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Article

First Molecular Identification of *Bulinus africanus* in Lake Malawi Implicated in Transmitting *Schistosoma* Parasites

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Abstract: The freshwater snail genus *Bulinus* plays a vital role in transmitting parasites of the *Schistosoma haematobium* group. A hybrid schistosome between *S. haematobium* and *S. mattheei* has been recently detected using DNA-based identification methods in school children along the Lake Malawi shoreline in Mangochi District. This finding raised the need for contemporary revaluation of local interactions between schistosomes and snails, with a particular focus on snail species within the *Bulinus africanus* group. In 2017 and 2018, malacological surveys sampled several freshwater sites in Mangochi District. Collected snails (n= 250) were characterised using cytochrome oxidase subunit 1 gene (*cox*1), with DNA barcoding of the 'Folmer' region and a rapid PCR-RFLP typing assay with double digestion with *Hae*III and *Sac*I restriction enzymes. DNA *cox*1 sequence analysis, with phylogenetic tree construction, suggested the presence of at least three *Bu. africanus* group taxa in Lake Malawi, *Bu. globosus*, alongside first reports of *Bu. africanus* and *Bu. angolensis*, which can be differentiated by PCR-RFLP methods. In addition, A total of 30 of the 106 *Bu. africanus* group snails (28.30%) were positive to the *Schistosoma*-specific screen using real-time PCR methods. This study provides new insight into the recent changes in the epidemiology of urogenital schistosomiasis as likely driven by a new diversity of *Bu. africanus* group snails within the lake

Keywords: *Bulinus; Schistosoma haematobium*; urogenital schistosomiasis

1. Introduction

The freshwater snail genus Bulinus play a crucial role in the transmission of species in the *S. haematobium* group that cause schistosomiasis. Meanwhile, The group included three human pathogens (*S. haematobium, S. guinieensis,* and *S. intercalatum*) and five others that could infect wild and domestic ruminants (*S. bovis, S. leiperi, S. curassoni, S. margrebowiei,* and *S. mattheei*). The interaction and relationship between schistosomes and snails are very precise, and compatibility may vary over relatively small geographical ranges (1). Recently, there was a detection of a hybrid schistosome between *S. haematobium* and *S. mattheei* in Mangochi District along Lake Malawi among local people (2). There is a lack of certainty about the compatibility of hybrid schistosome to snail species in Malawi. Some *Bu. africanus* group species are playing a role in the life cycle of *S. haematobium*, *S. bovis* and *S. mattheei* which would continue to drive the hybridisation of human and zoonotic schistosomes.

It is essential to have data of the intermediate host for schistosomiasis to control snails; the data include accurate snail identity, their roles in transmission infection, and map distribution (3). Several molecular characterisation methods are currently available to be used in the molecular identification of freshwater snails, such as sequence analysis. Mitochondrial region of cox1 sequences are commonly used for snail identification, making it a suitable comparison gene for general species identification (4). Fortunately, the relatively high genetic variation of *Bulinus* snails, particularly in the cox1 gene, is a

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sufficient marker to distinguish between species as it is challenging to identify some snails utilizing traditional taxonomy (5). A rapid RFLP assay is another molecular technique used for unknown snail discrimination at a low cost. This method can benefit from the published sequences in Genbank for developing markers through sequence analysis for published sequences for selected species.

Understanding the epidemiology of schistosomes requires a reliable taxonomy of the genus Bulinus (6). For example, some species from the *Bu. africanus* group are well known or suspected as the intermediate host for *S. haematobium, S. bovis* and *S. mattheei.* However, classification within *Bu. africanus* species group complex is probably the most problematic of the four Bulinus snail species groups (6) . As *Bu. globosus* is not common in open lake habitats, it was previously believed that the open shores of Lake Malawi were free from *S. haematobium.* Plus, the transmission was limited to a protected area where *Bu. globosus* can be found (7). Surprisingly, in 2001, Madsen et al.,(2001) reported that *Bu. nyassanus* from *truncatus/tropicus* complex could produce cercariae (8). This new epidemiology change linked this snail for the urogenital schistosomiasis transmission in some parts of the lake shoreline.

Species identification within the *Bu. africanus* group can be unreliable when based exclusively on morphology. The shell is often plastic, being subject to incongruent or eco-phenotypic variant (9). Brown, (1994) suggests that more populations and advanced application of molecular analysis are needed to distinguish between *Bulinus africanus* group species with more confidence to help in understanding their distribution and their roles in disease transmission (10). Some studies have investigated some species from this group using molecular identification methods in a different population (5, 6, 11). The findings obtained from these studies were interesting, and some results were unexpected results that reflect the need for further molecular identification methods for snail characterisation. For example, Allan et al. (2017) conducted a genetic investigation and found that *Bu. angolensis* should be reclassified to *Bu. africanus* group instead of originally documented in *Bu. truncatus/tropicus* complex (11).

The population of *Bu. africanus* group in Malawi and precisely in the Lake have not been investigated at a molecular level. Interestingly, during two malacological surveys in 2017 and 2018 in Malawi *Bu. africanus* group were found in the most visited sites in the Lake beside water bodies out the Lake in Mangochi District. Therefore, this study aims to investigate this population at a molecular level. By exploiting different molecular applications, this study aims to find an optimum method to distinguish between different species in Malawi. Although this study focuses on *Bu. africanus* group in Lake Malawi, we included some known Bulinus species collected from in and out the Lake for comparison.

The development of rapid identification techniques and the data provided in this study would contribute to following the recent changes in the Schistosoma intermediate host in Lake Malawi. Also, it helps in the reliable monitoring of the altering dynamic between the parasite and its vector. Further observations are needed to evaluate the epidemiological changes reported recently in Malawi and the potential concerns of these change.

2. Materials and Methods

2.1. Sample survey

Two malacological surveys were carried out in the Mangochi District along the southern lake shoreline and nearby water bodies in November 2017 (14 locations) and May 2018 (43 locations). *Bulinus* species samples were collected from the vast majority of visited sites, plus details of each site such as Global Positing System (GPS). Additional *Bu. globosus* samples collected by Prof. J. R. Stothard from other two Districts in Malawi were included in RFLP and sequencing analysis for comparison; the samples were collected from a river in Blantyre and a pond in Chikhwawa district located in the southern region of Malawi.

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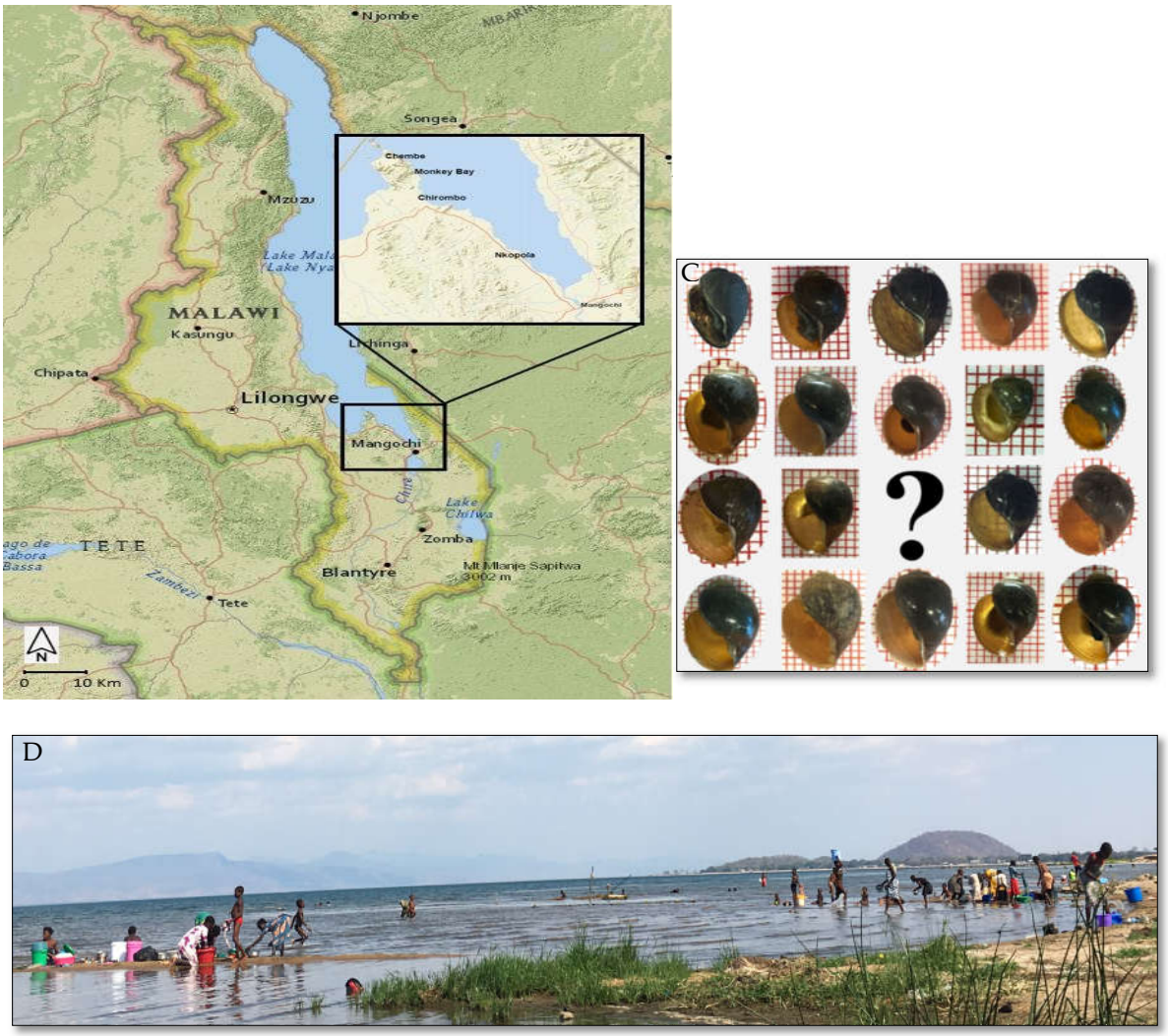

Figure 1. A. Map of Malawi adapted with a highlight of Mangochi District, where the malacological surveys conducted in 2017, 2018 were conducted. **B** and **D** . Pictures from locations visited in 2017 and 2018 showing the high water contact by human and animal. **C.** *Bulinus* shells collected from Lake Malawi were invistigated in this study.

2.2. Molecular characterization analysis

Snail tissue from the foot and head (about 2 mm) was cut, and then we placed them in a 24 well plate, having distilled water for approximately 5 minutes to diffuse out ethanol in the tissue. Then, DNA was extracted using the DNeasy Blood and tissue kit (Qiagen™), following the instructions. The quantity and quality of the DNA of samples were tested using spectrophotometry (Nanodrop™). Later, extracted DNA is diluted to 10ng/µL in elution buffer (AE).

Fifty samples were randomly slected for sequence analysis using *cox*1 gene ,Partial mitochondrial *cox*1 was amplified using BulCox1 and HCO2198 (6, 12) in PCR reaction for sequence analysis. Amplifications were performed using (Illustra puReTaq Ready-To-Go PCR beads (GE Healthcare) in 25µl total volume consisting of 1 µl of forward and reverse primers, 21µl of nuclease-free water and 2µl of DNA. As per the manufacturer's instructions, successfully amplified products were purified using MiniElute PCR purification kit (Qiagen™), following the manufacturer's instructions before being sent for sequencing. This step was also conducted for selected samples to validate the results from the RFLP rapid assay.

2.3. Restriction digestion

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The *cox1* fragment amplified from the 'Folmer' region in the snails were digested using SacI and HaeIII restriction enzymes (New England Biolabs) in CutSmart® Buffer (New England Biolabs). Based on sequenced samples used as a control in this study, two species from *Bu. africanus* group were identified as *Bu. africanus* and *Bu. globosus*. Thus, we developed two markers (restriction enzymes) in the RFLP essay to distinguish between the identified species through the sequences generated. SacI (GAGCT^C) is expected to cut only *Bu. globosus* samples, while HaeIII (GG^CC) only cuts *Bu. africanus*. The reaction contained 3.5µL of nuclease-free water, 5µL of DNA, 1µL of each enzyme, and 4.5µL of a buffer. Then, the mixture was incubated to digest 3 hours at 37.5°C before running on 1% agarose gel stained with SYBR™ Safe DNA gel stain at 100V for 40 mins. Additional *Bu. globosus* samples from the different areas were included in RFLP and sequencing analysis for comparison. Several samples identified as *Bu. truncatus/tropicus* complex from different sites were included as a negative control for amplification and RFLP to test the specificity of the primers and restriction enzymes used in this study.

2.4. Real-time PCR detection of schistosomes within snails

Real-time PCR to detect schistosome within snail was conducted to detect *Schistosoma* within *Bu. africanus* group samples using Ssp48F(5′-GGT CTA GAT GAC TTG ATY GAG ATG CT-3′), Ssp124R(5′-TCC CGA GCG YGT ATA ATG TCA TTA-3′), and the probe Ssp78T[FAM-5′-TGG GTT GTG CTC GAG TCG TGGC-3′ as described by (13).

2.5. Statistical and analytical methods

Molecular data were analysed using Geneious Prime 2019, National Centre for Biotechnology Information (NCBI) for BLAST and alignment using MUSCLE to investigate molecular identification and sequence variation. Plus, a neighbour-joining and maximum likelihood tree were created using Mega.

3. Results

Generally, the *Bu. globosus* shells collected from pond and stream has shown a higher range of measurements taken compared to shells of *Bu. afrianus* group collected from the Lake. The RFLP assay was utilised as the primary method to classify the species of the indivudal samples collected based on markers developed in this study. Thus, further investigation was conducted using *cox*1 markers in sequence analysis, resulting in an indication that four groups of *Bu.africanus* group exist within the samples examined, classified based on blast to *Bu. africanus, Bu. globosus, Bu. angolensis*, and *Bulinus* sp.

The specific band pattern was consistent in the known samples were identified and verified using DNA sequencing as controls. The rapid essay test RFLP used in this study aimed to distinguish between *Bu. globosus* and *Bu. africanus* using 250 samples, including twenty *Bu. truncatus* samples to test the sensitivity and specificity of the primers used in PCR and markers used in RFLP. As a result, the selected primers used to amplify the Folmer region worked successfully with *Bu. africanus,* while in *B. truncatus* samples, only a few were amplified. Although the high level of variation among samples, there was no overlap in the result of *Bu. globosus*, *Bu. africanus*, and *Bu. truncatus* samples. Interestingly, the RFLP assay unexpectedly reveals another group with no cut site found *Bu. angolensis* through further sequence analysis; there is no report of finding this species in Malawi. Plus, Another group of *Bu. africanus* group was found via the molecular identification.

3.1. PCR-restriction fragment length polymorphism assay

The PCR-RFLP gel result is illustrated in figure 2 shows that the *Bu. globosus* profile was two bands digested by SAC1 enzyme at about 400 and 200 bp, while *Bu. africanus* shows two bands at approximately 320 bp. The test confirmed the validity of the developed markers besides the specificity of the selected primers being utilised for *Bu. africanus* group population. For example, 20 samples from *Bu. truncatus/tropicus* group from different locations were used as non *Bu. africanus* group. Out of the The 20 samples, six samples

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illustrate four bands while no amplification found in 14 samples. Interestingly, this test reveals another species that shows no site cut, which has led to further molecular investigation for all taxa using sequence analysis and evolution analysis using phylogenic tree.

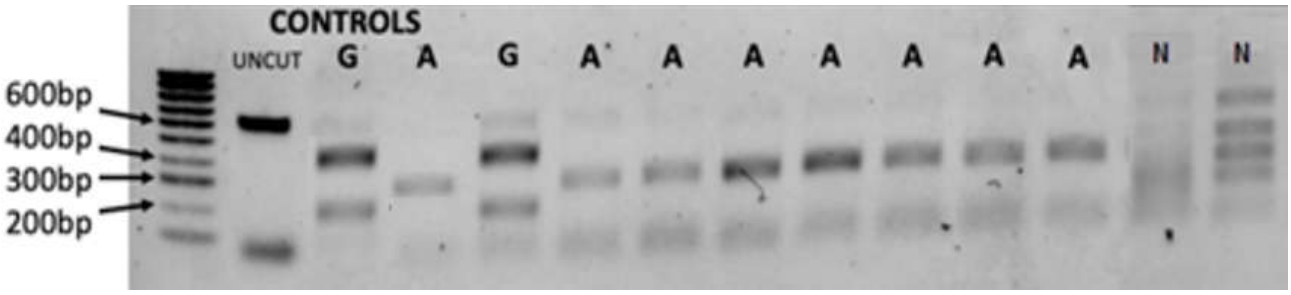

Figure 2. Gel images of the RFLP assay that shows typical PCR-RFLP variation profile.

1% agarose gel stained with SYBR™ Safe DNA showing the 'Folmer' region of *cox*1 restricted with double digestion enzymes Sac1 and HaeIII. Known controls of uncut DNA, *Bu. Africanus* (A) and *Bu. Globosus* (G), were used with hyperladder 100bp as control.. The uncut sample (UNCUT) and non *Bu.africanus* group (N) are also labelle.

3.2. Sequence analysis

The mitochondrial *cox*1 gene sequence generated for all *Bu. africanus* group samples in this study was found highly variant, in agreement with a similar study (6, 11). Alignment of 50 sequence samples in this study has revealed four taxa of *Bu.africanus* group population in Malawi

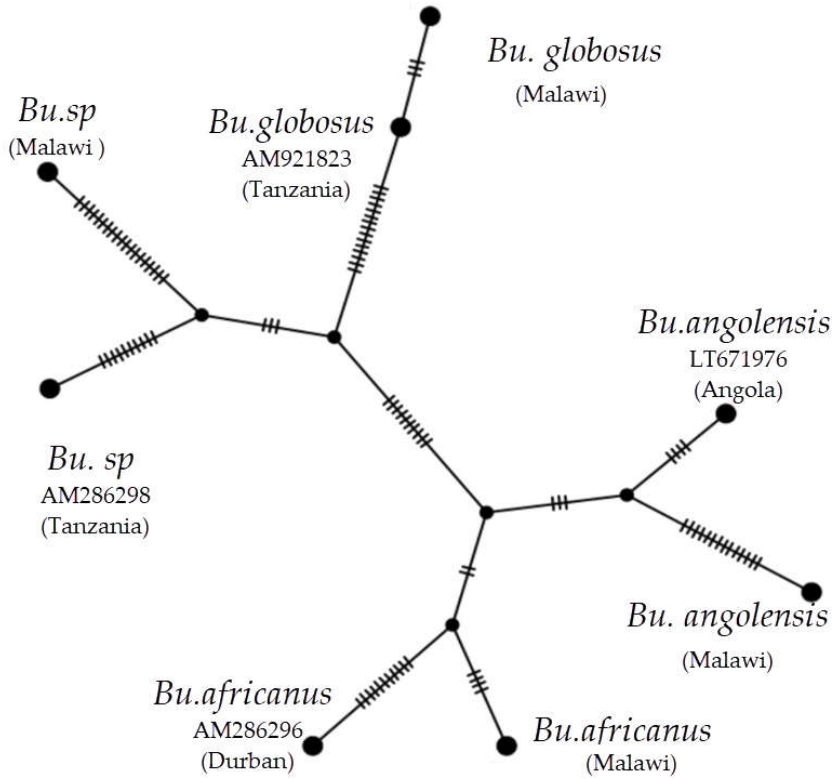

Figure 3. Minimum spanning network TCS network of the *cox*1 sequences for *Bu. africanus* group samples, *Bu. africanus*, *Bu. globosus*, *Bulinus* sp. and *Bu. angolensis.*

Minimum spanning network generated to identify the distance pairwise among a combination of four taxa from Malawi with four reference samples. Also, to identify the

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genetic diversity occur in Folmer region. Hatch mark indicates the different nucleotide in the pairwise alignment.

3.3. Distribution of Bu. africanus group snails on Lake Malawi

Although the surveys were carried out in two different seasons, *Bu. globosus* that was generally bigger in the shell was not found in the Lake, while all the other three main groups were collected from the Lake. *Bu. globosus* snails were observed only at a stream, river, and pond in Mangochi, Blantyre and Chikhwawa district, respectively. On the other hand, *Bu. africanus* snail was found in all visited sites in the Lake whereas *Bu. angolensis* were collected exclusively from three sites in the Lake, including one site visited in the eastern region.

3.4. The prevalence of Schistosoma DNA within Bu. africanus group

A total of 30 of the 106 *Bulinus* snails (28.3%) were positive to the Schistosoma-specific screen using real-time PCR methods. DNA *Schistosoma* were detected in *Bu. africanus* and *Bu. angolensis* samples in eastern and southern shores of the Mangochi district. The positives samples were collected from the lake, and connected waterbodies.

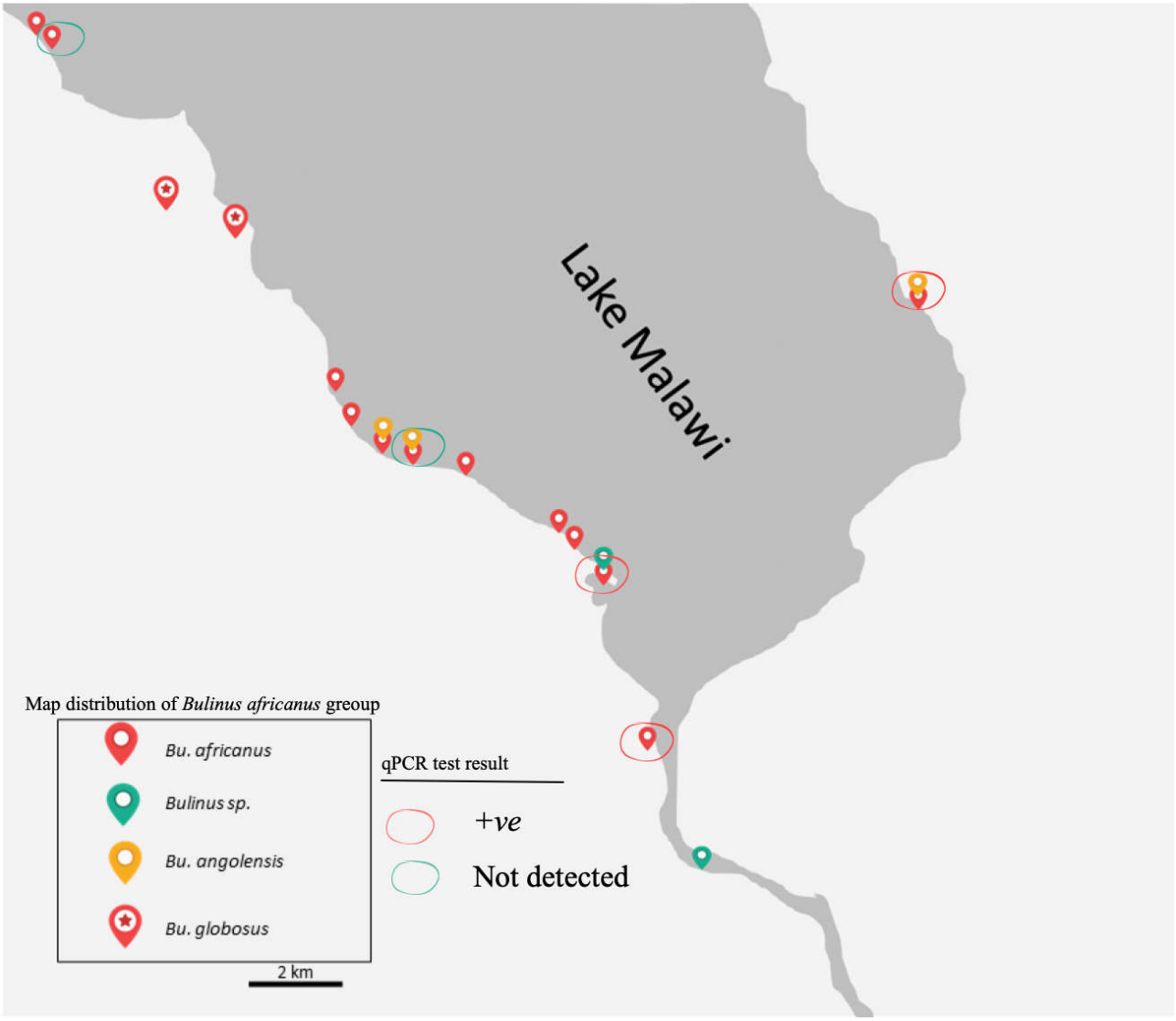

Figure 4. Map of the distribution of *Bu. africanus* group on the southern shoreline of Lake Malawi and its connected water bodies visited between 2017 and 2018. The red circle indicates samples were positive for real-time PCR, while the green circle indicates samples where DNA of *Schistosoma* were not detected whithin snail. Previously all snails were thought to be *Bu. globosus*.

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3.5. Phylogenetics analysis

Both Maximum Likelihood (ML) and Neighbour-Joining method (NJ) analyses of *cox*1 gene generated strongly supported phylogenies that show the presence of four species within the *Bulinus africanus* group in Lake Malawi in addition to two species from *Bu. truncatus* complex and *Bu. forskalii* group (Figure 4).

Based on sequence generated of fragment from the 'Folmer' *cox1,* I performed evolution analysis using phylogenic tree for fourteen *Bu. africanus* group samples. Seven samples represent four *Bu. africanus* group samples, and seven published sequenced from NCBI were included due to their similarities to the project's sequenced samples. Moreover, *Bu. forskalii*, *Bu. succcinoides*, and *Bu. nyassanus* collected from Lake Malawi were included as a non-*africanus* group. The phylogenetic trees illustrate similar results of genetic differentiation of *Bu. africanus* groups samples. In the *cox*1 tree, the *Bu. africanus* group specimens are split into four groups, namely *Bu. africanus*, *Bu. globosus*, *Bu. angolensis*, and *Bulinus* sp.

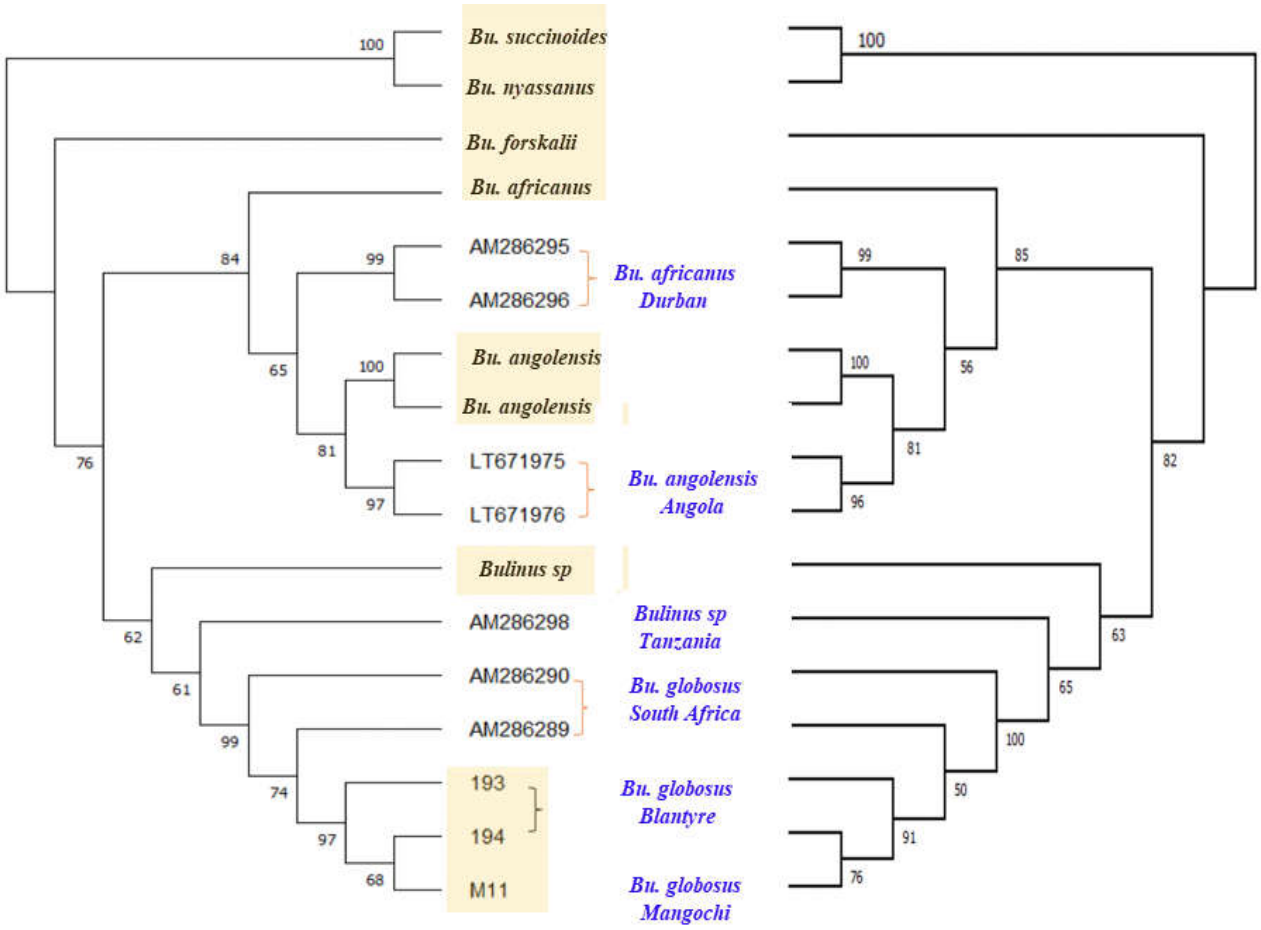

Figure 5. comparison between Maximum Likelihood tree and Neighbour-joining method for *Bulinus* from Lake Malawi.

Maximum likelihood using Tamura 3-parameter model and Neighbour-joining method using the Jukes-Cantor method. Both tree of a 467 bp fragment of the (*cox*1) gene for, *Bu. forskalii, Bu. succcinoides, Bu. nyassanus, Bu. africanus, Bu. globosus, Bu. angolensis*, and *Bulinus sp* (unidentified) collected in this study , Plus previously published sequences. The Numbers at the nodes indicate bootstrap proportion (1000 replications). Evolutionary analyses were conducted in MEGA X.

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3.6. The differences between molecular and morphological identification

Figure 6 generally shows a problematic identification based on the shell morphology among all four groups. Of note, the *Bu. globosus* (Figure.6A) from Mangochi, Blantyre, and Chikhwawa district populations are very similar by visual inspection of the shell; Despite far geographical distances and different environmental conditions. Moreover, the *Bu. globosus* shell was relatively higher than the other groups, which are very similar and overlap in obtained shell measurements.

Although the species of *Bulinus africanus* group seemed to be similar, a closer investigation demonstrated a subtle morphological distinction in *Bu. globosus* only. For example, curved columellar edge, basal margin join, and a more pronounced and distinctive notch on the inside *Bu. globosus* shell; in other species, most of the shells show no extra curvature in the basal margin, and the notch is absent (Figure 49 & Figure 50).

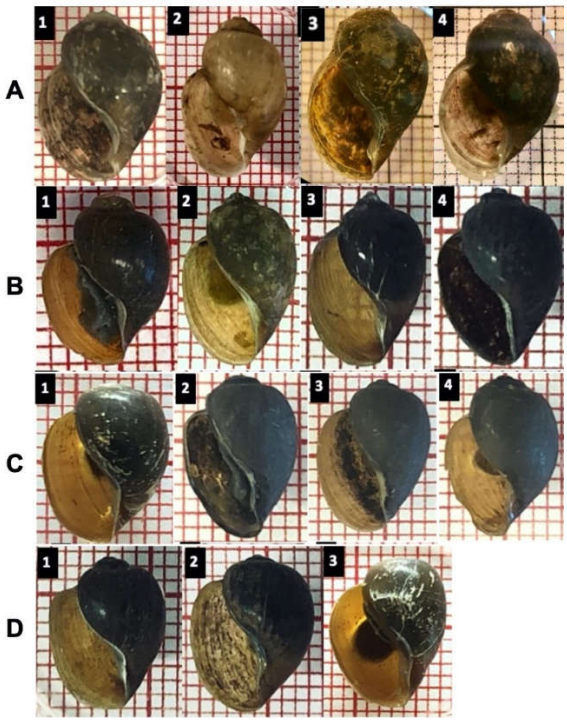

Figure 6. A visual comparison of selected shells indicates some subtle differences.

Several shells were identified using both RFLP and sequence analysis. **(A)**: *Bu. globosus* represent three different population habitats; A1-2: From Mangochi District, A3: from Blantyre, A4: Chikhwawa District (**B):** *Bu. africanus*. **(C):** *Bu. angolensis*, **(D):** *Bulinus* sp.

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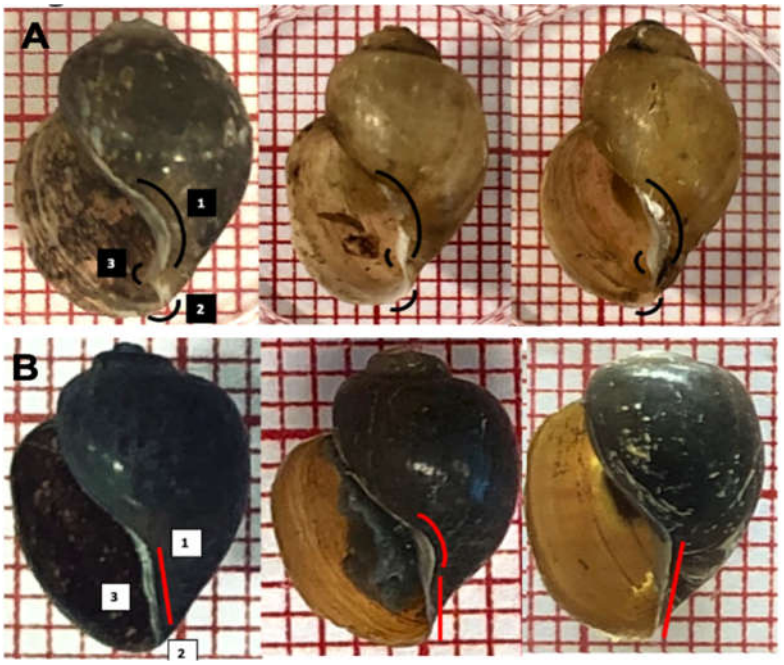

Figure 7. Subtle morphological differences in truncated columella region, or tooth, in the aperture of *Bu. globosus.* **(A)**: (1) curved columellar edge (2) basal margin join (3) distinctive notch on the inside **(B)**: shells and other *Bu. africanus* group (1 square =1mm^2).

4. Discussion

Due to the scale and spread of snails and their contribution to schistosomiasis transmission, updating information and monitoring is important to understand the disease and transmission dynamic. For example, detection of the intermediate host of *S. mansoni* in 2017 has led to increased disease surveillance and revealed the emergence of intestinal schistosomiasis in Mangochi District in 2018, followed by an outbreak of intestinal schistosomiasis in primary schools in Mangochi District in 2019 (14) (15). Additionally, detecting schistosomes hybrid in the same regionis a significant epidemiology change and reveals potential new facts about schistosomiasis changes occurring in Lake Malawi(2).

During the past decades, the identification of natural populations and species belonging to *Bu. africanus* group has been well-known as problematic and sometimes indecisive (10, 16).Although the attempts were made to use some morphology characters such as micro-sculpture of the shell, it was sometimes misleading rather than helpful (17) (18). However, the combination of morphological and molecular data analysis conducted in this study suggested that at least two separate medical important species from *Bu. africanus* group species are present in Mangochi District and need to be assessed through a taxonomic appraisal. To our knowledge, this is the first investigation that focuses on *Bu. africanus* group population from Malawi at a molecular level.

With regard to morpholog, The *B. globosus* shells in Malawi can be distinguished from *Bu. africanus* shells by the distinctive small notch or tooth in the truncated columella margin of the aperture inside (Figure 7). Striking findings, all molecular identification used in this study reveal that *Bu. africanus*, the intermediate host for *S. haematobium* and *S. mattheei*, is present in Lake Malawi; this species has not been reported before in Malawi. We validated the identification of *Bu. africanus* by two *cox*1 markers, ITS and RFLP. Finding a new intermediate host in Lake Malawi is considered a new significant alteration in the epidemiology of schistosomiasis transmission in Lake Malawi. The detectetion of DNA shcistosomes in real-time PCR screening test in *Bu. africanus* is expeted as it is known as intermediat host for schistosomiasis. In contrast, detectetion of DNA shcistosomes in *Bu. angolensis* was unexpected in order that *Bu. angolensis* has not been reprted as an intermediat host for schistosomiasis. Therefore, a future study is suggested to investigate the roles of *Bu. africanus* group in schistosomiasis in Lake Malawi.

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Two possible explanations of the colonisation of *Bu. africanus* observed in 2017 and 2018 in Lake Malawi might be suggested. The colonisation might have recently occurred, similar to the colonisation of *Biomphalaria,* which was observed in the same surveys (14). The second scenario is presumed to be because of mistaken identity for *Bu. globosus* in Lake Malawi in previous studies in light of no report of using molecular identification in this population. Additionally, *Bu. africanus* was not considered to go beyond the immediate southern African countries. Hence, the molecular applications used in this study, such as blast, sequence analysis, and RFLP rapid essay, sustains the need to develop molecular applications to identify the *Bu. africanus* group, particularly in distinguishing between *Bu. globosus* and *Bu. africanus*.

Molecular identification using *cox*1 gene is a popular method for freshwater snails species identification through sequence comparison with available reference data such as GenBank (19). Also, *cox*1 evolution is rapid enough to help discriminate species in (20). However, the genetic variation in the *cox*1 marker requires a good selection for a critical primers, particularly for *Bulinus* (19) (21). The selected primers for the *Bu. africanus* group and the sequences generated in this study provide information and data that can be used for comparison in different populations when trying to understand the distribution of *Bu. africanus* group species and their role in disease transmission.

Sequence analysis of restriction sites suggested that enzyme could be a species-specific marker for *Bu. globosus* while HaeIII enzyme is a marker for *Bu. africanus*. Upon SacI digestion, only *Bu. globosus* sequences were cut, whereas *Bu. africanus*, *Bu. angolensis*, *Bulinus* sp. remained intact. Besides, upon double digestion with Sac1 and HaeIII, *Bu. globosus* and *Bu. africanus* produced a restriction profile where two fragments were generated that were estimated to be approximately 400 bp and 220 bp for *Bu. globosus* and two bands at a similar size at 300 bp in *Bu. africanus*. These restriction data show that a stable genetic marker has been found for *Bu. globosus* from Malawi. It would now be interesting to ascertain whether the *Bu. africanus* group from different parts of Africa can be similarly differentiated.

Although the significant increase in *Bulinus* density after the rainy season in 2018, *Bu. globosus* was not present in the Lake in both surveys. Interestingly, the *cox*1 gene sequence generated for *Bu. globosus* samples collected from a river in Blantyre, stream in Chikwawa, stream in Mangochi district were identical through the distance and different environment. These data sustain the belief that *Bu. globosus* is not present in the Lake, at least in Mangochi District, reflecting environmental factors such as aquatic plants might play a role in its distribution.

The phylogenetic tree in this study used two methods to measure the evolutionary relatedness among the four taxa collected in this study, with two known species from *Bu. truncatus/tropicus* complex collected from Lake Malawi and reference samples from GenBank from *Bu. africanus* group.

The neighbour-joining and maximum likelihood tree, in addition to the minimum spanning network, inferred an evolutionary relationship between the four taxa groupings within the specimens investigated (Figure 3, and 5). Phylogenetic analysis of the *cox*I has confirmed that *Bu. africanus* from Lake Malawi can be separated from *Bu. globosus* collected from a different population in Malawi. Also, the tree is further evidence confirming three species from *Bu. africanus* group are found in Lake *Bu. africanus*, *Bulinus* sp., and *Bu. angolensis*. The latter could indicate a new species has not been reported before in Lake Malawi, which would probably reflect mistaken identification because of the similarity in shell morphology between *Bu. angolensis* and *Bu. globosus*. Our study provides further evidence for recent study has been suggested based on a genetic investigation that *Bu. angolensis* is more closely related to *Bu. africanus* group than originally documented belonging to *Bu. truncatus/tropicus* group. However, looking more closely, tree and sequence analysis in our study found that *Bu. angolensis* from Lake Malawi is closer to *Bu. africanus* than *Bu. globosus* . It is worthwhile noting that Brown., (1994) made the point that the treatment of *Bu. angolensis* is unclear partly as the molecular properties were unknown (10).

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Interestingly, about the few unidentified species found in Palm Beach and the Shire River, it shares a node in the phylogenetic tree with *Bu. africanus* and *Bu. globosus* specimens but is separate from these species . Although no medical report is known yet for *Bu. angolensis* and *Bulinus* sp., finding these species with evolutionary closeness to *Bu. africanus* group species is providing data that deserve further investigation and could open the door for potential medical importance for these two species.

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