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Cyathostoma (Cyathostoma) phenisci Baudet, 1937 (Nematoda: Syngamidae), a parasite of respiratory tract of African penguin *Spheniscus demersus*: Morphological and molecular characterisation with some ecological and veterinary notes

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ABSTRACT

Here we provide a morphological and molecular analysis of the taxonomic status of *Cyathostoma (Cyathostoma) phenisci* Baudet, 1937, a rare nematode parasite of African penguin *Spheniscus demersus*. Taxonomical evaluation is supplemented wi th ecological and epidemiological analysis of the nematode's occurrence in the African penguin's population. Tracheae and air sacs of 13 among the 94 necropsied birds (overall prevalence 13.8%) contained a total of 33 nematode specimens (20 females, 13 males). The highest prevalence was observed in juveniles (6 infected, 25%) and "blues" (6 infected, 14.3%), followed by nestlings (1 infected, 7.7%); no nematodes were found in adults. Our morphological and morphometric analysis shows that *C. phenisci* is closely related to another species, *Cyathostoma (Cyathostoma) verrucosum* (Hovorka & Macko, 1959). The doubtful status of the latter species was confirmed by molecular data: comparison of ITS2 sequence of *C. phenisci* with previously deposited sequences of *C. verrucosum* showed 96.3% similarity in this region. On this basis, we recognized *Cyathostoma (Cyathostoma) verrucosum* (Hovorka & Macko, 1959) as a synonym of *Cyathostoma (Cyathostoma) phenisci* Baudet, 1937.

1. Introduction

Nematodes of the family Syngamidae Leiper, 1912 (superfamily Strongyloidea) comprises parasites of respiratory, excretory, digestive tracts and body cavities of birds and mammals. Classification of this family has changed several times, in relation to different evaluation of observed morphological characters [1–6]. In the most recent classification of Syngamidae proposed by Lichtenfels [6], parasites of the respiratory system and body cavities of avian hosts occurring only in genera *Boydinema*, *Cyathostoma* and *Syngamus* in the subfamily Syngaminae Baylis & Daubney, 1926. The majority of species in these genera belong to the genus *Cyathostoma*: so far, more than 20 species have been described from several bird orders (Anseriformes, Charadriiformes, Casuariiformes, Ciconiiformes, Columbiformes, Coraciiformes, Galliformes, Gaviiformes, Gruiformes, Falconiformes, Passeriformes, Pelecaniformes, Psittaciformes, Strigiformes, Struthioniformes, Sphenisciformes)[1,2,5,7–17]. Lichtenfels [6] divided genus *Cyathostoma* into two subgenera: *Cyathostoma (Cyathostoma)* (Blanchard, 1849), in which the dorsal ray extends beyond the end of the copulatory bursa to form characteristic thorn-like projections and spicules that measure 0.08–0.4 mm, and *Cyathostoma (Hovorkonema)* Turemuratov, 1963 in which the dorsal ray does not extend beyond the end of the copulatory bursa and spicules measure 0.45–0.8 mm. In the system proposed by Baruš and Tenora [5] *Cyathostoma* and *Hovorkonema* have the status of independent genera. Parasites of fish-eating birds occur exclusively in nominative subgenus *Cyathostoma*, representing by five species: *Cyathostoma (C.) lari* recorded mainly from gulls in Europe and North America [9], *Cyathostoma (C.) microspiculum* noted in cormorants in Europe and Asia [16], *Cyathostoma (C.) phenisci* described from penguins from South America [18], *Cyathostoma (C.) verrucosum* noted in pelicans and storks in Europe and Asia [3,19–21] and *Cyathostoma (C.) trifurcatum* from *Cicionia nigra* in Europe [15,19].

C. phenisci was originally described by Baudet [18], based on nematode specimens collected from Peruvian penguin *Spheniscus humboldti* Meyen, 1834 (Aves: Sphenisciformes). These birds died soon after arrival from South America (Chile) to the zoo in Amsterdam. Since their description, the occurrence of this nematode has been reported only three times in avian hosts from North America [12,22,23], but authors did not give any morphological details about those nematodes. Thus, so far, detailed data concerning validity, morphology, ecology and distribution of *C. phenisci* are highly insufficient. Furthermore, nematodes from family Syngaminae occurring in avian hosts, usually have great veterinary importance: under certain conditions, they can cause serious pathological

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lesions and death of infected birds [24]. Thus, it is necessary to conduct detailed analysis of taxonomy and ecology of Syngamidae for economic (poultry farming) and conservation reasons (e.g., potential negative effect on population of vulnerable or endangered bird species, risks associated with translocations of wild animals) [25,26]. In our opinion, final confirmation of the validity of *C. phenisci*, irrespective of evaluation of different morphological and morphometric analysis, requires molecular methods. Taxonomic investigations based on molecular markers have proved to be invaluable in establishing systematic relationships at all taxonomic levels [27], however, within subfamily Syngaminae such investigations are scant [15].

Considering the current state of the presented problem, the main objective of this research was morphological and molecular identification of *C. phenisci*, a parasite of the endangered African penguin *Spheniscus demersus* from the eastern coast of South Africa, supplemented by ecological and epidemiological analysis of occurrence of this nematode among African penguin's population. Simultaneously, this is the first report of *C. phenisci* associated with the African penguin.

2. Material and methods

2.1. Host characteristics, sampling protocols and necropsy procedures

The African penguin *Spheniscus demersus* (L., 1758), is the only penguin species that breeds in Africa. Number of African penguins dramatically decreased from over 1.45 million individuals at the start of 20th century to 21,000 breeding pairs in 2009 and conservation status of this species has been re-classified from Vulnerable to Endangered [28,29]. African penguins, beached along the east coast of South Africa are brought to the Penguins Eastern Cape Marine Rehabilitation Centre (PEC) for rehabilitation and eventual release. Necropsies were carried out on penguins that die at PEC, as well as those die in transit to PEC or on fresh carcasses found on the beach. Birds were aged as nestlings, "blues" (chicks, that have left the nest, but not yet molted into juvenile plumage), juveniles (yearlings that will stay out the sea, until they are mature) and adults, and sexed based on the state of the gonads. In years 2006–2011 a total of 94 African penguins (13 nestlings, 42 "blues", 24 juveniles and 12 adults) were analyzed for a complete helminthological examination. In addition, there were three birds that we did not have verified data on their age and so they were excluded during statistical analysis. Nematodes found in tracheae and air sacs were washed in physiological salt solution, fixed in Petri dishes in 70% ethanol and stored in the same medium. Because determination of the sex failed in 35 of the necropsied penguins, we do not analyze sex-dependent relationships in *C. phenisci* occurrence.

2.2. Morphological identification, ecological terms and statistical analysis

Preserved nematodes were cleared in glycerine or lactophenol, examined and measured using a Leica DM2000 microscope with LAS 3.7 software and identified according to the original description [18]. Drawings were made with aid of a camera lucida. The ecological terms in present work are used as defined by Bush et al. [30]. Parameters of occurrence of *C. phenisci* in necropsied penguins in relation to their ages were analyzed using Fisher exact tests (for prevalence) and Moods median test (for mean intensity and mean abundance). Calculations were carried out using the software package Quantitative Parasitology 3.0 [31].

Voucher specimens have been deposited in the Polish Collection of Parasitic Helminths, Museum of Natural History, Wrocław University, Coll. No. 134271.

2.3. Molecular analysis

Irrespective of sampled specimens of *C. phenisci*, for comparative molecular analysis, we use individuals of *Cyathostoma (Cyathostoma) mirospiculum* (Skrjabin, 1913) collected from a typical host, the great cormorant *Phalacrocorax carbo* from northern Poland [16], fixed as above and stored in 70% ethanol.

Total genomic DNA was extracted from a single worm using DNeasy Blood and Tissue Kit (Qiagen, Düsseldorf, Germany). Specimens of *C. microspiculum* and *C. phenisci* were transferred from 70% ethanol to ATL lysis buffer with Proteinase K and incubated overnight at 56 °C. After digestion, the lysis buffer containing nucleic acids was transferred to a fresh Eppendorf tube and stored for DNA isolation according to the manufacturer's protocol. Amplification of two DNA markers, i.e. LSU of rDNA and ITS1-5.8SrDNA-ITS2 was done using the following primers: ITS complex [32] — NLF/NLR (5′-TTTGyACACACCGCCCGTCG-3′/5′-ATA TGCTTAArTTCAGCGGGT-3′) and LSU [33] — C1/D1 (5′-ACCCGCTGAAT TTAAGCAT-3′/5′-TCCGTGTTTCAAGACGG-3′) in the following thermocycling conditions: 95 °C/3 min — initial denaturation; 95 °C/30 s, 48 °C/30 s, 72 °C/45 s — 40 cycles; 72 °C/7 min — final extension. The PCR reaction (25 μl) was contained: 4 μl of genomic DNA, 10 mM Tris–HCl, 50 mM KCl, 1.5 mM MgCl2, 200 μM of each dNTP, 150 pmol of each primer and 2 units of Taq polymerase (EurX, Gdańsk, Poland). The amplification product was purified using QIAquick PCR purification kit (Qiagen, Düsseldorf, Germany) and sequenced on both directions (Genomed S.A., Poland). The obtained sequences were deposited in GenBank under following accession numbers KC764948–KC764951.

In order to elucidate any homologies with previously deposited sequences in GenBank, we conducted a BLAST search (www.ncbi.nih.gov/BLAST/). The multiple sequence alignment was carried out by the online version of MAFFT program ver. 7 with the L-INS-i option [34]. Phylogenetic analysis of *Cyathostoma* was inferred using the Maximum Likelihood method implemented by MEGA 5 package [35]. The tree has been rooted with *Toxocara canis* (JF837169).

3. Results

3.1. Necropsy results, morphological and morphometric features

In 13 out of 94 necropsied African penguins (overall prevalence 13.8%) a total of 33 nematode specimens (20 females, 13 males) were found. These were identified as *Cyathostoma (Cyathostoma) phenisci* Baudet 1937. The highest prevalence was observed in juveniles (6 infected, 25%) and "blues" (6 infected, 14.3%), before nestlings (1 infected, 7.7%); no nematodes were found in adult penguins. Similarly, the highest mean intensity was observed in "blues" penguins (2.83; mean abundance 0.4), and juveniles (2.5; mean abundance 0.62), and the lowest was found in nestlings (1; mean abundance 0.08). However, differences in *Cyathostoma* occurrence in relation to host ages were not significant (prevalence: Fisher exact test, $p = 0.23$; mean intensity and mean abundance: Moods median tests, $p > 0.99$).

Description: Adult nematodes small to intermediate size, brown–white after fixation. Cuticule smooth, translucent. Buccal capsule well developed, with thick chitinized wall. On the bottom of the buccal capsule five to six medium-size teeth. Esopagus relatively short, but strong and muscular, with characteristic widening at the distal end. Excretory pore and nerve ring encircling esopagus indistinct. The detailed comparison of selected biometrical and morphological characteristics of *C. phenisci* are provided in Table 1. The measurements (mean values) of the most representative structures, expressed in millimeters, are given below.

Male ($n = 6$). Total body length 12.22, maximum width on the level on mid-body 0.447. The mouth opening rounded. Buccal capsule wider than longer, cup shaped 0.287 × 0.244. Head collar usually absent, sometimes weakly developed (Fig. 1A). Esopagus 0.828 long and 0.171 wide. Copulatory bursa well developed. Dorsal ray at base is bifurcated into two wide rays, reaching about to the half of the length of copulatory bursa; approximately at 1/2 length dorsal ray is again bifurcated into two symmetrical lobes at each side, reaching the edge of the copulatory bursa. Dorsal ray at 2/3 length divided into two additionally thin and short symmetrically lobes, reaching the end of copulatory bursa; distal part of dorsal ray reaches beyond the end of copulatory bursa, to form a