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Short Communication

ITS2 variability of *Biomphalaria* (Mollusca, Planorbidae) species from the Paranapanema Valley (São Paulo State, Brazil): Diversity patterns, population structure, and phylogenetic relationships

Roseli Tuan and Priscila dos Santos

Laboratório de Bioquímica e Biologia Molecular, Superintendência de Controle de Endemias, Secretaria da Saúde do Estado de São Paulo, São Paulo, SP, Brazil.

Abstract

The ribosomal DNA internal transcribed spacer 2 (ITS2) has been shown to be a useful genetic marker for species identification and phylogenetic reconstruction in the genus of freshwater snails *Biomphalaria* (Preston 1910). Additionally, ITS2 studies in *Biomphalaria* have uncovered significant intra-specific genetic variability suggesting the presence of cryptic species complexes. We obtained ITS2 sequences for the *Biomphalaria* species *B. glabrata*, *B. tenagophila*, *B. occidentalis* and *B. peregrina* from the Paranapanema Valley (São Paulo State, Brazil) and compared them with a comprehensive set of published *Biomphalaria* ITS2 sequences using Bayesian inference of phylogeny. Analysis of the resulting trees showed that the newly obtained *B. glabrata* sequences did not cluster with those from other Brazilian localities and that sub-structuring occurred among Brazilian *B. tenagophila* populations. Moreover, although ITS2 sequences seem to indicate clear genetic differentiation within both *B. glabrata* and *B. tenagophila*, evidence in support of the occurrence of cryptic species is more compelling for the latter. We discuss the significance and implications of the detected patterns of ITS2 variability for taxonomic studies in *Biomphalaria*.

Key words: Bayesian inference, *Biomphalaria*, DNA sequences, internal transcribed spacer 2 (ITS2), ribosomal DNA.

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Species of planorbid snails belonging to the genus *Biomphalaria*, which includes the vectors of the blood fluke *Schistosoma mansoni*, have recently received increasingly attention in molecular systematic and phylogeography studies (Campbell *et al.*, 2000; Vidigal *et al*., 2000, 2004; DeJong *et al*., 2001; Mavárez *et al*., 2002; Pointier *et al*., 2005). These studies have largely supported the pivotal body of work on the taxonomy of Neotropical *Biomphalaria* carried out by malacologist Lobato Paraense (Paraense, 2001) and, additionally, uncovered interesting patterns of intraspecific variability (Mavárez *et al*., 2002; Vidigal *et al*., 2004).

Among the genetic markers used so far in studies on *Biomphalaria*, the nuclear ribosomal DNA (rDNA) internal transcribed spacers (ITS) have been shown to be particularly useful for species identification and phylogenetic reconstruction (Campbell *et al.*, 2000; Vidigal *et al*., 2000; Pointier *et al*., 2005). Furthermore, ITS sequence studies have revealed and/or confirmed sub-structuring within widely distributed species such as *B. glabrata* and *B. tenagophila* (Mavárez *et al*., 2002; Vidigal *et al*., 2004), adding to the body of evidence indicating that these nominal taxa may constitute complexes of cryptic species (Woodruff and Mulvey, 1997; Spatz *et al*., 1999). In these circumstances, the investigation of genetic variability patterns across the range of distribution of these species takes a prominent role in helping to elucidate how many independently evolving entities there are and which geographical areas they occupy.

In this study, we obtained nuclear ribosomal DNA (rDNA) internal transcribed spacer 2 (ITS2) sequences from *B. glabrata*, *B. tenagophila*, *B. occidentalis* and *B. peregrina* specimens collected in the Paranapanema Valley region (São Paulo State, Brazil). The newly obtained sequences were compared to all other ITS2 sequences published to date for the same species. In doing so, we addressed the following issues: polymorphism and divergence patterns, phylogenetic relationships, and the possible occurrence of species complexes in *Biomphalaria*.

We collected 16 *Biomphalaria* snails (Table 1) along the banks of different streams of the Paranapanema River in Ourinhos (lat -22.98, lon -49.87) and Ipauçu (lat -23.05, lon -49.62) and extracted total genomic DNA from the foot tissue of individual specimens using DNeasy Tissue Kits

Send correspondence to Roseli Tuan. Superintendência de Controle de Endemias, Rua Paula Souza 166, 5 ° andar 01027-000 São Paulo, SP, Brazil. E-mail: rtuan@sucen.sp.gov.br.

Table 1 - *Biomphalaria* rDNA ITS2 sequences used in the study.

Species and localities where collected	Sequence code	ITS2 GenBank accession numbers
B. glabrata		
Ourinhos (SP), Brazil	*BglOur 1 BglOur 2 BglOur 3*	DQ143952 DQ143953 DQ143954
Esteio (RS), Brazil	*BglEst 1 BglEst 2*	AF198660[1] AF198661[1]
Belo Horizonte (mg), Brazil	*BglBH*	AF449582[3]
Salvador (BA), Brazil	*BglSal 1 BglSal 2*	AY030376[2] AF449581[3]
Belém (PA), Brazil	*BglBel*	AF198659 [1]
Aragua, Venezuela	*BglAra*	AY030377[2]
Calicanto, Venezuela	*BglCal*	AF449589[3]
Caripe, Venezuela	*BglCar*	AF449583[3]
Chuao, Venezuela	*BglChu*	AF449588[3]
Los Naranjos, Venezuela	*BglLN 1 BglLN 2*	AF449584[3] AF449585[3]
Portuguesa, Venezuela	*BglPor*	AF198662[1]
Punta Cabito, Venezuela	*BglPC*	AF449591[3]
Tres Lances, Venezuela	*BglTL 1 BglTL 2*	AF449586 [3] AF449587[3]
Zuata, Venezuela	*BglZua*	AF449590[3]
Pierrot, Saint-Lucia	*BglPie*	AF449596[3]
Maupéou, Martinique	*BglMau*	AF449593[3]
Grand Camp, Guadeloupe	*BglGC*	AF449595[3]
Vallet, Guadeloupe	*BglVal*	AF449592 [3]
Vieux-Fort, Guadeloupe	*BglVF*	AF449594[3]
Rio Grande Town, Puerto Rico	*BglRG*	AY030375[2]
Jarabacoa, Dominican Republic	*BglJar*	AY030374[2]
B. tenagophila		
Ourinhos (SP), Brazil	*BttOur 1-2 BttOur 3 BttOur 4 BttOur 5*	DQ143955 DQ143956 DQ143957 DQ143958
Ipauçu (SP), Brazil	*BttIpa 1-4*	DQ143959
Imbé (RS), Brazil	*BttImb*	AF198655[1]
Taim (RS), Brazil	*BttTai*	AY631860[5]
Contagem (MG), Brazil	*BttCon*	AF449614[3]
Vespasiano (MG), Brazil	*BttVes*	AF198654[1]
Formosa (GO), Brazil	*BttFor 1 BttFor 2*	AF198656 [1] AY030388[2]
Itamaraju (BA), Brazil	*BttIta*	AY631859[5]
Asunción, Paraguay	*BttAsu*	AY030387[2]
Araza, Argentina	*BttAra*	AY425746[4]
Bonpland, Argentina	*BttBon*	AY425745[4]
Kinshasa, Democratic Republic of Congo	*BttKin*	AY631861[5]
B. t. guaibensis		
Esteio (RS), Brazil	*BtgEst*	AY425749[4]
Guaíba (RS), Brazil	*BtgGua*	AY425750[4]
Termas, Uruguay	*BtgTer*	AY425751[4]
B. occidentalis		
Ourinhos (SP), Brazil	*BocOur 1-2*	DQ143960
Caceres (MG), Brazil	*BocCac*	AY030389[2]
Capetinga (MG), Brazil	*BocCap*	AF198658[1]
Campo Grande (MS), Brazil	*BocCG*	AF198657[1]
Triângulo, Argentina	*BocTri*	AY425748[4]
Villa Chica, Argentina	*BocVC*	AY425747[4]
B. peregrina		
Ourinhos (SP), Brazil	*BpeOur 1 BpeOur 2*	DQ143961 DQ143962
Taim (RS), Brazil	*BpeTai*	AF198677[1]
Alfenas (MG), Brazil	*BpeAlf*	AF198676[1]
Bom Jesus da Penha (MG), Brazil	*BpeBJ*	AF198678[1]
Nova Lima (MG), Brazil	*BpeNL*	AY030401[2]
San Antonio, Uruguay	*BpeSA*	AY030400[2]

[1]Vidigal *et al.* (2000); [2]DeJong *et al.* (2001); [3]Mavárez *et al.* (2002); [4]Vidigal *et al.* (2004); [5]Pointier *et al.* (2005).
Acronyms for Brazilian states shown between parentheses: BA = Bahia; GO = Goiás; mg = Minas Gerais; PA = Pará; RS = Rio Grande do Sul; and SP = São Paulo.

(Qiagen). The nuclear rDNA ITS2 was amplified by the polymerase chain reaction (PCR) using the ITS2F and ETTS1 primers (Vidigal *et al*., 2000) in a 50 µL total volume of amplification mixture containing 10-100 ng of *Biomphalaria* DNA, 0.2 mM of each dNTP, 0.10 µm of each primer and 1 U of *Taq* DNA polymerase (BioLabs) with the supplied buffer. The following PCR temperature profile was used: a initial 4 min denaturation step at 94 °C followed by 35 iterations of 30 s at 94 °C, 1 min at 60 °C and 1 min at 72 °C plus a final 7 min extension at 72 °C. The PCR products were checked by agarose gel electrophoresis, cleaned using QIAquick PCR Purification Kits (Qiagen) and cloned using TOPO TA Cloning (Invitrogen) or pGEM-T (Promega) kits. One recombinant clone was sequenced per individual. Sequencing was carried out at the Human Genome Research Center (CEGH, São Paulo).

Sequences were corrected using the program Chromas (Technelysium Pty Ltd.), aligned using the program ClustalX version 1.8 (Thompson *et al*., 1997) and the alignments subsequently adjusted by eye using the program BioEdit version 7.0 (Hall, 1999). Two sequence alignments were built: one for the Ourinhos-Ipauçu data set, and another including a number of published sequences, also listed in Table 1. Nucleotide diversity (π, Nei, 1987, equations 10.5 or 10.6 and 10.7) and nucleotide divergence between sequence groups (D_{xy}, Nei, 1987, equation 10.20 using Jukes and Cantor correction) were calculated using the program DnaSP version 4.1 (Rozas *et al*., 2003).

The program MrModeltest version 2.0 (Nylander, 2004) was used to determine the best-fit model of DNA sequence evolution and model parameters for the sequence alignments. The data was used to choose the model form for Bayesian inference of phylogeny as implemented in the program MrBayes version 3.1 (Huelsenbeck and Ronquist, 2001). Two simultaneous independent searches were run for 1.5 x 10^6 generations, with trees saved every 100 generations, and the first 1,500 sampled trees of each search discarded as "burn-in". The data obtained in MrModeltest 2.0 was also used to obtain maximum-likelihood (ML) trees using the program PAUP* version 4.0 (Swofford, 2002), employing the heuristic search option and 100 random taxon addition replicates to improve tree searches. Clade support was assessed by 500 bootstrap pseudo-replicates, again with random addition of taxa (for the Ourinhos-Ipauçu data set only). Additionally, maximum parsimony (MP) trees were obtained using PAUP* 4.0 heuristic search option with 100 random additions, followed by computation of strict consensus trees and 1,000 bootstrap pseudo-replicates with random addition of taxa for measure of support. Gaps were treated as missing data. In all phylogenetic analyses *B. peregrina* was designated the outgroup.

The ITS2 fragments sequenced displayed a length range of 424-449 bp (primers excluded). The final alignment matrix for the 16 sequences (9 *B. tenagophila*, 3 *B. glabrata*, 2 *B. occidentalis* and 2 *B. peregrina*) consisted of 468 characters, 69 of which were parsimony informative. The best-fit model of sequence evolution suggested by MrModeltest 2.0 for this alignment was the general time reversible (GTR) plus G (gamma distribution for rate variation among sites). The majority rule consensus tree obtained from the convergence of two independent Bayesian runs is shown in Figure 1. Exactly the same topology was observed in the single resulting ML tree (- ln*L* = 1094.27). MP searches yielded four equally parsimonious trees of length 87, consistency index (CI) = 0.977 and retention index (RI) = 0.987. The strict consensus showed the same topology of the Bayesian/ML trees, except for the clade containing the *B. glabrata* sequences, which became an unresolved trichotomy.

The tree depicted in Figure 1 shows a close relationship between *B. tenagophila* and *B. occidentalis*, with this grouping being well differentiated from both *B. glabrata* and *B. peregrina* clades. These results are in accordance with previous ITS2 studies using the same species (Vidigal *et al*., 2000; DeJong *et al*., 2001; Pointier *et al.*, 2005). It is noteworthy, though, that some differentiation between *B. tenagophila* from the two collection sites was noticeable in the tree.

For comparison with previous studies, 48 *Biomphalaria* ITS2 sequences were added to the Ourinhos-Ipauçu data set, yielding a sequence alignment of 497 characters, 82 of which were parsimony informative. Identical sequences (in the Ourinhos-Ipauçu data set) and sites 474 to 497 were removed before analyses. Included in the alignment were all *B. glabrata*, *B. tenagophila*, *B. occidentalis* and *B. peregrina* ITS2 sequences presented in Vidigal *et al*. (2000, 2004), DeJong *et al*. (2001), Mavárez *et al*. (2002; except AF449580, due to sequence incompleteness) and Pointier *et al*. (2005). Figure 2 depicts the

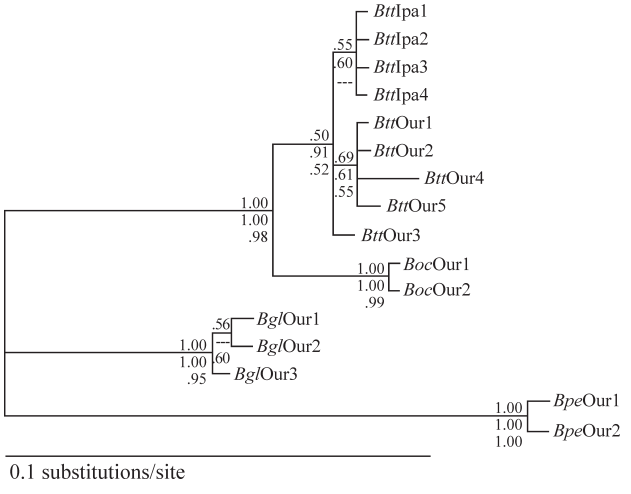

Figure 1 - Bayesian majority rule consensus phylogram for the Ourinhos-Ipauçu ITS2 data set. Values above nodes indicate clade posterior probabilities and those below refer to maximum parsimony (MP) and maximum likelihood (ML) bootstrap proportions, respectively (values lower than .50 are not shown).

Bayesian majority rule consensus tree obtained for this data set, using the parameters form of the GTR + G model, as recommended by MrModeltest 2.0. The ML analysis produced two trees of - ln*L* = 1610.16, differing minimally from one another: tree # 1 had *Bgl*BH sister to *Bgl*Est ½ and tree #2 displayed the same topology for the *B. glabrata* clade as the Bayesian one. Both ML trees also differed from the Bayesian one by a minor variation in the *B. peregrina* clade (*Bpe*BJ sister to *Bpe*Tai/*Bpe*SA). Evaluation of clade support for the ML trees via bootstrapping was precluded by computing limitations. The MP analysis yielded an incalculable number of trees of length 160 (CI = 0.863, RI = 0.977), but the strict consensus of the first 100,000 retained trees produced the same topology as the Bayesian tree, except that *Bgl*Est 1 and *Bgl*Est 2 did not cluster together. MP bootstrap values (maximum number of trees retained per iteration set to 200) are indicated in Figure 2.

All *B. glabrata* sequences were recovered in a monophyletic clade, containing a set of defined branches denominated V1, V2 and B in Figure 2. These branches were also characterized by Mavárez *et al*. (2002), using concatenated ITS2 and mitochondrial 16S rDNA sequences. Venezuela harbors two distinct ITS2 lineages (V1 and V2) and, interestingly, the Venezuelan sample reported in Vidigal *et al*. (2000) clearly belongs to subgroup V2, showing that this clade is more widespread than previously thought. The Brazilian clade (B), also reported by Mavárez *et al*. (2002) for two sequences (*Bgl*BH and *Blg*Sal 2), contained all other Brazilian sequences published to date (*Bgl*Sal 1, DeJong *et al*., 2001; *Bgl*Est 1-2, *Bgl*Bel, Vidigal *et al*., 2000) except the ones from Ourinhos (*Bgl*Our 1-3), these latter sequences appearing in an unstructured, more basal, region of the *B. glabrata* clade, along with the sequences from Central America (*Bgl*Pie, *Bgl*Mau, *Bgl*GC, *Bgl*Val, *Bgl*VF, *Bgl*RG, *Bgl*Jar).

In contrast to *B. glabrata*, the clade containing *B. tenagophila*, *B. t. guaibensis*, and *B. occidentalis* sequences displayed a polyphyletic pattern (the *B. tenagophila* complex, Spatz *et al*., 1999; Vidigal *et al*., 2004). The *B. tenagophila* sequences were observed in two different branches (T1 and T2). The two *B. occidentalis* samples from Argentina (*Boc*Tri and *Boc*VC) also appeared on separate branches, one clustered with *B. t. guaibensis* sequences (G/O), the other with the remaining *B. occidentalis* (O). However, unlike in Vidigal *et al*. (2004), support for joint monophyly of branches G/O and O was not obtained.

Interestingly, sub-structuring within the *B. tenagophila* T1 branch was observed, extending the differentiation characterized for the Ourinhos/Ipauçu data set to other samples (T1 groups a and b). Additionally, some underived *B. tenagophila* sequences were recovered, in the same fashion of the *B. glabrata* clade. In fact, substructuring within *B. tenagophila* T1 makes the nucleotide diversity in this branch only ($\pi = 0.893\% \pm 0.232$) comparable to that found in the entire *B. glabrata* data set

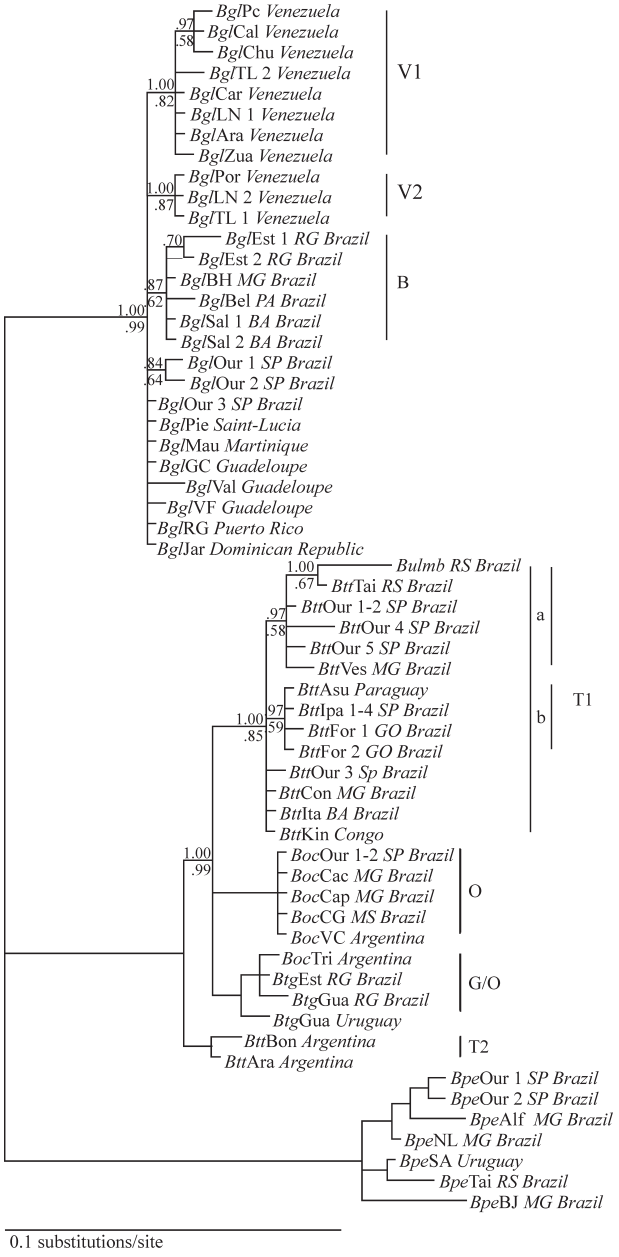

Figure 2 - Bayesian phylogenetic tree for *Biomphalaria* ITS2 sequences. Values above and below nodes indicate clade posterior probabilities and maximum parsimony (MP) bootstrap proportions, respectively.

($\pi = 0.729\% \pm 0.084$). Divergence between branches within *B. glabrata* and *B. tenagophila* T1 is also similar (*e.g.* $D_{V1\text{-}V2} = 1.277\% \pm 0.338$, $D_{T1a\text{-}b} = 1.215\% \pm 0.434$). However, when the entire *B. tenagophila* complex is considered it becomes apparent that not only is it comprised of highly diverse sequences ($\pi = 1.604\% \pm 0.176$) but also that divergence within the nominal *B. tenagophila* is far larger ($D_{T1\text{-}T2} = 2.029\% \pm 0.532$) than within the nominal *B. glabrata* (for which $D_{V1\text{-}V2}$ gives the highest pairwise value). Finally, the *B. peregrina* clade also encompasses a set of divergent sequences, which is reflected in a remark-

ably high level of nucleotide diversity (π = 1.823% ± 0.412).

Whereas cross validation with independent biological markers remains a critical step in assessing the nature and significance of ITS2 variability in *Biomphalaria*, the patterns of genetic differentiation revealed in ITS2 studies nonetheless provide significant information about barriers to gene flow. Thus, it is remarkable to observe fixed differences between *B. tenagophila* samples from Ourinhos and Ipauçu, localities no more than 30 km apart, and this finding may indicate low connectivity between these sites and deserves further investigation. It is also noteworthy that *B. glabrata* sequences from Ourinhos did not cluster with the remaining samples from Brazil, and in this case additional sampling within São Paulo and other Brazilian states will be needed to elucidate the significance of this finding on a broader phylogeographical scale. Nonetheless, we note that in both cases the low magnitude of change (π < 1%) seems to indicate these are likely to be examples of intraspecific polymorphism. Actually, a cohesive status for *B. tenagophila* T1 and *B. glabrata* samples is suggested not only by the magnitude, but also by the pattern of distribution of ITS2 variability. The latter is apparent in the structure of both clades, which exhibit a few distinct but short branches and a set of undifferentiated sequences displayed in a "rake" fashion – the typical pattern of continuous sequence variability characteristic of single species (Crandall and Templeton, 1993). We investigated this pattern further by examining statistical parsimony networks obtained with the program TCS (Clement *et al*., 2000) separately for *B. glabrata* and *B. tenagophila* T1 sequences. In both cases, the most undifferentiated (zero length branches) sequences (*Bgl*Our 3, *Bgl*Pie, *Bgl*Mau, *Bgl*GC, *Bgl*RG and *Bgl*Jar for *B. glabrata*, *Btt*Con, *Btt*Ita and *Btt*Kin for *B. tenagophila* T1) occupied central (ancestral) positions in the networks, holding most of the mutational connections with other sequences (data not shown). One direct implication of this finding is that, considering the presumably recent introduction of *B. tenagophila* into Africa (Pointier *et al*., 2005), ITS2 genotyping would offer limited power for source estimation of the Kinshasa population – the use of multiple and/or more sensitive genetic markers would be required in this case. However, since the reliability of network inferences (in particular the assignment of ancestral sequences) depends on dense populational sampling (Crandall and Templeton, 1993), and is, in general, an inadequate tool for use on data sets obtained mainly for taxonomic studies, these results must be regarded with caution, solely as preliminary conjectures to be tested with additional data.

When the *B. glabrata* clade was compared with the entire *B. tenagophila* complex clade, however, a more intricate picture emerged: *vis-à-vis*, the *B. glabrata* clade was shallower with less differentiated branches than the *B. tenagophila* complex clade (Figure 2). Here, both the degree and the pattern of differentiation of *B. tenagophila* T1 and T2 branches supply compelling evidence that these samples may belong to distinct, yet cryptic, species.

Finally, in our reappraisal of the ITS2 data it was interesting to identify that the highest level of sequence diversity among all the species analyzed was for *B. peregrina*. Historically, species of greater epidemiological importance such as *B. glabrata* have received more attention in population genetic studies, and their respective levels of genetic diversity, therefore, emphasized (*B. glabrata* was once called "the most differentiated mollusc species yet described", Woodruff and Mulvey, 1997). However, as data from multi-species studies accumulates it becomes evident that several Neotropical *Biomphalaria* species (such as *B. tenagophila* and *B. peregrina*) show remarkably high levels of genetic variability. This is a relevant finding from both epidemiological and evolutionary viewpoints and supports, for instance, the hypothesis of a Neotropical origin for the genus (Campbell *et al*., 2000; DeJong *et al*., 2001). Moreover, it highlights the need for further studies aimed at investigating patterns of population structure and species limits for these taxa.

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