



# First molecular identification of the trematode *Pulmonicola cochleotrema* (Platyhelminthes: Opisthotrematidae) in West Indian manatees (*Trichechus manatus*, Sirenia: Trichechidae) from Puerto Rico and Florida

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Received: 13 September 2023 / Accepted: 6 November 2023  
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**Abstract** West Indian manatees (*Trichechus manatus*) harbor a variety of endoparasites, including the nasal trematode *Pulmonicola cochleotrema*, which infects the respiratory tract, especially the nasal passages. Previous studies have described and identified this digenean using morphological data only. This study presents the first molecular identification of *P. cochleotrema* in West Indian manatees from Puerto Rico and Florida. Samples of the trematode were collected from seven manatees found stranded dead at both locations. The small subunit ribosomal DNA (18S rDNA) was amplified from each sample using universal primers for different regions of the gene, resulting in a consensus sequence of 1871 base pairs. The phylogenetic reconstruction was carried out using DNA sequences of other species of digenean parasites from other hosts, including a

trematode of the same taxonomic family from another sirenian species. Specimens collected from both locations show the same molecular identity using SSU rDNA sequence data. The identity of *P. cochleotrema* was confirmed using the Basic Local Alignment Search Tool from the National Center for Biotechnology Information database, yielding a high similarity of 98.8 % with *Opisthotrema dujonis* and 98.2 % with *Lankatrema mannarensis* located in the same clade in our analysis. The latter two digeneans belong to the Opisthotrematidae as does *P. cochleotrema* and previous studies reported them infecting the Eustachian tubes, esophagus, and digestive tract in dugongs (*Dugong dugon*). These findings evidence that the nasal trematode of manatees in Florida and Puerto Rico and the dugong, all inhabiting and feeding in marine environments, will have a marine mollusk as an intermediate host, probably a gastropod. The question remains, which species of nasal trematode are harbored by lotic-dwelling manatees in other parts of their distribution like South America.

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## Introduction

The West Indian manatee (*Trichechus manatus* Linnaeus) is an herbivorous aquatic mammal that is the final host for various helminths, including a gastric nematode (*Heterocheilus tunicatus* Diesing, 1839), two protozoans and various digenean trematodes (Beck & Forrester, 1988; Upton et al., 1989;

Mignucci-Giannoni et al., 1999a, b; Colón-Llavina et al., 2009). The two protozoans are *Eimeria nodulosa* Upton, Odell, Bossart & Walsh 1989, and *E. manatus* Upton, Odell, Bossart & Walsh 1989, and the five species of digenean are the intestinal trematodes *Nudacotyle undicola* Dailey, Vogelbein & Forrester, 1988, *Moniligerum blairi* Dailey, Vogelbein & Forrester, 1988, *Chiorchis fabaceus* (Diesing, 1838) and *C. groschafti* Coy-Otero 1989, and the nasal trematode *Pulmonicola cochleotrema* (Travassos & Vogelsang, 1931) (Price, 1932; Beck & Forrester, 1988; Dailey et al., 1988; Coy-Otero, 1989). This parasitic fauna has been documented for several centuries, mainly using morphological data from different life stages of the parasites (Lainson et al., 1983; Mignucci-Giannoni et al., 1999a, b; Mora-Pinto, 2000; Colón-Llavina et al., 2009; Bando et al., 2014; Wyrosdick et al., 2018). However, recent studies have begun to identify and characterize these species at the molecular level using eggs and adult specimens, specifically of *N. undicola*, *C. fabaceus*, *C. groschafti*, *E. nodulosa*, and *E. manatus* (Rivera-Pérez, 2018; Vélez et al., 2018; Vélez et al., 2019). However, *P. cochleotrema*, formerly known as *Cochleotrema cochleotrema* Travassos & Vogelsang, 1931, has not previously been molecularly identified in any of its parasitic life stages. Molecular identification of endoparasite species of manatees provides tools to understand and confirm their taxonomy and to evaluate their phylogenetic location with related helminths, both in the host, their environment, and even their feeding habits. We present the first molecular identification and phylogenetic placement of the digenean helminth *P.*

*cochleotrema* of West Indian manatees from Puerto Rico and Florida.

## Materials and methods

### Sample collection

Nasal trematodes were collected from seven West Indian manatees stranded dead in Puerto Rico (n = 3) and Florida (n = 4) (Table 1). The manatees examined included subadults (n = 2) and adults (n = 5) (age categories as in Mignucci-Giannoni et al., 2000), and carcass conditions were fresh (n = 3), moderately decomposed (n = 2) and in advanced decomposition (n = 2) (as in Geraci & Lounsbury, 2005). The digenean specimens were observed onsite (Figure 1A), removed from the nasal cavity, trachea, or bronchi, collected intact, and preserved in ethanol equal to or greater than 70%, as stipulated in a standardized protocol for the collection, preservation, and shipment of endo and ectoparasites of aquatic mammals by the Caribbean Manatee Conservation Center (available at <http://manatipr.org/parasiteprotocol/>). Each specimen was observed on a dissecting microscope (10x) for confirmation of morphological identification (Figure 1B), as per the description by Blair (1981), Bonde (1985), and Carvalho et al. (2009).

### Molecular and phylogenetic analyses

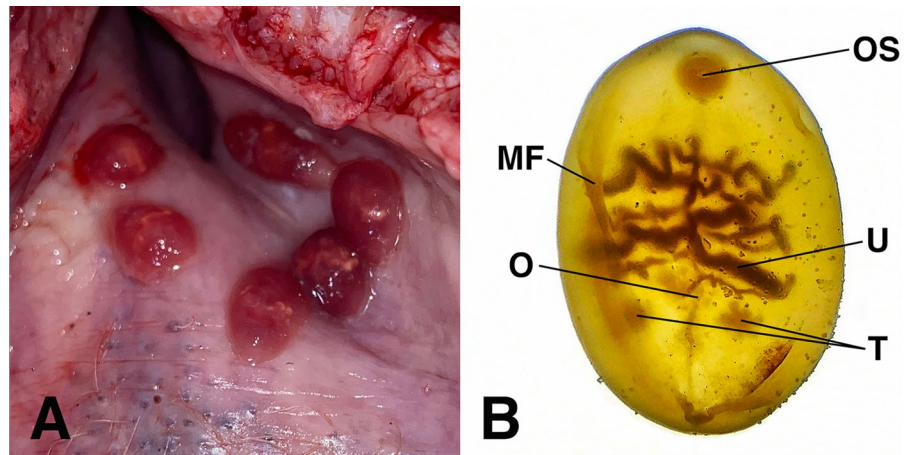
Total DNA was extracted from the trematode specimens using the EURx GeneMATRIX Tissue DNA Purification Kit (Gdańsk, Poland) following the

**Table 1** The helminth *Pulmonicola cochleotrema* collected from West Indian manatees (*Trichechus manatus*)

Host field number	Date of collection	Sex	Relative age	Geographic location	Trematode location in host
<b>Antillean manatee (<i>Trichechus manatus manatus</i>)</b>					
NEPST939	24 Aug 2010	F	SA	Carolina, PR	Nasal cavity
NEPST958	16 Jul 2013	M	SA	Río Grande, PR	Trachea
CCMPR160530Tm01	30 May 2016	M	A	Peñuelas, PR	Nasal cavity, bronchi, lungs
<b>Florida manatee (<i>Trichechus manatus latirostris</i>)</b>					
MSW1159	20 May 2011	F	A	Sarasota, FL	Nasal cavity
SWFTm1207b	18 Apr 2012	F	A	Indian River, FL	Nasal cavity
MNW18071	12 Jun 2018	F	A	Hillsborough, FL	Nasal cavity
MSW18158	15 Jun 2018	M	A	Lee, FL	Nasal cavity

F = female, M = male, SA = subadult, A = adult, PR = Puerto Rico, FL = Florida.

**Fig. 1** (A) The nasal trematode *Pulmonicola cochleotrema* found in the nasal cavity of a dead Antillean manatee (*Trichechus manatus manatus*) from Puerto Rico, and (B) the visualization through the dissecting microscope showing the oval body, muscular fringe (MF), oral sucker (OS), uterus (U), testes (T) and ovary (O) (10x).



manufacturer's instructions and eluted in 80  $\mu$ l of buffer and stored at  $-20^{\circ}\text{C}$ . The nucleic acid concentration and purity of each trematode sample were evaluated and quantified on a NanoPhotometer NP80.

Small subunit ribosomal DNA (18S rDNA) was amplified by polymerase chain reaction (PCR) using universal primers for different regions of the gene: 18e/870r, 390f/18gM, 390f/870r and 870f/18gM (Hillis & Dixon, 1991; Freeman et al., 2013). The PCRs were performed in an Eppendorf Mastercycler Nexus Thermal Cycler containing 20  $\mu$ L reaction volumes of 6.8  $\mu$ L of distilled water, 9  $\mu$ L of a master mix to give final concentrations of 35 mM  $\text{MgCl}_2$  and 0.2 mM deoxynucleotide triphosphates (dNTPs), 0.8  $\mu$ M of each primer, 0.2  $\mu$ L Taq polymerase and 1  $\mu$ L of DNA template. The thermocycler temperature profile was as follows: denaturation at  $94^{\circ}\text{C}$  for 4 min, followed by 35 cycles at  $95^{\circ}\text{C}$  for 30 s, annealing at  $55^{\circ}\text{C}$  for 30 s, extension at  $72^{\circ}\text{C}$  for 1 min, and final elongation at  $72^{\circ}\text{C}$  for 7 min. Aliquots of 4  $\mu$ L of each PCR product were electrophoresed in a 1% agarose with 1x Tris Acetate-EDTA buffer (TAE) gel, and this was stained using ethidium bromide solution. PCR products were purified using a EURx GeneMATRIX PCR Clean-Up Purification Kit (Gdańsk, Poland) and sequenced by Apical Scientific (Selangor, Malaysia).

Each sample was manually aligned with forward and reverse sequences, generating a final consensus sequence using Clustal X and BioEdit softwares (<http://www.clustal.org/clustal2/>, <https://bioedit.software.informer.com/>). Phylogenetic analysis of the 18S rDNA sequences of trematode samples for maximum

likelihood (ML) were performed using PhyML 3.0 software and the Hasegawa-Kishino-Yano (HKY) substitution model (Hasegawa et al., 1985), with 100 bootstrap repetitions (Guindon et al., 2010). Fig.Tree v1.4.3 (<http://tree.bio.ed.ac.uk/software/fig.tree/>) was used to visualize the phylogenetic tree.

## Results

### Molecular identification

DNA extraction followed by 18S rDNA amplification from the nasal trematodes collected in Puerto Rico and Florida were successfully performed. A single contiguous sequence of 1871 bases was obtained, which was identical in trematodes from both locations. Basic Local Alignment Search Tool (BLAST) searches (Altschul et al., 1990) in the National Center for Biotechnology Information (NCBI GenBank) database using our novel sequence revealed that the closest matches available with high similarities were *Opisthotrema dujonis* (Leuckart, 1874) (AY222117) and *Lankatrema mannarensis* Cruz & Fernand, 1954 (AY222116) at 98.8% and 98.3%, respectively, both related trematodes of the same taxonomic family infecting dugongs (*Dugong dugon* [Müller]) in Queensland, Australia (Olson et al., 2003).

The 18S rDNA sequences from *P. cochleotrema* specimens of West Indian manatees from Puerto Rico and Florida obtained in this study were deposited in GenBank under the following accession numbers, OR428573 and OR428574, respectively.

## Phylogenetic analysis

The phylogenetic reconstruction showed that *P. cochleotrema* specimens collected from two different geographic locations have the same molecular placement with respect to SSU rDNA sequences (Figure 2).

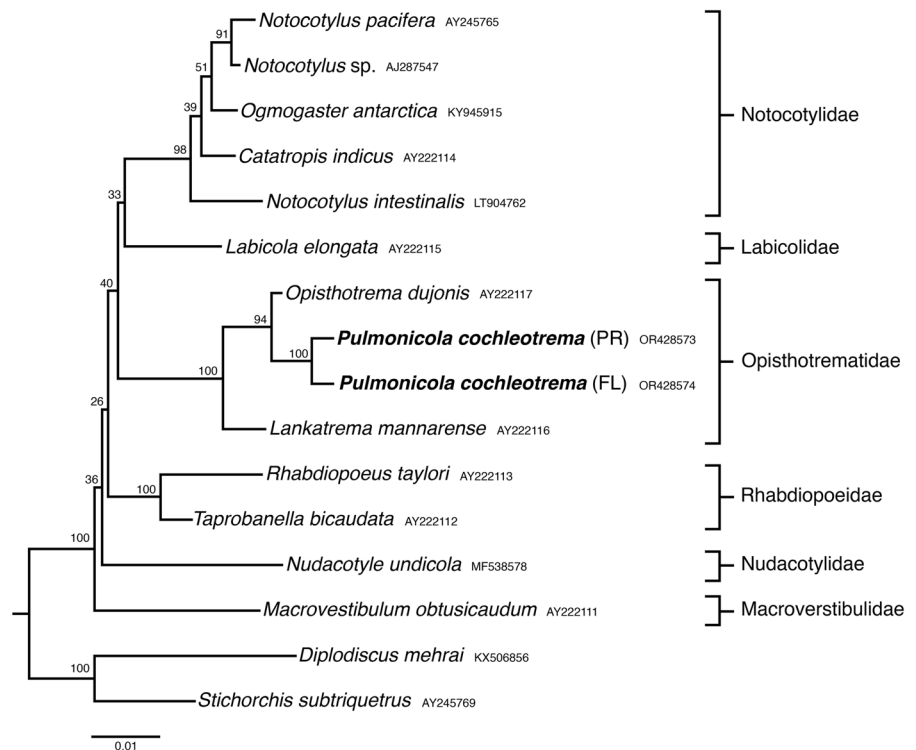
The sequences of *O. dujonis* (AY222117) and *L. mannarensis* (Y222116) were robustly placed in the same clade as *P. cochleotrema*, with full bootstrap support. *Rhabdiopoeus taylori* Johnston, 1913 (AY222113), *Taprobanella bicaudata* Cruz & Fernandez, 1954 (AY222112), and *Labicola elongata* Blair, 1979 (AY222115) are from dugongs, belong to the Opisthotrematidae, Rhabdiopoeidae, and Labicolidae, respectively (Olson et al., 2003) (Figure 2). The *N. undicola* (MF538578), an intestinal trematode of manatees, belongs to Nudacotylidae. The *Ogmogaster antarctica* Johnston, 1931 (KY945915), a parasite of sei whales (*Balaenoptera borealis* Lesson), and *Cat-*

1933) (AY245765) from Muscovy ducks (*Cairina moschata* [Linnaeus]), American coots (*Fulica americana* Gmelin) and domestic chickens (*Gallus domesticus* [Linnaeus]) (Olson et al., 2003; Chaisiri et al., 2011; Canaris & Waldmann, 2017), belong to the Notocotylidae. Two taxa were used as an outgroup selected from an identified sister clade: *Diplodiscus mehrai* Pande, 1937 (KX506856) and *Stichorchis subtriquetrus* (Rudolphi, 1814) (AY245769), belonging to the Diplodiscidae and Paramphistomidae. The genetic changes per site represented in the time-scale resulted in 0.01, considerably a low number of substitutions for this gene region.

## Discussion

West Indian manatees belong to the order Sirenia, along with dugongs, and two other species of manatees (*T. inunguis* Natterer and *T. senegalensis* Link).

**Fig. 2** Maximum-likelihood phylogenetic tree of alignments of 18S rDNA sequences of *Pulmonicola cochleotrema* collected from the West Indian manatees (*Trichechus manatus*) from Puerto Rico (PR) and Florida (FL) and sequences from most closely related digenean species from different hosts obtained through GenBank. The numbers in the nodes represent the bootstrap support values of each clade. *D. mehrai* and *S. subtriquetrus* were used as outgroup taxa.



*atropis indicus* Srivastava, 1935 (AY222114), *Notocotylus* sp. Diesing, 1839 (AJ287547), *N. intestinalis* Tubangu, 1932 (LT904762), and *N. pacifera* (Noble,

*Trichechus manatus* is subdivided into two subspecies, the Florida manatee (*T. m. latirostris* [Harlan]), in the southeast coast of the United States, and the

Antillean manatee (*T. m. manatus* Linnaeus), in the northern coast of Mexico, the eastern coast of Central America, the West Indies and the northern of South America (Self-Sullivan & Mignucci-Giannoni, 2012; Jefferson et al., 2015). Florida manatees live in marine, brackish, and freshwater habitats, while Antillean manatees live in marine ecosystems in the West Indies, Mexico, and Brazil, and in brackish and freshwater habitats in the remaining of its distribution in Central and South America. Both subspecies feed on submerged or floating marine and freshwater plants depending on the ecosystem they inhabit (Mignucci-Giannoni & Beck, 1998; Mora-Pinto, 2000; Allen et al., 2018), although they have been reported to utilize other food resources during shortages of seagrasses or aquatic plants (Vélez-Juarbe, 2014), including invertebrates and fish (Heinsohn et al., 1977; Courbis & Worthy, 2003; Keith-Diagne, 2014; Caicedo-Herrera et al., 2020), together with snails that live attached or on seagrasses and other aquatic plants.

Using molecular tools and phylogenetic reconstruction, the first molecular identification of *P. cochleotrema* was performed. The phylogenetic tree showed that the specimens collected from both subspecies of the West Indian manatee, share the same nasal trematode species and group in the same clade, supporting the morphological and general external identification from other studies (Gomes Borges et al., 2016; Wyrosdick et al., 2018). These results support that this endoparasite uses a common marine gastropod as an intermediate host, infecting both manatee subspecies. The trematode *O. dujonis* is most closely related to *P. cochleotrema*, indicating a shared ancestry (Figure 2). *Opisthotrema dujonis* infects the auditory tube, middle ear, and esophageal lumen of dugongs (Beck & Forrester, 1988; Blair, 2005). Also, it is evident that *L. mannaense* reportedly encapsulated in the digestive tract wall of dugongs (Crusz & Fernand, 1954; Blair, 2005) is closely related taxonomically to *O. dujonis* and *P. cochleotrema*, these three helminths belonging to the same family (Figure 2).

Olson et al. (2003) showed a similar phylogeny between the families of *O. dujonis*, *L. mannaense*, *R. taylori*, *T. bicaudata*, *L. elongata*, *C. indicus*, and *Notocotylus* sp. based on inter-relational representations of reduced phylogenetic trees using the *ssrDNA* and *lsrDNA* regions, both individually

and in combination. Thus, Notocotylidae is more closely related to Labicolidae, Opisthotrematidae, and Rhabdiopoeidae, than to Macroverstrubulidae. The phylogenetic relationship representations according to individuals of these families by Olson et al. (2003) are very similar to that presented in this study but without the presence of *P. cochleotrema*. This evidence supports and demonstrates the correct molecular identification of *P. cochleotrema*, the trematode of interest, and the phylogenetic position of the digeneans previously studied by Olson et al. (2003) and used in this study. However, the trematode *P. pulmonalis* (von Linstow, 1904) (previously known as *Opisthotrema pulmonale* von Linstow, 1904), also belonging taxonomically assigned to Opisthotrematidae and re-assigned to the genus *Pulmonicola*, reported in the nasal cavity and lungs of dugongs, has only been described morphologically (Yamaguti, 1971; Angsinco-Jiménez et al., 2013), and no specimens were available to study molecularly; therefore, it could not be included in this study. The digenean *N. undicola*, is the only species within the Nudacotylidae, despite infecting the same host as *P. cochleotrema* (Bando et al., 2014; Vélez et al., 2018; Wyrosdick et al., 2018), that it is not very close phylogenetically, compared with the dugong parasites used in our phylogenetic reconstruction.

The life cycle of these digenean parasites has not been fully elucidated, but it appears almost certain that an obligate molluscan gastropod intermediate host forms part of the life cycle (Williams & Williams, 1995, 1996; Rivera-Pérez, 2018). All the digeneans, which are closely related to *P. cochleotrema*, have a sirenian final host which lives or feeds in the marine environment. While eating seagrasses or marine plants, they are known to ingest potentially infected gastropods or perhaps plant leaves-encysted metacercariae of digeneans (Beck & Forrester, 1988). While the other digenean species, except for *O. antarctica*, which parasitizes a marine whale, use intermediate hosts that live and feed in freshwater or lotic environments (Olson et al., 2003), we can assume that in the case of marine-dwelling West Indian manatees and dugongs, the intermediate host for its nasal trematodes is a marine mollusk.

The molecular identification of *P. cochleotrema* provided by this study contributes to research on the parasitic fauna of manatees, for example, facilitating the identification and genetic comparisons of



this digenean in other manatee species and populations. Such comparisons are warranted among morphologically similar nasal trematodes of lotic-living West Indian manatees in Colombia and Venezuela to ascertain if they truly represent the same or a different species of *Pulmonicola*. As it has been shown in different helminths infecting other marine mammals (Mignucci-Giannoni et al., 1998; Colón-Llavina et al., 2009; Rivera-Pérez, 2018), their use a biological tag may provide an insight into the parasitic life cycles as some of these parasites have proven to differ in population distribution, habitat use, and the host's diet.

**Acknowledgments** We thank Martine de Wit, Andy Garrett, Brandon Bassett, and their team from the Florida Wildlife Conservation Commission Marine Mammal of Pathobiology Laboratory in Florida for their help collecting samples from Florida manatees. We also thank the technicians and rescue volunteers of the Caribbean Manatee Conservation Center at the Inter American University of Puerto Rico for their participation during the strandings and for assisting in collecting samples during the postmortem examinations. The parasite samples in Puerto Rico were collected under US Fish and Wildlife Service permit #LOAFC 231088-A and Puerto Rico Department of Natural and Environmental Resources (PRDNER)-Red Caribeña de Varamientos Letter of Agreement 2020-2025 and PRDNER Scientific permit 2021-EPE-035. Support for this research was granted by Ross University School of Veterinary Medicine Intramural grant (RUSVM grant ID # 43011-2022) and the Morris Animal Foundation grant (MAF grant ID #D22ZO-822).

**Author contributions** Carla I. Rivera-Pérez: Visualization, research, funding acquisition, methodology, sample collection, data analysis, original drafting, writing-reviewing & editing. Antonio A. Mignucci-Giannoni: Visualization, research, supervision, funding acquisition, writing-reviewing & editing. Michelle M. Dennis: Research, funding acquisition, writing-reviewing & editing. Mark A. Freeman: Visualization, conceptualization, funding acquisition, project management, supervision, methodology, original drafting, writing-reviewing & editing.

**Funding** For this publication, there has been no significant financial support that could have influenced its outcome.

#### Declarations

**Competing interests** The authors declare that they have no conflict of interest associated with this publication.

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