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Mitochondrial DNA Variations in Sibling Species of the *Bulinus truncatus/tropicus* Complex in Lake Albert, Western Uganda

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Allen Nalugwa, Thomas K. Kristensen, Silvester Nyakaana, and Aslak Jørgensen (2010) Mitochondrial DNA variations in sibling species of the *Bulinus truncatus/tropicus* complex in Lake Albert, western Uganda. *Zoological Studies* **49**(4): 515-522. Thirty-nine snail samples morphologically conforming to the *Bulinus truncatus/tropicus* species complex were collected from 5 populations (Booma, Bugoigo, Piida, Toonya, and Walukuba) on Lake Albert, western Uganda. Analysis of patterns of sequence variations and evolutionary relationships within and between localities using a 612 bp fragment of the mitochondrial cytochrome oxidase subunit I (COI) gene revealed 5 unique haplotypes defined by 21 polymorphic sites. The Bayesian and median-joining network phylogenetic reconstructions partitioned the haplotypes into 2 reciprocally monophyletic clades concordant with *B. truncatus* and *B. tropicus* separated by 18 mutational substitutions, corresponding to a 3.0% net sequence divergence. Sequence divergences between haplotypes ranged 0.2%-3.5%, and significant genetic differentiation was observed in 5 of the 6 population pairwise comparisons. The 2 sibling species were sympatric in only 1 locality, at Piida. Our results confirm the existence of 2 evolutionary lineages of the *Bulinus truncatus/tropicus* species in Lake Albert and underscore the utility of the mitochondrial COI gene in differentiating between sibling species of the *B. truncatus/tropicus* complex which are otherwise indistinguishable based on the shell morphology. http://zoolstud.sinica.edu.tw/Journals/49.4/515.pdf

Key words: *Bulinus truncatus/tropicus* species complex, Freshwater snails, COI gene, Genetic variation, Lake Albert.

According to current taxonomy, the *Bulinus truncatus/tropicus* complex is one of the 4 species groups that are recognized in the genus *Bulinus* (Brown 1994), and it is comprised of a polyploid series of morphologically almost indistinguishable diploid, tetraploid, hexaploid, and octoploid populations. Six of the 14 species that have so far been identified within the *B. truncatus/tropicus* complex are diploids (*B. depressus* (Haas 1936), *B. liratus* (Tristram 1863), *B. natalensis* (Küster 1841), *B. nyassanus* (Smith 1877), *B. succinoides* (Smith 1877), and *B. tropicus* (Krauss 1848)). The species complex has received much attention by malacologists as well as parasitologists because some of the species transmit the parasite *Schistosoma haematobium*, which causes urinary schistosomiasis, a disease that occurs in Africa. *Bulinus truncatus* (Andouin 1827) was found to be a tetraploid taxon (Brown and Shaw 1989) and associated with the transmission of *S. haematobium* parasites. The diploid species, *B. tropicus*, is not known to transmit the *S. haematobium* parasite, although *B. nyassanus* and *B. liratus* are implicated in its transmission.

The identification of species within this group has proven difficult when based on morphological characters owing to high levels of intraspecific variations in shell form (Brown 1994). In previous

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studies, the tetraploids *B. truncatus* and *B. permembranaceus* were distinguished from the diploid *B. tropicus* based on differences in enzyme characters (Wright and Rollinson 1981, Brown et al. 1991).

Recently characterization of *Bulinus* species within the species complex has been greatly improved by the use of phylogenetic analyses based on DNA sequence variations of the mitochondrial gene cytochrome oxidase subunit I (COI) (Kane et al. 2008, Stothard et al. 2001).

The COI gene is more robust in resolving taxonomic species-level affinities within *Bulinus* than the internal transcribed spacer 2 (ITS2) region of the nuclear ribosomal gene complex (Kane et al. 2008).

Currently, little information exists about the identity and genetic variations of *Bulinus* populations in Lake Albert, western Uganda. This study therefore used mitochondrial DNA sequence variations to characterize the otherwise morphologically indistinguishable snail populations of the *B. truncatus/tropicus* complex sampled in Lake Albert, western Uganda.

MATERIALS AND METHODS

Sample collection and DNA extraction

Samples of the *B. truncatus/tropicus* complex were collected in July 2007 at 5 localities from Lake Albert, western Uganda (Fig. 1). The taxonomic identity of the sampled snails was initially determined based on shell morphology using the field identification key of Kristensen (1987). All shells identified had a micro-sculpture consisting of transverse ribs (Fig. 2) a characteristic typical of the *B. truncatus/tropicus* complex.

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

Amplification of mtDNA COI, sequencing, and alignment

A 750 bp fragment of the mitochondrial COI gene was amplified by a polymerase chain

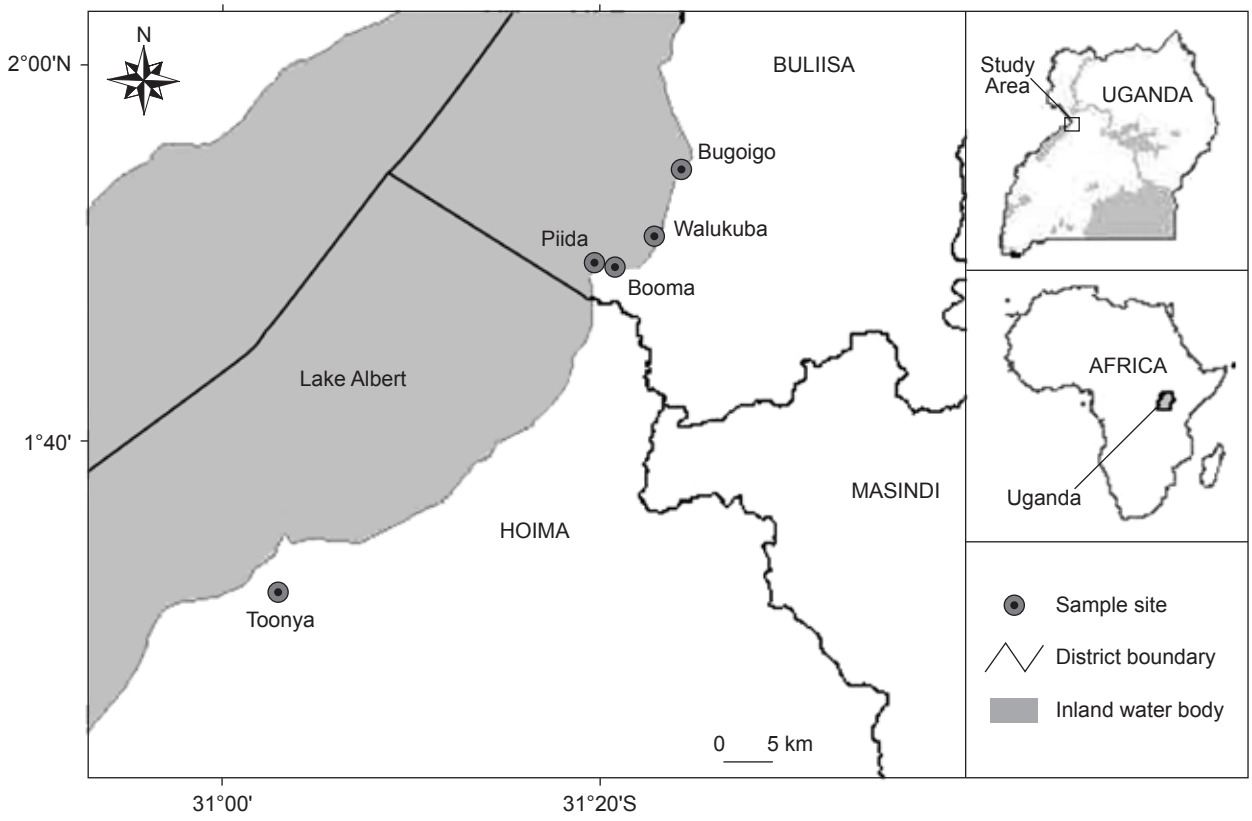


Fig. 1. Geographic distribution of *Bulinus truncatus* and *B. tropicus* collection sites at Lake Albert in western Uganda.

reaction (PCR) using primers LCO1490 (5'-GGTC AACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al. 1994). Amplification was performed with TaqDNA polymerase (Roche) in a 50 μL total reaction volume with standard reaction conditions. The PCR profile consisted of initial denaturation for 5 min at 95°C, followed by 35-40 cycles of denaturation for 2 min at 94°C, annealing for 2 min at 56-58°C, and extension for 2.5 min at 72°C, with a final extension for 5 min at 72°C. PCR products were purified using the Qiaquick PCR Kit (Qiagen) and sequenced in both directions using a Big Dye Terminator vers. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, Lincoln Centre Drive, USA) on an automated DNA sequencer (ABI PRISM® 3700).

Sequences were edited using BioEdit vers. 7.0.5.3 (Hall 1999) and multiple aligned with ClustalW vers. 1.4 (Thompson et al. 1994). All sequences were compared to other *Bulinus* sequences deposited in GenBank to ensure that the mtDNA COI region was correctly amplified.

Genetic variations

Genetic diversity was quantified as the number of distinct haplotypes per population, and number of haplotypes (h) and nucleotide diversity (π) within populations (Nei 1987) computed using the program DnaSP vers. 4.10 (Rozas et al. 2003). Sequence divergences between haplotypes and net sequence divergences between populations (percent sequence divergence between populations corrected for intrapopulation genetic diversity) were also estimated using the program POPSTR vers. 1.2 (H.R. Siegismund unpublished). Exact tests of population differentiation were estimated using the program ARLEQUIN vers. 3.11 (Excoffier et al. 2006).

Phylogenetic analyses

Phylogenetic relationships were estimated using a Bayesian analysis (MrBayes vers. 3.1; Huelsenbeck and Ronquist 2001) in which inferences of phylogeny are based upon posterior probabilities of phylogenetic trees. Posterior probabilities were estimated by sampling trees using a Markov chain Monte Carlo (MCMC) method based on the GTR+G nucleotide substitution model of evolution determined using the program MRMODELTEST 2.2 (Nylander 2004).

Eight different *Bulinus* haplotypes from 4 different species (*B. natalensis*, *B. nyassanus*, *B. tropicus*, and *B. truncatus*) were retrieved from GenBank and included in the phylogenetic analysis in order to examine the topology of the test haplotypes on the tree. The mitochondrial genomic sequence of COI for *B. forskalii* (GenBank accession no.: AM286310) was used as an outgroup. The inferred tree was visualized using

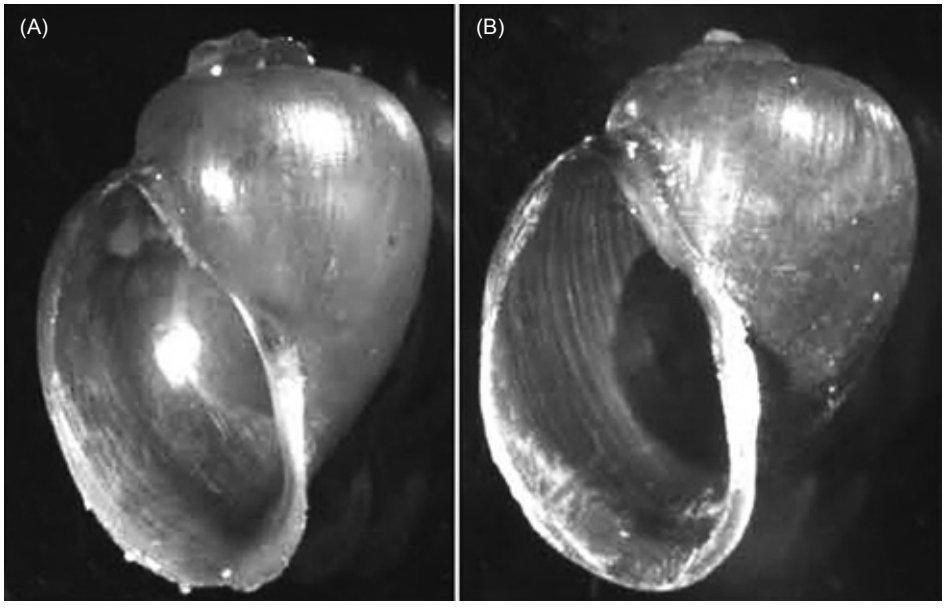


Fig. 2. Morphologically very similar *Bulinus* shells of 2 sibling snails *B. truncatus* (A) and *B. tropicus* (B), sampled from Toonya and Bugoigo, respectively, in Lake Albert.

the program TreeView (Win32) (Page 2001). The program NETWORK vers. 4.1 (Bandelt et al. 1999), was also used to construct a median-joining network of haplotypes showing the number of mutational steps between them.

RESULTS

Genetic variations and haplotype distributions

The resulting alignment of 612 bp comprised 5 unique haplotypes of *Bulinus* in the total sample characterized by 21 polymorphic sites, involving 17 transitions and 4 transversions. Sequence data of these haplotypes were submitted to GenBank with accession numbers GU176747-GU176751. In a comparative analysis of the 5 test haplotypes from Lake Albert (with 8 other *Bulinus* sequences retrieved from GenBank, 41 segregating sites were observed (Table 1). Haplotypes BO and BU were respectively specific to the Booma and Bugoigo sites. The most frequent haplotype was TO which occurred with a frequency of 41% and was shared among Tonya, Piida, and Bugoigo. The 2nd most frequent haplotype, 1PD, was found at Piida and Booma, whereas haplotype 2PD was shared by Piida, Bugoigo, and Walukuba. Haplotype 1PD differed from haplotype 2PD by 18 transitions and 1 transversion at position 446. The non-synonymous change that occurred at site 446 resulted in separation of all the haplotypes into 2 distinct groups, A and B (Table 1).

The nucleotide diversity (π) in the total sample was 1.3% and was generally low within each population (0%-0.1%), whereas haplotype diversity greatly varied from 0 (Toonya) to 0.59 (Piida) as shown in table 2. Sequence divergence between haplotypes varied 0.2%-3.5% (equivalent to 1 and 21 substitutions between haplotypes, respectively) as shown in table 3. Significant genetic differentiation was observed between all populations except Tonya and Piida (Table 4).

Phylogenetic relationships

The Bayesian analysis resolved the haplotypes from the different localities into 2 major clades of A and B (Fig. 3). All haplotypes in clade A were differentiated from those in clade B by a non-synonymous substitution at position 446 that led to a replacement of the amino acid threonine in clade A by the amino acid asparagine in clade B (Table 1). The median-joining haplotype network

Table 1. Sequence variation in the mitochondrial COI gene of *Bulinus truncatus/tropicus* complex in Lake Albert, the shaded area shows site of non-synonymous substitution. **TO**, **BO**, **BU**, **1PD** and **2PD** are the 5 new haplotypes. The rest of the eight haplotypes were retrieved from the GenBank

Haplotype	41 Polymorphic sites																																			Haplotype distribution				
	1	1	1	1	1	2	2	2	2	2	2	2	3	3	3	3	3	4	4	4	4	4	4	4	4	4	5	5	5	5	5	6	6	6	6	TO	PD	BO	BU	WA
	1	3	4	5	6	9	0	3	4	5	6	7	7	0	1	6	6	7	9	2	2	2	3	4	4	4	5	6	6	8	0	2	6	7	8	9	0	0	0	0	1
	2	2	4	3	5	8	4	4	7	5	4	0	9	3	8	3	9	8	6	3	6	9	2	4	6	7	6	2	8	1	5	8	7	9	8	7	0	3	4	9	2
1TRUC	T	T	A	G	T	C	T	A	T	G	T	C	G	T	A	A	T	G	G	C	T	A	G	T	C	T	A	T	T	C	T	A	C	C	T	C	T	T	T	T	G
2TRUC	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
3TRUC	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
4TRUC	.	.	.	.	.	.	.	.	A	.	.	.	C	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.
5TRUC	.	.	.	.	.	.	.	.	.	.	.	.	C	.	G	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.
TO	G	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.
1PD	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	A	C	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.
BO	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.
2PD	.	A	G	A	.	T	G	.	.	.	T	A	C	.	.	C	.	.	.	.	G	T	.	A	.	.	.	.	.	C	.	T	.	.	T	.	C	.	C	A	
BU	.	A	G	A	.	T	G	.	C	.	.	.	.	C	.	.	C	.	.	.	.	G	T	.	A	.	.	.	.	.	C	.	T	.	.	T	.	.	.	C	A	
TROP	.	A	G	A	.	T	A	.	.	.	.	T	A	C	.	T	C	.	.	.	.	G	T	.	A	.	.	.	.	.	C	.	T	T	.	T	.	.	.	C	A	
NAT	.	A	G	A	.	T	.	.	.	.	A	T	A	.	.	.	.	A	T	T	.	G	A	.	.	.	.	A	.	.	C	.	.	T	.	T	.	.	.	C	A	
NYA	.	A	.	A	C	T	A	T	.	.	.	T	A	C	.	.	C	.	.	.	.	G	.	.	.	.	G	.	.	T	C	.	.	T	A	T	A	.	C	C	A	
No. of bases	2	2	2	2	2	2	3	2	2	2	2	2	2	2	2	3	2	2	2	2	2	2	3	2	2	2	2	2	2	2	2	2	2	2	2	2	2	9	12	8	8	2

NYA- *B. nyassanus*, TRUC- *B. truncatus*, TROP- *B. tropicus*, NAT – *B. natalensis*. TO- Haplotypes from Toonya population, BO- Haplotypes from Booma population, BU- Haplotypes from Bugoigo population, PD- Haplotypes from Piida population.

analysis also supported the existence of these 2 major groups of haplotypes A and B separated by 18 mutational steps and a net genetic divergence of 3.0% (Fig. 4). The tetraploid *B. truncatus* (TRUC) retrieved from GenBank clustered with haplotypes BO, 1PD, and TO with a posterior probability of 0.65 while the diploid *B. tropicus*, *B. natalensis*, and *B. nyassanus* clustered together with a posterior probability support of 0.91, with *B. tropicus* forming a sister clade with haplotypes 2PD and BU supported by a posterior probability of 0.93 (Fig. 3). Haplotypes from the Piida (1PD and 2PD) population occurred in both clades. The 2 divergent evolutionary lineages represented by clades A and B therefore occur in sympatry at the Piida locality in Lake Albert.

DISCUSSION

Based on morphological observations of the shell (Fig. 2), the *Bulinus* samples collected from the 5 localities (Walukuba, Bugoigo, Piida, Toonya, and Booma) of Lake Albert were indistinguishable and were identified as belonging to the *B. truncatus/tropicus* complex (see Kristensen 1987). Placement of the samples in the *B. truncatus/tropicus* species complex was based on the presence of a micro-sculpture with transverse ribs. Previous attempts to delineate species within this complex confirmed that species within the *B.truncatus/tropicus* complex cannot easily be differentiated based on shell morphology alone due to high levels of variation in the shell form (Brown 1994).

Table 2. Genetic diversity of mitochondrial COI gene sequences in the *Bulinus* samples

Locality	No.	No. haplotypes	Segregating sites (s)	Nucleotide diversity % (π)	Haplotype diversity (h)
Tonya	9	1	0	0	0
Piida	12	3	20	0.072	0.59091
Booma	8	2	1	0.070	0.42857
Bugoigo	8	2	1	0.093	0.57143
Sum	37	5	21	1.343	0.72673

Table 3. Kimura's distances between the haplotypes are below the diagonal and numbers of substitutions between the haplotypes are above the diagonal

	TO	1PD	2PD	BO	BU
TO		2	20	3	21
1PD	3.3		19	1	20
2PD	3.4	3.2		18	1
BO	0.5	0.2	3.0		19
BU	3.5	3.4	0.2	3.2	

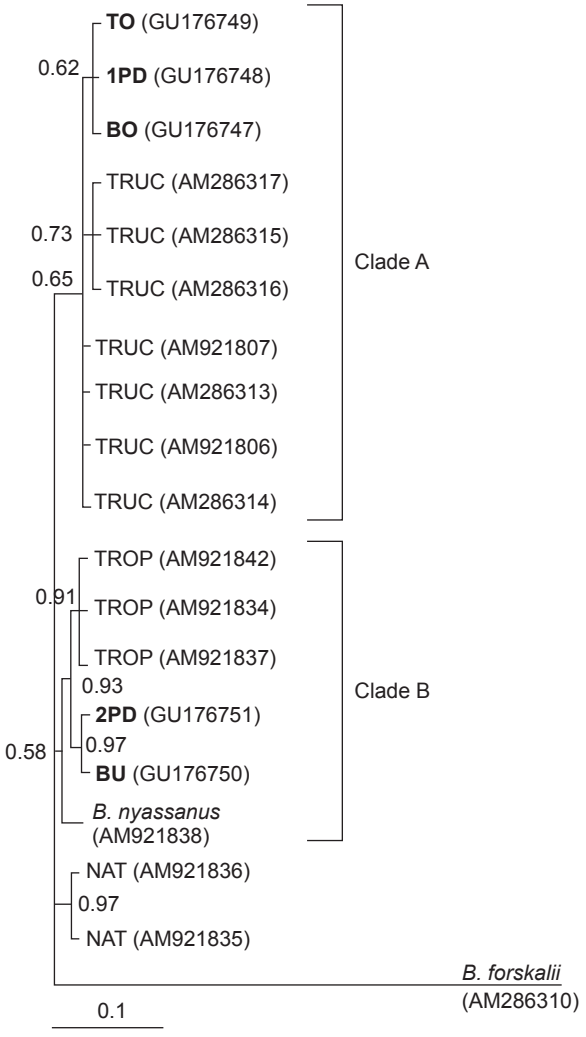
Table 4. F_{ST} (lower diagonal) and net per cent sequence divergence (upper diagonal) between populations based on mtDNA analysis

Population	Toonya (TO)	Piida (PD)	Booma (BO)	Bugoigo (BU)
TO	-	0.044	0.332	3.298
PD	0.082ns	-	0.111	2.635
BO	0.912 ***	0.183 *	-	3.059
BU	0.987 ***	0.854 ***	0.974 ***	-

*$p < 0.05$; ***$p < 0.001$, and ns = non significant

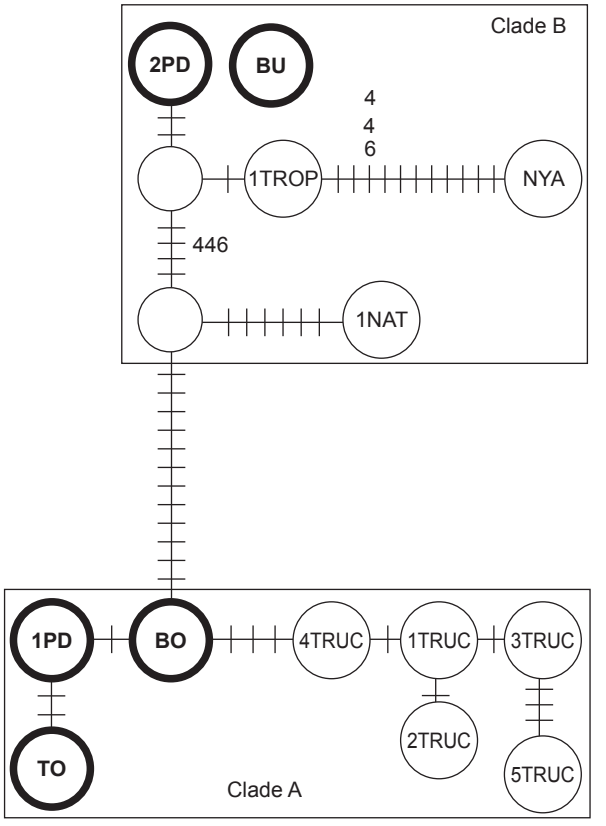
MtDNA sequence variations of the *Bulinus* samples analyzed in this study, however, revealed 2 different taxa occurring in Lake Albert despite their morphological similarities. The 2 taxa were delimited by a non-synonymous substitution at position 446 that resulted into the replacement of the amino acid asparagine in clade A by threonine in clade B (Table 1). The relatively low levels of net percent sequence divergence between populations represented in the same clades (0.044-0.332) and the very high sequence divergences between populations derived from different clades (2.635-3.298) (Table 3) further confirms that the 2 clades correspond to 2 evolutionarily divergent lineages of *B. truncatus* and *B. tropicus*.

The taxonomic assignment of haplotypes



BO - Haplotypes from Booms population
BU - Haplotypes from Bugoigo population
PD - Haplotypes from Piida population
TO - Haplotypes from Toonya population

Fig. 3. Bayesian-based phylogram showing posterior probability values for the different haplotype clades from Lake Albert compared to GenBank sequences *Bulinus natalensis* (NAT), *B. nyassanus*, *B. tropicus* (TROP), and *B. truncatus* (TRUC). The scale shows the expected changes per site. TO- Haplotypes from Toonya population, BO- Haplotypes from Booma population, BU- Haplotypes from Bugoigo population, PD- Haplotypes from Piida population



NYA - *B. Nyassanus*
TRUC - *B. truncatus*
TROP - *B. tropicus*
NAT - *B. natalensis*
446- (non-synonymous polymorphic site)
New haplotyps from Lake Albert

BO - Haplotypes from Booms population
BU - Haplotypes from Bugoigo population
PD - Haplotypes from Piida population
TO - Haplotypes from Toonya population

Fig. 4. Mitochondrial cytochrome oxidase subunit I (COI) gene haplotype network for *Bulinus truncatus/tropicus* samples from Lake Albert. Each connecting number corresponds to a single mutational step. Site 446 is a non-synonymous substitution.

2PD and BU to *B. tropicus* and haplotypes TO, 1PD, and BO to *B. truncatus* was further supported by the relatively high posterior probability values of 0.93 and 0.65, respectively (Fig. 3).

The 3.0% sequence divergence of mtCOI gene between the 2 reciprocally monophyletic clades A and B (*B. tropicus* vs. *B. truncatus*), although low it is close to the species threshold proposed by Johnson et al. (2000). Such mtCOI genetic divergence corresponds to an evolutionary divergence of approximately 1.5×10^6 yr (My), given a divergence rate of 2.0%/My. The fact that *B. truncatus* was suggested to be a hybrid of 2 diploid species, *B. tropicus* (2*n* = 36) being one of the parental species (Goldman et al. 1983), could also partly explain the low sequence divergence observed between the 2 sister clades of sibling species. Recent studies of DNA barcoding showed that sequence variations of > 2.0%-3.0% at the mtCOI locus can be used to delineate different species. For example, a similar species threshold of close to 3.0% sequence divergence was recently used to discriminate species within the Lepidoptera order, one of the most speciose orders of insects (Hebert et al. 2003). Comparable sequence divergences ranging 2.2%-3.3% were reported between putative species within the genera *Alphestes* and *Dermatolepis* (Craig et al. 2004).

The results from this study in conjunction with other molecular studies such as that of Brown and Shaw (1989) involving chromosome counts and that of Rollinson and Kane (1991) involving use of polymerase chain reaction rapid fragment length polymorphism (PCR-RFLP) techniques augment morphological delineation of sibling species within the otherwise morphologically indistinguishable individuals of the *B. truncatus/tropicus* complex. We recommend that future taxonomic studies should involve the complete sequencing of the COI gene whereby more taxon-specific barcodes can be identified to differentiate among the different sibling species within this complex.

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