Schistosoma kisumuensis n. sp. (Digenea: Schistosomatidae) from murid rodents in the Lake Victoria Basin, Kenya and its phylogenetic position within the *S. haematobium* species group

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SUMMARY

Schistosoma kisumuensis n. sp. is described based on 6 adult males and 2 adult females collected from the circulatory system of 3 murid rodent species, *Pelomys isseli, Mastomys natalensis*, and *Dasymys incomtus*. Specimens were collected from a single location, Nyabera Swamp, in Kisumu, Kenya in the Lake Victoria Basin. This new species is morphologically similar to members of the *S. haematobium* group, currently represented by 8 species parasitizing artiodactyls and primates, including humans. *Schistosoma kisumuensis* differs from these species by producing relatively small *Schistosoma intercalatum*-like eggs (135·2 × 52·9 μm) with a relatively small length to width ratio (2·55). Comparison of approximately 3000-base-pair sequences of nuclear rDNA (partial 28S) and mtDNA (partial *cox1*, *nad6*, 12S) strongly supports the status of *S. kisumuensis* as a new species and as a sister species of *S. intercalatum*. The *cox*1 genetic distance between these two species (6·3%) is comparable to other pairwise comparisons within the *S. haematobium* group. Separation of the Congo River and Lake Victoria drainage basins is discussed as a possible factor favoring the origin of this species.

Key words: Digenea, Schistosomatidae, *Schistosoma*, *Schistosoma kisumuensis* n. sp, rodent parasite, Kenya.

INTRODUCTION

The digenetic trematode genus *Schistosoma* Weinland, 1858, contains several species that are of medical or veterinary importance. These species cause schistosomiasis, a disease most common in Africa, Asia, and South America, afflicting at least 165 million domestic ruminants (De Bont and Vercruysse, 1997), and as many as 207 million humans (Steinmann *et al*. 2006). Worms within this genus form a monophyletic group with members of the genus *Orientobilharzia* (Farley, 1971) based on molecular data (Snyder and Loker, 2000; Li *et al*. 2008; Wang *et al*. 2009). All members of *Schistosoma* and *Orientobilharzia* inhabit the vascular system of mammalian definitive hosts.

Species of *Schistosoma* have historically been placed into 4 groups based on their egg morphology, intermediate and definitive hosts, and biogeography (Rollinson and Southgate, 1987). Although molecular evidence has required modification of the 4 species group concept, (Snyder and Loker, 2000; Lockyer *et al*. 2003; Morgan *et al*. 2003; Webster *et al*. 2006), the group traditionally referred to as the *Schistosoma haematobium* group has been supported by molecular data as monophyletic. In these species, the ovary is in the posterior half of the body, males contain 4–7 testes, the uterus contains numerous eggs, and all but *S. margrebowiei* produce eggs with a terminal spine (Rollinson and Southgate, 1987; Pagès *et al*. 2003). The *S. haematobium* group contains 8 species (Table 1), 4 of which can infect humans, and all of which use snails in the genus *Bulinus* as intermediate hosts (Brown, 1994).

Due to the economic and health impact of schistosomiasis, representatives of *Schistosoma* have been intensely studied for nearly a century. Despite this research focus, our understanding of their diversity and taxonomy remains incomplete as evidenced by recent reports of new species (Attwood *et al*. 2002) or lineages (Morgan *et al*. 2003). During a parasitological survey of rodents in and around Kisumu, Kenya, located in the Lake Victoria Basin, several schistosomes were recovered, including worms, which produced small uniquely shaped eggs.

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Table 1. Details of the *Schistosoma haematobium* group

(DRC, Democratic Republic of the Congo.)

Species of *Schistosoma*	Hosts		Intermediate (genus)	Geographical region	Egg ratio†
	Definitive				
	Normal	Others			
S. bovis	Artiodactyls	Perissodactyls, rodents	*Bulinus, Planorbarius*	Africa, Middle East, Europe	2·48
S. curassoni	Artiodactyls		*Bulinus*	Africa	2·36
S. guineensis	Primates	Rodents	*Bulinus*	SW Africa	3·27
S. haematobium	Primates	Artiodactyls, rodents	*Bulinus*	Africa, SW Asia, Europe	2·48
S. intercalatum	Primates	Artiodactyls, rodents	*Bulinus*	DRC, Uganda§	2·9
S. kisumuensis n. sp.	Rodents		Unknown	Kenya	2·55
S. leiperi	Artiodactyls	Perissodactyls	*Bulinus*	Africa	5·09
S. margrebowiei	Artiodactyls		*Bulinus*	Africa	1·4
S. mattheei	Artiodactyls, primates	Perissodactyls, rodents*	*Bulinus*	Africa	3·26

* Experimental laboratory exposure.
§ Locality needs to be verified.
† Length/width ratio.

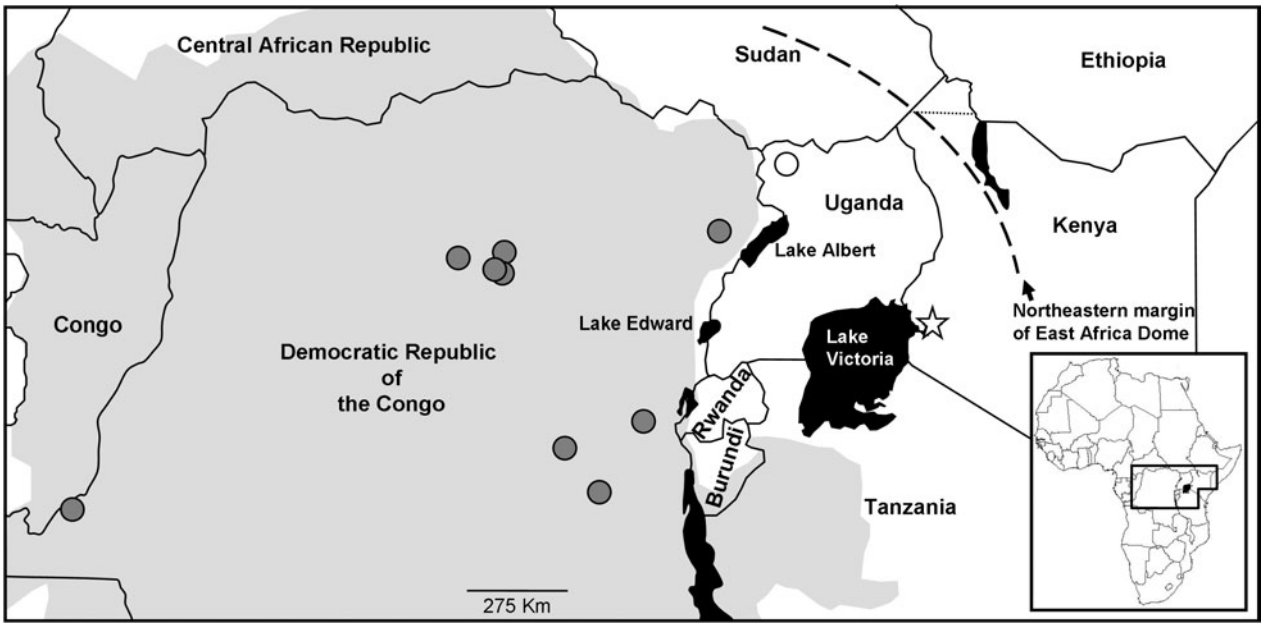

Fig. 1. Collection locality of *Schistosoma kisumuensis* n. sp. and the distribution of *S. intercalatum*. Inset map shows the area of detail. Circles represent reports for *S. intercalatum*: grey, confirmed infection foci; open, unconfirmed infection foci. Star represents collection locality of *S. kisumuensis*. Shaded area is the Congo River drainage Basin.

Adult worm anatomy and egg morphology most closely resemble members of the *S. haematobium* group. In the present study, we describe these distinctive worms as a new schistosome species based on morphological and molecular characters.

MATERIALS AND METHODS

Parasites

Schistosomes were collected from rodent definitive hosts as follows. Sherman live traps were placed in and around Nyabera swamp in Kisumu, Kenya (0·10914°S, 34·775°E) from October 2006 to December 2008 (Fig. 1). Samples were taken monthly with the exception of a period between January and August 2009, a time of civil unrest in Kenya. This site is located along the Kisumu-Kericho highway on the eastern outskirts of the city, is bordered on all but one side by homes and businesses, and continues approximately 6·5 kilometers to the south to Winam Gulf, part of Lake Victoria. The swamp has been used previously for rice cultivation, but since 2004

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Table 2. Measurements of adult *Schistosoma intercalatum* and *Schistosoma kisumuensis* n. sp.

(*S. kisumuensis* measurements are based on alcohol preserved specimens; S. *intercalatum* in both studies were recovered from experimentally infected rodents.)

Species Source	*S. intercalatum* Fisher, 1934	*S. intercalatum* Wright *et al*. 1972	*S. kisumuensis* n. sp. Present study
Male length	11–14 mm	11·5–14·5 mm	4·92 mm
Female length	10–14	13–24 mm	6·4 mm
Male width	0·3–0·4 mm	0·31–0·5 mm	0·24 mm
Female width	0·15–0·18 mm	0·2–0·25 mm	0·23 mm
Male oral sucker diameter	—	0·25 mm	113·3 μm
Male ventral sucker diameter	—	0·25–0·37 mm	179·6 μm
Female oral sucker diameter	—	0·03–0·05 mm	47 μm
Female ventral sucker diameter	—	0·02–0·42 mm	30 μm
Testis number	3–5	2–7	4–6
Testis diameter	0·07–0·1 mm	—	0·063 μm
Testicular chain length	0·22–0·25 mm	—	0·197 mm
Testicular chain width	0·11 mm	—	0·061 mm
Ovary length	0·25–0·5 mm	0·4–0·7 mm	0·33 mm
Ovary width	0·12–0·13 mm	—	0·123 mm

has lain fallow and has mainly been used as a source for several macrophyte species used for roof-thatching materials. Human defecation is common in and around the waters within the swamp, which is also used as a communal bathing area. *Schistosoma mansoni* Sambon, 1907 has been reported from this site (Steinauer *et al*. 2008*a*).

Sherman traps were baited with an even mixture of peanut butter and cupcakes (locally known as Queen Cakes). Traps were checked at dusk and dawn, and animals were returned live to the laboratory in Kisian, Kenya, 15 kilometers from Nyabera swamp. For all captured individuals, sex, weight, and several size measurements were taken. Animals were euthanized, injected with a mixture of heparin and sodium pentabarbitol, and worms were recovered by perfusion (Lewis, 1998). A liver smear was made from each animal to confirm infection status and to view eggs passed from the female's body, which will be referred to as 'expelled eggs'. Worms were placed in 100% ethanol, and stored at 4 °C for 2–10 weeks. The identity and prevalence of all schistosomes recovered from these hosts will be presented in a separate survey paper. The following description focuses only on the rodents infected with *S. kisumuensis* n. sp.

Snails were collected at Nyabera swamp monthly throughout the study period. Snails were isolated in individual wells of tissue-culture plates in aged tap water for 6–12 h and examined for shedding of cercariae in a natural light cycle. Infected snails were given an individual identification number and their cercariae were (1) used to infect mice (Swiss Albino, male and female, 6–7 weeks old) or hamsters (males, 10 weeks old), or (2) preserved in 100% ethanol for subsequent genetic analysis.

Livers containing eggs were minced by passing through a 212 μm mesh metal sieve, and placed in water for miracidial hatching for 1–12 h. Snails, collected at Nyabera swamp, *Bulinus globosus* Morelet 1868, *Bulinus forskalii* (Ehrenburg, 1831), and previously screened numerous times for cercarial shedding and found to be negative, were exposed to water from a liver mixture and/or to water containing liver fragments. Exposed snails were maintained in the laboratory for up to 10 weeks, and periodically checked for cercarial shedding.

Morphological examinations

Eggs were measured fresh, either as intrauterine eggs or from liver smears using 400x magnification on an Olympus BX2 microscope (Olympus Corp., Center Valley, PA, USA) and an Nikon Coolpix 990 digital camera. Adult worms were photographed, alive or after placement in 100% ethanol. All body measurements were based on worms placed in ethanol. Therefore, the presented size data must be interpreted with caution, since the preservation in alcohol may shrink worms. Worm body length was measured, before the posterior 10–15% of each worm was removed, retained in 100% ethanol, and saved for DNA analysis. Worms were then stained with Semichon's acetocarmine (Pritchard and Kruse, 1982), counterstained with fast green and mounted in Canada balsam. Morphometric data were recorded (Table 2). Drawings were done by image overlay in Microsoft PowerPoint (Microsoft Corp., Seattle, WA, USA). All measurements are in micrometers (μm) unless otherwise stated.

Molecular analyses

Genomic DNA from whole adults, parts of adults, or single cercariae, was extracted using a modified

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Table 3. List of taxa and sequences used in this study

(Taxa in bold type are previously unreported sequences.)

Species	GenBank Accession			
	*cox*1	*nad* 6	12S	28S
Austrobilharzia terrigalensis	AY157195			AY157249
Heterobilharzia americana	AY157192			AY157246
Schistosoma kisumuensis n. sp. A	**FJ897157**	**FJ897161**	**FJ897165**	**FJ897153**
Schistosoma kisumuensis n. sp. B	**FJ897158**	**FJ897162**	**FJ897166**	**FJ897154**
Schistosoma kisumuensis n. sp. C	**FJ897159**	**FJ897163**	**FJ897167**	**FJ897155**
Orientobilharzia turkestanicum	AY157200			EU436661
Schistosoma bovis Kenya	**FJ897160**	**FJ897164**	**FJ897168**	**FJ897156**
Schistosoma bovis Tanzania	AY157212			AY157263
Schistosoma curassoni	AJ519516	AJ416895	AJ419786	AY157264
Schistosomatium douthitti	AY157193			AY157247
Schistosoma edwardiense	AY197347			AY197344
Schistosoma guineensis Cameroon	AJ519522	AJ416902	AJ419787	AJ519528
Schistosoma guineensis NHM 1970	AJ519523	AJ416903	AJ419788	AJ519525
Schistosoma guineensis NHM 2758	AJ519517	AJ416896	AJ419783	AJ519526
Schistosoma haematobium	EU567129			EU567125
Schistosoma haematobium NHM 3572	AJ519520	AJ416900	AJ419782	EU567124
Schistosoma hippopotami	AY197346			AY197343
Schistosoma incognitum	AY157201			AY157255
Schistosoma indicum	AY157204			AY157258
Schistosoma intercalatum Congo	AJ519515	AJ416894	AJ419779	AJ519529
Schistosoma intercalatum NHM 3396	AJ519519	AJ416899	AJ419781	AJ519527
Schistosoma japonicum	AF215860	AF215860	AF215860	Z46504
Schistosoma leiperi	AY157207			AY157261
Schistosoma malayensis	AY157198			AY157252
Schistosoma mansoni	AF216698	AF216698	AF216698	Z46503
Schistosoma margrebowiei	AY157206			AY157260
Schistosoma mattheei	AJ519518	AJ416897	AJ419789	AY157265
Schistosoma mekongi	NC_002529	NC_002529	NC_002529	AF465922
Schistosoma nasale	AY157205			AY157259
Schistosoma rodhaini	AY157202			AY157256
Schistosoma sinensium	AY157197			AY157251
Schistosoma spindale	NC_008067	NC_008067	NC_008067	AY157257
Schistosoma sp.	AY197348			AY197345
Trichobilharzia ocellata	AY157189			AY157243
Trichobilharzia regenti	DQ859919			AY157244

HotShot method (Truett *et al*. 2000; Steinauer *et al*. 2008*b*). A single *Schistosoma bovis* (Sonsino, 1876) worm recovered from the rodent *Mastomys natalensis* (Smith, 1834) was also included in the subsequent analysis. DNA was amplified using protocols and primers to mirror the work by Kane *et al*. (2003). Partial fragments of *cox*1, *nad*6, 12S and 28S were amplified using GO Taq (Promega, Madison, WI) and a standard PCR protocol using the following primers: Cox1 Schist 5′ and Cox1 Schist 3′ for *cox1* (Lockyer *et al*. 2003), RKND6F and RKND6R3 for *nad6*, RK12SF and RK12SR2 for 12S (Kane *et al*. 2003), and LSU5' and LSU3' for 28S (Littlewood and Johnston, 1995). Extracted DNA was stored at 4 °C until PCR amplification. PCR reactions were analysed by agarose gel electrophoresis, with the use of 1·0% agarose gels, stained with 0·5% GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, California), and visualized on a UV transilluminator. Amplicons were purified by use of a Montage SEQ96 Cleanup Kit (Millipore, Billerica, Massachusetts). Purified DNA products were then sequenced with the use of the BigDye version 3.1 kit (Applied Biosystems, Foster City, California) in an ABI 3130x sequence analyser (Applied Biosystems). Both strands of the amplified DNA fragments were sequenced, edited in Sequencer version 4.7 (Gene Codes, Ann Arbor, Michigan), and manually corrected for ambiguous base calls. Sequences from other species of *Schistosoma* and outgroups were retrieved from GenBank (Table 3). Protein coding regions of *cox*1 and *nad*6 were aligned by eye. Alignments of 12S and 28S rRNA were done with ClustalX (Larkin *et al*. 2007), and refined by eye to minimize gap openings and to remove unalignable regions.

Two phylogenetics analyses were performed. First, we used combined *cox*1 and 28S sequence data to place the proposed new species within the larger context of the family Schistosomatidae (Snyder, 2004; Webster *et al*. 2006). The second analysis was performed with combined *cox*1-12S-*nad*6-28S regions to reconstruct relationships within the

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S. *haematobium* group, including our proposed new species (Table 3). Phylogenetic analyses using maximum parsimony (MP), maximum likelihood (ML), and minimum evolution (ME) were carried out using PAUP* ver 4.0b10 (Swofford, 2002) and Bayesian inference (BI) using MrBayes (Huelsenbeck *et al.* 2001; Ronquist and Huelsenbeck, 2003). jModeltest (Posada, 2008) was used to determine the best nucleotide substitution model for ML and ME analyses. In cases where the Bayesian Information Criteria (B.I.C) and Akaike Information Criteria (A.I.C) selected different models, both were used in analyses and in all cases, the tree topologies were the same. The model TIM + I + G, was selected for the *cox*1-28S data matrix, and GTR + I + G was selected for the *cox*1-12S-*nad*6-28S data matrix, for the ME and ML analyses. For BI, a mixed model approach was implemented to account for the potential differences in evolutionary model parameters between data partitions (both genes and codon positions). Parsimony trees were reconstructed using heuristic searches, random taxon-input order and tree-bisection and reconnection (TBR) branch swapping. Optimal ME and ML trees were determined from heuristic searches (500 replicates for MP and ME; 5 replicates for ML), random taxon-input order, and TBR. Nodal support was estimated by bootstrap (500 replicates) and was determined for the MP and ME trees (100 replicates for the ML bootstrap of the *cox*1-12S-*nad*6-28S matrix), using heuristic searches, each with random taxon-input order. In all BI the data were partitioned by codon position for *cox*1 and *nad*6, and by genes for 12S and 28S. For the BI of the *cox*1-28S data set, there were four partitions defined by *cox*1pos1, *cox*1pos2, *cox*1pos3 (Nst = 6 rates-invgamma ngammacat = 4), and 28S (Nst = 6 rates = gamma ngammacat = 4). For the BI of the *cox*1-12S-*nad*6-28S dataset, there were 8 partitions defined by first *cox*1pos1, *cox*1pos2, *cox*1pos3, *nad*6-pos1, *nad*6pos2, *nad*6pos3 (Nst = 6 rates-invgamma ngammacat = 4) and 12S and 28S (Nst = 6 rates = gamma ngammacat = 4). All parameters were unlinked between partitions. For all analyses, 4 chains were run simultaneously for 1×10^5 generations, trees were sampled every 100 cycles, the first 10 000 trees with pre-asymptotic likelihood scores were discarded as burnin, and the retained trees were used to generate 50% majority-rule consensus trees and posterior probabilities. Genetic distances of the *cox*1 were calculated with MEGA 2.0 (Kumar *et al.* 2001) using the p-distance options.

The methods used in this project were approved by the University of New Mexico's Institutional Animal Care and Use Committee (IACUC), and the Scientific Steering Committee of the Kenya Medical Research Institute, and the KEMRI/National Ethics Review Board of Kenya. All investigators/assistants in this study attained animal use certification regarding the ethical treatment of animals.

Table 4. Hosts collected with *Schistosoma kisumuensis* n. sp.

(Host No. – refers to the identification number in the National Museum of Kenya.)

			Worms	
Date collected	Host No.	Host	Males	Females
20 June 2007	169428	*Pelomys isseli*	1	
21 Sept 2007	16593	*Pelomys isseli*	1[A]	
19 Oct 2007	16695	*Mastomys natalensis*	1*	
13 Dec 2007	16791	*Pelomys isseli*	1	
14 Dec 2007	167811	*Pelomys isseli*	1[B]	
14 Dec 2007	167821	*Pelomys isseli*	1	
15 Dec 2007	167807	*Pelomys isseli*		2*
15 Dec 2007	167806	*Dasymys incomtus*	1[C]	
12 Sept 2008	168316	*Pelomys isseli*§	2	1
16 Sept 2008	168295	*Pelomys isseli*	1	1
16 Sept 2008	168313	*Pelomys isseli*	1*	

* Immature worm.
§ Male and 1 female worm collected *in copula*.
A–C, genotypes included in the phylogenetics analysis.

RESULTS

Parasites

Over 70 trap days and nights, 276 small mammals were caught and perfused, including 247 rodents of 16 species and 29 insectivores of 4 species. Hosts were deposited in the National Museum of Kenya, Nairobi (Table 4). Of these, 11 rodents of 3 species were found to contain 15 unidentified schistosomes (Table 4). All were collected from rodents of the family Muridae, most often in *Pelomys isseli* (de Beaux, 1924), Issel's groove-toothed swamp rat. One specimen each was also recovered from *Mastomys natalensis*, the Natal multimammate mouse, and *Dasymys incomtus* (Sundevall, 1847), the African marsh rat. Of these 15 worms, DNA was extracted from 2 whole worms and from posterior ends of 13 worms. Four of these worms were immature; 1 female worm was destroyed during the staining procedure. Measurements used for the following analyses were thus based on the remaining mature males ($n = 6$) and females ($n = 2$). Eggs were collected from the livers of 4 hosts, and measurements are based on 12 intrauterine eggs and 22 expelled eggs. Although all hosts with patent infections, defined by the presence of eggs in the liver, contained at least 1 worm, both male and female worms were recovered together from only 1 host, and were collected *in copula*. The other female from this host was unpaired. The reason for not finding both sexes in all patently infected rodents could be loss during perfusion due to the small worm size, or death of worms after oviposition. However, in several cases where only a single worm was recovered by perfusion, the

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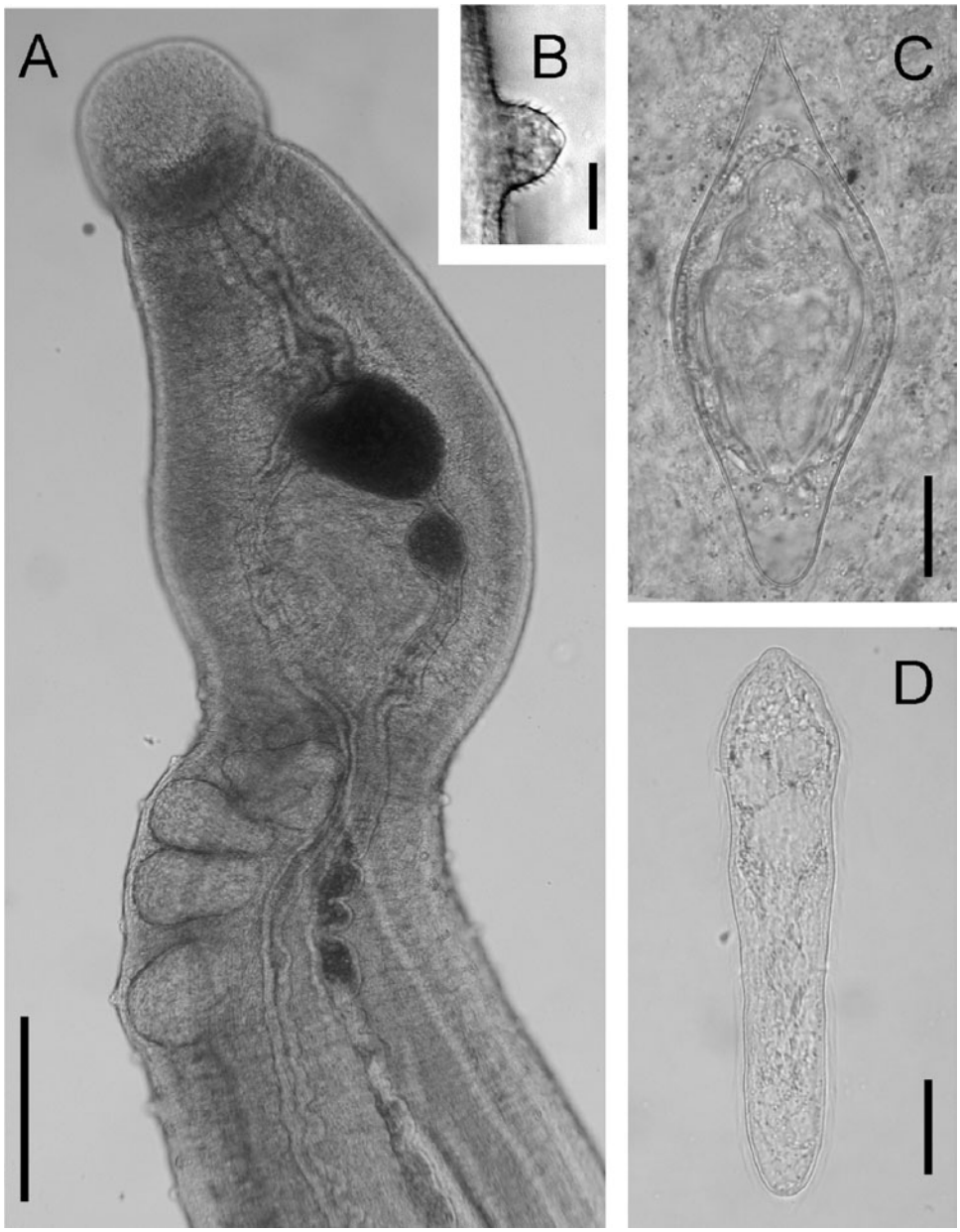

Fig. 2. *Schistosoma kisumuensis* n. sp. (A) Unfixed, live adult male, scale bar = 100 μm (note that the ventral sucker is below the specimen in a separate plane of view). (B) Tubercle with spines, scale bar = 5 μm. (C) Egg from liver, scale bar = 25 μm. (D) Miracidium, scale bar = 25 μm.

rodent was examined manually and in no cases were additional worms found in the vascular system or body cavity.

Snail collection at Nyabera swamp, based on shell morphology, consisted of the following gastropods: *Biomphalaria sudanica* (Martens, 1870), *Bulinus globosus*, *Bulinus forskalii*, *Ceratophallus natalensis* (Krauss, 1848), *Lymnaea natalensis* Krauss, 1848, and *Melanoides tuberculata* (Müller, 1774). Of 2066 *B. sudanica* snails collected at Nyabera swamp, 11 shed *S. mansoni* cercariae. Of 211 *B. globosus*, 3 shed mammalian cercariae, and of 194 *B. forskalii*, 1 shed mammalian cercariae. Genetic analysis of cercariae from these snails indicated that the infections were of *S. bovis* in *B. forskalii* and of *Schistosoma haematobium* (Bilharz, 1852) in *B. globosus*. The identity of these cercariae was confirmed by genetic analysis of adult worms retrieved from laboratory-infected mice and hamsters (data not shown). None of the other snail species shed mammalian schistosome cercariae (*C. natalensis*, $n = 190$; *L. natalensis*, $n = 124$; *M. tuberculata*, $n = 155$). Snails shedding *S. kisumuensis* were not found and we hope that future work may identify the natural intermediate host.

Schistosoma kisumuensis eggs were noted in 8 livers. In each of these livers, only 1 egg type was observed. Five attempts were made to hatch miracidia from these livers. Unhatched eggs were observed within the homogenized liver tissue. Miracidia hatched only from a single sample (Fig. 2D). None of the snails

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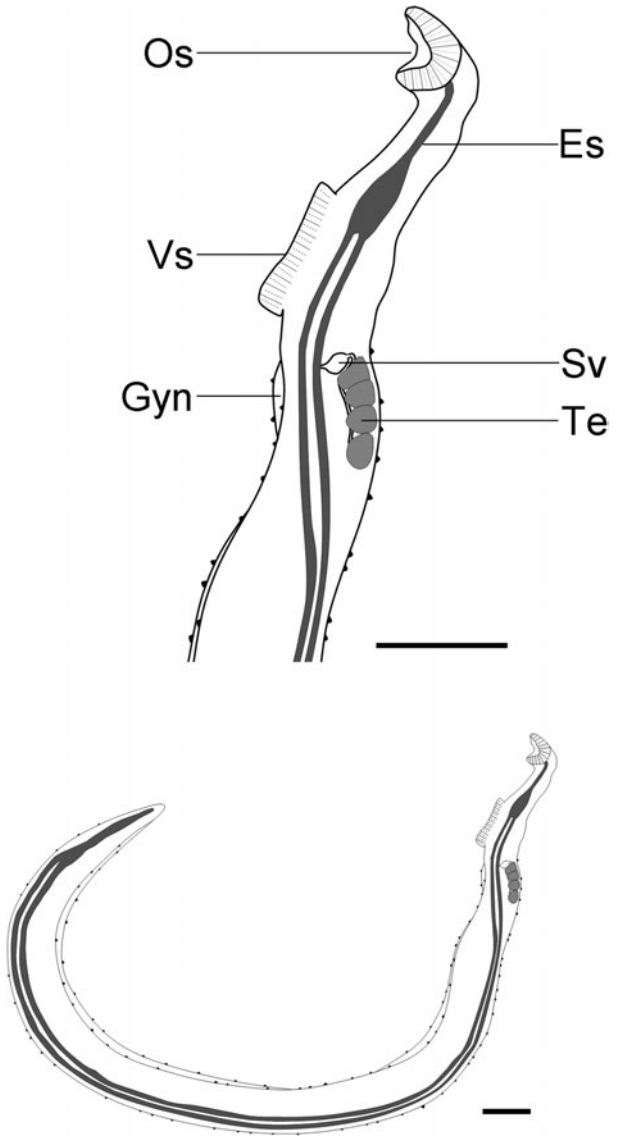

Fig. 3. Drawing summarizing the morphology of *Schistosoma kisumuensis* n. sp. adult male. Es, esophagus; Gyn, gynaecophoric canal; Os, oral sucker; Sv, seminal vesicle; Te, testes; Vs, ventral sucker. Scale bars = 200 μm.

exposed to miracidia or liver pieces shed cercariae within 10 weeks of exposure.

Morphology

Schistosoma kisumuensis n. sp.

Type-host: *Pelomys isseli* (de Beaux, 1924), Issel's groove-toothed swamp rat (Rodentia: Muridae).

Other hosts: *Mastomys natalensis* (Smith, 1834), the Natal multimammate mouse (Rodentia: Muridae), *Dasymys incomtus* (Sundevall, 1847), the African marsh rat (Rodentia: Muridae) deposited at the National Museum of Kenya (Table 1).

Intermediate host: unknown.

Type-locality: Nyabera swamp, Kisumu, Nyanza Province, Kenya, 0·10914°S, 34·775°E.

Type-specimens: Holotype (male) and allotype (female).

Site: Vascular system.

Type-material: The type series consists of 5 adult males, 1 adult female, 1 pair of adult worms *in copula*, 2 immature males and 1 immature female, all of which were preserved in ethanol. All types are deposited at the Parasitology Division, Museum of Southwestern Biology, University of New Mexico, New Mexico, USA, numbers MSBPARA164–MSBPARA173. The holotype (male) is MSBPARA165 and the paratype (female) is MSBPARA172.

Etymology: The species is named for the collection locality, Kisumu, Kenya.

Description (Figs 2–5)

Male. Mature male is elongate, but relatively short, from 3·2–6·9 mm long by 0·18–0·32 mm wide (Table 2, Figs 2A and 3). Well-defined gynaecophoric canal extends from behind ventral sucker to tip of the posterior extremity. Posterior to ventral sucker, tegument is covered with tubercles (Fig. 2B), decreasing in size and number posteriorly. Tubercles covered with fine spines. Constriction present at the anterior end of body, just posterior to ventral sucker (Fig. 1A). Oral sucker, 87–163, is funnel shaped. Ventral sucker, 151–223, is large and does not appear to be supported by a fleshy peduncle. Oesophagus extends posteriorly to the ventral sucker where it bifurcates into the gut caecae. The 2 branches reunite near the posterior end of the body. The testes lie dorsally and their anterior extent is at the same position as the anterior end of the gynaecophoric canal. Testes from 4–6, not arranged in linear manner, and often overlap. Testes size tends to decrease anterior to posterior, oval and round to ovoid (nearly triangular) in shape. Seminal vesicle lies immediately anterior to testes, opens into anterior margin of gynaecophoric canal immediately posterior to ventral sucker. Immature males had small body size, lacked fully developed testes.

Female. Body filiform, longer than male, 5·2 and 7·6 mm long, 0·22 and 0·23 mm wide, posterior half of body expanded (Fig. 4). Posterior extremity tapered and rounded. Tegument smooth without tubercles or spines. Oral sucker similar to that of male, except smaller 36 and 58. Ventral sucker not as well developed and much smaller 27 and 33·5, than that of male, appearing rudimentary. Caecum branches in region of the ventral sucker reunites posterior to ovary, and terminates near posterior extremity of worm. Ovary, 257 and 412 long, 122 and 125 wide, situated at same region where the body expands, just anterior to the body's midpoint. Uterus straight tube, starts at ovary and terminates just posterior to ventral sucker where it opens through genital pore. Uterus has greater diameter posteriorly, and expands in anterior 25% only when containing egg. Vitellaria

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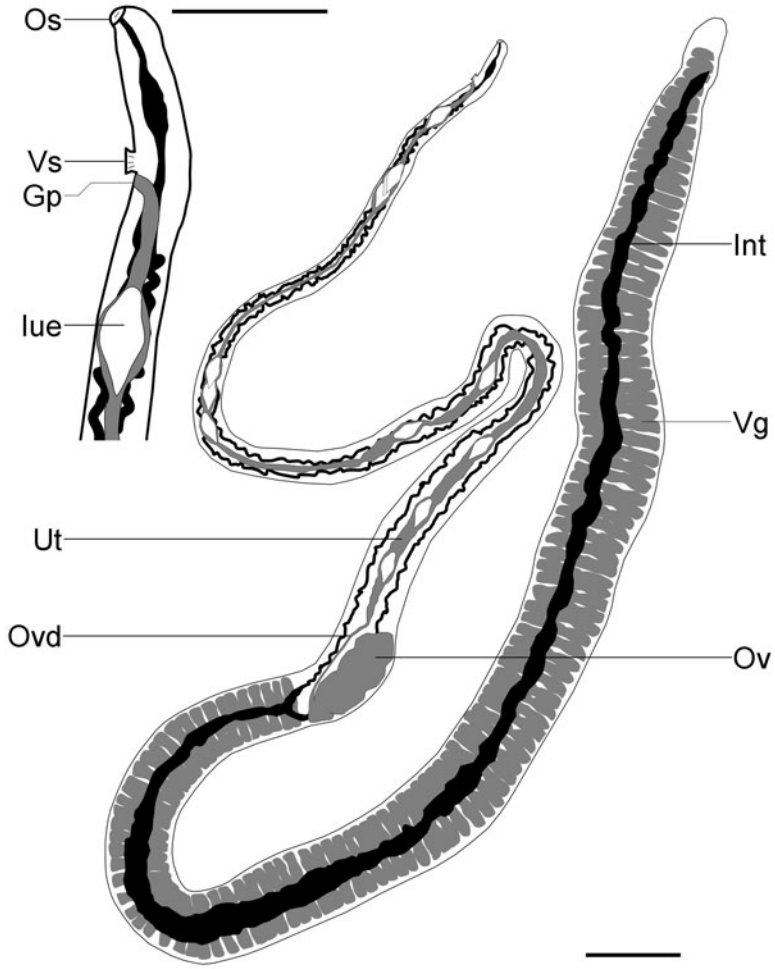

Fig. 4. Drawing summarizing the morphology of *Schistosoma kisumuensis* n. sp. adult female. Gp, genital pore; Int, gut caecum; Iue, intrauterine egg; Os, oral sucker; Ov, ovary; Ovd, oviduct; Ut, uterus; Vg, vitelline gland; Vs, ventral sucker. Scale bars: bottom = 250 μm; top = 125 μm.

extensive, occupying roughly the posterior 50% of worm and extend further posteriorly than intestine.

Eggs. Females contain 5 and 10 intrauterine eggs, which are smaller and differently shaped than expelled eggs. Expelled eggs were obtained from the liver of the hosts (Figs 2C and 5; Table 5). Intrauterine eggs 41·0 (34·7–45·3) wide and 96·1 (81·3–106·7) long, ovoid shape, pointed end but lacking distinct spine. Expelled eggs, relatively small, compared with other *S. haematobium* group eggs, and diamond shaped, 52·9 (49·0–58·0) wide and 135·2 (123·0–142·7) long, similar in shape to eggs of *S. intercalatum*. As compared to intrauterine eggs, expelled eggs larger and longer and more diamond-shaped. Intrauterine eggs have a broader size range than expelled eggs.

MOLECULAR ANALYSES

Fifteen worms of the proposed new species were sequenced for *cox*1 (1197 bp), 12S (779 bp), *nad*6 (610 bp), and 28S (1248 bp). The dataset used in the phylogenetic reconstructions was trimmed after alignment with the available sequence data in GenBank (Table 3) to include 1137 bp *cox*1, 729 bp 12S, 420 bp *nad*6, and 704 bp 28S. The combined 4 DNA regions of the 15 worms varied slightly between individuals (Genetic distances $<0{\cdot}01\%$); 3 of the more variable genotypes (Table 4) were included in the subsequent phylogenetic analyses, and are denoted as *Schistosoma kisumuensis* A, B, C (Figs 6 and 7).

Aligned *cox*1 and *nad*6 sequences appeared to be genuine mitochondrial sequence rather than nuclear copies, based on the lack of stop codons and the similarity of the translated protein sequences to those of close relatives. MP, ML, ME, and BI analyses each produced 1 tree per data matrix. Trees recovered from these analyses were largely congruent, and no conflicts received high support from bootstrapping or Bayesian posterior probabilities (Figs 6 and 7). *Schistosoma kisumuensis* was strongly supported as a clade. The *cox*1-28S tree supported the inclusion of *S. kisumuensis* within the family Schistosomatidae and within the clade containing species of

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Table 5. Measurements of eggs of *Schistosoma intercalatum* and *Schistosoma kisumuensis* n. sp.

(Data are mean followed by standard deviation or range; measurements are given in μm.)

Species	*S. kisumuensis* n. sp.	*S. intercalatum*	*S. intercalatum*§	*S. intercalatum*§
Source	Present study	Fisher, 1934	Bjorneboe and Frandsen, 1979	Wright *et al.* 1972
Intrauterine eggs				
N	12	*		*
Length	96·1 (±7·1)	130		140 (±10·7)
Width	41·0 (±3·4)	40		36·7 (±2·4)
Length/width ratio	2·34	3·25		3·81
Expelled eggs				
N	22	*	*	*
Length	135·2 (±5·5)	175 (140–240)	187·2 (±16·5)	167 (±17·8)
Width	52·9 (±2·27)	60 (50–85)	59·6 (±7·1)	58·4 (±8·8)
Length/width ratio	2·56	2·92	3·14	2·86

* Unknown data.
§ Eggs were derived from experimentally infected rodents.

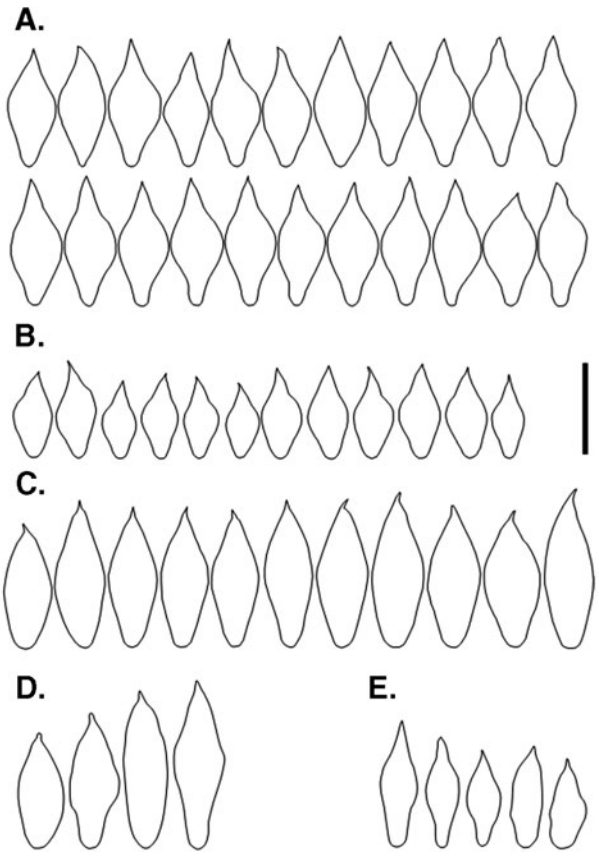

Fig. 5. A comparison of egg morphology of (A) expelled and (B) intrauterine *Schistosoma kisumuensis* n. sp. eggs; (C) mature *S. intercalatum* eggs from the feces of rodents (redrawn from Wright *et al.* 1972); and eggs from the original species description of *S. intercalatum* (D) expelled, and (E) intrauterine eggs (redrawn from Fisher, 1934). Scale bar = 100 μm, but note that D and E were drawn without scale bars but eggs are sized to average measurements as given by Fisher (1934).

Schistosoma and *Orientobilhazia* (Fig. 6). *Schistosoma kisumuensis* grouped within the clade defined by egg shape and by molecular data (Morgan *et al.* 2003; Kane *et al.* 2003; Webster *et al.* 2006) as the S. *haematobium* group. For a more detailed analysis, a smaller data matrix was created that included all available gene regions for the *S. haematobium* group. The *cox*1-12S-*nad*6-28S tree (Fig. 7) shows strong node support for *Schistosoma kisumuensis* as sister to *S. intercalatum* Fisher, 1934.

Genetic distances of *cox*1 among the 3 *S. kisumuensis* individuals were 0·1%, 0·2%, and 0·3%. The distance between *S. kisumuensis* and *S. intercalatum* is 6·3%, which falls within the pairwise distance values between other species of the *S. haematobium* group (Table 6).

DISCUSSION

Schistosoma kisumuensis n. sp. is member of the genus *Schistosoma* based on morphological and molecular characters. Genetic distance data indicate that *S. kisumuensis* is a distinct genetic lineage that merits recognition as a new species. Taken together, adult and egg morphology, and phylogenetic analyses firmly place this new species within the *S. haematobium* group, as the sister species to *S. intercalatum*.

Eight species comprise the *S. haematobium* group: *S. bovis*, *S. curassoni* Brumpt, 1931, *S. haematobium*, *S. intercalatum*, *S. leiperi* Le Roux, 1955, *S. margrebowiei* Le Roux, 1933, *S. mattheei* Veglia and Le Roux, 1929, and the most recently described *S. guineensis* Pagés, 2003. Previous studies have found that morphological features of adult flukes of the *S. haematobium* group vary and often overlap (Pitchford, 1965; Pagès *et al.* 2001). Adult morphology of *S. kisumuensis* closely resembles that of members of the *S. haematobium* group, with the exception of the small adult size of *S. kisumuensis*, a trait likely affected by the preservation of worms we collected in alcohol. Available male and female worms are 2–3 times smaller than other species within the

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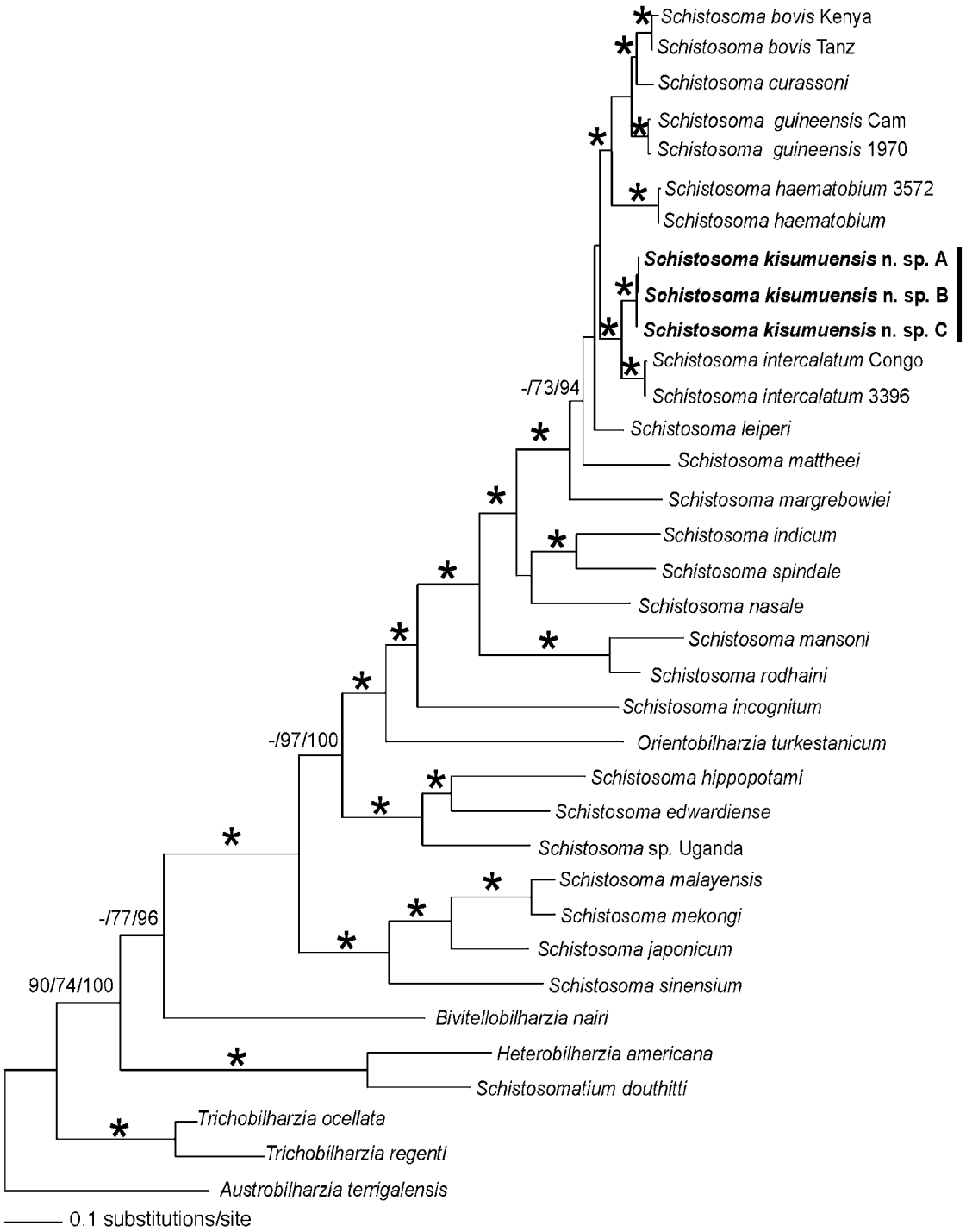

Fig. 6. Maximum likelihood tree based on the combined partial *cox*1 and 28S sequence data. *Schistosoma kisumuensis* n. sp. isolates are indicated in bold. Numbers at branch nodes indicate MP and ME bootstrap values and Bayesian PP, respectively. * Indicates MP and ME bootstrap values of >65 and Bayesian PP of 100. – Indicates <55% bootstrap support.

S. haematobium group (Loker, 1983). It should be noted that adult worm length of schistosomes is variable and is not always a suitable diagnostic trait, since size variation can occur with host species (Wright *et al.* 1972). Spined tubercles are shared by all members of the *S. haematobium* group, with the exception of *S. bovis* and *S. mattheei*, which possess tubercles without spines.

The eggs of *S. kisumuensis* are unique when considering their length to width ratio and size. Expelled eggs are about 75% the length of eggs of its sister species *S. intercalatum*. Similarly, intrauterine eggs are about 70% of the length of those of *S. intercalatum*. Expelled *S. kisumuensis* eggs are diamond-shaped, exhibiting blunt 'shoulders' on both ends and have a mean length to width ratio of 2·55, in

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Table 6. Mitochondrial (*cox*1) genetic distances of species within the *Schistosoma haematobium* group

Schistosoma species	1.	2.	3.	4.	5.	6.	7.	8.
1. *S. bovis*								
2. *S. curassoni*	0·052							
3. *S. guineensis*	0·064	0·061						
4. *S. haematobium*	0·103	0·106	0·106					
5. *S. kisumuensis* n. sp.	0·094	0·102	0·095	0·106				
6. *S. intercalatum*	0·109	0·114	0·122	0·106	0·063			
7. *S. leiperi*	0·098	0·093	0·928	0·095	0·092	0·090		
8. *S. mattheei*	0·122	0·123	0·128	0·133	0·129	0·127	0·126	
9. *S. margrebowiei*	0·133	0·125	0·131	0·138	0·132	0·128	0·125	0·147

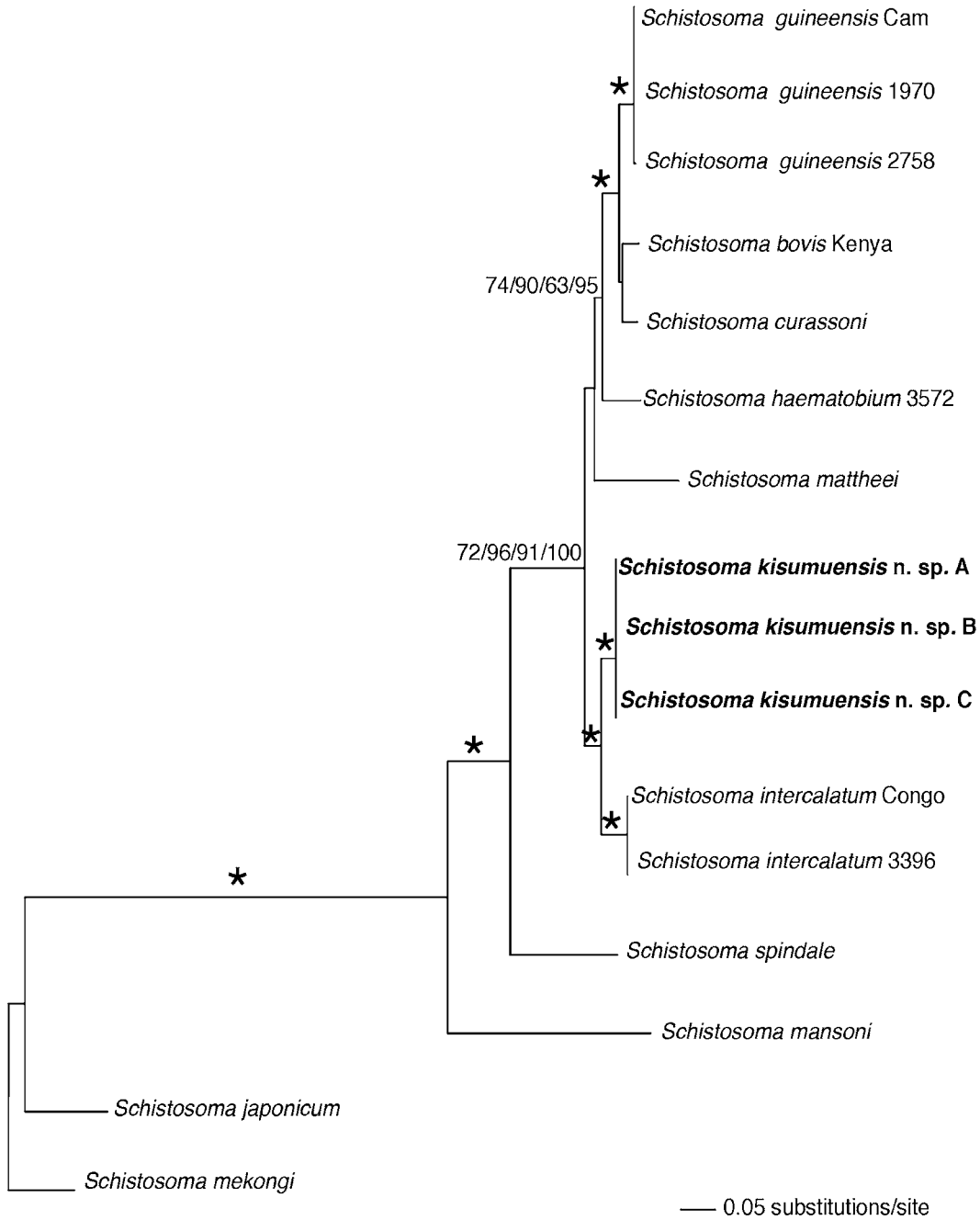

Fig. 7. Maximum likelihood tree based on the combined partial *nad*6, cox1, 12S, and 28S sequence data. Isolates of *Schistosoma kisumuensis* n. sp. are indicated in bold. Numbers at branch nodes indicate MP, ME, and ML bootstrap values and Bayesian PP, respectively. The '*' indicates MP, ME, and ME bootstrap values of >90 and PP of 100.

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contrast to mature *S. intercalatum* eggs, which are more elongate and oval, rarely contain shoulders, and have a mean length to width ratio of 2·9. The egg morphology of *S. kisumuensis* is more similar to some of the eggs of *S. intercalatum* drawn by Fisher (1934) who based his drawings on eggs collected from humans (Fig. 5D), and less similar to those of *S. intercalatum* drawn by Wright *et al.* (1972), who based his drawings on eggs collected from experimentally infected mice (Fig. 5C). Additionally, unlike *S. intercalatum*, eggs of *S. kisumuensis* do not contain a true spine; rather the anterior end tapers to a spine-like point.

The intermediate host of *S. kisumuensis* is unknown. It has been well established that other members of the *S. haematobium* group primarily use snails of the genus *Bulinus*; *Schistosoma intercalatum* uses bulinid snails of the *B. africanus* group (Brown, 1994). Thus, it is likely that *S. kisumuensis* also uses bulinid snails as an intermediate hosts, although the discovery of African members of *Schistosoma* in *Ceratophallus* snails (Morgan *et al.* 2003) suggests caution is warranted. *Biomphalaria sudanica*, *B. globosus*, *B. forskalii*, *C. natalensis*, *L. natalensis*, and *M. tuberculata* were all recovered from Nyabera, in proximity to where we found rodents infected with *S. kisumuensis*.

Schistosoma kisumuensis has only been found in murid rodents. Most of the species within the *S. haematobium* group are able to infect a relatively broad spectrum of definitive hosts, including rodents (Pitchford, 1977). For example, the sister species of *S. kisumuensis*, *S. intercalatum*, naturally infects humans (Fisher, 1934), experimentally infects sheep and goats (Schwetz, 1956*b*), and has been maintained in the laboratory in both hamsters (e.g. Wright *et al.* 1979) and mice (e.g. Frandsen, 1977). In one additional report, *S. intercalatum*-like eggs were found in naturally infected rodents, *Hybomys univittatus* (Peters, 1876), near Kisangani, Democratic Republic of the Congo (DRC), near the parasite's type locality (Schwetz, 1956*a*). Considering the fact that most species within the *S. haematobium* group can infect a relatively wide variety of hosts, it is conceivable that *S. kisumuensis* may also use hosts in addition to rodents, such as perissodactyls, artiodactyls, and primates.

Currently, *S. kisumuensis* has only been found in a single location in Kisumu, Kenya, located within the Lake Victoria Basin. Its sister species, *S. intercalatum*, appears to be confined to the adjacent Congo River Basin. *Schistosoma intercalatum* was first mentioned from a number of human intestinal schistosomiasis cases near Kisangani in what is now the Democratic Republic of the Congo (Chesterman, 1923), but was not described as a distinct species until nearly 10 years later (Fisher, 1934). Fisher (1934) deemed *S. intercalatum* as distinct from *S. bovis* and *S. haematobium*, based on egg shape and the observation that this schistosome produced terminal-spined eggs that were causing intestinal rather than urinary schistosomiasis in humans. Over the next decades, the range of *S. intercalatum* was extended to Lower Guinea forest regions, including Cameroon, Gabon, Equatorial Guinea, Nigeria and São Tomé (for review, see Tchuem Tchuente *et al.* 2003). However, all *S. intercalatum*-like worms collected outside of the DRC have been determined to be *S. guineensis*, a distinct and comparatively distantly related species (Webster *et al.* 2006). Since its original description from the infection focus within the upper reaches and tributaries of the Congo river, *S. intercalatum* has only been found in one other focus, Kinshasa city (De Clercq, 1987; Tchuem Tchuente *et al.* 1997), located about 300 kilometers from the delta of the Congo River.

Due to the sporadic nature of human infections, it has been suggested that *S. intercalatum* is a zoonotic parasite, and that its natural host is a 'forest-dwelling animal' (Wright *et al.* 1972). The distribution of many schistosomes is limited by that of their intermediate host species. However, *S. intercalatum*, remains highly restricted compared to the distribution of its intermediate hosts, members of the *B. africanus* group, found throughout large parts of Africa (Brown, 1994). Therefore, the distribution of this parasite has been suggested to be limited by its normal definitive hosts, thought to include monkeys, such as mangabeys, and forest dwelling rodents (Wright *et al.* 1972). *Schistosoma intercalatum* cercariae are shed during the day, concentrate at the water surface, congregate in large masses, and adhere easily to surfaces. Among mammalian schistosomes, this cercarial behaviour has been reported from *Schistosomatium douthitti* Cort, 1914), mainly a rodent parasite, *S. japonicum* Katsurada, 1904, which occurs in a large number of mammalian species, and *S. margrebowiei*, a parasite of antelopes. Thus, it is conceivable that *S. intercalatum* normally resides in a forest-dwelling mammal and only infects humans as a result of human incursions into pristine forests (Wright *et al.* 1972).

Based on available data, *S. intercalatum* and its sister species *S. kisumuensis* are confined to adjacent hydrographic basins. One explanation for this distribution pattern is that these two species derived from a common ancestor, possibly with a rodent definitive host, and formed one lineage in the Congo Basin and another in the Lake Victoria Basin. This idea is supported by geological events that shaped east central Africa. From 8·0–2·3 MYA (million years ago) Lake Obweruka encompassed present day Lake Edward, Lake Albert, and the basin in between (Pickford *et al.* 1993). During this time, east-to-west rivers, such as the Kafu, Katonga, and Kagera rivers, are hypothesized to have drained much of the East Africa Dome into Lake Obweruka,

the outlet of which flowed through the Beni Gap, into the Congo River and to the Atlantic (de Heinzelin, 1962; Pickford *et al.* 1993). During the Pleistocene (1·8 MYA – 10 000 years ago) fossil and geological evidence suggests that the connection to the Congo Basin of Lake Obweruka was severed, largely separating the Congo Basin and what would become the Lake Victoria Basin (Van Damme and Pickford, 1999). Finally, about 12 000 years ago, fault warping along the Central Rift Valley caused a regional tilt to the north in the Albert-Edward area, causing the east-to-west rivers to reverse course, creating Lake Victoria, while the northwards tilting of the Lake Albert and Lake Victoria Basins resulted in flows into the Nile system (Pickford *et al.* 1993). Thus, it is conceivable that *S. intercalatum* and *S. kisumuensis* were separated as long as 1·8 MYA or as recently as 10 000 years ago.

It is interesting to note that *S. kisumuensis* falls in the middle of a clade of schistosomes infective to humans. Although, no reports exist from the Lake Victoria Basin of humans passing terminal-spined eggs in their stools, fitting the description of *S. kisumuensis*, an intriguing report of such an infection focus stems from the Nile drainage, one that is contiguous with the Lake Victoria Basin. In this report, 6 patients were passing terminal-spined eggs in their stools in Rhino Camp, located in northwestern Uganda, approximately 500 miles northwest of Kisumu, Kenya (Odongo-Aginya *et al.* 1994). It has been suggested that these eggs could represent non-viable eggs produced parthenogenetically by the heterospecific pairing of an *S. mansoni* male and a *S. haematobium* female, resulting in the production of terminal-spined eggs in the feces (Tchuem Tchuente *et al.* 2003). However, since *S. haematobium* has not been reported from this area of Uganda, this explanation may not hold. Although the eggs recovered in Rhino Camp resemble those of *S. intercalatum*, their size was relatively small, $156 \times 59\,\mu$m, and the length to width ratio, 2·59, is much closer to that of *S. kisumuensis* (2·56) than to *S. intercalatum* (2·90). This raises the intriguing possibility that the infection focus described by Odongo-Aginya *et al.* (1994) could have been caused by *S. kisumuensis*. Studies on eggs from this part of Uganda using molecular tools to identify and possibly reveal cryptic species are urgently needed.

Although there are currently no definitive reports of humans passing these relatively small terminal-spined schistosome eggs, we do not find it impossible to think that this species could infect humans, especially since Nyabera swamp, the type locality for *S. kisumuensis*, supports many human activities. In the future, testing the human population specifically for infection with this new species in both Kenya and elsewhere may reveal whether it contributes to the problem of schistosomiasis in Africa.

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