Molecular evolution of freshwater snail intermediate hosts within the *Bulinus forskalii* group

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SUMMARY

Freshwater snails of the *Bulinus forskalii* group are one of four *Bulinus* species complexes responsible for the transmission of schistosomes in Africa and adjacent regions. The species status of these conchologically variable and widely distributed planorbids remains unclear, and parasite compatibility varies considerably amongst the eleven taxa defined, making unambiguous identification and differentiation important prerequisites for determining their distributions and evolutionary relationships. Random Amplified Polymorphic DNA (RAPD) analyses were used to investigate relationships between taxa, with particular emphasis on Central and West African representatives. RAPD-derived phylogenies were compared with those from other independent molecular markers, including partial sequences of mitochondrial cytochrome oxidase subunit I (COI) gene, and the nuclear ribosomal RNA internal transcribed spacer 1 region (ITS1). The phylogenetic reconstructions from the three approaches were essentially congruent, in that all methods of analysis gave unstable tree topologies or largely unresolved branches. There were large sequence divergence estimates between species, with few characters useful for determining relationships between species and limited within species differentiation. Nuclear and mtDNA sequence data from Central and East African representatives of the pan-African *B. forskalii* showed little evidence of geographical structuring. Despite the unresolved structure within the phylogenies, specimens from the same species clustered together indicating that all methods were capable of differentiating taxa but could not establish the inter-specific relationships with confidence. The limited genetic variation displayed by *B. forskalii*, and the evolution and speciose nature of the group, are discussed in the context of the increasingly arid climate of the late Miocene and early Pliocene of Africa.

Key words: *Bulinus forskalii*, mitochondrial DNA, nuclear ribosomal DNA, Randomly Amplified Polymorphic DNA, phylogeny, evolution.

INTRODUCTION

African freshwater snails of the *Bulinus forskalii* group serve as intermediate hosts of *Schistosoma* species, blood flukes responsible for human and animal schistosomiasis. Taxa of this group are amongst the most conchologically variable and widely distributed planorbids, with parasite compatibility varying considerably between taxa (Brown, 1994). It is essential to identify species reliably for a better understanding of the role of individual taxa in disease transmission. Defining the boundaries of species variation and reconstructing the phylogeny of freshwater snails of the *B. forskalii* group has been problematic as there is often ecophenotypic conchological variation between sites (Mandahl-Barth, 1957). Such localized variation is often exaggerated by the high incidence of inbreeding and self-fertilization, promoted by restricted dispersal between small, isolated water bodies. Although poorly represented in the fossil record (Van Damme, 1984) to date, eleven *B. forskalii* group species have been defined, with varying precision, nine according to morphological criteria (Brown, 1994).

Although the group is widely distributed throughout Africa only *B. forskalii* (Ehrenberg, 1831) has a truely pan-African distribution and is a host for *S. intercalatum*, the causative agent of one form of intestinal schistosomiasis; its range overlaps several other *B. forskalii* group taxa, which have comparatively restricted distributions. For instance, *B. senegalensis* Muller, 1781, a host for *S. haematobium* which causes urinary schistosomiasis, is found mainly in the sub-Saharan belt throughout Senegal, Gambia, Mauritania, Chad, Nigeria, Cameroon and Niger (Mimpfoundi & Slootweg, 1991). Where the distributions of *B. forskalii* and *B. senegalensis* are sympatric, unambiguous species identification from shells alone can be difficult (Goll, 1981; Betterton, Fryer & Wright, 1983), as is typical of some other *B. forskalii* taxa comparisons. Conversely, *B. camerunensis*, confined to two crater lake localities, Barombi Kotto (the type locality) and Lake Debundsha, in South West Cameroon, although conchologically

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distinct, is biochemically and genetically indistinct from *B. forskalii* (Mimpfoundi & Greer, 1989; Jones *et al.* 1997, 1999). A major problem limiting our understanding of the evolutionary ecology and host-parasite relationships within the *B. forskalii* group is that most surveys have necessarily been localized in their extent (Greer *et al.* 1990; Mimpfoundi & Greer, 1989, 1990), and although useful, for broader evolutionary questions and epidemiological surveys further data are required in the context of the group's pan-African distribution. The present study evaluates the application of RAPD analyses to generate polymorphic markers for both species discrimination and phylogenetic analysis of taxa within *B. forskalii* group from West and Central Africa.

To avoid major errors, it has been suggested that the application of RAPD analyses for phylogenetics be restricted to closely related taxa (Bowditch *et al.* 1993; Stothard & Rollinson, 1996), or even sibling species (Van de Zande & Bijlsma, 1995). Although *B. forskalii* group taxa share morphological characters, at present there are few pointers to their genetic relationships. With such limited information it is impossible to determine if these species are in fact closely or distantly related. To strengthen the analysis several RAPD fragments apparently shared between taxa were investigated by Southern blotting and hybridization. Phylogenies derived from RAPD analyses were compared with other independent molecular markers; partial sequences of mitochondrial DNA (mtDNA) cytochrome oxidase I (COI) gene and the nuclear ribosomal RNA internal transcribed spacer 1 region (ITS1).

MATERIALS AND METHODS

Samples

Study material (Table 1) comprised 6 species of the *B. forskalii* group taxa including *B. forskalii* (Ehrenberg 1831), *B. scalaris* (Dunker, 1845), *B. senegalensis* Muller, 1781, *B. camerunensis* Mandahl-Barth, 1957, *B. crystallinus* (Morelet, 1868), and *B. cernicus* (Morelet, 1867). Material was field-collected from Cameroon and Kenya or obtained from the Natural History Museum collections held in the Biomedical Parasitology Division. Field-collected specimens were picked from emergent aquatic vegetation using long-handled nets, relaxed for approximately 30 min by the addition of a drop of menthol-saturated ethanol to the collecting vial, and placed in 100% ethanol for long-term storage. Parasite-free first generation laboratory stocks of two *B. forskalii* populations were included for comparison with field collections for RAPD data sets (asterisked in Table 1).

B. forskalii group taxa were identified on the basis of shell characters (Brown, 1994) and distal genitalia morphology (Mandahl-Barth, 1957). *B. senegalensis* can usually be differentiated conchologically from extreme forms of *B. forskalii* by the complete absence of a carina, allowing initial identification based on the shell alone. Specimens with intermediate shell morphologies were identified by comparison of their RAPD profiles with those of specimens which had unequivocal shell morphology and confirmed using RAPD-derived species-specific primers (Jones *et al.* 1997).

DNA extraction and RAPD analyses

Tissue preserved in 95% ethanol was first vacuum dried and total genomic DNA extracted according to Vernon, Jones & Noble (1995). General RAPD and electrophoresis conditions are detailed in Jones, Okamura & Noble (1994). Positive controls used DNA samples with known banding patterns and negative controls omitted template DNA. Forty primers from kits Y, R & F from Operon Technologies, Du Pont, USA and three decamer primers from Okamura, Jones & Noble (1993) were initially screened for RAPD analysis of *Bulinus* snails. Twenty primers were chosen to characterize, in detail, 27 *B. forskalii* populations and 2 other *Bulinus* species. Primers chosen include three arbitrary decamers (Primer 02, 10 and 12) from Okamura *et al.* (1993), and the remaining seventeen were Operon primers (OPY14–18, OPR8–13 and OPF4–9). Several repeat amplifications were always performed for each primer to ensure profile reproducibility.

Negatives of the gel photos were examined on a light box and each RAPD fragment between 350 and 1400 base pairs (bp) was scored for presence (1) or absence (0) to produce a binary matrix for all taxa and primers examined. To be scored as present the fragments had to be strongly fluorescent and reproducible; all samples were coded and gels scored blind with no knowledge of the identity of each sample in each lane to avoid any scoring bias.

RAPDs are considered unsuitable for phylogeny inference using parsimony methods as the shared absence of a character in this instance cannot be considered informative (Backeljau *et al.* 1995). Hence a purely phenetic approach was adopted. Pairwise similarities (S) were calculated between taxa using Dice's coefficient (Jackson, Somers & Harvey, 1989). This similarity index was chosen because it is based upon sharing of present bands alone which is more robust than those which weight shared presence alleles equally with shared nulls (Rossetto, Weaver & Dixon, 1995). The similarity coefficients (S) were converted to distance (distance, $P = 1 - S$; percent sequence divergence is $P \times 100$; Swofford & Olsen, 1990).

Shared sequence identity and hybridization

Shared sequence identity between common bands between species was investigated by Southern analy-

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Table 1. Snails used in this study and the collection localities

Species	Population no. and locality	Latitude, longitude or NHM/Aberdeen† accession no.	Markers		
			RAPD	mtDNA	ITS1
Bulinus forskalii species					
B. senegalensis (type locality)	1. Podor, Senegal	1745	√	√	—
B. senegalensis	2. Diator, Senegal	1746	√	—	—
B. senegalensis	3. Nianga, near Podor, Senegal	1749	√	—	—
B. senegalensis	4. Lambata, near Mora, extreme north Cameroon	N11.02.017 E14.09.533	√	—	—
B. senegalensis	5. 3 km from Mora, extreme north Cameroon	N11.03.767 E14.09.319	√	√	√
B. senegalensis	6. Guidiguis, past Lara, extreme north Cameroon	N10.08.333 E14.41.622	√	—	—
B. senegalensis	7. Boro, Mali	1901	√	√	—
B. forskalii	8. Mbakhana, Senegal	1750	√	—	—
B. forskalii	9. Mbeyssus, Senegal	1751	√	—	—
B. forskalii	10. Lampsar, Senegal	1754	√	—	—
B. forskalii	11. Maklingay, Far North Cameroon	N10.52.059 E14.14.179	√	√	√
B. forskalii	12. Udkia, Mokolo, Far North Cameroon	N10.42.216 E13.50.285	√	—	—
B. forskalii	13. Yagoua, Maya Daray river, Far North Cameroon	CY9/93†	√	—	—
B. forskalii	14. Sangmelina, South Cameroon	N02.56.515 E11.58.953	√	√	—
B. forskalii	15. River Mabanga, Kumba, SW Cameroon	N04.38.161 E09.28.349	√	√	√
B. forskalii	16. Mabanga water, Kumba, SW Cameroon	N04.37.997 E09.25.608	√	—	—
B. forskalii	17. Loum, Southwest Cameroon	N04.42.411 E09.44.517	√	√	—
B. forskalii	18. Ebebda village, Sanaga River, S Cameroon	N04.21.845 E11.16.130	√	√	—
*B. forskalii**	19. Ebebda village, Sanaga River, S Cameroon, F1	1770	√	√	—
B. forskalii	20. Bafia, Ritob stream, South Cameroon	N04.45.001 E11.13.676	√	√	—
*B. forskalii**	21. Bafia, Ritob stream, South Cameroon, F1	1769	√	√	—
B. forskalii	22. Nigeria	1019	√	—	—
B. forskalii	23. Sao Tome	1625	√	√	√
B. camerunensis (type locality)	24. Barombi Kotto, Southwest Cameroon	N04.27.949 E09.15.435	√	√	√
B. forskalii mixed	25. Toukou, Far North Cameroon	N10.24.695 E15.14.836	√	—	—
B. senegalensis/*B. crystallinus*	26. Angola	247	√	√	—
B. cernicus	27. Mauritius	1687	√	√	√
B. scalaris	28. Kwanziuv stream, Nairobi, Kenya	KN2/95†	—	√	√
B. scalaris	29. Murrum, Kamagaga area, Kisumu, Kenya	S00.05.781 E34.57.832	—	√	√
B. scalaris/*B. forskalii* mixed	30. Murrum, Kamagaga area, Kisumu, Kenya	S00.05.008 E34.57.846	—	√	√
B. forskalii	31. Masongaleni, Kibwenzi, Nairobi, Kenya,	1900	—	√	√
Other *Bulinus* species					
B. truncatus (Outgroup)	32. Barombi Kotto, Southwest Cameroon	N04.27.949 E09.15.435	√	√	√
B. truncatus (Outgroup)	33. Senegal	2060	—	—	√
B. wrighti	34. Oman	1698	√	—	—

sis of RAPD gels using DNA isolated from these bands as probes. Amplified DNA fragments were separated by agarose gel electrophoresis and alkali blotted onto Hybond N+ nylon membrane (Amersham International, UK). Fragments to be used as probes were excised from the gel prior to Southern blotting and purified directly using Gene clean (Bio 101, Inc.). The purified DNA fragments were kept at −70 °C until required. These fragments were radiolabelled by either nick translation (fragments less than 400 bp; Rigby *et al*. 1977) or random priming (fragments greater than 400 bp; Feinberg & Vogelstein, 1983), with [α-^{32}P]dCTP (3000 Ci mmol^{-1}, Amersham International). Standard methods for Southern blots followed Sambrook, Fritsch, & Maniatis (1989). Blots were prehybridized in 1 % (w/v) BSA, 1 mM EDTA, 0·5 M Na_2HPO_4 pH 7·2, 7 % (w/v) SDS, according to Church & Gilbert (1984), at 65 °C for 4–6 h and hybridized overnight at 65 °C, by the addition of the labelled probe. After hybridization, blots were washed to a high stringency by briefly rinsing in 400 mM Na_2HPO_4 pH 7·2, at room temperature, and incubated twice in each of the following solutions of 400 mM, 100 mM, 40 mM, 10 mM and 1 mM Na_2HPO_4 pH 7·2, each with 0·1 % SDS, for 15 min at 65 °C. Autoradiography was carried out for using X-ray film (Hyperfilm, Amersham) for 24–72 h with no screens.

PCR amplification and sequencing

Primer sequences used to amplify a 450 bp section of the mtDNA COI gene were: forward 5′ TTTTTTGGGCATCCTGAGGTTTAT 3′ and reverse 5′ TAAAGAAAGAACATAATGAAAATG 3′, following Bowles, Blair & McManus (1992). A 569 bp fragment of the ITS1 was amplified using the primers ETTS2 (Kane & Rollinson, 1994) and ETTS16 (Stothard, Hughes & Rollinson, 1996). PCR cycling conditions for mtDNA COI and ITS1 followed Stothard & Rollinson (1997) and Stothard *et al*. (1996), respectively. PCR products were purified using Qiaquick PCR™ purification columns (Qiagen Inc.) and sequenced on both strands using the SequiTherm EXCEL™ II DNA sequencing kit-LC (Cambio, UK) and run on a Li-COR Long-ReadIR automatic sequencer (MWG Biotech, UK). Sequences were edited using *e-seq*™ (version 1.0), 5′ and 3′ ends aligned using Align IR (version 1.2) and multiple alignments performed in Clustal X (Thompson *et al*. 1997).

Phylogenetic analyses

Phylogenetic reconstruction was estimated using Fitch–Margoliash criteria that utilize a least squares optimization method (Fitch & Margoliash, 1967) for all data sets. Fitch-generated distance trees were achieved using PHYLIP 3.5 (Felsenstein, 1993), with options set to prohibit negative branch lengths, jumble input order and for global tree rearrangements. As the rate of transitional (Ts) nucleotide substitution is often higher than transversional (Tv) substitution, especially for mtDNA, the Kimura 2-Parameter (K2P) distance model was applied to the sequencing data sets; this model incorporates transitional bias and computes the numbers of transitional and transversional nucleotide substitutions per site and their variances.

Maximum likelihood (ML) methods (quartet puzzling; Strimmer & Von Haeseler, 1996), and maximum parsimony (Swofford, 1996) were additionally employed for the sequence data sets. All characters (nucleotide sites) were weighted equally. The heuristic search option of PAUP 4.0 v 3.c (Phylogenetic Analyses Using Parsimony; Swofford, 1996) was used with gaps (indels) scored as missing data and coded separately, with each gap representing one character with the states either present (1) or absent (0). Gaps which spanned more than one nucleotide position were scored as a single character only. Nodal branch support for distance and parsimony analyses was assessed using 1000 bootstrap replications (Felsenstein, 1985) for the sequencing data sets only. Computation of maximum likelihood trees using PUZZLE 3.1 was achieved without incorporating a molecular clock, but with substitution rates using the HYK model (which allows transitions and transversions to occur at different rates and allows base frequencies to vary as well) following Hasegawa, Kishino & Yano (1985), and the model of rate heterogeneity set at a uniform rate. Alternative models of substitution (e.g. Tamura-Nei) and different rates of heterogeneity (e.g. gamma distribution) were implemented, but had no impact upon the main branch topology of the tree or support values. The adequacy of the phylogenetic information content of the sequence data sets were assessed by the proportion of unresolved maximum likelihood quartets reported by PUZZLE 3.1. The program MEGA (Kumar, Tamura & Nei, 1993) was used to calculate nucleotide statistics such as nucleotide composition and number of Ts and Tvs.

Where possible representatives of the same in-group taxa were used for all analyses; samples of *B. scalaris* were not available when the RAPD analyses commenced, and despite several attempts, *B. crystallinus* samples failed to give good quality sequence data with the ITS1 primers. Outgroup taxa from Gastropoda, Pulmonata for the sequencing data sets included *Albinaria caerulea* and *Bulinus truncatus* (from the *B. truncatus/tropicus* complex). Further outgroups tested due to the availability of the additional sequences included *Cepaea nemoralis* for the mtDNA COI and *Biomphalaria pfeifferi* for the ITS1, data sets respectively. *B. truncatus* and *B.*

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wrighti were included as outgroups for the RAPD analyses.

RESULTS

Shared RAPD sequence identity and scoring

To test the assumption that co-migrating RAPD bands from different taxa can be scored as shared characters for phylogenetic analyses, selected fragments were examined for shared sequence identity. A sample of between 2 and 4 different fragments from each of 3 selected primers were investigated by Southern analysis of RAPD gels, using isolated 'shared' fragments as radiolabelled probes. Bands with high sequence homology to the probes were identified by washing the blots at high stringency. In nine out of ten probings homology of the fragments between taxa was as predicted; fragments shared between taxa and of equal fluorescence were shown to be homologous (Table 2). For example, probe 2/4 (1200 bp product from primer 2) showed similar sized fragments of equal fluorescence from *B. forskalii* and *B. camerunensis* to be homologous. Weakly amplifying fragments, with respect to the rest of the profile, of approximately the same size were not homologous. For example, a 330 bp fragment from *B. senegalensis* (Senegal) was highly fluorescent, whereas a similarly sized fragment was less fluorescent in *B. forskalii* and *B. camerunensis*. When this fragment was excised and used as a probe (2/2), it was not homologous to *B. forskalii* or *B. camerunensis* but was specific to *B. senegalensis* from both the type locality (Senegal), North Cameroon and Mali. In all cases, with one exception, hybridization signal of the probe and the fluorescent intensity of the original band on ethidium stained agarose gels corresponded. A single case of homoplasy was detected between fragments of the same size and intensity. Homology was detected as predicted for fragment 12/1 between *B. forskalii*, *B. senegalensis* and *B. camerunensis* but not in *B. truncatus*; this may be explained by their relative positions on the gel as the first three species were run in adjacent lanes and the latter was on the gel edge.

140 RAPD bands in total were included in the RAPD data set scored using the criteria of fragments of the same size and equal fluorescent intensity assumed to be homologous, from 20 primers, with an average of 7 bands each, from 27 *B. forskalii* group populations and two non-*B. forskalii* species as outgroups, *B. truncatus* and *B. wrighti*, respectively. Each primer generated characteristic fragment profiles for each species, comprising mainly of common species-diagnostic monomorphic bands, with few intraspecific polymorphic bands and with few similarities between taxa (Fig. 1). Mean percent sequence divergence estimates within and between each of 5 *B. forskalii* group taxa and 2 outgroup taxa reflect these observations from RAPD profiles, ranging from 0·0 to 15·8 % for within species, 9·9–88·4 % for between ingroup taxa and 71·8–90·3 % between ingroup and outgroup taxa comparisons (Table 3).

RAPD phylogeny

Analysis of the pairwise genetic distance matrix using the Fitch–Margoliash least squares criterion is shown in Fig. 2. This tree was rooted with *B. wrighti* and shows two main branches, with *B. senegalensis* branching earlier than *B. forskalii-cernicus-crystallinus* clade. Unexpectedly, *B. truncatus*, a non-*B. forskalii* group species falls within the latter ingroup, the *B. forskalii* clade. Within the main *B. forskalii* clade, *B. cernicus* branches before *B. crystallinus*, followed by the remaining *B. forskalii* specimens. Additionally, although identified as a separate species on morphology, *B. camerunensis* clearly clusters with specimens identified as true *B. forskalii* and is most closely related to the nearest *B. forskalii* population (locality 15, 16 from Table 1). Conversely, the molecular data clusters what has been described tentatively as an extreme geographical variant of *B. forskalii* from Sâo Tomé on shell morphology, with *B. crystallinus* from Angola. Within the *B. forskalii* branch no clear geographical structuring of samples was evident, with samples from Senegal falling amongst Cameroonian specimens. While geographically separate localities of *B. senegalensis* could be differentiated, clustering on separate lineages on the main *B. senegalensis* branch.

However, the tree topology is unstable with some significant changes occurring, for instance when the tree is rooted with *B. truncatus* (data not shown), causing the affinities of *B. wrighti*, *B. cernicus* and *B. crystallinus* to swap to the *B. senegalensis* clade.

Molecular sequence phylogenies

Phylogenetic reconstruction used 21 nucleotide sequences obtained from 6 species of *B. forskalii* group taxa, *B. truncatus* and 2 outgroups, for 350 aligned bases of the mtDNA COI gene. Comparison of *Bulinus* COI sequences showed that they were A : T rich (68·9 %) (nucleotide compositions: A = 27·8 %, C = 12·3 %, G = 18·8 %, T = 41·1 %), there were no major insertions or deletions and that 266 (76 %) of the 350 nucleotide sites were invariant. Of the 84 variable sites, there were 8 (9·5 %) at the first, none at the second and 76 (90·5 %) at the third codon position. This pattern of variation, most substitutions accumulating at the third codon position, is typical of sequences under strong functional constraints. 74 of the variable sites were phylogenetically informative. Seven of the eight variable first codon positions, including all of the phylogenetically informative ones, occurred in leucine codons and

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Table 2. Shared sequence identity of RAPD bands and species-specificity by hybridization analysis

Primer used	Fragment name	Size (in bp)	Species the fragment was derived from	Prediction; reason for probing[a]	Outcome
10	10/5	800	*B. senegalensis*	NH; similar sized fragment weakly amplified in *B. forskalii*	NH; specific to *B. senegalensis*
10	10/7	380	*B. senegalensis*	NH; weakly amplifying fragments in *B. forskalii* & *B. camerunensis*	NH; fragment specific to *B. senegalensis*
10	10/10	940	*B. forskalii*	H; similar sized fragment in *B. camerunensis*	H; fragment homologous in *B. camerunensis* & *B. forskalii*
2	2/2	330	*B. senegalensis*	NH; weakly amplifying fragments in *B. forskalii* & *B. camerunensis*	NH; fragment specific to *B. senegalensis*
2	2/3	1400	*B. senegalensis*	H; similar sized fragment in *B. forskalii* & *B. camerunensis*	H; fragment homologous in *B. forskalii*, *B. senegalensis* & *B. camerunensis*
2	2/4	1200	*B. camerunensis*	H; similar sized fragment in *B. camerunensis* & *B. forskalii*	H; fragment homologous from *B. camerunensis* & *B. forskalii*
2	2/5	1100	*B. camerunensis*	H; similar sized fragment in *B. forskalii*	H; fragment specific to *B. camerunensis*
12	12/1	750	*B. forskalii*	H; similar sized fragment in *B. senegalensis*, *B. camerunensis* & *B. truncatus*	NH/X[b]; fragment homologous in *B. forskalii*, *B. senegalensis* & *B. camerunensis* but not *B. truncatus*
12	12/2	420	*B. senegalensis*	H; similar sized fragment in *B. forskalii* & *B. camerunensis*	H; fragment homologous in *B. senegalensis*, *B. forskalii* & *B. camerunensis*

[a] H – To test shared sequence identity of similar sized fragments with similar fluorescent intensity from other taxa compared, predicting probe hybridization; NH – To test shared sequence identity of strongly amplifying with weakly amplifying fragments, predicting no probe hybridization; [b] NH/X – mixed outcome of probing indicating fragments of shared sequence identity in some taxa but not all.

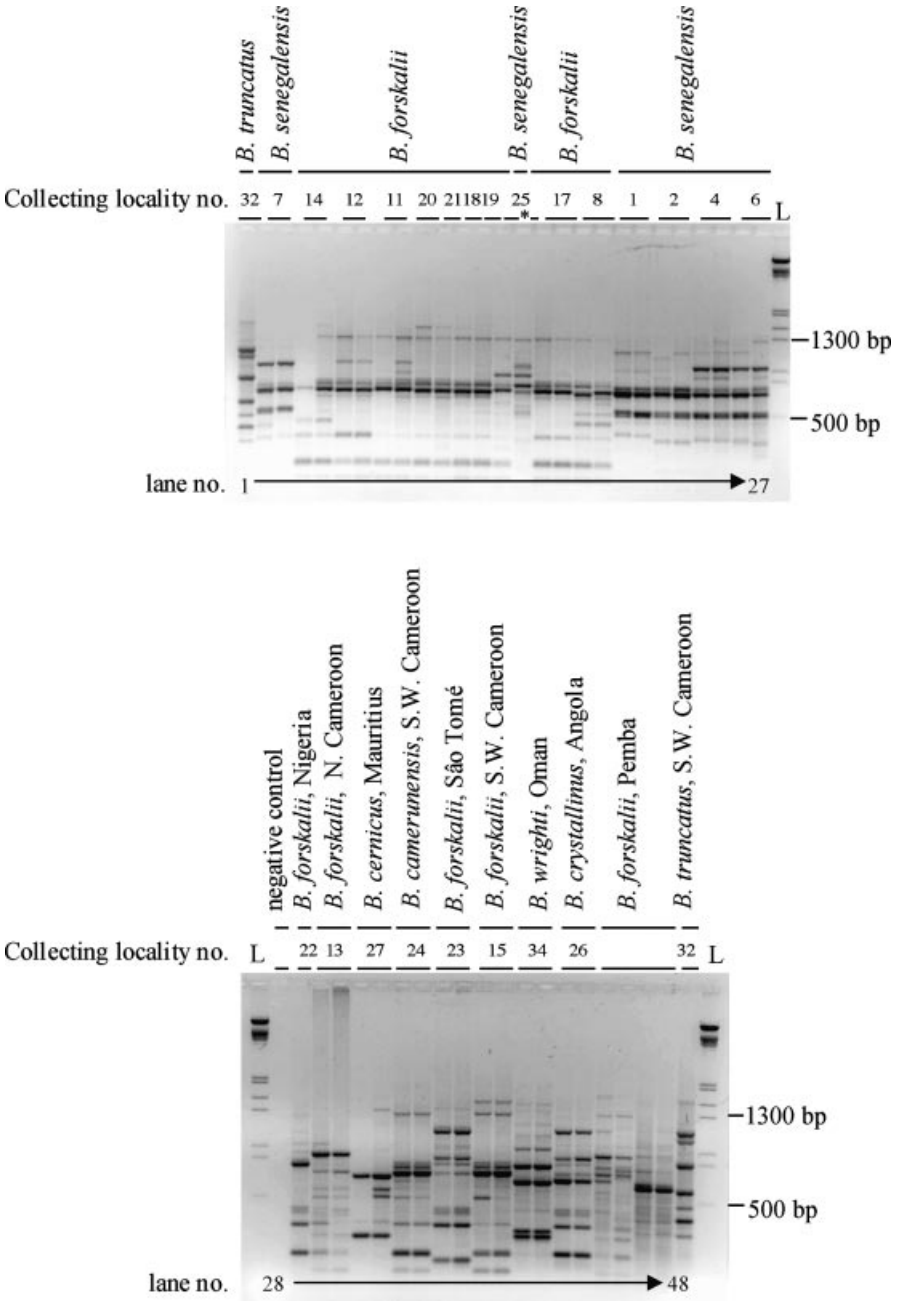

Fig. 1. Amplification products from RAPD primer OPF06 (Operon Technologies, Du Pont, USA) illustrating representative profiles from *B. forskalii* group taxa and outgroups. Lane designations (numbers in brackets refer to collecting localities listed in Table 1): 1. *B. truncatus*, S.W. Cameroon (32); 2–3. *B. senegalensis*, Mali (7); 4–5. *B. forskalii*, S. Cameroon (14); 6–7. *B. forskalii*, N. Cameroon (12); 8–9. *B. forskalii*, N. Cameroon (11); 10. *B. forskalii*, S. Cameroon (20); 11. *B. forskalii*, S. Cameroon (21); 12. *B. forskalii*, S. Cameroon (18); 13. *B. forskalii*, S. Cameroon (19); 14. *B. forskalii*, S. Cameroon (25)*; 15. *B. senegalensis*, S. Cameroon (25)*; 16–17. *B. forskalii*, S.W. Cameroon (17); 18–19. *B. forskalii*, Senegal (8); 20–23. *B. senegalensis*, Senegal (1, 2); 24–27. *B. senegalensis*, *B. forskalii*, N. Cameroon (4, 6); 28. negative control; 29. *B. forskalii*, Nigeria (22); 30–31. *B. forskalii*, N. Cameroon (13); 32–33. *B. cernicus*, Mauritius (27); 34–35. *B. camerunensis*, S.W. Cameroon (24); 36–37. *B. forskalii*, Sâo Tomé (23); 38–39. *B. forskalii*, S.W. Cameroon (15); 40–41. *B. wrighti*, Oman (34); 42–43. *B. crystallinus*, Angola (26); 44–45. *B. forskalii*, Pemba (NHM accession no. 1828); 46–47. *B. forskalii*, Pemba (NHM accession No. 1826); 48. *B. truncatus*, S.W. Cameroon (32). L. molecular weight ladder in base pairs (1KB ladder, BRL–Gibco, UK). Taxa depicted by asterisks indicate two distinct species from the same collecting locality.

were silent substitutions. The remaining first codon position change occurred in *B. truncatus*, changing a valine to an isoleucine.

The transition/transversion ratio across all codon positions was 1·74, indicating that there is a transitional bias, typical of animal mitochondrial DNA (Wakeley, 1996). The number of Ts and Tv substitutions across all codon positions was plotted against percent sequence divergence (*P*-distance $\times 100$) to investigate whether Ts substitutions

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Table 3. Estimates of RAPD mean percent sequence divergence (P distance × 100) within and between taxa (ranges given in brackets for within species comparisons)

	B. senegalensis	*B. forskalii*	*B. camerunensis*	*B. cernicus*	*B. crystallinus*	*B. truncatus*	*B. wrighti*
B. senegalensis	7·47 (1·40–15·8)	88·17	88·45	74·53	82·32	89·46	85·43
B. forskalii		8·02 (1·30–14·3)	7·96	75·00	69·53	71·69	87·95
B. camerunensis			0·00	74·78	69·07	71·83	87·87
B. carnicus				3·12 (0·00–3·12)	64·04	85·71	82·76
B. crystallinus					0·00	77·99	90·32
B. truncatus						0·00	89·47
B. wrighti							0·00

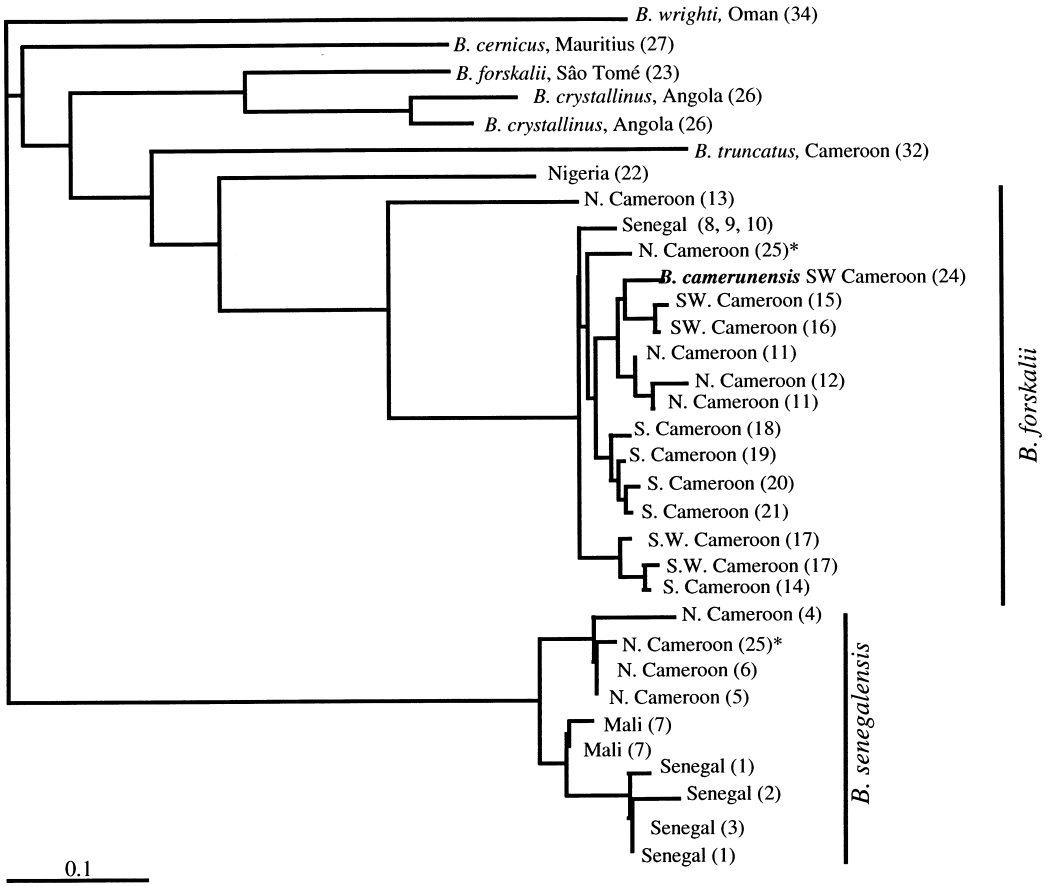

Fig. 2. Fitch-generated distance phylogram using the Kimura 2-parameter model depicting phylogenetic relationships among *B. forskalii* group species from Cameroon using the RAPD data. The scale denotes pairwise nucleotide substitution. Taxa depicted by asterisks indicate two separate species from the same collecting locality. *B. camerunensis* (in bold) falls within the *B. forskalii* clade. Numbers in brackets refer to collecting localities listed in Table 1.

become saturated with increasing sequence divergence (Fig. 3). The number of Ts substitutions is generally in excess of Tv across almost the entire sequence divergence range (up to 12%), significant linear regressions could be fitted to both Ts ($y = 1{\cdot}653x + 3{\cdot}396$; $R^2 = 0{\cdot}898$, $P = 0{\cdot}0001$) and Tv ($y = 1{\cdot}776x - 3{\cdot}625$; $R^2 = 0{\cdot}891$, $P = 0{\cdot}0001$) substitutions, suggesting that the data set was not significantly affected by 'saturation' of transitions. Subsequent phylogenetic analyses used distance estimates calculated using the K2P model, which incorporates transitional bias.

COI data set displayed largely congruent topologies for each phylogenetic methods employed. Parsimony analysis yielded 6 equally parsimonious trees (length = 294 steps, consistency index, CI = 0·701) and the 50% majority-rule consensus tree showed three unresolved branches: *B. truncatus*, *B.*

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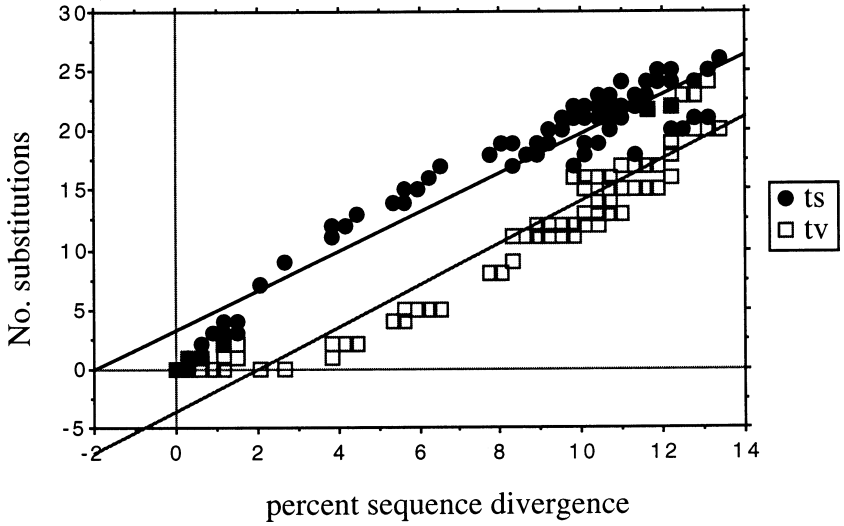

Fig. 3. Plot of transitions (Ts; shaded circles) and transversions (Tv; open boxes) against percent sequence divergence for the partial mtDNA COI sequences from *Bulinus*, depicting linear relationships.

senegalensis and a *B. forskalii-cernicus-scalaris-crystallinus* clade. Maximum likelihood analyses shows that *B. truncatus* and *B. senegalensis* branch earlier than an unresolved, weakly supported trichotomy (quartet puzzling value of 66) of *B. forskalii*, *B. crystallinus* with *B. forskalii* from Sâo Tomé and finally *B. cernicus* with *B. scalaris* (Fig. 4a). The low frequency of unresolved ML quartets (1·3 %) indicated a good phylogenetic signal in this data set. Distance methods resolve the latter main branch into 2 sister groups with *B. cernicus* clustering with *B. scalaris* and in the second group *B. crystallinus* clustering with *B. forskalii* from Sâo Tomé which branch earlier than the rest of the *B. forskalii* taxa. In all cases, *B. camerunensis* clusters tightly *B. forskalii*, with nodal bootstrap support values of 77 and 78 for parsimony and distance methods, respectively, and a quartet puzzling value of 100.

The ITS1 data matrix analysed here contained 15 sequences, representing 5 *B. forskalii* group species, *B. truncatus* and 2 outgroup taxa with 493 aligned bases. ITS1 sequences from 3 *B. forskalii* samples representing different geographical localities in Cameroon and *B. camerunensis* from South West Cameroon, proved identical and replicates were removed from the data set to speed up analyses. *Bulinus* ITS1 sequences were G:C rich (59·4 %) (nucleotide compositions: A = 19·5 %, C = 30·4 %, G = 29 %, T = 21·2 %), characterized by minor insertions and deletions, with mainly 1–4 gaps, but up to 8 were recorded for some taxa (*B. scalaris*). A total of 40 binary gap characters were scored, and added to the data set, with 28 being phylogenetically informative. 178 (36 %) of the 493 nucleotide characters were constant. Of the remaining variable sites, 183 were phylogenetically informative. Phylogenetic reconstruction using the ITS1 region is largely congruent with the mtDNA COI results. Parsimony analysis gave 2 equally parsimonious trees with 464 steps and a high CI value (0·916) suggesting a robust phylogeny, although 10 % of puzzling quartets were unresolved in the ML analyses. For all algorithms, two main branches were detected, one clade with *B. truncatus* and the other with the remaining taxa split into an unresolved trichotomy of *B. senegalensis*, *B. cernicus-scalaris* and *B. forskalii* taxa, with *B. forskalii* from Sâo Tomé branching earlier (Fig. 4b).

Mean pairwise percent sequence divergences for both the mtDNA COI and ITS1, are given in Table 4. Sequence divergence estimates for mtDNA COI indicate that the majority of *B. forskalii* group species are as distant from each other as the non-*B. forskalii* group taxa, *B. truncatus*. Values ranged from 3·9–14·8 % between *B. forskalii* ingroup taxa, 8·7–13·8 % for between the ingroup *B. forskalii* taxa and *B. truncatus* and 16·8–27·9 % between ingroup and outgroup taxa. Sequence divergence within *B. forskalii* ranged from 0·00 to 6·75 % (mean = 4·24 %). No geographical structuring of *B. forskalii* with COI sequences was evident using all phylogenetic approaches, with samples from Kenya, East Africa and Cameroon, Central Africa clustering together (Fig. 4a); sequence divergence between these 2 localities range from 0·89–6·75 % (mean = 4·47 %). Sequence divergence within *B. senegalensis* ranged from 0·59–2·68 % (mean = 1·78 %) with taxa separating according to major geographical locality.

The ITS1 sequence divergence estimates show that while ingroup taxa appear equally distant from each other, ranging from 0·44–9·43 %, there are larger differences between the ingroup *B. forskalii* taxa and *B. truncatus* (27·1–30·9 %) and between ingroup and outgroup taxa (39·4–57·8 %). No intraspecific sequence divergence was detected in *B. forskalii* from Cameroon, with only negligible variation between Kenyan and Cameroonian samples (0·44 %).

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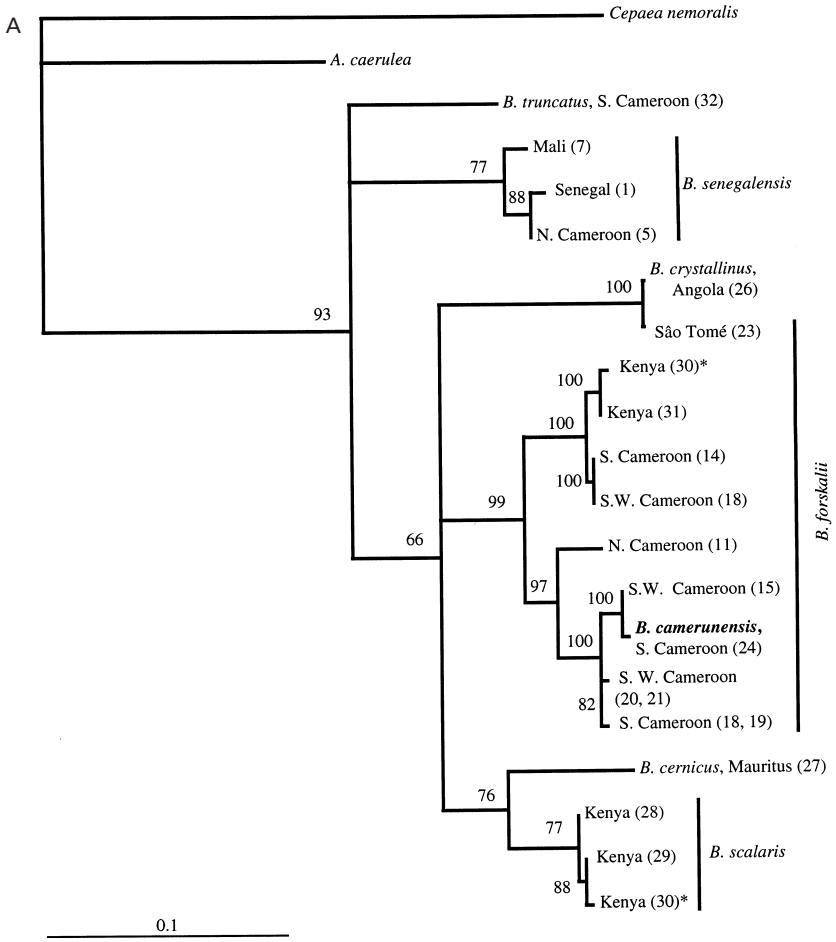

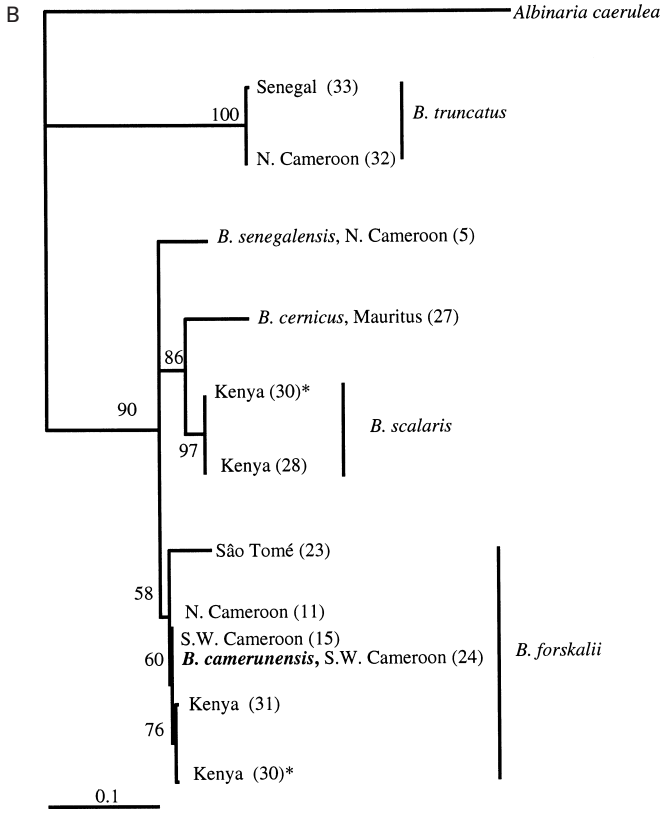

Fig. 4. Maximum likelihood neighbour-joining tree using quartet puzzling (Strimmer & Haeseler, 1996) depicting phylogenetic relationships among *B. forskalii* group species with sequences from (a) with partial mtDNA COI

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DISCUSSION

Phylogenetic reconstructions of the *B. forskalii* group species, based on RAPD analyses, partial mtDNA gene sequences (COI) and nuclear region sequences (ITS1 rDNA) were essentially congruent. With few exceptions all methods of analysis gave largely unresolved branches, with relatively large sequence divergence estimates between species and limited within species differentiation. The ITS1 and COI of pan-African *B. forskalii*, with representatives from Central and East Africa, showed little evidence of geographical structuring. Despite the unresolved or unstable nature of the phylogenies for all data sets, specimens from the same taxon clustered together, indicating that all methods were capable of differentiating species.

RAPDs – homology and phylogeny

The speed, simplicity and arbitrary sequence of primers has made RAPDs (Williams *et al*. 1990; Welsh & McClelland, 1990) one of the most frequently used molecular tools for identification and differentiation of many genetically anonymous groups (Rollinson & Stothard, 1994), including planorbid snails (Langand *et al*. 1993; Stothard & Rollinson, 1997; Jones *et al*. 1997; Kristensen, Yousif & Raahauge, 1999). Preliminary analyses of *B. forskalii* group snails (Jones *et al*. 1999), and data from this study, have demonstrated the utility of RAPDs for differentiation of morphologically similar species such as *B. forskalii* and *B. senegalensis*.

However, for estimation of nucleotide divergence between closely related taxa Clarke & Lanigan (1993) suggested RAPDs must conform strictly to a number of criteria, the most important of which is arguably verification of band homology. Co-migrating RAPD PCR products of similar molecular weight amplified from different species of the same genus may represent different sequences. Our limited Southern blot analysis of probes derived from excised RAPD fragments demonstrated that 90% of co-migrating bands in different taxa were of shared sequence identity, suggestive of homology, providing that fragments of the same size and fluorescence were compared from closely related species, a finding concordant with other studies (Smith *et al*. 1994; Rieseberg, 1996). However, unless loci are mapped to genomic locations it is impossible to distinguish whether these are paralogous or orthologous copies.

Phenetic analysis of RAPD data produced an apparently resolved tree, with *B. senegalensis* branching separately and earlier than the remaining *B. forskalii* group taxa. However, the tree topology was unstable, with affiliations of some taxa changing with the outgroup. This suggests it may be difficult to determine the relationships of *B. forskalii* group taxa using RAPDs. *B. truncatus* (a non-*B. forskalii* group species) was unexpectedly found clustered within the ingroup. This anomalous finding may be partially attributable to the large nucleotide divergence estimates between species, extending beyond the phylogenetic scope of RAPDs, concordant with the findings of Stothard & Rollinson (1997), who evaluated their use in phylogenetic analysis of *Bulinus* species groups. A reliable phylogenetic signal with RAPD markers is attainable if there is less than 10% sequence divergence between taxa, otherwise the variance in the estimate of divergence becomes too large to be informative (Clark & Lanigan, 1993).

Sequence phylogenies

COI is known to exhibit a relatively slow rate of evolution for a mtDNA gene and is subject to considerable selective constraints, the rate of amino acid substitutions being very low, even between taxa belonging to different phyla (Simon *et al*. 1994). Consequently, this marker exhibits taxonomically useful levels of variation for between species comparisons, including *Bulinus* (Stothard & Rollinson, 1997). Similarly, Porter & Collins (1991) noted that rDNA ITS sequences are probably free to accumulate substitutions at rates at least as high as those recorded for silent sites in protein coding genes. ITS sequences have often been shown to be phylogenetically informative at the species level (Jeandroz, Roy & Bousquet, 1997) including snail intermediate hosts (Stothard *et al*. 1996). Campbell *et al*. (2000) used COI and ITS1 gene sequences to reconstruct the phylogenetic relationships of species of the genus *Biomphalaria*, intermediate hosts of *S. mansoni* and both data sets gave concordant, resolved phylogenies, revealing that the Neotropical species *B. glabrata* had an African affinity.

In this study phylogenetic analyses of COI, ITS1 and RAPDs produce approximately concordant trees which are unresolved at the main branches. Despite this, relationships of some *B. forskalii* group members showed congruencies across all data sets, for all algorithms used. *B. forskalii* and *B. senegalensis* form

sequences and (b) nuclear rDNA ITS1 region. Numbers at the branch nodes indicates percentage quartet puzzling support values for 1000 puzzling steps. The scale denotes pairwise nucleotide substitution. Taxa depicted by asterisks indicate two separate species from the same collecting locality. *B. camerunensis* (in bold) falls within the *B. forskalii* clade. Numbers in brackets refer to collecting localities listed in Table 1. Sequences are deposited under GenBank accession numbers AF369729–AF369747, and AY–30345–AY030354, for the mtDNA COI and ITS1 data sets, respectively.

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Table 4. Estimates of mtDNA COI (above diagonal) and nuclear rDNA ITS1 region (below diagonal) percent sequence divergence with the two-parameter model of Kimura (1980)

	B. senegalensis	*B. forskalii*	*B. camerunensis*	*B. cernicus*	*B. scalaris*	*B. crystallinus*	*B. truncatus*	*A. caerulea*
B. senegalensis	—	14·58	14·48	12·59	13·63	14·83	12·72	27·03
B. forskalii	5·15	—	3·96	11·34	10·04	11·57	13·80	22·65
B. camerunensis	5·15	0·44	—	11·18	10·77	11·14	13·47	26·21
B. cernicus	9·43	8·16	8·16	—	8·39	13·67	12·98	27·98
B. scalaris	6·25	4·11	4·11	6·40	—	13·23	13·15	26·12
B. crystallinus	n/a	n/a	n/a	n/a	n/a	—	8·70	17·71
B. truncatus	29·74	27·12	26·89	30·82	28·70	n/a	—	16·79
B. pfeifferi	41·66	39·44	39·44	43·19	38·20	n/a	44·59	—
A. caerulea	53·89	51·13	51·13	57·88	53·60	n/a	57·60	—

distinct branches, demonstrating that these morphologically similar species are clearly differentiated by all molecular approaches. Conversely, cluster analyses place *B. camerunensis* with specimens unequivocally identified as *B. forskalii*, confirming the taxonomic status of *B. camerunensis* is debatable, supporting the observation that although conchologically distinct and compatible with a different parasite, it possesses no allozyme alleles additional to those of *B. forskalii* (Mimpfoundi & Greer, 1989). In particular its affinity with *B. forskalii* from nearby Kumba (the closest *B. forskalii* population to the *B. camerunensis* site) suggests that *B. camerunensis* has probably evolved *in situ* from local *B. forskalii* which became isolated in the crater lake. A further unexpected outcome of molecular analysis was the clustering of a conchologically extreme geographical variant of *B. forskalii* from Sâo Tomé (Brown, 1991), with Angolan *B. crystallinus*. Given the historical links between these two areas such movement of *B. crystallinus* seems plausible. It is important to test the affiliations between these taxa because *B. forskalii* from Sâo Tomé is an intermediate host for *S. intercalatum* while *B. crystallinus* is suspected to transmit *S. haematobium* in Angola (Wright, 1963) and maybe a host of *S. intercalatum* in Gabon (Jelnes & Highton, 1984). Finally, *B. cernicus* from the island of Mauritius forms another separate group and where included in the analyses, clusters with East African *B. scalaris*.

Divergence estimates

Owing to the paucity of the fossil record, the evolutionary history of *B. forskalii* must be inferred from extant species (Brown, 1994). Although grouped by similar morphological traits, there are few indicators of relationships between the *B. forskalii* group species. However, some indications may be achieved by applying a general time frame using the average rate of mtDNA divergence across the entire molecule; for most organisms 2–4 % per million years (Wilson, Ochman & Prager, 1987). A linear relationship of transitions against percent sequence divergence for the COI suggests that the data set was not significantly affected by 'saturation' or 'multiple hits', which could have caused an underestimate of sequence divergence. An average COI sequence divergence between the sympatric species *B. senegalensis* and *B. forskalii* is 14·58 %, indicating an approximate time-scale of 3·6–7·2 million years. Estimated sequence divergence between Central (Cameroon) and East African (Kenya) *B. forskalii* is 4·47 % dating an approximate divergence to 1·1–2·2 million years. Following Desprès *et al.* (1992) a time frame specific to the COI region sequenced in *Bulinus* can be calibrated with sequences homologous to this region from rat and mouse with a known divergence of 9–12 Mya (Jaeger,

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Tong & Denys, 1986). Calculated at 1·8–2·4% per million years this encompasses the lower mtDNA sequence divergence estimates assumed for the general mtDNA clock. However, these should be considered conservative estimates as mollusc mtDNA may evolve more rapidly (Hoeh *et al*. 1996) than vertebrates, suggesting that the use of upper range of the general mtDNA clock may be more appropriate.

Evolution of the B. forskalii *group*

As a group these taxa show little intra-specific variation, but are separated by large sequence divergences which produce unresolved branches on phylogenetic trees. Several scenarios involving taxon-wide catastrophic population crashes, a likely possibility for tropical freshwater snails, could be invoked to explain both range expansion of certain individual taxa and their genesis. East Africa began to cool and become progressively drier some 10 Mya, during the Miocene, leading to the eventual drying out of the lake basins and the extinction of many species (Van Damme, 1984). The palaeoclimate of the Late Miocene–Early Pliocene, about 4–8 Mya, would have provided a mosaic of extreme and temporally unstable habitats for freshwater snails (Roberts *et al*. 1993). This period correlates with the suggested divergence time of *B. forskalii* and *B. senegalensis* and several other *B. forskalii* group taxa suggesting that genesis of the species complex may have been concomitant with environmental change.

Environmental change can promote rapid genesis of novel self-compatible taxa, with little substantive genetic change or adaptive divergence (Gottlieb, 1978; Carson, 1971), often from single propagules. Sudden and severe environmental stress, of the kind probably experienced by freshwater snails during the Miocene, can lead to catastrophic selection of a very few robust individuals to produce derived taxa (Lewis, 1963; Davies, 1993). That the breeding system of pulmonate molluscs, unusual amongst higher invertebrates in their high incidence of self-compatibility, can lead to speciation via this route is reflected in the many taxonomic complexes of both terrestrial (Noble & Jones, 1996) and freshwater representatives, such as the *B. forskalii* group.

While phyletic gradualism inevitably produces a legacy of distinct intermediate forms, arising from common ancestral stock, rapid and abrupt saltational speciation constructs a derivative genome in a few generations, containing a limited repertoire of parental allelic variation and linkages (Noble & Jones, 1996). Molecular analysis of phyletic gradualism produces a well differentiated phylogenetic tree with most branches resolved reflecting the gradual differentiation which has taken place in contrast to the unresolved branches of genetically similar rapidly derived taxa. Some derived taxa (Gottlieb, 1978) may fortuitously isolate complexes of adaptive genes and so persist, eventually expanding their range, but most are ephemeral. The extreme ecological and physiological tolerance of many of the less widespread *B. forskalii* taxa is consistent with this scenario (Brown, 1994).

Anomalous transmission of Schistosoma *in the* B. forskalii *group*

Only the 'terminal spined' schistosomes use species of the genus *Bulinus* as intermediate hosts in Africa and the Middle East (Rollinson & Southgate, 1987; Rollinson *et al*. 1997). This group contains 11 species, 2 infecting man, *S. haematobium* and *S. intercalatum* both transmitted by species of the *B. forskalii* group and taxa from other *Bulinus* groups. Though *B. forskalii* has a wide distribution throughout much of tropical Africa its transmission of *S. intercalatum* is restricted to Cameroon, Sâo Tomé and Gabon. While *B. senegalensis*, with a more restricted, mainly Sahelian distribution from Senegambia through to Nigeria and Cameroon, transmits *S. haematobium* throughout much of its range. A natural hybridization event between *S. haematobium* and *S. intercalatum* has been documented from Loum, Southwest Cameroon (Southgate, Van Wijk & Wright, 1976; Tchuem Tchuente *et al*. 1997). *S. haematobium* has completely replaced *S. intercalatum* where originally only *S. intercalatum* was present, the shift occurring progressively over 30 years. Hybrid parasites exhibit heterosis, are more fecund and can infect both *B. truncatus* and *B. forskalii*, the local snail intermediate hosts.

This hybridization event emphasises the low fitness of *S. intercalatum* and affords an explanation of the restricted distribution of this parasite, despite the widespread distribution of the intermediate (*B. forskalii*) and definitive hosts (Tchuem Tchuente *et al*. 1996). The situation in Barombi Kotto, a crater lake in Southwest Cameroon, may reflect just such a parasite hybridization event, where *B. camerunensis*, effectively a geographical variant of *B. forskalii*, anomalously transmits *S. haematobium* and not the expected *S. intercalatum*. Comparison of nuclear and mtDNA sequences from schistosomes sampled from Loum and Barombi Kotto would substantiate their origin and afford an explanation for the anomalous transmission of *S. haematobium* in *B. forskalii*.

Within-species variation

Sequence analyses of COI and ITS1 from East and Central African *B. forskalii* show little differentiation within and between localities in Kenya and Cameroon, with no geographic structuring evident, suggesting no clear isolation by distance effect.

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Further, RAPD analyses from a more limited survey of West and Central Africa also show limited within-*B. forskalii* differentiation and no evidence of geographical structuring. Enzyme electrophoresis had previously indicated rather low genetic diversity in *B. forskalii* (Jelnes, 1980, 1986; Mimpfoundi & Greer, 1989, 1990), although some alleles showed a geographical pattern in Cameroon (Mimpfoundi & Slootweg, 1991). Further genetic analyses (allozymes – Mimpfoundi & Greer, 1990; microsatellites – Gow *et al*. in press) of wild-caught *B. forskalii* population samples suggests this taxon may be facultatively self-fertile under natural conditions. The lack of pan-African differentiation could be a consequence of the homogenizing effects of considerable gene flow, but this seems unlikely considering the snail's breeding system. Alternatively there could have been little genetic diversity available to the taxon upon its genesis, or selection for a generalist genotype capable of coping with climatic exigencies, might lead to a loss of genetic diversity (Mimpfoundi & Greer, 1990; Noble & Jones, 1996).

Rapid range expansion from a segment of the *B. forskalii* range would similarly result in a widely distributed, genetically depauperate taxon, as would the converse, severe and prolonged bottlenecking followed by range expansion. The very limited differentiation of ITS1 sequences from Cameroon and Kenya, combined with lack of geographical structuring of COI and RAPD data, suggest a rapid and recent origin is the most likely explanation for our observations of these populations.

Resolution of the evolutionary history of the *B. forskalii* complex shows its genesis is linked with the story of climatic change during the Late Tertiary of Africa. The breeding system of these snails, by permitting the rapid establishment of evolutionary novelties, allows the group to exploit marginal habitats. Establishment of novel gene complexes, protected by self-fertilization and experiencing only limited gene flow, clearly provides the most adaptive evolutionary strategy to a constantly changing abiotic or biotic environment. The less widespread *B. forskalii* taxa may represent successful colonization of marginal environments, whereas *B. forskalii* itself has expanded rapidly, coming to dominate more equable habitats.

However, the rapid genesis of taxa within this complex has led to incomplete phylogenetic reconstruction, demanding further sequence analysis and examination of additional taxa, including East African representatives such as *B. barthi* and *B. browni*, for a fuller resolution of the *B. forskalii* group.

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