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SHORT COMMUNICATION

Molecular Identification of Plerocercoids of *Clistobothrium montaukensis* (Cestoda: Phyllobothriidea) Parasitizing the King of Herrings *Regalecus glesne*

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Abstract

Purpose Endo-parasites of the bathypelagic king of herrings *Regalecus glesne* and oarfish *Regalecus russelii* are only known from few specimens opportunistically examined. As a consequence, there are few records of parasites from either *Regalecus* species. We report plerocercoid larvae of phyllobothriidean cestodes parasitizing an adult *R. glesne* stranded in Bahía de La Paz, Baja California Sur, Mexico.

Methods Sixty-three plerocercoids were obtained from the intestine of *R. glesne* and characterized using morphological and molecular methods (nuclear 28S rDNA and mitochondrial cytochrome c oxidase I gene sequences).

Results Following the morphological diagnostic criteria of scolex and muscle bands in the strobila, plerocercoids specimens were preliminary assigned to the genus *Clistobothrium.* Mitochondrial and nuclear DNA sequences indicate these plerocercoids correspond to *Clistobothrium montaukensis* Ruhnke, 1993.

Conclusion *Regalecus glesne* is a new host known for *C. montaukensis* and this report is a new geographical record of *C. montaukensis* parasitizing species of the genus *Regalecus* previously known only from California and Florida, USA.

Keywords Cestoda · Regalecidae · Oarfish · 28S rDNA · *Cox*1 · Gulf of California

Introduction

The genus *Regalecus* includes two species: king of herrings *Regalecus glesne* Ascanius, 1772 and oarfish *Regalecus russelii* (Cuvier 1816). Both species typically inhabit the bathypelagic strata (200–1000 m depth) in tropical and temperate oceans [1–3]. However, their planktonic eggs may drift over the continental shelf [4]. Both *Regalecus* species have been reported in the northwest region of Mexico, including the Gulf of California [5–10]. Parasites of *R. glesne* and *R. russelii* are scarcely known because the rare finding of specimens [2, 11–13]. Metazoan parasites of *R. glesne* have been reported only in a few studies, including: an unidentified tetraphyllidean cestode (plerocercoid) from Florida, USA [11], an adult digenean *Syncoelium regaleci* (Syncoeliidae) Villarreal and Dailey, 1993 from the Gulf of California, Mexico [12] and an unidentified ectoparasitic isopod observed in videotapes of living *R. glesne* in the northern Gulf of Mexico, USA [2]. A total of 20 plerocercoids of *Clistobothrium* cf. *montaukensis*, two larvae of *Contracaecum* sp. (Nematoda), and an unidentified adult acanthocephalan of

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the family Arhythmacanthidae were reported parasitizing *R. russelii* in California, USA [13].

The goal of the present study was to report Phyllobothriidae plerocercoids identified based on the morphology and nuclear (28S rDNA) and mitochondrial (cytochrome c oxidase subunit I gene, *cox*1gene) DNA sequences parasitizing an adult *R. glesne* found stranded at Bahía de La Paz, Gulf of California, Mexico.

Materials and Methods

Collection and Dissection of *R. glesne*

An adult female of king of herrings identified as *R. glesne* was found stranded on the beach at Bahía de La Paz, Baja California Sur, Mexico (24° 09′ 30″ N, – 110° 19′ 11″ W) in May 28, 2014. The specimen measured 5.4 m total length and was in fresh body condition (recently died). A sample of 63 plerocercoids were obtained from the intestine during the dissection, no other helminths were detected. Half of the cestodes were fixed in cold formalin 4% saturated with sodium borate for morphological identification and the other half were fixed in 96% ethanol for molecular purposes.

Morphological Identification of Plerocercoids

Plerocercoid specimens were photographed using a Canon Power Shot A2500 digital camera installed in a Carl Zeiss SV11 light stereoscope. Formalin fixed specimens were dehydrated through a series of gradual ethanol from 30 to 96%, then stained with Gömöri trichrome, cleared with clove oil and permanently mounted in synthetic resin (60% xylene) on a slide following the method described for the study of larval stages of Platyhelminthes [14, 15]. Specimens were measured using a calibrated micrometer installed in the eyepiece of the Carl Zeiss SV11 stereoscope. The length of the body, scolex and strobile of ten plerocercoid specimens were measured with a compound microscope (Leica DMLB, USA) equipped with a calibrated micrometer (Meyer Instruments). The mean length and range of all morphological measurements of the plerocercoids were reported in millimeters (mm). Plerocercoid biometry and morphology of the scolex were compared with previous records [11, 16]. Two plerocercoid specimens were observed with a Scanning Electron Microscope (SEM, Hitachi S-3000 N) following a standard protocol described in a previous study of helminths [17]. Nomenclature of microtriches in plerocercoids was used following standard criteria [18]. One plerocercoid specimen was deposited in Colección Parasitológica del Museo de Historia Natural, Universidad Autónoma de Baja California Sur, La Paz, Mexico (accession number: CPMHN-UABCS-724); and three specimens were deposited in Colección Nacional de Helmintos, Instituto de Biología, Universidad Nacional Autónoma de México, Mexico City (accession numbers: CNHE 11151–11153). No hologenophores were deposited in any of these two parasitological collections.

Molecular Identification (DNA Extraction, Gene Amplification, and Sequencing)

Total DNA of three plerocercoid specimens was extracted using the Kit QIAGEN at the Laboratorio Nacional de Biodiversidad (LANABIO, IB-UNAM, Mexico City) and used for the amplification of the D1-D3 region of the nuclear 28S rDNA gene (28S). DNA from other three plerocercoid specimens was extracted using the automated Glass Fiber protocol [19] in the Barcode of Life facilities located at Centro de Investigaciones Biológicas del Noroeste (CIBNOR, La Paz, Baja California Sur, Mexico) to amplify partial mitochondrial cytochrome c oxidase subunit I (*cox*1). Both genes were amplified through polymerase chain reaction (PCR). The primers JB3 5′ TTTTTTGGGCATCCTGAGGTTTAT 3′ [20] and CO1-R-Trema 5′ CAACAAATCATGATGCAA AAGG 3′ [21] were used for *cox*1 gene fragment and primers ZX-1 5′ ACCCGCTGAATTTAAGCATAT 3′ [22] and 1500R 5′ GCTATCCTGAGGGAAACTTCG 3′ [23] for the 28S gene fragment. Amplification reactions were performed in a thermo-cycler Eppendorf (Mastercycler Pro) following the next profile for *cox*1, 3 min at 96 °C, then 35 cycles 30 s at 94 °C, 2 min at 56 °C and 90 s at 72 °C, with a final extension of 5 min at 72 °C. The profile for nuclear 28S included 2 min at 94 °C, then 40 cycles of 30 s each at 94 °C, 30 s at 54 °C, 2 min at 72 °C, with a final extension of 7 min at 72 °C. Each PCR reaction included 1 µl of genomic DNA (10–30 ng/µl), 3.6 µl of 5X PCR Buffer, 0.9 µl of each primer (10 µM), and 0.15 µl of MyTaq (5U/µl, Bioline) for a total volume of 18 µl. Sequencing reactions were carried out in an Applied Biosystem 3500×1 sequencer (24 capillaries) (Life Technology Corporation, Thermo Fisher Scientific, Singapore) at the Laboratorio Nacional de Biodiversidad (LANABIO, IB-UNAM, Mexico City). The same primers used for *cox*1 PCR were used for sequencing reactions. For sequencing reactions of the 28S gene, in addition to the PCR primers, we used the internal primers 300F 5′ CAAGTA CCGTGAGGGAAAGTTG 3′ [24], ECD2 R 5′ CTTGGT CCGTGTTTCAAGACGGG 3′ [24], 1090F F 5′ TGAAAC ACGGACCAAGG 3′ [25], and 400R R 5′ GCAGCTTGA CTACACCCG 3′ [26].

Sequences were assembled and edited using GENEIOUS 11.1.4 software [27]. A BLAST analysis [28] was performed to compare with sequences deposited in GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) and BOLD Systems (www.boldsystems.org). However, the *cox*1 tree is here shown only for future comparative purposes. Taxon for the phylogenetic

analyses was selected based on Caira *et al*. [29–31] and sequences available in GenBank. DNA sequences of 28S and *cox*1 with > 95% identity similarity compared with the newly generated sequences were selected (Table S1, Supplemental information). *Thysanocephalum crispum* (Linton, 1889) Linton 1890 was selected to root the 28S analysis based on previous phylogenetic studies [30, 31] and *Acanthotaenia shipleyi* von Linstow, 1903 was selected to root the *cox*1 analysis based on BLAST results and a previous study [32]. Maximum Likelihood phylogenetic analyses of both genetic markers were performed using RaxML v. 8.2 [33] with the command line version, with 10,000 bootstrap replicates using the general time reversible model (GTR) with gamma distributed rate parameter and invariable regions model selected by default. 28S and *cox*1 gene sequences of plerocercoids parasitizing *R. glesne* generated in the present study were deposited at GenBank (Table S1). The lengths of the three 28S sequences deposited at GenBank were 972 bp (MT772143), 864 bp (MT772143), and 891 bp (MT772145), and the lengths of the three *cox*1 sequences deposited in BOLD System were 895 bp (MT772382, MT772383), and 539 bp (MT772384) (Table S1).

Results and Discussion

A sample of 63 plerocercoid specimens (Fig. 1A) were recovered alive from the anterior part of intestine of *R. glesne*. Morphological comparative morphometry and maturity stage of plerocercoid larvae parasitizing *R. glesne* are shown in Table S2 (Supplemental information). Plerocercoid specimens showed an apical sucker in the scolex and four longfoliose bothridia (two-dorsal and two-ventral bothridia) provided with an anterior round muscularized accessory sucker and posterior loculus (Fig. 1B, C). Loculus foliose is in form of folding flap of tissue (Fig. 1B), Strobila with distinct longitudinal band of muscles (Fig. 1A). These morphological traits are diagnostic of the larvae of the genus *Clistobothrium* (Ruhnke 1993) [16, 34]. Tegument of larval body was covered with acicular filitriches. Although the morphology of plerocercoid specimens analyzed in the present study was similar to the plerocercoid previously reported from *R. glesne* [11] and *R. russelii* [13, 34], several morphological differences in the measures of length of the scolex and the total length of the larval body were detected (Table S2). The total length of the single plerocercoid specimen (3.12 mm) reported previously [11] is shorter than the ten specimens measured in the present study (6.57–9.27 mm) (Table S2). However, based on morphological data, we assign these 63 plerocercoid specimens to the genus *Clistobothrium*. Kuris *et al*. [13] reported the plerocercoid of *Clistobothrium* cf. *montaukensis* parasitizing *R. russelii* from Santa Catalina Island, California, USA and noted that the morphology of the unidentified plerocercoid found parasitizing *R. glesne* in Florida, USA [11] was congruent with the *Clistobothrium* specimens of their study, and in general, congruent with the morphology of the specimens reported in the present study.

The final 28S gene matrix included 64 terminals and 972 aligned nucleotides. The log-likelihood of the optimal ML tree analysis was – 1544.320017. The phylogenetic analysis places the three sequences reported in the present study within a group of *C. montaukensis* and an unidentified tetraphyllidean from the squid *Illex coindetii* Vérany, 1839 collected in the Mediterranean Sea with a 61% bootstrap value (Fig. 2A). Importantly, the specimens of the present study group with sequences of *C. montaukensis* obtained from adult worms parasitizing *Isurus oxyrhynchus* from New York, USA. Therefore, based on this evidence, the plerocercoid specimens collected from *R. glesne* stranded in Bahía de La Paz, Baja California Sur, Mexico belong

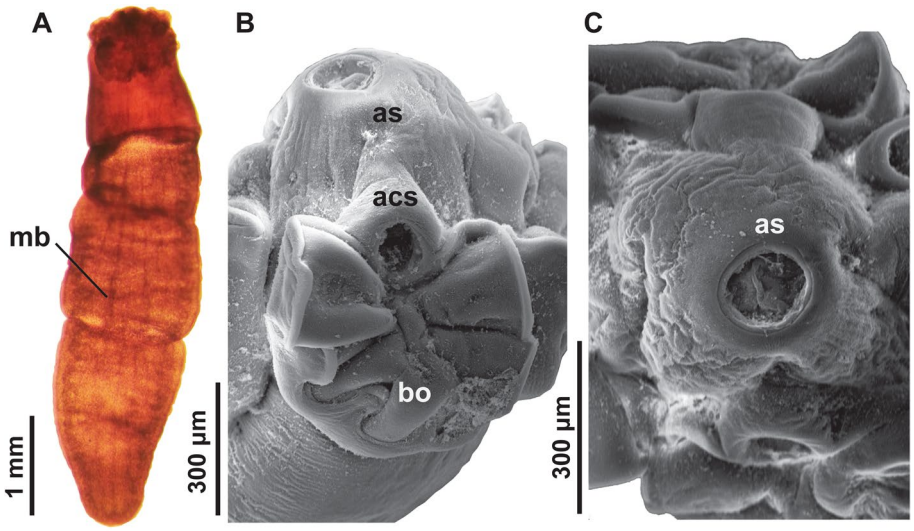

Fig. 1 *Clistobothrium montaukensis* obtained from the intestine of *R. glesne* found stranded at a beach of Bahía de La Paz, Gulf of California, Mexico. **A** *C. montaukensis* observed with Gömöri trichrome stain showing longitudinal muscle bands (mb) in the strobila. SEM images obtained at different magnifications (**B**) morphological detail of the scolex with the apical sucker (as), bothridia (bo) and the accessory sucker (acs) in each bothridium, **C** amplified of the apical sucker (as) of the scolex

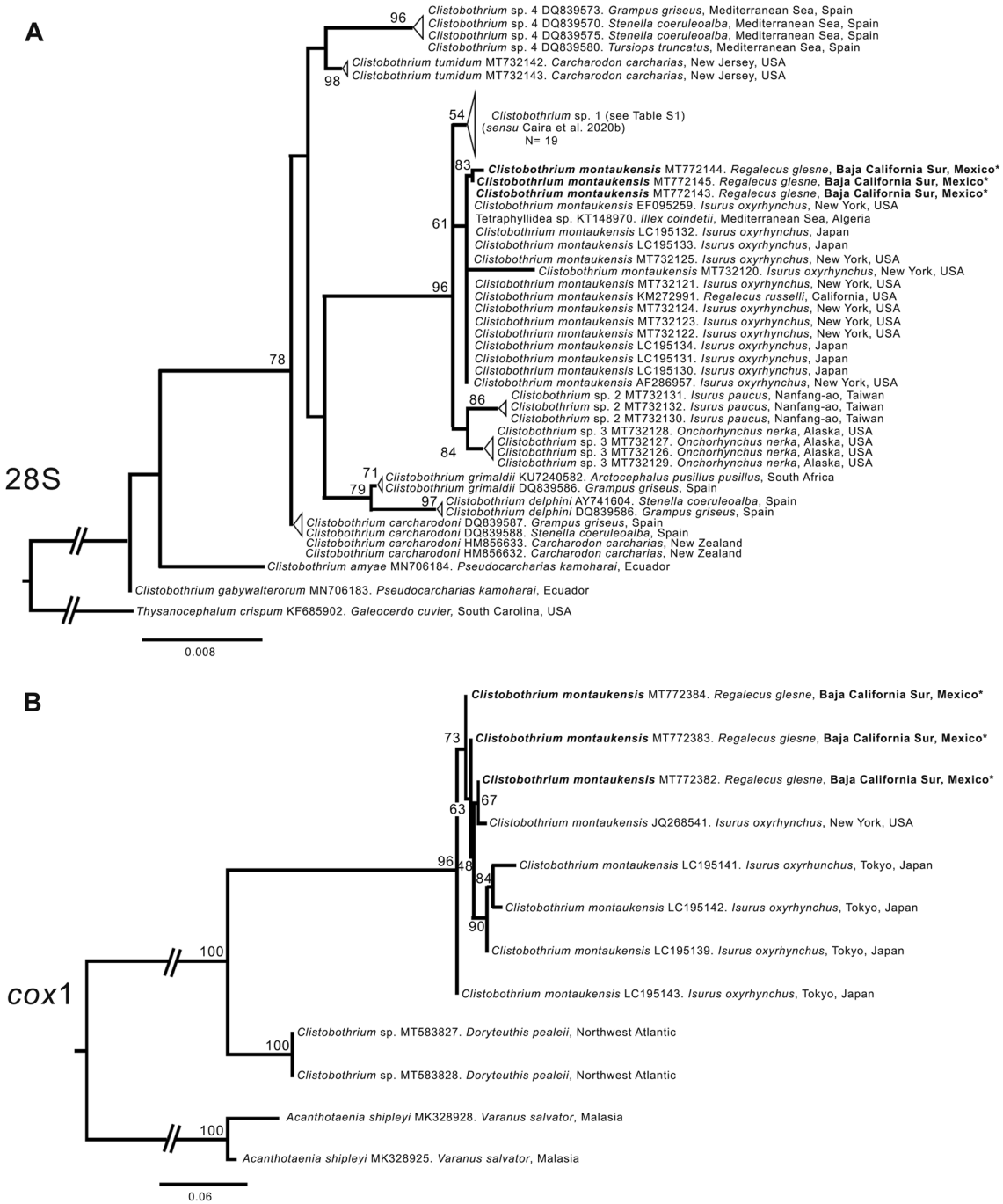

Fig. 2 Maximum likelihood phylogenetic tree, based on the analyses of: **A** the nuclear 28S rDNA gene (D1-D3 region) (28S) of cestodes representing major lineages according to Caira *et al*. [31] and **B** the partial mitochondrial cytochrome c oxidase subunit I gene (*cox*1). Values next to nodes indicate bootstrap values above 50%. For *Clistobothrium montaukensis* and species with a single representative, taxon names are followed by GenBank accession numbers. For species with more than one representative N indicates the number of DNA sequences included in the analysis. *Parasite collected from *R. glesne*; **Parasite collected from *R. russelli.* In bold, sequences generated in the present study. The genus and species of the host and the sampling location is shown for each sequence in 28S and *cox*1 gene trees

to *C. montaukensis*. Two samples obtained in the present study appeared forming a group; this variation is interpreted as intraspecific variation, especially after comparing the variation found among *C. montaukensis* and its congeners. Sister to *C. montaukensis* is a group formed by *Clistobothrium* sp. 1. These specimens were labeled as *Clistobothrium*

cf *montaukensis* from a squid host (*Doryteuthis pealeii*) reported in a previous study [35], but recently recognized as a separate species for which no formal description is still available [31] (Fig. 2A). Overall, the same groups recovered in the comprehensive study of Caira *et al*. [31] were also found in the present study, in particular the same clusters of samples representing distinct species (Fig. 2A). However, major differences in deeper nodes were found but with little support (< 70%). For example, in Caira *et al.* [31], *Clistobothrium amyae* and *C. gabywalterorum* form the sister group of all the species of *Clistobothrium* genus, whereas in the present analysis, only *C. amyae* is sister to all species of the genus *Clistobothrium*. It is important to mention that most of the internal nodes shown in the 28S tree of Caira *et al*. [31] and in the 28S tree of the present study have bootstrap values under 50%, indicating that the phylogenetic relationships within *Clistobothrium* still remain unresolved (Fig. 2A).

The comparison among the three newly generated *cox*1 sequences showed 15 variable sites, 14 of them correspond to third positions and only one was in a second position. Only two amino acid changes were detected when sequences are translated into proteins. The genetic distance among the three *cox*1 sequences was < 1% suggesting that the three analyzed specimens belong to the same species (Fig. 2B). BLAST comparisons recovered *Clistobothrium montaukensis* (JQ268541) infecting the shortfin mako shark *Isurus oxyrinchus* from New York, USA [36] as the closest match (98.4–99.5% of similarity) with a genetic distance between 0.5–1.7%. In the *cox*1 phylogenetic tree (Fig. 2B), the newly generated sequences from the present study form a group with the same parasite specimen found infecting the shortfin mako shark, together with four additional sequences identified as *C. montaukensis* for which no additional information is available (Fig. 2A). Interestingly, two unidentified samples of *Clistobothrium* were found forming a separate group (MT583827 and MT583827); these sequences were obtained from plerocercoids parasitizing the longfin inshore squid *Doryteuthis pealeii* collected in the Atlantic Northwest [35]. Fortunately, 28S sequences were generated from the same samples and they group with *Clistobothrium* sp.1 sensu Caira *et al*. [31]. Based on this information, this separate lineage of *Clistobothrium* found in the *cox*1 analysis most likely corresponds to *Clistobothrium* sp.1 sensu Caira *et al*. [31].

Life cycles of *Clistobothrium* species are partially characterized [31, 37, 38]. Previously, information of hosts and geographical distribution range of *Clistobothrium montaukensis* in plerocercoid larval stage includes cephalopods [39–41] and *Regalecus* species [11, 13] (Fig. S1A). Adult specimens of *Clistobothrium* species are known from large pelagic sharks of the families Lamnidae [16, 34, 42] and Pseudocarchariidae [31] (Fig. S1A). *Regalecus russelii* has been mentioned as paratenic hosts for species of *Clistobothrium* [13] and then be trophically transmitted to shortfin definitive hosts. Our study adds *R. glesne* to the lists of hosts for species of *Clistobothrium* and provides evidence that both *Regalecus* species might function as paratenic hosts for *Clistobothrium* spp. *Regalecus* species feed on euphausiid swarms, small herrings and squids [43, 44], and a previous study suggested that *R. russelii* becomes parasitized with phyllobothriidean procercoids after preying pelagic crustaceans infected with procercoids [13]. During the dissection of *R. glesne* analyzed in the present study, we observed small euphausiid crustaceans in the intestine; however, it was not possible from these semi-digested specimens to study if these crustacean were parasitized. Therefore, the host of *Clistobothrium* before parasitizing *R. glesne* is still to be confirmed in future studies.

The present study represents the first record of *C. montaukensis* parasitizing *R. glesne* (here identified based on morphological and molecular evidence) and this *C. montaukensis* report in Bahía de La Paz, Baja California Sur, Gulf of California (Fig. S1A, B) extends previous known biogeographic distribution of *Clistobothrium* parasitizing *Regalecus* in Florida and California, USA.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11686-021-00400-9.

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Declarations

Conflict of Interest The authors declare that they have not conflicts of interest.

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