J. Parasitol., 91(5), 2005, pp. 1040–1045

A MOLECULAR PHYLOGENETIC STUDY OF THE GENUS *RIBEIROIA* (DIGENEA): TREMATODES KNOWN TO CAUSE LIMB MALFORMATIONS IN AMPHIBIANS

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ABSTRACT: Species of *Ribeiroia* (Trematoda: Psilostomidae) are known to cause severe limb malformations and elevated mortality in amphibians. However, little is known regarding the number of species in this genus or its relation to other taxa. Species of *Ribeiroia* have historically been differentiated by slight differences among their larval stages. To better understand the systematics and biogeography of this genus and their potential relevance to the distribution of malformed amphibians, specimens identified as *Ribeiroia* were collected across much of the known range, including samples from 5 states in the United States (8 sites) and 2 islands in the Caribbean (Puerto Rico and Guadeloupe). A cercaria from East Africa identified as *Cercaria lileta* (Fain, 1953), with attributes suggestive of *Ribeiroia* (possibly *R. congolensis*), was also examined. The intertranscribed spacer region 2 (ITS-2) of the ribosomal gene complex was sequenced and found to consist of 429 nucleotides (nt) for *R. ondatrae* (United States) and 427 nt for *R. marini* (Caribbean), with only 6 base differences noted between the 2 species. The ITS-2 region of *C. lileta* (429 nt) aligned closely with those of the 2 other *Ribeiroia* species in a phylogenetic analysis that included related trematode genera. This evidence suggests that a third *Ribeiroia* species exists in tropical Africa. Variation in ITS-2 within *R. ondatrae* was nonexistent among the 8 populations from North America. Our study further suggests that *Ribeiroia* spp. originally parasitized *Biomphalaria* sp., and that a host switch to a closely related snail, *Helisoma* sp., may have occurred in the lineage represented by *R. ondatrae*. However, relationships within the Echinostomatidae are not understood well enough to make any robust conclusions at this time.

Severe declines in amphibian populations worldwide have been documented over the past 3 decades (Alford and Richards, 1999; Houlahan et al., 2000; Alford et al., 2001). Although the exact causes of such declines are controversial, the agents responsible generally involve either abiotic or biotic factors. Abiotic factors include, for example, UV-B radiation (Blaustein et al., 1997) and chemical contaminants (Kiesecker, 2002; Bridges and Boone, 2003), whereas biotic factors include predation (Bohl, 1997; Thiemann and Wassersug, 2000) and pathogens such as *Batrachochytrium dendrobatidis* (Longcore et al., 1999; Fellers et al., 2001; Bradley et al., 2002; Muths et al., 2003), iridoviruses (Daszak et al., 1999), bacteria (*Aeromonas hydrophila*) (Rollins-Smith et al., 2002; Taylor et al., 1999), and trematodes (*Ribeiroia* sp.) (Johnson et al., 2004).

Species of *Ribeiroia* have a typical 3-host life cycle involving 2 aquatic intermediate hosts and predacious definitive hosts. The first intermediate hosts are species of *Helisoma* and *Biomphalaria* and the second intermediate hosts are either freshwater fishes or, more commonly, amphibians (Johnson et al., 2004). The definitive hosts include piscivorous birds and mammals. In recent years, *Ribeiroia* sp. has achieved notoriety because of the malformations it induces in amphibians (reviewed by Johnson and Sutherland, 2003). In field and laboratory studies, *Ribeiroia* sp. has been linked to malformations, e.g., extra, missing, or malformed limbs, in a variety of frog, toad, and salamander species (Sessions and Ruth, 1990; Johnson et al., 1999; Johnson, Lunde, Haight et al., 2001; Johnson, Lunde, Ritchie et al., 2001; Johnson et al., 2002; Kiesecker, 2002; Schotthoefer et al., 2003). The factors responsible for the suspected increase in *Ribeiroia* sp. infection (Johnson and Sutherland, 2003), along with the long-term consequences of both infection and malformations, remain under investigation. Malformed amphibians rarely survive to sexual maturity, suggesting that malformations may have population-level consequences in severe cases.

Although *Ribeiroia* sp. flukes have sparked interest because of their ability to cause limb malformations in amphibians, little research has been conducted on the basic biology, distribution, and systematics of the genus (reviewed by Johnson et al., 2004). Trematodes of *Ribeiroia* sp. appear similar to echinostomes, but lack collar spines and exhibit characteristic esophageal diverticula. Since the description of the first species by Faust and Hoffman (1934) and of the genus by Travassos (1939), the group has had a confusing and controversial taxonomic history. This genus is variously assigned to either the Psilostomidae or Cathaemasiidae; however, the status of either family also remains conjectural (see Johnson et al., 2004). Of the 3 described species, *R. ondatrae* occurs in the Americas (Lumsden and Zischke, 1963), *R. marini* and a subspecies, *R. marini guadeloupensis*, are known from Caribbean islands (Puerto Rico, St. Lucia, and Guadeloupe) (Basch and Sturrock, 1969; Nassi, 1978), and *R. congolensis* was described from the Democratic Republic of Congo in Africa (Dollfus, 1950). Additionally, a cercaria (*Cercaria lileta*) with esophageal diverticula and a rose-colored organ strongly similar to *Ribeiroia* sp. was described by Fain (1953) from *Biomphalaria* sp. in east-central Africa and subsequently from Tanzania (Loker et al., 1981) and Kenya (E. Loker, unpubl. obs.). Although *C. lileta* may represent the larval form of *R. congolensis*, insufficient information is available to decisively address this question. Aside from geography, differences among the described species of *Ribeiroia* are few and subtle. Adult worms are virtually indistinguishable morphologically (e.g., Mettrick, 1963), and cited differences among species focus on variations in larval stages, particularly involving the plate pattern of miracidia and the density of cystogenous glands in cercariae (Basch and Sturrock, 1969; Johnson et al., 2004).

The current study was conducted in an attempt to overcome some of these problems and explicitly addresses the following questions: (1) how many species occur within *Ribeiroia*; (2) what is the geographic distribution of *Ribeiroia* species; (3) is

Received 10 September 2004; revised 10 March 2005; accepted 10 March 2005.
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TABLE I. Collection data and GenBank accession numbers of the parasites used in this study.

Parasite species	Tissue type*	Collection location†	Host	GenBank accession number
Cercaria lileta	C	Lake Victoria, Kisumu, Kenya	*Biomphalaria sudanica*	AY761143
Cathaemasia hians	A	Central Spain	*Ciconia nigra*	AY761146
Echinoparyphium sp.	C	Rio Grande Nature Center, Albuquerque, New Mexico	*Physa* sp.	AY761145
Echinostoma caproni				U58098
Echinostoma paraensei				U58100
Echinostoma revolutum				U58102
Echinostoma sp.				AF026791
Echinostoma trivolvis				U58102
Fasciola sp.				AB010979
Isthmiophora melis				AY168932
I. hortensis				U58101
Protechinostoma sp.	C	McGaffey Lake, McGaffey, New Mexico	*Stagnicola elodes*	AY761144
Ribeiroia ondatrae	C	(NM) Shady Lakes, Albuquerque, New Mexico	*Helisoma trivolvis*	AY761142
	C	(ORa) Aloha Pond, Oregon	*H. tenue*	
	C	(ORb) Spyglass Pond, Oregon	*H. subcrenatum*	
	C	(WIa, WIb) Liberty Creek, Wisconsin	*H. trivolvis*	
	M	(WIc) Wanoka Lake, Bayfield Co., Wisconsin	*Rana septentrionalis*	
	M	(NY) Iroquois, New York	*R. pipiens*	
	C	(WA) South America Pond, Washington	*H. subcrenatum*	
R. marini	C	(PR) Puerto Rico	*B. glabrata*	AY761147
R. m. guadeloupensis	C	(GD) Grand Etang Lake, Guadeloupe	*B. glabrata*	

* A = adult, C = cercarie, M = metacercariae
† Information in parentheses corresponds to sites listed in Figure 1.

Ribeiroia a monophyletic genus; and (4) what is the relationship of *Ribeiroia* to other trematode genera? Answers to these questions will not only advance our understanding of this understudied group of parasites, but will have immediate bearing on the amphibian malformation issue, particularly with respect to the geographic distribution of such deformities. Although *Ribeiroia* sp. has been recorded throughout the Americas, malformed amphibians associated with infection have been widely observed primarily in the northern latitudes of the United States and in southern Canada. Reasons for this pattern remain conjectural, but variations in *Ribeiroia* species, parasite abundance, or host–parasite–environment interactions are undoubtedly involved.

MATERIALS AND METHODS

Specimens of *Ribeiroia* were collected across much of the known range of the genus, including samples from the United States (8 sites), the Caribbean (2 sites), and Africa (1 site; Table I). Tissue was collected from these specimens as either metacercariae (removed from amphibian hosts) or as rediae and cercariae (isolated from infected snails). Identification was based on the esophageal diverticula characteristic of the genus (Beaver, 1939; Yamaguti, 1975; Johnson et al., 2004). Material was preserved in 95% ethanol and stored for DNA extraction. Ethanol-stored specimens were rinsed 3 times in 1× TE buffer (10 mM Tris Cl, 1 mM EDTA, pH 8.0) prior to extraction. DNA was extracted in a total final volume of 50–200 ml using the HotSHOT method (Truett et al., 2000). If unsuccessful, a standard phenol–chloroform method was used. The polymerase chain reaction (PCR) was employed to amplify regions of the nuclear rDNA (intertranscribed spacer region [ITS]-1 and ITS-2) using the following primers (forward listed first, then reverse): BD1 (5′ GTC GTA ACA AGG TTT CCG TA 3′; Bowles and McManus, 1993) and 4S (5′ TCT AGA TGC GTT CGA A(G/A)T GTC GAT G 3′; Bowles and McManus, 1993) for ITS-1; 3S (5′ GGT ACC GGT GGA TCA CGT GGC TAG TG 3′; Bowles et al., 1995) and ITS2.2 (5′ CCT GGT TAG TTT CTT TTC CTC CGC 3′; Hugall et al., 1999) for ITS-2. Initial PCR reactions were carried out in a total volume of 20 μl. Each reaction contained 0.5 μM of each primer pair, combined with 10–100 ng of template DNA, 10× Taq buffer, 0.8 mM dNTP, 3.0 mM magnesium, and 0.5 units of Taq polymerase (Promega, Madison, Wisconsin). This mix was placed in a Whatman Biometra T Gradient thermocycler for 30 cycles, programmed to ramp between temperatures at 1 C per sec. The initial cycle was 95 C for 120 sec, 53 C for 45 sec, and 72 C for 90 sec. This was followed by 29 shorter cycles (95 C for 30 sec, 53 C for 30 sec, and 72 C for 90 sec). The final step was an extension at 72 C for 7 min. After completion, the samples were held at 4 C. Products were viewed on an ethidium bromide-stained 1% agarose and TAE gel. Five 20-μl PCR reactions were repeated for each successful sample (100 μl total volume) to obtain enough DNA for sequencing.

Prior to sequencing, PCR products were concentrated and desalted using a Microcon centrifuge filter (Millipore, Billerica, Massachusetts). The concentration of DNA was quantified by running 2 μl of product on an ethidium bromide-stained 1% agarose TAE gel, with λ Hind III as a marker. Sequencing was carried out using an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, California) with half-volume (10 μl) reactions containing 0.5 μl of Big Dye Ready Reaction Mix, 3.5 μl of Big Dye buffer (200 mM Tris, pH 8.5, 5 mM $MgCl_2$), 15–20 nm of PCR product, and 3.2 pmol of 1 PCR primer. Reactions were cycled in a Whatman Biometra T-Gradient thermocycler (Göttingen, Germany) following recommended protocol for a TC1 thermocycler. This was followed by an ethanol/sodium acetate precipitation (using the protocol for microcentrifuge tubes). Products were run on an ABI 377 (PE Applied Biosystems) automated sequencer.

Both strands of PCR product were sequenced and were aligned and edited using Sequencher version 4.0.5 (Gene Codes, Ann Arbor, Michigan). Multiple sequences were aligned using Clustal X, version 1.8 (Thompson et al., 1997) then edited by eye in GeneDoc, version 2.6.02 (Nicholas and Nicholas, 1997). Nucleotide substitution models for maximum likelihood methods (ML) were evaluated using ModelTest 3.06 (Posada and Crandall, 1998). All phylogenetic analyses were carried out using PAUP*, version 4.0b10 (Swofford, 2001). These analyses included parsimony, distance, and ML. Parsimony analysis was used with default parsimony settings. Heuristic settings for parsimony analysis used stepwise addition to obtain the starting tree for branch swap-

TABLE II. Alignment of the 6 variable sites in the ITS-2 regions of the 2 species of *Ribeiroia* and *Cercaria lileta* used in this study.

	Nucleotide site					
Species	72	357	366	368	425	429
R. ondatrae	T	A	T	G	G	T
R. marini	A	G	C	A	—	—
C. lileta	A	G	C	G	G	T

ping; additional sequences were set at random. The swapping algorithm was set at tree-bisection-resection (TBR) with MulTrees in effect, and all zero length branches were collapsed. Using the same heuristic settings, the distance settings used the HKY85 model (Hasegawa et al., 1985), and Minimum Evolution.

As noted above, the family to which *Ribeiroia* sp. belongs is debated, with opinions split between Psilostomidae and Cathaemasiidae. Olson et al. (2003), in a phylogenetic analysis of the Digenea, hypothesized that the Psilostomidae is sister to the Echinostomatidae and Fasciolidae (Cathaemasiidae was not used). Therefore, in an attempt to find suitable groups to include in a phylogenetic analysis of *Ribeiroia* sp., GenBank was searched to include species within these families. The genera used included *Fasciola* (the outgroup), *Echinostoma*, and *Isthmiophora* (see Table I). *Isthmiophora hortensis* in GenBank is listed as *Echinostoma hortensis*; however, Kostadinova et al. (2003) reclassified this species to *Isthmiophora*. In addition, our study included samples of *Cathaemasia hians* isolated from black storks (*Ciconia negra*) in central Spain to represent Cathaemasiidae (see Merino et al., 2001).

Upon alignment of *Ribeiroia* spp. with the other genera, only 398 nucleotides (nt) were alignable, thus 29 nt (*R. marini* and *R. m. guadeloupensis*) and 31 nt (*R. ondatrae*) at the 3′ end of the ITS-2 region were not included for phylogenetic analyses.

RESULTS

This is the first sequence report of ITS sequence from either *Cathaemasia* or *Ribeiroia* spp. The ITS-2 region of *Ribeiroia* consisted of 429 nt for *R. ondatrae* and *C. lileta* and 427 nt for *R. marini* and *R. m. guadeloupensis*. Only 6 of these sites were variable between species (Table II), indicating that there is little sequence divergence between species in this genus. Variation within the ITS-2 region of *R. ondatrae* samples collected in the United States was nonexistent, and all 8 populations had identical ITS-2 sequences (Fig. 1). Phylogenetic analyses revealed that the 2 available described species of *Ribeiroia* (*R. ondatrae* and *R. marini*) form a monophyletic group that includes a third species, *C. lileta* (a possible synonym of *R. congolensis*) (Fig. 1). All phylogenetic methods provided similar topologies, with the placement of *R. marini* and *C. lileta* moving, but never clustering, within the *R. ondatrae* group or outside the *Ribeiroia* clade. The topology within *Ribeiroia* did not change significantly when phylogenetic analyses were run using a single *R. ondatrae* sequence (data not shown). Analyses using the entire ITS-2 sequence (using *I. melis* as an outgroup) and a single *R. ondatrae* sequence did not provide any resolution into the relationships within *Ribeiroia*. The sister species (given the current information) to *Ribeiroia* are *I. hortensis* and *I. melis* (Echinostomatidae) and *Cathaemasia hians* (Cathaemasiidae).

The 444 nt in the *R. ondatrae* ITS-1 region and 443 nt in the *R. marini* ITS-1 region did not add additional phylogenetic information as to the relationship within *Ribeiroia*. This region was monomorphic within each species and only had 7 variable sites between species (data not shown).

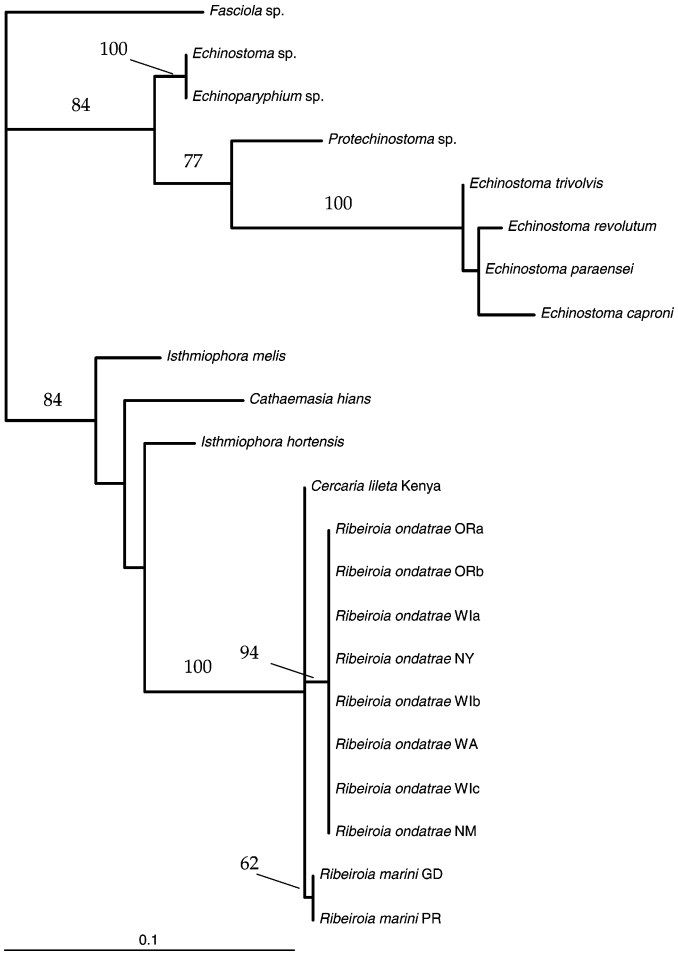

FIGURE 1. Maximum Likelihood (GTR + I + G) tree of *Ribeiroia* using the 398-bp portion of ITS-2. Bootstrap support is indicated at branches where bootstrap support was >50%. *Ribeiroia ondatrae*: NM = New Mexico; NY = New York; ORa = Aloha Pond, Oregon; ORb = Spyglass Pond, Oregon; WA = Washington; WIa and WIb = Madison, Wisconsin; WIc = Wanoka, Wisconsin; *R. marini* PR = Puerto Rico; *R. m. guadeloupensis* GD = Guadeloupe. For detailed information about localities and GenBank accession numbers see Table I.

DISCUSSION

Our data indicate the recognition of 3 species of *Ribeiroia*: *R. ondatrae* in North America, *R. marini* in the Caribbean, and an African species, possibly *R. congolensis*. It is quite possible, however, that additional species exist within this range. For example, no specimens from Canada, Central America, or South America were available for inclusion in this analysis, despite the occurrence of *Ribeiroia* sp. and ''*Ribeiroia*-like'' species from these regions (Johnson et al., 2004).

Surprisingly, no variation was found within *R. ondatrae* samples collected within the United States, despite the wide geographic range surveyed (Washington to New Mexico to New York). Similarly low levels of genetic variation among species in the Echinostomatoidea have been noted by others; for example, Morgan and Blair (1995) used the ITS region to discriminate among 5 species of *Echinostoma* and found that within some isolates the ITS-2 variation was as low as 0.5% (between *E. trivolvis* and *E. revolutum*) and as high as 2.6%

(between *E. revolutum* and *E. caproni*). The present study shows similar ITS-2 variation within *Ribeiroia* spp. The variation within *R. ondatrae* was 0%, between *R. ondatrae* and *C. lileta* 0.7%, and between *R. ondatrae* and *R. marini* 1.2%. Morgan and Blair (1998) stated that ND1 and CO1 might be better suited for species discrimination within *Echinostoma*. Future studies directed more toward describing intraspecific differences in widespread species such as *R. ondatrae* might profitably exploit such markers or, alternatively, microsatellite markers. Although we found no differences in the nucleotide sequences of *R. marini* (Puerto Rico) and *R. marini guadeloupensis* (Guadeloupe), we are hesitant to question the validity of the subspecies status without an examination of additional markers. This is due to the distinctly different host patterns observed for these groups, with *R. marini guadeloupensis* relying almost exclusively on mammalian rather than avian definitive hosts (Nassi, 1978).

This is the first study to show that *C. lileta* is almost certainly a species of *Ribeiroia*. Our study is not, however, the first report of *Ribeiroia* sp. from Africa. Dollfus (1950) described adults of *R. congolensis* from Goliath Herons in the Democratic Republic of the Congo. Unfortunately, adult specimens of *R. congolensis* were unavailable, precluding a direct comparison of *R. congolensis* and *C. lileta*, the latter of which is known only from rediae and cercariae. *Cercaria lileta* has been recovered from *B. sudanica* and *B. choanomphala* near Lake Albert, Tanzania (Fain, 1953) and the Mwanza region of Tanzania where Loker et al. (1981) found *C. lileta* in 3 out of 1913 (0.2%) of the *B. pfeifferi* surveyed. The specimens used for the present study came from *B. sudanica* from Lake Victoria, Kisumu, Kenya. Thus, *Ribeiroia* spp. may have a greater geographical distribution than previously thought.

Examination of the snail species utilized as first intermediate hosts by *Ribeiroia* spp. may offer some additional insights regarding the group's evolutionary history. Species of *Biomphalaria* have a broad geographic distribution that includes the southeastern United States, South America, the Antilles, and Africa (DeJong et al., 2001). *Helisoma* spp. snails, in contrast, have a historical distribution restricted to the New World, including Mexico, Ecuador, Peru, Haiti, United States, Nicaragua, Guatemala, Costa Rica, and Belize (Paraense, 1976, 2003). This distribution of snail species suggests that *Ribeiroia* spp. may have first utilized species of *Biomphalaria* as the first intermediate host then switched to *Helisoma* spp. with an expansion into new regions. However, relationships within the Echinostomatidae are not understood well enough to make any robust conclusions at this time. This might also suggest that the infection of amphibians, and the consequent induction of malformations, is also a derived characteristic, particularly as the infection of amphibians has not yet been observed outside of the United States. Some support for this hypothesis can be drawn from the strong and effective immune response observed in fish challenged with *Ribeiroia* sp. cercariae, whereas comparable responses in amphibians are rare (Johnson et al., 2004). However, it could also be argued that this observation indicates the opposite, i.e., that avoidance of amphibian hosts' immune systems by *Ribeiroia* suggests an older, more established relationship between parasite and host. Whether *Ribeiroia* sp. originated in South America, the probable origin of *Biomphalaria* spp. (DeJong et al., 2001), or in Africa also remains an open and intriguing question for subsequent investigation.

Also in need of further study is the possibility that additional species of *Ribeiroia* exist, possibly in South America, Africa, or the southeastern United States. The southeastern United States is one area where *R. marini* and *R. ondatrae* might cooccur. As stated by Malek (1977) while investigating *Ribeiroia* sp., ''. . . *R. ondatrae* occurs throughout the Americas . . . most of the localities where the parasite has been found in the United States and the Caribbean are on bird migratory routes. . . . It is very likely, therefore, that birds carry the infection with the same species from one place to the other . . . '' More specimens from these border zones are obviously needed to evaluate the ranges and overlap of *Ribeiroia* species. Malek (1977) observed natural infections of *R. ondatrae* in *Biomphalaria obstructa* in Louisiana. This observation is intriguing because most records of *R. ondatrae* involve *Helisoma* species whereas *R. marini* is commonly recorded in *Biomphalaria* sp. Although classified as *R. ondatrae*, *Ribeiroia* sp. specimens from South America are both geographically and ecologically closer to *R. marini*, at least insofar as snail hosts are concerned (all *Ribeiroia* sp. records in South America involve *Biomphalaria* sp. [Johnson et al., 2004]). Intriguingly, malformations in amphibians associated with *Ribeiroia* sp. infection have primarily been recorded in northern parts of the United States and are notably absent from the southeastern United States, the Caribbean, and South America. This raises the tantalizing possibility that the distribution of malformations follows the distribution of *R. ondatrae*. However, such geographic patterns could just as easily be related to phenology, parasite abundance, amphibian ecology, or biases in observation effort.

There needs to be a better knowledge of the species included in the Psilostomidae, which are close to those of *Ribeiroia* sp. in the sense that they do not have collar spines (the presence of collar spines and their number constitute a very important parameter for the determination of the species); unfortunately, members of the Psilostomidae (including many species belonging in *Psilochasmus*, *Psilorchis*, *Psilostomum*, *Psilotrema*, or *Sphaeridiotrema*) were not used and it will be difficult to conclude on the placement of *Ribeiroia* spp. in this family. It could even be hypothesized that *Ribeiroia* spp. do not belong to Cathaemasiidae, because in this family, species like *Cathaemasia hians* have collar spines; *Guaicaipura parapseudoconcilia* sp. (Nasir and Silva, 1972) was placed in the Cathaemasiidae and also has spines. What is also interesting is that *Ribeiroia* spp. seem to have an ancestor that includes a worm with collar spines, like the echinostome, *Isthmiophora* sp. (Echinostomatidae); thus, species of *Ribeiroia* would have lost the spines during evolution.

In conclusion, results of our phylogenetic analysis recognize at least 3 closely related species of *Ribeiroia* found in parts of the Americas (*R. ondatrae*), the Caribbean (*R. marini*), and Africa (*C. lileta*). *Cercaria lileta* is recognized here as belonging within *Ribeiroia* spp.; however, an evaluation of the correspondence between *C. lileta* and *R. congolensis*, while likely synonymous, requires further study. The significance of species of host snails, i.e., *Biomphalaria* (vs.) *Helisoma*, in supporting the different species of *Ribeiroia* is highlighted as a rich area for future research. The significance of *Ribeiroia* species and their distributions in driving geographic patterns associated with am-

phibian malformations is further emphasized for future research. Additional studies are needed to clarify to which digenean family *Ribeiroia* spp. belongs (or perhaps the validity of Psilostomidae and Cathaemasiidae as separate families) by using multiple genera from each family including Psilostomidae and Cathaemasiidae. The present study shows that given the current data, species of either *Cathaemasia* or *Isthmiophora* are the likely sisters to *Ribeiroia* spp., but additional interpretation on the placement of *Ribeiroia* spp. into either Psilostomidae or Cathaemasiidae cannot be made at this time. The species diversity and geographic distribution will also change when areas such as South America are surveyed for *Ribeiroia* spp. infections.

ACKNOWLEDGMENTS

We would like to thank Kevin Lunde for aid in collecting samples in the United States, Sharon File for providing specimens from Puerto Rico, Rebecca Cole for providing additional samples from Wisconsin, Santiago Merino for specimens of *Cathaemasia hians*, J. P. Pointier for assistance in Guadeloupe, Randy DeJong and Jessica Morgan for providing technical assistance, and 2 anonymous reviewers who made important suggestions.

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