Identification of Planorbids from Venezuela by Polymerase Chain Reaction Amplification and Restriction Fragment Length Polymorphism of Internal Transcriber Spacer of the RNA Ribosomal Gene

Roberta L Caldeira, Teofânia HDA Vidigal*, Liboria Matinella, Andrew JG Simpson***, Omar S Carvalho/+**

Centro de Pesquisas René Rachou-Fiocruz, Av. Augusto de Lima 1715, 30190-002 Belo Horizonte, MG, Brasil *Instituto de Ciências Biológicas, UFMG, Belo Horizonte, MG, Brasil **Laboratório de Malacologia, Ministerio de Sanidad y Asistencia Social, Aragua, Venezuela ***Laboratório de Genética de Câncer, Instituto Ludwig de Pesquisas sobre o Câncer, São Paulo, SP, Brasil

Snails of the genus Biomphalaria *from Venezuela were subjected to morphological assessment as well as polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) analysis. Morphological identification was carried out by comparison of characters of the shell and the male and female reproductive apparatus. The PCR-RFLP involved amplification of the internal spacer region ITS1 and ITS2 of the RNA ribosomal gene and subsequent digestion of this fragment by the restriction enzymes* Dde*I*, Mnl*I*, Hae*III and* Msp*I. The planorbids were compared with snails of the same species and others reported from Venezuela and present in Brazil, Cuba and Mexico. All the enzymes showed a specific profile for each species, that of* Dde*I being the clearest. The snails were identified as* B. glabrata, B. prona *and* B. kuhniana.

Key words: *Biomphalaria glabrata - Biomphalaria kuhniana - Biomphalaria prona* - snails - internal transcribed spacer - ribosomal DNA

The endemic area for schistosomiasis in Venezuela includes the Federal District of Caracas and the states of Aragua, Carabobo, Miranda and Guárico. One third of the country's population lives in this area, where the prevalence of infection with *Schistosoma mansoni* is 2% (Balzán 1992).

To date, seven species of *Biomphalaria* have been reported in Venezuela: *B. glabrata* (Martens 1873), *B. straminea* (Martens 1873), *B. prona* (Martens 1873), *B. kuhniana* (Baker 1930), *B. havanensis* (Hubendick 1961), *B. schrammi* (Chrosciechowski 1968) and *B. obstructa* (Chrosciechowski 1988). The first of these species has been shown to be responsible for the transmission of *Schistosoma* in this country (Balzán 1992).

The specific identification of these planorbids is based on comparison of morphological characters of the shell and male and female reproductive organs, as described by Deslandes (1951), Paraense (1975, 1988, 1990), Paraense and Deslandes (1958) and Paraense et al. (1992). Identification is complicated in many cases by morphological variation in these characters, for example when the *B. prona* population from Lake Valencia (Venezuela), is compared with populations of the same species that occur outside this lake (Paraense et al. 1992).

Moreover, identification is also complicated by morphological similarities among some species of snail, for example the *B. straminea* (Paraense 1988, Caldeira et al. 1998) and *B. tenagophila* complexes (Spatz et al. 1998, 1999).

The polymerase chain reaction amplification and restriction fragment length polymorphism (PCR-RFLP) technique have been used successfully to distinguish several medically important snails of the genera *Oncomelania* (Hope & McManus 1994), *Bulinus* (Stothard et al. 1996, 1997) and *Biomphalaria* (Vidigal et al. 1998a, b, Caldeira et al. 1998, Spatz et al. 1998, 1999). This technique is based on amplification of the internal transcribed spacer region of the rRNA followed by digestion of the resulting fragment with restriction enzymes.

This is an easy technique to perform and gives profiles that are simple to analyse. In the present study snails of the genus *Biomphalaria* obtained from different regions of Venezuela were identified using both morphological and molecular criteria.

Work partially supported by Fapemig, Pibic/Fiocruz and OPS.
+Corresponding author. Fax: + 55-31-295.3115. E-mail: omar@netra.cpqrr.fiocruz.br
Received 25 August 1999
Accepted 20 October 1999

MATERIALS AND METHODS

Snail populations - Snails from different localities of Venezuela were used in the study together with specimens previously identified as *B. obstructa* from Isla del Carmen, Mexico (type locality); *B. havanensis* from Havana, Cuba; *B. glabrata*, *B. straminea* and *B. kuhniana* from Brazil. All these species have been reported from Venezuela (Table). All specimens used were collected in the field, with the exception of *B. obstructa* from Isla del Carmen which had been maintained in the Departament of Malacology of the Instituto Oswaldo Cruz, Rio de Janeiro and *B. havanensis* from Guatao, Cuba reared in the Departament of Malacology of the Instituto Pedro Kouri (Havana). All the specimens obtained from the field were examined for cercariae of *Schistosoma mansoni* and found to be negative. Two individuals from each locality were used for the molecular analysis.

Morphological identification of snail populations - Ten specimens of each population were killed, fixed (Deslandes 1951, Paraense 1976) and the feet removed for subsequent DNA extraction. After fixation, each specimen was identified by comparative morphology of the reproductive organs and shell as described by Deslandes (1951), Paraense and Deslandes (1958), Paraense (1975, 1988, 1990) and Paraense et al. (1992).

DNA extraction - Total DNA was extracted from the feet of the snails using the Wizard Genomic DNA Purification Kit (Promega) with some modifications. Each foot was mechanically disrupted in 200 µl of nucleic lysis solution and incubated at 37°C for 4 h or overnight with 50 µg/ml of proteinase K, then 80 µl of protein precipitation solution was added to the initial mix. The solution was shaken vigorously and centrifuged at 13,000 rpm for 5 min. The supernatant containing DNA was transferred to a clean tube with 200 µl of isopropanol for DNA precipitation. The mixture was gently homogenized by inversion for 20 min and centrifuged at 13,000 rpm for 5 min. The precipitated DNA was washed with 300 µl of 70% ethanol and centrifuged for 10 min. The pellet treated with 25 µl 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.

rDNA-ITS amplification - The entire internal transcriber spacer (ITS) (which includes the 5.8S rDNA gene together with the flanking ITS1 and ITS2 spacers) was amplified using the primers

TABLE

Localities of *Biomphalaria* species from Venezuela used in the study

Species	Locality	State, country
B. glabrata	Villa de Cura	Aragua, Venezuela
	Manuare	Carabobo, Venezuela
	Guacara	Carabobo
	Belén	Carabobo
	Caripe	Monagas, Venezuela
	Chabasquén	Portuguesa, Venezuela
	Anzoátequi	Lara, Venezuela
	Belém	Pará, Brazil
	Esteio	Rio Grande do Sul, Brazil
B. kuhniana	San Casimiro	Aragua, Venezuela
	Villa de Cura	Aragua
	Pao de Zárate	Aragua
	Villa de Cura	Aragua
	Laguna del Parque Recreacional	Carabobo, Venezuela
	Tucuruí	Pará, Brazil
B. prona	San Casimiro	Aragua, Venezuela
	Lake de Valencia	Aragua
	Laguna del Parque Recreacional	Carabobo, Venezuela
	Canal La Pista, Tinaquillo	Cojedes, Venezuela
	San Carlos	Cojedes
	Represa El Guamo	Monagas, Venezuela
B. straminea	Belém	Pará, Brazil
	Picos	Piauí, Brazil
	Florianópolis	Santa Catarina, Brazil
B. havanensis	Guatao	Cuba
B. obstructa	Isla del Carmem	Mexico

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). PCR amplification was undertaken in a volume of 10 µl consisting of: 1-10 ng of template DNA, 10 mM Tris-HCl, pH 8.5, 200 µM each dNTP, 1.5 mM $MgCl_2$, 0.8 U of Taq DNA polymerase (Cenbiot, RS, Brazil), 50 mM KCl, together with 1 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 5 min at 95°C, and then 32 cycles with: annealing of 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 visualized on 0.8% ethidium bromide stained agarose gels to check the quality of amplification. The remaining 7 µl were mixed with 43 µl of water and divided into 10 µl samples for enzyme digestion.

RFLP analysis - To evaluate possible enzymes that might yield informative RFLPs of the ITS region, four restriction enzymes (Amersham Life Science) were used: the four base cutters *Hae*III and *Msp*I, the five base cutter *Dde*I and the 11 base cutter *Mnl*I. One microliter (10-12 units) of each enzyme was used for each digestion, together with 1.2 µl of the respective enzyme buffer and 10 µl of the diluted amplification product in a final volume of 12.2 µl. The digestion was performed for 3.5 h at 37°C and products were evaluated on 6% silver stained polyacrylamide gels (Santos et al. 1993, Sanguinetti et al. 1994) after phenol/chloroform extraction to remove protein. The results were recorded with Polaroid film 667 (St Albans, UK). A control for the activity of each enzyme was performed by digesting 150 ng of pUC18 simultaneously with the samples being evaluated.

RESULTS

Morphological identification of snail populations - The results of morphological identifications of the snails examined are shown in the Table.

RFLP analysis - DNA amplification with the primers ETTS1 and ETTS2 generated a fragment of approximately 1.300 bp for all the specimens. Fig. 1 shows the profiles obtained with *Dde*I enzyme which grouped together: the *B. glabrata* snails from Brazil and Venezuela (lanes 1 to 16), *B. prona* populations from the Lake Valencia and from outside this lake (lanes 17 to 28), *B. straminea* from Brazil (lanes 29 to 31) and *B. kuhniana* from Venezuela and Brazil (lanes 32 to 43).

Fig. 2A, B and C shows the profiles obtained with *Hae*III, *Msp*I and *Mnl*I respectively. The pro-

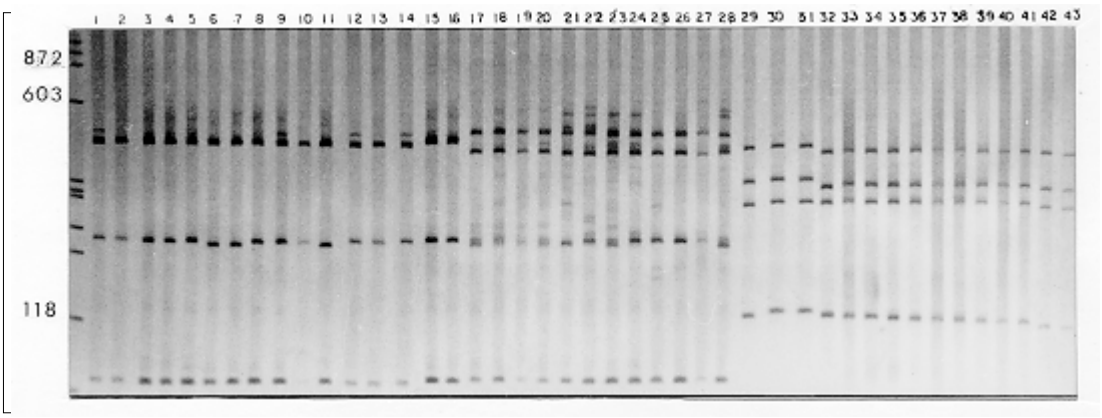

Fig. 1: silver-stained polyacrylamide gels (6%) showing restriction fragment length polymorphism profiles obtained following digestion of the rDNA internal transcriber spacer (ITS) with *Dde*I enzyme. The snail specimens were *Biomphalaria glabrata:* lane 1 Belém (State of Pará, Brazil); 2 Esteio (State of Rio Grande do Sul, Brazil); 3-4 Villa de Cura, El Cortijo (State of Aragua, Venezuela); 5-6 Manuare (State of Carabobo, Venezuela); 7-8 Belén, Las Tinajitas (State of Carabobo, Venezuela); 9-10 Guacara (State of Carabobo, Venezuela); 11-12 Caripe, La Elvira Quebrada (State of Monagas, Venezuela); 13-14 Chabasquén (State of Portuguesa, Venezuela); 15-16 Anzoátequi, Valle Lindo la Rinconada (State of Lara, Venezuela). *B. prona*: lanes 17- 18 Lake de Valencia, Pan de Azúcar, Club Bahia Paraíso (State of Aragua, Venezuela); 19-20 San Casimiro, El Loro Rio (State of Aragua, Venezuela); 21-22 Laguna del Parque Recreacional Sur de Valencia (State of Carabobo, Venezuela); 23-24 Canal La Pista, Tinaquillo (State of Cojedes, Venezuela); 25-26 Represa El Guamo (State of Monagas, Venezuela); 27-28 San Carlos (State of Cojedes, Venezuela). *B. straminea*: lane 29 Belém (State of Pará, Brazil); 30 Picos (State of Piauí, Brazil); 31 Florianópolis (State of Santa Catarina, Brazil). *B. kuhniana*: lanes 32-33 San Casimiro, La Barquera Quebrata 2 (State of Aragua, Venezuela); 34-35 Villa de Cura, El Cortijo (State of Aragua, Venezuela); 36-37 Pao de Zárate (State of Aragua, Venezuela); 38-39 Laguna del Parque Recreacional, Sur de Valencia (State of Carabobo, Venezuela); 40-41 Villa de Cura (State of Aragua, Venezuela); 42-43 Tucuruí (State of Pará, Brazil).

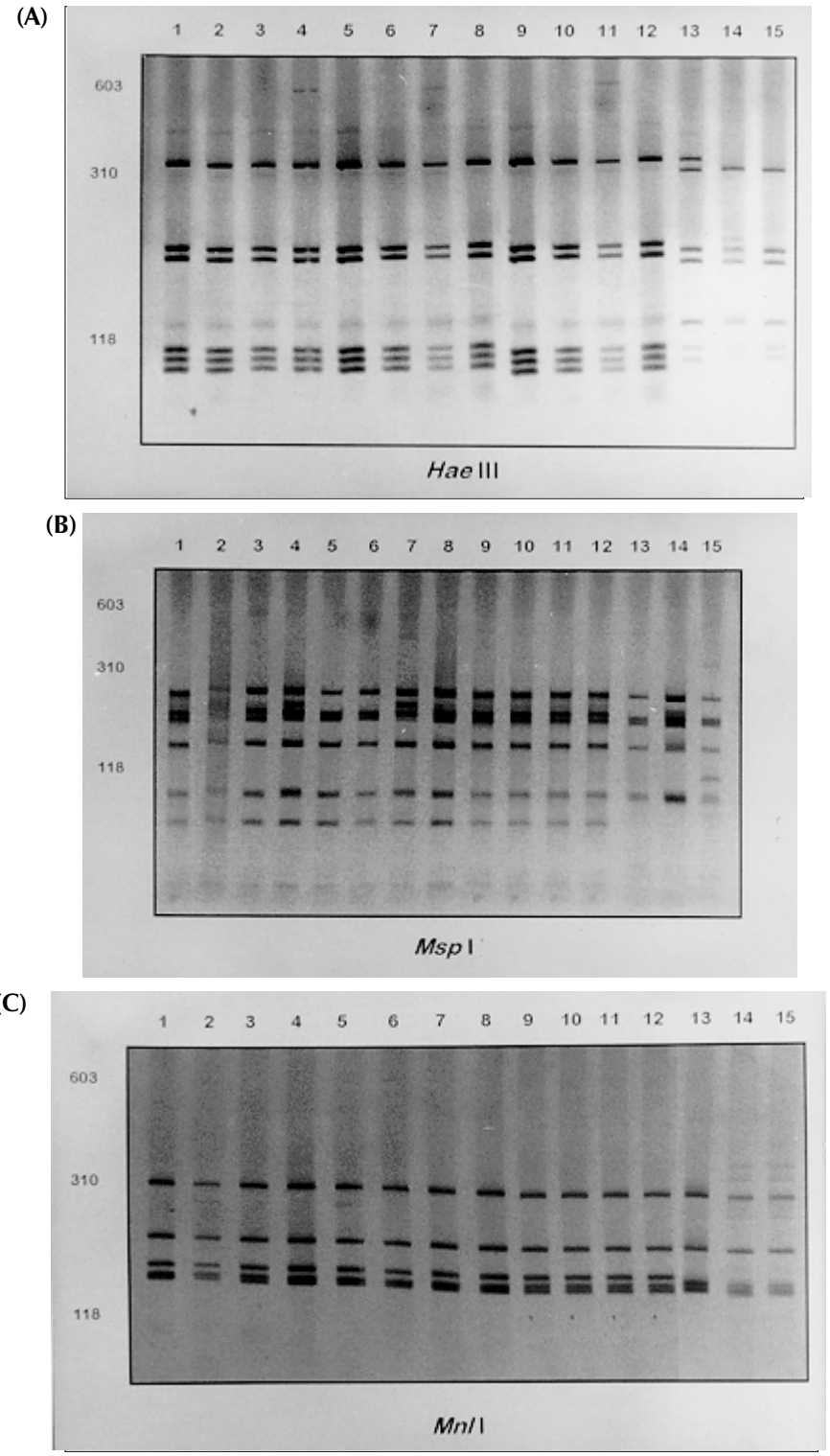

Fig. 2: silver-stained polycrylamide gels (6%) showing the restriction fragment length polymorphism profiles obtained by digesting the rDNA internal trancriber spacer (ITS) with *Hae*III (A), *Msp*I (B) and *Mnl*I (C) enzymes. In each gel the snails specimens were: *Biomphalaria kuhniana* lanes 1-2 San Casimiro, La Barquera Quebrada 2 (State of Aragua, Venezuela); 3-4 Villa de Cura, El Cortijo (State of Aragua, Venezuela); 5-6 Pao de Zárate (State of Aragua, Venezuela); 7-8 Laguna del Parque Recreacional, Sur de Valencia (State of Carabobo, Venezuela); 9-10 Villa de Cura (State of Aragua, Venezuela); 11-12 Tucuruí (State of Pará, Brazil). *B. straminea* lane 13 Belém (State of Pará, Brazil); 14 Picos (State of Piauí, Brazil); 15 Florianópolis (State of Santa Catarina, Brazil).

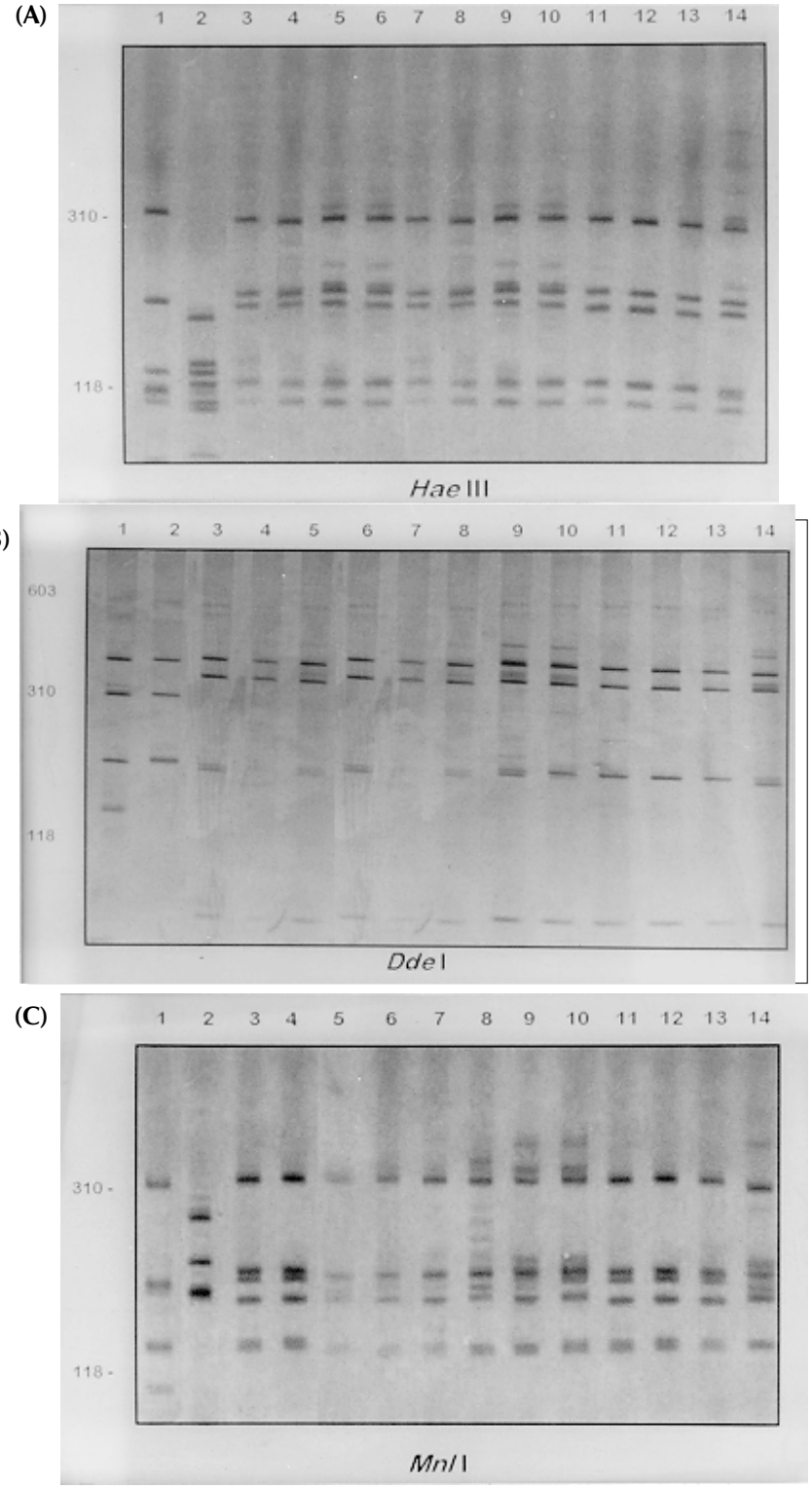

Fig. 3: silver-stained polycrylamide gels (6%) showing the restriction fragment length polymorphism profiles obtained by digesting of the rDNA internal trancriber spacer (ITS) with *Hae*III (A), *Dde*I (B) and *Mnl*I (C) enzymes. In each gel the snails specimens were: *Biomphalaria havanensis:* lane 1 Guatao (Cuba). *B. obstructa:* lane 2 Isla del Carmem (Mexico). *B. prona:* lanes 3-4 Lake de Valencia, Pan de Azúcar, Club Bahia Paraíso (State of Aragua Venezuela); 5-6 San Casimiro, El Loro Rio (State of Aragua, Venezuela); 7-8 Laguna del Parque Recreacional, Sur de Valencia (State of Carabobo, Venezuela); 9-10 Canal La Pista, Tinaquillo (State of Cojedes, Venezuela); 11-12 Represa El Guamo (State of Monagas, Venezuela); 13-14 San Carlos (State of Cojedes, Venezuela).

files obtained with theses enzymes grouped together the *B. kuhniana* from Venezuela and Brazil (lanes 1 to 12) and separated *B. straminea* from Brazil (lanes 13 to 15). Theses results confirmed the morphological identification and the molecular data showed above with *Dde*I enzyme.

Fig. 3A, B and C shows profiles obtained with *Hae*III, *Dde*I and *Mnl*I, respectively, which demonstrated that theses enzymes permit the separation of *B. havanensis* (Cuba), *B. obstructa* (Mexico) of the *B. prona* populations from Venezuela.

DISCUSSION

The snails *B. kuhniana* and *B. straminea* present tenuous morphological differences, making the distinction of these two species the subject of diverse taxonomic problems. Paraense (1988) currently groups them with *B. intermedia* in the *B. straminea* complex. Caldeira et al. (1998) were able to separate these two species with the PCR-RFLP technique, with a small genetic distance (0.58) being observed between them in the several populations studied. In the present study, populations from Venezuela previously identified as *B. straminea* were identified as *B. kuhniana* based on their morphology and later confirmed by molecular analysis. The data obtained by PCR-RFLP using the enzymes *Dde*I, *Hae*III, *Mnl*I and *Msp*I confirmed the morphological findings. In fact, all the populations exhibited similar profiles to those of *B. kuhniana* from Tucuruí (Brazil) and were distinct from those of *B. straminea* from different regions of Brazil.

The only reference in the literature to the presence of *B. kuhniana* in Venezuela was made by Baker in 1930. This record is noteworthy from an epidemiological viewpoint since *B. kuhniana* is considered to be refractory to *S. mansoni* (Floch & Fauran 1954) while *B. straminea* is an intermediate host of this trematode.

Paraense and Deslandes (1958), found *B. prona* (Martens 1873) among material originating from Lake Valencia (Venezuela). Despite the fact that Paraense et al. (1992) later found specimens with morphology and biotypes apparently distinct from the Lake Valencia population in other areas, subsequent morphological comparisons and isoenzyme studies enabled all these snail populations to be identified as *B. prona*. Although the enzymes used in our PCR-RFLP analysis generated similar profiles for the populations and provide confirmation of this grouping it appears that this species possesses great phenotypic plasticity.

The enzymes *Hae*III, *Msp*I and *Mnl*I presented complexes profiles, however they were distinct for each species.

From the technical point of view PCR-RFLP proved to be effective in the specific identification of these snails, facilitating distinction between *Biomphalaria* species.

ACKNOWLEDGMENTS

To Dr W Lobato Paraense, Departament of Malacology, Instituto Oswaldo Cruz, Rio de Janeiro, for help in the identification of *B. kuhniana* and for providing *B. obstructa*. To Dr Gloria Perera Puga, Departament of Malacology of the Pedro Kori Institute, Havana, Cuba, for providing *B. havanensis*

REFERENCES

Baker HB 1930. The mollusca collected by the University of Michigan Williamson Expedition in Venezuela. *Occ Pap Mus Zool Univ Mich 210*: 1-94.

Balzán CB 1992. Un nuevo enfoque en la lucha contra la esquistosomiasis, XXXIX Asamblea anual ordinaria y Jornadas científicas Maracay, Aragua.

Caldeira RL, Vidigal THDA, Paulinelli ST, Simpson AJG, Carvalho OS 1998. Molecular identification of similar species of the genus *Biomphalaria* (Mollusca: Planorbidae) determined by a PCR-RFLP. *Mem Inst Oswaldo Cruz 93:* 219-225.

Chrosciechowski P 1968. Conocimiento actual de los caracoles de la familia planorbidae (Mollusca, Gastropoda, Pulmonata) de Venezuela. *Bol Inform Direc Malariol Saneam Ambiental VIII:* 3-10.

Chrosciechowski P 1988. *Hospedador Intermediario,* Venezuela, mimiog, 33 pp.

Deslandes N 1951. Técnica de dissecação e exame de planorbídeos. *Rev Serv Esp Saúde Pub 4*: 371-382.

Floch H, Fauran P 1954. Essais infructueux d'infection expérimentale de *Tropicorbis kuhnianus* (Clessin) par *Schistosoma mansoni*. *Bull Soc Pathol Exot 47*: 452-459.

Hope M, McManus DP 1994. Genetic variations in geographically isolated populations and subspecies of *Oncomelania hupensis* determined by a PCR-based RFLP method. *Acta Trop 57:* 75-82.

Hubendick B 1961. Studies on Venezuelan Planorbidae. *Meddel Goteborgs Mus Zool Avdeln 132:* 17-23.

Kane RA, Rollison D 1994. Repetitive sequences in the ribosomal DNA internal transcribed spacer of *Schistosoma haematobium, Schistosoma intercalatum* and *Schistosoma mattheii*. *Mol Biol Parasit 63*: 153-156.

Martens E 1873. Die Binnenmollusken Venezuela's, p. 157-225. In Festschrift zur Feier des hundertjährigen Bestehens der Gesellschaft Naturforschender Freunde zu Berlin. Ferd. Dummlers Verlagsbuchhandlung, Harrwitz und Gossmann, Berlin, apud Hubendick B 1961. Studies on Venezuelan Planorbidae. *Meddel Goteborgs Mus Zool Avdeln 132*: 17-23.

Paraense WL 1975. Estado atual da sistemática dos planorbídeos brasileiros. *Arq Mus Nac 55*: 105-128.

Paraense WL 1976. A natural population of *Helisoma duryi* in Brazil. *Malacology 15*: 360-376.

Paraense WL 1988. *Biomphalaria kuhniana* (Clessin, 1883), planorbid mollusc from South America. *Mem Inst Oswaldo Cruz 83*: 1-12.

Paraense WL 1990. *Biomphalaria obstructa* (Morelet, 1849): a study of topotypic specimens (Mollusca: Pulmonta: Planrobidae). *Mem Inst Oswaldo Cruz 85*: 391-399.

Paraense WL, Deslandes N 1958. Observations on "*Taphius havanensis*" (Pulmonata, Planorbidae). *Rev Bras Biol 18*: 87-91.

Paraense WL, Pointier JP, Delay B, Pernot AF, Incani RN, Balzan C, Chrosciechowski P 1992. *Biomphalaria prona* (Gastropoda: Planorbidae): a morphological and biochemical study. *Mem Inst Oswaldo Cruz 87*: 171-179.

Sanguinetti CJ, Neto ED, Simpson AJG 1994. Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. *Biotechniques 17*: 915-918.

Santos FR, Pena SDJ, Epplen TJ 1993. Genetic populational study of a Y-linked tetranucleotide repeat DNA polymorphism. *Human Genetics 90*: 655-656.

Spatz L, Vidigal THDA, Caldeira RL, Dias Neto E, Cappa SMG, Carvalho OS 1998. Molecular study of similar *Biomphalaria* species. *Mem Inst Oswaldo Cruz 93:* 169-170.

Spatz L, Vidigal THDA, Caldeira RL, Dias Neto E, Cappa SMG, Carvalho OS 1999. Study of *Biomphalaria tenagophila, B. t. guaibensis* and *B. occidentalis* by polymerase chain reaction amplification and restriction enzyme digestion of the ribosomal RNA gene intergenic spacer. *J Moll Stud 65*: 143-149.

Stothard JR, Hughes S, Rollinson D 1996. Variation within the internal transcribed spacer (ITS) of ribossomal DNA genes of intermediate snail hosts within the genus *Bulinus* (Gastropoda: Planorbidae). *Acta Trop 61:*19-29.

Stothard JR, Rollinson D 1997. Molecular characterization of *Bulinus globosus* and *B. nasutus* on Zanzibar, and an investigation of their roles in the epidemiology of *Schistosoma haematobium. Trans R Soc Trop Med Hyg 91*: 353-357.

Vidigal THDA, Dias Neto E, Spatz L, Nunes ND, Pires RE, Simpson AJG, Carvalho OS 1998a. Genetic variability and identification of the intermediate snail hosts of *Schistosoma mansoni. Mem Inst Oswaldo Cruz 93*: 103-110.

Vidigal THDA, Spatz L, Nunes DN, Simpson AJG, Carvalho OS, Neto E 1998b. *Biomphalaria* spp: identification of the intermediate snail hosts of *Schistosoma mansoni* by polymerase chain reaction simplification and restriction enzyme digestion of the ribossomal RNA gene intergenic spacer. *Exp Parasitol 89*: 180-187.