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# MORPHOLOGIC AND MOLECULAR IDENTIFICATIONS OF DIGENETIC TREMATODES IN DOUBLE-CRESTED CORMORANTS (*PHALACROCORAX AURITUS*) FROM THE MISSISSIPPI DELTA, USA

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ABSTRACT: Increasing numbers of Double-crested Cormorants (*Phalacrocorax auritus*) in the Mississippi River Delta, USA, have been observed over the past few decades. This piscivorous bird is a definitive host for numerous digenetic trematodes, some of which may cause pathology in a fish host. We conducted a 2-yr survey of intestinal trematodes in 35 Double-crested Cormorants collected in the Mississippi Delta. We counted gastrointestinal trematodes, identified them to species using morphometric and molecular techniques, and sequenced the 18S and cytochrome oxidase I (COI) genes. We collected 4,909 trematodes, representing five digenetic species: *Drepanocephalus spathans*, *Hysteromorpha triloba*, *Pseudopsilostoma varium*, *Austrodiplostomum ostrowskiae*, and *Ascocotyle longa*. The most prevalent trematode of the Double-crested Cormorants was *D. spathans* (91%), followed by *H. triloba* (78%), *P. varium* (74%), *A. ostrowskiae* (57%), and *A. longa* (29%). Among these, the life cycles are only known for *H. triloba* and *A. longa*. Novel DNA sequences of the COI gene were obtained for *D. spathans*, *A. ostrowskiae*, *P. varium*, and *A. longa* adults. Using these DNA sequences, the identification and confirmation of the larval stages of these parasites in the fish and snail hosts will be possible.

*Key words:* Digenean, Double-crested Cormorant, *Phalacrocorax auritus*, trematode.

## INTRODUCTION

Channel catfish (*Ictalurus punctatus*) aquaculture is a major industry in Mississippi, USA. In 2009, sales of the Mississippi channel catfish industry were over $197 million, with approximately 78,300 water acres in production (USDA 2012). Ponds used in channel catfish aquaculture are ideal feeding and loafing grounds for a variety of piscivorous birds. The birds most often observed feeding on these ponds include the Double-crested Cormorants (*Phalacrocorax auritus*), American White Pelicans (*Pelecanus erythrorhynchos*), Great Egrets (*Ardea alba*), and Great Blue Herons (*Ardea herodias*), with the Double-crested Cormorants and Great Blue Herons being the most commonly reported species feeding on commercial catfish ponds (Glahn and King 2004).

The steady increase in the Double-crested Cormorant populations since the 1970s has been partially attributed to the year-round, readily available food source of commercial catfish these open ponds provide. Predation on these catfish has been linked to increases in survivability of juvenile birds (Duffy 1995). Additionally, premigratory cormorants that feed on catfish ponds are in better body condition than cormorants that reside in nonaquaculture areas (Glahn et al. 1999). In addition, some cormorant populations have altered their migratory patterns (King et al. 2010) and are remaining in the Mississippi Delta year-round. Small breeding colonies have also been established in this region (Reinhold et al. 1998).

Double-crested Cormorants also serve as definitive hosts for a variety of trema-

todes, many of which are pathogenic in fish. Currently, there is little information on the helminth populations in Double-crested Cormorants foraging on the ponds in the Mississippi Delta or the potential impact these parasites may have on local fish populations. Four surveys of parasites in Double-crested Cormorants have been reported in the US. A survey was completed on two Double-crested Cormorant populations in Florida; however, the majority of birds collected were *Phalacrocorax auritus floridanus*, a nonmigratory subspecies residing in Florida year-round (Threlfall 1982). Another survey quantified the parasite load of 12 Double-crested Cormorants in Texas, an area that is included in the wintering grounds of the interior population of Double-crested Cormorants (Fedynich et al. 1997). Parasites were also collected from two Double-crested Cormorants around catfish ponds in North Carolina (Flowers et al. 2004). In 2009, a survey of *Austrodiplostomum ostrowskiae* prevalence in Double-crested Cormorants and Neotropical Cormorants (*Phalacrocorax brazilianus*) was performed in Texas (Dronen 2009).

The increasing numbers of Double-crested Cormorants in the Mississippi Delta, potentially infected with high numbers of several digenetic trematode species, could have a profound impact on the fish populations in this region. Through life-history studies or annotated lists of multihost species surveys, other authors (Chandler and Rausch 1948; Hugghins 1956; Hutton and Sogandares-Bernal 1960; Hutton 1964; Forrester and Spalding 2003) have reported various digeneans from *P. auritus* in the US. To address this potential impact, the digenetic trematode population and the level of parasite infections in this bird population need to be determined. We conducted a 2-yr survey of adult trematodes in the gastrointestinal tracts of Double-crested Cormorants collected in the Mississippi Delta. DNA sequencing of the trematodes collected from the Double-crested Cormorant could aid in the further identification of the larval stages of these parasites.

## METHODS

### Bird collection and necropsy

We collected 35 adult Double-crested Cormorants, January–May 2003 and January–March 2004, in the Mississippi Delta region. Five birds were collected each month during these periods when possible. Birds were killed and transported on ice to the parasitology laboratory at the College of Veterinary Medicine at Mississippi State University for necropsy on day of collection.

The gastrointestinal tract from esophagus to cloaca was removed from each cormorant. The stomach and intestines were separated and processed individually. Stomach and intestines were opened longitudinally, and contents were rinsed into a brass sieve with an aperture of 75 μm using reverse osmosis water. The mucosal surfaces of the stomachs and intestines were carefully scraped to ensure all trematodes were dislodged and collected.

Stomach contents were preserved in 10% formalin for later parasite collection and identification. At each collection, the intestinal contents of three of the five birds were immediately examined microscopically, and all live parasites were collected. Representatives of each parasite species were placed in 70% molecular-grade ethanol for future DNA analysis or staining for morphologic analysis. Intestinal contents of the remaining two birds were preserved in 10% formalin for parasite collection and identification. Preserved intestinal and stomach samples were washed through a #200 stainless-steel screen (aperture = 75 μm) to remove all formalin, and the wash was examined using a dissecting microscope (Olympus SZ60, Olympus Imaging America, Inc., Center Valley, Pennsylvania, USA) at 4× magnification. All parasites were removed and placed in 70% molecular-grade ethanol for identification and enumeration.

### Parasite identification

For morphologic identification, the trematodes were stained with acetocarmine (Fisher Scientific, Pittsburgh, Pennsylvania, USA). Specimens were placed in the stain for 8–10 hr and destained with 1% acid alcohol until organs became visible. The specimens were

progressed through a series of dehydrating ethanol washes (70%, 95%, and 100% ethanol). Each dehydration step lasted approximately 1 hr. The trematodes were cleared with Citri-solve (Omega Laboratories, Inc., Houston, Texas, USA) and mounted on slides using Permount (ProSciTech, Thuringowa Central, Queensland, Australia). Stained specimens were identified using taxonomic keys (Yamaguti 1958; Schell 1985; Gibson et al. 2002, 2005, 2008). The molecular identification of the digenetic trematodes collected was based on DNA sequencing of both the nuclear ribosomal 18S gene and the mitochondrial cytochrome oxidase I (COI) gene. Prior to extraction, specimens were rinsed in nuclease-free water. DNA was extracted from representative specimens previously preserved in 70% molecular-grade ethanol using the Dneasy Blood and Tissue Kit (Qiagen, Valencia, California, USA) according to the manufacturer's protocol. The purified genomic DNA was suspended in 400 μL of AE elution buffer solution (10 mM Tris-Cl, 0.5 mM ethylenediaminetetraacetic acid [EDTA]) (Qiagen).

To ensure adequate coverage of the 18S gene, single PCR reactions were performed using the following three sets of primers (Littlewood and Olson 2001): Worm A and Worm B, 18S-7 and 18S-A27 and 600F and 18S-11. The total PCR volume was 25 μL, which contained 10× buffer, 2.0 μL of template, 0.625 U of *Taq* polymerase (Hot Start Taq; Takara Bio, Inc., Shiga, Japan), 200 nM of each primer, and 200 μM of each deoxynucleotide triphosphate. The PCR conditions were 94 C for 5 min followed by 40 cycles of 94 C for 30 sec, 54 C for 30 sec, 72 C for 2 min, with a final cycle of 72 C for 5 min. PCR reactions were performed using a PTC-100 Peltier Thermal Cycler (MJ Research, Watertown, Massachusetts, USA).

Amplification and sequencing of the COI gene were performed using the COI specific primers and PCR protocols of Moszczynska et al. (2009). All genomics were screened using two sets of primers; Plat-diploCOX1F and Plat-diploCOX1R and MplatCOX1dF and MplatCOX1dR. The Plat-diploCOX1F and Plat-diploCOX1R are specific for the family Diplostomatidae, while the MplatCOX1dF and MplatCOX1dR are generic platyhelminthes primers. In most cases, each genomic sample was amplified using only one of the two primer sets, depending on the species. The total PCR volume was 25 μL, which contained 1× buffer, 2.5 mM of $MgCl_2$, 2.0 μL of template, 0.625 U of Platinum *Taq* Polymerase (Invitrogen, Carlsbad, California, USA), 200 nM of each primer, and 200 μM of each deoxynucleotide triphosphate. The PCR conditions were 94 C for 2 min and 35 cycles of 94 C for 30 sec, 50 C for 30 sec, 72 C for 1 min, with a final extension of 72 C for 10 min.

All PCR products were visualized with Gelstar nucleic acid stain (Cambrex BioScience Rockland, Inc., Rockland, Maine, USA) on a 1.2% agarose gel. PCR products were purified using a Montage PCR Centrifugal Filter device (Millipore, Billerica, Maryland, USA) prior to sequencing at Arizona State University DNA Laboratory (Tempe, Arizona, USA). Sequences were assembled using the SeqMan of the Lasergene version 8.0 software package (DNASTAR, Madison, Wisconsin, USA) and were edited manually. Results underwent a database search using Basic Local Alignment Search Tool (Altschul et al. 1990).

Prevalence and mean infection intensity were calculated as described by Margolis et al. (1982) and Bush et al. (1997). Prevalence is the number of individuals infected with a particular parasite species divided by the number of hosts examined. Mean infection intensity is the total number of individuals of a particular parasite species divided by the number of infected hosts (Bush et al. 1997).

## RESULTS

From all birds, we collected 4,909 trematodes, which were distributed among five digenetic species: *Ascocotyle longa*, *Austrodiplostomum ostrowskiae*, *Drepanocephalus spathans*, *Hysteromorpha triloba*, and *Pseudopsilostoma varium*. All birds had trematodes, and the number of trematode species per bird ranged from one to five. Two Double-crested Cormorants (6%) were hosts to a single trematode species. Five birds (14%) were infected with two species of trematodes; 11 birds (31%) were infected with three species; 15 birds (43%) were infected with four species; two birds (6%) were infected with all five species of trematodes. Prevalences and infection intensities by trematode species are provided in Table 1.

TABLE 1. Gastrointestinal digenetic trematodes found in 35 Double-crested Cormorants (*Phalacrocorax auritus*) from the Mississippi River Delta, Mississippi, USA, 2003–04.

| Trematode species | Prevalence (%) | Mean infection intensity, $\bar{X}$±SE | Range | Site of infection[a] | Percentage in stomach |
|---|---|---|---|---|---|
| *Ascocotyle longa* | 29 | 14.8±6.0 | 1–61 | S, I | 10.1 |
| *Austrodiplostomum ostrowskiae* | 57 | 11.3±3.0 | 1–44 | I | 0 |
| *Drepanocephalus spathans* | 91 | 81.5±18.1 | 1–346 | S, I | 8.9 |
| *Hysteromorpha triloba* | 78 | 32.5±9.4 | 1–184 | S, I | 20.4 |
| *Pseudopsilostoma varium* | 74 | 40.5±18.0 | 1–466 | S | 100 |

[a] S = stomach; I = intestine.

The collected specimens of *Drepanocephalus spathans* concurred with the morphologic descriptions by Dietz (1909) and Kostandinova et al. (2002). DNA sequencing of this parasite supported the morphologic identification. Resultant sequences of the 18S gene were a 99% match to the published *D. spathans* sequence in GenBank (GenBank accession AY245762). The COI gene sequence of *D. spathans* in this study had no close matches to any published sequences. A partial COI gene sequence of *D. spathans* has been published in GenBank (Griffin et al. 2012). We attribute the lack of homology between the COI gene sequences of *D. spathans* in this study and that of Griffin et al. (2012) to the use of different primer sets, which amplified different portions of the gene.

The collected specimens of *Hysteromorpha triloba* matched the morphologic descriptions of Rudolphi (1819) and Lutz (1931). Our sequences from *H. triloba* were a 100% match for both the 18S and COI genes of the *H. triloba* sequences published in GenBank (Locke et al. 2010, 2011).

Our specimens of *Pseudopsilostoma varium* matched the morphologic descriptions of Linton (1928). There are no previous sequencing data on this parasite published in GenBank. The 18S gene sequence of *P. varium* collected in this study (GenBank accession JX468068) was a 97% match to *Fascioloides magna*, *Petasiger phalacrocoracis*, *Fasciola gigantica*, *Euparyphium melis*, and *Isthmiophora hortensis*. The sequence of the COI gene for *P. varium* in this study (GenBank accession JX468064) had no close matches to any digenean sequence currently published in GenBank.

The specimens of *Austrodiplostomum ostrowskiae* collected in this survey matched the morphologic description of Dronen (2009). Our sequence of the 18S gene was a 100% match to *Diplostomum spathaceum* (GenBank accession AY245761). However, our COI gene sequence (GenBank accession JX468066) was only an 88% match to *Diplostomum* sp. (Locke et al. 2010). The COI sequence for *A. ostrowskiae* is not published in GenBank, so no comparisons could be made with our sequence.

Our morphologic identifications of *Ascocotyle longa* matched those of Ransom (1920). Our 18S gene sequence was a 99% match to the published sequence of *Phagicola longa* (Dzikowski et al. 2004). The sequence of the COI gene for this parasite is not published in GenBank, and therefore no comparisons were made.

## DISCUSSION

We found five adult gastrointestinal trematode species in this population of Double-crested Cormorants. This number of trematode species is similar to those found in three previous surveys (Table 2). Fedynich et al. (1997) described six species of intestinal trematodes in Double-crested Cormorants wintering in Texas, including *Ascocotyle* sp., *Austrodiplostomum mordax*, *D. spathans*, *Maritrema*

TABLE 2. Comparison among four studies of prevalence and mean infection intensity of digeneans in Double-crested Cormorants, *Phalacrocorax auritus*.[a]

| Trematode species | Current study | Threlfall (1982) | Fedynich et al. (1997) | Dronen (2009) |
|---|---|---|---|---|
| *Ascocotyle longa* | | | | |
| Prevalence (%) | 29 | 38–56 | NR[a] | NR |
| Mean infection intensity | 14.8 | 54–106 | | |
| *Austrodiplostomum ostrowskiae* | | | | |
| Prevalence (%) | 57 | NR | 33 | 100 |
| Mean infection intensity | 11.3 | | 4.2 | 135 |
| *Drepanocephalus spathans* | | | | |
| Prevalence (%) | 91 | 9.20 | 1/12[b] | NR |
| Mean infection intensity | 81.5 | 263 | | |
| *Hysteromorpha triloba* | | | | |
| Prevalence (%) | 78 | 17 | NR | NR |
| Mean infection intensity | 32.5 | 4,169 | | |
| *Pseudopsilostoma varium* | | | | |
| Prevalence (%) | 74 | NR | 35[c] | NR |
| Mean infection intensity | 40.5 | | 27.7 | |

[a] NR = not reported.

[b] Reported as published.

[c] Neotropic Cormorants (*Phalacrocorax brazilianus*).

sp., *Mesoophorodiplostomum pricei*, and *Phocitremoides butionis*. However, Dronen (2009) concluded that the trematodes identified as *A. mordax* in the Texas survey were *A. ostrowskiae*. Threlfall (1982) described four species in Double-crested Cormorants that reside in Florida year-round, including *D. spathans*, *A. longa*, *Mesostephanus appendiculatoides*, and *H. triloba.* Only three digenetic trematodes were described from Double-crested Cormorants residing in North Carolina (*D. spathans*, *Austrodiplostomum compactum*, and *H. triloba*; Flowers et al. 2004); however, only two cormorants were sampled, and no quantitative data were reported.

The sequencing results of *A. ostrowskiae* demonstrated that sequencing the 18S gene alone could be misleading or result in an inaccurate identification. Although the 18S gene sequence for *A. ostrowskiae* was a 100% match to that of *D. spathaceum*, the differences in both the morphology of these trematodes and the COI gene sequences indicate that they are different species. Adult *D. spathaceum* are found in the intestines of gulls (Whyte et al. 1987) and are morphologically distinct from *A. ostrowskiae* (Niewiadomska 1984). The COI gene sequence for *D. spathaceum* is not published on GenBank and cannot be compared to the COI gene sequence of *A. ostrowskiae* found in the Mississippi Delta population of Double-crested Cormorants. However, the COI gene sequence of *A. ostrowskiae* is only an 88% match to *Diplostomum* sp. (Locke et al. 2010), which provides further evidence that the 18S gene is too highly conserved to provide adequate data for molecular identification at the species level.

*Drepanocephalus spathans* was both highly prevalent and abundant in the Double-crested Cormorants in the Mississippi Delta. *Drepanocephalus spathans* has recently been reported to infect channel catfish in both natural and artificial infections; however, the pathol-

ogy and impact of this digenean on channel catfish aquaculture have yet to be elucidated (Griffin et al. 2012).

*Pseudopsilostoma varium* was found only in the stomachs of the Mississippi Delta Double-crested Cormorants. None of the previous surveys reported this trematode in any of the Double-crested Cormorants sampled. Little is known about this digenetic trematode, and the only quantitative data for it are from a population of Neotropic Cormorants, *Phalacrocorax brazilianus* residing in Texas (Fedynich et al. 1997). Little information exists on the life cycle of *P. varium*. The prevalence and abundance of this parasite in the Mississippi Delta population of Double-crested Cormorants strongly suggest that the second intermediate host is a fish species; however, no studies have elucidated the life cycle of this parasite. The impact of this trematode on fish populations in the Mississippi Delta is unknown.

Previous surveys of Double-crested Cormorants reported that *D. spathans*, *H. triloba*, and *A. longa* have only been found in the intestines of the birds. However, we found that a small number of these parasites may be present in the stomachs (Table 1). Although transported on ice, it was possible that the 3 hr that lapsed between euthanasia and necropsy could have resulted in the parasites' migration to aberrant sites.

We are the first to document the prevalence and intensities of trematodes in the Double-crested Cormorants wintering in the Mississippi Delta. The predation impact of this piscivorous bird on the commercial catfish industry in this region has been well documented; however, the parasites in this bird population and the impact of these parasitisms are unknown. This 2-yr survey demonstrated that adult Double-crested Cormorants in this region are hosts to high numbers of five species of trematodes, causing no demonstrated pathology. The impact such a high level of parasitism may have on juvenile cormorants is unknown. Many of these digeneans, however, are capable of infecting a wide variety of freshwater fish species, often causing pathology. The variation seen in helminth profiles of this population of cormorants compared to the Double-crested Cormorants surveyed in Texas and Florida may have been due to differences in habitat and diet. Most of the Mississippi Delta population of Double-crested Cormorants does not migrate further south than this region; subsequently, brackish water and saltwater fish and crustaceans are not usually components of their diet. Thus, these birds would not have the opportunity to ingest the metacercariae of trematodes that utilize these potential intermediate hosts.

We obtained novel sequences of the COI gene for several digenetic trematodes that have been reported in the Double-crested Cormorant. The COI sequences for *D. spathans*, *A. ostrowskiae*, *P. varium*, and *A. longa* are new additions to the GenBank database. The addition of these sequences to the molecular data available on digenetic trematodes will provide information that can be used in the further understanding of the life histories and the phylogenetic relationships of these parasites.

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