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Full Length Research Paper

Molecular phylogeny of *Bulinus* (Gastropoda: Planorbidae) reveals the presence of three species complexes in the Albertine Rift freshwater bodies

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In this study, partial mitochondrial DNA cytochrome oxidase subunit I (mtCOI) sequences (612 bp) of *Bulinus* snails sampled from 31 freshwater bodies in the Albertine Rift were analyzed to investigate the extent of genetic variation and phylogenetic relationships. Bayesian phylogenetic inferences clustered the samples into three species groups; *Bulinus truncatus/tropicus*, *Bulinus forskalii* and *Bulinus africanus*. Twenty-two haplotypes were identified within the *B. truncatus/tropicus* species group which clustered into two well-differentiated lineages; with 2.7% sequence divergence between them. Significant genetic variation was also observed within the *B. forskalii* group, with the Maramagambo forest haplotype being separated by 55 mutational changes from the rest of the haplotypes. The *B. truncatus/tropicus* species group showed early divergence from the two *B. forskalii* and *B. africanus* species groups which were more closely related. A single species *B. globosus* in the *B. africanus* species group was identified in the Albertine Rift. We report the presence of five *Bulinus* species in the Albertine Rift; two in the *B. truncatus/tropicus* group, two in the *B. forskalii* group (one species yet to be identified) and one species in the *B. africanus* group. The findings of this study highlight the limitations of relying solely on shell characteristics to delineate snail species within the genus *Bulinus*.

Key words: *Bulinus* species, cytochrome oxidase c subunit I, mitochondrial DNA, phylogenetic relationships, Albertine Rift.

INTRODUCTION

Freshwater snails of the genus *Bulinus* are widely distributed in Africa, the East African islands, the Middle East and some Mediterranean countries. *Bulinus* is comprised of 37 recognized species (Brown, 1994) categorized into four species groups; the *Bulinus forskalii* group (11 species), *Bulinus truncatus/tropicus* complex (14 species), *Bulinus africanus* group (10 species) and *Bulinus reticulatus* group (2 species). Taxonomic studies of *Bulinus* are on the increase due to the taxon's role as intermediate hosts in the transmission of schistosomiasis in humans, domestic and wild animals. The Albertine Rift is the western arm of the Great Rift Valley and it is occupied for more than half of its length by water, forming the five great Lakes Albert, Edward, George, Kivu and Tanganyika (Kaufman et al., 1996). The Albertine Rift also harbors a number of volcanic crater lakes found along the eastern side of the Rift valley in western Uganda between Kabarole and Kasese districts.

The Albertine Rift harbors more endemic mammals, birds and amphibians than any other region in Africa and has consequently been declared a biodiversity hotspot (Myers et al., 2000). The geological events that created

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the mountains of this hotspot have also yielded some of the world's most extraordinary lakes that harbor a number of *Bulinus* species on which very little information exists regarding their identity, molecular genetic diversity and phylogenetic relationships. Earlier morphological work to characterize the genus *Bulinus* at the species level proved problematic due to high levels of variation especially in shell form within and among populations (Mandahl-Barth, 1965; Brown, 1994). Techniques such as morphometrics (Kristensen and Christensen, 1989) and biochemical studies (Rollinson and Southgate, 1979; Rollinson and Wright, 1984; Jelnes, 1986; Mimpfoundi and Greer, 1990), cytogenetic studies (Burch, 1960; Goldman et al., 1980; Brown and Shaw, 1989) and molecular genetic studies (Stothard and Rollinson, 1997; Raahauge and Kristensen, 2000; Stothard et al., 2002; Jorgensen et al., 2007a; Kane et al., 2008) have all enabled researchers to search for genetic differences that may help to solve the taxonomic questions in *Bulinus*.

Over the past two decades, molecular approaches have increasingly proven to be valuable not only in resolving phylogenetic uncertainties, but also in providing an insight into the time scales of evolutionary divergence. Unlike the relatively slow evolving nuclear rRNA genes that have been widely used in studies attempting to resolve relationships among groups that have a long history of evolutionary divergence, the more rapidly evolving mitochondrial coding genes are increasingly being employed to infer relationships among groups with a more recent ancestry.

Mitochondrial cytochrome oxidase subunit I (mtCOI) and cytochrome b (cyt b) have so far been the favorite candidate genes for this purpose. Although both genes show a high incidence of base substitutions at third position nucleotides thereby allowing the discrimination of closely related species, mtCOI possesses two important advantages over cyt b, both associated with its slower rate of molecular evolution. Firstly, the universal primers for this gene are very robust, enabling the recovery of its 5' end from most animal species and secondly, mtCOI has a greater taxonomic signal range than cyt b. These characteristics have therefore made mtCOI a popular molecular marker for resolving both recent and deeper taxonomic affinities between taxa (Remigio and Hebert, 2003).

Mitochondrial DNA COI sequence variation has increasingly been widely employed in phylogenetic studies of the genera *Bulinus*, *Biomphalaria* and other freshwater gastropods (Stothard and Rollinson, 1997; Davis et al., 1998; Campbell et al., 2000; Remigio and Hebert, 2003; Sørensen et al., 2005; Jørgensen et al., 2007a, b, 2008; Plam et al., 2008; Sengupta et al., 2009). Different other studies have also used sequence diversity of the COI as a DNA barcode for the identification of different animal species (Hebert et al., 2004a, b; Kane et al., 2008).

In this study, partial mitochondrial DNA cytochrome oxidase subunit I (mtDNA COI) was also used to determine phylogenetic affinities between *Bulinus* species groups from different localities within the Albertine Rift water bodies. New data on the geographical distribution and genetic diversity gathered on the potential *Bulinus* intermediate host snails will contribute towards the identifycation of target areas for focal schistosomiasis control in the Albertine Rift.

MATERIALS AND METHODS

Sample collection and DNA extraction

Snail samples were collected from 26 localities across the five great lakes (Albert, Edward, George, Kivu, Tanganyika) and ten crater lakes as well as permanent and temporary ponds found in the Albertine Rift as shown in Table 1 and Figure 1. The taxonomic status of the sampled individuals was assessed by shell morphology using the field identification key of Kristensen (1987) (depicted in Figure 2).

The sorted *Bulinus* snails were later preserved in 80 % ethanol and stored in the laboratory at -80°C. The frozen samples were thawed at room temperature prior to DNA extraction. Genomic DNA was extracted from individual snails using the DNeasy Tissue Kit (Qiagen, Inc. Valencia, CA), following the manufacturer's instructions.

Amplification of mtDNA COI and sequencing

The mitochondrial cytochrome oxidase subunit I (mtDNA COI) fragment was amplified using universal primers LCO1490 (5´-GGT CAA CAA ATC ATA AAG ATA TTG G-3´) HCO2198 (5´-TAA ACT TCA GGG TGA CCA AAA AAT CA-3´) (Folmer et al., 1994). Amplification was performed with *Taq*DNA polymerase (Roche) in 50 µL total reaction volume using the following PCR profiles: initial denaturation (5 min at 95°C), followed by 35 - 40 cycles of denaturation (2 min at 94°C), annealing (2 min at 56 - 58°C), extension (2 min 30 s at 72°C) and final extension (5 min at 72°C). PCR products were purified using the QIAquick PCR purification Kit (QIAGEN) and sequenced in both directions using Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on an automated ABI PRISM® 3700 DNA sequencer (Applied Biosystems).

Genetic variation and phylogenetic analyses

Nucleotide sequences were multiple aligned using ClustalW v1.4 (Thompson et al., 1994) and edited using BioEdit v7.0.5.3 (Hall, 1999). Sequence similarity with other gastropod sequences in the GenBank was determined using BLAST (Basic Local Aligned Search Tool, hptt://www.ncbi/BLAST /index/html) to exclude the possibility of amplification of the nuclear pseudogene copies of mtDNA COI region.

The COI sequences were also translated into amino acid sequences using the invertebrate mitochondrial genetic code and inspected for stop codons using BioEdit v7.0.5.3 (Hall, 1999) to further eliminate the possibility of amplification of numts. Nucleotide variations in the mtDNA COI region sequences were estimated using the program POPSTR version 1.2 (H. R. Siegismund, unpublished).

Phylogenetic analyses were performed using PAUP software Version 4.0 (Swofford, 1998) and the best fit model was estimated using MrModeltest 2.2 (Nylander, 2004). A rooted neighbor - joining (NJ) haplotype tree was constructed using maximum likelihood settings and 100 Bootstrap replicates, incorporating a gamma-

Table 1. Details of sampling localities in the Albertine Rift freshwater bodies; *B.t/t = Bulinus truncatus/tropicus* group samples, *B.f = Bulinus forskalii* group samples *B.a = Bulinus africanus* group samples.

Sample site	Localities	Position	*B.f*	*B.t/t*	*B.a*
Lake Kivu	Kakondo	S 2.040361, E 28.14477		1	
	Ikondere	S 2.044203, E 28.14537		4	
	Cibale	S 2.036428, E 28.14388		12	
	Ceya	S 2.034058, E 28.14310		3	
Lake Albert	Booma	N 1.136356, E 31.05799	3	8	
	Bugoigo	N 1.151164, E 31.06784	5	8	
	Piida	N 1.136686, E 31.05503		8	
	Toonya	N 0.097783, E 31.01584	13	9	
	Walukuba	N 1.141136, E 31.06342		2	
Crater Lake	Nkuruba	S 0.103300, E 30.06975		7	
	Nyamirima	S 0.144336, E 30.08908		4	
	Mafuru	S 0.074708, E 30.02906		10	
	Kanyigwe	S 0.125006, E 30.07561		1	
	Kasenda	S 0.119897, E 30.08101		5	
	Rwenzogoro	S 0.075317, E 30.02250		8	
	Nyungu	S 0.070850, E 30.02651		8	
	Nyinabunga	S 0.184667, E 30.08375		1	
	Mwamba	S 0.127083, E 30.07586		1	
	Nyabikere	S 0.138547, E 30.09046		1	
Swamp	Katosho	S -4.141950,E 29.10945	18	21	
Lake Tanganyika	Mwakizenga	S -5.012967,E 29.13302	5		
	Bulombora	S -5.004561,E 29.13068	10		
Lake Edward	Mweya C.S	S 0.1073600,E 29.53749	20		
Lake George	Kasenyi	S 0.0174500,E 30.08986	7		
Forest	Maramagambo	S 0.2270000,E 29.52438	3		
Stream	Tutwe	N 1.4434300,E 31.23920			10
Total no. of samples			88	122	10

corrected Hasegawa-Kishino-Yano (HKY+I+G) model of evolution (Rodriguez et al., 1990). The mtDNA COI sequence of *Indoplanorbis exustus* (GenBank accession no. AY577511), the closest relative of the genus *Bulinus* (Morgan et al., 2002), was used as an out–group.

Evolutionary relationships between the unique haplotypes were also estimated using the Kimura 2-parameter model genetic distances as implemented in MEGA 3.1 (Kumar et al., 2004) with 1000 bootstrap replications. In this analysis, twelve different *Bulinus* haplotypes corresponding to different species that belong to the four species groups recognized within *Bulinus* were retrieved from the GenBank and included in the phylogenetic analysis in order to examine the topology of the test haplotypes on the tree.

An unrooted statistical parsimony network connecting the haplotypes was inferred with TCS v1.21 (Clement et al., 2000) and manually constructed indicating the number of mutational changes between haplotypes.

RESULTS

Morphology and sequence variation

Assessment of the taxonomic status of snails based on shell structure showed that three morphologically distinct forms do exist in the Albertine Rift (Figure 2): a high spired shell characteristic of *B. forskalii* species, a short obtuse shell apex found in *B. globosus* snails and a thin fragile shell with a narrow columellar margin characteristic of snails found within the *B. truncatus/tropicus* species complex.

The 613 bp fragments of the mtDNA COI gene aligned for 220 *Bulinus* samples from 26 localities revealed 35 different haplotypes characterized by 147 polymorphic

Figure 1. Map showing the different sampled localities for the genus *Bulinus* species in the Albertine Rift, east Africa.

sites (Table 2). The polymorphic sites were represented by 4 changes on the first codon position, 2 on the second codon position and 141 on the third codon position. Transversions were greater (56.4%) than transitions (43.6%) and the mutational changes resulted into a total number of 6 amino acid substitutions. The 35 unique haplotypes clustered into three distinct mitochondrial lineages A, B and C that correspond to three of the four species groups in the genus *Bulinus*; the *B. truncatus/ tropicus* species complex, the *B. forskalii* group and the *B. africanus* group, respectively.

A rooted neighbor-joining reconstruction of the *Bulinus* unique haplotypes resulted into three distinct groups A, B and C (Figure 3). The *B. truncatus/tropicus* complex (Group A) is further split into two subclades A1 and A2;

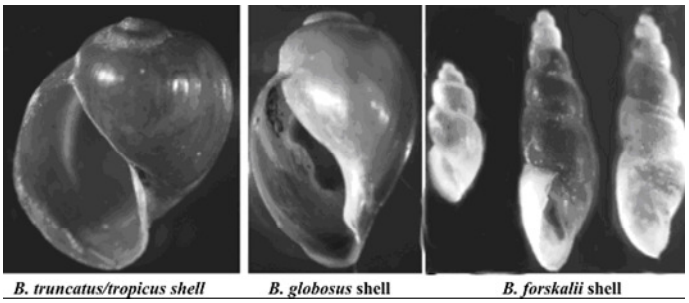

Figure 2. Sinistral shells of the different species groups in the genus *Bulinus* found in the Albertine Rift.

subclade A1 constituted by haplotypes A1, A2, A3 from Lake Albert, T1, T2 from Tanzania and one haplotype K1 from Lake Kivu, clustered together to form a monophyletic lineage supported by 61% (Figure 3) bootstrap value. Within subclade A2 three more clusters composed of Crater Lake haplotypes (C) are observed. Haplotypes A4 and A5 from Lake Albert also form a cluster of 64% support.

In a different rooted neighbor-joining tree with inclusion of more reference sequences from the GenBank, the topology of the tree was more resolved into four distinct groups corresponding to *B. truncatus/tropicus* species group (with subgroups appearing as sister clades); *B. reticulatus* group; *B. forskalii* group (with two distinct subclades B1 and B2) and *B. africanus* group (Figure 4). The Maramagambo forest haplotype M1 grouped with various *Bulinus* forskalii group species in subclade B1, whereas the rest of the haplotypes in clade B grouped with the *B. forskalii* species in subclade B2. The single haplotype U1 clustered with all other species in the *B. africanus* group in clade C. No haplotypes from the Albertine Rift grouped with the *B. reticulatus* species group sequences from the GenBank. The same topology of evolutionary affinities was confirmed in the minimum spanning network of haplotypes (Figure 5).

Within the *B. truncatus/tropicus* species complex

A total of 36 variable positions in the mitochondrial COI gene defined 22 haplotypes within the *B. truncatus/ tropicus* group (Table 3). Haplotypes locality were specific with 4 major groups being recognized; two Katosho swamp haplotypes, five Lake Albert haplotypes, one Lake Kivu haplotype and 14 haplotypes from the different crater lakes; no haplotypes were shared among the four haplogroups (Table 3).

A non-synonymous substitution occurred at position 446 between two subclades A1 and A2 replacing the amino acid asparagine with threonine. The sister clades A1 and A2 were separated from each other by 14 mutations (Figure 5) equivalent to a sequence divergence of approximately 2.7%. Subclade A1 comprised of haplotypes

Table 2. Polymorphic positions in the *Bulinus* haplotypes; a period denotes a matching base with the top most sequence. Regions A, B and C show the different species complexes. A (1 and 2) - *Bulinus truncatus/tropicus* species complex; B- *Bulinus forskalii* species group and C- *Bulinus africanus* species group.

147 POLYMORPHIC SITES

	11111111111111111111222222222222222222222222222222223333333333333333333333334444444444444444444444444444455555555555555555555555666	
	1112222333445567889992334455566777888899000111222333444555666777888990001122334455666677788991222333444555556677788889001112233445677888999001	
	134925812573465778021403962847369291240369581470396891470472581470392581703689273625143469058476973692581467036795814701699150365817095709258147392	
A1	TAGTGATATTATTAACTTATTTTTTTTAAAGTAACTCTTTTTACTTATTACTTAAATATAGTTTACAGTAAATATTGCAAATATATACTTTTGAAGAACTAGAAACCTAAATAATAATACTTTACAATAAAAGAACACAATATCTTG	
A2	....T..A...	
A3	....T..A...T...	A1
K1	....T..T........C..............................T...	
T1	....T...C..............................T........C.........T......................	
T2	....T...C..............................T........C.........T....................CA	
A4	....T.....................A.G.A............T.G...................T.A......C.A............C..........GT...TA............................T.......TCCA	
A5	....T.....................A.G.A............T.G............C......T.A......C.A............C..........GT...TA............................T.......TCCA	
C1	....T.....................A.G..............T.A...................T.A........A............C..........GT...TA.................T..........T.......T.CA	
C2	....T.....................A.G..............T.A...................T.A........A............C...........T...TA.................T..........T.......T.CA	
C3	....T.....................A.G..............T.A...................T.A........A............C..........GT...TA.........G.......T..........T.......T.CA	
C4	....T.....................A.G..............T.G...................T.A......C.A............C..........GT...TAC.....G........C.T..........T.T.....T.CA	
C5	....T.....................A.G..............T.G...................T.A......C.A............C..........GT...TAC.....G..........T..........T.T.....T.CA	A2
C6	....T.....................A.G..............T.G...................T.A......C.A............C..........GT...TAC................T............T.....T.CA	
C7	....T.....................A.G..............T.G...................T.A......C.A............C..........GT...TAC................T............T.....T.CA	
C8	....T.....................A.G..............T.G...................T.A......C.A............C..........GT...TAC................T..........T.T.....T.CA	
C9	....T.....................A.G..............T.G...................T.A......C.A............C..........GT...TA.................T..........T.T.....T.CA	
C10	....T.....................A.G.A............T.A...................T.A......C.A.G.............A.......GT...TA............T....T..........T.T.....T.CA	
C11	....T................A....A.G.A............T.A...................T.A........A............C..A.......GTG..TA............................T.T.....T.CA	
C12	....T................A....A.G.A............T.A...................T.A........A............C..A.......GTG..TA..............................T.....T.CA	
C13	....T................A....A.G.A............T.A...................T.A......C.A............C..A.......GTG..TA.....................G........T.....T.CA	
C14	....T..........T.....A....A.G.A............T.A...................T.A......C.A............C..A.....T.GTG..TA..............................T.....T.CA	
A6	C..AATCT....CT..ACC..A.ACA..G.T...TATA.CAC.T.A....TACTTTA...AACA.T.A.GTTATCC...T.ATA..T....ATTTTTTTC..TTTT..GG..GG.TGATTCA...T.A.TTT..TT.TT.A.GT.CA	
A7	C..AATCT....CT..ACC..A.ACA..G.T...TATA.CAC.T.A....TACTTTA...AACA.T.A.GTTATCC...T.ATA..T....ATTTTTTTC..TTTT..GG..GG.TGATTCA...T.A.TTT...T.TT.A.GT.CA	
A8	C..AATCTC..ACT..ACT..A.ACA..G.T...TATA.CAC.T.A....TACTTTA...AACA.T.A..TTAT.C...T.ATC..T....ATTTTTTTC..TT.T..GG..GG.CGATTCA.G.T.A.TTT..TT.TT.A.GT.CA	
E1	C..AATCTC..ACT..ACT..A.AC...G.T...TATA.CAC.T.A....TACTTTA...AACA.T.A..TTAT.CA..T.ATC..T....ATTTTTTTC..TTTT..GG..GG.TGATTCA.G.T.A.TTT..TT.TT.A.GT.CA	
E2	C..AATCTC..ACT..ACT..A.ACA..G.T...TATA.CAC.T.A....TACCTTA..GAACA.T.A..TTATCC...TGATC..T....A.TTTTTTC..TTTT..GG..GG.TGATTCA.G.T.A.TTT..TT.TT.A.GT.CA	
G1	C..AATCTC..ACT..ACT..A.ACA..G.T...TATA.CAC.T.A....TACTTTA...AACA.T.A.GTTATCC...T.ATA..T....ATTTTTTTC..TTTT..GG..GG.TGATTCA.G.T.A.TTT..TT.TT.A.GT.CA	B
G2	C..AATCTC..ACT..ACT..A.ACA..G.T...TATA.CAC.T.A....TACTTTA...AACA.T.A..TTAT.CA..T.ATC..T....ATTTTTTTC..TTTT..GG..GG.TGATTCA.G.T.A.TTT..TT.TT.A.GT.CA	
T3	C..AATCT...ACT..ACT..A.ACA..G.T...TATA.CAC.T.A....TACCTTA..GAACA.T.A.GTTATCC...TGATC..T....A.TTTTTTC..TTTT..GG..GG.TGATTCA.G.T.A.TTT..TT.TT.A.GT.CA	
T4	C..AATCTC..ACT..ACT..A.ACA..G.T...TATA.CA..T.A....TACCTTA..GAACA.T.A.GTTATCC...T.ATC..T....A.TTTTTTC..TTTT..GG..GG.TGATTCA.G.T.A.TTT..TT.TT.A.GT.CA	
T5	C..AATCTC..ACT..ACT..A.ACA..G.T...TATA.CAC.T.A....TACCTTA..GAA.A.T.A.GTTAT.C...T.ATC..T.. .A.CTTTTT...TTTT..GG..GG.TGATTCA.G.T.A.TTT.GTT..T.A.GT.CA	
T6	C..AATCTC..ACT..ACT..A.ACA..G.T...TATA.CAC.T.A....TACCTTA..GAA.A.T.A.GTTAT.C...T.ATC..T....A.CTTTTTC..TTTT..GG..GG.TGATTCA.G.T.A.TTT.GTT.TT.A.GT.CA	
M1	..A.A.CT...ACTT....C..AA.A.GCGAC.GTATA..A.TTCAGCC.TACTTTGG..AA.AGGGAA.TTGTC..T.TTGTAG.T...CA.TT.TTT..ATTGT.A....G..T.A.T.A..TGGAGTTTA..TGTT.AGAT..A	
U1	CG..T....CTA.....CT.CA.G....T.ACG.TATAAA...TAA...GTA..TTA...AA.A.A.TAT.G.T..T..TTA..GGTTA..CTT.T..T..ATTTT....GC...T...T..CTTT.A.T....TTTT.TA.GT..A	C

from Lake Kivu (K1), Lake Albert (A1, A2, A3) and from the Katosho swamp (T1, T2) in Kigoma with 100% support (Figure 3). Subclade A2 comprised of all the crater lakes haplotypes plus two other haplotypes A4 and A5 from Lake Albert. Haplotypes from the different crater lakes all cluster with subclade A2 although haplotypes C5 and C6 from Lake Rwenzogoro show non-synonymous changes at sites 47 and 322, respectively.

Within *Bulinus forskalii* species group

A sequence alignment of the *B. forskalii* group COI fragment resulted in 12 haplotypes with 76 polymorphic sites (Table 4). The most frequent haplotype T3 from Katosho swamp in Tanzania was also shared in Lake Tanganyika at second Bulombora and in Lake Albert at Tonya. The commonest haplotype, G2, was shared between lakes George and Edward. Haplotype M1 was unique to Maramagambo forest and shows genetic discontinuity from the rest of the *Bulinus forskalii* species group (Figure 5) with 55 of the polymorphic sites unique to Maramagambo forest (Table 4). A comparison of the amino acid sequences using the invertebrate mitochondrial code and based on reading frame1 revealed three non-synonymous substitutions (changes at sites 4,

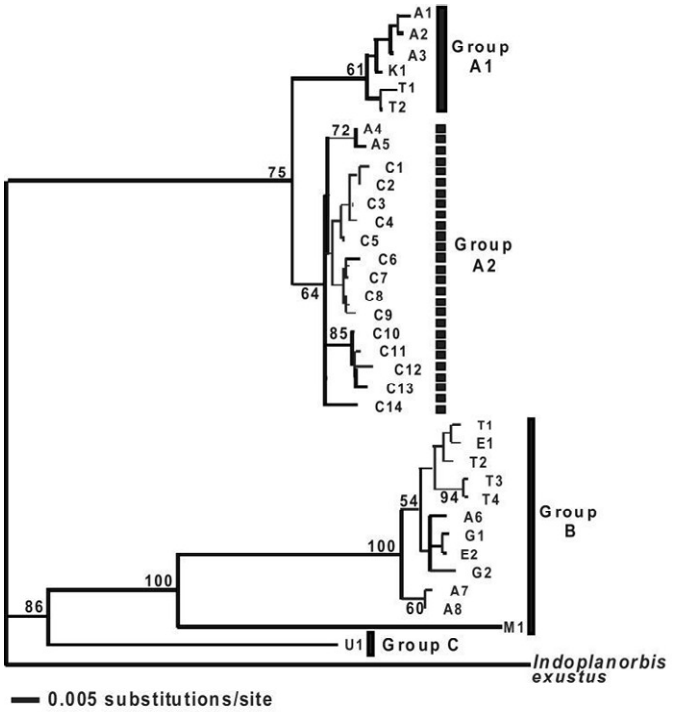

Figure 3. Maximum likelihood (ML) phylogram illustrating haplotype relationships of the Bulinus species in the Albertine Rift**.** The tree is rooted with the *Indoplanorbis exustus* mitochondrial sequence (AY577511). The numbers on the branches are bootstrap support values based on 100 replications.

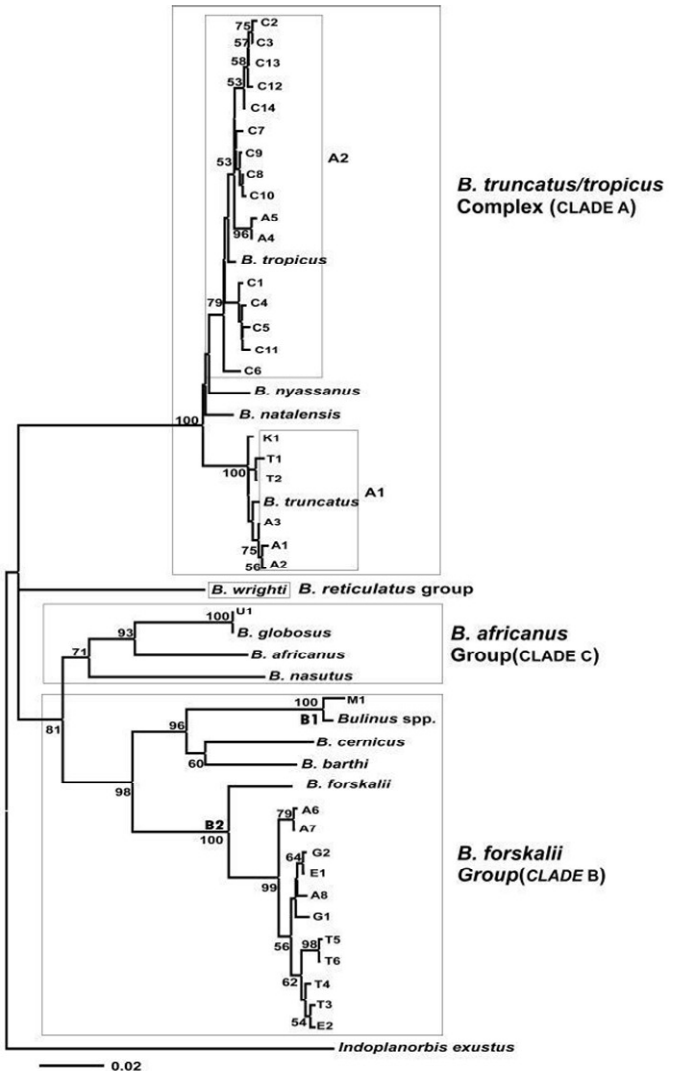

Figure 4. A rooted neighbour-joining tree of the Albertine Rift samples for mtDNA COI using the Kimura 2-parameter distance. Both transitions and transversions were included and the values on the branches are bootstrap support based on 1000 replications.

244 and 319) that were unique between haplotype M1 and the rest of the *B. forskalii* group haplotypes (Table 4); the substitutions occurred at the first codon position. Haplotype A7 a supposedly *B. forskalii* species from Lake Albert is separated from the Maramagambo haplotype by 64 mutation changes which is equivalent to a percent sequence divergence of 11.2% (Figure 5). Further comparison of haplotype M1 sequence from Maramagambo forest with Bulinus sequences retrieved from the GenBank showed that it is closely related with a sequence similarity of 99% to a *Bulinus* spp. (Accession No. AM921832; Kane et al., 2008) sampled from Pemba Island in Tanzania. The two haplotype differ at sites 4 and 319 (Table 5) which give rise to non-synonymous changes. The rest of the substitutions between the B. forskalii haplotypes were synonymous**.**

DISCUSSION

Analyses based on shell morphology indicate that the *Bulinus* species in this study belong to three species groups; *B. truncatus/tropicus*, *B. forskalii* and *B. africanus*. Earlier studies using morphological characters have categorized the genus *Bulinus* species into four species groups (Brown, 1980). In this study, different analyses of the *B. truncatus/tropicus* species complex consistently recovered two reciprocally monophyletic sister subclades A1 and A2 (Table 2, Figures 3, 4 and 5); corresponding to *B. truncatus* and *B. tropicus* respect-tively. Subclade A1 comprised all haplotypes that possessed Threonine at position 446 and subclade A2 comprised all that had asparagine.

Although *B. tropicus* and *B. truncatus* are morphologically indistinguishable, a number of different studies have shown that they differ in the number of chro-mosomes they possess; *B. tropicus* is a diploid (2n= 36) whereas *B. truncatus* is a tetraploid (2n = 72) (Brown and Shaw, 1989). Five other species out of the 14 *Bulinus* species in the *B. truncatus/tropicus complex* are diploid, namely, *B. depressus*, *B. natalensis, B. liratus, B. nyassanus*, and *B. succinoides* (Burch, 1960; 1967a; Goldman et al., 1980; and Burch, 1978). Our results clearly indicate that the crater lakes of Western Uganda

Table 3. List of 36 polymorphic sites of the 22 mtDNA COI *B. truncatus/tropicus* haplotypes. The shaded area shows the site (446) of genetic discontinuity between the two species *B. truncatus* and *B. tropicus*.

36 Polymorphic sites		Haplotype distribution and frequency																				
	1111222233333444444444444555556666	KSW	Lake Albert						Lake Kivu				Crater Lakes									
	1483459047701267223344466678902679001																					
	2742438470938298392546758419557973 92	KSW	ATO	ABU	ABO	APA	APD	AWA	IKK	CIK	CEK	KOK	KUC	YAC	DAC	ASC	YBC	WBC	ANC	AFC	YUC	WEC
A1	GCTTAGCTTCGTGATGCAGACCTATACTCACCCTTG		9			4	3															
A2	T.....A..				6		4															
A3	T.....A.T.				2																	
K1	T.....T.C.....T.								5	11	3	1										
T1	T.....C.....T...C...T.....	20																				
T2	T.....C.....T...C...T.....CA	1																				
A4	T..AGATG.TACA.C..GT.TA.....T.TCCA			4			1	2														
A5	T..AGATGCTACA.C..GT.TA.....T.TCCA			4																		
C1	T.AAGATA.TA.A.CA.GTGTA.....TTT.CA												1									
C2	T..AG.TG.TACA.C..GT.TACG...CT.TTT.CA												3									
C3	T..AG.TG.TACA.C..GT.TACG....T.TTT.CA												2	1								
C4	T.AAGATA.TA.A.CA.GTGTA.....TT.CA												1	2								
C5	TTAAGATA.TACA.CATGTGTA.....TT.CA																					5
C6	T..AGATA.TACAG.A.GT.TA....T.T.TTT.CA																			2	2	3
C7	T..AG.TA.TA.A.C...T.TAC.....T.TTT.CA													1			1					
C8	T..AG.TA.TA.A.C..GT.TA.....T.T.T.CA														1							
C9	T..AG.TA.TA.A.C...T.TA.....T.T.T.CA																	1				
C10	T..AG.TA.TA.A.C..GT.TA...G..T.T.T.CA																		1			
C11	T.AAGATA.TACA.CA.GTGTA.....G.TT.CA																			8	6	
C12	T..AG.TG.TACA.C..GT.TAC.....T..TT.CA															1						
C13	T..AG.TG.TACA.C..GT.TAC.....T.TTT.CA															2						
C14	T..AG.TG.TACA.C..GT.TA.....T.TTT.CA															2						
		21	9	8	8	4	8	2	5	11	3	1	7	4	1	5	1	1	1	10	8	8

are inhabited by *B. tropicus* while both *B. truncatus* and *B. tropicus* are sympatric in lake Albert and the sampled localities of Patterson Lake Kivu and Katosho swamp near Lake Tanganyika are inhabited by only *B. truncatus.* The non-synonymous mutations that occurred at sites 47 and 322 of haplotypes C5 and C6 were conservative and the two respective haplotypes are considered genetically similar to the *B. tropicus* which they group with in subclade A2. The amino acids substituted for at both sites; Threonine with Methionine species and Valine with Isoleucine were all found to be hydrophobic with similar chemical properties. In this study, we have found out that the two species *B. truncatus* and *B. tropicus* occur in sympatry in Lake Albert; a mixed population containing both species found at Piida in Lake Albert. The two species have been reproductively isolated for more than 1.3 Mya as depicted by the 2.7% sequence divergence between them. The is 2.7% sequence divergence of mtCOI gene between the two reciprocally monophyletic subclades A1 and A2 though low, is close to a species threshold as proposed by Johnson et al. (2000). It has also been suggested that animal species, at most, exhibit 2-3 % difference among conspecifics (Hebert et al., 2004). A group of individuals that exhibited DNA difference greater than the 2% threshold limit would potentially represent different species. The amount of genetic variation corresponds to the level found in a clade of *Biomphalaria* another schistosome intermediate host snail in East Africa (Jørgensen et al., 2007b Plam et al., 2008).

Another major finding in this study is the outstanding divergence between the Maramagambo forest haplotype (M1) and the rest of the *B. forskalii* haplotypes, which implies that it represents a different species in the Albertine Rift. The *Bulinus* spp. from Maramagambo forest is closely related to another unidentified *Bulinus* spp. (AM921832 Kane et al., 2008) from Pemba Islan Tanzania while the rest of the haplotypes in subclade B2 are confirmed to be *B. forskalii* species. The extreme divergences between the Maramagambo gambo Forest species and other *B. forskalii* species could be interpreted as being indicative of yet another unidentified species within the *B.*

Table 4. Polymorphic sites among haplotypes at mtCOI in the *Bulinus forskalii* species group, Dots represent nucleotide variants identical to the first sequence. The shaded regions show sites 4, 244 and 319 where non-synonymous changes took place. Haplotype GB represents the GeneBank sequence AM921832.

	76 polymorphic sites	HAPLOTYPE DISTRIBUTION IN THE TEN POPULATIONS										
	1111111111122222222222222333333333344444444445555555555556											
	1234556789922344556889001134456677889001133457789234455677880011244577990											
	1495235780240969847362395170310421703257368936510846621703514061503509509149	KSW	TMW	TBU	ABU	ATO	ABO	EDC	GEK	MFO	GBK	Total
T3	CGATTAAACTTATCAAAGATTACCATATTCAAGCATATGACCGCGACATGTTCGTTGGGTGTCGCTAAGATATAGC	17		9		3						29
T4	...C.....T.....A.....	1										1
A6	....T...C.....T..A.....A.A..T.....A.....				4							4
A7	....T...C.....T..A.....A.A..T.....A.....A.....				1							1
A8	...C.....T..A.....A.T..A....T....A....C.....			1		10	3					14
T5	...C.....T.....T..A.....C.....G..C..		3									3
T6	...C.....T.....T..A.....C.....G.....		2									2
G1	...C.....T..A.....A.A..T.....								1			1
G2	...C.....T..A.....A.T.A.A....T.....							18	6			24
E1	...C.....T.....T..A.....A.T.A.A....T.....							1				1
E2	...C.....A....A.....							1				1
M1	TATA..TTTACTAT..GCGACGTTTCGCCTGGATGGGAAG.T.TTGAGC..GTAGAAAA.AATATGGGA.AG.GAT									3		3
GB	T.TA..TTTACTAT.GGTGACG.TTC.CCTGGATGGGAAG.T..TGAGC..GTAGAAAA.AATATGGGA.AG.GAT										1	1
	TOTAL NUMBER OF SAMPLES IN EACH POPULATION	18	5	10	5	13	3	20	7	3	1	85

forskalii species group.

Blast searches of the GenBank confirm that the *B. africanus* species sampled in the Albertine Rift is *B. globosus* as confirmed by the similarity Index 100% when compared to *B. globosus* sampled from Uganda (Accession No. AM921848; Kane et al., 2008). This study illustrates that though to a certain extent *Bulinus* snails may be identified on the basis of shell characters, identification at the species level needs other more reliable methods (for instance the use of DNA analysis) to reach a more conclusive result. Despite the uniformity I the shell morphology of the characterized snails within both the *B. forskalii* and *B. truncatus/ tropicus* species groups the molecular analysis shows the presence of more than one genetically different species in each of the groups.

Conclusion

The mtCOI fragment analyzed in this study has been very useful in differentiating the species within the three species groups found in the Albertine rift and here we report the presence of five *Bulinus* species in the Albertine rift; B. *globosus*, *B. forskalii*, *B. truncatus*, *B. tropicus* and another yet to be identified *Bulinus* sp. within the *forskalii* group found in Maramagambo forest. The findings of this study highlight the delimitations of relying solely on shell characteristics to delineate snail species.

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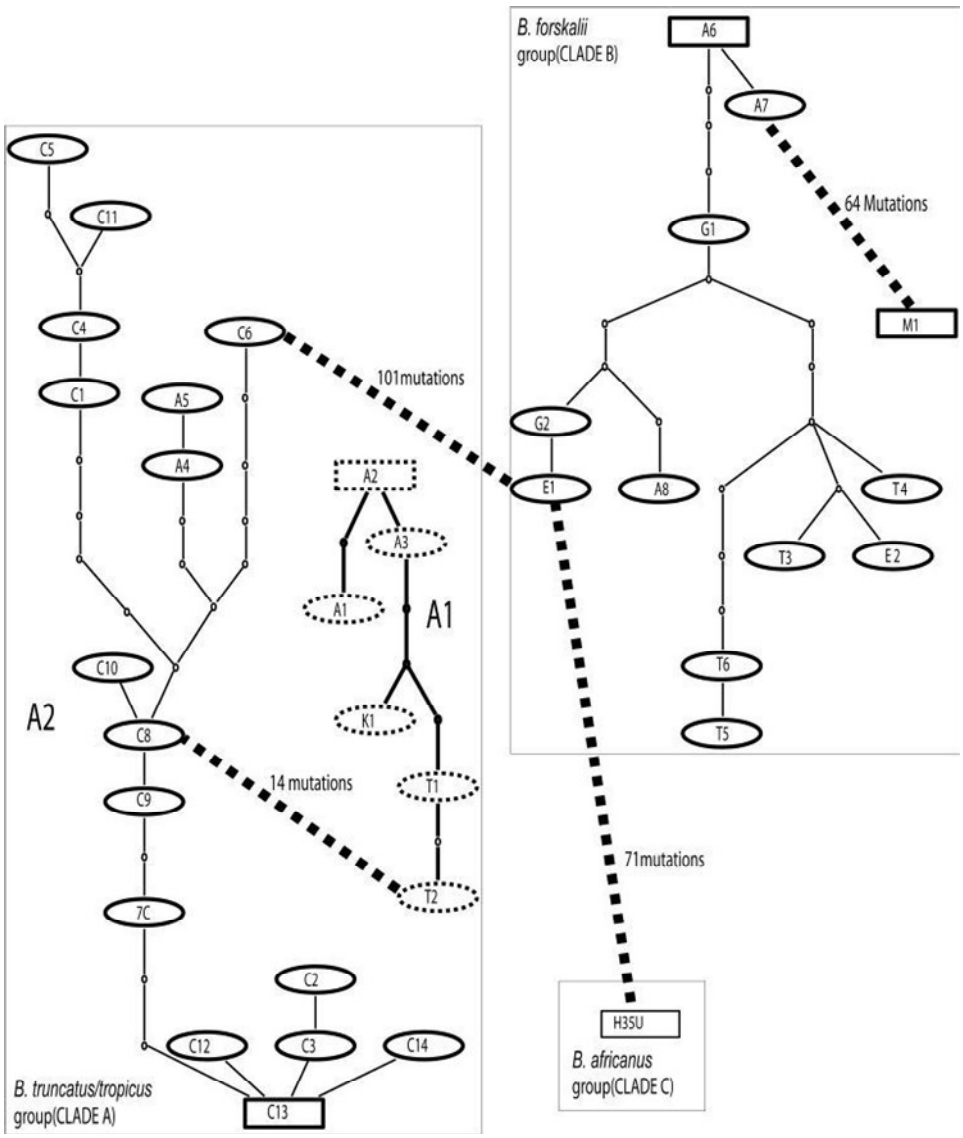

Figure 5. Mitochondrial COI haplotype network for *Bulinus* samples from the Albertine Rift. C = Crater lakes, A = Lake Albert, K = Lake Kivu, T = Tanzania. E = Lake Edward, G = Lake George, M = Maramagambo forest, U = Tutwe stream

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