Genetic diversity and evolution within the genus *Bulinus* and species-level interactions with the transmission of *Schistosoma haematobium* group parasites

by

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DEDICATION

I would like to dedicate this thesis to the memory of Simon Bush, a dear friend and life mentor, whose extraordinary can-do attitude in every aspect of life will forever be an inspiration.

PREFACE

During completion of this doctorate, I have produced the following first and co-authored publications, which are additional to the work presented in this thesis:

Allan, F., Ame, S.M., Tian-Bi, Y.T., Hofkin, B.V., Webster, B.L., Diakité, N.R., N'Goran, E.K., Kabole, F., Khamis, I.S., Gouvras, A.N., Emery, A.M., Pennance, T., Rabone, M., Kinung'hi, S., Hamidou, A.A., Mkoji, G.M., McLaughlin, J.P., Kuris, A.M., Loker, E.S., Knopp, S., Rollinson, D. (2020) Tackling the snail control dilemma in Africa: snail related contributions from the SCORE program including xenomonitoring, focal mollusciciding, biological control and modelling. *American Journal of Tropical Medicine*, 103(1), 66-79.

Archer, J., Barksby, R., Pennance, T., Rostron, P., Bakar, F., Knopp, S., Allan, F., Kabole, F., Ali, S. M., Ame, S. M., Rollinson, D., Webster, B. (submitted *Molecules*) Analytical and clinical assessment of a portable, isothermal recombinase polymerase amplification (RPA) assay for the molecular diagnosis of urogenital schistosomiasis.

Gouvras, A.N., Allan, F., Kinung'hi, S., Rabone, M., Emery, A., Angelo, T., Pennance, T., Webster, B., Nagai, H. and Rollinson, D. (2017) Longitudinal survey on the distribution of *Biomphalaria sudanica* and *B. choanomophal*a in Mwanza region, on the shores of Lake Victoria, Tanzania: implications for schistosomiasis transmission and control. *Parasites & Vectors*, 10:*316*.

Pennance, T., Ame, S., Amour, A., Suleiman, K., Allan, F., Rollinson, D., Webster, B. (2018) Occurrence of *Schistosoma bovis* on Pemba Island, Zanzibar: implications for urogenital schistosomiasis transmission monitoring. *Parasitology,* 145.13: 1727-1731.

Pennance, T., Allan, F., Emery, A., Rabone, M., Cable, J., Garba, A.D., Hamidou, A.A., Webster, J.P., Rollinson, D. and Webster, B.L. (2020) Interactions between *Schistosoma haematobium* group species and their *Bulinus* spp. intermediate hosts along the Niger River Valley. *Parasites & Vectors*, 13:268.

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SUMMARY

Schistosomiasis is a Neglected Tropical Disease (NTD) prevalent across much of Africa, and also regions in South America, Asia, the Middle East and parts of Southern Europe. Caused by infections with dioecious parasitic flatworms of the *Schistosoma* genus, schistosomiasis results in an undue degree of suffering for humans and animals, particularly in Sub-Sharan Africa. Transmission occurs in freshwater containing specific intermediate host snails that act as vectors for the parasites; hosts becoming infected when the larval stages, released (shed) from the snails, burrow through the skin of the mammalian hosts that come into contact with the freshwater. Therefore, in humans this is a preventable water borne disease disproportionately affecting those that do not have access to safe water sources and good sanitation infrastructures.

Mass drug administration (MDA) for the control of human schistosomiasis, which can occur in both intestinal and urogenital forms, is the recommended strategy by the World Health Organization (WHO), however, various factors contribute to the maintenance of schistosomiasis transmission in endemic areas of Africa. Potentially the biggest cause for persistence is the reservoir of infection that remains in freshwater intermediate host snails. Establishing the geographical distribution of intermediate host species in endemic regions and determining their associations with different schistosome species is paramount during schistosomiasis control programmes. Following years of neglect, a resurgence of interest in snail focussed surveillance and control is currently underway, with the WHO even recognising it as a critical action point in their 2020 road map for schistosomiasis control and elimination goals by 2030. However, rigorously tested and refined tools necessary for incorporating snails into schistosomiasis control programmes and the fundamental knowledge about the diversity of the snails and schistosomes underpinning such developments, are not available.

Focusing on two endemic regions for urogenital schistosomiasis: the Niger River Valley, and the elimination setting of the Zanzibar Archipelago, this study provides new insights into the genetic diversity of *Bulinus* spp. and importantly, the associations they have with multiple *S. haematobium* group species in these regions. Furthermore, the first large-scale approach for snail surveillance protocols are tested, showing the advantages that this approach will provide in certifying future elimination and in monitoring disease endemicity. Additionally, novel mitogenome data produced for *Bulinus* spp. from across Sub-Saharan Africa and the Indian Ocean islands are analysed, which sheds light on the evolutionary radiation of this important genus and the cryptic species diversity that has traditionally confounded taxonomy. The findings can be applied to implement new policies on snail surveillance in schistosomiasis control, whilst also providing a valuable resource for future research investigating the *Bulinus* genus.

Chapter 1. Snails and schistosomes in Sub-Saharan Africa: rationale and aims for investigating *Schistosoma haematobium* group species and their interactions with *Bulinus* spp. intermediate hosts

This thesis focuses on understanding the relationships between snails of the *Bulinus* genus and their associated schistosomes of the *Schistosoma haematobium* group species, the etiological agents for urogenital schistosomiasis and the majority of animal schistosomiasis across Sub-Saharan Africa. The rationale for the current study focussing on *S. haematobium* stems from the fact that major advances have been made in understanding the transmission dynamics of human intestinal schistosomiasis caused by *S. mansoni*, but very little has been investigated with the *S. haematobium* system (Rollinson, 2009). The availability of genome and mitochondrial genome sequencing of *S. mansoni* and the snail intermediate host, *Biomphalaria* spp., respectively (DeJong *et al.*, 2004; Berriman *et al.*, 2009; Crellen *et al.*, 2016; Zhang *et al.*, 2018) and the identification of resistant markers through transcriptomics in the intermediate host (Ittiprasert and Knight, 2012; Kenny *et al.*, 2016; Allan *et al.*, 2018, 2019) have created multiple avenues of research for the *S. mansoni* system. In contrast, although the genome of *S. haematobium* has been sequenced (Young *et al.*, 2012; Oey *et al.*, 2019), sequence data for *Bulinus* spp. beyond a few partial mitochondrial and nuclear gene regions are not available.

In this thesis, two typical urogenital schistosomiasis endemic regions are investigated, the highly endemic Niger River Valley and the elimination setting of the Zanzibar Archipelago. *Bulinus* spp. snails, and any associated *Schistosoma* spp. parasites found infecting them, from these two regions were collected during multiple malacological surveys spanning several years, allowing for multiple avenues of investigation to be explored using these specimens. In addition, *Bulinus* spp. snails available from other studies across Sub-Saharan Africa and Madagascar accessioned in the Natural History Museum (London) collections were used to explore the diversity and phylogenetic relationships between these taxa.

The overarching aims and objectives for this thesis were: a) to further our understanding of the transmission biology and diversity of the *S. haematobium* group parasites (including their hybrid species) and the *Bulinus* spp. transmitting them, b) develop and test xenomonitoring assays for identifying non-patent *S. haematobium* group species infections in *Bulinus* spp. in the elimination setting of Zanzibar to enable targeted interventions, and ultimately provide a tool for certification of schistosomiasis elimination., c) to use an NGS approach to make phylogenetic inferences of *Bulinus* species diversity, identify cryptic taxa and inspect the evolutionary radiation of *Bulinus*. These objectives were aimed at providing data relevant for policy decisions on schistosomiasis surveillance and control. This could not come at a better time, since in the ‘road map’ to the 2030 schistosomiasis elimination goals,

the WHO has renewed its interest in targeting the intermediate host snails for control and surveillance of schistosomiasis (WHO, 2020a).

These aims are explored across five self-contained data chapters (Chapters 3 to 7), which are preceded by a more detailed introduction to schistosomiasis and the *Bulinus* genus in Sub-Saharan Africa (Chapter 2) before concluding with a final discussion chapter (Chapter 8). The chapters and their specific aims for each, including any further information and acknowledgements, are as follows:

1.1 Outline and objectives

Chapter 2. The neglected intermediate hosts of a neglected tropical disease: snails and schistosomiasis in Sub-Saharan Africa

A detailed introduction to schistosomiasis and its intermediate hosts is provided followed by a review of the available literature (up to July 2020) relating to the species diagnostics, taxonomy and phylogenetic analysis of *Bulinus* spp. Following publication of the relevant data chapters (see below), this chapter combined with components of Chapter 8, will be adapted and submitted for publication as a review article.

Chapter 3. Interactions between *Schistosoma haematobium* group species and their *Bulinus* spp. intermediate hosts along the Niger River Valley

This study set out to establish the *Bulinus* spp. involved in the transmission of *S. haematobium*, *S. bovis* and *S. haematobium* group hybrids in the Niger River Valley region. This study used novel techniques to infer the number of schistosome infections present in each snail, identifying same-species and multiple-species infections to aid in the surveillance of schistosomiasis and establish schistosome interaction/compatibilities and biology.

*This data chapter is a modified version of a manuscript published in the peer-reviewed journal Parasites & Vectors (*Pennance et al., *2020), presented here to fit thesis guidelines. I would therefore like to thank the co-authors of this manuscript for their useful comments. I would also like to acknowledge that the funding for the snail and parasite collections and data acquisition were provided by the Schistosomiasis Consortium for Operational Research and Evaluation (SCORE). The collections of snails and parasites from the field were obtained by Omar Barkire, Salem Boukary, Amadou Djibril, Omar Garba, Ali Maiga, Bachir Madougou, Mohamed Ousmane, Samira Souley, Tankari and Dan Kountche, (Réseau International Schistosomoses, Environnement, Aménagement et Lutte). The sample repositories and*

curation were handled by Ms Muriel Rabone, Dr Aidan Emery and Dr Fiona Allan of the Wellcome Trust Schistosomiasis Collection at the Natural History Museum (SCAN Project, grant ID 104958/Z/14/Z). All data analysis and interpretation were conducted by the author during this doctorate.

Chapter 4. Transmission and diversity of *Schistosoma haematobium* and *S. bovis* and their freshwater intermediate hosts (*Bulinus globosus* and *B. nasutus*) in Zanzibar

This study aimed to update and describe the transmission biology of schistosomiasis across the Zanzibar Archipelago in light of continuing efforts to eliminate urogenital schistosomiasis across the islands. Through performing malacological and parasitological surveys it was hoped to elucidate the role of a potential second species of intermediate host (*B. nasutus*) in the transmission of *S. haematobium*, and also identify the occurrence of *S. bovis* across the island. The occurrence of *S. bovis* on Pemba has been reported separately, and is published (Pennance *et al*., 2018). All sample and data analysis/interpretation were conducted by the author during this doctorate.

Samples were available for this study through conducting fieldwork on Unguja and Pemba Island supported by a Wellcome Trust Seed Award (Grant ID: 207728, awarded to Dr Bonnie Webster, Natural History Museum) between 2017 and 2019. Additionally, support was received from the London Centre for Neglected Tropical Disease Research (LCNTDR) travel award to conduct feasibility fieldwork in 2016, which partly led to the development of this study and project award, and through field trips supported by the current doctorate budget provided through the Natural Environment Research Council (NERC) GW4+ DTP. Additional collections were also available through the SCAN Project (grant ID 104958/Z/14/Z) sourced from the SCORE Elimination of schistosomiasis project (ZEST, Unguja and Pemba Island, sub-award no. RR374-053/4893196). I would also like to acknowledge the assistance of Mr Mtumweni Ali Muhsin for helping to coordinate the malacological surveys we conducted in the south of Unguja Island.

Chapter 5. Rapid diagnostic assay distinguishing the urogenital schistosomiasis intermediate snail hosts *Bulinus globosus* and *B. nasutus* on Zanzibar

As a follow up to Chapter 4, it was deemed necessary to develop a rapid diagnostic assay to distinguish the two intermediate snail hosts of the Zanzibar Archipelago, *B. globosus* and *B. nasutus*, using a single step PCR. This was to facilitate cheap, rapid and robust differentiation

of these morphologically cryptic species whose allopatric distribution on Zanzibar dictate the degree of schistosomiasis across these Indian Ocean islands.

Chapter 6. Snail xenomonitoring on Pemba Island: a surveillance approach for schistosomiasis control and elimination programmes

This chapter aimed to validate a recently developed xenomonitoring marker (Pennance, Archer, *et al.*, 2020). This chapter was produced as part of this doctorate for the detection of non-patent *S. haematobium* and *S. bovis* infections in *Bulinus* spp. in the elimination setting of Zanzibar, whilst also modelling environmental predictors dictating *Bulinus* spp. abundance and infection prevalence.

The study was only possible thanks to Dr Steffi Knopp and Prof. David Rollinson who kindly shared the urogenital schistosomiasis prevalence data collected during the ZEST study (SCORE sub-award no. RR374-053/4893196) so that study sties could be selected. I would like to acknowledge that the field work, data collection and molecular work for this chapter was funded by a Wellcome Trust Seed Award (Grant ID: 207728, awarded to Dr Bonnie Webster, Natural History Museum) between 2017 and 2019. Additionally, I was initially supported by the London Centre for Neglected Tropical Disease Research (LCNTDR) travel award to conduct feasibility fieldwork in 2016, which led to the development of the final study and award. I am also grateful for the assistance in field work and data collection from Dr Bonnie Webster (NHM) and researchers of the Public Health Laboratory (Chake Chake, Pemba Island): Dr Shaali Ame, Amour Khamis Amour and Khamis Rashid Suleiman. I would also like to acknowledge the help of NHM research assistants employed on the Wellcome Trust Seed Award (Grant ID: 207728); Mr John Archer, Dr Penny Rostron, Dr Elena Luigi and Mr Felix Llanwarne, for their role in performing snail DNA extractions and PCRs. I would also like to acknowledge the support of Ms Galina Jönsson for their guidance in selecting appropriate models for the modelling of Bulinus *spp. abundance and schistosome infections.*

Chapter 7. Mitogenome genetic diversity of medical and veterinary important *Bulinus* spp. snails in Sub-Saharan Africa and the Indian Ocean Islands

This data chapter aimed to investigate the genetic diversity of 71 *Bulinus* spp. collected from across Sub-Saharan Africa and the Indian Ocean Islands using mitochondrial genome and ribosomal operon sequence data, with particular emphases on identifying cryptic species within the *B. africanus* group and investigating divergence of taxa from island populations.

This study would have not been possible without the award from the NHM Departmental Investment Fund for conducting part of the molecular work or the donation and access to specimens in the SCAN Project (grant ID 104958/Z/14/Z). Samples were sourced from a variety of collaborators and projects associated with the NHM, including; SCORE Elimination of schistosomiasis (Unguja and Pemba Island, sub-award no. RR374-053/4893196), SCORE Gaining and Sustaining (Niger, sub-award no. RR374-053/5054146 and RR374-053/4785426), SCORE snail xenomonitoring (Tanzania, sub-award no. RR37-053/4787466, SUB00000691), Wellcome Trust Seed Award for snail xenomonitoring (Pemba Island, Grant ID: 207728), SCHISTO_PERSIST ERC University of Glasgow (Uganda, Grant ID: 680088), WISER-EPSRC (Ethiopia, EP/P028519/1) and collections from other contributors to the collections; Prof. Russell Stothard (Madagascar, Malawi, Mafia Island, Liberia), Mohammad Alharbi (Malawi), Khadija Said (Tanzania), David Rollinson, José C. Sousa-Figueirdo and Fiona Allan (Angola, Gulbenkian funded project), James Rudge (Unguja Island), Chelsea Wood and Sanna Sokolow (Senegal, Upstream Alliance). Contributors played no role in either the design of the study or interpreting the findings. I would also like to acknowledge the support from Dr Andrew Briscoe (NHM) for providing a reference mitogenome from another collaborative project, along with Dr Andrea Waeschenbach (NHM) for providing much appreciated advice on conducting the data analysis.

Chapter 8. Future outlooks and research in *Bulinus* spp. snails and schistosomiasis in Sub-Saharan Africa

This final discussion chapter highlights some areas of interest concerning research into *Bulinus* spp. and schistosomiasis in sub-Saharan Africa that are not covered in the introductory or data chapters of this thesis.

I am grateful to Mr Zikmund Bartoníček and Mr John Archer for very useful discussion on the environmental detection of snails and schistosomes and diagnostics for human infection, respectively.

1.2 Author contributions

I collected, analysed and wrote up all data within this thesis under the supervision and guidance of Dr Bonnie Webster and Prof. Jo Cable, with additional contributions from those who will be co-authors on resulting manuscripts from these chapters. For instance; thanks to Prof. David Rollinson and Dr Fiona Allan for their expertise on *Bulinus* species and *S. haematobium* transmission (Chapters 2, 4 and 7), Dr Steffi Knopp for their expertise on urogenital schistosomiasis elimination on Zanzibar (Chapter 4), Dr Andrew Briscoe and Dr

Andrea Waeschenbach for their expertise in the phylogenetic analysis of invertebrate mitochondrial genomes (Chapter 7) and Ms Galina Jönsson for their expertise in ecological modelling (Chapter 6), all of whom will be co-authors of papers resulting from these studies.

1.3 Ethical statement

Local approval was gained for all malacological studies in endemic regions. No direct collection of data from humans was acquired for this thesis, and studies involving parasitological data from humans were embedded in activities of the SCORE operational research projects. The SCORE studies in Zanzibar received ethical approval from the Zanzibar Medical Research Ethics Committee in Stone Town, Zanzibar (ZAMREC, reference no. ZAMREC 0003/Sept/011), the "Ethikkommission beider Basel" (EKBB) in Basel, Switzerland (reference no. 236/11) and the Institutional Review Board of the University of Georgia in Athens, United States of America (project no. 2012- 10138-0). For SCORE studies in Niger, ethical approval was obtained from the Niger Republic National Consulate for ethical review (reference no. 012/2010/CCNE) and from the Imperial College Research Ethic Committee (ICREC_8_2_2) and the Institutional Review Board of the University of Georgia in Athens, United States of America (project no. 2012- 10431-0). All participants of the SCORE study, and in the case of children their parents, provided written informed consent. Snails were removed and killed as part of this study, but as important vectors of disease, ethically once collected they should never be returned to natural waterbodies. For the published manuscript (Appendix 1), *S. haematobium* experimental infections were conducted at the Biomedical Research Institute – Schistosomiasis Resource Centre (Rockville, MA, USA) animal facility maintained with AAALAC full accreditation (Site # 000779) and operates under the National Institutes of Health's Office of Laboratory Animal Welfare (OLAW) # A3080-01. *S. haematobium* parasite material is collected from male LVG Syrian golden hamsters following percutaneous exposure to cercariae. Hamster use was approved by the Institutional Animal Care and Use Committee (IACUC) of the Biomedical Research Institute for the Animal Use Protocol, #18-01.

Chapter 2. The neglected intermediate hosts of a Neglected Tropical Disease: snails and schistosomiasis in sub-Saharan Africa

Abstract

Parasitic digeneans, capable of infecting a wide range of vertebrate hosts, utilise molluscs as a first, and sometimes second, intermediate host. The diseases caused by infection with these metazoan endoparasites cause a significant economic, medical and veterinary health burden on society. Schistosomes, currently represented by 23 recognised species within the *Schistosoma* genus, are considered the most important snail transmitted pathogens due to the resulting urogenital and intestinal schistosomiasis impact on humans and intestinal schistosomiasis in animals. The main burden of disease is observed in sub-Saharan Africa, where *S. mansoni* (transmitted by *Biomphalaria* spp. snails) and *S. haematobium* (transmitted by *Bulinus* spp. snails) are the etiological agents for intestinal and urogenital schistosomiasis, respectively.

This review of schistosomiasis considers the current status of research into *S. haematobium* group parasites and their *Bulinus* spp. intermediate hosts, highlighting that the *S. mansoni* and *Biomphalaria* system continues to be much more extensively studied. Although genome information for some *S. haematobium* group species is now available, relatively few studies have investigated their associated *Bulinus* spp. intermediate hosts using the next generation of molecular tools now available. An updated taxonomy of *Bulinus* spp. and identification of those species involved in the transmission of *S. haematobium* group parasites will significantly improve future disease surveillance. This is urgent given the current WHO guidelines to incorporate snail focussed interventions for schistosomiasis control and monitoring.

Molecular phylogenies of *Bulinus* spp. snails have demonstrated significant genetic diversity, highlighting cryptic diversity within the currently recognised ~37 species. Questions still remain on the basal topology of this genus, with species subcategorised into four well defined species groups, but the relationship between three groups remains elusive. The evolutionary history of these species has also not been adequately studied, but is of interest both in terms of understanding the radiation of these freshwater gastropods across Africa, including the African Indian Ocean Islands, the Mediterranean basin and parts of the Middle East, and because of the associations observed between the *S. haematobium* group parasites, and their co-evolving hybrids.

2.1 Digenean trematodes and the role of snails as disease vectors

The subclass Digenea (Class Trematoda) consists of approximately 148 families within the Phylum Platyhelminthes (Caira and Littlewood, 2013). The lifecycle of almost all digeneans,

otherwise known as parasitic flatworms or flukes of which there are >18,000 described species, involves endoparasitism in various organ systems of vertebrate definitive hosts. They are considered the largest and most 'successful' group of internal metazoan parasites (Cribb *et al.*, 2001; Littlewood *et al.*, 2015). The ubiquitous nature of the Digenea means they can be found parasitising almost all major vertebrate groups across a broad geographical reach, with gastropods and other mollusc groups serving as the first, and in many instances the adopted second, intermediate hosts (Cribb *et al.*, 2003).

The life-cycles of digenean trematodes are diverse and quite remarkable, which in contrast to other Platyhelminthes, such as the Cestoda that are transmitted entirely via trophic transmission, have at least one life cycle stage that actively finds and penetrates its next host. This almost always involves a gastropod intermediate host, but can involve other mollusc groups (Caira and Littlewood, 2013). Due to the great variety of intermediate and definitive hosts these parasites must navigate between and within, a remarkable diversity of anatomical features are observed in both adult worms (e.g. acetabulum, adhesive papillae, gynecophoral canal, oral sucker) and the multiple larval forms (miracidia, sporocyst, rediae, cercaria) (Caira and Littlewood, 2013). These host specific associations and morphologies have enabled investigations into the evolution of this group and the hosts they infect, with the uptake of targeted gene sequencing and phylogenetic analyses bolstering inferences of the evolutionary histories, including host switching and life history traits, taxonomic status and relationships between groups/taxa within digeneans (Cribb *et al.*, 2003; Olson *et al.*, 2003; Blasco-Costa *et al.*, 2010; Fraija-Fernández *et al.*, 2015). With the increasing availability of genome and transcriptome data of flatworms, and other parasitic worms, such as the nematodes (roundworms), the evolution of digeneans, and parasitism in general, is continually being explored (Briscoe *et al.*, 2016; Coghlan *et al.*, 2019). However, the specific life-cycles of some trematodes, primarily related with intermediate snail host use have still not been fully elucidated (for example, see Cumberlidge *et al.,* 2018). This shows the necessity for continued field collecting and accessioning of samples into natural history collections (Harmon *et al.*, 2019).

Diseases caused by infection with snail transmitted digeneans impose a significant economic, medical and veterinary health burden on society (Lockyer *et al.*, 2004). Roughly 300 million people (Adema *et al.*, 2012) and an unknown number of domestic livestock (De Bont and Vercruysse, 1997; Keiser and Utzinger, 2009) are currently suffering from snail-transmitted infections globally. Of these, schistosomiasis and food-borne trematodiasis, receive the most attention in terms of research and control efforts due to their widespread distribution and associated morbidity (Table 2.1), including the significant disability adjusted life years (or years lived with disability) imposed on humans (Keiser and Utzinger, 2009; Fürst *et al.*, 2012; GBD-2016, 2017). Avoiding infection in daily life can be almost impossible, and

appropriate treatments are not readily available for all those in need (WHO, 2017b). Complications also arise due to the wide definitive host range of the parasites causing these snail-transmitted diseases, necessitating a One Health approach to be employed for pooling research efforts and disease control (Robertson *et al.*, 2014; Jenkins *et al.*, 2015; Webster *et al.*, 2016).

Table 2.1. Snail transmitted digeneans of significant medical and veterinary importance and their geographical distribution

	Infectious Agent	Geographical Distribution
Schistosomiasis	**African urogenital schistosomiasis**	
	Schistosoma haematobium	Africa, Madagascar, the Middle East, Corsica (France)
	African human intestinal schistosomiasis	
	Schistosoma mansoni	Africa, Middle East, Caribbean, South America (partial)
	Schistosoma intercalatum *Schistosoma guineensis*	Rain forest areas of central Africa
	African veterinary schistosomiasis	
	S. mattheei	Southern Europe, southern Asia, and Africa
	S. bovis	North, East and West Africa, Mediterranean Europe and the Middle East
	S. curassoni	West Africa
	Asian human and veterinary intestinal schistosomiasis	
	S. japonicum	China, Indonesia and the Philippines
	Schistosoma mekongi	Cambodia and the Lao People's Democratic Republic
Food-borne trematodiases	**Opisthorchiidae** *Clonorchis sinensis* *Opisthorchis viverinni* *Opisthorchis felineus*	Asia
	Troglotrematidae *Paragonimus* spp	Africa, Asia and Latin America
	Fasciolidae *Fasciola gigantica* *Fasciola hepatica* *Fasciolopsis buski*	Global
	Heterophyidae *Haplorchis* spp. *Heterophyes* spp. *Metagonimus* spp.	Global (but patchy distribution)
	Echinostomatidae *Echinostoma* spp.	Global

This thesis expands upon the current biological knowledge of *Schistosoma haematobium* group parasites (Class Trematoda, Family Schistosomatidae) and particularly their associated intermediate snail hosts of the genus *Bulinus* (Class Gastropoda, Family Bulinidae). Several species of this *S. haematobium* group, of which there are currently nine described, are responsible for human urogenital schistosomiasis and bovine intestinal schistosomiasis in Sub-Saharan Africa, parts of the Middle East and Southern Europe (Table

2.1). Other species within this group also infect artiodactyls and rodents (Webster *et al.*, 2006; Hanelt *et al.*, 2009; Standley, Dobson, *et al.*, 2012). However, there are currently 23 recognised schistosome species in total (Webster *et al.*, 2006), each capable of infecting humans and/or animals causing a whole spectrum of diseases referred to, maybe incorrectly, under the same 'umbrella' term schistosomiasis (Gryseels *et al.*, 2006; Standley, Dobson, *et al.*, 2012; Colley *et al.*, 2014). This includes other species endemic to Africa and South America, but also Asia where the zoonotic schistosome *S. japonicum* infects a wide range of mammalian hosts (Table 2.1), as well as other closely related species such as *S. mekongi*, *S. malayensis* and *S. sinensium* (see Webster *et al.,* 2006). These Asian schistosomes are remarkably different to those in Africa and South America in terms of their lifecycle and intermediate host use, and therefore for the purpose of this overview, the focus will only be on those species endemic to sub-Saharan Africa (i.e. *S. mansoni* and *S. haematobium* groups).

This introductory chapter is formed of two parts. Part 1 provides a broad overview of African schistosomiasis, from the epidemiology to current strategies for controlling the transmission of schistosomes. Part 2 is a review of the historic and current knowledge surrounding the *Bulinus* intermediate snail hosts transmitting *S. haematobium* group parasites, with a focus on how the taxonomy of this genus has changed over the years and led to an improved understanding of the diversity and snail-schistosome associations.

PART 1

2.2 Schistosomiasis in sub-Saharan Africa

2.2.1 Epidemiology

With >700 million people at risk and causing the annual loss of ~3.3 million disability adjusted life years (WHO, 2017b), human schistosomiasis ranks only second to malaria among human parasitic diseases (Steinmann *et al.*, 2006; Hotez *et al.*, 2008). Most recent estimates suggest that between ~189-229 million people require treatment for schistosomiasis, and over 90% of these live in sub-Saharan Africa (GBD-2016, 2017; WHO, 2020a). Schistosomiasis prevalence and infection intensities are typically highest in young adolescents due to behavioural risk factors, i.e. contact with freshwater where infection occurs (King *et al.*, 1988; Rudge *et al.*, 2008). High prevalence though can persist in subpopulations of adults that have frequent water contact during their daily activities (Karanja *et al.*, 2002; Matthys *et al.*, 2007). Of those infected, roughly 60% are symptomatic and 10% have severe disease (Chitsulo *et al.*, 2000). Through recent advances in schistosomiasis diagnostics and methods for assessing disease morbidity, it is apparent that schistosomiasis infection can have multiple other negative effects on human health and well-being, such as those associated with sexual health, infertility and developmental stunting (Leutscher *et al.*, 2000; Kjetland *et al.*, 2014; Kayuni, Corstjens, *et al.*, 2019; Mduluza-Jokonya *et al.*, 2020; Stothard, Odiere, *et al.*, 2020).

As well as the *S. haematobium* group present in sub-Saharan Africa, the *S. mansoni* group causing African human intestinal schistosomiasis (Table 2.1) and several types of wildlife schistosomiasis (Steinauer *et al.*, 2008; Catalano *et al.*, 2018, 2020) are present across much of sub-Saharan Africa (as well as the Caribbean and South America). The *S. mansoni* group are all transmitted by snails of the genus *Biomphalaria* (Class Gastropoda, Family Planorbidae). Co-endemicity and sympatric transmission of these *Schistosoma* spp. can therefore occur in regions where both *Bulinus* and *Biomphalaria* spp. cohabit, however the associated disease morbidity, diagnostics and prevention differ due to the different biology of these schistosomes (Colley *et al.*, 2014). A third group of *Schistosoma* are restricted to regions of Asia, the *S. japonicum* group, and although not discussed further here due to their geographical isolation from *S. haematobium* and *S. mansoni* species, they are also a cause of Asian human and veterinary intestinal schistosomiasis (Table 2.1). The most widespread species of Asian schistosome, *S. japonicum*, is a truly zoonotic parasite, infecting humans, cattle, dogs, pigs and rodents (Gryseels *et al.*, 2006).

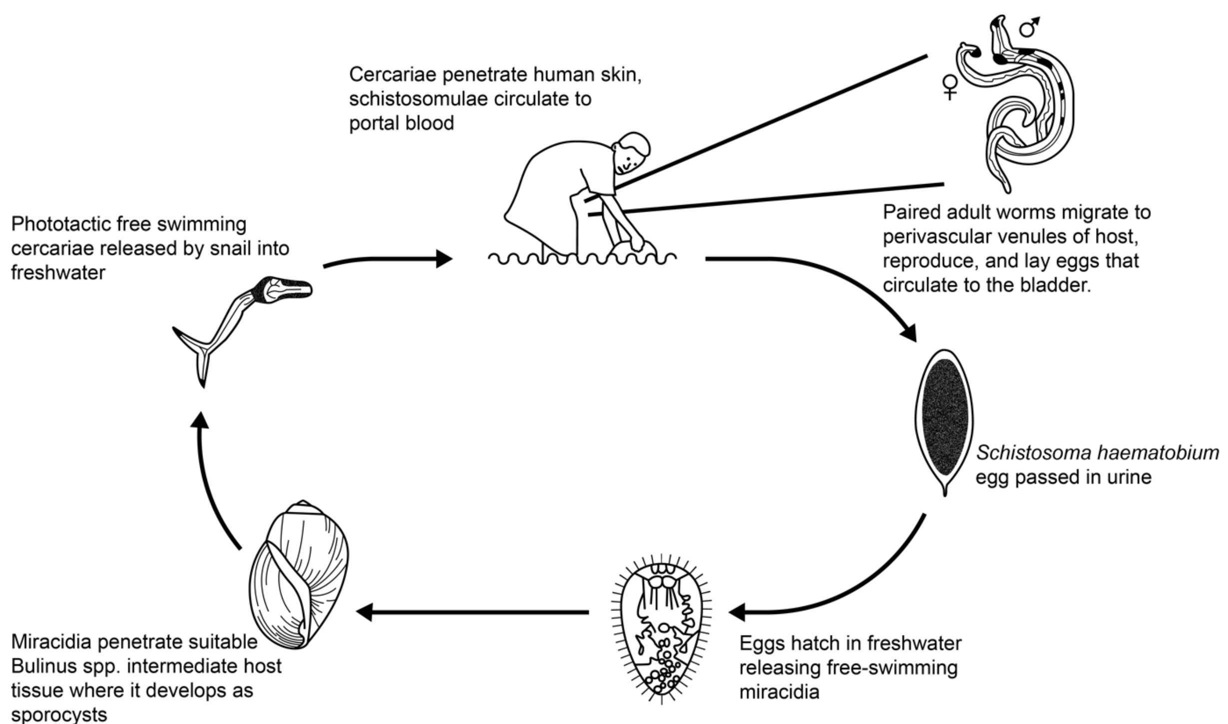


Figure 2.1. The lifecycle of *Schistosoma haematobium*, the causative agent of urogenital schistosomiasis in humans, endemic across much sub-Saharan Africa.

2.2.2 Schistosome lifecycle

Schistosomiasis has a highly focal distribution since transmission can only occur in freshwater in the presence of specific intermediate hosts where contamination with infected urine / faeces occurs. The lifecycle of schistosomes (Figure 2.1) involves the paired male and female adult worms living within the perivascular (urogenital *S. haematobium*) or mesenteric (intestinal mainly *S. mansoni* and *S. japonicum*) venules of the host producing hundreds to thousands

of fertilised eggs capable of being released back into the environment via urine or faeces (Colley *et al.*, 2014). The eggs are the only stage of the parasite's lifecycle responsible for host pathology (see below), as they become trapped and retained in host tissues inducing inflammatory responses leading to tissue becoming fibrotic, instigating severe morbidity and mortality if left untreated. The eggs that do reach freshwater hatch to release a ciliated larva, miracidium, adapted to seeking and infecting a suitable snail host.

Miracidia penetrate through the snail's head-foot tissue, and if a compatible snail species is infected the larva can develop into a mother sporocyst (Pan, 1963). The mechanisms that underpin this compatibility is determined by the response of snail haemocytes and humoral factors to the schistosome (Pila *et al.* 2016a, b), however little is understood regarding to what extent other predispositions including age, concurrent infections and environmental stressors have on these coordinated responses, although this is an advancing and topical field in schistosomiasis research (Tennessen, Bonner, *et al.*, 2015; Pila *et al.*, 2017; Allan *et al.*, 2018). If successfully avoiding any snail immune response, the mother sporocyst asexually produces daughter sporocysts that migrate to other parts of the snail's body, primarily the digestive gland, where they produce thousands of cercariae through asexual reproduction. The 'prepatent or latent period' between initial penetration of the snail and production of cercariae is species specific and dependant on environmental conditions, such as temperature (Foster, 1964; El-Hassan, 1974), but is between 4-6 weeks.

Cercariae infect their vertebrate host when they are in contact with water, by penetrating the skin and entering the blood system. The presence of freshwater is therefore essential for transmission, so transmission can be seasonal if endemic areas experience extreme dry and rainy seasons. Maturation time from schistosomula entering the blood to the adults attached and mating inside the venules is species specific and ranges from 5-7 weeks. During this time, schistosomes migrate through the heart, lungs and finally the liver where maturation and mate finding occurs (Nation *et al.*, 2020). Schistosomes are unusual compared to the majority of digenean trematodes, in that they are not hermaphroditic. Dioecious adult worms are sexually dimorphic and form pairs to mate (Figure 2.1), with the smaller female enveloped inside the male's gynaecophoric canal (Skelly *et al.*, 2014). Once schistosome pairing has occurred, worms migrate against the blood flow to reach their preferred egg laying sites, which differs between species (Nation *et al.*, 2020). Adult worms can live for 20-30 years (Jordan and Webbe, 1982), although the average life is likely to be somewhere between 5-10 years (Fulford *et al.*, 1995). Adult schistosomes that pair and mate are mostly monogamous (Steinauer, 2009), however it seems clear that they likely compete with each other for mates and also change partners, particularly when mixed infections are involved (Tchuenté *et al.*, 1995; Webster *et al.*, 1999).

2.2.3 Schistosomiasis disease morbidity and mortality in sub-Saharan Africa

The eggs of schistosomes, rather than the adult worms, induce pathogenesis and morbidity in the definitive host. Many eggs (estimated 20 to 50%) produced by the adult female worms are not excreted from the definitive host (Moore and Sandground, 1956; Fan and Kang, 2003), and during migration through the body become permanently trapped in any host tissue they may reach, but mainly those surrounding the bladder and urogenital system (urogenital schistosomiasis) or the intestines or liver (intestinal schistosomiasis) (Costain *et al.*, 2018). Host immune responses trap these eggs by inducing granuloma formation surrounding the egg, which although preventing tissue necrosis leads to chronic inflammation of the tissue surrounding the egg and the typical manifestations of schistosomiasis (Peterson and von Lichtenberg, 1965; Colley *et al.*, 2014). The annual mortality rate of non-functioning kidney relating to *S. haematobium* infection was estimated to be around 150,000, whilst haematemesis due to *S. mansoni* is around 130,000 (van der Werf *et al.*, 2003), although these estimates need updating since treatment has become increasingly available.

The burden of African schistosomiasis on human health is drastically underestimated, with disease surveillance often only being performed during large national control programmes, typically with a focus on school aged children, or in under-resourced national healthcare systems that do not have the capacity to assess infection severity (King *et al.*, 2005). Both these factors mean that several 'hidden' morbidities / pathologies and negative health consequences associated with *Schistosoma* spp. infection go unnoticed, or at least are difficult to associate when estimating the disability caused by these parasites. Sexual and reproductive health is particularly pertinent in the case of urogenital schistosomiasis, which due to egg deposition by the adult *S. haematobium* worms within the human body can manifest as female genital schistosomiasis (FGS) and male genital schistosomiasis (MGS). Host inflammation caused by the eggs deposited in the tissues of the genital tracts produces intravaginal lesions that result in genital itching and pain, bleeding and dyspareunia (Downs *et al.*, 2011; Kjetland *et al.*, 2014; Woodall and Kramer, 2018). Women suffering from female genital schistosomiasis are at an almost three-fold higher risk of HIV (Kjetland *et al.*, 2006), and can accelerate the disease progression of HIV (Mbabazi *et al.*, 2011). Eggs deposited in the fallopian tubes and uterus can result in infertility (Downs *et al.*, 2011; Woodall and Kramer, 2018). This can all lead to social stigma, depression and marital discord whilst at the same time being misdiagnosed as sexually transmitted infections and not receive the required treatment (Hotez *et al.*, 2019). Similarly, MGS can manifest in several ways and can lead to pelvic pain, erection and ejaculation discomfort / dysfunction, infertility and plausibly increase the risk of HIV transmission (Kayuni, Lampiao, *et al.*, 2019).

2.3 Schistosomiasis control and elimination

Morbidity control of schistosomiasis using Mass Drug Administration (MDA) with Praziquantel - the only registered chemotherapeutic drug for treating all forms of schistosomiasis - has been deemed achievable by implementing national scale treatment programmes of a reasonable (~75%) coverage (Fenwick *et al.*, 2009; Colley *et al.*, 2020). This has been supported by a Praziquantel donation commitment from Merck KGaA (Darmstadt, Germany); since 2007 the company has donated over 1 billion tablets and are now committed to providing 250 million tablets annually (figures taken July 2020: https://www.merckgroup.com). With ground gained on reducing schistosomiasis prevalence through MDA, a move from control to elimination of schistosomiasis in selected regions of Africa was deemed possible, particularly considering the success of elimination campaigns in parts of Asia (King, 2009; Rollinson *et al.*, 2013; Stothard *et al.*, 2017). Elimination of schistosomiasis as a public health problem (defined as the "reduction of *Schistosoma* prevalence to <1% heavy infections based upon direct egg-detection methods in school aged population in a given population", see Rollinson *et al.,* 2013) has been proposed an achievable goal when multidisciplinary interventions are focused and goal driven (Utzinger *et al.*, 2003; Knopp *et al.*, 2012; Rollinson *et al.*, 2013; Tchuenté *et al.*, 2017). These interventions target several potential break points in the life cycle of schistosomes.

2.3.1 Behavioural interventions

Educating at risk populations about the biology and control of schistosomiasis provides knowledge on how to avoid high schistosomiasis transmission risk activities (Knopp *et al.*, 2011; Rollinson *et al.*, 2013; Celone *et al.*, 2016; Person *et al.*, 2016). Water, Sanitation and Hygiene (WASH - https://www.unicef.org/wash/) is an encompassing initiative representing the infrastructure and education required to alleviate infectious disease transmission. There are obvious applications to schistosomiasis control, mainly that increasing access to safe water should prevent human contact with freshwater (potentially containing infected snails) and also sanitation improvements will deter the contamination of freshwater with sewage (i.e. open defecation) so that snails do not become infected (Secor, 2014). Evidence strongly supports this, with for example improved piped water supply to communities being shown to decrease the intensity of reinfection of *S. haematobium* significantly (Mogeni *et al.*, 2020). Programmes aiming to further address the current gaps in knowledge concerning water treatment and improved water supply on schistosomiasis transmission are currently taking place (see https://www.wiserschisto.com).

2.3.2 Treatment

Since 2002 large scale Preventive Chemotherapy (PC) with Praziquantel has been implemented (Fenwick *et al.*, 2009; Webster *et al.*, 2014). Morbidity control has been generally successful in many sub-Saharan African countries, and this has led, in part, to a revision of the WHO's strategic plan and to a vision for "a world free of schistosomiasis" (WHO, 2012, 2013). The WHO still leads its schistosomiasis control message with providing MDA, and have set the goal of elimination as a public health problem by 2030 (WHO, 2020a), which has already seen a delay of 5 years from previous aims of reaching this target by 2025 (WHO, 2017b). A persisting issue in the delivery of effective MDA coverage, which is primarily delivered during school based deworming programs, is the treatment gap observed in pre-school (3 months to 6 years) aged children (Bustinduy *et al.*, 2016; Faust *et al.*, 2020). Despite the evidence that treatment with Praziquantel in this age group is safe (Coulibaly *et al.*, 2018) and reverses morbidity (Osakunor *et al.*, 2018), access to the drug for this age group is low and morbidity is regularly observed (Stothard *et al.*, 2013; Mduluza and Mutapi, 2017; Mduluza-Jokonya *et al.*, 2020). By raising awareness of this issue, campaigns called for the development of a paediatric formulation for Praziquantel to be developed, with the first doses being delivered in endemic countries in 2019 (Phase III trials) and depending on the success of these trials will hopefully be available to all endemic countries in 2022 (see https://www.pediatricpraziquantelconsortium.org).

2.3.3 Snail control

Molluscicides have largely been replaced due to the success of population-based chemotherapy. In addition, efficient application of molluscicide requires substantial logistical planning, community sensitization, human effort and material resources (Gryseels *et al.*, 2006; King and Bertsch, 2015; Allan *et al.*, 2020). The use of molluscicides for control and eventually elimination of schistosomiasis is a divisive topic, with some labelling it as a tool 'far in the past' (Fenwick and Savioli, 2011), and others regarding it as an important component of control strategies (King *et al.*, 2006; Gray *et al.*, 2010; King and Bertsch, 2015). A recent cross-sectional cluster-randomised trial in Zanzibar, however, showed that snail control did not significantly boost the impact of MDA (Knopp, Person, *et al.*, 2019). Other than mollusciciding, biological snail control through the reintroduction of predator species (freshwater prawns) in Senegal demonstrated that these could also be effective methods (Sokolow *et al.*, 2015, 2017). In further support of snail control, an evaluation of large-scale schistosomiasis control attempts compared those that did and did not involve snail control and concluded that its addition to control programmes had been the most effective way of reducing schistosomiasis prevalence (Sokolow *et al.*, 2016). It is evident that further research is needed to better understand the pros and cons of incorporating snail control into schistosomiasis intervention

strategies (Allan *et al.*, 2020). New WHO guidelines are available for the field application of molluscicides (WHO, 2017a) and recommendations for implementing targeted snail control are outlined for reaching the WHO 2030 goals (WHO, 2020a). Therefore one should expect to see a resurgence in the snail focussed control, potentially including gene drive technologies for schistosomiasis transmission control (Pila *et al.*, 2017; Maier *et al.*, 2019), over the next 10 years.

2.3.4 Case study: A history of urogenital schistosomiasis control on Zanzibar and the Zanzibar Elimination of Schistosomiasis Transmission (ZEST)

Zanzibar, an archipelago of two islands, Unguja to the south and Pemba to the north, is situated off the east coast of Tanzania in the Indian Ocean. Pemba and Unguja islands have a long history of urogenital schistosomiasis research and control, which dates back almost a century. The control of Zanzibar fell into the arms of the British Empire in 1890, and from 1920 annual colonial reports were produced for the Zanzibar Protectorate, with 'Public Health' becoming an agenda item from the reporting year of 1927 (Crofton, 1928). This first inclusion of Public Health was likely spurred on due to the presence of "the Economic Biologist" then present in Zanzibar, and although not named was likely Dr. W. Mansfield-Aders, a zoologist with the Zanzibar Government Service, who also produced a report for the Biological Division of the British Government during that year (Mansfield-Aders, 1928). Both of these governmental reports and another, eight years later (Govt.Office, 1936), paint a familiar picture of helminthiasis on Zanzibar, with Crofton (1928) stating that "practically all natives suffer from ankylostomiasis [*hookworm*] and bilharzia [*schistosomiasis*], and ascaris [*roundworm*] occur with some frequency in certain districts" and that "bilharziasis is found among a very large proportion of school children especially in Pemba". Both of these statements acknowledged the spatial heterogeneity of schistosomiasis infection still observed in Zanzibar today, further confirmed in parasitological surveys reporting a prevalence of 11.9-36.2% during these early observations (Mansfield-Aders, 1928; McCarthy, 1930). The incidence of infection in *Bulinus* spp. collected and identified for infection from dissection, was reported as 1.8% (McCarthy, 1930).

The first evidence of public health campaigns directed towards helminthiasis in Zanzibar is reported in 1931; "propaganda measures take the form of pamphlets, popular lectures, and the erection of model latrines in principal villages and at the district dispensaries" (Govt.Office, 1932). Long before the use of Praziquantel to treat schistosomiasis, lucanthone hydrochlorine (Nilodin) was trialled in 1954 to treat school children suffering with *S. haematobium* infection in Unguja, the results reported as "encouraging but by no means uniformly successful" (Govt.Office, 1955). Other drugs, including niridazole and metrifonate, were also trialled for their therapeutic effect of *S. haematobium* infection in Zanzibar (Forsyth,

1966; Macdonald *et al.*, 1968; Mgeni *et al.*, 1990), each study contributing to the search for therapeutic drugs to treat schistosomiasis. Early examples of environmental modification for snail control and comprehensive mapping for investigating spatial heterogeneity of snail species related with disease prevalence were also conducted in Zanzibar (Mozley, 1939; Goatly and Jordan, 1965). Since then, the snail fauna responsible for schistosomiasis transmission (i.e. *Bulinus* spp.) have been extensively investigated in relation to distribution patterns, species diagnostic markers, host-parasite interactions and xenomonitoring (Stothard *et al.*, 1997; Stothard, Loxton, *et al.*, 2000; Allan *et al.*, 2013). Ground truthing for low-cost diagnostics, such with microhaematuria reagent strips coupled with treatment using Praziquantel were also demonstrated on Zanzibar; for the first time employing such tests on a large scale (Savioli and Mott, 1989). More recently, validation of diagnostic tests has relied on Zanzibar as an ideal, self-contained, test location (Knopp *et al.*, 2015; Rostron *et al.*, 2019). Identification and characterisation of persistent hotspots of schistosomiasis have also been conducted on the islands (Savioli *et al.*, 1989; Pennance *et al.*, 2016), creating a wave of interest (see below) in how best to tackle persistent hotspots during schistosomiasis elimination programs (Sang *et al.*, 2014; Kittur *et al.*, 2019, 2020; Assaré *et al.*, 2020; Walker *et al.*, 2020).

Following on from this long history and current developments in research and control of urogenital schistosomiasis, Pemba and Unguja are currently targeted for elimination. Most recently with the Zanzibar Elimination of Schistosomiasis Transmission (ZEST) project trialling integrated interventions with biannual MDA (Knopp *et al.*, 2012, 2013; Knopp, Ame, *et al.*, 2019; Knopp, Person, *et al.*, 2019). Between 2011-2017, the cluster randomised trial conducted in selected shehias (smallest administrative region on Zanzibar) tested the combined impact of two intervention arms: biannual MDA + snail control (administering the molluscicide niclosamide to *B. globosus* infested freshwater) and biannual MDA + behavioural interventions (improving WASH facilities and providing education to communities and schools on schistosomiasis) on the prevalence of schistosomiasis compared to a third intervention arm of biannual MDA alone (Knopp *et al.*, 2012, 2013; Knopp, Ame, *et al.*, 2019; Knopp, Person, *et al.*, 2019). Following the ZEST trial, prevalence was significantly reduced across the islands in the selected shehias, but transmission was not completely interrupted (Knopp, Ame, *et al.*, 2019). Considerable spatial heterogeneity of *S. haematobium* infection was observed across the shehias in Zanzibar irrespective of treatment coverage (percentage of population receiving Praziquantel) and intervention arm (Knopp, Ame, *et al.*, 2019), with the prevalence of schistosomiasis decreasing in some shehias and schools, but remaining high in others. Combined strategies may be necessary in persistent hotspots of schistosomiasis (see below) and are being planned for the next stages of elimination trials (Knopp personal communication).

As is the concern for any infectious disease elimination program, the focus of ZEST is now turning towards the complete interruption of transmission (i.e. no new case or resurgence in transmission) and how to certify accurately that elimination has been reached. Sensitive diagnostic tools (see below) and targeted administration of preventive chemotherapy are needed to eliminate schistosomiasis (Amoah *et al.*, 2020). However, since this situation of schistosomiasis elimination in a sub-Saharan African setting is so unusual, best implementation methods (i.e. Which diagnostics to use in a test and treat scenario? Should snail xenomonitoring be coupled with elimination surveillance?) have still not been fully established.

2.4 Persistent hotspots of schistosomiasis

Persistent hotspots of schistosomiasis transmission have been identified across multiple settings during schistosomiasis control and elimination campaigns (Kittur *et al.*, 2020). Despite frequent interventions and adequacy of reported MDA coverage, these areas maintain a persistently high or even increasing prevalence of schistosomiasis, signifying high levels of ongoing transmission and reinfection (Pennance *et al.*, 2016). Based on analyses of multiple longitudinal studies, definitions for persistent hotspots (i.e. the percentage changes in prevalence or infection intensity in endemic regions) need to be assessed based on the different capacities and goals of the local control programmes (i.e. control versus elimination) and the setting (rural, peri-urban or urban settings) (Kittur *et al.*, 2017, 2019, 2020; Walker *et al.*, 2020). Another important outcome of these analyses was that it is possible to predict the presence of persistent hotspots in the third year of MDA, with the data for mean infection intensity (measured in *S. mansoni* eggs per gram of faeces in this instance), prevalence of heavy infections (≥400 eggs per gram) and the overall prevalence of infection being the best predictors (Kittur *et al.*, 2020).

Multiple factors can significantly increase the risk of infection with schistosomes, such as: close proximity to freshwater bodies containing intermediate host snail species (Clennon *et al.*, 2006; Steinmann *et al.*, 2006; Rudge *et al.*, 2008; Pennance *et al.*, 2016; Mutuku *et al.*, 2019), frequent and intense contact of humans with natural freshwater (Rudge *et al.*, 2008; Sousa-Figueiredo *et al.*, 2015; Assaré *et al.*, 2020), lack of drinking water resources and bad quality latrines in schools (Sousa-Figueiredo *et al.*, 2015). The construction of agricultural water schemes has been shown to decrease the risk of infection with schistosomes though (Chitsulo *et al.*, 2000). Infected individuals that remain potentially untreated and treated individuals that are re-infected and harbour heavy infections may also contribute to the perpetuation or resurgence of transmission in these persistent hotspots (King, 2009; Rollinson *et al.*, 2013). It is necessary to adopt a multidisciplinary approach targeting the multiple intervention points that will be appropriate for curtailing transmission in these areas (Knopp,

Ame, *et al.*, 2019). However, it needs to be determined how these can be implemented in a cost-effective manner to optimize the interventions in hotspots whilst dialling back the efforts in areas responding well to MDA alone (Knopp, Ame, *et al.*, 2019; Kittur *et al.*, 2020).

2.5 Diagnostics for schistosomiasis infection and transmission

The WHO states that measures for schistosomiasis mapping, starting treatment, stopping treatment and post-treatment surveillance "*exist, but either require major modification or [are] considered inadequate to reach 2030 targets*" (WHO, 2020a). The standard diagnosis for active schistosomiasis across all these surveillance stages is by observing viable eggs in the urine or faeces, however due to the low sensitivity of these methods, infection cannot be ruled out if a patient is deemed egg negative. This is particularly true in areas of low endemicity where the number of eggs excreted may be very low (e.g. <5 eggs / 10 ml urine) (Knopp *et al.*, 2018). The sample preparation and handling of potentially hazardous excreta, and also the specialist equipment and technician training required to perform these diagnostics also present issues for their implementation. Molecular techniques targeting the DNA of schistosomes in samples, particularly collected through non-invasive methods (Archer *et al.*, 2019), offer a more sensitive diagnostic platform and are more convenient / low risk in their use (Sousa-Figueiredo *et al.*, 2013; Adriko *et al.*, 2014; Knopp *et al.*, 2015). These are also being developed with use at the point of care being kept in mind, allowing for results to be interpreted easily and negating the use of large equipment.

2.6 Animal and zoonotic schistosomiasis and hybridization

Although the burden of human schistosomiasis, caused by infection with ~6 species is well reported, significantly less is known about the *Schistosoma* spp. infecting wildlife and domestic animals comprising the other ~17 species of *Schistosoma* (see Webster *et al.* 2006). As aforementioned, the one exception is *S. japonicum* in Asia, since this represents a typical zoonotic parasite, that can switch between a wide range of mammalian hosts, with zoonotic reservoirs that range from water buffalo to rodents (Rudge *et al.*, 2013).

Nevertheless, schistosomes are known to infect domestic livestock throughout Africa, the Middle East, Asia, and some countries bordering the Mediterranean Sea. At least 165 million cattle are infected worldwide and it is recognised that this is a gross underestimation (De Bont and Vercruysse, 1997). The schistosomes infecting livestock and other ungulates in Africa and Europe, which are all represented by species within the *S. haematobium* group, are often overlooked (Standley, Dobson, *et al.*, 2012; Standley, Mugisha, *et al.*, 2012; Calavas and Martin, 2014; Gower *et al.*, 2017; Pennance *et al.*, 2018), yet it is known that infection incurs economic costs from the loss of meat and milk products from infected livestock (De Bont and Vercruysse, 1998; Legesse *et al.*, 2014) and may be cause for concern in

conservation (Standley, Dobson, *et al.*, 2012). The *Schistosoma* species involved in the majority of these infections include: *S. bovis*, *S. mattheei* and *S. curassoni*, however several other species, namely *S. margrebowiei*, *S. leiperi* and the recently described *S. kisumuensis*, also infect animals (Webster *et al.*, 2006; Hanelt *et al.*, 2009; Standley, Dobson, *et al.*, 2012; Beechler *et al.*, 2017). A single species from the less diverse *S. mansoni* group, *S. rodhaini* (Brumpt, 1931), is transmitted by rodents.

Increasing interest of the schistosomes infecting the animals in Africa has been brought about by the identification of *S. haematobium* group hybrids, first reported based on atypical *Schistosoma* egg morphologies collected from humans suggesting *S. haematobium*-*S. mattheei* hybrids in Zimbabwe (Alves, 1948). Several other earlier reports of unusual egg morphologies, particularly in South and West Sub-Saharan Africa, gave further evidence of potential inter-species hybridisation between closely related *Schistosoma* species (Le Roux, 1954; Pitchford, 1959; Wright, 1974). However, in many cases viability of these eggs was rarely assessed with many of the observations being dismissed as misleading identifications or misdiagnoses (as reviewed in Léger and Webster, 2017). With the use of biochemical markers, such as isoelectric-focussing of enzymes, experimental hybridization between *S. haematobium* and *S. mattheei* was shown to be successful (Wright and Ross, 1980). This also led to the confirmation of hybridisation between certain *S. haematobium* group species in nature (Brémond *et al.*, 1990, 1993; Rollinson, Southgate, *et al.*, 1990).

The use of DNA markers has led to further investigations into the extent of *S. haematobium* group hybrids infecting humans and intermediate hosts (Huyse *et al.*, 2009, 2013; Webster, Diaw, *et al.*, 2013; Léger *et al.*, 2016; Pennance, Allan, *et al.*, 2020). Multi-locus approaches are employed by utilising *cox*1 and the nuclear rDNA regions of the internal transcribed spacer (ITS) and 18S ribosomal RNA (both containing species specific SNPs) so both maternal and paternal inheritance can be observed (see Pennance, Allan, *et al*., 2020). These species specific SNPs allow for the detection of introgression between parental copies of nuclear DNA, before complete homogenization of DNA occurs through concerted evolution (Webster, Diaw, *et al.*, 2013). With the regular detection of hybridisation between human and animal *Schistosoma* species, growing concern that the zoonotic transmission of these parasites may occur and impact control (Borlase *et al.*, 2017; Léger and Webster, 2017). These hybrids are routinely identified from humans and snails in West Africa (Tian-Bi *et al.*, 2019; Pennance, Allan, *et al.*, 2020; Webster *et al.*, 2020), and also in focal areas of East Africa (Webster *et al.*, 2019). However, there is still much debate about the role of *S. haematobium* group hybrids in zoonotic transmission, with currently only a single report of *S. haematobium* group hybrids infecting cattle (Savassi *et al.*, 2020). However, the zoonotic transmission of the human infecting schistosomes *S. mansoni* and *S. haematobium* has been demonstrated through wild rodents (Catalano *et al.*, 2018, 2020), apes and monkeys (Červená

et al., 2016) and ungulates (Phiri *et al.*, 2011), although for *S. haematobium*, animal infections are much rarer likely due to their higher specificity for the human urinary system. With further research, it may be relevant to revise the transmission model for *S. haematobium* group species to account for overlapping transmission potentials in the definitive and intermediate hosts (Stothard, Kayuni, *et al.*, 2020).

A difficulty in accurately assessing the prevalence, epidemiology and distribution of schistosomes infecting animals, particularly those from large ungulates, has been the lack of a sensitive diagnostic method that captures these parasites for genetic analysis. This is due to the unfortunate matter that these large animals produce equally large amounts of faeces, and therefore detecting parasite eggs using classical methods designed for human faecal samples (e.g. filtration and concentration of eggs using Pitchford funnels and Kato-Katz slides) is not sensitive (Giovanoli Evack *et al.*, 2020; Savassi *et al.*, 2020). Most of our understanding of schistosomes infecting cattle therefore derive from post-mortem autopsies (Léger and Webster, 2017).

PART TWO

2.7 Intermediate snail hosts of urogenital schistosomiasis in sub-Saharan Africa

The 'umbrella' term for the disease schistosomiasis in sub-Saharan Africa, not only varies in terms of the individual species causing different disease morbidities in various definitive hosts, but also in the intermediate freshwater snail hosts that transmit them. Species of the *S. haematobium* group are transmitted through freshwater snails of the *Bulinus* genus (Family Bulinidae) whereas species of the *S. mansoni* group utilise intermediate hosts of the *Biomphalaria* genus (Family Planorbidae). The distribution of these snails is paramount in understanding the distribution and transmission of urogenital and intestinal schistosomiasis, as well as animal schistosomiasis. This is particularly true since snail range and habitats do not always overlap with for example *Bulinus* being more tolerant of higher temperatures and temporary habitats (Brown, 1994; Rollinson, 2009). Although in some habitats, conditions are suitable for cohabitation of these gastropod hosts, and therefore co-endemicity of both *S. haematobium* and *S. mansoni* group parasites can occur (Garba *et al.*, 2010).

In Africa, there are ~37 species of *Bulinus*, whilst there are ~12 species of *Biomphalaria* (with many additional species of *Biomphalaria* are present in South America). Both the *Bulinus* and *Biomphalaria* are arranged into species groups, classically based on distinguishing morphological characteristics, (shell, copulatory organs, radula) and habitat preferences. These groups serve practical purposes, facilitating the identification of taxa to the species group level, although further specification to species level without the use of molecular / biochemical assays is renowned for its difficulty. For *Bulinus* spp. at least, the four species groups; *B. africanus* group, *B. truncatus/tropicus* complex, *B. forskalii* group and *B.*

reticulatus group (Brown, 1981) have been supported by the use of molecular markers (Kane *et al.*, 2008), and in doing so this has uncovered the cryptic diversity of this genus. These freshwater snails are diverse, colonising anything from small seasonal water bodies to larger lakes and irrigation systems across Africa, Asia, Arabia and Mediterranean regions (Figure 2.2).

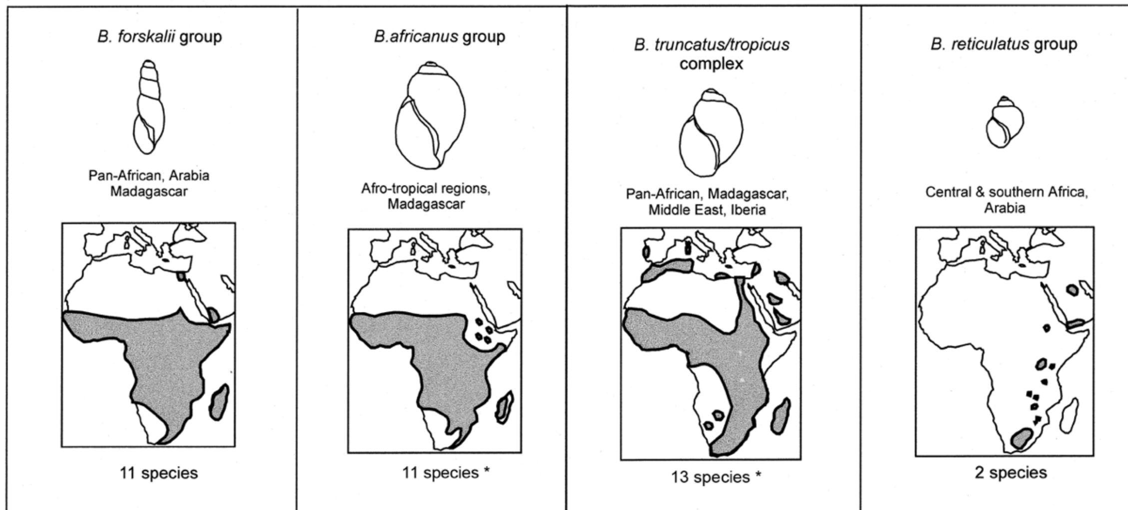


Figure 2.2. Overview of distribution of *Bulinus* in Africa, the Middle East and the Mediterranean Basin area based upon the taxonomy and maps of Brown (1994) (adapted from Rollinson *et al.*, 2001 incorporating change in species group of *B. angolensis* as reported F. Allan *et al.*, 2017*).

Delimitation of *Bulinus* to species level is vital, since the compatibility or rejection of schistosomes in snails is dependent on the finely balanced interactions between parasites maximising transmission through snail hosts whilst minimising the impact of the parasite on the host (Lockyer *et al.*, 2004). This has inevitably led to host-parasite co-evolution and co-speciation making the relationships between taxa very specific. Considerable variation in intermediate host specificity of parasite species can be observed over small geographical areas, for example, the transmission of *S. haematobium* and *S. bovis* on Zanzibar is primarily associated with freshwater bodies harbouring *Bulinus globosus*, and not those harbouring the closely related *B. nasutus* (Stothard, Mgeni, *et al.*, 2002a; Pennance *et al.*, 2018). In Niger, the transmission of *S. haematobium* and *S. bovis* occurs through *B. truncatus* and *B. forskalii*, although *S. bovis* can also be transmitted by *B. globosus* here (Labbo *et al.*, 2007; Pennance, Allan, *et al.*, 2020). The discrimination between bulinid species is therefore necessary to determine the schistosomiasis transmission risks in these focal regions. Molecular assays and *cox*1 barcoding are implemented in some malacological surveys to identify species (Stothard and Rollinson, 1996; Stothard *et al.*, 2001; Kane *et al.*, 2008) and in doing so have refined our understanding of the molecular evolution of these species (Zein-Eddine *et al.*, 2014; F. Allan *et al.*, 2017).

Rollinson (2009) highlighted that a disproportionate number of studies have focussed on *S. mansoni* over *S. haematobium* (almost a 10-fold difference). This disparity was due to

the widespread adoption of a *S. mansoni* and *Biomphalaria glabrata* model system to study schistosomiasis in the laboratory (Rollinson, 2009). Likewise in the last 20 years, all major advances in African snail-schistosome research have focussed on the *S. mansoni* and *Biomphalaria* spp. system, to mention just a few: publication of the *S. mansoni* genome (Berriman *et al.*, 2009) with insights into its evolutionary history (Crellen *et al.*, 2016), availability of the *B. glabrata* genome (Adema *et al.*, 2017) and transcriptome (Kenny *et al.*, 2016), along with associated *S. mansoni* infections (Lockyer *et al.*, 2008; Kenny *et al.*, 2016; Buddenborg *et al.*, 2019) all of which support the investigation of *Biomphalaria* spp. resistance and immunity studies (Knight *et al.*, 2014; Tennessen, Bonner, *et al.*, 2015; Tennessen, Théron, *et al.*, 2015; E. R. O. Allan *et al.*, 2017; Pila *et al.*, 2017; Allan *et al.*, 2018) and finally comparative mitogenomics (although very few taxa) investigating the genomic diversity of *Biomphalaria* spp. (Zhang *et al.*, 2018). Although whole-genome sequences of *S. haematobium* (see Young *et al.,* 2012) and *S. bovis* (see Oey *et al.,* 2019) are now available, nothing significant has been published on the *Bulinus* spp. intermediate hosts advancing from the phylogenetic studies investigating partial regions of mitochondrial and nuclear DNA (Kane *et al.*, 2008; Zein-Eddine *et al.*, 2014). Mitogenome/genome data and further evidence on host-parasite associations are needed for these bulinids to support the development of research areas, particularly *S. haematobium* control and unravelling the genetic diversity of this medically important group of snails.

Within each of the four species groups of *Bulinus*, species act as intermediate hosts for one or more species of *S. haematobium* group schistosomes, and many of the hosts for veterinary species are still largely unknown (Moné *et al.*, 1999; Hanelt *et al.*, 2009). Infection success of *Bulinus* by schistosomes is dependent upon geographical origin, with sympatric combinations of snails and parasites producing more compatible combinations than allopatric populations (McCullough, 1959; Wright and Knowles, 1972; Manning *et al.*, 1995). Additionally, hybrid schistosomes inherit multi-host compatibility and increased infectivity over parental species (Wright, 1974; Webster and Southgate, 2003). With continuing research investigating these host-parasite associations, we are aware that both snail susceptibility and trematode infectivity influence compatibility. However, there is still much to discover about the mechanisms controlling host specificity (Rollinson *et al.,* 2001) such as: What are the true compatibility ranges and restrictions of *S. haematobium* group species and *Bulinus* species? What are the environmental preferences of *Bulinus* species? Which methods can reliably detect schistosome presence in *Bulinus*? How does parasite and snail genetic diversity vary within and between habitats, regions and countries?

Difficulty in answering these questions is often rooted in the fact that species identification of *Bulinus* is difficult, and classically requires (an almost lost artform in the world of medical malacology) dissections of the soft body tissues to examine radula and copulatory

organs (Figure 2.3). As reviewed briefly here, the taxonomic history and literature of *Bulinus* is complicated by synonym taxa, splitting of taxons, and changes in subgenera / species groups of *Bulinus*. The taxonomy also suffers through the use of biological species concepts for *Bulinus*, which have evolved over the years as new parts of Africa were explored and species discovered. However, as the ability to explore diversity through the advances in molecular techniques has improved in recent years, there is an urgent need to re-explore the species of this medically important group of snails in light of genetic and phylogenetic species concepts, not only to understand their evolution and dispersal, but also to support on-going efforts to control and eliminate schistosomiasis in sub-Saharan Africa.

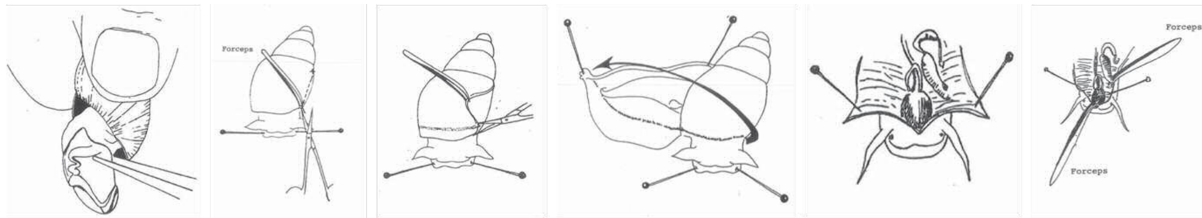


Figure 2.3. Sketches demonstrating the dissection and preparation of freshwater snails to expose the radula and copulatory organs for further preparations, adapted from methodology reported in the Danish Bilharzia Laboratory: WHO Collaborating Centre for Applied Malacology – 'A field guide to African freshwater snails: Introduction' (1988).

2.8 The *Bulinus* genus from the taxonomist's perspective

Michel Anderson (1757) gave the name '*Le Bulin*' to small freshwater snails collected in Senegal that had a small bubble of air and floated on the water surface (*bull* (French) = a bubble). *Le Bulin*, then type species of *Bulinus senegalensis*, was later named by Müller in 1781. Serious attempts to classify large collections of *Bulinus* into species and sub-species began around 60 years ago (Mandahl-Barth, 1957), at a time when research in identifying the African snails of medical importance flourished. Unfortunately, from this point onwards there were consistency issues in the morphological and ecological characters used to assign species names, resulting in revisions of *Bulinus* taxonomy in quick succession (Mandahl-Barth, 1960, 1965). The result is a rather confusing taxonomy, caused by the blurred lines between species boundaries from the snails micro-geographical [evolutionary] races occurring in African freshwater bodies (Mandahl-Barth, 1965) and the desire by early malacologists to bestow new names on unusual forms (Wright, 1961). A general issue with species level taxonomy in *Bulinus* (as discussed further below), as for many other gastropods (Prévot *et al.*, 2013), is the difficulty in determining a species-concept for facultatively selfing taxa, even though many species concepts exist (Mayden, 1997; Richards, 2014). Differentiating *Bulinus* of evolutionary taxonomic units from fixed polymorphisms due to sustained selfing and isolation presents a complex issue.

Probably the most comprehensive and systematic guide to the genus *Bulinus* is the *magnum opus* of Dr David Brown (1935-2004), in his book 'Freshwater Snails of Africa and

their Medical Importance', now in its second edition (Brown, 1994). This guide revised Mandahl-Barth's (1965) original *Bulinus* taxonomy, in which 27 species were described within four groups, split into two subgenera based on characters of the shell and anatomy of the soft body. Brown (1994) increased the number of species to 37 through refuting synonymy and naming those previously unrecognised (Brown, 1981), concluding, in contrary to some (Biocca *et al.*, 1979), that the *Bulinus* genus is split into four groups, each group since having support for a monophyletic origin through phylogenetic analysis (Figure 2.4) (Kane *et al.*, 2008; Jørgensen *et al.*, 2011; Zein-Eddine *et al.*, 2014). Although Brown's systematic guide to *Bulinus* is by far the most complete and comprehensive, it is not without its inevitable flaws due to the vague species descriptions that had to rely on interpreting mixed morphological characters (often of the shell and components of the copulatory organ), egg protein and enzyme analysis, since this was all that was available (for example, see page 221 of Brown (1994) for discussion on differentiating *B. africanus* and *B. globosus*).

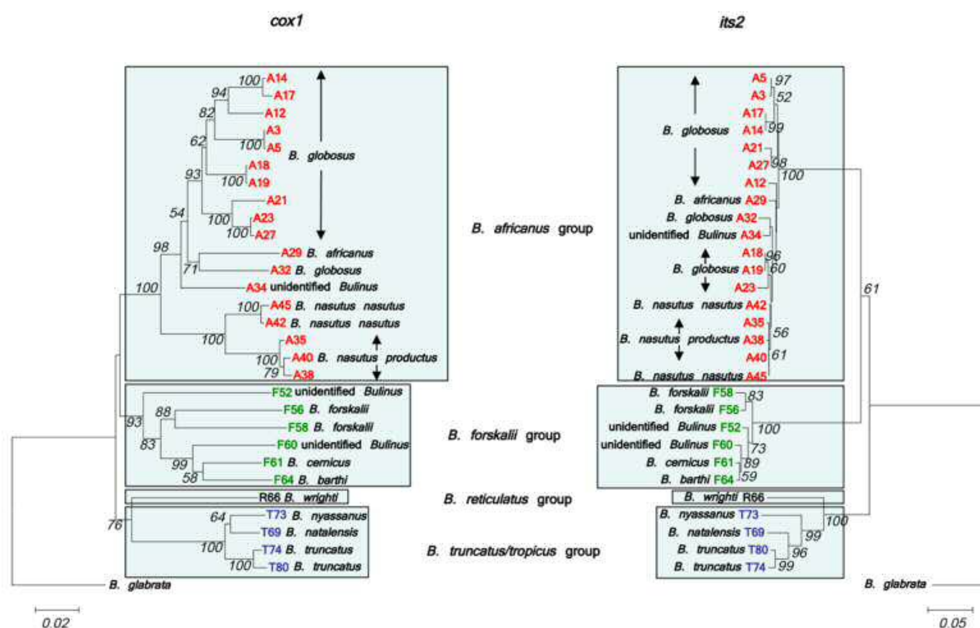


Figure 2.4. *Bulinus* phylogenies: Neighbour-Joining trees based on *cox*1 and ITS2 from Kane *et al.*, (2008) showing support for the four *Bulinus* species groups (although weak support for sister groups between *B. africanus* and *B. forskalii* group).

The ~37 *Bulinus* species in the four groups: *B. africanus*, *B. forskalii, B. reticulatus* and *B. truncatus/tropicus* complex, are still recognised today (Table 2.2), and specimens can be placed in one of these groups with relative ease through basic morphological observations (see below). This is important as identification to the species group level can help infer *S. haematobium* transmission; for example, it is apparent that *B. africanus* group species, and not those of the *B. truncatus/tropicus* complex, transmit *S. haematobium* in East Africa whilst in West Africa both species seem to play a role in transmission, as well as members of the *B.*

forskalii group (Rollinson and Southgate, 1987; Brown, 1994). Differentiation of species within each group is difficult, even for a trained malacologist. Additionally, a lack of sampling in certain regions indicates that the genus *Bulinus* may have a wider range than currently anticipated. It is therefore likely that the taxonomically difficult assemblage of *Bulinus* may change further until a satisfactory classification is reached.

Table 2.2. The ~37 recognised species of the *Bulinus* genus in four species groups, with additional species not currently recognised. Species names shown in bold are those with evidence of natural transmission (i.e. not including experimental infections) of *Schistosoma haematobium* group species, and those additionally underlined for the medically important *S. haematobium*. 1 = *B. angolensis* previously of *B. truncatus*/*tropicus* complex (see F. Allan *et al*., 2017). 2 = *B. productus*, previously identified as a form of *B. nasutus* (*B. nasutus productus*), although strong molecular evidence for splitting species (see Kane *et al*., 2008 and Raahauge and Kristensen, 2000).

	Bulinus species group / complex			
	B. africanus	*B. forskalii*	*B. reticulatus*	*B. truncatus*/*tropicus* complex
Recognised species				
	<u>B. abyssinicus</u>	*B. barthi*	*B. reticulatus*	*B. depressus*
	<u>B. africanus</u>	*B. bavayi*	***<u>B. wrighti</u>***	*B. hexaploidus*
	B. angolensis [1]	***<u>B. beccarii</u>***		*B. liratus*
	<u>B. globosus</u>	*B. browni*		*B. natalensis*
	B. hightoni	***<u>B. camerunensis</u>***		*B. nyassanus*
	<u>B. jousseaumei</u>	*B. canescens*		*B. octoploidus*
	<u>B. nasutus</u>	***<u>B. cernicus</u>***		*B. permembranaceus*
	<u>B. obtusispira</u>	***B. crystallinus***		*B. succinoides*
	B. obtusus	***B. forskalii***		*B. transversalis*
	B. ugandae	***B. scalaris***		***B. trigonus***
	<u>B. umbilicatus</u>	***<u>B. senegalensis</u>***		*B. tropicus*
				<u>B. truncatus</u>
				B. yemenensis
Additional species not currently recognised				
	B. productus [2]			

As recognised by Brown (Figure 2.5), DNA based methods would enable refinements, including species additions and movement of species within the *Bulinus* spp. groups. Since then, there have however only been minor changes in the taxonomy listed by Brown (1994), although it is suspected that this is due to the very few molecular studies investigating these snails (see below) and the absence of taxonomists working on *Bulinus*. The two currently recognised changes based on molecular data support the transition of *B. angolensis* from the *B. truncatus*/*tropicus* complex to the *B. africanus* group (F. Allan *et al.*, 2017), and as discussed by Brown (1994) a form of *B. nasutus*, previously named *B. nasutus productus*, having support for its consideration as a closely related species *B. productus* (see Kane *et al.,* 2008). The diversity past the current list of ~37 species is evident even from the few published studies using molecular analysis of target genes/DNA regions. However, interpreting these molecular results into tangible species descriptions has not been possible due to the absence of a species concept fitting *Bulinus* and the lack of multilocus genetic data to develop such sophisticated phylogenetic species delimitations (see below).

In light of the rationale for the current study, this review provides a basis for the current knowledge of *Bulinus* diversity across sub-Saharan Africa, with a particular emphasis of those in the *B. africanus* group. Since morphological and early molecular characterisation methods are reviewed extensively elsewhere (Brown, 1994; Rollinson *et al.*, 2001), this chapter will only touch on these techniques, but instead focus on research and advances in technology over the past 20 years that have enabled further study on *Bulinus* spp..

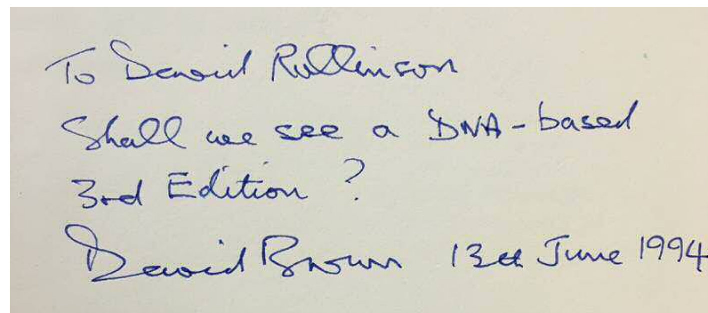


Figure 2.5. Message from Dr David Brown to Prof. David Rollinson on a copy of Brown's monographic piece; Freshwater Snails of Africa and their Medical Importance, 2nd Edition (1994).

2.9 The need for delimiting *Bulinus* species

2.9.1 Brief history of experimental taxonomy at the Natural History Museum: investigating Bulinus *species and their interactions with* S. haematobium *group species*

Before diving into the current challenges of working with snail vectors of schistosomiasis, it is worth considering the historic perspectives. The Natural History Museum (NHM, London) has for over half a century been involved in the field of *Bulinus* and *Schistosoma* spp. taxonomy and associations. This division of research at the museum was led by Dr. Christopher Amyas Wright (1928-1983), who following his appointment in the Department of Zoology in 1954 eventually led the establishment of the Experimental Taxonomy unit at the NHM in 1964 that went on to make huge contributions to the field of schistosomiasis. By 1964, Wright had already been working for over ten years on the snail vectors of schistosomiasis. Remarkably, the discussions that were taking place between Wright and his contemporaries working on snail intermediate hosts are much the same as those had between medical malacologists today (see Adema *et al.,* 2012). For example, in a letter from George Mandahl-Barth (then curator of *Danmarks Akvarium* and Chief WHO Snail Identification Centre, Charlottenlund, Denamrk) to Wright, Mandahl-Barth states:

"*I am happy to know that you have taken up the study of Bilharzia vector snails, because much more people must work on this field before we can clean up this mess."*

3rd January 1954

G. Mandahl-Barth

NHM archive: DF268/3/1/15

This initial letter opened up several years of correspondence (NHM archive: DF268/3/1/15-23), and visits between Wright and Mandahl-Barth to discuss *Bulinus* and ongoing taxonomy work, although not always in agreement with one another! After a few years of working on this topic, Wright was keen to take things further, with an *aide memoire* of the Dr. Francis Charles Fraser (at the time, the newly appointed Keeper of Zoology at the NHM between 1957-1964) stating:

"*Dr. Wright told me that he had been speaking to one of the W.H.O. people who seems to have developed some ideas about developing a central organization for the co-ordination of African Parasitic Helminthology. I indicated to Dr. Wright that in my opinion if such an organization was got going it would almost certainly come under the control of the School of Parasitology and Tropical Medicine."*

24th September 1957

F.C. Fraser

NHM archive: DF/ZOO/206/54

Presumably unimpressed by this reply or after further discussions, Wright sent a more formal letter a few months later to Fraser dated on New Year's Eve of 1957, detailing further that:

"*…there is a need for a centralised laboratory in Europe where fundamental research in medical and veterinary malacology can be carried out… The majority of the biological problems in the field of schistosomiasis centre around taxonomic work… this museum would by the ideal headquarters for such work to be carried out.*"

31st December 1957

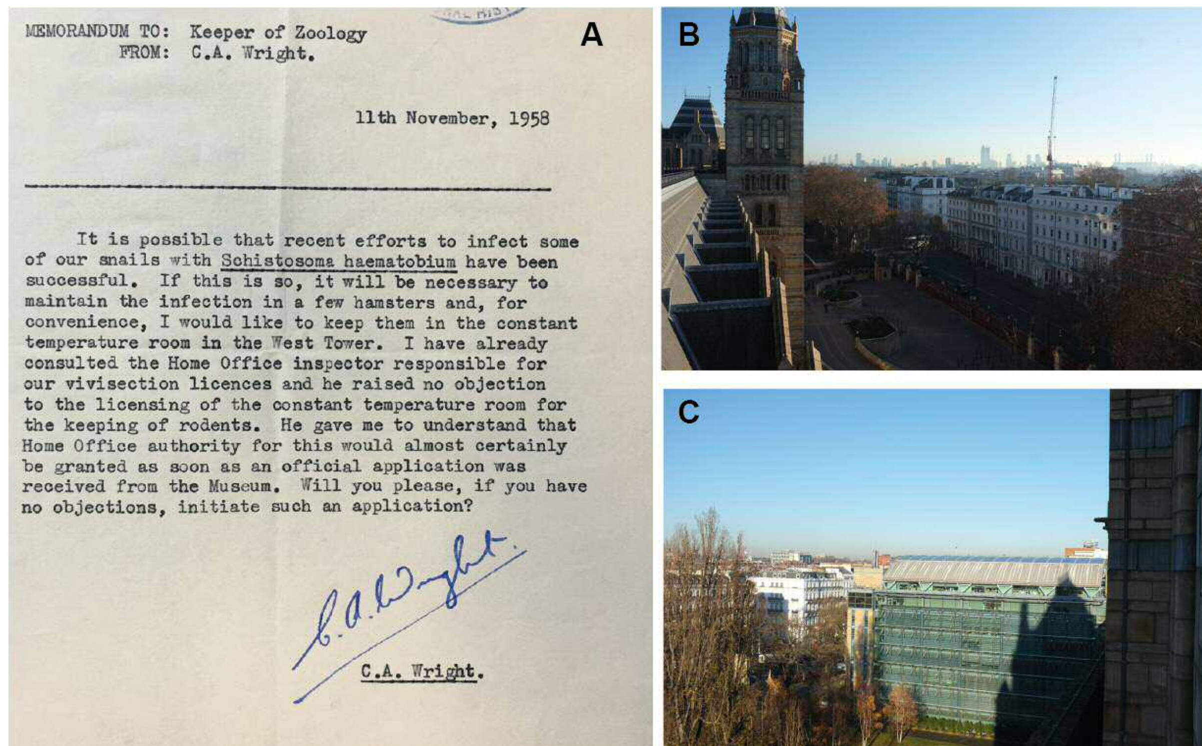
C. A. Wright

NHM archive: DF/ZOO/206/54

The work Wright envisaged was very forward thinking for the time, planning on using paper chromatography and paper electrophoresis for taxonomic work. He also planned to conduct growth studies on *Bulinus*, since he was not satisfied (rightly so) that absolute size of *Bulinus* should be used as an important specific character, and that gerontic changes in shell form should also be investigated since many species by this point had been described from a single, large, aberrant specimen and may therefore not be representative of the species.

Evidently, this worked, since almost a year later on the 11th of November 1958, correspondences between Wright and Fraser report that efforts to infect *Bulinus* spp. with *S. haematobium* had been successful (Figure 2.6A), and also that the NHM restaurant manager was providing the outer leaves of lettuces to feed the snails. Along with several others working with Wright at the Natural History Museum (notably Vaughan Southgate, David Brown, David Rollinson), the taxonomy of *Bulinus* and the understanding of interactions with *S. haematobium* group species where taken from its elementary stages to a significantly increased understanding based in the West Tower of the NHM (Figures 2.6B and C).

Now under the guise of the 'Parasites and Vectors' division, many of the questions proposed by Wright are still under investigation at the NHM, and keeping in with Wright's vision, utilising modern and novel technologies to answer questions in *Bulinus* taxonomy and schistosomiasis control in sub-Saharan Africa.



MEMORANDUM TO: Keeper of Zoology
FROM: C.A. Wright.

11th November, 1958

It is possible that recent efforts to infect some of our snails with Schistosoma haematobium have been successful. If this is so, it will be necessary to maintain the infection in a few hamsters and, for convenience, I would like to keep them in the constant temperature room in the West Tower. I have already consulted the Home Office inspector responsible for our vivisection licences and he raised no objection to the licensing of the constant temperature room for the keeping of rodents. He gave me to understand that Home Office authority for this would almost certainly be granted as soon as an official application was received from the Museum. Will you please, if you have no objections, initiate such an application?

C.A. Wright.

Figure 2.6. A: Memorandum from C. A. Wright to F. C. Fraser stating the first *Schistosoma haematobium* infections of *Bulinus* spp. had been performed at the Natural History Museum (NHM, London). B: View East from the West Tower of the NHM where the Experimental Taxonomy Unit established by Wright was based, overlooking Cromwell Road (South Kensington, London) and the main entrance to the NHM. C: View North-West from the West Tower overlooking the Darwin Centre 1 building where directly in the shadows of the West Tower the majority of the Parasites and Vectors division is now based.

2.9.2 Demonstrating the importance of Bulinus *taxonomy*

Although *Bulinus* are an interesting group in respect to investigating the diversity of freshwater gastropods in sub-Saharan Africa, their diversity and taxonomy is also of extreme importance when considering the control and transmission of *S. haematobium* group species. Schistosomiasis control strategies neglect the fact that malacological surveys can refine interventions for maximum return of investment. For example, as species within *B. africanus* group and *B. truncatus/tropicus* complex snails do not usually transmit the same species of schistosome in the same geographic area, it is of course only those sites containing the incriminated taxa that contribute towards transmission and require targeting (McCullough, 1959; Wright and Knowles, 1972; Chu *et al.*, 1978; Vera *et al.*, 1990; Rollinson *et al.*, 1997; J. R. Stothard *et al.*, 2002a).

Rising temperatures and an increase in travel have led to the migration of hosts, parasites and vectors with their associated tropical diseases into Europe (Bouzid *et al.*, 2014).

Snails are unable to quickly spread and colonise vast areas like winged vectors, but since *B. truncatus* are endemic in Southern European regions, such as Corsica in France (Doby *et al.*, 1966), it is possible for schistosomiasis outbreaks to occur here (Boissier *et al.*, 2016). Indeed in 2013, following several case reports (Berry *et al.*, 2014; Holtfreter *et al.*, 2014; Boissier *et al.*, 2015), at least 124 cases of schistosomiasis were reported by French nationals who swam in the Cavu River (Boissier *et al.*, 2016). Furthermore, an acute case of schistosomiasis was acquired during 2015, and several more in 2016, showing that the infection had persisted for at least two years in a non-endemic area (Berry *et al.*, 2016; Bisoffi *et al.*, 2016; Ramalli *et al.*, 2018). Hybridisation between *S. haematobium* parasites along with ecological and environmental changes are likely to have led to the outbreak of schistosomiasis in more temperate European climates (Moné *et al.*, 2015; Boissier *et al.*, 2016; Le Govic *et al.*, 2019). The hybrid status of the parasite also increases the risk of infection spreading further into Europe, by utilising other intermediate host snails (Webster and Southgate, 2003; Boissier *et al.*, 2015).

Modelling schistosomiasis distribution in relation to climate change has predominantly focussed on *S. mansoni* (see Mangal, Paterson and Fenton, 2008; Stensgaard *et al.*, 2013) transmitted by the less diverse *Biomphalaria* genus (Brown, 1994). Comparable risk maps and climate change models for *S. haematobium* infecting *Bulinus* spp. cannot be created yet because empirical data is lacking. Temperature will impact each stage of the schistosome lifecycle and the survival of snails through aestivation will play a large part in identifying risk areas (McCreesh and Booth, 2013; Mulero *et al.*, 2019). Since *Bulinus* spp. show different capacities for survival through aestivation and associated *Schistosoma* spp., it is important to consider this when establishing risk areas where transmission may occur (Rubaba *et al.*, 2016). The multiple environmental stressors caused by climate changes are likely to influence both parasite and intermediate host fitness, which may have additive, synergistic or even antagonistic effects on parasite transmission (Cable *et al.*, 2017).

Dam construction inevitably leads to environmental change. Apart from expanding existing and creating new habitats for snails (Southgate, 1997) dams can reduce predators, such as prawns (Sokolow *et al.*, 2015, 2017) and fish (Slootweg *et al.*, 1994), causing a freshwater snail population explosion. This snail population and habitat expansion may contribute towards the increase in risk of schistosomiasis in surrounding areas (Steinmann *et al.*, 2006), although a dramatic increase in the number of intermediate host snails following dam construction in Côte d'Ivoire did not increase schistosomiasis prevalence (Diakité *et al.*, 2017). Was this due to the lower overall starting prevalence of *S. haematobium* (13.9%) not driving transmission, or it could be due to a lack of compatible intermediate host (*B. globosus* and *B. truncatus*) proliferation in human water contact sites suited for *S. haematobium* transmission?

Assessing how variable environment conditions relate to the distribution of *Bulinus* genotypes has not been attempted but would be useful in estimating transmission risk of specific human water contact sites. For example, two morphologically and ecologically distinct *Biomphalaria* taxa (*Biom. sudanica* and *Biom. choanomphala*) endemic in the Lake Victoria region, could be ecophenotypes of one species (Standley *et al.*, 2011) although *B. sudanica* has higher prevalence of schistosome infection (Gouvras *et al.*, 2017). Therefore, their involvement in transmission of *S. mansoni* is more likely because of their environmental settings rather than species difference. Similarly, our recent observations (Pennance and Webster unpublished observations) uncovered morphologically and ecologically distinct *Bulinus* in the same Lake Victoria region. This now requires investigation of micro-geographical factors and species status to infer whether the environment or snail species is dictating schistosome infection here. Also in Zanzibar, two separate species of *Bulinus* are thought to inhabit either permanent (*B. globosus* and *B. nasutus*) or seasonal (*B. nasutus* only) freshwater bodies (Stothard and Rollinson, 1997a). As the latter is more resilient to desiccation during drought but the former is the predominant intermediate host for *S. haematobium*, it is important to differentiate these species when considering where transmission can take place, and also monitor any switch in intermediate host use that may result in seasonal transmission of schistosomes in some waterbodies through *B. nasutus*.

2.10 Methods for species delimitation within the *Bulinus* genus

2.10.1 Morphological identification of Bulinus

Conchology of *Bulinus* shell characteristics (Figure 2.7) such as size, shape and ornamentation of the shell and aperture, degree of umbilication and form of the columellar margin (the lip and inner margin of the aperture bordering the columella) have been used as the basis of taxonomic descriptions for this genus (Brown, 1994). Soft body anatomy, and especially morphology and shape of the radula (teeth) and reproductive system (Table 2.3) have also aided species diagnostics (Mandahl-Barth, 1957). Species identification based on morphology alone is, however, unreliable due to the plastic nature of discriminatory factors, gerontic variation, absence of an adult 'form' since the snails grow until they die, species descriptions based on aberrant forms and being subject to ecophenotypic variation creating incongruence with species descriptions (Jørgensen *et al.*, 2013). Extreme morphotypes with conflicting character states are also observed in natural populations (Stothard *et al.*, 1997), and the remarkable difference in morphology has led to malacologists bestowing new species names, or incorrect assignment with genetically different specimens (Akinwale *et al.*, 2015). To reiterate, this is also not aided by the vagueness of species descriptions and the uncertainty of what underpinning theories (and species concepts, see below) separate species from each other (e.g. shell microsculptures, ploidy, habitat preferences). Planorbids (of which *Bulinus* is

included) are known for their morphological disparity, and still little is understood regarding the causes for this when considering the contributions of ecophenotypic versus genetic diversity (Clewing *et al.*, 2015). A perfect example of this type of ecophenotypic plasticity was observed from the 'corkscrew' like *Bulinus* collected from Lake Malawi; despite obvious morphological differences in the shell structure they showed little genetic differentiation from morphologically 'normal' species (Clewing *et al.*, 2020). It seems apparent that SEM-based examination of shell structures, particularly those of juvenile forms rather than highly variable adult shell forms, should be used when trying to unravel the systematic position of *Bulinus* based on morphology, although currently this is completely absent for extant taxa and has only been performed on fossil specimens of the genus (Neubauer *et al.*, 2017).

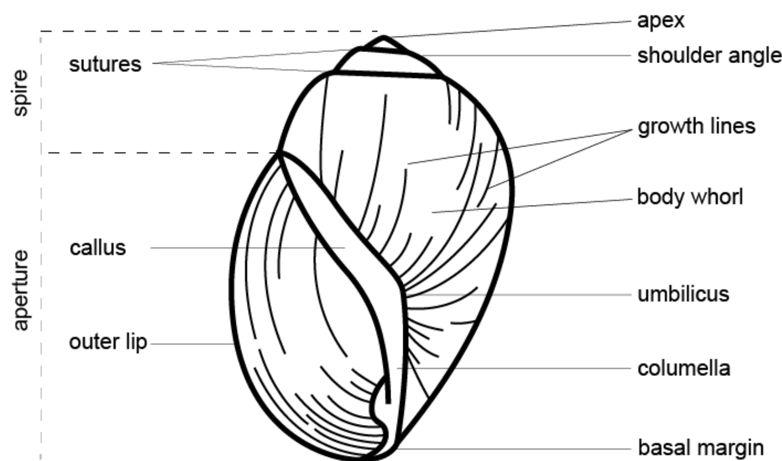


Figure 2.7. Morphological features of a freshwater snail shell (*Bulinus globosus*, Pemba Island, Zanzibar) used in conchology.

2.10.2 The development of molecular techniques for Bulinus *identification*

Molecular techniques have provided new insights into *Bulinus* taxonomy, and over the last 60 years our understanding of the species within and among the four *Bulinus* species groups (Table 2.2) has vastly improved. Determining chromosome numbers provided early evidence for polyploidy of the *B. truncatus/tropicus* complex, differentiating them from other *Bulinus* species (Burch, 1960; Goldman *et al.*, 1983). This was followed by electrophoretic techniques (Rollinson and Southgate, 1979; Wright and Rollinson, 1981) assessing enzyme profiles, which for example revealed the occurrence of cryptic sibling species (Jelnes, 1979, 1986). Enzyme profiles also inferred that intra-specific variation was greater than that usually considered for geographically isolated subspecies (Rollinson and Wright, 1984; Rollinson, Kane, *et al.*, 1990).

With the development of modern DNA profiling methods in the mid 1980's, 'DNA fingerprinting' methods were used for the first time to identify the four *Bulinus* groups (Strahan *et al.*, 1990; Rollinson and Kane, 1991), then microsatellites to explore genetic diversity (Jarne *et al.*, 1994; Viard *et al.*, 1996; Gow *et al.*, 2001, 2005; Emery *et al.*, 2003; Nalugwa *et al.*, 2011; Nyakaana *et al.*, 2013; Zein-Eddine *et al.*, 2017), and then Random Amplified

Polymorphic DNA markers (RAPDs) and Restriction Fragment Length Polymorphism (RFLP) analysis to discriminate species and also assess diversity (Williams *et al.* 1990; Langand *et al.* 1993; Stothard and Rollinson 1997a; Raahauge and Kristensen 2000). PCR-RFLP is still being used 20 years later as a cheap and quick alternative when sequencing facilities are not available (Hassan *et al.*, 2016). Additionally, SNaPshot™, a rapid technique for single nucleotide mutation detection using fluorescence was used to distinguish *B. africanus* species from coastal Kenya, and successfully identified *B. globosus* and *B. nasutus,* based on *cox*1 mutations (Stothard *et al.* 2002). However, although informative in terms of showing substantial genetic heterogeneity within a given population (Stothard *et al.*, 1997; Davies *et al.*, 1999), the utility and robustness of these methods in delineating species within the *Bulinus* genus is restricted (Stothard and Rollinson, 1996) and their reliability for continued use should be tested in comparison with DNA sequences for unequivocal proof of species identifications, rather than just comparing between PCR-RFLP and RAPD markers (Raahauge and Kristensen, 2000). With the affordability and access to Sanger sequencing technologies ever improving, DNA sequencing and inferences from nucleotide sequences has gradually become the standard for investigating relationships between taxa.

2.10.3 DNA sequencing of Bulinus*: Inferring basal topologies and species groups*

With the introduction of more advanced commercially available DNA sequencing kits and DNA sequencers in the late 1980's, nuclear and mitochondrial DNA fragments were starting to be sequenced from *Bulinus* collections (see Stothard *et al.* 1996; Stothard and Rollinson 1997b). These sequencing technologies provided the opportunity to further investigate intra- and inter-species diversity, geographical and environmental variation, evolution, infer phylogenies and molecularly define species involved in the transmission of schistosomes in endemic regions. It also allowed for the sister taxa relationship of *Bulinus* genus (classified under the Bulininae) the Planorbinae (within the Planorbidae) to be confirmed in molecular phylogenies (Morgan *et al.*, 2002; Albrecht *et al.*, 2004, 2007). The *Bulinus* genus also forms a well-supported ancestral position in a clade with *Indoplanorbis* (Figure 2.8), a genus of freshwater snail predominantly across Asia. The emergence of the *Bulinus* and *Indoplanorbis* genera have been associated with the breakup of India and Africa ~130 Ma, geographical isolation driving evolution (Stothard *et al.*, 2001; Morgan *et al.*, 2002; Albrecht *et al.*, 2007), and therefore the ancestral position for these taxa seems likely to be in East Africa. The most recent phylogenetic tree including a representative of the Bulininae was performed by Albrecht *et al*. (2007), and those with more extensive taxon sampling including other Planorbidae (Dayrat *et al.*, 2011), but not *Bulinus*. The relationship of *B. tropicus* with *Indoplanorbis exustus* demonstrated by Albrecht *et al*. (2007) (Figure 2.8) may suffer from insufficient taxon sampling considering the diversity seen within the *Bulinus* genus, and previous hypotheses for the

genus to be split into 3-4 genera as supported by Biocca *et al*. (1979). For example, the inclusion of a *B. africanus* group or *B. forskalii* group species rather than a *B. truncatus/tropicus* group species (i.e. *B. tropicus*) may result in different tree topology when constructing a phylogeny with higher level genera within Planorbidae.

Table 2.3. Summary of methods used to delineate *Bulinus* species and their ability to infer inter- and intra-specific variation. * mitochondrial genomes of *Bulinus* species are not published, however have been produced in this study and reported in Chapter 7.

Identification Method	Description	Ability to separate species groups	Ability to separate species within groups	Intra-specific variation
Morphology				
Conchology	Shell characteristics: size, shape, ornamentation, aperture, columellar	Yes	Maybe	Maybe - can observe extreme morphologies
Anatomy	Morphology of radula (teeth) and reproductive system	Yes	Maybe	Maybe - can observe extreme morphologies
Molecular methods				
Ploidy	Number of chromosomes in a cell	Yes	No - too conserved	No - too conserved
Enzyme profiles	Isoelectric focussing of enzymes	No - too variable	No - too conserved	Yes
'DNA fingerprinting'	Restriction fragment length polymorphism (RFLP) using analysis of DNA	No - too variable	Maybe but needs proving	Yes
Random Amplified Polymorphic DNA markers (RAPDs)	Randomly amplifying fragments of DNA using universal primer sets to observe electrophoretic amplicon profiles	No - too variable	No - too conserved	Yes
Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFP)	Amplifying DNA region, and cut the amplicon with specific restriction enzymes to produce a genetic profile that can be visualised by gel electrophoresis	No - too variable	Maybe but needs proving	Yes
Complementary bases changes (CBCs)	Observing CBCs in the secondary structure of the rDNA ITS2 region	Yes	Yes	Yes/No – cryptic variation postulated
SNaPshot™	Single nucleotide mutation detection using fluorescence	No - too variable	Probably but needs proving	Yes
Sequencing				
Nuclear DNA	Sanger sequencing of nuclear genes to observe SNPs	Yes	No - too conserved	No - too conserved
Mitochondrial cytochrome oxidase 1 (*cox*1)	Sanger sequencing of mitochondrial genes to observe SNPs	Yes	Yes	Yes
Compensatory base changes (CBCs)	Changes in nucleotides at pairing positions altering secondary structures restricting breeding between species	Maybe - insufficient evidence	Maybe - insufficient evidence	Maybe - insufficient evidence
Next Generation Sequencing*	High-throughput genome sequencing	Yes*	Maybe – insufficient data*	Yes*

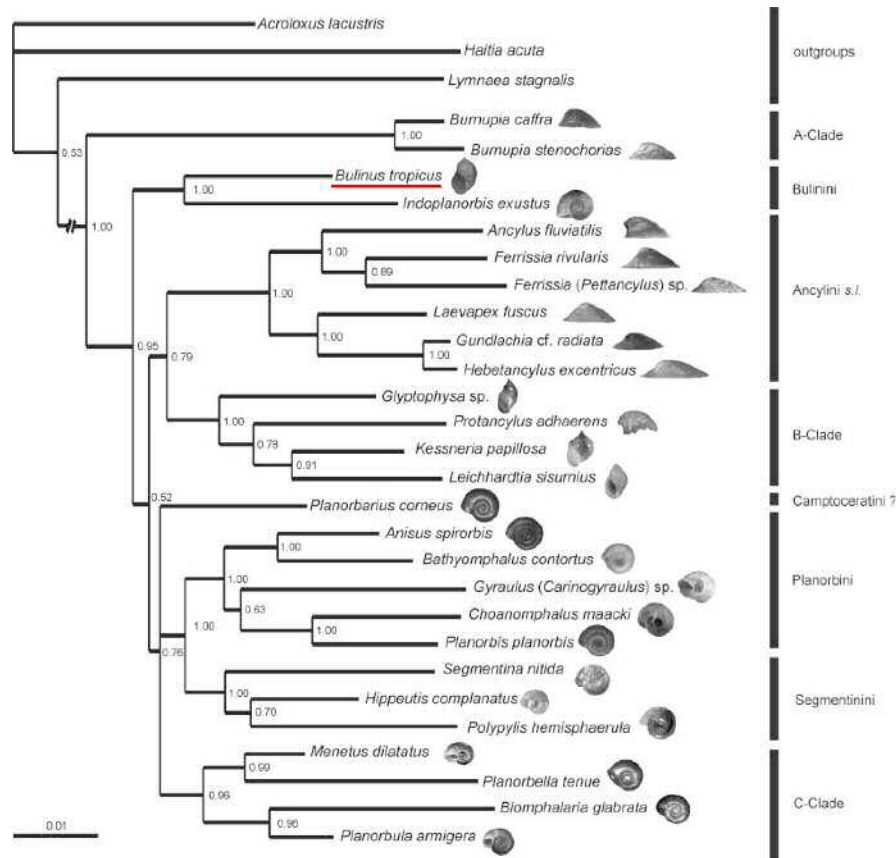


Figure 2.8. Bayesian inference phylogram for planorboidean taxa based on concatenated *cox*1 and 18S data, showing position of *Bulinus tropicus* (underlined red), forming an ancestral clade with *Indoplanorbis exustus* (taken from Albrecht *et al*. 2007).

Although the monophyletic origins of each *Bulinus* species group have now been well supported, the initial degree of nucleotide divergence first observed in rDNA questioned whether species of *Bulinus* should be considered within the same genus (Stothard *et al.*, 1996). This would have supported the theories of separating the *Bulinus* into different genera (Biocca *et al.*, 1979), however this has since been refuted and the inclusion of all groups under one genus remains the most widely supported inference (Brown, 1994), which is additionally supported by molecular data (see below).

There are currently 19 published studies that include phylogenetic analysis of *Bulinus* spp. using sequence data and using several different gene/rDNA/rRNA regions (as listed in Table 2.4). From these, several inferences have been made on the basal topology between three of the four *Bulinus* species groups; *B. truncatus*/*tropicus* complex, *B. africanus* group and *B. forskalii* group species (*nota bene*: position of *B. reticulatus* group as sister taxa to *B. truncatus/tropicus* complex continually well supported). Inferences of topology have even shown to be incongruent depending on how sequence data is handled (i.e. by including / excluding gaps and poorly aligned regions; Jørgensen *et al.,* 2013) or which partial region of the target gene is used for phylogenetic inferences (Kane *et al.*, 2008). To summarise the 12 studies that include representatives from these three species groups (Table 2.4) with

supporting phylogenetic topologies, three different basal topologies are reported, depicted in Figure 2.9. The strongest support is for the *B. africanus* being a sister group to the *B. truncatus/tropicus* complex, with this clade sister to the *B. forskalii* species (Jørgensen *et al.*, 2011, 2013; Zein-Eddine *et al.*, 2014), i.e. topology 2; Figure 2.9. However, basal topology of the *Bulinus* species groups is still speculative when using both rDNA and *cox*1 data (Table 2.4), for example recent studies including a large number of taxa suggested that *B. forskalii* and *B. africanus* species are sister taxa (Tumwebaze *et al.*, 2019; Clewing *et al.*, 2020). It seems evident that a broader range of samples and comparing concatenated data matrices of both conserved and variable gene regions is required to help resolve this issue.

2.10.4 DNA sequencing of Bulinus*: Investigating inter-/intra-specific variation and phylogeography*

To further resolve *Bulinus* taxonomy within species groups, an extended mitochondrial *cox*1 barcode was trialled by Kane *et al*. (2008), three times larger than the region previously used, containing both Folmer (Folmer *et al.*, 1994) and Asmit regions (Bowles *et al.*, 1992). The combined sequences could be edited to remove transitional saturation (Stothard *et al.*, 2001), whilst still providing sufficient molecular diversity to identify an extensive range of species / isolates (Kane *et al.*, 2008). The superiority of the species resolution when using this *cox*1 barcode over the nuclear ITS2 region, or other species delineation methods (Table 2.3), was immediately apparent, with 13 species delineated from multiple geographical areas (Kane *et al.*, 2008). In contrast to European freshwater snail species (Bargues *et al.*, 2001), the ITS2 does not robustly identify species within groups (Kane *et al.*, 2008; Jørgensen *et al.*, 2013).

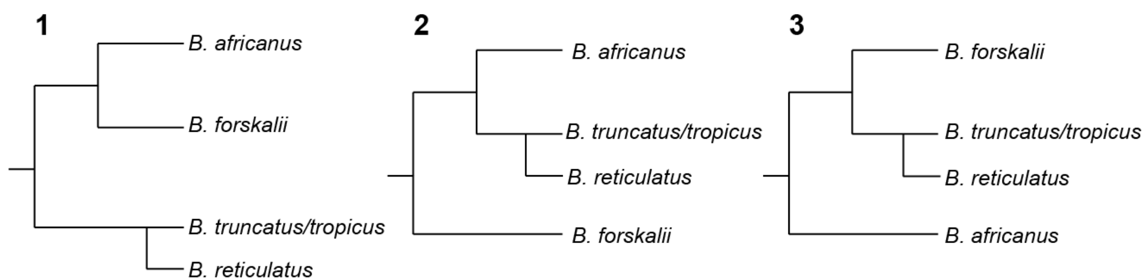


Figure 2.9. The three different basal divergences of *Bulinus* species groups observed from 12 currently published phylogenetic studies including these species groups (as listed in Table 2.4).

Table 2.4. (see next page) All phylogenetic studies published using DNA sequence data of *Bulinus* species as of July 2020. [1]Country that taxa originate for which sequence data generated: ANG; Angola, BKF; Burkina Faso, CAM; Cameroon, DRC; Democratic Republic of Congo, EGY; Egypt, ETH; Ethiopia, GAB; Gabon, IR; Iran, KEN; Kenya, MAD; Madagascar, MLW; Malawi, MAU; Mauritius, SUD; Sudan, NGE; Nigeria, NIG; Niger, OM; Oman, POR; Portugal, SA; South Africa, SAR; Sardinia, SEN; Senegal, ST; São Tomé, TZ; Tanzania, ZIM; UGA; Uganda, Zimbabwe, ZNZ; Zanzibar. [2]Species identification: Morphological guides used to determine species (important for when novel sequences generated without a species reference). [3]*Bulinus* species groups: *B.a.*; *B. africanus*, *B.f.*; *B. forskalii, B.t.*; *B. truncatus/tropicus* complex, *B.r.*; *B. reticulatus*.

Reference	County[1]	Species identification[2]	*Bulinus* Species Groups included[3]	Marker (bp)	Resulting basal topology for *Bulinus* species groups (see Figure 2.9)
Abe *et al*. (2018)	EGY, SUD, ZIM	Brown (1994)	*B.t.*	*cox*1 (737) ITS1 (580)	2
Akinwale *et al*. (2015)	NGE	Brown (1994)	*B.a.* & *B.f.*	ITS (~1,300)	NA
F. Allan et al. (2017)	ANG	Brown (1994) & reference material (NHM)	*B.a.* & *B.f.*	*cox*1 (~600)	NA
Clewing et al. (2020)	MLW	NA	*B.a.* & *B.f.* & *B.t.*	*cox*1 (390) 16S (460)	1
Jones et al. (2001)	SEN, CAM, MLW, NIG, ST, ANG, MAU, KEN	Brown (1994) & Mandahl-Barth (1957) genitalia & Jones et al. (1997) RAPD	*B.f.*	*cox*1 (350) ITS1 (493)	NA
Jørgensen et al. (2007)	MLW	?	*B.a.* & *B.f.* & *B.t.*	*cox*1 (340) 16S (430)	2
Jørgensen et al. (2011)	ETH, SEN, IR, TZ, MAD, MLW, OM, UGA, BKF, MAU, CAM	?	*B.a.* & *B.f.* & *B.t.* & *B.r.*	18S (1703) 28S (2122) H3 (375)	2
Jørgensen et al. (2013)	TZ, UGA, SEN, KEN, SA, NIG, ANG, MAU, OM	See Kane *et al.* 2008	*B.a.* & *B.f.* & *B.t.* & *B.r.*	ITS2 (638)	1 and 2
Kane *et al*. (2008)	TZ, ZNZ, UGA, KEN, SA, BKF, NIG, SEN, CAM, ST, ANG, MAU, OM, SAR, POR	Multiple (refer to article)	*B.a.* & *B.f.* & *B.t.* & *B.r.*	*cox*1 (1010) ITS2 (394)	1 and 2
Morgan *et al.* (2002)	KEN, EGY, TZ	?	*B.a.* & *B.f.* & *B.t.*	28S (1488) actin exon 2 (526)	3
Mutsaka *et al.* (2020)	ZIM	Brown (1994)	*B.a.* & *B.t.*	*cox*1 (607)	NA
Nalugwa *et al.* (2010a)	UGA	Kristensen (1987)	*B.t.*	*cox*1 (612)	NA
Nalugwa *et al.* (2010b)	UGA, DRC, TZ	Kristensen (1987)	*B.a.* & *B.f.* & *B.t.*	*cox*1 (612)	1
Pennance *et al.* (2020)	NIG	Brown (1994)	*B.a.* & *B.f.* & *B.t.*	*cox*1 (414 - 653)	NA
Stothard *et al.* (1996)	ZAM, MAL, MAU	? Brown (1994)	*B.a.* & *B.f.* & *B.t.*	ITS (~1,300)	2
Stothard & Rollinson (1997)	ZNZ	? Brown (1994)	*B.a.*	*cox*1 (340)	NA
Stothard *et al.* (2001)	MAD	? Brown (1994)	*B.a.* & *B.f.* & *B.t.*	*cox*1 (340) 16S (~400)	3
Tumwebaze *et al.* (2019)	UGA	?	*B.a.* & *B.f.* & *B.t.*	*cox*1 (655)	1
Zein-Eddine *et al.* (2014)	CAM, SEN, GAB	Kristensen (1987)	*B.a.* & *B.f.* & *B.t.*	*cox*1 (1031) ITS (600-990) 18S (852) 28S (855)	2

The mitochondrial *cox*1 region also provided support for delimiting two sub-species, *B. nasutus nasutus* and *B. nasutus productus* (see Figure 2.3) as *B. nasutus* and *B. productus* (Kane *et al.,* 2008). Further analysis also proposed (Kane *et al.*, 2008) that the first 644 bp of the *cox*1 barcode (the Folmer region; Folmer *et al.*, 1994) was the most suitable region for species identification, as inferred tree topology closely mirrored that of minimum evolution and neighbour joining trees produced by the full ~1000 bp *cox*1 barcode. Use of the Folmer *cox*1 region has led to re-description of the *B. africanus* group, with the inclusion of *B. angolensis* (formerly *B. truncatus/tropicus* complex; Wright 1963) after it unequivocally aligned with *B.*

globosus (*B. africanus* group) specimens collected from Angola (F. Allan *et al.*, 2017). It has also enabled investigation into cryptic morphology, with the analysis of morphologically indistinguishable *Bulinus* samples from Lake Albert in Western Uganda identifying two evolutionary lineages and confirmed the existence of two species, *B. truncatus* and *B. tropicus*, partitioned into two monophyletic clades (Nalugwa, Kristensen, *et al.*, 2010). Similarly, cryptic variation has been observed in *B. globosus* collected from close proximity to one another in Zimbabwe (Mutsaka-Makuvaza *et al.*, 2020). However, substitutional saturation of mitochondrial genes in *Bulinus* may limit the phylogenetic power for analysis of these species when including many taxa, and additional methods such as observing secondary structures of the ITS2 may provide secondary basis for support (Jørgensen *et al.*, 2013). Alternatively, when *cox*1 sequences are used in conjunction with the nuclear markers (such as ITS, 18S and 28S), they have been shown to reliably infer a phylogeny of seven *Bulinus* species (*B. camerunensis, B. forskalii, B. globosus, B. senegalensis*, *B. tropicus*, *B. truncatus*, *B. tropicus* and *B. umbilicatus*) collected from Cameroon, Senegal and Egypt (Zein-Eddine *et al.*, 2014).

When considering phylogeography, inferences from *cox*1 sequences have provided the most insight. For example, sequencing of the Folmer *cox*1 region revealed extreme variation, with high haplotype diversity but strong haplotype and geographical clustering within species from West Africa (Zein-Eddine *et al.*, 2014). Also, a 340 bp fragment of *cox*1 provided sufficient phylogenetic information to clearly split the two taxa *B. globosus* and *B. nasutus* from Unguja and Pemba islands (Zanzibar), on both a species and geographical level (Stothard and Rollinson, 1997b). Molecular evolution and colonisation times of *Bulinus* (*B. obtusispira*, *B. liratus* and *B. bavayi B. forskalii*) endemic on Madagascar (Stothard *et al.*, 2001), and the relationship between two endemic species (*B. nyassanus* and *B. succinoides*) in Lake Malawi (Jørgensen *et al.*, 2007) were also inferred using both *cox*1 and 16S (rRNA) DNA regions.

In terms of investigating the relationships of *Bulinus* taxa based on sequence variation, there is no question that the *cox*1 region is the best target for identification, not only due to the abundance of reference sequences available from online sequence repositories, but also because of the availability of developed primers and most importantly the inferences that can be made from phylogenetic analyses. However, inferences made from *cox*1 analysis reach a limit, as although one can reliably split taxa based on *cox*1 haplotypes, if adding information about geography and evaluating the level of reproductive isolation between populations, it does not offer the statistical power to test for species delimitation. Cryptic variation can therefore be observed form the molecular sequence data obtained through gene targeting, but larger DNA regions and concatenated sequence matrices are needed to confidently make species delimitations (Prévot *et al.*, 2013). This has been particularly true for the *B. africanus*

group species *B. globosus*, potentially the most widespread and medically important species of *Bulinus* because of its pan-African distribution to its role in the transmission of *S. haematobium* (see Brown 1994; Rollinson 2009). In several studies, it is apparent from tree topologies that phylogenetically distinct *B. globosus* haplotypes are present across Africa and, from a taxonomic standpoint, may require splitting (Stothard and Rollinson, 1997b; Kane *et al.*, 2008; Jørgensen *et al.*, 2013; Mutsaka-Makuvaza *et al.*, 2020). To avoid everlasting confusion of freshwater snail taxonomy in Africa this has not been attempted but may become necessary with increasing molecular data.

2.10.5 Next Generation Sequencing of Bulinus

The *Biomphalaria* mitochondrial genome recently published provides an important resource allowing for the design of new molecular markers and new molluscicides that target gene products involved in gene regulation/reproduction/metabolism (Adema *et al.*, 2017). Two on-going projects at the Natural History Museum involve next generation sequencing (NGS) of the mitochondrial genome of ~20 *Bulinus* species (see Chapter 7). The data will assess inter- and intra-specific diversity using diverse taxa from multiple countries, but will also provide genome wide data to uncover regions of DNA that could be targeted for control as proposed for *Biomphalaria* (see Adema *et al.* 2017). In the current study (Chapter 7), the data was used to assess the cryptic diversity of *B. africanus* spp. (mainly *B. globosus*) from East Africa including the Zanzibar Archipelago, assess ancestral positions of Madagascan taxa, and investigate the possibility of delimiting new species based on molecular data within this group.

2.11 Developing a species concept for *Bulinus*

As discussed throughout this introductory chapter, the taxonomy of *Bulinus* has long suffered due to difficulties in applying a typical species concept to delimiting species within the genus. A biological species concept for example, bound by sexual reproduction and parentage (Dobzhansky, 1970), can not apply to a hermaphroditic organism. A morphological species concept, relying on consistently and persistently distinct and distinguishable features, as has been attempted by the works of Mandahl-Barth (1957, 1965) and Brown (1994), is rendered useless in the microtaxonomy of *Bulinus* due to the unusual forms and overlapping morphology encountered. With the ever-increasing abundance of genetic data being produced for *Bulinus*, could a phylogenetic species concept, i.e. identifying the smallest biological entities that are diagnosable and/or monophyletic (Mayden, 1997), be applied to *Bulinus*?

A few things need to be considered in regard to inferring taxonomy through phylogeny, starting with the fundamental schools of systematics, cladistics and phenetics. Cladistics concerns the pathways of evolution, through using synapomorphies and emphasising holophyly to form classifications and reconstruct rooted phylogenetic trees (cladograms),

phenetics on the other hand has its emphasis on the quantitative assessment of overall similarity between taxa regardless of evolutionary relation (Stuessy, 2013). Due to the ignorance of phenetics in considering ancestry, gene trees are often constructed using commonly used tree-building algorithms that have their foundations in cladistic approaches (such as maximum likelihood, parsimony and Bayesian inference), although many still exist that originate in phenetic principles (neighbour-joining and UPGMA). However, many of these tree-building algorithms originate from a combination of both cladistic and phenetic approaches, and biologists are therefore more concerned with the statistical fit of the tree and the computational time/power required to analyse the dataset, rather than the classification of the tree (Stuessy, 2013).

Following the development of Markov chain Monte Carlo (MCMC) methods (a class of algorithms that sample from a probability distribution) in Bayesian statistics, it has been possible to compute much larger genetic datasets, increasing the popularity of this method and its use in investigating *Bulinus* relationships (Chibwana *et al.*, 2015; Tumwebaze *et al.*, 2019; Clewing *et al.*, 2020; Pennance, Allan, *et al.*, 2020). Although sophisticated, Bayesian phylogenies can still be confounded (as for other methods) due to genetic peculiarities, such as: false similarity (homoplasy), locus duplication (gene paralogy), incomplete lineage sorting, allopatric lineages, mutational saturation effects, polyploidy and introgressive hybridisation that create complex reticulate relationships between taxa, all of which can mask a correct phylogeny (Stuessy, 2013). These complications are likely to have an effect when considering *Bulinus* phylogenies since allopatric lineages of *Bulinus* are uncovered (see Chapter 4 and 7) and polyploidy is known to exist for species of *Bulinus* particularly in the *B. truncatus/tropicus* complex (Brown and Wright, 1972). Polyploidy in *Bulinus* may have occurred as an adaptation to altitude (Brown, 1976) and hybridisation (Goldman *et al.*, 1983), either way acting as a reproductive barrier between *Bulinus* species of differing ploidy which may not be apparent from a gene tree. Introgressive hybridisation also occurs in some of the *S. haematobium* group species that *Bulinus* transmit (Léger *et al.*, 2016; Platt *et al.*, 2019), although whether these hybrids relate directly to the origination of a new lineage (i.e. reticulate evolution) or are a result of introgression and repeated backcrossing within the same population needs to be inspected further. Quantitative tools for examining these kinds of networks and reticulate relationships are required (Huson *et al.*, 2010). As for tree building algorithms within phylogenetic analyses, evolutionary nucleotide substitution models are also determined based on statistical tests in packages such as MrModelTest (Nylander, 2004), jModelTest (Darriba *et al.*, 2012) and PartitionFinder (Lanfear *et al.*, 2016). Allowing computational algorithms and models to dictate the phylogenetic analysis being performed isn't necessarily due to a careless or naïve systemicist, but as stated by Stuessy (2013) is "due to the overwhelming challenge

of volumes of DNA data, we are using whatever algorithm we need to find patterns of relationship".

Another advantage for following a phylogenetic species concept for *Bulinus*, is that as is the same for a lot of freshwater pulmonates, *Bulinus* are poorly represented in the fossil record. Interpreting the evolution of species and ancestral forms to infer phylogeny is therefore possible predominantly from extant taxa. An extensive fossil record of *Bulinus* could allow for investigation into the morphological changes and potentially infer speciation over time providing a basis to determine extant taxa. The available fossil record is however very geographically limited for *Bulinus*, and the most recent and detailed descriptions of *Bulinus* fossils are from Central Europe (Harzhauser *et al.*, 2012; Neubauer *et al.*, 2017), adding to some older records of *Bulinus* outside of Africa (Neumayr, 1883) (Figure 2.10). As discussed by Neubauer *et al.* (2017), these *Bulinus* of Central Europe are likely an extraordinary introduction of an ancestral species via long distance dispersal, since there was no hydrological connection between Africa and Europe during this early Miocene period when *Bulinus* were known to already be present in Africa. It is still considered therefore that *Bulinus* very likely originated from Africa, where the earliest fossil records date back to 19-20 Ma (Pickford, 2008). It is however extremely likely that the *Bulinus* genus is much older than this considering the possibility of *B. obtusispira* and *B. bavayi* on Madagascar, which separated from East Africa ~158 Ma and again from India ~84 Ma (Briggs, 2003), being 'relic' *Bulinus* species here (Wright, 1971; Stothard *et al.*, 2001; Jørgensen *et al.*, 2011), Unfortunately, the fossil record is likely to hold few answers for inferring speciation due to *Bulinus* being a relatively thin shelled gastropod that will not fossilize well like many of the other more robust snails found in Africa (West *et al.*, 1991), Secondly, living specimens have proven problematic based on shell characteristics to describe, and therefore soft tissue anatomy would be required which is not preserved during fossilisation anyway (Neubauer *et al.*, 2013). Thirdly, we know from the current distribution and habitat preferences that *Bulinus* have a propensity to inhabit either quickly changing or even temporary freshwater bodies, with only particular species inhabiting the long-lived lakes that offer a better setting for preservation through lacustrine deposits. The fossil record may be quite biased by these factors, and overall not provide the required information to develop time calibrated trees and resolve potential sources of error in phylogenetic trees (Forest, 2009).

2.12 The future of *Bulinus* systematics

As discussed here and further by Brown (1994), a huge difficulty when working with *Bulinus* stems from there being no "simple species-concept to a hermaphroditic organism such as *Bulinus*, capable of self-fertilisation as well as outcrossing and living in discrete patches of habitat, where isolation favours genetic differentiation among populations". The description of

nearly all *Bulinus* species therefore derives from a traditional taxonomic approach following the morphological species concept. Although with exceptions, since other variables, such as the capacity for schistosome infection, have also been used in species delimitation. For example, the split of *B. globosus* and *B. ugandae* that were recovered from the same areas was supported by the differences in susceptibility to *S. haematobium* (see Mandahl-Barth, 1957, 1965). Whether this is an alternative and reliable species concept to develop for delimiting *Bulinus* species has not however been discussed in detail. It is complicated by the fact that species names of *Bulinus* have been used indiscriminately when conducting infection studies (Joubert *et al.*, 1990, 1991) and it also seems that geographical strains of schistosome and *Bulinus*, rather than different species, can determine infection success (Zumstein, 1983). Confirmation of species status has also been studied through investigations of environmental tolerance, with for example *B. africanus* being better adapted to low temperatures and less tolerant to warmth compared to *B. globosus* (Joubert *et al.*, 1984, 1986). As aforementioned, Chromosome number has also been used for species delimitation, particularly between species of the *B. truncatus/tropicus* complex where ploidy variation is high (Burch, 1960; Goldman *et al.*, 1983).

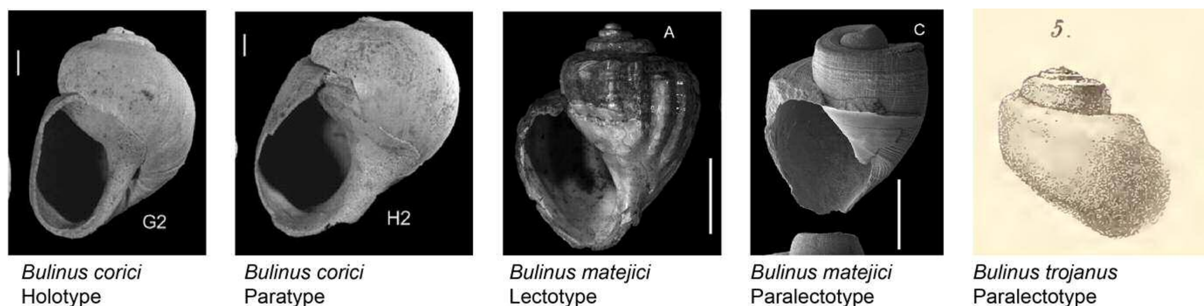


Figure 2.10. Images and illustrations available for three extinct *Bulinus* species (Middle Miocene – 13.65 – 15.97 Ma), *B. corici* (Jauring, Austria), *B. matejici* (Madare, Serbia), and *B. trojanus* (Behramkale, Turkey) collected from Central Europe. Images taken from: Neubauer e*t al*. (2017); *B. corici*, Harzhauser *et al*. (2012); *B. matejici*, Neeumayr (1883); *B. trojanus*.

It is therefore difficult to conclude what species concept to move forward with when investigating *Bulinus*, especially since the general concept of what operational criteria species should be recognised and delimited is still being discussed (De Queiroz, 2007; Hausdorf, 2011). With a general shift of biologists towards newer and continuously improving phylogenetic methods, and the search for cryptic speciation within these datasets (Bickford *et al.*, 2007), this may be the most agreeable and discussion worthy method for the time being. Although not currently available for *Bulinus*, as is the case for some other taxa, the huge quantities of DNA deriving from large genomic studies will create future challenges for those investigating evolutionary relationships between species as the methods underpinning the analysis of this data still requires improving (Mooi and Gill, 2010; Stanton *et al.*, 2019),

including methods of handling gene tree discordance (Degnan and Rosenberg, 2009). Extra consideration should be given when inferring results from these large datasets, as discussed briefly here and extensively by Mooi and Gill (2010), the statistical programmes and optimisations used in molecular systematics have become the new authority on determining phylogeny by the trees they produce, and often at the cost of ignoring character homology. Although it is hoped that further assessment of the molecular diversity across the whole range of *Bulinus* spp. may provide support for species inferences as before (Kane *et al.*, 2008), it is also necessary to balance this with ecological and phenotypic data that form the cornerstone of *Bulinus* taxonomy (Brown, 1994).

Chapter 3. Interactions between *Schistosoma haematobium* group species and their *Bulinus* spp. intermediate hosts along the Niger River Valley

Abstract

Urogenital schistosomiasis, caused by infection with *Schistosoma haematobium,* is endemic in Niger but complicated by the presence of *S. bovis*, *S. curassoni* and *S. haematobium* group hybrids along with various *Bulinus* snail intermediate host species. Establishing the schistosomes and snails involved in transmission aids disease surveillance whilst providing insights into snail-schistosome interactions/compatibilities and biology. Snail and schistosome species from Niger need better characterisation. Infected *Bulinus* spp. were collected from 16 villages north and south of the Niamey region, Niger. From each *Bulinus* spp., 20-52 cercariae shed were analysed using microsatellite markers and then a subset were identified using the mitochondrial *cox*1 and nuclear ITS1-2 and 18S DNA regions. Infected *Bulinus* spp. were identified morphologically and molecularly by analysis of partial mt *cox*1 region.

From 87 infected *Bulinus* from 26 sites*,* 29 were molecularly confirmed as *B. truncatus,* three as *B. forskalii* and four as *B. globosus.* The remaining samples were morphologically identified as *B. truncatus* (n=49) and *B. forskalii* (n=2). Microsatellite analysis of 1,124 cercariae revealed 186 cercarial multi locus genotypes (MLGs). Identical cercarial genotypes were frequently (60%) identified from the same snail (clonal populations from a single miracidia), however several (40%) of the snails had cercariae of different genotypes (2-10 MLG's) indicating multiple miracidial infections. The bovid schistosome *S. bovis* was shed from 57 of the *B. truncatus* and all of the *B. forskalii* and *B. globosus.* The other *B. truncatus* were shedding the human schistosomes, *S. haematobium* (n=6) and the *S. haematobium* group hybrids (n=13). Two *B. truncatus* had co-infections with *S. haematobium* and *S. haematobium* group hybrids whilst no co-infections with *S. bovis* were observed.

This study has advanced our understanding of human and bovid schistosomiasis transmission in the Niger River Valley region. Human *Schistosoma* species/forms (*S. haematobium* and *S. haematobium* hybrids) were found transmitted in the five villages north of Niamey whereas those causing veterinary schistosomiasis (*S. bovis*), found across all villages. *B. truncatus* was most abundant, transmitting all *Schistosoma* species, while the less abundant *B. forskalii* and *B. globosus,* only transmitted *S. bovis.* Our data suggests that species-specific biological traits may exist in relation to co-infections, snail-schistosome compatibility and intramolluscan schistosome development.

3.1 Introduction

Schistosomiasis is a snail-borne Neglected Tropical Disease (NTD) associated with poverty, poor sanitation and lack of safe water (Steinmann *et al.*, 2006; Hotez *et al.*, 2014).

Schistosomes are diverse, with 23 species currently recognised within the genus *Schistosoma* that exhibit preferences for both their intermediate snail and mammalian hosts, all of which governs their distribution. In sub-Saharan Africa there are four species that cause human schistosomiasis, whilst there are many more that infect livestock and/or wildlife (Webster and Littlewood, 2012).

Schistosomes can be identified by species-specific phenotypic characteristics, particularly associated with the adult worms and their eggs. Additionally, epidemiological and ecological characteristics, such as geographic region and intermediate snail and definitive mammalian hosts associations, are used as proxies for species identification at a given focus (Rollinson and Southgate, 1987). Supported by new field collection and sample preservation methods (Emery *et al.*, 2012; Webster *et al.*, 2015), molecular data from schistosome collections have revealed new species distributions (Pennance *et al.*, 2018), inter-species hybridisation (Huyse *et al.*, 2013; Webster, Diaw, *et al.*, 2013; Léger *et al.*, 2016; Oey *et al.*, 2019), and unexpected host associations (Catalano *et al.*, 2018), all of which highlight the need to incorporate molecular analyses into disease surveillance. Molecular data are particularly pertinent for free-living schistosome cercarial larvae, which have limited species-specific morphologies (Frandsen and Christensen, 1984) and also for schistosomes that overlap in their snail intermediate host use.

Aside from schistosomes, snail host identification can present/pose complications as it often relies on species-specific morphological characteristics, including shell characteristics, chromosome counts and reproductive organs (Brown, 1994). However, morphologically similar snails can be very difficult to identify, with phenotypic plasticity, including, for example, overlap in shell morphology potentially confounding identification (Stothard *et al.*, 1997). Molecular analyses provide greater accuracy and also enable interrelationship, phylogenetic and population diversity analyses (Kane *et al.*, 2008). Moreover, precise snail identifications are needed to determine the true hosts involved in *Schistosoma* transmission. This is vital for mapping and monitoring disease transmission and in determining infection risk to human and livestock populations surrounding snail habitats (Adema *et al.*, 2012).

Of the estimated 3-4 million people at risk of human schistosomiasis in Niger, the majority of the disease is urogenital, caused by *Schistosoma haematobium* (including *S. haematobium* group hybrids), with transmission relying on freshwater *Bulinus* spp. snails (Garba and Aboubacar, 2000; Garba *et al.*, 2010). There is also a relatively unknown burden of veterinary schistosomiasis caused by the schistosomes *Schistosoma bovis* and *S. curassoni,* and also *S. bovis-curassoni* hybrids (Mouchet *et al.*, 1989; Brémond *et al.*, 1990, 1993), all of which are transmitted by *Bulinus* spp. (Mouchet *et al.*, 1989, 1992; Brémond *et al.*, 1990). Additionally, the occurrence of *S. haematobium* group hybrids, involving a mixture of human and veterinary schistosome species (*S. haematobium-S. bovis*, *S. haematobium-S.*

curassoni, *S. haematobium-bovis-curassoni*) have been reported from humans in Niger (Mouchet *et al.*, 1988; Brémond *et al.*, 1993; Léger *et al.*, 2016), and are common in other African countries (Webster and Southgate, 2003; Huyse *et al.*, 2009, 2013; Webster, Diaw, *et al.*, 2013; Boon *et al.*, 2017; Tian-Bi *et al.*, 2019; Webster *et al.*, 2019). This complicates transmission and epidemiology, whilst raising many questions regarding the general biology and host (snail and mammalian) specificities of these closely related schistosome species.

Our current understanding of *S. haematobium* and *S. bovis* interactions with the intermediate hosts in Niger is based on morphological identifications (cercariae and their snail host), cercarial emergence patterns, and experimental infections. Regarding the intermediate hosts of human schistosomiasis, *Bulinus senegalensis*, *B. forskalii* and *B. truncatus* have previously been reported to transmit *S. haematobium* (Vera *et al.*, 1990, 1992, 1995; Labbo *et al.*, 2003, 2007) whilst *B. umbilicatus* and *B. senegalensis* have been reported as shedding the veterinary schistosomes, *S. curassoni* and *S. bovis* respectively (Mouchet *et al.*, 1992; Vera *et al.*, 1992). However, molecular data are needed to support these predominantly morphological observations for both snail and schistosome, providing a more accurate assessment of snail-schistosome relations and epidemiology (Labbo *et al.*, 2007; Léger *et al.*, 2016). The relationship between *S. haematobium* group species and hybrids with their different *Bulinus* hosts needs further clarification in Niger and in other endemic populations (Tian-Bi *et al.*, 2019), including the outcomes of intramolluscan schistosome co-infections and concurrent infections (Laidemitt *et al.*, 2019).

Aggregation of parasites within intermediate host snails, i.e. the number of schistosomes that successfully infect a snail, including co-infections, has been investigated in both laboratory and field based studies in: *Biomphalaria* spp. (see Minchella *et al.,* 1995; Sire *et al.,* 1999; Davies *et al.,* 2001; Webster *et al.,* 2001; Davies *et al.,* 2002; Eppert *et al.,* 2002; Gower & Webster 2004)*, Bulinus* spp. (see Woolhouse *et al.,* 1990; Dabo *et al.,* 1997; Davies *et al.,* 1999)*, Oncomelania* spp. (see Yin *et al.*, 2008; Lu *et al.*, 2010; Gu *et al.*, 2020), and for other trematodes (Semyenova *et al.*, 2005). Although snail infection prevalence is usually low for schistosomiasis, infected snails often harboured high parasite loads, referred to as 'super shedding' snails, that probably contribute most to local transmission (Eppert *et al.*, 2002). This tendency for metazoan parasites to be aggregated (overdispersed) is well established (Crofton, 1971; Anderson and May, 1978, 1979; Poulin, 1993; Woolhouse *et al.*, 1997), and in snail-schistosome relationships that may be compounded by individual fine-scale genetic compatibility (Mitta *et al.*, 2017; Gu *et al.*, 2020). However, it has not been definitely established whether the causal factor for this is due to new combinations of snail and schistosome genotypes arising through selection, migration or environmental modification allowing for intramolluscan-schistosome proliferation, or whether multiple miracidia infections

alter cercarial production through competition and increased virulence as has been observed during intra-molluscan competition (Davies *et al.*, 2002).

Using a combination of methods, this study investigates the biogeography, snail-schistosome relationships and infection dynamics of *S. haematobium* group species and their *Bulinus* snail hosts along the Niger River Valley to: (i) identify the *Bulinus* species involved in schistosomiasis transmission in the Niamey region of Niger; (ii) identify the schistosome species being transmitted by specific *Bulinus* spp. in these areas; and (iii) investigate the infection dynamics of different co-infecting schistosome species within individual snails.

3.2 Methods

3.2.1 Study area and sample collection

This study was part of the urogenital schistosomiasis control project in Niger, incorporated into the wider Schistosomiasis Consortium for Operational Research and Evaluation (SCORE) programme (Ezeamama *et al.*, 2016). Malacological surveys coupled with snail schistosome infection screening were conducted at potential urogenital schistosomiasis transmission sites, in 16 villages located approximately 60 km upstream (North) and downstream (South) of Niamey, along the Niger River basin.

In total, 68 potential transmission/water contact sites were surveyed monthly from July 2011 to January 2016 (Rabone *et al.*, 2019), the majority of sites were located in the north (n=41) with fewer in the south (n=27). At each site, two collectors searched and collected snails, morphologically identified as *Bulinus* spp. (Brown, 1994) using scoops and by picking them directly from freshwater vegetation for 15-30 minutes. Individual snails were then checked for patent schistosome infections by cercarial shedding. Furcocercous cercariae (having a forked tail) were morphologically identified from descriptions of *Schistosoma* spp. (Frandsen and Christensen, 1984), which were collected and preserved for molecular characterisation on Whatman-Indicating FTA Classic Cards (GE Healthcare Life Sciences, Buckinghamshire, UK) by pipetting individual cercariae onto the card in 2-3 µL (Tian-Bi *et al.*, 2019), and the corresponding infected snails were preserved in 100% ethanol for future molecular identification.

All information on collection dates and site localities containing infected snails can be found in Supplementary Table 3.1.

3.2.2 Molecular Identification and mitochondrial cox*1 analyses of the* Schistosoma *infected* Bulinus

The soft tissue of the individual snails was removed from the shell, and DNA extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Manchester, UK) as described in Pennance *et al.* (2018). Due to the genetic diversity within and between *Bulinus* spp., different primer

combinations were used as described in Table 3.1 (Folmer *et al.*, 1994; Kane *et al.*, 2008). Partial mitochondrial *cox*1 DNA regions were amplified in a reaction volume of 25 µl using 1 µl of template snail DNA and 1 µl of each primer [10 µmol] and brought to a volume of 25 µl with RNA free water, the reaction mixture being combined with Illustra 'Ready-To-Go' PCR beads (GE Healthcare Life Sciences, Buckinghamshire, UK) as described previously by Pennance *et al.* (2018). Different primer combinations were used to amplify a 397-653bp region of the *cox*1, a region used previously for *Bulinus* spp. barcoding studies (Kane *et al.*, 2008), including primers previously published; LCO1490, HCO2198 (Folmer *et al.*, 1994), BulCox1, BulCox7 (Kane *et al.*, 2008), and a newly developed forward primer designed to match identically and anneal at the 5' end of previously sequenced *B. globosus cox*1 (GenBank Accession: AM286294, Kane *et al*. 2008) from Niger; Bglob_CoxF (Table 3.1) was used with reverse primer HCO2198. PCR cycling conditions for each primer combination are outlined in Table 3.1. In addition, two reverse primers; Bt-26R (5'-CGTTCTATAGTAATTCCTGGTGCC-3') and Bt-30R (5'-GGACAAATAATTCCTGGTGCCC-3') were designed directly from sequence alignments to specifically target the regions of variance (as described in the results) and two cycling conditions trialled (PCR cycles for Primer combinations A and B, Table 3.1) .

Table 3.1. PCR primers and cycling conditions for amplification of mitochondrial and nuclear DNA regions of *Schistosoma* and Folmer *cox*1 region of *Bulinus.*

Primer combinations	Primer	Primer sequence (5' - 3')	Forward / Reverse	Marker	Target organism in this study	Reference	PCR cycle 95ºC 5 mins, 40 cycles (95ºC 30 sec, AºC 30sec, 72ºC E mins), 72ºC 10 mins.
A	LC1490	GGTCAACAAATCATAAAGATATTGG	F	*cox*1	*Bulinus* spp.	Folmer et al., 1994	A=44ºC E=1.30 mins
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	R				
B	Bglob_CoxF	TGGTAGGAACCGGACTTTCA	F	*cox*1	*Bulinus globosus*	This study *	A=50ºC E=1.30 mins
	HCO1490	TAAACTTCAGGGTGACCAAAAAATCA	R			Folmer et al., 1994	
C	BulCox1	TTTTTGGWGTTTGATGTGG	F	*cox*1	*Bulinus* spp.	Kane et al., 2008	A=44ºC E=1.30 mins
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	R				
D	BulCox7	GCAATAGGTCCTCTTAAAGG	F	*cox*1	*Bulinus* spp.	Kane et al., 2008	A=44ºC E=1.30 mins
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	R				
E	Asmit1	TTTTTTGGTCATCCTGAGGTGTAT	F	*cox*1	*Schistosoma haematobium & Schistosoma bovis*	Webster et al., 2009	A=58ºC E=1 min
	SbR	CACAGGATCAGACAAACGAGTACC	R				
	ShR	TGATAATCAATGACCCTGCAATAA	R				
F	ETTS2	TAACAAGGTTTCCGTAGGTGAA	F	ITS1-2	*Schistosoma haematobium* group species	Kane and Rollinson, 1994	A=40ºC E=1.30 mins
	ETTS1	TGCTTAAGTTCAGCGGGT	R				
G	WA	GCGAATGGCTCATTAAATCAG	F	18S	*Schistosoma haematobium* group species	Webster et al., 2006	A=44ºC E=1 min
	300R	TCAGGCTCCCTCTCCGGA	R				

Amplicons (4 µl) were visualised on a 2% GelRed agarose gel electrophoresis (90V for 1 hour), and positive reactions were purified using QiaQuick PCR purification columns (Qiagen). Sanger sequencing was performed in both directions using the same primers as used for PCR amplification (except for LCO1490 that consistently produced poor sequence for several samples) and conducted on an Applied Biosystems 3730xl DNA analyser, using a dilution of the original PCR primers. Sequences were manually edited, aligned and trimmed using Sequencher v.5.4.6 (GeneCodes Corp.). The edited *cox*1 consensus sequence data from each snail were collapsed together to identify individuals with identical sequences (haplotypes). The number of unique *cox*1 haplotypes was recorded for each *Bulinus* species. Each haplotype sequence was analysed using the BLASTn search (NCBI) to confirm species

identity. Additionally, each *cox*1 sequence was translated (using the invertebrate mitochondrial genetic code) and the data was analysed using the BLASTx search (NCBI) to further confirm species identity and to check for the occurrence of non-coding data which indicates the presence of mitochondrial pseudogene data. This was performed due to nucleotide deletions observed in the *cox*1 regions from some of the samples.

To analyse the mitochondrial *cox*1 diversity of sequenced *Bulinus* spp., two methods were used. First, a haplotype network was generated using PopART (Clement *et al.*, 2000) to investigate haplotype relationships and diversity of *Bulinus* species. Second, haplotype sequences were imported into Geneious v11.1.4 (www.geneious.com) for phylogenetic analysis together with reference data available from GenBank: Nigerien *Bulinus* spp. accession numbers: AM286308 (*B. forskalii*), AM286294 (*B. globosus*), AM286316 (*B. truncatus*), AM286317 (*B. truncatus*) (see Kane *et al*., 2008). *Biomphalaria glabrata* (AY380531; DeJong *et al.,* 2004) was included as the outgroup. Haplotype alignments were performed and cross checked for consistency using the MAFFT v7.388 (Katoh *et al.*, 2002; Katoh and Standley, 2013), MUSCLE v3.8.425 (Edgar, 2004) and ClustalW v2.1 (Larkin *et al.*, 2007) tools in Geneious v11.1.4. Alignments were executed in PAUP* (Swofford, 2001) and then an appropriate evolutionary nucleotide substitution model was selected in MrModelTest v2.4 (Nylander, 2004) using the Akaike Information Criterion. Bayesian Inference analysis was performed using MrBayes v3.2.7a (Ronquist *et al.*, 2012). The burn-in was defined as the point at which the average standard deviation of split frequencies (ASDOSF) reported from MrBayes output was at least <0.01 over 5 million generations, although a burn-in of 3.5 million generations was used for consistency. Clades were considered to have high nodal support if Bayesian inference posterior probability was ≥0.95, therefore tree nodes with <0.95 were collapsed in SumTrees v4.4.0 (Sukumaran and Holder, 2010) to give the final tree topology.

3.2.3 Genetic profiling and molecular species identification of the Schistosoma *cercariae*

DNA was prepared from individual cercariae on FTA cards using an alkaline elution method as previously described by Webster *et al.,* (2015). The microsatellite reactions were performed using the Type-it® Microsatellite PCR Kit (Qiagen) in 12.5 µl reactions containing 2 µl of alkaline eluted schistosome DNA, 0.2 µM of each primer and 1.25 µl of the Type-it® Microsatellite PCR Kit Q-Solution. The primers and PCR cycle are the same as those reported in Webster *et al*., (2015). Amplicons (4 µl) were visualised by gel electrophoresis on a 3% GelRed agarose gel (90V for 1 hour). Positive reactions were diluted 1 in 10 before being mixed with 0.35 µl of GS500Liz size standard (Applied Biosystems, USA) denatured and injected at 3 kV and run at 12 kV for 12 seconds into the Applied Biosystems 3130xl DNA analyser for analysis and the data were analysed using Geneious 11.1.4 (www.geneious.com)

using the microsatellite plugin (Biomatters Ltd. V1.4.6). A minimum of 20 (except for one snail, where only 4 cercariae were recovered) and a maximum of 52 cercariae were analysed from each shedding snail (Supplementary Table 3.1). Individual cercariae were genotyped using the Panel 1 microsatellite loci (Table 3.2) previously developed by Webster *et al*., (2015).

Allele calls were checked and edited in Geneious 11.1.4 (www.geneious.com) using the microsatellite plugin (Biomatters Ltd. V1.4.6). Out of the nine microsatellite loci (Table 3.2), six provided good quality data for all cercariae analysed. The three loci (C102, C111 and Sh7) (Table 3.2) not included in the analysis proved species specific to *S. haematobium.* Matching cercarial microsatellite profiles from each snail (indicating clonal cercariae) were then grouped together into individual MLGs for each infected snail. We recorded the number of different cercarial MLG's originating from each snail and the number of cercariae within each MLG. To check for potential sampling bias in relation to the numbers of cercarial MLG's found per snail, a Pearson's correlation test was performed in R version 3.5.2 (R Core Team, 2018) comparing the number of cercarial genotypes observed per infected *Bulinus* spp. with the number of cercariae analysed.

Table 3.2. Microsatellite loci and primers (from Webster *et al.,* 2015) used for identification of *Schistosoma* cercariae genotypes.

Loci	Forward Primer (5'- 3')	Reverse Primer (5'- 3')	Dye	Size Range (bp)	Repeat	PCR Cycle
C102	TGTCTCTGTGAATGACCGAAT	TTAGATGAATAATAATGTTGAAACCAC	VIC	184-199	ATT	95ºC 5 mins, 32 cycles (95ºC 30 sec, 54ºC 90 sec, 72ºC 3 mins), 60ºC for 39 mins
Sh1	GCATCCAATTTCGTACAC	CCACATTAGGCCAACAAG	VIC	245-284	AAT	
Sh14	GTCCTCCTTCCCTCTTTG	CACATTCGTCCTAGATATCG	NED	184-240	ACTC	
C131	CTTGTCATTTGGGCATTGTG	CATGGTGAGGTTCAAACGTG	NED	253-265	AAT	
Sh6	GGTGGATTACGCAATAG	TTTAATCAACCGGGTGTC	NED	309-321	AAT	
Sh9	GGGATGTATGCAGACTTG	TTGTTTGGCTGCAGTAAC	6-FAM	197-227	AAT	
Sh3	GCTGAGCTTGAGATTG	CTTCTGTCCCATCGATACC	6-FAM	270-366	AAT	
C111	CCCTTGTCTTCAATGCGTTA	GAACGTCTAACTGGCGATCA	PET	201-225	ATT	
Sh7	TCCAAGCACCATTATCAAG	ACGGAAACTTGTTGAAATG	PET	293-311	AAT	

3.2.4 Schistosoma *cercariae species and hybrid identification*

Both mitochondrial and nuclear DNA regions were analysed to determine the schistosome species or hybrid status of each MLG (Huyse *et al*., 2009; Webster, Diaw, *et al*., 2013). All *cox*1, ITS and 18S reactions were performed using PuReTaq Ready-To-Go PCR beads (GE Healthcare Life Sciences, Buckinghamshire, UK) in 25 µl reactions composed of 3 µl of alkaline eluted schistosome DNA, 1 µl of each primer at a concentration of 10 µmol and the remaining volume composed of RNA free water. PCR cycling conditions differed for the organism and target gene as shown in Table 3.1. The diagnostic *cox*1 PCR, that distinguishes between *S. bovis* (306 bp) and *S. haematobium* (543 bp) based on the size of the *cox*1 amplicon (Webster *et al*., 2010), was used to screen all the selected cercariae MLG's (Table 3.1 and Supplementary Table 3.1). A subset of the amplicons was sequenced to confirm the mitochondrial genotypes and to enable preliminary haplotype analyses.

The nuclear ITS1+2 rDNA region (including the 5.8S gene region) was also amplified (~915 bp) (Table 3.1) and sequenced to identify the species-specific SNP's (Table 3.3). Additionally, a fragment of the 5' end of a second rDNA region (18S) (Webster *et al.*, 2006) containing additional species specific SNPs (Table 3.3) was amplified (289 bp) and sequenced for a subset of the cercariae.

Sequence identity was confirmed using NCBI BLASTn and / or by comparison to reference data (Webster *et al.*, 2010). Following successful sequencing and analysis, both mitochondrial (cox1) and nuclear (ITS + 18S) profiles were asigned to each cercarial MLG to determine species or hybrid status.

Table 3.3. Expected and observed *Schistosoma* species specific SNP positions in nuclear ITS (1+2) and 18S (WA-300R) genes sequenced from cercariae collected during study.

Expected Schistosome Species Specific SNP positions (bp)

		ITS (1+2)					**18S (WA-300R)**		
***Schistosoma* species**	**mt *cox* 1**	**50**	**120**	**170**	**225**	**877**	**138**	**163**	**210**
Schistosoma haematobium	*S. h*	*S. h* (G)	*S. h* (C)	*S. h* (G)	*S. h* (C)	*S. h* (T)	*S. h* (T)	*S. h* (C)	*S. h* (C)
Schistosoma bovis	*S. b*	*S. b* (A)	*S. b* (T)	*S. b* (A)	*S. b* (T)	*S. b* (C)	*S. b* (C)	*S. b* (C)	*S. b* (T)
Schistosoma curassoni	*S. c*	*S. c* (A)	*S. c* (T)	*S. c* (A)	*S. c* (T)	*S. c* (T)	*S. c* (T)	*S. c* (T)	*S. c* (T)

Observed Schistosome Species Specific SNP positions (bp)

		ITS (1+2)					**18S (WA-300R)**		
***Schistosoma* species**	**mt *cox* 1**	**50**	**120**	**170**	**225**	**877**	**138**	**163**	**210**
Schistosoma haematobium	*S. h*	*S. h* (G)	*S. h* (C)	*S. h* (G)	*S. h* (C)	*S. h* (T)	*S. h* (T)	*S. h* (C)	*S. h* (C)
Schistosoma bovis	*S. b*	*S. b* (A)	*S. b* (T)	*S. b* (A)	*S. b* (T)	*S. b* (C)	*S. b* (C)	*S. b* (C)	*S. b* (T)
Hybrid 1 (*S. h-b*)	*S. b*	*S. h* (G)	*S. h* (C)	*S. h* (G)	*S. h* (C)	*S. h* (T)	*S. h* (T)	*S. h* (C)	*S. h* (C)
Hybrid 2 (*S. h-b-c*)	*S. b*	*S. h-c* (G/A)	*S. h-c* (C/T)	*S. h-c* (G/A)	*S.h-c* (C/T)	*S. h-c* (T)	*S. h* or *S. c* (T)	*S. h or S. c* (C/T)	*S. h or S. c* (T)

3.2.5 Schistosoma *cercariae microsatellite population structure analysis*

To gain insights into the population structure of the cercariae, the microsatellite data were subjected to Principal Component Analysis (PCA) using the "adegenet" v2.1.1 package (Jombart, 2008) in R version 3.5.2 (R Core Team, 2018). PCA was performed on two datasets with all loci including the missing data (from the 3 *S. haematobium* specific loci C102, C111, Sh7) and excluding the missing data. Each cercarial MLG analysed was grouped into the *Schistosoma* spp. identified: *S. haematobium*, *S. bovis,* or *S. haematobium* group hybrids. Analyses were run both including and excluding missing microsatellite loci data which occurred due to the specificity of the microsatellite primers to *S. haematobium*.

Genetic variability of each locus was estimated in all genotyped cercariae by calculating allelic richness (*Ar*), expected heterozygosity (*He*) and observed heterozygosity (*Ho*) using the "adegenet" v2.1.1 package (Jombart, 2008) in R version 3.5.2 (R Core Team, 2018). Results were calculated for: *S. haematobium*, *S. bovis,* or *S. haematobium* group hybrids, and additionally for the two populations of *S. bovis* collected from the North and South of Niamey. Plots of *He* against *Ho* were produced for each *Schistosoma* species and *S. bovis* population to compare potential effects of inbreeding across species and geographically

isolated populations. A PCA plot following the same methods as above was also produced for *S. bovis* populations collected from the North and South of Niamey was also produced.

3.2.6 Distribution of infected Bulinus *and* Schistosoma *species and environmental factors influencing their relationships*

Bulinus and *Schistosoma* spp. distribution data were visualised using QGIS v3.0.1 Girona (http://qgis.osgeo.org). Collection sites were grouped by village and points scaled to the number of infected *Bulinus* spp. Digital shape files for Niger administrative regions and Inland water areas were obtained from DIVA-GIS (https://www.diva-gis.org).

To investigate the effect snail habitats (river, pond, secondary irrigation canal, tertiary irrigation canal, branching stream), the species of schistosome infecting each snail and the species of snail on the number of cercarial genotypes released from each snail, a Linear Mixed-Effects Model (LMM) was selected for best fit of the data. Data for snails collected from streams, rice paddies and spillways were excluded, as there was only a single record of infected *Bulinus* spp. from each of these habitat types. As such, the total number of snails used in the analyses was 84. Snail habitats, snail species and schistosome species were selected as fixed variables and Village as a random variable. The response variable (number of cercarial genotypes released from each snail) was log transformed to increase model fit. The Linear Mixed-Effects Model (LMM) was fitted using the "lme4" package v1.1-12 (Bates *et al.*, 2015). The effects of fixed variables were tested with a type II Wald chi-square test using the "car" package v3.0-6 (Fox and Weisberg, 2019). All analyses were performed using R version 3.5.2 (R Core Team, 2018).

3.3. Results

3.3.1 Distribution and abundance of the Schistosoma *infected* Bulinus *spp.*

Of the 15,288 *Bulinus* spp. snails collected from the study sites in Niger, 137 had patent schistosome infections. At multiple time points between 2011 and 2015, schistosome infected snails were collected at 26 of the 68 sites surveyed from villages both in the North and South of Niamey (Figure 3.1). *Schistosoma* cercariae and snail molecular data were obtained from 87 of the 137 infected *Bulinus* spp. collected from 24 of the 68 sites (50 snails could not be included in the study due to specimen degradation, Table 3.4). The 87 infected *Bulinus* spp. included were found in several different habitat types surveyed: rivers (n=9), ponds (n=44), streams (n=1), rice paddies (n=1), secondary irrigation canals (n=10), tertiary irrigation canals (n=10), branching streams (n=11) and spillways (n=1).

3.3.2 Molecular identification and cox*1 analyses of the* Schistosoma *infected* Bulinus *species*

Of the 87 infected snails included in the analyses, 82 were morphologically identified at the time of collection as *B. truncatus* and five as being from the *B. forskalii* group (Table 3.4). Molecular analysis of 61 of the morphologically identified *B. truncatus,* confirmed that 57 were *B. truncatus* but four were *B. globosus*. Three of the five *B. forskalii* were also confirmed by molecular data. For the remaining 23 infected *Bulinus* spp., poor DNA quality precluded molecular analysis, and therefore identifications were made solely by morphology (Table 3.4): *B. forskalii* (n=2)*, B. truncatus* (n=21). The geographical distributions of the different snail species are shown in Figure 3.1.

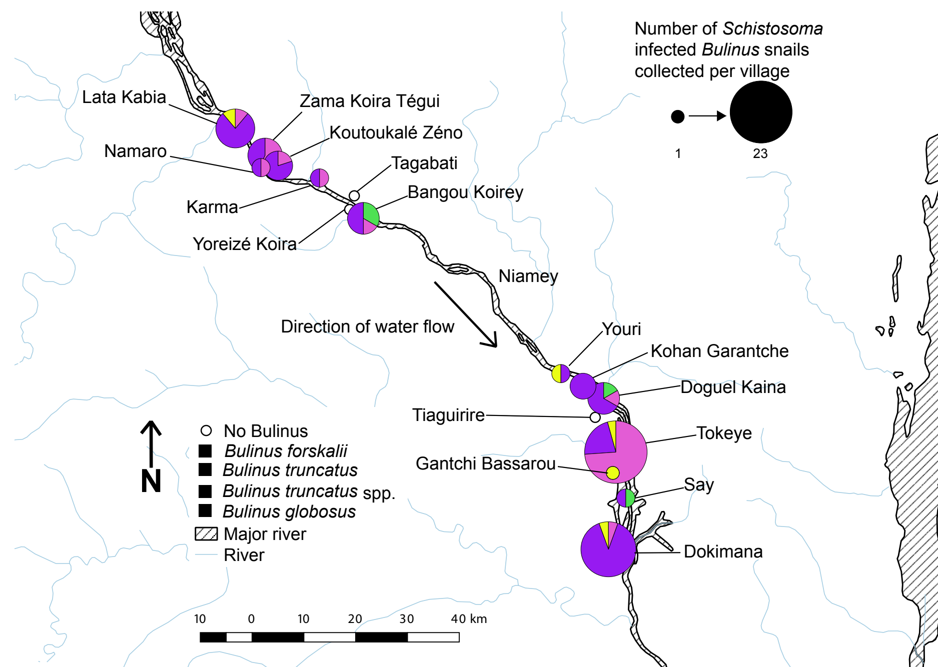


Figure 3.1. Infected *Bulinus* spp. snail distribution in Niamey region of Niger.

Table 3.4. *Schistosoma* spp. infections identified from *Bulinus* snails identified using morphology and molecular techniques from Niger. mol = molecularly confirmed, and mor = morphologically confirmed. *Co-infected snails shedding more than one schistosome species / hybrid type. ^*Bulinus truncatus* spp. based on morphology and preferential amplification of non-coding mitochondrial genes.

			Bulinus with each *Schistosoma* infection				*Bulinus* species identified using molecular and morphological methods			
	Village	No. of infected *Bulinus* spp.	*Schistosoma bovis*	*Schistosoma haematobium*	*Schistosoma haematobium* group hybrids	Co-infection*	*Bulinus forskalii* (mol / mor)	*Bulinus globosus* (mol / mor)	*Bulinus truncatus* (mol / mor)	*Bulinus truncatus* spp.^
North	Zama Koira Tégui	7	0	2	3	2	0 / 0	0 / 0	2 / 1	4
	Yoreizé Koira	0	0	0	0	0	0 / 0	0 / 0	0 / 0	0
	Tagabati	0	0	0	0	0	0 / 0	0 / 0	0 / 0	0
	Bangou Koirey	6	6	0	0	0	0 / 0	2 / 0	3 / 0	1
	Namaro	2	1	0	1	0	0 / 0	0 / 0	1 / 0	1
	Lata Kabia	9	1	3	3	2	1 / 0	0 / 0	3 / 4	1
	Koutoukalé Zéno	5	1	1	1	2	0 / 0	0 / 0	0 / 4	1
	Karma	2	1	0	1	0	0 / 0	0 / 0	0 / 1	1
South	Youri	2	2	0	0	0	0 / 1	0 / 0	0 / 1	0
	Tokèye	23	23	0	0	0	0 / 1	0 / 0	1 / 4	17
	Gantchi Bassarou	1	1	0	0	0	1 / 0	0 / 0	0 / 0	0
	Dokimana	18	18	0	0	0	1 / 0	0 / 0	12 / 4	1
	Say	2	2	0	0	0	0 / 0	1 / 0	0 / 1	0
	Kohan Garantche	4	4	0	0	0	0 / 0	0 / 0	4 / 0	0
	Doguel Kaina	6	6	0	0	0	0 / 0	1 / 0	3 / 1	1
	Tiaguirire	0	0	0	0	0	0 / 0	0 / 0	0 / 0	0
	Total (North)	31	10	6	9	6	1 / 0	2 / 0	9 / 10	9
	Total (South)	56	56	0	0	0	2 / 2	2 / 0	20 / 11	19
	Total (Overall)	87	66	6	9	6	3 / 2	4 / 0	29 / 21	28

Eight different *cox*1 haplotypes were identified from 29 of the 57 infected *B. truncatus* (Table 3.5). For the remaining 28 *B. truncatus* samples, only non-coding mitochondrial *cox*1 sequences were generated, possibly showing the occurrence of pseudogenes. These data could not be translated (contained many stop codons) and contained deletions (10-18 bp). Attempts to obtain the functional partial *cox*1 DNA region, using alternative primers and combinations including designed primers specifically targeting the functional *cox*1 copy (Bt-26R) and the non-coding gene (Bt-30R), either failed to amplify the target region, or produced mixed sequence profiles. The resulting data, however, were sufficient to confirm the snail species as within the *B. truncatus* species complex (see Table 3.4). The three *B. forskalii* fell into two haplotypes and the four *B. globosus* fell into three different haplotypes (Table 3.5). The *Bulinus cox*1 haplotype network revealed clear species division and a diverse population with no structuring or dominant haplotypes (Figure 3.2). Phylogenetic tree topology (Figure 3.3) showed the three expected monophyletic groups: 1) *B. forskalii*, 2) *B. globosus,* and 3) *B. truncatus.* There was no support for significant intra-species variation between the haplotypes in each clade, further confirming species assignment.

Table 3.5. *Bulinus* species haplotypes identified and the numbers of snails representing each with GenBank accession numbers. nc = probably translocated pseudogenes.

Bulinus species	Bulinus species haplotype	Number of samples	Sequence length (number of bp)	GenBank Submission
B. truncatus	*B. truncatus* 1	3	653	TBC
	B. truncatus 2	10	652	TBC
	B. truncatus 3	7	653	TBC
	B. truncatus 4	4	636	TBC
	B. truncatus 5	2	653	TBC
	B. truncatus 6	1	653	TBC
	B. truncatus 7	1	397	TBC
	B. truncatus 8	1	636	TBC
	B. truncatus 9 (nc)	6	NA	NA
	B. truncatus 10 (nc)	16	NA	NA
	B. truncatus 11 (nc)	3	NA	NA
	B. truncatus 12 (nc)	3	NA	NA
B. forskalii	*B. forskalii* 1	2	651	TBC
	B. forskalii 2	1	651	TBC
B. globosus	*B. globosus* 1	2	600	TBC
	B. globosus 2	1	589	TBC
	B. globosus 3	1	414	TBC

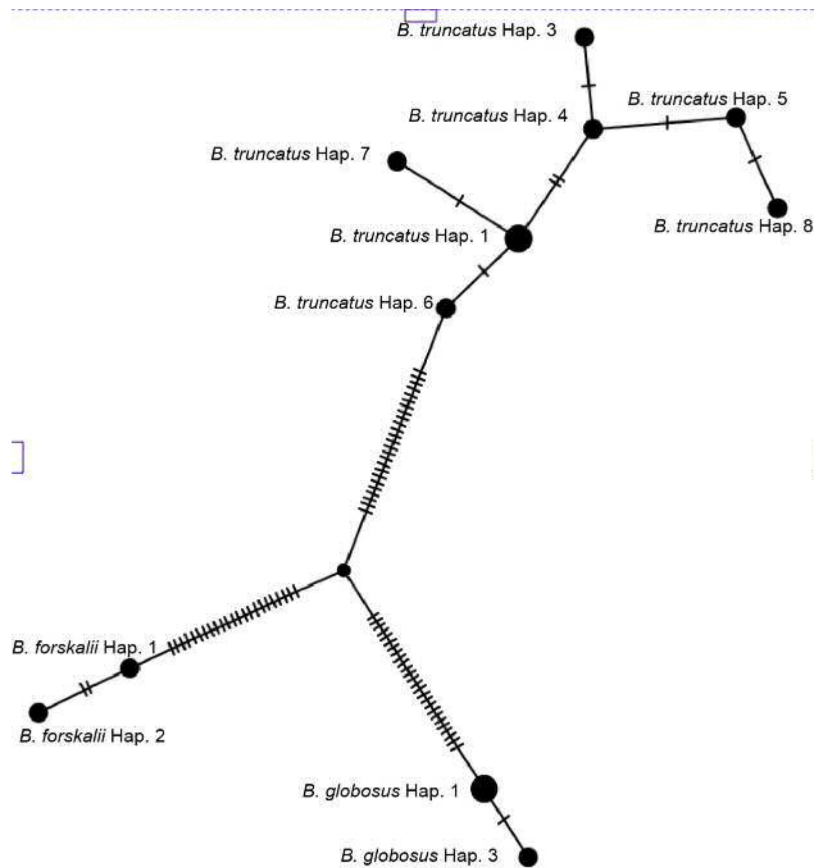


Figure 3.2. Haplotype network of unique *cox*1 haplotypes found for each *Bulinus* species generated using PopArt (Clement *et al*., 2000).

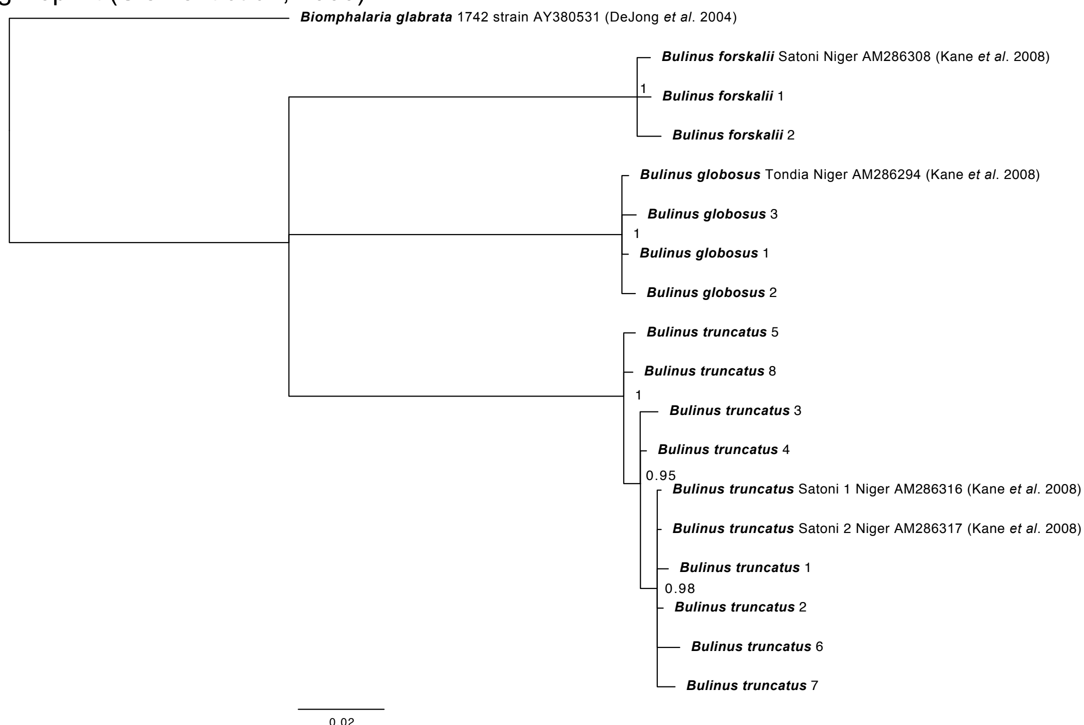


Figure 3.3. Bayesian analysis of the partial mitochondrial *cox*1 haplotype dataset from *Bulinus* spp. Phylogenetic tree constructed using MrBayes v3.2.7A (Ronquist *et al*., 2012) under the GTR+G model; 5,000,000 generations; 3,500,000 generations burn-in. Posterior probabilities shown and all branches with <0.95 posterior probability have been collapsed. The branch length scale bar indicates the number of substitutions per site.

3.3.3 Schistosoma *cercariae microsatellite analysis*

In total, 1,124 cercariae were characterised from the 87 infected *Bulinus* spp. (Supplementary Table 3.1), identifying 186 unique cercarial MLGs. Cercarial genotypes differed between snails, with identical genotypes frequently identified from individual cercariae shed from the same snail, due to the clonal replication of the schistosome larval stages (Supplementary Table 3.1). However, in several cases cercariae originating from the same snail had different genotypes, indicating co-infections of the same (mono-) or different (co-) species. The number of different cercarial MLGs per individual snail ranged from 1 to 10, with 52 (60%) of the infected *Bulinus* shedding cercariae with a single MLG and 35 (40%) shedding cercariae with >1 MLG. Sample size analyses showed that the number of cercarial genotypes observed per infected *Bulinus* spp. was not dependent on the number of cercariae analysed (see Figure 3.4, Pearson's correlation coefficient, r = 0.04).

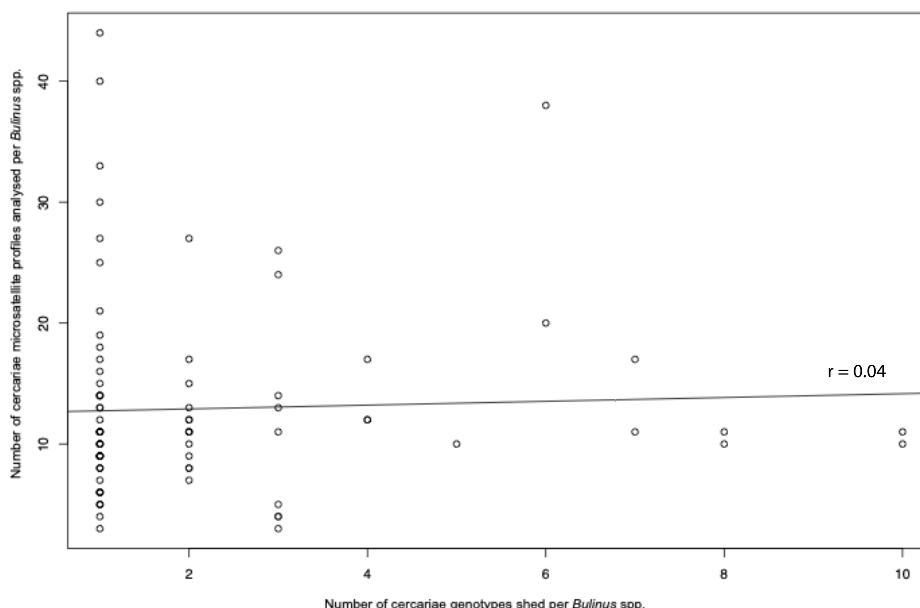


Figure 3.4. Pearson's correlation test comparing the number of cercarial genotypes shed per *Bulinus* spp. to the cercarial microsatellite profiles amplified and analysed per *Bulinus* spp.

Table 3.6. *Schistosoma haematobium* group infections observed in *Bulinus* spp. from Niger villages.

Bulinus species	Snail - *Schistosoma* infection profile	Number of snails	Suspected definitive host
B. truncatus	*S. bovis*	57	Bovidae
	S. haematobium	6	Human
	Hybrid 1 (*S.h-b*)	9	Human
	Co-infection: *S. haematobium* & Hybrid 1 (*S.h-b*)	1	Human
	Co-infection: *S. haematobium* & Hybrid 2 (*S.h-b-c*)	1	Human
	Co-infection: Hybrid 1 (*S.h-b*) & Hybrid 2 (*S.h-b-c*)	4	Human
B. forskalii	*S. bovis*	5	Bovidae
B. globosus	*S.bovis*	4	Bovidae

3.3.4 Schistosoma *cercariae species / hybrid identification*

Of the infected snails, 66 (76%) were shedding the livestock schistosome *S. bovis* only*,* six (7%) were shedding the human schistosome *S. haematobium*, and 9 (10%) the *S. haematobium* group hybrids. The remaining six snails (7%) harboured co-infections with different *S. haematobium* group hybrids and/or *S. haematobium* (Table 3.6).

Of the hybrid cercariae identified, two hybrid genetic profiles were observed (Table 3.3). The most common hybrid (*S. haematobium-bovis, S.h-b*), displaying a *S. bovis* mt *cox*1 and *S. haematobium* nuclear profile, was shed from 14 (16%) of the infected *Bulinus.* The second hybrid genetic profile was more unusual, consisting of cercariae with a *S. bovis* mt *cox*1 and a mixed *S. haematobium / S. curassoni* nuclear profile. The unusual genetic profiles of these rare human infecting schistosomes were further confirmed by analysis of the partial 5' end of the 18S nuclear ribosomal gene region, which contains further *S. bovis / S. curassoni* inter-species SNP's (Table 3.3). Schistosome co-species snail infections were observed with: *S. haematobium* + hybrid 1 (*S.h-b*), *S. haematobium* + hybrid 2 (*S.h-b-c*) or hybrid 1 (*S.h-b*) + hybrid 2 (*S.h-b-c*). Mono-species/hybrid infections of snails were observed with hybrid 1 (*S.h-b*), *S. haematobium* or *S. bovis.* No co-species infections were found in snails infected with *S. bovis.*

*Cox*1 haplotype data was obtained from 18 *S. bovis cox*1 amplicons, of which 15 were from cercariae identified as *S. bovis* and three from hybrid cercariae (*S.h-b, S.h-b-c*). Four *S. bovis* mt *cox*1 haplotypes were identified (Sb-Hap-1, Sb-Hap-2, Sb-Hap-3, Sb-Hap-4), with all the hybrid cercariae presenting the same *cox*1 haplotype (Sb-Hap-2) whereas all the *S. bovis* cercariae presented all haplotypes (1-4). Seven *S. haematobium* cercariae shed from six *B. truncatus* presented two *cox*1 haplotypes (Sh-Hap-1, Sh-Hap-2). Both the *S. haematobium* mt *cox*1 haplotypes are common haplotypes found across Africa (Webster *et al.*, 2012) with Sh-Hap-1 being the most common and representing the H1 pan African haplotype (Webster *et al*., 2012). Although the sample sizes are biased towards *S. bovis,* the data suggests that the mitochondrial *cox*1 diversity is higher for *S. bovis* compared to *S. haematobium,* supporting observations from Webster *et al.,* (2010). Cercariae with multiple *cox*1 haplotypes were also observed from cercariae shed from the same snail (see snail: LA79, Supplementary Table 3.1), supporting the observations of multiple mono-species infections of individual snails inferred from cercarial MLG data.

3.3.5 Schistosoma *species and hybrid associations with the different* Bulinus *species.*

All of the *B. forskalii* (n=5), *B. globosus* (n=4) and 73% of the *B. truncatus* (n=57) were only shedding *S. bovis* cercariae (Table 3.6). Six of the *B. truncatus* (8%) were only shedding *S. haematobium* and nine of the *B. truncatus* (12%) were only shedding the *S. haematobium-bovis* hybrid 1 (*S.h-b*). All other *B. truncatus* (n=6) had co-infections, four involving both hybrid

1 (*S.h-b*) + hybrid 2 (*S.h-b-c*), one involving *S. haematobium* + hybrid 1 (*S.h-b*) and one involving *S. haematobium* + hybrid 2 (*S.h-b-c*) (Table 3.6). No co-infections or hybrid infections were observed in *B. globosus* or *B. forskalii*.

3.3.6 Multi Locus Genotypes and snail / Schistosoma *species associations*

The number of cercarial MLGs per snail showed differences by snail species, for example; two *B. truncatus* were found shedding *S. bovis* cercariae of up to ten MLGs, one *B. forskalii* was shedding *S. bovis* cercariae with up to six MLGs whereas all four *B. globosus* snails were shedding *S. bovis* cercariae of a single genotype. However, the sample sizes of *B. forskalii* and *B. globosus* were very small in comparison to *B. truncatus* and so this observation may change with increased sample sizes (Supplementary Table 3.1).

The highest numbers of mono-species MLG's found per snail were associated with *S. bovis* infections, indicating potential multiple co-infections with different *S. bovis* miracidia. A wide range of these multiple mono-species co-infections with different *S. bovis* miracidia were observed, with MLG's per snail ranging from 1-10 (Supplementary Table 3.1). However, again these observations may be affected by sample size due to the relatively high numbers of *S. bovis* infected snails (Table 3.4).

For the mono-species infections involving *S. haematobium* or the *S. haematobium* group hybrids, only low numbers of MLG's were identified per snail (1 or 2 MLG per snail for *S. haematobium* and 1 per snail for *S. haematobium* group hybrids). This indicates that infections only involved 1 or 2 miracidia for these species/hybrids.

3.3.7 Microsatellite population structure of human and bovine Schistosoma *cercariae*

PCA analysis including and excluding missing data showed the same population clustering patterns. The PCA plot (Figure 3.5) reveals two population clusters one that included *S. haematobium* and the *S. haematobium* group hybrids and a second that included only *S. bovis* suggesting no gene flow between the populations. Allelic richness, *He* and *Ho* and the size ranges of each locus varied between the *S. haematobium* group species and between geographically isolated populations of *S. bovis* (Table 3.7 and Figure 3.6). *S. bovis* had the largest allelic size range at each locus when compared to *S. haematobium* and the *S. haematobium* group hybrids, although ranges still overlapped (Table 3.7). *S. bovis* also on average displayed a *He* slightly greater than *Ho* (Figure 3.6A). However, it was observed that the populations of *S. bovis* collected south of Niamey (where the majority of *S. bovis* infected snails were collected) contributed to this the most, with on the other hand *He* being slightly less than *Ho* at several loci when considering *S. bovis* cercariae collected from North of Niamey (Figure 3.6B). The PCA plot revealed that a single population cluster of *S. bovis* was

present with little structuring dividing the populations from the North and South of Niamey (Figure 3.7).

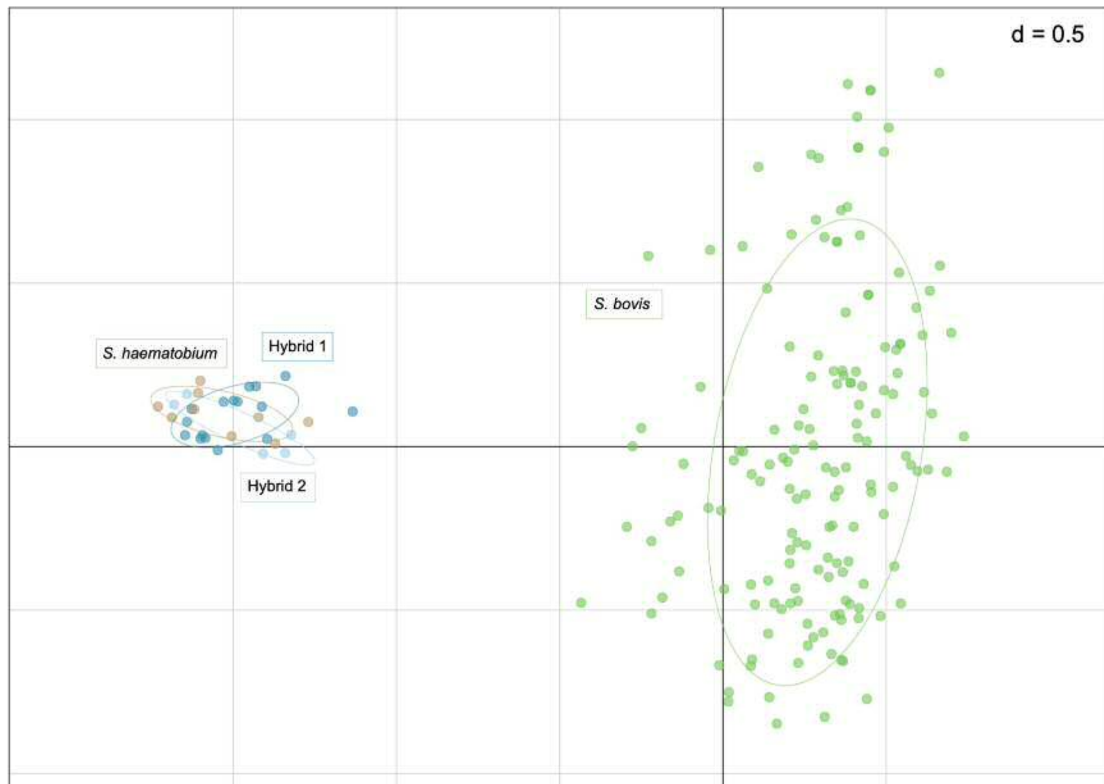


Figure 3.5. Biplot of a principal components analysis on the allele frequencies of *Schistosoma* spp. cercariae shed from *Bulinus* spp. collected in the Niger River Valley (186 observations). PCA produced using six microsatellite loci (excluding three *Schistosoma haematobium* specific loci). The cumulative proportion is 19.8 for the first two principal components (PC1: 14.1%; PC2: 5.7%). Observations are coloured by the *Schistosoma* species profile (see Table 3.3).

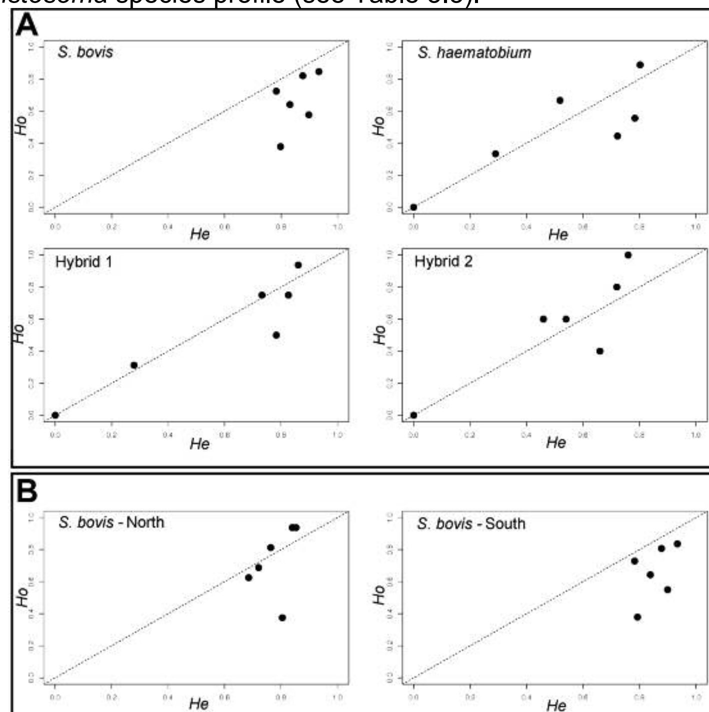


Figure 3.6. Observed (*Ho*) and expected (*He*) heterozygosity plots of 6 microsatellite loci genotyped from *Schistosoma haematobium* group cercariae from the Niger River Valley.

Table 3.7. Summary statistics for 6 microsatellite loci from 186 genotyped *Schistosoma haematobium* group cercariae from the Niger River Valley giving allele size range, allelic richness (*Ar*), expected (*He*) and observed (*Ho*) heterozygosity for each locus. Showing summary statistics dividing *S. bovis* populations (grey fill) collected from the North and South of Niamey (see Figure 3.8). n = number of cercariae multi locus genotypes for each population. Microsatellite loci from Webster *et al*. (2015).

Microsatellite loci*	Sh9	Sh3	Sh1	Sh14	C131	Sh6
***S. bovis* (n = 156)**						
Allele size range	194 - 248	294 - 366	242 - 287	186 - 237	253 - 296	302 - 338
Ar	24	22	16	19	15	24
He	0.7981	0.9340	0.8768	0.7830	0.8313	0.8983
Ho	0.3782	0.8462	0.8205	0.7244	0.6410	0.5769
***S. haematobium* (n = 9)**						
Allele size range	196 - 218	282 - 312	254 - 278	194 - 220	253 - 253	309 - 318
Ar	6	7	5	6	1	3
He	0.7222	0.8025	0.5185	0.7840	0	0.2901
Ho	0.4444	0.8889	0.6667	0.5556	0	0.3333
Hybrid 1 (n = 16)						
Allele size range	196 - 229	276 - 309	247 - 278	188 - 232	253 - 253	309 - 321
Ar	8	10	8	10	1	4
He	0.7832	0.8262	0.7324	0.8613	0	0.2793
Ho	0.5000	0.7500	0.7500	0.9375	0	0.3125
Hybrid 2 (n = 5)						
Allele size range	196 - 212	276 - 300	257 - 269	196 - 220	253 - 253	309 - 318
Ar	4	5	3	5	1	3
He	0.6600	0.7200	0.5400	0.7600	0	0.4600
Ho	0.4000	0.8000	0.6000	1.0000	0	0.6000
***S. bovis* North (n = 16)**						
Allele size range	194 - 218	309 - 351	247 - 278	188 - 200	259 - 268	304 - 326
Ar	8	11	8	7	6	9
He	0.8047	0.8535	0.8398	0.7207	0.6855	0.7637
Ho	0.3750	0.9375	0.9375	0.6875	0.6250	0.8125
***S. bovis* South (n = 140)**						
Allele size range	194 - 248	294 - 366	242 - 287	186 - 237	253 - 296	302 - 338
Ar	24	22	16	18	15	24
He	0.7927	0.9342	0.8779	0.7826	0.8383	0.8993
Ho	0.3786	0.8357	0.8071	0.7286	0.6429	0.5500

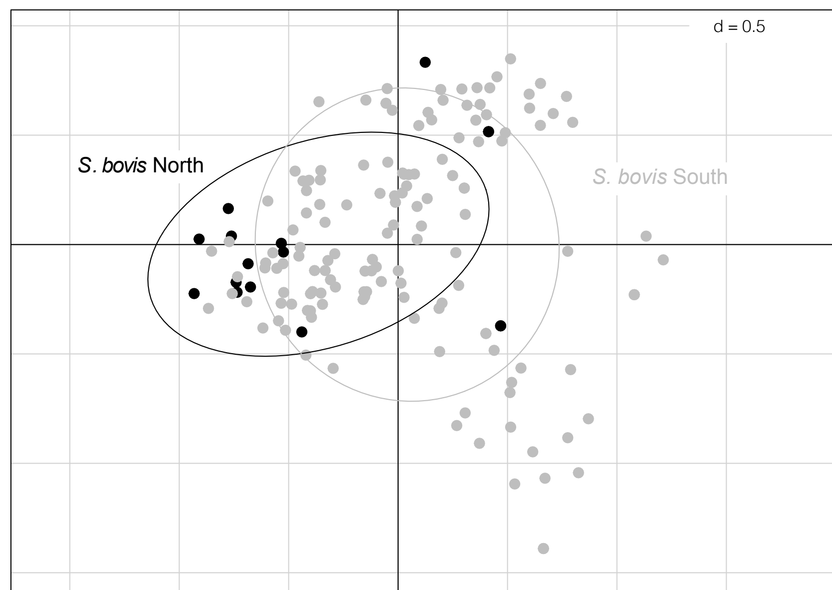


Figure 3.7. Biplot of a principal component analysis on the allele frequencies of *Schistosoma bovis* cercariae from *Bulinus* spp. collected in the North and South of Niamey in the Niger River Valley (156 observations). PCA produced using six microsatellite loci. The cumulative proportion is 14.2 for the first two principal components (PC1: 7.4%; PC2: 6.8%). Observations are coloured by populations of *S. bovis* collected from the North and South of Niamey (see Table 3.7).

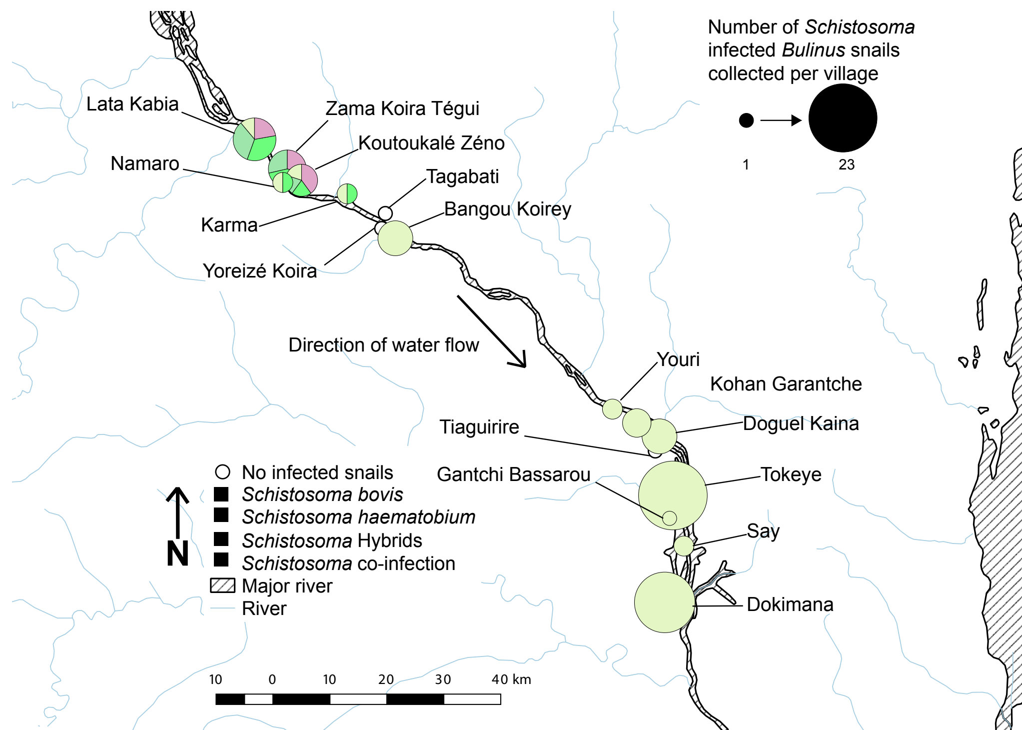


Figure 3.8. Distribution of *Schistosoma* spp. cercariae shed from *Bulinus* spp. collected North and South of Niamey in the Niger River Valley.

3.3.8 Distribution of infected Bulinus *and the* Schistosoma *species*

S. haematobium and the *S. haematobium* group hybrids involved in human urogenital schistosomiasis infections were only detected in five villages north of Niamey (Figure 3.7). *S. bovis* occurred throughout the study villages in the north and south (except Yoreizé Koira and Tagabati) but was the only schistosome species found in the southern sites, where the majority (n=56) of infected snails infected were collected (Table 3.4), predominantly *B. truncatus* collected from two villages: Tokeye (n=23) and Dokimana (n=18) (Figure 3.1). Considerably fewer snails were involved in the transmission of human urogenital schistosomiasis (n=21) compared to those involved in the transmission of the bovid schistosomes (Table 3.6). The infected *B. globosus* and *B. forskalii* were present in low numbers in both the northern and southern sites, transmitting *S. bovis*.

There was no significant effect of snail habitat type (χ^2=0.53, *df*=4, *P*=0.26), *Bulinus* species (χ^2=0.75, *df*=3, *P*=0.86) nor schistosome species (χ^2=2.58, *df*=3, *P*=0.46) on the number of cercariae MLGs found per infected *Bulinus* spp.

3.4 Discussion

Molecular identification of schistosome cercariae and their snail hosts has proved vital for identifying the species of schistosome being transmitted in the Niger River Valley, enabling the future mapping of human and animal schistosomiasis transmission and risk. Additionally, unravelling the relationships between the different schistosome species and their snail host

species at a focal level, allows us to gain a better understanding of disease transmission and snail-schistosome epidemiology. Here we have identified the sympatric transmission of *S. haematobium* and *S. bovis,* causing human and livestock schistosomiasis respectively, in the northern area of the Niger River Valley region, and allopatric distribution of bovid schistosomes in southern sites (Figure 3.8). We also detected *S. haematobium-bovis* hybrids, known human pathogens, adding to the geographical range of these hybrids now reported in several countries, Senegal, Niger, Mali, Côte d'Ivoire, Malawi (Huyse *et al.*, 2009; Léger *et al.*, 2016; Soentjens *et al.*, 2016; Webster *et al.*, 2019) and also imported into Corsica, France (Moné *et al.*, 2015). Additionally, *S. haematobium-bovis-curassoni* hybrid cercariae were identified, confirming the transmission of this unusual hybrid combination previously reported from humans in Niger (Léger *et al.*, 2016).

The microsatellite data analysis showed no gene flow between the human (*S. haematobium* and the hybrids) and cattle (*S. bovis*) schistosome populations analysed (Figure 3.5). This suggests that strong reproductive barriers exist between the two populations and that the observed hybrids are not first-generation resulting from zoonotic and / or zooanthroponotic inter-species interactions. These hybrids appear to be introgressed forms, with parts of the *S. bovis* genome introgressed into *S. haematobium*, leaving two differentiated parental populations that are not panmictic or leading to hybrid speciation. This was also reported in recent studies in Senegal (Boon *et al.*, 2019) and Niger (Platt *et al.*, 2019), and indicates that more research is warranted to understand *S. haematobium* group species hybridisation, the effect of hybridisation on definitive host range (Catalano *et al.*, 2018) and the potential human and veterinary impacts (Léger and Webster, 2017).

Mitochondrial *cox*1 analysis of the *Bulinus* snail samples identified the three species, *B. truncatus*, *B. globosus* and *B. forskalii,* involved in schistosome transmission in Niger. These three *Bulinus* species show diverse intra-species populations with no clustering by geographical region or in relation to transmission. Additionally, non-coding mitochondrial DNA was amplified in several (28) of our *B. truncatus* samples, the preferential sequencing of which could be attributed to unsuitable primer selection or specimen degradation. As these were non-coding, they are dissimilar to *NUMTs* described in other molluscs (Williams *et al.*, 2017) and may instead be related to the high degree of polyploidy known to occur in *B. truncatus/tropicus* group snails (Brown and Wright, 1972; Brown, 1976; Goldman *et al.*, 1983) promoting mitochondrial heteroplasmy.

Strong support for these non-coding sequences arising through mitochondrial heteroplasmy have been identified from mitochondrial sequences of a *B. truncatus* isolate from Niger sequenced on a Next Generation Sequencing (NGS) platform that is currently being analysed in collaboration with colleagues at the Natural History Museum (NHM) (Briscoe *et al.*, unpublished). In brief, sequence data were produced on a MiSeq (600 cycles) and

unassembled reads were iteratively assembled to NHM derived *Bulinus* spp. *cox*1 sequences until resulting contigs could be circularised and annotated using MITOS (Bernt *et al.*, 2013). When investigating a specific site of interest where a considerable 10 bp deletion had been observed in the non-coding *cox*1 of the *B. truncatus* sequenced here it was noted that in a single read of the data there was a match with these 'alternative' sequences. This finding provides evidence that at least within the *B. truncatus* species, somatic mutations can occur leading to a genetically mosaic individual. Although the mechanism by which heteroplasmic mitochondrial DNA mutations can lead to predominating tissues in comparison to the functional genes (as seems the case with the preferentially sequenced non-coding *cox*1 sequences of *B. truncatus* observed in the current study) are poorly understood, they will progressively erode mitochondrial function until a threshold is reached, which when concerning mtDNA diseases for example, would lead to the onset of symptoms (Wallace and Chalkia, 2013). This raises multiple questions considering that non-functional copies of *cox*1 were observed in just under half (28 of 57) of the *B. truncatus* analysed suggesting that these mutations are not leading to any obvious evolutionary selective pressure. It could be of interest however to compare this rate of non-coding mtDNA occurrence in schistosome infected *B. truncatus* with those from the same sites not harbouring patent schistosome infections, to establish if any link to schistosome susceptibility is worth investigating further. Although initially ruled out, further investigation into whether these non-coding sequences may be occurring due to the presence of *NUMTs* should also be performed.

The main purpose for sequencing a region of the *cox*1 in the current study was simply to easily obtain species identifications since it has been established mitochondrial barcoding is useful for exploring genetic diversity and relationships of *Bulinus* spp. (Kane *et al.*, 2008; Zein-Eddine *et al.*, 2014). However, the preferential sequencing of non-coding genes here highlights difficulties in using such a marker for species identification and the need for using other informative DNA regions.

B. globosus, B. forskalii and *B. truncatus* were confirmed as transmitting *S. bovis* supporting previous reports that *S. bovis* can utilize a wide variety of *Bulinus* hosts (Southgate and Knowles, 1975; Tian-Bi *et al.*, 2019). Conversely, *S. haematobium* and the *S. haematobium* group hybrids appeared more specific and were only transmitted by *B. truncatus* (Table 3.3). This is consistent with historical findings from this region showing that *B. globosus* and *B. forskalii* were not compatible with *S. haematobium* (Vera *et al.*, 1990) and questions the previous reports of *B. forskalii* snails, that could have been morphologically confused with *B. senegalensis*, as infected with *S. haematobium* (see Vera *et al.*, 1992; Labbo *et al.*, 2007).

Proportional to the total number of snails collected (n=15,288), few had patent schistosome infections (0.56-0.90%) (see Rabone *et al*., 2019), however 40% (n=35) of the infected snails examined were shedding multiple (2-10) cercarial genotypes. Of these 35, 17%

had co-species infections, confirming that they had been infected multiple times by miracidia of different species/strains. The remaining 83% had mono-species infections, suggesting that they had either been infected by multiple miracidia of the same species, or that the different genotypes may have arisen through genetic mutations during clonal parasite replication from a single miracidial infection. Near identical MLG cercariae observed from individual snails has been identified previously for *S. japonicum* (see Shrivastava *et al.*, 2005; Yin *et al.*, 2008; Lu *et al.*, 2010; Huo *et al.*, 2016), with the conclusion that somatic mutation occurs during schistosome sporocystogenesis, resulting in genetically different cercariae originating from a single miracidium. This has also been shown for *S. mansoni*, with significant intra-clonal variation of cercariae resulting from single miracidial snail infections (Grevelding, 1999), and also sporocysts cultured *in vitro*, suggesting that mitotic recombination events occur during intramolluscan larval development (Bayne and Grevelding, 2003). For *S. haematobium,* intramolluscan replication of daughter sporocysts does occur and has been observed in *B. truncatus* (see Kechemir & Théron, 1989); however, the occurrence of mitotic recombination and / or somatic mutation during replication has not been investigated.

If our MLG data do correlate to multiple individual miracidial infections, it is clear that multiple *S. bovis* infections are high in these snails, suggesting that these are high transmission zones, or that *S. bovis* egg deposition is focally more concentrated than that of humans at transmission sites. This is also supported by the finding of all three of the snail species (*B. truncatus, B. forskalii and B. globosus*) being infected, however only the former two species harboured high numbers of cercarial genotypes, with *B. globosus* only emitting cercariae of a single genotype (Figure 3.2). This may be due to a low sample size of *B. globosus* (n = 4), or that this snail species is less suitable for *S. bovis* transmission in this region (Vera *et al.*, 1990; Brémond *et al.*, 1993; Labbo *et al.*, 2003, 2007). Inter schistosome species comparisons also show that compared to *S. bovis, S. haematobium* / hybrids infections only showed few (≤3) cercarial genotypes from individual *B. truncatus* (Figure 3.2). Although this again may be a consequence of sample size and the fact that far fewer snails were found shedding *S. haematobium* and / or the hybrids, it might also reflect biological species differences during intramolluscan development, such as variation in sporocystogenesis regulatory mechanisms (Kechemir and Théron, 1980; Jourdane, 1983; Touassem and Théron, 1986). Differences in intramolluscan replication may also be an explanation for the higher degree of diversity observed in *S. bovis* populations with *S. haematobium* showing extremely low diversity levels within and between endemic zones (Webster *et al.* 2012; Djuikwo-Teukeng *et al.* 2019). However, these differences could also be due to the transmission of *S. bovis* being more frequent than that of human schistosomes due to; larger numbers of parasites released in the faecal matter of infected ruminants, more frequent freshwater use of definitive hosts, and / or due to the lack of chemotherapeutic

treatment for animals, compared to that of the human populations previously reported (Ezeamama *et al.*, 2016). The genetic variability (interpreted by *Ho* and *He*) of *S. bovis* populations collected South of Niamey (where no *S. haematobium* was collected) was lower than that of cercariae collected in the North, indicating a potentially higher degree of inbreeding within the populations South of Niamey. In the North however, *Ho* was higher than *He* at some loci, indicating a higher genetic variability than might be expected. However, this may be a direct result of sample size, since few *S. bovis* unique MLGs were collected in the North which would impact the accuracy of the population genetic analyses (Hale *et al.*, 2012).

Interestingly, no co-infections were found between *S. haematobium* and *S. bovis* or between *S. bovis* and the hybrids, although a co-infection involving *S. haematobium* and a hybrid were found as has been previously reported in Côte d'Ivoire (Tian-Bi *et al.*, 2019). Considering the identified sympatric distribution of these *Schistosoma* species at the village level in the north of Niamey and the absence of *S. haematobium* in the seven villages south (Figure 3.8), this observation raises further questions worth exploring. The lack of *S. haematobium* and *S. bovis* co-infections may be due to the transmission sites of human and cattle schistosomiasis in this region of the Niger River Valley being for the most part separated, since cattle and human schistosomes were only found together at two transmission sites.

Alternatively, there could be more complex intramolluscan mechanisms that inhibit the occurrence of this co-infection, such as the antagonism/relationship that has been observed between *S. mansoni* and *S. haematobium* with *Calicophoron* spp. trematode parasites (Southgate *et al.*, 1989; Laidemitt *et al.*, 2019), or the induced immunoregulation and adaptive immunity of the snail during multiple schistosome infection challenges (Sire *et al.*, 1998; Portela *et al.*, 2013; Pinaud *et al.*, 2016, 2019), including those of the same *Schistosoma* species. The role that hybridisation plays in relation to these co-infections adds an extra element of complexity, with *S. haematobium* group hybrids being observed to co-infect with both *S. bovis* (Tian-Bi *et al.*, 2019) and *S. haematobium*, signifying again the expanded range of compatibility between schistosome hybrids and their intermediate snail hosts warranting further investigation.

Several limitations of the current study are worth considering while making conclusions. First of all, just over a third of infected *Bulinus* spp. and their associated schistosomes collected in the field could not be included due to sample degradation reducing the samples available for this study. The inclusion of these collections in the present study under better circumstances may have revealed other host-parasite relationships not considered here. Second, for several of the *Bulinus* spp. included, *cox*1 sequences to distinguish snail species could not be analysed due to amplification of non-coding DNA or poor sequencing results. Although morphological observations of the shell were attributed to *B. truncatus* and *B. forskalii* species, there may be hidden genetic diversity within these

species currently undetermined. In addition, the two snails morphologically identified as *B. forskalii* may be those of a closely related species such as *B. senegalensis* reported as present in Niger (Vera *et al.*, 1992). Thirdly, it should be reiterated that the inferences made in the current study regarding multiple schistosome miracidia infections per *Bulinus* spp. were established primarily using microsatellite markers alone and are therefore in need of further support. As aforementioned, we need to conduct controlled experimental infections of *Bulinus* spp. with *Schistosoma* spp. to gage how these microsatellite loci may be affected during intramolluscan replication when somatic mutations may occur. During such infection experiments, microsatellite markers should be analysed alongside other variable regions of schistosome DNA (as was demonstrated for one snail in the current study where different *cox*1 sequences haplotypes from each MLG confirmed multiple infections) to give further support for the occurrence of multiple miracidia infections and/or somatic mutation in field collected intermediate hosts.

Detailed snail and schistosome sampling coupled with molecular analyses has advanced our understanding of human and bovid schistosomiasis transmission in the Niger River Valley region. Schistosomes found to infect humans (*S. haematobium* and *S. haematobium* hybrids) were restricted to the north sites but were much less abundant than those causing veterinary schistosomiasis (*S. bovis*) across the region. No genetic overlap was observed between human and bovine schistosomes, supporting population structure and division. *B. truncatus,* the most abundant snail species (Rabone *et al.*, 2019), was involved in transmission of all schistosomes, whilst the less abundant *B. forskalii* and *B. globosus* were only involved in the transmission of *S. bovis.* The data suggest that species-specific biological traits may exist in relation to co-infections, snail-schistosome compatibility and intramolluscan schistosome development which might affect transmission dynamics and genetic outcomes of the different schistosome populations. The scarcity of human infecting schistosomes to the comparatively abundant livestock schistosomes, even in highly endemic settings such as this, shows the necessity from a public health view to identify species accurately to assess the presence and level of human schistosomiasis transmission.

Chapter 4. Transmission and diversity of *Schistosoma haematobium* and *S. bovis* and their freshwater intermediate hosts (*Bulinus globosus* and *B. nasutus*) in Zanzibar

Abstract

Schistosomiasis is a parasitic disease of medical and veterinary importance in sub-Saharan Africa. The Zanzibar Archipelago (Pemba and Unguja islands) is endemic for human urogenital schistosomiasis and focal transmission of bovine schistosomiasis, caused by infection with trematode species, *Schistosoma haematobium* and *S. bovis* respectively. The extent of *S. bovis* transmission here is unknown, and this complicates future surveillance of *S. haematobium* in the shared snail intermediate host, *Bulinus globosus*. The compatibility of a second intermediate host for *S. haematobium*, *B. nasutus*, further complicates our understanding of *S. haematobium* transmission on Zanzibar. Changes to *Schistosoma* spp. transmission on Zanzibar will have significant implications on current elimination targets for the disease. Through performing malacological and parasitological surveys between 2016 and 2019, and using archived specimens, the current geographical distribution and associations of *Bulinus* spp. and *Schistosoma* spp. were investigated across Zanzibar.

Phylogenetic analysis of *cox*1 haplotype groups using a partial *cox*1 region from 433 *B. globosus* and 77 *B. nasutus* revealed that three genetically distinct populations of the predominant *B. globosus* are present, two with an overlapping distribution on Pemba Island and one on Unguja Island. For *B. nasutus*, only a single genetically similar clade with matching haplotypes was observed across the restricted sites where this species was present in Pemba and Unguja Islands. By analysing a partial *cox*1 region and the complete ITS (1 + 2) rDNA region of 179 larval schistosome cercariae collected from 89 *Bulinus* spp., 20 from Pemba Island were confirmed as *B. globosus* infected with *S. haematobium* (n = 12), *B. globosus* infected with patent *S. bovis* (n = 7) and *B. nasutus* infected with *S. haematobium* infection (n = 1). Snail identification was not possible for the remaining 69 *Bulinus* spp., however all shed *S. haematobium* cercariae with 18 different *S. haematobium* haplotypes representing populations associated with mainland Africa and the Indian Ocean Islands.

The unexpected finding of *B. nasutus* infected with *S. haematobium* demonstrates that this species also plays a role in the transmission of *S. haematobium* on Pemba Island. Due to the genetic similarity of both *B. nasutus* and *S. haematobium* populations on Unguja and Pemba islands, and our past knowledge of schistosomiasis in Zanzibar, it is concluded that transmission through *B. nasutus* likely occurs on Unguja Island too. Further investigation to establish the role that intraspecific genetic diversity of schistosomes and snails plays in the compatibility and transmission of schistosomiasis will allow for better informed schistosomiasis monitoring and implementation of control interventions in other schistosomiasis endemic regions.

4.1 Introduction

Schistosomiasis is a snail-borne neglected tropical disease, causing severe morbidity and mortality in both humans and wildlife (De Bont and Vercruysse, 1998; Colley *et al.*, 2014). *Schistosoma haematobium* and *S. mansoni*, causing human urogenital and intestinal schistosomiasis respectively, are the two species contributing most to disease in sub-Saharan Africa. Community or school-based treatment of schistosomiasis using the only recommended preventive chemotherapeutic drug currently available, Praziquantel (Merck KGaA), is the most common and effective means of alleviating disease burden (King, Binder, *et al.*, 2020; King, Kittur, *et al.*, 2020). As prevalence of the disease moves towards elimination levels in parts of sub-Saharan Africa following sustained treatment programmes, it is apparent that transmission is still being sustained by the snail intermediate hosts acting as a reservoir of infection (Allan *et al.*, 2020; WHO, 2020a). Monitoring snails during control programmes and during pre/post elimination surveillance, either complimentary to parasitology surveys or as a stand-alone risk establishment mechanism, will aid in certifying that either *Schistosoma* spp. transmission is persisting, interrupted or re-established following elimination.

Unguja and Pemba Islands, collectively known as Zanzibar (United Republic of Tanzania), are endemic for urogenital schistosomiasis. Zanzibar has a long history of pioneering urogenital schistosomiasis research and control dating back to the 1920's. This ranges from investigating the freshwater snails involved in disease transmission to early trials of schistosomacidal drugs and assessing disease prevalence through low cost diagnostics (Mansfield-Aders, 1928; McCarthy, 1930; Mozley, 1939; Goatly and Jordan, 1965; Forsyth and MacDonald, 1966; Macdonald *et al.*, 1968; Savioli and Mott, 1989; Savioli *et al.*, 1989; Mgeni *et al.*, 1990). Most recently, these islands have been targeted for elimination with the Zanzibar Elimination of Schistosomiasis Transmission (ZEST) project implemented between 2011 and 2017, trialling integrated interventions with mass drug administration (Knopp *et al.*, 2012, 2013; Knopp, Ame, *et al.*, 2019; Knopp, Person, *et al.*, 2019). Following the ZEST project, prevalence was significantly reduced across the islands, but not eliminated (Knopp, Ame, *et al.*, 2019), leaving focal endemicity in hotspot areas that require new methods of surveillance and tailored interventions (Knopp, Person, *et al.*, 2019; Kittur *et al.*, 2020)

Of the four endemic species of *Bulinus* on Zanzibar, it was previously concluded that only *Bulinus globosus* acted as a compatible intermediate host (Stothard and Rollinson, 1997a; Stothard, Loxton, *et al.*, 2000; Allan *et al.*, 2009, 2013). Urogenital schistosomiasis was therefore considered to be restricted across the islands to areas where *B. globosus* was present (Stothard, Loxton, *et al.*, 2000). Genetic diversity of *S. haematobium* on Zanzibar is however very diverse in comparison to mainland Africa, with the presence of both Group 1 (mainland Africa) and the more diverse Group 2 (Indian Ocean Islands) strains (Webster *et al.*, 2012; Webster, Culverwell, *et al.*, 2013). This prior knowledge on both snail and parasite

underpinned the ZEST control programme, assuming that: (i) all *Schistosoma* infections from humans and snails were *S. haematobium*, and (ii) regions of freshwater waterbodies (e.g. southern part of Unguja Island) previously identified as containing only *Bulinus nasutus* (a closely related freshwater snail species to *B. globosus*) were free of *Schistosoma* transmission (Figure 4.1 and as reviewed in Chapter 2). It is now apparent, however, that *B. globosus* can also host *S. bovis* on Pemba Island (Pennance *et al.*, 2018), and therefore larval schistosomes shed from *B. globosus* in Zanzibar cannot be assumed to be *S. haematobium*. Additionally, there is new evidence that *B. nasutus* can harbour *Schistosoma* spp. in Zanzibar (Ame, 2018). Both these observations require further investigation since bovine schistosomiasis has not been identified in any potential definitive hosts on Zanzibar (i.e. cattle) and the *Schistosoma* species potentially transmitted by *B. nasutus* need to be established as either *S. haematobium, S. bovis* or potentially both, to make further inferences about how this might impact schistosomiasis surveillance and control.

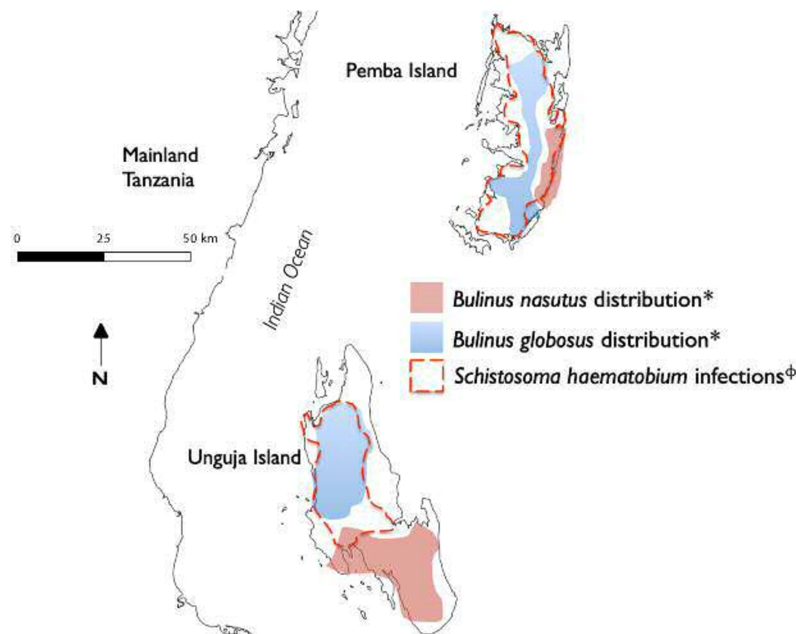


Figure 4.1. Predicted distribution of *Bulinus* spp. and *Schistosoma haematobium* endemicity on Unguja and Pemba islands based on previous findings. * *Bulinus* spp*.* distribution inferred from Stothard *et al.* (2000) and Pennance *et al*. (2016, 2018). [ϕ] *Schistosoma haematobium* infection distribution interpreted from Knopp, Ame, *et al*., (2019).

If not controlled bovine schistosomiasis can cause significant health problems for cattle, such as haemorrhagic enteritis, anaemia, emaciation and death, ultimately affecting local farming practices and the economy (De Bont and Vercruysse, 1998). Schistosomiasis diagnosis in animals however remains problematic, with methods relying on stool microscopy for eggs or miracidial hatching, which both lack sensitivity (Giovanoli Evack *et al.*, 2020). Miracidial hatching from bovine stool samples has been used in preference to faecal egg counts on slides for the diagnosis of schistosome infections in cattle as it enables the use of much larger faecal samples that can improve sensitivity and also ensues viability of eggs found

in faeces (Lawrence, 1977; De Bont *et al.*, 1996; De Bont and Vercruysse, 1998; Savassi *et al.*, 2020). It is important, therefore, to continue trialling protocols for the diagnosis of *S. bovis* in cattle, and begin monitoring the distribution of infected animals in Zanzibar where *S. bovis* infection has not previously been studied (Pennance *et al.*, 2018).

Despite several studies demonstrating the incompatibility of *S. haematobium* with *B. nasutus* on Unguja Island (Stothard and Rollinson, 1997a; Stothard, Loxton, *et al.*, 2000; Allan *et al.*, 2009, 2013), overlapping areas of *B. nasutus* and urogenital schistosomiasis endemicity exist predominantly on Pemba Island (Figure 4.1) and have done historically on Unguja Island (Mozley, 1939; Goatly and Jordan, 1965; Stothard, Loxton, *et al.*, 2000). This suggests that *B. nasutus* is an intermediate host for *S. haematobium* on Pemba Island, as is the case in neighbouring regions of Kenya (Kariuki *et al.*, 2004) and Tanzania (Webbe, 1962; Kinoti, 1964; Loker *et al.*, 1981; Sarda *et al.*, 1985; Lwambo, 1988; Angelo *et al.*, 2018), as well as the closely related *B. (nasutus) productus* (McCullough *et al.,* 1968; Pennance *et al.,* unpublished observations).

This study aimed to investigate the current transmission status of urogenital and bovine schistosomiasis on Pemba and Unguja Islands by monitoring the genetic diversity of *B. globosus* and *B. nasutus* and where possible their associated schistosomes. We additionally assessed the occurrence of *S. bovis* in cattle by trialling two miracidial hatching methods.

4.2 Methods

4.2.1 Sampling of Bulinus *spp. on Pemba and Unguja Islands*

Following approval to conduct surveys by the Sheha, a community leader that locally governs each shehia, human-freshwater contact sites were located from previous accounts of water body locations or with the help of local residents. Coordinates were taken at 112 freshwater sites across 20 shehias (Figure 4.2) using a Garmin GPSMAP 62sc device (Garmin, Kansas City, USA) and each water body was surveyed for the presence of intermediate host snails. Between 1 and 5 surveys were conducted at each human-freshwater contact site across Pemba and Unguja islands in October 2016, October 2017, February, July and November 2018 and January 2019 (Table 4.1).

At each site, *Bulinus* snails were collected by hand predominantly from submerged vegetation and tree roots around the water's edge that were neighbouring access points to the freshwater sites. Each site was surveyed for 15 minutes by three snail collectors. Snails were placed in collection pots and transported back to either the Public Health Laboratory (Chake Chake, Pemba Island) or the Neglected Diseases Program laboratory (Stone Town, Unguja Island) where they were morphologically identified, counted and housed in plastic trays with bottled water and covered by a glass lid overnight to acclimatise. The following morning (before 08:00), snails were washed and examined for cercarial shedding by being placed

individually in wells of 12-well ELISA plates filled to approximately two thirds with bottled water and placed in indirect sunlight. Each well was checked using a dissection microscope two hours and again eight hours after first sunlight to capture schistosomes of different shedding patterns (Mouahid *et al.*, 1991). An experienced microscopist distinguished furcocercous schistosome cercariae from others using descriptions of *Schistosoma* spp. under a dissecting microscope (Frandsen and Christensen, 1984); any shed cercariae were captured and individually pipetted in 3.5 µl onto Whatman FTA cards (Whatman, Part of GE Healthcare, Florham Park, USA) for molecular characterisation. Infected snails were preserved in 100% ethanol for subsequent molecular characterisation as previously described by Pennance *et al.,* (2018).

Additionally, a targeted malacological survey was conducted in late January 2019 to collect *B. nasutus* snails at one site in Kangagani on Pemba Island previously identified as inhabited by *B. nasutus* (see Ame 2018) (Figure 4.2). These snails were maintained in lab aquaria (dimensions 45x30x30cm, 40.5L, filled to approximately two thirds full with water from the collection site and equipped with an air pump for continuous aeration) at a maximum density of 100 *Bulinus* per aquarium. Snails were re-checked for shedding of schistosome cercariae 3 weeks later in an effort to capture any infections that may have been pre-patent during the initial screen. Freshwater for these snails were replaced biweekly using water from the site of collection (Kangagani) after being stored for at least 48 hours in transparent 15L water containers (allowing for any sediment to settle and eliminate the risk of introducing live schistosome miracidia into the aquaria). Any dead snails were removed from the aquaria daily.

4.2.2 Additional samples included in the analysis

Bulinus samples, from Unguja and Pemba, accessioned within the Schistosomiasis Collection at the Natural History Museum (SCAN) (Emery *et al.*, 2012) were included in the study providing material from areas not covered in the malacological surveys described above. Samples were only included if associated geographical information was available. Six snails were included, five from Unguja Island (Jendele, Miwani, Kinyasini, Mtopepo, Chaani) and one from Pemba Island (Wingwi) (Table 4.1). Two of these snails from Miwani and Kinyasini on Unguja Island (Table 4.1) were patent with *S. haematobium* when they were collected, species being determined by cercarial molecular analysis (see below). The remaining four *Bulinus* snails were negative for patent *Schistosoma* infections (see Supplementary Table 4.1). One of the infected *B. globosus* identified in the SCAN repository (MCF389B0F0286, Supplementary Table 4.1) could not be associated with its own *S. haematobium* cercariae as it was preserved together with two other *B. globosus* also infected with *S. haematobium* from the same site.

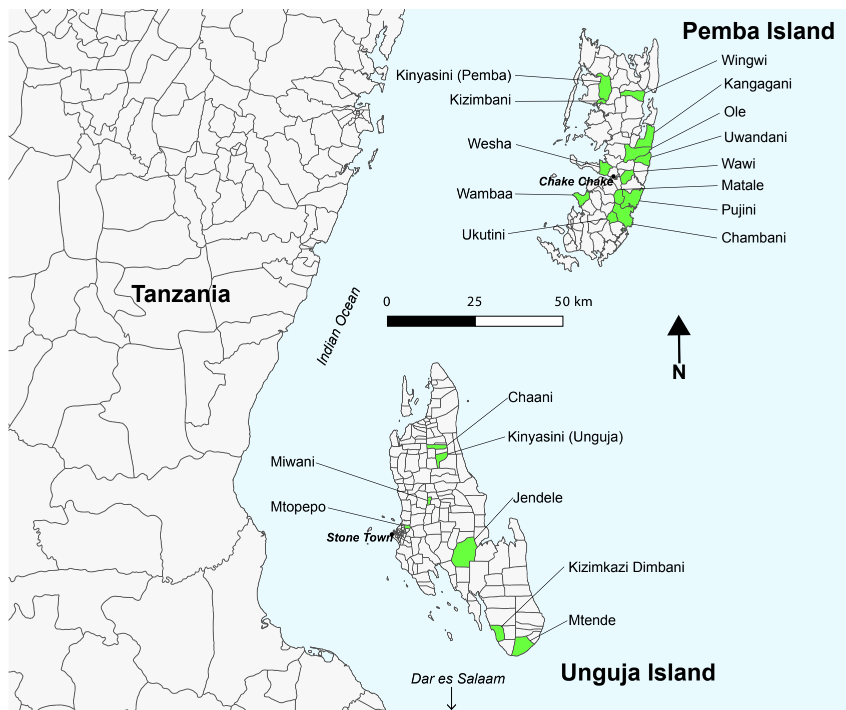


Figure 4.2. Map of Pemba and Unguja islands (Zanzibar, United Republic of Tanzania) showing shehias (in green) where malacological surveys were conducted to collect *Bulinus* spp. and faecal samples collected from cattle (Kinyasini and Kizimbani – Pemba Island).

4.2.3 Bulinus *spp. molecular characterisation*

To identify the species of each *Bulinus* snail, the shell was removed from the preserved sample and gDNA from whole snail tissue was extracted using either the Qiagen BioSprint 96 DNA Blood Kit following manufacturer's instructions (Qiagen, Manchester, UK) or the Qiagen DNeasy Blood & Tissue Kit modified protocol (Qiagen, Manchester, UK) using double volumes of lysis buffers (see Pennance *et al*., 2018). Since not all snails could be identified using molecular characterisation, a minimum of three non-patent snails per site per malacological survey (except for those collected on Pemba Island during October 2016 and from Chambani and Uwandani in 2017/2018) were randomly selected for identification. Molecular characterisation was performed for all snails with patent *Schistosoma* infections and snails retrieved from the SCAN.

DNA was extracted from a total of 510 *Bulinus* spp. from Unguja (11 snails collected from 8 sites) and Pemba (499 snails collected from 63 sites). A 623 bp partial region of mitochondrial *cox*1 DNA was amplified and Sanger sequenced following previously described methods (see Pennance *et al.,* 2018). Sequence data was edited, manually trimmed to 463-621 bp and aligned in Sequencher v5.4.6 (GeneCodes Corp., Michigan, USA) before being collapsed into *cox*1 haplotype groups. Species identification were confirmed by alignment and phylogenetic analysis (see section 4.2.6) of *Bulinus cox*1 haplotype groups (Table 4.2) with reference data for *B. globosus* and *B. nasutus* (see Kane *et al.* 2008).

4.2.4 *Bovine faecal sampling and collection of* S. bovis *miracidia*

Fresh stool samples were collected from six cows across two sites in Kinyasini (Pemba Island) in mid-February 2019. Three cows were sampled in the area surrounding site Kinya6 (coordinates: -5.03560, 39.73972), a stream surrounded by marsh previously reported for the occurrence of several *S. bovis* infected *B. globosus* (see Pennance *et al.,* 2018). Fresh stool samples were also taken from three cows from a second stream site, Kinya9 (coordinates: -5.03077, 39.73333), 800 m north-west of the first site. Using a spatula, a 300 ml collection pot was filled and transported back to the Public Health Laboratory (Chake Chake, Pemba Island) and stored at 4°C. Within 24 hours of collection, each faecal sample was individually washed with 0.85% saline solution through four sieves of decreasing mesh size (1.4 mm, 710 µm, 355 µm, 212 µm) to remove large debris and the sediment was collected in a final sieve (125 µm). This final collection was subdivided in half to trial two different miracidia hatching methods, using either a Pitchford funnel or sedimentation flask.

For hatching miracidia using the Pitchford funnel method, a final wash through a Pitchford funnel (Visser *et al.*, 1972; Pitchford and Visser, 1975) (mesh sizes: inner sieve 200 µm, outer sieve 40 µm) using bottled water concentrated the faecal sediment, which was then placed equally in three separate 90 mm petri dishes (Appleton Woods Ltd, AB260). Bottled water was added to each petri dish and placed in indirect sunlight outside (28-34ºC) for at least one hour to induce miracidia hatching. Petri dishes were checked for swimming miracidia at 4, 8, 20 and 24 hours after plating.

For the sedimentation flask method, the faecal sediment was placed directly in individual 1L sedimentation flasks, filled with room temperature bottled water to the 1L mark, mixed using a wooden spatula and left in indirect sunlight for 1 hour. Each sedimentation flask was then completely covered in tin foil, except for the top 100 ml (i.e. between 900-1000 ml) which was left exposed to light from a desk lamp. Between 5 and 10 ml of liquid was removed and placed in a petri dish to check for swimming miracidia from the top of the flask following 4, 8, 20 and 24 hours. For both methods, any swimming *S. bovis* miracidia observed in petri dishes using a dissection microscope were captured and individually pipetted in 3.5 µl onto Whatman FTA cards (Whatman, Part of GE Healthcare, Florham Park, USA) for molecular characterisation.

4.2.5 Larval Schistosoma *spp. molecular characterisation*

From each infected snail two to six *Schistosoma* cercariae were characterised using molecular methods, as well as miracidia collected from any bovine stool sample. Following elution of parasite DNA from Whatman FTA cards (see Webster *et al.,* 2015), schistosome species identification was performed by DNA amplification following previous methods of a partial *cox*1 region (956 bp) and the complete ITS (1 + 2) rDNA region (967 bp) (see Webster *et al.,* 2012,

2013). The *cox*1 sequence data were manually edited and trimmed to 750 bp, and the ITS sequence data to 880 bp, in Sequencher v5.4.6 (GeneCodes Corp., Michigan, USA) before being compared with reference sequence databases for Zanzibar *Schistosoma* (see Pennance *et al.,* 2018; Webster, Culverwell, *et al.,* 2013). Cercariae and miracidia of identical *cox*1 sequences were collapsed into *cox*1 haplotype groups for further phylogenetic analysis (Table 4.3). Two larval schistosome cercariae captured from each of the 69 infected snails (i.e. 138 in total) collected as part of the ZEST study (Knopp *et al.*, 2012, 2013; Knopp, Ame, *et al.*, 2019), stored on Whatman FTA cards and accessioned in SCAN (Emery *et al.*, 2012) were molecularly identified using the same methods. ITS data was not used for phylogenetic analysis since no intra-species diversity was observed.

4.2.6 Phylogenetic cox*1 analysis of* Bulinus *spp. and* Schistosoma *spp.*

Bulinus haplotype sequences were imported into Geneious v11.1.4 (Kearse *et al.*, 2012) for phylogenetic analysis together with reference data for *B. nasutus* and *B. globosus* collected previously from East Africa (Zanzibar, Tanzania, Kenya, Uganda, Mafia Island; (Kane *et al.*, 2008) and an outgroup of *Biomphalaria glabrata* available from GenBank (Accession: NC005439) (DeJong *et al.*, 2004). Haplotype alignments were performed using ClustalW v2.1 (Larkin *et al.*, 2007) executed in PAUP* (Swofford, 2001) and then an appropriate evolutionary nucleotide substitution model (HKY + I + G; -lnl 2020.2144, AIC 4052.4287) was selected in MrModelTest v2.4 (Nylander, 2004) using the Akaike Information Criterion. Bayesian inference was performed using MrBayes v3.2.7a (Ronquist *et al.*, 2012). The burn-in was set at 3.5 million generations for consistency after confirming that the average standard deviation of split frequencies (ASDOSF) reported from MrBayes output was at least <0.01 by this point. Clades were considered to have high nodal support if Bayesian inference posterior probability was ≥0.95; tree nodes with <0.95 were collapsed in SumTrees v4.4.0 (Sukumaran and Holder, 2010). A TCS haplotype network was also produced in PopART (Clement *et al.*, 2002; Leigh and Bryant, 2015) using the same sequence alignment, however any short non-overlapping sequences were removed and other haplotypes trimmed to only overlapping sequences in PopART.

Larval *Schistosoma* haplotype phylogenetic analyses were performed as for *Bulinus* spp., except for using a different outgroup of *S. curassoni* reference sequence (AY157210; Lockyer *et al.* 2003). Alignments also included published *S. haematobium* haplotypes from Zanzibar (GU257334 – GU257360 Webster, Culverwell *et al.,* 2013), and *S. bovis* from Pemba Island (MH014042 – MH014043 Pennance *et al.,* 2018), mainland Tanzania (AY157212, Lockyer *et al.,* 2003) and Cameroon (MH647141, Djuikwo-Teukeng *et al.,* 2019), all trimmed to a maximum of 750 bp. A TCS haplotype network was also produced in PopART (Clement *et al.*, 2002; Leigh and Bryant, 2015) using the same sequence alignment.

4.2.7 *Spatial distribution of* Bulinus *and* Schistosoma *species*

Bulinus and *Schistosoma* spp. distribution data were visualised using QGIS v3.0.1 Girona (http://qgis.osgeo.org) and mapped for each site. Digital shape files for Unguja and Pemba Island administrative regions were obtained from DIVA-GIS (https://www.diva-gis.org).

4.2.8 *Statistical analysis*

Chi-squared tests were performed in R v.4.0.0 (R Core Team, 2018) to investigate differences in the abundance of Group 1 and 2 *S. haematobium* cercariae (Webster *et al.*, 2012) shed from *Bulinus* spp. identified from partial *cox*1 regions on Unguja and Pemba islands.

4.3 Results

4.3.1 Bulinus *infections*

Over the six malacological surveys conducted on Pemba Island, a total of 11,110 *B. globosus* and *B. nasutus* were collected from 62 of the 109 sites. The majority of these (n = 9,501) based on a subset of identifications from each site (see section 3.2) were *B. globosus*, with the remaining being *B. nasutus* (n = 1,485) and some ambiguous snails (n = 124) that could be either species since these originated from a site containing a mixture of both species (see section 3.2). Of these 0.18% (20 snails) shed schistosome cercariae. These were collected from eight sites: one in Kinyasini (n = 16), two in Kizimbani (n = 2), one in Chambani (n = 1) and one in Kangagani (n =1) (Table 4.1). The infected snail from Kangagani was collected during the targeted malacological *B. nasutus* survey, in which 198 individual *Bulinus* spp. were collected. Although having no observed patent schistosome infections during the first round of shedding, 21 days later (at which point 90 snails had died in culture) one *B. nasutus* was identified with a patent schistosome infection. Malacological surveys on Unguja Island were conducted at three sites in two shehias (Mtende & Kizimbani Dimbani) (Table 4.1). Only six *B. nasutus* were collected in total and none shed *Schistosoma* cercariae (Supplementary Table 4.1).

Table 4.1. (see next page) *Bulinus* spp. collected in Pemba and Unguja islands (Zanzibar, United Republic of Tanzania) and samples analysed from previous collections and accessioned in the Schistosomiasis Collection at the Natural History Museum (SCAN)*. NA represents that no *Bulinus* snails were collected or identified during these surveys. a = Five *S. bovis* patent *B. globosus* also collected. b = Two *S. bovis* patent *B. globosus* also collected. FWBs = Freshwater bodies.

Shehia	Collection Dates	Number FWBs surveyed	*Bulinus* spp. collected	*Bulinus* spp. identified	*Bulinus* spp.	Number of *S. haematobium* infected *Bulinus* spp.
Pemba						
Ukutini	21/02/2018	10	750	29	*B. globosus*	0
	18/07/2018	10	996	29	*B. globosus*	0
	19/01/2018	10	724	23	*B. globosus*	0
Pujini	11/10/2017	10	210	20	*B. nasutus*	0
	15/02/2018	10	268	17	*B. nasutus*	0
	22/07/2018	10	467	19	*B. globosus* & *B. nasutus*	0
	19/11/2018	10	0	0	NA	0
Kizimbani	25/10/2016	3	199	6	*B. globosus*	0
	10/10/2017	3	289	9	*B. globosus*	2
	16/02/2018	4	283	14	*B. globosus*	0
	20/07/2018	4	102	12	*B. globosus*	0
	22/11/2018	4	75	11	*B. globosus*	0
Kinyasini	20/10/2016	9	463	17	*B. globosus*	1[a]
	11/10/2017	11	744	27	*B. globosus*	0
	14/10/2018	11	627	31	*B. globosus*	7
	19/07/2018	11	369	25	*B. globosus*	0
	22/11/2018	11	241	25	*B. globosus*	1 [b]
Wambaa	26/10/2016	8	79	3	*B. globosus*	0
	08/10/2017	9	1033	24	*B. globosus*	0
	19/02/2018	10	267	25	*B. globosus*	0
	21/07/2018	11	356	18	*B. globosus*	0
	21/11/2018	11	348	22	*B. globosus*	0
Wawi	22/10/2016	2	149	3	*B. globosus*	0
	05/10/2017	3	183	7	*B. globosus*	0
	13/02/2018	3	10	4	*B. globosus*	0
	17/07/2018	3	1	1	*B. globosus*	0
	20/11/2018	3	4	3	*B. globosus*	0
Ole	27/10/2016	11	0	0	NA	0
	06/10/2017	13	4	3	*B. globosus*	0
	12/02/2018	13	3	3	*B. globosus*	0
	17/07/2018	13	0	0	NA	0
	23/11/2018	13	0	0	NA	0
Matale	27/10/2016	10	0	0	NA	0
	09/10/2017	11	420	16	*B. globosus*	0
	19/02/2018	11	0	0	NA	0
	23/07/2018	11	212	16	*B. globosus*	0
	20/11/2018	11	167	14	*B. globosus*	0
Chambani	24/10/2016	9	291	10	*B. globosus* & *B. nasutus*	1
	07/10/2017	13	91	0	NA	0
	09/02/2018	13	0	0	NA	0
	24/07/2018	2	16	0	NA	0
	19/11/2018	2	16	0	NA	0
Uwandani	19/10/2016	9	136	10	*B. nasutus*	0
	05/10/2017	9	261	1	*B. nasutus*	0
	20/02/2018	10	18	0	NA	0
	17/07/2018	10	28	0	NA	0
	26/11/2018	2	12	0	NA	0
Kangagani	22/01/2019	1	198	1	*B. nasutus*	1
Wingwi	unknown	1*	NA	1	*B. globosus*	0
Wesha	22/10/16	6	0	0	NA	0
Unguja						
Jendele	unknown	1*	NA	1	*B. nasutus*	0
Miwani	06/02/2013	1*	NA	1	*B. globosus*	1
Kinyasini	10/10/2016	1*	NA	1	*B. globosus*	1
Mtopepo	unknown	1*	NA	1	*B. nasutus*	0
Chaani	06/07/2005	1*	NA	1	*B. globosus*	0
Mtende	17/07/2018	1	4	4	*B. nasutus*	0
Kizimkazi Dimbani	23/07/2018	1	2	2	*B. nasutus*	0

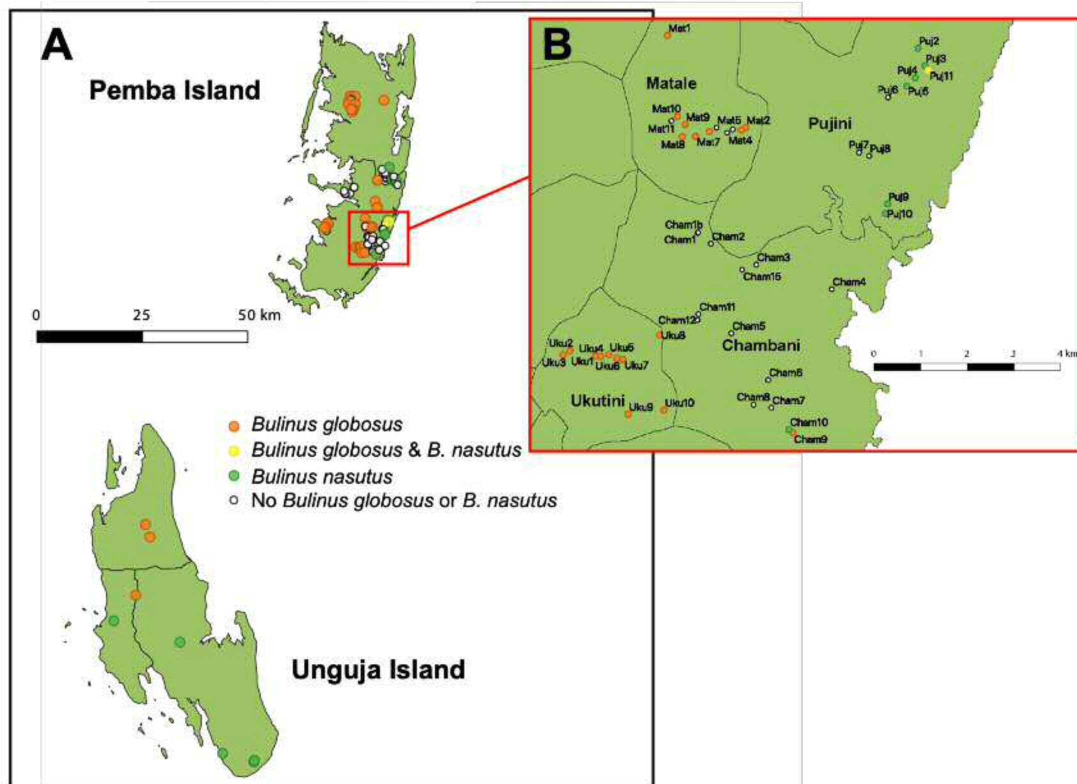


Figure 4.3. A: *Bulinus globosus* (orange) and *B. nasutus* (green_ distribution on Unguja and Pemba Islands (Zanzibar, United Republic of Tanzania) identified by *cox*1 sequencing. B: South East region of Pemba Island, displaying human freshwater contact sites in four shehias (Matale, Pujini, Chambani, Ukutini) and the single freshwater body cohabited by *B. globosus* and *B. nasutus* (Puj11).

4.3.2 Bulinus *spp. genetic diversity and distribution*

From the *Bulinus* spp. successfully sequenced, 433 out of 510 from 52 sites across Unguja and Pemba islands were *B. globosus*. The remaining 77 snails were identified as *B. nasutus* collected from 20 Unguja and Pemba sites (Supplementary Table 4.1). *B. nasutus* was found only from freshwater bodies to the east coast of Pemba and the southern districts up to the central west areas of Unguja (Figure 4.3). Where >1 snail was identified per site, each site was either inhabited by *B. globosus* or *B. nasutus*, with the exception of one waterbody on Pemba (Puj11, Figure 4.3B), where both species co-occurred in the same seasonal pond (Supplementary Table 4.1). The close proximity of two sites harbouring different snail species in Chambani during one survey (preliminary survey), with one (Cham9) harbouring *B. nasutus* and the other (Cham10) having *B. globosus* was also observed (Figure 4.3B).

All 11 *B. globosus* and 9 out of 10 *B. nasutus* haplotypes were unique to either Unguja or Pemba, with one exception being *B. nasutus* haplotype three that was detected on both islands (Table 4.2). A single clade of *B. nasutus* specimens from both Unguja and Pemba was observed, whereas the *B. globosus* isolates from Zanzibar fell into two distinct paraphyletic clades (Figure 4.4). All *B. globosus* from Pemba fell into one of these two paraphyletic clades,

with those previously identified from this island, whereas the three haplotypes from Unguja fell into a second clade containing *B. globosus* from Eastern Kenya and those previously identified from Unguja (see Figure 4.4). As only complete sequence data can be used when producing TCS haplotype networks, *Bulinus* spp. sequences had to be trimmed to the same length (i.e. no 'loose ends') and haplotype *B. globosus* 3c (Unguja) removed to leave a 477 bp alignment for use in a haplotype network. The clades of *Bulinus* were less well defined in the TCS haplotype network, due to the restricted length of *cox*1 sequence excluding important SNP sites (Figure 4.5).

4.3.3 Bovine schistosomiasis detection and identification from faecal samples

From the six cow stool samples processed from Kinyasini, all were negative using the sedimentation method, but one sample was positive for miracidial hatching using the Pitchford funnel method. From this positive sample, two swimming miracidia were recovered and molecularly identified as *S. bovis* with two *cox*1 haplotypes (Sb2 and Sb3).

4.3.4 Schistosoma *spp. cercariae identification*

From the cercariae identified from 89 of the infected *Bulinus*, 82 were shedding *S. haematobium* with 18 different haplotypes and the remaining seven snails were shedding *S. bovis* cercariae of two haplotypes (Table 4.3 & Supplementary Table 4.2). Both Group 1 and 2 haplotypes of *S. haematobium* representing mainland African and Indian Ocean islands respectively were identified (Webster *et al.*, 2012; Webster, Culverwell, *et al.*, 2013) (Figures 4.6 & 4.7). Including *Schistosoma* co-infections, of which there were seven determined by multiple *cox*1 haplotypes (Table 4.4), the number of snails shedding *S. haematobium* Group 1 cercariae (n = 41) was similar to those shedding Group 2 cercariae (n = 45). However, the majority of Group 1 *S. haematobium* infections occurred in Unguja (n = 35), with significantly fewer (n = 6) from Pemba (χ^2=10.0, *df*=1, P< 0.01); in contrast, Group 2 *S. haematobium* cercariae were distributed evenly across the islands (Unguja n = 23 and Pemba n = 22). Most (n = 13) haplotypes were unique to either Pemba or Unguja, but five were present across both islands (Table 4.3).

Seven snails were confirmed as shedding schistosome cercariae with multiple *cox*1 haplotypes of *S. haematobium* (n = 6) and *S. bovis* (n = 1), indicating they had been infected by multiple miracidia (Table 4.4). Furthermore, five of the six *S. haematobium* infected snails simultaneously shed both Group 1 and Group 2 *S. haematobium* haplotypes, only one snail was identified shedding two haplotypes of Group 2. Another *B. globosus* was coinfected with *S. haematobium* Group 1 and *Euclinostomum* sp. (Table 4.4).

ITS profiles from *S. haematobium* cercariae showed no hybridisation between *S. haematobium* and *S. bovis* or intraspecies variation from those previously sequenced from

Africa and the Indian Ocean Islands (Webster *et al.*, 2012). Likewise, the ITS profiles from the two *S. bovis* miracidia collected from a cow were identical and showed no intraspecies variation to those already sequenced from Africa and Indian Ocean Islands (Pennance *et al.*, 2018).

Table 4.2. *Bulinus* spp. partial *cox*1 haplotypes observed from Pemba and Unguja islands, also showing *Schistosoma* spp. observed infecting each snail haplotype. *It was not possible to associate this snail with its *S. haematobium* cercariae *cox*1 haplotypes as it was preserved with two other *S. haematobium* infected *B. globosus*. ^*Schistosoma* haplotype group represents whether cercariae were identified as mainland Africa (1) or Indian Ocean Island (2) haplotypes (as described in Webster *et al*. 2012).

Bulinus species	Haplotype	Number identified	Unguja / Pemba islands	*Schistosoma* species transmitted (haplotype group ^)	Sequence length *cox*1 (bp)	GenBank
Bulinus globosus	1a	257	Pemba	*S. haematobium* (2)	621	MT380541
	1b	2	Pemba	-	568	MT380542
	1c	2	Pemba	-	621	MT380545
	1d	1	Pemba	-	621	MT380546
	1e	6	Pemba	-	621	MT380543
	1f	2	Pemba	-	621	MT380544
	2a	159	Pemba	*S. haematobium* (1+2) & *S. bovis*	621	MT380547
	2b	1	Pemba	-	621	MT380548
	3a	1	Unguja	*S. haematobium* (2)	574	MT380549
	3b	1	Unguja	*S. haematobium* (?*)	612	MT380550
	3c	1	Unguja	-	463	MT380551
Bulinus nasutus	1	29	Pemba	-	612	MT380552
	2	26	Pemba	-	619	MT380553
	3	3	Unguja & Pemba	*S. haematobium* (1)	619	MT380554
	4	7	Pemba	-	485	MT380558
	5	2	Pemba	-	611	MT380556
	6	1	Unguja	-	572	MT380561
	7	3	Unguja	-	621	MT380557
	8	3	Unguja	-	621	MT380555
	9	2	Pemba	-	601	MT380559
	10	1	Pemba	-	598	MT380560

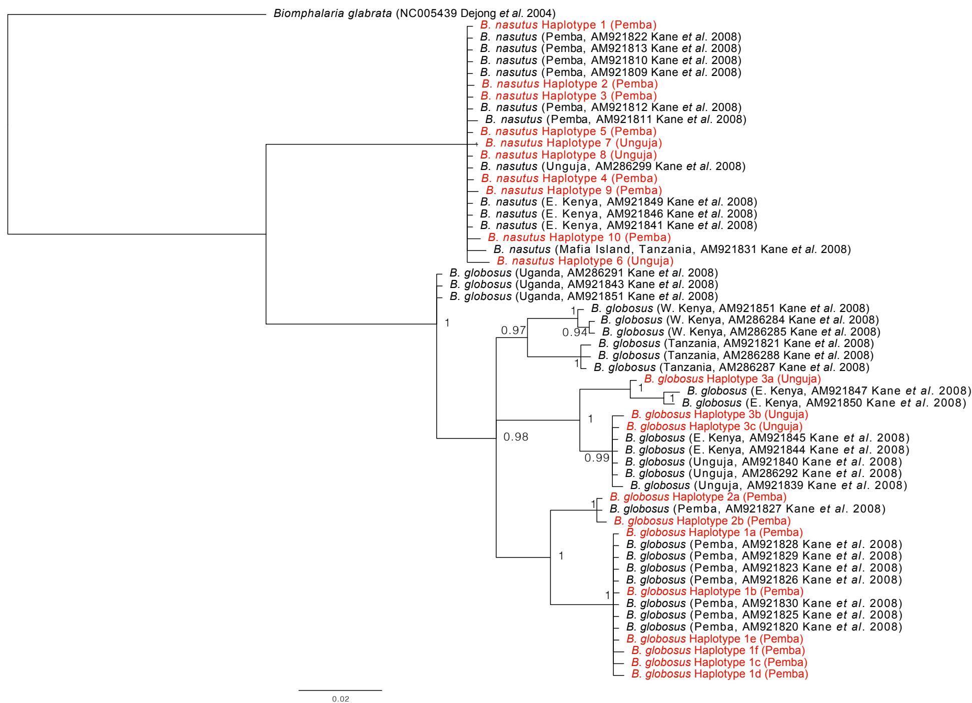


Figure 4.4. Bayesian inference of the partial mitochondrial *cox*1 haplotype dataset of *Bulinus nasutus* and *Bulinus globosus* collected from Unguja and Pemba island and reference data from East Africa produced using Bayesian inference using MrBayes v3.2.7A (Ronquist *et al*. 2012) under the HKY+I+G model (-lnl 2020.2144, AIC 4052.4287). Branches <0.95 posterior probability collapsed. The branch length scale bar indicates the number of substitutions per site. Text in red represents *Bulinus* haplotypes generated from the current study.

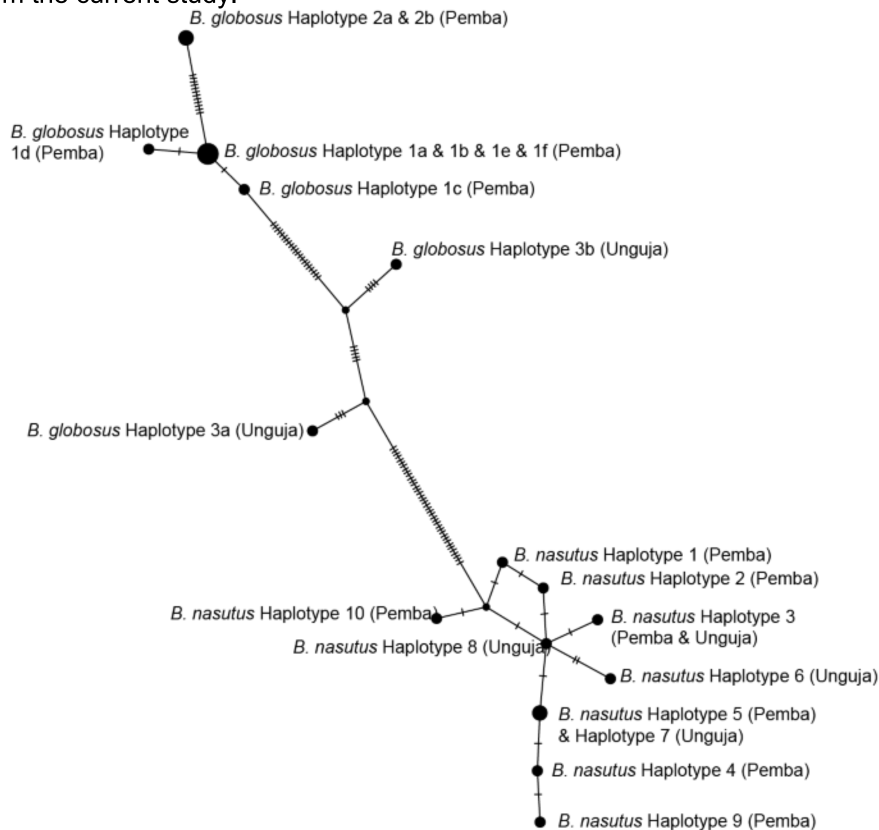


Figure 4.5. TCS haplotype network of *Bulinus globosus* and *B. nasutus* partial *cox*1 DNA sequences (477 bp) produced using PopArt (Leigh & Bryant, 2015).

Table 4.3. *Schistosoma cox*1 haplotypes identified from cercariae and miracidia. Sample; C = cercariae, M = miracidia. Island; U = Unguja, P = Pemba. **Schistosoma* haplotype group represents whether cercariae were identified as mainland Africa (1) or Indian Ocean Island (2) haplotypes (as described in Webster *et al*. 2012)

Schistosoma spp.	*Group	Haplotype	Sample	Number of samples per haplotype	Number of snail samples from	Island	GenBank
Schistosoma haematobium	1	Sh1	C	42	30	U & P	MT380523
		Sh2	C	5	3	U	MT380529
		Sh3	C	6	3	U & P	MT380524
		Sh4	C	2	1	P	MT380525
		Sh5	C	2	1	U	MT380526
		Sh6	C	2	1	U	MT380530
		Sh7	C	1	1	U	MT380527
		Sh8	C	1	1	U	MT380528
	2	Sh9	C	33	16	U & P	MT380535
		Sh10	C	21	12	U & P	MT380531
		Sh11	C	7	4	U	MT380533
		Sh12	C	5	3	P	MT380539
		Sh13	C	4	2	P	MT380536
		Sh14	C	2	1	U	MT380538
		Sh15	C	1	1	P	MT380540
		Sh16	C	1	1	P	MT380532
		Sh17	C	16	5	U & P	MT380537
		Sh18	C	3	1	P	MT380534
Schistosoma bovis	NA	Sb1	C	11	4	P	MT380520
		Sb2	C & M	11	4		MT380521
		Sb3	M	1	NA		MT380522

Table 4.4. Trematode coinfections of *Bulinus* spp. from Unguja and Pemba islands. a = snail identified as *B. globosus*. b = *Schistosoma* haplotype group represents whether cercariae were identified as mainland Africa (1) or Indian Ocean island (2) *cox*1 haplotypes (as described in Webster *et al*. 2012). c = coinfection represents the *Schistosoma cox*1 haplotype group (as described in Webster *et al*. 2012) infection profile of each *Bulinus* spp. Sh1 = *Schistosoma haematobium* mainland African haplotype group 1, Sh2 = *S. haematobium* Indian Ocean haplotype group 2, Sb1= *S. bovis* haplotype 1, Sb2= *S. bovis* haplotype 2, E = *Euclinostomum* sp.

	Shehia	Snail ID	Cercariae ID	Cercariae species	Schistosoma *cox*1 haplotype (group [b])	Coinfection[c]
Unguja Island	Mwera	Mwera 98	ZEST 9	*S. haematobium*	Sh7 (1)	Sh1 & E
			ZEST 10	*Euclinostomum* sp.	NA	
	Mchangani	Tingatinga S11	ZEST 21	*S. haematobium*	Sh1 (1)	Sh1 & Sh2
			ZEST 22	*S. haematobium*	Sh10 (2)	
		Tingatinga S61	ZEST 25	*S. haematobium*	Sh9 (2)	Sh1 & Sh2
			ZEST 26	*S. haematobium*	Sh1 (1)	
	Kinyasini	A2	ZEST 51	*S. haematobium*	Sh1 (1)	Sh1 & Sh2
			ZEST 52	*S. haematobium*	Sh9 (2)	
	Kitope	C4	ZEST 79	*S. haematobium*	Sh10 (2)	Sh1 & Sh2
			ZEST 80	*S. haematobium*	Sh1 (1)	
Pemba Island	Mkanyageni	6284	ZEST 59	*S. haematobium*	Sh1 (1)	Sh1 & Sh2
			ZEST 60	*S. haematobium*	Sh9 (2)	
	Kinyasini	SP_Kin2.3.1 [a]	SP_Kin2.3.1_2	*S. haematobium*	Sh17 (2)	Sh2 & Sh2
			SP_Kin2.3.1_4	*S. haematobium*	Sh9 (2)	
	Kinyasini	SP_Kin4.5.10 [a]	SP_Kin4.5.10_4	*S. bovis*	Sb2 (Sb2)	Sb1 & Sb2
			SP_Kin4.5.10_5	*S. bovis*	Sb1 (Sb1)	
			SP_Kin4.5.10_6	*S. bovis*	Sb2 (Sb2)	
			SP_Kin4.5.10_7	*S. bovis*	Sb2 (Sb2)	

4.3.5 Bulinus *observed shedding* Schistosoma haematobium *and* S. bovis

Of the 20 infected snails collected in Pemba, 12 were identified as *B. globosus* infected with *S. haematobium* and seven as *B. globosus* infected with *S. bovis* (Table 4.5). The remaining patent *Bulinus* collected during targeted surveys in Kangagani was confirmed as *B. nasutus* (haplotype 3), matching that previously reported on Pemba Island (GenBank Accession: AM921812, Kane *et al.,* 2008) and the two cercariae identified from this snail were *S. haematobium* of a single *cox*1 haplotype (Sh3). This schistosome haplotype has been previously identified as 'Group 1' representing those from mainland Africa and Zanzibar (Figure 4.6) (GenBank Accession: GU257343, Webster, Culverwell *et al.* 2013). The infected snail from Unguja Island from the SCAN repository was shedding Group 2 cercariae (Table 4.5).

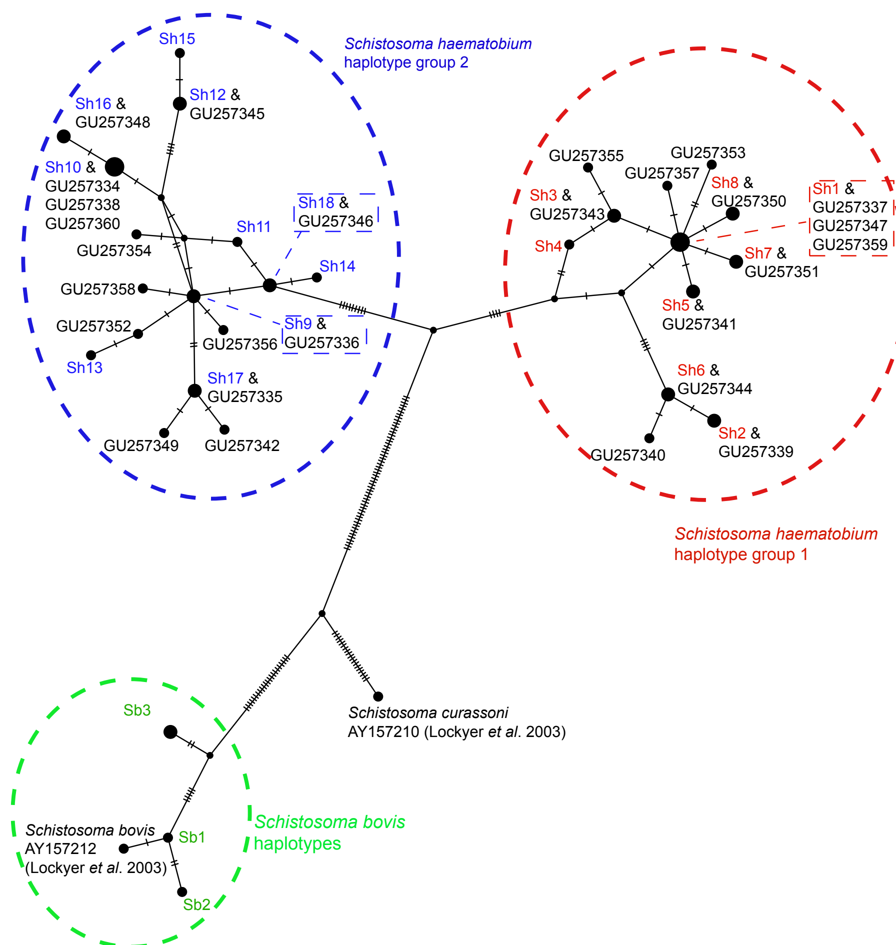


Figure 4.6. TCS haplotype network of *Schistosoma* spp. partial *cox*1 DNA sequences (750 bp) produced using PopArt (Leigh & Bryant, 2015). *Schistosoma haematobium* haplotype groups representing mainland Africa (1) or Indian Ocean island (2) haplotypes (as described in Webster *et al.*, 2012). Hatches represent SNP differences from joined nodes and size of nodes is scaled to the number of identical haplotypes listed. *S. haematobium* reference haplotypes (GU257334 – GU257360) from Webster, Culverwell *et al.*, (2013).

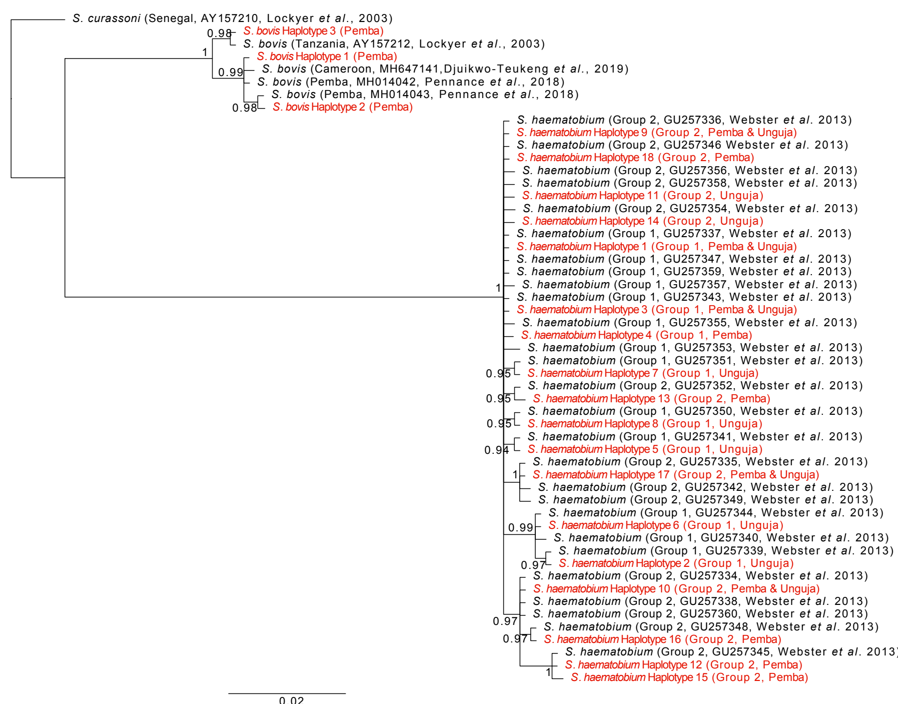


Figure 4.7. Bayesian inference of the partial mitochondrial *cox*1 haplotype dataset of *Schistosoma haematobium* and *S. bovis* collected from Unguja and Pemba Island using MrBayes v3.2.7a (Ronquist *et al*., 2012) under the HKY + I model (-lnL = 1809.8890, AIC 3629.7781, ASDOSF < 0.01 at 1,791,000 generations). Branches <0.95 posterior probability collapsed. The branch length scale bar indicates the number of substitutions per site. Text in red represents *Schistosoma* haplotypes generated in the current study.

Table 4.5. Infected *Bulinus* spp. identified from Unguja and Pemba islands (Zanzibar, United Republic of Tanzania). * *Schistosoma* haplotype group represents whether cercariae were identified as mainland Africa (1) or Indian Ocean Island (2) haplotypes (as described in Webster *et al*. 2012).

Island	Region	Snail ID	*Bulinus* species (haplotype)	Schistosome species	*Schistosoma* haplotype (group*)
Pemba	Chambani	SP_Cham10.1a	*B. globosus* (2a)	*S. haematobium*	Sh13 (2)
	Kangagani	Kangagani-4.1+ve	*B. nasutus* (3)	*S. haematobium*	Sh3 (1)
	Kinyasini	SP_Kin2.3.1	*B. globosus* (1a)	*S. haematobium*	Sh17 & Sh9 (2)
		SP_Kin4.5.3	*B. globosus* (2a)	*S. bovis*	Sb1
		SP_Kin4.5.10	*B. globosus* (2a)	*S. bovis*	Sb1 & Sb2
		SP_Kin4.6.7	*B. globosus* (2a)	*S. bovis*	Sb2
		SP_Kin4.8.3	*B. globosus* (2a)	*S. bovis*	Sb1
		SP_Kin4.9.11	*B. globosus* (2a)	*S. bovis*	Sb_hap2
		S2_Kinya6.1	*B. globosus* (2a)	*S. haematobium*	Sh18 (2)
		S4_Kin_6.1+ve	*B. globosus* (2a)	*S. bovis*	Sb1
		S4_Kin_6.2+ve	*B. globosus* (2a)	*S. bovis*	Sb2
		S4_Kin_6.3+ve	*B. globosus* (2a)	*S. haematobium*	Sh1 (1)
		S2_Kinya9.1	*B. globosus* (2a)	*S. haematobium*	Sh9 (2)
		S2_Kinya9.2	*B. globosus* (2a)	*S. haematobium*	Sh17 (2)
		S2_Kinya9.3	*B. globosus* (2a)	*S. haematobium*	Sh9 (2)
		S2_Kinya9.4	*B. globosus* (2a)	*S. haematobium*	Sh17 (2)
		S2_Kinya9.5	*B. globosus* (2a)	*S. haematobium*	Sh9 (2)
		S2_Kinya11.1	*B. globosus* (2a)	*S. haematobium*	Sh17 (2)
	Kizimbani	S2_Kiz4.1	*B. globosus* (2a)	*S. haematobium*	Sh9 (2)
		S2_Kiz5.1	*B. globosus* (2a)	*S. haematobium*	Sh10 (2)
Unguja	Miwani	UNG-Miwani-123.1	*B. globosus* (3a)	*S. haematobium*	Sh10 (2)

4.4. Discussion

The primary aim of the current study was to update *Schistosoma* species distribution and risk maps of schistosomiasis in Zanzibar for use as a resource in schistosomiasis surveillance, whilst also investigating specific associations between *Bulinus* and *Schistosoma* spp. on the islands. Of the *Bulinus* spp. collected from Pemba Island between 2016 and 2019, 0.18% were infected with schistosomes; *B. globosus* shed *S. haematobium* (n = 9) and *S. bovis* (n = 7) and *B. nasutus* shed *S. haematobium* (n = 1), the latter host-parasite relationship only the first confirmed on Zanzibar following on from findings by Ame (2018). The distribution of *B. globosus* and *B. nasutus* across Pemba and Unguja islands confirmed previous findings (Stothard, Loxton, *et al.*, 2000; Pennance *et al.*, 2016, 2018); however the two species overlapped at two sites along the east coast of Pemba Island where only *B. nasutus* was expected. Identification of a second compatible intermediate snail host for *S. haematobium* (*B. nasutus*) changes our understanding of the snail and schistosome biology on Zanzibar. In addition, the confirmed presence of *S. bovis,* a schistosome species of veterinary importance, infecting cattle on Pemba Island, is cause for concern since increased transmission could lead to significant animal health and economic impact, as well as a potential risk for hybridisation with *S. haematobium* (see Huyse *et al.,* 2009; Savassi *et al.,* 2020).

4.4.1 Distribution and diversity of B. globosus *and* B. nasutus

Cohabitation of *Bulinus globosus* and *B. nasutus* was only observed at one site on Pemba Island (Puj11) so their presence / absence in freshwater bodies may not always be dictated by ecological factors determined by the geological zones of Zanzibar as previously determined (Kent *et al.*, 1971; Stothard, Mgeni, *et al.*, 2002a). Cohabitation of the two species though may have occurred through translocation of snails between disconnected freshwater sites by vector animals, such as predation by birds as has been previously demonstrated (Wada *et al.*, 2012; Salawu and Odaibo, 2013).

The 11 *B. globosus cox*1 haplotypes identified from the snails collected across Zanzibar fell into two distinct clades (Stothard and Rollinson, 1997a). The *B. globosus* from Unguja Island were more closely related to those previously identified from East Kenya (Kane *et al.*, 2008), whilst those from Pemba Island form an independent group distinct from the other East African isolates, suggesting independent origins. This agrees with our current understanding of Zanzibar's geological formation, whereby Pemba Island separated from mainland Africa much earlier, during at least the early Pliocene compared to Unguja Island during the Pleistocene (Kent *et al.*, 1971; Prendergast *et al.*, 2016). In contrast, there was no phylogenetic distinction between the *B. nasutus* from Unguja Island, Pemba Island and mainland East Africa, with samples from each region all forming a single clade of multiple haplotypes. As recorded previously, *B. globosus* is more genetically diverse than *B. nasutus*

(see Stothard & Rollinson 1997b). Identical haplotypes of *B. nasutus* were also present on Unguja and Pemba islands. This is surprising due to the hermaphroditic nature of *Bulinus* that are capable of self-fertilisation and outcrossing, and geographically isolated populations, typically favouring genetic differentiation (Kane *et al.*, 2008; Zein-Eddine *et al.*, 2014). Genome-wide analyses will hopefully resolve these issues of *Bulinus* evolution in the future.

The *B. nasutus* infected with *S. haematobium* observed here in Pemba matched haplotypes of *B. nasutus* in the south of Unguja, so these could also act as intermediate hosts of urogenital schistosomiasis, as is the case in Kenya (Kariuki *et al.*, 2004, 2017) and Tanzania (Webbe, 1962; Kinoti, 1964; Loker *et al.*, 1981; Sarda *et al.*, 1985; Lwambo, 1988; Angelo *et al.*, 2018).

4.4.2 A 'new' intermediate host of Schistosoma haematobium *on Pemba Island:* Bulinus nasutus

Bulinus nasutus infected with *S. haematobium* on Pemba Island is a somewhat unexpected finding since this snail species was considered refractory to infection on Zanzibar (Stothard and Rollinson, 1997a; Stothard, Loxton, *et al.*, 2000; Allan *et al.*, 2013). However this is perhaps not surprising considering the close proximity of Pemba Island to coastal Kenya where *B. nasutus* is involved in transmission (Kariuki *et al.*, 2004). Indeed, pre-patent *Schistosoma* spp. infections previously observed in *B. nasutus* from Pemba Island might have been *S. haematobium* (see Ame, 2018). The current finding also questions whether cercariae collected from the same site in Kangagani during the ZEST study identified here as *S. haematobium*, were shed from *B. nasutus* also (snail identification was not possible, see Supplementary Table 4.2). If so, this would mean that transmission through *B. nasutus* may have been occurring in this region much earlier.

Schistosoma haematobium cercariae shed from intermediate snail hosts, analysed here, fall into two haplotype groups (1 and 2) previously identified from miracidia collected from infected humans (Webster *et al.*, 2012; Webster, Culverwell, *et al.*, 2013). The cercariae shed from *B. nasutus* was identified as a Group 1 parasite, a group predominantly associated with African mainland schistosome populations. Possibly only Group 1 schistosomes are compatible with Pemba *B. nasutus*, since this same snail species acts as an intermediate host in East Africa (Webbe, 1962; Kinoti, 1964; Loker *et al.*, 1981; Sarda *et al.*, 1985; Lwambo, 1988; Kariuki *et al.*, 2004; Angelo *et al.*, 2018). Previous infection trials apparently refuting compatibility of Zanzibar *B. nasutus* may have unknowingly challenged this snail with a putative non-compatible Indian Ocean Island Group 2 *S. haematobium* (see Stothard & Rollinson, 1997a). Such strain dependency or local adaptation reflects the patchy compatibility of *Bulinus* snail hosts of *Schistosoma* generally (Rollinson *et al.*, 2001), discussed previously with *S. haematobium* and *B. nasutus* in Tanzania, where experimental infections of *B. nasutus*

using a local strain *S. haematobium* that usually infects *B. globosus* was unsuccessful (Zumstein, 1983). Although *B. globosus* is the main host of *S. haematobium* on Zanzibar, *B. nasutus* may still play a significant role in transmission especially if a shift towards transmission of Group 1 *S. haematobium* occurs. Further experimental cross infection studies challenging *B. globosus* and *B. nasutus* from Zanzibar with these different *S. haematobium* group parasites are needed to test this hypothesis, and particularly with the *B. nasutus* population (Haplotype 3) identified here. This will also allow for further investigation into this study population and determine whether this infected *B. nasutus* is a complete anomaly, and infection has occurred due to other reasons than simply a 'genetic' compatibility. Using miracidia already available in SCAN collected from humans in these endemic regions where *B. nasutus* is the dominant intermediate host (e.g. Pujini, Uwandani, Kangagani), it could be established through DNA barcoding if Group 1 *S. haematobium* parasites are more common than those of Group 2, which would further suggest local transmission in these regions of Group 1 parasites by *B. nasutus*. Previous molecular characterisation of *Schistosoma* miracidia from Zanzibar though focussed on samples from Unguja (Webster, Culverwell, *et al.*, 2013), therefore this hypothesis cannot be explored with currently available data.

In shehias, such as Muyuni and Mtende in the south of Unguja Island, *B. nasutus*, but not *B. globosus,* is present (see Stothard, Mgeni, *et al.*, 2002b). Since human urogenital schistosomiasis prevalence is low in Muyuni, the few cases present may be due to migrants from endemic areas, rather than there being any local transmission (Stothard, Mgeni, *et al.*, 2002b). In contrast, the prevalence in Mtende has historically oscillated (from 89% highs to 2.5% lows) and therefore is considered an area of 'unstable' or 'intermittent' schistosomiasis transmission (Mansfield-Aders, 1928; Mozley, 1939; Goatly and Jordan, 1965; McCullough and Krafft, 1976; Stothard, Mgeni, *et al.*, 2002b). The naturally infected *B. nasutus* with *S. haematobium* observed here offers some explanation for how transmission may have been maintained in the absence of *B. globosus*. The low levels of *S. haematobium* prevalence in Muyuni could have been driven by successful early attempts at snail control through environmental modification and molluscicidng in this area (Mansfield-Aders, 1928; Mozley, 1939; Stothard, Mgeni, *et al.*, 2002b). This may have also caused a change in snail taxa by removing the more successful intermediate host *B. globosus*, possibly driven by the changes in aquatic vegetation and the pond no longer being permanent throughout the year (Stothard *et al.* 2002b). It should be noted that the *B. nasutus* identified in Mtende during this study, and also a *B. nasutus* isolate from Muyuni not included in the current study (personal observations), are of the same *cox*1 haplotype group as the *S. haematobium* infected *B. nasutus* on Pemba Island, and that Group 1 *S. haematobium* parasites are found across Unguja as well as Pemba. However, due to the dynamic nature of schistosome transmission, regulated by changing freshwater use, water chemistry, improved sanitation, snail control and

other control measures such as behavioural interventions and treatment through mass drug administration, hypotheses related to the changing transmission of *S. haematobium* here on Zanzibar are likely confounded by multiple other aspects at play. These kinds of questions can only be answered through a multi-faceted study incorporating both historical and current knowledge of freshwater usage/WASH and biological factors.

4.4.3 Schistosome haplotype groups

Both Group 1 and Group 2 *S. haematobium* parasites, representing mainland Africa and Indian Ocean groups respectively (Webster *et al.*, 2012; Webster, Culverwell, *et al.*, 2013), were equally abundant on Unguja Island, but Group 2 schistosomes were more common in Pemba Island. As for the snail hosts, these differences might reflect the earlier formation and separation of Pemba Island from mainland Africa compared to Unguja Island (Kent *et al.*, 1971; Prendergast *et al.*, 2016), indicating the Indian Ocean type schistosomes have persisted on Pemba Island without frequent introduction of mainland African types. Alternatively, this variance could reflect present-day differences between the islands, connectivity between mainland Tanzania and Zanzibar is mainly via Unguja Island due to its proximity to Dar es Salaam. Therefore, imported Group 1 *S. haematobium* infections from mainland Tanzania may have occurred more frequently on Unguja Island than Pemba Island. Pemba Island is connected with Kenya through ferry ports (personal communication F. Allan), but not to the degree of Unguja Island's mainland connections.

Five out of six *Bulinus* had *S. haematobium* intraspecific coinfections of Groups 1 and 2 cercariae, with only one snail harbouring an infection of Group 2 cercariae (Table 4.4). As only six *Bulinus* spp. were observed with coinfections, and the minimum number of cercariae analysed per snail was small (n = 2), it is unknown whether this pattern of predominantly mixed *S. haematobium* group coinfections occurred through chance, or host regulation. For example, multiple *S. haematobium* group infections may be dictated by snail immunoregulation and potentially adaptive immunity, whereby an initial infection can prime the immune system causing encounters with the same pathogen to fail (Sire *et al.*, 1998; Pinaud *et al.*, 2016). However, it seems that, at least for *B. glabrata* and *S. mansoni*, this immune memory protects only against schistosomes of the same genetic lineage, and secondary infections with an increasing genetic distance have increased chances of establishing an infection (Portela *et al.*, 2013; Pinaud *et al.*, 2016, 2019). Therefore, due to the genetic distance between these schistosome strains (Webster *et al.*, 2012; Webster, Culverwell, *et al.*, 2013), coinfection of Groups 1 and 2 *S. haematobium*, as observed here, may be more likely than multiple Group 1 or Group 2 infections.

4.4.4 Detection and diversity of S. bovis *in cattle and* B. globosus

Our trial in recovering bovine schistosomes following miracidial hatching was successful in that we detected, collected and identified two *S. bovis* miracidia using this non-invasive sampling method. This low number of miracidia is expected (Giovanoli Evack *et al.*, 2020). Although high *S. bovis* egg excretion is often associated with severe morbidity of cattle, it does decrease with host age (De Bont and Vercruysse, 1998). These sampling techniques need to be tested though on a larger number of stool samples, and in combination with another diagnostic test (such as an antigen based method) to establish sensitivity of this technique (de la Torre-Escudero *et al.*, 2012). Currently, no gold standard/reference test exists for the diagnosis of bovine schistosomiasis making comparisons of diagnostic sensitivity near impossible.

The *S. bovis* cercariae and miracidia collected from seven snails and one cow in Kinyasini were of only three different haplotypes, two of which had been previously identified. The third new haplotype (Sb3) from the cow was identical to laboratory passaged *S. bovis* isolates originating from Iringa, coastal Tanzania (Lockyer *et al.*, 2003), providing some support that these bovine schistosomes may have been introduced recently through the increased importation of cattle to Zanzibar rather than being a distinct Pemba Island population (Mdoe, 2003; Pennance *et al.*, 2018). Investigating the origin of *S. bovis* being transmitted on Pemba, by more extensive genetic comparison with other mainland strains of *S. bovis* such as those from coastal Kenya where cattle have been exported from previously (D. Rollinson, personal communication), may help elucidate how this parasite has been imported to Zanzibar, potentially by comparison with parasites from coastal Kenya. Since the eradication of the Tsetse fly, the vector of human and African trypanosomiasis, on Unguja Island (Vreysen *et al.*, 2000), there has been an increase in cattle farming (Mdoe, 2003) facilitated by the importation of cattle under strict guidelines of the United Republic of Tanzania's Animal Resources Management Act (1999). Bovine schistosomiasis, however, is widely ignored/unknown as a veterinary health problem, and therefore is currently not included in these guidelines, despite the risk to livestock health and the current economic importance of livestock farming to the archipelago's economy (OCGS, 2019) as well as the potential risk to artiodactylids (Standley, Mugisha, *et al.*, 2012), such as Ader's duiker (*Cephalophis adersi*) endemic to Zanzibar.

4.4.5 Study limitations and future work

Several limitations are apparent in the current study. First, few *Bulinus* spp. samples were available for analysis from Unguja Island, so further malacological surveys and collections here would be beneficial to confirm snail species distributions across the island. Second, since the majority of infected *Bulinus* spp. collected during the ZEST studies were not accessioned

with their associated schistosomes, complete inferences on snail-schistosome relationships were impossible here. Third, it would have been beneficial to identify >2 cercariae per snail infection, as this may have significantly increased the number of coinfections observed from these *Bulinus* spp. and also helped to draw conclusions on the association of Group 1 and Group 2 *S. haematobium* coinfections. Although cercariae identification was predominantly used here to identify schistosome species, it would be of interest using these cercarial collections from Zanzibar to explore the population genetics of these intramolluscan infections in the future.

Regarding bovine schistosomiasis, it is essential to sample more cows to identify whether the parasite is present in other regions of Pemba Island rather than just Kinyasini. Also, it is necessary to trial various inner sieve sizes in the Pitchford funnel. For example, the inner sieve size used here (200 μm) may not have been ideal for collecting longer more slender *S. bovis* eggs that can be >200 μm, and an inner sieve size of 300 μm may have been more suitable (Giovanoli Evack *et al.*, 2020). It would also be of interest to trial alternative miracidial hatching techniques, such as homogenization and filtration, that have also succeeded in high positive rates (Savassi *et al.*, 2020).

4.4.6 Implications for future monitoring of schistosomiasis on Zanzibar

The findings discussed here provide implications for future control and elimination efforts of schistosomiasis on Zanzibar (Knopp, Ame, *et al.*, 2019; Knopp, Person, *et al.*, 2019). First, it is suggested here that the Ministry of Health should conduct periodic surveys in areas associated with *B. nasutus* distribution on Unguja and Pemba islands, including both malacological collections and combined human urine collections with questionnaires (including questions on freshwater usage locally and elsewhere in Zanzibar), followed by targeted treatment of infected individuals, to reduce any ongoing transmission. Second, genetic analysis has to be performed on cercariae shed from *Bulinus* spp. snails on Zanzibar to differentiate bovine and human schistosomiasis, and monitor any potential hybridization events between *S. haematobium* and *S. bovis* that may be occurring in cattle (Savassi *et al.*, 2020). Third, further investigation is required to identify the distribution and impact of bovine schistosomiasis on local cattle to assess the requirement for starting livestock treatment to curtail transmission considering the importance of livestock farming to the archipelago's economy (OCGS, 2019).

Chapter 5. Rapid diagnostic assay distinguishing the urogenital schistosomiasis intermediate snail hosts *Bulinus globosus* and *B. nasutus* on Zanzibar

Abstract

As the prevalence of schistosomiasis, a snail-borne neglected tropical disease predominantly in sub-Saharan Africa, moves towards elimination in certain endemic areas, it has become apparent that risk mapping is fundamental for identifying focal transmission areas in order to target intervention strategies. The Zanzibar Archipelago remains endemic for urogenital schistosomiasis despite a long history of urogenital schistosomiasis control through school based and community wide treatment, and more recently additional small-scale focal snail control and behavioural change interventions, aimed at interrupting transmission and reaching eventual elimination. A shift of tac is required now to focus resources into areas of persisting schistosomiasis prevalence (hot-spots) where frequent human-freshwater contact takes place in the presence of suitable snail intermediate hosts.

Of the four *Bulinus* species potentially acting as intermediate hosts in Zanzibar, only one, *Bulinus globosus*, is responsible for the majority of transmission. A closely related freshwater snail species also present on Zanzibar, *B. nasutus*, is morphologically cryptic to *B. globosus* but is generally regarded as refractory to *S. haematobium* infection on the archipelago. However, *B. nasutus* is capable of transmitting the parasite elsewhere in Sub Saharan Africa and evidence is growing to suggest it may play a minor role in transmission on Zanzibar. To facilitate distribution mapping of these two snail intermediate hosts, this study developed a rapid and low-cost diagnostic molecular assay to differentiate these species without the need for DNA sequencing. A multiplex PCR assay was developed for the 5.8S rDNA and ITS2, amplifying two different size fragments for *B. globosus* and *B. nasutus*. This assay provided species specific and consistent differentiation of *B. globosus* (n = 30) and *B. nasutus* (n = 11) tested from Zanzibar and Tanzania. The assay allows cheap and rapid differentiation of two morphologically cryptic species, whose allopatric distribution on Zanzibar may dictate the degree of schistosomiasis transmission across these Indian Ocean islands.

5.1 Introduction

The advent of single dose Praziquantel for schistosomiasis treatment almost 40 years ago lead to its use in widespread preventive chemotherapy programs that continues to this day (Fenwick *et al.*, 2009; Rollinson *et al.*, 2013). Before this, freshwater snail control and the associated research of the intermediate hosts was considered essential to supporting schistosomiasis control programs (King *et al.*, 2015; Sokolow *et al.*, 2016). This mindset was somewhat lost due to the considerable achievements made alleviating morbidity and suffering caused by schistosomiasis through Mass Drug Administration (MDA). However, models

predicting the effects of schistosomiasis control strategies suggest that breaking transmission and reaching the end goal of elimination requires interventions focussed on, or guided by, the snail intermediate hosts role (King and Bertsch, 2015; King *et al.*, 2015; Sokolow *et al.*, 2016, 2018; Allan *et al.*, 2020). The World Health Organisation (2020) also acknowledges the necessity for complementary measures to be used with preventive chemotherapy. It will therefore be vital to use the findings of operational research programs, particularly in sub-Saharan Africa where in comparison to China little has been done to effectively implement these additional strategies (Knopp, Ame, *et al.*, 2019; Allan *et al.*, 2020).

The islands of Pemba and Unguja (Zanzibar Archipelago, United Republic of Tanzania) have a long history of urogenital schistosomiasis (*Schistosoma haematobium*) research and control (see Chapter 2), and are now targeted for elimination through the use of integrated interventions with MDA (Knopp *et al.*, 2012, 2013; Knopp, Ame, *et al.*, 2019; Knopp, Person, *et al.*, 2019). Although prevalence has significantly reduced across the islands in many shehias, transmission had not been completely interrupted (Knopp, Ame, *et al.*, 2019). Considerable spatial heterogeneity of *S. haematobium* infection was observed across Zanzibar irrespective of treatment coverage (percentage of population receiving Praziquantel) and intervention arm, with the prevalence of schistosomiasis decreasing in some shehias and schools, but remaining high in others (Pennance *et al.*, 2016; Knopp, Ame, *et al.*, 2019).

Persistent hotspots of schistosomiasis on Zanzibar likely exist due to frequent human contact with freshwater bodies containing the appropriate intermediate host snails, with contributing factors of close proximity of houses and schools to these water bodies, their attractiveness for farming, household and leisure activities, and the inconvenience of safe water access (Rudge *et al.*, 2008; Pennance *et al.*, 2016; Knopp, Ame, *et al.*, 2019). Other factors related to the snail fauna may also contribute to persisting schistosomiasis prevalence in endemic areas, such as the presence of multiple intermediate host snail species (Mutuku *et al.*, 2019). In future elimination efforts on Zanzibar, it is clear that MDA alone is not sufficient to reduce prevalence enough to break transmission, particularly in persistent hotspots. It is likely that more intensive and sustained interventions such as behavioural change and snail control should be combined with MDA and, if possible, improvements to safe water and sanitation to move towards the ultimate goal of transmission interruption where rapid test and treat strategies can be effectively employed (Knopp, Person, *et al.*, 2019).

Infection with urogenital schistosomiasis on the Zanzibar Archipelago occurs predominantly when humans come into contact with freshwater bodies containing the intermediate snail host *Bulinus globosus* (Stothard and Rollinson, 1997a; Stothard, Loxton, *et al.*, 2000; Allan *et al.*, 2009, 2013), with the same snail species also being incriminated for its role in *S. bovis* transmission (Pennance *et al.*, 2018). However, differentiation of *B. globosus* from *B. nasutus* that is also present on the Zanzibar Archipelago is complicated by the

significant degree of morphological overlap between these two species (Wright, 1961; Goatly and Jordan, 1965; Mandahl-Barth, 1965; Stothard *et al.*, 1997). A diagnostic marker utilising species specific DNA sequences may therefore offer a more reliable method for species identification. Genes within the nuclear ribosomal DNA (rDNA), a tandemly repeated array of 18S, internal transcribed spacer (ITS)-1, 5.8S, ITS-2, 28S and the non- and external transcribed spacers, are often used in molecular systematics, both for *Bulinus* (refs) and for higher taxonomic level analysis of the Planorboidea (Albrecht *et al.*, 2007). The use of rDNA sequences in phylogenetic studies exploring relationships at both the species and genera level such as these assume that intraspecific variation is low and that the tandemly repeated rDNA arrays are homogenous within a single genome, the copies being homogenised through a process of 'concerted evolution' (Arnheim *et al.*, 1980). However, both in *Bulinus* and other freshwater gastropods such as *Lymnaea*, a substantial degree of intra-specific genetic variability and intragenomic heterogeneity is known to exist (Stothard and Rollinson, 1997a; Raahauge and Kristensen, 2000; Stothard, Brémond, *et al.*, 2000), potentially jeopardising the reliability of a rDNA species diagnostic marker.

By exploring newly generated sequence data of the complete 5.8S-ITS2 region of the rDNA of *B. globosus* and *B. nasutus* from Zanzibar, the aim of this study was to develop and validate a single step diagnostic PCR to enable the low cost, rapid, simple and low resource identification of *B. globosus* and *B. nasutus* from Pemba Island whilst avoiding complications with potential intra-genomic heterogeneities. To investigate the geographic range and specificity of the assay, a subset of available *Bulinus* species collected from elsewhere in East and West Africa were also tested.

5.2. Methods

5.2.1 Molecular diagnostic assay to rapidly distinguish Bulinus globosus *and* B. nasutus

Ribosomal DNA (rDNA) sequence data was available from a single *B. globosus* and *B. nasutus* specimen collected from Unguja Island, Zanzibar available through the Schistosomiasis Collection at the Natural History Museum (SCAN) (Emery *et al.*, 2012). The sequence data was obtained from a previous MiSeq (600 cycles) run of multiple snail samples as part of a collaborative Natural History Museum research project (Briscoe *et al*. unpublished). The rDNA fragments were assembled by mapping them to available ribosomal sequence data on GenBank for *Bulinus* and annotated as described by Briscoe *et al.* (2016). The 5.8S rDNA and the internal transcribed spacer 2 (ITS2) were extracted (635 bp for *B. globosus* and 628 bp for *B. nasutus*) from the dataset and, following annotation, an alignment was created in Geneious v.11.1.4 (Biomatters) using MAFFT v7.388 (Katoh and Standley, 2013).

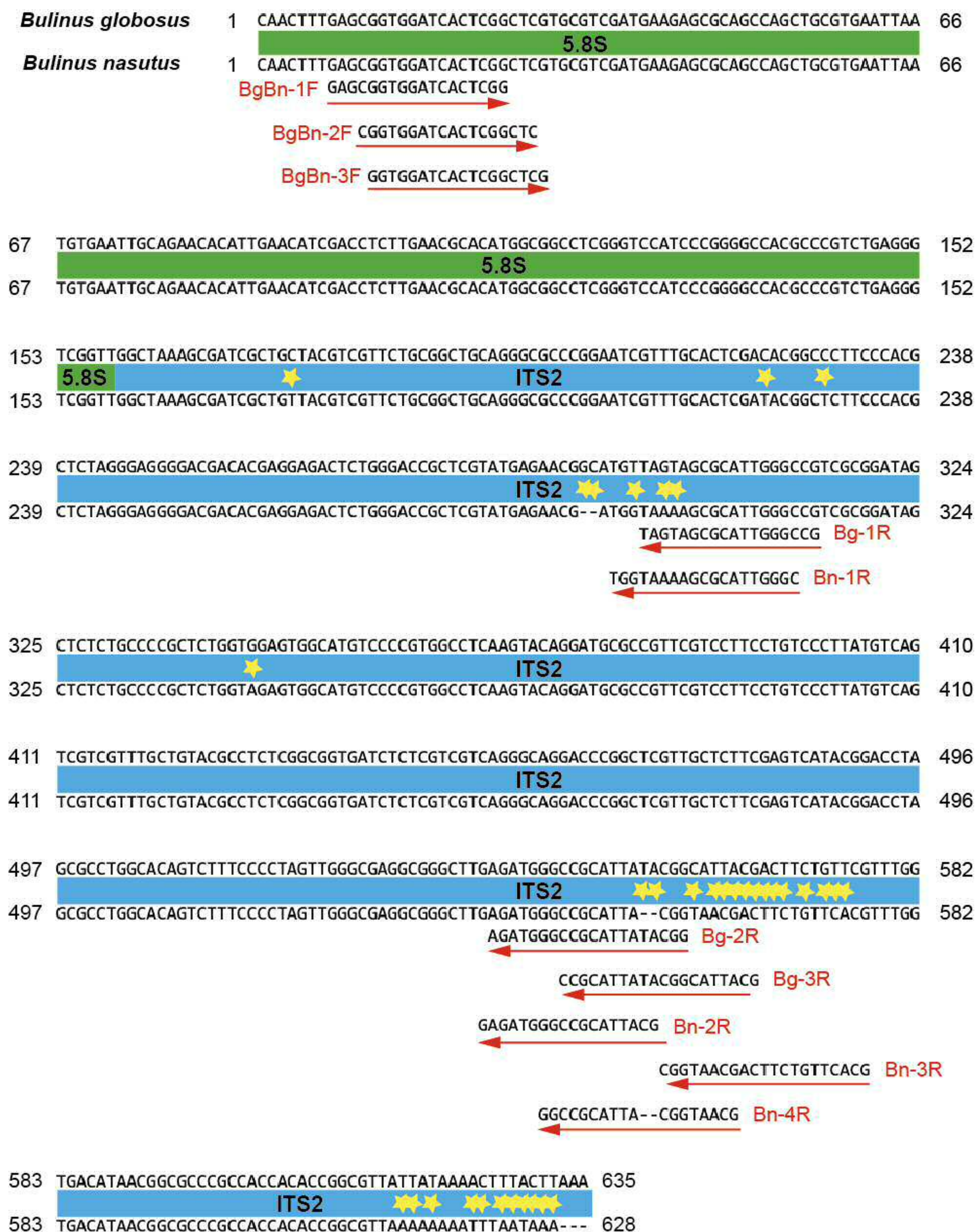


Figure 5.1. Sequence alignment of 5.8S rDNA and ITS2 of *Bulinus globosus* and *B. nasutus* (Zanzibar isolates) showing the positions of universal forward and species-specific reverse primers. Reverse primers labelled Bn- and Bg- represent those designed specifically for *B. nasutus* and *B. globosus* respectively. Yellow stars indicate SNPs or indels.

The alignment was inspected for regions of interspecies nucleotide variability and conservation to enable the design of species-specific and universal primers for the

development of an amplicon based diagnostic assay. The alignment contained two regions of significant polymorphism between the *B. globosus* and *B. nasutus* isolates in the ITS2 (Figure 5.1). The 5.8S rDNA region was conserved across species, and therefore universal forward primers were selected within this region. These three primer sites were manually selected and targeted for primer design using the Primer3 (version 2.3.7) tool within Geneious v.11.1.4 (Biomatters). Three suitable universal forward primers were identified in the 5.8S gene region and seven 'species-specific' reverse primers from the two regions of high SNP density in the ITS2 (Figure 5.1 and Table 5.1) were also deemed suitable (high %GC, containing a 'GC clamp' at the 3' end and melting temperature within 3ºC of each other). All primers were visualised for their positioning on the DNA alignment to evaluate amplicon size using Sequencher v5.1 (Figure 5.1), and the custom oligos were produced by Sigma-Aldrich (UK).

Table 5.1. Primer sequences and expected amplicon lengths for *Bulinus nasutus* and *B. globosus*. Forward primers are universal for both *B. nasutus* and *B. globosus*. Reverse primers labelled with 'Bn-' and 'Bg-' were designed to be specific for *B. nasutus* and *B. globosus* respectively *Amplicon size is shown for when the reverse primers are used in conjunction with the forward primer BgBn-1F and includes primer sequence. NA = no amplification. NS = non-specific amplification.

				Expected amplicon size *		Actual amplicon size from singleplex reactions *	
Primer	Primer sequence (5' - 3')	Forward / Reverse	Primer melting temp. (ºC)	*Bulinus globosus*	*Bulinus nasutus*	*Bulinus globosus*	*Bulinus nasutus*
BgBn-1F	GAGCGGTGGATCACTCGG	F	67.2				
BgBn-2F	CGGTGGATCACTCGGCTC	F	67.2				
BgBn-3F	GGTGGATCACTCGGCTCG	F	67.2				
Bg-1R	CGGCCCAATGCGCTACTA	R	67.1	307	NA	307	307
Bg-2R	CCGTATAATGCGGCCCATCT	R	67.7	552	NA	552	552
Bg-3R	CGTAATGCCGTATAATGCGG	R	64.8	559	NA	559	559
Bn-1R	GCCCAATGCGCTTTTACCA	R	67.9	NA	303	NS	303
Bn-2R	CGTAATGCGGCCCATCTC	R	66.6	NA	547	547	547
Bn-3R	CGTGAACAGAAGTCGTTACCG	R	64.5	NA	566	NS	566
Bn-4R	CGTTACCGTAATGCGGCC	R	66.2	NA	553	553	NA

5.2.2 Singleplex and duplex assay testing

All 21 forward and reverse primer combinations were trialled with single samples of *B. globosus* from Kizimbani (Pemba Island) and *B. nasutus* from Uwandani (Pemba Island), previously extracted and identified through *cox*1 barcoding (see Chapter 4), to provide an overview of primer performance with both species (Supplementary Table 5.1). PCR amplifications were performed in a total reaction volume of 25 µl using GE Healthcare (Amersham, Buckinghamshire, UK) 'Ready-To-Go' PCR beads, 1 µl of each 10 µM primer, 1 µl of template DNA (derived from known *B. globosus* or *B. nasutus* sample) and 22 µl of RNA-free PCR water. A negative control was also performed for each primer set reaction performed, using 1 µl of RNA-free PCR water in replacement of template DNA. Thermocycling was performed in an Applied Biosystems GeneAmp PCR system 9700 thermal cycler

(ThermoFisher, Waltham, MA, USA) with PCR conditions for all primer combinations involving denaturing of 95°C for 5 min followed by 40 cycles of, 95°C for 30 sec, 60°C for 30 sec, 72°C for 45 sec, followed by a final 7 min extension at 72°C. The melting temperature of all primers ranged from 64.5 to 67.7ºC, so a 60ºC annealing temperature was chosen to maximise specificity over lower annealing temperatures. A 4 µl aliquot of each PCR amplicon was loaded with 1 µl of a Bioline (London, UK) 5x DNA Loading Buffer Blue/gel red mix on a 2% agarose gel for 1 hour at 90V and imaged using a GBOX-Chemi-XRQ gel documentation system (Syngene, Cambridge, UK). All gels were run with BioLine (London, UK) HyperLadder IV to assess band sizes in 100 bp increments.

Selected PCR amplicons giving expected amplicon band size were purified using AxyPrep Bead Magnetic Bead Purification Kits (Axygen, part of Corning group, New York, USA). Bi-directional Sanger sequencing was performed on successful amplicons, on an Applied Biosystems 3730xl DNA analyser, using a dilution of the original PCR primers. Sequences were manually edited, aligned and trimmed using Sequencher v.5.4.6 (GeneCodes Corp.) before being compared to the reference sequence data (Briscoe *et al*., unpublished data) to confirm that amplicons were indeed the target amplicon and snail species.

After screening all singleplex primer combinations and confirming band identity through sequencing, those that gave robust, reproducible and desirable species-specific band sizes for *B. globosus* and *B. nasutus* from Pemba Island (Table 5.1), were selected for testing with further field isolates from Pemba Island and other *Bulinus* species from East Africa (mainland Tanzania) and West Africa (Niger) (Table 5.2 and Supplementary Table 5.1). To check for *Schistosoma* cross reactivity, this included specimens with *S. haematobium* or *S. bovis* patent schistosome infections, identified previously through the molecular identification of the shed cercariae (see Chapter 4 and Supplementary Table 5.1). Duplex reactions were trialled utilising a single universal forward primer (BgBn-1F) and each reverse primer. Duplex reactions were performed using the same singleplex thermocycling conditions (described above) but with the addition of 3 primers instead of 2 (1 universal forward primer and 2 species specific reverse primers), therefore reducing the RNA-free PCR water in the reaction to 21 µl of to account for the extra 1 µl of primer.

5.2.3 Specificity assessment with Bulinus *spp. from Sub Saharan Africa*

Primer combinations were tested to validate their performance on *B. globosus* and *B. nasutus* samples from endemic regions other than Pemba Island. This included samples of *B. globosus, B. nasutus, B. nasutus productus, B. africanus* sp*., B. truncatus* and *B. forskalii* from Niger (see Chapter 3) and mainland Tanzania (see Supplementary Table 5.1), previously identified by *cox*1 molecular analysis. The specimens included additional *B. globosus* and *B.*

nasutus, *B. nasutus productus* and unidentified *B. africanus* group species closely related to *B. nasutus* and *B. globosus* respectively (Kane *et al.*, 2008), and more distantly related *Bulinus* spp.; *B. truncatus* and *B. forskalii* (Table 5.2). Adverse or false positive reactions were deemed as those which produced PCR amplicons of the same molecular weight as those observed from *B. globosus* or *B. nasutus*.

Table 5.2. Summary of the *Bulinus* species used and those that could be determined during the species diagnostic primer trials. *Adverse species determination were samples that produced PCR amplicons of the same or similar molecular weight as those observed from *B. globosus* or *B. nasutus* judged by the gel electrophoresis.

Bulinus species group	*Bulinus* species	Location	Number of samples	Species determined?*
Bulinus africanus group	*Bulinus globosus*	Pemba Island	30	Yes
		Tanzania	1	Yes
		Niger	1	Yes
	Bulinus nasutus	Pemba Island	12	Yes
	Bulinus (nasutus) productus	Tanzania	2	No (adverse)
	Bulinus sp. (africanus group)	Tanzania	1	No (adverse)
Bulinus truncatus/tropicus complex	*Bulinus truncatus*	Niger	1	No
Bulinus forskalii group	*Bulinus forskalii*	Niger	1	No

5.3. Results

5.3.1 Molecular diagnostic assay primer design

The 5.8S gene (158 bp) of *B. globosus* and *B. nasutus* was conserved between species, allowing the design of three universal forward primers at the 5' end of this region (Figure 5.1 and Table 5.1). Two regions within the ITS2 (470 bp *B. nasutus* and 477 bp *B. globosus*) showed inter-species mutations that enabled the design of seven (four for *B. nasutus* and three for *B. globosus*) species specific primers (Figure 5.1 and Table 5.1). Two primers were designed to generate a 'short' amplicon for *B. globosus* (Bg-1R) and *B. nasutus* (Bn-1R) and five primers were designed to generate a 'long' amplicon for *B. globosus* (Bg-2R and Bg-3R) and *B. nasutus* (Bn-2R, Bn-3R, Bn-4R) when used in conjunction with one of the universal forward primers identified in 5.8S (Figure 5.1). Primer sequences and desired amplicon lengths for the selected primer pairs for *B. globosus* or *B. nasutus* are displayed in Table 5.1.

5.3.2 Singleplex and duplex assay testing

The majority of the 21 primer combinations tested, generated PCR products for both *B. globosus* and *B. nasutus* samples from Pemba Island (Table 5.1, Figure 5.2). As well as amplifying the targeted *B. globosus* rDNA, the *B. globosus* specific primers (Bg-1R – Bg-3R)

also generated amplicons of the same size for *B. nasutus* (Table 5.1). Two of the *B. nasutus* specific primers (Bn-1R and Bn-3R) resulted in non-specific banding for *B. globosus* samples but expected amplicons for *B. nasutus*, another (Bn-2R) giving the same amplicon size for both *B. globosus* and *B. nasutus*, and the final primer (Bn-4R) unexpectedly amplified *B. globosus* instead of *B. nasutus* with the desired amplicon size (orange box, Figure 5.2). From these results, Bn-1R and Bn-4R were considered to give *B. globosus* and *B. nasutus* specific bands, importantly of different amplicon sizes. However, further trialling of these primers on other *B. globosus* and *B. nasutus* samples showed that amplification of *B. nasutus* and *B. globosus* also occurred with these primers in duplex reactions and therefore species could not be differentiated (results not shown). Bn-4R was replaced by Bn-3R to increase specificity for *B. nasutus*, however this then failed to amplify *B. globosus* samples from mainland Tanzania and also gave multiple bands for *B. nasutus* (Figure 5.3)

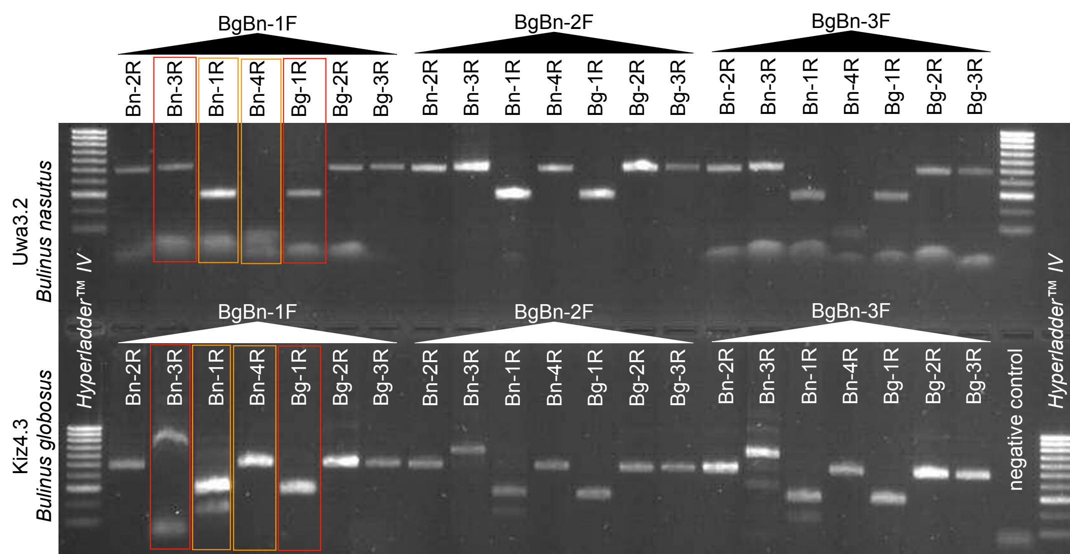


Figure 5.2. Gel electrophoresis showing PCR amplicon products of the partial 5.8S and ITS2 genes of *Bulinus nasutus* (top row) and *B. globosus* (bottom row) using 21 primer combinations aimed at rapidly distinguishing the two species. Forward primers are shown in horizontal text and reverse vertical text for each reaction. HyperLadder™ IV = 100bp bands. Uwa3.2 = *Bulinus nasutus* from Pemba Island. Kiz4.3 = *B. globosus* from Pemba Island.

A third duplex reaction was therefore trialled using Bg-1R, which originally amplified a 307 bp fragment for both *B. globosus* and *B. nasutus* and Bn-3R that amplified an expected 553 bp fragment of *B. nasutus* and produced non-specific banding for *B. globosus* (Table 5.1, and red boxes in Figure 5.2). Although initially Bg-1R was not specific for *B. globosus* due to amplification of *B. nasutus* DNA (see Table 5.1 and Figure 5.2), it was predicted that in the presence of two primers, preferential amplification of the most suitable primer pair for each species would take place. This duplex reaction (BgBn-1F + Bg1R + Bn3R) was first trialled using samples of *B. globosus* from Pemba Island and mainland Tanzania and one *B. nasutus productus* from Tanzania (Supplementary Table 5.1) giving the desired differential banding

patterns (Figure 5.3).

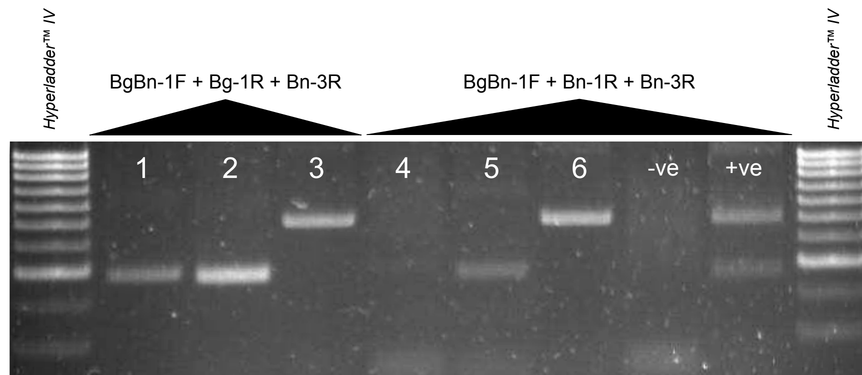


Figure 5.3. Gel electrophoresis showing PCR amplicon products of partial 5.8S and ITS2 genes of *Bulinus globosus* from Tanzania (Lane 1 & 4) and Pemba Island (Lane 2 & 5) and *B. nasutus productus* from Tanzania (Lane 3 & 6). -ve = RNA free water. +ve = *B. nasutus* from Pemba Island. See Supplementary Table 5.1 for full details of each sample per lane.

The tested duplex assay (BgBn-1F + Bg-1R + Bn-3R) was therefore performed on multiple *B. globosus* and *B. nasutus* from three shehias on Pemba Island (Pujini, Wambaa, Matale), *Bulinus* sp. and *B. globosus* from Tanzania and *B. forskalii* from Niger (Figure 5.4). Expected banding patterns were observed from all *B. globosus* and *B. nasutus*, a faint secondary band of the larger *B. nasutus* specific amplicon was observed for the *Bulinus* sp. from Tanzania, and no amplification was observed from *B. forskalii* as expected. As predicted, the amplicons produced using BgBn-1F + Bg-1R with *B. nasutus* observed in initial primer tests (see Figure 5.2) was not observed when reactions performed with the *B. nasutus* Bn-3R primer.

This duplex primer combination (BgBn-1F + Bg-1R + Bn-3R) showed no adverse amplifications with *B. globosus* with patent *S. haematobium* (Lanes 1 and 7-16 Figure 5.5) and *S. bovis* (Lane 2-6 Figure 5.5) infections, single banding was observed for *B. productus* with a *S. haematobium* infection (Lane 21 Figure 5.5) and non-specific banding for non-target *B. truncatus* species infected with *S. haematobium* (Lane 22 Figure 5.5). The primers also amplified *B. globosus* positive for *S. haematobium* infection from Niger (Lane +ve Figure 5.5).

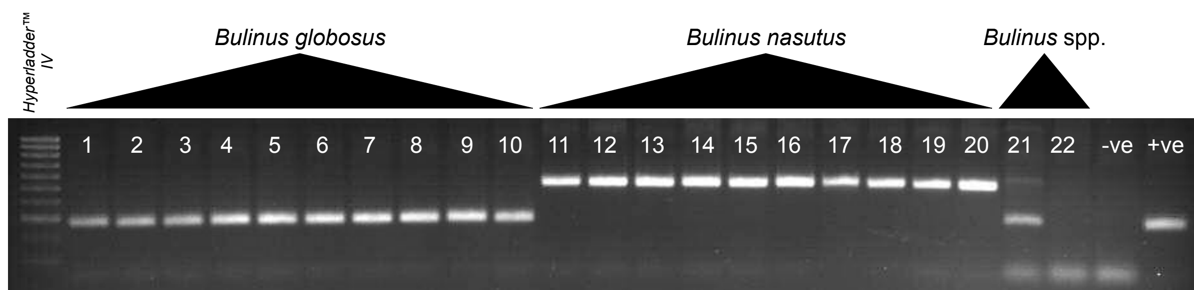


Figure 5.4. Gel electrophoresis showing PCR amplicon products of partial 5.8S and ITS2 genes using primer combination BgBn-1F + Bg-1R + Bn-3R of *Bulinus globosus* from Pemba Island (Lane 1-10), *B. nasutus* from Pemba Island (Lane 11-20), an undescribed *Bulinus* sp. (africanus group) from Tanzania

(Lane 21) and a *B. forskalii* isolate from Niger (Lane 22). Legend: -ve RNA free water. +ve = *B. globosus* from Tanzania. See Supplementary Table 5.1 for full details of each sample per lane.

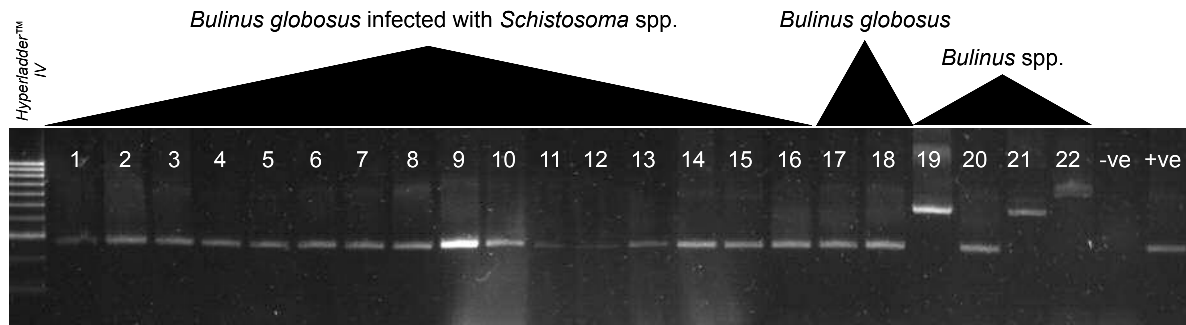


Figure 5.5. Gel electrophoresis showing PCR amplicon products of partial 5.8S and ITS2 genes using primer combination BgBn-1F + Bg-1R + Bn-3R of *Bulinus globosus* with patent *Schistosoma haematobium* (Lane 1 & Lane 7-16) and *S. bovis* (Lane 2-6) infections, *B. nasutus productus* with a patent *S. haematobium* infection (Lane 21), a non-target species *B. truncatus* with a patent *S. haematobium* (Lane 22) and also uninfected *B. globosus* (Lane 17, 18 & 20) and uninfected *B. nasutus* (Lane 19). Legend: -ve RNA free water. +ve = *B. globosus* from Niger with a patent *S. bovis* infection. See Supplementary Table 5.1 for full details of each sample per lane.

5.4 Discussion

To employ effective malacological surveys in an endemic setting such as Pemba Island, it is essential to fully understand schistosomiasis transmission dynamics in relation to the *Bulinus* spp. (*B. globosus* and *B. nasutus*) that act as intermediate hosts. Here, we have developed a duplex PCR assay using the primers BgBn-1F + Bg-1R + Bn-3R (Table 5.1) targeting both the conserved (5.8S gene) and variable (ITS2) rDNA regions to accurately differentiate, by amplicon size, *B. globosus* and *B. nasutus,* both from East and West Africa. There was no cross reactivity with distantly related *Bulinus* spp. also present in sub-Saharan African (*B. truncatus* and *B. forskalii*). However, banding patterns for *B. nasutus* and its closely related *B. nasutus productus* from Tanzania were the same, and an undescribed *Bulinus* sp. from Tanzania gave the same banding pattern as the closely related *B. globosus*. Therefore, the assay can differentiate between the *B. nasutus* and *B. globosus* tested from Pemba where these other closely related species are not present, but results of the assay could be confused with non-target species if applied on mainland Africa.

Other than circumventing unreliable morphological identifications, there are several advantages to using this diagnostic assay. The protocol is simple and involves few steps allowing high numbers of samples to be processed. The full protocol (including snail tissue digestion and DNA extraction) and interpretation of results (by visualising amplicons using gel electrophoresis) only uses basic laboratory equipment (incubator, centrifuge, PCR machine, agarose gel electrophoresis) and does not require a cold chain for reagents or any additional steps requiring enzymes, such as for restriction digest (Stothard & Rollinson 1997a, b;

Stothard *et al.,* 1997; Tchami Mbagnia *et al.,* 2020). The assay involves just a single PCR for species determination, halving the number of reactions required in the previously described taxon specific diagnostic assay (Stothard, Mgeni, *et al.*, 2002a). Species identification can therefore be performed locally by researchers and technicians in Zanzibar using basic molecular laboratories. This all contributes towards a cost-effective and easy to implement protocol for mapping snail distribution and potential transmission risk areas in Zanzibar.

During field-based malacological surveys investigating urogenital schistosomiasis transmission, collected bulinids are often identified based on shell morphology alone, with schistosome identification taking precedence due to this being the etiological agent (Labbo *et al.*, 2007). The absence of basic and low cost (i.e. without sequencing costs and sequence analysis) protocols for snail species delimitation, as is available for certain *S. haematobium* group schistosomes (Webster *et al.*, 2010), may be a reason for this. The accessibility and simplicity of this diagnostic marker for *S. haematobium* parasites enabled the discovery of hybrid *S. haematobium* group parasites from snails in West Africa (Tian-Bi *et al.*, 2019; Pennance, Allan, *et al.*, 2020) and led to the discovery of *S. bovis* on Zanzibar (Pennance *et al.*, 2018). The *Bulinus* spp. diagnostic assay reported here offers a tool for future intervention planning, being particularly pertinent considering the updated WHO recommendations for snail control and monitoring to be integrated as a complementary measure (WHO, 2017a, 2020a).

This duplex assay showed no cross reactivity for distantly related non-target *Bulinus* species; *B. truncatus* and *B. forskalii*. To aid in interpreting these negative results (i.e. no PCR amplicon observed), the assay could be improved by including an internal control capable of amplifying a universal region of all *Bulinus* spp. DNA. Also, considering that misidentification of other *Bulinus* spp. is commonplace (e.g. *B. globosus* misidentified as *B. truncatus* from Niger; Pennance *et al.,* 2020), it would be useful to expand this duplex into a multiplex assay to also include *B. truncatus*. Although not necessary on the Zanzibar Archipelago, this would be particularly useful in West Africa where *B. globosus* and *B. truncatus* are widespread and can inhabit the same freshwater body (Brown, 1994) as shown in Niger (Rabone *et al.*, 2019; Pennance, Allan, *et al.*, 2020) and Senegal (Ndione *et al.*, 2018, 2019). Although to a trained malacologist these two species are distinct in their shell morphology, misidentification by less experienced snail collectors is possible, particularly when dealing with juvenile snails. In order to produce another species-specific marker for *B. truncatus*, available ribosomal DNA data could be assembled and annotated for *B. truncatus* (Briscoe *et al.* personal communication), further sites of variance explored and *B. truncatus* specific primers designed.

Unlike species of the *B. truncatus/tropicus* complex, species belonging to the *B. forskalii* group have very distinct shell morphology (having a slender shell and a spire higher than the aperture) compared to snails of the *B. africanus* group (*B. globosus* and *B. nasutus*)

that have a broader shell with a spire height less than that of the aperture. Because of this, it was deemed unnecessary to distinguish the *B. forskalii* group species present on Pemba Island in this assay from those of the *B. africanus* group. However, significant morphological overlap does exist between species within the *B. forskalii* group, such as *B. forskalii* and *B. senegalensis*, that are host to *S. haematobium* group parasites, particularly in West Africa (Frandsen, 1979; Ngonseu *et al.*, 1992; Labbo *et al.*, 2003, 2007), therefore a separate diagnostic assay to separate species within this group, improving on those previously developed (Jones *et al.*, 1997), may offer a useful solution for mapping freshwater snails in this region.

The assay was performed on two isolates of *B. nasutus productus* and one currently undescribed *Bulinus* sp. from Tanzania, which are closely related to *B. nasutus* and *B. globosus* respectively (Kane *et al.*, 2008). Both *B. nasutus* and *B. nasutus productus* act as intermediate hosts of *S. haematobium* in regions of East Africa (Webbe, 1962; Kinoti, 1964; McCullough *et al.*, 1968; Loker *et al.*, 1981; Sarda *et al.*, 1985; Lwambo, 1988; Kariuki *et al.*, 2004; Angelo *et al.*, 2018), and a 'typical' form of each can allow for distinguishing between species (see Brown, 1994). Therefore, differentiation of these two species using a molecular assay may be less important. However, the undescribed *Bulinus* sp. with a currently undetermined role in *S. haematobium* transmission present in Tanzania, could be confused with *B. globosus* due both to its overlapping morphology and similar amplicon size (albeit with a secondary larger non-specific amplicon also produced, the consistency of which was not tested). Therefore, the diagnostic marker described here should be used with caution outside of the Zanzibar Archipelago. It should also not be ignored that several other *B. africanus* group snails are present in East Africa, such as *B. africanus*, that could produce similar results to *B. globosus* or *B. nasutus* with this marker. The diversity of species of these *B. africanus* group species across mainland Africa, and their role in the transmission of schistosomiasis, is less well understood than on Zanzibar, and therefore the necessity for such species diagnostics needs to be determined.

Although *B. globosus* and *B. nasutus* from Unguja were not trialled with this assay, the primers used in this assay were developed using sequence data from *B. globosus* and *B. nasutus* from isolates collected in Unguja (Briscoe *et al.* unpublished). It is therefore almost certain that the assay will function as with the snails distinguished from Pemba Island, but tests are needed to confirm this. Further tests on a broader sample range are particularly necessary due to the erratic nature of the ITS loci of *Bulinus*, i.e. intra-genomic non-homologous rDNA sequences (Stothard and Rollinson, 1997a). The developed primers may therefore behave in an allele specific manner if heterogeneous copies of the ITS-2 are present in individuals that interfere with the selected primers, although this was not observed from the *B. globosus* and *B. nasutus* from Pemba tested here, this may occur in populations included

from a wider sample range. However, assuming that feasibility to use this marker across the Zanzibar Archipelago is established through testing on more snails from Zanzibar, it may be deemed beneficial for future risk planning and interventions (Knopp, Ame, *et al.*, 2019; Knopp, Person, *et al.*, 2019) to produce a comprehensive and updated *Bulinus* species distribution map to assess potential at risk areas with greater accuracy.

To aid in targeting control interventions on Zanzibar to those areas with continuing *S. haematobium* transmission, it is important to improve surveillance methods used for the detection of *S. haematobium*. Diagnosing human infections of *S. haematobium* rapidly using sensitive diagnostics, at the point of care, will be vital at avoiding reintroduction and recrudescence in areas that may become free of transmission (Rosser *et al.*, 2015; Archer *et al.*, 2019; Rostron *et al.*, 2019). Snail xenomonitoring protocols to identify *Schistosoma* spp. infections within snails will allow for the identification of 'active' transmission sites (Abbasi *et al.*, 2017; Pennance *et al.*, 2018; Schols *et al.*, 2019), but accurately determining and assessing freshwater snail species will additionally allow for infection risk mapping in endemic regions for targeting schistosomiasis control and interventions.

Chapter 6. Snail xenomonitoring on Pemba Island: a surveillance approach for schistosomiasis control and elimination programmes

Abstract

Surveillance of freshwater snails has been discussed as a predictive tool for assessing schistosomiasis transmission, particularly in the context of surveillance in elimination programs. The current study validated a high-throughput xenomonitoring protocol for the detection of non-patent *Schistosoma haematobium* (causing urogenital schistosomiasis) and *S. bovis* (causing bovine schistosomiasis) infections in *Bulinus* spp. in the elimination setting of Pemba Island (Zanzibar, United Republic of Tanzania).

Bulinus spp. were collected and checked for patent schistosome infections across four surveys (2017-2018) from 52 human freshwater contact sites located in eight shehias (smallest administrative regions) on Pemba Island. Shehias varied from high (persisting) to low (declining) schistosomiasis prevalence, but with variable abiotic conditions. Of the 9,430 *Bulinus* spp. collected, 5,465 were screened for non-patent *Schistosoma* infections. The xenomonitoring assay identified 2.72% (149) *S. haematobium* and 0.24% (13) *S. bovis* non-patent infections from those extracted, whilst only 0.11% (10) *S. haematobium* and 0.02% (2) *S. bovis* patent infections were observed using standard parasitological methods. *Bulinus* abundance significantly decreased, whilst the probability of *S. haematobium* infection increased in warmer water temperatures. *Bulinus* abundance was also significantly lower at sites in the lowest transmission shehias. The probability of *S. haematobium* infection was significantly higher when snails were collected from normal compared to flooded or even low water levels.

The xenomonitoring assay was up to ~24 times more sensitive at detecting *S. haematobium* infections than standard parasitological procedures. Non-patent infections suggest that *S. bovis* transmission occurs in several focal areas on Pemba Island, with a wider distribution than previously thought. Water level and temperature contributed to the persistence of urogenital schistosomiasis transmission. The identification of schistosome transmission in low prevalence settings shows the feasibility for using xenomonitoring assays in transmission monitoring and elimination surveillance.

6.1 Introduction

Schistosomiasis is a neglected tropical disease (NTD) leading to severe morbidity and mortality caused by infection with parasitic worms of the genus *Schistosoma*. An estimated 229 million people are infected with many more at risk, ~90% of whom live in Africa (WHO, 2020c). In Africa there are two forms of the disease, urogenital and intestinal schistosomiasis

caused by *Schistosoma haematobium* and *S. mansoni,* respectively, each having their own disease pathology and lifecycles (Colley *et al.*, 2014). Urogenital and intestinal schistosomes are transmitted through a specific intermediate host of the *Bulinus* or *Biomphalaria* genus. Infection occurs when humans come into contact with freshwater containing these infected gastropods, that act as disease reservoirs, shedding cercariae. Although the adult parasites can be removed from humans using Praziquantel, reinfection by coming into contact with larvae shed from freshwater snails is immediately possible, and immature worms are not effectively killed by the treatment in the human host, so prevalence can return to baseline levels once chemotherapy ceases (Gray *et al.*, 2010). Each schistosome species has a specific geographical distribution defined by their intermediate snail host's habitat range (Brown, 1994).

The most recent WHO recommendations for progressing towards the elimination of schistosomiasis as part of the 2030 NTD goals (WHO, 2020a), highlight the need for increased snail-focused interventions such as molluscicidimg to combat the disease (WHO, 2017a). Snail surveillance is a potential predictive tool for assessing transmission levels of schistosomiasis, which should facilitate the ultimate goal of parasite elimination (King *et al.*, 2006; Rollinson *et al.*, 2013). Identifying freshwater bodies where the specific species of *Bulinus* host is present and observing patent infections, whereby schistosome cercariae are actively being shed from the snail, are the first stages in assessing infection risk (Allan *et al.*, 2013). The distribution of intermediate host snails and the likelihood of observing patent schistosome infections, is determined by a mosaic of abiotic and biotic variables (DeWitt, 1955; Chu *et al.*, 1966; Purnell, 1966; El-Hassan, 1974; Pennance *et al.*, 2016; Kalinda *et al.*, 2017a, 2017b; Rabone *et al.*, 2019). This can make identifying areas of transmission challenging, in addition to snail and larval schistosome species being morphologically cryptic and the prevalence of patent schistosome infections being low (Allan *et al.*, 2013; Pennance *et al.*, 2016; Pennance, Allan, *et al.*, 2020), especially in regions nearing elimination (see Chapter 4). Furthermore, as well as patent schistosome infections, snails that have been exposed to *Schistosoma* (signifying waterbody contamination with miracidia) can result in other infection 'outcomes' observed at the time of snail collection, such as pre-patent (early stage of snail infection before cercarial shedding occurs) or failed infections (where the larval schistosome has been unable to establish an infection in the challenged intermediate host). Both of these outcomes, collectively referred to as non-patent infections from here on, are missed through monitoring of cercarial shedding, however they provide important information that schistosomiasis transmission is still occurring. It is therefore proposed that molecular detection of *Schistosoma* DNA in *Bulinus*, including molecular xenomonitoring of intermediate hosts, will be a more efficient and definitive approach to monitor snail infections and certifying elimination / interruption of transmission in endemic areas (Rollinson *et al.*, 2013).

Molecular xenomonitoring refers to the collection and testing of vectors for the presence of pathogen DNA to monitor the transmission of these etiological agents of medical and veterinary importance (Minetti *et al.*, 2016). The technique is often associated with diseases transmitted by hematophagous insects, such as lymphatic filariasis in mosquito vectors (Schmaedick *et al.*, 2014; Pilotte *et al.*, 2016, 2017; Cook *et al.*, 2017) and trypanosomes in tsetse flies (Cunningham *et al.*, 2016; Garrod *et al.*, 2020). Several assays have been developed for detecting trematodes in freshwater snails (Kaplan *et al.*, 1995; Born-Torrijos *et al.*, 2014; Rathinasamy *et al.*, 2018), including medically important schistosome species; *S. japonicum* (Kumagai *et al.*, 2010; Tong *et al.*, 2015), *S. mansoni* (Hamburger, Ramzy, *et al.*, 1998; Hamburger, Xu, *et al.*, 1998; Melo *et al.*, 2006; Abbasi *et al.*, 2010; Kane *et al.*, 2013; Fernández-Soto *et al.*, 2014; Gandasegui *et al.*, 2016; Lu *et al.*, 2016; Caldeira *et al.*, 2017; Schols *et al.*, 2019; Casotti *et al.*, 2020) and *S. haematobium* (Hamburger *et al.*, 2001, 2004; Abbasi *et al.*, 2010, 2017; Allan *et al.*, 2013; Kane *et al.*, 2013; Schols *et al.*, 2019; Kaiglová *et al.*, 2020). The first developed assay for the molecular detection of *S. haematobium* DNA in *Bulinus* employed the highly repetitive *Dra*1 sequence and this has been the marker of choice for the majority of these xenomonitoring studies for urogenital schistosomiasis (Hamburger *et al.*, 2001). However, the specificity of *Dra*1 and interpretation of results are not reliable, with false positive and negative results often observed (Pennance, Archer, *et al.*, 2020). Hence, new markers with high sensitivity and specificity for schistosomes have been developed (Schols *et al.*, 2019), including a high-throughput assay (Pennance, Archer, *et al.*, 2020). This assay is a two-step screening method with the first multiplex PCR, incorporating an internal snail control, enabling the quick identification of an infection and then a second targeted PCR for a more detailed analysis of the infection. The next step is to test the field applicability of this newly developed xenomonitoring assay for the detection of patent and non-patent *S. haematobium* infections in snails.

Zanzibar, an archipelago of two islands (Unguja and Pemba) in the United Republic of Tanzania, has been targeted with multidisciplinary interventions in an effort to eliminate schistosomiasis (Knopp *et al.*, 2012, 2013; Knopp, Ame, *et al.*, 2019; Knopp, Person, *et al.*, 2019). Although schistosomiasis prevalence has significantly decreased across the islands, focal areas of high schistosomiasis prevalence, referred to as persistent transmission hot-spots, remain (Pennance *et al.*, 2016; Knopp, Ame, *et al.*, 2019). Regions across the islands with different schistosomiasis transmission intensities provide an ideal system for testing a xenomonitoring marker, since *S. haematobium* prevalence in *Bulinus* species is expected to vary with local transmission intensities. Additionally, the presence of two intermediate hosts for *S. haematobium* (*B. globosus* and *B. nasutus*, see Chapter 4), and the occurrence of *S. bovis* in one region on Pemba Island (Pennance *et al.*, 2018), allows for further investigation into the distribution of these infections across the island.

The primary aim of the current study was to demonstrate the feasibility and capacity of a high-throughput xenomonitoring assay for the detection of non-patent *S. haematobium* infections in large snail populations collected between 2017 and 2018 across Pemba Island (Pennance, Archer, *et al.*, 2020). This was investigated in different *S. haematobium* transmission settings, the inferred highest (persisting schistosomiasis hot-spot areas) and lowest (persisting very low / intermittent prevalence areas) transmission settings established following biannual preventive chemotherapy and additional interventions conducted between 2012 and 2016 (Knopp, Ame, *et al.*, 2019). The secondary aim was to model the effect seasonality and ecological variables had on the abundance of *Bulinus* species, as well as the infection status of snail populations. Finally, the scale and depth of these investigations enabled further insights into the distribution of *S. haematobium* and *S. bovis* across the Island and the role of the different *Bulinus* spp. (*B. globosus* and *B. nasutus*) in transmission.

6.2 Methods

6.2.1 Study area selection and characterisation

Urogenital schistosomiasis infection prevalence data of 9-12 year old schoolchildren from Pemba Island (Knopp, Ame, *et al.*, 2019; Knopp, Person, *et al.*, 2019) was used to select xenomonitoring study shehias by assessing the change in prevalence from 2012 to 2016. This allowed the majority of shehias on Pemba Island to be categorised into one of four ‘transmission scenarios’ (Table 6.1).

Table 6.1. Schistosomiasis transmission levels on Pemba Island (Zanzibar) based on the prevalence of *Schistosoma haematobium* in 9 to 12-year-old school children from 2012 to 2016 in shehias. *Urogenital schistosomiasis prevalence provided by Knopp, Ame, *et al.*, 2019.

	Urogenital schistosomiasis prevalence (egg positive) *	
Transmission level	2012	2016
High non-responder (HNR)	> 10%	< 50% reduction in prevalence
High decliner (HD)	> 10%	> 50% reduction in prevalence
Low non-responder (LNR)	> 0% to < 5%	< 50% reduction in prevalence
Low decliner (LD)	> 0% to < 5%	> 50% reduction in prevalence

Following the inclusion criteria outlined in Figure 6.1 and feasibility (primarily pre-existing knowledge on access to freshwater bodies for malacological surveys), two shehias per transmission category (Table 6.1) were selected for inclusion in the study (Table 6.2). In each shehia, four surveys were performed in October 2017 (Survey 1), February 2018 (Survey 2), July 2018 (Survey 3) and November 2018 (Survey 4). Surveying in the rainy seasons (between March to May and November to December) was avoided where possible, because many sites were inaccessible, and the high and fluctuating water levels disperses and disrupts *Bulinus* species distribution.

Initially during malacological Survey 1, Uwandani was included as a high non-responder shehia. However, between Surveys 1 and 2, Uwandani was impacted by the activity

of a different research group trialling focal mollusciciding at freshwater bodies, which significantly reduced snail populations at these sites. Therefore, Uwandani was replaced with Ukutini in Survey 2 and therefore no malacological surveys or collections were made in Ukutini during Survey 1.

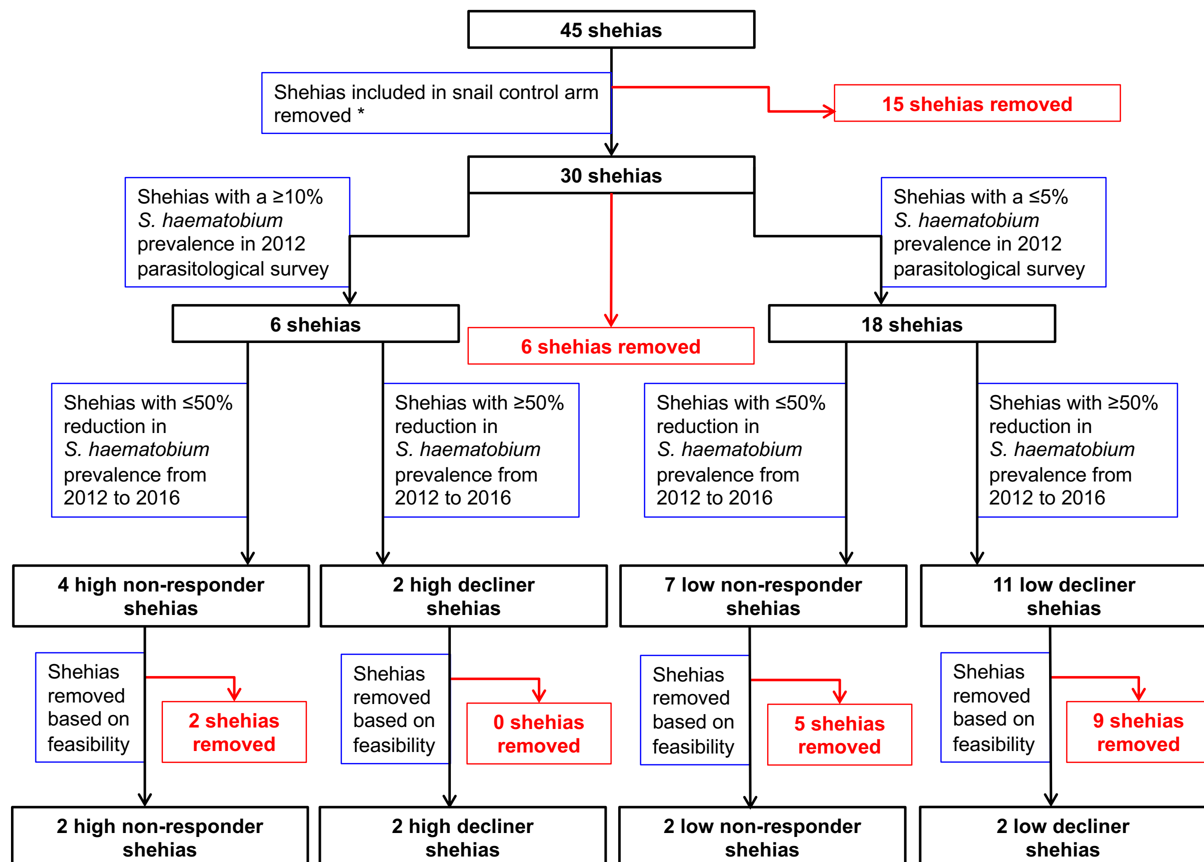


Figure 6.1. Flowchart showing the inclusion criteria for high non-responder, high decliner, low non-responder and low decliner shehias (smallest administrative region) on Pemba Island. *Shehias were excluded in the first stage of selection if included in the snail control arm of the schistosomiasis elimination project (see Knopp *et al*., 2012).

Table 6.2. Shehias of Pemba Island selected for inclusion in xenomonitoring study, showing schistosomiasis prevalence transmission type (HNR = high non-responder, HD = high decliner, LNR = low non-responder, LD = low decliner), ZEST study arm (PC only = biannual preventive chemotherapy, PC plus Behaviour = biannual preventive chemotherapy and behavioural interventions), *Schistosoma haematobium* prevalence (%) and infection intensity (eggs/10ml urine) shown in italics in 9 – 12 year old schoolchildren from each year between 2012 – 2016 during the ZEST study (Knopp, Ame, *et al.*, 2019) and the percentage change of prevalence and infection intensity from 2012 to 2016.

		Urogenital schistosomiasis prevalence (% egg+ve) & *infection intensity (arithmetic mean egg count, eggs/10ml urine)*											
Shehia	ZEST arm	2012		2013		2014		2015		2016		Percentage difference 2012 to 2016	
Ukutini (HNR)	PC	14.5	*15.7*	9.8	*10.6*	9.6	*9.6*	8.0	*12.4*	8.5	*8.5*	-41.4	*-45.9*
Pujini (HNR)	B	10.7	*10.7*	15.6	*14.8*	4.2	*2.5*	9.6	*11.2*	15.1	*17.8*	+41.1	*+66.4*
Kizimbani (HD)	B	29.2	*29.6*	20.7	*21.6*	20.7	*24.5*	13.7	*15.4*	8.7	*11.4*	-70.2	*-61.5*
Kinyasini (HD)	B	28.0	*35.4*	25.2	*23.4*	17.8	*20.3*	12.4	*13.3*	11.4	*12.3*	-59.3	*-65.3*
Wambaa (LNR)	PC	5.0	*7.9*	6.4	*5.5*	9.6	*13.2*	20.5	*22.3*	7.8	*10.7*	+56.0	*+35.4*
Wawi (LNR)	PC	2.1	*3.1*	2.9	*7.8*	5.4	*6.4*	5.8	*7.8*	2.0	*2.0*	-4.8	*-35.5*
Ole (LD)	PC	4.1	*11.2*	3.3	*4.1*	3.6	*5.3*	2.5	*3.3*	0.9	*4.7*	-78.0	*-58.0*
Matale (LD)	PC	3.9	*6.9*	1.8	*0.9*	1.7	*4.4*	0.9	*2.7*	0.8	*2.5*	-79.5	*-63.8*

6.2.2 Malacological surveys and snail shedding

At each human freshwater contact site over the four surveys, water body type (pond, lake, dam, rice paddy, marsh, stream, river, canal), water permanency (1, permanent; 2, non-permanent water source with the latter defined as dry or very low/nearly dry during any survey), water level (1, Flooded; 2, Normal; 3, Low; 4, Dry), flow rate (>1m/s, 0.5-1m/s, <0.5m/s, still), substrate (predominantly mud, sand, gravel, rock, concrete or roots), and water vegetation (lilies, rice, palm fronds, water hyacinth, grass) were recorded. GPS coordinates were also recorded at each site along with water temperature (ºC), pH, conductivity (mS/cm), total dissolved solids (g/L), dissolved oxygen (%) and salinity (g/L). Sites within each shehia were excluded from further surveying if salinity was >35g/L (3.5%), signifying brackish/saline water that is unfavourable for the survival of *Bulinus* (see Donnelly *et al.,* 1983). At each site, domestic (e.g. cow, goat, donkey) and wild animal (e.g. ungulate, water bird) presence was recorded along with any human schistosomiasis 'risk' behavioural activities observed or determined by asking locals (wudu, bathing, washing clothes/dishes/car/bike, collecting water, swimming, fishing, rice cultivation, other farming, sanitation).

At each human freshwater contact site, three experienced collectors searched for *Bulinus* snails (*B. globosus, B. nasutus* and *B. forskalii* group species) by scooping or hand searching of submerged debris/foliage for 15 minutes. Any *Bulinus* spp. collected were transported in collection 'pots' separated by site to the Public Health Laboratory in Chake Chake, Pemba Island. Other freshwater snail species (*Lymnaea* spp., *Melanoides* spp., *Cleopatra* spp., *Pila* spp., *Lanistes* spp., *Thiara* spp.) were noted as either present or absent at each site by morphological identification, but not collected.

In the laboratory, *Bulinus* spp. individuals were counted from each site (ranging from 1 to 316 individuals per site) and housed in plastic trays with bottled water covered by a glass lid overnight to acclimatise. The following morning (before 08:00), snails were rinsed in bottled water and separated into individual wells of 12-well ELISA plates filled to approximately two thirds full with bottled water and placed in indirect sunlight to induce cercarial shedding (Allan *et al.*, 2013). Each well was checked by an experienced microscopist using a dissection microscope two and eight hours after first sunlight. The microscopist distinguished furcocercous schistosomes from other cercariae using descriptions of *Schistosoma* spp. (Frandsen and Christensen, 1984) and any shed cercariae were individually pipetted in 3.5 µl aliquots onto Whatman FTA cards (Whatman, GE Healthcare, Florham Park, USA) for long-term DNA storage. After transporting the Whatman FTA cards back to the Natural History Museum, these *Schistosoma* cercariae were molecularly characterised to identify species and investigate genetic diversity (see Chapter 4).

After screening for patent infections, the snails were preserved in glass vials using 100% ethanol. Shedding snails were preserved individually but non-shedding snails were

grouped by collection site for each survey. All shedding snails as well as three non-patent snails per site and survey, were extracted for further molecular characterisation to confirm species identifications of either *B. globosus* or *B. nasutus* and determine genetic diversity (see Chapter 4). Based on these results, an assumption was made on the identity of the *Bulinus* sp. present at each site as it was not feasible to conduct species identifications on all snails, and the two snail species generally follow an allopatric distribution, not sharing or switching freshwater sites.

6.2.3 Selection of snails for analysis

From Surveys 1 to 4, total DNA from whole snail soft tissue was extracted from 95-111 *B. globosus* and/or *B. nasutus* individuals collected during each survey from 18 sites, and all snails were extracted from 39 sites with ≤94 collected during each survey. For Surveys 2 and 3, total DNA from whole snail soft tissue was extracted from 51-72 *B. globosus* and/or *B. nasutus* individuals collected during each survey from 13 sites, and all snails were extracted from 28 sites with ≤50 individuals collected during each survey. In total across the four surveys, 5,465 (of the 9,430 collected) snails were analysed. An additional 12 snails collected from Kinyasini and Kizimbani during the surveys with patent *S. haematobium* (n = 10) and *S. bovis* (n = 2) infections were also included in the analyses (see Chapter 4).

6.2.4 Molecular xenomonitoring to identify non-patent Schistosoma *infections*

DNA was extracted from individual snails, eliminating cross contamination during the process, by: removing the shell using sterilised tweezers and new petri dishes for each snail, rehydrating tissue for ~30 minutes using bottled water in individual wells of sterilised 24 well ELISA plates (ThermoFisher, Massachusetts, USA), chopping tissue using a new surgical blade and glass slide for each (Swann-Morton, Sheffield, UK) and lysing tissue overnight in double lysis buffers (see Pennance *et al*., 2018). The final stage of DNA extraction was performed using the Thermo Qiagen BioSprint 96 Workstation and Qiagen BioSprint 96 DNA Blood / Tissue Kit (Qiagen, Manchester, UK), providing a high-throughput system for large scale analyses. Each sample was then analysed for *Schistosoma* spp. infection using the recently developed two step ITS xenomonitoring methodology (Pennance, Archer, *et al.*, 2020). The first multiplex ITS xenomonitoring (MIX) PCR enables the rapid screening of the samples for infection and incorporates an internal control, amplifying snail DNA, to prevent false negatives in relation to extraction errors (Figure 6.2). The secondary *Schistosoma* ITS xenomonitoring (SIX) PCR specifically targets the *Schistosoma* spp. amplicon of 538 bp (Figure 6.2) and allows for infection and *Schistosoma* species-level identification (see below). PCR's were performed in a 96 well plate format to facilitate high-throughput processing.

Figure 6.2. Electrophoretic agarose gel profile showing the amplicons produced using the initial multiplex ITS xenomonitoring (MIX) PCR (see Pennance, Archer *et al*. 2020) to screen for *Schistosoma* spp. infections when template gDNA of *Bulinus wrightii* (1 and 2), *Schistosoma haematobium* (3), *S. bovis* (4), and combinations of *B. wrightii* + *S. haematobium* (5) and *B. wrightii* + *S. bovis* (6) used. Snail internal control (~1200 bp), trematode (~1000 bp) and *Schistosoma* spp. (~538 bp) amplicon positions shown.

The first stage multiplex PCRs were performed in a total volume of 25 µl using illustra™ PuReTaq Ready-To-Go™ PCR Beads (GE Healthcare, UK), with 1 µl template gDNA, 1 µl of each primer (ETTS1, ETTS2 (Kane and Rollinson, 1994) and ITS2_Schisto_F (Schols *et al.*, 2019), see Table 6.3) at 10 µM and 21 µl of nuclease free water. PCR cycling conditions were: initial denaturation 5 minutes at 95ºC followed by 40 cycles of 30 seconds at 95ºC, 30 seconds at 58ºC, 90 seconds at 72ºC and a final extension of 10 minutes at 72ºC. PCR products were visualised by gel electrophoresis for 90 minutes at 90 V in a 2% agarose gel with 7.5 µl of PCR product and 2 µl of Bioline (London, UK) 5x DNA Loading Buffer Blue/gel red mix and visualised using a GBOX-Chemi-XRQ gel documentation system (Syngene, Cambridge, UK). All gels were run with BioLine (London, UK) HyperLadder I and HyperLadder IV to assess amplicon band sizes. The resulting agarose gel profiles of each sample were interpreted and categorised as either: 1) snail band only (Figure 6.2: 1 and 2) snail and trematode band, 3) snail, trematode and *Schistosoma* spp. band (Figure 6.2: 5 and 6), 4) failed PCR, 5) snail and *Schistosoma* spp. band or 6) non-specific banding (i.e. none of the expected amplicon sizes observed and significantly different size amplicons produced).

6.2.5 Identification of non-patent Schistosoma *species*

Following the high-throughput MIX PCR screening (see above) those samples that were identified to contain *Schistosoma* spp. were subjected to the SIX (*Schistosoma* targeted) PCR specifically to produce a *Schistosoma* amplicon for further analysis. PCRs were performed as

described above but only containing the specific ITS2_Schisto_F and the universal ETTS1 primers (Table 6.3) to produce the 538 bp *Schistosoma* specific amplicon which was visualised by gel electrophoresis as above. Amplicons were purified using the QiaQuick PCR Purification Kit (Qiagen) and Sanger sequenced in a single direction using ITS2_Schisto_F (Schols *et al.*, 2019). Sequences were trimmed to 466 bp, edited and aligned in Sequencher v5.4.6 (GeneCodes Corp., Michigan, USA) and compared to reference data for *S. haematobium* and *S. bovis* to confirm species identity by analysis of the four ITS2 species-specific SNPs (Webster *et al.*, 2010).

Table 6.3. Details of primers used in the xenomonitoring assay. Universal (U) and specific (S) denotes whether the primer universally targets both *Schistosoma* and *Bulinus* DNA or just specifically targets *Schistosoma* DNA.

Primer (direction)	Primer Sequence (5'-3')	Primer position	State	Reference
ETTS1 (Reverse)	TGCTTAAGTTCAGCGGG	28S 5' end (ITS2 3' flanking region)	U	Kane *et al.* (1994)
ETTS2 (Forward)	TAACAAGGTTTCCGTAGGTGA	18S 3' region (ITS1 5' flanking region)	U	Kane *et al.* (1994)
ITS2_Schisto_F (Forward)	GGAAACCAATGTATGGGATTATTG	ITS1 3' end (5.8S 5' flanking region)	S	Schols *et al.* (2019)

6.2.6 Abiotic and biotic variables impacting non-patent Schistosoma *infections and abundance of* Bulinus

Two separate Generalised Linear Mixed Effects Models (GLMMs) were fitted to investigate the effects of several environmental and abiotic factors, schistosomiasis transmission level (as defined in Table 6.1), season (proxied by the time of year each survey was conducted) and various human behaviours on *S. haematobium* infection status among collected *Bulinus* spp. individuals (Supplementary File 6.1: GLMM 1), and *Bulinus* abundance (Supplementary File 6.1: GLMM 2). The environmental and abiotic factors tested here were: waterbody type, waterbody permanency, water level, flow rate, vegetation, substrate, temperature, conductivity, pH, total dissolved solids and salinity, as measured during each survey; whilst the human behaviours tested were: swimming/playing, bathing, washing dishes, rice cultivation, collecting water or farming summarised, summarised as either observed or not across all surveys for each site. A third GLMM exploring an interaction between temperature and water level was also fitted to investigate the factors determining *Bulinus* abundance, however as this did not increase model fit, it is not reported further (Supplementary File 6.1: GLMM 3).

All analyses were conducted in R v4.0.0 (R Core Team, 2018), and all models were fitted using the "lme4" v1.1.23 package (Bates *et al.*, 2015). GLMM 1 was modelled with a binomial error distribution (with a logit link function; for justification see Warton and Hui, 2011) and infection status per snail (i.e. infected or not) as the response variable. GLMM 1 used the "BOBYQA" (Powell, 2007, 2009) optimizer to reduce computational time and the optimiser method was validated with the function "allFit" from the package "lme4" v1.1.23 (Bates *et al.*,

2015). GLMM 2 was fitted with a negative binomial error distribution (the "glmer.nb" function in the "lme4" package v1.1.23 (Bates *et al.* 2015)) and the total number of snails collected per survey as the response variable. The choice of error distribution for GLMM 2 was based on whether a higher deviance was explained by distribution type (negative binomial vs Poisson) through assessing model convergence and AIC values. Shehia and site were treated as random factors in both models, with sites being nested within shehias to account for unobserved heterogeneity given the sampling structure (Harrison, 2015).

For both GLMM 1 and GLMM 2, a maximal to minimal model approach was conducted whereby initially, all environmental/abiotic factors, season and all observed human behaviours were modelled as fixed effects; thereafter, both models were reduced by iteratively removing fixed effects with the highest p values, until only significant ($p < 0.05$) or marginally significant ($p < 0.1$) fixed effects remained; hence, balancing power and Type I error rate (Matuschek *et al.*, 2017). In accordance with previous methodologies for scaling regression inputs for binary response variables (Gelman, 2008), fixed effects of GLMM1 were scaled and standardised using the package "arm" v1.11.1 (Gelman *et al.*, 2008). The schistosomiasis transmission category (high non-responder, high decliner, low non-responder and low decliner) and water level (1, Flooded; 2, Normal; 3, Low; 4, Dry) were treated as ordered factors in both models. Significance of fixed effects were assessed with Type II Wald χ^2 tests in the R package "car" v3.0.7 (Fox and Weisberg, 2019). Estimated marginal means (EMMs) were found for significant and marginally significant fixed effects in the R package "emmeans" v1.4.7 (Lenth *et al.*, 2018) to investigate the probabilities of *S. haematobium* infections in intermediate host snails.

For GLMM 1, the final model included the following fixed effects: water level, water temperature and pH as fixed effects (Supplementary File 6.1: GLMM 1). Of the 5,477 snail records, 194 were omitted since water temperature and water chemistry measurements at the site were not recorded at the time. Freshwater sites with <10 snails processed using the multiplex ITS xenomonitoring assay over the four surveys (of which six sites fit this category) were also removed as these data clearly represented outliers within the dataset. The final data set for analysis therefore included 5,259 individual snails.

For GLMM 2, the final model (Supplementary File 6.1: GLMM 2), included the following variables: waterbody level, water temperature, the schistosomiasis transmission category and survey (proxy for season) as fixed effects. Of the 296 observations in this dataset (74 sites over 4 surveys), 46 observations were removed since collections were not conducted at some sites in particular surveys (e.g. Ukutini not surveyed in Survey 1), water temperature / chemistry were not recorded at the time of collection, or the water body was dry, leaving 250 observations included in the analysis.

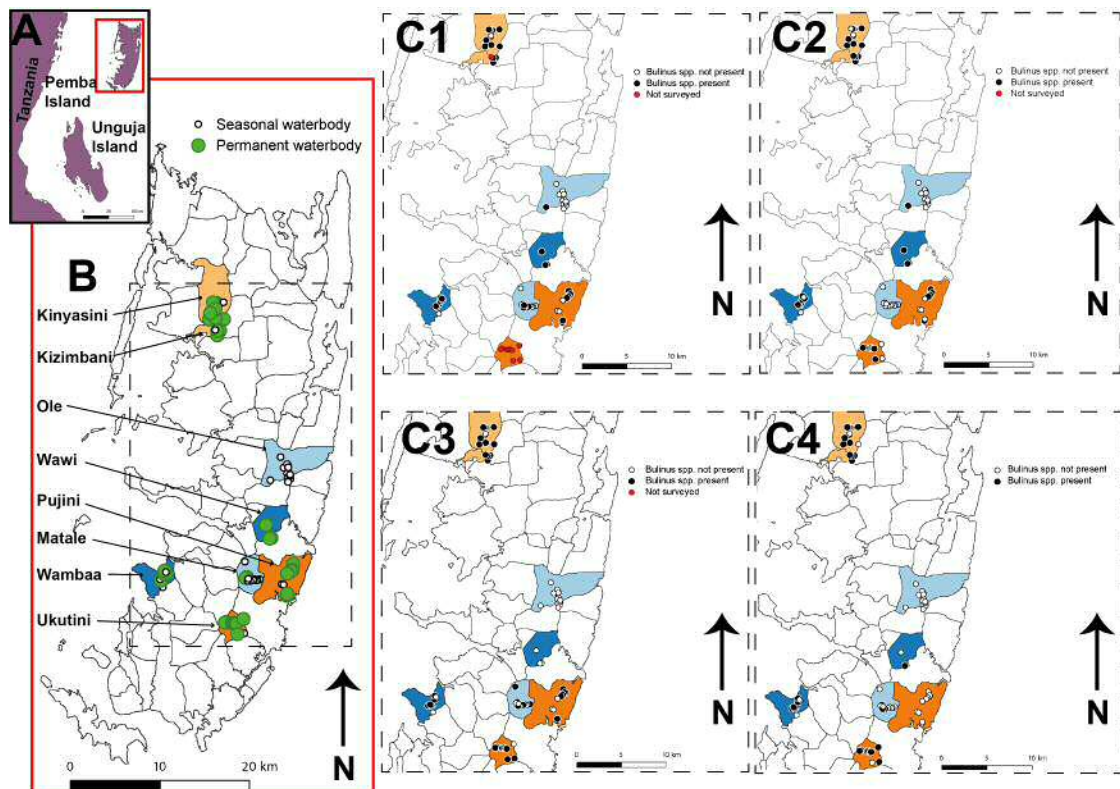


Figure 6.3. A: East coast of Tanzania and the Zanzibar Archipelago (Pemba and Unguja islands). **B**: Pemba Island (United Republic of Tanzania) showing the location of selected shehias categorised based on *Schistosoma haematobium* prevalence from 2012 to 2016 (Knopp, Ame *et al*., 2019) as high non-responders (dark orange), high decliners (light-orange), low non-responders (dark-blue) and low decliners (light-blue). Location of seasonal (i.e. temporary, white circles) and permanent (i.e. never dry, green circles) freshwater bodies shown on map. **C1 – C4**: Freshwater bodies where *Bulinus globosus* and/or *B. nasutus* were present (black circles) or absent (white circles) or not surveyed (red circles) during malacological Survey 1 (**C1**), Survey 2 (**C2**), Survey 3 (**C3**) and Survey 4 (**C4**).

6.3 Results

6.3.1 Malacological surveys and snail infections

A total of 74 human freshwater contact sites (HWCSs) were identified and surveyed in the eight xenomonitoring shehias (Figure 6.3). Among these, 38 were permanent and 36 were seasonal (i.e. completely dry or almost dry at some point during the year). The number of HWCSs per shehia ranged from 3 (Wawi) to 13 (Ole) (Figure 6.4). *B. globosus* or *B. nasutus* were present at least once over the 4 surveys in 70% (n = 52) of the HWCSs. Streams were the most common HWCS (n = 33) on Pemba and were also the majority of sites where *Bulinus* were collected (n = 27). The majority of stream sites were in Wambaa (n=10) and Kinyasini (n=10). Ponds were also abundant (n=22, mainly in Ole n=11 and Pujini n=8), although *Bulinus* snails were only collected from nine of these. Rice paddies (n=4), marshland (n=1), canals (n=2) and springs (n=3) were also surveyed and all contained *Bulinus* snails. Freshwater bodies making up 10 of the 12 HWCSs surveyed in Matale were dynamic throughout the year, changing from disconnected ponds at the end of the dry season (February - March) to connected stream or marshland sites following the end of the rainy season (July - August), the

majority of these (n = 7) contained *Bulinus* snails at some point over the four surveys.

In total, 406 snails were identified as *B. globosus* (n = 359) and *B. nasutus* (n = 47) from the sites surveyed for the xenomonitoring surveys (see Chapter 4). All *B. nasutus* were collected from eight separate pond sites in Pujini and co-occurred with *B. globosus* at one site (Puj11, see Chapter 4). All other sites containing *Bulinus* (n = 44) were identified as *B. globosus* (see Chapter 4). Out of 9,430 *B. globosus* / *B. nasutus* collected from each site across the 4 xenomonitoring surveys, 12 (0.12%) patent schistosome infections were observed from *B. globosus* in Kinyasini (n=10) and Kizimbani (n=2) collected in Surveys 2 and 4 (Table 6.4, Figures 6.5B and C). Ten of these schistosome infections were identified as *S. haematobium* and two as *S. bovis* (see Chapter 4: Table 6.3).

The temperatures tested here (average: 27.5ºC: range: 22.3-37.4ºC) showed a negative effect (χ^2 = 4.1697, *df* = 1, *p* = 0.041) of increasing temperature on snail abundance, indicating that for every one degree increase in temperature, the number of snails collected at a site will decrease by a factor of 0.84 (SE = 0.07).

The effect of the ordered schistosomiasis transmission categories on *Bulinus* abundance showed a negative quadratic trend (χ^2 = 19.1575, *df* = 3, *p* < 0.001) from high to low transmission categories, signifying that the abundance of *Bulinus* decreases and that this decrease accelerates at a certain point (Supplementary File 6.1:GLMM 2). As is evident from overlapping 95% CIs, no difference in *Bulinus* abundance was detected between the three highest transmission categories (high non-responder, high decliner, low non-responder) and on average, *Bulinus* abundance was 36.65 (SE: 31.49), 22.48 (SE: 20.27) and 21.17 (SE: 19.68) across the three transmission categories respectively, with an overall average of 26.8 snails collected per site (Supplementary File 6.1: GLMM 2). However, the abundance of *Bulinus* in the lowest transmission category (i.e. low decliner) was on average 0.27 (SE = 0.25), approximately a hundredfold less than the remaining three categories.

Season affected *Bulinus* abundance (χ^2 = 18.0197, *df* = 2, *p* < 0.001); when compared to Survey 1 (October, end of long dry season), *Bulinus* abundance was significantly lower in Survey 2 (*p* = 0.003) (February, end of short dry season), 3 (*p* = 0.001) (July, following long rain season) and 4 (*p* < 0.001) (November, end of long dry season / start of short rain season) (Supplementary File 6.1: GLMM 2). There was no difference in *Bulinus* abundance between these Surveys 2, 3 and 4 (Supplementary File 6.1: GLMM 2).

There was a significant negative linear trend between water level and *Bulinus* abundance (GLMM 2: E = -1.01 +/-0.44, *p* = 0.02 see Supplementary File 6.1), such that at each decreasing water level (ordered from flooded to normal and low), *Bulinus* abundance declined by a factor of 0.35 (SE = 0.20). However, according to the Type II Wald χ^2 test, the effect of water level was on the boundary of significance (χ^2 = 5.7265, *df* = 2, $\chi^2 p$ = 0.057),

and 95% confidence intervals (CIs) overlapped for all three water level categories' estimated effects (see Supplementary File 6.1: GLMM 2).

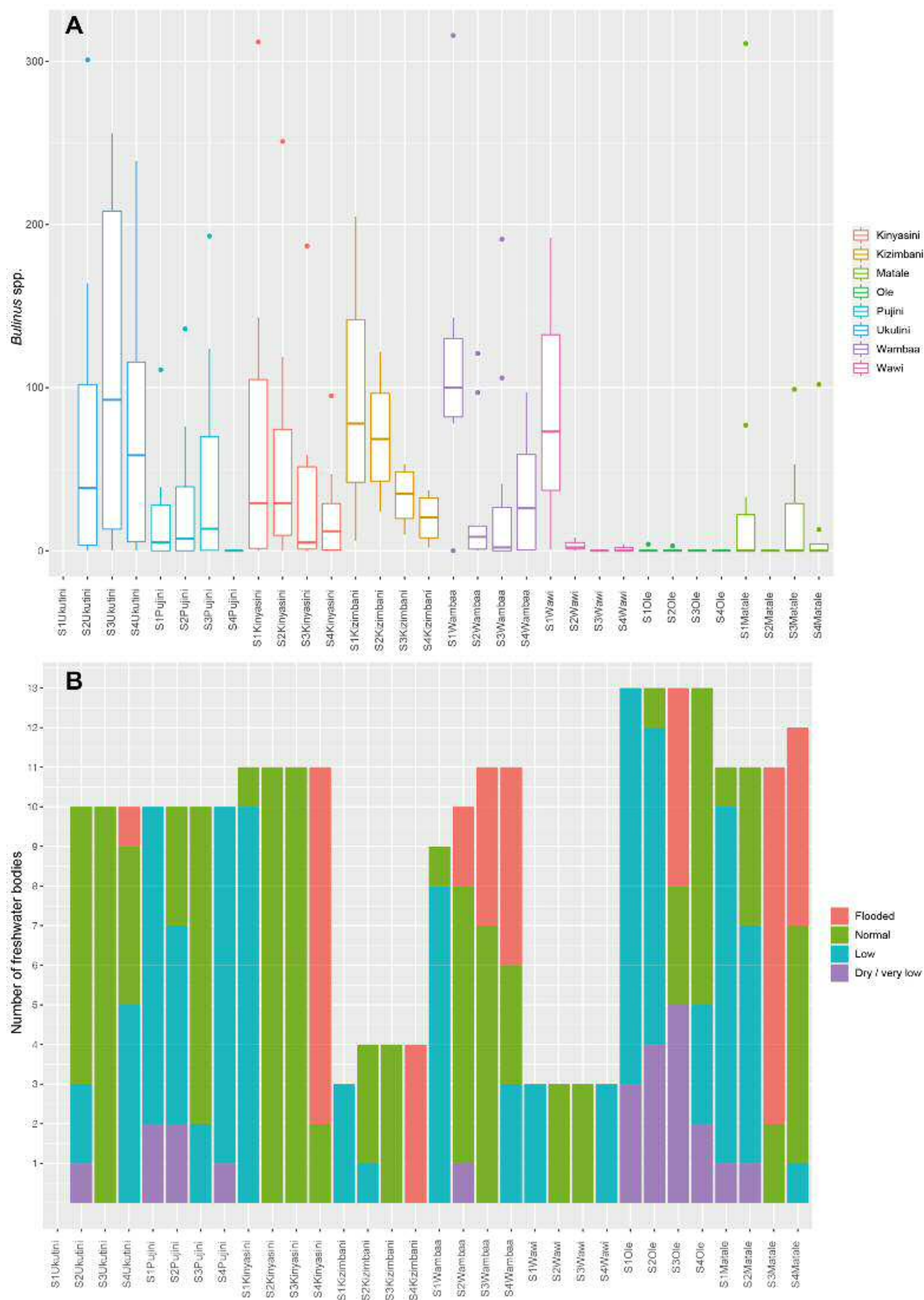


Figure 6.4. A: Boxplot showing the median, upper and lower quartiles (hinges), variability (whiskers) and outliers (points) of the number of *Bulinus globosus* and *B. nasutus* collected at freshwater bodies in each shehia during each malacological survey (labelled S1-S4 i.e. Survey 1 to Survey 4). **B**: The water level at freshwater bodies in each shehia during four malacological surveys (labelled S1-S4 i.e. Survey 1 to Survey 4).

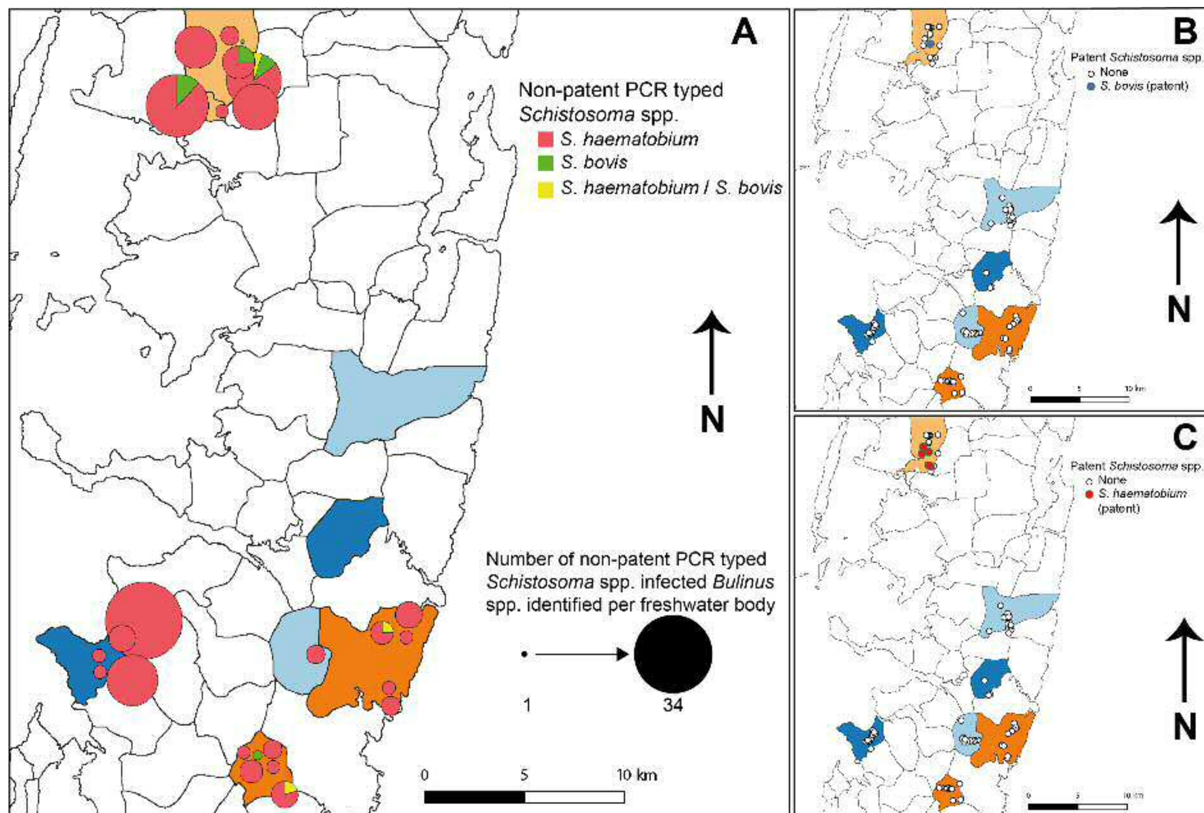


Figure 6.5. Location of selected shehias on Pemba (United Republic of Tanzania) categorised based on *Schistosoma haematobium* prevalence as high non-responders (dark orange), high decliners (light-orange), low non-responders (dark-blue) and low decliners (light-blue). **A**: Non-patent PCR typed *Schistosoma* spp. infections of *Bulinus* identified at freshwater bodies. Circle area is proportional to the number of non-patent PCR typed *Schistosoma* spp. infections identified from each site across all surveys. Yellow represent *S. haematobium* / *S. bovis* co-infections or hybrids. **B**: Patent *S. bovis* infections *of B. globosus* from one site in Kinyasini, and absence across remaining sites on Pemba Island. **C**: Patent *S. haematobium* infections of *B. globosus* identified from five sites in Kinyasini (n = 3) and Kizimbani (n = 2), and absence across remaining sites on Pemba Island.

Table 6.4. *Bulinus globosus* observed with patent *Schistosoma haematobium* and *Schistosoma bovis* infections collected from Kinyasini and Kizimbani (Pemba Island, United Republic of Tanzania) between 2017 and 2018. *Site permanency: P =. Permanent freshwater body i.e. site never dry during survey periods. T = Temporary freshwater body i.e. site dry or almost dry during at least one survey.

Survey	**ID**	**Shehia**	**Site name (* *site permanency*)**	***Schistosoma* spp.**
S2	Kiz4.1	Kizimbani	Kiz4 (*P)*	*S. haematobium*
S2	Kiz5.1	Kizimbani	Kiz5 (T*)*	*S. haematobium*
S2	Kinya9.1	Kinyasini	Kin9 (*P)*	*S. haematobium*
S2	Kinya9.2	Kinyasini	Kin9 (*P)*	*S. haematobium*
S2	Kinya9.3	Kinyasini	Kin9 (*P)*	*S. haematobium*
S2	Kinya9.4	Kinyasini	Kin9 (*P)*	*S. haematobium*
S2	Kinya9.5	Kinyasini	Kin9 (*P)*	*S. haematobium*
S2	Kinya11.1	Kinyasini	Kin11 (*P)*	*S. haematobium*
S2	Kinya6.1	Kinyasini	Kin6 (*P)*	*S. haematobium*
S4	Kin6.3	Kinyasini	Kin6 (*P)*	*S. haematobium*
S4	Kin6.1	Kinyasini	Kin6 (*P)*	*S. bovis*
S4	Kin6.2	Kinyasini	Kin6 (*P)*	*S. bovis*

6.3.2 Xenomonitoring of Schistosoma *infections*

In total, 9,430 snails were collected from the 74 xenomonitoring sites over the four malacological surveys across the eight shehias. Of these, 5,465 (58%) non-patent *Bulinus* spp. (i.e. excluding 12 *B. globosus* with patent schistosome infections) were screened for non-patent *Schistosoma* infections. The assay failed to amplify DNA from 1.8% (n = 95) of these samples and ambiguous results were observed for 1.5% (n = 81) and were therefore excluded from further analysis. Of the remaining 5,289 samples, single snail bands were detected from 90.1% (n = 4766), double snail and trematode bands from 2.4% (n = 126), triple snail, trematode and schistosome bands from 1.5% (n = 79) and double snail and schistosome bands were observed from 6.0% (n = 318). The 12 patent *B. globosus* all displayed triple snail, trematode and schistosome bands using the xenomonitoring assay.

In total, 7.5% (397) of successfully amplified samples gave a schistosome specific band (i.e. triple: snail, trematode and schistosome, n = 79, or double: snail and schistosome, n = 318) and therefore were subject to a second schistosome DNA targeted PCR. Of these 397 positive samples, 47.4% (188) successfully amplified the schistosome specific band and were therefore submitted for sequencing. Sequencing of these amplicons revealed 149 *S. haematobium*, 13 *S. bovis* and three *S. haematobium / S. bovis* mix infections (hybrid or co-infections) (Table 6.6). Schistosome species identifications could not be confirmed from 23 of the 188 samples due to failed sequencing following at least two repeated attempts. Of the 318 snails that amplified only the snail and schistosome band, only 98 (30.8%) were confirmed as either *S. haematobium* (n = 90) or *S. bovis* (n = 8), whereas 64 of the 79 (81%) of the snails with snail, trematode and schistosome banding patterns were confirmed as *S. haematobium* (n = 59) and *S. bovis* (n = 5).

Non-patent PCR typed *S. haematobium* infections were identified at 23 of the 52 sites where *B. globosus* and/or *B. nasutus* were collected in all shehias except for Ole and Wawi (Figure 6.5A and Table 6.5). Proportional to the number of snails extracted, the highest number of non-patent *S. haematobium* infections was observed in Kizimbani (5.48%), followed by Wambaa (4.14%), Kinyasini (3.26%) and Pujini (2.04%). A lower prevalence of non-patent *S. haematobium* infections were observed from sites in the low decliner shehias (Ole, Matale) than in high decliner shehias (Kinyasini, Kizimbani), however the prevalence in low non-responder shehias (Wambaa, Wawi) was higher than that observed in high non-responder shehias (Kinyasini, Kizimbani) (Table 6.5). The low decliner transmission category had both the least snails collected (n = 774) and the fewest positive *S. haematobium* PCRs (0.40%), whereas the high non-responder shehias had the highest abundance of snails collected (n = 3,550) but the highest positive *S. haematobium* PCR detection level was seen in the high decliner shehias (Table 6.5).

Snail infection was positively correlated with temperature (average: 27.5ºC, range:

22.3-37.4ºC) (χ^2 = 20.1058, *df* = 1, p< 0.001) such that the odds of snails being infected increased by a factor of 1.25 (SE = 0.06) with every degree Celsius rise in water temperature (Supplementary File 6.1: GLMM 1). The probability of infections with *S. haematobium* was highest when snails were collected at a normal water level since water level (ordered from flooded to normal and low) was fitted as a statistically significant positive linear (p = 0.016) and negatively quadratic (p < 0.001) trend (χ^2 = 62.0918, *df* = 2, p < 0.001) (Supplementary File 6.1: GLMM 1). This means the probability of the snails being infected with *S. haematobium* is highest at normal water levels (EMM = 0.018; SE = 0.0070), whilst the probability of infection decreases at both flooded (EMM = 0.0013; SE = 0.00073) and low (EMM = 0.0033; SE = 0.0015) water levels (Supplementary File 6.1: GLMM 1). Overall, pH (average: 7.01 pH; range: 5.55 – 8.53) negatively affected the probability of *S. haematobium* infection (χ^2 = 6.8943, *df* = 1, p = 0.008), where with each unit increase in pH, the odds of snails being infected decreased by a factor of 0.43 (SE = 0.16) (Supplementary File 6.1: GLMM 1). Although snails were present, no *S. haematobium* infections (patent or non-patent) were seen at pH levels >8.24.

Table 6.5. Non-patent PCR typed *Schistosoma haematobium* infections of *Bulinus globosus* and *B. nasutus* identified using the xenomonitoring assay and confirmed from sequence analysis of the 538 bp *Schistosoma* specific amplicon. * Number of human-freshwater contact sites per shehia, and number of permanent freshwater bodies shown in brackets. [1] Three *S. bovis* non-patent infections also identified. [2] Five *S. bovis* non-patent infections also identified. [3] Two *S. bovis* non-patent infections also identified. [4] One snail sample showed a mixed *S. haematobium / S. bovis* infection (coinfection or hybrid). HNR = high non-responder, HD = high decliner, LNR = low non-responder, LD = low decliner.

		Number of *Bulinus globosus* and *B. nasutus* analysed: No. of *S. haematobium* infected snails detected / No. of snails tested (*Total number of snails collected*)					*S. haematobium* snail infection prevalence	
Shehia (transmission category)	Sites*	S1 October 2017	S2 February 2018	S3 July 2018	S4 November 2018	Total		
Pujini (HNR)	10 (6)	4[4] / 161 (210)	2 / 153 (*302*)	5 / 225 (*469*)	0 / 0 (0)	11 / 539 (*981*)	2.04	1.30
Ukutini (HNR)	10 (9)	na / na (na)	6 / 301 (*750*)	5[2,4] / 331 (*1095*)	0 / 523 (*724*)	11 / 1155 (*2569*)	0.95	
Kinyasini (HD)	11 (10)	18[1] / 466 (*744*)	21[1] / 309 (*630*)	0 / 218 (*369*)	1 / 235 (*241*)	40 / 1228 (*1984*)	3.26	3.94
Kizimbani (HD)	4 (3)	11[4] / 166 (*289*)	4 / 174 (*283*)	10[3] / 127 (*133*)	5 / 80 (*80*)	30 / 547 (*785*)	5.48	
Wambaa (LNR)	11 (5)	7 / 631 (*1047*)	11 / 144 (*267*)	2 / 168 (*356*)	35 / 384 (*387*)	55 / 1327 (*2057*)	4.14	3.64
Wawi (LNR)	3 (3)	0 / 170 (*266*)	0 / 9 (*10*)	0 / 0 (*0*)	0 / 4 (*4*)	0 / 183 (*280*)	0.00	
Matale (LD)	12 (2)	2 / 216 (*432*)	0 / 0 (*0*)	0 / 160 (*212*)	0 / 116 (*123*)	2 / 492 (*767*)	0.41	0.40
Ole (LD)	13 (0)	0 / 4 (*4*)	0 / 2 (*3*)	0 / 0 (*0*)	0 / 0 (*0*)	0 / 6 (*7*)	0.00	
Total	74 (38)	42 / 1814 (*2992*)	44 / 1092 (*2245*)	22 / 1229 (*2634*)	41 / 1342 (*1559*)	149 / 5477 (*9430*)		
S. haematobium infection prevalence per survey		2.32	4.03	1.79	3.06	2.72		

The 538 bp region was successfully amplified from both *S. haematobium* and *S. bovis* infections and revealed six different sequence profiles, including those differentiated from a

fifth, currently unreported SNP at position 360 (Table 6.6). Double peaking was observed in three samples at the four *S. haematobium* and *S. bovis* species-specific sites representing either co-infections with *S. haematobium* and *S. bovis* or *S. haematobium-bovis* hybrid infections. (Table 6.6).

Non-patent infections of bovine *S. bovis* were observed in Kinyasini (n = 6), where patent infections have been observed previously (Pennance *et al.*, 2018). Non-patent *S. bovis* infections were also detected in snails at sites in the neighbouring shehia, Kizimbani (n = 2), but also a geographically distant site, Ukutini (n = 5), located further south on the island (Figure 6.5B).

Due to allopatric distributions of the different snail hosts the majority of non-patent *S. haematobium* group infections were identified from snails predicted to be *B. globosus* (n = 153, *S. haematobium* = 138, *S. bovis* = 13, *S. haematobium-bovis* = 2), but snails also predicted to be *B. nasutus* from Pujini also had non-patent *S. haematobium* group infections (n = 11, *S. haematobium* = 10, *S. haematobium-bovis* = 1). One snail harbouring *S. haematobium* originated from the aforementioned site in Pujini where both *B. globosus* and *B. nasutus* were sympatrically present (see Chapter 4), and it therefore cannot be assumed from the current data which intermediate host species this was (*B. globosus* or *B. nasutus*).

Table 6.6. Observed *Schistosoma* species-specific SNP positions (including base position) in the ITS2 region. The 360 SNP in unclassified and it not recognised as a species-specific SNP.

Schistosoma species	ITS 2 Schistosome Species-Specific SNP positions (bp)				
	SNP1 (90)	SNP2 (145)	SNP3 (195)	(360 SNP)	SNP4 (265)
S. haematobium	*S. h* (G)	*S. h* (C)	*S. h* (G)	(T)	*S. h* (C)
S. haematobium	*S. h* (G)	*S. h* (C)	*S. h* (G)	(C)	*S. h* (C)
S. haematobium	*S. h* (G)	*S. h* (C)	*S. h* (G)	(C / T)	*S. h* (C)
S. bovis	*S. b* (A)	*S. b* (T)	*S. b* (A)	(T)	*S. b* (T)
S. haematobium / *S. bovis*	*S. h* / *S. b* (G / A)	*S. h* / *S. b* (C / T)	*S. h* / *S. b* (G / A)	(C)	*S. h* / *S. b* (C / T)
S. haematobium / *S. bovis*	*S. h* / *S. b* (G / A)	*S. h* / *S. b* (C / T)	*S. h* / *S. b* (G / A)	(T)	*S. h* / *S. b* (C / T)

6.4 Discussion

Here, we validate a recently developed xenomonitoring assay targeting *Schistosoma haematobium* and *S. bovis* DNA within *Bulinus* spp. intermediate hosts (Pennance, Archer, *et al.*, 2020) as an effective tool for detecting the presence of schistosomes in endemic regions on Pemba Island. The xenomonitoring assay was up to 24 times more sensitive at detecting *S. haematobium* infections compared to traditional parasitological shedding methods. Importantly, the assay detected transmission of urogenital schistosomiasis in areas where

patent infections were not identified, including low prevalence areas where both human and snail infections are difficult to detect showing the feasibility of the assay for the surveillance of schistosomiasis transmission in elimination settings (WHO, 2020a). By detecting such infections, it allows us to show that interventions are still needed to prevent schistosomiasis resurgence in low prevalence areas where active transmission is evidently still taking place. Non-patent infection prevalence in snails, however, does not directly compare with levels of human disease, although *Bulinus* abundance in freshwater bodies did relate to human schistosome prevalence. Both *B. globosus* and *B. nasutus* were observed with non-patent *S. haematobium* infections, and to a lesser extent *S. bovis*, showing that both these snail species are exposed to schistosomes in this endemic setting. The presence of *Schistosoma* DNA in freshwater samples (Fornillos *et al.*, 2019; Alzaylaee, Collins, Shechonge, *et al.*, 2020) or in the intermediate host snails signifies that waterbody contamination with miracidia is occurring. This added information should be used to inform schistosomiasis control strategies, such as behavioural interventions to educate against contamination behaviours (in this case open urination) or snail control measures, to target specific transmission areas (Knopp, Ame, *et al.*, 2019; Knopp, Person, *et al.*, 2019).

6.4.1 Abiotic variables impacting Bulinus *spp. abundance*

Bulinus abundance decreased at low water level sites, probably due to snail death or aestivation (Cawston 1927a, b; Wilkinson *et al.,* 2007). Temperature is an important determinant for *Bulinus* distribution and population sizes due to the effects it has on several biological processes; egg production, hatching, juvenile maturation and even adult death rates (El-Hassan 1974; Kalinda *et al.,* 2017a, b). Here, it is demonstrated that with each degree increase in temperature from 22.3-37.4ºC, the abundance of *Bulinus* decreased. This was previously noted for *Bulinus* (see Rabone *et al.,* 2019) where water bodies are above the optimal survival temperature of 26-27ºC; (Shiff, 1964). The abundance of *Bulinus* was significantly lower in low-decliner shehias compared to the three higher transmission categories, and therefore *Bulinus* abundance potentially plays a role in curtailing transmission. It should also be noted that only two of the 25 human water contact sites surveyed across these two shehias contained permanent freshwater, with the temporary nature of freshwater likely having both a negative impact on snail abundance and likelihood for schistosomiasis transmission.

6.4.2 Abiotic variables impacting S. haematobium *infections of* Bulinus

During flooding, *Bulinus* spp. were more abundant but the probability of *S. haematobium* infection was lower than at a normal water levels, probably due to a dilution effect. During low water levels, the probability of *S. haematobium* infection was lower than normal, which

probably links to human behaviour, since accessibility to freshwater and therefore usage (e.g. for playing, washing, sanitation etc) decreases.

Temperature impacts both snails and the larval stages of *S. haematobium* (see Shiff, 1974; Tchounwou *et al.*, 1992; Kalinda, Chimbari, Malatji, *et al.*, 2018; Stensgaard *et al.*, 2019). Here the probability for *S. haematobium* infection in snails was higher for those collected from warmer waters. This finding from the field correlates well with previous laboratory research demonstrating that the rate of infection with *Schistosoma* spp. increases when snails are exposed to miracidia at higher temperatures up to a limit where snail mortality outweighs infection success (DeWitt, 1955; Chu *et al.*, 1966; Purnell, 1966). With our increasing understanding on how snail resistance phenotypes can be modulated by temperature (Ittiprasert and Knight, 2012), it will be of great interest to see how stressors such as temperature may affect snail immune factors due to changing transcripts in wild caught populations (Pila *et al.*, 2017).

Although it was not possible to include water permanency in the *S. haematobium* infection models here (Supplementary File 6.1: GLMM 1), water permanency is likely correlated with water level (since sites reaching low water levels are more likely to be non-permanent). At low water levels, there was a decreased probability of *S. haematobium* infection. *Bulinus* spp. undergoing the additional stressors of aestivation (caused by drying of freshwater sites) stand a significantly lower chance of survival than those that do not (Kalinda, Chimbari, Malatji, *et al.*, 2018), particularly when infected (Woolhouse and Taylor, 1990; Rubaba *et al.*, 2016). So, although habitat recolonization of *Bulinus* will occur following drying and refilling of freshwater bodies (Wilkinson *et al.*, 2007), the odds are against the survival of schistosomes here, which may in turn lead to fewer snails reaching patency. Indeed, only one infected *B. globosus* was identified from a temporary water body and all shehias that started with a high urogenital schistosomiasis prevalence in 2012, had a higher proportion of permanent freshwater bodies within that shehia. The data suggests that the water permanency of environments in Kinyasini and Kizimbani accentuate the transmission of *S. haematobium* (and *S. bovis*) by promoting the occurrence of patent *B. globosus* infections, which may have led to the high prevalence of urogenital schistosomiasis in these shehias before the onset of ZEST (Knopp *et al.*, 2012).

Previous studies have demonstrated a profound effect of pH on the survival of *S. mansoni* miracidia, concluding that survival is better in slightly alkaline environments (Upatham, 1972; Tchounwou *et al.*, 1992). *S. haematobium* miracidia, however, reportedly can tolerate a relatively wide pH range (Lo, 1972). The data suggests the former; the probability of *S. haematobium* infections was higher in alkaline environments, which may be a knock on effect of increased and prolonged miracidia survival in slightly alkaline freshwater (Upatham, 1972; Tchounwou *et al.*, 1992). Due to the different geological zones and

associated water conductivity that dictate the distribution of *B. globosus* and *B. nasutus* on Zanzibar (Kent *et al.*, 1971; Stothard, Loxton, *et al.*, 2000), investigating further whether pH is significantly different at sites containing *B. globosus* and *B. nasutus* is a priority for future research, which may be a contributing factor to *B. globosus* distribution across Zanzibar and its likelihood to act as an intermediate host if it is found in more alkaline habitats. However, experimental infections of both species controlling for water chemistry and therefore miracidia survival, do not support this theory (Stothard, Loxton, *et al.*, 2000).

6.4.3 Urogenital schistosomiasis transmission through B. nasutus

From the *Bulinus* spp. identifications performed on a subset of samples (see Chapter 4), it can be assumed that non-patent *S. haematobium* infections were observed in at least 10 *B. nasutus* collected in Pujini. This further confirms that this snail species is penetrated by *S. haematobium* in Zanzibar (Allan *et al.*, 2009). It also provides support that this species may play a role in the transmission of *S. haematobium* in multiple regions on Pemba Island (see Chapter 4), especially considering that Pujini, where *B. nasutus* was in high abundance, is an area of high persisting human infection prevalence suggesting sustained local transmission through *B. nasutus*. Although the distribution of *B. globosus* and *B. nasutus* is relatively well described for Pemba Island (see Chapter 4), it would still be beneficial to combine a *Bulinus* species diagnostic such as those already developed (see Chapter 5) with this xenomonitoring methodology. This would provide species identifications for both intermediate host and schistosome in parallel methods, or simultaneously as is conducted in other infectious disease monitoring tools (Tedrow *et al.*, 2019).

6.4.4 Distribution of S. bovis *transmission and the occurrence of co-infections with* S. haematobium

The presence of *S. bovis* DNA within five *B. globosus* collected in Ukutini demonstrates that *S. bovis* transmission is also occurring in this southern region in addition to the northern regions (where patent infections in livestock and snails has been confirmed, see Pennance *et al.*, 2018) of Pemba (Kinyasini/Kizimbani). Further studies on the transmission of *S. bovis* on Pemba Island is needed to elucidate the presence of bovine schistosomiasis and the risk to cattle health island wide and not just in the focal regions of Kinyasini (see Chapter 4). This also poses the risk of *S. haematobium-bovis* hybridisation, a worrying scenario that may impact human health. In fact, mixed profiles of *S. haematobium* and *S. bovis* were observed within three snails from three different shehias: Kizimbani, Ukutini and Pujini. Without being able to screen individual larvae this data is difficult to interpret since it could represent either a co-infection of *S. haematobium* and *S. bovis* or a *S. haematobium-bovis* hybrid (Webster *et al.*, 2010; Pennance, Allan, *et al.*, 2020), although hybrids have not been detected in human

infections (personal observations). *S. haematobium-bovis* hybrids commonly occur in West Africa (as reviewed in Léger and Webster, 2017), however, more recently hybrids have also been identified in human infections in Malawi suggesting that they may be more wide spread than previously thought (Webster *et al.*, 2019). Additionally, Zanzibar was until recently considered to be allopatric for *S. haematobium,* with *S. bovis* probably imported more recently with the movement of cattle. Therefore, it seems more likely that these mixed profiles represent *S. haematobium* / *S. bovis* co-infections, as observed in some *Bulinus* in West Africa, although these co-infections are rare (Tian-Bi *et al.*, 2019). The presence of *S. haematobium* and *S. bovis* on Pemba, and the current absence of hybrid species offers a useful place to study the evolution of hybridisation of these species, as this may provide a baseline for introgression of these two species. From the three snails observed with mixed *S. haematobium-bovis* profiles in the current study, two originated from Kizimbani and Ukutini, where non-patent *S. bovis* infections were also detected in snails (see above), whilst the third originated from one site in Pujini, where only *S. haematobium* infections were detected. The snail co-infected with *S. haematobium* and *S. bovis* from Pujini is assumed to be *B. nasutus*, suggesting that this species could also play a role in *S. bovis* transmission on Zanzibar if infections reach patency, as is the case on mainland Africa (Brown, 1994), although this needs confirming in future work.

6.4.5 Advantages and limitations of incorporating xenomonitoring into schistosomiasis surveillance

A primary aim of this study was to assess the feasibility of using a xenomonitoring assay in schistosomiasis surveillance and elimination following new WHO recommendations (WHO 2020a). As for other xenomonitoring assays (Cook *et al.*, 2015; Garrod *et al.*, 2020), the primary advantage is that the xenomonitoring assay used here offers a more sensitive method to detect *Schistosoma* transmission in intermediate hosts than conventional methods of parasitological shedding. A second advantage is that species-level identification of the schistosomes being transmitted by the snail can be deduced from sequence analysis, and are not required to be inferred by cercarial shedding patterns (Mouahid *et al.*, 1991).

There are certain disadvantages to using a snail xenomonitoring assay which are particularly apparent when conducted on a large scale. The cost of PCR reagents and consumables may be difficult to justify for a regional disease control programme manager, especially if additional costs are needed for a basic laboratory set up. Testing pooling strategies, such as those used in arthropod vectors before or after DNA extraction (Schmaedick *et al.*, 2014; Cook *et al.*, 2017; Ramesh *et al.*, 2018), to maximise the number of snails processed and minimise costs should be trialled. The processing of samples pre-PCR is also time consuming, with a major bottleneck being the time taken to extract DNA from each

snail. Following the protocol described here, it was possible for an experienced technician to perform 94 snail extractions in 24 hours. Moreover, interpreting xenomonitoring assay gel electrophoresis profiles is open to human error. A higher proportion of schistosome infections were confirmed when the triple banding pattern (snail, trematode, schistosome) was observed (81%) compared to when the trematode band was absent (30.8%). A lack of primer specificity must therefore be present in the first xenomonitoring PCR, resulting in non-specific binding of primers ETTS2 with either ETTS1 or ITS2_Schisto_F, producing an amplicon of a similar size to those expected from *Schistosoma* spp. Further attempts should be made to improve the assay to avoid these initial false positives and to streamline screening. However, trematode diversity is high in African freshwater bodies and current research is constantly expanding on the range of trematode species encountered (Laidemitt *et al.*, 2017; Outa *et al.*, 2020). Species-specific molecular assays need constant specificity testing against the backdrop of hidden trematode diversity. This is particularly important for assays set to certify transmission interruption where false positives pose a major stumbling block and it may be a constant need to sequence amplicons for species confirmation. Sensitivity is also key, as any missed infections could lead to an assumption that transmission is not occurring. The assay used here does present the required limit of detection of a single larval stage at the initiation of infection, however, the sensitivity in terms of how many snails need to be collected and analysed and at what time intervals represents a continuing conundrum which will differ by site, settings and species.

To circumvent the requirements for sequencing of amplicons to confirm schistosome species, diagnostic PCRs to differentiate *S. haematobium* and *S. bovis* using amplicon sizes are available (Webster *et al.*, 2010; Abbasi *et al.*, 2017) for miracidia and cercariae, although these have not been consistent in trials on infected snails in terms of PCR success and failure (Pennance, Archer, *et al.*, 2020) with *S. haematobium* infections often failing to amplify. In future iterations of schistosome xenomonitoring assays, alternative post-PCR analysis to differentiate species, such as those used in other disease systems, could be used (Tedrow *et al.*, 2019). Other protocols such as qPCR or real-time PCR may also be beneficial if access to the required equipment is possible (Kane *et al.*, 2013; Garrod *et al.*, 2020). Portable nucleic acid based diagnostic assays such as rapid polymerase amplification (RPA) protocols have shown to be sensitive diagnostic tests for human infections from urine (Rosser *et al.*, 2015; Rostron *et al.*, 2019), and these point-of-need diagnostic methodologies could be trialled for snail xenomonitoring.

6.4.6 The future of schistosomiasis surveillance

Although improvements need to be made before the use of a schistosomiasis xenomonitoring protocol for schistosomiasis surveillance on a large scale, the findings here offer good

progress towards developing such a protocol. The results demonstrate that it is possible to detect urogenital schistosomiasis transmission by collecting only intermediate host snails in both low and high prevalence areas. Environmental DNA (eDNA) xenomonitoring protocols for the detection of snail and schistosome DNA within freshwater samples may also offer valuable schistosomiasis surveillance tools, however, further improvements to protocols and the demonstration of field applicability is required (Fornillos *et al.*, 2019; Alzaylaee, Collins, Rinaldi, *et al.*, 2020; Alzaylaee, Collins, Shechonge, *et al.*, 2020; Mulero, Boissier, *et al.*, 2020). The results presented here also show how infections fluctuate seasonally and can be restricted to specific freshwater bodies. In addition, the data on *S. haematobium* prevalence in snails provides unique insights into the relationships between schistosomes, snails and the environment where transmission occurs and also the level of endemic disease prevalence. The data collected from this study not only improves our understanding of schistosomiasis on Pemba Island at a critical time when elimination is targeted, but it also provides data for those interested in modelling schistosomiasis transmission and incorporating data on the intermediate host infections.

Chapter 7. Mitogenome genetic diversity of medical and veterinary important *Bulinus* spp. snails in Sub-Saharan Africa and the Indian Ocean Islands

Abstract

Species of the *Bulinus* genus are a group of medically and veterinary important freshwater snails (~37 species) involved in the transmission of *Schistosoma haematobium* group parasites, causing human urogenital and animal intestinal schistosomiasis. Taxonomy of these snails has always been problematic, and historically relies on interpreting often overlapping morphological and ecological characters. Most recently, the identification of cryptic taxa and varied opinions on basal topologies have also been discussed. Here, 70 complete mitogenomes and ribosomal operon sequences (rDNA) were generated from *Bulinus* collected across Sub-Saharan Africa (Angola, Ethiopia, Liberia, Malawi, Niger, Senegal, Tanzania and Uganda) and the Indian Ocean Islands (Pemba, Unguja and Madagascar).

The relationship between *B. africanus* group species and *B. truncatus/tropicus* complex species as sister taxa was supported, with *B. bavayi* (a Madagascan *B. forskalii* species) diverging earlier. Taxa sequenced from the Madagascan islands were basal to *B. africanus* and *B. truncatus/tropicus* complex species groups, which included identification of a new species of the *B. truncatus/tropicus* complex. Cryptic diversity was observed from *Bulinus* collected in both East and West Africa, with up to five new *B. truncatus*/*tropicus* complexes and up to 11 new *B. africanus* group species identified. Support is given for splitting *B. globosus* into at least four sperate species, representing taxa from the type locality of Angola, West Africa, mainland East Africa and Unguja, and Pemba. Phylogenetic support was also provided for demarcating *B. nasutus* and *B. productus* as species as previously suggested, although species delimitation analysis using a Bayesian Poisson tree process was not in agreement. Specimens collected from the great lakes of Central East Africa were particularly diverse and may relate to the age of these waterbodies and diverse habitats promoting evolutionary radiation. This unique data set should help promote future research into *Bulinus*, which has been severely neglected in comparison to *Biomphalaria* spp., the intermediate hosts of *S. mansoni*.

7.1 Introduction

Freshwater snails of the *Bulinus* genus are a diverse group of medically important taxa with a pan-African distribution. They act as the intermediate host of *Schistosoma haematobium* group species, a group of blood flukes responsible for causing human and animal schistosomiasis across much of sub-Saharan Africa. The 37/38 currently recognised species

of *Bulinus*, separated into four species groups, were only established after decades of confusing taxonomy due to their variable and often overlapping morphological forms (Wright, 1961; Brown, 1994). Current understanding of the taxa has not significantly advanced since the last major review of the genus performed by Brown (1994) over a quarter of a century ago; the evolutionary relationship between three of the basal species groups is still uncertain, and it is likely that a lot of cryptic diversity exists within each species (Wright, 1961; Brown, 1994). DNA sequencing has resolved some taxonomic uncertainties within the genus, and helped to delimit taxa involved in the transmission of schistosomes in some regions (Stothard, Loxton, *et al.*, 2000; Stothard *et al.*, 2001; F. Allan *et al.*, 2017). At the same time, exploring the phylogenetic relationships between taxa has created an abundance of other issues, and occasional contradictions, particularly concerning the basal topology. Improving the taxonomy of these species will not only improve interpretations of the evolutionary history of freshwater molluscs across Africa but will also create further insight into the associations between these planorbid intermediate hosts and the schistosomes they transmit.

As Brown (1994) predicted, the use of molecular tools has uncovered much cryptic diversity and potential species. Demarcating these cryptic species has however proven difficult, complicated by the absence of a species concept for *Bulinus*. However, the accessibility and availability of next generation sequencing (NGS) technologies to produce large and informative sequence datasets has significantly improved the capacity for inferring relations between molluscan taxa, with mitochondrial genome sequencing often being the method of choice in creating phylogenies (Williams *et al.*, 2017). Mitochondrial genomes, with high copy number, can resolve deep and shallow nodes across molluscan taxa including cephalopods, bivalves and gastropods (Allcock *et al.*, 2011; Williams *et al.*, 2014, 2017; Uribe *et al.*, 2016).

Freshwater molluscs such as *Bulinus*, are interesting groups to investigate from an evolutionary perspective, considering the geographical isolation which exists between many of the populations that prevent regular admixture. Dry land acts as an isolating barrier to freshwater snail populations, which can eventually lead to genetically distinct populations if divergent evolution occurs. For a species, such as those within the *Bulinus* genus, that can withstand ecological pressures such as desiccation, are hermaphroditic and in many cases also capable of self-fertilisation, the colonisation of new habitats is likely to take place rather regularly, probably explaining their widespread dispersal across much of Africa. The only possibility of interchange between different populations across mainland Africa is through translocation of snails (e.g. through accidental dispersal such as predators; Wada *et al.* 2012) or through geological changes where once disconnected water bodies become conjoined. Therefore, admixing if it occurs, will occur at different rates between populations. The degree of genetic divergence between snail populations is therefore heavily influenced by these

ecological changes, and those in more geologically unstable environments may lead to the regular separation, and in some case re-joining, of species. This will either lead to crossbreeding of populations, or independent breeding of sympatric species if divergence has been long enough, potentially creating a complex web of related species in freshwater bodies such as the African great lakes across central East Africa (Salzburger *et al.*, 2014). However, several species of *Bulinus* are isolated on islands neighbouring the African continent, such as the species endemic to the Indian Ocean islands of Madagascar and the Zanzibar Archipelago (Wright, 1971; Stothard and Rollinson, 1997a; Stothard *et al.*, 2001; Stothard, Loxton, *et al.*, 2002). These populations potentially offer a completely isolated population where divergent evolution may have taken place for some time resulting in differentiation between ancestral taxa and those more recently introduced.

Bulinus globosus is currently considered the most medically important species of *Bulinus* due to its capacity for transmitting *S. haematobium* and expansive range across sub-Saharan Africa (Brown, 1994; Rollinson, 2009) from the small ponds on Pemba Island (disconnected from mainland Africa for ~5.3-2.5) to the large riverine systems of the Niger River Valley (Pennance *et al.*, 2018; Pennance, Allan, *et al.*, 2020), both a considerable distance from the type locality in Angola (Wright, 1963; F. Allan *et al.*, 2017). Although with some support from recent molecular characterisation of *B. globosus* populations (Kane *et al.*, 2008; Jørgensen *et al.*, 2013; F. Allan *et al.*, 2017; Mutsaka-Makuvaza *et al.*, 2020), the status quo for decades of urogenital schistosomiasis snail surveillance has been to treat these geographically isolated populations of *B. globosus* as a single species (i.e. a taxonomic inertia by 'lumping' species together; see Stanton *et al.*, 2019). With an appreciation of how different snail-schistosome species associations can impact the transmission of schistosomes (Rollinson *et al.*, 2001; Lockyer *et al.*, 2004), it is of great importance to explore the diversity and resulting taxonomy of *B. globosus*, and other *Bulinus* taxa, especially in light of the renewed interest in implementing snail control and surveillance in continued efforts to eliminate schistosomiasis (WHO, 2020a).

As mitochondrial genome data is not currently available for any *Bulinus* species, but is for some intermediate hosts of intestinal schistosomiasis *Biomphalaria* (see DeJong *et al.,* 2004; Zhang *et al.,* 2018), a primary aim of the current study was to generate robust mitogenomes, and ribosomal operon sequences, for *Bulinus* taxa representing key species capable of *S. haematobium* transmission. By including representatives from *B. africanus* group, *B. forskalii* group and *B. truncatus*/*tropicus* complex species, it was hoped to explore the basal topology of the *Bulinus* genus. The utility of exploring inter-species and intra-species variation between taxa of these groups, and performing species delimitation analysis, was also conducted, with a particular focus on those of the *B. africanus* group because of their important role in urogenital schistosomiasis endemicity. By including specimens from a broad

geographical range, including those of the Indian Ocean islands, the phylogeography of *Bulinus* species was also explored to allow interpretations on the evolutionary history of species groups and the establishment of unique populations.

7.2. Methods

7.2.1 Sample collection

In total 71 snails representing eight *Bulinus* species were sequenced in this study to obtain mitochondrial genomes and ribosomal operon sequences for each. Snails were obtained through multiple projects and collaborators (see Chapter 1 and Supplementary Table 7.1) and all samples and resulting DNA extracts were archived in the Schistosomiasis Collection at the Natural History Museum, London (Emery *et al.*, 2012). The snails included in this study were first morphologically identified based on shell characteristics to the species level (Brown, 1994).

Since morphological identifications based of shell characteristics lacks specificity, particularly within species groups, identification of the selected samples was further clarified to the species / species group level by Sanger sequencing the 'Folmer' partial *cox*1 region and comparing to reference sequences (Kane *et al.*, 2008). PCRs to amplify this ~623 bp DNA region used either the forward HCO or BC1 primers in combination with the reverse LCO primers following previously reported PCR cycling conditions (Folmer *et al.*, 1994; Kane *et al.*, 2008). Therefore, it was possible to confidently select taxa to cover key species groups for investigation within the *Bulinus* genus, and also select those from Unguja and Pemba islands based on genetic divergence (i.e. to capture all *cox*1 haplotypes) and geographical locality.

Three of the four species groups were represented, including: *B. africanus* group (n = 58), *B. truncatus/tropicus* complex (n = 12) and *B. forskalii* group (n = 1) (Table 7.1). The samples were provided from a range of localities (Figure 7.1) and freshwater bodies in sub-Saharan Africa (Angola, Ethiopia, Liberia, Malawi, Niger, Senegal, Tanzania and Uganda) and the Indian Ocean Islands (Pemba, Unguja and Madagascar), preserved in 100% ethanol (Table 7.1). Malacological surveys and the associated collections span over ~21 years, from 1997 (Madagascar) to 2018 (Pemba Island) (Supplementary Table 7.1). Samples were not available from the *B. reticulatus* group.

The regions with the highest number of taxa sequenced were *B. globosus* (n = 20) and *B. nasutus* (n = 8) originating from Unguja and Pemba islands (Zanzibar Archipelago, United Republic of Tanzania). *B. globosus* were also included from West (Niger, Senegal, Liberia) and East Africa (Tanzania, Malawi), and the type locality in Angola (Table 7.1). Other *B. africanus* group species sequenced, included: *B. angolensis* (n = 2) with one isolate also from their type locality in Angola, *B. obtusispira* (n = 1) an endemic species to Madagascar, *B. africanus* (n = 1) from Malawi, *B. nasutus productus* (n = 2) from Tanzania, and several

unidentified *B. africanus* group species (n = 13), that could not be confidently identified from the partial *cox*1 sequence analysis or shell characteristics (Brown, 1994). Two other species endemic to Madagascar were included: *B. bavayi* of the *B. forskalii* group (n = 1), and *B. liratus* (n = 5) of the *B. truncatus/tropicus* complex group. Other *B. truncatus/tropicus* complex group species were also sequenced, including: *B. truncatus* from Niger (n = 1) and Uganda (n = 3), *B. truncatus/tropicus* complex lacustrine species from Lake Victoria shores in Uganda (n = 1) and Tanzania (n = 1), and *B. truncatus/tropicus* complex species collected in Ethiopia. GPS coordinates associated with each specimen were visualised in QGIS v3.0.1 Girona (http://qgis.osgeo.org) and mapped for each site. Digital shape files for mapping the samples to their origins in Africa were obtained from DIVA-GIS (https://www.diva-gis.org).

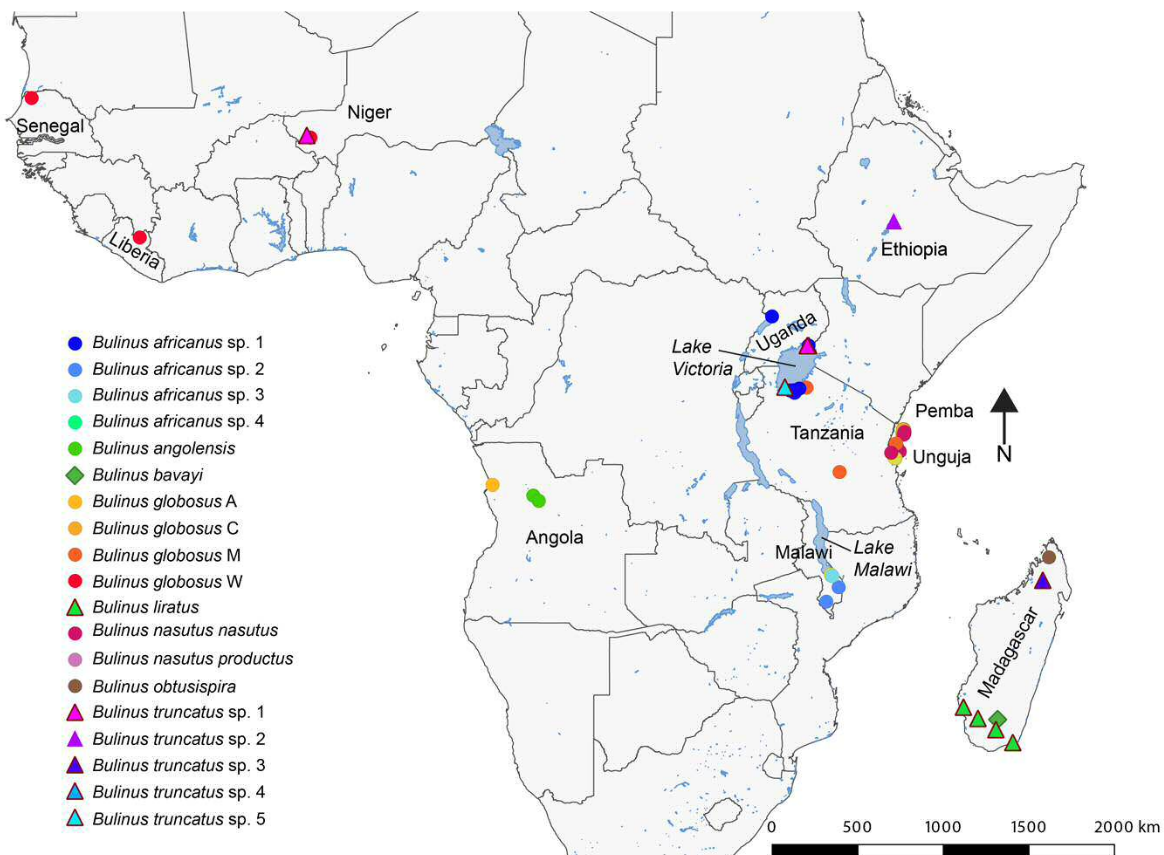


Figure 7.1. Collection locations of *Bulinus* taxa sequenced in the current study categorised by *B. africanus* group species (circles), *B. bavayi* (diamond) and *B. truncatus/tropicus* complex species (triangles).

Several of the *Bulinus* analysed were identified as infected with *Schistosoma* species upon collection by observing, collecting and molecularly identifying the schistosome cercariae shed (see Chapters 3-4). These included: *S. haematobium* infected *Bulinus* from Pemba (Table 7.1: Taxa ID 2, 11, 15, 21), Unguja (Table 7.1: Taxa ID 25 and 26) and Niger (Table 7.1: Taxa ID 75), *S. bovis* infected *Bulinus* from Pemba (Table 7.1: Taxa ID 12) and Niger (Table 7.1: Taxa ID 63 and 73) and *S. kisumuensis* (unpublished observations) infected

Bulinus from the Lake Victoria region of Tanzania (Table 7.1: Taxa ID 35). Unidentified wildlife / veterinary patent trematode infections that were not *Schistosoma* spp., were also observed in three snails, included in the study, from Tanzania (Table 7.1: Taxa ID 41 and 42) and Madagascar (Table 7.1: Taxa ID 54).

Following selection, total gDNA was extracted from the whole snail tissue from each specimen and sequenced to obtain mitochondrial genome and ribosomal operon (see below).

Table 7.1. Details of *Bulinus* species sequenced in the present study; country of collection, the *Bulinus* species group following taxonomic positions of Brown (1994) except for change of *B. angolensis* to *B. africanus* group as supported by F. Allan *et al*., (2017), morphological identifications, species or molecular operational taxonomic unit (MOTU) determined by phylogenetic analysis of mitochondrial genome and ribosomal operon sequence data (NGS), number of taxa per country/MOTU (N), the unique numerical identifier for each sample (Taxa ID) and the number of highly supported species / cryptic species delimited using Bayesian Poisson posterior tree probability (bPTP > 0.91, see Zhang *et al*., 2013).

Country	*Bulinus* Species group	Morphological species identification	Molecular species identification / MOTU(NGS)	N	Taxa ID	Number of supported species delimited (bPTP)
Pemba Island	*B. africanus*	*B. globosus*	*B. globosus* (C)	17	1,3,6,7,8,9, 10,11,12,13, 14,15,16,17, 18,19,21	1
		B. nasutus	*B. nasutus*	5	2,4,5,20,22	0
Unguja Island	*B. africanus*	*B. globosus*	*B. globosus* (M)	3	25,26,28	2
		B. nasutus	*B. nasutus*	3	23,24,27	0
Tanzania	*B. africanus*	*B. globosus*	*B. globosus* (C)	2	36, 37	1
		B. globosus	*B. globosus* (M)	2	38, 40	2
		B. nasutus	*B. nasutus*	1	39	0
		B. nasutus productus	*B. nasutus productus*	2	41,42	0
		Bulinus sp.	*B. africanus* sp. 1	7	35,43,44,45, 46,47,48	0
	B. truncatus / tropicus	*Bulinus* sp.	*B. truncatus* sp. 5	1	49	1
Uganda	*B. africanus*	*B. africanus* sp.	*B. africanus* sp. 1	2	30,33	0
	B. truncatus / tropicus	*B. truncatus*	*B. truncatus* sp. 1	2	29,32	0
		B. truncatus sp.	*B. truncatus* sp. 4	1	31	1
Madagascar	*B. africanus*	*B. obtusispira*	*B. obtusispira*	1	50	1
	B. truncatus / tropicus	*B. liratus*	*B. liratus*	4	52,54,55,56	1
		B. liratus	*B. truncatus* sp. 3	1	53	1
	B. forskalii	*B. bavayi*	*B. bavayi*	1	51	1
Malawi	*B. africanus*	*B. globosus*	*B. globosus* (C)	1	60	0
		B. africanus	*B. africanus* sp. 2	3	57,59,62	3
		B. africanus sp.	*B. africanus* sp. 3	1	61	1
		B. africanus sp.	*B. africanus* sp. 4	1	58	1
Ethiopia	*B. truncatus / tropicus*	*B. truncatus* sp.	*B. truncatus* sp. 2	2	68,69	1
Angola	*B. africanus*	*B. globosus*	*B. globosus* (A)	1	67	1
		B. angolensis	*B. angolensis*	2	65,66	1
Niger	*B. africanus*	*B. globosus*	*B. globosus* (W)	2	63,73	1
	B. truncatus / tropicus	*B. truncatus*	*B. truncatus* sp. 1	1	75	1
Senegal	*B. africanus*	*B. globosus*	*B. globosus* (W)	1	70	1
Liberia	*B. africanus*	*B. globosus*	*B. globosus* (W)	1	64	1

7.2.2 DNA extraction, library preparation and sequencing

Before initial gDNA extraction, photographs were taken of each specimen using a camera mounted dissection microscope for later morphological comparisons. gDNA was extracted

from whole snail, or partial snail tissue from each sample using either the DNeasy Blood & Tissue kit (Qiagen, Manchester, UK) or the Thermo Qiagen BioSprint 96 Workstation and Qiagen BioSprint 96 DNA Blood / Tissue Kit (Qiagen, Manchester, UK) protocol following manufacturers protocol's (double lysis buffers) for whole snail tissue extracts adapted by Pennance *et al.,* (2018).

Genomic libraries were prepared using the Nextera DNA Flex Library Prep (Illumina, California, USA) and each sample was indexed following manufacturer's instructions. The Nextera DNA Flex Library Prep (Illumina, California, USA) supports a broad DNA input range of 1-500 ng and utilises a modified transposon for cleaving and tagging DNA. In preparation for tagmentation of the gDNA, whereby transposons cleave DNA to an optimal size of between 200-1000 bp and tag it with transposomes, a Qubit® fluorometer (Invitrogen, California, USA) and dsDNA Broad Range (BR) Assay Kit (Molecular Probes, Life Technologies) assay kit was used to accurately determine the amount of double stranded DNA (ng) per sample. Each sample was then normalised to 20 ng of input material using nuclease-free water to a total volume of 30 µl to avoid under/over tagmentation of gDNA and improve cluster densities of libraries. The recommended Illumina protocol was followed for the tagment amplification step and eight library PCR cycles were performed (as DNA was normalised to 20 ng).

Following clean up, indexed sample concentrations were manually quantified on the Qubit® fluorometer (Invitrogen, California, USA) with the dsDNA Broad Range (BR) Assay Kit and library insert size checked using genomic DNA screen tape on an Agilent 2200 TapeStation System (Agilent Technologies, California, USA). Using this information, a size corrected concentration (nM) was calculated and used to normalise sample concentration for the final pooled libraries. Two pools were made, one for individual samples with a size corrected concentration of <10 nM with a final library concentration of 3 nM (low pool), and a second for samples with a size corrected concentration of >10 nM with a final diluted library concentration of 10 nM (high pool). The libraries were then immediately stored at -20ºC ready for sequencing. All 71 samples were sequenced on 1/4 of a flowcell on an Illumina Highseq platform (v.2 chemistry; 2 x 150 paired-end).

7.2.3 Mitochondrial genome assembly

Following on-instrument demultiplexing, raw sequencing reads were analysed and assembled in Geneious v11.1 (Kearse *et al.*, 2012). The data were trimmed allowing no ambiguous base calls and removing bases from the terminal ends of reads with an error probability of >0.05 (5%). The trimmed reads were first assembled to reference mitochondrial genomes of *B. globosus* (Briscoe *et al*., unpublished). Unassembled reads were then iteratively mapped and reassembled to the mitochondrial sequences until the resulting contig could be circularised, and a final stringent reassembly was performed to give a final mitochondrial sequence. If

circularized mitochondrial genomes could not be assembled in Geneious, then data were trimmed using Trimmomatic v0.39 (Bolger *et al.*, 2014) and a de-novo assembly using SPAdes v3.13.0 (Bankevich *et al.*, 2012) was performed on paired trimmed read files. Resulting contigs were imported into Geneious and a BLAST search was performed on contigs between 8-15 kb. Contigs were then selected based on longest sequence length and best coverage when BLAST searched to planorbid mitochondrial genomes (Zhang *et al.*, 2018). Overlapping regions of the 5' and 3' ends of linear sequences resulting from de novo assembly of the circular mitochondrial genome were identified to produce finalised circularised mitochondrial genomes where possible.

7.2.4 Mitochondrial genome annotation

No available transcriptomic data is available for any *Bulinus* spp. to verify gene boundaries and therefore ribosomal and protein coding gene annotations were putatively identified using MITOS2 (Bernt *et al.*, 2013). The conserved motifs and gene boundaries identified from MITOS2 were then verified by visualization of open reading frames and comparison to alignments of the mitochondrial genomes of closely related *Biomphalaria pfeifferi*, *B. sudanica* and *B. choanomphala* (see Zhang *et al.,* 2018). Annotations from MITOS2 were then visually checked for each sequence to identify any potential erroneous gene duplications, split genes or missing genes that can sometimes occur in the *de novo* annotation. Any peculiarities in annotations were investigated further to establish whether these were *bona fide* through performing iterative trimmed sequence assemblies and remapping of trimmed sequence data across the region of interest in Geneious v11.1 (Kearse *et al.*, 2012). Predicted protein coding gene annotations were edited at the 5' and 3' ends to incorporate conserved initiation and termination codons that were identified by comparing gene boundaries across taxa and, with reference to the *Biom. pfeifferi* mitogenome (Zhang *et al.*, 2018). The methodology used by Zhang et al. (2018) and resulting annotated mitochondrial genomes of *Biomphalaria* spp. suggests that the longest reading frame from start to stop codon of each individual mitochondrial gene was used, however due to the large sample size in the present study, gene boundaries were refined conservatively based on matching conserved start (Methionine/Leucine/Isoleucine) and stop codons (TAG / TAA) across the taxa. When no clearly conserved termination codon was observed, truncated stop codons (T / A) were used where necessary to avoid large overlapping gene boundaries (this was performed primarily for genes: *nad*4l, *cob, cox*2, *cox*3, *nad*3). Short overlaps with tRNA genes were however deemed acceptable (personal communication, A. Briscoe and A. Waeschenbach). Using this conservative method of gene boundary annotation, overlap between protein coding genes was avoided.

Conserved protein-coding gene boundaries were checked by extracting individual gene sequences for each taxa and aligning amino acid sequence data for each gene in the online tool; TranslatorX (Abascal *et al.*, 2010), using the invertebrate mitochondrial genetic code and Muscle alignment program (Edgar, 2004). Resulting nucleotide and amino acid alignments were visualised in Mesquite v3.61 (Maddison and Maddison, 2019) to establish conserved gene regions, as well as the nucleotide sequences of both large and small rRNA subunits (rrnL and rrnS). Both rrnL and rrnS sequences were refined only after finalising all protein coding genes and for consistency were extended from the end of the previous gene to the start of the following gene. The rRNA subunit sequences were then aligned in MAFFT v7.429 (Katoh *et al.*, 2002; Katoh and Standley, 2013) before being visualised in Mesquite v3.61 (Maddison and Maddison, 2019) to identify conserved regions.

Sequences corresponding to 21-22 transfer RNA sequences (tRNAs) were putatively identified using MITOS2 (Bernt *et al.*, 2013) and ARWEN v1.2 (Laslett and Canbäck, 2007), and boundaries were confirmed by comparison with secondary structures. No further editing or analysis was performed with tRNAs in the current study.

7.2.5 Nuclear ribosomal operon assemblies and annotations

The ribosomal RNA gene repeat region (rDNA: 18S-ITS1-5.8S-ITS2-28S) was assembled using a reference sequence of *Bulinus globosus* from Zanzibar (Briscoe *et al*. unpublished). Regions of low coverage, or gaps in rDNA sequences were resolved by iteratively assembling sequences from both 5' and 3' directions of the issue in Geneious v11.1 (Kearse *et al.*, 2012) until overlapping contigs could then be assembled. The ribosomal operon contigs were annotated by copying annotations from the reference dataset (Briscoe *et al*. unpublished) and editing the 5' and 3' ends of DNA regions to conform with the reference dataset and to remove unreliable and variable sequence data, forming the external transcribed spacer and non-transcribed spacers. The ITS1 and ITS2 fragments are poor markers for species discrimination (Kane *et al.*, 2008; Zein-Eddine *et al.*, 2014; Akinwale *et al.*, 2015), and were therefore excluded. The 18S, 5.8S and 28S were further confirmed by comparing with results of the ribosomal operon contigs when submitted to the online tool RNAmmer using the Eukaryote settings. Ambiguous bases were identified in rDNA sequences, which may occur through either recent hybridisation events or due to polymorphisms occurring during concerted evolution of these taxa, that have not been rapidly homogenized to maintain the highly identical repeat arrays observed in other species (Ganley and Kobayashi, 2007). Therefore, sequences with ambiguous bases were left unresolved, although these sequences were later excluded (see section 7.2.6). The 5.8S gene was highly conserved across all taxa (3 single nucleotide polymorphisms in 158 bases) and was therefore excluded from further analysis.

7.2.6 Phylogenetic analysis: whole mitogenome species relationships and delimitation

The 71 new mitogenomes and cytoplasmic rRNA (18S and 28S) for taxa were included for phylogenetic analyses along with an outgroup of *Biomphalaria pfeifferi* (Accession: mitochondrial genome: NC038059, rRNA: MG461588, Zhang *et al*., 2018). Gblocks v. 0.91b (Castresana, 2000) was used to select conserved sites in alignments and eliminate poorly aligned and overly saturated regions of all sequences, using the settings for less stringent selection. These exclusion criteria were then specified when conducting further phylogenetic model tests and analysis. Of the 71 *Bulinus* mitogenomes produced, it was not possible to obtain a complete circular mitochondrial genome from one of the *B. africanus* group species (bul61), due to missing atp6, atp8, *cox*3, nad2, nad3, nad4 and only had a partial *cox*1 sequence. One *B. truncatus/tropicus* complex species (bul31 Mayuge, Uganda) had a unique duplication event, with two copies of *cox*2 present in the mitochondrial genome; only the second one, in a different position in the mitochondrial genome, was functional (i.e. protein coding sequence) and was used in analyses. Apart from these exceptions, all other genes were used in analyses (Table 7.2). The final alignment was 18,751 nucleotides in length before Gblocks exclusion, and 17,861 when accounting for exclusions (Table 7.2). Only the dataset accounting for these exclusions was used in further phylogenetic analyses.

Table 7.2. Number of nucleotides (longest sequence for each gene) in alignment of *Bulinus* spp. before and after removal of non-conserved positions with Gblocks.

	Original Length (nucleotides)	Length after Gblocks (nucleotides)	% nucleotides remaining
Protein coding genes			
*atp*6	681	639	93.8
*atp*8	162	105	64.8
cob	1185	1073	90.6
*cox*1	1557	1527	98.0
cox2	669	618	92.4
cox3	801	774	96.6
*nad*1	894	894	100
nad2	954	891	93.4
nad3	360	342	95.0
nad4	1359	1303	95.9
*nad4*l	315	312	99.0
nad5	1671	1621	97.0
nad6	519	465	89.6
Ribosomal RNA			
rrnL	1140	978	85.8
rrnS	768	685	89.2
Ribosomal DNA			
18S	1851	1850	99.9
28S	3865	3784	97.9
All	18751	17861	95.3

Best fit partitioning schemes and models of molecular/DNA evolution for phylogenetic analyses were identified in PartitionFinder2 (Guindon *et al.*, 2010; Lanfear *et al.*, 2012, 2016). To identify the best fit evolutionary model for DNA sequences to interpret using Bayesian and maximum likelihood methods (see below) using PartitionFinder2, genes were first grouped into partitions of all protein coding mitochondrial genes, mitochondrial rRNA (rrnS and rrnL) and nuclear rDNA (18S and 28S). The GTR invariant gamma (GTR+I+Γ) substitution model was selected as the best fit for all partitions and therefore used in all following analyses.

Phylogenetic analyses of this complete dataset including the 71 *Bulinus* species and one reference *Biomphalaria pfeifferi* were undertaken in four parts. First, a Randomized Axelerated Maximum Likelihood (RAxML) tree produced using RAxML v.8.2.12 (Stamatakis, 2014) for all taxa. Second, a Bayesian inference (BI) analysis and tree construction using MrBayes v3.2.6 (Ronquist *et al.*, 2012) for all taxa. Third, a Bayesian species tree estimation was performed using *BEAST (Heled and Drummond, 2009; Bouckaert *et al.*, 2014) on taxa that were inferred as the same species / molecular operational taxonomic units (MOTUs) in ML and BI analysis. Fourth, a species delimitation analysis was conducted on the supported BI tree following Bayesian Poisson tree processes (bPTP) (Zhang *et al.*, 2013). Bootstrap values for 100 replicates were obtained using two threads in ML analyses, and two BI runs were performed over 5,000,000 generations and sampled every 1,000 generations. Clades were considered to have high nodal support if bootstrap values in ML were ≥70% and posterior probability in BI were ≥0.95; nodes with <70% bootstrap support or <0.95 posterior probability were collapsed in SumTrees v4.4.0 (Sukumaran and Holder, 2010) to give final tree topology. The burnin for BI was set as 3,500,000 million generations for consistency, at which point the average standard deviation of split frequencies (ASDOSF) reported from MrBayes output was <0.01 over 5 million generations.

To investigate relationships between the individual species identified in RAxML and BI analyses (Table 7.3) that were represented by two or more taxa (the minimum that can be used for this analysis), Bayesian inference to co-estimate multilocus gene trees embedded in a shared species tree was conducted using *BEAST (Heled and Drummond, 2009) in BEAST v2.6.2 (Bouckaert *et al.*, 2014). Following the removal of species represented by only one taxon (n = 8), 11 species / MOTUs were included in the analysis. Tree models of mitochondrial genes and rRNA were linked together but separated from the 18S/28S. Parameter priors were set as the default, meaning no assumptions of birth-death rates or population means were provided to the model since these were not established priori (Heled and Drummond, 2009). The analysis was run for 10 million generations and resulting tree files were viewed and edited using DensiTree v2.2.7 (Bouckaert and Heled, 2014), a burn in of 5 million trees (50%) was set. Mean highest posterior density (HPD) and 95% HPD were displayed graphically for clades

with a posterior support >18%. Lines of consensus trees were coloured by the clade they represented, and a root canal was added. Multiple parameters in the *BEAST analysis for further species delimitation failed to reach acceptable Effective Sample Sizes (ESSs) of >200, and therefore was deemed not to represent the posterior distribution well with this current dataset. However, a species consensus tree was produced with the available data (see Supplementary Figure 7.1).

To provide evidence for delimiting species from one another based on the tree outputs of BI analysis, the final tree topology (i.e. post collapsing clades with low support) was submitted to the bPTP server (https://species.h-its.org/ptp/: Zhang *et al.,* 2013) and was run for 500,000 generations, with a burn-in of 0.1. Convergence was assessed by viewing the likelihood plot, and visually determining that the MCMC chain remained at high likelihood locations, only occasionally exploring low likelihood locations. Accuracy for each species delimitation was interpreted by the Bayesian posterior probability of the given taxa, with values ≥0.91 being considered as strong support for the delimitation as recommended (Zhang *et al.*, 2013). This bPTP method is a close relative of the generalized mixed yule coalescent (GMYC) approach (Fujisawa and Barraclough, 2013) often used for species delimitation, however bPTP comes with the advantage that ultrametric trees are not required, removing the difficulties and errors that can occur in producing time calibrated trees or arbitrarily ultrametricising non-ultrametric trees such as those resulting from ML and BI here.

7.2.7 Phylogenetic cox*1 data analysis and species identification*

As reference mitogenome sequences are currently not available for any *Bulinus* species outside this dataset (and one used for reference; Briscoe *et al.* unpublished), it was necessary to conduct a Bayesian inference of species with reference *cox*1 data available from previous studies including isolates from similar geographic regions (Stothard *et al.*, 2001; Kane *et al.*, 2008; F. Allan *et al.*, 2017). Full *cox*1 sequences (i.e. not excluding any sites) were extracted from the mitogenomes sequenced here and aligned with the reference data set. Reference sequences ranged in nucleotide length from 340 bp (Stothard *et al.*, 2001) to 1,548 bp (Zhang *et al.*, 2018)) and were aligned using the ClustalW (Larkin *et al.*, 2007) plugin in Geneious v11 (Kearse *et al.*, 2012). BI analysis and tree construction was performed using MrBayes v3.2.6 (Ronquist *et al.*, 2012), following the same criteria as above (5 million generations, nodes collapsed with <0.95 posterior probability in SumTrees v4.4.0 (Sukumaran and Holder, 2010), and a burnin of 3.5 million generations to produce final tree topology). Taxa sequenced in the current study that did not form clades with the reference sequences included here were individually searched for on the BLAST (NCBI) database. Sequence data from four *Bulinus* taxa: *B. globosus* (bul1, Pemba), *B. nasutus* (bul4, Pemba), *B. bavayi* (bul51, Madagascar) and *B. truncatus* (bul75, Niger) was translated using the invertebrate mitochondrial code and

an amino acid (AA) alignment was produced using the MUSCLE algorithm in TranslatorX (Edgar, 2004; Abascal *et al.*, 2010). The number of variable AA sites between species was investigated.

7.3 Results

7.3.1 General features of the mitochondrial genomes

Complete mitochondrial genomes and nuclear rRNA were sequenced for 70 of the 71 *Bulinus* specimens. The additional specimen, a *B. africanus* sp. (bul61 Palm Beach, Malawi), was left incomplete (8,277 bp) as it could not be circularised unambiguously. The smallest complete genome was 13,584 (*B. obtusispira*; bul50), with short small (12S) and large (16S) ribosomal subunits, 695 and 1,007 bp respectively, compared to the other 69 *Bulinus* spp. analysed. The largest genome was 14,543 bp (in *B. truncatus* sp.; bul31), which contained a possible gene duplication of *cox*2 (see below).

All the complete *Bulinus* mitochondrial genomes consisted of 13 metazoan protein coding genes, two rRNA genes and the ribosomal RNA repeat region. Protein coding genes, rRNA and tRNA genes were transcribed on the forward and backwards strand (i.e. in a forward and reverse direction Table 7.3). The gene order of both the protein coding and the rRNA genes were confirmed to be conserved among 68 of the 70 complete mitogenomes in this study (Table 7.3), and was identical to that observed in the *Biomphalaria* spp. genomes available (DeJong *et al.*, 2004; Zhang *et al.*, 2018). One of the two exceptions in gene order were that in one *B. truncatus* sp. (bul49, Bulolo, Tanzania) a potential gene rearrangement for *nad*4, *nad*4l, *cob* and *cox*2 was observed (Table 7.3). The second exception was observed in another *B. truncatus* sp. (bul31, Mayuge, Uganda), which contained a duplication of *cox*2, resulting in a non-functional copy in the normal gene position for *cox*2 compared to other *Bulinus* spp. analysed, and a second functional copy between *nad*3 and *nad*4 genes that was protein coding (Table 7.3).

7.3.2 Gene boundaries

Protein coding genes varied in length from 3 to 59 nucleotides, with the greatest length variation seen in *cob* and *nad*6 (Table 7.3). Using the conservative methodology for predicting gene boundaries (i.e. with using potentially conserved truncated stop codons, see methods), no overlap of protein coding genes was present here. However, potential large overlaps between protein coding genes and tRNA molecules were observed if gene regions were elongated to reach stop codons in most taxa as observed in *Biomphalaria* spp. (see Zhang *et al.,* 2018).

Table 7.3. Details of mitochondrial genome for 70 *Bulinus* spp. (excluding one partial mitochondrial genome): direction of protein-coding genes and ribosomal RNA forward (F) or reverse (R), order of protein coding genes (Ord), range of the minimum initial base pair (Min), maximum final base pair (Max) and length of gene in nucleotides (Len). Gene order rearrangements of bul31 and bul49 are highlighted in green shading.

Species	*Bulinus* spp. (68 taxa summary)				*Bulinus truncatus* sp. 4				*Bulinus truncatus* sp. 5			
Sample ID	bul1-bul30, bul32-bul33, bul35-bul60, bul62-bul70, bul73, bul75				bul31				bul49			
Gene (direction)	Ord	Min	Max	Len	Ord	Min	Max	Len	Ord	Min	Max	Len
*atp*6 (R)	7	5232-5542	5865-6181	634-640	6	5099	5738	640	5	4377	5016	640
*atp*8 (R)	6	5058-5355	5163-5474	106-120	5	4619	5029	111	4	4201	4311	111
cob (F)	4	2829-3088	3913-4164	1069-1128	4	2853	3928	1076	10	11281	12361	1081
*cox*1 (F)	12	10392-10807	11918-12342	1521-1536	12	11299	12825	1527	9	8234	9760	1527
*cox*2 (F)	5	4035-4296	4652-4913	613-640	8	7174	7818	645	12	12935	13562	628
*cox*3 (R)	10	8557-8972	9331-9746	775-778	10	9449	10226	778	7	6382	7159	778
*nad*1 (F)	2	1623-1880	2510-2773	888-894	2	1644	2534	891	2	1639	2529	891
*nad*2 (F)	11	9436-9854	10336-10748	895-904	11	10334	11240	907	8	7271	8174	904
*nad*3 (R)	8	6735-7110	7080-7454	343-349	7	6638	6983	346	6	5922	6261	340
*nad*4 (F)	9	7187-7567	8485-8878	1297-1312	9	8061	9366	1306	3	2590	3891	1302
*nad*4l (F)	3	2516-2775	2828-3087	313	3	2540	2852	313	11	12584	12892	309
*nad*5 (F)	1	11 -12	1619-1641	1609-1630	1	11	1640	1630	1	12	1635	1624
*nad*6 (F)	13	13117-13610	13581-14074	450-492	13	14091	14540	450	13	13769	14221	453
rrnL rRNA (F)	-	11963-12386	12969-13445	1007-1060	-	12872	13923	1052	-	9805	10847	1043
rrnS rRNA	-	5981-6304	6675-7011	695-717	-	5859	6575	717	-	5137	5858	722

7.3.3 Molecular phylogeny: relationships, identification and delimitation

The phylogenetic analysis based on the complete mitogenome and nuclear rDNA data resulted in strongly supported species trees. The topology of the consensus trees resulting from the two Bayesian analysis runs were identical (Figure 7.2), with the one RAxML tree only varying in terms of basal support for the three *Bulinus* species groups being lost (Supplementary Figure 7.2). Both BI and RAxML tree's displayed high posterior probability values and bootstrap support respectively for all clades. Inferences made here relating to the relationship between these taxa are therefore well supported. In addition, the species delimitation analysis (bPTP), resulted in a high likelihood plot (Supplementary Figure 7.3), showing strong support for model convergence. The bPTP model gave strong support (≥0.91

posterior probability) for 22 species and weak support (>0.5 and <0.91 posterior probability) for a further nine species (see Supplementary File 7.1).

The BI tree topology of the full mitogenome and rDNA combined data set showed strong support for the early division of the *B. forskalii* group specimen (*B. bavayi*), the *B. truncatus/tropicus* complex and the later diverging *B. africanus* group species (Figure 7.2). Although this support for the divergence of *B. forskalii* and *B. truncatus/tropicus* complex species in comparison to *B. africanus* spp. was lost when *cox*1 data was analysed alone or when RAxML analysis was performed on the whole dataset, where all species groups fell in an unresolved, early diverging position (Supplementary Figures 7.2 and 7.3). Within both the *B. truncatus/tropicus* complex and *the B. africanus* group species clades, the earliest diverging lineages were species from Madagascar, *B. truncatus* sp. 3 (bul53) and *B. obtusispira* (bul50) respectively, both being ancestral to the species groups of taxa analysed here. Unresolved positions for taxa were not observed in the mitogenome data (Figure 7.2), however many were present in the *cox*1 dataset (Figure 7.3).

The ancestral Madagascan *B. truncatus* sp. 3 (bul53) in the *B. truncatus/tropicus* complex is clearly distinct from *B. liratus*, which represents the other endemic taxa in this species group, in terms of morphology (Figure 7.4) and position in tree topology (Figures 7.2 and 7.3). As well as this Madagascan isolate, two additional taxa in this complex, both collected from the lacustrine habitats on the shores of Lake Victoria in Uganda and Tanzania; *B. truncatus* sp. 4 and *B. truncatus* sp. 5 respectively, did not form clades with the other taxa sequenced in this study (Figure 7.2), or with the reference taxa in the *cox*1 dataset (Figure 7.3). No highly similar *cox*1 sequences were identified from (BLAST) searches of this partial region (maximum of 95.8-96.4% similarity). Each of these three species were delimited as individual species within our mitogenome dataset by bPTP analysis (posterior probability = 1).

The remaining taxa in the *B. truncatus/tropicus* complex fell into three clades, one representing taxa from West Africa (Figure 7.2: *B. truncatus* sp. 1), a clade of two taxa from Uganda (Figure 7.2: *B. truncatus* sp. 2) and those identified as *B. liratus* (Figure 7.2: *B. liratus*). When compared with *cox*1 reference data, specimens identified as *B. truncatus* sp. 1 in the full mitogenome and rDNA combined data set grouped, or was closely positioned as a neighbouring clade, with *B. truncatus* previously identified from West Africa and Europe (Figure 7.3). The *B. truncatus* from Niger (bul75) was supported as a unique species within the complete dataset (Figure 7.2) through bPTP analysis (posterior probability = 0.942). The *B. liratus* specimens analysed here also grouped with those previously identified as *B. liratus* species from Madagascar based on short 340 bp *cox*1 sequences (Stothard *et al.*, 2001), supporting its original identification (Figure 7.3). The two *Bulinus* sequenced from Ethiopia (*B. truncatus* sp. 2), did not fall directly into a clade with any of the reference data used for the *cox*1 analysis and through bPTP was delimited as a unique species within the dataset (Figure

7.2). However, from the *cox*1 topology, these samples formed a sister clade to those previously identified as *B. tropicus* from Tanzania (Figure 7.3). Therefore, based on this high sequence similarity and morphological similarities (Brown, 1994), these should be considered as Ethiopian isolates of *B. tropicus*.

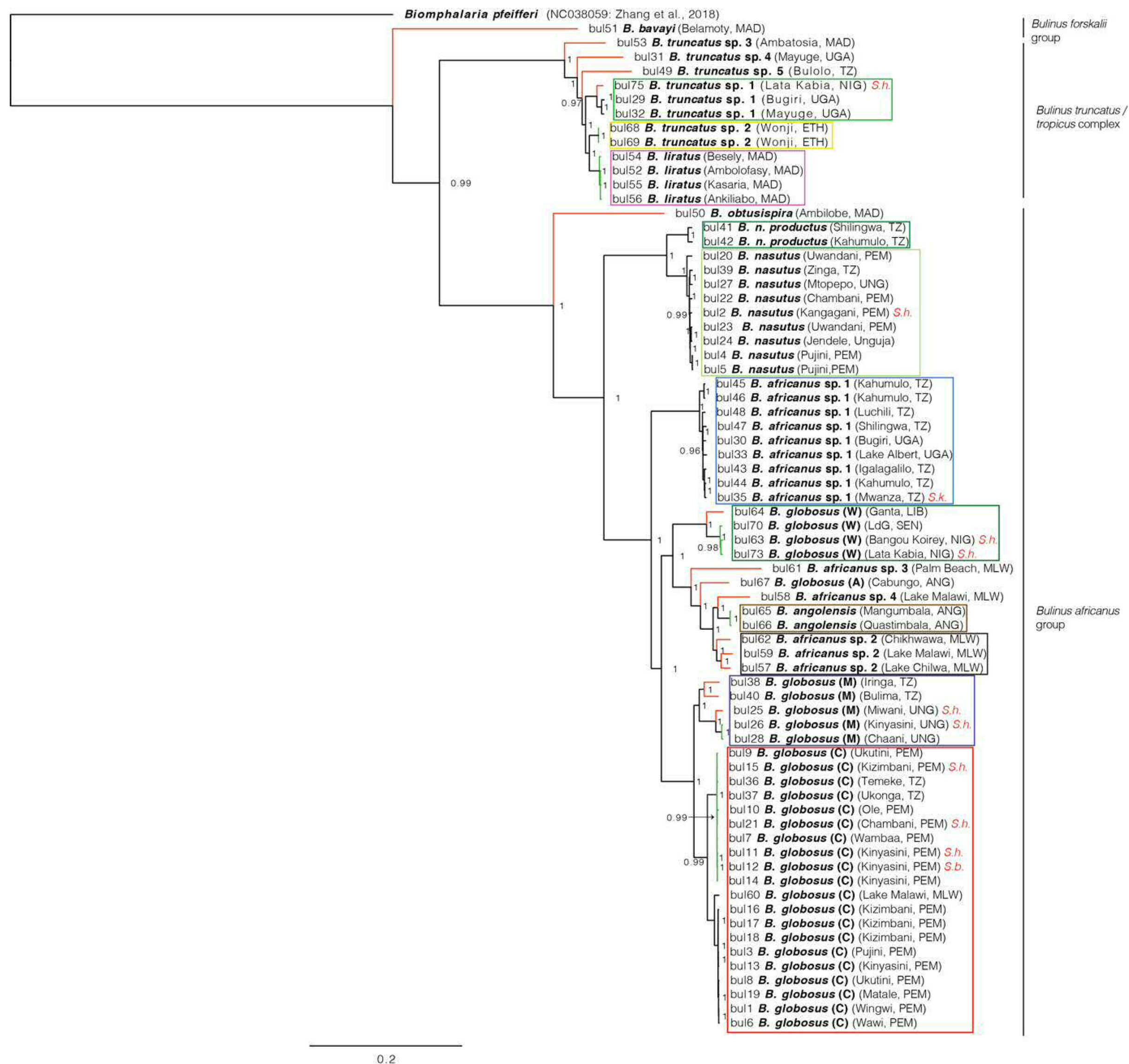


Figure 7.2 Bayesian analysis of the concatenated complete mitogenome (13 protein coding genes and the small and large subunits of rRNA) and nuclear rDNA 18S + 28S dataset of *Bulinus* species constructed using MrBayes version 3.2.2 under the GTR+I+Γ model; 5,000,000 generations; 3,500,000 generations burn-in. Posterior probabilities are given on each branch. All nodes with <0.95% posterior probability have been collapsed. The branch length scale bar indicated the number of substitutions per site. Following Taxa id (bul*n*), species name / molecular operation taxonomic unit (MOTU) determined by tree position are given in bold and coloured boxes denote each species/MOTU. Collection locality is given in the brackets as well as the country: ANG, Angola; ETH, Ethiopia; LIB, Liberia; MAD, Madagascar; MLW, Malawi; NIG, Niger; PEM, Pemba Island, SEN, Senegal; TZ, Tanzania; UGA, Uganda; UNG, Unguja. If present, *Schistosoma* species infections for each taxon are displayed in red: *S.h.*, *S. haematobium*; *S.b.*, *S. bovis*; *S.k.*, *S. kisumuensis*. Branches coloured orange (single taxa) and nodes coloured green (multiple taxa), represent taxa/taxon delimited as unique species through Bayesian Poisson Tree Processes posterior probabilities being >0.91.

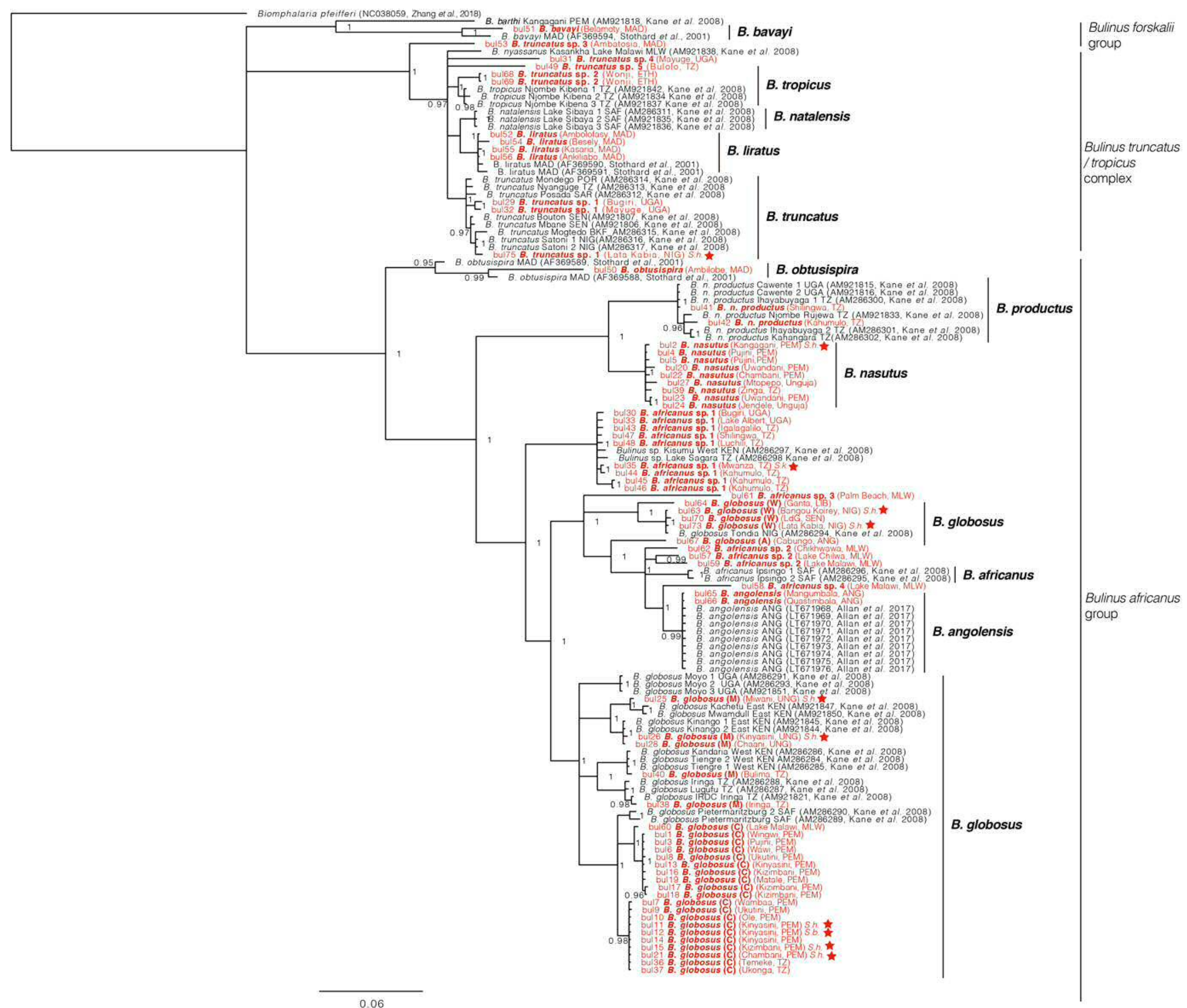


Figure 7.3. Bayesian analysis of complete and partial *cox*1 dataset of *Bulinus* species constructed using MrBayes version 3.2.2 under the GTR+I+Γ model; 5,000,000 generations; 3,500,000 generations burn-in. Posterior probabilities are given on each branch. All nodes with <0.95% posterior probability have been collapsed. The branch length scale bar indicated the number of substitutions per site. Sequences produced in the current study are displayed in red, and reference sequences obtained (Stothard *et al.*, 2001; Kane *et al.*, 2008; F. Allan *et al.*, 2017) in black. Following Taxa id (buln), species name / molecular operation taxonomic unit (MOTU) of samples sequenced in the current study determined by tree position in the full mitogenome dataset are given in bold. Collection locality is given for each taxa as well as the country of origin: ANG, Angola; BKF, Burkina Faso; ETH, Ethiopia; LIB, Liberia; MAD, Madagascar; MLW, Malawi; NIG, Niger; PEM, Pemba Island, POR, Portugal; SAF, South Africa; SAR, Sardinia; SEN, Senegal; TZ, Tanzania; UGA, Uganda; UNG, Unguja. Data generated during the current study are shown in red text. If present, *Schistosoma* species infections for each taxon are displayed and marked with a star: S.h., *S. haematobium*; S.b., *S. bovis*; S.k., *S. kisumuensis*. Where relevant, *Bulinus* species identifications for clades, by comparing with reference sequences are shown in bold.

The remainder and bulk of the tree was composed of representatives of the *B. africanus* group. Ancestral to the group was *B. obtusispira* from Madagascar (Figures 7.2 and 7.3). The other early diverging lineages in this group were the taxa representing *B. nasutus* and *B. nasutus productus* species (Figures 7.2 and 7.3), which clearly split into two clades as recognised previously (Brown, 1994; Kane *et al.*, 2008). However, neither *B. nasutus productus* (bPTP posterior probability = 0.583) nor *B. nasutus* (bPTP posterior probability =

0.542) were strongly supported as unique species in the current dataset. The clade representing the *B. africanus* sp. 1 was well supported in ML and BI topologies, however this was not deemed a highly supported species within the dataset (bPTP posterior probability = 0.557). These taxa also formed an unresolved clade with other unidentified *B. africanus* species in the *cox*1 analysis, and it therefore seems as previously concluded by Kane *et al*. (2008) that this species represents an undescribed mainland species of the *B. africanus* group. However, 28S sequences from these species (bul46) had highest identity matches to *B. ugandae* on GenBank (AF435660 (Morgan *et al.*, 2002)) whilst another (bul45) had highest match to *B. africanus* (AF435658 (Morgan *et al.*, 2002). Further inferences of which species these represent are therefore restricted from the data available.

Using the full mitogenome and rDNA combined data set, another set of taxa grouped as *B. africanus* sp. 2, which all originated from Malawi, formed a clade together (Figure 7.2), although this same clade was left unresolved in the *cox*1 tree topology (Figure 7.3). Two other taxa also from Malawi, labelled here as *B. africanus* sp. 3 and *B. africanus* sp. 4, were also very distinct from the other *B. africanus* species analysed (Figures 7.2 and 7.3). Each of these five taxa from Malawi were delimited as unique species within the dataset (bPTP posterior probability = 1 for all), and the closest similarity based on comparison with the available partial *cox*1 data was the *B. africanus* previously collected and sequenced from the type locality in Durban, South Africa (Kane *et al.*, 2008). In terms of tree topology, *B. africanus* sp. 4 was observed as a sister taxon for *B. angolensis*, although the *B. angolensis* analysed, one of which was collected from the type locality (bul65), was delimited as a unique species (bPTP posterior probability = 0.994).

The remaining snails in the tree were all named under the 'species umbrella' of *B. globosus*, and included taxa collected from several countries across sub-Saharan Africa (Table 7.1). *B. globosus* (A) (taxa ID: bul61) collected from the type locality of Bengo stream, Cabungo, Angola (Brown, 1994; F. Allan *et al.*, 2017), appeared divergent from the other *B. globosus*, and was also a well-supported unique species (bPTP posterior probability = 1). *B. globosus* from West Africa formed a separate clade (*B. globosus* (W)), although within this group there are also two well supported species, one being the individual snail collected from Liberia (bPTP posterior probability = 1), and the second comprising samples collected in Senegal and Niger (bPTP posterior probability = 0.987). The snail from Liberia was highly similar based on *cox*1 sequences to *B. globosus* previously sequenced from Burkina Faso (AM286293; Kane *et al.*, 2008). The *B. globosus* samples collected from East Africa, were also very diverse, however for convenience these were loosely clustered into two groups termed 'coastal' (C) and 'mainland' (M) species. The *B. globosus* (C) formed two well supported clades (Figure 7.2), representing the two *B. globosus* haplotypes that have been identified through *cox*1 barcoding on Pemba Island (and have an overlapping distribution, see

Supplementary Figure 7.4), but also contained snails from Tanzania (bul36, bul37) in one clade and Malawi (bul60) in another. The *B. globosus* (C) clade containing isolates from Tanzania and Pemba (Figure 7.2) had high support for being a single delimited separate species (bPTP posterior probability = 0.950). However, the second *B. globosus* (C) clade containing snails from Malawi and Pemba (Figure 7.2) did not have support for a delimited species (bPTP posterior probability = 0.663). Much more divergence was observed in the species grouped as *B. globosus* (M), which consisted of snails from Unguja Island and mainland Tanzania, showing a closer relationship of *Bulinus* on Unguja to those from mainland Tanzania than to those on Pemba (Figure 7.2). Four separate species were delimited from the five snails within this clade (bPTP posterior probabilities = 0.975; bul26 & bul28, 0.982; bul25, 1; bul38 and bul40 individually). As for the *B. globosus* on Pemba, there appeared to be two distinct *B. globosus* variants on Unguja, which for Unguja at least showed support for being two species, whereas only one was supported on Pemba Island (Figure 7.2).

When comparing amino acid alignment of four *Bulinus* species, a total of 22 nonsynonymous missense mutations between the species were detected (Table 7.4). When compared to the amino acid sequence of *B. globosus*, the number of amino acid variable positions of other species reflected the phylogenetic distance observed in *cox*1 phylogenies (Figure 7.3), with *B. bavayi*, *B. truncatus* and *B. nasutus*, having 18 (3.5%), 13 (2.6%) and 7 (1.4%) variable positions in 509 amino acid positions respectively.

Table 7.4. 22 variable *cox*1 amino acid positions of four *Bulinus* species from the complete 509 amino acid alignment of the *cox*1, aligned using MUSCLE (Edgar, 2004). Yellow highlights show amino acid replacements different to those of *B. globosus*. *B. globosus* = bul1 (Pemba), *B. nasutus* = bul4 (Pemba), *B. bavayi* = bul51 (Madagascar), *B. truncatus* sp. 1 = bul75 (Niger).

	250	334	394	399	429	443	451	464	472	476	477	478	480	481	482	483	484	485	486	491	501	506
B. globosus	V	F	V	C	S	F	F	V	M	T	V	V	P	E	F	S	S	M	T	L	L	S
B. nasutus	V	Y	A	S	S	F	F	I	M	T	V	V	P	E	V	S	S	L	T	L	S	S
B. bavayi	V	Y	V	S	S	F	Y	I	V	S	S	L	S	L	-	-	N	V	S	M	S	A
B. truncatus	I	Y	V	S	A	Y	Y	V	M	T	S	L	P	V	T	S	S	L	T	V	S	S

7.4 Discussion

The *Bulinus* genus is currently described as consisting of ~37 species, split into four species groups, distributed over various freshwater habitats mainly in sub-Saharan Africa. Here, relationships between taxa were investigated by using complete mitochondrial genome and ribosomal operon sequence data for the first time. The resulting phylogenetic relationships, genetic diversity and species delimitations provide evidence that the number of species in this genus may be much higher. This is particularly true for the cryptic species identified under the 'umbrella' species name *B. globosus*. We also provide evidence for a previously undescribed species of *B. truncatus/tropicus* species group that shows high support for being ancestral to the *B. africanus* group.

Figure 7.4. *Bulinus* from sub-Saharan Africa, (1) *B. bavayi*, bul51, Belamoty, Madagascar, (2-9) *B. truncatus/tropicus* complex: (2) *B. truncatus* sp. 1, bul75, Lata Kabia, Niger, (3) *B. truncatus* sp. 1, bul29, Bugiri, Uganda, (4) *B. truncatus* sp. 1, bul32, Mayuge, Uganda, (5) *B. truncatus* sp. 3, bul53, Ambatosia, Madagascar, (6) *B. truncatus* sp. 4, bul31, Mayuge, Uganda, (7) *B. truncatus* sp. 5, bul49, Bulolo, Tanzania, (8) *B. liratus*, bul52, Ambolofasy, Madagascar, (9) *B. liratus*, bul56, Ankiliabo, Madagascar. (10-30) *B. africanus* species group: (10) *B. obtusispira*, bul50, Ambilobe, Madagascar, (11) *B. productus*, bul41, Shilingwa, Tanzania, (12) *B. productus*, bul42, Kahumulo, Tanzania, (13) *B. nasutus*, bul2, Kangagani, Pemba, (14) *B. nasutus*, bul5, Pujini, Pemba, (15) *B. nasutus*, bul27, Mtopepo, Unguja, (16*) B. nasutus*, bul39, Zinga, Tanania, (17) *B. africanus* sp. 1, bul30, Bugiri, Uganda, (18) *B. africanus* sp. 1, bul35, Mwanza, Tanzania, (19) *B. africanus* sp. 1, bul43, Igalagalilo, Tanzania, (20) *B. africanus* sp. 2, bul57, Lake Chilwa, Malawi, (21) *B. africanus* sp. 2, bul62, Chikhwawa, Malawi, (22) *B. africanus* sp. 3, bul61, Palm Beach, Malawi, (23) *B. globosus* C, bul16, Kizimbani, Pemba, (24) *B. globosus* C, bul10, Ole, Pemba, (25) *B. globosus* C, bul37, Ukonga, Tanzania, (26) *B. globosus* M, bul25, Miwani, Unguja, (27) *B. globosus* M, bul26, Kinyasini, Unguja, (28*) B. globosus* M, Chaani, Unguja, (29) *B. globosus* W, bul63, Bangou Koirey, Niger, (30*) B. globosus* W, Ganta, Liberia.

7.4.1 Basal topology of Bulinus *species groups*

Although the position of *B. reticulatus* group species as sister taxa to *B. truncatus/tropicus* complex species has been well supported from previous studies (Kane *et al.*, 2008; Jørgensen *et al.*, 2011, 2013), the relationship of this group to the *B. africanus* group and *B. forskalii* group remains unresolved due to phylogenetic studies showing three different sister taxa relationships of either; *B. truncatus/tropicus* and *B. forskalii* (Stothard *et al.*, 2001; Morgan *et al.*, 2002), *B. africanus* group species and *B. forskalii* group species (Kane *et al.*, 2008; Nalugwa, Joslash, *et al.*, 2010; Jørgensen *et al.*, 2013; Tumwebaze *et al.*, 2019; Clewing *et al.*, 2020) or *B. truncatus/tropicus* and *B. africanus* species (Stothard *et al.*, 1996; Jørgensen *et al.*, 2007, 2011, 2013; Kane *et al.*, 2008; Zein-Eddine *et al.*, 2014) (reviewed in Chapter 2). Although the current dataset lacks multiple representatives from the *B. forskalii* group, the tree topology shows support for the latter, that *B. africanus* and *B. truncatus* are sister taxa, with *B. forskalii* group species (namely *B. bavayi* in this instance) being an earlier diverging group. The amino acid alignment and variability between representatives of each species group also supported this. It would however be of interest to include data available for other *B. forskalii* group species (Briscoe *et al*. unpublished data) in future phylogenies to improve support for this, particularly since this basal topology was collapsed in the RAxML tree.

7.4.2 B. africanus *group species: the who, where and what of* B. globosus

The *B. africanus* group is well established as genetically diverse (Kane *et al.*, 2008), especially in East Africa where it is believed this taxon radiated from (Mandahl-Barth, 1960; Wright, 1961). As predicted (Jørgensen *et al.*, 2011), the ancestral species to this group was *B. obtusispira*, one of the known intermediate hosts for *S. haematobium* endemic to Madagascar (Stothard *et al.*, 2001). It is likely therefore that the *Bulinus* on Madagascar represent ‘relic’ species (Wright, 1971; Stothard *et al.*, 2001; Jørgensen *et al.*, 2011), which may have been present on the Madagascan island since the separation from East Africa ~158 Ma, and again from India ~84 Ma (Briggs, 2003). The clade, including *B. nasutus* and *B. productus,* also represents an early divergence from the other *B. africanus* group taxa included here. Compared to other species with similar distribution ranges (i.e. *B. globosus*, see below), *B. nasutus* showed an extremely low level of diversity across the taxa sampled, with those across the Zanzibar Archipelago and coastal Tanzania being highly similar, despite potentially several millennia separating these populations since the split of Zanzibar ~5 - 2 Ma (Kent *et al.*, 1971). Given the slowly evolving *B. nasutus* taxa and the clear divergence in tree topology from *B. productus*, further support is provided for the distinction of *B. productus* and *B. nasutus* as separate species as has been suggested previously (Jelnes *et al.*, 2003; Kane *et al.*, 2008), rather than just sub-species as they are currently considered (Brown, 1994). However, this was not supported by the bPTP species delimitation analysis conducted in this dataset, and

therefore other species delimitations, such as generalised mixed Yule-coalescent (GMYC) or a Bayesian multispecies coalescent approach, could be tested as an alternative. However, bPTP has demonstrated the tendency to overestimate rather than underestimate species annotations (Luo *et al.*, 2018), so the use of other more conservative measures may yield a similar outcome for not splitting *B. nasutus* and *B. productus*.

There were several *B. africanus* group specimens that could not be delimited to the species level either through morphology, or with alignment to reference data, and therefore likely represent several unidentified species of the *B. africanus* group species on mainland Africa. The largest and most widely distributed clade were those represented as *B. africanus* spp. 1, with isolates from Tanzania, Uganda, and Kenya in *cox*1 analysis all displaying a close relationship. These specimens could potentially be either the previously described *B. ugandae* (reliable reference *cox*1 sequence data with accurate species description not available for this taxon currently, and this was not concluded as the species identification to describe genetically similar snails by Kane *et al.,* 2008), or more likely a new *B. africanus* group species across parts of East Africa. This group of snails was of particular interest since one snail collected in the marsh habitats bordering Lake Victoria in the Mwanza region of Tanzania had been incriminated with the transmission of *S. kisumuensis*. This is the first report identifying the intermediate host species infected and also the first report of this rodent parasite being present outside of Kenya where only the adult stage schistosome have been described from three murid rodent species (Hanelt *et al.*, 2009). It was also observed that between three to five unidentified *B. africanus* spp. were identified from the specimens collected in Malawi, from both Lake Malawi and Lake Chilwa (*B. africanus* sp. 2, 3 and 4). This hub of diversity in Lake Malawi for *Bulinus* spp. can be expected, since the same patterns of evolutionary radiation and explosive speciation have been observed for the other endemic fauna from this meromictic lake, that in terms of age (~ 5 Ma) and time since last severe drought (~100 ka) is only surpassed by Lake Tanganyika in this Albertine Rift system (Salzburger *et al.*, 2014). None of these unique species from Lake Malawi grouped well with reference data of *B. africanus* species when comparing *cox*1 sequences. Although none of the snails examined from Lake Malawi here were infected with schistosomes, the diversity of these taxa may potentially aid the transmission of schistosomes in this region, particularly recently identified hybrid schistosomes that are known to have an expanded intermediate host range (Webster *et al.*, 2019; Stothard, Kayuni, *et al.*, 2020). Much more attention should be paid to the *B. africanus* species through the Albertine Rift, as the majority of interest has previously been on the *B. truncatus/tropicus* complex species from this region of central east Africa (Nalugwa, Joslash, *et al.*, 2010; Nalugwa, Kristensen, *et al.*, 2010; Tumwebaze *et al.*, 2019; Clewing *et al.*, 2020), despite these species not being as important in terms of transmission.

The species *B. globosus* is currently incriminated with causing the majority of urogenital schistosomiasis transmission across Africa (Rollinson, 2009). Even in the *B. globosus* samples processed in the current study, seven were infected with *S. haematobium* from both East and West Africa. The single specimen of *B. globosus* sequenced here from the type locality in Angola was considered as the 'true' *B. globosus*, since this specimen was identified in comparison to reference material from Wright's (1961) descriptions of *B. globosus* from the same type locality available from the NHM collections (F. Allan, personal communication). Therefore, this species was used as a reference point for the other species termed *B. globosus* in this study, which were initially assumed to be closely related. However, none of the other identified *B. globosus* sequenced here were closely related to this type locality specimen. In fact, this 'true' *B. globosus* was more closely related to taxa, considered as different *Bulinus* species, collected from areas geographically close by, such as the *B. angolensis* from Angola and a currently unrecognised species (*B. africanus* sp. 2) from Malawi. Complicating matters further, was that clearly divergent clades of *B. globosus* collected from East and West Africa were present, and clearly not the same species with multiple of these also being inferred as unique species within each clade. The existence of different forms of *B. globosus* in West Africa has long been postulated based on size and compatibility with *S. haematobium*, and the identification of a large specimen delimited as a unique species from Liberia within this *B. globosus* (W) clade suggests that these taxa may represent different West African species (Hira, 1968; Fryer *et al.*, 1987). One of these *B. globosus* forms may even represent the elusive *B. jousseaumei* (designated as a synonym of *B. globosus* by Brown 1994) or *B. umbilicatus* species, the former being contested as either a geographical variant of *B. globosus* based on internal morphology and enzyme analysis (Wright, 1957; Jelnes, 1986) or a true species from shell morphometry and microsculpture (Mandahl-Barth, 1957; Kristensen and Christensen, 1989). The latter (*B. umbilicatus*), is supported from both morphology and enzyme analysis (Jelnes, 1986; Brown, 1994). However, no reference sequence data is currently available for snails identified as *B. jousseaumei*, and available phylogenies including *B. umbilicatus* do not support a close relationship with snails previously identified as *B. globosus* from West Africa (Stothard *et al.*, 2001). Although our specimens are dissimilar to those described in Mandahl-Barth (1957) and Brown (1994), we cannot establish definitively from the current dataset that species reported as *B. globosus* are *B. jousseaumei*, or new species previously undescribed.

B. globosus from East Africa were additionally split into two other well supported recently diverging clades, one representing taxa of mainland East Africa and Unguja Island, and then those from Pemba Island (and coastal Tanzania and Malawi), likely reflecting the earlier separation of Pemba from East Africa (~ 6) Ma compared to Unguja Island (maybe as little as 10-18 thousand years), where *Bulinus* population admixing would have continued for

longer. This is in contrast to evidence from terrestrial molluscs on Pemba, where the different isolation times of each island have not been reflected in the snail fauna (Rowson *et al.*, 2010). The presence of snails collected from mainland Africa (Tanzania and Malawi) in this otherwise strictly Pemba clade of *B. globosus* is most unusual but may represent a translocation of snails from Pemba to mainland Africa in the recent evolutionary history of this taxa. In addition, the snails representing the two well defined clades of *B. globosus* from Pemba Island (i.e. the sister groups of *B. globosus* (C), one being delimited as a group of unique species through bPTP analysis) represent the two *cox*1 haplotypes of *Bulinus* readily found across Pemba Island (Pennance *et al.*, 2018), and these *cox*1 haplotypes overlap significantly in their distribution (Supplementary Figure 7.4 and see Chapter 4). Other studies collecting *B. globosus* from sites within a close proximity in Zimbabwe, showed low genetic variation between taxa even with predicted limited gene flow between sites, and the formation of one population (Mutsaka-Makuvaza *et al.*, 2020). Therefore, it could be hypothesised that the two populations on Pemba represent two fixed haplotypes that have independent origins on Pemba Island, but since have spread to colonise similar (in some cases the same) freshwater bodies and both play a role in *S. haematobium* transmission on the island (see Chapter 4). Whether these two closely related forms/species can out-cross when they occur in sympatry needs to be determined experimentally, as judging from the currently available data it is unclear what barriers, if any, sperate these.

7.4.3 B. truncatus/tropicus *complex species*

One of the more interesting findings within the *B. truncatus/tropicus* group was the presence of a currently undescribed, but ancestral species, of *B. truncatus/tropicus* complex species collected from the north of Madagascar (*B. truncatus* sp. 3, Ambatosia, Madagascar). The specimen was identified from its quite different shell morphology (large aperture, non-exserting spire) to other endemic *Bulinus* species. Interestingly, Wright postulated the possibility of cryptic species currently described as *B. liratus* from Madagascar, but concluded it was unlikely from his experience of dealing with the extreme morphological variants of the African mainland (Wright, 1971). This species may represent this variant, and is therefore in addition to the other endemic *B. truncatus/tropicus* species, *B. liratus,* present on Madagascar, which we also identified here based on the similar shell characteristics to those described by Brown (1994), and genetic similarity to the partial *cox*1 sequences produced by Stothard *et al.* (2001). The relatively recent divergence of *B. liratus* from the *B. truncatus* sp. 2 also agrees with the inference that this is a recently introduced species to Madagascar, however our tree topology refutes the inference that *B. obtusispira* colonised Madagascar before *B. bavayi* (Stothard *et al.*, 2001). As for *B. obtusispira* and *B. bavayi*, the presence of an ancestral *B. truncatus/tropicus* species on Madagascar suggests its presence here for quite some time,

and maybe since Madagascar's separation from East Africa (once Gondwana) some ~158 Ma (Briggs, 2003), denoting that ancestral forms of all three species groups would have been present in this region. We also show by comparison with reference *cox*1 sequences, that this new species, *B. truncatus* sp. 3, on Madagascar is likely ancestral to the group of *B. truncatus*/*tropicus* complex, rather than *B. nyassanus* as has been previously suggested (Kane *et al.*, 2008; Jørgensen *et al.*, 2011).

The remaining taxa in the *B. truncatus/tropicus* group here consisted of two snails from Ethiopia (*B. truncatus* sp. 2), likely variants of *B. tropicus*, two other unidentified *B. truncatus/tropicus* species collected from Lake Victoria on the Ugandan (*B. truncatus* sp. 4) and Tanzanian (*B. truncatus* sp. 5) shores and snails identified as *B. truncatus* from Uganda and Niger. *B. truncatus* is a host of *S. haematobium* in West Africa, and its apparent relatively recent divergence from snails in East Africa was somewhat unexpected (see below).

7.4.4 Phylogeography: geographical isolation as a diagnostic between Bulinus *species?*

Overall, the geographical location provided a certain degree of diagnostic power for estimating similar species, for example clades usually represented species from East or West Africa. However, this was confounded by two points of reference. The first being the close relationship between *B. truncatus* sp. 1 that were collected in Niger and Uganda (see above). This was the only example of *Bulinus* species being highly similar sister taxa based on their position in the tree between the East and West of the continent (however, the *B. truncatus* from Niger was supported as a unique species in bPTP). A second unusual finding was the grouping of a specimen from Malawi, being a close sister taxon, to a highly supported clade of *B. globosus* from Pemba Island. This suggests that an ancestral taxon must have been translocated between these sites at some point in recent evolutionary history. We also detect connectivity between great lake sites, with taxa identified here as *B. africanus* sp. 1 being observed in both Lake Albert and Lake Victoria. Lake Victoria is a relatively young lake (forming ~0.4 Ma) compared to the other great lakes in the Albertine Rift valley (e.g. Lake Malawi formed 5 Ma), and has gone through at least three major desiccations in the past 100,000 years, the most recent being 18,000-14,000 years ago (Johnson *et al.*, 2000; Stager and Johnson, 2008). Therefore, it is likely that *Bulinus* spp. were introduced into Lake Victoria recently from other waterbodies such as Lake Malawi. Unique and diverse *Bulinus* species are not expected in Lake Victoria since the time for evolutionary radiation here has been considerably shorter (Salzburger *et al.*, 2014). In contrast, and as aforementioned, the genetic diversity in the older Lake Malawi was extremely high with potentially multiple species being present within relatively close proximity, making inferences on species here much more difficult. All in all, the micro-evolutionary races often on small geographical ranges make interpreting *Bulinus*

species based on geography very difficult, and species are likely to be present in focalised regions rather than having a pan-Africa distribution as is often believed.

7.4.5 Features of the Bulinus *mitochondrial genomes and ribosomal operon*

The mitochondrial genome was comparable to that of *Biomphalaria* spp. in terms of size and gene order (DeJong *et al.*, 2004; Zhang *et al.*, 2018), except for two taxa which showed unique gene order rearrangements and a duplicated gene copy for which more investigation is necessary. Gene rearrangements have been observed in some *Biomphalaria* spp. (*Briscoe et al.,* unpublished) and since gene duplicates can arise through several mechanisms (Zhang, 2003) and large regions of non-coding sequence have been observed in other gastropods (Williams *et al.*, 2017), it is of no surprise that some of these features were identified in *Bulinus*. Another interesting similarity with the *Biomphalaria* mitogenomes was that overlapping protein coding genes are potentially present in the *Bulinus* mitochondrial genome, with at least five genes (*nad*4l, *cob, cox*2, *cox*3, *nad*3) having a significant degree of overlap with other genes if they were not truncated at what were interpreted as the conserved truncated stop codon sites. This potentially suggests that polycistronic transcription of protein coding genes occurs in *Bulinus*, as has been discussed for other gastropods (Fourdrilis *et al.*, 2018). Unfortunately, this was not eluded to by Zhang *et al*. (2018) when discussing the presence of overlapping protein coding genes in *Biomphalaria*. Transcriptomic data is certainly needed both to confirm the gene boundaries interpreted for *Bulinus* here, and also investigate the existence of these polycistronic transcriptions.

The 18S rDNA region (and 5.8S although not reported previously) is well conserved between *Bulinus* taxa with very few SNP sites compared to the more variable 28S. As discussed in previous studies, these genes may be beneficial for exploring the basal relationships between *Bulinus* taxa (Morgan *et al.*, 2002; Jørgensen *et al.*, 2011). Both the ITS regions between these genes however were extremely variable and, in some taxa, ambiguous bases could not be resolved. These ambiguities might be a result of variance within the nuclear rDNA repeat region of *Bulinus* that persist in some taxa through concerted evolution (Ganley and Kobayashi, 2007). For convenience, the ITS regions were excluded for phylogenetic analysis in this study, as the presence of ambiguous bases may have complicated the data analysis, however it would be of interest to explore these regions further and compare phylogenies with other available reference data sets (Jones *et al.*, 2001; Jørgensen *et al.*, 2013).

Although the high genetic diversity within *B. globosus* has been identified before (Stothard and Rollinson, 1997a; Kane *et al.*, 2008; F. Allan *et al.*, 2017; Mutsaka-Makuvaza *et al.*, 2020), the generation and analysis of a much larger sequence data matrix here has provided the statistical power to confidently delimit molecularly divergent taxa. This can be

concluded both from the BI tree topology and also the bPTP species delimitation, however stricter phylogenetic techniques for species delimitation should also be performed since the bPTP performed here could be interpreted as rather 'relaxed' when comparing tree topology with species inferences. This tendency for overestimation has also been demonstrated in simulated and case study datasets where both bPTP and GMYC species delimitation methods delimit higher MOTUs in comparison to Bayesian multispecies coalescent approach, designed specifically for multilocus data (Luo *et al.*, 2018). Differences in methods may yield different results however, since approaches using Poisson tree processes, such as bPTP, follow the assumption that the number of substitutions between species is significantly higher than the number of substitutions within, which is comparable in certain aspects to the GMYC approach that identifies significant changes in the pace of branching events, however, different in that GMYC performs this according to time rather than the number of substitutions (Zhang *et al.*, 2013). Further comparisons of methods for molecular species delimitation for use with large multilocus datasets, and potentially the development of new methods specifically for use with mitochondrial genomes in combination with non-mitochondrial DNA markers, will aid in resolving complex phylogenies such as those observed in *Bulinus*, where traditional taxonomic and species concepts do not apply*.* Taking into consideration other biological and ecological variables associated with each specimen will also improve interpretation, especially in considering when speciation is 'complete' to avoid over or under taxonomic inflation (Stanton *et al.*, 2019). With the current evidence, it seems absolutely necessary to split the *B. globosus* species into at least East and West African species as previously discussed (Kane *et al.*, 2008), but potentially with further sub-divisions (i.e. multiple species or sub-species) in each region. Changing the nomenclature of *B. globosus*, and other *B. africanus* group species, should be given serious consideration, and further investigation into how these multiple species may play different roles in *S. haematobium* group species transmission due to how species perform as intermediate hosts and impact host/parasite fecundity across sub-Saharan Africa should be conducted (Rollinson, 2009). The questions surrounding what species concepts to use for *Bulinus*, as discussed by Wright (1961) and Brown (1994), still remain. Although the extensive genetic data provides unequivocal evidence for population genetic divergence, fundamental ecological and biological factors for these snail species need to be established to truly determine an appropriate species concept (Mayden, 1997).

Chapter 8. Future outlooks and research on *Bulinus* spp. snails and schistosomiasis in Sub-Saharan Africa

The contributions of technical knowledge, motivation, drugs and funding to treat and prevent Neglected Tropical Diseases (NTDs) are continually increasing. Great progress has been made in alleviating the suffering of more than a billion people impacted by NTDs thanks to the coordination and international collaboration of organisations and governments. Although the goal posts for NTDs such as schistosomiasis are moving due to unforeseen setbacks and difficulties in controlling such a parasitic disease (WHO, 2012, 2020a), it is clear that novel methods in surveillance and control will significantly improve the chances of eventually meeting public health and elimination goals.

This thesis has primarily focussed on the snail-schistosome associations present in sub-Saharan Africa, demonstrating again that by investigating these we can better understand parasite transmission, disease endemicity and improve surveillance of schistosomiasis. However, other areas of schistosomiasis research, such as improvements in human diagnostics and other environmental surveillance techniques, will also be vital in curtailing the transmission of schistosomiasis. New hurdles for schistosomiasis control are also appearing, the most obvious whilst writing this thesis during the global COVID-19 pandemic, being the direct and indirect effects that other public health and governmental measures might impose on schistosomiasis control and elimination. In this final chapter, some of these priorities for future research and questions arising from current control measures are briefly discussed.

8.1 Environmental surveillance for intermediate snails hosts and schistosomes

Throughout this thesis, schistosomiasis transmission and infection risk has been inferred by searching for and then collecting snails from freshwater bodies, followed by appropriate DNA extraction and species identification using sequencing platforms (Chapters 3, 4 and 6) or rapid diagnostic assays (Chapter 5). To further establish whether schistosome contamination has taken place at these freshwater bodies (signifying infected definitive hosts) additional steps of parasite shedding, or targeting schistosome DNA in non-patent infections (Abbasi *et al.*, 2010; Amarir *et al.*, 2014; Schols *et al.*, 2019), as also performed in Chapter 6, are necessary. However, biomonitoring methods harnessing environmental DNA (eDNA) could potentially mitigate these processes (Wilcox *et al.*, 2018). Such methods are less time consuming and destructive since the target organisms do not have to be directly captured and removed from the environment. The target eDNA can be either extracellular or encapsulated in shed cells. For the free-living larval stages of schistosomes, this could technically include free floating DNA, cell bound or even whole larvae (which are often referred to as 'eDNA' in the

schistosome literature), whereas for snails this could include sloughed cells, mucus, or waste products.

The detection of whole schistosome larvae from filtered water samples (i.e. without capturing the snail host) has been demonstrated in several studies (Hamburger, Xu, *et al.*, 1998; Hertel *et al.*, 2004; Hung and Remais, 2008; Worrell *et al.*, 2011), although this comes with the obvious flaw that the larval parasite has to be captured, therefore complicating sampling. Although there are several studies reporting the detection of schistosome eDNA utilising targeted real-time/qPCR (Sato *et al.*, 2018; Fornillos *et al.*, 2019; Sengupta *et al.*, 2019; Alzaylaee, Collins, Rinaldi, *et al.*, 2020; Alzaylaee, Collins, Shechonge, *et al.*, 2020) and droplet digital PCR methods (Mulero, Boissier, *et al.*, 2020), none of these studies have demonstrated that cercariae shed detectable eDNA rather than just larval parasites being detected. These methods, however, have not been extensively tested on environmental water samples collected directly from freshwaters, and have only been tested for *S. mansoni* (see Sato *et al.* 2018; Sengupta *et al.* 2019) and *S. japonicum* (see Fornillos *et al.,* 2019). Therefore, most research currently has involved having collected snails in tanks and then sampling water from these (i.e. concentrating schistosome density; Alzaylaee *et al.* 2020b). Better evidence has been provided for the presence of eDNA in the environment from intermediate host snails, demonstrated by conducting a metabarcoding approach on water samples on the Corsican islands detecting *B. truncatus* (see Mulero, Toulza, *et al.*, 2020).

A key difference between the eDNA approach and snail xenomonitoring approach tested here (Chapter 6), is that the eDNA includes the detection of schistosomes that have not located an intermediate host, or the eDNA left behind (if this occurs, see above) by the presence of these larval stages. How long this eDNA can be detected in the environment following egg/miracidia/cercaria presence is unknown, although it is established from other studies that freely floating DNA can degrade within hours or days in aquatic environments (Barnes *et al.*, 2014; Strickler *et al.*, 2015). Further questions arise such as, will eDNA methods also detect non-patent schistosome infections in snails? From the study conducted here (Chapter 6), it has been demonstrated that these non-patent infections form the bulk of snail infections, which would potentially underestimate disease risk using an eDNA approach (although equally, snail xenomonitoring also misses environmental schistosome/DNA stages). A priority for future research is comparing these surveillance approaches by conducting a multi-faceted study using snail targeted molecular xenomonitoring and environmental DNA monitoring side by side (i.e. sampling from the same site at the same time), to establish the most sensitive diagnostic practice.

8.2 Diagnostics for human infection

As demonstrated during this thesis (Chapter 6), the prevalence of schistosome infections in snails is drastically underestimated following traditional parasitological methods of shedding for patent infections. Likewise, the standard diagnosis for active schistosomiasis by observing viable eggs in the urine or faeces lacks sensitivity, and therefore infection cannot be ruled out if urine samples are deemed egg-negative. This is particularly true in areas of low endemicity, such as the Zanzibar Archipelago, where the number of eggs excreted may be very low (e.g. <5 eggs / 10 ml urine) (Utzinger *et al.*, 2015; Knopp *et al.*, 2018), but individuals excreting low numbers of eggs are still able to maintain disease transmission, or even re-introduce transmission where previously eliminated. Developed molecular diagnostic methods for schistosomiasis include immunoassays for the detection of anti-helminth antibodies (Elhag *et al.*, 2011; Sheele *et al.*, 2013) and helminth derived antigens (Stothard *et al.*, 2006; Knopp *et al.*, 2015; Peralta and Cavalcanti, 2018), although these still present issues with sensitivity (Stothard *et al.*, 2009; Tchuem Tchuenté *et al.*, 2012; Knopp *et al.*, 2015), especially those designed for point of care use where robust laboratory infrastructure is not available.

Attention has also moved towards diagnostics capable of detecting cell-free DNA (cfDNA) of schistosomes, which can be identified through several types of nucleic acid amplification tests and have all reported higher sensitivity than egg microscopy (as reviewed in Archer *et al.,* 2019; Minetti *et al.,* 2016; Weerakoon *et al.,* 2018). This includes tests developed specifically for point of care use that require minimal reagents, are easy to interpret and equipment is transportable (Rosser *et al.*, 2015; Rostron *et al.*, 2019). Following further refinement, these tests offer future scope for use in control and elimination programmes moving towards a test and treat strategy, where a ‘one size fits all’ approach of MDA is not warranted. Combined with snail xenomonitoring, incorporating these field based molecular DNA based tests into schistosomiasis control programmes will certainly be crucial in the future surveillance of elimination settings where every infection counts, and any missed infections may lead to rapid resurgence.

8.3 Compatibility and resistance

As discussed across this thesis, the role of snail immune factors and/or the schistosome infection capacity that dictates compatible schistosome infections has puzzled researchers for decades. Compatibility and resistance have been investigated here by considering species identifications of snail and schistosomes (see Chapters 3, 4 and 7), with the potential that genetic distance between taxa can infer schistosome susceptibility (i.e. different snail species often do not transmit the same schistosome species in the same geographical region).

More recently, advances in genomic tools and technologies have revealed genetic mechanisms (gene expression levels) that determine resistance of *S. mansoni* infection in the

typical laboratory model *Biomphalaria glabrata* (Hanington *et al.*, 2010, 2012; Ittiprasert and Knight, 2012; Tennessen, Bonner, *et al.*, 2015; Tennessen, Théron, *et al.*, 2015; Pila, Gordy, *et al.*, 2016; Pila, Tarrabain, *et al.*, 2016). *Biom. glabrata* is only responsible for *S. mansoni* transmission in South America, and the presence of the four loci identified in *B. glabrata* at which allelic variation influences resistance (FREP3, BgTLR, BgGRN, Hsp90) have not yet been assessed in any *Biomphalaria* species of sub-Saharan Africa, even though these are responsible for the majority of global *S. mansoni* transmission (for example: *B. sudanica, B. pfeifferi* and *B. choanomphala*) (WHO, 2020c). Due to the absence of a well-established lab model of *S. haematobium* and *Bulinus*, searching for similar resistant factors in *Bulinus* species is not even in its infancy, with currently no available transcriptomic or genomic data available for any *Bulinus* species to get this off the ground. Conducting genome wide association studies (GWAS) on these African snail vectors is a priority and resulting genomes can be interrogated for regions associated with resistance. By combining schistosome resistance with phylogenetic studies, it will also be possible to understand the evolution of these resistance genes across snail taxa, which may point towards how so many micro-geographical differences in snail-schistosome compatibility have occurred.

Combined with the body of knowledge around the developing CRISPR/Cas and drive mechanisms, there is speculation that identifying sites of resistance in snails could lead to the development of novel schistosomiasis control (Famakinde, 2018, 2020; Maier *et al.*, 2019). Altering target snail genes to infer resistance / reduced susceptibility as a heritable characteristic could lead to developing strategies for the interruption of transmission of schistosomiasis to humans by releasing resistant snail hosts into the environment. This technique offers an exciting opportunity to move away from the predominant form of snail intervention, chemical mollusciciding, which is indiscriminately toxic and requires repeated applications (Secor, 2014; King and Bertsch, 2015). However, how successful gene drive will be in the case of highly selfing intermediate host snail species (e.g. *Bulinus truncatus*) will also be a priority for investigation if it comes to implementation.

8.4 Intramolluscan schistosome diversity

In Chapter 3 of this thesis, the intramolluscan diversity of schistosomes was investigated using population genetic markers (microsatellites) on shed cercariae to establish multiple infections (i.e. by identifying clonal cercariae). A key factor to establish first is whether new mutations occur during intramolluscan sporocystogenesis for all species of *Schistosoma*. If mutations do occur, this would potentially distort the inferences on multiple miracidial infections made in Chapter 3 using microsatellite markers to identify multiple genotypes. It would also impact previous studies that have examined shed cercariae and passaged adult worms to identify multiple schistosome infections using RAPD markers (Dabo *et al.*, 1997; Davies *et al.*, 1999;

Sire *et al.*, 1999; Semyenova *et al.*, 2005), polymorphic mtVNTR markers (Minchella *et al.*, 1995; Eppert *et al.*, 2002) or W chromosome repetitive DNA elements (Grevelding, 1999). Other molecular techniques such as identifying if there are any sex biases or mixed sex infections may help inferences on these multiple infections (Kincaid-Smith *et al.*, 2016). The proportion of snails naturally harbouring multiple genotypes according to this past literature varies from ~11% of *Biomphalaria glabrata* in Guadeloupe (Sire *et al.*, 1999) to 67% of *B. globosus* collected from Zimbabwe (Davies *et al.*, 1999). The study in Chapter 3 showed similarly high proportions of multiple genotypes per snail; 40% of *Bulinus* spp. harbouring multiple schistosome populations.

It was interesting to note the schistosome species differences in multiple infections from the data in Chapter 3, since snails inferred with multiple infections >3 were all infected with *S. bovis* whereas snails infected with *S. haematobium* / hybrids had ≤3. This disparity could be a result of variation in the intramolluscan development of *S. haematobium* and *S. bovis*, in that sporocystogenesis in *S. haematobium* occurs in either one mechanism; after cessation of cercariogenesis, or in a second mechanism; by direct sporocystogenesis (Kechemir & Théron 1980; Jourdane 1983; Touassem & Théron 1986), whereas in *S. bovis* this can occur in a third mechanism where sporocystogenesis occurs simultaneously with cercariogenesis (Touassem and Théron, 1986). Therefore, *S. bovis* may be more likely to have multiple daughter sporocysts that are simultaneously shedding cercariae compared to *S. haematobium*. If significant levels of genetic mutation occurs during sporocystogenesis as suggested above, this higher degree of intramolluscan replication may also be an explanation for the higher degree of diversity observed in *S. bovis* compared to *S. haematobium* (see Webster *et al.,* 2012; Djuikwo-Teukeng *et al.,* 2019). Alternatively, this species difference could be indicative that transmission of *S. bovis* between intermediate and definitive hosts is more frequent than that of human schistosomes due to: larger numbers of parasites released in the faecal matter of infected ruminants; more frequent freshwater use of definitive hosts, or; lack of chemotherapeutic treatment for domesticated animals in the region (Ezeamama *et al.*, 2016).

To test these biological hypotheses of intramolluscan schistosome mutation, experimental infection studies are required to directly assess the *Schistosoma* cercarial diversity from snails experimentally challenged with one, two and or multiple miracidia. By analysing the resulting cercarial diversity of these fixed miracidia exposures using microsatellites in the same fashion as the methodology used in Chapter 3, it can be established whether; a lower (signifying failed sporocyst establishment), the same (signifying that cercarial MLGs relate directly to number of miracidia establishing infection) or higher (suggesting somatic mutation occurring during daughter sporocyst generation) number of cercariae multi-locus genotypes are observed during snail infections. Coupled with exposed

snail dissections to quantify number of mother and daughter sporocysts, this study could give strong support for any one of these hypotheses.

8.5 Climate change

It has been predicted that climate change will exacerbate the effect of NTDs in endemic regions of sub-Saharan Africa (Bryson *et al.*, 2020). The impact that climate change may have on the distribution of schistosomiasis, mainly linked to temperature, precipitation and adverse weather events, has been discussed for ~20 years (Martens *et al.*, 1995, 1997), but predictions are still confounded by significant knowledge gaps in how these will affect the most vulnerable areas in sub-Saharan Arica (Stensgaard *et al.*, 2019). Since climate change will have different impacts at different geographical locations, climate change could either result in more suitable or less favourable environmental conditions for schistosomiasis transmission. In the study presented here (Chapter 6), water temperature had a significant impact on transmission, with increasing temperatures reducing *Bulinus* abundance (less favourable conditions for snail survival) but increasing the *S. haematobium* infection likelihood (a more suitable environment for schistosome infections to occur). This study provides much needed empirical evidence for the affect that temperature change may have on *S. haematobium* transmission in addition to those previously conducted (Kalinda *et al.*, 2017a, b; Kalinda, Chimbari, Grant, *et al.*, 2018; Kalinda, Chimbari, Malatji, *et al.*, 2018).

As observed in a review by Stensgaard *et al*., (2019), studies investigating the effects of climate change on urogenital schistosomiasis transmission were far outweighed by those investigating both Asian and African intestinal schistosomiasis. This is despite the fact that *S. haematobium* is currently the species we are already seeing a shift in geographical range, with emerging transmission in Europe (Boissier *et al.*, 2015) that may be due to the shifts in temperature promoting overwintering survival of snails and schistosomes (Mulero *et al.*, 2019). It is evident that more empirical evidence on the effects temperature, precipitation, drought and flooding have on snail and schistosome biology in endemic settings are needed to improve mathematical models predicting future change (McCreesh and Booth, 2013).

8.6 Schistosomiasis control in the face of a pandemic: The impact of COVID-19

Following weeks of monitoring and tracking the spread of COVID-19, the WHO assessed that COVID-19 could be characterized as a pandemic in March 2020, almost a week before, the first case was announced in WHO's African Region (Algeria). Concerns began to rise on the impact a respiratory pathogen such as COVID-19 could have on the several public health interventions already taking place across Africa, and the potential co-morbidities of the disease with neglected tropical diseases that are not yet possible to establish. WHO issued a general recommendation on April 1st 2020 that due to the COVID-19 pandemic, public health

measures for neglected tropical including community based surveys, active case finding and mass treatment campaigns be postponed until further notice (see: https://www.who.int/neglected_diseases/news/COVID19-WHO-interim-guidance-implementation-NTD-programmes/en/ accessed 1/4/2020). However, critical interventions intended to support prompt diagnosis, treatment and care of neglected tropical diseases for patients presenting to health care facilities, and the implementation of essential vector control would continue wherever possible. The WHO also recognise that the pandemic has affected interventions in three main areas: implementation at country level, supply chain management of health products (including delivery of medicine and diagnostics) and financial support that will have considerable impact on the 2030 goals (WHO, 2020a).

It is clear that delays in MDA will increase infections, increase morbidity, and in turn increase the time and total rounds of MDA it will take to reach agreed public health targets. The NTD Modelling Consortium (https://www.ntdmodelling.org) presented a document, entitled: 'Impact of COVID-19 on NTD programmes progress', during an online WHO meeting (5/6/2020) covering 'Mitigation and recovery from COVID-19 - associated delays: a research agenda for NTD programs' (available: https://www.who.int/neglected_diseases/news/Impact-COVID-19-NTD-programmes.pdf?ua=1). The findings are only relevant to intestinal schistosomiasis (*S. mansoni*) but predicted that only minor impacts would be seen in achieving the 2030 goals in regions with a <10% *S. mansoni* prevalence, while a maximum of <2 years delay may be seen in high transmission regions with >50% prevalence. It was concluded that additional treatment rounds, and the inclusion of adult treatments would have to be included to stay on track for 2030 goals in high transmission settings where the adult burden of infection is also high.

Schistosomiasis is not covered in the WHO CoV Essential Health Services report (WHO, 2020b), as although the prolonged delays in delivering NTD interventions will likely lead to a resurgence of schistosomiasis cases, these will not be fatal or lead to profound disability if not promptly diagnosed. Resources need to be best placed for those diseases requiring case management where symptoms can rapidly progress and cause life changing morbidities, such as those associated with lymphatic filariasis, trypanosomiasis and onchocerciasis. With already restricted budgets and the financial strains of other public health campaigns, this pandemic is likely going to impose a bigger challenge for the healthcare systems in Africa to recover than those of developed nations. All that can be hoped for from a global health perspective is that ground gained on the control of NTDs in Africa is not lost.

Table 8.1. The primary snail surveillance and/or snail controls that can be immediately added to schistosomiasis control programmes. RD-PCR: Rapid diagnostic PCR. *The pros & cons for implementing snail control by gene drive are hypothetical since there is currently no evidence for the efficacy of such methods.

	Pros (√) and cons (x)
1. Snail surveys	√ Requires only basic equipment (collection pots, dissection microscope) √ Establish presence of potential intermediate hosts (morphology) and increase understanding of transmission √ Can identify patent schistosome infections √ Cheap × Cannot identify snail or schistosome species × Cannot infer infection risk map based on species identity × Misses pre-patent and non-patent infections
2. Snail surveys + snail/schistosome identification (molecular)	√ Unambiguous species identification √ Only basic molecular laboratory needed if using RD-PCR (gel electrophoresis) √ Develop a schistosomiasis risk map based on snail species presence; target additional interventions to risk areas √ Differentiate human and animal schistosomes, favours One Health × Additional costs for sample DNA extraction and PCR (and sequencing if required) × Requires basic molecular consumables and equipment × Requires access to Sanger sequencing platform if no appropriate RD-PCR
3. Snail surveys + molecular xenomonitoring	√ Detect patent, pre-patent and non-patent infections: higher sensitivity for detecting contamination at sites √ Indirect effect on transmission, can modify intervention targets (e.g. behavioural change interventions) to highest risk/contamination sites. √ Differentiate human and animal schistosomes, favours One Health √ Provides elimination certification × Sanger sequencing necessary to differentiate human and animal schistosomes × Large numbers of snail extractions currently time consuming × Potentially large ecological impact through non discriminate molluscicide effects
4. Snail surveys + snail control (molluscicide)	√ Directly impacts schistosomiasis transmission by reducing snail abundance / removing snail populations √ Application relatively quick and easy × Repeated application necessary, snails can quickly repopulate × Potential nontarget impact (e.g. lethal effects to vertebrates) × Inappropriate for large water bodies (sub-lethal doses not effective) × Additional costs
5. Snail surveys + snail control (environmental modification)	√ Directly impacts schistosomiasis transmission by reducing snail abundance / removing snail populations √ Requires only a single intervention √ Long lasting, potentially permanent, impact × Initially costly × Requires access to multiple resources and disciplines (e.g. engineering)

8.7 Finding a place for medical malacology in schistosomiasis surveillance and control

As discussed throughout this thesis, malacological surveys provide a wealth of information that can be used to best implement schistosomiasis control strategies. However, control programme managers will have to make decisions based on financial and logistical constraints whether to implement additional snail surveys and/or control (Table 8.1), and if so, in which manner? This is particularly topical since current WHO guidelines state the need for implementing additional snail surveillance and control (WHO, 2020a), however it is not stated specifically which methods should be implemented. This is primarily due to the current absence of on the ground implementation of these snail surveillance and control measures, except for more historic insights into the effect of chemical mollusciciding (Sokolow *et al.*, 2018). Although updated guidelines for the field use of molluscicides have recently been

published (WHO, 2017a), their uptake in sub-Saharan Africa has not been widespread in recent years due to the increased costs of chemicals, the reduced cost/donation of preventive chemotherapy, concerns over pollution and other biological impacts to non-target organisms (Savioli *et al.*, 2015; Sokolow *et al.*, 2016) (Table 8.1). In an endemic area where no previous medical malacology has been performed, but is implementing preventive chemotherapy, snail surveillance should be first implemented to map the schistosomiasis transmission risk through snail surveys and identifying snails and schistosomes to the species level (Table 8.1), following which, snail xenomonitoring can be performed to establish where continuing transmission is taking place and interventions (such as educational/behavioural change programmes) can be focalised. Only after establishing this foundation should snail control be considered and protocols be tailored to appropriate focal spatial and temporal epidemiology identified through the data collected during malacological surveys.

The uptake of phylogenetic methodologies in identifying and exploring intermediate host snail distributions and diversity has provided both answers and spurred new questions in schistosomiasis surveillance and control. Comparing DNA sequences to delimit snail species and then incriminate *Schistosoma* transmission with one or more of these has improved our ability to accurately map freshwater bodies in terms of *Schistosoma* transmission risk, a clear benefit. However, the huge amount of underlying diversity in genera such as *Bulinus*, as demonstrated throughout this thesis in both reviewed literature and new results, muddles our understanding of what separates one species from another (i.e. which species concepts to employ). This is important from a public health perspective since schistosomiasis control programmes and policies need clear guidelines supported by the scientific knowledge of how *Schistosoma* transmission takes place in endemic areas. Placing further emphasis on the need for malacological surveys during schistosomiasis projects, employing new *Schistosoma* surveillance techniques and pursuing studies into the taxonomy of intermediate host species is the only way to demonstrate the importance of medical malacology.

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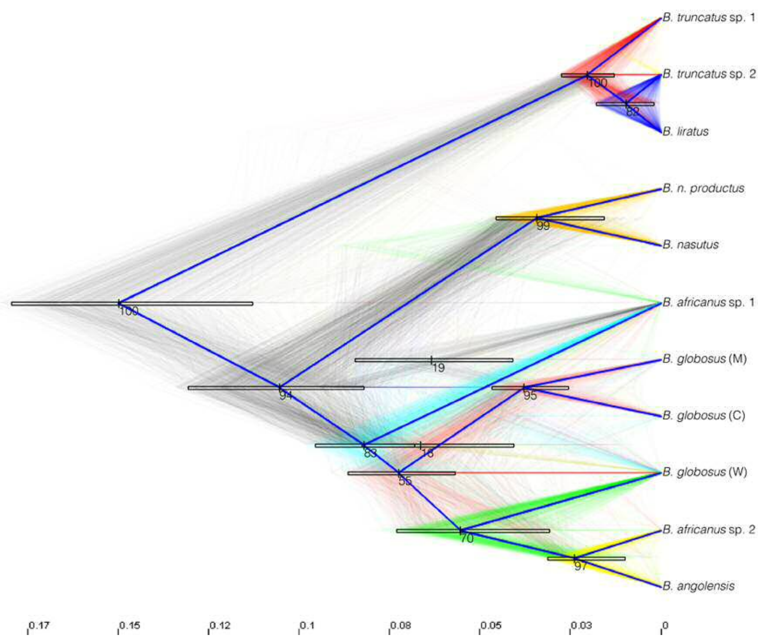
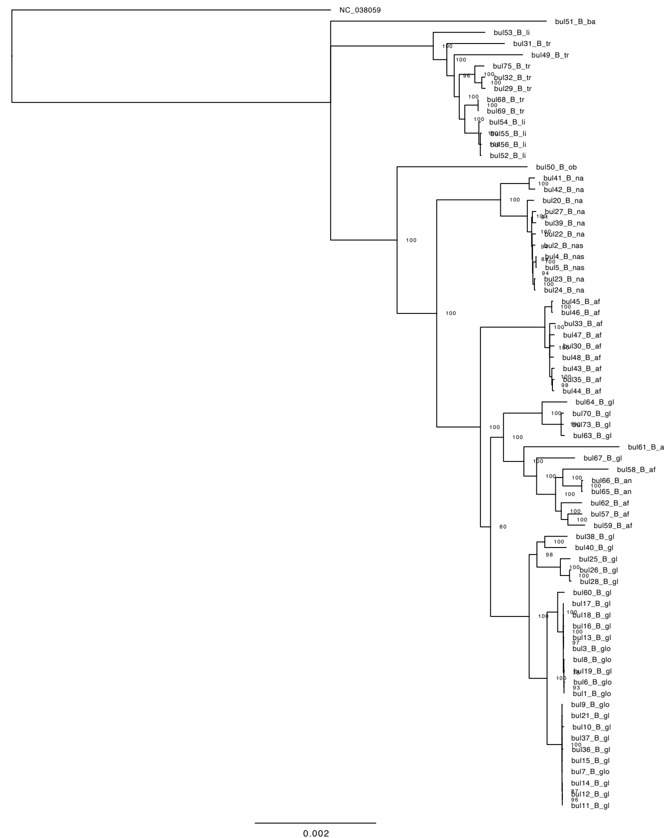
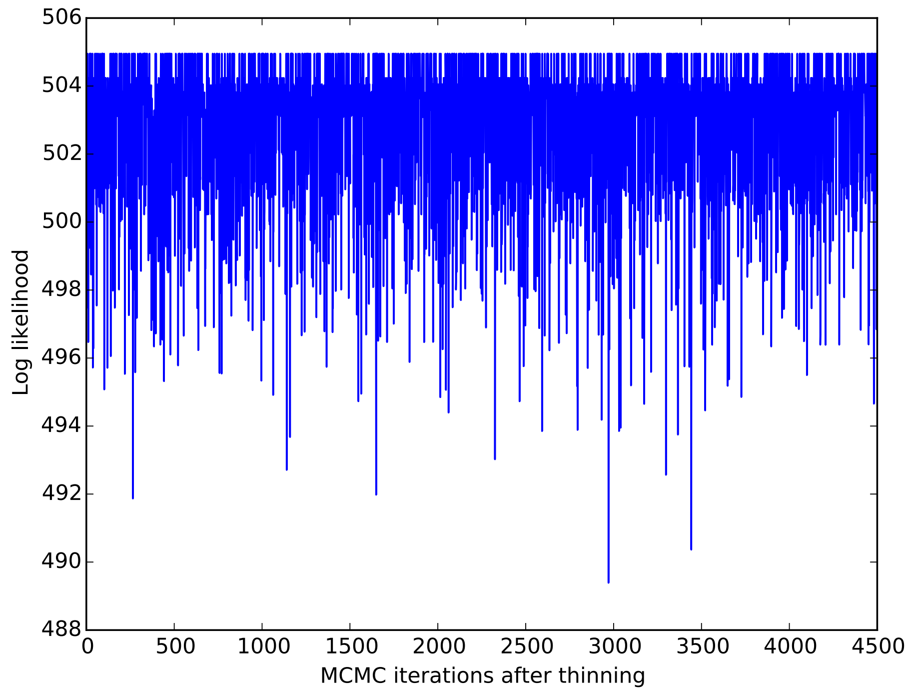
SUPPLEMENTARY MATERIAL



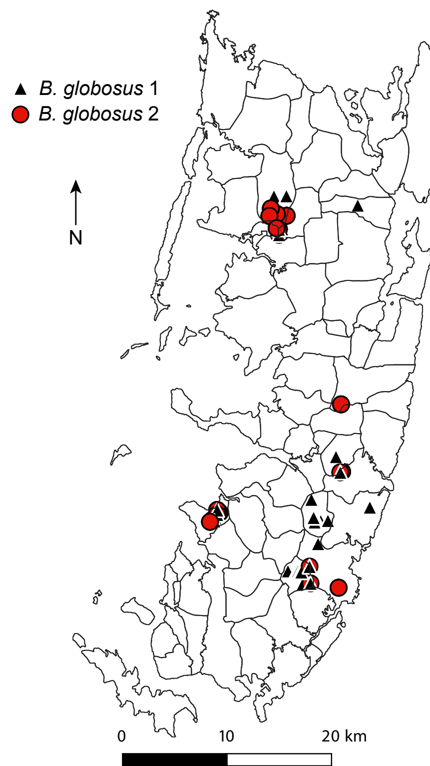
Supplementary Figure 7.1. Bayesian species tree estimation of *Bulinus* spp. performed using *BEAST (Heled and Drummond, 2009; Bouckaert *et al*., 2014) using taxa inferred as the same species / molecular operational taxonomic unit (MOTU) in maximum likelihood and Bayesian inference analysis and only including MOTUs represented by >2 taxa (n = 11). Analysis run for 10 million generations with a burn in of 5 million generations. Mean highest posterior density (HPD) and 95% HPD displayed graphically for clades with a posterior support >18%. Lines of consensus trees are coloured by the clade they represent.



Supplementary Figure 7.2. Maximum likelihood (RAxML) tree of 70 *Bulinus* spp. displaying Bootstrap support for all nodes.



Supplementary Figure 7.3. Bayesian species delimitation analysis (bPTP) model convergence plot.



Supplementary Figure 7.4. Overlapping distribution of the two *Bulinus globosus cox*1 haplotype groups (1 and 2) observed on Pemba Island.

Supplementary Table 3.1. Complete dataset used in this study providing snail collection information (from malacological survey), *Bulinus* and *Schistosoma* species identifications (including SNPs where necessary) and *Schistosoma* microsatellite loci data including genotypes identified from each snail.

Snail_id	Worm_Genotype	Village	Site_Name	Site_type	Year_collected	Latitude	Longitude	Snail_cox1_haplotpye	Bulinus_species_confirmed	Number_of_MLGs_per_Sn	Number_of_cercariae_per_	Worm_Genotype_Number	Schistosome_Species_per	Number_cercariae_process	Schistosome_genotype_sp	Schistosome_Diagnostic_c	Schistosome_cox1_haplotype	Schistosome_ITS1-2_result	ITS1 - 52	ITS2 - 90	ITS2 - 145	ITS2 - 195	ITS2 - 265	18S - 138	18S - 163	18S - 210
DO8	Dokimana.1	Dokimana	Dokimana 1 Mare Nord -Est	pond	2011	13.03523	2.34320	B.truncatus-Hap-12	*Bulinus truncatus* spp.	2	12	1	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
DO8	Dokimana.2	Dokimana	Dokimana 1 Mare Nord -Est	pond	2011	13.03523	2.34320	B.truncatus-Hap-12	*Bulinus truncatus* spp.	2	3	2	Sb	24	Sb4	Sb	Sb2	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
K1	Karma.1	Karma	Karma 1 Fleuve	river	2011	13.66431	1.82411	NA	*Bulinus truncatus*	1	21	1	Sb	36	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO7	Tokeye0083.1	Tokeye	Tokoye 2 canal secondaire	secondary irrigation canal	2011	13.19226	2.36143	NA	*Bulinus forskalii*	1	12	1	Sb	20	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO9	Tokeye0084.1	Tokeye	Tokoye 1 Mare	pond	2011	13.19668	2.35522	B.truncatus-Hap-9	*Bulinus truncatus* spp.	1	14	1	Sb	20	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
DK10	Doguel14.1	Doguel Kaina	Doguel Kaina 1 Bras Du Fleuve	branching stream	2012	13.28931	2.32526	B.globosus-Hap-1	*Bulinus globosus*	1	10	1	Sb	20	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
DK15	Doguel13.1	Doguel Kaina	Doguel Kaina 1 Bras Du Fleuve	branching stream	2012	13.28931	2.32526	NA	*Bulinus truncatus*	1	8	1	Sb	20	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
DO4	Dokimana15.1	Dokimana	Dokimana 1 Mare Nord -Est	pond	2012	13.03523	2.34320	B.truncatus-Hap-3	*Bulinus truncatus*	1	14	1	Sb	20	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
DO5	Dokimana16.1	Dokimana	Dokimana 1 Mare Nord -Est	pond	2012	13.03523	2.34320	B.truncatus-Hap-3	*Bulinus truncatus*	1	10	1	Sb	20	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
KK17	Koutoukale3.1	Koutoukale Zeno	Koutoukale Zeno 1 Mare	pond	2012	13.63161	1.74466	NA	*Bulinus truncatus*	4	3	1	Sb	36	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
KK17	Koutoukale3.2	Koutoukale Zeno	Koutoukale Zeno 1 Mare	pond	2012	13.63161	1.74466	NA	*Bulinus truncatus*	4	6	2	Sb	36	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
KK17	Koutoukale3.3	Koutoukale Zeno	Koutoukale Zeno 1 Mare	pond	2012	13.63161	1.74466	NA	*Bulinus truncatus*	4	7	3	Sb	36	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
KK17	Koutoukale3.4	Koutoukale Zeno	Koutoukale Zeno 1 Mare	pond	2012	13.63161	1.74466	NA	*Bulinus truncatus*	4	1	4	Sb	36	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
KK6	Koutoukale2.1	Koutoukale Zeno	Koutkoukale 2 canal secondaire	secondary irrigation canal	2012	13.66634	1.74466	NA	*Bulinus truncatus*	3	20	1	Cl	36	Hy2	Sb	0	Sh/Sc mix	Sh/Sc (A)	Sh/Sc (G/A)	Sh/Sc (C/T)	Sh/Sc (G/A)	Sh/Sc (C/T)	0	0	0

KK6	Koutoukale2.2	Koutoukale Zeno	Koutkoukale 2 canal secondaire	secondary irrigation canal	2012	13.66634	1.74466	NA	*Bulinus truncatus*	3	3	2	CI	36	Hy1	Sb	Sb2	Sh	Sh (A)	Sh (G)	Sh (C)	Sh (G)	Sh (C)	0	0	0
KK6	Koutoukale2.3	Koutoukale Zeno	Koutkoukale 2 canal secondaire	secondary irrigation canal	2012	13.66634	1.74466	NA	*Bulinus truncatus*	3	1	3	CI	36	Hy1	Sb	0	Sh	Sh (A)	Sh (G)	Sh (C)	Sh (G)	Sh (C)	0	0	0
LA8	Lata8.1	Lata Kabia	Lata Kabia canal tertiare	tertiary irrigation canal	2012	13.74133	1.68262	B.forskalii-Hap-2	*Bulinus forskalii*	1	8	1	Sb	20	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO24	Tokeye5.1	Tokeye	Tokoye 1 Mare	pond	2012	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	1	27	1	Sb	36	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
DK34	DK4.1	Doguel Kaina	Doguel Kaina 1 Bras Du Fleuve	branching stream	2013	13.28931	2.32526	B.truncatus-Hap-4	*Bulinus truncatus*	1	3	1	Sb	4	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
DK35	DK34.1	Doguel Kaina	Doguel Kaina 1 Bras Du Fleuve	branching stream	2013	13.28931	2.32526	B.truncatus-Hap-4	*Bulinus truncatus*	1	9	1	Sb	20	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
DK36	DK36.1	Doguel Kaina	Doguel Kaina 1 Bras Du Fleuve	branching stream	2013	13.28931	2.32526	B.truncatus-Hap-4	*Bulinus truncatus*	1	33	1	Sb	54	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
DO13	DO13.1	Dokimana	Dokimana 1 Mare Nord -Est	pond	2013	13.03523	2.34320	B.truncatus-Hap-3	*Bulinus truncatus*	1	40	1	Sb	54	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
GB16	GB16.1	Gantchi Basarou	Gantchi Bassarou 1 Mare	pond	2013	13.17606	2.35497	B.forskalii-Hap-1	*Bulinus forskalii*	2	10	1	Sb	32	Sb4	Sb	Sb1	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
GB16	GB16.2	Gantchi Basarou	Gantchi Bassarou 1 Mare	pond	2013	13.17606	2.35497	B.forskalii-Hap-1	*Bulinus forskalii*	2	17	2	Sb	32	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
K15	K15.1	Karma	Karma 1 Fleuve	river	2013	13.66431	1.82411	B.truncatus-Hap-11	*Bulinus truncatus spp.*	1	44	1	Hy	52	Hy1	Sb	0	Sh	Sh (A)	Sh (G)	Sh (C)	Sh (G)	Sh (C)	0	0	0
KK36	KK36.1	Koutoukale Zeno	Koutoukale 3 canal tertiaire	tertiary irrigation canal	2013	13.68620	1.74518	NA	*Bulinus truncatus*	2	16	1	CI	34	Hy2	Sb	0	Sh/Sc mix	Sh/Sc (A)	Sh/Sc (G/A)	Sh/Sc (C/T)	Sh/Sc (G/A)	Sh/Sc (C/T)	Sh/Sc (T)	*Sh/Sc (C/T)*	Sh/Sc (T)
KK36	KK36.2	Koutoukale Zeno	Koutoukale 3 canal tertiaire	tertiary irrigation canal	2013	13.68620	1.74518	NA	*Bulinus truncatus*	2	1	2	CI	34	Sh	Sh	Sh-Hap-1	Sh	Sh	Sh	Sh	Sh	Sh	Sh	Sh	Sh
KK37	KK37.1	Koutoukale Zeno	Koutoukale 3 canal tertiaire	tertiary irrigation canal	2013	13.68620	1.74518	NA	*Bulinus truncatus*	1	14	1	Sh	20	Sh	Sh	Sh-Hap-1	Sh	Sh	Sh	Sh	Sh	Sh	0	0	0
N11	N11.1	Namaro	Namaro 3 Mare	pond	2013	13.70971	1.69698	B.truncatus-Hap-12	*Bulinus truncatus spp.*	1	6	1	Sb	20	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
N12	N12.1	Namaro	Namaro 3 Mare	pond	2013	13.70971	1.69698	B.truncatus-Hap-6	*Bulinus truncatus*	1	19	1	Hy	34	Hy1	Sb	0	Sh	Sh (A)	Sh (G)	Sh (C)	Sh (G)	Sh (C)	0	0	0
SA17	SA17.1	Say	say 2 deversoir	spillway	2013	13.10468	2.35263	NA	*Bulinus truncatus*	2	7	1	Sb	20	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
SA17	SA17.2	Say	say 2 deversoir	spillway	2013	13.10468	2.35263	NA	*Bulinus truncatus*	2	1	2	Sb	20	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
SA20	SA20.1	Say	Say 1 ruisseau	stream	2013	13.11028	2.34223	B.globosus-Hap-3	*Bulinus globosus*	1	25	1	Sb	52	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0

TO29	TO29.1	Tokeye	Tokoye 1 Mare	pond	2013	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	1	30	1	Sb	40	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO30	TO30.1	Tokeye	Tokoye 1 Mare	pond	2013	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	3	23	1	Sb	36	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO30	TO30.2	Tokeye	Tokoye 1 Mare	pond	2013	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	3	1	2	Sb	36	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO30	TO30.3	Tokeye	Tokoye 1 Mare	pond	2013	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	3	2	3	Sb	36	Sb2	Sb	Sb3	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO31	TO31.1	Tokeye	Tokoye 1 Mare	pond	2013	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	1	11	1	Sb	20	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO32	TO32.1	Tokeye	Tokoye 1 Mare	pond	2013	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	1	13	1	Sb	20	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO44	TO44.1	Tokeye	Tokoye 1 Mare	pond	2013	13.19668	2.35522	Failed	*Bulinus truncatus*	7	3	1	Sb	34	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO44	TO44.2	Tokeye	Tokoye 1 Mare	pond	2013	13.19668	2.35522	Failed	*Bulinus truncatus*	7	1	2	Sb	34	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO44	TO44.3	Tokeye	Tokoye 1 Mare	pond	2013	13.19668	2.35522	Failed	*Bulinus truncatus*	7	2	3	Sb	34	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO44	TO44.4	Tokeye	Tokoye 1 Mare	pond	2013	13.19668	2.35522	Failed	*Bulinus truncatus*	7	2	4	Sb	34	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO44	TO44.5	Tokeye	Tokoye 1 Mare	pond	2013	13.19668	2.35522	Failed	*Bulinus truncatus*	7	1	5	Sb	34	Sb1	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO44	TO44.6	Tokeye	Tokoye 1 Mare	pond	2013	13.19668	2.35522	Failed	*Bulinus truncatus*	7	1	6	Sb	34	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO44	TO44.7	Tokeye	Tokoye 1 Mare	pond	2013	13.19668	2.35522	Failed	*Bulinus truncatus*	7	1	7	Sb	34	Sb2	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	Sb	Sb	Sb
Y24	Y24.1	Youri	Youri 3 Fleuve	river	2013	13.33371	2.24556	NA	*Bulinus forskalii*	6	7	1	Sb	52	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
Y24	Y24.2	Youri	Youri 3 Fleuve	river	2013	13.33371	2.24556	NA	*Bulinus forskalii*	6	2	2	Sb	52	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
Y24	Y24.3	Youri	Youri 3 Fleuve	river	2013	13.33371	2.24556	NA	*Bulinus forskalii*	6	7	3	Sb	52	Sb4	Sb	Sb1	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
Y24	Y24.4	Youri	Youri 3 Fleuve	river	2013	13.33371	2.24556	NA	*Bulinus forskalii*	6	3	4	Sb	52	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
Y24	Y24.5	Youri	Youri 3 Fleuve	river	2013	13.33371	2.24556	NA	*Bulinus forskalii*	6	3	5	Sb	52	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
Y24	Y24.6	Youri	Youri 3 Fleuve	river	2013	13.33371	2.24556	NA	*Bulinus forskalii*	6	16	6	Sb	52	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0

BK21	BK21.1	Bangou Koirey	Bangou Koirey 2 canal secondaire	branching stream	2014	13.60475	1.90509	B.truncatus-Hap-12	*Bulinus truncatus spp.*	2	12	1	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
BK21	BK21.2	Bangou Koirey	Bangou Koirey 2 canal secondaire	branching stream	2014	13.60475	1.90509	B.truncatus-Hap-12	*Bulinus truncatus spp.*	2	1	2	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
BK22	BK22.1	Bangou Koirey	Bangou Koirey 2 canal secondaire	branching stream	2014	13.60475	1.90509	B.truncatus-Hap-1	*Bulinus truncatus*	1	18	1	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
BK23	BK23.1	Bangou Koirey	Bangou Koirey 2 canal secondaire	branching stream	2014	13.60475	1.90509	B.globosus-Hap-1	*Bulinus globosus*	1	14	1	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
BK24	BK24.1	Bangou Koirey	Bangou Koirey 2 canal secondaire	branching stream	2014	13.60475	1.90509	B.truncatus-Hap-2	*Bulinus truncatus*	3	11	1	Sb	22	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
BK24	BK24.2	Bangou Koirey	Bangou Koirey 2 canal secondaire	branching stream	2014	13.60475	1.90509	B.truncatus-Hap-2	*Bulinus truncatus*	3	1	2	Sb	22	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
BK24	BK24.3	Bangou Koirey	Bangou Koirey 2 canal secondaire	branching stream	2014	13.60475	1.90509	B.truncatus-Hap-2	*Bulinus truncatus*	3	2	3	Sb	22	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
DK60	DK60.1	Doguel Kaina	Doguel Kaina 4 Fleuve	river	2014	13.29273	2.32335	B.truncatus-Hap-11	*Bulinus truncatus spp.*	1	11	1	Sb	20	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
DO43	DO43.1	Dokimana	Dokimana 1 Mare Nord -Est	pond	2014	13.03523	2.34320	B.forskalii-Hap-1	*Bulinus forskalii*	1	16	1	Sb	24	Sb3	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	Sb	Sb	Sb
DO51	DO51.1	Dokimana	Dokimana 1 Mare Nord -Est	pond	2014	13.03523	2.34320	NA	*Bulinus truncatus*	1	10	1	Sb	20	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
DO52	DO52.1	Dokimana	Dokimana 1 Mare Nord -Est	pond	2014	13.03523	2.34320	NA	*Bulinus truncatus*	2	5	1	Sb	20	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
DO52	DO52.2	Dokimana	Dokimana 1 Mare Nord -Est	pond	2014	13.03523	2.34320	NA	*Bulinus truncatus*	2	6	2	Sb	20	Sb2	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
DO53	DO53.1	Dokimana	Dokimana 1 Mare Nord -Est	pond	2014	13.03523	2.34320	B.truncatus-Hap-3	*Bulinus truncatus*	1	11	1	Sb	20	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
DO54	DO54.1	Dokimana	Dokimana 1 Mare Nord -Est	pond	2014	13.03523	2.34320	B.truncatus-Hap-3	*Bulinus truncatus*	2	7	1	Sb	20	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
DO54	DO54.2	Dokimana	Dokimana 1 Mare Nord -Est	pond	2014	13.03523	2.34320	B.truncatus-Hap-3	*Bulinus truncatus*	2	1	2	Sb	20	Sb4	Sb	Sb1	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
DO56	DO56.1	Dokimana	Dokimana 1 Mare Nord -Est	pond	2014	13.03523	2.34320	NA	*Bulinus truncatus*	1	6	1	Sb	20	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
LA30	LA30.1	Lata Kabia	Lata Kabia 2 canal tertiaire	tertiary irrigation canal	2014	13.7419	1.68262	B.truncatus-Hap-1	*Bulinus truncatus*	1	9	1	Sh	22	Sh	Sh	0	Sh	Sh	Sh	Sh	Sh	Sh	0	0	0
LA36	LA36.1	Lata Kabia	Lata Kabia 2 canal tertiaire	tertiary irrigation canal	2014	13.74193	1.68262	B.truncatus-Hap-2	*Bulinus truncatus*	1	9	1	Hy	24	Hy1	Sb	0	Sh	Sh (A)	Sh (G)	Sh (C)	Sh (G)	Sh (C)	0	0	0
LA45	LA45.1	Lata Kabia	Lata Kabia 2 canal tertiaire	tertiary irrigation canal	2014	13.74193	1.68262	B.truncatus-Hap-1	*Bulinus truncatus*	1	15	1	Hy	20	Hy1	Sb	0	Sh	Sh (A)	Sh (G)	Sh (C)	Sh (G)	Sh (C)	0	0	0

LA 54	LA54.1	Lata Kabia	Lata Kabia 2 canal tertiaire	tertiary irrigation canal	20 14	13.7 4193	1.68 262	NA	*Bulinus truncatus*	1	9	1	H y	2 0	H y 1	S b	0	Sh	Sh (A)	Sh (G)	Sh (C)	Sh (G)	Sh (C)	0	0	0
TO 48	TO48.1	Tokeye	Tokoye 1 Mare	pond	20 14	13.1 9668	2.35 522	NA	*Bulinus truncatus*	3	1	1	S b	2 0	S b 4	S b	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO 48	TO48.2	Tokeye	Tokoye 1 Mare	pond	20 14	13.1 9668	2.35 522	NA	*Bulinus truncatus*	3	2	2	S b	2 0	S b 4	S b	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO 48	TO48.3	Tokeye	Tokoye 1 Mare	pond	20 14	13.1 9668	2.35 522	NA	*Bulinus truncatus*	3	1	3	S b	2 0	S b 4	S b	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO 49	TO49.1	Tokeye	Tokoye 1 Mare	pond	20 14	13.1 9668	2.35 522	B.truncatu s-Hap-10	*Bulinus truncatus spp.*	4	7	1	S b	2 0	S b 4	S b	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO 49	TO49.2	Tokeye	Tokoye 1 Mare	pond	20 14	13.1 9668	2.35 522	B.truncatu s-Hap-10	*Bulinus truncatus spp.*	4	2	2	S b	2 0	S b 4	S b	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO 49	TO49.3	Tokeye	Tokoye 1 Mare	pond	20 14	13.1 9668	2.35 522	B.truncatu s-Hap-10	*Bulinus truncatus spp.*	4	2	3	S b	2 0	S b 4	S b	Sb1	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO 49	TO49.4	Tokeye	Tokoye 1 Mare	pond	20 14	13.1 9668	2.35 522	B.truncatu s-Hap-10	*Bulinus truncatus spp.*	4	1	4	S b	2 0	S b 4	S b	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO 51	TO51.1	Tokeye	Tokoye 1 Mare	pond	20 14	13.1 9668	2.35 522	B.truncatu s-Hap-10	*Bulinus truncatus spp.*	3	1	1	S b	2 0	S b 4	S b	0	Sb	?	Sb	Sb	Sb	Sb	0	0	0
TO 51	TO51.2	Tokeye	Tokoye 1 Mare	pond	20 14	13.1 9668	2.35 522	B.truncatu s-Hap-10	*Bulinus truncatus spp.*	3	2	2	S b	2 0	S b 4	S b	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO 51	TO51.3	Tokeye	Tokoye 1 Mare	pond	20 14	13.1 9668	2.35 522	B.truncatu s-Hap-10	*Bulinus truncatus spp.*	3	1	3	S b	2 0	S b 4	S b	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO 55	TO55.1	Tokeye	Tokoye 1 Mare	pond	20 14	13.1 9668	2.35 522	B.truncatu s-Hap-10	*Bulinus truncatus spp.*	4	1	1	S b	2 0	S b 4	S b	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO 55	TO55.2	Tokeye	Tokoye 1 Mare	pond	20 14	13.1 9668	2.35 522	B.truncatu s-Hap-10	*Bulinus truncatus spp.*	4	3	2	S b	2 0	S b 4	S b	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO 55	TO55.3	Tokeye	Tokoye 1 Mare	pond	20 14	13.1 9668	2.35 522	B.truncatu s-Hap-10	*Bulinus truncatus spp.*	4	7	3	S b	2 0	S b 4	S b	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO 55	TO55.4	Tokeye	Tokoye 1 Mare	pond	20 14	13.1 9668	2.35 522	B.truncatu s-Hap-10	*Bulinus truncatus spp.*	4	1	4	S b	2 0	S b 4	S b	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO 56	TO56.1	Tokeye	Tokoye 1 Mare	pond	20 14	13.1 9668	2.35 522	NA	*Bulinus truncatus*	2	9	1	S b	2 0	S b 4	S b	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO 56	TO56.2	Tokeye	Tokoye 1 Mare	pond	20 14	13.1 9668	2.35 522	NA	*Bulinus truncatus*	2	1	2	S b	2 0	S b 4	S b	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO 58	TO58.1	Tokeye	Tokoye 1 Mare	pond	20 14	13.1 9668	2.35 522	B.truncatu s-Hap-10	*Bulinus truncatus spp.*	2	3	1	S b	2 2	S b 4	S b	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO 58	TO58.2	Tokeye	Tokoye 1 Mare	pond	20 14	13.1 9668	2.35 522	B.truncatu s-Hap-10	*Bulinus truncatus spp.*	2	8	2	S b	2 2	S b 4	S b	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0

TO61	TO61.1	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	8	1	1	Sb	24	Sb2	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO61	TO61.2	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	8	1	2	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO61	TO61.3	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	8	1	3	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO61	TO61.4	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	8	2	4	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO61	TO61.5	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	8	1	5	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO61	TO61.6	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	8	1	6	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO61	TO61.7	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	8	2	7	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO61	TO61.8	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	8	1	8	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO62	TO62.1	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	8	2	1	Sb	22	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO62	TO62.2	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	8	1	2	Sb	22	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO62	TO62.3	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	8	2	3	Sb	22	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO62	TO62.4	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	8	1	4	Sb	22	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO62	TO62.5	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	8	1	5	Sb	22	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO62	TO62.6	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	8	2	6	Sb	22	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO62	TO62.7	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	8	1	7	Sb	22	Sb4	Sb	Sb1	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO62	TO62.8	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	8	1	8	Sb	22	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO65	TO65.1	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	10	1	1	Sb	24	Sb2	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO65	TO65.2	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	10	1	2	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO65	TO65.3	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	10	1	3	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0

TO65	TO65.4	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	10	1	4	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO65	TO65.5	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	10	1	5	Sb	24	Sb2	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO65	TO65.6	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	10	1	6	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO65	TO65.7	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	10	1	7	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO65	TO65.8	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	10	1	8	Sb	24	Sb1	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO65	TO65.9	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	10	1	9	Sb	24	Sb4	Sb	Sb4	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO65	TO65.10	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	10	1	10	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO66	TO66.1	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-7	*Bulinus truncatus*	5	1	1	Sb	22	Sb2	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO66	TO66.2	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-7	*Bulinus truncatus*	5	5	2	Sb	22	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO66	TO66.3	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-7	*Bulinus truncatus*	5	1	3	Sb	22	Sb2	Sb	Sb2	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO66	TO66.4	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-7	*Bulinus truncatus*	5	2	4	Sb	22	Sb2	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO66	TO66.5	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-7	*Bulinus truncatus*	5	1	5	Sb	22	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO68	TO68.1	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	10	1	1	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO68	TO68.2	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	10	1	2	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO68	TO68.3	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	10	1	3	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO68	TO68.4	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	10	1	4	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO68	TO68.5	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	10	1	5	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO68	TO68.6	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	10	1	6	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO68	TO68.7	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	10	1	7	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0

TO68	TO68.8	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	10	2	8	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO68	TO68.9	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	10	1	9	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO68	TO68.10	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	10	1	10	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO70	TO70.1	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	3	9	1	Sb	22	Sb4	Sb	Sb1	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO70	TO70.2	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	3	2	2	Sb	22	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO70	TO70.3	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	3	2	3	Sb	22	Sb3	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO72	TO72.1	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	NA	*Bulinus truncatus*	7	2	1	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO72	TO72.2	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	NA	*Bulinus truncatus*	7	1	2	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO72	TO72.3	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	NA	*Bulinus truncatus*	7	3	3	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO72	TO72.4	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	NA	*Bulinus truncatus*	7	6	4	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO72	TO72.5	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	NA	*Bulinus truncatus*	7	1	5	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO72	TO72.7	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	NA	*Bulinus truncatus*	7	3	7	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO72	TO72.8	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	NA	*Bulinus truncatus*	7	1	8	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO73	TO73.1	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	6	7	1	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO73	TO73.2	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	6	5	2	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO73	TO73.3	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	6	3	3	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO73	TO73.4	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	6	1	4	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO73	TO73.5	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	6	3	5	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
TO73	TO73.6	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	6	1	6	Sb	24	Sb3	Sb	Sb2	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0

TO76	TO76.1	Tokeye	Tokoye 1 Mare	pond	2014	13.19668	2.35522	B.truncatus-Hap-10	*Bulinus truncatus spp.*	1	11	1	Sb	24	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
ZKT22	ZKT22.1	Zama Koira Tagui	Zamakoira Tegui 4 canal secondaire	secondary irrigation canal	2014	13.70432	1.72169	B.truncatus-Hap-5	*Bulinus truncatus*	2	10	1	Sh	22	Sh	Sh	Sh-Hap-1	Sh	Sh	Sh	Sh	Sh	Sh	0	0	0
ZKT22	ZKT22.2	Zama Koira Tagui	Zamakoira Tegui 4 canal secondaire	secondary irrigation canal	2014	13.70432	1.72169	B.truncatus-Hap-5	*Bulinus truncatus*	2	2	2	Sh	22	Sh	Sh	Sh-Hap-1	Sh	Sh	Sh	Sh	Sh	Sh	0	0	0
ZKt26	ZKt26.1	Zama Koira Tagui	Zamakoira Tegui 4 canal secondaire	secondary irrigation canal	2014	13.70432	1.72169	B.truncatus-Hap-9	*Bulinus truncatus spp.*	1	6	1	Hy	20	Hy1	Sb	0	Sh	Sh (A)	Sh (G)	Sh (C)	Sh (G)	Sh (C)	0	0	0
ZKt27	ZKt27.1	Zama Koira Tagui	Zamakoira Tegui 4 canal secondaire	secondary irrigation canal	2014	13.70432	1.72169	B.truncatus-Hap-11	*Bulinus truncatus spp.*	2	4	1	Cl	20	Hy1	Sb	0	Sh	Sh (A)	Sh (G)	Sh (C)	Sh (G)	Sh (C)	0	0	0
ZKt27	ZKt27.2	Zama Koira Tagui	Zamakoira Tegui 4 canal secondaire	secondary irrigation canal	2014	13.70432	1.72169	B.truncatus-Hap-11	*Bulinus truncatus spp.*	2	5	2	Cl	20	Hy2	Sb	0	Sh /Sc mix	Sh /Sc (A)	Sh /Sc (G/A)	Sh /Sc (C/T)	Sh /Sc (G/A)	Sh /Sc (C/T)	Sh /Sc (T)	*Sh /Sc (C/T)*	Sh /Sc (T)
BK40	BK40.1	Bangou Koirey	Bangou Koirey 2 Bras Du Fleuve	branching stream	2015	13.6048	1.90509	B.globosus-Hap-2	*Bulinus globosus*	1	5	1	Sb	20	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
BK49	BK49.1	Bangou Koirey	Bangou Koirey 2 Bras Du Fleuve	branching stream	2015	13.60475	1.90509	B.truncatus-Hap-2	*Bulinus truncatus*	1	6	1	Sb	24	Sb4	Sb	Sb1	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
DO59	DO59.1	Dokimana	Dokimana 1 Mare Nord -Est	pond	2015	13.03523	2.34320	B.truncatus-Hap-2	*Bulinus truncatus*	3	5	1	Sb	20	Sb4	Sb	Sb1	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
DO59	DO59.2	Dokimana	Dokimana 1 Mare Nord -Est	pond	2015	13.03523	2.34320	B.truncatus-Hap-2	*Bulinus truncatus*	3	4	2	Sb	20	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
DO59	DO59.3	Dokimana	Dokimana 1 Mare Nord -Est	pond	2015	13.03523	2.34320	B.truncatus-Hap-2	*Bulinus truncatus*	3	2	3	Sb	20	Sb2	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
DO60	DO60.1	Dokimana	Dokimana 1 Mare Nord -Est	pond	2015	13.03523	2.34320	B.truncatus-Hap-2	*Bulinus truncatus*	1	10	1	Sb	20	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
DO63	DO63.1	Dokimana	Dokimana 1 Mare Nord -Est	pond	2015	13.03523	2.34320	B.truncatus-Hap-2	*Bulinus truncatus*	1	11	1	Sb	20	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
DO64	DO64.1	Dokimana	Dokimana 1 Mare Nord -Est	pond	2015	13.03523	2.34320	NA	*Bulinus truncatus*	1	8	1	Sb	20	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
DO65	DO65.1	Dokimana	Dokimana 1 Mare Nord -Est	pond	2015	13.03523	2.34320	B.truncatus-Hap-3	*Bulinus truncatus*	3	2	1	Sb	20	Sb4	Sb	Sb1	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
DO65	DO65.2	Dokimana	Dokimana 1 Mare Nord -Est	pond	2015	13.03523	2.34320	B.truncatus-Hap-3	*Bulinus truncatus*	3	1	2	Sb	20	Sb2	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
DO65	DO65.3	Dokimana	Dokimana 1 Mare Nord -Est	pond	2015	13.03523	2.34320	B.truncatus-Hap-3	*Bulinus truncatus*	3	2	3	Sb	20	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
DO66	DO66.1	Dokimana	Dokimana 1 Mare Nord -Est	pond	2015	13.03523	2.34320	B.truncatus-Hap-2	*Bulinus truncatus*	2	10	1	Sb	20	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
DO66	DO66.2	Dokimana	Dokimana 1 Mare Nord -Est	pond	2015	13.03523	2.34320	B.truncatus-Hap-2	*Bulinus truncatus*	2	2	2	Sb	20	Sb4	Sb	Sb1	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0

DO67	DO67.1	Dokimana	Dokimana 1 Mare Nord -Est	pond	2015	13.03523	2.34320	B.truncatus-Hap-3	*Bulinus truncatus*	1	7	1	Sb	20	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
DO68	DO68.1	Dokimana	Dokimana 1 Mare Nord -Est	pond	2015	13.03523	2.34320	B.truncatus-Hap-2	*Bulinus truncatus*	1	9	1	Sb	20	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
KG19	KG19.1	Kohan Garantche	Kohan Garantche 1 Fleuve	river	2015	13.31996	2.29661	B.truncatus-Hap-5	*Bulinus truncatus*	1	9	1	Sb	20	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
KG20	KG20.1	Kohan Garantche	Kohan Garantche 1 Fleuve	river	2015	13.31996	2.29661	B.truncatus-Hap-2	*Bulinus truncatus*	1	9	1	Sb	20	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
KG21	KG21.1	Kohan Garantche	Kohan Garantche 1 Fleuve	river	2015	13.31996	2.29661	B.truncatus-Hap-2	*Bulinus truncatus*	1	9	1	Sb	20	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
KG22	KG22.1	Kohan Garantche	Kohan Garantche 1 Fleuve	river	2015	13.31996	2.29661	B.truncatus-Hap-4	*Bulinus truncatus*	1	4	1	Sb	20	Sb4	Sb	0	Sb	Sb	Sb	Sb	Sb	Sb	0	0	0
KK92	KK92.1	Koutoukale Zeno	Koutoukale Zeno 2 canal tertiaire	*tertiary irrigation canal*	2015	13.68637	1.74520	B.truncatus-Hap-9	*Bulinus truncatus spp.*	1	13	1	Hy	20	Hy1	Sb	0	Sh	Sh (A)	Sh (G)	Sh (C)	Sh (G)	Sh (C)	0	0	0
LA57	LA57.1	Lata Kabia	Lata Kabia 2 canal tertiaire	tertiary irrigation canal	2015	13.74193	1.68262	NA	*Bulinus truncatus*	2	6	1	Cl	20	Hy2	Sb	0	Sh/Sc mix	Sh/Sc (A)	Sh/Sc (G/A)	Sh/Sc (C/T)	Sh/Sc (G/A)	Sh/Sc (C/T)	0	0	0
LA57	LA57.3	Lata Kabia	Lata Kabia 2 canal tertiaire	tertiary irrigation canal	2015	13.74193	1.68262	NA	*Bulinus truncatus*	2	1	3	Cl	20	Hy1	Sb	0	Sh	Sh (A)	Sh (G)	Sh (C)	Sh (G)	Sh (C)	0	0	0
LA63	LA63.1	Lata Kabia	Lata Kabia 2 canal tertiaire	tertiary irrigation canal	2015	13.74193	1.68262	NA	*Bulinus truncatus*	1	6	1	Sh	20	Sh	Sh	0	Sh	Sh	Sh	Sh	Sh	Sh	0	0	0
LA70	LA70.1	Lata Kabia	Lata Kabia 3 Riziere	rice paddy	2015	13.74199	1.6826	NA	*Bulinus truncatus*	1	5	1	Sh	20	Sh	Sh	0	Sh	Sh	Sh	Sh	Sh	Sh	0	0	0
LA79	LA79.1	Lata Kabia	Lata Kabia 8 Canal Secondaire	secondary irrigation canal	2015	13.76385	1.66861	B.truncatus-Hap-9	*Bulinus truncatus spp.*	3	1	1	Cl	24	Hy1	Sb	0	Sh	Sh (A)	Sh (G)	Sh (C)	Sh (G)	Sh (C)	0	0	0
LA79	LA79.2	Lata Kabia	Lata Kabia 8 Canal Secondaire	secondary irrigation canal	2015	13.76385	1.66861	B.truncatus-Hap-9	*Bulinus truncatus spp.*	3	1	2	Cl	24	Sh	Sh	Sh-Hap-1	Sh	Sh	Sh	Sh	Sh	Sh	0	0	0
LA79	LA79.3	Lata Kabia	Lata Kabia 8 Canal Secondaire	secondary irrigation canal	2015	13.76385	1.66861	B.truncatus-Hap-9	*Bulinus truncatus spp.*	3	1	3	Cl	24	Hy1	Sb	Sb2	Sh	Sh (A)	Sh (G)	Sh (C)	Sh (G)	Sh (C)	0	0	0
Y46	Y46.1	Youri	Youri 3 Fleuve	river	2015	13.33371	2.24556	NA	*Bulinus truncatus*	1	5	1	Sb	20	Sb4	Sb	0	Sb	?	Sb	Sb	Sb	Sb	0	0	0
ZKt34	ZKt34.1	Zama Koira Tagui	Zamakoira Tegui 1 canal secondaire	secondary irrigation canal	2015	13.70169	1.71942	B.truncatus-Hap-8	*Bulinus truncatus*	2	8	1	Cl	20	Hy1	Sb	0	Sh	Sh (A)	Sh (G)	Sh (C)	Sh (G)	Sh (C)	0	0	0
ZKt34	ZKt34.2	Zama Koira Tagui	Zamakoira Tegui 1 canal secondaire	secondary irrigation canal	2015	13.70169	1.71942	B.truncatus-Hap-8	*Bulinus truncatus*	2	3	2	Cl	20	Hy2	Sb	Sb2	Sh/Sc mix	Sh/Sc (A)	Sh/Sc (G/A)	Sh/Sc (C/T)	Sh/Sc (G/A)	Sh/Sc (C/T)	0	0	0
ZKt40	ZKt40.1	Zama Koira Tagui	Zamakoira Tegui 4 canal secondaire	secondary irrigation canal	2015	13.70432	1.72169	NA	*Bulinus truncatus*	1	11	1	Sh	20	Sh	Sh	Sh-Hap-1	Sh	Sh	Sh	Sh	Sh	Sh	0	0	0
ZKt41	ZKt41.1	Zama Koira Tagui	Zamakoira Tegui 4 canal secondaire	secondary irrigation canal	2015	13.70432	1.72169	B.truncatus-Hap-9	*Bulinus truncatus spp.*	1	17	1	Hy	20	Hy1	Sb	0	Sh	Sh (A)	Sh (G)	Sh (C)	Sh (G)	Sh (C)	0	0	0

ZKt 42	ZKt42.1	Zama Koira Tagui	Zamakoira Tegui 4 canal secondaire	secondary irrigation canal	2015	13.70432	1.72169	B.truncatus-Hap-9	*Bulinus truncatus spp.*	1	10	1	Hy	20	Hy1	Sb	0	Sh	Sh (A)	Sh (G)	Sh (C)	Sh (G)	Sh (C)	0	0	0

Supplementary Table 4.1. Complete *Bulinus* spp. dataset used in this study providing snail collection information and species identifications.

ID	Species_ID	Species_simplified	Country	Region	Site	Lat_cor	Lon_cor	Patent trematode infection?	Schisto_haplotype	schisto_haplotype_gr
Pem_Wingwi_1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wingwi	Wingwi	-5.02793	39.80836	no		
UNG-Jendele-134.1	B_nasutus_7	*Bulinus nasutus nasutus*	Unguja Island	Jendele	Forodhani	-6.18935	39.37102	no		
UNG-Miwani-123.1	B_globosus_3a	*Bulinus globosus*	Unguja Island	Miwani	Kinumoshi	-6.08857	39.27673	*S. haematobium*	Sh10	2
UNG-Kinya-Ziwani-A+ve (MCF389B0F0286)	B_globosus_3b	*Bulinus globosus*	Unguja Island	Kinyasini (Unguja)	Ziwani A (Mrogoro)	-5.96415	39.30687	*S. haematobium*	?	?
UNG-Mfenesini-135.1	B_nasutus_6	*Bulinus nasutus nasutus*	Unguja Island	Mtopepo	Mfenesini	-6.14353	39.22935	no		
UNG-Chaani-12-17.1	B_globosus_3c	*Bulinus globosus*	Unguja Island	Chaani	Chaani site 12	-5.93717	39.29730	no		
UNG-Mtende-ZJ-1	B_nasutus_8	*Bulinus nasutus nasutus*	Unguja Island	Mtende	Ziwa Jangwa	-6.44600	39.52938	no		
UNG-Mtende-ZJ-2	B_nasutus_7	*Bulinus nasutus nasutus*	Unguja Island	Mtende	Ziwa Jangwa	-6.44600	39.52938	no		
UNG-Mtende-Mta.-1	B_nasutus_8	*Bulinus nasutus nasutus*	Unguja Island	Mtende	Mtajabuni	-6.44242	39.53072	no		
UNG-Mtende-Mta.-2	B_nasutus_8	*Bulinus nasutus nasutus*	Unguja Island	Mtende	Mtajabuni	-6.44242	39.53072	no		
UNG-KizDim-Hur.-1	B_nasutus_3	*Bulinus nasutus nasutus*	Unguja Island	Kizimkazi Dimbani	Hurumzi	-6.42775	39.46262	no		
UNG-KizDim-Hur.-2	B_nasutus_7	*Bulinus nasutus nasutus*	Unguja Island	Kizimkazi Dimbani	Hurumzi	-6.42775	39.46262	no		
SP_Kin2.3.1	*B_globosus_1a*	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya2	-5.02033	39.73855	*S. haematobium*	Sh 17 & Sh 9	2
SP_Kin4.5.3	*B_globosus_2a*	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya6	-5.03560	39.73850	*S. bovis*	Sb1	NA
SP_Kin4.5.10	*B_globosus_2a*	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya6	-5.03560	39.73850	*S. bovis*	Sb1 & Sb2	NA
SP_Kin4.6.7	*B_globosus_2a*	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya6	-5.03560	39.73850	*S. bovis*	Sb2	NA
SP_Kin4.8.3	*B_globosus_2a*	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya6	-5.03560	39.73850	*S. bovis*	Sb1	NA
SP_Kin4.9.11	*B_globosus_2a*	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya6	-5.03560	39.73850	*S. bovis*	Sb2	NA
SP_Cham10.1a	*B_globosus_2a*	*Bulinus globosus*	Pemba Island	Chambani	Cham10	-5.35805	39.79182	*S. haematobium*	Sh13	2
SP_Kiz2.1	*B_globosus_1a*	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz2	-5.04835	39.74305	*Euclinostomum spp.*		
SP_Wam10.1	*B_globosus_2a*	*Bulinus globosus*	Pemba Island	Wambaa	Wam10	-5.30045	39.68438	no		
SP_Wam4.1	*B_globosus_1a*	*Bulinus globosus*	Pemba Island	Wambaa	Wam4	-5.30087	39.68325	no		
SP_Wam4.2	*B_globosus_1a*	*Bulinus globosus*	Pemba Island	Wambaa	Wam4	-5.30087	39.68325	no		
SP_Cham9.1	*B_nasutus_5*	*Bulinus nasutus nasutus*	Pemba Island	Chambani	Cham9	-5.35862	39.79255	no		
SP_Cham9.2	*B_nasutus_5*	*Bulinus nasutus nasutus*	Pemba Island	Chambani	Cham9	-5.35862	39.79255	no		
SP_Cham10.1b	*B_globosus_1a*	*Bulinus globosus*	Pemba Island	Chambani	Cham10	-5.35805	39.79182	no		
SP_Cham10.2	*B_globosus_2a*	*Bulinus globosus*	Pemba Island	Chambani	Cham10	-5.35805	39.79182	no		
SP_Cham10.3	*B_globosus_2a*	*Bulinus globosus*	Pemba Island	Chambani	Cham10	-5.35805	39.79182	no		
SP_Cham10.4	*B_globosus_2a*	*Bulinus globosus*	Pemba Island	Chambani	Cham10	-5.35805	39.79182	no		
SP_Cham10.5	*B_globosus_2a*	*Bulinus globosus*	Pemba Island	Chambani	Cham10	-5.35805	39.79182	no		
SP_Cham10.6	*B_globosus_2a*	*Bulinus globosus*	Pemba Island	Chambani	Cham10	-5.35805	39.79182	no		
SP_Cham1.1	*B_globosus_1a*	*Bulinus globosus*	Pemba Island	Chambani	Cham1	-5.32000	39.77412	no		
SP_Kiz1.1	*B_globosus_1b*	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz1	-5.05337	39.74040	no		
SP_Kiz2.2	*B_globosus_1a*	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz2	-5.04835	39.74305	no		
SP_Kiz4.1	*B_globosus_2a*	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz4	-5.04872	39.74020	no		
SP_Kiz4.2	*B_globosus_2a*	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz4	-5.04872	39.74020	no		
SP_Kiz4.3	*B_globosus_1a*	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz4	-5.04872	39.74020	no		
SP_Wawi1.1	*B_globosus_1a*	*Bulinus globosus*	Pemba Island	Wawi	Waw1	-5.24493	39.78955	no		
SP_Wawi1.2	*B_globosus_1a*	*Bulinus globosus*	Pemba Island	Wawi	Waw1	-5.24493	39.78955	no		
SP_Wawi1.3	*B_globosus_1a*	*Bulinus globosus*	Pemba Island	Wawi	Waw1	-5.24493	39.78955	no		
SP_Uwa2.1	*B_nasutus_4*	*Bulinus nasutus nasutus*	Pemba Island	Uwandani	Uwa2	-5.19583	39.82658	no		
SP_Uwa3.1	*B_nasutus_4*	*Bulinus nasutus nasutus*	Pemba Island	Uwandani	Uwa3	-5.19865	39.82723	no		

SP_Uwa4.1	*B_nasutus_4*	*Bulinus nasutus nasutus*	Pemba Island	Uwandani	Uwa4	-5.19762	39.83002	no		
SP_Uwa5.1	*B_nasutus_4*	*Bulinus nasutus nasutus*	Pemba Island	Uwandani	Uwa5	-5.20082	39.82897	no		
SP_Uwa5.2	*B_nasutus_1*	*Bulinus nasutus nasutus*	Pemba Island	Uwandani	Uwa5	-5.20082	39.82897	no		
SP_Uwa6.1	*B_nasutus_9*	*Bulinus nasutus nasutus*	Pemba Island	Uwandani	Uwa6	-5.20398	39.83438	no		
SP_Kinya1.1	*B_globosus_1a*	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya1	-5.02062	39.73972	no		
SP_Kinya1.2	*B_globosus_1f*	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya1	-5.02062	39.73972	no		
SP_Kinya2.1	*B_globosus_1e*	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya2	-5.02033	39.73855	no		
SP_Kinya6.1	*B_globosus_2a*	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya6	-5.03560	39.73850	no		
SP_Kinya6.2	*B_globosus_2a*	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya6	-5.03560	39.73850	no		
SP_Kinya6.3	*B_globosus_2a*	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya6	-5.03560	39.73850	no		
SP_Kinya6.4	*B_globosus_2a*	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya6	-5.03560	39.73850	no		
SP_Kinya6.5	*B_globosus_2a*	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya6	-5.03560	39.73850	no		
SP_Kinya9.1	*B_globosus_2a*	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya9	-5.03077	39.73333	no		
SP_Kinya11.1	*B_globosus_2a*	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya11	-5.03717	39.73208	no		
SP_Kinya12.1	*B_globosus_2a*	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya12	-5.03705	39.74692	no		
SP_Uwa3.2	*B_nasutus_4*	*Bulinus nasutus nasutus*	Pemba Island	Uwandani	Uwa3	-5.19865	39.82723	no		
SP_Uwa4.2	*B_nasutus_10*	*Bulinus nasutus nasutus*	Pemba Island	Uwandani	Uwa4	-5.19762	39.83002	no		
SP_Uwa5.3	*B_nasutus_4*	*Bulinus nasutus nasutus*	Pemba Island	Uwandani	Uwa5	-5.20082	39.82897	no		
SP_Uwa6.2	*B_nasutus_9*	*Bulinus nasutus nasutus*	Pemba Island	Uwandani	Uwa6	-5.20398	39.83438	no		
S2_Kinya9.1	*B_globosus_2a*	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya9	-5.03077	39.73333	*S. haematobium*	Sh9	2
S2_Kinya9.2	*B_globosus_2a*	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya9	-5.03077	39.73333	*S. haematobium*	Sh17	2
S2_Kinya9.3	*B_globosus_2a*	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya9	-5.03077	39.73333	*S. haematobium*	Sh9	2
S2_Kinya9.4	*B_globosus_2a*	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya9	-5.03077	39.73333	*S. haematobium*	Sh17	2
S2_Kinya9.5	*B_globosus_2a*	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya9	-5.03077	39.73333	*S. haematobium*	Sh9	2
S2_Kinya6.1	*B_globosus_2a*	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya6	-5.03560	39.73850	*S. haematobium*	Sh18	2
S2_Kinya11.1	*B_globosus_2a*	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya11	-5.03717	39.73208	*S. haematobium*	Sh17	2
S2_Kiz5.1	*B_globosus_2a*	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz5	-5.04782	39.73830	*S. haematobium*	Sh10	2
S2_Kiz4.1	*B_globosus_2a*	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz4	-5.04872	39.74020	*S. haematobium*	Sh9	2
S2_Uku2.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku2	-5.34282	39.74940	no		
S1_Mat6.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat6	-5.30038	39.77640	no		
S2_Puj2.1	*B_nasutus_1*	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj2	-5.28427	39.81677	no		
S2_Ole13.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Ole	Ole13	-5.19982	39.79393	no		
S2_Uku3.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku3	-5.34355	39.74808	no		
S1_Mat8.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat8	-5.30138	39.77112	no		
S2_Puj3.1	*B_nasutus_2*	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj3	-5.28767	39.81813	no		
S2_Uku4.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku4	-5.34390	39.75533	no		
S2_Uku4.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku4	-5.34390	39.75533	no		
S2_Uku5.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku5	-5.34355	39.75690	no		
S2_Uku6.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku6	-5.34422	39.75848	no		
S2_Uku7.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku7	-5.34450	39.75967	no		
S2_Uku9.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku9	-5.35512	39.76063	no		
S1_Mat9.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat9	-5.29903	39.77168	no		
S1_Mat10.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat10	-5.29747	39.77023	no		
S1_Puj4.1	*B_nasutus_2*	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj4	-5.29000	39.81627	no		
S2_Puj5.1	*B_nasutus_1*	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj5	-5.29160	39.81463	no		
S1_Puj9.1	*B_nasutus_1*	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj9	-5.31440	39.81087	no		
S2_Puj10.1	*B_nasutus_1*	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj10	-5.31627	39.81052	no		
S2_Puj10.2	*B_nasutus_2*	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj10	-5.31627	39.81052	no		
S3_Kinya3.1	*B_globosus_1a*	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya3	-5.02032	39.73722	no		
S1_Kinya4.1	*B_globosus_1a*	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya4	-5.02008	39.73597	no		
S3_Kinya5.1	*B_globosus_1a*	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya5	-5.01983	39.74673	no		
S3_Mat1.1	*B_globosus_1a*	*Bulinus globosus*	Pemba Island	Matale	Mat1	-5.28180	39.76830	no		
S3_Puj11.1	*B_nasutus_2*	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj11	-5.28853	39.81882	no		
S3_Uku1.1	*B_globosus_1a*	*Bulinus globosus*	Pemba Island	Ukutini	Uku1	-5.34380	39.75430	no		
S4_Uku8.1	*B_globosus_1a*	*Bulinus globosus*	Pemba Island	Ukutini	Uku8	-5.33978	39.76675	no		
S3_Uku10.1	*B_globosus_1a*	*Bulinus globosus*	Pemba Island	Ukutini	Uku10	-5.35428	39.76762	no		

S3_Wam11.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam11	-5.30117	39.68070	no		
S4_Kin_6.1+ve	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya6	-5.03560	39.73850	*S. bovis*	Sb1	NA
S4_Kin_6.2+ve	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya6	-5.03560	39.73850	*S. bovis*	Sb2	NA
S4_Kin_6.3+ve	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya6	-5.03560	39.73850	*S. haematobium*	Sh1	1
S3_Wawi5.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wawi	Waw5	-5.25847	39.79327	no		
S3_Mat2.1	*B_globosus_1a*	*Bulinus globosus*	Pemba Island	Matale	Mat2	-5.29962	39.78343	no		
S4_Mat7.1	*B_globosus_1a*	*Bulinus globosus*	Pemba Island	Matale	Mat7	-5.30133	39.77372	no		
S4_Mat12.1	*B_globosus_1a*	*Bulinus globosus*	Pemba Island	Matale	Mat12	-5.30005	39.78260	no		
S2_Wam2.1	*B_globosus_1a*	*Bulinus globosus*	Pemba Island	Wambaa	Wam2	-5.29622	39.68607	no		
S1_Wawi4.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wawi	Waw4	-5.25796	39.79491	no		
S1_Uwa8.1	*B_nasutus_4*	*Bulinus nasutus nasutus*	Pemba Island	Uwandani	Uwa8	-5.20052	39.83583	no		
S2_Wam3.1	*B_globosus_1a*	*Bulinus globosus*	Pemba Island	Wambaa	Wam3	-5.29837	39.68533	no		
S2_Wam5.1	*B_globosus_1a*	*Bulinus globosus*	Pemba Island	Wambaa	Wam5	-5.29975	39.68252	no		
S2_Wam6.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam6	-5.29487	39.68693	no		
S2_Wam7.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam7	-5.29338	39.68913	no		
S2_Wam8.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam8	-5.29100	39.68697	no		
S1_Wam9.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam9	-5.29228	39.68833	no		
1_ xS1_Wam2.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam2	-5.29622	39.68607	no		
2_ xS1_Wam2.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam2	-5.29622	39.68607	no		
3_ xS1_Wam2.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam2	-5.29622	39.68607	no		
4_ xS1_Wam3.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam3	-5.29837	39.68533	no		
5_ xS1_Wam3.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam3	-5.29837	39.68533	no		
6_ xS1_Wam3.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam3	-5.29837	39.68533	no		
7_ xS1_Wam4.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam4	-5.30087	39.68325	no		
8_ xS1_Wam4.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam4	-5.30087	39.68325	no		
9_ xS1_Wam4.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam4	-5.30087	39.68325	no		
10_ xS1_Wam5.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam5	-5.29975	39.68252	no		
11_ xS1_Wam5.2	Unknown	Unknown	Pemba Island	Wambaa	Wam5	-5.29975	39.68252	no		
12_ xS1_Wam5.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam5	-5.29975	39.68252	no		
13_ xS1_Wam6.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam6	-5.29487	39.68693	no		
14_ xS1_Wam6.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam6	-5.29487	39.68693	no		
15_ xS1_Wam6.3	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam6	-5.29487	39.68693	no		
16_ xS1_Wam7.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam7	-5.29338	39.68913	no		
17_ xS1_Wam7.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam7	-5.29338	39.68913	no		
18_ xS1_Wam7.3	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam7	-5.29338	39.68913	no		
19_ xS1_Wam8.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam8	-5.29100	39.68697	no		
20_ xS1_Wam8.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam8	-5.29100	39.68697	no		
21_ xS1_Wam8.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam8	-5.29100	39.68697	no		
22_ xS1_Wam9.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam9	-5.29228	39.68833	no		
23_ xS1_Wam9.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam9	-5.29228	39.68833	no		
24_ xS1_Wam9.3	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam9	-5.29228	39.68833	no		
25_ xS1_Mat6.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat6	-5.30038	39.77640	no		
26_ xS1_Mat6.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat6	-5.30038	39.77640	no		
27_ xS1_Mat6.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat6	-5.30038	39.77640	no		
28_ xS1_Mat8.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat8	-5.30138	39.77112	no		
29_ xS1_Mat8.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat8	-5.30138	39.77112	no		
30_ xS1_Mat8.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat8	-5.30138	39.77112	no		
31_ xS1_Mat9.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat9	-5.29903	39.77168	no		
32_ xS1_Mat9.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat9	-5.29903	39.77168	no		
33_ xS1_Mat9.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat9	-5.29903	39.77168	no		
34_ xS1_Mat10.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat10	-5.29747	39.77023	no		
35_ xS1_Mat10.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat10	-5.29747	39.77023	no		
36_ xS1_Mat10.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat10	-5.29747	39.77023	no		
37_ xS1_Puj2.1	B_nasutus_2	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj2	-5.28427	39.81677	no		
38_ xS1_Puj2.2	B_nasutus_2	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj2	-5.28427	39.81677	no		
39_ xS1_Puj2.3	B_nasutus_2	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj2	-5.28427	39.81677	no		

40_ xS1_Puj3.1	B_nasutus_1	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj3	-5.28767	39.81813	no		
41_ xS1_Puj3.2	B_nasutus_2	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj3	-5.28767	39.81813	no		
42_ xS1_Puj3.3	B_nasutus_2	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj3	-5.28767	39.81813	no		
43_ xS1_Puj4.1	B_nasutus_2	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj4	-5.29000	39.81627	no		
44_ xS1_Puj4.2	B_nasutus_2	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj4	-5.29000	39.81627	no		
45_ xS1_Puj4.3	B_nasutus_2	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj4	-5.29000	39.81627	no		
46_ xS1_Puj5.1	B_nasutus_1	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj5	-5.29160	39.81463	no		
47_ xS1_Puj5.2	B_nasutus_2	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj5	-5.29160	39.81463	no		
48_ xS1_Puj5.3	B_nasutus_2	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj5	-5.29160	39.81463	no		
49_ xS1_Puj9.1	B_nasutus_1	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj9	-5.31440	39.81087	no		
50_ xS1_Puj9.2	B_nasutus_1	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj9	-5.31440	39.81087	no		
51_ xS1_Puj9.3	B_nasutus_1	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj9	-5.31440	39.81087	no		
52_ xS1_Puj10.1	B_nasutus_1	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj10	-5.31627	39.81052	no		
53_ xS1_Puj10.2	B_nasutus_1	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj10	-5.31627	39.81052	no		
54_ xS1_Puj10.3	B_nasutus_2	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj10	-5.31627	39.81052	no		
55_ xS1_Kinya1.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya1	-5.02062	39.73972	no		
56_ xS1_Kinya1.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya1	-5.02062	39.73972	no		
57_ xS1_Kinya1.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya1	-5.02062	39.73972	no		
58_ xS1_Kinya3.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya3	-5.02032	39.73722	no		
59_ xS1_Kinya3.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya3	-5.02032	39.73722	no		
60_ xS1_Kinya3.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya3	-5.02032	39.73722	no		
61_ xS1_Kinya4.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya4	-5.02008	39.73597	no		
62_ xS1_Kinya4.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya4	-5.02008	39.73597	no		
63_ xS1_Kinya5.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya5	-5.01983	39.74673	no		
64_ xS1_Kinya5.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya5	-5.01983	39.74673	no		
65_ xS1_Kinya5.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya5	-5.01983	39.74673	no		
66_ xS1_Kinya6.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya6	-5.03560	39.73850	no		
67_ xS1_Kinya6.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya6	-5.03560	39.73850	no		
68_ xS1_Kinya6.3	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya6	-5.03560	39.73850	no		
69_ xS1_Kinya9.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya9	-5.03077	39.73333	no		
70_ xS1_Kinya9.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya9	-5.03077	39.73333	no		
71_ xS1_Kinya9.3	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya9	-5.03077	39.73333	no		
72_ xS1_Kinya11.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya11	-5.03717	39.73208	no		
73_ xS1_Kinya11.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya11	-5.03717	39.73208	no		
74_ xS1_Kinya11.3	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya11	-5.03717	39.73208	no		
75_ xS1_Kinya12.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya12	-5.03705	39.74692	no		
76_ xS1_Kinya12.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya12	-5.03705	39.74692	no		
77_ xS1_Kinya12.3	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya12	-5.03705	39.74692	no		
78_ xS1_Kiz1.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz1	-5.05337	39.74040	no		
79_ xS1_Kiz1.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz1	-5.05337	39.74040	no		
80_ xS1_Kiz1.3	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz1	-5.05337	39.74040	no		
81_ xS1_Kiz2.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz2	-5.04835	39.74305	no		
82_ xS1_Kiz2.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz2	-5.04835	39.74305	no		
83_ xS1_Kiz2.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz2	-5.04835	39.74305	no		
84_ xS1_Kiz4.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz4	-5.04872	39.74020	no		
85_ xS1_Kiz4.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz4	-5.04872	39.74020	no		
86_ xS1_Kiz4.3	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz4	-5.04872	39.74020	no		
87_ xS1_Kinya9.4	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya9	-5.03077	39.73333	no		
88_ xS1_Kinya9.5	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya9	-5.03077	39.73333	no		
89_ xS1_Kinya9.6	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya9	-5.03077	39.73333	no		
90_ xS1_Waw1.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wawi	Waw1	-5.24493	39.78955	no		
91_ xS1_Waw1.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wawi	Waw1	-5.24493	39.78955	no		
92_ xS1_Waw1.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wawi	Waw1	-5.24493	39.78955	no		
93_ xS1_Waw4.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wawi	Waw4	-5.25796	39.79491	no		
94_ xS1_Waw4.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wawi	Waw4	-5.25796	39.79491	no		
95_ xS1_Waw4.3	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wawi	Waw4	-5.25796	39.79491	no		

96_ xS1_Ole13.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Ole	Ole13	-5.19982	39.79393	no		
97_ xS1_Ole13.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Ole	Ole13	-5.19982	39.79393	no		
98_ xS1_Ole13.3	B_globosus_2a	*Bulinus globosus*	Pemba Island	Ole	Ole13	-5.19982	39.79393	no		
99_ xS2_Wam2.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam2	-5.29622	39.68607	no		
100_ xS2_Wam2.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam2	-5.29622	39.68607	no		
101_ xS2_Wam2.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam2	-5.29622	39.68607	no		
102_ xS2_Wam3.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam3	-5.29837	39.68533	no		
103_ xS2_Wam3.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam3	-5.29837	39.68533	no		
104_ xS2_Wam3.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam3	-5.29837	39.68533	no		
105_ xS2_Wam4.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam4	-5.30087	39.68325	no		
106_ xS2_Wam4.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam4	-5.30087	39.68325	no		
107_ xS2_Wam4.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam4	-5.30087	39.68325	no		
108_ xS2_Wam5.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam5	-5.29975	39.68252	no		
109_ xS2_Wam5.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam5	-5.29975	39.68252	no		
110_ xS2_Wam5.3	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam5	-5.29975	39.68252	no		
111_ xS2_Wam6.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam6	-5.29487	39.68693	no		
112_ xS2_Wam6.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam6	-5.29487	39.68693	no		
113_ xS2_Wam6.3	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam6	-5.29487	39.68693	no		
114_ xS2_Wam8.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam8	-5.29100	39.68697	no		
115_ xS2_Wam8.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam8	-5.29100	39.68697	no		
116_ xS2_Wam8.3	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam8	-5.29100	39.68697	no		
117_ xS2_Wam10.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam10	-5.30045	39.68438	no		
118_ xS2_Puj2.1	B_nasutus_2	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj2	-5.28427	39.81677	no		
119_ xS2_Puj2.2	B_nasutus_2	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj2	-5.28427	39.81677	no		
120_ xS2_Puj2.3	B_nasutus_3	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj2	-5.28427	39.81677	no		
121_ xS2_Puj3.1	B_nasutus_2	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj3	-5.28767	39.81813	no		
122_ xS2_Puj3.2	B_nasutus_2	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj3	-5.28767	39.81813	no		
123_ xS2_Puj3.3	B_nasutus_1	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj3	-5.28767	39.81813	no		
124_ xS2_Puj5.1	B_nasutus_1	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj5	-5.29160	39.81463	no		
125_ xS2_Puj5.2	B_nasutus_1	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj5	-5.29160	39.81463	no		
126_ xS2_Puj5.3	B_nasutus_1	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj5	-5.29160	39.81463	no		
127_ xS2_Puj10.1	B_nasutus_1	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj10	-5.31627	39.81052	no		
128_ xS2_Puj10.2	B_nasutus_1	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj10	-5.31627	39.81052	no		
129_ xS2_Puj10.3	B_nasutus_1	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj10	-5.31627	39.81052	no		
130_ xS2_Kinya1.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya1	-5.02062	39.73972	no		
131_ xS2_Kinya1.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya1	-5.02062	39.73972	no		
132_ xS2_Kinya1.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya1	-5.02062	39.73972	no		
133_ xS2_Kinya2.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya2	-5.02033	39.73855	no		
134_ xS2_Kinya2.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya2	-5.02033	39.73855	no		
135_ xS2_Kinya2.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya2	-5.02033	39.73855	no		
136_ xS2_Kinya3.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya3	-5.02032	39.73722	no		
137_ xS2_Kinya3.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya3	-5.02032	39.73722	no		
138_ xS2_Kinya3.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya3	-5.02032	39.73722	no		
139_ xS2_Kinya5.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya5	-5.01983	39.74673	no		
140_ xS2_Kinya5.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya5	-5.01983	39.74673	no		
141_ xS2_Kinya5.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya5	-5.01983	39.74673	no		
142_ xS2_Kinya6.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya6	-5.03560	39.73850	no		
143_ xS2_Kinya6.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya6	-5.03560	39.73850	no		
144_ xS2_Kinya6.3	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya6	-5.03560	39.73850	no		
145_ xS2_Kinya9.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya9	-5.03077	39.73333	no		
146_ xS2_Kinya9.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya9	-5.03077	39.73333	no		
147_ xS2_Kinya9.3	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya9	-5.03077	39.73333	no		
148_ xS2_Kinya11.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya11	-5.03717	39.73208	no		
149_ xS2_Kinya11.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya11	-5.03717	39.73208	no		
150_ xS2_Kinya11.3	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya11	-5.03717	39.73208	no		
151_ xS2_Kinya12.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya12	-5.03705	39.74692	no		

152_ xS2_Kinya12.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya12	-5.03705	39.74692	no		
153_ xS2_Kinya12.3	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya12	-5.03705	39.74692	no		
154_ xS2_Kiz1.1	B_globosus_1b	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz1	-5.05337	39.74040	no		
155_ xS2_Kiz1.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz1	-5.05337	39.74040	no		
156_ xS2_Kiz1.3	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz1	-5.05337	39.74040	no		
157_ xS2_Kiz2.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz2	-5.04835	39.74305	no		
157_ xS2_Kiz2.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz2	-5.04835	39.74305	no		
159_ xS2_Kiz2.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz2	-5.04835	39.74305	no		
160_ xS2_Kiz4.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz4	-5.04872	39.74020	no		
161_ xS2_Kiz4.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz4	-5.04872	39.74020	no		
162_ xS2_Kiz4.3	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz4	-5.04872	39.74020	no		
163_ xS2_Kiz5.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz5	-5.04782	39.73830	no		
164_ xS2_Kiz5.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz5	-5.04782	39.73830	no		
165_ xS2_Kiz5.3	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz5	-5.04782	39.73830	no		
166_ xS2_Waw1.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wawi	Waw1	-5.24493	39.78955	no		
167_ xS2_Waw1.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wawi	Waw1	-5.24493	39.78955	no		
168_ xS2_Waw1.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wawi	Waw1	-5.24493	39.78955	no		
169_ xS2_Waw5.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wawi	Waw5	-5.25847	39.79327	no		
170_ xS2_Ole13.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Ole	Ole13	-5.19982	39.79393	no		
171_ xS2_Ole13.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Ole	Ole13	-5.19982	39.79393	no		
172_ xS2_Ole13.3	Lanistes	*Lanistes*	Pemba Island	Ole	Ole13	-5.19982	39.79393	no		
173_ xS2_Uku2.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku2	-5.34282	39.74940	no		
174_ xS2_Uku2.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku2	-5.34282	39.74940	no		
175_ xS2_Uku2.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku2	-5.34282	39.74940	no		
176_ xS2_Uku3.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku3	-5.34355	39.74808	no		
177_ xS2_Uku3.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku3	-5.34355	39.74808	no		
178_ xS2_Uku3.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku3	-5.34355	39.74808	no		
179_ xs2_Uku4.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku4	-5.34390	39.75533	no		
180_ xS2_Uku4.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku4	-5.34390	39.75533	no		
181_ xS2_Uku4.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku4	-5.34390	39.75533	no		
182_ xS2_Uku5.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku5	-5.34355	39.75690	no		
183_ xS2_Uku5.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku5	-5.34355	39.75690	no		
184_ xS2_Uku5.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku5	-5.34355	39.75690	no		
185_ xS2_Uku6.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku6	-5.34422	39.75848	no		
186_ xS2_Uku6.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku6	-5.34422	39.75848	no		
187_ xS2_Uku6.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku6	-5.34422	39.75848	no		
188_ xS2_Uku7.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku7	-5.34450	39.75967	no		
189_ xS2_Uku7.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku7	-5.34450	39.75967	no		
190_ xS2_Uku7.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku7	-5.34450	39.75967	no		
191_ xS2_Uku9.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku9	-5.35512	39.76063	no		
192_ xS2_Uku9.2	B_globosus_1c	*Bulinus globosus*	Pemba Island	Ukutini	Uku9	-5.35512	39.76063	no		
193_ xS2_Uku9.3	B_globosus_1c	*Bulinus globosus*	Pemba Island	Ukutini	Uku9	-5.35512	39.76063	no		
194_ xS3_Wam4.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam4	-5.30087	39.68325	no		
195_ xS3_Wam4.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam4	-5.30087	39.68325	no		
196_ xS3_Wam4.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam4	-5.30087	39.68325	no		
197_ xS3_Wam5.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam5	-5.29975	39.68252	no		
198_ xS3_Wam5.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam5	-5.29975	39.68252	no		
199_ xS3_Wam5.3	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam5	-5.29975	39.68252	no		
200_ xS3_Wam6.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam6	-5.29487	39.68693	no		
201_ xS3_Wam6.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam6	-5.29487	39.68693	no		
202_ xS3_Wam6.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam6	-5.29487	39.68693	no		
203_ xS3_Wam8.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam8	-5.29100	39.68697	no		
204_ xS3_Wam8.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam8	-5.29100	39.68697	no		
205_ xS3_Wam8.3	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam8	-5.29100	39.68697	no		
206_ xS3_Wam10.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam10	-5.30045	39.68438	no		
207_ xS3_Wam10.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam10	-5.30045	39.68438	no		

208_ xS3_Wam11.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam11	-5.30117	39.68070	no		
209_ xS3_Wam11.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam11	-5.30117	39.68070	no		
210_ xS3_Wam11.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam11	-5.30117	39.68070	no		
211_ xS3_Mat1.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat1	-5.28180	39.76830	no		
212_ xS3_Mat1.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat1	-5.28180	39.76830	no		
213_ xS3_Mat1.3	B_globosus_1d	*Bulinus globosus*	Pemba Island	Matale	Mat1	-5.28180	39.76830	no		
214_ xS3_Mat2.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat2	-5.29962	39.78343	no		
215_ xS3_Mat2.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat2	-5.29962	39.78343	no		
216_ xS3_Mat2.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat2	-5.29962	39.78343	no		
217_ xS3_Mat6.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat6	-5.30038	39.77640	no		
218_ xS3_Mat6.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat6	-5.30038	39.77640	no		
219_ xS3_Mat6.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat6	-5.30038	39.77640	no		
220_ xS3_Mat8.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat8	-5.30138	39.77112	no		
221_ xS3_Mat8.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat8	-5.30138	39.77112	no		
222_ xS3_Mat10.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat10	-5.29747	39.77023	no		
223_ xS3_Mat10.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat10	-5.29747	39.77023	no		
224_ xS3_Mat10.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat10	-5.29747	39.77023	no		
225_ xS3_Puj2.1	B_nasutus_1	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj2	-5.28427	39.81677	no		
226_ xS3_Puj2.2	B_nasutus_2	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj2	-5.28427	39.81677	no		
227_ xS3_Puj2.3	B_nasutus_2	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj2	-5.28427	39.81677	no		
228_ xS3_Puj3.1	B_nasutus_2	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj3	-5.28767	39.81813	no		
229_ xS3_Puk3.2	B_nasutus_2	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj3	-5.28767	39.81813	no		
230_ xS3_Puj3.3	B_nasutus_2	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj3	-5.28767	39.81813	no		
231_ xS3_Puk4.1	B_nasutus_1	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj4	-5.29000	39.81627	no		
232_ xS3_Puj4.2	B_nasutus_1	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj4	-5.29000	39.81627	no		
233_ XS3_Puj4.3	B_nasutus_2	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj4	-5.29000	39.81627	no		
234_ xS3_Puj5.1	B_nasutus_1	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj5	-5.29160	39.81463	no		
235_ xS3_Puj5.2	B_nasutus_1	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj5	-5.29160	39.81463	no		
236_ xS3_Puj9.1	B_nasutus_1	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj9	-5.31440	39.81087	no		
237_ xS3_Puj10.1	B_nasutus_1	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj10	-5.31627	39.81052	no		
238_ xS3_Puj10.2	B_nasutus_1	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj10	-5.31627	39.81052	no		
239_ xS3_Puj10.3	B_nasutus_1	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj10	-5.31627	39.81052	no		
240_ xS3_Puj11.1	B_nasutus_2	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj11	-5.28853	39.81882	no		
241_ xS3_Puj11.2	B_nasutus_1	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj11	-5.28853	39.81882	no		
242_ xS3_Puj11.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Pujini	Puj11	-5.28853	39.81882	no		
243_ xS3_Kinya2.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya2	-5.02033	39.73855	no		
244_ xS3_Kinya2.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya2	-5.02033	39.73855	no		
245_ xS3_Kinya2.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya2	-5.02033	39.73855	no		
246_ xS3_Kinya3.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya3	-5.02032	39.73722	no		
247_ xS3_Kinya3.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya3	-5.02032	39.73722	no		
248_ xS3_Kinya3.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya3	-5.02032	39.73722	no		
249_ xS3_Kinya4.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya4	-5.02008	39.73597	no		
250_ xS3_Kinya4.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya4	-5.02008	39.73597	no		
251_ xS3_Kinya4.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya4	-5.02008	39.73597	no		
252_ xS3_Kinya5.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya5	-5.01983	39.74673	no		
253_ xS3_Kinya5.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya5	-5.01983	39.74673	no		
254_ xS3_Kinya5.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya5	-5.01983	39.74673	no		
255_ xS3_Kinya6.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya6	-5.03560	39.73850	no		
256_ xS3_Kinya6.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya6	-5.03560	39.73850	no		
257_ xS3_Kinya6.3	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya6	-5.03560	39.73850	no		
258_ xS3_Kinya9.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya9	-5.03077	39.73333	no		
259_ xS3_Kinya9.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya9	-5.03077	39.73333	no		
260_ xS3_Kinya9.3	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya9	-5.03077	39.73333	no		
261_ xS3_Kinya11.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya11	-5.03717	39.73208	no		
262_ xS3_Kinya11.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya11	-5.03717	39.73208	no		
263_ xS3_Kinya11.3	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya11	-5.03717	39.73208	no		

264_ xS3_Kinya12.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya12	-5.03705	39.74692	no		
265_ xS3_Kinya12.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya12	-5.03705	39.74692	no		
266_ xS3_Kiz1.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz1	-5.05337	39.74040	no		
267_ xS3_Kiz1.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz1	-5.05337	39.74040	no		
268_ xS3_Kiz1.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz1	-5.05337	39.74040	no		
269_ xS3_Kiz2.1	B_globosus_1e	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz2	-5.04835	39.74305	no		
270_ xS3_Kiz2.2	B_globosus_1e	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz2	-5.04835	39.74305	no		
271_ xS3_Kiz2.3	B_globosus_1e	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz2	-5.04835	39.74305	no		
272_ xS3_Kiz4.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz4	-5.04872	39.74020	no		
273_ xS3_Kiz4.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz4	-5.04872	39.74020	no		
274_ xS3_Kiz4.3	B_globosus_1e	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz4	-5.04872	39.74020	no		
275_ xS3_Kiz5.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz5	-5.04782	39.73830	no		
276_ xS3_Kiz5.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz5	-5.04782	39.73830	no		
277_ xS3_Kiz5.3	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz5	-5.04782	39.73830	no		
278_ xS3_Uku1.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku1	-5.34380	39.75430	no		
279_ xS3_Uku1.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku1	-5.34380	39.75430	no		
280_ xS3_Uku1.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku1	-5.34380	39.75430	no		
281_ xS3_Uku2.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku2	-5.34282	39.74940	no		
282_ xS3_Uku2.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku2	-5.34282	39.74940	no		
283_ xS3_Uku2.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku2	-5.34282	39.74940	no		
284_xS3_Uku3.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku3	-5.34355	39.74808	no		
285_xS3_Uku3.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku3	-5.34355	39.74808	no		
286_xS3_Uku3.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku3	-5.34355	39.74808	no		
287_xS3_Uku4.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku4	-5.34390	39.75533	no		
288_xS3_Uku4.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku4	-5.34390	39.75533	no		
289_xS3_Uku4.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku4	-5.34390	39.75533	no		
290_xS3_Uku5.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku5	-5.34355	39.75690	no		
291_xS3_Uku5.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku5	-5.34355	39.75690	no		
292_xS3_Uku5.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku5	-5.34355	39.75690	no		
293_xS3_Uku6.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku6	-5.34422	39.75848	no		
294_xS3_Uku6.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku6	-5.34422	39.75848	no		
295_xS3_Uku6.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku6	-5.34422	39.75848	no		
296_xS3_Uku7.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku7	-5.34450	39.75967	no		
297_xS3_Uku7.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku7	-5.34450	39.75967	no		
298_xS3_Uku7.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku7	-5.34450	39.75967	no		
299_xS3_Uku9.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku9	-5.35512	39.76063	no		
300_xS3_Uku9.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku9	-5.35512	39.76063	no		
301_xS3_Uku9.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku9	-5.35512	39.76063	no		
302_xS3_Uku10.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Ukutini	Uku10	-5.35428	39.76762	no		
303_xS3_Uku10.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku10	-5.35428	39.76762	no		
304_xS3_Uku10.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku10	-5.35428	39.76762	no		
305_xS4_Wam2.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam2	-5.29622	39.68607	no		
306_xS4_Wam2.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam2	-5.29622	39.68607	no		
307_xS4_Wam2.3	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam2	-5.29622	39.68607	no		
308_xS4_Wam3.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam3	-5.29837	39.68533	no		
309_xS4_Wam3.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam3	-5.29837	39.68533	no		
310_xS4_Wam3.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam3	-5.29837	39.68533	no		
311_xS4_Wam4.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam4	-5.30087	39.68325	no		
312_xS4_Wam4.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam4	-5.30087	39.68325	no		
313_xS4_Wam4.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam4	-5.30087	39.68325	no		
314_xS4_Wam5.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam5	-5.29975	39.68252	no		
315_xS4_Wam5.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam5	-5.29975	39.68252	no		
316_xS4_Wam5.3	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam5	-5.29975	39.68252	no		
317_xS4_Wam6.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam6	-5.29487	39.68693	no		
318_xS4_Wam6.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam6	-5.29487	39.68693	no		
319_xS4_Wam6.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam6	-5.29487	39.68693	no		

320_xS4_Wam8.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam8	-5.29100	39.68697	no		
321_xS4_Wam10.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam10	-5.30045	39.68438	no		
322_xS4_Wam10.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam10	-5.30045	39.68438	no		
323_xS4_Wam10.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wambaa	Wam10	-5.30045	39.68438	no		
324_xS4_Wam11.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam11	-5.30117	39.68070	no		
325_xS4_Wam11.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam11	-5.30117	39.68070	no		
326_xS4_Wam11.3	B_globosus_2a	*Bulinus globosus*	Pemba Island	Wambaa	Wam11	-5.30117	39.68070	no		
327_xS4_Mat7.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat7	-5.30133	39.77372	no		
328_xS4_Mat7.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat7	-5.30133	39.77372	no		
329_xS4_Mat7.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat7	-5.30133	39.77372	no		
330_xS4_Mat8.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat8	-5.30138	39.77112	no		
331_xS4_Mat8.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat8	-5.30138	39.77112	no		
332_xS4_Mat8.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat8	-5.30138	39.77112	no		
333_xS4_Mat10.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat10	-5.29747	39.77023	no		
334_xS4_Mat10.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat10	-5.29747	39.77023	no		
335_xS4_Mat10.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat10	-5.29747	39.77023	no		
336_xS4_Mat12.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat12	-5.30005	39.78260	no		
337_xS4_Mat12.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat12	-5.30005	39.78260	no		
338_xS4_Mat12.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Matale	Mat12	-5.30005	39.78260	no		
339_xS4_Kinya1.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya1	-5.02062	39.73972	no		
340_xS4_Kinya2.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya2	-5.02033	39.73855	no		
341_xS4_Kinya2.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya2	-5.02033	39.73855	no		
342_xS4_Kinya2.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya2	-5.02033	39.73855	no		
343_xS4_Kinya3.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya3	-5.02032	39.73722	no		
344_xS4_Kinya3.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya3	-5.02032	39.73722	no		
345_xS4_Kinya3.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya3	-5.02032	39.73722	no		
346_xS4_Kinya4.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya4	-5.02008	39.73597	no		
347_xS4_Kinya4.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya4	-5.02008	39.73597	no		
348_xS4_Kinya4.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya4	-5.02008	39.73597	no		
349_xS4_Kinya5.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya5	-5.01983	39.74673	no		
350_xS4_Kinya5.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya5	-5.01983	39.74673	no		
351_xS4_Kinya5.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya5	-5.01983	39.74673	no		
352_xS4_Kinya6.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya6	-5.03560	39.73850	no		
353_xS4_Kinya6.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya6	-5.03560	39.73850	no		
354 _xS4_Kinya6.3	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya6	-5.03560	39.73850	no		
355_xS4_Kinya9.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya9	-5.03077	39.73333	no		
356_xS4_Kinya9.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya9	-5.03077	39.73333	no		
357_xS4_Kinya9.3	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya9	-5.03077	39.73333	no		
358_xS4_Kinya11.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya11	-5.03717	39.73208	no		
359_xS4_Kinya11.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya11	-5.03717	39.73208	no		
360_xS4_Kinya11.3	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kinyasini	Kinya11	-5.03717	39.73208	no		
361_xS4_Kiz1.1	B_globosus_1f	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz1	-5.05337	39.74040	no		
362_xS4_Kiz1.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz1	-5.05337	39.74040	no		
363_xS4_Kiz2.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz2	-5.04835	39.74305	no		
364_xS4_Kiz2.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz2	-5.04835	39.74305	no		
365_xS4_Kiz2.3	B_globosus_1e	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz2	-5.04835	39.74305	no		
366_xS4_Kiz4.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz4	-5.04872	39.74020	no		
367_xS4_Kiz4.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz4	-5.04872	39.74020	no		
368_xS4_Kiz4.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz4	-5.04872	39.74020	no		
369_xS4_Kiz5.1	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz5	-5.04782	39.73830	no		
370_xS4_Kiz5.2	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz5	-5.04782	39.73830	no		
371_xS4_Kiz5.3	B_globosus_2a	*Bulinus globosus*	Pemba Island	Kizimbani	Kiz5	-5.04782	39.73830	no		
372_xS4_Waw5.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wawi	Waw5	-5.25847	39.79327	no		
373_xS4_Waw5.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wawi	Waw5	-5.25847	39.79327	no		
374_xS4_Waw5.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Wawi	Waw5	-5.25847	39.79327	no		
375_xS4_Uku2.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku2	-5.34282	39.74940	no		

376_xS4_Uku2.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku2	-5.34282	39.74940	no		
377_xS4_Uku2.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku2	-5.34282	39.74940	no		
378_xS4_Uku3.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku3	-5.34355	39.74808	no		
379_xS4_Uku3.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku3	-5.34355	39.74808	no		
380_xS4_Uku3.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku3	-5.34355	39.74808	no		
381_xS4_Uku4.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku4	-5.34390	39.75533	no		
382_xS4_Uku4.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku4	-5.34390	39.75533	no		
383_xS4_Uku4.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku4	-5.34390	39.75533	no		
384_xS4_Uku5.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku5	-5.34355	39.75690	no		
385_xS4_Uku5.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku5	-5.34355	39.75690	no		
386_xS4_Uku5.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku5	-5.34355	39.75690	no		
387_xS4_Uku6.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku6	-5.34422	39.75848	no		
388_xS4_Uku6.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku6	-5.34422	39.75848	no		
389_xS4_Uku6.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku6	-5.34422	39.75848	no		
390_xS4_Uku7.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku7	-5.34450	39.75967	no		
391_xS4_Uku7.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku7	-5.34450	39.75967	no		
392_xS4_Uku7.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku7	-5.34450	39.75967	no		
393_xS4_Uku8.1	B_globosus_2b	*Bulinus globosus*	Pemba Island	Ukutini	Uku8	-5.33978	39.76675	no		
394_xS4_Uku8.2	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku8	-5.33978	39.76675	no		
395_xS4_Uku8.3	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku8	-5.33978	39.76675	no		
396_xS4_Uku10.1	B_globosus_1a	*Bulinus globosus*	Pemba Island	Ukutini	Uku10	-5.35428	39.76762	no		
Kangagani-Aqua-4.1+ve	B_nasutus_3	*Bulinus nasutus nasutus*	Pemba Island	Kangagani	Kanga1	-5.17184	39.82031	*S. haematobium*	Sh3	1

Supplementary Table 4.2. Complete *Schistosoma* spp. cercariae dataset used in this study providing collection information and species identification for each sample.

SCAN_barcode_label_ID	country	Shehia	site_name	Lat_cor	Lon_cor	coll_date	Snail ID	Sequencing_ID	schisto_cox3-5_haplotype	sch_cox_hap_new	schisto_haplotype_group	coinfection	sp_post_bc
MCF03050E0055	Zanzibar: Unguja	Mwera	Mwera pre-baseline site D	-6.15796	39.26299	2011-02-19	Mwera river #1 S100	ZEST_cerc_1	Zan14	Sh1	1	0	*S. haematobium*
MCF03050E0055	Zanzibar: Unguja	Mwera	Mwera pre-baseline site D	-6.15796	39.26299	2011-02-19	Mwera river #1 S100	ZEST_cerc_2	Zan14	Sh1	1	0	*S. haematobium*
MCF03050E0055	Zanzibar: Unguja	Mwera	Mwera pre-baseline site D	-6.15796	39.26299	2011-02-19	Mwera river #2 S101	ZEST_cerc_3	Zan31_new	Sh14	2	0	*S. haematobium*
MCF03050E0055	Zanzibar: Unguja	Mwera	Mwera pre-baseline site D	-6.15796	39.26299	2011-02-19	Mwera river #2 S101	ZEST_cerc_4	Zan31_new	Sh14	2	0	*S. haematobium*
MCF03050E0056	Zanzibar: Unguja	Mwera	Mwera pre-baseline site D	-6.15796	39.26299	2011-02-19	Mwera 96	ZEST_cerc_5	Zan5	Sh10	2	0	*S. haematobium*
MCF03050E0056	Zanzibar: Unguja	Mwera	Mwera pre-baseline site D	-6.15796	39.26299	2011-02-19	Mwera 96	ZEST_cerc_6	Zan5	Sh10	2	0	*S. haematobium*
MCF03050E0056	Zanzibar: Unguja	Mwera	Mwera pre-baseline site D	-6.15796	39.26299	2011-02-19	Mwera 97	ZEST_cerc_7	Zan28_new	Sh11	2	0	*S. haematobium*
MCF03050E0056	Zanzibar: Unguja	Mwera	Mwera pre-baseline site D	-6.15796	39.26299	2011-02-19	Mwera 97	ZEST_cerc_8	Zan28_new	Sh11	2	0	*S. haematobium*
MCF03050E0057	Zanzibar: Unguja	Mwera	Mwera pre-baseline site D	-6.15796	39.26299	2011-02-19	Mwera 98	ZEST_cerc_9	Zan18	Sh7	1	2	*S. haematobium*

MCF03050E0057	Zanzibar: Unguja	Mwera	Mwera pre-baseline site D	-6.15796	39.26299	2011-02-19	Mwera 98	ZEST_cerc_10	Euclinosomum heterostomum	NA	NA	2	*Euclinosomum heterostomum*
MCF03050E0057	Zanzibar: Unguja	Mwera	Mwera pre-baseline site D	-6.15796	39.26299	2011-02-19	Mwera 99	ZEST_cerc_11	Zan4	Sh1	1	0	*S. haematobium*
MCF03050E0057	Zanzibar: Unguja	Mwera	Mwera pre-baseline site D	-6.15796	39.26299	2011-02-19	Mwera 99	ZEST_cerc_12	Zan14	Sh1	1	0	*S. haematobium*
MCF03050E0058	Zanzibar: Unguja	Mchangani	Tingatinga	-5.95635	39.30783	2011-02-15	TT S15	ZEST_cerc_13	Failed	NA	NA	0	*x*
MCF03050E0058	Zanzibar: Unguja	Mchangani	Tingatinga	-5.95635	39.30783	2011-02-15	TT S15	ZEST_cerc_14	Zan14	Sh1	1	0	*S. haematobium*
MCF03050E0058	Zanzibar: Unguja	Bandamaji	Bandamaji pre-baseline site D	-5.95565	39.29576	2011-02-15	Bandamaji S9/TTS15	ZEST_cerc_15	Zan28_new	Sh11	2	0	*S. haematobium*
MCF03050E0058	Zanzibar: Unguja	Bandamaji	Bandamaji pre-baseline site D	-5.95565	39.29576	2011-02-15	Bandamaji S9/TTS15	ZEST_cerc_16	Zan28_new	Sh11	2	0	*S. haematobium*
MCF03050E0067	Zanzibar: Unguja	Mchangani	Tingatinga	-5.95635	39.30783	2011-02-12	Tingatinga S1	ZEST_cerc_17	Zan_DP	NA	NA	0	*S. haematobium*
MCF03050E0067	Zanzibar: Unguja	Mchangani	Tingatinga	-5.95635	39.30783	2011-02-12	Tingatinga S1	ZEST_cerc_18	Zan26	Sh1	1	0	*S. haematobium*
MCF03050E0068	Zanzibar: Unguja	Mchangani	Tingatinga	-5.95635	39.30783	2011-02-12	Tingatinga S2	ZEST_cerc_19	x	NA	NA	0	*x*
MCF03050E0068	Zanzibar: Unguja	Mchangani	Tingatinga	-5.95635	39.30783	2011-02-12	Tingatinga S2	ZEST_cerc_20	Zan5	Sh10	2	0	*S. haematobium*
MCF03050E0075	Zanzibar: Unguja	Mchangani	Tingatinga	-5.95635	39.30783	2011-02-15	Tingatinga S11	ZEST_cerc_21	Zan4	Sh1	1	1	*S. haematobium*
MCF03050E0075	Zanzibar: Unguja	Mchangani	Tingatinga	-5.95635	39.30783	2011-02-15	Tingatinga S11	ZEST_cerc_22	Zan1	Sh10	2	1	*S. haematobium*
MCF03050E0092	Zanzibar: Unguja	Mchangani	Tingatinga	-5.95635	39.30783	2011-02-15	Tingatinga S60	ZEST_cerc_23	x	NA	NA	0	*x*
MCF03050E0092	Zanzibar: Unguja	Mchangani	Tingatinga	-5.95635	39.30783	2011-02-15	Tingatinga S60	ZEST_cerc_24	Zan10	Sh3	1	0	*S. haematobium*
MCF03050E0092	Zanzibar: Unguja	Mchangani	Tingatinga	-5.95635	39.30783	2011-02-15	Tingatinga S61	ZEST_cerc_25	Zan3	Sh9	2	1	*S. haematobium*
MCF03050E0092	Zanzibar: Unguja	Mchangani	Tingatinga	-5.95635	39.30783	2011-02-15	Tingatinga S61	ZEST_cerc_26	Zan4	Sh1	1	1	*S. haematobium*
MCF03050E0093	Zanzibar: Unguja	Mchangani	Tingatinga	-5.95635	39.30783	2011-02-15	Tingatinga S69	ZEST_cerc_29	Zan_DP	NA	NA	0	*S. haematobium*
MCF03050E0093	Zanzibar: Unguja	Mchangani	Tingatinga	-5.95635	39.30783	2011-02-15	Tingatinga S69	ZEST_cerc_30	Zan_DP	NA	NA	0	*S. haematobium*
MCF03050E0620	Zanzibar: Unguja	Miwani	Kinumoshi	-6.08857	39.27673	2013-08-13	A5	ZEST_cerc_35	Zan3	Sh9	2	0	*S. haematobium*
MCF03050E0620	Zanzibar: Unguja	Miwani	Kinumoshi	-6.08857	39.27673	2013-08-13	A5	ZEST_cerc_36	Zan3	Sh9	2	0	*S. haematobium*
MCF03050E0621	Zanzibar: Unguja	Miwani	Kinumoshi	-6.08857	39.27673	2013-08-13	D1	ZEST_cerc_37	Zan_DP	NA	NA	0	*x*
MCF03050E0621	Zanzibar: Unguja	Miwani	Kinumoshi	-6.08857	39.27673	2013-08-13	D1	ZEST_cerc_38	Zan5	Sh10	2	0	*S. haematobium*
MCF03050E0621	Zanzibar: Unguja	Miwani	Kinumoshi	-6.08857	39.27673	2013-08-13	B5	ZEST_cerc_39	Zan11	Sh6	1	0	*S. haematobium*
MCF03050E0621	Zanzibar: Unguja	Miwani	Kinumoshi	-6.08857	39.27673	2013-08-13	B5	ZEST_cerc_40	Zan11	Sh6	1	0	*S. haematobium*
MCF03050E0622	Zanzibar: Unguja	Kinyasini	Ziwani B (Eneo Jipya)	-5.96495	39.30573	2013-08-27	C4	ZEST_cerc_41	Zan26	Sh1	1	0	*S. haematobium*
MCF03050E0622	Zanzibar: Unguja	Kinyasini	Ziwani B (Eneo Jipya)	-5.96495	39.30573	2013-08-27	C4	ZEST_cerc_42	Zan14	Sh1	1	0	*S. haematobium*
MCF03050E0625	Zanzibar: Unguja	Kinyasini	Ziwani B (Eneo Jipya)	-5.96495	39.30573	2013-08-27	B4	ZEST_cerc_47	Failed	NA	NA	0	*x*
MCF03050E0625	Zanzibar: Unguja	Kinyasini	Ziwani B (Eneo Jipya)	-5.96495	39.30573	2013-08-27	B4	ZEST_cerc_48	Zan4	Sh1	1	0	*S. haematobium*

MCF03050E0626	Zanzibar: Unguja	Kinyasini	Ziwani A (Mrogoro)	-5.96415	39.30687	2013-08-27	A1	ZEST_cerc_49	Zan14	Sh1	1	0	*S. haematobium*
MCF03050E0626	Zanzibar: Unguja	Kinyasini	Ziwani A (Mrogoro)	-5.96415	39.30687	2013-08-27	A1	ZEST_cerc_50	Zan26	Sh1	1	0	*S. haematobium*
MCF03050E0627	Zanzibar: Unguja	Kinyasini	Ziwani A (Mrogoro)	-5.96415	39.30687	2013-08-27	A2	ZEST_cerc_51	Zan14	Sh1	1	1	*S. haematobium*
MCF03050E0627	Zanzibar: Unguja	Kinyasini	Ziwani A (Mrogoro)	-5.96415	39.30687	2013-08-27	A2	ZEST_cerc_52	Zan3	Sh9	2	1	*S. haematobium*
MCF03050E0629	Zanzibar: Unguja	Miwani	Kinumoshi	-6.08857	39.27673	2013-08-13	D2	ZEST_cerc_55	Failed	NA	NA	0	*x*
MCF03050E0629	Zanzibar: Unguja	Miwani	Kinumoshi	-6.08857	39.27673	2013-08-13	D2	ZEST_cerc_56	Zan4	Sh1	1	0	*S. haematobium*
MCF03050E0630	Zanzibar: Unguja	Miwani	Kinumoshi	-6.08857	39.27673	2013-08-13	A1	ZEST_cerc_57	Zan1	Sh10	2	0	*S. haematobium*
MCF03050E0630	Zanzibar: Unguja	Miwani	Kinumoshi	-6.08857	39.27673	2013-08-13	A1	ZEST_cerc_58	Zan_DP	NA	NA	0	*x*
MCF03050E0926	Zanzibar: Pemba	Mkanyageni	Muanzini	-5.39428	39.67078	2013-08-01	6284	ZEST_cerc_59	Zan4	Sh1	1	1	*S. haematobium*
MCF03050E0926	Zanzibar: Pemba	Mkanyageni	Muanzini	-5.39428	39.67078	2013-08-01	6284	ZEST_cerc_60	Zan3	Sh9	2	1	*S. haematobium*
MCF03050E0927	Zanzibar: Pemba	Mkanyageni	Muanzini	-5.39428	39.67078	2013-08-01	6285	ZEST_cerc_61	Zan14	Sh1	1	0	*S. haematobium*
MCF03050E0927	Zanzibar: Pemba	Mkanyageni	Muanzini	-5.39428	39.67078	2013-08-01	6285	ZEST_cerc_62	x	NA	NA	0	*x*
MCF03050E0928	Zanzibar: Pemba	Mkanyageni	Muanzini	-5.39428	39.67078	2013-08-01	6286	ZEST_cerc_63	Zan10	Sh3	1	0	*S. haematobium*
MCF03050E0928	Zanzibar: Pemba	Mkanyageni	Muanzini	-5.39428	39.67078	2013-08-01	6286	ZEST_cerc_64	Zan10	Sh3	1	0	*S. haematobium*
MCF51F7510250	Zanzibar: Unguja	Kitope	Trein	-6.02043	39.24647	2014-07-23	C3	ZEST_cerc_67	x	NA	NA	0	*x*
MCF51F7510250	Zanzibar: Unguja	Kitope	Trein	-6.02043	39.24647	2014-07-23	C3	ZEST_cerc_68	Zan26	Sh1	1	0	*S. haematobium*
MCF51F7510251	Zanzibar: Unguja	Kitope	Trein	-6.02043	39.24647	2014-07-24	C6	ZEST_cerc_69	Zan_DP	NA	NA	0	*x*
MCF51F7510251	Zanzibar: Unguja	Kitope	Trein	-6.02043	39.24647	2014-07-24	C6	ZEST_cerc_70	short	NA	NA	0	*S. haematobium*
MCF51F7510252	Zanzibar: Unguja	Kitope	Trein	-6.02043	39.24647	2014-07-24	D2	ZEST_cerc_71	Zan6	Sh2	1	0	*S. haematobium*
MCF51F7510252	Zanzibar: Unguja	Kitope	Trein	-6.02043	39.24647	2014-07-24	D2	ZEST_cerc_72	Zan6	Sh2	1	0	*S. haematobium*
MCF51F7510253	Zanzibar: Unguja	Kitope	Mkaratini	-6.01178	39.24893	2014-07-24	A3	ZEST_cerc_73	Zan1	Sh10	2	0	*S. haematobium*
MCF51F7510253	Zanzibar: Unguja	Kitope	Mkaratini	-6.01178	39.24893	2014-07-24	A3	ZEST_cerc_74	Zan5	Sh10	2	0	*S. haematobium*
MCF51F7510254	Zanzibar: Unguja	Kitope	Kitope Bridge	-6.01200	39.24743	2014-07-23	D3	ZEST_cerc_75	Zan14	Sh1	1	0	*S. haematobium*
MCF51F7510254	Zanzibar: Unguja	Kitope	Kitope Bridge	-6.01200	39.24743	2014-07-23	D3	ZEST_cerc_76	Zan4	Sh1	1	0	*S. haematobium*
MCF51F7510255	Zanzibar: Unguja	Kitope	Mkaratini	-6.01178	39.24893	2014-07-23	A2	ZEST_cerc_77	Zan6	Sh2	1	0	*S. haematobium*
MCF51F7510255	Zanzibar: Unguja	Kitope	Mkaratini	-6.01178	39.24893	2014-07-23	A2	ZEST_cerc_78	Zan_DP	NA	NA	0	*x*
MCF51F7510256	Zanzibar: Unguja	Kitope	Kitope bridge	-6.01200	39.24743	2014-07-24	C4	ZEST_cerc_79	Zan1	Sh10	2	1	*S. haematobium*
MCF51F7510256	Zanzibar: Unguja	Kitope	Kitope bridge	-6.01200	39.24743	2014-07-24	C4	ZEST_cerc_80	Zan14	Sh1	1	1	*S. haematobium*
MCF51F7510257	Zanzibar: Unguja	Chaani	Darajani (Unguja)	-5.93100	39.29995	2014-07-14	B1	ZEST_cerc_81	Failed	NA	NA	0	*x*

MCF51F7510257	Zanzibar: Unguja	Chaani	Darajani (Unguja)	-5.93100	39.29995	2014-07-14	B1	ZEST_cerc_82	Zan3	Sh9	2	0	*S. haematobium*
MCF51F7510258	Zanzibar: Unguja	Chaani	Darajani (Unguja)	-5.93100	39.29995	2014-07-14	A4	ZEST_cerc_83	Zan26	Sh1	1	0	*S. haematobium*
MCF51F7510258	Zanzibar: Unguja	Chaani	Darajani (Unguja)	-5.93100	39.29995	2014-07-14	A4	ZEST_cerc_84	Zan14	Sh1	1	0	*S. haematobium*
MCF51F7510259	Zanzibar: Unguja	Kinyasini	Kwa Khamis Seif (Eneo Jipya)	-5.97068	39.30220	2014-07-04	C4	ZEST_cerc_85	Zan4	Sh1	1	0	*S. haematobium*
MCF51F7510259	Zanzibar: Unguja	Kinyasini	Kwa Khamis Seif (Eneo Jipya)	-5.97068	39.30220	2014-07-04	C4	ZEST_cerc_86	Zan4	Sh1	1	0	*S. haematobium*
MCF51F7510260	Zanzibar: Unguja	Kinyasini	Kwa Khamis Seif (Eneo Jipya)	-5.97068	39.30220	2014-07-04	B5	ZEST_cerc_87	Failed	NA	NA	0	*x*
MCF51F7510260	Zanzibar: Unguja	Kinyasini	Kwa Khamis Seif (Eneo Jipya)	-5.97068	39.30220	2014-07-04	B5	ZEST_cerc_88	Zan14	Sh1	1	0	*S. haematobium*
MCF51F7510262	Zanzibar: Unguja	Kinyasini	Ziwani B (Eneo Jipya)	-5.96495	39.30573	2014-07-11	B6	ZEST_cerc_91	x	NA	NA	0	*x*
MCF51F7510262	Zanzibar: Unguja	Kinyasini	Ziwani B (Eneo Jipya)	-5.96495	39.30573	2014-07-11	B6	ZEST_cerc_92	Zan26	Sh1	1	0	*S. haematobium*
MCF51F7510263	Zanzibar: Unguja	Kinyasini	Ziwani B (Eneo Jipya)	-5.96495	39.30573	2014-07-11	B4	ZEST_cerc_93	Zan14	Sh1	1	0	*S. haematobium*
MCF51F7510263	Zanzibar: Unguja	Kinyasini	Ziwani B (Eneo Jipya)	-5.96495	39.30573	2014-07-11	B4	ZEST_cerc_94	Zan4	Sh1	1	0	*S. haematobium*
MCF51F7510264	Zanzibar: Unguja	Kinyasini	Ziwani B (Eneo Jipya)	-5.96495	39.30573	2014-07-11	D1	ZEST_cerc_95	Zan30_new	Sh1	1	0	*S. haematobium*
MCF51F7510264	Zanzibar: Unguja	Kinyasini	Ziwani B (Eneo Jipya)	-5.96495	39.30573	2014-07-11	D1	ZEST_cerc_96	Zan30_new	Sh1	1	0	*S. haematobium*
MCF51F7510265	Zanzibar: Unguja	Kinyasini	Ziwani B (Eneo Jipya)	-5.96495	39.30573	2014-07-11	A6	ZEST_cerc_97	Zan5	Sh10	2	0	*S. haematobium*
MCF51F7510265	Zanzibar: Unguja	Kinyasini	Ziwani B (Eneo Jipya)	-5.96495	39.30573	2014-07-11	A6	ZEST_cerc_98	Zan1	Sh10	2	0	*S. haematobium*
MCF51F7510267	Zanzibar: Unguja	Kinyasini	Ziwani B (Eneo Jipya)	-5.96495	39.30573	2014-07-11	A3	ZEST_cerc_101	Zan28_new	Sh9	2	0	*S. haematobium*
MCF51F7510267	Zanzibar: Unguja	Kinyasini	Ziwani B (Eneo Jipya)	-5.96495	39.30573	2014-07-11	A3	ZEST_cerc_102	Zan_DP	NA	NA	0	*x*
MCF51F7510268	Zanzibar: Unguja	Kinyasini	Ziwani B (Eneo Jipya)	-5.96495	39.30573	2014-07-11	A4	ZEST_cerc_103	Zan14	Sh1	1	0	*S. haematobium*
MCF51F7510268	Zanzibar: Unguja	Kinyasini	Ziwani B (Eneo Jipya)	-5.96495	39.30573	2014-07-11	A4	ZEST_cerc_104	x	NA	NA	0	*x*
MCF51F7510269	Zanzibar: Unguja	Kinyasini	Ziwani B (Eneo Jipya)	-5.96495	39.30573	2014-07-11	C2	ZEST_cerc_105	Zan_DP	NA	NA	0	*x*
MCF51F7510269	Zanzibar: Unguja	Kinyasini	Ziwani B (Eneo Jipya)	-5.96495	39.30573	2014-07-11	C2	ZEST_cerc_106	Zan4	Sh1	1	0	*S. haematobium*
MCF51F7510270	Zanzibar: Unguja	Bandamaji	Mwanamanju B	-5.95642	39.29953	2014-06-28	A1	ZEST_cerc_107	Zan3	Sh9	2	0	*S. haematobium*
MCF51F7510270	Zanzibar: Unguja	Bandamaji	Mwanamanju B	-5.95642	39.29953	2014-06-28	A1	ZEST_cerc_108	Zan_DP	NA	NA	0	*x*
MCF51F7510272	Zanzibar: Unguja	Kinyasini	Abdalla Said - Shuweni	-5.98192	39.30640	2014-07-10	C6	ZEST_cerc_109	Zan17	Sh8	1	0	*S. haematobium*
MCF51F7510272	Zanzibar: Unguja	Kinyasini	Abdalla Said - Shuweni	-5.98192	39.30640	2014-07-10	C6	ZEST_cerc_110	Zan_DP	NA	NA	0	*x*
MCF51F7510271	Zanzibar: Unguja	Kinyasini	Abdalla Said - Shuweni	-5.98192	39.30640	2014-07-10	D6	ZEST_cerc_111	Zan6	Sh2	1	0	*S. haematobium*
MCF51F7510271	Zanzibar: Unguja	Kinyasini	Abdalla Said - Shuweni	-5.98192	39.30640	2014-07-10	D6	ZEST_cerc_112	Zan6	Sh2	1	0	*S. haematobium*

MCF51F75102 73	Zanzibar: Unguja	Kinyasini	Abdalla Said - Shuweni	- 5.98192	39.3064 0	2014-07-10	D2	ZEST_cerc_113	Zan28_new	Sh11	2	0	*S. haematobium*
MCF51F75102 73	Zanzibar: Unguja	Kinyasini	Abdalla Said - Shuweni	- 5.98192	39.3064 0	2014-07-10	D2	ZEST_cerc_114	x	NA	NA	0	*x*
MCF76346903 26	Zanzibar: Pemba	Shumba Viamboni	Darajani (Pemba)	- 5.00320	39.7564 5	2014-08-10	6314	ZEST_cerc_115	Zan12	Sh12	2	0	*S. haematobium*
MCF76346903 26	Zanzibar: Pemba	Shumba Viamboni	Darajani (Pemba)	- 5.00320	39.7564 5	2014-08-10	6314	ZEST_cerc_116	Zan12	Sh12	2	0	*S. haematobium*
MCF76346903 28	Zanzibar: Pemba	Michewen i	Kikangue stream	- 5.01980	39.7467 0	2014-12-19	6316	ZEST_cerc_119	Zan32_new	Sh13	2	0	*S. haematobium*
MCF76346903 28	Zanzibar: Pemba	Michewen i	Kikangue stream	- 5.01980	39.7467 0	2014-12-19	6316	ZEST_cerc_120	Zan19	Sh13	2	0	*S. haematobium*
MCFB63CC202 78	Zanzibar: Pemba	Shumba Viamboni	Darajani (Pemba)	- 5.00320	39.7564 5	2011-07-11	SAR/SV/1	ZEST_cerc_129	Zan12	Sh12	2	0	*S. haematobium*
MCFB63CC202 78	Zanzibar: Pemba	Shumba Viamboni	Darajani (Pemba)	- 5.00320	39.7564 5	2011-07-11	SAR/SV/1	ZEST_cerc_130	Zan12	Sh12	2	0	*S. haematobium*
MCFB63CC202 79	Zanzibar: Pemba	Vitongoji	Ziwa Kichanje	- 5.21743	39.8311 5	2011-07-22	2k/v/2	ZEST_cerc_131	x	NA	NA	0	*x*
MCFB63CC202 79	Zanzibar: Pemba	Vitongoji	Ziwa Kichanje	- 5.21743	39.8311 5	2011-07-22	2k/v/2	ZEST_cerc_132	Zan1	Sh10	2	0	*S. haematobium*
MCFB63CC202 80	Zanzibar: Pemba	Vitongoji	Ziwa Kichanje	- 5.21743	39.8311 5	2011-07-22	2k/v/1	ZEST_cerc_133	Zan33_new	Sh15	2	0	*S. haematobium*
MCFB63CC202 80	Zanzibar: Pemba	Vitongoji	Ziwa Kichanje	- 5.21743	39.8311 5	2011-07-22	2k/v/1	ZEST_cerc_134	x	NA	NA	0	*x*
MCFB63CC202 81	Zanzibar: Pemba	Shumba Viamboni	Darajani (Pemba)	- 5.00320	39.7564 5	2011-07-11	DAR/SV/2	ZEST_cerc_135	Zan12	Sh12	2	0	*S. haematobium*
MCFB63CC202 81	Zanzibar: Pemba	Shumba Viamboni	Darajani (Pemba)	- 5.00320	39.7564 5	2011-07-11	DAR/SV/2	ZEST_cerc_136	Zan_DP	NA	NA	0	*x*
MCFB63CC202 83	Zanzibar: Pemba	Vitongoji	Muwau	- 5.24713	39.8309 8	2011-12-15	MU1	ZEST_cerc_137	Zan3	Sh9	2	0	*S. haematobium*
MCFB63CC202 83	Zanzibar: Pemba	Vitongoji	Muwau	- 5.24713	39.8309 8	2011-12-15	MU1	ZEST_cerc_138	Zan3	Sh9	2	0	*S. haematobium*
MCFB63CC202 84	Zanzibar: Pemba	Vitongoji	Muwau	- 5.24713	39.8309 8	2011-12-15	MU2	ZEST_cerc_139	Zan15	Sh16	2	0	*S. haematobium*
MCFB63CC202 84	Zanzibar: Pemba	Vitongoji	Muwau	- 5.24713	39.8309 8	2011-12-15	MU2	ZEST_cerc_140	x	NA	NA	0	*x*
MCFB63CC202 85	Zanzibar: Pemba	Piki	Darajani Kijito Songoro	- 5.11670	39.7665 2	2012-01-04	6329	ZEST_cerc_141	Zan29_new	Sh4	1	0	*S. haematobium*
MCFB63CC202 85	Zanzibar: Pemba	Piki	Darajani Kijito Songoro	- 5.11670	39.7665 2	2012-01-04	6329	ZEST_cerc_142	Zan29_new	Sh4	1	0	*S. haematobium*
MCFF9A0BB01 10	Zanzibar: Unguja	Mchangan i	Tingatinga	- 5.95635	39.3078 3	2012-10-22	6331	ZEST_cerc_145	Zan26	Sh1	1	0	*S. haematobium*
MCFF9A0BB01 10	Zanzibar: Unguja	Mchangan i	Tingatinga	- 5.95635	39.3078 3	2012-10-22	6331	ZEST_cerc_146	Zan_DP	NA	NA	0	*x*
MCFF9A0BB01 11	Zanzibar: Unguja	Miwani	Kinumoshi	- 6.08857	39.2767 3	2013-02-06	6332	ZEST_cerc_147	Zan5	Sh10	2	0	*S. haematobium*
MCFF9A0BB01 11	Zanzibar: Unguja	Miwani	Kinumoshi	- 6.08857	39.2767 3	2013-02-06	6332	ZEST_cerc_148	Zan1	Sh10	2	0	*S. haematobium*
MCFF9A0BB01 12	Zanzibar: Pemba	Shumba Viamboni	Badala	- 5.00132	39.7647 0	2012-08-03	6333	ZEST_cerc_149	Zan5	Sh10	2	0	*S. haematobium*
MCFF9A0BB01 12	Zanzibar: Pemba	Shumba Viamboni	Badala	- 5.00132	39.7647 0	2012-08-03	6333	ZEST_cerc_150	Zan1	Sh10	2	0	*S. haematobium*
MCFF9A0BB01 14	Zanzibar: Pemba	Mkanyage ni	Muanzini	- 5.39428	39.6707 8	2012-08-15	6335	ZEST_cerc_153	Zan3	Sh9	2	0	*S. haematobium*
MCFF9A0BB01 14	Zanzibar: Pemba	Mkanyage ni	Muanzini	- 5.39428	39.6707 8	2012-08-15	6335	ZEST_cerc_154	Zan3	Sh9	2	0	*S. haematobium*
MCF8EC4B401 84	Zanzibar: Unguja	Kinyasini	Ziwani B (Eneo Jipya)	- 5.96495	39.3057 3	2016-01-05	B6	ZEST_cerc_155	Zan30_new	Sh1	1	0	*S. haematobium*

MCF8EC4B40184	Zanzibar: Unguja	Kinyasini	Ziwani B (Eneo Jipya)	-5.96495	39.30573	2016-01-05	B6	ZEST_cerc_156	Zan30_new	Sh1	1	0	*S. haematobium*
MCF2E37330083	Zanzibar: Unguja	Kinyasini	Ziwani A (Mrogoro)	-5.96415	39.30687	2016-10-10	B4	ZEST_cerc_159	Zan4	Sh1	1	0	*S. haematobium*
MCF2E37330083	Zanzibar: Unguja	Kinyasini	Ziwani A (Mrogoro)	-5.96415	39.30687	2016-10-10	B4	ZEST_cerc_160	x	NA	NA	0	*x*
MCF2E37330084	Zanzibar: Unguja	Kinyasini	Ziwani A (Mrogoro)	-5.96415	39.30687	2016-10-10	A2	ZEST_cerc_161	Zan8	Sh5	1	0	*S. haematobium*
MCF2E37330084	Zanzibar: Unguja	Kinyasini	Ziwani A (Mrogoro)	-5.96415	39.30687	2016-10-10	A2	ZEST_cerc_162	Zan8	Sh5	1	0	*S. haematobium*
MCF2E37330085	Zanzibar: Unguja	Kinyasini	Ziwani A (Mrogoro)	-5.96415	39.30687	2016-10-10	A1	ZEST_cerc_163	Zan_DP	NA	NA	0	*x*
MCF2E37330085	Zanzibar: Unguja	Kinyasini	Ziwani A (Mrogoro)	-5.96415	39.30687	2016-10-10	A1	ZEST_cerc_164	Zan2	Sh17	2	0	*S. haematobium*
MCF28EEAF0775	Zanzibar: Unguja	Mchangani	Tingatinga	-5.95635	39.30783	2011-10-06	B1	ZEST_cerc_167	Zan3	Sh9	2	0	*S. haematobium*
MCF28EEAF0775	Zanzibar: Unguja	Mchangani	Tingatinga	-5.95635	39.30783	2011-10-06	B1	ZEST_cerc_168	Zan3	Sh9	2	0	*S. haematobium*
MCF28EEAF0775	Zanzibar: Unguja	Chaani	Mtopweza	#REF!	#REF!	2011-10-06	A5	ZEST_cerc_169	Failed	NA	NA	0	*x*
MCF28EEAF0775	Zanzibar: Unguja	Chaani	Mtopweza	#REF!	#REF!	2011-10-06	A5	ZEST_cerc_170	Zan3	Sh9	2	0	*S. haematobium*
MCFA3F02B0970	Zanzibar: Unguja	Chaani	Mtopweza	#REF!	#REF!	2018-07-27	x10002	ZEST_cerc_177	Zan28_new	Sh11	2	0	*S. haematobium*
MCFA3F02B0970	Zanzibar: Unguja	Chaani	Mtopweza	#REF!	#REF!	2018-07-27	x10002	ZEST_cerc_178	Zan28_new	Sh11	2	0	*S. haematobium*
MCF51F7510265	Zanzibar: Unguja	Kinyasini	Ziwani B (Eneo Jipya)	-5.96495	39.30573	2014-07-11	D3	ZEST_cerc_181	Zan30_new	Sh1	1	0	*S. haematobium*
MCF51F7510265	Zanzibar: Unguja	Kinyasini	Ziwani B (Eneo Jipya)	-5.96495	39.30573	2014-07-11	D3	ZEST_cerc_182	Failed	NA	NA	0	*x*
MCF51F7510267	Zanzibar: Unguja	Kinyasini	Ziwani B (Eneo Jipya)	-5.96495	39.30573	2014-07-11	C4	ZEST_cerc_183	Zan14	Sh1	1	0	*S. haematobium*
MCF51F7510267	Zanzibar: Unguja	Kinyasini	Ziwani B (Eneo Jipya)	-5.96495	39.30573	2014-07-11	C4	ZEST_cerc_184	Failed	NA	NA	0	*x*
	Zanzibar: Pemba	Kinyasini	Kinya2	-5.02033	39.73855		SP_Kin2.3.1	SP_Kin2.3.1_2	Zan2	Sh17	2	1	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya2	-5.02033	39.73855		SP_Kin2.3.1	SP_Kin2.3.1_4	Zan3	Sh9	2	1	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya6	-5.03560	39.73850		SP_Kin4.5.3	SP_Kin4.5.3_1	Sb1	Sb1	Sb1	0	*S. bovis*
	Zanzibar: Pemba	Kinyasini	Kinya6	-5.03560	39.73850		SP_Kin4.5.3	SP_Kin4.5.3_2	Sb1	Sb1	Sb1	0	*S. bovis*
	Zanzibar: Pemba	Kinyasini	Kinya6	-5.03560	39.73850		SP_Kin4.5.3	SP_Kin4.5.3_3	Sb1	Sb1	Sb1	0	*S. bovis*
	Zanzibar: Pemba	Kinyasini	Kinya6	-5.03560	39.73850		SP_Kin4.5.3	SP_Kin4.5.3_3a_1	Sb1	Sb1	Sb1	0	*S. bovis*
	Zanzibar: Pemba	Kinyasini	Kinya6	-5.03560	39.73850		SP_Kin4.5.10	SP_Kin4.5.10_4	Sb2	Sb2	Sb2	1	*S. bovis*
	Zanzibar: Pemba	Kinyasini	Kinya6	-5.03560	39.73850		SP_Kin4.5.10	SP_Kin4.5.10_5	Sb1	Sb1	Sb1	1	*S. bovis*
	Zanzibar: Pemba	Kinyasini	Kinya6	-5.03560	39.73850		SP_Kin4.5.10	SP_Kin4.5.10_6	Sb2	Sb2	Sb2	1	*S. bovis*
	Zanzibar: Pemba	Kinyasini	Kinya6	-5.03560	39.73850		SP_Kin4.5.10	SP_Kin4.5.10_7	Sb2	Sb2	Sb2	1	*S. bovis*
	Zanzibar: Pemba	Kinyasini	Kinya6	-5.03560	39.73850		SP_Kin4.6.7	SP_Kin4.6.7_10	Sb2	Sb2	Sb2	0	*S. bovis*
	Zanzibar: Pemba	Kinyasini	Kinya6	-5.03560	39.73850		SP_Kin4.6.7	SP_Kin4.6.7_11	Sb2	Sb2	Sb2	0	*S. bovis*

	Zanzibar: Pemba	Kinyasini	Kinya6	-5.03560	39.73850		SP_Kin4.8.3	SP_Kin4.8.3_12	Sb1	Sb1	Sb1	0	*S. bovis*
	Zanzibar: Pemba	Kinyasini	Kinya6	-5.03560	39.73850		SP_Kin4.8.3	SP_Kin4.8.3_13	Sb1	Sb1	Sb1	0	*S. bovis*
	Zanzibar: Pemba	Kinyasini	Kinya6	-5.03560	39.73850		SP_Kin4.8.3	SP_Kin4.8.3_14	Sb1	Sb1	Sb1	0	*S. bovis*
	Zanzibar: Pemba	Kinyasini	Kinya6	-5.03560	39.73850		SP_Kin4.9.11	SP_Kin4.9.11_15	Sb2	Sb2	Sb2	0	*S. bovis*
	Zanzibar: Pemba	Kinyasini	Kinya6	-5.03560	39.73850		SP_Kin4.9.11	SP_Kin4.9.11_16	Sb2	Sb2	Sb2	0	*S. bovis*
	Zanzibar: Pemba	Chambani	Cham10	-5.35805	39.79182		SP_Cham10.1a	SP_Cham10.1a_2	Zan19	Sh13	2	0	*S. haematobium*
	Zanzibar: Pemba	Chambani	Cham10	-5.35805	39.79182		SP_Cham10.1a	SP_Cham10.1a_3	Zan19	Sh13	2	0	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya9	-5.03077	39.73333		S2_Kin9.1	S2_Kin9.1_1	Zan3	Sh9	2	0	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya9	-5.03077	39.73333		S2_Kin9.1	S2_Kin9.1_2	Zan3	Sh9	2	0	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya9	-5.03077	39.73333		S2_Kin9.1	S2_Kin9.1_3	Zan3	Sh9	2	0	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya9	-5.03077	39.73333		S2_Kin9.1	S2_Kin9.1_4	Zan3	Sh9	2	0	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya9	-5.03077	39.73333		S2_Kin9.1	S2_Kin9.1_5	Zan3	Sh9	2	0	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya9	-5.03077	39.73333		S2_Kin9.2	S2_Kin9.2_1	Zan34_new	Sh17	2	0	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya9	-5.03077	39.73333		S2_Kin9.2	S2_Kin9.2_2	Zan2	Sh17	2	0	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya9	-5.03077	39.73333		S2_Kin9.2	S2_Kin9.2_3	Zan2	Sh17	2	0	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya9	-5.03077	39.73333		S2_Kin9.2	S2_Kin9.2_4	Zan34_new	Sh17	2	0	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya9	-5.03077	39.73333		S2_Kin9.2	S2_Kin9.2_5	Zan2	Sh17	2	0	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya9	-5.03077	39.73333		S2_Kin9.3	S2_Kin9.3_1	Zan3	Sh9	2	0	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya9	-5.03077	39.73333		S2_Kin9.3	S2_Kin9.3_2	Zan3	Sh9	2	0	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya9	-5.03077	39.73333		S2_Kin9.3	S2_Kin9.3_3	Zan3	Sh9	2	0	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya9	-5.03077	39.73333		S2_Kin9.3	S2_Kin9.3_4	Zan3	Sh9	2	0	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya9	-5.03077	39.73333		S2_Kin9.3	S2_Kin9.3_5	Zan3	Sh9	2	0	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya9	-5.03077	39.73333		S2_Kin9.4	S2_Kin9.4_2	Zan2	Sh17	2	0	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya9	-5.03077	39.73333		S2_Kin9.4	S2_Kin9.4_3	Zan2	Sh17	2	0	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya9	-5.03077	39.73333		S2_Kin9.4	S2_Kin9.4_4	Zan2	Sh17	2	0	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya9	-5.03077	39.73333		S2_Kin9.4	S2_Kin9.4_5	Zan2	Sh17	2	0	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya9	-5.03077	39.73333		S2_Kin9.5	S2_Kin9.5_1	Zan3	Sh9	2	0	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya9	-5.03077	39.73333		S2_Kin9.5	S2_Kin9.5_3	Zan3	Sh9	2	0	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya9	-5.03077	39.73333		S2_Kin9.5	S2_Kin9.5_4	Zan3	Sh9	2	0	*S. haematobium*

	Zanzibar: Pemba	Kinyasini	Kinya9	-5.03077	39.73333		S2_Kin9.5	S2_Kin9.5_5	Zan3	Sh9	2	0	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya6	-5.03560	39.73850		S2_Kin6.1	S2_Kin6.1_2	Zan13	Sh18	2	0	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya6	-5.03560	39.73850		S2_Kin6.1	S2_Kin6.1_3	Zan13	Sh18	2	0	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya6	-5.03560	39.73850		S2_Kin6.1	S2_Kin6.1_5	Zan13	Sh18	2	0	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya11	-5.03717	39.73208		S2_Kin11.1	S2_Kin11.1_1	Zan2	Sh17	2	0	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya11	-5.03717	39.73208		S2_Kin11.1	S2_Kin11.1_2	Zan2	Sh17	2	0	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya11	-5.03717	39.73208		S2_Kin11.1	S2_Kin11.1_3	Zan2	Sh17	2	0	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya11	-5.03717	39.73208		S2_Kin11.1	S2_Kin11.1_4	Zan2	Sh17	2	0	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya11	-5.03717	39.73208		S2_Kin11.1	S2_Kin11.1_5	Zan2	Sh17	2	0	*S. haematobium*
	Zanzibar: Pemba	Kizimbani	Kiz5	-5.04782	39.73830		S2_Kiz5.1	S2_Kiz5.1_1	Zan1	Sh10	2	0	*S. haematobium*
	Zanzibar: Pemba	Kizimbani	Kiz5	-5.04782	39.73830		S2_Kiz5.1	S2_Kiz5.1_2	Zan1	Sh10	2	0	*S. haematobium*
	Zanzibar: Pemba	Kizimbani	Kiz5	-5.04782	39.73830		S2_Kiz5.1	S2_Kiz5.1_3	Zan1	Sh10	2	0	*S. haematobium*
	Zanzibar: Pemba	Kizimbani	Kiz5	-5.04782	39.73830		S2_Kiz5.1	S2_Kiz5.1_4	Zan1	Sh10	2	0	*S. haematobium*
	Zanzibar: Pemba	Kizimbani	Kiz5	-5.04782	39.73830		S2_Kiz5.1	S2_Kiz5.1_5	Zan1	Sh10	2	0	*S. haematobium*
	Zanzibar: Pemba	Kizimbani	Kiz4	-5.04872	39.74020		S2_Kiz4.1	S2_Kiz4.1_3	Zan3	Sh9	2	0	*S. haematobium*
	Zanzibar: Pemba	Kizimbani	Kiz4	-5.04872	39.74020		S2_Kiz4.1	S2_Kiz4.1_4	Zan3	Sh9	2	0	*S. haematobium*
	Zanzibar: Pemba	Kizimbani	Kiz4	-5.04872	39.74020		S2_Kiz4.1	S2_Kiz4.1_5	Zan3	Sh9	2	0	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya6	-5.03560	39.73850		S4_Kinya6.1	S4_Kinya6.1_2	Sb1	Sb1	Sb1	0	*S. bovis*
	Zanzibar: Pemba	Kinyasini	Kinya6	-5.03560	39.73850		S4_Kinya6.1	S4_Kinya6.1_3	Sb1	Sb1	Sb1	0	*S. bovis*
	Zanzibar: Pemba	Kinyasini	Kinya6	-5.03560	39.73850		S4_Kinya6.1	S4_Kinya6.1_4	Sb1	Sb1	Sb1	0	*S. bovis*
	Zanzibar: Pemba	Kinyasini	Kinya6	-5.03560	39.73850		S4_Kinya6.2	S4_Kinya6.2_1	Sb2	Sb2	Sb2	0	*S. bovis*
	Zanzibar: Pemba	Kinyasini	Kinya6	-5.03560	39.73850		S4_Kinya6.2	S4_Kinya6.2_2	Sb2	Sb2	Sb2	0	*S. bovis*
	Zanzibar: Pemba	Kinyasini	Kinya6	-5.03560	39.73850		S4_Kinya6.2	S4_Kinya6.2_4	Sb2	Sb2	Sb2	0	*S. bovis*
	Zanzibar: Pemba	Kinyasini	Kinya6	-5.03560	39.73850		S4_Kinya6.3	S4_Kinya6.3_2	Zan4	Sh1	1	0	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya6	-5.03560	39.73850		S4_Kinya6.3	S4_Kinya6.3_3	Zan4	Sh1	1	0	*S. haematobium*
	Zanzibar: Pemba	Kinyasini	Kinya6	-5.03560	39.73850		S4_Kinya6.3	S4_Kinya6.3_4	Zan4	Sh1	1	0	*S. haematobium*
	Zanzibar: Pemba	Kangagani	Kanga1	-5.17184	39.82031		Kangagani-Aqua-4.1	Kangagani-Aqua-4.1.1	Zan10	Sh3	1	0	*S. haematobium*
	Zanzibar: Pemba	Kangagani	Kanga1	-5.17184	39.82031		Kangagani-Aqua-4.1	Kangagani-Aqua-4.1.2	Zan10	Sh3	1	0	*S. haematobium*
	Zanzibar: Pemba	Kangagani	Kanga1	-5.17184	39.82031		Kangagani-Aqua-4.1	Kangagani-Aqua-4.1.3	Zan10	Sh3	1	0	*S. haematobium*

Supplementary Table 5.1. Complete *Bulinus* spp. dataset used in this study providing collection locality, species identification and gel image references for Figures 5.3 – 5.6.

Country	**Snail_id**	**Village**	**Site_Name**	**Latitude**	**Longitude**	**Snail_Morpho**	***Snail_cox1_hap***	**Infected?**	**Figure 5.3**	**Figure 5.4**	**Figure 5.5**	**Figure 5.6**
Pemba Island	SP_Kiz4.3	Kizimbani	Kiz4	-5.04872	39.74020	*Bulinus globosus*	*B_globosus_1a*	no	Y	Y (2+5)		
Pemba Island	SP_Uwa3.2	Uwandani	Uwa3	-5.19865	39.82723	*Bulinus nasutus*	*B_nasutus_4*	no	Y	Y (+ve)		
Pemba Island	SP_Kinya6.2	Kinyasini	Kinya6	-5.03560	39.73850	*Bulinus globosus*	*B_globosus_2a*	no				Y (20)
Pemba Island	SP_Uwa4.1	Uwandani	Uwa4	-5.19762	39.83002	*Bulinus nasutus*	*B_nasutus_4*	*no*				Y (19)
Tanzania	Tz144+ve-4	Shilingwa (TZ 144)	Site 6	-2.43632	33.091	*Bulinus nasutus.productus*	B.n.p._TZ_Hap_3	*S. haematobium*				Y (21)
Tanzania	Tz011-Bul-4	Bulima_(Tz011)	Nyahanga mwaloni site 1	-2.36423	33.5762	*Bulinus globosus*	*B_globosus_TZ_Hap _1*	*no*		Y (1+4)	Y (+ve)	
Pemba Island	SP_Kin2.3.1	Kinyasini	Kinya2	-5.03560	39.73850	*Bulinus globosus*	*B_globosus_1a*	*S. haematobium*				Y (1)
Pemba Island	SP_Kin4.8.3	Kinyasini	Kinya6	-5.03560	39.73850	*Bulinus globosus*	*B_globosus_2a*	*S. bovis*				Y (5)
Pemba Island	S2_Kinya9.2	Kinyasini	Kinya9	-5.03560	39.73850	*Bulinus globosus*	*B_globosus_2a*	*S. haematobium*				Y (9)
Niger	BK40	Bangou Koirey	Bangou Koirey 2 Bras Du Fleuve	13.60475	1.90509	*Bulinus truncatus*	B.globosus-Hap-2	*S. bovis*				Y (+ve)
Tanzania	Tz144-Bul-?+ve-1	Shilingwa (TZ 144)	Igusa site 1	-2.42583	33.1238	*Bulinus nasutus.productus*	B.n.p._TZ_Hap_2	non-schisto		Y (3+6)		
Niger	GB16	Gantchi Basarou	Gantchi Bassarou 1 Mare	13.17606	2.35497	*Bulinus forskalii*	B.forskalii-Hap-1	*S. bovis*			Y (22)	
Pemba Island	SP_Kin4.5.3	Kinyasini	Kinya6	-5.03560	39.73850	*Bulinus globosus*	*B_globosus_2a*	*S. bovis*				Y (2)
Pemba Island	SP_Kin4.5.10	Kinyasini	Kinya6	-5.03560	39.73850	*Bulinus globosus*	*B_globosus_2a*	*S. bovis*				Y (3)
Pemba Island	SP_Kin4.6.7	Kinyasini	Kinya6	-5.03560	39.73850	*Bulinus globosus*	*B_globosus_2a*	*S. bovis*				Y (4)
Pemba Island	SP_Kin4.9.11	Kinyasini	Kinya6	-5.35805	39.79182	*Bulinus globosus*	*B_globosus_2a*	*S. bovis*				Y (6)
Pemba Island	SP_Cham10.1a	Chambani	Cham10	-6.08857	39.27673	*Bulinus globosus*	*B_globosus_2a*	*S. haematobium*				Y (7)
Pemba Island	S2_Kinya9.1	Kinyasini	Kinya9	-5.03077	39.73333	*Bulinus globosus*	*B_globosus_2a*	*S. haematobium*				Y (8)
Pemba Island	S2_Kinya9.3	Kinyasini	Kinya9	-5.03717	39.73208	*Bulinus globosus*	*B_globosus_2a*	*S. haematobium*				Y (10)
Pemba Island	S2_Kinya9.4	Kinyasini	Kinya9	-5.04782	39.73830	*Bulinus globosus*	*B_globosus_2a*	*S. haematobium*				Y (11)
Pemba Island	S2_Kinya9.5	Kinyasini	Kinya9	-5.04872	39.74020	*Bulinus globosus*	*B_globosus_2a*	*S. haematobium*				Y (12)
Pemba Island	S2_Kinya6.1	Kinyasini	Kinya6	-5.03077	39.73333	*Bulinus globosus*	*B_globosus_2a*	*S. haematobium*				Y (13)
Pemba Island	S2_Kinya11.1	Kinyasini	Kinya11	-5.02033	39.73855	*Bulinus globosus*	*B_globosus_2a*	*S. haematobium*				Y (14)
Pemba Island	S2_Kiz5.1	Kizimbani	Kiz5	-5.03560	39.73850	*Bulinus globosus*	*B_globosus_2a*	*S. haematobium*				Y (15)
Pemba Island	S2_Kiz4.1	Kizimbani	Kiz4	-5.03560	39.73850	*Bulinus globosus*	*B_globosus_2a*	*S. haematobium*				Y (16)

Pemba Island	SP_Wam4.1	Wambaa	Wam4	-5.30087	39.68325	*Bulinus globosus*	B_globosus_1a	no				Y (17)
Pemba Island	SP_Cham10.1b	Chambani	Cham10	-6.08857	39.27673	*Bulinus globosus*	*B_globosus_2a*	*no*				Y (18)
Niger	ZKT22	Zama Koira Tagui	Zamakoira Tegui 4 canal secondaire	13.70432	1.72169	*Bulinus truncatus*	B.truncatus-Hap-5	*S. haematobium*				Y (22)
Pemba Island	17_ xS1_Wam7.2	Wambaa	Wam7	-5.29338	39.68913	*Bulinus globosus*	B_globosus_2a	*no*			Y (1)	
Pemba Island	18_ xS1_Wam7.3	Wambaa	Wam7	-5.29338	39.68913	*Bulinus globosus*	B_globosus_2a	*no*			Y (2)	
Pemba Island	19_ xS1_Wam8.1	Wambaa	Wam8	-5.29100	39.68697	*Bulinus globosus*	B_globosus_1a	*no*			Y (3)	
Pemba Island	20_ xS1_Wam8.2	Wambaa	Wam8	-5.29100	39.68697	*Bulinus globosus*	B_globosus_1a	*no*			Y (4)	
Pemba Island	21_ xS1_Wam8.3	Wambaa	Wam8	-5.29100	39.68697	*Bulinus globosus*	B_globosus_1a	*no*			Y (5)	
Pemba Island	22_ xS1_Wam9.1	Wambaa	Wam9	-5.29228	39.68833	*Bulinus globosus*	B_globosus_2a	*no*			Y (6)	
Pemba Island	23_ xS1_Wam9.2	Wambaa	Wam9	-5.29228	39.68833	*Bulinus globosus*	B_globosus_2a	*no*			Y (7)	
Pemba Island	24_ xS1_Wam9.3	Wambaa	Wam9	-5.29228	39.68833	*Bulinus globosus*	B_globosus_2a	*no*			Y (8)	
Pemba Island	25_ xS1_Mat6.1	Matale	Mat6	-5.30038	39.77640	*Bulinus globosus*	B_globosus_1a	*no*			Y (9)	
Pemba Island	26_ xS1_Mat6.2	Matale	Mat6	-5.30038	39.77640	*Bulinus globosus*	B_globosus_1a	*no*			Y (10)	
Pemba Island	43_ xS1_Puj4.1	Pujini	Puj4	-5.29000	39.81627	*Bulinus nasutus*	B_nasutus_2	*no*			Y (11)	
Pemba Island	44_ xS1_Puj4.2	Pujini	Puj4	-5.29000	39.81627	*Bulinus nasutus*	B_nasutus_2	*no*			Y (12)	
Pemba Island	45_ xS1_Puj4.3	Pujini	Puj4	-5.29000	39.81627	*Bulinus nasutus*	B_nasutus_2	*no*			Y (13)	
Pemba Island	46_ xS1_Puj5.1	Pujini	Puj5	-5.29160	39.81463	*Bulinus nasutus*	B_nasutus_1	*no*			Y (14)	
Pemba Island	47_ xS1_Puj5.2	Pujini	Puj5	-5.29160	39.81463	*Bulinus nasutus*	B_nasutus_2	*no*			Y (15)	
Pemba Island	48_ xS1_Puj5.3	Pujini	Puj5	-5.29160	39.81463	*Bulinus nasutus*	B_nasutus_2	*no*			Y (16)	
Pemba Island	49_ xS1_Puj9.1	Pujini	Puj9	-5.31440	39.81087	*Bulinus nasutus*	B_nasutus_1	*no*			Y (17)	
Pemba Island	50_ xS1_Puj9.2	Pujini	Puj9	-5.31440	39.81087	*Bulinus nasutus*	B_nasutus_1	*no*			Y (18)	
Pemba Island	51_ xS1_Puj9.3	Pujini	Puj9	-5.31440	39.81087	*Bulinus nasutus*	B_nasutus_1	*no*			Y (19)	
Pemba Island	52_ xS1_Puj10.1	Pujini	Puj10	-5.31627	39.81052	*Bulinus nasutus*	B_nasutus_1	*no*			Y (20)	
Tanzania	TZX-059.9.1	Kahumulo	Kahumulo_(Tz059)	-2.68010	32.80136	*Bulinus* sp. *africanus*	B.sp.glob_TZ_Hap_1	*S. kisumuensis*			Y (21)	

Supplementary Table 7.1. All *Bulinus* spp. mitogenome dataset providing collection information and species identification.

bul#	Sample_ID	Species_ID (cox1 haplotype)	Species_morphological	Species_NGS_MOTU	Country	Region	Site	Parasite?	Lat_cor	Lon_cor	Collector	Assembly_progress_SNAIL
1	Pem_Wingwi_1	B_globosus_1a	*Bulinus globosus*	*Bulinus globosus C*	Pemba Island	Wingwi	Wingwi	no	-5.02793	39.80836	Russell Stothard	COMPLETE
2	PEM_Kanga_4.1	B_nasutus_3	*Bulinus nasutus nasutus*	*Bulinus nasutus nasutus*	Pemba Island	Kangagani	Kangagani1	*Schistooma haematobium*	-5.17184	39.82031	Tom Pennance	COMPLETE
3	242_ xS3_Puj11.3	B_globosus_1a	*Bulinus globosus*	*Bulinus globosus C*	Pemba Island	Pujini	Puj11	no	-5.28853	39.81882	Tom Pennance	COMPLETE
4	240_ xS3_Puj11.1	B_nasutus_2	*Bulinus nasutus nasutus*	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj11	no	-5.28853	39.81882	Tom Pennance	COMPLETE
5	241_ xS3_Puj11.2	B_nasutus_1	*Bulinus nasutus nasutus*	*Bulinus nasutus nasutus*	Pemba Island	Pujini	Puj11	no	-5.28853	39.81882	Tom Pennance	COMPLETE
6	166_ xS2_Waw1.1	B_globosus_1a	*Bulinus globosus*	*Bulinus globosus C*	Pemba Island	Wawi	Waw1	no	-5.24493	39.78955	Tom Pennance	COMPLETE
7	108_ xS2_Wam5.1	B_globosus_2a	*Bulinus globosus*	*Bulinus globosus C*	Pemba Island	Wambaa	Wam5	no	-5.29975	39.68252	Tom Pennance	COMPLETE
8	192_ xS2_Uku9.2	B_globosus_1c	*Bulinus globosus*	*Bulinus globosus C*	Pemba Island	Ukutini	Uku9	no	-5.35512	39.76063	Tom Pennance	COMPLETE
9	393_xS4_Uku8.1	B_globosus_2b	*Bulinus globosus*	*Bulinus globosus C*	Pemba Island	Ukutini	Uku8	no	-5.33978	39.76675	Tom Pennance	COMPLETE
10	170_ xS2_Ole13.1	B_globosus_2a	*Bulinus globosus*	*Bulinus globosus C*	Pemba Island	Ole	Ole13	no	-5.19982	39.79393	Tom Pennance	COMPLETE
11	S2_Kinya6.1	B_globosus_2a	*Bulinus globosus*	*Bulinus globosus C*	Pemba Island	Kinyasini	Kinya6	*Schistosoma haematobium*	-5.0356	39.7385	Tom Pennance	COMPLETE
12	SP_Kin4.8.3	B_globosus_2a	*Bulinus globosus*	*Bulinus globosus C*	Pemba Island	Kinyasini	Kinya6	*Schistosoma bovis*	-5.0356	39.7385	Tom Pennance	COMPLETE
13	131_ xS2_Kinya1.2	B_globosus_1a	*Bulinus globosus*	*Bulinus globosus C*	Pemba Island	Kinyasini	Kinya1	no	-5.02062	39.73972	Tom Pennance	COMPLETE
14	75_ xS1_Kinya12.1	B_globosus_2a	*Bulinus globosus*	*Bulinus globosus C*	Pemba Island	Kinyasini	Kinya12	no	-5.03705	39.74692	Tom Pennance	COMPLETE
15	S2_Kiz5.1	B_globosus_2a	*Bulinus globosus*	*Bulinus globosus C*	Pemba Island	Kizimbani	Kiz5	*Schistosoma haematobium*	-5.04782	39.7383	Tom Pennance	COMPLETE
16	154_ xS2_Kiz1.1	B_globosus_1b	*Bulinus globosus*	*Bulinus globosus C*	Pemba Island	Kizimbani	Kiz1	no	-5.05337	39.7404	Tom Pennance	COMPLETE
17	361_xS4_Kiz1.1	B_globosus_1f	*Bulinus globosus*	*Bulinus globosus C*	Pemba Island	Kizimbani	Kiz1	no	-5.05337	39.7404	Tom Pennance	COMPLETE
18	271_ xS3_Kiz2.3	B_globosus_1e	*Bulinus globosus*	*Bulinus globosus C*	Pemba Island	Kizimbani	Kiz2	no	-5.04835	39.74305	Tom Pennance	COMPLETE
19	213_ xS3_Mat1.3	B_globosus_1d	*Bulinus globosus*	*Bulinus globosus C*	Pemba Island	Matale	Mat1	no	-5.2818	39.7683	Tom Pennance	COMPLETE
20	SP_Uwa_5.1	B. nasutus_4	*Bulinus nasutus nasutus*	*Bulinus nasutus nasutus*	Pemba Island	Uwandani	Uwa5	no	-5.20082	39.82897	Tom Pennance	COMPLETE
21	SP_Cham10.1a	B_globosus	*Bulinus globosus*	*Bulinus globosus C*	Pemba Island	Chambani	Cham10	*Schistosoma haematobium*	-5.35805	39.79182	Tom Pennance	COMPLETE
22	SP_Cham9.1	B. nasutus_5	*Bulinus nasutus nasutus*	*Bulinus nasutus nasutus*	Pemba Island	Chambani	Cham9	no	-5.35862	39.79255	Tom Pennance	COMPLETE
23	U-Mtende-ZJ-2	B_nasutus	*Bulinus nasutus nasutus*	*Bulinus nasutus nasutus*	Unguja Island	Mtende	Ziwa Jangwa	no	-6.44507	39.52943	Tom Pennance	COMPLETE
24	UNG-Jendele-134.1	B_nasutus_NA	*Bulinus nasutus nasutus*	*Bulinus nasutus nasutus*	Unguja Island	Jendele	Forodhani - IRN: 384805	no	-6.18935	39.37102		COMPLETE
25	UNG-Miwani-123.1	B_globosus	*Bulinus globosus*	*Bulinus globosus M*	Unguja Island	Miwani	Kinumoshi - IRN: 384814	*Schistosoma haematobium*	-6.08857	39.27673		COMPLETE

26	UNG-Kinya-Ziwani-A+ve-286.1	B_globosus	*Bulinus globosus*	*Bulinus globosus M*	Unguja Island	Kinyasini	Ziwani A (Mrogoro) IRN: 384846	*Schistosoma haematobium*	-5.96415	39.30687		COMPLETE
27	UNG-Mfenesini-135.1	B_nasutus_NA	*Bulinus nasutus nasutus*	*Bulinus nasutus nasutus*	Unguja Island	Mtopepo	Mfenesini	no	-6.14353	39.22935	Fiona Allan	COMPLETE
28	UNG-Chaani-12-17.1	B_globosus	*Bulinus globosus*	*Bulinus globosus M*	Unguja Island	Chaani	Chaani site 12 - ‚ÄúSharp/Bulubulu‚Äù	no	-5.93717	39.2973	James Rudge	COMPLETE
29	UGA_Waka1	B_truncatus	*Bulinus truncatus*	*Bulinus truncatus sp 1*	Uganda	Bugiri	Waka Waka 0M	no	0.32253	33.71113	Christina Faust	COMPLETE
30	UGA_Waka4	Bulinus sp. (africanus)	*Bulinus sp. (africanus)*	*Bulinus africanus sp 1*	Uganda	Bugiri	WA150M	no	0.32372	33.71055	Christina Faust	COMPLETE
31	UGA_Bugoto6	Bulinus sp. (truncatus)	*Bulinus sp. (truncatus)*	*Bulinus truncatus sp 4*	Uganda	Mayuge	Bugoto Site E	no	0.32033	33.62859	Christina Faust	COMPLETE
32	UGA_Bugoto8	B_truncatus	*Bulinus truncatus*	*Bulinus truncatus sp 1*	Uganda	Mayuge	Bugoto Site F	no	0.3184	33.62732	Christina Faust	COMPLETE
33	UGA_Wanseko-14.1	Bulinus sp. (africanus)	*Bulinus sp. (africanus)*	*Bulinus africanus sp 1*	Uganda	Lake Albert	Wanseko	no	2.1798	31.37838		COMPLETE
35	TZ059.9-1	Bulinus sp. (africanus)	*Bulinus sp. (africanus)*	*Bulinus africanus sp 1*	Tanzania	Mwanza	Majini	*Schistosoma kisumuensis*	-2.66965	32.8174	Bonnie Wesbter	COMPLETE
36	TEM-1	B_globosus_2a	*Bulinus globosus*	*Bulinus globosus*	Tanzania	Temeke	Dar	*? Schistosoma bovis ?* Prepatent	-6.88736	39.25727	Khadija Said / Steffi Knopp	COMPLETE
37	TZ_Ukonga_primary-29.1	B_globosus_2	*Bulinus globosus*	*Bulinus globosus*	Tanzania	Ukonga	Ukonga Primary School	no	-6.85744	39.19306		COMPLETE
38	TZ-Iringa-97.1	B_globosus	*Bulinus globosus*	*Bulinus globosus M*	Tanzania	Iringa	Dar	no	-7.75709	35.6871		COMPLETE
39	TZ_Zinga-16.1	Bulinus nasutus nasutus	*Bulinus nasutus nasutus*	*Bulinus nasutus nasutus*	Tanzania	Zinga	Dar	no	-6.52671	38.98549		COMPLETE
40	TZ011-Nyahanga-Sc-S2_1	Bulinus globosus	*Bulinus globosus*	*Bulinus globosus M*	Tanzania	Bulima	Nyahanga mwaloni site 1	no	-2.36423	33.5762		COMPLETE
41	TZ144-site6-Sc-S3_+1	Bulinus nasutus productus	*Bulinus nasutus productus*	*Bulinus nasutus productus*	Tanzania	Shilingwa	Site 6	non-schisto	-2.43632	33.091		COMPLETE
42	TZ059-Lus1-Sc-S1_+3	Bulinus nasutus productus	*Bulinus nasutus productus*	*Bulinus nasutus productus*	Tanzania	Kahumulo	Lusesebela site 1	non-schisto	-2.67758	32.7875		COMPLETE
43	TZ038-Msikitini-Sc-S2_1	Bulinus sp. (africanus)	*Bulinus sp. (africanus)*	*Bulinus africanus sp 1*	Tanzania	Igalagalilo	Msikitini	no	-2.5342	32.7554		COMPLETE
44	TZ059-Malila-Sc-S2_1	Bulinus sp. (africanus)	*Bulinus sp. (africanus)*	*Bulinus africanus sp 1*	Tanzania	Kahumulo	Malila	no	-2.67122	32.8169		COMPLETE
45	TZ059-Katani-Sc-S2_1	Bulinus sp. (africanus)	*Bulinus sp. (africanus)*	*Bulinus africanus sp 1*	Tanzania	Kahumulo	Katani site 1	no	-2.69238	32.8189		COMPLETE
46	TZ059-Mkama-Sc-S2_1	Bulinus sp. (africanus)	*Bulinus sp. (africanus)*	*Bulinus africanus sp 1*	Tanzania	Kahumulo	Mkama site 1	no	-2.66922	32.8359		COMPLETE
47	TZ144-Igusa2-Sc-S2_1	Bulinus sp. (africanus)	*Bulinus sp. (africanus)*	*Bulinus africanus sp 1*	Tanzania	Shilingwa	Igusa site 2	no	-2.42508	33.1245		COMPLETE
48	TZ087-Kanyen2-Sc-S2_1	Bulinus sp. (africanus)	*Bulinus sp. (africanus)*	*Bulinus africanus sp 1*	Tanzania	Luchili	Kanyenyela site 2	no	-2.54493	32.5136		COMPLETE
49	TZ012-Ujaroni-Dr-S2_2	Bulinus tropicus / nyassanus	*Bulinus sp. (truncatus)*	*Bulinus truncatus sp 5*	Tanzania	Bulolo	Ujaroni site 3	no	-2.32725	32.1965		COMPLETE
50	M3-Ambilobe-Bul-1	B_obtusispira	*Bulinus obtusispira*	*Bulinus obtusispira*	Madagascar		Ambilobe	no	-13.20747	49.05596	Russell Stothard	COMPLETE

51	M2-Belamoty-Bul-1	B_bavayi	*Bulinus bavayi*	*Bulinus bavayi*	Madagascar		Belamoty	no	-23.56683	45.77686	Russell Stothard	COMPLETE
52	M5-Ambolofasy-Bul-1	Bulinus sp. (truncatus)	*Bulinus sp. (truncatus)*	*Bulinus liratus*	Madagascar		Ambolofasy	no	-25.03487	46.74643	Russell Stothard	COMPLETE
53	MAD-Ambatosia2-90.1	Bulinus sp. (truncatus)	*Bulinus sp. (truncatus)*	*Bulinus truncatus sp 3*	Madagascar		Ambatosia 2	no	-14.66555	48.66018		COMPLETE
54	MAD-Besely-92.1	Bulinus sp. (truncatus)	*Bulinus sp. (truncatus)*	*Bulinus liratus*	Madagascar		Besely	Trematode	-23.4906	44.52076		COMPLETE
55	MAD-Kasaria-93.1	Bulinus sp. (truncatus)	*Bulinus sp. (truncatus)*	*Bulinus liratus*	Madagascar		Kasaria	no	-24.2064	45.66405		COMPLETE
56	MAD-Ankiliabo-94.1	Bulinus sp. (truncatus)	*Bulinus sp. (truncatus)*	*Bulinus liratus*	Madagascar		Ankiliabo	no	-22.7721	43.5967		COMPLETE
57	Lake_Chilwa_1	Bulinus africanus	*Bulinus africanus*	*Bulinus africanus sp 2*	Malawi	Lake Chilwa		no	-15.11698	35.63106	Russell Stothard	COMPLETE
58	SS1	Bulinus sp. (africanus)	*Bulinus sp. (africanus)*	*Bulinus africanus sp 4*	Malawi	Lake Malawi	Malawi_01	no	-14.36875	35.17667	Russell Stothard	COMPLETE
59	SM1	Bulinus sp. (africanus)	*Bulinus africanus*	*Bulinus africanus sp 2*	Malawi	Lake Malawi	Malawi_01	no	-14.36875	35.17667	Russell Stothard	COMPLETE
60	M11	B_globosus	*Bulinus globosus*	*Bulinus globosus*	Malawi	Lake Malawi	Malawi_11	no	-14.3117	35.13551	Russell Stothard	COMPLETE
61	Malawi_Palm_Beach_1	Bulinus sp. (africanus)	*Bulinus sp. (africanus)*	*Bulinus africanus sp 3*	Malawi	Palm Beach	Malawi_12	no	-14.39361	35.222	Russell Stothard	MISSING GENES
62	Chikhwawa_1	Bulinus sp. (africanus)	*Bulinus africanus*	*Bulinus africanus sp 2*	Malawi	Chikhwawa	Mpangeni oxbow lake	no	-16.03819	34.84222	Russell Stothard	COMPLETE
63	BK23	B. globosus	*Bulinus globosus*	*Bulinus globosus W*	Niger	Bangou Koirey	Bangou Koirey 2 canal secondaire	*Schistosoma bovis*	13.60475	1.90509	Niger team	COMPLETE
64	L2-Ganta-Bul-1	B. globosus	*Bulinus globosus*	*Bulinus globosus W*	Liberia			no	7.22499	-8.98131	Russell Stothard	COMPLETE
65	BA-56.1	B. angolensis	*Bulinus angolensis*	*Bulinus angolensis*	Angola	Source of River Cota	TYPE LOCALITY. Malanje River Mangumbala	no	-9.26186	16.13693	David Rollinson + J C. S-F - Gulbenkian funded project	COMPLETE
66	BA-59.2	B. angolensis	*Bulinus angolensis*	*Bulinus angolensis*	Angola	River Quastimbala	Type locality for Biom. Salinarium	no	-9.59848	16.48999	David Rollinson + J C. S-F - Gulbenkian funded project	COMPLETE
67	BG-5.9	B. globosus	*Bulinus globosus*	*Bulinus globosus A*	Angola	Cabungo stream	TYPE LOCALITY. Bengo stream Cabungo	no	-8.57579	13.53737	David Rollinson + J C. S-F - Gulbenkian funded project	COMPLETE
68	E2	B. trunc group	*Bulinus sp. (truncatus)*	*Bulinus truncatus sp 2*	Ethiopia	Wonji		no	8.25078	39.14097	Fiona Allan WISER EPSRC	COMPLETE
69	E3	B. trunc group	*Bulinus sp. (truncatus)*	*Bulinus truncatus sp 2*	Ethiopia	Wonji		no	8.25078	39.14097	Fiona Allan WISER EPSRC	COMPLETE
70	D3-33-44-B6	B. globosus	*Bulinus globosus*	*Bulinus globosus W*	Senegal	Lac de Guiers	Malla Tack 2	no	16.13378	-15.88548	Chelsea Wood / Sanna Sokolow - Upstream Alliance	COMPLETE
73	BK40	B. globosus	*Bulinus globosus*	*Bulinus globosus W*	Niger			*Schistosoma bovis*	13.60475	1.90509		COMPLETE
75	LA30	B. truncatus	*Bulinus sp. (truncatus)*	*Bulinus truncatus sp 1*	Niger			*Schistosoma haematobium*	13.74193	1.68262		COMPLETE

Supplementary File 6.1: Generalised Linear Mixed Effects Models (GLMMs) outputs testing Abiotic and biotic variables impacting non-patent schistosome infections and abundance of *Bulinus*

GLMM 1: Presence/absence of *Schistosoma haematobium* infections per *Bulinus* spp. snail

GLMM1: Model output

```
Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)
[glmerMod]

 Family: binomial  ( logit )
Formula: Sh_all ~ (1 | shehia.x/site.x) + wlevel + temp + ph
   Data: df.na.rem
Control: glmerControl("bobyqa")

     AIC      BIC   logLik deviance df.resid
  1170.3   1216.3   -578.1   1156.3     5252

Scaled residuals:
    Min      1Q  Median      3Q     Max
-0.7302 -0.1826 -0.0817 -0.0474 31.1000

Random effects:
 Groups          Name        Variance  Std.Dev.
 site.x:shehia.x (Intercept) 3.487e+00 1.867e+00
 shehia.x        (Intercept) 1.897e-14 1.377e-07
Number of obs: 5259, groups:  site.x:shehia.x, 46; shehia.x, 7
```

Table S1. Summary table of GLMM1, displaying; fixed effects estimate (E), fixed effects standard error (SE) and the exponential transformations as well as p value. Significant fixed effects p values ($p < 0.05$) in bold.

	E	SE	E (transformed exponential)	SE (transformed exponential)	*p* value
(Intercept)	-5.70162	2.87293	0.00334	0.05575	0.04719
wlevel.L	0.66604	0.27789	1.94651	0.62355	**0.01654**
wlevel.Q	-1.75884	0.22952	0.17224	0.04444	**1.81E-14**
temp	0.22253	0.04963	1.24923	0.06356	**7.33E-06**
ph	-0.83789	0.31911	0.43262	0.16262	**0.00865**

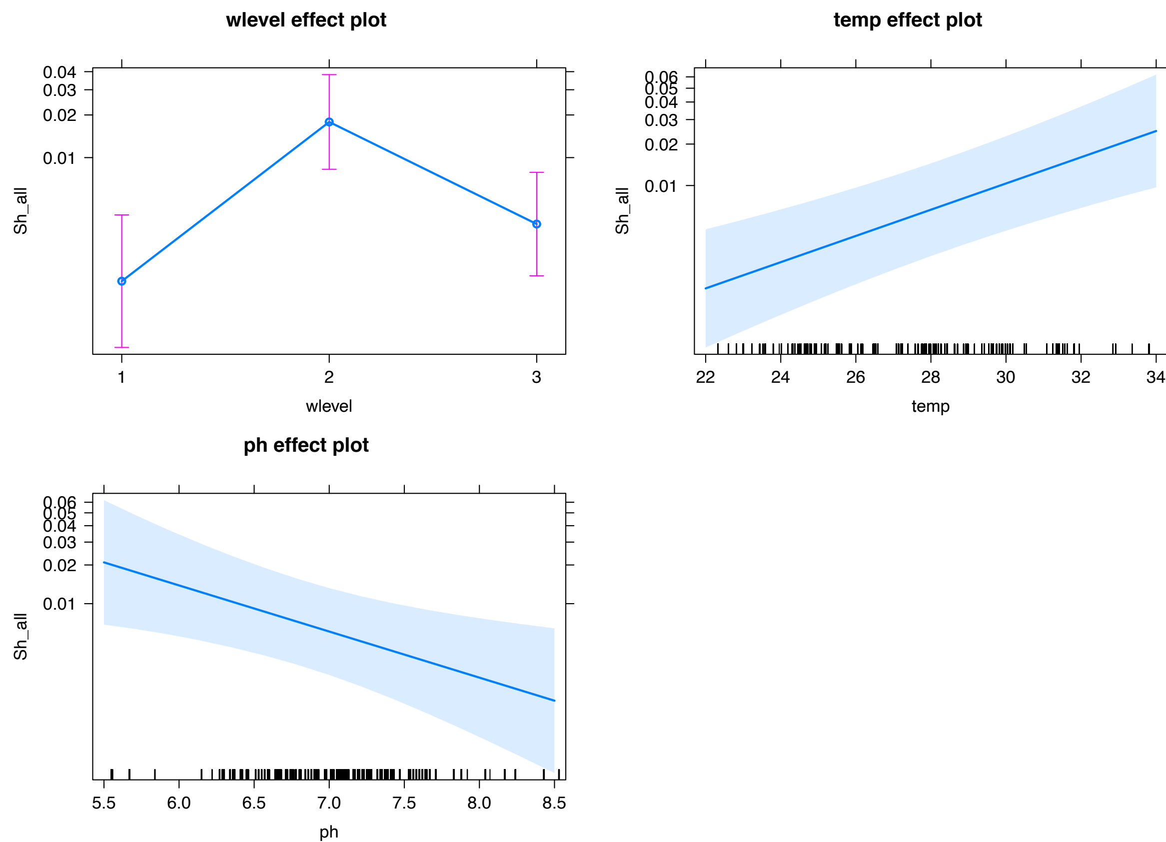


Figure S1. Estimated fixed effects plots for GLMM1 produced using the “car” v3.0.7 (Fox and Weisberg, 2019) package in R v4.0.0 (R Core Team, 2018) displaying 95% confidence intervals.

GLMM 2: *Bulinus* spp. abundance per site

GLMM2: Model output

```
Generalized linear mixed model fit by maximum likelihood (Laplace
  Approximation) [glmerMod]
 Family: Negative Binomial(0.392)  ( log )
Formula: BgBn_number ~ wlevel + type + survey + temp + (1 | shehia/site)
   Data: siteDF

     AIC      BIC   logLik deviance df.resid
  1743.6   1789.4   -858.8   1717.6      237

Scaled residuals:
    Min      1Q  Median      3Q     Max
-0.6250 -0.5029 -0.2673  0.1924  3.0168

Random effects:
 Groups      Name        Variance Std.Dev.
 site:shehia (Intercept) 3.3700   1.8358
 shehia      (Intercept) 0.9299   0.9643
Number of obs: 250, groups:  site:shehia, 73; shehia, 8
```

Table S2. Summary table of GLMM2, displaying; fixed effects estimate (E), fixed effects standard error (SE) and the exponential transformations as well as p value. Significant fixed effects p values ($p < 0.05$) in bold.

	E	SE	E (transformed exponential)	SE (transformed exponential)	*p* value
(Intercept)	8.48248	2.57008	4829.41200	58275.88556	0.000965
wlevel.L	-1.01431	0.43806	0.36265	0.19935	**0.020589**
wlevel.Q	0.21199	0.31904	1.23614	0.46455	0.506395
type.L	-3.30766	0.88789	0.03660	0.05234	**0.000195**
type.Q	-1.93669	0.89809	0.14418	0.20977	**0.031048**
type.C	-1.05775	0.91179	0.34724	0.51696	0.246014
surveyS2	-1.54016	0.52552	0.21435	0.14819	**0.003382**
surveyS3	-2.29989	0.69894	0.10027	0.10143	**0.001**
surveyS4	-2.15312	0.52497	0.11612	0.08017	**4.11E-05**
temp	-0.17426	0.08534	0.84008	0.07484	**0.041154**

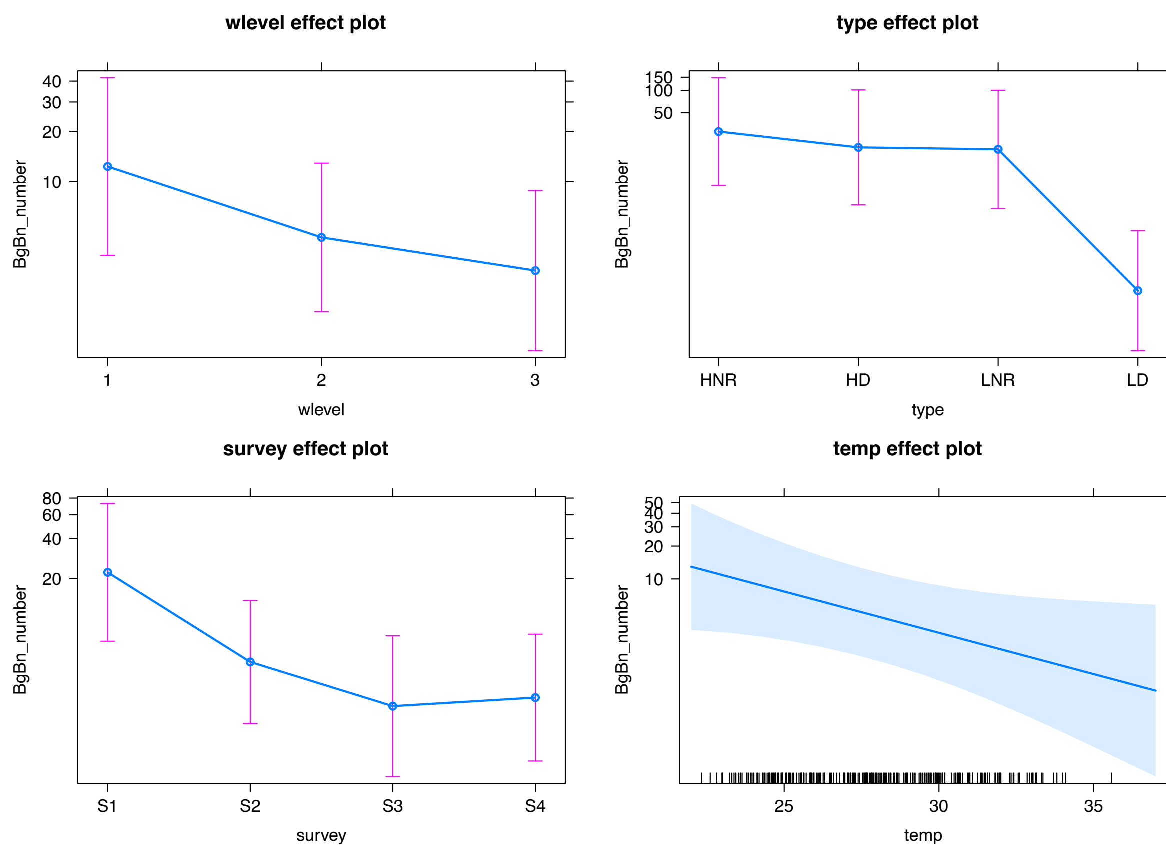


Figure S2. Estimated fixed effects plots for GLMM2 produced using the “car” v3.0.7 (Fox and Weisberg, 2019) package in R v4.0.0 (R Core Team, 2018) displaying 95% confidence intervals.

GLMM3: *Bulinus* spp. abundance per site with temperature – water level interaction

GLMM3: Model output

```
Generalized linear mixed model fit by maximum likelihood (Laplace
  Approximation) [glmerMod]
 Family: Negative Binomial(0.392)  ( log )
Formula: BgBn_number ~ wlevel * temp + type + survey + (1 | shehia/site)
   Data: siteDF

     AIC      BIC   logLik deviance df.resid
  1747.2   1800.0   -858.6   1717.2      235

Scaled residuals:
    Min      1Q  Median      3Q     Max
-0.6251 -0.5112 -0.2724  0.1718  3.0561

Random effects:
 Groups      Name        Variance Std.Dev.
 site:shehia (Intercept) 3.305    1.8179
 shehia      (Intercept) 0.884    0.9402
Number of obs: 250, groups:  site:shehia, 73; shehia, 8

Fixed effects:
              Estimate Std. Error z value Pr(>|z|)
(Intercept)    9.15472    2.86662   3.194 0.001405 **
wlevel.L      -2.14748    3.50560  -0.613 0.540152
wlevel.Q       2.02779    3.00375   0.675 0.499620
temp          -0.19657    0.09613  -2.045 0.040883 *
type.L        -3.35649    0.87579  -3.833 0.000127 ***
type.Q        -1.97055    0.88387  -2.229 0.025784 *
type.C        -1.03523    0.89678  -1.154 0.248341
surveyS2      -1.62913    0.54541  -2.987 0.002818 **
surveyS3      -2.33407    0.71730  -3.254 0.001138 **
surveyS4      -2.14991    0.53210  -4.040 5.33e-05 ***
wlevel.L:temp  0.04095    0.12377   0.331 0.740762
wlevel.Q:temp -0.06662    0.10915  -0.610 0.541610
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Supplementary File 7.1. Species delimitation analysis (bPTP) output displaying posterior probability for the species recognised.

```
# Most supported partition found by simple heuristic search
Species 1 (support = 1.000)
    bul51_B_bavayi
Species 2 (support = 1.000)
    bul50_B_obtusispira
Species 3 (support = 1.000)
    bul61_B_africanus_spp
Species 4 (support = 1.000)
    bul49_B_truncatus_spp
Species 5 (support = 1.000)
    bul31_B_truncatus_spp
Species 6 (support = 1.000)
    bul53_B_liratus
Species 7 (support = 1.000)
    bul58_B_africanus_spp
```

Species 8 (support = 0.994)
bul65_B_angolensis,bul66_B_angolensis
Species 9 (support = 1.000)
bul67_B_globosus
Species 10 (support = 1.000)
bul64_B_globosus
Species 11 (support = 0.987)
bul70_B_globosus,bul63_B_globosus,bul73_B_globosus
Species 12 (support = 1.000)
bul38_B_globosus
Species 13 (support = 1.000)
bul40_B_globosus
Species 14 (support = 1.000)
bul62_B_africanus_spp
Species 15 (support = 0.950)
bul9_B_globosus,bul15_B_globosus,bul36_B_globosus,bul37_B_globosus,bul7_B_globosus,bul11_B_globosus,bul12_B_globosus,bul14_B_globosus,bul10_B_globosus,bul21_B_globosus
Species 16 (support = 1.000)
bul59_B_africanus_spp
Species 17 (support = 1.000)
bul57_B_africanus
Species 18 (support = 0.987)
bul54_B_liratus,bul52_B_liratus,bul55_B_liratus,bul56_B_liratus
Species 19 (support = 0.955)
bul68_B_truncatus_spp,bul69_B_truncatus_spp
Species 20 (support = 0.982)
bul25_B_globosus
Species 21 (support = 0.975)
bul26_B_globosus,bul28_B_globosus
Species 22 (support = 0.704)
bul60_B_globosus
Species 23 (support = 0.663)
bul16_B_globosus,bul17_B_globosus,bul18_B_globosus,bul8_B_globosus,bul19_B_globosus,bul1_B_globosus,bul6_B_globosus,bul3_B_globosus,bul13_B_globosus
Species 24 (support = 0.942)
bul75_B_truncatus_spp
Species 25 (support = 0.854)
bul29_B_truncatus_spp,bul32_B_truncatus_spp
Species 26 (support = 0.557)
bul48_B_africanus_spp,bul47_B_africanus_spp,bul30_B_africanus_spp,bul33_B_africanus_spp,bul43_B_africanus_spp,bul44_B_africanus_spp,bul35_B_africanus_spp
Species 27 (support = 0.591)
bul45_B_africanus_spp,bul46_B_africanus_spp
Species 28 (support = 0.583)
bul41_B_nasutus_productus
Species 29 (support = 0.583)
bul42_B_nasutus_productus
Species 30 (support = 0.544)
bul20_B_nasutus
Species 31 (support = 0.542)
bul39_B_nasutus,bul27_B_nasutus,bul22_B_nasutus,bul2_B_nasutus,bul23_B_nasutus,bul24_B_nasutus,bul4_B_nasutus,bul5_B_nasutus

APPENDIX

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Article

Development of a Molecular Snail Xenomonitoring Assay to Detect *Schistosoma haematobium* and *Schistosoma bovis* Infections in their *Bulinus* Snail Hosts

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Abstract: Schistosomiasis, a neglected tropical disease of medical and veterinary importance, transmitted through specific freshwater snail intermediate hosts, is targeted for elimination in several endemic regions in sub-Saharan Africa. Multi-disciplinary methods are required for both human and environmental diagnostics to certify schistosomiasis elimination when eventually reached. Molecular xenomonitoring protocols, a DNA-based detection method for screening disease vectors, have been developed and trialed for parasites transmitted by hematophagous insects, such as filarial worms and trypanosomes, yet few have been extensively trialed or proven reliable for the intermediate host snails transmitting schistosomes. Here, previously published universal and *Schistosoma*-specific internal transcribed spacer (ITS) rDNA primers were adapted into a triplex PCR primer assay that allowed for simple, robust, and rapid detection of *Schistosoma haematobium* and *Schistosoma bovis* in *Bulinus* snails. We showed this two-step protocol could sensitively detect DNA of a single larval schistosome from experimentally infected snails and demonstrate its functionality for detecting *S. haematobium* infections in wild-caught snails from Zanzibar. Such surveillance tools are a necessity for succeeding in and certifying the 2030 control and elimination goals set by the World Health Organization.

Keywords: bovine; control; elimination; schistosomiasis; urogenital; surveillance; disease; parasite

1. Introduction

Schistosomiasis is a disease affecting an estimated 229 million people worldwide caused by infection with parasitic worms of the genus *Schistosoma*, leading to severe morbidity and mortality due to the associated complications of worm presence [1]. *Schistosoma* spp. in Africa are transmitted through specific freshwater snail intermediate hosts of the *Bulinus* and *Biomphalaria* genera [2]. Infections occur when humans or animals come into contact with freshwater containing infectious larval stages (cercariae) shed from the infected snails. Human schistosomiasis in Africa, where at least ~90% of the people requiring treatment live [3], consists of two forms of the disease—urogenital and intestinal schistosomiasis, caused predominantly by *Schistosoma haematobium* and *Schistosoma mansoni*, respectively [1]. Bovine, ovine, and caprine schistosomiasis is also of significant veterinary and economic importance across sub-Saharan Africa [4,5] and is caused by infection of cattle, sheep, and goats with species closely related to *S. haematobium* (termed *S. haematobium* group species), primarily *Schistosoma bovis, Schistosoma curassoni,* and *Schistosoma mattheei*. Overlapping geographical distribution of multiple schistosome and intermediate snail host species strains complicates disease transmission surveillance in (co)endemic zones [2,6,7].

The World Health Organization (WHO) aims for the elimination of human schistosomiasis as a public health problem, defined as >1% of the population with heavy intensity infections (≥50 schistosome eggs per 10 mL of urine, or ≥400 schistosome eggs per gram of feces [8]), in all endemic countries by 2030 [9]. Despite great advances in schistosomiasis control mainly via preventative chemotherapy (praziquantel), the lack of protection against rapid re-infection together with prolific asexual replication of schistosomes within their intermediate snail host presents substantial hurdles to achieving the targeted elimination of schistosomiasis. Very quickly, snails can become infected by eggs emanating from untreated humans, leading to a rapid resurgence of transmission [10]. Therefore, adaptive treatment strategies that take into account the transmission dynamics of *Schistosoma* spp. with their snail hosts are required to control and eliminate the disease [11].

To better understand the local transmission dynamics of different *Schistosoma* species, allowing both human and bovine schistosomiasis to be monitored, a need exists for methodologies that detect schistosome infections in the intermediate host snails. These tools for assessing *Schistosoma* transmission could eventually be used during elimination programs to identify focal areas of persisting transmission or certify elimination and/or transmission interruption [12–14]. Defining ongoing transmission in snail populations through traditional methods of observing cercariae shed from snails is particularly challenging in an elimination setting, such as the Zanzibar Archipelago, where few snails (0.5–2.3%) are observed shedding cercariae [6,15]. Snails with non-patent (including pre-patent) infections are missed using these approaches. Additionally, larval schistosomes are not easily identifiable to a species level using morphological characteristics (although the relative position of sensory receptors is of some value [16,17]).

Molecular xenomonitoring is a DNA-based method that has been developed to monitor the transmission of several vector-borne diseases, including trypanosomiasis [18,19], filariasis and malaria [20], helminthiases [21], and fascioliasis [22], including to some extent schistosomiasis [23–29]. Screening snails provides evidence on the extent of environmental contamination (i.e., schistosome miracidia penetrating snails), as well as environmental infection risk (i.e., schistosome sporocysts and cercariae developing inside the (pre-patent) snails, eventually emerging from the (patent) snail. Most of the available snail-schistosome xenomonitoring assays do not include internal controls [23,28,30], an important feature in any diagnostic tool that helps prevent false-negative results [27]. Many assays will assume that a negative result means non-infection, not necessarily reaction failure.

In the current study, we adapted available universal [31] and *Schistosoma*-specific [27] internal transcribed spacer (ITS) rDNA primers to design a three primer multiplex assay and tested this as a simple, robust, and rapid xenomonitoring PCR assay to enable the large-scale screening of *Bulinus* snails for *Schistosoma* infections (*S. haematobium* and *S. bovis*). We used a conventional PCR-based approach focused on simplicity, ease of data interpretation, sensitivity, and specificity, with a primary aim to provide a xenomonitoring tool for monitoring *S. haematobium* transmission in endemic settings.

2. Results

2.1. *In silico and in vitro Primer Evaluation*

Bulinus globosus and *Bulinus nasutus* rDNA sequence data showed conserved primer binding sites for the universal primers ETTS2 and ETTS1 [31] at the 3′ end of the 18S and 5′ end of the 28S, the flanking regions of the ITS, respectively. ETTS1 gave a 100% match, and the ETTS2 primer showed just a single base pair mismatch. The resulting snail amplicon size predicted from these alignments was between 1232 and 1263 bp and served as an internal snail control during PCR amplification.

Alignments of the *Schistosoma*-specific ITS primers (ITS2_Schisto_F and ITS2_Schisto_R [27]) showed 100% and 90% (2 mismatches) homology to *S. haematobium* and *S. bovis*, respectively, with no cross-reactivity to the *Bulinus* reference rDNA data. When paired with their opposing universal primers (ITS2_Schisto_F + ETTS1 or ITS2_Schisto_R + ETTS2), the amplicon sizes of 538 and 835 bp were predicted, respectively, for *Schistosoma*. With the addition of the other universal primer to each combination (ETTS2 and ETTS1, respectively), the three-primer multiplex ITS xenomonitoring (MIX) reactions were predicted to be able to produce distinct amplicon profiles for non-infected snails (a single snail amplicon) and snails infected with *Schistosoma* spp. (three-band profile). This was confirmed by *in vitro* testing of the primer combinations (Figure 1).

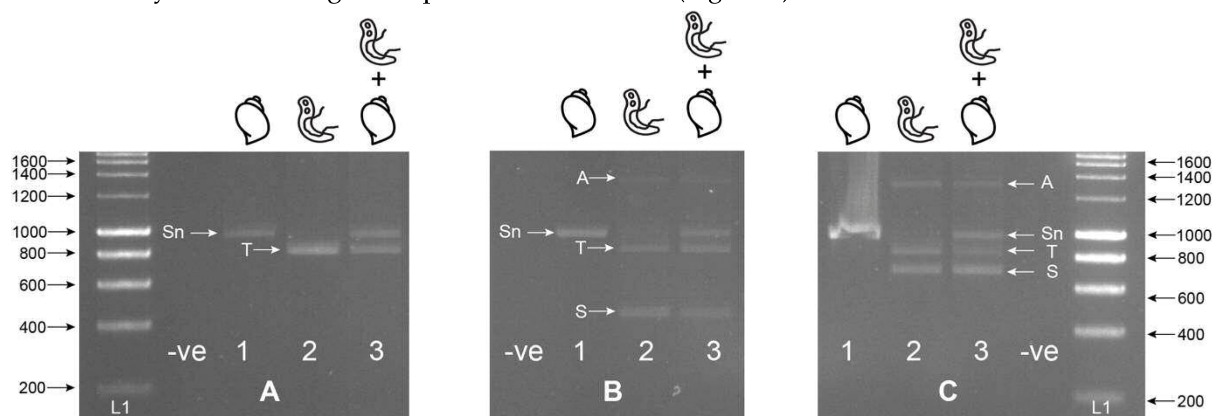


Figure 1. Singleplex (**A**; ETTS2 + ETTS1) and multiplex (**B**; multiplex ETTS2 + ETTS1 + ITS2_Schisto_F, **C**; ETTS2 + ETTS1 + ITS2_Schisto_R) PCRs on laboratory-bred *Bulinus wrighti* (*B.w.*) and *Schistosoma haematobium* (*S.h.*) gDNA separately (1; *B.w.*, 2; *S.h.*) and combined (3; *B.w.* + *S.h.*). When *B.w.* and *S.h.* DNA was combined (A3, B3, C3), two amplicons were produced by the ETTS1 + ETTS2 primers, a larger snail amplicon (Sn) (~1200 bp) and a smaller *Schistosoma* amplicon (T) (~1000), with the additional *Schistosoma*-specific primers producing either a 538 bp (B3; ITS2_Schisto_F) or 835 bp (C3; ITS2_Schisto_R) amplicon (S). A larger amplicon (A) (~1400–1600 bp) was also observed to be amplified in some reactions, and this was thought to be a PCR artifact or additional primer targets in the *Schistosoma* gDNA. L1 = HyperLadder I (Bioline, London, UK). -ve = negative, no template control. ITS = internal transcribed spacer.

To maximize amplification efficiency/sensitivity and to provide good amplicon size differentiation, the multiplex PCR incorporating the internal ITS2_Schisto_F (Figure 1B) was selected for further development and testing. This primer combination was also selected as it targeted the ITS2 region for *Schistosoma* containing four species-specific SNPs, enabling species identification (Table 1).

The MIX assay proved robust at varying annealing temperatures (55 °C, 60 °C Figure 2A, 58 °C Figure 2B), with 58 °C proving to be the most efficient, maximizing specificity without decreasing sensitivity. Each of the three amplicons was extracted from the gel and sequenced, confirming the band identity and specificity of the primers to their target gDNA amplicon. These three bands have been described as the snail (Sn) (1232–1263 bp), trematode (T) (~1000 bp), and *Schistosoma* (S) (538 bp) bands going forward. The secondary *Schistosoma* ITS xenomonitoring (SIX) PCR, solely targeting the *Schistosoma* amplicon, proved robust, enabling single amplicon generation and sequencing (Figure 3).

This provided a two-step PCR methodology with the MIX PCR for the initial high-throughput screening of the samples and the secondary SIX PCR to target specific samples for further infection clarification by *Schistosoma* species identification through DNA sequencing.

Table 1. *Schistosoma* species-specific SNP positions (including base position) in the internal transcribed spacer (ITS)2 region.

Schistosoma Species	ITS 2 Schistosome Species-Specific SNP Positions (bp)			
	SNP1 (90)	SNP2 (145)	SNP3 (195)	SNP4 (265)
S. haematobium	*S. h* (G)	*S. h* (C)	*S. h* (G)	*S. h* (C)
S. bovis	*S. b* (A)	*S. b* (T)	*S. b* (A)	*S. b* (T)

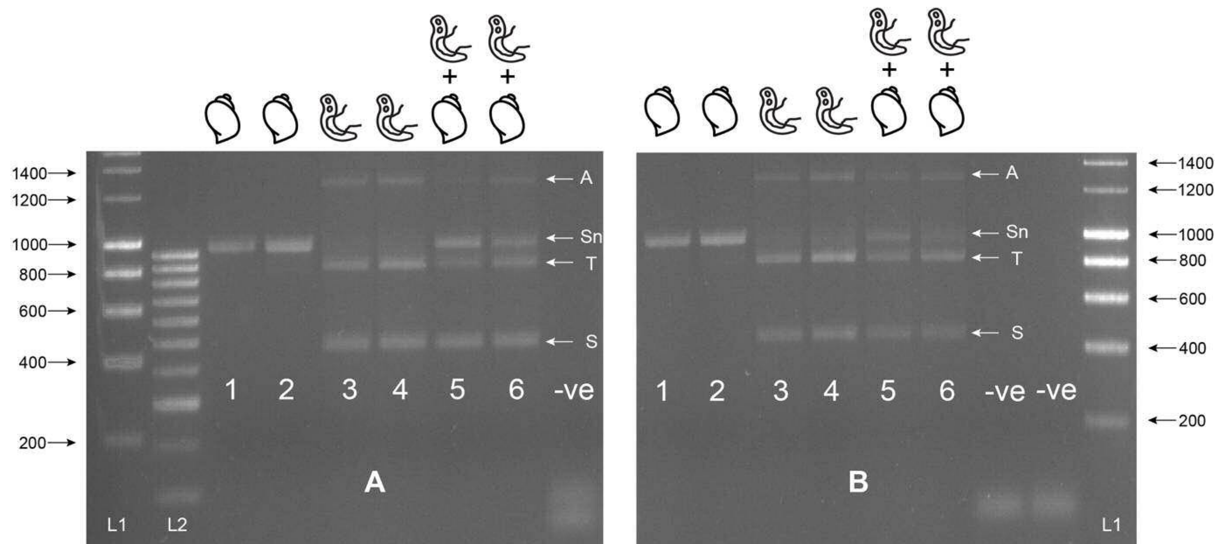


Figure 2. Multiplex ITS xenomonitoring assay trial at 55 °C (**A**) and 60 °C (**B**). Includes gDNA of *Bulinus wrighti* of both BioSprint (Lane 1 and 5) and DNeasy extractions (Lane 2 and 6) and gDNA of *Schistosoma haematobium* (Lane 3 and 5) and *S. bovis* (Lane 4 and 6). Combinations of *B. wrighti* and *S. haematobium* (Lane 5) or *S. bovis* (Lane 6) gDNA shown. Sn = snail amplicon, T = trematode amplicon, S = *Schistosoma* amplicon, and A = non-specific amplicon or artifact. L1 = HyperLadder I. L2 = HyperLadder IV (Bioline, London, UK). -ve = negative, no template control.

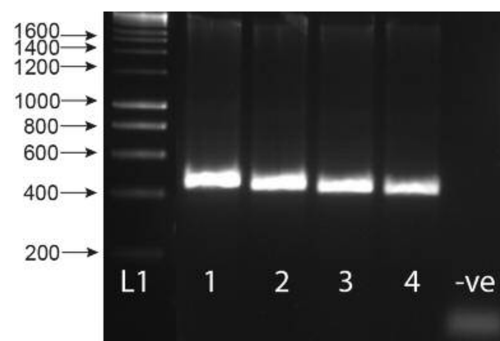


Figure 3. Gel showing the secondary singleplex ITS xenomonitoring (SIX) PCR for 1) *Schistosoma haematobium* gDNA; 2) *S. bovis* gDNA; 3) *S. haematobium* + *B. wrighti* gDNA; 4) *S. bovis* + *B. wrighti* gDNA. -ve = non-template negative control. L1 = HyperLadder I (Bioline, London, UK).

2.2. Analytical Sensitivity

The assay proved highly sensitive with a limit-of-detection (LoD) of 0.02 ng and 0.002 ng of gDNA for *S. bovis* and *S. haematobium*, respectively (Figure 4). Sensitivity appeared higher for *S. haematobium* (Figure 4), but in both the cases, the assay's sensitivity was above that necessary to detect gDNA from a single miracidium, which ranges from 1.6–3.65 ng/μL [32]. At lower *Schistosoma* DNA concentrations, the 1005 bp trematode band (T) lost sensitivity compared with the smaller *Schistosoma*-specific band.

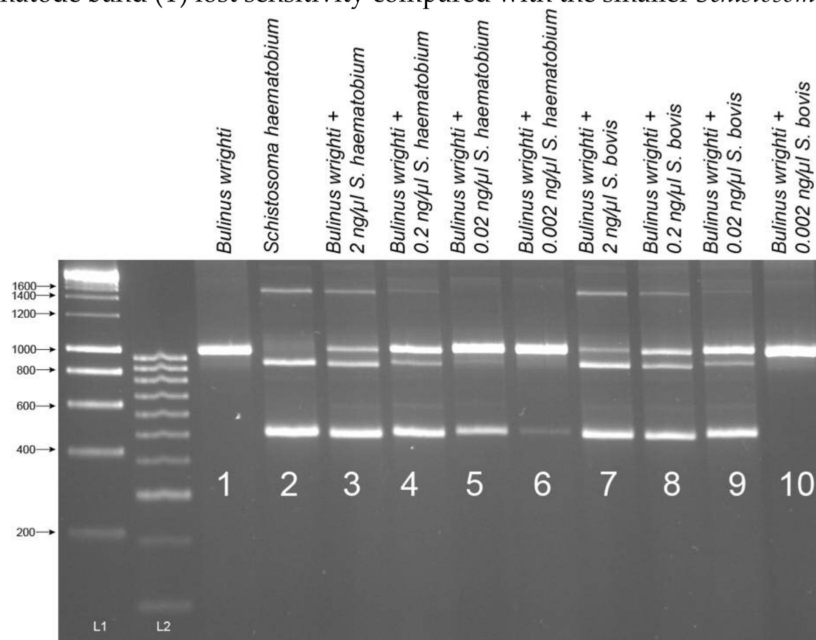
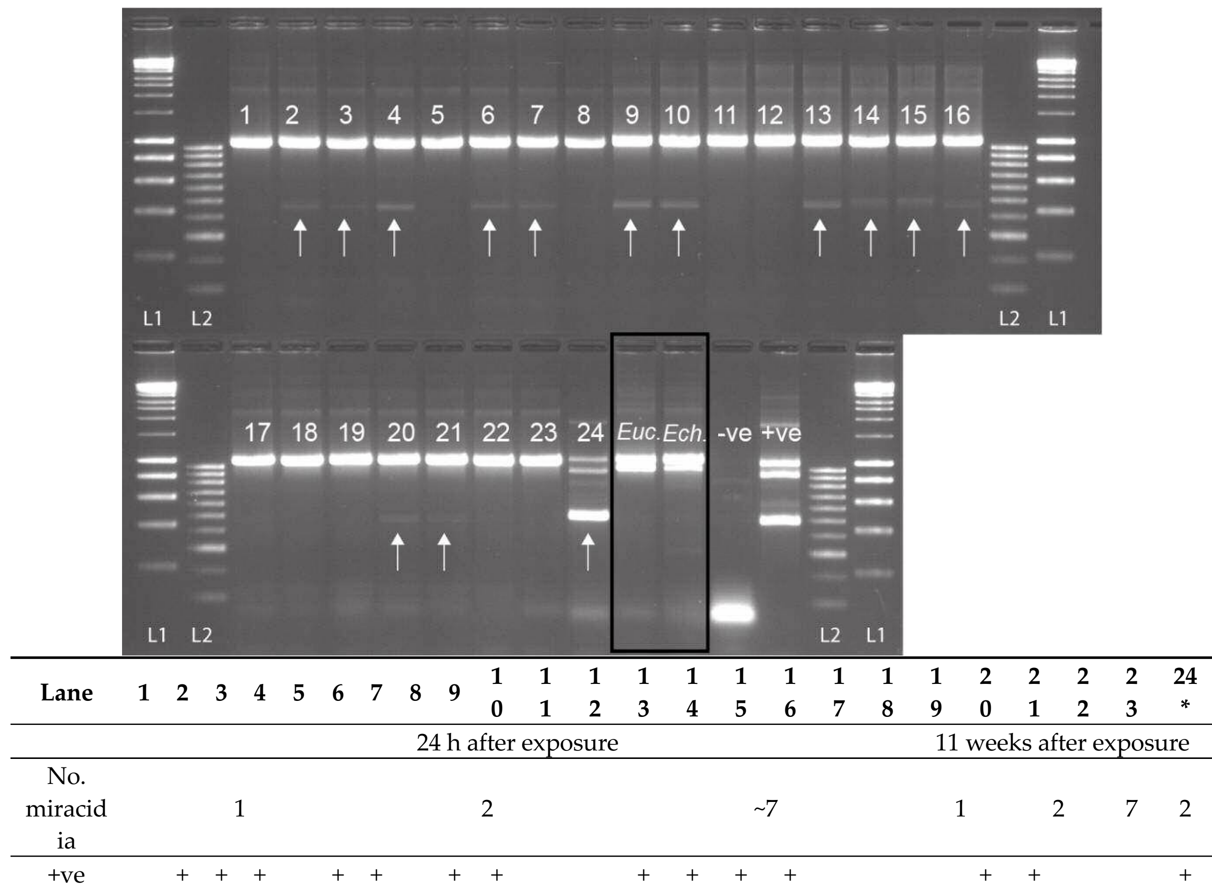


Figure 4. Sensitivity tests of ITS1-2-F PCR performed with serial dilutions of *Schistosoma haematobium* and *S. bovis* gDNA in the presence of *Bulinus wrighti* gDNA. L1 = HyperLadder I. L2 = HyperLadder IV (Bioline, London, UK).

2.3. Experimental Snail Infections

For the non-patent infections of *Bulinus truncatus* with *S. haematobium*, preserved 24 h after exposure, 61.1% (11 out of 18) of the snails were observed to be penetrated by the *S. haematobium* miracidia, presenting the *Schistosoma*-specific ITS2 band (Figure 5). Infections were detected in snails exposed to 1, 2, and 7 miracidia. Two of the five (40%) *B. truncatus,* exposed to one or two miracidia and left for 11 weeks, did not reach patency but were also confirmed to be penetrated by *S. haematobium* miracidia (Figure 5: Lanes 20 and 21). The secondary SIX PCR was performed on all 13 non-patent infected snails, and the single amplicons were sequenced and confirmed as *S. haematobium*. Out of all the snails infected that survived until the end of the experiment (11 weeks), 15% (nine out of 62) reached patency, of which two had been infected with two miracidia, and seven with seven miracidia. One of these samples, infected with two miracidia, was analyzed using the MIX PCR, giving the expected triple banding pattern (snail, trematode, and *Schistosoma*) (Figure 5: Lane 24). All three amplicons from this sample (Figure 5: Lane 24) were gel extracted and sequenced, confirming their identity. Interestingly, in all the non-patent infections, the large trematode amplicons (ETTS2-ETTS2) did not amplify (Figure 5) due to the low level of *Schistosoma* DNA present in the snails that did not reach patency.



Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24*
										24 h after exposure										11 weeks after exposure				
No. miracidia				1						2					2 ~7				1		2		7	2
+ve		+	+	+		+	+		+	+			+	+	+	+				+	+			+

LANE	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24*
	24 HOURS AFTER EXPOSURE																		11 WEEKS AFTER EXPOSURE					
NO. MIRACIDIA	1						2						~7						1		2		7	2
+VE		+	+	+		+	+		+	+			+	+	+	+				+	+			+

Figure 5. Experimental infections of *Bulinus truncatus* with *Schistosoma haematobium* (1–24), field-collected *B. globosus* infected with *Euclinostomum* sp. (*Euc.*) and field-collected *B. nasutus* shedding *Echinostoma* sp. cercariae (*Ech.*). The *S. haematobium* DNA amplicon was present (+ve) in 13 of the 23 non-patent snails (11 at 24 h post-exposure, and two at 11 weeks post-exposure), highlighted by the arrow. Lane 24 = *B. truncatus* sample that was shedding *S. haematobium* cercariae 11 weeks after exposure. The positive control (+ve) is a mix of *B. wrighti* and *S. haematobium* control gDNA. L1: HyperLadder I, L2: HyperLadder IV (Bioline, London, UK).

2.4. Specificity Testing

The *B. globosus* with patent *S. haematobium* ($n = 2$) and *S. bovis* ($n = 5$) infections showed the expected triple banding pattern (snail, trematode, and *Schistosoma* amplicons, results not shown), and following gel extraction and sequencing the data matched those from the cercariae collected from these samples (GenBank Accessions: MH014047 and MH014044, see [6]).

When the MIX PCR was tested on snails confirmed to be infected with other commonly found trematode species (*B. globosus* infected with *Euclinostomum* sp., and the *B. nasutus* infected with *Echinostoma* sp. (Figure 5: Lanes *Euc.* and *Ech.*)), no *Schistosoma* amplicon was observed. However, there was strong amplification of the trematode band together with the snail band. These trematode

amplicons were gel extracted, sequenced, and the infections were confirmed as *Euclinostomum* sp. and *Echinostoma* sp., matching data from the cercariae originally collected from each snail.

2.5. Testing on Field Samples

From the 94 field-collected *B. globosus,* 33 were shown to be infected with *Schistosoma* spp. with amplification of the *Schistosoma*-specific band (Figure 6). Among them, eight also presented the trematode band. The internal snail control was amplified in all samples apart from one. The one that failed was predicted to be due to poor sample preservation, gDNA extraction, or PCR error, and so was disregarded (Figure 6). Of all the samples that gave the *Schistosoma*-specific band, the secondary SIX PCR (ITS2_Schisto_F + ETTS2) was conducted, and all amplicons were Sanger sequenced. Two failed to amplify, but the remaining 31 produced the *Schistosoma* amplicon that all sequenced as *S. haematobium*. One sample also gave the trematode band without the *Schistosoma* band, indicating a non-*Schistosoma* trematode infection.

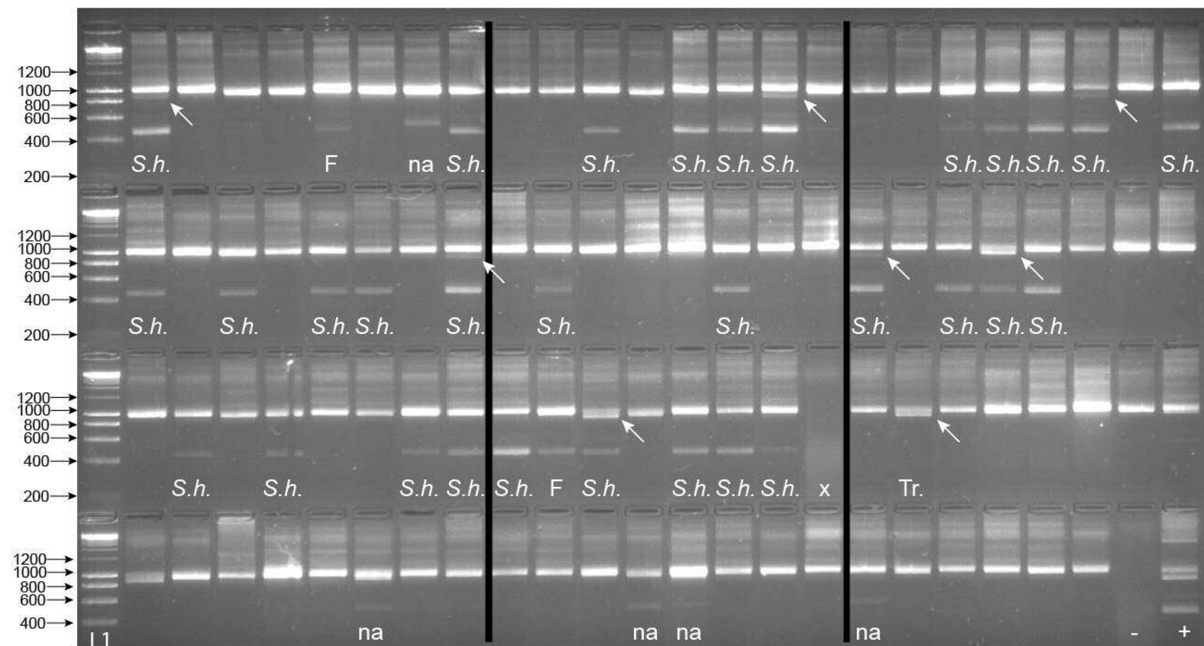


Figure 6. Gel images for the multiplex ITS xenomonitoring (MIX) PCR amplicons for 94 non-patent *Bulinus globosus* collected from Wambaa, Pemba, United Republic of Tanzania. The text under each amplicon denotes the outcome of the *Schistosoma* sp. targeted sequencing where relevant (i.e., presence of 538 bp amplicon), which resulted in either *S. haematobium* (*S.h.*) or sequencing failure (F). The presence of a trematode band without the presence of the *Schistosoma* band indicated a non-*Schistosoma* trematode infection (Tr.). Other non-specific bands, in this case, larger bands (NA), were also observed in these snail populations, which did not amplify with the secondary SIX PCR. x = sample failure with no control amplicon. Arrows highlight the presence of the ~1000 bp trematode band when present (n = 8). *B. globosus* with a patent *S. haematobium* infection (Cham10.1 see [6]) was run as a positive control (+ve) and also represented the amplicon profile obtained for the seven patent *B. globosus* snails (five and two with *S. bovis* and *S. haematobium* infections, respectively (see Section 2.4). -ve = the non-template negative control. L1—HyperLadder IV (Bioline, London, UK).

2.6. Schistosoma *spp.* cox*1 RD-PCR*

Despite trying different annealing temperatures and gDNA template amounts used, the *cox*1 RD-PCR, developed by Webster et al. (2010) [33], tested on the patent *S. haematobium* and *S. bovis*-infected *B. globosus,* only generated the species-specific amplicon for *S. bovis*-infected snails. PCRs for the *S. haematobium*-infected snails repeatedly failed to produce a clear amplicon. The *cox*1 amplicons produced for the *S. bovis*-infected snails were sequenced, and the data matched that obtained from the cercariae collected and analyzed from the snails (see [6]).

3. Discussion

Pre-patent and non-patent snail screening methods for schistosomes, such as molecular xenomonitoring, offer a higher sensitivity over traditional snail shedding methods that can only detect patent infections by the observation of schistosome cercariae. Molecular xenomonitoring better helps to assess the impact of schistosomiasis control interventions in local communities, particularly where local elimination is being achieved, and certification of the absence of transmission is required at specific foci. However, the diversity of schistosomes circulating in co-endemic areas means that species-specific methods are needed to prevent false-positive data due to non-target species cross-reactivity.

Here, we describe the development and application of a molecular xenomonitoring pipeline for the detection and differentiation of *S. haematobium* and *S. bovis* patent and non-patent infections in *Bulinus* freshwater snails, using three previously developed primers [27,31]. The MIX assay screens for *Schistosoma* and other trematode species, while also incorporating an internal control, in this case, gastropod DNA, an important feature for any molecular diagnostic assay. The MIX PCR generates clearly identifiable amplicons, of different sizes, for each target (snail, trematode, *Schistosoma*), which are visible by simple agarose gel electrophoresis. However, the trematode target lacks sensitivity at low DNA concentrations, probably due to its large size and PCR biases for small amplicons at reduced gDNA concentrations. Interestingly, a PCR artifact (~1400–1600 bp) is also observed when using the MIX assay in the presence of *Schistosoma* DNA, suggesting that the primers may have a secondary binding site. However, this artifact is clearly identifiable from the main target amplicons and does not mislead the interpretation of the results.

3.1. The Sensitivity of MIX PCR Assay

Our *in silico* and *in vitro* testing of the MIX assay shows that the presence of *S. haematobium* and *S. bovis* DNA can be routinely detected at low concentrations and also is able to identify non-patent *Schistosoma* infections in snails where the level of DNA varies depending on the development of the infection. The LoD for *Schistosoma* DNA is ≤0.02 ng/μL, which is 80-fold lower than the minimum amount of gDNA usually observed from a single miracidium [32]. This is also demonstrated by the assay's ability to detect pre-patent snail infections 24 h after exposure to a single miracidium. This provides sufficient sensitivity for the LoD needed to detect any stage of snail infection, from initial miracidia penetration of a single miracidium to full patency, in natural settings. The fact that not all the snails tested from the experimental snail infections give positive results is corroborative with observations that, even in the experimental systems, many snails avoid penetration or destroy the miracidia rapidly upon invasion. The MIX and SIX methodologies also prove robust when used to screen 'wild-caught' snails from Pemba, with uninfected, pre-patent *S. haematobium*-infected snails, and non-*Schistosoma* trematode infections are clearly identified.

3.2. Benefits of an Updated Molecular Xenomonitoring Protocol for Schistosomiasis Surveillance

The molecular xenomonitoring protocol requires few consumables and no cold chain, and results can be interpreted using basic molecular laboratory equipment (thermocycler and gel electrophoresis), making the molecular assay accessible in lower resource settings, such as schistosomiasis endemic regions. The molecular xenomonitoring approach described here, therefore, provides a useful tool for monitoring schistosomiasis transmission, as has been outlined as a necessary method for leading toward the WHO 2030 goals for schistosomiasis control and elimination [9].

Molecular xenomonitoring surveillance techniques are often associated with parasites transmitted by hematophagous insects, such as lymphatic filaria in mosquito vectors [20,34–36] and trypanosomes in tsetse flies [18,19]. However, several assays have been developed for detecting trematode species in freshwater snails, including *Fasciola* spp. [22,37–44], other wildlife trematode species [45], and medically important schistosome species—*S. japonicum* [46,47], *S. mansoni* [24,27,28,48–54], and *S. haematobium* [23,26,27,30,52,54,55]. The first developed assay for the molecular detection of *S. haematobium* DNA in *Bulinus* employs the highly repetitive *Dra*1 target, and this has been the marker of

choice for studies investigating *S. haematobium* infections in snails due to its high sensitivity [55]. However, the specificity of the *Dra*1 and the interpretation of results can be problematic due to the frequent false-positive and -negative results, lack of internal control, and difficulties in interpreting the amplicon patterns. Furthermore, this marker does not allow for species identification. Kane et al. (2013) [54] employed the use of another repetitive marker, intergenic spacer (IGS), for the detection of snail infections, and a post-amplification restriction digest allowed for the downstream species identification of *S. haematobium* and *S. bovis*. However, the method lacks internal controls. In addition, many of these assays use quantitative-PCR (qPCR), rather than conventional PCR/gel electrophoresis. Although able to quantify levels of DNA within a sample, qPCR is more arduous to carry out and lesser suited for use in endemic settings. However, recent technological advances in sample preparation and DNA extraction methods have demonstrated robust field setting methodologies to conduct qPCR analysis capable of detecting avian trematodes and host species in Canadian lakes [56–58], which could potentially be modified to suit the detection of human and bovine schistosomes in sub-Saharan Africa, although cost and throughput would need to be considered.

A recent assay designed by Schols et al. (2019) [27] is a six primer multiplex PCR, which incorporates an internal snail control and offers a xenomonitoring tool for *S. haematobium* group species that are transmitted by *Bulinus* snail hosts. Our study simplifies the multiplex process, reducing the primer numbers and mitigating against PCR competition and some of the biases that may occur with multiple primer combinations. It also allows for greater amplicon size differentiation (as amplicon sizes can be more easily distinguished based on size), making results more interpretable. The ITS rDNA is a favorable target within the repeat ribosomal operon of *Bulinus* and *Schistosoma* spp., easily detected within small quantities of DNA due to the high copy number of rRNA clusters within eukaryote genomes. Another key feature of the target relates to specificity. The ITS regions of *Schistosoma* and *Bulinus* spp. can be routinely amplified using conventional PCR, thanks to its small size (~1000 bp) and highly conserved flanking regions (5′18S and 3′28S), enabling the use of universal primers (ETTS1 + 2) for multiple species [31]. However, interspecies heterogeneity and, to a lesser extent, intraspecies heterogeneity (Pennance et al., unpublished observations) of the ITS regions allow for differentiation between species, such as those of the *S. haematobium* group [7,33]. The internal *Schistosoma*-specific primer is situated in a conserved ITS region within the *Schistosoma* genus, with 100% conservation between several African species, suggesting that it could be utilized for several *Schistosoma*-snail transmission systems.

3.3. Limitations of Molecular Xenomonitoring Approaches for Schistosomiasis Surveillance

From our study, we have identified two limiting factors for the practical use of this method. First, the laborious nature of testing each individual snail adds time and cost. Further sensitivity testing should be performed to support the development of pooling strategies. This would help to determine whether infections are still detected when the *Schistosoma* DNA is diluted in the presence of much higher concentrations of snail DNA, which may inhibit the reaction. Pooling strategies have been successful for arthropod xenomonitoring protocols [18] and would allow for higher throughput of samples required for screening large populations of snails, such as those encountered for schistosomiasis.

Second, a limitation does come with the need for the secondary screening (SIX PCR) of the *Schistosoma* amplicon, via sequencing, to confirm species. Despite best efforts, rapid species diagnostics, such as the rapid diagnostic *cox*1 RD-PCR developed by Webster et al. (2010) [33] to determine adult worm and larval stage species identity, is not robust when snail DNA is present, particularly for *S. haematobium* infections. The *cox*1 RD-PCR was suggested as a secondary screening method by Schols et al. (2019) [27], but it was only theoretically examined as part of that study. Clearly, further 'wet lab' testing on infected snails is needed. In regions where *Schistosoma* hybridization occurs, mitochondrial DNA analysis would be necessary since both nuclear and mitochondrial DNA are required for hybrid identification [33]. Unfortunately, as with most diagnostics, there is a balance between sensitivity and specificity, with sensitivity increasing and specificity decreasing, usually due to the nature of the

biomedical targets. Here, rapid screening with high sensitivity is a priority due to the low levels of infections in our study sites, with secondary species-specific screening only required on a subset of samples that are identified as infected. Moreover, Zanzibar previously thought to be a *S. haematobium* transmission only zone, although, with the recent report of *S. bovis* transmission [6], the additional species-specific screening is warranted. However, the need for the secondary screening step for *Schistosoma* species identification does need further exploration, such as trialing more direct methods that negate DNA sequencing, for example, amplicon enzyme restriction digestion demonstrated in Kane et al. (2013) [54]. However, it is also important to gather detailed information, as is obtained through DNA sequencing and analysis [6,7]. It is likely that xenomonitoring methods may need to be adapted to optimize focal surveillance strategies to specific endemic zones due to geographical genetic differences of the target organisms and potential unidentified species.

4. Materials and Methods

4.1. Primer Selection and in silico *Evaluation*

The universal primer pair, ETTS2 and ETTS1 (Table 2 and Figure 7), was selected for the development of the internal control for the assay. They anneal to conserved flanking regions either side of the ITS(1 + 2) rDNA region of *Schistosoma* spp., amplifying the full ITS rDNA regions, resulting in an amplicon of ~1005 bp [6,7,31,33]. These primers have also been demonstrated to amplify the full ITS rDNA region of other organisms, including intermediate gastropod hosts. Primer's cross-reactivity with the target *Bulinus* snail hosts was further confirmed through alignments of the ETTS2 and ETTS1 primers with *B. globosus* and *B. nasutus* rDNA regions, available from ongoing projects (Briscoe et al. unpublished data, Pennance et al. unpublished data).

To develop the *Schistosoma*-specific target, two *Schistosoma* specific primers (ITS2_Schisto_F and ITS2_Schisto_R), published by Schols et al. (2019) [27], were selected, targeting the internal ITS1 and ITS2 rDNA regions of *Schistosoma* (Figure 7). These were further tested *in silico* for specificity by stringently aligning them with rDNA sequence data (Briscoe et al., unpublished data; Pennance et al., unpublished data) of a single *B. globosus* and *B. nasutus* from both Unguja and Pemba island (Zanzibar, United Republic of Tanzania) and those previously published for *Schistosoma* spp. [59,60].

All alignments were performed using Sequencher v5.4.6 (Gene Codes Corporation, Michigan, USA), and the primer positions were used to predict the specific amplicon sizes that would result, following amplification of snail and schistosome DNA using the different primer combinations of ETTS1, ETTS2, ITS2_Schisto_F, and ITS2_Schisto_R.

Table 2. Details of the primers selected for the development of the xenomonitoring assay. Universal (U) and specific (S) denote whether the primers universally target both *Schistosoma* and snail or just specifically target *Schistosoma* DNA.

Primer (Direction)	Primer Sequence (5′-3′)	Primer Position	State	Reference
ETTS1 (Reverse)	TGCTTAAGTTCAGCGGG	28S 5′ end (ITS2 3′ flanking region)	U	Kane et al. (1994) [31]
ETTS2 (Forward)	TAACAAGGTTTCCGTAGGTGA	18S 3′ region (ITS1 5′ flanking region)	U	Kane et al. (1994) [31]
ITS2_Schisto_F (Forward)	GGAAACCAATGTATGGGATTATTG	ITS1 3′ end (5.8S 5′ flanking region)	S	Schols et al. (2019) [27]
ITS2_Schisto_R (Reverse)	ATTAAGCCACGACTCGAGCA	ITS2 (middle)	S	Schols et al. (2019) [27]

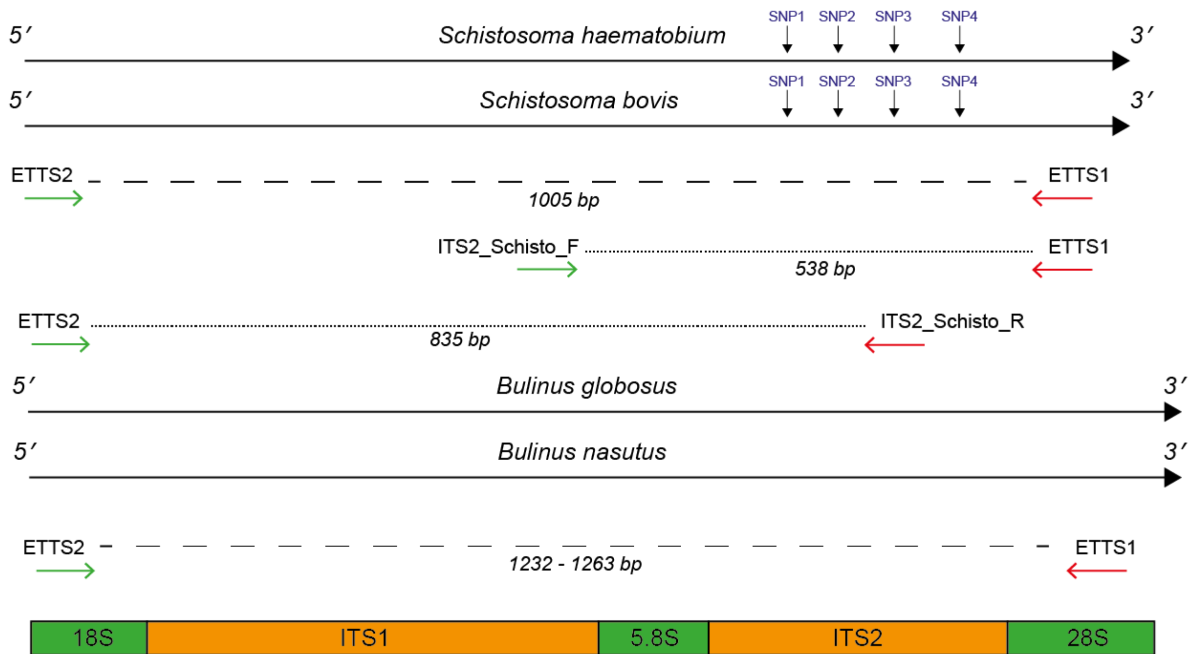


Figure 7. Primer annealing positions flanking and internal to the ITS1 + 2 rDNA targets. Primer positions were mapped to *Schistosoma haematobium* and *S. bovis* ITS1 + 2 reference data [59] and to a *Bulinus globosus* and *B. nasutus* DNA reference (Pennance et al., unpublished data). For *Schistosoma* DNA, the primer combinations produced two fragments; 1) ETTS2–ETTS1 (1005 bp) and either 2) ITS2_Schisto_F-ETTS1 (538 bp) or 3) ITS2_Schisto_R-ETTS2 (835 bp). For *Bulinus* DNA, the primer combinations produced one fragment, ranging in size between 1232 and 1263 due to interspecies variation. For *Schistosoma* species identification, four SNPs were present at bp positions 90, 145, 195, and 265 in the ITS2 rDNA region, allowing differentiation of *S. haematobium* and *S. bovis* following ITS2 sequencing.

4.2. Bulinus *and* Schistosoma *Genomic DNA Extractions*

Whole soft tissue from *Bulinus* samples (as detailed below) available through the Schistosomiasis Collection at the Natural History Museum (SCAN) [61] and other ongoing projects, including laboratory and field samples, infected/non-infected and patent/non-patent, were used for the assay development and validation. Genomic DNA (gDNA) from all *Bulinus* samples was extracted using a modified tissue lysis protocol [6]. Two kits were then used to extract total gDNA from the lysed snail tissue—the BioSprint 96 DNA Blood Kit (Qiagen, Manchester, UK) for high-throughput multiple sample processing, and the DNeasy Blood & Tissue Kit (Qiagen, Manchester, UK) for single sample processing. Protocols were carried out according to the manufacturer's instructions.

Positive control *Schistosoma* gDNA was obtained from adult worms; *S. haematobium* (single female worm from Zanzibar) and *S. bovis* (single male worm from Senegal) were available from SCAN. DNA was extracted following the DNeasy Blood & Tissue Kit protocol according to manufacturer's instructions (Qiagen, Manchester, UK) [60].

4.3. PCR Conditions, Amplicon Visualization, and Sequencing

All PCR amplifications were performed in 25 μL reactions using illustra™ PuReTaq Ready-To-Go™ PCR Beads (GE Healthcare, UK) with 1 μL of each primer, in their different combinations, as stated in each section, at a concentration of 10 μM. gDNA templates (*Schistosoma* and/or *Bulinus* sp.) were added at different volumes and concentrations, as detailed below. The PCR cycling conditions for all multiplex and singleplex reactions were as follows: initial denaturation 5 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 58 °C (unless stated otherwise), 90 s at 72 °C, and a final extension of 10 min at 72 °C. The visualization of all PCR products was performed by running 7.5 μL of each PCR product, mixed with 2 μL of Bioline 5x DNA Loading Buffer Blue (London, UK) and GelRed for

visualization under UV light, on a 2% agarose gel for 90 min at 90 V. HyperLadder I and HyperLadder IV were run alongside the PCR amplicons to assess fragment sizes. Gels were visualized using a GBOX-Chemi-XRQ gel documentation system (Syngene, Cambridge, UK).

To validate amplification specificity, selected PCR amplicons from multiplex PCRs, where multiple amplicons were present, were cut from agarose gels and sequenced following purification using the QiaQuick Gel Purification Kit (Qiagen, Manchester, UK) following manufacturer's instructions. For singleplex reactions, resulting in a single amplicon, PCR products were purified using the QiaQuick PCR Purification Kit (Qiagen, Manchester, UK) following the manufacturer's instructions. The amplicons were sequenced in both directions using dilutions of the PCR primers. The sequence data were manually edited using Sequencher v5.4.6 (Gene Codes Corporation, Michigan, USA), and the amplicon identification was confirmed by comparison to *Schistosoma* reference data [59] and by BLAST analysis (BLAST: Basic Local Alignment Search Tool, NCBI).

4.4. In vitro *Primer Testing and Assay Validation*

All gDNA extractions from laboratory-bred *Bulinus wrighti* (not exposed to any trematodes and, therefore, negative for infection) and from the *S. haematobium* and *S. bovis* adult worms were quantified using a Qubit® Fluorometer using the dsDNA Broad Range (BR) Assay Kit (Molecular Probes, Life Technologies). The gDNA extracts from the single adult *S. haematobium* and *S. bovis* worms were normalized, using nuclease-free water, to 2 ng/µL (± 0.05 ng/µL). The gDNA extract of a *B. wrighti* snail control was recorded and kept at 31.3 ng/µL. Template gDNA (1 µL) was used in each PCR separately or combined and used to test the different primer combinations (shown in Figure 1). The primers were tested as singleplex PCRs for the internal control (ETTS2 + ETTS1), targeting both snail and *Schistosoma* gDNA, and then as multiplex PCR's incorporating each of the internal *Schistosoma*-specific primers (ETTS2 + ETTS1 + ITS2_Schisto_F or ITS2_Schisto_R). All test PCRs were initially performed at an annealing temperature of 55 °C.

The multiplex primer combination ETTS2 + ITS2_Schisto_F + ETTS1 was selected and taken forward for further development and validation. This is referred to as the multiplex ITS xenomonitoring (MIX) PCR. The MIX PCR was further tested at annealing temperatures of 58 °C and 60 °C to enhance assay specificity, with 58 °C taken forward for further experiments. Additionally, a secondary *Schistosoma* ITS xenomonitoring (SIX) PCR, incorporating just the *Schistosoma*-specific primer (ITS2_Schisto_F) and its universal reverse primer (ETTS1), was validated, targeting just the 538 bp *Schistosoma* DNA amplicon. The SIX PCR was developed to obtain more targeted schistosome species data amplicon sequence analysis, of positive samples, following an initial high-throughput screening of snail populations with the multiplex PCR, which incorporates the internal snail control.

4.5. Sensitivity Testing

The analytical sensitivity and LoD for *Schistosoma* DNA in the MIX PCR were performed using serial dilutions of *S. haematobium* and *S. bovis* gDNA. The *S. haematobium* and *S. bovis* gDNA, normalized to 2 ng/µL (± 0.05 ng/µL), was diluted using nuclease-free water by one in ten (0.2 ng/µL), one in one hundred (0.02 ng/µL), and one in one thousand (0.002 ng/µL). 1 µL of each *Schistosoma* gDNA dilution was used in each multiplex PCR together with 1 µL of the *B. wrighti* gDNA (31.3 ng/µL).

Sensitivity was tested using controlled laboratory snail infections. Infections were performed by the Schistosomiasis Resource Center (SRC) (Biomedical Research Institute, Maryland, USA [62]) using their *B. truncatus*/*S. haematobium* (Egyptian strain) model lifecycle system. Juvenile *B. truncatus* (2–3 mm, n = 133) was divided into three groups, with individual snails in each group being exposed to either 1, 2, or several (~7) *S. haematobium* miracidia, respectively (Table 3). Miracidia, hatched in freshwater from eggs collected from *S. haematobium*-infected male LVG Syrian golden hamsters (see Ethical Statement), were added to individual wells of 24-well ELISA plates containing the *B. truncatus* snails. A fine-tipped Pasteur pipette was used under a dissection microscope to capture and deliver either an individual miracidium (for 1 and 2 miracidia exposures) or several (~7) miracidia at a time,

following the standard operating procedures (SOPs) conducted at SRC (see: https://www.afbr-bri.org/schistosomiasis/standard-operating-procedures/).

The snails were kept in their individual wells until no miracidia were observed swimming under a binocular microscope, assumed to have penetrated the snail (~2 h). Following 24 h after initial exposure to the miracidia, half of each infection group was preserved in 100% ethanol for molecular analysis. The remaining exposed *B. truncatus* were maintained in their separate infection groups for 11 weeks to allow the infections to mature, and since this was the first opportunity to conduct sampling of the infected snails. Snails were maintained according to the SRC's SOP's (see above). Snails that died were recorded and promptly removed from the group. At 11 weeks post-exposure, the remaining snails were individually induced to shed cercariae by exposure to freshwater and light. Once it had been determined if the snails were infected and patent, they were washed, to remove any cercariae, and preserved in 100% ethanol for molecular analysis.

The MIX PCR was performed using gDNA (1 μL) extracted from six individual *B. truncatus* from each group, which were preserved after 24 h—two non-patent snails from group 1 and 2, and one non-patent snail from group 3 (11 weeks post-exposure), and one patent (shedding) snail from group 2 (11 weeks post-exposure) (Table 3). The secondary SIX PCR was performed on selected *Schistosoma* positive samples, to amplify the 538 bp *S. haematobium*-specific amplicon for sequence analysis to confirm that the MIX PCR was not a false-positive.

Table 3. Groups of *Bulinus truncatus* (*B.t.*) experimentally challenged with either 1, 2, or ~7 *S. haematobium* (*S.h.*) miracidia and preserved 24 h post-exposure or checked for patent *S.h.* infections and preserved 11 weeks (wks) post-exposure.

Infection Group	No. of *B.t.* Exposed	No. of *S.h.* Miracidia Used	No. of *B.t.* Preserved at 24 h	No. of *B.t.* Checked for Patency at 11 wks and Preserved (no. Shedding + ve)
1	45	1	22	22 [1] (0)
2	43	2	21	19 [1] (2)
3	45	~7	23	21 [1] (7)

[1] One *B. truncatus* died from each infection group during the 11 weeks post miracidia exposure.

4.6. Specificity Testing and Validation on Field Samples

As part of a longitudinal xenomonitoring project on Pemba in relation to urogenital schistosomiasis transmission [6], the 'wild-caught' *B. globosus* and *B. nasutus* field isolates were available for further validation of the MIX assay. Individual snails had been collected during malacological surveys, individually checked for patent trematode infections by cercarial shedding, and then preserved in 100% ethanol for molecular analysis [6]. Cercariae from infected *B. globosus* were preserved on Whatman FTA cards and identified using molecular methods as *S. haematobium* or *S. bovis* from two and five snails, respectively [6]. In addition, individual *B. globosus* and *B. nasutus* (also collected from Pemba), which were shedding two other trematode species, *Euclinostomum* sp. and *Echinostoma* sp., respectively (unpublished data), were tested to investigate assay specificity. Additionally, 94 *B. globosus* snails from Wambaa (Pemba) collected during November 2018, which were not shedding any trematode cercariae, were tested for infections by PCR.

All samples, which gave the 538 bp *Schistosoma*-specific amplicon (Figure 7), were further subjected to the SIX PCR assay with the resulting amplicons purified and sequenced to confirm the species of the infection. The identity of the *S. haematobium* and *S. bovis* species was confirmed by analysis of the four species' SNPs that exist in the ITS2 region [7] between *S. haematobium* and *S. bovis* (Table 1).

4.7. Testing the Schistosoma cox1 Rapid-Diagnostic PCR (RD-PCR) for Secondary Species Identification

The patent *B. globosus* snails collected from Pemba shedding either *S. haematobium* (*n* = 2) or *S. bovis* (*n* = 5) (see [6]), as detailed above, were further tested using the published multiplex RD-PCR (see [27,33]) with an aim to provide a secondary species-specific screening method, as described in Schols

et al. (2019) [27]. This multiplex RD-PCR, capable of differentiating *S. bovis* and *S. haematobium* by species-specific amplicon size (*S. haematobium* (543 bp), *S. bovis* (306 bp)), was performed following the published protocol and cycling conditions described by Webster et al. (2010) [33]. Different amount of gDNA (1 μL, 2 μL, and 3 μL) and PCR annealing temperatures (58 °C, 62 °C, and 65 °C) were trialed to investigate sensitivity and specificity. The amplicons were purified, and Sanger sequenced, as described above, using the species-specific reverse primers to confirm species/amplicon identification.

4.8. Ethical Statement

Schistosoma haematobium experimental infections were conducted at the Biomedical Research Institute – Schistosomiasis Resource Center (Rockville, MA, USA) animal facility maintained with AAALAC full accreditation (Site # 000779), operating under the National Institutes of Health's Office of Laboratory Animal Welfare (OLAW) # A3080-01. *S. haematobium* parasite material was collected from male LVG Syrian golden hamsters following percutaneous exposure to cercariae. Hamster's use was approved by the Institutional Animal Care and Use Committee (IACUC) of the Biomedical Research Institute for the Animal Use Protocol, #18-01.

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Sample Availability: Samples (DNA extracts of snails and parasites) are available from the authors upon appropriate request.

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