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Record of *Renicola sloanei* Wright, 1954 (Plagiorchiida: Renicolidae) in the Atlantic puffin *Fratercula arctica* (Linnaeus, 1758) arrived at the Canary Islands (Spain)

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Abstract

In the winter of 2022–2023, hundreds of the Atlantic puffins (*Fratercula arctica*) appeared dead in the coast of the Canary Islands, a rare event considering their cold-living habits, normally occupying the North Atlantic Ocean. In this work, investigation about the parasites present in the Atlantic puffins found in the biggest islands of the Archipelago was carried out from a population portion. Necropsies of 39 birds were made and, during the examination of the urinary tracts, helminths were found. Morphoanatomical analysis under microscope allowed to identify them into *Renicola* genus with high similarity to *Renicola sloanei.* After that, DNA was extracted and NADH dehydrogenase subunit 1 gene were amplificated by a polymerase chain reaction method followed by sequencing and phylogenetic analysis. The molecular results demonstrated that in fact *R. sloanei* was the helminth parasite present in the urinary tracts of the Atlantic puffins found in the Canary Islands.

Introduction

Seabird populations worldwide have been declining dramatically over the past decades as a result of a range of environmental and anthropogenic stressors. The Atlantic puffin, *Fratercula arctica* Linnaeus, 1758 (Charadriiformes: Alcidae) has been designated as vulnerable to extinction globally and listed as endangered in Europe (Kersten, 2023). These birds breed in isolated cliff slopes and islands in the North Atlantic Ocean (Greenland, Iceland, Scandinavian Peninsula, British Islands), in late summer they leave their colonies on migration and distribute all around the North Atlantic, having even been seen in the Mediterranean Sea (Clairbaux et al., 2021). Nevertheless, during the winter of 2022–2023, numerous puffins (hundreds or even thousands as estimated) were found dead on the shores of the Canary Islands (Spain), located near the coast of Morocco, belonging to the Macaronesia region in 13°23′–18°80′ W and 27°37′–29°24′N. The reason for this change in their migration route is not clear, but adverse weather phenomena and changes in food availability due to climate change are the most likely causes (Guilford et al., 2011; Dorresteijn et al., 2012).

Because of this rare event, investigation about the parasites present in *F. arctica* appeared in the Canarian coasts were performed in cooperation with 'Dirección General de Lucha Contra el Cambio Climático y Medio Ambiente' as part of the Canarian Network for the Surveillance of the Wildlife Health (Red Vigía, Orden No. 134/2020 of 26 May 2020) and 'Consejería de Transición Ecológica, Lucha contra el Cambio Climático y Planificación Territorial' as part of 'Estudio de patógenos en aves migratorias y en especies exóticas en un escenario de cambio climático' project (No. 248/2020 of 4 December 2020). The aim of this work was to investigate renal parasites in these birds, following previous findings of renal digeneans in the same species (Hill, 1954).

Materials and methods

Animal management

A total of 39 *F. arctica* (21 adults, 17 juveniles and one age undetermined) found in coastal areas of Tenerife and Gran Canaria islands (Canary Islands, Spain) were dissected for the present study. The kidneys and ureters were examined using stereoscopic microscope and dissection equipment, searching for helminths.

Morphological identification

The helminths found were preserved in 70% ethanol at room temperature. Each one was stained with Semichon acetocarmine, differentiated in 50% acid-alcohol solution, dehydrated in an ethanol series and with 2-propanol, cleared in clove oil and, finally, mounted on slides with Canadian balsam. After that, they were examined under optical microscope. Because of the poor condition of the parasites found, only a single specimen could be measured (see the following section for morphometric data).

DNA extraction

Genomic DNA was obtained from renal tissue portions and the helminths found in bad condition for morphometric analysis, following López et al. (2015) protocol. The samples were transferred to 1.5-ml tubes containing 250 µl of lysis solution (10 mM EDTA, 30 mM Tris-HCL pH 8.0 and 0.4% SDS) and 3 µl of proteinase K (20 mg/ml, PanReac AppliChem ITW Reagents, Monza, Italy); after that, they were incubated at 56°C overnight. The following day, 250 µl of 4M ammonia acetate was added, mixed thoroughly and consequently kept for 30 min at room temperature (15–25°C). The mixture was centrifuged at 13,000 rpm for 10 min, and the pellet was discarded. DNA was then precipitated with ethanol, and the sediment was suspended in 20–80 µl of molecular grade water (PanReac AppliChem ITW Reagents). The quantity and quality of genomic DNA was verified using DeNovix DS-11 + Spectrophotometer (DeNovix Inc., Wilmington, DE, USA).

Polymerase chain reaction amplification

The NADH dehydrogenase subunit 1 gene (ND1) was amplified using the primers ND1J and ND1J2A described by Bray et al. (1999) and Morgan and Blair (1995). The polymerase chain reaction mix contained 1X buffer (VWR International, Haasrode, Belgium), 1.5 mM MgCl2 (VWR International), 0.2 mM of each dNTP (VWR International), 2 µM of each primer (Condalab, Madrid, Spain) and 20–40 ng of total genomic DNA in a total volume of 25 µl filled with molecular grade water. Amplification was performed using a XP Cycler (Bioer Technology, Hangzhou, China) with the following parameters: 95°C for 5 min; 30 cycles at 94°C for 1 min, 50°C for 1 min, 72°C for 1 min; and a final extension step at 72°C for 7 min. The resulting amplicons were visualised on 1.5% agarose gel at 90 V for 1 h.

Sequencing and sequencing data analysis

The polymerase chain reaction products with the expected size (500 bp) were sequenced at Macrogen (Madrid, Spain) along with ND1J and ND1J2A primers, using the Sanger method. The

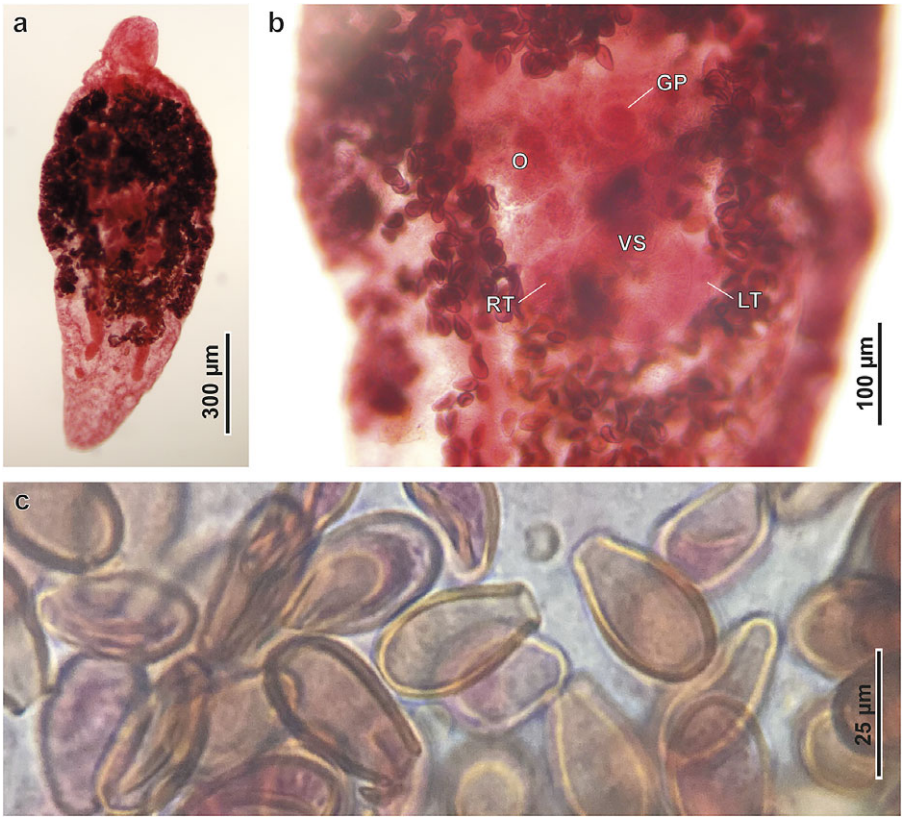

Figure 1. *Renicola sloanei* found in kidneys and ureter tracts of *Fratercula arctica* arrived at the Canary Islands. a: Whole mount specimen. b: Detail of gonads. c: Eggs. GP, genital pore; LT, left testis; O, ovary; RT, right testis; VS, ventral sucker.

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sequences obtained were studied with MEGA X software (Kumar et al., 2018), applying the multiple alignment program ClustalW and compared with different *Renicola* Cohn, 1904 species sequences from GenBank database, with minor corrections made by hand. Afterwards, they were analyzed using the Basic Local Alignment Search Tool (BLAST), and their identity was confirmed by homology comparison.

Phylogenetic relationships were stablished based on the maximum likelihood method with the Kimura 2-parameter model (Kimura, 1980) and 1,000 bootstrap replications, exploring the relationships among different *Renicola* species using MEGA X software. *Echinostoma ilocanum* Garrison, 1908 sequence (Acc. Number: MN549984.1) was used as outgroup.

Results and discussion

One and three morphologically similar helminth specimens were found in the urinary tract of two (5%) of the 39 birds examined.

The recovered digeneans were identified as belonging to the genus *Renicola* according to specialised literature (Wright, 1954; Gibson, 2008; Heneberg et al., 2016; Matos et al., 2019). Body oval to pyriform, round-shaped anteriorly and attenuated posteriorly. Body length 1682 µm, maximum width 671 µm. Body length to width ratio 2.5:1. Oral sucker subterminal, 195 × 193 µm. Ventral sucker postequatorial, 144 × 136 µm. Oral sucker to ventral sucker length ratio 1:0.74 Oral sucker to ventral sucker width ratio 1.42. Pharynx muscular, well-developed 118 × 121 µm. Two testes symmetrical, located at the ventral sucker level. Ovary lobed, preacetabular. Vitellarium consist of two lateral fields, each with numerous follicles. Vitelline fields extend approximately from the pharynx level to the posterior body part, ending well behind the posterior edge of the testes. Eggs 28-31 × 15-18 µm (29.2 × 16.8 µm) (n=5).

These morphological characteristics (Figure 1) show similarities with those of *Renicola sloanei* Wright, 1954. Moreover, our specimens were clearly differentiated from *Renicola lari* Timon-David, 1933, *Renicola sternae* Sitko & Heneberg in Heneberg, Sitko, Bizos & Horne, 2016 and Renicola *pinguis* Mehlis in Creplin, 1846. The main difference between the specimens found in the present study and these three species is related to the vitellarium. Thus, in *R. lari*, *R. pinguis* and *R. sternae* the number of vitelline follicles is low, whereas in our specimens, as occurs in *R. sloanei*, the number of vitelline follicles is much higher and more extended in the worm's lateral margins. Comparative measures of *R. sloanei* found in different hosts are shown in table 1.

A 285-bp sequence from ND1 gene was obtained. The BLAST analysis showed great homology with *R. sloanei* (Acc. No.: MK463858.1, Query Cover: 88%, Identity: 100.0%, e-value 6e-126). The nucleotide sequence obtained in this study were submitted to the GenBank database under the accession number PP764027.

The results of the maximum likelihood method analysis based on the obtained fragment ND1 gene is shown in Figure 2. The sequence obtained grouped with *R. sloanei* species and was clearly separated from other *Renicola* species with high bootstrap value (100%).

Renicolidae digeneans are parasites of the urinary system of aquatic birds feeding on infected bivalves and fishes. The intramolluscan stages of this parasite develop in marine and brackish-water gastropods, whereas metacercariae develop in molluscs and fishes (Galaktionov et al., 2023). Following the molecular analyses of Galaktionov et al. (2023), *R. sloanei* belong to the second branch (clade II) of Renicolidae, a group of species that use sea birds as the

Table 1. Measurements (in µm, except ratios) of *Renicola sloanei* from different seabirds in diverse localities

Hosts	*Pygoscelis antarcticus* and *Eudyptes chrysolophus*	*Uria aalge*	*Puffinus puffinus*	*Fratercula arctica*
Localities	South Georgia and Edinburgh Zoo (UK)	Sussex (UK)	Paraná State (Brazil)	Tenerife and Gran Canaria Islands (Spain)
References	Wright (1954)	Wright (1954)	Matos et al. (2019)	Present study
Body length	1470–2710	930–1820	1287–3096	1682
Maximum width	690–1260	420–710	631–1659	671
Maximum width:body length			1:1.6–2.9	1:2.5
OS length	257–329	129–143	158–536	195
OS width	229–286	186–228	115–666	193
VS length	114–129*	73–100*	111–270	144
VS width			115–267	136
OS:VS length			1:0.2–1	1:0.74
OS:VS width			1:0.2–1.5	1:0.70
Pharynx length	114	47–85	68–158	118
Pharynx width	114	73–85	92–173	121
Eggs length	28–34	32–38	24–35	28–31
Eggs width	16–18	16.5–19.5	12–19	15–18

OS, oral sucker; VS, ventral sucker.
*Diameter.

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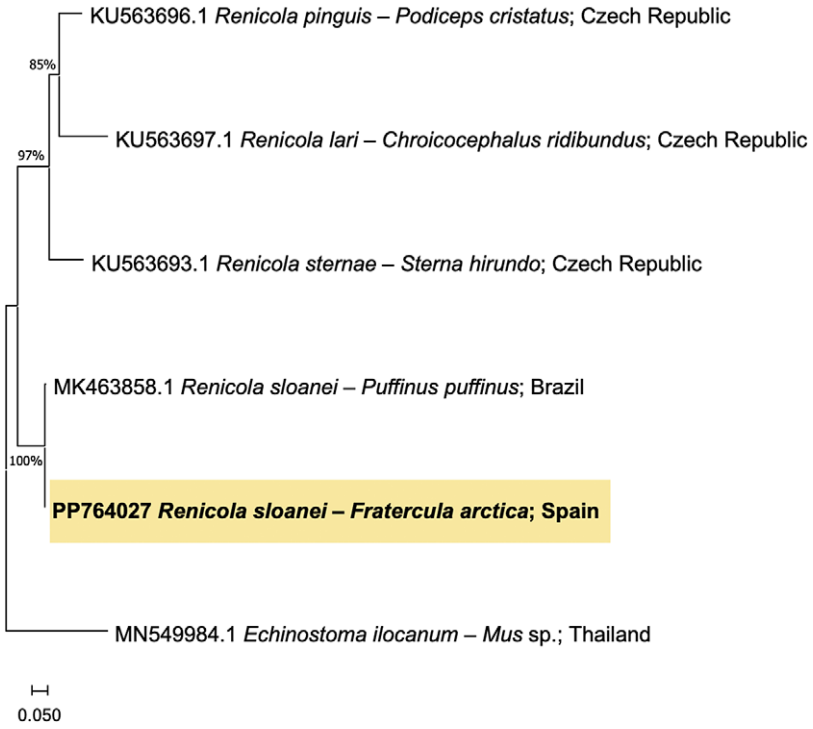

Figure 2. Phylogenetic relationships between sequences of the NADH dehydrogenase subunit 1 of *Renicola* species, including the nucleotide sequence obtained in the present study (showed in bold and highlighted in yellow). The tree was built using the Maximum Likelihood method with p-distance and 1,000 bootstrap replications. *Echinostoma ilocanum* was used as outgroup.

definitive host, and their cercariae belong to the Rhodometopa group or to 'transitional morphotype'.

Nevertheless, the status, life cycle and classification of some renicolids are still considered uncertain and several descriptions are incomplete due to the non-observation of some crucial characters, namely ventral sucker, intestine, testes, ovary and vitellarium. Moreover, nothing is known accurately related to the degree of specificity of the different morphological characters used for identification (Heneberg, 2016). According to Galaktionov et al. (2023), molecular biology tools are needed to perform a correct identification of the species, using DNA sequencing methods to upgrade the sequences database, especially on morphologically similar species. A precise description of the life cycles and host ranges of *Renicola* digeneans, as well as a detailed analysis of their morphological features according to their different life stages is necessary for a better understanding of these parasites.

The genetical analysis based on ND1 gene of the digeneans found in this study confirm the morphological identification of *R. sloanei*. To date, only a previous single finding of an unidentified species of the genus *Renicola* in *F. arctica* has been notified, concretely in Britain in 1953 (Hill, 1954), which was picked up in bad condition in London docks and died the day after from renal failure. Thereby, our record represents the second report of this genus of flukes in this bird species. Moreover, present record represents a new host for *R. sloanei*; the species was previously found parasitizing other species of seabirds, namely *Pygoscelis antarcticus*, *Eudyptes chrysolophus*, *Uria aalge* and *Puffinus puffinus* (see Table 1).

In conclusion, this work reports the occurrence and first identification, by morphoanatomical analysis and genetic studies, of *R. sloanei* in the urinary tract of *F. arctica*. Further investigation is required to better understand the morphological and molecular characteristics of Renicolidae species, together with the determination of their life cycles.

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Competing interest. The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Ethical standards. Ethical approval was not necessary because all the animals used in this study were already dead before the study. Statements on consent to participate and consent to publish are not applicable.

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