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Clinostomum album n. sp. and *Clinostomum marginatum* (Rudolphi, 1819), parasites of the great egret *Ardea alba* L. from Mississippi, USA

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Abstract Members of the genus *Clinostomum* Leidy, 1856, colloquially known as yellow grubs, are cosmopolitan parasites of piscivorous birds, freshwater snails, fish and amphibians. In the southeastern United States, piscivorous birds present a continuous challenge for producers of farm-raised catfish. Ciconiiform birds are common hosts of *Clinostomum* spp. in North America and are endemic on most commercial catfish operations. The great egret *Ardea alba* L. is an avian predator often found foraging on commercial catfish operations, but to date the trematode fauna of great egrets preying on catfish ponds remains mostly understudied. Thirteen great egrets were captured from commercial catfish ponds in northeast Mississippi, and examined for trematode infections. Two morphologically distinct *Clinostomum* spp. were observed in the great egrets sampled, one morphologically consistent with *Clinostomum marginatum* (Rudolphi, 1819) and one morphologically unique species. These morphological descriptions were supplemented with molecular sequence data (*c*.4,800 bp of ribosomal DNA and *c*.600 bp of mitochondrial DNA). Gene sequences confirmed the identification of *C. marginatum*. However, the second species differed significantly from its congeners in both morphology and DNA sequence. Given these distinct morphological and molecular characters we propose this second species as *Clinostomum album* n. sp.

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Introduction

Digenetic trematodes of the genus *Clinostomum* Leidy, 1856 are widely distributed parasites of piscivorous birds, molluscs, fishes and amphibians (Kanev et al., 2002). Adults are found in the oral cavity or oesophagus of the bird hosts, the cercariae develop in freshwater snails and metacercariae encyst in freshwater fish or amphibians (Dias et al., 2003). The debate on the number of species is ongoing, but contemporary studies investigating the genetic variation between and among species have revealed new

insights into the taxonomy of the genus (Locke et al., 2015). As with other groups of digeneans, discriminatory morphological characters that differentiate closely related species are lacking. This has led to an underestimation of true species richness, which was revealed primarily through molecular DNA sequencing (Caffara et al., 2011; Locke et al., 2015; Rosser et al., 2016a). Supplemental molecular data coupled with detailed morphological descriptions have facilitated the identification of new species and offer more sound support of amended descriptions of established species. Genetic markers typically used to distinguish between species include ribosomal (e.g. internal transcribed spacer regions) and mitochondrial (e.g. cytochrome *c* oxidase subunit 1) genes (Caffara et al., 2011; Gustinelli et al., 2010; Sereno-Uribe et al., 2013; Locke et al., 2015).

The great egret *Ardea alba* L. (Pelecaniformes: Ardeidae) is a species of piscivorous bird ranging throughout the USA, southern Canada, Central America and South America. Given the frequent occurrence of the great egret on commercial catfish operations in the southeastern USA, the great egret is widely considered a nuisance species in catfish aquaculture (Glahn & King, 2004). While the effect of predatory foraging by great egrets on catfish aquaculture has been documented (Glahn et al., 1999; Werner et al., 2001), the impact of the trematodes they introduce to catfish production systems is largely understudied.

In North America the number of *Clinostomum* species continues to expand. *Clinostomum heluans* Braun, 1899 was reported in the great blue heron *Area herodias* L. and *Clinostomum intermedialis* Lamont, 1920 in the Brandt's cormorant *Phalacrocorax penicillatus* Brandt from Mexico (Bravo-Hollis, 1947). In a survey of the helminth parasites of great egrets in Florida, USA, two species of *Clinostomum* were reported, *Clinostomum attenuatum* Cort, 1913 and *Clinostomum complanatum* (Rudolphi, 1814) (see Sepúlveda et al., 1999). Overstreet & Curran (2004) reported *Clinostomum marginatum* (Rudolphi, 1819) from herons, egrets and catfish obtained from production ponds in Louisiana and Mississippi, USA. In Mexico, *C. complanatum* has been found in great egrets (Violante-González et al., 2012); however recently Sereno-Uribe et al. (2013) suggested that previous records of *C. complanatum* in Mexico are likely *C. marginatum* or the more recently recognized *Clinostomum tataxumui* Sereno-Uribe, Pinacho-Pinacho, García-Varela & Pérez-Ponce de León, 2013 from great egret, great blue heron, and bare-throated tiger heron *Tigrisoma mexicanum* Swainson. Furthermore, Caffara et al. (2011) combined morphological and molecular data to differentiate adult and metacercaria stages of *C. complanatum* and *C. marginatum* and concluded that *C. complanatum* is the "European" species and is not present in the Americas.

A recent collection of great egrets from catfish production operations in the northeastern area of Mississippi was evaluated for *Clinostomum* spp. infection and a novel species is described herein.

Materials and methods

Trematode collection and morphological characterisation

Thirteen great egrets were collected from commercial catfish operations in Noxubee County, Mississippi using soft catch leg hold traps and euthanized using CO_2. Immediately following euthanasia, the oral cavity and sublingual area were inspected for adult *Clinostomum* spp. These were removed manually with sterile featherweight forceps (BioQuip Products, Rancho Dominguez, California) and placed in 0.09% sterile saline. The oesophagus and trachea were separated and opened longitudinally, the contents emptied into a 38-μm aperture brass sieve and washed with dechlorinated water. The intestinal lining was scraped manually and the contents rinsed onto the screen. The entire screen contents were then examined in a lined Petri dish under a dissecting stereomicroscope (Olympus SZ60, Olympus Optical Co. Ltd., Tokyo, Japan). Remaining intestinal contents were removed and examined for additional trematodes. All *Clinostomum* spp. adults were washed into containers with 0.09% saline.

Adult trematodes were relaxed in slightly boiling saline and fixed in 70% ethanol. A subsample of each suspected species was stained with acetocarmine for at least 5 h, destained in 1% acidic ethanol, and rinsed in increasing concentrations of ethanol (70–100%) for at least 1 h each. Specimens were cleared in Hemo-De (Fisher Scientific, Pittsburgh, Pennsylvania, USA) and mounted on clean glass microscope slides using Permount™ Mounting Medium (Fisher Scientific, Pittsburgh, Pennsylvania, USA). Representative

specimens were submitted to the Smithsonian Institution, National Museum of Natural History, Washington, DC, USA under accession numbers: USNM 1422013–1422018. Line drawings of each species were made with the aid of a camera lucida and digitized using Adobe Illustrator CC 2014 (Adobe, San Jose, California). Photomicrographs of adult specimens were captured using an Olympus DP72 digital camera and DP-2-Twain/cellSens software (Olympus Optical Co. Ltd., Tokyo, Japan). Morphological characteristics of the collected *Clinostomum* spp. were compared to other species within the genus (Caffara et al., 2011). Measurements are presented as the range followed by the mean in parentheses and are reported in micrometres.

DNA extraction and molecular characterisation

Genomic DNA was extracted from three adult specimens of each species with the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, California, USA). Approximately 4,800 bp of ribosomal DNA, including the 18S rRNA gene, ITS1 region, 5.8S rRNA gene, ITS2 region, and partial 28S rRNA gene, was amplified by polymerase chain reaction (PCR) from one representative adult for each species. To identify the isolate to a lower taxonomic level, an approximately 600 bp sequence of the cytochrome *c* oxidase subunit 1 (*cox*1) gene was amplified for all adult specimens from both species. Primers used for each region are listed in Table 1. Briefly, each PCR reaction contained 22 µl of Platinum® PCR Supermix (Invitrogen, Carlsbad, California, USA), 10 pmol of forward and reverse primer, and 1 µl of gDNA (*c*.15 ng/µl) as template. For Barker3/Barker4 primers the thermal cycling program consisted of 94°C for 3 min, 35 cycles of 94°C for 30 s, 50°C for 30 s, followed by 72°C for 1 min. Parameters for the 1F/5R, BD1/BD2, and LSU5/1500R primer combinations were similar, but employed an annealing temperature of 45°C and an elongation step of 1 min 30 s. Likewise, the PCR thermal cycling program for Diplo1795F/Diplo2549R, Diplo2617F/Diplo3170R, 28S 3431F/28S 4779R, and 28S 4759F/28S 5699R primer combinations used previous parameters with an annealing temperature of 55°C. Finally, the thermal cycling protocol for the cox1_schist 5′/acox650r primer set was the same as above, but used a 45°C annealing temperature. Amplification products were electrophoresed through 0.8% agarose gels stained with ethidium bromide (0.5 µg/ml) and visualized under ultraviolet fluorescent light. Each gel was run concurrently with a molecular weight ladder (HyperLadder™ 50 bp, Bioline, London, UK) to confirm the presence of appropriate sized bands.

Amplicons were excised and purified using the QIAquick Gel Extraction Kit (QIAGEN Inc., Valencia, California, USA) and sequenced commercially (Eurofins MWG Operon LLC, Huntsville, Alabama, USA) using the same forward and reverse primers used to generate the amplicons. Ambiguous base calls were annotated manually from respective chromatograms in SeqMan™ (DNAStar, Madison, Wisconsin, USA). The contiguous rRNA and *cox*1 gene sequences for each species were compared to other sequenced *Clinostomum* species by a Blastn search of the National Center for Biotechnology Information non-redundant nucleotide database (NCBI nr/nt) (Altschul et al., 1990).

Published *cox*1 gene sequences from the genus *Clinostomum* available in the NCBI nr/nt database were downloaded and ClustalW aligned and trimmed in MEGA6 (Tamura et al., 2013). The final dataset contained a total of 427 positions across 88 sequences. Accession numbers for sequences used in phylogenetic analysis are provided in Supplementary Table S1. The best-fit nucleotide substitution model for phylogenetic analysis was determined using the Bayesian Information Criterion as the Hasegawa Kishino-Yano (HKY) model including gamma distribution site variation (Nei & Kumar, 2000). Bayesian inference analysis was performed in MrBayes 3.2.6 with Markov chain Monte Carlo searches of two simultaneous runs of four chains. Chain sampling occurred every 100th tree over 10,000,000 generations (Ronquist & Huelsenbeck, 2003) and the first 25% were discarded as 'burn-in' with the posterior probabilities calculated from the remaining trees. The consensus tree was visualized in FigTree 1.4.2 (Rambaut, 2014) and annotated in Adobe Illustrator (Adobe, San Jose, California, USA). Pairwise distances were calculated in MEGA6 based on the alignment of the two *Clinostomum* spp. encountered in this study with those used to construct the phylogenetic tree.

Results

Two distinct species of *Clinostomum*, characterised morphologically and molecularly, were observed in

Table 1 Primers used in amplification of ribosomal genes and mitochondrial cytochrome *c* oxidase subunit 1 gene of *Clinostomum* spp.

Primer	Sequence (5′-3′)	Target gene	Reference
Barker3	TTAGAGTGTTCAAAGCAG	SSU rRNA	Barker et al. (1993)
Barker4	GATCCTTCTGCAGGTTCACCTAC	SSU rRNA	Barker et al. (1993)
1F	TACCTGGTTGATCCTGCCAGTAG	SSU rRNA	Carranza et al. (1997)
5R	CTTGGCAAATGCTTTCGC	SSU rRNA	Carranza et al. (1997)
Diplo1795F	CGTCGCTACTACCGATTGAA	SSU rRNA and ITS	Rosser et al. (2016a)
Diplo2549R	AGTGATCCACCGCTCAGAGT	SSU rRNA and ITS	Rosser et al. (2016a)
BD1	GTCGTAACAAGGTTTCCGTA	ITS	Morgan & Blair (1995)
BD2	TATGCTTAAATTCAGCGGGT	ITS	Morgan & Blair (1995)
Diplo2617F	CATCGACATCTTGAACGCATA	ITS and 28S rRNA	Rosser et al. (2016a)
Diplo3170R	GCTGGACTTAGGATGGAGCA	ITS and 28S rRNA	Rosser et al. (2016a)
LSU5	TAGGTCGACCCGCTGAAYTTAAGCA	28S rRNA	Littlewood et al. (2000)
1500R	GCTATCCTGAGGGAAACTTCG	28S rRNA	Littlewood et al. (2000)
28S 3431F	TCAGAGGTAAACGGGTGGAG	28S rRNA	This study
28S 4779R	CTCAGCTTGCAATGACGGTA	28S rRNA	This study
28S 4759F	GTCTTGAAACACGGACCAAG	28S rRNA	This study
28S 5699R	TACCACCAAGATCTGCACCT	28S rRNA	This study
Cox1_schist 5′	TCTTTRGATCATAAGCG	*cox*1	Lockyer et al. (2003)
Acox650r	CCAAAAAACCAAAACATATGCTG	*cox*1	Kudlai et al. (2015)

the oral cavity and occasionally the oesophagus of 11/13 (overall prevalence of 85%) great egrets. *Clinostomum marginatum* was identified in 10/13 (prevalence of 77%) and a second morphologically and molecularly distinct species in 4/13 (prevalence of 31%) great egrets. No *Clinostomum* spp. were observed in the lower intestinal tracts of any bird.

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

Clinostomum album n. sp.

Type host: Great egret *Ardea alba* Linnaeus (Pelecaniformes: Ardeidae).
Type-locality: Noxubee County, Mississippi, USA.
Type-material: Holotype USNM 1422013 and 2 paratypes USNM 1422014–1422015 are deposited in the Smithsonian Institution, National Museum of Natural History, Washington, D.C., USA
Site in host: Oral cavity (sublingual) and oesophagus.
Infection parameters: Prevalence: 31% (4 out of 13 birds); abundance: range 0–6, mean 0.9 worms per bird; mean intensity 3.0 worms per infected bird.
Representative DNA sequences: GenBank KU708008 (ribosomal genes) and KU708010 (*cox*1).
Etymology: The specific epithet is in reference to the host specific name.

Description (Figs. 1A, 2A)

[Based on the holotype and 5 paratypes. All measurements were taken from stained and mounted gravid adult worms.] Body, linguiform, slender anterior region, widest at level of gonads, 4,402–5,929 × 969–1,108 (5,269 × 1,047). Anterior extremity with distinct oral collar-like fold typical of the genus, 357–507 × 526–690 (428 × 617). Oral sucker small, 207–307 × 234–344 (256 × 263). Pharynx present, intestine bifurcates just posterior to oral sucker and intestinal caeca laterally extend almost to posterior of body. Ventral sucker, large, located in lower anterior third of the body, 480–648 × 485–610 (578 × 560). Distance between oral and ventral sucker 360–827 (656).

Testes, tandem, located in upper region of posterior third of body. Anterior testis triangular, lobed, laterally compressed by cirrus-sac on right margin, 266–469 × 449–535 (375 × 488). Posterior testis

Fig. 1 Photomicrographs of stained *Clinostomum* spp. from *Ardea alba* collected on commercial catfish operations in Noxubee County, Mississippi, USA. A, *Clinostomum album* n. sp.; B, *Clinostomum marginatum*. *Scale-bar*: 800 μm

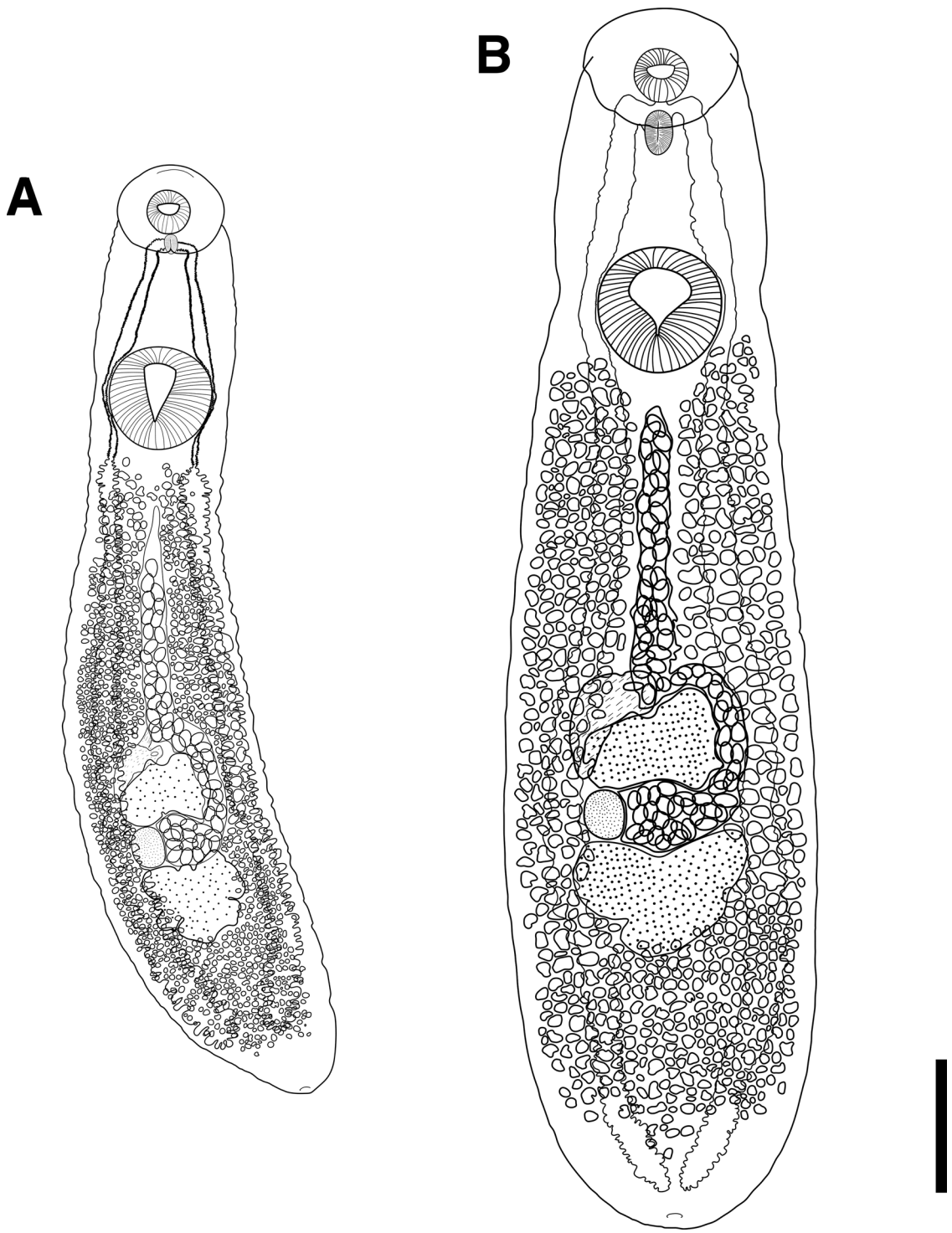

Fig. 2 Line drawing of *Clinostomum* spp. from *Ardea alba* collected on commercial catfish operations in Noxubee County, Mississippi, USA. A, *Clinostomum album* n. sp.; B, *Clinostomum marginatum*. *Scale-bar*: 800 μm

larger, triangular, lobed, 313–473 × 416–571 (425 × 501). Distance between testes 264–354 (298). Cirrus-sac, laterodorsally surrounds right margin of anterior testis, 140–307 × 117–162 (222 × 146).

Ovary small, intertesticular, dextral, ovoid, 201–281 × 180–254 (235 × 199). Uterine duct intracaecal, extending anteriorly along left margin of anterior testis before opening into uterine sac. Uterine sac filled with eggs, occupies lower field between ventral sucker and anterior testis. Metraterm muscular, leads into uterus close to genital atrium. Genital pore pretesticular. Vitelline fields restricted mostly to lateral margins of body, begin just posterior to ventral sucker and extend to posterior extremity of body. Eggs yellowish, 90–108 × 53–67 (100 × 61), on average 70–82 (75) in number, often located within uterine sac, uterine duct, and oötype region.

Molecular data

Molecular analysis of *c*.4,800 bp of ribosomal DNA from a single adult showed a high level of conservation between the two species collected in this study, as they were 99.4% (4,801/4,832 bp) similar across the five ribosomal targets examined. When compared to other sequences deposited in the GenBank database, *C. album* n. sp. shared 99.6% (1,952/1,960) sequence similarity at the 18S rRNA gene with an unpublished sequence of *C. marginatum* from North America (AY245760). Additionally, *C. album* n. sp. shared 96.5–97.2% sequence similarity with *C. complanatum* (AY245701 and FJ609420; Dzikowski et al., 2004; Gustinelli et al., 2010), *Clinostomum cutaneum* Paperna, 1964 (GQ339114 and FJ609421; Gustinelli et al., 2010), and *Clinostomum phalacrocoracis* Dubois, 1931 (FJ609422–FJ609423; Gustinelli et al., 2010) across partial 18S rDNA, complete ITS1, 5.8S rDNA, ITS2, and partial 28S rDNA sequences.

A 604 bp sequence of the mitochondrial *cox*1 gene from three individual adults was identical for all three specimens and revealed that *C. album* n. sp. shared limited sequence similarity with any *Clinostomum* spp. in the GenBank database. The highest sequence similarity was with *C. detruncatum* at 85.3–85.4% (KP110517–KP110519; Locke et al., 2015). *Clinostomum album* n. sp. shared 85.1% sequence similarity with *C. attenuatum* (KP150305–KP150306; Locke et al., 2015), and <85% sequence similarity with two unidentified species of *Clinostomum*, designated as *Clinostomum* sp. 4 from *Apistogramma* sp. Regan in Peru (KP110531; Locke et al., 2015) and *Clinostomum* sp. from *Rana clamitans* Latreille and *Rana pipiens* Schreber in Canada (JF718587 & JF718585; Caffara et al., 2011). Similarly, *C. album* n. sp. shared only 82.9–84.9% sequence similarity with isolates of *C. marginatum* available in the GenBank database and from great egrets in this study.

Remarks

Morphologically *C. album* n. sp. was distinct from *C. marginatum* infecting the great egrets examined in this study. Although similar in mean length, *C. album* n. sp. was considerably narrower than *C. marginatum* (1,047 *vs* 1,562 μm). Furthermore the oral sucker, ventral sucker, testes, and cirrus-sac of *C. album* n. sp. were all smaller on average when compared to those of *C. marginatum* observed in this study and in previous records of this species from North American Ardeidae (Caffara et al., 2011). *Clinostomum album* n. sp. also tended to have less diffuse vitelline follicles, usually confined to the lateral margins of the body compared to the more expansive vitelline follicles of *C. marginatum*. Eggs of *C. album* n. sp. are roughly the same size as *C. marginatum*, although overall body length and width are considerably smaller. Morphological data of the *Clinostomum* spp. of North America are presented in Table 2.

***Clinostomum marginatum* (Rudolphi, 1819)**

Host: Great egret *Ardea alba* (Linnaeus) (Pelecaniformes: Ardeidae).
Locality: Noxubee County, Mississippi, USA.
Site in host: Oral cavity (sublingual) and oesophagus.
Infection parameters: Prevalence: 77% (in 10 out of 13 birds); abundance: range 0–29, mean 5 worms per bird; mean intensity 6.5 worms per infected bird.
Voucher material: Vouchers USNM 1422016–1422018 are deposited in the Smithsonian Institution, National Museum of Natural History, Washington, D.C., USA
Representative DNA sequences: GenBank KU708007 (ribosomal genes) and KU708009 (*cox*1).

Description (Figs. 1B, 2B)

[Based on 7 stained and mounted gravid adult worms.] Body, stout, linguiform, 5,132–6,210 × 1,350–1,820

Table 2 Morphological data for *Clinostomum* spp. from great egrets in this study and North American species of *Clinostomum*

Parasite	*Clinostomum album* n. sp.	*Clinostomum marginatum*	*Clinostomum attenuatum*	*Clinostomum complanatum*	*Clinostomum heluans*	*Clinostomum intermedialis*	*Clinostomum marginatum*	*Clinostomum tataxumui*
Host	*Ardea alba* L.	*Ardea alba* L.	*Phalacrocorax auritus floridanus*	*Egretta garzetta* L.; *Ardea cinerea* L.; *Ardea purpurea* L.	*Ardea herodias* L.	*Phalacrocorax penicillatus* Brandt	*Ardea herodias* L.; *Bubulcus ibis* L.	*Ardea alba* L.; *Ardea herodias* L.; *Tigrisoma mexicanum* Swainson
Locality	Mississippi, USA	Mississippi, USA	Florida, USA	Italy	Mexico	Mexico	Canada; Florida & Texas, USA	Mexico
Reference	This study	This study	Hutton & Sogandares-Bernal (1960)	Caffara et al. (2011)	Bravo-Hollis (1947)	Bravo-Hollis (1947)	Caffara et al. (2011)	Sereno-Uribe et al. (2013)
BL	4,402–5,929 (5,269)	5,132–6,210 (5,697)	6,900–7,358	3,400–6,300 (4,900)	20,706–26,145	6,615–11,100	5,900–8,200 (7,000)	3,360–9,800 (4,500)
BW	969–1,108 (1,047)	1,350–1,820 (1,562)	2,080–2,650	1,500–2,700 (1,900)	2,640–3,444	2,037–2,880	1,300–2,800 (2,000)	800–3,000 (1,200)
BL/BW	4.2–6.1 (5.0)	3.2–4.0 (3.7)	–	–	–	–	–	–
OCL	357–507 (428)	432–793 (561)	–	–	840–1,000	600–1,480	–	330–990 (460)
OCW	526–690 (617)	742–963 (823)	–	–	1,000–1,440	567–1,360	–	570–1570 (760)
OSL	207–307 (256)	246–299 (268)	–	190–570 (422)	640–800	300–500	171–394 (311)	170–350 (206)
OSW	234–344 (263)	237–318 (267)	310–380	320–850 (557)	580–700	–	252–501 (399)	170–300 (208)
OSW/BW	0.2–0.4 (0.3)	0.15–0.19 (0.2)	–	–	–	–	–	–
VSL	480–648 (578)	550–694 (612)	–	600–900 (760)	880–1,100	840–1,200	601–918 (764)	420–850 (536)
VSW	485–610 (560)	589–677 (611)	790–864	620–900 (737)	880–1,100	798–1,240	583–966 (756)	420–777 (524)
VSW/OSW	1.5–2.4 (2.2)	2.0–2.5 (2.3)	–	–	–	–	–	–
VSW/BW	0.5–0.6 (0.5)	0.37–0.44 (0.4)	–	–	–	–	–	–
DBS	360–827 (656)	640–928 (770)	–	–	–	–	–	–
ATL	266–469 (375)	373–526 (453)	436–500	550–750 (694)	860–900	945–1,320	290–743 (519)	220–650 (341)
ATW	449–535 (488)	534–737 (667)	900–1000	360–600 (456)	1,700–1,840	735–1,060	569–1,141 (802)	370–1005 (506)

Table 2 continued

Parasite	*Clinostomum album* n. sp.	*Clinostomum marginatum*	*Clinostomum attenuatum*	*Clinostomum complanatum*	*Clinostomum heluans*	*Clinostomum intermedialis*	*Clinostomum marginatum*	*Clinostomum tataxumui*
Host	*Ardea alba* L.	*Ardea alba* L.	*Phalacrocorax auritus floridanus*	*Egretta garzetta* L.; *Ardea cinerea* L.; *Ardea purpurea* L.	*Ardea herodias* L.	*Phalacrocorax penicillatus* Brandt	*Ardea herodias* L.; *Bubulcus ibis* L.	*Ardea alba* L.; *Ardea herodias* L.; *Tigrisoma mexicanum* Swainson
Locality	Mississippi, USA	Mississippi, USA	Florida, USA	Italy	Mexico	Mexico	Canada; Florida & Texas, USA	Mexico
Reference	This study	This study	Hutton & Sogandares-Bernal (1960)	Caffara et al. (2011)	Bravo-Hollis (1947)	Bravo-Hollis (1947)	Caffara et al. (2011)	Sereno-Uribe et al. (2013)
ATW/ATL	1.1–1.7 (1.3)	1.2–1.8 (1.5)	–	–	–	–	–	–
PTL	313–473 (425)	319–589 (424)	400–518	600–940 (791)	640–840	580–940	166–587 (461)	240–420 (346)
PTW	416–571 (501)	569–826 (700)	774–980	300–510 (410)	1,440–1,740	1,050–1,400	379–1,414 (790)	450–1220 (599)
PTW/PTL	1.0–1.3 (1.2)	1.4–1.9 (700)	–	–	–	–	–	–
DBT	264–354 (298)	252–378 (312)	–	–	–	–	–	–
OVL	201–281 (235)	187–261 (236)	65–73	220–310 (256)	640–840	320–480	118–306 (175)	160–420 (218)
OVW	180–254 (199)	176–256 (217)	109–115	140–300 (213)	360–714	260–380	101–267 (186)	150–360 (196)
OVW/OVL	0.8–0.9 (0.8)	0.8–1.04 (0.9)	–	–	–	–	–	–
CSL	140–307 (222)	316–544 (418)	–	350–400 (382)	–	400–640	151–795 (327)	250–520 (350)
CSW	117–162 (146)	154–298 (220)	–	100–200 (162)	–	546–800	166–741 (497)	130–300 (172)
CSL/BL	0.02–0.1 (0.04)	0.06–0.09 (0.1)	–	–	–	–	–	–
EGGL	90–108 (100)	94–105 (101)	–	100–125 (114)	114–136	104–120	101–109 (105)	–
EGGW	53–67 (61)	63–72 (68)	–	65–90 (74)	70–80	76–82	63–79 (69)	–
EGG#	70–82 (75)	37–94 (68)	–	–		–	–	–

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 testes; OVL, ovary length; OVW, ovary width; CSL, cirrus sac length; CSW, cirrus sac width; EGGL, egg length; EGGW, egg width; EGG#, no. of eggs

(5,697 × 1,562). Anterior end possessing oral collar-like fold typical of the genus, 432–793 × 742–963 (561 × 823), surrounding oral sucker. Oral sucker, small, 246–299 × 237–318 (268 × 267). Ventral sucker, large, located near anterior of the body, 550–694 × 589–677 (612 × 611). Intestinal caeca bifurcate immediately behind oral sucker and extend to the terminal end of the body. Distance between the oral and ventral sucker, 640–928 (770).

Testes, tandem, located toward the middle or upper portion of the posterior of the body. Anterior testis, triangular, lobed, 373–526 × 534–737 (453 × 667) and laterally compressed on the right margin by cirrus sac. Posterior testis, triangular, lobed, 319–589 × 569–826 (424 × 700). Distance between testes 252–378 (312). Cirrus sac, laterodorsally surrounds the right margin of the anterior testis, 316–544 × 154–298 (418 × 220).

Ovary, ovoid, intertesticular, dextral, 187–261 × 176–256 (236 × 217). Uterine duct lies at the level of the caecae and extending anteriorly along the left margin of the anterior testis before opening into the uterine sac. Uterine sac occupies almost the entire area of the body between the ventral sucker and anterior testis. Metraterm, muscular, joins the uterus close to the genital atrium. Genital pore, when observed, pretesticular. Vitelline follicles diffuse, concentrated in the lateral regions of the body and beginning at the level of the ventral sucker and extending to the end of the body. Eggs, yellow in color, 94–105 × 63–72 (101 × 68), 37–94 (68) eggs throughout the uterine ducts and sac, and also often obscuring the oötype region.

Remarks

Morphologically *C. marginatum* specimens identified in this study were consistent with those previously described for the species (Cort, 1913; Caffara et al., 2011). *Clinostomum marginatum* collected from great egrets in this study shared 98.9–100% sequence similarity at ribosomal genes available for *C. marginatum* in the GenBank database, with most archived sequences covering the ITS1, 5.8S rRNA, and ITS2 region. Additionally, *C. marginatum* shared 97.5–96.9% sequence similarity with > 4,500-bp of ribosomal DNA from *C. cutaneum* (GQ339114 & FJ609421; Gustinelli et al., 2010), *C. complanatum* (AY245701 & FJ609420; Dzikowski et al., 2004; Gustinelli et al., 2010), and *C. phalacrocoracis* (FJ609422–FJ609423; Gustinelli et al., 2010).

Moreover, the 612-bp *cox*1 sequence of three *C. marginatum* specimens was > 99% similar to isolates of *C. marginatum* in the GenBank database. The morphological description and limited interspecific variation at the *cox*1 gene support the identification as *C. marginatum.*

*Cox*1 phylogeny of *Clinostomum* spp.

Genetic divergence of *cox*1 sequences (Table 3) of *C. album* n. sp. with other species of the genus ranged on average 15.29–19.86% (14.12–20.0%). Whereas, *C. marginatum* had minimal intraspecific genetic divergence at the *cox*1 sequence when compared to other isolates of *C. marginatum.* The genetic divergence of *C. marginatum* to other isolates of *C. marginatum* was 1.49% (0.47–8.71%). Bayesian inference based on *cox*1 sequences demonstrated distinct clustering of the *Clinostomum marginatum* from this study with other isolates obtained throughout North America (Fig. 3) and as sister taxa to *C. attenuatum*. *Clinostomum album* n. sp. was basally located within a clade containing *C. tataxumui*, *Clinostomum* sp. 5, *Clinostomum* sp. 2, *Clinostomum* sp. 1, *Clinostomum* sp. 3, and *Clinostomum* sp. 4. Topology of the tree was similar to previously published phylogenetic trees of *cox*1 sequences of clinostomes (Locke et al., 2015) and well supported. For an uncollapsed tree, see Supplementary Figure S1.

Discussion

Clinostomum species are cosmopolitan parasites of avian, mollusc and amphibian or fish hosts. These digeneans are of significant commercial and ecological importance as parasites of ecologically threatened species of amphibians, as well as wild and farm-raised fish (Paperna, 1991). In North America, six named species have been reported (*C. attenuatum*, *C. complanatum*, *C. heluans*, *C. intermedialis, C. marginatum* and *C. tataxumui*) from avian, mollusc, fish and amphibian hosts (Bravo-Hollis, 1947; Stuart et al., 1972; McAllister, 1990; Sepúlveda et al., 1994, 1996, 1999; Kinsella et al., 2004; Caffara et al., 2011; Sereno-Uribe et al., 2013). The larval stages of

Table 3 Pairwise distances (in %) between cytochrome *c* oxidase subunit 1 sequences of *Clinostomum album* n. sp. and *Clinostomum marginatum* from this study and other *Clinostomum* spp

	Clinostomum album n. sp.	*Clinostomum marginatum* (this study)
Clinostomum complanatum (Rudolphi, 1814)	19.86 (19.29–20.00)	16.77 (06.47–06.94)
Clinostomum cutaneum Paperna, 1964	18.00 (17.88–18.12)	17.06 (16.94–17.18)
Clinostomum detruncatum Braun, 1899	15.37 (15.29–15.53)	14.20 (14.12–14.35)
Clinostomum marginatum (Rudolphi, 1819)	15.39 (14.82–16.94)	01.49 (00.47–08.71)
Clinostomum phalacrocoracis Dubois, 1931	19.84 (19.53–20.00)	19.37 (19.06–19.53)
Clinostomum philippinense Velasquez, 1960	19.76	18.35
Clinostomum tataxumui Sereno-Uribe, Pinacho-Pinacho, García-Varela & Pérez-Ponce de León, 2013	18.09 (17.88–18.12)	16.21 (16.00–16.24)
Clinostomum sp. 1 of Locke et al. (2015)	15.29	16.00
Clinostomum sp. 2 of Locke et al. (2015)	16.00 (16.00–16.00)	15.06 (15.06–15.06)
Clinostomum sp. 3 of Locke et al. (2015)	16.47	15.53
Clinostomum sp. 4 of Locke et al. (2015)	14.12	15.76
Clinostomum sp. 5 of Locke et al. (2015)	16.00 (16.00–16.00)	16.47 (16.47–16.47)
Clinostomum sp. 6 of Locke et al. (2015)	19.29	17.41
Clinostomum sp. 8 of Locke et al. (2015)	19.24 (19.06–19.53)	16.41 (16.24–16.71)

Values are presented as average number of base differences per site followed by the range in parentheses

C. marginatum have been reported from hosts in commercial catfish ponds, marsh ramshorn snails *Planorbella trivolvis* (Say) and channel catfish *Ictalurus punctatus* (Rafinesque) (Lorio, 1989) collected from commercial catfish operations. Moreover, Overstreet & Curran (2004) reported *C. marginatum* in egrets and herons in the southeastern United States. Herein we report two species, *C. marginatum* and *C. album* n. sp., from great egrets foraging on catfish aquaculture operations in Mississippi.

Morphologically *C. album* n. sp. was distinct from other *Clinostomum* spp. reported from North American avian hosts. The isolate was consistently smaller across numerous features. Gonads were diminutive and placement of vitelline follicles was restricted to the lateral margins of the body rather than the more diffuse vitelline follicles of *C. marginatum*. *Clinostomum* spp. from South America and Mexico, specifically *C. detruncatum* and *C. heluans* differ considerably, not only in their much larger size, but also placement of the gonads at the posterior of the body (Bravo-Hollis, 1947; Travassos et al., 1969). In addition, eggs of *C. album* n. sp. were similar in size to *C. marginatum*, even though the body and other morphological features of *C. album* n. sp. are markedly smaller.

While morphological descriptions have been the basis of identification of digeneans over the past two centuries, molecular identification has afforded more precise differentiation of morphologically similar species (Caffara et al., 2011). While unremarkable at the *c*.4,800 bp rDNA region, *C. album* n. sp. was markedly divergent at the *cox*1 gene from all other species of *Clinostomum* available in the GenBank database. That said, the interspecific variability between *C. album* n. sp. and *C. marginatum* was consistent with intrageneric variability described for the genus (Caffara et al., 2011; Locke et al., 2015). Additionally, the limitation of ribosomal genes as the only molecular marker for species delimitation was exemplified in this study. The *c*.4,800-bp of *C. album* n. sp. ribosomal DNA demonstrated less than 0.7% divergence from *C. marginatum*, while demonstrating less than 4% divergence from other *Clinostomum* species in the GenBank database. These results are consistent with those reported by Gustinelli et al. (2010), where ribosomal DNA sequences of *C. cutaneum* were less than 3% divergent from other closely related species of *Clinostomum*. In order to fully appreciate the species richness of the *Clinostomum*, further sampling from avian, fish and mollusc hosts from different continents is needed, coupling

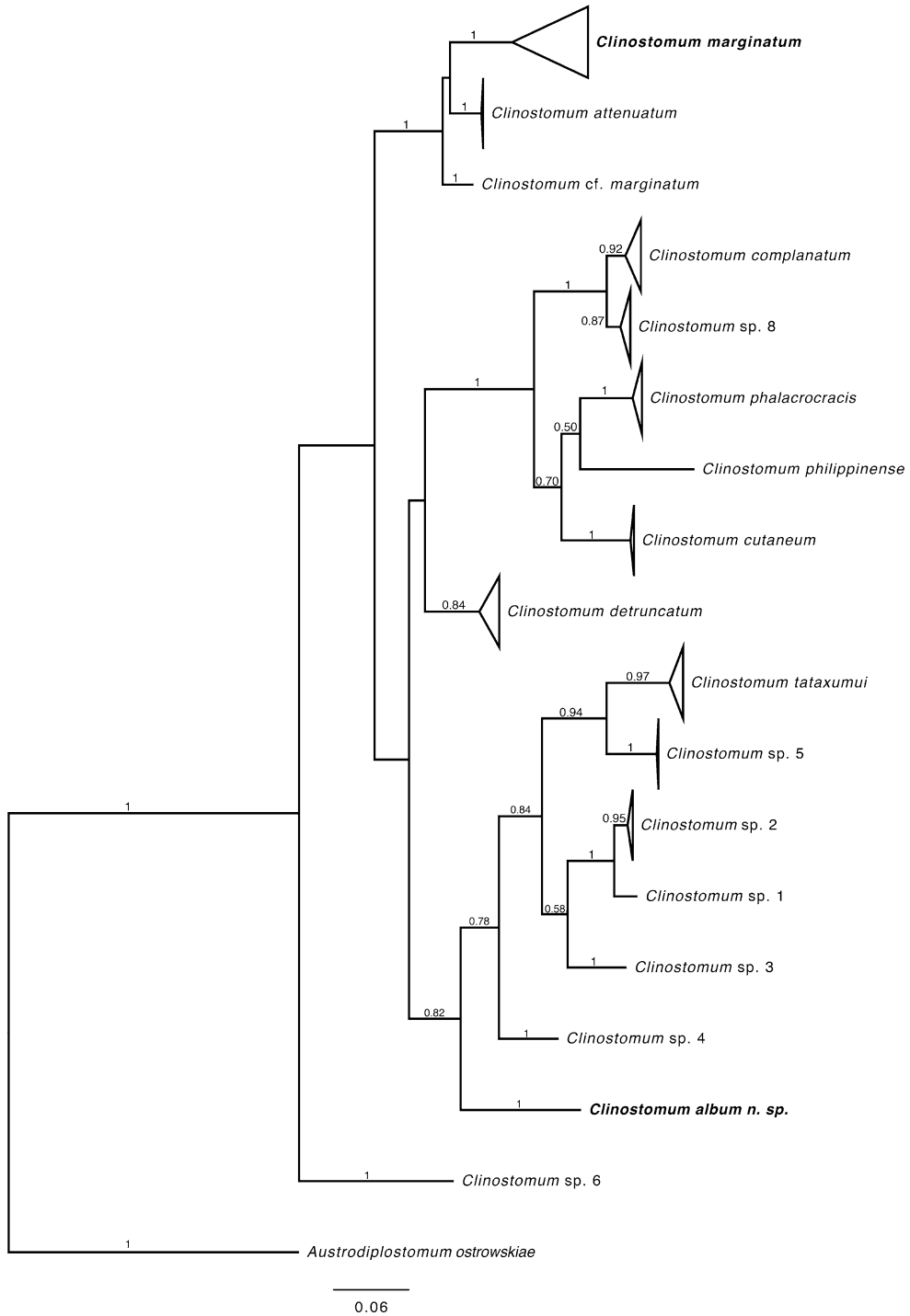

Fig. 3 Bayesian inference tree for *Clinostomum* spp. based on the cytochrome *c* oxidase subunit 1 gene dataset. Numbers above branches represent posterior probability values (values < 0.50 are not shown). *Clinostomum* species observed in this study are in bold

detailed morphological descriptions with sequences from both conserved (ribosomal) and fast evolving genes (mitochondrial).

At present, the importance of *C. album* n. sp. as a pathogen of amphibians or fish is unclear as the intermediate hosts involved in the life-cycle are

unknown. *Clinostomum marginatum* has been reported from farm-raised catfish in the southeastern United States, where infections may lead to unmarketable fish at processing. However, *C. marginatum* infections are rare and generally of little consequence to catfish aquaculture compared to other more damaging digeneans (Lorio, 1989; Wise et al., 2008; Griffin et al., 2012).

In catfish production ponds, marsh ramshorn snails serve as intermediate hosts for *C. marginatum*, and possibly other *Clinostomum* spp. (Hunter & Hunter, 1934; Lorio, 1989; Overstreet & Curran, 2004). In Brazil, planorbid snails in the genus *Biomphalaria* Preston serve as the first intermediate host for a *Clinostomum* sp., suggesting that other planorbid snails may be suitable hosts in the life-cycle of *Clinostomum* spp. (Pinto et al., 2015). In catfish production ponds in Mississippi, USA, there are at least two species of planorbid snail, namely *P. trivolvis* and *Biomphalaria havanensis* (L. Pfeiffer), that host digeneans infecting farmed catfish (Yost et al., 2009; Rosser et al., 2016a, b). The importance of *B. havanensis* as a first intermediate host in the life-cycle of *Clinostomum* spp. is currently unknown, but *B. havanensis* has been shown to host several genera of diplostomids including, *Austrodiplostomum ostrowskiae* Dronen, 2009, an uncharacterised *Austrodiplostomum* sp., *Bolbophorus damnificus* Overstreet, Curran, Pote, King, Blend & Grater 2002, *Drepanocephalus auritus* Kudlai, Kostadinova, Pulis & Tkach 2015, and an unidentified *Tylodelphys* sp. (Alberson et al. unpublished data; Rosser et al., 2016a, b).

Herein we report the clinostomid trematodes of great egrets collected from commercial catfish operations in the northeastern part of Mississippi, USA. *Clinostomum album* n. sp. represents the fourth named species of *Clinostomum* described in North America. Molecular sequencing data will allow further elucidation of life-cycle stages of *C. album* n. sp. as they are discovered. Additionally *C. marginatum* was observed and molecularly confirmed as a parasite of the great egret. The *Clinostomum* species of other piscivorous birds foraging from commercial catfish ponds and their effects on catfish production warrant further study.

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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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