A genetically distinct *Schistosoma* from *Radix luteola* from Nepal related to *Schistosoma turkestanicum*: a phylogenetic study of schistosome and snail host

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Abstract

During a survey of freshwater snails in the Terai region of southern Nepal, 16 of 2,588 specimens of *Radix luteola* from 4 different habitats were found to be shedding schistosome cercariae. None of the 1,411 specimens of *Radix acuminata* we collected were positive for schistosomes. Analysis of 28S, *cox1*, 16S and 12S sequences indicated that all the *R. luteola*-derived schistosomes were genetically very similar to one another and, although unambiguously grouping most closely to the widespread Asian species *Schistosoma turkestanicum*, were clearly genetically distinct from it. We lack information from other life cycle stages to verify the specific identity of these cercariae, but it is possible they are of *Schistosoma bomfordi* or *Schistosoma dattai*, both species previously known only from northern India, the latter species known to infect *R. luteola*. This study provides sequence evidence for a third genetically distinct lymnaeid-transmitted *Schistosoma* lineage in Asia (to go along with *S. turkestanicum* and *S. incognitum*). As a close relative of *S. turkestanicum*, it provides the first direct molecular evidence to accompany morphological results from earlier studies for the presence of a *S. turkestanicum* species group in Asia. It increases to five the number of known or suspected mammalian schistosome species to be present in the Terai region of Nepal. *Radix luteola* and *R. acuminata* were identified and differentiated using conchological features and by molecular phylogenetic analyses of *cox1* and 16S genes.

Keywords: Schistosomiasis, mammalian schistosomes, *Schistosoma dattai*, *Schistosoma bomfordi*, *Radix luteola*, host-parasite relationships, Nepal

1. Introduction

The species diversity and host-parasite relationships among mammalian schistosomes in Asia require further study and clarification. Although some species like *Schistosoma japonicum* are relatively well known because of their role in causing serious disease in humans, other species are more poorly known, in some cases literally having been found on only a single occasion. Part of the problem stems from inadequate sampling across large geographic areas of Asia, with one of the countries most under-represented in prior sampling being Nepal.

The presence of *Bivitellobilharzia nairi* in both Asian elephants and the Asian one horned rhino was documented (Devkota et al., 2014) as part of a study directed primarily towards collection of schistosome cercariae from snails and, secondarily, of schistosome eggs from some mammal species in the Terai region of southern Nepal. Furthermore, three distinct lineages of mammalian schistosomes from snails of the *Indoplanorbis exustus* species group have been retrieved from the Terai region. One unequivocally groups with *Schistosoma nasale* and another with *S. spindale*, both of which along with *S. indicum* comprise the so-called *S. indicum* group of schistosomes from Asia (Devkota et al., 2015). The finding of a third lineage of *I. exustus*-transmitted schistosome from Nepal by Devkota et al. (2015) was surprising. The third lineage clustered with *S. spindale* yet was distinct from it or from the species for which the group is named, *S. indicum*. The survey results from Nepal indicated that additional surprises may lie ahead, and more information is needed before the full picture is revealed with respect to mammalian schistosome diversity in Asia.

In the present study we examined Nepalese lymnaeid snails of two species, *Radix acuminata* and *Radix luteola*, for schistosome cercariae. *Radix acuminata* and closely allied forms are distributed widely across Eurasia, and extend into northern India. *Radix luteola* is

known from Nepal, Bangladesh, India, southern China, Iran, Malaysia, Myanmar, Pakistan, Sri Lanka and Thailand (Ramakrishna and Dey, 2007). Snails of the family Lymnaeidae are well-known for their role in hosting both avian and mammalian schistosomes (Davis, 2006; Brant and Loker, 2009). Regarding their role in transmission of mammalian schistosomes in Asia, *R. luteola* is implicated in transmitting *Schistosoma incognitum*. This species is known from southeastern India (Bengal), Java and Thailand and infects pigs, dogs and a variety of rodents (Attwood et al., 2007). Also known to be, or suspected of being, transmitted by lymnaeid snails are the four commonly recognized species of *Schistosoma* once considered to be representatives of a separate genus, *Orientobilharzia*. The principal morphological characteristic separating members of this genus from *Schistosoma* is the large number of testes: 10 or less in the case of *Schistosoma* and 37-80 for *Orientobilharzia* (Khalil, 2002; Aldhoun and Littlewood, 2012). As discussed by Aldhoun and Littlewood (2012), *Orientobilharzia* has been relegated to the status of a junior synonym to *Schistosoma*, so these four species are now referred to as *Schistosoma turkestanicum*, *S. bomfordi*, *S. dattai*, and *S. harinasutai*. *Schistosoma turkestanicum*, the only species in this group for which sequence data have been obtained, is widely distributed from Turkey, Iraq and Iran in the west, across Russia to Mongolia, northern India and northeast China. It is a parasite mainly of domestic ruminants and has been reported from a number of lymnaeid species including *Radix auricularia*, *R. gedrosiana* and *Radix peregra*, although not from *R. luteola* (see Kumar and deBurbure, 1986; Wang et al., 2009). *Schistosoma bomfordi* has been reported only once from cattle in Muktesar (now Mukteshwar) in northern India (Montgomery, 1906). *Schistosoma dattai*, also a parasite of artiodactyls, especially buffaloes, was first found in India (Dutt and Srivastava, 1952), and all life cycle stages have been well-described (Dutt and Srivastava, 1961, 1962a,b). Studies of both natural and experimental infections have confirmed

R. luteola to be the snail intermediate host (Dutt and Srivastava, 1962b). The parasite is known from Hissar, in northern India, about 400 km to the west of Muktesar. It has only been reported from India. The final species, *S. harinasutai*, is known only from Thailand and southern Laos where it infects water buffaloes and *Radix rubiginosa* snails (Kruatrachue et al., 1965).

Below we discuss the *R. luteola*-transmitted schistosome we found in Terai in southern Nepal, including its relationships to mammalian schistosomes based on 28S, *cox1*, 16S and 12S sequence data. We also include a sequence-based analysis of the specimens of *R. luteola* we found to be infected with this parasite, and how they relate to other lymnaeid species recovered from Nepal and elsewhere.

2. Materials and Methods

2.1 Collection, morphological identification and screening of freshwater snails

Lymnaeid snails were collected from 31 freshwater habitats in different areas of Nepal (Table 1). Identification of snails followed Subba Rao (1989) and Glöer and Bössneck (2013). Snails were isolated and exposed to light to stimulate cercarial shedding. Cercariae were identified by means of cercarial keys (Frandsen and Christensen, 1984), ethanol fixed, measured and photographed using a digital camera fitted to the compound microscope. Snails that did not shed cercariae were also re-examined for infection the following day.

Some cercariae were preserved in RNAlater (Ambion The RNA Company, Life Technologies, Grand Island, New York, USA) or in 96% ethanol. All preserved samples were hand-carried with the permission (ref. number 44, 25 July 2011) of the Nepal Health Research Council to the Department of Biology at the University of New Mexico, Albuquerque, New Mexico, USA for molecular and further morphological analysis.

Table 1. List of localities in Nepal sampled for lymnaeid snails from 2007 to 2014. The number of snails in parentheses indicates the number of *Radix luteola* positive for non-ocellate schistosome cercariae.

Locality Name	District	Habitat Type	Co-ordinates	Number of snails collected	
				<i>Radix luteola</i>	<i>Radix acuminata</i>
Baghmara Community Forest	Chitwan	Marsh	N 27°35'22.0", E 84°28'52.4"	3	-
Budhi Rapti River (near Elephant Breeding Center)	Chitwan	River	N 27°34'57.8", E 84°27'56.6"	42	27
Chitwan National Park 1	Chitwan	Marsh	N 27°33'22.6", E 84°29'5.40"	9	9
Chitwan National Park 2	Chitwan	Marsh	N 27°32'43.1", E 84°30'08.1"	3	-
Chitwan National Park 3	Chitwan	Marsh	N 27°33'59.3", E 84°30'14.9"	57	37
Chitwan National Park 4	Chitwan	Marsh	N 27°33'37.5", E 84°29'24.5"	16	24
Chitwan National Park 5	Chitwan	Marsh	N 27°33'37.0", E 84°30'09.1"	71	42
Jamunapur, Jutpani-5	Chitwan	Rice field	N 27°39'42.48", E 84°30'57.43"	51	-
Jankauli, Bachhauli-7	Chitwan	Marsh	N 27°34'29.28", E 84°30'48.64"	141(1)	-
Khageri River (near Panchakanya Community Forest)	Chitwan	River	N 27°39'34.9", E 84°29'00.2"	61	93
Kuchkuche Community Forest, Kathar	Chitwan	River	N 27°34'26.85", E 84°36'28.88"	25	-
Kuchkuche Community Forest (near Rapti dam), Kathar	Chitwan	Marsh	N 27°34'02.28", E 84°37'16.55"	-	4
Kumrose	Chitwan	Marsh	N 27°34'03.42", E 84°32'22.87"	-	31
Kumrose (near Dhumre River)	Chitwan	Marsh	N 27°34'34.11", E 84°31'02.21"	750(10)	265
Panchakanya Community Forest	Chitwan	Fish pond	N 27°39'28.1", E 84°29'18.7"	-	66
Rapti River, Sauraha	Chitwan	River	N 27°34'52.24", E 84°28'56.12"	43	99
Shishuwar bagar, Bachhauli-3	Chitwan	Marsh	N 27°35'31.76", E 84°30'00.31"	573(3)	3
Tikauli, Ratnanagar-7	Chitwan	River	N 27°37'45.52", E 84°29'21.57"	261(2)	344
Twenty Thousand Lakes	Chitwan	Lake	N 27°36'54.0", E 84°26'19.9"	12	3
Chisapani Village 1	Dhanusa	Rice field	N 26°55'51.5", E 86°08'45.8"	7	-
Chisapani Village 2	Dhanusa	Rice field	N 26°56'26.09, E 86°08'51.19"	55	4
Dhad River, Tulsi Chauda	Dhanusa	River	N 27°00'57.75", E 85°55'27.00"	108	58
Kumaraura	Dhanusa	Pond	N 26°46'36.76", E 85°56'05.50"	45	-
Ramaidaiya Bhawadi Village	Dhanusa	Marsh	N 26°49'26.31", E 85°57'05.80"	3	-
Rato River, Gauribash (west of Tulsi Chauda Village)	Dhanusa	River	N 27°01'43.56", E 85°55'21.30"	135	21
Tulsi Chauda Village	Dhanusa	River	N 27°00'50.37", E 85°55'31.80"	108	58
Jagdishpur reservoir, Niglihawa	Kapilvastu	Water reservoir	N 27°36'59.67" E 83°05'49.36"	-	27
Begnas Lake	Kaski	Lake	N 28°09'58.00", E 84°05'34.50"	-	2
Phewa Lake, Pokhara	Kaski	Lake	N 28°12'31.27, E 83°57'19.42"	1	-
Nawalpur, Hetauda	Makawanpur	Stream	N 27°25'55.06", E 89°58'57.02"	-	115
Ghansikuwa	Tanahun	Marsh	N 28°00'33.4", E 84°08'04.6"	8	79

2.2 Molecular and phylogenetic analysis

Alcohol- or RNAlater-preserved cercarial samples were subjected to DNA extraction either by using the Qiagen DNeasy Blood and Tissue Kit, or the Qiagen QIAamp DNA Micro Kit (Valencia, California, USA) according to the guidelines given by manufacturer. Amplifications of the nuclear ribosomal 28S and mitochondrial cytochrome oxidase I (*cox1*), 12S and 16S genes were done by polymerase chain reaction using Takara Ex Taq kit (Takara Biomedicals, Otsu, Japan) and previously published primers (U178; 5'-GCA CCC GCT GAA YTT AAG-3' and L1642; 5'-CCA GCG CCA TCC ATT TTC A -3' for 28S sequences, CO1F6; 5'-TTT GTY TCT TTR GAT CAT AAG CG-3' and Cox1 3; 5' -TAA TGC ATM GGA AAA AAA CA- 3' for *cox1* sequences P12SF; 5' – TTT GTC CAC AGT TAT AAC TGA AAG G -3' AND P12SR; 5' – GAT TCT TCA AGC ACT ACC ATG TTA CGA C -3' for 12S sequences and R16SF 5' - TGT TTT TTT CCK ATG CAT TA - 3' and R16SR 5' - GGC TTA CAC CGG TCT TAA CT - 3' for 16S sequences) (Attwood et al., 2002; Lockyer et al., 2003; Morgan et al., 2003; Devkota et al., 2015). PCR products were purified either with Omega E.Z.N.A Cycle-Pure Kit (Omega Bio-Tek, 400 Pinnacle Way Ste 450 Norcross, GA 30071) or USB ExoSAP-IT PCR Product Cleanup (Affymetrix, Inc. 26111 Miles Road, Cleveland Ohio 44128, USA) according to the manufacturer's guidelines. Sequencing reactions were performed with Applied Biosystems BigDye direct sequencing kit, version 3.1 (Applied Biosystems, Foster City, California) at UNM Molecular Biology Facility.

DNA from *Radix luteola* and *Radix acuminata* snails were extracted from alcohol preserved samples by using either the Qiagen DNeasy Blood and Tissue Kit (Valencia, California, USA) or the Omega E.Z.N.A Mollusc DNA Kit (Omega Bio-Tek, 400 Pinnacle Way Suite 450 Norcross, GA 30071), following the manufacturers' guidelines. The partial sequences of the two

mitochondrial genes; cytochrome oxidase I (*cox1*) and 16S were amplified by polymerase chain reaction using Takara Ex Taq kit (Takara Biomedicals, Otsu, Japan) and previously published primers (LCO1490; 5' GGT CAA CAA ATC ATA AAG ATA TTG G 3' and HCO2198; 5' TAA ACT TCA GGG TGA CCA AAA AAT CA 3' for *cox1* sequences and 16Sar; 5' CGC CTG TTT ATC AAA AAC AT 3' and 16Sbr; 5' CCG GTC TGA ACT CAG ATC ACG T 3' for 16S sequences) (Palumbi et al., 1991; Folmer et al., 1994). PCR products were purified and sequencing reactions performed as described above for cercariae.

Phylogenetic analysis of schistosome cercariae was performed with the 28S, 12S, 16S and *cox1* gene fragments as well as a combined analysis of 28S-12S-*cox1* using Bayesian inferences (BI) with MrBayes v 3.1.2 (Huelsenbeck and Ronquist, 2001). Analyses were performed using default priors. Model selection was estimated using ModelTest (Posada and Crandall, 1998). For the analysis of the 28S dataset, Nst = 6 rates = invgamma ngammacat = 4. For the BI analysis of the *cox1* dataset, each codon was modelled separately with GTR+I+G and rates allowed to vary separately (preset applyto=(all) ratepr=variable). In the combined analysis our data were partitioned by gene (28S, 12S) and by codon position (*cox1*) and modeled as above, except for the 12S where GTR+G was used. The taxa included were those for which sequence data, for all three of the above loci, were available in GenBank. For both 28S and *cox1* and combined 28S-12S-*cox1* datasets, 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. Outgroups for the schistosome datasets included members of the related *S. japonicum* species group. DNA

sequence data for the remaining taxa on the tree were obtained from the literature and its associated sequences already published in GenBank.

For analyses of the phylogenetic position of the snail host *Radix luteola*, two mitochondrial genes, the 16S and *cox1* fragments, were subjected to phylogenetic analyses using Bayesian inferences (BI) with MrBayes (Huelsenbeck and Ronquist, 2001). Outgroups for the snail data sets included species of *Lymnaea* in a lineage sister to the *Radix* lineage of Correa et al., (2010). DNA sequence data from snails were also deposited in GenBank (GenBank accession number KT160288-KT160298 for *cox1* sequences and KT160299- KT160308 for 16S sequences).

3. Results

From 2007 to 2014, we collected and screened 1,411 *Radix acuminata* and 2,588 *Radix luteola* from 31 habitats in the Terai and hilly region of Nepal (Table 1). Sixteen specimens of *R. luteola* from 4 different locations shed non-ocellate schistosome cercariae (Figure 1). Other trematodes recovered from *R. luteola* and *R. acuminata*, respectively, listed as percent of snails infected, include xiphidiocercariae (2.36%, 3.91%), strigeids (0.15%, 0.43%) and gymnocephalus cercariae (0.08%, 0.14%). *Radix acuminata* also shed echinostome (0.29%) and clinostomoid cercariae (0.14%).

Figure 1. Schistosome cercaria obtained from *Radix luteola*.

Measurements of the schistosome cercariae from Nepalese *R. luteola* were compared with those from other Asian schistosome species available from other studies (Table 2). The Nepalese *R. luteola*-derived specimens were notable in having relatively wide body and tail stem measurements. The former may simply reflect measurements for cercariae with bodies that had contracted because body length measurements were frequently shorter than noted for other species. Regarding the tail stem measurements, the recorded tail stem length is comparable to that of other species and therefore does not seem to indicate measurements reflecting an unusual degree of contraction. This suggests the relatively wide tail stem is a real feature of the cercariae we recovered from *R. luteola*.

Table 2. Comparison of cercarial measurements between the potential members of the *S. turkestanicum* species group and the members of *S. indicum* group along with *S. incognitum*.

	Body		Tail stem		Tail furca		Snail host	Reference
	Length	Width	Length	Width	Length	Width		

Schistosome cercariae from <i>Radix</i> snail (n=10, Alcohol preserved)	150-165 (158±5.01)	75-80 (78±2.45)	250-270 (263±7.5)	40-50 (45±4.5)	95-110 (103±6)	15-20 (19±2)	<i>Radix luteola</i>	This study
<i>S. turkestanicum</i> (Heat-killed)	178	62	187	22.6	45	13	<i>Radix auricularia</i>	Machattie, 1936
<i>S. bomfordi</i>	-	-	-	-	-	-	<i>Radix luteola</i>	Montgomery, 1906
<i>S. dattai</i> (Heat-killed)	135-200 (168)	44-65 (58)	210-316 (258)	26-39 (32)	71-116 (97)	-	<i>Radix luteola</i>	Dutt and Srivastava, 1962b
<i>S. harinasutai</i> (Hot formalin-fixed)	150-220	40-70	240-350	25-40	45-100	15-25	<i>Lymnaea rubiginosa</i>	Kruatrachue et al., 1965
<i>S. incognitum</i> (Heat killed)	157-207	42-71	185-307	28-49	85-135	-	<i>Lymnaea luteola</i>	Sinha and Srivastava, 1960
<i>Schistosoma</i> sp. (W557) (n=10, Alcohol Preserved)	160-170 (164±4.4)	50-60 (56.5±4.5)	250-260 (256.5±4.5)	30-40 (34.5±4.7)	100-105 (101±2)	12-20 (18.2±3)	<i>Indoplanorbis exustus</i>	Devkota et al., 2015
<i>S. spindale</i> (W804) (n=10, Alcohol Preserved)	120-160 (148.5±11.4)	50-60 (57.5±4)	280-300 (289.5±6.5)	30-40 (36.5±4.5)	120-140 (123±9.8)	15-20 (19.5±1.5)	<i>I. exustus</i>	Devkota et al., 2015
<i>S. nasale</i> (W542) (n=5, Alcohol Preserved)	155-190 (171±12.8)	50-60 (56±3.7)	290-320 (305±10)	35-40 (39±4.5)	95-110 (100±5.5)	20-25 (21±2)	<i>I. exustus</i>	Devkota et al., 2015

We attempted to obtain *cox1*, 12S, 16S and 28S sequences from all 16 cercarial isolates. We were able to obtain at least one of the above sequence regions (Table 3) from all of our samples. We also included as much pertinent corresponding sequence that we could find from schistosomes (especially *S. turkestanicum*) from elsewhere in Asia or Europe, and the lymnaeid-transmitted *S. incognitum* from India, Bangladesh, Thailand and Indonesia.

Table 3. List of the schistosome specimens for which DNA sequence data was obtained and used in the phylogenetic analysis.

Taxon or Sample number	GenBank Accession number			
	28S sequences	12S sequences	<i>cox1</i> sequences	16S sequences
W526	KT022106	-	-	-
W527	KT022107	-	KT022091	-
W529	KT022108	-	KT022092	-
W530	KT022109	KT022124	KT022093	-

W536	KT022110	-	KT022094	-
W543	KT022111	-	KT022095	-
W544	KT022112	-	KT022096	-
W552	KT022113	-	KT022097	-
W553	KT022114	KT022125	KT022098	-
W554	KT022115	KT022126	KT022099	-
W555	KT022116	KT022127	KT022100	KT022122
W559	KT022117	-	KT022101	-
W560	KT022118	-	KT022102	-
W797	KT022119	KT022128	KT022103	-
W802	KT022120	KT022129	KT022104	KT022123
W803	KT022121	KT022130	KT022105	-
<i>Schistosoma turkestanicum</i>	EU702749 (Hungary), AF167092, AY157254 (Iran), AJ313461, EU436659 (China)	HQ283100	EU221230, EU221231, EU436657, EU177877 (China), AY157200 (Iran), JX467182 (Hungary)	-
<i>S. kisumuensis</i>	FJ897155	-	-	-
<i>S. bovis</i>	AY157266	FJ897168	FJ897160	-
<i>S. intercalatum</i>	AY157262	AJ419779	AJ519519	-
<i>S. haematobium</i>	AY157263	DQ157222	DQ157222	-
<i>S. matthei</i>	AY157265	AJ419789	AJ519518	-
<i>S. curassoni</i>	-	AJ419786	-	-
<i>S. leiperi</i>	AY157261	-	AY157207	-
<i>S. mansoni</i>	AY157173	HE601612	HE601612	-
<i>S. rodhaini</i>	AY157256	-	AY157202	-
<i>S. incognitum</i>	JQ408705	EF534279	AY157201	-
<i>S. edwardiense</i>	AY197344	-	AY197347	-
<i>S. hippopotami</i>	AY197343	-	AY197346	-
<i>S. japonicum</i>	Z46504	KP793878	EU340357	-
<i>S. mekongi</i>	AY157253	AF217449	AF217449	-
<i>S. sinensium</i>	AY157251	AF465918	AY157197	-
<i>Bivitellobilharzia loxodontae</i>	JN579949	-	-	-
<i>B. nairi</i>	AY858888	-	-	-
<i>Schistosoma indicum</i>	AY157258	EF534276	AY157204	-
<i>S. spindale</i>	Z46505	EF534281	AY157203	-
<i>S. nasale</i>	AY157259	EF534280	AY157205	-

First, regarding the Bayesian analysis of *cox1* schistosome sequences (Figure 2), our *R. luteola*-derived schistosomes always clustered together with strong support, but distinct from and a sister clade to, *Schistosoma turkestanicum*. Our *cox1* sequences differed from those of *S. turkestanicum* by 19-20% (uncorrected *p*-distance). A similar tree topology was also found for other genes (28S in Figure 3, 12S in Figure 4 and for the combined gene analysis in Figure 5), supporting the monophyly of the *R. luteola*-derived schistosomes distinct from *S. turkestanicum*.

Figure 2. Bayesian phylogenetic tree incorporating *cox1* (1029 bp) sequences from schistosome cercariae collected from *Radix luteola*. Samples in bold are those collected in this study. Node support is indicated by Bayesian posterior probabilities.

Figure 3. Bayesian phylogenetic tree including 28S (1125 bp) sequences from schistosome cercariae collected from *Radix luteola*. Samples in bold are those collected in this study. Node support is indicated by Bayesian posterior probabilities.

Figure 4. Bayesian phylogenetic tree including 12S sequences from schistosome cercariae collected from *Radix luteola*. Samples in bold are those collected in this study. Node support is indicated by Bayesian posterior probabilities.

Figure 5. Bayesian phylogenetic tree based on a combined analysis of 28S (1528 bp), 12S (311 bp) and *cox1* (1027 bp) sequences from schistosome cercariae collected from *Radix luteola*. Samples in bold are those collected in this study. Node support is indicated by Bayesian posterior probabilities.

Regarding the phylogenetic position of the Nepalese lymnaeids we collected, sequences for *cox1* were obtained from 5 specimens of *R. acuminata* and 6 specimens of *R. luteola*, including 3 specimens that were infected with schistosomes (Figure 6). All of our specimens of *R. acuminata* formed a distinct clade comprised exclusively of snails we collected. The six isolates of *R. luteola* we collected also grouped closely with two isolates designated “*Radix* sp. clade 11” collected from Nepal by von Oheimb et al. (2011). We were not able to compare our *R. luteola* *cox1* sequences with other sequences for the same species as there are no *R. luteola* *cox1* sequences in GenBank.

Figure 6. Bayesian phylogenetic tree based on *Radix* snail *cox1* (482 bp) sequences. Samples in bold are those collected in this study. Node support is indicated by Bayesian posterior probabilities.

Sequences for 16S were obtained from 3 specimens of *R. acuminata* and 7 specimens of *R. luteola*, including 4 specimens that were infected with schistosomes (Figure 7). Our three specimens of *R. acuminata* grouped closely with *Radix natalensis* isolate 6120 from Malawi. These three snails along with *R. natalensis* isolate 6120 from Malawi and isolates designated as “*Radix* sp. clade 5” collected from Nepal and India (von Oheimb et al., 2011) formed a distinct clade of *Radix* snails in our analysis. The seven isolates of *R. luteola* we collected grouped closely with isolates designated “*Radix* sp. clade 11” collected from Nepal or Myanmar or Thailand by von Oheimb et al. (2011) and *Radix swinhoei*. Our *R. luteola* were more distantly related to the snails designated as *R. luteola* from Sri Lanka included in the analysis by Correa et al. (2010). These authors also noted a close relationship between *R. luteola* from Sri Lanka with *R. natalensis* from La Réunion Island.

4. Discussion

A previously unreported, well-supported, genetically distinct lineage of lymnaeid-transmitted *Schistosoma* occurs in Nepal, bringing to five the number of known or suspected species of mammalian schistosome species in the country. The snail intermediate host for the elephant and Asian one-horned rhino schistosome *B. nairi* is unknown (Brant et al., 2013; Devkota et al., 2014). For the *S. indicum* group representatives, all thus far have been reported as parasites of artiodactylids, mostly domestic ruminants like water buffalo, cattle and goats and infect snails of the *Indoplanorbis exustus* species complex. The report of *Schistosoma mansoni*-like eggs from human stool samples from Terai (Sherchand et al., 1999), as yet unconfirmed by the lack of sequenced specimens or lack of evidence for the presence of *Biomphalaria* in Nepal, if confirmed, would represent a sixth species of Nepalese mammalian schistosome.

Figure 7. Bayesian phylogenetic tree based on *Radix* snail 16S sequences. Samples in bold are those collected in this study. Node support is indicated by Bayesian posterior probabilities.

The lymnaeid-transmitted *Schistosoma* we report here is a close relative of *S. turkestanicum*, a geographically widespread Asian schistosome, and the only species for which sequence data are available among the four species of what can be called the *S. turkestanicum*

species group. A likely possibility is that our Nepalese specimens belong to one of two members of this species group, either *S. bomfordi* or *S. dattai*. The former species was reported from cattle from Mukteshwar, India, only 600 km west of Chitwan National Park by Montgomery (1906), and has not been reported since. The males of this species are reported as having 68 testes. Other details regarding the biology of *S. bomfordi* are unknown. *Schistosoma dattai* is hosted by *R. luteola* and water buffaloes, and is known from Hissar, northern India, about 1000 km from Chitwan. Males of this species have 37-66 testes (Dutt and Srivastava, 1952). Unfortunately, no sequence data have been forthcoming for verifiable adult worms of either species. The remaining species *S. harinasutai* seems a less likely candidate as it is known only from Thailand and Laos (Kruatrachue et al., 1965), though it is suspected to occur in India (Agrawal and Rao, 2011).

The cercariae we recovered have a wider tail stem than that reported for *S. dattai*, but such differences have to be interpreted carefully as measurements may vary considerably based on use of different methods of fixation and preparation of specimens. Further study is clearly warranted, including provision of sequence data for adults identified as *S. bomfordi* or *S. dattai*.

Genetic variation among the *cox1* gene of 15 different isolates of schistosomes from *R. luteola* we sequenced was minimal (0-1%), and reminiscent of *S. nasale* in this regard (Devkota et al., 2015). However, unlike the specimens for *S. nasale* which came from distant locations (Sri Lanka and Nepal), all the isolates we recovered from *R. luteola* came from a circumscribed region of Nepal with a radius of about 50 kilometers. Consequently, it is likely we have sampled only a small part of this unknown species' geographic range. The extent of the geographic range of *S. bomfordi* or of *S. dattai* in India or elsewhere is unknown.

Our results provide the first sequence-based evidence that close relatives for *S. turkestanicum* exist, supporting the validity of an *S. turkestanicum* species group (the former

members of the genus *Orientobilharzia*) based on their common possession of a large numbers of testes, Asian distribution and use of lymnaeid snail hosts. As noted by Agrawal and Rao (2011) and Devkota et al. (2015), the mammalian schistosomes of south central Asia are poorly known, and many additional geographic reference points and samples are needed before we gain a more accurate impression for their host spectra, abundances and geographic ranges.

With respect to the Nepalese lymnaeid snails we sampled, they could be placed into two groups, one identifiable as *Radix acuminata* and the second as *R. luteola* based on standard conchological references (Subba Rao, 1989; Glöer and Bössneck, 2013). Only one species, *R. luteola* (Figure 8A), was found infected with schistosomes. Based on analysis of 16S and *cox1* sequence data, neither of the lymnaeid species we recovered grouped closely with *R. luteola* from Sri Lanka as defined by Correa et al. (2010). Our *R. luteola* did group closely with three different isolates of “*Radix* sp. clade 11” collected from either Chitwan or Banke, Nepal or from Myanmar by von Oheimb et al. (2011). Similarly, what we called *R. acuminata* (Figure 8B) grouped with “*Radix* sp. Clade 5” from Taplejung Nepal or Uttarakhand, India reported by von Oheimb et al. (2011).

Figure 8. *Radix* snails from Nepal from which sequence data were obtained. A) A specimen of *Radix luteola* that was shedding the schistosome cercariae discussed in this paper. B) A specimen of *Radix acuminata*.

The apparent discrepancies and uncertainties noted in Correa et al. (2010), von Oheimb et al. (2011) and the present study with respect to relating reference sequences to specific individual snails with documented shell, anatomical, or infection features, highlights the need for much further study of the lymnaeid species of southern Asia and their roles in transmission of parasites of veterinary significance. At least five species of mammalian schistosomes (the four members of the *S. turkestanicum* group as well as *S. incognitum*) and possibly others yet to be found, and both *Fasciola hepatica* and *F. gigantica* are all transmitted by lymnaeid snails. Species such as *Radix acuminata*, *R. luteola*, *R. rubiginosa*, *R. gedrosiana*, *R. auricularia* and *R. peregra* are all mentioned as possible hosts of these important parasites. How many of these

species will prove to be valid, and how many of them will be shown to extend into southern Asia and be involved in the transmission of these and other parasites remains to be ascertained using a combination of conchological, anatomical and sequence-based characters. This effort will greatly clarify the host-parasite relationships and epizootiology of the parasites mentioned above. Furthermore, continued diligence is needed in considering the possibility that some of the schistosomes mentioned above not only are capable of causing dermatitis in humans, but in some cases may actually initiate egg-producing infections in people (Baugh, 1978; Agrawal and Rao, 2011). The time has come to greatly increase our knowledge base for the many species of schistosomes infecting the mammals of southern Asia.

In conclusion, the unknown mammalian schistosome of the *S. turkestanicum* species group we obtained from *Radix luteola* is of veterinary and potentially medical interest and in need of further study. It is probably either *S. dattai* or *S. bomfordi*, and consequently is being reported for the first time from Nepal. Or, it may represent a new species to science. Again, access to means to acquire adult worms that match the sequences we acquired from cercariae derived from lymnaeid snails are needed to pin down more fully the identity of this species.

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