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Morphological and molecular characterization of the metacercaria of *Paragonimus caliensis*, as a separate species from *P. mexicanus* in Costa Rica

Roderico Hernández-Chea [a,*], Ana Eugenia Jiménez-Rocha [b], Ruth Castro [a], David Blair [c], Gaby Dolz [a,b]

[a] Maestría en Enfermedades Tropicales, Posgrado Regional en Ciencias Veterinarias Tropicales, Universidad Nacional, Campus Benjamín Nuñez, Barreal de Heredia, P.O. Box 86, 3000 Heredia, Costa Rica
[b] Escuela de Medicina Veterinaria, Universidad Nacional, Campus Benjamín Núñez, Barreal de Heredia, Costa Rica
[c] College of Marine and Environmental Sciences, James Cook University, Townsville, QLD, 4811, Australia

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ABSTRACT

The trematode *Paragonimus mexicanus* is the etiological agent of paragonimiasis, a food-borne zoonotic disease in Latin America. This species, as well as *Paragonimus caliensis*, have been reported from Costa Rica, but it is not known if the two are synonymous. Two types of *Paragonimus* metacercariae from freshwater pseudothelphusid crabs from several localities in Costa Rica were recognized by light microscopy. Morphologically, these corresponded to descriptions of *P. mexicanus* and *P. caliensis*. Metacercariae of the former species lacked a membrane or cyst and their bodies were yellow in color. Those of *P. caliensis* were contained in a transparent thin cyst and were pink in color. Morphotypes of metacercariae were determined using scanning electron microscopy (SEM). Based on the number and distribution of papillae in the ventral sucker, three morphotypes were found for *P. mexicanus* and two for *P. caliensis*. Analysis of DNA sequences (nuclear ribosomal 28S and ITS2 genes, and partial mitochondrial *cox*1 gene) confirmed the presence of *P. mexicanus* and provided the first molecular data for *P. caliensis*. The two species are phylogenetically distinct from each other and distant from the Asian species. The confirmation of *P. caliensis* as a separate species from *P. mexicanus* raises several questions about the ecology, biological diversity, and epidemiology of the genus *Paragonimus* in Costa Rica.

1. Introduction

Paragonimiasis is a food-borne zoonotic disease, which is listed among the most important trematodiases in Asia, Africa and Latin America. It is caused by several species of the genus *Paragonimus*. Around 50 species have been described since this genus was erected by Braun in 1899 [1]. Of these, about ten are pathogenic for human beings. The best-known human pathogen is *Paragonimus westermani*, widely distributed in eastern and southern Asia, but others occur in Asia (*P. skrjabini* and *P. heterotremus*), Africa (*P. africanus* and *P. uterobilateralis*) and in the Americas (*P. kellicotti* and *P. mexicanus*) [2]. Eight species of *Paragonimus* have been named from the Americas [3]. The earliest of these was *P. rudis* (Diesing, 1850), adult worms of which were found in the lungs of the Brazilian otter, *Ptenourura brasiliensis*, on a single occasion in 1828 [4]. In recent years, *P. rudis* has been considered a *nomen nudum*, because the original description was inadequate and the type specimens are poorly preserved and do not allow a clear morphological identification [4,7]. The first report of a lung fluke from Costa Rica was by Caballero [5], who described adult worms he regarded as representing *P. rudis* in a gray fox, *Urocyon cinereoargenteus*. Brenes et al. [6] later described a non-encysted metacercaria of a "*Paragonimus* sp". The first report of *P. mexicanus* in Costa Rica was in 1968 [7]. It is most likely that the worms Caballero described as *P. rudis* from Costa Rica and Guatemala in fact belonged to *P. mexicanus* [6]. Rojas et al. [8] reported a morphologically different type of metacercaria that matched with the description of the metacercaria of *P. caliensis*, first discovered in Colombia by Little [9]. *Paragonimus caliensis* is often considered a morphological variant of *P. mexicanus* [10,11].

Given the relative paucity of morphological characters available for distinguishing among *Paragonimus* species, scanning electron microscopy has often been used to reveal details of subtle morphological features, such as tegumentary papillae, that might assist in taxonomy [12]. This approach has been used only rarely for metacercariae of *Paragonimus* species in Latin America. Hernandez and Monge [13] reported two different morphotypes of metacercariae in an ultrastructural study of the papillae on the ventral sucker. These morphotypes were indistinguishable using light microscopy. Given this background, it is important to discern whether or not different morphotypes of

* Corresponding author.
E-mail address: roderik123animal@gmail.com (R. Hernández-Chea).

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metacercariae represent different species, a task well suited to molecular studies.

To date, molecular studies have identified four complexes of *Paragonimus* species in Asia [1]. Among these are the *P. westermani* complex, including *P. westermani* and *P. siamensis* and the *P. ohirai* complex, including a number of nominal species such as *P. ohirai* and *P. harinasutai* [14]. It is not known whether *P. mexicanus* constitutes a complex of species in Latin America, or if it is the only species as suggested by Tongu [10].

The first reported human cases of paragonimiasis in Costa Rica were in two young girls with pleural effusion [15]. Since then 28 cases have been described, primarily in children from rural areas. The most recent case occurred in an eight-year-old boy with cerebral paragonimiasis in 2013, who fortunately recovered after neurosurgery [Adriana Yock, Hospital Nacional de Niños, personal communication, July 5, 2014]. Despite the long history and morphological descriptions of lung flukes in Costa Rica, there is no molecular evidence of the presence of this trematode in the country.

The objective of this study was to determine the species of *Paragonimus* present in freshwater crabs in Costa Rica through morphological, molecular, and phylogenetic analyses.

2. Materials and methods

2.1. Crab collection and dissection

Three hundred and forty-seven pseudothelphusid crabs were collected between March and November of 2015, in streams of the Atlantic and Pacific slopes of Costa Rica, in the provinces of Limón (Veragua: 9.9258 N, −83.1909 W, Guácimo: 10.1252 N, −83.64838 W, Talamanca: 9.5586 N, −82.8555 W), Heredia (Sarapiquí: 10.2442 N, −83.9693 W), Alajuela (Upala: 10.7262 N, −85.0562 W), and Puntarenas (Buenos Aires: 9.2821 N, −83.37071 W, Coto Brus 8.8229 N, −83.0193 W; Parrita 9.6048 N, −84.2323 W). Crabs were collected by lifting rocks and organic material on the edges of the streams. The cephalothorax of each crab was separated from the body, leaving soft tissues exposed. The organs (false lung, gills, heart and hepatopancreas) and muscle tissue were placed individually on Petri plates with 0.9% saline solution, while the chelae and limbs were crushed and then placed on Petri plates with 0.9% saline solution. Metacercariae were isolated from four genera of crabs: *Ptychophallus* spp., *Achlidon* spp., *Potamocarcinus* spp., and *Allacanthos* spp. Genera of crabs were identified by the morphological appearance of the gonopods by Dr. Ingo S. Wehrtmann and Fresia Villalobos, Centro de Investigación en Ciencias del Mar y Limnología (CIMAR), Universidad de Costa Rica.

2.2. Morphological characterization of metacercariae

All the Petri plates with the organs and tissues were directly examined using a stereoscope. Visible metacercariae were collected with a dropper and stored in tubes with 0.9% saline solution until processed. Some were fixed with 70% ethanol for observation using a light microscope (Nikon Eclipse E200, objectives 10× and 40×). Various measurements were taken; length and width of the body, length and width of the oral sucker, and length and width of the ventral sucker. For scanning electron microscopy, metacercariae were fixed in 80% glutaraldehyde, 10% paraformaldehyde, and 10% phosphate buffer, pH 7.2 for 2 min, then left to dry at room temperature for 5 min. The scanning electron microscope used was a Hitachi TM 3000. The ventral sucker was located, and the metacercariae morphotype was determined based on the number and distribution of papillae on this structure.

2.3. DNA extraction, PCR amplification, and sequencing

Total DNA was extracted from each metacercaria using the Dneasy® Blood & Tissue kit (QIAGEN 2006), following the manufacturer's recommendations. A portion of the nuclear 28S ribosomal DNA (rDNA) gene was amplified using the primers TSD2 (5′ GTACCGTGAGGGAAAGTTG-3′) and D4AR (5′-GTCCGTGTTTCAAGAC-GGG-3′) [16]. The nuclear ribosomal ITS2 region was amplified using the primers 3S (5′-CGG TGG ATC ACT CGG CTC GT-3′) and A28 (5′-CCT GGT TAG TTT CTT TTC CTC CGC-3′) [17], and a portion of the mitochondrial *cox*1 gene was amplified using primers FH5 (5-TTT TTT GGG CAT CCT GAG GTT TA-3′) and FH3 (5-TAA AGA AAG AAC ATA ATG AAA ATG-3′) [17]. Each PCR reaction contained 12.5 μl of Dream Taq™ PCR Master Mix (Fermentas®), 2.25 μl (10 pmol/μl) of each primer, 3 μl of genomic DNA and 5 μl of nuclease-free water, for a final volume of 25 μl. The DNA of an adult *Paragonimus heterotremus* was used as a positive control (kindly donated by Dr. Paron Dekumyoy, Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand). A reaction lacking only genomic DNA was used as a negative control. For the 28S rDNA gene, cycling conditions were an initial denaturation at 94 °C for 5 min, then 30 cycles (94 °C for 30 s, annealing of the primers at t 50 °C for 30 s, extension at 72 °C for 45 s), and a final extension at 72 °C for 5 min. For the ITS2 and *cox*1 regions, amplification conditions consisted of 35 cycles of denaturation at 94 °C for 1 min, annealing of the primers at 50 °C (*cox*1) or 52 °C (ITS2) for 1 min, extension at 72 °C for 2 min, with a final extension at 72 °C for 10 min. PCR products were separated by electrophoresis in 1% agarose gel, in TBE 1× buffer (Tris base, boric acid, EDTA, pH 8, 0.5 M), and stained with GelRed™ Nucleic Acid Gel Strain. The GeneRuler 100 pb DNA Ladder Plus (Fermentas®, Ontario, Canada) was used as a molecular weight marker. The expected products of 560, 285 and 335 bp for 28S rDNA, ITS2 and *cox*1, respectively, were sent to Macrogen Inc. (Seoul, Korea) for sequencing.

2.4. Sequence analyses and construction of phylogenetic trees

The similarity of each sequence to those available in the data base (GenBank) of the U.S. National Center for Biotechnology Information was assessed using the BLASTn algorithm [18]. Sequence editing and alignment were performed using BioEdit 7.2 sequence editor program [19] and ClustalW [20] multiple alignment, respectively. The best nucleotide substitution model was estimated independently for each dataset using jModelTest 2.1.3 [21]. The best-fitting model for the 28S rDNA sequence alignment was the General Time Reversible (GTR + G), and for the ITS2 and *cox*1 datasets it was the Hasegawa-Kishino-Yano plus Gamma (HKY + G) model. Phylogenetic reconstruction was performed using maximum likelihood (ML) analysis with MEGA 6 [22], and Bayesian inference (BI) was conducted using MrBayes [23,24]. In MrBayes, two parallel runs, each of four chains, were run for 1000,000 generations and sampled every 1000 generations, the first 25% of the sampled trees were discarded as "burn-in" for each dataset. Convergence of runs was considered complete when the average standard deviation of split frequencies was <0.01. The consensus trees were drawn and labeled using the program Figtree v.1.4 [25]. Percentage similarities between sequences were calculated using the sequence identity matrix in BioEdit. A pairwise distance matrix of the 28S rDNA sequences was calculated with MEGA 6 [22]: the analysis involved 25 nucleotide sequences and all ambiguous positions were removed for each sequence pair.

3. Results

3.1. Morphology of metacercariae by light microscopy

Five hundred and ninety-nine metacercariae were collected from a total of 347 crabs examined. Two types of metacercariae of the genus *Paragonimus* could be distinguished using light microscopy (Fig. 1A). The first type of metacercaria (588 specimens found) was observed freely moving in the crab tissues (without membrane or cyst) and was yellow in body color but contained reddish granules, and yellow caeca (Fig. 1B). The morphometric details of a total of 20 metacercariae of