Genetic Variability in Brazilian Populations of *Biomphalaria straminea* Complex Detected by Simple Sequence Repeat Anchored Polymerase Chain Reaction Amplification

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Biomphalaria glabrata, B. tenagophila *and* B. straminea *are intermediate hosts of* Schistosoma mansoni, *in Brazil. The latter is of epidemiological importance in the northwest of Brazil and, due to morphological similarities, has been grouped with* B. intermedia *and* B. kuhniana *in a complex named* B. straminea. *In the current work, we have standardized the simple sequence repeat anchored polymerase chain reaction (SSR-PCR) technique, using the primers* $(CA)_8RY$ *and K7, to study the genetic variability of these species. The similarity level was calculated using the Dice coefficient and genetic distance using the Nei and Li coefficient. The trees were obtained by the UPGMA and neighbor-joining methods. We have observed that the most related individuals belong to the same species and locality and that individuals from different localities, but of the same species, present clear heterogeneity. The trees generated using both methods showed similar topologies. The SSR-PCR technique was shown to be very efficient in intrapopulational and intraspecific studies of the* B. straminea *complex snails.*

Key words*:* snails - Planorbidae - *Biomphalaria straminea - Biomphalaria kuhniana - Biomphalaria intermedia* - SSR anchored PCR amplification - genetic variability

The genus *Biomphalaria* (Preston, 1910) includes some species that transmit *Schistosoma mansoni* in Brazil: *Biomphalaria glabrata* (Say, 1818), *B. tenagophila* (Orbigny, 1835) and *B. straminea* (Dunker, 1848), while two other species, *B. amazonica* Paraense 1966 and *B. peregrina* (Orbigny, 1835), are considered to be potential hosts of the parasite (Corrêa & Paraense 1971, Paraense & Corrêa 1973).

Paraense and Corrêa (1989) reported that despite the low efficiency of *B. straminea* as a host (less than 1% of these snails are found naturally infected, and experimental infection rates are less than 4%), this species is an important schistosomiasis vector in the northwest of Brazil. Indeed, this species has a prevalence of parasitism over 50%, in some localities, in Pernambuco. This species is found in almost all hydrographic basins of Brazil, and the open spaces on distribution maps are due to the lack of research in these regions (Paraense 1986). Thus, the wide distribution (Paraense 1972, 1983, 1986) increases the risks of expansion of schistosomiasis to areas currently free of the disease.

B. straminea shares many morphological similarities with *B. intermedia* and *B. kuhniana*, frequently causing taxonomic confusion. *B. intermedia* is found in Brazil in the states of São Paulo, Minas Gerais and Mato Grosso do Sul while *B. kuhniana* is restrict to the Tucuruí region of Pará. Because of their morphological similarities, these three species were grouped by Paraense (1988) in the *B. straminea* complex. This species complex had been studied by Caldeira et al. (1998) using polymerase chain reaction amplification and restriction fragment length polymorphism (PCR-RFLP). These authors reported great genetic similarity among the three species, supporting Paraense's observations (1988).

The availability of methodologies based on molecular analysis has enabled the access to more consistent information on the population structure of the Planorbidae of the genus *Biomphalaria*. Studies on genetic variability have permitted correlation of the origins, colonization processes and dispersion of the populations and species of *Biomphalaria* genus (Woodruff et al. 1985, Mulvey et al. 1988, Woodruff & Mulvey 1997).

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Stothard and Rollinson (1996) and Rollinson et al. (1998) analyzed nine *Bulinus* species by arbitrarily primed polymerase chain reaction (AP-PCR) technique showing that there is a high genetic divergence among species, in addition to an intraspecific variation. Vidigal et al. (1994) used the same technique and observed a high genetic variability in Brazilian *B. glabrata* populations. Simpson et al. (1995) compared the high genetic variability found in different *B. glabrata* populations with the low polymorphism presented by *S. mansoni* suggesting later that the intermediate host genetics could play a more important role in schistosomiasis epidemiology than that of the parasite itself.

Jarne et al. (1994), using microsatellites as genetic markers to study *B. truncatus*, detected greater genetic variability than that estimated by Njiokou et al. (1993) for the same species using isoenzymes. This technique was also used for other mollusks such as: *Bulinus*, *Melania* and *Littorina* (Viard et al. 1997a, b, c, Samadi et al. 1999, Tie et al. 2000). Jones et al. (1999) reported the isolation and characterization of the first microsatellite *loci* in *B. glabrata*, and also demonstrated divergence between resistant and susceptible populations to *S. mansoni*. Mavárez et al. (2000) characterized nine microsatellite *loci* in *B. glabrata* populations, from Venezuela, detecting at least eight suitable *loci* for studies on populational structure, reproduction systems and resistance to *S. mansoni*.

Zietkiewicz et al. (1994) introduced the simple sequence repeat anchored PCR (SSR-PCR) technique in the study of several eukariotic species. This technique is based on the anchorage of the primers at the 3' or 5' ends of the microsatellites and its advantage lies on the reduction of the number of other possible targets for annealing. Among the seven tested primers the $(CA)_8RY$ presented the best results.

Wu et al. (1994) used the same concept to study plants of the genus *Arabidopsis*. These authors anchored four nucleotides at the 5'end of the CT repetitive primer with another random primer of only 10mer, resulting in a technique named random amplified microsatellite polymorphism (RAMP).

Oliveira et al. (1997) used SSR-PCR with $(CA)_8RY$ primer to study the intraspecific variability of *Trypanosoma cruzi*, *Leishmania braziliensis* and *S. mansoni*, showing that the patterns obtained were comparable to those resulting from AP-PCR. Gomes et al. (1998) observed similar results when using SSR-PCR and AP-PCR with isolated *T. cruzi* strains, from chronic patients with Chagas disease.

Due to its applicability and the quality of the results obtained, we have used SSR-PCR to study the intrapopulational, intraspecific and interspecific variability of the Brazilian populations of *B. straminea*, *B. intermedia* and *B. kuhniana*.

MATERIALS AND METHODS

Snail populations - The snail species, number of samples, localities, abbreviations and geographic coordinates are presented in the Table. All specimens were directly field-collected, examined for the presence of *S. mansoni* cercariae and were found to be negative. The snails were killed, fixed in Railliet-Henry's fluid for further dissection (Deslandes 1951, Paraense 1976) and the foot of each specimen removed for subsequent DNA extraction (Vidigal et al. 1994). The specimens were identified by means of comparative morphology accordingly to Paraense (1975, 1988). In each of

TABLE

Species, localities, geographical coordinates and abbreviations of the *Biomphalaria* populations used

Species	No. of	Locality samples	Abbreviations	Geographical coordinates
B. straminea	20	Belém, Pará	BE	01s27/48w30
	5	Monte Carmelo, Minas Gerais	MC	18s43/47w29
	5	Passos, Minas Gerais	PA	20s43/46w36
	5	São Lourenço da Mata, Pernambuco	SM	07s58/35w02
	5	Brasília, Distrito Federal	BS	15s46/47w55
	5	Icém, São Paulo	IC	20s20/49w11
B. kuhniana	20	Tucuruí, Pará	TU	03s46/49w40
B. intermedia	20	Jales, São Paulo	JA	20s16/50w32
	5	Paulo Faria, São Paulo	PF	20s01/49w23
	5	Pindorama, São Paulo	PN	21s11/48w54
	5	Planura, Minas Gerais	PL	20s08/48w42
	5	Campina Verde, Minas Gerais	CV	19s32/49w29
	5	Itapagipe, Minas Gerais	IT	19s54/49w22

the experiments one *B. glabrata* specimen from Esteio (RS) was used as an outgroup.

DNA extraction - Total DNA was extracted from the foot of each snail, utilizing the Wizard Genomic DNA Purification Kit (Promega), with some modifications (Vidigal et al. 2000).

SSR-PCR amplification - The protocol used was basically that of Oliveira et al. (1997) with slight modifications. The PCR amplification using the primer $(CA)_8$RY 5'CACACACACACACACARY 3' (Fig. 1A) was undertaken in a volume of 20 µl containing: 1-10 ng 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, 2% formamide (v/v), together with 5 pmol of primer. The reactions were covered with a drop of mineral oil and subjected to the following cycle program: initial denaturation for 3 min at 94°C, and then 26 cycles with annealing of 52°C for 45 sec, extension at 72°C for 1 min and denaturation at 94°C for 30 sec. The final extension step was at 72°C for 7 min. For the primer K7 5'CAACTCTCTCTCTCT 3' (Fig. 1B), the protocol was described as above, except that formamide was not added to the reaction and the annealing temperature was 42°C.

A negative control (no template DNA) was included in all experiments and 5 µl of the products were separated on 6% silver stained polyacrylamide gels (Santos et al. 1993, Sanguinetti et al. 1994).

Quantitative analysis - The bands generated by both primers for the different populations were used to construct a taxon/character matrix for each species. The bands observed in 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 analyzed with TREECON for Windows (Version 1.2 – Van de Peer & De Watchter 1994). The genetic distance was calculated using the coefficient of Nei and Li (1979). These data were clustered with NJ (Saitou & Nei 1987, Studier & Keppler 1988) and used for the construction of the tree of genetic distance. The reliability of the NJ trees was assessed by the bootstrap method (Felsenstein 1985) with 1,000 pseudoreplications. Only bootstrap values higher than 70% were considered significant (Hillis & Huelsenbeck 1992).

The data obtained were also analyzed with Numerical Taxonomy and Multivariate Analysis System-NTSYSpc (Version 2.0). The percentage of shared bands was calculated using the Similarity Coefficient of Dice (Dice 1945). These data were clustered with UPGMA (Sneath & Sokal 1962) and used for the construction of a phenetic tree. The average percentage of shared bands among all possible pairs was calculated and marked on the tree with a dotted line (phenon line).

The comparison was made among individual snails of the same population and among populations of snails from different localities.

RESULTS

Six *B. straminea*, six *B. intermedia* populations and one *B. kuhniana* population were analyzed. The gel shown in Fig. 2 illustrates the reproducibility of the profiles obtained with the SSR-PCR technique using 20 specimens of *B. kuhniana*

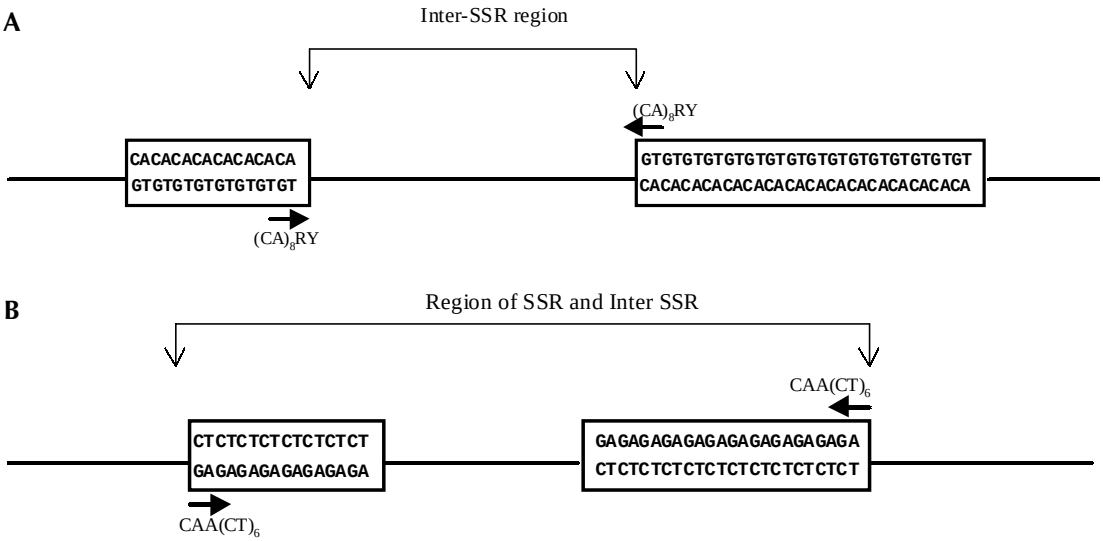

Fig. 1-A: schematic representation of the inter-simple sequence repeat region, primer $(CA)_8$RY (Zietkiewiez et al. 1994); B: schematic representation of simple sequence repeat and inter-simple sequence repeat regions, primer K7 (Wu et al. 1994)

with the primer K7. The profiles obtained using K7 and $(CA)_8RY$ from 20 specimens of *B. kuhniana* and 20 specimens from one population of *B. intermedia* and *B. straminea* (data not shown), randomly chosen, showed to be homogenous.

When we analyzed the trees, generated by both methods with the primers, it can be observed that the trees obtained using the primer K7 are more variable than with the primer $(CA)_8RY$ (data not shown). The outgroup formed a separated group in all trees.

Intraspecific genetic variability

Biomphalaria straminea - Figs 3A and B shows the amplification profiles of five specimens from each of the six populations of *B. straminea*, with the primers $(CA)_8RY$ and K7, respectively. The profiles produced, using both primers, were homogenous within a population, but quite heterogeneous when different populations were compared.

The mean percentage of bands shared among all possible pairs from each population, obtained by the coefficient of Dice, ranged from 83 to 98%, while among all the possible pairs of the different populations, using both primers, was 68%. This is marked on the tree as the phenon line (Fig. 4A).

The trees shown in the Figs 4A and B, produced by the UPGMA and NJ methods, respectively, mirror the similarities and genetic distance among *B. straminea* populations. These trees present similar topologies clustering specimens from the same locality, except the population specimens from Icém/SP (IC) and São Lourenço da Mata/PE (SM), which clustered in the NJ and UPGMA trees, but with an insignificant bootstrap value (12%). We have also observed that the populations from Belém/PA (BE) are more related to those from Brasília/DF (BS), supported by a bootstrap value of 60%, and the populations from Monte Carmelo/MG (MC) are more related to those from Passos/MG (PA), presenting a similarity coefficient of approximately 67%.

Biomphalaria intermedia - The amplification profiles of five specimens from each of the six populations of *B. intermedia* with the primers $(CA)_8RY$ and K7, respectively, are showed in Figs 5A and B. The profiles produced were homogenous within a population but heterogeneous when the populations were compared.

The average percentage of bands shared among all possible pairs from each population, obtained by the coefficient of Dice, ranged from 91 to 97%. The percentage among all possible pairs from the different populations, using both primers, was 63%. This is marked on the tree as the phenon line (Fig. 6A).

The trees, in Figs 6A and B, produced by UPGMA and NJ methods, mirror the similarity and genetic distance, respectively, among *B. intermedia*

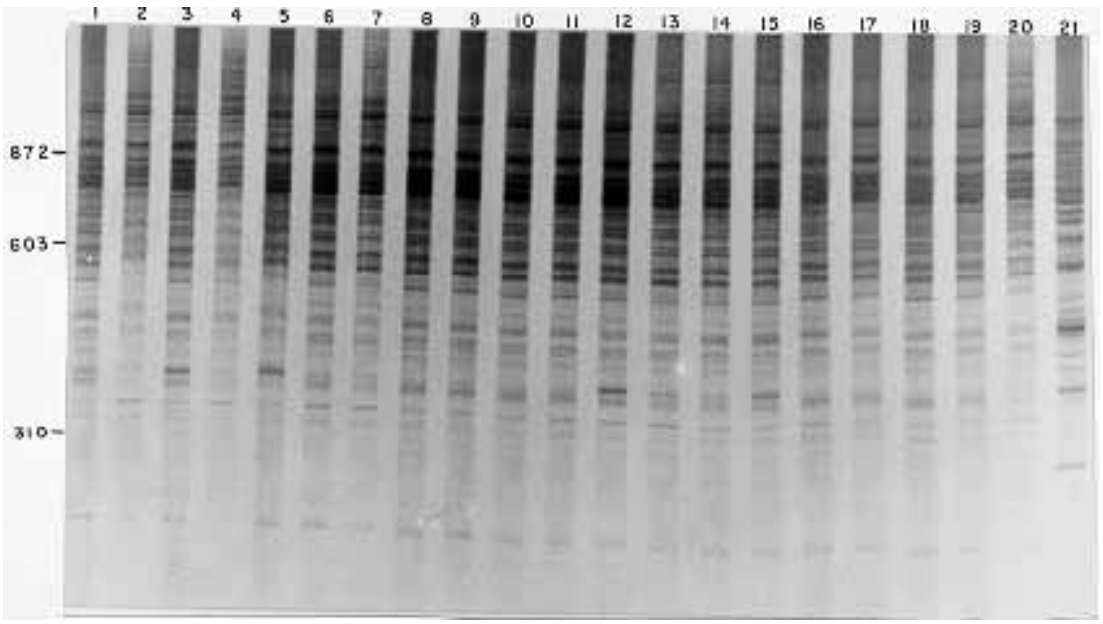

Fig. 2: 6% silver-stained polyacrylamide gels showing simple sequence repeat-anchored PCR amplification profiles obtained with the K7 primer from 20 individuals of *Biomphalaria kuhniana* lanes 1 to 20, from Tucuruí (Pará, Brazil) and one outgroup, *B. glabrata* lane 21, from Esteio (Rio Grande do Sul, Brazil). Molecular size markers are shown on the left of each gel.

populations. Such trees presented similar topologies, clustering specimens from the same locality with the formation of two groups, supported by a 100% bootstrap value; the first clustered the Campina Verde/MG (CV), Itapagipe/MG (IT), Pindorama/SP (PN) and Jales/SP (JA) populations, despite being supported by a low bootstrap value (24%), and the second grouped the populations from Paulo Faria/SP (PF) and Planura/MG (PL), also supported by a low bootstrap value (43%).

Interspecific genetic variability

The gels produced, using both primers for two individuals from each of the 13 populations, showed that the interspecific profiles were very heterogeneous (data not shown). However, as previously observed, the specimens from the same populations presented homogenous profiles. The trees obtained with all populations, with both primers and with each primer separately, showed different topologies, clustering populations of different species (data not shown).

DISCUSSION

The snails of the genus *Biomphalaria* exhibit considerable morphological (Paraense 1957) and molecular variability (Vidigal et al. 1994, Simpson et al. 1995). This variability is responsible for the great phenotypic plasticity shown by some snail species as verified by Paraense et al. (1992) and Caldeira et al. (2000) in *B. prona* populations obtained from Lake Valencia, Venezuela and in surrounding watercourses. On the other hand, morphologically similar species such as those in the current study as well as *B. tenagophila*, *B. t. guaibensis* and *B. occidentalis* have been clustered

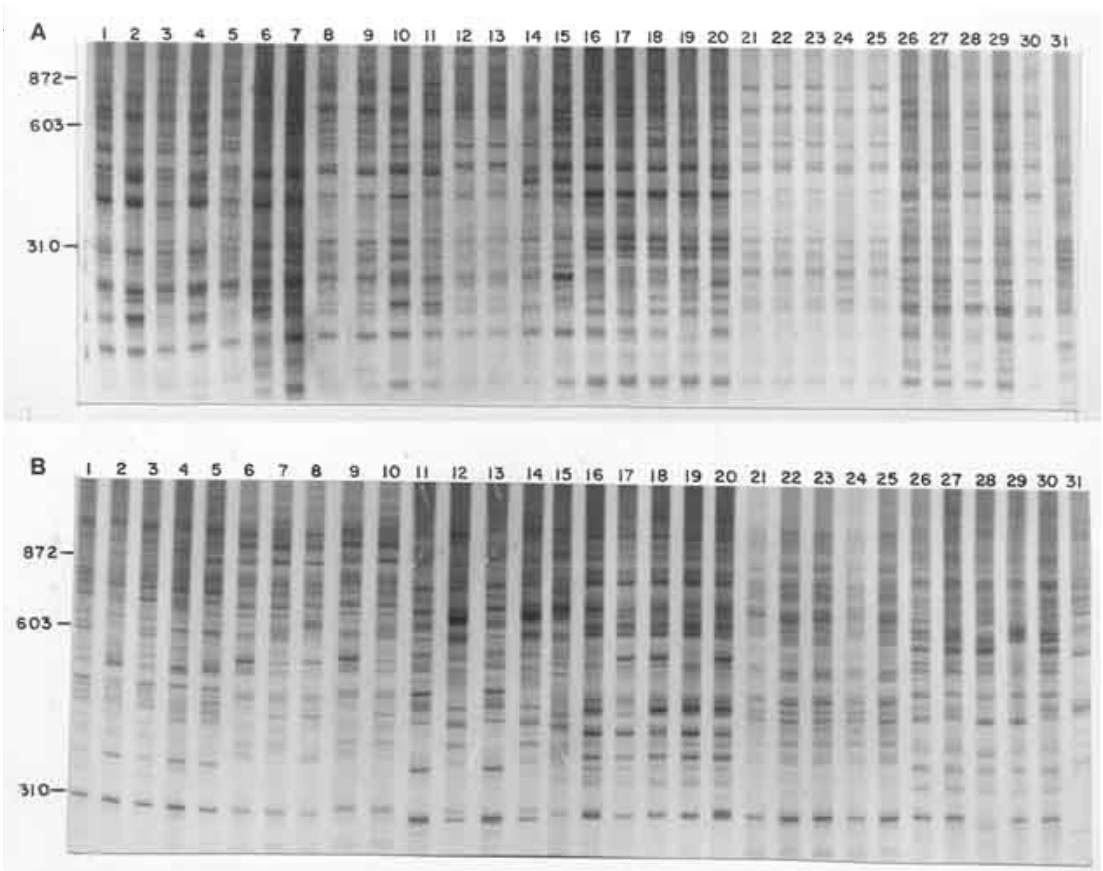

Fig. 3A: 6% silver-stained polyacrylamide gels showing simple sequence repeat- anchored PCR amplification profiles obtained with the $(CA)_8RY$ primer from 30 *Biomphalaria straminea* lanes 1 to 5 from Belém (Pará, Brazil); lanes 6 to 10 from Monte Carmelo (Minas Gerais, Brazil); lanes 11 to 15 from Passos (Minas Gerais, Brazil); lanes 16 to 20 from São Lourenço da Mata (Pernambuco, Brazil); lanes 21 to 25 from Icém (São Paulo, Brazil); lanes 26 to 30 from Brasília (Distrito Federal, Brazil) and one outgroup, *B. glabrata* lane 31 from Esteio (Rio Grande do Sul, Brazil). Molecular size markers are shown on the left of each gel; B: 6% silver-stained polyacrylamide gels showing simple sequence repeat to anchored PCR profiles obtained with the K7 primer. The legend is as shown above.

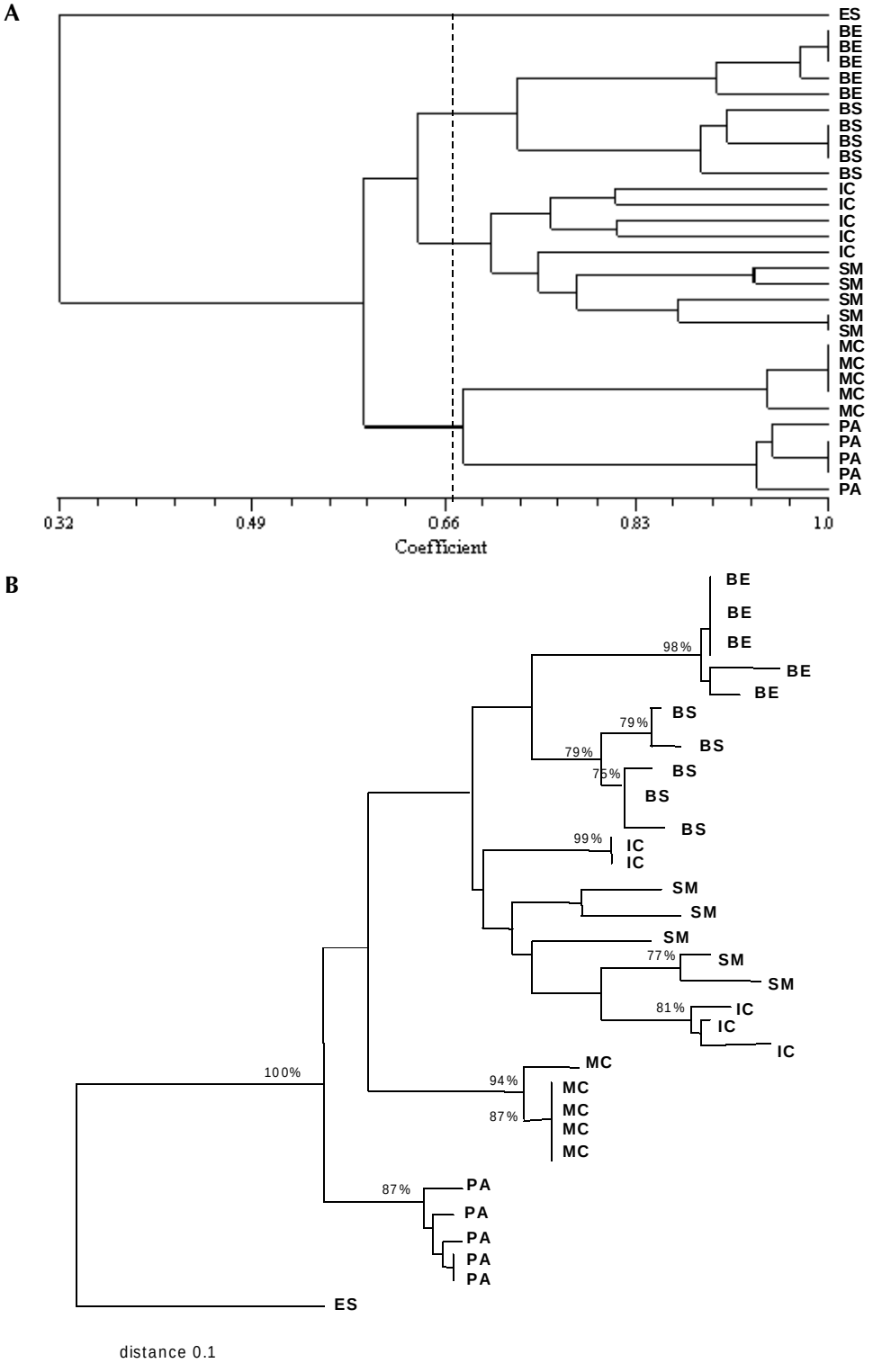

Fig. 4-A: UPGMA tree of 30 *Biomphalaria straminea* and one outgroup *B. glabrata*, constructed using the simple sequence repeat-anchored PCR amplification profiles produced with the two primers. Population names follow in the Table. The numbers shown are similarity indices. The dotted line represents the phenon line; B: neighbor-joining tree rooted with the outgroup of 30 *B. straminea* and one *B. glabrata*, constructed using the SSR-PCR profiles produced with the two primers. Population names follow in the Table and in the text. The numbers are bootstrap percent values based on 1,000 pseudoreplications.

in complexes named *B. straminea* (Paraense 1988) and *B. tenagophila* (Spatz et al. 1999), respectively.

We have observed here, for all the species studied, that the profile of bands among specimens from the same locality was homogenous. These qualitative data were supported by the quantitative analysis performed with the clearest bands of the gels, generated by the two primers. The mean percentage of shared bands among all possible pairs from the same locality was over 83%. This intrapopulational homogeneity observed in the three species studied suggests the existence of uniform populations. Indeed, the observations of the trees indicate that the most related individuals were from the same locality. In contrast, *B. straminea* and *B. intermedia* presented intraspecific genetic heterogeneity, 68% and 63%, respectively. It is very likely that these results are due to the genetic recombination, genetic drift, low gene flow among populations and a founder effect, as suggested by Paraense (1957) and Jarne and Delay (1991) of the genus *Biomphalaria*. It is relevant to remark here that, despite the variability, at the molecular level, those species are morphologically, in accordance with the classical taxonomy.

Similar results were achieved, through AP-PCR, using *B. glabrata*, from Brazil (Vidigal et al. 1994). The mean percentage of shared bands among all possible pairs are over 91% and 43% for intra and interpopulational studies, respectively. Mulvey and Vrijenhoek (1982), studying *B. glabrata* from Puerto Rico, through isoenzyme methodology, observed that only 4 out of 26 examined *loci* were polymorphic, suggesting a low intrapopulational genetic variability. On the other hand, the low heterozygosity levels, observed

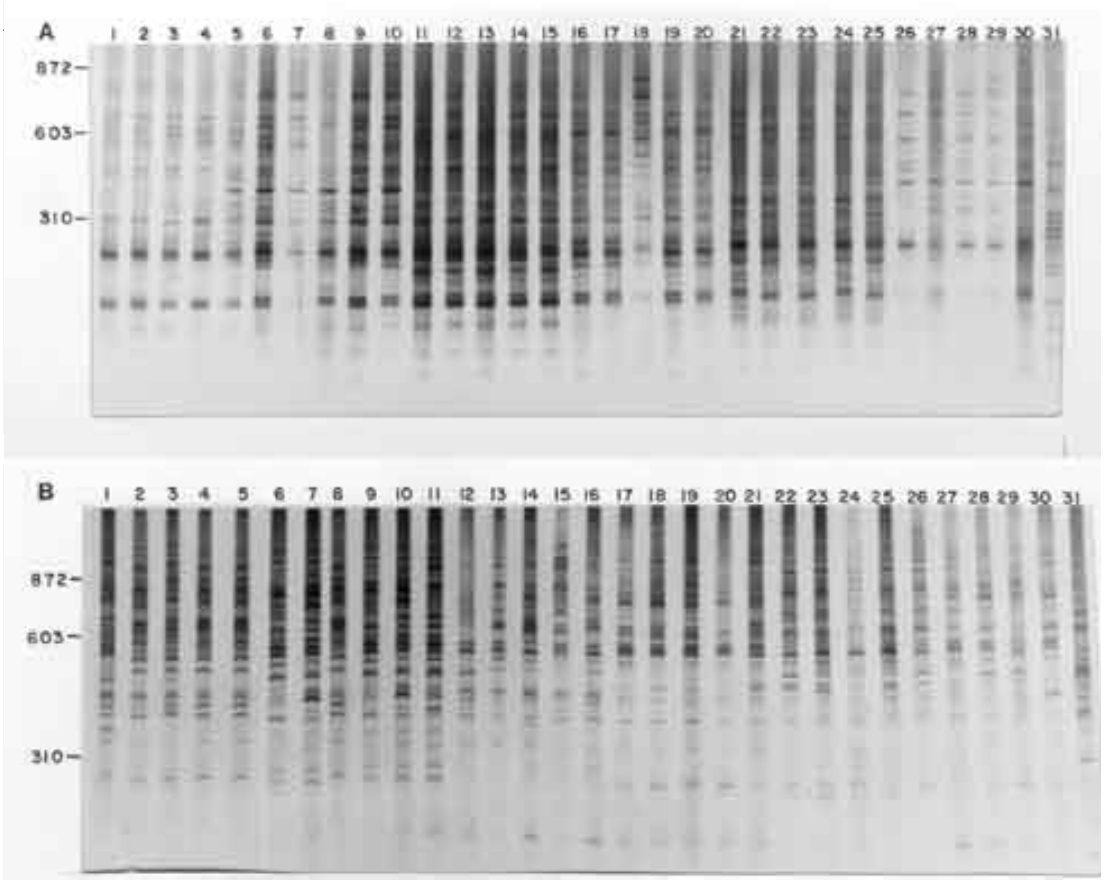

Fig. 5A: 6% silver-stained polyacrylamide gel showing simple sequence repeat - anchored PCR amplification profiles obtained with the $(CA)_8RY$ primer from 30 *Biomphalaria intermedia* lanes 1 to 5 from Planura (Minas Gerais, Brazil); lanes 6 to 10 from Paulo Faria (São Paulo, Brazil); lanes 11 to 15 from Jales (São Paulo, Brazil); lanes 16 to 20 from Pindorama (São Paulo, Brazil); lanes 21 to 25 from Campina Verde (Minas Gerais, Brazil); lanes 26 to 30 from Itapagipe (Minas Gerais, Brazil) and one outgroup, *B. glabrata* lane 31 from Esteio (Rio Grande do Sul, Brazil). Molecular size markers are shown on the left of each gel. B: 6% silver-stained polyacrylamide gel showing simple sequence repeat-anchored PCR profiles obtained with the K7 primer. The legend as shown above.

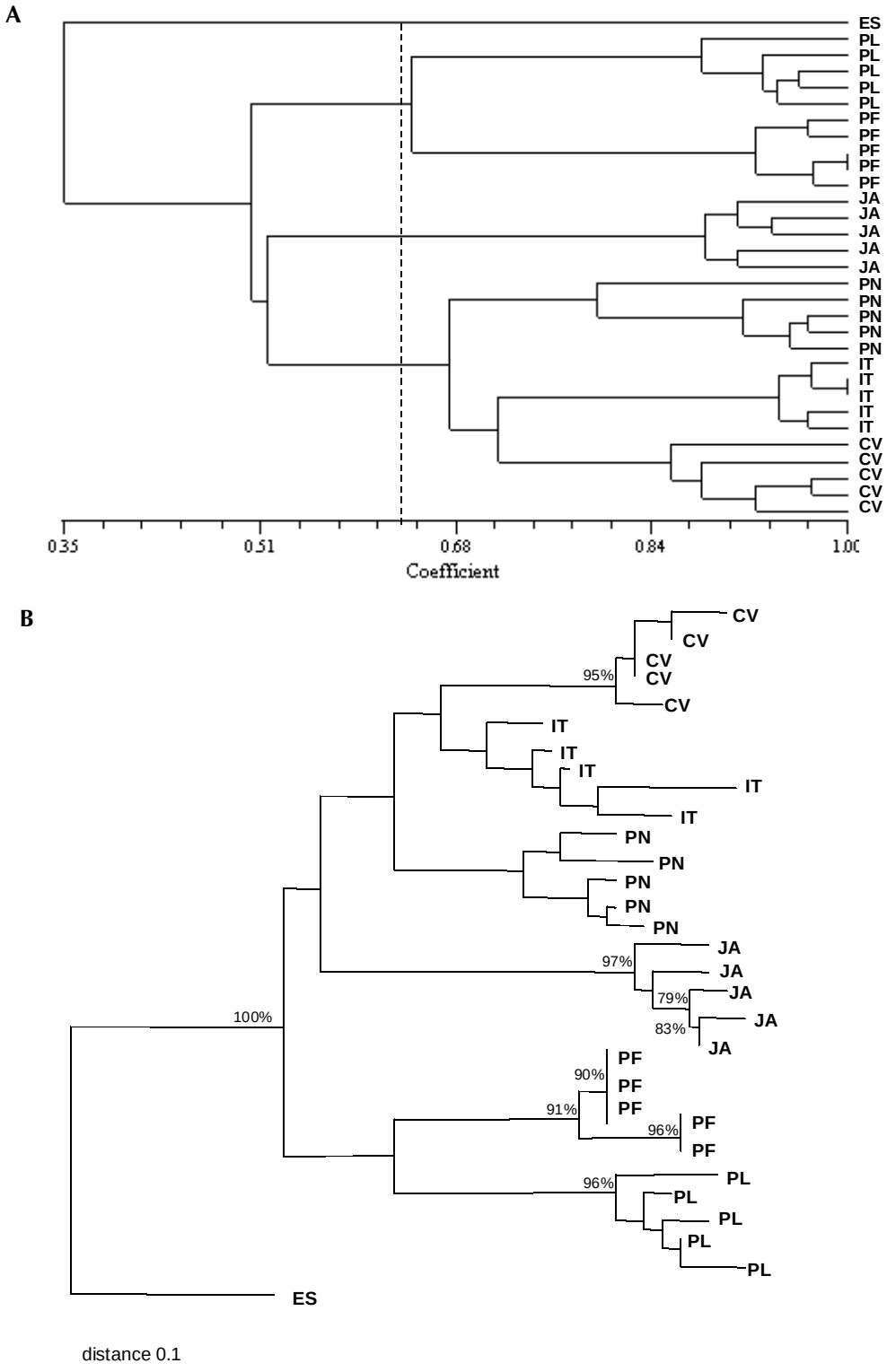

Fig. 6-A: UPGMA tree of 30 *Biomphalaria intermedia* and one outgroup *B. glabrata*, constructed using the simple sequence repeat- anchored PCR amplification profiles produced with the two primers. Population names follow in the Table. The numbers shown are similarity indices. The dotted line represents the phenon line; B: neighbor-joining tree rooted with the outgroup of 30 *B. intermedia* and one *B. glabrata*, constructed using the SSR-PCR profiles produced with the two primers. Population names follow in the Table and in the text. The numbers are bootstrap percent values based on 1,000 pseudoreplications.

among seven populations, indicated a high interpopulational variability.

The profiles obtained with both primers were used to construct phenetic trees through UPGMA, assuming that all lineage have diverged on equal amounts. On the other hand, the NJ method, conceptually related to the traditional cluster analysis, does not make that assumption (Swofford et al. 1996). In spite of this, regardless the methodology, the trees presented similar topologies (Figs. 4A, B; 6A, B) and, in most situations, individuals from the same locality could be clustered. Such groups were supported by high bootstrap values (over 70%), except for the *B. straminea* populations, from São Paulo and Pernambuco that are most volatile in terms of their position on the trees (IC and SM).

The SSR-PCR has been shown not to be the best technique for interspecific studies, since it clustered populations of different species and did not present reproducibility and robustness in their trees. It can be explained by the kind of methodology applied, which involves the study of the whole genome, being likely the overlap of the studied regions, in the species. The PCR-RFLP was shown to be more suitable for such analysis (Caldeira et al. 1998).

The SSR-PCR technique has been described as an alternative method to the AP-PCR for polymorphism studies in the eukaryote genome, as it presents good reproducibility due to the high stringency conditions used (Zietkiewiez et al. 1994). Oliveira et al. (1997) remarked that this technique has a higher discrimination power than the AP-PCR, as it focuses on microsatellites, which are known polymorphic regions, scattered in the genome.

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