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Where are the South American freshwater turtle blood flukes (Trematoda: Spirorchiidae)? The first morphological and molecular analysis of spirorchiid cercariae from freshwater snails in Brazil

Hudson Alves Pinto [a,*], Alan Lane de Melo [a], Sara Vanessa Brant [b]

[a] Laboratório de Taxonomia e Biologia de Invertebrados, Departamento de Parasitologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Brazil
[b] University of New Mexico, Department of Biology, Albuquerque, NM 87111, USA

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ABSTRACT

Trematodes belonging to the family Spirorchiidae are blood parasites mainly of turtles with a worldwide distribution. These flukes were recently reported in some marine turtles from South America, where the occurrence of spirorchiids in freshwater definitive and intermediate hosts is so far unknown. In the present study, three morphotypes of brevifurcate apharyngeate distome cercariae found in freshwater molluscs from an urban reservoir in Brazil were used for morphological and molecular (nuclear 28S rDNA) evaluation. Two morphotypes of cercariae, probably congeneric species, were found in 12/17,465 specimens of *Biomphalaria* spp. and differ from each other by body size and sequences (0.1%). They present morphology similar to North American freshwater spirorchiids (*Spirorchis* spp.), however surprisingly molecular data reveals that these lineages are more closely related to marine spirorchiids. A third species found in 2/777 *Pomacea* sp. differs morphologically from all previously described spirorchiid cercariae and genetically from spirorchiids with available sequences (16–19%), grouping in the phylogenetic tree with freshwater North American species. This is the first report of freshwater spirorchiids in South America and the first molecular confirmation of the involvement of a caenogastropod in the life cycle of spirorchiids.

1. Introduction

The trematode species belonging to the family Spirorchiidae Stunkard, 1921 are parasites of the circulatory system of reptiles with a worldwide distribution. This family of blood flukes includes 19 genera and more than 100 species described from turtles [1–3], and a single dioecious species (*Griphobilharzia amoena* Platt and Blair, 1991) from crocodile in which inclusion in this family is supported by molecular phylogenetic data [4]. In turtles, parasitism by these blood flukes can cause severe pathology, including miliary granuloma formation around the eggs of the spirorchiids in different organs and cardiovascular lesions, which may result in the death of infected hosts [5–8].

The life cycle of spirorchiids, known for a few freshwater species and one marine species, has two hosts, a gastropod as intermediate and a vertebrate as definitive, like their sister family of blood flukes, Schistosomatidae. As is currently known, freshwater spirorchiids are transmitted by pulmonate snails, in which cercariae are produced by sporocysts and after emergence, they penetrate into the oral and ocular mucosa of the turtle definitive hosts [9]. Given the difficulties inherent in maintaining laboratory life cycle studies of these parasites as well as inability to identify larvae to species, molecular tools have been shown as an ideal approach in order to identify new biological interactions between molluscs and spirorchiids [10–12]. In this sense, the molecular study of larval stages found in the potential intermediate hosts in the life cycle of spirorchiids can provide evidence of unknown species, species diversity, host use and distribution. These data will also provide a baseline for spirorchiid diversity from which helminthological studies on turtles can be compared, and life cycles revealed, that will connect such studies across time and space.

Most studies on spirorchiids have been focused in North America, Africa and Asia [1]. Despite the species diversity within Spirorchiidae, there have been few studies in South America. In fact, only 7 species of spirorchiids were reported in marine definitive hosts from Brazil in the last decade [13,14] and there are no reports of spirorchiids from freshwater turtles. Regarding larval stages, despite the diversity of furcocercariae reported in snails from Argentina, Brazil and Venezuela [15–17], none of these cercariae were until now linked to spirorchiids. The scarcity of helminthological studies on these cold blooded vertebrate hosts is surprising and is in contrast to the species diversity of turtles recorded from this subcontinent.

As a result of a long term survey of larval trematodes found in gastropods in Brazil, we report for the first time the morphological, molecular and phylogenetic identification of freshwater spirorchiids in South America. Moreover, the first confirmed report of a caenogastropod snail as intermediate host of spirorchiids is presented.

* Corresponding author.
E-mail address: hudsonalves13@ig.com.br (H.A. Pinto).

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2. Material and methods

As part of the initiative to expand our knowledge of trematodes transmitted by freshwater molluscs in Brazil, malacological surveys were carried out at Pampulha Reservoir, an urban artificial eutrophic reservoir located in Belo Horizonte, state of Minas Gerais (19′51′77″S; 43′58′54 W), between 2009 and 2013. Among a diversity of larval trematodes found in molluscs, three morphotypes of brevifurcate apharyngeate distome cercariae were found and used for morphological and molecular studies.

The molluscs were collected with the aid of a D-shaped nylon hand net. In the laboratory they were placed individually in wells of culture cell plates containing 2 mL of chlorine-free water and subjected to artificial photostimulation followed by examination under a stereomicroscope to detect infection with free swimming larval trematodes. Emerged cercariae were characterized preliminarily under optical microscopy with the aid of vital stains (0.05% neutral red and Nile blue sulfate) and after heat killing in water at 70 °C, fixed in 10% formalin. Measurements were made with the aid of a micrometer eyepiece and are presented, in micrometers, as the mean followed by the standard deviation and the range in parentheses. Some cercariae were stained with alum acetocarmine, dehydrated in increasing series of ethanol, cleared in beechwood creosote, and mounted as permanent slides in Canada balsam. Samples of cercariae were deposited in the collections of the Laboratory of Taxonomy and Biology of Invertebrates, Brazil (accession number DPIC 2580, 2565, 2567) and Museum of Southwestern Biology Division of Parasites, University of New Mexico, USA (accession number MSB:Para: 20804–20807).

For the genetic studies, samples were fixed in 90% ethanol and subsequently a pool of cercariae (5 larvae) were subjected to DNA extraction followed by polymerase chain reaction (PCR) of the 5′ region of the nuclear 28S ribosomal DNA (1000 bp). The following primer pair was used for PCR U178/L1642 then added to those, ECD2 and DIG12 were used for sequencing, from Lockyer et al. [18]. DNA extraction, PCR conditions, sequencing and sequence analysis methods were as described by Brant et al. [10]. The sequences obtained were compared to those reported for other spirorchiids and schistosomatids available in GenBank. Phylogenetic analyses were performed with Bayesian inference (BI) using MrBayes [19]. The BI analyses were as follows: nst = 6 rates = invgamma ngammacat = 4. Four chains were run simultaneously for 5×10^5 generations, with 4 incrementally heated chains sampled at intervals of 100 generations. The first 5000 trees with preasymptotic likelihood scores were discarded as burnin, and the retained trees were used to generate 50% majority-rule consensus trees and posterior probabilities. The outgroup was designated as Sanguinicolidae [10]. The sequences obtained were deposited in GenBank (accession numbers KT235798–KT235800).

3. Results

From 17,465 specimens of *Biomphalaria* spp. screened, 12 were found infected with larvae here designated as Spirorchiidae sp. 1 (Fig. 1A–C) [2/606 (0.16%) *Biomphalaria glabrata* (Say, 1818), 8/16,235 (0.04%) *Biomphalaria straminea* (Dunker, 1848), and 1/644 (0.15%) *Biomphalaria tenagophila* (d'Orbigny, 1835) (Planorbidae)]. A morphologically closely related species designated as Spirorchiidae sp. 2 (Fig. 1D–F) was found in 1/606 specimens of *B. glabrata*. A third species of spirorchiid cercaria emerged from 2/707 (0.28%) of the ampullarid *Pomacea* sp., which is here designated as Spirorchiidae sp. 3 (Fig. 1G–I). Four other species of molluscs, i.e., 323 specimens of *Pseudosuccinea columella* (Say, 1817) (Lymnaeidae), 2384 specimens of *Physa acuta* Draparnaud, 1805, 199 specimens of *Stenophysa marmorata* (Guilding, 1828) (Physidae) and 11,468 specimens of *Melanoides tuberculata* (Müller, 1774) (Thiaridae), were not found harboring larval spirorchiids.

Both types of cercariae found in planorbids of the genus *Biomphalaria* present general morphology similar with that described for other spirorchiids. This includes the presence of a brevifurcate tail with furcae presenting prominent fin-folds extending beyond tip and with dense striated lines or rays (similar to spines) in the entire extension (Fig. 1C and F), a pair of spherical pigmented eyespots, pharynx absent, ventral sucker equatorial, six pairs of laterally arranged penetration glands, and flame cell formula 2 [(1 + 1 + 1) + (1 + 1 + 1)] = 12. When at rest, the tails of the cercariae remain bent into a U-shape. Although the general morphological traits are similar, the cercariae of Spirorchiidae sp. 2 are larger than Spirorchiidae sp. 1 (Table 1).

The cercariae of Spirorchiidae sp. 3 found in *Pomacea* sp. differ significantly from these two cercariae found in *Biomphalaria* in the present study as well other spirorchiid cercariae characterized previously by

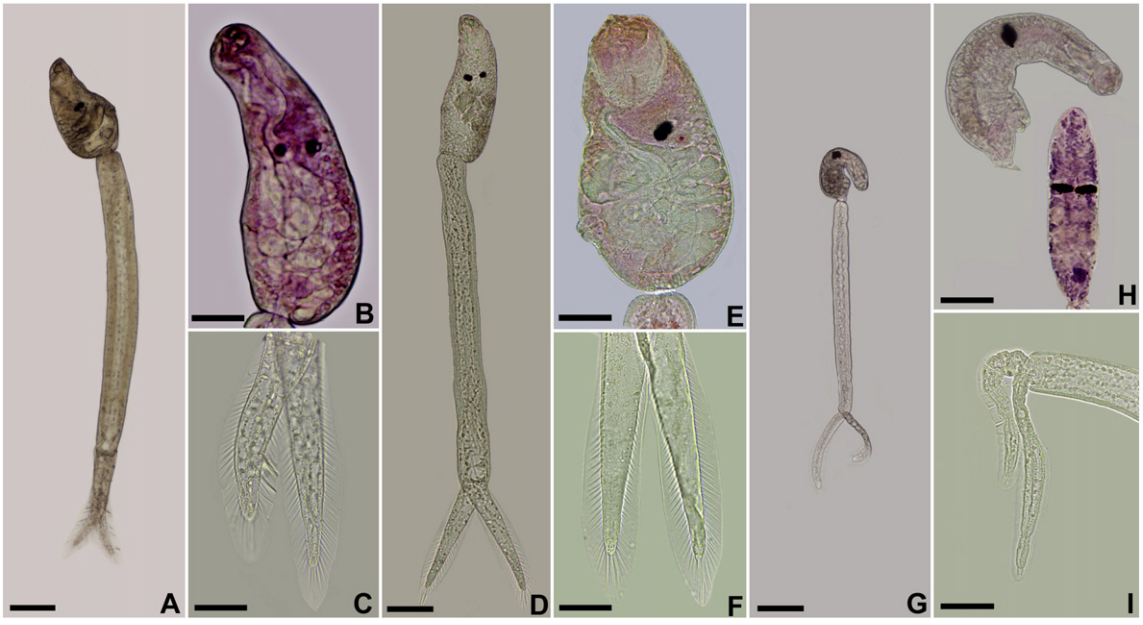

Fig. 1. Larval spirorchiids found in freshwater snails from Brazil. (A–C) Spirorchiidae sp. 1 and (D–F) Spirorchiidae sp. 2 from *Biomphalaria straminea*. (G–I) Spirorchiidae sp. 3 from *Pomacea* sp. Panels B, E, H show details of the cercarial body; and panels C, F, I show the detail of the furcae. Scale bars A, D, G = 100 μm; all other = 50 μm.