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▣ ORIGINAL ARTICLE

Genetic Diversity of *Schistosoma haematobium* Eggs Isolated from Human Urine in Sudan

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Abstract: The genetic diversity of *Schistosoma haematobium* remains largely unstudied in comparison to that of *Schistosoma mansoni*. To characterize the extent of genetic diversity in *S. haematobium* among its definitive host (humans), we collected *S. haematobium* eggs from the urine of 73 infected schoolchildren at 5 primary schools in White Nile State, Sudan, and then performed a randomly amplified polymorphic DNA marker ITS2 by PCR-RFLP analysis. Among 73 *S. haematobium* egg-positive cases, 13 were selected based on the presence of the *S. haematobium* satellite markers A4 and B2 in their genomic DNA, and used for RFLP analysis. The 13 samples were subjected to an RFLP analysis of the *S. haematobium* ITS2 region; however, there was no variation in size among the fragments. Compared to the ITS2 sequences obtained for *S. haematobium* from Kenya, the nucleotide sequences of the ITS2 regions of *S. haematobium* from 4 areas in Sudan were consistent with those from Kenya (> 99%). In this study, we demonstrate for the first time that most of the *S. haematobium* population in Sudan consists of a pan-African *S. haematobium* genotype; however, we also report the discovery of Kenyan strain inflow into White Nile, Sudan.

Key words: *Schistosoma haematobium*, Sudan, ITS2, PCR-restriction fragment length polymorphism (PCR-RFLP)

INTRODUCTION

Schistosomiasis is a chronic and debilitating disease, second only to malaria in terms of parasite-induced human morbidity and mortality. Among the species of schistosomes infecting humans, *Schistosoma haematobium* is responsible for the largest number of infections in sub-Saharan Africa; an estimated 112 million people are infected with this species, which is more than double the estimated figure for *Schistosoma mansoni* [1]. In Sudan, *S. haematobium* infection is dominant, although both *S. haematobium* and *S. mansoni* are found [2,3].

Studies of the genetic diversity of natural *Schistosoma* populations are complicated by the fact that adult worms are inaccessible in the cardiovascular system of the mammalian host. Molecular epidemiological studies of schistosomiasis have provided opportunities to investigate many important topics such as the contribution of parasite genetics to variation in disease burden and pathology, the genetic consequences of various control activities for parasite populations, patterns of recruitment and transmission in endemic areas, and the likely evolution and spread of drug resistance [4,5]. Previous reports have demonstrated that the second internal transcribed spacer (ITS2) of *S. haematobium* sequences from Kenya were nearly identical (99%) to conspecific sequences from Egypt, Mali, and Niger [6], and *Schistosoma* japonicum in China was highly genetically diverse by cloning ITS1-ITS2 sequences according to the location [7]. Recently, it was reported that a high level of genetic variability of *S. haematobium* in the populations from Mali and Nigeria [8].

However, the genetic diversity of *S. haematobium* remains largely unstudied in comparison to *S. mansoni* [9], primarily because of the more demanding conditions for laboratory maintenance and lack of available molecular markers [10]. Moreover, there were no reports about the extent of genetic diversity of *S.*

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haematobium within its definitive host, humans in Sudan. Here, to characterize the genotype of *S. haematobium* in an infected population and to identify potential associations with parasite diversity, we collected *S. haematobium* eggs from 73 infected children at 5 schools in Sudan and performed genotyping using a polymorphic microsatellite marker, ITS2 region.

MATERIALS AND METHODS

Ethical statement

This study protocol was reviewed and approved by the institutional review board of the Korea Association of Health Promotion (acceptance no. 12-C-01) and was also approved by the National Control Program for Schistosomiasis and Soil-Transmitted Helminths, Federal Ministry of Health, Sudan. Before collecting the urine samples, informed verbal consent was obtained from each child with the presence of school teachers.

Study areas and collection of *S. haematobium* eggs

S. haematobium eggs were collected from 73 children at 5 primary schools in February of 2013. Five primary schools were selected in the Al Jabalain locality of White Nile, Sudan. All schools were located adjacent to the White Nile River, and they were located at Al Hidaeb, Al Zealet, Jazeera Aba, Khour Ajwal, and Al Sidding villages. From each child, 10-15 ml of urine specimen was collected, and transferred to the Schistosomiasis Control Center established by the Korea International Cooperation Agency (KOICA) in Kosti, White Nile State, Sudan. In the Center, urine samples were centrifuged at 1,500 rpm for 5 min, and the pellets were examined for eggs of *S. haematobium* by microscopy.

Preparation of *S. mansoni* genomic DNA

Adult *S. mansoni* specimens were obtained from Department of Environmental Medical Biology and Institute of Tropical Medicine, Yonsei University College of Medicine, Korea, and the fluke was stored at -70℃. For DNA isolation, adult *S. mansoni* was cut into 20 mg tissue samples using a sterile scalpel. Genomic DNA was isolated from adult *S. mansoni* worms using a G-DEX™ genomic DNA extraction kit (iNtRON Biotechnology, Seoul, Korea) according to the manufacturer's instructions.

Multiplex PCR assay

Genomic DNA was isolated from *S. haematobium* eggs using a G-DEX™ genomic DNA extraction kit (iNtRON Biotechnology) according to the manufacturer's instructions. PCR was performed with primers specific for 2 microsatellites markers of *S. haematobium*, A4 and B2, as described previously [7]. All amplifications were performed using TaKaRa Ex Taq DNA Polymerase (Takara Bio Inc., Otsu, Japan) with the following primers: A4(F) (5′-CGA ACT CCA ACG AGC ATC-3′), A4(R) (5′-GGG TGT GGG AAT GAC TTG-3′), B2(F) (5′-AAG CCG ACC ATT TGA CTC-3′), and B2(R) (5′-GTT GCT GTT GAT GAC GAT G-3′). The following conditions were carried out with an initial denaturation step of 94℃ for 30 sec, followed by 30 cycles of denaturation at 98℃ for 10 sec, annealing at 60℃ for 30 sec, and extension at 72℃ for 30 sec. The multiplex PCR products were then electrophoresed on a 2% agarose gel, stained with ethidium bromide, and visualized under UV light.

Amplification of the ITS2 region by PCR

PCR amplification of the ITS2 fragment (ITS2-PCR) from *S. haematobium* eggs was performed in a 100-μl volume, which consists of 100 ng of genomic DNA from each sample, 10 μl of 10× Ex Taq buffer, 8 μl of a dNTP mixture (2.5 mM each dNTP), 5 μM each of ITS2F (5′-GAA TTA ATG TGA ACT GCA TAC TGC TT-3′) and ITS2R (5′-TTC CTC CGC TTA TTG ATA TGC TT-3′), and 0.5 μl of 5 U/μl of TaKaRa Ex Taq DNA Polymerase, using a TaKaRa PCR Thermal Cycler (Takara Bio Inc.). All PCR assays were performed using 1 cycle of 94℃ for 30 sec and 30 cycles of 98℃ for 10 sec, 60℃ for 30 sec, and 72℃ for 30 sec, followed by 1 cycle at 72℃ for 7 min and a final hold at 4℃. Agarose gel electrophoresis (1.5%) with ethidium bromide staining was used to visualize the ITS2-PCR products.

Restriction fragment length polymorphism (RFLP) analysis of the ITS2 region

To evaluate interspecific restriction enzyme cutting site variation within the ITS2 region, this region was singled out for further study. The ITS2-PCR products were purified using a QIAquick™ PCR purification kit (Qiagen, Valencia, California, USA). To reveal the RFLP pattern, the purified PCR products were digested with *Sau*3A1 (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. The *Sau*3A1 reactions were incubated at 37℃ for 4 hr, at which point an additional 1 μl of *Sau*3A1 enzyme was added to each reaction. The reactions were then incubated overnight. The RFLP products

were separated on 2% agarose gels, stained with ethidium bromide, photographed, and analyzed for fragment sizes.

Sequencing and analysis of the ITS2 region

The purified ITS2-PCR products were ultimately sequenced directly by SolGent (Daejeon, Korea) using the primers described previously. The complete ITS2 sequences for *S. haematobium* from 13 samples were compared to those isolated from Kenya (GenBank accession no. AF146038) using the Clone Manager software (Sci-Ed Software, Cary, North Carolina, USA).

Analysis of ITS-2, *cox1*, and *nad1* genes of *S. haematobium* and *S. mansoni* using PCR

The mitochondrial cytochrome oxidase subunit 1(*cox1*), NADH dehydrogenase subunit 1 (*nad1*) and ITS-2 genes of *S. haematobium* and *S. mansoni* were analyzed using adult worms using PCR amplification. Briefly, genomic DNA was extracted from the adult worm using G-DEX™ genomic DNA extraction kit according to the manufacturer's instructions. The primers used for PCR amplification were used the following primers: *S. haematobium cox1* (DQ677664, 110 bp), forward 5′-AAA AGC TGT GGG TCT CGT GT-3′, reverse 5′-AAT GAA GAA GCG GAG AAA GC-3′; *S. mansoni cox1* (NC_002545, 598 bp),forward 5′-TCA ATT TGA GAG GGG TCT GG-3′, reverse 5′-TGG GAT CTA AAA CCC GCA TA-3′; *S. haematobium nad1* ((JQ595404,431 bp), forward 5′-GGC TGA TGT TCG TGA TCA AA-3′, reverse 5′-CGA AGT CGA GAA AAT GAA CCA-3′; *S. mansoni nad1* (AF216698, 194 bp) forward 5′-TGG GGA GTG TGA GAG TGA GC-3′, reverse 5′-GAA AAG ACC CAC GCA TCA TT-3′; and *S. mansoni* ITS-2 (JQ289745, 104 bp) forward 5′-GAC GCA CAT TAA GTC GTG GA-3′, reverse 5′-CTG TGG CCG GAT TAT TAG GA-3′. Genomic DNA from *S. haematobium* eggs and *S. mansoni* adult worms were used for detection of specific genes. The PCR mixture for the PCR amplification contained 5 µl genomic DNA (200 ng/µl), 2 µl each of forward and reverse primers (10 µM), 4 µL dNTP (2.5 mM each), 5 µl 10× Ex Taq buffer, 0.25 µl Ex Taq polymerase (5 U/µl), and 31.75 µl DDW.PCR assays were performed with an initial denaturation step of 94℃ for 30 sec, followed by 30 cycles of denaturation at 98℃ for 10 sec, annealing at 56℃ for 30 sec, and extension at 72℃ for 60 sec, followed by 1 cycle at 72℃ for 10 min and a final hold at 4℃.

Amplifications were generated using a TaKaRa PCR Thermal Cycler (Takara Bio Inc.). Agarose gel electrophoresis (1.5%) with ethidium bromide staining was used to visualize the *cox1*, *nad1*, and ITS2 of PCR products.

RESULTS

Results of multiplex PCR amplification

To select samples for the RFLP analysis of *S. haematobium* eggs from 73 Sudanese schoolchildren, multiplex PCR was performed using genomic DNA isolated from patients with microscopically confirmed urinary schistosomiasis. According to our multiplex PCR results, we selected 13 schistosomiasis-positive samples, each of which produced the expected 292-bp A4 and 266-bp B2 fragments (Fig. 1). Also, *S. haematobium* eggs were confirmed by PCR using *cox1* and *nad1* genes as the markers of genetic diversity. They showed species-specific *cox1* and *nad1* in comparison of those of *S. mansoni* (Fig. 2). These positive samples were used in the following genotyping study.

Detection and RFLP analysis of the ITS2 region

Using DNA from the 13 isolates collected in 5 areas of Sudan, PCR amplification using ITS2-specific primers produced 468-bp PCR product. Further, we examined species-specific variation in the enzyme cutting sites. *Sau*3A1 cut the ITS2-containing PCR product of *S. haematobium* in 3 places, producing fragments that were 237, 98, 83, and 50 bp in length (Fig. 3). The number and location of these restriction enzyme sites were found to be consistent in both GenBank sequences isolated from Kenya. The selection of *Sau*3A1 for our RFLP analysis was based on this lack of intraspecific variation in the presence and location of the restriction enzyme cutting sites. The RFLP patterns of the 13 specimens were similar, indicating that the *S. haematobium* isolated from 5 different areas of Sudan were genetically identical in comparison to those collected in Kenya.

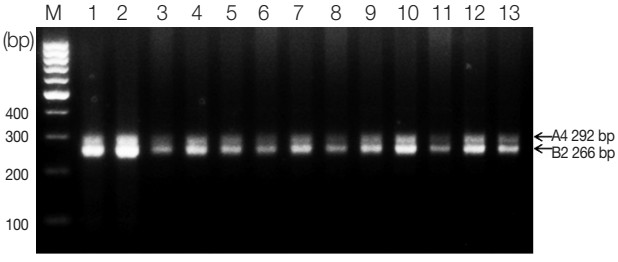

Fig. 1. Agarose gel electrophoresis of multiplex PCR products containing 2 microsatellite markers of *Schistosoma haematobium* (A4 and B2). Two bands were amplified from each sample: 292 bp for A4 and 266 bp for B4. M, 100-bp ladder. Lanes 1-2, Al Hidaeb; lanes 3-4, Al Zealet; lanes 5-6, Jazeera Aba; lanes 7-10, Khour Ajwal; lane 11, Al Sidding; and lanes 12-13, Al Zealet.

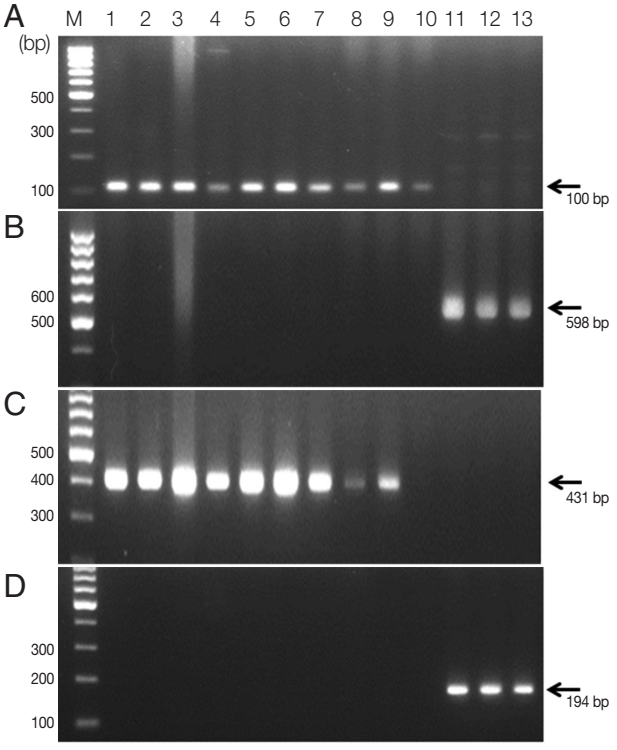

Fig. 2. Agarose gel electrophoresis of PCR products containing the mitochondrial cytochrome oxidase subunit 1 (*cox1*) and NADH dehydrogenase subunit 1 (*nad1*) of *S. haematobium* and *S. mansoni*. A, *S. haematobium cox1* (DQ677664, 110 bp); B, *S. mansoni* ***cox1*** (NC_002545, 598 bp); C, *S. haematobium nad1* (JQ595404, 431 bp); D, *S. mansoni nad1* (AF216698, 194 bp). M, 100 bp marker; lanes 1-10, *S. haematobium* eggs; lane 11-13, *S. mansoni* adult worms.

ITS2 sequencing and analysis

The 468-bp ITS2-PCR fragments were sequenced along both strands and compared to isolates from Kenya (AF146038, position 1-400) and other African countries including Guinea-Bissau (JQ397404), Senegal (FJ588861), Cameroon (JQ397406), Tanzania (GU257398), Egypt (JQ397407), Madagascar (JQ397414), and Malawi (JQ397410) (position 518-927). The ITS2 sequences of *S. haematobium* obtained from other African countries including Guinea-Bissau, Senegal, Cameroon, Tanzania, Egypt, Madagascar, and Malawi area were 100% identical (data not shown). As shown in Fig. 4, when the nucleotide sequences of the ITS2 region of *S. haematobium* from 13 samples (position 1-400) obtained in 5 different areas of Sudan were compared to the ITS2 sequences obtained for *S. haematobium* from Kenya (position 1-400) and other African countries (including Guinea-Bissau, Senegal, Cameroon, Tanzania, Egypt, Madagascar, and Malawi (position 518-927), there was only a single intraspecific base change at position 326 ('T' in 4 areas of Sudan) compared with Kenya sequence at position, while 100% was identical with those obtained from other African countries including Guinea-Bissau, Senegal, Cameroon, Tanzania, Egypt, Madagascar, and Malawi (position 518-927). The samples obtained from the Al Sidding area were 100% identical with *S. haematobium* from Kenya; however, there was only a single intraspecific base change at position 326 ('C') compared with other African countries sequences at position 843 'T'.In addition, to compare the ITS2 gene between *S. haematobium* and S. mansoni, their ITS2 PCR fragments were sequenced. As shown in Fig. 5, the nucleotide sequence of the PCR products from *S. mansoni* were 99.0%, 99.0%, and 97.1% identical, respectively, to the ITS2 sequences of JQ289745 (position 619-722). However, ITS2 sequence of *S. haematobium* (AF146038, position 112-215) was just 92.3% identical to the ITS2 sequences of *S. mansoni* (JQ289745, position 619-722) (Fig. 6). Thus, the ITS2 of *S. haematobium* was different from that of S. mansoni.

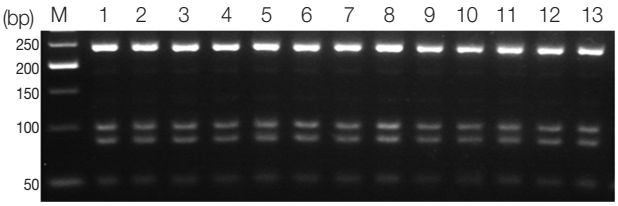

Fig. 3. Agarose gel stained with ethidium bromide showing the band patterns obtained by digestion of the 468-bp PCR product containing the second internal transcribed spacer (ITS2) with *Sau*3AI. *Sau*3A1 cut the ITS2-PCR product of *S. haematobium* in 3 places, producing visible fragments of 237, 98, 83, and 50 bp.

DISCUSSION

The genetic diversity of human schistosome infections is influenced by a number of factors. Overlapping contact sites and snail and human movements may support the coexistence of a large number of genotypes in a given area. They can also engender the development of new parasitic strains or families through genetic interchange and recombination between local and introduced genotypes [11]. Investigation of the population genetics of *S. haematobium* is important for understanding its variable disease manifestations and epidemiology, and developing new strategies for treatment, vaccination, and diagnosis [4,5]. Dabo et al. [12] found infections with multiple genotypes of *S. haematobium* in the intermediate host of the

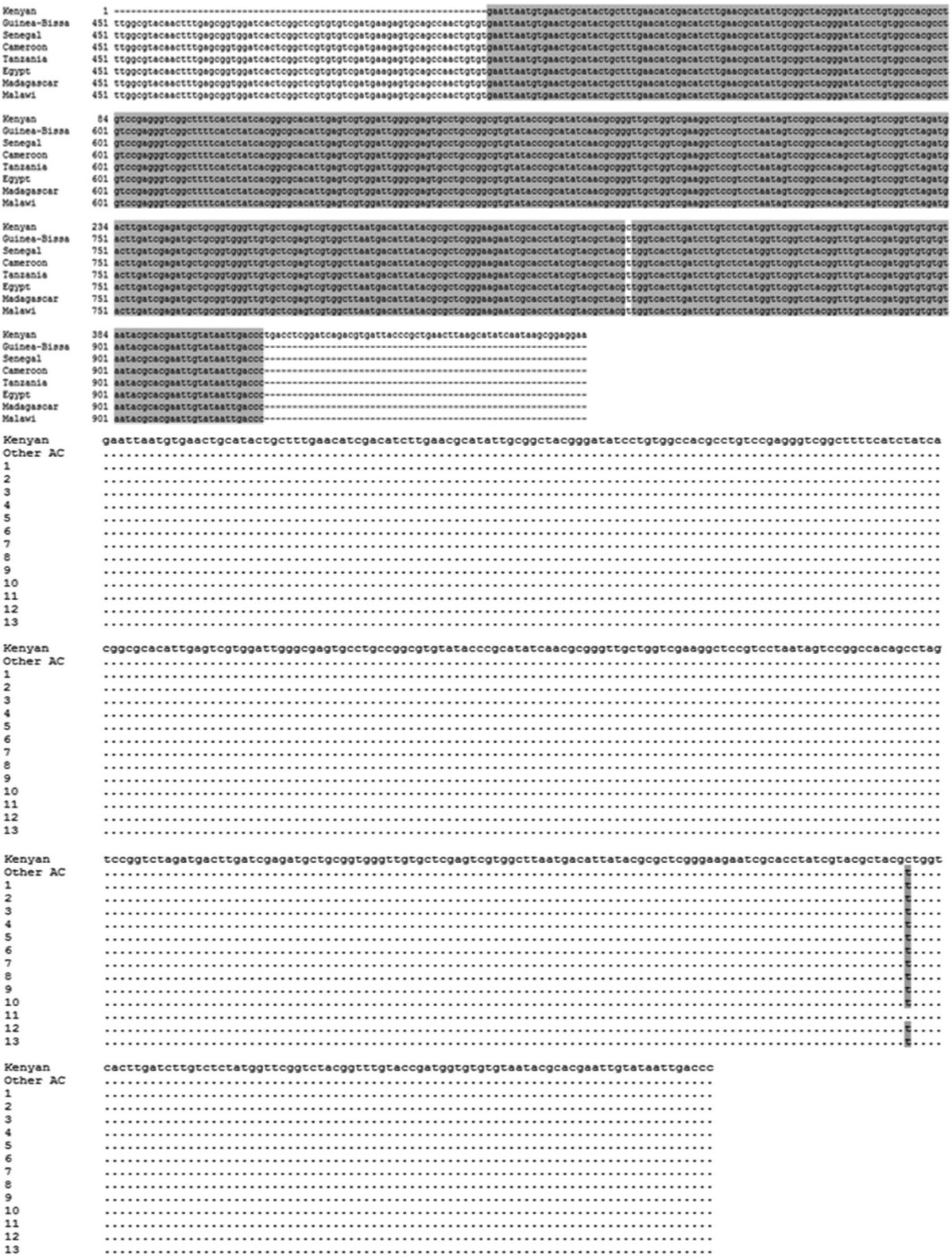

Fig. 4. The ITS2 nucleotide sequences of *S. haematobium* from 13 positive samples obtained from PCR products compared with a GenBank sequence isolated from Kenya (accession no. AF146038) and other African countries. Base homologies are indicated with a dot (·); base changes are shown in orange.

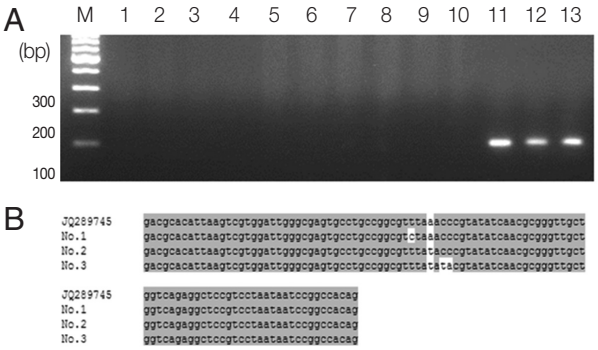

Fig. 5. The ITS2-PCR products and nucleotide sequences of *S. mansoni* adult worms. (A) Agarose gel electrophoresis of PCR products containing the ITS2 of the ribosomal gene complex from *S. haematobium* and *S. mansoni*. M, 100 bp marker; lane 1-10, *S. haematobium* eggs; lane 11-13: *S. mansoni* adult worms. (B) *S. mansoni* ITS2 nucleotide sequences of 3 positive samples obtained from PCR products compared with a GenBank sequence (accession no. JQ289745). The position of the *S. mansoni* ITS2 region was 619-722.

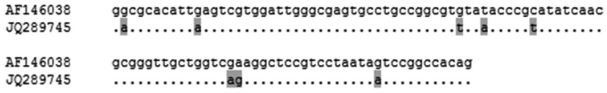

Fig. 6. The ITS2 nucleotide sequences of *S. haematobium* (AF146038, position 112-215) were compared with ITS2 sequences of *S. mansoni* (JQ289745, position 619-722). Base homologies are indicated with a dot (·); base changes are shown in orange.

organism (bulinid snails) using randomly amplified polymorphic DNA markers.

In this study, we first tested *S. haematobium* egg samples isolated from the urine of 73 infected children using the microsatellite markers A4 and B2 by PCR. A4 and B2 are useful for distinguishing *S. haematobium* from other *Schistosoma* species. However, our multiplex PCR results showed that 60 of the 73 samples did not produce the expected PCR band patterns for *S. haematobium*, suggesting that the number of *S. haematobium* eggs was insufficient for isolation of genomic DNA, and that the eggs were not from *S. haematobium*, or that polymorphisms in A4 and B2 exist among *S. haematobium* in Sudan (data not shown). Therefore, we grouped the samples according to their PCR band patterns and identified 13 samples that produced the expected PCR product size for markers A4 and B2; these were analyzed for genetic variation.

To determine the genotype of *S. haematobium* within the final hosts, we examined the ITS2 region. Internal transcribed spacers are pieces of non-functional RNA situated between structural ribosomal RNAs on a common precursor transcript. ITS2 patterns can be used to rapidly differentiate *S. haematobium* from *Schistosoma bovis* by PCR-RFLP [6]. However, the *Sau*3A1-RFLP was not found in our selected groups, indicating that the eggs were indeed *S. haematobium*. Next, to exclude the possibility of an unknown single nucleotide polymorphism in the ITS2 region, we sequenced the complete ITS2 region in our tested samples. According to our sequencing results, most of the A4- and B2-positive samples of *S. haematobium* from Sudan were of a single origin and genotype that is dominant in African countries, including Guinea-Bissau, Senegal, Cameroon, Tanzania, Egypt, Madagascar, Mauritius Zambia, and Malawi, except for the sample from the Al Sidding region, which has experienced an inflow of Kenyan *S. haematobium*. Although a limited number of *S. haematobium* eggs with a stable genotype from 5 regions of Sudan were available, the finding of a single nucleotide alteration (326 C > T), which has been reported in Kenyan *S. haematobium*, suggests that it is not a naturally occurring point mutation or polymorphism; instead, it suggests the inflow of Kenyan *S. haematobium* into Sudan. Webster et al. [13] have observed that according to DNA 'barcoding' study, using the *cox1* and *nad1* genes, low sequence variation was found among 41 localities representing 18 countries across Africa and the Indian Ocean Islands. Similarly, no obvious change in genetic diversity was detected on Zanzibar over a 4-year period [14]. These studies provided evidence that if we analyze the genetic diversity using these mitochondrial genes of *cox1* and *nad1*, there will not appear change in genotyping diversity.

According to geographic data, there is a direct connection between Lake Victoria in Kenya and Sudan (especially north Sudan) through the White Nile River, which passes through Uganda and South Sudan, suggesting that the spread of Kenyan *S. haematobium* to Sudan might be based on water or a water-dwelling intermediate host of *Schistosoma*. Monitoring of the abundance of Kenyan *S. haematobium* along the White Nile River may help determine the inflow route of the strain. Another possible route for the inflow of Kenyan *Schistosoma* to Sudan is by the migration of infected Kenyan people to Sudan. Whether the symptoms of schistosomiasis caused by the Kenyan strain of *S. haematobium* differ in their severity compared to the strains in Sudan or other African countries requires further study; however, Brouwer et al. [11] reported that the ge-

netic diversity in parasite populations was considerable among children, and that this variability may impact acquired immunity and the clinical outcome of the infection.

Based on the results of genotyping with high-resolution and microsatellite markers, we found most of the *S. haematobium* population in Sudan consists of a pan-African *S. haematobium* genotype, and also we uncovered the possible inflow of a Kenyan strain of *S. haematobium* into Sudan. These data may serve as a baseline for future research, including population genetic analyses of *S. haematobium* in this region of Sudan.

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CONFLICT OF INTEREST

We have no conflict of interest related to this work.

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