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Schistosoma haematobium in Lake Malaŵi: susceptibility and molecular diversity of the snail hosts *Bulinus globosus* and *B. nyassanus*

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Abstract

Intermediate hosts of *Schistosoma haematobium*, the causative agent of urinary schistosomiasis, in Lake Malaŵi include: *Bulinus globosus*, a member of the *B. africanus* group and *B. nyassanus*, a diploid member of the *B. truncatus/tropicus* species complex. We compared genetic variability between isolates of *S. haematobium* from the southern part of the lake (Cape Maclear), where both *B. globosus* and *B. nyassanus* play a role as intermediate hosts, and isolates from the northern part, where only *B. globosus* is host. Data show that the *S. haematobium* isolates from these two areas of Lake Malaŵi cannot be distinguished using nuclear or mitochondrial sequences and are capable of cross-infections.

Introduction

Historically, *Bulinus globosus* was the only known snail host for *Schistosoma haematobium* in Lake Malaŵi. This snail typically inhabits inland, slow-moving waters and limited areas within the lake that are protected and, therefore, it was suspected that *S. haematobium* transmission in Lake Malaŵi was limited to such habitats (Stauffer *et al.*, 1997). Beginning in the mid-1980s, reports of schistosomiasis among tourists suggested that, for the first time, transmission was occurring in the open waters of Lake Malaŵi (Harries *et al.*, 1986; Whitworth, 1993; Pollner *et al.*, 1994; Cetron *et al.*, 1996). It was clear that the epidemiology of *S. haematobium* in Lake Malaŵi was changing, and the open waters of Lake Malaŵi were no longer free from urinary schistosomes.

Madsen *et al.* (2001) discovered an endemic snail, *Bulinus nyassanus*, infected with human schistosomes in the open waters of Nankumba Peninsula in southern Lake Malaŵi. Extensive sampling efforts (Stauffer *et al.*, 2006) yielded no infected *B. nyassanus* outside of Nankumba Peninsula; thus, we believed that open-water transmission was limited to this area. The school-aged children residing in Chembe Village (Nankumba Peninsula) had a very high prevalence of urinary schistosomiasis (>87% in 1998; Madsen *et al.*, 2001) and currently the highest found in villages along Lake Malaŵi (Stauffer *et al.*, 2006). Therefore, we postulated that the current high prevalence of human infection at Chembe Village is linked to *B. nyassanus* being an intermediate host in this portion of Lake Malaŵi.

The host specificity of *S. haematobium* can be divided as follows: strains most closely adapted to *B. truncatus*, a tetraploid member of the *B. truncatus/tropicus* species complex (North Africa and West Africa), strains most closely adapted to members of the *B. africanus* species group (West Africa, East Africa and southern Africa), and strains most closely adapted to the *B. forskalii* species group (Brown, 1994; Southgate *et al.*, 2000;

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Rollinson *et al.*, 2001). The finding of *B. nyassanus*, endemic to Lake Malaŵi and a diploid member of the *B. tropicus/truncatus* species group, as intermediate host in Lake Malaŵi was therefore unexpected.

We compare the genetic variation between *S. haematobium* from the southern part of the lake (Cape Maclear), where both *B. globosus* and *B. nyassanus* are intermediate hosts, and from the northern part (Likoma Island), and investigate the compatibility of the *S. haematobium* from Likoma Island with *B. nyassanus* from Chembe Village. Because infected *B. nyassanus* were collected only around Nankumba Peninsula in Lake Malaŵi, we concluded that *B. globosus* was the only intermediate host at Likoma Island (Stauffer *et al.*, 2006).

Methods

Bulinus globosus and *B. nyassanus* were collected from Cape Maclear (Nankumba Peninsula). Snails were placed in 6 ml of water in 12.5 ml beakers and exposed to light to stimulate cercarial shedding. Cercariae shed from each snail were collected and preserved in 99% ethanol for DNA extraction.

DNA extraction, DNA sequencing and phylogenetic analysis

Cercariae collected from three snails of each species were used for molecular analysis. The ethanol-preserved cercariae were centrifuged in a 1.5-ml Eppendorf tube at 1300 rpm for 3 min to collect the cercariae at the bottom of the tube. Most of the ethanol was pipetted from the sample, which was then left to air dry for 10–30 min, allowing any residual ethanol to evaporate from the sample. Once dry, total genomic DNA was extracted from each sample using the DNeasy™ Tissue Kit (Qiagen, West Sussex, UK) according to the manufacturer's protocol.

To identify any genetic variation between the samples, two nuclear DNA and four mitochondrial DNA partial regions were amplified by polymerase chain reaction (PCR), as shown in table 1. Amplifications were performed in a total reaction volume of 25 μl using Ready-to-go PCR Beads (Amersham Pharmacia Biotech, Buckinghamshire, UK), each containing 1.5 units DNA *Taq* polymerase, 10 mM Tris–HCl (pH 9), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP and stabilizers, including bovine serum albumin (BSA), 0.4 μM of each primer and 2 μl of DNA (~10 ng). Thermal cycling in a Perkin Elmer 9600 Thermal Cycler used the following PCR conditions: 5 min denaturing at 95°C: 40 cycles of 30 s at 95°C, 30 s at 40–55°C, 1–2 min at 72°C; followed by a final extension period of 7 min at 72°C. PCR products were visualized on a 0.8% ethidium bromide agarose gel and then purified using Qiagen PCR Purification Kits (Qiagen) according to the manufacturer's protocol. Sequencing was performed using Fluorescent Dye Terminator Sequencing Kits (Applied Biosystems, Foster City, USA) and the sequencing reactions run on either an Applied Biosystems 377 or a 373A automated sequencer. PCR products were sequenced directly using the original PCR primers; and for COX1, 28S and 18S, internal sequencing primers were also used to obtain the full sequence of each fragment from both strands (table 2). The sequences were assembled and manually edited using Sequencher version 4.5 (GeneCodes Corp., Ann Abor, USA) and submitted to EMBL/GenBank. Each DNA region from each sample was aligned using Sequencher and sequence differences were checked by visualization of the sequence chromatograms.

The 28S and COX1 sequences were aligned by eye in Maclade 4.05 (Sinauer Associates, Sunderland, Connecticut, USA) to sequence alignments of several other *Schistosoma* species and strains. Phylogenetic analyses were conducted separately using a neighbour-joining (NJ) algorithm to examine the evolutionary distance relationships among the samples.

Compatibility testing

Schistosoma haematobium eggs were obtained from urine samples from 10–15 infected children from Cape Maclear

Table 1. DNA regions utilized in this study and the primers used to amplify them. COX1, 16S, 28S and 18S regions were amplified as they have proved useful in phylogenetic studies, and the variable regions ND1 and a small section between Proline and COX1 were amplified as a result of preliminary data from studies by Littlewood, Webster & Huyse (pers. comm.) on *Schistosoma haematobium* mitochondrial genomes.

Region	Size (bp)	Forward primer	Reverse primer
Mitochondrial DNA			
COX1	1164	Cox1_Schist_5′ 5′-TCTTTRGATCATAAGCG-3′	Cox1_Schist_3′ 5′-TAATGCATMGGAAAAAAACA-3′
16S	897	16S_Schist_3′ 5′-GATAAGAACCAACCTGGC-3′	16S_Schist_5′ 5′-CTCGATGTTGGCTTGTTG-3′
ND1	464	S. haem_ND1_F CACCTTTAGCTTTTATTGCATGG	S. haem_ND1R TTAGAATGCTTCCGGCGTTA
Proline–COX1	242	S. haem_Pro_COX1F 5′-GAGTTTATAGTGAGTTGGTTAG-3′	S. haem_Pro_COX1R 5′-GAAAGCAAGCAGTCTTCTAAC-3′
Nuclear DNA			
28S	1110	LSU5 5′-TAGGTCGACCCGCTGAAYTTAAGCA-3′	1500R 5′-CGAAGTTTCCCTCAGGATAGCAAC-3′
18S	2552	WA 5′-GCGAATGGCTCATTAAATCAG-3′	WB 5′-GGAAGTAAAAGTCGTAACAAG-3′

Table 2. Internal sequencing primers.

Primer	Direction	Sequence 5′–3′
28S sequencing		
ECD2	R	CTTGGTCCGTGTTTCAAGACGGG
300F	F	CAAGTACCGTGAGGGAAAGTTG
1200R	R	CCGAAAGATGGTGAACTATGC
1200F	F	CCCGAAAGATGGTGAACTATGC
18S sequencing		
600R	R	TCAGGCTCCCTCTCCGGA
600F	F	AGGGTTCGATTCCGGAG
1200F	F	CAGGTCTGTGATGCCC
1200R	R	GGGCATCACAGACCTG
COX1 sequencing		
CO1560	F	TTTGATCGGAATTTTGGTAC
CO1800	R	CCAACCATAAACATGTGATG

and Likoma Island. Urine samples were washed three times in saline water and once in fresh water (filtered lake water; 20 μm filter). After sedimentation in conical flasks for 25 min in the dark, the supernatant in the absence of light was discarded and the flask filled with more washing medium (saline or filtered lake water). After washing in filtered lake water, the eggs were transferred to Petri dishes and more filtered lake water added. Hatching was stimulated by exposing the Petri dishes to direct sunlight. Miracidia were captured with a tapering glass pipette and each snail was exposed to 5–20 miracidia. The uninfected *B. nyassanus* and *B. globosus* that were exposed came from Chembe Village. We exposed 25 *B. globosus* and 25 *B. nyassanus* to miracidia from eggs collected from children at Chembe Village and 25 *B. globosus* to miracidia from eggs from children at Likoma Island. Subsequently, we

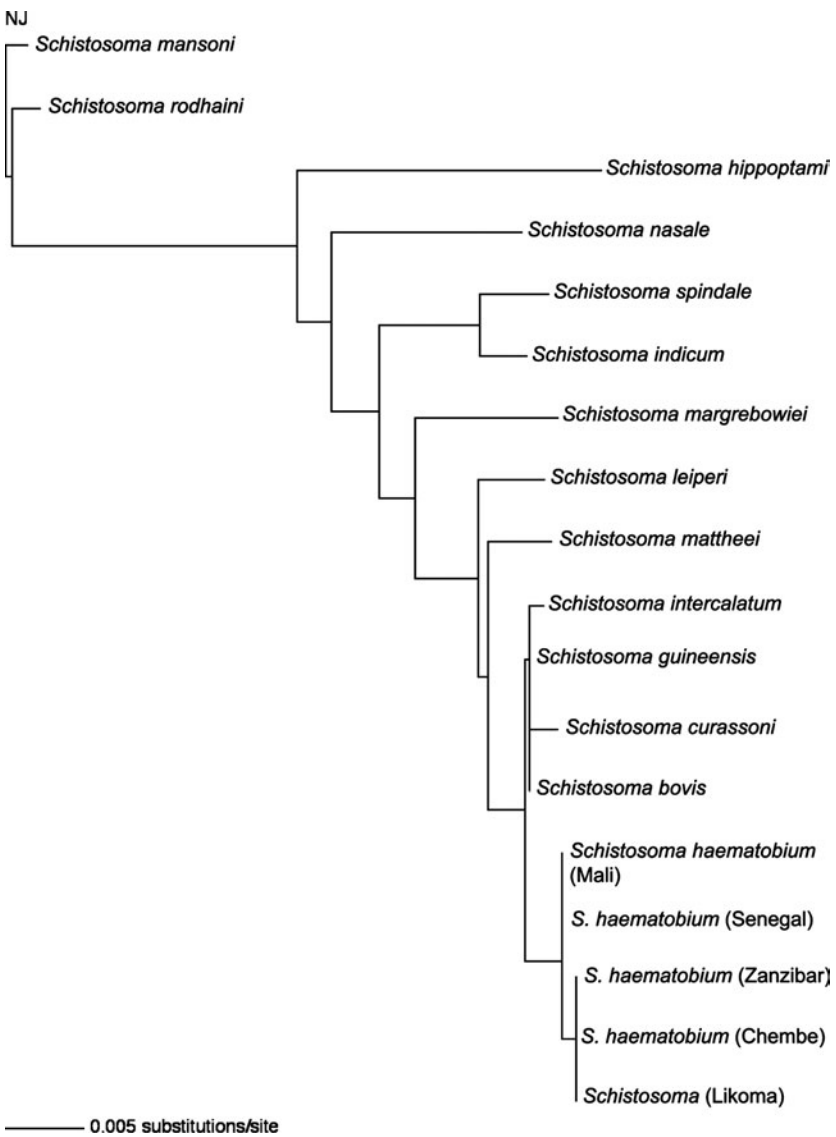

Fig. 1. Neighbour joining (distance tree); 28S (nuclear DNA).

repeated the exposure of 25 *B. nyassanus* from Chembe Village to miracidia from eggs captured from Likoma Island children. The exposed snails were kept in separate beakers under light for 6 h to facilitate infection, and maintained in aquaria (Madsen *et al.*, 2001). From 5 to 8 weeks after exposure, snails were checked individually once a week for cercarial release: snails were transferred to beakers as described above and exposed to indirect sunlight for 2 h. The water in each beaker was visually checked for the presence of cercariae.

Results

The 28S and 18S sequences from all the Lake Malaŵi isolates recovered from snails were identical, showing no nuclear genetic variation. The phylogenetic analysis of the 28S sequences showed that there is very little variation between the *S. haematobium* isolates incorporated into the tree, and the isolates from Lake Malaŵi match the Zanzibar strain of *S. haematobium* (fig. 1).

In all mitochondrial DNA sequences, there was very little variation between the Lake Malaŵi isolates (table 3). All nucleotide differences were also non-synonymous, suggesting that there is no genetic difference between the different isolates. The phylogenetic analysis of the COX1 sequences shows that there is very little variation between the different *S. haematobium* isolates incorporated into the tree. The Malaŵi isolates clearly group with other *S. haematobium* strains, but there is not enough genetic variation between the isolates to give clear resolution (fig. 2).

In the first exposure experiment, 13 *B. globosus* were infected from eggs that were captured from Chembe Village children, 10 *B. globosus* were infected from eggs that were captured from Likoma Island, and no *B. nyassanus* were infected. When we repeated the exposure of *B. nyassanus* to miracidia from eggs captured from Likoma Island children, one snail was infected.

Discussion

The lack of genetic variation between the *S. haematobium* isolates from the two different intermediate snail hosts was surprising, especially due to the fact that *B. nyassanus* (*B. truncatus/tropicus* complex) is an unusual intermediate host for this parasite. Within the *B. truncatus/tropicus* complex, most diploid species seem to be resistant to infection in nature (Brown, 1994), although *B. liratus* on the island of Madagascar appears to be an exception (Stothard *et al.*, 2001). The fact that *B. nyassanus* is also susceptible is therefore another interesting exception to the rule, because it is diploid (Madsen *et al.*, 2001), and is a species within the *B. truncatus/tropicus* complex (Jørgensen *et al.*, 2007). Both *B. tropicus* and *B. truncatus* are known to occur in other parts of Malaŵi and, given the infectivity of Lake Malaŵi strains of *S. haematobium* to *B. nyassanus*, it is recommended that further compatibility studies are conducted to assess the possible risk of the spread of schistosomiasis transmission to other areas.

Preliminary mitochondrial genome data from different strains of *S. haematobium* have suggested that surprisingly little genetic variation occurs between different strains and even between strains from very distant geographical areas (Littlewood, Webster & Huyse, pers. comm.). In this study, we amplified, together with some conserved regions, the most variable regions of the mitochondrial genomes, aiming to capture any genetic variation that might be occurring between the isolates. We postulated that the miracidia collected from children in Chembe Village would infect both *B. globosus* and *B. nyassanus*, since we have collected infected individuals of both snail species on Nankumba Peninsula. Although we have examined some 700 *B. nyassanus* (3.0–9.1 m depth) from sites on Likoma Island and sites along the mainland shore in the northern part of the lake for cercarial shedding, we have not found a single individual that was infected. Based on these data, we concluded that all of the urinary schistosomiasis found in inhabitants of Likoma Island originated from cercariae shed from *B. globosus*. Therefore, it is significant that we were able to infect one *B. nyassanus* with eggs that originated from children from Likoma Island. The fact that we exposed 50 *B. nyassanus* collected from Chembe Village, and only had one infected snail is consistent with the field observations that showed that of 24,775 *B. nyassanus* collected, only 87 (0.4%) were infected (Stauffer *et al.*, 2006). The fact that the *S. haematobium* from Lake Malaŵi can utilize *Bulinus* species from both the *B. truncatus/tropicus* and the *B. africanus* group is somewhat unique. Furthermore, the fact that miracidia isolated from children at Likoma Island, where the only infected snails found were *B. globosus*, suggests that the Lake Malaŵi *S. haematobium* always had the potential to infect both *B. globosus* and *B. nyassanus*.

Certainly, along the lake shore, where both *B. globosus* and *B. nyassanus* shed cercariae, the prevalence of infection in school-aged children is 2–3 times higher than where only *B. globosus* is a host (Stauffer *et al.*, 2006). Our current studies suggest that only in the waters of Nankumba Peninsula have population numbers of *B. nyassanus* reached a critical level in shallow waters, enabling it to become involved in the schistosome life cycle.

Table 3. Mitochondrial DNA nucleotide differences of *S. haematobium* isolates from different intermediate hosts and localities (number of single nucleotide polymorphisms) (accession numbers EU567124–EU567144) compared to the Likoma isolate.

Sample area and host	COX1	16S	ND1	Pro-COX1
Chembe, *B. globosus*				
1	0	0	0	0
2	1	2	3	3
3	0	0	0	0
Chembe, *B. nyassanus*				
1	0	0	1	0
2	0	0	0	1
3	0	0	0	0
Likoma, *B. globosus*				
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0

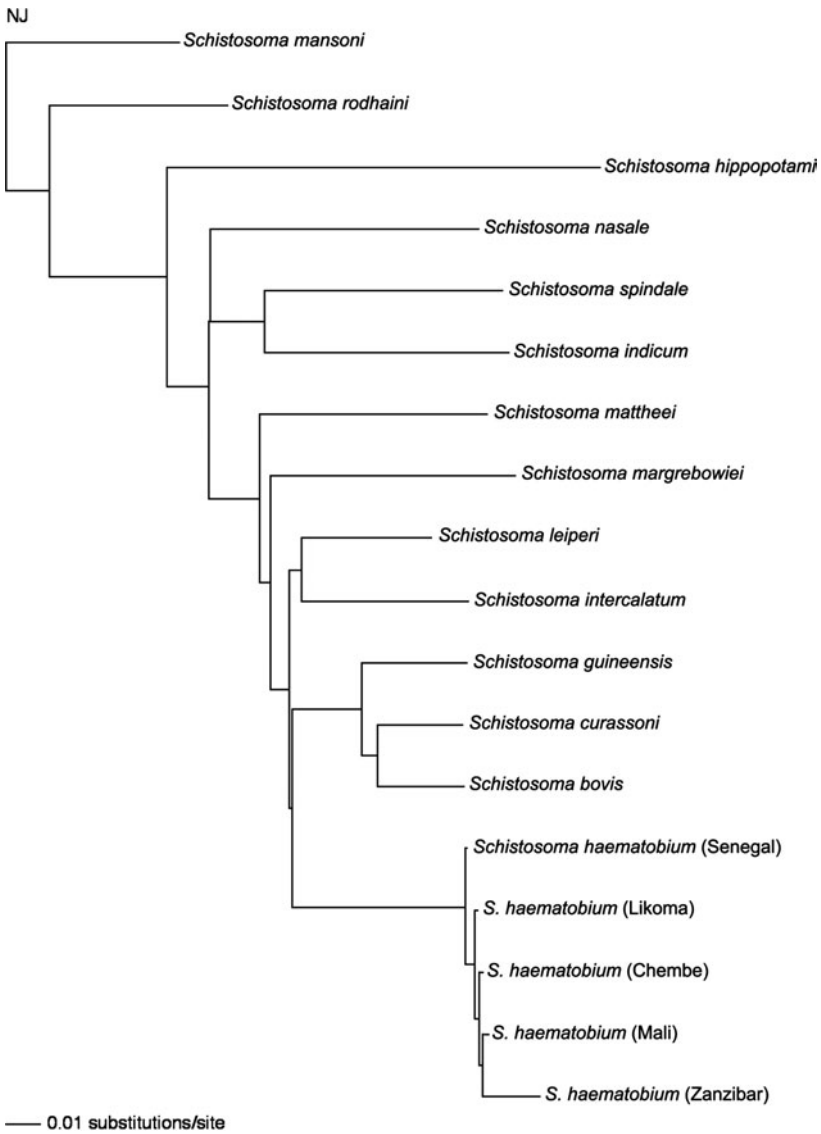

Fig. 2. Neighbour joining (distance tree); COX1 (mitochondrial DNA).

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