Populations of *Biomphalaria spp.* Snails Isolates in Kenya Show Variable Resistance to Schistosome Infection

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Abstract

Fresh water snails of genus *Biomphlaria, Bulinus* and *Ocomelinia* are important vectors for human schistosomiasis. Human contact patterns with water infested with cercaria are important factors in transmission of schistosomiasis and these conditions are associated with tropical climate wetlands. However, some snail strains within the genera that transmit cercaria are resistant to infection, providing means where these traits can be harnessed for biological control. PCR rDNA probes were used to determine miracidia/cercaria infected *Biomphalaria* snail isolates within Kenya. The derived sequences together with similar Genbank datasets were applied in phylogenetic analysis. PCR results showed that susceptibility/resistance trait occurs in proportion of the field sampled snails and also those maintained in laboratory culture. Phylogenetic analysis of sampled snails in this study together with composite dataset of similar rDNA sequences across Africa showed that *Biomphalaria spp* population structure is composed of distinct monophyla lineages and clusters of closely related isolates or clones. Further, the analysis indicated that nomenclature of *Biomphalaria spp.* classification needs revision. This provides evidence for resistance to cercaria transmission in some isolates of *Biomphalaria spp.* within Kenya but the trait does not cluster together in phylogenetics that is based on rDNA gene.

Keywords: *Snails; Phylogeny; Schistosomiasis; Resistance*

Introduction

Fresh water snails of genus *Biomphlaria, Bulinus* and *Oncomelania* are important vectors for human schistosomiasis. The disease also infects vertebrate mammals including primates, cattle, birds and crocodilians [1]. In human, the chronic disease (Bilharziasis) is debilitating in early childhood, therefore the main impact of schistosomiasis is low productivity in terms of human resources.

World Health Organization (WHO) global epidemiological data shows that schistosomiasis is endemic in 78 tropical and subtropical countries with 779 million people are at risk of schistosomiasis. More than 230 million people are infected with 20 million suffering from debilitating illnesses [2,3]. Due to the association of the disease incidence with poverty, Africa accounts for majority of the infections.

The life cycle of schistosomes has initial developmental stages in the invertebrate snails and further developments and maturity in a vertebrate host. Schistosome eggs that are released into fresh water by vertebrate host hatch to miracidia larvae that infect snails. After several weeks of sporocysts asexual multiplication in the snail tissue, the next larva called cercaria, are released into the water where they infects vertebrate host upon contact. Therefore, snail infection and human contact patterns with water infested with cercaria are important factors in transmission of schistosomiasis [4,5]. Therefore, ecological conditions favoring transmission cycle are tropical climate, fresh water wetlands, presence of intermediate snail and lack of proper sanitation. Wetlands provide a valuable focal point for domestic water consumption, irrigation agriculture and fishing dams, as well as source of water for wild and domestic animals [6]. Farming wetlands are valuable but also focal point of disease transmission [7,8].

The species of schistosomes of economic importance include *Schistosoma mansoni,* originally from Africa and later spread to South America; *S. haematobium*, found in Africa and adjacent Middle East regions and *S. japonicum* found in South-East Asia. *S. intercalatum* and *S. mekongi* have more localized geographic location while *S. bovis* is an important parasite for angulates [9,10]. Fresh water snails of the genus *Oncomelania* are the important vectors for *S. japonicum* while the pulmonate snails of the genus *Bulinus* and *Biamphalaria* transmit *S. haematobium* and *S. mansoni,* respectively [11].

Biological control using competitor fresh water snails in the reduction of schistosomiasis transmission has achieved appreciable successes in the Caribbean Islands and in Brazil [12]. In their studies in West Indies, Pointier et al. and Guimareas et al. showed that *B. glabrata* and *B. straminea* can be eliminated using competitor thiarid snail, *Melanoides tuberculata* [13,14]. Other approach in biological control of transmitting snails is by exploiting snails genetically resistant to schistosomes. *B. glabrata* and *B. tenagophila* are important in transmission of *S. mansoni* in Brazil, but the Taim strain of *B. tenagophila* consistently shows absolute resistance against the parasite [15]. Several studies on challenge infection of Taim strain with miracidia shows systematic resistance of this strain to *S. mansoni* [16,17] and the resistance trait was dominant during crossbreeding with susceptible *B. tenagophila* strains [18].

In the African region, it has been noted that not all species within the genus *B. africanus* act as intermediate host for *S. haematobium* and similarly, *S. mansoni* miracidia infects only certain strains of *B. glabrata* and *B. ugandae* species while some strains of are refractory to infection [19]. This study explores population variability of *Biomphalaria* snails samples from within Kenya and tests PCR probe for cercaria transmission to determine the susceptibility or resistant trait in these snails.

Methods and Materials

Sampling and specimen identification

Field work sampling of *Biamphalaria* snails was carried out in two schistosomiasis endemic regions: Mwea irrigation farmlands that drain from Mt Kenya in central Kenya highlands and another site, Kibwezi dam in Eastern part of Kenya towards the coastal region. Sampling involved a technologist from Institute of Primate Research (IPR) schistosomiasis program to guide in determining microhabitat sites and vector snails by morphology. Snails were collected alive and placed on wet cotton wool in perforated containers then transported to snail culture facility at IPR within 24hrs.

Snail infection with miracidia

To standardize infections, snail were put in de-clorinated tap water in 200 ml beakers containing miracidia (at 5/ml-10/ml) that had been obtained from stool from on-going schistosomiasis experiments in non-human primates at IPR. After period of 3 to 6 weeks of incubation, cercaria shedding was done by procedure of direct light illumination where individual snails were put in 10ml water in beaker and cercaria shedding determined by observation on 10 X magnification stage microscope.

DNA extraction and preservation

Biopsy tissues from snails were obtained and subjected to DNA extraction using Qiagen kit (Qiagen Inc. MD, USA) to maximize yield. Briefly, 20 μl Proteinase K and 200 μl of digestion/lysis buffer was added to sample and incubated at 56°C water-bath for 1hr. The lysate was pelleted to remove debris by centrifugation (X1000 g) and the supernatant aspirated into silica matrix spin column to bind DNA. After initial centrifugation at 10,000 g for 2 min the DNA in the column matrix was 2X cleaned in wash buffer centrifugations then eluted in 200 μl nuclease free distilled de-ionized water (ddH$_2$0), as described in owner's manual. DNA was visually analyzed on 1% agarose gel with Ethdium bromide under utra-violet illumination and quantity determined by 260/280 nm ratio. The DNA samples were labelled and stored at -20°C for subsequent analysis.

Polymerase chain reaction (PCR) amplification

PCR was carried out using specific primers dependent on target gene of the extracted DNA, *Schistosoma spp* cercaria and *Biomphalaria spp* ribosomal target, respectively (TGCATACTGCTTTGAACATTC; CCTGACTAGGCTGGT) (TCGAAGCGCACGAACGCG; GGAAGGATCATTAAAGGCTT). The PCR amplification was done using approximate 70ng/ul of the template DNA, 0.5 μM of each primer, 200 μM each dNTP, 10 X buffer with 2.0 mM MgCl$_2$ and 0.5 U Takara Taq polymerase, made up to 25 μl with ddH$_2$O. Cycling conditions were: Taq polymerase activation 92°C, 3 min; then 30 cycles profile: denaturation, 92°C, 1 min; annealing 52°C-57°C, 1 min; polymerase nucleotide elongation 70°C, 1 min; final polymerase extension step 70°C, 3 min and final storage in the PCR machine at 8°C.

Sequencing and phylogenetic analysis

Gene clean procedures on the PCR products were carried out according to innuPREP procedures (Lifescience Jena, Germany) and samples sent for custom sequencing (Macrogen Inc., Denmark). The raw sequences were analyzed and edited in BioEdit Suite (BioEdit v7.0.5, Tom Hall Ibis Therapeutics) to get consensus sequences and trim 3' and 5'ends. Verification for cercaria derived sequences was infection was previously done via BLAST analysis to determine highly related sequences in NCBI nucleotide database. In another set of gene clean and sequencing procedures, Biomphalaria rDNA ITS1 sequences were

deposited via BankIt procedures in NCBI to GenBank (Accession: 579768.1-579823.1). To determine snail species, subspecies and strains population structure, analysis was performed in Phylip tree [20] and test of neutrality tested using Tajima-D [21] using program implemented in MEGA Version 6 software [22].

Results

Standardized sensitivity PCR detection of cercaria

PCR done on cercaria DNA using pair of primers franking the ITS 1 schistosoma rDNA yielded a distinct band approximate 500bps (**FIG.1**, arrow). Detection tested by limiting dilution was sensitive at picoMole/ femtoMole concentrations (1×10^{-12}-1 $\times 10^{-15}$g) of DNA. The PCR product had previously been verified through sequencing to be schistosome rRNA gene.
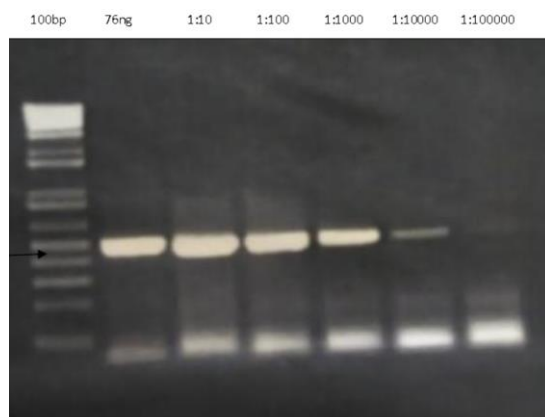

FIG. 1. **Limiting dilution test of sensitivity of PCR probes for cercaria detection, Lane 1, 100bp marker; lane 2-7, limiting dilution PCR tests.**

Field PCR screening of cercaria infection in snails

PCR on DNA extracted from snails after exposure to infection by miracidia produced specific amplification of target size band of approximately 400 bps in rDNA ITS gene (**FIG. 2,** arrow) but there were amorphous amplification, possibly degraded target. In the target amplification, the results were not uniform, with some snails demonstrating higher positive titre of sporocyst/cercaria DNA while others had limiting titre while others were negative.

Phylogenetic structure of *Biomphalaria* Snails in Kenya

The 450 bps PCR products obtained using primers for *Biomphalaria* rDNA 5.8 s, ITS1 were sent for custom sequencing and edited sequences subsequently submitted to GenBank (Accession: 579768.1-579823.1). The phylogenetic structure showed that certain lineages of *Biomphalaria* are monophyletic with long distance time separation lineages I, II and IIIA **(FIG. 3)** while the majority of the sampled snails populations clusteres were phylogenetically indistinguishable (IB, IIIB and other clonal off shoots within cluster II and III).

Phylogeographic structure of Biomphalaria snails within Africa

Snails rDNA sequences obtained in this study together with similar sequences datasets from across Africa obtained in GenBank showed that most snail are clusters of closely related populations, isolates or are derived from clonal propagation

(**FIG. 4**, curly brackets) with few monophyletic lineages (bold). While nomenclature may need revision, four clusters are evident (brackets I, II, III, and IV). *B. pfeiffeferi* is the most abundant with other representative populations in *B. sudanica*, *B. choanomphala*, *B. stanleyi*, *B. alexandria* and *B. glabrata*. Majority of Kenyan sampled snails (*italized*) belong to *B. pfeferri* but there is ample representation in the other clusters.
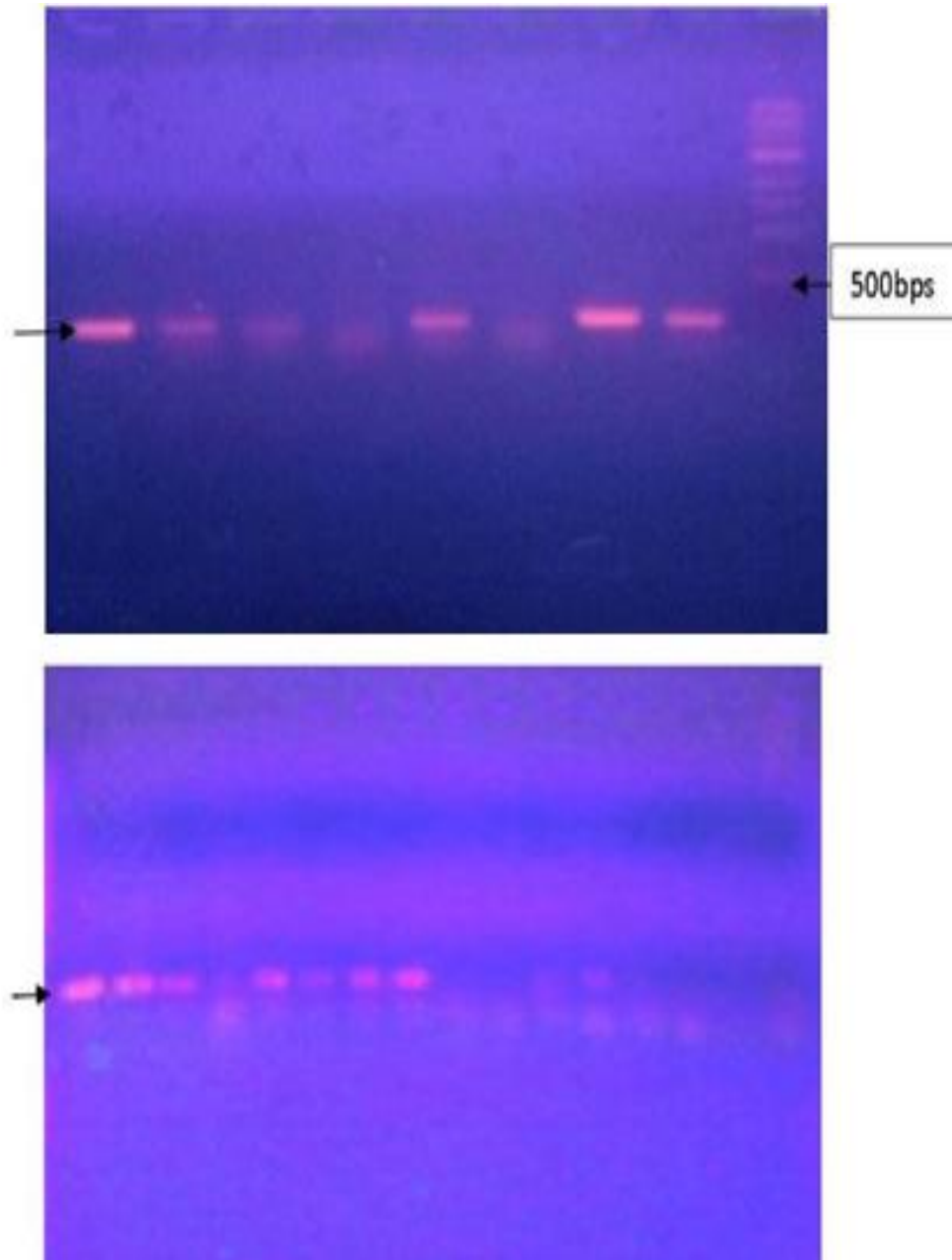

FIG. 2. **PCR detection of cercaria in snail samples collected during fieldwork within Kenya. Note arrow indicating 500bp maker and arrows indicating 400bp product. Amplification in samples is shown by arrow. Samples were either positive amplification, partial amplification or no amplification.**
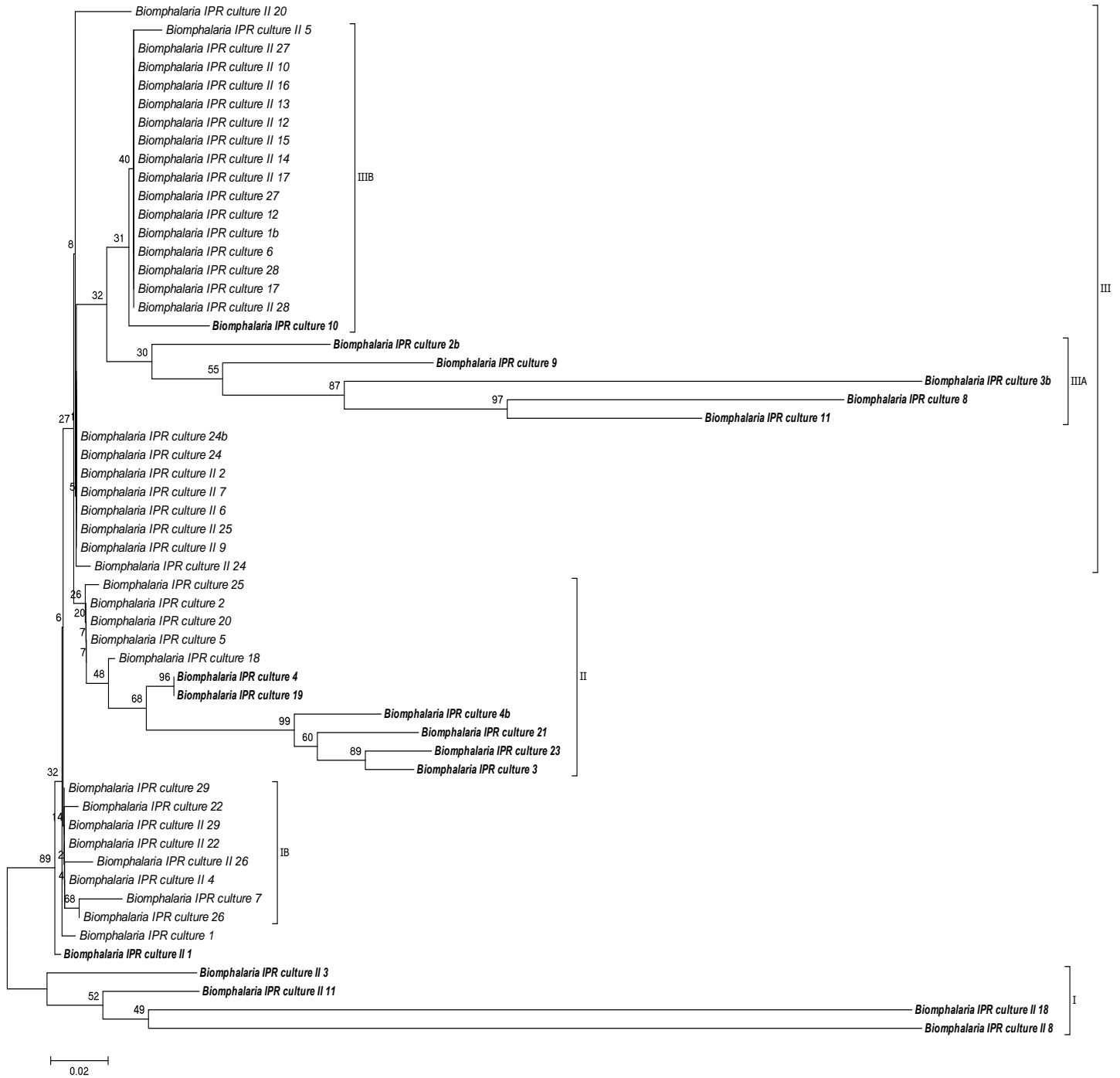
FIG. 3. **Evolutionary history of *Biomphalaria* snails population within Kenya was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown above the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 56 nucleotide sequences. There were a total of 207 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.**

FIG. 4. **Evolutionary history relationships of *biomphalaria* snails within Africa was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches . The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 83 nucleotide sequences with were a total of 207 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.**

Biomphalaria snails population drift

Test of drift from neutrality was performed using Tajima's D. When population is at equilibrium neutrality, the nucleotide diversity (π) and the number of nucleotide segregating sites (Θ) are indistinguishable. In the tested *Biomphalaria* populations (**TABLE 1**), Θ is significantly greater than π resulting in pronounced negative Tajima's D.

TABLE 1. **Tajima's Neutrality Test for *Biomphalaria* snails populations.**

Population	m	S	p_s	Θ	π	D
Kenya regional *Biomphalaria*	56	146	0.705314	0.153542	0.080617	-1.681654
Africa composite *Biomphalaria*	83	131	0.632850	0.126823	0.051698	-2.003619

Note: The analysis involved 56 and 83 nucleotide sequences representation of Kenya and Africa regions, respectively. There were a total of 207 positions in each of the final dataset. m=number of sequences, n=total number of sites, S=number of segregating sites, ps=S/n, Θ=ps/a1, π=nucleotide diversity, and D is the Tajima test statistic (π and S/a1 both estimate Θ, where E (expected) E [π]=Θ, E [S]=a1 Θ), software default significant at P < 0.10. Evolutionary analyses were conducted in MEGA6.

Discussion

Human population growth and erratic weather patterns have prompted the continued expansion of irrigated agricultural schemes in Africa hence transmission of schistosomiasis in this region is expected to increase [7,8]. Moreover, there is no effective vaccine against schistosomiasis [23,24]. It is therefore, important that various public health control measures for schistosomiasis are carried out in concert.

Freshwater *Biomphalaria* snails are the vector for *Schistosoma mansoni* and strategies to interrupt the disease at vector stage need more emphasis [25]. These snails show variability in susceptibility to transmit the human infective larvae stage, cercaria and some studies implicate macrophage-like hemocytes cellular immunity for the resistance to infection [26-28]. A necessary aspect in determining transmission is provision of tools to detect infection of snails by cercaria. The traditional demonstration of snail infection via shedding of cercaria upon direct illumination is easy and inexpensive but requires long and variable pre-patent periods from three weeks to two month for snails to readily shed [29]. Molecular detection of snail infection by cercaria has been done using PCR targeting NADH subunit 5 gene, Cytochrome c oxidase gene and rDNA ITS regions [30,31]. In this study we applied PCR primers franking schistosomes rDNA ITS1 that were specific and highly sensitivity (**FIG. 1**), demonstrated at femtomole concentrations similar to detection levels obtained by [30]. Much higher sensitivity can be achieved by designing nested primers for the target DNA fragment and this can provide a simple schistosomes infection detection PCR kit for epidemiological survey of schistosomiais transmission [32].

Isolates of *Biomphalaria spp* and *Bulinus spp* snails vary in their ability to transmit schistosomes based on their susceptibility or resistance traits that is sometimes ascribed to hemocyte immune cells [26,33,34]. For example, *S. mansoni* miracidia infects only some strains of *B. glabrata* and *B. ugandae* while some other strains are refractory to infection. Similarly, it has been

noted that not all species within the genus *Bulinus africanus* act as intermediate host for *S. haematobium* [19,35]. Snail culture at our IPR laboratory used in this study composed of different species and strains sampled from across Kenya. We applied PCR primers targeting schistosomes rDNA ITS region to detected the pre-patent infections of the snails by cercaria. The results **(FIG. 2)** show that not all the snail samples that had been exposed to miracidia sustained infections and the titres of the pathogen were different. Some snails were miracia/cercaria free and the blurred smear could be poor amplifications in the degraded pathogen. This indicates that some variant snails in every sampling resist schistosome infections. In fact, after long term culture of snails exposed to miracidia infection, the resistant snails become the dominant population.

Snails that transmit human schistosomiasis in Kenya are *Bulinus spp,* for *S. haematobium* that is mainly found in the coastal region and the adjoining eastern regions and *Biompalaria spp,* for *S. mansoni* in the central and western parts of the country although there are intermixtures of both species in some regions [19,35,36]. Detailed studies on phylogenetics and classification systematics for these snail vectors of schistosomiasis is an important prerequisite in defining isolates and strains that are susceptible or resistant to schistosomes [30,37]. Species and strains identification using the snail shell are not completely reliable hence the need for revised nomenclature combining morphological features and molecular markers [19,36].

In this study, phylogenetics analysis of *Biomphalari*a isolates using rDNA ITS sequences identified at six clusters **(FIG. 3)** within Kenya with some lineages that are monophyletic over long evolutionary time (cluster I, II and IIIA) while other populations reflected closely related clonal progenies (cluster IB, IIB) that are offshoots from these monophylectic lineages. This characteristics is similarly observed in the composite phylogenetic analysis of *Biomphalaria spp* from across Africa drainages **(FIG. 4)** suggesting that some field isolates are likely to be monophyla lineages (in bold) while other clades are clonal or very closely related isolates (**FIG. 4**, curly brackets). The nomenclature while representative of the various isolates of *Biomphalaria spp* appears uncoordinated and may need revision [19]. Kenya isolates used in this study (italized in **FIG. 3,4**) show diverse genetic structure representative of six major isolates from across Africa comprising of *B. pfeiferri, B. sudanica*, *B. choanomphala*, *B. stanleyi*, *B. alexandria* and *B. glabrata* (**FIG. 4**, brackets I, II, III, IVA and IVB). This is possibly due to Kenya's geographic location in relation to the main drainages in Africa: Lake Victoria and the Nile basin, river Congo basin and drainages from Eastern and Southern Africa Highlands to Indian Ocean. The disruption and redistribution of river systems with the formation of East African Rift Valley in Miocene period provides a guide to tropical Africa fauna phylogeographic affinities in this region [38,39].

Population structure of these fresh water snails is likely the result of isolated wetlands separated by geographic barriers. Tajima neutrality test gave significant negative values (-1.681654 and -2.003619) for Kenyan and Africa regional isolates, respectively (**TABLE 1**). Tajima-D values close to zero indicate that the nucleotide diversity is near neutrality and the population from which samples were drawn is almost in equilibrium with respect to drift and mutation [21]. The datasets showed negative Tajima-D value implying purifying selection against certain deleterious alleles or population expansion where new selected-for alleles are still in low frequency resulting in low heterozygozity. The results indicate that the isolated wetland habitat for freshwater snails possibly represents snail populations undergoing recent expansions.

Conclusion

Biompalaria snail populations sampled in this study comprise of monophyletic lineages and clusters of closely related isolates or clonal expansion. Similar population structure was observed when Africa regional sequence datasets were analyzed.

The PCR detection of pre-patent cercaria infection showed that some snails are susceptible while others were resistant to schistosomes infection.

Acknowledgement

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