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BIOMPHALARIA TENAGOPHILA: GENETIC VARIABILITY WITHIN INTERMEDIATE SNAIL HOSTS SUSCEPTIBLE AND RESISTANT TO *SCHISTOSOMA MANSONI* INFECTION

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Summary:

DNA analysis by molecular techniques has significantly expanded the perspectives of the study and understanding of genetic variability in molluscs that are vectors of schistosomiasis. In the present study, the genetic variability of susceptible and resistant *B. tenagophila* strains to *S. mansoni* infection was investigated using amplification of their genomic DNA by RAPD-PCR. The products were analyzed by PAGE and stained with silver. The results showed polymorphism between tested strains with four different primers. We found two bands of 1,900 and 3,420 bp that were characteristic of the susceptible strains with primer 2. The primers 9 and 10 identified a single polymorphic band that was also characteristic of (3,136 and 5,041 bp, respectively) susceptible snails. Two polymorphic bands were detected by primer 15: one with 1,800 bp was characteristic of the resistant strain and the other with ≃ 1,700 bp in the susceptible one. These results provide additional evidence showing that the RAPD-PCR technique is adequate for the study of polymorphisms in intermediate hosts snails of *S. mansoni*. The obtained results are expected to expand the knowledge about the genetic variability of the snails and to permit the future identification of genomic sequences specifically related to the resistance/susceptibility of *Biomphalaria* to the larval forms of *S. mansoni*.

KEY WORDS : *Biomphalaria*, schistosomiasis, RAPD-PCR, genetic variability, polymorphism.

Résumé : *BIOMPHALARIA TENAGOPHILA*: VARIABILITÉ GÉNÉTIQUE DES MOLLUSQUES HÔTES INTERMÉDIAIRES SENSIBLES ET RÉSISTANTS À L'INFECTION PAR *SCHISTOSOMA MANSONI*

Les analyses de l'ADN par le moyen de techniques moléculaires augmentent de façon significative les perspectives des études et les connaissances sur la variabilité génétique de mollusques hôtes intermédiaires de la schistosomiase. Dans cette étude, on a étudié la variabilité génétique de la résistance et de la sensibilité de B. tenagophila *à l'infection par* S. mansoni, *en utilisant l'amplification du matériel génomique par RAPD-PCR. Les produits ont été analysés par PAGE et colorés au nitrate d'argent. Les résultats ont montré des polymorphismes avec quatre "primers" examinés. Il a été trouvé beaucoup de bandes polymorphiques dans les lignées de* B. tenagophila *résistantes et sensibles à l'infection par* S. mansoni. *On s'attend à ce que les résultats obtenus augmentent les connaissances en matière de variabilité génétique des mollusques et permettent la future identification des ordres génomiques liés à la résistance/sensibilité de* Biomphalaria *aux formes larvaires de* S. mansoni.

MOTS CLÉS : Biomphalaria, *schistosomiase, RAPD-PCR, variabilité génétique, polymorphisme.*

INTRODUCTION

The biological processes involved in the interaction of *Schistosoma* with its hosts and the modulation of susceptibility/resistance of the vector molluscs have been investigated in several studies. In 1966, Saoud suggested that one of the determinant factors of *Schistosoma* pathogenicity may be the degree of infectivity of the invertebrate host. Studies by Zanotti-Magalhães *et al.* (1991, 1993, 1995, 1997) and Dias *et al.* (1988) have shown that the degree of infectivity of certain *S. mansoni* strains may be a reflex of how easily the larvae of this parasite develop in their invertebrate hosts.

The susceptibility of *Biomphalaria* to *Schistosoma mansoni* infection depends on a series of cellular and humoral immunological mechanisms (Raticliffe, 1985; Allegretti., 1991) and several studies have shown that susceptibility is a genetic trait with a not yet very well defined pattern of inheritance (Newton, 1953; Richards & Merritt, 1972; Santana *et al.*, 1978; Nabih & El-Ansary, 1980; Richards *et al.*, 1992; Larson *et al.*, 1996). As proposed by Mascara *et al.* (1999) the resistance could be arisen from a selection process in different loci whereas each gene would take incompatibility at different periods during the relation parasite-vector. The resistant phenotype would result from the alteration in any of the different loci. It occurs that, in short, a complex net of interactions or sequential events must be processed in order to be established the infection. Among those events it is worth to point out: the attraction and

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penetration of parasite (Yoshino *et al.*, 1993; Yousif *et al.*, 1993), the development and production of cercaries (Moné, 1991; Coustau & Yoshino, 1994). The relations parasite-vector are, therefore, resulting from complex biological events (Preston & Southgate, 1994; Manning *et al.*, 1995), from which genetic basis or inheritance patterns have not been, up to present, well established.

DNA analysis by molecular biology techniques has significantly expanded the perspectives for the study and the genetic knowledge of schistosomiasis vectors. The randomly amplified polymorphic DNA by polymerase chain reaction (RAPD-PCR) technique proposed at the beginning of the 1990's (Welsh & McClelland, 1990; Williams *et al.*, 1990) has proved to be highly useful for the analysis of polymorphisms in different species of the genus *Biomphalaria* (Vidigal *et al.*, 1994; Larson *et al.*, 1996; Knight *et al.*, 1999).

The analysis of *B. tenagophila* strains susceptible and resistant to *S. mansoni* infection was first carried out by Abdel-Hamid *et al.* (1999). The authors used five primers, in three of which they observed variation between strains susceptible and resistant to infection. According to these investigators, analysis of genetic variability by RAPD-PCR represents "an efficient pathway" for the comparison of the genomes of strains susceptible and resistant to *S. mansoni* infection.

In the present study it was employed the RAPD-PCR technique to study the genetic variability and the occurrence of specific genetic markers associated to susceptibility and/or resistance of the vector mollusk *B. tenagophila* to *S. mansoni* infection.

MATERIALS AND METHODS

SNAILS MAINTENANCE

Adult mollusks from the SJ lineage from *B. tenagophila* (proceeding from São José dos Campos City, S.P., Brazil) were utilized. They were kept in the Parasitology laboratory at Campinas University (Campinas, S.P., Brazil) under controlled conditions. The snails were selected to character susceptibility by self fertilization of the parental generation according to the method described by Zanotti-Magalhães *et al.* (1997).

A total number of 100 (N = 100) adult mollusks were used after a careful selection on the basis of health and age. The snails were exposed individually to 10 miracidia in flasks containing 5 ml water for two hours. A dose of 10 miracidia can be considered as a high dose, minimizing the risk of the residual variability of the strain. Snails were maintained in a glass aquaria containing declorinated water and they were examined at 30 days post exposure and checked for infection through cercarial emergence. Examination of snails for emergence of cercariae were performed at 4-10 weeks after exposure to avoid missing delayed development of parasites. Any snails in which infections were observed were considered susceptible and those that remain uninfected after two exposures were considered resistant. In selecting for resistant stocks, snails that remained uninfected after two exposures were isolated and reared singly for selfing. Also, in selecting for susceptible stocks, unexposed progeny of snails from which exposed test groups yielded high infection frequencies, were isolated and reared singly for selfing. Resistant and susceptible *B. tenagophila* snails were used in the present study to identify RAPD fragments that might be associated with resistance.

DNA EXTRACTION

DNA was extracted from the tip of the head foot region of individual snails (resistant and susceptible strains), using lysis buffer containing 2 % CTAB (Winnepenninckx *et al.*, 1993; Abdel-Hamid *et al.*, 1999), as previously described by Spada *et al.* (2002). The DNA concentration and purity was determined spectrophotometrically (Spectrophotometer Ultrospec III – Pharmacia, UK) at absorbances of 260 and 280 nm, and also by 2.0 % agarose gel electrophoresis using the gel photodocumentation system (EDAS) DC 120 Zoom Digital Camera (Eastman Kodak, NY, USA).

DNA AMPLIFICATION

The extracted DNA was amplified using RAPD-PCR as the method of Simpson *et al.* (1993), with some modifications. Two nanograms (ng) of genomic DNA obtained from the snails was amplified with a PTC 200 Peltier Thermal Cycler (MJ Research - USA). Each reaction was carried out in a final volume of 20 µl containing one unit Taq DNA polymerase (Gibco-BRL, MD, USA), 1X PCR buffer, 0.2 mM of each dNTP, and 7 pmol of each random primer (10 bp) (Gibco-BRL). In the amplifications of pool it was employed 1 ng of DNA from each of the analysed samples. The amplification conditions were as follows: one cycle at 95°C for five minutes, two cycles at 95°C for 30 secondes, at 30°C for two minutes and at 72°C for one minute, and 33 cycles during which the annealing temperature was changed to 40°C and the time of the extension step was increased to five minutes during the final cycle. As control, PCR reactions were run without genomic DNA. Five primers (GIBCO-BRL) were utilised and they were defined from an ample checking of the correlative works (Table I).

Primer	Séquence (5'-3')	Reference
2	CTGATGCTAC	Vidigal *et al.*, 1994
4	AGTGCTACGT	Vidigal *et al.*, 1994
9	CAGGCCCTTC	Larson *et al.*, 1996
10	GGTCCCTGAC	Larson *et al.*, 1996
15	TGCCGAGCTG	Abdel-Hamid *et al.*, 1999

Table I. – Primers used in the RAPD-PCR reactions.

POLYACRILAMIDE GEL ELECTROPHORESIS

The PCR products were analyzed by polyacrylamide gel electrophoresis (PAGE) and visualized by silver staining as follows: 4 µl of each DNA amplification reaction was added to 2.5 µl sample buffer (0.125 % bromophenol blue, 0.125 % xylene cianol and 15 % glycerol) and the mixture was submitted to 8 % polyacrylamide (29/1 acrylamide-bisacrylamide) gel electrophoresis in TBE (2 mM Tris-borate, EDTA, pH 8) at 60 volts. The gels were fixed in 10 % ethanol and 0.5 % (v/v) acetic acid for 15 minutes, stained with 0.2 % silver nitrate for 15 minutes, washed with deionized water for five minutes, and developed with 0.75 M NaOH and 0.1 M formaldehyde for 15 minutes (*Santos et al.*, 1993).

POLYMORPHISM ANALYSIS

Genetic variability of the susceptible and resistant strains was evaluated by analyzing the electrophoretic band patterns obtained on the gels and by determining the similarity coefficient as described by Dice (1945).

RESULTS

SUSCEPTIBILITY/RESISTANCE OF *B. TENAGOPHILA* TO *S. MANSONI* INFECTION

Susceptibility or resistance to *S. mansoni* infection within the same snail species *B. tenagophila* were studied starting 30 days post infection and continuing thereafter weekly up to 10 weeks after miracidia exposure. The susceptibility/resistance behavior of *B. tenagophila* snail population tested in this work (N = 100) to *S. mansoni* infection is shown in Figure 1. The results pointed out that about 83 % of the examined snails were resistant, in contrast with 17 % of these snails were susceptible. The curve representing percentages of susceptible snails started at 6th week post infection and reached the highest peak by approximately the 9th week, while the curve of refractory snails gradually decreasing toward the 9th week and up to the 9th week post-infection no changes in percentages of refractory snails occurred (Fig. 1). No differences were noted at that time up to the 10th week. The ratio of susceptibility/resistance at a time was fixed, although the number of snails could changes depending on the increasing of mortality within susceptible ones (Fig. 1). The electrophoretic pattern of the bands presented in Figures 2 to 5 tally with a representative sample of a

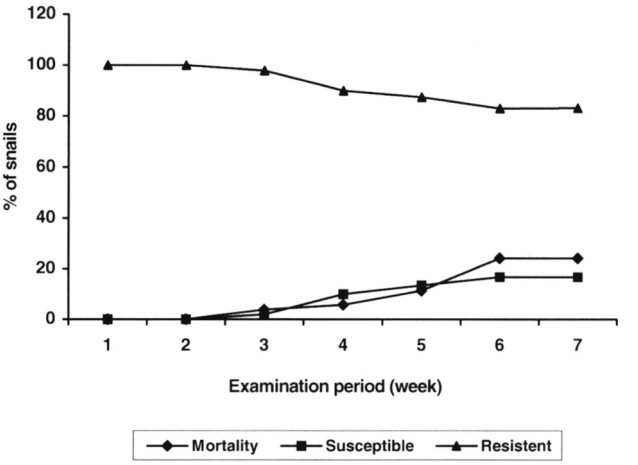


Fig. 1. – Diagramatic representation od susceptibility/resistance of *Biomphalaria tenagophila* to *Schistosoma mansoni* infection.

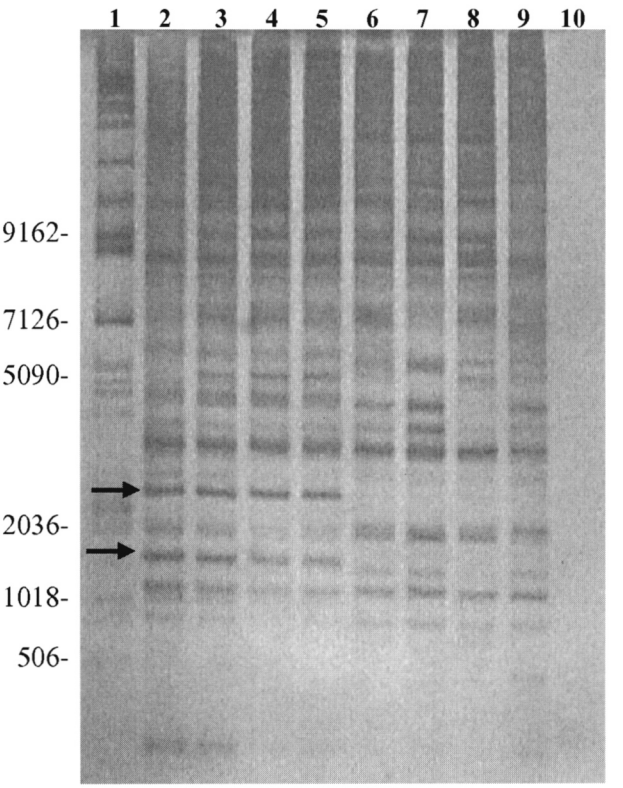


Fig. 2. – Random-amplified PCR from genomic DNA of *Biomphalaria tenagophila* strains resistant and susceptible using arbitrary primer 2 (5'-CTGATGCTAC-3'). Lane 1 (1-Kb DNA ladder); lane 2 (S1, susceptible); lane 3 (S2, susceptible); lane 4 (S3, susceptible); lane 5 (pool of S1, S2 and S3); lane 6 (pool of R1, R2 and R3); lane 7 (R1, resistant); lane 8 (R2, resistant); lane 9 (R3, resistant); lane 10 (negative control, amplification without DNA). Samples were analyzed electrophoresis through a 8 % polyacrylamide gel and visualized by silver staining. Polymorphic bands are indicated by arrow.

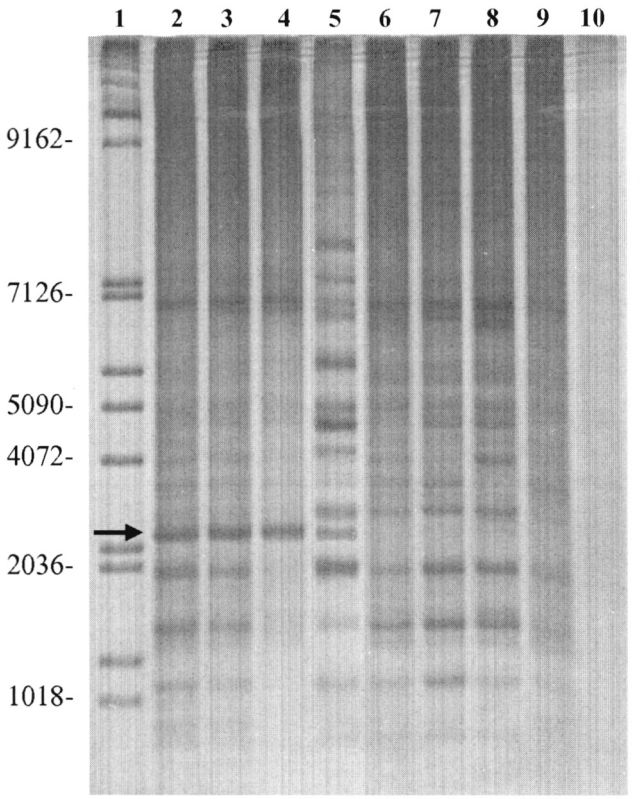


Fig. 3. – Random-amplified PCR from genomic DNA of *Biomphalaria tenagophila* strains resistant and susceptible using arbitrary primer 9 (5'-CAGGCCCTTC-3'). Lane 1 (1-Kb DNA Ladder); lane 2 (S1, susceptible); lane 3 (S2, susceptible); lane 4 (S3, susceptible); lane 5 (pool S, S1, S2 and S3); lane 6 (pool R, R1, R2 and R3); lane 7 (R1, resistant); lane 8 (R2, resistant); lane 9 (R3, resistant); lane 10 (negative control, amplification without DNA). Samples were analyzed electrophoresis through a 8 % polyacrylamide gel and visualized by silver staining. Polymorphic band is indicated by arrow.

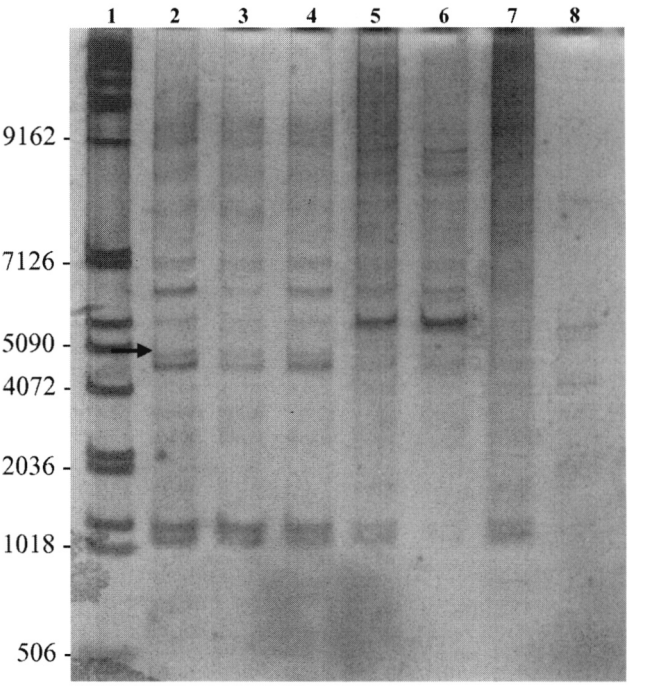
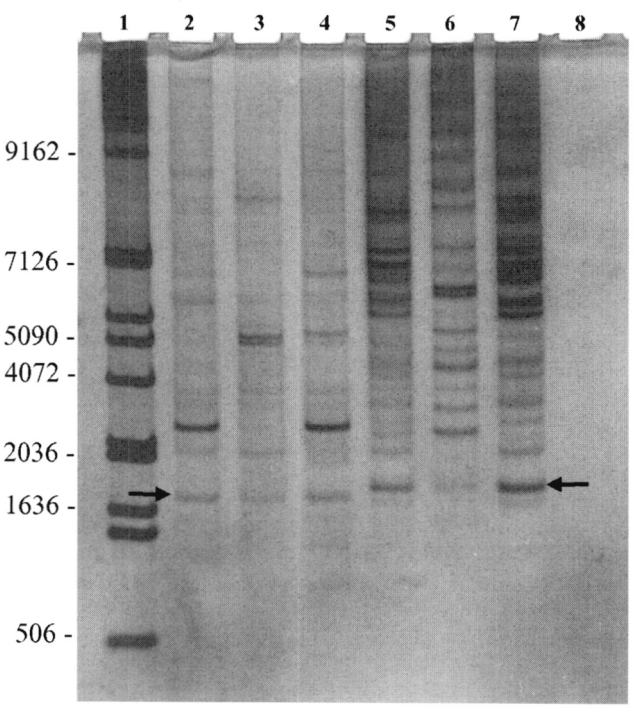


Fig. 4. – Random-amplified PCR from genomic DNA of *Biomphalaria tenagophila* strains resistant and susceptible using arbitrary primer 10 (5'-GGTCCCTGAC-3'). Lane 1 (1-Kb DNA ladder); lane 2 (S1, susceptible); lane 3 (S2, susceptible); lane 4 (pool S, S1 and, S2); lane 5 (pool R, R1 and R2); lane 6 (R1, resistant); lane 7 (R2, resistant) and lane 8 (negative control, amplification without DNA). Samples were analyzed electrophoresis through a 8 % polyacrylamide gel and visualized by silver staining. Polymorphic band is indicated by arrow.



series of amplifications (five or more) to each tested primer. As pointed out before, these primers were used in prior works in which specific polymorphic markers were detected in *B. glabrata*, *B. tenagophila* or in the parasite *S. mansoni* (Vidigal *et al.*, 1994; Larson *et al.*, 1996 and Abdel-Hamid *et al.*, 1999).

Since the genetic variability of schistosomiasis vector snails is high (Vidigal *et al.*, 1996, 1998), due to the large number of polymorphic bands, for each primer used, we adopted the use of duplicates or triplicates and a pool. This pool represents the amplification of the DNA mixture from two individual snails (duplicates) or three individual snails (triplicates) from each strain. This pool was used as an additional control of

Fig. 5. – Random-amplified PCR from genomic DNA of *Biomphalaria tenagophila* strains resistant and susceptible using arbitrary primer 15 (5'-TGCCGAGCTG-3'). Lane 1 (1-Kb DNA ladder); lane 2 (S1, susceptible); lane 3 (S2, susceptible); lane 4 (pool S, S1 and, S2); lane 5 (pool R, R1 and R2); lane 6 (R1, resistant); lane 7 (R2, resistant) and lane 8 (negative control, amplification without DNA). Samples were analyzed electrophoresis through a 8 % polyacrylamide gel and visualized by silver staining. Polymorphic bands are indicated by arrow.

Primers	P2	P4	P9	P10	P15
Number of shared bands between susceptible and resistant snails (a)	13	12	11	12	11
Number of bands in susceptible but not in resistant snails (b)	2	0	1	1	1
Number of bands in resistant but not in susceptible snails (c)	0	0	0	0	1
Similarity coefficient (S)	0.92	1.00	0.96	0.96	0.92

(*) S = 2a/2a + b + c.

Table II. – Dice's similarity coefficient (*) between susceptible and resistant *B. tenagophila* snails.

reproducibility of bands. The identification of polymorphic bands was based on the comparison of the band patterns on the same gel for the two strains and only those detected in all individuals of the same strain and in the pool (and absent in the other strain) were considered polymorphic, as proposed by Larson *et al.* (1996). The electrophoretic pattern of the bands identified by primer 2 revealed two polymorphic bands of 3,420 and 1,900 bp, indicated by the arrows in Figure 2, that are characteristic of the susceptible strain. Despite the homogeneity of most bands, primers 9 and 10 revealed a single polymorphic band, approximately 3,136 and 5,041 bp, respectively (Figs 3 and 4), that is characteristic in snails of the susceptible strain. In experiments performed with primer 15, the gels mostly exhibited electrophoretic profiles with uniform bands for individuals of the same strain. However, two polymorphic bands of approximately 1,800 and 1,700 bp (Fig. 5) were identified in snails of the resistant and susceptible strain, respectively. The bands observed in the negative control (Fig. 4, lane 8) may have been due to the amplification of secondary structures formed between the primers, or to nonspecific contamination amplification in the reaction (Larson *et al.*, 1996).

As it may be seen in Table II, although the lineages being studied present differences relating susceptibility to infection by *S. mansoni,* the two strains studied here showed no remarkable genetic heterogeneity expressed by the Dice's coefficient of similarity.

DISCUSSION

Some studies have been performed to define specific markers for susceptibility in *B. glabrata* by means of isoenzymes and pigmentation analysis (Mulvey & Vrijenhoek, 1981; Mulvey & Wooddruff, 1985). Knight *et al.* (1999) analyzed by means of RAPD-PCR the parental descents and the progeny of backcross (F1 *versus* parental). This approach led to detection of two segregation markers that were found only in resistant strain BS-90 of *B. glabrata*. In the species *B. tenagophila*, however, few studies have been conducted to analyze the genetic variability and to date no specific marker has been detected that might be safely associated with resistance or susceptibility in this species.

Within the general context, the electrophoretic profile of the RAPDs, of the snails of the two strains studied here, with the five primers, showed no remarkable individual differences in the amplified bands, as it may be concluded from the results presented in Table II. These results are also in agreement with others previous observations. In this way, it has been shown, along the years that, although the genetic heterogeneity among populations from different localities is expressive (Paraense, 1959), the genetic variability among intra-population or individuals of the same isolate tends to be small or restricted (Vidigal *et al.*, 1994; Vidigal *et al.*, 1998). As it could be seen in Table II, although the lineages being studied present differences relating susceptibility to infection by *S. mansoni*, the two strains studied here showed no remarkable genetic heterogeneity, expressed by the Dice's coefficient of similarity.

Larson *et al.* (1996) used oligonucleotide sequence (OPA-06), equivalent to primer 4 (5'-GGTCCCTGAC-3') in the present study, to differentiate genetically defined lines of *B. glabrata*. He detected a series of specific markers for the resistant and susceptible strains using RAPD-PCR analysis. In the present study, a polymorphic band of about 5,041 bp was obtained with the same primer. The detection of polymorphism using primers 9, 10 and 15, of relatively high molecular weight, is a curious feature since in general, as observed in other studies, the polymorphisms of *Biomphalaria* tend to be located in the 150 to 1,500 bp range (Larson *et al.*, 1996).

Using the same methodology, *i.e.*, a primer with a sequence equivalent to that of primer 15 (5'-TGCC-GAGCTG-3'), as well as the *B. tenagophila* (SJ strain), Abdel-Hamid *et al.* (1999) did not identify any polymorphic marker and obtained a similarity index of 1.0 between the resistant and susceptible strains. Two polymorphic bands were obtained in this study, an 1,800 bp band which characteristically occurred only in the resistant strain, and 1,700 bp band occurring in

the susceptible strain. The similarity index obtained was 0.92. On this basis, although slightly elevated, the similarity index does not coincide with that obtained by Abdel-Hamid *et al.* (1999). It is possible that the resolution of the gels obtained by these authors did not permit the identification of the polymorphic bands present in the strains analyzed. It must also be considered that the possibility of results divergency may be attributed to genetic drift, founder effect or other evolutive factors, although the individuals used in this work were from the same population and maintained in standardized lab conditions and so, submitted to similar and little intense selective pressures.

Figures 2 to 5 (gel of polyacrilamide) correspond to samples of eight (10 %) resistant individuals and four (24 %) susceptible ones, taken at random from the initial population of 83 resistant individuals and 17 individuals susceptible to infection by *S. mansoni*. As previously referred to, the electrophoretic patterns presented are representative of, at least, five amplifications of each sample of DNA (eight resistant individuals and four susceptible ones). So, at least 40 amplifications for each primer tested in the resistant lineages and 20 amplifications, for each primer in the susceptible lineage, were realised. Therefore, along that research, different approaches of experimental control were realised as, at least, five amplifications of each sample of DNA for each of the five tested primers, running electrophoresis with two or three samples from each individual, apart from a pool, which corresponds to amplification of a mixture of equal parts of DNA of two individuals (at the experiments with duplicates) or three individuals (in the experiments with triplicates). In that manner, one intended to assure the reproducibility and consistency of results (pattern of bands) obtained.

Several restrictions have been raised relating the RAPD-PCR technique, mainly concerned to an artifactual variation and an excess of uninformative bands (Riedy *et al.*, 1992; Elssvorth *et al.*, 1993; Matioli & Brito, 1995; Jones *et al.*, 1999). On the other hand, it has been proposed that this methodology is useful for the analysis of genetic variability and the identification of biologically important phenotypes such as resistance to infection (Barral *et al.*, 1993; Vidigal *et al.*, 1994; Larson *et al.*, 1996; Abdel-Hamid *et al.*, 1999; Knight *et al.*, 1999). The results reported here support the idea that the RAPD-PCR technique is adequate for the analysis of genetic variability and the identification of polymorphic markers.

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