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Aporocotyle michaudi n. sp. (Digenea: Aporocotylidae) from the emerald rock cod, *Trematomus bernacchii* (Teleostei: Perciformes) in Antarctica

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ABSTRACT

Aporocotyle michaudi n. sp. is described from the gill blood vessels of the emerald rock cod *Trematomus bernacchii* in the Ross Sea, Antarctica. It is distinguished from all other species of *Aporocotyle* by its body tegument showing single conical spines, spinous buccal capsule, and genital atrium positioned medially; all congeners described to date are characterized by clusters of tegumental spines, unspined buccal capsule and genital atrium located in the lateral part of the body. *Aporocotyle michaudi* n. sp. clearly differs from *A. notothenia* (the only other species of *Aporocotyle* found in a perciform fish) in its shape and arrangement of tegumental spines, buccal capsule features, location of genital atrium, body size, ratio of esophagus/body length, anterior caeca/posterior caeca ratio, number of testes, cirrus sac and ovary size and shape, and host. The new species is easily distinguished from *A. argentinensis* (the species that most closely resembles *A. michaudi*) by the shape and arrangement of tegumental spines, buccal capsule features, genital atrium location, left anterior caecum longer than right, esophagus/body length ratio, number of testes, cirrus sac size and shape, host and molecular analyses. Phylogenetic analyses of partial 28S rDNA genetic data showed that sequences representing the new species form a distinct clade with all other sequences for species of *Aporocotyle* and appear basal within the genus. *Aporocotyle michaudi* n. sp. represents the only species of genus described in Antarctica.

1. Introduction

The Ross Sea of Eastern Antarctica is considered to be a "pristine ecosystem", supporting one of the most diverse and species-rich environments in the southern oceans [1]. This species diversity is also reflected in the parasite communities and according to Zdzitowiecki [2] and Rocka [3] the Digenea are the most species-rich helminth taxa found as adult stages infecting Antarctic teleost fishes, with at least 44 taxa reported from the gastrointestinal tract alone. During a parasitological survey on notothenioid fishes in the Ross Sea Santoro et al. [4,5] identified digenean flukes in the gill blood vessels of the emerald rock cod *Trematomus bernacchii* Boulenger (Perciformes: Nototheniidae). Initial morphological examination determined the parasite to be a previously undescribed species of *Aporocotyle* Odhner 1900, a genus within the socioeconomically important blood fluke family Aporocotylidae Odhner 1912 (Digenea), which infect extraintestinal sites of marine and freshwater fishes [6,7].

The Aporocotylidae have a global distribution and include five nominal species that infect cartilaginous fishes (Chondrichthyes) [8], plus >100 species that infect bony fishes (Actinopterygii: Teleostei) [9,10], and the genus *Aporocotyle* parasitizes fish belonging to five teleost orders, including Gadiformes, Ophidiiformes, Perciformes, Pleuronectiformes, and Scorpaeniformes [11–25]. Currently, there are 17 known species of *Aporocotyle* infecting the heart, bulbus arteriosus and blood vessels of fishes from the Atlantic Ocean, Pacific Ocean, Indian Ocean, Japan Sea, and Baltic Sea [11–25]. All species within the genus have been described based on morphological characters (i.e. presence/absence and architecture of tegumental spines, position of posterior caecum) and host specificity. These morphological characteristics were first used to differentiate the type species *A. simplex* Odhner 1900, the first blood fluke collected from the gill of flounder, *Platichthys flesus* Linnaeus (Pleuronectiformes: Pleuronectidae) and to be reported as an ectoparasite [see 19]. However, owing to the well documented problems of species delineation based only on morphological characters there has been a recent increase in the use of molecular phylogenetic approaches to disentangle genera and species in the Aporocotylidae as highlighted by Holzer et al. [26]. The sequencing of ribosomal rDNA genes has been favored to identify different species within the Aporocotylidae and recently employed to differentiate *A. mariachristinae* Hernández-Orts et al. 2012 and *A. ymakara* Villalba and Fernández 1986 from each other in co-infections of the Argentinian pink cusk-eel *Genypterus blacodes* Forster. To date, there are no full descriptions of species of *Aporocotyle*

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from Antarctica, therefore, the aim of the current study was to provide a full taxonomic description of a previously undescribed Antarctic species, using a combination of traditional morphological criteria and molecular phylogenetic analysis of 28s rDNA sequences.

2. Materials and methods

2.1. Sample collection and morphological analysis

A total of 426 notothenioid fishes (Perciformes), including 180 *T. bernacchii*, 73 *T. hansoni* Boulenger, 30 *T. newnesi* Boulenger, 12 *T. pennelli* Regan, eight *Lindbergichthys nudifrons* Lönnberg (Nototheniidae), 14 *Prionodraco evansii* Regan, two *Cygnodraco mawsoni* Waite (Bathydraconidae), seven *Artedidraco skottsbergi* Lönnberg (Artedidraconidae), and 100 *Chionodraco hamatus* Lönnberg (Channichtyidae), were collected on February 2012 and January 2013 and 2014 from Terra Nova Bay in the Ross Sea. Fishes were collected by dredges, hand line, hook line or net, at benthic depths ranging from 50 to 250 m in front of the Mario Zucchelli Italian Station. The heart, bulbus arteriosus, and gill blood vessels of fresh fishes were examined under a dissecting microscope for blood flukes. Flukes were fixed in 70% ethanol for morphological examination, 100% ethanol for the molecular analysis, or 4% formalin for SEM. Ten fluke specimens were used for the morphological study. These were stained with Mayer's acid carmine, dehydrated in ethanol, cleared in methyl salicylate and permanently mounted in Canada balsam. Specimens were examined and illustrated using a compound microscope with a drawing tube. Measurements were obtained using a calibrated ocular micrometer and are herein expressed in micrometers (μm) as mean followed, in parentheses, first by the range and then by the number of specimens measured for each feature. Three specimens were used for SEM. These were transferred from 4% formalin to 40% ethanol (10 min), rinsed in 0.1 M cacodylate buffer, postfixed in 1% OsO4 for 2 h and dehydrated in ethanol series. The specimens were then coated with gold in a JEOL JFS 1200 fine coater and examined using a JEOL JSM 5510 microscope at 10 kV.

For morphological comparison, specimens of *A. argentinensis* Smith 1969 (collection no. 1994.6.7.30–34) collected from *Merluccius hubbsi* Marini from the Falkland Island and deposited in the Natural History Museum in London were studied. Unfortunately, comparisons with *A. nototheniae* Paruckhin 1985 [16] were not possible as access to the specimens could not be obtained.

2.2. Molecular analysis and phylogenetics

Whole genomic DNA was extracted from a single worm using the Qiagen DNeasy™ blood and tissue kit following the manufacturer's instructions. Owing to the phylogenetic properties of the large ribosomal subunit gene (28S rDNA) and its ability to provide accurate insights into the evolutionary relationships of Digenea [27], a partial fragment of approximately 1400 bps was amplified using the primers and protocols described by Olson et al. [27]. Unlike in other studies on the taxonomy of Aporocotilydae the 28S rDNA was favored over the internal transcribed region in this study as there are no ITS sequences available for comparison between species within *Aporocotyle* [26–29]. Polymerase chain reaction (PCR) amplifications were performed in 25 μl reactions using Ready-To-Go™ (Amersham Pharmacia Biotech) PCR beads (each containing, 1.5 units Taq DNA polymerase, 10 mM Tris–HCl at pH 9, 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM of each dNTP and stabilizers, including BSA) with 3 μl of genomic DNA and 10 mM of each primer (forward: LSU-5 5′-TAG GTC GAC CCG CTG AAY TTA AGC A-3′; reverse: 1500R 5′-GCT ATC CTG AGG GAA ACT TCG-3′). Reactions were performed using a Veriti 96 well thermal cycler (Applied Biosystems™) PCR machine using the following thermocycling conditions: 3 min initial denaturation at 94 °C; 40 cycles of 30 s at 94 °C, 30 s at 55 °C, 2 min at 72 °C with a final extension hold of 7 min at 72 °C. In order to visualize the amplicon, 5 μl of complete PCR reaction was run on a 1% agarose gel stained with GelRed (Bioline). The remaining 20 μl of positive PCR products was sequenced at the DNA sequencing facility of the Natural History Museum, London, using fluorescent dye terminator sequencing kits (Applied Biosystems™), these reactions were then run on an Applied Biosystems 3730KL automated sequencer.

Resultant forward and reverse 28S rDNA sequences were assembled and edited using Bioedit [30] and the consensus sequence subjected to a BLAST search using blastn (http://blast.ncbi.nlm.nih.gov/) which enabled an initial identification of the fluke to be within the Aprocotylidae and similar to sequences from species within the genus *Aporocotyle*. Detailed phylogenetic analysis of the obtained sequences was performed using alignments with published 28S rDNA, in comparison with 20 species of other fish blood flukes. *Carettacola hawaiiensis* Dailey et al. 1991 (AY604709), *Learedius learedi* Price 1934 (AY604707) and *Hapalotrema mehrai* Rao 1976 (AY604708) were used as an out group as in Holzer et al. [26]. DNA sequence alignments were performed using the MUSCLE sequence alignment tool (http://www.ebi.ac.uk) and the Gblocks server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) [31] was used to remove any alignment gaps and ambiguities with default parameters for block selection to allow smaller final blocks without gaps. Phylogenetic analysis was performed on a final alignment of 886 bp using both maximum likelihood methods (ML) and maximum parsimony methods (MP) implemented in MEGA6 [32]. A suitable nucleotide substitution model, also estimated in MEGA6 [32] based upon the lowest Bayesian information criterion scores relative to the other models tested, was used to perform the ML phylogenetic reconstruction. The ML analysis was performed under the conditions of the general time reversal (GTR) model including among site heterogeneity over a discrete gamma distribution (G) and with estimates of invariable sites (I). The MP phylogenetic trees were obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 5 in which initial trees method obtained by random addition of sequences (10 replicates). In both ML and MP reconstructions all positions containing gaps and missing data were eliminated from the analysis and nodal support values were estimated using 1000 bootstrap replicates.

3. Results

3.1. General data

A total of 20 blood flukes were found from nine of 180 (5%) emerald rock cod, including two males and seven females (fork length ranging from 18.8 to 24 cm), collected at benthic depths ranging from 80 to 120 m.

3.2. Species description

3.2.1. Aporocotyle michaudi n. sp. (Figs. 1 and 2)

Description based on 10 adult whole mounted (Fig. 1) and three SEM adult prepared specimens (Fig. 2).

3.2.2. General morphology

Body flat, lanceolate, pointed at anterior end and blunt at posterior end, 4016 (3048–5060, $n = 13$) long and 336 (283–420, $n = 13$) wide. Maximum body width approximately at mid-length. Tegument papillate (Fig. 2E). Anterior end with seven concentric rows of single conical spines, 1.1 (0.9–1.2, $n = 10$) long and 0.5 (0.4–0.5, $n = 10$) wide at basis level (Fig. 2C). Body tegument entirely covered by single conical spines 4.9 (4.3–5.4, $n = 10$) long and 0.3 (0.3–0.4, $n = 10$) wide at basis level (Figs. 1C, D, 2B, E). Oral sucker absent. Mouth ventral leading to a spinous buccal capsule 58 (42–68, $n = 8$) long and 34 (23–44, $n = 8$) wide (Fig. 2A, D). Nerve commissure ventral, approximately at first fifth of esophagus. Nerve cords extending anteriorly from buccal capsule in parallel with body margin, indistinct posteriorly (Fig. 1A). Esophagus 783 (632–935, $n = 9$) long or 18.8% (15–20.5%,