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ORIGINAL PAPER

Alligator wrestling: morphological, molecular, and phylogenetic data on *Odhneriotrema incommodum* (Leidy, 1856) (Digenea: Clinostomidae) from *Alligator mississippiensis* Daudin, 1801 in Mississippi, USA

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Abstract Based on specimens collected from harvested American alligator *Alligator mississippiensis* Daudin, 1801 in Mississippi, USA, novel molecular data for both nuclear ribosomal genes (18S, ITS1-5.8S, ITS2, and 28S) and mitochondrial genes (cytochrome *c* oxidase subunit 1 and nicotinamide adenine dinucleotide dehydrogenase subunit 1) are provided for *Odhneriotrema incommodum* (Leidy, 1856), a trematode of the family Clinostomidae Lühe, 1901 infecting *A. mississippiensis* and the Florida spotted gar *Lepisosteus platyrhincus* DeKay, 1842. This represents the first sequencing data available for the genus *Odhneriotrema* and the subfamily Nephrocephalinae Travassos, 1928. Additionally, the results of phylogenetic analyses, additional morphometric data, a photomicrograph, and a line drawing supporting the present identification of *O. incommodum* are provided. These data will aid in elucidating the life cycle of *O. incommodum* through molecular identification of larval stages as well as understanding the evolutionary history of Clinostomidae and its subfamilies. Implications for the currently accepted organization of the Clinostomidae are discussed.

Keywords *Alligator mississippiensis* · Clinostomidae · Clinostomoidea · Nephrocephalinae · *Odhneriotrema incommodum*

Introduction

The American alligator *Alligator mississippiensis* is an abundant top predator and keystone species that can structure aquatic ecosystems throughout their range, spanning the Coastal Plain of the southeastern United States (Mazzotti and Brandt 1994). Acting as an ecosystem engineer, *A. mississippiensis* can be important predators and prey, mobilizing nutrients, manipulating hydrology, and influencing plant communities within aquatic and adjoining terrestrial ecosystems (Mazzotti et al. 2009; Rosenblatt and Heithaus 2011). *A. mississippiensis* exist in fresh, brackish (salinity 5–30‰), and marine (salinity > 30‰) systems (Elsey 2005; Rosenblatt and Heithaus 2011). Despite the array of systems occupied by *A. mississippiensis*, the parasite community associated with this species is considered relatively similar throughout this species' range (Tellez 2014; Tellez and Nifong 2014). Interest in and taxonomic categorization of these parasites has a long history, riddled with change.

Odhneriotrema incommodum (Leidy 1856) (= *Monostomum incommodum*; *Distoma oricola*; *Distomum incommodum*; *Clinostomum incommodum*; *Homoscaphis incommodum*) was first described by Leidy (1856) as *M. incommodum* based on five specimens reportedly recovered from the feces of *A. mississippiensis* Daudin, 1801 in Florida, USA. Later, Leidy (1884) described an additional species, *D. oricola* based on eight worms collected from the mouth of the type host species from Florida. He also noted scarring of the tongue apparently associated with chronic infection. Leidy (1890) later concluded these two trematodes

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were conspecifics and named the species *Distomum incommodum*. Pratt (1902) and Ward and Whipple (1918) speculated the species likely belonged in the family Clinostomidae, but indicated insufficient data were available to define its taxonomic relationship within this family. Harrah (1922) moved the species into the genus *Clinostomum*, thus erecting *C. incommodum*.

In his description of four archived specimens reportedly collected from the thorax of the type host species from Florida, Canavan (1933) provided the first detailed morphological characterization of the species, although he mistook the pharynx for an oral sucker and thus erroneously concluded the species lacks a pharynx. It must be noted, however, that all subsequently examined adults of the species (McIntosh 1935), as well as observations in the present study, reveal *O. incommodum* does possess a well-developed, muscular pharynx. In addition to his description of the species, Canavan also erected the novel genus *Homoscaphis* based on the placement of the genital pore, large body size and dorsal convexity, relatively large acetabulum, placement and arrangement of the testes and ovary, lack of vitelline follicle confluence posterior to the reproductive organs, uterus arrangement, prominent caecal outpocketings, and host species, thus naming the trematode *H. incommodum* (Canavan, 1933).

Based on similarity of measured characters of three specimens collected from *A. mississippiensis* in Florida as well as one of Leidy's original specimens, McIntosh (1935) noted the species was congeneric with *Odhneriotrema microcephala*, thus establishing the currently accepted *O. incommodum*. McIntosh (1935) notes the presence of a well-developed pharynx in all four specimens. Thus, prior to the present study, adults of this trematode have been described based on only 20 specimens collected from naturally infected *A. mississippiensis*, all from Florida.

Subsequent to these examinations, the family Clinostomidae, and thus *O. incommodum*, has been subject to a number of taxonomic rearrangements. Dollfus (1931) asserted the family Clinostomidae should be raised to the rank of a superfamily, the Clinostomoidea Witenberg, 1925, with the family Clinostomidae containing only the genera *Clinostomum* Leidy, 1856, *Euclinostomum* Travassos, 1928, and *Ithyclinostomum* Witenberg, 1926, while the family Opisthophallidae Travassos, 1926 should contain the genera *Odhneriotrema* Travassos, 1928, and *Opisthophallus* Baer, 1921. While agreeing with this organization within the family Clinostomidae, Baer (1933) contended the Clinostomidae should not be raised the rank of a superfamily and the latter two genera did not warrant placement in a distinct family. Skrjabin (1947) maintained the placement of the genus *Odhneriotrema* in the subfamily Opisthophallinae within the family Clinostomidae based on the presence of a *pars prostatica*, maturity of miracidia at the point of oviposition, and having crocodilians as definitive hosts. Dollfus (1950) reasserted his prior placement of *Odhneriotrema* and *Opisthophallus* into a distinct family, Nephrocephallidae (= Opisthophallidae), within the superfamily Clinostomoidea, distinguished from the Clinostomidae by a number of morphological differences and having reptiles as definitive hosts. Yamaguti (1958) moved *Odhneriotrema* into the subfamily Clinostominae Pratt, 1902 with *Clinostomoides* Dollfus, 1950, *Clinostomatopsis* Dollfus, 1932, and *Clinostomum*. Conversely, Tavassos et al. (1969) erected an entirely separate subfamily, Odhneriotrematinae Travassos, Freitas, and Kohn 1969 within the family Clinostomidae for *Odhneriotrema*, distinguishing it from the Clinostominae based on non-confluence of the vitellaria posterior to the gonads, the appearance of the uterus, and intertesticular distance. Yamaguti (1971), considering the work of Tavassos et al. (1969) too narrow in scope, reasserted his placement of *Odhneriotrema* within the subfamily Clinostominae, distinguished from the Nephrocephalinae by the intertesticular placement of the cirrus sac. Conversely, Feizullaev and Mirzoeva (1983) supported the placement of the genus within its own family within the superfamily Clinostomoidea as proposed by Tavassos et al. (1969). *Keys to the Trematoda* (Kanev et al. 2002) uses a similar system to that proposed by Yamaguti (1971), but places *Odhneriotrema* in the subfamily Nephrocephalinae within the family Clinostomidae based on host species, genital pore placement, and oral sucker size.

To aid in clarification of the long-disputed taxonomy of this species, the present study provides the first molecular description and phylogenetic analyses of *O. incommodum* in addition to morphometric and ecological data based on adults collected from an alligator processor in Mississippi, USA. Additionally, morphometric data, consisting of previously reported measurements for the species, and ecological data, consisting of the standard parasitological parameters of intensity, abundance, and prevalence are provided.

Materials and methods

Specimen collection

During Mississippi, USA's 10-day alligator hunting season in August through September of 2016, 11 partial specimens of *A. mississippiensis* were collected from an alligator processor in Port Gibson, Mississippi. Of these 11 *A. mississippiensis*, 8 (1.37 to 3.58 m in length) still possessed tongues. Tongues and attached glottal regions of the buccal cavities were separated from hosts and transported in 0.9% saline separately to the Parasitology Laboratory of the Mississippi State University College of Veterinary Medicine, Mississippi State, Mississippi, USA, where they were grossly examined for the presence of parasites. Detected trematodes were removed from the tongues with forceps, relaxed in 0.9% hot saline (~ 95 °C), and stored in

70% molecular grade ethanol for morphological and molecular characterization.

Calculation of intensity, abundance, and prevalence

Intensity, defined as the number of parasites per infected host, abundance, defined as the number of parasite per host in all examined hosts, and prevalence, defined as the percentage of hosts examined found to be infected, were calculated. These definitions are consistent with those provided by Bush et al. (1997) and Rózsa et al. (2000). These parasitological parameters were calculated using Quantitative Parasitology 3.0 (Rózsa et al. 2000). Confidence limits of 95% for intensity, abundance, and prevalence were calculating using the program's implementation of the Clopper-Pearson method (Clopper and Pearson 1934) with 2000 bootstrap replications.

Morphological identification and characterization

Thirteen ethanol fixed adult *O. incommodum* excised from three different *A. mississippiensis* were stained for 1 week in Semichon's acetocarmine or a concentrated formulation of Van Cleave's hematoxylin (5 mL of Delafield's hematoxylin, 5 mL of Ehrlich's hematoxylin, 6 g potassium aluminum sulfate, and 100 mL of distilled deionized water). After 1 week, specimens were destained in acidic ethanol until the parenchyma was pale and internal organs were distinct. Specimens were then transferred to alkaline ethanol, dehydrated in a series of four ethanol washes of increasing concentrations from 70 to 100%. Specimens were then cleared in Hemo-De (Scientific Safety Solvents, Texas, USA), and mounted on glass slides in Canada balsam (Aldon Corporation, New York, USA). Measurements of stained and mounted trematodes were taken using an Olympus BX50 microscope (Olympus Corporation, Tokyo, Japan) with an Olympus DP72 camera attachment and associated cellSens Standard 1.12 software. A line drawing was made with the aid of a camera lucida and digitized using Adobe Illustrator CC 2017.1 (Adobe Systems, San Jose, CA, USA). A photomicrograph was taken using an Olympus BX41 with an attached Nikon DS-Fi1 camera (Nikon Corporation, Tokyo, Japan).

Molecular analysis

Genomic DNA was extracted from a total of three specimens of *O. incommodum*, two from one *A. mississippiensis*, and one from a second using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Ribosomal genes (spanning partial 18S rRNA gene, internal transcribed spacer region 1, 5.8S rRNA gene, ITS 2 region, and partial 28S rRNA gene) were amplified using the following primer sets: ERIB1/ERIB10, Dipo1795F/Diplo2549R, BD1/BD2, Diplo2617F/Diplo3170R, and LSU5/1500R. Mitochondrial cytochrome *c* oxidase subunit 1 (CO1) and nicotinomide adenine dinucleotide dehydrogenase subunit 1 (NAD1) sequences were amplified using the following primer sets: modified versions of Dice1F/Dice14R with T3 and T7 tails removed, cox1_schist5'/modified Dice14R, JB3/JB4.5, and NDJ11/NDJ2a. Primer sequences and references for each primer are listed in Table 1. Each 20-μL reaction consisted of 10 μL of Phusion Green Hot Start II High-Fidelity PCR Master Mix (ThermoFisher, Waltham, MA, USA), 10 pmol/μL of each primer, 1 μL of DNA (> 10 ng/uL), and 7 μL of nuclease free water to volume. Thermal cycling profiles are indicated in Table 2.

To obtain pairwise distances between top BLASTN (Altschul et al. 1990) hits and sequences generated in the present study, sequences were aligned using the MAFFT algorithm (Katoh and Standley 2013) using GUIDANCE2 (Landan and Graur 2008; Sela et al. 2015). The resultant alignments were then trimmed by eye and gaps removed using MEGA7 (Kumar et al. 2016). Finally, pairwise distances were calculated using MEGA7.

To determine intraspecific variability for each gene target between sequences generated in the present study, sequences were aligned using the MUSCLE algorithm (Edgar 2004), trimmed by eye, and pairwise distances between them calculated in MEGA7.

Phylogenetic analysis

Similar to the methods of Caffara et al. (2016), ribosomal (ITS regions) and CO1 sequences belonging to members of the family Clinostomidae, as well as the diplostomids *Alaria mustelae* Bosma, 1931 and *Diplostomum baeri* Dubois, 1937 (as outgroups) were downloaded from the NCBI nr/nt database (Supplemental Table 1). Regions of the ITS1, 5.8S, and ITS2 genes were extracted using ITSx 1.0.11 (Bengtsson-Palme et al. 2013), subjected to gap removal, aligned using the MAFFT algorithm as implemented in Geneious 10.2 (Kearse et al. 2012), and concatenated with CO1 sequences in MEGA7 for a final alignment containing 1169 positions. Best-fitting models for each position were selected using the Bayesian Information Criterion: CO1 codon position 1 (TN93 + I; 176 positions), CO1 codon position 2 (HKY; 176 positions), CO1 codon position 3 (TN93 + G; 176 positions), ITS1 region (K2 + G; 402 positions), 5.8S rRNA gene (JC; 157 positions), and ITS2 region (JC + G; 70 positions). Phylogenetic inferences were made with MrBayes 3.2.6 (Ronquist and Huelsenbeck 2003; Altekar et al. 2004) by using Markov chain Monte Carlo searches of two simultaneous runs of four chains with sampling every 100th tree for 1×10^6 generations. This number of generations ensured the value of the standard deviation of split frequencies reached < 0.01. After the first 25% of trees were discarded as burn-in, posterior probability values were calculated from the

Table 1 Primers used for DNA amplification

Primer name	Primer sequence (5′-3′)	Gene target	Reference
ERIB1	ACCTGGTTGATCCTGCCAG	18S	Barta et al. (1997)
ERIB10	CTTCCGCAGGTTCACCTACGG	18S	Barta et al. (1997)
Diplo1795F	CGTCGCTACTACCGATTGAA	18S and ITS	Rosser et al. (2016)
Diplo2549R	AGTGATCCACCGCTCAGAGT	18S 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	Rosser et al. (2016)
Diplo3170R	GCTGGACTTAGGATGGAGCA	ITS and 28S	Rosser et al. (2016)
LSU5	TAGGTCGACCCGCTGAAYTTAAGCA	28S	Littlewood et al. (2000)
1500R	GCTATCCTGAGGGAAACTTCG	28S	Tkach et al. (2003)
Cox1_schist5’	TCTTTRGATCATAAGCG	CO1	Lockyer et al. (2002)
Acox650r	CCAAAAAACCAAAACATATGCTG	CO1	Kudlai et al. (2015)
Modified Dice1F	TTWCNTTRGATCATAAG	CO1	Steenkiste et al. (2015)
Modified Dice14R	CCHACMRTAAACATATGATG	CO1	Steenkiste et al. (2015)
JB3	TTTTTTGGGCATCCTGAGGTTTAT	CO1	Bowles et al. (1995)
JB4.5	TAAAGAAAGAACATAATGAAAATG	CO1	Bowles et al. (1995)
NDJ11	AGATTCGTAAGGGGCCTAATA	NAD1	Morgan and Blair (1998)
NDJ2a	CTTCAGCCTCAGCATAAT	NAD1	Kostadinova et al. (2003)

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 1000 pseudoreplicates. Trees were annotated in FigTree 1.4.3 (Rambaut 2016) and Adobe Illustrator 2017.1.

Results

Prevalence and intensity

Odhneriotrema incommodum was detected in five of eight *A. mississippiensis* buccal cavities examined, yielding a prevalence of 62.5% (95% bootstrap BC_a confidence limit, 24.5–

Table 2 Thermal cycling parameters used for DNA amplification

Primer	Denaturation	Cycling	Extension/elongation
ERIB1/ERIB10	98 °C; 10 min	30 cycles of 98 °C for 10 s, 51 °C for 30 s, 72 °C for 1 min	72 °C; 10 min
BD1/BD2 LSU5/1500R	98 °C; 10 min	45 cycles of 98 °C for 10 s, 60 °C for 30 s, 72 °C for 1 min	72 °C; 10 min
Diplo1975F/Diplo2549R Diplo2617F/Diplo3170R	98 °C; 10 min	35 cycles of 98 °C for 10 s, 58 °C for 30 s, 72 °C for 1 min	72 °C; 10 min
JB3/JB4.5	98 °C; 10 min	35 cycles of 98 °C for 10 s, 53 °C for 30 s, 72 °C for 1 min	72 °C; 10 min
Cox1_schist5’/Dice14R Dice1F/Dice11R Dice1F/Dice14R	98 °C; 10 min	3 cycles of 98 °C for 10 s, 54 °C for 30 s, 72 °C for 30 s, 5 cycles dropping the annealing temperature 1 °C each cycle from 53 to 49 °C, 35 cycles with an annealing temperature of 48 °C	72 °C; 10 min
Cox1_schist5’/acox650r NDJ11/NDJ2a	98 °C; 10 min	40 cycles of 98 °C for 10 s, 48 °C for 30 s, 72 °C for 1 min	72 °C; 5 min

91.5%). The number of parasites in each infected host ranged from 1 to 16. Mean intensity was 5.4 (95% bootstrap BC_a confidence limit, 1.8–11.4) *O. incommodum* per buccal cavity while the median intensity was 4.0 *O. incommodum* per buccal cavity. Mean abundance of *O. incommodum* per *A. mississippiensis* was 3.4 (95% bootstrap BC_a confidence limit, 1–9.1).

Morphological characterization

All measurements given in micrometers unless otherwise stated. Measurements are given in the format range (mean ± standard deviation). Measurements for individual worms are also available (Supplementary Table 2). Measurements were consistent with those previously reported for the species while not consistent with those reported for the only congenerous species, *Odhneriotrema microcephala* (Table 3). Voucher specimens are deposited in the Smithsonian Institution, Museum of Natural History, Washington, District of Columbia, USA (USNM 1457205, 1457207).

Body elongate, dorsally convex, and ventrally flattened, 8.7–19.2 (14.8 ± 3.6) mm × 1.3–2.3 (1.8 ± 0.3) mm (Figs. 1, 2). Anterior end of body possesses reniform collar surrounding the oral sucker. Collar 0.4–1.4 (0.8 ± 0.3) mm × 0.8–1.4 (1.1 ± 0.2) mm. Oral sucker ovoid to circular, 222.4–559.7 (366.8 ± 94.4) × 286.2–452.9 (381.6 ± 60.5). Pharynx ovoid, immediately posterior to oral sucker, 315.8–562.8 (454.5 ± 72.3) × 181.8–389.0 (299.8 ± 49.5). Caeca lacking distinct diverticula in most specimens, bifurcate just posterior to pharynx, marginal until terminating posterior to anterior testis and anterior to excretory pore. Acetabulum large, muscular 1.0–1.7 (1.4 ± 0.2) mm × 0.6–1.5 (1.2 ± 0.2) mm. Acetabulum muscular ring 238.3–598.5 (413.9 ± 116.2) in thickness. Acetabulum inner orifice 235.0–832.6 (642.2 ± 150.9) in diameter. Distance from anterior end of acetabulum to anterior end of body 2.7–5.2 (3.9 ± 0.7) mm. Acetabulum diameter to pharynx diameter ratio 3.2–4.6 (3.9 ± 0.4). Distance from anterior border of acetabulum to middle of body 0.6–3.3 (2.2 ± 1.1) mm. Testes, two, irregularly pyramidal, unlobed. Anterior testis, 324.6–880.2 (656.6 ± 199.0) × 99.2–545.1 (358.5 ± 126.21). Distance from anterior testis to posterior testis 0.6–1.4 (1.0 ± 0.3) mm. Posterior testis 268.3–956.6 (555.8 ± 196.0) × 218.7–586.9 (426.9 ± 112.2). Cirrus sac large, ovoid, contains *pars prostatica*, intertesticular, 402.8–1830.9 (1299.4 ± 479.1) × 126.7–364.4 (259.4 ± 84.9). Distance from posterior end of posterior testis to posterior end of the body 0.7–1.6 (1.1 ± 0.3) mm. Vitelline follicles extend from just posterior to point of caecal bifurcation to acetabulum, then extend laterally from posterior to the acetabulum for the length of the ceaca, not convergent posterior to reproductive organs. Ovary round, 97.1–394.8 (295.1 ± 98.8) × 148.1–600.5 (298.5 ± 124.8). Uterus extends anteriorly from ovary, loops posteriorly just before ventral sucker, meets the metraterm at level of anterior testis, contains eggs too numerous to count. Genital pore opening in right margin of body between ovary and posterior testis. Distance from genital pore to posterior testis 40.8–426.6 (147.3 ± 101.5). Distance from center of genital pore to posterior end of body 0.9–2.1 (1.5 ± 0.4) mm. Y-shaped excretory canal at posterior end of body, terminates at excretory pore. Eggs (n = 90, 10 measured from each gravid specimen), elliptical, average 58.4–110.7 (95.9 ± 15.7) × 42.6–59.2 (52.8 ± 5.2), mature eggs containing developed miracidia were located nearest genital pore.

Molecular characterization

NCBI accession numbers for sequences generated from three specimens in the present study are as follows: MF766001-MF766003: cytochrome *c* oxidase subunit 1; MF765998-MF766000: 18S, ITS1-5.8S, ITS2, and 28S rRNA; and MF766004-MF766006: nicotinamide adenine dinucleotide dehydrogenase subunit 1.

Top BLASTN hits for the 784–1051-bp CO1 sequences for Clinostomidae were *Clinostomum attenuatum* Cort, 1913 metacercariae from *Lithobates* sp. Fitzinger, 1843 (Locke et al. 2015; KP150306), *Clinostomum marginatum* (Rudolphi, 1819) metacercariae from yellow perch *Perca flavescens* Mitchill, 1814 (Caffara et al. 2011; JF718610), *Clinostomum detruncatum* Braun, 1899 metacercariae from marbled swamp eels *Synbranchus marmoratus* Bloch, 1785 (Locke et al. 2015; KP110519), *Clinostomum tataxumui* Sereno-Uribe et al., 2013 metacercariae from Pacific sleepers *Gobiomorus maculatus* Günther, 1859 (Locke et al. 2015; KP110551), and *Clinostomum cutaneum* Paperna, 1964 metacercariae from Nile tilapia *Oreochromis niloticus* Linnaeus, 1758 (Locke et al. 2015; KP110516). Pairwise distances between *Odhneriotrema incommodum* CO1 sequences and top BLASTN hits are shown in Table 4. Intraspecific variability at CO1 was 0–0.13%.

Top BLASTN hits identified to species level for ribosomal sequences for Clinostomidae were *Clinostomum marginatum* metacercariae from *Notropis* sp. Rafinesque, 1818 (Sereno-Uribe et al. 2013; JX631101), *Clinostomum album* Rosser et al., 2017 adults from Great Egrets *Ardea alba* Linnaeus, 1758 (Rosser et al. 2017; KU708008), *Clinosotmum complanatum* (Rudolphi, 1814) metacercariae from Nile tilapia *Oreochromis niloticus* (Gustinelli et al. 2010; FJ609420), *Clinostomum phalacrocoracis* Dubois, 1931 metacercariae from Nile tilapia *Oreochromis niloticus* (Gustinelli et al. 2010; FJ609422), and *Clinostomum tataxumui* Sereno-Uribe et al., 2013 adults from Bare-throated Tiger Herons *Tigrisoma mexicanum* Swainson, 1834 (Pérez-Ponce de

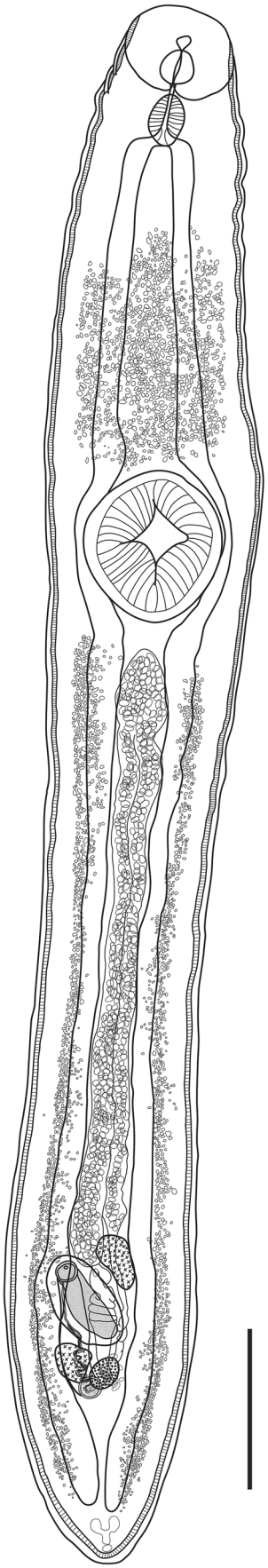
Table 3 Morphological measurements from previous accounts of *Odhneriotrema* species and this study. Measurements from the present study are indicated in italics

	Odhneriotrema inocommodum				*Odhneriotrema microcephala*	
	Alligator mississippiensis				*Caimen sclerops*	
Reference	Leidy 1856	Leidy 1884	Canavan 1933	This paper	Travassos 1922	Travassos 1928
BL	9 lines	15–20 mm	17.2 mm	*8.69–19.19 mm*	9 mm	12–15 mm
BW	1.5 lines	3 mm	3 mm	*1.29–2.25 mm*	3.5 mm	3–5 mm
CL	–	–	0.418 mm	*0.37–1.38 mm*	–	–
CW	–	–	1.0 mm	*0.81–1.4 mm*	–	–
OSL	–	–	–	*222.37–559.69*	–	–
OSW	–	–	0.4–0.45 mm	*0.29–0.45 mm*	0.74 mm	0.7–1 mm
PhL	–	–	–	*315.75–562.78*	0.53 mm	–
PhW	–	–	–	*181.75–389.01*	0.43 mm	0.40–0.50 mm
AcL	–	–	–	*1.03–1.75 mm*	–	–
AcW	–	–	1.54–1.63 mm	*0.64–1.5 mm*	1.8 mm	1.8 mm
AcMRT	–	–	365–550	*238.27–598.51*	–	–
AcIOD	–	–	530–810	*234.99–832.64*	–	–
Ac:Ph	–	–	4	*3.20–4.62*	–	–
Ac-Body	–	–	4.2 mm	*2.65–5.16 mm*	–	–
Ac-Mid	–	–	4.7 mm	*0.58–3.29 mm*	–	–
GP-Body	–	–	2.27 mm	*0.91–2.12 mm*	–	–
GP-PT	–	–	100	*40.79–426.63*	–	–
CSL	–	–	1.95 mm	*0.40–1.83 mm*	–	–
CSW	–	–	327–390	*126.66–364-46*	–	–
ATL	–	–	1.272 mm	*0.32–0.88 mm*	0.87 mm	–
ATW	–	–	0.910 mm	*0.10–0.55 mm*	0.95 mm	0.3–0.4 mm
AT-PT	–	–	1.365 mm	*0.57–1.43 mm*	–	–
OVL	–	–	636	*97.09–394.81*	340	–
OVW	–	–	550	*148.10–600.48*	450	–
PTL	–	–	1.09 mm	*268.26–956.62*	0.78 mm	–
PTW	–	–	0.910 mm	*218.74–586.92*	1 mm	0.3–0.4 mm
PT-Body	–	–	1.575 mm	*0.67–1.55 mm*	–	–
EgLAVG	–	–	92–108	*58.41–110.74*	134	134
EgWAVG	–	–	44–48	*42.56–59.23*	63	63

BL body length, *BW* body width, *CL* collar length, *CW* collar width, *OSL* oral sucker length, *OSW* oral sucker width, *PhL* pharynx length, *PhW* pharynx width, *AcL* acetabulum length, *AcW* acetabulum width, *AcMRT* acetabulum medial ring thickness, *AcIOD* acetabulum inner orifice diameter, *Ac:Ph* ratio of acetabulum diameter to pharynx diameter, *Ac-Body* distance from posterior border of acetabulum to posterior border of the body, *Ac-Mid* distance from posterior border of the acetabulum to the midpoint of the body, *GP-Body* distance from middle of the genital pore to the posterior end of the body, *GP-PT* distance from the middle of the genital pore to the anterior border of the posterior testis, *CSL* cirrus sac length, *CSW* cirrus sac width, *ATL* anterior testis length, *ATW* anterior testis width, *AT-PT* distance from posterior border of the anterior testis to the anterior border of the posterior testis, *OVL* ovary length, *OVW* ovary width, *PTL* posterior testis length, *PTW* posterior testis width, *PT-Body* distance from the posterior border of the posterior testis to the posterior border of the body, *EgLAVG* average length of 10 eggs from each gravid specimen, *EgWAVG* average width of 10 eggs from each gravid specimen

León et al. 2016; JX631050). Pairwise distances between *Odhneriotrema incommodum* ribosomal sequences and top BLASTN hits are shown in Table 5. Intraspecific variability for ribosomal genes was 0%.

The only Clinostomidae sequence a BLASTN search identified to species level for the NAD1 sequences was that of a complete mitochondrial genome, reportedly from *Clinostomum complanatum* metacercariae (74.24–74.42% sequence similarity) from goldfish *Carassius auratus* Linnaeus, 1758 (Chen et al. 2016; KM923964). Comparison of cytochrome *c* oxidase subunit 1 sequences from the same specimens to this genome showed sequence similarity of 79.35–80.20%. Intraspecific variability at NAD1 was 0–0.46%.



◀ **Fig. 1** Line drawing of *Odhneriotrema incommodum*. Bar = 2 mm

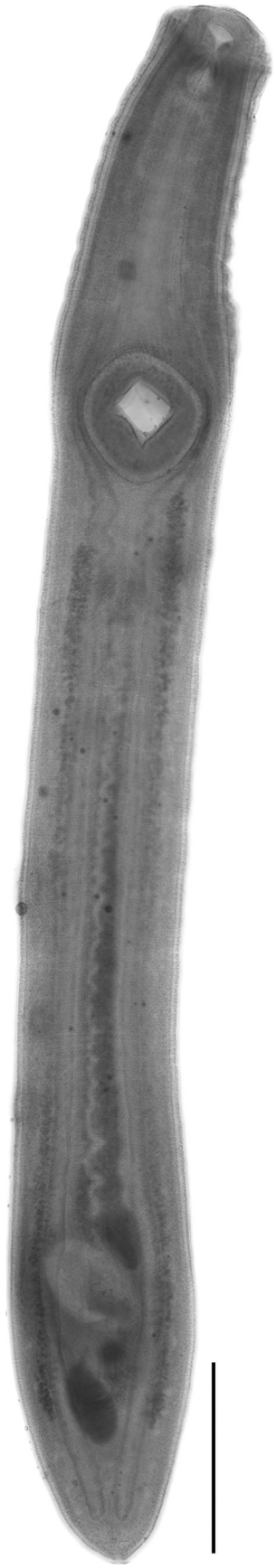
Phylogenetic analysis

Phylogenetic inference based on both conserved (ribosomal) and fast-evolving (mitochondrial) sequencing data placed *Odhneriotrema incommodum* within the Clinostomidae basal to *Euclinostomum*, which formed a clade basal to *Clinostomum*, while the diplostomids *Alaria mustelae* and *Diplostomum baeri* were chosen as the outgroup (Fig. 3). While the phylogenetic placement of *Odhneriotrema* is novel to the present study, placement of *Euclinostomum* is consistent with analyses carried out in previous studies (Senapin et al. 2014; Caffara et al. 2016). These results are consistent with the systematic arrangement of the family Clinostomidae as reported by Kanev et al. (2002).

Discussion

Odhneriotrema incommodum collected in the present study was morphologically consistent with previous accounts of the species (Leidy 1856; McIntosh 1935), including those that provided standard measurements of morphological characters (Leidy 1884; Canavan 1933). However, the measurements of specimens of *O. incommodum* collected in the present study were not consistent with measurements for the only congenerous species; *O. microcephala* (Travassos 1922, 1928), collected from the mouth of the spectacled caiman *Caiman crocodilus* Linnaeus, 1758.

Leidy (1856) described the species as collected from the feces of *A. mississippiensis* while all subsequent descriptions of adult *O. incommodum* have been based on specimens collected only from the buccal cavities of *A. mississippiensis* (Leidy 1884; Canavan 1933; McIntosh 1935). *Odhneriotrema incommodum* was recovered only from the buccal cavities in the present study despite a full examination of the gastrointestinal tract for the collection of other helminths. Given these findings, coupled with the relationship of *O. incommodum* to the Clinostomidae, which are typically associated with the mouth, buccal cavity, and esophagus of their various definitive host species (Ukoli 1966), it is probable the specimens Leidy described had died or failed to attach to their host properly and were incidentally ingested. It is also possible that his specimens were simply mislabeled. In his redescription of the species, Canavan (1933) lists their location in the host as "thorax." Because Canavan's description is based on archived specimens labeled as having been recovered from the thorax, he likely repeated another's erroneous labeling. Barring these oddities in reported infection sites, observations presented in the current study are consistent with historical accounts of this species.



◀ **Fig. 2** Photomicrograph of *Odhneriotrema incommodum*. Stained with Semichon's acetocarmine. *Bar* = 2 mm

While the complete life history of *O. incommodum* is unknown, encapsulated metacercariae have been briefly described as 6–8 mm orange cysts in the gonads, mesentery, and fatty tissues of Florida spotted gar *Lepisosteus platyrhincus* DeKay 1842 (Leigh 1960). Subsequently *L. platyrhincus* was experimentally confirmed as a viable second intermediate host by Leigh (1978) who force-fed metacercariae collected from naturally infected fish to uninfected *A. mississippiensis* and observed the development of the adult worms in the buccal cavities of the definitive hosts, noting the formation of fibrotic lesions at attachment sites. The finding of numerous fibrotic lesions within the buccal cavities of the *A. mississippiensis* in the present study supports Leigh's (1963, 1978) hypothesis that worms relocate to new attachment sites in the buccal cavity after lesions form. Fibrotic lesions on host tongues were often more numerous than the number of *O. incommodum* detected.

Given that the current geographic range of *L. platyrhincus* (IUCN 2013) does not overlap with the host locations for *A. mississippiensis* in the present study, it is unlikely *L. platyrhincus* is the only naturally occurring second intermediate host species for *O. incommodum*. More plausible is that other gar species are capable of serving as second intermediate hosts in the life cycle of *O. incommodum.* Gar species common to Mississippi include the shortnose gar *L. platostomus* Rafinesque, 1820, the spotted gar *L. oculatus* Winchell, 1864, the longnose gar *L. osseus* Linnaeus, 1758, and the alligator gar *Atractosteus spatula* Lacepède, 1803 (Ross 2002). Stomach content analyses carried out on *A. mississippiensis* have shown several of the aforementioned species to be prey items including *Atractosteus spatula* (Gabrey 2010), unidentified gar species (Overstreet et al. 1985), and *Lepisosteus* spp. (McNease and Joanen, 1977). Thus, parasitological examinations of potential second intermediate hosts species, particularly of the genera *Lepisosteus* and *Atractosteus*, are needed to identify additional hosts, provide a more detailed morphological description of the metacercariae, and to carry out histopathological analyses of

Table 4 Pairwise distances (in %) in cytochrome *c* oxidase subunit 1 sequences between most similar NCBI BLASTN results and *Odhneriotrema incommodum* sequences from the present study

	Odhneriotrema incommodum
Clinostomum attenuatum KP150306	17.33–17.53
Clinostomum detruncatum KP110519	18.13–18.33
Clinostomum marginatum JF718610	18.53–18.73
Clinostomum cutaneum KP110516	18.73–18.92
Clinostomum tataxumui KP110551	20.12–20.32

Table 5 Pairwise distances (in %) in ribosomal sequences between most similar NCBI BLASTN results and *Odhneriotrema incommodum* sequences from the present study

	Odhneriotrema incommodum
Clinostomum marginatum JX631101	11.16
Clinostomum album KU708008	11.70
Clinostomum tataxumui JX631050	11.70
Clinostomum phalacrocoracis FJ609422	11.81
Clinostomum complanatum FJ609420	12.68

infections in second intermediate hosts. Previous surveys of *L. oculatus* have shown higher prevalence of *O. incommodum* in females with metacercariae being found encysted chiefly in the ovaries. Additionally, metacercariae were found in the testes of males and the mesenteries of both sexes (Leigh 1960, 1978). Histopathological examinations of fish infected with metacercariae of other species have been shown to cause inflammation in host ovarian tissues (Blazer 2002), which may have implications for host fecundity, as has been suggested for other helminth taxa infecting this site in fish hosts (Clarke et al. 2006).

Although the adult and metacercaria of *O. incommodum* are both described, the cercaria and first intermediate host species remain unknown. Experimental exposure of snails of the species *Planorbella duryi* (= *Helisoma duryi*) Wetherby, 1879 and *Physa pumilia* Conrad, 1834 to miracidia failed to produce infected snails, possibly indicating these particular snail species are not suitable hosts for *O. incommodum* (Leigh 1978).

Given that larval stages of Clinostomidae have been previously reported from aquatic planorbid snails of the genera *Biomphalaria* Preston, 1910 (Pinto et al. 2015; Fernández et al. 2016), *Planorbella* Halderman, 1842 (Hunter and Hunter 1934), and *Bulinus* Müller, 1781 (Feizullaev and Mirzoeva 1983) as well as the lymnaeid *Radix auricularia* Linnaeus, 1758 (Chung et al. 1998), viable snail hosts may be in these genera. Planorbid species susceptible to infection with clinostomids are known to inhabit the range of *A. mississippiensis* (Hunter and Hunter, 1934). Further malacological surveys, coupled with morphological and molecular analysis of collected cercariae, are needed to identify the natural snail host of *O. incommodum* to elucidate the complete life cycle. Experimental infections may also reveal additional first intermediate host species. Snails of the Neotropical genus *Biomphalaria* have recently invaded the range of *A. mississippiensis* (Pointier et al. 2005) and may be able to serve as hosts for this and other clinostomids, as *Biomphalaria* spp. have been shown to serve as hosts for *Clinostomum* sp. (Pinto et al. 2015).

Kanev et al. (2002) present two historical classification systems for the family Clinostomidae, those of Skrjabin (1947) and Yamaguti (1958, 1971). For their part, Kanev et al. employ their own system most similar to that of Yamaguti. Additionally, Feizullaev and Mirzoeva (1983) provide another reclassification. Skrjabin placed *Odhneriotrema* in the subfamily Ophisthophallinae with *Ophisthophallus* based on the presence of a prostatic gland in the cirrus sac, whereas Yamaguti placed it within the subfamily Clinostominae with *Clinostomum, Clinostomatopsis*, and *Clinostomoides* based on the structure of the excretory system. Finally, Feizullaev and Mirzoeva (1983) placed the *Odhneriotrema* species in their own family, the Odhneriotremidae, based on definitive host species. Feizullaev and Mirzoeva also state they have based their reassessment of the superfamily on statistical analysis of morphometric data between and within species, but these data are not available for independent analysis, though keys to the families were provided based on said analyses (Feizullaev and Mirzoeva 1986). While significant efforts have been made to better determine these relationships using molecular data within the genus *Clinostomum* (Locke et al. 2015; Caffara et al. 2017), similar efforts have not been made to determine the systematics of the family as a whole. Molecular data from other taxa within the Clinostomidae are needed to more accurately determine the evolutionary relationships between genera within the Clinostomidae. It is hoped the molecular data provided here will be useful for such a task as it is the first for a member of the Clinostomidae not belonging to *Clinostomum, Euclinostomum*, or *Clinostomoides*. It is notable that the present analyses place a member of a Clinostomid subfamily which uses a reptile as a definitive host basal to species which rely on avian definitive hosts. Should future phylogenetic analyses carried out on other members of the Nephrocephalinae place them similarly, this may suggest a parasite evolutionary history that mirrors that of the hosts. However, in the absence of analyses employing calibrated molecular clock techniques, this is highly speculative.

Greater intrafamilial sequence variability at NAD1 than at CO1 suggest this region may be of use for molecular confirmation of species within the Clinostomidae with greater resolution than CO1, as has been shown for other trematode families (Morgan and Blair 1998). To establish this, more sequencing data are needed for these regions from other members of the family Clinostomidae and other closely related members of the superfamily. It should be noted however, that the reported species identity for the sequence of *C. complanatum* to which sequences from the present study were compared is somewhat doubtful. A BLASTN search using the cytochrome *c* oxidase subunit 1 barcoding region from this mitochondrial genome sequence reveals a 100% match to be *Clinostomum* sp. 8 (KP110536; Locke et al., 2010) and only a 96% match to *C. complanatum* (KU236382; Gaglio et al. 2016). Unfortunately, the sequences of both Chen et al. (2016) and Locke et al. (2015) are based on

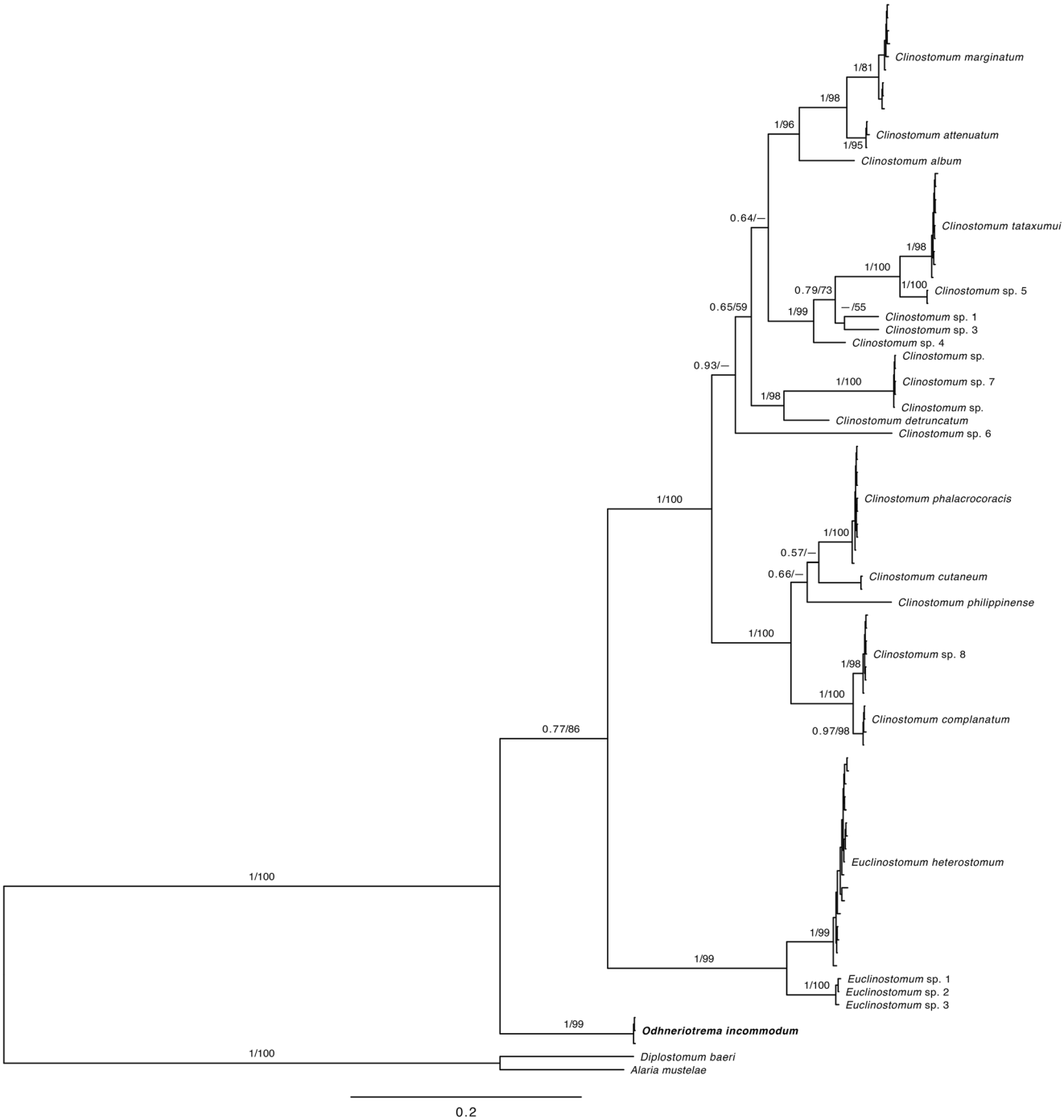


Fig. 3 Phylogenetic tree constructed using an alignment of concatenated ribosomal and cytochrome *c* oxidase subunit 1 sequences. Topology based on Bayesian inference analysis. Numbers above branches indicate Bayesian posterior probabilities/bootstrap values. Posterior probabilities less than 0.5 and bootstrap values less than 50 not shown. Redundant species names in each clade are also omitted. Scale bar represents the number of substitutions per site

metacercariae and no morphometric data justifying specific diagnosis is included..

Although Feizullaev and Mirzoeva (1983) did not provide data to justify their reordering of the taxonomy of the Clinostomidae, their observation that many morphometric characters employed have not had their taxonomic utility rigorously assessed is nonetheless compelling. The advent of DNA sequencing provides a useful way to validate the utility of these characters. By using sequencing data to validate ranges of these measurements, which are thought to define particular species, it should be possible to determine which are of more use in distinguishing between taxa. With this in

mind, the measurements from individual specimens are provided here (Supplemental Table 2) to better facilitate statistical comparisons of measured characters within and between species in the hopes that the utility of individual morphological characters, diagnostic of species identity and confirmed with molecular data, can be quantitatively assessed. The authors of the present study would encourage other researchers, particularly those working with taxa within the family Clinostomidae, to do the same. Authors who seldom report morphometric data may be more inclined to do so if the utility of those measurements were quantified and not unduly cumbersome to collect.

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