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MORPHOLOGICAL AND ITS2 MOLECULAR CHARACTERIZATION OF *RIBEIROIA* CERCARIAE (DIGENEA: PSILOSTOMIDAE) FROM *BIOMPHALARIA* SPP. (GASTROPODA: PLANORBIDAE) IN NORTHERN ARGENTINA

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ABSTRACT: Species of *Ribeiroia* use planorbid snails as intermediate host. Since there is little information about these digenean parasites in South America, we aimed to assess whether *Ribeiroia* cercariae from 3 north Argentina locations belonged to the same species and differed from *Ribeiroia* cercariae described elsewhere. Specimens were obtained from *Biomphalaria tenagophila* and *Biomphalaria orbignyi* (Salta Province), and *Biomphalaria occidentalis* (Corrientes Province). Morphological traits of cercariae were analyzed, as well as their sequence of the ribosomal internal transcribed spacer 2 (ITS2). The ITS2 region consisted of 426 nucleotides identical in all samples, suggesting that all specimens belong to the same species in spite of their morphological differences and first intermediate host species. Comparison of the ITS2 region with GenBank database records showed that specimens from Argentina were different from *Ribeiroia ondatrae* (0.9% divergence), *Ribeiroia marini* (0.7% divergence), and *Cercaria lileta* (0.2% divergence). In summary, morphological, ecological, and ITS2 molecular data suggest that specimens from Argentina belong to a different species.

The genus *Ribeiroia* was established by Travassos (1939) for *Ribeiroia insignis*. Price (1942) considered this species synonymous with *Psilostomum ondatrae* Price, 1931 (originally described from *Ondathra zibetica*), recognized the new genus, and formed the new combination *Ribeiroia ondatrae*. Its life cycle was described by Beaver (1939). Dollfus (1950) described *Ribeiroia congolensis* from *Ardea goliath* in Africa, while *Cercaria lileta* Fain, 1953, was considered to be perhaps the corresponding cercaria by Johnson et al. (2004). Basch and Sturrock (1969) described *Ribeiroia marini*, a species experimentally obtained starting with a cercaria identical to *Cercaria marini* Faust and Hoffman, 1934. Later on, Nassi (1978) described the life cycle of *Ribeiroia marini guadaloupensis*, a subspecies of *R. marini*, which uses rats rather than birds as definitive hosts. More recently, Johnson et al. (2004) considered 3 valid species in the genus: *R. ondatrae*, with *R. insignis* as its junior synonym, *R. marini*, and *R. congolensis*. Kostadinova (2005) cited *R. insignis* as type species, without mentioning its relationship with *R. ondatrae*.

Ribeiroia spp. are characterized by a pair of esophageal diverticula in cercariae, metacercariae, and adults. These trematodes use planorbid snails as their first intermediate host, from which emerge cercariae that encyst in fish and amphibians; these second intermediate hosts are ingested by birds and mammals, and the life cycle is completed (Johnson et al., 2004). *Trifolium* sp. Travassos, 1922, also has esophageal diverticula, but life cycles of species of this genus have not been described.

Cercariae identified as *Ribeiroia* sp. in South America have been found in *Biomphalaria prona*, *Biomphalaria occidentalis* (Ostrowski de Núñez, 1981; Ostrowski de Núñez et al., 1991), and *Biomphalaria straminea* (Pinto et al., 2013).

Ribeiroia ondatrae infections have been reported to cause limb malformation in amphibians, particularly in the United States, where infection levels rise higher than 20% (Johnson et al., 2001; Blaustein and Johnson, 2003; Johnson and Sutherland, 2003; Schotthoefer et al., 2003; Johnson and Hartson, 2009). In Argentina, Fabrezi (1999) reported duplications in the left forelimb of *Lepidobatrachus llanensis* larvae from a Departamento San Martín waterbody, Salta Province, where *Biomphalaria* snails were also present.

Identification of cercariae based on morphological characters is difficult and is best corroborated with infection of a suitable definitive host that yields the adult. Selection and infection of a definitive host are not always straightforward endeavors. For this reason, the examination of molecular markers such as the internal transcribed spacer 2 (ITS2) region (Nolan and Cribb, 2005) is an alternative solution to determine species. In that regard, Wilson et al. (2005) characterized specimens of *Ribeiroia* spp. from several locations in North America, along with the Caribbean and Africa, based on their ITS2 sequences.

As part of a larger endeavor of describing the parasitic helminths in planorbids from north Argentina, we needed to determine what species of *Ribeiroia* are present. Using morphological characters of cercariae and analysis of the ITS2 fragment, we examined cercariae from 3 regions in northern Argentina and explored their phylogenetic link with the ITS2 sequences of *R. ondatrae*, *R. marini*, and *C. lileta*.

MATERIALS AND METHODS

Morphological characterization

Cercariae of *Ribeiroia* spp. were obtained from *Biomphalaria* spp. collected from the following locations: 638 specimens of *Biomphalaria tenagophila* (D'Orb, 1835) from reservoir Campo Alegre (24°34′S, 65°81′W), 943 specimens of *Biomphalaria orbignyi* Paraense, 1975, from reservoir Puerta de Díaz (25°16′S, 65°31′W) in Salta Province (both during 2005–2007), and 1 specimen of *B. occidentalis* Paraense, 1981, from Paiva Pond (27°30′S, 58°45′W; collected on 27 April 2007) in Corrientes Province. Snails were transported to the laboratory, where they were either placed in dechlorinated water at 18–27 C, exposed to light, fed lettuce ad libitum, and examined for the emergence of cercariae, or they were crushed and examined for redia stages. Identification of *Ribeiroia* cercariae was based on the esophageal diverticula characteristic of the genus (Beaver, 1939; Yamaguti, 1975; Johnson et al., 2004). Except specimens from Corrientes, the number of infected and uninfected snails

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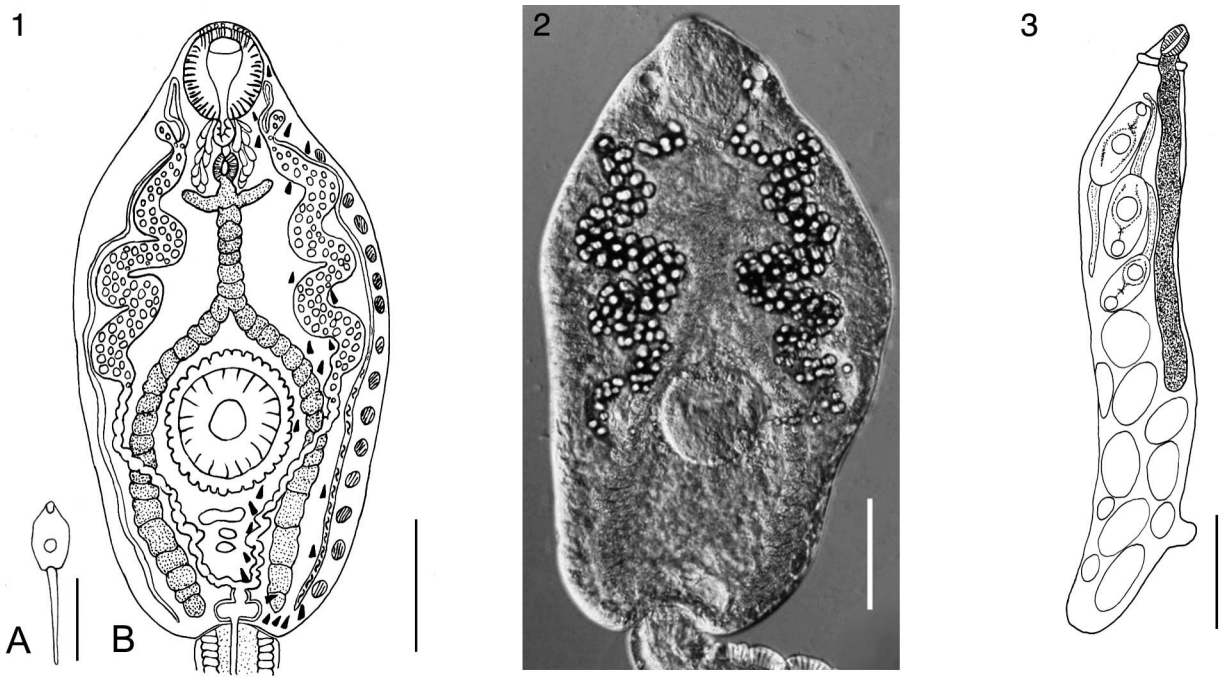

FIGURES 1–3. Larval stages of *Ribeiroia* sp. **(1)** Cercaria from *Biomphalaria orbignyi* of Puerta de Diaz reservoir, Salta Province. (**A**) Cercaria in resting position; scale bar: 600 μm. (**B**) Cercaria body in detail; scale bar: 100 μm. **(2)** Cercaria body from *Biomphalaria occidentalis* of Pavia Pond, Corrientes Province; scale bar: 100 μm. **(3)** Redia stage from *Biomphalaria orbignyi* of Puerta de Diaz reservoir, Salta Province; scale bar: 200 μm.

was recorded, and prevalence of parasitic infections was defined according to Bush et al. (1997).

Observation of morphology was conducted on living specimens following methods outlined in Ostrowski de Núñez et al. (1991). Briefly, cercariae were stained with neutral red and Nile blue sulfate vital stains.

Fish (*Astyanax* sp., n = 10; *Corydoras paleatus*, n = 1; and *Hoplias malabaricus*, n = 1) and tadpoles of *Rhinella arenarum* (n = 13, Gosner stages 25–28) were collected from a site without gastropods, exposed to 2–50 emerged cercariae from *B. orbignyi* or *B. tenagophila* for 24 hr in small vials, and dissected 8 hr to 12 days postexposure (PE).

Drawings were made with camera lucida. For measurements, cercariae were fixed and mounted in hot 4% formalin, and placed between slide and coverslip without pressure. Measurements are in micrometers, with the minimum and maximum followed by the arithmetic mean and standard deviation in parentheses. Samples from each snail species were fixed and conserved in ethanol 96% for molecular analysis. The single *B. occidentalis* specimen collected from Paiva Pond in 2007 was fixed in ethanol 96%. Specimens have been deposited in the Museum Bernardino Rivadavia (MACN-PA 573/1-3 and 573/4-7) and Invertebrates Collection of the Facultad de Ciencias Naturales, National University of Salta, Argentina (UNSA-FCN-P 113, 121–123).

To assess parasite morphological variation, principal component analysis (PCA) and discriminant function analysis (DFA) were carried out with XLStat. We employed 8 morphological traits measured in 31 *Ribeiroia* sp. cercariae recovered from the 3 natural first intermediate host species. Analyzed traits included length and width of body, tail, oral sucker, and ventral sucker. The 3 most important characters obtained from PCA were then subjected to DFA to evaluate the morphometric variation of cercariae released by different hosts. In addition, the effect of host species on these 8 morphological traits was statistically analyzed using 1-way ANOVA with Tukey post-test (GraphPad Prism 5.0).

Molecular analysis

DNA extraction followed the protocol of QIAamp® DNA Mini Kit for Tissue and Body Fluids (Qiagen, Valencia, California). The ITS2 rRNA gene was amplified by PCR, using the same primers and conditions published by Wilson et al. (2005): primer forward 5′ GGT ACC GGT GGA TCA CGT GGC TAG TG 3′, and reverse 5′ CCT GGT TAG TTT CTT TTC CTC CGC 3′. Each reaction contained 1X buffer, 10.5 mM $MgCl_2$, 0.23 mM dNTPs, 0.6 μM each primer, 1.5 GoTaq polymerase enzyme units (Promega, Madison, Wisconsin) and 100 ng of DNA. The mix was placed in a Boeco (Boeckel and Co., Hamburg, Germany) thermocycler under the following amplifying conditions: a first cycle of 95 C for 120 sec, 59 C for 45 sec, 72 C for 90 sec, and 29 repetitions of 95 C for 30 sec, 58 C for 30 sec, 72 C for 90 sec, finalized by a cycle of 72 C for 7 min, 4 C for 4 min, and 15 C on hold. PCR products were observed in 2% agarose gels stained with ethidium bromide. Amplified fragments were precipitated with 70% ethanol and sequenced on both strands (University of Texas Medical Branch [UTMB] Genomics Core Facility) by standard protocols with the same primers used in the initial amplification.

Each chromatogram was visually inspected peak by peak using Chromas Lite version 2.0 (http://www.technelysium.com.au/chromas_lite.html). Sequences obtained for each locus were aligned using SeaView software (Galtier et al., 1996). Nucleotide substitution models for maximum likelihood methods were evaluated using jModelTest 0.1.1 (Posada, 2008). To determine the similarity with other species, the maximum likelihood tree was performed using MEGA 5 software (Tamura et al, 2011), including different parasite species used by Wilson et al. (2005). Branch support was calculated by bootstrap based on 1,000 replications. Sequences have been deposited on GenBank (accession KF525784.1).

RESULTS

Morphology of Cercariae and Rediae of *Ribeiroia* sp.

Cercaria (Figs. 1–2): Drawing and description are based on cercariae that emerged from naturally infected *B. orbignyi* from Puerta de Díaz; measurements are given in Table I. Oval body, tapered anteriorly, with maximal width just anterior to ventral sucker, truncated posteriorly. Four pairs of penetration gland ducts, anterior to oral sucker; penetration glands not discernible. Oral sucker smaller than ventral sucker. Conspicuous pink pear-shaped prepharyngeal body situated immediately posterior to oral sucker. Long prepharynx with oval pharynx well developed. On either side of prepharynx and pharynx, small glands with granular

TABLE I. Comparative measurements (μm) of *Ribeiroia* sp. cercariae obtained in the present study from different hosts. Significant P values ($P < 0.05$) for $F_{(2,28)}$ are indicated with a symbol (‡). The same symbols between columns denote significant differences according to Tukey post-test. Abbreviations: L: length, W: width.

	Ribeiroia sp.			
Host	*Biomphalaria orbignyi*	*Biomphalaria tenagophila*	*Biomphalaria occidentalis*	Analysis 1-way ANOVA
Locality	Puerta de Díaz (Salta)	Campo Alegre (Salta)	Paiva Pond (Corrientes)	
Body, L	354–560 (480 ± 64.612)*	403–452 (420 ± 15.213)*	422–520 (456 ± 32.171)	$F = 4.994$, $P = 0.0248$‡
Body, W	147–236 (204 ± 28.492)*	140–167 (156 ± 8.412)*†	196–236 (210 ± 13.256)†	$F = 23.823$, $P < 0.0001$‡
Oral sucker, L	69–88 (77 ± 6.032)*	51–61 (56 ± 3.277)*†	64–86 (77 ± 8.006)†	$F = 40.544$, $P < 0.0001$‡
Oral sucker, W	51–69 (59 ± 5.425)*	54–61 (57 ± 2.841)†	66–78 (69 ± 3.616)*†	$F = 23.464$, $P < 0.0001$‡
Pharynx, L	22–37 (29 ± 3.959)	22–37 (29 ± 3.733)	–	–
Pharynx, W	20–25 (22 ± 1.915)	20–25 (22 ± 1.806)	–	–
Ventral sucker, L	69–110 (92 ± 10.111)*	61–86 (75 ± 7.413)*†	76–110 (92 ± 10.837)†	$F = 11.218$, $P = 0.0003$‡
Ventral sucker, W	86–108 (96 ± 6.937)*	59–78 (69 ± 7.217)*†	78–98 (93 ± 6.477)†	$F = 46.873$, $P < 0.0001$‡
Tail, L	442–795 (650 ± 87.515)*	579–658 (625 ± 23.238)†	687–874 (798 ± 54.587)*†	$F = 21.490$, $P < 0.0001$‡
Tail, W	59–108 (70 ± 13.539)	54–74 (63 ± 6.331)†	59–88 (77 ± 10.142)†	$F = 3.939$, $P = 0.0339$‡

contents present. Solid long esophagus, with 2 anterior diverticula, which extend laterally and anteriorly to pharynx level. Intestinal ceca reaching end of body. Ventral sucker in anterior part of posterior half of the body, protrusible, with festooned edge. Cystogenous gland cells with bar-shaped contents. Excretory vesicle divided into 2 chambers, anterior smaller than posterior; main excretory tubes containing about 100–140 calcareous concretions of 8–15 (11 ± 2.062) μm diameter, dilate between pharynx and ventral sucker, and flow into anterior chamber at its proximal border. Secondary excretory tubes ciliated internally. Flame cells difficult to count, at least 24 pairs. Two groups of cells, 1 dorsal to ventral sucker and 1 posterior to ventral sucker connected by a chain of cells, represent reproductive anlagen. Tail simple, caudal excretory tube bifurcating in first third of tail, opening in pores on lateral borders.

Freely emerged cercariae from naturally infected *B. tenagophila* were similar to the former, differing in the presence of pink area instead of the conspicuous pink gland posterior to the oral sucker, and cystogenous glands with granular content instead of bar-shaped content. Measurements are given in Table I.

Cercariae from *B. occidentalis* collected in 2007 (Fig. 2; measurements in Table I) were considered morphologically identical to that described by Ostrowski de Núñez et al. (1991), except for the presence of tegumental spines, which could not be observed in our specimens. These cercariae differed from the ones shed by *B. orbignyi* in their content of cystogenous glands, which was granular instead of bar-shaped. In cercariae from *B. occidentalis*, the pink tissue was absent.

Redia (Fig. 3): Description and drawing are based on specimens from *B. orbignyi* from Puerta de Díaz, with measurements on 14 rediae. Body 921 (638–1,238) long by 152 (128–196) wide. Head collar present, and 2 locomotor appendages. Pharynx 51 (37–61) long by 43 (37–49) wide. Intestinal ceca filled with brown material, 543 (324–687) long. Rediae were yellow to orange, containing up to 4 mature cercariae and several groups of germ cells. They were found on host gonads and the digestive gland.

ANOVA showed statistically significant differences in measurements of cercariae emerged from different hosts (Table I). Tukey post-test indicated that cercariae shed by *B. tenagophila* and *B. orbignyi* were significantly different in body length and width, oral and ventral sucker length, and ventral sucker width (Tukey test: $P = 0.010$, $P < 0.0001$, $P < 0.0001$, $P = 0.001$, and $P < 0.0001$, respectively). Cercariae shed by *B. tenagophila* and *B. occidentalis* showed statistically significant differences in 7 out of 8 analyzed parameters (Tukey test: body width, $P < 0.0001$; tail length, $P < 0.0001$; tail width, $P = 0.024$; oral sucker length, $P < 0.0001$; oral sucker width, $P < 0.0001$; ventral sucker length, $P = 0.001$; and ventral sucker width, $P < 0.0001$). Cercariae shed by *B. orbignyi* and *B. occidentalis* showed statistically significant differences in tail length (Tukey HSD test: $P < 0.0001$) and oral sucker width ($P < 0.0001$).

PCA showed that 97.62% of cumulative variance was explained by components I–III. Tail length had the highest coefficient in the first principal component, and body length strongly contributed

TABLE II. PCA scores of 8 morphological traits based on 31 cercariae of *Ribeiroia* sp. shed by 3 host species (values are shown for only 3 principal components). BL: body length, BW: body width, TL: tail length, TW: tail width, OSL: oral sucker length, OSW: oral sucker width, VSL: ventral sucker length, VSW: ventral sucker width.

Component	Proportion of variance	Cumulative proportion
PCI	73.23	73.23
PCII	18.37	91.59
PCIII	6.03	97.62
	Eigenvectors	
Character	PCI	PCII	PCIII
BL	0.025	0.965	−0.212
BW	0.155	0.130	0.866
TL	0.983	−0.063	−0.168
TW	0.025	0.011	0.172
OSL	0.049	0.137	0.191
OSW	0.046	−0.009	0.040
VSL	0.032	0.120	0.136
VSW	0.053	0.123	0.300

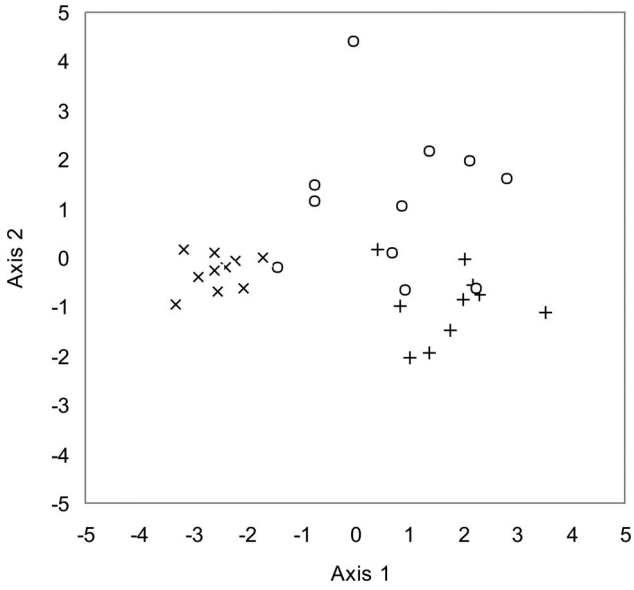

FIGURE 4. AFD scatterplot based on body length, body width, and tail length on cercariae of *Ribeiroia* sp. from *Biomphalaria tenagophila* (x), *Biomphalaria orbignyi* (o), and *Biomphalaria occidentalis* (+).

to the second principal component, while body width contributed to the third principal component (Table II).

Body length, tail length, and body width were employed to carry out DFA. As a result, 100% of variance was explained by 2 functions, which were able to discriminate the 3 observed groups (Wilk's lambda = 0.115; df = 6; $P = 0.0001$). The first canonic function was able to discriminate cercariae shed by *B. tenagophila* from those shed by *B. occidentalis* (80.7% of total variance). The second canonic function separated cercariae shed by *B. orbignyi* from the other 2 snail species (19.32% of total variance). Body length and width showed a strong correlation with the first axis, and tail length was associated with discrimination along the second axis of the bidimensional plan. All cercariae from *B. tenagophila* specimens were correctly assigned a priori to their group. There was 1 cercaria from *B. orbignyi* incorrectly assigned as shed by *B. occidentalis* (3.23%), and 1 cercariae from *B. occidentalis* wrongly assigned as shed by *B. orbignyi* (3.23%). This result explained the dispersion and overlapping of size among cercariae from *B. orbignyi* and *B. occidentalis* (Fig. 4).

Remarks

Prevalence of *Ribeiroia* sp. in *Biomphalaria orbignyi* was 0.64% (6:943) and in *B. tenagophila* was 0.16% (1:638). In Paiva Pond, prevalence in the present study was not determined, but it was reported to be 2.4% (2:82) by Ostrowski de Núñez et al. (1991).

Cercariae moved actively in the middle of the water column by vigorous lateral waving of the tail. Active swimming was alternated with periods of inactivity during which the body directed upward and the tail extended straight back. Only 1 metacercaria was found in a tadpole; 2 dead cercariae were found in another tadpole, and 1 dead cercaria was found in a specimen of *Astyanax* sp. The remainder of exposed hosts showed negative results.

TABLE III. ITS2 variable sites from specimens of *Ribeiroia* sp. from Argentina and *Ribeiroia ondatrae*, *Ribeiroia marini*, *and Cercaria lileta*. Differences in nucleotides from GenBank sequences are marked in bold.

	Nucleotide site					
Species	72	261	357	366	368	425
Ribeiroia ondatrae	**T**	**T**	**A**	**T**	G	G
Ribeiroia marini	A	**T**	G	C	**A**	**T**
Cercaria lileta	A	**T**	G	C	G	G
Ribeiroia sp.*	A	C	G	C	G	G
Ribeiroia sp.†	A	C	G	C	G	G
Ribeiroia sp.‡	A	C	G	C	G	G

* From *Biomphalaria occidentalis*.
† From *Biomphalaria tenagophila*.
‡ From *Biomphalaria orbignyi*.

Molecular analysis

Amplification products of PCR targeting the ITS2 region included 426 nucleotides (nt) and were identical in all samples (Campo Alegre, Puerta de Díaz, and Paiva Pond) despite their geographical origin. Sequences from cercariae of *Ribeiroia* sp. collected in Argentina were compared with those of *Ribeiroia ondatrae* (429 nt), *R. marini* (427 nt), and *C. lileta* (429 nt) available in GenBank, and this allowed the identification of only 6 polymorphic sites between samples from Argentina and other *Ribeiroia* spp. (Table III). Variations in PCR conditions that led to different fragment size may account for the 3-nt difference between Argentinean samples and *R. ondatrae* and *C. lileta*, and 1-nt difference in *R. marini*, as they occurred at the end of the sequences. Therefore, comparisons were made on the 426-nt sequence length shared by all species. A unique nucleotide at position 261 was different between *Ribeiroia* sp. from Argentina and *C. lileta* (0.2% divergence). Cercariae from Argentina were different from *R. ondatrae* in nucleotides at positions 72, 261, 357, and 366, which represents 0.9% divergence. Samples from Argentina differed from *R. marini* at positions 261, 368, and 425 (0.7% divergence).

A TPM2uf+G selection model was selected by jModelTest. The maximum likelihood tree showed that the ITS2 genotype corresponding to Argentinean samples was different from *R. ondatrae*, *R. marini*, and *C. lileta* described previously by Wilson et al. (2005) (Fig. 5).

DISCUSSION

Quantitative assessment, including ranges and mean values of *Ribeiroia* sp. cercariae from Argentina, indicated that there was variation in body size (Table I). Traditionally, dimensions are considered useful criteria in taxonomy. In certain cases, like cercariae and adult schistosomes, measurements can be variable because worm contractility is altered by fixative compounds, host size, and temperature. Additionally, using PCA, Podhorský et al. (2009) demonstrated that morphometric criteria are inadequate for species determination in *Trichobilharzia*. In *Ribeiroia* specimens from Argentinean locations, PCA analysis showed that body length and width, and tail length were the main morphometric parameters that would differentiate cercariae coming from 3 different hosts. DFA allowed differentiation of

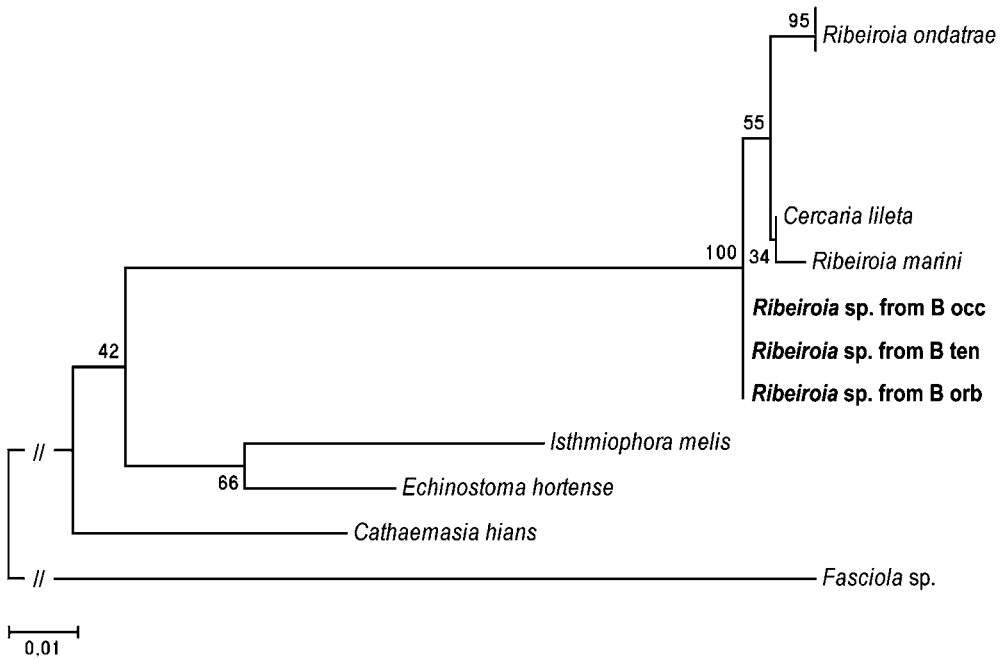

FIGURE 5. Phylogenetic analysis of *Ribeiroia* sp. from Argentina (highlighted) and their relationship to other *Ribeiroia* species based on its 426-bp portion of ITS2 using maximum likelihood (TPM2uf+G) analysis.

cercariae emerged from 3 host species using body length and width, and tail length. These results were confirmed by Tukey test. Moreover, there were morphological differences in cercariae from the 3 northern Argentina populations concerning the pink tissue between the oral sucker and pharynx (present in samples from reservoirs Campo Alegre and Puerta de Díaz, but absent in samples from Paiva Pond), and cystogenous gland cells (granular content in samples from Paiva Pond and Campo Alegre vs. rod content in Puerta de Díaz). These differences suggest that either specimens belong to different species, or they represent phenotypic variations due to utilization of different hosts, geographical variations, or fixation artifacts (Blankespoor, 1974; Galazzo et al., 2002; Nolan and Cribb, 2005; Caffara et al., 2011). Morphological differences of cercariae from Argentina in comparison with *R. ondatrae*, *R. marini*, *C. lileta*, and *Ribeiroia* sp. (Pinto et al., 2013) are given in Table IV.

The identity within the ITS2 region would suggest that the studied specimens from northern Argentina belong to the same species, in spite of their morphological variations and the geographical distance between populations (approximately 900 km from Salta to Corrientes). This finding is in agreement with results published by Wilson et al. (2005), who did not find variation in ITS2 sequences of *R. ondatrae* cercariae and metacercariae from different host species in several North American populations. However, the authors did not mention morphological characteristics in the samples that were genetically analyzed.

The differences between sequences of specimens from Argentina and those published in GenBank were 0.2% for *C. lileta*, 0.7% for *R. marini*, and 0.9% for *R. ondatrae*, suggesting that cercariae from Argentina belong to a different species. Among Echinostomatidae, a family related to Psilostomidae, Morgan and Blair (1998) reported 2.2% variation in ITS1 sequences among 5 species of *Echinostoma* with 37 spines; Vilas et al. (2005) reported differences in the ITS2 region ranging between 0.5 and 12.4% in sibling species of Echinostomatidae; and Tantrawatpan et al. (2013) found 2.29% variation in ITS2 fragments of 2 species of synonymized *Artyfechinostomum*, indicating that they would be different species. However, in the Psilostomidae family, Wilson et al. (2005) reported low variation among species of the genus

TABLE IV. Comparison between *Ribeiroia* cercariae. (1) Beaver (1939), (2) Basch and Sturrock (1969), (3) Fain (1953), (4) Pinto et al. (2013), (5) Ostrowski de Núñez et al. (1991), and (6) and (7) present study.

Species	Locality	Tegument	Acetabulum	Pink tissue	Cystogenous glands/content
Ribeiroia ondatrae (1)	USA	Without spines	79–81 hooks	Rose area	Few
Ribeiroia marini (2)	Puerto Rico	Without spines	Without teeth	Absent	Numerous/rods
Cercaria lileta (3)	Kenya	Without spines	With veil	Pink organ	Numerous/rods or granular
Ribeiroia sp. (4)	Brazil	Without spines	With veil	Rose area	Few/granular
Ribeiroia sp. (5)	Argentina	With spines	With veil	Absent	Few/granular
Ribeiroia sp. (6)	Argentina	Without spines	With veil	Pink organ	Few/with rods
Ribeiroia sp. (7)	Argentina	Without spines	With veil	Rose area	Few/granular

Ribeiroia in their ITS2 region: *R. ondatrae* and *R. marini* showed 1.2% variation, whereas the difference of *C. lileta* with *R. marini* and *R. ondatrae* was 0.7%. Altogether, these reports suggest that the ITS2 region alone does not always provide enough information to discriminate among closely related species (Georgieva et al., 2013). In that regard, molecular differences found between samples from Argentina versus *C. lileta*, *R. marini*, and *R. ondatrae* range between 0.2 and 0.9%, but considering evidence of morphological variation and geographical location, specimens from Argentina might be considered a different species. Moreover, the species from Argentina could be conspecific with *Ribeiroia* sp. cercariae emerging from *B. straminea* recently found in Brazil by Pinto et al. (2013). Analyzing ITS1 sequences, they found differences with *R. marini* and *R. ondatrae*, in agreement with our results using ITS2 data. These variations in genetic sequences between species of the Northern Hemisphere (*R. ondatrae*, *R. marini*) and cercariae of the Southern Hemisphere (*Ribeiroia* sp. after Pinto et al., 2013; the present study) suggest that biogeographical implications should be considered, that more than 1 species in South America might exist, and that *R. insignis* may not be synonymous with *R. ondatrae*. In this sense, the presence of *R. ondatrae* in Argentina as reported by Ostrowski de Núñez (1968), Boero et al. (1972), Labriola and Suriano (1998), and Drago et al. (2011) becomes a matter of revision as more data on life cycles and molecular characteristics are available.

The poor infection success with the *Ribeiroia* cercariae in our experiments, detecting dead cercariae in *Rh. arenarum* tadpoles, may indicate that amphibians act as second intermediate host, but the immune response of *Rh. arenarum* rejected the parasite invasion. Subsequent experiments are needed to assess the adequate secondary intermediate host to *Ribeiroia* sp. in northern Argentina.

In conclusion, our work represents the first ITS2 sequence study combined with morphological analysis of *Ribeiroia* spp. cercariae from South America. The identity of ITS2 sequences among *Ribeiroia* spp. from Argentina suggests that all specimens belong to the same species, in spite of their morphological differences. Comparison of the ITS2 region with GenBank database showed that the specimens from Argentina are different from *R. ondatrae*, *R. marini*, and *C. lileta*. Additional information about other genetic sequences (as used by Littlewood, 2008; Marcilla, 2009; Locke et al., 2010; and Georgieva et al., 2013), linked to morphological description, host records, and collection localities, is needed. This information will also be valuable to understand the biogeography and phylogeny of *Ribeiroia* spp.

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