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# GENETIC AND MORPHOLOGIC CHARACTERIZATION OF *DIASCHISTORCHIS PANDUS* (DIGENEA: PRONOCEPHALIDAE) TREMATODES EXTRACTED FROM HAWKSBILL TURTLES, *ERETMOCHELYS IMBRICATA* (TESTUDINES: CHELONIIDAE), IN GRENADA, WEST INDIES

**Daniel M. Fitzpatrick[1], Monica A. Tetnowski[1], Thomas G. Rosser[2], Rhonda D. Pinckney[1], David P. Marancik[1], and Brian P. Butler[1]**

[1] Department of Pathobiology, School of Veterinary Medicine, St. George's University, P.O. Box 7, True Blue Campus, True Blue, St. George Parish, Grenada, West Indies.

[2] Department of Comparative Biomedical Sciences, College of Veterinary Medicine, Mississippi State University, P.O. Box 6100, Mississippi State, Mississippi 39762.

Correspondence should be sent to Daniel M. Fitzpatrick (http://orcid.org/0000-0002-1411-4178) at: *dfitzpat@sgu.edu*

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Pronocephalidae
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Turtles
*Eretmochelys imbricata*
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West Indies

**ABSTRACT**

The hawksbill turtle *Eretmochelys imbricata* is a critically endangered species with a worldwide distribution. Limited information is available about the naturally occurring intestinal parasites of this species and what impact these parasites may have on the health of the hawksbill turtle. *Diaschistorchis pandus* was identified postmortem in 5 hawksbill turtles from Grenada, West Indies, using morphologic characterization. Sanger sequencing was performed for conserved ribosomal regions (*5.8S*, *ITS2*, *28S*) and the mitochondrial cytochrome *c* oxidase subunit 1 gene (*COI*). Phylogenetic analysis of the *28S* rRNA gene sequence data shows *D. pandus* clustering with other trematodes in the family Pronocephalidae, corroborating morphological classification. No genetic sequences have been previously reported for this trematode species, which has limited the collection of objective epidemiological data about this parasite of marine turtles.

Grenada, a tri-island country in the West Indies, supports nesting and foraging populations of hawksbill turtles *Eretmochelys imbricata*. This species is listed as critically endangered by the International Union for Conservation of Nature's Red List of threatened species (Mortimer and Donnelly, 2008). Numerous factors have contributed to their global decline including legal and illegal hunting, commercial fishing by-catch, coastal development, pollution, and pathogens (Wallace et al., 2011). Of the last, trematodes are a common parasite of sea turtles, including hawksbill turtles, which may pose a health threat to this critically endangered species.

Trematodes are the most prevalent and diverse parasite of sea turtles (Greiner, 2013). Multiple families of trematodes have been documented in hawksbill turtles with varying levels of pathogenicity. Digenean trematodes belonging to the family *Spirorchiidae* are best known for their migration into major vessels and potential to cause vasculopathy, aneurysm, and granulomatous inflammation (Werneck et al., 2015b; Chapman et al., 2019; Jerdy et al., 2020). However, the majority of known parasites of hawksbill turtles reside within the gastrointestinal tract (Dyer et al., 1995; Greiner, 2013; Santoro et al., 2015; Werneck et al., 2015a). These include trematodes from the families Pronocephiladae, Plagiorchiidae, Brachycoeliidae, Styphlotrematidae, Calycodidae, Rhytidodidae, and Telorchiidae (Glazebrook and Campbell 1990; Dyer et al., 1995; Greiner, 2013; Werneck et al., 2015a).

Based on limited postmortem studies of wild sea turtle populations, it is suspected that mild infection by gastrointestinal parasites has minimal clinical significance on healthy turtles. However, it is presumed that more severe health impacts may occur with higher intensities or host debilitation or with more pathogenic parasitic species. In hawksbill turtles, gastrointestinal trematodes within the family Pronocephalidae have been associated with disease; *Diaschistorchis* sp. was observed attached to the gastrointestinal mucosa of a hawksbill turtle with petechial hemorrhages on its gastric wall (Glazebrook and Campbell, 1990). Similarly, Santoro et al. (2015) reported a wild-caught hawksbill turtle with nodular lesions on the serous membrane of the intestines as well as granulomatous lesions containing spirorchiid eggs on the host's mucosal, submucosal, and muscular layers of stomach and intestine; disease was attributed to the trematode *Carettacola stunkardi*.

In other species, namely, green turtles *Chelonia mydas*, trematodes (including their eggs) are associated with more severe

lesions including ulcerative esophagitis and gastroesophagitis due to *Rameshwarotrema uterocrescens* (Santoro et al., 2007a, 2007b; Ribeiro et al., 2017; Jerdy et al., 2019), hemorrhagic and ulcerative gastritis associated with 2 *Charaxicephaloides* spp. trematodes (Santoro et al., 2007a), and multiorgan inflammatory response due to spirorchiids eggs and cholecystitis associated with *Carettacola stunkardi* trematodes (Werneck et al., 2019). Werneck et al. (2018) also reported cystitis associated with *Plesiochorus cymbiformis* trematodes in a loggerhead turtle *Caretta caretta*. In many of these instances, factors associated with disease pathogenesis in wild marine turtles are difficult to establish due to a lack of perimortem health indicators of the host and incomplete morphological and/or molecular identification of the parasite species.

The diversity of trematodes found in the gastrointestinal tract of sea turtles can complicate accurate identification in the absence of thorough morphological characterization. Historically, diagnosis of trematodes has been based on morphology, although this can be difficult due to postmortem autolysis and variability in specimen preparation before microscopic evaluation (Ulmer, 1952; Dyer et al., 1995). Molecular methods, such as multi-locus sequence typing, offer more sensitive methods of species identification and can improve the recognition and classification of important trematode species and epidemiologic data. In addition to disease diagnostics, quantification of parasite prevalence and burden is an effective tool where sentinel animals are used as indicator species for ecosystem health, underlying the need for accurate identification of parasites in marine turtles (Sures et al., 2017).

The goal of this study was to provide comprehensive morphologic and genomic sequence data for a commonly observed trematode parasite from hawksbill turtles in Grenada, West Indies. This report describes trematodes identified as *Diaschistorchis pandus* found in 5 necropsied hawksbill turtles and associated gross and histomorphologic observations. These results expand our shared data for identifying trematodes in sea turtles and shed light on parasite distribution and pathogenic potential of trematodes in hawksbill turtles in the southern Caribbean Sea.

## MATERIALS AND METHODS

### Postmortem examination

Five adult male hawksbill turtles were found dead and confiscated by government officials after being captured in an illegally placed net off the shore of Grenada in May 2014. The turtles were submitted to the necropsy service at St. George's University, School of Veterinary Medicine, Grenada, West Indies, for post-mortem examination. The carcasses were frozen in the interval between their collection and post-mortem exam to prevent autolysis and putrefaction before carcass relocation and examination. The post-mortem examination followed the guidelines set forth by the sea turtle stranding and salvage network gross necropsy report (Norton and Mettee, 2020). All examined turtles were identified as male based on external anatomic characteristics and observation of mature testes internally. Mean curved carapace length from notch to tip (CCL-nt) was 81.9 cm ± 1.9 standard deviation (SD), and mean curved carapace width (CCW) was 71.9 ± 1.9 SD. Carapace measurements trend toward those described for mature hawksbill turtles (van Dam and Diez, 1998; Meylan et al., 2011). Although growth curves have not been described for hawksbill turtles in Grenada, the interpretation that these were mature animals is consistent with the mature testes observed grossly.

Upon post-mortem examination, all 5 turtles contained low to moderate numbers (n = 10–50) of adult flukes within the duodenum and/or proximal small intestine. The parasites were readily visible upon examination of the intestinal contents and were subsequently collected using a sieve, laboratory forceps, and spatulas. The flukes were preserved in 70% ethanol for later morphological and molecular characterization. A full set of tissues from each turtle was fixed by immersion in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm, stained with hematoxylin and eosin (HE) using standard histologic techniques, and examined by light microscopy by 2 veterinary pathologists.

### Morphologic characterization of trematodes

Ethanol preserved flukes were stained using Semichon's acetocarmine or Van Cleave's hematoxylin, dehydrated in an increasing ethanol series, and mounted permanently in Canada balsam (Pritchard and Kruse, 1982). The family and genus were identified using taxonomic keys (Blair, 2005), and primary literature was used to determine species identity (Braun, 1901; Johnston, 1913; Rohde, 1962; Mehrotra and Gupta, 1976). Mounted specimens were examined using an Olympus BX-53 microscope (Olympus Optical Co. Ltd., Tokyo, Japan) with differential interference contrast optics and an Olympus DP74 digital camera (Olympus). Measurements were taken using the accompanying CellSens software (Olympus) and are reported in micrometers.

### Genetic sequencing of trematode DNA

Whole genomic DNA was extracted from 2 single adult flukes using the Qiagen DNeasy Blood and Tissue (Qiagen, Hilden, Germany) extraction kit per the manufacturer's instructions. Polymerase chain reaction (PCR) was used to amplify regions of the extracted DNA. Regions targeted for amplification were the *5.8S* rRNA gene, internal transcribed spacer region 2 (*ITS2*), partial *28S* rRNA gene, and partial mitochondrial cytochrome *c* oxidase subunit 1 (*COI*) gene. The specific primers used for each region are listed in Table I. PCR reactions for 3S/A28,300F/1200R, and 900F/1500R primer sets were 20 μl in total volume, using 10 pmol of each primer, and 1.5 mM $MgSO_4$. PCR conditions were as follows: 94 C for 2 min; 40 cycles of 94 C for 30 sec, 50 C for 30 sec, 72 C for 2 min; and a final extension of 72 C for 7 min. The PCR reaction for Modified Dice 1F/11R primer set was 10 μl in total volume, using 10 pmol of each primer, and 3.5 mM $MgSO_4$. PCR conditions were as follows: 95 C for 2 min; 43 cycles of 95 C for 30 sec, 48 C for 30 sec, 72 C for 30 sec; and a final extension of 72 C for 7 min. PCR products were separated and visualized using gel electrophoresis. Amplicons of sizes consistent with the primers used were excised, and DNA was extracted using the Qiagen Gel Extraction kit (Qiagen). These were sequenced bidirectionally with the primers used to generate the amplicon sequence. Chromatograms were assembled into contiguous sequences, and ambiguous bases were edited manually using version 2.6.6 of the Chromas Lite™

**Table I.** Primers used in this study.

| Primer | Sequence (5′-3′) | Target gene | Reference |
|---|---|---|---|
| 3S | GGTACCGGTGGATCACTCGGCTCGTG | *5.8S* and *ITS2 rRNA* | Bowles et al. (1995) |
| A28 | GGGATCCTGGTTAGTTTCTTTTCCTCCGC | *5.8S* and *ITS2 rRNA* | Blair et al. (1997) |
| 900F | CCGTCTTGAAACACGGACCAAG | *28S rRNA* | Lockyer et al. (2003) |
| 1500R | GCTATCCTGAGGGAAACTTCG | *28S rRNA* | Olson et al. (2003) |
| 300F | CAAGTACCGTGAGGGAAAGTTG | *28S rRNA* | Lockyer et al. (2003) |
| 1200R | GCATAGTTCACCATCTTTCGG | *28S rRNA* | Lockyer et al. (2003) |
| Modified Dice 1F | TTWCNTTRGATCATAAG | *COI* | Van Steenkiste et al. (2015) |
| Modified Dice 11R | GCWGWACHAAATTTHCGATC | *COI* | Van Steenkiste et al. (2015) |

software (http://technelysium.com.au/wp/chromas/). Overlapping portions of the consensus sequences produced by 300F/1200R and 900F/1500R were 100% identical and were thus concatenated to produce a combined sequence. Sequences were compared to other trematode sequences publicly accessible in the National Center for Biotechnology Information genetics database by using the Nucleotide Basic Local Alignment Search Tool (BLASTn). Unambiguous sequence data for all 3 gene regions were available for only 1 trematode; these sequences were submitted to the GenBank sequence database. Some *28S* rRNA sequence data were available for 2 trematode specimens, and pairwise comparison of the unambiguous portions of these sequence data demonstrated 100% identity between specimens.

### Phylogenetic analysis

Sequences of the *28S* rRNA gene region from trematodes within the superfamily Pronocephaloidea were downloaded and aligned using the MAFFT plugin (MAFFT v. 1.4.0) in Geneious Prime® 2019.2.3 (https://www.geneious.com/). Positions containing any ambiguities or gaps in the alignment were masked for a final alignment with 751 positions. The Bayesian Information Criterion in MEGA7 was used to determine the DNA substitution model that best fit the data as the General Time Reversible (GTR + G) model with gamma-distributed sites. Bayesian inference and maximum likelihood analyses were used to construct phylogenetic trees to infer the evolutionary history of the trematodes in this study based on the *28S* rRNA alignment. MrBayes 3.2.6 was used for Bayesian inference analysis by Markov chain Monte Carlo searches of 2 simultaneous runs of 4 chains with sampling occurring every 100th tree for 1,000,000 generations or when convergence was reached with a deviation of split frequency value $< 0.01$ (Ronquist et al., 2012). Maximum likelihood analysis was carried out using IQ-Tree on the IQ-Tree webserver (Nguyen et al., 2015; Trifinopoulos et al., 2016). Topology support was assessed using ultrabootstrap support based on 1,000 pseudoreplicates (Minh et al., 2013). Trees were viewed in FigTree 1.4.2 (Rambaut, 2012) and edited for publication in Adobe Illustrator 2020.

## RESULTS

Parasite specimens were dead upon collection due to the postmortem interval of the host, as well as freezing and thawing of tissues. Subsequently, the most highly detailed morphological features of trematode and host tissues were at least minimally obscured or lost.

### Postmortem examination: Gross and histopathologic observations

All 5 turtle carcasses had moderate autolysis with an estimated post-mortem interval of 24–48 hr followed by placement into a freezer. Upon gross examination, all turtles were in good body condition with adequate stores of adipose tissue and a minimal accumulation of epibiota. One of the 5 turtles had a linear regionally extensive area of subcutaneous hemorrhage and edema on the lateral aspect of the neck that was interpreted as traumatic injury from the fishing net used during illegal capture. Another turtle was noted to have a previously healed amputated rear flipper which was interpreted as a likely predation injury or other trauma such as a boat strike. No further evidence of external trauma or overt disease was grossly observed. Within each turtle, a variable number of total parasites ranging from an estimate of 10–50 adult trematodes was observed within the duodenum and proximal small intestine. Adult trematodes were morphologically consistent with the descriptions of *Diaschistorchis pandus* (Braun, 1901; Johnson, 1913; Fischthal and Acholonu, 1976; Mehrotra and Gupta, 1976) for all trematodes found. No observable gross lesions of the intestinal mucosa were associated with the presence of the luminal intestinal flukes. No other trematodes or intestinal parasites were found.

Histopathological changes in the gastrointestinal tract indicated that there was no severe injury to the intestinal mucosa associated with the presence of the luminal trematodes. However, the degree of autolysis within the examined intestinal tissues precluded a detailed evaluation of the superficial mucosal epithelium. No additional findings were observed microscopically to indicate significant disease in other organ systems that may be attributed to the presence of the intestinal flukes.

The presence of mild vascular spirorchidiasis was confirmed microscopically predominantly in the intestines, with lesser involvement of other abdominal viscera and lungs, and these infections were associated with low numbers of parasite eggs surrounded by small foci of granulomatous inflammation. The eggs were irregularly round to oval in shape, often collapsed on multiple sides, and ranged in size from approximately 100–250 μm in greatest diameter. Additional morphologic features of the eggs within tissue sections were not discernable. This finding was not interpreted as a debilitating or life-threatening cardiovascular fluke infection since the lesions were mild and all turtles were found in good body condition. A previous study focused on

spirorchids in Mediterranean loggerhead turtles described a fatal case of spirorchidiasis resulting in cachexia and severe tissue damage associated with parasite egg emboli within the pancreas, intestine, and brain (Santoro et al., 2017). The lesions observed in our present study were very mild and the turtles were not cachexic.

## Morphologic trematode identification and description: Taxonomic summary

### Trematoda: Pronocephalidae: *Diaschistorchis pandus* (Braun, 1901) Johnston, 1913

*Host:* Hawksbill turtle (*Eretmochelys imbricata*).

*Locality:* GPS coordinates unknown, caught in a fishing net in waters near Grenada, West Indies.

*Materials deposited:* Two vouchers are deposited in the Harold W. Manter Laboratory of Parasitology, University of Nebraska State Museum, Lincoln, Nebraska (HWML 216389).

*Site of infection:* Duodenum and/or proximal small intestine.

*Intensity of infestation:* Approximately 10–50 trematodes/turtle (n = 5 turtles examined).

*Representative DNA sequences:* GenBank MN810921–MN810922 (ribosomal regions); MN810923 (*COI*) (derived from 1 specimen).

*Description based on 3 ovigerous adults, stained and mounted in Canada balsam; see Fig. 1. All measurements in microns:* Body linguiform, aspinose, dorsoventrally flattened and ventrally concave, widest at the level of the gonads and attenuating slightly anteriorly, 10,381–11,602 × 2,414–3,180 (10,814 × 2,751). Collar inconspicuous. Oral sucker, large, ovoid, subterminal. Esophagus, short, with inconspicuous esophageal bulb. Intestinal caeca branching in the anterior of the body and running laterally until almost reaching the posterior margin. Finer details of the intestinal caeca and their diverticula are difficult to discern. Testes (n = 14–17 in number; n = 46 measured), lobulate and arranged in a U-shape in the posterior third of the body with most lobules being lateral in position and ventral to the intestinal caeca, 126–242 × 239–272 (181 ± 28 × 199 ± 31). External seminal vesicle loosely winding anteriorly along the right margin of the worm. Cirrus sac, elongate, obliquely positioned, and located medially in the posterior margin of the anterior third of the body, 1,496–2,011 × 258–336 (1,738 × 292). Elongate cirrus, protruding from genital pore in all specimens. Ovary, lobulated, submedian, slightly dextral, and located in the anterior region of posterior third of the body, 292–303 × 233–312 (299 × 284). Mehlis' gland, distinct, sinistral to ovary, 206–328 × 251–338 (275 × 289). Uterus, intercaecal, reaching the posterior portion of the anterior third of the body. Two longitudinal rows of vitelline follicles ventral to intestinal ceca (poorly visible) that overlap the anterior testicular follicles, extending from the anterior testicular area to body mid-region. Genital pore, sinistral, often extracaecal. Eggs (n = 45 measured) operculate, ovoid, tapering anteriorly, without terminal projections, 40–44 × 21–24 (41 ± 1 × 23 ± 1).

### Remarks

Specimens collected in this study are in line with the morphological description of *D. pandus*, the type-species for the genus *Diaschistorchis* and documented in other marine chelonians and hawksbill turtles (Johnston, 1913; reviewed in Mehrotra and Gupta, 1976). Using the key to species of Mehrotra and Gupta (1976), specimens in this study are separated from congeners by the distribution of the vitelline follicles and their beginning in the body mid-region, well posterior to the genital pore.

*Phylogenetic analysis:* With limited sequence data available from members of the family Pronocephalidae, phylogenetic analysis of partial *28S* rRNA gene (accession number MN810922) was performed. Results with the highest degrees of similarity were those within the superfamily Pronocephaloidea (Fig. 2).

**Table II.** Review of published information on *Diaschistorchis pandus* trematode hosts and locations.*

| Turtle host | Location | Reference(s) |
|---|---|---|
| *Caretta caretta* | Egypt | Sey (1977) |
| | Florida, USA | Pratt (1914); Greiner (2013) |
| | Italy | Braun (1901) |
| *Chelonia mydas* | Australia | Johnston (1913) |
| | Brazil | Werneck and da Silva (2015) |
| | Florida, USA | Greiner (2013) |
| | Italy | Mehra (1932) |
| *Eretmochelys imbricata* | Australia | Johnston (1913) |
| | Bermuda | Barker (1922) |
| | Brazil | Werneck (2015a) |
| | Cuba | Pérez-Vigueras (1935, 1955) |
| | Florida, USA | Greiner (2013) |
| | Grenada | Present study |
| | India | Chattopadhyaya (1972) |
| | Puerto Rico, USA | Fischthal and Acholonu (1976); Dyer et al. (1995) |
| Unspecified | Japan | Hughes et al. (1942); Meira Filho et al. (2017) |

* Mehra (1932) mentions a previous occurrence of *D. pandus* infesting *C. mydas* but does not provide a citation. Hughes et al. (1942) and Meira Filho et al. (2017) cite previous reports of *D. pandus* infestations in the above-mentioned hosts but do not specify which species.

## DISCUSSION

Currently, the genus *Diaschistorchis* contains 5 nominal species described from freshwater and marine chelonians. The emended generic diagnosis and key to species provided by Mehrotra and Gupta (1976) remains the more current and thorough efforts on accessing species diversity within the genus. Adult trematodes collected from the small intestine of the examined hawksbill turtles were morphologically consistent with descriptions of *Diaschistorchis pandus* from the loggerhead turtles *Caretta caretta* (Braun, 1901), hawksbill and green sea turtles *Chelonia mydas* in Australia (Johnson, 1913), and hawksbill sea turtles from India (Mehrotra and Gupta, 1976) and the Atlantic Ocean (Fischthal and Acholonu, 1976), suggesting relatively low host specificity and likely worldwide distribution (Table II).

Based on this study, *D. pandus* appears to be a relatively common inhabitant of hawksbill turtles within the coastal waters of Grenada. Therefore, it may be pertinent to further explore prevalence and parasite loads between various species of marine turtles within the greater Caribbean region. Further epidemiologic characterization may help assess the potential health impact of intestinal flukes on these at-risk sea turtle populations.


**Figure 1.** Photomicrograph of stained *Diaschistoris pandus* collected from *Eretmochelys imbricata* from Grenada, West Indies. Color version available online.

Comprehensive morphologic and genomic characterization of these and other observed gastrointestinal parasites provide a baseline to more reliably identify this type of data.

Currently, the life cycle of *D. pandus* is unknown. Related trematodes within the family Pronocephalidae are suspected to infect their definitive hosts through ingestion of metacercariae encysted on plants (Pérez-Ponce de León et al., 1996). The diet of hawksbill turtles is thought to primarily consist of sponges and other invertebrates, though algae and grasses are occasionally reported within the stomach contents during postmortem examination (Meylan, 1988). Molecular surveillance for *D. pandus* DNA may contribute to future investigation into possible intermediate hosts and parasite life stages of this species.

The absence of gross and histopathologic gastrointestinal changes associated with the trematode infection was not unexpected. The healthy status of the turtles before the anthropogenic cause of death suggests there was adequate host tolerance to the relatively low intensity of infection. The effect the parasite may have on more debilitated and chronically sick turtles

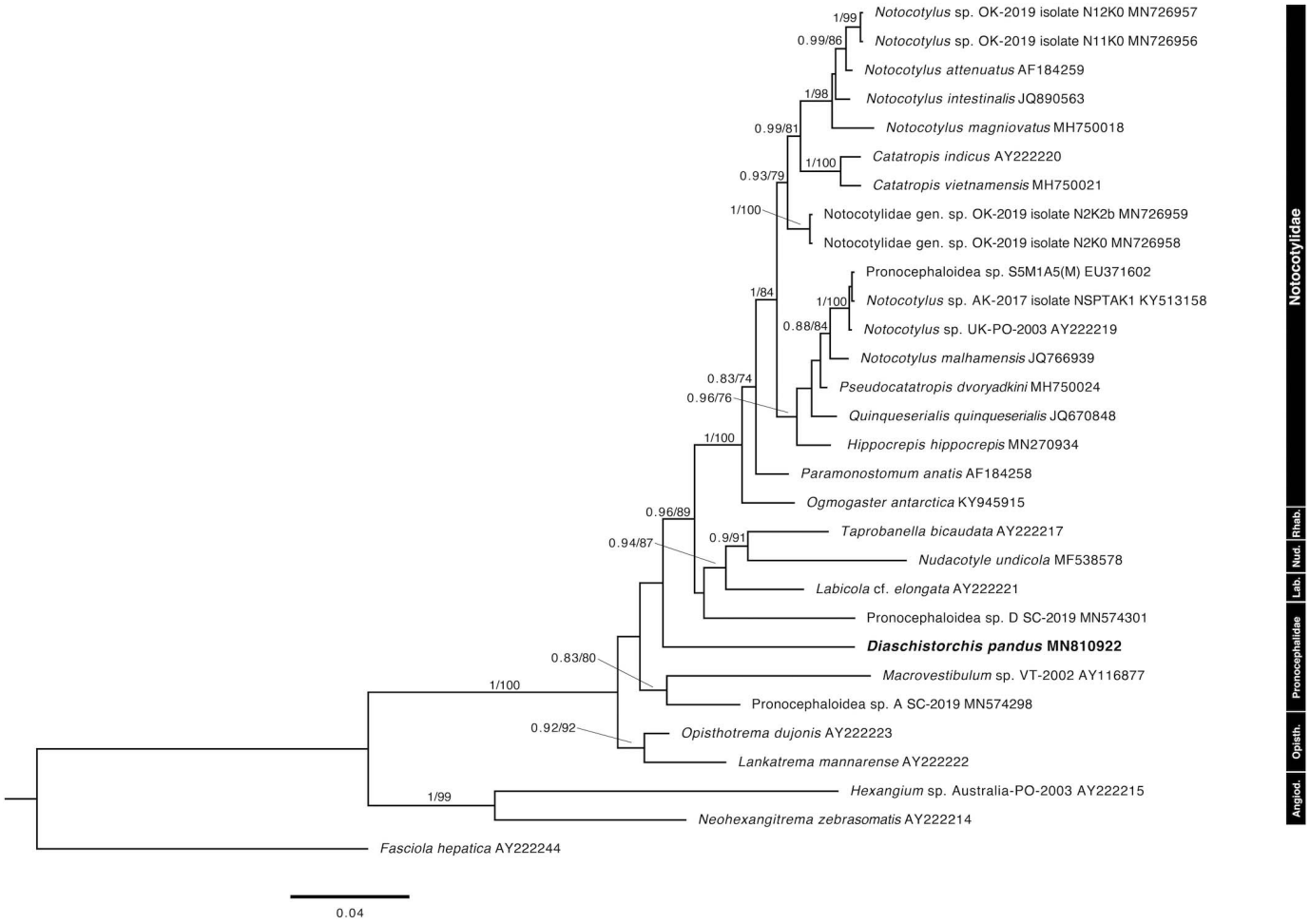


**Figure 2.** Phylogenetic tree of trematodes in the superfamily Pronocephaloidea based upon *28S* rRNA sequence data. Angiod. = Angiodictyidae. Lab. = Labicolidae. Nud. = Nudacotylidae. Opisth = Opisthotrematidae. Rhab. = Rhabdiopoeidae. Values at each node represent Bayesian posterior probabilities and maximum likelihood bootstrap values based on 1,000 pseudoreplicates. Values $\leq 0.7/70$ omitted. Scale bar represents the number of nucleotide substitutions per site.

is unknown. Increased gastrointestinal parasite loads and pathogenic effects in immunosuppressed and debilitated animals have been described in other species through natural and experimental infection (Chai et al., 1995; Pinto et al., 2015; Aragaw and Tilahun, 2019). Although turtles in these conditions are encountered in Grenada, antemortem assessment of parasite load and associated lesions is challenging in sea turtles. Additionally, turtles that present for necropsy often have an extended interval between death and necropsy, which makes it difficult to assess tissues within the gastrointestinal tract, which often have advanced stages of post-mortem autolysis and bacterial proliferation.

Phylogenetic analysis of the *28S* rRNA gene from *D. pandus* and other members of the superfamily Pronocephaloidea corroborates findings in Assis et al. (2019) and Ebert et al. (2020), whose trees were also constructed using single-locus analysis (*28S* rRNA in Assis et al. [2019]; *18S* rRNA in Ebert et al. [2020]). However, in our analysis, *D. pandus* formed a separate clade from *Macrovestibulum* sp., the only other member of the family Pronocephalidae with *28S* rRNA gene sequences deposited in GenBank, with an observed p-distance of 0.10. At present, multilocus analysis beyond the few species already covered in the phylogram in Olson et al. (2003) will not be comprehensive due to the paucity of publicly available sequence data for pronocephaloid trematodes. Future molecular analyses of type-species of other pronocephaloid genera will aid in the resolution of evolutionary relationships within these trematodes.

Expansion of trematode-specific morphologic and genomic data provides a means to reliably identify parasites of sea turtles. This improves our ability to perform pathogen surveillance and increases our understanding of the disease epidemiology and health status of these ecologically important species.

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