Interactions between intermediate snail hosts of the genus *Bulinus* and schistosomes of the *Schistosoma haematobium* group

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SUMMARY

Within each of the four species groups of *Bulinus* there are species that act as intermediate hosts for one or more of the seven species of schistosomes in the *Schistosoma haematobium* group, which includes the important human pathogens *S. haematobium* and *S. intercalatum*. *Bulinus* species have an extensive distribution throughout much of Africa and some surrounding islands including Madagascar, parts of the Middle East and the Mediterranean region. Considerable variation in intermediate host specificity can be found and differences in compatibility between snail and parasite can be observed over small geographical areas. Molecular studies for detection of genetic variation and the discrimination of *Bulinus* species are reviewed and two novel assays, allele-specific amplification (ASA) and SNaPshot™, are introduced and shown to be of value for detecting nucleotide changes in characterized genes such as cytochrome oxidase 1. The value and complexity of compatibility studies is illustrated by case studies of *S. haematobium* transmission. In Senegal, where *B. globosus*, *B. umbilicatus*, *B. truncatus* and *B. senegalensis* may act as intermediate hosts, distinct differences have been observed in the infectivity of different isolates of *S. haematobium*. In Zanzibar, molecular characterization studies to discriminate between *B. globosus* and *B. nasutus* have been essential to elucidate the roles of snails in transmission. *B. globosus* is an intermediate host on Unguja and Pemba. Further studies are required to establish the intermediate hosts in the coastal areas of East Africa. Biological factors central to the transmission of schistosomes, including cercarial emergence rhythms and interactions with other parasites and abiotic factors including temperature, rainfall, water velocity, desiccation and salinity are shown to impact on the intermediate host-parasite relationship.

Key words: *Bulinus*, *Schistosoma haematobium* group, compatibility, transmission, molecular characterization.

INTRODUCTION

The interactions observed between snails of the genus *Bulinus* and parasites within the *Schistosoma haematobium* group provide an excellent example of how host-parasite compatibility may change over space and time. The co-evolution of these host-parasite relationships is reflected by the present day geographical distribution of human and animal schistosomiasis caused by *S. haematobium* and related parasites in Africa and adjacent regions. Observations on parasites and snails isolated from different parts of their ranges reveal that the interplay between them has resulted in the emergence of many different intermediate host specificities. This applies not only on a large geographical scale but may be observed at a local level, with parasites being generally more infective to sympatric hosts than to allopatric hosts of the same species.

Perhaps to enhance the genetic diversity required for selection processes to act upon, both snails and parasites have reproductive strategies that are striking in their complexity. *Bulinus* are freshwater planorbids and, for the most part, are true hermaphrodites being able to outcross as both male and female. They can also self-fertilise and some species preferentially appear to do so, whereas others predominantly outcross. One of the four *Bulinus* species groups exists in different ploidy states, from diploids, tetraploids, hexaploids to octoploids, and some populations consist of aphallic individuals that are unable to cross-fertilise as males. Most digenean parasites are hermaphrodite but schistosomes are unusual in possessing separate sexes. Male and female worms pair in the vasculature system of the definitive host, and intriguing mating strategies have evolved that may result in mating competition and even hybridization between different species in the *S. haematobium* group. In the snail host, schistosomes multiply prodigiously and asexually placing considerable demands on the snail's resources. Place this complicated scenario of multiplication, gene flow and development against a continually changing environmental backdrop, with temperature and water levels oscillating through the seasons, together with various ecological parameters including predators and other parasites and it becomes apparent that multiple factors are involved in shaping the interactions and evolution of the host-parasite relationship.

Currently some 37 species of *Bulinus* are recognized (Brown, 1994), which have been divided into

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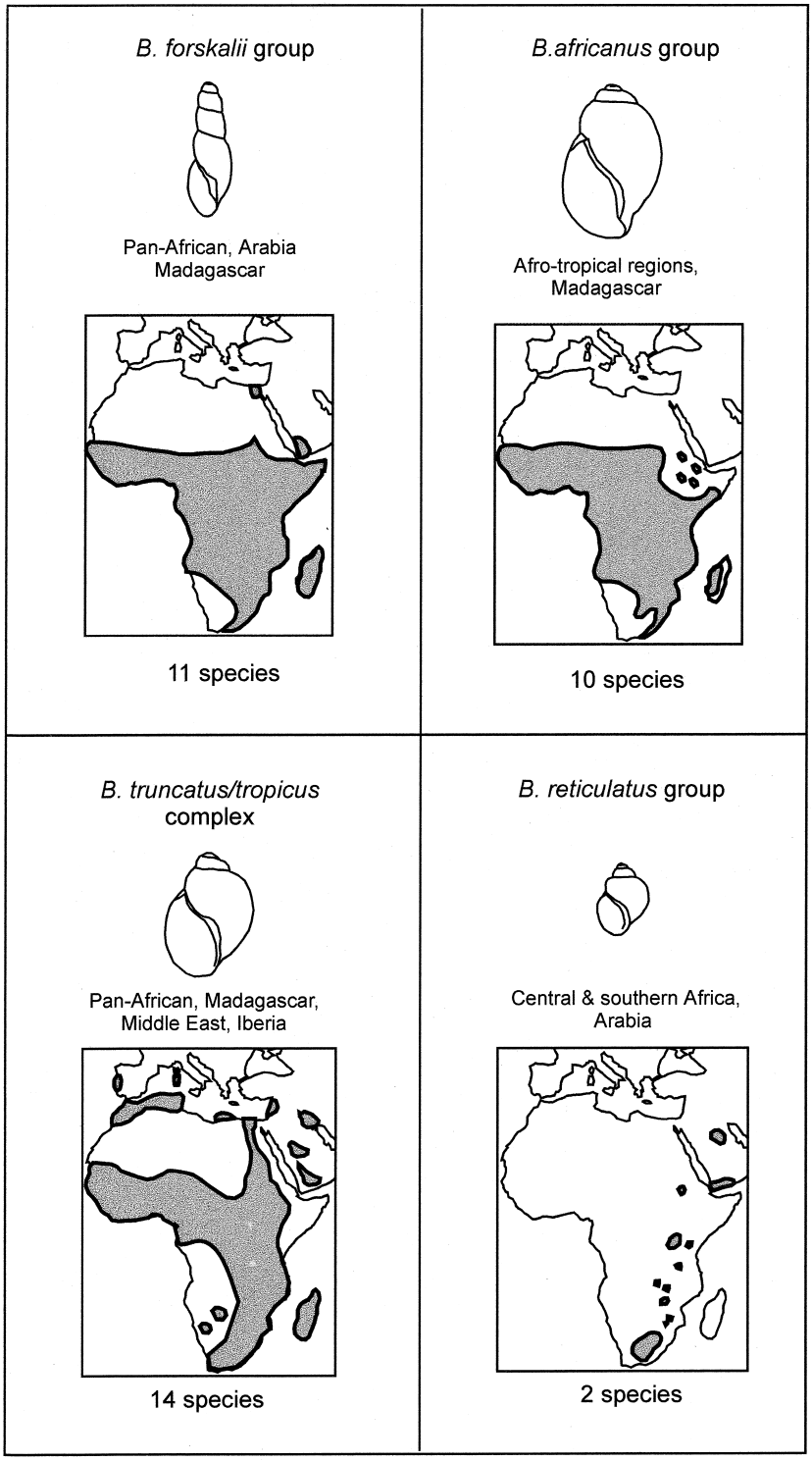

Fig. 1. Sketch map of the general distribution of species groups of *Bulinus* in Africa, the Middle East and in the Mediterranean Basin area based upon the maps of Brown (1994). A total of 37 species is presently recognized which are partitioned into four species groups. A shell, representing the characteristic adult morphology of a typical species within each of the species groups is presented and scaled accordingly; the shell of an adult rarely exceeds 2·5 cm in length and is usually between 0·5–2 cm. Within each of the species groups, there are snails capable of natural transmission of human schistosomes.

four species groups. 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;

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the two species included in the *B. reticulatus* group both have restricted distributions (Fig. 1). The species groups are for the most part well differentiated and early enzyme studies revealed large genetic distances within the genus (Biocca *et al*. 1979). The proposal was made to divide the genus into three genera but this classification has not been generally accepted and was considered to have serious disadvantages (Brown, 1981, 1994). Interestingly, substantial nucleotide variation of the internal transcribed spacer (ITSI) of the ribosomal RNA gene (rRNA) also indicated large divergence between representatives of the species groups (Stothard, Hughes & Rollinson, 1996).

Within each of the species group of *Bulinus* there are species that act as intermediate hosts for one or more of the seven species of schistosomes in the *S. haematobium* group in part or all of their geographical range (Rollinson & Southgate, 1987). Two of these species, *S. haematobium* and *S. intercalatum*, are primarily parasites of man. *S. haematobium*, responsible for urinary schistosomiasis, is found in 53 countries in the Middle East and Africa including the islands of Madagascar and Mauritius, the majority of cases being located in the Afrotropical region. *S. intercalatum* causes a form of intestinal schistosomiasis and has a much more restricted distribution primarily in Equatorial Guinea, Zaire, Gabon, São Tomé, Nigeria and Cameroon. Recent studies suggest that the two recognized biological strains of *S. intercalatum*, which display quite different intermediate host specificities, may well be distinct species (Pagès *et al*. 2001). The closely related *S. bovis*, *S. mattheei* and *S. curassoni* can be found in sheep and cattle, whereas *S. margrebowiei* and *S. leiperi* are more commonly found in antelopes. Many molecular studies point to the close relationship of the *S. haematobium* group species (see Rollinson *et al*. 1997*a*) which is further emphasised by observations on natural hybridization between species infecting the same definitive host and the ease at which both pairing and mating take place in experimental studies (see Southgate, Jourdane & Tchuem Tchuenté, 1998). The ability of different species within the *S. haematobium* group to develop in the same species of *Bulinus* in sympatric situations and infect the same definitive host (e.g. *S. haematobium* and *S. mattheei* in *B. globosus* in Zimbabwe and South Africa) creates conditions where hybridization occurs, resulting in gene introgression with the resulting epidemiological consequences (Jourdane & Southgate, 1992).

Unravelling the interplay between snails and schistosomes throughout their range has been central to the understanding of the geographical distribution and transmission of schistosomiasis. In a paper published in 1974 concerning experiments with hybrid schistosomes and observations on the inheritance of infectivity, Wright posed the fundamental question ''Snail susceptibility or trematode infectivity?''. He showed that the progeny of a cross between *S. mattheei* males X *S. intercalatum* females were equally infective to *B. globosus* and *B. scalaris*, whereas the parental forms were restricted in the case of *S. mattheei* to *B. globosus* and *S. intercalatum* to *B. scalaris* (Wright, 1974). Now we are fully aware that compatibility comprises both infectivity of the parasite and susceptibility of the snail but there is still much to learn concerning genetically-based variation among and within populations of both snail and schistosomes and the genes that influence the host parasite relationship (Woolhouse & Webster, 2000).

Aided by an increasing armoury of molecular and morphological techniques to identify and describe the genetic makeup of parasites and hosts, significant advances in our understanding of the snails responsible for transmission and the epidemiology of *S. haematobium* group schistosomes have been made (see Rollinson *et al*. 1998; Davies *et al*. 1999; Jones *et al*. 1997, 1999). In this paper the identification and discrimination of *Bulinus* species based on techniques of molecular characterization are briefly considered and new methods of detecting known nucleotide changes are introduced. Snails responsible for the transmission of *S. haematobium* are reviewed and examples from our recent studies in both east and west Africa are used to illustrate the importance of compatibility studies in understanding the changing epidemiology of urinary schistosomiasis. Environmental factors important in the transmission of schistosomes are considered and, although the *Bulinus*/*Schistosoma* model is not as amenable to laboratory study as others, the current state of knowledge concerning the internal defence mechanisms of *Bulinus* is highlighted as this is important in our understanding of snail parasite interactions.

MOLECULAR CHARACTERIZATION OF *BULINUS*

Reliable methods for the differentiation and identification of *Bulinus* species have been sought in order to determine those species and strains playing a major role in schistosomiasis transmission. Early descriptions of taxa relied upon analysis of morphological variation, including observations on shell shape and size, on the radula and on the reproductive system. Knowledge of a particular snail species was often confined to collections of snails from a few localities in one geographical area. These data sets were added to by determination of chromosome number and the use of biochemical characters. 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, 1986;

Njiokou *et al*. 1993; Rollinson & Southgate, 1979; Rollinson & Wright, 1984). More recently, studies on ribosomal RNA genes (rRNA), random amplified polymorphic DNA (RAPDs) and the mitochondrial gene cytochrome oxidase 1 (CO1) have added further insight into the relationships between species and provided new markers for species differentiation as described below.

DNA probes and microsatellites

Early explorations of DNA methods for identification of *Bulinus* were made by Strahan, Kane & Rollinson (1991) and Rollinson & Kane (1991). Shotgun-cloned genomic DNA from *B. cernicus* was used in both dot and Southern blot methodologies to screen recombinant DNA inserts for 'species' specificity. A representative selection of *Bulinus* species was used to investigate the probes potential for cross hybridisation. Four recombinant clones were characterized; two clones appeared population specific while the remainder were either non-specific or showed some species group resolution (Strahan *et al*. 1991). Rollinson & Kane (1991) investigated the use of restriction fragment length polymorphism (RFLP) analysis and Southern blotting to identify variation within the ribosomal internal transcribed spacer (ITS) between species of *Bulinus*. 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 hybridized to the schistosome derived probe pSM889. This probe is a 4·4 kb fragment that encompasses part of the small rRNA, internal transcribed spacer and large rRNA gene. As it contains highly conserved regions it shows homology with a wide variety of organisms and will readily bind to restricted fragments of snail DNA. 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 as was later shown by Stothard *et al*. (1996).

Population genetic analysis of *B. globosus* and *B. truncatus* using human minisatellite sequences in a multi-locus profiling approach was introduced by Jarne *et al*. (1990, 1992). More detailed population studies have been made possible by the development of microsatellites for *Bulinus*. Microsatellites are useful tools for investigating selfing rates in highly inbred species such as selfing snails. Enzyme studies by Njiokou (1993) on the tetraploid *B. truncatus* showed little variation in this predominantly selfing species, but Viard *et al*. (1996) demonstrated higher levels of polymorphism using microsatellites. Low genetic variation was also detected in *B. forskalii* using allozymes (Mimpfoundi & Greer, 1990), but Gow *et al*. (in press) have recently isolated and characterized eleven polymorphic microsatellites in *B. forskalii*, which will allow further elucidation of the population structure and mating system.

RAPD assays

(Langand *et al*. (1993) were the first to apply RAPD-PCR to populations of *Bulinus*. RAPD profiles could differentiate samples of *B. globosus* and *B. umbilicatus*, the number of shared or co-migrating fragments between profiles was 33%. Amplified fragments within a profile varied across populations of *B. forskalii* from West Africa. In a slightly more formal use of RAPD profiles for identification purposes using congruence between different sets of primers and electrophoretic methods, Stothard & Rollinson (1996) showed that in principle, RAPDs would not be ultimately reliable within all groups of the genus. Phenetic analysis of RAPD data showed that distance estimates between species were often non-additive, indicative of non-metric data, resulting in unstable, conflicting dendrograms. Whilst RAPDs could clearly show differences between species, it was unlikely that there would be species-diagnostic profiles for all species (Stothard & Rollinson, 1996). It was concluded that the nucleotide divergence within the genus went beyond the phylogenetic scope of the technique which is limited to samples of less than 10% divergence across actual or potential RAPD priming sites [see simulations of Clark & Lanigan (1993) and Stothard (1997)].

Nevertheless, on a regional level or within a species group of *Bulinus*, RAPDs have been useful especially when used in conjunction with other methods of identification. The levels of heterogeneity detected with RAPDs vary considerably within the species groups of *Bulinus*. For example, there is substantial diversity within and between populations of *B. globosus* (Stothard *et al*. 1997; Davies *et al*. 1999), a member of the *B. africanus* group. However, in a recent survey of *B. truncatus* (*B. truncatus*/*tropicus*) complex species from Sudan nearly all snails inspected had identical RAPD profiles (J. R. Stothard, unpublished data). RAPDs have been used in local comparisons of *B. africanus* group species in Zanzibar (Stothard *et al*. 1997) and around Kisumu, western Kenya (Raahauge & Kristensen, 2000).

Genetic population structures of both *S. haematobium* and *B. globosus* from the Zimbabwean highveld were compared using RAPDs by Davies *et al*. (1999). The observations reflected the dispersal mechanisms of snails and schistosomes with the genetic distance of snail populations correlating to position on river systems. In contrast *S. haematobium* from different sites within a river system were not differentiated, although populations from different

river systems appeared to be, suggesting that the definitive host plays an important role in dispersal of the parasite.

One potential drawback with RAPD analysis is the problem of homology of amplified fragments such that co-migrating fragments may be of identical size but of totally different sequence (Jones *et al*. 1999). After inspection of RAPD profiles from *B. forskalii* group snails, Jones *et al*. (1997) selected RAPD fragments to which further PCR fragments were designed. These new primer sets allowed amplification of the characterized regions using conventional PCR with two primers of specific sequence. This new assay was easier to interpret as only single amplified fragments of known sizes were amplified. The procedure named RAPD-SCARs, Sequence Characterized Amplified Regions provides a useful method for differentiating *B. forskalii* and related taxa.

The ribosomal internal transcribed spacer (*ITS*)

Parallel with the developments utilizing PCR with random primers, universal primers have been of value for studies on *Bulinus*. Using primers developed by (Kane & Rollinson (1994) to amplify the ribosomal internal transcribed spacer (ITS) from schistosomes, amplification products from all *Bulinus* tested were of approximately 1·3 kbp (Stothard *et al*. 1996). Direct digestion of these products with a battery of restriction enzymes produced RFLP profiles that could be easily visualized by agarose electrophoresis and ethidium bromide staining. Substantial variation between species groups was recorded both by inspection of the RFLP profiles themselves and by DNA sequencing of the ITS1, an approximately 500 bp sub-region within the ITS. The levels of divergence questioned the systematic placement of all such species within a single genus (Stothard *et al*. 1996). One restriction assay proved particularly useful. Digestion of the ITS with *Rsa*I clearly differentiated *B. truncatus* from *B. tropicus*. Separation of these two species often requires detailed inspection of radulae preparations or chromosomal squashes from egg mass material, *B. truncatus* is tetraploid whereas *B. tropicus* is diploid. PCR-RFLP analysis of the ITS has been used by Sène & Southgate (1998) for *B. truncatus* from Mali and Senegal. More recently a RFLP assay proved to be useful for differentiation of *B. forskalii* group snails from Mafia Island, Tanzania using digestion with *Sac*I (Stothard, Loxton & Rollinson, unpublished).

Nucleotide divergence within the *B. africanus* group was detectable with PCR-RFLP of the ITS and originally RFLP patterns appeared to be stable, offering potential for identification purposes for nearly all species within this group (Stothard *et al*. 1996). Further sampling of *B. africanus* group snails from Zanzibar revealed greater variation suggesting the occurrence of multiple ITS types within an individual snail (Stothard & Rollinson, 1997*a*). Whilst the profiles could differentiate *B. globosus* and *B. nasutus*, interpretation of RFLP patterns had to be conducted with care as the multiple or paralogous ITS types could potentially confound comparisons. Raahauge & Kristensen (2000) similarly found multiple ITS types within *B. africanus* group snails from western Kenya. This intragenomic heterogeneity was thought to be localized within the ITS2 region. Further PCR primers were designed to amplify only the ITS1 and restriction digestion of this product differentiated *B. globosus* from *B. nasutus* and *B. africanus*. It failed, however, to detect variation between *B. nasutus* and *B. africanus* (Raahauge & Kristensen, 2000).

Cytochrome Oxidase 1

Stothard & Rollinson (1997*b*) amplified a 450 bp region within the COI and determined by DNA sequencing that 33 positions within the sequence were variable between *B. globosus* and *B. nasutus*. Moreover, using double digestion with *Rsa*I and *Alu*I, RFLP patterns were produced that could differentiate *B. globosus* from *B. nasutus*. In addition, a species-specific restriction enzyme site *Ssp*I was identified such that only *B. globosus* sequences would be cut whilst those from *B. nasutus* or *B. africanus* remained intact (Stothard & Rollinson, 1997*b*). A simple restriction test such as this had certain advantages. Firstly, as the COI was amplified in all snails there was no need for further experimental controls during PCR and secondly, positive controls to check for restriction digestion could be performed with either *Alu*I or *Rsa*I or both. The assay appeared to be validated on three *B. africanus* group species originating from Zanzibar, Kenya, Zimbabwe and South Africa. However, as further snail populations were inspected from further afield, e.g. *B. globosus* from Niger, it become clear that this *Ssp*I site, whilst present in some individuals, was not universally shared (Rollinson & Hughes, unpublished data). Restriction enzymes are highly specific, hence their value decreases with increased sampling of genetic diversity and the detection of point mutations.

Point mutation detection: *ASA and SNaPshot*™

Molecular assays may have advantages and disadvantages when compared against each other (see Table 1 of Jones *et al*. 1999). New techniques under development may be valuable for devising routine procedures for the detection of single nucleotide changes in known sequences. One possibility is the use of allele-specific amplification (ASA). During the PCR amplification, if there is a base pair mismatch at the 3′ end of the PCR primer, the

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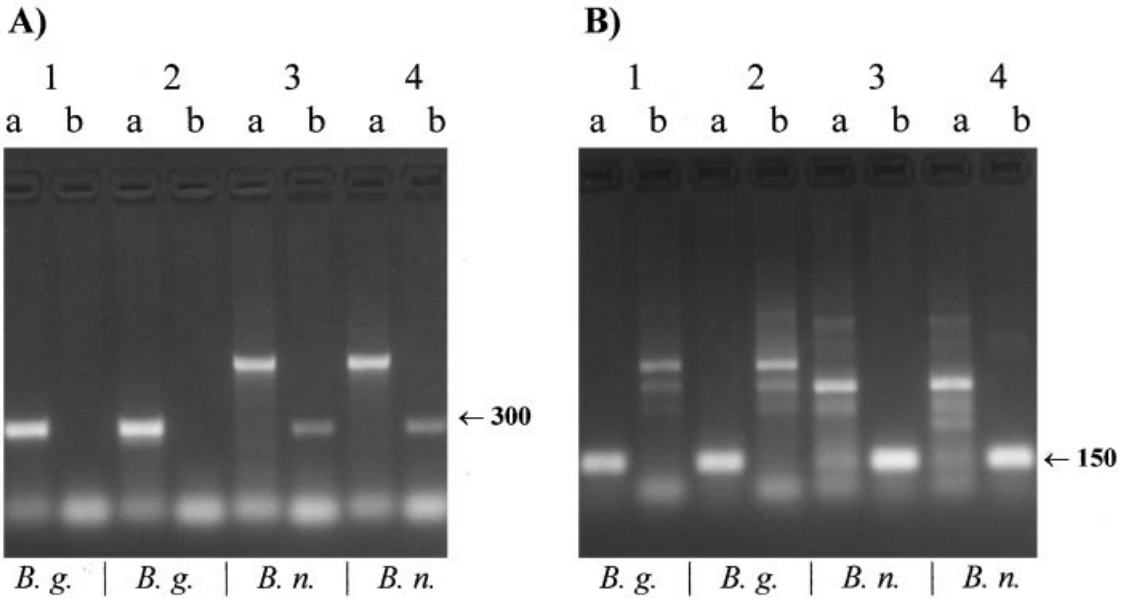

Fig. 2. Allele Specific Amplification (ASA) assay using a gel-based detection method for discrimination of *Bulinus* using point mutations within the CO1. Fig. 2A. ASA primers specific for either *B. globosus* (lanes a) or *B. nasutus* (lanes b) are used separately upon genomic DNA preparations from 4 single snails. An amplification product of specific size (300 bp) is targetted (Tracks l and 2 *B. globosus*, Tracks 3 and 4 *B. nasutus*). Presence or absence of the 300 bp product allows differentiation of the two taxa but in lanes 3a and 4a, a spurious amplification product is also produced. Fig. 2B. ASA primers specific for either *B. globosus* (lanes a) or *B. nasutus* (lanes b) are used separately upon genomic DNA preparations from 4 single snails and an amplification product of specific size (150 bp) is now targetted (Tracks l and 2 *B. globosus*, Track 3 & 4 *B. nasutus*). Whilst the target 150 bp amplification product is specific to each taxon with each ASA primer-pair, further spurious amplification products are seen e.g. lanes 1b and 2b and 3a and 4a.

enzyme Taq will not extend this oligonucleotide (Ugozzoli & Wallace, 1991). If the DNA variants/mutations are known, PCR primers can therefore be designed to match only one allele or variant. Providing that this allele or variant is specific to a species, ASA can provide a useful taxonomic assay but it is limited to the extent of sequence data available for candidate loci.

A good candidate locus is the COI, where there are numerous sequence differences between species but only a few are detectable with restriction enzymes. After inspection of several DNA point mutations that differentiated *B. nasutus* and *B. globosus*, four variant positions were selected to which ASA primers were designed to match either species. Initially non-gel based methods were used by incorporation of ethidium bromide into the PCR reaction itself, however, false positives were obtained because of primer-dimer formation and (or) the amplification of non-specific bands. After agarose electrophoresis, non-specific bands or primers-dimers could be discernible from the desired amplification product as its exact size was known (Fig. 2). Whilst ASA is highly discriminatory, it still is affected by additional mutations near the tested nucleotide that might also cause mis-priming and reaction failure. More importantly, the assay is biased towards false negatives if less than four primers are used to type each position. For example, specific primers for the nucleotide A would fail for all non-A nucleotides therefore mutations with C, G or T are not discernible unless additional ASA primers are used, or the region is later sequenced.

SNaPshot™ (Applied BioSystems Inc., U.K.) has recently been marketed as a fluorescent-based, primer extension assay and offers an alternative approach. In brief, after the gene target is amplified by PCR, a short oligonucleotide primer, or probe, is used to abut next to the variant position to be typed (Fig. 3). The SNaPshot primer can be of the same sequence as an ASA primer except that the 3′ terminal nucleotide is omitted. The SNaPshot primer is then used in a cycle sequencing reaction that has only dideoxynucleotides terminators with fluorescent labels and AmpliTaq DNA polymerase. In essence, a sequencing reaction is performed for the variable, single base. Upon completion of this reaction, the SNaPshot primer now carries a fluorescent label complementary to the nucleotide typed. This labelled primer is then separated on a standard denaturing polyacrylamide gel and analysed with GeneScan (analysis software on an automated DNA sequencer, e.g. ABI PRISM 377). The whole procedure is very quick, less than a working day and has the potential to be multiplexed where several variant positions, not necessarily from the same gene target, are genetically typed.

Preliminary results using SnaPshot™ typing of

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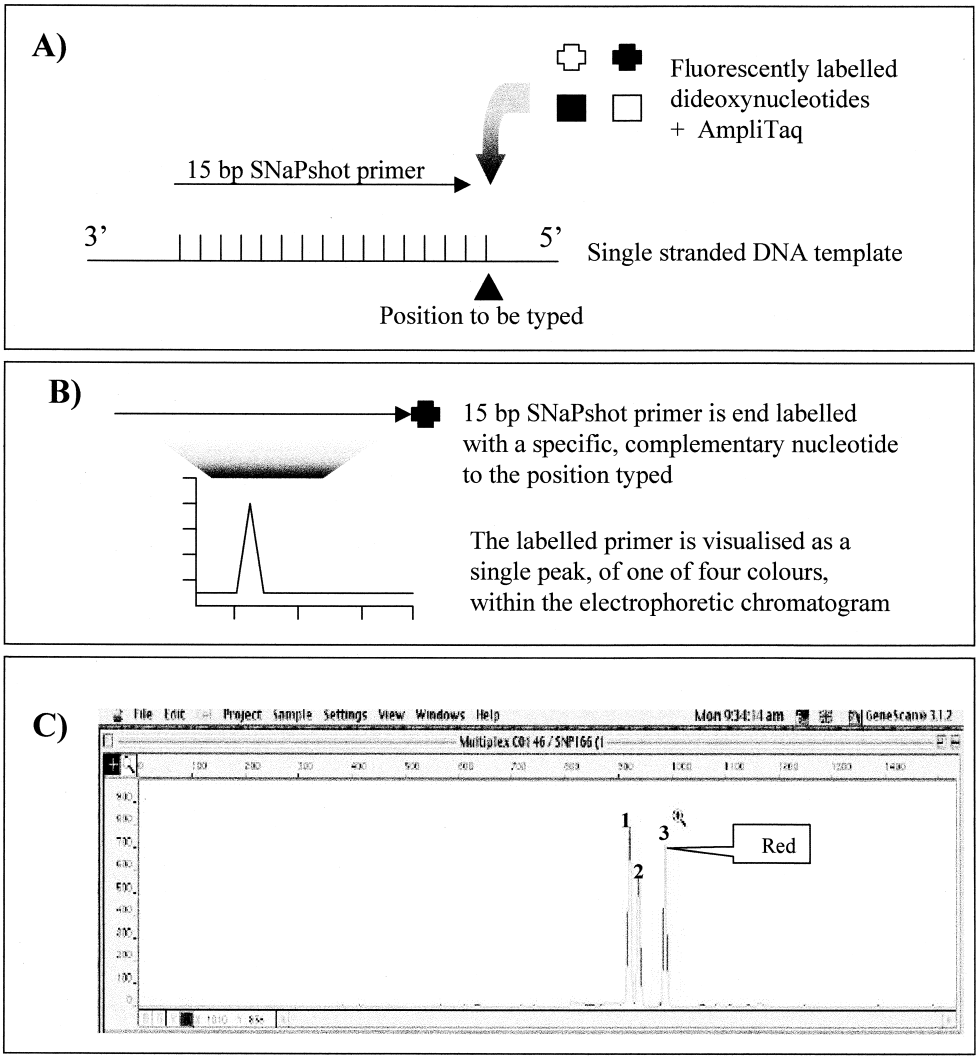

Fig. 3. SNaPshot™ offers a new primer extension based assay to type mutations at specific locations within an amplification product. Fig. 3A. A purified amplification product undergoes the addition of a 3í fluorescent label complementary to the variant position to be typed in a SNaPshot™ reaction using a 15 bp primer. Fig. 3B. The SNaPshot™ primer is now fluorescently labelled and subjected to denaturing electrophoresis on an ABI 377 automated DNA sequencer. This primer can be visualised as a single peak within the electrophoretic chromatogram and is of a specific colour to the incorporated dideoxynucleotide. Fig. 3C. A typical SNaPshot™ reaction as visualised using GeneScan software. In this instance, three reactions are sequentially loaded at 2 and 4 minute intervals. Multiplexing of several reactions within a single lane can considerably reduce costs. Up to 96 individuals can be typed individually for several point mutations in several target amplification products and analysed within a single gel.

variant nucleotides within the COI have shown the potential of this method as mutations can be quickly recognized and typed as well as detection of 'mixed templates' or heterozygotes (Fig. 3). Further optimization and use of multiplexing might make this methodology ideal for routine identification.

SNAIL HOSTS OF *S. HAEMATOBIUM*

Species and distributions of the intermediate hosts of *S. haematobium* have been summarized by Brown (1994). Modifications to the list of species would include the addition of *B. truncatus* for Jordan (Arbaji *et al.* 1998) and Senegal (Southgate *et al.* 2000). Intermediate hosts can be found in all of the species groups but most commonly in the *B. africanus* group and the *B. truncatus/tropicus* complex. The existence of strains of *S. haematobium* has been recognized for some time based primarily on compatibility with different species of *Bulinus*. The main divisions seem to lie between strains adapted more closely to *B. truncatus*, to *B. globosus* and to members of the *B. forskalii* group. Many experiments have reported the differences in parasite infectivity with *S. haematobium* from *B. truncatus* failing to develop in *B. africanus* group snails and *vice versa* (McCullough, 1959; Wright & Knowles, 1972; Chu, Kpo & Klumpp, 1978). A population study of the compatibility between *S. haematobium* and its potential snail hosts in Niger, exposing populations of snails from the same focus (sympatric)

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and populations from other foci (allopatric), emphasise the range of the 'compatibility polymorphism' within the *S. haematobium–Bulinus* system (Vera *et al*. 1990).

In some endemic areas the situation is simple with only one species of *Bulinus* being implicated in transmission. For example, in areas of North Africa including Algeria, Egypt, Libya, Morocco and Tunisia, *B. truncatus* is the only intermediate host, in Mauritius *B. cernicus* is the sole host: although variation in parasite compatibility may occur over small areas it is possible that only *B. globosus* acts as a host in Zambia, Zimbabwe and Zanzibar (Manning, Woolhouse & Ndamba, 1995; Mukaratirwa *et al*. 1996). Elsewhere more than one potential snail host may occur and transmission may be through more than one species. For example Fryer & Probert (1988) showed that in north-eastern Nigeria *S. haematobium* derived from *B. truncatus* and from *B. globosus* could also develop in *B. senegalensis*. The following two case studies illustrate how changes in compatibility can occur over a small geographical range.

Bulinus *and* Schistosoma *spp. in Senegal*

In Senegal there have been dramatic increases in the prevalence and intensity of human infection with *S. mansoni* and to a lesser extent *S. haematobium* (see Picquet *et al*. 1996; Southgate, 1997 and Sturrock *et al*. this supplement) following the construction of the Diama Dam on the Senegal River and the Manantali Dam on the Bafang River, a tributary of the Senegal River, in Mali. In the Lower and Middle Valleys, *S. haematobium* is transmitted primarily by *B. globosus* and *B. senegalensis* respectively (Vercruysse *et al*. 1994) and yet in the Upper Valley in Mali the main host appears to be *B. truncatus* (Rollinson *et al*. 1997*b*).

Interestingly, *B. truncatus* from the Lower and Middle Valleys were shown experimentally to be susceptible to *S. haematobium* isolated from the urines of children in Tenegue, Office du Niger, Mali where *B. truncatus* is the intermediate host. However they were not susceptible to a parasite isolated from the Lower Valley where natural transmission is normally associated with *B. globosus*. The authors drew attention to the possible appearance of a *B. truncatus*-borne parasite in the Middle and Lower Valleys (Rollinson *et al*. 1997*b*).

Southgate *et al*. (2000) carried out further snail infection experiments with *S. haematobium* from various sites in Senegal using laboratory-bred and wild-caught snails. They were able to show that isolates of *S. haematobium* from the Middle Valley do show some compatibility with *B. truncatus* although the experimental snails were from Mali and not Senegal. However, M. Sène (unpublished observations) has recently found *B. truncatus* from the Middle Valley naturally infected with *S. haematobium*. Therefore it seems that the situation in the Lower and Middle Valley of the SRB may be changing in that there is the likelihood that the currently most widespread bulinid snail, *B. truncatus*, may become more important in the epidemiology of urinary schistosomiasis in the Senegal River Basin (SRB) in addition to *B. senegalensis* and *B. globosus*. In the Tambacounda region of Senegal *B. umbilicatus* also acts as a host for *S. haematobium* and *S. curassoni* (Rollinson *et al*. 1998).

When other schistosome species are taken into account it can be seen that the situation is more complex with some snails potentially acting as hosts for more than one schistosome parasite. For example, *S. curassoni* from Senegal is incompatible with *B. truncatus*, marginally compatible with *B. senegalensis* and compatible with *B. umbilicatus*, whereas *S. bovis* is compatible with all three species (Southgate *et al*. 1985*a*). Therefore, in the SRB it is necessary to be able to differentiate cercariae of *S. bovis* and *S. haematobium* shed from naturally infected *B. truncatus*. *S. bovis* has a natural wide intermediate host range and is compatible experimentally with species in all four *Bulinus* species groups (for review see Moné, Mouahid & Morand, 2000).

Bulinus *and* S. haematobium *transmission in Zanzibar*

In East Africa hosts for *S. haematobium* are found in the *B. africanus* group. Four species of the *B. africanus* group are currently recognized in Kenya and Tanzania: *B. globosus*, *B. nasutus*, *B. africanus* and *B. ugandae* (Brown, 1994) and all except *B. ugandae* have been implicated in transmission. Two of the taxa, *B. globosus* and *B. nasutus*, are difficult to identify, as morphometric variation within each species appears to form a continuum and in the north eastern region of Tanzania specimens from either taxa almost appear conspecific (Mandahl-Barth, 1957). To resolve the taxonomic dilemma between *B. globosus* and *B. nasutus* a detailed study of the snails was conducted in Zanzibar with individual snails being characterized by three methods: shell morphometry, enzyme analysis and molecular analysis with RAPDs.

Zanzibar incorporates two islands Unguja, more commonly known as Zanzibar island, and the more northerly island of Pemba. Schistosomiasis is major public health problem on both Pemba and Unguja and these islands have been the focus of a WHO-funded disease control programme through chemotherapy (Savioli *et al*. 1989). Both islands lie close to the coastal region of mainland Tanzania where earlier Rollinson & Southgate (1979) found three

enzyme systems which appeared to discriminate *B. africanus* group species.

On Zanzibar it became clear that the two species could be differentiated by isoenzyme analysis and DNA typing (Stothard *et al*. 1997; Stothard & Rollinson, 1997*a*, *b*). Detailed surveys suggest that the distributions of *B. globosus* and *B. nasutus* are allopatric although *B. forskalii* may be found in association with both species. On Unguja, *B. globosus* is restricted to northern areas whereas *B. nasutus* is confined mainly to the South. On Pemba, *B. globosus* is widespread whereas *B. nasutus* is associated with the eastern border of the central region (Stothard *et al*. 1997). With clear species markers it was possible to assess the roles played by the snails in transmission of *S. haematobium*. *B. nasutus* was refractory to experimental infection and no evidence of natural infection has been observed. In contrast, *B. globosus* has been found naturally infected and showed a high infection rate on experimental challenge with miracidia hatched directly from infected urines. In the north of Unguja the high prevalence of infection in school children fits with the known distribution of *B. globosus* (Stothard & Rollinson, 1997*b*: Stothard *et al*. 2000).

It must now be clarified as to whether *B. nasutus* is playing a role in transmission on the African mainland especially in the coastal areas of Kenya and Tanzania (Webbe & Msangi, 1958; Sturrock, 1965; Pringle *et al*. 1971). This will be possible if morphological identifications are supplemented with enzyme and DNA markers. Also observations on naturally infected snails will need to take into account that *B. africanus* group snails may also transmit *S. bovis*, hence cercariae emerging from naturally infected snails will need to be identified. If *S. haematobium* from coastal areas is shown to be transmitted by *B. nasutus* it will be of interest to determine whether the parasite is compatible with *B. nasutus* from Zanzibar.

OBSERVATIONS ON TRANSMISSION

Chronobiology

There is considerable interspecific diversity in cercarial emergence rhythms within the genus *Schistosoma*, with emergence peaks occurring at different times of the nycthemere (a 24 h photocycle), ranging between 08·00 and 09·00 h for the earliest, to 22·00 h and 23·00 h for the latest (Combes *et al*. 1994). The rhythmic emergence of cercariae is synchronized by exogenous factors to which the cercariae respond, and photoperiod is the most important factor. Furthermore, generally there is a clear correlation between the maximum shedding period of cercarial and water contact patterns of the definitive host, thus enhancing the chances of infection of the definitive host and continuation of the parasite's life cycle. The cercarial emergence rhythms limit their temporal dispersion by concentrating them in the period of the nycthemere when the chances of meeting the host are greatest. Thus, cercarial rhythms may be considered as an adaptive behaviour increasing the probabilities of a meeting between parasite and host (Combes *et al*. 1994). Intraspecific variation in cercarial emergence rhythms has been well documented in cases where the parasite has adapted to different hosts which themselves possess dissimilar water contact patterns. Indeed, experimental cross-mating experiments between different species and populations with different chronobiological phenotypes demonstrate that the rhythms are genetically controlled (Théron, 1989; Théron & Combes, 1988).

There is less information available regarding intraspecific variation in cercarial emergence patterns with the same definitive host. A study by N'Goran *et al*. (1997) on nine isolates of *S. haematobium* that utilized either *B. truncatus* or *B. globosus* from the Ivory Coast as intermediate hosts demonstrated the existence of intraspecific variation in cercarial emergence rhythms. A statistical analysis on the data demonstrated that the nine isolates fell into 3 groups which corresponded to 3 different climatic and vegetal zones: (i) sub-Sudanian zone with aquatic habitats in the wooded savannah; (ii) transitory zone between savannah and forest and (iii) Guinea climatic zone where transmission sites are located in the dense forest. Illumination is the primary factor influencing cercarial shedding, and the cercarial emergence of *S. haematobium* shows rapid reaction to variation in light intensity (Raymond & Probert, 1987). N'Goran *et al*. (1997) postulated that *S. haematobium* cercariae from the forest foci in Ivory Coast may be more sensitive to light than those from the savannah habitat, thus enabling the cercariae from the forest foci to maintain the shadow response, that is, the stimulation of cercarial emergence and increased activity of cercariae caused by human activity forming a shadow. Thus, the response to a shadow will increase the chances of infection. N'Goran *et al*. (1997) considered the differential sensitivity to light to be an ecological adaptation to maintaining the shadow response.

Studies by Pitchford *et al*. (1969) examined the influence of changing seasons on the chronobiology of cercarial emergence of a number of schistosome species, including *S. haematobium*, *S. mattheei* and *S. bovis*. These authors noted interspecific differences and seasonal differences in shedding patterns: for example, *S. mattheei* and *S. bovis* shed between 06·00 h and 08·00 h, whereas *S. haematobium* shed between 11·00 h and 15·00 h. Temperature was also found to influence the numbers of cercariae released, generally more cercariae were shed at higher temperatures, fewer at lower temperatures. *S. mattheei* was found to be also a

nocturnal shedder, in addition to the early morning shed, at lower temperatures. *S. curassoni*, a parasite of domestic stock, has a shedding pattern which peaks in the early morning (Mouchet *et al*. 1992), whereas *S. intercalatum*, a parasite of man, peaks at the middle of the day (Pagès & Théron, 1990). However, *S. margrebowiei* is unusual in that it possesses an ultradian shedding rhythm with two distinct emergence peaks, at dawn and at dusk. The two peaks of emergence pattern coincide with visits to watering places by antelopes and waterbuck (Raymond & Probert, 1991).

Other parasites can influence compatibility

A polyploid series occurs within the *B. truncatus/tropicus* group, with diploid, tetraploid, hexaploid and octoploid species. The diploid species are found in the Afrotropical region and, although there is some overlap between the tetraploid and diploid species, the tetraploid species have a more northern distribution. Hexaploid and octoploid species are associated with streams at high altitude in the Ethiopian highlands. *B. tropicus*, a diploid species, was generally considered to be incompatible with schistosome parasites until *Schistosoma margrebowiei* had been shown to be naturally transmitted by *B. tropicus* in Lochinvar National Park, Zambia (Southgate *et al*. 1985*b*). Around the same time Southgate *et al*. (1985*c*) reported that of 112 *B. tropicus* collected from the Mau Escarpment, Kenya, 10 were infected with *S. bovis* and an amphistome parasite. Exposure of laboratory-bred *B. tropicus* from the Mau Escarpment, Kenya to *S. bovis* proved negative, thus suggesting that the presence of amphistome infection may have a suppressive effect on the immune system of the snail, thereby allowing *S. bovis* to develop. Subsequent infection studies in the laboratory demonstrated that it is possible to infect *B. tropicus* with *S. bovis*, but only if the snails had been exposed to miracidia of an amphistome, *Calicophoron microbothrium* (Southgate *et al*. 1989). Thus the laboratory studies supported the field studies that a primary infection of another trematode is necessary for *B. tropicus* to be susceptible to *S. bovis*. Studies by (Loker, Bayne & Yui (1986) on a different host parasite relationship showed that when cytotoxic *Biomphalaria glabrata* haemocytes were co-incubated with *S. mansoni* sporocysts and *E. paraensi* rediae, the echinostome depressed the cytotoxic capacity of the haemocytes for the larval schistosomes. Thus, it is possible that larval amphistomes secrete interfering factor(s) that somehow alter the behaviour of the *Bulinus* haemocytes, preventing them from recognizing and encapsulating the young *S. bovis* sporocysts.

Internal defence mechanisms in Bulinus

The recognition of non-self by the snail is suspected to be mediated by lectins or other humoral factors which potentially function as opsonins or recognition agents that promote antigen clearance or encapsulation by haemocytes. Functional differences of haemocytes between various snail species are known; for example, peroxidases can be lacking (Adema, Harris & Van Deutekom-Mulder, 1992), and there appears to be at least two morphologically distinct types (Preston & Southgate, 1994).

With regard to the immuno-biology of *Bulinus*, a different set of molecules are thought to operate from those in *Biomphalaria*. For example, in *Biomphalaria glabrata* there appears to be two families of lectins, which can be differentially induced. These are broadly placed within two groups: G1M and G2M – carbohydrate binding proteins of 150–220 kDa and 75–130 kDa, respectively (Couch, Hertel & Loker, 1990). These molecules are also able to bind mammalian erythrocytes, hence have agglutinin properties, and are able to differentiate non-self epitopes. Agglutinins have been shown to be present in two species of *Bulinus*, *B. nasutus*, and *B. truncatus*, though surprisingly, of the other species of snail tested, none was detected.

Harris (1990) studied the binding of these agglutinins in *Bulinus* and found they were not inhibited by either monosaccharides or disaccharides, hence were unlikely to be lectins: the agglutinating activity was later traced to a glycoprotein (Harris, 1990). In the reduced form on SDS-PAGE gels this protein was shown to be approximately 135 kDa and inducible upon infection with miracidia. It was also shown to bind to invading miracidia but not to mother sporocysts, daughter sporocysts or to emerging cercariae. It is speculated that this molecule is involved in humoral surveillance (Harris, 1990). Proteins of similar M_r to the haemagglutinin of *B. nasutus* were shown to be present in a different species of bulinid tested, and Cleveland mapping of the M_r 135 kDa proteins revealed identical polypeptide patterns thus suggesting that the primary structure of these proteins is similar (Harris, Preston & Southgate, 1993). The glycoprotein is thought to have similar primary structure and could be categorized into two groups: agglutinin or non-agglutinin. Further subgroups could be recognized immunologically: polyclonal antisera were obtained from BALB/c mice immunized with the 135 kDa protein from *B. truncatus* and these antibodies were used as probes in Western blots against 135 kDa polypeptides from several other *Bulinus* species (Harris *et al*. 1993). The antibody cross-reactivity was confined to species within the *B. truncatus/tropicus* complex. Despite there being some primary sequence homology between species groups, the protein epitopes recognized by mice were not shared across species groups of *Bulinus* (Harris *et al*. 1993). Since this protein is glycosylated, it may potentially allow considerable

variation in the shape of this molecule and may be of considerable importance in immuno-surveillance (Preston & Southgate, 1994).

ABIOTIC FACTORS AFFECTING TRANSMISSION

Numerous abiotic factors (see below) are important in how they affect the distribution patterns of snails, and influence the life cycles of snails, population dynamics and hence patterns of transmission. One of the remarkable features of the biology of *Bulinus* is the high rate of increase in populations which is related to the ability to reproduce by both cross-fertilization (outcrossing) and self-fertilization (selfing) and responding to environmental stimuli (e.g. temperature, rainfall etc.).

For example, the life cycle of *B. globosus* has been described by many authors (O'Keefe, 1985*a*, *b*; (Marti, 1986; Woolhouse & Chandiwana, 1989, 1990*a*, *b*). Young snails commence egg-laying at an age of 5–7 weeks and a height of 6–7 mm and continue breeding while growing to a height of 12–17 mm. Eggs develop immediately and hatch about one week after being laid.

Temperature

Temperature is considered as the most important abiotic factor influencing the distribution of snails in lentic environments. Snails may be killed by temperatures above or below lethal limits. However, the assessment of the effects of temperature in the field is difficult because there are considerable temperature gradients within a water body and snails can seek microhabitats where temperature is most favourable. Temperature influences snail distribution through its effects on reproduction and growth of juveniles, as well as on the survival of adults.

For cohorts of *B. globosus* reared in reservoirs on the Kenyan coast, r (the intrinsic increase in a population) was inversely related to increasing mean water-temperature above 25 °C (O'Keefe, 1985*a*). Nevertheless, *B. globosus* seems better adapted to high temperatures than *Biomphalaria pfeifferi* which is absent from the coastal area of Kenya. Mark recapture data show the recruitment rate of *B. pfeifferi* into populations in Zimbabwe to increase over the range 13–24 °C; therefore, under the cooler climatic conditions of southern Africa the breeding season is short. Although optimal temperature of *B. globosus* in the laboratory lies at about 25 °C, whereas for *B. pfeifferi* it is between 20–27 °C, it seems as though *B. globosus* is more tolerant of higher temperatures. For example, in a cohort of *B. globosus* egg production was delayed at 18 °C until snails were about 23 weeks old, more than twice the age of first oviposition at 25 °C (Shiff, 1964*a*). In cohorts maintained at different temperatures, 18, 22·5, 25 and 27 °C, the most rapid increase of growth was observed at 25 °C (Shiff, 1964*b*). Laboratory investigations have demonstrated that although the temperatures at which snails attained an optimal r were similar, the range at which r remained high varied for different species. The marked peak in r attained by *B. globosus* at 25 °C and 27 °C probably enables it to take advantage of harsh environmental conditions found in temporary water bodies, for example, multiplying rapidly at the optimal temperature range. These examples illustrate how temperature plays a vital role in the biology of pulmonate snails by influencing growth and reproduction.

Rainfall and water velocity

Rainfall has a marked effect on snail populations by causing population fluctuations through drought and flooding, and influencing rates of oviposition and survival. For example, Woolhouse & Chandiwana (1990*a*, *b*) investigated the population dynamics of *B. globosus* in Zimbabwe and reported that year-to-year fluctuations were correlated with the effects of sudden spates, which washed away the snails from some sites and deposited them in others. Gradual increases in water level can encourage breeding probably through reducing the population density thereby removing density-dependent inhibition of reproduction. O'Keefe (1985*a*, *b*) noted peaks of egg production in reservoirs on the coast of Kenya following rainfall in the cooler months.

Water velocity is determined by geomorphic factors, especially the resistance offered by different types of bedrock: it is recognized as the most important factor in lotic environments determining the distribution of intermediate hosts. Measuring currents in the field experienced by snails is difficult because of variations in time and space. Appleton (1975) estimated tolerance ranges with upper limits of 0·3 m s^{-1} for *Bulinus*, but where the bedrock is hard a river may contain many boulders providing refuges and protection for snails in currents above 0·3 m s^{-1}. In small rivers and streams sudden spates following heavy rainfall will cause major fluctuations in population density and may be an important factor in the dispersal of snails. In such habitats, the rainy season will coincide with the reduction in snail population and transmission. Southgate *et al*. (1994) carried out a survey of *B. forskalii* on the island of São Tomé and noted that the snails were confined to the north east of the island where water velocity was considerably less than elsewhere on the island. In order to investigate the limiting factor(s) of the distribution of *B. forskalii* on São Tomé, a survey was conducted at 4 sites along the Agua Serra, taking physical measurements and searching for freshwater snails, and again snails were only found in one locality, Bombom, where the water velocity was

much reduced. A one year study at two weekly intervals from 5 different sites showed that the snail populations generally increased during the dry period of June, July and August, and during the wet season snails were completely absent from two localities for two months and one month, respectively and were much reduced in the other three localities, with the obvious implications for reduced rates of transmission (Southgate *et al*. 1994).

Desiccation

Desiccation is a catastrophe for a snail population and it is the major restraint on the number of species that live in seasonal water bodies. There is a distinction between aestivation (prolonged survival by dormant snails out of water) and diapause (spontaneous climbing out of water and entering a state of dormancy). A third type of behaviour has been recognized, water-quitting. Diapause and water quitting are of interest in relation to the avoidance by snails of molluscicide. Aestivation is important because some species of snail, for example, *B. senegalensis* and *B. nasutus*, are able to survive without water for between 5–8 months. This ability enables these species to inhabit ephemeral water bodies which become transmission foci only during the rainy season. There is a general correlation between success in aestivation and a high capacity for increase (r), enabling rapid repopulation of a habitat when water returns. Field observations on pre-aestivation behaviour by *Bulinus* indicate two different strategies. *B. nasutus* and *B. senegalensis* aestivated around the margins of temporary pools, whereas *B. globosus* and *B. truncatus* aestivated towards the bottom of drying out pools. Aestivation around the margins of the pools prevents snails emerging when revived by isolated showers; in fact, the snails only emerge when pools are well filled. This strategy is advantageous preventing the depletion of the emerging population. It is known that some snails are able to carry over an infection after a period of aestivation (Webbe, 1962).

Salinity

Salinity (also known as total dissolved chemical content or total electrolytes) is commonly estimated as electrical conductivity. The concentration of dissolved salts limits the distribution of pulmonate snails when it is unusually high or low. For example, in West Africa the intrusion of sea water into rivers in the dry season is known to limit the distribution of intermediate hosts. There is a progressive elimination of gastropod species at salinities of as little 1‰. Donnelly, Appleton & Schutte (1983) demonstrated that the fecundity and survival of *B. africanus* was adversely affected with salinities of 1‰ with the most significant reductions occurring between 3·5‰ and 4·5‰. The severe outbreak of *S. mansoni* and *S. haematobium* in the Senegal river basin is correlated with reductions in salinity due to the construction of a barrage at Diama, approximately 40 km from the sea, on the Senegal River thus preventing the intrusion of sea water into the Senegal River during the dry season (Southgate, 1997).

Recent studies on Zanzibar Island (Unguja) demonstrated a clear distinction in the distribution of *B. globosus* and *B. nasutus* on the island and this appears to be correlated to the sharp division of the geological zones of the island and water conductivity (Stothard *et al*. 2000). *B. globosus*, which is highly compatible with *S. haematobium* is found only in the north of the island on clayey soils with subordinate limestone, whereas *B. nasutus* is found primarily in the south and is confined to fossiliferous limestone/marly sand substratum. There is some overlap of the two species in the central region, but no occurrences of sympatric populations. *B. nasutus* on Zanzibar Island is refractory to *S. haematobium*.

CONCLUDING REMARKS

The identification and characterization of *Bulinus* spp. is sometimes fraught with difficulties, especially in those cases where there is considerable overlap of characters. The development of molecular techniques has added significantly to the armoury of morphological, cytological and biochemical techniques that have been used in the identification and characterization of species and populations of *Bulinus*, and has facilitated a more detailed elucidation of the intermediate host parasite relationship. More specifically studies on ribosomal genes (rRNA), random amplified polymorphic DNA (RAPDs) and mitochondrial gene cytochrome oxidases 1 (CO1) have been used to determine insights into relationships between species and provide markers for species differentiation. Morphometric variation poses problems in distinguishing *B. globosus* from *B. nasutus* on Zanzibar Island and Pemba, but these have been solved by the use of enzymes and DNA typing. More detailed population studies have been made possible by the development of microsatellites. RAPDs-PCR have been shown to be useful in certain situations for diagnosis, but it is thought unlikely that this approach will lead to a species specific diagnosis for all species of *Bulinus*. A further refinement, RAPD-SCARs, sequence amplification of characterized regions, has enabled amplification of characterized regions using PCR with two pairs of specific sequence. Additional techniques are being developed and investigated to refine diagnosis such as the allele specific amplification (ASA) and SNaPshot™ typing of variant nucleotide within CO1 where mutations can be quickly recognized and scored. It seems likely molecular techniques will continue to develop in the area of diagnosis. The epidemiology and *Bulinus*/*S. haematobium* group

relationships are complex. For example, some species of *Bulinus* within the 4 species groups may act as an intermediate host for *S. haematobium* but strains of *S. haematobium* show considerable variation in levels of compatibility or indeed incompatibility with different species of *Bulinus*. It is important that this intermediate host parasite specificity is recognized to gain a further understanding of the intricacies of epidemiology. The intermediate host response has both humoral and cellular components, and Preston & Southgate (1994) suggested that a glycosylated protein found in *Bulinus* spp. may be of importance in immunosurveillance. It is interesting that the 'normal' intermediate host response to an incompatible schistosome may be modified by earlier infection with a different parasite. The epidemiological implications of such interactions and modification of host response have yet to be fully elucidated. Examples are given to demonstrate how environmental factors such as temperature, rainfall, water velocity and desiccation influence the distribution of *Bulinus* spp. and how different species adapt in varying ways to environmental changes. The influence of abiotic factors on the distribution of *Bulinus* spp. inevitably has a bearing on the distribution of the parasites. The situation in the SRB is given as an example where environmental changes (primarily reductions in levels of salinity) have given rise to marked increases in the spread, prevalence and intensity of *S. mansoni* and to a lesser extent *S. haematobium* (Picquet *et al*. 1996). Recent field and laboratory data support the view that changes are occurring in the intermediate host–parasite relationships between *Bulinus* spp. and *S. haematobium* in the SRB possibly through the introduction and establishment of different strains of *S. haematobium*: such changes emphasise the dynamic nature of the intermediate host-parasite relationship. With the ever-increasing mobility of the definitive host (man) and environmental changes induced by increases in the human population it is not surprising that new intermediate host parasite relationships are being initiated resulting in changes in epidemiology.

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