J. Parasitol., 103(1), 2017, pp. 75–82

PHYLOGENETIC PLACEMENT OF A SCHISTOSOME FROM AN UNUSUAL MARINE SNAIL HOST, THE FALSE LIMPET (*SIPHONARIA LESSONI*) AND GULLS (*LARUS DOMINICANUS*) FROM ARGENTINA WITH A BRIEF REVIEW OF MARINE SCHISTOSOMES FROM SNAILS

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ABSTRACT: In the blood fluke family Schistosomatidae, marine snails are well known as intermediate hosts. Eight families of marine snails have thus far been reported to host schistosomes across the world, most of which have been implicated in human cercarial dermatitis (HCD) outbreaks. As part of our larger effort to define the species diversity and biology of schistosomes in Argentina, in particular their role in causing HCD, we searched in the marine pulmonate snail (*Siphonaria lessoni*) for a schistosome species described previously from *S. lessoni* from southern Argentina. Additionally, gulls (*Larus dominicanus*) collected from a different project locality (inland) were examined, because they are known to spend time in the intertidal regions. Schistosome sporocysts were found in *S. lessoni*, and a small worm fragment was retrieved from a gull. Molecular phylogenies for 28S, ITS1-5.8S-ITS2, and *cox*1 genes revealed that the specimens from the gull and *S. lessoni* grouped closely together, suggesting they are conspecifics. Also, ITS1-5.8S-ITS2 sequences suggested one of the schistosomes from *S. lessoni* and a schistosome from a South African penguin were also conspecifics. Further study is needed to verify if these specimens comprise a distinct marine clade within the larger avian schistosome clade that is comprised mostly of species using freshwater snail hosts. Thus far, it appears this group of marine schistosomes may be more likely found in the southern hemisphere. It is unclear if the observed distribution pattern of schistosomes in *Siphonaria* is a result of sampling bias and/or indicative of a specific bird–snail–schistosome association. It is clear they are sharply differentiated from the basal marine clade of avian schistosomes that includes *Austrobilharzia*.

In both aquatic and terrestrial environments, gastropods are important intermediate hosts of parasites, particularly as first intermediate hosts for digenetic trematodes. Some families of digeneans include related genera that use marine or freshwater snails (e.g., Heterophyidae, Microphallidae, Echinostomatidae, Schistosomatidae) inviting a stimulating question as to whether disparate habitat use (marine to freshwater or vice versa) evolved once or on multiple occasions within a particular family. In the blood fluke family Schistosomatidae, marine snails are well known as intermediate hosts. In fact, 8 families of marine snails have thus far been reported to host schistosomes across the globe (Table I), most of which have been implicated in marine human cercarial dermatitis (HCD) outbreaks (Penner, 1950, 1953a, 1953b; Chu, 1952; Hutton, 1952; Stunkard and Hinchliffe, 1952; Leigh, 1953, 1955; Bearup, 1955; Ito, 1956; Grodhaus and Keh, 1958; Ewers, 1961; Short and Holliman, 1961; Martin, 1972; Chauhan et al., 1973; Rohde, 1977; Canestri-Trotti et al., 2001; Appleton, 2003; Abdul-Salam and Sreelatha, 2004; Walker, 2005; Alda and Martorelli, 2009; Brant et al., 2010).

We know very little about the natural history of most of the species of marine snails, except for *Austrobilharzia variglandis*. This is in part because of the slow progress in recovering marine schistosomes because prevalence of infections in snails is typically very low. For most of the marine snail families, their associated schistosomes are usually known from only cercariae (Penner, 1950; Hutton, 1952; Ewers, 1961; Martorelli, 1989; Appleton, 2003; Alda and Martorelli, 2009; Brant et al., 2010). One such family of marine snail hosts, the pulmonate Siphonariidae, specifically members of *Siphonaria* or false limpets, is one such family that hosts schistosomes (Ewers, 1961; Appleton, 2003; Alda and Martorelli, 2009). Species of *Siphonaria* are distributed worldwide in tropical and temperate seas.

As part of our effort to describe schistosome diversity in Argentina, we revisited the collecting locality of Alda and Martorelli (2009) at Comodoro Rivadaria, where schistosome-positive *Siphonaria* were found. Our goal was to obtain new schistosome samples for genetic characterization to complement the authors' detailed morphological description. Herein we describe the phylogenetic position of the schistosome cercariae from *Siphonaria lessoni* as well as an adult schistosome from *Larus dominicanus*, which was collected from a different locality, a large inland lake, as part of a different project. Biodiversity studies such as these add important host and distribution records to develop our global understanding of parasite diversity, and also provide the foundations for more targeted local studies oriented toward improved understanding of parasite biology, including life cycles, transmission and control.

MATERIALS AND METHODS

A total of 402 *S. lessoni* were collected, 300 from Comodoro Rivadavia Chubut (45°52′S, 67°28′W) and 102 from Caleta Cordova Chubut (45°45′S, 67°22′W) Argentina in February 2011. Each snail was dissected and examined with the aid of a stereoscopic dissecting microscope to look for parasite larvae. Cercariae and sporocysts obtained were preserved in 95% alcohol.

A total of 40 individuals of *L. dominicanus* (gulls) were collected in the National Park Nahuel, under permit 1296 granted by the Administration of the Nahuel Huapi National Park, from

Received 7 April 2016; revised 31 August 2016; accepted 2 September 2016.

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DOI: 10.1645/16-43

TABLE I. A review of schistosomes that use marine snails as intermediate hosts and their distribution.

Schistosome species	Snail host family	Snail hosts	Avian host	Locality	Reference
Austrobilharzia variglandis	Nassariidae	*Ilyanassa obsoleta*		North America	Grodhaus and Keh (1958), Curtis (1997), Leighton et al. (2004)
			Merganser serrator	North America	Penner (1953b)
			Larus	North America	Keppner (1973), Barber and Caira (1995)
			Branta canadensis	North America	Barber and Caira (1995)
			Phalacrocorax auritus	North America	Barber and Caira (1995)
Austrobilharzia sp.	Planaxidae	*Planaxis sulcatus*		Kuwait Bay	Abdul-Salam and Sreelatha (2004)
	Potomididae	*Cerithidia cingulata*		Kuwait Bay	Al-Kandari et al. (2012)
			Larus	South Africa	Appleton (1982, 1986),
	Nassariidae	*Illyanasa reticulatus*		Italy	Canestri-Trotti et al. (2001)
		Cerithidia		North America	Martin (1972)
	Littorinidae	*Littorina*		North America (Hawaii)	Chu (1952)
			Onychoprion fuscatus	North America (Hawaii)	Chu (1954)
			Anous minutus	North America (Hawaii)	Chu (1954)
	Littorinidae	*Littorina planaxis*		North America	Penner (1950)
			Pelecanus occidentalis	North America	Courtney and Forrester (1974)
			Gavia immer	North America	Kinsella and Forrester (1999)
Austrobilharzia terrigalensis	Potomididae	*Velacumantus australis*		Australia	Appleton (1984)
			Larus	Australia	Johnston (1917, 1941) Appleton (1984)
			Larus	Red Sea	Witenberg and Lengy (1967)
	Planaxidae	*Planaxis sulcatus*	*Egretta*	Australia	Rohde (1977)
			Larus	Australia	Rohde (1977)
Austrobilharzia penneri	Potomididae	*Cerithidia scalariformis*		North America	Holliman (1961)
	Potomididae	*Cerithidia scalariformis*		North America	Short and Holliman (1961)
Ornithobilharzia canaliculata			*Larus*	Red Sea	Witenberg and Lengy (1967)
	Battilariidae	*Batillaria minima*		North America	Penner (1953a), Morales et al. (1971)
			Aythya marila	North America	Chapin (1924)
			Eudocimus albus	North America	Bush and Forrester (1976)
Avian Schistosome *Haminoea*	Haminoeidae	*Haminoea antillarum*		North America	Hutton (1952), Leigh (1953, 1955)
	Haminoeidae	*Haminoea japonica*		North America	Brant et al. (2010)
Avian Schistosome *Siphonaria*	Siphonariidae	*Siphonaria denticulata*		Australia	Ewers (1961)
	Siphonariidae	*Siphonaria lessoni*		Argentina	Alda and Martorelli (2009)
	Siphonariidae	*Siphonaria capensis*		South Africa	Appleton (2003)
Avian schistosome			*Larus*	South Africa	Appleton (1986), Appleton and Randall (1986)
Avian schistosome	Cochliopidae	*Heleobia conexa*		Argentina	Martorelli (1989)

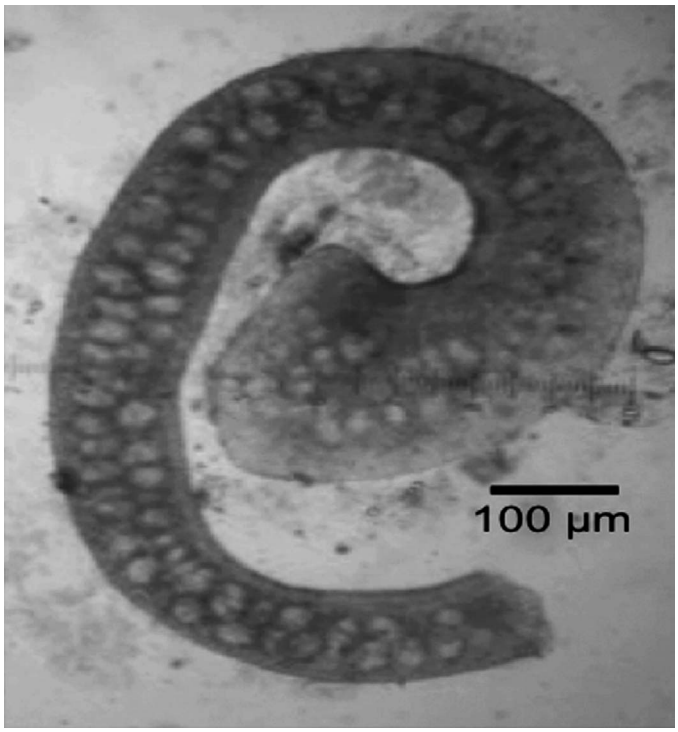

FIGURE 1. Image of the posterior end of worm fragment from *Larus dominicanus*. The spatulate end is a unique feature for some species of avian schistosomes. This is the same worm fragment from which the material was taken for genetic analysis.

October 2013 to March 2015. They were collected from a freshwater lake for a study on HCD in that area, and thus were not collected from the same locality as the *Siphonaria* (about 771 km between localities). The gulls were examined immediately after death. The hepatic portal vein and liver were removed and washed in a series of decantation steps to isolate adult schistosomes and the mesenteric veins were examined with a dissecting microscope.

DNA was extracted from ethanol-preserved sporocysts from *S. lessoni* and from a small piece of the fragment of an adult worm fragment from *L. dominicanus* with the DNeasy Tissue Kit (Qiagen, Valencia, California) according to manufacturer's guidelines. DNA was amplified by polymerase chain reaction, PCR (TaKara *Ex Taq* kit, Takara Biomedicals, Otsu, Japan) and sequenced with the use of previously published primers. For 28S rDNA we used the primers and conditions listed in Brant et al. (2006), ITS1-5.8S-ITS2 region with the primers BDF1, BDR2, 3S and 4S (Bowles and McManus, 1993; Bowles et al., 1995), and for *cox*1 we used the primers and conditions listed in Brant and Loker (2009). PCR products were purified with E.Z.N.A. Cycle Pure Kit (Omega Bio-Tek, Norcross, Georgia) and sequenced with the use of the Applied Biosystems BigDye direct sequencing kit, version 3.1 (Applied Biosystems, Foster City, California).

Phylogenetic analyses for the data sets were carried out with Bayesian inference (BI) with the use of MrBayes (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) and were set up as follows: with default priors, 28S and ITS1-5.8S-ITS2 (Nst = 6 rates = gamma ngammacat = 4) and *cox*1 (parameters unlinked so each partition has its own set of parameters, partitioned by codon; Nst = 6 rates-invgamma. We also allowed the partitions by codon to evolve under different rates (preset applyto = (all) ratepr = variable). Model selection was estimated with the use of ModelTest (Posada and Crandall, 1998). Four chains were run simultaneously for 5×10^5 generations, the first 5,000 trees with preasymptotic likelihood scores were discarded as burn in, and the retained trees were used to generate 50% majority-rule consensus trees and posterior probabilities. Outgroups for 28S rDNA were marine spirorchiids, the sister group to Schistosomatidae according to Snyder (2004). Currently there is no clear sister group to the ingroup taxa, thus the outgroup for *cox*1 was selected from the most recent and most inclusive taxonomically results of our 28S analysis (Flores et al., 2015).

RESULTS

It was found that 4/300 (1.3%) *S. lessoni* from Comodoro Rivadavia and 1/102 (0.9%) from Caleta Cordova had single species schistosome infections. Of the birds examined, 1/40 gulls were infected with a schistosome found in the veins of the large intestine (Fig. 1). Vouchers were deposited in the Museum of Southwestern Biology Division of Parasites (MSB:Para 18934–18938). The samples used for the phylogenetic analysis were MSB:Para 18934 (W636), MSB:Para 18938 (W640), and MSB:Para:24529 (W829). The associated tissues from infected and uninfected snails were deposited in the Museum of Southwestern Biology Division of Parasites MSB: Host: 15401–15403, 21184.

The phylogenetic analysis of the partial 28S data set (1,302 base pairs [bp]) shows that the cercariae from *S. lessoni*, the worm fragment from *L. dominicanus* and the schistosomes from the marine snail *Haminoea* from coastal California (Brant et al., 2010) group together and formed a monophyletic marine schistosome clade that clustered within the larger freshwater avian schistosomes clade (Fig. 2).

Phylogenetic analysis of the ITS1-5.8S-ITS2 regions (1,183 bp) was also performed because the schistosome sample from the penguin has only 28S and ITS gene regions available, plus it provides a more variable nuclear DNA region for comparisons. Like the 28S results, the ITS analysis recovered a monophyletic clade consisting only of marine schistosome lineages (Fig. 3). Unfortunately, only 1 of our samples from *Siphonaria* and *Larus* could be sequenced for ITS, which grouped with the schistosome from the South African penguin. The genetic distance is low, suggesting they are conspecifics (0.3% uncorrected *p*-distance). The clade containing the *Siphonaria* plus South African penguin (Aldhoun and Horne, 2015) schistosomes and the schistosomes from *Haminoea* was 5.8% different, suggesting they may not be congeners. This genetic distance is within the range of values noted between existing genera within the large freshwater avian schistosomes clade (Brant and Loker, 2009). As an example from this clade, *Gigantobilharzia huronensis* and *Dendritobilharzia pulverulenta,* both common in North America freshwater ponds, are 7.2% different. Genetic differences are used as an approximate gauge of variation until we can obtain more data on morphology and life cycles. Thus far, it appears our sample belongs to a new genus.

The results from the analysis using the more variable mtDNA gene *cox*1 (947 bp) show the adult worm fragment from *L. dominicanus* groups with schistosome cercariae from *S. lessoni*, suggesting they are likely conspecifics (Fig. 4). Genetic distance is

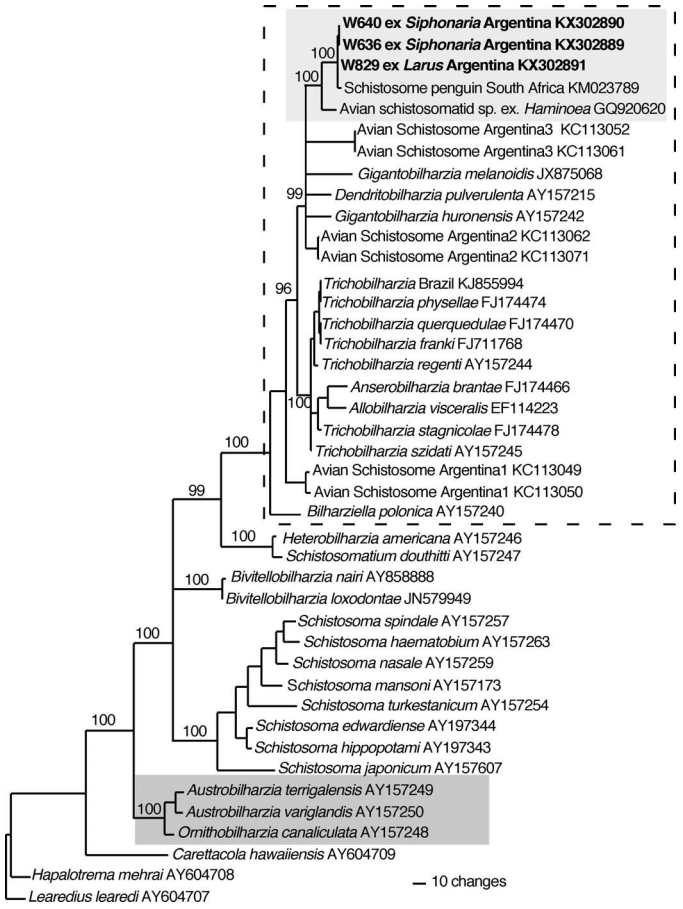

FIGURE 2. Phylogenetic tree based on Bayesian Inference of 28S rDNA sequences. The schistosomes samples from *Siphonaria lessoni* and *Larus dominicanus* in bold. The major avian schistosome clade is outlined in the dashed box. For comparison, marine schistosome taxa are in a shaded gray box. Nodal support is indicated by posterior probabilities. GenBank accession numbers follow the taxon name.

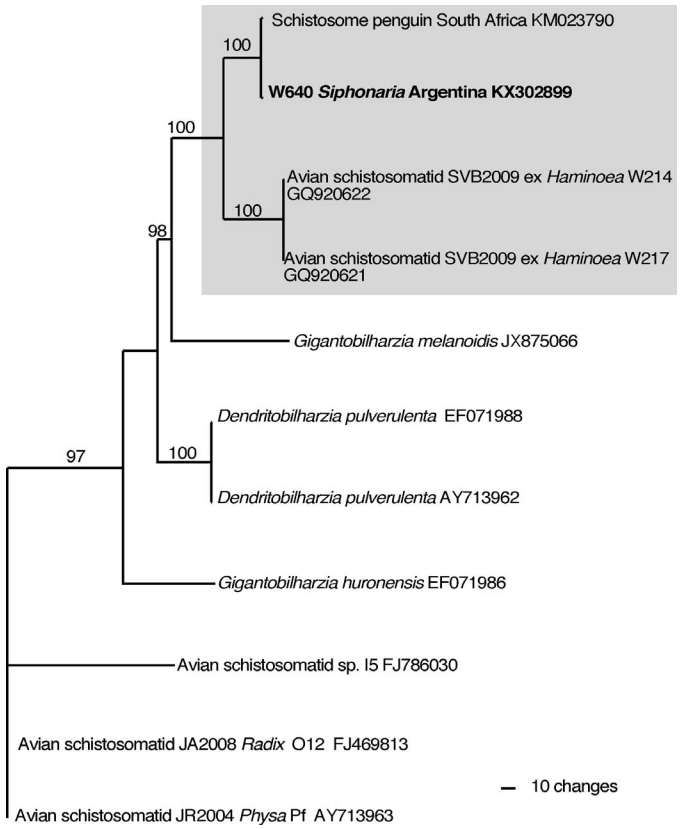

FIGURE 3. Phylogenetic tree based on Bayesian Inference of ITS1-5.8S-ITS2 rDNA sequences. The schistosome sample from *Siphonaria lessoni* is bolded. The marine taxa are highlighted in a gray box. Nodal support is indicated by posterior probabilities. GenBank accession numbers follow the taxon name.

relatively low between the sample from *L. dominicanus* and the two from *S. lessoni* (*cox*1 uncorrected *p*-distance 0.5% and 0.7%) and between the cercariae from *S. lessoni* (*p*-distance 0.2%). However, the schistosome samples from the 2 different marine gastropods, *Siphonaria* and *Haminoea,* are likely not congeners since the average genetic distance (uncorrected *p*-distance) is 18.6%, a value within the range observed to demarcate differences between known genera. Again, for reference, *G. huronensis* and *D. pulverulenta* are 18.3% different, result consistent with the nuclear data (Brant et al., 2006; Brant and Loker, 2009).

The phylogenetic analysis of the *cox*1 data set supports the conspecific relationship of worms from *S. lessoni* and *L. dominicanus*, but otherwise does not show a sister clade grouping with the cercariae from the marine snail *H. japonica* as was revealed by the 28S and ITS analysis (Fig. 4). The topology and lack of resolved nodes is the same if we run the analysis removing the third codon position or remove taxa and rerun the analysis (results not shown). There are several reasons for this observation: the problem of putative missing taxa, different taxon composition among the 3 gene trees, these are gene trees, not species trees. We can say that while we did not recover a clade of marine schistosomes, the *cox*1 phylogeny is still correlated to the nuclear gene trees and that the lack of resolution does not mean absence of a relationship. Thus, based on their common Argentine origin, their genetic similarity and their distinctiveness from other known marine schistosomes, the schistosomes recovered from *Siphonaria* and a gull in Argentina (as well as the sample from the South Africa penguin), likely comprise a distinct species within a new genus, a species with confirmed hosts in the wild.

DISCUSSION

This is the third record of a marine schistosome species that groups within the large clade of freshwater avian schistosomes rather than with the basal marine avian schistosome clade (Fig. 2; Snyder, 2004; Brant et al., 2006). The first report was from the marine snail *Haminoea japonica* in the United States and the second was from a marine penguin (*Spheniscus demersus*) in South Africa (Brant et al., 2010; Aldhoun and Horne, 2015). The schistosome reported here was from the pulmonate gastropod genus *Siphonaria*, and thus far is the only marine heterobranch snail other than *Haminoea* spp. (Hutton, 1952; Brant et al., 2010) reported to have schistosomes (Ewers, 1961; Martorelli, 1989; Appleton, 2003; Alda and Martorelli, 2009). There have been a few other reports of schistosomes from *Siphonaria*, curiously to date, only from the southern hemisphere: *Siphonaria denticulata* from Australia (Ewers, 1961), *Siphonaria capensis* from South

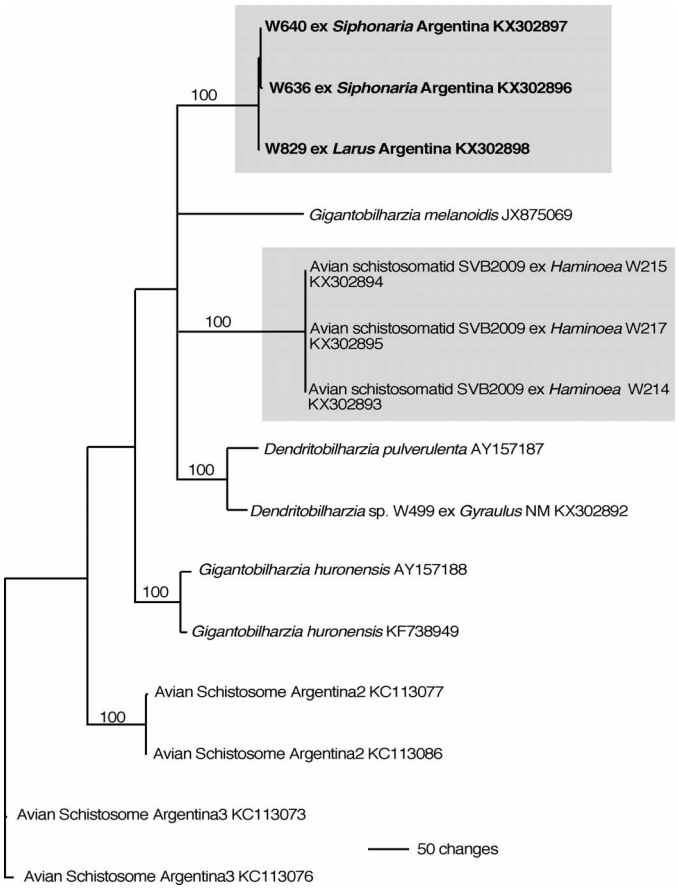

FIGURE 4. Phylogenetic tree based on Bayesian Inference of *cox*1 sequences. The samples from *Siphonaria lessoni* are in bold. The marine taxa are highlighted in gray boxes. Nodal support is indicated by posterior probabilities. GenBank accession numbers follow the taxon name.

Africa (Appleton, 2003), and most recently *Siphonaria lessoni* from Argentina (Alda and Martorelli, 2009). It is unclear if the observed southern hemispheric distribution pattern of schistosomes in *Siphonaria* is a result of sampling bias and/or is indicative of specific bird–snail–schistosome associations, in part because thus far there are very few parasite surveys of *Siphonaria* (Hodgson et al., 1993; Alda and Martorelli, 2009; Gilardoni et al., 2011).

The sequence results here suggest that the samples from *Siphonaria* from Argentina group with the sample from a South African penguin, *S. demersus* (Aldhoun and Horne, 2015), and are likely conspecifics. Interestingly also from South Africa is 1 report of a putative avian schistosome from *S. capensis* (Appleton, 2003) as well as reports of eggs from *S. demersus*. Whether or not these 2 reported specimens are conspecific or related to each other or to the *Siphonaria*/*Larus* sample from Argentina and that from the penguin by Aldhoun and Horne (2015) is not known. The South African penguin schistosome sequenced by Aldhoun and Horne (2015) was from the same penguin species that was examined by Appleton (1986), although Aldhoun and Horne (2015) stated that their egg size was smaller and thus not similar enough to be considered conspecific.

The 1 small worm fragment we recovered from *L. dominicanus* was not of the size or shape of males or females of the larger-bodied, strongly dimorphic genera *Austrobilharzia* or *Ornithobilharzia* (Fig. 1). It is presumed that intact adult worms of the Argentine species are long and thin (Fig. 1). With 2 exceptions, *Bilharziella*, *Dendritobilharzia*, long and thin adult bodies predominate in this large clade of avian schistosomes (Loker and Brant, 2006). Fortunately, the posterior worm fragment recovered is somewhat helpful diagnostically, as there are a few species of *Gigantobilharzia* that have a widened posterior end and most of these have been described from *Larus*.

Species in *Gigantobilharzia* likely represents at least 5 morphologically distinct genera based on combinations of presence or absence of the oral sucker, the ventral sucker and/or the gynaecophoric canal, and thus do not represent a monophyletic group. However, until there is a major taxonomic revision, the following species have a widened posterior end like the specimen from *Larus* in this study; these are *Gigantobilharzia monocotylea* and *Gigantobilharzia acotylea* from Europe (Szidat, 1930; Akramova et al., 2010), *Gigantobilharzia* sp. from Australia (Rohde, 1978), and *Gigantobilharzia* sp. from North America (Ulmer, 1968). Our specimen is likely not *G. acotylea*, because it was described as using freshwater snails (Akramova et al., 2010). But the *Gigantobilharzia* sp. from Australia and North America appear to be the most similar with respect to their posterior ends to our specimen fragment from *Larus* (Rohde, 1978; Ulmer, 1968). The posterior ends of *G. huronensis* from passerine birds and physid snails and *Gigantobilharzia melanoidis* from *Melanoides tuberculata* snails (bird host not known) represented in the tree figures is not widened in either males or females (Najim, 1956; Schuster et al., 2014).

Unfortunately, there are no adult specimens for the *Gigantobilharzia* sp. from penguins, only eggs (Aldhoun and Horne, 2015; Appleton, 1986) and the description of *Gigantobilharzia huttoni* from *Haminoea antillarum* did not include a description or drawing of the posterior end (Leigh, 1955). There are also reports of schistosome eggs from other collections of *L. dominicanus* from South Africa that do not resemble eggs of either *Austrobilharzia* or *Ornithobilharzia* known to infect gulls. These distinctive, unknown eggs might represent the species (or possibly multiple related species) transmitted by *Siphonaria* (Appleton, 1982, 1986; Appleton and Randall, 1986). Likewise, Rohde (1978) found eggs similar to those from South Africa in *Larus novaehollandiae* from Australia. The morphology of these distinctive but unknown eggs (Rohde, 1978) is very similar to the eggs described for *G. huttoni* (a species with long, thin adults) obtained from an experimental infection using cercariae derived from the marine snail *H. antillarum* (Leigh, 1953, 1955). Because of this similarity, the distinctive eggs recovered from gulls were described as *Gigantobilharzia* sp. (Appleton, 1982, 1986; Rohde, 1978). Even though the gull from the present study was collected from an inland lake, gulls are known to travel large distances and several species spend time in both marine and freshwater (lakes, rivers) habitats (e.g., Kilpi and Saurola, 1983; Capllonch, 2004), though some are also resident birds (e.g., Whittington et al., 2009).

Certain species of gulls may represent a common host for these *Gigantobilharzia*-like schistosomes, because the snail hosts (*Haminoea, Siphonaria*) are common in intertidal zones, areas where many common gull species prefer to feed and rest (this is also similar to some species of penguins). The families of snails that host *Austrobilharzia* and *Ornithobilharzia* are also common in intertidal regions (Table I), where gulls are infected regularly with

Austrobilharzia, in particular (e.g., Johnston, 1941; Rohde, 1977; Appleton, 1984; Barber and Caira, 1995; Brant et al., 2010). At least for *Austrobilharzia,* other families of marine birds can also serve as hosts and this genus is still by far the most often recovered in surveys of marine intertidal birds (Table I). The present report though is the first to show a genetic match of an adult worm from a bird with cercariae from a snail that does not fall within the basal clade occupied by *Austrobilharzia* and *Ornithobilharzia*.

The schistosomes found in *Siphonaria* spp., gulls and penguins around the southern hemisphere likely comprise the same, or a closely related species. With new collections permitting additional analyses of morphological and genetic data we will be able to verify species status as well as learn more about the natural history of these worms. The use of molecular data to aid in species characterization within Schistosomatidae has greatly improved our understanding of the phylogenetic relationships among the species as well as their host associations (Snyder and Loker, 2000; Brant et al., 2006). Based on genetic data, *Austrobilharzia* and *Ornithobilharzia* from caenogastropod snails form the basal clade within Schistosomatidae and are exclusively marine (Snyder, 2004). Yet, unexpectedly, the schistosomes emerging from the marine heterobranch snails *H. japonica* and *S. lessoni* cluster within the major avian schistosome clade comprised of freshwater species instead of with the basal marine schistosomes (Brant et al., 2010; Brant and Loker, 2013).

As far as is known, the basal branches of this clade including the *Siphonaria* and *Haminoea* avian schistosomes are all species found in freshwater snails (Brant and Loker, 2013) thus the results herein suggest there has been at least 1 secondary colonization of marine habitats by schistosomes. Such a pattern could be a result of one or both possible scenarios: (1) switching from use of strictly freshwater gastropods to related species, such as *Heleobia*, that live in transitional estuarine habitats; and/or (2) by hosts such as gulls that regularly circulate between marine and freshwater habitats. These 2 scenarios are not mutually exclusive and are likely because some species or populations of gulls do frequent water bodies of varying salinity. Although the gulls and other shorebirds can switch among different salinities in their habitat, the snails do not. Thus it is not difficult to imagine such a bird with a freshwater schistosome would over time encounter different ecologies of snail hosts to which their worms would be exposed, at least initiating the encounter of the 2 hosts and the parasites. There are at least 7 species of schistosome that have been reported for gulls and/or terns from around the world, but for none of these named species do we have genetic data (excluding species of *Austrobilharzia* and *Ornithobilharzia*). There may be a case where we have genetic data without adult morphology for confirmation, such as with the schistosomes from *Haminoea* (Brant et al., 2010). With such data for these species, plus relevant snail host data, we might begin to piece together the trajectory of what is predominantly a freshwater group of schistosomes into the marine environment. Discovering the sister group to the marine schistosomes would also reveal a possible origin of the lineage.

ACKNOWLEDGMENTS

In Argentina the study was supported by Agencia de Promoción Científica y Técnica PICT 1288-2011 and CONICET PIP No.: 11220110100550 to V.F. The University of New Mexico supported this study through a National Science Foundation grant to SVB (DEB 1021427) and a National Institutes of Health grant to ESL (RO1 A144913). Technical assistance at UNM Molecular Biology Facility was supported by NIH grant 1P20RR18754 from the Institute Development Award program of the National Center for Research Resources. We thank two anonymous reviewers who took the time to write constructive reviews.

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