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Use of Isozyme Patterns in the Identification of *Biomphalaria tenagophila* (D'Orbigny, 1835) and *B.occidentalis* (Paraense, 1981) (Gastropoda: Planorbidae)

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Two sibling species of Biomphalaria, B. tenagophila *and* B. occidentalis *were identified using isozyme patterns obtained by horizontal gel electrophoresis. Six diagnostic enzymatic loci were identified in digestive gland homogenates. The results enable us to distinguish the species, calculate the Nei's coefficient of genetic similarity, and provide a basis for making inferences about the pattern of these two planorbid species colonization and distribution.*

Key words: *Biomphalaria tenagophila - Biomphalaria occidentalis - jisoenzyme patterns - sibling species - biochemical taxonomy*

Biomphalaria occidentalis and *B. tenagophila* are species which are indistinguishable from the shell, but separable by characteristics of some of the genital organs (Bailey et al. 1986). Paraense (1981) demonstrated in laboratory studies complete reproductive isolation between these species. These two species are sympatric in part of their ranges (Teles 1989), and have been found together in breeding sites in localities in the State of São Paulo, Brazil (Teles 1988). Since *B. occidentalis* is not susceptible to infestation by the trematode *Schistosoma mansoni* (Paraense & Côrrea 1982), the identification of these species is important for the epidemiological study of schistosomiasis. The objective of the present study was to utilize starch and acrylamide gel electrophoresis to define isozyme patterns that might contribute to the identification of these planorbids.

MATERIALS AND METHODS

Samples - Population samples were collected in the State of São Paulo. Collections were made at 14 localities between May 1987 and August 1988. Nine samples of *B. tenagophila* and six of *B. occidentalis* were obtained (Fig. 1).

The collected specimens were maintained in 25-liter aquaria containing non-chlorinated water at room temperature and fed on fresh lettuce leaves.

The specimens were observed for 40 days to determine whether they released any type of cer-

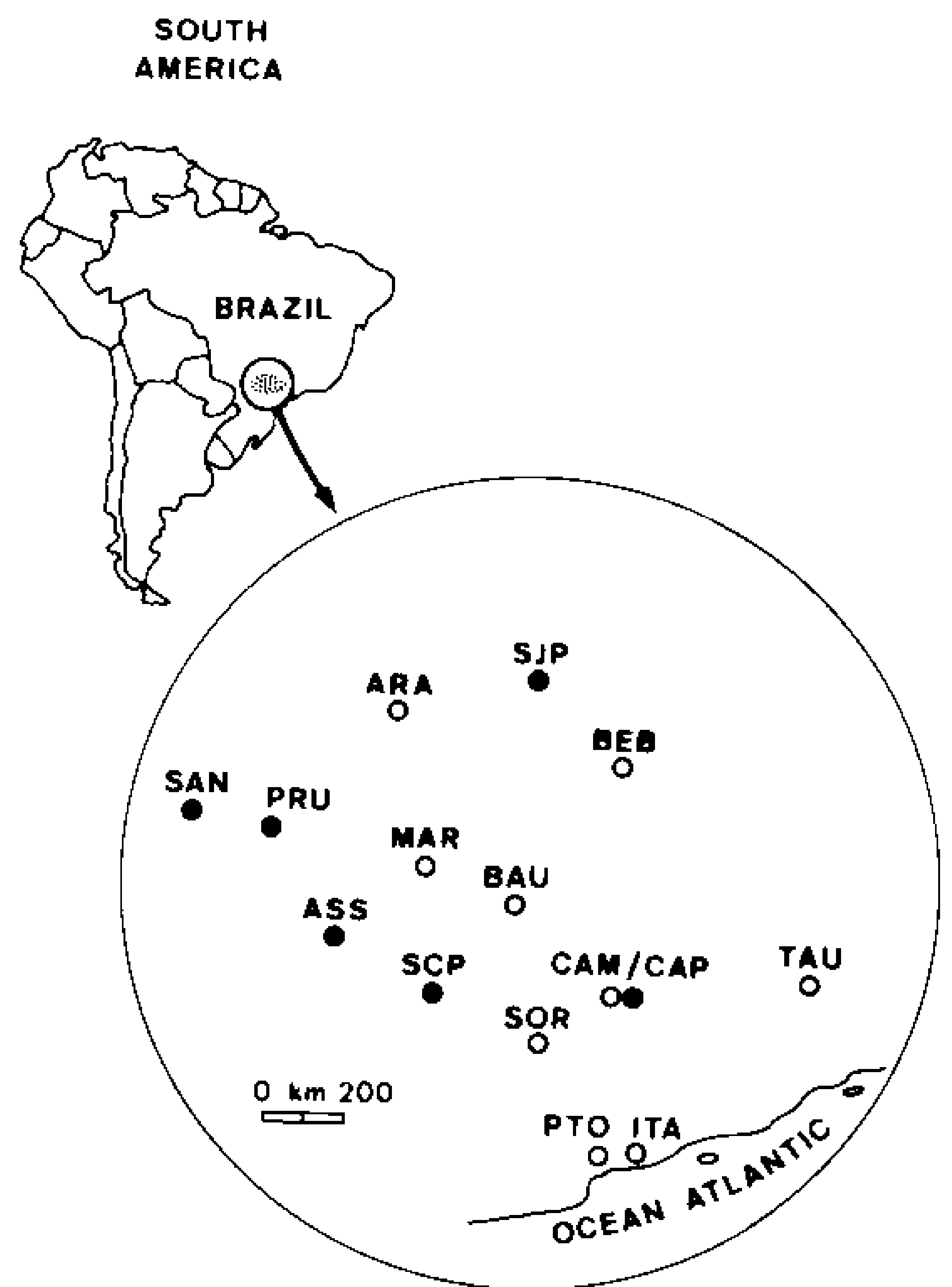

Fig. 1: distribution of populations examined in this study. *Biomphalaria tenagophila* and *B. occidentalis* were collected at nine and six sites, respectively (right). Empty circles represent the samples of *B. tenagophila*: Taubaté (TAU), Itariri (ITA), Pedro de Toledo (PTO), Campinas (CAM), Sorocaba (SOR), Bauru (BAU), Bebedouro (BEB), Marilia (MAR), Araçatuba (ARA) and filled circles the samples of *B. occidentalis:* Campinas (CAP), Santa C. do Rio Pardo (SCP), Assis (ASS), São José do R. Preto (SJP), Presidente Prudente (PRU), Santo Anastácio (SAN).

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caria. When this did not occur, the snails were used to prepare homogenates for electrophoresis.

Electrophoresis - Digestive gland homogenates were prepared in 0.5% saline, placed in 0.25ml tubes and stored at -70°C until electrophoretic analysis was performed. The homogenates were collected with Whatman paper No. 3 and applied to the electrophoretic gel.

Analysis was carried out by horizontal electrophoresis. Starch or acrylamide gels were employed according to the enzyme system analyzed using the technique adapted by Malavasi and Morgante (1982). At least 30 specimens per enzymatic locus were analyzed according to the criterion of Hames and Rickwood (1987). A locus was considered to be polymorphic when the frequency of the rarest allele was 1% or higher.

The following enzyme systems were analyzed: alcohol dehydrogenase (ADH, Enzyme Comission number - E.C.1.1.1.1), ß-hydroxybutyrate dehydrogenase (HBDH, E.C.1.1.1.30), malate dehydrogenase (MDH, E.C.1.1.1.37), glutamate-oxaloacetate transaminase (aspartate aminotransferase) (GOT, E.C.2.6.1.1), phosphoglucomutase (PGM, E.C.2.7.5.1), α-esterase (EST, E.C.3.1.1.1), alkaline phosphatase (APH, E.C.3.1.3.1), leucine aminopeptidase (LAP, E.C.3.4.11.1) and glucose phosphate isomerase (PGI, E.C.5.2.1.9).

Data analysis - The methodology described by Nei (1972) was used to calculate the level of similarity (S). The gene frequency values were analyzed by the unweighted pair-group centroid clustering (UPGMC) method of Sneath and Sokal (1973).

RESULTS

Nine enzyme systems were analyzed for isozyme electrophoretic pattern and 14 enzymatic loci were observed and scored. In order to detect the amplitude of variation existing in the geo-

TABLE I

Eletrophoretic analysis for nine enzymatic systems. Comparative painting between *Biomphalaria tenagophila* and *B. occidentalis*

Classification of loci	Enzymatic systems	Allele number
Polymorphic I[a]:		
MDH2	Malate dehydrogenase	5
APH	Alkaline phosphatase	4
EST3	Esterase	5
Polymorphic II[b]:		
HBDH	B-Hydroxybutyrate dehydrogenase	3
EST1	Esterase	5
EST4	Esterase	5
Monomorphic I[c]:		
EST2	Esterase	1
PGI	Glucose phosphate isomerase	1
GOT1	Glutamate oxaloacetate transaminase	1
GOT2	Glutamate oxaloacetate transaminase	1
MDH1	Malate dehydrogenase	1
Monomorphic II[d]:		
ADH	Alcohol dehydrogenase	2
LAP	Leucine aminopeptidase	2
PGM	Phosphoglucomutase	2

[a]: variation in populations. Same alleles between the species
[b]: variation in populations. Distints alleles between the species
[c]: same patterns in two species
[d]: distincts patterns between the species

TABLE II

Allelic frequency in polymorphic loci in at least one of the species. *Biomphalaria tenagophila* (**BT**) and *B. occidentalis* (**BO**)

Loci	Samples	N	Alleles	Allele frequency				
				1.50	**1.20**	**1.00**	**0.90**	
APH	BT	366	3	.353	.028	.619	-	
	BO	228	2	-	-	.977	.023	
				1.20		**1.00**	**0.75**	
HBDH	BT	366	2	-		.694	.306	
	BO	228	1	1.00		-	-	
				1.20	**1.10**	**1.05**	**1.00**	**0.90**
MDH2	BT	366	3	-	.005	.033	.962	-
	BO	228	3	.175	-	-	.152	.673
				-1.80	**-1.20**	**-1.00**	**-0.70**	**-0.40[a]**
EST1	BT	366	2	-	-	.781	.219	-
	BO	228	3	.175	.482	-	-	.342
				1.20	**1.00**	**0.90**	**0.85**	**0.80**
EST3	BT	366	3	.004	.991	.005	-	-
	BO	228	3	-	.828	-	.018	.154
				1.10	**1.05**	**1.00**	**0.95**	**0.90**
EST4	BT	366	3	-	.645	-	.037	.321
	BO	228	2	.154	-	.846	-	-

[a]: (-) Anodic displacement

graphic region where the two species, *B. tenagophila* and *B.occidentalis*, occur, the allelic variation observed in the 15 populations sampled was summarized (Table I). According to this analysis, 6 of the 14 loci studied were polymorphic.

For polymorphic loci the allelic frequency for each species is presented in Table II. The polymorphic loci APH, MDH2 and EST3 share common mobility patterns in the two species. However, the HBDH, EST1 and EST4 loci always showed distinct alleles between species.

The results showed 6 diagnostic loci between species, 3 of them being polymorphic and 3 monomorphic (Table III; Fig. 2).

At the locations investigated, *B. occidentalis* consisted of predominantly monomorphic populations, although the alleles might differ among populations (Table IV). The results obtained for *B. tenagophila* (intrapopulation variation) have been analyzed in a previous study (Mascara & Morgante 1991). An estimate of the level of genetic variability in the two species of *Biomphalaria* is presented in Table V.

On the basis of these values, it was possible to calculate genetic similarity (S) between species, as well as among the populations of each species (Table VI). Among the *B. tenagophila* populations, S ranged from 0.714 (BAU-CAM and BAU-TAU) to 1.000 (CAM-TAU). The intrapopulation similarity of *B. occidentalis* ranged from 0.714 (SJP-PRU) to 0.999 (CAP-SAN and ASS-SCP). However, the highest S value between the two species was 0.514 (SOR-SAN and ARA-SAN).

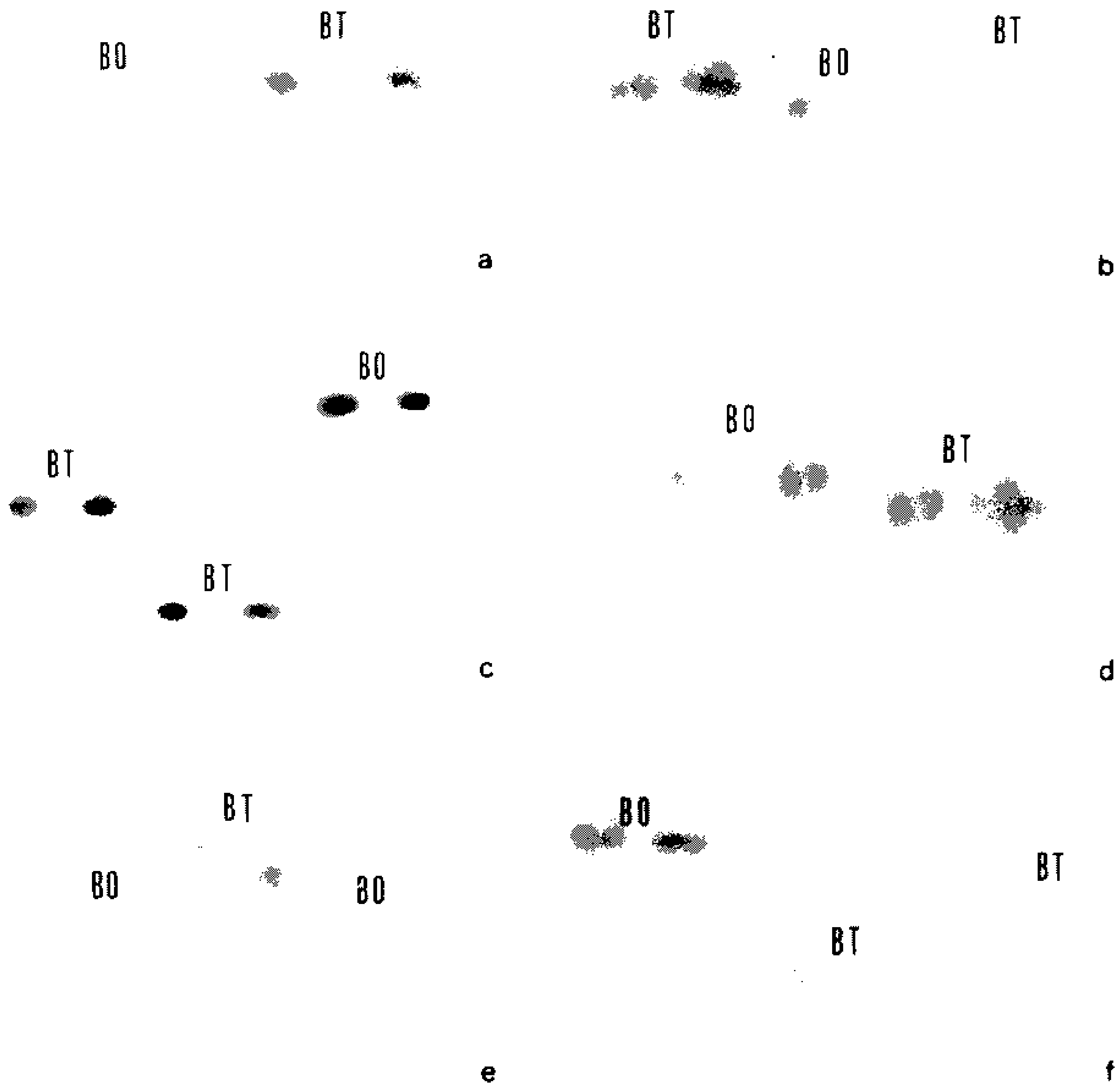

Fig. 2: isozyme patterns for the diagnostic loci for *Biomphalaria tenagophila* (BT) and *B. occidentalis* (BO) observed on the electrophoresis gels. **a**: alcohol dehydrogenase (ADH); **b**: phosphoglucomutase (PGM); **c**: β-hydroxybutyrate dehydrogenase (HBDH); **d**: leucine aminopeptidase (LAP); **e**: α- esterase 4 (EST4); **f**: α-esterase 1 (EST1).

The genetic similarity results among isozyme patterns permitted us to group the two species into distinct clusters (Fig. 3).

Henriksen and Jelnes (1980), when justifying biochemical systematics, pointed out that the results of isozyme analysis are of great importance when employed to separate morphologically close or cryptic species. Despite its limitations, the information related to isozyme variability permits to estimate genetic similarity among the samples analyzed.

TABLE III

Allelic frequencies of diagnostics loci for *Biomphalaria tenagophila* and *B. occidentalis*

Loci	Allelomorphs	*B. tenagophila* N = 366	*B. occidentalis* N = 228
ADH	1.20	1.000	-
	1.00	-	1.000
LAP	1.20	-	1.000
	1.00	1.000	-
PGM	1.10	1.000	-
	1.00	-	1.000
	1.20	-	1.000
HBDH	1.00	.694	-
	0.75	.306	-
	1.80	-	.175
	1.20	-	.482
EST1	1.00	.781	-
	0.70	.219	-
	0.40	-	.342
	1.10	-	.154
	1.05	.642	-
EST4	1.00	-	.846
	0.95	.037	-
	0.90	.321	-

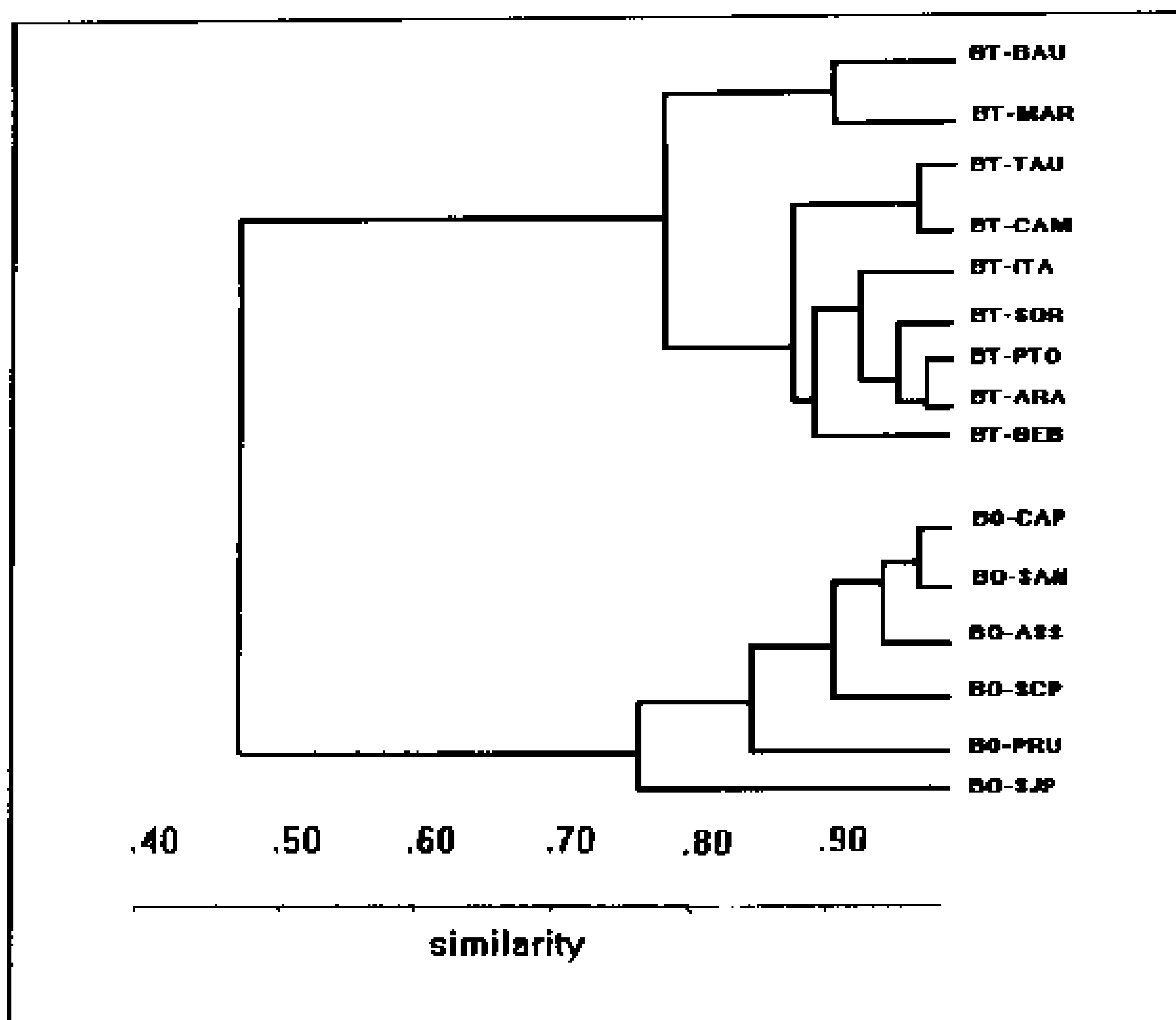

Fig. 3: dendrogram for the *Biomphalaria tenagophila* and *B. occidentalis* populations studied in the State of São Paulo, Brazil.

In this study the isozyme technique permitted not only species characterization but also, and importantly, the identification of populations originating from different locations (Malek & File 1971, Narang et al. 1981, Jelnes 1982, Woodruff et al. 1985).

The present study allowed the identification of species or genetic populations (Lewontin 1985) by the determining of the allelic divergence existing among populations. It is possible to estimate genetic divergence by the variation observed on the electrophoretic gel as long as a strategy of geographic sampling of natural populations is used (Bush & Kitto 1978, Buth 1984, Mascara & Morgante 1991). Furthermore, a comparative analysis of the results obtained makes it possible to correlate the isozyme variation observed with the level of genetic similarity existing among populations (Nei 1972).

However, the use of any taxonomic identification technique, especially isozyme variation, is of systematic value only when the variability of the trait studied is estimated in natural populations. Thus, we may affirm that in the area of contact between species, the loci reported to be diagnostic allow the distinction of populations of both species.

Analysis of the level of species variability (Table V) based on results obtained for each sample, presents a distortion resulting from the genetic structure of the populations. *Biomphalaria tenagophila* and especially *B. occidentalis* form predominantly monomorphic populations as the result of the founder effect in the colonization of breeding sites (Mascara & Morgante 1991). Table V shows the difference between mean heterozygosity (H) calculated from the variation at each locus and H obtained by the mean heterozygosity of each population.

Populations with distinct fixed alleles have a high rate of heterozygosity when the H value is obtained by variation per locus, however, such value is unreal since the populations are monomorphic. Thus, the method for obtaining the real level of variability of planorbid species is the calculation of the mean variability for each population.

The level of genetic similarity (Table III) obtained from the survey of isozyme variation was calculated among populations of both species. This method of analysis permitted us to individualize the two species populations (Fig. 3), which reinforces the relevance of the technique employed. Nonetheless, it did not allow a delimitation of geographic areas having a uniform gene set. All pulmonate molluscs are hermaphrodite

TABLE IV

Allele frequencies for the polymorphic loci among populations of *Biomphalaria tenagophila* and *B. occidentalis*

		Biomphalaria tenagophila											*Bionphalaria occidentalis*			
Loci	Alleles	BAU N = 45	TAU N = 38	ITA N = 35	SOR N = 68	PTO N = 35	CAM N = 40	MAR N = 40	BEB N = 35	ARA N = 30	CAP N = 30	SAN N = 45	SJP N = 35	ASS N = 40	PRU N = 40	SCP N = 38
	1.50	1.00	-	1.00	-	0.22	-	1.00	0.04	-	-	-	-	-	-	-
APH	**1.20**	-	-	-	0.15	-	-	-	-	-	-	-	-	-	-	-
	1.00	-	1.00	-	0.85	0.78	1.00	-	0.96	1.00	1.00	1.00	1.00	1.00	0.87	1.00
	0.90	-	-	-	-	-	-	-	-	-	-	-	-	-	0.13	-
	1.20	-	-	-	-	-	-	-	-	-	1.00	1.00	1.00	1.00	1.00	1.00
HBDH	**1.00**	-	1.00	1.00	0.64	1.00	1.00	0.06	1.00	0.84	-	-	-	-	-	-
	0.75	1.00	-	-	0.36	-	-	0.94	-	0.16	-	-	-	-	-	-
	1.20	-	-	-	-	-	-	-	-	-	-	-	-	-	1.00	-
	1.10	-	-	-	-	-	-	0.05	-	-	-	-	-	-	-	-
MDH2	**1.05**	-	-	0.13	0.10	-	-	-	0.02	-	-	-	-	-	-	-
	1.00	1.00	1.00	0.87	0.90	1.00	1.00	0.95	0.98	1.00	-	0.04	0.94	-	-	-
	0.90	-	-	-	-	-	-	-	-	-	1.00	0.96	0.06	1.00	-	1.00
	-1.80	-	-	-	-	-	-	-	-	-	-	-	-	-	1.00	-
	-1.20	-	-	-	-	-	-	-	-	-	1.00	1.00	1.00	-	-	-
EST1	**-1.00**	1.00	-	1.00	0.97	1.00	-	1.00	1.00	1.00	-	-	-	-	-	-
	-0.70	-	1.00	-	0.03	-	1.00	-	-	-	-	-	-	-	-	-
	-0.40	-	-	-	-	-	-	-	-	-	-	-	-	1.00	-	1.00
	1.20	-	-	-	0.02	-	-	-	-	-	-	-	-	-	-	-
	1.00	0.96	1.00	1.00	0.98	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	1.00	1.00	0.89
EST3	**0.90**	0.04	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.85	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.11
	0.80	-	-	-	-	-	-	-	-	-	-	-	1.00	-	-	-
	1.10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1.05	-	0.98	1.00	0.88	1.00	1.00	-	0.07	0.85	-	-	-	-	-	-
EST4	**1.00**	-	-	-	-	-	-	-	-	-	1.00	1.00	-	1.00	1.00	1.00
	0.95	-	0.02	-	0.12	-	-	-	-	0.15	-	-	-	-	-	-
	0.90	1.00	-	-	-	-	-	1.00	0.93	-	-	-	-	-	-	-

TABELA V

Summary statistics of genetic variation among populations of *Biomphalaria tenagophila* (**BT**) and *B. occidentalis* (**BO**)

	Mean sample size per loci	Mean No. of alleles per loci	Mean Heterozygosity expected	Loci Polymorphic (%)
BT	316.4 ± 20.22	2.0 ± 0.3	0.186 ± 0.07[a] 0.023 ± 0.025[b]	42.8[a] 13.5[b]
BO	234.6 ± 3.4	1.6 ± 0.3	0.120 ± 0.07[a] 0.007 ± 0.005[b]	30.0[a] 4.8[b]

[a]: this values have been estimated by genic frequencies for loci in each species
[b]: this values have been obtained by heterozygosity mean for each population

(Geraerts & Joosse 1984) and planorbid snails may also present a reproductive behavior which allows cross-fertilization and even self-fertilization. Thus, the recolonization of the breeding site may start from a single genome.

The results obtained in the present study are related to the colonization process which is consistent with the dynamics of natural populations of the genus *Biomphalaria*. Maked fluctuations in density detected in natural populations (Paraense 1956, Selander & Kaufman 1973). The possibility of recolonizing the environment by self-fertilization of the remaining specimens explains the observation of different fixed alleles in close populations, as well as the existence of a random geographic distribution of gene sets.

The variation in allele frequency among the populations analyzed (Table IV) may reflect the dispersal of strains by colonization of one or few individuals (Mascara & Morgante 1991). However, no selective effect was observed with respect to the enzymatic polymorphism detected. Thus, variation does not represent differences in the adaptative value of populations.

This evidence indicates that, in the genus *Biomphalaria*, the geographic isolation of genetic strains and the consequent allopatric speciation of these populations may represent the most probable form of species differentiation.

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TABLE VI

Genetic similarity (S) between *Biomphalaria tenagophila* and *B. occidentalis* from the State of São Paulo

Populations	*Biomphalaria tenagophila*									*Biomphalaria occidentalis*					
	BAU	TAU	ITA	SOR	PTO	CAM	MAR	BEB	ARA	CAP	SAN	SJP	ASS	PRU	SCP
Bauru (BAU)	-														
Taubaté (TAU)	0.714	-													
Itariri (ITA)	0.854	0.854	-												
Sorocaba (SOR)	0.837	0.917	0.924	-											
P. de Toledo (PTO)	0.811	0.924	0.954	0.986	-										
Campinas (CAM)	0.714	1.000	0.855	0.918	0.924	-									
Marília (MAR)	0.999	0.721	0.862	0.842	0.818	0.721	-								
Bebedouro (BEB)	0.861	0.862	0.864	0.927	0.929	0.861	0.868	-							
Araçatuba (ARA)	0.812	0.924	0.922	0.995	0.993	0.924	0.818	0.939	-						
Campinas (CAP)	0.427	0.501	0.432	0.511	0.491	0.501	0.432	0.502	0.511	-					
Sto. Anastácio (SAN)	0.431	0.505	0.436	0.514	0.495	0.504	0.436	0.506	0.514	0.999	-				
S.J.R. Preto (SJP)	0.427	0.498	0.421	0.502	0.489	0.498	0.426	0.498	0.507	0.793	0.798	-			
Assis (ASS)	0.427	0.501	0.432	0.511	0.491	0.501	0.432	0.502	0.511	0.929	0.928	0.722	-		
P. Prudente (PRU)	0.431	0.495	0.436	0.506	0.487	0.495	0.435	0.497	0.504	0.855	0.857	0.714	0.855	-	
S.C.R. Pardo (SCP)	0.422	0.496	0.427	0.506	0.486	0.496	0.427	0.497	0.505	0.927	0.927	0.727	0.999	0.853	-

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