Patterns and processes of diversification in African pulmonate gastropods of zoonotic, biomedical and veterinary importance

Muster und Prozesse der Diversifikation in afrikanischen Lungenschnecken von zoonotischer, biomedizinischer und veterinärmedizinischer Bedeutung

Dissertation for the award of the degree "Doctor rerum naturalium (Dr. rer. nat.)"
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English

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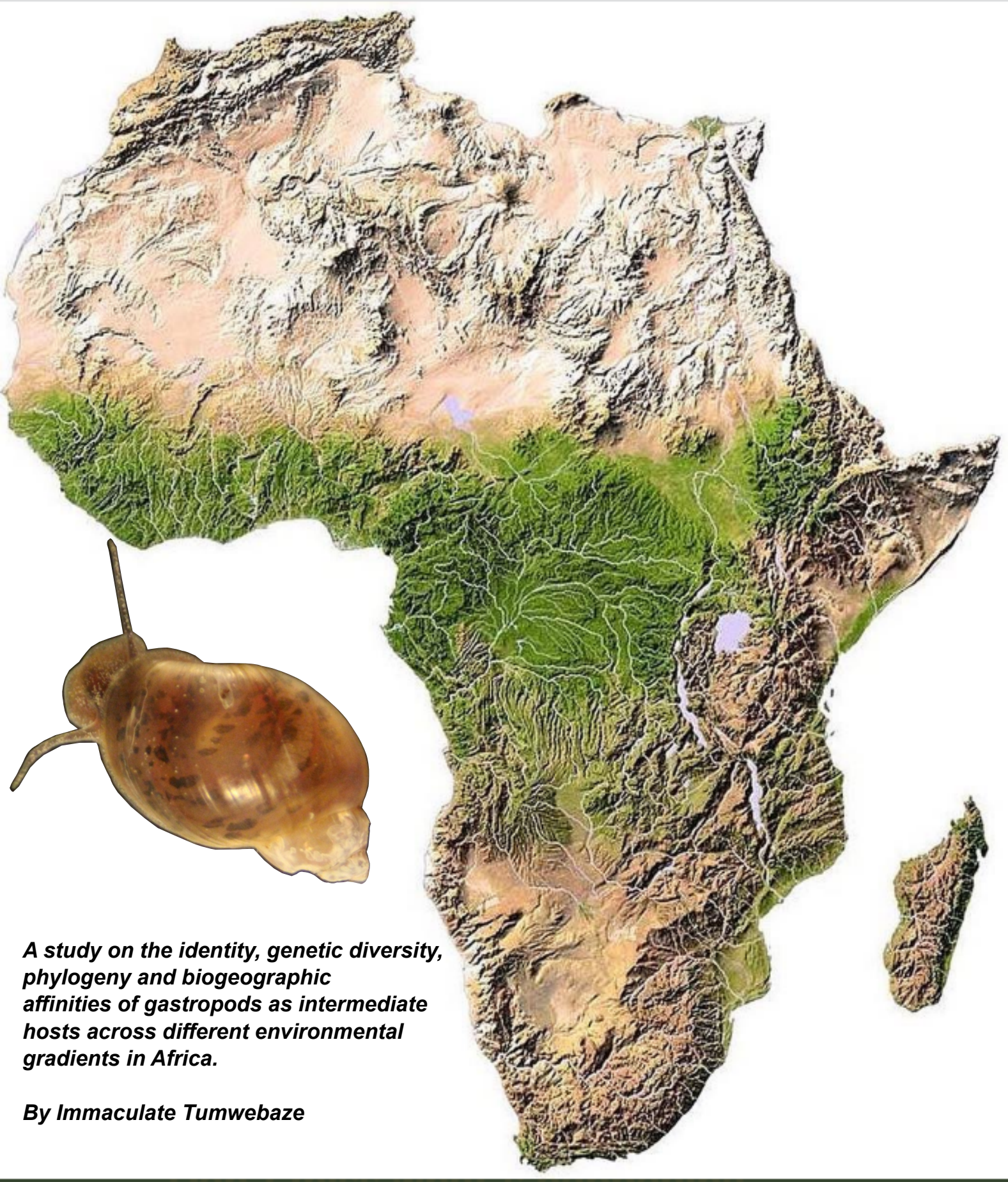

A study on the identity, genetic diversity, phylogeny and biogeographic affinities of gastropods as intermediate hosts across different environmental gradients in Africa.

By Immaculate Tumwebaze

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Contents

i. Summary (English)

Vector-borne tropical diseases cause significant impacts on the ecological, economic, and social aspects of society. It is estimated that hundreds of millions of people, particularly in sub-Saharan Africa, are affected directly or indirectly by these diseases. Freshwater gastropods play an important role as intermediate hosts (IHs) for the causative agents of parasitic diseases such as schistosomiasis (bilharzia) and fascioliasis. While many aspects of the diseases can be considered relatively well studied, comparatively little is known about the biology, ecology, and eco-evolutionary dynamics of the intermediate hosts. A shift in the prevalence distribution threshold of schistosomiasis to higher altitudes above 1,600 m in crater lakes of western Uganda, moreover assumed to be linked to climate change, raised concerns. It is precisely the dynamics of the intermediate hosts that determine the disease prevalence and so focusing on these is regarded as the most promising control strategy for the declared goal of the complete elimination of schistosomiasis and fascioliasis. Especially with regard to climate change, dramatic shifts in the occurrence of intermediate hosts with important consequences for tropical diseases are predicted.

The present dissertation, therefore, addresses aspects of evolutionary biology such as speciation patterns and processes, phylogenetic and biogeographical relationships, and dynamics of selected intermediate hosts of different genera. In particular, historical but also recent ecological factors that are taken into account have led to the current occurrence patterns and thus to the occurrence or non-occurrence of parasites and diseases. Various case studies, primarily on the genus *Bulinus*, but also on *Biomphalaria* and *Galba*, were carried out on a regional and continental scale. The sampling area covers various landscapes and altitudinal ranges, and a variety of geological and ecological aspects. The snail intermediate hosts of human and veterinary importance for dozens of crater lakes in western Uganda were molecularly identified and phylogenetically investigated. The interplay between biotic and abiotic elements that regulate the presence of intermediate hosts was analysed. In this system, *Biomphalaria* snails the intermediate hosts for the *Schistosoma mansoni* that causes the intestinal form of schistosomiasis dominate, while *Bulinus* snails though equally dominant, are mostly of a species (*Bulinus tropicus*) that hosts *Schistosoma bovis* parasites that affect livestock. A high level of diversification was realized in these small and relatively young crater lakes. The small-scale analysis of the biotic and abiotic factors revealed that the intermediate hosts *Biomphalaria* and *Bulinus* require simply a suitable freshwater habitat just like other gastropods. The colonization of these East African rift valley freshwater systems is attributed to the surrounding great lakes system. The study was expanded to include the Lake Victoria system, which has been noted as one of the significant biodiversity source reservoirs for the crater lakes. Despite being quite vast and having a variety of ecological niches characterized by shallow and ecologically suited shorelines and banks of the main waterbody and islands, Lake Victoria displays an array of *Bulinus* populations that is less diverse. Generally, there is more species diversity and genetic diversity in the genus *Bulinus* and therefore the potential for urogenital schistosomiasis is much higher than previously known.

Two studies on a continental scale were carried out for the genera *Bulinus* and *Galba* with a special focus on Afromontane regions, as these will play an important role in the future occurrence of tropical diseases in the course of the predicted climate change. Multigene phylogenies were recronstructed for both genera based on an Africa-wide data set. A previously unknown cryptic species was found for the genus *Galba*, which acts as an intermediate host for the liver fluke (*Fasciola hepatica*) throughout sub-Saharan Africa. For both genera, the various mountain regions were already settled several times in the Plio-Pleistocene. Though the high altitude

colonization for species *B. truncatus/tropicus* complex is comparatively more recent, they have more extensively evolved in higher altitudes than the other *Bulinus* species groups. As a result, the study defines a new high altitude distribution threshold of ~4000 m above sea level. Several unknown lineages and species, particularly at extreme altitudes on Mt. Elgon/Uganda and in Lesotho, have been identified. The multimarker phylogenetic analysis also helped to finally resolve the disputed relationship between the four *Bulinus* species groups.

All work in this dissertation contributes to a better understanding of the biodiversity and evolution of intermediate hosts. They thus represent an important basis for scenarios for the future development of occurrence and prevalence as well as more successful strategies to combat the neglected but extremely important tropical diseases schistosomiasis and fasciolosis.

ii. Zusammenfassung

Vektorübertragene Tropenkrankheiten haben erhebliche Auswirkungen auf die ökologischen, ökonomischen und sozialen Aspekte der Gesellschaft. Schätzungen zufolge sind Hunderte Millionen Menschen, insbesondere in Subsahara-Afrika, direkt oder indirekt von diesen Krankheiten betroffen. Süßwasserschnecken spielen eine wichtige Rolle als Zwischenwirte (IHs) für die Erreger parasitärer Erkrankungen wie Bilharziose (Schistosomiasis) und Fasziolose. Während viele Aspekte der Krankheiten als relativ gut untersucht angesehen werden können, ist vergleichsweise wenig über die Biologie, Ökologie und öko-evolutionäre Dynamik der Zwischenwirte bekannt. Eine Verschiebung der Prävalenzverteilungsschwelle der Bilharziose in höhere Lagen über 1600 m in Kraterseen im Westen Ugandas, von der außerdem angenommen wird, dass sie mit dem Klimawandel zusammenhängt, gab Anlass zur Sorge bei Verantwortungsträgern. Gerade die Dynamik der Zwischenwirte bestimmt die Krankheitsprävalenz und so gilt ihre Fokussierung als erfolgversprechendste Bekämpfungsstrategie für das erklärte Ziel der vollständigen Eliminierung von Bilharziose und Fasziolose. Gerade im Hinblick auf den Klimawandel werden dramatische Verschiebungen im Auftreten von Zwischenwirten mit erheblichen Folgen für Tropenkrankheiten prognostiziert.

Die vorliegende Dissertation befasst sich daher mit Aspekten der Evolutionsbiologie wie Artbildungsmuster und prozesse, phylogenetische und biogeografische Beziehungen und Dynamik ausgewählter Zwischenwirte verschiedener Gattungen. Insbesondere historische, aber auch rezente ökologische Faktoren, die berücksichtigt werden, haben zu den aktuellen Vorkommensmustern und damit zum Auftreten oder Nichtauftreten von Parasiten und Krankheiten geführt. Auf regionaler und kontinentaler Ebene wurden verschiedene Fallstudien vor allem zur Gattung *Bulinus*, aber auch zu *Biomphalaria* und *Galba* durchgeführt. Das Probenahmegebiet umfasst verschiedene Landschaften und Höhenlagen sowie eine Vielzahl geologischer und ökologischer Aspekte. Für Dutzende von Kraterseen in Westuganda wurden die Zwischenwirte von human- und veterinärmedizinischer Bedeutung molekular identifiziert und phylogenetisch analysiert. Die Kombination von biotischen und abiotischen Faktoren, die das Vorhandensein der Zwischenwirte kontrollieren, wurde bestimmt. In diesem System dominieren *Biomphalaria*-Schnecken, die Zwischenwirte für *Schistosoma mansoni*, die intestinale Form der Bilharziose, während *Bulinus*-Schnecken, obwohl ebenso dominant, meist zu einer Art (*Bulinus tropicus*) gehören, die mit *Schistosoma bovis*-Parasiten befallen sein können, die Nutztiere infizieren. In diesen kleinen und relativ jungen Kraterseen wurde ein hohes Maß an Diversifizierung realisiert. Die kleinräumige Analyse der biotischen und abiotischen Faktoren ergab, dass die Zwischenwirte *Biomphalaria*

und *Bulinus* ebenso wie andere Gastropoden lediglich ein geeignetes Süßwasserhabitat benötigen. Die Besiedlung dieser Süßwassersysteme des ostafrikanischen Grabenbruchs wird dem umliegenden System der großen Seen zugeschrieben. Die Studie wurde auf das Lake Victoria-System ausgeweitet, das als eines der bedeutenden Besiedlungsquellen für die Kraterseen gilt. Obwohl der Viktoriasee sehr groß ist und eine Vielzahl von ökologischen Nischen aufweist, die durch flache und ökologisch geeignete Küstenlinien und Ufer des Hauptwasserkörpers und der Inseln gekennzeichnet sind, leben im Viktoriasee eine Anzahl von *Bulinus*-Populationen, die weniger vielfältig sind. Generell hat die umfangreiche Studie aber ergeben, dass die Gattung *Bulinus* eine größere Artenvielfalt und genetische Vielfalt aufweist und daher das Potenzial für eine urogenitale Bilharziose viel höher ist als bisher bekannt.

Für die Gattungen *Bulinus* und *Galba* wurden zwei Studien auf kontinentaler Ebene mit besonderem Fokus auf afromontane Regionen durchgeführt, da diese eine wichtige Rolle für das zukünftige Auftreten von Tropenkrankheiten im Zuge des Klimawandels spielen werden. Für beide Gattungen wurden auf der Grundlage eines afrikaweiten Datensatzes die bisher umfangreichsten Multigen-Phylogenien erstellt. Für die Gattung *Galba*, die in ganz Afrika südlich der Sahara als Zwischenwirt für den Leberegel (*Fasciola hepatica*) fungiert, wurde eine bisher unbekannte kryptische Art gefunden. Für beide Gattungen wurden die verschiedenen Bergregionen bereits im Plio-Pleistozän mehrfach besiedelt. Obwohl die Besiedelung in großer Höhe für die Arten des *B. truncatus/tropicus*-Komplexes vergleichsweise jünger ist, haben sie sich in größeren Höhen als die anderen *Bulinus*-Artengruppen entwickelt. Als Ergebnis definiert die Studie eine neue Höhenverbreitungsschwelle von ~4000 m über dem Meeresspiegel. Mehrere unbekannte Abstammungslinien und Arten, insbesondere in extremen Höhen auf dem Mt. Elgon/Uganda und in Lesotho, wurden identifiziert. Die phylogenetische Multimarker-Analyse trug auch dazu bei, die Verwandtschaft zwischen den vier *Bulinus*-Artengruppen endgültig aufzuklären.

Alle Arbeiten in dieser Dissertation tragen zu einem besseren Verständnis der Biodiversität und Evolution von Zwischenwirten bei. Sie stellen damit eine wichtige Grundlage für Szenarien zur zukünftigen Entwicklung von Vorkommen und Prävalenz sowie erfolgreichere Strategien zur Bekämpfung der vernachlässigten, aber äußerst wichtigen Tropenkrankheiten Bilharziose und Fasziolose dar.

1. Synthesis

1.1. Introduction

1.1.1. Freshwater-associated trematodiases focusing on schistosomiasis and fascioliasis endemism in Africa

The zoonotic diseases schistosomiasis and fascioliasis are both grouped under neglected tropical diseases (NTDs) and affect millions of people and ruminants (livestock and wildlife) (Kouadio, et al., 2020; Ojeda-Robertos, et al., 2022; Standley, et al., 2012a). Thus they impose devastating economic and public health impacts worldwide but especially in developing regions (Lu, et al., 2018). The prevalence of human fascioliasis is not yet well investigated in most African countries although the disease in other animals such as livestock is relatively well known (Dermauw, et al., 2021; Mas-Coma, 2020). It is caused mainly by *Fasciola hepatica* and *Fasciola gigantica* (Dermauw, et al., 2021). Schistosomiasis on the other hand, though prevalent in livestock (Standley, et al., 2012b), is more pronounced in humans. Two main forms of human schistosomiasis, i.e. intestinal schistosomiasis and urogenital schistosomiasis are caused by *Schistosoma mansoni* and *Schistosoma haematobium*, respectively in Africa (Aula, et al., 2021; Colley, et al., 2014). Though potentially more prevalent (Van der Werf, et al., 2003) urogenital schistosomiasis has been given less attention compared to intestinal schistosomiasis (Rinaldi, et al., 2011; Rollinson, 2009). Moreover, urogenital schistosomiasis has often been linked to the spread of other infectious diseases such as HIV/Aids in women (Zirimenya, et al., 2020) and cancer of the bladder (Dematei, et al., 2017; Efared, et al., 2022). Both fascioliasis and schistosomiasis greatly affect the quality of life, especially in school-age children (Gryseels, et al., 2006; Hotez & Kamath 2009; Mas-Coma, 2005; Rubaihayo, et al, 2008) leading to disability-adjusted life years (stunted cognitive and physical growth, anemia and reduced physical fitness) or even death (Van der Werf, et al., 2003) in severe cases.

Infection with *Schistosoma* is mainly via skin penetration by cercariae during contact with unsafe water sources while infection (Figure 1) with *Fasciola* is associated with consumption of raw vegetables or livestock products as well as drinking unsafe water infested with miracidia (Danso-Appiah, 2016; Fentie, et al., 2013). Thus based on their mode of infection, the risk of human infection with schistosomiasis is relatively higher than that for fascioliasis. That is, avoiding *Schistosoma* infection would mean avoiding contact with such infected water sources, which is relatively impossible in settings such as those in most African countries that mostly lack safe municipal water supplies (Evan Secor, 2014). In addition, co-infection of both fascioliasis and schistosomiasis within the definitive host such as humans is possible (Fentie, et al., 2013).

The general control and eradication intervention strategies for such parasitic diseases appear to be insufficient so far due to the existing knowledge gaps. The most common approach is Mass Drug Administration (MDA). This however does not help to break the infection cycle (the re-infection and re-emergence). The use of vaccines is also being proposed and even tested but it requires more research (McManus, 2020; Molehin, 2020). The life cycle of these trematodes involves freshwater gastropods as intermediate hosts, in the family Lymnaeidae and Planorbidae for *Fasciola* and *Schistosoma* respectively (Correa, et al., 2010; Nelwan, 2019; Sturrock, 2001). A break in the life cycle by targeting the snails may enable control of as well as elimination of the trematodiases (Lu, et al., 2018). Control of snails using molluscicides (Zheng, et al., 2021) or natural predators of snails (Biological control) in addition to MDA, however, raises concerns as regards to their unspecificity (Evan Secor,

2014) and consequently the impact on the ecosystem functions (Lu, et al., 2018). Successful control or complete elimination would require attention to all the parties involved in the transmission, i.e. a so-called one health approach (Bergquist, et al., 2017; WHO, 2022) all of which still demands more research. Therefore these parasitic diseases especially schistosomiasis which is the second most important (after malaria) continue to remain a public health problem mostly in the continent of Africa (Tchuem Tchuenté, et al., 2017). It is thus selected to be the main focus of this PhD research project, with emphasis on the snail intermediate hosts as illustrated in Figure 2.

1.1.2. Freshwater snails (Gastropoda: Pulmonata) as intermediate hosts for disease causing trematodes, *Fasciola* and *Schistosoma*

Air-breathing freshwater gastropods are comprised of three main families; Physidae, Lymnaeidae and Planorbidae (Los Huertos, 2020; Strong, et al., 2008). These gastropods occupy a variety of habitats such as ponds (hence lymnaeids termed as pond snails), lakes, rivers, streams, or marshy areas of both man-made and natural, temporary or permanent ecosystems in different environments (Brown, 1994; Strong, et al., 2008). Gastropods, especially of families Lymnaeidae and Planorbidae, are of great medical and veterinary importance as intermediate hosts in the transmission of specific parasitic diseases (Dodangeh, et al., 2019; Mohammed, et al., 2016). In Africa, *F. hepatica* the main causative agent of human fascioliasis, and *F. gigantica* are transmitted by snail vectors *Galba* (*Lymnaea*) *truncatula* and *Radix natalensis* respectively (Mas-Coma, et al., 2022) but see Mahulu et al., 2019; while *S. mansoni* and *S. haematobium* group parasites are transmitted by snails in the genera *Biomphalaria* and *Bulinus* respectively (Brown, et al., 1994). These trematodes are highly specific with regard to the snail species used as intermediate hosts (Brown, 1994; Mas-Coma, et al., 2022; Sturrock 2001). There are 12 *Biomphalaria* species in Africa informally grouped into four groups; *B. pfeifferi*, *B. choanomphala*, *B. alexandrina* and *B. sudanica* (Mandahl-Barth, 1957). They are all susceptible to *S. mansoni* infection (Brown, 1994). *Bulinus* snails are a diverse group of species with varying degree of susceptibility to schistosome infection. They are divided into four groups, *B. africanus*, *B. forskalii*, *B. reticulatus* and *Bulinus truncatus/tropicus* complex (Brown, 1994). Apart from the latter group, the species in the rest are all potential intermediate hosts for human infective *S. haematobium* group parasites. Some of the species in *Bulinus truncatus/tropicus* complex serve as intermediate hosts for humans schistosome pathogens (Mohammed, et al., 2016; Sène, et al., 2004), others for schistosomes infectious to other animals such as livestock while others are not known as potential intermediate hosts at all (Brown, 1994). Indeed the *Bulinus truncatus/tropicus* group is a complex combination of species with various characteristics such as high degree of polymorphism, genetic diversity, and a peculiar constitution of polyploidy (Tumwebaze, et al., 2019; Zein-Eddine, et al., 2014). Such a constellation provides an opportunity to test various epidemiological and evolutionary hypotheses.

The schistosome abundance is a function of the population densities of the intermediate (snails) and definitive (vertebrates) hosts, and the likelihood of successful transmission upon the interaction between them (Mari, et al., 2017; Opisa, et al., 2011). Moreover, the geographical distribution of the parasitic disease depends on the snail distribution (Habib, et al., 2021). The population densities and the geographical distribution of the freshwater gastropods are determined by the reproduction potential, dispersal and successful colonization of habitats in different environments. These gastropods are hermaphrodites capable of self- and cross-fertilization with short generation times and high reproduction rate (Brown, 1994; Strong, et al., 2008) which affects the

genetic structure of the population. The pulmonate families Lymnaeidae and Planorbidae are highly speciose and have a wide distribution which is attributed to their high tolerance to a wide range of environmental conditions and thus exhibit a low level of endemism. The intermediate host-parasite susceptibility and/or infectivity also varies across small geographical scales (Rollinson, et al., 2001). Therefore, understanding the environmental requirements and the response of the intermediate hosts to climate-related changes in the environment can help to understand the disease dynamics and to develop effective control strategies.

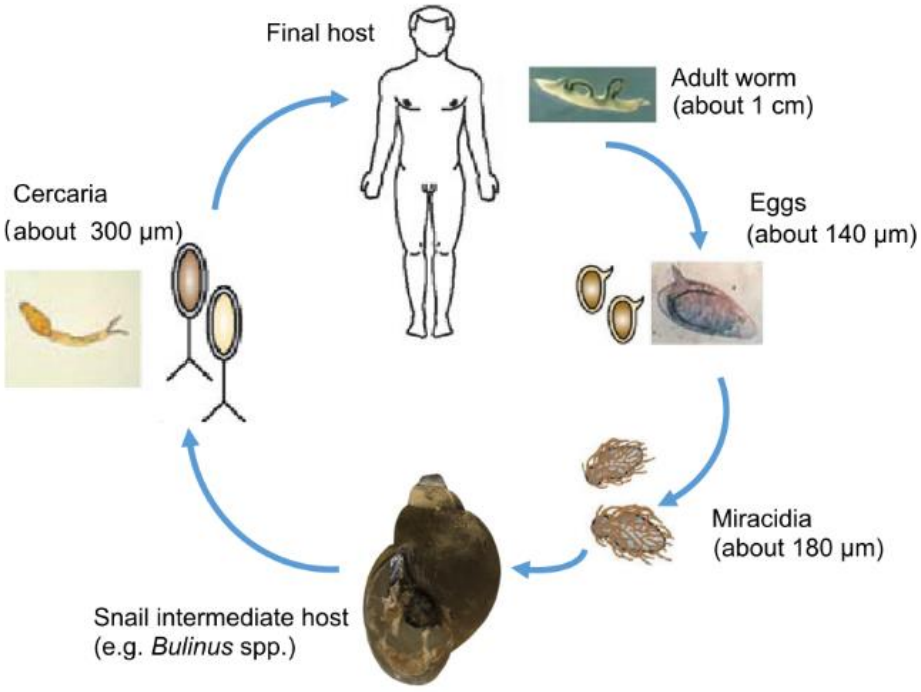

Figure 1. An overview of the *Schistosoma haematobium* parasite's life cycle with *Bulinus* snails serving as the intermediate hosts. Adopted from Danso-Appiah (2016).

1.1.3. Biotic and abiotic freshwater habitat factors causing the heterogeneity across different landscapes and their evolutionary and epidemiological implication

In this study, a large geographical area has been intermittently studied to collectively account for altitudinal and latitudinal range variation as well as heterogeneity and connectivity of freshwater habitats in the biogeography of freshwater trematode intermediate host snails.

Ancient biogeological activities have led to ecological habitat variation directly influencing biodiversity (Cardinale, et al., 2018). For instance, the formation of the Great African Rift Valley led to the flow of water on the rift valley floor forming rivers and lakes. Accompanying volcanism resulted in variation in the nature and characteristics of surface waters spread across different elevation gradients, i.e. on low lands as well as a series of high mountains and highlands termed as Afromontane archipelago. Such freshwaters include the small and unique crater lakes of western Uganda (Melack, 1978; Tumwebaze, et al., 2018), the relatively large East African great lakes (Odada & Olago, 2006), including Lake Victoria the world's second largest lake (Chibwana, et al., 2020; Kayombo & Jorgensen, 2006; Krishnamurthy & Ibrahim, 1973) and high mountain and highland water sources (Tumwebaze, et al., 2022).

Crater lakes are one of the lentic water habitats formed as a result of the filling of maars formed through volcanic activity. There are about 90 maar crater lakes in Western Uganda grouped into four crater fields (Melack, 1978), spread along a gradient from 914 to 1,566 m elevation and from semi-arid to sub-humid climate regimes (Rumes, et al., 2011). They are however small and thus sometimes neglected by researchers and the wider public, yet water-related diseases such as schistosomiasis affect people in the surrounding communities (Kabatereine, et al. 2004). Although freshwater habitats are often associated with comparatively low land levels, high mountains and highlands host water ecosystems of varying physical and chemical characteristics. A series of unique but interconnected high mountains and highlands are said to form an island ecosystem (Costanzi & Steifetten, 2019) often termed sky-islands. The Afromontane sky-islands species possess phylogenetic and biogeographical characteristics that are important for conservation as well as other interdisciplinary research (Mairal, et al., 2017). The spatial-temporal variation between the highlands combined with climate change and the associated physical and chemical properties of the water ecosystems across a range of elevations above sea level shapes the biodiversity (Benito, et al., 2020). This is why mountains and highlands are regarded as biodiversity hotspots (Myers et al., 2000).

The unique paleolimnological history, climate change and associated Anthropocene activities of freshwater habitats has evidence of important evolutionary aspects such as species endemism and so provide an opportunity for research (Odada, et al., 2003; Odada & Olago, 2006). For example they are good models for phylogeographical studies in the context of island biogeography theory (e.g., Mairal, et al., 2017), in which a number of hypotheses regarding species colonization, diversification and thus evolution as well as climate change and its impact to the biodiversity (e.g. Knowles, 2000; McCormack, et al. 2008) can be studied. The theory of island biogeography can also be extended to insular systems formation within large lakes such as Victoria (Chibwana, et al., 2022), in which the complex interaction between different organisms across a range of habitat characteristics within these systems affects biodiversity.

The effect of climate change both globally and locally on biodiversity has been investigated mostly for terrestrial organisms. However, freshwater biodiversity, species distribution ranges, and patterns as well as evolution dynamics have also been found to exhibit changes in response to climate change. Such changes include extinction, extirpations, and range shifts (Heino et al., 2009; Prakash & Srivastava, 2019). It was pointed out that climate change (global warming) would affect the distribution and survival rate of parasite vectors and also directly influence the reproduction and maturation rate of parasites (McCarthy, 2001). Climate particularly climate change influences mollusk diversity (Marcogliese, 2008; Stensgaard, et al., 2019). Associated with climate are environmental variables such as temperature, rainfall and/or desiccation. Together with water velocity, conductivity or salinity have been shown to impact the geographical distribution range, host-parasite interaction and consequently the prevalence of water-borne diseases (Rollinson, et al., 2001). For instance, mountains are characterized by decreased temperature, decreased pressure, decreased oxygen and increased insolation directly related to elevation changes. *Fasciola* vectors have a high adaptive power to various kinds of environmental conditions (Mas-Coma, 2004) hence the wide distribution. On the other hand, schistosomiasis prevalence is affected by the environmental conditions, due to the restricted environmental tolerance range of the parasites and the vectors thus endemic in subtropic and tropical regions (De Leo, et al., 2020). Increasing human population densities, altered land use, and aquaculture efforts have strong effects on the properties of a freshwater ecosystem. For example, they cause increased eutrophication and sedimentation, consequently affecting the population of macroinvertebrates (Van Bocxlaer, et al., 2014).

In this study, I focus on the biogeography of gastropods in crater lakes of western Uganda and Afromontane ecosystems as island models putting into consideration the altitudinal variation in the distribution of the disease hosts to predict disease risks. Included is a joint study on the population of snails in Lake Victoria and the biodiversity distribution across different zones of the large lake ecosystem, from one location to the other.

1.1.4. Freshwater intermediate host species identification and characterization

Freshwater species identification has been based mainly on shell morphology, radula (feeding structure), reproductive organs, chromosome number, enzyme characteristics and geographical distribution (Brown, 1994; Rollinson, et al., 2001). These techniques however have had a number of constraints. For instance, morphological species distinction of the snails in the two families Lymnaeidae and Planorbidae especially to the species level is difficult due to a high diversity or polymorphism of features such as the shell or radula termed as eco-phenotypic plasticity (Pfenninger, et al., 2006) that is often linked to the vagaries of the environment. Such challenges have consequently affected the reliability of the species differentiation, with subsequent changes or regroupings, and synonym problems as was also anticipated by the earlier researchers themselves (e.g. Brown, 1994), all leading to taxonomic confusion. Molecular techniques have revolutionized species identification in modern systematics and biodiversity studies, aiming for consistent and reliable species identification in an effort to achieve a stable taxonomy (Correa, et al., 2010). The molecular techniques have so far predominantly involved the use of partial nuclear and mitochondrial genes, both offering a better understanding of phylogenetic relationships and speciation dynamics. It is, for instance, proposed that a multilocus gene tree generated from a combination of both nuclear and mitochondrial markers is capable of serving as a species tree (Kane, et al., 2008). A barcoding gene for cytochrome *c* oxidase subunit 1 (*cox1*) has been particularly beneficial for identifying species and reconstructing phylogenetic relationships between intermediate hosts (Chibwana et al., 2020; Kane et al., 2008; Mahulu et al., 2019; Tumwebaze, et al., 2019). Annotating the entire genomes of these intermediate hosts has also been initiated with the onset of next-generation sequencing. This is particularly useful when groups of species cannot be amplified using universal markers for Sanger sequencing, as has been the case with *B. globosus's* behavior towards *cox1*. So far a full mitochondrial and nuclear genome for *B. truncatus* species is available (Young, et al., 2021; Zhang, 2022). Another diagnostic tool the multiplex PCR has been established in which genotyping of both the parasite and the host is done in a 'one health' context (Alda, et al., 2018; Schols, et al., 2019), which further supports monitoring the dynamics of parasitic diseases. These have helped to gain a better understanding of the evolutionary changes and dynamics of these organisms in the environment.

1.2. The Scientific Problem/Rationale

There are considerably fewer freshwater biodiversity studies in less developed regions of the world (Faghihinia, et al., 2021) mainly due to a lack of proper resources such as infrastructure, funds, expertise and/or sensitization. As such, not only is it difficult to develop a comprehensive account of freshwater diversity and dynamics, but it also creates gaps in knowledge in the fight against freshwater-related diseases, such as waterborne Neglected

Tropical Diseases (NTDs). For instance, fascioliasis was previously considered a disease of livestock but has only recently been recognized as a zoonotic disease by the World Health Organisation (WHO) (Molyneux, 2013) and a priority for control. The 2012 London Declaration on NTDs put schistosome parasites on the list of ten NTDs to be eliminated, eradicated, or controlled by 2020 (Adenowo, et al., 2015). However, this objective has not been achieved yet and the spread of the parasitic disease keeps increasing, infesting new areas. With an estimated 200 million cases and 500–600 million people at constant risk of infection worldwide, schistosomiasis is considered the second most important parasitic disease after malaria (Bergquist, et al., 2017). Extension of the prevalence threshold of schistosomiasis to higher altitudes in the crater lakes of Western Uganda was reported (Rubaihayo, et al., 2008). However, no comprehensive study of the intermediate hosts or direct parasite field studies had been conducted in this region although local infection was presumed. Unfortunately, this is also true in the case of the high mountain and highland regions of most parts of the pan-African region. Genetic diversity and population structure of intermediate host snails have been shown to be drivers of transmission dynamics (Standley, et al., 2011).

To explore this subject, we studied freshwater snail species representatives of two families Lymnaeidae and (especially) Planorbidae of the superfamilies Lymnaeoidea and Planorboidea respectively and in the superorder Hygrophila subgroup of Pulmonates (Gastropoda; Mollusca). The family Planorbidae is composed of three important genera in the transmission of the second most important parasitic disease, schistosomiasis; *Bulinus*, *Biomphalaria*, or *Indoplanorbis*. Although both *Bulinus* and *Biomphalaria* snails of the subfamilies Bulininae and Planorbinae respectively are the important schistosome intermediate hosts in Africa, the latter has received most of the research attention. However, *Bulinus* snails are more diverse and show great variation in their compatibility with and susceptibility to schistosomes (Brown, 1994). Furthermore, the genus *Bulinus* has faced the majority of taxonomic difficulties. Therefore, this study's attention was drawn more to the genus *Bulinus*.

Bulinus snails and Lymnaeids exhibit a wide distribution across different climatic regimes (Brown, 1994). However, the driving forces behind their wide distribution and mode and extent of the simultaneous speciation has not been properly investigated. Changes in *Bulinus* species composition along the altitudinal gradient as one of the confounding effects of environmental variables should be studied. Besides, the effect of altitude per se has also not well been studied and understood. The exact lineages of intermediate hosts need to be identified in order to make meaningful inferences and risk predictions. Freshwater habitats have a number of confounding factors that are mostly unknown or uncontrolled. These include climate change, changes in the environment and limnology, like regional eutrophication due to intensified land-use practices, and ecological measures (e.g., productivity, salinity), that are independent of climatic variables. In the event of the ongoing climate change dynamics, it would be interesting to find out to what extent these *Bulinus* snails would evolve in space and time.

In this study, the *S. haematobium* intermediate hosts, the *Bulinus* snails' diversity, population structure, and distribution were assessed. Along an altitudinal gradient, the study area included the western Uganda crater lakes and the afromontane freshwater ecosystems. The impact of climate variability and other environmental and historical factors on both the *Schistosoma* intermediate hosts was tested using the crater lakes as a model system. The Afromontane *Bulinus* species distribution analysis was supplemented by a study of the Lymnaeids snails found on high-altitude mountain ranges for comparison with the specificity of the response of *Bulinus* snails to the environment.

Therefore, on the basis of regional versus continental geographical scales, I intended to:

(i) identify lineages and quantify genetic diversities of the trematode intermediate hosts,

(ii) determine the phylogenetical and biogeographical affinities and dynamics of intermediate host gastropod species along environmental gradients, and

(iii) assess the variation in physico-chemical water parameters, long-term climatic factors such as temperature and rainfall, and habitat characteristics potentially driving the presence, distribution and (genetic) diversity of intermediate host snails and thus estimate the risk of the parasitic diseases in different environments.

Using case studies of representative intermediate host genera, I discuss patterns and processes of diversification in the African pulmonate gastropods of zoonotic, biomedical and veterinary importance.

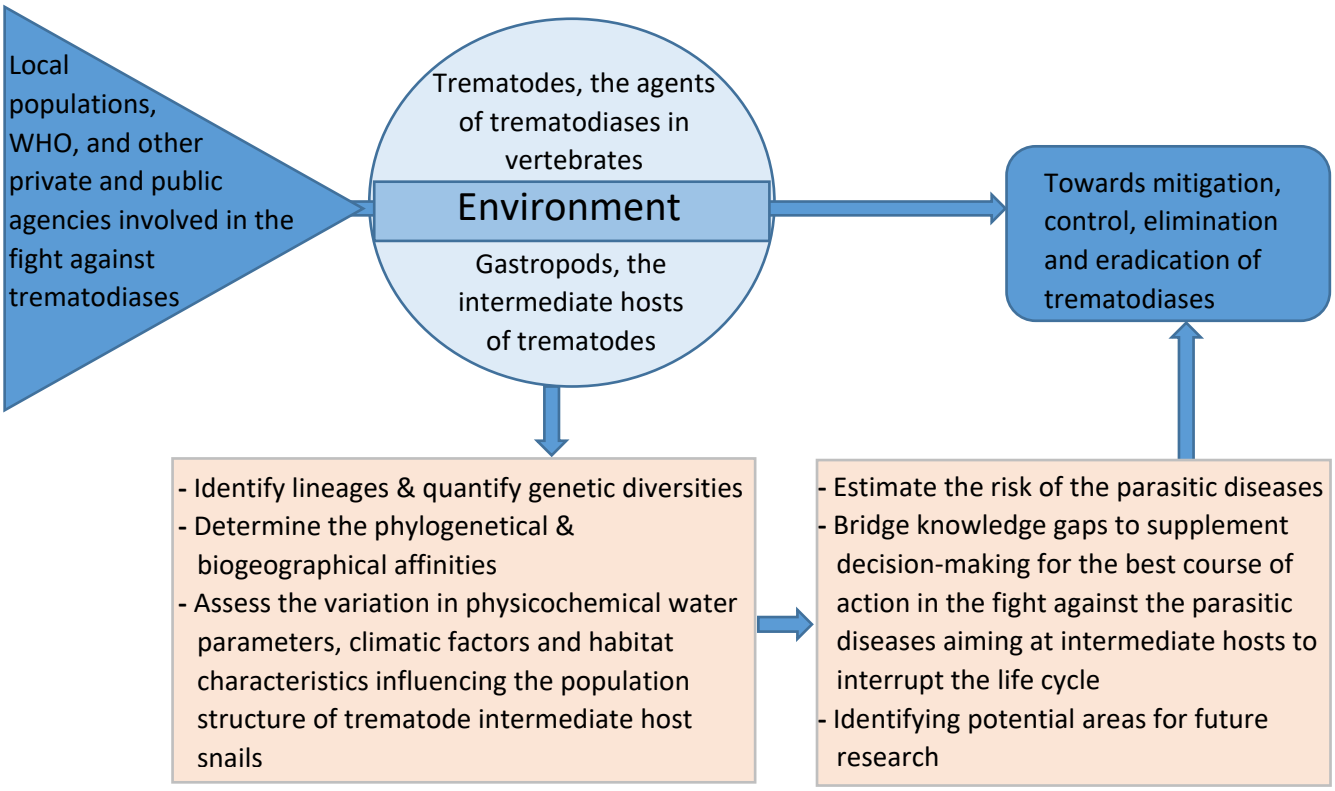

Figure 2. Schematic illustration of the study concept highlighting the current focus of investigation on the intermediate host gastropods as a basis for all the stake holders to control the trematodes in the environment by breaking the life cycle.

1.3. Materials and Methods

In this study, freshwater gastropods were primarily sampled from sub-Saharan Africa's rift valley lakes, including Lakes Albert, Edward, George, Kivu, Tanganyika, and the western Uganda crater lakes (Tumwebaze, et al., 2019), in the western Albertine rift valley, Lake Victoria (Chibwana, et al., 2020), Lake Malawi in the southern rift valley, and high mountain/highlands such as the Lesotho Highlands (Maloti-Drakensberge), Ethiopian Highlands (Abyssinian Massif), Kenyan Highlands (Aberdares ranges including Kinangop Plateau,

Mt. Kenya/Laikipia), Tanzanian Eastern Arc Mts. (Udzungwa, East Usambara), and Rwenzori Foothills (Figure 3). The snail sampling was stretched to cover altitudinal ranges between ca. 900 and ca. 4000 m.

DNA was isolated from the foot of these ethanol-preserved snail samples using the CTAB method (Wilke, et al. 2006) and DNeasy blood and tissue kit according to the manufacturer's guidelines. Different DNA loci have different evolutionary characteristics (Rubinoff & Holland, 2005), and therefore a combination of different genetic markers helps to control/limit biases in phylogenetic reconstructions (Young, et al., 2021) due to the incongruence of DNA loci. In this study, a combination of both mitochondrial and nuclear markers (up to six markers) were amplified. The bidirectional Sanger DNA sequencing for amplicons was performed on an ABI 3730xl DNA analyzer using the BigDye Terminator Kit (Life Technologies, LGC Genomics GmbH, Berlin, Germany). The newly obtained sequences were complemented with sequences from the National Center for Biotechnology Information (NCBI) genbank database.

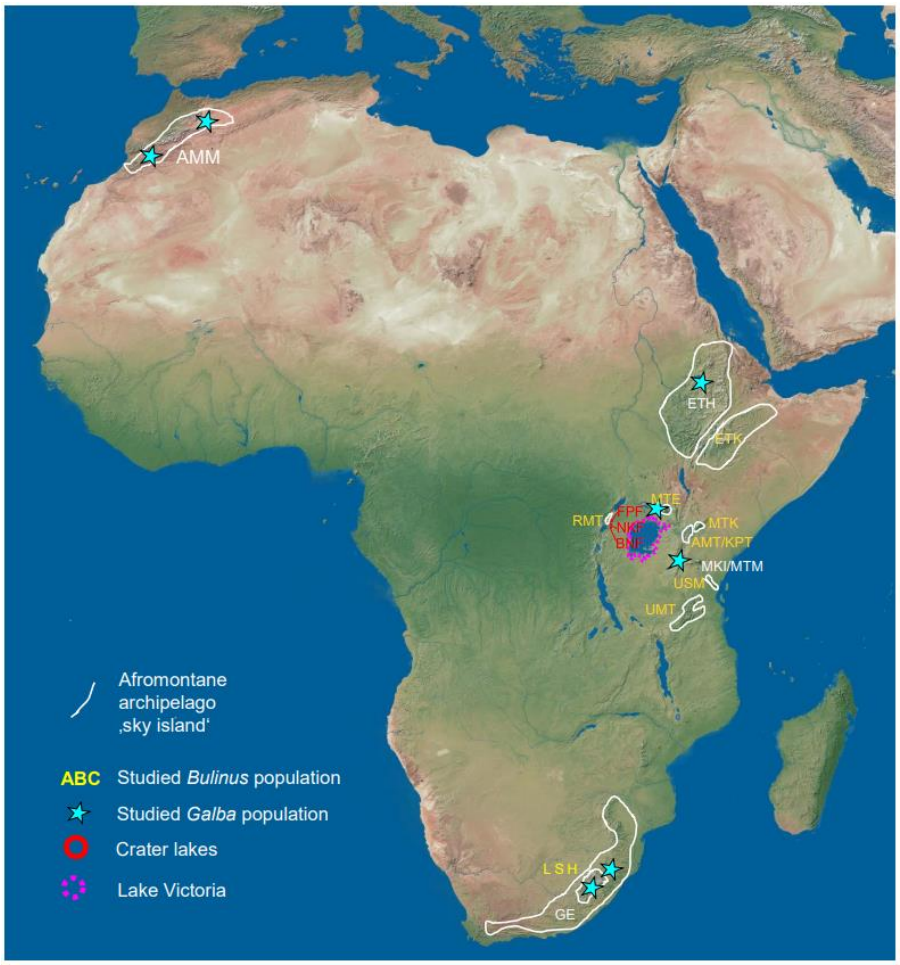

Figure 3. Principal study areas of *Bulinus* and *Galba* populations: Western Uganda Crater Lakes (Tumwebaze et al., 2019; Tabo et al., 2022), Afromontane / high altitudes (Tumwebaze et al., 2022; Mahulu et al., 2019).

Abbreviations:
MTK_ Mount Kenya,
UMT_ Udzungwa Mountains,
KPT_ Kinangop Plateau,
ETH_ Ethiopian Highlands,
LSH_ Lesotho Highlands,
AMT_ Aberdares Mountains,
MTE_ Mount Elgon,
MKI _ Mt. Kilimanjaro
MTM_Mt. Meru,
GE_ Great Escarpment,
USM_ Usambara Mts,
AMM_ Atlas Mountains in Morocco.

A set of statistical inference methods were employed in the genealogical analysis. These are Bayesian inference (BI) as implemented in BEAST (Drummond, et al., 2012) and MrBayes (Ronquist & Huelsenbeck, 2003) using MCMC (Suchard, et al., 2018), maximum likelihood (ML) implemented in Randomized Accelerated Maximum Likelihood (RAxML) and Molecular Evolutionary Genetics Analysis (MEGA) (see Chibwana et al., 2020, Mahulu et al., 2019, Tumwebaze et al., 2022 and Tumwebaze et al., 2019 for details). In all the cases, best-fit substitution models were selected using jModelTest 0.1.1 (Posada, 2008). MEGA was also used to calculate the uncorrected genetic p-distances. In order to examine the spatial-temporal evolution dynamics of the freshwater

gastropods, a molecular clock was used to generate time-calibrated trees using fossil calibration as used in the estimation of divergence times. Statistical parsimony network analysis implemented in TCS software was also used to explore the phylogeographical affinities. The phylogenetic analysis was also supplemented by DNA-based species delimitation methods General Mixed Yule Coalescent (GMYC) model and Bayesian Poisson tree processes (bPTP) for purposes of species diversity determination and species identification. To test the hypothesis that a different set of parameters control the distribution of intermediate hosts and differ between genera and across the geographical scale, the combination of biotic and abiotic factors that control the presence of the *Schistosoma* IHs was thus investigated using a model system of the western Uganda crater lakes region. Extensive data for limnological and ecological as well as environmental and climatic parameters were compiled to cover recent natural conditions and past impacts on the freshwater habitats. Parameters such as habitat characteristics, inflow and outflow regime, substrate, slope and shoreline development, surface water temperature, dissolved oxygen, conductivity and surface pH, depth and Secchi depth, total phosphorous, and Calcium and Magnesium content have been measured on ground. Given the great inconsistencies pertinent in both the published record and public databases as to the basic geographical characteristics of the lakes concerned, the current lake surface area (A) in this study we determined using LandSat radar mission satellite images in QGIS v. 2.10.1 (QGIS Development Team, 2015). Altitude was determined in Google Earth 7.1.2.204 (Google, Inc.). In addition, land use categories and settlement densities around the lakes were determined from satellite images using the QGIS.

1.4. Publication outlines

Paper 1. Tumwebaze, I., Clewing, C., Dusabe, M. C., Tumusiime, J., Kagoro-Rugunda, G., Hammoud, C., & Albrecht, C. (2019). Molecular identification of *Bulinus* spp. intermediate host snails of *Schistosoma* spp. in crater lakes of western Uganda with implications for the transmission of the *Schistosoma haematobium* group parasites. Parasites & Vectors, 12(1), 1-23.

This study focuses on *Bulinus* species as intermediate hosts in crater lakes of western Uganda. Large-scale field sampling was conducted in western Uganda and the adjacent areas. Using Bayesian inference and parsimony network analyses of the of the mitochondrial barcoding gene cytochrome *c* oxidase subunit 1 (*cox1*) sequences, this study sought to identify and establish the diversity, population structure, phylogeographical patterns, and phylogenetic affinities of *Bulinus* species as potential hosts for *Schistosoma* spp. The crater lakes were dominated by *Bulinus tropicus* species, a non-potential host for human infecting *S. haematobium* parasites. These species are however important for schistosomiasis in livestock and wild ruminants. With 31 different haplotypes present in the crater lakes, these species showed a high degree of species diversity. *Bulinus truncatus* and *Bulinus forskalii*, the important hosts for human urogenital schistosomiasis in some regions of Africa, were found in one lake and two lakes respectively. These three species of *Bulinus* snails were found in 34 out of 58 crater lakes surveyed. The crater lakes haplotypes were clustered together with those of the surrounding sources implying species source pool for the crater lakes. This is the first comprehensive malacological analysis of the *Bulinus* species population in the crater lake systems in western Uganda. The *Bulinus* species richness revealed low risk of human urogenital schistosomiasis but rather a high risk *S. bovis* in the crater lakes, as seen from high

dominance of the potential intermediate hosts, *B. tropicus* species. Given that livestock keeping is an important economic and subsistence activity in this region and moreover the study area comprised of mainly a protected area for tourism and conservation, precautions should be taken to protect wild and domestic ruminants in this region as the population benefits from these animals.

Declaration of author contribution: As a lead author and corresponding author, I participated in the study design, and fieldwork work. I did the processing of the molecular data and analyses, wrote the draft manuscript including tables and figures.

Paper 2. Tumwebaze, I., Clewing, C., Chibwana, F. D., Kipyegon, J. K., & Albrecht, C. (2022). Evolution and biogeography of freshwater snails of the genus *Bulinus* (Gastropoda) in Afromontane extreme environments. Frontiers in Environmental Science, 572.

This article addresses the evolution of *Bulinus* species in the Afrotropical Mountains and highlands commonly known as the Afromontane archipelago, which are frequently distinguished by remarkable biodiversity and high plant and animal species endemism. In order to understand how the current climate and previous geological factors affect species distributions and evolutionary processes, we analyzed pan-African *Bulinus* species from a variety of habitats across different altitudinal ranges of the high mountains categorized as "sky islands". The purpose of the study was to ascertain the frequency and timing of extreme altitude colonization, the biogeographical affinities and degree of isolation of high-altitude species, the lineages that diverged and developed endemism in the afromontane environments, and whether or not the afromontane regions exhibit a "sky islands'' ecological nature. These objectives were assessed by combining two mitochondrial genes (*cox1*, 16S) with two nuclear genes (ITS2 and H3) in a multigene phylogeny that was calibrated using available *Bulinus* fossil data. Employing the Bayesian phylogenetic inference method and the molecular clock analysis a strongly supported phylogeny of the genus *Bulinus*, resolving for the first time the relationships between the four *Bulinus* groups was achieved. Some species within the *Bulinus truncatus/tropicus* complex were the only ones identified to have evolved the extreme altitude limits of this study, while most others including of the rest of the groups evolved in low to mid-altitudinal ranges. The high altitudes were noted to have been colonized on several occasions independently in the Plio-Pleistocene era. Though conclusions regarding endemism are to be made with reservations for even more thorough sampling, the tendency was towards the highlands of Ethiopian Kenya and Lesotho, the latter of which additionally exhibited cryptic diversity. Most interesting though is the discovery of a new species at the extreme altitude of ~4,000 m a.s.l. on Mt. Elgon/Uganda which extends the formerly known altitudinal maximum distribution range of the genus by roughly 900 m. This peculiar species was determined to have diverged in the Pliocene (~4 myr) and is currently characterized by low genetic diversity.

Declaration of author contribution: I participated in the designing of the study, collected in the field for some of the samples, did lab work and compilation of the data from literature, analyses, formulation of tables and figures and writing of the manuscript.

Paper 3. Chibwana, F. D., Tumwebaze, I., Mahulu, A., Sands, A. F., & Albrecht, C. (2020). Assessing the diversity and distribution of potential intermediate hosts snails for urogenital schistosomiasis: *Bulinus* spp. (Gastropoda: Planorbidae) of Lake Victoria. Parasites & Vectors, 13(1), 1-18.

Lake Victoria is one of the most important freshwater ecosystems not only in East Africa but the whole continent. Moreover, it is the second largest lake in the whole world, after Lake Superior. Comparatively, and like other freshwater ecosystems especially in the region, Lake Victoria has faced significant transformations mostly due to anthropogenic activities but also climate change effects. Effects to the macrobenthic community, though known for the fishes is less investigated for other species such as gastropods species which are important, especially as vectors of water-borne diseases. The *Bulinus* snails as one of the genera important moreover for one of the important diseases is less studied compared to its counterpart, *Biomphalaria* snails. Given the adaptive potential of the *Bulinus* snails and the Lake Victoria ecosystem size with potentially a number of ecological differentiation, the population structure/pattern of these species was investigated to determine their diversity, phylogenetic and phylogeographical affinities and thus assess the risk of schistosomiasis in light of climate change dynamics. The analyses of the Lake Victoria *Bulinus* population revealed phylogenetic differentiation whereby species of the same group appeared to cluster according to the habitat type. In spite of this, there was also high gene flow between habitats. The gene pool analysis also showed recent genetic diversification (expansion) thus have not reached equilibrium. This study further helps to untangle the taxonomic confusion about *Bulinus transversalis* and *Bulinus ugandae* by earlier researchers.

Declaration of author contribution: I participated in part of the field sampling, labwork, analyses and reviewing of the manuscript prior to submission.

Paper 4. Mahulu, A., Clewing, C., Stelbrink, B., Chibwana, F. D., Tumwebaze, I., Stothard, J. R., & Albrecht, C. (2019). Cryptic intermediate snail host of the liver fluke *Fasciola hepatica* in Africa. Parasites & Vectors, 12(1), 1-11.

Lymnaeids consist of snail species that act as intermediate hosts for the liver flukes that cause fascioliasis a public health problem for especially the livestock but also and especially recently the human beings. Although they are well known for their cosmopolitan distribution, including the high altitudes, their population structure and distribution in Africa have not well been studied especially by using modern molecular analytical techniques. In addition to the Pan-African sampling, samples from Europe and Asia were also added to aid the reconstruction of the origin and diversification of these species. Using a combination of mitochondrial and nuclear markers, the phylogenetic and phylogeographic affinities were reconstructed. *Galba truncatula* species were shown to be restricted to Palaearctic regions, specifically Morocco, while the rest of the continent was composed of a distinct species identified here as *Galba mweruensis* (Connolly, 1929), a relatively recently diversified species (Plio-Pleistocene) morphologically indistinguishable from *Galba truncatula* and *Galba schirazensis* of Northern Africa. The species colonized Africa in the Pliocene and Miocene periods, thus the slightly insinuated dispersal from Europe excluding human beings as agents for the initial colonisation. Just like the high-altitude *Bulinus* species, the *Galba mweruensis* population of Mount Elgon were highly distinct. This study together with the one on high-altitude *Bulinus* species highlight that there is still a high potential of cryptic

species in high altitudes that should be further investigated in order to understand the patterns of species diversification in light of climate change and the subsequent changes in the environment.

Declaration of author contribution: I participated in the mobilization of some of the received material, analytical discussion and interpretation of the data, and review of the manuscript.

Paper 5. Tabo, Z., Neubauer, T. A., Tumwebaze, I., Stelbrink, B., Breuer, L., Hammoud, C., & Albrecht, C. (2022). Factors controlling the distribution of intermediate host snails of *Schistosoma* in crater lakes in Uganda: A machine learning approach. Frontiers in Environmental Science, 10, 871735.

A model system of 56 crater lakes was used to test the effect of extrinsic variables in freshwater ecosystems on the prevalence of *Schistosoma* intermediate hosts of genera *Bulinus* and *Biomphalaria*. The geographical scale distribution of the crater lakes of western Uganda presents an ecological setting of habitat characteristics in terms of isolation, altitudinal ranges, anthropogenic influence and hydrological properties. Physical and chemical properties of the crater lakes were assessed for their importance in schistosomiasis transmission singly or synergistically using statistical methods, i.e. random forest algorithm to determine the drivers of the distribution of two important *Schistosoma* intermediate hosts *Biompharia* and *Bulinus* snails. These were climatic variables temperature and precipitation, chemical properties such as pH, dissolved oxygen, water temperature, conductivity of magnesium and calcium, depth and transparency, surface area, competitors of the mollusk fauna community, geographical location coordinates, altitude and isolation. Land use measures and human population density around each lake were included to account for anthropogenic pressure. The measures (quantity and quality) of the properties were obtained either during the field sampling, i.e., measured on-site, or obtained from literature as well as the online databases. Climatic variables (temperature & rainfall), geographical location, and mollusk fauna species richness were reconstructed as the major drivers of *Biomphalaria* while the latter two together with water chemical properties influence the distribution of *Bulinus* snails. Overall, the presence and distribution of both genera showed tendency towards mollusk diversity and geography and thus an emphasis on the significance of a suitable environment. Epidemiologically, such an approach can help in prediction of disease incidences risks in a certain area, thus helping to control as well as eventually eradicate spread of the disease.

Declaration of author contribution: I participated in the design of the study, fieldwork, conceptualizations of the findings and review of the manuscript.

1.5. Results and Discussion

There are five articles that make up this thesis. The freshwater gastropod snail species that serve as intermediate hosts for trematodes of human and veterinary importance, particularly schistosomiasis and fascioliasis were studied. Various case studies, mainly on the genus *Bulinus*, but also on *Biomphalaria* and *Galba*, were carried out on a regional and continental scale sampling. The species diversity, evolutionary relationships, and biogeographical affiliations were determined primarily in the dozens of crater lakes of Western Uganda, and then the study expanded to include the Lake Victoria system, which was noted as one of the significant

biodiversity source reservoirs for the crater lakes. Two studies on a continental scale were carried out for the genera *Bulinus* and *Galba* with a special focus on Afromontane regions, as these will play an important role in the future occurrence of tropical diseases in the course of climate change. The discussion is focused mainly on the genus *Bulinus* the intermediate hosts for *S. haematobium* group parasites important for humans as well as domestic and wildlife to conceptualize the risk of schistosomiasis in the study region.

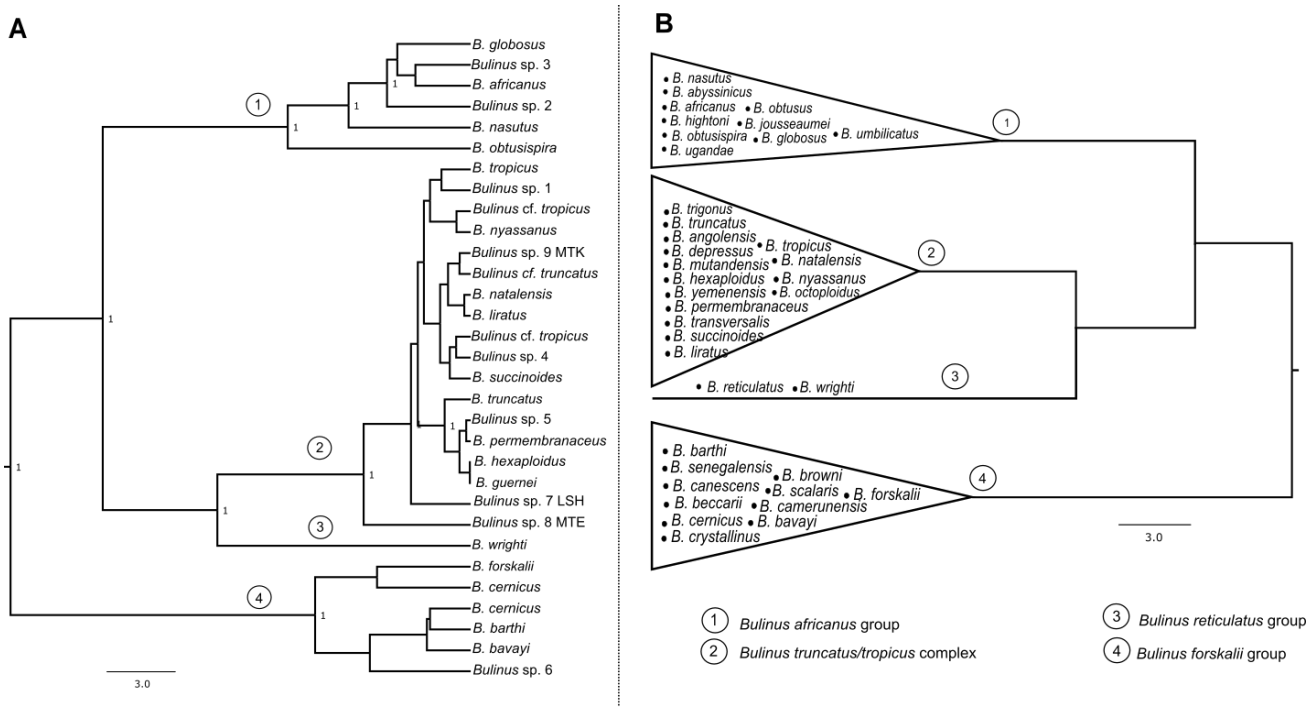

Figure 4. Phylogenetic trees: (A) List of the MOTUs as recovered by this project. Here, species are named according to earlier determinations or with a genus name followed by a qualifier for those species that have not yet been described integratively. The posterior probabilities for the highly supported reconstructed relationships are shown. The outgroup was extracted off a posteriori: (B) *Bulinus* groups species composition according to earlier determination by Brown (1994) and others.

1.5.1. The intermediate host gastropods' phylogenetic affinity and species diversity

The wide sampling of the genus *Bulinus*, coupled with the multimarker phylogenetic reconstruction resulted in a well-supported phylogeny of four major clades representing the four *Bulinus* groups; the *Bulinus africanus* group, *Bulinus forskalii* group, *Bulinus reticulatus* group, and the *Bulinus truncatus/tropicus* complex. This is important since there have been discussions as to whether all four groups belong to one genus or if they should rather be split into different genera, let alone the phylogenetic relationship of the whole genus (Zhang et al., 2022). Different published articles have presented a variety of *Bulinus* phylogenies in which a consensus could not be reached as regards to the phylogenetic relationship between the four groups of *Bulinus* (Jørgensen et al., 2011, 2013; Kane et al., 2008; Tumwebaze et al., 2019). This can be attributed mainly on the single marker approach usually employed.

Overall, six *Bulinus* species were found across the entire research area. *Bulinus tropicus* were the most dominant in the Albertine rift valley crater lakes region, while a species of the *B. africanus* group that appeared morphologically conformable to *B. ugandae* rather than *B. globosus* a complex group of species as has often been reported (Brown, 1994; Nyakaana 2013; Zhang et al., 2022), along with *B. truncatus* were dominant in

Lake Victoria (Chibwana et al., 2022). The presence of *B. globosus* in Lake Victoria cannot be ruled out since it has been reported on the shores of Kisumu, Kenya (Opisa, et al., 2011), a region that was not sampled extensively for this study. *Bulinus forskalii*, though barely present were also found in two crater lakes and the foothills of Mount Elgon. A species, likely *B. trigonus,* was found co-existing with physid species on the sand beaches of Lake Victoria. The high mountain and highlands' phylogenetic study indicated the presence of cryptic species from Lesotho and Mt. Elgon. The Mt. Elgon species emerged ancestral to all the rest of the thus far known composition of the *Bulinus truncatus/tropicus* complex. The closest genetic relationship to other *Bulinus* species in the Genbank database was 92% thus representing a species new to science (Tumwebaze et al., 2022). Interestingly the genotyped specimens of the afromontane *Galba* population revealed similar phylogenetic relationship patterns in which the distinctiveness of Mount Elgon samples from the rest of the species was equally outstanding (Mahulu et al., 2019). Further examination of the distinctive *Bulinus* snails on the summit of Mount Elgon found a population with very little variability (i.e. made up of only one haplotype). This could suggest that in such a harsh environment, evolutionary factors are unique. Earlier reports claimed that the *B. tropicus* species lived in the highlands of Lesotho (Brown, 1994). It must still be confirmed whether the cryptic specimens collected from the same area fit the former classification or if they represent a distinct species.

Out of about 37 described species of the genus *Bulinus* (Brown, 1994; Figure 4B), mostly phenotypically characterized species of *Bulinus,* the overall species delimitations retrieved roughly 30 molecular taxonomic units (MOTUs) (Figure 4A). Moreover, since most of the MOTUs could not be identified with a specific established name due to their unclear identity the number of lineages is potentially lower. This demonstrates that this genus (*Bulinus*) still has a low molecular representation, which is one of the factors contributing to the failure in achieving a reliable and stable *Bulinus* taxonomy.

It has been found that molecular techniques are more reliable than morphological techniques for species delimitation down to the species level (Alda et al., 2021; Kane et al., 2008). Although morphological methods have been widely employed to identify species, they frequently suffer from ambiguity due to a lack of clear separation between species, which is sometimes attributed to ecophenotypic plasticity (Clewing et al., 2015). Consequently, the molecular phylogenetic analysis of the *Galba* specimens revealed that, in contrast to the previously known distribution of *Galba* species in the sub-Saharan Africa region (Brown, 1994), the distinct species *Galba mweruensis* dominates as the major potential intermediate host for *Fasciola hepatica* (Mahulu et al., 2019). The research on the *Bulinus* population of Lake Victoria also draws attention to potential taxonomic issues with *B. transversalis*, whose topotypic material molecularly conforms to *B. ugandae* (Chibwana et al., 2020). This could be because the previous population has been replaced or because morphological traits have been mixed up. By studying the *B. transversalis* holotype's molecular makeup, this problem could be resolved. Generally, the taxonomy of freshwater gastropods is still largely under scrutiny partly due to the discordance between the classical morphological taxonomic approaches and the current molecular techniques and insufficient field surveys especially in the face of the changing climate as well as environmental conditions. *Lymnaea* and *Biomphalaria* snails were also discovered throughout the entire research region (Tabo et al., 2022; Tumwebaze et al., 2022). *Biomphalaria pfeifferi* was the most common species throughout the entire crater lakes region. Based on DNA sequencing and shell morphology, the crater lakes area contained two species of *Biomphalaria*, namely *B. pfeifferi* and *B. sudanica* (Tumwebaze, unpublished data).

1.5.2. Biogeography and phylogeographic affinities of the intermediate hosts

All three of the intermediate hosts for *Schistosoma* and *Fasciola*, namely *Bulinus, Biomphalaria,* and *Lymnaea/Radix*, as well as six other molluscan genera, *Gabbiella, Pila, Segmentorbis, Melanoides, Afrogyrorbis,* and *Gyraulus*, were discovered to be present throughout the primary study area, the crater lakes region (Tabo et al., 2022). Although the study covered in this dissertation was focused on *Bulinus*, a variety of these species have also been documented in Lake Victoria and other places (Darwall et al., 2011; Mwambungu 2004). The three intermediate host genera were also discovered along the slope of Mount Elgon (Howell et al., 2012, Tumwebaze et al., 2022). This suggests that the region's gastropods, which are important for veterinary and medical fields, are widely distributed. It has been established in this study that the presence of the *Schistosoma* IH generally depends on the presence of other mollusk genera implying that they prefer favorable environmental conditions regardless of the genus (Tabo et al., 2022) which comes as no surprise. As a result, the reported sympatric occurrence between the taxa was noted across the research area (Tabo et al., 2022).

The small lakes are dispersed throughout the entire crater lakes field systems and do not have direct hydrological/hydrogeological connectivity, which is consistent with the hypothesis of island biogeography (Itescu, 2019). The lakes, pools, and ponds in the highlands and mountains are also island like systems. However, Lake Victoria, the continent's largest freshwater body, is distinct from the aforementioned systems. The shallow shorelines and banks of the islands and the main waterbody provide a variety of ecological niches that are ideal for macrobenthic species. It is therefore categorized as a hotspot for biodiversity with several endemic species (Sayer et al., 2018). In this lake, there was a higher *Bulinus* species richness than in the other freshwater categories studied. Contrary to expectations, the genetic population analysis revealed no genetic differentiation between the *Bulinus* populations in the lake, indicating high gene flow between populations and suggesting that there is no habitat differentiation, or that the *Bulinus* species is not truly niche-conserving (ecological niche endemism) in low environment differentiation. However, the genetic equilibrium revealed that the species are still expanding. Within the crater lakes, species endemism has also been noted before. Brown (1994) indicated that *Bulinus tropicus toroensis* is endemic to the crater lakes in western Uganda, just as *Bulinus cameroonensis* is to the crater lakes of Cameroon (Darwall et al., 2011).

Despite the limnological singularity of the crater lakes system, the haplotypes were not phylogenetically monophyletic but rather clustered with other samples from the vicinity. Furthermore, samples from far-off places like Cameroon also appeared to be grouped together with some samples from Uganda crater lakes, and several crater lakes also had haplotypes that were distantly related. Because *Bulinus* snails are very adaptable (Brown, 1994), it is not surprising that geography as a factor was found to have less of an impact on their spread in contrast to *Biomphalaria* snails (Tabo et al., 2022). This could imply that the *Biomphalaria* snails are less difficult to control compared to *Bulinus* snails, and as a result, the corresponding schistosomiasis varieties as is already the case in some regions (e.g. Joof et al., 2021). Based on a supra-regional altitudinal analysis of crater lakes, an initial attempt to explain the evolution of the high altitude species indicated a tendency with a unimodal species distribution, with the bulk of haplotypes in the middle ranges and comparatively few in the higher and lower altitudes (Tumwebaze et al., 2019). Species range shifts from lower warmer altitudes to higher cooler altitudes due to climate change lead to the formation of climate refugia (Couet et al., 2022) for organisms like freshwater macroinvertebrate species that have limited mobility. The environmental conditions in these climate

refugia may facilitate an adjusted mode of speciation for example mostly allopatric speciation or ecological speciation and in some cases peripatric or adaptive radiation (Couvreur et al., 2021; Cox et al., 2014).

Though altitude was found slightly significant for *Bulinus* taxa (Tabo et al., 2022), it was generally of low value to the IH species distribution. Altitudinal gradients are associated with variations in climate and other parameters that are often linked to drivers of speciation and species extinction (Kohler et al., 2014; Rahbek et al., 2019). Given the observed high rate of altitudinal range shifts for the species as a whole as a result of climate change (Chen et al., 2011) the finding of lesser significance of altitude (Tabo et al., 2022) appears a little bit implausible. A benefit of the doubt however should be attached to the limited geographic scope range the machine learning prediction model was subjected to, warranting errors. Moreover, altitude as a factor has been found significant in other studies but on a large scale (Marcogliese, 2008). This shows that, in addition to monitoring the ongoing altitudinal range niche alterations, it is also important to look into the dynamics of the evolutionary adaptation to high altitudes, which were previously thought to be unfavorable.

In the follow-up investigation expanding the sampling across a wide altitudinal range, a number of obscure and unique species have been found in high-altitude ecosystems including the Lesotho Highlands and the region close to Mount Elgon's summit (Mahulu et al., 2019; Tumwebaze et al., 2022) as well as in Lake Victoria (Chibwana et al., 2020). The existence of these high-altitude genetically distant species can be attributed to the isolation of these species due to geography and/or ecology, leading to an allopatric diversification that may be related to the harsh climate at these elevations. In order to avoid issues with the biodiversity taxonomy and subsequent inconsistency in biodiversity conservation and management (Delić, et al., 2017; Fišer et al., 2018), more research accompanied by formal species descriptions and nomenclature should be done. Besides, the species endemism for the tarns in high mountains and highlands is not yet fully conceived though these may be potential candidates for species endemism (Oliver et al., 2017). High elevations of mountains and highlands are very sensitive to climate change (Colwell & Rangel, 2010; Trew & Maclean, 2021) due to direct exposure to the climate effects making such species vulnerable to extinction. It may also be appropriate to consider the idea of an evolutionary trap that limits gene flow and genetic drift customized to the species-specific life-history features.

One of the most important findings of the study about the *Bulinus* species is the expansion of the elevation distribution threshold to roughly 4000m a.s.l. These were species of the *Bulinus truncatus/tropicus* complex which is not completely unheard of for these species' higher elevations distribution. *Bulinus octoploidus, Bulinus hexaploidus,* and *Bulinus permembranaceus* are all high mountain species (Ethiopia and Kenya) (Brown, 1994). The oscillations between dry and wet cycles associated with the Holocene climate, as well as the future predictions (Martinez-Meyer, 2005; Rodó et al., 2013) will most likely favor the expansion of *B. truncatus/tropicus* complex species distribution. The polyploidy of the *Bulinus truncatus/tropicus* group, which is regarded to be an adaptation to high elevations, is one striking feature that sets this group apart from the others. Basically, the higher the polyploidy status, the higher the altitude distribution of the species (Brown & Wright, 1972; Brown, 1994). Further research should be done to understand the significance of polyploidization in high altitude evolution and diversity in relation to the interplay between the biology of the snails and their environment. Based on the well-supported current phylogeny (Tumwebaze et al., 2022), *Bulinus forskalii* group species emerged ancestral to all the other three groups of *Bulinus* snails. Their MRCA were shown to have lived in low to intermediate altitudes (0–2,000 m). Investigating the life history traits of this group may be ideal in understanding the evolutionary relationships and trend with the rest of the *Bulinus* groups and the subsequent high altitude evolution.

With a focus on evolutionary dynamics, species endemism, dispersal, and colonization history, the phylogeographic patterns of the two genera *Bulinus* and *Galba* in the various habitat types were explored. The *Bulinus tropicus* species in the crater lakes showed a high haplotype diversity (Tumwebaze et al., 2019) compared to the species diversity in Lake Victoria, a much larger ecosystem (Chibwana et al., 2022). Given the young age of the crater lakes (8,000–10,000) (Schumann et al., 2015) and the generally low *cox1* gene mutation rate, the observed species diversity may not be a result of intralacustrine speciation (Wilke et al., 2009). Rather, a high influx of haplotypes from the numerous nearby big lake systems such as Lake Victoria through passive means by humans and/or animals, likely birds, in a form of a stochastic colonization process is implicated. Thus, the intermediate hosts species richness in Lake Victoria is higher due to the active supply provided by the numerous and significant rivers (Yin & Nicholson, 1998).

The characteristics of the habitat can be responsible for the disparity in species richness amongst the various ecosystems (Brown et al., 2007). For example, *B. truncatus* is rare in crater lakes but is abundant in Lake Victoria, where they are primarily associated with shallow open waters, rocks, and sand beaches (Chibwana et al., 2020). These conditions are not present in the crater lakes, which are typically deep and with steep slopes (Rumes et al., 2011). Furthermore, climate variation especially temperature and human activities, together with other confounding environmental factors, have significantly impacted freshwater ecosystems in diverse ways (Marchant & Lane, 2014; Sirami et al., 2017). Calamities due to climate change such as the prolonged drought of freshwaters pose different stresses on the ecosystem's macrobenthic communities. For instance, complete desiccation has occurred to Lake Victoria (Salzburger et al., 2014; Stager et al., 2008) and crater lakes as seen from the history of satellite images due to prolonged drought and is expected to have had an effect on the macroinvertebrate population.

Other external factors such as population density, and the accompanying land use intensity, and limnological factors were found to have little or no impact on the distribution of IH in the crater lakes although they have been found significant elsewhere (Olkeba et al., 2020). The model predicted that the farther away from the large lake, the more likely it was that *Schistosoma* IH snails would be encountered in the particular crater lake (Tabo et al., 2022). Although this is a little counterintuitive, it could be because water basins that are relatively far away, like Lake Victoria, are also the ones that have a high potential for serving as source pool. It was determined that a haplotype from the Ndali-Kasenda crater field was the ancestor of the remaining *Bulinus tropicus* individuals, while a large number of distinct haplotypes were also present in this crater lake field (Tumwebaze et al., 2019). This region is also where tourism is concentrated because of national parks, hence a high population pressure. Human population geographical migration to high altitudes in attraction to resources, favorable environment for comfort and support of livelihoods is a problem on a global scale and has also been noted in the crater lakes region. This is as a result of environmental changes brought on by climate change, as well as population expansion/growth (Gelorini et al., 2012; Hongtao & Ting, 2021). The effect of population pressure on Lake Victoria due to fishing related activities, poor management/disposal of municipal effluents, and other human influences have already been observed (Verschuren et al., 2002; Sayer et al., 2018). In Lake Victoria, the potential intermediate hosts of the *Bulinus africanus* group were found associated with marshes on islands and shorelines, habitat enhanced by water hyacinth (Plummer, 2005). Although the population pressure model tested on the crater lakes showed little effect on IH distribution (Tabo et al., 2022), consequences on the IH species population dynamics and disease epidemiology should be monitored. In the case of Mount Elgon,

the human settlement was found up to 1200 meters. However, the high altitude regions face less anthropogenic pressure compared to low altitudes due to the stiff terrane that does not favor human settlement.

According to the molecular clock estimates, the diversification of *Bulinus* snails from their most recent common ancestor (MRCA) began in the early Miocene with the *Bulinus forskalii* group, and continued through the Plio-Pleistocene to produce the individual species complexes (Mahulu et al., 2019; Tumwebaze et al., 2022). It should be noted here that the paleoclimate in tropical Africa at the time coincides with this temporal diversification (Gasse, 2006; Jones et al., 2001). It was also discovered that the most recent common ancestor had resided in all altitudinal ranges, from low to high altitudes. The colonization of the afromontane for both genera took place during the Pleistocene and Miocene epochs. Therefore, the hypothesis of species range dispersal by humans as it may be the case for the Anthropocene epoch is minimized and skewed more towards other agents such as birds as has been proposed on several occasions (Clewing et al., 2013). Dispersal between mountains and highlands as 'sky islands' facilitated by the habitat corridors (the low-lying land masses) has been reported for terrestrial species (Mairal et al., 2021; 2017), but this does not explain the case of Mount Elgon and Lesotho lineages given their reconstructed evolutionary ages. It would imply an ancient immigration followed by in-situ speciation, also known as vicariant speciation, thus an evolutionary trap scenario (Schlaepfer et al., 2002). However, the volcanic and glaciation history in several highlands such as Mt. Elgon (Hamilton & Perrott, 1978) postdates the reconstructed colonization periods. This is further supported by another case in which the palaeontological history of the *Bulinus* species endemic to Madagascar (Tumwebaze et al., 2022) postdates the formation of the island (Masters et al., 2021). *Galba*'s long-distance spread from Europe is only loosely projected (Mahulu et al. 2019), but it is difficult to pinpoint the origin of the *Bulinus* species/lineages of Lesotho and Mt. Elgon due to the absence of close relatives. Additionally, *Galba* species have a more global distribution and are not restricted in either tropical or temperate regions. The effect of factors such as climate change on these species may be difficult to test compared to *Bulinus* species. In both cases, however, the species sampling is certainly not exhaustive and conclusions regarding the isolation should be done with caution.

1.5.3. Evaluation of the risk of schistosomiasis in comparison to fascioliasis

The crater lakes region as the primary study area was dominated by *Bulinus tropicus*, a species important for livestock schistosomiasis but refractory to the human infective varieties (Brown, 1994; Madsen, 2017). The human important *Schistosoma* IHs were evident throughout the rest of the study region. *Bulinus globosus* species, the initially and probably the most important intermediate hosts for *S. haematobium* in Africa (Brown, 1994; Mandahl-Barth, 1965), though rare in the crater lakes were found in Lake Kyaninga, one of the lakes at the highest altitude in the Fort Portal crater lake field and predominated Mount Elgon's lower elevations. This strengthens the theory of a possible local infection, as demonstrated by Lachish et al. (2013) and explains the epidemiological shift/extension of *Schistosoma* infections in that region to high altitudes (Rubaihayo et al., 2008). Although genetic drift frequently affects its population structure (Njiokou et al., 1994), *B. globosus* species seem to prefer comparatively cold environments (Manyangadze et al., 2016). Despite *B. globosus* being the most widely distributed of all the species in the *B. africanus* group (Brown, 1994), it was found that *B. ugandae* significant for livestock schistosomiasis rather than the human-infective *S. haematobium* parasites (Stothard et al., 2017), predominated in Lake Victoria. However, the distinction between *B. ugandae* and *B. globosus* species is still ambiguous, and the existence of the latter in Lake Victoria, and consequently a high

risk of urogenital schistosomiasis cannot be discounted (Zhang et al., 2022). Some species in the in *Bulinus truncatus/tropicus* complex such as *Bulinus tropicus*, and the *Bulinus* species found in the high altitudes, that is, *B. permembranaceus* and *B. hexaploidus* are so far not known to be susceptible to human infective schistosomes. However, these are important for livestock schistosomiasis for instance (Brown, 1994) which depicts still the disease burden particularly in the crater lakes region. Although the problem of urogenital schistosomiasis does not seem apparent in the crater lakes region based on the potential IH species distribution and the entire region of Uganda as earlier reported by Adriko et al. (2018), the surrounding great lakes system is reported to possess potential intermediate hosts of *S. haematobium* for urogenital schistosomiasis in humans (Nalugwa et al., 2010). In addition, the immediate surrounding regions such as DRC and Tanzania are faced with the problem of urogenital more than intestinal schistosomiasis (Brooker et al., 2001; Madinga et al., 2015). Therefore, it might only be a matter of time before the disease spreads to the area.

Overall, co-existance of all the three genera (*Bulinus, Biomphalaria* and *Galba* or *Radix*) was common across the study area. This overlapping distribution increases the chances of co-infection, thus aggravating the disease effects and the control (Ojo et al., 2021). All the species of *Biomphalaria* snails are potential IHs for human infective *Schistosoma mansoni* (Brown, 1994). Moreover, *Biomphalaria* snails were the most dominant implying that the intestinal form of schistosomiasis is more prevalent in this region. When screened for schistosomes, the cryptic species of Mt. Elgon did not show any proof of infection; however, this warrants additional examination. The population dynamics as well as the vulnerability to infection of the IHs, however, may be impacted by the changes in environmental factors brought on by climate change (Erkano, 2021). For instance, whereas increase of temperatures in high altitudes may affect the gastropod species adaptation to cold climates, this might increase the problem of trematodes originally thermally inhibited by cold temperatures for example the schistosomes (Kalinda et al., 2017). As a result, a shift in the endemism of trematodiases is anticipated, with an increase in high altitudes where the diseases are less expected and a likely disappearance from lower altitudes (Mas-Coma et al., 2009; Stensgaard et al., 2019).

Trematodiases in animals, such as livestock and wildlife, have long been disregarded because it was believed that they did not directly endanger human health. Zoonotic diseases such as schistosomiasis are however a major problem especially in developing countries (Bidaisee et al., 2014). There is a growing interest in these health issues and their repercussions facilitated by the development of sophisticated diagnostic and analytical methods, with some diseases—such as fascioliasis—now being recognized as zoonotic diseases, in contrast to earlier times (Dinnik & Dinnik, 1957; Robinson & Dalton, 2009). In this study, *Radix natalensis* the intermediate host for *Fasciola gigantica* that causes livestock fascioliasis dominated in the crater lakes region (Tabo et al., 2022). *Galba mweruensis* an intermediate host for both *Fasciola gigantica* and *Fasciola hepatica* that causes the disease in both livestock and humans was identified in the highland regions of Sub-Saharan Africa such as Usambara mountains, Mt. Elgon, Ethiopia, Lesotho and Kenya (Mahulu et al., 2019). The crater lakes region case study showed a high abundance of *Bulinus tropicus* a major intermediate host for livestock *Schistosoma* infective species *S. margrebowiei, S. bovis, S. curassoni* and *Calicophoron microbothrium* (Brown, 1994; Southgate et al., 1985a,b). Hybridization and introgression between livestock and human infective *Schistosoma* species such as *S. bovis* and *S. haematobium* are considered potential drivers of a zoonotic problem (Borlase et al., 2021) necessitating a one health approach (Gower et al., 2017). Hybridization is linked to alterations in the genetic diversity of the trematodes (Huyse et al., 2009; Teukeng et al., 2022) and so to the compatibility/specificity of infectivity for both the intermediate hosts and the definitive hosts (Webster et al., 2013).

1.6. Recommendation and Concluding remarks

Freshwater resources and water-borne diseases are vitally important subjects that relate to all people living in Africa. Basic research in freshwater-borne diseases especially under global change scenarios is therefore of utmost importance. The transferability of theoretical and empirical evolutionary and ecological research to applied (site-specific) schistosomiasis mitigation measures not only benefit local people, but the economy nationwide, for it traditionally has depended on aquatic resources in a variety of ways, e.g. as a source of animal protein to the population, and as a source of water for domestic purpose. Using these vital sources, however, should be risk-free, i.e. without obtaining the notorious disease schistosomiasis or fascioliasis. Since most of the studies done about schistosomiasis have been based on epidemiological studies in the definitive hosts and others by using models, a field study in the natural environment helps to understand the nature of the origin of schistosomiasis and so can help health workers and policymakers in making informed decisions about how the disease can be controlled. A number of recommendations are given as a result of the current findings.

First of all, epidemiological surveys and regular monitoring should be conducted regularly to prevent the disease spread and potential outbreaks in some areas not yet affected, given the evident presence of the potential intermediate hosts throughout the study region. The crater lakes region is an important tourist center in Uganda with one of the famous National Parks (Queen Elizabeth) and cases such as the outbreak of infection in travelers to one of the crater lakes, Nyinambuga and the accompanying consequences should be avoided. Therefore, the stakeholders should exercise great caution for conservation purposes and to safeguard not just the local population but also wildlife and livestock, thereby safeguarding the tourism sector and the long-term viability of the local community. More research to understand the evolutionary dynamics, specificity and sensitivity of the IHs to *Schistosoma* should be enhanced. The biological elements of polyploidization and hybridization, as well as the compatibility changes that result from these processes are crucial and so should be further investigated. Molecular evaluation of the available type species (morphospecies) of the genus to substantiate the mitochondrial lineages could further help to substantiate the real diversity of *Bulinus* snails so as to have a stable and sustainable *Bulinus* taxonomy.

On the other hand, the perspective of the initially perceived Pan-African intermediate snail host distribution for trematodes that cause fascioliasis an important disease for livestock but also for humans is now slightly changed. *Galba mweruensis* instead of *Galba truncatula* was more prevalent especially in the high altitudes. Although the species has been shown to transmit both *F. hepatica* and *F. gigantica*, this status is not yet ascertained and requires further investigation by for instance applying xenomonitoring approaches such as Multiplex-PCR based screening, likewise to schistosomiasis and other trematodiases across all freshwater ecosystems. Moreover, the distinctness of *Galba* and *Bulinus* species in highland ranges especially those defined as extreme environments should be investigated for their adaptation to such environments, even for other species that have not been studied. Lastly, the importance of the suitable ecological conditions for the distribution of the intermediate hosts as observed in this study is an indication that the ecosystem environment should also be put into consideration in the strategies for controlling the waterborne trematodiases, although the strategies should be sustainable ones considering the overall ecosystem functions. A combination of predictor variables and species occurences in the machine learning approach should be spatially extended to help in prediction of disease risks and gain more insights into IHs-parasite dynamics at the continental scale.

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2. Publications

2.1. Molecular identification of *Bulinus* spp. intermediate host snails of *Schistosoma* spp. in crater lakes of western Uganda with implications for the transmission of the *Schistosoma haematobium* group parasites

Parasites & Vectors

RESEARCH Open Access

Molecular identification of *Bulinus* spp. intermediate host snails of *Schistosoma* spp. in crater lakes of western Uganda with implications for the transmission of the *Schistosoma haematobium* group parasites

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Abstract

Background: Human schistosomiasis is the second most important tropical disease and occurs in two forms in Africa (intestinal and urogenital) caused by the digenetic trematodes *Schistosoma mansoni* and *Schistosoma haematobium*, respectively. A proposed recent shift of schistosomiasis above a previously established altitudinal threshold of 1400 m above sea level in western Ugandan crater lakes has triggered more research interest there.

Methods: Based on extensive field sampling in western Uganda and beyond and employing an approach using sequences of the mitochondrial barcoding gene cytochrome *c* oxidase subunit 1 (*cox*1) this study aims were: (i) identification and establishment of the phylogenetic affinities of *Bulinus* species as potential hosts for *Schistosoma* spp.; (ii) determining diversity, frequency and distribution patterns of *Bulinus* spp.; and (iii) establishing genetic variability and phylogeographical patterns using Bayesian inference and parsimony network analyses.

Results: Out of the 58 crater lakes surveyed, three species of *Bulinus* snails were found in 34 crater lakes. *Bulinus tropicus* was dominating, *Bulinus forskalii* was found in two lakes and *Bulinus truncatus* in one. The latter two species are unconfirmed potential hosts for *S. haematobium* in this region. However, *Bulinus tropicus* is an important species for schistosomiasis transmission in ruminants. *Bulinus tropicus* comprised 31 haplotypes while both *B. forskalii* and *B. truncatus* exhibited only a single haplotype in the crater lakes. All species clustered with most of the haplotypes from surrounding lake systems forming source regions for the colonization of the crater lakes.

Conclusions: This first detailed malacological study of the crater lakes systems in western Uganda revealed presence of *Bulinus* species that are either not known or not regionally known to be hosts for *S. haematobium*, the causing agent of human urogenital schistosomiasis. Though this disease risk is almost negligible, the observed dominance of *B. tropicus* in the crater lakes shows that there is a likelihood of a high risk of infections with *Schistosoma bovis*. Thus, extra attention should be accorded to safeguard wild and domestic ruminants in this region as the population benefits from these animals.

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Keywords: *Bulinus forskalii*, *Bulinus tropicus*, *Bulinus truncatus*, *Schistosoma haematobium*, *Schistosoma bovis*, Neglected tropical disease, Schistosomiasis surveillance

Background

Schistosomiasis is an important tropical disease especially in sub-Saharan Africa, with more than 90% of the disease burden [1] and the second most important public health disease after malaria [1, 2]. Schistosomiasis is a parasitic disease transmitted by planorbid gastropods. Human schistosomiasis in Africa occurs in two forms (intestinal and urogenital), caused by the digenetic trematodes *Schistosoma mansoni* and *Schistosoma haematobium*, respectively. Urogenital schistosomiasis accounts officially for two-thirds of all cases [3], a figure that might be too optimistic as the real prevalence of the disease is potentially underestimated by a factor of three [4]. The already important direct impact of urogenital schistosomiasis is worsened by its established role in cancer epidemics and AIDS epidemics in Africa (reviewed in [5]), besides the long recognized roles in other pathologies and diseases such as haematuria and female genital schistosomiasis (reviewed in [6]). Interestingly, *S. haematobium* is the least studied of the major human schistosomes [7, 8].

Unlike in many other regions of sub-Saharan Africa, in Uganda, intestinal rather than urogenital schistosomiasis is considered a major public health problem [9]. Urogenital schistosomiasis, though present has long been assumed to be restricted to a few areas of eastern and northern Uganda [10]. Schistosomiasis studies in Uganda have so far been focused on regional intestinal schistosomiasis [11–14] around the great lake systems of Lake Victoria and Lake Albert. A negligible amount of studies have been conducted on urogenital schistosomiasis and *S. haematobium* and their planorbid host snails belonging to the genus *Bulinus*. Today there is no official declaration about any region of Uganda to be completely free of urogenital schistosomiasis. Thus, the status of urogenital schistosomiasis remains an enigma in Uganda.

A recent study [15] has indicated that intestinal schistosomiasis actually occurs above an earlier designated threshold of 1400 m above sea level (a.s.l.), specifically in crater lakes in western Uganda. For instance, a high rate of intestinal schistosomiasis in travelers after a brief exposure to the high-altitude crater Lake Nyinambuga was reported in 2012 [16]. The Albertine Rift valley region of western Uganda is dominated by mainly two types of freshwater bodies; the three great lakes Albert, Edward, George and about 90 small crater lakes of

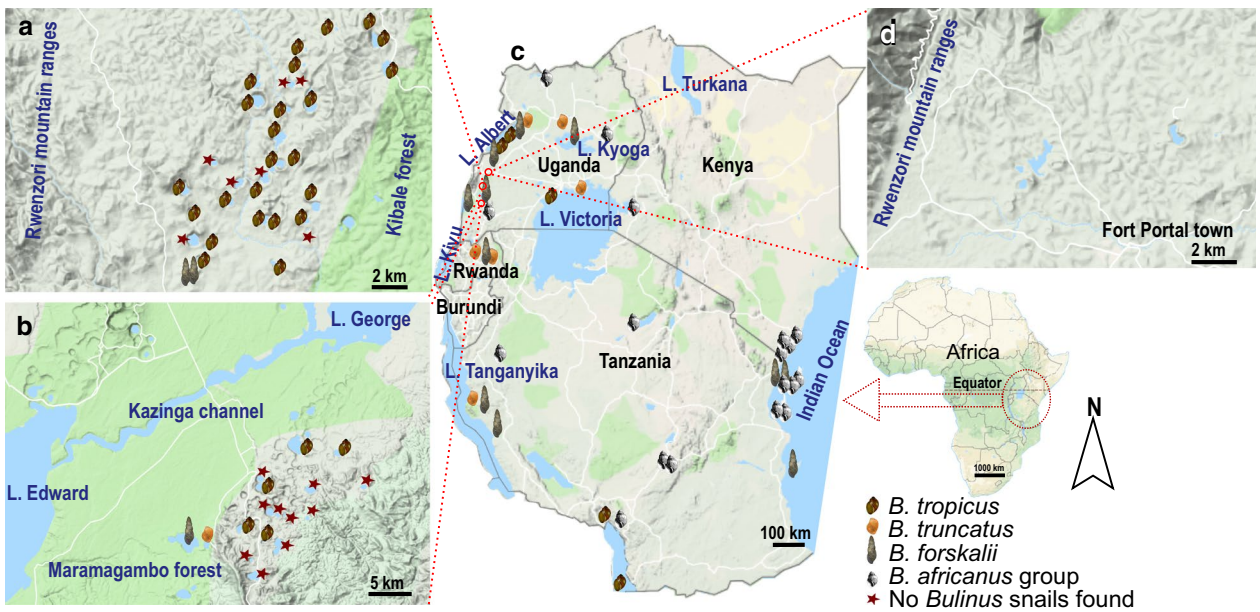


Fig. 1 Sampling sites in the three crater lakes fields in Uganda and at the supra-regional scale. **a** Ndali-Kasenda crater lakes. **b** Bunyaruguru crater lakes. **c** Populations from East Africa. **d** Fort Portal crater lakes. Snail symbols indicate localities of *Bulinus* populations studied, whereas a star indicates when sampling did not yield a *Bulinus* population in the respective crater lake. Note that data for some populations used were retrieved from GenBank (see Table 1)

varying sizes scattered throughout the region. The crater lakes have originally been divided into four geographical fields of Fort Portal, Ndali-Kasenda, Katwe-Kikorongo and Bunyaruguru [17] (see Fig. 1). They straddle the equator between and are spread along the regional rift valley gradient from 914 m to 1566 m elevation [18], with varying limnological characteristics [19] and climatic gradient. The region is one of the most densely populated rural areas in sub-Saharan Africa [20] and is also a tourist destination, attracting local and international travelers.

The potential of urogenital schistosomiasis in Uganda has been neglected, despite the fact that the disease is common in regions nearby such as the Democratic Republic of the Congo (DRC) [21], Tanzania [22] and South Sudan [23]. It has recently been shown that targeting the schistosome intermediate hosts is the most effective of all elimination strategies combating the burden of schistosomiasis [24]. A first step in targeting regional transmission foci is the correct identification of the intermediate hosts [25]. This is particularly true for the *Bulinus* spp./*Schistosoma haematobium* system, since *Bulinus* is a very diverse freshwater gastropod genus of currently recognized 37 species belonging to four species complexes that are morphologically variable [26, 27]. Although there are still some taxonomic issues involved, it has repeatedly been shown that these species complexes can be identified using molecular genetic tools [28–31]. Three of the species complexes are known to occur in regions along the Albertine Rift, namely the *B. truncatus/B. tropicus* complex, the *B. forskalii* group and the *B. africanus* group [32]. These regions are potentially species source pools for the crater lakes. Given that these groups include potential hosts for *S. haematobium*, it is important to survey the crater lakes region for snails transmitting human urogenital schistosomiasis. Therefore, enhanced simultaneous mapping and monitoring of strains of urogenital schistosomiasis and their intermediate hosts populations are both necessary to control this disease in areas where it has not been known to occur before. Very little information, however, exists on the mollusc fauna of the crater lakes [33]. This necessitates an assessment of the status of potential host snail species and thus urogenital schistosomiasis in the region.

Based on extensive field sampling in the crater field region and beyond and employing an approach using sequences of the barcoding gene of mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) this study aims are: (i) identification and establishment of the phylogenetic affinities of *Bulinus* species as potential hosts for *Schistosoma* spp.; (ii) determining diversity, frequency and distribution patterns of *Bulinus* spp.; and (iii) establishing genetic variability and phylogeographical patterns.

Methods

Study area

This study was conducted in lakes of the three main crater fields in Uganda, between Fort Portal region in the north, Ndali-Kasenda in the middle and Bunyaruguru in the south (Fig. 1). The region is bordered by the vast Rwenzori Mountains in the north-west, the southern shores of Lake Albert in the north and the region of the Queen-Elizabeth National Park (Lake Edward-George) in the south. Most of the crater lakes were formed by faulting and volcanic eruption some 8000 to 10,000 years ago [34]. Bunyaruguru lakes lie on the southern side of the Edward-George system while the rest are located on its northern side. The climate, hydrology, water chemistry and landscape settings are highly heterogeneous between crater fields. Lakes in Fort Portal crater field lie at higher altitude (above 1500 m a.s.l), than those in the Ndali-Kasenda crater field and Bunyaruguru. Lake Kyaninga (Fig. 2a) is one of the deepest (220 m) known crater lake in western Uganda [35], although the crater lakes are generally shallow. Some of the lakes are embedded in a still rather natural setting whereas the surroundings of many of the lakes studied are highly disturbed by anthropogenic activities. The lakes are commonly exploited as a water source for humans and livestock consumption (Fig. 2). Furthermore, these lakes are also a main source of food for the local communities using them for fishing. Some of the fish species are introduced. For example, *Tilapia zillii*, *Orechromis leucosticus* and *Poecilia reticulata* were introduced in Lake Nkuruba [36].

Sampling

Out of 58 crater lakes sampled, 19 were from Bunyaruguru, 33 from Ndali-Kasenda and the 6 remaining from Fort Portal crater field (Table 1). We purposively selected these lakes to cover a range from low altitude (1033 m a.s.l) to high altitude (1569 m a.s.l). The selection of a lake and/or sampling site was based on representation of lake field size, lake size class, utilization and lake type as well as accessibility. Lakes of Katwe-Kikorongo field are known to be mainly saline [19] and were therefore not included in this study.

Since the access to the crater lakes is often made difficult by their steep escarpments, we collected snails from one to two localities per crater lake. We used scoop netting and/or dredging sampling techniques to capture snails along the edges of the access point of the lake. A maximum of 40 min sampling time per lake was used. Sampling also involved visual inspection of shoreline vegetation and hand-picking of snails. Samples were derived from depths down to a maximum

Fig. 2 Impressions from selected crater lakes in western Uganda. **a** Lake Kyaninga (Fort Portal). **b** Lake Nyinambuga (Ndali-Kasenda). **c** Lake Ekikoto (Fort Portal). **d** Lake Ntambi (Ndali-Kasenda). **e** Lake Nyamugosani (Ndali-Kasenda). **f** Lake Kayihara (Fort Portal). **g** Lake Kako (Bunyaruguru) (Photo credit: C. Albrecht (**a**, **c**, **f**); D. Engelhardt (**b**); C. Dusabe (**d**, **e**); I. Tumwebaze (**g**))

of 1.5 m, covering all major habitat types present. The collected snails were fixed in 80% ethanol and stored at −20 °C for subsequent genetic analyses.

DNA isolation, amplification and sequencing

Prior to DNA isolation, we photographed all specimens with a digital microscope system (KEYENCE VHX-2000; Keyence Deutschland GmbH, Neu-Isenburg, Germany). Genomic DNA was isolated using the CTAB method of DNA extraction [37]. In a few cases, DNA was isolated using DNeasy Blood & Tissue Kit (Qiagen, Mississauga, ON, Canada) following the provided instructions. A fragment of the mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) with a target length of 655 bp was amplified using the Folmer region primers LCO1490 [38] and COR722B [39]. PCR reactions were run according to Albrecht et al. [37]. Sanger DNA sequencing was performed on an ABI 3730xl DNA analyzer using the BigDye Terminator Kit (Life Technologies, LGC Genomics GmbH, Berlin, Germany). Vouchers (shells and DNA) are deposited in the University of Giessen Systematics and Biodiversity collection (UGSB, [40]).

Table 1 Summary on localities and haplotypes found in the crater lakes and other regions studied

Crater field	Location	Lake code	Coordinates		Altitude (m)	Species	No. of haplotypes[a]	Specimen code[a]	Specimen voucher	Prep. no.	GenBank ID [reference]
			Latitude	Longitude							
Bunyaruguru	Lake Bugwagyi, Uganda	BGJ	−0.19680°N	30.18663°E	1070	*B. tropicus*	1	BGJ1.1	UGSB 21302	25644	MN551518
						B. tropicus		BGJ1.2	UGSB 21303	25645	MN551519
	Lake Chema[b], Uganda		−0.25229°N	30.11444°E	1268	–			–	–	–
	Lake Kabarogyi[b], Uganda		−0.22692°N	30.21375°E	1352	–			–	–	–
	Lake Kako[b], Uganda		−0.30484°N	30.09728°E	1405	–			–	–	–
	Lake Kamunzuku[b], Uganda		−0.26422°N	30.15410°E	1272	–			–	–	–
	Lake Kamweru[b], Uganda		−0.26023°N	30.12223°E	1270	–			–	–	–
	Lake Kariya[b], Uganda		−0.23010°N	30.16624°E	1256	–			–	–	–
	Lake Kasiriya[b], Uganda		−0.25673°N	30.13180°E	1265	–			–	–	–
	Lake Katinda[b], Uganda		−0.22237°N	30.10978°E	1033	–			–	–	–
	Lake Kigezi, Uganda	KGZ	−0.28589°N	30.11084°E	1323	*B. tropicus*	1	KGZ1.1	UGSB 21308	25648	MN551520
						B. tropicus		KGZ1.2	UGSB 21309	25649	MN551521
	Lake Kyamwiga, Uganda	KMG	−0.19196°N	30.15009°E	1035	*B. tropicus*	1	KMG1.1	UGSB 21296	25640	MN551516
						B. tropicus		KMG1.2	UGSB 21297	25641	MN551517
	Lake Kyasanduka, Uganda		−0.28745°N	30.04731°E	1016	*B. truncatus*			UGSB 23628	27178	MN551575
						B. truncatus			UGSB 23629	27179	MN551576
	Lake Mafuro, Uganda	MFR	−0.26749°N	30.10385°E	1279	*B. tropicus*	3	MFR1.1	UGSB 21287	25634	MN551510
						B. tropicus		MFR1.2	UGSB 21288	25635	MN551511
						B. tropicus		MFR2.1			HQ121571 [32]
	Lake Mugogo[b], Uganda		−0.28262°N	30.12591°E	1323					–	
	Lake Murambi, Uganda	MRM	−0.22512°N	30.10789°E	1079	*B. tropicus*		MRM1.1	UGSB 21290	25636	MN551512
						B. tropicus		MRM1.2	UGSB 21291	25637	MN551513
	Lake Nyamusingire[b], Uganda		−0.28455°N	30.03994°E	985	–			–	–	–
	Lake Nkugute[b], Uganda		−0.32068°N	30.10341°E	1404	–			–	–	–
	Lake Nyungu, Uganda	NGU	−0.25500°N	30.09524°E	1196	*B. tropicus*	2	NGU1.1	UGSB 21293	25638	MN551513
						B. tropicus		NGU1.2	UGSB 21294	25639	MN551515
						B. tropicus		NGU2.1			HQ121571 [32]
	Lake Rwijongo[c], Uganda	RWG	−0.27122°N	30.08909°E	1262	*B. tropicus*	2	RWG2.2			HQ121570 [32]
						B. tropicus		RWG2.3			HQ121571 [32]
Ndali Kasenda	Lake Kanyabuterere[b], Uganda		0.41485°N	30.28823°E	1201	–			–	–	–
	Lake Kanyamukali, Uganda	KML	0.40699°N	30.23669°E	1167	*B. tropicus*	2	KML1.1	UGSB 21434	25839	MN551526
						B. tropicus		KML1.2	UGSB 21435	25840	MN551527
						B. tropicus		KML1.3	UGSB 16767	22523	MN551503

Table 1 (continued)

Crater field	Location	Lake code	Coordinates		Altitude (m)	Species	No. of haplotypes[a]	Specimen code[a]	Specimen voucher	Prep. no.	GenBank ID [reference]
			Latitude	Longitude							
						B. tropicus		KML1.4	UGSB 16768	22524	MN551504
						B. tropicus		KML1.5	UGSB 16853	22571	MN551508
	Lake Kanyangwe, Uganda	KAG	0.45041°N	30.27586°E	1280	*B. tropicus*	3	KAG1.1	UGSB 22111	26335	MN551554
						B. tropicus		KAG1.2	UGSB 22112	26336	MN551555
						B. tropicus		KAG2.1			HQ121576 [32]
	Lake Kasenda, Uganda	KSD	0.43285°N	30.29179°E	1248	*B. tropicus*	5	KSD1.1	UGSB 21431	25837	MN551524
						B. tropicus		KSD1.2	UGSB 21432	25838	MN551525
						B. tropicus		KSD2.1			HQ121577 [32]
						B. tropicus		KSD2.2			HQ121578 [32]
						B. tropicus		KSD2.3			HQ121579 [32]
	Lake Kibungo, Uganda	KIG	0.39237°N	30.23338°E	1140	*B. forskalii*	1	KIG1.1	UGSB 22375	26620	MN551573
	Lake Kifuruka[b], Uganda		0.48912°N	30.28845°E	1407	–			–	–	–
	Lake Kitere, Uganda	KTR	0.39731°N	30.27346°E	1144	*B. tropicus*	2	KTR1.1	UGSB 21570	25956	MN551541
						B. tropicus		KTR1.2	UGSB 21571	25957	MN551542
	Lake Kyanga, Uganda	KYG	0.40022°N	30.23221°E	1167	*B. tropicus*	2	KYG1.1	UGSB 21455	25853	MN551532
						B. tropicus		KYG1.2	UGSB 21456	25854	MN551533
	Lake Lugembe, Uganda	LGB	0.44722°N	30.28123°E	1298	*B. tropicus*	1	LGB1.1	UGSB 22123	26343	MN551560
						B. tropicus		LGB1.2	UGSB 22124	26344	MN551561
	Lake Lyantonde[b], Uganda		0.48662°N	30.28024°E	1404	–			–	–	–
	Lake Mirambi, Uganda	MRB	0.38902°N	30.22906°E	1144	*B. forskalii*	1	MRB1.1	UGSB 22120	26341	MN551559
						B. forskalii		MRB1.2	UGSB 22372	26618	MN551574
	Lake Mubiro[b], Uganda		0.44144°N	30.25545°E	1212	–			–	–	–
	Lake Muligamire, Uganda	MRR	0.42302°N	30.28884°E	1208	*B. tropicus*	1	MRR1.1	UGSB 21561	25950	MN551538
						B. tropicus		MRR1.2	UGSB 21562	25951	MN551539
	Lake Mwamba, Uganda	MBA	0.45746°N	30.27303°E	1307	*B. tropicus*	4	MBA1.1	UGSB 22093	26323	MN551547
						B. tropicus		MBA1.2	UGSB 22364	26610	MN551571
						B. tropicus		MBA1.3	UGSB 22365	26611	MN551570
						B. tropicus		MBA2.1			HQ121575 [32]
	Lake Mwengenyi, Uganda	MGY	0.48757°N	30.25972°E	1410	*B. tropicus*	2	MGY1.1	UGSB 22117	26339	MN551557
						B. tropicus		MGY1.2	UGSB 22118	26340	MN551558
	Lake Ndicho[b], Uganda		0.44525°N	30.26904°E	1272	–			–	–	–
	Lake Njarayabana, Uganda	NJN	0.42805°N	30.24747°E	1204	*B. tropicus*	2	NJN1.1	UGSB 22366	26612	MN551569
						B. tropicus		NJN1.2	UGSB 22367	26613	MN551568

Table 1 (continued)

Crater field	Location	Lake code	Coordinates		Altitude (m)	Species	No. of haplotypes[a]	Specimen code[a]	Specimen voucher	Prep. no.	GenBank ID [reference]
			Latitude	Longitude							
	Lake Nkuruba, Uganda	NRB	0.51720°N	30.30324°E	1517	*B. tropicus*	6	NRB1.1	UGSB 21549	25942	MN551534
						B. tropicus		NRB1.2	UGSB 21550	25943	MN551535
						B. tropicus		NRB1.3	UGSB 16761	22517	MN551501
						B. tropicus		NRB1.4	UGSB 16762	22518	MN551502
						B. tropicus		NRB2.1			HQ121568 [32]
						B. tropicus		NRB2.2			HQ121567 [32]
						B. tropicus		NRB2.3			HQ121566 [32]
						B. tropicus		NRB2.4			HQ121569 [32]
	Lake Ntambi[b], Uganda		0.40729°N	30.22946°E	1155	–			–	–	–
	Lake Ntanda/Katanda, Uganda	KTD	0.47775°N	30.26165°E	1341	*B. tropicus*	1	KTD1.1	UGSB 22108	26333	MN551552
						B. tropicus		KTD1.2	UGSB 22109	26334	MN551553
	Lake Nyabikere, Uganda	NKR	0.50101°N	30.32792°E	1389	*B. tropicus*	3	NKR1.1	UGSB 21440	25843	MN551528
						B. tropicus		NKR1.2	UGSB 21441	25844	MN551529
						B. tropicus		NKR2.1			HQ121572 [32]
						B. tropicus		NKR2.2			HQ121574 [32]
	Lake Nyahira, Uganda	NHR	0.49914°N	30.28737°E	1458	*B. tropicus*	1	NHR1.1	UGSB 21552	25944	MN551536
						B. tropicus		NHR1.2	UGSB 21553	25945	MN551537
	Lake Nyamirima, Uganda	NMM	0.52001°N	30.32035°E	1497	*B. tropicus*	2	NMM1.1	UGSB 21720	26080	MN551543
						B. tropicus		NMM1.2	UGSB 21721	26081	MN551544
						B. tropicus		NMM2.1			HQ121568 [32]
	Lake Nyamugosani, Uganda	NGS	0.42498°N	30.23139°E	1237	*B. tropicus*	2	NGS1.1	UGSB 21428	25835	MN551522
						B. tropicus		NGS1.2	UGSB 21429	25836	MN551523
						B. tropicus		NGS1.3	UGSB 22099	26327	MN551550
	Lake Nyamugoro[b], Uganda		0.44809°N	30.24233°E	1272	–			–	–	–
	Lake Nyamuteza, Uganda	NTZ	0.43509°N	30.22866°E	1261	*B. tropicus*	2	NTZ1.1	UGSB 21734	26089	MN551545
						B. tropicus		NTZ1.2	UGSB 21735	26090	MN551546
	Lake Nyanswiga, Uganda	NSG	0.50733°N	30.28825°E	1479	*B. tropicus*	1	NSG1.1	UGSB 21567	25954	MN551540
						B. tropicus		NSG1.2	UGSB 22359	26606	MN551572
	Lake Nyinabulita, Uganda	NBT	0.50760°N	30.32533°E	1424	*B. tropicus*	2	NBT1.1	UGSB 21446	25847	MN551530
						B. tropicus		NBT1.2	UGSB 21447	25848	MN551531
	Lake Nyinambuga, Uganda	NNG	0.48109°N	30.28773°E	1372	*B. tropicus*	3	NNG1.1	UGSB 22106	26332	MN551551
						B. tropicus		NNG1.2	UGSB 22368	26614	MN551567
						B. tropicus		NNG1.3	UGSB 22369	26615	MN551566

Table 1 (continued)

Crater field	Location	Lake code	Coordinates		Altitude (m)	Species	No. of haplotypes[a]	Specimen code[a]	Specimen voucher	Prep. no.	GenBank ID [reference]
			Latitude	Longitude							
						B. tropicus		NNG1.4	UGSB 17784	23160	MN551500
						B. tropicus		NNG2.1			HQ121573 [32]
	Lake Rukwanzi, Uganda	RKZ	0.47481°N	30.27925°E	1345	*B. tropicus*	1	RKZ1.1	UGSB 22115	26338	MN551556
						B. tropicus		RKZ1.2	UGSB 22370	26616	MN551565
						B. tropicus		RKZ1.3	UGSB 22371	26617	MN551564
	Lake Rwandakara, Uganda	RKR	0.41567°N	30.27054°E	1171	*B. tropicus*	2	RKR1.1	UGSB 22126	26345	MN551562
						B. tropicus		RKR1.2	UGSB 22127	26346	MN551563
	Lake Rwenjuba, Uganda	RJB	0.43922°N	30.26577°E	1257	*B. tropicus*	2	RJB1.1	UGSB 22096	26325	MN551548
						B. tropicus		RJB1.2	UGSB 22097	26326	MN551549
	Lake Wankenzi, Uganda	WKZ	0.41834°N	30.26326°E	1158	*B. tropicus*	3	WKZ1.1	UGSB 16770	22526	MN551505
						B. tropicus		WKZ1.2	UGSB 16839	22557	MN551507
						B. tropicus		WKZ1.3	UGSB 16854	22572	MN551509
Fort Portal	Lake Balama[b], Uganda		0.67959°N	30.23345°E	1566				–	–	–
	Lake Ekikooto[b], Uganda		0.70142°N	30.31342°E	1536				–	–	–
	Lake Kayihara[b], Uganda		0.70256°N	30.31590°E	1549				–	–	–
	Lake Kyaninga, Uganda		0.70070°N	30.29919°E	1553				–	–	–
	Lake Saaka[b], Uganda		0.68700°N	30.23954°E	1569				–	–	–
	Lake Wabikere[b], Uganda		0.68826°N	30.23518°E	1550				–	–	–
Others (non crater lakes)	Angola, Bengo, Cabungo					*B. globosus*					LT671918 [81]
						B. globosus					LT671947 [81]
	Angola, Bengo, Burgalheira escola					*B. globosus*					LT671925 [81]
	Angola, Luanda, Candimba/ Muxima					*B. globosus*					LT671928 [81]
	Angola, Kwanza Norte, Cacuso					*B. globosus*					LT671936 [81]
	Angola, Luanda, Cassefo					*B. forskalii*					LT671955 [81]
	Angola, Luanda, Caquila					*B. forskalii*					LT671956 [81]
	Angola, Bengo, Porto mangueiras					*B. forskalii*					LT671950 [81]
						B. forskalii					LT671951 [81]
	Angola, Kwanza Norte, N'Dalatando					*B. forskalii*					LT671957 [81]
	Angola, Bengo, Icau Wando					*B. forskalii*					LT671966 [81]

Table 1 (continued)

Crater field	Location	Lake code	Coordinates		Altitude (m)	Species	No. of haplotypes[a]	Specimen code[a]	Specimen voucher	Prep. no.	GenBank ID [reference]
			Latitude	Longitude							
						B. forskalii					LT671949 [81]
	Angola, Malanje, Carlanga					*B. forskalii*					LT671961 [81]
						B. forskalii					LT671962 [81]
	Angola, Malanje, Calandula					*B. forskalii*					LT671964 [81]
	Angola, Quifangondo					*B. forskalii*					AM286306 [30]
	Cameroon, Peptonoum-west	CAM	5.63306°N	10.63528°E		*B. tropicus*	2	CAM1.1			KJ157495 [54]
						B. tropicus		CAM1.2			KJ157496 [54]
						B. tropicus					KJ157497 [54]
	Burkina Faso, Mogtedo barrage		12.30647°N	−0.82783°E		*B. forskalii*					AM286310 [30]
						B. globosus					AM286293 [30]
						B. truncatus					AM286315 [30]
	Cameroon, Peptonoum-east					*B. tropicus*					KJ157492 [54]
						B. tropicus					KJ157494 [54]
	Cameroon, Bertoua		4.58889°N	13.68111°E		*B. truncatus*					KJ135287 [54]
	Cameroon, Mourtous					*B. globosus*					KJ157471 [54]
	Cameroon, Yagoua					*B. globosus*					KJ157472 [54]
	Cameroon, Kaprissi					*B. globosus*					KJ157475 [54]
	Cameroon, Gounougou					*B. globosus*					KJ157473 [54]
	Cameroon, Ouroudoukoudje					*B. globosus*					KJ157474 [54]
	Cameroon, Kaele					*B. senegalensis*					KJ157481 [54]
						B. senegalensis					KJ157480 [54]
	Cameroon, Kekem					*B. truncatus*					KJ135289 [54]
	Cameroon, Mokolo					*B. truncatus*					KJ135291 [54]
	Cameroon, Loum					*B. truncatus*					KJ135295 [54]
	DR Congo, Lake Kivu					*B. truncatus*					HQ121561 [32]
	Egypt, Quena		26.17306°N	32.16611°E		*B. truncatus*					KJ135304 [54]
	Giza, Egypt		30.14139°N	31.07694°E		*B. truncatus*					KJ135300 [54]
	Iran, Khouzestan					*B. truncatus*					KT365867
	Kenya, Nimbodze					*B. nasutus nasutus*					AM921841 [30]
	Kenya, Kisumu, Kandaria dam					*B. globosus*					AM286286 [30]
	Kenya, Kinango					*B. globosus*					AM921844 [30]
	Kenya, Kachetu					*B. globosus*					AM921847 [30]

Table 1 (continued)

Crater field	Location	Lake code	Coordinates		Altitude (m)	Species	No. of haplotypes[a]	Specimen code[a]	Specimen voucher	Prep. no.	GenBank ID [reference]
			Latitude	Longitude							
	Kenya, Mwamduli					*B. globosus*					AM921850 [30]
	Malawi, Lake Malawi	LM				*B. nyassanus*	1	LM1.1			AM921838 [30]
						B. africanus					AM286296 [30]
	Niger, Satoni					*B. forskalii*					AM286308 [30]
						B. truncatus					AM286316 [30]
	Niger, Tonida					*B. globosus*					AM286294 [30]
						B. globosus					AM921808 [30]
	Nigeria, Ibaro, Ogun		7.15000°N	3.11667°E		*B. truncatus*					FN546781 [80]
	Nigeria, Ilie		8.25000°N	4.96667°E		*B. truncatus*					FN546797 [80]
						B. truncatus					FN546797 [80]
	Nigeria, Oshogbo		8.08333°N	4.66667°E		*B. truncatus*					FN546805 [80]
						B. truncatus					FN546805 [80]
	Nigeria, Oju Alaro					*B. globosus*					FN546815 [80]
	Nigeria, Ipogun					*B. globosus*					FN546814 [80]
	Nigeria, Imala, Odo					*B. truncatus*					FN546787 [80]
	Nigeria, Awuru					*B. truncatus*					FN546786 [80]
	Oman					*B. wrighti*					AM286318 [30]
	Rwanda, rice scheme dam lake		−1.28652°N	30.31509°E	1349	*Bulinus* sp.			UGSB 16778	22532	MN551579
						B. truncatus			UGSB 16777	22531	MN551578
	Rwanda, Lake Muhazi		−1.85912°N	30.49039°E	1452	*B. truncatus*			UGSB 16755	22511	MN551581
	Rwanda, Lake Muhazi		−1.84843°N	30.47826°E	1455	*B. truncatus*			UGSB 4936	22549	MN551580
	Sardinia, Posada		40.63487°N	9.67537°E		*B. truncatus*					AM286312 [30]
	Sao Tome and Principe, Sao Tome Island					*B. forskalii*					AM286305 [30]
	Senegal, Dakar					*B. forskalii*					AM286307 [30]
	Senegal, Diama		15.43611°N	16.23389°E		*B. truncatus*					KJ135306 [54]
	Senegal, Toukar					*B. senegalensis*					KJ157483 [54]
	Senegal, Diohine					*B. senegalensis*					KJ157484 [54]
						B. senegalensis					KJ157485 [54]
	Senegal, Poudaye					*B. senegalensis*					KJ157486 [54]
	South Africa, Lake Sibaya	SA				*B. natalensis*	1	SA1.1			AM286311 [30]
	South Africa, Pietermaritzburg					*B. globosus*					AM286289 [30]
						B. globosus					AM286290 [30]
	South Africa, Durban Isipingo					*B. africanus*					AM286295 [30]

Table 1 (continued)

Crater field	Location	Lake code	Coordinates		Altitude (m)	Species	No. of haplotypes[a]	Specimen code[a]	Specimen voucher	Prep. no.	GenBank ID [reference]
			Latitude	Longitude							
	Tanzania, Muyuni, Unguja		−6.37845°N	39.46415°E		*B. nasutus productus*					AM286299 [30]
	Tanzania, Njombe Kibena	TZ	−9.20382°N	34.78402°E		*B. tropicus*	1	TZ1.1			AM921834 [30]
						B. tropicus		TZ1.2			AM921837 [30]
						B. tropicus		TZ1.3			AM921842 [30]
	Tanzania, Lake Sagara,		−5.25140°N	31.08518°E		*Bulinus* sp.					AM286298 [30]
	Tanzania, Ihayabuyaga					*B. nasutus productus*					AM286300 [30]
						B. nasutus productus					AM286301 [30]
	Tanzania, Njombe Rujewa					*B. nasutus productus*					AM921833 [30]
	Tanzania, Zanzibar Vitonguji, Pemba Island		−5.23378°N	39.82843°E		*B. nasutus productus*					AM921809 [30]
	Tanzania, Zanzibar, Pemba Island		−5.17120°N	39.82198°E		*B. nasutus productus*					AM921811 [30]
	Tanzania, Zanzibar, Pemba Island		−4.92803°N	39.73785°E		*Bulinus* sp.					AM921832 [30]
	Tanzania, Zanzibar, Pemba Island					*B. globosus*					MH014041 [82]
	Tanzania, Zanzibar, Pemba Island, Kangagani					*B. barthi*					AM921818 [30]
	Tanzania, Zanzibar, Mafia Island					*B. nasutus nasutus*					AM921831 [30]
	Tanzania, Zanzibar, Mafia Island, Kanga swamp					*B. barthi*					AM921814 [30]
						B. globosus					AM921823 [30]
	Tanzania, Iringa					*B. globosus*					AM286288 [30]
						B. globosus					AM921821 [30]
	Tanzania, Ungunja, Kinyasini					*B. globosus*					AM921839 [30]
	Uganda, Lake Victoria	LV	−0.30371°N	32.28927°E	1228	*B. tropicus*	1	LV1.1	UGSB 16774	22530	MN551506
	Uganda, Lake George	LG				*B. forskalii*	2	LG1.1			HQ121586 [32]
						B. forskalii		LG1.2			HQ121585 [32]
	Uganda, Lake Edward	LE				*B. forskalii*	2	LE1.1			HQ121583 [32]
						B. forskalii		LE1.2			HQ121584 [32]
	Uganda, Lake Albert	LA				*B. forskalii*	4	LA1.1			HQ121582 [32]

Table 1 (continued)

Crater field	Location	Lake code	Coordinates		Altitude (m)	Species	No. of haplotypes[a]	Specimen code[a]	Specimen voucher	Prep. no.	GenBank ID [reference]
			Latitude	Longitude							
						B. forskalii		LA1.2			HQ121580 [32]
						B. forskalii		LA1.3			HQ121581 [32]
						B. forskalii		LA1.4			HQ121587 [32]
						B. tropicus	2	LA1.5			HQ121564 [32]
						B. tropicus		LA1.6			HQ121565 [32]
						B. tropicus		LA1.7			GU176751 [50]
						B. tropicus		LA1.8			GU176750 [50]
	Uganda, Toonya, Lake Albert					*B. truncatus*					GU176749 [50]
	Uganda, Booma, Lake Albert					*B. truncatus*					GU176747 [50]
	Uganda, Katosho swamp	KS				*B. forskalii*	2	KS1.1			HQ121588 [32]
						B. forskalii		KS1.2			HQ121587 [32]
						B. truncatus					HQ121563 [32]
						B. truncatus					HQ121562 [32]
	Uganda, Lake Tanganyika	LT				*B. forskalii*	3	LT1.1			HQ121590 [32]
						B. forskalii		LT1.2			HQ121589 [32]
						B. forskalii		LT1.3			HQ121587 [32]
	Uganda, Albert Nile River		3.47032°N	31.92267°E		*B. globosus*					AM286291 [30]
	Uganda, Lake Kyoga		1.82235°N	33.53725°E		*B. nasutus productus*					AM921815 [30]
						Bulinus sp.					AM921819 [30]
	Uganda, Kahirimbi		−08205° N	30.8559 °E	1249	*Bulinus* sp.			UGSB 24296	19415	MN551577
	Uganda, Maramagambo Forest					*Bulinus* sp.					HQ121591 [32]
	Uganda, Lake Victoria		0.13707°N	33.60149°E	1135	*B. truncatus*			UGSB 16757	22513	MN551582
						B. truncatus			UGSB 16758	22514	MN551583
	Uganda, Nile River		1.69249°N	32.09664°E	1035	*B. truncatus*			UGSB 16760	22516	MN551585
						B. truncatus			UGSB 16759	22515	MN551584
	Zimbabwe, laboratory strain	ZW				*B. tropicus*	1	ZW1.1			AY282583 [37]

Notes: Data include the crater field region, geographical coordinates, altitude (obtained from GoogleEarth Pro 1.0.0.1), number of haplotypes, specimen code (used for the network analyses), species, DNA preparation number (prep. no.), specimen voucher (UGSB, University of Giessen Systematics and Biodiversity collection) and GenBank accession number. *Bulinus* specimens from Lake Kyaninga could not be amplified. Note that a few GenBank numbers of other authors are occurring in more than one locality because they represent same haplotypes. Locality information and geographical coordinates of GenBank sequences are provided as they were published

[a] Number of haplotypes and specimen codes are only given for specimens used in the network analyses

[b] Lakes that did not yield populations of *Bulinus*

[c] Crater lake where currently no *Bulinus* were found but sequences on GenBank were available

Alignment and dataset composition

The study comprised specimens from all crater lakes where *Bulinus* occurred. Furthermore, additional specimens from surrounding watershed and other major aquatic systems were included in order to better trace regional affinities. These are samples from a rice scheme and lakes Muhazi (Rwanda), Mburo, Victoria and the Nile River system in Uganda. In total, material from 43 sampling localities and 85 specimens was used for DNA analyses. Sequences were edited and aligned in BioEdit version 7.0.5 [41]. All 84 newly generated sequences were Blast-searched against the GenBank sequence database. The newly generated sequences were supplemented with all previously published relevant sequences on GenBank. The resulting dataset was aligned using the ClustalW multiple alignment tool in BioEdit after removing redundant haplotypes.

Phylogenetic and phylogeographical analyses

Bayesian inference analysis was based on a total of 152 sequences (unique haplotypes) originating from our new sampling, as well as from GenBank data. The analysis was performed utilizing MrBayes version 3.2.2 [42] using the following settings: ngen = 4,000,000, samplefreq = 200, 'burn-in' = 5000 (25%) and HKY+I+Γ as the best-fit substitution model (selected using jModelTest version 2.1.4 [43] under the AIC, AICc and BIC criteria). The effective sample size (ESS) values were examined in Tracer version 1.5.0 [44] indicating for all major parameters values > 800. Statistical parsimony network analyses for all major clades found to contain crater lake specimens were conducted using TCS version 1.21 [45]. The sub-datasets were selected according to the results of the phylogenetic analysis, two specific clades were selected: Clade 1 and 2). The connection limit was set to either 95% (Clade 1) or 90% (Clade 2).

Genetic diversity

The final cluster analysis of crater lake similarity was based on presence/absence of 31 haplotypes of *Bulinus*, the Bray-Curtis similarity measure was used (PAST version 3.22) [46].

The relationship of altitude and distribution of haplotype diversity across the crater lake fields was tested using correlation analysis implemented in PAST version 3.22 [46].

Results

Host species identification and diversity

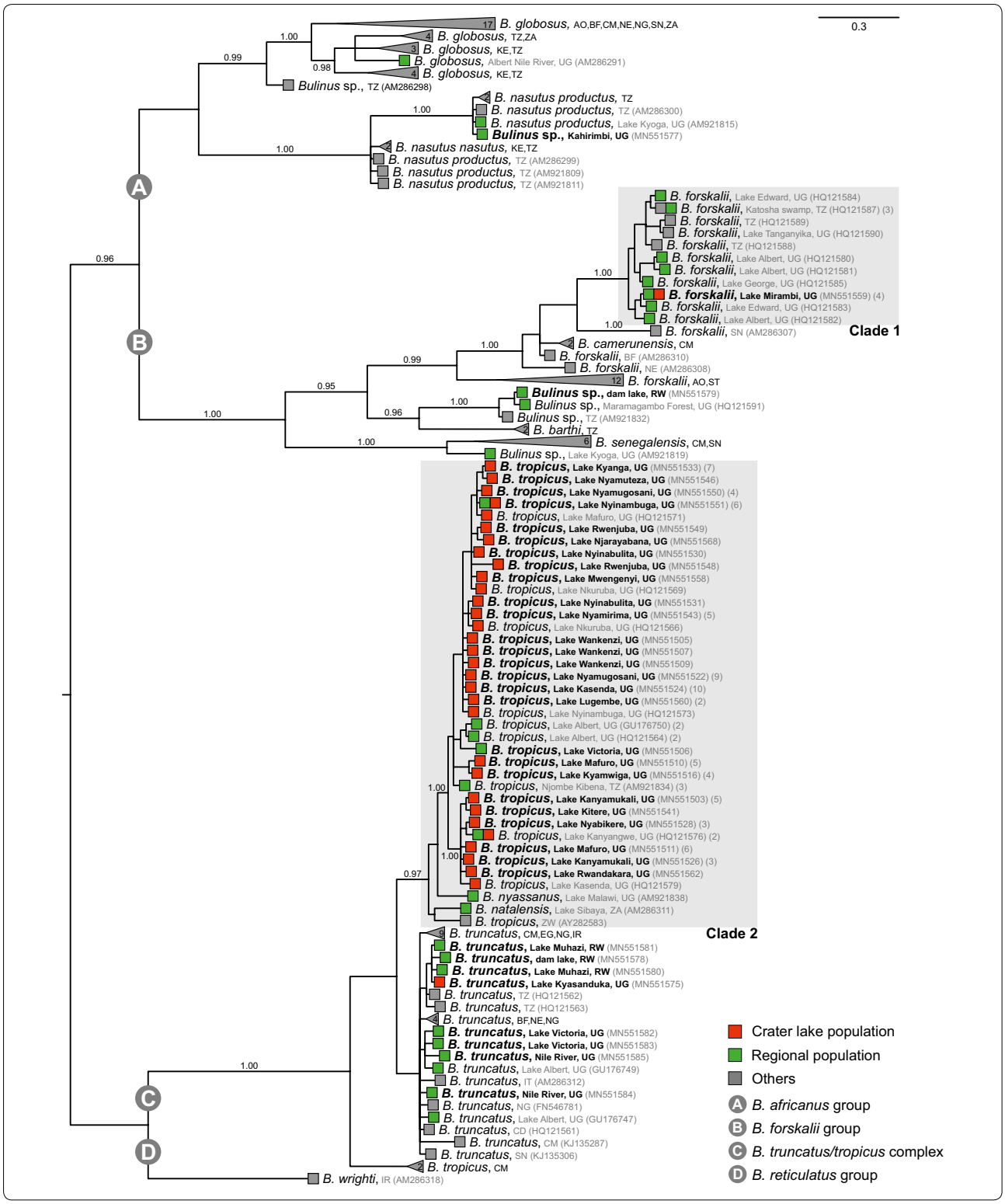
Out of 58 crater lakes sampled, *Bulinus* spp. snails were found in 34 belonging to the two crater fields Bunyaruguru and Ndali-Kasenda. Although *Bulinus* spp. snails were sampled in Lake Kyaninga (Fort Portal), the samples did not yield any DNA for analysis due to technical issues. However, there is a high likelihood that the *Bulinus* spp. from this lake belong to one of the important *S. haematobium* hosts, *Bulinus globosus*, based on the shell shape (Additional file 1: Figure S1c). Lakes Rwijongo (Bunyaruguru), Mubiro and Kanyabutetere (Ndali-Kasenda) yielded *Bulinus* spp. shells only during the sampling. For Lake Rwijongo, sequences from the GenBank database were available and were included in the analyses. The rest of the crater lakes had either other gastropods or no snails at all. *Bulinus* spp. co-occurred with *Biomphalaria* spp. in 28 lakes. Three species of *Bulinus* were found, i.e. *B. forskalii*, *B. truncatus* and *B. tropicus*. The first was found in only two crater lakes (Mirambi and Kibungo), which are in close proximity located in the Ndali-Kasenda crater field. *Bulinus truncatus* exclusively occurred in Lake Kyasanduka in the Maramagambo Forest (Bunyaruguru). *Bulinus tropicus* was dominant, found in the rest of the crater lakes that harbored *Bulinus* spp. (Table 1). In addition, neither *B. forskalii* nor *B. truncatus* occurred sympatrically with *B. tropicus*. All the three *B. forskalii* and the four *B. truncatus* specimens genotyped showed the same haplotypes. *Bulinus tropicus* portrayed a high variability within and from one lake to another across the crater lakes fields. In total, this study is composed of 33 haplotypes in 34 crater lakes (one haplotype for *B. forskalii* and *B. truncatus* and 31 for *B. tropicus*).

Phylogenetic relationships and biogeographical affinities

The Bayesian inference analysis showed that *Bulinus* specimens genotyped for this study are distributed across three of the four *Bulinus* species groups/complex, specifically the *B. forskalii* and *B. africanus* groups and the *B. truncatus/B. tropicus* complex (Fig. 3). *Bulinus tropicus* from the crater lakes clustered exclusively within a

(See figure on next page.)

Fig. 3 Bayesian inference phylogenetic tree for *Bulinus* spp. based on *cox*1 gene sequences. Specimens are given with locality information as to country of origin and localities in some cases. The DNA preparation numbers are provided. Crater lake names are provided and the two specific clades of *B. forskalii* (Clade 1) and *B. tropicus* (Clade 2) are highlighted with light grey boxes. Crater lake populations are represented at the end of the branch by red boxes, while regional and non-regional (= others) populations are demonstrated by green and grey boxes, respectively. The outgroup *Indoplanorbis exustus* is not shown. The tree has been partly graphically collapsed (for the full tree see Additional file 2: Figure S2). Bayesian posterior probabilities (pp) are given for deeper nodes (when pp ≥ 0.95). The scale-bar represents substitutions per site according to the model of sequence evolution. The number of individuals per haplotype is shown in parentheses for the two specific clades (for details see Figs. 4, 5)



highly supported *B. tropicus* clade (Clade 2, pp = 0.97, see Fig. 3 and Additional file 2: Figure S2) of the *B. tropicus/B. truncatus* complex (pp = 1.00). However, *B. tropicus* specimens from the crater lakes did not form a monophyletic group. Instead, the clade comprising the crater lake samples also included *B. tropicus* from

Lake Victoria (MN551506), Lake Albert (GenBank: HQ121564, GU176750) and Njombe Kibena in Tanzania (GenBank: AM921834). Specimens from Lake Malawi (GenBank: AM921838), South Africa (GenBank: AM286311) and a laboratory strain from Zimbabwe (GenBank: AY282583) clustered in a basal position to the *B. tropicus* clade that comprised the crater lake samples. The specimen from Lake Kyasanduka and some specimens of regional populations clustered within the *B. truncatus* assemblage. These populations derived from Lake Victoria, Nile River, Lake Muhazi and other places in Rwanda, which are in close proximity to the crater field systems in western Uganda (Figs. 1, 3). The *B. truncatus* assemblage also includes populations from locations as far away as Nigeria, Cameroon, or Egypt and Burkina Faso.

The *B. forskalii* group was genetically very diverse as evidenced by the branch lengths variation with the two crater lake populations clustering with other Ugandan populations from lakes Albert, Edward and George. A distinct and highly supported clade (clade 1, pp = 1.00, see Fig. 3) also comprised populations from the Katosho swamp (Tanzania) and Lake Tanganyika (Tanzania). A *Bulinus* sp. from a dam lake connected to a rice field irrigation system in Rwanda (MN551579) belonged to the *Bulinus forskalii* group but clustered in a different subclade. It also included another *Bulinus* sp. from Maramagambo Forest, Uganda (GenBank: HQ121591), a place not far from the crater lake fields. Yet another *Bulinus* sp. from Lake Kyoga clustered with the *B. senegalensis* subclade of the *B. forskalii* group.

Another *Bulinus* sp. (MN551577) from Kahirimbi in Lake Mburo/Nakivale wetland system, about 120 km south of the crater lakes, belonged the *B. africanus* species group (Fig. 3). It was part of a clade that comprised *B. nasutus* from Lake Kyoga, Uganda and other regions in Tanzania. *Bulinus globosus* from Nile River (Moyo, Uganda) was the geographically closest occurrence of this species to the crater lakes in our dataset. Both resolution and support values were low within *B. tropicus* and *B. forskalii* clades. The phylogeographical structure for those clades were thus specifically analysed with a parsimony network analysis.

Phylogeographical patterns

Bulinus forskalii from the crater lakes formed a single network with GenBank haplotypes from the surrounding lakes, i.e. Lake Albert in the north, Lake Edward in the west and Lake George in the east. A few haplotypes from regions outside Uganda, such as Lake Tanganyika and nearby Katosho swamp also appeared in the network, whereas others from Rwanda or the Maramagambo Forest east of Lake Edward did not (Fig. 4). All the three

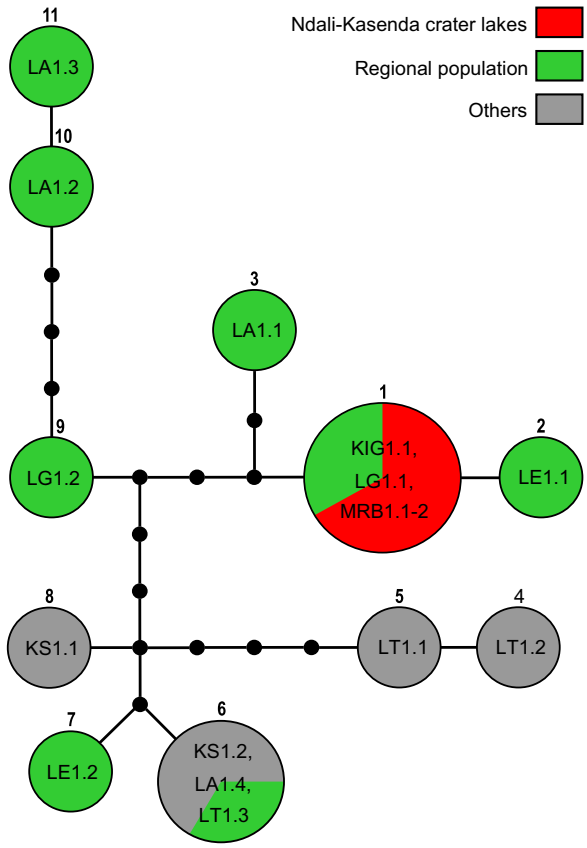


Fig. 4 Statistical parsimony network of *cox*1 sequences for *Bulinus forskalii*. The network corresponds to the *B. forskalii* clade highlighted in Fig. 3. The connecting limit was 95%. The Ndali-Kasenda crater lake specimens are indicated in red. Haplotypes originating from regional and non-regional (= others) are in green and grey, respectively. The most probable ancestral haplotype is indicated by a bold circle

crater lake specimens represented one haplotype and together with a haplotype (GenBank: HQ121586) from Lake George formed the most probable ancestral haplotype for the network. Haplotypes from far away regions were also reconstructed as distantly related. For example, there were 11 and 10 mutational steps between haplotypes from Lake Tanganyika (GenBank: HQ121590, HQ121589). On the other hand, haplotypes from nearby regions such as Lake Edward and Lake George were reconstructed either as relatively similar (e.g. GenBank: HQ21582, HQ21583, HQ121586) or as relatively far distant in terms of mutational steps (e.g. GenBank: HQ121580).

The single haplotype network of *B. tropicus* based on a 90% connection limit contained 38 haplotypes (Fig. 5). Haplotype 11 (Fig. 5) was present in six lakes in Ndali-Kasenda was reconstructed as the most probable ancestral haplotype. The haplotypes of both the Ndali-Kasenda and the Bunyaruguru crater fields were very diverse

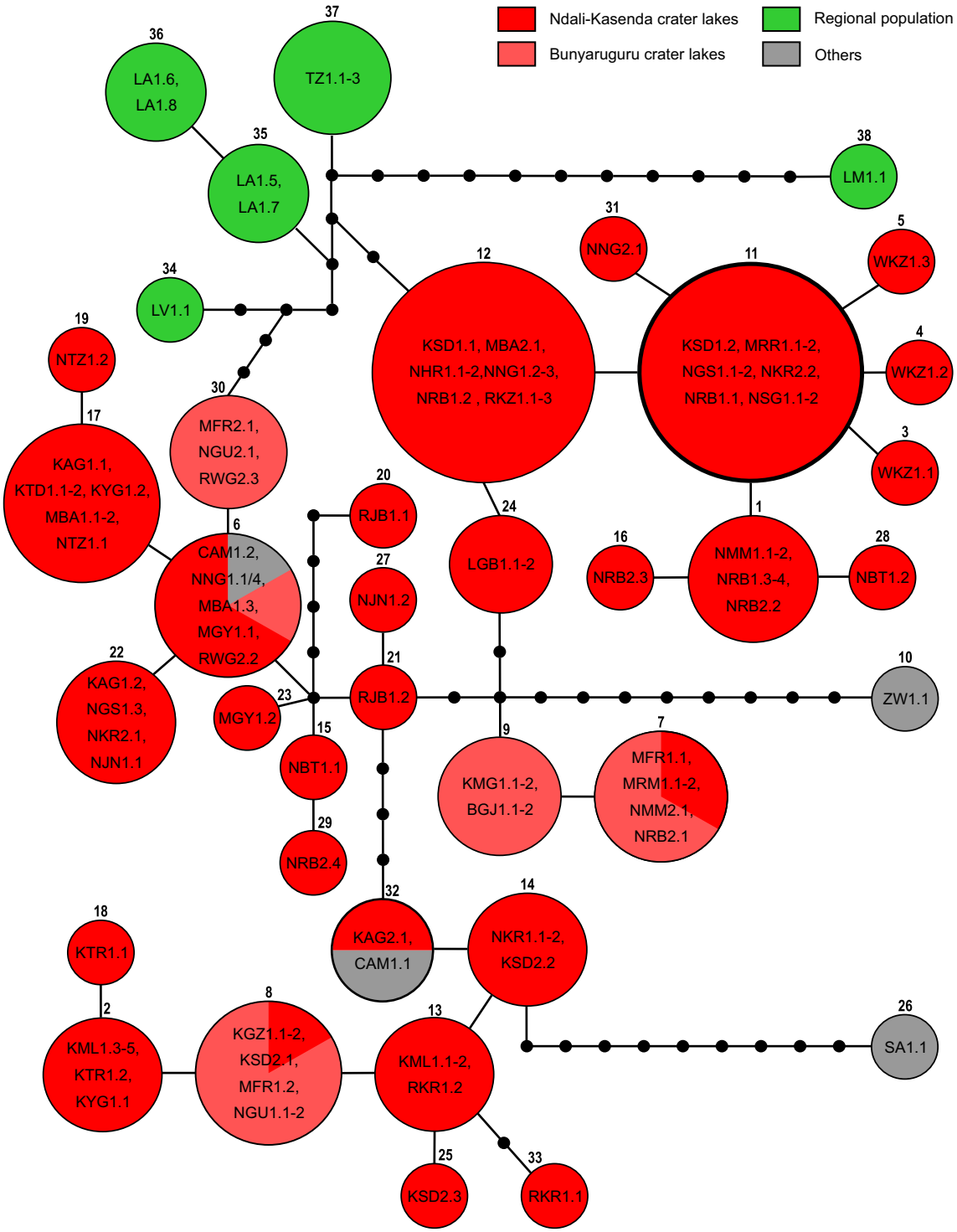


Fig. 5 Statistical parsimony network of *cox*1 sequences for *Bulinus tropicus*. The network corresponds to the *B. tropicus* clade highlighted in Fig. 3. The connecting limit was 90%. The two crater lakes fields of Ndali-Kasenda and Bunyaruguru are coloured red and light red, respectively. Haplotypes connected but occurring in other systems are represented in green and grey for regional and non-regional (= others) populations respectively. The most probable ancestral haplotype is indicated by a bold circle

ranging from a few or no mutational steps, to as many as 17.

In most cases, haplotypes of the Ndali-Kasenda crater field were unique with a few exceptions where a haplotype was shared with either samples from the Bunyaruguru crater field (haplotypes 7 and 8 in Fig. 5), outside the region (haplotype 32 in Fig. 5) or both (haplotype 6 in Fig. 5). Lakes Kyamwiga and Bugwagyi of the Bunyaruguru crater field had exclusive haplotypes. Some lakes had quite distantly genetically related haplotypes such as MN551511, MN551510 and HQ121571, all of which are from Lake Mafuro located in the Bunyaruguru crater field. A haplotype from as far as Cameroon was identical to three crater lake samples of the Ndali-Kasenda crater field that are in close proximity to one another and Lake Rwijongo of the Bunyaruguru crater field (haplotype 6 in Fig. 5). Except for the Cameroonian, all haplotypes from outside crater lake systems are unique. These are haplotypes from Lake Victoria, Lake Albert and Tanzania. They belonged to a single group connected to crater lake haplotypes by a minimum of four mutation steps to the Ndali-Kasenda haplotypes *via* a Tanzanian haplotype (haplotype 37 in Fig. 5), Lake Albert (haplotype 35 in Fig. 5) and five mutation steps to a Bunyaruguru haplotype *via* a Lake Victoria haplotype (haplotype 34 in Fig. 5). Three haplotypes were extremely distant from the core network, i.e. samples from South Africa (haplotype 26 in Fig. 5), *B. nyassanus* from Lake Malawi (haplotype 38 in Fig. 5) and the laboratory strain from Zimbabwe (haplotype 10 in Fig. 5).

Genetic diversity

The cluster analysis based on the presence/absence of haplotypes did not result in a clear pattern (Fig. 6). Whereas some lakes clustered together, others remained unclustered. Lakes Nyungu (NGU), Kigezi (KGZ) and Mafuro (MFR) all belong to the Bunyaruguru crater field cluster together. Other lakes belonging to that crater field such as lakes Bugwagyi (BGJ) Kyamwiga (KMG) were clustered too, whereas Rwijongo (RWG) did not cluster together with any of the two groups in the same crater field. The numerous lakes of the Ndali-Kasenda field formed three main clusters. Lakes Wankenzi (WKZ), Lugembe (LGB), Rwenjuba (RJB) and Nyinabulitwa (NBT) did not cluster to any of the other groups. Some lakes in the two crater fields tended to cluster

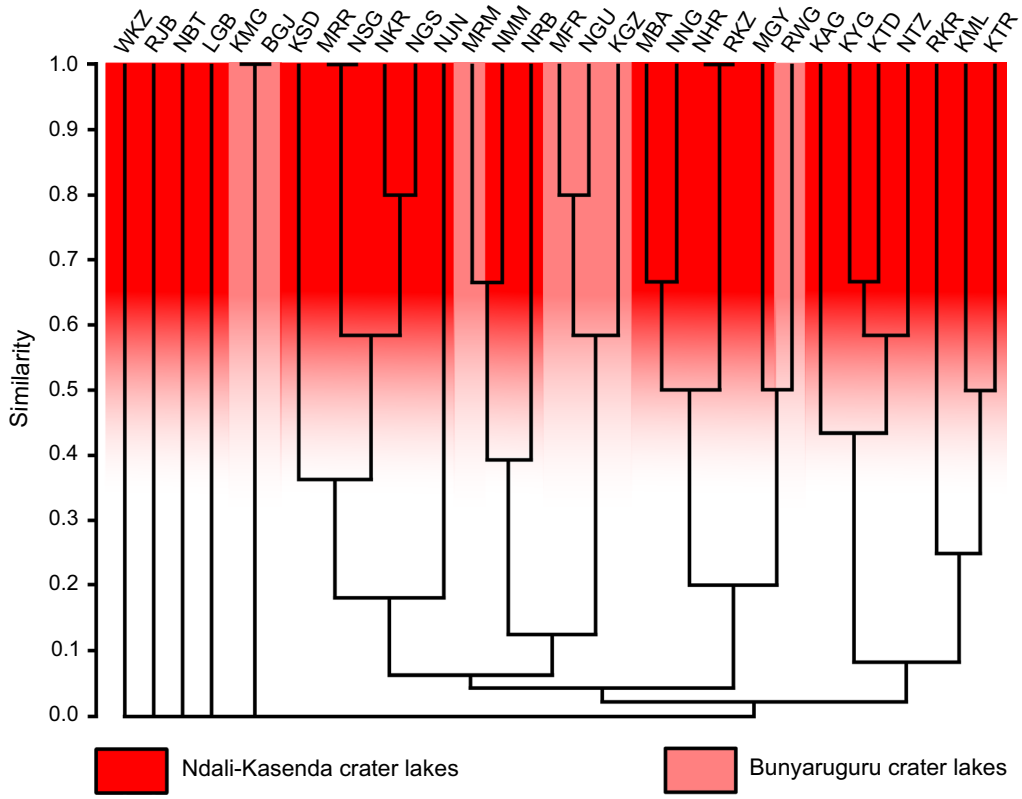


Fig. 6 Cluster analysis of crater lake similarity based on presence/absence of 31 haplotypes of *Bulinus tropicus*. The Bray-Curtis similarity measure was used. Three letter codes refer to the respective lakes in Table 1. The haplotype matrix is given in Additional file 3: Table S1

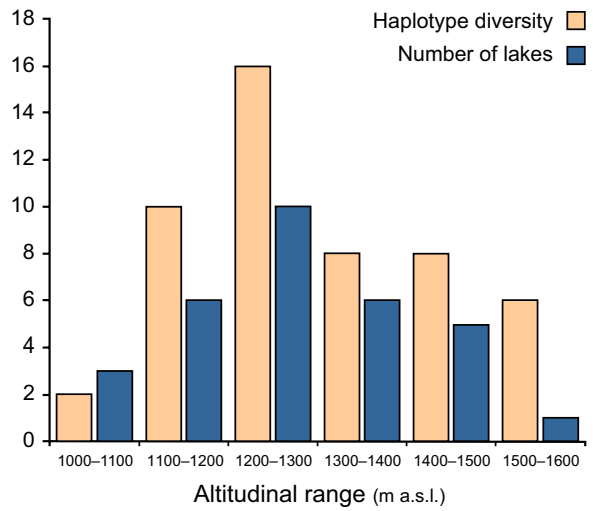


Fig. 7 Haplotype diversity *versus* increase in altitude according to 100 m altitudinal bands for 31 unique haplotypes in 31 crater lakes

together, for example Lake Nyamirima (NMM) with Lake Murambi (MRM) and Lake Rwijongo (RWG) with Lake Mwengenyi (MGY). Lakes that came out to be more similar according to this analysis, for example lakes Nyahira (NHR) and Rukwanzi (RKZ), or lakes Nyanswiga (NSG) and Muligamire (MRR) are not geographically related.

Haplotype diversity across the crater lakes' altitudinal gradient

The distribution of haplotype diversity along the altitude gradient was unimodal (Fig. 7). A high haplotype diversity was realized in an altitude range between 1200 to 1300 m a.s.l., with 16 different haplotypes. Lake Nkuruba with the most haplotype diversity and situated at an altitude of 1517 m a.s.l) was represented by eight specimens and six unique haplotypes. The lakes in the Bunyaruguru crater field, which are at the lowest altitudes exhibited limited haplotype diversity ($n=6$). Based on the present dataset, the haplotype diversity was not correlated with altitude ($r=0.26813$, $P=0.12787$).

Discussion

Bulinus species in the crater lakes

To date, historical records provide very restricted information on molluscs in crater lakes in Uganda [33]. Although *Bulinus tropicus* has been present there for quite a while already, not much more was known hitherto, except for the study by Nalugwa et al. [32] on *Bulinus* species complexes in the Albertine Rift, which included a few crater lakes of western Uganda. In this study, *Bulinus tropicus* by far dominates in the crater lakes, whereas *B. forskalii* was exclusively found in lakes of Mirambi and Kibungo that are in close proximity. *Bulinus forskalii* is essentially an Afrotropical species that often occurs in small and even temporary water bodies [26]. It seems less common in colder climates of highlands in eastern Africa and has been found up to 1800 m a.s.l. in Ethiopia [47]. *Bulinus forskalii* is not known to be naturally infected with *S. haematobium* (see [48]). *Bulinus tropicus* is a widespread species in eastern and southern Africa, but unlike its sibling species *B. truncatus*, is apparently not acting as intermediate host for *S. haematobium*, the parasite causing urogenital schistosomiasis [26, 27]. *Bulinus tropicus* is known to occur up to 2700 m a.s.l. in Kenya [26]. This species is extremely flexible ecologically, i.e. it exhibits a high tolerance towards cooling and drought conditions and even to only temporary availability of habitat. Such conditions might exist in the crater lakes of western Uganda, where extensive lake level fluctuations over seasonal periods have been documented ([49]; Tumwebaze, own observations from historical satellite images).

This ecological flexibility might be linked to another very interesting finding of the present study, which is the extremely high diversity of haplotypes of *B. tropicus* in the crater lakes. It almost matches the total range of genetic diversity hitherto known for this species [30]. Given the fact that the present study was not designed as a population study, the real variability might be even higher.

The fact that so far, the majority of the studied *Bulinus* spp. populations belonged to *B. tropicus*, does not mean *S. haematobium* strains would not occur in the entire crater lakes region. The sibling tetraploid species *B. truncatus*, a major intermediate host for *S. haematobium* in many regions of Africa, has been found in various places along the Albertine Rift [32, 50] and our dataset included populations from nearby areas in Uganda and Rwanda (Fig. 3). Although we found it exclusively in one crater lake, sympatric occurrences of the two species are possible [32]. A presence of tetraploid *B. truncatus* can therefore not be ruled out without such detailed molecular surveys such as the ones conducted in the present study. The recurrent almost complete absence from the crater lakes of *B. truncatus*, an ecologically largely flexible species with high colonizing capacity [26], remains somewhat enigmatic. This species has been found confined to altitudes of 2100 m a.s.l., rarely up to 2400 m a.s.l. in Ethiopia [26]. It was present in our study in Lake Victoria, the Nile River and Lake Muhazi, Rwanda, all locations in a radius of just *c.*250 km. *Bulinus globosus*, another potential host species, is known from the Nile River, Moyo Province, Uganda [30]. We found another *B. nasutus*-like species at the Lake Mburo-Nakivale system in southwestern Uganda. *B. nasustus* has also been found in

previous work in Lake Kyoga [30] (Fig. 3). It is therefore perhaps just a matter of time and or chance until other species of *Bulinus* acting as intermediate hosts for human schistosomiasis are to be found in the crater lakes. The absence of members of the *B. africanus* group from the crater lakes is noteworthy though. Prediction as to occurrences of specific snail lineages is difficult since not only time and isolation matter, but also ecology of the small lakes. They are very different from littoral conditions in better studied large lakes such as Albert, Edward and Victoria, for which factors favoring a mitigation of the proliferation of snail populations have been determined to a much greater extent than in other lotic or lentic natural aquatic systems in East Africa [14, 51–53]. The absence of *Bulinus* in some of the studied crater lakes might be attributed to limnological conditions [18, 19]. However, 19 lakes where *Bulinus* was not collected contained other molluscs (Tumwebaze & Albrecht, unpublished data). Repeated sampling during different seasons might help constructing a more complete picture of *Bulinus* spp. communities in these lakes.

Phylogenetic relationships and biogeographical affinities

The phylogenetic study of *Bulinus* spp. corroborated earlier findings that three major species complexes exist in the Albertine Rift region [32], although only *B. tropicus* was found to be present then in the crater lakes. Our field sampling complements the previously limited knowledge of the *Bulinus* spp. communities of the crater lakes based on the previous effort of Nalugwa et al. [32] on a smaller subset of lakes. The phylogenetic affinities of specimens genotyped from potential source populations, i.e. close geographical proximity, revealed a wide range of genetic variability which is interpreted as reflecting the high morphological and ecological flexibility of the species, as well as its extraordinary dispersal capacities. The analyses of the *B. tropicus* subclade (Fig. 3) resulted in few well-supported branches which made tracing a single origin of the crater lakes populations difficult from a phylogenetic analysis. However, the findings support the hypothesis of highly dynamic fluctuations of populations coming into the crater lakes on a potentially frequent basis. It was unexpected to find haplotypes that are known from other places far away in East Africa or even western Africa (Cameroon) in the crater lakes studied here. For the present dataset, the origin of the haplotype network was reconstructed for a haplotype occurring in six lakes of the Ndali-Kasenda lake region, likely reflecting the extraordinary diversity in the DNA data. Whereas environmental parameters might account for phenotypic plasticity observed in the *B. truncatus/B. tropicus* complex, no such direct relationships have been shown for the degree of genetic variation. It might be in fact one of the reasons why *B. tropicus* is refractory to human-infecting schistosomes [26]. Both *B. forskalii* and *B. truncatus* exhibit less genetic variation on similar geographical scales [25, 54, 55]. *Bulinus forskalii* from the crater lakes clearly belongs to a very distinct clade of Albertine Rift valley populations and colonization likely happened from nearby sources such as Lakes George, Albert and Edward. Interestingly, this clade also comprises haplotypes from further south, namely Lake Tanganyika. Other species of the *B. forskalii* group that are geographically closer, such as Lake Kyoga or Rwanda or even the Maramagambo Forest in Queen Elizabeth National Park, Uganda, do represent quite distinct lineages.

Phylogeography and lake patterns

Since the crater lakes in western Uganda are roughly 8000 to 10,000 years-old [34], the variation observed is not likely to have developed in that setting given the general mutation rate of the molecular marker *cox*1, even if we consider potentially higher rates under tropical conditions [56]. It is worth noting that the unique haplotypes often differed by one mutational step only. We must, however, also keep in mind that the coverage of samples of *B. tropicus* throughout its vast African range is far from being representative and therefore the 'endemicity' of the unique haplotypes cannot be evaluated with all certainty. The variability in haplotypes might also be reflected in shell shapes as outlined by an example from Lake Mafuro, in which two distinct haplotypes corresponded to distinct shell morphs (Additional file 1: Figure S1a, b). The *Bulinus* snails from Lake Kyaninga have shells that are quite distinct from the rest in the region (Additional file 1: Figure S1c).

One question relates to colonization history, i.e. where the lineages come from. In the case of *B. tropicus*, our results identified populations from across Africa as potential sources based on genetic affinities, both from nearby source of the Victoria-Nile-Albert system or places considerably far away in Tanzania or even West Africa. The co-occurrence of distantly related haplotypes in a single lake (e.g. Nkuruba or Nyabikere) points towards multiple colonization of the same lake system likely fostered by high propagule pressure. Sharing haplotypes regionally hints towards population dispersal by passive means since most of the lakes studied have no hydrological connection. A similar pattern has been found for fishes in the Fort Portal region [57]. Machado-Schiaffino et al. [58] studying the Bunyaruguru crater lakes discovered strong genetic and morphological differentiation whereby geographically close lakes tend to be genetically more similar. Such a general trend was not obvious when comparing the lakes based on the haplotype distribution in our study.

It is important to notice that altitude reflecting climate parameters as earlier predicted [15], did not correlate with occurrence and diversity of snail populations. Rather, a more complex interplay of land use, lakes limnology, community resistance and stochasticity might account for the presence or absence of certain snail species and certain haplotypes in the crater lakes.

Parasitological implications

This study did not find an immediate risk for urogenital schistosomiasis based on the *Bulinus* snails identified. However, the identification of up to six partly highly divergent haplotypes in small and young isolated systems such as the crater lakes in Uganda, might hint to either extremely fast evolution or multiple invasions by vectors from various source populations. This involves humans most likely. If this is the case, other species of *Bulinus* and *Biomphalaria* might also potentially be introduced. Not only is the probability of the introduction of host snails likely to increase given increased mobility of people in Uganda and international migrations, such as refugees from the crisis regions in surrounding countries, but also are such human flows capable of dispersing non-native parasite strains with them. It should be kept in mind that for example in the neighboring Nile Province of South Sudan prevalence of *S. haematobium* infection was found to be more than 70% in school children [23] and that the few modelling attempts for urogenital schistosomiasis transmission risk suggest dynamic patterns for the near future [22].

In order to establish an enhanced model of schistosomiasis prevalence in the crater lakes region, a dedicated survey of infection rates among households adjacent to the lakes that are actually using the water resources of the lakes for various purposes should be conducted. The various ways of how and to what extent water is used directly or indirectly should be assessed and quantified, as the information available with regard to these activities is very limited. The role of human (indirect) transport of both host snails and parasites is likely to be more important than previously considered, due to the important flows of human populations in the region. Movement from regions with high infection risk sites around Lake Albert and Lake Victoria or other inland water bodies infested with schistosomiasis might enhance the prevalence of schistosomiasis in the western region of Uganda. There is also need for increased surveillance of new schistosomiasis outbreaks in the crater lakes region especially at higher altitudes in the face of the projected increase in temperature in the near future [59, 60] since crater lakes have shown to be sensitive to climate change [61, 62].

A largely neglected aspect here relates to schistosomiasis as a disease in livestock. *Bulinus tropicus* and *B. forskalii* found in the crater lakes are well-known intermediate hosts for bovine schistosome species such as *S. bovis* [63–67]. This parasite is responsible for a large proportion of livestock trematode infections [68], and the parasite's distribution overlaps largely with *S. haematobium*. Moreover, these two *Schistosoma* species have been shown to hybridize repeatedly, which triggered considerable parasitological and public health concern [69, 70]. Thus, future surveys are suggested to include molecular screening of schistosome infections [71]. *Schistosoma bovis* infections of cattle have been confirmed from western Uganda [31]. *Bulinus tropicus* and *B. forskalii* are also intermediate hosts for *Schistosoma margrebowiei* and *Calicophoron microbothrium* [72, 73], with *B. forskalii* transmitting a wide range of parasites in Ethiopia [74]. Several trematode infections have been reported in *B. forskalii* [75]. Loker et al. [76] detected cercariae of seven species from naturally infected snails in north-west Tanzania. Our study thus points to a significant concern since livelihoods of people in the crater lake region of western Uganda predominantly depend on cows, sheep and goats, which are all susceptible to schistosomiasis and other trematodiases hosted by *B. tropicus* and *B. forskalii* [77]. The crater lakes are in close proximity to nature reserves and national parks that are home to one of the most diverse primate populations in Africa. It is thus noteworthy that zoonotic schistosomiasis is a significant concern at the human-wildlife interface that is currently largely underestimated [78], which makes the crater lake region further interesting for parasitological studies in addition to the relevance for increased intestinal schistosomiasis [79].

Conclusions

This first detailed malacological study of the crater lakes systems in western Uganda revealed a dominance of *Bulinus* species that are either not known at all (*B. tropicus* and *B. forskalii*) or not known to act as intermediate hosts of *S. haematobium*, the causative agent of human urogenital schistosomiasis in this region of Africa (*B. truncatus*). The risk of contracting this form of schistosomiasis is thus currently very low. However, potential sources for intermediate host species and known regions with high prevalence rates, have been identified in comparatively close proximities to the study region (within a radius of *c.*250 km). The epidemiology of urogenital schistosomiasis is very dynamic and there is a potential for near-future occurrence in this part of Uganda. It is thus advisable to conduct more in-depth epidemiological studies in conjunction with the activities related to intestinal schistosomiasis. There is need for coordinated

effort to document the genetic diversity of schistosome intermediate hosts from small-scale (in western Uganda) to large-scale (from Uganda as a country, to east Africa and the whole of Africa), so that an effort to eradicate the parasites *via* snail control from the natural system is based on informed grounds. A cautionary note is raised in terms of the veterinary importance of the gastropod species found. They both act as intermediate host for a variety of parasites including the species causing the majority of cases of livestock schistosomiasis, *S. bovis*. The impact of this finding is potentially of major importance but currently unstudied in the region. Such studies are needed as well as investigations into factors driving the presence of hosts and parasites in regions and ecosystems so far largely neglected but with the potential of becoming major transmission sites. This is significant, especially under the projected climate changes that will shift altitudinal limits of one of the most notorious tropical diseases that continues to be a major burden especially in sub-Saharan Africa.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13071-019-3811-2.

Additional file 1: Figure S1. Photographs of *Bulinus tropicus* from Lake Mafuro (a, b) and a *Bulinus* species resembling *B. globosus* of Lake Kyaninga (c) showing variation in shell morphology. Both snails from Lake Mafuro are 11 mutation steps apart in the *cox1* network.

Additional file 2: Figure S2. Bayesian inference phylogenetic tree for *Bulinus* spp. based on *cox1*. Specimens are given with locality information (country of origin and localities in some cases). The DNA preparation numbers are provided. Crater lake names are provided and the two specific clades of *B. forskalii* (Clade 1) and *B. tropicus* (Clade 2) are highlighted with light grey boxes. Crater lake populations are represented at the end of the branch by red boxes, while regional and non-regional (= others) populations are demonstrated by green and grey boxes, respectively. Outgroup taxa are not shown. This tree is the full version of the collapsed tree in Fig. 3. Bayesian posterior probabilities (pp) are given for deeper nodes (when pp $\geq$ 0.5). The scale-bar represents substitutions per site according to the applied model of sequence evolution. The number of individuals per haplotype is shown in parentheses for the two specific clades (for details see Figs. 4, 5).

Additional file 3: Table S1. Haplotype sequence matrix for *Bulinus tropicus* in 31 crater lakes of western Uganda. *Abbreviations*: NL, total number of haplotypes per lake; NH, total number of haplotype frequency (based on a 95% connection limit). For details of 'lake codes' see Table 1.

Abbreviations

AIDS: acquired immune deficiency syndrome; a.s.l.: above sea level; *cox*1: mitochondrial cytochrome *c* oxidase subunit 1 gene; CTAB: cetyl trimethyl ammonium bromide; DNA: deoxyribonucleic acid; DRC: Democratic Republic of the Congo; MEGA: molecular evolutionary genetics analysis; NTDs: neglected tropical diseases; PAST: paleontological statistics; PCR: polymerase chain reaction; UGSB: University of Giessen Systematics and Biodiversity collection.

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Authors' contributions

IT and CA conceived the study. IT did field work, produced and analyzed the molecular data and drafted the tables/figures. IT and CA drafted the initial manuscript, while the latter is the overall supervisor of the study. CC was involved in data analyses, drafting the manuscript and fine-tuning the tables/figures. MCD and JT conducted significant parts of the field sampling. GKR was involved in planning and organizing the study and helped with permits and logistics in the field as well as in drafting the initial manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Data supporting the conclusions of this article are included within the article and its additional files. The newly generated sequences were submitted to the GenBank database under the accession numbers MN551500-MN551585. The datasets generated and analysed during the present study are available in the University of Giessen Systematics and Biodiversity repository and are available upon reasonable request.

Ethics approval and consent to participate

This study was approved by Uganda National Council for Science and Technology (UNCST) (research clearance reference number NS20ES).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Publisher's Note

2.2. Cryptic intermediate snail host of the liver fluke *Fasciola hepatica* in Africa

Parasites & Vectors

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Cryptic intermediate snail host of the liver fluke *Fasciola hepatica* in Africa

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Abstract

Background: Snails such as *Galba truncatula* are hosts for trematode flukes causing fascioliasis, a zoonosis that is a major public health problem. *Galba truncatula* has recently been shown to be a cryptic species complex. African populations of *Galba* spp. are not yet studied using molecular assessments and is imperative to do so and reconstruct the centre of origin of *Galba* and to understand when and by what means it may have colonized the highlands of Africa and to what extent humans might have been involved in that process.

Methods: Samples from all known sub-ranges throughout Africa and new samples from Europe and Asia were obtained. We used a combination of two mitochondrial (*cox*1 and *16S*) and one nuclear (ITS2) markers and phylogenetic, divergence time estimates and phylogeographical methods to determine the identity and biogeographical affinities. We also reconstructed the colonization history including the likely mode of dispersal and tested for the presence of cryptic *Galba* species in Africa.

Results: *Galba truncatula* is restricted to the Palaearctic region of the continent, namely Morocco. All sub-Saharan populations proved to be a distinct species according to the phylogenetic analyses and genetic distance. We propose to use the existing name *Galba mweruensis* (Connolly, 1929) for this species which is morphologically indistinguishable from the other two species hitherto known to occur in northern Africa, i.e. *G. truncatula* and *G. schirazensis*. Sub-tropical Africa has been colonized only once in either the Pliocene and possibly Miocene. Diversification within *G. mweruensis* is dated to the Plio-Pleistocene and thus human-mediated dispersal can be ruled out for the initial colonization of the isolated mountain ranges. There are potentially even more cryptic species in high altitude areas of Africa as outlined by the distinctness of the population found at the top of Mt. Elgon, Uganda.

Conclusions: From a novel genetic inspection of available African material, a hitherto neglected distinct species, *G. mweruensis*, now appears a major host of *F. hepatica* throughout sub-Saharan Africa. A closer examination of trematode parasites hosted by this species is needed in order to understand transmission patterns in highlands throughout eastern and southern Africa. We encourage future studies to inspect other high altitudes areas in Africa in light of parasites of either veterinary or medical importance.

Keywords: Fascioliasis, Medical malacology, Cryptic species, *Galba truncatula*, Lymnaeidae, Dispersal, Islands-in-the-sky

Background

Parasitic disease caused by the liver flukes of the genus *Fasciola* affects hundreds of millions of people and livestock worldwide. Collectively, they cause considerable economic damage. Indeed, fascioliasis, a very debilitating snail-borne disease, is widespread across the globe; however, in the subtropical/cooler regions it is caused by *Fasciola hepatica* [1] whereas in the tropical/warmer regions is caused by *Fasciola gigantica* [2].

To complete the life-cycle, the two species of liver fluke are tied to a variety of intermediate freshwater pulmonate snail hosts of the family Lymnaeidae [3].

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Until relatively recently, the taxonomy of snails was consolidated to a single genus *Lymnaea* with remarkable morphological diversity; however, with application of molecular phylogenetics a multi-generic nomenclature has become favoured with *Galba* and *Radix* now used in preference [4]. In Africa, for example, *Galba truncatula* (also known as *Lymnaea truncatula*) is involved in the transmission of *F. hepatica* while *Radix natalensis* is involved in the transmission of *F. gigantica* with any epidemiological cross-over considered to be rare [4]. As an intermediate host of *F. hepatica*, the liver fluke largely responsible for human disease, *G. truncatula* is characterized by its amphibious lifestyle, adaptation to cooler habitats, and its ability to withstand drought events and other harsh environmental conditions in unstable waterbodies [5]. It has been found in high altitudes in South America, where it can reach up to 4100 m [6] and it is thus among the few gastropods reaching extreme habitats on high elevations [7].

The taxonomy of lymnaeid gastropods continues to be debated [4, 8], but recent molecular phylogenetic studies improved the understanding of the evolution of this major freshwater gastropod family [3, 9–11]. The species *G. truncatula* has been treated as belonging to *Lymnaea* and *Fossaria* in North America and is thus a prime example of taxonomic confusion in lymnaeid systematics. *Galba truncatula* as the type-species of the genus is conceived to be mainly a Holarctic species [12], with a wide distribution range throughout North America and Eurasia, where it reaches as far as India [13]. The scattered occurrences in South America have been interpreted as recent introductions [14]. However, the real extent of the distribution of *G. truncatula* on a global scale is potentially masked by the occurrence of cryptic species that are morphologically indistinguishable from *G. truncatula*. Among these species are *Lymnaea cubensis* [15] and *Lymnaea schirazensis*, two species that have been previously confused with *G. truncatula* prior to the introduction of molecular methods of characterisation. Such a confusing situation has important implications to parasite transmission and epidemiology because the cryptic species may differ in their competence for transmission of *F. hepatica*.

Given the importance of these species for veterinary and human parasitology, a number of attempts have been made to identify species based on molecular markers. As a result, a relatively rich record of sequences of several mitochondrial and nuclear molecular markers is available for comparative analyses of material studied recently [3]. On the population level, SNPs [16] and microsatellites have been published [17]. A recent study proposed an easy and inexpensive PCR-based approach to distinguish among three cryptic *Galba* species [15].

Despite the variety of applicable molecular diagnostic markers, there is a significant gap of knowledge about snails referred to as *G. truncatula* on the African continent. Here, the *Galba truncatula*-like snails have a disjunct distribution with four largely isolated sub-ranges: in the mountainous parts of the Maghreb states in northern Africa [18], the highlands of Ethiopia [19], some highland areas in East Africa such as Mt. Elgon [20], Usambara Mt. [21], the Kitulo Plateau [22], the highlands of Lesotho [23], and temperate coastal, i.e. cooler, regions of South Africa [24].

When compared to the other native lymnaeid species in Africa, such as *Radix natalensis* the main host of *Fasciola gigantica*, the distribution pattern of what is considered *G. truncatula* is particularly striking (Fig. 1) being confined to allopatry in higher altitudes [20]. The discontinuous range of *G. truncatula* has been hypothesized to be the result of passive dispersal by migratory birds, being more likely perhaps than an alternative of much longer historical associations with geological vicariance of uplifted African high highlands [25]. Given scattered subfossil records in the Sahara, the Near East and Namibia [21], this could represent a range of ancestral or relic habitats isolated for eons. Another possibility would be a human- or livestock-mediated introduction, given the well-recognized anthropophily of the species [26]. In fact, historical records in the eastern part of the DR Congo have been attributed to human introductions [13]. Records of the Nile Delta in Egypt recently turned out to represent populations of *Lymnaea schirazensis* [27] and thus raise questions as to a potential camouflaged invasion in other parts of the continent. The only populations of *Galba* spp. that were identified by molecular DNA to be *G. truncatula* inhabited Mt. Elgon [20] and the Kitulo Plateau in southern Tanzania [22]. Both studies, however, used short fragments of the highly conservative nuclear ribosomal *18S* gene. Whereas, this genetic marker is sufficient to delimit *Galba* spp. from *Radix natalensis*, it is not suitable for intra-generic studies. Given this situation, it remains currently unclear whether the high-altitude African populations of *Galba* spp. indeed represent *Galba truncatula*. Moreover, it is unknown how these populations are related to populations in Europe, Asia and the Americas. Due to the complete absence of molecular assessments (but see [22]) it is, to date, impossible to reconstruct the centre of origin of *Galba* spp. and to understand when and by what means *Galba* spp. may have colonized Africa and to what extent humans might have been involved in that process.

To shed new light on the phylogeography of *Galba* spp. populations, and its impact on snail-borne

diseases, we examine several African populations using combination of mitochondrial and nuclear DNA markers to determine the identity and biogeographical affinities, reconstruct the colonization history including the likely mode of dispersal, and test for the presence of cryptic *Galba* species in Africa.

Methods

Sampling

The snail specimens studied were collected in Africa between 2010 and 2018. Field trips were conducted in the Atlas Mountains in Morocco, the highlands of Ethiopia, the Eastern Arc Mountains of Tanzania, Mt. Elgon in Uganda and the highlands of Lesotho in southern Africa (Table 1). In addition, material from outside Africa available in the collection of University of Giessen Systematics and Biodiversity (UGSB) was also used. This included material from the type-locality of *G. truncatula* in Thuringia, Germany. Snails were manually collected using a scoop net in stable pools, ponds, marshes, swamps and slow-running waters. Specimens were fixed in 80% ethanol prior to DNA extraction.

DNA extraction, amplification and sequencing

In most cases, DNA was extracted from two *Galba* specimens per locality. DNA extraction from ethanol-preserved snails was performed following the CTAB protocol of [28]. The primers used to amplify a fragment of the *cox*1 gene with a target length of 658 bp were LCO1490 and HCO2198 [29]. Amplification of the *LSU* rRNA fragment (*16S*) with a target length of 500 bp was performed with primers 16Sar and 16Sbr [30]. For the nuclear internal transcribed spacer ITS2, primers LT1 and ITS2-RIXO were used [9, 31].

PCR conditions were as described in [32]. Bidirectional sequencing was performed on an ABI 3730 XL sequencer at LGC Genomics, Berlin, Germany. *Galba* spp. samples successfully sequenced comprised two specimens from Germany, three specimens from Greece, two specimens from Slovenia, five specimens from Russia, six specimens from Nepal, one specimen from Ethiopia, five specimens from Lesotho, nine specimens from Morocco, four specimens from Tanzania, and six specimens from Uganda (Table 1).

Phylogenetic analyses

DNA sequences were edited using MEGA v.7.0 [33]. The resulting dataset was complemented with other *Galba* spp. and *Lymnaea* spp. sequences available on GenBank (Table 1). The final dataset comprised a total of 19 specimens. The *16S* partition was aligned using the online program MAFFT [34], whereas Prankster [35] was used to align the ITS2 partition. The final concatenated alignment was 1494 bp long (*16S*: 434 bp; *cox*1: 655 bp; ITS2: 405 bp). Two outgroups were used for rooting the tree, *Radix natalensis* and *Pseudosuccinea columella* (Table 1).

We used jModelTest v.2.1.4 [36] to identify the best-fit substitution model for running phylogenetic analyses based on Bayesian inference (BI) as implemented in MrBayes v.3.2.6 [37]. Based on the corrected Akaike's information criterion (AICc), the best-fit models were: GTR+Γ for *16S*, GTR+I+Γ for *cox*1, and GTR+Γ for ITS2. We ran two independent Markov Chain Monte Carlo (MCMC) searches (each with four chains) for 1 million generations and sampled every 50th tree and applied a 'burn-in' of 50%. Convergence of the two independent runs was examined *a posteriori* in Tracer 1.5 [38]. Effective sample size (ESS) values of > 200 indicated adequate sampling of posteriors distributions. In addition, a maximum likelihood (ML) analysis was conducted using RAxML-HPC2 8.2.10 [39] on the CIPRES Science Gateway [40] by applying the GTR+Γ model to all partitions and using a stop rule for the bootstrap analysis as recommended.

Estimation of divergence times

Because of the scanty fossil record of *Galba* spp. and lymnaeids in general [4] and given the absence of a specific substitution rate for Lymnaeidae or freshwater pulmonate gastropods in general, we adopted a very conservative approach of dating the molecular phylogeny. We used two substitution rates for *cox*1, i.e. 1%/myr and 2%/myr and estimated divergence times using BEAST v.1.8.4 [41]. Analyses were run for 20 million generations, sampling every 1000th tree. Convergence of runs was analyzed using Tracer v.1.5. Because convergence was not reached and ESS values were < 200, we applied the less complex HKY substitution model to the different partitions (i.e. *16S*: HKY+Γ; *cox*1: HKY+I+Γ; and ITS: HKY+Γ). The maximum clade credibility (MCC) tree was identified using TreeAnnotator v.1.8.4 (BEAST package) by applying a 'burn-in' of 50%.

Phylogeographical analyses

Phylogeographical analyses were carried out for the subset of samples from sub-Saharan Africa. The datasets consisted of 11 sequences for *cox*1, 11 sequences for *16S*, and 16 sequences for ITS2 and were individually analyzed. Relationships between haplotypes were calculated using a statistical parsimony network analysis performed using the software tool TCS v.1.21 [42] with a connection limit of 95%. Uncorrected genetic p-distances were calculated in MEGA v.7.0 [33] for within and among major *cox*1 clades inferred from the phylogenetic analyses.

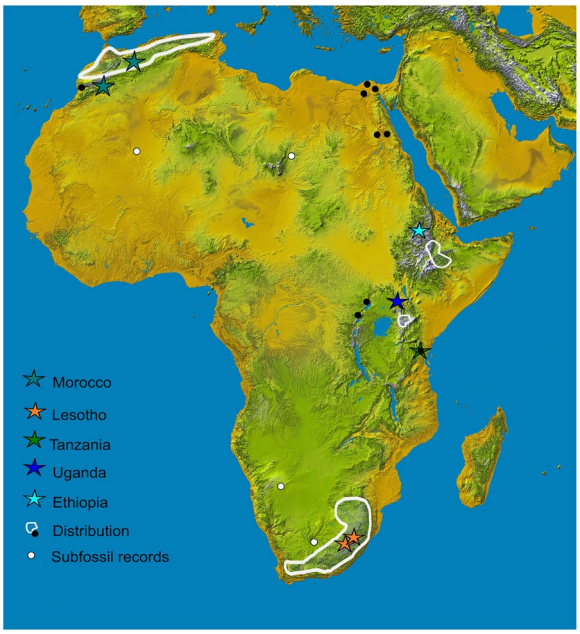

Fig. 1 Distribution map of *Galba* in Africa including the sampling for the present study (see Table 1 for details). Four sub-ranges hitherto known of *Galba truncatula* are indicated (adopted from [21] and modified from [24, 26]. Note that occurrences on the Arab Peninsula are not shown here. Black dots denote isolated occurrences; white dots represent subfossil records. Localities of the newly obtained material are shown as coloured stars

Results

Phylogenetic analyses and divergence time estimation

The phylogenetic analyses conducted resulted in a generally highly supported phylogeny (Fig. 2) including a highly supported clade (ML bootstrap values, bs = 96; MrBayes posterior probability; pp = 1.00, BEAST posterior probability; bpp = 1.00) represented by *G. truncatula* comprising samples from Europe (including the type-locality in Thuringia, Germany), Asia, and a single specimen from Morocco. The remaining African samples formed a highly supported monophyletic clade (bs = 98; pp = 1.00; bpp = 1.00) that is referred to as *G. mweruensis* hereafter, which is possibly sister to *G. truncatula* (bs = 77, pp = 0.81, bpp = 1.00). *Galba mweruensis* (Connolly, 1929) is an available name for that clade ([43]; see Discussion). The distinction of *G. mweruensis* from *G. truncatula* is further corroborated by a more comprehensive *cox*1-based phylogeny (Additional file 1: Figure S1) and genetic distances (Table 2). However, both phylogenetic approaches (MrBayes and BEAST) revealed slightly different topologies. According to the MrBayes analysis, a clade of *Lymnaea humilis* and *L. cousini* was sister to the two *Galba* species. They together formed the sister-group to the remaining South American species (*L. cubensis*, *Lymnaea* sp., and *L. viatrix*). The cryptic species *G. schirazensis* from Iran and *L. diaphana* are more distantly related. In contrast, the BEAST analysis suggests a closer relationship of *G. schirazensis* (Iran) and *Lymnaea* sp. (Colombia) to *L. truncatula* and *L. mweruensis* and also found differences in the more basal phylogenetic relationships.

The split between *G. truncatula* and *G. mweruensis* was estimated to have occurred between *c*.3.9 (95% highest posterior density, 95% HPD: 5.6–10.2) and *c*.7.8 (95% HPD: 2.8–5.1) million years ago (Ma) depending on whether a clock rate of 2%/myr or 1%/myr was used (Additional file 2: Figure S2 and Additional file 3: Figure S3). The diversification of *G. mweruensis* started between *c*.1.7 (95% HPD: 1.1–2.3) and *c*.3.4 (95% HPD: 2.3–4.6) Ma.

Phylogeographical analysis

The *cox*1 haplotype network consisted of six haplotypes, two of which belonged to populations from Tanzania and Lesotho each, whereas the single specimens from Ethiopia and Uganda represented unique haplotypes. These geographical haplotypes were all connected except for the populations from Mt. Elgon (Uganda) that were separated by at least 22 mutational steps from the remaining haplotypes and thus represented a distinct haplotype network based on the 95% connection limit (Fig. 3). Similar patterns were also revealed by the *16S* and ITS2 datasets. Populations from Tanzania and Ethiopia seem to be more closely related in the two mitochondrial networks, whereas the ITS2 dataset suggested a closer relationship between populations from Ethiopia, Lesotho and Tanzania. The individuals from Mt. Elgon were also not connected with the remaining populations in the *16S* network (separated by at least 14 mutational steps) and were separated by 8 mutational steps from the other haplotypes in the ITS2 network based on the 95% connection limit.

The genetic distance within *G. truncatula* was higher (4.4%) than within *G. mweruensis* (1.9%). The uncorrected genetic p-distance between both groups was considerably high (9.0%).

Discussion

Identity of *Galba* in Africa and phylogenetic affinities

This study found two geographically separated species of *Galba* in Africa. *Galba truncatula* is restricted based on the available evidence to the Palaearctic zone of the continent, namely Morocco. All sub-Saharan populations proved to be a distinct species according to the phylogenetic analyses and genetic distance to the sister species

Table 1 Locality, voucher (UGSB no.), and GenBank accession information for the species studied. UGSB is the acronym of the University of Giessen Systematics and Biodiversity collection

Species	Locality	Latitude	Longitude	Altitude (masl)	Code	UGSB no.	GenBank ID		
							*cox*1	*16S*	ITS2
Galba mweruensis	Lesotho, Mantsonyane	29.51682°S	28.29032°E	2212	Gmw15772	23470	MN601402	MN602685	MN602657
					Gmw15773	23471	MN601403	MN602686	MN602658
					Gmw15775	23473	MN601405	MN602688	MN602660
					Gmw15776	23474	MN601406	MN602689	MN602661
	Tanzania, Lushoto	04.44859°S	38.17837°E	1639	Gmw25316	20983	MN601423	MN602698	MN602674
					Gmw25317	20984	MN601424	MN602699	MN602675
					Gmw25318	20985	MN601425	MN602700	MN602676
					Gmw25319	20986	MN601426	MN602701	
	Ethiopia, Adi Aba Musa, Lake Ashenge	12.58650°N	39.52100°E	2409	Gmw22773	17407	MN601410	MN602707	MN602665
	Uganda, Budadiri, Mt. Elgon, Jackson's Pool	01.14951°N	34.51054°E	3939	Gmw19054	12151	MN601409	MN602706	MN602664
	Uganda, Mt. Elgon	01.14954°N	34.54736°E	3792	Gmw26767	22833			MN602677
					Gmw26769	22835			MN602678
					Gmw26770	22836			MN602679
					Gmw26771	22837			MN602680
					Gmw26772	22838			MN602681
Galba truncatula	Morocco, Marrakech-Safi	31.15573°N	07.86678°W	2100	Gtr25298	18267	MN601412	MN602690	MN602666
	Morocco, Timdighas	32.68417°N	05.33972°W	1982	Gtr25297	18265	MN601411		
	Morocco, Marlay youssef Dam	31.39272°N	07.15383°W	167	Gtr25304	20971	MN601415		
					Gtr25305	20972	MN601416		
	Germany, Thuringia, Ilm River	50.89112°N	11.24089°E	289	Gtr15785	23475	MN601407	MN602704	MN602662
					Gtr15786	23476	MN601408	MN602705	MN602663
	Greece, Rhodos Island, 7 springs dam lake, on mud	36.25464°N	28.11596°E	232	Gtr25308	20975	MN601419	MN602694	MN602670
					Gtr25306	20973	MN601417		
					Gtr25307	20974	MN601418	MN602693	MN602669
	Russia, Ilovlya, river near Ilovlya Town	49.31367°N	43.97659°E	43	Gtr25312	20979	MN601420	MN602695	MN602671
	Russia, Moscow Region, Oka River	na	na		Gtr25313	20980	MN601421	MN602696	MN602672
					Gtr25314	20981	MN601422	MN602697	MN602673
	Slovenia, Vrhnika, creek Obrh	45.69906°N	14.51176°E	376	Gtr25299	18543	MN601413	MN602691	MN602667
					Gtr25301	18860	MN601414	MN602692	MN602668
	Nepal, Karnali	29.26667°N	82.15933°E	2300	Gtr11234	23477	MN601399	MN602702	MN602654
					Gtr12653	23478		MN602703	MN602656
	Nepal, Bagmati	29.30000°N	82.36667°E	2700	Gtr11235	23479	MN601400	MN602684	MN602655
	Nepal, Bheri	29.10717°N	82.58867°E	2625	Gtr11237	23481	MN601401		
	France, Limoges				GB2			HQ283236	HQ283262
Lymnaea schirazensis	Iran, Gilan Province, Taleb-Abad River				GB1		JF272607	JF272605	
Lymnaea humilis	USA, New York				GB3			FN182195	FN182191
Lymnaea cousini	Venezuela, Mucubají				GB4			HQ283237	HQ283266
Lymnaea cubensis	USA, South Carolina				GB5			FN182204	
Lymnaea diaphana	Argentina, Lago Escondido				GB6			HQ283241	HQ283260
Lymnaea sp.	Colombia, Antioquia				GB7			HQ283235	HQ283263
Lymnaea viatrix	Argentina, Rio Negro				GB8			HQ283239	HQ283265
Radix natalensis	Kenya, Kisumu, Lake Victoria	00.12739°S	34.74232°E	1140	Rna15771	23483	MN601427	MN602708	MN602708
Pseudosuccinea columella	South Africa, Mpumalanga	24.84539°S	30.83879°E	1374	Pco15787	23484	MN601428	MN602709	MN602683

Abbreviations: na, not available; masl, meters above sea level

G. truncatula from Europe and Asia. Interestingly, no *G. schirazensis* was found at the examined localities, which further supports the hypothesis that mountain ranges of tropical Africa are inhabited by a species different from *G. truncatula* and its cryptic counterpart *G. schirazensis* has not had opportunity to disperse into these areas or is unable to do so. We therefore propose to use the existing name *G. mweruensis* (Connolly, 1929) for this species that was described based on shell features and size measures (for a comparison of the original type-material and our new populations see Additional file 4: Figure S4; Additional file 5: Table S1). Moreover, it is morphologically indistinguishable from the other two species hitherto known to occur in Africa, i.e. *G. truncatula* and *G. schirazensis* (Additional file 6: Figure S5). *Galba mweruensis* is not the oldest available name for African *Galba* species for which even the section name *Afrogalba* had been introduced by Kruglov & Starobogatov [44]. Another taxon described earlier is *Galba umlaasianus* (Küster, 1862) from the Umlaas River, South Africa. Recent repeated attempts to obtain material from *terra typica* in the Kwa Zulu Natal Province of South Africa unfortunately failed. However, *G. umlaasiana* originally has been referred to as a lowland species of the temperate zones along the coastal regions of South Africa, whereas *G. mweruensis* has been described from mountainous terrain from Mweru town (type-locality) at the foothills of Mt. Kenya, which is somewhat in the core range of the species we found to occur widely in tropical Africa. Attempts to locate a population in the Mweru region in central Kenya in 2010 unfortunately failed. Moreover, Vinarski [45] compared both *G. mweruensis* and *G. umlaasiana* with the newly described *G. robusta* from Yemen and found the former two species to be morphologically different. We therefore propose to use the name *G. mweruensis* for mountainous *Galba* populations until it can be compared with topotypic material of *G. umlaasianus*. The latter taxon might even represent another distinct species given its different altitudinal range and may potentially co-occur with *R. natalensis* in the lower altitudes. Such a co-occurrence has not been observed for *G. mweruensis* in the studies that were conducted in the highlands of Lesotho (as *G. truncatula* in [24]), the Kitulo Plateau in Tanzania [22], and Mt. Elgon in Uganda [20]. In South Africa, however, either *G. truncatula* (*G. umlaasianus*), *L. natalensis* or the invasive *P. columella* have been reported to occur sympatrically [24].

Among the newly genotyped specimens of this species, the population from Mt. Elgon in Uganda is of particular interest. Mandahl-Barth [46] identified a small form of *Galba* at Mt. Elgon at 2770 m and attributed it to *G. mweruensis*. According to the present analyses, this population turned out to be sister to the remaining populations from Ethiopia, Lesotho and Tanzania, and the Mt. Elgon population was very distantly related to the remaining groups in the phylogeographical analyses. A more detailed analysis that investigates morphological and anatomical characters is needed in order to establish the status of the Mt. Elgon populations compared to their sub-Saharan counterparts. Hubendick [26] had material from the Kenyan slopes of Mt. Elgon and found similarities to *G. truncatula* but treated it as *G. mweruensis*. Isolated records of *Galba* spp. from the eastern part of the DR Congo west of Lake Albert and at Lake Kivu from considerably lower altitudes have not been confirmed during the last decades [21, 47].

The genetic diversity within *G. mweruensis* is comparable to that of other distinct *Galba* species such as *G. shirazenzis* [26]. Given the continuous and by far greater distributional range of *G. truncatula*, the higher degree of genetic differentiation in *G. truncatula* compared to *G. mweruensis* is not surprising. Nevertheless, the comparatively high genetic diversity within *G. mweruensis* raises the question as to how this diversity in isolated patches scattered over Africa has evolved and how these areas have been colonized. Further study in detail of several life-history traits for survival in cooler zones could be illuminating.

Colonization history

Our study indicates that subtropical Africa has been colonized only once in either the Pliocene or even Miocene if one considers the age of the most recent common ancestor of *G. truncatula* and *G. mweruensis* as indicative of colonization time. Diversification within the African species *G. mweruensis* is dated to the Plio-Pleistocene and thus human-mediated dispersal can be ruled out for the initial colonization of the mountain ranges. We here applied commonly used substitution rates for mitochondrial markers in invertebrates, i.e. 1%/myr and 2%/myr (i.e. divergence rates of 2%/myr and 4%/myr). Assuming that *Galba* may have evolved with an extremely fast substitution rate of 4%/myr, the split would, of course, become younger (*c*.2 Ma). However, this would not change our conclusions that the hypothesis of human-mediated dispersal can be rejected. However, the data do not currently allow drawing a final conclusion as to whether Africa has been colonized from Europe, the Near East or South America. The tree topology may favour a colonization scenario out of Europe; however, Asian and especially Near East samples of *G. truncatula* are scarce and *G. robusta* (Yemen) could not be included. Subfossil records in Africa are also not very helpful as they originate from less mountainous regions and are not very informative given the small morphospace occupied by all *Galba* species. However, recent and subfossil

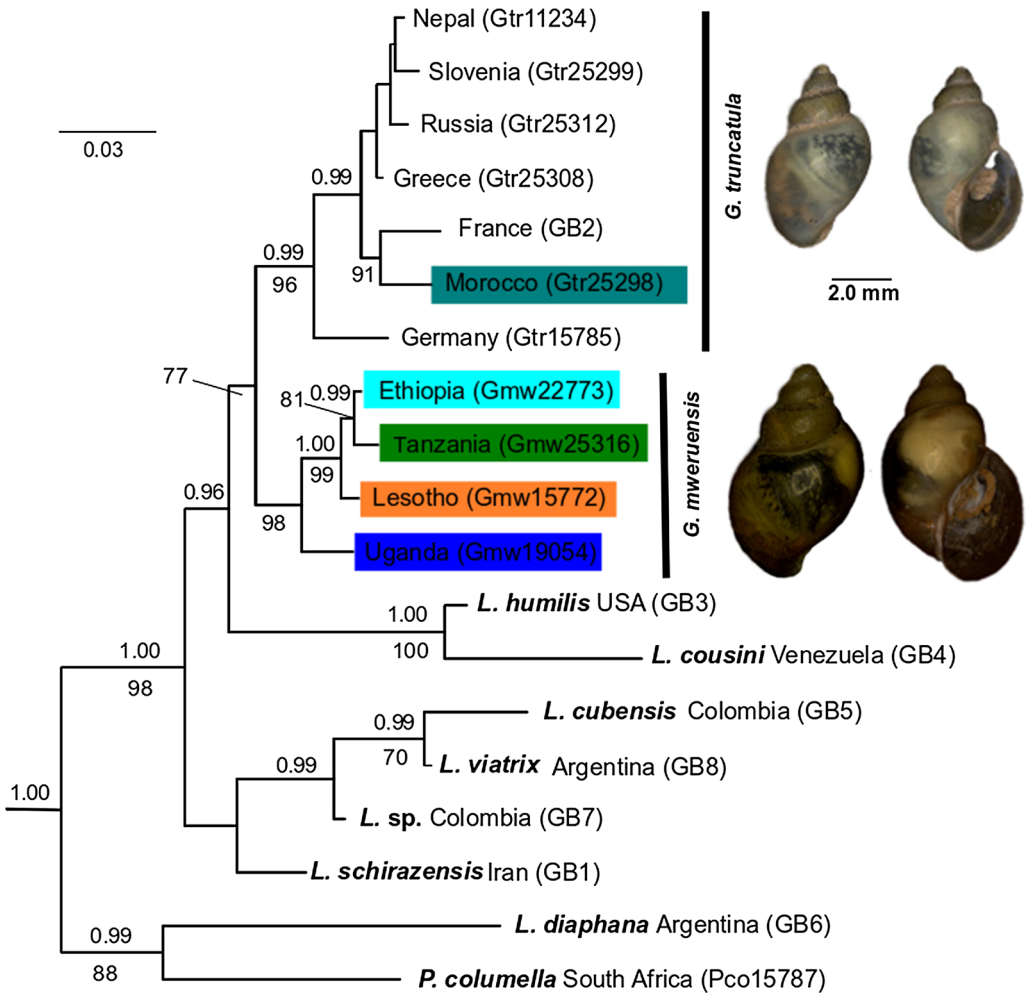

Fig. 2 Bayesian inference phylogram based on concatenated *cox*1, *16S* and ITS2 sequences. The two outgroups have been removed *a posteriori*. Bayesian posterior probabilities are provided next to each node (top: MrBayes, bottom: RAxML). Sequences obtained from GenBank are labelled as GB1–GB8 (see Table 1). Nodes 1 and 2 indicate the nodes for which divergence time estimates are discussed. Colour codes used for species represent the origin of African samples and refer to those used in the map in Fig 1. Shell images are from *Galba truncatula* (Morocco, Gtr25298) and *Galba mweruensis* (Tanzania, Gmw25316). The scale-bar indicates substitutions per site according to the applied models of sequence evolution

Table 2 Genetic distances of *Galba mweruensis* and *Galba truncatula* based on the *cox*1 dataset

	Uncorrected p-distance (%)			K2P model		
	G. mweruensis	*G. truncatula*	*G. mweruensis* vs *G. truncatula*	*G. mweruensis*	*G. truncatula*	*G. mweruensis* vs *G. truncatula*
Minimum	0.2	0.0	7.1	–[a]	–[a]	–[a]
Maximum	4.2	7.8	9.7	–[a]	–[a]	–[a]
Mean	2.3	3.2	8.4	2.4	3.4	9.0

Note: Uncorrected genetic p-distances and genetic distances based on the K2P model were calculated in MEGA v.7.0 [33]

[a] Not calculated

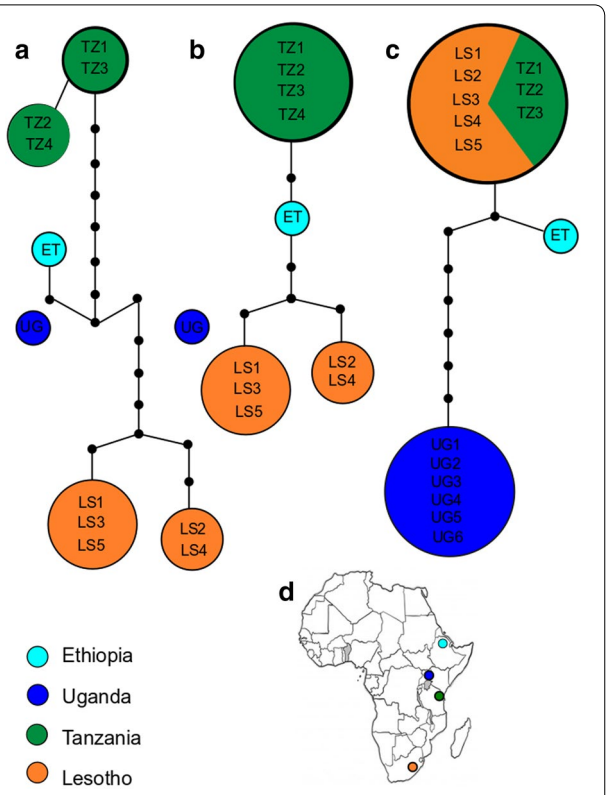

Fig. 3 TCS maximum parsimony network of *Galba mweruensis* based on *cox1* (**a**), *16S* (**b**) and ITS2 sequences (**c**). **d** Map showing the locations of the studied populations. The possible ancestral haplotypes are highlighted in bold, and the size of the circles corresponds to the number of individuals belonging to the respective haplotypes. Mutational steps representing missing haplotypes are displayed as small black circles

Saharan records [18, 21] may indicate a stepping-stone dispersal for the northern Africa *G. truncatula* populations. The generally much higher lymnaeid diversity in the northern hemisphere makes an 'out of Africa' alternative for the *Galba* less likely. However, given the existence of the cryptic *G. schirazensis* in Egypt [27], no conclusion can be drawn here. On the intra-continental scale, a closer relationship between the Northeast and East African populations in comparison to the populations of the highlands of Lesotho would be expected. However, according to our analyses, specimens from Mt. Elgon are genetically more distinct compared to the remaining sub-Saharan haplotypes.

Dispersal by water birds, also at high altitudes, has been commonly shown to be a major factor in range evolution for freshwater molluscs in general [48] and pulmonate snails in particular [49]. To which extent water birds might have been involved in the colonization of these isolated mountain ranges can only be speculated. If such dispersal is as frequent as demonstrated in other regions [50, 51], *G. mweruensis* should be more widespread across different mountain ranges in sub-Saharan Africa.

Africa has experienced severe climatic fluctuations since the late Miocene and especially in the Plio-Pleistocene [52]. The patchy distribution pattern observed may thus reflect the emergence of climatic refugia in these mountain ranges that acted as islands in the sky [53]. Such relictary species distributions in African mountain ranges have been documented for diverse taxa such as birds [54], flightless insects [55] and frogs [56]. Although the status of *G. umlaasiana* has not been assessed yet, a correlation of cooler climates and the occurrence of *G. mweruensis* is apparent. Alternatively, the presence of the omnipresent and thus potentially competitive *R. natalensis* may considerably restrict the distribution of *G. mweruensis* to more temperate areas. Although mountain ranges are sometimes acting as refugia, they are also sensitive to climate changes [57]. Small and isolated populations might thus go through repeated bottlenecks and might experience local disappearance as found for the *Galba* population on Kitulo Plateau, Tanzania. A recent field survey (FC in October 2018) showed that the swampy habitats where the species earlier occurred [22] had completely dried out. A high estivating potential for *Galba* is, however, reported from highlands of Ethiopia [58].

Parasitological implications of cryptic *Galba* species in Africa

Despite its patchy continental distribution, *G. mweruensis* is well established, especially in the extensive sub-ranges (Fig. 1). We here confirmed its presence in regions where it has not been observed for decades such as the Usambara Mountains (Tanzania) or Mt. Elgon in Uganda. It is also the predominant snail species in the highlands of Ethiopia and Lesotho and thus should be the intermediate host for livestock fascioliasis and potentially other trematode infections in that region [19, 59]. Dinnik & Dinnik [60] already pointed out that *G. mweruensis* is the intermediate host of both liver flukes, *F. hepatica* and *F. gigantica*, and thus not only represent major threats for livestock. For livestock, considerable economic losses are known from several African countries [61]. We suggest that there is a need to now ascertain the level of snail-parasite compatibility of *G. mweruensis* with several isolates of *F. hepatica* and *F. gigantica*, especially where these snails are found in cattle farmed areas.

Although estimating the prevalence of human fascioliasis is challenging [62], infection risks should be considered high wherever the intermediate host occurs [22]. Outbreaks can happen quickly, and the extent is often

underestimated as recently outlined for the mountains in northern Tanzania [63]. Unlike with other human snail-borne diseases such as schistosomiasis, there is a high prevalence in high altitude regions. A prime example is the endemic in the Andean Altiplano [14, 64]. Although high mountainous regions are still considerably remote and less densely populated in Africa, there is a growing demand for land and thus humans increasingly occupying high elevations [65]. Even touristic activities such as trekking and mountain climbing are on the rise in basically all the mountain ranges where *G. mweruensis* occurs so further surveillance is warranted. Therefore, more dedicated surveys on infection and prevalence rates and the study of parasites actually hosted by *G. mweruensis* are necessary in all the areas where this species is established [20]. Whereas *G. schirazensis* is not particularly involved in transmission of *F. hepatica* [27], high rates of infection have been reported for *G. mweruensis* (originally *G. truncatula*) populations from Lesotho and Ethiopia [58, 66].

Conclusions

This study has identified a hitherto neglected distinct species, *G. mweruensis*, as a host of *F. hepatica* throughout sub-Saharan Africa. It had previously been considered to be conspecific with Eurasian *G. truncatula*, a well-known and globally intermediate host species for several trematode parasites. Following our findings, a closer examination of the parasite communities hosted by *G. mweruensis* is needed in order to understand transmission patterns in highlands throughout eastern and southern Africa. Other high altitudes areas in Africa are to be surveyed for this species and veterinary and human health concerns have to be evaluated under the new precondition. It would be also interesting to study host specificity and potential climatic adaptations of both the host and the preferred temperature range of *F. hepatica* in Africa. The nature of striking non-overlap in occurrences between the omnipresent *R. natalensis* and *G. mweruensis* deserves more scientific attention because of its evolutionary implications and possible epidemiological cross-over as implicated host of *F. gigantica* and *F. hepatica*.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13071-019-3825-9.

Additional file 1: Figure S1. Bayesian inference phylogram based on *cox*1. The two outgroups have been removed *a posteriori*. Bayesian posterior probabilities are provided next to each node (top: MrBayes, bottom: RAxML). Sequences obtained from GenBank are labelled plain whereas new sequences from this study are bold. Nodes 1 and 2 indicate the nodes for which divergence time estimates are discussed.

Additional file 2: Figure S2. BEAST molecular clock tree based on an HKY model and a substitution rate of 1%.

Additional file 3: Figure S3. BEAST molecular clock tree based on an HKY model and a substitution rate of 2%.

Additional file 4: Figure S4. Shell measurements of *Galba mweruensis* populations in comparison to the type specimen as described in Connolly, 1929 (p. 175).

Additional file 5: Table S1. Shell measurements of *Galba mweruensis* in the highlands of Lesotho, Tanzania and Mt. Elgon in Uganda.

Additional file 6: Figure S5. Shell, soft body anatomy and reproductive organs of *Galba mweruensis* from Lesotho (Mantsonyane). *Abbreviations*: BC, bursa copulatrix; PHT, phallotheca; PRP, praeputium; VD, vas deferens.

Abbreviations

asl: above sea level; ESS: effective sample size; Gtr: *Galba truncatula*; Gmw: *Galba mweruensis*; Pco: *Pseudosuccinea columella*; PCR: polymerase chain reaction; Rna: *Radix natalensis*; UGSB: University of Giessen Systematics and Biodiversity collection.

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Authors' contributions

AM and CA conceived the study. AM produced the sequences and performed data analyses, with the help of CC and BS. CA, CC and FC collected part of the material, and all authors were involved in data interpretation. AM produced the figures. All authors critically reviewed the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in the article and its additional files. The newly generated sequences were submitted to the NCBI GenBank database under the accession numbers MN601399–MN601428 for *cox*1, MN602684–MN602709 for *16S*, and MN602654–MN602683 for ITS2.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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2.3. Assessing the diversity and distribution of potential intermediate hosts snails for urogenital schistosomiasis: *Bulinus* spp. (Gastropoda: Planorbidae) of Lake Victoria

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Assessing the diversity and distribution of potential intermediate hosts snails for urogenital schistosomiasis: *Bulinus* spp. (Gastropoda: Planorbidae) of Lake Victoria

Fred D. Chibwana[1,2*], Immaculate Tumwebaze[1], Anna Mahulu[1], Arthur F. Sands[1] and Christian Albrecht[1]

Abstract

Background: The Lake Victoria basin is one of the most persistent hotspots of schistosomiasis in Africa, the intestinal form of the disease being studied more often than the urogenital form. Most schistosomiasis studies have been directed to *Schistosoma mansoni* and their corresponding intermediate snail hosts of the genus *Biomphalaria*, while neglecting *S. haematobium* and their intermediate snail hosts of the genus *Bulinus*. In the present study, we used DNA sequences from part of the cytochrome *c* oxidase subunit 1 (*cox*1) gene and the internal transcribed spacer 2 (ITS2) region to investigate *Bulinus* populations obtained from a longitudinal survey in Lake Victoria and neighbouring systems during 2010–2019.

Methods: Sequences were obtained to (i) determine specimen identities, diversity and phylogenetic positions, (ii) reconstruct phylogeographical affinities, and (iii) determine the population structure to discuss the results and their implications for the transmission and epidemiology of urogenital schistosomiasis in Lake Victoria.

Results: Phylogenies, species delimitation methods (SDMs) and statistical parsimony networks revealed the presence of two main groups of *Bulinus* species occurring in Lake Victoria; *B. truncatus*/*B. tropicus* complex with three species (*B. truncatus*, *B. tropicus* and *Bulinus* sp. 1), dominating the lake proper, and a *B. africanus* group, prevalent in banks and marshes. Although a total of 47 *cox*1 haplotypes, were detected within and outside Lake Victoria, there was limited haplotype sharing (only Haplotype 6 was shared between populations from Lake Victoria open waters and neighbouring aquatic systems) – an indication that haplotypes are specific to habitats.

Conclusions: The *Bulinus* fauna of Lake Victoria consists of at least *B. truncatus*, *B. tropicus*, *Bulinus* sp. 1 (*B. trigonus*?) and *B. ugandae*. The occurrence and wide distribution of *Bulinus* species in Lake Victoria potentially implies the occurrence of urogenital schistosomiasis in communities living along the shores and on islands of the lake who depend solely on the lake for their livelihood. More in-depth studies are needed to obtain a better picture of the extent of the disease in the Lake Victoria basin.

Keywords: African lakes, Epidemiology, Phylogeography, Neglected tropical diseases, *Schistosoma haematobium*

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Background

Schistosomiasis is a parasitic disease caused by digenean trematodes of the genus *Schistosoma* and is a socio-notable disease in tropical and subtropical regions. It is prevalent in more than 78 countries and territories infecting more than 250 million people worldwide, most of whom inhabit sub-Saharan Africa [1, 2]. Although more than 20 *Schistosoma* species are recognised, only *Schistosoma mansoni* and *S. haematobium* are ubiquitously known in sub-Saharan Africa due to their capability to cause intestinal and urogenital schistosomiasis, respectively [1, 3, 4]. The highest infections and disease burdens are frequently found in school-aged children, particularly in settings with poor hygiene and sanitary facilities [5]. Human hosts infected with these *Schistosoma* species experience acute hyperaemia, abnormal growth, internal haemorrhaging, fibrosis and tissue thickening [6]. As a result, infection with *S. mansoni* culminates with liver fibrosis, portal hypertension and ascites, while bladder cancer is the final stage of a *S. haematobium* infection [7]. Furthermore, genital schistosomiasis complications associated with *S. haematobium* infections include hypertrophic and ulcerative lesions of the female genital tract [8]. *Schistosoma* species, like other digenean trematodes, utilise pulmonate snails to complete their two-host life-cycles; i.e. *Biomphalaria* spp. for *S. mansoni* and *Bulinus* spp. for *S. haematobium* [1, 3, 4].

The Lake Victoria ecoregion of the East African Rift System, is characterized by a wealth of extraordinary freshwater biodiversity that has accumulated throughout the Quaternary, including almost 700 species of cichlid fishes [9, 10]. Major geological and climatological changes occurred in this region during this period. These changes are linked to the development of the East African Rift. More recently, anthropogenic pressures in the Lake Victoria ecoregion have grown exponentially due to multifactorial stressors such as habitat degradation, pollution, exploitation, the introduction of invasive species, ecosystem modifications and climate change [11, 12]. Insights into the consequences of recent and historic environmental changes in the region are crucial to understanding the diversification dynamics of freshwater biota. Effects of ecosystem changes on the community composition and demography of benthic organisms remain poorly assessed since few studies have been conducted apart from cichlid fishes and the schistosome intermediate host snail genus *Biomphalaria* [13].

Lake Victoria is endowed with a remarkable mollusc fauna, although it is less diverse than in lakes Malawi and Tanganyika, perhaps due to its younger age and relative shallowness [10, 14]. Despite its young age of about 400,000 years, Lake Victoria has experienced three major desiccation events within the last 100,000 years [15, 16]. The current water body arose about 14,600 years ago [15, 16], which is relatively shorter for snail species radiation [10, 17]. Nevertheless, Brown et al. [17] listed 28 gastropod species in Lake Victoria, of which six are medically significant species within genera *Bulinus* (4 species) and *Biomphalaria* (2 species). Lake Victoria, which is shared between Tanzania, Uganda and Kenya, therefore plays a significant role in the persistence of schistosomiasis in these surrounding countries [18–20]. Despite the increasing efforts to control schistosomiasis with praziquantel through mass drug administration (MDA) programmes, East African countries are still among the hotspots for this parasitic disease. Herein, the majority of schistosomiasis cases are reported from fishing communities and particularly in school-aged children surrounding Lake Victoria [21–24]. The vast majority of studies focusing on *Schistosoma* and their intermediate hosts in Lake Victoria and neighbouring aquatic systems have mainly focused on *S. mansoni* and *Biomphalaria* spp. [13, 18, 25] while overlooking *Bulinus* spp. and their potential role in the urogenital schistosomiasis transmission (i.e. *S. haematobium*). However, identifying these potential *Bulinus* hosts is an initial step in estimating the extent and relevance of urogenital schistosomiasis in the given area [26, 27].

The genus *Bulinus* consists of 37 species occurring mainly in Africa, the Middle East and in the Mediterranean Area [17]. The recognized *Bulinus* species fall into four groups, namely the *Bulinus africanus*, *B. reticulatus*, and *B. forskalii* species groups, and the *B. truncatus*/*B. tropicus* species complex. Many species within these groups except, for example, *B. tropicus* and *B. ugandae* are involved in the transmission of *S. haematobium* [28, 29]. Moreover, *B. africanus* group species play an important role in the transmission of *S. haematobium* and *S. bovis* in Central East Africa [17]. However, precise species identification of snails of the genus *Bulinus* is often difficult because of strong morphological similarities and overlap among species, the coexistence of different forms and groups in a narrow area and the lack of well-defined criteria by which to distinguish species [17, 29]. Additionally, some studies have also reported the existence of cryptic species in some localities [30, 31], which further exacerbates the taxonomic uncertainties within genus *Bulinus*.

The knowledge of the number of *Bulinus* species occurring within or nearby Lake Victoria is obscure. For instance, Mandahl-Barth [29] recognised *B. trigonus* and *B. transversalis* as independent species, but Brown [17] viewed them as lacustrine morphs of *B. tropicus* and *B. truncatus* or synonyms of unnamed *Bulinus* species (*Bulinus* sp.). Moreover, there is a scarcity of information on the geographical distribution patterns of *Bulinus* species in the lake. *Bulinus trigonus* and *B. transversalis*

have their type-localities in the Tanzanian side of Lake Victoria, while *B. ugandae* was first found in Jinja Bay, Uganda [17]. Surveys by Mwambungu [32] reported the occurrence of *B. ugandae* in the Speke Gulf of the lake in Tanzania, Ngupula & Kayanda [33] found *B. ugandae* and *B. transversalis* in Uganda and Opisa et al. [34] and Nyakaana et al. [35] reported the existence of *B. globosus* in the lake shores in Kenya and Uganda. Although the separation of *B. ugandae* from *B. globosus* is dubious and the overall taxonomy of *Bulinus* spp. in Lake Victoria is uncertain [17], it is not clear if all the four *Bulinus* groups are represented in the lake. Moreover, knowledge of how the four groups may be spatially distributed remains questionable. Moreover, *Bulinus* species such as *B. ugandae* and *B. trigonus*, whose type-materials come from Lake Victoria, are not endemic to the lake, similar morphs have been recorded in lakes Mutanda and Edward as well [17].

In many biological cases where conventional analyses have failed to identify species, molecular techniques, particularly phylogenetic approaches using DNA sequence data, have proven successful. For example, the application of markers, such as cytochrome *c* oxidase subunit 1 (*cox*1) and nuclear genes such as the internal transcribed spacer (ITS) regions, *28S* and *18S*, have facilitated species identification of *Bulinus* spp. [31, 36, 37]. In the present study we, therefore, used two more variable genetic markers, *cox*1 and ITS2, to investigate the phylogeography of *Bulinus* species occurring in Lake Victoria. This information is invaluable in improving our understanding of *Bulinus* species identities and phylogenetic relationships, as well as the epidemiology of the potential urogenital schistosomiasis.

Therefore, we combine mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) markers to investigate *Bulinus* populations obtained from a longitudinal survey in Lake Victoria and neighbouring aquatic systems to (i) determine the identity, diversity and phylogenetic position of the species, (ii) reconstruct phylogeographical affinities and (iii) determine the population structures of the species. We discuss the results and their implications for the potential transmission and epidemiology of urogenital schistosomiasis in Lake Victoria.

Methods

Source of material for genomic DNA

Pulmonate snails of the genus *Bulinus* were collected from 20 locations around Lake Victoria and (for comparative purposes) from an additional four locations in the neighbouring aquatic systems of the River Nile and Lake Mburo-Nakivale (Fig. 1, Table 1). Sampling was carried out in open waters, on shoreline banks, around islands and in bordering marsh habitats where water was either stagnant or relatively calm. Specimens were hand-picked off water plants, rocks, stones or the floor bottom where they were more easily accessible or collected with strainers, long handheld scoops and dredges in more challenging situations (e.g. deeper waters). Dredging was carried out repeatedly per site in depths from 2 m down to approximately 25 m in the Kenyan and Ugandan part of the lake. In most of the sites, sampling was carried out close to active anthropogenic activities (e.g. fish landing sites or ferry docks) and for at least 30–60 min. All specimens were collected during various field trips from 2010–2019 and snails identified as *Bulinus* spp. were preserved in 80% ethanol.

DNA extraction, amplification and sequencing

Genomic DNA was extracted using the CTAB method [38] from 2–5 specimens per locality for a total of 74 specimens. A 655-bp target fragment of the mtDNA *cox*1 gene was amplified using primers and PCR conditions given by Folmer et al. [39]. In a few cases, the region was amplified using the primers LCO1490 [39] and COR722B [40] and PCR conditions as detailed by Kane et al. [37]. Primers LT1 and ITS2-RIXO and PRC conditions stated by Almeyda-Artigas et al. [41] and Bargues et al. [42] were used to amplify the rDNA ITS2 region. Sanger sequencing was performed by LGC Genomics GmbH (Berlin, Germany).

Phylogenetic analyses

Chromatograms were assembled and inspected using Geneious version 8.0.6 (Biomatters, Auckland, New Zealand; Kearse et al. [43]). Multiple alignments were generated for each marker, with the ClustalW tool [44] implemented in BioEdit version 7.0.5.3 [45]. Newly generated sequences from 74 specimens were combined with 57 additional available sequence data from GenBank to expand our datasets (Additional file 1: Table S1). The online program MAFFT [46], was used to align the ITS2 partition. The phylogenetic trees of the concatenated datasets of 620 bp *cox*1 and ITS2 were estimated using Maximum Likelihood (ML) and Bayesian Inference (BI) analyses. The *cox*1 and ITS2 partitions were concatenated using Sequences Matrix version 1.2.8 [47]. In both cases, *Indoplanorbis exustus* was used as the outgroup. The best sequence evolutionary model to each partition was evaluated with jModelTest version 2.1.4 [48]. Based on the Akaike's information criterion (AIC), HYK + G and GTR+G were selected as the best evolutionary models for *cox*1 and ITS2 datasets, respectively. ML analysis was conducted using Randomized Accelerated Maximum Likelihood (RAxML version 7.0.4; [49]) with a bootstrap of 1000 replicates. Bayesian inference analysis, to obtain an ultrametric tree for the General Mixed Yule

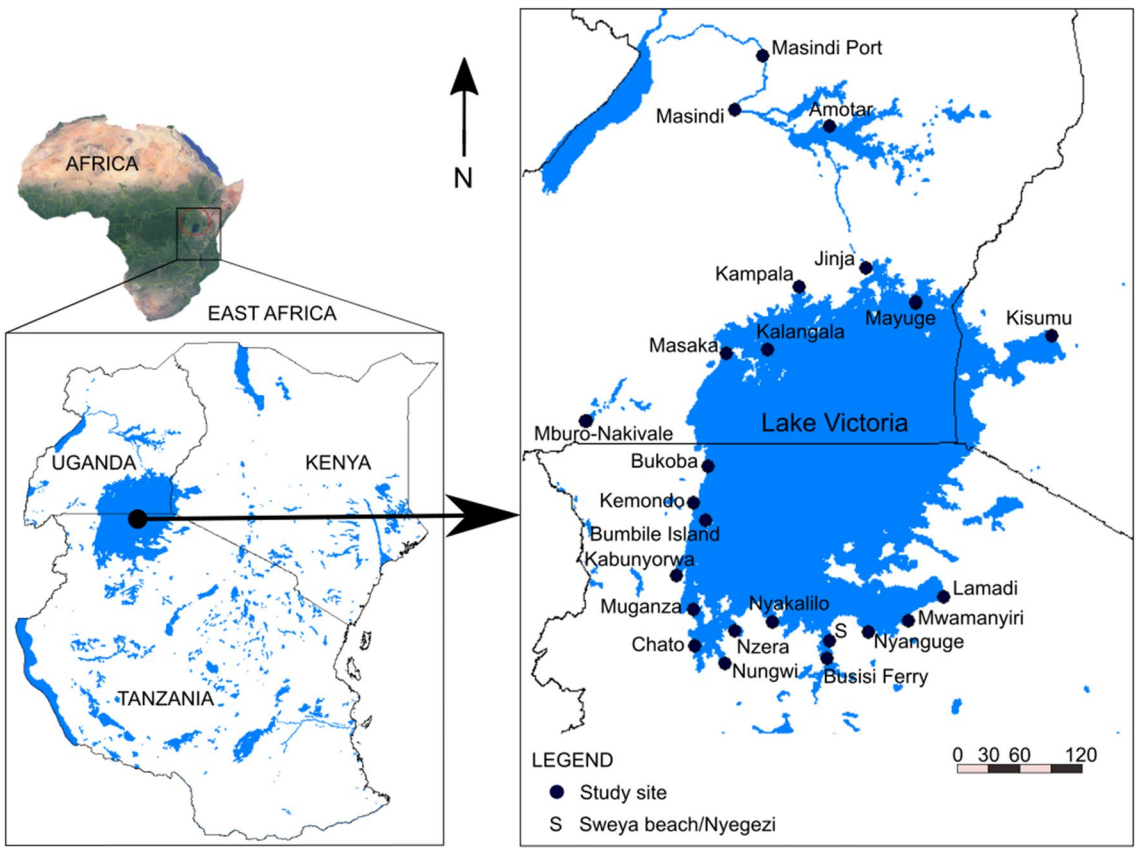

Fig. 1 Study area map of East African countries that share Lake Victoria and the sampling sites for this study are shown

Coalescent (GMYC) model of species delimitation [50], was carried out using BEAST version 1.8.4 [51]. Runs consisted of 5,000,000 MCMC generations, sampling every 500th tree. Validation of convergence and mixing was assessed in Tracer 1.5 [52] to ensure that all effective sample size (ESS) values were > 200. We used TreeAnnotator 1.8.4 (BEAST package) to identify the maximum clade credibility (MCC) tree by discarding 50% of the trees as 'burn-in'.

We applied two DNA-based species delimitation methods (SDMs) with single and multiple delimiting thresholds to resolve the species boundaries in *Bulinus* specimens incorporated. These were the Poisson Tree Process (PTP [53]) and the GMYC method as mentioned above. Both mPTP (maximum likelihood, PTP and Bayesian, bPTP) and GMYC analyses were carried out with the web-based service at https://species.h-its.org/.

Phylogeographical and population analyses

Phylogeographical analyses were performed for the novel *cox*1 sequences of the *Bulinus* specimens from Lake Victoria and the neighbouring systems (i.e. Lake Mburo-Nakivale and the River Nile). The dataset consisted of the 74 sequences generated herein. The relationships between haplotypes were identified through a statistical parsimony network constructed in TCS version 1.21 [54] with 95% confidence.

For genetic diversity, differentiation and population expansion or shrinkage *cox*1 sequences belonging to the *Bulinus* specimens from Lake Victoria basin were split into two groups representing *B. truncatus* and *Bulinus* sp. 2. *Bulinus truncatus* sequences were divided into three subpopulations based on habitat, namely, lentic sand substrate, lentic stones and rock substrates and lotic habitats. The sequences forming the *Bulinus* sp. 2 group were also divided into three subpopulations based on lentic habitats; islands, papyrus swamps and marshes (water hyacinth). We estimated haplotype diversity (h) and nucleotide diversity (π) [55] using DnaSP version 6.12.03 [56]. Moreover, we performed analyses of molecular variance (AMOVA), to examine the amount of genetic variability within and between populations, using Arlequin version 3.5.2.2 [57].

The mitochondrial DNA sequence data were also tested for deviation from neutral expectations (e.g. population expansion events). Genetic equilibrium was assessed

Table 1 Locality, voucher, sequence and haplotype information for the *Bulinus* spp. from Lake Victoria studied

Species	Locality	Country	Latitude	Longitude	Voucher No.	Sequence ID	Habitat	Haplotype ID	GenBank ID	
									*cox*1	ITS2
B. truncatus	Igabiro	Tanzania	−1.17769	31.87792	UGSB 22907	Bkt26885	Stone and rocks	BKT1	MT707360	MT707212
B. truncatus	Igabiro	Tanzania	−1.17769	31.87792	UGSB 22908	BKt26886	Stone and rocks	BKT2	MT707361	
B. truncatus	Kemondo	Tanzania	−1.47796	31.7498	UGSB 22909	Ket26887	Stone and rocks	KET1	MT707362	MT707222
B. truncatus	Kemondo	Tanzania	−1.47796	31.7498	UGSB 22910	Ket26888	Stone and rocks	KET2	MT707363	
Bulinus sp. 2	Bumbire Island	Tanzania	−1.61476	31.85625	UGSB 22911	Bit26889	Island	BIT1	MT707364	
Bulinus sp. 2	Bumbire Island	Tanzania	−1.61476	31.85625	UGSB 22912	Bit26890	Island	BIT2	MT707365	MT707234
Bulinus sp. 2	Bumbire Island	Tanzania	−1.61476	31.85625	UGSB 22946	Bit26924	Island	BIT3	MT707366	
Bulinus sp. 2	Bumbire Island	Tanzania	−1.61476	31.85625	UGSB 23464	Bit27073	Island	BIT4	MT707367	
Bulinus sp. 2	Bumbire Island	Tanzania	−1.61476	31.85625	UGSB 23465	Bit27074	Island	BIT5	MT707368	
Bulinus sp. 2	Kabunyorwa	Tanzania	−2.06018	31.61382	UGSB 22913	Kbt26891	Papyrus	KBT1	MT707369	
Bulinus sp. 2	Kabunyorwa	Tanzania	−2.06018	31.61382	UGSB 22914	Kbt26892	Papyrus	KBT2	MT707370	MT707235
B. truncatus	Muganza	Tanzania	−2.33702	31.75166	UGSB 22915	Mut26893	Open water	MUT1	MT707371	
B. truncatus	Muganza	Tanzania	−2.33702	31.75166	UGSB 22916	Mut26894	Open water	MUT2	MT707372	MT707220
Bulinus sp. 2	Muganza	Tanzania	−2.33702	31.75166	UGSB 22940	Mut26918	Water Hyasin	MUT3	MT707373	MT707236
Bulinus sp. 2	Muganza	Tanzania	−2.33702	31.75166	UGSB 22941	Mut26919	Water Hyasin	MUT4	MT707374	
B. truncatus	Muganza	Tanzania	-2.33702	31.75166	UGSB 22945	Mut26923	Open water	MUT5	MT707375	
Bulinus sp. 2	Chato	Tanzania	−2.63292	31.76368	UGSB 23463	Cht27072	Papyrus	CHT1	MT707376	
Bulinus sp. 2	Nungwe	Tanzania	−2.77446	32.0136	UGSB 22919	Nut26897	Papyrus	NUT1	MT707377	
Bulinus sp. 2	Nungwe	Tanzania	−2.77446	32.0136	UGSB 22920	Nut26898	Papyrus	NUT2	MT707378	MT707233
Bulinus sp. 2	Nungwi	Tanzania	−2.77446	32.0136	UGSB 23466	Nut27075	Papyrus	NUT3	MT707379	
B. tropicus	Nzera	Tanzania	−2.51209	32.09845	UGSB 22921	Nzt26899	Sand beach	NZT1	MT707380	
B. tropicus	Nzera	Tanzania	−2.51209	32.09845	UGSB 22922	Nzt26900	Sand beach	NZT2	MT707381	MT707229
B. truncatus	Nyakalilo	Tanzania	−2.43669	32.41158	UGSB 22923	Nyt26901	Stone beach	NYT1	MT707382	MT707221
Bulinus sp. 2	Nyakalilo	Tanzania	−2.43669	32.41158	UGSB 22924	Nyt26902	Papyrus	NYT2	MT707383	MT707237
Bulinus sp. 2	Busisi	Tanzania	−2.72626	32.87034	UGSB 22925	But26903	Water Hyasin	BUT1	MT707384	MT707230
Bulinus sp. 2	Busisi	Tanzania	−2.72626	32.87034	UGSB 22926	But26904	Water Hyasin	BUT2	MT707385	
Bulinus sp. 2	Nyegezi A	Tanzania	−2.585	32.88541	UGSB 22927	Sat26905	Water Hyasin	SAT1	MT707386	MT707238
B. truncatus	Nyegezi A	Tanzania	−2.585	32.88541	UGSB 22928	Sat26906	Stone and rocks	SAT2	MT707387	MT707223
B. truncatus	Nyegezi B	Tanzania	−2.58434	32.88331	UGSB 22929	Sbt26907	Stone and rocks	SBT1	MT707387	MT707216
B. truncatus	Nyegezi B	Tanzania	−2.58434	32.88331	UGSB 22930	Sbt26908	Stone and rocks	SBT2	MT707387	
B. tropicus	Nyegezi C	Tanzania	−2.58388	33.51714	UGSB 22942	Sct26920	Sand beach	SCT1	MT707390	MT707228
Bulinus sp. 1	Nyegezi C	Tanzania	−2.58388	33.51714	UGSB 22943	Sct26921	Sand beach	SCT2	MT707391	
Bulinus sp. 1	Nyegezi C	Tanzania	−2.58388	33.51714	UGSB 22944	Sct26922	Sand beach	SCT3	MT707392	MT707226
Bulinus sp. 2	Nyanguge	Tanzania	−2.51911	33.20884	UGSB 22933	Ngt26911	Marshes/papyrus	NGT1	MT707393	

Table 1 (continued)

Species	Locality	Country	Latitude	Longitude	Voucher No.	Sequence ID	Habitat	Haplotype ID	GenBank ID	
									*cox*1	ITS2
Bulinus sp. 2	Nyanguge	Tanzania	−2.51911	33.20884	UGSB 22934	Ngt26912	Marshes/papyrus	NGT2	MT707394	MT707232
Bulinus sp. 2	Nyanguge	Tanzania	−2.51911	33.20884	UGSB 23467	Ngt27076	Marshes/papyrus	NGT3	MT707395	
Bulinus sp. 2	Mwamanyiri	Tanzania	−2.43022	33.5424	UGSB 22935	Mwt26913	Marshes/papyrus	MWT1	MT707396	MT707231
Bulinus sp. 2	Mwamanyiri	Tanzania	−2.43022	33.5424	UGSB 22936	Mwt26914	Marshes/papyrus	MWT2	MT707397	
Bulinus sp. 2	Mwamanyiri	Tanzania	−2.43022	33.5424	UGSB 23468	Mwt27077	Marshes/papyrus	MWT3	MT707398	
Bulinus sp. 2	Lamadi	Tanzania	−2.23738	33.84236	UGSB 22937	Lat26915	Marshes/papyrus	LAT1	MT707399	
Bulinus sp. 2	Lamadi	Tanzania	−2.23738	33.84236	UGSB 22938	Lat26916	Marshes/papyrus	LAT2	MT707400	MT707240
Bulinus sp. 2	Lamadi	Tanzania	−2.23738	33.84236	UGSB 23469	Lat27078	Marshes/papyrus	LAT4	MT707401	
Bulinus sp. 2	Kisumu	Kenya	−0.12739	34.74232	UGSB 23446	Kik27055	Water Hyasin	KIK1	MT707402	
Bulinus sp. 2	Kisumu	Kenya	−0.12739	34.74232	UGSB 23447	Kik27056	Water Hyasin	KIK2	MT707403	MT707239
Bulinus sp. 2	Kisumu	Kenya	−0.12739	34.74232	UGSB 23448	kik27057	Water Hyasin	KIK3	MT707406	
B. truncatus	Nile	Uganda	0.42084	33.19639	UGSB 23452	Niu27061	Open water	JIU1	MT707407	MT707214
B. truncatus	Mayuge	Uganda	0.14067	33.60258	UGSB 16758	Myu22513	Open water	MYU1	MT707404	
B. truncatus	Mayuge	Uganda	0.14067	33.60258	UGSB 16757	Myu22514	Open water	MYU2	MT707405	
B. truncatus	Mayuge	Uganda	0.14067	33.60258	UGSB 23453	Myu27062	Open water	MYU3	MT707408	
B. truncatus	Mayuge	Uganda	0.14067	33.60258	UGSB 23454	Myu27063	Open water	MYU4	MT707409	
B. truncatus	Mayuge	Uganda	0.14067	33.60258	UGSB 23603	Myu27108	Open water	MYU5	MT707411	
B. truncatus	Mayuge	Uganda	0.14067	33.60258	UGSB 23604	Myu27109	Open water	MYU6	MT707410	MT707218
B. truncatus	Masindi	Uganda	2.12852	32.32919	UGSB 23457	Msu27066	Nile river	MSU1	MT707412	
B. truncatus	Masindi	Uganda	2.12852	32.32919	UGSB 23458	Msu27067	Nile river	MSU2	MT707413	
B. truncatus	Masindi	Uganda	2.12852	32.32919	UGSB 23459	Msu27068	Nile river	MSU3	MT707414	
B. truncatus	Masindi	Uganda	2.12852	32.32919	UGSB 23460	Msu27069	Nile river	MSU4	MT707415	MT707217
B. truncatus	Masindi	Uganda	2.12852	32.32919	UGSB 23607	Msu27112	Nile river	MSU5	MT707416	
B. truncatus	Masindi Port	Uganda	1.69249	32.09664	UGSB 16759	Mpu22515	Nile river	MPU1	MT707417	
B. truncatus	Masindi Port	Uganda	1.69249	32.09664	UGSB 16760	Mpu22516	Nile river	MPU2	MT707418	
B. truncatus	Masindi Port	Uganda	1.69249	32.09664	UGSB 23610	Mpu27115	Nile river	MPU3	MT707419	MT707219
Bulinus sp. 2	Masaka	Uganda	−0.27263	32.02691	UGSB 23461	Mku27070	Water Hyasin	MKU1	MT707420	
Bulinus sp. 2	Kampala	Uganda	−0.27263	32.02691	UGSB 23596	Kau27101	Water Hyasin	KAU1	MT707421	MT707241
B. truncatus	Kampala	Uganda	−0.27263	32.02691	UGSB 23598	Kau27103	Open water	KAU2	MT707422	MT707215
Bulinus sp. 2	Kampala	Uganda	−0.27263	32.02691	UGSB 23599	Kau27104	Water Hyasin	KAU3	MT707423	
Bulinus sp. 2	Amotar	Uganda	1.55822	32.88828	UGSB 23613	Amu27118	Marshes/papyrus	AMU1	MT707424	
B. truncatus	Kalangala	Uganda	0.30371	32.28927	UGSB 16774	Klu22530	Open water	KLU1	MT707425	
B. truncatus	Kalangala	Uganda	0.30371	32.28927	UGSB 23616	Klu27121	Open water	KLU2	MT707426	MT707213

Table 1 (continued)

Species	Locality	Country	Latitude	Longitude	Voucher No.	Sequence ID	Habitat	Haplotype ID	GenBank ID	
									*cox*1	ITS2
B. truncatus	Lake Mburo	Uganda	−0.638	30.9528	UG3	BspJDUG3		MNU1	MT707427	
B. truncatus	River Rwizi	Uganda	−0.6863	30.8856	UG19	BspJUG19		MNU2	MT707428	
B. truncatus	River Rwizi	Uganda	−0.6863	30.8856	UG22	BspJUG22		MNU3	MT707429	
B. truncatus	Lake Mburo	Uganda	−0.6951	30.8514	UG27	BspJUG27		MNU4	MT707430	
B. truncatus	Lake Nakivale	Uganda	−0.8205	30.8559	UG7	BspJDUG7		MNU5	MT707431	
Bulinus sp. 2	Lake Nakivale	Uganda	−0.8205	30.8559	UG98	BspJUG98		MNU6	MT707432	
B. forskalii	Lake Nakivale	Uganda	−0.8205	30.8559	UG76	BspJUG76		MNU7	MT707403	
B. truncatus	Lake Albert	Uganda			A1	HQ121558		LAU1	HQ121558	
B. truncatus	Lake Albert	Uganda			A2	HQ121559		LAU2	HQ121559	
B. truncatus	Lake Albert	Uganda			A3	HQ121560		LAU3	HQ121560	
B. truncatus	Katosho swamp	Tanzania			T1	HQ121562		KST1	HQ121562	
B. truncatus	Lake Albert	Uganda			BO (Booma)	GU176747		LAU4	GU176747	
B. truncatus	Lake Albert	Uganda			1PD (Piida)	GU176748		LAU5	GU176748	
B. truncatus	Lake Albert	Uganda			TO (Toonya)	GU176749		LAU6	GU176749	
B. truncatus	Nyanguge	Tanzania			Nyanguge	AM286313		NGT	AM286313	
Bulinus sp. 2	Kisumu	Kenya			ADC farm	AM286297		AFK1	AM286297	
B. truncatus	Lake Sagara	Tanzania			T04em43A	AM286298		LST	AM286298	

Abbreviation: UGSB, University of Giessen Systematics and Biodiversity collection

using Arlequin version 3.5.2.2 [57] by calculating Tajima's *D* [58] and Fu's *Fs* [59]. Under the assumption of selective neutrality, Arlequin version 3.5.2.2 was also used for mismatch distribution analysis of pairwise differences within and between populations. The relative population sizes (θ_0 and θ_1) and relative time since population expansion (τ) were estimated also using Arlequin version 3.5.2.2. The estimated τ value was used to estimate time since expansion using the formula $\tau = 2\ \mu t$, where μ is the mutation rate per site per generation and τ is the time since population expansion [60]. In the present study, the substitution rate of $1.22 \pm 0.27\%$ per million years was applied for the mtDNA (*cox*1) region [61]. Additionally, a Mantel test for matrix correspondence between genetic and geographical distances was performed using GenAlEx version 6.5. [62] to test the isolation by distance (IBD). The input matrices for genetic distance were constructed in Mega X [63].

Results

Species identification and phylogenetic relationships

Both Maximum Likelihood (ML) and Bayesian Inference (BI) analyses of concatenated genes (*cox*1 and ITS2) generated strongly supported phylogenies that revealed the presence of two main *Bulinus* groups in Lake Victoria (Fig. 2). Clade I comprised of *B. truncatus/tropicus* complex and Clade II contained the *B. africanus* group Moreover, Clade I exhibited a complex structure that corresponded to *Bulinus* specimens that inhabited open waters and sandy beaches of Lake Victoria. For instance, specimen labelled Sct26922, collected from Nyegezi on the Tanzanian side of the lake, was found in shallow waters near sandy beaches coexisting with a physid species. Both species delimitation methods (SDMs), PTP and GMYC, categorised the specimen as a unique molecular operational taxonomic unit (MOTU; *Bulinus* sp. 1.). Clade I also contained *Bulinus* samples collected outside the lake albeit within the lake basin i.e. the Lake Mburo-Nakivale and Nile River ecosystems, denoting that these species are not endemic to Lake Victoria. Moreover, combined phylogenetic and SDMs analyses revealed the presence of *B. truncatus* and *B. tropicus* in Lake Victoria, although *B. truncatus* are more widely distributed than *B. tropicus*.

Novel sequences forming subclade I (SCI, Fig. 2) were isolated from *Bulinus* specimens collected from the banks and marshes surrounding the lake and small islands, particularly Bumbire in Tanzania and Mayuge in Uganda. Although these *Bulinus* specimens formed a well-defined and supported clade in both analyses (ML = 100% and BI = 1.0), they did not intermingle with other species within the clade; they formed a definite group of their own (*Bulinus* sp. 2). As shown in Fig. 2, despite the complexity or the presence of cryptic species in GenBank sequences designated as *B. globosus*, SDMs treated *Bulinus* specimens from the banks and surrounding marshes, regardless of the location they were collected, as one species (MOTU). The specimens from the banks matched only with *Bulinus* sp. T04em43A (GenBank: AM286298) from Lake Sagara in Tanzania, and accordingly, SDMs placed them under the same MOTU. The phylogeny and SDMs from *cox*1 also identified *Bulinus nasutus productus* and *B. forskalii* collected from Lake Mburo-Nakivale system within the Lake Victoria basin. Similar results are shown for *cox*1 analyses (Additional file 2: Figure S1)

Phylogeographical and population analyses

Although the phylogeographical analysis of the present study did not acquire sufficient samples from the Kenyan side and small islands, in particular, TCS networks supported the phylogenies (Fig. 3) that Lake Victoria is dominated by two distinct clades of *Bulinus* species; species occurring in the lake proper and those inhabiting the banks and surrounding marshes. However, at the confidence limit of 0.95, the dataset comprising specimens from the banks and marshes represented *B. africanus* group species (A in Fig. 3) while those from the open water (lake proper) revealed three separate networks: *B. truncatus*/*B. tropicus* complex, i.e. *B. truncatus* (B in Fig. 3), *B. tropicus* (C in Fig. 3) and an undefined species *Bulinus* sp. 1 (D in Fig. 3). *Bulinus nasutus productus* and *B. forskalii* from Lakes Mburo-Nakivale systems formed separate networks (E and F in Fig. 3, respectively). Similar to phylogeny and SDMs, TCS analysis revealed a *Bulinus* specimen collected from Nyegezi in Tanzania (Haplotype 47) as a distinct species (D in Fig. 3). Generally, the TCS analysis showed that *Bulinus* species had shared haplotypes distributed throughout Lake Victoria, indicating that these species are not localised in the lake (Fig. 4). Moreover, the TCS analysis corroborated phylogenies and SDMs that specimens sampled from the banks and surrounding marshes of Lake Victoria relate to potentially undescribed bulinid species, *Bulinus* sp. T04em43A (GenBank: AM286298) from Lake Sagara, Tanzania and *Bulinus* sp. K3.03 (GenBank: AM286297) from Lake Victoria in Kisumu, Kenya.

The mtDNA loci showed high overall haplotype (*h*) and nucleotide (π) diversity among populations (0.984 and 0.071). The population analysis of *B. truncatus* revealed 22 haplotypes, out of which 2 haplotypes were shared between sand beaches and river systems (Table 2). On the other hand, *Bulinus* sp. 2 (Clade II) population consisted of 17 haplotypes and a least one haplotype was shared

between two habitats i.e. islands, papyrus and water hyacinths. Nevertheless, no haplotype was shared among the three habitats; an indication that haplotypes are specific to habitats. Nucleotide and haplotype diversities were also high within each habitat (Table 2).

The inbreeding coefficients (F_{ST}), defined from the AMOVA, for *B. truncatus* and *Bulinus* sp. 2 populations were 0.034 ($P=0.045$) and 0.064 ($P=0.020$) respectively. These F_{ST} values demonstrate an apparently low genetic differentiation between habitats. Table 2 summarises the genetic variations of the *Bulinus* in these groups occurring in Lake Victoria. Generally, F_{ST} values (0.021–0.023) between and within habitats groups were low (Table 2) indicating that the gene flow among *Bulinus* species populations and subpopulations within the Lake Victoria is high. The AMOVA concurs with the haplotype network, in which there was no clear demarcation between the localities where a given specimen was collected and its genetic affiliation with other haplotypes (Figs. 3, 4).

The estimates of Tajima's *D* and Fu's *Fs* test of *Bulinus* populations from Lake Victoria (i.e. within the lake, banks and surrounding marshes) were negative and statistically significant (Table 2), which denotes that the *Bulinus* species in the lake have undergone a recent population expansion. With a 95% confidence interval (CI), estimates of θ_0 and θ_1 for *Bulinus* species indicated that populations expanded, both demographically and spatially, from a compact to a considerable size (Table 3). Using the tau values (τ) of 3.787 and 4 for the *B. truncatus* in the open water and *Bulinus* sp. 2 occurring in the banks and marshes of Lake Victoria, we roughly estimate the starting time for *Bulinus* rapid population expansion to be between 207,694 (±107,823) and 464,678 (±278,312) years ago (Table 3).

There was no significant correlation between genetic and geographical distances within the *Bulinus* population (*B. truncatus*) inhabiting the proper lake ($r^2=0.018$, $P>0.05$) or those (*Bulinus* sp. 2) from the banks, islands and marshes ($r^2=0.0038$, $P>0.05$). Overall all *Bulinus* samples from Lake Victoria did not exhibit any correlation between genetic variations and distance ($r^2=0.0175$, $P>0.05$), indicating the variation in genetic distance is mainly due to taxonomic differences as already shown by both phylogeny and parsimony networks.

Discussion

Identity of *Bulinus* in Lake Victoria and their phylogenetic affinities

The present study, to our knowledge, is the first to apply molecular techniques on the longitudinally surveyed *Bulinus* species occurring in Lake Victoria. A majority of studies on molluscs in Lake Victoria have been conducted on *Biomphalaria* species for their role in the spread of intestinal schistosomiasis [13, 18, 25]. The present study provides molecular-based evidence on the presence of two *Bulinus* groups in the lake; *B. truncatus/B. tropicus* occupying the open waters, covering sand beaches, stones and submerged rocks, while *B. africanus* group dominates the banks, small islands and surrounding marshes. Although the number of species determined by PTP and GMYC was slightly indecisive, the present study supports previous findings [27, 31, 35–37] that molecular methods could delineate the monophyletic subclade comprising of *B. truncatus* and its sibling *B. tropicus* (Fig. 2), which are morphologically difficult to distinguish [17].

From Mandahl-Barth [64] to present, the taxonomy of *Bulinus* species in Lake Victoria is in scrutiny. According to Brown [17], four species of *Bulinus* occur in Lake Victoria and the most common are the coexisting diploid and tetraploid populations forming the *B. truncatus/B. tropicus* complex that lack an apparent taxonomic boundary. Other *Bulinus* material was classified as *B. trigonus* and *B. transversalis* [64], though Brown [17] suggested that they might be lacustrine morphs of *B. tropicus* and *B. truncatus*. However, the present molecular analysis of material from Bumbire Island, the type-locality for *B. transversalis* [17], grouped the material with *Bulinus* sp. 2, which is regarded by the present study as *B. ugandae*. Nonetheless, the specimens from the island were smaller than those collected from the banks and marshes elsewhere. Although potentially topotypic material was collected and a single species only occurred there, we cannot conclude that *Bulinus* sp. 2 is, in fact, *B. transversalis*. Morphological characteristics of the snails studied here suggest that nowadays the waters around the island are rather inhabited by *B. ugandae*.

Phylogenetic analysis accompanied by SDMs also revealed a unique MOTU of *Bulinus*, *Bulinus* sp. 2, in Lake Victoria (Fig. 2, Clade II/Subclade I), which was strongly supported as sister to *B. globosus* in the *B. africanus* group. Although our phylogenetic analyses did not find sequences of *Bulinus* from Lake Victoria in the GenBank database to compare with, our sampling is reasonable to relate the *Bulinus* sp. 2 to *B. ugandae*. In our perusal of the literature regarding genus *Bulinus* in Lake Victoria, only *B. ugandae* shares similar features to the present material. Both Mandahl-Barth [29] and Brown [17] while scrutinising the morphological characters of *B. ugandae*, they questioned its taxonomic position in relation to *B. globosus*. Loker et al. [65] also acknowledged the challenging task of separating accurately *B. globosus* and *B. ugandae* from the Lake Victoria region. Moreover, Mwambungu [32] encountered *B. ugandae* in the Speke Gulf of the lake on the Tanzanian side and Ngupula & Kayanda [33] found *B. ugandae* and *B. transversalis* in

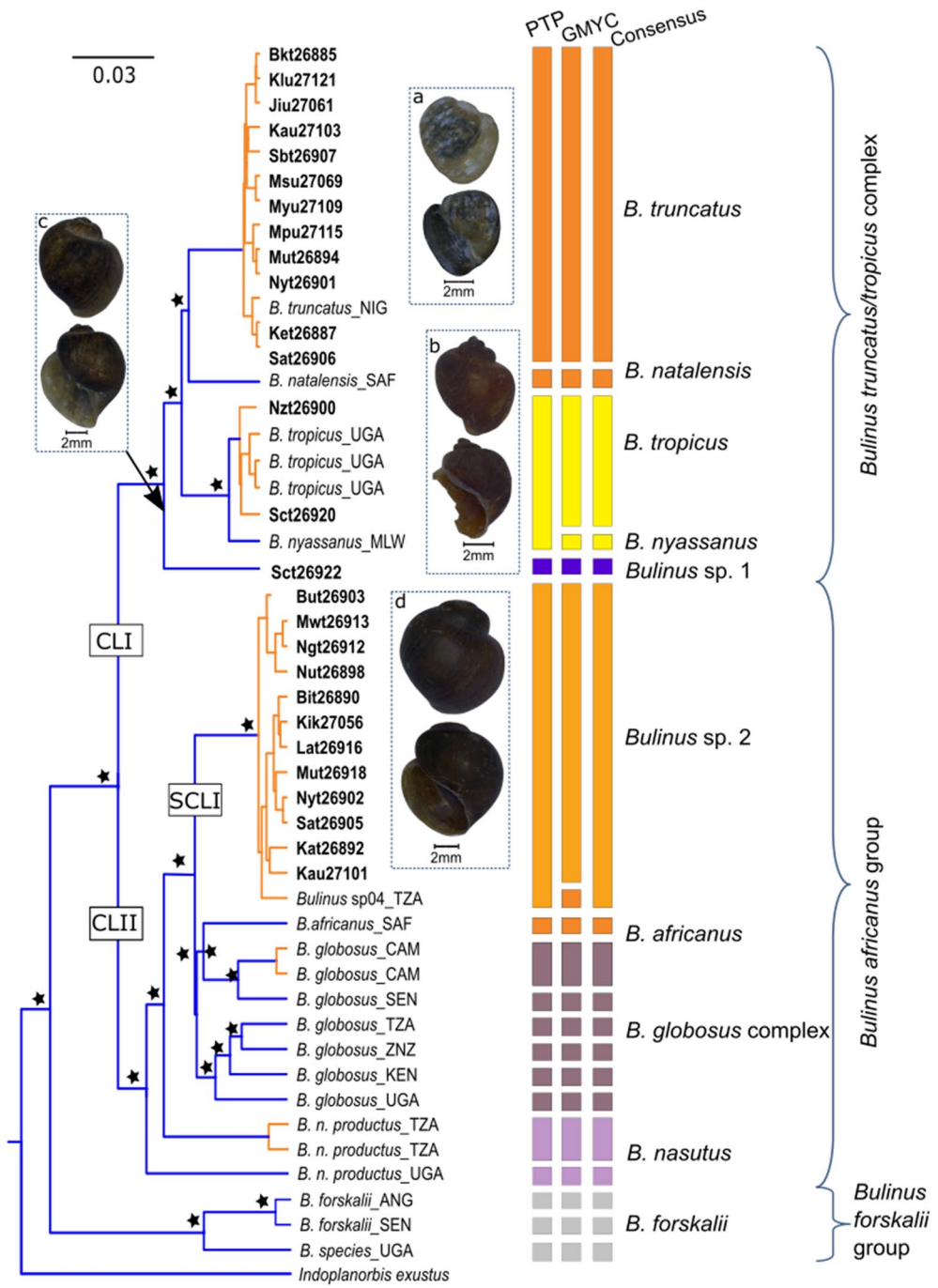

Fig. 2 The BI phylogenetic tree of *Bulinus* species with bars, on the right, denoting different species delimitation results, based on the dataset of concatenated *cox*1 and ITS2 sequences. Within the phylogeny, nodes supported and shared between BI and ML methods are marked with stars where support equates to 90–100% (ML) and 0.95–1 (BI). Names in bold are for specimens collected in the present study and the rest have been retrieved from GenBank: *B. truncatus* (**a**); *B. tropicus* (**b**); *Bulinus* sp. 1 (**c**); *Bulinus* sp. 2 (**d**). Locality details are provided in Table 1. *Abbreviations*: CLI, Clade I; CLII, Clade II; SCI, Subclade I; SCII, Subclade II. The three-letter abbreviation for countries is also given. *Notes*: the blue colour represents different species, while green stands for the same species according to species delimitation methods. The three-letter abbreviations represent countries: NIG, Nigeria; SAF, South Africa; UGA, Uganda; MLW, Malawi; TZA, Tanzania; CAM, Cameroon; SEN, Senegal; ZNZ, Zanzibar; KEN, Kenya; ANG, Angola. The information for sequences retrieved from the GenBank is presented in Additional file 1: Table S1

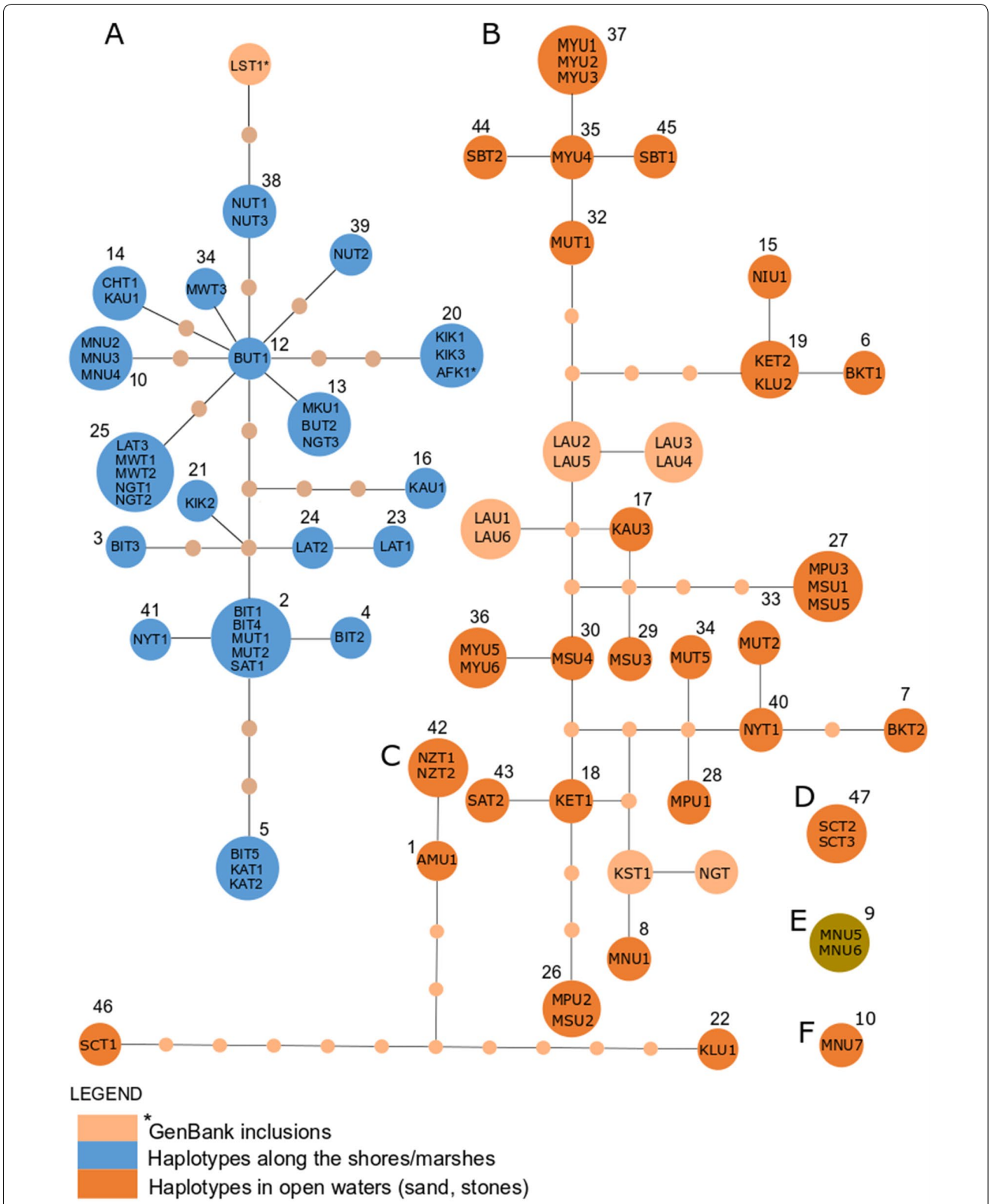

Fig. 3 Statistical parsimony network of *cox*1 sequences (connecting limit: 95%) of *Bulinus* species from Lake Victoria; *Bulinus* sp. 2 (**a**), *B. truncatus* (**b**), *B. tropicus* (**c**), *Bulinus* sp. 1 (**d**), *B. nasutus* (**e**) and *B. forskalii* (**f**). The size of the circles corresponds to the number of individuals belonging to the respective haplotype. Mutational steps for the missing haplotypes are presented as small circles, and numbers correspond to the number of individuals with a given haplotype. Green stands for GenBank material

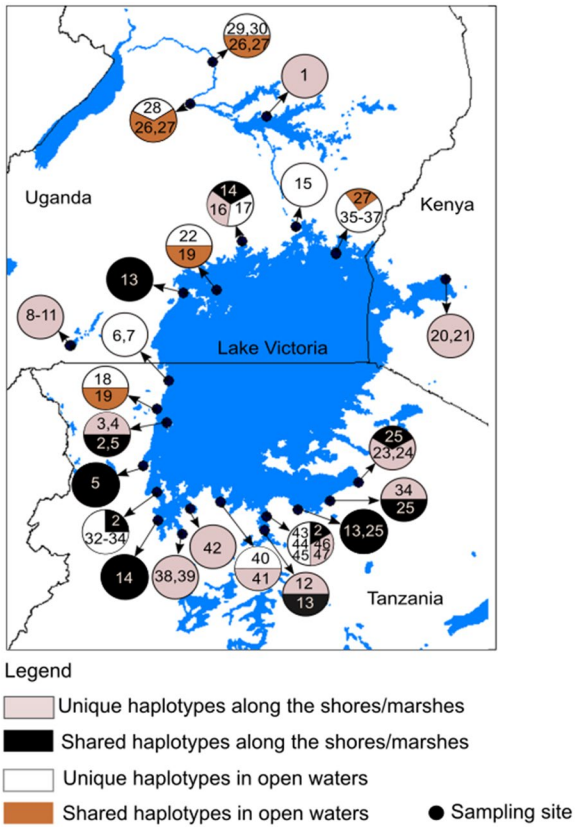

Fig. 4 A map of Lake Victoria showing *Bulinus* species haplotypes distribution. Specimen details are provided in Table 1

Uganda. A study Opisa et al. [34] also found *B. globosus* distributed along the shores of Lake Victoria in Kisumu, Kenya. The close relatedness of the present specimen and *Bulinus* sp. (GenBank: AM286297) from Kisumu in Kenya [26] further shows a wide distribution of *B. ugandae* in Lake Victoria. While the separation of *B. ugandae* from *B. globosus* morphologically is paradoxical [17, 29], most workers used the names interchangeably.

In this analysis, we also found a unique MOTU of *Bulinus* (*Bulinus* sp. 1) which were collected in the southern part of Lake Victoria at Sweya beach in Nyegezi, Mwanza. The strong phylogenetic support for *Bulinus* sp. 1 (BS = 100%, PP = 1.00; Fig. 2) within the *B. truncatus/B. tropicus* clade and the separation of *B. truncatus* and *B. tropicus* haplotypic networks (Fig. 3), is a clear indication that *Bulinus* sp. 1 is a different species. The closest match to the *cox*1 sequences of *Bulinus* sp. 1 was 97.22% with *B. tropicus* (GenBank: KJ157492) from Cameroon [36]. Morphologically, *Bulinus* sp. 1 were similar to other members of the *B. truncatus/B. tropicus* species complex except that they were found co-existing with *B. tropicus* and physids in much shallower water on the mud-covered sand beach. Given that this species is neither *B. truncatus* nor *B. tropicus* nor *B. transversalis* (see above), we remain with *B. trigonus* as the sole known member of the *B. truncatus/B. tropicus* complex for Lake Victoria. More research is, however, needed to decide whether *Bulinus* sp. 1 indeed represents *B. trigonus*.

It is noteworthy that the shallow lake systems west of Lake Victoria harbour at least two different *Bulinus* species (i.e. *B. nasutus productus* and *B. forskalii*; see Figs. 2, 3, 4). Summarizing the current *Bulinus* diversity (Table 4), the Lake Victoria fauna consists of at least four species: *B. truncatus*; *B. tropicus*; *Bulinus* sp. 1 (*B. trigonus*?); and *B. ugandae* (*Bulinus* sp. 2).

Genetic population analysis

The genetic variation, analysis of molecular variance (AMOVA), and isolation by distance showed *Bulinus*

Table 2 Results of genetic diversities, AMOVAs and mismatch distribution for populations of *Bulinus* spp. in Lake Victoria

	Bulinus truncatus				*Bulinus* sp. 2			
	Mean ($n=29$)	Lentic sand substrate ($n=14$)	Lentic stone & rocks substrate ($n=8$)	River systems ($n=7$)	Mean ($n=31$)	Islands ($n=5$)	Swamp papyrus ($n=15$)	Marshes water hyacinth ($n=11$)
Haplotype (h)	22	11	8	5	17	4	10	7
Haplotype diversity (h)	0.978 ± 0.005	0.956 ± 0.0156	1.000 ± 0.022	0.905 ± 0.040	0.940 ± 0.005	0.900 ± 0.016	0.895 ± 0.022	0.909 ± 0.041
Nucleotide diversity (π)	0.01067	0.01134	0.01014	0.08387	0.00753	0.005	0.010	0.084
F_{ST} (P-value)	0.034 (0.045)	0.014 (0.297)	0.048 (0.072)	0.047 (0.027)	0.064 (0.020)	0.077 (0.081)	− 0.016 (0.432)	0.080 (0.000)
Tajima's D (P-value)	− 0.126 (0.484)	− 1.092 (0.144)	0.096 (0.575)	0.618 (0.733)	− 1.158 (0.112)	− 1.162 (0.058)	− 0.606 (0.279)	− 0.317 (0.414)
Fu's FS (P-value)	− 1.735 (0.237)	− 2.549 (0.097)	− 3.273 (0.022)	0.617 (0.585)	− 5.439 (0.014)	− 0.445 (0.277)	− 2.088 (0.149)	0.644 (0.312)

Note: The ordering of specimens was based on the habitats they were found

Table 3 Results of the mismatch distribution analyses for the demographic and spatial expansions of the *Bulinus* species from Lake Victoria populations and time since expansion

	Bulinus truncatus						*Bulinus* sp. 2					
	Demographic expansion			Spatial expansion			Demographic expansion			Spatial expansion		
	Lentic sand substrate	Lentic stones & rock substrates	Lotic habitats	Lentic sand substrate	Lentic stones & rock substrates	Lotic habitats	Islands	Swamp papyrus	Marshes[a]	Islands	Swamp papyrus	Marshes[a]
SSD (P-value)	0.095 (0.280)	0.079 (< 0.0001)	0.024 (0.160)	0.095 (0.430)	0.069 (0.040)	0.020 (0.600)	0.023 (0.130)	0.087 (0.010)	0.069 (0.170)	0.021 (0.370)	0.059 (0.100)	0.063 (0.410)
RI (P-value)	0.310 (0.280)	0.219 (< 0.0001)	0.073 (0.280)	0.310 (0.510)	0.219 (0.110)	0.073 (0.640)	0.032 (0.450)	0.092 (0.580)	0.213 (0.200)	0.032 (0.680)	0.092 (0.430)	0.213 (0.390)
Theta0/ Theta	0.000	1.900	0.000	0.05589	0.001	0.001	0.000	5.500	1.622	0.010	0.010	1.180
Theta1	16.211	3414.978	34.961				46.951	3414.978	49.882			
τ (CI)	3.469 (2.258-6.211)	4.000 (2.822–7.725)	5.556 (2.994–7.803)	3.233 (1.555-6.146)	5.811 (2.309–7.509)	5.363 (2.788–7.639)	6.277 (3.646–8.352)	5.000 (3.555–13.682)	5.438 (3.344–10.984)	6.121 (3.805–7.578)	7.234 (2.901–8.282)	5.453 (2.619–10.173)
T in years	222,824	256,951	356,946	207,694	373,288	344,497	403,243	321,188	349,293	393,184	464,678	350,287
ΔT in years	± 77,788	± 75,655	± 164,610	± 107,823	± 224,936	± 165,400	± 169,000	± 92,843	± 134,498	± 148,784	± 278,312	± 182,033

Abbreviations: SSD, sum of squared deviations; RI, raggedness index; CI, 95% confidence interval; τ, population parameter Tau; T, time since expansion

[a] Water hyacinth

species populations in Lake Victoria to be panmictic. The overall F_{ST} value (0.034) in *cox*1 was significantly low, which may be explained by high gene flow rates among *Bulinus* populations in Lake Victoria to favour the evolution of phenotypic plasticity within species [66]. Also, AMOVA produced F_{ST} values within populations ranging from 0.00–0.080, meaning *Bulinus* species in Lake Victoria consist of overlapping populations. However, the ranges of genetic differentiation between populations (0.00–0.08) are comparable to previous studies on *Bulinus* species [67–69], who attributed the variations to self-fertilization within the populations. Given the size of the lake and high gene flow observed, it can be hypothesized that *Bulinus* species in Lake Victoria could be both cross and self-fertilizers. The cross-fertilization and pathogenesis in the banks and surrounding marshes may be increased due to intrusion of water weeds water hyacinth (*Eichhornia crassipes*), which are implicated in creating new habitats for snails [70, 71]. Moreover, our findings corroborate Standley et al. [13] who argued about the impossibility of sudden demographical events that would influence the genetic diversity and population structure of snail populations in Lake Victoria.

Studies in Lake Victoria have shown that, despite its large size, it is one of the youngest large lakes in the African Rift and has existed only 400,000 years ago with three complete desiccations in between, and the current water body was refilled about 14,600 years ago [15]. In contrast, our findings showed the *Bulinus* populations in Lake Victoria began spatial and demographic expansion about 99,700–743,000 years before the present. The explanation may be twofold, (i) the snails colonized the lake from neighbouring aquatic systems during the last refilling and (ii) the lake did not completely dry to reflect the 100,000 years of Milankovitch climate forcing cycles [10, 15]. Both scenarios could be associated with the low levels of genetic variation and population structure indices at the intrapopulation level within the *Bulinus* species in Lake Victoria [29]. Our results, however, support the scenario that the current biota in Lake Victoria recolonized the refilling lake from refugia as argued by Nalugwa et al. [72] given that about 100,000 years ago Lake Victoria probably collected its waters from regions near Lake Tanganyika [10]. The occurrence of *Bulinus* species in Lake Sagara in the Ugalla-Malagarasi drainage system in western Tanzania [37] and *B. truncatus* in Lakes Kivu and Tanganyika (Katosho swamp) [73], respectively, similar to those found in Lake Victoria, further supports the invasion theory.

Ecological aspects

Lake Victoria experienced tremendous ecological perturbations in the Anthropocene, and human activities nowadays might contribute significantly to the mixing of populations across the lake and adjacent aquatic ecosystems [74]. Even though we found no indication of such human effects for the *Bulinus* populations studied, future studies employing more sensitive markers should focus on these potentially confounding factors affecting population structures across the lake. Differential impacts of human disturbances on snail existence and abundances have been demonstrated in the Kenyan part of Lake Victoria [75]. Whereas some species might disappear, others, including intermediate host snails, i.e. pulmonates generally, might be even favoured by eutrophication processes and as such might increase the risks of transmission [75, 76]. The general abundance of pulmonate snails is high throughout the lake and marsh systems (FC and CA, personal observations). This in concert with reduced predator pressure from molluscivorous fishes might account for the comparatively high biomasses of certain gastropod species including some of the *Bulinus* spp. There is evidence for the roles of habitats in shaping (eco-) morphotypes in the less diverse *Biomphalaria* in Lake Victoria [77]. Such effects remain to be studied in detail for *Bulinus*, although our results so far indicated a link between habitat types and genetic diversity.

Parasitological implications of *Bulinus* species in Lake Victoria

Lake Victoria is one of the most well-known hotspots of schistosomiasis worldwide with fishing communities and school-aged children reported to be the most infected demographic groups in the surrounding countries of Kenya, Tanzania and Uganda [18–20, 23, 24]. However, a vast majority of reports on schistosomiasis in the lake and banks have focused on *Biomphalaria* species and their consequential *S. mansoni* [13, 18, 25]. There are two specific or subspecific forms of *Biomphalaria* species that preserve transmission of schistosomiasis in the lake: (i) *B. sudanica*, mainly found along the shores and surrounding marshes and swamps; and (ii) *B. choanomphala*, a more in-depth water inhabitant of Lake Victoria (Stanley et al. [18], but see Zhang et al. [25] for a discussion on species identified). The present findings showed that two dominant taxa of *Bulinus* occur in the lake: (i) *B. ugandae* (*Bulinus* sp. 2), mainly found along the banks and surrounding marshes and swamps in the mainland and islands; and (ii) members of *B. truncatus/B. tropicus* complex, which are found in open water habitats.

Although the present study did not test the collected snails for patent and prepatent infections with *Schistosoma* spp. or other digenean trematodes, the presence of certain *Bulinus* species in Lake Victoria potentially implies the presence of *S. haematobium*. Both *B. truncatus/B. tropicus* complex, *B. africanus* and *B.*

Table 4 Species diversity of the genus *Bulinus* in the Lake Victoria basin

Species	Occurrence	Role as host	Reference	Present study
B. africanus	Near LV in Kenya, Mwanza, Tanzania	Main host in South Africa, NW Tanzania	Brown [17]	Not found
B. globosus	Mwanza, LV, Kisumu	Southern Africa, Main host in NW Tanzania	Loker et al [65]; Opisa et al [34]	Not found
B. forskalii	LV	not confirmed	Brown [17]	*B. forskalii* (not found in lake proper)
B. nasutus productus	Eastern shore LV	Main host in NW Tanzania	Brown [17] Mandahl-Barth [14]	*B. nasutus productus* (not found in lake proper)
B. tropicus	Not mentioned before	Not known		*B. tropicus*
B. reticulatus	Near Kisumu and Mwanza	Not known	Brown [17]; Loker et al [65]	Not found
B. trigonus	LV and Lake Edward	*B. truncatus*: main host in NE, W and N Africa	Brown [17]	*B. trigonus?*
B. transversalis	LV and Victoria Nile	Not known	Brown [17]; Mandahl-Barth [14]	Not found
B. ugandae	LV, NW Tanzania	Not known	Brown [17]; Mandahl-Barth [14]	*B. ugandae*

Notes: Taxa mentioned in the literature, their distribution, assumed or proven roles as intermediate hosts for *S. haematobium* are provided. Where possible, findings from the recent study are compared to the previous information

Abbreviation: LV, Lake Victoria

forskalii group members have already been implicated in the transmission of *S. haematobium* elsewhere in Africa [17, 31, 78]. *Bulinus nasutus productus* has been known to occur around the eastern shore of the lake [33] and was now also found in the west. This species has been shown to be involved in *S. haematobium* transmission [12]. Even if *B. tropicus* is not known to be an intermediate host for *Schistosoma* species [17], the present findings are particularly important because hitherto the morphological distinction within *B. truncatus/B. tropicus* complex is challenging [17]. *Bulinus truncatus* is not yet known to be a host in equatorial Africa; however, there is potential [17] since it is the main host in the regions up the Nile river (Nile Province of South Sudan) where high prevalences of *S. haematobium* infections have been reported [79]. *Bulinus ugandae* is apparently not known to host *S. haematobium* but screening for *B. globosus* should continue in and around Lake Victoria. Given that *B. africanus* group members are found close by (*B. nasutus* and *B. forskalii* in satellite lakes that are hydrologically connected to Lake Victoria), there is a hidden risk for the prevalence of *S. haematobium*. Therefore, the occurrence and wide distribution of *Bulinus* species in Lake Victoria potentially threaten the health of communities living along the shores and on islands of the lake who depend on the lake for their livelihood. This situation is even triggered by the increasing pollution of the lake, which has recently been demonstrated to worsen the infection risks [80], this is yet another factor complicating the combat of schistosomiasis in this hotspot [24]. Future studies should undertake more experimental approaches to snail transmission. Another promising tool in predicting and identifying transmission potential (contamination and exposure) is the environmental DNA approach [81]. This has very recently been successfully used for environmental surveillance of schistosomiasis [82].

Previous studies on the prevalence of *S. mansoni* and *S. haematobium* showed the species were partitioned according to distance from the lake, i.e. *S. mansoni* occurred close to the lake and *S. haematobium* further on the hinterland [83]. Additionally, the spatial distribution of *S. haematobium* was in line with the presence of streams and ponds [79]. These observations imply that intermediate host species of *Biomphalaria* and *Bulinus*, the respective intermediate hosts for *S. mansoni* and *S. haematobium*, likely occur inside and outside the lake, respectively [18]. Our results, on the other hand, corroborate the previous observations that arrange of *Bulinus* species are present in the lake and are confirmed here to be widespread, but their role in *S. haematobium* transmission remains uncertain. A widely neglected aspect relates to schistosomiasis as a disease of veterinary concern [27]. *Bulinus tropicus* and *B. ugandae* are a well-known host for *S. bovis*, a parasite extensively infecting livestock [81]. Zoonotic schistosomiasis is currently largely underestimated [84] but could be studied in the setting of Lake Victoria in the future. Zoonotic schistosomiasis could be of high concern for both livestock and also wildlife existing in the adjacent world-famous national parks.

Conclusions

This study has reported two major *Bulinus* groups and at least four species occurring in Lake Victoria, *B. truncatus/B. tropicus* complex and *B. africanus* inhabiting vegetation-free sand and stone beaches, and banks and surrounding marshes/papyrus beds on the mainland and islands. These findings reflect previous findings on *Biomphalaria* species. Since in this study, we did not trace how far deep *B. truncatus/B. tropicus* complex can occur, we recommend a depth abundance relationship analysis for *Bulinus* species be carried out. Our findings also conclude that the assumed *B. ugandae* dominates the banks and surrounding marshes. *Bulinus trigonus* might indeed be a separate species whereas the *B. transversalis* remains to be studied genetically. Following our findings, a parasitological examination of *Bulinus* species around the lake is paramount to understanding their role in the epidemiology of urogenital schistosomiasis and its subsequential control. It is also recommended to study in parallel patterns in co-occurring *Biomphalaria* spp. throughout seasonal cycles and along environmental gradients.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13071-020-04281-1.

Additional file 1: Table S1. Summary of additional sequence data from the crater lakes and other regions retrieved from GenBank with the localities and haplotypes noted. Additionally, the locality, voucher, sequence and haplotype information for the *Bulinus* species from Lake Victoria studied for the first time herein are also given. *Abbreviation*: UGSB, University of Giessen Systematics and Biodiversity.

Additional file 2: Figure S1. The BI phylogenetic tree of *Bulinus* species with bars, on the right, denoting different species delimitation results, based on the dataset of concatenated *cox*1 sequences. Within the phylogeny, nodes supported and shared between BI and ML methods are marked with stars where support equates to 90–100% (ML) and 0.95–1 (BI). Names in bold denote specimens collected in the present study and the rest have been retrieved from the GenBank. Locality details are provided in Table 1. Blue colour represents different species, while green represents the same species as resolved by species delimitation methods. The information for sequences retrieved from the GenBank is presented in Additional file 1: Table S1. *Abbreviations*: SC1, Subclade 1; SC2, Subclade 2; SC3, Subclade 3; SC4, Subclade 4. The three-letter abbreviations represent countries: NIG, Nigeria; SAF, South Africa; UGA, Uganda; MLW, Malawi; TZA, Tanzania; CAM, Cameroon; SEN, Senegal; ZNZ, Zanzibar; KEN, Kenya; ANG, Angola; EGY, EGYPT; DRC, Democratic Republic of Congo.

Abbreviations

SDMs: Species delimitation methods; PTP: Poisson tree processes; GMYC: Generalized mixed Yule coalescent; AMOVA: Analysis of molecular variance; ML: Maximum likelihood; BI: Bayesian inference; NCBI: The National Center for Biotechnology Information.

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Authors' contributions

FC and CA conceived the study. FC carried out the sampling in the Tanzanian side of Lake Victoria. FC also produced the sequences and performed data analyses, with the help of AM, IT and AFS. IT and CA collected part of the material from Kenyan and Ugandan sides, and all authors were involved in data interpretation. Figures were produced by FC. All authors critically reviewed and approved the final manuscript.

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Availability of data and materials

All data generated or analysed in the course of this study are included in the article, its additional files or have been deposited in the University of Giessen Systematics and Biodiversity (UGSB) repository, which are available upon request. Additionally, newly generated sequences were deposited in the GenBank database under the accession numbers MT707360-MT707433 (*cox*1) and MT707212-MT707241 (ITS2).

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Publisher's Note

2.4. Factors controlling the distribution of intermediate host snails of *Schistosoma* in crater lakes in Uganda: A machine learning approach

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Factors Controlling the Distribution of Intermediate Host Snails of *Schistosoma* in Crater Lakes in Uganda: A Machine Learning Approach

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Schistosomiasis affects over 700 million people globally. 90% of the infected live in sub-Saharan Africa, where the trematode species *Schistosoma mansoni* and *S. haematobium* transmitted by intermediate hosts (IH) of the gastropod genera *Biomphalaria* and *Bulinus* are the major cause of the human disease burden. Understanding the factors influencing the distribution of the IH is vital towards the control of human schistosomiasis. We explored the applicability of a machine learning algorithm, random forest, to determine significant predictors of IH distribution and their variation across different geographic scales in crater lakes in western Uganda. We found distinct variation in the potential controls of IH snail distribution among the two snail genera as well as across different geographic scales. On the larger scale, geography, diversity of the associated mollusk fauna and climate are important predictors for the presence of *Biomphalaria*, whereas mollusk diversity, water chemistry and geography mainly control the occurrence of *Bulinus*. Mollusk diversity and geography are relevant for the presence of both genera combined. On the scale of an individual crater lake field, *Biomphalaria* is solely controlled by geography, while mollusk diversity is most relevant for the presence of *Bulinus*. Our study demonstrates the importance of combining a comprehensive set of predictor variables, a method that allows for variable selection and a differentiated assessment of different host genera and geographic scale to reveal relevant predictors of distribution. The results of our study contribute to making realistic predictions of IH snail distribution and schistosomiasis prevalence and can help in supporting strategies towards controlling the disease.

Keywords: schistosomisis, biotic and abiotic predictors, mollusks, random forest, Africa

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INTRODUCTION

Human schistosomiasis (bilharzia) is the second most important tropical parasitic disease after malaria (World Health Organization, 2016) and ranked the most important water-borne disease (Steinmann et al., 2006). It poses a global burden to humankind with over 700 million individuals in 78 countries at risk of infection, claiming over 200,000 lives annually (World Health Organization, 2016). In addition, more than 240 million people are infected worldwide, predominantly in sub-Saharan Africa (World Health Organization, 2016), where the disease burden is up to 90% of the global infections due to poor standards of living (Bergquist et al., 2017). Thus, schistosomiasis is commonly referred to as "the disease of the poor". Countries in sub-Saharan Africa face a challenge of high population growth, and most people live in rural or semi-rural settings associated with poverty, poor sanitation and no access to clean water (Gray et al., 2010; King, 2010; Payne and Fitchett, 2010). In such geographical settings, people might continuously be in contact with water contaminated with schistosome eggs (Stothard et al., 2005), and a large part of the population is at risk of infection.

Despite schistosomiasis being one of the most prevalent tropical diseases (Steinmann et al., 2006), it is also probably the most neglected and was given little priority by the funding bodies compared to HIV/AIDS, malaria and tuberculosis (Hotez et al., 2007; Utzinger et al., 2009). Nevertheless, a recent growing interest in neglected tropical diseases including schistosomiasis has been observed over the last decade (World Health Organization, 2012; Shiff, 2017; King et al., 2020).

So far, strategies to control the spread of the disease *via* the provision of schistosomicides and/or WASH (water, sanitation, hygiene) programmes have shown limited effectiveness, and were consequently leading to disease re-emergences in spite of the interventions (Gryseels and Polderman, 1991; Chitsulo et al., 2000; Fenwick et al., 2009). Schistosomiasis is caused by trematode worms of the genus *Schistosoma* being transmitted through intermediate host (IH) snails. The reproductive cycle of *Schistosoma* trematodes starts with parasitic eggs released into freshwater through faeces and urine by infected humans. Eventually, motile larvae called miracidia hatch from the eggs and swim in search of snails to infect as intermediate host. The parasite then reproduces asexually within the snail, before shedding to the water as cercariae, larvae that penetrate the skin of the human host to complete the cycle and eventually cause the disease (Colley et al., 2014).

Sustainable vector snail control has been suggested as a more reliable approach to the schistosomiasis problem (Gryseels et al., 2006; Steinmann et al., 2006; Wang et al., 2008; Colley et al., 2014). The control aims at interrupting the transmission and stopping the spread of infection (Rollinson et al., 2013; Walz et al., 2015; Sokolow et al., 2016), by interrupting the *Schistosoma* life cycle through eliminating potential host snails from local habitats (King and Bertsch, 2015). Yet, this approach relies on the availability of high-quality snail distribution data, which represents a major knowledge gap in most developing countries in sub-Saharan Africa.

In sub-Saharan Africa, *Schistosoma mansoni* and *S. haematobium* are the major cause of the human disease burden in Africa (Chitsulo et al., 2000; Gryseels et al., 2006). *Schistosoma mansoni* is transmitted by snails of the genus *Biomphalaria* (Planorbidae) and causes human intestinal schistosomiasis. In contrast, *S. haematobium* is transmitted by species of *Bulinus* (Bulinidae) and cause human urogenital schistosomiasis (Wang et al., 2008; Colley et al., 2014). *Schistosoma mansoni* and *S. haematobium* are mainly distributed in and around a variety of freshwater habitats such as dams, lakes and rivers (Brown, 1994; Steinmann et al., 2006; Appleton and Madsen, 2012). *Bulinus* species in particular can live in permanent or seasonal pools, rice fields and ditches. In addition, there are several other species of *Schistosoma* that are of significant veterinary importance causing schistosomiasis in livestock. They are either hosted by *Bulinus* species (*S. bovis*) or selected species of the genus (*S. magrebowiei*) (Standley et al., 2012).

Previous studies dealing with the factors that influence the distribution of *Schistosoma* IH snails included water depth (Prah and James, 1978; Utzinger and Tanner, 2000; Boelee and Laamrani, 2004), altitude (John et al., 2008; Stanton et al., 2017), temperature, precipitation, pH level, forest cover and distance to nearest water bodies (Stensgaard et al., 2013), heat waves, droughts and floods (McCreesh and Booth, 2013; Pedersen et al., 2014), calcium and salinity (Hoverman et al., 2011; Maes et al., 2021). Although the relationships with these potential predictors have been examined and tested individually, they were not quantitatively assessed for their combined importance. The contribution of these factors to the distribution of IH snails and the prevalence of schistosomiasis accordingly across different regions is far from understood.

This study focuses on two main goals: 1) to assess the significance of extrinsic (geographical, hydrological, climatic, faunistic and anthropogenic) factors for the distribution of intermediate IH snails of the genera *Bulinus* and *Biomphalaria*, being the major causes of human infections in Africa, 2) to test for differences of potential predictors and their varying impact across different geographical scales. To do so, we used a model system of 56 crater lakes in western Uganda that variably contain IH species of the two genera, along with a diverse set of geographical, climatic, hydrological, faunistic and anthropogenic parameters. We used a machine learning approach (random forest, RF) to assess the importance of the individual parameters and how they differ across the study area. Considering the geographic variation of population density (Gelorini et al., 2012; Hartter et al., 2012), land use intensity (Hartter et al., 2015), and limnological characteristics of the lakes (De Crop and Verschuren, 2019), we hypothesize that a different set of parameters is relevant in the different regions examined. Moreover, we expect that different parameters control the distributions of the two genera, since they do have different dispersal and drought resistance capacities (Brown, 1994).

MATERIALS AND METHODS

Study Area

The study area is located on the hilly uplands (>1,600 m a.s.l.) of the Edward-George branch of the East African Rift valley in western Uganda (**Figure 1**). It is bordered by the Rwenzori

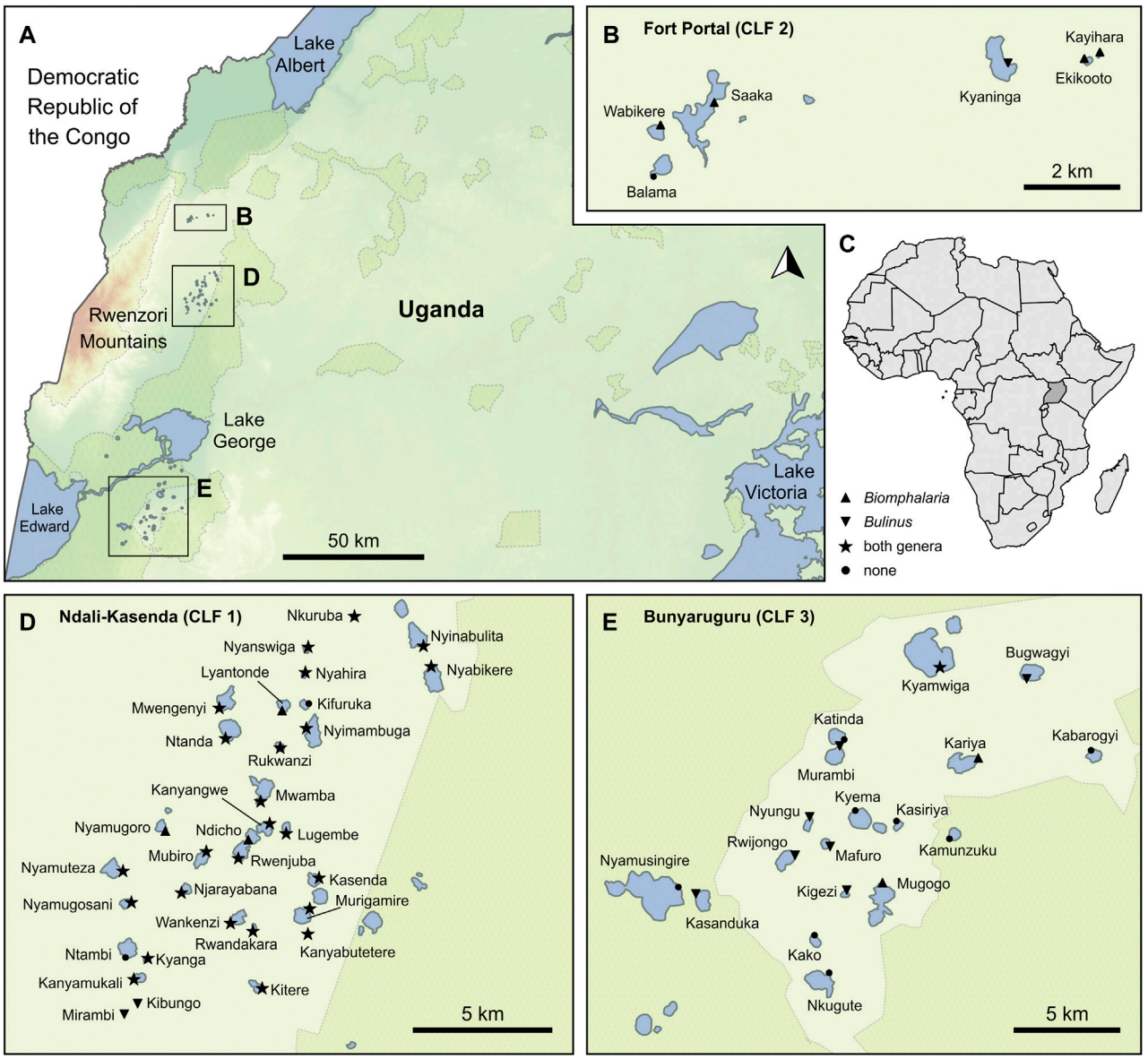

FIGURE 1 | Study area and occurrences of intermediate host (IH) snails. Three crater lake fields (CLF1–3) **(B,D,E)** in western Uganda **(A)** in Africa **(C)** were studied. Symbols at sampled lakes indicate the presence of *Biomphalaria* (triangle), *Bulinus* (inverted triangle), and both genera combined (dark star), whereas lakes marked with a small circle indicate localities where no IH snail was found.

Mountain range in the North-West, Lake Albert in the North and Queen Elizabeth National Park in the South. It contains over 90 small crater lakes, most of which formed between 4,000 and 10,000 years ago as a result of faulting and volcanic eruptions (Vinogradov, 1980; Schumann et al., 2015). Lake Ntambi, which formed c. 50,000 years ago is an exception, (Dirk Verschuren, pers. comm.).

The crater lakes of western Uganda have been repeatedly promoted as an ideal model system for studying large environmental gradients in limnological characteristics in a setting that allows meaningful comparisons due to shared geological history (e.g. Melack, 1978; Mills and Ryves, 2012; Saulnier-Talbot and Lavoie, 2018). The crater lakes region is one of the most densely populated rural areas in sub-Saharan Africa (Hartter et al., 2012), with a population growth rate of 3.3% annually (Gelorini et al., 2012). The population growth is coupled with increasing human impact through settlement, fishing, agriculture on the fertile volcano soils, swimming and water extraction for irrigation and domestic use. As a result of the high population density, the prevalence of (human) schistosomiasis in the region has dramatically increased (Kabatereine et al., 2004; Kabatereine et al., 2006; John et al., 2008; Stanton et al., 2017). In 2014, the crater lakes region covered parts of the 73 out of 112 districts of Uganda with prevalence of

schistosomiasis (Loewenberg, 2014). Nationwide, four million people are estimated to be infected and almost 20 million are at risk of infection (Loewenberg, 2014). The only preventative measures in the area are massive drug administration while environmental transmission interruption is rarely emphasized (Loewenberg, 2014).

Melack (1978) classified the region into four crater lake fields (CLFs, **Figure 1**). The Ndali-Kasenda field is locatedin the central part of the region, ~20 km from Fort Portal field in the North and ~65 km from Bunyaruguru field in the South. The fourth field of Katwe-Kikorongo contains lakes with saline waters (Rumes et al., 2011), unsuitable for mollusks (Tumwebaze et al., 2019).

Our dataset combines information from a total of 56 crater lakes in the three fields, including 32 lakes of the Ndali-Kasenda field (CLF 1), six lakes of the Fort Portal field (CLF 2) and 18 lakes of the Bunyaruguru field (CLF three; see **Supplementary Table S1**).

Data Collection

Malacological Field Data

Snails were sampled across the three regions in random months between 2010 and 2019 to account for a range of weather conditions, but mostly in dry seasons at normal or low water levels between 2010 and 2019 (**Figure 1**). Field work periods were aligned to times when highest population densities of the intermediate host snails were expected. Confounding effects by flooding and restricted accessibility during rainy seasons were avoided. Due to the steep and slippery escarpments of the crater lakes, sampling in the wet season was also avoided. Sampling methods involved dredging and/or scoop netting up to a maximum depth of 1.5 m or hand-picking snails found attached to shoreline vegetation and any solid substrates. The sampling time per lake was 40 min, and snails were collected in one to two localities (depending on the size of the respective lake). In some cases, lakes were visited more than once to ensure comprehensive representation of the local fauna. At each locality, we identified and counted the IH snails as well as the associated mollusk fauna on the genus level. The survey also revealed the presence of seven other non-host snail genera (*Radix, Gabbiella, Pila, Melanoides, Segmentorbis, Afrogyrorbis* and *Gyraulus*) as well as the bivalve genus *Sphaerium*.

Climatic and Environmental Data

We included air temperature and precipitation as proxies, because they have previously been shown to influence the distribution of freshwater mollusks (e.g., Hauffe et al., 2016a; Georgopoulou et al., 2016) and particularly those of IH snails (Appleton, 1978; Rowel et al., 2015). Specifically, temperature influences the survival and reproduction rates of snails (Paull and Johnson, 2011; McCreesh et al., 2014; Kalinda et al., 2017). Precipitation is associated with organic matter input and nutrient supply, which affects snail growth and fecundity (Madsen et al., 1987; Camara et al., 2012; Nyström Sandman et al., 2013). We retrieved the climatic data (averaged for the period 1970–2000) from the WorldClim two global database (Fick and Hijmans, 2017). We used mean annual temperature (BIO1), temperature of warmest month (BIO5), temperature of the coldest month (BIO6), annual precipitation (BIO12), precipitation of the wettest month (BIO13) and precipitation of the driest month (BIO14) to account for potential selectivity of the IH snail species to climatic fluctuations. Since the different temperature and precipitation parameters showed a similar range of variation, we calculated principal component analyses and used the first principal component for each of the two sets.

Water chemistry also plays a vital role for the occurrence and abundance of freshwater gastropods. This concerns pH, oxygen, conductivity, surface water temperature, magnesium and calcium (Rumes et al., 2011; Marie et al., 2015; Mahmoud et al., 2019; Alhassan et al., 2020; Olkeba et al., 2020). Surface water temperature, dissolved oxygen, pH and conductivity were measured using a handheld multi-meter probe. Calcium and magnesium data were retrieved from Rumes et al. (2011) and Nankabirwa et al. (2019).

Previous studies have shown the relevance of depth (both absolute lake depth and Secchi depth, i.e., a measure of water transparency) for the occurrence of both *Bulinus* and *Biomphalaria*. Absolute depth was retrieved from De Crop and Verschuren (2019), and water transparency (i.e., Secchi depth) was measured at the sampling points using a Secchi disk.

The crater lakes are characterized by seasonal fluctuations in water levels, and some lakes occasionally dry out (e.g., Lake Mirambi and Lake Kibungo). We included lake surface area and surface area variance over time as parameters in this study. The lake surface area was retrieved from satellite images from Google Earth Pro v. 7.3 taken in 2019. The lake area variance was calculated as the variance of four time slots of lake surface areas traced from satellite images taken in 2003, 2008, 2013 and 2018. Information for a few satellite images was missing, because either some lakes dried out or no records were captured in Google Earth. In such cases, we used the image of the closest time prior or after a given time slot to retrieve lake surface area.

Geographical Data

Longitude and latitude of the sampling sites were included as variables to account for potential variation in the geographical distribution of the IH snails. Altitude has been also proved relevant in the occurrence of snail hosts (John et al., 2008; Stanton et al., 2017) and was therefore considered in our study. Longitude, latitude and altitude were measured with a handheld Garmin GPS eTrex 20 device.

We used two measures for geographical distance to serve as proxies of biogeographical isolation, which might impact colonization and thus the IH presence in the area: 1) distance from a crater lake to the nearest other crater lake, and 2) distance from a crater lake to the nearest larger lake in the surrounding (i.e., lakes Victoria, Edward, Albert and George; **Figure 1**). All distances were measured "as the crow flies" in Google Earth.

Human Impact

To obtain a measure of human impact, we distinguished and quantified the proportion of land use. Since no data are available from online databases, we used the total percentage of cultivatable fields (cropland, fallow land and plantations) as a proxy and visualized a square of 0.0625 km^2 (0.25 × 0.25 km) around each

TABLE 1 | Predictor variables used in this study. Note that temperature and precipitation each represent the first principal component calculated from three climate parameters; see text for details. Sources: 1—Rumes et al. (2011), 2—Nankabirwa et al. (2019), 3—De Crop and Verschuren (2019), 4—Fick and Hijmans (2017).

Category	Predictor variables	Sources
Fauna	Species richness of associated mollusk fauna	This study
Environment/hydrology	Surface water temperature (°C)	This study
	Water pH	This study
	Dissolved oxygen (mg/L)	This study
	Electric conductivity (μS/cm)	This study
	Magnesium concentration (mg/L)	1, 2
	Calcium concentration (mg/L)	1, 2
	Secchi depth (m)	This study
	Lake depth (m)	3
	Lake surface area (km^2)	This study
	Lake area variance	This study
Climate	Temperature (°C)	4
	Precipitation (mm)	4
Geography	Longitude (°E)	This study
	Latitude (°N)	This study
	Altitude (m a.s.l.)	This study
	Distance to the nearest crater lake (km)	This study
	Distance to the nearest large lake (km)	This study
Human impact	Land use (% of area)	This study
	Population density (number of houses)	This study

crater lake in Google Earth (centered around the lake centroid). Final percentages were arcsine/square-root transformed according to Warton and Hui (2011) to limit the influence of outliers.

Additionally, the number of people living in the surroundings of a lake directly relates to the risk of schistosomiasis infection. Due to the lack of population census records in the region, we counted the number of houses in a standardized area of 0.25 km^2 (0.5 × 0.5 km) around each lake using satellite images from Google Earth taken in 2019 as a measure of population density.

A total of 20 predictor variables belonging to five categories were used in our study, 15 of which were retrieved in the course of the present survey, two were obtained from online databases and three were taken from the literature (**Table 1**, **Supplementary Table S1**).

Data Analysis

We applied a machine learning approach, i.e., random forest (RF; Breiman, 2001), to assess the combined impact of the chosen set of predictors on the distribution of IH snails. Machine learning approaches, and particularly RFs, have gained prominence in classification and regression analyses across various fields of science in recent years (e.g., Huang and Boutros, 2016; Pang et al., 2017; Schonlau and Zou, 2020; Collin et al., 2021; Georganos et al., 2021; Ruiz-AÍvarez et al., 2021). In classification problems, RFs have been demonstrated to give more accurate predictions than other approaches, such as logistic regression (Boulesteix et al., 2012; Bunyamin and Tunys, 2016; Couronné et al., 2018; Xia et al., 2019; Zhang et al., 2020). Since it is a non-parametric technique, the RF algorithm is not affected by multicollinearity among the predictor variables (Boonprong et al., 2018), which is a common problem in ecology. Also, many RF software packages come with convenient solutions to deal with missing values (Brieuc et al., 2018).

We conducted separate RF analyses to variably predict the presence of *Bulinus*, *Biomphalaria* and both genera combined. To assess variation of the potential predictors across different geographical scales, at a larger geographical extent, we ran analyses for the overall and complete dataset combining data from all the three crater lake fields. On the scale of individual crater lakes regions, we ran analyses for two subsets of CLF 1 and CLF 3. The analysis for Fort Portal field (CLF 2 subset) was not performed because it contains only six lakes. All analyses were done in the R statistical environment v. 4.0.3 (R Core Team, 2020), using the packages randomForest v. 4.6-14 (Liaw and Wiener, 2002), rfUtilities v. 2.1-5 (Evans and Murphy, 2019) and rfPermute v. 2.1.81 (Archer, 2020).

We performed imputation to fill missing data prior to further data analyses, using the function "rfImpute" in the package randomForest, which uses the RF algorithm to obtain weighted averages of the available observations. This was done for the predictors; calcium, magnesium and water depth, for which no data were available for 18, 16 and 16 lakes, respectively. Overall, missing data added up 4.7%. The "rfPermute" algorithm was used to assess variable importance in each RF model *via* permutation. The algorithm creates decision trees from the original dataset by random sampling of rows (i.e., lakes) without replacement. At each node, two-thirds of the rows are taken as training data to create the model, the remaining one-third is taken as so-called out-of-bag (OOB) sample and is used to make predictions and test for the performance of the model (Breiman, 1996; Breiman, 2001). Variables were permuted 100 times over 1,000 decision trees.

Model performance was additionally assessed *via* cross validation. This approach was chosen over the standard train–test data procedure because of the comparably low number of lakes in the dataset. Cross validation is a commonly used resampling method to assess the

TABLE 2 | Error rates and results of cross validation for all runs of the random forest (RF) models. Validation agreement was evaluated in accordance with Viera and Garrett (2005). The co-existence model for CLF three had insufficient data and is not included here. OOB, out-of-bag error.

Dataset	RF model	Error rates			Cross validation		
		OOB error	Error presence	Error absence	Kappa coefficient *K*	Validation error	Validation agreement
Complete dataset	*Biomphalaria*	0.143	0.111	0.200	0.911	0.089	excellent
	Bulinus	0.250	0.111	0.500	0.956	0.044	excellent
	Co-existence	0.179	0.148	0.207	0.919	0.081	excellent
Ndali-Kasenda (CLF 1)	*Biomphalaria*	0.063	0.000	0.500	0.633	0.367	substantial
	Bulinus	0.188	0.037	1.000	0.800	0.200	substantial
	Co-existence	0.188	0.040	0.714	0.633	0.367	substantial
Bunyaruguru (CLF 3)	*Biomphalaria*	0.222	1.000	0.067	0.633	0.367	substantial
	Bulinus	0.556	0.750	0.400	0.800	0.200	substantial

generalization potential of a model and to avoid overfitting (Berrar, 2019). We report here the kappa coefficient *K*, which determines the model's predictive accuracy, i.e., it gives the percentage of the data that is in agreement with the model (and is thus the opposite of the validation error). We adopted the suggestion of Viera and Garrett (2005), who regarded kappa values of 0.81-1 as excellent and 0.61–0.80 as substantial agreement, to evaluate our model performances.

The relevance of individual parameters to the overall RF models was assessed using two importance metrics, i.e., the mean decrease in accuracy (MDA) and the mean decrease Gini (MDG) (Calle and Urrea, 2011; Huang and Boutros, 2016). Due to discrepancies in ranking results between MDA and MDG, the results of a single metric are not completely exhaustive (Strobl et al., 2007; Liu et al., 2011). Therefore, we included both metrics but discussed in detail only those parameters that are found significant by both MDA and MDG. We used partial dependence plots to visualize the relationships and marginal effects of individual predictor variables (Friedman, 2001; Evans et al., 2011).

RESULTS

Models converged and were stable across all datasets, with those for *Biomphalaria* always performing better than those for *Bulinus* or both genera combined (**Supplementary Figure S1**). The RF models resulted in excellent to substantial classification successes for the presences of *Biomphalaria* and *Bulinus* in relation to the chosen set of predictor variables (**Table 2**). Classification errors were generally higher for false negatives compared to false positives, which is probably a result of the low number of absences in all datasets. Nonetheless, the cross validation showed that the classification agreements ranged from substantial (CLF subsets) to excellent (total dataset; **Table 2**).

For the overall and complete dataset, geographical, water chemistry and biotic parameters were the most important predictors, but their relative importance and contributions varied across the three RF models, i.e., *Biomphalaria* vs. *Bulinus* vs. both genera combined (**Figure 2**). The distribution of *Biomphalaria* was mainly controlled by latitude, longitude, diversity of the associated mollusk fauna and distance to large lake, as well as by precipitation to some extent; oxygen was only found significant by MDA. For *Bulinus*, the diversity of the associated mollusk fauna, water conductivity and latitude were most important, whereas only MDG identified altitude as a significant predictor. For the combined model, the diversity of the associated mollusk fauna, latitude, longitude and distance to large lake were found relevant. Distance to the next crater lake, magnesium and calcium played a minor role and were only found significant by MDA. Other predictors such as human impact, water pH, surface area and temperature had very little effect and were not significantly impacting the IH species distribution in the region (**Figure 2**).

A different set of parameters was found to be important for the presence of IH snails in individual crater lake fields. For CLF 1, the distance to the next crater lake was the sole important parameter for the distribution of *Biomphalaria* (**Supplementary Figure S2**). In turn, the diversity of the associated mollusk fauna seemed to be the most relevant factor shaping the distribution of *Bulinus*, in addition to distance to the next large lake and lake surface area (MDA only). The co-existence of both genera was controlled by the diversity of the associated mollusk fauna and distance from the next crater lake (**Supplementary Figure S2**). The model for CLF three yielded comparably high error rates, concerning both overall errors as well as classification errors for presences and absences (**Table 2**). Consequently, any association found for CLF 3 with individual parameters is unreliable and will not be discussed further.

The predicted probabilities for the occurrence of IH snails show non-linear relationships with individual predictor variables, with a generally positive trend being apparent in many cases (**Figure 3**). For *Biomphalaria*, the predicted probabilities of its occurrence increases with a rise in latitude, longitude, distance to the large lake (with a slight increase at the end), diversity of the associated mollusk fauna and precipitation. Increasing mollusk diversity also links with an increased probability to encounter *Bulinus*. The parameter conductivity shows a more complex relationship, featuring a steep probability increase up to ~400 µS/cm followed by a weakly, more or less gradually declining trend towards higher values (**Figure 3**). For both genera combined, similar positive trends are revealed for the relationship with the diversity of the associated mollusk fauna, latitude and longitude. The association with distance to the nearest large lake is more complex but indicates an increasing probability to encounter both genera above a distance of ~35 km.

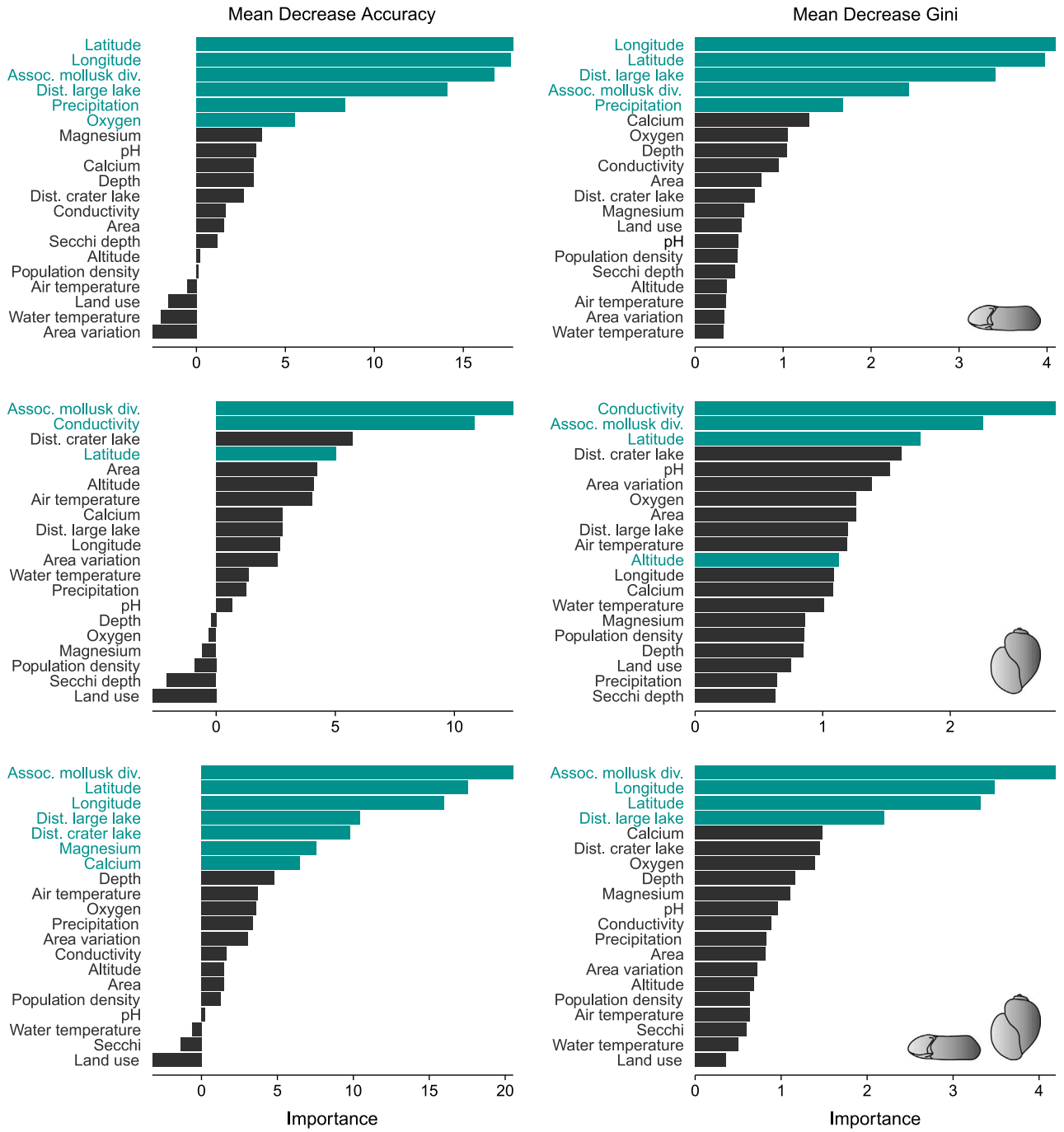

FIGURE 2 | Importance scores of predictor variables for *Biomphalaria* (upper panels), *Bulinus* (middle panels) and both genera combined (lower panels) for the complete dataset. The green bars indicate the significant predictors as determined *via* permutation tests. See **Supplementary Figure S2** for the importance scores of the subset CLF 1.

DISCUSSION

Understanding the factors influencing the distribution of snails acting as IH for *Schistosoma* is crucial for the control of human schistosomiasis. In this study, we explored the applicability of a machine learning algorithm to determine significant predictors of IH distribution and whether they differ among different Ugandan crater lakes. We found distinct variation in the potential controls of IH snail distribution. A different set of parameters is found to influence the distribution of the different genera and across different geographical scales. On the larger scale, geography, diversity of the associated mollusk fauna and

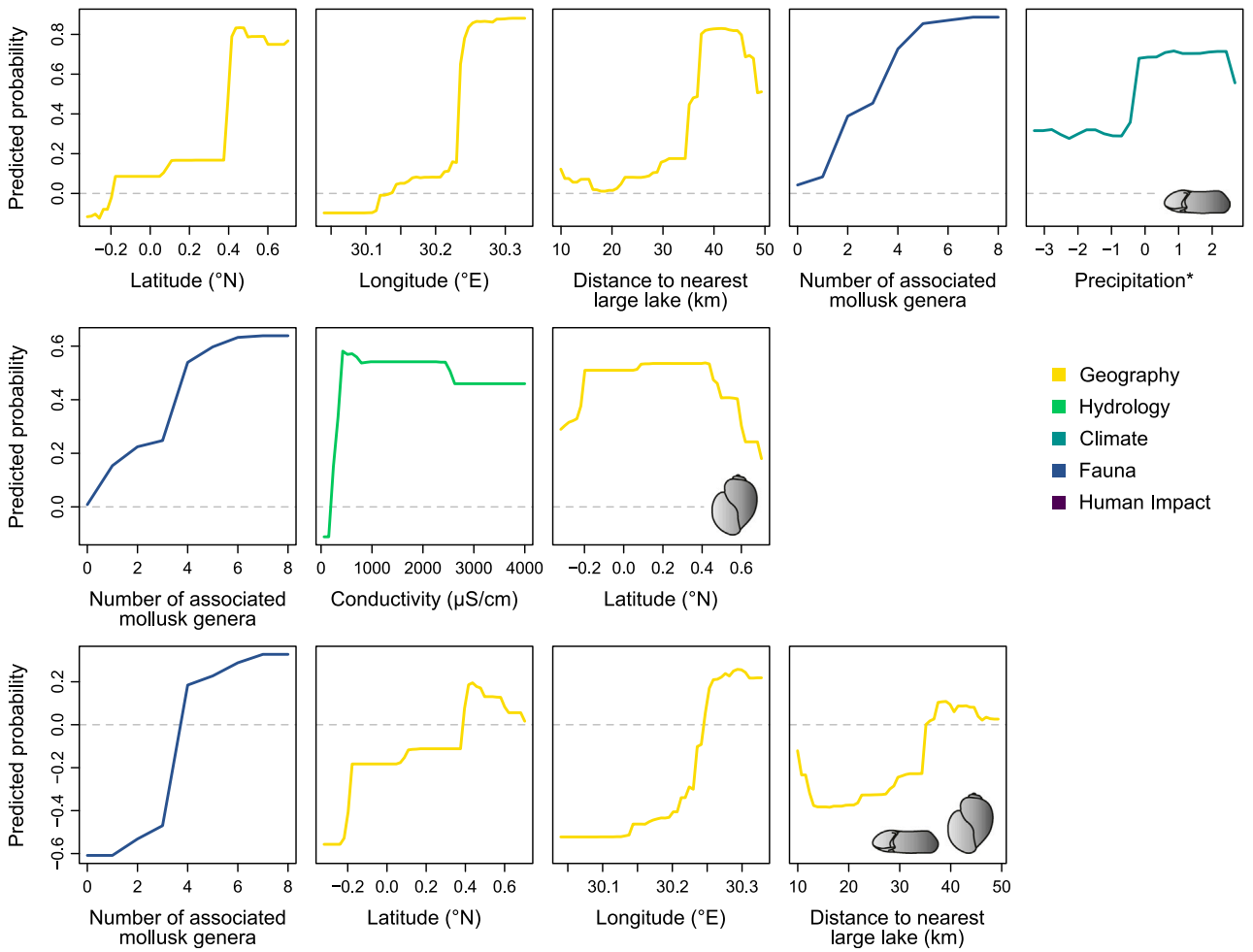

FIGURE 3 | Predicted probabilities for the presence of *Biomphalaria* (upper panels), *Bulinus* (middle panels) and both genera combined (lower panels) in relation to selected predictor variables from each dataset. Only the contributors that were found significant by both importance metrics (MDA and MDG) for the entire dataset are shown (compare **Figure 2**; see **Supplementary Figures S3–S8** for the remaining predictors as well as for the predicted probabilities for CLF 1). The asterisk denotes scaled parameters, i.e., the *x*-axis does not denote the original extent of the variable.

climate are important predictors for the presence of *Biomphalaria*, whereas mollusk diversity and water chemistry control the occurrence of *Bulinus*. Finally, mollusk diversity and geography are relevant for the presence of both genera. On the scale of an individual crater lake field (CLF1) geography (yet with a different variable) and mollusk diversity are relevant for the presence of *Bulinus* and both genera while *Biomphalaria* is solely controlled by geography. These results confirm our hypothesis that a different set of parameters is relevant on different geographical scales.

In the following sections, we discuss the relationships between IH snail occurrence and individual factors and groups of factors (geography, hydrology/water chemistry, climate, biotic and human impact) in an ecological context. Specifically, we discuss the distribution of the two genera with respect to metacommunity assembly processes, i.e., dispersal limitation, environmental filtering and biotic interactions, which limit the successful establishment of species in an ecosystem (see, e.g., Hauffe et al., 2016b for another freshwater gastropod example).

Geography: A Driver on Various Spatial Scales

Geographical variables were found to be among the most important controls for IH snail distribution, but the individual parameters depend on the scale of observation and the taxon in question. Latitude, longitude and distance to the nearest large lake are relevant for the occurrence of *Biomphalaria* as well as both genera combined. In contrast, *Bulinus* is much less influenced by geographical variables. Although latitude and altitude were identified significant, no positive relationship was found for the first (**Figure 3**), whereas the latter was of comparatively low importance (**Figure 2**). A similar trend is found in the Ndali-Kasenda crater lake field (CLF 1), where the distribution of *Biomphalaria* is strongly shaped by the distance to the nearest

crater lake, whereas the presence of *Bulinus* is mostly related to the diversity of the associated mollusk fauna (**Supplementary Figure S2**).

The strong positive relationship between the presence of *Biomphalaria* and latitude as well as longitude reflects the uneven occurrence of the genus across the study area (**Figure 1**). Particularly the rarity of the genus in the Bunyaruguru crater lake field (CLF 3) indicates that dispersal limitations and/or environmental filtering (e.g., Hauffe et al., 2016b) might constrain its distribution.

The generally positive association between the occurrence of *Biomphalaria* and distance to the nearest large lake is surprising at first, considering that distance is related to biogeographical isolation and colonization potential (Covich, 2010). Generally, these snails have a high passive dispersal capacity, a high reproduction rate and short generation times (Brown 1994). This combination is pronounced particularly in pulmonate snails such as *Biomphalaria* and makes them prime colonizers (Kappes and Haase, 2012; Kappes et al., 2014). The crater lakes are hydrologically disconnected, the possible longitudinal dispersal means are natural vectors such as birds (Kappes and Haase, 2012) and humans, through the attachment to fishing nets and/or boats.

A possible explanation for the positive association with distance concerns the taxonomic resolution. Here, we investigated the presence of genera, which may overprint patterns of dispersal and colonization of individual species of *Bulinus* and *Biomphalaria*. Also, Euclidean shore-to-shore distances might not reflect real dispersal means or pathways, because hydrological connectivity varies considerably between the large lakes and the crater lake fields. For example, the Fort Portal field (CLF 2) is connected to the Lake George–Lake Edward system *via* the Mpanga River but not to the geographically closer Lake Albert. The pattern may also results from choosing only four major lakes but disregarding smaller ones and river systems. Finally, in colonization processes, stochastic components might play an important role. For instance, regular episodes of shifting mixing regimes in some crater lakes (De Crop and Verschuren, 2021) could lead to low dissolved oxygen and consequently to the demise of most aquatic life. Based on genetic relationships of *Bulinus*, a previous study suggested that the large lakes acted as potential sources for (re)-colonization of the crater lakes (Tumwebaze et al., 2019), a scenario that cannot be tested here given the lack of genetic data. Fast re-appearance or aestivation in fluctuating environments is an intrinsic ecological feature of many *Bulinus* species, including the ones found in the study area (Watson, 1958; Brown, 1994). As such, the colonization and re-colonization patterns might be more complex and faster than previously anticipated.

Altitude was found to have limited influence on the distribution of IH snails. Several previous studies have indicated a shift in recent years of intestinal human schistosomiasis transmission towards higher altitudes (Kabatereine et al., 2004; John et al., 2008; Stanton et al., 2017). This is also supported by our findings showing that IH snails occur up to approx. 1,600 m a.s.l., and thus considerably higher than the previously presumed threshold of 1,400 m a.s.l. (Kabatereine et al., 2004). Future studies will have to establish an upper limit for both forms of schistosomiasis, because both IH snail genera have been found at altitudes above 2,000 m a.s.l. in Uganda (Stanton et al., 2017, unpubl. data).

Associated Mollusk Diversity

A strong control for the distribution of both *Bulinus* and *Biomphalaria* is the diversity of the associated mollusk fauna. The strong positive relationships in all three models (**Figure 3**) indicate that both genera are more likely encountered in generally diverse systems. One may expect the opposite, that a higher number of species (especially in a small crater lake) results in higher competition (e.g. Svanbäck and Bolnick, 2007; Hauffe et al., 2016b) and thus a lower chance of successful establishment. Instead, the positive association with diversity indicates that the IH snail genera are present in environments that provide favorable conditions for mollusks generally.

The associated mollusk fauna consists predominantly of pulmonates (*Radix natalensis* and several species of planorbids other than *Biomphalaria*), which are good colonizers in general and are characterized by a high productivity and shorter generation times (Kappes et al., 2014). Malacological surveys showed the presence of *Biomphalaria sudanica*, *Biomphalaria pfeifferi*, *Bulinus forskalii*, *Bulinus globosus*, and *Bulinus tropicus* (Tumwebaze et al., 2019; Tumwebaze et al., unpubl. data). All *Biomphalaria* species in the region are regarded susceptible to *S. mansoni*. Only *B. forskalii* and *B. globosus* were identified as host species for human schistosomes and the majority of *Bulinus* in the crater lakes region were *B. tropicus*, which is an important IH snail for livestock schistosomes. The non-pulmonate species are common and widespread regional species with no particular ecological requirements (Brown, 1994). More specialized taxa such as *Bellamya* or *Cleopatra* or unionid bivalves were not found in the crater lakes. The mollusk association in the crater lakes could still be seen as depauperate. The most obvious variation in habitat conditions and thus potentially determining mollusk associations is in the limnological characteristics of the crater lakes. Although we gathered a variety of different parameters concerning water chemistry and climatic conditions, the associations with individual parameters do not allow drawing a multifactorial picture. Moreover, the parameters measured for each crater lake do not cover seasonal variability (except for climate and lake area variation).

Hydrology and Water Chemistry

Only selected hydrochemical variables had an influence on the distribution of IH snails in our study region. Water conductivity was a significant driver across the entire study region and in CLF one for *Bulinus*. For *Biomphalaria*, oxygen was found a significant driver by one metric (MDA), but its relative importance was low (**Figure 2**). Similarly, MDA found the presence of both genera linked to magnesium and calcium, but again with low importance values.

Water conductivity determines the ionic strength of the concentration of dissolved solids including calcium and magnesium (Cormier et al., 2013). An expanding distribution with increasing conductivity was also suggested by Camara et al. (2012). This might reflect the presence of dissolved ions (e.g., calcium), which stimulate shell development for snail species

(Dillon, 2000; see also below). The non-linear relationship with a sharp increase around ~400 µS/cm followed by a nearly steady but weak decline indicates that a certain threshold must be met to allow the establishment of *Bulinus* in a lake. Higher electric conductivity is, in turn, less favorable. Conductivity as a complex factor integrating several chemical components has been identified as a determinant for *Bulinus* mortality and thus poses an important constraint on its occurrence (Brown, 1994; Marie et al., 2015).

The predicted probabilities for the presence of both genera in relation to magnesium and calcium concentrations, which were, however, only found significant by one importance metric (MDA), demonstrate that a certain threshold must be surpassed in both factors to promote the establishment of IH snails. Especially the relationship with calcium is not surprising, considering that a low concentration would constrain snail growth, fecundity, survival rate and reproduction, which, in turn, limit snail distribution (Dillon, 2000; Brodersen and Madsen, 2003). The concentration of calcium and magnesium and their ratio in the water both affect the presence and life cycle performances in southern African streams with thresholds at the lower and higher ends of concentrations (Brown, 1994).

Climate

Climatic factors seem to have surprisingly little effect on the distribution of IH snails in Ugandan crater lakes. Precipitation was found a significant but rather weak predictor in the model with *Biomphalaria*, whereas air and water temperature do not appear influential. Generally, climatic conditions and climate change are known to be important predictors for mollusk species distribution on larger geographical scales (Marcogliese, 2008; Stensgaard et al., 2019) and were also found to be relevant for IH snails. Temperature influences the survival and reproduction rates of the snails (Paull and Johnson, 2011; McCreesh et al., 2014; Kalinda et al., 2017). Higher rainfall causes more runoff into freshwater ecosystems increasing the supply of organic matter serving as food for the snails, which, in turn, promotes growth and fecundity (Madsen et al., 1987; Camara et al., 2012; Nyström Sandman et al., 2013; but see also discussion in David et al., 2018 for opposite associations). This may also explain the relationship with precipitation in our case. However, the lack of a (strong) association in our models is likely owed to the constrained geographical scale of our observations. The crater lakes are located in the same climatic zone in western Uganda, which is why precipitation and air temperature vary only little across the study region. Whereas air temperature seems to be irrelevant on that scale, the relationship with precipitation indicates that even small variations might have a significant impact on the IH snail distribution.

Non-Significant Drivers

A series of parameters was found to be non-significant in any of our models. Here, we briefly discuss the potential reasons for these findings, especially in the light of conflicting results reported in the literature. In contrast, previous studies found relationships between the distribution of *Biomphalaria* and water pH, whereas both oxygen and water pH have been suggested to determine the occurrence of *Bulinus* (Yirenya-Tawiah et al., 2011; Stensgaard et al., 2013; Marie et al., 2015; Mahmoud et al., 2019; Alhassan et al., 2020). However, no such influences were noticed despite of the influential factors of high oxygen concentration and water pH of the lakes studied (**Supplementary Table S1**). Possibly, the high carbon dioxide emission from decomposing submerged vegetation and organic matter, together with the presence of other dissolved ions could have indirectly affected such relationships (Tchakonté et al., 2014). Lake area variation, being a measure of ecosystem stability, seems also of little importance. Apparently, most of the lakes show minimal fluctuations through time, and the few that occasionally dry up could not influence the distribution or recolonization, which is facilitated by the short distances between the crater lakes. The mollusk associations of mostly opportunistic species found is also supporting this interpretation (see above).

Human impact, quantified by both land use and population density around the crater lakes, has apparently no impact on snail distribution. This result is rather unexpected, because humans are often involved in introduction of snails into new environments as passive dispersal vectors (Kappes and Haase, 2012). In the Ugandan crater lake fields, extensive anthropogenic activities (e.g., multipurpose water fetching, watering for livestock, littering and pollution) are limited to lower, relatively flat and thus more easily accessible shores of most of the crater lakes. As such, one may have expected an impact on snail occurrences. Moreover, only about 26% of the crater lakes are located in national parks (Queen Elizabeth NP and Kibale NP), thus three-quarter of the lakes are accessible and utilizable by humans. Despite these constraints, some reserves and parks like Kibale NP increasingly face migration of settlers and extensive cultivation (Hartter et al., 2015). Perhaps the prevailing population pressure affects the prevalence of infected snails, but it does not influence their presence in general. Follow-up studies should focus specifically on this aspect for mollusks communities and the prevalence of schistosomiasis. Interestingly, in other non-gastropod taxa such as cladocerans (Rumes et al., 2011) and fungi communities (Gelorini et al., 2012), negative effects of land use change and anthropogenic pressure have already been demonstrated for the crater lakes region. With increasing human activities in the Ugandan crater lakes region (including tourism), we expect further changes in biological communities of the lakes as well as increasing cases of human schistosomiasis among both local communities and visiting travelers (Lachish et al., 2013) in the near future.

Methodological Implications and Limitations

Despite the numerous advantages machine learning approaches like RFs offer, one disadvantage concerns the non-linearity of the approach, resulting in a limited prediction power outside the data range. Therefore, we cannot extrapolate our findings to other datasets or regions. Furthermore, small datasets (e.g. CLF 2,

Biomphalaria subset with CLF3), decisively limit RF classification due to insufficient data. Optimally, future studies need to include data from a larger geographical scale, involving a greater variation in the ranges of predictor variables, to provide more general predictions for the controls of IH snail distributions. Using machine-learning algorithms like RFs on a comprehensive dataset will eventually facilitate more general conclusions about the importance of individual predictors (or sets of predictors) for the presence of IH snails. Future studies may focus on applying this approach to map infection risk areas, especially in comparison to areas with actual prevalence records and those where preventative measures are in place.

CONCLUSION

The results indicate that *Biomphalaria* is mainly controlled by geography, associated mollusk diversity and climate, while fauna, hydrology and to some extent geography controlled the presence of *Bulinus*. Geography (*Biomphalaria*) and mollusk diversity (*Bulinus*) were the only significant predictors on the scale of an individual crater lake field. The intricate relationship between IH snail distribution and geography likely reflects dispersal limitations and/or environmental filtering on the hand and a complex pattern of (re-)colonization on the other hand. The positive association with the diversity of accompanying mollusks, as well as the relationship with water conductivity, indicates that IH snails are common in ecosystems offering favorable conditions for mollusks in general.

Our machine learning approach helped disentangling relevant factors in IH snail distribution. The results of this study provide baseline data that assist future research towards controlling schistosomiasis.

DATA AVAILABILITY STATEMENT

All data used in the study is available in the paper or the **Supplementary Material**.

AUTHOR CONTRIBUTIONS

CA, ZT, and IT conceived the study. CA and IT conducted field work. ZT and TN performed the analyses. ZT wrote the manuscript with contributions from TN and CA. TN, BS, and CH prepared the figures. LB and CA supervised and critically revised the study. All authors reviewed the manuscript and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2022.871735/full#supplementary-material

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2.5. Evolution and biogeography of freshwater snails of the genus *Bulinus* (Gastropoda) in afromontane extreme environments

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Evolution and Biogeography of Freshwater Snails of the Genus *Bulinus* (Gastropoda) in Afromontane Extreme Environments

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Mountains are considered island-like systems often characterized by exceptional biodiversity and endemism. There are many highly isolated mountain ranges in Africa that collectively have been called the Afromontane archipelago. Freshwater snails of the genus *Bulinus* are composed of many veterinary and biomedical important species. These intermediate hosts for schistosomiasis parasites are wide spread and some of the species are considered to be highly adapted to very harsh environmental conditions such as droughts or low temperatures. However, the extent to which the *Bulinus* have adapted to live in high-altitudes and factors influencing these range shifts are not well investigated. In this study, we analyzed pan-African *Bulinus* species from various habitats across different altitudinal ranges, focusing on the high mountains or "sky islands" to examine how the contemporary climate and historical geological factors affect species distributions and evolutionary processes. Using a fossil-calibrated multigene phylogeny composed of two mitochondrial genes (*cox1, 16S*) and two nuclear genes (*ITS2* and *H3*), we tested: 1) how often and when extreme altitudes were colonized, 2) what are the biogeographical affinities and degree of isolation of high-altitude species, 3) which lineages diversified and evolved endemism in the Afromontane environments, and 4) whether the Afromontane regions represent "sky islands". Bayesian phylogenetic inference employing a fossil-calibrated molecular clock resulted in a strongly supported phylogeny resolving the relationships between the four *Bulinus* groups. High-altitude colonization exists exclusively within the *Bulinus truncatus/tropicus* complex. Several independent colonization events occurred in the Pliocene and Pleistocene throughout Africa, mostly from nearby regions of the respective mountain ranges. Most species evolved in low to mid-altitudinal ranges. Endemism is pronounced in the Ethiopian Highlands and those of Kenya and Lesotho. A previously unknown species was found at an extreme altitude (~4,000 m a.s.l.) on Mt. Elgon/Uganda extending the formerly known altitudinal maximum of the genus by roughly 900 m. The endemic species has already diverged in the Pliocene (~4 myr) and is currently characterized by low genetic diversity. There is further cryptic diversity in mountain ranges of Lesotho. Our findings are discussed in a biogeographical, conservation and biomedical context.

Keywords: Afrotropics, endemism, sky islands, biodiversity hotspots, schistosomiasis, climate change, high-altitude, intermediate host snails

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INTRODUCTION

Mountains worldwide are well-known cradles of biodiversity additionally characterized by elevated levels of endemism (Perrigo et al., 2020). Mountains are also island-like systems, surrounded by low-lying land masses (Itescu, 2019). A number of mountains are thus termed "sky islands" (e.g., McCormack et al., 2009; Mairal et al., 2017). The complex nature of these sky islands and the isolation between them restrict dispersal, thus affecting species distribution, richness and abundance (Gillespie et al., 2009). Colonisation for sky islands, unlike real islands is through immigration from low lands (altitudinal range niche shifts) or short and long dispersal from the nearby sky islands (latitudinal range shift) (McCormack et al., 2009). These immigration events, followed by *in-situ* speciation may give rise to cryptic species and in some cases species endemism. Consequently, these resulting narrow-range species are often under threat (Mairal et al., 2017; Martín-Queller et al., 2017). Besides historical events such as mountain-building, other factors have been "implicated" in shaping species richness (Georgopoulou et al., 2016). These are primarily temperature, species richness, isolation and area (Steinbauer et al., 2016). Varying climates along steep altitudinal gradients are often seen as prime drivers of isolation of populations, eventually leading to speciation on mountain tops (Rahbek et al., 2019). Mountain species are regularly characterized by pronounced niche conservatism (Antonelli et al., 2018). As isolated geographical features with characteristic species composition and distribution along pronounced altitudinal and thus climatic gradients, they are often used as models in climate change research (e.g., Fischer et al., 2011).

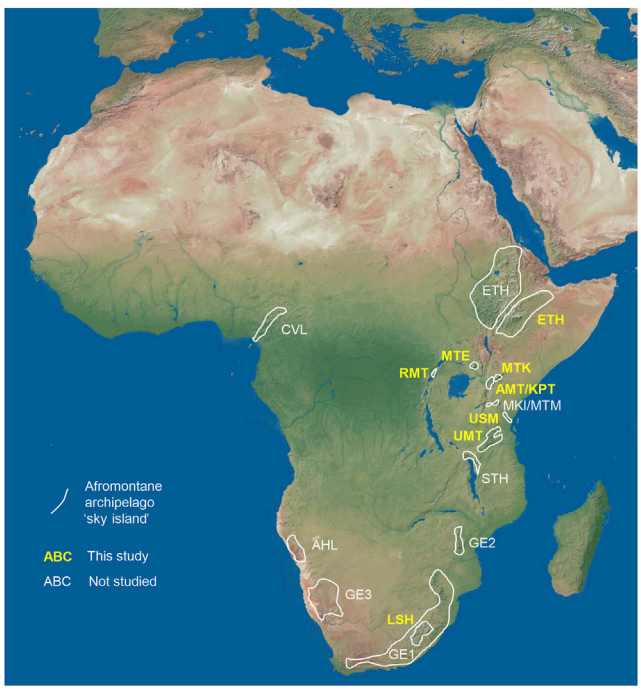

FIGURE 1 | Afromontane "sky islands" studied. The mountain ranges in sub-Saharan Africa covered in the current study are italicized and colored in yellow. Abbreviations: AHL, Angolan Highlands; AMT, Aberdares Mts.; CVL, Cameroon Volcanic Line; ETH, Ethiopian Highlands; GE1–3, Great Escarpment; Kinangop Plateau (KPT)-; LSH, Lesotho Highlands; MKI, Mt. Kilimanjaro; MTK, Mt. Kenya; MTM, Mt. Meru; MTE, Mt. Elgon; STH, Southern Tanzania Highlands; USM, Usambara Mts.; UMT, Udzungwa Mts.

There are many highly isolated mountain ranges in Africa, a topographically diverse continent significantly shaped by the rifting processes involving volcanism. These processes, in turn, produced several prominent volcanic mountains such as iconic Kilimanjaro, Mt. Kenya, Mt. Elgon, or the Cameroon Volcanic line, a long chain of volcanoes. The Ethiopian Highlands, Maloti-Drakensberge, Kenyan-Tanzanian Highlands, Eastern Arc Mts., and the Angolan Highlands represent other important mountain ranges that collectively have been called the Afromontane archipelago, i.e., a collection of widely scattered sky islands (**Figure 1**). They have been studied repeatedly regarding their diversity, endemicity and interconnectivity (e.g., Measey and Tolley, 2011; Mairal et al., 2017). Remarkably, they represent both cradles and refugia for biodiversity (Perrigo et al., 2020). Contrasting hypotheses have been proposed as to corridors connecting these sky islands in close proximity but also across the whole continent, e.g., from the Kenya-Tanzania Highlands to the Cameroon Volcanic line (Allen et al., 2021). Pleistocene corridors of Afromontane forest belts have been proposed (Mairal et al., 2021) but remain to be tested for most taxa, as the results of hitherto conducted studies are equivocal (e.g., Brühl, 1997; Cox et al., 2014). For mountain biota in Africa, montane refugia are often invoked as speciation mechanism, followed by montane gradient speciation and, to a lesser extent, peripatric speciation and rapid adaptive radiation, whereas the role of polyploidization as a genomic mechanism is not well understood (Couvreur et al., 2021).

Many patterns seen in biodiversity of the Afromontane archipelago have been attributed to changing topography and ecology triggered by geological rifting (e.g., Kingdon, 1989; Menegon et al., 2014). In addition, Pleistocene climatic changes produced multiple refugia, including cold-climate areas such as in sky-islands. These often acted as cradles for diversity on the population (Mairal et al., 2017) and species levels (Cox et al., 2014). The African Rift System and associated mountain ranges and beyond are thus an ideal model to study the interacting forces of climatic changes and geographic barriers on genetic biodiversity patterns over evolutionary times (Platts et al., 2013). Such studies are also critical in times of enormous pressure on the remaining montane biodiversity hotspots throughout the continent (White, 1981; Burgess et al., 2007). Even though these outstanding biodiversity hotspots have long been recognized (Mittermeier et al., 2011), most knowledge stems from either plants such as grasses (Mairal et al., 2021) or trees (DeBusk, 1998) and vertebrates such as small mammals, birds and amphibians (e.g., Cox et al., 2014; Loader et al., 2014). Invertebrates are less frequently studied and if so, mostly mobile organisms such as butterflies (but see flightless insects; Brühl, 1997). The overwhelming majority though represent terrestrial taxa, freshwater organisms are exceptionally scarcely studied (e.g., Daniels et al., 2020; Musonge et al., 2020).

Freshwater molluscs are very suitable for biogeographical studies of high-altitude ecosystems since they are less mobile, often highly habitat specific and have a moderate level of diversity but are comparatively well-known taxonomically (Brown, 1994), unlike most other invertebrates groups in tropical regions. African sky islands are only inhabited by a limited number of freshwater mollusc genera, namely pea-clams (Sphaeriidae; e.g., Kuiper, 1966; Clewing et al., 2022) and pulmonate snails (some Bulinidae, Planorbidae, Lymnaeidae; Brown, 1994). Mollusc species living in high-altitudes (roughly above 2,500 m above sea level) may experience extreme environmental conditions. These include, for example, higher radiation, desiccation of habitat, short periods for reproduction, shortage of food or nutrient availability, cold temperatures including freezing and low abundances (Bößneck, 2012).

The pulmonate gastropod genus *Bulinus* is, arguably, the most widespread pulmonate snail species in Afromontane regions. The genus is composed of many veterinary and biomedical important species. Many *Bulinus* species act as intermediate hosts for *Schistosoma* trematodes that cause, especially, human urogenital schistosomiasis, a disease affecting at least 130 million people in Africa alone (Brindley and Hotez, 2013). Factors that affect species distributions in Africa have not been investigated for many of the approximately 35 extant *Bulinus* species (Brown, 1994), especially in high-altitude freshwater habitats. Generally, climate change has been predicted to cause changes in species distribution and consequently changes in endemism, evolutionary processes such as speciation and extinction dynamics (Hua and Wiens, 2013), including molluscs (Böhm et al., 2021). Some species of the genus *Bulinus* are considered to be highly adapted to very harsh environmental conditions such as desiccation (e.g., *B. globosus*) or low temperatures (e.g., *B. africanus*) (Brown, 1994). The role of polyploidization has been discussed in this context (Brown, 1994). However, the extent to which *Bulinus* snails have adapted to high-altitudes in general and extreme altitude above 3,000 m a.s.l., in particular, is not yet accounted for. Here, we explore how and when species of *Bulinus* have evolved altitudinal ranges. This is important in order to understand the climate-related dynamics of host-parasite interactions. Furthermore, it is a prerequisite for potential predictions of how the ongoing climate change throughout sub-Saharan Africa will affect occurrence of intermediate host snails (Stensgaard et al., 2019; De Leo et al., 2020). In turn, this has direct implications for parasite prevalence and infections of both humans and livestock in areas where diseases have previously been absent (Stanton et al., 2017). Predictions for schistosomiasis in Africa foresee disappearance regionally in lowlands due to extended droughts (shortage of water and thus habitat). Contemporarily, however, increase in schistosomiasis in mountainous regions is predicted due to the expansion of suitable conditions, given the warming experienced (Stensgaard et al., 2019). As a consequence, the altitudinal threshold for schistosomiasis is expected to rise, potentially putting more people and an unrecognized part of the populations at risk (Stanton et al., 2017). Currently, there are no empirical studies available on this subject.

In this study, we analyzed pan-African *Bulinus* species from various habitats across different altitudinal ranges, focusing on the high mountains or "sky islands" to examine how the contemporary climate and historical geological factors affect species distributions and evolutionary processes. High-altitude samples from most Afromontane regions were collected and altitudinal ranges compiled from our own continent-wide sampling, the literature and databases.

Using a multigene phylogeny of three mitochondrial and two nuclear markers, molecular clock and character evolution analyses, we tested several evolutionary and biogeographical hypotheses for the evolution of the altitudinal niches for all species groups of *Bulinus*.

Specifically, we tested:

1) how often and when extreme altitudes were colonized,
2) what are the biogeographical affinities and degree of isolation of high-altitude species,
3) which lineages diversified and evolved endemism in the Afromontane environments, and
4) whether the Afromontane regions represent "sky islands".

Our findings are discussed in the context of biogeographical and diversification history and the dominant mode of speciation. Potential future developments are evaluated given changing climate predictions as well as biomedical and conservation implications.

MATERIALS AND METHODS

Sampling

Specimens were collected from mountains and highland regions throughout Africa between 2010 and 2018. These areas included the Lesotho Highlands (Maloti-Drakensberge), Ethiopian Highlands (Abyssinian Massif), Kenyan Highlands (Aberdares range including Kinangop Plateau, Mt. Kenya/Laikipia), Tanzanian Eastern Arc Mts. (Udzungwa, East Usambara), and Rwenzori Foothills (**Supplementary Table S1**; **Figure 1**). We also obtained samples from lower altitudes, primarily from Uganda, Malawi, Tanzania, the Democratic Republic of Congo, and Cameroon, for comparative purposes and maximized taxonomic representation. Sampling was done using mainly a scoop net, and in some occasions, handpicking from pools, wetlands marshes, lotic and lentic waterbodies. The material was fixed and preserved in 70%–80% ethanol. Coordinates and the altitude records were obtained using a hand-held Garmin etrex V global position system (GPS) device, and were later verified with Google Earth Pro version 7.3.4.

DNA Extraction, Amplification and Sequencing

At least two snails of each of the *Bulinus* population were selected for DNA isolation, resulting in a total of 104 *Bulinus* specimens.

Prior to DNA isolation, each of the selected snails was photographed using a Keyence digital microscope system (KEYENCE VHX-2000; Keyence Deutschland GmbH, Neu-Isenburg, Germany). Genomic DNA was then extracted from a small piece of the foot muscle (~2 mg) using a CTAB method (Wilke et al., 2006) or a DNeasy Blood and Tissue Kit (Qiagen, Mississauga, ON, Canada) following the manufacturer's protocol. The specimen vouchers (shells and DNA) are stored in the University of Giessen Systematics and Biodiversity collection (UGSB; Diehl et al., 2018). DNA amount and quality was checked using a NanoDrop 2000 (Thermo Fisher Scientific Inc., Waltham, MA, United States). Mitochondrial cytochrome *c* oxidase subunit I (*cox1*; "Folmer" and "Asmit" regions) and large subunit ribosomal RNA (*16S*) gene fragments were amplified using primers LCO1490 and HCO2198 (Folmer et al., 1994) or COR722b (Wilke and Davis, 2000) for the "Folmer" region, Asmit1, Asmit2 for the "Asmit" region (Bowles et al., 1992), and 16Sar and 16Sbr for *16S* (Palumbi et al., 1991). The internal transcribed spacer 2 (*ITS2*) and histone 3 (*H3*) were amplified using primers LT1 (Bargues et al., 2001), ITS2-RIXO (Almeyda-Artigas et al., 2000) and H3F and H3R (Colgan et al., 2000), respectively.

PCR conditions and primer details are described in the **Supplementary Table S4**. Sanger sequencing was performed on an ABI 3730xl DNA analyzer using the BigDye Terminator Kit (Life Technologies, LGC Genomics GmbH, Berlin, Germany).

Dataset Compilation, Alignment, and Substitution Models

DNA sequences were edited and aligned using BioEdit version 7.0.5 (Hall, 1999). For phylogenetic analyses, additional sequences for all five genetic markers were downloaded from the NCBI database, resulting in a dataset composed of 145 ingroup taxa. After the sequences were reduced to unique haplotypes (**Supplementary Figure S1** and **Supplementary Table S1**), the final dataset consisted of 96 taxa and was used for subsequent analyses. *Indoplanorbis exustus*, the sister species to *Bulinus* spp. (Albrecht et al., 2007; Albrecht et al., 2019), was used as an outgroup. Multiple sequence alignment for *cox1* (both the Folmer and Asmit regions) and *H3* was conducted using ClustalW tool implemented in BioEdit software program while the gap forming partitions *16S* and *ITS2* were aligned using MAFFT an online alignment tool (Katoh et al., 2019). Gblocks program version 0.91b (http://molevol.cmima.csic.es/castresana/Gblocks_server.html), was used to remove poorly aligned regions, applying less stringent settings. The final alignments contained 655 bp, 390 bp, 432 bp, 452 bp and 328 bp for *cox1* (Folmer region), *cox1* ("Asmit" region), *16S*, *ITS2*, and *H3*, respectively. All new sequences have been deposited in NCBI GenBank (**Supplementary Table S1**).

The concatenated sequence dataset was created using SequenceMatrix version 1.7.8 (Vaidya et al., 2011). The software jModelTest version 2.1.10 (Darriba et al., 2012) was used to select the best-fit substitution models for each partition, with the number of substitution schemes set to 3.

Fossil-Calibrated Molecular Phylogeny

The estimated substitution models were used for the subsequent Bayesian inference analysis employing a molecular-clock approach for estimating species divergence using BEAST version 1.8.4 (Drummond et al., 2012). The analyses were run on the CIPRES Science Gateway V 3.3 (Miller et al., 2010). A fossil calibration was used to estimate divergence times in *Bulinus* using BEAST version 1.8.4 (Drummond et al., 2012). We used the oldest *Bulinus* fossil (c. 20 myr; Pickford, 2008) to date the most recent common ancestor (MRCA) of the ingroup.

Based on the Akaike information criterion (AIC), the best-fit substitution models for the concatenated dataset were: HKY + I + Γ, HKY + I + Γ, GTR + I+Γ, GTR + I + Γ and SYM + I for *cox1* (Folmer region), *cox1* ("Asmit" region), *16S*, *ITS2*, and *H3*, respectively.

The Markov chain Monte Carlo (MCMC) in BEAST analysis was run for 40 million generations, sampling every 1000th tree, using both a strict-clock and a relaxed-clock model with a birth–death tree prior (Gernhard, 2008) and a gamma prior for the fossil calibration point with the settings: offset = 19, shape = 1.0, and scale = 2.0. The log file was checked in Tracer version 1.5 (http://tree.bio.ed.ac.uk/software/tracer) to examine the parameter convergence. The maximum clade credibility (MCC) tree was generated using TreeAnnotator version 1.8.4 (BEAST package) with a burnin of 50%.

Whereas most of the parameters revealed effective sampling size (ESS) values for the strict-clock model slightly better (ESS of most parameters >300) than with the relaxed-clock model (ESS of most parameters <150), convergence for some of the strict clock model parameters was not reached (i.e., cases in which ESS values were <200). Therefore, the maximum clade credibility (MCC) tree obtained from the strict-clock analyses was used for subsequent analyses, with adjusted substitution models as follows: HKY + I + Γ, HKY + I + Γ, HKY + I + Γ, HKY + I + Γ and HKY (all base frequencies equal), for *cox1* (Folmer region), *cox1* ("Asmit" region), *16S*, *ITS2*, and *H3* respectively, resulting in sufficient ESS values (>200). We also tested a general invertebrate molecular substitution rate (mitochondrial) clock (Wilke et al., 2009) for the HKY + I + Γ, model of sequence evolution (1.57% My^{-1}) in the preliminary analyses (hence data not shown), to check the suitability of the fossil in estimating the divergence time of the *Bulinus* phylogeny., and this did not result in much difference.

Species Delimitation

Specimens were preliminarily determined based on their shell morphology and ecological setting information such as habitat and geographical position. However, we also used approaches for molecular species delimitation recently conducted (Chibwana et al., 2020; Clewing et al., 2020; Mahulu et al., 2021). Molecular operational taxonomic units (MOTUs) were identified in the BEAST MCC tree using this approach and computational methods for species delimitation; specifically the Generalized Mixed Yule Coalescent (GMYC) for multiple thresholds (Fujisawa and Barraclough, 2013; Pons et al., 2006) and Poisson Tree Process (PTP; both maximum likelihood and highest Bayesian supported solutions; Zhang et al., 2013). The

MOTUs were finally assessed in an integrated approach considering the preliminary identifications and named by integrating as many nominal taxa as possible. Doubtful cases or apparently new lineages were simply labelled as *Bulinus* sp., sometimes with a qualifier (e.g., *Bulinus* sp. 8 MTE for Mt. Elgon).

Reconstruction of Ancestral Altitudinal Ranges

Two sets of altitudinal information were compiled. Each specimen had a specific sampling altitude, either measured by ourselves in the field or taken from the literature for the GenBank sequences. Google Earth Pro was used to determine the altitudes in cases when only locality names were available in the original literature. In the cases of BtroAY18 and BtruAY19 (**Supplementary Table S1**), the altitude ranges of their countries of origin were used since no more specific locality information was available.

The second set of altitudinal information consisted of published altitudinal occurrences for all nominal species of *Bulinus*, which we screened for minima and maxima (**Supplementary Table S2**). This information was compiled through literature research in comprehensive publications (Mandahl-Barth, 1957; Mandahl-Barth, 1965; Brown, 1994) or species-specific accounts (e.g., De Kock and Wolmarans, 2005a; De Kock and Wolmarans, 2005b). In addition, online databases such as Global Biodiversity Information Facility (GBIF), International Union for Conservation of Nature (IUCN) Redlist, NHM London Zoological Specimen database were checked. A character matrix with six categories was created: A) 0–500 m, B) 501–1,000 m, C) 1,001–1,500, D) 1,501–2,000 m, E) 2,001–2,500, and F) > 2,500 m. The altitude ranges of all the MOTUs were coded as present (1) or absent (0) in these categories. In very few cases, our own sampling served for character coding. Character evolution of the altitudinal range distribution was traced along the phylogenetic tree using stochastic character mapping (Huelsenbeck et al., 2003) as implemented in phytools 1.0-1 (Revell, 2012) for the R statistical environment 4.1.2 (R Core Team, 2021). Three models of character evolution, ARD (all rates different), SYM (symmetric rates), and ER (equal rates), were tested by calculating 100 stochastic character maps and finally compared with AIC.

RESULTS

Species Diversity, Phylogenetic Relationships, and Divergence Times

The automated species delimitation methods yielded 24 and 38 MOTUs for bPTP and 33 for GMYC (**Supplementary Figure S1**). Our integrative approach resulted in a final set of 30 MOTUs. *Bulinus wrighti* is the sole representative of the *B. reticulatus* group. The *B. forskalii* group comprises *B. forskalii*, *B. bavayi*, *B. cernicus*, *B. barthi,* and *Bulinus* sp. 6. The *B. africanus* group primarily consists of *B. globosus* and *B. nasutus*, but also *B. africanus* and *B. obtusispira*. In addition, it contains *Bulinus* sp. 2 (of Chibwana et al., 2020) and a MOTU which could not be assigned to nominal names (*Bulinus* sp. 3). Most species belong to the *B. truncatus/tropicus* complex, of which a total of 18 MOTUs correspond to species-level taxa. Names cannot be applied to all of them, but instead nine nominal taxa could be assigned. *Bulinus* sp. 1 was previously reported in Chibwana et al. (2020). A further two have been labelled *Bulinus* sp. 4 and *Bulinus* sp. 5 for convenience. *Bulinus* sp. 7 LSH (= Lesotho) and *Bulinus* sp. 8 MTE (= Mt. Elgon) were previously unknown species. The MOTU called *Bulinus* sp. 9 MTK (= Mt. Kenya) might represent a small species complex of closely related taxa (*B. rumrutiensis/laikipiensis*). Both *B. truncatus* and *B. tropicus* were not found monophyletic, instead there where other MOTUs resembling them morphologically (*B.* cf. *truncatus*, *B.* cf. *tropicus*).

The phylogenetic reconstruction (**Figure 2**) revealed four major clades, which are strongly supported. These clades correspond to the four traditional species groups of *Bulinus*, i.e., the *B. forskalii*, *B. africanus*, *B. reticulatus* groups, and the *B. truncatus/tropicus* complex. *Bulinus reticulatus* and the *B. truncatus/tropicus* complex are well supported sister-groups (PP 1.0) and *B. africanus* is the sister group to them (PP 1.0). The *B. forskalii* group is ancestral to the other three groups (PP 1.0).

High-mountain lineages from the Aberdares Mts. and the Mt. Kenya region are sister groups in *B. tropicus*, whereas the Udzungwa Mts. lineage is not closely related to it. It clusters with regional lineages (**Figure 2**; **Supplementary Figure S1**). *Bulinus* sp. 9 MTK is sister to a specimen from Malawi (BtruAY19), but this relationship is not supported. This is also the case for *Bulinus* sp. 4 the sister of which is BtroAY18, a specimen from Zimbabwe. *Bulinus permembranaceus* from the Kinangop Plateau is closest to *Bulinus* sp. 5, represented by specimens from Cameroon and the D.R. Congo. *Bulinus hexaploidus* from the Ethiopian Highlands is sister to *B. guernei*.

According to our molecular-clock analysis, *Bulinus* started to diversify in the Early Miocene, which is also when the *B. forskalii* group diverged from the remaining groups. In the mid-Miocene (~16 Ma), the *B. africanus* group diverged from the *B. reticulatus* group and the *B. truncatus/tropicus* complex. The latter two diverged from each other around 11 Ma. Within the *B. truncatus/tropicus* complex, *Bulinus* sp. 8 MTE from Mt. Elgon originated in the Pliocene (4.71 Ma) (**Figure 2**). A series of divergence events leading to all other lineages in the complex happened near the Plio-Pleistocene border at 2.62 Ma (**Figure 2**). This marks the split of *Bulinus* sp. 7 LSH from the remaining *B. truncatus/tropicus* complex. All other divergence events for the *B. truncatus/tropicus* complex are dated to the Pleistocene, concerning all high mountain linages and also *B. liratus* from Madagascar. Much more lineages were accumulated in the Pliocene in both the *B. africanus* and *B. forskalii* groups. Here, the *B. obtusispira* from Madagascar is sister to the remaining members of the *B. africanus* group from which it split 8.05 Ma (**Figure 2**; **Supplementary Figure S1**).

Biogeographical Patterns

Extreme altitudes (>2,500 m) have been colonized by *Bulinus* at least three times independently and exclusively in the *B. truncatus/tropicus* complex: 1) The highlands of Lesotho are

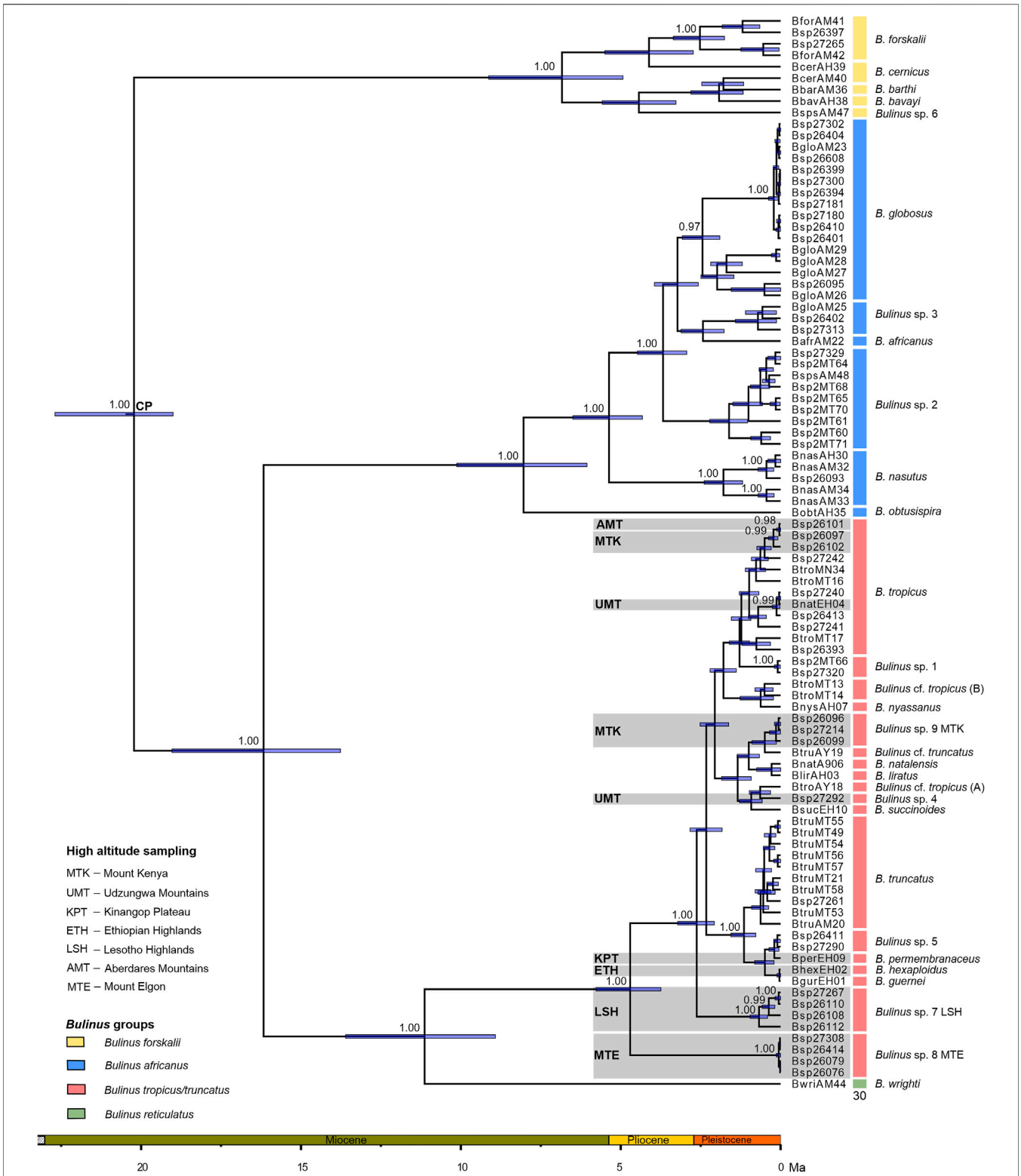

FIGURE 2 | BEAST MCC tree with the posterior probabilities above 0.95 given. 95% highest probability densities (blue bars) and the calibration point (CP) are provided. The outgroup was removed *a posteriori*. Specimen details can be seen in **Supplementary Table S1**.

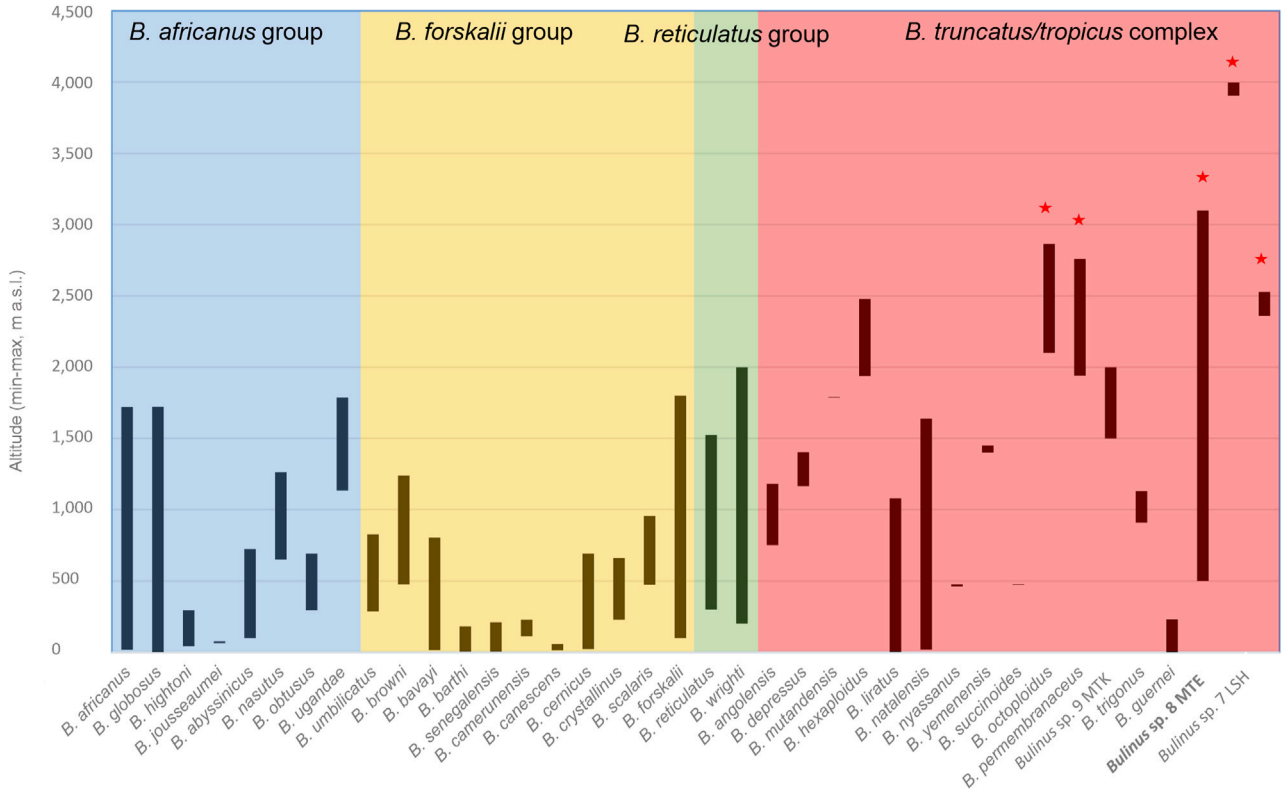

FIGURE 3 | Altitudinal distribution of members of the *Bulinus* groups (highlighted with colors) throughout their entire range compiled from various literature and collection sources. Red stars mark species occurring in extreme altitudes above 2,500 m a.s.l. Note the extraordinary high occurrence of *Bulinus* sp. 8 MTE on Mt. Elgon, Uganda. This species is new to science and is only distantly related to the *B. truncatus/tropicus* complex. Further details on altitudinal ranges can be found in the **Supplementary Tables S2, S3**.

inhabited by an apparently endemic lineage (*Bulinus* sp. 7 LSH, up to 2,528 m a.s.l.); 2) *Bulinus* sp. 8 MTE (up to ~4,000 m a.s.l.) represents an absolute extreme for *Bulinus* (**Figure 3**); 3) The Ethiopian Highlands are represented by a specimen of *B. hexaploidus* from 2,592 m (note that *B. octoploidus* is missing in the phylogeny).

Distinct colonizations occurred on Mt. Elgon and the Lesotho Highlands, spatially and temporally independent from one another and the other Afromontane regions. Diversification on Mt. Elgon is very recent though (0.06 Ma), whereas populations in Lesotho are roughly 0.67 myr old. The colonization patterns of the Aberdares Mts. and other Kenyan Highlands as well as the Udzungwa Mts. (Eastern Arc Mts.) are more complex with the respective species and lineages being widely scattered among the *B. truncatus/tropicus* complex. *Bulinus hexaploidus* is closely related to *B. permembranaceus* from the Kinangop Plateau in Kenya, though they are not sister species. There are two distinct MOTUs from the Mt. Kenya region, representing independent colonizations of the region (*B. tropicus* and *Bulinus* sp. 9 MTK). The Udzungwa Mts. as part of the old Eastern Arc Mts. complex are inhabited by at least two MOTUs (*B. tropicus* and *Bulinus* sp. 4) (**Figure 2**). All Ethiopian, Kenyan, and Tanzania MOTUs occurring in higher altitudes represent comparatively young lineages, having diversified in the late Pleistocene (**Figure 2**). No species of either the *B. forskalii*, nor in the *B. africanus* group occur in the second highest altitudinal band from 2,001 to 2,500 m. *Bulinus wrighti* has been described from low altitudes up to 2,000 m.

Altitudinal Range Evolution

The MOTUs in this study range in altitudinal distribution from sea level to almost 4,000 m (**Figure 3**; **Supplementary Table S2**). However, the majority occurs from sea level to 1,500 m with 22 MOTUs in 0–500 m, 15 in 501–1,000 m band and 17 MOTUs found in band 1,001–1,500 m. Much fewer occur in band 1,501–2,000 m (10 MOTUs) and 2,001–2,500 m (six MOTUs), whereas only four MOTUs are found at altitudes above 2,500 m (**Supplementary Table S3**). Considering altitudinal ranges of all *Bulinus* spp. including the new MOTUs of this study, the average range is 639 m (5–2,600 m). The average minimum altitude is 678 m (0–3,905 m) and the average maximum is 1,317 m (57–3,997 m) (**Figure 3** and **Supplementary Table S2**).

Most species evolved in low to mid-altitudinal ranges and only approximately nine times evolved adaptations to Afromontane regions. The Equal rates model (ER) was the best-fit model, assuming equal rates for the transition between altitudinal range bands (AIC: ARD: 148.99, ER: 109.51; SYM: 136.27). As many species of *Bulinus* occur in more than a single altitudinal range

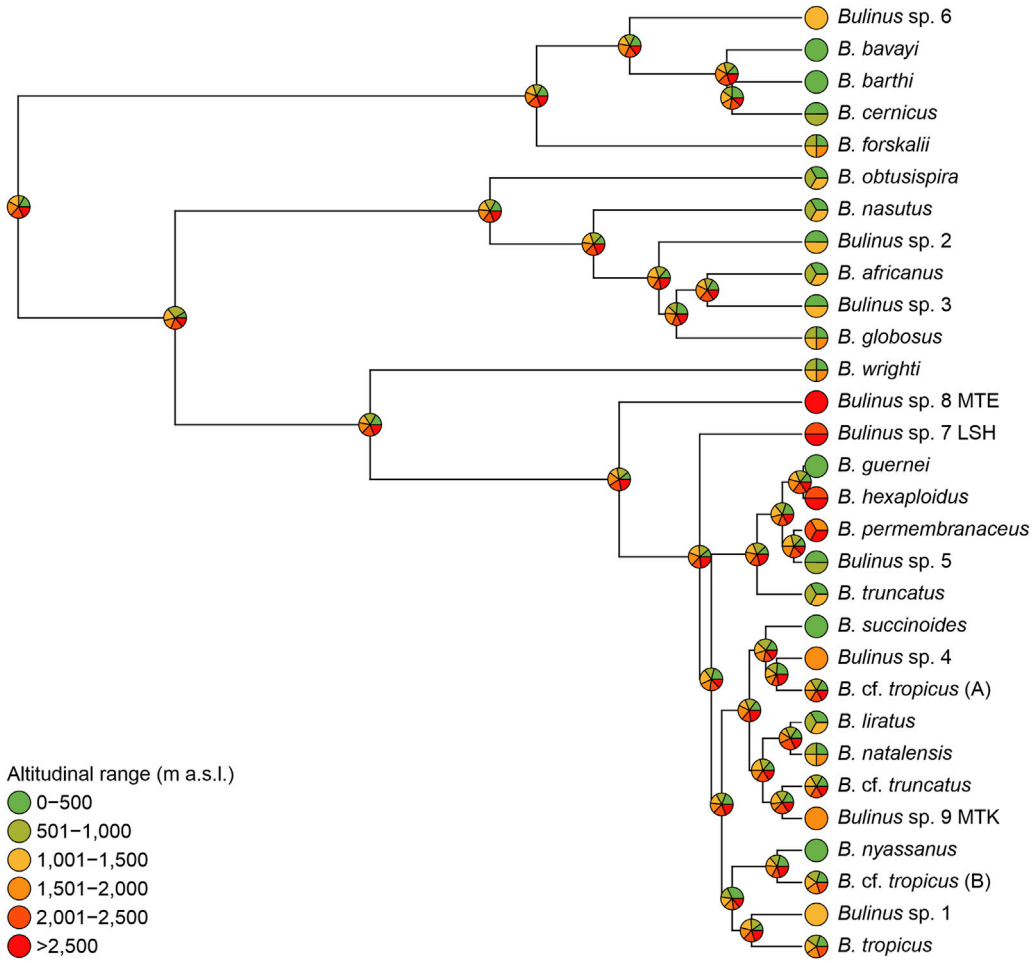

FIGURE 4 | Character evolution of altitudinal bands along the BEAST MCC MOTU-like tree. Six classes of altitudinal occurrences are used and pie charts represent probabilities of ancestral states at the respective nodes (see also **Supplementary Tables S2, S3**).

band, estimation of ancestral ranges is equivocal for each node in the phylogeny (**Figure 4**). The MRCA of the *B. forskalii* group most likely lived in low to intermediate altitudes (0–2,000 m) whereas the MRCA of the *B. africanus* group most likely lived in altitudes from 0 to 1,500 m, except for *B. globosus* which extends its distribution up to 2,000 m. *Bulinus reticulatus* group was only represented by *B. wright*, and the MRCA of this group has already lived in altitudes covering four bands from low- to mid-altitudes (0–2,000 m). The MRCA of *B. truncatus/tropicus* complex is shown to have lived in all the six altitudinal bands, and is from which the *Bulinus* sp. 8 MTE in the extreme altitude at Mt. Elgon has evolved (**Figure 4**).

DISCUSSION

Phylogenetic Relationships and Species Delimitation

Based on the increased taxon and character sampling of the new multi-gene phylogeny, all previously defined subgroups of *Bulinus* spp. were recovered. Unlike in previous phylogenies based on single markers and less extensive sampling (Kane et al., 2008; Jørgensen et al., 2007; Jørgensen et al., 2011; Jørgensen et al., 2013), intergroup relationships were also well supported in our phylogeny. This is important since the relationship among the *Bulinus* groups has been extensively debated in the past (e.g., Jørgensen et al., 2011; Jørgensen et al., 2013). Our findings strongly support that the *B. reticulatus* and the *B. truncatus/tropicus* complex are sister-groups and *B. africanus* forms the sister group to the first two whereas the *B. forskalii* group is ancestral to the other three groups. A sister relationship of the *B. reticulatus* group and the *B. truncatus/tropicus* complex has been well supported before (Kane et al., 2008; Jørgensen et al., 2011; Jørgensen et al., 2013). Various constellations of this group relative to the *B. africanus* and *B. forskalii* groups had been proposed and none conforms with the one found here (Stothard et al., 1996; Stothard et al., 2001; Jørgensen et al., 2007; Kane et al., 2008; Nalugwa et al., 2010; Jørgensen et al., 2011; Jørgensen et al., 2013; Zein-Eddine et al., 2014; Tumwebaze et al., 2019; Clewing et al., 2020).

The species delimitation overall found a match of morphologically identified species and MOTUs in many cases, however, there were also several cases of non-monophyly and mismatches. Most often this occurred in the *B. tropicus/truncatus* complex, which has been known for notorious difficulties in species assignments (e.g., Brown, 1994; Kane et al., 2008). *Bulinus globosus* was another case, known to be an "umbrella" name for various lineages of similar shell morphology (Pennance, 2020). In our conservative approach, we preferred to label unclear specimens as "sp." rather than assigning names to these lineages in order to not further complicate the situation of potentially wrongly labelled sequence information in GenBank. A major obstacle in *Bulinus* systematics is to disentangle wrongly identified specimens from cases where indeed cryptic species are involved in potentially rapidly diverging lineages (Tumwebaze et al., 2019). The complexity of the species level systematics stems from different taxonomic values of character used and even species concepts applied by the various authors. Here, either shell characters or anatomy, chromosome numbers, parasite susceptibility and the pre-condition of hermaphroditic reproduction mode have been implemented to various extents over time (Mandahl-Barth, 1957; Mandahl-Barth, 1965; Brown, 1994). Given these circumstances, a comparatively consistent level of MOTUs that correspond to nominal taxa has been found in this study. It should be further substantiated in future studies, which should strictly focus on type or topotypic material of all recognized species. In order to disentangle the real diversity of *Bulinus* and also the roles that certain processes play in diversification (such as polyploidization), genomic approaches should be used, especially since mitochondrial and nuclear genomes are already available for *B. truncatus* providing the much needed baseline (Young et al., 2021; Young et al., 2022; Zhang et al., 2022).

The sparse fossil record of Bulinidae has been discussed elsewhere (Jørgensen et al., 2013; Albrecht et al., 2019). The fossil-calibrated tree might potentially underestimate the real age of the group. Given that external substitution rates are often taxon- and gene-specific and may be saturated over time (see, e.g., Wilke et al., 2009), it was still worthwhile to explore it in the case of *Bulinus*. It should be noted though that the fossil-independent dating strategy using a universal invertebrate molecular clock rate did not yield substantially different age estimates for the major splits in the phylogeny (data not shown). As such, the ages of the endemics on Madagascar (*B. liratus* and *B. obtusispira, B. bavayi*) are interesting. Although *B. bavayi* and *B. obtusipira* represent comparatively old lineages in their respective *Bulinus* groups, their splitting from other lineages by far postdates the separation of Madagascar from East Africa and the Indian subcontinent (e.g., Masters et al., 2021). The *Bulinus* spp. on Madagascar thus do likely not represent vicariant forms (Wright, 1971; Stothard et al., 2001; Jørgensen et al., 2011). Interestingly, a similar pattern has been found for another gastropod species of the genus *Lanistes* endemic to Madagascar (Mahulu et al., 2021). The now available temporal framework of *Bulinus* evolution also allows specific colonization patterns and evolutionary patterns of endemism to be evaluated.

Colonization History and Evolution of Endemism

Members of the genus *Bulinus* have colonized altitudes from sea level to real alpine altitudes in some Afromontane regions. This study has extended the known altitudinal range of the genus by 900 m to around 4,000 m on the top of Mt. Elgon, where a new species of *Bulinus* has been characterized for the first time from such an extreme environment. Interestingly only four species (14% of the currently recognized biodiversity), all belonging to the *B. truncatus/tropicus* complex, evolved very high altitude occurrences (>2,500 m). All except *B. tropicus* are "true" high-altitude species whereas *B. tropicus* has an exceptional wide altitudinal range (500–3,100 m), the widest of all *Bulinus* species. To which extent the high-altitude populations of this species indeed belong to *B. tropicus* or also represent independent lineages needs to be shown in future studies. *Bulinus* sp. 7 LSH, previously considered to belong to *B. tropicus*, is a good example of how cryptic morphology can mask true phylogenetic and biogeographical patterns. The Bokong bogs (Lesotho) at 3,100 m, was recorded as the highest place for *B. tropicus* so far (Brown, 1994). It remains to be seen whether this indeed represents *B. tropicus* or rather *Bulinus* sp. 7 LSH as identified in this study. Although the sampling was certainly limited, it is noteworthy that no *B. tropicus* lineage was found in the highlands of Lesotho. Colonization might have occurred via a continuous upwards dispersal since *Bulinus* populations are usually found in all intermediate altitudes if suitable habitats are present and the slopes are not too steep. A notable exception it's the conically shaped Mt. Elgon, where intermediate altitudes are free of *Bulinus* (Howell et al., 2012).

Altitudinal endemism is pronounced in the case of the Ethiopian species as well as the Kenyan Highlands. Here, phylogeographical sampling within these mountain ranges has to further disentangle the species status of several nominal taxa such as *B. octoploidus* in Ethiopia and *B. permembranaceus* in Kenya. This specifically applies to the *Bulinus* sp. 9 MTK which potentially includes the taxa *B. laikipiensis, alluaudi, rumrutiensis* (currently fallen in synonymy). It would also be interesting to test the hypothesis that polyploidisation might be linked to the ability to adapt to extreme altitudes (Brown, 1994). A striking finding of this study is the fact that high altitude *Bulinus* sp. 8 MTE from Mt. Elgon and *Bulinus* sp. 7 LSH from Lesotho are representing old lineages (= long branches), very isolated in the *B. truncatus/tropicus* complex with no immediate sister species currently known. The genetic distinctness of *Bulinus* sp. 8 MTE is very pronounced and might even warrant separation from the *B. truncatus/tropicus* complex. Even though this "stand alone" situation could potentially be a sampling artefact, it is the East African and Southern African regions that are comparatively well represented in previous phylogenetic studies of *Bulinus* spp. (Kane et al., 2008; Jørgensen et al., 2011; Jørgensen et al.,

2013; Pennance, 2020), which render missing major lineages less likely. Given the phylogenetic pattern and acknowledging the sampling available to date, it appears as if living in high-altitudes is a condition already present in the MRCA of the *B. truncatus*/*tropicus* complex. It was an equally likely ancestral condition in the MRCA of the *B. reticulatus* group and the *B. truncatus*/*tropicus* complex. The character tracing revealed an equally likely colonization from altitudes from 0 to 2,000 m for *Bulinus* sp. 8 MTE. The Lesotho Highlands in contrast were colonized from either low (0–500 m) or high (>2,500 m) altitudes. Given the wide altitudinal ranges many *Bulinus* species occupy and their easy colonization capabilities, it is challenging to reconstruct ancestral conditions using phylogenetic approaches. Here, phylogeographical studies of species or species-groups might help to further elucidate the evolution of rare adaptations to extreme altitudes.

Role of Paleogeography and Paleoclimate

The Afromontane mountains are recognized as the most isolated mountain ranges in the world (see Mairal et al., 2021). This isolation has led to speciation and endemism in floras and faunas (Levin et al., 2020; Cuypers et al., 2022) and is also seen in *Bulinus* spp. It is noteworthy that isolation is apparently more pronounced in the Lesotho Mts. and on Mt. Elgon, where the oldest lineages of the *B. truncatus*/*tropicus* complex are found. There is no indication of hopping dispersal from "sky island" to "sky island" in these areas. Similarity in floras and faunas of Afromontane regions has been attributed to the existence of habitat corridors in the Holocene potentially facilitating dispersal between the various montane regions (Cooper, 2021). In *Bulinus*, this has likely not happened between the southern mountains and East Africa. To what extent such biotic exchange occurred between the Ethiopian Highlands and the Kenyan and Eastern Arc Mts. cannot be answered conclusively with the data at hand. Judging from the branching patterns and the molecular clock estimates it is less likely that such exchange happened as recently as after the last glacial maximum. Inter-mountain long distance dispersal has been shown for various taxa, the majority of which are terrestrial (Mairal et al., 2017; Mairal et al., 2021). This is also less likely in the case of *Bulinus* since no direct sister-group relationships were found. However, it is interesting since in a similar case of a lymnaeid gastropod (*Galba mweruensis*) such a pattern connecting widely isolated Afromontane populations was demonstrated, though the actual dispersal mechanism remained unknown (Mahulu et al., 2019). In the case of *Bulinus* it is likely that adaptation to cold climates (= high-altitudes) represents a form of niche conservatism which makes these lineages or species trapped in their specific environment with subsequent *in situ* speciation. This has been shown for pulmonate snails in other high-altitude regions (Albrecht et al., 2022). It is again noteworthy that such adaptations happened considerably earlier in the southern African Mts. and Mt. Elgon, i.e., in the Pliocene or Plio-Pleistocene transition times. Interestingly, this older or longer separation of populations from Lesotho and Mt. Elgon has also been found in *Galba* (Mahulu et al., 2019), with those populations from Mt. Elgon to be the most distinct ones as well. A striking feature remains regarding the roles of volcanic activities and glaciation of Mt. Elgon. Extensive icefields and glaciers existed on Mt. Elgon in the Late Pleistocene (Osmaston, 2004). Earlier (around 3 Ma), volcanic eruptions occurred on Mt. Elgon (Scott, 1998). Both conditions argue against a Pliocene colonization and uninterrupted existence of *Bulinus* populations on Mt. Elgon. Alternatively, a post-glacial long dispersal from a yet unknown source must be assumed, possibly located in East Africa. The current study clearly demonstrated that Afromontane regions represent "sky islands" for freshwater organism, here *Bulinus* gastropods. Rifting processes and associated climatic fluctuations have impacted the distribution patterns of *Bulinus* lineages at least since mid-Pliocene times. It is likely that recurrent climatic changes (Kohler et al., 2014) might lead to range shifts, reduction or even extinctions in high mountain populations. These populations have been postulated to be particularly sensitive to changing climates (Trew and Maclean, 2021). Such range shifts might also have significant implications for species of *Bulinus* that act as intermediate host for trematode diseases.

Implications for Schistosomiasis

Whereas *Bulinus* snails adapt to cold conditions, this is not necessarily the case for their parasites. Though our knowledge is still scanty for most trematode parasites, species of the genus *Schistosoma* received considerable attention due to their role in causing, e.g., cancerogenic urogenital schistosomiasis in humans (e.g., Rollinson, 2009). With warming climates, regions in altitudes where schistosomiasis was previously unknown, might become suitable for thermally restricted schistosomes. Warming means that known intermediate hosts might occur in high places in the future and that species not yet susceptible to schistosomes might become susceptible or get into contact with the parasites. A multiplex-PCR based screening of the specimens of *Bulinus* sp. 8 MTE did not detect infections (data not shown). These populations at altitudes of around 4,000 m are less likely to be visited by final hosts. Lower altitudes between 2,000 and 3,000 m are much more frequented in Afromontane regions. *Bulinus permembranaceus* and *B. hexaploidus* are currently not known to be intermediate hosts for human schistosomes (Brown, 1994). *Bulinus octoploidus* on the other hand could be experimentally infected with *S. haematobium* (Lo et al., 1970) and is naturally infected with *S. bovis* a major parasite causing livestock schistosomiasis (Brown, 1994). *Schistosoma bovis* is also known from *B. tropicus* and thus potentially also from *Bulinus* sp. 7 LSH. Future assessments of high-altitude species should use the newly developed xenomonitoring approaches (Pennance et al., 2020; Hammoud et al., 2022) to enhance our knowledge of snail-trematode communities in these particular species. This is relevant since the above mentioned shift of prevalence towards higher altitudes have already been documented for the *Biomphalaria-S. mansoni* system (John et al., 2008; Stanton et al., 2017). It is also desirable to carry out transect assessments along altitudinal gradients in all Afromontane regions (Howell et al., 2012; Stanton et al., 2017) as a baseline for tracing future shift of both snail and parasite communities. It would also help to more precisely define the actual and potential altitudinal limits of the disease that is so pertinent for both humans and livestock throughout Africa.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, GenBank accession numbers of newly sequenced samples are provided in the **Supplementary Table S1**.

AUTHOR CONTRIBUTIONS

IT and CA designed the study, CA and IT collected in the field and compiled data and wrote the draft manuscript. IT conducted lab work. IT and CC performed the analyses. FC organized field work in Tanzania and helped in the lab. JK facilitated work in Kenya. CA secured funding and did project administration. All authors read, contributed to the initial drafts, and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2022.902900/full#supplementary-material

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3. Appendix

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