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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★ Note: Nucleotide sequence data reported in this paper are available in the GenBank database under Accession Nos. **KR423832-KR423867**, **KR607213-KR607293** and **KR811315-KR811361**.

17 Abstract

18 From 2007-2014, 19,360 freshwater snails from the Terai and hilly regions of Nepal were
19 screened for cercariae of mammalian schistosomes. Based on analysis of mitochondrial cytochrome
20 oxidase I (*cox1*), 12S, 16S and 28S sequences (3,675 bp) of the cercariae recovered, we provide, to our
21 knowledge, the first report of the *Schistosoma indicum* species group in Nepal. Five samples of
22 *Schistosoma nasale*, nine of *Schistosoma spindale* and 17 of *Schistosoma* sp. were recovered, all from
23 the snail *Indoplanorbis exustus*. The last-mentioned lineage failed to group in any of our analyses with
24 *S. nasale*, *S. spindale* or *S. indicum*. It diverged in *cox1* sequence from them by 16%, 13% and 13%,
25 respectively, levels of difference comparable to well-studied species pairs of *Schistosoma*. Analysis of
26 *cox1*, 16S and internal transcribed spacer 1 (ITS1) sequences (1,874 bp) for Nepalese specimens of *I.*
27 *exustus* was also surprising in revealing the presence of four genetically distinct clades. They diverged
28 from one another at levels comparable to those noted for species pairs in the sister genus *Bulinus*.
29 There was no obvious pattern of use by Nepalese *Schistosoma* of the *Indoplanorbis* clades. We found
30 high support for a close relationship between *S. indicum* and *Schistosoma haematobium* groups, but
31 failed to retrieve support for a clean separation of the two, with a tendency for *S. nasale* to fall as the
32 most basal representative. If this pattern holds, hypotheses for the origin of the Asian *Indoplanorbis*-
33 transmitted *S. indicum* group from the *Bulinus*-transmitted *S. haematobium* group may require
34 modification, including consideration of more contemporaneous origins of the two groups. The Indian
35 subcontinent is under-studied with respect to schistosome diversity and our current knowledge of the *S.*
36 *indicum* and *I. exustus* species groups is inadequate. Further study is warranted given the ability of
37 *indicum* group species to cause veterinary problems and cercarial dermatitis, with a worrisome
38 potential in the future to establish infections in humans.

39 **Keywords:** Schistosomes, *Schistosoma indicum* group, *Indoplanorbis exustus*, Host-parasite
40 relationships, Nepal, Host switch, Schistosomiasis

41

42 **1. Introduction**

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

53 The *Schistosoma indicum* species group as currently recognized is comprised of three species:
54 *S. indicum*, *Schistosoma nasale* and *Schistosoma spindale* (Attwood et al., 2007). Although
55 *Schistosoma incognitum* was formerly included in this group, it has been shown that it is a more distant
56 relative (Agatsuma et al., 2002; Attwood et al., 2002; Morgan et al., 2003; Webster et al., 2006;
57 Lawton et al., 2011). Among the four customarily recognized and prominent species groups, our
58 overall knowledge of the *S. indicum* group lags far behind the other three. The primary reason for this
59 is that the members of this group, although they can and do cause dermatitis in people (Anantaraman,
60 1958; Narain et al., 1998; Agrawal et al., 2000), rarely if ever establish patent human infections
61 (Agrawal et al., 2000; Agrawal and Rao, 2011), so consequently they have attracted less attention.
62 They are however of considerable veterinary significance and they pose a number of interesting
63 questions regarding the evolution and biogeography of schistosomes.

64 Overviews of the biology of the three *indicum* group species can be found in Kumar and
65 deBurbure (1986), Rollinson and Southgate (1987) and Agrawal (2012). Briefly, all three species are

66 hosted by the bulinine planorbid snail, *Indoplanorbis exustus*. This snail is widely distributed across
67 southern Asia (see map range from Budha, P.B., Dutta, J., Daniel, B.A., 2012. *Indoplanorbis exustus*.
68 The IUCN red list of threatened species. Version 2014.3. Available from www.iucnredlist.org.), and has
69 since been introduced into exotic locales such as West Africa and the Caribbean islands by human
70 activities. As noted by Liu et al. (2010), and as we discuss further below, *I. exustus* actually represents
71 a complex of cryptic species. The extent of genetic diversification within what at least superficially
72 looks like a fairly morphologically homogeneous gastropod species was surprising, and will surely
73 improve our overall understanding of the evolution and diversification of the *S. indicum* group and of
74 other dependent trematodes. Some literature also implicates lymnaeid snails in transmission of
75 members of the *indicum* group, but when careful experimental infections have been performed, only *I.*
76 *exustus* has been experimentally infected (Dutt and Srivastava, 1968; De Bont et al., 1991). In our
77 view, it is likely that the Asian mammalian schistosomes recovered from lymnaeids and attributed to
78 the *S. indicum* group are most probably representatives of the lymnaeid-transmitted *Schistosoma*
79 *turkestanicum* species group or of *S. incognitum*.

80 The most widely distributed member of the *S. indicum* group is *S. spindale* which causes
81 intestinal schistosomiasis with its most prominent hosts being water buffaloes, cattle, sheep, and goats.
82 It can also infect equines and wild rodents. It is known from India, Bangladesh, Sri Lanka, Malaysia,
83 Vietnam, Laos, Indonesia and Thailand. It produces characteristic elongated, spindle-shaped eggs with
84 a terminal spine. The males of this species are atuberculate (Rollinson and Southgate, 1987; Gupta and
85 Agrawal, 2002).

86 *Schistosoma indicum* also causes intestinal schistosomiasis in buffaloes, cattle, sheep, goats,
87 camels and horses (Srivastava and Dutta, 1951). This species, as is best known, is confined in its
88 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, *S. 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. 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, Bangladesh, 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 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 *Schistosoma 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 *Schistosoma japonicum* group.

113 The number of *indicum* group specimens available for molecular analysis has to date been
114 limited. They consist of *S. indicum* from Bangladesh, *S. spindale* from Bangladesh, Sri Lanka and
115 Thailand, and *S. nasale* from Bangladesh and Sri Lanka. Specimens of *S. incognitum* from
116 Bangladesh, Indonesia and Thailand have thus far been examined. Mitochondrial gene order has been
117 determined for all four of the above species and found to be of the “derived” type (Lockyer et al.,
118 2003; Littlewood et al., 2006; Sato et al., 2008; Webster and Littlewood, 2012). Remarkably, to date
119 no specimens of the *S. indicum* group from India have been subjected to molecular phylogenetic
120 analysis in a published study.

121 No specimens from the *S. indicum* group have previously been reported from Nepal, although
122 the presence of mammalian schistosome cercariae from *I. exustus* has been reported (Devkota et al.,
123 2011). The elephant schistosome *Bivitellobilharzia nairi* occurs in Nepal in both elephants and
124 rhinoceros (Devkota et al., 2014a), and at least two species of avian schistosomes also occur there
125 (Devkota et al., 2014b), but our knowledge of schistosomes present in Nepal is otherwise rudimentary.
126 Laterally-spined schistosome eggs closely resembling the eggs of *Schistosoma mansoni* have been
127 reported from human fecal samples from the Terai region of southern Nepal (Sherchand et al., 1999),
128 but no additional reports of a schistosome with laterally-spined eggs from Nepal have since come to
129 light.

130 The same freshwater snails discussed by Devkota et al. (2014b) were also screened for
131 cercariae of mammalian schistosomes. These collections took place over a 7-year time interval, mostly
132 in the Terai region of southern Nepal, in and around Chitwan National Park. Here we present the first
133 known report from Nepal of the presence of cercariae representing the *S. indicum* group, all of which
134 were recovered from *I. exustus*. Sequence data for the mitochondrial cytochrome oxidase I (*cox1*), 12S,
135 16S and nuclear 28S gene regions were acquired from most of the specimens and were subjected to
136 molecular phylogenetic analyses. Additionally, for both uninfected *I. exustus*, and for some of the

137 schistosome-positive *I. exustus* specimens, we also acquired *cox1*, 16S and internal transcribed spacer
138 1 (ITS1) (including parts of adjacent 18S and 5.8S genes) sequence data. This information has been
139 used to examine patterns of diversification within *I. exustus*, and to relate those to both other studies of
140 this snail and phylogenetic results obtained for the *S. indicum* group.

141

142 **2. Materials and methods**

143 *2.1. Collection and morphological identification of freshwater snails*

144 Snails were collected from 40 freshwater habitats in different areas of Nepal (Table 1,
145 Supplementary Table S1) using kitchen sieves and triangular scoops mounted on long bamboo handles.
146 The collected snails were kept moist and shaded prior to separation and cleaning. Identification of
147 snails was made using conchological and morphological features (Subba Rao, 1989) and some of the
148 snails were preserved in 96% ethanol for molecular identification.

149

150 *2.2. Screening of infected snails and morphological identification of cercariae*

151 Collected snails were isolated in individual wells of a 24-well tissue culture plate or in Petri
152 dishes containing clean water. The isolated snails were exposed to window light or artificial
153 illumination to stimulate cercarial shedding. Approximately 1 h later, snails were individually screened
154 for shed cercariae using a dissecting microscope. If cercariae were observed, a few were transferred to
155 a microscope slide and observed with the aid of a compound microscope. The cercariae were identified
156 morphologically by means of cercarial keys (Frandsen and Christensen, 1984). Cercariae were ethanol-
157 preserved, measured and photographed using a digital camera fitted to the compound microscope.
158 Snails that did not shed cercariae in the first hour were re-examined for shedding of cercariae at least
159 twice within the following 24 h.

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

165

166 2.3. Molecular and phylogenetic analyses

167 DNA from schistosome cercariae was extracted from alcohol- or RNAlater©-preserved
168 cercarial samples either by using the Qiagen DNeasy Blood and Tissue Kit or the Qiagen QIAamp
169 DNA Micro Kit (Valencia, California, USA). Cercariae were digested for 2 – 3 h or overnight. The
170 nuclear ribosomal 28S and mitochondrial *cox1*, 16S and 12S genes were amplified by PCR by using a
171 Takara Ex *Taq* kit (Takara Biomedicals, Otsu, Japan) and previously published primers (U178; 5'-
172 GCA CCC GCT GAA YTT AAG-3' and L1642; 5'-CCA GCG CCA TCC ATT TTC A -3' for 28S
173 sequences, *cox1*F6; 5'-TTT GTY TCT TTR GAT CAT AAG CG-3' and *cox1* 3; 5' -TAA TGC ATM
174 GGA AAA AAA CA- 3' for *cox1* sequences and P12SF; 5' – TTT GTC CAC AGT TAT AAC TGA
175 AAG G -3' and P12SR; 5' – GAT TCT TCA AGC ACT ACC ATG TTA CGA C -3') (Attwood et al.,
176 2002; Lockyer et al., 2003; Morgan et al., 2003). New primers were designed to amplify the 16S gene
177 (R16SF 5' - TGT TTT TTT CCK ATG CAT TA - 3' and R16SR 5' - GGC TTA CAC CGG TCT TAA
178 CT - 3'). PCR products were purified either with an Omega E.Z.N.A Cycle-Pure Kit (Omega Bio-Tek,
179 Norcross, GA 30071, USA) or USB ExoSAP-IT PCR Product Cleanup (Affymetrix, Inc., Cleveland
180 Ohio 44128, USA) according to the manufacturers' guidelines. Sequencing reactions were performed
181 with Applied Biosystems BigDye direct sequencing kit, version 3.1 (Applied Biosystems, Foster City,
182 California, USA).

183 DNA from snails was extracted from alcohol-preserved samples either by using the Qiagen
184 DNeasy Blood and Tissue Kit or the Omega E.Z.N.A Mollusc DNA Kit following the manufacturers'
185 guidelines. The partial sequences of a nuclear ITS1 and two mitochondrial genes; *cox1* and 16S genes
186 were amplified by PCR by using a Takara Ex Taq kit and previously published primers (ITS1-S; 5'
187 CCA TGA ACG AGG AAT TCC CAG 3' and 5.8S-AS 5' TTA GCA AAC CGA CCC TCA GAC 3'
188 for ITS1; LCO1490; 5' GGT CAA CAA ATC ATA AAG ATA TTG G 3' and HCO2198; 5' TAA ACT
189 TCA GGG TGA CCA AAA AAT CA 3' for *cox1* sequences and 16Sar; 5' CGC CTG TTT ATC AAA
190 AAC AT 3' and 16Sbr; 5' CCG GTC TGA ACT CAG ATC ACG T 3' for 16S sequences) (Palumbi et
191 al., 1991; Folmer et al., 1994; DeJong et al., 2001). PCR products were purified either with an Omega
192 E.Z.N.A Cycle-Pure Kit or USB ExoSAP-IT PCR Product Cleanup according to the manufacturers'
193 guidelines. Sequencing reactions were performed with Applied Biosystems BigDye direct sequencing
194 kit, version 3.1.

195 The schistosome cercariae 28S, 16S, 12S and *cox1* gene fragments and the snail host *I. exustus*
196 ITS1, 16S and *cox1* gene fragments were used in phylogenetic analyses using Bayesian inferences (BI)
197 with the use of MrBayes, v 3.1.2 (Huelsenbeck and Ronquist, 2001). NEXUS files used with MrBayes
198 to create alignments have been made public as Supplementary Data S1 - S11. Model selection was
199 estimated using ModelTest (Posada and Crandall, 1998). The BI analyses were as follows: the 28S and
200 ITS1 dataset, Nst = 6 rates = invgamma ngammacat = 4; the *cox1* dataset, for codons one and two Nst
201 = 2 and for codon three Nst = 6 rates = gamma, ngammacat = 4; for 12S and 16S combined Nst = 6
202 rates = invgamma ngammacat = 4 (parameters were unlinked among partitions). Four chains were run
203 simultaneously for 5×10^5 generations, with four incrementally heated chains sampled at intervals of
204 100 generations. The first 5000 trees with pre-asymptotic likelihood scores were discarded as burn-in
205 using Tracer v1.6 (available from <http://beast.bio.ed.ac.uk/Tracer>), and the retained trees were used to
206 generate 50% majority-rule consensus trees and posterior probabilities. Outgroups for the schistosome

207 *cox1*, 12S, 16S and 28S datasets included members of the *S. mansoni* species group (sister to the *S.*
208 *indicum* + *S. haematobium* groups), and in some cases also included members of the *S. turkestanicum*
209 and *S. japonicum* species groups (Supplementary Table S2). The remaining taxa used in the tree were
210 obtained from the published literature (Attwood et al., 2002, 2007; Lockyer et al., 2003). Outgroups
211 for the phylogenetic analysis of *Indoplanorbis* were selected as the sister genus *Bulinus* (GenBank
212 accession numbers AM921851, AM921835, AM921842, GU176747, KJ157497, EF489313,
213 AY029546, EU076730, EU076727, EU076726), *Planorbarius* (GenBank accession numbers
214 AY282590, AY577473) and *Biomphalaria* (GenBank accession numbers DQ084823, AY126606,
215 AY198103, JQ886409) snails as outgroups. The character variation in the ingroup for the alignments
216 used to create each phylogenetic tree is given in Supplementary Table S3. All DNA sequence data
217 were deposited in GenBank (Tables 2, 3).

218

219 3. Results

220 3.1. Snail survey results

221 From 2007-2014, in the 40 locations listed in Table 1 and Supplementary Table S1, we
222 collected and screened 19,360 freshwater snails for trematodes. The nine families represented 14
223 different species and their numbers were: *Bellamya bengalensis* (429), *Brotia costula* (15), *Gabbia*
224 *orcula* (1,374), *Gyraulus* spp. (4,454), *Indoplanorbis exustus* (7,286), *Lymnaea acuminata* (1,380),
225 *Melanoides pyramis* (194), *Melanoides tuberculata* (254), *Physa* sp. (16), *Pila globosa* (74), *Radix*
226 *luteola* (2,588), *Segmentina* spp. (529), *Succinea* sp. (14), and *Thiara* spp. (753). Only specimens of *I.*
227 *exustus* shed schistosome cercariae subsequently shown to be consistent with affiliations to the *S.*
228 *indicum* group. We found 34 (0.47%) *I. exustus* snails from 11 different localities (Fig. 1, Table 1,
229 Supplementary Table S1) to be infected. As indicated in the phylogenetic analyses that follow (Fig. 3),
230 one location (#35) yielded two schistosome lineages (*S. spindale* and *Schistosoma* sp.) collected at the

231 same time point from two different snails. Another location (#38) yielded schistosomes from all three
232 lineages (*S. spindale*, *S. nasale* and *Schistosoma* sp.), but at different times within the same year or in
233 different years (see Supplementary Table S1 for details).

234

235 3.2. Morphological features of the schistosome cercariae recovered

236 Measurements of representatives of cercariae from each of the three lineages from Nepalese *I.*
237 *exustus* were compared with those available from other studies of the *S. indicum* group, and for one
238 species (*S. incognitum*) formerly included in the group (Supplementary Table S4). Although
239 differences in preservation techniques make rigorous comparisons among studies difficult, we found
240 all three kinds of cercariae that were recovered to be somewhat smaller than the cercariae of *S.*
241 *incognitum*. With respect to tail stem length, which in our experience is useful for facilitating
242 discrimination among species (Morgan et al., 2003), the relative order (shortest to longest) from other
243 studies of the *S. indicum* group was: *S. indicum*, *S. spindale* and *S. nasale*. From shortest to longest tail
244 stem length, the Nepalese specimens were ranked *Schistosoma* sp. (Fig. 2), *S. spindale* and *S. nasale*.

245

246 3.3. Phylogenetic analyses of the schistosomes recovered

247 With respect to our phylogenetic studies of the schistosome cercariae, we attempted to obtain
248 *cox1*, 12S, 16S and 28S sequences from all specimens. At least one of the above sequence regions
249 (Table 2) for all but three of our specimens was obtained. We also included as much pertinent
250 corresponding published sequence that could be found from closely-related schistosomes from
251 elsewhere in Asia or Africa.

252 First, regarding the Bayesian analysis of *cox1* schistosome sequences (Figs. 3A B), several
253 features are noteworthy, many of them recapitulated in the analyses of other genes. The two trees
254 differed in that Fig. 3B incorporated additional sequences from GenBank, but these sequences were

255 shorter (372 bp) compared with sequences used in Fig. 3A (1,125 bp). In Fig. 3A, for which better
256 overall resolution was obtained, sequences representing seven members of the *S. haematobium* group
257 consistently grouped together with strong support, and this group was sister (0.91 posterior probability)
258 to a group containing *S. indicum* from Bangladesh, *S. spindale* from Sri Lanka and Nepal, and
259 *Schistosoma* sp. from Nepal. Lying sister to these two lineages with strong support in the analysis of
260 the longer *cox1* sequences, was a tightly grouped clade consisting solely of specimens of *S. nasale*
261 from both Sri Lanka and Nepal.

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

268 Two specimens of *S. spindale* from Sri Lanka grouped with high support with seven Nepalese
269 isolates and although further study is warranted, particularly from intervening geographic regions, all
270 are likely to be *S. spindale*. However, there is an appreciable degree of genetic distance (approximately
271 8%, Table 4, see Supplementary Table S3 as well for a summary of variation found for particular
272 genes) between the specimens from the two countries. Also included in the analysis are nine closely-
273 related Nepalese isolates that consistently formed a distinct, well-supported group that diverged in
274 *cox1* sequence from *S. nasale* or *S. spindale* by 16% and 13%, respectively. We initially assumed this
275 third lineage would correspond by sequence similarity to *S. indicum*. We were surprised to note (as in
276 Fig. 3A), however, that the few available sequences of *S. indicum* from GenBank consistently failed to
277 group with the nine Nepalese isolates in this third lineage. Furthermore, the genetic distance separating
278 *S. indicum* Bangladesh and our third lineage was 13%. This suggested to us that this third Nepalese

279 lineage was distinct from all three recognized members of the *S. indicum* group, and it is referred to
280 here as *Schistosoma* sp.

281 The tree in Fig. 3B was made to allow inclusion of a second available short *cox1* sequence for
282 *S. indicum* from Bangladesh, and although there was some tendency for Nepalese *Schistosoma* sp. to
283 group with the two specimens of *S. indicum* from Bangladesh in this tree, support was not strong.
284 Many nodes in this tree were not strongly supported and no support was found for uniting presumptive
285 *S. spindale* from Nepal with *S. spindale* from elsewhere.

286 The nodal support provided by the analyses of 28S, 12S and 16S sequences (Fig. 3C,
287 Supplementary Fig. S1 - S3) was generally not as strong as noted for Fig. 3A, but this may be expected
288 as these genes are less variable than *cox1*. However, many patterns similar to those noted for the *cox1*
289 analyses were again observed using these other gene regions. The 28S analysis shown in Fig. 3C again
290 resolved *S. nasale* as separate from either the remainder of the *S. indicum* group or the *S. haematobium*
291 group, but was unable to resolve relative branch orders among these three groups. A Bayesian analysis
292 based on combined *cox1*, 16S and 12S sequences (Fig. 3D) revealed the following patterns in the data:
293 i) weakly supported basal position of *S. nasale*; ii) strong support (0.94 posterior probability) for a
294 clade containing two major lineages, the *S. haematobium* group and the remainder of the *S. indicum*
295 group consisting of the Bangladesh samples of *S. indicum*, *S. spindale*, and *Schistosoma* sp. from
296 Nepal; iii) substantial variation among lineages ascribed to *S. spindale* from Sri Lanka and Nepal; and
297 iv) the *Schistosoma* sp. lineage from Nepal was divergent from the one set of sequences available for
298 *S. indicum* from Bangladesh.

299 Of the 20 specimens for which we obtained both a 28S nuclear sequence and at least one
300 mitochondrial sequence (*cox1*, 16S or 12S), none revealed a nuclear sequence characteristic of one
301 species and a mitochondrial sequence characteristic of another.

302

303 3.4 Phylogenetic analyses of the *Indoplanorbis exustus* snails recovered

304 Regarding the phylogenetic analyses for *Indoplanorbis exustus*, 30 *cox1*, 31 16S and 17 ITS1
305 (the latter also including parts of 18S and 5.8S genes) sequences were obtained from specimens of this
306 snail, mostly from the Terai region (Table 3). For two of these samples, snail sequences were actually
307 amplified from extracts of schistosome cercariae (W798 and W804) originally derived from *I. exustus*
308 but for which the snails were no longer available. In addition, we compared our sequences with those
309 from 14 additional specimens acquired from GenBank (Table 3, Fig. 4). Included in the analyses are
310 sequences for other planorbid snails including species of *Bulinus*, the genus believed to be the most
311 closely related to *Indoplanorbis*.

312 Results of the *cox1* analysis (Fig. 5A) provide two especially noteworthy features. The first,
313 and most surprising, is the presence of four differentiated clades of *I. exustus* (designated I - IV), two
314 of which (Clades I and IV), including the basal group in our *cox1* analysis (Clade I), are comprised
315 exclusively of snails from Nepal. Representatives of each of the four groups are found in Nepal, and
316 three of the four groups (Clades I, III and IV) included specimens infected with members of the *S.*
317 *indicum* group. Strong nodal support was obtained for a clade uniting all identified *I. exustus*
318 specimens. Notably lacking in this analysis are snail specimens from India or Pakistan, or Iran in the
319 western part of the natural range of *I. exustus*.

320 In the 16S analyses (Supplementary Fig. S4), all specimens of *I. exustus* were again shown to
321 group together with high support. Once again four major clades of *I. exustus* were discerned (each
322 containing specimens from Nepal) but with more variable support, and in this case there was not a
323 strongly supported basal clade. The pattern of relationships among the three remaining clades was
324 often poorly resolved, although the members within each clade grouped together with strong support
325 (0.92-1 posterior probability). Regarding the analysis of 1,036 bp of combined *cox1* and 16S sequence
326 (Fig. 5B), strong support for the monophyly of the *Indoplanorbis* lineages was again retrieved. Strong

327 support at most nodes was retrieved for the four major clades, although the topology differed from that
328 noted in Fig. 5A. This analysis also showed strong support for the sister relationship between *Bulinus*
329 and *Indoplanorbis*.

330 The *p*-distance values for both *cox1* and 16S genes among the four different lineages of *I.*
331 *exustus* (Table 5, Supplementary Table S3) are in the same range (*cox1* 0-14% and 16S 0-8%) as
332 reported for species pairs in *Bulinus*. Due to a lack of ITS1 data for *Indoplanorbis* in GenBank we
333 were unable to compare our data with *I. exustus* group specimens from other localities. In our ITS1
334 analysis (Supplementary Fig. S5), we were unable to resolve the four major clades shown in our other
335 analyses.

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

339

340 4. Discussion

341 This study was first stimulated by a report of *S. mansoni*-like eggs from human stool samples
342 from the Terai region of Nepal (Sherchand et al, 1999). Although we could not provide corroborating
343 evidence for the presence of either *S. mansoni* or *Biomphalaria*, the survey work that followed
344 revealed four lineages of *Schistosoma* in Nepal: the three lineages of the *S. indicum* group discussed
345 here, all of which were recovered from *I. exustus*, and a fourth lineage of lymnaeid-transmitted
346 *Schistosoma* we will discuss in a separate paper.

347 We were unable to examine adult schistosomes as part of this study because buffaloes and
348 especially cattle are maintained for milk production and are not commonly slaughtered in the Terai
349 region. Government sanctioned abattoirs are not present in the area and we found owners of private
350 abattoirs were unwilling to grant permission to examine viscera of slaughtered animals. Also, mice or

351 hamsters are not readily available for experimental infections and sanctioned animal care committees
352 and procedures are either not in place or lack the ability to articulate with animal care committees in
353 the USA. The lack of adult worms and associated eggs, the usual benchmarks whereby species of
354 schistosomes are described and delineated, means that what is said about the specific identity of the *S.*
355 *indicum* group cercariae that were recovered, has to be qualified.

356 One consistent feature of our analyses was that *S. nasale* was retrieved as separate from, and
357 often basal to, members of the combined *S. haematobium*-*S. indicum* groups. Furthermore, relative to
358 the other lineages we found, the amount of genetic diversity present in this species was minimal, both
359 for specimens retrieved from Nepal and when specimens from Sri Lanka or Bangladesh were included.
360 With respect to the remaining two *S. indicum* group lineages we found in Nepal, one is almost
361 certainly *S. spindale*, although the Nepalese specimens diverge genetically from the few *S. spindale*
362 specimens available for comparison from elsewhere in Asia. Thus, whereas *S. nasale* from Nepal, Sri
363 Lanka and Bangladesh exhibit 0% *p*-distance values for their *cox1* sequences, specimens of *S. spindale*
364 from Nepal and Sri Lanka differ by 8% in *p*-distance values for the same target gene (Table 4).
365 Nonetheless, strong posterior probability support is obtained for grouping Nepalese specimens of
366 presumptive *S. spindale* with *S. spindale* from other locations.

367 We refer to the third *S. indicum* group lineage in Nepal as “*Schistosoma* sp.” Although we
368 originally expected this third lineage to be *S. indicum*, it never grouped closely in any of our trees with
369 available sequences for *S. indicum* from GenBank. Nepalese *Schistosoma* sp. differs substantially from
370 known *S. indicum* with respect to *p*-distance values: 13% for the *cox1* gene, a value commensurate
371 with typical species differences within both the *S. indicum* and *S. haematobium* groups (Table 4). A
372 note of caution is in order here as very few specimens of *S. indicum* for which sequence data exist
373 (potentially only two worms represented) are available. Furthermore, they are from the same locality in
374 Bangladesh. Only small fragments of 16S (60 bp) and 28S (346 bp) of *S. indicum* from India were

375 available in GenBank. The Bangladesh and Indian specimens may not be representative of the genetic
376 variation within *S. indicum*, or it is possible *S. indicum* is broadly distributed and encompasses a
377 particularly wide range of genetic variation. It is certainly a possibility that Nepalese *Schistosoma* sp.
378 is actually a distinct species, possibly one confined to the northern part of the collective range of the *S*
379 *indicum* group. It is hoped that this possibility will stimulate additional study including verification
380 with specimens of adult worms and eggs, and experimental infection studies.

381 Although *Schistosoma* sp. from Nepal does not cluster with previously identified *S. indicum*, it
382 persistently groups with *S. spindale* as a close but distinct relative in our trees (*p*-distance value 13%
383 for *cox1*, Table 4). Even though worms of both lineages likely inhabit the mesenteric veins of domestic
384 ruminants in Nepal, we found no evidence in our samples suggestive of hybridization of *Schistosoma*
385 sp. with *S. spindale*. There was also no indication of hybridization of either lineage with *S. nasale*.
386 Does hybridization occur in the *S. indicum* group as now seems to be commonly the case with the *S.*
387 *haematobium* group (Rollinson et al., 1990; Webster et al., 2005, 2013; Huyse et al., 2009)? Although
388 we saw no evidence for similar admixture in the *S. indicum* group, this was not the main objective of
389 our work, so further study is needed to settle this point.

390 Regarding *I. exustus*, our results are in agreement with Liu et al. (2010) in showing a surprising
391 amount of structure within what has traditionally been considered a single widespread snail species.
392 Liu et al. (2010) identified three major clades, one of which includes specimens from localities as
393 diverse as Oman, Malaysia, Indonesia, Thailand, the Philippines and Nepal, and corresponds to clade
394 II identified in our trees. The other two clades from the study of Liu et al. (2010) were more closely
395 related to one another, were from Bangladesh and northern India, and corresponded to clade III in our
396 *cox1* analysis. The sample from Bangladesh was moved to clade I in our 16S and combined *cox1* and
397 16S analyses. Our clade IV only includes specimens from Nepal. The specimen from Bangladesh
398 noted by Liu et al. (2010) was relatively divergent from those we collected from Nepal, suggesting it

399 might be appropriate to recognize even a fifth lineage. Our results indicate multiple clades can inhabit
400 the same specific habitat (#38) from which clades I, II and IV of *Indoplanorbis* were all found.

401 The *p*-distance values for both *cox1* and 16S genes among the four different lineages of *I.*
402 *exustus* we identified (Table 5) are not dissimilar from values recorded for species pairs within the
403 probable sister genus *Bulinus*. Samples of this snail from many other parts of its known range have yet
404 to be included in molecular phylogenetic studies, so the overall diversity inherent in the *I. exustus*
405 species complex is bound to increase. In particular, specimens from north of the Himalayas, most of
406 the western portion of the range including much of India, Pakistan, Afghanistan and Iran, island
407 populations from the Indian Ocean, and specimens from Africa are bound to provide more surprises.

408 Caution is required in interpreting both the relative abundance of the various *I. exustus* lineages
409 in Nepal and the role each plays in schistosome transmission. Snails from lineage I, for which
410 representatives are mostly known from the current study, were commonly collected and two
411 echinostome infections and two *Schistosoma* sp. infections were recovered from them. Lineage II,
412 which includes specimens from several countries (including one specimen from Nepal) collected by
413 others, was also found by us but not commonly, and of the three specimens we sequenced, two had
414 double trematode infections (xiphidiocercariae and strigeids) and one had a sanguinicolid infection.
415 Lineage III, which also includes specimens collected by others from Assam, or Assam and Bangladesh
416 depending on the tree, was less common in Nepal but included one snail positive for *S. nasale*.
417 Finally, Lineage IV, known from only our collections to date, was probably the most common lineage
418 in our collections, and in addition to infections with strigeids, sanguinicolids and xiphidiocercariae,
419 samples of all three of the *S. indicum* group lineages were recovered: five *S. spindale*, two *S. nasale*,
420 and one *Schistosoma* sp. In Nepal, three of the four major *I. exustus* lineages supported at least one
421 member of the *S. indicum* group, Lineage IV supported all three schistosome lineages, and Lineage II
422 was not found to support any schistosomes although it is likely snails of this lineage support the *S.*

423 *indicum* group elsewhere in Asia where they seem to be common (Liu et al., 2010). Lineage II may
424 also transmit schistosomes in Nepal where, based on admittedly incomplete sampling, it appears to be
425 uncommon.

426 As noted in several other studies (Lockyer et al, 2003; Morgan et al., 2003; Webster et al.,
427 2006; Lawton et al, 2011; Webster and Littlewood, 2012), we found clear support for a close
428 relationship between the *S. haematobium* and *S. indicum* species groups. As was also noted in the most
429 recent study of the *S. indicum* group by Attwood et al. (2007), we found the two groups did not
430 separate cleanly and there was a persistent tendency for the *S. haematobium* group to nest within the *S.*
431 *indicum* group, with *S. nasale* basal, albeit often without strong support, e.g. the *S. indicum* group as
432 traditionally considered appears to be paraphyletic (see also Attwood et al., 2007). The results may be
433 dependent on the particular sequences selected for study as other investigations (Morgan et al., 2003;
434 Webster et al., 2006; Webster and Littlewood, 2012) have indicated separation of the *S. indicum* and *S.*
435 *haematobium* groups, but bootstrap support for the basal position of *S. nasale* within the *S. indicum*
436 group is always reported as relatively weak, again raising questions as to the delineation of these two
437 groups. Provision of more sequence data may resolve what looks like an obvious anomaly in
438 schistosome systematics, given that members of the recognized *S. haematobium* groups all parasitize
439 *Bulinus* snails and are predominantly African and southwest Asian in distribution, whereas members of
440 the recognized *S. indicum* group are transmitted by the *I. exustus* species group and are Asian in
441 distribution. The close relationship between the two species groups of schistosomes is supported by the
442 close relationship of their snail hosts. *Bulinus* and *Indoplanorbis* are united by the presence of a
443 distinctive synapomorphy, the ultrapenis, and by molecular systematic studies that place them in a
444 bulinine clade distinct from other planorbid snails (Fig. 5B, Morgan et al., 2002; Albrecht et al., 2007),
445 but note the difficulties in documenting a sister group relationship mentioned by Jørgensen et al.
446 (2011).

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

454 This scenario may need to change somewhat, however, especially if further study shows that *S.*
455 *nasale* retains its basal position relative to the remainder of the combined *S. indicum*-*S. haematobium*
456 group. If so, this is noteworthy for at least two reasons. One is that the original snail host for this
457 combined lineage may have been *Indoplanorbis*, not *Bulinus*. Second, given the present-day
458 distribution of *Indoplanorbis* in southern Asia (African representatives of *I. exustus* appear to be
459 relatively recent colonists), it raises a question as to where the origins of this larger group of
460 schistosomes might actually lie. Today, aside from recent human-influenced range changes, the ranges
461 of *Indoplanorbis* and *Bulinus* are contiguous in regions of Iraq and Iran, and may overlap in some
462 areas there. In the past, especially considering the occurrence of several pluvial periods in northern
463 Africa and southwest Asia at various periods ranging from 3.2 million years ago (mya) to 12,500 ya
464 (Lawler, 2014), opportunities for intermingling of both definitive and intermediate hosts may have
465 been much more extensive in the Levant, the Arabian peninsula, Mesopotamia and present-day Iran
466 than they are today.

467 That *I. exustus* is actually a species complex with much more genetic structure than previously
468 considered is particularly germane with respect to the evolution of the *S. indicum* group. Particular
469 lineages within the *I. exustus* complex, assuming they were already in existence, may have played a
470 critical role in the transition of *Schistosoma* into *Indoplanorbis*. Additional study is needed to better

471 characterize the full diversity within both the *S. indicum* group and the *I. exustus* species complex, as is
472 a thorough analysis of how the two species groups interact. For example, are particular species in the *S.*
473 *indicum* group confined to certain lineages of the *Indoplanorbis* group, or do some lineages of
474 *Indoplanorbis* host all, whereas others host none, of the *indicum* group species? Our results are not
475 suggestive of congruent patterns of cospeciation as all three schistosome species from Nepal were
476 found in *I. exustus* Lineage IV, and two schistosome species were found in two different *I. exustus*
477 lineages. Our results suggest that Lineage IV is important in transmission in Nepal, but more extensive
478 sampling of both worms and snails across a broader geographic area may well change this story.

479 In considering the timing involved in the emergence of the *S. indicum* group, it is noteworthy
480 and fortuitous that members of the *S. indicum* group do not establish patent infections in humans,
481 unlike at least two members of the *S. haematobium* group in Africa. This tends to suggest that the time
482 available for members of the *S. indicum* group to adapt to and infect humans has been limited relative
483 to members of the *S. haematobium* group, which, by virtue of their origin in Africa, would have had
484 opportunity for continual contact with hominins. Also, the dependency of *indicum* group worms on
485 *Indoplanorbis* may similarly have prevented them from gaining any longterm access to hominins as
486 they evolved and diversified, because *Indoplanorbis* is an Asian genus. Unlike *Bulinus*-transmitted
487 schistosomes, *indicum* group worms did not evolve in Africa, the acknowledged location of origin and
488 diversification for hominins.

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

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

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

512

513 **Acknowledgments**

514 This study was supported primarily by funds provided by the University of New Mexico
515 (UNM), USA to ESL to support travel and specimen collection for RD. Technical assistance and
516 financial support at the UNM Molecular Biology Core Facility were provided by National Institutes of
517 Health (NIH), USA grant P30GM110907 from the Institute Development Award program of the
518 National Center for Research Resources and a National Science Foundation, USA grant to SVB (DEB

519 1021427). We are grateful to the officials of the Department of National Parks and Wildlife
520 Conservation, Chitwan National Park, Nepal and the Nepal Health Research Council (permit no. 44)
521 for their cooperation in carrying out this research.

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655 **Legends to Figures**

656

657 **Fig. 1.** Map of the study area showing sites positive for *Indoplanorbis exustus*-transmitted mammalian
658 schistosomes, with numbers corresponding to locality data in Table 1 and Supplementary Table S1.
659 (A) Map of Nepal and (B) expanded map of the Chitwan district within Nepal where many of the
660 sampling sites were located.

661

662 **Fig. 2.** Cercaria of a *Schistosoma* sp. obtained from an *Indoplanorbis exustus* snail.

663

664 **Fig. 3.** Bayesian phylogenetic trees for *Schistosoma*. (A) Tree based on mitochondrial cytochrome
665 oxidase I (*cox1*) sequences (1125 bp). This tree includes the only *Schistosoma indicum* sequence of
666 this length available in GenBank. (B) Tree based on *cox1* sequences (372 bp). This tree includes two *S.*
667 *indicum* sequences available in GenBank. (C) Tree based on 28S sequences (1515 bp). (D) Tree based
668 on combined analysis of *cox1*, 16S and 12S sequences (1740 bp). Samples in bold are those collected
669 in this study. Node support is indicated by Bayesian posterior probabilities.

670

671 **Fig. 4.** Map showing localities from which specimens of *Indoplanorbis exustus* were collected, and for
672 which 16S and mitochondrial cytochrome oxidase I (*cox1*) sequences are available in GenBank from
673 Albrecht et al. (2004) and Liu et al. (2010).

674

675 **Fig. 5.** Bayesian phylogenetic trees for *Indoplanorbis*. (A) Tree based on mitochondrial cytochrome
676 oxidase I (*cox1*) sequences (620 bp). (B) Tree based on *Indoplanorbis cox1* and 16S combined
677 sequences (1036 bp). In both trees samples in bold are those collected in this study. Node support is

678 indicated by Bayesian posterior probabilities. The “L” followed by a number designates the location
679 number indicated in Table 1.
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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 *Schistosoma indicum* group schistosomes. The identifications for the schistosomes provided are derived from the phylogenetic analyses that follow (see Fig. 3), and the designation “*Schistosoma* sp.” reflects the genetic distinctiveness of these worms from the few available specimens of *S. indicum* in GenBank. 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	Number of <i>Indoplanorbis</i> collected	Species identity and W number ^a
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)	<i>Schistosoma</i> 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 <i>Schistosoma spindale</i> (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)	<i>Schistosoma</i> 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 <i>Schistosoma</i> 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)	<i>Schistosoma nasale</i> (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)	<i>S. spindale</i> (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)	<i>Schistosoma</i> sp. (W557)
23.	Kuchkuche Community forest, near Rapti dam Kathar, Chitwan	340	N 27°34'02.28", E 84°37'16.55"	154 (1)	<i>S. spindale</i> (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)	<i>S. nasale</i> (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"	1,321 (11)	6 <i>Schistosoma</i> sp. (W380, W381, W383, W531, W534, W541), 2 <i>S. spindale</i> (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"	1,890 (12)	6 <i>Schistosoma</i> sp. (W517, W518, W519, W550, W798, W799); 3 <i>S. spindale</i> (W464, W538, W804); 3 <i>S. nasale</i> (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	-

689 ^aW number indicates schistosome cercariae sample number; the cercariae of different sample numbers come from different individual *Indoplanorbis*

690 *exustus* snails

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695 **Table 2.** List of the *Schistosoma indicum* group schistosome specimens and GenBank accession
696 numbers for which DNA sequence data was obtained for phylogenetic analysis.

Taxon or Sample number	GenBank Accession number			
	28S sequences	12S sequences	cox1 ^a 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
<i>S. indicum</i>	AY157258 (Bangladesh)	EF534276 (Bangladesh)	AY157204 (Bangladesh)	EF534284 (Bangladesh)
<i>Schistosoma spindale</i>	Z46505 (Sri Lanka); AY157257 (Sri Lanka); AF465925 (Thailand); AF465926 (Thailand)	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)
<i>Schistosoma nasale</i>	AY157259 (Sri Lanka)	EF534280 (Bangladesh)	AY157205 (Sri Lanka)	-

^a*cox1*, mitochondrial cytochrome oxidase I

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699 **Table 3.** List of the sources of *Indoplanorbis exustus* DNA sequence data used in this study. The SW
700 number indicates the snail from which a particular schistosome (designated by a W followed by the
701 same number) was obtained.

Taxon	Co-ordinates	Collection date	GenBank accession number		
			<i>cox1</i> ^a	16S	ITS1 ^b
Snail infected with <i>Schistosoma nasale</i> (SW439)	N 26°46'36.76", E 85°56'05.50"	9 th January 2007	KR811332	KR607278	KR811325
Snail infected with <i>Schistosoma spindale</i> (SW464)	N 27°37'45.52", E 84°29'21.57"	4 th July 2010	KR811347	KR607268	KR811315
Snail infected with <i>S. spindale</i> (SW525)	N 27°34'57.8", E 84°27'56.6"	2 nd July 2010	KR811348	KR607263	KR811316
Snail infected with <i>Schistosoma</i> sp. (SW528)	N 27°33'37.5", E 84°29'24.5"	9 th July 2010	KR811338	KR607284	-
Snail infected with <i>S. spindale</i> (SW538)	N 27°37'45.52", E 84°29'21.57"	4 th July 2010	KR811349	KR607272	KR811321
Snail infected with <i>S. nasale</i> (SW545)	N 27°34'34.11", E 84°31'02.21"	12 th May 2011	KR811350	KR607273	KR811319
Snail infected with <i>S. nasale</i> (SW551)	N 27°37'45.52", E 84°29'21.57"	22 nd July 2010	KR811351	KR607274	KR811318
Snail infected with <i>Schistosoma</i> sp. (SW557)	N 27°34'26.85", E 84°36'28.88"	3 rd July 2012	KR811352	KR607276	KR811320
Snail infected with <i>Schistosoma</i> sp. (SW798)	N 27°37'45.52", E 84°29'21.57"	1 st August 2014	KR811339	KR607285	-
Snail infected with <i>S. spindale</i> (SW804)	N 27°37'45.52", E 84°29'21.57"	29 th July 2014	KR811354	KR607271	-
Snail infected with <i>S. spindale</i> (SW558)	N 27°34'02.28", E 84°37'16.55"	3 rd 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"	9 th 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"	6 th 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"	8 th July 2010	KR811337	KR607283	KR811327

Two snails infected with echistosoma 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"	9 th 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"	11 th 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"	7 th 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"	8 th 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"	23 rd July 2010	KR811343	KR607289	-
<i>I. exustus</i> (Janakpur, Nepal) ^c	-	-	GU451739	GU451732	-
<i>I. exustus</i> (Assam, India) ^c	-	-	GU451744	GU451726	-
<i>I. exustus</i> (Mymensingh, Bangladesh) ^c	-	-	GU451745	GU451727	-
<i>I. exustus</i> (Kekirawa, Sri Lanka) ^c	-	-	GU451742	GU451735	-
<i>I. exustus</i> (Bintulu, Borneo, Malaysia 1) ^c	-	-	GU451746	GU451728	-
<i>I. exustus</i> (Kampang Pelegong, Malaysia 2) ^c	-	-	GU451738	GU451731	-
<i>I. exustus</i> (Bogor, Indonesia) ^c	-	-	GU451747	GU451729	-
<i>I. exustus</i> (Xang, Laos) ^c	-	-	GU451750	GU451751	-
<i>I. exustus</i> (Wadi Bani Khaled, Oman 1) ^c	-	-	GU451740	GU451733	-
<i>I. exustus</i> (Wadi Qab, Oman 2) ^c	-	-	GU451741	GU451734	-
<i>I. exustus</i> (Bulan, Philippines) ^c	-	-	GU451748	GU451730	-
<i>I. exustus</i> (Khon Kaen, Thailand 1) ^c	-	-	GU451743	GU451736	-
<i>I. exustus</i> (Phitsanulok, Thailand 2) ^c	-	-	HM104223	HM104222	-
<i>I. exustus</i> (Maenam Loei, Thailand 3) ^d	-	-	AY282587	-	-

^acox1, mitochondrial cytochrome oxidase I; ^bTTS1, internal transcribed spacer 1; ^centries in the table are from Liu et al. (2010); ^dentry in the table is from Albrecht et al. (2004)

707 **Table 4.** *p*-distances for the mitochondrial cytochrome oxidase I (*cox1*), 12S, 16S and 28S sequences
708 among representative cercariae samples which we collected, and compared with other mammalian
709 schistosomes.

Taxa	<i>cox1</i>	12S	16S	28S
W798 (<i>Schistosoma</i> sp.) – <i>Schistosoma indicum</i> (Bangladesh)	13%	7%	15%	0%
W804 (<i>Schistosoma spindale</i>) – <i>S. spindale</i> (Sri Lanka)	8%	2.5%	6%	0%
W551 (<i>Schistosoma nasale</i>) – <i>S. nasale</i> (<i>cox1</i> and 28S-Sri Lanka, 12S- Bangladesh)	0%	0%	-	0%
W798 (<i>Schistosoma</i> sp.) - W804 (<i>S. spindale</i>)	13%	6.7%	13%	0%
W551 (<i>S. nasale</i>) - W798 (<i>Schistosoma</i> sp.)	16%	8.6%	17%	2%
W551 (<i>S. nasale</i>) - Our sample W804 (<i>S. spindale</i>)	16%	1%	17%	2%
W528 (<i>Schistosoma</i> sp.) – W531 (<i>Schistosoma</i> sp.)	0%	0%	0%	0%
W558 (<i>S. spindale</i>) – W804 (<i>S. spindale</i>)	0%	0%	1%	0%
W545 (<i>S. nasale</i>) - W551 (<i>S. nasale</i>)	0%	0%	0%	0%
<i>S. indicum</i> – <i>S. spindale</i>	14% (<i>S. indicum</i> Bangladesh, <i>S. spindale</i> SL)	7% (Both from Bangladesh)	17% (Both from Bangladesh)	0% (<i>S. indicum</i> Bangladesh, <i>S. spindale</i> SL)
<i>S. spindale</i> – <i>S. nasale</i>	16% (Both from SL)	9% (Both from Bangladesh)	-	2% (Both from SL)
<i>S. indicum</i> – <i>S. nasale</i>	16% (<i>S. indicum</i> Bangladesh, <i>S. nasale</i> SL)	9% (Both from Bangladesh)	-	2% (<i>S. indicum</i> Bangladesh, <i>S. nasale</i> SL)
<i>Schistosoma mansoni</i> - <i>Schistosoma rodhaini</i>	11.5%	-	-	0%
<i>Schistosoma mattheei</i> – <i>Schistosoma haematobium</i>	13%	4%	-	1%
<i>Schistosoma intercalatum</i> – <i>S. haematobium</i>	11%	4%	-	0%

710 Note: W number indicates the schistosome cercariae sample number; the cercariae of different sample numbers come from different individual
711 *Indoplanorbis exustus* snails

712 **Table 5.** Uncorrected *p*-distances for the mitochondrial cytochrome oxidase I (*cox1*) and 16S
713 sequences among representative snail samples we collected, and with other snails. The SW number
714 indicates the snail from which a particular schistosome (designated by a W followed by the same
715 number) was obtained.

Taxa	<i>cox1</i>	16S
Within <i>Indoplanorbis</i>	0-14%	0-8%
Within <i>Bulinus</i>	2-15%	1-13%
<i>Indoplanorbis</i> infected with sanguinicolid Chitwan National Park – <i>Indoplanorbis exustus</i> Nepal (Liu et al., 2010)	2%	4%
<i>Indoplanorbis</i> uninfected Chitwan National Park (location 12) – <i>I. exustus</i> Nepal (Liu et al., 2010)	14%	7%
<i>Indoplanorbis</i> Chisapani, Dhanusa – <i>Indoplanorbis</i> infected with sanguinicolid Chitwan National Park	13%	7%
<i>Indoplanorbis</i> Chisapani, Dhanusa – <i>Indoplanorbis</i> infected with <i>Schistosoma spindale</i> (SW538)	12%	8%
<i>Indoplanorbis</i> infected with <i>S. spindale</i> (SW538) – <i>Indoplanorbis</i> infected with <i>Schistosoma nasale</i> (SW439)	9%	5%
<i>Indoplanorbis</i> infected with <i>Schistosoma</i> sp. (SW528) – <i>Indoplanorbis</i> infected with <i>S. nasale</i> (SW439)	13%	7%
<i>Indoplanorbis</i> infected with <i>Schistosoma</i> sp. (SW528) - <i>Indoplanorbis</i> infected with <i>S. spindale</i> (SW538)	12%	8%
<i>Indoplanorbis</i> infected with <i>S. spindale</i> (SW464) - <i>Indoplanorbis</i> infected with <i>S. spindale</i> (SW525)	0%	0%
<i>Bulinus tropicus</i> – <i>Bulinus nasutus</i>	15%	9%
<i>Bulinus globosus</i> – <i>Bulinus. forskali</i>	12%	13%
<i>Biomphalaria glabrata</i> – <i>Biomphalaria peregrine</i>	10%	7%

716

Figure 1 black and white

Figure 2 colour

Figure 2 black and white

50 μm

Figure 3

Figure 4 colour

Figure 4 black and white

Figure 5