Identification of Snails within the *Bulinus africanus* Group from East Africa by Multiplex SNaPshot™ Analysis of Single Nucleotide Polymorphisms within the Cytochrome Oxidase Subunit I

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Identification of populations of Bulinus nasutus *and* B. globosus *from East Africa is unreliable using characters of the shell. In this paper, a molecular method of identification is presented for each species based on DNA sequence variation within the mitochondrial cytochrome oxidase subunit I (COI) as detected by a novel multiplexed SNaPshot™ assay. In total, snails from 7 localities from coastal Kenya were typed using this assay and variation within shell morphology was compared to reference material from Zanzibar. Four locations were found to contain* B. nasutus *and 2 locations were found to contain* B. globosus*. A mixed population containing both* B. nasutus *and* B. globosus *was found at Kinango. Morphometric variation between samples was considerable and UPGMA cluster analysis failed to differentiate species. The multiplex SNaPshot™ assay is an important development for more precise methods of identification of* B. africanus *group snails. The assay could be further broadened for identification of other snail intermediate host species.*

Key words: *Bulinus* - cytochrome oxidase - single nucleotide polymorphism - schistosomiasis - *Schistosoma haematobium*

The freshwater pulmonate snail genus *Bulinus* is divided into four species groups: *B. africanus* group, *B. forskalii* group, *B. reticulatus* group and the *B. truncatus/ tropicus* complex (Brown 1994). Despite limited morphological divergence within species groups, there is considerable molecular divergence (Jones et al. 2001, Stothard et al. 2001). Within the *B. africanus* group 10 species are recognised and distributed throughout Afro-tropical regions and Madagascar. Several *B. africanus* group species are known, or suspected, to act as intermediate snail hosts for *Schistosoma haematobium*, a trematode parasite that causes urinary schistosomiasis.

Interactions between *B. africanus* group species and *S. haematobium* can be complex. Not all snail species act as intermediate hosts e.g. *B. ugandae* appears refractory to infection, or only certain snail species act as hosts in specific areas (Rollinson et al. 2001). Lack of clear-cut morphological characters hinders identification of natural populations (Mandahl-Barth 1965). For accurate separation of these snail species it is necessary to use biochemical (Rollinson & Southgate 1979) or molecular DNA methods (Rollinson et al. 2001). There are many single nucleotide polymorphisms (SNPs) within the mitochondrial cytochrome oxidase subunit I (COI) gene which may be exploited for species identification (Fig. 1). Taxon specific polymerase chain reaction (PCR) primers for *B. globosus* and *B. nasutus* have been designed based on genetic variation within the COI (Stothard et al. in press).

Whilst taxon specific primers are highly discriminatory, the PCR assay is limited within the known scope of detected sequence variation; further sequence variation may lead to false negatives. SNaPshot™ is a commercially available product from PE Biosystems, UK for genotyping SNPs using a fluorescent based, primer extension assay (Rollinson et al. 2001). Makridakis and Reichardt (2002) have taken the SNaPshot™ assay a step further by multiplexing SNaPshot™ primers of differing length coining the terminology 'multiplex automated primer extension analysis' (MAPA). Although this multiplex assay requires a semi-automated DNA sequencer, the assay has certain key advantages; many snails can be individually typed simultaneously for several key SNPs, and detection and precise characterisation of further DNA variation is possible.

This paper reports on the development and implementation of a multiplexed SNaPshot™ assay to type simultaneously four SNPs within the COI (Fig. 2) of *Bulinus* species. The assay is then used for identification of *B. africanus* group snails collected from 7 localities within coastal Kenya. The Kenyan shell material is compared to a selection of *B. globosus* and *B. nasutus* shells from Zanzibar to ascertain if there is any morphological divergence.

MATERIALS AND METHODS

Snail material - A total of 147 *B. africanus* group snails from 7 collecting localities from coastal Kenya: Kinango (n = 28), Mbovu (n = 24), Msambweni (n = 23), Mazeras (n = 6), Nguzo (n = 3), Ramisi (n = 25) and Timboni (n = 38), were examined. The snails were kindly provided in 70% ethanol by Dr M Otieno, National Museums of Kenya, Nairobi, Kenya. Genomic DNA was extracted from each snail for PCR analysis (see below) following stan-

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dard methodology (Stothard et al. 1997) and the shell was retained for morphometric analysis. A collection of 79 dry shells as reported by Stothard et al. (1997) of *B. africanus* group snails from Zanzibar [*B. globosus* Unguja (n = 18), Pemba (n = 22) and *B. nasutus* Unguja (n = 18), Pemba (n = 21)] was used as reference material for morphometric comparison with Kenyan material.

PCR and SNaPshot™ typing of COI - A subregion (~ 450 bp) of the COI was amplified from each snail following conditions described by Stothard and Rollinson (1997). The amplification product was then purified using a Qiagen PCR clean-up spin column and adjusted to an approximate concentration of 0.2 pmol/µl.

Four SNaPshot™ primers were designed to type SNPs at positions 49, 109, 166 and 255 (Fig. 1) in the alignment of COI sequences described by Stothard and Rollinson (1997). The primer sequences were as follows:

Position 255 (29mer) 5' – TAAAAAGAAAAATAAAYCC TAAYACTCAA

Position 166 (34mer) 5' – ACACCTTAATTCCTGTTGG TACAGCAATAATTAT

Position 109 (38mer) 5' – GCTCGAGTATCCACATCTATT CCHACAGTAAATATATG

Position 49 (45mer) 5' – TAAACCTAAAATTCCAATT GAAACTATWGCATAAATTATTCCTAA

1µl of purified amplification product was added to a SNaPshot™ reaction mixture containing 0.15 pmols of each primer. The reaction mixture (10 µl) was incubated for 25 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 30 sec in a standard thermal cycler. The SNaPshot™ reaction mixture was purified by removal of excess fluorescent dye terminators by incubation with 1unit of calf intestinal alkaline phosphatase according to manufacturer's instructions.

1.5 µl of purified SNaPshot™ reaction was combined with 1.5 µl deionised formamide/blue dextran and the total volume was loaded onto an ABI 377 machine using a standard 5% long ranger, 6M urea sequencing gel. Electrophoresis was conducted according to standard separation conditions detailed in the PE Biosystems' electrophoresis manual. As the 4 SNaPshot™ primers were of differing length, upon separation by denaturing gel electrophoresis each SNP position could be assigned according to the order in which the now fluorescently labelled primers were separated (Fig. 2). The subsequent gel file was visually inspected and also processed with GeneScan software version 2.1 (PE Biosystems, UK).

DNA sequencing - In addition to SNaPshot™ analysis, the DNA sequence of the COI was determined for a total of 21 snails taken across the 7 sampling localities [Kinango (n = 7), Mbovu (n = 3), Msambweni (n = 3), Mazeras (n = 3), Nguzo (n = 1), Ramisi (n = 2) and Timboni (n = 2)] using direct DNA cycle sequencing of purified amplification product and separated on an ABI377 semi-automated DNA sequencer.

Morphometric analysis - A total of 226 snail shells were examined. Identification of Kenyan *B. globosus* and *B. nasutus* was based upon the results of the SNaPshot™ COI profile. Digital images of the shells (aperture facing) at either x8 or x12 magnification were collected using a Leica MZ6 dissecting microscope with an attached digital camera and DIC-E image capture software (World Precision Instruments, UK). A 10 mm scale bar was included within each shell image for size calibration. Shell microsulpture was also noted according to descriptions given by Kristensen et al. (1987). Image analysis was conducted with SigmaScanPro 4.0 software package (Jandel Scientific, UK).

Nine measurements were taken for each shell: 5 linear, 3 area and 1 angular. These were, *linear* (mm) L: total length of shell, W: width of shell (perpendicular from aperture apex to opposite edge), WSp: basal width of shell

	7	**14**	**16**	**19**	**22**	**28**	**31**	**35**	**37**	**46**
B. nasutus	T	Y	G	A	Y	K	T	C	T	R
B. globosus	R	Y	A	T	T	T	A	M	Y	G
	49^{Y}	**67**	**76**	**91**	**103**	**109 ^{Y}***	**124**	**127**	**136**	**151**
B. nasutus	A	A	A	T	G	C	T	W	A	Y
B. globosus	Y	W	W	W	R	T	A	A	R	T
	154	**166 ^{Y}***	**199**	**226**	**241**	**250**	**255 ^{Y}***	**262**	**268**	**286***
B. nasutus	C	A	G	A	T	A	A	G	R	A
B. globosus	Y	T	R	R	Y	R	T	R	R	T
	292	**304**	**322**	**328**	**336**					
B. nasutus	T	T	Y	A	Y					
B. globosus	K	K	T	R	T					

Y - SNP variant used for SNaPshotTM typing
* - SNP variant used for taxon specific PCR

Fig. 1: a summary of the presently known variation within the 340 bp sequenced COI region between *Bulinus globosus* and *B. nasutus*. For the original DNA alignment see Stothard and Rollinson (1997). The shaded variant positions are SNP variants utilised for SNaPshot™ [positions denoted ^{Y} (49, 109, 166, 255)] and (or) taxon specific PCR assays [positions denoted * (109, 166, 255, 286)].

spire, LAp: length of aperture, WAp: width of aperture; *area* (mm^2), TA: total shell area, Asp: area of spire and AAp: area of aperture; and *angular* (degrees): angle subtended from longitudinal axis of shell rotation to maximum width point on the outer body whorl edge.

Before analysis, area measurements were transformed by taking the square root. All measurements were then standardised using the geometric mean of Log_{10} adjusted ratios following Clarke et al. (1999). Euclidean distances between each individual were calculated using the program SYN-TAX 5.0 (J Podani, Scientia Publishing, Budapest) and a dendrogram was generated using unweighted pair-group arithmetic average (UPGMA) analysis to evaluate phenetic groups.

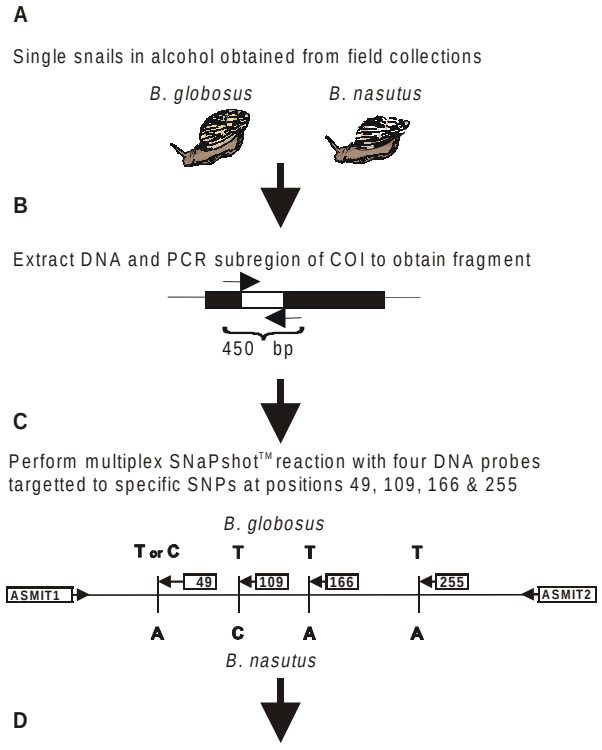

Fig. 2: design of the multiplex SNaPshot™ assay for identification of *Bulinus globosus* and *B. nasutus*. In brief, after the gene target is amplified by PCR, a short oligonucleotide primer, or probe, is used to abut next to the variant position to be typed. The SNaPshot™ primer can be of the same sequence as a taxon specific primer except that the 3' terminal nucleotide is omitted. It is this nucleotide only that is then added to the SNaPshot™ primer upon the primer extension reaction. A: snails are collected from the field; B: a subregion of the COI is amplified using standard PCR conditions; C: four SNPs are targeted within the COI which differentiate between species; D: after the SNaPshot™ reaction the 4 SNaPshot™ primers, now one nucleotide longer and flourescently labelled, are separated by electrophoresis to reveal a characteristic coloured barcode pattern typical for each species.

RESULTS

Identification and COI variation - SNaPshot™ COI reactions from all snails could be readily assigned to the expected profile for either species (Fig. 3), and no further variation within the 4 variant positions was detected. From the SNaPshot™ COI profile *B. globosus* was encountered at Timboni and Mazeras while *B. nasutus* was encountered at Mbouv, Msambweni, Nguzo and Ramisi. A mixed population of *B. globosus* and *B. nasutus* was found at Kinango. Of the 21 COI sequences obtained and in comparison to previous COI data from *B. africanus* group snails, 8 novel COI sequences were encountered. Three *B. globosus* and 5 *B. nasutus* sequences have been deposited in GenBank, accession numbers AF507035-AF507042. The majority of *B. globosus* from Kenya did not have a *SspI* restriction enzyme cutting site within the COI.

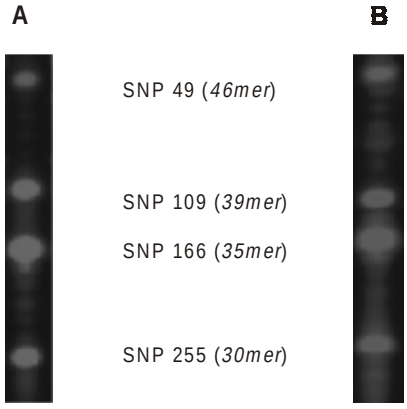

Fig. 3: SNaPshot™ gel profile of the Kenyan snails corresponded to the expected profiles for either *Bulinus globosus* or *B. nasutus*, no additional variation was encountered. A: profile typical of *B. globosus*. Note that the C/T polymorphism at position 49 within *B. globosus* from Zanzibar is absent within the Kenyan material. All snails possessed a C at this position; B: profile typical of *B. nasutus*.

Morphometric variation - The Table details the mean value with 1 standard deviation for shell measurements. A significant difference ($p < 0.05$) was detected between *B. globosus* and *B. nasutus* with an unpaired student t test for ASp and AAp. Generally, *B. globosus* had larger aperture and smaller spire areas than *B. nasutus*. Plotting a combination of shell variables in bivariate plots against shell length did not reveal the existence of two discrete distributions of points (Fig. 4).

UPGMA analysis of morphometric variation within Kenyan and Zanzibarian snails resulted in a dendrogram containing 8 major clusters, designated A to H (Fig. 5). The largest clusters, B and H, contained 64 and 65 snails respectively. No single cluster contained exclusively *B. globosus* or *B. nasutus*. Both species were distributed

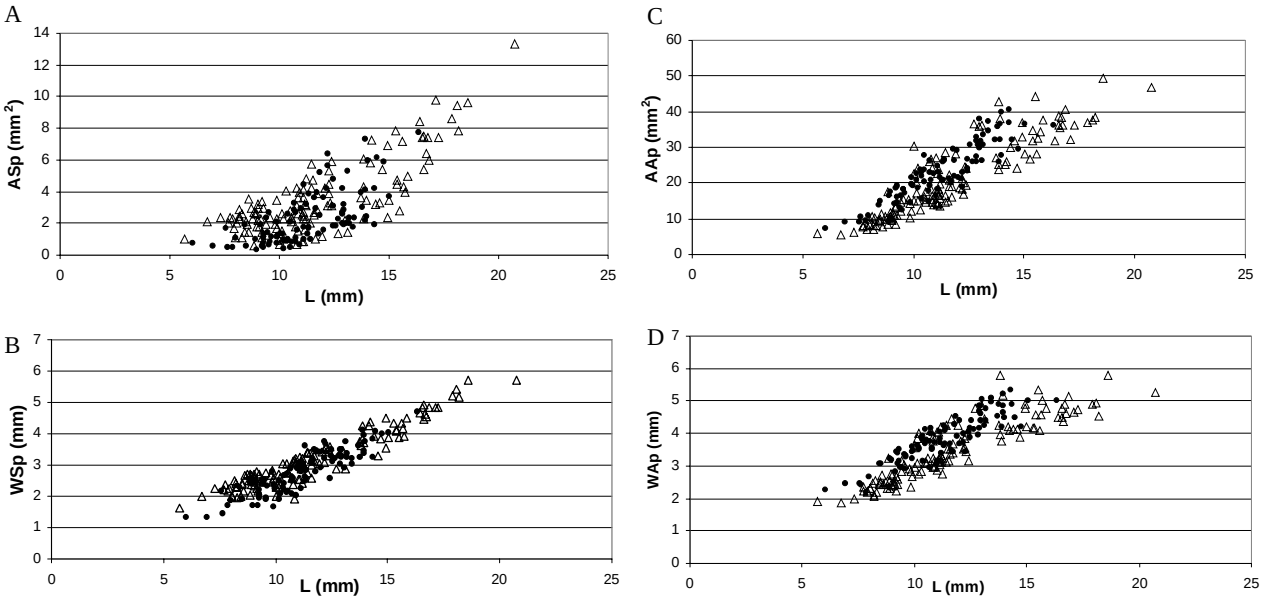

Fig. 4: bivariate plots of shell variation of Kenyan and Zanzibarian snails reveals no discernible groups between *Bulinus globosus* (•) and *B. nasutus* (△). A: area of spire against shell length; B: width of spire against shell length; C: area of aperture against shell length; D: width of aperture against shell length.

TABLE

Shell measurements 5 linear (mm), 3 area (mm^2) and 1 angular (^{o}) with means and 1 standard deviation

	Coastal Kenya		Zanzibar	
	Bulinus globosus	*B. nasutus*	*B. globosus*	*B. nasutus*
L	10.8 ± 1.9	11.7 ± 3.0	11.9 ± 1.9	11.5 ± 2.9
W	5.4 ± 1.1	5.7 ± 1.5	6.0 ± 1.1	5.5 ± 1.3
WSp	2.5 ± 0.6	3.1 ± 0.9	3.2 ± 0.6	3.2 ± 0.9
LAp	8.5 ± 1.4	8.6 ± 2.1	8.4 ± 1.3	8.1 ± 1.9
WAp	3.9 ± 0.8	3.6 ± 1.0	3.8 ± 0.7	3.3 ± 0.8
TA	60.0 ± 20.6	64.8 ± 32.5	65.1 ± 21.3	57.5 ± 29.8
AAp	23.7 ± 8.0	21.9 ± 11.0	22.9 ± 7.4	17.6 ± 8.9
ASp	1.5 ± 0.9	3.2 ± 2.1	3.6 ± 1.7	4.1 ± 2.4
	28.5 ± 2.8	28.8 ± 2.7	29.9 ± 2.4	31.2 ± 3.2

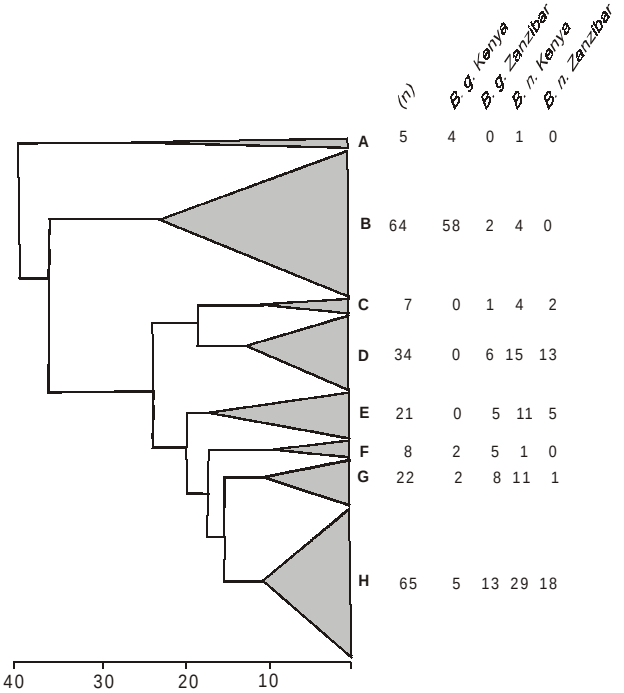

Fig. 5: cluster analysis of shell variation revealed 8 major groups labelled A to H. Within each group there were *Bulinus globosus* and *B. nasutus* intermingled as denoted by the adjacent table for each cluster. Whilst group B contained predominately *B. globosus* there was no discrete separation evidenced between species.

across clusters although the cluster B was predominately composed of Kenyan *B. globosus*. Following from the identification equations proposed by Kristensen et al. (1987) using presence of microsculpture and aperture banding, a specificity of 53% for identification of *B. globosus* was found. Approximately 1 in 2 *B. globosus* snails would have been identified correctly within this sample.

DISCUSSION

Multiplex SNaPshot™ assay - The multiplex SNaPshot™ assay is a robust methodology for typing SNPs within the COI from individual snails. Analysis and interpretation of the resultant SNaPshot™ profile is less labour intensive than inspection of a corresponding DNA sequencing chromatogram as only 4 fragments are produced (Fig. 2). Each characteristic colour bar-code is immediately apparent from the corresponding gel file picture (Fig. 3). As such identification of many snails can be quickly accomplished and as this technology requires minimal liquid handling steps, snails can be processed in microtitre plate format. This multiplex assay has great potential for regular, high-through-put DNA typing in a convenient single tube reaction. Multiplex SNaPshot™

assays may also simultaneously screen for variation within other candidate genes e.g. ribosomal 18S and Internal Transcribed Spacer, and once sufficient DNA sequence data has been accrued for representatives of *Bulinus*, the assay could no doubt be extended. The methodology might also be of use for identification of problematic populations of *Lymnaea* (Bargues et al. 1997) or *Biomphalaria* (Morgan et al. 2001) or even in the detection of *Schistosoma* sequences from infected snails.

Snail populations - Accurate identification of *B. globosus* and *B. nasutus* populations is important. Recent work on Zanzibar has shown that *B. nasutus* plays no role in transmission of local *S. haematobium* and the distribution of urinary schistosomiasis in schoolchildren is clearly linked with the distribution of *B. globosus* (Stothard et al. in press). A similar transmission picture might also exist in Kenyan and Tanzanian coastal regions nearby.

Both *B. globosus* and *B. nasutus* have been encountered within the Kenyan material. While 6 of the 7 samples contained a single species, the sample from Kinango contained a mixed population of both species. This situation contrasts somewhat with Zanzibar where mixed populations have yet to be encountered (Stothard et al. 1997). In Zanzibar, *B. globosus* appears to be associated with harder water while *B. nasutus* is associated with softer water but the range of water hardnesses between species does overlap (Stothard et al. in press); potentially, mixed populations could occur in nature. As water conductivity values were not recorded at the time of collection of this Kenyan material, it might be expected that water conductivity at Kinango could be within this overlapping range.

The DNA divergence within the COI between Kenyan and Zanzibarian species is confined to several further point mutations. It is worthy of note that the PCR-RFLP methodology designed by Stothard and Rollinson (1997) to differentiate *B. globosus* and *B. nasutus* in Zanzibar on the presence and absence of a *Ssp*I cutting site respectively (recognition sequence AATATT) does not work consistently on Kenyan *B. globosus*. Coastal Kenyan *B. globosus* has two predominant sequence types present within this selected 6 bp region: AATATT (*Ssp*I site) and GATATT (non-*Ssp*I site), while all *B. nasutus* have AATATA. Whilst this assay can positively identify certain *B. globosus* by the presence of an *Ssp*I site, in this case the snails from Kinango, the absence of a *Ssp*I restriction site cannot now be interpreted as exclusive to *B. nasutus*. Confident typing of the 3' T in *B. globosus* and 3' A in *B. nasutus* which differentiates the species, is best performed by SNaPshot™ or taxon specific PCR primers assays.

Shell morphology - It is known that the shell of *B. africanus* group snails from East Africa can be highly variable (Brown 1994). The problem was identified by Mandahl-Barth (1965) as long-spired and short-spired forms of either species 'overlapped' making separation of populations and individuals of either species problematic. Morphometric analysis presented here has confirmed that it is difficult to separate either species on shell characters alone. The bivariate plots are particularly illustrative of the overlapping nature of variation. Whilst the mean aperture area (AAp) of each species is significantly different, for example aperture area is greater in *B. globosus* than *B. nasutus* for a typical shell, this measurement is not capable of precisely differentiating species (Fig. 4).

Variation within other shell variables has been inspected with the hope of finding clear-cut divisions between species. Kristensen et al. (1987) developed a simple, powerful morphometric test which could differentiate *B. africanus* group species from the Lake Victoria region of Kenya. Using these equations, however, for the Kenyan and Zanzibarian material there is incongruence between molecular and morphological methods of identification. This has also been recently reported by Raahauge and Kristensen (2000) upon analysis of *B. africanus* group snails from Kisumu region Kenya. The presence and absence of shell microsculpture has been used to differentiate *B. nasutus* and *B. globosus* respectively from Lake Victoria but upon inspection of further snails from different areas it seems that this character becomes less species specific. In Zanzibar shell microsculpture does not help to differentiate the species. Certain populations confidently typed by both genetic and biochemical methods as *B. globosus* do have microsulpture, as well as, aperture bands whereas others do not. Inspection of the clusters generated by UPGMA analysis of shell measurements (Fig. 5) adds further weight to the suggestion made by Stothard et al. (1997) that there may be few, if any, conchological measurement useful for reliable identification.

Conclusion - Correct identification of populations of *B. africanus* group snails requires the use of biochemical or molecular DNA methods. The multiplex SNaPshot™ assay is a promising new tool for species identification. New data are required concerning the distribution and transmission status of potential intermediate hosts in East Africa in order to gain a fuller understanding of the distribution of urinary schistosomiasis.

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