

Further Studies on the Molecular Systematics of *Biomphalaria* Snails from Brazil

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The polymerase chain reaction and restriction fragment length polymorphism (RFLP) of the internal transcribed spacer (ITS) region of the rRNA gene, using the enzyme DdeI were used for the molecular identification of ten species and one subspecies of Brazilian Biomphalaria. Emphasis is given to the analysis of B. oligoza, B. schrammi and B. amazonica. The RFLP profiles obtained using this enzyme were highly distinctive for the majority of the species and exhibited low levels of intraspecific polymorphism among specimens from different regions of Brazil. However, B. peregrina and B. oligoza presented very similar profiles that complicated their identification at the molecular level and suggested a very close genetic similarity between the two species. Others enzymes including HaeIII, HpaII, AluI and MnlI were tested for their ability to differentiate these species. For B. amazonica three variant profiles produced with DdeI were observed. The study demonstrated that the ITS contains useful genetic markers for the identification of these snails

Key words: *Biomphalaria* - ribosomal DNA - internal transcribed spacer - polymerase chain reaction - Brazil

Ten species and one subspecies of *Biomphalaria* are recognized in Brazil: *B. glabrata* (Say, 1818), *B. tenagophila* (Orbigny, 1835), *B. tenagophila guaibensis* Paraense, 1984, *B. straminea* (Dunker, 1848), *B. peregrina* (Orbigny, 1835), *B. kuhniiana* (Clessin 1883), *B. schrammi* (Crosse, 1864), *B. amazonica* Paraense 1966, *B. oligoza* Paraense, 1974, *B. intermedia* (Paraense & Deslandes, 1962) and *B. occidentalis* Paraense 1981. Only, *B. straminea*, *B. t. tenagophila* and *B. glabrata* are found naturally infected with *Schistosoma mansoni*. Among these, *B. glabrata* is the most important due to its wide spread distribution and its high susceptibility to *S. mansoni* (Paraense & Corrêa 1963). In addition *B. peregrina* and *B. amazonica* are considered potential hosts of this parasite based on experimental infection (Corrêa & Paraense 1971, Paraense & Corrêa 1973).

The morphological identification of freshwater snails of medical importance, is greatly complicated by the extensive intraspecific variation of the morphological characteristics used for classical identification (Paraense 1975, 1981, 1984,

1988). To overcome this problem, the use of molecular techniques as additional tools for the identification of these snails has been proposed. In previous studies we tested a low stringency polymerase chain reaction technique (LS-PCR) (Dias Neto et al. 1993) which permitted the differentiation of *B. glabrata* and *B. t. tenagophila* and also the identification of very similar species such as *B. t. tenagophila* and *B. occidentalis* (Vidigal et al. 1996, Pires et al. 1997).

Recently, we have explored the possibility of using sequence polymorphism in the internal transcribed spacer region (ITS) of the rDNA of *Biomphalaria* snails (which includes the 5.8S rDNA gene together with the flanking ITS1 and ITS2 spacers) by means of PCR amplification and digestion with different restriction enzymes. This method was successfully used to construct the molecular key for *Aedes* species (West et al. 1997). It was also used to distinguish closely related parasitic worms and others different organisms (Newton et al. 1998, Wu et al. 1999) and on studies of genetic variation and identification of snails of the genera *Oncomelania*, *Bulinus* and *Biomphalaria* (Hope & McManus 1994, Stothard et al. 1996, Stothard & Rollinson 1997, Rollinson et al. 1998, Vidigal et al. 1998a,b, Caldeira et al. 1998, Spatz et al. 1998, 1999).

The aim of the present study was to investigate the use of restriction profiles resulting from digestion of the ITS spacer of rDNA with *Dde* I for sepa-

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ration of the majority *Biomphalaria* species from Brazil, with special attention to *B. oligoza*, *B. schrammi* and *B. amazonica* species which were being studied for the first time. We found difficulty to differentiate between *B. peregrina* and *B. oligoza* with *DdeI*, however we propose the use of others restriction enzymes such as *HpaII*, *Hae III* and *AluI* for identification of these species.

MATERIALS AND METHODS

Snail populations - Snail populations from different Brazilian localities were used (Fig. 1). The number of snails used and the geographical localization from each population are shown in the Table. The snails were identified by means of comparative morphology based on the reproductive organs and shells accordingly to Deslandes (1951) and Paraense (1975, 1981, 1984, 1988).

DNA extraction - Total DNA was extracted from the feet of snails using the Wizard Genomic DNA Purification Kit (Promega) with some modifications. Briefly, the tissues were mechanically disrupted in 200 μ l of nucleic lysis solution and incubated at 37°C for 4 h or overnight with 50 μ g/

ml proteinase K. Thereafter, 80 μ l of protein precipitation solution was added to the initial mix. The mixture was vortex vigorously for 10-20 sec and centrifuged at 13,000 rpm for 5 min. The supernatant was transferred to a microcentrifuge tube containing 200 μ l of room temperature isopropanol for DNA precipitation. The mixture was gently mixed by inversion for 20 min and centrifuged at 13,000 rpm for 5 min. The DNA pellet was washed with 300 μ l of 70% ethanol and centrifuged for 1 min. The pellet was treated with 25 μ l of DNA rehydration solution for 30 min at 65°C and stored at -20°C. The DNA concentrations were estimated by comparison with known standards on 2% ethidium bromide stained agarose gels.

Amplification by PCR - The entire ITS was amplified using the primers ETTS2 (5'-TAACAAGGTTTCCGTAGGTGAA-3') and ETTS1 (5'-TGCTTAAGTTCAGCGGGT-3') anchored respectively in the conserved extremities of the 18S and 28S ribosomal genes (Kane & Rollinson 1994). The PCR amplification was undertaken in a volume of 10 μ l consisting of: 1-10 ng template DNA, 10mM Tris-HCl, pH 8.5, 200



Fig. 1: map showing the localization of the snail populations used. The legend shows the abbreviation of each species mentioned in the map. *Biomphalaria glabrata* (Bg); *B. tenagophila tenagophila* (Btt); *B. straminea* (Bs); *B. occidentalis* (Boc); *B. t. guaiabensis* (Btg); *B. amazonica* (Ba); *B. intermedia* (Bi); *B. kuhniiana* (Bk); *B. peregrina* (Bp); *B. oligoza* (Bo); *B. schrammi* (Bsch).

µm each dNTP, 1.5 mM MgCl₂, 0.8 U of Taq DNA polymerase, 50 mM KCl, together with 5.0 pmol of each primer. The reactions were covered with a drop of mineral oil and subjected to the following cycle program: initial denaturation step for 3 min at 95°C, and then 32 cycles with annealing at 54°C for 1 min, extension at 72°C for 2 min, denaturation at 95°C for 45 sec and a final extension step at 72°C for 5 min. A negative control (no template DNA) was included in all experiments. Three microliters of the amplification products were visualised on 0.8% ethidium bromide stained agarose gels to check the quality of amplification. The remaining 7 µl were mixed with 53 µl of water, and divided into 10 µl aliquots for enzyme digestion.

Production and evaluation of the rDNA-ITS RFLP profiles - In the initial experiments the enzyme *DdeI* (Amersham, Life Science) was evaluated for its ability to differentiate all *Biomphalaria* species from Brazil. Additional enzymes were tested including *AluI*, *MnII*, (Amersham, Life Science) *HaeIII* and *HpaII* (Promega Co, USA) in situations when *DdeI* was not effective in the separation of some species. One microliter (10-12 units) was used for each digestion reaction, together with 1.2 µl of the respective enzyme buffer in a final volume of 12.2 µl. The digestion was performed for 3.5 h at 37°C, and the digestion products were evaluated on 6 or 8% silver stained polyacrylamide gels (Sanguinetti et al. 1994) after phenol/chloroform extraction. The results were recorded on Polaroid film 667 (St Albans, UK). A control for the activity of each enzyme was performed by digesting 150 ng of pUC18.

RESULTS

The PCR amplification of the *Biomphalaria* ITS region with the primers ETTS2 and ETTS1 resulted in a product of approximately 1.3kb (Fig. 2A). However, *B. oligoza* (lanes 18 and 19), *B. peregrina* (lanes 20 and 21) and *B. amazonica* (lanes 25 at 28) presented an ITS product of approximately 1.0kb. Fig. 2B shows the rDNA-ITS RFLP profiles produced by digestion with *DdeI* of two to four specimens of all *Biomphalaria* species from Brazil. The profiles obtained with this enzyme permit the easy separation of the majority of the species analysed with exception of *B. occidentalis* and *B. t. guaibensis* (Fig. 2B lanes 7, 8 and 9, 10, respectively) and *B. oligoza* and *B. peregrina* (Fig. 2B lanes 18, 19 and 20, 21, respectively) which presented very similar profiles. The reproducibility of the profiles obtained with *DdeI* for *B. oligoza*, *B. peregrina*, *B. schrammi* and *B. amazonica* were previously evaluated by increasing of the number of specimens for each spe-

cies obtained from different localities (Table). Fig. 3A shows the profiles obtained using *DdeI* with *B. oligoza* collected in Rio Grande do Sul (lanes 1 to 4) and Santa Catarina (lanes 5 to 10). The profiles were very homogeneous for all specimens. Fig. 3B shows the profiles obtained for *B. peregrina* specimens collected from different localities in Minas Gerais. The profiles obtained with *B. peregrina* were homogeneous but, as mentioned above, similar to the those obtained from *B. oligoza* (Fig. 3A).

Fig. 4 shows the profiles of *B. schrammi* obtained with *DdeI*. This enzyme produced a simple profile with six fragments, which was clearly different from those seen with other species as observed in Fig. 2B (lanes 22 to 24). *B. amazonica* presented three variant profiles with three to nine fragments when treated with *DdeI* (Fig. 5). In fact in the snails from one locality (Benjamin Constant, Amazonas) three profiles were observed: lanes 1, 2 and 4 show the profiles characterized by presence of three bands, lane 3 shows five fragments and lanes 5, 6 and 7 show the profiles with nine fragments. In the snails obtained from Barão do Melgaço, Mato Grosso (lanes 8 to 10) only one profile with five fragments was observed. The specimens with variant profiles, for this species, were included in Fig. 2B (lanes 25 to 28). The profiles obtained with *DdeI* for the ten species and one subspecies of the Brazilian *Biomphalaria* snails are represented in Fig. 6.

To obtain an improved molecular separation of *B. oligoza* and *B. peregrina* other enzymes were tested (*HpaII*, *MnII*, *AluI* and *HaeIII*). Fig. 7A shows the results obtained with *HpaII* which produced the best results with distinct profiles observed for each species. Both *HaeIII* (Fig. 7B) and *AluI* (Fig. 7C) produced similar profiles with only subtle differences. *B. oligoza* and *B. peregrina* *HaeIII* profile shows six fragments two of which are species-specific for *B. oligoza* (Fig. 7B lanes 4 to 12). Using *AluI* *B. peregrina* presented one band of approximately 200bp distinct from *B. oligoza* (Fig. 7C). The enzyme *MnII* did not permit the separation of these species (data not shown).

DISCUSSION

The possibility of using molecular tools for identification of snails of medical importance has contributed to increased knowledge of the genus *Biomphalaria*. Previously, we have explored other molecular methods to obtain species-specific markers. Initially we observed that the arbitrarily primed-PCR (AP-PCR) mirrored the high genetic variability in *B. glabrata* species (Vidigal et al. 1994, 1998b). These results demonstrated that this methodology does not provide good markers for molecular identification of *Biomphalaria* species.

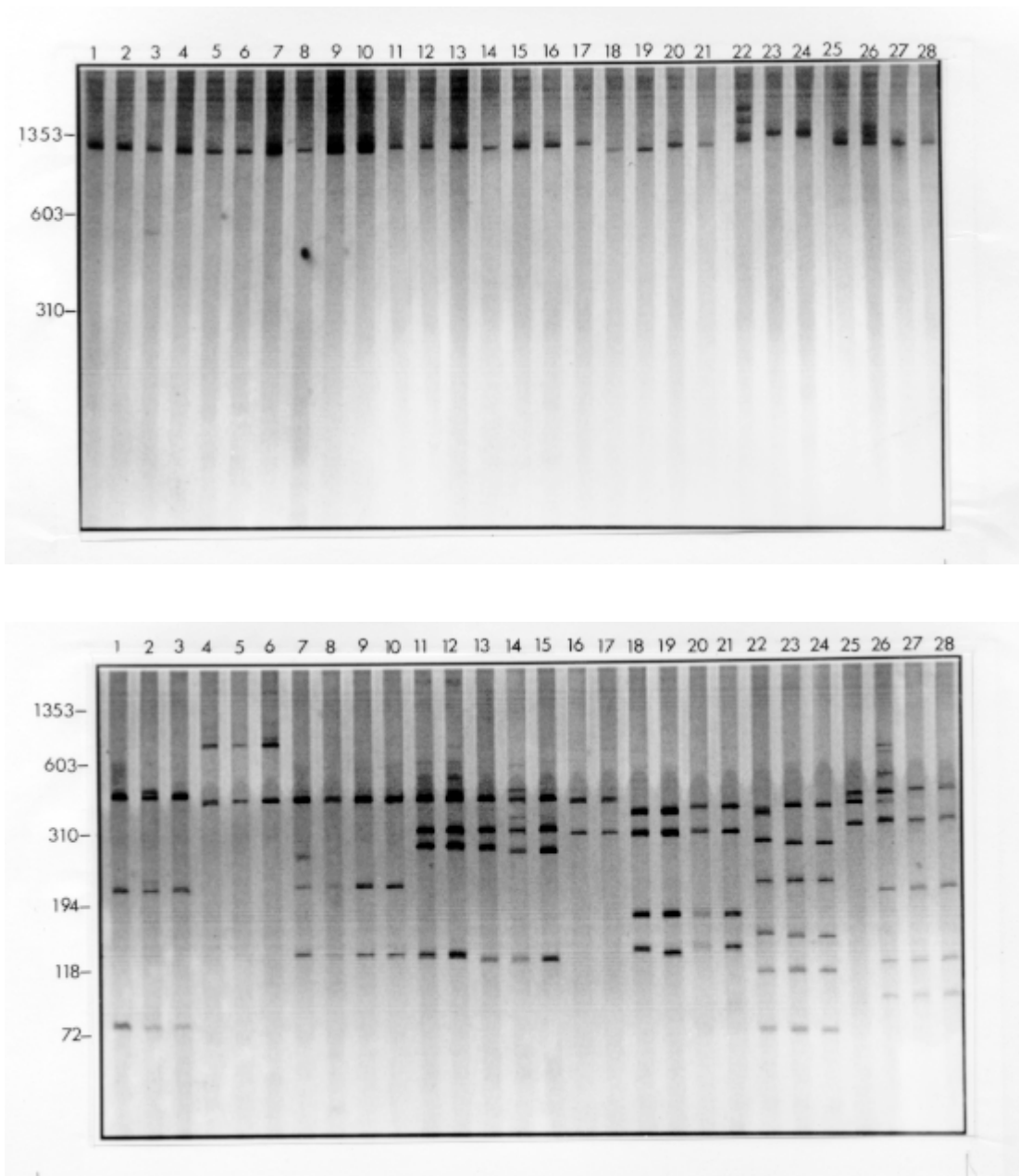


Fig. 2A: silver stained polyacrylamide gel (6%) showing the products of polymerase chain reaction amplification of internal transcriber spacer (ITS) region of *Biomphalaria* species. Lane 1, *B. glabrata* from Belém, PA; lane 2, *B. glabrata* from, Sabará, MG; lane 3, *B. glabrata* from, Esteio, RS; lane 4, *B. tenagophila* from Formosa, GO; lane 5, *B. tenagophila* from Vespasiano, MG; lane 6, *B. tenagophila* from Imbé, RS; lane 7, *B. occidentalis* from Campo Grande, MS; lane 8, *B. occidentalis* from Capetinga, MG; lane 9, *B. t. guaiabensis* from Esteio, RS; lane 10, *B. t. guaiabensis* from Eldorado do Sul, RS; lanes 11 and 12, *B. kuhniiana* from Tucuruí, PA; lane 13, *B. straminea* from Picos, PI; lane 14, *B. straminea* from Guaíra, PR; lane 15, *B. straminea* from Florianópolis, SC; lane 16, *B. intermedia* from Itapagipe, MG; lane 17, *B. intermedia* from Pindorama, SP; lane 18, *B. peregrina* from Alfenas, MG; lane 19, *B. peregrina* from Bom Jesus da Penha, MG; lane 20, *B. oligoza* from Florianópolis, SC; lane 21 *B. oligoza* from Eldorado do Sul, RS; lane 22, *B. schrammi* from Itaú de Minas, MG; lane 23, *B. schrammi* from Cássia, MG; lane 24, *B. schrammi* from Ilícinea, MG; lanes 25 and 26, *B. amazonica* from Bejamin Constant, AM; lanes 27 and 28, *B. amazonica* from Barão de Melgaço, MT. Molecular size markers are shown on the left of each gel; B: silver stained polyacrylamide gel (8%) showing the restriction fragment length polymorphism profiles obtained following digestion of rDNA ITS with *Dde*I. The legend is the same as that shown above.

TABLE
Species, localities and localization of the populations of snails used

Species	Localities (State)	Latitude/longitude
<i>Biomphalaria glabrata</i>	Belém (Pará)	01s27/48w30
	Esteio (Rio Grande do Sul)	29s51/51w10
	Sabará (Minas Gerais)	19s53/43w48
<i>B. tenagophila</i>	Vespasiano (Minas Gerais)	19s41/43w55
	Formosa (Goiás) ^a	15s32/47w20
	Imbé (Rio Grande do Sul) ^a	29s58/50w07
<i>B. straminea</i>	Picos (Piauí) ^a	07s04/41w28
	Florianópolis (Santa Catarina)	27s35/48w32
	Guaíra (Paraná) ^a	24s04/54w15
<i>B. intermedia</i>	Pindorama (São Paulo)	21s11/48w54
	Itapagipe (Minas Gerais)	19s54/49w22
<i>B. peregrina</i>	Alfenas (Minas Gerais)	21s25/45w56
	Guimarânia (Minas Gerais)	18s50/46w47
	Juruáia (Minas Gerais)	21s15/46w34
<i>B. schrammi</i>	Bom Jesus da Penha (Minas Gerais)	21s01/46w31
	Ilicínea (Minas Gerais)	20s56/45w49
	Itaú de Minas (Minas Gerais)	20s44/46w45
	Cássia (Minas Gerais)	20s34/46w55
<i>B. khuniana</i>	Tucuruí (Pará)	03s46/49w40
<i>B. occidentalis</i>	Capetinga (Minas Gerais)	20s36/47w03
	Campo Grande (Mato Grosso do Sul) ^a	20s26/54w38
<i>B. t. guaibensis</i>	Esteio (Rio Grande do Sul)	29s51/51w10
	Eldorado do Sul (Rio Grande do Sul)	30s05/51w36
<i>B. oligoza</i>	Eldorado do Sul (Rio Grande do Sul)	30s05/51w36
	Florianópolis (Santa Catarina)	27s35/48w32
<i>B. amazonica</i>	Benjamin Constant (Amazonas)	04s22/70w01
	Barão de Melgaço (Mato Grosso) ^a	16s11/55w41

a: laboratory populations

The LS-PCR technique produced a great number of bands complicating the identification of the species-specific LSP markers (LS products) (Vidigal et al. 1996, Pires et al. 1997). In the present study we demonstrate that PCR-RFLP of the ITS region of *Biomphalaria* rDNA, using *DdeI*, permits the differentiation of the majority of the ten Brazilian *Biomphalaria* species examined. In previous studies we demonstrated that restriction profiles resulting from digestion with *DdeI* permit the identification of *B. glabrata*, *B. straminea* and *B. tenagophila* (Vidigal et al. 1998a,b). We also used this technique in the study of very similar species such as *B. straminea*, *B. intermedia*, *B. khuniana*, *B. peregrina* (Caldeira et al. 1998) and *B. t. tenagophila*, *B. t. guaibensis* and *B. occidentalis* (Spatz et al. 1998, 1999).

The morphological separation of typical specimens of *B. peregrina* and *B. oligoza* are easy tasks for the specialist in the identification of *Biomphalaria* snails. The principal characteristic for

separation of these species is the presence of the small number of prostatic diverticula (1 to 6, rarely 7), or absence in *B. oligoza* (Paraense 1974, 1975). In *B. peregrina* the presence of 8 to 22 prostatic diverticula is reported. However, when specimens from both species show similar number of diverticula (*B. oligoza* maximum 7 and *B. peregrina* minimum 8) morphological separation of the species becomes more difficult. In this case only observation of the prostatic diverticula covering the spermathecal body of the *B. peregrina* can be considered as a diagnostic character (Paraense 1966, 1975). Here, the molecular results obtained with PCR-RFLP suggested a high similarity between these species based on the similarity of profiles obtained with *DdeI* and *AluI* and the identical profiles produced with *MnII*. Although the similarity among these profiles, *AluI*, *HpaII*, *HaeIII* (7A, B, C) permit the molecular separation of these species and can be used in cases when the morphological identification of the *B. oligoza* and *B. peregrina* is not effective. It is noteworthy

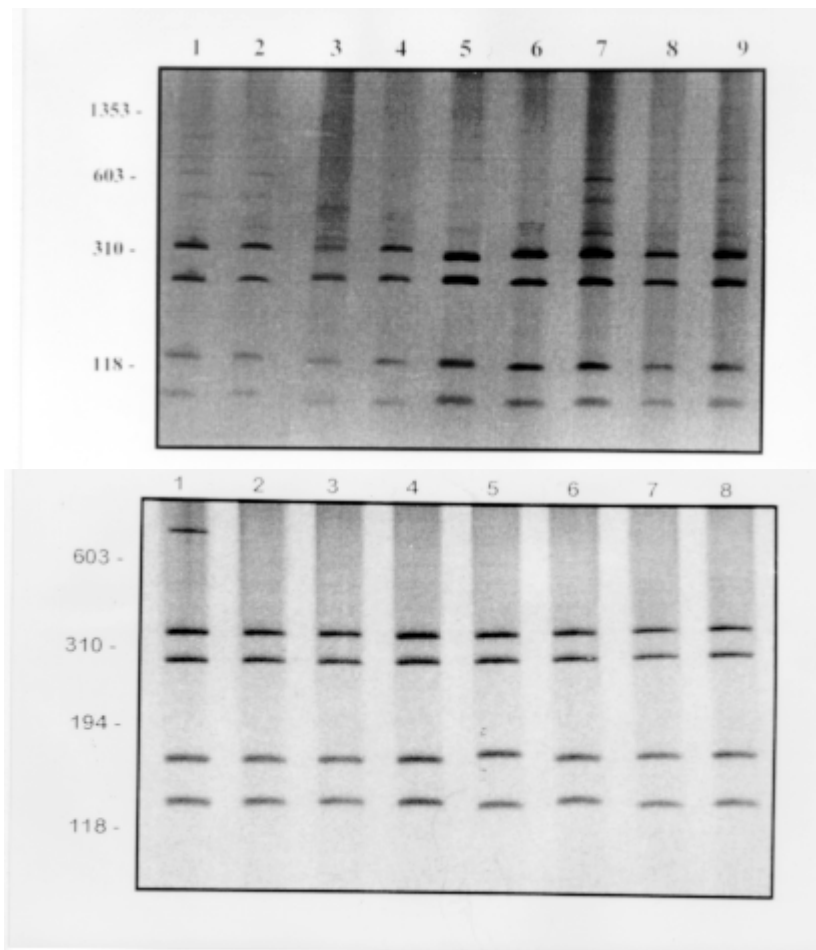


Fig. 3: silver stained polyacrylamide gel (8%) showing restriction fragment length polymorphism profiles produced using *DdeI* to digest rDNA internal transcriber spacer (ITS) amplified from specimens of *Biomphalaria oligoza* and *B. peregrina* from different localities in Brazil. A: *B. oligoza*: lanes 1 to 4, snails from Esteio, RS; lanes 5 to 10, snails from Florianópolis, SC; B: *B. peregrina*: lanes: 1 to 3 snails from Guimarães, MG; lanes 4 to 6 snails from Alfenas, MG; lane 7, snail from Juruáia, MG; lane 8 snail from Bom Jesus da Penha, MG. Molecular size markers are shown on the left of each gel.

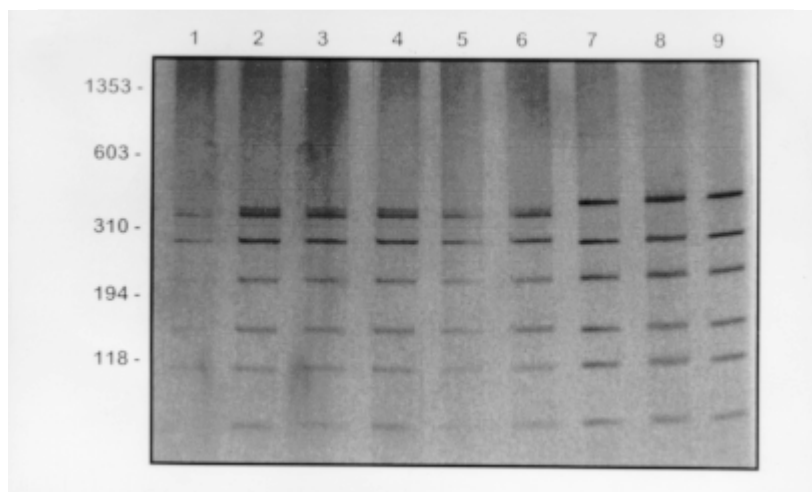


Fig. 4: silver stained polyacrylamide gel (6%) showing the restriction fragment length polymorphism profiles obtained by digestion of rDNA internal transcriber spacer (ITS) with *DdeI*. The DNA of individual snails of *Biomphalaria schrammi* obtained from: lanes 1 to 3, Cássia, MG; lanes 4 to 6, Itaú de Minas, MG; lanes 7 to 9, Ilícinea, MG. Molecular size markers are shown on the left of the gel.

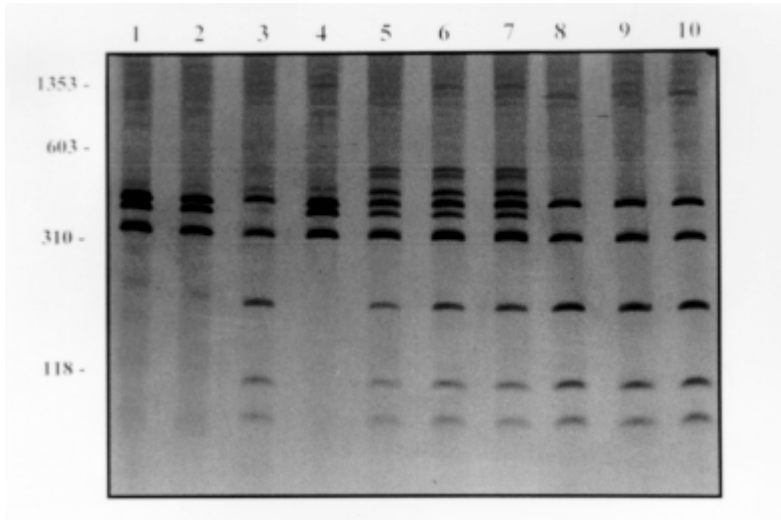


Fig. 5: silver stained polyacrylamide gel (6%) showing the restriction fragment length polymorphism profiles obtained by digestion of rDNA internal transcriber spacer (ITS) with *DdeI*. The DNA of individual snails of *Biomphalaria amazonica* obtained from lanes 1 to 7, snails from Benjamin Constant, AM and lanes 8 to 10, snails from Barão de Melgaço, MT. Molecular size markers are shown on the left of the gel.

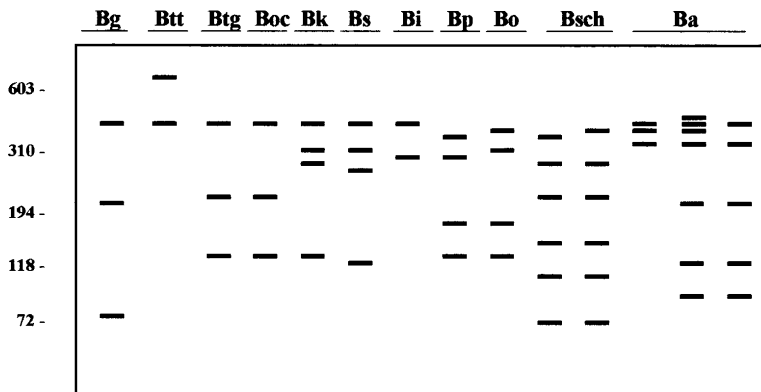


Fig. 6: schematic representation of the internal transcribed spacer rDNA restriction patterns of ten Brazilian *Biomphalaria* species and one subspecies produced with *DdeI*. The legend shows the abbreviation of each species: *Biomphalaria glabrata* (Bg); *B. t. tenagophila* (Btt); *B. occidentalis* (Boc); *B. t. guaibensis* (Btg); *B. straminea* (Bs); *B. intermedia* (Bi); *B. kuhniiana* (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.

that *B. peregrina* is one of the most widespread planorbid species in the Neotropical region hence, according to Paraense (1975), the origin to its scientific name (from the latin *peregrinus*, meaning wanderer, in reference to its wide geographical distribution).

B. schrammi is distributed in most regions of Brazil, but has not been found in Amazonas, Paraná, Rio Grande do Sul or Santa Catarina (Paraense 1975). The molecular characters shown here (Fig. 4) very clearly identified specimens belonging to this species. The distribution of *B. amazonica* in Brazil is concentrated in Amazonas,

Acre, Mato Grosso do Sul and Rondônia (Paraense 1983). The snails analyzed here were obtained from two distant localities and showed three variant profiles (Fig. 5). Accordingly the molecular identification of this species requires that attention is given to this diversity of profiles. The schematic representation of the ITS restriction patterns obtained for Brazilian *Biomphalaria* snails using *DdeI* can be used as model for comparative analysis (Fig. 6).

The sequences of ITS regions of the Brazilian *Biomphalaria* species will be analyzed in the future to better understand the intraspecific and interspecific relationships among this species.

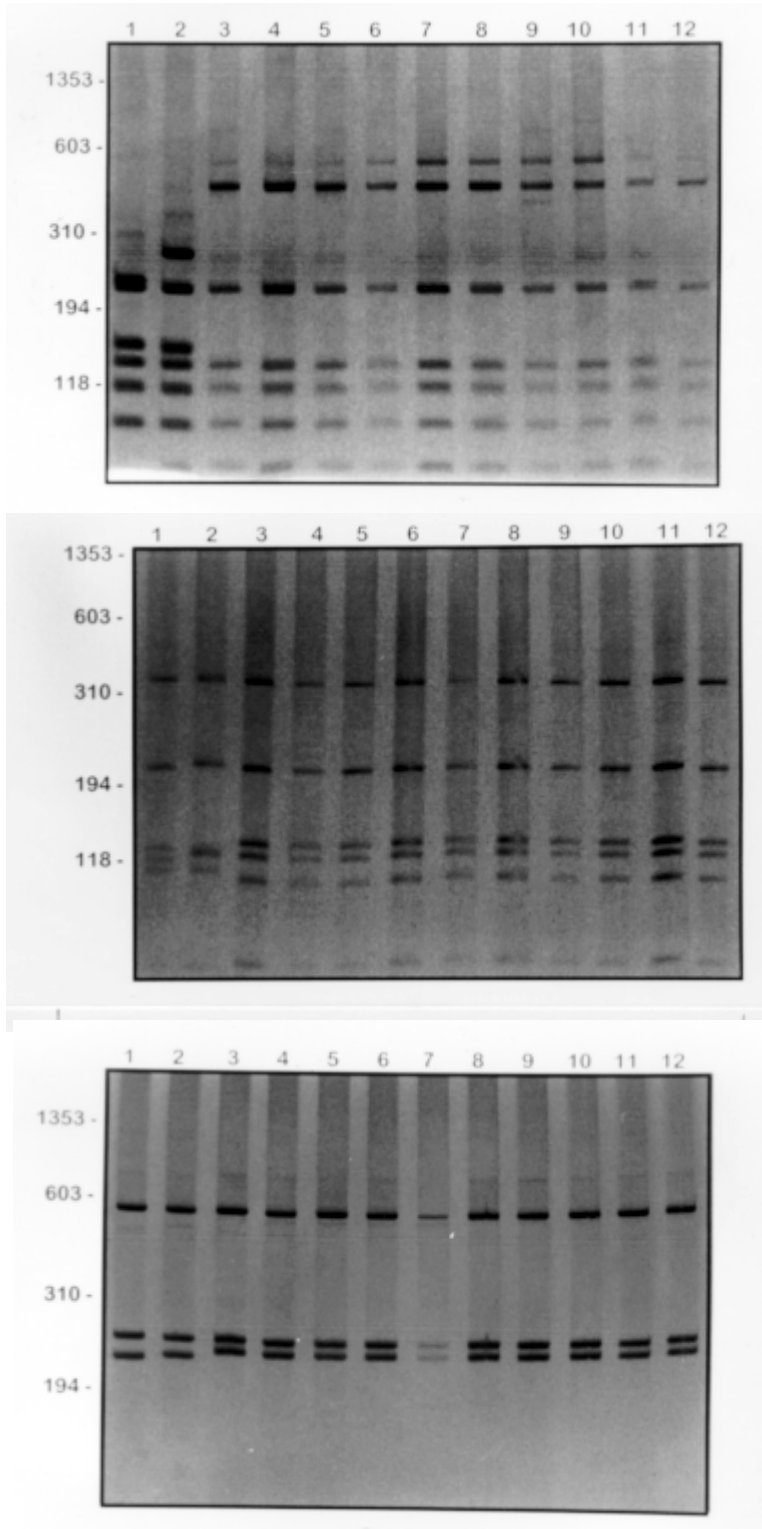


Fig. 7: (*AluI*) silver stained polyacrylamide gel (6% or 8%) showing the restriction fragment length polymorphism profiles obtained following digestion of rDNA internal transcribed spacer with *HpaII* (A), *HaeIII* (B), *AluI* (C) amplified from DNA extracted from *Biomphalaria peregrina* and *B. oligoza*. In each gel the snails specimens are: lane 1, *B. peregrina* from Alfenas, MG; lane 2, *B. peregrina* from Bom Jesus da Penha, MG; lanes 3 at 7, *B. oligoza* from Eldorado do Sul, RS; lanes 8 at 12, *B. oligoza* from Florianópolis, SC. Molecular size markers are shown on the left of the gel.

We show here that PCR-RFLP is a simple and rapid technique representing an important advance for studies of Brazilian *Biomphalaria* species which can be used as an auxiliary tool to morphological identification. The study demonstrated that ITS contains useful genetic markers for the identification of these snails. The results obtained with PCR-RFLP are concordant with the actual morphological systematics proposed for the Brazilian *Biomphalaria* snails by Paraense (1975, 1981, 1984, 1988).

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