Molecular Identification of Similar Species of the Genus *Biomphalaria* (Mollusca: Planorbidae) Determined by a Polymerase Chain Reaction-Restriction Fragment Length Polymorphism

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The freshwater snails Biomphalaria straminea, B. intermedia, B. kuhniana *and* B. peregrina, *are morphologically similar; based on this similarity the first three species were therefore grouped in the complex* B. straminea*. The morphological identification of these species is based on characters such as vaginal wrinkling, relation between prepuce: penial sheath:deferens vas and number of muscle layers in the penis wall. In this study the polymerase chain reaction restriction fragment length polymorphism technique was used for molecular identification of these molluscs. This technique is based on the amplification of the internal transcribed spacer regions ITS1 e ITS2 of the ribosomal RNA gene and subsequent digestion of these fragments by restriction enzymes. Six enzymes were tested:* Dde *I,* Mnl *I,* Hae *III,* Rsa *I,* Hpa *II e* Alu *I. The restriction patterns obtained with* Dde*I presented the best profile for separation of the four species of* Biomphalaria*. The profiles obtained with all the enzymes were used to estimate the genetic distances among the species through analysis of common banding patterns.*

Key words: *Biomphalaria straminea - Biomphalaria kuhniana - Biomphalaria intermedia - Biomphalaria peregrina* - snails - polymerase chain reaction - restriction fragment length polymorphism

Schistosomiasis mansoni is endemic in several countries of the Americas and Africa (WHO 1993). It is caused by *Schistosoma mansoni* (Sambon 1907), the intermediate hosts of which are freshwater snails of the genus *Biomphalaria*. Ten species and one subspecies of this genus are known in Brazil (Paraense 1975, 1981, 1984, 1988), of which only *B. glabrata, B. tenagophila* e *B. straminea* have been found with natural infections. Two other species (*B. amazonica* and *B. peregrina),* can also be infected experimentally and are considered to be potential hosts of the trematode (Corrêa & Paraense 1971, Paraense 1973).

The specific identification of *B. straminea, B. kuhniana, B. intermedia* and *B. peregrina* is based on very similar morphologically characters. Because of this similarity, Paraense (1988) grouped the first three species in a complex named *B. straminea*. Hofman (1987) and Pointier et al. (1993) used isoenzymatic techniques for the differentiation of some of these species. However Steindel et al. (1994) pointed out that this technique had certain limitations, such as the requirement for large quantities of biological material, and the small number of loci examined consequently providing relatively little information at the intraspecific level.

Knight et al. (1991), used RFLP (restriction fragment length polymorphism) in the molecular differentiation of species of the genus *Biomphalaria,* while Vidigal et al. (1996) and Pires et al. (1997) utilized LS-PCR (low stringency polymerase chain reaction) for identification of species of this genus. Langand et al. (1993) and Vidigal et al. (1994) used AP-PCR (arbitrarily primed- polymerase chain reaction) to study genetic variability of *Bulinus* and *Biomphalaria* respectively.

Hope and McManus (1994) analyzed restriction polymorphisms in the internal transcribed spacer region of ribosomal RNA gene generated through the polymerase chain reaction (PCR-RFLP), to distinguish subespecies of *Oncomelania hupensis.* This technique has also been used for the differentiation of *Bulinus* (Stothard et al.

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1996, Stothard & Rollison 1997) and *Biomphalaria* (Vidigal et al. 1998).

We here describe the identification of *B. straminea*, *B. kuhniana*, *B. intermedia* and *B. peregrina* using classical morphological identification as well as the PCR-RFLP technique in order to differentiate molecularly these species and to estimate the genetic distances among them.

MATERIALS AND METHODS

Snails populations - The snail species used are listed in Fig. 1, together with their distribution in Brazil (Paraense 1986, 1988, Carvalho et al. 1994, 1997). Populations of snails were collected in the field, except *B. straminea* from Paraná, Bahia and Piauí specimens which were obtained from the Departamento de Malacologia of Instituto Oswaldo Cruz, Rio de Janeiro. All field-collected specimens were examined for *S. mansoni* and found to be negative. The snails were killed, fixed (Deslandes 1951, Paraense 1976) and the foot of each specimen removed for subsequent DNA extraction.

Selection of the restriction enzymes was done using three specimens from each of the following species and localities: *B. kuhniana* from Tucuruí (PA); *B. straminea* from Belém (PA), Monte Carmelo (MG) and Guaíra (PR); *B. intermedia* from Jales (SP), Paulo Farias (SP) and Planura (MG); and *B. peregrina* from Taim (RS), Guimarania (MG) and Alfenas (MG).

DNA extraction - Total DNA was extracted from the foot of each snail as described by Vidigal et al. (1994) and Steiner et al. (1995) with some modifications. The foot of each snail was lysed mechanically with 200 ml of 50 mM Tris-HCl at pH 8.0, 100 mM NaCl, 50 mM EDTA and 0.5% SDS then incubated overnight at 37°C with 50mg/ml proteinase K. Following incubation, 200 ml of a buffer solution containing 10 mM Tris-HCl and 2.5 mM EDTA at pH 8.0 and 1% polyvinyl-polypyrrolidone (PVPP, insoluble in water) was added and incubated for 20 min at 95°C. This solution was subsequently placed in ice for 5 min, then centrifuged at 14,000 rpm for 10 min. After centrifugation the DNA was transferred to tubes of 1.5 ml and precipitated by the addidtion of 0.1 vol. Na acetate (3 M, pH 7) and 3 vol. absolute ethanol. The precipitated DNA was washed in 70% ethanol, resuspended in 20 ml water and cooled to -20°C.

Fig. 1: geographical distribution of four *Biomphalaria* species. (●) *Biomphalaria straminea;* (■) *B. peregrina;* (▲) *B. intermedia;* (★) *B. kuhniana.* Solid symbols represent field-collected snails and open symbols represent snails from laboratory.

The rDNA-ITS amplification - The entire ITS (which includes the 5.8S rDNA gene together with the flanking ITS1 and ITS2 spacers) was amplified using the primers ETTS2 (5/-TAACAAGG TTTCCGTAGGTGAA-3/) and ETTS1 (5/-TGC TTAAGTTCAGCGGGT-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 µl consisting of: 1-10 ng of template DNA, 10mM 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 1.0 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 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 µl 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 93 µl of water and divided into 10 µl aliquots for subsequent enzyme digestion.

RFLP analysis - To evaluate possible enzymes that might yield informative RFLP of the ITS regions, six restriction enzymes (Amersham Life Science) were used: four base cutters *Alu*I, *Hae*III, *Hpa*II, *Rsa*I, five base cutters *Dde*I and 11 base cutters *Mnl*I. One µl of each enzyme (10-12 units) was used for each digestion reaction, 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 hr at 37°C, and products were separated 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 (Sigma Albans, UK). A control for the activity of each enzyme was performed by digesting 150 ng of pUC18 simultaneously with the samples being evaluated.

Quantitative analysis of restriction profiles - The bands observed on each lane were compared with all the other lanes of the same gel. A matrix of taxon/character was constructed based on the presence/absence of each band. The most easily distinguishable bands were considered for analysis. The data obtained were analysed with TREECON for Windows (Version 1.2 - Van de Peer & De Wachter 1994) and NTSYS-PC (Version 1.6 - Rohlf 1990). The genetic distance was calculated using the coefficient of Nei and Li (1979). These data were clustered with Neighbor-joining - NJ (Saitou & Nei 1987, Studier & Keppler 1988) and used for the construction of tree of the genetic distance. The reliability of the NJ trees is assessed by the bootstrap method (Felsenstein 1985) with 1,000 pseudoreplications. The percentage of shared bands was calculated using the similarity coefficient of Dice (Dice 1945). These data were clustered with UPGMA, unweighted pair group method analysis (Sneath & Sokal 1962) and used for the construction of tree of the similarity. The average similarity among all the individuals in the group was calculated and marked on the tree as the phenon line. Divergence below the phenon line indicates separation of distinct groups. The comparison was made among individual snails of the same species from different localities and among snails from different species.

RESULTS

RFLP analysis - DNA amplification with the ETTS1 and ETTS2 primers generated a fragment of approximately 1.300 pb. Among the enzymes used, *Dde* I presented the best profile for the separation of the snail species (Fig. 2). The enzyme *Rsa* I presented the same profile for *B. straminea* and *B. intermedia* (data not shown), while the remaining enzymes presented a polymorphic profile for *B. peregrina* (Fig. 3).

In order to confirm the reproducibility of the profiles obtained for D*de* I two samples were used from each locality, as well as additional specimens from the following species and localities: *B. straminea* from Picos (PI), Varzea do Porto (BA) and Icém (SP) and *B. intermedia* from Tupaciguara (MG) (data not shown).

Quantitative analysis of restriction profiles - The trees were constructed using 79 bands produced by six enzymes, reflecting the genetic distance (Fig. 5a) and similarity (Fig. 5b) among the 12 examples of the four snail species. The genetic distance matrix is shown in the Fig. 4. The bootstrap methods showed that these clades are confirmed with high statistical reliability. Intraespecific distance was observed only among specimens of *B. peregrina.* The degree of intraspecific similarity was 100% for *B. kuhniana*, *B. intermedia*, *B. straminea* and 70% for *B. peregrina*. The mean percentage of bands shared among all the possible pairs was 44%, represented in the dendrogram by the dotted dividing line.

DISCUSSION

The separation of *B. straminea*, *B. kuhniana*, *B. intermedia* and *B. peregrina* (Paraense 1975, 1988) is based on subtle morphological differences in characters such as the degree of wrinkling of the vaginal wall (absent in *B. peregrina*, intermediate in *B. kuhniana* and *B. intermedia* and con-

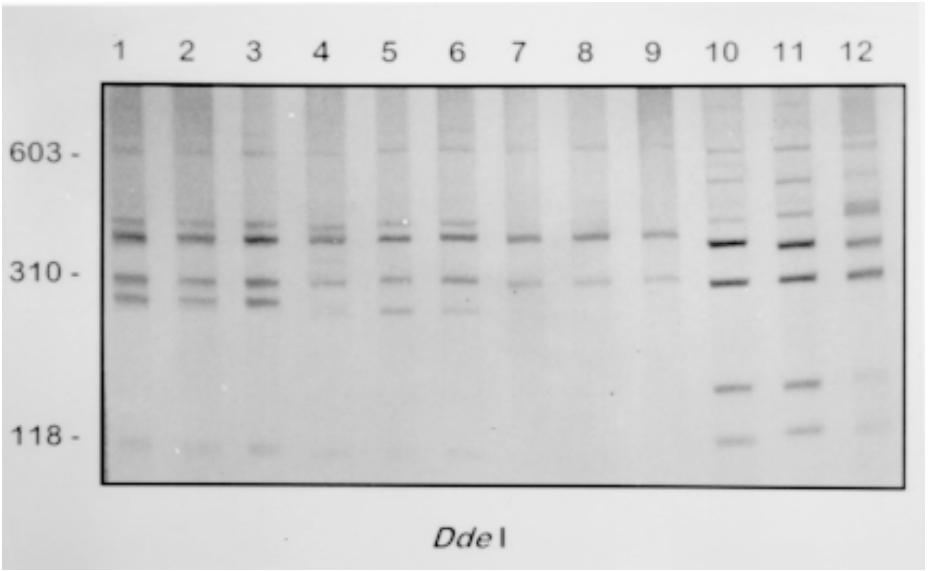

Fig. 2: 6% silver stained polyacrylamide gels showing the RFLPs profiles obtained following the digestion of the rRNA ITS with *Dde*I. Snail specimens are: lane 1, 2 and 3 *Biomphalaria kuhniana* from Tucuruí (PA); lane 4, *B. straminea* from Belém (PA); lane 5, *B. straminea* from Monte Carmelo (MG); lane 6, *B. straminea* from Guaíra (PR); lane 7, *B. intermedia* from Jales (SP); lane 8, *B. intermedia* from Paulo Farias (SP); lane 9, *B. intermedia* from Planura (MG); lane 10, *B. peregrina* from Taim (RS); lane 11, *B. peregrina* from Guimarania (MG); lane 12, *B. peregrina* from Alfenas (MG). Molecular size markers are shown in the figure.

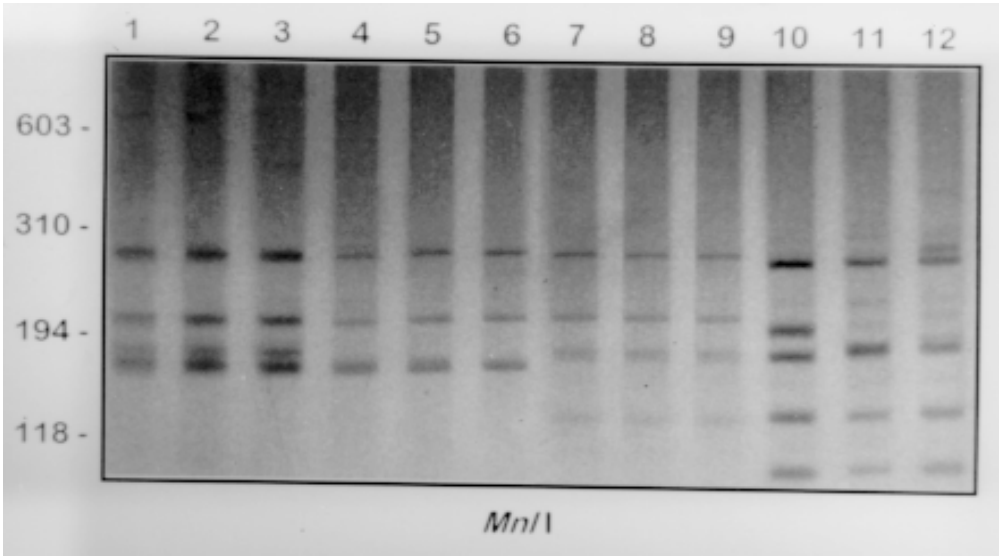

Fig. 3: 6 % silver stained polyacrylamide gels showing the RFLPs profiles obtained following the digesting of the rRNA ITS with *Mnl* I. Snail specimens are: lane 1, 2 and 3 *B. kuhniana* from Tucuruí (PA); lane 4, *B. straminea* from Belém (PA); lane 5, *B. straminea* from Monte Carmelo (MG); lane 6, *B. straminea* from Guaíra (PR); lane 7, *B. intermedia* from Jales (SP); lane 8, *B. intermedia* from Paulo Farias (SP); lane 9, *B. intermedia* from Planura (MG); lane 10, *B. peregrina* from Taim (RS); lane 11, *B. peregrina* from Guimarania (MG); lane 12, *B. peregrina* from Alfenas (MG). Molecular size markers are shown in the figure.

spicuous in *B. straminea)* and the diameter of the median portion of the penis sheath (in *B. peregrina* this is greater than the diameter of the vas deferens, while in the other species the diameters are equal). In addition, *B. straminea* has three muscle layers in the wall of the penis (longitudinal, oblique and circular), differing from *B. kuhniana*, *B. intermedia* and *B. peregrina* that have only two (longitudinal and circular) (Paraense 1975, 1988).

The separation of these species is further com-

	B.k.1	B.k.2	B.k.3	B.s.1	B.s.2	B.s.3	B.i.1	B.i.2	B.i.3	B.p.1	B.p.2	B.p.3
B.k.1	0.00	0.00	0.00	0.58	0.58	0.58	0.80	0.80	0.80	0.96	0.97	0.99
B.k.2	0.00	0.00	0.00	0.58	0.58	0.58	0.80	0.80	0.80	0.96	0.97	0.99
B.k.3	0.00	0.00	0.00	0.58	0.58	0.58	0.80	0.80	0.80	0.96	0.97	0.99
B.s.1				0.00	0.00	0.00	0.82	0.82	0.82	0.98	0.99	1.0
B.s.2				0.00	0.00	0.00	0.82	0.82	0.82	0.98	0.99	1.0
B.s.3				0.00	0.00	0.00	0.82	0.82	0.82	0.98	0.99	1.0
B.i.1							0.00	0.00	0.00	0.90	0.91	0.93
B.i.2							0.00	0.00	0.00	0.90	0.91	0.93
B.i.3							0.00	0.00	0.00	0.90	0.91	0.93
B.p.1										0.00	0.68	0.64
B.p.2											0.00	0.59
B.p.3												0.00

Fig. 4: genetic distance matrix. The letters refer to species and the localities from which the snails originate. Abreviations used in the figure: B.k.1, B.k.2 and B.k.3 = *Biomphalaria kuhniana* from Tucuruí (PA); B.s.1 = *B. straminea* from Guaíra (PR); B.s.2 = *B. straminea* from Monte Carmelo (MG); B.s.3 = *B. straminea* from Belém (PA); B.i.1 = *B. intermedia* from Planura (MG); B.i.2 = *B. intermedia* from Paulo Farias (SP); B.i.3 = *B. intermedia* from Jales (SP); B.p.1 = *B. peregrina* from Alfenas (MG); B.p. 2 = *B. peregrina* from Guimarania (MG); B.p. 3 = *B. peregrina* from Taim (RS).

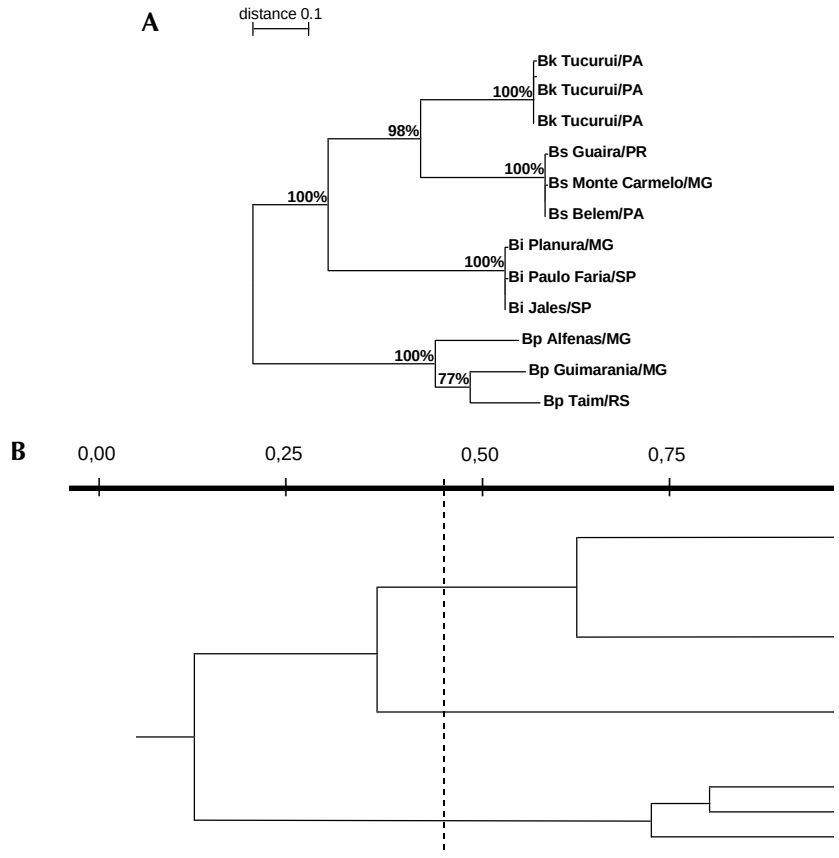

Fig. 5a: neighbor-joining tree unrooted of *Biomphalaria kuhniana*, *B. straminea*, *B. intermedia and B. peregrina* constructed using the PCR-RFLPs profiles produced with all enzymes used. The numbers are bootstrap percent values based on 1,000 pseudoreplications. Fig. 5b: UPGMA tree of *B. kuhniana*, *B. straminea*, *B. intermedia and B. peregrina* constructed using the PCR-RFLPs profiles produced with all enzymes used. The numbers shown on top are indices of similarity. The vertical line is the phenon line, which shows the average level of similarity between all of the pair samples analyzed.

plicated by the high genetic variability observed in the genus *Biomphalaria* and by the small size of the specimens, making dissection and perception of morphological differences difficult (Paraense 1975, Vidigal et al. 1994).

Pointier et al. (1993), using isoenzyme technique observed that *B. kuhniana* presented slight genetic differences in relation to *B. straminea*, concluding that these differences could be attributed to geographical isolation or due to the fact that *B. straminea* was a complex of species rather than a single taxonomic entity. In this study, a similarity of 64% and a smaller genetic distance (0.58) between the two species was found supporting the results of the previous authors.

To confirm our results, two cluster analysis methods were used, the UPGMA which assume that all lineages (OTU's) have diverged equal amounts and the NJ wich is conceptually related to traditional cluster analysis, but removes the assumption that all lineages have diverged equal amounts (Swofford et al. 1996).

The trees produced by these methods showed the same topology with the formation of three distinct groups: the first clustered *B. straminea* and *B. kuhniana* species, the second consisted by only specimens of *B. intermedia* and the third only by specimens of *B. peregrina*. However, *B. intermedia* is more related to *B. straminea* and *B. kuhniana* than to *B. peregrina*. This molecular results reinforces data of Paraense (1988) which grouped *B. straminea B. kuhniana* and *B. intermedia* in *B. straminea* complex.

The intraspecific genetic distance seen in *B. peregrina* may be attributed to its dispersion in the world, deriving its scientific name "from the latin *peregrinus*, meaning wanderer, in reference to its wide geographical distribution " (Paraense 1975). The sequences of ITS regions will be analyzed in the future to better understand the intraspecific polymorphism of this species.

The PCR-RFLP technique, using the ITS region fragment restricted by the *Dde* I enzyme, will be an auxiliar tool for the identification of morphologically similar species such as the members of these studies.

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