Genetic variability and molecular identification of Brazilian *Biomphalaria* species (Mollusca: Planorbidae)

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SUMMARY

Freshwater snails belonging to the genus *Biomphalaria* are intermediate hosts of the trematode *Schistosoma mansoni* in the Neotropical region and Africa. In Brazil, one subspecies and ten species of *Biomphalaria* have been identified: *B. glabrata*, *B. tenagophila*, *B. straminea*, *B. occidentalis*, *B. peregrina*, *B. kuhniana*, *B. schrammi*, *B. amazonica*, *B. oligoza*, *B. intermedia* and *B.t. guaibensis*. However, only the first three species are found naturally infected with *S. mansoni*. The classical identification of these planorbids is based on comparison of morphological characteristics of the shell and male and female reproductive organs, which is greatly complicated by the extensive intra-specific variation. Several molecular techniques have been used in studies on the identification, genetic structure as well as phylogenetic relationships between these groups of organisms. Using the randomly amplified polymorphic DNAs (RAPD) analysis we demonstrated that *B. glabrata* exhibits a remarkable degree of intra-specific polymorphism. Thus, the genetics of the snail host may be more important to the epidemiology of schistosomiasis than those of the parasite itself. Using the simple sequence repeat anchored polymerase chain reaction (SSR-PCR) in intra-populational and intra-specific studies we have demonstrated that snails belonging to the *B. straminea* complex (*B. straminea*, *B. kuhniana* and *B. intermedia*) clearly presented higher heterogeneity. Using the low stringency polymerase chain reaction (LS-PCR) technique we were able to separate *B. glabrata* from *B. tenagophila* and *B. tenagophila* from *B. occidentalis*. To separate all Brazilian *Biomphalaria* species we used the restriction fragment length polymorphism (PCR-RFLP) of the internal transcribed spacer region (ITS) of the DNA gene. The method also proved to be efficient for the specific identification of DNA extracted from snail eggs. Recently we have sequenced the ITS2 region for phylogenetic studies of all *Biomphalaria* snails from Brazil.

Key words: *Biomphalaria*, Brazil, polymerase chain reaction, genetic variability, identification, ribosomal RNA, internal transcribed spacer, phylogeny.

BIOMPHALARIA SNAILS: IMPORTANCE AND BIOLOGICAL CHARACTERIZATION

Freshwater snails, belonging to the genus *Biomphalaria* Preston, 1910 (Mollusca: Pulmonata, Planorbidae) are intermediate hosts for the parasitic trematode *Schistosoma mansoni* in the Neotropical region and Africa. According to Baker (1945), geological records of the Planorbidae prove its presence in Europe and United States since the Jurassic Period.

These molluscs can be found in a variety of natural and artificial (lakes, dams, river streams and irrigation ditches) environments. Thus, such snails readily adapt to different environmental conditions and are able to withstand a range of variation in the physical, chemical and biological characteristics in the environment in which they live (Paraense, 1972).

Biomphalaria snails are hermaphrodites but usually reproduce by cross-fertilization when paired. Such biological characteristics are evolutionarily important, providing the organism with the ability to establish colonies from a single individual while maintaining genetic variation through sexual reproduction (Paraense, 1955). Most populations are under significant environmental pressure (rain and drought) that can dramatically reduce population levels, requiring re-colonization from a low number of remaining snails, founder effect (Paraense, 1955). Paraense, Pereira & Pinto (1955) comment that the fixation of populations may occur in a very short period of time, due to the high reproductive potential of the species, enhanced by the absence of intra-specific competition. On the other hand, limited gene flow (a lack of interconnecting waterways) between different populations could enable the formation of local strains, resulting in inter-populational variability. The observation of several levels of reproductive isolation, detected in allopatric conspecific individuals, also indicated extensive genetic heterogeneity among populations of *Biomphalaria* snails from different localities (Paraense, 1959).

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BRAZILIAN *BIOMPHALARIA* SNAILS: SPECIES, GEOGRAPHICAL DISTRIBUTION AND SUSCEPTIBILITY

In Brazil, ten species and one subspecies of *Biomphalaria* are recognised: *B. glabrata* (Say, 1818), *B. tenagophila* (Orbigny, 1835), *B. straminea* (Dunker, 1848), *B. peregrina* (Orbigny, 1835), *B. schrammi* (Crosse, 1864), *B. kuhniana* (Clessin, 1883), *B. intermedia* Paraense & Deslandes, 1962, *B. amazonica* Paraense, 1966, *B. oligoza* Paraense, 1974, *B. occidentalis* Paraense, 1981 and *B. t. guaibensis* (Paraense, 1984). However, only the first three species are found naturally infected with *S. mansoni*. *Biomphalaria peregrina* and *B. amazonica* are considered potential hosts based on experimental infection (Paraense & Corrêa, 1973; Corrêa & Paraense, 1971).

Biomphalaria glabrata is considered the most important *S. mansoni* intermediate host in the Neotropical region, due to its wide distribution and high susceptibility to the trematode (Paraense & Corrêa, 1963). On the other hand, *B. straminea* plays an important role as a vector in the Northwest of Brazil (Paraense, 1986; Paraense & Corrêa, 1989) and *B. tenagophila* in some areas in the South of Brazil (Paraense & Corrêa, 1987).

Richards & Shade (1987) reported that *Biomphalaria* susceptibility to *S. mansoni* infection varies among geographic areas, populations in the same area, individuals within the same population and also among snails of different ages. In fact, Richards (1984) showed that in *B. glabrata* adults resistance to infection is controlled by a single gene, the inheritance of which follows simple Mendelian genetics, with dominance to resistance. In juvenile snails, however, resistance appears to be a polygenic trait (Richards & Merrit, 1972). Richards & Shade (1987) point out that the genetic variation in snail susceptibility and parasite infectivity result in a variety of host-parasite relations.

Thus, studies on the parasite and intermediate host genome remain important for the study of the epidemiology and control of schistosomiasis.

MOLECULAR STUDIES OF *BIOMPHALARIA* SNAILS

Molecular studies to further understand the genetic variability and population structure of *Biomphalaria* snails are needed due to: (1) intraspecific variation being detected at both morphological (Paraense, 1975*a*) and genetic levels (Knight *et al*. 1991; Vidigal *et al*. 1994), and (2) differences in susceptibility and compatibility among *Biomphalaria* snails to infection with *S. mansoni* (Paraense & Corrêa, 1963; Souza, Passos-Jannoti & Freitas, 1995).

Isoenzyme techniques have been shown to be useful in the identification of *Bulinus*, *Oncomelania*, *Lymnaea* and *Biomphalaria* as well as for the investigation of phylogenetic relationships and the genetic structure of these groups of organisms (Bandoni, Mulvey & Loker 1995; Mascara & Morgante, 1995; Zahab-Jabbour *et al*. 1997; Zhou & Kristensen, 1999; Mukaratirwa *et al*. 1998).

Knight *et al*. (1991) used the ribosomal RNA gene to detect restriction fragment length polymorphisms (RFLPs) and demonstrated the variability between resistant and susceptible *B. glabrata* snails to *S. mansoni*. The development of the polymerase chain reaction (PCR) has allowed considerable progress in the molecular study of *Biomphalaria*. The arbitrarily primed polymerase chain reaction (AP-PCR–Welsh & McClelland, 1990; Williams *et al*. 1990) has been used to study genomic variability in different groups of organisms (Gomes *et al*. 2000; De Sousa, Dutari & Gardenal, 1999). Vernon, Jones & Noble (1995) showed the potential of RAPDs as molecular markers for analysis of fertilisation in wild-type *B. glabrata*. In addition, promising results concerning resistance and susceptibility to *S. mansoni* were reported by Larson *et al*. (1996), Lewis, Knight & Richards (1997), Knight *et al*. (1999) and by Knight, Ongele & Lewis (2000). Abdel-Hamid *et al*. (1999) also employed RAPD analysis for the study of susceptility and resistance to *S. mansoni* in *B. tenagophila*. These studies showed genetic variability between the susceptible and resistant strains. A high level of genetic variability among populations of *B. pfeifferi*, obtained along a 6 km stretch of the Zimbabwe river, was demonstrated by Hoffmam *et al*. (1998). On Guadalupe Island, with a limited area of 45 km^2, a correlation between genetic distance and geographic distance was reported by Langand *et al*. (1999), with a high level of genetic variability.

Microsatellites (MS) are useful genetic markers due to their frequencies within eukaryotic genomes, their ease of amplification by PCR and their degree of polymorphism. Microsatellites have been widely used for studies of genetic variability in many organisms (Donnelly *et al*. 1999; Ferdig & Su, 2000) including snails such as *Bulinus*, *Melania* and *Littorina* (Viard *et al*. 1997*a*, *b*, *c*; Samadi *et al*. 1999; Tie, Boulding & Naish, 2000).

Recently, Jones *et al*. (1999) reported the identification and characterization of the first microsatellite *loci* in *B. glabrata* and also demonstrated divergence between resistant and susceptible stocks to *S. mansoni*. These authors indicated that microsatellite markers could contribute to the elucidation of evolutionary relationships among African and Neotropical *Biomphalaria* species. Indeed, *B. pfeifferi* was found to be more polymorphic than the neotropical species (*B. straminea*, *B. occidentalis* and *B. tenagophila*). Mavárez *et al*. (2000) characterized nine microsatellite *loci* in *B. glabrata* populations from Venezuela and also detected at least eight *loci*

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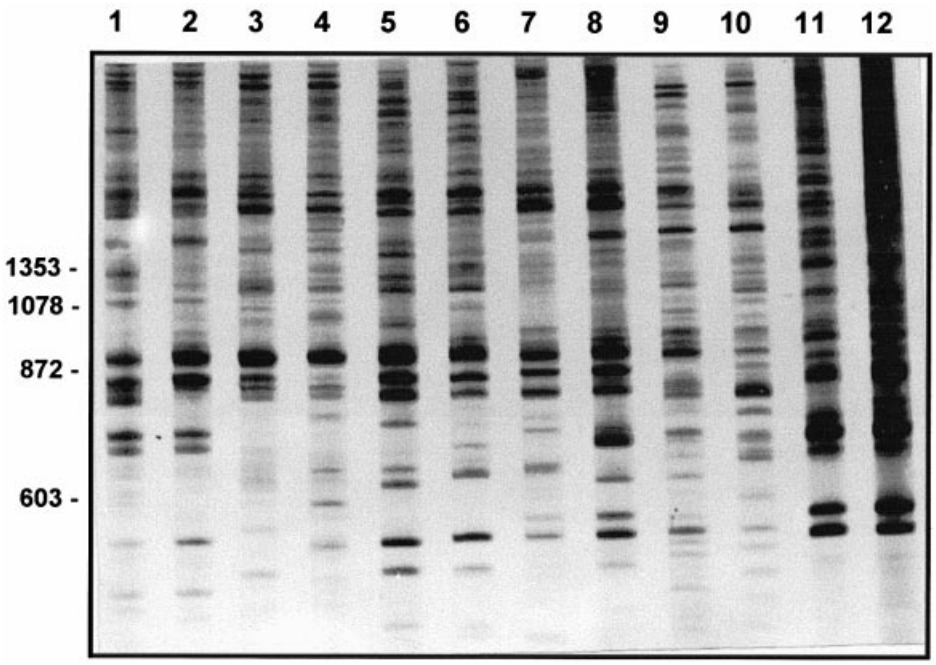

Fig. 1. Silver-stained polyacrylamide gel (4 %) showing AP-PCR profiles obtained with primer 3307 and twelve Brazilian *Biomphalaria glabrata* specimens. Lanes 1 and 2: Specimens from Belém (Pará); lanes 3 and 4: from Cururupu (Maranhão); lanes 5 and 6: from Touros (Rio Grande do Norte); lanes 7 and 8: from Pontezinha (Pernambuco); lanes 9 and 10: from Aracaju (Sergipe); lanes 11 and 12: from Jacobina (Bahia). Molecular size markers are shown on the left of the gel. Vidigal *et al*. (1994).

suitable for studies of population structure, reproductive systems and resistance to *S. mansoni*.

GENETIC VARIATION IN BRAZILIAN *BIOMPHALARIA*

The available methodologies, based on molecular analysis, have permitted the generation of more consistent information concerning the population structure of *Biomphalaria*. Such studies have allowed inference of the origin, colonization processes and dispersion of populations and species of the *Biomphalaria* genus (Woodruff, Mulvey & Yipp, 1985; Mulvey, Newman & Woodruff, 1988; Woodruff & Mulvey, 1997).

By means of AP-PCR, an estimate of intra and interpopulation variability of *B. glabrata*, from different localities, in Brazil, was achieved (Vidigal *et al*. 1994). The comparison among specimens, from different field populations, presented a limited intrapopulation but high interpopulation heterogeneity. Such results suggest that individual localized populations are homogenous but that the global *B. glabrata* population is highly heterogeneous. Snails, which had been laboratory reared and maintained for different periods of time, were also analysed and showed no differences in intrapopulation variability as compared to field populations. This rules out the possibility that selective breeding in the laboratory produced inbred strains, regardless of the time period of maintenance in the laboratory. The high level of genetic variability found in *B. glabrata* was confirmed by analysis of two randomly selected individuals from each of six populations, both from laboratory and field, using the AP-PCR primers 3307 (Fig. 1) and 3302. Less than 10 % of the amplified fragments were present in all analysed samples. The average percentage of shared bands between each pair from the same locality was 74·5 % and between all possible pairs, 43 %. These results are in accordance with those obtained for *B. glabrata* from Puerto Rico and *B. prona* from Venezuela using isoenzymes (Mulvey & Vrijenhoek, 1982; Mulvey *et al*. 1988; Paraense *et al*. 1992), or for *Bulinus* snails by RAPD analysis (Langand *et al*. 1993). In summary, the studies using AP-PCR demonstrated that it is appropriate for the study of *Biomphalaria* at the genetic level, and that it allowed confirmation of the wide genetic variability of these snails, previously observed in isoenzyme and morphological studies.

Zietkiewicz, Rafalski & Labuda (1994) introduced the simple sequence repeat anchored PCR (SSR-PCR) technique to the study of several eukariotic species. This technique is based on the anchoring of PCR primers at the 3′ or 5′ ends of microsatellites. Its advantage lies in the reduction of the number of other possible targets for annealing and hence results are easier to interpret. Among the seven tested primers the $(CA)_8RY$ gave the best results. Oliveira *et al*. (1997) used SSR-PCR with the $(CA)_8RY$ primer to study the intraspecific variability of *Trypanosoma cruzi*, *Leishmania braziliensis* and

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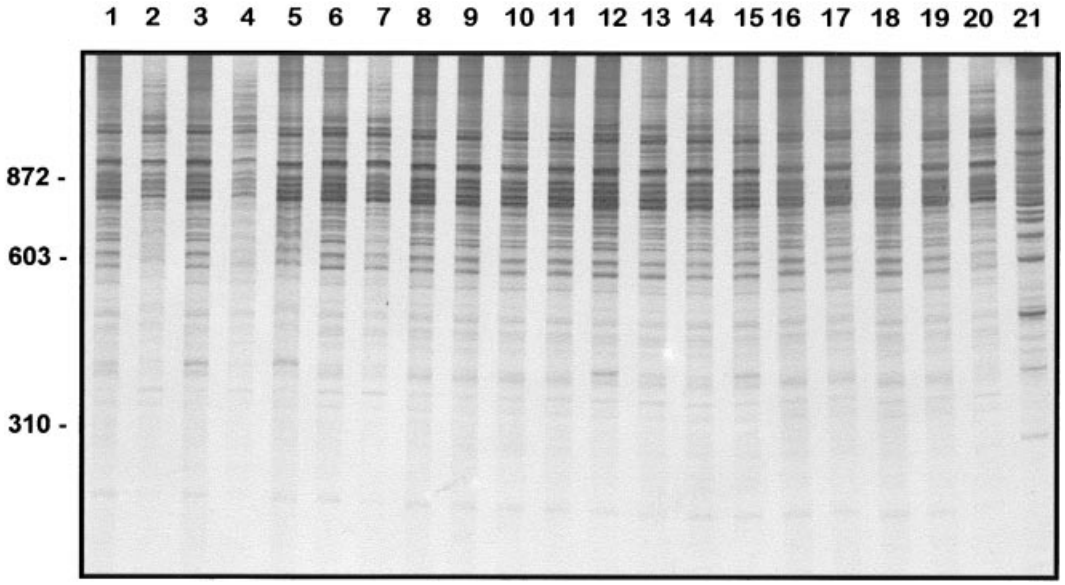

Fig. 2. Silver-stained polyacrylamide gel (6%) showing simple sequence repeat-anchored polymerase chain reaction amplification profiles obtained with the K7 primer and twenty Brazilian *Biomphalaria kuhniana* specimens. Lanes 1 to 20: from Tucuruí (Pará, Brazil) and one outgroup, *B. glabrata* specimen lane 21: from Esteio (Rio Grande do Sul, Brazil). Molecular size markers are shown on the left of the gel. Caldeira *et al.* (2001).

S. mansoni, showing that the patterns obtained were comparable to those resulting from AP-PCR. Due to its applicability and the quality of the results obtained, we have standardized the SSR-PCR technique using the primers $(CA)_8RY$ and K7 for the study of genetic variability among Brazilian populations of *B. straminea*, *B. intermedia* and *B. kuhniana* (Caldeira *et al.*, in press). Fig. 2 illustrates the reproducibility of the profiles obtained with the SSR-PCR technique using 20 specimens of *B. kuhniana* and the primer K7. The trees obtained by UPGMA and neighbour-joining methods showed similar topologies. We observed greater heterogeneity between populations than within. The mean percentage of shared bands among all possible pairs from the same locality was 83%. This result suggests, in the three species studied, the existence of uniform populations. In contrast, *B. straminea* and *B. intermedia* displayed intraspecific genetic heterogeneity.

SPECIFIC IDENTIFICATION OF *BIOMPHALARIA* SNAILS

The morphological identification of medically important freshwater snails is greatly complicated by the extensive intraspecific variation in anatomical and morphological characteristics commonly used for classical identification (Paraense, 1975*a*, *b*, 1981, 1984, 1988). To overcome this problem, the use of molecular techniques, in conjunction with morphological characters, has been proposed for the identification of these snails. The remarkable degree of intraspecific polymorphism detected in *B. glabrata*, through AP-PCR, suggests that this technique is not suitable for identification of this species (Vidigal *et al.* 1994). Testing the low stringency polymerase chain reaction technique (LS-PCR–Dias Neto *et al.* 1993), Vidigal *et al.* (1996) used primers (NS1 and ET1) designed to amplify a portion of the rDNA gene, which permitted the differentiation of *B. glabrata* and *B. tenagophila* (Fig. 3) from six localities in Brazil. The primers produced a complex pattern of bands with 4 species-specific fragments for *B. glabrata* and 3 for *B. tenagophila*. Pires *et al.* (1997), using the same technique, were able to differentiate *B. tenagophila* from *B. occidentalis*, which is not possible using morphological characters done (Paraense, 1981). Fig. 4 shows different profiles of *B. tenagophila* specimens with the primers NS1 and ET1. Note the presence of a double 500 bp band (T1) and two fragments of 400 and 310 bp (T2 and T3). *B. occidentalis* specimens were characterized by a band of 130 bp (O1). The profiles were polymorphic but were found to be reproducible in all specimens from each species.

Preliminary experiments with *B. straminea* populations, using the same methodology, suggest that this species has higher levels of interpopulation variability than *B. glabrata*, *B. tenagophila* or *B. occidentalis*. No fragments were shared among all studied specimens (Vidigal *et al.* 1996; Pires *et al.* 1997). Although LS-PCR was able to differentiate the species studied, polymorphic profiles were produced making the interpretation of results very difficult.

Vidigal *et al.* (1998) examined sequence polymorphism within the internal transcribed spacer region (ITS) of the rDNA which includes the 5·8S

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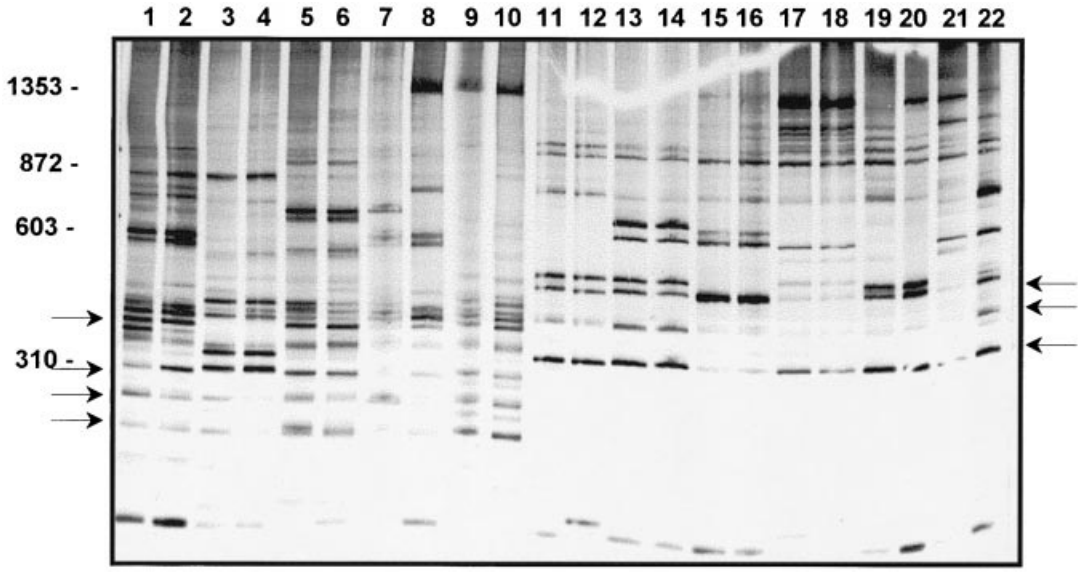

Fig. 3. Silver-stained polyacrylamide gel (4%) showing LS-PCR profiles obtained with primers NS1-ET1 and Brazilian *Biomphalaria* species. Lanes 1 and 2: *B. glabrata* from Belém (Pará); lanes 3 and 4: *B. glabrata* from Cururupu (Maranhão); lanes 5 and 6: *B. glabrata* from Touros (Rio Grande do Norte); lanes 7 and 8: *B. glabrata* from Pontezinha (Pernambuco); lanes 9 and 10: *B. glabrata* from Aracaju (Sergipe); lanes 11 and 12: *B. tenagophila* from Paracambi (Rio de Janeiro); lanes 13 and 14: *B. tenagophila* from Imbé (Rio Grande do Sul); lanes 15 and 16: *B. tenagophila* from Joinville (Santa Catarina); lanes 17 and 18: *B. tenagophila* from Araçatuba (São Paulo); lanes 19 and 20: *B. tenagophila* from Formosa (Goiás); lanes 21 and 22: *B. tenagophila* from Vila Velha (Espirito Santo). The species diagnostic bands are indicated by arrows, and the molecular size markers are shown on the left of the gel. Vidigal *et al*. (1996).

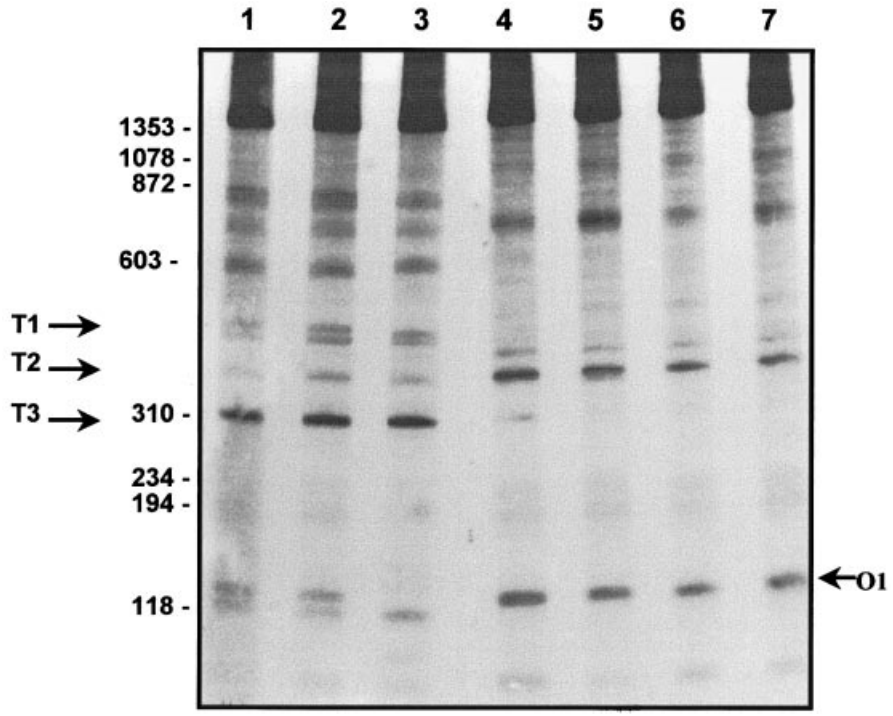

Fig. 4. Comparison of LSP of profiles of *Biomphalaria occidentalis* and *B. tenagophila* obtained with the primer pair NS1-ET1 and 1 ng of DNA template. Lanes 1 to 3: *B. tenagophila* specimens from Vespasiano, MG; Vitória, ES and Joinville, SC, respectively. Lanes 4 to 7: *B. occidentalis* specimens from Campo Grande, MS; Ladário, MS; Assis, SP and Dracema, SP, respectively. The LS-PCR amplification products were visualized in a 4% polyacrilamide gel stained with silver. The species diagnostic bands are indicated by arrows, and the molecular size markers are shown on the left of the gel. Pires *et al*. (1997).

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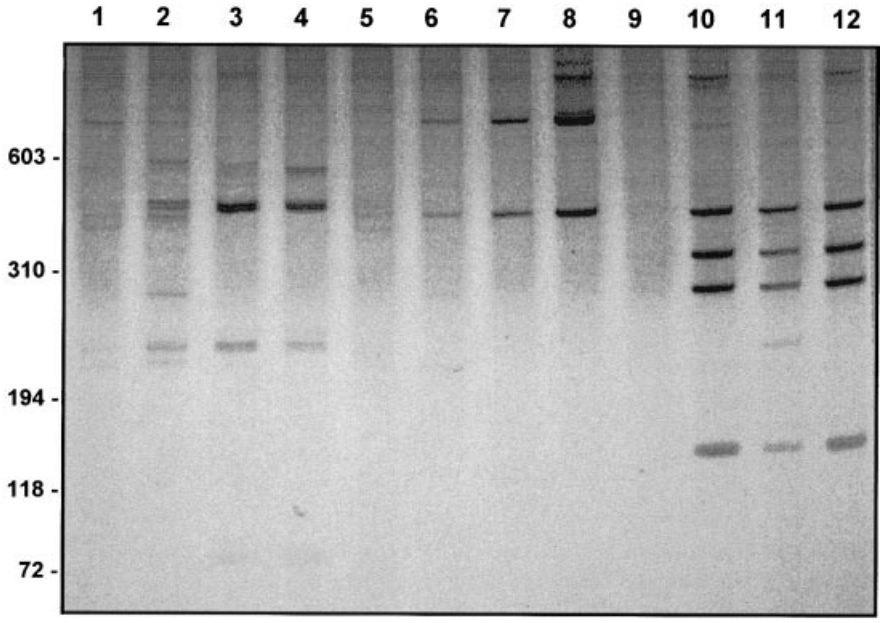

Fig. 5. Silver stained polycrylamide gel (6%) showing the RFLP profiles obtained by digesting the DNA ITS with *Dde*I. The DNA used was extracted from the eggs of *Biomphalaria glabrata*, *B. tenagophila* and *B. straminea* on different days and compared with the profile produced with adult control DNA. Lane 1: 1 day eggs of *B. glabrata*; lane 2: 2 day eggs of *B. glabrata*; lane 3: 3 day eggs; lane 4: adult *B. glabrata*; lane 5: 1 day eggs of *B. tenagophila*; lane 6: 2 day eggs of *B. tenagophila*, lane 7: 3 day eggs of *B. tenagophila*; lane 8: adult *B. tenagophila*; lane 9: 1 day eggs lane 10: 2 day eggs; lane 11: 3 days layed eggs; lane 12: adult *B. straminea*. Vidigal *et al.* (1998).

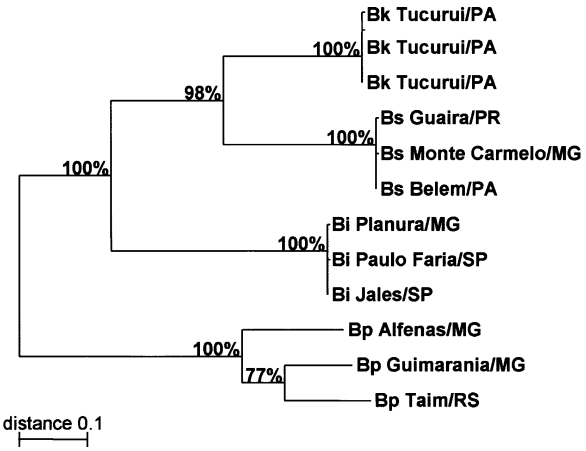

Fig. 6. Neighbour-joining tree unrooted of *Biomphalaria kuhniana*, *B. straminea*, *B. intermedia and B. peregrina* constructed using the PCR-RFLPs profiles produced with all enzymes. The numbers are bootstrap percent values based on 1000 replications. Caldeira *et al.* (1998).

rDNA gene together with the flanking ITS1 and ITS2 spacers. They used PCR amplification followed by digestion with several restriction enzymes (RFLP). This method was employed in systematic studies of snails within the genera *Oncomelania*, *Bulinus* and *Stagnicola* (Hope & McManus, 1994; Stothard, Hughes & Rollinson, 1996; Stothard & Rollinson, 1997; Remigio & Blair, 1997). Firstly, different *B. glabrata*, *B. tenagophila* and *B. straminea* populations from Brazil were analysed. The entire ITS region was amplified using the primers ETTS1 and ETTS2 anchored respectively in the conserved extremities of the 18S and 28S ribosomal genes (Stothard, Hughes & Rollinson, 1996; Kane & Rollinson, 1994). The PCR-specific amplification of the *Biomphalaria* ITS resulted in a product of approximately 1·3 kb. The amplified ITS region, from all species studied, contains sites for a variety of restriction enzymes including *Alu*I, *Dde*I, *Hae*III, *Mnl*I, *Msp*I, *Rsa*I and *Sau*3*a*I. The most promising RFLP profiles were those produced with *Hae*III and *Dde*I which included species-specific fragments for the three species studied (data not shown). *Biomphalaria glabrata* is characterized by the presence of three fragments of approximately 500, 220 and 80 bp. *Biomphalaria tenagophila* had two fragments of approximately 800 and 470 bp. The majority of the *B. straminea* snails were characterized by the presence of four fragments of approximately 470, 310, 280 and 120 bp, although two specimens from Porto Alegre showed only two fragments (470 and 310 bp). The reproducibility of the *Dde*I–ITS RFLP profiles was demonstrated by analysis of several specimens obtained from distinct localities within Brazil. The possibility of using eggs as a source of DNA was reported by Knight *et al.* (1992) and our data confirmed the usefulness of this approach (Fig. 5). This possibility has important practical implications when the number of collected specimens is low, permitting the maintenance of live adults in the laboratory for further studies.

Using the same methodology Caldeira *et al.* (1998) studied morphologically similar species *B. straminea*, *B. intermedia*, *B. kuhniana* and *B. peregrina*. Six

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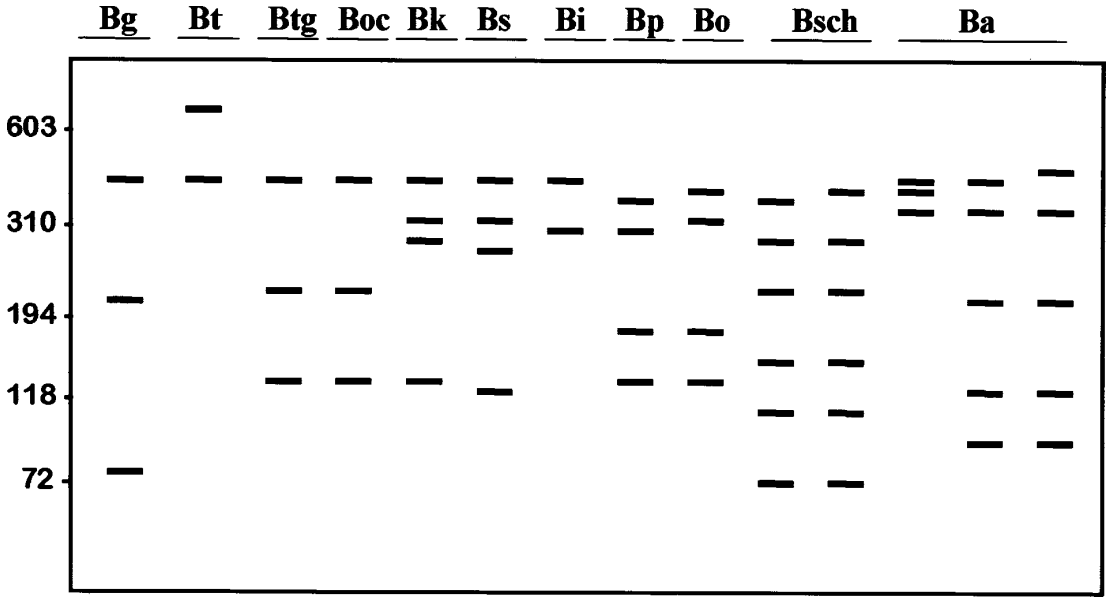

Fig. 7. Schematic representation of the ITS rDNA restriction patterns of 10 Brazilian *Biomphalaria* species and one subspecies produced with *Dde*I. The abbreviation for each species is: *B. glabrata* (Bg); *B. tenagophila* (Bt); *B. occidentalis* (Boc); *B. t. guaibensis* (Btg); *B. straminea* (Bs); *B. intermedia* (Bi); *B. kuhniana* (Bk); *B. peregrina* (Bp); B. *oligoza* (Bo); *B. schrammi* (Bsch) and *B. amazonica* (Ba). Molecular size markers are shown on the left of the gel. Vidigal *et al*. (2000).

enzymes were tested (*Dde*I, *Mnl*I, *Hae*III, *Rsa*I, *Hpa*II and *Alu*I) and the best profile for the differentiation of these species was obtained with *Dde*I. Two methods of clusters and two coefficients (NJ and UPGMA; distance coefficient of Nei and Li and similarity coefficient of Dice) were used. Trees with the same topology identifying three distinct groups were produced: (1) *B. straminea* and *B. kuhniana*; (2) *B. intermedia*; (3) *B. peregrina*. Fig. 6 shows the tree constructed with NJ and distance coefficient of Nei and Li from 79 bands generated by six enzymes. Branches reflect the shared percentages of bands among the 12 specimens of the four snail species. The mean percentage of bands shared among all the possible pairs was 44%. The three groups were supported by high bootstrap values (98% and 100%). We verified a high level of similarity between the first and second groups, which reinforces the data from Paraense (1988) who, based on the morphology characters, grouped *B. straminea*, *B. kuhniana* and *B. intermedia* in the *B. straminea* complex.

Using PCR-RFLP Spatz *et al*. (1999) studied *B. tenagophila*, *B. t. guaibensis* and *B. occidentalis* which are indistinguishable on the basis of shell morphology and are similar across most organs of the genital system. Snails from different localities in Brazil, Argentina and Uruguay were analysed using seven enzymes (*Hae*III, *Dde*I, *Alu*I, *Mnl*II, *Hpa*II, *Hfa*I, *Rsa*I). The *Alu*I profiles were the most informative and distinguish these species (data not shown). Profiles obtained with the other enzymes did not permit species identification as extensive intraspecific polymorphism was observed. Restriction profiles obtained with all enzymes were used to calculate the percentage of shared bands between all possible snail pairs and these data were used for a cluster analysis (data not shown). A closer relationship was observed between *B. occidentalis* and *B. t. guaibensis* than between *B. tenagophila* and the subspecies *B. t. guaibensis*. Based on previous morphological and molecular data, it was proposed that *B. tenagophila*, *B. occidentalis* and *B. t. guaibensis* should be grouped into a complex named *B. tenagophila*.

PCR-RFLP was also used for the identification of ten Brazilian species and one subspecies of *Biomphalaria* with emphasis on the analysis of *B. oligoza*, *B. schrammi* and *B. amazonica* (Vidigal *et al*. 2000). The profiles obtained with *Dde*I (data not shown) allowed the ready separation of the majority of species studied, with the exception of *B. occidentalis* and *B. t. guaibensis*, and *B. oligoza* and *B. peregrina* which have very similar profiles. The correct identification of *B. oligoza* and *B. peregrina* species is also difficult at the morphological level. *Biomphalaria peregrina* is widely distributed in South America and is considered a potential intermediate host of *S. mansoni*. Thus, with the aim of obtaining a suitable molecular separation between *B. oligoza* and *B. peregrina* other enzymes were tested (*Hpa*II, *Mnl*I, *Alu*I and *Hae*III). Despite the similarity among these profiles, *Alu*I, *Hpa*II, *Hae*III permitted the molecular separation of these species. The profiles obtained with *Dde*I for the 10 species and one subspecies of the Brazilian *Biomphalaria* snails are all represented in the diagram in Fig. 7. We have been routinely using this schematic representation of

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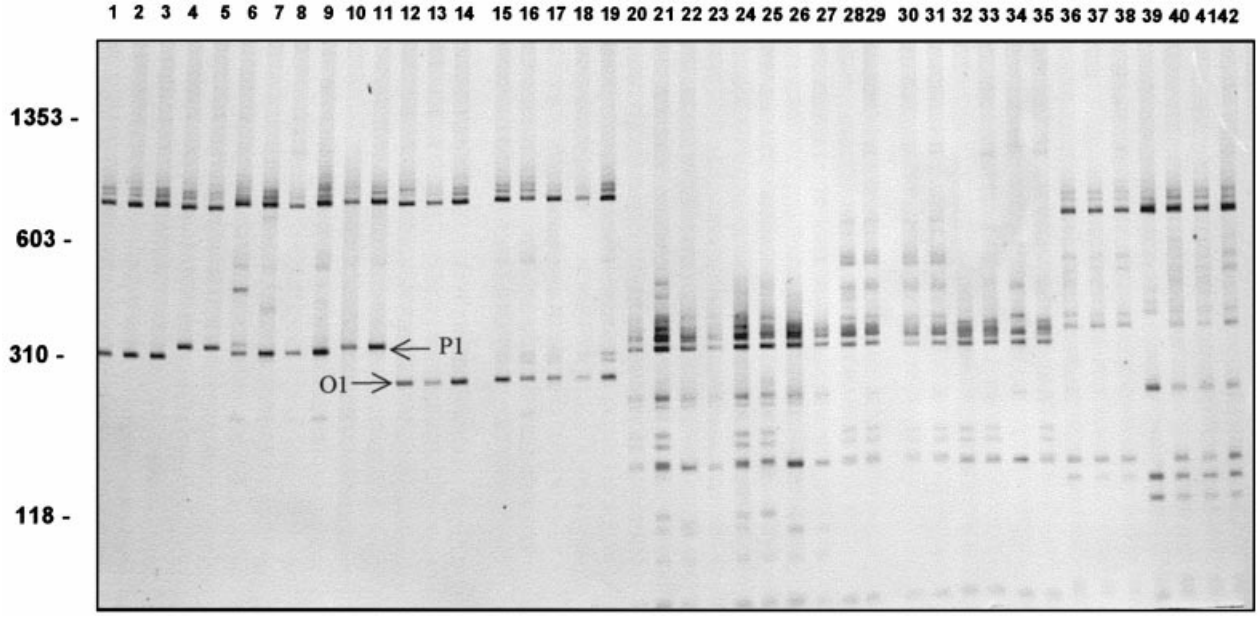

Fig. 8. Silver stained polyacrylamide gel (6%) showing the polymerase chain reaction and restriction fragment length polymorphism profiles obtained following the digestion of the rDNA internal transcribed spacer with *Mva*I. Lane 1: *Biomphalaria peregrina* from Alfenas, Minas Gerais, (Brazil); lane 2: *B peregrina* from Bom Jesus da Penha, Minas Gerais (Brazil); lane 3: *B. peregrina* from Alfenas, Minas Gerais (Brazil); lanes 4–5: *B. peregrina* from Buenos Aires (Argentina); lanes 6–7: *B. peregrina* from Paso de los Toros (Uruguay); lanes 8–9: *B. peregrina* from Cholila (Argentina); lanes 10–11: *B. peregrina* from Córdoba Province (Argentina); lanes 12–13: *B. oligoza* from Florianópolis, Santa Catarina (Brazil); lanes: 14–15: *B. oligoza* from Eldorado do Sul, Rio Grande do Sul (Brazil); lanes 16–19: *B. oligoza* from Córdoba Province (Argentina); lanes 20–25: *B. orbigny*i from San Roque Corrientes Province (Argentina); lanes 26–27: *B. orbignyi* Termas, Arapey (Uruguay); lanes 28–31: *B. orbignyi* Sierra Quijadas, San Luis Province (Argentina); lanes 32–34: *B. orbignyi* La Carlota, Córdoba Province (Argentina); lanes 35–38: *B. orbignyi* from Chamical, La Rioja Province (Argentina); lanes 39–42: *B. orbignyi* from Patquia, La Rioja Province (Argentina). Molecular size markers are shown on the left of the gel. The arrows indicate species-specific fragments of *B. oligoza* (O1) and *B. peregrina* (P1). Spatz *et al.* (2000).

ITS restriction patterns in our laboratory as a model for comparative molecular identification of Brazilian *Biomphalaria* populations.

Using several enzymes Spatz *et al.* (2000) studied *B. oligoza* and *B. peregrina* from Brazil, Argentina and Uruguay. *Biomphalaria orbignyi* (Paraense, 1975*b*) was included due to its high morphological similarity to *B. peregrina*. This species was originally described in Argentina and has been shown to be refractory to *S. mansoni* infection (Paraense, 1975*b*). The enzyme *Mva*I (Fig. 8) produced two distinct profiles for *B. peregrina* and *B. oligoza* snails. These profiles exhibit (a) one fragment of approximately 700 bp, shared between *B. peregrina* and *B. oligoza*, (b) one fragment of approximately 300 bp (P1) specific for *B. peregrina* and (c) one fragment specific for *B. oligoza* of approximately 200 bp (O1). This enzyme produced polymorphic profiles for *B. orbignyi* specimens (profile 1: lanes 20 to 35, profile 2: lanes 36 to 42). Similarity and genetic distance analyses of the three species were performed using 146 bands obtained with six restriction enzymes. The phenetic trees, obtained using UPGMA and NJ analysis suggested that *B. peregrina* and *B. oligoza* are the most closely related species (data not shown).

The ITS region contains useful genetic markers for the identification of Brazilian *Biomphalaria* snails. PCR-RFLP is a simple and rapid technique that will improve the precision of snail population surveys undertaken in South America. The efficiency and utility of this methodology was confirmed when snail populations from Venezuela, previously identified as *B. straminea*, were characterized as *B. kuhniana*, at the molecular level (Caldeira *et al.* 2000).

In recent years, the eukaryotic ribosomal RNA locus has been extensively utilised for phylogenetic reconstruction. Sequence comparisons of the two internal transcribed spacers (ITS1 and ITS2) have been used for phylogenetic reconstruction of closely related species from a wide range of organisms. In molluscs, ITS1 sequences have been used to study the relationship of different genera such as: *Isabellaria*, *Albinaria*, *Bulinus* and *Stagnicola* (Schilthuizen, Gittenberger & Gultyaev 1995; Stothard *et al.* 1996; Remigio & Blair, 1997).

Vidigal *et al.* (2000), generated data for 27 *Biomphalaria* snails from Brazil, two from Venezuela and two specimens of *Helisoma duryi* from Brazil in order to determine the molecular relationships among Brazilian *Biomphalaria* snails (Table 1). This study was performed by ITS2 sequence analysis,

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Table 1. Species, localities, geographical coordinates, abbreviations and GenBank accession number of the rRNA ITS2 for the snail populations studied

Species	Specimens	Locality	Geographical coordinates	Abbreviation	GenBank accession number
Biomphalaria glabrata	01	Belém, PA, Brazil	01s27/48w30	B.glPA	AF198659
	02	Esteio, RS, Brazil	29s51/51w10	B.glRS-1	AF198661
				B.glRS-2	AF198660
	01	Portuguesa, Chabasquen, Venezuela		B.glVEN	AF198662
B. tenagophila	01	Vespasiano, MG, Brazil	19s41/43w55	B.tenMG	AF198654
	01	Imbé, RS, Brazil*	29s58/50w07	B.tenRS	AF198655
	01	Formosa, GO, Brazil*	15s32/47w20	B.tenGO	AF198656
B. straminea	01	Picos, PI, Brazil*	07s04/41w28	B.strPI	AF198672
	02	Porto Alegre, RS, Brazil*	29s58/51w17	B.strPA-1	AF198669
				B.strPA-2	AF198670
	01	Florianópolis, SC, Brazil	27s35/48w32	B.strSC	AF198668
	01	Belém, PA, Brazil	01s27/48w30	B.str PA	AF198671
B. intermedia	01	Jales, SP, Brazil	20s16/50w32	B.int SP-1	AF198674
	01	Pindorama, SP, Brazil	21s11/48w54	B.int SP-2	AF198675
	01	Itapagipe, MG, Brazil	19s54/49w22	B.int MG	AF198673
B. peregrina	01	Alfenas, MG, Brazil	21s25/45w56	B.per MG-1	AF198676
	01	Taim, RS, Brazil	32s29/52w34	B.per RS	AF198677
	01	Bom Jesus da Penha, MG, Brazil	21s01/46w31	B.perMG-2	AF198678
B. schrammi	02	Ilicinea, MG, Brazil	20s56/45w49	B.schMG-1	AF198681
				B.schMG-2	AF198682
B. kuhniana	01	Tucurui, PA, Brazil	03s46/49w40	B.kun PA	AF198666
	01	Aragua, Villa de Cura, Venezuela		B.kun VEN	AF198667
B. occidentalis	01	Capetinga, MG, Brazil	20s36/47w03	B. ocMG	AF198658
	01	Campo Grande, MS, Brazil*	20s26/54w38	B. ocMT	AF198657
B. oligoza	01	Eldorado do Sul , RS, Brazil	30s05/51w36	B.oliRS	AF198680
	01	Florianópolis, SC, Brazil	27s35/48w32	B.oli SC	AF198679
B. amazonica	02	Bejamin Constant, AM, Brazil	04s22/70w01	B.aAM-1	AF198663
				B.aAM-2	AF198664
	01	Barão de Melgaço, MT, Brazil*	16s13/55w58	B.aMT-1	AF198665
Helisoma duryi	02	Uberaba, MG, Brazil	19s45/47w56	H.duryi-3	AF267503
				H.duryi-5	AF267504

* Laboratory populations. From Vidigal *et al*. (2000).

which could be confidently aligned. The multiple sequence alignment revealed a substantial degree of variation between snails from different species with substitutions, insertions and deletions being present. However, there was minimal sequence variation between snails from the same species collected in different localities. The phylogenetic analysis of ITS2 sequences, performed with the three different methods of phylogenetic reconstruction (distance, maximum parsimony and maximum likelihood), generated very similar trees. However, only the parsimony and neighbour-joining algorithm (NJ) with the addition of *Helisoma* were showed (Fig. 9A and B). Parsimony (MP) analysis yielded 1274 equally parsimonious trees of length 281. Only one of the trees is shown, however any bifurcating branches that are not present in the strict consensus of all trees are indicated in bold (Fig. 9A). All trees were found on a single island. The tree generated by maximum-likelihood (ML) has a log likelihood of −2199·87746. In the case of distance analyses, the trees were constructed using the neighbour-joining algorithm (NJ) with and without the additional taxa. In all cases, the trees were unrooted for analysis, however *B. glabrata* was specified as the outgroup followed the arguments of Bandoni *et al*. (1995) and Woodruff & Mullvey (1997). All four trees show the same major groupings: I–VI (Fig. 9A). The branch uniting the members of group VI and the branch uniting groups I–III are quite long and separate these groups well, however the branch uniting groups IV and V is shorter and less well supported by bootstrap analysis (63%). Some species show invariant relationships to each other in all trees

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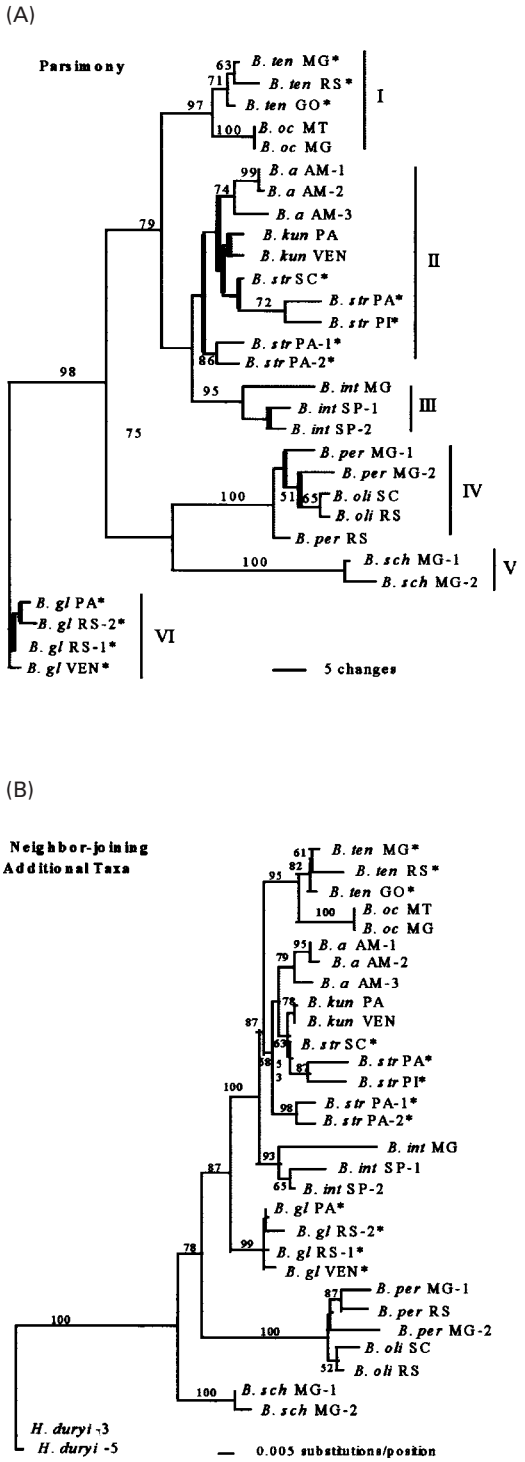

Fig. 9. Trees resulting from phylogenetic analysis of ITS2 sequences of the *Biomphalaria species*. Species abbreviations are defined in Table 1. Bootstrap values above 50% are listed above branches. Scale bars are as indicated. Species names followed by an asterisk* are natural hosts for the parasite *Schistosoma mansoni*. Roman numerals (I–VI) shown in (A) define the major lineages found in both trees. (A) maximum parsimony analysis, bold vertical bars represent bifurcations not present on the strict consensus of 1274 equally parsimonious trees; (B) neighbour-joining analysis using additional taxa. Vidigal *et al*. (2000).

(*B. tenagophila*, *B. occidentalis*, *B. amazonica*, *B. intermedia* and *B. schrammi*) however, others are variable. The pairs of species that are most volatile in terms of their position on the trees are *B. straminea*/*B. kuhniana* and *B. peregrina*/*B. oligoza*. In both cases, the specimens from each of these species pairs are intermingled and do not form monophyletic groups by any of the methods used. The ITS2-based trees exhibit very similar overall topologies that are compatible with the traditional taxonomy based on morphology.

Of interest is the placement of *B. glabrata* in the interior of the tree when *H. duryi* is specified as the outgroup (Fig. 9B). This tree clearly places *B. schrammi* outside of *B. glabrata* and is basal to the remaining *Biomphalaria* species. Our inspection of the sequence alignment supports this finding. *Biomphalaria glabrata* is clearly distinct from the other species examined. This distinction has been made previously by Bandoni *et al*. (1995) and Woodruff & Mulvey (1997). The results obtained by the first group led them to suggest that *B. glabrata* was a sister taxon to the African species. They demonstrated African affinities of *B. glabrata* obtained from Brazil and the Dominican Republic. These results are in accordance with those of Woodruff & Mulvey (1997), which show greater affinity of the American *B. glabrata* with African *Biomphalaria* species than with other American species. The fact that the *B. glabrata* specimens cluster differently in the NJ tree than they do in the MP (Fig. 9A) and ML trees can be explained by genetic variability attributed to this species as mentioned above or by the marker used. However, our results suggested that *B. glabrata* was not the most distant *Biomphalaria* species in Brazil (Fig. 9B). The three species that are natural hosts of *S. mansoni* do not form a monophyletic group. There is at least one potential or natural intermediate host species for *S. mansoni* in four of the *Biomphalaria* groups. In group I: *B. tenagophila*; in group II: *B. straminea*; in group IV: *B. peregrina* and in group VI: *B. glabrata*. The latter shows the highest infection rates both naturally and experimentally and is almost always associated with the presence of schistosomiasis. Thus, the ability to act as an intermediate host for *S. mansoni* may be 'easily' acquired.

We believe that molecular-based methodologies may be very useful in furthering understanding of *Biomphalaria* species by means of systematic and population genetics studies. Indeed, Simpson *et al*. (1995), analyzing the *Schistosoma* and *B. glabrata* genomes, observed that while parasite genetic intraspecific polymorphism is limited, that of the mollusc is very extensive, suggesting that the genetics of the intermediate host play a more important role in the epidemiological control of schistosomiasis than those of the parasite itself.

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