GENETIC CHARACTERIZATION OF *Biomphalaria pfeifferi* FROM ASAO STREAM, KISUMU COUNTY KENYA: IN RELATION TO SEASONAL VARIATION, SELFING AND REFRACTORY PROPERTIES

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

I dedicate this work to my husband, Samuel Omari, for his technical support in the laboratory; to our son, Sage Neville, for his love and motivation; to my father, Mr. Jackton Oduor, and my siblings, Yvee, Faith, Aaron, and Donna, for their encouragement throughout my study period; and in honour of my late mother, Mildred Akinyi Samo.

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ABBREVIATIONS AND ACRONYMS

AMOVA	Analysis of Molecular Variance
CBRD	Centre for Biotechnology Research and Development
CGHR	Centre for Global Health Research
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
FIS	Fixation Index Within Subpopulations (Inbreeding coefficient among Subpopulations)
FST	Fixation Index among Subpopulations (Genetic Differentiation).
gDNA	genomic Deoxyribonucleic Acid
GRC	Guadeloupe Resistance Complex
Ho	Observed Heterozygosity
HWE	Hardy Weinberg Equilibrium
ILRI	International Livestock Research Institute
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KEMRI	Kenya Medical Research Institute
MDA	Mass Drug Administration
MLG	Multilocus Genotypes
NTD	Neglected Tropical Diseases
PCR	Polymerase Chain Reaction
PE	Post Exposure

PPE	Personal Protective Equipment
PZQ	Praziquantel
SERU	Scientific and Ethics Review Committee
SSLP	Simple Sequence Length Polymorphism
SSR	Simple Sequence Repeats
STR	Short Tandem Repeats
TR	Tandem Repeats
UNM	University of New Mexico
WHO	World Health Organization

DEFINITION OF OPERATIONAL TERMS

Seasonal variation	Seasonal variation refers to changes in rainfall, temperature, water quality, water flow, food availability, and vegetation.
Sampling time	This refers to the preferred sample collection duration used to capture different seasonal variations throughout the year.
Shedders	*Biomphalaria pfeifferi* snails that tested positive for *Schistosoma mansoni* infection.
Non-shedders	*Biomphalaria pfeifferi* snails that tested negative for *Schistosoma mansoni* infection.

ABSTRACT

Biomphalaria pfeifferi, a predominantly self-fertilizing freshwater snail, is the world's most important intermediate host for *Schistosoma mansoni*, one of the causative agents of schistosomiasis, which affects about 200 million people in sub-Saharan Africa. Understanding the genetic variation, selfing rates, and refractory traits of *B. pfeifferi* is important for improving snail-based strategies to interrupt *S. mansoni* transmission. The use of multilocus genotypes (MLGs) is important in identifying unique lineages within the snail population. This study sought to determine: (i) if lineages of *B. pfeifferi* within Asao stream could persist through different seasons of the year, (ii) if infections by *S. mansoni* or other trematodes occur within the persistent lineages, (iii) if *B. pfeifferi* from the Asao stream are selfers and finally (iv) if there are refractory genotypes among snails from Asao stream. *B. pfeifferi* snails were collected from Asao Stream in six bi-monthly (every other month) sampling times between November 2018 and September 2019. Snails were isolated and screened for *S. mansoni* and other trematode infections using the shedding method, cercarial morphology was examined microscopically. Successive infection and breeding experiments was done to determine refractory genotypes. Using 14 microsatellite markers in a multiplex Polymerase Chain Reaction (PCR), *B. pfeifferi* snails were genotyped. Data analysis was done using GENALEX software and R studio software; poppr and coin packages. For the first three objectives, a total of 502 *B. pfeifferi* snails were collected. Genetic differentiation among the snails was low, ranging from F_{ST} = 0.019, p = <0.05 (November and March) to F_{ST} = 0.372, p = <0.001) (July and March). Analysis of molecular variance (AMOVA) showed a small variation (8%) among sampling times. A total of 319 MLGs were identified, 26 of which were present at two or more sampling times. Four MLGs persisted across the 10-month sampling period, one of which was represented by 17 individuals. Of the trematode species *S. mansoni* was the most common at 64.79%, other trematodes included; Echinostomes (3.29%), Xiphidiocercariae (6.10%), Paramphistomoids (23.47), Sangunicolids (0.94%) and Strigeids (1.41%). The persistent MLGs had more trematode infections compared to those found only at a single sampling time. There was high inbreeding coefficients (Mean F_{IS} =0.639) and low heterozygosity (Mean H_o=0.145), with selfing rates estimated at 77.77%. Objective four began with 300 non shedding snails, the breeding experiment generated 10 Fillial 3(F3) *B. pfeifferi* non-shedding snails. These findings suggest that *B. pfeifferi* in Asao stream is predominantly self-fertilizing, with persistent genotypes that may sustain *S. mansoni* transmission across seasons. The identification of 10 F3 non-shedding snails suggests the possible presence of refractory snails. This study highlights the need for year-round snail control and monitoring of potentially refractory snail genotypes should be considered in control strategies.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Biomphalaria pfeifferi is planorbid gastropod that is commonly found in freshwater ecosystems across sub-Saharan Africa (Brown, 1994). It is the single most important intermediate host for *Schistosoma mansoni*, the most prevalent of the *Schistosoma* spp causing human intestinal schistosomiasis (Bu *et al*., 2023).

Worldwide, human schistosomiasis accounts for over 231 million cases, with the majority cases in sub-Saharan Africa, where the disease affects over 200 million people (Onasanya *et al.,* 2021; WHO, 2024). Several factors continue to facilitate the persistence of schistosomiasis in sub-Saharan Africa. These include poverty, poor sanitation, use of untreated water from lakes and rivers for drinking and washing, and Limited access to health care (Rinaldo *et al*., 2021). Effective control strategies for schistosomiasis rely on a multifaceted approach such as such as snail control, improved sanitation and hygiene, clean water supply, and health education for complete elimination of schistosomiasis. Interrupting the parasite's life cycle requires an understanding of the biology, the underlying genetics, and transmission potential of the intermediate freshwater snail hosts (WHO, 2022).

In Kenya studies have been conducted on *B. pfeifferi* focusing on compatibility with *S. mansoni*, trematode diversity and ecology. Mutuku et al. (2014) demonstrated a highly compatible association between Kenyan *S. mansoni* and *B. pfeifferi*, with some snails capable of shedding cercariae for more than one year, thereby sustaining transmission. Other studies in western Kenya by Laidemitt et al. (2019) reported a high diversity of trematodes infecting *B. pfeifferi*, highlighting the ecological importance of the snail in maintaining transmission of schistosomes and other trematodes.

Previous studies focusing on the population structure of *B. pfeifferi* have consistently provided strong evidence of preferential selfing associated with

inbreeding, with relatively low levels of intrapopulation variation when compared to interpopulation variation (Nguema *et al*., 2013; Kengne-Fokam *et al*., 2016). In some areas, where opportunities for colonization are limited, such as Oman (Nguema *et al*., 2013), or where recent population expansions have occurred, as in Senegal (Campbell *et al*., 2010), individual *B. pfeifferi* populations often show low levels of genetic diversity as compared to populations where opportunities for colonization are higher, as reported in Madagascar (Charbonnel *et al*., 2005).

These population genetics studies, though, do not directly address the issue of the extent to which, as a consequence of selfing, distinct clusters of related individuals that share multilocus genotypes (MLGs) exist within the selfing populations of *B. pfeifferi*. Importantly, it remains a gap on whether particular MLGs that represent the progeny produced by self-fertilization can be identified, and if they persist over time. Although these clusters of individuals would not necessarily be expected to be genetically identical because of the possibility of some recombination occurring during selfing, the members of a particular MLG might nonetheless share important attributes, such as being especially susceptible or refractory, to particular parasites, including *S. mansoni* (Stetsenko & Roze, 2022).

Previous studies in Senegal have suggested that in areas not previously occupied by *B. pfeifferi*, a colonization event occurred, originally consisting of a single *B. pfeifferi* lineage, one in this case highly susceptible to *S. mansoni* infection and that favoured an outbreak of intestinal schistosomiasis (Campbell *et al*., 2010). This reinforces the idea that different *B. pfeifferi* MLGs exhibit varying levels of susceptibility to *S. mansoni*, possibly including some that are refractory to schistosome infection. If *S. mansoni*-resistant MLGs are present, they might limit transmission and potentially could be identified and exploited in novel snail resistance-based control programs. The idea of using snail immune-based resistance to *S. mansoni* has been developed using the related schistosome vector *Biomphalaria glabrata* as a model, for which genetically based resistance and inbred *S. mansoni*-resistant lines are known (Bu *et al*., 2022; Pila *et al*., 2017; Richards, 1970). Better understanding of the potential variation in susceptibility among *B. pfeifferi* MLGs holds significant value for schistosomiasis control.

If *B. pfeifferi* MLGs persist over time, it is also conceivable through Red Queen dynamics which describe the continuous evolutionary adaptation between hosts and parasites, that local parasites might adapt to and become more numerous in locally common genotypes (Mutuku *et al*., 2017). Such dynamics could be prevented from developing, though, because particular habitats may be dramatically altered by flooding or drought, leading to the elimination of established MLGs (Mutuku *et al*., 2017). The underlying reason for the existence of selfing may be most apparent in favouring rapid colonization of habitats frequently "reset" by flooding, drought, or other environmental changes (McElderry *et al*., 2022). As both annual and long-term abiotic environmental changes are well-known to have major effects on populations of aquatic schistosome vector snails, including favouring both expansions and collapse of snail populations, such effects will have to be taken into account for resistance-based strategies to succeed (Haggerty *et al*., 2022; Bakhoum et al., 2021; Sokouri *et al*., 2024).

Microsatellite markers were used in this study to identify MLGs. Microsatellites are powerful genetic tools because they have dense distribution in the genome, have co-dominant inheritance, are fairly simple to identify, and have high variability (Noble, 1999). Their high level of variation makes them extremely useful for detecting differences within a population and between individual organisms (Marwal & Gaur, 2020).

1.2 Problem Statement

Biomphalaria pfeifferi, the main intermediate host of *Schistosoma mansoni* in sub-Saharan Africa, has maintained the transmission of the disease for a long time now (Mutuku et al., 2014). In Kenya, *S. mansoni* is highly prevalent around the Lake Victoria basin, Lake Jipe, and irrigation schemes such as Mwea, Ahero, and Bura, where suitable intermediate host snails occur abundantly (Mutuku et al., 2014; Odiere et al., 2012). Recent national reports estimate that over six million Kenyans are infected with schistosomiasis, while approximately 25 million people remain at risk of infection (MOH, 2023). The Lake Victoria basin remains the

major transmission focus for intestinal schistosomiasis in Kenya, with some shoreline and island communities reporting infection rates of more than 50% among school-aged children and fishing communities (Odiere et al., 2012; Mwinzi et al., 2012).

Schistosomiasis continues to be a public health burden despite efforts to control or eliminate it (Colley et al., 2014). These control efforts, which primarily focus on mass drug administration (MDA) using praziquantel (PZQ), seem inadequate to eliminate the disease due to the fact that PZQ does not prevent reinfection and also has no effect on the juvenile worms (Colley et al., 2014; Rollinson et al., 2013). Snail control can rapidly interrupt the transmission of schistosomiasis. However, the snail control strategies currently available, such as molluscicide, are costly and not environmentally friendly; therefore, they are not sustainable (King & Bertsch, 2015).

Although the snail is known for its preferential selfing mode of reproduction, the selfing rates of *B. pfeifferi* in Asao stream has not been determined (Charbonnel et al., 2002; Campbell et al., 2010). The impact of selfing for the survivability of the snail is not clearly understood, considering that the snail experiences seasonal variations like adverse weather conditions (drought and/or flooding). Given that under natural conditions, generally, only a few snails from a collection shed cercariae, i.e., are infected with *S. mansoni*, the exact reason underlying this phenomenon is not known (Rowel *et al*., 2015). While a variety of reasons could be responsible for this phenomenon, it is also possible that there could be a biological effect that makes some of the snails susceptible to *S. mansoni* infection while others remain refractory. Snail genetics is strongly suggested to be one of the causes, with particular multilocus genotypes (MLGs) suspected to influence susceptibility or resistance to infection (Sokolow *et al*., 2016; Bizimana *et al*., 2019).

1.3 Justification of the Study

Biomphalaria pfeifferi, a stream-based species with wide distribution in Kenya, including the Lake Victoria basin, is an important transmitter of *Schistosoma mansoni* in Kenya and the entire sub-Saharan Africa region (Adenowo *et al*., 2015). *B. pfeiferi* snails account for most of schistosomiasis cases worldwide (Adenowo *et al*., 2015). The World Health Organization (WHO) has emphasized the need for integrated schistosomiasis control approaches, including interruption of transmission through snail control, in order to achieve elimination targets by 2030 WHO, 2022). Kisumu County remains one of the major endemic regions for intestinal schistosomiasis due to continued human-water contact and persistence of infected *B. pfeifferi* populations. Therefore, A snail control approach that guarantees cost-effectiveness and sustainability can help complement chemotherapy and contribute significantly to efforts to eliminate schistosomiasis (Odiere et al., 2012; Mwinzi et al., 2012).

From previous studies, it has been determined that the Nyakatch region where Asao stream is located is endemic for *S. mansoni* infection (Mutuku et al., 2014). The Asao stream experiences periodic flooding and drought, making it a suitable study site for the study of variation and adaptability of *B. pfeifferi* snails (Lu et al., 2016). *B. pfeifferi* is a hermaphroditic snail and can either self-fertilize or cross-fertilize, and it seems selfing is the most preferred form of mating for the snail (Charbonnel *et al*., 2005). Selfing is crucial because it can lead to the fixation of certain genotypes that have a survival fitness for a particular habitat. A better understanding of the mode of reproduction of *B. pfeifferi* snails, their seasonal variation, and genetic characteristics that may cause susceptibility and/or refractoriness to infection with *S. mansoni* will both improve the knowledge of snail biology and may offer valuable insights into the development of better schistosomiasis control strategies (Mutuku et al., 2014; Lu et al., 2016)

1.4 Research Questions

1) Do the genetic characteristics of *B. pfeifferi* vary with seasons in the Asao stream?
2) Do infections by *S. mansoni* or other trematodes occur among persistent genotypes?
3) What is the selfing rate of the *B. pfeifferi* population present in the Asao stream?
4) Are there refractory strains in the *B. pfeifferi* population present in the Asao stream?

1.5 Study Objectives

1.5.1 General Objectives

To assess the genetic characteristics of the *B. pfeifferi* population in Asao stream, Kisumu County, in relation to seasonal variation, selfing, and refractory properties

1.5.2 Specific Objectives

1) To assess the genetic variation of *B. pfeifferi* across the different seasons of the year in the Asao stream.
2) To determine the occurrence of *S. mansoni* and other trematode infections among persistent *B. pfeifferi* genotypes in the Asao stream.
3) To determine Selfing rates of *B. pfeifferi* snails collected from the Asao Stream.
4) To characterize genotypes of the progeny of refractory *B. pfeifferi* from Asao stream.

CHAPTER TWO

LITERATURE REVIEW

2.1 Human Schistosomiasis

Human schistosomiasis, a neglected tropical disease (NTD), ranks second to malaria in the tropics and subtropics regions of the world in terms of its adverse effect on public health (Barry *et al*., 2013). Schistosomiasis is caused by blood flukes of the genus *Schistosoma* and remains one of the most important parasitic diseases globally (Colley et al., 2014). The disease is transmitted through contact with freshwater contaminated with cercariae released by infected freshwater snails, which act as intermediate hosts. The major human schistosome species include *Schistosoma mansoni*, *S. haematobium*, and *S. japonicum*, with *S. mansoni* being responsible for intestinal schistosomiasis (McManus et al., 2018).

Global estimates of human schistosomiasis indicate that the disease actively infects at least 251.4 million individuals, with an estimated 779 million people living at continuous risk in endemic regions (WHO, 2026). Most schistosomiasis cases globally occur in sub-Saharan Africa, with 42 countries affected (WHO, 2024). In Kenya, approximately 9 million people are actively infected and an additional 17.4 million individuals are living at continuous risk of contracting the disease (Odiere et al., 2011). Communities that live along the Lake Victoria shores, the Athi and Tana Rivers, large-scale irrigation schemes like Mwea, and local dams suffer from schistosomiasis, while those on the islands of Lake Victoria bear a substantial burden of the disease (Odiere *et al*., 2012; Woodhall *et al*., 2013). Both *S. mansoni* and *S. haematobium* are endemic in Kenya and are significant public health problems. Chronic infection may result in abdominal pain, diarrhoea, anaemia, hepatosplenomegaly, impaired growth, and reduced cognitive development, particularly among school-aged children (Colley et al., 2014).

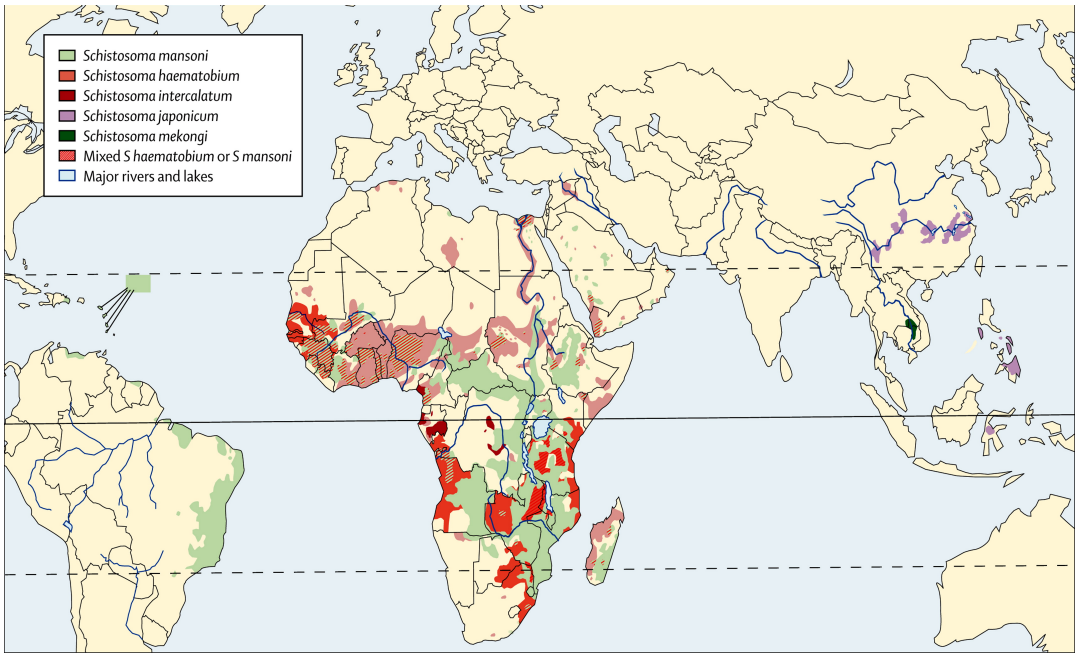

Figure 2.1: Global Geographic Distribution of Schistosomiasis

(Colley et al., 2014)

2.2 *Schistosoma mansoni* Life Cycle

Adult schistosomes have separate sexes, and the adult male and female *Schistosoma mansoni* live paired in the mesenteric veins of their definitive host, where they mate and lay eggs. The laid eggs then migrate into the intestinal lumen of the host and are excreted in the faeces. Under appropriate conditions, the eggs hatch upon contact with freshwater, releasing miracidia (Collins *et al.*, 2013), which swim freely in the water in search of the *Biomphalaria* snail intermediate host, penetrating it to initiate infection. Inside the snail host, the miracidium transforms into a mother sporocyst, within which daughter sporocysts develop. Within these, larval forms known as cercariae are formed, which are infective to the definitive human host (Gryseels *et al.*, 2006). Once fully developed, *S. mansoni* cercariae are released from an infected *Biomphalaria* snail under light stimulation approximately five weeks after initial exposure to the miracidia. The cercariae released from the snail swim freely in the water, searching for a definitive host. They enter the body of the definitive host by penetrating intact skin, a process facilitated by proteolytic enzymes (McKerrow & Salter, 2002). The

cercariae lose their tails during skin penetration, transform into schistosomula, and enter the blood circulatory system. The schistosomula then migrate to the mesenteric veins, where they mature into adults, pair up, mate, and reproduce (Figure 2.1) Gryseels et al., 2006).

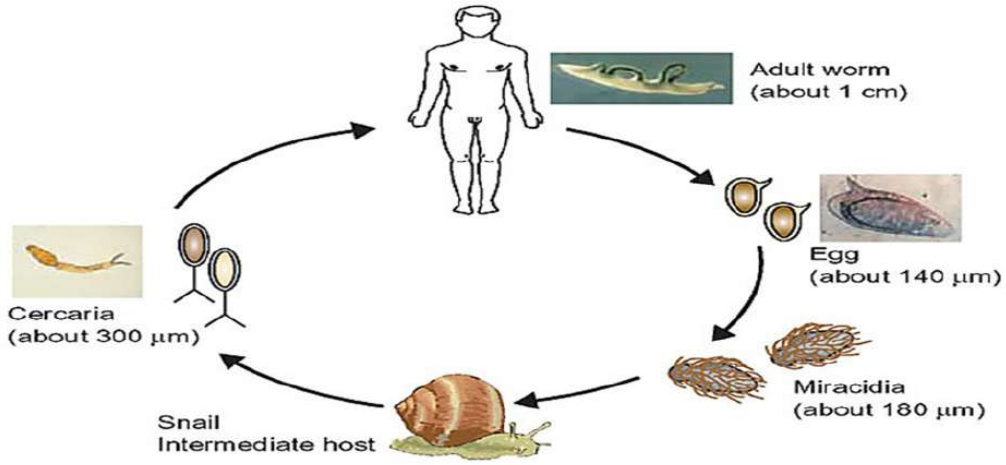

Figure 2.2: Life Cycle of Schistosomes

(Danso-Appiah et al., 2024)

2.3 Prevention and Control of Schistosomiasis

Chemotherapy using mass drug administration (MDA) with 40 mg/kg of praziquantel has been the primary strategy for controlling schistosomiasis alongside health education, improved sanitation, and snail control (Rollinson et al., 2013; Ross *et al*., 2014). Although chemotherapy rapidly reduces the burden of infection in infected individuals it has limited efficacy against juvenile stages of the parasite, hence, reinfections rapidly occur, making it challenging to sustain control efforts. An integration of the existing control strategies and targeting the life cycle stages, should be integrated by the policy makers for the complete elimination of schistosomiasis (Bethony *et al*., 2011; Ross *et al*., 2014).

Removal of infected snails from transmission habitats to reduce the number of cercariae from the habitat has been observed to reduce the risk of reinfection (Owiny *et al*., 2019; Wan *et al*., 2016). Several control programs have

implemented snail control by using chemical molluscicides such as niclosamide. Still, there are concerns about chemical toxicity, pollution of the environment, and the cost of molluscicide (McManus *et al*., 2018). In most wealthy countries, snail control is the most preferred method and accounts for up to 92% prevalence elimination as it is probably the most effective way to reduce schistosomiasis transmission (McManus *et al*., 2018; Ngigi *et al*., 2018). Therefore, the effective elimination of schistosomiasis should include snail control (Sokolow *et al*., 2016).

2.4 Overview and Biology of *Biomphalaria pfeifferi*

Biomphalaria pfeifferi is a freshwater pulmonate snail belonging to the family Planorbidae and serves as the most important intermediate host of *Schistosoma mansoni*, a causative agent of intestinal schistosomiasis (Brown, 1994; Rollinson et al., 2001). The snail plays a critical role in the life cycle of the parasite by supporting the development and release of cercariae that infect humans through contact with contaminated water. Due to its high compatibility with *S. mansoni*, *B. pfeifferi* is considered one of the most epidemiologically important snail hosts involved in schistosomiasis transmission (Bu et al., 2023). The snail thrives in freshwater habitats such as streams, ponds, irrigation canals, dams, and slow-moving water bodies rich in aquatic vegetation, which provide favourable conditions for breeding and survival (Appleton, 1978; Sturrock, 1965).

Apart from its role in schistosomiasis transmission, *B. pfeifferi* also supports a diverse range of digenetic trematodes, making it an ecologically important component of freshwater ecosystems. Trematode infections may influence the survival, reproduction, and susceptibility of the snail to other parasite species through competitive interactions within the host (Laidemitt et al., 2019a; Laidemitt et al., 2019b). Environmental factors such as temperature, rainfall, flooding, water flow, and habitat disturbance have been shown to influence the abundance and distribution of *B. pfeifferi*, ultimately affecting transmission dynamics of *S. mansoni* and other trematodes (Woolhouse & Chandiwana, 1990; Mutuku et al., 2014). Recent genomic studies have further highlighted the importance of understanding the biology and

genetics of *B. pfeifferi* in developing novel snail control approaches aimed at interrupting schistosomiasis transmission (Bu et al., 2023)

2.5 Trematode Diversity and the Intermediate Host Role of *Biomphalaria pfeifferi* in Kenya

In Kenya, *B. pfeifferi* supports a wide range of digenetic trematodes, including medically and veterinary important species. Among these, *Schistosoma mansoni* is the most significant because of its role in human intestinal schistosomiasis. Previous studies have shown that *B. pfeifferi* populations from endemic areas frequently harbour *S. mansoni* infections, confirming their major role in maintaining transmission within local communities (Mutuku et al., 2014; Laidemitt et al., 2019a).

Apart from *S. mansoni*, *B. pfeifferi* in Kenya is associated with several other trematodes, including echinostomes, paramphistomoids, strigeids, and xiphidiocercariae. These trematodes compete for the same snail host and may influence schistosome transmission through antagonistic or facilitative interactions. Studies conducted in western Kenya, particularly within the Asao stream and Lake Victoria basin, have reported mixed trematode infections in *B. pfeifferi*, (Laidemitt et al., 2019b). The occurrence of multiple trematode species within the same snail populations highlights the ecological importance of *B. pfeifferi* in maintaining parasite diversity and transmission dynamics in endemic freshwater habitats.

Previous work has also suggested that susceptibility to trematode infection may vary among snail genotypes. Some *B. pfeifferi* individuals appear highly compatible with *S. mansoni*, while others may show reduced susceptibility or possible resistance. Mutuku et al. (2014) reported that some Kenyan *B. pfeifferi* infected with *S. mansoni* were capable of prolonged cercarial shedding for more than one year, suggesting strong parasite compatibility. Recent genomic studies have identified genes potentially associated with schistosome resistance in African snail vectors, highlighting the growing importance of snail genetics in understanding transmission dynamics (Mutuku et al., 2025).

2.6 Geographic Distribution of *Biomphalaria pfeifferi* in Kenya

Biomphalaria spp. inhabit freshwater habitats and are found in Africa, Madagascar, the Middle East, and the Americas by 2008, 34 extant species were recognized (22 American species and 12 species in Africa, Madagascar, and the Middle East) (Majoros *et al*., 2008). *Biomphalaria* species have been observed to expand their distributional range partly through human activities and possibly due to climate change (Ngigi *et al*., 2018). In Kenya *B*. *pfeifferi* is widely distributed in western Kenya along the feeder streams of Lake Victoria, in the Mwea irrigation scheme in central Kenya (within the Tana River basin), in habitats within the Athi River basin, and in Taita-Taveta (in southwest Kenya), where they occur in irrigation canals, in small man-made impoundments, and in both seasonal and perennial streams throughout the country (Adekiya *et al*., 2019; Mutuku *et al*., 2017).

One of the most important streams that habours *B pfeifferi* is Asao stream. It is located within the Lake Victoria basin in western Kenya and has long been recognized as an active transmission site for *Schistosoma mansoni* (Loker, 1993; Laidemitt et al., 2019a). Asao stream experiences periodic flooding and seasonal environmental fluctuations, yet *B. pfeifferi* populations persist throughout the year. The site is frequently used by local communities for domestic and water contact activities, increasing opportunities for schistosome transmission. Previous studies conducted in Asao stream have demonstrated high prevalence of *S. mansoni* and other trematode infections in *B. pfeifferi*, making the stream an important site for studying snail–parasite interactions, compatibility, and transmission dynamics (Laidemitt et al., 2019b)

2.7 Environmental Influences on *Biomphalaria pfeifferi* in Kenya

Biomphalaria pfeifferi thrives well in warm, stable conditions (Kalinda *et al*., 2017) and occurs in a wide variety of water bodies (Rowel *et al*., 2015). However, the snail is mainly found in small man-made impoundments, seasonal streams, and irrigation canals (De Kock *et al*., 2004). Snail populations respond to temperature fluctuations in the aquatic environment. Studies have shown that snail abundance

is generally high within temperature ranges of 15–31°C. Outside this temperature range, snail numbers drop sharply (McCreesh & Booth, 2014). The temperature has also been found to influence fecundity, growth, survival, and parasite development in the snail, thus dictating the time it takes for the parasite to complete its life cycle and transmission (Kalinda *et al*., 2017). Environmental influences on fecundity and maturity are also observed, where parasites from permanent habitats mature at small sizes and are more fecund than those from temporary habitats (Tian-Bi *et al*., 2013).

Physico-chemical factors such as temperature, pH, slight wave action, water depth, and conductivity may influence *B. pfeifferi* infections and population density (Rowel *et al*., 2015). There is evidence that environment and geography influence the spatial patterning of *B. pfeifferi*. Also, spatial substructure based on water bodies suggests a significant gene flow across these geographic sites (Steinauer *et al*., 2009). Intense rainfall and flooding have huge effects on the transmission of schistosomiasis, as such conditions reduce the number of cercariae present in transmission sites (Wu *et al*., 2008). During heavy rainfall and flooding, most snails are washed downstream. They can be dispersed into new habitats, establishing new colonies and facilitating the spread of infected and non-infected snails (Gurarie *et al*., 2018). Flooding, drought, and the effects of climate change have been found to play a huge role in determining the actual range of schistosomiasis distribution and snail abundance (Adekiya *et al*., 2019; McCreesh & Booth, 2013).

2.8 *Biomphalaria pfeifferi* Mating Preferences and Resistance to *Schistosoma mansoni* Infection

Biomphalaria pfeifferi has a higher invasive ability and compatibility with *Schistosoma mansoni*; hence, it can colonize diverse and inconstant habitats. This can be attributed to the fact that *B. pfeifferi is* a preferential self-fertilizer or selfer which means *B. pfeifferi* favours self-reproduction over outcrossing, a characteristic that supports its colonization and the sustainability of *the S. mansoni* life cycle (Jarne and Theron, 2001; Charbonnel *et al*., 2005; Campbell *et*

al., 2010*).* Besides being highly susceptible to *S. mansoni* infection*, B. pfeifferi* also produces cercariae in greater abundance than the non-selfing "out-crossing" *Biomphalaria sudanica* (Mutuku *et al*., 2017).

Analysis of microsatellite markers in *B. pfeifferi* has also confirmed that this species is a selfer and exhibits limited variation within populations (Tian-Bi *et al*., 2013). Analysis of the polymorphic microsatellite of *B. pfeifferi* collected from the Senegal River Basin revealed a genetic variation in a locus (Campbell *et al*., 2010). The snails from the basin exhibited minimal genetic differentiation compared to those from natural habitats, which had lower infection rates. These findings suggest that genetic variation may contribute to resistance against infection (Ngigi *et al*., 2018). Other studies have shown that *B. pfeifferi* populations exhibit genetic structuring and high genetic diversity, which may contribute to resistance to infection by schistosomes due to low compatibility between certain snail genotypes and parasite strains (Standley *et al.,* 2014). These gene variations have been associated with water availability, which can drive phenotypic and genetic differentiation of traits in snails (Tian-Bi *et al*., 2013). *B. pfeifferi* has been shown to have high compatibility with *Schistosoma* infections, unlike other species. This observation is crucial in the search for a control mechanism (Lu et al., 2016).

2.9 Red Queen Dynamics in *Biomphalaria pfeifferi*

Red Queen dynamics refer to a coevolutionary process driven by continuous, reciprocal genetic adaptation. Because parasites constantly evolve to infect the most common host genotypes. (Woolhouse et al., 2002). Red Queen dynamics are known to occur in freshwater snails capable of selfing and exposed to heavy trematode burdens, Similarly, *B. pfeifferi* meets this criterion because it is subjected to intense trematode infection pressure and demonstrates the capacity for both self-fertilization and outcrossing.

In the freshwater snail *Potamopyrgus antipodarum,* diploid individuals capable of sexual reproduction and triploid females that reproduce by parthenogenesis might be present, the latter resulting in the production of distinct clones of snails. Snails

within a clone are capable of rapid population expansion (all individuals can produce progeny), but the genetic homogeneity within clones potentially renders them vulnerable to attack by parasites, more so than progeny produced by sexual reproduction, which differ genetically from one another (Van Baalen & Beekman, 2006). Progeny resulting from sexual reproduction might harbour unique combinations of resistance genes, which might diminish parasite success rates. In its native lacustrine habitats, given that trematode pressure is highest around lakeshores as compared to deeper water, for example, *P. antipodarum* tends to engage in sexual reproduction in shoreline habitats, whereas snails from deeper waters, where parasite pressures are less, tend to reproduce parthenogenetically (Vergara *et al*., 2014).

2.10 Application of Microsatellite Markers in *Biomphalaria pfeifferi* Characterization

Microsatellites are subcategories of tandem repeats (TRs) that make up repetitive genomic regions. T.R.s are evolutionarily relevant due to their instability, which refers to their high rate of mutation through the gain or loss of repeat units (Ellegren, 2004). This instability occurs during DNA replication when the enzyme DNA polymerase copies these highly repetitive, identical sequences (Ellegren, 2004; Leclercq et al., 2010). The instability also generates high genetic polymorphism, making them incredibly useful markers for studying population structure, mapping genes, and tracking evolutionary changes over relatively short timeframes (Selkoe & Toonen, 2006; Vieira et al., 2016). Microsatellites, also known as Simple Sequence Repeats (SSR), Short Tandem Repeats (STR), and Simple Sequence Length Polymorphisms (SSLP), are found in prokaryotes and eukaryotes (Ellegren, 2004; Vieira et al., 2016). They are widely distributed throughout the genome, especially in the euchromatin of eukaryotes and coding and non-coding nuclear and organellar DNA (Gemayel *et al*., 2012; Phumichai *et al*., 2015). Microsatellites are codominant, highly polymorphic, easily typed, and undergo Mendelian inheritance. These properties make them very suitable for studying population structure and pedigree analysis and for detecting differences among closely related species. Polymerase Chain Reaction (PCR) for

microsatellites can be automated for identifying simple sequence repeat polymorphism. Most microsatellites are non-coding, and therefore variations are independent of natural selection. These properties make microsatellites ideal genetic markers for species analysis (Vieira *et al*., 2016).

2.11 Knowledge Gaps in *Biomphalaria pfeifferi* Genetics

Although *B. pfeifferi* is the most important intermediate host of *Schistosoma mansoni*, limited information exists regarding the population genetics of Kenyan *B. pfeifferi* populations, particularly within natural stream habitats such as Asao stream. Previous studies have mainly focused on snail compatibility, cercarial shedding patterns, trematode diversity, and ecological distribution, with few studies examining the genetic structure of *B. pfeifferi* populations over time (Mutuku et al., 2014; Laidemitt et al., 2019a). Consequently, the extent of genetic variation and persistence of *B. pfeifferi* populations across seasons in Asao stream had not been clearly determined.

Although multilocus genotypes (MLGs) are increasingly being used to study population structure in selfing organisms, their association with transmission of *S. mansoni* and other trematodes in *B. pfeifferi* populations had not been well established in Kenya. It remained unclear whether particular MLGs persist over time and whether some genotypes are more susceptible to trematode infections than others. In addition, selfing rates of *B. pfeifferi* within Asao stream had not previously been quantified despite evidence suggesting that the species predominantly reproduces through self-fertilization (Campbell et al., 2010; Charbonnel et al., 2002). Understanding selfing rates is important because selfing influences genetic diversity, population structure, and host–parasite interactions.

Resistant or refractory strains of *B. pfeifferi* had not been documented within Asao stream. Although some studies have suggested that certain snails may show reduced susceptibility to *S. mansoni*, little information existed regarding whether resistant genotypes occur naturally within Kenyan *B. pfeifferi* populations or whether such traits can persist across generations (Mutuku et al., 2014). The absence of such information limited understanding of the role of snail genetics in sustaining

schistosomiasis transmission and constrained the development of genetically informed snail control strategies.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Approval and Ethical Review

Ethical approval of this study was obtained from the KEMRI Scientific and Ethical Review Unit (SERU) under reference No. KEMRI/SERU/CBRD/224/4252. A research license was also obtained from the National Commission for Science, Technology and Innovation (NACOSTI) under Reference No. 622444. Proposal approval by JKUAT's Board of Postgraduate Studies was granted under reference No. JKU/2/11/TM305-1540/2019. Written consents were obtained from the parents/guardians of the school children who were enrolled in the study to obtain faecal samples for isolating *Schistosoma mansoni* eggs needed in the experiments described in this study. In addition, the children who agreed to provide a faecal sample and who were older than 13 years provided assent to participate. Before participant recruitment, selection, screening, or treatment of any children, permission was obtained from the district health and education authorities, and the purpose of the study was explained to the school administration and parents. Involvement of human subjects in this project was limited to collection of faecal samples, which is a non-invasive procedure with no perceived risks and which is routinely used in clinical investigations of intestinal infections. Any child found positive for *S. mansoni* infection was offered the standard treatment with praziquantel (40 mg/kg body weight), and those found positive for geohelminths were also treated with albendazole (500 mg) under the supervision of a trained and qualified clinician. Praziquantel and albendazole are remarkably safe drugs and have been administered to millions of children in endemic countries, with no transient side effects. To ensure confidentiality, each participant was given a unique identifier, and all references to information/data obtained from each participant were referred to by this number.

3.2 Study Site

Biomphalaria pfeifferi snails for this study were Collected from Asao stream, which is located in Asao village, Nyakach district, within Kisumu County, in western Kenya, GPS coordinates -0.318086, 35.006904 (Figure 3.1). Asao village is located 45 kilometres south of Kisumu City on the Kisumu-Sondu Road, in western Kenya. Asao stream originates from the Mau escarpment and flows into Lake Victoria through Asao village within the lake basin plainlands. The area experiences hot and humid climatic conditions. Generally, the average temperature range in the region is 16°C (at night) - 30°C, with a bimodal rain seasonality, characterized by long rains between February and May and short rains between October and December. The average rainfall ranges between 800 and 1900 mm. However, especially considering ongoing climate change, variability in these weather patterns from year to year is to be expected (KMD, 2020). Asao Stream was first identified as a *B. pfeifferi* habitat in which *S. mansoni* transmission occurs, over 30 years ago and has since been the site of other studies of schistosomes and snails (Laidemitt et *al*., 2019a; Loker, 1993).

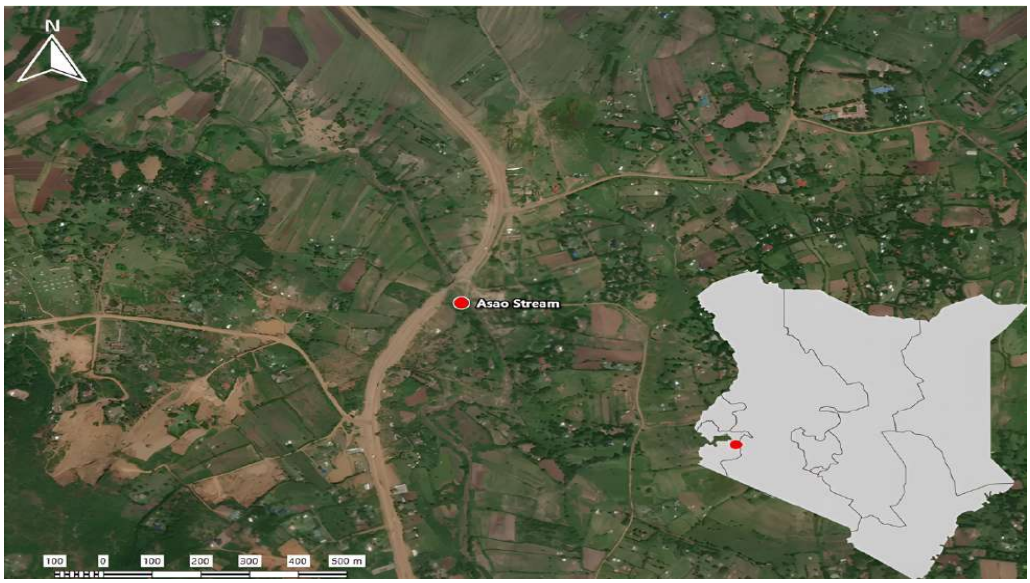

Figure 3.1: Map of Kenya Showing the Location of the Study Site, in Asao Stream. (The map was created using ExpertGPS version 9.3 (ExpertGPS, 2025)

3.3 Study Design

This study was both a laboratory-based and a field-based study involving juvenile and young adult *Biomphalaria pfeiferi* snails. Archived *B. pfeifferi* samples used in objectives one, two and three were obtained from a larger 'Mother' study whose approval number was SERU-3540 and titled "Snail-related studies of transmission and control of schistosomiasis in Kenya", PIs: E.S Loker (UNM)& G.M. Mkoji (KEMRI-CBRD). The samples were collected from Asao stream bimonthly between November 2018 and September 2019: Specifically, November 2018, January 2019, March 2019, May 2019, July 2019 and September 2019. Trematode infection status was determined through cercarial shedding before preservation in 70% ethanol. For objective four the snails were freshly collected from Asao stream the snails were enumerated and screened for schistosome infections. All laboratory work for this study was done in the KEMRI facilities based in Nairobi at the Centre for Biotechnology Research and Development (CBRD) and in Kisian, western Kenya, at KEMRI's Centre for Global Health Research (CGHR). Fragment analysis of the resulting products was done at the Molecular Laboratories of the International Livestock Research Institute (ILRI), based in Kabete, Nairobi.

3.4 Sample Size Determination

To ensure adequate statistical power for both the population genetic analyses and the parasitological screening, the sample size for this study was determined based on empirical standards for microsatellite markers.

For Objectives One, Two and Three standard molecular ecology protocols recommend a baseline target of at least 25 to 30 individual snails per sampling cohort to accurately estimate allele frequencies without sampling bias (Foster et al., 2021; Gow et al., 2005; Hale et al., 2012).

For Objective Four, which required young and juvenile *Biomphalaria pfeifferi* snails that were uninfected with schistosomes at the time of collection, a total baseline pool of 300 field-collected snails was utilized at the start of the experiment. This sample

size was selected to serve as an experimental buffer. Because field-collected snails in endemic regions carry variable background infection rates and cercarial shedding rates, which can occasionally exceed 30% depending on the locality and season (Alharbi et al., 2023).

3.5 Inclusion and Exclusion Criteria

3.5.1 Inclusion Criteria

For objectives one, two and three archived young adult and juvenile *Biomphalaria pfeifferi* samples collected bi-monthly between November 2018 and September 2019 were used. For objective four, fresh young adult and juvenile *B. pfeifferi* snails that were not infected with schistosomes at the time of collection were prospectively collected and used for refractory studies.

3.5.2 Exclusion Criteria

For objectives one, two and three snails that had degraded due to poor preservation were excluded from the study. For objective three, unhealthy snails, including those with cracked shells, white patches, or retracted bodies within the shell, were excluded from the refractory experiments.

3.6 Snail Collection and Screening

Biomphalaria pfeifferi snails were collected in the morning hours (between 9 and 10 am) with the assistance of two experienced snail collectors using a long-handled snail scoop made from a stainless-steel sieve with a mesh size of 2 × 2 mm, supported on an iron frame mounted on a 1.5-meter-long wooden handle (see Figure 3.2). It was used to sweep aquatic vegetation along the shoreline of Asao stream at the same designated location (under a bridge where the stream crosses under the Kisumu-Sondu Road) over a 30-minute collection period. From previous studies, this site is known to reliably contain *B. pfeifferi* and is a contact point frequented by the residents of this locality and their domestic animals (Mutuku *et al*., 2014). All *B. pfeifferi* collected were rinsed with dechlorinated water and then isolated in individual wells of 24-well culture plates (see Figure

3.3) and exposed to indirect sunlight for 1-2 hours between 10:00 a.m. and 2:00 p.m. to induce shedding. Shedding (or cercarial shedding) is the process where a snail releases the free-swimming, infectious larval stage of a trematode parasite such as *Schistosoma* cercariae into the surrounding water. Shedding was observed using a dissecting microscope.

Figure 3.2: A Snail Scoop for Collecting Snails

Figure 3.3: *Biomphalaria pfeifferi* Snails in Wells of A 24-Well Culture Plate Set Up for Screening to Identify Schistosome Cercariae and Other Trematodes

3.6.1 Trematode Identification

After shedding, the cercariae were collected with a glass pipette, placed on a glass microscope slide, stained with Lugol's iodine, and then identified morphologically at X40 or X100 with the aid of a compound microscope. Cercariae were identified according to trematode keys (Frandsen et.al., 1984; Shell et al., 1985) and were broadly placed into the following groups: *Schistosoma mansoni*, strigeids, paramphistomoids, echinostomes, xiphidiocercariae, and sanguinicolids.

3.6.2 Establishment of Laboratory Colonies of Snails

The snails were held outdoors, in "semi-field" ambient conditions, in a simple, roofed, open-sided structure containing wooden benches at KEMRI's Centre for Global Health Research (CGHR) at Kisian, Kisumu (Figure 3.4). Plastic aquaria measuring 18 cm long X 8 cm wide X 6 cm deep were used for rearing the snails within the structure (Figure 3.5). The aquaria were placed on the benches, and sterile, clean, crushed oyster shells measuring approximately 2 cm X 2 cm were placed in each aquarium as a supplement for calcium for the snails to cover the entire bottom of the aquarium. Plants from the Asao stream was introduced into the aquaria, which were filled with water. Each aquarium was aerated by connecting a 0.4-millimeter-thick pipe to an air pump to enable more oxygen to dissolve into the water and keep it fresh. The water in the aquarium was changed once a week by pouring it through a sieve to prevent loss of the snails. The snails were fed on slightly boiled store-bought lettuce, supplemented with a fish meal as a source of calcium. The goal was to promote more natural survivorship rates of exposed snails by using environmental conditions much similar to what snail's experience in the field, where they thrive well.

Figure 3.4: An Outdoor, "Semi-Field" Ambient Structure Where the Tanks Containing *Biomphalaria pfeifferi* Snails Were Stored

Figure 3.5: Plastic Aquaria where *Biomphalaria pfeifferi* Snails Were Reared and Maintained

3.6.3 Faecal Sample Collection from Children, Isolation of *Schistosoma mansoni* Eggs, and Hatching of Eggs

School-going children aged 6-14 years from Obuon Primary School in Asao, Nyakach Sub-County, Kisumu County, in western Kenya were enrolled in the study. This age group was preferred because most of these children tend to go swimming and bathing in the stream (see Figure 3.6); hence, they are at a higher risk for exposure to *S. mansoni* cercariae (CDC, 2018). The exclusion criteria were the children whose parents/guardians did not consent or those children over 13 years who did not assent to participate in the study. Once the parents/guardians provided written consent, a faecal sample was collected from each enrolled child. A thick faecal sample smear was then prepared on a glass microscope slide using the Kato-Katz procedure described by Cheesbrough (2005). Briefly, a small amount of fresh stool is sieved to remove debris, then pressed into a hole on a Kato-Katz template placed on a microscope slide. Once filled, the template is removed, and a glycerol-soaked cellophane strip is placed over the sample. The slide is gently pressed to evenly spread the stool and then left to clear for 30–60 minutes; the smear was subsequently examined under the compound microscope for *S. mansoni* eggs.

Figure 3.6: Human Water Contact Activity at Asao Stream, Predisposing the Local Population to *Schistosoma mansoni* Infection

Eight *S. mansoni*-heavily infected (≥400 eggs per gram of stool) children provided enough faecal samples for isolating parasite eggs for experimental use in infecting *Biomphalaria pfeifferi* snails. For egg isolation, pooled faecal samples from the *S. mansoni*-positive children were homogenized in dechlorinated water, and the resulting homogenate was strained through a series of nested sieves of different pore sizes in descending order (710 µM, 425 µM, 212 µM, 125 µM, and 45 µM); (see Figure 3.7 A) following established parasitological protocols (Mutuku *et al*., 2014). The schistosome eggs were collected in the last sieve of pore size 45 µM, with the eggs being subsequently washed out from this sieve with clean dechlorinated water into a conical flask, and the egg suspension topped up with more dechlorinated water. Exposure of the flask containing the egg homogenate to light for 2 hours induced hatching of the eggs into miracidia. For ease of collecting the miracidia, the flask was covered with aluminium foil up to the flask neck; (see Figure 3.7 B). Because miracidia are phototrophic, they move up the water column to the uncovered top area of the flask, making it easier to pick the miracidia using a Pasteur pipette. After 30 min, the miracidia in the flask were transferred into a petri dish. Five hatched miracidia were used to infect each single *B. pfeifferi* snail.

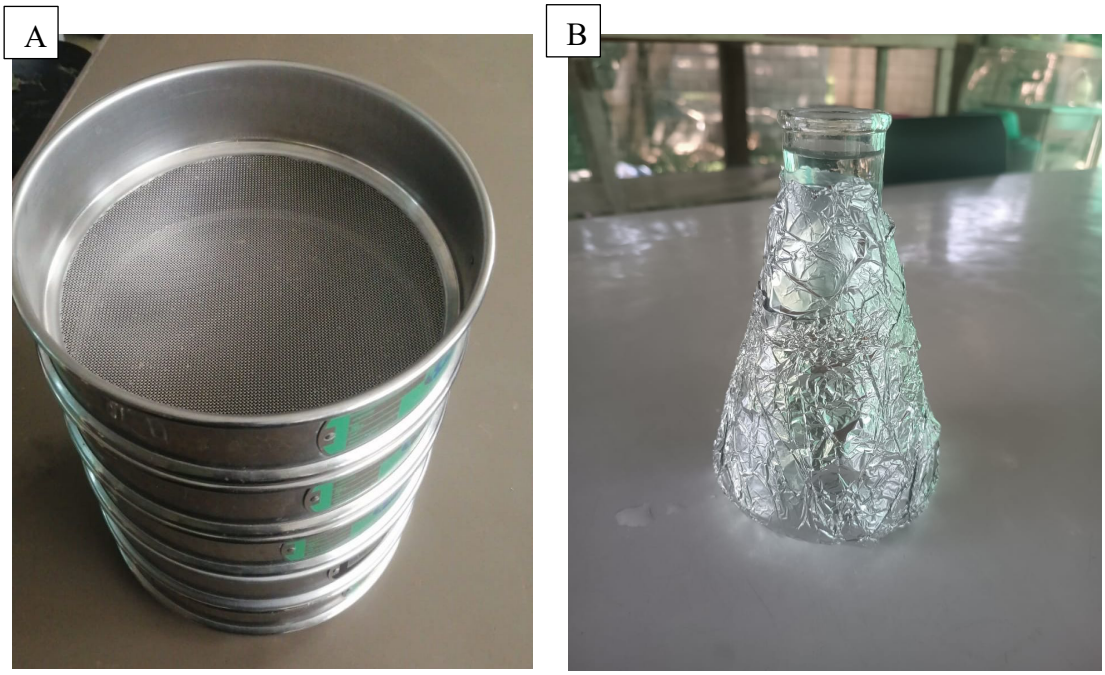

Figure 3.7: A- Nested Sieves of Different Pore Sizes in Descending Order for Straining Faecal Samples, B- Conical Flask Covered with Alluminium Foil

3.7 Molecular Procedures

3.7.1 DNA Extraction

The Hotshot method, originally described by Truett (Truett *et al*., 2000), was employed to extract total DNA from the head-foot tissue of *Biomphalaria pfeifferi* snails. This method offers a rapid and efficient way to isolate genetic material. Briefly, head-foot tissue was severed from each snail with a razor blade and then was transferred into an individual well of a 96-well plate using sterile pipette tips. A total of 75 µl of HotSHOT lysis solution (25 mM NaOH and 0.2 mM disodium ethylenediaminetetraacetic acid (EDTA)) was added to each well. The plates were then vortexed to ensure thorough mixing and then centrifuged for 20 seconds at 16,000 g. To prevent evaporation and contamination, the plates were sealed with a silicone mat before being placed in an Eppendorf Master cycler Systems thermocycler with a heated lid for incubation at 95°C for one hour. After this incubation step, the reaction was stopped by adding 75 µl of HotSHOT

neutralization reagent (40 mM Tris-HCl, pH 5.0) to each well. The concentration of each DNA isolate was determined by measuring the absorbance of 1 µl of the sample at 260/280 nm using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific).

3.7.2 DNA Amplification Targeting Microsatellite Markers

DNA amplification was carried out in a multiplex PCR format using two panels of microsatellites, both of which are specific to *Biomphalaria pfeifferi* (Charbonnel et al., 2000; Jones et al., 1999; Nguema et al., 2013; Palumbi, 1996). Panel 1 consisted of Bpf4, Bpf8, Bpf12, U2, Rg3, Bpf1, and Bpf2 primers, while the second panel contained Rg1, U-7, Rg4, Rg5, Bpf5, Rg8, and Rg6 primers (see Table 3.1). The QIAGEN Multiplex PCR Kit was used for amplification in a final reaction volume of 14 µl reactions according to the manufacturer's instructions. Briefly, 5 µl QIAGEN Multiplex ready mix, 2 µl forward primer, 2 µl reverse primer, and 5 µl DNA were prepared in a 0.2 ml reaction plate. The primer concentration per reaction was at 1 µM. The concentration of DNA varied across samples with an average of 696 ng/µl.

Table 3.1: Target Microsatellite Loci, Primer Oligo Sequences, Approximate Product Length (In Base Pairs), Fluorescent Dyes and Genbank Accession

Marker	Primer sequence	Amplicon size range (bp)	Fluorescent dyes	Accession No
Bpf4	F: GAATTCAGTCCTTACAGCAGC	317-329	VIC	AF189701
	R: CTTAAGCTGCGCTATCTGTGG			
Bpf8	F: GGTTCCCATAGCATACAGTGC	193-225	6FAM	AF189704
	R: GGCTTACAAAGAAACAGGCATAC			
Bpf12	F: GACACAAAGAAAGAGATAAGCA	307-334	6FAM	AF189708
	R: GTCGACCTCCCACTCTTC			
U-2	F: TCAATTTGCAAAGTTCAAGAGC	140-148	PET	AY425953
	R: GTAATCCAAAGCAACCGACTG			
Rg3	F: TGACACGGCTAGTGATGAGG	180-182	HEX	JN205162
	R: AGAACAGGGATGAACAATGG			
Bpf 1	F: TCCTATCCTTGTAACTTCTCCAC	203-207	HEX	AF189698
	R: CGAAACCATGCAAATCAG			
Bpf 2	F : GCAGCTTCATTCAACATTCC	139-153	VIC	AF189699
	R: AAATTAACATTTCGCTGAAACAG			
Rg1	F: TGGGCAGTTCCAATGAGG	125-131	6FAM	JN205160
	R: TGAGAAAGCCAACAAAGG			
U-7	F: TCATGACATCTAATGGGAGAAG	222-246	6FAM	AY425954
	R: GGGCTCAGAGAAATGAATGG			
Rg4	F: GGTCACTTTTATTTGGCAACAG	185-187	NED	JN205163
	R: GTGGATAGATGGGGAGTTAGGG			
Rg5	F: GTCAAAATATCTGCACTGATGG	196-198	6FAM	JN205164
	R: GAAACCTGCCTTTTTACATGC			
Bpf 5	F: TGTATGCTGACACTTAAAGAAACC	175-187	HEX	AF189702
	R: GCTACGCCACTGCTTATGAC			
Rg8	F: GCAAGTCATTAGGAGGATGAGG	264-268	VIC	JN205167
	R: GACACTGCGATTAGGTCAAGG			
Rg6	F: GATTTTGTCTCACGGAAACG	233-244	PET	JN205165

(Charbonnel et al., 2000; Jones et al., 1999; Nguema et al., 2013; Palumbi, 1996).

The thermal cycling profile included an initial denaturation step at 95°C for 15 minutes, followed by 35 cycles for 30 seconds at 94°C, an annealing temperature

specific for each multiplex panel (52°C for Panel 1, 55°C for Panel 2) for 60 seconds, an extension at 72°C for 60 seconds, and a final step of 10 minutes at 72°C using an Eppendorf Mastercycler Systems thermocycler. Multiplexed PCR products were then diluted in N, N'-dimethyl formamide with Genescan® – 500 [LIZ 500] as an internal size standard and genotyped using an ABI3500XL automated sequencer (Applied Biosystems) and scored with GeneMaker® version 3.0.1 software (Soft Genetics). All genotype calls were verified manually.

3.8 Determining the Genotypes of *Biomphalaria pfeifferi* Across Different Seasons of the Year in The Asao Stream

Biomphalaria pfeifferi snails collected bi-monthly between November 2018 and September 2019 at Asao stream were retrieved from the archive where they had been preserved in 70% ethanol. Snails were partitioned chronologically according to the documented historical sampling dates. Individual records were organized into distinct temporal cohorts. The snails were soaked overnight in double-distilled water to wash off the ethanol used in storing them. DNA extraction, PCR, and fragment analysis were done as described in section 3.7 above. Data was then exported from the GeneMaker software to an Excel sheet for data analysis. The genetic variation was determined by genetic differentiation, heterozygosity, analysis of molecular variance, and by comparing the multi-locus genotypes (MLGs) across the time intervals. Multi Locus Genotypes are unique combinations of alleles across all loci within an organism, for snails to be considered the same MLG, they must be identical at all microsatellite loci. The baseline genetic composition of the population was tracked over time.

3.9 Determining the occurrence of Trematode Infection among Persistent Genotypes

Individual records of data from section 3.8 were screened and filtered based on documented infection status, specifically the snails were categorized as "infected" (confirmed to be shedding cercariae of *Schistosoma mansoni* or other trematode groups) or "uninfected."

The infection profiles were mapped against the hosts genetic profile to determine the occurrence of trematode infection among specific multilocus genotypes.

3.10 Determination of Selfing Rates

Selfing rate is the proportion of offspring in a population produced through self-fertilization. Data from *Biomphalaria pfeifferi* snails from section 3.8 were used to determine the selfing rate. Selfing known to be independent of the mutation model was inferred by analysing the distribution and frequency of homozygous and heterozygous loci recorded across the dataset, the formulars used were FIS = $(H_e-H_o)/H_e$ and $s = 2F_{IS} / (1 + F_{IS})$ (Pollak, 1987; Rousset, 1996).

3.11 Characterizing Genotypes of Refractory *Biomphalaria pfeifferi* and their Progeny

Young adult *B. pfeifferi* snails were collected from Asao stream, screened, and *B. pfeifferi* shedding *Schistosoma mansoni* were discarded while non-shedders (Negative for *S. mansoni*) were kept for 4 weeks to allow any early infections to develop. The snails were then screened for prepatent infections; this helped detect infections that were missed during the initial screening. To generate refractory snails, two hundred and one (n=201) confirmed non-shedders were infected with five miracidia each, and after 8 weeks, the shedders were discarded while the non-shedders were bred to produce filial one (F1) generation. The F1 snails were then infected, and after 8 weeks the shedders were discarded while non-shedders were bred to result in the 2nd filial generation (F2). This was repeated for the filial three generations (F3), to generate refractory snails (Figure 3.8). DNA extraction, PCR, and fragment analysis of the refractory snails was performed as described in section 3.5 above.

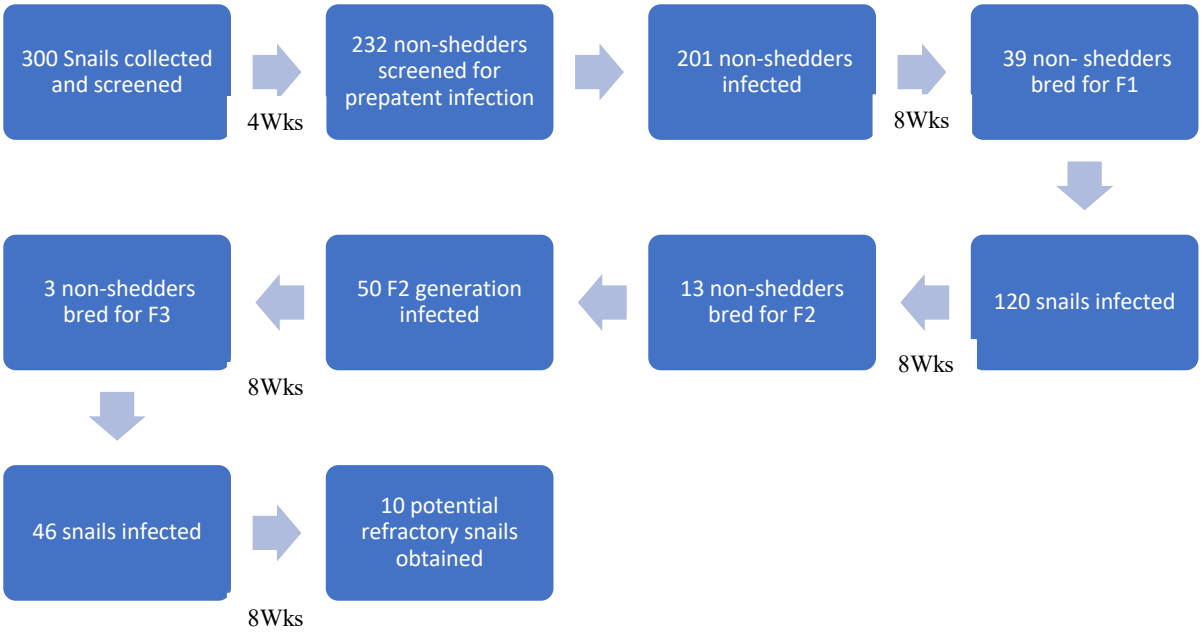

Figure 3.8: Breading of *Biomphalaria pfeifferi* Snails to Generate Refractory Genotypes

3.12 Data Analysis

Microsoft Excel 2016 (Coşkunserçe, 2017), GenAlex version 6.5 software (Peakall & Smouse, 2012), and R studio-based packages poppr version 2.9.6 and Coin version 4.4 (Hothorn *et al*., 2008; Kamvar *et al*., 2014; R Core Team, 2023; RStudio Team, 2020) were used to analyse data collected in this study. Poppr was utilized to determine the number of multilocus genotypes (MLGs) and MLGs that crossed sampling times. The MLGs were determined using 14 codominant microsatellite loci (Table 3.1) and they were calculated by using a rank function on strings of alleles. To control for potential bias arising from cloned genotypes, clone correction was done during identification of MLG that cross sampling times. This process removed duplicated MLGs within population strata. Phylogenetically uninformative loci (monomorphic across all individuals and loci with excessive missing data) were also eliminated. Coin was used to calculate a permutation tests. Excel was used to create graphs for visualization and to calculate descriptive statistics (Coşkunserçe, 2017). GenAlEx provides a range of analysis tools for handling genetic data (Liu *et al*., 2023) and was used to calculate allelic

frequencies, population assignment tests, expected heterozygosity (He), values of F_{ST} analysis (999 permutations), the number of alleles per sampling time, percentage of polymorphic loci and analyses of molecular variance (AMOVA). A Mantel test was done, and an isolation-by-distance test was performed to assess the correlation between FST values and temporal distance (999 permutations)**.** The unbiased estimator $\hat{f}$ of Wright's inbreeding coefficient F_{IS} was calculated using $F_{IS} = (H_e\text{-}H_o)/H_e$ according to Weir & Cockerham (Weir & Cockerham, 1984). The selfing rate, known to be independent of the mutation model, was estimated using the relationship $s = 2F_{IS} / (1 + F_{IS})$ (Pollak, 1987; Rousset, 1996).

CHAPTER FOUR

RESULTS

4.1 *Biomphalaria pfeifferi* Snails Collected from Asao Stream

A total of 502 archived *B. pfeifferi* snails were collected during the six bi-monthly sampling times (Figure 4.1). The relative abundance of *B. pfeifferi* varied over the collection period, with the lowest values being in July 2019 (n=22 snails) and the highest values being in November 2019 (n=120 snails).

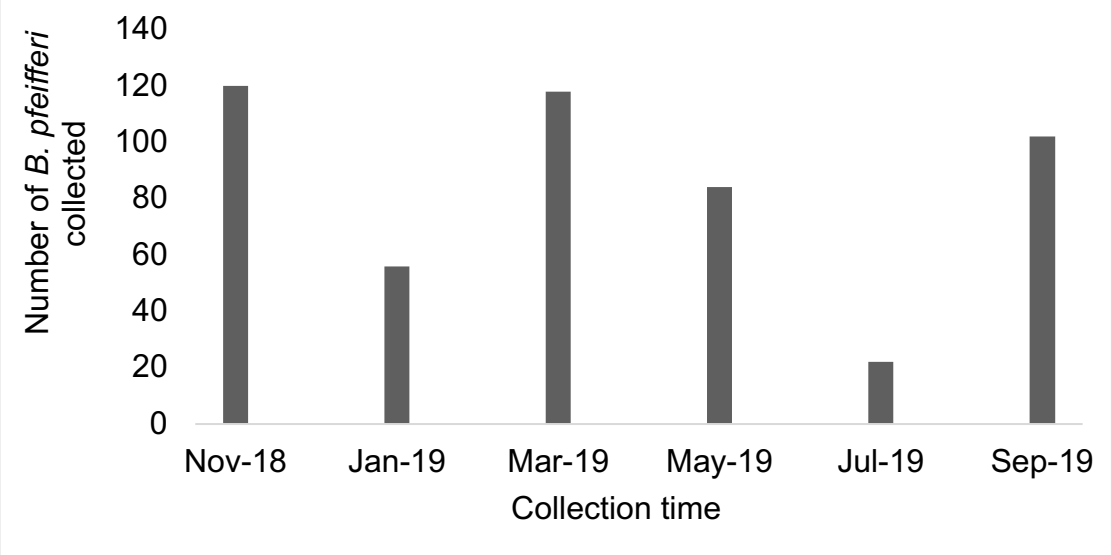

Figure 4.1: *Biomphalaria pfeifferi* Collected Per Sampling Time (November 2018-September 2019).

4.2 Genetic Variation of *Biomphalaria pfeifferi* across the Different Seasons of the Year in the Asao Stream

4.2.1 Genetic Diversity of *Biomphalaria pfeifferi*

The genetic diversity of *B. pfeifferi* collected from Asao stream over the six sampling points, including the levels of heterozygosity, allelic variation, and percentage of polymorphic loci, is (Table 4. 1). Expected heterozygosity ranged from 0.328 in July 2019 to 0.486 in September 2019, while observed heterozygosity was consistently lower than expected heterozygosity across all

sampling periods, with the lowest value recorded in May 2019 (0.118) and the highest in March 2019 (0.173). The results indicate a deficit of heterozygotes. The mean number of alleles per locus (Na) varied between 2.643 and 4.143, with the highest value observed in September 2019. The proportion of polymorphic loci (NP) showed substantial variation, peaking at 0.500 in March 2019 and dropping to a low of 0.071 in May 2019. The proportion of polymorphic loci remained high throughout the sampling periods, with complete polymorphism (100%) observed in most cases, except in March (92.86%) and July (71.43%). The data indicates low genetic diversity from the *B. pfeifferi* snails collected from Asao stream.

Table 4.1: Population Genetics Data for *Biomphalaria pfeifferi* Snails Collected from Asao Stream

Sampling Time	N	He	Ho	N_a	NP	P (%)
Nov-18	120	0.380	0.143	4.000	0.357	100.0
Jan-19	56	0.440	0.157	3,643	0.214	100.0
Mar-19	118	0.385	0.173	4.000	0.500	92.86
May-19	84	0.437	0.118	3.714	0.071	100.0
Jul-19	22	0.328	0.110	2.643	0.214	71.43
Sep-19	102	0.486	0.167	4.143	0.214	100.00

N- number of snails per sampling time, He-expected heterozygosity, Ho- observed heterozygosity, Na-Average number of alleles, NP—Average number of private alleles P (%) percentage of polymorphic loci

4.2.1.1 Hardy-Weinberg Equilibrium

A total of 14 loci were assessed for deviation from Hardy-Weinberg equilibrium (HWE) across six sampling periods (Table 4.2). Significant deviation ($p < 0.05$) was observed consistently throughout the year, with nearly all polymorphic loci deviating from equilibrium. This persistent, near-total deviation from HWE across

at almost all loci suggests high rates of non-random mating, or localized population bottlenecks that allow specific *Biomphalaria pfeifferi* genotypes to persist in the Asao stream.

Table 4.2: Summary of Chi-Square Test for Hardy-Weinberg Equilibrium Test for *Biomphalaria pfeifferi* Snails Collected in Asao Stream

Sampling Time	*n*Loci	ploci	mLoci	S ($p < 0.05$)	NS
Nov-18	14	14	0	12	2
Jan-19	14	14	0	13	1
Mar-19	14	13	1	13	0
May-19	14	14	0	13	1
Jul-19	14	11	3	10	1
Sep-19	14	14	0	13	1

nLoci-number of loci tested, p Loci-number of polymorphic loci, mLoci- number of monomophic loci, S(P<0.05)- significant deviations, NS- not significant

4.2.2 Population structure

4.2.2.1 Population Differentiation (FST)

FST is used to estimate the level genetic differentiation among population among sampling times. Pairwise FST values ranged from 0.008 (Sept-19 vs May-19) to 0.372 (July-19 vs March-19), with all comparisons being statistically significant ($p < 0.05$, Table 4.3). The lowest genetic differentiation was observed between September-19 and May-19 ($F_{ST} = 0.008$, $p = 0.003$). The highest genetic differentiation was between July and March 2019 ($F_{ST} = 0.372$, $p = 0.001$). This low overall genetic differentiation (FST < 0.5) indicates that high gene flow from passive downstream dispersal effectively maintains genetic connectivity and keeps the *Biomphalaria pfeifferi* population genetically similar across the Asao stream.

There was no significant correlation between F_{ST} and time revealed by the Mantel test (see Figure 4.2), evidenced by a weak negative correlation (r = -0.079, p = > 0.05). This indicates that genetic differentiation does not steadily increase or decrease over time.

Table 4.3: Pairwise Fst Analysis of *Biomphalaria pfeifferi* per Sampling Time

	Nov-18	**Jan-19**	**Mar-19**	**May-19**	**Jul-19**	**Sep-19**
Nov-18		0.001	0.003	0.001	0.001	0.001
Jan-19	0.117		0.001	0.001	0.001	0.001
Mar-19	0.019	0.157		0.001	0.001	0.001
May-19	0.047	0.051	0.064		0.001	0.031
Jul-19	0.354	0.166	0.372	0.222		0.001
Sep-19	0.050	0.046	0.052	0.008	0.184	

F_{ST} values are below diagonal (black), P values are above diagonal(red).

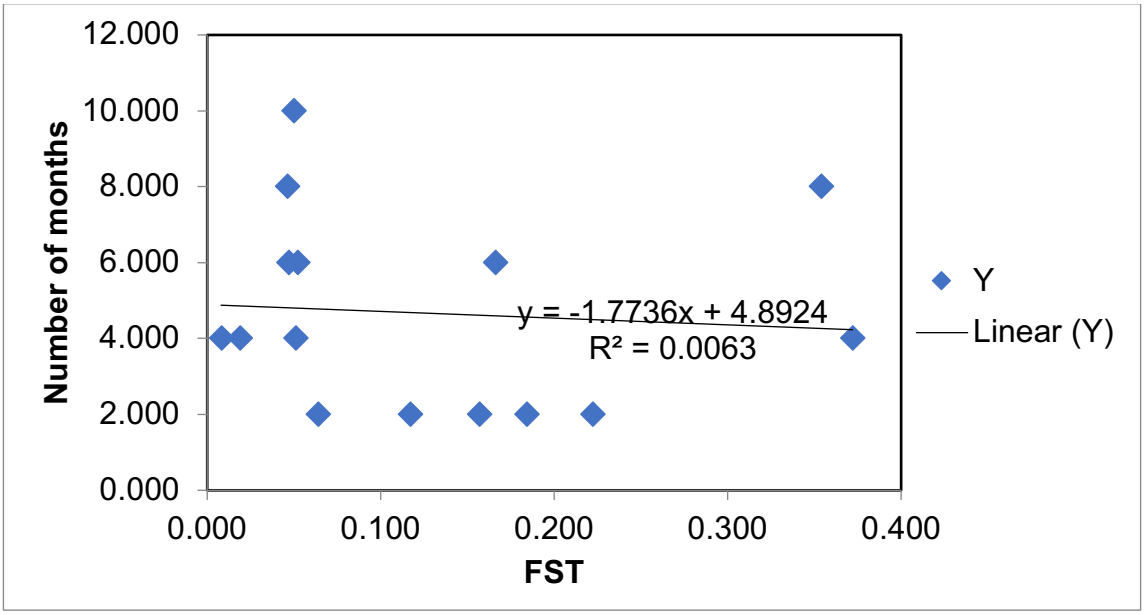

Figure 4.2: Correlation between Fst and Time of *Biomphalaria pfeifferi*

4.2.2.2 Population Assignment Test in *Biomphalaria pfeifferi* in Asao Stream

Population assignment analysis for *B. pfeifferi* showed that at each sampling time, individuals were assigned either to their own sampling time (Self) or to a different sampling time (Other) based on their genetic profiles (Table 4.4). The total number of individuals assigned to self and to other sampling times were 180 (36%) and 322 (64%), respectively, meaning there is considerable carryover of genotypes during the 10-month period represented by the samples. The July samples had all its individuals (22) assigned to their original sampling time, while September had only 2 of its individuals out of 102 assigned to their original sampling time.

Table 4.4: Summary of Population Assignment Test of the 502 *Biomphalaria pfeifferi* Samples

Pop	Self-Pop	Other Pop
Nov-18	39 (32.5%)	81 (67.5%)
Jan-19	26 (46.4%)	30 (53.6%)
Mar-19	83 (70.4%)	35 (29.6%)
May-19	8 (9.5%)	76 (90.5%)
Jul-19	22 (100%)	0 (0.0%)
Sep-19	2 (2.0%)	100 (98.0%)
Total	180 (36%)	322 (64%)

4.2.2.3 Analysis of Molecular Variance (AMOVA) of *Biomphalaria pfeifferi* within Asao Stream

Analysis of molecular variance for the six sampling times of *Biomphalaria pfeifferi* was conducted using 999 permutations and was estimated using standard F-statistics showing the sources of genetic variation (Table 4.5). The results showed that most of the genetic variation (58%) is found among individuals within sampling times, a significant portion (33%) is found within individuals, and a smaller portion (8%) is found among sampling times.

Table 4.5: Analysis of Molecular Variance for the Six Sampling Times of *Biomphalaria pfeifferi*

Source	Df	SS	MS	Est. Var.	%	p-value
Among Times	5	240.881	48.176	0.269	8%	<0.001
Among Individuals	496	2352.463	4.743	1.843	58%	<0.001
Within Individuals	502	530.500	1.057	1.057	33%	<0.001
Total	1003	3123.844		3.169	100%	

Source- Source of variation, Df- Degree of freedom, SS- Sum of squares, MS- Mean Square, Est.Var.- estimated variance, % - percentage of total genetic variance attributed to the source.

4.2.2.4 Principles of Coordinates (PCoA) of *Biomphalaria pfeifferi* within Asao Stream

The percentage of variation explained by the first three axes of a principal coordinates analysis (PCoA) was determined for *B. pfeifferi* sampled (Table 4.6, Figure 4.3). The data show that the first axis explains a large proportion of the variation in the dataset (77.93%), while the second and third axes explain smaller proportions (14.71% and 4.81%, respectively). The cumulative percentage of variation explained by the first three axes is 97.45%.

Table 4.6: Principles of Coordinates Results for *Biomphalaria pfeifferi.*

Percentage of variation explained by the first 3 axes			
Axis	**1**	**2**	**3**
%	77.93	14.71	4.81
Cum %	77.93	92.64	97.45

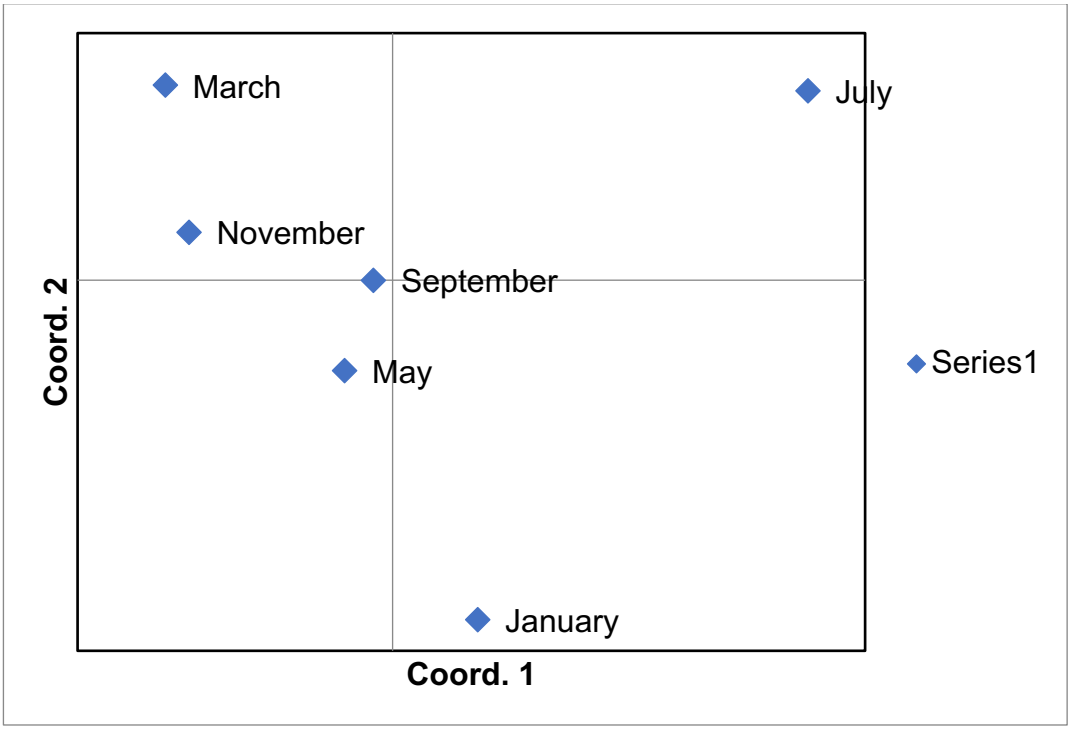

Figure 4.3: Principal Coordinates (PCoA) for *Biomphalaria pfeifferi* Populations within Asao Stream

4.2.3 Multilocus Genotypes for *Biomphalaria pfeifferi*

Among the 502 *B. pfeifferi examined,* 319 distinct MLGs were found across the six sampling times, with the number of MLGs ranging from 13 in July 2019 to 82 in March 2019. A total of 26 MLGs were found in more than one sampling time point, indicating repeated occurrence of certain genotypes within the population. Table 4.7 and Figure 4.4 provide overviews of the distribution of MLGs for the *B. pfeifferi* samples collected from Asao at each time point.

Table 4.7: Multilocus Genotypes Data for *Biomphalaria pfeifferi* Snails Collected from Asao Stream for Each Month

Sampling Time	MLG	MLG cst	MLG ≥2	MLGms
Nov-18	80 [120]	09[20]	21[48]	22[47]
Jan-19	39[56]	04 [04]	17[34]	17[34]
Mar-19	82 [118]	19[45]	17[57]	10[20]
May-19	67[84]	16[27]	11[28]	06[12]
Jul-19	13[22]	00[00]	09[18]	09[18]
Sep-19	74 [102]	14[27]	19[46]	15[30]

MLG: multilocus genotypes; MLG cst: multilocus genotypes found at two or more sampling times; MLG ≥2: multilocus genotypes represented by 2 or more individuals; MLG ms: multilocus genotypes shared by multiple individuals but found only at a single time point. Numbers in brackets represent the number of snails represented by the MLGs.

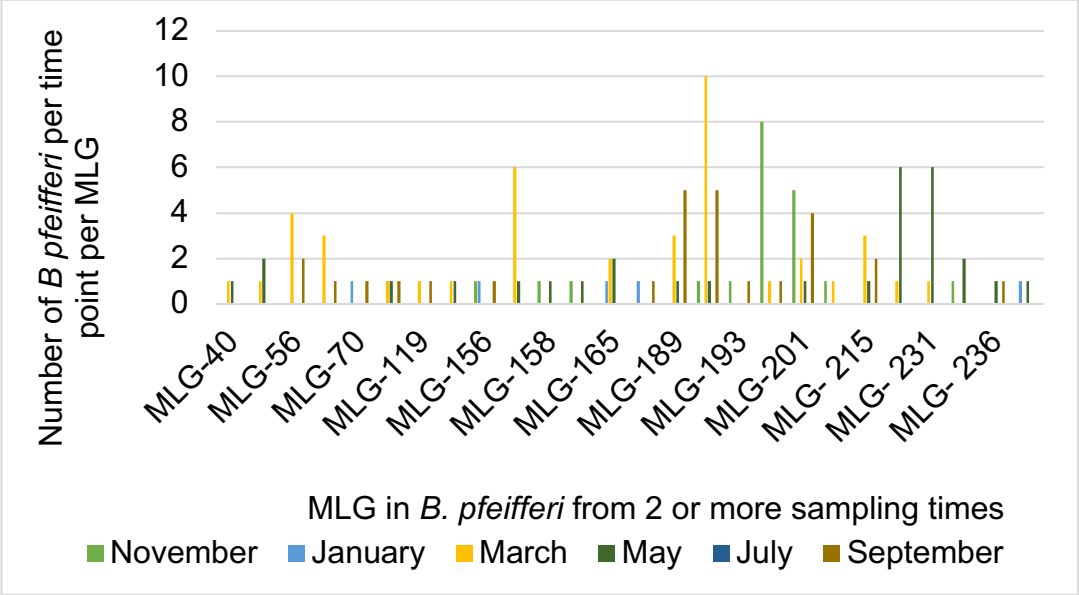

Figure 4.4: *Biomphalaria pfeifferi* Represented for each of the 26 Multilocus Genotypes (MLGs) that Were Found at Two or More of the Different Sampling Times

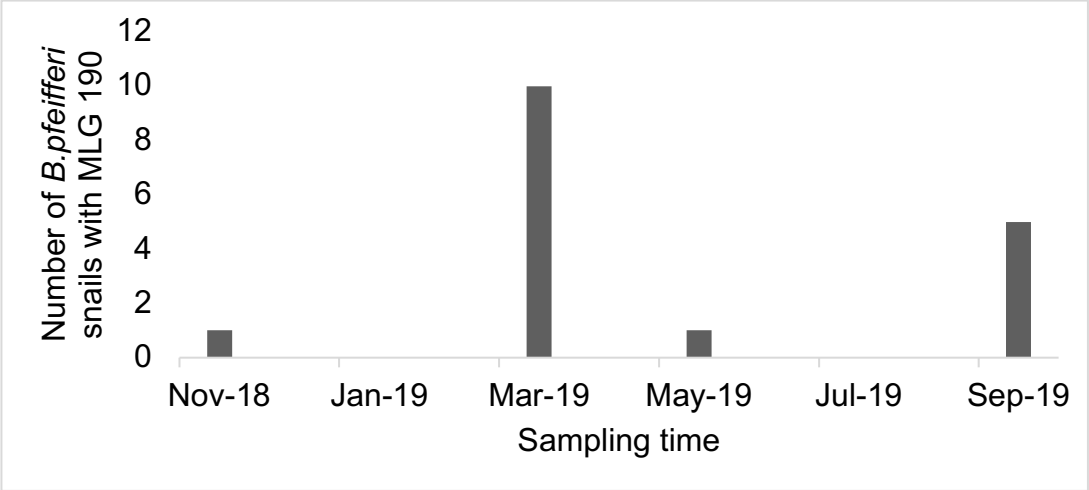

Figure 4.5: Number of *Biomphalaria pfeifferi* Snails Present Per Sampling Time with MLG 190

The 26 MLGs encountered 2 or more times represent 124 of the 502 (24.7%) *B. pfeifferi* that were collected. MLGs varied in their persistence and abundance. For instance, MLG 190 was found ten times in March and appeared at

four sampling times and in 17 individuals overall (see Figure 4.4 and Figure 4.5). Similarly, MLGs 190, 193 and 200, were present in both the initial and final sampling times, demonstrating persistence throughout the study period. MLGs 40, 70, 119, 152, 158, 160, 188, 193, 203, 236, and 237 were each restricted to only two time points and had a single occurrence within each (see Figure 4.4 and Figure 4.6). MLGs 40, 49, 152, 157, 227, and 231 were only detected in two successive sampling points, spanning three months coincidentally between March and May (see Figure 4.4 and figure 4.7). The latter MLGs (40, 49, 152, 157, 221, and 231) may be less prevalent and thus less frequently sampled or may truly be more transient over time. The July sample did not harbour any MLGs that were shared with other sampling times.z

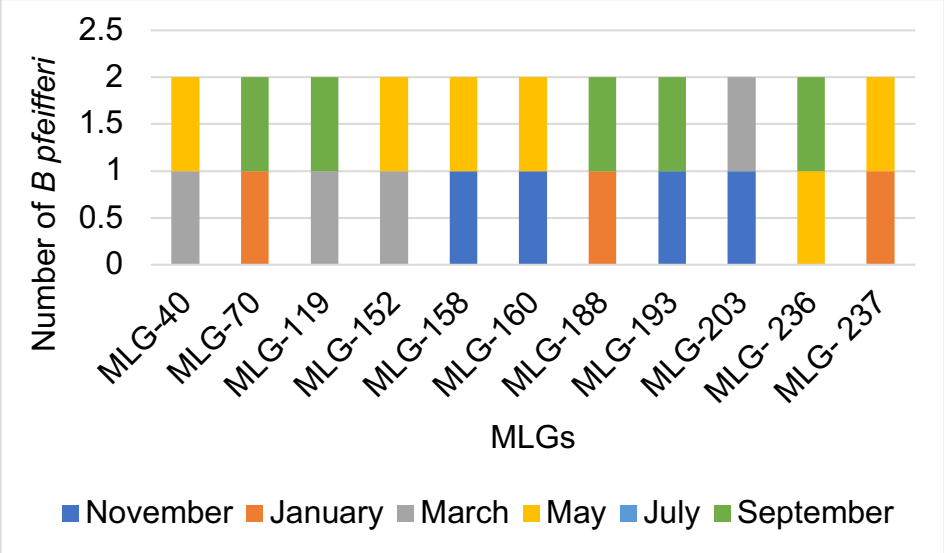

Figure 4.6: MLGs Restricted to Only Two Sampling Time Points and Had a Single Occurrence

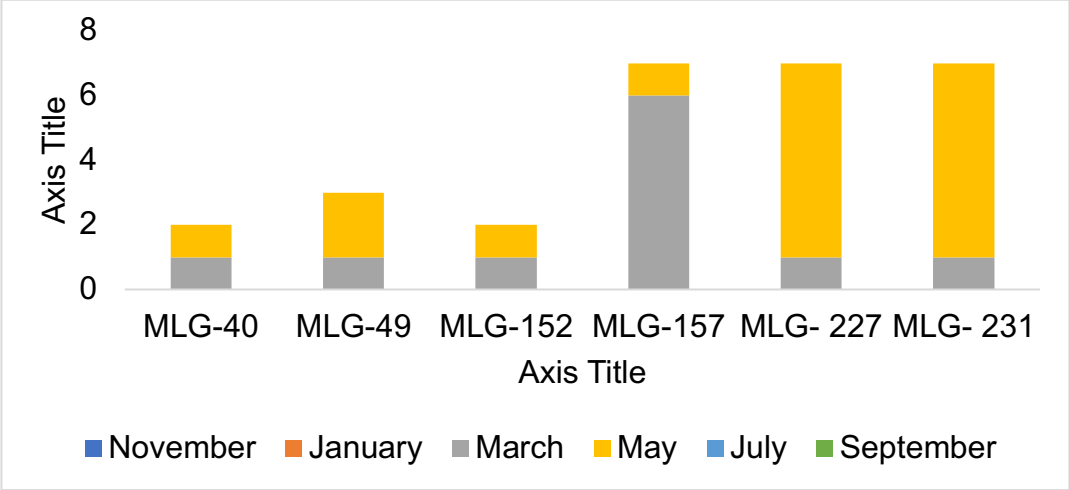

Figure 4.7: MLGs Detected Iin Two Successive Sampling Points Only

The duration of persistence of these MLGs ranged from 3 to 10 months (see Figure 4.8). These findings demonstrate the presence of persistent multilocus genotypes within the *B. pfeifferi* population Asao stream.

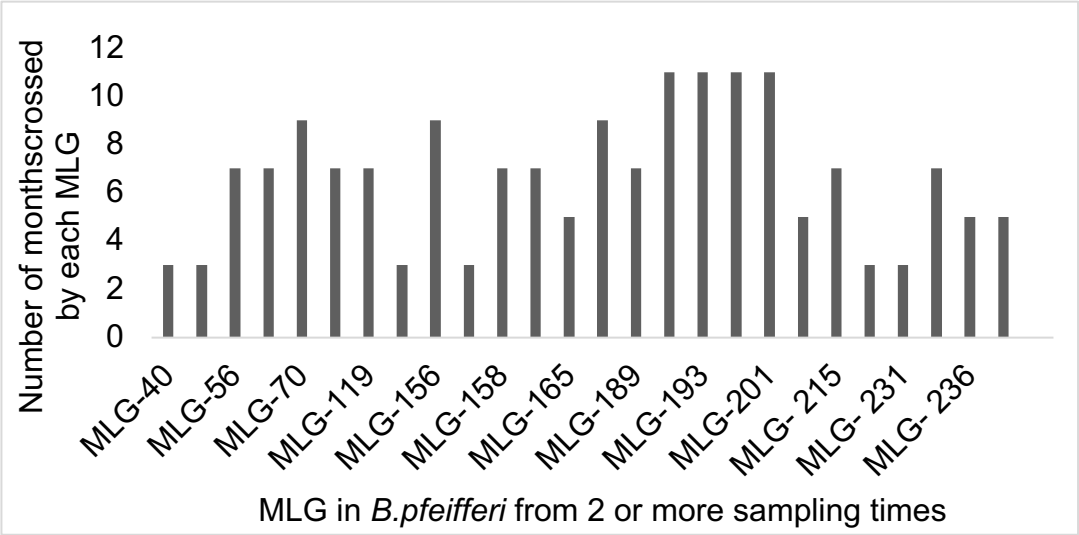

Figure 4.8: Number of Months Crossed by Multilocus Genotypes (MLG) in *Biomphalaria pfeifferi*

4.3 Occurrence of *Schistosoma mansoni* and other Trematode Infections among Persistent *Biomphalaria pfeifferi* Genotypes in the Asao Stream

4.3.1 Prevalence of Trematode Infections in *Biomphalaria pfeifferi* Snails from Asao Stream

At each of the six sampling points, the number of *B. pfeifferi* with or without patent trematode infections (shedding/not shedding cercariae) was determined. Trematode infections were identified as being *Schistosoma mansoni*, strigeid, paramphistomoids, xiphidiocercariae, echinostomes, and sanguinicolid. While the November 2019 collection yielded the most snails (120 snails), it also had relatively few infected snails; only 3 with paramphistomoids. March 2019 collections had the most infected snails (a total of 90), harboring *S. mansoni*, strigeids, paramphistomoids, xiphidiocercariae, echinostomes, and sangunicolids. July had the fewest snails with no trematode infections present (see Figure 4.9 and Table 4.8). Of the 502 snails collected, 138 (27.5%) were infected with *S. mansoni* (Table 4.8). The overall prevalence of recovered trematode infections was 42.4% (213/502).

Table 4.8: *Biomphalaria pfeifferi* Collections at each Sampling Time and their Infection Status

	Sampling time	N	Infection status	*n*
1.	November (2018)	120	No trematodes	117
			Paramphistomoids	3
2.	January (2019)	56	No trematodes	36
			S. mansoni	19
			Echinostomes	1
3.	March (2019)	118	No trematodes	28
			S. mansoni	51
			Paramphistomoids	23
			Xiphidiocercariae	12
			Echinostomes	3
			Sangunicolids	1
4.	May (2019)	84	No trematodes	38
			S. mansoni	31
			Paramphistomoids	9
			Strigeids	3
			Echinostomes	2
			Sangunicolids	1
5.	July (2019)	22	No trematodes	22
6.	September (2019)	102	No trematodes	48
			S. mansoni	37
			Paramphistomoids	15
			Echinostomes	1
			Xiphidiocercariae	1

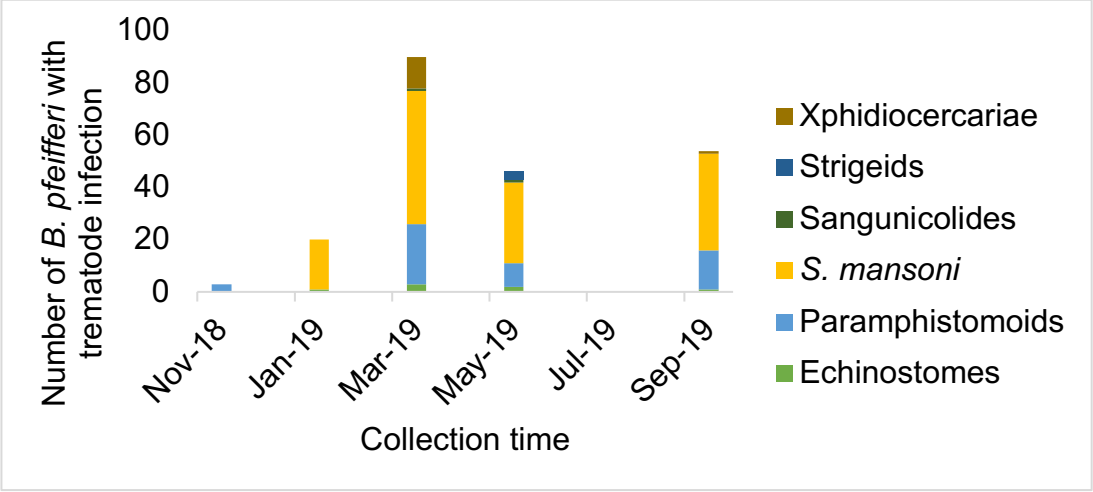

Figure 4.9: Total Number of *Biomphalaria pfeifferi* Snails with Trematode Infections (determined based on morphology) at each Sampling Time

4.3.2 Occurrence of Trematode Infections among *Biomphalaria pfeifferi* Multilocus Genotypes

Schistosoma mansoni was the most prevalent trematode species at this study site and was found in 22 out of the 26 snail MLGs recovered in more than one sampling period (see Figure 4.10). Paramphistomoids, another prevalent group of trematodes, were present in 17 out of 26 MLGs. Eight MLGs (40, 49, 57, 156, 158, 193, 236, and 237) exhibited exclusive infections with *S. mansoni.* MLGs 160, 188, and 203 were found to harbour only paramphistomoids. MLG 190, one of the persistent MLGs identified throughout the study period, exhibited the highest number of trematode infections. 16 out of 17 snails were positive (see Figure 4.10). The association between MLG 190 snails and snail infections was examined using a permutation test. There was a significant difference in the proportion of MLG 190 snails among infected snails (p= 0.0001), indicating that the observed distribution is unlikely due to random chance. A total of 103 of all 213 snails with patent trematode infections were in the 124 snails from the repeat 26 MLGs (overall prevalence of 83.1%). The overall prevalence of all trematode infections among the *B. pfeifferi* not in MLGs found in more than one sampling time was 29.1% (110 out of 378 snails). For *S. mansoni* specifically, a total of 65 infections were from the 26 repeat MLGs (prevalence of 65 of 124, or 52.4%) as

compared to the overall prevalence of *S. mansoni* of 138 out of all 502 snails (27.5%) or the prevalence of *S. mansoni* among the *B. pfeifferi* not in MLGs found in more than one sampling time (73 of 378 snails, or 19.3%).

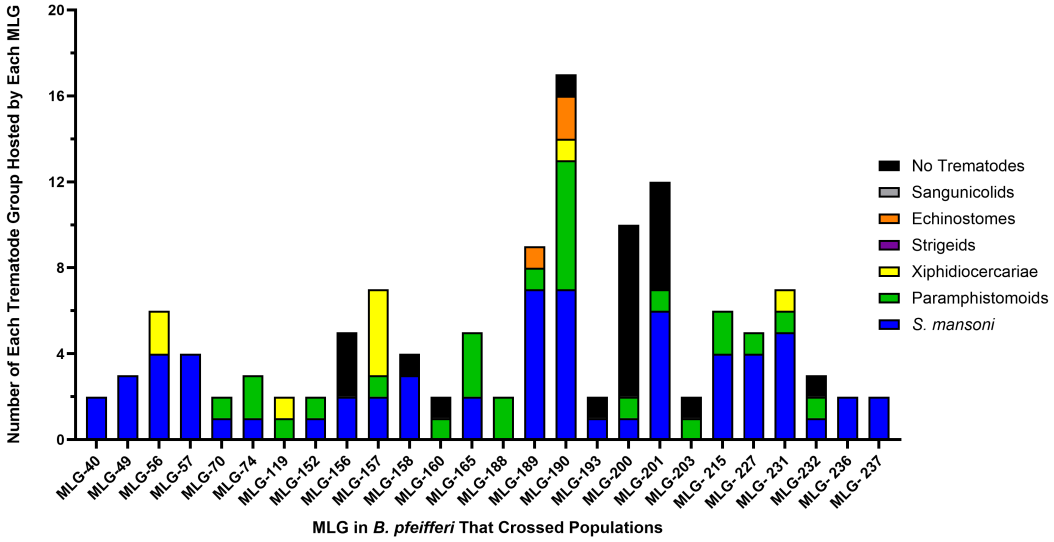

Figure 4.10: Distribution of MLGs in *Biomphalaria pfeifferi* Recovered from Two or More Sampling Times and their Associations with Trematodes

Summing across all patent (shedding) trematode infections, 213 of 502 snails were positive. Table 4.9 provides an overview of the distribution of MLGs for the *B. pfeifferi* samples harbouring trematodes collected at each time point. The table also reveals the number of MLGs harbouring trematodes that are represented at two or more sampling time points.

Table 4.9: Multilocus Genotypes Data for *Biomphalaria pfeifferi* Snails from Asao Stream that Harboured Trematode Infections

Sampling Time	N	MLG	MLG cst
Nov-18	03	03	01[01]
Jan-19	20	20	04 [04]
Mar-19	90	68	32[55]
May-19	46	39	21[24]
Jul-19	00	00	00[00]
Sep-19	54	44	17[24]

N: number of individuals harbouring trematodes; MLG: number of multilocus genotypes harbouring trematodes; MLG cst: multilocus genotypes harbouring trematodes that were recovered from two or more sampling times. Numbers in

4.4 Selfing Rates of *Biomphalaria pfeifferi* Snails Collected from Asao Stream

The observed heterozygosity measures the total number of heterozygotes over the total number of samples and the average was 0.1445 (see Table 4.1) which indicates a deficit of heterozygotes which is an indicator of predominant self-fertilization. The deviation from Hardy Weinberg equilibrium (HWE) across almost all loci (Table 4.2) is an indication of elevated self-fertilization (selfing). The inbreeding coefficient (F_{IS}) measures the degree of inbreeding within a subpopulation relative to the total population. The data revealed that the snails exhibited high levels of inbreeding at all six sampling points, as evidenced by positive global F_{IS} values (see Table 4.10). F_{IS} was used to estimate the degree to which selfing (S) contributes to the overall inbreeding in the population. From the results, the selfing rates accounted for 77.77%, 95% CI [74%, 84%] inbreeding.

Table 4.10: Inbreeding Coefficient and Selfing Rates for Samples of *Biomphalaria pfeifferi* Collected at the Six Different Time Points

Sampling Time	N	F_{IS}	S
Nov-18	120	0.624	0.768
Jan-19	56	0.643	0.783
Mar-19	118	0.517	0.682
May-19	84	0.730	0.844
Jul-19	22	0.665	0.799
Sep-19	102	0.653	0.790

F_{IS}-inbreeding coefficient, S-selfing rate

4.5 Genotypes of the Refractory *Biomphalaria pfeifferi* and their Progeny

The refractory experiment yielded refractory *B. pfeifferi* snails per generation (see Table 4.11) and a final of 10 *B. pfeifferi* snails at Filial 3 (F3) generation, all bred from non-shedding lineages. The 10 F3 *B. pfeifferi* snails, were genotyped to assess the genetic profiles of the refractory population. The analysis revealed multiple distinct genotypes among the individuals per generation (see Figure 4.11). Genotypes R-5 and R-8 were identical, while genotypes R-1, R-2, R-3, R-4, R-6, R-7, and R-9 were closely related to R-5/R-8, differing at only one to four loci. Genotype R-10 was the most divergent, appearing more distantly related to the rest. All genotyped snails had survived experimental infection with *Schistosoma mansoni* through three successive generations and were confirmed to be non-shedders at the time of screening.

Table 4.11: Number of Infected and Non-Shedding *Biomphalaria pfeifferi* Snails Per Generation

Generation	Infected	Non-shedders
Initial	201	39
F1	120	13
F2	50	3
F3	46	10

Pop	Genotype
R_8	176176322322174174215215145149135135235235169169293293233233g
R_5	176176322322174174215215145149135135235235169169293293233233g
R_7	176176322322168174215215145149135135235235175175293293233233g
R_1	176176322322170174215215145145135135235235169169293293233233g
R_6	176176322322174174207215145145135135235235169169293293233233g
R_2	176176322322174174215215145149135135235235169169291293233233g
R_9	176176322322174174215215145149135135235235175175293293233233g
R_3	176176322322174174215215145149137137235235169169293293233233g
R_4	178178322322178178215215145149135135235235169175293293233233g
R_10	180180310322174174207215145145135135235235169169291291233233g

Figure 4.11: Genotypic Profiles of Ten Refractory Snails

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 Discussion

Biomphalaria pfeifferi supports a complex community of digenetic trematodes including a high prevalence of *Schistosoma mansoni* infections in Asao stream, (Laidemitt *et al*., 2019a; (Laidemitt *et al*., 2019b; Laidemitt *et al*., 2017a). The stream is a site of active water contact and use by the local community. This study aimed to assess the genetic variation of the snail across different seasons in Asao stream, to determine the occurrence of *S. mansoni* and other trematodes among persistent *B. pfeifferi* genotypes, determine selfing rates within the snail population, and characterize genotypes of the progeny of potentially refractory *B. pfeifferi*.

Rainfall and temperature variation during the sampling period may have influenced snail abundance, which varied across the six sampling periods. The highest numbers of *B. pfeifferi* collected in November 2018 (n = 120) and the lowest in July 2019 (n = 22). McCreesh et al. (2015) showed that changes in temperature and rainfall can influence *S. mansoni* transmission in East Africa, supporting the need to consider seasonal environmental variation when interpreting snail population patterns. Although this study did not test a direct cause-and-effect relationship between seasonal variation and snail population changes, the 10-month sampling period from November 2018 to September 2019 covered both wet and dry conditions, making it sufficient to assess how *B. pfeifferi* genotypes persisted across naturally varying environmental conditions in Asao village.

This study observed that *B. pfeifferi* from Asao had a deficiency of heterozygotes (Table 4.2). Expected heterozygosity (He), a measure of genetic diversity within a population, considers the probability of finding two different alleles at a given locus in an individual, while observed heterozygosity (Ho) reflects the actual heterozygosity observed in the analysed samples. The average number of alleles

(A) and the average number of private alleles (NP) provide additional measures of genetic diversity within sampling times. These data indicate that the snails investigated have relatively low numbers of alleles; However, the presence of private alleles in some of the sampling times suggests that there may be some unique genetic variation that is not shared.

The low levels of genetic variability evidenced by AMOVA (Table 4.6) and the population assignment test (Table 4.5) in the present study are similar to the findings of studies on genetic diversity on *B. pfeifferi* populations reported from Madagascar using microsatellite markers (Charbonnel *et al*., 2002). Weak differentiation among samples from different time points is demonstrated by low values of F_{ST} (as low as 0.010). This is not surprising given that all samples were drawn from the same location but at different sampling times. These F_{ST} values were lower than the values reported by Charbonnel *et al*., 2002, at a similar geographic location, and consistent with values reported in other selfing snail species as reported by Viard *et al*., 1997.

Using 14 codominant microsatellite loci and employing clone correction, 319 unique multilocus genotypes among the *B. pfeifferi* samples were identified. This study indicates that recognizable MLGs can be identified and that some persisted over the 10 months of the study. The presence of particular MLGs, such as MLG 190, 193, 200, and 201 over 10 months, indicates their ability to persist in varying environmental conditions. Although *B. pfeifferi* can survive in the laboratory for as long as 100 weeks (Sturrock, 1966), and laboratory survival of individuals infected with *S. mansoni* has been recorded for over a year (Mutuku *et al*., 2014), average life expectancy in the field is considerably lower, lasting 2 to 3 weeks (Woolhouse, 1992). If this is so, the 10-month duration in this study may have been long enough to have captured individuals with the same MLG but representing different generations.

With respect to MLGs of six *B. pfeifferi* populations known to bear a heavy parasite burden, the study identified *S. mansoni*, and several other trematodes including strigeids, paramphistomoids, xiphidiocercariae, echinostomes, and sanguinicoloids which are commonly associated with wildlife, amphibians, birds,

and livestock suggesting that Asao stream supports complex host–parasite interactions involving both human and animal hosts. Previous studies have suggested that competition among trematodes and host immune responses may alter compatibility relationships between schistosomes and their snail hosts (Combes, 2001; Laidemitt et al., 2019). The overall prevalence of larval digenean infections among the 502 *B. pfeifferi* examined at Asao collection site was indeed high, 42.4%, with over half of the infections recorded being of the medically important trematode, *S. mansoni* (138 of 502 snails, a prevalence of 27.5%).The high prevalence of *S. mansoni* observed in Asao stream is consistent with previous studies identifying the area as an active transmission focus within the Lake Victoria basin of western Kenya (Mutuku et al., 2014; Laidemitt et al., 2019).

There are intriguing indications to hint that the more common or persistent MLGs are prone to trematode infection, supporting both a high prevalence of infection and diverse trematodes. For 26 MLGs that were recovered at two or more sampling times, in aggregate representing 124 snails, the prevalence of infection for all trematodes was 103 of 124 snails (83.1%), in contrast to the prevalence in snails with an MLG found in only one sampling time or that had unique genotypes not found in other snails, 110 of 378 snails, or 29.1%. Furthermore, the prevalence of *S. mansoni* among the snails representing the 26 MLGs found more than once was higher (65 of 124, or 52.4%) as compared to snails with an MLG found at only one sampling time or that had unique genotypes (73 of 378, or 19.3%). Similarly, for the most commonly recovered MLG, MLG 190, one that persisted throughout the 10-month study, 16 of 17 (94.1%) snails were positive for trematode infections, including the broadest representation of trematode diversity seen for any MLG; additionally, 7 of these 17 (41.2%) were positive for *S. mansoni*. These results highlight the potential existence of persistent *B. pfeifferi* MLGs prone to transmitting a diverse range of trematodes, including *S. mansoni* (Laidemitt *et al*., 2017b) (M.R. *et al*., 2017) (Cupit *et al*., 2011).

In comparison with the multilocus genotype data, 4 of the 26 MLGs found more than once were not found to harbour *S. mansoni*, though they were not among the most common repeat MLGs found. Furthermore, although the prevalence of *S.*

mansoni infection among MLGs found only once or in snails with unique genotypes was considerably lower at 19.3% than the 52.4% prevalence in MLGs found more than once, the former group comprised, in aggregate, more *S. mansoni* infections (n = 73) than the latter (n = 65). Infection rates for different MLGs may be influenced by inherent differences in resistance, longevity (with longer-lived MLGs eventually accumulating more infections), or post-infection survivorship. Trematode infection imposes considerable fitness costs on infected snails, which are typically castrated and suffer higher mortality (Negovetich & Esch, 2008).

These results suggest that basic Red Queen dynamics as exemplified by the *Potamopyrgus antipodarum* might exist in *B. pfeifferi* as well, although more studies are required to learn if common or long-standing MLGs come to have higher trematode loads than individuals from rare or newly appearing MLGs (Vergara *et al*., 2014). If so, this may be because they become more susceptible over time as trematodes adapt to them, or it is possible they inherently live longer and so have higher chances of acquiring trematode infections. It should not be forgotten that *B. pfeifferi* is capable of cross-fertilization (Kengne-Fokam *et al*., 2016a; Charbonnel *et al*., 2005b), and the frequency with which this might occur in habitats like Asao is not known. Repeated episodes of seasonal flooding, followed by periods of drought, occur at Asao Stream, though it has not been observed to completely dry out since it was initially identified as a *B. pfeifferi* habitat where *S. mansoni* transmission took place 32 years ago (Loker, 1993a). Flooding may fundamentally reset the situation in Asao with respect to MLG representation, thereby preventing Red Queen dynamics from ever becoming sufficiently strong to favour sexual reproduction. What is clear, though, is that the *B. pfeifferi* population at Asao remains remarkably robust, in spite of the high levels of trematode parasitism exhibited at this site.

One biological aspect of *B. pfeifferi* is that it is a predominantly self-fertilizing species (Campbell *et al*., 2010b), with attendant consequences for its population structure. This study also confirms, using F_{IS}, that *B. pfeifferi* from Asao are selfers, with selfing rates as high as 77%. The low levels of observed heterozygosity and the deviation from Hardy Weinberg equilibrium across almost

all loci are also an indicator of inbreeding. Self-fertilization is common in hermaphroditic organisms like *B. pfeifferi* and often leads to increased homozygosity over generations (Olson et al., 2025).

Owing to its high selfing rate, individual *B. pfeifferi* might give rise to distinctive MLGs that are then maintained across generations by repeated selfing, and the aggregate *B. pfeifferi* population in a given area might consist of multiple MLGs that potentially differ in their susceptibility to *S. mansoni* and other parasites. It is also hypothesized that the populations are able to survive the fluctuation in weather conditions by selfing to recolonize the habitat. According to the "dead-end hypothesis" (Jullien et al., 2021;Stebbins, 1957), selfing populations are expected to accumulate deleterious mutations (Awad et al., 2014), and could lack the genetic diversity required to adapt to changing environmental conditions (Abu Awad & Roze 2020; Porcher & Lande, 2016). Therefore, we can expect frequent catastrophic demographic events, with strong bottlenecks or even extinctions followed by recolonization accompanied by strong founder effects (Dudash & Fenster, 2010).

With respect to thoughts that some *B. pfeifferi* genotypes might be found that are refractory to *S. mansoni*, ten F3 *Biomphalaria pfeifferi* snails, all derived from non-shedding lineages, revealed moderate genetic diversity, with a dominant cluster of closely related genotypes (R-1 to R-9) and a single outlier (R-10). These findings are consistent with studies in *B. glabrata*, where resistance to *S. mansoni* is often inherited and associated with specific genomic regions, such as the Guadeloupe Resistance Complex (GRC), which harbours dominant resistance alleles (Tennessen et al., 2015). While no molecular expression data were available in this study, the clustering of genotypes may reflect similar structural variations or shared immune traits underlying refractoriness, as seen in resistant *B. glabrata* lines (Locker et al., 2012). The identification of *B. pfeifferi* MLGs that appear less susceptible to *S. mansoni* infection may have important implications for snail-based control strategies. If such potentially refractory genotypes are confirmed through further experimental studies, they could help identify natural

resistance patterns within Asao stream and guide future interventions aimed at reducing transmission in similar African freshwater ecosystems.

5.2 Conclusion

1. The study revealed genetic variation among *Biomphalaria pfeifferi* snails across the 10-month sampling period. Several MLGs were detected, with some persisting across different seasons, suggesting they may possess adaptive traits that support survival under varying environmental conditions.
2. Occurrence of trematodes among specific MLGs among *Biomphalaria pfeifferi* snails was evident. The most common and persistent MLGs were likely to harbour trematode infection compared to the less common MLGs, these MLGs can be targeted for the intervention of *Schistosoma mansoni* in Asao stream,
3. Analysis of genetic data indicated high levels of global fixation index (FIS) or inbreeding coefficient among the *Biomphalaria pfeifferi* snails, with a selfing rate of over 77%, confirming the preferential self-fertilization (selfing) within this population. This reproductive mode likely contributes to the stability and recurrence of specific genotypes across generations.
4. The present study also characterized genotypes from the Filial 3 (F3) generation of *Biomphalaria pfeifferi* snails that remained uninfected after exposure to *S. mansoni*. These refractory snails displayed distinct MLG profiles, indicating potential natural resistance to schistosome infection. The identification and manipulation of such resistant MLG variants may prove useful in intervention strategies to control schistosomiasis in Asao stream and similar African ecosystems.

5.3 Recommendation

5.3.1 Recommendation for control of schistosomiasis

1. Schistosomiasis control in Kenya should include year-round snail control, as adaptive *Biomphalaria pfeifferi* populations persist across seasons an occurrence likely caused by high selfing rate which maintain transmission.
2. Targeted monitoring of potentially refractory genotypes in snails within Kenya should be included as part of the control strategies.

5.3.2 Recommendation for future studies

1. In order to account for longer and more frequent seasonal fluctuations, future research should increase the study period to include several years and a cause-and-effect analysis should be done to compare the weather patterns and the snail abundance.
2. The results would be more broadly applicable in many ecological contexts if the sampling were extended to more sites.
3. The study's potential for controlling *S. mansoni* would be expanded if it included other Biomphalaria species, such as B. sudanica and B. choanomphala.

5.4 Study Limitation

The research was limited to the Asao Stream, limiting its generalizability to regions with different ecological conditions. Cause and effect analysis of weather and snail abundance was not determined, though the collection times were spread out to ensure the sampling of snails covered the varying seasons of the year. Because of *Schistosoma mansoni's* morphological similarity to *S. rodhaini*, the cercariae were not genetically verified, raising the risk of species misidentification; this study overcome this by shedding the snails at the time-of-day coinciding with *S. mansoni* shedding time (10:00 am).

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APPENDICES

Appendix I: Ethics Review Committee

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel: (254) 2722541, 2713349, 0722-205901, 0733-400003, Fax: (254) (020) 2720030
Email: director@kemri.org, info@kemri.org, Website. www.kemri.org

KEMRI/RES/7/3/1 **August 18, 2021**

TO: **NOEL ADHIAMBO ODUOR,**
PRINCIPAL INVESTIGATOR.

THROUGH: **THE DEPUTY DIRECTOR, CBRD,**
NAIROBI.

Dear Madam,

RE: **KEMRI/SERU/CBRD/224/4252** ***(RESUBMISSION OF INITIAL SUBMISSION):*** **GENETIC CHARACTERIZATION OF *BIOMPHALARIA PFEIFFERI* IN ASAO STREAM KISUMU COUNTY IN RELATION TO SEASONAL VARIATION, SELFING AND REFRACTORY PROPERTIES** ***(VERSION 1.4 DATED 02 AUGUST 2021)***

Reference is made to your undated letter. The KEMRI Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised study documents on August 07, 2021.

This is to inform you that the Committee notes that the issues raised during 313th Committee B meeting of the KEMRI Scientific Ethics Review Unit (SERU) held on **July 21, 2021** have been adequately addressed.

Consequently, the study is **granted approval** for implementation effective this day, **August 18, 2021** for a period of **one (1) year**. Please note that authorization to conduct this study will automatically expire on **August 17, 2022.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **July 06, 2022.**

Please note that only approved documents including (informed consents, study instruments, Material Transfer Agreement) will be used. You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received. Any unanticipated problems resulting from the implementation of this study should be brought to the attention of SERU and you should advise SERU when the study is completed or discontinued.

Prior to commencing your study, you will be expected to obtain a research license from National Commission for Science, Technology and Innovation (NACOSTI) https://oris.nacosti.go.ke and also obtain other clearances needed.

Yours faithfully,

PROF. CHARLES OBONYO,
THE ACTING HEAD,
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT.

In Search of Better Health

Appendix II: National Commission for Science, Technology and Innovation (NACOSTI) Study Approval

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE, TECHNOLOGY & INNOVATION

Ref No: 622444

Date of Issue: 13/September/2021

RESEARCH LICENSE

This is to Certify that Miss.. Noel Adhiambo Oduor of Kenya Medical Research Institute, has been licensed to conduct research in Kisumu on the topic: GENETIC CHARACTERIZATION OF BIOMPHALARIA PFEIFFERI FROM ASAO STREAM, KISUMU COUNTY IN RELATION TO SEASONAL VARIATION, SELFING AND REFRACTORY PROPERTIES for the period ending : 13/September/2022.

License No: **NACOSTI/P/21/12802**

622444

Applicant Identification Number

Director General
NATIONAL COMMISSION FOR SCIENCE, TECHNOLOGY & INNOVATION

Verification QR Code

NOTE: This is a computer generated License. To verify the authenticity of this document, Scan the QR Code using QR scanner application.

Appendix III: University Approval of Research Proposal

JOMO KENYATTA UNIVERSITY
OF
AGRICULTURE AND TECHNOLOGY

OFFICE OF THE DIRECTOR, BOARD OF POSTGRADUATE STUDIES

P.O. BOX 62000, 00200 • NAIROBI •KENYA•TEL: (067)-5870001-4• Email: director@bps.jkuat.ac.ke

REF: JKU/2/11/TM305-1540/2019 17TH FEBRUARY, 2022

ODUOR NOEL ADHIAMBO
C/o SOBMS
JKUAT

Dear, Ms. Adhiambo,

RE: APPROVAL OF RESEARCH PROPOSAL AND APPOINTMENT OF SUPERVISORS

Kindly note that your MSc. research proposal entitled: **"GENETIC CHAACTERIZATION OF *BIOMPHALARIA PFEIFFERI* FROM ASAO STREAM, KISUMU COUNTY IN RELATION TO SEASONAL VARIATION, SELFING AND REFRACTORY PROPERTIES"** has been approved. The following are your approved supervisors:-

1. Dr. Daniel Kariuki
2. Dr. Eric Lelo
3. Dr. Gerald Mkoji

Yours sincerely,

PROF. LOSENGE TUROOP
DIRECTOR, BOARD OF POSTGRADUATE STUDIES
Copy to: Dean, SOBMS
/cm

Appendix IV: Publication from this Study

Oduor *et al. Parasites & Vectors* (2025) 18:235
https://doi.org/10.1186/s13071-025-06881-1

Parasites & Vectors

RESEARCH Open Access

Evidence for persistent multilocus genotypes of *Biomphalaria pfeifferi* in a natural population in Kenya, with implications for transmission of *Schistosoma mansoni*

Noel A. Oduor[1,2*], Daniel W. Kariuki[1], Gerald M. Mkoji[2], Polycup O. Oraro[2], Martina R. Laidemitt[4], Michelle L. Steinauer[3], Eric S. Loker[4] and Eric L. Agola[2]

Abstract

Background *Biomphalaria pfeifferi*, a predominantly self-fertilizing freshwater snail, is the world's most important intermediate host for *Schistosoma mansoni*, one of the causative agents of schistosomiasis, a neglected tropical disease affecting millions of people in sub-Saharan Africa. We sought to determine whether we could identify distinct and persistent lineages of *B. pfeifferi* within a natural stream habitat in western Kenya, indicative of their asexual descent. We also sought to determine whether infections by *S. mansoni* or other trematodes were associated with particular lineages.

Methodology Utilizing 14 microsatellite markers in a multiplex polymerase chain reaction (PCR) format, we genotyped 502 *B. pfeifferi* collected in six bimonthly (every other month) sampling times from the same locality in a single habitat (Asao Stream, western Kenya). Snails were isolated and screened for infection with *S. mansoni* and other trematodes using the shedding method followed by microscopical examination of any cercariae found.

Results We identified 26 multilocus genotypes (MLGs), that were present at two or more sampling times. Four MLGs persisted across the entire 10-month sampling period, one of which was represented by 17 individuals. These persistent lineages harbored a variety of trematode species, with *S. mansoni* being the most common. The persistent MLGs were more likely to have trematode infections than those found only at a single sampling time. Low genetic differentiation was observed between November and March (fixation index among subpopulations [F_{ST}] = 0.019; $p = < 0.05$). The highest genetic differentiation was observed between July and March ($F_{ST} = 0.372$; $p = < 0.001$). Analysis of molecular variance (AMOVA) showed higher variation among individuals within sampling times (58%) than within individuals (33%), and a smaller variation (8%) was found among sampling times.

Conclusions By identifying the presence of persistent MLGs and their associations with trematode transmission, this study highlights the importance of considering *B. pfeifferi* MLGs, some of which could be resistant to infection, when developing strategies to control schistosomiasis transmission within Asao Stream and similar ecosystems across sub-Saharan Africa.

*Correspondence:
Noel A. Oduor
noeladhis@gmail.com
Full list of author information is available at the end of the article

Appendix V: Approval for Archived Samples

GERALD M. MKOJI

Centre for Biotechnology Research and Development, Kenya Medical Research Institute (KEMRI), PO Box 54840-00200 City Square, NAIROBI; Phone: (020) 2722541; Cell Phone: 0721-585 696; E-mail: gmkoji5@gmail.com; gmkoji@kemri.org

Head
SERU-KEMRI

August 6, 2021

Thro' DD, CBRD

RE: SERU-4252 Genetic Characterization of *Biomphalaria pfeifferi* from Asao Stream, Kisumu County in Relation to Seasonal Variation, Selfing and Refractory Properties, PI: Noel A. Oduor

This Memo is to confirm that the research proposal referenced above is nested within the main proposal referenced **SERU-3540** and titled **"Snail-related studies of transmission and control of schistosomiasis in Kenya", PIs: E.S Loker & G.M. Mkoji (CBRD)**, an on-going project funded through the US National Institutes of Health (NIH).

Ms. Noel A. Oduor, the PI of the above referenced project is a Master's degree student enrolled at the Jomo Kenyatta University of Agriculture and Technology (JKUAT) through the KEMRI Graduate School. To facilitate the research for her studies, she will have access to samples that are routinely being collected through the "mother project", including those that were collected during the period 2018-2019.

Please accord her the support she needs from SERU.

Yours sincerely,

Gerald M. Mkoji
KEMRI Project PI

Appendix VI: Informed Consent Agreement for Parents/Guardians

I, Mr./Mrs./Miss _____, being an adult aged 18 years and over, and being the parent/guardian of:
Master/Miss (Child's Name) _____ Aged_ _____, who attends:
_____School, do hereby give permission to Prof/Dr./Mr./Mrs./Miss _____for my child to take part in the new study known as **"Snail-Related Studies of Transmission and Control of Schistosomiasis in Kenya"** which has been explained to me in _____, a language I speak fluently and understand clearly, and now, I know what the study is all about, the tests to be done on my child, the benefits my child will receive for taking part in the study, the medications he/she will be given, if found to be sick with bilharzia or other intestinal illnesses caused by parasites, the side effects he/she could suffer from the medication, which I have been told, are mild, temporary, and should not cause any harm to my child. I was given an opportunity to ask questions and to seek clarification of the issues I had not understood clearly about the study, and I am satisfied with the answers and the explanations I was given. I have also, been told that if I have additional questions or concerns about the study later, I can contact the researcher in charge of the study (**Gerald Mkoji, Kenya Medical Research Institute P.O.Box;54840-00200,Phone:0721585696, or email: gmkoji@Kemri.org** and if I have questions or concerns about my child's rights as a participant in this study, **I can contact: contact person is: The Committee Chairperson, KEMRI Scientific and Ethics Review Unit, P. O. Box 54840-00200, Nairobi; Telephone numbers: 020-2722541, 0717719477; Email address: seru@kemri.go.ke**.

I consent for my child to take part in this study, and agree that he/she can give stool or urine samples (or both) for the tests needed in this study. I have been told that my child can leave the study any time he/she decides to do so, and I have been assured that he/she will not suffer any penalty or loss of benefits that he/she should get through this study. All these things have been explained to me and my

child in _____, a language we speak fluently, and understand clearly. I agree to allow the researchers to remove the eggs of the bilharzia parasites from the stool and/or urine samples my child will give, and they can take these eggs or the resulting adult worms, abroad for further investigations and research related to this current project. I have been informed that all identifiable information (e.g., your name, email) will be removed from the specimen/information collected from my child if enrolled in this project. After removal of all identifiers, the specimen/information collected from my child may be used for future research or shared with other researchers without my additional informed consent.

Regarding use of my child's specimen/information in future research or sharing it with other researchers for research unrelated to the present study:

……I agree for my child's specimen/information to be used for future research and shared with other researchers without my additional consent as long as identifiers have been removed

……I do not agree for my child's specimen/information to be used for future research or shared with other researchers with or without identifiers

_____

Signature (or Thumb Print) of Parent/Guardian

_____

Date

_____

Name of the Person Obtaining Consent and Signature

_____

Name and Signature (or Thumb Print) of Witness

Appendix VII: Assent for Children

You are being asked to take part in a study called "**Snail-Related Studies of Transmission and Control of Schistosomiasis in Kenya"** being carried out by researchers from the Kenya Medical Research Institute (KEMRI), the University of New Mexico, America and Western University of Health Sciences, America The purpose of the study is to enable us better understand the role snails play in transmitting the bilharzia parasites, and to see how we can develop ways to destroy the bilharzia parasites present in the snail host, as means to control bilharzia. Bilharzia is transmitted through water snails, and makes millions of people all over the world, especially children, sick.

If you agree to take part in this study, we will ask you to give stool and/or urine samples so that we can check to see if you have eggs of bilharzia worms in your body. If you are found to have bilharzia or other intestinal parasites, you will be given medication by the doctor to get rid of the bilharzia worms, or the other intestinal parasites present in your body, free of charge. You do not have to give a stool or urine sample for this study, if you don't want to, but there will be no harm if you gave a sample. By giving stool or urine or both, we can get to check if you have bilharzia or other intestinal parasites. Actually, giving stool and/or urine samples will not harm you in any way. Do you agree to take part in this study and give stool **and/or urine** samples? If you **agree to take part in this study and give stool and/or urine samples, please** put a tick (√) next to the answer **"YES"**, in the space given below, and sign your name in the space provided:

YES, _____I agree to take part in this study and provide stool and/or urine samples. I also, agree that the researchers can send the eggs of the bilharzia worms they find in the stool and/or urine sample I give to America for more analyses and studies related to this current project. I have been informed that all identifiable information (e.g., your name, email) will be removed from the specimen/information collected from me if enrolled in this project. After removal of all identifiers, the specimen/information collected from me may be used for future research or shared with other researchers without my additional informed assent.

Regarding use of my specimen/information in future research or sharing it with other researchers for research unrelated to the present study:

……I agree for my specimen/information to be used for future research and shared with other researchers without my additional assent as long as identifiers have been removed

……I do not agree for my specimen/information to be used for future research or shared with other researchers with or without identifiers

_____

_____

Name of the Child Signature or Thumb Print

_____

_____

Name of the Person Obtaining Consent and Signature (or Thumb Print)

_____

_____

Name and Signature (or Thumb Print) of the Witness

Appendix VIII: Plagiarism Report

Plagiarism Detector v. 2867 - Originality Report 08/08/2025 12:16:01

Analyzed document: NOEL ADHIAMBO ODUOR Thesis Revised.docx Licensed to: Jomo Kenyatta University of Agriculture and Technology

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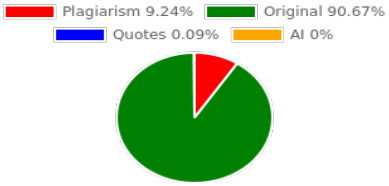
Check type: Internet Check

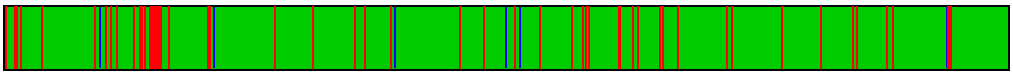
TEE and encoding: DocX n/a

Detailed document body analysis:

Relation chart:



Distribution graph:



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