginatum* (99.5% sequence similarity; 100% query coverage; KU708007; Rosser et al., 2017) and *Clinostomum album* (99.3% sequence similarity; 100% query coverage; KU708008; Rosser et al., 2017) from a great egret in Mississippi, USA; *Clinostomum cutaneum* Paperna, 1964 (97.6% sequence similarity; 95–96% query coverage; GQ339114 and FJ609421; Gustinelli et al., 2010); and *Clinostomum phalacrocoracis* Dubois, 1931 (97.6% sequence similarity; 96% query coverage; FJ609422-FJ609423; Gustinelli et al., 2010) from the grey heron *Ardea cinerea* L. and Nile tilapia *Oreochromis niloticus* L. in Kenya. Additionally, *C. poteae* n. sp. shared 99.6% (38% query coverage; AY222095; Olson et al., 2003) sequence similarity at the 28S rRNA gene with an unresolved *Clinostomum* sp. metacercaria from the North American bullfrog *Lithobates catesbeiana* Shaw in Tennessee, USA.

The partial c.550 bp *cox1* sequences of the four specimens were identical and no sequences in the NCBI database shared significant similarity sufficient to suggest conspecificity. The top hits of the BLASTn search were *cox1* sequences of *C. attenuatum* metacercariae from *Lithobates clamitans* Latreille and *Lithobates pipiens* Schreber in the USA and Quebec, Canada (88.2–88.5% sequence similarity; 94–98% query coverage; KP150305-KP1150306 and JF718585 and JF718587; Caffara et al., 2011; Locke et al., 2015).

For the ITS regions, interspecific variability was low between the *Clinostomum* spp. described from the Americas the application of ITS sequence data in species delineation is limited (Table 4). However, pairwise distances calculated from *cox1* sequence data

Table 3 Morphological data of *Clinostomum* spp. metacercariae from intermediate hosts in North America

Species	<i>Clinostomum album</i>	<i>Clinostomum</i> sp.	<i>Clinostomum heluans</i>	<i>Clinostomum marginatum</i>		<i>Clinostomum tatxumui</i>	
Host	<i>Pimephales promelas</i>	<i>Poecilia reticulata</i>	<i>Cichla temensis</i>	Perciformes	<i>Dormitator maculatus</i>	<i>Menidia beryllina</i>	<i>Dormitator maculatus</i>
Locality	Mississippi, USA	Brazil	Brazil	Canada	Mexico	Mississippi, USA	Mexico
Reference	This study	Pinto et al. (2015)	Vicente et al. (1978)	Caffara et al. (2011)	Sereno-Uribe et al. (2013)	Rosser et al. (2016)	Sereno-Uribe et al. (2013)
BL	4,081–4,938 (4,509)	3,610–5,553 (4,224)	2,000–2,200	4,215–6,346 (5,402)	2,770–4,100 (3,300)	3,128–5,407 (3,968)	2,390–3,950 (2,940)
BW	737–874 (805)	1,031–1,616 (1,215)	640–740	979–1,532 (1,329)	600–850 (730)	904–1,303 (1,068)	340–840 (593)
OsL	227–267 (247)	170–239 (213)	120–150	238–599 (312)	160–220 (183)	192–281 (222)	140–210 (160)
OsW	268–297 (283)	232–273 (251)	120–140	194–586 (290)	170–220 (196)	191–241 (215)	150–210 (174)
VsL	593–666 (629)	602–746 (685)	200–420	510–757 (669)	430–620 (496)	468–666 (560)	350–560 (420)
VsW	548–673 (610)	602–756 (684)	230–290	571–804 (708)	450–620 (530)	485–606 (542)	320–570 (431)
DS	–	515–860 (659)	–	959–1,491 (1,243)	380–700 (539)	–	350–610 (468)
ATL	270–294 (282)	137–206 (159)	56	231–413 (307)	180–370 (224)	130–242 (190)	120–250 (171)
ATW	333–334 (333)	137–226 (142)	84–120	264–498 (389)	200–350 (247)	213–361 (278)	140–275 (200)
PTL	273–364 (318)	178–259 (217)	56	233–457 (327)	170–270 (214)	153–230 (189)	115–230 (172)
PTW	287–301 (294)	116–311 (172)	84–120	295–492 (405)	250–400 (280)	159–306 (239)	180–320 (230)
OvL	144–148 (146)	89–147 (110)	50–110	85–142 (115)	80–120 (98)	121–171 (144)	72–130 (96)
OvW	125–132 (128)	68–120 (85)	90–150	62–97 (80)	60–90 (73)	169–275 (233)	42–111 (70)
CSL	–	150–205 (183)	–	172–343 (256)	180–220 (200)	–	137–225 (179)
CSW	–	89–164 (121)	–	119–153 (137)	90–120 (106)	–	65–125 (91)

Abbreviations: BL, body length; BW, body width; OsL, oral sucker length; OsW, oral sucker width; VsL, ventral sucker length; VsW, ventral sucker width; DS, distance between oral and ventral sucker; ATL, anterior testis length; ATW, anterior testis width; PTL, posterior testis length; PTW, posterior testis width; OvL, ovary length; OvW, ovary width; CSL, cirrus-sac length; CSW, cirrus-sac width

for *C. album* cercaria and metacercaria reported here, and an adult *C. album* from previous work (Rosser et al., 2017) had high (> 10%) interspecific diversity

and low (< 1%) intraspecific variability compared to the other *Clinostomum* spp. (Table 5). The values reported here are consistent with previous studies



Fig. 6 Photomicrographs of Mayer's carmalum-stained *Clinostomum album* metacercariae from *Pimephales promelas* collected in Lowndes County, Mississippi, USA. A, Voucher; B, Hologenophore. Scale-bar: 500 μ m

involving representatives of the Clinostomoidea suggesting the cercariae and metacercaria from this study and adult stages of *C. album* are conspecific (Sereno-

Uribe et al., 2013; Senapin et al., 2014; Locke et al., 2015; Caffara et al., 2017; Woodyard et al., 2017). Likewise, pairwise distances for the *nad1* between the *Clinostomum* spp. in this study had low intraspecific variability and a greater interspecific variability compared to *cox1*, consistent with previous studies investigating genetic diversity of the mitochondrial genes of the Echinostomatidae Looss, 1899 (Morgan & Blair, 1998; Detwiler et al., 2010) (Table 6). The molecular data presented here evince the cercaria and metacercaria in this study represent previously unidentified stages in the life-cycle of *C. album* in Mississippi, USA.

Bayesian inference and maximum likelihood analyses of the concatenated *cox1*, ITS1, 5.8S rRNA gene, and ITS2 DNA sequences were consistent with previous phylogenies of the Clinostomidae (Figure 7). *Clinostomum poteae* n. sp. forms a clade within the larger grouping of American clinostomids, *Clinostomum marginatum*, *C. attenuatum* and *C. album*, but was basal to *C. marginatum* and *C. attenuatum*. Branches were strongly supported by high posterior probability and bootstrap values.

Discussion

For almost 200 years researchers have debated the systematics of clinostomids. The advent of molecular techniques and the ability to supplement morphological data with nucleotide sequence data has identified the clinostomids as a truly diverse group of trematodes, with new species described regularly (Caffara et al., 2011; Sereno-Uribe et al., 2013; Locke et al., 2015; Pérez-Ponce de León et al., 2016; Rosser et al., 2017). Currently, six *Clinostomum* spp. are recognised in North America based on detailed morphological descriptions of stained and mounted adults recovered from avian hosts (Bravo-Hollis, 1947; Hutton & Sogandares-Bernal, 1960; Caffara et al., 2011; Pérez-Ponce de León et al., 2016; Rosser et al., 2017). Of these, few *Clinostomum* spp. are described or observed in American cormorants and even fewer are documented from the trachea. Herein *C. poteae* n. sp. from the trachea of a double-crested cormorant in Mississippi, USA, is described and the larval stages of the life-cycle of *C. album* are identified.

Clinostomum poteae n. sp. differs from many of the *Clinostomum* spp. in the Americas by location within the host (trachea vs oesophagus and oral cavity), but

Table 4 Pairwise distances calculated from ITS region sequences of *Clinostomum poteae* n. sp. and *Clinostomum album* from the alignment in this study. Values are presented as average number of base differences per site followed by the range in parentheses

	<i>Clinostomum poteae</i> n. sp.	<i>Clinostomum album</i>
<i>Clinostomum poteae</i> n. sp.	0.000 (0.000–0.000)	0.007 (0.007–0.007)
<i>Clinostomum album</i>	0.007 (0.007–0.007)	0.000 (0.000–0.000)
<i>Clinostomum attenuatum</i>	0.002 (0.002–0.002)	0.005 (0.005–0.005)
<i>Clinostomum complanatum</i>	0.042 (0.042–0.042)	0.049 (0.049–0.049)
<i>Clinostomum cutaneum</i>	0.034 (0.034–0.034)	0.041 (0.041–0.041)
<i>Clinostomum detruncatum</i>	0.029 (0.029–0.029)	0.029 (0.029–0.029)
<i>Clinostomum heluans</i>	0.024 (0.024–0.024)	0.022 (0.022–0.022)
<i>Clinostomum marginatum</i>	0.002 (0.002–0.002)	0.005 (0.005–0.005)
<i>Clinostomum phalacrocoracis</i>	0.036 (0.036–0.036)	0.042 (0.042–0.042)
<i>Clinostomum philippinense</i>	0.037 (0.037–0.037)	0.044 (0.044–0.044)
<i>Clinostomum tataxumui</i>	0.022 (0.022–0.022)	0.025 (0.025–0.025)
<i>Clinostomum tilapiae</i>	0.032 (0.032–0.032)	0.039 (0.039–0.039)
<i>Clinostomum</i> sp. 1	0.019 (0.019–0.019)	0.022 (0.022–0.022)
<i>Clinostomum</i> sp. 3	0.019 (0.019–0.019)	0.022 (0.022–0.022)
<i>Clinostomum</i> sp. 4	0.017 (0.017–0.017)	0.020 (0.020–0.020)
<i>Clinostomum</i> sp. 5	0.022 (0.022–0.022)	0.025 (0.025–0.025)
<i>Clinostomum</i> sp. 7	0.034 (0.034–0.034)	0.037 (0.037–0.037)
<i>Clinostomum</i> sp. 8	0.044 (0.044–0.044)	0.051 (0.051–0.051)

Table 5 Pairwise distances calculated from *cox1* sequences of *Clinostomum poteae* n. sp. and *Clinostomum album* from the alignment in this study. Values are presented as average number of base differences per site followed by the range in parentheses

	<i>Clinostomum poteae</i> n. sp.	<i>Clinostomum album</i>
<i>Clinostomum poteae</i> n. sp.	0.000 (0.000–0.000)	0.174 (0.172–0.179)
<i>Clinostomum album</i>	0.174 (0.172–0.179)	0.004 (0.000–0.008)
<i>Clinostomum attenuatum</i>	0.121 (0.121–0.211)	0.149 (0.148–0.152)
<i>Clinostomum complanatum</i>	0.171 (0.170–0.172)	0.203 (0.201–0.209)
<i>Clinostomum cutaneum</i>	0.176 (0.175–0.177)	0.180 (0.150–0.187)
<i>Clinostomum detruncatum</i>	0.149 (0.149–0.149)	0.155 (0.150–0.158)
<i>Clinostomum heluans</i>	0.181 (0.181–0.181)	0.196 (0.195–0.199)
<i>Clinostomum marginatum</i>	0.138 (0.136–0.136)	0.156 (0.150–0.164)
<i>Clinostomum phalacrocoracis</i>	0.174 (0.172–0.175)	0.190 (0.183–0.193)
<i>Clinostomum philippinense</i>	0.201 (0.201–0.201)	0.204 (0.201–0.207)
<i>Clinostomum tataxumui</i>	0.151 (0.150–0.152)	0.180 (0.175–0.185)
<i>Clinostomum tilapiae</i>	0.177 (0.177–0.177)	0.192 (0.187–0.197)
<i>Clinostomum</i> sp. 1	0.142 (0.142–0.142)	0.146 (0.144–0.150)
<i>Clinostomum</i> sp. 3	0.148 (0.148–0.148)	0.159 (0.158–0.162)
<i>Clinostomum</i> sp. 4	0.138 (0.138–0.138)	0.143 (0.140–0.148)
<i>Clinostomum</i> sp. 5	0.156 (0.156–0.156)	0.162 (0.160–0.166)
<i>Clinostomum</i> sp. 7	0.179 (0.179–0.179)	0.211 (0.207–0.216)
<i>Clinostomum</i> sp. 8	0.172 (0.170–0.172)	0.196 (0.191–0.201)

Table 6 Pairwise distances calculated from *nad1* sequences of *Clinostomum poteae* n. sp. and *Clinostomum album* from the alignment in this study. Values are presented as average number of base differences per site followed by the range in parentheses

	<i>Clinostomum poteae</i> n. sp.	<i>Clinostomum album</i>
<i>Clinostomum poteae</i> n. sp.	0.000 (0.000–0.000)	–
<i>Clinostomum album</i>	0.196 (0.195–0.198)	0.0005 (0.000–0.0028)
<i>Clinostomum marginatum</i>	0.180 (0.178–0.181)	0.175 (0.173–0.176)

also based on morphometric and molecular data. *Clinostomum intermedialis* was described by Lamont (1920) from the oesophagus of a single Neotropic cormorant from Lake Valencia, Venezuela. In Lamont's description, few morphological characters were measured although the description is detailed and informative when considering the placement of the organs. *Clinostomum intermedialis* differs considerably from other *Clinostomum* spp. in having an ovoid anterior testis located in the left margin of the body compared to *C. poteae* n. sp. in which the anterior testis is distinctly lobed and situated near the body midline, similar to other *Clinostomum* congeners (Caffara et al., 2011; Sereno-Uribe et al., 2013; Rosser et al., 2017). Although similar in size, we believe these to be distinctly different from *C. intermedialis*, as all mounted *C. poteae* n. sp. specimens were consistent in the placement and size of the reproductive organs. Later, Bravo-Hollis (1947) supplemented the descriptions of *C. intermedialis* and *C. heluans* with new specimens collected from the pharynx of Brandt's cormorant from Nuevo León, Mexico. Molecular data have yet to be provided for *C. intermedialis* to support these morphological differences, although now *C. intermedialis* is considered a species of *Clinostommatopsis* (Lunaschi & Drago, 2009).

Clinostomum attenuatum has been reported in Florida cormorants from Florida, USA (Hutton & Sogandares-Bernal, 1960; Hutton, 1964). In Mexico, *Clinostomum tataxumui* was recently recognised as a novel species in the buccal cavity of great egrets, great-blue herons *Ardea herodias* L. and the bare-throated tiger heron *Tigrisoma mexicanum* Swainson (see Sereno-Uribe et al., 2013). *Clinostomum marginatum* has also been reported from the trachea and lungs of double-crested cormorants in Florida, although no morphological data were reported to justify this species level designation and no molecular

data exist from these specimens (Threlfall, 1982). Additionally, *C. marginatum* has been reported in the oral cavity of the Neotropic cormorant in Brazil (Travassos et al., 1969). When compared to specimens of *C. marginatum* and *C. album* collected from great egrets in Mississippi, *C. poteae* n. sp. is noticeably larger across many morphological characters and is found within the trachea. Meanwhile both *C. marginatum* and *C. album* were recovered from the oral cavity and oesophagus (Rosser et al., 2017). In light of the often morphologically ambiguous nature of *Clinostomum* spp. (and other trematode genera) adults and larval forms, the examination of multiple specimens and the inclusion of a hologenophore creates a more compelling case for species identification. Although morphological analysis should be the basis of identification of these organisms at first glance, supporting molecular data offers a more robust identification and hologenophores are recommended in novel species descriptions moving forward.

In addition to morphological differences, the specimens of *C. poteae* n. sp. were molecularly distinct from other *Clinostomum* spp. for which *cox1* sequencing data exists. The interspecific variability at the *cox1* gene was consistent with that previously described for the genus (Locke et al., 2015; Caffara et al., 2017) and phylogenetic analyses demonstrated *C. poteae* n. sp. formed a distinct clade, sister to other *Clinostomum* spp. collected from the Americas (Caffara et al., 2011; Locke et al., 2015; Rosser et al., 2017).

Remarkably, given the number of described species and their importance as parasites of fish and amphibians, few *Clinostomum* spp. life-cycles are known. In Brazil, the life-cycle of a *Clinostomum* sp. was described from naturally infected avian, molluscan and fish hosts (Dias et al., 2003). Adult specimens designated as *C. complanatum* were collected from *Ardea cocoi* L., *A. alba*, *Egretta thula* Molina, and *P.*



Fig. 7 Phylogenetic tree constructed from concatenated *cox1* gene and ITS regions alignment. Numbers above the branches represent Bayesian posterior probabilities and maximum likelihood bootstrap values based on 1,000 pseudoreplicates (< 0.5 are not shown). *Clinostomum* species obtained in this study are in bold. Scale-bar: average number of nucleotide substitutions per site

brasiliensis in the Americas, but the identity of these isolates is debated. A malacological survey performed in this study identified the planorbid snail *Biomphalaria peregrina* as the only snail species releasing clinostomid cercariae (0.75% prevalence). When laboratory raised banded knife fish *Gymnotus carapo* L. were exposed to these cercariae, a metacercaria consistent with *C. complanatum* was observed developing on an operculum 42 dpi (Dias et al., 2003). Metacercariae were also reported from several wild siluriform fish, including *Loricariichthys platymetopon*, Isbrücker & Nijssen, *Parauchenipterus galeatus* L., *Hoplosternum littorale* Hancock and *Loricaria* sp. L. Metacercariae consistent with *C. complanatum* were also observed in a single characiform fish, *Hoplias malabaricus* Block (see Dias et al., 2003).

In the USA, significant research investment regarding the life-cycle of *C. marginatum* has been made over the past century. The planorbid snail *P. trivolvis* has been identified as the first intermediate host and development of larval stages within the snail have been extensively studied, both from wild, naturally infected populations, as well as under experimental conditions. Edney (1950) demonstrated that laboratory reared marsh rams-horn snails as young as one day to two months were susceptible to infection, but older cohorts (1–2 years) were not capable of being infected. Snails are possibly infected early in life and are capable of maintaining infections for several years, continually producing rediae and cercariae (Edney, 1950). Consistent with previous studies concerning the pathology of larval trematode infections in snails, the digestive gland and gonad were severely atrophied in *C. album*-infected *Planorbella trivolvis*. Little to no inflammation or necrosis was observed, suggesting that atrophy is associated with mechanical destruction attributed to direct ingestion of host tissues or the appropriation of nutrients from the host (Crews, 1987). Although rediae were not observed actively feeding on snail tissues, the presence of intestinal black pigment suggests these stages were feeding on host tissues. Parasites were present in the connective tissues and blood spaces of the snails, which is also typical of many snail-trematode parasitic relationships. The presence of cercariae in the gizzard and intestine is not usually noted, and this may be due to the snail feeding on cercariae shed into the environment. The metacercariae of *C. marginatum* are widely distributed throughout the Americas and have been reported from

a myriad of fish and amphibian hosts (Hoffmann, 1999; McAllister, 1990; Miller et al., 2004), though many of these are not supported by molecular data and could represent currently unidentified species. The ability to distinguish *Clinostomum* spp. based on cercaria morphometric data alone is likely unreliable, especially when conducting experimental challenges or elucidating life-cycles with specimens collected from naturally infected hosts in areas where multiple species share a given geographical locality or population of hosts. The usefulness of molecular data obtained from larval stages is already known from previous studies of *Clinostomum* spp. (Gustinelli et al., 2010; Caffara et al., 2017).

Comparably, based on molecular data obtained here, the life-cycle of *C. album* includes the ramshorn snail as a first intermediate host, fathead minnows as a second intermediate host, and great egrets as a definitive host (Rosser et al., 2017). Given the diverse number of potential hosts for any given *Clinostomum* sp., it is likely that other hosts exist but have yet to be identified and the health impacts on baitfish species such as the fathead minnow remain unknown. Experimental infections in channel catfish were unsuccessful and did not result in detectable metacercariae up to 60 dpi. Comparably, similar studies in catfish have demonstrated development and persistence of *C. marginatum* metacercariae for periods > 100 days post-exposure (Doffitt, 2011).

The larval stages of *C. poteae* n. sp. remain unknown, but molecular data generated in this study will aid in the elucidation of the life-cycle when cercaria and metacercaria stages are encountered in future studies of clinostomids in mollusc, fish, or amphibian hosts. The utility of molecular data in parasitological studies remains in its infancy but will likely continue to aid in the elucidation of life-cycles of various parasitic groups and should remain an integral component of novel species descriptions. Further work examining the diversity and effects on fish and amphibian health of clinostomids in their avian hosts in the southeastern United States is warranted.

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Compliance with ethical standards

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

Ethical approval All applicable institutional, national and international guidelines for the care and use of animals were followed (IACUC QA 2458).

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Supplementary material for

***Clinostomum poteae* n. sp. (Digenea: Clinostomidae), in the trachea of a double-crested cormorant *Phalacrocorax auritus* Lesson, 1831 and molecular data linking the life-cycle stages of *Clinostomum album* Rosser, Alberson, Woodyard, Cunningham, Pote & Griffin, 2017 in Mississippi, USA**

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Supplementary Table 1 Primers used in amplification of ribosomal genes and mitochondrial genes of *Clinostomum* spp. in this study

Primer	Sequence (5'-3')	Gene target	Reference
1F	TACCTGGTTGATCCTGCCAGTAG	SSU rRNA	Carranza et al. (1997)
5R	CTTGGCAAATGCTTTCGC	SSU rRNA	Carranza et al. (1997)
Barker3	TTAGAGTGTTCAAAGCAG	SSU rRNA	Barker et al. (1993)
Barker4	GATCCTTCTGCAGGTTACCTAC	SSU rRNA	Barker et al. (1993)
Diplo1795F	CGTCGCTACTACCGATTGAA	SSU rRNA and ITS	Rosser et al. (2016)
Diplo2549R	AGTGATCCACCGCTCAGAGT	SSU rRNA and ITS	Rosser et al. (2016)
BD1	GTCGTAACAAGGTTTCCGTA	ITS	Morgan and Blair (1995)
BD2	TATGCTTAAATTCAGCGGGT	ITS	Morgan and Blair (1995)
Diplo2617F	CATCGACATCTTGAACGCATA	ITS and 28S rRNA	Rosser et al. (2016)
Diplo3170R	GCTGGACTTAGGATGGAGCA	ITS and 28S rRNA	Rosser et al. (2016)
LSU5	TAGGTCGACCCGCTGAAYTTAAGCA	28S rRNA	Littlewood et al. (2000)
1500R	GCTATCCTGAGGGAAACTTCG	28S rRNA	Littlewood et al. (2000)
28S 3431F	TCAGAGGTAAACGGGTGGAG	28S rRNA	Rosser et al. (2017)
28S 4779R	CTCAGCTTGCAATGACGGTA	28S rRNA	Rosser et al. (2017)
28S 4759F	GTCTTGAAACACGGACCAAG	28S rRNA	Rosser et al. (2017)
28S 5699R	TACCACCAAGATCTGCACCT	28S rRNA	Rosser et al. (2017)
Cox1_schist 5'	TCTTTRGATCATAAGCG	<i>cox1</i>	Lockyer et al. (2003)
Acox650r	CCAAAAAACCAAAACATATGCTG	<i>cox1</i>	Kudlai et al. (2015)
NDJ11	AGATTCGTAAGGGGCCTAATA	<i>nd1</i>	Morgan and Blair (1998)
NDJ2a	CTTCAGCCTCAGCATAAT	<i>nd1</i>	Kostadinova et al. (2003)

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Supplementary Table 2 Sequences used in phylogenetic analyses

Species	Genbank Accession No.		Isolate	Host	Stage	Locality	Reference
	ITS	<i>cox1</i>					
<i>Clinostomum album</i>	KU708008	KU708010	C-2	<i>Ardea alba</i>	A	Mississippi, USA	Rosser et al. 2017
<i>Clinostomum attenuatum</i>	JF718622	JF718587	C.M.Rp.1.1	<i>Lithobates pipiens</i>	M	Canada	Caffara et al. 2011
<i>Clinostomum attenuatum</i>	JF718620	JF718585	Cm.Rcl.BBA8.1.1	<i>Lithobates clamitans</i>	M	Quebec, Canada	Caffara et al. 2011
<i>Clinostomum attenuatum</i>	KP150307	KP150305	CL.Bc.R.p.VPH.1.1	<i>Lithobates pipiens</i>	M	USA	Locke et al. 2015
<i>Clinostomum marginatum</i>	JF718634	JF718600	Cl.RM.B08.P.1F.2	<i>Perca flavescens</i>	M	Quebec, Canada	Caffara et al. 2011
<i>Clinostomum marginatum</i>	MH282555	KU707946	Cm_1-C-1	<i>Menidia beryllina</i>	M	Mississippi, USA	Rosser et al. 2016
<i>Clinostomum marginatum</i>	JF718636	JF718604	Cl.GRI.Ah.1F.3	<i>Ardea herodias</i>	A	Quebec, Canada	Caffara et al. 2011
<i>Clinostomum marginatum</i>	JF718635	JF718602	Cl.GRI.Ah.1F.1	<i>Ardea herodias</i>	A	Quebec, Canada	Caffara et al. 2011
<i>Clinostomum marginatum</i>	KU708007	KU708009	C-1	<i>Ardea alba</i>	A	Mississippi, USA	Rosser et al. 2017
<i>Clinostomum marginatum</i>	JF718640	JF718612	Cl.RON.B.S.1.1	<i>Lepomis gibbosus</i>	M	Quebec, Canada	Caffara et al. 2011
<i>Clinostomum marginatum</i>	MH282554	MH282538	Cm_Lm_1	<i>Lepomis macrochirus</i>	M	Mississippi, USA	This study
<i>Clinostomum marginatum</i>	JF718641	JF718615	Cl.RM.D.P.18.1	<i>Perca flavescens</i>	M	Quebec, Canada	Caffara et al. 2011
<i>Clinostomum marginatum</i>	JF718633	JF718599	Cm.M.2.R.5.2	<i>Ambloplites rupestris</i>	M	Quebec, Canada	Caffara et al. 2011
<i>Clinostomum marginatum</i>	JF718632	JF718598	Cm.M.2.R.5.1	<i>Ambloplites rupestris</i>	M	Quebec, Canada	Caffara et al. 2011
<i>Clinostomum marginatum</i>	JF718631	JF718597	Cl.LM.B08.Ms.1F.3	<i>Micropterus salmoides</i>	M	Quebec, Canada	Caffara et al. 2011
<i>Clinostomum poteae</i>	MH282567	MH282550	R02-Clino-1	<i>Phalacrocorax auritus</i>	A	Mississippi, USA	This study
<i>Clinostomum poteae</i>	MH282568	MH282551	R02-Clino-2	<i>Phalacrocorax auritus</i>	A	Mississippi, USA	This study
<i>Clinostomum poteae</i>	MH282569	MH282552	R02-Clino-3	<i>Phalacrocorax auritus</i>	A	Mississippi, USA	This study
<i>Clinostomum poteae</i>	MH282570	MH282553	R02-Clino-4 hologenophore	<i>Phalacrocorax auritus</i>	A	Mississippi, USA	This study

<i>Clinostomum album</i>	MH282566	MH282549	Ca_Pt_1	<i>Planorbella trivolvis</i>	C	Mississippi, USA	This study
<i>Clinostomum album</i>	MH282565	MH282548	Ca_Pt_2	<i>Planorbella trivolvis</i>	C	Mississippi, USA	This study
<i>Clinostomum album</i>	MH282564	MH282547	Ca_Pt_3	<i>Planorbella trivolvis</i>	C	Mississippi, USA	This study
<i>Clinostomum album</i>	MH282563	MH282546	Ca_Pt_4	<i>Planorbella trivolvis</i>	C	Mississippi, USA	This study
<i>Clinostomum album</i>	MH282562	MH282545	Ca_Pt_5	<i>Planorbella trivolvis</i>	C	Mississippi, USA	This study
<i>Clinostomum album</i>	MH282561	MH282544	Ca_Pt_6	<i>Planorbella trivolvis</i>	C	Mississippi, USA	This study
<i>Clinostomum album</i>	MH282560	MH282543	Ca_Pt_7	<i>Planorbella trivolvis</i>	C	Mississippi, USA	This study
<i>Clinostomum album</i>	MH282559	MH282542	Ca_Pt_8	<i>Planorbella trivolvis</i>	C	Mississippi, USA	This study
<i>Clinostomum album</i>	MH282558	MH282541	Ca_Pt_9	<i>Planorbella trivolvis</i>	C	Mississippi, USA	This study
<i>Clinostomum album</i>	MH282557	MH282540	Ca_Pt_10	<i>Planorbella trivolvis</i>	C	Mississippi, USA	This study
<i>Clinostomum album</i>	MH282556	MH282539	Ca_Pp_1	<i>Pimephales promelas</i>	M	Mississippi, USA	This study
<i>Clinostomum tataxumui</i>	KP110588	KP110544	C.BC.Ep.TRP4	<i>Eleotris picta</i>	M	Guerrero, Mexico	Locke et al. 2015
<i>Clinostomum tataxumui</i>	KP110593	KP110559	C.BC.DI.TRP.1	<i>Dormitator latifrons</i>	M	Guerrero, Mexico	Locke et al. 2015
<i>Clinostomum tataxumui</i>	KP110590	KP110546	C.BC.Ct.TRP2	<i>Cichlasoma trimaculatum</i>	M	Guerrero, Mexico	Locke et al. 2015
<i>Clinostomum tataxumui</i>	KP110597	KP110563	C.BC.Ep.TRP3	<i>Eleotris picta</i>	M	Guerrero, Mexico	Locke et al. 2015
<i>Clinostomum tataxumui</i>	KP110596	KP110562	C.BC.Ep.TRP2	<i>Eleotris picta</i>	M	Guerrero, Mexico	Locke et al. 2015
<i>Clinostomum tataxumui</i>	KP110595	KP110561	C.BC.Ep.TRP1	<i>Eleotris picta</i>	M	Guerrero, Mexico	Locket et al. 2015
<i>Clinostomum tataxumui</i>	KP110592	KP110551	C.LV.Vent.1.4	<i>Gobiomorus maculatus</i>	M	Oaxaca, Mexico	Locke et al. 2015
<i>Clinostomum tataxumui</i>	KP110591	KP110550	C.LV.P.Vent.1.1	<i>Dormitator latifrons</i>	M	Oaxaca, Mexico	Locke et al. 2015
<i>Clinostomum tataxumui</i>	KP110594	KP110560	C.BC.DI.TRP.2	<i>Dormitator latifrons</i>	M	Guerrero, Mexico	Locke et al. 2015
<i>Clinostomum</i> sp. 5 SAL-2015	KP110576	KP110533	C.G.Cb.SCR.4.a	<i>Cichlasoma boliviense</i>	M	Santa Cruz, Bolivia	Locke et al. 2015
<i>Clinostomum</i> sp. 5 SAL-2015	KP110575	KP110532	C.Oes.Cb.SCR.4.c	<i>Cichlasoma boliviense</i>	M	Santa Cruz, Bolivia	Locke et al. 2015
<i>Clinostomum</i> sp. 1 SAL-2015	KP110571	KP110524	Cl.Rg.CIH.X.1	<i>Rhamdia guatamalensis</i>	M	Yucatan, Mexico	Locke et al. 2015
<i>Clinostomum</i> sp. 3 SAL-2015	KP110573	KP110530	C.G.Pm.ABA.X.1	<i>Poecilia mexicana</i>	M	Veracruz, Mexico	Locke et al. 2015
<i>Clinostomum</i> sp. 4 SAL-2015	KP110574	KP110531	C.Asp.ITA.1.1	<i>Apistogramma</i> sp.	M	Iquitos, Peru	Locke et al. 2015
<i>Clinostomum</i> sp. 6 SAL-2015	KP110577	KP110534	C.F.Cb.SCR.4.b	<i>Cichlasoma boliviense</i>	M	Santa Cruz, Bolivia	Locke et al. 2015
<i>Clinostomum</i> sp. 7	KJ789384	KJ818259	43/13_1	<i>Poecilia reticulata</i>	M	Brazil	Pinto et al. 2015
<i>Clinostomum</i> sp. 7	KJ789385	KJ818261	43/13_2	<i>Poecilia reticulata</i>	M	Brazil	Pinto et al. 2015
<i>Clinostomum</i> sp. 7	KJ789386	KJ818262	43/13_3	<i>Poecilia reticulata</i>	M	Brazil	Pinto et al. 2015

<i>Clinostomum detruncatum</i>	KP110566	KP110517	C.Sm.ITA.1.1	<i>Synbranchus marmoratus</i>	M	Parana, Brazil	Locke et al. 2015
<i>Clinostomum phalacrocoracis</i>	KJ786979	KJ786971	20/13_B1	Cichlid fingerling	M	Israel	Caffara et al. 2014
<i>Clinostomum phalacrocoracis</i>	KJ786975	KJ786967	20/13_A1	Cichlid fingerling	M	Israel	Caffara et al. 2014
<i>Clinostomum phalacrocoracis</i>	KJ786976	KJ786968	20/13_A2	Cichlid fingerling	M	Israel	Caffara et al. 2014
<i>Clinostomum phalacrocoracis</i>	KP110567	KP110521	Cp.On.KYA.X.1	<i>Oreochromis niloticus niloticus</i>	M	Kenya	Locke et al. 2015
<i>Clinostomum phalacrocoracis</i>	KJ786982	KJ786974	20/13_B7	Cichlid fingerling	M	Israel	Caffara et al. 2014
<i>Clinostomum phalacrocoracis</i>	KJ786981	KJ786973	20/13_B3	Cichlid fingerling	M	Israel	Caffara et al. 2014
<i>Clinostomum phalacrocoracis</i>	KJ786980	KJ786972	20/13_B2	Cichlid fingerling	M	Israel	Caffara et al. 2014
<i>Clinostomum phalacrocoracis</i>	KP110568	KP110522	Cp.O.Ac.KYA.X.1	<i>Ardea cinerea</i>	A	Kenya	Locke et al. 2015
<i>Clinostomum phalacrocoracis</i>	KJ786977	KJ786969	20/13_A3	Cichlid fingerling	M	Israel	Caffara et al. 2014
<i>Clinostomum phalacrocoracis</i>	KJ786978	KJ786970	20/13_A7	Cichlid fingerling	M	Israel	Caffara et al. 2014
<i>Clinostomum tilapiae</i>	KY649353	KY649361	172/15_7	<i>Synodontis batensoda</i>	M	Nigeria	Caffara et al. 2017
<i>Clinostomum tilapiae</i>	KY649355	KY649363	172/15_21	<i>Synodontis batensoda</i>	M	Nigeria	Caffara et al. 2017
<i>Clinostomum tilapiae</i>	KY649356	KY649364	172/15_22	<i>Synodontis batensoda</i>	M	Nigeria	Caffara et al. 2017
<i>Clinostomum tilapiae</i>	KY649349	KY649357	172/15_A1	<i>Synodontis batensoda</i>	M	Nigeria	Caffara et al. 2017
<i>Clinostomum tilapiae</i>	KY649350	KY649358	172/15_A2	<i>Synodontis batensoda</i>	M	Nigeria	Caffara et al. 2017
<i>Clinostomum tilapiae</i>	KY649352	KY649360	172/15_B1	<i>Synodontis batensoda</i>	M	Nigeria	Caffara et al. 2017
<i>Clinostomum tilapiae</i>	KY649351	KY649359	172/15_A3	<i>Synodontis batensoda</i>	M	Nigeria	Caffara et al. 2017
<i>Clinostomum tilapiae</i>	KY649354	KY649362	172/15_20	<i>Synodontis batensoda</i> <i>Oreochromis niloticus niloticus</i>	M	Nigeria	Caffara et al. 2017
<i>Clinostomum cutaneum</i>	KP110565	KP110516	Cc.On.KYA.X.1	<i>Oreochromis niloticus niloticus</i>	M	Kenya	Locke et al. 2015

<i>Clinostomum cutaneum</i>	KP110564	KP110515	Cc.Ac.KYA.X.1	<i>Ardea cinerea</i>	A	Kenya	Locke et al. 2015
<i>Clinostomum philippinense</i>	KP110570	KP110523	C.Tm.ITA.1.2	<i>Trichogaster microlepis</i>	M	Thailand	Locke et al. 2015
<i>Clinostomum</i> sp. 8 SAL-2015	KP110583	KP110539	C.G.Ca.CHU.4.1	<i>Carassius auritus</i>	M	Hubei, China	Locke et al. 2015
<i>Clinostomum</i> sp. 8 SAL-2015	KP110580	KP110536	C.Op.Ca.Lzi.3	<i>Carassius auritus</i>	M	Hubei, China	Locke et al. 2015
<i>Clinostomum</i> sp. 8 SAL-2015	KP110581	KP110537	C.Op.Ca.Lzi.4	<i>Carassius auritus</i>	M	Hubei, China	Locke et al. 2015
<i>Clinostomum</i> sp. 8 SAL-2015	KP110585	KP110541	C.Op.Ci.HUB.1	<i>Ctenopharyngodon idella</i>	M	Hubei, China	Locke et al. 2015
<i>Clinostomum</i> sp. 8 SAL-2015	KP110582	KP110538	C.Op.Ca.Lzi.5	<i>Carassius auritus</i>	M	Hubei, China	Locke et al. 2015
<i>Clinostomum</i> sp. 8 SAL-2015	KP110586	KP110542	C.Op.Ca.Lzi.1	<i>Carassius auritus</i>	M	Hubei, China	Locke et al. 2015
<i>Clinostomum</i> sp. 8 SAL-2015	KP110579	KP110535	C.Op.Ci.HUB.2	<i>Ctenopharyngodon idella</i>	M	Hubei, China	Locke et al. 2015
<i>Clinostomum complanatum</i>	JF718624	JF718589	C.S.ITA5.1	<i>Lepomis gibbosus</i>	M	Veneto, Italy	Caffara et al. 2011
<i>Clinostomum complanatum</i>	KM518258	KM518252	228/11_2	Newts: <i>Triturus carnifex</i> , <i>Lissotriton vulgaris</i>	M	Italy Emilia-Romagna,	Caffara et al. 2014
<i>Clinostomum complanatum</i>	JF718623	JF718588	C.Sc.ITA3.6	<i>Squalius cephalus</i>	M	Italy	Caffara et al. 2011
<i>Clinostomum complanatum</i>	KM518259	KM518253	228/11_1	Newts: <i>Triturus carnifex</i> , <i>Lissotriton vulgaris</i>	M	Italy	Caffara et al. 2014
<i>Euclinostomum heterostomum</i>	KP721427	KP721409	349/13_7	Cichlids	M	Israel	Caffara et al. 2016
<i>Euclinostomum heterostomum</i>	KP721434	KP721416	55/14_7	Cichlids	M	Israel	Caffara et al. 2016
<i>Euclinostomum heterostomum</i>	KP721435	KP721417	55/14_8	Cichlids	M	Israel	Caffara et al. 2016
<i>Euclinostomum heterostomum</i>	KP721429	KP721412	55/14_2	Cichlids	M	Israel	Caffara et al. 2016
<i>Euclinostomum heterostomum</i>	KP721424	KP721406	349/13_4	Cichlids	M	Israel	Caffara et al. 2016
<i>Euclinostomum heterostomum</i>	KP721436	KP721418	55/14_9	Cichlids	M	Israel	Caffara et al. 2016
<i>Euclinostomum heterostomum</i>	KP721423	KP721405	349/13_3	Cichlids	M	Israel	Caffara et al. 2016

<i>Euclinostomum heterostomum</i>	KP721439	KP721421	55/14_12	Cichlids	M	Israel	Caffara et al. 2016
<i>Euclinostomum heterostomum</i>	KP721428	KP721410	349/13_8	Cichlids	M	Israel	Caffara et al. 2016
<i>Euclinostomum heterostomum</i>	KP721430	KP721413	55/14_3	Cichlids	M	Israel	Caffara et al. 2016
<i>Euclinostomum heterostomum</i>	KP721431	KP721414	55/14_4	Cichlids	M	Israel	Caffara et al. 2016
<i>Euclinostomum heterostomum</i>	KP721426	KP721408	349/13_6	Cichlids	M	Israel	Caffara et al. 2016
<i>Euclinostomum heterostomum</i>	KP721437	KP721419	55/14_10	Cichlids	M	Israel	Caffara et al. 2016
<i>Euclinostomum heterostomum</i>	KP721422	KP721404	349/13_2	Cichlids	M	Israel	Caffara et al. 2016
<i>Euclinostomum heterostomum</i>	KP721433	KP721415	55/14_6	Cichlids	M	Israel	Caffara et al. 2016
<i>Euclinostomum heterostomum</i>	KP721425	KP721407	349/13_5	Cichlids	M	Israel	Caffara et al. 2016
<i>Euclinostomum heterostomum</i>	KP721438	KP721420	55/14_11	Cichlids	M	Israel	Caffara et al. 2016
<i>Euclinostomum</i> sp. 1 SS-2014	KC894793	KC894795	Parasite no. 1	<i>Trichopsis vittata</i>	M	Thailand	Senapin et al. 2014
<i>Euclinostomum</i> sp. 2 SS-2014	KC894799	KC894796	Parasite no. 2	<i>Trichopsis schalleri</i>	M	Thailand	Senapin et al. 2014
<i>Euclinostomum</i> sp. 3 SS-2014	KC894800	KC894797	Parasite no. 3	<i>Trichopsis vittata</i>	M	Thailand	Senapin et al. 2014
<i>Diplostomum baeri</i>	AY123042	GQ292501	5DM24	<i>Larus delawarensis</i>	A	Canada New Hampshire,	Galazzo et al. 2002; Locke et al. 2010
<i>Alaria mustelae</i>	JF769478	JF904529	A.Rc.OXB.3	<i>Lithobates clamitans</i>	M	USA	Locke et al. 2011

Abbreviations: A, adult; C, cercaria; M, metacercaria

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Supplementary Table 3 Morphological data of *Clinostomum* spp. redia from aquatic snails in the Americas

	<i>Clinostomum album</i>	<i>Clinostomum</i> sp.	<i>Clinostomum golvani</i>	<i>Clinostomum marginatum</i>
Host	<i>Planorbella trivolvis</i>	<i>Biomphalaria glabrata</i> , <i>Biomphalaria straminea</i> , <i>Biomphalaria tenagophila</i>	<i>Biomphalaria glabrata</i>	<i>Planorbella anceps</i>
Locality	Mississippi, USA	Minas Gerais, Brazil	Guadeloupe, West Indies	Maryland, USA
Reference	This study	Pinto et al. (2015)	Nassi & Bayssade-Dufour (1980)	Krull 1934*
BL	271–819 (455)	*1375–2321 (1826)	2000–3100	490
BW	97–235 (150)	150–205 (177)	–	140
PhL	30–57 (47)	38–50 (46)	67	36
PhW	31–58 (46)	42–53 (46)	–	27
CL	162–523 (291)	683–1203 (878)	2/3 – 4/5 body length	–
CW	28–59 (41)	25–47 (30)	–	–

Abbreviations: BL, body length; BW, body width; PhL, pharynx length; PhW, pharynx width; CL, caecum length; CW, caecum width.

*Measurements from the single largest redia.

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Supplementary Figure 1. *Clinostomum poteae* n. sp. hologenophore. Mayer's carmalum stain.
Scale bar: 1 mm.



Supplementary Figure 2. *Clinostomum marginatum* hologenophore from bluegill *Lepomis macrochirus*. Mayer's carmalum stain. *Scale bar:* 1 mm.



Supplementary Figure 3. Phylogenetic tree constructed from concatenated *cox1* gene and ITS regions alignment. Numbers above the branches represent Bayesian posterior probabilities and maximum likelihood bootstrap values based on 1,000 pseudoreplicates (<0.5 are not shown). *Clinostomum* species obtained in this study are in bold. *Scale-bar*: average number of nucleotide substitutions per site.

