Bulinus species on Madagascar: molecular evolution, genetic markers and compatibility with *Schistosoma haematobium*

J. R. STOTHARD[1]*, P. BRÉMOND[2], L. ANDRIAMARO[2], B. SELLIN[2], E. SELLIN[2] *and* D. ROLLINSON[1]

[1] *Wolfson Wellcome Biomedical Laboratories, Department of Zoology, The Natural History Museum, Cromwell Road, London, SW7 5BD*
[2] *Equipe Schistosomoses I.R.D., BP 434, 101 Antananarivo, Madagascar*

SUMMARY

Of the four species of *Bulinus* found on Madagascar, three species: *B. obtusispira*, *B. liratus* and *B. bavayi* are endemic while the fourth, *B. forskalii*, is probably a recent introduction from the African mainland. The evolutionary relationships of these species with *Bulinus* species from Africa were studied by phylogenetic analysis of DNA sequence variation at two mitochondrial loci: cytochrome oxidase subunit I (COI) and large ribosomal subunit (LSU) or 16S. The observed levels of nucleotide divergence within *Bulinus* were substantial but may underestimate the true levels as there was evidence of 'saturation' of transitional substitutions at both loci. A putative secondary structure model for the sequenced segment of the 16S was developed. Subsequent phylogenetic analysis using transversional changes only for both loci, showed that there were contrasting levels of divergence within the four species groups. *B. obtusispira* was consistently placed within the *B. africanus* group, appearing ancestral to this group and was closest to the basal node within *Bulinus*. Together with *B. bavayi*, the two species appear to have been isolated on Madagascar for a long time, contrasting with both *B. liratus* and *B. forskalii* that appear more recent colonisers; however, estimate of exact times of divergence is problematic. A PCR-RFLP assay was developed to enable identification and discrimination of *B. obtusispira* and *B. liratus* using discriminatory variation within the COI. To enable population genetic analysis within *B. obtusispira*, microsatellite markers were developed using an enrichment method and 8 primer pairs are reported. Laboratory infection experiments using Madasgacan *S. haematobium* from the Mahabo area showed that certain populations of *B. obtusispira*, *B. liratus* and *B. bavayi* were compatible.

Key words: Madagascar, *Bulinus*, *Schistosoma haematobium*, mitochondrial DNA, phlylogeny, microsatellites.

INTRODUCTION

Madagascar is the fourth largest island in the world and is located off the Mozambique coast of Africa (Fig. 1). The human population of the Republic of Madagascar numbers over 16 million (Anon, 1999) and the Malagasy are of mainly Malay-Indonesian origin (Preston-Mafham, 1991). Schistosomiasis is endemic on Madagascar with estimates of 2 million infections of *Schistosoma mansoni* and 0·5 million of *S. haematobium* (Ollivier, Brutus & Cot, 1999). With one or two exceptions, the two forms of the disease follow a strong east-west division. Intestinal schistosomiasis is found in eastern and central regions, where it is also spreading (Ollivier *et al.* 1999). Urinary schistosomiasis is in central and western regions but is absent in the east (Doumenge *et al.* 1987).

The pulmonate snail family Planorbidae is widely distributed throughout the world with origins thought to be within the Triassic (Baker, 1945). The subfamily Bulininae contains two genera, *Bulinus* and *Indoplanorbis*. The genera are conchologically different, physoid and discoid respectively, but are both associated with the transmission of schistosomes (Rollinson & Southgate, 1987). Several of the 37 species of *Bulinus* act as intermediate hosts for parasites of the *S. haematobium* group throughout Africa, Madagascar and adjacent regions (Brown, 1994). Whilst *Indoplanorbis* is absent from the African mainland save for recent introductions (Brown, 1994), *I. exustus* is an intermediate host for members of the *S. indicum* group throughout India and south-east Asia (Rollinson & Southgate, 1987).

Many elements of the fauna of Madagascar are strongly associated with endemicity (Jolly, Oberle & Albignac, 1984). The paleogeographic history of Madagascar is firmly established based upon sedimentological, structural and paleomagnetic data (Rabinowitz, Coffin & Falkey 1983; Piqué, 1999). The island was once part of Gondwana (Fig. 1, inset A) and separated from Africa at a present position close to Kenya/Tanzania (Piqué, 1999). The conjoined Madagascar and India subsequently drifted southwards towards the island's present position around 120 MYA (Storey *et al.* 1995). Madagascar finally separated from India at a time when dis-

* Address for correspondence: e-mail: j.r.stothard@nhm.ac.uk

Parasitology (2001), **123**, S261–S275.
DOI: 10.1017/S003118200100806X Printed in the United Kingdom

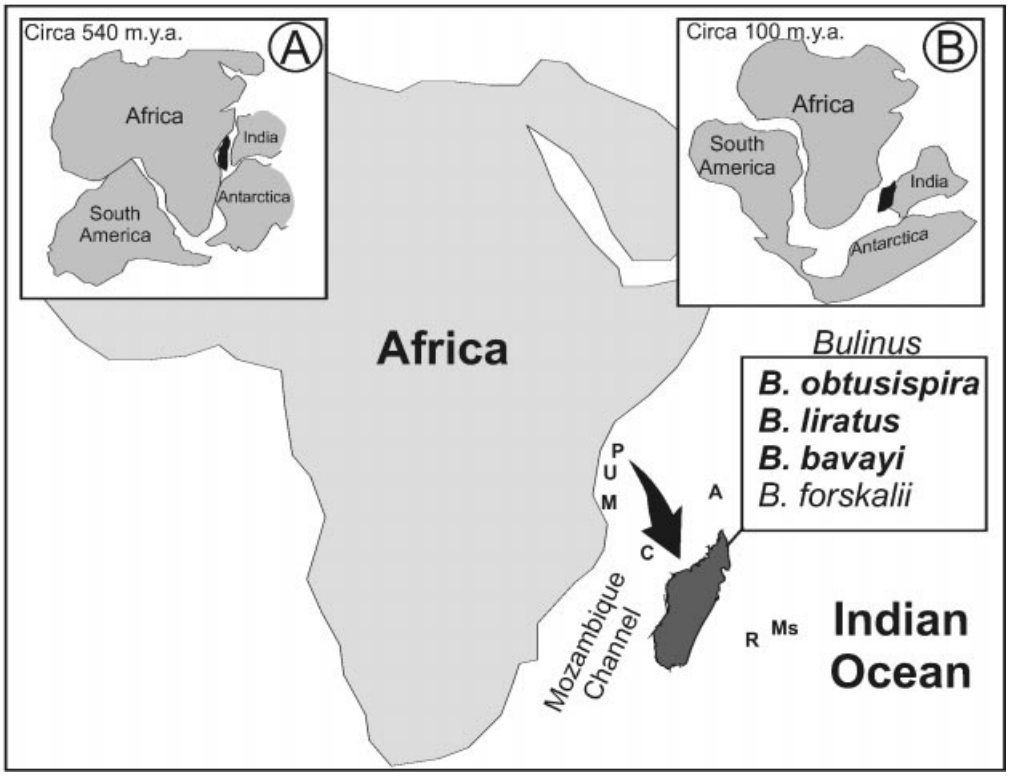

Fig. 1. Sketch map of the geography and geological evolution of Madagascar with present species of *Bulinus*. Insert A – depiction of Madagascar's placement in the land mass of Gondwana during the Protozeroic taken from Piqué (1999). Insert B – depiction of continental break up of Gondwana land masses during the Cretaceous taken from Smith *et al*. (1994). The three species of *Bulinus* in bold are endemic to Madagascar, *B. obtusispira* (*B. africanus* group), *B. bavayi* (*B. forskalii* group) and *B. liratus* (*B. truncatus/tropicus* complex). Key of Indian Ocean Islands: P–Pemba, U–Unguja (Zanzibar Island), M–Mafia, C–Comoros, A–Aldabra, R–Réunion, Ms–Mauritius.

integration of virtually all of the individual Gondwanan land masses had begun, circa 88 MYA (Mildenhall, 1980), Fig. 1, inset B. At that time, although physically isolated, dispersal pathways for many organisms via Antarctica to South America were thought to remain up to the late Eocene, circa 40 MYA (Raven, 1979).

Tristram (1863) provided the first account of freshwater snails on Madagascar. Since then there have been several taxonomic revisions, see Starmühlner (1969) & Wright (1971*a*). While Starmühlner (1969) recognized only two species of *Bulinus*, *B. liratus* and *B. mariei*, with the latter now treated as synonymous with *B. forskalii* (Brown, 1994), four species are accepted today. The four species have affinities with three of the four species groups of *Bulinus* (Fig. 1). Two species, *B. obtusispira* and *B. liratus* are restricted to Madagascar, whereas *B. bavayi* is also found on Aldabra (Wright, 1971*a*), see Fig. 1. In contrast, *B. forskalii* has a pan-African distribution.

B. obtusispira is placed within the *B. africanus* group due to immunological and biochemical assays (Wright, 1971*a*, *b*; Brown & Wright, 1978; Wright & Rollinson, 1979; Jelnes, 1984). Conchologically and anatomically it can be confused with members of the *B. truncatus/tropicus* complex in which *B. liratus* is placed (Wright, 1971*b*). Its specific status was reconsidered when a collection of *B. liratus*, highly compatible with *S. haematobium*, was encountered (Brygoo & Moreau, 1966). Wright (1971*b*) concluded that *B. obtusispira* was a 'relic' species of *Bulinus*, perhaps paralleling the unusual evolution of other endemic Madagascan groups, and proposed that ancestral *B. obtusispira* became isolated on Madagascar at a time when the *B. africanus* group had only recently diverged from other bulinids.

Wright (1971*b*) noted that while *B. obtusispira* was highly compatible with *B. forskalii* group-borne *S. haematobium* and compatible with *B. truncatus*-borne *S. haematobium*, it was curiously incompatible with *B. africanus* group-borne *S. haematobium*. The sporocyst development of Madagascan *S. haematobium* in *B. obtusispira* has some unusual features (Jourdane, 1983). The transmission status of *B. liratus* was less clear. Wright (1971*a*) concluded that like other diploids of the *B. truncatus/tropicus* complex it did not prove to be susceptible to any strain of *S. haematobium* to which it had been exposed. In the Mangoky region, however, Degrémont (1973), who also used immuno-diffusion tests for snail identifications similar to those of Wright (1971*a*), found naturally infected *B. liratus*. By laboratory infections, Degrémont (1973) was also able to demonstrate compatibility with local *S. haematobium*. Similarly the transmission status of *B.*

https://doi.org/10.1017/S003118200100806X Published online by Cambridge University Press

Table 1. Details of *Bulinus* populations used for COI* and 16S sequences

Marker	Species/Country	Population (NHM no.)	GenBank no.
COI	*B. obtusispira*/Madagascar	Andohaviana (2000)	AF369588
	B. obtusispira/Madagascar	Ambilobe (1998)	AF369589
	B. liratus/Madagascar	Befeno (1966)	AF369590
	B. liratus/Madagascar	Ranohira (1967)	AF369591
	B. forskalii/Madagascar	Vohémar (2092)	AF369592
	B. bavayi/Madagascar	Ranohira (1967)	AF369593
	B. bavayi/Madagascar	Nosibe (2093)	AF369594
	B. globosus/Zanzibar Is.	Kisongoni (2019)	AF369595
	B. globosus/Niger	Liboré (3003)	AF369596
	B. globosus/Ivory Coast	Kan Gare (3002)	AF369597
	B. nasutus/Mafia Is.	Site M15 (2013)	AF369598
	B. umbilicatus/Niger	Baban Tabkin (3001)	AF369599
	B. umbilicatus/Niger	Bouboute (3000)	AF369600
	B. africanus/South Africa	Pietermaritzburg (2094)	AF369601
	B. tropicus/Kenya	Lanet (1253)	AF369602
	B. tropicus/Zambia	Kalumba (1638)	AF369603
	B. truncatus/Malawi	Nyemba (1087)	AF369604
	B. truncatus/Sudan	Keriab (2095)	AF369605
	B. truncatus/Sudan	Abu-Ushar (2096)	AF369606
	B. truncatus/Sudan	Gamooeya (2097)	AF369607
	B. truncatus/Sudan	Faki Hashem (2098)	AF369608
	B. truncatus/Sudan	Managil (2099)	AF369609
	B. forskalii/Mafia Is.	Site M3 (2012)	AF369610
	B. forskalii/Pemba Is.	Kinowe (2012)	AF369611
	B. sp./Mafia Is.	Kilindoni (2014)	AF369612
	B. cernicus/Mauritius	Vallée Pitot (1689)	AF369613
	B. wrighti/France	Lab. stock (1697)	AF369614
	B. wrighti/Oman	Baushar (1698)	AF369615
16S	*B. obtusispira*/Madagascar	Andohaviana (2000)	AY029542
	B. liratus/Madagascar	Befeno (1966)	AY029543
	B. bavayi/Madagascar	Ranohira (1967)	AY029544
	B. forskalii/Madagascar	Vohémar (2092)	AY029545
	B. globosus/Zanzibar Is.	Kisongoni (2019)	AY029546
	B. nasutus/Zanzibar Is.	Kibonde (2019)	AY029547
	B. truncatus/Burundi	Cyohoha (1087)	AY029548
	B. truncatus/Malawi	Nyemba (1087)	AY029549
	B. forskalii/Mafia Is.	Site M3 (2012)	AY029550
	B. sp./Mafia Is.	Kilindoni (2014)	AY029551
	B. wrighti/Oman	Baushar (1698)	AY029552

* Other COI sequences were obtained from Stothard & Rollinson (1997*a*).

bavayi remained uncertain; whilst Degrémont (1973) was unable to show compatibility with local *S. haematobium*, Wright (1971*a*), using isolates of *S. haematobium* from Mauritius, found *B. bavayi* to be compatible.

The studies in this paper stem from a collaborative project concerned with the distribution and transmission status of *Bulinus* species on Madgascar. This paper will focus upon the molecular taxonomy and evolution of the *Bulinus* species encountered and on laboratory compatibility studies. Phylogenetic analysis of DNA sequences from mitochondrial cytochrome oxidase subunit I (COI) was chosen since this gene target has been useful in interspecific comparisons within *Bulinus* (Stothard & Rollinson, 1997*a*; Jones *et al*. 1999) and other gastropods (Davis *et al*. 1998; Campbell *et al*. 2000). In addition, variation in the mitochondrial ribosomal large subunit (16S) was analysed and a secondary structure model for this region in *Bulinus* is proposed. To compare the genetic structures of populations of *B. obtusispira* across the island, recent progress in isolation of microsatellite marker loci is reported.

MATERIALS AND METHODS

Snail identification

Identification of snails was by conchological examination and inspection of isoenzyme profiles for four enzyme systems: glucose phosphate isomerase (GPI), malate dehydrogenase (MDH), acid phosphatase (ACP) and phosphoglucomutase (PGM) following separation by iso-electric focusing (Wright & Rollinson, 1979). Morphological descriptions, enzyme profiles and distribution of snail species on Madagascar will be described later by Brémond *et al*. (unpublished).

https://doi.org/10.1017/S003118200100806X Published online by Cambridge University Press

Amplification COI, 16S and phylogenetic analysis

The COI was amplified according to methods detailed in Stothard & Rollinson (1997*a*). In total a 340 bp data set covering the region of the COI was analysed. From the 35 sequences generated in this study, 28 have been deposited in GenBank and are detailed in Table 1 to which other, existing COI data were added (Stothard & Rollinson, 1997*a*). The sequences were aligned by eye since there were no insertions or deletions.

A sub-region of the 16S was amplified using the conserved primers 16Sar-L [5′-cgcctgtttatcaaaaacat] and 16Sbr-H [5′-ccggtctgaactcagatcacgt] detailed by Thollesson (1999) and Remigio & Blair (1997). A PCR product of approximately 450 bp was produced, extracted from an agarose gel using QIAEX II (Qiagen, Germany) according to manufacturer's instructions and then sequenced using ABI PRISM BigDye cycle sequencing with dye terminators (ABI, U.K.). The 11 sequences of the 16S generated in this study have been deposited in GenBank and are detailed in Table 1. With the addition of 16S data from two outgroup taxa, *Biomphalaria pfeifferi* and *Lymnaea stagnalis* (Remigio & Blair, 1997), the sequence data were aligned using Clustal-V (Higgins, Bleasby & Fuchs, 1992) using default parameters. This alignment was subsequently modified with the use of secondary structure models that are available electronically from ''http://www.rna.icmb.utexas.edu''. Stem and loop regions were identified and sequence nomenclature followed that proposed by Kjer (1995). The secondary structure, however, within the region between L10–L11 was not easily identified by direct comparison to other taxa and an investigation of the intra-strand folding of this region was conducted with the program RNAdraw 1·0 (Matzura & Wennborg, 1996). A sliding window analysis over the 16S alignment, window size – 7 bp, was performed to identify the most variable regions in the alignment.

Basic nucleotide statistics (nucleotide composition, transition (Ts) and transversion (Tv) frequencies, number of variable and parsimony informative sites) were calculated for the COI and 16S in MEGA (Kumar, Tamura & Nei, 1993). Analysis of the COI and 16S data, treated separately, was conducted using distance methods with programs PHYLIP (Felsenstein, 1993) and MEGA (Kumar *et al*. 1993), maximum parsimony with PAUP* (Swofford, 1999), and maximum likelihood with PAUP* (Swofford, 1999) and PUZZLE 3·1 (Strimmer & Von Haeseler, 1997). The resultant phylograms were compared between methods and assessed for congruence. Tests for phylogenetic signal such as skewness of observed tree length to that of randomly generated trees and Tajima's test for neutrality were also conducted (Tajima, 1989).

Microsatellite isolation

Twenty individual *B. obtusispira* from Miandrivazo (site 20, Table 4) were selected and total genomic DNA was extracted following protocols of Stothard, Hughes & Rollinson (1996) for each snail and then pooled, adjusting for DNA concentrations, to form a genomic stock. This genomic stock was digested with *Mbo*I and microsatellites were isolated from this preparation using a single-strand-capture (SSC) enrichment method as described by Hammond *et al*. (1998). Mirocosatellite hybridization probes of either $(ca)_{18}$ and $(ct)_{18}$ sequence that were 5′ biotin labelled were used for SSC. Post-enrichment preparations were then blunt-end cloned into *E. coli* (Stratagene, U.K.). Clones containing repetitive regions were identified after hybridisation to microsatellite probes. Briefly, bacterial colonies were transferred onto nylon membranes followed by cell lysis then hybridisation to 5′ poly-digoxigenin-labelled $(ca)_{18}$ and $(ct)_{18}$ probes. Microsatellite containing clones were identified after autoradiography using chemiluminescence detection methods according to the manufacturer's instructions (Roche Molecular Biochemicals, U.K.). Those clones with positive inserts after hybridisation were subsequently amplified by PCR using plasmid primers, purified and sequenced. Cloned inserts found to contain microsatellite motifs, with sufficient flanking sequence to anchor PCR primers, were selected to which PCR primers sets were designed. A single clone containing a minisatellite motif 'AATCATGCTC' was also found to which primers were designed. Primers sets were tested and optimised by varying either $MgCl_2$ or annealing temperature of the PCR upon the original genomic stock from Miandrivazo and DNA preparations from individual snails from the same population.

Snail infections and parasite isolation

Snail infection experiments were conducted from December 1996 to July 1999 as laboratory snail material became available. All snails used for infections originated from laboratory colonies that ranged from first to eighth generation stocks. The colonies had been established from field collected snails. The snail colonies were identified by morphological examination and, where possible, isoenzyme and(or) DNA analysis. The majority of exposed snails were either of second or third generation. Snails were individually exposed to *S. haematobium* collected from the Mahabo area (the villages: Antsakoavaky, Soaserana, Andohaviana, Soafoasa) using approximately five miracidia per snail. Schistosome eggs were directly obtained from infected urines that were taken, after the patient's informed consent, as part of routine epidemiological surveillance of urinary schistosomiasis in Mahabo

https://doi.org/10.1017/S003118200100806X Published online by Cambridge University Press

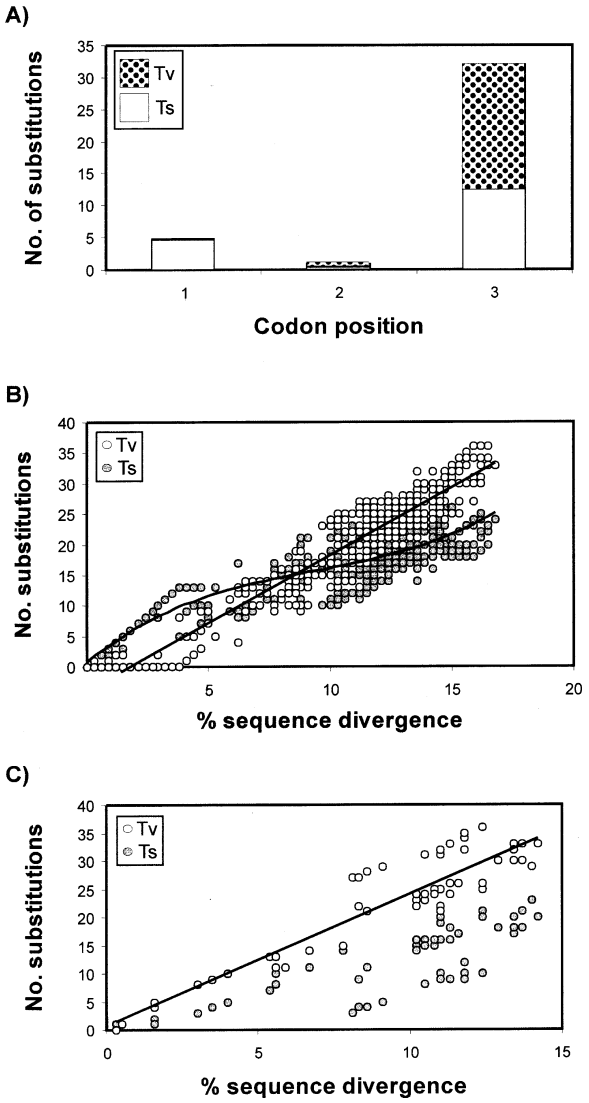

Fig. 2. Plots of transitions (Ts) and transversions (Tv) for *Bulinus* with respect to codon position and % sequence divergence (p-distance) show (non)linear relationships. Fig. 2A. Variation of Ts and Tv at 1st, 2nd and 3rd codon positions in the COI. Fig. 2B. Plot of Ts and Tv against % sequence divergence for the COI shows nonlinear relationship for Ts and linear relationship Tv. Fig. 2C. Plot of Ts and Tv against % sequence divergence for the 16S shows a linear relationship for Tv, and that there are less Ts than Tv substitutions.

area. Patients found infected with schistosomes were provided with a curative dose of praziquantel. The schistosome eggs were concentrated by sedimentation in saline, eggs were hatched after exposure to freshwater. After 28 days post-challenge, snails were visually inspected for shedding of schistosome cercariae once a week until 50 days after exposure.

RESULTS

COI

The 40 aligned COI sequences from *Bulinus* were found to be A:T rich (70·5%), with nucleotide compositions of: T (42%), C (11·9%), A (28·5%) and G (17·6%). Of the 340 nucleotide positions, 218 (64%) were found to be invariant. A total of 122 (36%) sites varied within *Bulinus*; 104 were parsimony informative while the remainder (18) were singletons. Using the *Drosophilia* mitochondrion codon table, 17 (15%) positions of the 113 amino acids across *Bulinus* would be variable. Tajima's test of neutrality showed that substitutions did not deviate significantly from that expected under neutral evolution ($D = -0{\cdot}21$, $P > 0{\cdot}1$).

The number of Transitions (Ts) and Transversions (Tv) substitutions was plotted against codon position (Fig. 2A). Within the aligned data set there were on average ± one standard deviation, 4·8 ± 2·3 substitutions in the 1st position, 1 ± 0·8 in the 2nd and 32·1 ± 12 in the 3rd. The Ts 'v' Tv ratio differed considerably at each of the codon positions: 1st = 23, 2nd = 0·67 & 3rd = 0·64. The total number of Ts and Tv substitutions was plotted against % sequence divergence (p-distance × 100) to investigate (non)linear relationships (Fig. 2B). Up to 5% sequence divergence the number of Ts substitutions is in excess to that of Tv, however, at values > 5%, there is a greater number of Tv than Ts substitutions. A linear regression line could be fitted for Tv substitutions ($y = 0{\cdot}81x–3{\cdot}5$) with a high coefficient of determination ($R^2 = 0{\cdot}9$). The trend of Ts against % sequence divergence does not follow a linear relationship, with a 3rd order polynomial ($y = 0{\cdot}0005x^3 - 0{\cdot}04x^2 + 1{\cdot}05x + 0{\cdot}84$) the most predictive but with a low coefficient of determination ($R^2 = 0{\cdot}64$).

Inspection of Ts 'v' Tv plots suggests that 'multiple hits' or 'saturation' of transitions was occurring at the 3rd codon position. Subsequent phylogenetic analysis either omitted the 3rd position entirely or analysed Tv substitutions alone since a linear relationship could be demonstrated. Phylogenetic signal could be shown for Tv substitutions by comparison with the observed tree length to those of 1000 random trees. The most justifiable analysis for this data is presented, Fig. 3, and was conducted as follows: calculation of Kimura-2-Parameter (K-2-P) distance only considering Tv substitutions, neighbour-joining clustering algorithm to infer the topology of the phylogram with bootstrap support of each node (1000 replicates). The phylogram's distances were linearised to enable comparison of the relative order of bifurcations. Where bootstrap values were below 65% the nodes were collapsed into a polytomy. Similar phylograms recovered with maximum parsimony and maximum likelihood, using HYK85 substitution model, with the exceptions that the relationships of *B. umbilicatus* and *B. africanus* within the *B. africanus* group were sometimes reversed. In consideration of Kimura-2-Parameter distance using Tv substitutions alone, there are contrasting levels of sequence divergence

https://doi.org/10.1017/S003118200100806X Published online by Cambridge University Press

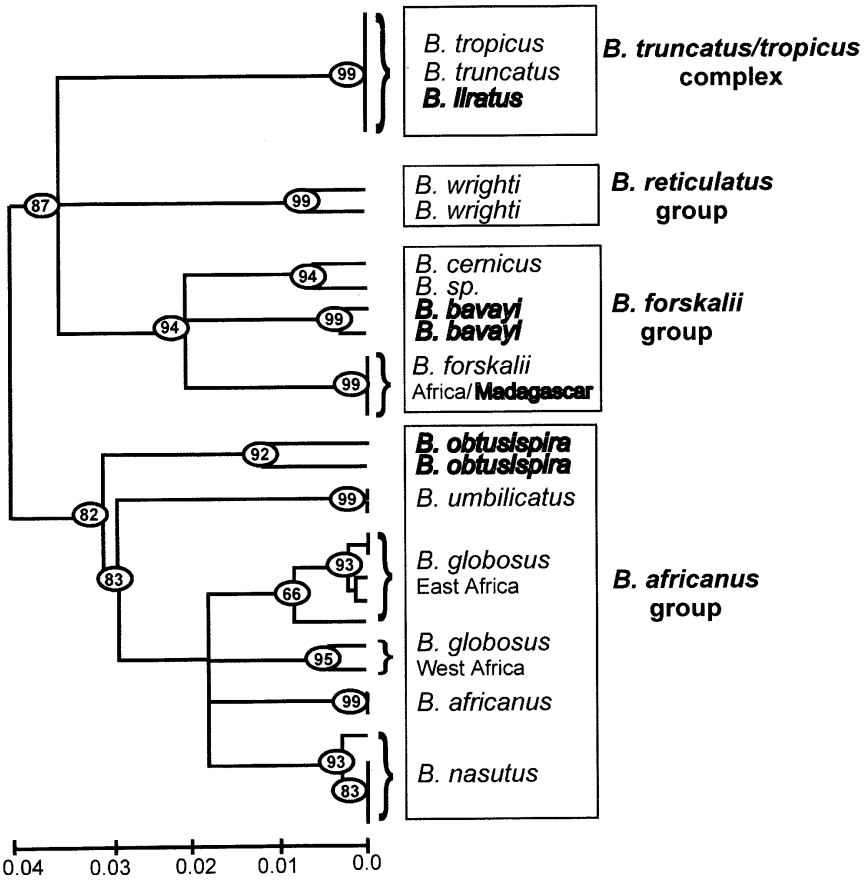

Fig. 3. A phylogram of relationships within *Bulinus* inferred from the COI calculated from distance data taken from Tv substitutions alone. Bootstrap values are placed on each node, the distances on the neighbor-joining phylogram were linearised, topology unchanged, with respect to relative time and the tree is unrooted. [If both Ts and Tv were considered many of the interspecific relationships that were previously resolvable became polytomies. With the exception that relationships within the *B. truncatus/tropicus* complex could now be determined]. Madagascan species are in bold typeface.

Table 2. Nucleotide divergence (K-2-P) between species groups of *Bulinus*. Above diagonal, Tv & Ts considered, below diagonal Tv alone, with standard deviation (estimated by bootstrapping)

	B. forskalii group	*B. africanus* group	*B. reticulatus* group	*B. truncatus/tropicus* complex
B. forskalii group		0·157 ± 0·019	0·150 ± 0·020	0·136 ± 0·018
B. africanus group	0·090 ± 0·013		0·167 ± 0·021	0·139 ± 0·018
B. reticulatus group	0·075 ± 0·013	0·096 ± 0·015		0·124 ± 0·018
B. truncatus/tropicus complex	0·076 ± 0·014	0·080 ± 0·013	0·064 ± 0·014	

within each of the species groups of *Bulinus* that were surveyed. In a descending order, *B. africanus* group (0·041), *B. forskalii* group (0·031), *B. reticulatus* group (0·015) and *B. truncatus/tropicus* complex (0·0). Table 2 shows the Kimura-2-parameter distance between the species groups calculated for all substitutions (above diagonal) and Tv alone (below diagonal).

The Madgascan species are clearly assigned to the species groups of *Bulinus*. Madgascan *B. forskalii* is almost identical in sequence to that sampled from mainland Africa, differing by only two substitutions across the alignment. Similarly, *B. liratus* is placed with other *B. truncatus/tropicus* species and, as there were no Tv substitutions found, these species cannot be differentiated. If, however, Ts substitutions were considered then relationships reanalysed, the species could be differentiated giving the following relationships ((*B. tropicus* [Kenya & Zambia], (*B. truncatus* [Malawi], *B. liratus*)), (*B. truncatus* [Sudan])) with a mean nucleotide divergence within these species of 2·3 %. In contrast the divergence of *B. bavayi* and *B. obtusispira* to other members of the same species group was much greater. However, whilst *B. bavayi* is placed within the *B. forskalii* group, the relationships of this species with other members were

https://doi.org/10.1017/S003118200100806X Published online by Cambridge University Press

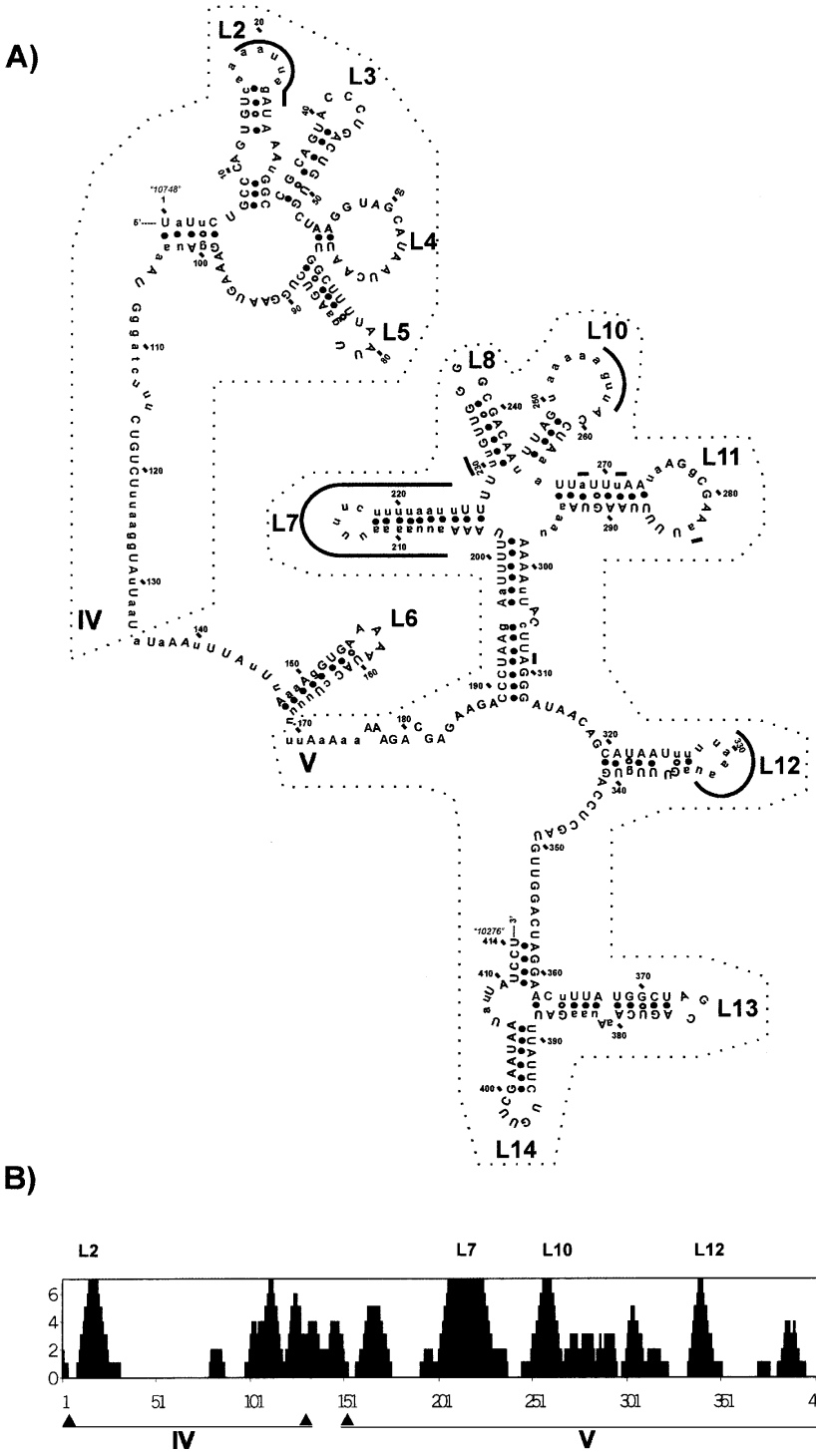

Fig. 4. Putative secondary structure of the sequenced 16S region for *Bulinus* and sliding window analysis to detect heterogeneity across this region. Fig. 4A. Secondary structure for *B. liratus* that approximately covers 10748–10276 bp of the mitochondrial genome of *Katharina tunicata* (MIKTTU098). Nucleotides in caps denote invariant positions within *Bulinus*, nucleotide in lower case denote positions that vary within *Bulinus*. The broken line arcs refer to domains IV and V, the unbroken line arcs denote regions of the 16S that varied within *Bulinus* attributable to insertions/deletions. Fig. 4A. Sliding window analysis reveals heterogeneity across regions of the 16S, the stem and loop regions of L2, L7, L10 and L12 are particularly variable.

not resolvable i.e. a deep polytomy was encountered. *B. obtusispira* was consistently placed within the *B. africanus* group and appears to be ancestral to other members. Moreover it is the species lineage placed closest to the basal node within the phylogram.

16S

The length of the homologous region of the 16S between species of *Bulinus* varied considerably, by 32 bp, with a mean of approximately 400 bp. *B.*

https://doi.org/10.1017/S003118200100806X Published online by Cambridge University Press

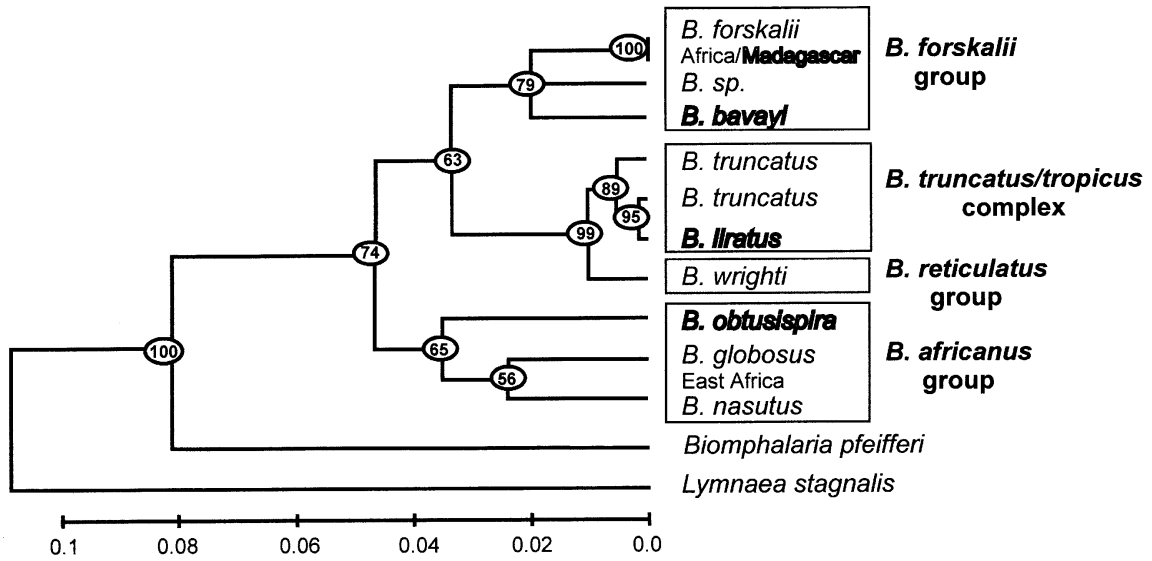

Fig. 5. A phylogram of relationships with *Bulinus* and *Biomphalaria*/*Lymnaea* inferred from the 16S calculated from distance data from Tv substitutions alone. Bootstrap values are placed on each node, the distances on the neighbour-joining phylogram were linearised, topology unchanged, with respect to relative time and the tree is rooted to the outgroups. Madagascan species are in bold typeface.

Table 3. Microsatellite primer pair sets for *B. obtusispira* with predicted size and type of repeat motif

Locus	Expected size (bp)	Type & Repeat	Primers
7S.22*	190	Broken $(GT)_3 \ldots (GT)_{13}$	F 5′-AGACCTAGGTCATGGTGAAA R 5′-CCCTTGCGATCCAATATAC
7S.25	171	Broken $(CA)_4 \ldots (CA)_7$	F 5′-CACTCACACTCACAAACAC R 5′-AGAGGGTGTATATTGTGGTGA
7S.26	243	Broken $(GT)_7 \ldots (GT)_{10}$	F 5′-TCAAGAAATTAATACACACATGA R 5′-ATTTTCCCATTGCAAATTGC
7S.29	190	Broken $(CT)_{10} \ldots (CT)_3$	F 5′-TGTATTCATAATGTGCATGC R 5′-TTTCATCAAACACATTTTACG
7S.33	247	Pure $(GT)_4$	F 5′-AGAACCATAGGAATTTTCCC R 5′-GGTGGGCTTAGTACAGCTTA
7S.34*	264	Broken $(CA)_{14} \ldots (CA)_8$	F 5′-GTCATTGGAATGAGGGATAC R 5′-GGCTTTTATAGTCTCCCTTG
7S.35	325	Broken, compound $(GT)_3 \ldots (CA)_4$	F 5′-ACCAATCAAAACACGAATCT R 5′-GGTTGGTGGATCACATACTC
7S.36*	140	Minisatellite $(AATCATGCTC)_5$	F 5′-ATCGATCTTTGTTTTGGTGA R 5′-CCAAGGGGTGATAACCAAC

* size variation detected in initial primer screen.

truncatus from Senegal possessed the longest sequence (419 bp) while *B. obtusispira* possessed the shortest (387 bp). The 16S sequences from *Bulinus* could be aligned over 419 positions and the nucleotide composition was A:T rich (71·1 %) with individual nucleotide compositions of T (34·7 %), C (11·9 %), A (36·4 %) and G (17 %). Since the 16S does not code for a polypeptide, an analysis of substitutions at each codon position is not possible; however, an analysis across the total number of variable sites (112) was possible and is presented in Fig. 2C. The number Tv was in excess of Ts, a linear relationship of Tv against % sequence divergence could be shown ($y = 2{\cdot}34x + 0{\cdot}77$) with an acceptable coefficient of determination ($R^2 = 0{\cdot}85$). Across the alignment (419 bp), within *Bulinus*, there were 112 variable positions in terms of both insertions/deletions and substitutions. Of the substitutional changes, 72 were parsimony informative and 39 were singletons.

A putative secondary structure model for this part of the 16S is presented (Fig. 4). Further sequence data are required to confirm complementary base pairings over region IV 107–134 bp, region V 169–183 bp and 350–357 bp as these are presumed to pair with bases outside this sequenced region. A sliding window analysis of *Bulinus* sequences across the sequence reveals considerable heterogeneity (Fig. 4B). L2, L7, L10 and L12 are particularly variable where there were numerous insertions or deletions

https://doi.org/10.1017/S003118200100806X Published online by Cambridge University Press

Table 4. Transmission status of laboratory populations of *Bulinus*

Site	Locality	Species	GPS Latitude	GPS Longitude	Exposed (n)	Survival (%)	Infected (%)
1	Vohémar	*B. forskalii* group	13·25·518 S	49·57·316 E	107	49·0	0·0
2	Ambilobe	*B. obtusispira*	13·12·001 S	49·09·832 E	214	57·0	30·3
3	Bealanana 2	*B. liratus*	14·33·456 S	48·43·875 E	297	65·7	0·0
4	Ambatosia 2	*B. liratus*	14·39·933 S	48·39·611 E	158	67·1	0·0
5	Marotandrano #	*B. liratus*	16·10·849 S	48·50·821 E	240	62·5	78·0
6	Route Brieville	*B. liratus*	17·38·455 S	47·45·077 E	25	56·0	0·0
7	Bemokotra	*B. obtusispira*	17·03·423 S	46·41·955 E	249	55·4	5·8
8	Andranomena *	*B. liratus*	18·50·800 S	47·33·500 E	7	71·4	0·0
9	Anosizato-Ouest	*B. forskalii* group	18·56·000 S	47·30·200 E	22	45·5	0·0
10	1800m alt	*B. liratus*	18·53·320 S	46·41·864 E	12	58·3	0·0
11	Fonoraty	*B. forskalii* group	18·56·017 S	46·16·483 E	48	37·5	0·0
12	Manandona #	*B. liratus*	18·53·320 S	46·41·864 E	193	55·4	7·5
13	Sakay	*B. liratus*	19·00·310 S	46·26·990 E	27	48·1	0·0
14	Belanitra	*B. liratus*	19·36·580 S	46·26·393 E	142	69·7	1·0
15	Ambohimanga	*B. liratus*	19·51·073 S	46·55·000 E	46	52·2	0·0
16	Ireninoro	*B. liratus*	19·51·423 S	46·54·833 E	50	74·0	2·7
17	Andoharano	*B. liratus*	19·55·691 S	46·55·806 E	49	49·0	0·0
18	Bemasoandro	*B. liratus*	19·55·069 S	46·55·379 E	62	43·5	0·0
19	Miarinatsimo	*B. liratus*	19·55·197 S	46·55·834 E	12	41·7	0·0
20	Miandrivazo	*B. obtusispira*	18·56·983 S	45·13·583 E	449	46·5	31·1
21	Tsitelo*	*B. forskalii* group	20·15·800 S	44·22·000 E	48	60·4	0·0
22	Andranovorogise	*B. forskalii* group	20·20·495 S	44·33·300 E	89	14·6	0·0
23	Nosibe	*B. forskalii* group	20·15·770 S	44·45·486 E	60	53·3	0·0
24	Manombo	*B. obtusispira*	20·14·308 S	44·47·825 E	437	69·3	26·7
25	Anjamahitsy	*B. forskalii* group	20·17·998 S	44·43·006 E	12	58·3	0·0
26	Tanandava I*	*B. forskalii* group	20·22·200 S	44·41·000 E	36	11·1	0·0
27	Analamitsivalana	*B. forskalii* group	20·19·125 S	44·41·403 E	115	54·8	0·0
28	Tsinjorano-toby	*B. forskalii* group	20·20·520 S	44·35·117 E	42	47·6	0·0
29	Mahabo	*B. forskalii* group	20·22·235 S	44·40·259 E	6	66·7	0·0
30	Bemokoty	*B. forskalii* group	20·21·715 S	44·41·069 E	131	35·9	0·0
31	Soaserana	*B. forskalii* group	20·21·199 S	44·40·877 E	221	23·1	5·9
32	Ankilimida*	*B. forskalii* group	20·21·600 S	44·43·700 E	66	30·3	0·0
33	Ampanihy #	*B. forskalii* group	20·24·196 S	44·43·790 E	19	21·1	50·0
34	Andohaviana	*B. obtusispira*	20·28·980 S	44·38·101 E	178	28·1	82·0
35	Andriamilato	*B. liratus*	21·39·862 S	47·02·774 E	50	66·0	3·0
36	Tsaramandroso	*B. forskalii* group	21·23·361 S	48·01·757 E	16	56·3	0·0
37	Samangoky*	*B. obtusispira*	21·43·000 S	43·45·600 E	145	39·3	0·0
38	Ankiliabo #	*B. liratus*	22·46·326 S	43·35·802 E	74	85·1	9·5
39	Ankililoaka #	*B. liratus*	22·46·684 S	43·36·759 E	252	75·0	2·1
40	Sakaraha	*B. forskalii* group	22·54·926 S	44·32·110 E	96	39·6	0·0
41	Sakaraha	*B. liratus*	22·54·926 S	44·32·110 E	92	85·9	0·0
42	Ranohira #	*B. liratus*	22·34·130 S	45·25·982 E	150	50·7	6·6
43	Ranohira	*B. forskalii* group	22·34·130 S	45·25·982 E	124	36·3	26·7
44	Besely #	*B. liratus*	23·29·436 S	44·31·246 E	53	66·0	20·0
45	Kasaria #	*B. liratus*	24·12·384 S	45·39·843 E	413	64·4	8·3
46	Ampanihy (Sakatovo)	*B. liratus*	24·39·925 S	44·42·780 E	102	68·6	7·1
47	Andrahora	*B. liratus*	24·20·342 S	45·37·631 E	18	83·3	0·0
48	Kariera	*B. liratus*	25·00·039 S	46·36·568 E	135	70·4	35·8
49	Ambolofasy	*B. liratus*	25·02·092 S	46·44·786 E	210	65·7	11·6
50	Ampasy	*B. liratus*	25·02·181 S	46·44·404 E	86	37·2	21·9
51	Befeno #	*B. liratus*	25·01·737 S	46·46·379 E	129	79·8	12·6
52	Sonjorano	*B. liratus*	22·34·220 S	45·52·690 E	17	35·3	0·0
53	Analavoka	*B. liratus*	22·32·760 S	46·29·500 E	18	100·0	0·0

* approximate locations from FTM maps.
\# snails from these locations were also challenged with two isolates of *S. haematobium* from Senegal and Zambia.

(Fig. 4A & 4B). *B. obtusispira* was exceptional in that the L7 region is completely truncated. To enable a direct phylogenetic comparison of the 16S and COI, phylogenetic analysis of the 16S was conducted in the same manner as that for COI. A K-2-P distance only considering Tv substitutions was calculated but gamma corrected ($\gamma = 2$) to account for heterogeneity between sites. Neighbour-joining algorithm was used to infer the topology of the phylogram with bootstrap support of each node (1000 replicates).

https://doi.org/10.1017/S003118200100806X Published online by Cambridge University Press

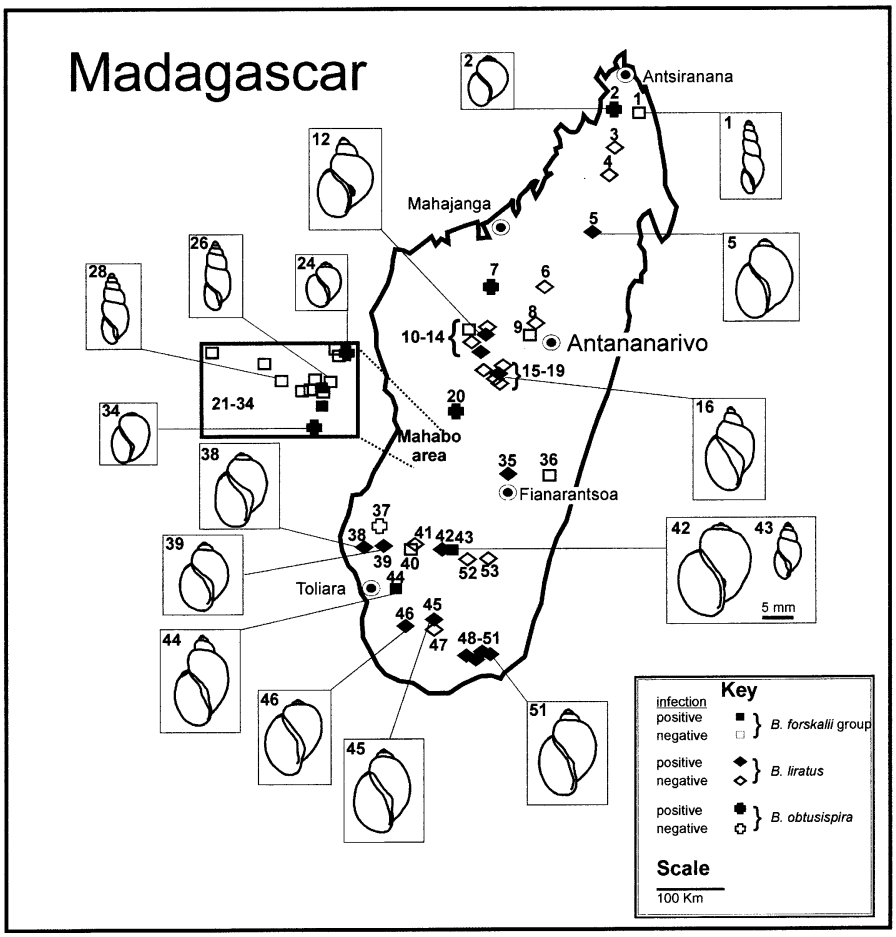

Fig. 6. The compatibility status and approximate distribution of *Bulinus* on Madagascar determined after laboratory challenge to progeny of field-collected populations. Outline shell drawings were made of representatives from some populations/species.

The phylogram distances were linearised to enable comparison of the relative order of bifurcations and two outgroup taxa are also considered (Fig. 5).

The four species groups of *Bulinus* are again obvious and there are contrasting levels of sequence divergence within species groups but there is resolution between the relationships of *B. forskalii* group, *B. truncatus/tropicus* complex and *B. reticulatus* group. There is minimal divergence between Madagascan and African *B. forskalii*, only a single substitutional change was detected. Slightly greater divergence was detected between *B. liratus* and *B. truncatus*, approximately 1·5 % when both Ts and Tv were considered. The relationships within the *B. forskalii* group remain unresolveable. *B. obtusispira* is clearly placed within the *B. africanus* group and appears ancestral within this group and a taxon placed towards the basal node of *Bulinus*.

Microsatellites

A total of 150 transformed, recombinant colonies were picked and probed for microsatellite motifs. Approximately 15 % of transformed colonies were positive by hybridisation. Twenty-two inserts were sequenced in both +ve and –ve directions. From the 20 sequences that could be obtained, 11 primer pairs were designed and tested against genomic DNA from *B. obtusispira*. Upon the basis of an amplification product of approximately the correct size in an agarose gel, 8 primer pairs appeared successful while 3 primers pairs gave non-specific amplification smears and could not be optimised to yield specific amplification products. Size variation, ± 50 bp, of the amplification products within the genomic pool was detectable for 3 of the primer pairs. The successful primer pairs and their microsatellite motifs are detailed in Table 3.

Compatibility with S. haematobium

In total the compatibility status of 53 populations of *Bulinus* has been determined: 29 *B. liratus*, 6 *B. obtusispira* and 18 *B. forskalii* group (the distinction between *B. bavayi* and *B. forskalii* was not made), Table 4. Percentage infection was calculated from the proportion of surviving snails found to be shedding cercariae. Where possible, outline drawing of representative shells were made. The distribution of the populations whose progeny were experimentally infected is shown (Fig. 6). Fifteen *B. liratus*

https://doi.org/10.1017/S003118200100806X Published online by Cambridge University Press

populations were compatible with *S. haematobium*, percent infection of survivors ranged from 2–35 % but with one population, Marotandrano (site 5), with an infection rate of 78 %. *B. liratus* from Befeno and Ranohira were used for DNA analysis (see above). With the exception of Samangoky (site 37), all of the *B. obtusispira* populations were compatible 6–82 %. *B. obtusispira* from Andohaviana (site 34) and Ambilobe (site 2) were used for DNA analysis (see above). The majority of the *B. forskalii* group populations were incompatible. Three populations, however, were able to act as hosts: Ampanihy (site 33)–50 %, Ranohira (site 43)–27 % and Soaserana (site 31)–6 %. Snails from Ranohira were identified as *B. bavayi* and were used for DNA analysis (see above).

DISCUSSION

Molecular evolution

Phylogenetic analysis of the COI and 16S has clarified that *B. obtusispira* is a member of the *B. africanus* group. It appears that this lineage could also be one of the earliest members of the group. Moreover it is placed closest to the basal node of *Bulinus*. Wright's (1971*a*, *b*) supposition that the *B. obtusispira* was a 'relic' species of *Bulinus* that became isolated on Madagascar at a time when the *B. africanus* group had only recently diverged from other bulinids is supported. The topology of the phylograms indicates that *Bulinus* could have colonised Madagascar on at least four separate occasions, with a relative temporal order of proto-*B. obtusispira* first, -*B. bavayi* second, -*B. liratus* third then -*B. forskalii* fourth (Fig. 5). The levels of sequence divergence between Madagascan and African taxa can also be ranked in the reverse of this order, minimal divergence of *B. forskalii* to considerable divergence of *B. obtusispira*.

Whilst the relative temporal placement of nodes can be inferred, one potential problem, however, is placing these events within a specific time-scale, as there are no extrinsic data to calibrate nodes with actual times, neither within *Bulinus*, nor those between *Bulinus* and *Biomphalaria* or *Lymnaea*. If we apply an approximate general mitochondrial molecular clock rate of 2–4 % Myr^{-1} (million years) utilised by Campbell *et al*. (2000) for COI sequences from *Biomphalaria*, then divergence between *B. obtusispira* and other *B. africanus* taxa could have been at the end of the Miocence/beginning of the Pliocene, circa 7 Myr^{-1}. This would be inferred if both transitions and transversions are considered [K-2-P, 16S ~ 10 %, COI ~ 14 %]. Presumably shortly before this time, *Bulinus* would have undergone a radiation of species such that the present day dispersal of populations does not reflect the long-term patterns of vicariances. Even if the general molecular clock is true the observed nucleotide distances between *Bulinus* may be wrong and a gross underestimate, since Ts introduce unacceptable errors to the data, so the time frame of the Miocence/Pliocene is too early.

Inspection of the non-linear relationships of transitions against percent sequence divergence is indicative of strong 'saturation' or 'multiple hits'. Transitional saturation has been associated with long diveregence times (Moritz, Dowling & Brown, 1987). Despite application of a correction factor, the K-2-P distance, the calculated divergence will still, however, be an underestimate of the true value. If this value is incorrect by approximately one order of magnitude then a vicariance theory incorporating Gondwana break-up may better explain the distribution of *B. obtusispira*. It would also point towards an early invasion of *B. bavayi* to Madagascar from nearby Africa with much later invasions of proto-*B. liratus* and *B. forskalii*. There are perhaps two other observations that favour the latter interpretation. Firstly, comparisons between populations of *B. truncatus/tropicus* species followed the classical Ts:Tv ratio $\gg 1$, whereas for interspecific comparisons within other species groups, this ratio often inverted i.e. < 1 (Fig. 2B). Secondly, when transversions alone were considered, there was good resolution between *Bulinus* and the outgroup taxa *Biomphalaria* and *Lymnaea*. If both Ts and Tv are considered, the molecular data implies the most recent common ancestor of *Bulinus* and *Biomphalaria/Lymnaea* to have been during the early Miocene, circa 20 Myr^{-1} which would be clearly at odds with the fossil record (Baker, 1945) that places the ancestral lymnaeid stock in the Permian, circa 250 Myr^{-1} (Remigio & Blair, 1997). Sampling of 16S variation within *Indoplanorbis* will help to date the relationships within *Bulinus* as the division of these two taxa might also be linked to continental break-up during the Cretaceous. For example, *Indoplanorbis* might have arrived to present-day Asia via the northward migrating Indian land-mass.

Molecular markers

Previous molecular and biochemical evidence has pointed towards *Bulinus* as a divergent group of snails that do not neatly fit within the variation shown by a genus (Biocca *et al*. 1979; Jelnes, 1987; Stothard *et al*. 1996). Morphologically, however, the snails are perhaps not differentiated enough to justify a generic revision (Brown, 1981). The lack of clear cut morphological characters also hampers specific identifications. For example, whilst *B. globosus* and *B. nasutus* on Zanzibar are clearly differentiated upon molecular characters (Stothard *et al*. 1997; Stothard & Rollinson, 1997*a*, *b*) and phylogenetic analysis (Stothard & Rollinson, 1997*a*), conchologically they can be confused since there appears to

https://doi.org/10.1017/S003118200100806X Published online by Cambridge University Press

be overlapping variation of the shell (Stothard *et al*. 1997). Even placement of species, e.g. *B. obtusispira* and *B. umbilicatus*, to a species group of *Bulinus* is problematic if morphological characters alone are considered. The problematic morphology calls for molecular characters for identification and for assays to be developed that can easily differentiate taxa e.g. *B. forskalii* group (Jones *et al*. 1999) or *B. africanus* group (Rollinson, Stothard & Southgate, this supplement).

Better methods of identification and discrimination of *B. obtusispira* and *B. liratus* are required especially where populations exhibit atypical morphology or there is only access to alcohol-preserved specimens that would preclude isoenzyme analysis. By taking advantage of the variable nucleotides within COI a PCR-RFLP assay can be developed by selecting restriction enzymes that cut specifically at sites that differentiate the two taxa. For example, upon *Hpa*II digestion of the COI, two fragments of approximately 240 and 210 bp are produced for *B. obtusispira*, while *B. liratus* amplification products would remain intact since there are no cutting sites; conversely, *Rsa*I digestion would produce two fragments of approximately 350 and 100 bp for *B. liratus*, while *B. obtusispira* amplification products would remain intact. As both *Hpa*II and *Rsa*I can be combined within the same digestion reaction, double digestions would be a powerful, simple method to differentiate each taxa at these enzyme cutting sites.

To enable calculation of Hardy-Weinberg equilibrium and (or) gene flow between populations of snails, nuclear markers that vary within populations are required. Microsatellites have several advantages as markers for detailed genetic analysis (Schlötterer & Pemberton, 1994) and have been used to examine mating systems and genetic structure of *Bulinus truncatus* (Jarne *et al*. 1994; Viard & Jarne, 1997). Microsatellite primers designed on one species, however, generally do not cross-amplify microsatellites in other species. To initiate such a population genetics study for a previously uncharacterized species, PCR primers have to be designed afresh (Jarne & Lagoda, 1996). This stage of development is recognized to be time-consuming. Even after isolation and primer design there can be significant loss of primer sets since they may fail to amplify the locus from genomic DNA, null alleles are encountered or the loci proves to be invariant. The 8 primer sets designed to *B. obtusispira* pass the first requirements, discrete amplification product(s) were obtained. For two of the primer sets, size variation was also apparent. Further analysis of populations of *B. obtusispira* is ongoing, as well as the use of denaturing polyacrylamide gels for better fragment sizing, to determine if all primers sets will be useful for detailed population genetic analysis. Using these markers, it would be interesting to assess the genetic diversity of *B. obtusispira* populations across Madagascar and the effect that parasite pressure has upon them.

Compatibility studies

After laboratory infections, three species: *B. obtusispira*, *B. liratus* and *B. bavayi* have been shown to be able to act as intermediate hosts for *S. haematobium* from the Mahabo area. However, our ongoing malacological surveys of *Bulinus* have so far failed to find evidence of natural infections of human schistosomes; this contrasts with *Biomphalaria* where natural infections are often encountered. Degrémont (1973) found natural infections in both *B. obtusispira* and in a single population of *B. liratus*. From laboratory infection studies, compatibility of both species could be shown while *B. bavayi* appeared refractory (Degrémont, 1973). Wright (1971*a*), however, was able to show that *B. bavayi* was compatible with a *B. cernicus*-strain of *S. haematobium* from Mauritius and with a strain from South Africa (Wright & Knowles, 1972).

Given the specific characteristics of the Malagasy fauna, Degrémont (1973) concluded that it would be dangerous to systematically eliminate *B. liratus* from the epidemiology of urinary schistosomiasis on Madagascar only on the pretext that diploid *B. truncatus/tropicus* complex species do not transmit human schistosomes in Africa. The COI data suggest that *B. liratus* is closely related but different from African *B. tropicus* and despite being diploid, shares a slightly greater affinity to *B. truncatus* from Malawi than other species examined. Expanding upon Degrémont's findings, there appear to be populations of *B. liratus* on Madagascar that have some compatibility with *S. haematobium*. To examine this compatibility further, nine populations (sites 5, 12, 33, 38, 39, 43, 44, 45 and 51) were exposed to two *B. globosus*-borne strains of *S. haematobium* from Senegal and Zambia. No compatibility could be shown with either of these strains despite twice repeating exposure with 5 miracidia per snail (J. R. Stothard, unpublished observations). It would be interesting to challenge *B. liratus* against other isolates of African *S. haematobium*.

The potentially broad compatibility of Madagascan *S. haematobium* with three of the species groups of *Bulinus* is rather unusual as normally strains of *S. haematobium* that develop in *B. africanus* group snails are not compatible with *B. truncatus/tropicus* complex snails and *vice versa* (see Rollinson, Stothard & Southgate, this supplement). The development of Madagascar *S. haematobium* in *B. obtusispira* is unusual since many secondary sporocysts remain in the snail foot, an adaptation to enhance production of cercariae (Jourdane, 1983). It will be of interest to make molecular comparisons of parasite isolates from Madagascar and mainland Africa as it is not yet known from where, or when,

https://doi.org/10.1017/S003118200100806X Published online by Cambridge University Press

the parasite was introduced. This could be as recently as during the last Millennium. Madagascar was only 'discovered' in the 15th Century by the Portuguese, although other human settlements pre-date. The first humans arrived, after leaving their Malaysian-Indonesian homeland, somewhere between 500 BC and 500 AD (Preston-Mafham, 1991).

The presence of compatible species of *Bulinus* precedes that of human movements to the island so that once *S. haematobium* was introduced, natural transmission could be sustained. The distribution of *S. mansoni* on Madagascar is spreading (Ollivier *et al*. 1999) but it is not clear when its intermediate snail host *Biomphalaria pfeifferi* first arrived. Other schistosome intermediate hosts have either failed to colonise the island or are yet to be introduced; despite the influx of people from the Far East infected with *S. japonicum*, this form of the disease has not established since there are no suitable intermediate snail hosts (Brygoo, 1972). The snail compatibility studies suggest that *S. haematobium* with different intermediate host specificities may have been introduced to Madagascar and (or) that Madagascan *Bulinus* in the absence of parasite pressure lost, or never possessed, the internal defence systems that evolved in African *Bulinus*. Further studies on natural transmission of *S. haematobium* and defence mechanisms of *Bulinus* will help clarify the intermediate host range on Madagascar.

ACKNOWLEDGEMENTS

JRS gratefully acknowledges funding from The Wellcome Trust. We thank the staff of Institut Pasteur, Antananarivo and I.R.D. for assistance with malacological surveys and maintenance of snail laboratory cultures. We thank Mike Anderson and Viv Tuffney for snail maintenance and Julia Bartley for running the ABI automated sequencer at the NHM. The study benefited from computer facilities at the Human Genome Mapping project ''www.hgmp.mrc.ac.uk''.

REFERENCES

ANON. (1999). Country file: Madagascar. *Geographical* **71**, 40.

BAKER, F. C. (1945). *The Molluscan Family Planorbidae*. Urbana, University of Illinois Press, p. 530.

BIOCCA, F., BULLINI, L., CHABAUD, A., NASCETTI, G., ORECCHIA, P. & PAGGI, L. (1979). Subdivisions su base morfologica e genetica del genre *Bulinus* in tre generi: *Bulinus* Müller, *Physopsis* Kraus e *Mandahlnarthia gen. Nov*. Rendiconti della classe di Scicenze Fisiche. Mateematiche e Naturali. *Accademia Nazionale del Lincei* **66**, 276–282.

BROWN, D. S. (1981). Generic nomenclature of freshwater snails commonly classified within the genus *Bulinus* (Gastropoda: Basommatophora). *Journal of Natural History* **15**, 909–915.

BROWN, D. S. (1994). *Freshwater Snails of Africa and their Medical Importance*, 2nd Edition. London, Taylor & Francis, p. 609.

BROWN, D. S. & WRIGHT, C. A. (1978). A new species of *Bulinus* from temporary freshwater pools in Kenya. *Journal of Natural History* **12**, 217–229.

BRYGOO, E. R. (1972). Human diseases and their relationship to the environment. In *Biogeography and Ecology in Madagascar*, pp. 703–725. (ed. Battistini, R. & Richard-Vindard, G.) The Hague, Dr. W. Junk B. V., Publishers.

BRYGOO, E. R. & MOREAU, J. P. (1966). *Bulinus obtusispira* (Smith, 1882), hôte intermédiarire de la Bilharziose à la *Schistosoma haematobium* dans le nord-ouest de Madagascar. *Bulletin de la Société de Pathologie Exotique* **59**, 835–839.

CAMPBELL, G., JONES, C. S., LOCKYER, A. E., HUGHES, S., BROWN, D., NOBLE, L. R. & ROLLINSON, D. (2000). Molecular evidence supports an African affinity of the neotropical freshwater gastropod, *Biomphalaria glabrata*, Say 1818, an intermediate host for *Schistosoma mansoni*. *Proceedings of the Royal Society of London*, *B* **267**, 2352–2358.

DAVIS, G. M., WILKE, T., SPOLSKY, C., QIU, C.-P., QIU, D.-C., XIA, M.-Y., ZHANG, Y. & ROSENBERG, G. (1998). Cytochrome oxidase I-based phylogenetic relationships among the Pomatiopsidae, Hydrobiidae, Rissoidae and Truncatellidae (Gastropoda: Caenogastropoda: Rissoacea). *Malacologia* **40**, 251–266.

DEGRÉMONT, A. E. (1973). *Mangoky Project : Campaign against Schistosomiasis in the Lower-Mangoky* (*Madagascar*). Basle, Swiss Tropical Institute, pp. 261.

DOUMENGE, J. P., MOTT, K. E., CHEUNG, C., VILLENAVE, D., CHAPUIS, O., PERRIN, M. F. & REAUD-THOMAS, G. (1987). *Atlas de la Répartition Mondiale des Schistosomiases/Atlas of the Global Distribution of Schistosomiasis*. Talence, CEGET-CNRS; Genève, OMS/WHO; Talence, PUB, pp. 400.

FELSENSTEIN, J. (1993). *PHYLIP – Phylogeny Inference Package*, Version 3.5. Seattle, Washington: Department of Genetics, University of Washington.

HAMMOND, R. L., SACCHERI, I. J., CIOFI, C., COOTE, T., FUNK, S. M., MCMILLAN, W. O., BAYES, M. K., TAYLOR, E. & BRUFORD, M. W. (1998). Isolation of Microsatellite Markers in Animals. In *Molecular Tools for Screening Biodiversity*, pp. 279–296. (ed. Karp, A., Isaace, P. & Ingram, D.) London, Chapman & Hall.

HIGGINS, D. G., BLEASBY, A. J. & FUCHS, R. (1992). CLUSTAL-V: improved software for multiple sequence alignment. *Computer Applications in the Biological Sciences* **8**, 189–191.

JARNE, P. & LAGODA, P. J. L. (1996). Microsatellites, from molecules to populations and back. *Trends in Ecology and Evolution* **11**, 424–429.

JARNE, P., VIARD, F., DELAY, B. & CUNY, G. (1994). Variable microsatellites in the highly selfing snail *Bulinus truncatus* (Basommatophora: Planorbidae). *Molecular Ecology* **3**, 527–528.

JELNES, J. E. (1984). Taxonomie expérimental de *Bulinus*. 6. Possibilité d'utiliser des charactères enzymatiques pour la distinction entre des espèces *Bulinus liratus* et *B. obtusispira* à Madagascar. *Archives de l'Institut Pasteur de Madagascar* **51**, 89–96.

JELNES, J. E. (1987). Enzymelektroforese avendt til belysning af afrikanse og amerikanske bilharziosesnegles systematik. *Privatety published* (in Danish with English summary).

JOLLY, A., OBERLE, P. H. & ALBIGNAC, R. (1984). Introduction. In *Madagascar* (eds. Jolly, A., Oberle, P. H. & Albignac, R.) Oxford, Pergamon Press Ltd., p. 239.

JONES, C. S., NOBLE, L. R., OUMA, J., KARIUKI, H. C., MIMPFOUNDI, R., BROWN, D. S. & ROLLINSON, D. (1999). Molecular identification of schistosome intermediate hosts: case studies of *Bulinus forskalii* group species (Gastropoda: Planorbidae) from central and East Africa. *Biological Journal of the Linnean Society* **68**, 215–240.

JOURDANE, J. (1983). Mise en évidence d'un processus original de la reproduction asexuée chez *Schistosoma haematobium*. *Comptes Rendus de l'Académie des Sciences, Paris* **296**, 419–424.

KJER, K. M. (1995). Use of rRNA secondary structure in phylogenetic studies to identify homologous positions: an example of alignment and data presentation from the frogs. *Molecular Phylogenetics and Evolution* **4**, 314–330.

KUMAR, S., TAMURA, K. & NEI, M. (1993). *MEGA: Molecular Evolutionary Genetics Analysis, Ver. 1.1.* University Park, PA, The Pennsylvania State Univ.

MATZURA, O. & WENNBORG, A. (1996). RNAdraw: an integrated program for RNA secondary structure calculation and analysis under 32-bit Microsoft Windows. *Computer Applications in the Biological Sciences* **12**, 247–249.

MILDENHALL, D. C. (1980). New Zealand Late Cretaceous and Cenozoic plant biogeography: a contribution. *Palaeogeography Palaeoclimatology Palaeoecology* **31**, 197–233.

MORITZ, C., DOWLING, T. E. & BROWN, W. M. (1987). Evolution of animal mitochondrial DNA: relationships for population biology and systematics. *Annual Review of Ecology and Systematics* **18**, 269–292.

OLLIVIER, G., BRUTUS, L. & COT, M. (1999). Schistosomiasis due to *Schistosoma mansoni* in Madagascar: spread and focal patterns. *Bulletin de la Société de Pathologie Exotique* **92**, 99–103.

PIQUÉ, A. (1999). The geological evolution of Madagascar: an introduction. *Journal of African Earth Sciences* **28**, 919–930.

PRESTON-MAFHAM, K. (1991). *Madagascar: A Natural History*. Oxford, Facts on File, p. 224.

RABINOWITZ, P. D., COFFIN, M. F. & FALVEY, D. (1983). The separation of Madagascar and Africa. *Science* **220**, 67–69.

RAVEN, P. H. (1979). Plate tectonics and Southern Hemisphere biogeography. In *Tropical Botany* (ed. Larsen, K.), pp. 1–24. London, Academic Press Ltd.

REMIGIO, E. A. & BLAIR, D. (1997). Molecular systematics of the freshwater snail family Lymnaeidae (Pulmonata, Basommatophora) utilising mitochondrial ribosomal DNA sequences. *Journal of Molluscan Studies* **63**, 173–185.

ROLLINSON, D. & SOUTHGATE, V. R. (1987). The genus *Schistosoma*: a taxonomic appraisal. In *The Biology of Schistosomes: Genes to Latrines* (eds. Rollinson, D. & Simpson, A. J. G.), pp. 1–49. London, Academic Press Ltd.

ROLLINSON, D., STOTHARD, J. R. & SOUTHGATE, V. R. (2001). Interactions of *Bulinus* and schistosomes of the *Schistosoma haematobium* group. *Parasitology*, in press.

SCHLÖTTERER, C. & PEMBERTON, J. (1994). The use of microsatellites for genetic analysis of natural populations. In *Molecular Ecology and Evolution: Approaches and Applications*. (eds. Schierwater, B., Streit, B., Wagner, G. P. & DeSalle, R.) Basel, Birkhäuser Verlag.

SMITH, A. G., SMITH, D. G. & FUNNELL, B. M. (1994). *Atlas of Mesozoic and Cenozoic Coastlines*. Cambridge, Cambridge University Press.

STARMÜHLNER, F. (1969). Die gastropoden der Madagassischen binnengewässer. *Malacologia* **8**, 1–434.

STOREY, M., MAHONEY, J. J., SAUNDERS, A. D., DUNCAN, R. A., KELLEY, S. P. & COFFIN, M. F. (1995). Timing of hot spot-related volcanism and the break-up of Madagascar and India. *Science* **267**, 852–855.

STOTHARD, J. R., HUGHES, S. & ROLLINSON, D. (1996). Variation within the Internal Transcribed Spacer (ITS) of ribosomal DNA genes of intermediate snails hosts within the genus *Bulinus* (Gastropoda: Planorbidae). *Acta Tropica* **61**, 19–29.

STOTHARD, J. R., MGENI, A. F., ALAWI, K. S., SAVIOLI, L. & ROLLINSON, D. (1997). Observations on shell morphology, enzymes and random amplified polymorphic DNA (RAPD) in *Bulinus africanus* group snails (Gastropoda: Planorbidae) in Zanzibar. *Journal of Molluscan Studies* **63**, 489–503.

STOTHARD, J. R. & ROLLINSON, D. (1997*a*). Partial DNA sequences from the mitochondrial cytochrome oxidase subunit I (COI) gene can differentiate the intermediate snail hosts *Bulinus globosus* and *B. nasutus* (Gastropoda: Planorbidae). *Journal of Natural History* **31**, 727–737.

STOTHARD, J. R. & ROLLINSON, D. (1997*b*). Molecular characterisation of *Bulinus globosus* and *B. nasutus* on Zanzibar, and an investigation of their roles in the epidemiology of *Schistosoma haematobium*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **91**, 353–357.

STRIMMER, K. & VON HAESELER, A. (1997). *Puzzle. Maximum Likelihood Analysis for Nucleotide and Amino Acid Alignments*. Munchen, Germany, Zoologisches Institut.

SWOFFORD, D. L. (1999). *PAUP*4.0. Phylogenetic Analysis Using Parsimony* (*and other methods*). Sunderland, MA, Sinauer.

TAJIMA, F. (1989). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* **123**, 585–595.

THOLLESSON, M. (1999). Phylogenetic analysis of dorid nudibranchs (Gastropoda: Doridacea) using the mitochondrial 16S rRNA gene. *Journal of Molluscan Studies* **65**, 335–353.

TRISTRAM, H. B. (1863). Note on some freshwater shells sent from Madagascar by J. Caldwell, Esq. *Proceeding of the London Zoological Society*, 60–61.

VIARD, F. C. & JARNE, P. (1997). Selfing, sexual polymorphism and microsatellites in the

hermaphroditic freshwater snail *Bulinus truncatus*. *Proceedings of the Royal Society London B* **264**, 39–44.

WRIGHT, C. A. (1971*a*). *Bulinus* on Aldabra and the subfamily Bulininae in the Indian Ocean area. *Philosophical Transactions of the Royal Society London B* **260**, 299–313.

WRIGHT, C. A (1971*b*). *Flukes and Snails*. Science of Biology Series, No. 4. London, George Allen and Unwin Ltd, p. 168.

WRIGHT, C. A. & KNOWLES, R. J. (1972). Studies on *Schistosoma haematobium* in the laboratory III. Strains from Iran, Mauritius and Ghana. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **66**, 108–118.

WRIGHT, C. A. & ROLLINSON, D. (1979). Analysis of enzymes in the *Bulinus africanus* group (Mollusca: Planorbidae) by isoelectric focusing. *Journal of Natural History* **13**, 263–273.

https://doi.org/10.1017/S003118200100806X Published online by Cambridge University Press