7-1-2015

SCHISTOSOMES OF NEPAL

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SCHISTOSOMES OF NEPAL

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DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy
Biology

The University of New Mexico
Albuquerque, New Mexico

July, 2015

ACKNOWLEDGMENTS

With affection and appreciation, I would like to thank my supervisor Dr. Eric S. Loker for his guidance and arrangement of financial support that made this work possible. I am grateful to Dr. Sara V. Brant for her incredible support and advice. I would like to express my deep gratitude to the other members of my dissertation committee Dr. Coenraad M. Adema, Dr. Howard L. Snell and Dr. Randall J. DeJong for their guidance and patience.

Thank you to my lab members Sarah Buddenborg, Erika Gendron, Martina Laidemitt and Dr. Ben Hanelt not only for their help, but also for encouragement and camaraderie.

Thank you to my parents, the Late Vim Kumari Devkota and Lila Ram Devkota, for their love, guidance and support and for pushing me to challenge my intellectual boundaries. My greatest thanks goes to my wife Rupa Sapkota Devkota and son Agrim Devkota, for their unlimited support and love to finish this work.

I would like to thank the Molecular Biology Facility and the University of New Mexico for technical assistance. I am grateful to the officials of the Department of National Parks and Wildlife Conservation, Chitwan National Park and the Nepal Health Research Council (Permit No. 44) for their cooperation with this research. I am also indebted to Bikas Sapkota, Arjun Thapa, Sanjan Bahadur Thapa and Ramesh Ghimire for providing field support to collect the samples.

I would also like to thank Dr. Jeevan Bahadur Sherchand, Professor, Tribhuvan University Teaching Hospital; Prem Bahadur Budha, Lecturer, Tribhuvan University,

Central Department of Zoology and Hari Bahadur Rana, Tribhuvan University, Institute of Agriculture and Animal Science for their constant advise and support to carry out this research.

The words of appreciation go to my other family members, relatives and friends for their affection and constant encouragements to achieve this success.

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ABSTRACT

Globally, digenetic trematodes called schistosomes (Family Schistosomatidae) have enormous public and veterinary health significance because they cause debilitating and chronic infections collectively called schistosomiasis. Schistosomes have a life cycle featuring a bird or mammal definitive host in which adult worms are found, and freshwater snail intermediate hosts in which larval stages are found and free-swimming cercariae are produced which infect the definitive host. Although schistosomes are known to be common in tropical and subtropical Asia, their presence in Nepal, a small south Asian country situated between India and China, was almost completely unknown at the beginning of this study. Consequently, from 2007-2014, we investigated the presence of schistosomes in and around Chitwan National Park (CNP) in the Terai and hilly regions of Nepal. We sampled both the dung of selected mammals for schistosome eggs, and screened 19,360 freshwater snails for cercariae of schistososmes.

As reported in Chapter 2 and published in the *Journal of Helminthology*, because elephants are known hosts for schistosomes elsewhere, dung samples from both domestic and wild Indian elephants (*Elephus maximus*) were examined, and were found to be

positive for eggs of the elephant schistosome *Bivitellobilharzia nairi*. Surprisingly, we also found similar eggs in dung samples of the endangered Asian or greater one-horned rhinoceros (*Rhinoceros unicornis*) from CNP. Subsequent study of *cox*1 and 28S DNA sequences obtained from eggs from both host species confirmed them to be those of *B. nairi*. This represents the first sequence-verified identification of an elephant schistosome from another mammal, the first sequence-verified report of a schistosome from any rhinoceros, and the only report indicating elephants and rhinos, even though unrelated, both transmit the same parasite in and around CNP in Nepal.

Regarding the snails collected during our study, as reported in Chapter 3 and published in *Parasitology International*, only two snails were found to harbor avian schistosome cercariae. Phylogenetic analyses of both 28S rDNA and *cox*1 sequences showed that the cercaria recovered from the snail *Radix luteola* grouped in a distinctive and previously unknown lineage within *Trichobilharzia*, a genus well-known for causing dermatitis outbreaks elsewhere. The second schistosome cercaria recovered from the snail *Indoplanorbis exustus* clustered most closely with *Macrobilharzia macrobilharzia*, a schistosome with unknown snail host, although the Nepalese specimens seem to represent a distinct lineage. This is the first sequence-verified documentation of an avian schistosome reported from *I. exustus*, and the first report of any sequence-verified avian schistosomes from south Asia.

Chapter 4 (accepted for publication in *The International Journal for Parasitology*) provides the first molecular evidence for the presence of the *Schistosoma indicum* species group in Nepal. Schistosomes of this group are of considerable veterinary importance and cause dermatitis or potentially even more advanced infections in people. Based on

analysis of *cox*1, 12S, 16S and 28S genes, two recognizable lineages were found, *S. spindale* and *S. nasale*. Unexpectedly, we also found a third distinct lineage that was not *S. indicum* as predicted, but rather one that was previously uncharacterized and that failed to group with any known members of *S. indicum* group. This suggests the presence of a new species of the *S. indicum* group in Nepal. Analysis of *cox*1, 16S and ITS1 sequences for the snail host *I. exustus* was also surprising in revealing the presence of four genetically distinct clades of this snail in Nepal, providing further evidence that *I. exustus* is actually a complex of related species. This finding will stimulate a general reevaluation of the role of this snail species complex in disease transmission.

In chapter 5 (in preparation for submission) we report that 16 of 2,588 specimens of *Radix luteola* from 4 different Nepalese habitats were found to be shedding mammalian schistosome cercariae. Based on 28S, *cox*1, 16S and 12S sequences analysis, these cercariae were found to be very similar to one another and to cluster with *Schistosoma turkestanicum*, although they were clearly genetically distinct from it. This study provides sequence-verification for a third lymnaeid-transmitted *Schistosoma* lineage in Asia, validates the existence of the *S. turkestanicum* species group, and reveals the presence of a distinct but unknown species of that group within Nepal.

This study concludes that schistosomes, and by extension schistosomiasis, are common in Nepal: the Terai region of Nepal alone harbors at least five distinct species of mammalian schistosomes and two distinct species of avian schistosomes. Our study was limited to a small portion of Nepal so we recommend more extensive sampling from different areas of the country, which may yield even more surprising results. Further study is also required to examine the adult stages of Nepalese schistosomes though

cultural and religious practices there make it difficult to get access to adult worms. These results also highlight that the Indian subcontinent in general needs much more additional study with respect to schistosome diversity and that our current knowledge of the schistosomes in this area is very poor. We need to remain mindful of the possibility that some of the schistosomes present may be able to infect humans in some locations.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

LIST OF TABLES

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER ONE

INTRODUCTION

Study Area

Nepal is a small landlocked country situated between two giant Asian countries, China (Tibet) on one side (north) and India on three sides (east, west and south). It is noted for its magnificent Himalayas that extend from east to west in the northern side of the country. Its shape is roughly rectangular, about 885 kilometers long (east to west) and about 200 kilometers wide (south to north), and comprises a total area of 147,181 square kilometers (56,827 square mile).

Albeit small in area, Nepal has great physical and cultural diversity. Geographically, Nepal is divided into three regions: the Mountain Region (27% of total land area) in the north, the Hill Region (56% of total land area), and the lowland tropical to sub-tropical Terai Region (17% of total land area) in the southern part of the country. These three geographic regions parallel each other, and expand from east to west as continuous geographic belts, occasionally divided by the country's major river systems like the Narayani, Karnali and Koshi. Mostly it is in the tropical to sub-tropical lowlands of the Inner Terai where this study was undertaken. The Inner Terai area in Nepal is situated between the Himalayan foothills, the 600–900 meter high Siwalik or Churia range and the 2,000-3,000 meter high Mahabharat range further north (Karki, 2011).

The climate of the Inner Terai region is hot and humid (temperatures can exceed 36^0C), and the region has a pronounced annual cycle of rainfall, divided into four major seasons: pre-monsoon (April and May) characterized by the highest temperatures; the summer monsoon (early June to September) noted for about 80% of annual rainfall

(Maskey et al., 2001); the post-monsoon (lasts until about December); and the winter monsoon (January through March) marked by occasional, short rainfalls in the lowlands and plains and snowfalls in the high-altitude areas.

Chitwan National Park (CNP) and its periphery were the major study sites for this research. CNP is the first national park in Nepal and was established in 1973. This national park is also designated as a World Natural Heritage Site by UNESCO. It covers an area of 932 square kilometer (93,200 hectares) in the subtropical Inner Terai lowlands of south-central Nepal in Chitwan, Parsa, Makwanpur and Nawalparasi districts (Bhuju et al., 2007).

The Narayani-Rapti river system forms a natural boundary to human settlements and dominates the overall ecology of the park (Karki, 2011). The rivers swell greatly during the monsoon season. In 1997, a buffer zone of 766.1 km^2 was added to the north and west of the river system, and between the south-eastern boundary of the park and the international border to India. In the CNP, the monsoon starts in mid-June and eases off in late September. During these 14–15 weeks most of the 2,500 mm yearly precipitation falls. After mid-October the monsoon clouds retreat, humidity drops off, and the top daily temperature gradually subside from ±36°C to ±18°C.

The typical vegetation of the Inner Terai is predominantly Sal (*Shorea robusta*) trees covering about 70% of the national park area. Riverine forest covers about 7% and is dominated by Catechu (*Acacia catechu*), Indian Rosewood (*Dalbergia sissoo*), Kapok (*Bombax ceiba*) and Rhino Apple trees (*Trewia nudiflora*). Similarly, grasslands cover about 20% of the park's area (Karki, 2011). More than 50 species of grasses are found here including some of the world's tallest grasses. Seasonal bushfires, flooding and

erosion evoke an ever-changing mosaic of riverine forest and grasslands along the riverbanks. The wide range of vegetation types in the CNP favors more than 700 species of vertebrates and a not yet fully surveyed number of invertebrate species.

The park harbors more than 50 species of mammals (Karki, 2011) including famous residents such as Indian one-horned rhinos (*Rhinoceros unicornis*), Bengal tigers (*Panthera tigris*) and Indian elephants (*Elephas maximus*). Other important mammals of the park include leopards, fishing cats, jungle cats, the rare marbled cats, golden jackals, spotted linsangs, palm civets, yellow-throated martens, wild boars, several species of flying squirrels, black-naped hares, endangered hispid hares and striped hyenas (Jnawali et al., 2011). CNP has the world's highest population density of sloth bears and gaurs (*Bos gaurus*), the latter the world's largest wild cattle species.

CNP and its vicinity is also the home for 26 different species of snakes (including the king cobra and rock python) (Pandey, 2012), Indian starred tortoise and golden monitor lizards. The Narayani and Rapti rivers, their small tributaries and myriads of oxbow lakes are the habitat for 113 recorded native species of fishes (Edds, 1986), gharials (*Gavialis gangeticus*) and Mugger crocodiles (*Crocodylus palustris*) and an unknown number of species of freshwater snails and bivalves.

This national park is also the home for 543 bird species (BCN and DNPWC, 2011). The park is very important for Indian spotted eagle as this site is among the few known breeding sites of this globally threatened bird. In addition to the large number of residential birds, this park and its periphery is also home for the large number of migrating and vagrant bird species during winter seasons.

CNP is one of the most popular tourist destinations in Nepal. In the fiscal year 2010/2011, 90,722 foreigners and 31,309 Nepali visited the park (Poudel, 2011). The area around the park is extensively cultivated particularly for rice, maize, mustard, lentils and wheat. Large populations of water buffalo occupy the area and are used extensively for milk and compost manure. The nearby town of Sauraha is a tourist attraction and domesticated elephants are a conspicuous feature in and around the town. Large populations of cattle, horses and goats are also present in the area. Chitwan district covers an area of 2,218 km^2 and has a population of 472,048. The literacy rate (6 years and above) is 63.0 percent and 79.3 percent for females and males respectively. Brahmin (hill) (29.3 %), Tharu (12.7 %), Chhetri (11.0 %), and Tamang (7.4 %) are the main caste/ethnic group of this district. Dengue, malaria, bacterial diarrhea and typhoid fever are some of the conspicuous human infectious diseases in this district.

We focused our studies on the Chitwan area because we were more familiar with this area, we know there were several trematodes present, and snails and the definitive hosts we wish to study were abundant in this low land region of southern Nepal.

Schistosomes in general

Schistosoma Weinland, 1858 is a genus of blood flukes within the phylum Platyhelminthes, Class Trematoda, Subclass Digenea (the digenetic trematodes) and family Schistosomatidae. Schistosomes are distinct from other digenetic trematodes because they are dioecious, often with pronounced sexual dimorphism. The family Schistosomatidae is comprised of 14 described genera (Brant and Loker, 2013), four of which reside in mammals including humans. Schistosomes are responsible for causing

schistosomiasis (Loker and Brant, 2006; Gryseels, 2012) and cercarial dermatitis or swimmer's itch (Kolářová, 2007; Soldánová et al., 2013). Schistosomiasis is one of the great neglected diseases of the tropical world and considered the second most devastating parasitic disease after malaria (CDC, last updated November 7, 2012). Schistosomes have a two host life cycle featuring a snail as an intermediate host and vertebrate as a definitive host (Fig. 1).

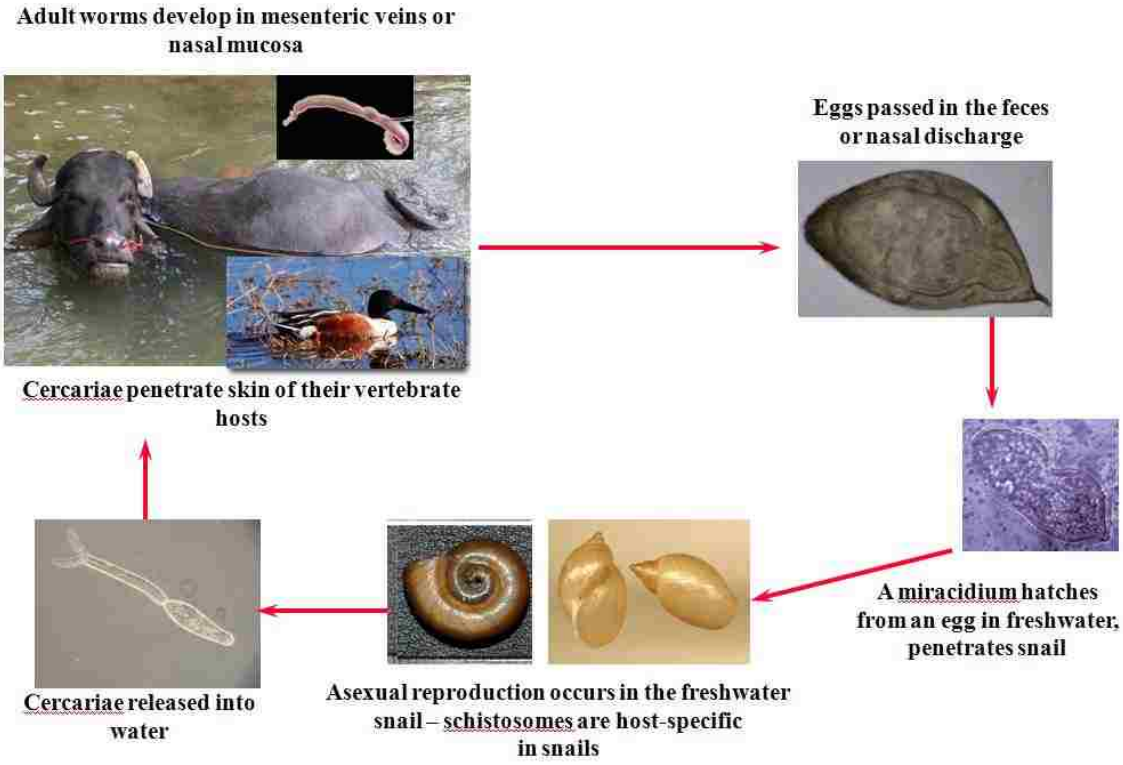

Figure 1. General life cycle of schistosomes

Schistosomes known from Asia and of potential relevance to Nepal

Several species of schistosomes are known from a variety of hosts in countries near to Nepal (Agrawal, 2012), but there is little published evidence for their presence in Nepal except Ghimire (1987) who reported the presence of internal schistosomiasis in

cattle and buffaloes in Surkhet district in western Nepal, Sherchand et al., (1999) who reported the possible presence of a human schistosome in southern Nepal, and Devkota (2008) who undertook a survey of cercariae from snails including schistosome cercariae and also screened elephant dung samples to find *Bivitellobilharzia nairi* eggs from the central Terai. The present study was initially undertaken because of the desire to seek more information regarding the report of suspected cases of human schistosomiasis in southern Nepal (Sherchand et al., 1999). This lead me to a broader study of the schistosomes present in southern Nepal. The schistosomes known to be present in areas near Nepal are briefly outlined below, and discussed in more detail in the chapters that follow.

As noted in the discussion of Chitwan National Park above, many wild vertebrate species occur in Nepal, and based on studies of schistosomes from elsewhere, including countries near to Nepal (Agrawal, 2012), it is known, or is reasonable to expect that, many of these species harbor schistosomes. Prominent among them is *Bivitellobilharzia nairi*, a schistosome common in Asiatic elephants (Agatsuma et al., 2004; Devkota et al., 2014). Eggs of *B. nairi* have been recovered from elephants in Nepal (Devkota, 2008). Chapter two discusses our studies of *B. nairi* from Nepal. A tremendous diversity of schistosomes infect birds around the globe (Brant and Loker, 2013), and given the richness of the avifauna of Nepal (BCN and DNPWC, 2011), it is reasonable to expect this is also the case in Nepal. Although it is difficult to secure permitting to examine birds directly for specimens of adult schistosomes, we have surveyed potential avian schistosome intermediate hosts in Nepal, and Chapter 3 details some of our results regarding the presence of *Trichobilharzia* and *Macrobilharzia*-like species in Nepal.

Avian schistosomes are also noteworthy with respect to their public health significance as it is likely that they can cause dermatitis upon penetration of human skin (Brant and Loker, 2009), and could potentially be implicated in causing outbreaks of cercarial dermatitis or swimmer's itch.

Schistosomes are of particular relevance to veterinary medicine in Nepal, because based on studies from nearby Asian countries, at least seven species of schistosomes are known to commonly afflict domestic ruminants in the region (Agrawal and Rao, 2011). Furthermore, these species can cause a variety of health problems for domestic animals, ranging from poor weight gain and failure to thrive, to major dysfunction of organs like the intestine and liver, to bizarre problems like the snoring disease exhibited by cattle infected with *Schistosoma nasale* (Agrawal and Southgate, 2000). Three of these species are close relatives and are said to comprise the *Schistosoma indicum* species group (Attwood et al., 2007), and include *S. indicum*, *S. spindale* and *S. nasale*. Furthermore, an additional group of ruminant schistosomes previously placed in the genus *Orientobilharzia* is also present in Asia. These include *O. turkestanicum*, *O. harinasutai*, *O. dattai*, and *O. bomfordi*. This genus, demarcated from *Schistosoma* by the presence of a greater number of testes in male worms, has recently been declared invalid, mostly on the basis of limited genetic divergence with *Schistosoma*, so these constituent species are all now referred to as members of *Schistosoma* (*Schistosoma turkestanicum*, etc.) (Aldhoun and Littlewood, 2012). Remarkably, none of these species are known to produce patent, egg-producing infections in humans (Sahba and Malek, 1979; Horák et al., 2015). The extent to which these six species of *Schistosoma*, or perhaps as yet

unknown members of the genus actually occur in Nepal was unknown at the start of this study. Chapters 4 and 5 of this document discuss these species in more detail.

Lastly, schistosomes from in or around nearby Nepal may be of direct relevance with respect to their potential to cause human schistosomiasis. Currently more than 207 million people worldwide are infected with schistosomes, with an estimated 700 million people at risk in 74 endemic countries (WHO, 2010). Over 90% of the world's cases of human-infecting schistosomes now occur in sub-Saharan Africa, mostly caused by *S. mansoni* and *S. haematobium* (Gryseels et al., 2006). However, human schistosomiasis, mostly caused by *S. japonicum* and its relatives, does occur in Asia, especially China, the Philippines and parts of Indonesia (Gryseels et al., 2006). Owing to a lack of appropriate snail intermediate hosts, this group of schistosomes is not known to occur on the Indian subcontinent. One report of a *S. haematobium*-like parasite from southeast India has been reported, and although follow-up studies have not found it to be present, it has been noted that more survey work is needed to definitively rule out its presence (Agrawal and Rao, 2011). Although the public health infrastructure in Nepal strongly suggests that human schistosomiasis is not present in the country, some parts of the country are remote and have never been fully investigated in this regard. Sherchand et al. (1999) reported eggs resembling those of *Schistosoma mansoni* from human stools in Dhanusha district of the Terai region, southern Nepal. Their study also suggested snails resembling *Biomphalaria* sp. were present in Terai, and could be the intermediate host of the *S. mansoni*-like parasite from Nepal. It remains a possibility that unknown species of schistosomes occur in remote areas of tropical Asia, with unknown impacts on human health.

As noted above, another public health impact for schistosomes in the Asian subcontinent is for their potential for causing cercarial dermatitis. In addition to avian schistosomes, the cercariae of which are frequently implicated in dermatitis outbreaks around the world (Brant and Loker, 2009; Horák et al., 2015), it is also known that the several species of mammalian-infecting schistosomes can also cause dermatitis in people, even though the worms are apparently incapable of developing into full-blown schistosomiasis cases featuring adult worms in people (but see persistent reports of human infections cited in Agrawal and Rao, 2011). Some schistosomes in tropical Asia cause "swimmer itch" in people, with a background prevalence of as high as 12.5% (Rao et al., 2007). Members of the *S. indicum* species group have been implicated of human cercarial dermatitis in India and in Malaysia (Singh et al., 1997: Narain et al., 1998).

In conclusion, several species of schistosomes were suspected of occurring in Nepal when this study was initiated. I was motivated to learn more about the schistosomes of Nepal for several reasons: 1) to learn if the presence of human-infecting schistosomes could be found; 2) to gain an appreciation for the species of schistosomes present and the patterns of their host use; 3) to provide additional information contributing to the general puzzle for why cases of human schistosomiasis are rare on the Indian subcontinent even though several species of schistosomes are present; 4) provide new insights on the diversification of schistosomes, including patterns in their use of *Indoplanorbis* snails; 5) provide information potentially of relevance to schistosome control; and 6) provide basic molecular knowledge that can be used to further research work on the overall schistosome and digenetic trematode fauna from Nepal and elsewhere.

References

Agatsuma, T., Rajapakse, R.P.V.J., Kuruwita, V.Y., Iwagami, M., Rajapakse, R.C. (2004) Molecular taxonomic position of the elephant schistosome, *Bivitellobilharzia nairi*, newly discovered in Sri Lanka. Parasitology International 53, 69–75.

Agrawal, M.C. (2012) Schistosomes and schistosomiasis in South Asia. Springer India.

Agrawal, M.C., Rao, V.G. (2011) Indian Schistosomes: A need for further investigations. Journal of Parasitology Research 2011, 1-4.

Agrawal, M.C., Southgate V.R. (2000) *Schistosoma spindale* and bovine schistosomiasis Journal of Veterinary Parasitology 14, 95-107.

Aldhoun, J.A., Littlewood, D.T. (2012) *Orientobilharzia* Dutt & Srivastava, 1955 (Trematoda: Schistosomatidae), a junior synonym of *Schistosoma* Weinland, 1858. Systematic Parasitology 82, 81-88.

Attwood, S.W., Fatih, F.A., Mondal, M.N., Alim, M.A., Fadjar, S., Rajapakse, R.P., Rollinson, D. (2007) A DNA sequence-based study of the *Schistosoma indicum* (Trematoda: Digenea) group: population phylogeny, taxonomy and historical biogeography. Parasitology 134, 2009-2020.

BCN, DNPWC (2011) The state of Nepal's birds 2010. Bird Conservation Nepal and Department of National Parks and Wildlife Conservation, Kathmandu.

Bhuju, U.R., Shakya, P.R., Basnet, T.B., Shrestha, S. (2007) Nepal biodiversity resource book. Published by International Centre for Integrated Mountain Development, Ministry of Environment, Science and Technology, in cooperation with United

Nations Environment Programme, Regional Office for Asia and the Pacific, Kathmandu, Nepal.

Brant, S.V., Loker, E.S. (2009) Schistosomes in the Southwest United States and their potential for causing cercarial dermatitis or 'swimmer's itch'. Journal of Helminthology 83, 191-198.

Brant, S.V., Loker, E.S. (2013) Discovery-based studies of schistosome diversity stimulate new hypothesis about parasite biology. Trends in Parasitology 29, 449-459.

CDC (last updated November 7, 2012) Parasite – Schistosomiasis retrieve from http://www.cdc.gov/parasites/schistosomiasis/ Accessed on March 23, 2015.

Devkota, R. (2008) Inventory of trematode cercariae infections in freshwater snails in Chitwan and Nawalparasi districts and trematodes infections in domestic elephants of Sauraha [MSc thesis], Kathmandu: Central Dept Zoology, Tribhuvan University. 41 p.

Devkota, R., Brant, S.V., Thapa, A., Loker E.S. (2014) Sharing schistosomes: the elephant schistosome *Bivitellobilharzia nairi* also infects the greater one-horned rhinoceros (*Rhinoceros unicornis*) in Chitwan National Park, Nepal. Journal of Helminthology 88, 32-40.

Edds, D.R. (1986) The fishes of Royal Chitwan National Park. A record of 113 native fish species known to occur in Nepals RCNP. Journal of Natural History Museum (Nepal) 10, 1-12.

Ghimire, N.P. (1987) Incidence of common disease of cattle and buffaloes in Surkhet district. Bulletin of Veterinary Science and Animal Husbandry, Nepal, 15, 95-101.

Gryseels, B. (2012) Schistosomiasis. Infectious Disease Clinics of North America 26, 383-397.

Gryseels, B., Polman, K., Clerinx, J., Kestens L. (2006) Human schistosomiasis. Lancet 368, 1106-1181.

Horák, P., Mikeš, L., Lichtenbergová, L., Skála, V., Soldánová, M., Brant, S.V. (2015) Avian schistosomes and outbreaks of cercarial dermatitis. Clinical Micribiology Reviews 28, 165-190.

Jnawali, S. R., Lee, S., Baral, H. S., Upadhyay, G. P., Pandey, M., Shrestha, R., Griffiths, J., Joshi, D., Amin, R. (2011) The Status of Nepal's Mammals: The National Red List Series, Department of National Parks and Wildlife Conservation, Ministry of Forests and Soil Conservation, Government of Nepal, Kathmandu.

Karki, J.B. (2011) Occupancy and abundance of tigers and their prey in the Terai Arc Landscape, Nepal. A Ph.D. thesis submitted to the forest research institute, University of Dehradun, Uttarakhand, India.

Kolářová, L. (2007) Schistosomes causing cercarial dermatitis: a mini-review of current trends in systematics and of host specificity and pathogenicity. Folia Parasitologica 54, 81-87.

Loker, E.S., Brant S.V. (2006) Diversification, dioecy and dimorphism in schistosomes. Trends in Parasitology 22, 521-528.

Maskey, S.L., Bhattarai, S., Peoples, M.B., Herridge, D.F. (2001) On-farm measurements of nitrogen fixation by winter and summer legumes in the hill and terai regions of Nepal. Field Crops Research 70, 209-221.

Narain, K., Rajguru, S.K., Mahanta, J. (1998) Incrimination of *Schistosoma spindale* as a causative agent of farmer's dermatitis in Assam with a note on liver pathology in mice The Journal of Communicable Diseases 30, 1-6.

Pandey, D.P. (2012) Snakes in the vicinity of Chitwan National Park, Nepal. Herpetological Conservation and Biology 7, 46−57.

Poudel, R.K. (2011, July 29) 'Rise in tourist flow increases National Park's revenue', *República*, Retrieve from http://www.myrepublica.com/portal/index.php?action=news_details_id=34027 Accessed on 17 Nov 2011.

Rao, V.G., Dash, A.P., Agrawal, M.C., Yadav, R.S., Anvikar, A.R., Vohra, S., Bhondeley, M.K., Ukey, M.J., Das, S.K., Minocha, R.K., Tiwari, B.K. (2007) Cercarial dermatitis in central India: an emerging health problem among tribal communities. Annals of Tropical Medicine and Parasitology 101, 409-413.

Sahba, G.H., Malek, E. A. (1979) Dermatitis caused by cercariae of *Orientobilharzia turkestanicum* in the Caspian Sea area of Iran. American Journal of Tropical Medicine and Hygiene 28, 912-913.

Sherchand, J.B., Ohara, H., Sherchand, S., Matsuda, H. (1999) The suspected existence of *Schistosoma mansoni* in Dhanusha district, southern Nepal. Annals of Tropical Medicine and Parasitology 93, 273-278.

Singh K.I., Krishnasamy M., Ambu S., Rasul R. & Chong N.L. (1997) Studies on animal schistosomes in Peninsular Malaysia: record of naturally infected animals and additional hosts of *Schistosoma spindale*. The Southeast Asian Journal of Tropical Medicine and Public Health 28, 303-307.

Soldánová, M., Slebach, C., Kalbe, M., Kastadinova, A., Sures, B. (2013) Swimmer's itch: etiology, impact, and risk factors in Europe. Trends in Parasitology 29, 65-74.

WHO (Feb 2010) 'Scistosomiasis, Fact Sheet No 115', World Health Organization. Available at http://www.who.int/mediacentre/facesheets/fs115/en/index.html. Accessed on Nov 17,2011.

CHAPTER TWO

This material in this chapter has been published and has the following citation:

Devkota, R., Brant, S.V., Thapa, A., Loker, E.S., 2014. Sharing schistosomes: the elephant schistosome *Bivitellobilharzia nairi* also infects the greater one-horned rhinoceros (*Rhinoceros unicornis*) in Chitwan National Park, Nepal. Journal of Helminthology 88, 32-40.

Sharing schistosomes: the elephant schistosome *Bivitellobilharzia nairi* also infects the greater one-horned rhinoceros (*Rhinoceros unicornis*) in Chitwan National Park, Nepal

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Abstract

Because the digenetic trematode fauna of Nepal is poorly known, we began to search for schistosomes in and around Chitwan National Park (CNP) of southern Nepal. Both domestic and wild Indian elephants (*Elephus maximus*) are present, and we found one of two dung samples from wild elephants and 1 of 22 (4.5%) dung samples from domestic elephants to be positive for schistosome eggs. The morphology of the eggs and both *cox*1 and 28S sequences derived from the eggs/miracidia were consistent with *Bivitellobilharzia nairi*, reported here for the first time from Nepal. Also, 7 of 14 faecal samples from the Asian or greater one-horned rhinoceros (*Rhinoceros unicornis*) contained viable eggs indistinguishable from those of *B. nairi*. This identification was confirmed by comparison with both *cox*1 and 28S sequences from *B. nairi* eggs/miracidia derived from Nepalese and Sri Lankan elephants. This represents the first sequence-verified identification of a schistosome from any species of rhinoceros, and the first verified occurrence of a representative of *Bivitellobilharzia* (a genus of 'elephant schistosomes') in mammals other than elephants. Our work suggests that elephants and rhinos share *B. nairi* in CNP, even though these two members of the 'charismatic megafauna' belong to unrelated mammalian families. Their shared life style of extensive contact with freshwater habitats likely plays a role, although the snail intermediate host and mode of definitive host infection for *B. nairi* have yet to be documented. This report also supports *Bivitellobilharzia* as a monophyletic group and its status as a distinct genus within Schistosomatidae.

1. Introduction

Among the many factors that potentially conspire against conservation of the world's biodiversity, including charismatic and popular large mammalian species, are infectious diseases, including those caused by metazoan parasites (Zhang et al., 2008). In general, we need an improved understanding of the parasites that infect these increasingly rare animals. At the same time, large host species can be viewed as arks that support a variety of unique symbiotic species, often including relatively host-specific parasites that may occur nowhere else in the world, and that are potentially susceptible to co-extinction events. Acknowledgment of the existence of this form of biodiversity and the need to preserve it adds even more incentive to characterizing and understanding the biology of the parasites of large, rare mammals.

This study focuses on two unrelated but prominent mammals of Chitwan National Park (CNP) in subtropical Nepal. The first is the Indian rhinoceros, also known as the greater one-horned rhinoceros or the Asian one-horned rhinoceros (*Rhinoceros unicorn*is Linnaeus, 1758), which is listed by the International Union for the Conservation of Nature (IUCN) as 'vulnerable'. The second is the Asian, or Indian, elephant *Elephas maximus* Linnaeus, 1758, listed as 'endangered' by IUCN. Whereas the biology and host–parasite relationships of vulnerable large mammals have been more intensively studied in other locations, such as African national parks (Southwell, 1921; Thapar, 1925; Fitzsimmons, 1962; Zumpt, 1964; Penzhorn et al., 1994; Kinsella et al., 2004; Brant et al., 2012), the study of the wild mammals of Nepal and their parasites is in its infancy. Our study focuses on schistosomes or blood flukes we have recovered from faecal samples of both elephants and rhinos in and around CNP.

The family Schistosomatidae is comprised of 14 recognized genera, five of which occur in mammals. Among the mammal-infecting genera is *Bivitellobilharzia* Dutt and Srivastava, 1955, with two described species, heretofore known only from elephants. *Bivitellobilharzia loxodontae* Vogel and Minnig, 1940 occurs in African forest elephants *Loxodonta cyclotis* Matschie, 1900 from the Democratic Republic of Congo (Vogel & Minning, 1940; Kinsella et al., 2004) and from the Central African Republic (Brant et al., 2012). There have been no definitive reports of schistosomes from the African savanna elephant, *Loxodonta africana* Blumenbach, 1797. *Bivitellobilharzia nairi* (Mudaliar & Ramanujachary, 1945) Dutt and Srivastava, 1955 occurs in Asian elephants *Elephas maximus* Linnaeus, 1758 from India (Vogel & Minning, 1940; Mudaliar & Ramanujachary, 1945; Rao & Hiregauder, 1953; Dutt & Srivastava, 1961), Sri Lanka (Agatsuma et al., 2004; Brant et al., 2006), Republic of the Union of Myanmar (Sundaram et al., 1972) and Nepal (Devkota, 2008; Karki & Manandhar, 2008). There are at least three recognized subspecies of Asian elephants: *Elephas maximus maximus* from Sri Lanka, the Indian elephant or *Elephas maximus indicus* from mainland Asia (including Nepal), and *Elephas maximus sumatranus* from the island of Sumatra. To our knowledge *B. nairi* has only been reported from the first two subspecies.

Adult males of *Bivitellobilharzia* are characterized by the presence of a tuberculated tegument, and by possession of up to 52 testes. Females are without tubercles and the ovary lies in the anterior fourth of the worm (Mudaliar & Ramanujachary, 1945). The eggs are asymmetrical and bear a terminal spine. We are otherwise largely ignorant of the biology of *Bivitellobilharzia*. We do not know the identity of the natural snail intermediate hosts and are poorly aware of the extent of their

geographic distributions or patterns of host use. In molecular phylogenetic reconstructions, *Bivitellobilharzia* is basal to the prominent *Schistosoma* + *Orientobilharzia* Dutt and Srivastava, 1955 clade of schistosomes that occurs predominately in ruminants, primates and rodents (Brant et al., 2006, 2012). Until recently, most of our current molecular knowledge of *B. nairi* has been from elephants in Sri Lanka (Agatsuma et al., 2004; Brant et al., 2006) but very little is known about this species in other parts of its range. A recent study has provided the first molecular sequence data for *B. loxodontae*, confirming that the two species in the genus are distinct from one another and united within a monophyletic group, thus upholding *Bivitellobilharzia* as a distinct schistosome genus (Brant et al., 2012).

Whereas elephants are well known for their role in hosting schistosomes, there has been but one prior report of schistosomes from any of the world's five extant species of rhinoceroses. Tiuria et al. (2006) reported the eggs of *Schistosoma* spp. (199.4 £ 111.8mm) in a faecal sample from the Java rhino *Rhinoceros sondaicus* Desmarest, 1822 collected from Ujung Kulon National Park, Banten, Java, Indonesia. Further information to identify this schistosome species is lacking, and no images were included in the report. Other surveys of rhino parasites, whether from African or Asian rhinoceroses have not reported schistosome eggs (Zumpt, 1964; Silberman & Fulton, 1979; Palmieri et al., 1980; Dutta et al., 1990; Chakraborty & Islam, 1993; Penzhorn et al., 1994; Chakraborty & Gogoi, 1995; Muryani et al., 2008).

Given the vulnerable or endangered status of the world's rhinoceros and elephant species, this report represents an effort to characterize their schistosome parasite fauna using modern, non-invasive approaches before these animals are no longer available to

study. Also, it is important to identify the parasite species harbored should they ever pose health problems for their hosts. Although there is some evidence that *B. nairi* infection can compromise survival of elephant calves (Anonymous, 1984), there has been little study of the impact schistosomes might have on the health of elephants or rhinoceroses, especially in the wild. This study also represents part of our ongoing effort to learn more about the poorly characterized schistosome genus *Bivitellobilharzia*, and the overall trematode fauna of Nepal.

2. Materials and methods

2.1 *Collection and examination of faecal samples*

Collections of fresh faecal samples from domestic or wild elephants, or from wild rhinoceroses, were made between 2007 and 2011, in and around CNP near Sauraha (27 30’0”N, 84 20’0”E) in south central Nepal. Fresh faecal samples were washed through a nested series of sieves (mesh sizes 450 µm, 150 µm, 75 µm and 32 µm) and eggs are retained on the 32 mm mesh screen. Sieved samples were either examined for unhatched eggs or were suspended in freshwater in 50 ml centrifuge tubes and exposed to sunlight to hatch the eggs. The upper layer of water in these tubes (1–2 ml) was collected and transferred to Petri dishes where miracidia were collected with the aid of a dissecting microscope. Fresh eggs were measured with the aid of a calibrated ocular micrometer at 400X magnification using a compound microscope. Eggs or miracidia were preserved in RNAlater (Ambion The RNA Company, Life Technologies, Grand Island, New York, USA) or 96% ethanol. Each sample collected consisted of a small number (10–50) of individual miracidia or eggs, all recovered from the same faecal sample. 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 analysis.

2.2 *Molecular and phylogenetic analyses*

DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit (Valencia, California, USA). Schistosome eggs were digested overnight, whereas miracidia were digested for 2–3 h. The nuclear ribosomal gene 28S and the mitochondrial gene cytochrome oxidase I (*cox*1) were amplified by polymerase chain reaction. Methods and primers were as described in Brant et al. (2012). There are only two known species of *Bivitellobilharzia* and to confirm that our samples belonged to *B. nairi* and that they were different from *B. loxodontae*, we compared *cox*1 pairwise sequence differences. This allowed us to also relate our results with species differences among closely related species of *Schistosoma*.

The 28S and *cox*1 gene fragments were used in phylogenetic analyses using maximum parsimony (MP), maximum likelihood (ML) and minimum evolution (ME), carried out in PAUP* ver. 4.0b10 (Swofford, 2002). Bayesian inferences (BI) were made with the use of MrBayes (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). jModel test was used to determine the best fit nucleotide substitution model for ML and ME analyses (Posada, 2008). Optimal MP, ME and ML trees were reconstructed using heuristic searches: for the 28S dataset we ran 100 replicates for MP and ME and 10 replicates for ML; and for the *cox*1 dataset we ran 500 replicates for MP and ME and 100 replicates for ML. Nodal support was estimated by bootstrap: for the 28S dataset we ran 200 replicates with 10 addition sequence replicates for MP and ME and for the cox1

dataset we ran 500 replicates with 10 addition sequence replicates for MP and ME, and 200 replicates with 5 addition sequence replicates for ML. For the BI analysis of the 28S dataset, the parameters were unlinked: Nst ¼ 6 rates ¼ invgamma ngammacat ¼ 4. For the BI analysis of the cox1 dataset, the parameters were unlinked; data were partitioned by gene, for codons one and two Nst ¼ 2 and for codon three Nst ¼ 6 rates ¼ gamma, ngammacat ¼ 4. For both 28S and *cox*1 datasets, four chains were run simultaneously for 5,000,000 generations, trees were sampled every 100 cycles, the first 5000 trees with pre-asymptotic likelihood scores were discarded as burn-in, and the retained trees were used to generate a 50% majority-rule consensus trees and posterior probabilities.

3. Results

From 2007 to 2011 we collected 22 fresh domestic elephant faecal samples and two fresh wild elephant faecal samples. The latter samples were determined to be from a wild elephant because they were recovered deep within CNP and had a plant composition typical of that consumed by wild elephants. Additionally, fresh faecal material was taken from each of 14 different rhinoceros faecal middens, all located in and around CNP. Rhinos use these middens to mark their territories. The locality information from our collections is summarized in table 1. Among them, two elephant samples (one domestic and one wild), and seven rhinoceros samples were found positive for schistosome eggs/miracidia.

All eggs resembled those of *B. nairi*. Those from *R. unicornis* samples measured 132–156 mm (average 144 mm) in length (excluding spine) by 81.6–86.4mm (average 83.4mm) wide with a spine length of 9.6–12mm (average 11.4mm) (fig. 1). Dimensions

of the schistosome eggs from elephants were not obtained, but these eggs were similar in size and shape to eggs obtained from rhinos. In table 2, the dimensions of schistosome eggs obtained from our rhino samples are compared to egg measurements from the literature for schistosomes from elephant and rhinoceros faecal samples.

Table 1. Host, status with respect to schistosome infection, date of collection and location (including co-ordinates) of the positive faecal samples from the Chitwan National Park (CNP) and surrounding sites; one sample was collected from each site unless otherwise stated.

Host	Date Collected	Location	Co-ordinates
Elephas maximus	23 September 2007	Sauraha, Chitwan	N $27^0 34' 38.6"$ E $84^0 29' 38.7"$
*E. maximus**	8 July 2010	CNP	N $27^0 33' 22.6"$ E $84^0 29' 54.0"$
Rhinoceros unicornis	4 July 2010	CNP	Unknown
*R. unicornis**	6 July 2010	CNP	N $27^0 33' 36.6"$ E $84^0 30' 09.1"$
*R. unicornis**	7 July 2010	CNP	N $27^0 34' 02.3"$ E $84^0 30' 37.6"$
*R. unicornis**	7 July 2010	CNP	N $27^0 33' 00.6"$ E $84^0 30' 16.4"$
R. unicornis	3 March 2011	CNP	N $27^0 33' 59.3"$ E $84^0 30' 14.9"$
R. unicornis	27 March 2011	CNP	N $27^0 33' 34.4"$ E $84^0 30' 05.4"$
*R. unicornis**	13 May 2011	CNP	N $27^0 34' 33.2"$ E $84^0 29' 37.2"$

*Samples sequenced successfully

DNA sequence data were deposited in GenBank, under accession numbers JQ975005 and JQ975006 for the 28S (1310 bp) data set and JQ975007–JQ975011 for the *cox*1 (1071 bp) data sets. The results of the analyses used to reconstruct the phylogenetic relationships of our samples to known samples were congruent other than variation in

nodal support (figs 2 and 3). *Bivitellobilharzia* grouped as a sister clade to *Schistosoma*, and our Nepalese rhinoceros and elephant samples grouped with the known sample of *B. nairi* from Sri Lanka, to the exclusion of *B. loxodontae* from the Central African Republic. The *cox*1 tree (fig. 3) included four samples from *R. unicornis* and one sample from wild *E. maximus*; these samples were not significantly different from each other, indicating that it is unlikely there is a separate schistosome species in each of the two host species. All samples grouped with, but were genetically different from (*cox*1 2.7–3.2%), the sample of *B. nairi* from Sri Lanka.

Based on our *cox*1 data, *B. nairi* and *B. loxodontae* were 12–12.5% different, a value similar to those of closely related species pairs: for example, *Schistosoma indicum–S. spindale* 14.4%, *S. mansoni–S. rodhaini* 11.7% (this is based on the sequence data we used to generate the phylogenetic tree). The average pairwise difference among the *B. nairi* we recovered and species of *Schistosoma* was 21–25%. The similarity in *cox*1 mtDNA sequences between schistosomes from the two large mammal species (0.0–0.5% divergence) suggests they share the same schistosome in CNP.

Figure 1. The schistosome egg obtained from fecal samples of *R. unicornis* from Chitwan National Park.

4. Discussion

The recovery of *B. nairi* from both domestic elephants that live adjacent to CNP and from wild elephants that live within the park represents the most northerly records for this species, which is otherwise known from India, Sri Lanka and the Republic Union of Myanmar (Vogel & Minning, 1940; Mudaliar & Ramanujachary, 1945; Rao & Hiregauder, 1953; Dutt & Srivastava, 1961; Sundaram et al., 1972; Agatsuma et al., 2004). Domestic elephants have a relatively low prevalence of *B. nairi*, perhaps because they spend relatively little time in natural snail habitats compared to wild elephants or rhinoceroses.

Table 2. Schistosome eggs obtained from rhino samples compared to egg measurements from the literature for schistosomes from elephant and rhinoceros fecal samples from other localities.

	Length	Width	Spine Length	References
R. unicornis	132-156 (144)**	81.6-86.4 (83.4)	9.6-12 (11.4)	This study (CNP, Nepal)
R. sondaicus	199.4***	111.8	Unknown	Tiuria *et al.* (2006) (Java, Indonesia)
E. maximus	138-183 (161)*	70-91 (84)	9-25 (16)	Vogel & Minning (1940) (infected elephants from Burma were transported to Hamburg, Germany in early 1939)
E. maximus	141.9–181.5 (155.6)***	66-108.9 (81.04)	6.6-13.2 (9.2)	Sundaram *et al.* (1972) (Kerala, India)
E. maximus (in utero)	80***	30	Unknown	Mudaliar and Ramanujachary (1945) (Coimbatore District of South India)
E. maximus	140-160***	65-80	Unknown	Rao & Hiregaudar (1953) (North Kanara District, Bombay, India)

Terminal spine: *included in measurement; **not included in measurement; ***not known if terminal spine was included in measurement

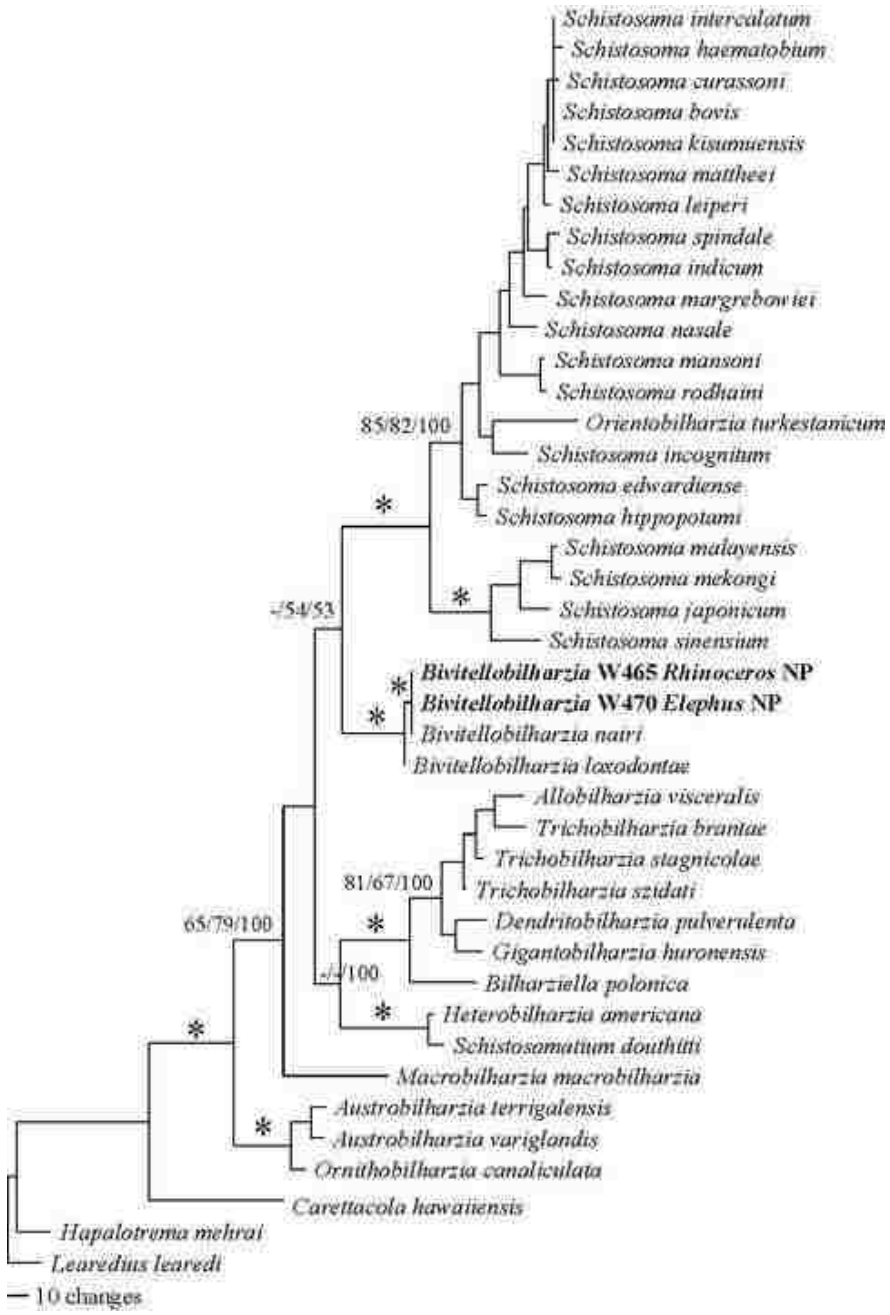

Figure 2. Bayesian inference tree based on 28S sequences. Samples in bold are those collected for this study. Node support is indicated by MP, ME bootstrap values and Bayesian posterior probabilities (PP), respectively. The asterisks indicate MP and ME bootstrap values of .90 and PP of .97. The dashes indicate no significant node support.

We were surprised to find readily hatching schistosome eggs derived from Asian rhino dung samples collected from characteristic rhino dung middens in CNP. This is the first report of a schistosome infection in *R. unicornis*. Molecular signatures obtained from rhino-derived eggs or miracidia indicated they were very similar with respect to both cox1 and 28S sequences to *B. nairi* eggs or miracidia obtained from CNP elephant dung samples. The degree of cox1 sequence divergence (genetic distance) between specimens of *B. nairi* recovered from elephants and rhinos in CNP (0.0–0.5%) is low in comparison to the 1.4% degree of variability observed within a locality for *S. mansoni*, another mammalian schistosome parasite (Stothard et al., 2009). Nothing about the sequence data we collected suggests that rhinos and elephants are supporting separate species or variants of *B. nairi* in CNP, although further study would be helpful to confirm this point.

In contrast, the divergence for *cox*1 between *B. nairi* collected from Sri Lanka and Nepal (2.7–3.2%) is appreciable. Although this probably merely reflects geographic variation in *B. nairi* between the two localities, which are separated by over 2300 km, the possibility that more discrete differences occur between worms from the two localities should not be ignored, especially given that the distribution of elephants in southern Asia is not continuous, and the Sri Lankan specimens are from an island population.

A second reason to keep an open mind regarding the possibility of additional diversity in Asian *Bivitellobilharzia* stems from the one prior report of schistosome eggs from *Rhinoceros sondaicus* from Java (Tiuria et al., 2006). This is because the egg sizes reported are larger (table 2) than those reported here for *B. nairi* from *R. unicornis*. Although no additional data are available (and may never be, given the rarity of Javan rhinos in the wild), given the disparity in egg sizes, further study is needed to determine if

this schistosome is likely to be *B. nairi* as well. To our knowledge, elephants are not present in the part of Java in which the infected Javan rhino, or rhinos, were found.

In general, based on what we know now, our results are in agreement with a recent study of *B. loxodontae* from African forest elephants (Brant et al., 2012): the Nepalese elephant/rhino schistosome supports the concept of two separate species within *Bivitellobilharzia*, and that this genus represents a distinct lineage within the Schistosomatidae. Whereas *Bivitellobilharzia* has been traditionally considered a genus of 'elephant schistosomes', our report of *B. nairi* from Indian rhinos, and the earlier report of a putative *Bivitellobilharzia* species from Javan rhinos (Tiuria et al., 2006), indicate *Bivitellobilharzia* can occur in animals other than elephants.

It is intriguing that Indian elephants and Asian rhinos both host the same schistosome in CNP, yet are not close relatives. A recent phylogenetic study of mammals suggests rhinos (Rhinoceratidae) and elephants (Elephantidae) last shared a common ancestor at about 100 million years ago (Meredith et al., 2011). Both species spend a considerable amount of time in water, a factor expected to predispose them to schistosome infection. However, both species also have very thick skin, which raises the question as to how elephants and rhinos become infected in the first place.

The answer to this question would become clearer if we knew the identity of the natural intermediate host for *B. nairi*. Vogel & Minning (1940) recovered *B. nairi* cercariae from experimentally exposed lab-reared snails of Planorbidae: *Biomphalaria pfeifferi*, originally from Africa, and *Planorbis* sp. from Germany. Despite considerable searching among thousands of freshwater snails recovered both within and outside CNP, we have failed to find any snails shedding *B. nairi* cercariae. Experimental infections of

some of the more prominent freshwater snail species from the area with miracidia derived from either rhinos or elephants have also failed to result in infections. Keeping experimentally exposed snails alive in the heat of the Terai region is a persistent challenge. Although it would be unprecedented for schistosomes and for most digenetic trematodes except sanguinicolids, perhaps the intermediate host for *B. nairi* is not a snail, or perhaps not an aquatic snail. It is also conceivable that infected intermediate hosts are actually ingested along with vegetation eaten by these large herbivores. That this is even a possibility is supported by the finding of mollusc shell contents in *R. unicornis* faecal samples from CNP (Laurie, 1978). In such a case, cercariae might never be shed from the intermediate host as is typical for other schistosomes. Dissections of the majority of snails collected have not, however, yielded cryptic infections. Our preferred hypothesis, suggested by Vogel & Minning's (1940) work, is that the life cycle is a conventional one, but that snail infections simply happen to be extremely rare. The longevity of rhinos and elephants would favor persistence of *B. nairi* in nature in two ways: these large animals could slowly accrue worms and acquire bisexual infections, and once infected, would be available to produce eggs to infect intermediate hosts – even at a potentially low level – over a span of decades.

The report of *B. nairi* from one, and possibly two, rhino species from Asia and from the Indian elephant raises the issue as to whether this is originally a rhino parasite that has colonized elephants, or vice versa. The presence of the only known congener, *B. loxodontae* in African forest elephants, suggests that *Bivitellobilharzia* worms are first and foremost elephant parasites. To our knowledge there are no reports of schistosomes in African rhinos, which in general are less aquatic than their Asian counterparts. Given

the long and complex history of both elephants and rhinos, and that the extant species we see today are but a small proportion of the many species that once existed, it may be very difficult to fully answer this question. Also, it is difficult to resolve whether *Bivitellobilharzia* originated in Africa or Asia.

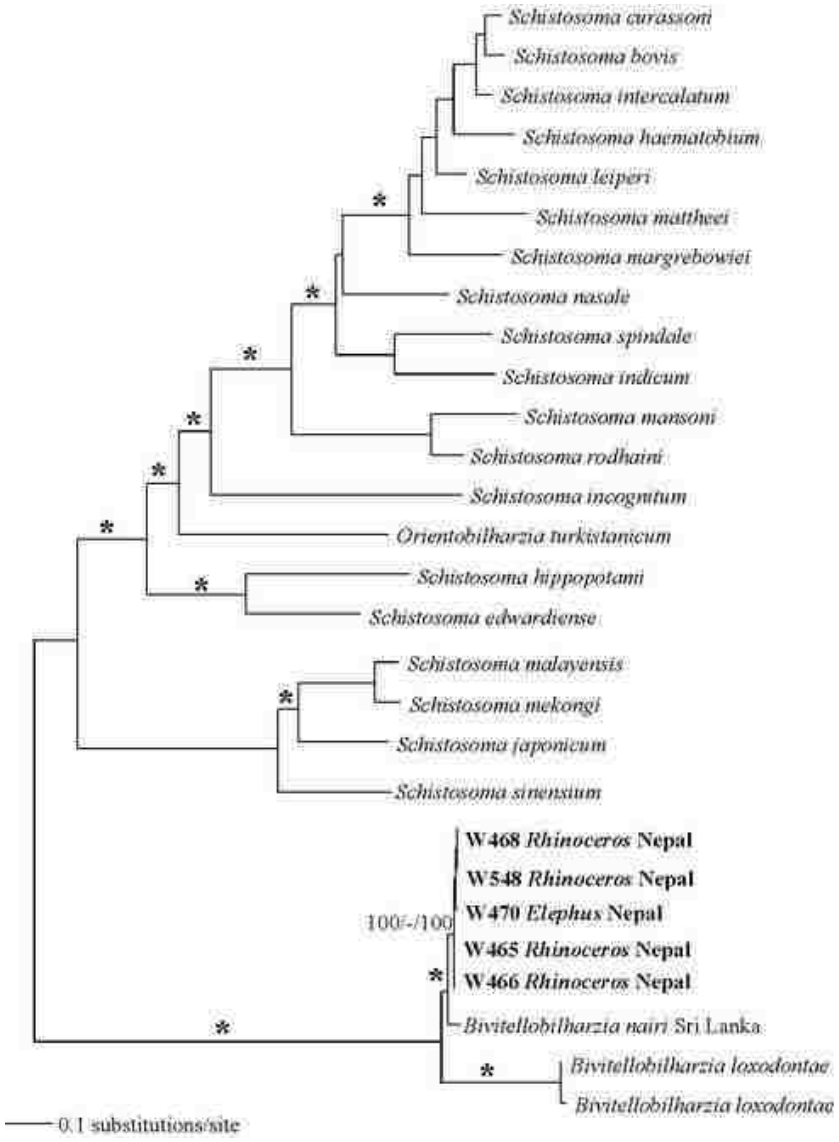

Figure 3. Maximum likelihood tree based on partial *cox*1 sequences. Samples in bold are those collected for this study. Node support is indicated by MP and ME bootstrap values and Bayesian PP, respectively. The asterisks indicate bootstrap values of .90 and a PP of .97. The dash indicates no significant node support.

The presence of a shared schistosome between Indian elephants and Asian rhinos also raises a question as to whether each species is able to maintain *B. nairi* in nature, or whether transmission really depends primarily on the eggs derived from one species or the other. Certainly the presence of *B. nairi* in elephants from locations, like Sri Lanka, where rhinos are absent suggests that elephants are able to propagate the infection themselves. Whether rhinos can do the same in the few localities where they currently exist without elephants remains to be determined. Once again, their longevity may be a factor that favors their ability to maintain *B. nairi* on their own. It is also conceivable that additional mammalian species transmit *B. nairi* in CNP. We examined 10 faecal samples from chital deer (*Axis axis*) and they were negative (unpublished observations). Gaur (*Bos guaros*) are also present, but rare, and we have not had the opportunity to examine faecal specimens from this species. Domestic buffaloes (*Bubalus bubalis*) and cattle (*Bos primigenius indicus*) are generally absent inside the park but we have had a chance to examine 100 faecal samples of domestic buffalo in areas adjacent to CNP. All were negative for *B. nairi* infection.

Finally, the presence of a shared potential pathogen between elephants and rhinos is noteworthy from a management point of view. Schistosomes are capable of causing considerable pathology, so it is important to keep in mind that both host species in Nepal could be affected by *B. nairi*. Translocations or population increases of one host species may result in unexpected transfer of *B. nairi* to the other species. Perhaps the two host species share other parasites as well, possibly even including the pathogenic liver fluke *Fasciola jacksoni* or amphistome flukes, both known from elephants. We must also

remain alert to the possibility that *B. nairi* may also infect other valuable hoof stock in CNP.

Acknowledgements

The University of New Mexico supported this study, through a National Institutes of Health grant to E.S.L. (RO1 A144913) and a National Science Foundation grant to S.V.B. (DEB 1021427). 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 are grateful to the officials of the Department of National Parks and Wildlife Conservation, Chitwan National Park and the Nepal Health Research Council (Permit No. 44) for their cooperation with this research. We are also indebted to Miss Susma Dhakal, Mr Sulav Dhakal, Mr Mohan Pandey, Mr Bikas Sapkota and Mr Ramesh Ghimire for providing field support to collect the samples.

References

Agatsuma, T., Rajapakse, R.P.V.J., Kuruwita, V.Y., Iwagami, M. & Rajapakse, R.C. (2004) Molecular taxonomic position of the elephant schistosome, *Bivitellobilharzia nairi*, newly discovered in Sri Lanka. Parasitology International 53, 69–75.

Anonymous (1984) Parasite killed elephant. 9 September 1984, Associated Press.

Brant, S.V., Morgan, J.A.T., Mkoji, G.M., Snyder, S.D., Rajapakse, R.P.V.J. & Loker, E.S. (2006) An approach to revealing blood fluke life cycles, taxonomy, and diversity: provision of key reference data including DNA sequence from single life cycle stages. Journal of Parasitology 92, 77–88.

Brant, S.V., Pomajbı´kova´,K.,Modry, D., Petrzˇelkova´, K.J., Todd, A. & Loker, E.S. (2012) Molecular phylogenetics of the elephant schistosome, *Bivitellobilharzia loxodontae* (Trematoda: Schistosomatidae) from the Central African Republic. Journal of Helminthology 86, 1–6.

Chakraborty, A. & Gogoi, A.R. (1995) Parasites of rhinoceros (*Rhinoceros unicornis*). Indian Journal of Animal Sciences 65, 421–422.

Chakraborty, A. & Islam, S. (1993) A survey of gastrointestinal parasitic infection in free-living rhinoceros of Kaziranga National Park. Indian Journal of Animal Sciences 63, 155–156.

Devkota, R. (2008) Inventory of trematode cercariae infections in freshwater snails in Chitwan and Nawalparasi Districts and trematode infections in domestic elephants of Sauraha (minor study). A thesis submitted to Central Department of

Zoology, Institute of Science and Technology, Tribhuvan University, Kirtipur, Kathmandu, Nepal.

Dutt, S.C. & Srivastava, H.D. (1961) On the morphology and life history of a new mammalian blood fluke, *Ornithobilharzia dattai* n. sp. (preliminary report). Indian Journal of Parasitology 13, 61–73.

Dutta, G.C., Bordoloi, G.C., Pathak, M.& Choudhury, A. (1990) Incidence of intestinal helminthic infection in *Rhinoceros unicornis* in captivity. Zoos' Print 5, 14.

Fitzsimmons, W.M. (1962) *Parabronema roundi* n. sp. (Spiruridae: Nematoda) from *Rhinoceros bicornis* in Kenya. Journal of Helminthology XXXVI, 39–44.

Huelsenbeck, J.P. & Ronquist, F. (2001) MrBayes: Bayesian inference of phylogenetic tress. Bioinformatics 17, 754–755.

Karki, K.&Manandhar, P. (2008) Incidence of gastrointestinal helminthes in captive elephants in wildlife reserves of Nepal. Articlesbase, Free Online Articles Directory. Available at website http://www.articlesbase.com/health-articles/incidence-of-gastrointestinal-helminthesin-captive-elephants-in-wildlife-reserves-of-nepal-335689.html (accessed 4 February 2012).

Kinsella, J.M., Deem, S.L., Blake, S. & Freeman, A.S. (2004) Endoparasites of African forest elephants (*Loxodonta africana cyclotis*) from the Republic of Congo and Central African Republic. Comparative Parasitology 71, 104–110.

Laurie, A.L. (1978) The ecology and behavior of the great one horned rhinoceros. A dissertation submitted to the University of Cambridge for the degree of doctor of philosophy, Selwyn College, Cambridge.

Meredith, R.W., Janecˇka, J.E., Gatesy, J., Ryder, O.A., Fisher, C.A., Teeling, E.C., Goodbla, A., Eizirik, E., Simaˆo, T.L.L., Stadler, T., Rabosky, D.L., Honeycutt, R.L., Flynn, J.J., Ingram, C.M., Steiner, C., Williams, T.L., Robinson, T.J., Burk-Herrick, A., Westerman, M., Ayoub, N.A., Springer, M.S. & Murphy, W.J. (2011) Impacts of the cretaceous terrestrial revolution and KPg extinction on mammal diversification. Science 334, 521–524.

Mudaliar, S.V. & Ramanujachary, G. (1945) *Schistosoma nairi* n. sp. from an elephant. Indian Veterinary Journal 12, 1–4.

Muryani, A., Tiuria, R., Andriansyah & Agil, M. (2008) Helminthes parasite at feces of Sumatran Rhinoceros (*Dicerorhinus sumtrensis*) and Sumatran Elephant (*Elephas maximus surnatranus*) inWay Kambas National park Lampung (semi in situ). Proceedings of Asian Zoo/Wildlife Medicine and Conservation, 19–22 August, Bogor, Indonesia.

Palmieri, J.R., Purmono & Ammann, H. (1980) Parasites of the lesser one-horned rhinoceros (*Rhinoceros sondaicus* Desmarest). Journal of Parasitology 66, 1031.

Penzhorn, B.L., Krecek, R.C., Horak, I.G.,Verster, A.J.M., Walker, J.B., Boomker, J.D.F., Knapp, S.E. & Quandt, S.K.F. (1994) Parasites of African rhinos: a documentation. Proceedings of a Symposium on 'Rhinos as Game Ranch Animals', 9–10 September, Onderstepoort, Pretoria, South Africa.

Posada, D. (2008) jModeltest: Phylogenetic model averaging. Molecular Biology and Evolution 25, 1253–1256.

Rao, R. & Hiregauder, L.S. (1953) Schistosomiasis in elephants in Bombay State. Indian Veterinary Journal 30, 241–242.

Ronquist, F. & Huelsenbeck, J.P. (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.

Silberman, M.S. & Fulton, R.B. (1979) Medical problems of captive and wild rhinoceros: a review of the literature and personal experiences. The Journal of Zoo Animal Medicine 10, 6–16.

Southwell, T. (1921) A new species of cestode (*Anoplocephala vulgaris*) from an African Rhinoceros. Annals of Tropical Medicine and Parasitology 14, 355–364.

Stothard, J.R., Webster, B.L., Weber, T., Nyakaana, S., Webster, J.P., Kazibwe, F., Kabatereine, N.B. & Rollinson, D. (2009) Molecular epidemiology of *Schistosoma mansoni* in Uganda:DNAbarcoding reveals substantial genetic diversity within Lake Albert and Lake Victoria populations. Parasitology 136, 1813–1824.

Sundaram, R.K., Padmanabha Iyer, R., Peter, C.T. & Alwar, V.S. (1972) On *Bivitellobilharzia nairi* (Mudaliar and Ramanujachari, 1945) Dutt and Srivastava, 1955 (Trematoda: Schistosomatidae) parasitic in Indian elephants (*Elaphas maximus*) with a redescription of the species. Indian Veterinary Journal 49, 1–10.

Swofford, D.L. (2002) PAUP*: Ver 4.0. Phylogenetic Analysis Using Parsimony (*and other methods). Sunderland, Sinauer Associates.

Thapar, G.S. (1925) On some new members of the Genus Kiluluma from the African Rhinoceros. Journal of Helminthology III, 63–80.

Tiuria, R., Primawidyawan, A., Pangihutan, J., Warsito, J., Hariyadi, A.R.S., Handayani, S.U. & Priosoeryarito, B.P. (2006) Identification of endoparasites from faeces of Javan rhino (*Rhinoceros sondaicus*) in Ujung Klon National Park, Indonesia. The

Second Symposium on the Asian Zoo and Wildlife Medicine and the First Workshop on Zoo and Wildlife Pathology (AZWMP). Bangkok, Thailand, Faculty of Veterinary Science, Chulalongkorn University.

Vogel, H. & Minning,W. (1940) Bilharziose bei Elefanten. Archiv fu¨ r Schiffs und Tropenhygiene 44, 562–574.

Zhang, J.,Daszak,P.,Huang,H.,Yang,G., Kilpatrick,A.M. &Zhang,S. (2008) Parasite threat to panda conservation. EcoHealth 5, 6–9.

Zumpt, F. (1964) Parasites of the white and black rhinoceroses. The Lammergeyer 3, 59–70.

CHAPTER THREE

The material in this chapter has been published and has the following citation:

Devkota, R., Brant, S.V., Thapa, S., Loker, E.S. 2014. Two avian schistosomes cercariae from Nepal, including a *Macrobilharzia*-like species from *Indoplanorbis exustus*. Parasitology International 63: 374–380.

Two avian schistosome cercariae from Nepal, including a *Macrobilharzia*-like species from *Indoplanorbis exustus*

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Abstract

As part of a global survey of schistosomes, a total of 16,109 freshwater snails representing 14 species were collected from lakes, ponds, rivers, rice fields and swamps mostly in the Terai region of southern Nepal. Only two snails were found to harbor avian schistosome cercariae even though Nepal is well known for its rich avian diversity. One schistosome infection was from an individual of *Radix luteola* and on the basis of phylogenetic analyses using 28S rDNA and cox1 sequences, grouped as a distinctive and previously unknown lineage within *Trichobilharzia*. This genus is the most speciose within the family Schistosomatidae. It includes 40 described species worldwide, and its members mostly infect anseriform birds (ducks) and two families of freshwater snails (Lymnaeidae and Physidae). The second schistosome cercaria was recovered from an individual of *Indoplanorbis exustus* thatwas also actively emerging a *Petasiger*-like echinostome cercaria. Although *I. exustus* is commonly infected with mammalian schistosomes of the *Schistosoma indicum* species group on the Indian subcontinent, this is the first specifically documented avian schistosome reported in this snail. Both cercariae reported here are among the largest of all schistosome cercariae recovered to date. The *I. exustus*-derived schistosome clustered most closely with *Macrobilharzia macrobilharzia*, although it seems to represent a distinct lineage. Specimens of *Macrobilharzia* have thus far not been recovered from snails, being known only as adult worms from anhingas and cormorants. This study is the first to characterize by sequence data avian schistosomes recovered from Asian freshwater habitats. This approach can help unravel the complex of cryptic species causing cercarial dermatitis here and elsewhere in the world.

Keywords: Schistosomiasis, Avian schistosomes, Host–parasite relationships, Cercarial dermatitis, Nepal

1. Introduction

The cercariae of most and possibly all schistosome species are capable of causing cercarial dermatitis or ‘swimmer's itch’ [1]. Most of the species involved in causing cercarial dermatitis are probably avian schistosomes, which as a whole are poorly-known as compared to schistosomes developing in mammals. Furthermore, the species involved as potential causes of dermatitis in many parts of the world have not been investigated or adequately characterized. The advent of molecular tools to aid identification and discrimination among previously cryptic species of schistosomes and other trematodes [2] offers the prospect of substantially improving our global understanding of the epidemiology of cercarial dermatitis.

With this long-term goal in mind, we have undertaken a concerted search for avian schistosomes in Nepal. Although there are a few published studies of mammalian schistosomes from Nepal [3], there are no previous studies of Nepalese avian schistosomes or cercarial dermatitis. Extensive works on trematode cercariae in freshwater gastropods and schistosomes have been reported from surrounding countries like India [4], but in Nepal, studies on larval trematode infections in freshwater gastropods are very few [5]. As part of an ongoing effort to characterize the schistosome fauna of Nepal, and to relate it to the general diversity of schistosomes and trematodes throughout the world, we here report the results of a survey of Nepalese freshwater snails for trematode cercariae, and report on two species of avian schistosomes recovered, both of which appear based on genetic data to represent distinctive, unreported lineages.

2. Materials and methods

2.1. Collection and identification of freshwater snails

Freshwater snails were collected in 39 freshwater habitats in different areas of Nepal (Table 1) using kitchen sieves and triangular scoops mounted on long bamboo handles. The collected snails were kept moist and shaded prior to separation and cleaning. The identification of snails was done using conchological and morphological features [6].

Table 1. List of localities in Nepal sampled for freshwater snails.

	Locations	Number of snails examined	Co-ordinates
1.	Amreni, Tanahu	65	N 27˚59'15.9", E 84˚16'58.2"
2.	Baghmara Community forest, Chitwan	219	N 27˚35'22.0", E 84˚28'52.4"
3.	Baruwa, Tamasariya-9, Nawalparasi	93	N 27˚34'54.51", E 84˚01'17.41"
4.	Begnas Lake, Kaski	43	N 28˚09'58.00", E 84˚05'34.50"
5.	Budhi Rapti River near Elephant Breeding Center, Chitwan	844	N 27˚34'57.8", E 84˚27'56.6"
6.	Chisapani Village, Godar-2/3, Dhanusa	153	N 26˚55'51.5", E 86˚08'45.8"
7.	Chitwan National Park, Chitwan	95	N 27˚33'15.8", E 84˚21'19.7"
8.	Chitwan National Park, Chitwan	147	N 27˚33'22.6", E 84˚29'5.40"
9.	Chitwan National Park, Chitwan	51	N 27˚32'43.1", E 84˚30'08.1"
10.	Chitwan National Park, Chitwan	162	N 27˚33'59.3", E 84˚30'14.9"
11.	Chitwan National Park, Chitwan	422	N 27˚33'37.5", E 84˚29'24.5"
12.	Chitwan National Park, Chitwan	359	N 27˚33'37.0", E 84˚30'09.1"
13.	Dhad Khola, Tulsi Chauda-2/3, Dhanusa	390	N 27˚00'57.75", E 85˚55'27.00"
14.	Dhalkebar near Basai bridge, Dhanusa	75	N 26˚55'39.0", E 85˚57'58.2"
15.	Dhumre river, Kumrose, Chitwan	759	N 27˚34'34.11", E 84˚31'02.21"
16.	Fish Pond in Panchakanya Community Forest	339	N 27˚39'28.1", E 84˚29'18.7"
17.	Ghansikuwa, Tanahu	183	N 28˚00'33.4", E 84˚08'04.6"
18.	Jagdishpur reservoir, Niglihawa VDC, Kapilvastu	599	N 27 36'59.67" E 83 05'49.36"
19.	Jamunapur, jutpani-5, Chitwan	417	N 27˚39'42.48", E 84˚30'57.43"
20.	Jankauli, Bachhauli-7, Chitwan	419	N 27˚34'29.28", E 84˚30'48.64"
21.	Khageri river, Near Panchakanya Community Forest	837	N 27˚39'34.9", E 84˚29'00.2"
22.	Kuchkuche Community forest, Kathar, Chitwan	183	N 27˚34'26.85", E 84˚36'28.88"
23.	Kuchkuche Community forest, near Rapti dam Kathar, Chitwan	340	N 27˚34'02.28", E 84˚37'16.55"
24.	Kudauli, Pithauli-7, Nawalparasi	155	N 27˚39'12.21", E 84˚10'24.70"
25.	Kumaraura, Dhanusa	135	N 26˚46'36.76", E 85˚56'05.50"
26.	Kumrose, Chitwan	337	N 27˚34'03.42", E 84˚32'22.87"
27.	Phewa Lake, Pokhara	59	N 28˚12'31.27, E 83˚57'19.42"
28.	Pragatinagar-2, Nawalparasi	63	N 27˚40'04.72", E 84˚11'03.59"
29.	Ramaidaiya Bhawadi village, Dhanusa	26	N 26˚49'26.31", E 85˚57'05.80"
30.	Rapti river, Ghailari, Jagatpur-1	27	N 27˚33'25.03", E 84˚20'02.48"
31.	Rapti river, Sauraha, Chitwan	296	N 27˚34'52.24", E 84˚28'56.12"
32.	Rato river, Gauribash, west of Tulsi Chauda village, Dhanusa	181	N 27˚01'43.56", E 85˚55'21.30"
33.	Rice fields in Chisapani Village, Godar-2/3, Dhanusa	257	N 26˚56'26.09", E 86˚08'51.19"
34.	Shishuwar bagar, Bachhauli-3, Chitwan	2,585	N 27˚35'31.76", E 84˚30'00.31''
35.	Small canal in Sauraha, Chitwan	373	N 27˚34'59.55", E 84˚29'39.44''
36.	Tikauli marshy land, Ratnanagar- 7, Chitwan	95	N 27˚37'12.66", E 84˚28'13.87"
37.	Tikauli, Ratnanagar-7, Chitwan	3, 383	N 27˚37'45.52", E 84˚29'21.57"
38.	Tulsi Chauda village, Dhanusa	357	N 27˚00'50.37", E 85˚55'31.80"
39.	Twenty thousand lake, Chitwan	586	N 27˚36'54.0", E 84˚26'19.9"

2.2. Screening of infected snails and morphological identification of cercariae

Collected snails were cleaned, and each snail was isolated in a well of a 24-well tissue culture plate or in a Petri dish containing clean water. The isolated snails were exposed to window light or artificial illumination to stimulate cercarial shedding [7]. About an hour later, snails were individually screened using a stereomicroscope for shedding cercariae. If cercariae were observed, a few were transferred to a microscope slide and observed with the aid of a compound microscope. The cercariae were identified morphologically by means of cercarial keys [7]. Cercariae were ethanol fixed, measured and photographed using a digital camera fitted to the compound microscope. Snails that did not shed cercariae in the first hour were re-examined for shedding trematode cercariae at least twice within the following 24 h.

Cercariae were preserved in RNAlater (Ambion The RNA Company, Life Technologies, Grand Island, New York, USA) and 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 analysis and further morphological analysis.

2.3. Molecular and phylogenetic analysis

DNA was extracted from alcohol- or RNAlater©-preserved cercarial samples by using the Qiagen DNeasy Blood and Tissue Kit (Valencia, California, USA). Cercariae were digested for 2–3 h. The nuclear ribosomal 28S and mitochondrial cytochrome oxidase I (*cox*1) genes were amplified by polymerase chain reaction by 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 and 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) [8,9]. PCR products were purified with Omega E.Z.N.A Cycle-Pure Kit (Omega Bio-Tek, 400 Pinnacle Way Ste 450 Norcross, GA 30071) 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).

The 28S and *cox*1 gene fragments were used in phylogenetic analyses using maximum likelihood (ML) and maximum parsimony (MP) which were carried out in PAUP* ver. 4.0b10 [10]. Bayesian inferences (BI) were made with the use of MrBayes [11]. jModel Test was used to determine the best fit nucleotide substitution model (GTR + I + G) for ML analysis [12]. Optimal MP and ML trees were reconstructed using heuristic searches. For the 28S dataset we ran 20 replicates for MP and 5 replicates for ML. For the *cox*1 dataset we ran 50 replicates for MP and 10 replicates for ML, and for both data sets random taxon- input order and tree bisection and reconnection (TBR) branch swapping were used. For the BI analysis of the 28S dataset, the parameters were unlinked: Nst = 6 rates = invgamma ngammacat = 4. For the BI analysis of the *cox*1 dataset, the parameters were unlinked; for codons one and two Nst = 2 and for codon three Nst = 6 rates = gamma, ngammacat = 4. For both 28S and cox1 datasets, four chains were run simultaneously for 5 × 105 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 28S

dataset included members of the related blood fluke family Spirorchiidae. Outgroups for the *cox*1 analyses used sister taxa to *Trichobilharzia*, namely *Allobilharzia visceralis* and *Anserobilharzia brantae* [1,13]. Optimal ML trees were determined from heuristic searches (10 replicates), random taxon input order and TBR.

Nodal support was estimated by bootstrap: for the 28S dataset we ran 200 replicates with 5 addition sequence replicates and for MP and ML, we ran 100 replicates with 5 addition sequence replicates. For the *cox*1 dataset, for MP we ran 500 replicates with 10 additional sequence replicates, and for ML, 200 replicates with 5 addition sequence replicates.

DNA sequence data were deposited in GenBank, under accession numbers KF672860 and KF672861 for the 28S (1528 and 1496 bp) data set and KF672862 and KF672863 for the *cox*1 (605 and 1020 bp) data set. The remaining taxa used in the tree were obtained from literature already published [8,13].

3. Results

From 2007 to 2012 we collected and screened 16,109 freshwater snails of 14 different species for trematodes. The species collected and their numbers were *Bellamya bengalensis* (387), *Brotia costula* (13), *Gabbia orcula* (1160), *Gyraulus* spp. (3849), *Indoplanorbis exustus* (5471), *Lymnaea acuminata* (1245), *Melanoides pyramis* (153), *Melanoides tuberculata* (241), *Physa* sp. (16), *Pila globosa* (72), *Radix luteola* (2220), *Segmentina* spp. (528), *Succinea* sp. (11), and *Thiara* spp. (743) from 39 different freshwater habitats within Nepal (Fig. 1; Table 1).

During this survey, we found that only two snails, one *R. luteola* from habitat 31, and one *I. exustus* from habitat 18 were infected with avian schistosomes. The dimensions of these cercariae relative to the largest and smallest reported schistosome cercariae are compared in Table 2. The cercaria from *R. luteola* (Fig. 2A) was noteworthy for its large size, had prominent eyespots but lacked fin-folds on the furcae of the tail. They were released in large numbers in the morning light. The avian schistosome cercaria from *I. exustus* (Fig. 2B–C) was even larger, and is in fact the largest schistosome cercaria we know of. It was shed simultaneously along with a *Petasiger*-like echinostome cercaria (Fig. 2D). The *I. exustus* schistosome had prominent eyespots, furcal fin-folds and a membranous, pointed tip extended each furca. They were released in large numbers in the morning. They did not stick to the surface or side of the well but actively swam to the surface of the water, then sank down with the body held downward. The samples were vouchered in the Museum of Southwestern Biology Division of Parasitology (MSB Para 18709 for *Trichobilharzia*, MSB Para 18710 for the schistosome from *Indoplanorbis*).

The results of the reconstruction of the phylogenetic relationships of our samples with known schistosomes are shown in Figs. 3 and 4. The avian schistosome collected from *I. exustus* (W688) clustered with *Macrobilharzia macrobilharzia*. The genus *Macrobilharzia* is thus far known only as adult worms from anhingas and cormorants. Although W688 groups with *M. macrobilharzia*, it does so without significant bootstrap support, and is substantially different as indicated by the genetic distance data (see Table 3). The schistosome from *R. luteola*, on the basis of our phylogenetic reconstruction using 28S and cox1 sequences, nested within the genus *Trichobilharzia*. The genetic

distance data for this species support it being distinct from other previously collected and genetically characterized species of *Trichobilharzia* (Table 3).

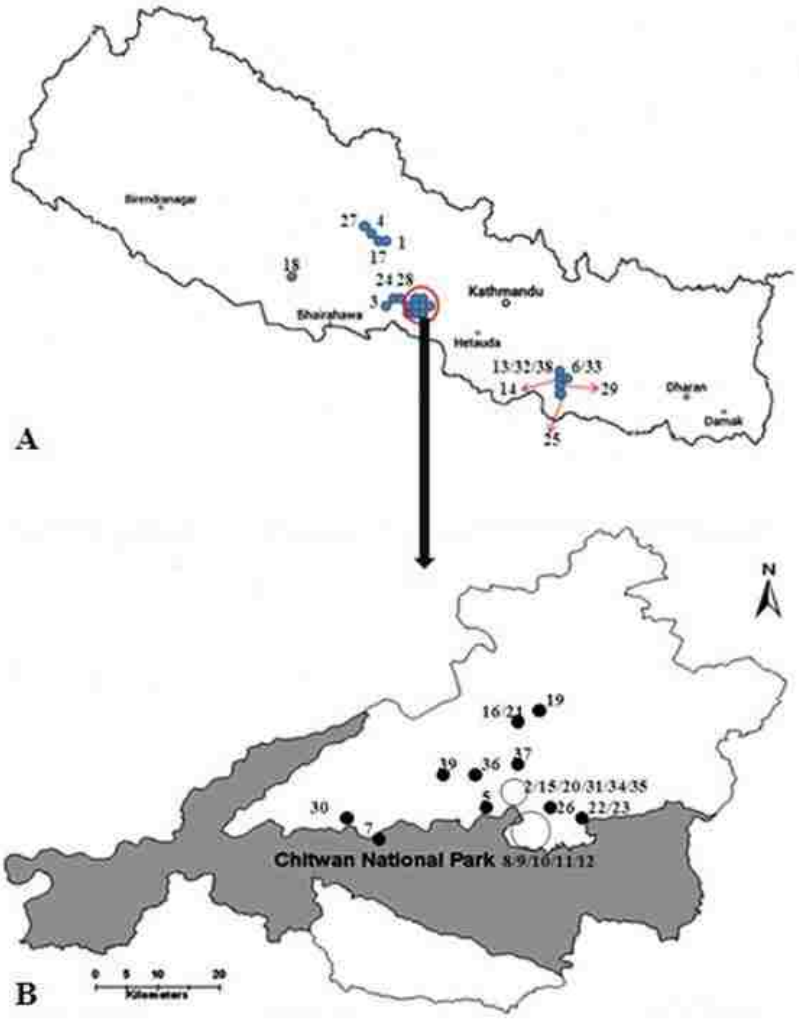

Figure 1. Map of the study area. A) Map of Nepal, with numbers indicating the different sampling sites corresponding to locality data in Table 1. B) Expanded map of Chitwan district within Nepal with the location of our major sampling sites in this area

Table 2. Comparison of cercarial dimensions of some schistosomes with our recent isolates obtained from *Radix* (W515) and *Indoplanorbis* (W688) (all measurements are in μm).

	Body		Tail stem		Tail furca		Snail host	Reference
	Length	Width	Length	Width	Length	Width		
Radix NP (W515) (average of 10)	267.8±12.09	83.5±5.3	566.5±20.7	51±7.8	233.5±9.7	41±7	*Radix luteola*	This study
Indoplanorbis NP (W688) (average of 10)	262.9±17.5	93±3.7	661.2±8.5	78.4±4.06	251.9±9.5	42.2±2.5	*Indoplanorbis exustus*	This study
Anserobilharzia brantae	290-350	75-120	550-570	40-60	220-250	-	*Gyraulus parvus*	[1]
Trichobilharzia australis	262	64	267	39.6	-	-	Lymnaeid snail	[14]
T. brevis	237	80	304	41	218	-	Lymnaeid snail (*L. rubiginosa*)	[15]
T. franki	307	-	419	-	234	-	*Radix auricularia*	[16]
T. physellae	270	-	352	-	188	-	*Physa gyrina*	[1]
T. szidati	305.7	72.3	431	44	247	24.3	Lymnaeid snail	[17]
Schistosoma hippopotami	213.1±20.1	76.0±17.5	411.6 ±18.3	46.6 ±4.2	151.9± 4.9	-	*Bulinus truncatus*	[9]
S. indicum	145-171	43-55	177-239	23-32	68-103	-	*Indoplanorbis exustus*	[18]
S. mansoni	168.6±4.4	63.7±4.4	248.0±18.8	39.2 ±5.4	87.5 ±6.7	-	*Biomphalaria sudanica*	[9]
S. nasale	151-224	42-71	204-284	25-32	75-117	48-66	*Indoplanorbis exustus*	[19]
S. turkestanicum	178	62	187	22.6	45	13	*Lymnaea* spp.	[20]
Heterobilharzia americana	147-182	51-67	101-138	23-32	37-53	-	*Lymnaea cubenisis*	[21]

4. Discussion

Based on the numbers of snails surveyed, our study showed that infections with larval avian schistosomes are not very common in Nepal, even though the country is renowned internationally for its rich avian diversity. A total of 867 bird species have been recorded in Nepal, accounting for over 8% of the world's known bird diversity. Of these, nearly 200 species are considered dependent on wetland habitats and many of these wetland birds are migratory [22]. This information is important as migratory wetland birds play an important role in the wider distribution of avian schistosomes.

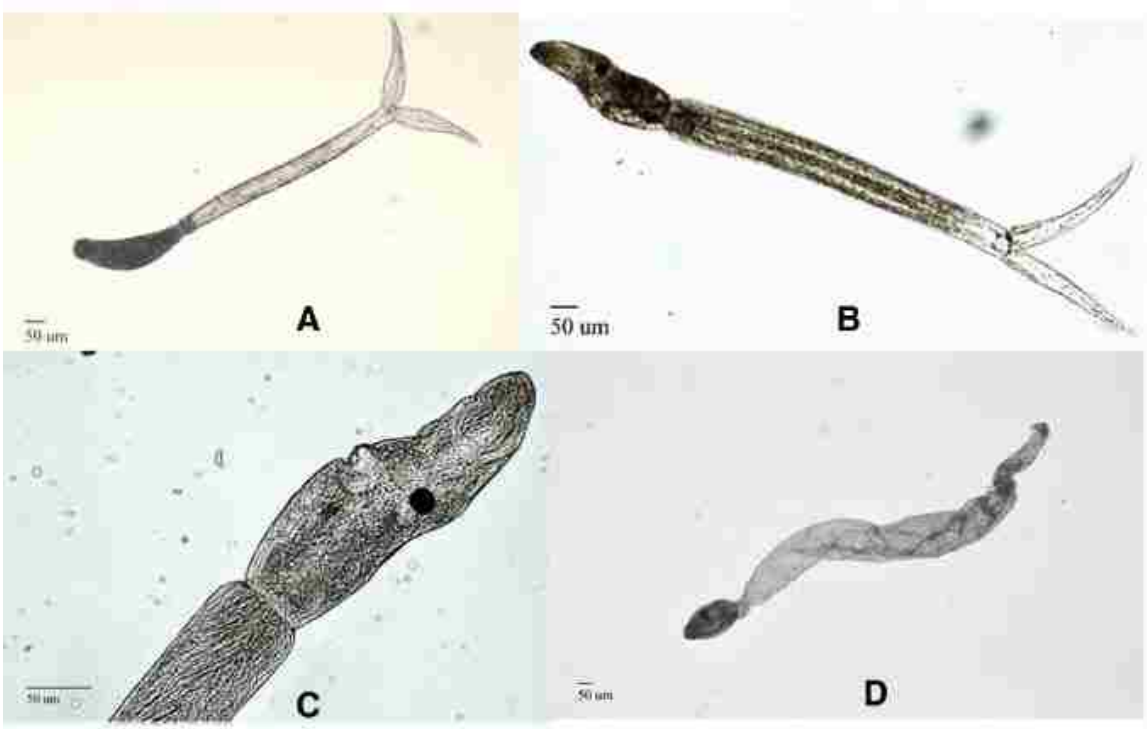

Figure 2. Two avian schistosome cercaria and a *Petasiger*-like echinostome cercariae recovered from Nepalese freshwater snails. A) The avian schistosome cercaria from *Radix luteola*; B) and C) two different views of the avian schistosome cercaria from *Indoplanorbis exustus*; and D) a *Petasiger*-like echinostome cercaria from the same *I. exustus* snail.

The low prevalence of avian schistosomes in our study sites may be because we missed major transmission sites, or because we sampled at the wrong time of year (in this study, mostly we collected our snail samples during the summer season). As large numbers of migratory birds reside in Nepal during the winter, perhaps spring or fall collections would yield more positive results. Here it is noteworthy that one of the positive specimens came from habitat 18, prominent for harboring migratory bird species. Further collections from this habitat are especially warranted. Although we will discuss mammalian schistosome cercariae recovered during this survey in a separate paper, we

note that many more snails positive for mammalian than avian schistosomes were found in our survey. Although none of the mammalian species recovered are known to develop to patency in people, all are known to cause dermatitis [23], so it seems very likely that mammalian schistosomes are more likely to cause dermatitis in Nepal than avian schistosomes.

Aquatic lymnaeid snails are very common in Nepal, and we examined 2220 specimens of *R. luteola* and 1245 specimens of *L. acuminata*, yet we were surprised that we found only one lymnaeid shedding avian schistosome cercariae. Our sequencing and associated genetic distance results suggest that the Nepalese avian schistosome from *R. luteola* is most similar to, but not identical with, *Trichobilharzia stagnicolae*. *Trichobilharzia* is a speciose genus, and from lymnaeid snails alone, 5 species have been reported from North America and 10 from Eurasia [24]. Until adult worms can be found that correspond genetically with the cercariae we have found and those worms can be compared to the formally described species, it will be difficult to assess whether the single lymnaeid-transmitted *Trichobilharzia* we have found in Nepal is a new species.

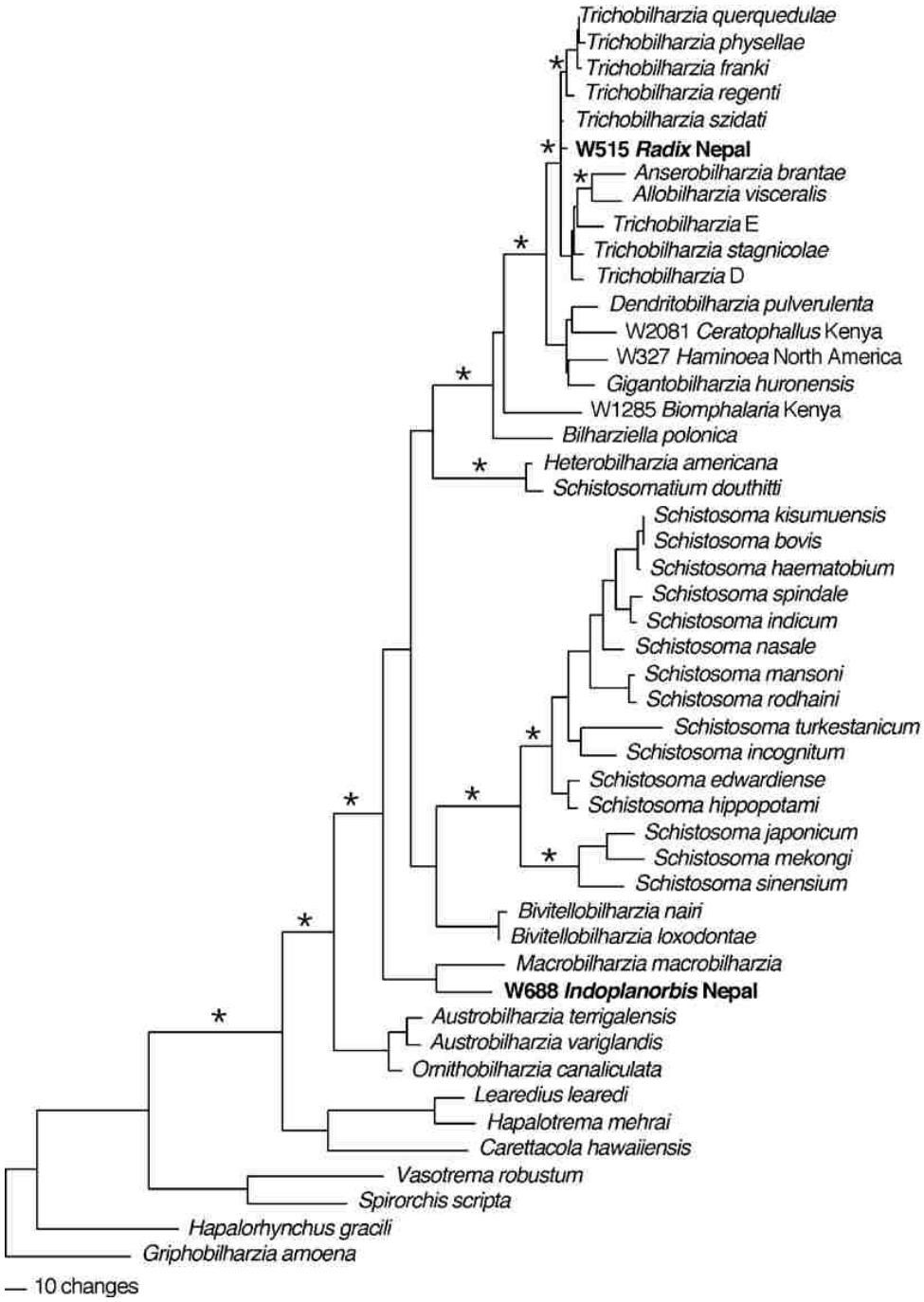

Figure 3. Bayesian phylogenetic tree based on 28S of our samples (in bold), incorporating known sequences from GenBank. '*' denotes significant nodal support for MP and ML (N90%) and Bayesian analysis (N0.95 posterior probability).

The avian schistosome cercaria recovered from *I. exustus* is unusual in several regards. First, although *I. exustus* is well-known for its role in hosting mammalian schistosomes of the *Schistosoma indicum* species group (*S. indicum, S. spindale* and *S. nasale*) in southern Asia, it is only rarely reported as an intermediate host for avian schistosomes [25] but nothing of a specific nature is known regarding the identity of the schistosome cercariae recovered in those studies. Our report shows unequivocally that this snail can host avian schistosomes. Secondly, the cercaria is noteworthy for its large size. It is one of the largest schistosome cercariae yet to be described (average 1.18 mm in length, including body, tail stem and furcal length). Three other similarly large schistosome cercariae recorded are those of *Anserobilharzia brantae* (average 1.115 mm in length, including body, tail stem and furcal length) [1], our sample W515 (average 1.066 mm in length, including body, tail stem and furcal length) and *Trichobilharzia australis* (average 0.992 mm in length, including body, tail stem and furcal length) [14]. Another report discusses a furcocercous cercaria from *I. exustus* that measures more than 1.5 mm in length [26] but this may actually be of a spirorchiid cercaria. The large size of the cercaria we report here is particularly intriguing given the results of the phylogenetic analysis that cluster it closely with sequences derived from adult worms of *M. macrobilharzia* derived from anhingas and cormorants in North America. Adults of this species are noteworthy for their large size.

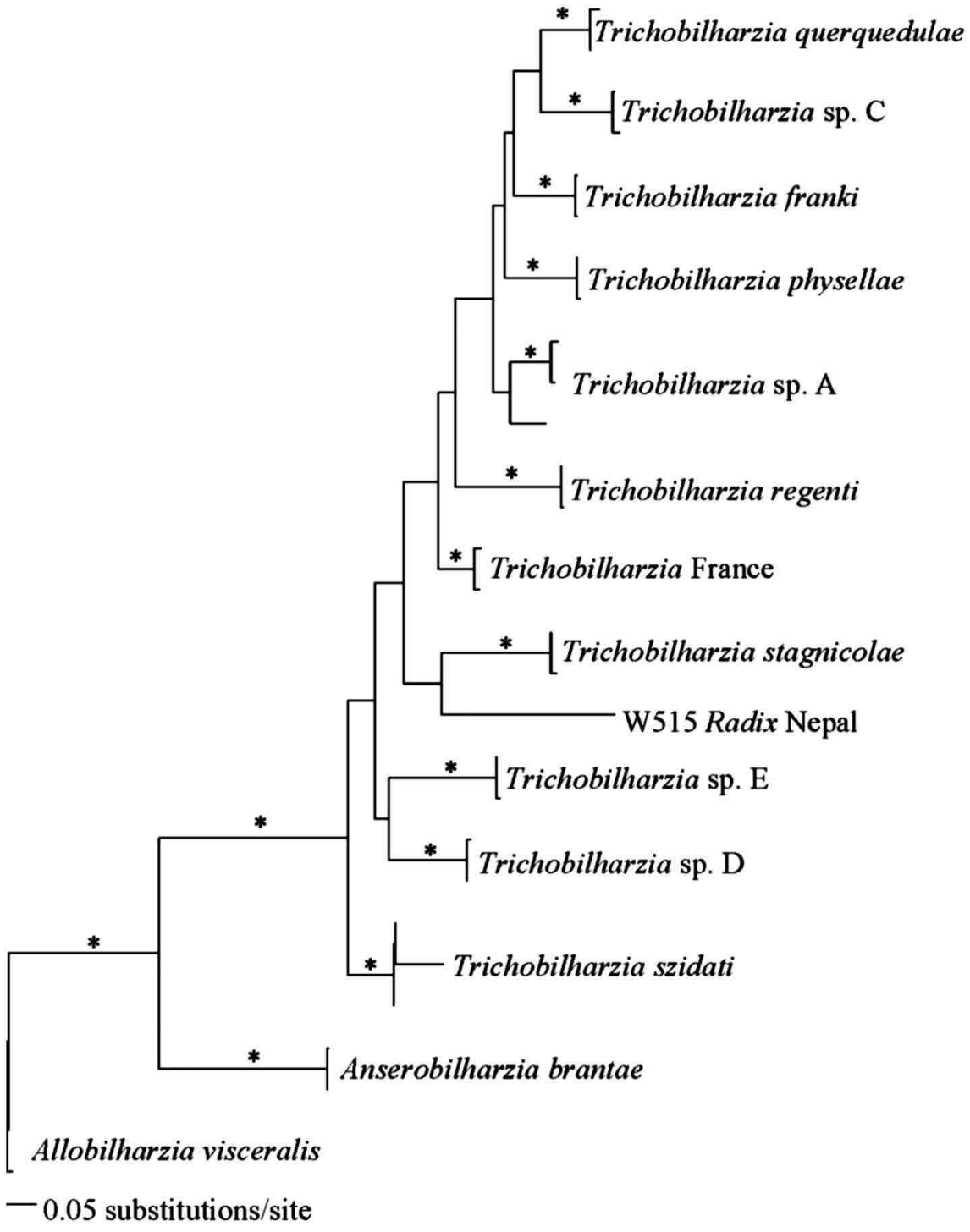

Figure 4. Maximum likelihood tree of *Trichobilharzia* spp. based on *cox*1. '*' denotes significant nodal support for MP and ML (N90%) and Bayesian analysis (N0.95 posterior probability).

Table 3. Genetic distances for *cox*1 and 28S among our samples and other schistosomes.

Taxa	*Cox*1	28S
Our sample W668 - *Macrobilharzia macrobilharzia*	20.5%	4.9%
Our sample W668 - *Gigantobilharzia huronensis*	22.3%	8.8%
Our sample W668 - *Austrobilharzia* sp. from Kuwait	53.4%	8.7%
Our sample W668 - *Dendritobilharzia pulverulenta*	23.3%	8.3%
Our sample W668 - *Ornithobilharzia canaliculata*	20.9%	8.1%
Our sample W668 - *Trichobilharzia stagnicolae*	22.9%	9.4%
Our sample W668 - *Bivitellobilharzia nairi*	21.4%	8.1%
Our sample W668 - *Schistosoma indicum*	22.6%	9.6%
Our sample W668 - *Schistosoma mansoni*	22.9%	10.0%
Our sample W515 - *Gigantobilharzia huronensis*	15.2%	3.3%
Our sample W515 - *Dendritobilharzia pulverulenta*	18.4%	3.4%
Our sample W515 - *Trichobilharzia stagnicolae*	9.0%	1.3%
Our sample W515 - *T. franki*	10.1%	1.2%
Our sample W515 - *T. physellae*	11.1%	1.2%
Our sample W515 - *T. regenti*	12.8%	1.7%

It is interesting that although *Macrobilharzia* adult worms have been known since 1922 [27] and are found in the Americas [28] and in the Old World [29], there are no known records of *Macrobilharzia* from snails. So, our report may prove of interest in helping to narrow the search for snail hosts for *Macrobilharzia* around the world. As one last comment about the *Macrobilharzia*-like cercaria, it is of interest that it came from a snail also actively shedding *Petasiger*-like cercariae that are produced in rediae. Although it is hard for us to know if one species was in the process of displacing another, or if the two species were stably coexisting in the snail, it is normally expected that a redia-producer would consume the sporocysts of another species if present together [30]. There is also a precedent for some schistosomes – such as representatives of the genus *Austrobilharzia* – being able to successfully colonize snails already infected with rediae of another trematode species [31]. Perhaps in the case of the schistosome we found in *I. exustus*, it too can thrive in the presence of rediae. This situation also raises the possibility that the evident rarity of snails infected with *Macrobilharzia* may be a consequence of the fact that the sporocysts of this species require the larvae of another

species (like *Petasiger*) to be present. These are interesting possibilities that can only be clarified by additional studies.

As a final point, we note that this is the first study to characterize by sequence data avian schistosomes recovered from Nepalese freshwater habitats. By providing reliable markers by which to identify avian schistosomes, this study can help unravel the complex of cryptic species causing cercarial dermatitis here and elsewhere in the world.

Acknowledgments

The University of New Mexico supported this study, through a National Science Foundation grant to SVB (DEB 1021427). Technical assistance at the UNM Molecular Biology Core Facility was supported by NIH grant 1P20RR18754 from the Institute Development Award program of the National Center for Research Resources.We are grateful to the officials of the Department of National Parks and Wildlife Conservation, Chitwan National Park and the Nepal Health Research Council (permit no. 44) for their cooperation to carry out this research.

References

[1] Brant SV, Loker ES. Schistosomes in the southwest United States and their potential for causing cercarial dermatitis or 'swimmer's itch'. J Helminthol 2009;83:191-8.

[2] Detwiler JT, Bos DH, Minchella DJ. Revealing the secret lives of cryptic species: Examining the phylogenetic relationships of echinostome parasites in North America. Mol Phylogenet Evol 2010;55:611–20.

[3] Devkota R, Brant SV, Thapa A, Loker ES. Sharing schistosomes: the elephant schistosome *Bivitellobilharzia nairi* also infects the greater one-horned rhinoceros (*Rhinoceros unicornis*) in Chitwan National Park, Nepal. J Helminthol 2012 Available on CJO2012. doi:10.1017/S0022149X12000697.

[4] Agrawal MC. Schistosomes and schistosomiasis in South Asia. India: Springer; 2012.

[5] Devkota R, Budha PB, Gupta R. Trematode cercariae infections in freshwater snails of Chitwan district, central Nepal. Him J Sci 2011;7(9):9-14.

[6] Subba Rao NV. Hand book of freshwater molluscs of India. Zoological Survey of India, Calcutta; 1989.

[7] Frandsen F, Christensen NO. An introductory guide to the identification of cercariae from African freshwater snails with special reference to cercariae of trematode species of medical and veterinary importance. Acta Trop 1984;41:181–202. [18] Srivastava HD, Dutta SC. Life history of *Schistosoma indicum* Montgomery, 1906 – a common blood-fluke of Indian ungulates. Curr Sci 1951;20:273-275.

[8] Lockyer AE, Olson PD, Østergaard P, Rollinson D, Johnston DA, Attwood SW et al.. The phylogeny of the Schistosomatidae based on three genes with emphasis on

the inter-relationships of Schistosoma Weinland, 1858. Parasitology 2003;126:203–24.

[9] Morgan JA, DeJong RJ, Kazibwe F, Mkoji GM, Loker ES. A newly-identified lineage of *Schistosoma*. Int J Parasitol 2003;33(9):977-85.

[10] Swofford DL. PAUP*: Ver 4.0. Phylogenetic Analysis Using Parsimony (*and other methods). Sunderland, Sinauer Associates; 2002.

[11] Huelsenbeck JP, Ronquist F. MrBayes: Bayesian inference of phylogenetic tress. Bioinformatics 2001;17:754–5.

[12] Posada D. jModeltest: Phylogenetic model averaging. Mol Biol Evol 2008;25:1253–6.

[13] Brant SV, Jouet D, Ferte H, Loker ES. *Anserobilharzia* gen. n. (Digenea, Schistosomatidae) and re-description of *A. brantae* (Farr and Blankemeyer, 1956) comb. N. (syn. *Trichobilharzia brantae*), a parasite of geese (Anseriformes). Zootaxa 2013;3670(2):193-206.

[14] Blair D, Islam KS. The life cycle and morphology of *Trichobilharzia australis* nsp from the nasal blood vessels of the black duck in Australia with a review of the genus *Trichobilharzia*. Syst Parasitol 1983;5:89-117.

[15] Basch PF. The life cycle of *Trichobilharzia brevis*, n. sp., an avian schistosome from Malaya. Z Parasitenkd 1966;27:242-51.

[16] Müller V, Kimmig P. *Trichobilharzia franki* n. sp. a causative agent of swimmer's itch in southwestern Germany. Appl Parasitol 1994;35:12-31.

[17] Neuhaus W. Biology and development of *Trichobilharzia szidati* n. sp. (Trematoda, Schistosomatidae), a parasite causing dermatitis in man.]. Z Parasitenkd 1952;15:203-66.

[18] Srivastava HD, Dutta SC. Life history of *Schistosoma indicum* Montgomery, 1906 – a common blood-fluke of Indian ungulates. Curr Sci 1951;20:273-5.

[19] Dutt SC. Studies on *Schistosoma nasale* Rao, 1933. I. Morphology of the adults, egg and larval stages. Indian J Vet Sci 1967;37:249-62.

[20] Machattie C. A preliminary note on the life history of *Schistosoma turkestanicum* Skrjabin, 1913. Trans R Soc Trop Med Hyg 1936;30(1):115-24.

[21] Lee H-F. Life history of *Heterobilharzia Americana* Price 1929, a schistosome of the raccoon and other mammals in Southeastern United States. J Parasitol 1962;48(5):728-39.

[22] Inskipp C, Inskipp T. A guide to the birds of Nepal. Second edition, Christopher Helm, London; 1991.

[23] Anantaraman M. On schistosome dermatitis: I. dermatitis in India caused by cercariae of *Schistosoma spindale* Montgomery, 1906. Indian J Helminthol 1958;X(I):46-52.

[24] Horák HP, Kolářová L, Adema CM. Biology of the schistosome genus *Trichobilharzia.* Adv Parasitol 2002;52:155-233.

[25] Agrawal MC, Gupta S, George J. Cercarial dermatitis in India. Bull World Health Organ 2000;78(2):278.

[26] Soparkar MB. Notes on some furcocercous cercariae from Bombay. Indian J Med Res 1921;9:23-32.

[27] Travassos L. Informacoes sobre a fauna helmintologica de Mato Grosso. Folha Medica 1922;3:187-90.

[28] Price EW. A synopsis of the trematode family Schistosomatidae with descriptions of new genera and species. Proc U.S. Nat Mus 1929;75:1-39**.**

[29] Fain A. Recherches sur les schistosomes d'oiseaux au Ruanda- Urundi (Congo Belge). Revue de Zool Bot Afri 1955b;51:373-87.

[30] Lie KJ, Basch PF, Umathevy T. Antagonism between two species of larval trematodes in the same snail. Nature 1965;206:422-3.

[31] Walker JC. *Austrobilharzia terrigalensis*: A schistosome dominant in interspecific interactions in the mollusc host. Int J Parsitol 1979;9:137-40.

CHAPTER FOUR

The material in this chapter has been accepted for publication in the International Journal for Parasitology.

The *Schistosoma indicum* species group in Nepal: presence of a new lineage of schistosome and use of the *Indoplanorbis exustus* species complex of snail hosts

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Abstract

From 2007-2014 we screened 19,360 freshwater snails from the Terai and hilly regions of Nepal for cercariae of mammalian schistosomes. Based on analysis of *cox*1, 12S, 16S and 28S sequences (3,675 bp) of the cercariae recovered, we provide the first report of the *Schistosoma indicum* species group in Nepal. Five specimens of *S. nasale*, 9 of *S. spindale*, and 17 of *Schistosoma* sp. were recovered, all from the snail *Indoplanorbis exustus*. The latter lineage failed to group in any of our analyses with *S. nasale*, *S. spindale* or *S. indicum*. It diverged in *cox*1 sequence from them by 16%, 13% and 13%, respectively, levels of difference comparable to well-studied species pairs of *Schistosoma*. Analysis of *cox*1, 16S and ITS1 sequences (1,874 bp) for Nepalese specimens of *I. exustus* was also surprising in revealing the presence of four genetically distinct clades. They diverged from one another at levels comparable to those noted for species pairs in the sister genus *Bulinus*. There was no obvious pattern of use by Nepalese *Schistosoma* of the *Indoplanorbis* clades. We found high support for a close relationship between *S. indicum* and *S. haematobium* groups, but failed to retrieve support for a clean separation of the two, with a tendency for *S. nasale* to fall as the most basal representative. If this pattern holds, hypotheses for the origin of the Asian *Indoplanorbis*-transmitted *S. indicum* group from the *Bulinus*-transmitted *S. haematobium* group may require modification, including to consider more contemporaneous origins of the two groups. The Indian subcontinent is understudied with respect to schistosome diversity and our current knowledge of the *S. indicum* and *I. exustus* species groups is inadequate. Further study is warranted given the ability of *indicum* group species to cause veterinary problems, and cercarial dermatitis, with a worrisome long-term potential to establish infections in humans.

Keywords:

Schistosomes, *Schistosoma indicum* group, *Indoplanorbis exustus*, host-parasite relationships, Nepal, host switch, schistosomiasis

1. Introduction

Within the medically important digenetic trematode genus *Schistosoma,* four prominent species groups are customarily recognized. These are the *japonicum* species group with 5 species, the *mansoni* group with 2 species, the *haematobium* species group with 9 species and the *indicum* species group with 3 species. In addition, other species groups can be considered including the schistosomes hosted by the hippopotamus (2 or 3 species), and what can be called the *turkestanicum* species group with 4 recognized species, although verification of their relatedness is required. The species groups are defined by their tendency to consistently separate as distinct molecularly-defined lineages, the identities and relatedness of their snail hosts, general geographic distributions and more loosely by the morphology of the eggs produced by the females (Rollinson and Southgate, 1987; Lockyer et al., 2003; Morgan et al., 2003; Attwood et al., 2007).

The *S. indicum* species group as currently recognized is comprised of three species: *Schistosoma indicum*, *S. nasale* and *S. spindale* (Attwood et al., 2007). Although *S. incognitum* was formerly included in this group, it has been shown that it is a more distant relative (Agatsuma et al., 2002; Attwood et al., 2002, Morgan et al., 2003; Webster et al., 2006: Lawton et al., 2011). Among the four customarily recognized and prominent species groups, our overall knowledge of the *S. indicum* group lags far behind relative to the other three. The primary reason for this is that the members of this group, although they can and do cause dermatitis in people (Anantaraman, 1958; Narain et al., 1998; Agrawal et al., 2000), they rarely if ever establish patent human infections (Agrawal et al., 2000; Agrawal and Rao, 2011), so consequently they have attracted less

attention. They are however of considerable veterinary significance, and they pose a number of interesting questions regarding the evolution and biogeography of schistosomes.

Overviews of the biology of the three *indicum* group species can be found in Kumar and deBurbure (1986), Rollinson and Southgate (1987) and Agrawal (2012). Briefly, all three species are hosted by the bulinine planorbid snail *Indoplanorbis exustus*. This snail is widely distributed across southern Asia (see map range from Budha et al., 2012), and has since been introduced into exotic locales like West Africa and the Caribbean islands by human activities. As noted by Liu et al. (2010), and as we discuss further below, *I. exustus* actually represents a complex of cryptic species. The extent of genetic diversification within what at least superficially looks like a fairly morphologically homogeneous gastropod species was surprising, and will surely improve our overall understanding of the evolution and diversification of the *S. indicum* group, and of other dependent trematodes. Some literature also implicates lymnaeid snails in transmission of members of the *indicum* group, but when careful experimental infections have been done, only *I. exustus* has been experimentally infected (Dutt and Srivastava, 1968; DeBont et al., 1991). It is likely that the Asian mammalian schistosome recovered from lymnaeids and attributed to the *S. indicum* group are really representatives of the lymnaeid-transmitted *S. turkestanicum* species group or of *S. incognitum*.

The most widely distributed member of the *S. indicum* group is *S. spindale* which causes intestinal schistosomiasis in a variety of mammals, with its most prominent hosts being water buffaloes, cattle, sheep, and goats. It can also infect equines and wild rodents. It is known from India, Bangladesh, Sri Lanka, Malaysia, Vietnam, Laos,

Indonesia, and Thailand. It produces characteristic elongated, spindle-shaped eggs with a terminal spine. The males of this species are atuberculate (Rollinson and Southgate, 1987; Gupta and Agrawal, 2002).

Schistosoma indicum also causes intestinal schistosomiasis in buffaloes, cattle, sheep, goat, camels and horses (Srivastava and Dutta, 1951). This species as best is known is more confined in its distribution, to the "Indian subcontinent," including India and Bangladesh. It is said to be present in every Indian state (Agrawal, 2012). The males are atuberculate and the eggs produced are oval and possess a terminal spine (Rollinson and Southgate, 1987).

Lastly, *Schistosoma nasale* is distinctive among mammalian schistosomes in occupying the veins of the nasal mucosa of ruminants, giving rise to nasal schistosomiasis, or "snoring disease" as affected animals, especially cattle, breathe with a distinctive snoring sound. The large, sinuous, terminally-spined eggs are passed from the body with nasal discharges. Buffaloes, apparently less prone to the large cauliflower-like granulomas that cause snoring in cattle, are also infected as are other ruminants like goats, which are also less prone to snore than cattle. Sheep do not reliably support patent infections of *S. nasale* (Agrawal and Rao, 2011). Adult males of this species have prominent tubercles, most of which lack spines (Southgate et al. 1990). This species is known from India, Bangledesh, Myanmar, and Sri Lanka (Dutt, 1967; Rollinson and Southgate, 1987; Agatsuma et al., 2002; Lockyer et al., 2003; Attwood et al., 2007).

A number of molecular phylogenetic studies have been undertaken to assess the position of the *S. indicum* group within *Schistosoma*. These studies have consistently shown that the lymnaeid-transmitted *S. incognitum* is not part of the *indicum* group even

though its distribution (India, Thailand, Indonesia) overlaps that of members of the *indicum* group (Agatsuma et al., 2002; Lockyer et al., 2003; Attwood et al., 2007). Furthermore, *S. indicum* and *S. spindale* are consistently retrieved as sister species with a high degree of support. This is interesting because the egg morphology of the two species is so divergent. The position of *S. nasale* relative to the other two species has been more problematic, and has been presented as either grouping with weak or equivocal support as basal to the other two species (Lockyer et al., 2003; Webster and Littlewood, 2012), or as being in a more distant position, with African species of the *Bulinus*-transmitted *S. haematobium* group intercalated, suggestive that the *S. indicum* group is paraphyletic (Attwood et al., 2007). Several lines of evidence, including mitochondrial gene order data (Agatsuma et al., 2002; Sato et al., 2008; Webster and Littlewood, 2012), consistently indicate that the *S. indicum* group is more closely related to the African schistosomes, particularly to the *S. haematobium* group, than to the Asian *S. japonicum* group.

The number of *indicum* group specimens available for molecular analysis has thus far been limited. They consist of *S. indicum* from Bangladesh, *S. spindale* from Bangladesh, Sri Lanka and Thailand, and *S. nasale* from Bangaladesh and Sri Lanka. Specimens of *S. incognitum* from Bangladesh, Indonesia and Thailand have thus far been examined. Mitochondrial gene order has been determined for all four of the above species and found to be of the "derived" type (Lockyer et al., 2003; Littlewood et al., 2006; Sato et al., 2008; Webster and Littlewood, 2012). Remarkably, as of this writing, no specimens of the *S. indicum* group from India have been subjected to molecular phylogenetic analysis in a published study, though 16S, 18S and 28S sequences for

Indian specimens of *S. indicum* and *S. spindale* from Kerala in southern India are now in GenBank.

No specimens from the *S. indicum* group have previously been reported from Nepal, though the presence of mammalian schistosome cercariae from *I. exustus* has been reported (Devkota, 2008; Devkota et al., 2011). The elephant schistosome *Bivitellobilharzia nairi* occurs in Nepal in both elephants and rhinos (Devkota, 2008; Devkota et al., 2014a), and at least two species of avian schistosomes also occur there (Devkota et al., 2014b), but otherwise our knowledge of schistosomes present in Nepal is rudimentary. Laterally-spined schistosome eggs closely resembling the eggs of *S. mansoni* have been reported from human fecal samples from the Terai region of southern Nepal (Sherchand et al., 1999), but no additional reports of a schistosome with laterally-spined eggs from Nepal have since come to light.

As part of the collecting effort that revealed the presence of two kinds of avian schistosome cercariae in Nepal (Devkota et al., 2014b), the same freshwater snails were also screened for cercariae of mammalian schistosomes. These collections took place, as circumstances allowed, over a seven-year time interval, mostly in the Terai region of southern Nepal, in and around Chitwan National Park. Here we present the first report from Nepal of the presence of cercariae representing the *S. indicum* group, all of which were recovered from *Indoplanorbis exustus*. Sequence data for the mitochondrial cytochrome oxidase I (*cox*1), 12S, 16S and nuclear 28S gene regions were acquired from most of the specimens and were subjected to molecular phylogenetic analysis. Additionally, for both uninfected *I. exustus*, and for some of the schistosome-positive *I. exustus* specimens, we acquired *cox*1, 16S and ITS1 (includes parts of adjacent 18S and

5.8S genes) sequence data as well. This information has been used to examine patterns of diversification within *I. exustus*, and to relate them both to other studies of this snail, and to phylogenetic results obtained for the *S. indicum* group. We also obtained genetically distinct mammalian schistosome cercariae from lymnaeid snails from the Terai region and will report on the results concerning these in a separate paper.

2. Materials and methods

2.1.Collection and morphological identification of freshwater snails

Snails were collected from 40 freshwater habitats in different areas of Nepal (Table 1 and supplementary Table 1) using kitchen sieves and triangular scoops mounted on long bamboo handles. The collected snails were kept moist and shaded prior to separation and cleaning. The identification of snails was done using conchological and morphological features (Subba Rao, 1989) and some of the snails were preserved in 96% ethanol for molecular identification.

2.2. Screening of infected snails and morphological identification of cercariae

Collected snails were isolated in individual wells of a 24-well tissue culture plate or in Petri dishes containing clean water. The isolated snails were exposed to window light or artificial illumination to stimulate cercarial shedding. About an hour later, snails were individually screened for shedding cercariae using a dissecting microscope. If cercariae were observed, a few were transferred to a microscope slide and observed with the aid of a compound microscope. The cercariae were identified morphologically by means of cercarial keys (Frandsen and Christensen, 1984). Cercariae were ethanol-preserved, measured and photographed using a digital camera fitted to the compound microscope.

Snails that did not shed cercariae in the first hour were re-examined for shedding cercariae at least twice within the following 24 hours.

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 of the Nepal Health Research Council (ref. number 44, 25 July 2011) to the Department of Biology at the University of New Mexico, Albuquerque, New Mexico, USA for molecular analysis and further morphological analysis.

Table 1. List of localities in Nepal sampled for freshwater snails from 2007 to 2014. The number of snails in parentheses indicates the number of *Indoplanorbis exustus* positive for *S. indicum* group schistosomes. The identifications for the schistosomes provided are derived from the phylogenetic analyses that follow, and that the designation "*Schistosoma* sp.*"* reflects the genetic distinctiveness of these worms from the few available specimens of *S. indicum* in GenBank. Also, we were unable to obtain sequence from three of the samples retrieved and these are referred to as "unidentified" in the table.

Locations	Number of snails examined	Co-ordinates	Number of *Indoplanorbis* collected *	Species identity and W number
1. Amreni, Tanahu	65	N 27˚59'15.9", E 84˚16'58.2"	65	-
2. Baghmara Community forest, Chitwan	219	N 27˚35'22.0", E 84˚28'52.4"	156	-
3. Baruwa, Tamasariya-9, Nawalparasi	93	N 27˚34'54.51", E 84˚01'17.41"	51 (1)	*Schistosoma* sp. (W379)
4. Begnas Lake, Kaski	43	N 28˚09'58.00", E 84˚05'34.50"	14	-
5. Budhi Rapti River near Elephant Breeding Center, Chitwan	844	N 27˚34'57.8", E 84˚27'56.6"	104 (2)	2 *S. spindale* (W525, W546)
6. Chisapani Village, Godar-2/3, Dhanusa	153	N 26˚55'51.5", E 86˚08'45.8"	1	-
7. Chitwan National Park, Chitwan	95	N 27˚33'15.8", E 84˚21'19.7"	59	-
8. Chitwan National Park, Chitwan	147	N 27˚33'22.6", E 84˚29'5.40"	22 (1)	*Schistosoma* sp. (W532)
9. Chitwan National Park, Chitwan	51	N 27˚32'43.1", E 84˚30'08.1"	29	-
10. Chitwan National Park, Chitwan	162	N 27˚33'59.3", E 84˚30'14.9"	49	-
11. Chitwan National Park, Chitwan	422	N 27˚33'37.5", E 84˚29'24.5"	231 (2)	2 *Schistosoma* sp. (W463, W528)
12. Chitwan National Park, Chitwan	359	N 27˚33'37.0", E 84˚30'09.1"	244	-
13. Dhad Khola, Tulsi Chauda-2/3, Dhanusa	390	N 27˚00'57.75", E 85˚55'27.00"	14	-
14. Dhalkebar near Basai bridge, Dhanusa	75	N 26˚55'39.0", E 85˚57'58.2"	-	-
15. Dhumre river, Kumrose, Chitwan	1,753	N 27˚34'34.11", E 84˚31'02.21"	658 (1)	*S. nasale* (W545)
16. Fish Pond in Panchakanya Community Forest	339	N 27˚39'28.1", E 84˚29'18.7"	27	-
17. Ghansikuwa, Tanahu	183	N 28˚00'33.4", E 84˚08'04.6"	15	-
18. Jagdishpur reservoir, Niglihawa VDC, Kapilvastu	802	N 27 36'59.67" E 83 05'49.36"	24	-
19. Jamunapur, jutpani-5, Chitwan	417	N 27˚39'42.48", E 84˚30'57.43"	218	-
20. Jankauli, Bachhauli-7, Chitwan	419	N 27˚34'29.28", E 84˚30'48.64"	248 (1)	*S. spindale* (W378)
21. Khageri river, Near Panchakanya Community Forest	837	N 27˚39'34.9", E 84˚29'00.2"	411	-
22. Kuchkuche Community forest, Kathar, Chitwan	183	N 27˚34'26.85", E 84˚36'28.88"	48 (1)	*Schistosoma* sp. (W557)
23. Kuchkuche Community forest,	340	N 27˚34'02.28", E	154 (1)	S. *spindale*

near Rapti dam Kathar, Chitwan		84˚37'16.55"		(W558)
24. Kudauli, Pithauli-7, Nawalparasi	155	N 27˚39'12.21", E 84˚10'24.70"	101	-
25. Kumaraura, Dhanusa	135	N 26˚46'36.76", E 85˚56'05.50"	49 (1)	*S. nasale* (W439)
26. Kumrose, Chitwan	337	N 27˚34'03.42", E 84˚32'22.87"	28	-
27. Nawalpur, Hetauda, Makwanpur	325	N 27˚25'55.06", E 89˚58'57.02"	66	-
28. Phewa Lake, Pokhara	59	N 28˚12'31.27, E 83˚57'19.42"	5	-
29. Pragatinagar-2, Nawalparasi	63	N 27˚40'04.72", E 84˚11'03.59"	63	-
30. Ramaidaiya Bhawadi village, Dhanusa	26	N 26˚49'26.31", E 85˚57'05.80"	-	-
31. Rapti river, Ghailari, Jagatpur-1	27	N 27˚33'25.03", E 84˚20'02.48"	21	-
32. Rapti river, Sauraha, Chitwan	296	N 27˚34'52.24", E 84˚28'56.12"	106	-
33. Rato river, Gauribash, west of Tulsi Chauda village, Dhanusa	181	N 27˚01'43.56", E 85˚55'21.30"	1	-
34. Rice fields in Chisapani Village, Godar-2/3, Dhanusa	257	N 26˚56'26.09, E 86˚08'51.19"	21	-
35. Shishuwar bagar, Bachhauli-3, Chitwan	3,023	N 27˚35'31.76", E 84˚30'00.31''	1321 (11)	6 *Schistosoma* sp. (W380, W381, W383, W531, W534, W541), 2 *S. spindale* (W535, W556), 3 unidentified (W384, W385, W533)
36. Small canal in Sauraha, Chitwan	373	N 27˚34'59.55", E 84˚29'39.44''	170	-
37. Tikauli marshy land, Ratnanagar- 7, Chitwan	95	N 27˚37'12.66", E 84˚28'13.87"	5	-
38. Tikauli, Ratnanagar-7, Chitwan	4, 674	N 27˚37'45.52", E 84˚29'21.57"	1890 (12)	6 *Schistosoma* sp. (W517, W518, W519, W550, W798, W799); 3 *S. spindale* (W464, W538, W804); 3 *S. nasale* (W542, W549, W551)
39. Tulsi Chauda village, Dhanusa	357	N 27˚00'50.37", E 85˚55'31.80"	6	-
40. Twenty thousand lake, Chitwan	586	N 27˚36'54.0", E 84˚26'19.9"	564	-

* The numbers in parentheses indicate the number of *Indoplanorbis* individuals positive for schistosomes at that particular location.

2.3. Molecular and phylogenetic analysis

DNA from schistosome cercariae was extracted from alcohol- or RNAlater©-preserved cercarial samples either by using the Qiagen DNeasy Blood and Tissue Kit or the Qiagen QIAamp DNA Micro Kit (Valencia, California, USA). Cercariae were

digested for 2–3 hours or overnight. The nuclear ribosomal 28S and mitochondrial *cox*1, 16S and 12S genes were amplified by polymerase chain reaction by 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, *cox*1F6; 5’-TTT GTY TCT TTR GAT CAT AAG CG-3′ and *cox*1 3; 5′ - TAA TGC ATM GGA AAA AAA CA- 3′ for *cox*1 sequences and 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’) (Attwood et al., 2002; Lockyer et al., 2003; Morgan et al., 2003). New primers were designed to amplify the 16S gene (R16SF 5' - TGT TTT TTT CCK ATG CAT TA - 3' and R16SR 5' - GGC TTA CAC CGG TCT TAA CT - 3'). PCR products were purified either with Omega E.Z.N.A Cycle-Pure Kit (Omega Bio-Tek, Norcross, GA 30071) or USB ExoSAP-IT PCR Product Cleanup (Affymetrix, Inc., 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).

DNA from snails was extracted from alcohol preserved samples either by using the Qiagen DNeasy Blood and Tissue Kit (Valencia, California, USA) or the Omega E.Z.N.A Mollusc DNA Kit (Omega Bio-Tek, Norcross, GA 30071) following the manufacturers’ guidelines. The partial sequences of a nuclear internal trascribed spacer region; ITS1 and two mitochondrial genes; *cox*1 and 16S genes were amplified by polymerase chain reaction by using Takara Ex Taq kit (Takara Biomedicals, Otsu, Japan) and previously published primers (ITS1-S; 5’ CCA TGA ACG AGG AAT TCC CAG 3’ and 5.8S-AS 5’ TTA GCA AAC CGA CCC TCA GAC 3’ for ITS1; 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 *cox*1 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; DeJong et al., 2001). PCR products were purified either with Omega E.Z.N.A Cycle-Pure Kit (Omega Bio-Tek, Norcross, GA 30071) or USB ExoSAP-IT PCR Product Cleanup (Affymetrix, Inc., 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).

The schistosome cercariae 28S, 16S, 12S and *cox*1 gene fragments and the snail host *Indoplanorbis exustus* ITS1, 16S and *cox*1 gene fragments were used in phylogenetic analyses using Bayesian inferences (BI) with the use of MrBayes v 3.1.2 (Huelsenbeck and Ronquist, 2001). The BI analyses were as follows (all parameters were unlinked): the 28S and ITS1 dataset, Nst = 6 rates = invgamma ngammacat = 4; the *cox*1 dataset, for codons one and two Nst = 2 and for codon three Nst = 6 rates = gamma, ngammacat = 4; for 12S and 16S combined Nst = 6 rates = invgamma ngammacat = 4. Four chains were run simultaneously for 5×10^5 generations, with 4 incrementally heated chains sampled at intervals of 100 generations. The first 5000 trees with preasymptotic likelihood scores were discarded as burnin, and the retained trees were used to generate 50% majority-rule consensus trees and posterior probabilities. Outgroups for the schistosome *cox*1, 12S, 16S and 28S datasets included members of the *S. mansoni* species group (sister to the *S. indicum* + *S. haematobium* groups), and in some cases also included members of the *S. turkestanicum* and *S. japonicum* species groups. The remaining taxa used in the tree were

obtained from the published literature (Attwood et al., 2002, Lockyer at al., 2003; Attwood et al., 2007). For the snail phylogenetic analysis we used different species of the sister genus *Bulinus* snails as outgroups. All DNA sequence data were deposited in GenBank (Table 3, Table 5).

3. Results

From 2007 to 2014, in the 40 locations listed in Tables 1 and S1, we collected and screened 19,360 freshwater snails for trematodes. The nine families represented 14 different species and their numbers were: *Bellamya bengalensis* (429), *Brotia costula* (15), *Gabbia orcula* (1374), *Gyraulus* spp. (4,454), *Indoplanorbis exustus* (7,286), *Lymnaea acuminata* (1,380), *Melanoides pyramis* (194), *Melanoides tuberculata* (254), *Physa* sp. (16), *Pila globosa* (74), *Radix luteola* (2,588), *Segmentina* spp. (529), *Succinea* sp. (14), and *Thiara* spp. (753). Only specimens of *I. exustus* shed schistosome cercariae subsequently shown to be consistent with affiliations to the *S. indicum* group. We found 34 (0.47%) *I. exustus* snails to be infected from 11 different localities (Figure 1, Tables 1 and S1). As indicated in the phylogenetic analyses that follow, one location (#35) yielded two schistosome lineages (*S. spindale* and *Schistosoma* sp.), collected at the same time point, from two different snails. Another location (#38) yielded schistosomes from all three lineages, but at different times within the same year or in different years (see supplementary table 1 for details).

Measurements of representatives of cercariae from each of the three lineages from Nepalese *I. exustus* are compared with those available from other studies of the *S. indicum* group, and for one species (*S. incognitum*) formerly included in the group (Table

2). Under any circumstances, cercariae of different species in this group are difficult to differentiate from one another morphologically owing to their broadly overlapping sizes and their consistent general morphological features (numbers and arrangement of flame cells and penetration glands). We found this to be true for the specimens we examined, including live cercariae. Although differences in preservation techniques make rigorous comparisons among studies difficult, we found all three kinds of cercariae we recovered to be somewhat smaller than the cercariae of *S. incognitum*. With respect to tail stem length which in our experience is useful for facilitating discriminating among species (Morgan et al., 2003), the relative order (shortest to longest) from other studies of the *S. indicum* group was: *S. indicum*, *S. spindale* and *S. nasale*. From shortest to longest tail stem length, the Nepalese specimens were ranked *Schistosoma* sp. (Figure 2), *S. spindale* and *S. nasale*.

Table 2. Comparison of measurements of representative mammalian-type (lacking eyespots) schistosome cercariae obtained from *Indoplanorbis exustus* (all measurements are in μm). The W number indicating the source and date collected is provided (Table 1, Supplementary Table 1). Identifications of the species from our study provided in the table are derived from the phylogenetic results discussed below).

	Body		Tail stem		Tail furca		Snail host	Reference
	Length	**Width**	**Length**	**Width**	**Length**	**Width**		
Schistosoma 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. exustus*	This study
S. spindale (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. exustus*	This study
S. nasale (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. exustus*	This study
S. indicum (Heat killed)	147-171	43-60	177-250	23-32	68-103	-	*I. exustus*	Srivastava and Datta, 1962
S. spindale (Formalin fixed)	133-183	42-62	226-265	25-36	70-97	-	*I. exustus*	Kohli, 1991
S. nasale (Formalin fixed)	151-224	42-71	204-284	25-32	75-117	-	*I. exustus*	Dutt, 1967
S. incognitum (Heat killed)	157-207	42-71	185-307	28-49	85-135	-	*Lymnaea luteola*	Sinha and Srivastava, 1960

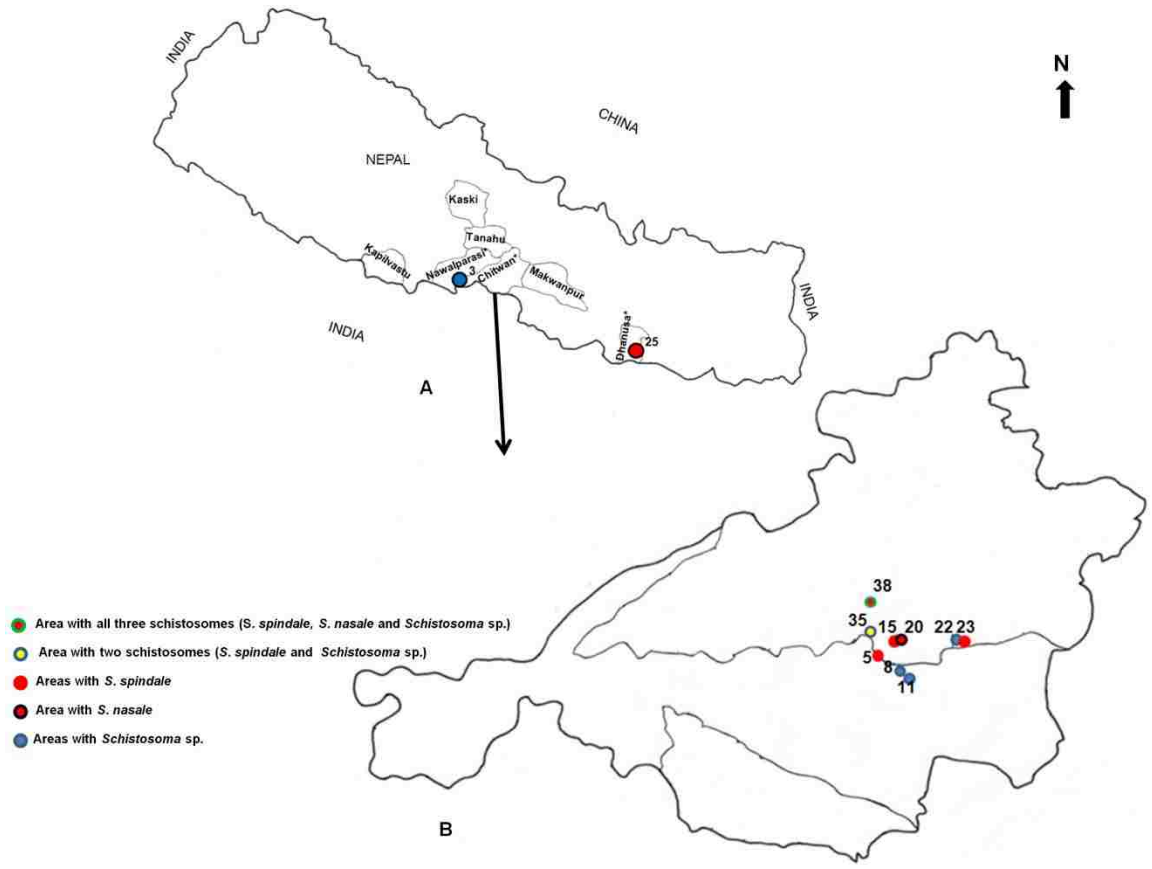


Figure 1. Map of the study area showing sites positive for *I. exustus*-transmitted mammalian schistosomes, with numbers corresponding to locality data in Table 1. A) Map of Nepal, and B) expanded map of Chitwan district within Nepal where many of our sampling sites were located.

With respect to our phylogenetic studies of the schistosome cercariae, we attempted to obtain *cox*1, 12S, 16S and 28S sequences from all specimens. We were able to obtain at least one of the above sequence regions (Table 3) for all but three of our specimens. We also included as much pertinent corresponding published sequence that we could find from closely-related schistosomes from elsewhere in Asia or Africa.

Figure 2. Cercaria of *Schistosoma* sp. obtained from *Indoplanorbis exustus* snail.

Table 3. List of the *S. indicum* group schistosome specimens for which DNA sequence data was obtained for phylogenetic analysis.

Taxon or Sample number	GenBank Accession number			
	28S sequences	**12S sequences**	***cox*1 sequences**	**16S sequences**
W378	KR423856	KR607250	KR607222	KR423839
W379	KR423845	KR607233	KR607213	KR423832
W380	-	KR607234	-	-
W381	-	KR607235	-	-
W383	KR423846	KR607236	-	-
W439	KR423863	-	-	-
W463	KR423847	KR607237	KR607214	-
W464	KR423857	KR607251	KR607223	-
W517	-	KR607238	-	-
W518	-	KR607239	KR607215	-
W519	-	KR607240	-	-
W525	KR423858	KR607252	KR607224	-
W528	KR423848	KR607241	KR607216	KR423833
W531	KR423849	KR607242	KR607217	KR423834
W532	KR423850	KR607243	KR607218	KR423835
W534	-	KR607244	-	-
W535	-	KR607253	-	-
W538	-	KR607254	-	-
W541	KR423851	KR607245	-	-
W542	KR423864	KR607259	KR607229	-
W545	KR423865	KR607260	KR607230	KR423843
W546	KR423859	KR607255	KR607225	-
W549	KR423866	KR607261	KR607231	-
W550	KR423852	KR607246	-	-
W551	KR423867	KR607262	KR607232	KR423844
W556	KR423860	KR607256	KR607226	KR423840
W557	KR423853	KR607247	KR607219	KR423836
W558	KR423861	KR607257	KR607227	KR423841
W798	KR423854	KR607248	KR607220	KR423837
W799	KR423855	KR607249	KR607221	KR423838
W804	KR423862	KR607258	KR607228	KR423842
Schistosoma indicum	AY157258 (Bangladesh); KF425714 (India)	EF534276 (Bangladesh)	AY157204 (Bangladesh)	EF534284 (Bangladesh)
S. spindale	Z46505 (Sri Lanka); AY157257 (Sri Lanka); AF465925 (Thailand); AF465926 (Thailand), KF425713(India)	EF534283 (Thailand); AF465920 (Thailand); AF465919 (Thailand); AF534282 (Sri Lanka); EF534281 (Bangladesh); DQ157223 (Lab strain originally from Sri Lanka)	AY157203 (Sri Lanka); DQ157223 (Lab strain originally from Sri Lanka)	EF534290 (Thailand); EF534289 (Sri Lanka); EF534288 (Bangladesh); DQ157223 (Lab strain originally from Sri Lanka)
S. nasale	AY157259 (Sri Lanka)	EF534280 (Bangladesh)	AY157205 (Sri Lanka)	-

Table 4. *P*-distances for *cox*1, 12S, 16S and 28S sequences among representative cercarial samples we collected, and with other mammalian schistosomes.

Taxa	*cox*1	12S	16S	28S
W798 (*Schistosoma* sp.) – *S. indicum* (Bangladesh)	13%	7%	15%	0%
W804 (*S. spindale*) – *S. spindale* (Sri Lanka)	8%	2.5%	6%	0%
W551 (*S. nasale*) – *S. nasale* (*cox*1and 28S- Sri Lanka, 12S- Bangladesh)	0%	0%	-	0%
W798 (*Schistosoma* sp.) - W804 (*S. spindale*)	13%	6.7%	13%	0%
W551 (*S. nasale*) - W798 (*Schistosoma sp.*)	16%	8.6%	17%	2%
W551 (*S. nasale*) - Our sample W804 (*S. spindale*)	16%	1%	17%	2%
W528 (*Schistosoma sp.*) – W531 (*Schistosoma sp.*)	0%	0%	0%	0%
W558 (*S. spindale*) – W804 (*S. spindale*)	0%	0%	1%	0%
W545 (*S. nasale*) - W551 (*S. nasale*)	0%	0%	0%	0%
S. indicum – S. spindale	14% (*S. indicum* Bangladesh, *S. spindale* SL)	7% (Both from Bangladesh)	17% (Both from Bangladesh)	0% (*S. indicum* Bangladesh, *S. spindale* SL)
S. spindale – S. nasale	16% (Both from SL)	9% (Both from Bangladesh)	-	2% (Both from SL)
S. indicum – S. nasale	16% (*S. indicum* Bangladesh, *S. nasale* SL)	9% (Both from Bangladesh)	-	2% (*S. indicum* Bangladesh, *S. nasale* SL)
S. mansoni- S. rodhaini	11.5%	-	-	0%
S. mattheei – S. haematobium	13%	4%	-	1%
S. intercalatum – S. haematobium	11%	4%	-	0%

Table 5. List of the sources of *Indoplanorbis exustus* DNA sequence data used in this study. The W number indicates the corresponding schistosome cercariae recovered from the snail. The last 14 entries in the table are from Liu et al. (2010) and Albrecht et al., (2004).

Taxon	Co-ordinates	Collection date	GenBank accession number		
			*cox*1	16S	ITS1
Snail infected with *S. nasale* (SW439)	N 26˚46'36.76", E 85˚56'05.50"	9th January 2007	KR811332	KR607278	KR811325
Snail infected with *S. spindale* (SW464)	N 27˚37'45.52", E 84˚29'21.57"	4th July 2010	KR811347	KR607268	KR811315
Snail infected with *S. spindale* (SW525)	N 27˚34'57.8", E 84˚27'56.6"	2nd July 2010	KR811348	KR607263	KR811316
Snail infected with *Schistosoma* sp. (SW528)	N 27˚33'37.5", E 84˚29'24.5"	9th July 2010	KR811338	KR607284	-
Snail infected with *S. spindale* (SW538)	N 27˚37'45.52", E 84˚29'21.57"	4th July 2010	KR811349	KR607272	KR811321
Snail infected with *S. nasale* (SW545)	N 27˚34'34.11", E 84˚31'02.21"	12th May 2011	KR811350	KR607273	KR811319
Snail infected with *S. nasale* (SW551)	N 27˚37'45.52", E 84˚29'21.57"	22nd July 2010	KR811351	KR607274	KR811318
Snail infected with *Schistosoma* sp. (SW557)	N 27˚34'26.85", E 84˚36'28.88"	3rd July 2012	KR811352	KR607276	KR811320
Snail infected with *Schistosoma* sp. (SW798)	N 27˚37'45.52", E 84˚29'21.57"	1st August 2014	KR811339	KR607285	-
Snail infected with *S. spindale* (SW804)	N 27˚37'45.52", E 84˚29'21.57"	29th July 2014	KR811354	KR607271	-
Snail infected with *S. spindale* (SW558)	N 27˚34'02.28", E 84˚37'16.55"	3rd July 2012	KR811353	KR607269	KR811317
Snail infected with strigeids and xiphidiocercariae Khageri river, Near Panchakanya Community Forest - location 21	N 27˚39'34.9", E 84˚29'00.2"	9th June 2011	KR811335	KR607281	-
Snail infected with strigeids and xiphidiocercariae Tikauli, Ratnanagar-7, Chitwan – location 38	N 27˚33'59.3", E 84˚30'14.9"	6th July 2010	KR811336	KR607282	KR811328
Snail infected with sangunicolids and xiphidiocercariae Chitwan National Park, Chitwan - location 9	N 27˚32'43.1", E 84˚30'08.1"	13 May 2011	KR811360	KR607270	KR811322
Snail infected with sangunicolids and xiphidiocercariae Chitwan National Park, Chitwan - location 7	N 27˚33'15.8", E 84˚21'19.7"	13 May 2011	KR811359	KR607267	-
Snail infected with sangunicolids Chitwan National Park, Chitwan - location 8	N 27˚33'22.6", E 84˚29'5.40"	8th July 2010	KR811337	KR607283	KR811327
Two snails infected with echistosome Baghmara Community forest, Chitwan - location 2	N 27˚35'22.0", E 84˚28'52.4"	22 July 2012	KR811341, KR811342	KR607288, KR607290	-
Snail infected with hooked-bodied strigeids Rapti river, Sauraha, Chitwan - location 32	N 27˚34'52.24", E 84˚28'56.12"	11 May 2011	KR811355	KR607277	KR811324
Two uninfected snails Kumaraura, Dhanusa - location 25	N 26˚46'36.76", E 85˚56'05.50"	9th January 2007	KR811333, KR811334	KR607279, KR607280	KR811326
Three uninfected snails Budhi Rapti River near Elephant Breeding Center, Chitwan - location 5	N 27˚34'57.8", E 84˚27'56.6"	11th May 2011	KR811356-KR811358	KR607264-KR607266	KR811323
Two uninfected snails Tulsi Chauda village, Dhanusa - location 39	N 27˚00'50.37", E 85˚55'31.80"	7th January 2007	KR811344, KR811345	KR607286, KR607292	KR811331
Two uninfected snail Rice fields in Chisapani Village, Godar-2/3, Dhanusa - location 34	N 26˚56'26.09, E 86˚08'51.19"	8th January 2007	KR811346	KR607287, KR607293	KR811329
Snail infected with sangunicolid Dhumre river, Kumrose, Chitwan - location 15	N 27˚34'34.11", E 84˚31'02.21"	12 May 2011	KR811361	KR607275	-
Uninfected snail Shishuwar bagar, Bachhauli-3, Chitwan - location 35	N 27˚35'31.76", E 84˚30'00.31''	2 July 2010	KR811340	KR607291	KR811330
Uninfected snail Chitwan National Park, Chitwan - location 12	N 27˚33'37.0", E 84˚30'09.1"	23rd July 2010	KR811343	KR607289	-
I. exustus, Janakpur, Nepal	-	-	GU451739	GU451732	-

I. exustus (Assam, India)	-	-	GU451744	GU451726	-
I. exustus (Mymensingh, Bangladesh)	-	-	GU451745	GU451727	-
I. exustus (Kekirawa, Sri Lanka)	-	-	GU451742	GU451735	-
I. exustus (Bintulu, Borneo, Malaysia 1)	-	-	GU451746	GU451728	-
I. exustus (Kampang Pelegong, Malaysia 2)	-	-	GU451738	GU451731	-
I. exustus (Bogor, Indonesia)	-	-	GU451747	GU451729	-
I. exustus (Xang, Laos)	-	-	GU451750	GU451751	-
I. exustus (Wadi Bani Khaled, Oman 1)	-	-	GU451740	GU451733	-
I. exustus (Wadi Qab, Oman 2)	-	-	GU451741	GU451734	-
I. exustus (Bulan, Philippines)	-	-	GU451748	GU451730	-
I. exustus (Khon Kaen, Thailand 1)	-	-	GU451743	GU451736	-
I. exustus (Phitsanulok, Thailand 2)	-	-	HM104223	HM104222	-
I. exustus (Maenam Loei, Thailand 3)	-	-	AY282587	-	-

First, regarding the Bayesian analysis of *cox*1 schistosome sequences (Figures 3A and B), several features are noteworthy, many of them recapitulated in our analyses of other genes. The two trees differed in that Figure 3B incorporated additional sequences from GenBank, but these sequences were shorter (372 bp) as compared to sequences used in Figure 2A (1125 bp). For Figure 3A for which better overall resolution was obtained, sequences representing seven members of the *S. haematobium* group consistently grouped together with strong support, and this group was sister (0.91 posterior probability) to a group containing *S. indicum* from Bangladesh, *S. spindale* from Sri Lanka and Nepal, and *Schistosoma* sp. from Nepal. Lying sister to these two lineages with strong support in the analysis of the longer *cox*1 sequences, was a tightly grouped clade consisting solely of specimens of *S. nasale* from both Sri Lanka and Nepal.

Interestingly, relative to the amount of diversity among the specimens of *S. indicum, Schistosoma* sp. and *S. spindale*, the amount of sequence diversity amongst the five representatives of *S. nasale* was minimal. The appearance of *S. nasale* as a lineage basal to both the *S. haematobium* group and to the remaining members of the *S. indicum* group was recovered in several of our analyses, with variable degrees of support. None of our analyses could significantly delineate the *S. haematobium* group from the *S. indicum* group as customarily defined.

Table 6. Uncorrected *p*-distances for the *cox*1 and 16S sequences among representative snail samples we collected, and with other snails

Taxa	*cox*1	16S
Within *Indoplanorbis*	0-14%	0-8%
Within *Bulinus*	2-15%	1-13%
Indoplanorbis infected with sanguinicolid Chitwan National Park – *Indoplanorbis exustus* Nepal (Liu et al., 2010)	2%	4%
Indoplanorbis uninfected Chitwan National Park (location 12) – *I. exustus* Nepal (Liu et al., 2010)	14%	7%
Indoplanorbis Chisapani, Dhanusa – *Indoplanorbis* infected with sanguinicolid Chitwan National Park	13%	7%
Indoplanorbis Chisapani, Dhanusa – *Indoplanorbis* infected with *S. spindale* (W538)	12%	8%
Indoplanorbis infected with *S. spindale* (W538) – *Indoplanorbis* infected with *S. nasale* (W439)	9%	5%
Indoplanorbis infected with *Schistosoma* sp. (W528) – *Indoplanorbis* infected with *S. nasale* (W439)	13%	7%
Indoplanorbis infected with *Schistosoma sp.* (W528) - *Indoplanorbis* infected with *S. spindale* (W538)	12%	8%
Indoplanorbis infected with *S. spindale* (W464) - *Indoplanorbis* infected with *S. spindale* (W525)	0%	0%
Bulinus tropicus – B. nasutus	15%	9%
B. globosus – B. forskali	12%	13%
Biomphalaria glabrata – B. peregrine	10%	7%

Two specimens of *S. spindale* from Sri Lanka grouped with high support with seven Nepalese isolates, and although further study is warranted particularly from intervening geographic regions, all are likely to be *S. spindale*. However, there is an appreciable degree of genetic distance (about 8%, Table 4) between the specimens from the two countries. Also included in the analysis are nine closely-related Nepalese isolates that consistently formed a distinct, well-supported group that diverged in *cox*1 sequence from *S. nasale* or *S. spindale* by 16% and 13%, respectively. We initially assumed this third lineage would correspond by sequence similarity to *S. indicum*. We were surprised to note (as in figure 3A), however, that the few available sequences of *S. indicum* from GenBank consistently failed to group with the nine Nepalese isolates in this third lineage. Furthermore, the genetic distance separating *S. indicum* Bangladesh and our third lineage was 13%. This suggested to us that this third Nepalese lineage was distinct from all three recognized members of the *S. indicum* group, and is referred to here as *Schistosoma* sp.

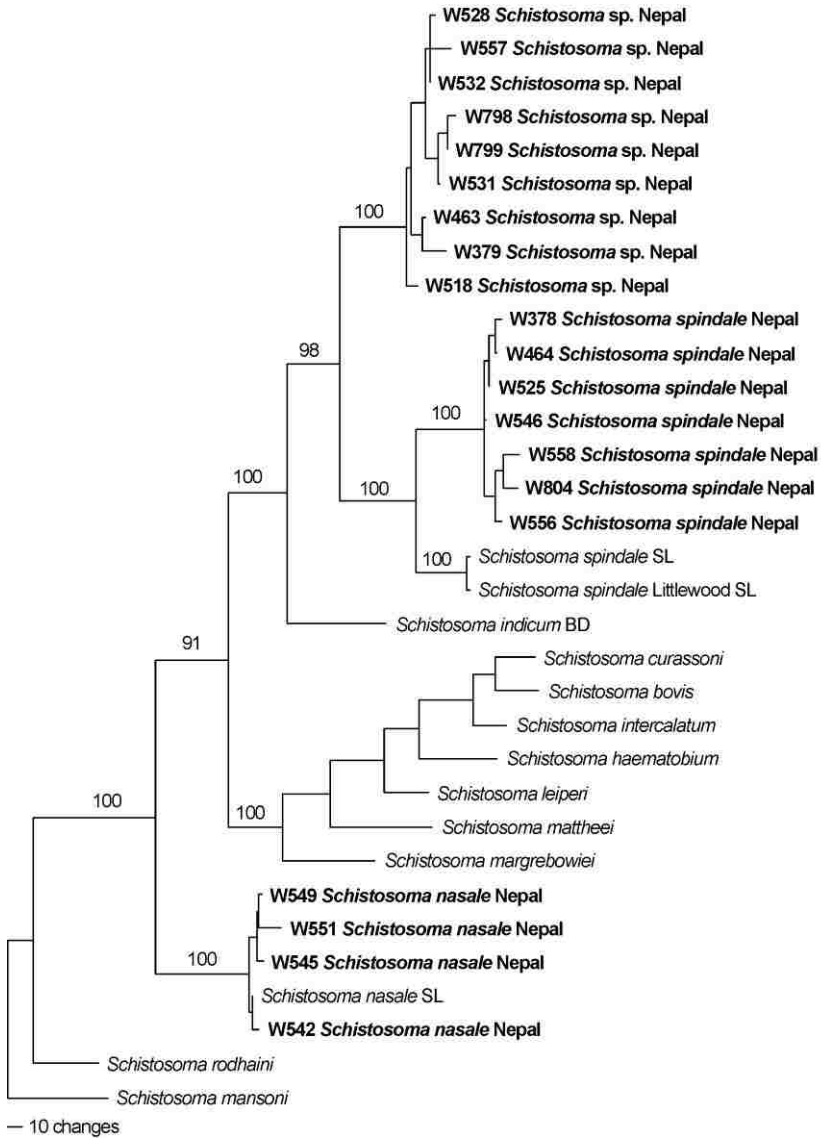


Figure 3A. Bayesian phylogenetic tree based on *Schistosoma cox*1 sequences (1125 bp). Samples in bold are those collected in this study. Node support is indicated by Bayesian posterior probabilities. This tree includes the only *Schistosoma indicum* sequence available in GenBank.

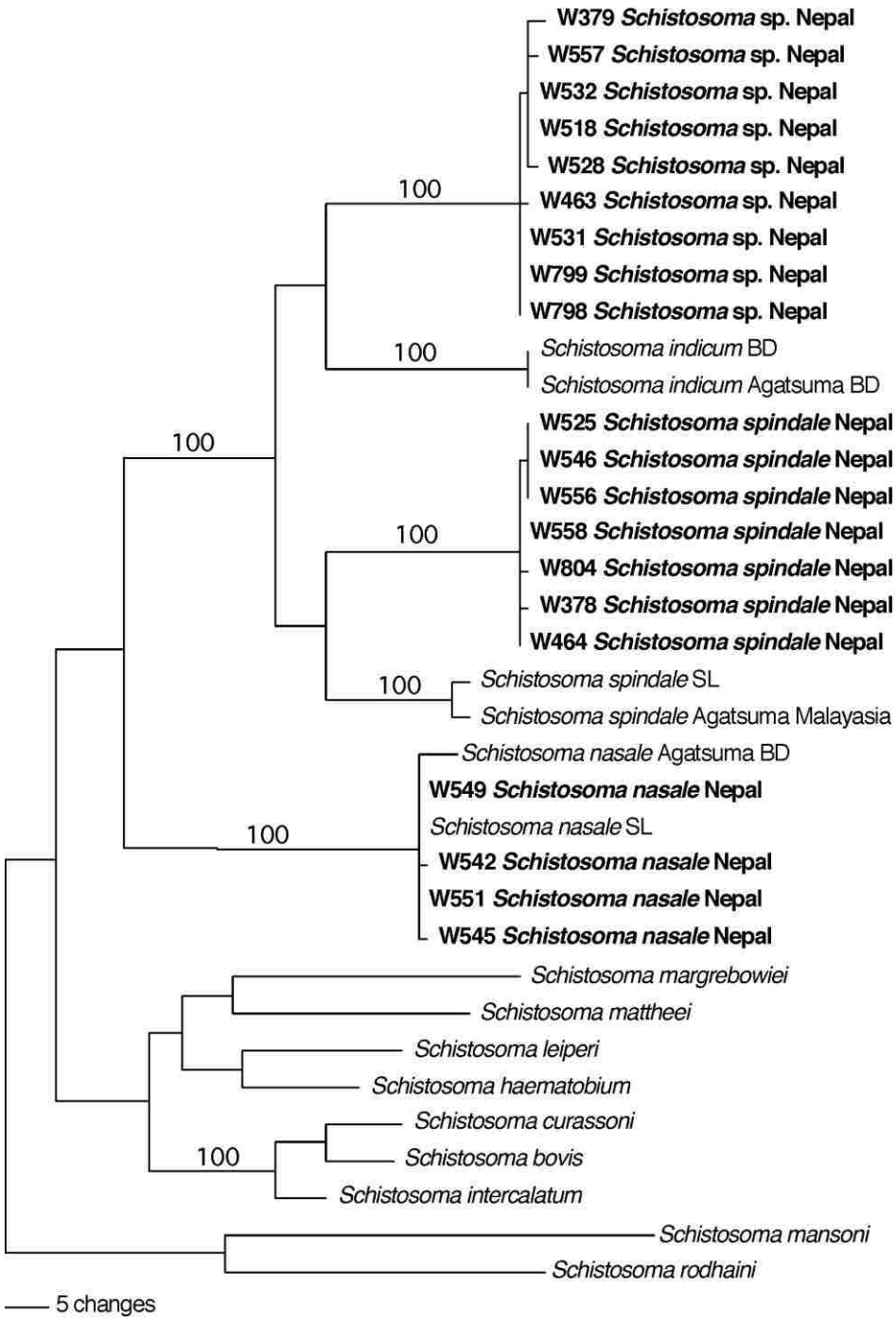


Figure 3B. Bayesian phylogenetic tree based on *Schistosoma cox*1 sequences (372 bp). Samples in bold are those collected in this study. Node support is indicated by Bayesian posterior probabilities. This tree includes two *Schistosoma indicum* sequences available in GenBank.

The tree in figure 3B was made to allow inclusion of a second available short *cox*1 sequence for *S. indicum* from Bangladesh, and although there was some tendency for Nepalese *Schistosoma* sp. to group with the two specimens of *S. indicum* from Bangladesh in this tree, support was not strong. Many nodes in this tree were not strongly supported, and no support was found for uniting presumptive *S. spindale* from Nepalese with *S. spindale* from elsewhere.

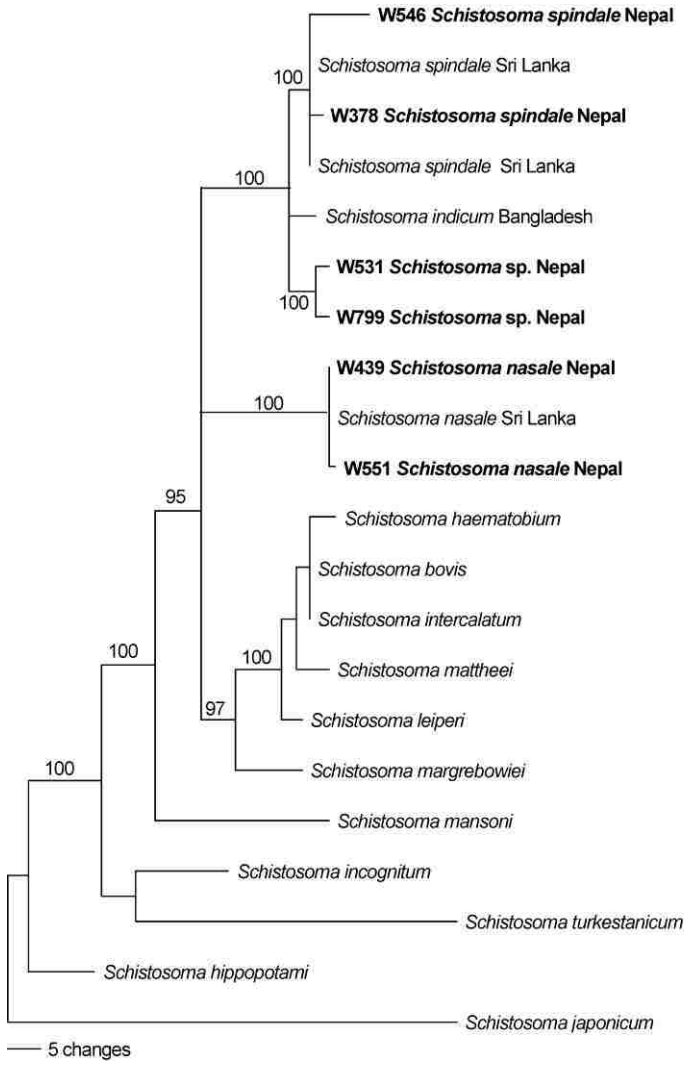


Figure 4. Bayesian phylogenetic tree based on *Schistosoma* 28S sequences (1515 bp). Samples in bold are those collected in this study. Node support is indicated by Bayesian posterior probabilities.

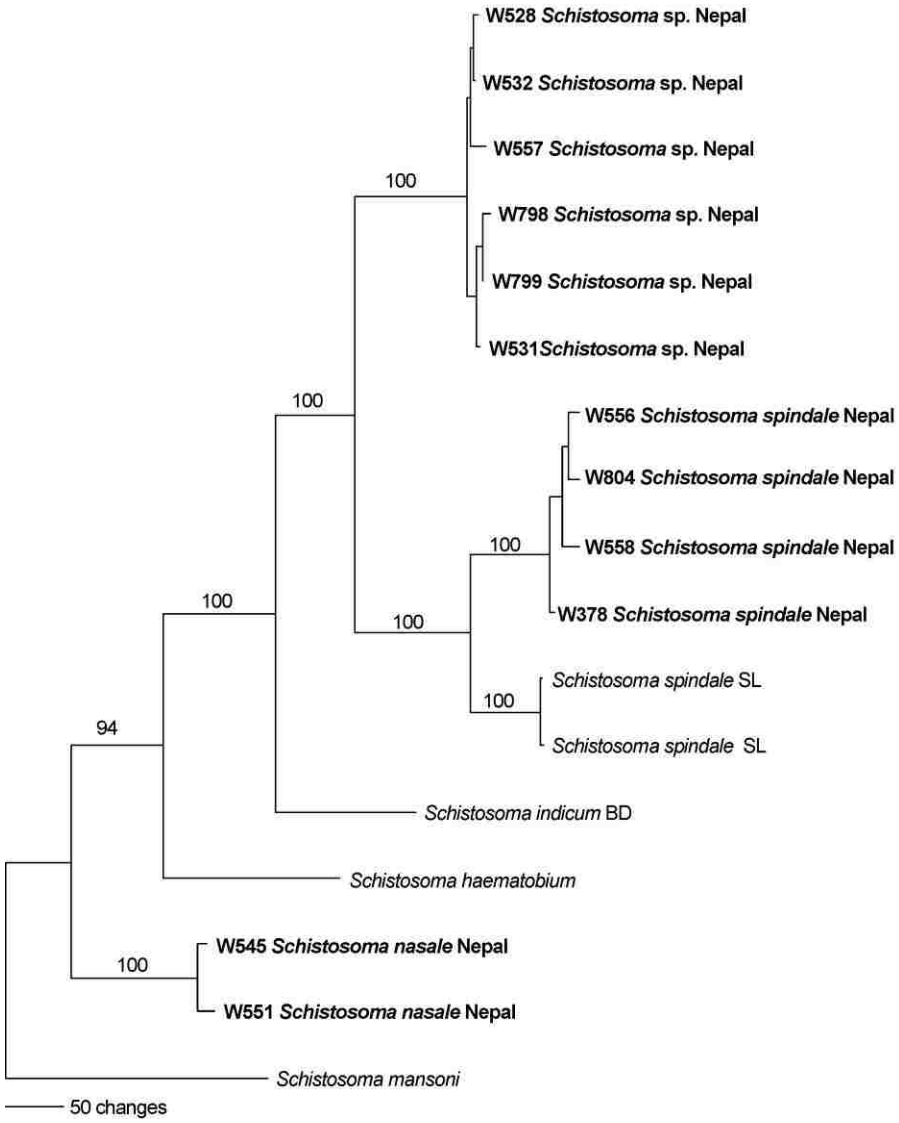


Figure 5. Bayesian phylogenetic tree based on combined *Schistosoma cox*1, 16S and 12S sequences (1740 bp). Samples in bold are those collected in this study. Node support is indicated by Bayesian posterior probabilities.

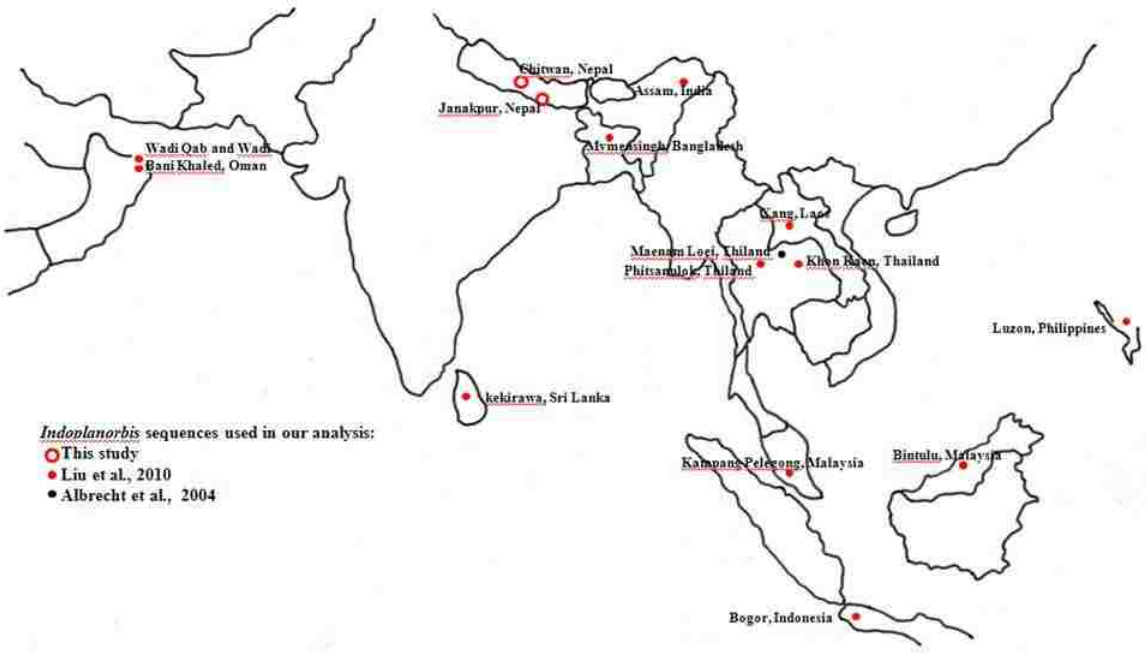


Figure 6. Map showing localities from which specimens of *Indoplanorbis exustus* were collected, and for which 16S and c*ox*1 sequences are available in GenBank from Albrecht et al., 2004 and Liu et al., 2010.

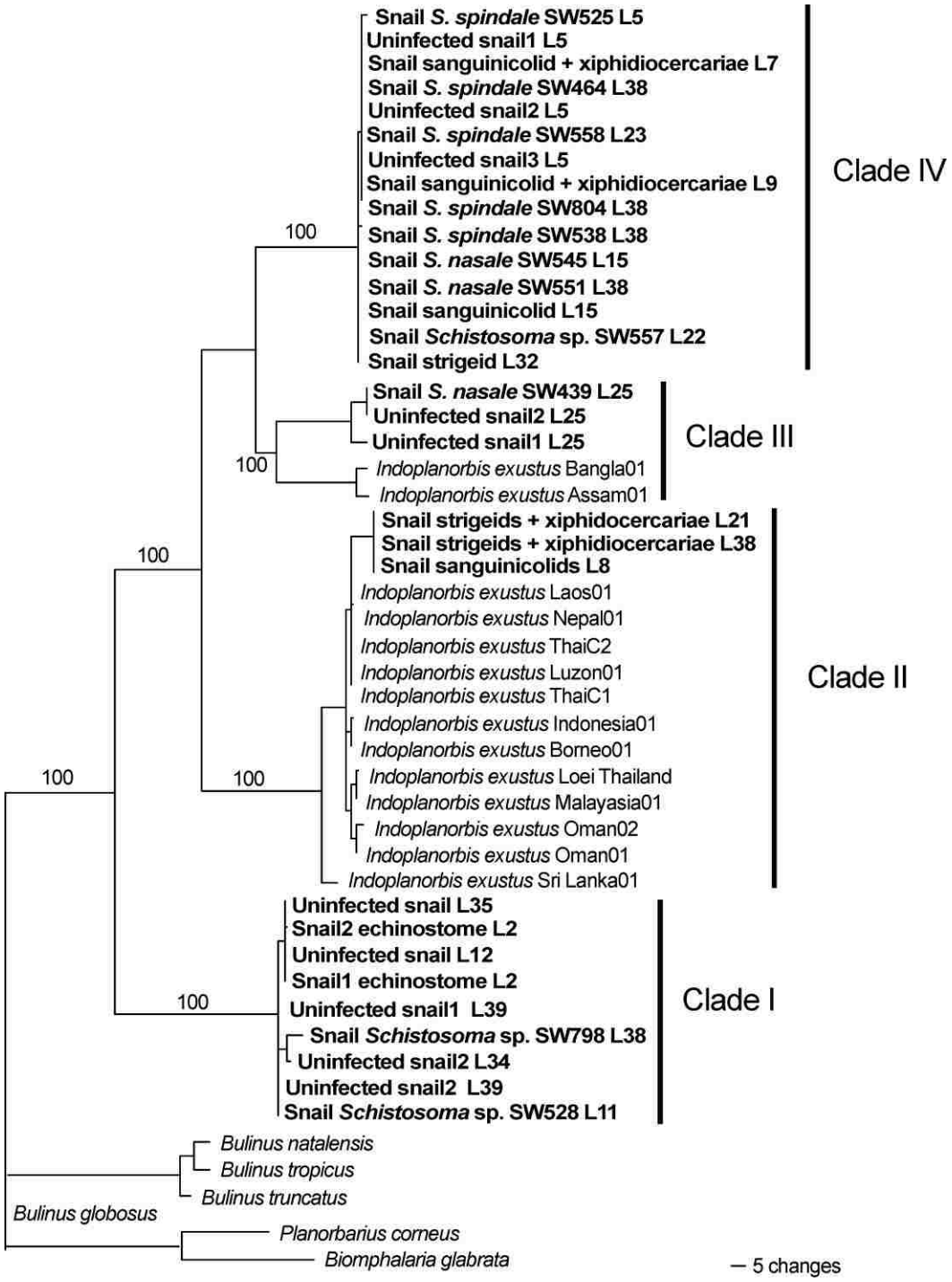


Figure 7. Bayesian phylogenetic tree based on *Indoplanorbis cox*1 sequences (620 bp). Samples in bold are those collected in this study. Node support is indicated by Bayesian posterior probabilities. The "L" followed by a number designates the location number indicated in Table 1.

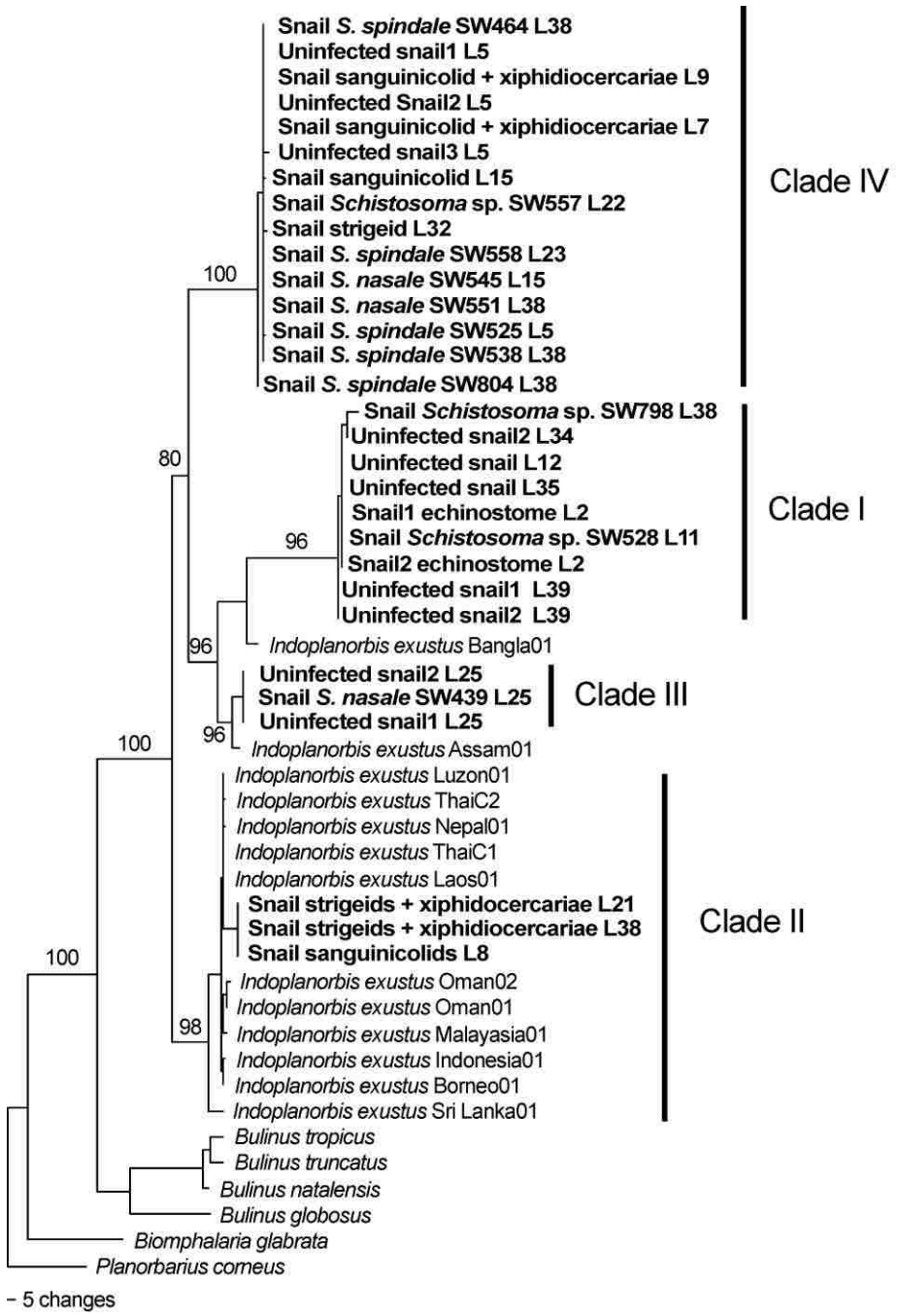


Figure 8. Bayesian phylogenetic tree based on *Indoplanorbis cox*1 and 16S combined sequences (1036 bp). Samples in bold are those collected in this study. Node support is indicated by Bayesian posterior probabilities. The "L" followed by a number designates the location number indicated in Table 1.

The nodal support provided by the analyses of 28S, 16S and 12S sequences (Figure 4, supplementary figures 1-3) was generally not as strong as noted for Figure 3A, but this may be expected as these genes are less variable than *cox*1. Yet, many similar patterns to those noted for the *cox*1 analyses were again observed using these gene regions. The 28S analysis shown in Figure 4 again resolved *S. nasale* as separate from either the remainder of the *S. indicum* group or the *S. haematobium* group, but was unable to resolve relative branch orders among these three groups. A Bayesian analysis based on combined *cox*1, 16S and 12S sequences (Figure 5) revealed the following patterns in the data: 1) weakly supported basal position of *S. nasale*; 2) strong support (0.94 posterior probability) for a clade containing two major lineages, the *S. haematobium* group and the remainder of the *S. indicum* group consisting of Bangladesh sample of *S. indicum*, *S. spindale*, and *Schistosoma* sp. from Nepal; 3) substantial variation among lineages ascribed to *S. spindale* from Sri Lanka and Nepal; and 4) the *Schistosoma* sp. lineage from Nepal was divergent from the one available set of sequences available for *S. indicum* from Bangladesh.

For the 20 specimens for which we obtained both a 28S nuclear sequence and at least one mitochondrial sequence (*cox*1, 16S or 12S), none revealed a nuclear sequence characteristic of one species and a mitochondrial sequence characteristic of another.

Regarding the phylogenetic analyses for *Indoplanorbis exustus*, 30 *cox*1, 31 16S and 14 ITS1 sequences were obtained from specimens of this snail, mostly from the Terai region (Table 5). For two of these samples, snail sequences were actually amplified from extracts of schistosome cercariae (W798 and W804) originally derived from *I. exustus* but for which the snails were no longer available. In addition, we compared our

sequences with those from 14 additional specimens acquired from GenBank (Table 5, Figure 6). Included in the analyses are sequences for other planorbid snails, including species of *Bulinus*, the genus believed to be the most closely related to *Indoplanorbis*.

Results of the *cox*1 analysis (Figure 7) provide two especially noteworthy features. The first, and most surprising, is the presence of four differentiated clades of *I. exustus* (designated I-IV), two of which (Clades I and IV), including the basal group in our *cox*1 analysis (Clade I), are comprised exclusively of snails from Nepal. Representatives of each of the four groups are found in Nepal, and three of the four groups (clade I, III and IV) included specimens infected with members of the *S. indicum* group. Strong nodal support was obtained for a clade uniting all identified *I. exustus* specimens. Notably lacking in this analysis are snail specimens from India or Pakistan, or Iran in the western part of the natural range of *I. exustus*. The second noteworthy feature is that the results do not provide support for a sister group relationship of *Indoplanorbis* with *Bulinus*.

The 16S analyses (Supplementary Figure 4) also did not retrieve strong support for a sister relationship between *Bulinus* and *Indoplanorbis,* and all specimens of *I. exustus* again were shown to group together with high support. Once again four major clades of *I. exustus* were discerned (each containing specimens from Nepal) but with more variable support, and in this case there was not a strongly supported basal clade. The pattern of relationships among the three remaining clades was often poorly resolved, although the members within each clade grouped together with strong support (0.92-1 posterior probability). In contrast to the separate analyses of *cox*1 and 16S, the analysis of combined *cox*1 and 16S sequence (1036 bp) (Figure 8) showed strong support for the

relationship between *Bulinus* and *Indoplanorbis*. Similarly strong support for the monophyly of the *Indoplanorbis* lineages was retrieved. Strong support at most nodes was retrieved for the four major clades, though the topology differed from that noted in Figure 6.

The *p*-distance values for both *cox*1 and 16S genes among the four different lineages of *I. exustus* (Table 6) are in the same range (*cox*1 0-14% and 16S 0-8%) as reported for species pairs in *Bulinus*. Due to lack of ITS1 data for *Indoplanorbis* in GenBank we were unable to compare our data with others. In our ITS1 ingroup analysis (Supplementary Figure 5), we were unable to resolve the four major clades shown in our other analyses.

We observed multiple clades (I, II and IV) of *Indoplanorbis* in one location (# 38) at different collecting times. Based on our limited number of snail sequences, we did not find any coexisting clades.

4. Discussion

This study was first stimulated by a report of *S. mansoni*-like eggs from human stool samples from the Terai region of Nepal (Sherchand et al, 1999). An initial visit to the area failed to provide corroborating evidence for the presence of either *S. mansoni* or *Biomphalaria* but highlighted the near complete lack of knowledge regarding the presence of schistosomes in Nepal. Consequently, we began a study of Nepalese schistosomes starting with an investigation of *Bivitellobilharzia nairi* eggs from both elephants and Asian rhinos from Nepal (Devkota et al., 2014a). Thereafter, we directed

our attention to schistosome cercariae obtained from Nepalese snails, including cercariae of avian schistosomes (Devkota et al., 2014b).

Our more extensive survey of schistosome cercariae that followed also failed to reveal either *Biomphalaria* snails or any cercariae identifiable as *S. mansoni*, nor did we find snails shedding *B. nairi* cercariae (Devkota et al., 2014a). We did find evidence of four lineages of *Schistosoma* in Nepal: the three lineages of the *S. indicum* group discussed here, all of which were recovered from *I. exustus*, and a fourth lineage of lymnaeid-transmitted *Schistosoma* we will discuss in a separate paper. Based on our survey results and on results of experimental infections (Dutt and Srivastava, 1968; De Bont et al., 1991), and given the difficulties of distinguishing among the mammalian schistosome cercariae purely on morphological features, in our view previous reports of *S. indicum* group cercariae from Asian lymnaeid snails probably represent *S. turkestanicum* or one of its relatives, or *S. incognitum*.

We were unable to study adult schistosomes as part of this study because buffaloes and especially cattle are maintained for milk production and are not commonly slaughtered in the Terai region. Government sanctioned abattoirs are not present in the area, and we found owners of private abattoirs were unwilling to grant permission to examine viscera of slaughtered animals. Second, small laboratory mammals like hamsters for experimental exposures are not readily available and administrative procedures to regulate care and maintenance of laboratory mammals are either not in place or lack any ability to articulate with animal care committees in the U.S.A. Similar constraints may explain why very few specimens of adult worms of the *S. indicum* group have been forthcoming from other countries in the region. The lack of adult worms and associated

eggs, the usual benchmarks whereby species of schistosomes are described and delineated, means that what we say about the specific identity of the *S. indicum* group cercariae we recovered has to be qualified.

One consistent feature of our analyses was that *S. nasale* was retrieved as separate from, and often basal to, members of the combined *S. haematobium-S.indicum* groups. Furthermore, relative to the other lineages we found, the amount of genetic diversity present in this species was minimal, both for specimens retrieved from Nepal and when specimens from Sri Lanka or Bangladesh were included. The habit of adult worms living in the nasal passages is found in several avian schistosomes (Horák et al., 2002), though is known with certainty only for *S. nasale* among mammalian schistosomes. With the exception of *S. haematobium*, all other members of both species groups live in mesenteric veins (Brant and Loker, 2013).

With respect to the remaining two *S. indicum* group lineages we found in Nepal, one is almost certainly *S. spindale*, though the Nepalese specimens diverge genetically from the few *S. spindale* specimens available for comparison from elsewhere in Asia. Thus whereas *S. nasale* from Nepal, Sri Lanka and Bangladesh exhibit 0% *p*-distance values for their *cox*1 sequences, specimens of *S. spindale* from Nepal and Sri Lanka differ by 8% in PI values for the same target gene (Table 4). Nonetheless, strong posterior probability support is obtained for grouping Nepalese specimens of presumptive *S. spindale* with *S. spindale* from other locations.

We refer to the third *S. indicum* lineage in Nepal as "*Schistosoma* sp." Although we originally expected this third lineage to be *S. indicum*, it never closely grouped in any of our trees with available sequences for *S. indicum* from GenBank. Nepalese

Schistosoma sp. differs substantially from known *S. indicum* with respect to *p*-distance values: 13% for the *cox*1 gene, a value commensurate with typical species differences within both the *S. indicum* and *S. haematobium* groups (Table 4). A note of caution is in order here as very few specimens of *S. indicum* for which sequence data exist (potentially only two worms represented) are available. Furthermore, they are from the same locality in Bangladesh. Only small fragments of 16S (60 bp) and 28S (346 bp) of *S. indicum* from India were available in GenBank. The Bangladesh and Indian specimens for some reason may not be representative of the genetic variation within *S. indicum*, or it is possible *S. indicum* is broadly distributed and encompasses a particularly wide range of genetic variation. It is certainly a possibility that Nepalese *Schistosoma* sp. is actually a distinct species, possibly one confined to the northern part of the collective range of the *S indicum* group. This possibility will hopefully stimulate additional study including verification with specimens of adult worms and eggs, and experimental infection studies.

Although *Schistosoma* sp. from Nepal does not cluster with previously identified *S. indicum*, it persistently groups with *S. spindale*, as a close but distinct relative in our trees (*p*-distance value 13% for *cox*1, Table 4). Even though worms of both lineages likely inhabit the mesenteric veins of domestic ruminants in Nepal, we found no evidence in our samples suggestive of hybridization of *Schistosoma* sp. with *S. spindale*. There was also no indication of hybridization of either lineage with *S. nasale*.

Regarding *I. exustus*, our results are in agreement with Liu et al. (2010) in showing a surprising amount of structure within what has traditionally been considered a single widespread snail species. Liu et al. (2010) identified three major clades, one of which includes specimens from localities as diverse as Oman, Malaysia, Indonesia,

Thailand, the Philippines and Nepal, and corresponded to clade II identified on our trees. The other two clades from the study of Liu et al. (2010) were more closely related to one another, were from Bangladesh and northern India, and corresponded to clade III in our *cox*1 analysis. The sample from Bangladesh was moved to clade I in our 16S and combined *cox*1 and 16S analyses. Our clade IV includes only specimens from Nepal. The specimen from Bangladesh noted by Liu et al. (2010) was relatively divergent from those we collected from Nepal, suggesting it might be appropriate to recognize even a fifth lineage. Our results indicate multiple clades can inhabit the same specific habitat (#38) from which clades I, II and IV of *Indoplanorbis* were all found.

The *p*-distance values for both *cox*1 and 16S genes among the four different lineages of *I. exustus* we identified (Table 6) are not dissimilar from values recorded for species pairs within the probable sister genus *Bulinus*. Samples of *I. exustus* from many other parts of this species' known range have yet to included in molecular phylogenetic studies, so the overall diversity inherent in what should more properly be considered the *I. exustus* species complex is bound to increase. In particular, specimens from north of the Himalayas, most of the eastern portion of the range including much of India, Pakistan Afghanistan and Iran, island populations from the Indian Ocean, and specimens from Africa are bound to provide more surprises.

We did not sequence characterize all of the *I. exustus* snails we collected, so caution is required in interpreting the relative abundance of the lineages in Nepal from our data. Likewise, we did not provide sequence for all snails shedding schistosome or other cercariae. Snails from lineage I, for which representatives are mostly known from our study, were commonly collected and two echinostome infections and two

Schistosoma sp. infections were recovered from them. Lineage II, which includes specimens from several countries (including one specimen from Nepal) collected by others, was also found by us but not commonly, and of the three specimens we sequenced two had a double trematode infections (xiphidiocercariae and strigeids) and one had a sanguinicolid infection. Lineage III, which also includes specimens collected by others from Assam, or Assam and Bangladesh depending on the tree, was less common in Nepal but included one snail positive for *S. nasale*. Finally, Lineage IV, known thus far from only our collections, was probably the most common lineage in our collections, and in addition to infections with strigeids, sanguinicolids, and xiphidiocercariae, specimens of all three of the *S. indicum* group lineages were recovered: 5 *S. spindale*, 2 *S. nasale*, and one *Schistosoma* sp. So, in Nepal, we can say that three of the four major *I. exustus* lineages we found were able to support at least one member of the *S. indicum* group, lineage IV supported all three schistosome lineages, and lineage II was not found to support any schistosomes though it is likely snails of this lineage support the *S. indicum* group elsewhere in Asia where they seem to be common (Liu et al., 2010). Lineage II may also transmit schistosomes in Nepal where, based on admittedly incomplete sampling, it appears to be uncommon.

As noted in several other studies (Lockyer et al, 2003; Morgan et al., 2003; Webster et al., 2006; Lawton et al, 2011; Webster and Littlewood, 2012), we find clear support for a close relationship between the *S. haematobium* and *S. indicum* species groups, but as also noted in the most recent study of the *S. indicum* group by Attwood et al (2007), we found the two groups do not separate cleanly and there was a persistent tendency for the *S. haematobium* group to nest within the *S. indicum* group, with *S.*

nasale basal, albeit often without strong support, e.g. the *S. indicum* group as traditionally considered appears to be paraphyletic (see also Attwood et al., 2007). The results may be dependent on the particular sequences selected for study as other investigations (Morgan et al., 2003; Webster et al., 2006; Webster and Littlewood, 2012) have indicated separation of the *S. indicum* and *S. haematobium* groups, but bootstrap support for the basal position of *S. nasale* within the *S. indicum* group is always reported as relatively weak, again raising questions as to the delineation of these two groups. Provision of more sequence may resolve what looks like an obvious anomaly in schistosome systematics, given that members of the recognized *S. haematobium* groups all parasitize *Bulinus* snails and are predominantly African and southwest Asian in distribution, whereas members of the recognized *S. indicum* group are transmitted by the *Indoplanorbis exustus* species group and are Asian in distribution. The close relationships between the two species groups of schistosomes is supported by the presumed sister group relationship of their snail hosts. *Bulinus* and *Indoplanorbis* are united by the presence of a distinctive synapomorphy, the ultrapenis, and by molecular systematic studies that place them in a bulinine clade quite distinct from other planorbid snails (Morgan et al., 2002; Albrecht et al., 2007). Here though it should be noted that recent studies have not documented strong support for the sister group relationship between *Bulinus* and *Indoplanorbis* (Jørgensen et al., 2011; this study, but see Figure 8). This in itself is an intriguing question and more extensive sampling and sequencing of *Indoplanorbis* and other planorbids may help to resolve this issue. What other planorbids may be close relatives though is not obvious as, for example, other possible bulinine snail candidates from Australia lack an ultrapenis (Walker, 1988).

Scenarios for the origins of the *S. indicum* group (see discussions in Barker and Blair 1996; Attwood et al., 2002; Lockyer et al., 2003; Attwood et al., 2007; Lawton et al., 2011) involve an origin in Africa in the Plio-Pleistocene from an artiodactylid-inhabiting schistosome with a bulinid snail host that colonized Asia via the Sinai and Levant. Land connections between Africa and Asia opened during the mid-Miocene with prominent faunal exchanges occurring thereafter. Among the animal groups in transit were bovids, which were well established by that time. Also, a host shift into *Indoplanorbis* is a likely possibility given the relatedness of the two snail genera.

This scenario may need to change some, however, especially if further study shows that *S. nasale* retains its basal position relative to the remainder of the combined *S. indicum-S. haematobium* group. If so, this is noteworthy for at least three reasons. One is that the original snail host for this combined lineage may have been *Indoplanorbis*, not *Bulinus*. Second, it is fascinating that the adult habitat for *S. nasale* in the nasal passages is so distinctively different from all other members of the combined *indicum-haematobium* lineage. Furthermore, given the present-day distribution of *Indoplanorbis* in southern Asia (African representatives of *I. exustus* appear to be relatively recent colonists), it raises a question as to where the origins of this larger group of schistosomes might actually lie. Today, aside from recent human-influenced range changes, the ranges of *Indoplanorbis* and *Bulinus* are contiguous in regions of Iraq and Iran, and may overlap in some areas there. In the past, especially considering the occurrence of several pluvial periods in northern Africa and southwest Africa at various periods ranging from 3.2mya to 12,500ya (Lawler, 2014), opportunities for intermingling of both definitive and

intermediate hosts may have been much more extensive in the Levant, the Arabian peninsula, Mesopotamia and present-day Iran than they are today.

That *I. exustus* is actually a species complex with much more genetic structure than previously considered is particularly germane with respect to the evolution of the *S. indicum* group. Particular lineages within the *I. exustus* complex, assuming they were already in existence, may have played a critical role in the transition of *Schistosoma* into *Indoplanorbis*. Additional study is needed to better characterize the full diversity within both the *S. indicum* group and the *I. exustus* species complex, as is a thorough analysis of how the two species groups interact. For example, are particular species in the *S. indicum* group confined to certain lineages of the *Indoplanorbis* group, or do some lineages of *Indoplanorbis* host all, whereas others host none, of the *indicum* group species? Our results are not suggestive of congruent patterns of cospeciation as all three schistosome species from Nepal were found in *I. exustus* lineage IV, and two schistosome species were found in two different *I. exustus* lineages. Our results suggest that lineage IV is important in transmission in Nepal, but more extensive sampling of both worms and snails across a broader geographic area may well change this story.

In considering the timing involved in the emergence of the *S. indicum* group, it is noteworthy and fortuitous that members of the *S. indicum* group do not establish patent infections in humans, unlike at least two members of the *S. haematobium* group in Africa. This tends to suggest that the time available for members of the *S. indicum* group to adapt to and infect humans has been limited relative to members of the *S. haematobium* group, which, by virtue of their origin in Africa, would have had opportunity for continual contact with hominins. Also, the dependency of *indicum* group worms on *Indoplanorbis*

may similarly have prevented them from gaining long duration access to hominins as *Indoplanorbis* is an Asian genus. Consequently, unlike *Bulinus*-transmitted schistosomes, *indicum* group worms were removed from the African center for human evolution and diversification.

The migration of *Homo erectus* out of Africa into southern Asia about 1.5-2 mya may have either preceded the presence of *S. indicum* group members in Asia, or may not have afforded the schistosomes ample opportunities to switch into hominin hosts. Similarly, the appearance of modern humans in southern Asia by 75,000 ya (Stanyon et al., 2009) may not have provided sufficient time for these worms to colonize humans. Although *S. japonicum* would also have had the same late access to human hosts relative to African schistosomes, it obviously was able to switch successfully into this important new host group. The generalist tendencies of *S. japonicum* with respect to its definitive hosts (over 40 species of mammals can be infected, ranging from rodents to buffaloes) may have favored human colonization relative to *S. indicum* group species, which seem to be far more limited in their host range, mostly to artiodactylids.

We note our survey focused on a relatively small part of Nepal. The presence there of three genetically distinct lineages of the *S. indicum* group (including one that is apparently new), and at least four well-differentiated clades of *I. exustus* indicate that Nepal and other parts of the northern Indian subcontinent are areas much in need of further study. The triculine snails in hilly regions of Nepal (Nesemann et al., 2007) warrant further study with respect to recovery of new schistosomes, given the role of triculines elsewhere in transmission of *Schistosoma japonicum* group parasites. Of particular interest will be to learn if members of the *S. indicum* group extend to areas

north of the Himalayas as *Indoplanorbis exustus* occurs in Tibet and China. The Himalayas have been shown to have a strong effect in isolating *Gyraulus* snails north of the mountains from those to the south (Oheimb et al., 2013) so might also have similar effects on *Indoplanorbis* and associated parasites. Once the *I. exustus* species complex has been better fleshed out, it will be interesting to determine if any of its lineages are susceptible to infection with any members of the *S. haematobium* group, or if *S. indicum* group parasites ever infect any (particularly Asian representatives of) bulinid snails. To our knowledge, such cross-infections have rarely been attempted (Agrawal and Rao, 2011). Wright (1971) exposed *I. exustus* from Socotra to *S. haematobium*, *S. bovis* and *S. mattheei*, without success.

Lastly, in considering schistosomes of the Indian subcontinent, at least three different species and maybe more (*S. spindale*, *S. indicum*, *S. turkestanicum* and relatives, possibly *Schistosoma* sp.) all inhabit the mesenteric veins of ruminants, creating opportunities for co-infections, and possibly hybridization (Agrawal and Rao, 2011). Ensuing competitive interactions may help to explain the predilection of *S. nasale* for its habitat in the nasal chambers. Does hybridization occur in the *S. indicum* group as commonly as now seems to be the case with the *S. haematobium* group (Rollinson et al., 1990; Webster, 2003; Webster et al., 2005; Huyse et al., 2009; Webster et al., 2013)? Although we saw no evidence for similar admixture in the *S. indicum* group, this was not the main objective of our work, and further study is needed. Studies of hybridization between *S. indicum* and S. *haematobium* group parasites are also warranted, particularly in areas of potential overlap in western Asia, as this might provide ways for more human-adapted worms to eventually be spread by *I. exustus*. As members of the *S. indicum* group

are frequently implicated in causing cercarial dermatitis in humans in southern Asia (Anantaraman, 1958; Narain et al., 1998; Agrawal et al., 2000), better characterization of the worms and snails involved in outbreaks may eventually help to alleviate this problem and highlight risk areas for possible emergence of human-adapted worms. With respect to where we began our studies of schistosomiasis in Nepal, it seems prudent to remain vigilant of the possibility of some of these worms adapting to and establishing patent infections in human hosts (Agrawal and Rao, 2011). Certainly there is no shortage of human contact with waters containing schistosome-infected snails.

Acknowledgments

This study was supported primarily by funds provided by the University of New Mexico to ESL to support travel and specimen collection for RD. Technical assistance and financial support at the UNM Molecular Biology Core Facility were provided by NIH grant P30GM110907 from the Institute Development Award program of the National Center for Research Resources and a National Science Foundation grant to SVB (DEB 1021427). We are grateful to the officials of the Department of National Parks and Wildlife Conservation, Chitwan National Park and the Nepal Health Research Council (permit no. 44) for their cooperation to carry out this research.

References

Agatsuma, T., Iwagami, M., Liu, C.X., Rajapakse, R.P.V.J., Mondal, M.M.H., Kitikoon, V., Ambu, S., Agatsuma, Y., Blair, D., Higuchi, T., 2002. Affinities between Asian non-human *Schistosoma* species, the *Schistosoma indicum* group, and the African human schistosomes. J. Helminthol. 76, 7-19.

Agrawal, M.C., 2012. Schistosomes and schistosomiasis in South Asia. Springer, India.

Agrawal, M.C., Gupta, S., George, J., 2000. Cercarial dermatitis in India. Bull. World Health Organ. 78, 278.

Agrawal, M.C., Rao, V.G., 2011. Indian schistosomes: a need for further investigations. J. Parasitol. Res. 2011, 1-4.

Albrecht, C., Kuhn, K., and Streit, B., 2007. A molecular phylogeny of Planorboidea (Gastropoda, Pulmonata): insights from enhanced taxon sampling. Zool. Scr. 36, 27-39.

Albrecht, C., Wilke, T., Kuhn, K., Streit, B., 2004. Convergent evolution of shell shape in freshwater limpets: the African genus *Burnupia*. Zool. J. Linnean Soc. 140, 577–586.

Anantaraman, M., 1958. On schistosome dermatitis: I. dermatitis in India caused by cercariae of *Schistosoma spindale* Montgomery, 1906. Indian J. Helminthol. 10, 46-52.

Attwood, S.W., Fatih, F.A., Mondal, M.M.H., Alim, M.A., Fadjar, S., Rajapakse, R.P.V.J., Rollinson, D., 2007. A DNA sequence based study of the *Schistosoma indicum* (Trematoda: Digenea) group: population phylogeny, taxonomy and historical biogeography. Parasitology 134, 2009-2020.

Attwood, S.W., Upatham, E.S., Meng, X.H., Qiu, D.-C., S. W., Southgate, V.R., 2002. The phylogeography of Asian *Schistosoma* (Trematoda: Schistosomatidae). Parasitology 125, 99-112.

Barker, S.C., Blair, D., 1996. Molecular phylogeny of *Schistosoma* species supports traditional groupings within the genus. J. Parasitol. 82, 292–298.

Brant, S.V., Loker, E.S., 2013. Discovery-based studies of schistosome diversity stimulate new hypotheses about parasite biology. Trends Parasitol. 29, 449-459.

Budha, P.B., Dutta, J., Daniel, B.A., 2012. *Indoplanorbis exustus*. The IUCN red list of threatened species. Version 2014.3. www.iucnredlist.org. Downloaded on 06 April 2015.

De Bont, J., Vercruysse, J., Van Aken, D., Southgate, V.R., Rollinson, D., 1991. Studies of the relationships between *Schistosoma nasale* and *S. spindale* and their snail host *Indoplanorbis exustus*. J. Helminthol. 65, 1-7.

DeJong, R.J., Morgan, J.A.T., Lobato, P.W., Pointier, J.-P., Amarista, M., Ayeh-Kumi P.F.K., Babiker, A., Barbosa, C.S., Bre´mond, P., Canese, A.P., de Souza C.P., Dominguez, C., File, S., Gutierrez, A., Incani, R.N., Kawano, T., Kazibwe, F., Kpikpi, J., Lwambo, N.J.S., Mimpfoundi, R., Njiokou, F., Poda, J.N., Sene, M., Vela´squez, L.E., Yong, M., Adema, C.M., Hofkin, B.V., Mkoji, G.M., Loker E.S., 2001. Evolutionary relationships and biogeography of *Biomphalaria* (Gastropoda: Planorbidae) with implications regarding its role as host of the human bloodfluke, *Schistosoma mansoni*. Mol. Biol. Evol. 18, 2225–2239.

Devkota, R., 2008. Inventory of trematode cercariae infections in freshwater snails in Chitwan and Nawalparasi districts and trematode infections in domestic elephants of Sauraha (minor study). A thesis submitted to Central Department of Zoology, Institute of Science and Technology, Tribhuvan University, Kirtipur, Kathmandu, Nepal pp.41.

Devkota, R., Brant, S.V., Thapa, A., Loker, E.S., 2014a. Sharing schistosomes: the elephant schistosome *Bivitellobilharzia nairi* also infects the greater one-horned rhinoceros (*Rhinoceros unicornis*) in Chitwan National Park, Nepal. J. Helminthol. 88, 32-40.

Devkota, R., Brant, S.V., Thapa, S., Loker, E.S., 2014b. Two avian schistosome cercariae from Nepal, including a *Macrobilharzia*-like species from *Indoplanorbis exustus*. Parasitol. Int. 63, 374-380.

Devkota, R., Budha, P.B., Gupta, R., 2011. Trematode cercariae infections in freshwater snails of Chitwan district, central Nepal. H. J. S. 7, 9-14.

Dutt, S.C. and Srivastava, H.D., 1968. Studies on *Schistosoma nasale* Rao, 1933, molluscan and mammalian host of the blood fluke. Indian Jour. Vet. Sci. and Anim. Husb. 38, 210-216.

Dutt, S.C., 1967. Studies on *Schistosoma nasale* Rao, 1933. I. Morphology of the adults, egg and larval stages. Indian J. Vet. Sci. 37, 249-262.

Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R., 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotechnol. 3, 294 - 299.

Frandsen, F., Christensen, N.O., 1984. An introductory guide to the identification of cercariae from African freshwater snails with special reference to cercariae of trematode species of medical and veterinary importance. Acta Trop. 41, 181 – 202.

Gupta, S., Agrawal, M.C., 2002. Comparative scanning electron microscopy of some Indian schistosomes. J. Parasitol. Appl. Biol. 11, 49-54.

Horák, P., Kolářová, L., Adema, C.M., 2002. Biology of the schistosome genus *Trichobilharzia.* Adv. Parasitol. 52, 155-233.

Huelsenbeck, J.P., Ronquist, F., 2001. MrBayes: Bayesian inference of phylogenetic tress. Bioinformatics 17, 754 – 755.

Huyse, T., Webster, B.L., Geldof, S., Stothard, J.R., Diaw, O.T., Polman, K., Rollinson, D., 2009. Bidirectional introgressive hybridisation between a cattle and human schistosome species. Plos Pathog. 5, e1000571.

Jørgensen, A., Madsen, H., Nalugwa, A., Nyakaana, S., Rollinson, D., Stothard, J.R., Kristensen, T.K., 2011. A molecular phylogenetic analysis of *Bulinus* (Gastropoda: Planorbidae) with conserved nuclear genes. Zool. Scr. 40, 126-136.

Kumar, V., deBurbure, G., 1986. Schistosomes of animals and man in Asia. Helminthological Abstracts (Series A) 55, 469-480.

Lawler, A., 2014. In search of green Arabia. Science. 345, 994-997.

Lawton, S.P., Hirai H., Ironside, J.E., Johnston, D.A., Rollinson, D., 2011. Genomes and geography: genomic insights into the evolution and phylogeography of the genus *Schistosoma*. Parasit. Vectors 4, 131.

Littlewood, D.T.J., Lockyer, A.E., Webster, B.L., Johnston, D.A., Le, T.H., 2006. The complete mitochondrial genomes of *Schistosoma haematobium* and *Schistosoma spindale* and the evolutionary history of mitochondrial genome changes among parasitic flatworms. Mol. Phylogenet. Evol. 39, 452-467.

Liu, L., Mondal, M.M.H., Idris, M.A., Lokman, H.S., Rajapakse, P.R.V.J., Satrija, F., Diaz, J.L., Upatham, E.S., Attwood, S.W., 2010. The phylogeography of *Indoplanorbis exustus* (Gastropoda: Planorbidae) in Asia. Parasit. Vectors 3, 57.

Lockyer, A.E., Olson, P.D., Østergaard, P., Rollinson, D., Johnston, D.A., Attwood, S.W., Southgate, V.R., Horak, P., Snyder, S.D., Le, T.H., Agatsuma, T., McManus, D.P., Carmichael, A.C., Naem, S., Littlewood, D.T.J., 2003. The phylogeny of the Schistosomatidae based on three genes with emphasis on the inter-relationships of *Schistosoma*Weinland, 1858. Parasitol. 126, 203–224.

Morgan, J.A.T., DeJong, R.J., Jung, Y., Khallaayoune, K., Kock S., Mkoji, G.M., Loker, E.S., 2002. A phylogeny of planorbid snails, with implications for the evolution of *Schistosoma* parasites. Mol. Phylogenet. Evol. 25, 477–488.

Morgan, J.A.T., DeJong, R.J., Kazibwe, F., Mkoji, G.M., Loker, E.S., 2003. A newly-identified lineage of *Schistosoma*. Int. J. Parasitol. 33, 977-985.

Narain, K., Rajguru, S.K., Mahanta, J., 1998. "Incrimination of *Schistosoma spindale* as a causative agent of farmer's dermatitis in Assam with a note on liver pathology in mice". The J. Commun. Dis. 30, 1–6.

Nesemann, H., Sharma, S., Sharma, G., Khanal, S.N., Pradhan, B., Shah, D.N., Tachamo, R.D., 2007. Aquatic invertebrates of the Ganga river system: Volume 1: Mollusca, Annelida, Crustacea (in part). Hasko Nesemann. 263 p

Oheimb, P.V.V., Albrecht, C., Riedel, F., Bössneck, U., Zhang, H., Wilke, T., 2013. Testing the role of the Himalaya Mountains as a dispersal barrier in freshwater gastropods (*Gyraulus* spp.) Biol. J. Linnean Soc. 109, 526-534.

Palumbi, S., Martin, A., Romano, S., McMillan, W.O., Stice, L., Grabowski, G., 1991. The simple fool's guide to PCR. Version 2.0. Honolulu, HL 96822: Department of Zoology and Kewalo Marine Laboratory, University of Hawaii. 1-45.

Rollinson, D., Southgate, V.R. 1987. The genus *Schistosoma*: a taxonomic appraisal. In the biology of Schistosomes. From Genes to Latrines (ed. Rollinson, D. and Simpson, A.J.G.) pp. 1-49. Academic Press, London

Rollinson, D., Southgate, V.R., Vercruysse, J., Moore, P.J., 1990. Observations on natural and experimental interactions between *Schistosoma bovis* and *Schistosoma curassoni* from West-Africa. Acta Trop. 47, 101–114.

Sato, Y., Le, T.H., Hiraike, R., Yukawa, M., Sakai, T., Rajapakse, R.P.V.J., Agatsuma, T., 2008. Mitochondrial DNA sequence and gene order of the Sri Lankan *Schistosoma nasale* is affiliated to the African/Indian group. Parasitol. Int. 57, 460-464.

Sherchand, J.B., Ohara, H., Sherchand, S., Matsuda, H., 1999. The suspected existence of *Schistosoma mansoni* in Dhanusa diatrict, southern Nepal By: Ann. Trop. Med. Parasitol. 93, 273-278.

Southgate, V.R., Rollinson, D., De Bont, J., Vercruysse, J., Van Aken, D., Spratt, J., 1990. Surface topography of the tegument of adult *Schistosoma nasale* Rao, 1933 from Sri Lanka. Syst. Parasitol. 16, 139-147.

Srivastava, H.D., Dutta, S.C., 1951. Life history of *Schistosoma indicum* Montgomery, 1906 – a common blood-fluke of Indian ungulates. Curr. Sci. 20, 273-275.

Stanyon, R., Sazzini, M., Luiselli, D., 2009. Timing the first human migration into eastern Asia. J. Biol. 8, 18.

Subba Rao, N.V., 1989. Hand book of freshwater molluscs of India. Zoological Survey of India, Calcutta.

Walker, J. C., 1988. Classification of Australian buliniform planorbids (Mollusca: Pulmonata). Rec. Aust. Mus. 40, 61–89.

Webster, B.L., 2003. On the interactions of *Schistosoma haematobium*, *Schistosoma guineensis* and their hybrids in the laboratory and in the field. PhD Thesis. University College London, London, United Kingdom.

Webster, B.L., Diaw, O.T., Seye, M.M., Webster, J.P., Rollinson, D., 2013. Introgressive hybridization of *Schistosoma haematobium* group species in Senegal: species barrier break down between ruminant and human schistosomes. PLOS Neg. Trop. Dis. 7, e2110.

Webster, B.L., Littlewood, D.T.J., 2012. Mitochondrial gene order change in *Schistosoma* (Platyhelminthes: Digenea: Schistosomatidae). Int. J. Parasitol. 42, 313-321.

Webster, B.L., Southgate, V.R., Littlewood, D.T., 2006. A revision of the interrelationships of *Schistosoma* including the recently described *Schistosoma guineensis*. Int. J. Parasitol. 36, 947-955.

Webster, B.L., Tchuem Tchuente´, L.A., Jourdane, J., Southgate, V.R., 2005. The interaction of *Schistosoma haematobium* and *S. guineensis* in Cameroon. J. Helminthol. 79, 193–197.

Wright, C.A., 1971. *Bulinus* on Aldabra and the subfamily Bulininae in the Indian Ocean area. Philos. Trans. R. Soc. London [Biol] 260, 299-313.

Supplementary Table and Figures

Supplementary Table 1. Detailed list of localities in Nepal sampled for freshwater snails from 2007 to 2014. The number of snails in parentheses indicates the number of *Indoplanorbis exustus* positive for *S. indicum* group schistosomes. The identifications for the schistosomes provided are derived from the phylogenetic analyses that follow, and that the designation "*Schistosoma* sp*."* reflects the genetic distinctiveness of these worms from the few other specimens of *S. indicum* in GenBank. Also, we were unable to obtain sequence data from three of our samples and these are referred to as "unidentified" in the table.

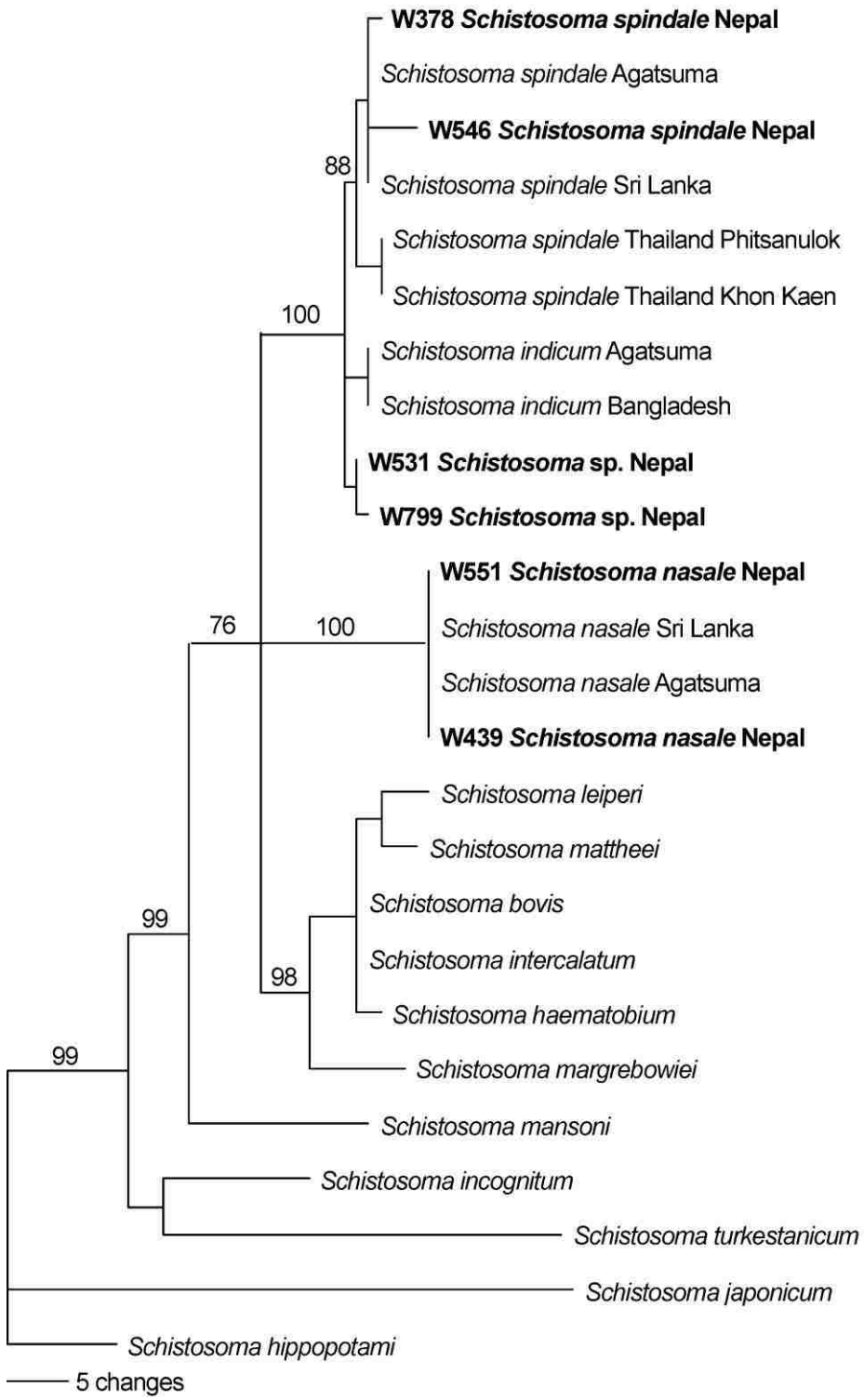
Locations	Number of snails examined	Co-ordinates	Collection date	Number of *Indoplanorbis* collected *	Species identity and W number
1. Amreni, Tanahu	65	N 27˚59'15.9", E 84˚16'58.2"	23rd July 2012	65	-
2. Baghmara Community forest, Chitwan	219	N 27˚35'22.0", E 84˚28'52.4"	22nd July 2012	156	-
3. Baruwa, Tamasariya-9, Nawalparasi	93	N 27˚34'54.51", E 84˚01'17.41"	23rd September 2007	51 (1)	*Schistosoma* sp. (W379)
4. Begnas Lake, Kaski	43	N 28˚09'58.00", E 84˚05'34.50"	23rd July 2012	14	-
5. Budhi Rapti River near Elephant Breeding Center, Chitwan	844	N 27˚34'57.8", E 84˚27'56.6"	8th October 2008	63	-
			2nd July 2010	5 (1)	S. s*pindale* (W525)
			11th May 2011	18 (1)	S. s*pindale* (W546)
			5th July 2012	18	
6. Chisapani Village, Godar-2/3, Dhanusa	153	N 26˚55'51.5", E 86˚08'45.8"	27th July 2012	1	-
7. Chitwan National Park, Chitwan	95	N 27˚33'15.8", E 84˚21'19.7"	28th February 2011	20	-
			13th May 2011	39	-
8. Chitwan National Park, Chitwan	147	N 27˚33'22.6", E 84˚29'5.40"	8th July 2010	22 (1)	*Schistosoma* sp. (W532)
9. Chitwan National Park, Chitwan	51	N 27˚32'43.1", E 84˚30'08.1"	8th July 2010	18	-
			3rd March 2011	6	-
			13th May 2011	5	-
10. Chitwan National Park, Chitwan	162	N 27˚33'59.3", E 84˚30'14.9"	6th July 2010	49	-
11. Chitwan National Park, Chitwan	422	N 27˚33'37.5", E 84˚29'24.5"	9th July 2010	162 (2)	*Schistosoma* sp. (W463, W528)
			6th July 2012	69	-

12. Chitwan National Park, Chitwan	359	N 27˚33’37.0”, E 84˚30’09.1”	23rd July 2010	244	-
13. Dhad Khola, Tulsi Chauda-2/3, Dhanusa	390	N 27˚00’57.75”, E 85˚55’27.00”	28th June 2012	14	-
14. Dhalkebar near Basai bridge, Dhanusa	75	N 26˚55’39.0”, E 85˚57’58.2”	25th June 2012	-	-
15. Dhumre river, Kumrose, Chitwan	1,753	N 27˚34’34.11”, E 84˚31’02.21”	4th July 2010	13	-
			5th February 2011	2	-
			28th February 2011	8	-
			3rd March 2011	5	-
			12th May 2011	274 (1)	*S. nasale* (W545)
			30th May 2011	65	-
			6th June 2011	90	-
			30th June 2012	17	-
			2nd July 2012	41	-
			5th July 2012	4	-
			22nd July 2012	28	-
			8th June 2014	7	-
			1st July 2014	20	
			17th July 2014	14	-
			31st July 2014	97	-
16. Fish Pond in Panchakanya Community Forest	339	N 27˚39’28.1”, E 84˚29’18.7”	29th January 2011	22	-
			27th May 2011	5	-
17. Ghansikuwa, Tanahu	183	N 28˚00’33.4”, E 84˚08’04.6”	23rd July 2012	15	-
18. Jagdishpur reservoir, Niglihawa VDC, Kapilvastu	802	N 27 36’59.67” E 83 05’49.36”	24th July 2012	17	
			5th July 2014	7	-
19. Jamunapur, jutpani-5, Chitwan	417	N 27˚39’42.48”, E 84˚30’57.43”	11th August 2007	156	-
			22nd September 2007	2	-
			5th October 2008	60	-
20. Jankauli, Bachhauli-7, Chitwan	419	N 27˚34’29.28”, E 84˚30’48.64”	26th September 2007	87 (1)	*S. spindale* (W378)
			28th July 2010	161	
21. Khageri river, Near Panchakanya Community Forest	837	N 27˚39’34.9”, E 84˚29’00.2”	1st June 2010	264	-
			29th January 2011	12	-
			29th May 2011	135	-
22. Kuchkuche Community forest, Kathar, Chitwan	183	N 27˚34’26.85”, E 84˚36’28.88”	3rd July 2012	30 (1)	*Schistosoma* sp. (W557)
			21st July 2012	18	-
23. Kuchkuche Community forest, near Rapti dam Kathar, Chitwan	340	N 27˚34’02.28”, E 84˚37’16.55”	3rd July 2012	89 (1)	S. *spindale* (W558)
			21st July 2012	65	-
24. Kudauli, Pithauli-7, Nawalparasi	155	N 27˚39’12.21”, E 84˚10’24.70”	23rd September 2007	101	-
25. Kumaraura, Dhanusa	135	N 26˚46’36.76”, E 85˚56’05.50”	9th January 2007	49 (1)	*S. nasale* (W439)
26. Kumrose, Chitwan	337	N 27˚34’03.42”, E 84˚32’22.87”	24th September 2007	28	-
27. Nawalpur,	325	N	28th June 2014	66	-

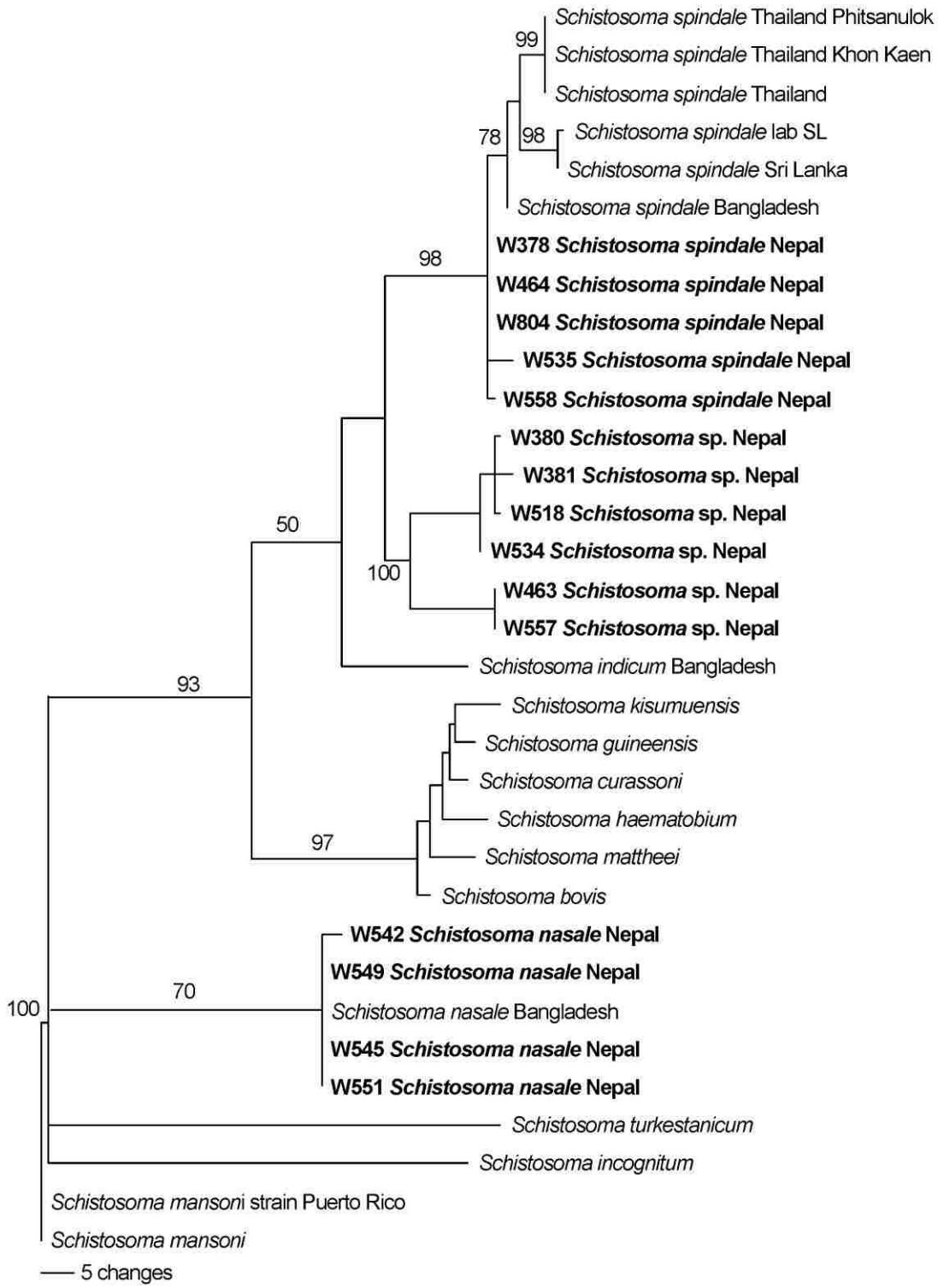
Hetauda, Makwanpur		27˚25'55.06", E 89˚58'57.02"			
28. Phewa Lake, Pokhara	59	N 28˚12'31.27, E 83˚57'19.42"	23rd July 2012	5	-
29. Pragatinagar-2, Nawalparasi	63	N 27˚40'04.72", E 84˚11'03.59"	23rd September 2007	63	-
30. Ramaidaiya Bhawadi village, Dhanusa	26	N 26˚49'26.31", E 85˚57'05.80"	9th January 2007	-	-
31. Rapti river, Ghailari, Jagatpur-1	27	N 27˚33'25.03", E 84˚20'02.48"	20th June 2010	21	--
32. Rapti river, Sauraha, Chitwan	296	N 27˚34'52.24", E 84˚28'56.12"	2nd July 2010	9	-
			11th May 2011	97	-
33. Rato river, Gauribash, west of Tulsi Chauda village, Dhanusa	181	N 27˚01'43.56", E 85˚55'21.30"	7th January 2007	1	-
34. Rice fields in Chisapani Village, Godar-2/3, Dhanusa	257	N 26˚56'26.09, E 86˚08'51.19"	8th January 2007	21	-
35. Shishuwar bagar, Bachhauli-3, Chitwan	3,023	N 27˚35'31.76", E 84˚30'00.31''	21st September 2007	34	-
			26th September 2007	33 (8)	4 *Schistosoma* sp. (W380, W381, W383,W534), *S. spindale* (W535), 3 unidentified (W384, W385, W533)
			10th July 2008	11	-
			8th October 2008	63	-
			27th July 2009	27 (1)	*Schistosoma* sp. (W541)
			2nd July 2010	94	
			4th July 2010	30	-
			26th July 2010	280 (1)	*Schistosoma* sp. (W531)
			12th August 2010	60	-
			30th May 2011	42	-
			6th June 2011	68	-
			4th August 2011	114	-
			14th August 2011	107	-
			30th June 2012	3	-
			4th July 2012	138 (1)	*S. spindale* (W556)
			1st July 2014	28	-
			10th July 2014	79	-
			17th July 2014	110	-
36. Small canal in Sauraha, Chitwan	373	N 27˚34'59.55", E 84˚29'39.44''	25th September 2007	53	-
			3rd July 2010	117	-
37. Tikauli marshy land, Ratnanagar-7, Chitwan	95	N 27˚37'12.66", E 84˚28'13.87"	24th September 2007	5	-
38. Tikauli, Ratnanagar-7, Chitwan	4, 674	N 27˚37'45.52", E 84˚29'21.57"	21st September 2007	47 (3)	*Schistosoma* sp. (W517, W518, W519)
			23rd September 2007	42	
			14th July 2008	14 (1)	*Schistosoma* sp. (W550)
			6th October 2008	100 (1)	*S. nasale*, (W549)
			20th June 2010	60	-
			4th July 2010	55 (2)	*S. spindale* (W464,

					W538)
			22nd July 2010	17 (1)	*S. nasale* (W551)
			11th August 2010	60	-
			11th March 2011	23	-
			18th March 2011	91	-
			16th April 2011	91	-
			23rd April 2011	67	-
			2nd May 2011	47	-
			22nd June 2011	98 (1)	*S. nasale* (W542)
			9th August 2011	116	-
			1st July 2012	128	-
			22nd June 2014	129	-
			15th July 2014	187	-
			20th July 2014	141	-
			29th July 2014	201(1)	*S. spindale* (W804)
			1st August 2014	176 (2)	*Schistosoma* sp. (W798, W799)
39. Tulsi Chauda village, Dhanusa	357	N 27˚00'50.37", E 85˚55'31.80"	7th January 2007	6	-
40. Twenty thousand lake, Chitwan	586	N 27˚36'54.0", E 84˚26'19.9"	24th September 2007	52	-
			12th October 2008	108	-
			22nd June 2010	33	-
			24th July 2010	87	-
			6th March 2011	7	-
			2nd June 2011	213	-
			4th July 2012	64	-

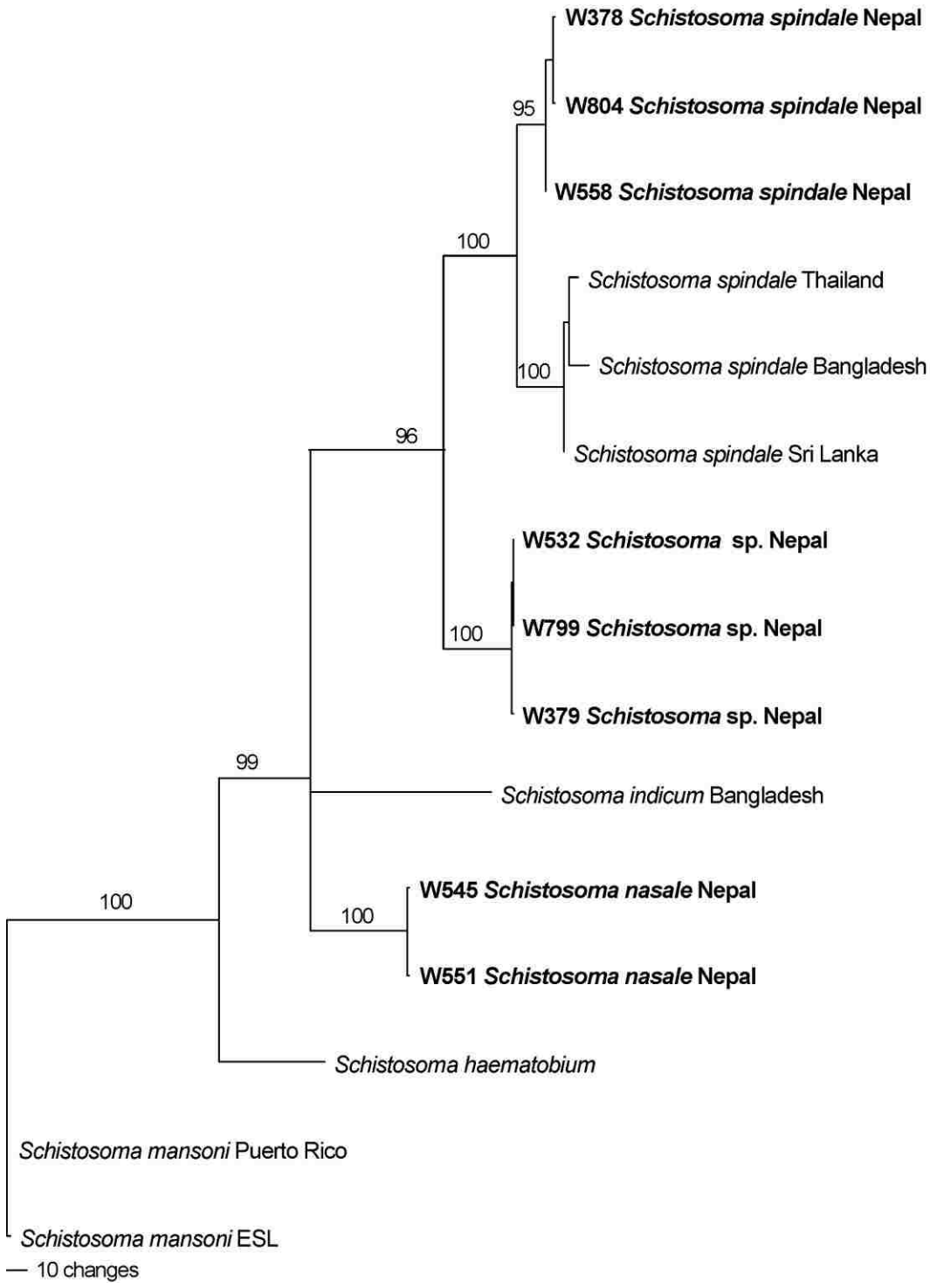
*The numbers in parentheses indicate the number of *Indoplanorbis* individuals positive for schistosomes at that particular location.



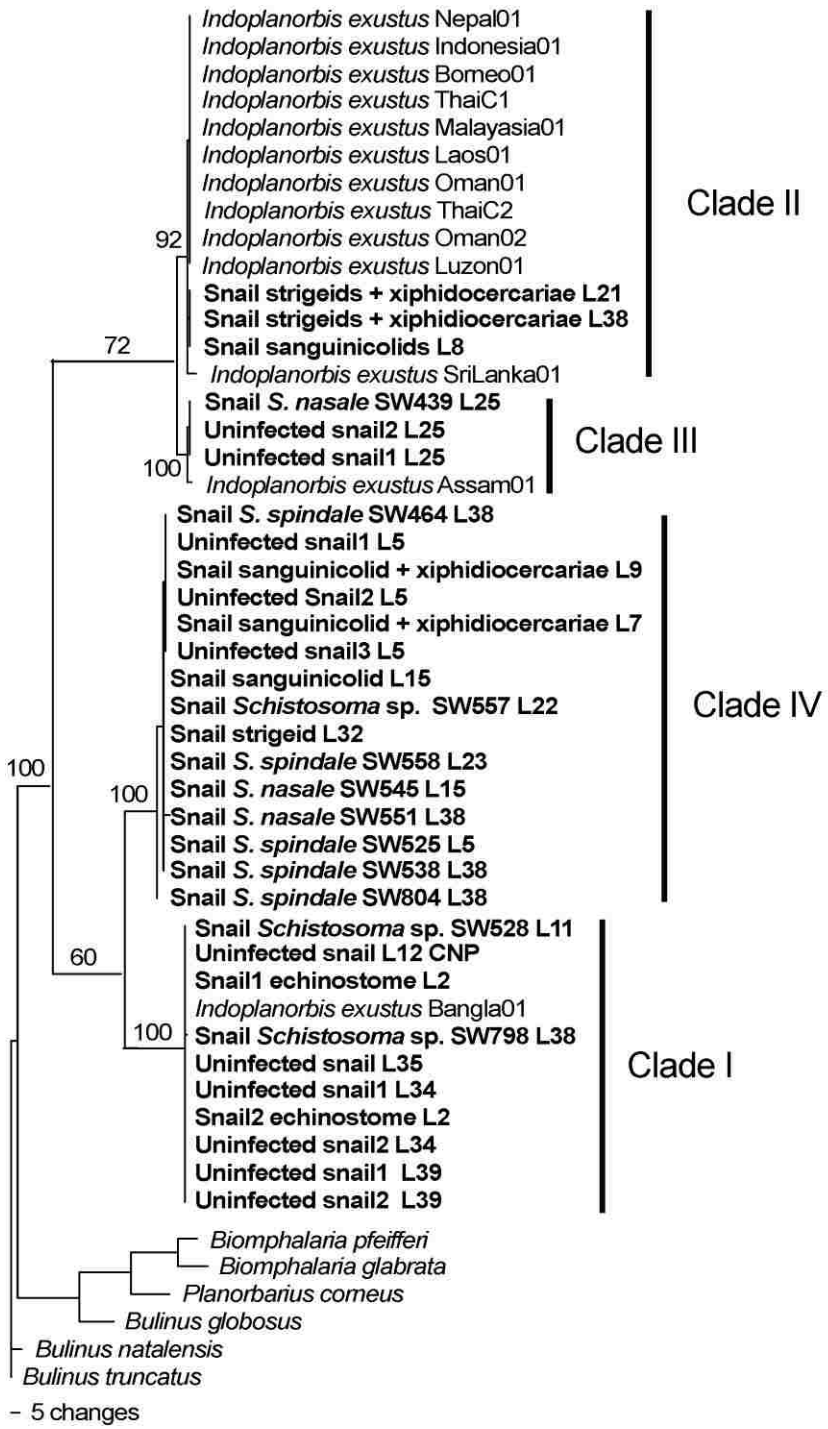
Supplementary Figure 1. Bayesian phylogenetic tree based on *Schistosoma* 28S sequences (477 bp). Samples in bold are those collected in this study. Node support is indicated by Bayesian posterior probabilities.



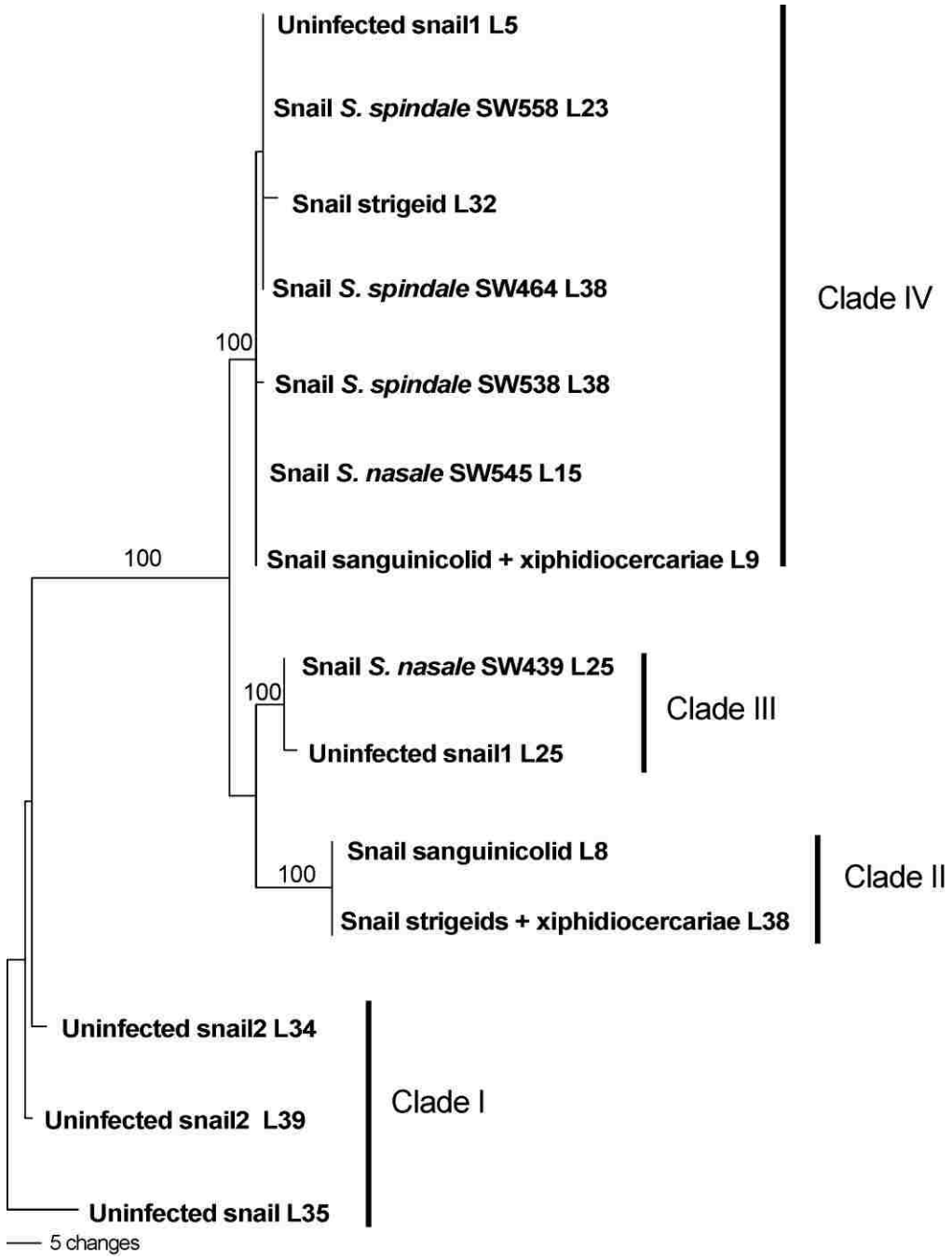
Supplementary Figure 2. Bayesian phylogenetic tree based on *Schistosoma* 12S sequences (386 bp). Samples in bold are those collected in this study. Node support is indicated by Bayesian posterior probabilities.



Supplementary Figure 3. Bayesian phylogenetic tree based on *Schistosoma* 16S sequences (649 bp). Samples in bold are those collected in this study. Node support is indicated by Bayesian posterior probabilities.



Supplementary Figure 4. Bayesian phylogenetic tree based on *Indoplanorbis* 16S sequences (420 bp). Samples in bold are those collected in this study. Node support is indicated by Bayesian posterior probabilities. The "L" followed by a number designates the location number indicated in Table 1.



Supplementary Figure 5. Bayesian phylogenetic ingroup analysis based on *Indoplanorbis* ITS1 sequences (834 bp includes parts of adjacent 18S and 5.8S genes). All the samples in analysis are collected in this study. Node support is indicated by Bayesian posterior probabilities. The "L" followed by a number designates the location number indicated in Table 1.

CHAPTER FIVE

A genetically distinct lymnaeid-transmitted *Schistosoma* from Nepal related to *S. turkestanicum*

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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 mammalian schistosome cercariae. None of the 1,380 specimens of *Radix acuminata* we collected were positive for this or any other schistosome. Analysis of 28S, *cox*1, 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*, this Nepalese cercaria 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.

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.

As part of a survey study directed mostly towards collection of schistosome cercariae from snails and secondarily, of schistosome eggs from some mammal species in the Terai region of southern Nepal, the presence of *Bivitellobilharzia nairi* in both Asian elephants and the Asian one horned rhino was documented (Devkota et al., 2014). 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, submitted). We were surprised to find a third lineage of *I. exustus*-transmitted schistosome from Nepal. It clustered with *S. spindale* yet was distinct from it or from the species for which the group is named, *S. indicum*. This study suggested that surprises may lie ahead, and more information is needed before the full picture is revealed with respect to mammalian schistosome diversity in Asia.

As part of our snail collections, we also 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 has 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 apparently from *R. luteola* (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 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 other mammalian schistosomes based on 28S, *cox*1, 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

Snails were collected from 40 freshwater habitats in different areas of Nepal (see Chapter 4 for details). 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.

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. Amplification of the nuclear ribosomal 28S and mitochondrial cytochrome oxidase I (*cox*1), 12S and 16S genes were done by polymerase chain reaction using Takara Ex Taq kit (Takara Biomedicals, Otsu, Japan) and previously published primers for the first three genes (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 *cox*1 sequences and 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’) (Attwood et al., 2002; Lockyer et al., 2003; Morgan et al., 2003). New primers (R16SF 5' - TGT TTT TTT CCK ATG CAT TA - 3' and R16SR 5' - GGC TTA CAC CGG TCT TAA CT - 3') were designed to amplify the 16S genes. 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 acumunata* 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 (*cox*1) 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 *cox*1 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 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 manufacturers' guidelines. Sequencing reactions were performed with Applied Biosystems BigDye direct sequencing kit, version 3.1 (Applied Biosystems, Foster City, California).

Phylogenetic analysis of schistosome cercariae was performed with the 28S, 12S, 16S and *cox*1 gene fragments using Bayesian inferences (BI) with MrBayes v 3.1.2 (Huelsenbeck and Ronquist, 2001). For the analysis of the 28S dataset, the parameters were unlinked: Nst = 6 rates = invgamma ngammacat = 4. For the BI analysis of the *cox*1

dataset, the parameters were unlinked; for codons one and two Nst = 2 and for codon three Nst = 6 rates = gamma, ngammacat = 4. For both 28S and *cox*1 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 *cox*1, 12S, 16S and 28S schistosome datasets included members of the related *S. japonicum* species groups. DNA sequence data were deposited in GenBank (See Table 3). The remaining taxa used in the tree were obtained from literature already published.

For analyses centered on the snail host *Radix luteola*, two mitochondrial genes, the 16S and *cox*1 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 *cox*1 sequences and KT160299-KT160308 for 16S sequences).

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 mammalian schistosomes.

S. N.	Locations	Co-ordinates	Number of snails collected	
			Radix luteola	*Lymnaea acuminata*
1	Baghmara Community forest, Chitwan	N 27˚35'22.0", E 84˚28'52.4"	3	-
2	Begnas Lake, Kaski	N 28˚09'58.00", E 84˚05'34.50"	-	2
3	Budhi Rapti River near Elephant Breeding Center, Chitwan	N 27˚34'57.8", E 84˚27'56.6"	42	27
4	Chisapani Village, Godar-2/3, Dhanusa	N 26˚55'51.5", E 86˚08'45.8"	7	-
5	Chitwan National Park, Chitwan	N 27˚33'22.6", E 84˚29'5.40"	9	9
6	Chitwan National Park, Chitwan	N 27˚32'43.1", E 84˚30'08.1"	3	-
7	Chitwan National Park, Chitwan	N 27˚33'59.3", E 84˚30'14.9"	57	37
8	Chitwan National Park, Chitwan	N 27˚33'37.5", E 84˚29'24.5"	16	24
9	Chitwan National Park, Chitwan	N 27˚33'37.0", E 84˚30'09.1"	71	42
10	Dhad Khola, Tulsi Chauda-2/3, Dhanusa	N 27˚00'57.75", E 85˚55'27.00"	108	58
11	Dhumre river, Kumrose, Chitwan	N 27˚34'34.11", E 84˚31'02.21"	750(10)	265
12	Fish Pond in Panchakanya Community Forest	N 27˚39'28.1", E 84˚29'18.7"	-	66
13	Ghansikuwa, Tanahu	N 28˚00'33.4", E 84˚08'04.6"	8	79
14	Jagdishpur reservoir, Niglihawa VDC, Kapilvastu	N 27 36'59.67" E 83 05'49.36"	-	27
15	Jamunapur, jutpani-5, Chitwan	N 27˚39'42.48", E 84˚30'57.43"	51	-
16	Jankauli, Bachhauli-7, Chitwan	N 27˚34'29.28", E 84˚30'48.64"	141(1)	-
17	Khageri river, Near Panchakanya Community Forest	N 27˚39'34.9", E 84˚29'00.2"	61	93
18	Kuchkuche Community forest, Kathar, Chitwan	N 27˚34'26.85", E 84˚36'28.88"	25	-
19	Kuchkuche Community forest, near Rapti dam Kathar, Chitwan	N 27˚34'02.28", E 84˚37'16.55"	-	4
20	Kumaraura, Dhanusa	N 26˚46'36.76", E 85˚56'05.50"	45	
21	Kumrose, Chitwan	N 27˚34'03.42", E 84˚32'22.87"		
22	Nawalpur, Hetauda, Makwanpur	N 27˚25'55.06", E 89˚58'57.02"	-	115
23	Phewa Lake, Pokhara	N 28˚12'31.27, E 83˚57'19.42"	1	-
24	Ramaidaiya Bhawadi village, Dhanusa	N 26˚49'26.31", E 85˚57'05.80"	3	
25	Rapti river, Sauraha, Chitwan	N 27˚34'52.24", E 84˚28'56.12"	43	99
26	Rato river, Gauribash, west of Tulsi Chauda village, Dhanusa	N 27˚01'43.56", E 85˚55'21.30"	135	21
27	Rice fields in Chisapani Village, Godar-2/3, Dhanusa	N 26˚56'26.09, E 86˚08'51.19"	55	4
28	Shishuwar bagar, Bachhauli-3, Chitwan	N 27˚35'31.76", E 84˚30'00.31''	573(3)	3
29	Tikauli, Ratnanagar-7, Chitwan	N 27˚37'45.52", E 84˚29'21.57"	261(2)	344
30	Tulsi Chauda village, Dhanusa	N 27˚00'50.37", E 85˚55'31.80"	108	58
31	Twenty thousand lake, Chitwan	N 27˚36'54.0", E 84˚26'19.9"	12	3

3. Results

From 2007 to 2014, we collected and screened 1,380 *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 mammalian-type (lacking eyespots) 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%). *R. acuminata* also shed echinostome (0.29%) and clinostomoid cercariae (0.14%).

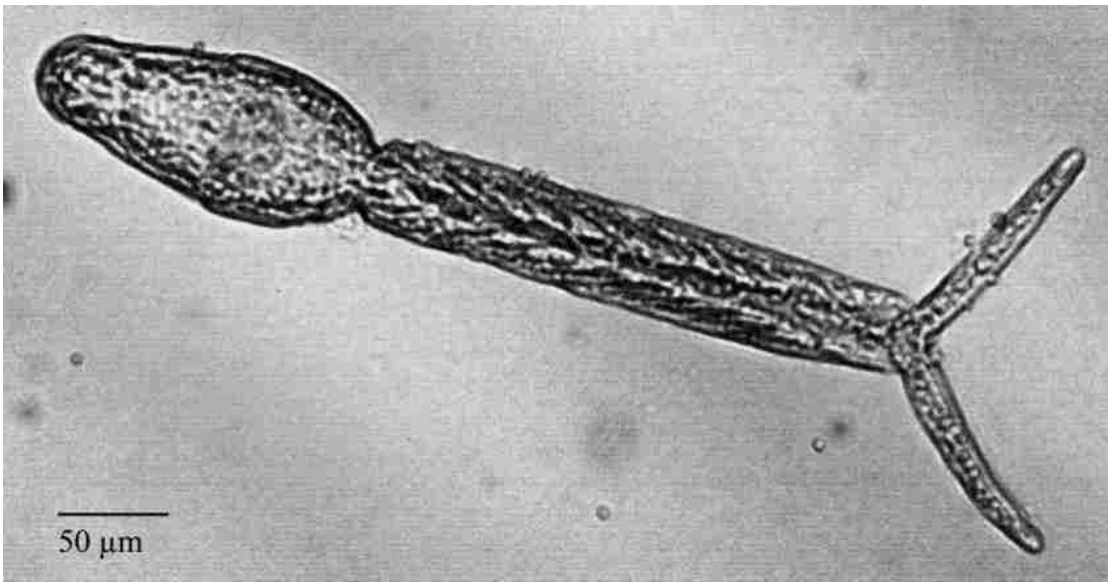

Figure 1. Schistosome cercaria obtained from *Radix luteola.*

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 *Radix* 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)	*Radix* sp.	This study
S. turkestanicum (Heat-killed)	178	62	187	22.6	45	13	*R. auricularia*	Machattie, 1936
S. bomfordi	-	-	-	-	-	-	*Radix luteola*	Montgomery , 1906
S. dattai (Heat-killed)	135-200 (168)	44-65 (58)	210-316 (258)	26-39 (32)	71-116 (97)	-	*Radix luteola*	Dutta and Srivastava, 1962
S. harinasutai (Hot formalin-fixed)	150-220	40-70	240-350	25-40	45-100	15-25	*Lymnaea rubiginosa*	Kruatrachue et al., 1965
S. incognitum (Heat killed)	157-207	42-71	185-307	28-49	85-135	-	*Lymnaea luteola*	Sinha and Srivastava, 1960
Schistosoma 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. exustus*	Devkota et al., unpublished
S. spindale (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. exustus*	Devkota et al., unpublished
S. nasale (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. exustus*	Devkota et al., unpublished

Measurements of the schistosome cercariae from Nepalese *R. luteola* are 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 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	*cox*1 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	-
Schistosoma turkestanicum	EU702749 (Hungary), AF167092, AY157254 (Iran), AJ313461, EU436659 (China)	HQ283100	EU221230, EU221231, EU436657, EU177877 (China), AY157200 (Iran), JX467182 (Hungary)	-
S. kisumuensis	FJ897155	-	-	-
S. bovis	AY157266	FJ897168	FJ897160	-
S. intercalatum	AY157262	AJ419779	AJ519519	-
S. haematobium	AY157263	DQ157222	DQ157222	-
S. mattheei	AY157265	AJ419789	AJ519518	-
S. curassoni	-	AJ419786	-	
S. leiperi	AY157261	-	AY157207	-
S. mansoni	AY157173	HE601612	HE601612	-
S. rodhaini	AY157256	-	AY157202	-
S. incognitum	JQ408705	EF534279	AY157201	-
S. edwardiense	AY197344	-	AY197347	-
S. hippopotami	AY197343	-	AY197346	-
S. japonicum	Z46504	KP793878	EU340357	-
S. mekongi	AY157253	AF217449	AF217449	-
S. sinensium	AY157251	AF465918	AY157197	-
Bivitellobilharzia loxodontae	JN579949	-	-	-
B. nairi	AY858888	-	-	-
Schistosoma indicum	AY157258	EF534276	AY157204	-
S. spindale	Z46505	EF534281	AY157203	-
S. nasale	AY157259	EF534280	AY157205	-

We attempted to obtain *cox*1, 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 closely-related schistosomes especially *S. turkestanicum* from elsewhere in Asia or Europe, and the lyamnaeid-transmitted *S. incognitum* from India, Bangladesh, Thailand and Indonesia.

First, regarding the analysis of *cox*1 schistosome sequences subjected to Bayesian analysis (Figure 2), our *R. luteola*-derived schistosomes always clustered together with, but distinct from, *Schistosoma turkestanicum*, with strong support. Our *cox*1 sequences differed from those of *S. turkestanicum* by 19-20%. A similar tree topology was also found for other genes (28S- Figure 3 and 12S- Figure 4) subjected to similar analyses.

Regarding the phylogenetic position of the Nepalese lymnaeids we collected, sequences for *cox*1 were obtained from 5 specimens of *R. acuminata* and 6 specimens of *R. luteola*, including 3 specimens that were infected with schistosomes (Figure 5). 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 cox*1 sequences with other sequences for the same species as there are no *R. luteola cox*1 sequences in GenBank.

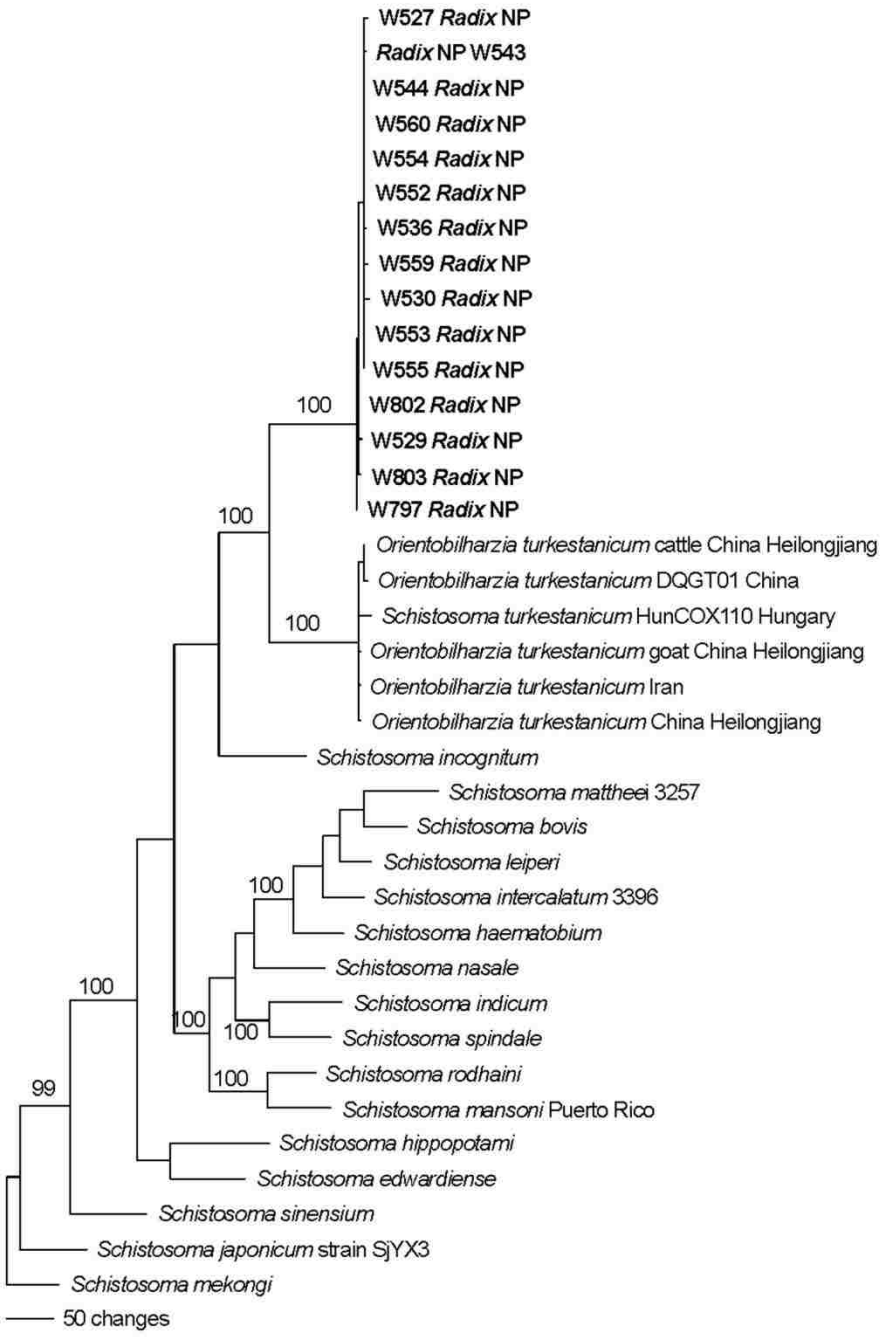

Figure 2. Bayesian phylogenetic tree based on Schistoeome cercariae collected from *Radix luteola cox*1 (1029 bp) sequences. Samples in bold are those collected in this study. Node support is indicated by Bayesian posterior probabilities (PP).

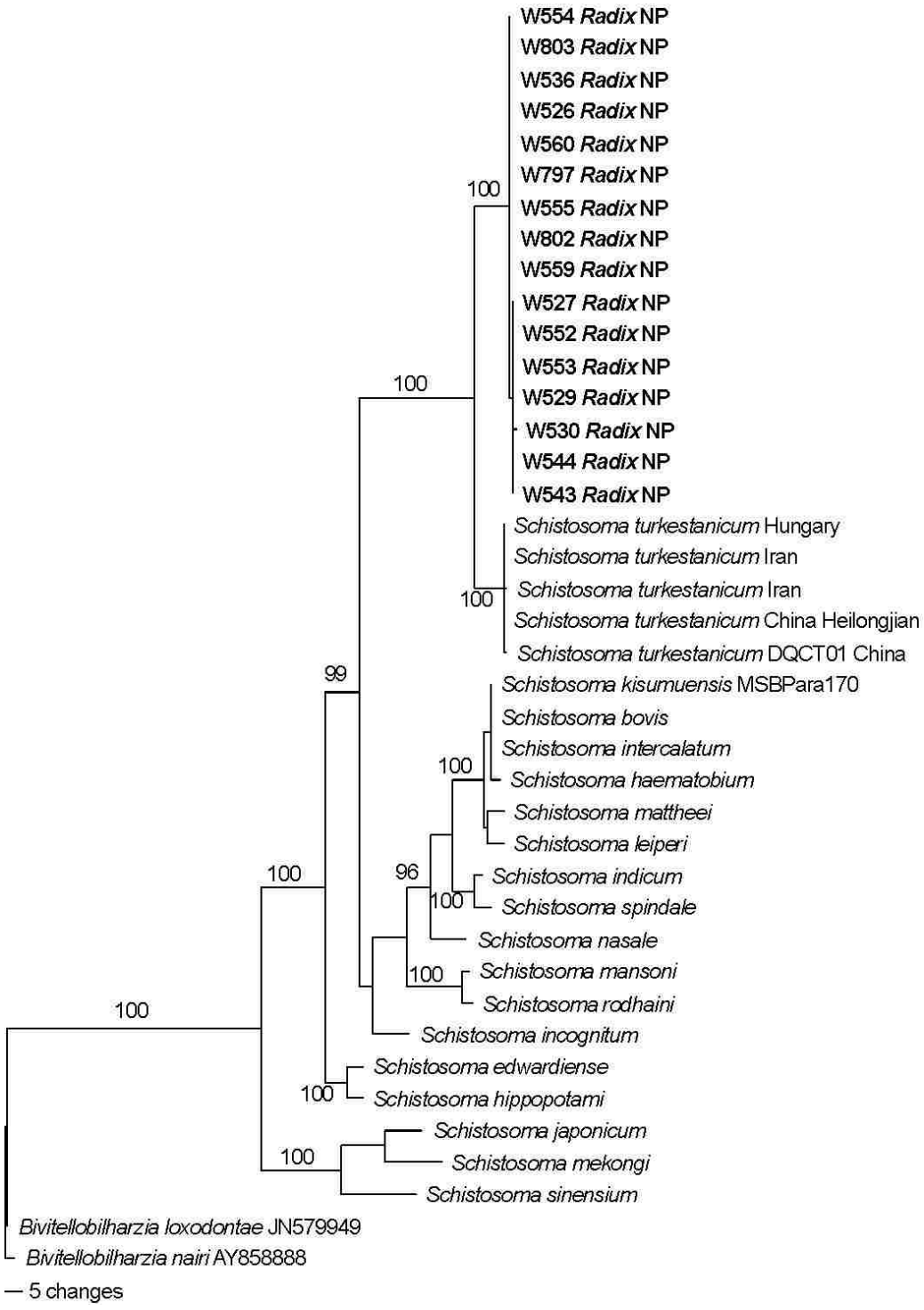

Figure 3. Bayesian phylogenetic tree based on Schistoeome cercariae collected from *Radix luteola* 28S (1125 bp) sequences. Samples in bold are those collected in this study. Node support is indicated by Bayesian posterior probabilities (PP).

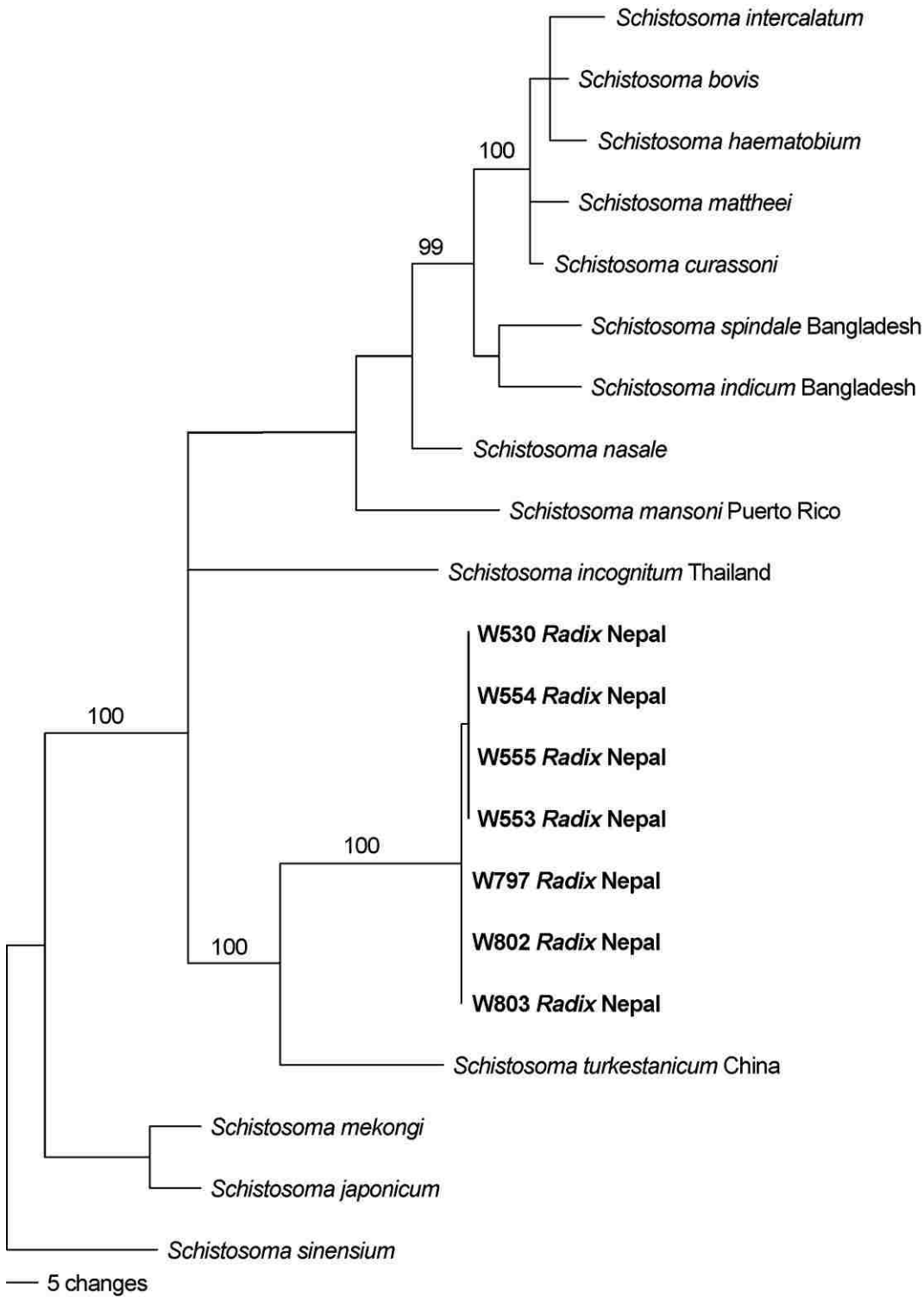

Figure 4. Bayesian phylogenetic tree based on Schistoeome cercariae collected from *Radix luteola* 12S sequences. Samples in bold are those collected in this study. Node support is indicated by Bayesian posterior probabilities (PP).

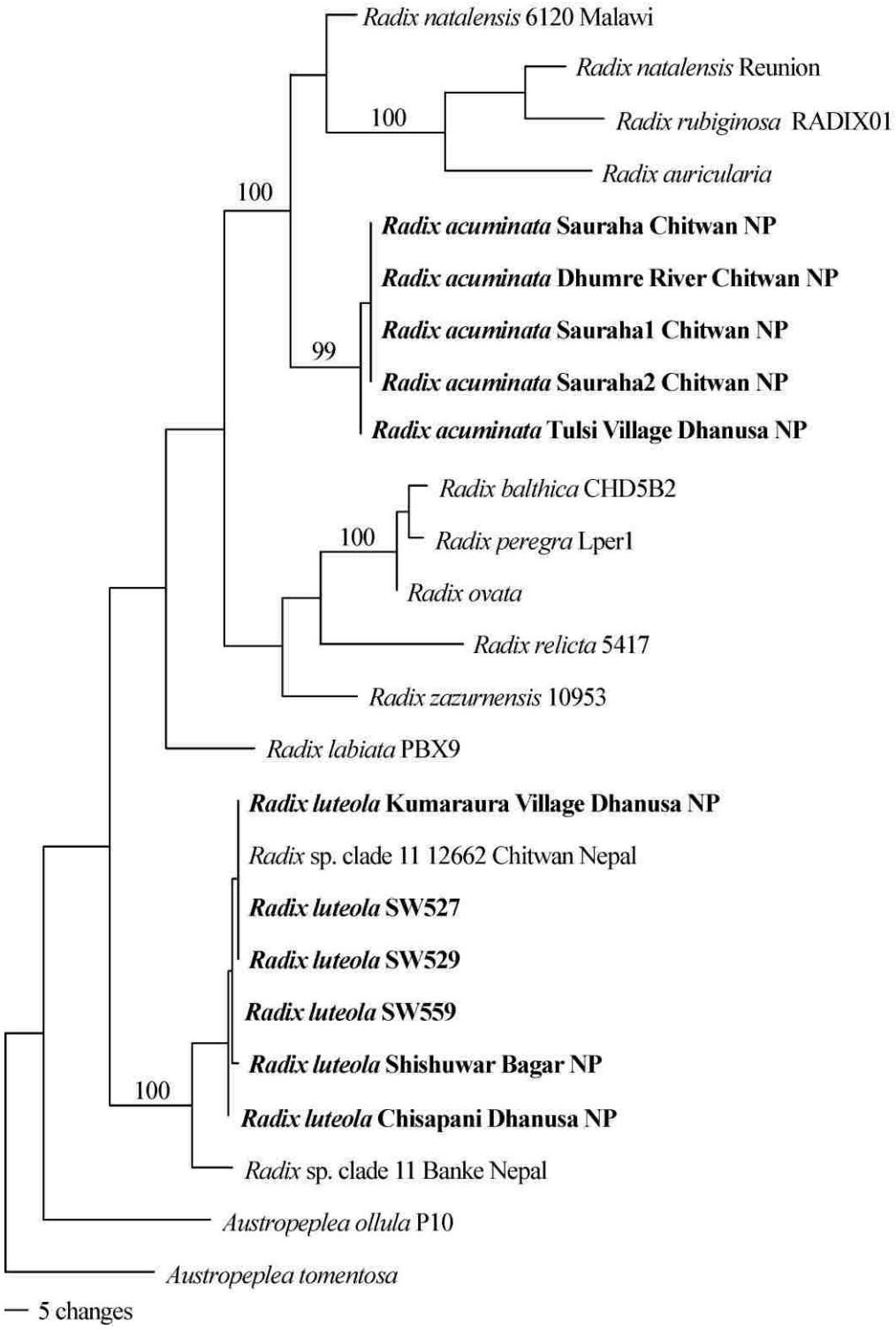

Figure 5. Bayesian phylogenetic tree based on *Radix* snail *cox*1 (482 bp) sequences. Samples in bold are those collected in this study. Node support is indicated by Bayesian posterior probabilities (PP).

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 6). 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 distantly removed on the tree from 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 R*é*union Island.

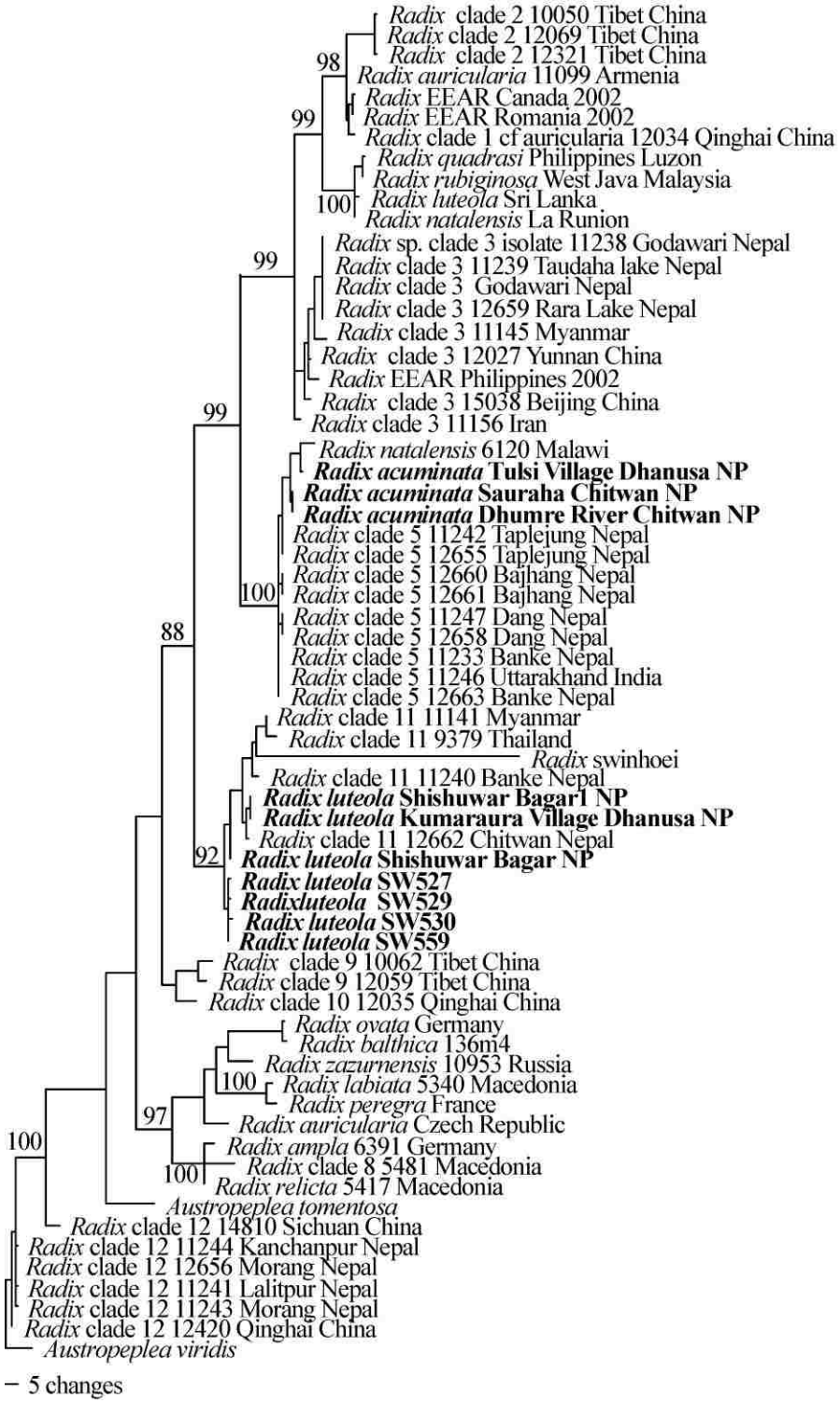

Figure 6. Bayesian phylogenetic tree based on *Radix* snail 16S sequences. Samples in bold are those collected in this study. Node support is indicated by Bayesian posterior probabilities (PP).

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 others are *Bivitellobilharzia nairi* (Devkota et al., 2014), and three members of the *S. indicum* species group, *S. spindale*, *S. nasale*, and a previously unknown genetically distinct lineage termed *Schistosoma* sp. (Devkota et al., submitted). The snail 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 are parasites of artiodactylids, mostly domestic ruminants like water buffalo, cattle and goats as best as is known. They 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.

The lymnaeid-transmitted *Schistosoma* we report here is a close relative of *S. turkestanicum*, a geographically widespread Asian schistosome. *S. turkestanicum* is the only species among four formerly placed in the genus *Orientobilharzia* for which sequence data are available. These four species, now all formally transferred to *Schistosoma* (Aldhoun and Littlewood, 2012), comprise what might be called an *S. turkestanicum* species group, and are *S. turkestanicum, S.dattai, S. bomfordi* and *S. harinasutai.* In our opinion, a likely possibility is that our Nepalese specimens are of either *S. bomfordi* or *S. dattai*. The former species was reported from cattle from

Muktesar (now 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 *cox*1 gene of 15 different isolates schistosomes from *R. luteola* we sequenced was minimal (0-1%), and reminiscent of *S. nasale* in this regard (Chapter 4). 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* 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 Devkota et al. (Chapter 4) and Agrawal and Rao (2011), 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, the *R. luteola* (Figure 7A), was found infected with schistosomes. Based on analysis of 16S and *cox*1 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 7B) grouped with "*Radix* sp. Clade 5" from Taplejung Nepal or Uttarakhand, India reported by von Oheimb et al., (2011).

Figure 7. *Radix* snails from Nepal. A) A specimen of *Radix luteola* that was shedding the schistosome cercariae discussed in this paper and for which sequence data were obtained. B) A specimen of *Radix acuminate*.

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 like *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.

Acknowledgments

This study was supported primarily by funds provided by the University of New Mexico to ESL to support travel and specimen collection for RD. Technical assistance and financial support at the UNM Molecular Biology Core Facility were provided by NIH grant P30GM110907 from the Institute Development Award program of the National Center for Research Resources and a National Science Foundation grant to SVB (DEB 1021427). We are grateful to the officials of the Department of National Parks and Wildlife Conservation, Chitwan National Park and the Nepal Health Research Council (permit no. 44) for their cooperation to carry out this research.

References

Agrawal, M.C. and Rao, V.G. 2011. Indian schistosomes: a need for further investigations. Journal of Parasitology Research. 2011: doi:10.1155/2011/250868

Aldhoun, J.A. and Littlewood, D.T.J. 2012. *Orientobilharzia* Dutt and Srivastava, 1955 (Trematoda: Schistosomatidae), a junior synonym of *Schistosoma* Weinland, 1858. Systematic Parasitology 82: 81-88.

Attwood, S.W., Fatih, F.A., Mondal, M.M.H., Alim, M.A., Fadjar, S., Rajapakse, R.P.V.J., Rollinson, D., 2007. A DNA sequence based study of the *Schistosoma indicum* (Trematoda: Digenea) group: population phylogeny, taxonomy and historical biogeography. Parasitology 134: 2009-2020.

Attwood, S.W., Upatham, E.S., Meng, X.H., Qiu, D.-C., S. W., Southgate, V.R., 2002. The phylogeography of Asian *Schistosoma* (Trematoda: Schistosomatidae). Parasitology 125: 99-112.

Baugh, S.C. 1978. A century of schistosomiasis in India: human and animal. Revista Iberica de Parasitologica 38: 435-472.

Brant, S.V., Pomajbikova, K., Modry, D., et al. 2013. Molecular phylogenetics of the elephant schistosome *Bivitellobilharzia loxodontae* (Trematoda: Schistosomatidae) from the Central African Republic. Journal of Helminthology 87: 102-107.

Brant, S.V and Loker, E.S. 2009. Molecular systematics of the avian schistosome genus *Trichobilharzia* (Trematoda: Schistosomatidae) in North America. Journal of Parasitology 95: 941-963.

Correa C. A., Escobar J. S., Durand P., Renaud F., David P., Jarne P., Pointier J.-P. & Hurtrez-Boussès S. (2010). Bridging gaps in the molecular phylogeny of the Lymnaeidae (Gastropoda: Pulmonata), vectors of Fascioliasis. BMC Evolutionary Biology 10: 381. doi:10.1186/1471-2148-10-381.

Davis, N.E. 2006. Identification of an avian schistosome recovered from *Aythya novaeseelandia* and infectivity of its miracidia to *Lymnaea tomentosa* snails. Journal of Helminthology 80: 225-233

Devkota, R., Brant, S.V., Loker, E.S. The *Schistosoma indicum* species group in Nepal: presence of a new lineage of schistosome and use of the *Indoplanorbis exustus* species complex of snail hosts. International Journal for Parasitology: submitted.

Devkota, R., Brant, S.V., Thapa, A., Loker, E.S. 2014. Sharing schistosomes: the elephant schistosome *Bivitellobilharzia nairi* also infects the greater one-horned rhinoceros (*Rhinoceros unicornis*) in Chitwan National Park, Nepal. Journal of Helminthology 88: 32-40.

Dutt, S.C. and Srivastava, H.D. 1952. On the morphology and life history of a new mammalian blood-fluke *Ornithobilharzia dattai* n. sp. (preliminary report). Parasitology 42: 144-150.

Dutt, S.C. and Srivastava, H.D. 1961. Morphology and life history of the mammalian blood fluke *Orientobilharzia dattai* (Dutt And Srivastava). I. Morphology of the adult, egg and miracidium. Indian Journal of Veterinary Science and Animal Husbandry 31: 288-303.

Dutt, S.C. and Srivastava, H.D. 1962a. Biological studies of *Orientobilharzia dattai* (Dutt and Srivastava, 1952) Dutt and Srivastava (1955) (Trematoda: Schistosomatidae). Indian Journal of Helminthology 13: 61-73.

Dutt, S.C. and Srivastava, H.D.1962b. Studies on the morphology and life history of the mammalian blood fluke *Orientobilharzia dattai* (Dutt and Srivastava) Dutt and Srivastava. II. The molluscan phases of the life-cycle and the intermediate host specificity. Indian Journal of Veterinary Science and Animal Husbendary 32: 33-43.

Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R., 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3: 294-299.

Frandsen, F. and Christensen, N.O. 1984. An introductory guide to the identification of cercariae from African freshwater snails with special reference to cercariae of trematode species of medical and veterinary importance. Acta Tropica 41: 181-202.

Glöer P. and Bössneck U. 2013. Freshwater molluscs from Nepal and North India with the description of seven new species (Gastropoda: Bithyniidae, Lymnaeidae, Planorbidae). Archiv für Molluskenkunde 142: 137-156.

Huelsenbeck, J.P., Ronquist, F., 2001. MrBayes: Bayesian inference of phylogenetic tress. Bioinformatics 17: 754-755.

Khalil, L.F. 2002. Family Schistosomatidae Stiles & Hassall, 1898. In: D.I. Gibson, A. Jones, and R.A. Bray (Eds) Keys to the Trematoda. Wallingford, UK: CABI Publishing, pp. 419-432.

Kruatrachue, M., Bhaibulaya, M., and Harinasuta, C. 1965. *Orientobilharzia harinasutai* sp. nov., a mammalian blood-fluke, its morphology and life cycle. Annals of Tropical Medicine and Parasitology 59: 181-188.

Kumar, V., deBurbure, G., 1986. Schistosomes of animals and man in Asia. Helminthological Abstracts (Series A) 55: 469-480.

Lockyer, A.E., Olson, P.D., Østergaard, P., Rollinson, D., Johnston, D.A., Attwood, S.W., Southgate, V.R., Horak, P., Snyder, S.D., Le, T.H., Agatsuma, T., McManus, D.P., Carmichael, A.C., Naem, S., Littlewood, D.T.J., 2003. The phylogeny of the Schistosomatidae based on three genes with emphasis on the inter-relationships of *Schistosoma*Weinland, 1858. Parasitology 126: 203-224.

Machattie, C., 1936. A preliminary note on the life history of *Schistosoma turkestanicum* Skrjabin, 1913. Transactions of the Royal Society of Tropical Medicine and Hygiene 1: 115-124.

Montgomery, R.E. 1906. Observations on bilharziosis among animals in India. II. Journal of Tropical Veterinary Science 1: 138-174, 2 pl.

Morgan, J.A.T., DeJong, R.J., Kazibwe, F., Mkoji, G.M., Loker, E.S., 2003. A newly-identified lineage of *Schistosoma*. International Journal of Parasitology 33: 977-985.

Palumbi, S., Martin, A., Romano, S., McMillan, W.O., Stice, L., Grabowski, G., 1991. The simple fool's guide to PCR. Version 2.0. Honolulu, HL 96822: Department of Zoology and Kewalo Marine Laboratory, University of Hawaii. 1-45.

Posada, D., 2008. jModeltest: Phylogenetic model averaging. Molecular Biology and Evolution 25: 1253-1256.

Ramakrishna; Dey, A., 2007. Handbook on Indian Freshwater Molluscs: 399 pp.

Subba Rao, N.V. 1989. Handbook of freshwater molluscs of India. Zoological Survey of India, Calcutta.

Sherchand, J.B., Ohara, H., Sherchand, S., Matsuda, H., 1999. The suspected existence of *Schistosoma mansoni* in Dhanusa diatrict, southern Nepal. Annals of Tropical Medicine and Parasitology 93: 273-278.

Swofford DL. 2002. PAUP*: Ver 4.0. Phylogenetic Analysis Using Parsimony (*and other methods). Sunderland, Sinauer Associates.

von Oheimb, P.V., Albrecht, C., Riedel, F., Du, L., Yang, J., et al. 2011. Freshwater biogeography and limnological evolution of the Tibetan Plateau – insights from a plateau-wide distributed gastropod taxon (*Radix* spp.). PLoS One 6: e26307. doi:10.1371/journal.pone.0026307

Wang, C. R., Chen, J., Zhao, J. P., et al. 2009. *Orientobilharzia* species: neglected parasitic zoonotic agents. Acta Tropica 109: 171-175.

Wang, Y., Wang, C.-R., Zhao, G.-H., Gao, J.-F., Li, M.-W. and Zhu, X.-Q. 2011. The complete mitochondrial genome of *Orientobilharzia turkestanicum* supports its affinity with African *Schistosoma* spp. Infection, Genetics and Evolution 11: 1964-1970.

CONCLUSIONS

Schistosomiasis is a major public and veterinary health problem in different part of the world. Human schistosomiasis afflicts over 220 million people, ranking second only to malaria as a eukaryotic parasite of medical concern (CDC, last updated November 7, 2012). Over 85% of those cases now occur in sub-Saharan Africa. Domestic animals in Africa and Asia bear heavy but largely unquantified burdens of schistosome infection as well (De Bont and Vercruysse, 1997; Ravindran et al., 2007). Although there have been many studies on schistosomiasis in Africa, East Asia, Europe, and North and South America, there are very limited modern studies available from South Asian countries. The study we undertook thus helped to fill a gap in our understanding of the schistosomes of a small south Asian country, Nepal. Prior to this study, there was but limited information about the presence of Nepalese schistosomes. We were initially motivated to undertake this study based on suspected cases of human schistosomasis caused by *S. mansoni* in Janakpur district of southern Nepal (Sherchand et al., 1999). Extensive snail collecting in the Terai region failed to find any evidence for the presence of the *Biomphalaria* snails that support *S. mansoni* development, so any report of *S. mansoni* in Nepal, particularly if indigenous transmission is considered, requires further confirmation in our view.

Although our investigations did not confirm the presence of this human schistosome in Nepal, they did reveal that schistosomes are by no means uncommon in the country. In a relatively limited sampling area in the Terai and associated foothills, one representing less than 10% of the area of the country, we found five distinct lineages of mammalian schistosomes and two different avian schistosome species, to be present. These include

Bivitellobilharzia nairi of Indian elephants and Asian one-horned rhinos; three species in the *Schistosoma indicum* group, including *Schistosoma spindale* and *S. nasale,* as well as one which possibly represents a new species; a member of the *S. turkestanicum* species group, likely either *S. dattai* or *S. bomfordi*, or another new species; and two avian schistosomes, one a new lineage of *Trichobilharzia* and one a *Macrobilharzia*-like parasite.

Several lines of investigation remain for future study. We tried and were unsuccessful in establishing successful infections of *B. nairi* in Nepalese snails. Consequently, the natural snail host for this species in the wild remains unknown and further exposure studies should be undertaken. For example, our discovery that there are multiple cryptic lineages in what can now be called an *Indoplanorbis exustus* species complex suggests that one likely approach to solving the riddle of the life cycle of *B. nairi* is to expose *I. exustus* snail from several locations with the thought that only one of the at least four lineages of this species now known to be present in Nepal may be susceptible.

We also found that avian schistosomes were relatively uncommon in our samples. This may be in part because we did not collect sufficiently in places with high bird diversity or where migratory birds congregate. We feel it is likely that more species of avian schistosomes will be revealed in time, and that the search will be worthwhile, given the novelty of the two species we did recover. The role of avian schistosomes in causing dermatitis should not be discounted as a potential public health problem.

Regarding our studies of the *S. indicum group,* we have expanded the overall sequence database for this group by about fifty-fold. Among our discoveries here was

that of a new lineage in the *S. indicum* group (designated *Schistosoma* sp.) in Nepal, for which adult anatomy and definitive host remain unknown. Here further progress will depend on getting more ready access to either carcasses of cattle or buffaloes at slaughter houses that can be searched for adult worms, or on getting colonies of laboratory rodents, particularly hamsters, set up for experimental life cycle studies. Our analyses consistently failed to find strong support for the *Schistosoma indicum* species group being monophyletic, and its evolutionary history is clearly closely entangled with the evolution of the *S. haematobium* group in Africa. More data will be needed to resolve the origins of these two prominent species groups. Our study proposed a complex origin for the *S. haematobium* and *S. indicum* groups in northeast Africa or southwest Asia. The relatively late arrival of hominids in Asia after the appearance of the *S. indicum* group there probably accounts for the lack of human-infecting members. Also, the fact that the *S. indicum* group is dependent on *I. exustus*, a group of snails that never colonized Africa extensively, and consequently that prevented the coevolution of *S. indicum* group schistosomes with hominid-adapted parasites, is another explanation.

As already noted, the snail host for the *S. indicum* group is not a single species *Indoplanorbis exustus*, but is better considered an *I. exustus* species complex. How the component species in this snail host group interact with schistosomes is still unclear and in need of further study.

The unknown mammalian schistosome of the *S. turkestanicum* species group we obtained from *Radix luteola* is also of 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 is 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 more fully pin down the identity of this species.

Our study has pointed out how little modern information exists regarding schistosomes across the broad swath of southern Asia. Nowhere is this shortage more glaring than for India. Many more collections of both cercariae and adult worms from across this area, followed by sequencing of the specimens acquired will be needed to clarify the extent of the geographic ranges and host preferences (both definitive and intermediate hosts) of the species involved. Also of interest is the fact that several species co-oocur in the same geographic area. We showed this to be the case for Nepal, and it is also known to be true in other regions like India. If these relatively closely related parasites are able to remain as distinct species or if the barriers between them break down in areas where co-infections are common remains an important point to clarify in the future. Also, as there are several reports of mammalian schistosomes causing dermatitis in humans, and some reports of humans from southern Asia passing schistosome eggs in either their feces or urine (Agrawal and Rao, 2011), we must remain alert to the possibility that some south Asian schistosome species have the potential to adapt to humans. Certainly the people of Nepal are constantly exposed to water containing snails that are shedding cercariae of mammalian schistosomes. This creates the opportunity for such host shifts to occur. This study has opened the door to many more exciting studies that will now hopefully follow regarding the biology of this fascinating group of parasites in Nepal.

References

Agrawal, M.C., Rao, V.G., 2011. Indian Schistosomes: A need for further Investigations. Journal of Parasitology Research 2011, 1-4.

CDC (last updated November 7, 2012) Parasite – Schistosomiasis. Retrieved from http://www.cdc.gov/parasites/schistosomiasis/ Accessed on March 23, 2015.

De Bont, J., Vercruysse, J., 1997. The epidemiology and control of cattle schistosomiasis. Parasitology Today 13, 255-62.

Ravindran, R., Lakshmanan, B., Ravishankar, C., Subramanian, H., 2007. Visceral schistosomiasis among domestic ruminants slaughtered in Wayanad, South India. Southeast Asian Journal of Tropical Medicine and Public Health 38, 1008-1010.

Sherchand, J.B., Ohara, H., Sherchand, S., Matsuda, H., 1999. The suspected existence of *Schistosoma mansoni* in Dhanusa diatrict, Southern Nepal. Annals of Tropical Medicine and Parasitology 93, 273-278.