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, New Mexico, 87111, USA.

* Corresponding author.

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

Abstract

Trematodes belonging to the family Spirorchiidae, 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 DNA 28S) 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.

Key-Words: cercariae, *Biomphalaria*, molecular phylogeny, *Pomacea*, Spirorchiidae, Brazil.

Introduction

The trematode species belonging to the family Spirorchiidae Stunkard, 1921 are parasites of the circulatory system of reptiles with a worldwide distribution including 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 a 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.

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 2mL 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 sulphate) 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. (2003) [18]. DNA extraction, PCR conditions, sequencing and sequence analysis methods were as described by Brant et al. (2006) [10]. The sequences obtained were compared to those reported for other spirorchiids and schistosomatids available in GenBank. Phylogenetic analyses reconstructed 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).

Results

From 17,465 specimens of *Biomphalaria* spp. screened, 12 were found infected with larvae here designated as Spirorchiidae sp. 1 (Figure 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 (Figure 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 (Figure 1G–I). Four other species of molluscs, i.e. 323 specimens of *Pseudosuccinea columella* (Say, 1817) (Lymnaeidae), 2,384 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 (Figure 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 generally 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 different authors (compiled by

Combes et al., 1980). These cercariae are smaller than the larvae found in planorbids and despite the presence of the brevifurcate tail with furcae presenting prominent fin-folds, extending beyond tip, the rays are not present. Moreover these cercariae present the pigmented rod-shaped eyespots and a ventral sucker located at a more posterior position. Similarly to other spirorchiid cercariae, those of *Spirorchiidae* sp. 3 also present six pairs of laterally arranged penetration glands and flame cell formula 2 $[(1+1+1) + (1+1+1)] = 12$. When at rest, larva remains with the tail straight and the body assumes a hook-shaped disposition. The morphometric data obtained in the present study compared to North American freshwater spirorchiid cercariae and some unidentified furcocercariae probably also spirorchiids, described in molluscs in South America are shown in Table 1.

Analysis of the 28S rRNA of the cercariae evaluated in the present study showed a clear relationship of the three species of spirorchiids described here with other members of family *Spirorchiidae* (Figure 2). The species found in planorbids in the present study (*Spirorchiidae* sp. 1 and *Spirorchiidae* sp. 2) differ genetically from each other (uncorrected *p*-distance 0.1% of 28S) and are probably congeneric species. In comparison, variation among the species of *Spirorchis* range from 0.2–0.4% (uncorrected *p*-distance average). Moreover, the phylogenetic analyses reveal that among available sequence data, *Spirorchiidae* sp. 1 and *Spirorchiidae* sp. 2 are most closely related to the marine spirorchiids (*Learedius*, *Hapalotrema*). Furthermore, despite their morphological similarities to North American species, they differ molecularly from species of the genus *Spirorchis* (uncorrected *p*-distance average 15.2%). Regarding *Spirorchiidae* sp. 3 from *Pomacea* sp., it grouped in the phylogenetic tree with freshwater North American species for which there are available sequences. Among these, *Spirorchiidae* sp. 3 formed a clade with *Unicaecum* found in the emydid

turtle *Trachemys scripta* [3], however the genetic distance is large (uncorrected *p*-distance 12.7%) and significant nodal support was not found.

Discussion

To date very few larval spirorchiids are known, most of them have been found in freshwater snails (planorbids, ancyliids physids from USA and India [9, 20–29]. The present study represents the first identification of spirorchiids in molluscs from South America.

The cercariae of Spirorchiidae sp. 1 and Spirorchiidae sp. 2 obtained in *Biomphalaria* spp. in the present study have general morphology similar to the larvae of *Spirorchis* spp. described from planorbid molluscs in North America [20, 21, 23, 24, 26, 27] and *Vasotrema robustum* Stunkard, 1928 found in *Physa* spp. also in the USA [22]. Moreover, the resting position and behavior of these Brazilian cercariae are similar to those described from *Spirorchis elegans* Stunkard, 1923 by Goodchild & Kirk (1960) [24]. Another unidentified spirorchiid cercaria described from the planorbid *Bulinus globosus* (Morelet, 1866) in Togo [30] and the larvae W1120 obtained from *Biomphalaria sudanica* (Martens, 1870) from Uganda [10] are smaller than those reported in the present study. Surprisingly though, based on genetic data, these two South American species transmitted by *Biomphalaria* spp. differ greatly from *Spirorchis* spp. and other unidentified spirorchiids transmitted by planorbids from North America and Africa for which there are available sequences. Reevaluating the described morphology of spirorchiid cercariae with these new results, still show that the species here reported maintain the general trait considered typical of larval spirorchiids, i.e. the presence of with rays in the whole extension of furcal fin-folds [9, 16]. Unidentified

larvae closely related to or maybe conspecific with those found in the present study were verified in *Biomphalaria* spp. collected at different locations from the state of Minas Gerais, Brazil (municipalities of Caeté, Januária, Montes Claros) (HAP unpublished data), and in *B. tenagophila* from Argentina and Brazil [31,32]. In addition, unidentified brevifurcate cercariae were reported from different species of molluscs in Argentina, Brazil and Venezuela [reviewed by 15–17] and it is possible that some of these larvae are spirorchiids.

Despite morphological similarities to the North American species of spirorchiids, the molecular phylogeny reveal that Spirorchiidae sp. 1 and 2 were found more closely aligned with the marine spirorchiids, whose cercarial morphology is unknown. By analogy to schistosomes, for which cercariae found in a marine environment present general traits similar to those observed in freshwater species [9, 34], it is possible that cercariae of some marine spirorchiids are similar to larvae reported here from *Biomphalaria* spp., and future studies may be enlightening.

All previous reports of freshwater spirorchiid cercariae in molluscs were based on larvae found in pulmonate snails. The verification of a caenogastropod in the life cycle of these blood parasites is reported for the first time, identified as Spirorchiidae sp. 3 found in *Pomacea* sp. This cercaria differs from all other spirorchiid cercariae described, by the smaller size and in lacking rays in the furcal fin-folds. It is similar and probably conspecific with *Cercaria II* found in *Pomacea canaliculata* (Lamarck, 1804) in Argentina that was putatively identified as a schistosome [33]. This is probably not a surprise as these cercariae, including the ones collected here, lack rays in the furcal fin-folds, a feature previously thought possessed by all spirorchiid cercariae.

Our results also reconfirm that Spirorchiidae is paraphyletic and presently could be considered a minimum of two different families, one of which includes both

freshwater and marine species that form the sister group to Schistosomatidae [3, 35]. In addition to the potential involvement of species of Vetigastropoda in the life cycle of marine spirorchiids [11], the grouping of cercariae emerging from *Biomphalaria* in this study with species of the marine spirorchiids may indicate the possibility of pulmonate marine snails (e.g. Amphiboloidea, Ellobioidea, Siphonarioidea) as intermediate hosts of these marine spirorchiids. As more spirorchiid diversity is uncovered, we can test if marine transmitted species are derived and evolved once, or if they have evolved more than once such as was found in Schistosomatidae [34]. Interestingly, based on the results of this work, the clade of *Learedius* + *Hapalotrema* does not group with the other marine genus, *Carettacola*. Overall there is low node support for the marine species + Spirorchiidae sp. 1 and 2, nonetheless, they are all well supported as sister clades to Schistosomatidae.

According to Smith (1997) [1] only 15% of the world's turtle species have been examined for spirorchiids and thus many species have yet to be discovered. In South America, this scenario is more critical given that only seven species of spirorchiids [*Amphiorchis caborojoensis* Fischthal & Acholonu, 1976; *Amphiorchis indicus* Mehrotra, 1973; *Amphiorchis solus* (Simha & Chattopadhyaya, 1970); *Carettacola stunkardi* Martin & Bamberger, 1952; *Hapalotrema postorchis* Rao, 1976; *Learedius learedi* Price 1934; *Monticellius indicum* Mehra, 1939] have been reported recently from 2 species of marine turtles found on the coast of Brazil (*Chelonia mydas* Linnaeus, 1758 and *Eretmochelys imbricata* (Linnaeus 1758)) [13–14].

Surprisingly there are no reports of species of Spirorchiidae from freshwater turtles collected in South America. Although 30 species of freshwater turtles have been reported in Brazil [36], some helminthological studies were performed on only 8 turtle species, which resulted in the identification of 14 species of trematodes in these hosts

[14, 37], but no spirorchiids. In Argentina, only 5 species of trematodes were reported from a single species of freshwater turtle, *Phrynops hilarii* (Duméril & Bibron, 1835) [38], but again, no spirorchiids. Thus, compounding the lack of reports of spirorchiids in turtles is both a scarcity of helminthological studies on freshwater turtles and the methodological impediments to finding these worms. These are small worms in large volumes of blood (sometimes clotted) making it a difficult if not impossible task to find them, which led to the development of specific techniques to increase the sensitivity for recovering parasites [39].

The diversity of species belonging to the family Spirorchiidae is underestimated, revealing the life cycles and molecular characterization of these parasites are in the initial phases. In fact, for 5 genera (*Unicaecum*, *Uterotrema*, *Cardiotrema*, *Hapalorhynchus*, *Spirhapalum*; 4 with representative species in our tree) of the 10 genera currently known of freshwater spirorchiids [2], there are no known life cycles. Thus, the possibility of Spirorchiidae spp. found here in *Biomphalaria* and *Pomacea* in Brazil to be new genera and species cannot be ruled out. A combined molecular and morphological approach of adult parasites recovered from naturally or experimentally infected freshwater turtles are needed to link the cercariae to its respective adults. Such integrated studies are urgently needed in South America to reveal the hidden diversity of spirorchiids in this subcontinent.

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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; panels C, F, I show the detail of the furcae. Scale bars A, D, G= 100µm; all other = 50 µm.

Fig 2. Phylogenetic tree constructed by Bayesian inference based on 5' region of 28S nuclear DNA sequence showing the relationships between larval trematodes found in snails from Brazil and representative species of Spirorchiidae and Schistosomatidae for which sequences were available. Outgroup includes members of Sangunicolidae. The specimens in bold are those collected for this study. The gray boxes include species

currently classified as Spirorchiidae. Node support is indicated as posterior probabilities.

FIGURES

Fig. 1

Fig. 2

TABLES

Table 1: Morphometric data of cercariae of Spirorchiidae found in freshwater snails from Brazil, and other similar larvae described by different authors. Measurements are presented in micrometers as the means followed by standard deviation and range in parentheses. Abbreviations: L = length, W = width. * Experimental host.

Reference		Spirorchidae sp.		Spirorchidae sp.		<i>Spirorchis parvus</i>		<i>S. artericola</i>	<i>S. elegans</i>
		Spirorchidae sp. 1	2	3			<i>S. scripta</i>		
		Present study	Present study	Present study	Wall, 1941 [20]	Holliman et al., 1971 [27]	Holliman & Fisher, 1968 [26]	Pieper, 1953 [23]	Goodchild & Kirk, 1960 [24]
Host		<i>Biomphalaria</i> spp.	<i>B. glabrata</i>	<i>Pomacea</i> sp.	<i>Planorbella trivolvis</i>	<i>Helisoma anceps</i>	<i>H. anceps</i>	<i>P. trivolvis</i> *	<i>H. anceps</i> *
					<i>P. campanulata</i>				<i>Menetus dilatatus</i>
Locality		Brazil	Brazil	Brazil	USA	USA	USA	USA	USA
Body	L	230 ± 16 (205-259)	326 ± 31 (273-375)	230 ± 16 (205-253)	244 (220-264)	251 (237-272)	209 (173-241)	180 (165-190)	209 (175-224)
	W	91 ± 7 (81-109)	97 ± 22 (75-143)	41 ± 4 (36-50)	79 (70-97)	62 (60-65)	67 (54-85)	65 (58-75)	64 (49-84)
Tail stem	L	695 ± 17 (655-717)	784 ± 43 (683-853)	378 ± 22 (334-410)	608(519-651)	612 (576-652)	553 (532-607)	615 (588-640)	605 (534-658)
	W	64 ± 7 (46-72)	63 ± 6 (53-79)	29 ± 3 (22-34)	62 (52-75)	54 (49-56)	49 (46-53)	45 (40-50)	-
Furcae	L	255 ± 16 (225-287)	316 ± 15 (280-341)	150 ± 8 (137-171)	226 (188-264)	254 (245-267)	160 (137-194)	189 (170-210)	174 (133-203)
	W	45 ± 5 (36-53)	43 ± 5 (29-51)	15 ± 2 (10-19)	50 (42-52)	38	31 (30-33)	-	-
Anterior organ	L	81 ± 5 (68-86)	106 ± 4 (94-120)	35 ± 6 (26-46)	69 (54-77)	81 (74-84)	65 (44-81)	-	-
	W	53 ± 4 (43-58)	52 ± 4 (46-62)	25 ± 3 (14-22)	33 (29-37)	41 (37-43)	39 (34-41)	-	-
Ventral sucker	L	27 ± 1 (24-29)	25 ± 2 (21-29)	20 ± 2 (14-22)	31 (22-35)	31 (28-32)	29 (25-31)	-	-
	W	28 ± 3 (26-34)	26 ± 2 (22-33)	19 ± 2 (15-22)	23 (21 25)	-	-	-	-
		<i>S. elephantis</i>	<i>Vasotrema</i>	<i>Cercaria II</i>	Spirorchidae	W1120	Furcocercaria 6	Furcocercaria 8	Cercária B8

<i>robustum</i>									
Reference		Wall, 1941 [21]	Wall, 1951 [22]	Ostrowski de Núñez, 1978 [33]	Bayssade-Dufour et al., 1989 [30]	Brant et al., 2006 [10]	Martorelli et al., 2013 [32]	Martorelli et al., 2013 [32]	Silva & Melo, 2013 [31]
Host		<i>P. trivolis</i>	<i>Physa</i> spp.	<i>P. canaliculata</i>	<i>Bulinis globosus</i>	<i>B. sudanica</i>	<i>B. tenagophila</i> .	<i>B. tenagophila</i> .	<i>B. tenagophila</i>
Locality		USA	USA	Argentina	Togo	Kenya	Argentina	Argentina	
Body	L	219 (194-233)	220 (189-260)	233-265	165 (150-178)	240-279	393 ± 80 (300– 450)	253 ± 45 (210– 300)	233 ± 15 (199- 253)
	W	71 (65-91)	96 (86-119)	31-42		119-120	152 ± 27 (125– 180)	97 ± 11 (90-105)	64 ± 6 (53-73)
Tail stem	L	652 (620-734)	573 (520-722)	360-381	460 (440-482)	655-658	823 ± 45 (780– 870)	612 ± 18 (600– 625)	756 ± 13 (738- 785)
	W	45 (35-56)	50 (34-60)	31-42		72-80			46 ± 4 (40-50)
Furcae	L	220 (196-225)	243 (206-258)	106-148	170 (168-178)	213-291	326 ± 25 (300– 350)	202 ± 32 (180– 225)	227 ± 11 (213- 239)
	W	56 (48-62)	37 (31-43)	10-21	-	29-35			-
Anterior organ	L	71 (48-81)	59 (46-65)	-	-	57	132 ± 7 (125-140)	58 ± 14 (50-75)	39 ± 4 (29-47)
	W	33 (29-38)	34 (31-42)	-	-	60	65 ± 7 (55-70)	43 ± 11 (30-50)	39 ± 5 (29-47)
Ventral sucker	L	28 (24-33)	23 (17-38)	18-21	-	-	28 ± 2 (25-30)	-	27-28
	W	-	24 (19-31)	-	-	-	28 ± 2 (25-30)	-	27-28

Graphical abstract

Molecular sequences of larval trematodes reveal of the occurrence of freshwater species of turtle blood flukes (Spirorchhiidae) in South America