# Molecular Characterisation of Intermediate Snail Hosts and the Search for Resistance Genes

**David Rollinson/+ , J Russell Stothard, Cathy S Jones*, Anne E Lockyer*, Cecilia Pereira de Souza**, Les R Noble***

The Natural History Museum, Cromwell Road, London SW7 5BD, UK *Zoology Department, Aberdeen University, AB9 2TN, UK **Centro de Pesquisas René Rachou-Fiocruz, Belo Horizonte, MG, Brasil

*The relationship between schistosomes and their intermediate hosts is an extremely intricate one with strains and species of the parasite depending on particular species of snail, which in turn may vary in their susceptibility to the parasites. In order to gain a better understanding of the epidemiology of the disease we have been investigating the use of molecular markers for snail identification and for studying host-parasite relationships. In this paper we will draw on examples concerning schistosomiasis in West and East Africa to illustrate how a molecular analysis can be used as part of a "total evidence" approach to characterisation of* Bulinus *species and provide insights into parasite transmission. Particular emphasis is given to ribosomal RNA genes (rRNA), random amplified polymorphic DNA (RAPDs) and the mitochondrial gene cytochrome oxidase I (COI). Snails resistant to infection occur naturally and there is a genetic basis for this resistance. In* Biomphalaria glabrata *resistance to* Schistosoma mansoni *is known to be a polygenic trait and we have initiated a preliminary search for snail genomic regions linked to, or involved in, resistance by using a RAPD based approach in conjunction with progeny pooling methods. We are currently characterising a variety of STSs (sequence tagged sites) associated with resistance. These can be used for local linkage and interval mapping to define genomic regions associated with the resistance trait. The development of such markers into simple dot-blot or specific PCR-based assays may have a direct and practical application for the identification of resistant snails in natural populations.*

Key words: *Bulinus* - *Biomphalaria* - molecular systematics - epidemiology

In recent years, new opportunities for the generation of molecular characters have been provided for the malacologist due to the introduction of various molecular techniques including the polymerase chain reaction (PCR) and DNA sequencing. This paper details some of our recent molecular approaches to the characterisation of *Bulinus* species in Africa and presents preliminary information concerning the search for resistance genes in *Biomphalaria glabrata*.

## CHARACTERISATION OF *BULINUS*

Freshwater snails of the genus *Bulinus* act as the intermediate hosts for *Schistosoma haematobium* and related species and occur commonly throughout much of Africa and adjacent regions. There are currently 37 species of *Bulinus* recognised (Brown 1994) but the specificity of the snail parasite interaction is such that only certain species are involved in transmission of the parasite. The genus can be divided into four major groups of species. The *B. forskalii* group contains 11 species with slender shells and usually high spires and is practically pan-African in distribution with species occurring on some of the surrounding islands and the Arabian peninsula. The *B. africanus* group has 10 species confined to the Afrotropical region. The *B. truncatus/tropicus* complex, which contains polyploid species, is again pan-African with 14 representatives extending into the Middle East, Mediterranean islands and the Iberian Peninsula; the two species included in the *B. reticulatus* group both have restricted distributions. Within each group there are species that act as intermediate hosts for schistosomes in part or all of their geographical range.

The host-parasite relationship can be viewed in terms of snail susceptibility and parasite infectivity. Some parasites appear to be genetically heterogeneous with regard to infectivity and are compatible with a wide variety of snail hosts whereas others are far more restricted in their choice of host (see Rollinson & Southgate 1987). For example,

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+Corresponding author. E-mail:D.Rollinson@nhm.ac.uk
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*S. haematobium,* the cause of urinary schistosomiasis in man, is known to be transmitted by at least 12 species of *Bulinus* in Africa and adjacent regions. In contrast, natural transmission of *S. intercalatum* appears to be confined to two different species, *B. globosus* and *B. forskalii.* Not only do differences exist between species of schistosome in their ability to infect snails but marked differences can exist between strains of the same species. Strains of *S. haematobium* which are compatible with *B. africanus* group snails are usually incompatible with *B. truncatus* and *vice versa.* Similarly, variation in susceptibility to a parasite can occur between populations of the same snail species.

Considerable efforts have been focused on providing reliable methods for the differentiation and identification of *Bulinus* species in order to determine those species and strains playing a major role in schistosomiasis transmission. Differences among taxa have been sought traditionally by analysis of morphological variation and determination of chromosome number. Enzyme electrophoresis has been used to supplement such studies and has proved useful for identification, elucidation of relationships between taxa and for studying aspects of reproductive biology and population structure (e.g. Biocca et al. 1979, Jelnes 1979, 1986, Rollinson & Southgate 1979, Rollinson & Wright 1984, Njiokou et al. 1993).

Various molecular approaches have been investigated for the identification and characterisation of *Bulinus* species including: the examination of variation in ribosomal RNA (rRNA) genes, as determined by conventional restriction fragment length polymorphism (RFLP) analysis and by PCR-RFLP of the internal transcribed spacer (ITS); the use of randomly amplified polymorphic DNA (RAPDs) and sequence analysis of mitochondrial genes.

*Ribosomal RNA genes* - The genomic ribosomal RNA (rRNA) gene complex is common to all eukaryotes and is well suited for taxonomic studies as it contains regions which evolve at different rates, thus permitting analysis of relationships over a wide taxonomic level. In general the spacer regions are less conserved than the coding regions. DNA probes have been shown to be of value for distinguishing schistosome species and particular use has been made of the rRNA gene probes pSM889, pSM890 and pSM389 derived from *S. mansoni* (Simpson et al. 1984, Johnston et al. 1993). The probe pSM889 has been used to study restriction enzyme digests of various species of *Bulinus.* This probe is a 4.4 kb fragment that encompasses part of the small rRNA, ITS and large rRNA gene. As it contains highly conserved regions it shows homology with a wide variety of organisms and will readily bind to restriction fragments of snail DNA. Clear differences in the sizes of restriction fragments between species of *Bulinus* representing the four species groups were observed when DNA was digested with either *Bam*HI or *Bgl*II and hybridised to pSM889. No differences were observed between samples of *B. tropicus* and *B. truncatus* but intraspecific variation was observed between samples of *B. forskalii* from São Tomé and Angola (Rollinson & Kane 1991). This work suggested that sequence variation in the spacer regions may be useful for discrimination. The entire ITS region including the 5.8S rRNA gene has now been amplified from species representing the four species groups following the methods of Kane and Rollinson (1994). The sequences for the primers used for PCR amplification were based on conserved regions of the 3' end of the 18S rRNA gene (ETTS1) and the 5' end of the 28S rRNA gene (ETTS2). The amplified product was digested with one of a number of 4-base or 6-base cutting restriction enzymes (*Rsa*I, *Alu*I, *Cfo*I, *Sma*I and *Sac*I). Characteristic RFLP patterns were obtained for the four species groups and, in the majority of cases, species within a species group could be differentiated (Stothard et al. 1996). Distinctions may be caused by variation in the number of restriction sites within the spacer region or by differences in sequence length between the restriction sites. The complete ITS1 spacer region has been sequenced for *B. globosus*, *B. cernicus* and *B. truncatus* and substantial nucleotide variation occurs indicating large divergence between the species groups (Stothard et al. 1996). The results are in agreement with earlier enzyme studies (Biocca et al. 1979) which also showed large divergence between the species groups of *Bulinus.*

*RAPD analysis* - Unlike conventional PCR-based analyses, RAPD approaches use single oligonucleotide primers of arbitrary sequence (between 5-20 bases) to initiate DNA strand synthesis under conditions of low stringency at a number of complementary binding sites scattered throughout the genome (Welsh & McClelland 1990, Williams et al. 1990). Discrete amplification products form where primer sites are orientated in an inverted repeat and are within an amplifiable distance of each other. Several amplification fragments may be generated within a single RAPD reaction and, when visualised by either agarose or polyacrylamide electrophoresis separation, give rise to a RAPD profile. Twenty-eight primers obtained from Operon Technologies Ltd and British Biotechnology were screened against genomic DNA extracted

from *B. sudanica* to identify primers which gave reproducible and informative RAPD profiles. Of these primers, eight (4 x 10 mers, 4 x 15 mers) were subsequently selected to study genetic variation within and between nine species of *Bulinus* (Stothard & Rollinson 1996). RAPD profiles were visualised using both polyacrylamide gel electrophoresis with silver staining and agarose gel electrophoresis with ethidium bromide staining. This approach allowed a direct comparison of the two methods and therefore permitted an evaluation of the stability of the derived phylogenetic relationships.

RAPD profiles were highly divergent between the species of *Bulinus* and intra-specific variation was also observed. PAGE/silver staining methods visualised a greater number of RAPDs in comparison with agarose/ethidium bromide methods. Phylogenetic tree construction was performed using a least squares method utilising the Fitch-Margoliash criterion. The resulting phenograms derived from both data sets had slightly different topologies and were almost constrained into a polytomy. It was concluded that this phenomenon was most likely attributable to nucleotide divergence between species exceeding 10%. Hence RAPD data have little value for comparisons between species groups and diagnostic RAPD profiles for a species throughout its range might be difficult to find. However, RAPDs do allow the identification of differences between species, populations and individuals on a regional basis and we have used this approach for members of the *B. forskalii* group.

Species of the *B. forskalii* group are not defined entirely satisfactorily. The present system is founded on characters of the shell and anatomy, particularly the shape of the mesocone on the first lateral radular tooth, and the presence or absence of a carina on the shell surface. However, the appearance of these features is variable and the absence of a carina is not always indicative. These morphological characters are not easily used by the non-specialist and many workers experience difficulty in differentiating species within the group. Currently there are 11 species recognised within the *B. forskalii* group, three of which are represented in Cameroon; *B. forskalii, B. senegalensis* and *B. camerunensis.* Mimpfoundi and Greer (1989) compared allozyme patterns among members of the *B. forskalii* group from Cameroon. Clear differences were found between *B. forskalii* and *B. senegalensis* but allozyme patterns for *B. camerunensis* were identical to those of *B. forskalii* from some localities. A more detailed study of 32 *B. forskalii* populations revealed intrapopulation variation in eight populations but heterozygotes were present in only two of these and neither population was in Hardy-Weinberg equilibrium (Mimpfoundi & Greer 1990). The results suggested low genetic diversity and indicated that *B. forskalii* reproduces principally by self-fertilisation.

In Cameroon, *B. forskalii* acts as a host for *S. intercalatum* whereas *B. senegalensis* and *B. camerunensis* have been implicated in the transmission of *S. haematobium.* Mixed populations of *B. forskalii* and *B. senegalensis* have been found in northern Cameroon. Twenty RAPD primers have now been used to characterize, in detail, a minimum of 10 individuals from 21 populations of *B. forskalii* taxa, including populations of *B. forskalii* from throughout Cameroon, several from Senegal and one from São Tomé; *B. senegalensis* from the type locality in Senegal and other localities, north Cameroon and Mali; *B. cernicus* from Mauritius and *B. crystallinus* from Angola; *B. camerunensis* from southwest Cameroon; a laboratory population of *B. wrighti* originally from Oman; and one population of *B. truncatus* from Cameroon used as an outgroup.

Few fragments were shared between *B. forskalii* and *B. senegalensis*, hence, unknown specimens conchologically assigned to these taxa may be identified to the specific level by RAPD profiling. No intraspecific polymorphisms were detected in the type locality material of *B. senegalensis* from Senegal. However, limited within and between population variation was found in *B. senegalensis* from north Cameroon. Similarly, limited within population variation was demonstrated in *B. forskalii*, but greater differences between populations were observed, especially between geographically separated localities.

Comparison of RAPD profiles is useful, although standardizing PCR parameters between laboratories and interpretation of the resulting banding patterns may be problematic. This difficulty may be resolved by identifying and excising 'unique' fragments which are then used as probes on full Southern blots of the RAPD gels, making species identification less ambiguous. Several such species-specific markers have been identified for *B. forskalii* and *B. senegalensis*. However, when hybridized to total genomic DNA dot blots species-specificity was lost. In order to develop a simple identification procedure, diagnostic RAPD products have been cloned and sequenced and a panel of species-specific primers have been designed for use in a PCR-based species identification test (Jones et al. 1997).

RAPD fragments 'shared' between taxa can be labelled as probes to check homology and then used as characters in cluster analysis. Phylogenetic analyses, using the Fitch-Margoliash criterion, sug-

gest that *B. forskalii* and *B. senegalensis* cluster on distinct branches, demonstrating that these morphologically similar species are clearly differentiated by molecular methods. Although identified as a separate species on morphology *B. camerunensis* clearly clusters with specimens identified as *B. forskalii*. In particular its affinity with *B. forskalii* from nearby Kumba, southwest Cameroon (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. Other *B. forskalii* group species, such as *B. cernicus* and *B. crystallinus*, cluster with *B. truncatus* to form a third distinct branch. Conversely, the molecular data suggest samples of what are tentatively described as an extreme geographical variant of *B. forskalii* from São Tomé on shell morphology, do in fact cluster with *B. crystallinus* from Angola. Given the historical links between these two places this seems plausible, but more samples are required to confirm this hypothesis.

*Mitochondrial DNA* - The mitochondrion exists as an organelle and is responsible for aerobic respiration within eukaryote cells. In addition to the genetic material found within each cell nucleus, the mitochondrion also contains its own DNA which codes for several proteins and transfer RNA molecules as well as two ribosomal RNAs. The mitochondrial genome of molluscs is circular and for the gastropods *Albinaria turrita* and *Cepaea nemoralis* is approximately 14 kb in length (Lecanidou et al. 1994, Terret et al. 1994). As this molecule does not recombine and is usually maternally inherited, sequence variation within the DNA of this molecule provide excellent characters for phylogenetic studies and, in particular, for cladistic analysis. One such gene coding for the cytochrome oxidase subunit I (COI) has had increasing attention as a phylogenetic marker as sequence variation within this gene is accumulating at a rate which allows the determination of relationships both within and between species. In addition, certain regions within this molecule are highly conserved enabling portions to be amplified by universal PCR primers.

Using such primer sequences, a 450 bp product has been amplified from three species within the *B. africanus* group and this partial COI sequences have been characterised by both restriction digestion and DNA sequencing. Two enzymes *Alu1* and *Rsa1*, upon double digestion, generate informative profiles which appear to discriminate *B. africanus*, *B. globosus* and *B. nasutus*. In addition, a species specific restriction site has been identified within *B. globosus* sequences from East Africa with the enzyme *Ssp1*. As these restriction assays are relatively simple and quick, they may be of value for routine identification purposes (Stothard & Rollinson 1997a). DNA sequencing of PCR products of COI obtained from *B. globosus* and *B. nasutus* has identified further variation within this molecule. In total, 33 variable sites were identified within a 330 bp sequenced region. Cladistic analysis of these COI sequences suggest that these taxa are evolutionary separate thereby firmly rejecting the possibility of conspecific taxa (Stothard & Rollinson 1997a). Interestingly, snails identified as *B. globosus* appear to be involved in transmission of *S. haematobium* in Zanzibar, no laboratory or field evidence was found implicating *B. nasutus* in transmission (Stothard & Rollinson 1997b).

**THE SEARCH FOR SCHISTOSOME RESISTANCE GENES IN *BIOMPHALARIA GLABRATA***

In order to investigate the genetic basis of parasite resistance we have turned to *B. glabrata* which acts as the intermediate host for *S. mansoni*. This is a more amenable system for laboratory culture and much more is known about the genetics of resistance in this snail. Resistance can take several forms, occurring because the parasite is not attracted to the snail, cannot penetrate it, penetrates the snail then degenerates immediately or after some development, or is actively destroyed by the snail defence system after penetration (Bayne 1991, Pereira de Souza et al. 1997). Experiments on selected lines of *B. glabrata* have established that schistosome resistance has a genetic basis (Richards et al. 1992) and genetic differentiation of laboratory maintained lines is possible (Larson et al. 1996). Identification and characterisation of molecular markers involved in parasite resistance in *B. glabrata* is of major importance and constitutes a critical first step towards the understanding of, not just the interaction between snail and schistosome, but more generally a variety of aspects of other host-parasite systems.

The regions of the *B. glabrata* genome involved in schistosome resistance can be targeted using RAPDs and bulked segregant analysis (BSA) (Michelmore et al. 1991) of a F2 population segregating for resistance and originating from crosses between resistant and susceptible snail lines. RAPD fragments linked to resistance are characterised by cloning and sequencing, to produce sequence characterised amplified regions (SCARs) which allow the design of primers specific to this genomic region. These may be developed into a codominant marker system if suitable restriction enzyme sites are present. In addition to markers linked to resistance, microsatellite markers may be used to

anchor linkage maps. Microsatellites are di-, tri-, or tetra-nucleotide repeats, the length of which varies between individuals. Dinucleotide repeat sequences have been identified in *B. glabrata* and developed into markers by the design of specific PCR primers (unpublished results). Thus, SCARs together with identified genomic regions, which exhibit sequence polymorphism, including RAPD fragments and microsatellite markers, can be used for local linkage and interval mapping to determine those genomic regions most closely involved in schistosome resistance. This strategy of using both RAPD-derived and microsatellite markers is ideal for mapping complex phenotypic traits (Lymbery 1996).

Adult F2 progeny of crosses between resistant and susceptible *B. glabrata* lines (see Godoy et al. 1997) were exposed to *S. mansoni* miracidia and checked from 3-5 weeks after exposure for shedding cercariae. The non-shedding (resistant) and heavy shedding (susceptible) snails from three families were pooled into six bulks. These were screened with over 200 random decamer primers. Four primers showed differences between non-shedders and heavy shedders which were repeatable and consistent across three families. RAPD primers giving resistant-specific fragments were also used to test the individuals which constitute a bulk. Several RAPD fragments have now been identified and await further characterisation by cloning, sequencing and radioactive hybridisation to confirm their presence in resistant RAPD profiles and absence from susceptible RAPD profiles.

## CONCLUDING REMARKS

By applying molecular methods to the characterisation and identification of intermediate snail hosts we hope to learn more about the relationships between the various species and gain a better insight into variation at the population level. The three molecular approaches detailed for *Bulinus* show promise for further characterisation studies: PCR-RFLP of the internal transcribed spacer region of the rRNA gene allows differentiation of the species groups and shows that sequence variation exists between species; RAPD profiles indicate high divergence between *Bulinus* species groups and allow in some cases the recognition of taxa as well as providing a means for examining intrapopulation variation and developing PCR based identification tests. Sequence analysis of the COI gene indicates that mitochondrial DNA is worthy of further study and may help to elucidate the relationships of morphologically variable species as demonstrated by studies on *B. africanus* group species. One common theme to emerge is that there is extensive divergence at the DNA level within the genus *Bulinus* and high levels of variability have been observed within some species.

The identification and characterisation of genomic regions associated with schistosome resistance, may provide insight into the mechanism of snail resistance and the role of the snail immune system, not just in *B. glabrata* but also in other snail intermediate hosts of schistosomes. In the long-term, understanding the genetic basis of schistosome resistance in snails may provide novel opportunities to develop alternative control strategies for schistosomiasis.

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