East African Medical Journal Vol. 95 No. 11 November 2018
MOLECULAR IDENTIFICATION OF LARVAL *SCHISTOSOMA* SPECIES (SCISTOSOMATIDAE: DIGENEA) IN INTERMEDIATE HOSTS (BIOMPHALARIA) FROM MWANZA GULF OF LAKE VICTORIA IN TANZANIA
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MOLECULAR IDENTIFICATION OF LARVAL *SCHISTOSOMA* SPECIES (SCISTOSOMATIDAE: DIGENEA) IN INTERMEDIATE HOSTS (BIOMPHALARIA) FROM MWANZA GULF OF LAKE VICTORIA IN TANZANIA

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ABSTRACT

Background: **Larval stages of *Schistosoma* species, like other digeneans in snail intermediate hosts, are challenging to delineate morphologically.**
Objective: **Thus, the main objective of the present study was to identify the morphologically described furcocercariae as *Schistosoma* species from snails *Biomphalaria* spp in the Mwanza Gulf of Lake Victoria using DNA barcoding region (cytochrome c oxidase, CO1) and large subunit (LSU) rRNA (28S).**
Design: **A retrospective clinical-laboratory study.**
Setting: **Mwanza Gulf, Lake Victoria**
Subjects: **Sporocysts and cercariae from different snail hosts were included in the analysis.**
Results: **The 28S ribosomal RNA gene identified the furcocercariae as *Schistosoma mansoni* by 100% match, while the barcoding region could not identify the specimens as *S. mansoni*. Conclusions: These results imply that the popular and highly promoted animal barcoding gene, CO1, when used alone may lead to misidentifications of the schistosome species. Thus, it must be considered with caution.**

INTRODUCTION

Schistosomiasis, one of the neglected tropical disease caused by parasitic flatworms of the genus *Schistosoma*, is a disease of major public health importance in tropical and subtropical regions. Schistosomiasis is endemic in more than 76 countries and territories infecting more than 200 million people worldwide, most of whom are in sub-Saharan Africa (1). Although there are more than 20 recognised species of Schistosoma, only three species, namely, *Schistosoma haematobium, S. mansoni*, and *S. japonicum* are commonly known to infect human beings (1,2). *Both S. mansoni* and *S. haematobium* are prevalent in Africa and the Middle East whereas *S. japonicum* occurs in Asia, mainly in China and the Philippines (3). Also, although there are more species which can cause human schistosomiasis, their distribution is much more localized. For instance, *Schistosoma mekongi* occurs in the Mekong River basin, and *S. guineensis* and *S. intercalatum* are prevalent in parts of the west and central Africa (2). *Schistosoma* species like other digenean trematodes are specific to their mollusc hosts. Consequently, their distribution is determined by their suitable snail intermediate hosts' habitat range. Freshwater snails of genera *Biomphalaria* and *Bulinus* are a requirement for the spread of, *S. haematobium* and *S. mansoni*, respectively; *S. japonicum* needs freshwater snails of genus *Oncomelania* (1,2).

In Tanzania, being part of Sub-Saharan Africa, schistosomiasis is endemic. The highest infections and disease burdens are frequently found in children particularly in settings with poor hygiene and sanitary facilities (3). The infection with *Schistosoma mansoni* and *S. haematobium*, which are causative agents of intestinal and urogenital schistosomiasis, respectively, is the most common (4,5). Reports have shown that urinary schistosomiasis is highly endemic in the coastal belt of Tanzania due to occurrence of *Bulinus* species [6] and references therein, whereas intestinal schistosomiasis is highly prevalent along the coast of Lake Victoria because of *Biomphalaria* species (4,7). The two species of *Biomphalaria* are implicated in the transmission of *S. mansoni* in Lake Victoria: *Biomphalaria sudanica* in the lake margins while *B. choanomphala* operates in the deep water [5,7]. However, both snail hosts and their parasites, cercariae recovered from snails upon artificial light stimulation, have been morphologically identified (8,9). As a result, the taxonomic status of *S. mansoni* remains debatable as it is unknown if *Biomphalaria sudanica* and *B. choanomphala* can be its hosts, or the snails are separate species (7,9).

However, in many biological cases where conventional analyses have failed to identify species, molecular techniques and in particular DNA barcoding approaches have proven successful. For instance, Standley et al. (9) revealed that the genetic variation of *Schistosoma mansoni* in Lake Victoria is much higher than previously envisaged. Similarly, an analytical view of cytochrome c oxidase 1 (CO1) new *Schistosoma* species described was purported to be a sister species to *S. intercalatum* in the Lake Victoria basin in the Kenyan side (10). Also, CO1 sequences of *S. mansoni* across sub-Saharan Africa found the highest diversity in East Africa; with samples forming several complicated cross connections between haplotypes on different branches; supporting substantial within locality diversity and geographical separation of genotypes (11).

In the present study, the DNA barcoding region was used to investigate the taxonomic status of trematode species occurring in freshwaters of Tanzania. The main objective was to use DNA methods to identify morphologically described *S. mansoni infecting Biomphalaria* spp in the Mwanza Gulf of Lake Victoria (9). DNA

barcoding region (cytochrome c oxidase, CO1) and large subunit LSU markers were used to compare with other congeners in the GenBank.

MATERIAL AND METHODS

Source of material for genomic DNA: Snails of the genus *Biomphalaria*, which are implicated in the transmission of schistosomiasis (8,9,12), were sampled from the Mwanza Gulf of Lake Victoria (situated at 2º 41′ S and 32º 51′ E) in Tanzania (Figure 1). The snails were mostly sampled along the shores in places where water was stagnant or slowly moving, using a strainer with a long handle or handpicked from the underneath of the waterweeds along the shores. Snails gathered from each site were placed in labelled separate plastic containers filled with lake water and lettuce or and transported to the Tanzania fisheries research institute laboratory for further processing. Because of some difficulty in separating *Biomphalaria* species, i.e. *B. sudanica* and *B. choanomphala* in Lake Victoria (7), the snails were not classified to species level. Cercarial shedding was induced by an intense artificial light and heat (60W) for a period of 6 to 48 hours in small vials of 12.5 ml or 50 ml containers. Both cercariae shading and non-shading snails were crushed for the examination of intramolluscan stages. More on morphological studies can be found in Chibwana and Nkwengulila (8). Both sporocysts and cercariae for DNA processing were fixed and preserved in 96% ethanol until needed. The snails' identification followed field guides for Africa freshwater snails [12].

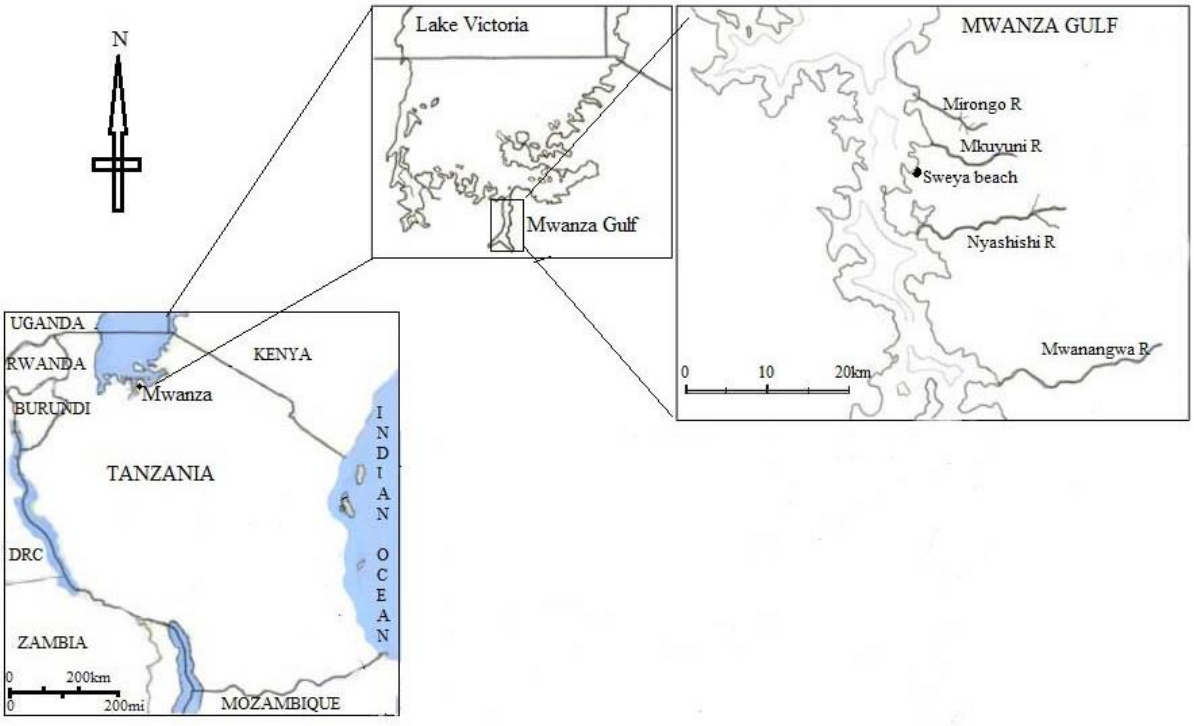

Figure 1 Localities where snails were collected in the present study

DNA extraction, amplification and sequencing: Genomic DNA was extracted using the Qiagen DNeasy Tissue and Blood Kits following the manufacture's protocol. DNA amplification of the barcode region of CO1 was performed using forward primer MplatCOX1dF (5′ TGTAAAACGACGGCCAGTTTWCITTRGA TCATAAG 3′) and reverse primer MplatCOX1dR (5′ CAGGAAACAGCTATGACTGAAAYAAY AIIGGATCICCACC 3′) with the following PCR conditions: 94 °C for 1 min, 5 cycles at 94 °C for 40 sec, 45 °C for 40 sec, and 72 °C for 1 min, followed by 35 cycles at 94 °C for 40 sec, 51 °C for 40 sec and 72°C for 1 min with a final extension at 72 °C for 5 min. Primers LSU-5 (5′-TAGGTCGACCC-GCTGAAYTTAAGCA-3′) and 1500R (5′-GCTATCCTGAGGGAAACTTCG-3′) were used for the 28S region as forward and reverse primers, respectively. Each rDNA PCR reaction consisted of 17.5 µl H2O, 2.5 µl 10X buffer, 1.25 µl MgCl2 (25 mM), 0.125 µl dNTP (10 mM), 0.25 µl PCR primers, 0.125 µl Taq DNA polymerase, and 3 µl of DNA template. The PCR conditions were as follows: 94 °C for 2 min, followed by 35 cycles at 94 °C for 1 min, 56 °C for 1 min and 72 °C for 2 min with a final extension at 72 °C for 5 min. In both CO1 and 28S reactions, the PCR products were held at 4°C. ABI Big Dye chemistry following the manufacturer's protocol was used for sequencing on an ABI Prism 3130xl automated sequencer.

Sequence analysis: Chromatograms were assembled and edited with Geneious (Biomatters Ltd., Auckland, New Zealand) and adjusted manually as needed, and sequences were subjected to Basic Local Alignment Search (BLAST), aligned with Clustal W implemented in MEGA X and analyzed using MEGA X. Sequences are archived at the GenBank with records; MK095609-MK095613 and MK085970-MK085971 for CO1 and LSU, respectively.

Newly obtained sequences for CO1 and LSU were aligned in two independent datasets. The CO1 dataset comprised of eight novel sequences obtained from furcocercariae from eight snails of the genus *Biomphalaria*. Only sequences that showed high similarity (Kimura 2-parameter distance of 0-2%) were analysed jointly with 18 sequences from GenBank in a neighbour-joining tree. Similarly, after an initial alignment of 8 new 28S furcocercariae sequences from the same snails, those that were closely related were used in the analysis. The 28S dataset was further supplemented with eleven sequences of other schistosomids from the GenBank. The details for the isolates used are provided in Table 1. *Austrobilharzia* species were used as outgroup in the neighbour-joining tree. Note also that sequences of LSU and CO1 analysed were not obtained from the same individual specimens but as isolates from the same molluscan hosts.

Table 1

Summary data for the Schistosoma CO1 and LSU sequences retrieved from the GenBank used in the analyses of the novel sequences

Species	Life cycle stage	Host species	Locality	28S /LSU Accession No	CO1 Accession No
Schistosoma bovis	A, C, E, M	*Human and snails*	Upper Senegal River Basin		FJ588855
Schistosoma bovis	A, C, E, M	*Human and snails*	Upper Senegal River Basin		FJ588856
Schistosoma bovis	A, E	Small mammals	Lake Victoria Basin, Kenya		GU294793
Schistosoma haematobium	A	*Mesocricetus auratus*	Mali		AY157209
Schistosoma haematobium	A, C, E, M	*Human and snails*	Upper Senegal River Basin		FJ588852
Schistosoma haematobium	A, C, E, M	*Human and snails*	Upper Senegal River Basin		FJ588853
Schistosoma mansoni	A	*Human*	Senegal		AJ519524
Schistosoma mansoni	C	*Biomphalaria pfeifferi*	Senegal		JQ289661
Schistosoma mansoni	C	*Biomphalaria pfeifferi*	Farako, Mali		JQ289621
Schistosoma mansoni	E, M	*Human*	Nder, Senegal		JQ289669
Schistosoma mansoni	E, M	*Human*	Namarigoungou, Niger		JQ289637
Schistosoma rodhaini	C	*Biomphalaria choanomphala*	Ngamba Island Chimpanzee Sanctuary, Lake Victoria, Uganda		JQ314103
Schistosoma rodhaini	A, E	Small mammals	Lake Victoria Basin, Kenya		GU294838
Schistosoma rodhaini	A, E	Small mammals	Lake Victoria Basin, Kenya		GU294839

Schistosoma japonicum	C, S	*Oncomelania hupensis*	China		KU196378
Schistosoma japonicum	C, S	*Oncomelania hupensis*	China		EU325891
Schistosoma japonicum	C, S	*Oncomelania hupensis*	China		EU340353
Schistosoma japonicum	C, S	*Oncomelania hupensis*	China		EU340352
Schistosoma rodhaini	A	*ex Mus musculus*	Lab strain (NHM)	AY157256	
Schistosoma intercalatum	A	*ex Mus musculus*	San Antonio, Sao Tome	AY157262	
Schistosoma haematobium	A	*ex Mus musculus*	Nigel delta, Mali	AY157263	
Schistosoma japonicum		*ex Mus musculus*	Originally from the Philippines	AY157607	
Schistosoma mansoni	A	*ex Mus musculus*	Lab strain (NHM)	AY157173	
Schistosoma mansoni	A		NMRI-Puerto Rica	Z46503	
Schistosoma indicum	A	*Bos indicus*	Bangladesh	LC224107	
Schistosoma bovis	A	Mamalian hosts (rodents)	Lake Victoria Basin, Kenya	FJ897156	
Schistosoma mansoni	Unknown	Unknown	Puerto Rico	XR_001974605	

RESULTS

Out of 675 snails belonging to the genus *Biomphalaria* examined (shedding and crushed), only eight were found to be infected with *Schistosoma* spp (Figure 1). However, only four of them were successfully sequenced to produce four novel CO1 sequences (50% sequencing success). The analysis of the CO1 sequences within 387 bp length based on p-distance showed that the mean divergence between materials from different snails was 0.3% on average (range, 0.00% to 2.42%). When the newly generated CO1 sequences were aligned with sequences of known schistosomids in the public domain (the GenBank), both NJ and ML resolutions strongly supported schistosomes of the present study to be a different species closely related to *Schistosoma mekongi* (Figure 2). Besides, the schistosomid species understudy did form one clade with *Schistosoma mansoni* as expected from morphology and snail host *Biomphalaria* spp., concluding that they could be genetically different species.

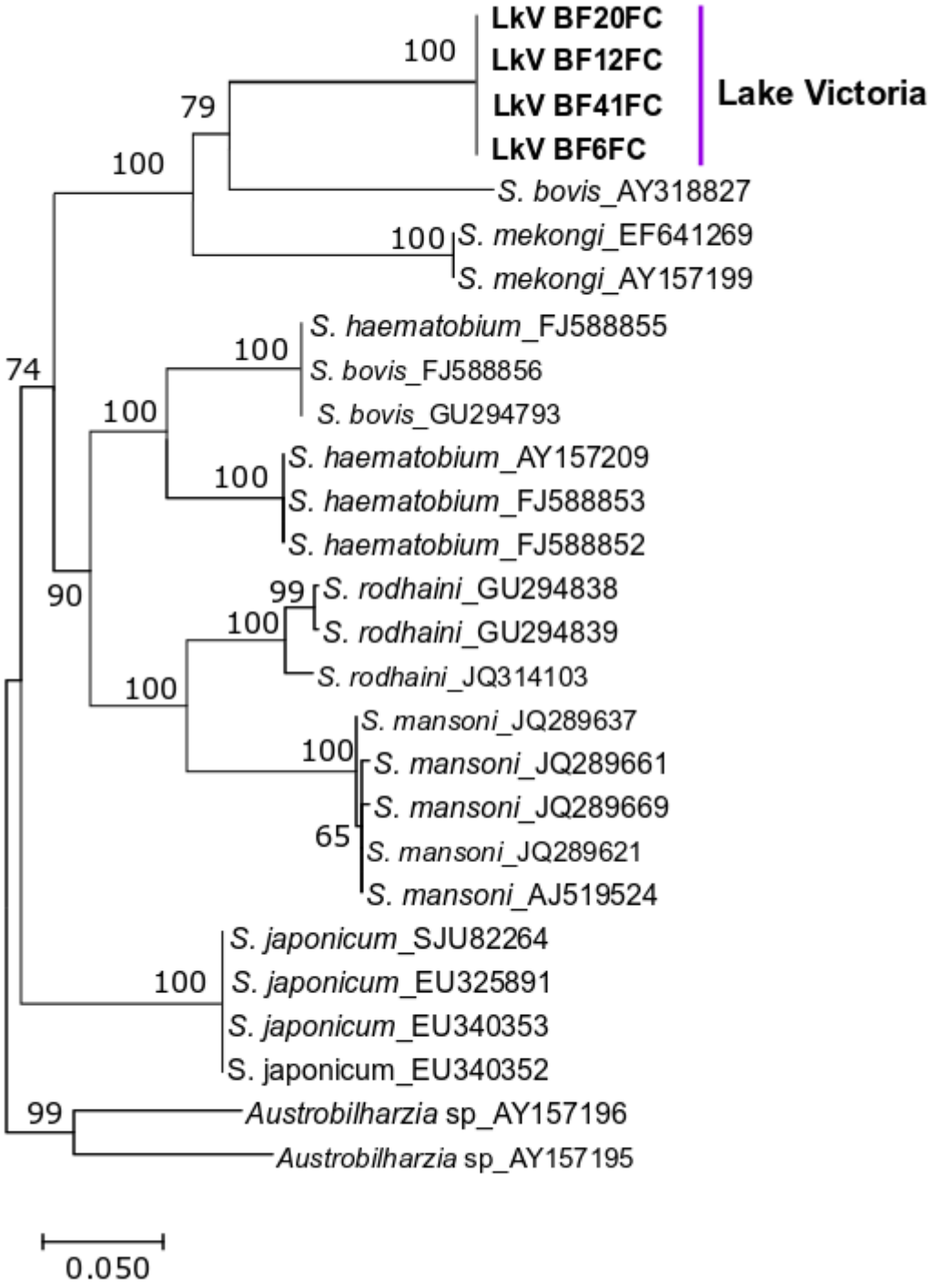

Figure 2: The evolutionary tree based on COI sequences inferred using the NJ and ML methods.

This study presents two (2) newly generated complete ribosomal DNA (28S) sequences out of 8 isolates recovered from the eight infected *Biomphalaria* snails from Mwanza Gulf of Lake Victoria. The similarity between the two sequences about p-distances was 100% (Table 2). Table 2 also shows that the two novel 28S sequences closely resemble *Schistosoma mansoni* by 100% and differ by 0.4% with *Schistosoma rodhaini*. When the newly generated 28S sequences were aligned with those of known schistosomes in the GenBank, their NJ and ML resolutions identified them as

Schistosoma mansoni (Figure 3). Moreover, *S. rodhaini* from experimental hosts *Mus musculus* is a sister clade to *S. mansoni* recovered from the Mwanza Gulf of Lake Victoria and GenBank instead of *S. mekongi* as in CO1 region. That means the analysis of 28S sequences recognizes the present material as *Schistosoma mansoni*.

Table 2
Estimates of the evolutionary divergence between LSU sequences

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1. LVBI12FC1												
2. LVBIFC6	0.000											
3. AY157173_*S. mansoni*	0.000	0.000										
4. Z46503_*S. mansoni*	0.000	0.000	0.000									
5. AY157256_*S. rodhaini*	0.004	0.004	0.004	0.004								
6. XR_001974605_*S. mansoni*	0.013	0.013	0.013	0.013	0.015							
7. AY157263_*S. haematobium*	0.033	0.033	0.033	0.033	0.034	0.042						
8. LC224107_*S. indicum*	0.032	0.032	0.032	0.032	0.034	0.041	0.017					
9. AY157258_*S. indicum*	0.032	0.032	0.032	0.032	0.034	0.041	0.017	0.000				
10. FJ897156_*S. bovis*	0.034	0.034	0.034	0.034	0.034	0.044	0.004	0.018	0.018			
11. AY157262_*S. intercalatum*	0.034	0.034	0.034	0.034	0.033	0.043	0.003	0.018	0.018	0.001		
12. JF742195_*Austrobilharzia*	0.103	0.103	0.103	0.103	0.100	0.112	0.103	0.105	0.105	0.102	0.101	
13. AY157250_*A. variglandis*	0.102	0.102	0.102	0.102	0.099	0.110	0.104	0.107	0.107	0.103	0.103	0.011

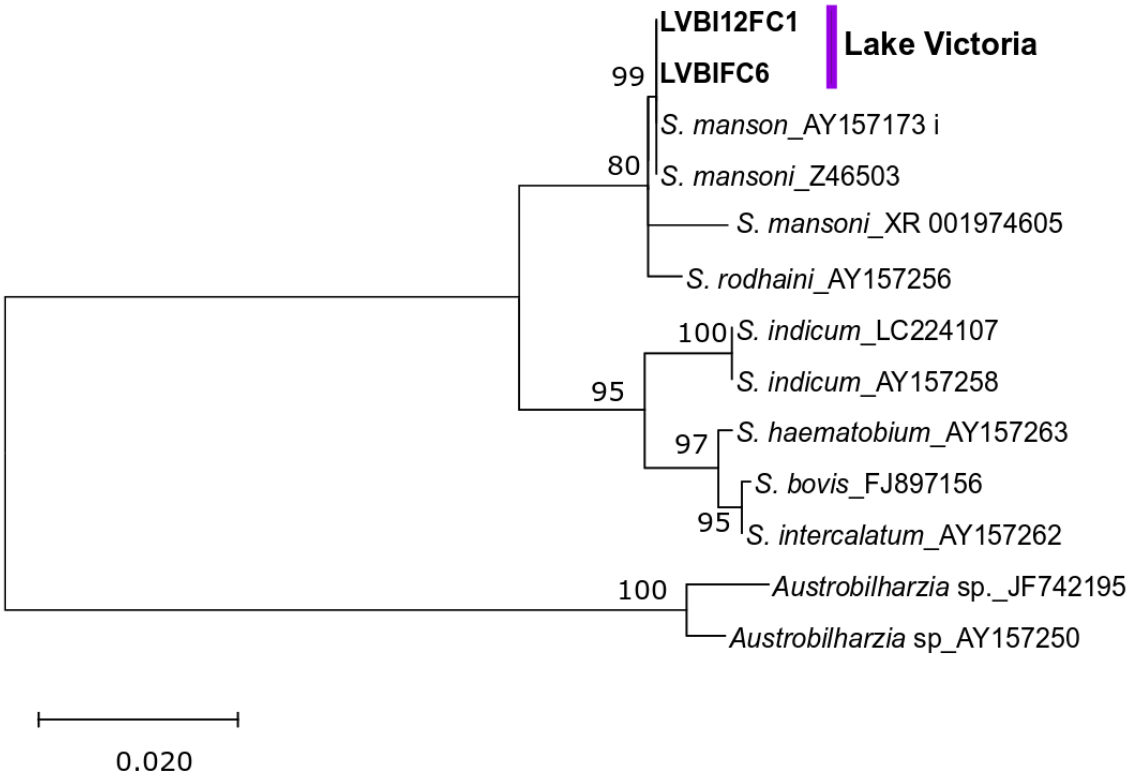

Figure 3: The evolutionary tree based on LSU (28S) sequences inferred using the NJ and ML methods.

DISCUSSION

The present study provides strong molecular-based evidence for the contrasting evolutionary patterns of 28S and barcoding region genes. The two markers identified morphologically established *Schistosoma mansoni* (8) as *Schistosoma mansoni* and an unknown *Schistosoma* species, perhaps a new species, closely related to *S. mekongi*, respectively. The molecular markers have been applied successfully in investigations of digenean species diversity (9,12), linking larval stages of parasites to their known adults (13), prospecting for cryptic species (14) and in the analyses of parasite community compositions (15). Although in many biological cases where conventional analyses have failed to identify species, molecular techniques and DNA barcoding approaches have proven successful, in the present study, CO1, unfortunately, was unsuccessful. However, although sequencing success coupled with a small number of specimens for some species affected the results of the present study, 28S has proved to be more robust in species identification than DNA barcode region, CO1, even in the absence of morphology.

The use of CO1 in the present study has revealed that the barcoding region is not an ideal marker in delimitating *Schistosoma* species. These results corroborate similar studies in the past (9,16). Standley et al. (10) argued that the level of genetic variation within *Schistosoma mansoni* in Lake Victoria is high because the considerable amount of diversity could be traced even within an individual host apart from the sampling locality. Although, Standley et al. (9) analysed more sequences than previous surveys, the stationary phase (asymptotic period) was not reached irrespective of geographical or numerical difference; and therefore a novel analysis was required to resolve the existence of high diversity in schistosomes. However, an analysis of the entire mitochondria genome sequences had observed that CO1 is not an ideal marker for either species identification (barcoding) or population studies in *Schistosoma* species (17). The present results are in accordance with this observation.

Likewise, partial 28S ribosomal RNA (rRNA) gene sequences have been successfully applied in the identification of 4 *Schistosoma* species, namely *S. mansoni*, *S. haematobium*, *S. spindale* and *S. japonicum* (18). The LSU (28S) has also been used effectively to discriminate 3 *Schistosoma* species; *Schistosoma intercalatum*, *S. haematobium* and *S. mansoni* (18). Also, the taxonomy of a new species *Schistosoma kisumuensis* in Lake Victoria Basin, Kenya, was a result of several markers including LSU (10). However, although 28S rRNA gene was used to benchmark species or genus-level boundaries, it is considered too conservative for this purpose (20). As a result, most authors prefer the highly popularized barcoding gene to CO1 28S gene.

CONCLUSION

DNA barcoding was proposed to provide an efficient method for species-level identifications based on a fast rate of evolution at variable domains from the 5' region of the mitochondrial cytochrome c oxidase (COI) gene (20). The present study, however, has shown that irrespective of the number of sequences, LSU is better for *Schistosoma* species identification than CO1. It cannot be overstated that correct identification of Schistosoma species at any developmental stage is central to many aspects of schistosomiasis, i.e., systematics, phylogeny, ecology diagnosis, epidemiology and control. As such the present study recommends that more similar studies should be carried out extensively in snail hosts to recover more unknown species and

determine the distribution of the already known species responsible for causing Schistosomiasis in Tanzania and Africa in general.

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