Molecular identification of schistosome intermediate hosts: case studies of *Bulinus forskalii* group species (Gastropoda: Planorbidae) from Central and East Africa

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African freshwater snails of the genus *Bulinus* act as intermediate hosts for schistosomes, trematode parasites responsible for medical and veterinary forms of schistosomiasis. The relationship between these snails and their parasites is an intricate one, with particular species of snail susceptible to infection only by certain species of schistosome. In common with other self-fertile hermaphrodite gastropods, *Bulinus* consists of a number of closely related species complexes with restricted gene flow between populations of each taxon. Consequently, despite their medical and veterinary importance as intermediate hosts, unambiguous identification and differentiation of planorbid snails such as these remains problematic, often confounding attempts to define the distribution and evolutionary relationships of conchologically similar taxa. Here we consider how morphological methods of discrimination can be used in conjunction with molecular based approaches to improve snail identification, thereby achieving a better understanding of the epidemiology of schistosomiasis. Data are presented from Central and East African taxa which illustrate how PCR-based methods have begun to be used in combination with traditional analyses in an integrated approach to characterize the genus *Bulinus*, specifically the *B. forskalii* species group. Particular emphasis is given to the analyses of Random Amplified Polymorphic DNA (RAPDs) and the mitochondrial gene cytochrome oxidase I (COI).

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ADDITIONAL KEY WORDS:—mitochondrial DNA — cytochrome oxidase subunit I — COI — Randomly Amplified Polymorphic DNA — RAPDs — arbitrary primers — phylogenetics — morphology — morphometrics.

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INTRODUCTION

Why study snail taxonomy?

Schistosomiasis is a debilitating disease currently afflicting over 200 million people in Africa, China and the Neotropics and like many infections its occurrence has become increasingly widespread due to the changing demography of human populations. The move from hunter-gatherer to established agriculturalist societies probably provided an opportunity for the first sustained widespread infections, especially where irrigation was practised. Certainly both man and livestock of early civilizations in ancient Egypt were plagued by this parasitic disease; during the reign of 4th Dynasty pharaoh Khufu (2600 BC) the most obvious symptom of urinary schistosomiasis, haematuria (blood passed in the urine), was common enough to be depicted as a hieroglyph on tombs (Farooq, 1973). However, the significance of freshwater snails in the transmission of schistosomiasis was not firmly established until nearly four and a half millennia later, when Miyairi & Suzuki (1914) discovered their role as intermediate hosts, essential for the completion of the lifecycle of Schistosoma japonicum. Leiper (1915) went on to identify the snail intermediate hosts of S. mansoni and S. haematobium in Egypt; these findings engendered a prevailing interest in snail identification and eradication.

Several decades ago it was perceived that taxonomic research was essential if intermediate hosts of trematodes were to be reliably identified and classified to determine the epidemiological biogeography of schistosomes. This realization encouraged classical conchological and morphological studies, many of which were necessarily parochial and failed to record the complexity of interactions between an intermediate host and its parasites throughout their range (Brown, 1996). Individual host-parasite relationships are a consequence of snail susceptibility and parasite infectivity, some parasites appear to be heterogeneous with regard to infectivity, compatible with a wide variety of snail hosts, whereas others show greater host fidelity (Rollinson & Southgate, 1987); conversely, parasite susceptibility can vary even between populations of the same snail species. Nevertheless, basic epidemiological and malacological research were discouraged as the theoretical goal of preventing disease transmission seemed attainable by introduction of effective programmes of chemotherapy to eliminate adult worms in the human host, and proper sanitation concurrent with the indiscriminant application of broad band molluscicides to exterminate freshwater snails, regardless of species. However, although such integrated programmes have had dramatic success in some areas (for instance oases in Tunisia) the sustained effort required for their maintenance in contiguous water
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bodies requires considerably more resources than are available in many affected areas (World Health Organization, 1993; Brown, 1994). Additionally, synthetic molluscicides can cause serious environmental damage to freshwater ecosystems, upon which many afflicted agrarian communities depend for food.

Water development schemes, increasingly demanded by expanding populations of developing countries, may encourage growth and spread of intermediate host species, creating a need for further fundamental epidemiological research. It has become increasingly apparent that, valuable though classical approaches are, the introduction of new molecular based methodologies have the potential to greatly improve identification of target species. Accurate identification permits both potential and actual local foci of infection to be well-defined, making snail control more cost-effective, practicable and precise, so minimizing collateral damage to other components of freshwater ecosystems. Furthermore, the greater resolving power of molecular genetic approaches allows consideration of the evolutionary relationships between host taxa, providing an opportunity to address issues concerning evolution of parasite susceptibility.

In Africa, two genera of snails, *Bulinus* and *Biomphalaria* (family Planorbidae), are associated with schistosome transmission. Of the important parasites infecting man, *Bulinus* species transmit *S. haematobium* the cause of urinary schistosomiasis, and *S. intercalatum* responsible for intestinal schistosomiasis, whereas *Biomphalaria* species act as intermediate hosts only for *S. mansoni*, another intestinal form. Currently, 37 species of *Bulinus* are recognized and commonly grouped into four species complexes (Brown, 1994). With 14 representatives, the *B. truncatus/tropicus* complex is practically pan-African, extending into the Middle East, Mediterranean islands and the Iberian Peninsula. The *B. reticulatus* group comprises only two species, both with restricted distributions, and the *B. africanus* group has ten species all confined to the Afrotropical region. Finally, the pan-African *B. forskalii* group is the most conchologically variable and widely distributed; its representatives are also found in the Arabian peninsula and on some Indian Ocean islands. The precise number of taxa that should be given specific status within the *B. forskalii* group remains unclear; to date, 11 species have been defined with varying degrees of precision.

The taxonomic complexity of *Bulinus* can be ascribed to two characteristics common to many freshwater snails, hermaphroditism coupled with self-compatibility, attributes which permit an isolated individual to become a founding propagule; promoting rapid colonization following dispersal or rapid recovery following a catastrophic population crash. A self-compatible breeding system can act to restrict geneflow within and between populations, encourage rapid evolutionary change and incipient speciation (Templeton, 1981). Consequently, self-compatibility often generates complexes of 'cryptic species', taxa which exhibit subtle morphological and genetic differences, perhaps replacing each other along ecological and environmental gradients, or harbouring different parasites. Our attempts to identify and establish the relationships of some African intermediate hosts in the genus *Bulinus*, with particular emphasis on the *Bulinus forskalii* group, illustrate some of the problems inherent in identification and classification of freshwater snails.

*Advent of PCR approaches and suitability of markers*

Considerable effort has focused on providing reliable methods for the differentiation and identification of *Bulinus* species. Traditionally differences among taxa
have been sought through detailed descriptions of shell morphology, and internal anatomy and determination of chromosome number (reviews in Sturrock, 1993; Brown, 1994). However, morphological markers from the shell, radula and genitalia, suffer age and environmental artefacts and/or require skilled preparation; consequently they are not easily used by the non-malacologist. Protein electrophoresis supplemented such studies and produced reliable markers for distinguishing at least some of the *Bulinus* species (e.g. Wright & Ross, 1966; Jelnes, 1979; Mimpfoundi & Greer, 1989; Jelnes, 1986). This approach, however, requires fresh or frozen material and adult or large snails, often impractical collecting considerations in the tropics.

Clearly there was a need for a methodology with greater resolution, one that can utilize minute ethanol-preserved tissue samples in quick, simple, and robust protocols, and is not confounded by age or environmentally induced artefacts. Molecular approaches utilizing the Polymerase Chain Reaction (PCR) seemed wholly appropriate. However, the lack of snail-specific sequences from which to design primers for specific amplification reactions has hampered the application of molecular tools to the study of these and other molluscan taxa. These problems have been largely overcome by the use of universal PCR primers, which amplify targets in nuclear and mitochondrial genomes across a broad range of taxa (Simon *et al.*, 1994; Palumbi, 1996), and the introduction of arbitrary primer mediated DNA fingerprinting (Williams *et al.*, 1990; Welsh & McClelland, 1990; Caetano-Anolles, Bassam & Gresshof, 1991; Vos *et al.*, 1995). The choice of appropriate universal and arbitrary primer methods for species identification and elucidation of relationships in tropical freshwater snails is discussed below and summarized in Table 1.

Eukaryotic ribosomal DNA (rDNA) is a tandemly arrayed, multigene family. Each repeat unit is characterized by three coding genes—18S, 5.8S and 28S—which are generally more highly conserved than the intervening Internal Transcribed Spacer regions (ITS1 and ITS2) and the External Transcribed Spacer (ETS) located at the 5' end of the transcribed RNA. The ITS regions may prove useful for examining relationships between closely related organisms as they constitute rapidly evolving nuclear sequences (Hillis, Mable & Moritz, 1996). Universal PCR primers can be designed from the conserved gene regions to amplify the intervening variable ITS regions in a wide array of taxa (Hills & Dixon, 1991). Concerted evolution, in which unequal crossing over and biased gene conversion of repeats allows the spread and fixation of mutations (Dover, 1986; Hillis *et al.*, 1991), can homogenize tandem arrays within individuals and populations. Consequently, the many hundreds of copies are often regarded as a single locus that can be compared among species (reviewed in Hillis & Dixon, 1991). However, complications can occur if there is extensive copy variation within individuals.

Using primers for the conserved 18S and 28S ribosomal genes (Kane & Rollinson, 1994), the entire ITS region has been successfully amplified, including the 5.8S rRNA and the ITS1 and ITS2, from representative taxa of each of the four *Bulinus* species group (Stothard, Hughes & Rollinson, 1996). Simply restricting amplification products from the entire ribosomal ITS region and visualizing the digests directly on ethidium stained agarose gels is sufficient to differentiate closely related taxa. Characteristic RFLP (Restriction Fragment Length Polymorphisms) patterns were obtained for the four *Bulinus* groups and, in the majority of cases, taxa within each could be discerned (Stothard *et al.*, 1996). Using the same approach, Vidigal *et al.*, (1998) showed that it was only possible to reliably differentiate three South American species of *Biomphalaria* with a single restriction enzyme (DdeI) out of eight tested.
### Table 1. Summary of advantages and disadvantages of some PCR-based approaches used in species characterization

<table>
<thead>
<tr>
<th>Technique</th>
<th>Rationale</th>
<th>Ease of use</th>
<th>Cost</th>
<th>Reproducibility</th>
<th>Recommended utility</th>
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<tr>
<td>RAPDs</td>
<td>use of short arbitrary PCR primers (10 bp); multilocus fragment profiles</td>
<td>+ + + *</td>
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<tr>
<td>DAF/AP-PCR</td>
<td>use of arbitrary PCR primers (very short 5-8 bp/long&gt;18 bp); multilocus fragment profiles</td>
<td>+ + b</td>
<td>Moderate</td>
<td>Low d</td>
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<tr>
<td>AFLPs</td>
<td>use of arbitrary* PCR primers; multilocus fragment profiles</td>
<td>+ + b</td>
<td>Moderate</td>
<td>Moderate</td>
<td>++ + + + + + + + + + + + +</td>
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<tr>
<td>mtDNA sequencing</td>
<td>use of universal PCR primers; high resolution sequence data</td>
<td>+</td>
<td>High</td>
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<tr>
<td>mtDNA PCR-RELPs</td>
<td>use of universal PCR primers; simple, unambiguous fragment profiles</td>
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<td>Low</td>
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<tr>
<td>rDNA ITS PCR-RFLPs</td>
<td>use of universal PCR primers; simple fragment profiles</td>
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<td>Low</td>
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<tr>
<td>RAPD SCARs</td>
<td>use of species-specific PCR primers; simple presence-absence assay</td>
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<td>Low</td>
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*technically simple, straightforward method, fragments visualized on ethidium bromide stained agarose gels; bproducts separated using technically more demanding polyacrylamide gel systems, radionlabelled or silver stained; although technically simple to use, development of primers is costly and time consuming; a reproducibility concerns reduced using good quality, intact genomic DNA and strict standardization of all reaction parameters; d complications occur if there is extensive copy variation within individuals; e evolutionary relationships estimated between closely related species only; f Southern blot analyses can be used to verify fragment homology, or fragment restriction profiles can be compared (Riesenberg, 1996); gPCR primers are made which are complementary to the adapter sequence and the restriction enzyme sequences used to digest the genomic DNA as well as three randomly selected nucleotides at the 3' end (Vos et al., 1995).

However, substantial intraspecific variation observed in Bulinus (Stothard et al., 1996) and other freshwater gastropods, including Oncomelania (Hope & McManus, 1994) and Biomphalaria (Vidigal et al., 1998), suggests a high mutation rate within the ITS region of these intermediate hosts. Additionally, Vidigal et al. (1998) have reported the presence of extra bands in rDNA RFLP profiles which they attributed to sequence heterogeneity (the presence of two or more ITS sequences in an individual) suggesting that there is insufficient gene flow between subpopulations to ensure effective intraspecific homogenization of rDNA tandem arrays, clearly degrading the utility of rDNA as an effective taxon diagnostic tool. For this reason rDNA was
not chosen as a tool for studies of *Bulinus forskalii* as its predisposition to self-fertilization limits gene flow, potentially engendering copy heterogeneity in populations.

In recent years, mitochondrial DNA (mtDNA) has provided the markers of choice for a wide range of taxonomic, population and evolutionary studies (Avise, 1994). Characteristics which make this a valuable molecular tool include its ease of isolation, high copy number, lack of recombination, conservation of sequence and structure across metazoan and different mutation rates in different gene regions. The cytochrome oxidase subunit I (COI) has been shown to exhibit taxonomically useful levels of variation between closely related taxa, while showing very low levels of variation within and among closely related populations (Sperling & Dickey, 1994; Wuster et al., 1995; Pedersen, 1996). Different regions within COI evolve at different rates, with some regions being highly conserved due to functional constraints on the protein, allowing the design of universal PCR primers (Lunt et al., 1996). A 450 base pair product has been amplified from three species within the *Bulinus africanus* group using universal primers (Stothard & Rollinson, 1997). PCR-RFLPs from this approach show potential as a rapid, cost effective and reliable species identification method at least in some *Bulinus* taxa (Stothard & Rollinson, 1997) and other molluscs (Baldwin et al., 1996).

The combination of speed, simplicity and arbitrary sequence of the primers has made arbitrary primer mediated DNA fingerprinting methods one of the most appropriate and frequently used molecular tools for the identification and differentiation of large numbers of genetically unknown taxa, including many pathogens and pests (Rollinson & Stothard, 1994), and gastropods (Noble & Jones, 1996; Stothard & Rollinson, 1996; Rollinson, Jones & Noble, 1996; Rollinson et al., 1998). Four arbitrary methods are now widely used: Randomly Amplified Polymorphic DNAs (RAPDs; Williams et al., 1990), Arbitrarily Primed Polymerase Chain Reaction (AP-PCR; Welsh & McClelland, 1990), DNA Amplification Fingerprinting (DAF; Caetano-Anolles et al., 1991) and Amplified Fragment Length Polymorphisms (AFLPs; Vos et al., 1995). The first three methods are very similar, and unlike conventional PCR-based analyses, all use single oligonucleotide primers of arbitrary sequence (between 5 and 20 bases) to initiate DNA strand synthesis under conditions of low stringency at a number of complementary binding sites scattered randomly throughout the genome. The simultaneous amplification of large numbers of polymorphic DNA fragments in a single assay is one of the greatest advantages of these methods. The primary difference between these three approaches concerns the length of arbitrary primers and the fragment visualization methods (see Table 1).

By contrast, AFLPs combine PCR and RFLPs to assay anonymous sequences over the entire genome but with the major advantage of being more robust and reliable than other arbitrary primer mediated DNA fingerprinting methods because amplification is performed under more stringent reaction conditions (Vos et al., 1995). Although the AFLP technique was developed for mapping plant genomes it has been successfully utilized for species identification purposes in plants, bacteria and fungi (Lin & Kuo, 1995; Janssen et al., 1996; Majer et al., 1996). However, the drawbacks to this approach compared to the RAPD assay include the relative complexity of the procedure, the use of radioactivity and the requirement of larger quantities of genomic DNA.

Nevertheless, application of all four arbitrary primer mediated DNA fingerprinting
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methods has been complicated by concerns over reproducibility, dominant inheritance patterns and questions of fragment homology. As the RAPD assay is the most widely used and technically simplest approach it best illustrates discussion of the problems inherent in arbitrary primer mediated methods. Most reproducibility problems stem primarily from inadequately prepared DNA and can be easily overcome (Jones, Okamura & Noble, 1994; Vernon, Jones & Noble, 1995; see Fig. 1A, B for typical examples of reproducible RAPD profiles). Strict standardization of all reagents and protocols is required due to sensitivity to template, magnesium chloride and primer concentrations (Grosberg, Levitan & Cameron, 1996). Although dominant markers lack the utility and resolving power of co-dominant systems for pedigree and population genetic analyses they remain useful in taxonomic/systematic studies, where a technically simple method sampling anonymous genomes at an almost inexhaustible number of loci is invaluable in estimating genetic similarity of taxa. However, fragment homology remains an important consideration. AFLPs are the least affected by this problem as co-migrating fragments are more likely to be homologous than those obtained by other methods because this approach is mediated by restriction enzymes.

Homology (common ancestry) is generally assumed among characters or genes used for phylogenetic reconstruction. Yet, with arbitrarily amplified loci there is no reason to believe that comigrating bands in two species actually represent homologous regions. The inference of homology is strong only when many RAPD bands are shared between taxa with high sequence similarity. With increasing genetic distance between species it becomes increasingly unlikely that fragments will be shared, calling the homology of co-migrating bands into question. To avoid major errors, it has been suggested that the application of RAPD analyses in phylogenetics be restricted to closely related organisms and that systematic relationships at higher taxonomic levels should not be inferred (Clark & Lanigan, 1993; Bowditch et al., 1993).

Homology errors using RAPDs can be reduced through increased agarose gel resolution, by separating products out further or, a less satisfactory solution in terms of simplicity and speed, changing to a polyacrylamide gel system. More laborious methods of determining homology include Southern hybridization using the isolated ‘shared’ bands as radiolabelled probes. Sequence homology can be assumed following Southern blot analysis if the target DNAs hybridize to a common probe. Cross hybridizing bands of identical size could legitimately be scored as a shared character. Alternatively verification of homology can be obtained by comparison of individual fragment restriction profiles (Rieseberg, 1996).

The utility of our selected molecular approaches for species identification and clarification of genetic relationships among planorbid snails are illustrated by the following case studies of *Bulinus forskalii* group taxa in Central and East Africa.

CASE STUDIES

**B. forskalii group intermediate hosts (Gastropoda: Planorbidae)**

Definitions of the *B. forskalii* group taxa are not entirely satisfactory. The present system is founded on characters of the shell and internal anatomy, particularly the
Figure 1. RAPD profiles obtained using primer 10 (5' TAGCAGCGGG 3') illustrating reproducibility of the low stringency protocol (A & B). Optimal RAPD profiles consist of discrete fragments, consistent within and between PCR runs. Template DNA was checked to ensure it remained undegraded, PCR conditions were made more stringent to reduce primer-template mismatching by increasing the annealing temperature to 38°C, reducing the number of cycles from 45 to 40, and lowering the MgCl₂ concentration from 4 mM to 2 mM. Template concentration was increased from 5 to 20 ng per reaction (quantified by fluorometry), as low quantities produced inconsistent profiles of spurious variant
shape of the mesocone on the first lateral radular tooth, and the presence or absence of a carina (a conspicuous spiral ridge running around the shell periphery). However, the appearance of these features is variable and the absence of a carina is not always indicative. Hence, the precise number of taxa that should be afforded specific status remains unclear. Currently there are 11 species recognized within the *B. forskalii* group; nine are defined according to morphological characters (Brown, 1994): *B. forskalii* (Ehrenberg, 1831), *B. scalaris* (Dunker, 1845), *B. canesens* (Morelet, 1868), *B. senegalensis* (Müller, 1781), *B. camerunensis* (Mandahl-Barth, 1957), *B. crystallinus* (Morelet, 1868), *B. beccarii* (Paladilhe, 1872), *B. cernicus* (Morelet, 1867), and *B. bavayi* (Dautzenberg, 1894). *B. forskalii* is possibly of sufficient biological complexity to merit subdivision and indeed Jelnes (1979, 1980) erected two additional species, *B. browni* and *B. barthi*, defined solely by ‘distinctive alleles’ at certain soluble enzyme loci (see Kenyan case study below).

*B. forskalii* from Cameroon, Central Africa

In Cameroon, Central Africa, the *B. forskalii* group is represented by three species: *B. forskalii*, *B. senegalensis* and *B. camerunensis*. *B. forskalii* acts as a host for *S. intercalatum* in the equatorial climate of south-western Cameroon but is thought to carry no known schistosome where its distribution overlaps with that of *B. senegalensis* in the drier sahelian region of the Far North (Mimpfoundi & Greer, 1989; Greer et al., 1990). *B. senegalensis* and *B. camerunensis* have been implicated in the transmission of *S. haematobium*. *B. camerunensis* is known from only two crater lakes, Barombi Kotto (the type locality) and Lake Debundsha, both in SW Cameroon.

The distribution of *B. senegalensis* is believed to extend throughout the sub-Saharan belt, including Senegal, from where it was first reported (type locality is Podor; Adanson, 1757), Gambia, Mauritania, Chad, Nigeria, Cameroon and Niger, where it inhabits temporary water bodies (Mimpfoundi & Slootweg, 1991). As *B. senegalensis* is an important intermediate host for *S. haematobium*, undetected populations may be extremely important in transmission (Wright, 1959) making it essential to define this species accurately in order that epidemiological boundaries can be constantly revised. However, unambiguous identification of *B. senegalensis* from shells alone is problematic as it closely resembles *B. forskalii*, especially in areas where the two species are sympatric (Betterton, Fryer & Wright, 1983; Brown, 1994).

Mimpfoundi & Greer (1989) compared allozyme patterns among members of the *B. forskalii* group from Cameroon, and demonstrated that *B. senegalensis* and *B. forskalii* have distinct allozyme profiles. However, although of some utility, protein electrophoresis is not ideal for the reasons already discussed. Hence, the RAPD assay, an approach amenable to small or juvenile, ethanol preserved field samples,
or museum collections, was evaluated as a tool for characterizing taxa within *B. forskalii* group from Central and West Africa. Twenty-eight RAPD primers were used to characterize, a minimum of 10 individuals from 28 populations of *B. forskalii* taxa, including *B. forskalii* from throughout Cameroon, several from Senegal and one from São Tomé; *B. senegalensis* from the type locality in Senegal and other localities, far N Cameroon and Mali; *B. cernicus* from Mauritius and *B. crystallinus* from Angola; *B. camerunensis* from SW Cameroon; a laboratory population of *B. wrighti* originally from Oman; and an outgroup of one *B. truncatus* population from Barombi Kotto, SW Cameroon. The majority of specimens could, in the first instance, be readily identified using shell characters. Individual snails with intermediate shell morphologies could be identified by comparison of their RAPD profiles with those of specimens with an unequivocal shell morphology. Additionally, samples were collected from localities reported to yield only a particular taxon.

The RAPD assay readily distinguished *B. forskalii* from the conchologically similar *B. senegalensis*; few fragments were shared, suggesting specimens ambiguously assigned on conchology may be identified to the specific level by RAPD profiling (Rollinson et al., 1996). No polymorphisms were detected in the type locality material of *B. senegalensis*, but limited variation within and between populations was found in samples from far N Cameroon. Similarly, limited within population variation was demonstrated in *B. forskalii*, but greater differences between populations were observed, especially between those from widely separated localities. RAPD profiles of *B. camerunensis* were very similar to those obtained for *B. forskalii*, falling within the range of variability characteristic of adjacent *B. forskalii* populations, although several distinct *B. camerunensis* fragments were detected (Fig. 1A, lanes 16–19).

Owing to the poor fossil record of the genus, the evolutionary history of *Bulinus* must be inferred from extant species. Although grouped by similar morphological traits, at present there are few other indicators of relationship between the *B. forskalii* group species, making it difficult to confirm if these species are in fact genetically similar. Phylogenetic analysis of genetically closely related taxa is possible using RAPD derived characters, if the assumption of fragment homology can be verified. RAPDs are considered unsuitable for phylogeny inference using parsimony methods as this approach requires that the amplification products are homologous, independent, variable and assumes the shared absence of a character to be informative (for a review see Backeljau et al., 1995). Conversely, phenetic analysis using the Dice coefficient (Jackson, Somers & Harvey, 1989), or Nei similarity index (Nei & Li, 1979), considers only the presence of shared RAPD fragments informative; shared bands are more likely to represent homologous alleles than are nulls (Grosberg et al., 1996). However, even a purely phenetic approach to reconstructing genealogies based on overall similarity in band-sharing, can be confounded by the problem of co-migration of non-homologous fragments. Hence, to accurately characterize phylogenetic relationships it is essential to establish if co-migrating fragments in different individuals are homologous. To test this assumption selected co-migrating RAPD bands from different *B. forskalii* taxa were excised from the gels, radiolabelled and used as probes to identify homologous fragments in Southern analyses (Fig. 2).

Hybridization of probes derived from RAPD products has demonstrated that some co-migrating bands in different species are indeed homologous (Hadrys, Balrick & Schierwater, 1992; Smith et al., 1994; Jones et al., in prep), but some clearly are not (Smith et al., 1994). In the majority of probings of *B. forskalii* taxa fragment homology was as predicted; fragments shared between taxa and of equal fluorescence...
Figure 2. Strategy for verifying homology of co-migrating fragments. Total genomic DNA is extracted and amplified with several single, arbitrary RAPD primers at low stringency. Discrete amplification products are run on ethidium bromide stained agarose gels. Homology between sequences representing common bands either within or between species is confirmed by Southern analysis of RAPD gels using isolated bands as probes radiolabelled by random priming (Feinberg & Vogelstein, 1983). Homologous characters scored as binary data (present/absent) are used to calculate distance estimates for phylogenetic analyses.

were shown to be homologous. A typical case is that of probe 10/10 of approximately 940 base pairs (Fig. 3A, derived from arbitrary primer 10), which showed similar sized fragments from *B. forskalii* and *B. camerunensis* to be homologous (Fig. 3C). However, weakly amplifying fragments, with respect to the rest of the profile, of approximately the same size were not homologous. An 800 base pair fragment produced by primer 10 (10/5) from *B. senegalensis* (Senegal) was highly fluorescent, whereas a similarly sized fragment was less fluorescent in *B. forskalii* (see Fig. 3A). When fragment 10/5 was excised and used as a probe, it was not homologous to *B. forskalii* (Fig. 3B), but was specific to *B. senegalensis* from both the type locality (Podor, Senegal) and far North Cameroon. Few studies have undertaken these time consuming checks, hence, there is a general lack of data on RAPD fragment homology within and between species. In this study only a single case of nonhomology was detected between fragments of the same size and intensity, however, the problem may lie in the difficulty of scoring fragments that are not in close proximity on a gel. This exemplifies one of the drawbacks to working with the complex multilocus profiles characteristic of RAPDs.

Once ambiguous fragments were verified as homologous they were used as phylogenetic characters. Analyses using the Fitch–Margoliash criterion, suggest that *B. forskalii* and *B. senegalensis* form distinct branches, demonstrating that these morphologically very similar species are clearly differentiated by RAPDs (Rollinson et al., 1996). Conversely, cluster analyses place *B. camerunensis* with specimens unequivocally identified as *B. forskalii*, confirming the taxonomic status of *B. camerunensis* is debatable and clarifying why although conchologically distinct and compatible with a different parasite, it possesses no allozyme alleles additional to those of *B. forskalii* (Mimpfoundi & Greer, 1989). Its affinity with *B. forskalii* from
Figure 3. Homology of PCR fragments with arbitrary primer 10. A, RAPD profiles. Open arrowheads indicate fragments 10/5 (from *B. senegalensis*, Senegal e.g. lane 17) and 10/10 (from *B. forskali*, Udkia, Far North Cameroon e.g. lane 3), taken from repeat samples excised from gel and radiolabelled for use as probes. Lanes: 1 & 2, *B. forskali*, Sangmelina, South Cameroon; 3–6, *B. forskali*, Udkia, Far North Cameroon; 7–10, *B. forskali*, Maklingay, Far North Cameroon; 11–13, *B. camerunensis*, Barombi.
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nearby Kumba, S W 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. A further unexpected outcome of molecular analysis was the clustering of a conchologically extreme geographical variant of B. forskalii from São Tomé with Angolan B. crystallinus. Given the historical links between these two areas such movement of B. crystallinus seems plausible, but more samples are required to confirm this hypothesis. Hence, RAPDs can elucidate the evolutionary relationships and differentiate sibling species in the B. forskalii group, being especially useful in taxa for which there is limited sequence information.

However, low stringency amplification often makes standardization and interpretation of complex RAPD profiles between laboratories difficult (Penner et al., 1993), impairing their value as a robust taxonomic tool since precisely determined optimal PCR conditions must be strictly maintained to avoid artifactual variation and obtain reliable, reproducible profiles (Ellsworth, Rittenhouse & Honeycutt, 1993; Meunier & Grimont, 1993). Black (1993) suggested that even storage conditions of samples can seriously affect the reproducibility of RAPD profiles; patterns are unlikely to be reproducible in dried or otherwise preserved specimens where template degradation has been extensive. This is a serious consideration in taxonomic work, where much material is necessarily derived from museum voucher specimens. Converting RAPD markers into Sequence Characterized Amplified Regions, SCARS (Paran & Michelmore, 1993), overcomes these problems. A high stringency PCR-based identification system can be produced by cloning and sequencing diagnostic RAPD fragments and designing a panel of highly specific primers (Fig. 4). These primers are sensitive only to annealing temperature and give reproducible species-specific product(s). Generation of high resolution PCR markers for species identification in micro-organisms (Argenton et al., 1996), and insects (Garnier & Slavicek, 1996) has followed this approach. This methodology has been applied to freshwater snails to develop a diagnostic molecular tool allowing conchologically similar species of the B. forskalii group from Central and West Africa to be distinguished (Jones et al., 1997).

An invariant fragment characteristic of a specific group is a prerequisite for a reliable diagnostic assay, and must be ascertained by screening panels of representative groups, species and individuals to establish the degree of intra- relative to interspecific variation. Jones et al. (1997) designed three primer pairs each of which give a single PCR product in simple presence-absence assays allowing unambiguous differentiation of B. forskalii and B. senegalensis (Fig. 5A – example of B. senegalensis specific primers). Another pair gave reproducible and readily interpretable multibanded species-specific profiles (Fig 5B). Specificity of these RAPD derived primers was reconfirmed by widespread sampling throughout each species’ range. Interestingly the primers used to identify B. forskalii also amplify a fragment from B. camerunensis, again calling the taxonomic status of the latter into question.

Kotto, SW Cameroon; 14 & 15, B. senegalensis, Mora, Far North Cameroon; 16 & 17, B. senegalensis, type locality material, Podor, Senegal. B, Southern blot analysis of RAPD gel in (A) probed with band 10/5 (derived from B. senegalensis profile) which hybridizes to B. senegalensis only (lanes 14–17). C, Southern blot analysis of RAPD gel (A) probed with band 10/10 (derived from B. forskalii profile) hybridizes to B. forskalii populations throughout Cameroon (lanes 1–10) and to B. camerunensis (lanes 11–13).
Figure 4. Strategy for isolating species-specific PCR primers. Species-specific markers from RAPD primers which give reliable, reproducible profiles (on several repeat amplifications) are cloned and sequenced. Many populations of the same species from a wide geographic area are screened to ensure fragments excised are not restricted to a population or geographical region. Candidate fragments are excised from the agarose gel, purified, cloned and double stranded plasmid DNAs are alkali-denatured and sequenced. A stretch (200–300 bases) of each end of the cloned fragments is sequenced for species-specific primer design. Primers are tested on a representative panel of specimens from widely separated localities. Identification depends upon primers consistently amplifying the same species-specific fragment(s) throughout a species’ geographic range. The quality of template DNA is assessed by a positive control using primers which routinely amplify. Ideal species-specific PCR primers are those which give unambiguous results when used in high stringency protocols; for example, PCR products present in species B but absent in A, although multibanded species diagnostic profiles are still useful.

However, it must be noted that tests involving presence or absence of single PCR products are not ideal since many factors can cause the PCR to fail. Using multiple markers can resolve this problem, indeed together the above mentioned four primer sets afford a means of differentiating *B. forskalii* and *B. senegalensis* from across Africa. Primers reliably amplifying the many copies of the entire ITS region of ribosomal RNA genes (Kane & Rollinson, 1994) in all *Bulinus* species (Stothard & Rollinson, 1997) were employed as a positive control. Although not a wholly appropriate control for a single copy marker it should be noted that the copy number of the species-specific products is unknown.

*Bulinus forskalii* group intermediate hosts from Kenya, East Africa

This case study extends our observations to East Africa, allowing further comparison of *B. forskalii* and related taxa. Four *B. forskalii* group species have been described from Kenya; *B. forskalii*, *B. scalaris*, *B. browni* and *B. barthi*, the relationships and identities of these species are far from clear (Jelnes, 1979; Brown, 1975, 1994). Although it would seem that these snails do not transmit human schistosomiasis in Kenya, *B. forskalii* and *B. browni* are implicated in the transmission of *S. bovis*. 
Figure 5. Species-specific amplification product(s) run on 2% agarose gels, stained with ethidium bromide. A, *B. senegalensis* species-specific primers (8f/r; derived from a *B. senegalensis* profile from the original RAPD gel) produced a 564 bp product in all *B. senegalensis* samples (NC, Far North Cameroon, lanes 7–10; S, Senegal, lanes 11–13; M, Mali, lanes 14–16), failed to amplify *B. forskali* template DNA from several populations (SWC, south-west Cameroon, lanes 1 & 2; S, Senegal, lanes 5 & 4; NC, Far North Cameroon, lanes 3 & 6). B, primers 6f/r (designed from a *B. forskali* fragment from the original RAPD gel) gave consistent species-diagnostic profiles irrespective of annealing temperatures: 60°C (lanes 1–7) or 65°C (lanes 8–14). *B. senegalensis* Far North Cameroon, lanes 1–3 & 8–10; *B. forskali* Far North Cameroon lanes 4–7 & 11–14. M = molecular weight marker (100 bp ladder, Pharmacia).
The primary objective of this study was to supplement diagnostic morphological characters with unambiguous molecular markers which differentiate East African *B. forskalii* group taxa, and compare this material with West and Central African specimens to allow the taxonomic status of *B. forskalii* species to be placed in a pan-African context.

Initial sampling included the Kano Plain, near Kisumu, Kenya, from where *B. forskalii*, *B. browni* and *B. scalaris* have been recorded, including from the Nyanza Province, Obtuso, type locality of *B. browni*. *B. forskalii* is probably the most abundant and widespread aquatic snail on the Kano Plain and characteristically inhabits small transitory water bodies, such as yaos, borrowpits and roadside ditches (Brown, 1975); it is found less abundantly in slow-flowing streams and major swamps where it may co-occur with *B. scalaris*. Conchologically, fully grown *B. forskalii* are characterized by slender, high-spired shells, with the usually carinate whorls showing some degree of shouldering, with strong ribs commonly present; additionally, they may bear fringes of periostracum. *B. scalaris* can be distinguished from *B. forskalii* by a broader shell, with the later whorls more smoothly curved, lacking a shoulder and regular ribbing. However, marked ontogenetic changes in shell shape occur in *B. forskalii*, and the shells of juveniles are comparatively broad and low-spired. This, in addition to within species variation in size and form of adults, has led to the synonymization of many named species (Mandahl-Barth, 1957). Hence, some difficulties are encountered when attempting to differentiate *B. forskalii* and *B. scalaris* solely on a conchological basis. Nevertheless, morphology of the distal genitalia is diagnostic of *B. forskalii* and *B. scalaris* (Mandahl-Barth, 1957); the latter has a larger copulatory organ with the penis sheath much swollen by a long and coiled epiphallus, unfortunately a character which can be measured only in fully mature specimens. This dearth of useful morphological characters led Jelnes (1979) to rely upon ‘distinctive enzyme alleles’ in some East African populations of *B. forskalii*. Two new species, *B. browni* and *B. barthi* were described based on these criteria (Jelnes 1979, 1980), although these specimens exhibited no morphological characters which distinguished them from *B. forskalii*.

A search for *B. forskalii* group snails from the coastal region, particularly at the Mariakani Dam, Mombasa, the type locality for *B. barthi* proved unproductive. Unfortunately no tissue of the type material exists for *B. barthi* or *B. browni* because it was destroyed during electrophoretic analysis (Jelnes, 1979). Collections were also made of *B. forskalii* taxa from around Nairobi. Earlier widespread sampling in Cameroon provided *B. forskalii* for comparison with East African specimens, along with samples from São Tomé, Mali and Senegal. Other *B. forskalii* group species including *B. senegalensis* (Senegal), *B. camerunensis* (Cameroon) and *B. crystallinus* (Angola) were also included in the molecular and morphometric analyses. The RAPD assay is clearly concordant with morphological analyses, differentiating *B. forskalii* and *B. scalaris* from Kenya (Fig. 6). Further, diagnostic *B. forskalii* fragments from Cameroonian material were also present in Kenyan samples identified as *B. forskalii* on conchological grounds, supporting the notion that Central and East African *B. forskalii* represent a single species transmitting different schistosomes throughout its range.

Supplementary data was provided by sequences of a 450 base pair region of the mtDNA COI gene from a panel of *B. forskalii* group taxa used for RAPD analysis; 64 specimens were examined, representing six *B. forskalii* group species from East, West and Central Africa and a non-*forskalii* outgroup, *B. truncatus*. Phylogenetic
analyses were performed on aligned nucleotide and amino acid sequences using distance and parsimony methods included in the package PHYLIP (Felsenstein, 1993) and the programme Phylogenetic Analysis Using Parsimony (PAUP) version 3.1 (Swofford, 1993). The robustness of internal branches was tested by bootstrapping ($n = 1000$). The most statistically robust phylogenies of mitochondrial sequences were identified and compared with taxonomies derived from morphological and RAPD data.

Figure 7 shows the 50% majority-rule consensus tree produced by the maximum parsimony method using partial mtDNA COI sequences representing 26 haplotypes of *B. forskalii* group species together with an outgroup sequence from *B. truncatus*. The high values for consistency and retention indices (CI & RI, respectively) indicate that the tree is a good representation of the sequence data. Further, the homoplasy index (HI) is quite low indicating that these sequences contain few homoplastic characters. The general topology of this tree is comparable to that observed with distance methods of phylogenetic inference, and has analogous bootstrap values. Further, mtDNA COI tree topologies were essentially the same as those based on the RAPD assay (Jones *et al.*, in prep.). Thus, all molecular phylogenetic analyses employed thus far are clearly concordant, and confirm species assignments proposed on morphological grounds.

The most striking feature of all RAPD and mtDNA based phylogenetic analyses is the distinct *B. senegalensis* branch, the remaining five taxa branching off to form two clades, the first comprising three sister taxa, *B. forskalii*, *B. camerunensis* and *B. crystallinus* and the second, *B. scalaris* and *B. cernicus*. Although representative samples of each putative taxon were identified on morphological criteria, in order that the accepted taxonomy may be adhered to, the relationships
Figure 7. Bootstrap 50% majority-rule consensus tree produced by the maximum parsimony method using partial mtDNA COI sequences representing 26 haplotypes of *B. forskalii* group species together with the outgroup sequence from *B. truncatus*. Percentage bootstrap values (>70%) for 1000 replicates are indicated. Consistency index (CI) excluding uninformative sites is 0.667, Retention Index (RI) 0.892, Homoplasy Index (HI) 0.333. Typical shell morphologies (drawn 2 x natural size) are indicated alongside each major taxon from the *B. forskalii* group (redrawn from Mandahl-Barth, 1965): *B. senegalensis* (Müller), Senegal; *B. crystallinus*, Angola; *B. forskalii* (Ehrenberg), Kenya; *B. camerunensis*, Mandahl-Barth, Cameroon; *B. cernicus*, Mauritius; and *B. scalaris* (Dunker), Kenya. Note, although *B. camerunensis* is morphologically distinct it is genetically similar to *B. forskalii*. 
realized from molecular data are at variance with those expected solely from a consideration of morphology; which places *B. forskalii* and *B. senegalensis* as sibling species. Similarly, *B. forskalii* and *B. camerunensis* are more dissimilar at the morphological than at the molecular level. At the regional level molecular analyses of Kenyan samples show two different taxa were collected from Kisumu and Nairobi. All Kenyan putative *B. forskalii* cluster with the Central African true *B. forskalii*, while the Kenyan *B. scalaris*, unequivocally identified from the large copulatory organ of mature specimens, form a separate branch with *B. cernicus*. There was no evidence of any taxon which might represent *B. browni* among populations sampled from sites previously reported positive for *B. browni*. As this species has only been described by protein electrophoresis explicit confirmation of its presence in the ethanol preserved material used in this study was not possible.

Additional molecular markers which readily differentiate *B. forskalii* and *B. scalaris* were sought from the mtDNA COI gene sequences. Two diagnostic RFLP markers between *B. forskalii* and *B. scalaris* from Kenya were identified upon restriction of these sequences with *RsaI* and *SacI*—each cutting at a single site in *B. scalaris*. PCR products from an additional 40 snails representing the two species were restricted individually with both enzymes, confirming each cleaved *B. scalaris* sequence once only and did not digest Kenyan *B. forskalii*. However, *SacI* also digested *B. forskalii* samples from some localities in Cameroon, relegating the use of this particular restriction enzyme to differentiation of the two species on a regional basis. Where diagnostic enzymes cut in only one species, but do not cleave the DNA of another, it is advisable to check whether the DNA of the latter species is actually restrictable by attempting digestion with other frequently cutting enzymes. Other restriction enzymes (such as *AluI*) were shown to cleave both *B. forskalii* and *B. scalaris* sequences several times, producing a number of more complex but diagnostic profiles in Kenyan samples of each species. Further study of a geographically representative panel of individuals may confirm the utility of COI PCR-RFLPs in these taxa as species-specific markers outwith Kenya. *B. forskalii* specific PCR primers derived from RAPDs of Central African material further confirmed our diagnoses of Kenyan material; a 553 base pair product was present in *B. forskalii* specimens and absent in *B. scalaris*. Amplification of the ITS region was in all cases used as a positive control.

In a morphometric analysis of 13 conchological characters using stepwise discriminant function and Principal Component Analyses, Kristensen (1992) found it possible to distinguish *B. forskalii* from *B. scalaris*. However, with the exception of a single *B. browni* population from Tanzania neither *B. browni* nor *B. barthi* could be differentiated from the other two taxa on morphological grounds. Morphometric characterization of our Kenyan taxa concentrated primarily on shell characters, on which the current taxonomy is based, in addition, approximately one third of snails from each site were dissected and the copulatory organ examined. Our PCA, incorporating several additional populations of *B. forskalii* and *B. scalaris* from Kenya and *B. forskalii* from Cameroon, Central Africa, was largely concordant with Kristensen (1992) in that many *B. scalaris* individuals had shell morphologies distinct from *B. forskalii* (Jones *et al.*, in prep). However, some *B. scalaris* individuals clustered within the range of variation of *B. forskalii*, which was shown to have a very variable shell morphology in juveniles and
adults, perhaps attributable to ecophenotypic variation. Despite their similar shell morphology *B. scalaris* and *B. forskalii* are so clearly distinguished on the copulatory organ (Mandahl-Barth, 1957) that recognition of *B. scalaris* is unquestionable a finding concordant with molecular evidence.

**DISCUSSION**

*Definitive molecular tools for species identification of snail intermediate hosts?*

The continuing development of unambiguous and reliable molecular methods of species identification remains an important goal for schistosomiasis control. RAPD analyses effectively differentiate the morphologically cognate *B. forskalii* group, and more generally have proven useful in the identification of taxa within the genus *Bulinus*, either by direct comparison of profiles (Langand et al., 1993), or by cluster analyses of derived distance estimates (Stothard & Rollinson, 1996). The case studies discussed above illustrate that taxon-diagnostic profiles contain many species-specific fragments, but some primers emphasize the regional nature of differences within a taxon, making it more difficult to identify taxon-specific profiles across regions, although identification of differences between species, populations and individuals within remain straightforward (Rollinson et al., 1998). For example, the distinctly different RAPD profiles of *B. forskalii* and *B. senegalensis* allowed specimens from far North Cameroon conchologically assigned to *B. forskalii/B. senegalensis* to be readily identified to the specific level. Although Kenyan and Cameroonian *B. forskalii* shared diagnostic fragments their profiles were sufficiently different to require cluster analysis of genetic distance estimates to establish their relationship, confirming it may be difficult to unequivocally assign specimens of a species throughout its range solely on profile pattern. Throughout this study all individuals analysed clustered as discrete species, and ‘unknowns’ analysed alongside patterns from identified specimens could be confidently assigned to conspecific clusters. By screening RAPD primers, and choosing those which exhibit adequate levels of variation, profiles can be produced that allow taxa to be accurately grouped and classified.

However, these findings cannot be generalized to all freshwater snail intermediate hosts. Although RAPDs have proven extremely useful for the study of population structure (Vidigal et al., 1994), breeding systems (Vernon et al., 1995) and differentiation of genetically defined lines (Larson et al., 1996), they were unable to generate molecular markers for the identification of South American *Biomphalaria* species due to remarkably high levels of intraspecific genetic variability. However, some differentiation between laboratory isolates of three African *Biomphalaria* species has been demonstrated (Kristensen, Raahauge & Wilken, 1998). A related methodology, termed low stringency PCR (LS-PCR), first described by Dias-Neto et al., (1993), utilizes specific primers, in contrast to RAPDs where the choice of primer is arbitrary, under low stringency PCR conditions. Utilizing this approach with primers designed to amplify a portion of the 18S rDNA gene significantly improved attempts to identify species of *Biomphalaria* (Vidigal et al., 1996; Pires et al., 1997). However, the technique remains problematic in *Biomphalaria* because of the complex profiles produced; critically it requires identification of a few diagnostic bands from a plethora of uninformative ones. PCR-RFLP of the rDNA ITS region may be
another promising approach, although drawbacks include detection of multiple sequence types within an individual degrading the utility of this region as an effective taxon diagnostic tool (Vidigal et al., 1998).

The development of species-specific PCR primers from species diagnostic RAPD fragments (Jones et al., 1997) greatly simplifies species identification, providing a robust, easy to use protocol applicable to degraded material, justifying the costly, time consuming isolation of primer pairs for each species of interest. Using this approach it was possible to obtain identifications from even badly broken shells, and partially degraded ethanol preserved material; particularly valuable for African taxa when fresh material may be more difficult to obtain. Unambiguous differentiation of B. forskalii and B. senegalensis, and extension of species-specific PCR analysis to other systematically difficult snail taxa, will permit accurate revision of their distribution, and a better understanding of their respective roles in the transmission of schistosomiasis in Central, West, and East Africa.

Sequence analyses of the COI gene indicate that mitochondrial DNA deserves further study, and may help to elucidate the relationships of morphologically similar species. Further, mtDNA PCR-RFLPs from this gene region show promise as a tool for species differentiation at a regional level, but species-specific restriction sites have yet to be identified throughout a species’ range. The availability of the entire mitochondrial sequence of two gastropods (Albinaria turritae – Hatzoglou, Rodakis & Lecanidou, 1995 and Cepaea nemoralis – Terrett, Miles & Thomas, 1996) should allow the design of further PCR primers for other mitochondrial genes for future taxonomic studies in Bulinus.

Towards an integrated approach

Although molecular data are advantageous for assessment of relationship, being free from age or environmental artefacts, for phylogenetic or taxonomic applications it is unwise to consider data from a single source. Sequences from both nuclear and mitochondrial genomes should be studied in order to derive a true identification and an organismal rather than a gene phylogeny (Degnan, 1993). Disparities between data sets may indicate introgression between closely related taxa, suggesting a necessary review of their species status. The data sets reviewed here were all concordant, suggesting taxa appear to be acting as good species over the greater part of their ranges.

Also, complementary morphological characterization of snail populations is essential in order that the molecular data may make a maximum contribution to the taxonomy and systematics of species groups by correlating known morphological characters with the results of the molecular analyses (Noble & Jones, 1996). Whatever molecular characters are examined it is important to remember that the traditional system of classification is founded on morphology (Brown, 1996). Imperfect though this formal system of names and rules is, it provides a reference framework essential for organizing knowledge of the organisms. Consequently, all molecular studies should include some morphological reference point(s) which allow comparison of current work with earlier findings, or deposit voucher specimens where they may be readily accessed for future work. This practise has greatly added to the value of non-morphological approaches, allowing the morphology of cryptic species differentiated by molecular and other techniques to be re-visited, when suitable
morphological characters may be recognized. The case studies illustrate the value of this approach. For instance, *B. scalaris* and *B. forskalii* in Kenya initially could only be reliably differentiated on the size of the copulatory organ, a character restricted to adults. However, establishment of a reference set of individuals permitted molecular identification of juveniles, and other individuals which it was not feasible to dissect.

**Cryptic speciation and future directions**

The habitats of African freshwater snails are dominated by two aspects, isolation and variation, tending towards species formation, and a third, temporal instability, opposing it. Although the temporary nature of many habitats probably prevents species formation, the bottlenecks, inbreeding and restricted gene flow experienced by many of these facultatively self-fertile hermaphrodites may produce a population structure similar to that suggested by Wright (1951, 1978) to promote rapid speciation. Reproductive isolation, although not selected *per se*, may arise as a pleiotropic consequence of selection in different environments (Templeton, 1981 and refs. therein), becoming increasingly likely in the face of temporary barriers to gene flow. *B. camerunensis* may have evolved in this manner, its crater lake environment effectively restricting gene flow, although the question of its reproductive isolation remains unresolved. Mandahl-Barth (1965) suggested there was a lack of ‘good’ species amongst *Bulinus*, conditions favouring the evolution of a series of microgeographic races. Interestingly the geographic distribution and limited potential for interaction between these ‘races’ is reminiscent of a metapopulation; population structure has important implications for the coevolution of snail-schistosome interactions. Natural populations of snail-schistosome systems are promising models for the study of evolutionary interactions between host and parasite. The introduction of molecular techniques for accurate identification of snail hosts, may encourage development of further molecular markers (such as microsatellites; Viard, Justy & Jarne, 1997), which may facilitate more detailed population genetic and evolutionary analyses of host-parasite interactions.

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