Phylogenetic analysis of *S. mansoni* in an infected population from two governorates in Egypt

Original Article

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ABSTRACT

Background: Data scarcity significantly hinders comprehensive phylogenetic analyses of *S. mansoni*. This limitation impedes our understanding of its evolutionary history, population structure, and the emergence of drug resistance. Addressing this gap through increased genomic sampling is crucial for robust evolutionary inferences and effective control strategies.

Objective: The present work aimed to appraise the genetic diversity of *S. mansoni* in two Egyptian governorates.

Subjects and Methods: *Schistosoma* eggs were detected using the Kato–Katz method in a total of 238 fecal samples from the two areas. From 33 positive samples, cytochrome c oxidase (*cox-1*) gene of 9 random cases was detected using conventional PCR, and sequenced from the mitochondrial DNA of *S. mansoni*. The alignment results were subsequently used to construct a phylogenetic tree *via* the neighbor-joining method in the MEGA6 program.

Results: The overall detection rate of *S. mansoni* in the study population was 13.9% representing 14.1%, and 13.6% in Kafr El Sheikh and El-Behira governorates, respectively. Alignment analysis of *S. mansoni* in the two governorates revealed three closely related clusters. One of the sequences was submitted to GenBank under accession number MW784615 (https://blast.ncbi.nlm.nih.gov/Blast.cgi#alnHdr_532139118).

Conclusion: Phylogenetic analysis revealed minimal genetic diversity of *S. mansoni* isolates from the two examined populations in Kafr Elsheikh and Behira Governorates.

Keywords: *cox-1* gene; Egypt; gene bank; phylogenetic analysis; risk factors; *S. mansoni*.

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INTRODUCTION

Schistosomiasis is a human parasitic disease endemic to tropical and subtropical areas with limited access to fresh water and adequate sanitation. Globally, it is the most prevalent neglected tropical disease (NTD) in sub-Saharan Africa. There are more than 250 million schistosomiasis cases worldwide, mostly in sub-Saharan Africa, and 95 million adults are estimated to be at risk of infection[1]. The number of DALYs lost due to schistosomiasis was estimated at over 2.2 million in the year 2000, decreasing to 2.1 million in 2010 and to an estimated 1.6 million in 2019 (24% reduction from 2000)[2]. In Egypt, infection rates have dropped: 4.7% and 1.25% prevalence of *S. haematobium* and *S. mansoni*, respectively, in rural areas[3].

For accurate and precise diagnosis of schistosomiasis, an endemic infection in many low-income countries, microscopy remains the gold standard for fast, easy, and cheap identification of *Schistosoma* spp. eggs in stool and urine samples[4]. Molecular methods proved to be increasingly important for studying schistosomiasis epidemiology, enabling more accurate examination of the *Schistosoma* spp. structure, genetic subdivision and response to control efforts. The *cox-1* genetic fragments are valuable for illustrating the phylogenetic diversity of *S. mansoni* from animals and human at the intraspecific level, *i.e.*, there is evolutionary differences within a single species[5,6]. The specific genotypes of *S. mansoni* within an individual host have crucial implications on both the transmission dynamics and the overall genetic diversity of schistosomes in the host population[7].

Although mitochondria undergo rapid evolution, the *cox-1* fragment is characterized by low evolution and rates and can thus be used for genetic characterization[8]. Our study aimed to sequence the *cox-1* gene from the mitochondrial DNA of *S. mansoni* eggs isolated from two different infected populations in Kafr Elsheikh and Behira governorates, Egypt. These sequences were aligned and compared to information from databases (BLAST.com), that might help in the phylogenetic analysis of the *S. mansoni* based on *cox-1* gene.

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SUBJECTS AND METHODS

This observational analytical study was conducted at Parasitology Department, Medical Research Institute (MRI), Alexandria University from June 2019 to February 2023.

Study design: The study was carried out on subjects recruited from primary health care centres or hospitals in Egypt (Elshekhloba village and Damanhor city). Diagnosis of schistosomiasis *mansoni* was performed by microscopic examination of the Kato–Katz prepared slides. From positive samples, *cox-1* gene was amplified using conventional PCR, and it was sequenced from the mitochondrial DNA. The alignment results were subsequently used to construct a phylogenetic tree via the neighbour-joining method in the MEGA6 program.

Study area: Figure (1) shows the locations of the study area.

Fig. 1. Map showing location of Elshekhloba village and Damanhor city.

Subjects: Participants (No.=238) were divided into two groups. Group (1) included 103 subjects from the Elshekhloba area, Kafr Elsheikh Governorate, while group (2) included 135 subjects from Damanhor city, Al-Behira Governorate. All participants mainly worked as farmers, fishermen, or were students or housewives. Participants and/or guardians completed a questionnaire and received health education about schistosomiasis. Table (1) shows the demographic characteristics of the study participants.

Table 1. Demographic characteristics of the participants in the two studied areas.

Characters	Kafr Elsheikh Governorate, Elshekhloba village (No. = 103)	Al-Behira Governorate Damanhour city (No. = 135)
	No. (%)	No. (%)
Age		
< 20	47 (45.6)	50 (37.04)
20 –	47 (45.6)	49 (36.30)
40 –	9 (8.7)	30 (22.22)
>60	0 (0.1)	6 (4.44)
Gender		
Male	56 (54.4)	78 (57.80)
Female	47 (45.6)	57 (42.20)
Occupation		
Student	47 (45.63)	48 (35.50)
Housewife	20 (19.42)	34 (25.20)
Fisherman	30 (29.12)	9 (6.70)
Farmer	6 (5.83)	44 (32.60)

Samples examination: Fresh stool samples were collected from all participants. The Kato–Katz technique[9] and microscopic examinations were implemented to detect *S. mansoni* eggs.

Amplification of *S. mansoni cox-1* gene: The DNA extraction from positive *S. mansoni* samples was performed according to the manufacturer' instructions for the ZYMO fecal isolate DNA[10]. Conventional PCR was performed for *cox-1* gene amplification[11] using the primer pairs JB3 (5` TTT GGG CAT CCT GAG GTT TAT 3) and JB4.5 (5` TAA AGA AAG AAC ATA ATG AAA ATG 3`)[12]. The PCR mixture was prepared using 0.5 mM of each primer (forward and reverse), 10 µl of Taq HS Red Mix, and 20 ng of DNA sample, and the volume was increased to 20 µl using nuclease-free H_2O. Amplification was performed using the recommended thermal cycling conditions: one cycle at 95°C for 1 min; 40 cycles at 95°C for 15 sec, 57°C for 1 min and 72°C; and a final extension at 72°C for 5 min. Amplified DNA was visualized using an electrophoresis apparatus with a 1.5 g% agarose gel stained with ethidium bromide[12]. Fragment bands of the PCR products were purified from the agarose gel using a Thermo DNA Purification Kit (K0701) according to the manufacturer's instructions.

Sequencing: The sequencing analysis was performed on randomly selected positive samples with Big Dye Terminator V3.1 cycle sequencing kit (Applied Biological Materials "ABM" Richmond, BC, Canada) using the forward primer JB3 and reverse primer JB4.5 (200 µM, 60°C Tm) to confirm the species identification.

Multiple sequence alignment (MSA): The alignment involved our five sequences, and 147 additional sequences of *S. mansoni* retrieved from the NCBI database[13]. These 147 sequences were selected since they showed high identity (98-100%) to our sequences, *i.e.*, they had very low *E*-values indicating strong similarity.

Poorly aligned positions and divergent regions of a DNA alignment were eliminated by the Gblocks server so that the sequence became more suitable for phylogenetic analysis. The evolutionary history was derived by using the maximum likelihood method and the Tamura 3-parameter model[14]. The bootstrap consensus tree deduced from 100 replicates[15] was constructed to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 60% bootstrap replicates were collapsed. Initial tree(s) for the experimental search were obtained automatically by applying the neighbour-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura 3 parameter model and then selecting the topology with the superior log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among sites [5 categories (+G, parameter = 0.8436)]. This analysis involved 95 nucleotide

sequences. Evolutionary analyses were conducted in MEGA11[16]. An evolutaionary analysis of 95 nucleotide sequences was conducted in Molecular Evolutionary Genetics Analysis Version 11 (MEGA11)[16], and a coloured phylogenetic tree was constructed using the interactive tree of life (ITOL) server.

Statistical analysis: The SPSS software version 22 statistical program (Feeney, 2012) was utilized for both data presentation and statistical analysis of the results. Categorical data were presented using frequency and percent. Association between categorical variables was assessed using Chi square test, or Monte Carlo significance test in more than 2x2 table if assumption is not met. Odds ratio with confidence interval were calculated. Level of statistical significance was set at 0.5.

Ethical consideration: An ethical approval was obtained from the Research Ethical Committee of the Medical Research Institute (MRI), Alexandria University (IORG000812). All performed procedures involving human participants were in accordance with the ethical standards of the institutional, national research committee and with the 1964 Helsinki declaration and its later amendments. Informed consents were obtained from participants after explaining purpose of the study. The confidentiality of the collected information and participants' identity were assured throughout the study.

RESULTS

Microscopic examination: Table (2) shows the rate of infection among the two studied groups diagnosed by Kato–Katz technique. Difference of detection rates between participants from the two areas proved to be insignificant. Among the 238 participants, 33 were positive for *S. mansoni* eggs by the Kato-Katz technique (Fig. 2).

Table 2. Detection rate of schistosomiasis *mansoni* among the two studied groups diagnosed by microscopy.

Kato-katz technique	Elshekhloba village (No. = 103)	Damanhour city (No. = 135)	Statistical analysis
	No. (%)	No. (%)	
Positive	14 (13.6%)	19 (14.1%)	X^2: 0.11
Negative	89 (86.4%)	116 (85.9%)	*P*: 0.915

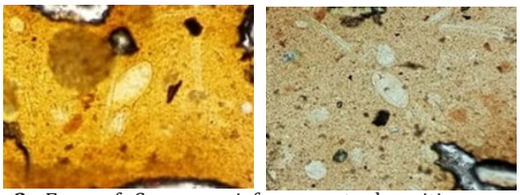

Fig. 2. Eggs of *S. mansoni* from a stool-positive sample examined microscopically after the Kato-Katz technique (10X).

Risk factors associated with schistosomiasis: Table (3) shows the risk factors associated with schistosomiasis *mansoni* in the examined participants. According to age, the highest infection rate was observed in the 20-40 age group (57.6%), followed by the <20 age group (30.3%), while the lowest infection rate was shown in the 40-<60 age group (12.1%), and no infection occurred in the >60 age group (0%). Regarding sex, the percentage of *S. mansoni* patients was significantly greater in males (75.8%) than in females (24.2%). Being a male increased the risk of acquiring schistosomiasis 3-fold (OR=3.279). With respect to occupation, the highest infection rate was observed among farmers (51.5%), followed by fishermen (24.2%), and the lowest infection rates were 21.2% and 3.1% for students and housewives, respectively. Farmers had a 2.9-fold greater risk of acquiring infection. Regarding contact with fresh body water, all positive patients had a history of contact with water. Individuals who had contact with water were 7 times more likely to have acquired the infection than those who had no contact with water (OR=7.9, *P*=0.008).

Table 3. Risk factors associated with schistosomiasis in the examined participants diagnosed by the Kato-Katz technique.

Characters	Positive cases (N=33)	Statistical analysis		
	No. (%)	95% CI (LL-UL)	OR	*P* value
Age				
< 20	10 (30.3)	0.471-2.173	1.0116(0.976)	^{MC}P= 0.665
20 –	19 (57.6)	0.615 -2.72	1.292 (0.498)	
40 –	4 (12.1)	0.104-2.06	0.464 (0.313)	
>60	0 (0.0)	-	-	
Gender				
Male	25 (75.8)	1.393 – 7.717	3.279*(0.007*)	0.002*
Female	8 (24.2)	1.863 (0.330)	1.863 (0.330)	
Occupation			1.354 (0.514)	
Student	7 (21.2)	0.544 – 3.371	0.426 (0.418)	
Housewife	1 (3.1)	0.054 – 3.355		
Fisherman	8 (24.2)	0.431 – 2.403	1.018 (0.966)	0.0001*
Farmer	17 (51.5)	0.457 – 1.992	2.954 (0.900)	
Contact with water source				
Yes	33 (100)	1.277 – 5.814	7.948 (0.728)	0.008*
No	0 (0.0)	-	-	

CI: Confidence interval; **LL:** Lower limit; **UL:** Upper limit; **OR:** Odds ratio; **MC:** Monte Carlo test for significance.

Conventional PCR: Successful amplification of the *S. mansoni* gene was confirmed by the detection of a clear band at 451 bp in a 1.6% agarose gel electrophoresis gel stained with ethidium bromide. Results revealed that six samples were positive in group I, and seven in group II (Fig. 3).

Phylogenetic analysis: Five isolates were sequenced (n1, n10, n24, n72, and n92). The common ancestor of the four isolates (n1, n10, n24, and n72) was the n92 isolate, as the ancestral homeland for the infection. One of the present sequences was submitted to GenBank under accession number MW784615 (https://blast.ncbi.nlm.nih.gov/Blast.cgi#alnHdr_532139118). Additionally, the sequences from five isolates fell within a cluster with *S. mansoni* H321 Homo sapiens KC964809.1; Uganda strain, *S. mansoni* chimpanzee JF508492; Uganda strain, *S. mansoni* H123 GQ415308; Uganda strain, *S. mansoni* chimpanzee JF508499; Uganda strain, *S. mansoni* H72 GQ415255; Uganda strain; and *S. mansoni* TA1d JQ289694; and the Tanzania strain (Table 4, Figures 4, 5).

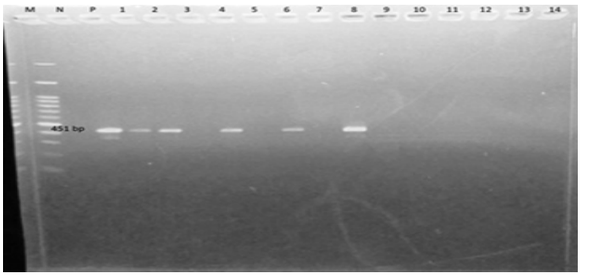

Fig. 3. A light photograph showing a 1.6% agarose gel stained with ethidium bromide represents the PCR amplification of the *cox-1* gene from *S. mansoni*-positive samples. **Lane M:** DNA marker; **lane N:** negative control (nuclease-free water); **lane P:** known positive DNA extracted from positive cases; **lanes 1, 2, 4, 6, 8:** positive specific band 451 bp for the *S. mansoni* mitochondrial cox 1 gene extracted from stool; and lanes 3, 5, 7, and 9: negative samples for the *S. mansoni* mitochondrial *cox-1* gene (showing no bands).

Table 4. Identification of strains associated with the studied positive molecular products by sequencing and accession numbers of the reference strains.

Sample code	Accession No.	Host pr isolation source	Origin country	*E* value
1	KC964809.1	*Homo sapiens*	Uganda	0.0
2	JF508492	Chimpanzee	Uganda: Ngamba Island, Lake Victoria	0.0
3	GQ415308	Lake Victoria	Uganda	0.0
4	JF508499	Chimpanzee	Uganda: Ngamba Island, Lake Victoria	0.0
5	GQ415255	Lake Victoria	Uganda	0.0
6	JQ289694	NA	Tanzania	0.0

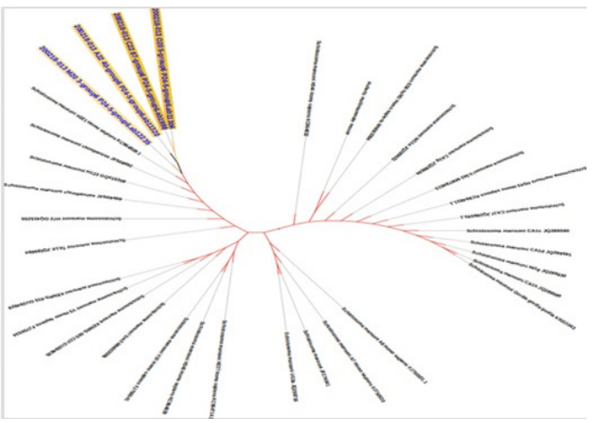

Fig. 4. Phylogenetic tree of *S. mansoni* aligned with 100% matched sequences in BLAST database.

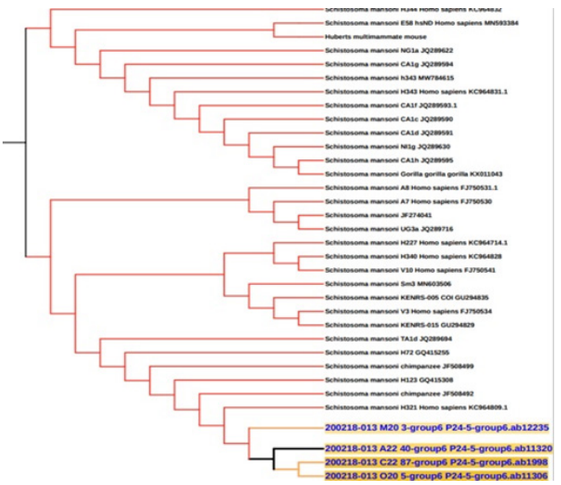

Fig. 5. The dendrogram was constructed by using NJ method. The distance of similarities was based on the molecular basis evolution of minisatellite sequence regions in the genomic DNA.

DISCUSSION

In *Schistosoma* spp., sexual reproduction with release of eggs inside the definitive hosts (humans, other primates, rodents) allows for parasite genotypic diversity. The phylogeny and evolution of the genus *Schistosoma* are of great interest and have been the subject of several molecular studies[17-19]. Although *S. mansoni* is morphologically considered a uniform species, differences between parasite strains or populations have been observed in terms of several biological characteristics, such as infectivity, virulence, response to treatment and fecundity[8]. Understanding the genetic structure of *S. mansoni* will lead to a better understanding of the variation in natural populations and the transmission dynamics of schistosomes between hosts across geographic areas. Accordingly, the current work aimed to update the rates of predisposition to infection and genotypic differences in *S. mansoni* infection in two different infected populations in the Kafr Elsheikh and Behira governorates, Egypt.

The overall detection rate of schistosomiasis *mansoni* was 14.1% (n=19), and 13.9% (n=14) in the Elshkhloba and Dammanhur areas, respectively. The observed

slightly high infection rate in our study from these localities might be due to the water contact behaviour of the study participants, the environmental sanitation, socioeconomic status, the ecological distribution of snails and local endemicity. These results are also similar to those of Ahmed *et al.*[3] who reported that colonic schistosomiasis is still prevalent among the Egyptian Nile Delta's symptomatic rural inhabitants at a rate of 12.4%. In agreement, earlier WHO reports estimated the prevalence of *S. mansoni* in the Delta region's general population at 14.8% in 1993, using a single Kato-Katz thick smear. This prevalence subsequently fell to 1.5% by 2006 as a result of successful control programs[20].

The molecular analysis revealed that 39.39% of Kato–Katz-positive samples were positive for *S. mansoni*. However, a high number of Kato–Katz-positive patients was observed compared to those detected *via* PCR. This could be attributed to presence of inhibitors in the samples; samples may not have the minimum number of eggs required to obtain DNA during the extraction; or the samples were taken from individuals from low-endemicity areas with low infection loads. Another possibility is the effect of variation in egg production and the consequent uneven egg distribution within the collected samples. Uneven egg distributions are typically minimized through fecal homogenization; however, the quantity removed from the sample for microscopic examination may have removed the few eggs present in the sample. Of note, relatively few studies addressed the detection of *S. mansoni* DNA in clinical samples[22-24]. Interestingly, schistosomiasis was diagnosed in the stool by real-time PCR (44%), followed by Kato–Katz (42%) and loop-mediated isothermal amplification (LAMP) (36%) among 50 schoolchildren from a rural area in Alexandria, Egypt[25].

Evolutionary relationships among all creatures are depicted as a branching tree, reflecting descent from a common ancestor. Genetic diversity drives the divergence of lineages, represented as terminal branches. Phylogeny, the historical pattern of descent, is visualized through phylogenetic trees (dendrograms), which illustrate evolutionary relationships and distances between taxa. These trees, while based on analytical hypotheses, provide a simplified framework for studying phylogenetic relationships. Clades, representing closely related groups, are positioned on proximal branches, indicating a higher degree of relatedness. Phylogenetic analysis involves the interpretation of these trees to accurately reconstruct evolutionary processes[26].

With respect to the sequence alignment results, the analysis investigated the population diversity of *S. mansoni* in the two governorates and revealed three closely related clusters and one outgroup. However, it is interesting that our samples were in cluster with 5 strains from Uganda, and one from Tanzania This finding showed low genetic diversity of *S. mansoni* and agreed with the study origin of the Egyptian haplotypes, which suggested that *S. mansoni*, established in Egypt, originally came from Central West Africa[27]. However, a former study stated that the Egyptian haplotype is more closely related to the Brazilian strain[8].

Additionally, phylogenetic analysis of the mitochondrial *cox-1* gene of 9 isolates collected from Kafr Elsheikh and Behira revealed that they are of the same genotype. This could be explained by the fact that the two governorates are geographically close to each other, and that the geographic topologies of the two areas are the same. The similarity of infections in both areas, despite being caused by a similar genotype, and exhibiting the same level of genetic variability, could be attributed to the ongoing National Schistosomiasis Control Program. This program likely reduced the genetic variability of *S. mansoni* by consistently lowering prevalence and intensity in both human and snail populations[28]. In conclusion, according to the results of phylogenetic alignment, the present study denied the genetic diversity of isolates from infected fishermen in the Kafr Elsheikh population and infected farmers in the Behira population. Accordingly, it was hypothesized that both areas exhibit the same ancestral genotype, same degree of genetic variability, and possibly common sources of infection.

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Authors contributions: Abd El-Latif NF participated in the conception and implementation of work, interpretation of the results, and writing of the manuscript. Elsayad M conceived and designed the experiments; reviewed the presentation and interpretation of the results. Tolba MM participated in the design, conception and implementation of work, interpretation of the results, and writing of the manuscript Thoul CT collected the stool samples and participated in the implementation of the work Mohamed SA reviewed the presentation and interpretation of the results.

Conflict of interest: We declare that no personal, or professional relationships or affiliations existed that could have influenced the design, conduct, or reporting of this research.

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