Genetic Variability and Identification of the Intermediate Snail Hosts of *Schistosoma mansoni*

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Studies based on shell or reproductive organ morphology and genetic considerations suggest extensive intraspecific variation in Biomphalaria *snails. The high variability at the morphological and genetic levels, as well as the small size of some specimens and similarities between species complicate the correct identification of these snails. Here we review our work using methods based on polymerase chain reaction (PCR) amplification for analysis of genetic variation and identification of* Biomphalaria *snails from Brazil, Argentina, Uruguay and Paraguay. Arbitrarily primed-PCR revealed that the genome of* B. glabrata *exihibits a remarkable degree of intraespecific polymorphism. Low stringency-PCR using primers for 18S rRNA permited the identification of* B. glabrata, B. tenagophila *and* B. occidentalis. *The study of individuals obtained from geographically distinct populations exhibits significant intraspecific DNA polymorphism, however specimens from the same species, exhibit some species specific LSPs. We also showed that PCR-restriction fragment of length polymorphism of the internal transcribed spacer region of* Biomphalaria *rDNA, using DdeI permits the differentiation of the three intermediate hosts of* Schistosoma mansoni*. The molecular biological techniques used in our studies are very useful for the generation of new knowledge concerning the systematics and population genetics of* Biomphalaria *snails.*

Key words: *Biomphalaria* - snails - genetic variability - identification - polymerase chain reaction

Biomphalaria snails are found both in Africa and in the Americas. In both continents members of this genus serve as intermediate hosts for the parasitic trematode *Schistosoma mansoni*. In Brazil ten species of *Biomphalaria* are recognized although only *B. straminea, B. tenagophila* and *B. glabrata* are found naturally infected with the parasite. Of these, the latter is the most important due to its wide distribution and its high susceptibility to *S. mansoni* (Paraense & Corrêa 1963). These snails are hermaphrodites and can self-fertilize but prefer to reproduce by cross-fertilization when paired. These biological characteristics are evolutionarily very important, providing the organism with the ability to establish colonies from an individual organism while maintaining genetic variation through sexual reproduction (Paraense 1955). Most populations are under significant environmental pressures (rains and droughts) that can dramatically reduce population levels, requiring recolonization from the low number of remaining snails (Paraense 1955). There are two points to be highlighted: (1) the re-building of a new population from a few surviving individuals (founder effect) could explain low intrapopulation variability. Jarne and Delay (1991) reported that self-fertilization can be selected under certain circunstances such as low population density or local environmental conditions, also supporting the low intra population variability. In addition, the fixation of these populations may occur in a very short period of time, due to the high reproduction potential of the species, enhanced by the absence of intra-specific competition (Paraense et al. 1955); (2) the limited gene flow (a lack of interconnecting waterways) between different populations could facilitate the formation of local strains, resulting in interpopulational variability. It has been proposed that genetic drift working in populations under strong environmental pressures, could be responsible for the genetic heterogenety, patchy distribution of susceptibility to *S. mansoni* among snail populations and also lead to a population structure characterized by relative genetic homogeneity within, and differentiation among local snails populations (Paraense 1955, Michelson & Dubois 1978, Mulvey & Vrijenhoek 1982). The observation of several levels of reproductive iso-

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lation detected in allopatric conspecific individuals also indicate extensive genetic heterogeneity among populations of *Biomphalaria* snails from separate localities (Paraense 1959).

The identification of *Biomphalaria* species is important for epidemiological studies of schistosomiasis. For a long time malacologists have been looking for characters that could aid in the specific identification of these snails. Their morphological identification is based mainly on the reports of Deslandes (1951) and Paraense (1975, 1981, 1984, 1988) which use shell and reproductive organ morphology. The high variability of these characters, the environmental influence on shell size and shape, the small size of specimens, and the similarity between some species, complicate specific identification, specially for non-experts.

Isozyme patterns have been useful for the specific identification of *Bulinus* and *Biomphalaria* snails as well as for investigating the phylogenetic relationships and genetic structure of these groups (Mulvey & Vryenhoeck 1981, Jelnes 1986, Mascara & Morgante 1991, 1995, Bandoni et al. 1995). Recently, molecular biology techniques have been introduced for the study of freshwater snails (Jarne et al. 1990, 1992, Knight et al. 1991, Strahan et al. 1991, Langand et al. 1993, Stothard & Rollinson 1996, Stothard et al. 1996, Stothard & Rollinson 1997, Remigio & Blair 1997, Lewis et al. 1997). We here describe our work with *Biomphalaria* snails using three techniques based on the polymerase chain reaction (PCR).

ANALYSIS OF GENETIC VARIATION IN BRAZILIAN ISOLATES OF *B. GLABRATA* SNAILS BY AP-PCR

AP-PCR (arbitrarily primed polymerase chain reaction - Welsh & McClelland 1990, Williams et al. 1990) has been used to study genomic variability in different groups of organisms (Dias Neto et al. 1993 b,c, Noyes et al. 1996, Barral et al. 1996) including molluscs (Crossland et al.1993, Langand et al. 1993, Larson et al. 1996, Stothard & Rollinson 1996). A recent report by Lewis et al. (1997) showed some promising results with the AP-PCR approach in analysis of *B. glabrata* populations resistant and susceptible to infection with *S. mansoni*, where a 1.3 kb marker appears in nearly all resistant progeny and 1.1 kb marker appears in all susceptible progeny. We used AP-PCR to estimate intra and interpopulation variability of *B. glabrata* from different localities of Brazil (Vidigal et al. 1994). We first compared specimens of two field populations from different localities and found a limited intrapopulation heterogenity in contrast to the high interpopulation heterogenity observed. These results suggest that each population is highly homogeneous but that the global *B. glabrata* population is highly heterogeneous. This was found to be true when any two of the isolates were compared using any of the five different primers tested. Snails reared and mantained for different periods of time were also analyzed, showing no differences in intrapopulation variability levels when compared with field populations. This rules out the possibility that selective breeding in the laboratory had produced inbred strains, irrespective of the length of time of maintenance in the laboratory. The high level of genetic variability found in *B. glabrata* was confirmed by amplifying two individuals selected at random from each of six populations, either from laboratories or the field (Fig. 1) from different regions of the country, using primers 3307 and 3302 (Fig. 2). Less than 10% of the amplified fragments were present in all of the analyzed samples. The average percentage of shared bands between each pair from the same locality was 74.5% and between all possilble pairs from different localities was 43%. These results agree with those obtained for *B. glabrata* in Puerto Rico and in *B. prona* using isoenzymes (Mulvey & Vrijenhoek 1982, Mulvey et al. 1988, Paraense et al. 1992), or for *Bulinus* snails using RAPD analysis (Langand et al. 1993). The high variability of *B. glabrata* (90%) is in contrast with that of *Schistosoma* (10%). The fact that only approx-imately 5% of RAPDs products obtained for the different schistosome strains are polymorphic (Barral et al. 1993, Dias Neto et al. 1993b), would indicate the higher relevance of molluscan genetics over parasite genetics in determing the epidemiology of the disease (Simpson et al. 1995).

From the technical point of view, our data showed that AP-PCR analysis is highly appropiate for the study of *Biomphalaria* genetics since it confirmed the extensive variability that had been observed by isoenzyme analysis and morphological studies in *B. glabrata*.

SPECIFIC IDENTIFICATION OF *BIOMPHALARIA* SNAILS BY LS-PCR

The remarkable degree of intraspecific polymorphism detected in *B. glabrata* by AP-PCR suggested us that this technique would be extremely difficult to use for species identification. We tested the low stringency polymerase chain reaction (LS-PCR) which uses two specific primers and low stringency annealing conditions since products obtained from the highly conserved rRNA genes have less intraspecific variation than those from randomly amplified sequences (Dias Neto et al. 1993a, Vidigal et al. 1996). Several primers were tested for their ability to group specimens of the same species. Primers NS-1 and ET-1 gave the best results when they were tested with *B. glabrata*

and *B. tenagophila* samples taken from six localities in Brazil (Vidigal et al. 1996). Even though these primers produced a complex pattern of bands in LS-PCR analysis, at least four LS products for each *Biomphalaria* were species-specific, independent of the origin of the snails (Fig. 3). We could also confirm that *B. glabrata* is more heterogeneous than *B. tenagophila*. Using this technique, it was also possible to distinguish *B. tenagophila* from *B. occidentalis* (Pires et al. 1997). These two species are not distinguishable by most morphologic characters (Paraense 1981) but only *B. tenagophila* is an intermediate host of *S. mansoni*. Previous experiments using this technique with *B. straminea* suggested that this species presents higher interpopulation levels of variability than *B. glabrata*, *B. tenagophila* or *B. occidentalis*. No sharing of LS products was observed on analyzing several *B. straminea* specimens from different localities (data not shown). Although LS-PCR did not allow specific identification of *B. straminea* snails, they can not be mistaken with *B. glabrata*, *B. tenagophila* or *B. occidentalis*. In summary we show that LS-PCR permits the differentiation *B. glabrata* and *B. tenagophila* and very similar species such as *B. tenagophila* and *B. occidentalis* (Vidigal et al. 1996, Pires et al. 1997).

SPECIFIC IDENTIFICATION OF *BIOMPHALARIA* SNAILS BY PCR-RFLP

Although very useful for identifying some species of *Biomphalaria*, the complexity of the patterns produced by LS-PCR represents a problem as it requires the correct identification of a few bands out of dozens. To obtain a less variable marker for the study of these molluscs, we have analyzed possible sequence polymorphisms in the ITS (internal transcribed spacer) region of the rDNA (which includes the 5.8S rDNA gene together with the flanking ITS1 and ITS2 spacers) by amplification using PCR and digestion with several restriction enzymes (restriction fragment length polymorphism - RFLP) (Vidigal et al. 1998). This method has been successfully employed to distinguish closely related parasitic worms (Gasser et al. 1996, Cunningham 1997) and in studies of genetic variation and identification of species of snails such as *Oncomelania hupensis*, *Bulinus* and *Stagnicola* (Hope & McManus 1994, Stothard et al.1996, Stothard & Rollinson 1997, Remigio & Blair 1997). We first analyzed different *B. glabrata*, *B. tenagophila* and *B. straminea* populations in Brazil. The entire ITS was amplified using the primers ETTS1 and ETTS2 anchored respectively in the conserved extremities

Fig. 1: geographical distribution of the snails used.

of the 18S and 28S ribosomal genes, according to described techniques (Kane & Rollinson 1994, Stothard et al. 1996). The PCR specific amplification of the *Biomphalaria* ITS resulted in a product of approximately 1.3 kb independent of the species. The ITS region amplified from all the species studied has sites for the restriction enzymes *Alu*I, *Dde*I, *Hae*III, *Mnl*I, *Msp*I, *Rsa*I and *Sau3a*I but not for *Hinf*I. Restriction fragments were separated by 6% or 8% polyacrylamide gel electrophoresis and stained with silver. Profiles obtained with *Alu*I, *Mnl*I, *Sau3a*I, *Msp*I and *Rsa*I did not permit species identification as extensive intraspecific polymorphism or invariant RFLP profiles were detected. The most promising RFLP profiles were those produced both with *Hae*III and *Dde*I which included invariant species-specific products for three representative specimens of *B. glabrata*, *B. tenagophila* and *B. straminea* (Fig. 4). The reproducibility of the *Dde*I - ITS RFLP profiles was demonstrated by analysis of diverse *Biomphalaria* specimens obtained from distinct localities within Brazil. Additional snails from Argentina (*B. straminea* and *B. tenagophila*), Uruguay (*B. straminea*) and Paraguay (*B. tenagophila*) were included in the analysis. The restriction profiles of *B. straminea* from Argentina and Uruguay, also showed the same two characteristic fragments present in the Brazilian isolates (data not shown). When *B. tenagophila* from different localities within Argentina, were compared with specimens from Brazil and Paraguay, the profiles of the specimens from Argentina appear quite distinct with two additional fragments that are absent in the other *B. tenagophila* specimens tested. However, in all *B. tenagophila* specimens the presence of a band of approximately 470 bp, was observed allowing identification (Fig. 5). Our results also showed the possibility of using eggs for snail

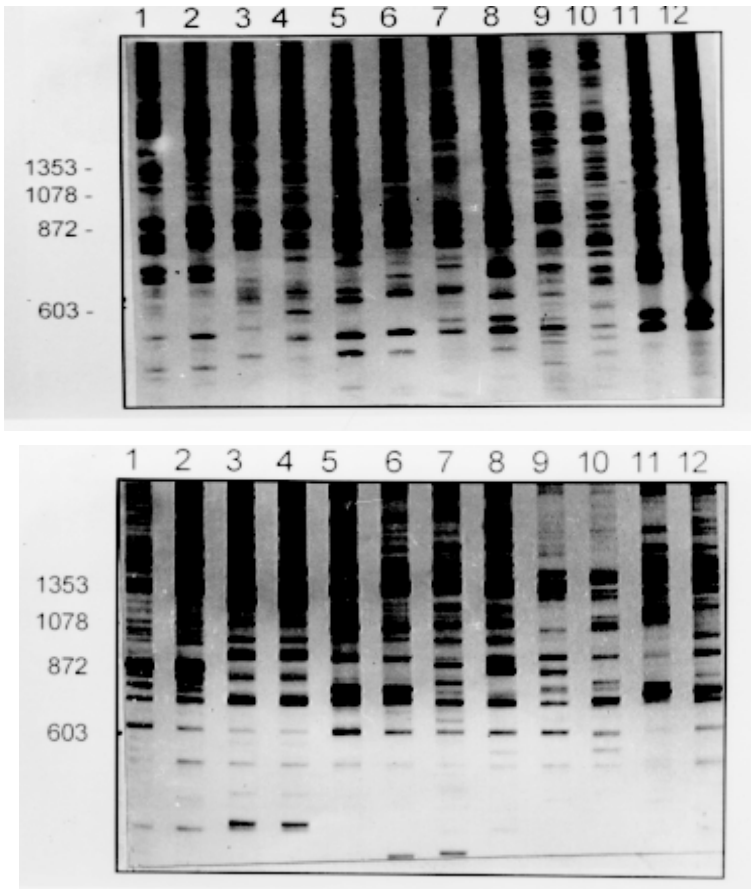

Fig. 2: RAPD profiles of two distinct *Biomphalaria glabrata* specimens from six different geographical regions using one ng of template and primers 3307(A) and 3302 (B). Lanes 1 and 2: two individuals from Belém, PA; lanes 3 and 4: two individuals from Cururupu, MA; lanes 5 and 6: two individuals from Touros, RN; lanes 7 and 8: two individuals from Pontezinha, PE; lanes 9 and 10: two individuals from Aracaju, SE; lanes 11 and 12: two individuals from Jacobina, BA. The PCR products were resolved by electrophoresis through a 4% polyacrylamide gel followed by silver staining.
(Source: *Exp Parasitol 79*: 187-194, 1994).

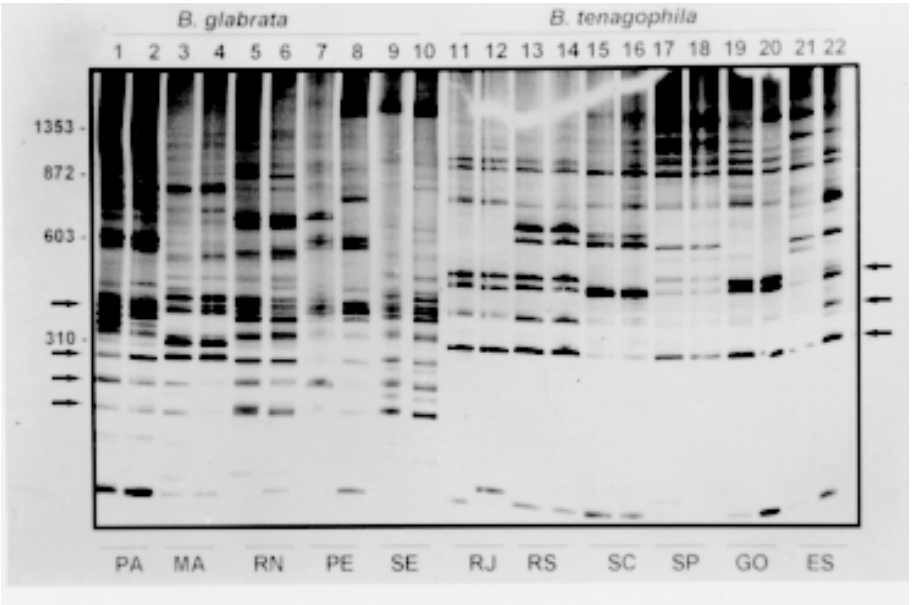

Fig. 3: silver-stained 4% polyacrylamide gel showing the LS-PCR amplification products obtained with primers NS1-ET1 and 1ng of DNA extracted from snails obtained from different localities. Lanes 1 and 2: *Biomphalaria glabrata* from Belém, PA; lanes 3 and 4: *B. glabrata* from Cururupu, MA; lanes 5 and 6: *B. glabrata* from Touros, RN; lanes 7 and 8: *B. glabrata* from Pontezinha, PE; lanes 9 and 10: *B. glabrata* from Aracaju, SE; lanes 11 and 12: *B. tenagophila* from Paracambi, RJ; lanes 13 e 14: *B. tenagophila* from Imbé, RS; lanes 15 and 16: *B. tenagophila* from Joinville, SC; lanes 17 and 18: *B. tenagophila* from Araçatuba, SP; lanes 19 and 20*: B. tenagophila* from Formosa, GO; lanes 21 and 22: *B. tenagophila* from Vila Velha, ES. The species diagnostic bands are indicated by arrows, and the molecular weight markers, are as shown.
(Source: *Mem Inst Oswaldo Cruz 91*: 739-744, 1996).

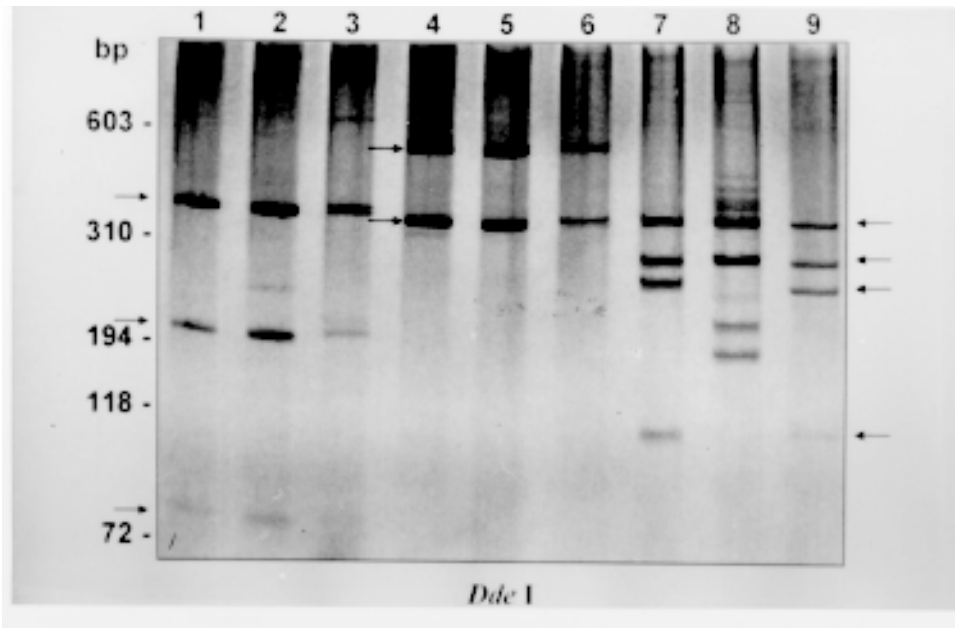

Fig. 4: 6% silver stained polyacrylamide gels showing the RFLP profiles obtained following the digestion of the rDNA ITS with *Dde*I. The snail species are: lane 1: *Biomphalaria glabrata* from Belém, PA; lane 2: *B. glabrata* from Aracaju, SE; lane 3: *B. glabrata* from, Sabará, MG; lane 4: *B. tenagophila* from Formosa, GO; lane 5: *B. tenagophila* from Vespasiano, MG; lane 6, *B. tenagophila* from Vitória, ES; lane 7: *B. straminea* from Picos, PI; lane 8: *B. straminea* from Porto Alegre, RS; lane 9: *B. straminea* from Várzea do Poço, BA. Molecular size markers are shown on the left of each gel. The arrows indicate species specific fragments.
(Source: *Exp Parasitol 89*: 180-187, 1998).

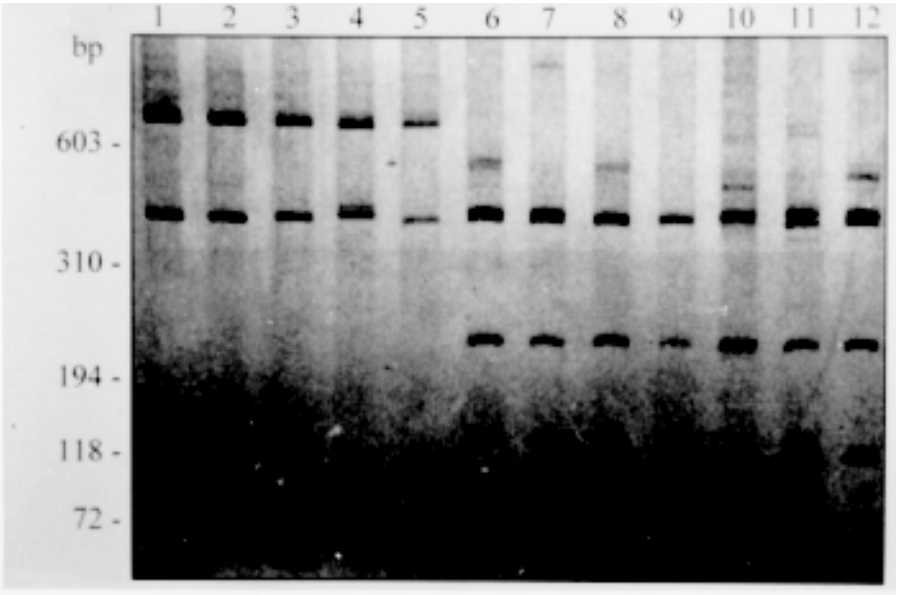

Fig. 5: 6% silver stained polyacrylamide gel showing the RFLP profiles obtained by digesting the rDNA ITS with *DdeI*. The DNA was extracted from individual *Biomphalaria tenagophila snails* derived from: lane 1, Formosa, State of Goiás; lane 2, Vespasiano, State of Minas Gerais; lane 3, Imbé, State of Rio Grande do Sul; lanes 4 and 5, Assuncion, Paraguay; lanes 6 to 8, Resistência (Chaco), Argentina; lanes 9 to 12, Corrientes, Argentina. Molecular size markers are shown on the left of the gel.
(Source: *Exp Parasitol 89*: 180-187, 1998)

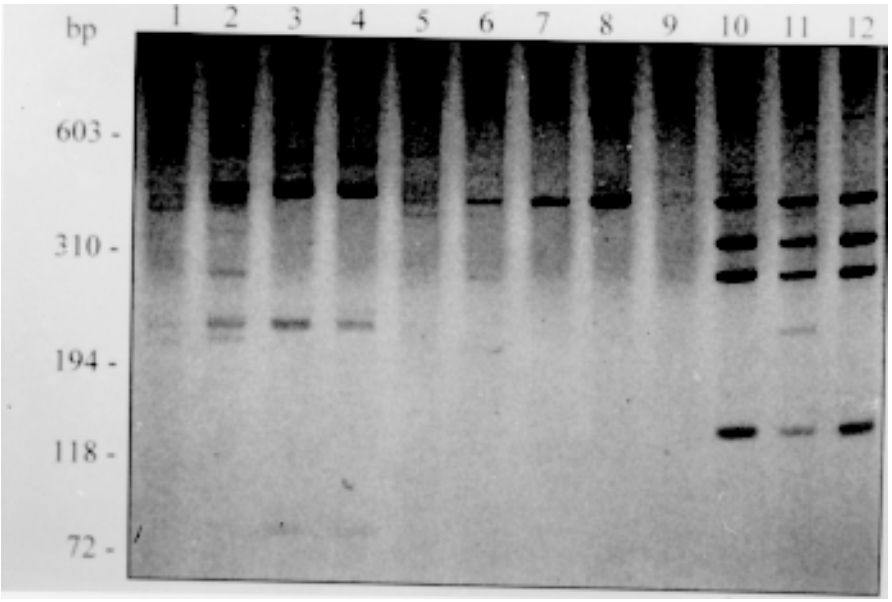

Fig. 6: 6% silver stained polyacrylamide gel showing the RFLP profiles obtained after digesting of the rDNA ITS with *DdeI*. The DNA used was extracted from the eggs of *Biomphalaria glabrata*, *B. tenagophila* and *B. straminea* on different days and compared with the profile produced with adult control DNA. Lane 1, day one eggs of *B. glabrata*; lane 2, day two eggs of *B. glabrata*; lane 3, day three eggs of *B. glabrata*; lane 4, adult *B. glabrata*; lane 5, day one eggs of *B. tenagophila*; lane 6, day two eggs of *B. tenagophila*; lane 7, day three eggs of *B. tenagophila*; lane 8, adult *B. tenagophila;* lane 9, day one eggs of *B. straminea*; lane 10, day two eggs of *B. straminea*; lane11, day three eggs of *B. straminea*; lane 12, adult *B. straminea.*
(Source: *Exp Parasitol 89*: 180-187, 1998)

identification with *Dde*I (Fig. 6). 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. In summary we show here that PCR-RFLP of the ITS region using a single enzyme (*Dde*I), permits the differentiation of the three intermediate hosts of *S. mansoni* from South America. The technique is simple and rapid and may represent an important advance in the precision of snail population surveys undertaken in South America. To understand better the relationships between the different species, as well as the relations between the isolates that resulted in more polymorphic profiles (as seen in *B. tenagophila* from Argentina or in *B. straminea* from Porto Alegre) the sequencing of the ITS region is being undertaken. The molecular biological techniques used in our studies are very useful for the generation of new knowledge concerning the systematics and population genetics of *Biomphalaria* snails.

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