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SMALL-SUBUNIT RIBOSOMAL RNA GENE IDENTIFICATION OF THE HUMAN PATHOGEN *SCHISTOSOMA* SP. IN EGYPT

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ABSTRACT

The polymerase chain reaction and restriction fragment length polymorphism (RFLP) of the 18S rRNA gene, utilizing the enzyme BspMI, were used for the molecular identification of the two species of *Schistosoma* (*haematobium* and *mansoni*). The RFLP profiles extracted using this enzyme were highly characteristic of the two species and exhibited low levels of intraspecific polymorphism among samples from different regions of Egypt. However, *S. haematobium* and *S. mansoni* showed very similar profiles that complicated their identification at the molecular level and promised a very close genetic similarity between the two species. Other enzymes including AccI, AvaII and SnaBI were tested for their ability to differentiate between these species. The study pointed out that the 18S rRNA gene contains effective genetic markers for the identification of these *Schistosoma* spp..

INTRODUCTION

In areas where schistosomiasis is endemic, infections are heterogeneous; low and high levels of infection may be recorded in peoples living in similar conditions of exposure to the parasite. Schistosomiasis is a major public health problem, which has afflicted more than 300 million people throughout 76 countries, especially in the tropics (Iida *et al.*, 1999). Human schistosomiasis is a permanent and continues to be one of the most common parasitic infections in tropical and subtropical environments (WHO, 1999). Owing to its chronic nature, the disease impacts labor capacity, thus having a major negative impact on the socio-economic development of endemic regions (Tanner, 1989). Schistosomiasis is directly related to

water sources; in view of the large number of water resource schemes being developed, it is expected that the disease will gain more recognition (Mott *et al.*, 1995). Schistosomiasis remains the most prevalent parasitic disease causing a major public health problem in Egypt. Both *Schistosoma mansoni* and *S. haematobium* are found in Egypt (WHO 1999). Egypt currently has the highest frequency of bladder cancer in the world, due to urinary schistosomiasis(Savioli et al., 1990; WHO 1994; and El-Rifai *et al.*, 2000). *S. mansoni* and *S. haematobium* have been more commonly identified in cases of spinal cord lesions (Haribhai *et al.*, 1991; Selwa *et al.*, 1991; Ueki *et al.*, 1995; Scully *et al.*, 1996; Iida *et al.*, 1999 and Leite *et al.*, 2000), with the infections characterized by mass lesions resulting from a dramatic host inflammatory response to ova deposited in the brain (Scrimgeour & Gajdusek, 1985 and Pitella 1991; 1997). *Schistosoma mansoni* is a chronic infection which leads to intestinal schistosomiasis, liver fibrosis, cirrhosis,hepatosplenic schistosomiasis and hepatocellular carcinoma in a large percentage of untreated individuals (WHO, 1993; McCully *et al.*, 1996 and Iida *et al.*, 1999).

The morphological identification of freshwater *Schistosoma mansoni* and *S. haematobium* is of great medical importance. The process, however, is complicated by the extensive intraspecific variation of the morphological characteristics used for classical identification. A previous study has proven it possible to distinguish different geographical isolates by allozyme electrophoresis (Chilton *et al.*, 1997).In another approach, Johansen *et al.*(1997) were successful in the in-vivo radiolabelling of cercariae of *Schistosoma.* To solve this problem, the use of molecular techniques as additional tools for the identification of these organisms has been suggested. It involves using the 18S rDNA of such organisms by means of PCR amplification and digestion with different restriction enzymes. This method was successfully employed to construct the molecular key for *Aedes* species West *et al.*, (1997). It was also used to distinguish closely related parasitic worms and other different organisms (Wu *et al.*, 1999) and. instudies of genetic variation and identification of snails of the genera *Oncomelania, Bulinus* and *Biomphalaria* (Hope & McManus 1994; Stothard *et al.*, 1996; Stothard & Rollinson, 1997; Caldeira *et al.*, 1998; Rollinson *et al.*, 1998; Vidigal *et al.*, 1998a,b and Spatz *et al.*, 1998; 1999).

The aim of the present study was to investigate the use of restriction profiles resulting from digestion of the 18S rDNA of *S. mansoni* and *S. haematobium* including some restriction enzymes

such as *Bsp*MI, *Acc*I, *Ava*II and *Sna*BI for identification of these species.

MATERIAL AND METHODS

Biological Materials – Cercariae of *S. mansoni* and *S. haematobium* were collected in 750 ml of distilled water from infected *Biomphalaria* sp. and *Bulinus* sp. snails attained from fresh water streams in four different provinces in Egypt (El-Qualyobia, El-Sharkaia, El-Dakahlia and Asuit), using light induction method (Lim *et al.*, 1999). Cercariae were shed from *Biomphalaria* sp. and *Bulinus* sp. snails in water and collected by sedimentation for about 20 minutes on ice.

DNA extraction – The current molecular biology study was carried out in Faculty of Science-Zagazig University-Benha branch. Total DNA was extracted from the cercariae of *S. mansoni and S. haematobium* using the Wizard Genomic DNA Purification Kit (Promega) with some modifications. Briefly, the tissues were mechanically dispersed in 200 µl of nucleic lysis solution and incubated at 37°C for 4 h or overnight with 50 µg/ ml proteinase K. Thereafter, 80 µl of protein precipitation solution was added to the initial mix. The mixture was vortex vigorously for 10-20 sec and centrifuged at 13,000 rpm for 5 min. The supernatant was transferred to a microcentrifuge tube containing 200 µl of room temperature isopropanol for DNA precipitation. The mixture was gently mixed by inversion for 20 min and centrifuged at 13,000 rpm for 5 min. The DNA pellet was washed with 300 µl of 70% ethanol and centrifuged for 1 min. The pellet was treated with 25 µl of DNA rehydration solution for 30 min at 65°C and stored at -20°C. The DNA concentrations were estimated by comparison with known standards on 2% ethidium bromide stained agarose gels.

Amplification by PCR-The entire nuclear srDNAwas amplified, using the primers SSU1 (5'-CGACTGGTTGATCCTGCCAGTAG-3') and SSU2 (3'-TCCTGATCCTTCTCAGGTTCAC-5') (Amresco)anchored respectively in the conserved extremities of the 18S ribosomal gene (Stothard & Rollinson, 1997). The PCR amplification was undertaken in a volume of 10 µl consisting of: 1-10 ng template DNA, 10mM Tris-HCl, pH8.5,200 µm each dNTP, 1.5 mM MgCl 2,0.8 U of Taq DNA polymerase,50 mM KCl, together with 5.0 pmol of each primer. The reactions were covered with a drop of mineral oil and subjected

to the following cycle program: initial denaturation step for 3 min at 95°C, and then 32 cycles with annealling at 54°C for 1 min, extension at 72°C for 2 min, denaturation at 95°C for 45 sec and a final extension step at 72°C for 5 min. A negative control (no template DNA) was included in all phases of the experiment. Three microlitres of the amplification products were visualized on 0.8% ethidium bromide stained agarose gels to check the quality of amplification. The remaining 7 µl were mixed with 53 µl of water, and divided into 10 µl aliquots for enzyme digestion.

Production and Evaluation of the Nuclear rDNA-18S RFLP Profiles-In the initial experiments, the enzyme *Bsp*MI (Amersham, Life Science) was evaluated for its ability to differentiate all *Schistosoma* species within Egypt. Additional enzymes were tested including AccI, AvaII, (Boehringer Mannheim) *Sna*BI (Sigma Co, USA) in cases when *Bsp*MI was not effective in the separation of the two species. One microlitre (10-12 units) was used for each digestion reaction, together with 1.2 µl of the respective enzyme buffer for a final volume of 12.2 µl. The digestion was performed for 3.5 h at 37°C, and the digestion products were evaluated on 2% TBE-agarose (FMC Bioproducts) gels and stained with ethedium bromide. Bands were detected upon ultraviolet transillumination and photographed (35mm Kodak Film, England).

RESULTS

DNA genome was extracted from *S. mansoni* and *S. haematobium* and collected from the four provinces and represented in Figure 1. This shows DNA genome from *S. mansoni* and *S. haematobium* isolates, lanes 1 - 4 represent *S. mansoni* DNA genome which were isolated from El-Qualyobia, El-Sharkaia, El-Dakahlia and Asuit provinces, respectively and lanes 6-9 represent *S. haematobium* DNA genome which were isolated all from the same provinces. The full-length nuclear small subunit ribosomal RNA (srRNA) gene PCR products from *S. mansoni* and *S. haematobium* with primers SSU1 and SSU2 resulted in a product of approximately ~2 kb (Fig. 2). Figure 2 symbolized full-segment srDNA of *Schistosoma* sp. isolates, lanes 1 – 4 represented srDNA of *S. mansoni* from El-Qualyobia, El-Sharkaia, El-Dakahlia and Asuit provinces, respectively, lanes 5 – 8 represented srDNA of *S. haematobium* from the same provinces. *Schistosoma* sp. isolates, which were collected from all provinces, give the same results when their nuclear rDNA was digested with the

different restriction endonucleases. All isolates were digested with BspMI. Figure 3 shows the representative RFLPs patterns from *S. mansoni* and *S. haematobium* from each isolate (province) with BspMI, which produced roughly the same fragments (2 bands, ~500 and ~1500 bp, for all isolates). This enzyme produced a simple profile with two fragments, which were clearly similar for all isolates. AccI restriction enzyme digested the nsrDNA of *S. mansoni* into 3 different band sizes (~450, ~500, and~950 bp,lanes 1-) rom ll provinces's isolates, (Fig. 4). The same restriction enzyme cut the nsrDNA of *S. haematobium* to 2 bands (~500 and ~1500 bp, lanes 5 - 8) from all provinces's isolates, (Fig. 4).In analysis of the nuclear srRNA gene, AvaII enzyme resulted in two RFLPs with *S. mansoni* (~800 and ~1200 bp, lanes 1 – 4), but digested nsrDNA of *S. haematobium* into three patterns (~500, ~600 and ~900), figure 5. The nuclear gene of *Schistosoma* sp. isolates was identified by one restriction enzyme. The nuclear gene of *S. mansoni* was digested uniquely by SnaBI (~500 and ~1500 bp, lanes1– 4),whereas *S.haematobium* nuclear gene was not digested at all by this restriction enzyme,(Fig. 6 lanes5 – 8).

DISCUSSION

In this research, it has been attempted to determine whether the two isolates of *S. mansoni* and *S. haematobium*, from El-Qualyobia, El-Sharkaia, El-Dakahlia and Asuit provinces (with any mutational differences among the isolates), that have been identified, belong together to a single species, or they are isolates distantly related. The problem is important because *S. mansoni* and *S. haematobium* are human pathogens and there were many difficulties with classification of pathogenic *Schistosoma* strains or isolates in the past. There was an indication in the past, however, that it might be monophyletic, but there was a need for more definitive test of this possibility. Our approach to this problem was to use a modern method of studying relationships among *S. mansoni* and *S. haematobium* isolates in which phylogeny is reconstructed on the basis of nuclear srDNA RFLPs. Differences in electrophoretic patterns are known as RFLPs (Restriction Fragment Length Polymorphisms). The contribution was to find the sites in the nuclear srRNA genes where different endonucleases enter. Pires *et al.*(1997), Caldeira *et al.* (1998), Vidigal *et al.*(1998), Spatz *et al.*(1999) Wu *et al.*(1999)and

Vidigal *et al.* (2000) used restriction fragment length polymorphisms (RFLPs) of Schistosomal snails DNA as a basis for examining relationships among strains. The possibility of using restriction enzyme analysis of the nuclear srRNA genes as an experimental tool to identify the two strains proved valid (*S.mansoni* and *S.haematobium*) and to find specific enzymes to identify individual strains.

BspMI restriction enzyme did not differentiate nsrDNA of *S. mansoni* and *S. haematobium* isolates that digested the genes into approximately two similar band lengths of all isolates (Fig. 3). This indicated that *S. mansoni* and *S. haematobium* isolates may be of the same species, or monophyletic. There are two restriction enzymes (AccI and AvaII) that differentiated nsrDNA of *S. mansoni* and *S. haematobium* of all isolates.AccI restriction enzyme it digested nsrDNA of *S. mansoni* to three RFLPs profile(~450, ~500,and ~950 bp), whereas it digested *S. haematobium* to two fragments (~500 and ~1500 bp),(Fig. 4).AvaII restriction enzyme cut nsrRNA genes of *S. mansoni* to two restriction patterns(~800 and~1200), but digested that of *S.haematobium* into three bands(~500, ~600, and ~900 bp), (Fig. 5).Thus, AccI and AvaII restriction enzymes are the best restriction enzymes for differentiation of *S. mansoni* and *S. haematobium*. This indicates that *S. mansoni* and *S. haematobium* isolates might be different species. It has been found that we can identify *S. mansoni* isolate by RFLPs obtained by using specific endonuclease to digest nsrDNA PCR product. There is one restriction enzyme, SnaBI, that gave unique RFLPs for *S. mansoni* through digestion of nsrDNA and gave a negative result with *S. haematobium* (Fig. 6).

So, PCR-RFLP is a simple and rapid technique representing an important advance for studies of Egyptian *Schistosoma* species, which can be used as a complementary tool to morphological identification. The study demonstrated that nsrDNA (nsrRNA genes) contain useful genetic markers for the identification of these *Schistosoma* (*mansoni* and *haematobium*). The present data proved that *S. mansoni*, which was collected from four provinces of Egypt, have similar strains and so are *S. haematobium*. The results obtained with PCR-RFLP accord with the actual morphological systematics proposed for the Egyptian *Schistosoma*.

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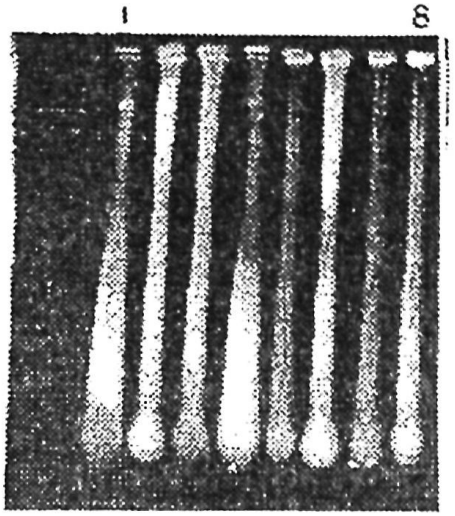

Figure 1. DNA genome from *S. mansoni* and *S. haematobium* isolates, lanes 1 - 4 represent the DNA genome of *S. mansoni* which was isolated from El-Qualyobia, El-Sharkaia, El-Dakahlia and Asuit provinces, respectively and lanes 6-9 represent the DNA genome of *S. haematobium* which was isolated all from the same provinces.

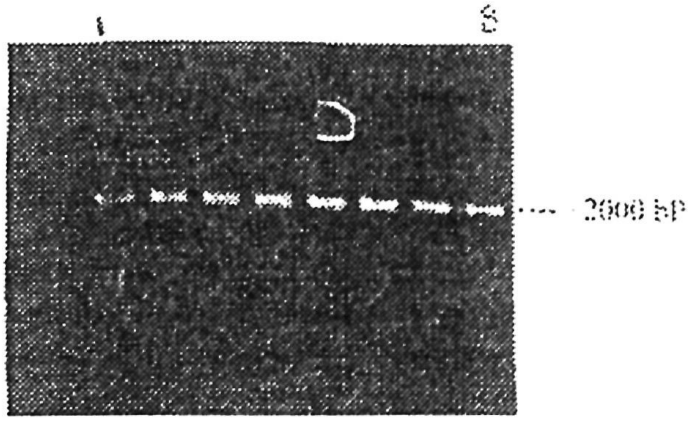

Figure 2. symbolized full-segment srDNA of *Schistosoma* sp. isolates, lanes 1 – 4 represented srDNA of *S. mansoni* from El-Qualyobia, El-Sharkaia, El-Dakahlia and Asuit provinces, respectively, lanes 5 – 8 represented srDNA of *S. haematobium* from the same provinces.

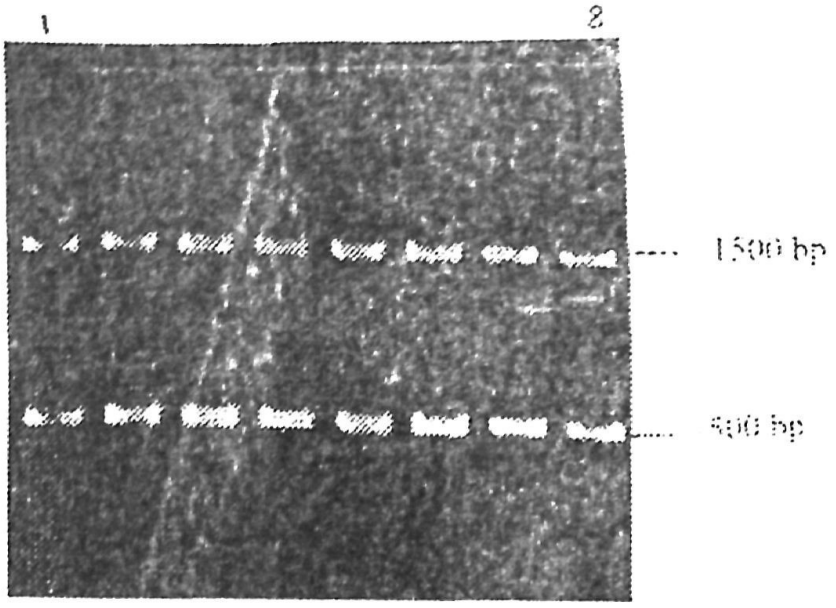

Figure 3. shows the representative RFLPs patterns from *S. mansoni* and *S. haematobium* from each isolate (province) with BspMI, which produced roughly the same fragments (2 bands, ~500 and ~1500 bp, for all isolates).

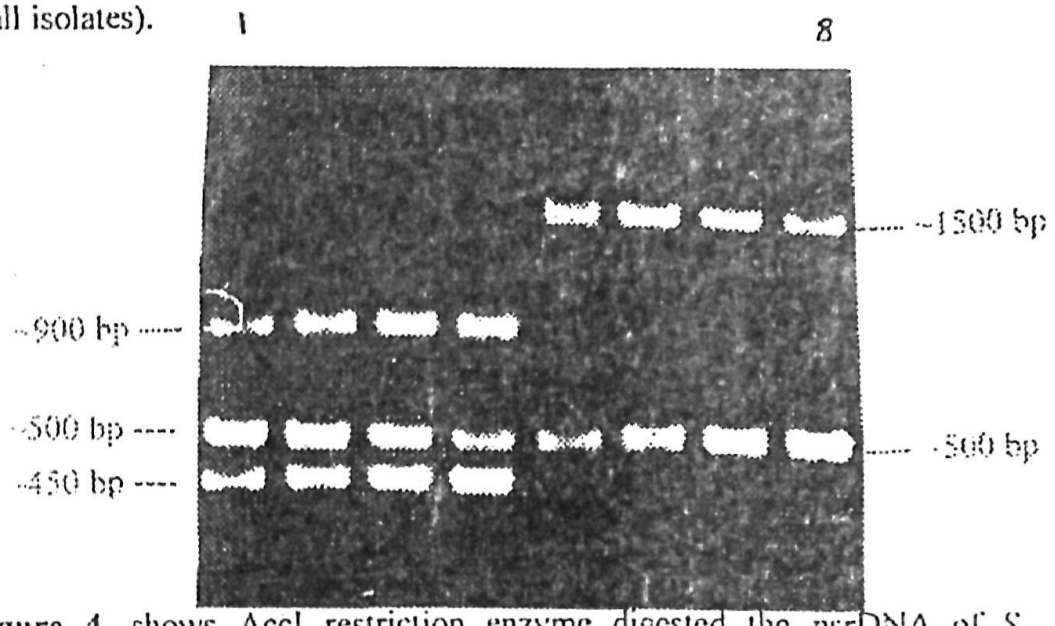

Figure 4. shows AccI restriction enzyme digested the nsrDNA of *S. mansoni* into 3 different band sizes (~450, ~500, and ~950 bp, lanes 1 - 4) from all provinces's isolates. The same restriction enzyme cut the nsrDNA of *S. haematobium* to 2 bands (~500 and ~1500 bp, lanes 5 - 8) from all provinces's isolates.

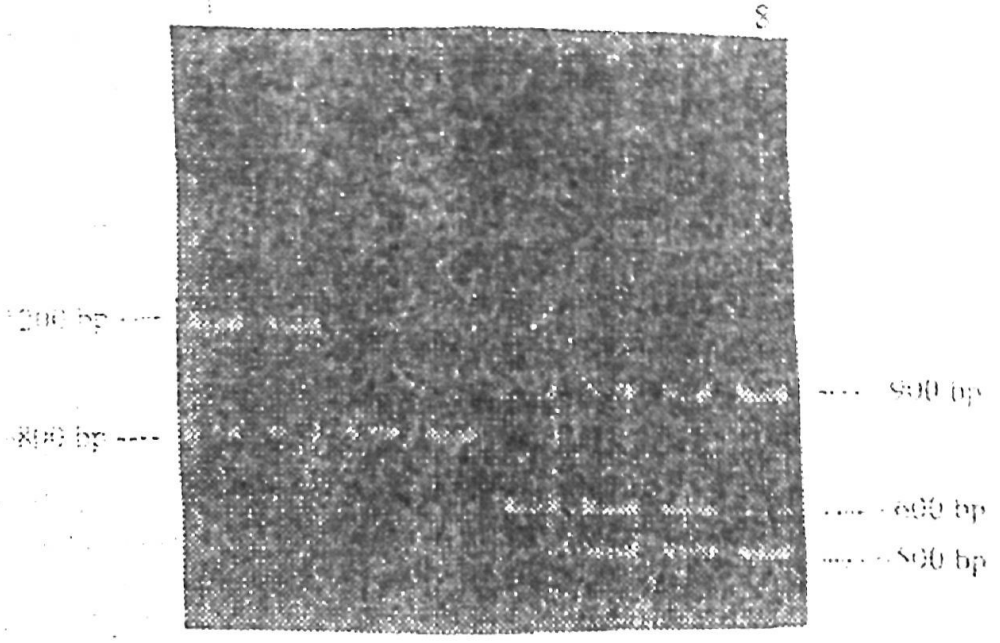

Figure 5. The analysis of the nuclear srRNA gene, AvaII enzyme resulted in two RFLPs with *S. mansoni* (~800 and ~1200 bp, lanes 1 – 4), but digested nsrDNA of *S. haematobium* into three patterns (~500, ~600 and ~900).

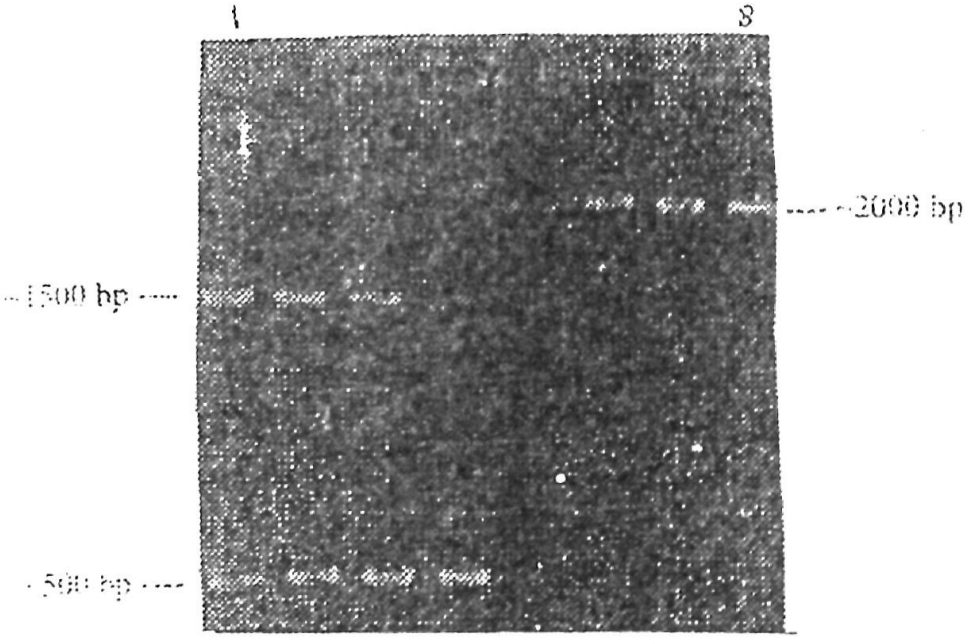

Figure 6. The nuclear gene of *S. mansoni* digested uniquely by SnaBI (~500 and ~1500 bp, lanes 1 – 4), whereas *S. haematobium* nuclear gene was not digested at all by this restriction enzyme (lanes 5 – 8).