FOLIA MALACOLOGICA
Folia Malacol. 26(4): 221–229
ISSN 1506-7629 (print) • ISSN 2300-7125 (on-line)
The Association of Polish Malacologists
Faculty of Biology, Adam Mickiewicz University
Bogucki Wydawnictwo Naukowe
Poznań, December 2018
https://doi.org/10.12657/folmal.026.019

GENETIC DIVERSITY OF THE FRESHWATER SNAIL *BIOMPHALARIA TENAGOPHILA* (D'ORBIGNY, 1835) (GASTROPODA: HYGROPHILA: PLANORBIDAE) ACROSS TWO COASTAL AREAS OF SOUTHEAST BRAZIL

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ABSTRACT: This study uses two mitochondrial (*COI, 16SrRNA*) and one nuclear marker (*ITS2*) to explore genetic diversity between populations of *B. tenagophila* (d'Orbigny), the most common intermediate host for *Schistosoma mansoni* in southeast Brazil. The snails were collected in freshwater bodies along the southern coastal region of Ribeira do Iguape river basin and along the northern coastal region of the state of São Paulo, Brazil. The estimates of genetic differentiation between the populations (Φ) indicate that there is no genetic isolation between northern and southern populations. The haplotype diversity observed in *B. tenagophila* shows a complex pattern of distribution: in the southern region, the genetic variation in *B. tenagophila* is partitioned into small and more heterogeneous mitochondrial haplotypes, whereas in the northern region there were one to three homogeneous haplotypes. The results may suggest a different colonisation history across the study area.

KEY WORDS: *Biomphalaria tenagophila*, genetic differentiation, *16SrRNA* mitochondrial DNA, *ITS2*, *COI*, schistosomiasis

INTRODUCTION

The freshwater snail *Biomphalaria tenagophila* (d'Orbigny, 1835) is widely distributed in the Neotropics acting as an intermediate host for *Schistosoma mansoni* in southeast Brazil (PARAENSE 2001). As other Hygrophila, *B. tenagophila* is able to self- and cross-fertilise. Under experimental conditions, B. *tenagophila* showed high rates of self-fertilisation (GUIMARÃES et al. 2016) suggesting that this mode of reproduction is an important and efficient component of the mating system in the species.

There is evidence of partition of genetic variation in the genus *Biomphalaria* in different mitochondrial and nuclear haplotypes on a fine geographical scale, indicating that even geographically close populations are genetically differentiated (MULVEY & VRIJENHOEK 1982, LANGAND et al. 1999, MAVÁREZ et al. 2002, TUAN & SANTOS 2007, TUAN et al. 2012). The structuring of the genetic variability of *Biomphalaria* populations on a fine geographical scale may be a result of reproductive characteristics and physiological adaptations that allow the snails to survive droughts and floods through desiccation by reducing their metabolic activity (BARBOZA et al. 1985, VIANEY-LIAUD & LANCASTRE 1986, TUAN & SIMÕES 1989, BARBOSA et al. 2012, WEIR & SALICE 2012).

In São Paulo state, Brazil, there are two distinct drainage regions. In the northern region, the freshwater ecosystems are formed by independent rivers which start in the Serra do Mar mountain range and flow into the coastal plain (HENRIQUES et al. 2015). In

Ministry of Science and Higher Education
Republic of Poland

Folia Malacologica is funded by the Ministry of Science and Higher Education, Republic of Poland, under agreement no 534/P-DUN/2018 of April 4th, 2018 allocated to the activities for disseminating science:
Task 1: Preparation of English versions of publications (sum funded by DUN 12,000 PLN) and
Task 2: Digitalisation of publications and scientific monographs to enable their open access in the Internet (sum funded by DUN 11,070 PLN).

the southern region, the Serra do Mar gives way to the Ribeira do Iguape valley which consists of large uninterrupted plains, dominated by the Ribeira do Iguape river, that flow from the foothills of Paranapiacaba mountains in southern Brazil to the Atlantic Ocean (Sevá Filho & Kalinowski 2012). The tributaries of the Ribeira do Iguape flow side-by-side in close proximity (Theodorovicz & Theodorovicz 2007). These regions are geographically separated by the Jureia Ecological Reserve, an area with intact habitats (Por & Imperatriz-Fonseca 1984).

The restricted natural level of connectivity amongst these drainage systems thereby offers an opportunity to speculate on the reasons why the freshwater discontinuity could impact the genetic variability of *B. tenagophila*, a species spread over both the southern and the northern freshwater systems.

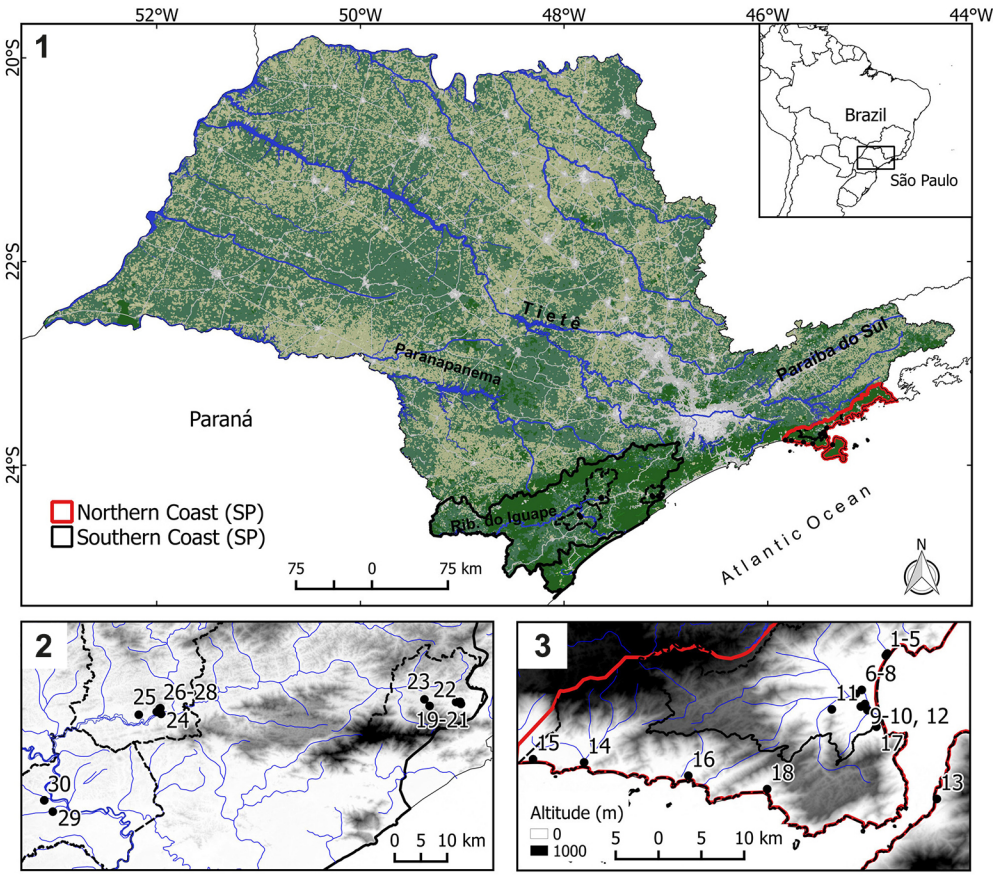
In this study, PCR-amplified sequences from *COI* and *16SrDNA* (*16S*) mitochondrial genes and the second internal transcribed spacer of nuclear ribosomal DNA (*ITS2*) were used to analyse the genetic variation in *B. tenagophila*. The mitochondrial genes were used because of their efficacy in inferring genetic patterns in populations associated with the effects of demographic variation within a species (Avise et al. 1984) and *ITS2* because it had previously yielded robust phylogenetic signals in the characterisation of genetically differentiated lineages of *B. tenagophila* (Tuan & Santos 2007).

Our findings show genetic population fragmentation of *B. tenagophila* in different population units within the southern and northern regions rather than between them.

MATERIAL AND METHODS

STUDY AREA

A total of 1,601 specimens were collected in thirty sites in freshwater ditches, streams and canals in the northern and southern regions along the coast of the state of São Paulo. In the northern region, snails were collected in Caraguatatuba, São Sebastião and Ilhabela, a municipality on the island of São Sebastião, which is 2.4 km from the mainland. In the southern region, snails were collected in Registro, Itariri and



Figs 1–3. Geographical location of the sampling sites: 1 – northern and southern regions; 2 – southern region (Itariri: 19–23; Juquia: 24–28, Registro: 29–30); 3 – northern region (Caraguatatuba: 1–12; Ilhabela: 13; São Sebastião: 14–18)

Table 1. Coordinates of sampling sites along the southern Atlantic coast in the state of São Paulo, Brazil. Code GenBank of 60 sequences of *16S*, 38 sequences of *COI* and 31 sequences of *ITS2*

Region	sampling site*	Coordinates		Code	*16S*	*COI*	*ITS2*
Northern	1–5	23°38'03.2"S	45°25'14.3"W	N10	KF840610	KF926106	KF840649
				N11	KF840615	KF926105	
		23°37'59.6"S	45°25'11.4"W	N17	KF840601		
				N18	KF840587		
		23°37'55.7"S	45°25'08.8"W	N24	KF840619	KF926218	
				N29	KF840612	KF926221	KF840655
		23°38'04.2"S	45°25'14.7"W	N30	KF840614	KF926217	
				N34	KF840620		
	6–8	23°40'26.1"S	45°26'54.3"W	N13	KF840605	MF380482	KF840652
				N21	KF840590		
		23°40'42.2"S	45°27'18.5"W	N22	KF840591		
				N27	KF840594	KF926214	
				N31	KF840597	KF926215	
	9–10	23°41'24.3"S	45°26'41.2"W	N12	KF840586		
				N14	KF840613		KF840648
		23°41'34.8"S	45°26'58.1"W	N23	KF840592		KF840654
				N26	KF840595	KF926216	
	11	23°41'46.4"S	45°28'57.9"W	N19	KF840588		KF840653
				N20	KF840589		
				N25	KF840593		
				N28	KF840596	KF926220	
	12	23°41'49.5"S	45°26'30.8"W	N1	KF840606	KF926136	
				N2	KF840611	KF926138	KF840656
				N8	KF840608		KF840650
				N15	KF840603	KF926222	KF840651
				N33	KF840598		
				N35			KF840657
	13	23°47'56.4"S	45°21'44.0"W	N16	KF840599	KF926212	
				N32	KF840600	KF926213	KF840664
	14	23°45'25.3"S	45°46'00.7"W	N7	KF840607	MF380479	KF840661
				N9	KF840618	MF380481	KF840660
	15	23°45'12.2"S	45°49'31.9"W	N6	KF840617	MF380478	KF840663
	16	23°46'20.2"S	45°38'50.5"W	N3	KF840604	MF380475	KF840658
	17	23°42'58.4"S	45°25'53.7"W	N5	KF840602	MF380477	KF840662
	18	23°47'15.6"S	45°33'24.9"W	N4	KF840609	MF380476	KF840659
Southern	19	24°18'13.6"S	47°04'31.7"W	S17	KF840636	KF926210	KF840678
	20	24°18'11.8"S	47°04'04.1"W	S15	KF840627	KF926209	KF840676
				S16	KF840645		
	21	24°18'26.3"S	47°03'58.9"W	S12	KF840644	MF380480	KF840674
				S19	KF840646	KF926207	
				S20	KF840647		
	22	24°18'39.9"S	47°07'31.4"W	S13	KF840628	KF926206	KF840675
				S14	KF840635		KF840677
	23	24°17'55.0"S	47°08'06.8"W	S18	KF840638	KF926211	KF840679
	24	24°19'33.6"S	47°37'53.5"W	S5	KF840630	KF926147	KF840673
				S6	KF840633	KF926151	
				S7	KF840634	KF926149	KF840672
	25	24°19'39.5"S	47°40'25.0"W	S24	MF380486	KT225580	
	26	24°18'56.7"S	47°38'03.1"W	S8	KF840631	KF926148	KF840669
				S9	KF840632		KF840671
				S10	KF840629		
	27	24°18'55.1"S	47°37'58.6"W	S21	MF380483	KT225577	
				S22	MF380485	KT225579	
				S23	MF380484	KT225578	
				S25	MF380487		
				S26	MF380488		
	28	24°19'19.7"S	47°38'22.9"W	S11	KF840623		KF840670
	29	24°30'37.0"S	47°50'07.9"W	S1	KF840637	MF380474	KF840667
	30	24°29'20.1"S	47°51'06.1"W	S2	KF840626		KF840666
				S3	KF840625	KF926153	
				S4	KF840624	KF926152	KF840668

*sampling sites: Caraguatatuba (1–12), Ilhabela (13), São Sebastião (14–18), Itariri (19–23), Juquia (24–28), Registro (29–30).

Juquiá. The collection points were geo-referenced with a Garmin eTrex 10 (Table 1). Both regions are subject to flooding when the river channels on the plains overflow (Souza 2005) (Figs 1–3, Table 1).

DNA EXTRACTION, PCR AMPLIFICATIONS, GENETIC, POPULATION STRUCTURE AND PHYLOGENETIC ANALYSIS

A portion of each live specimen's foot, large enough to extract DNA using a Qiagen Blood and Tissue kit, was excised. Each snail was then identified taxonomically using the anatomical characteristics of the reproductive system following Paraense (1975, 1981). The specimens used for taxonomic identification and genetic study were preserved in Fixative Railliet-Henry's fluid. The genomic DNA obtained from each specimen was stored in −18 °C freezers in the Molecular and Biochemical Laboratory at SUCEN. The different kinds of molecular markers used in this study were obtained from the same specimen.

Part of the *COI* gene (~600 bp) was amplified with the LCO1490/HCO2198 primers (Folmer et al. 1994) as described in Palasio et al. (2017). The *16S* mitochondrial gene was amplified by PCR using the 16SAr and 16SBr primers (Palumbi 1996) as described in Tuan et al. (2012) and *ITS2* was amplified with the ITS2F and ETTS1 primers (Vidigal et al. 2000) following the amplification protocol described in Tuan & Santos (2007). All polymerase chain reactions (PCR) were carried out in a total volume of 50 μl as described in Palasio et al. (2017). The amplified *ITS2* fragments were cloned using pGEM-T (Promega). Five clones were sequenced for each animal. All amplification reactions were carried out in an MJ Research thermal cycler. PCR products were visualised in agarose gel (1.4%) stained with Gel Red. DNA fragments were purified with a Qiagen purification kit. DNA concentration was estimated using a Low DNA Mass Ladder (Invitrogen). PCR products were purified with a Qiagen purification kit and then sequenced in the Biotechnology Centre at the Butantan Institute in an ABI3100 automated sequencer (Applied Biosystems®). The sequences were aligned in ClustalX (Thompson et al. 1997) and then corrected manually with BioEdit 7.0.5.3 (Hall 1999).

The analyses of genetic parameters in the northern and southern regions were considered separately through genetic diversity measures based on the differences calculated in different sequences. First, genetic differences between *16S* sequences were analysed under the Kimura 2-parameter nucleotide-substitution model using PAUP (Swofford 2002). A neighbour-joining consensus tree was generated with 1,000 bootstrap replicates. The tree was used to select sequences for which the *COI* and *ITS2* would be analysed. Sequences that met the following criteria were chosen: belonging to differentiated mitochondrial lineages and having come from different collection points.

All the measures were calculated using DnaSP 5.1 (Librado & Rozas 2009). The data set with 38 *COI*, 60 *16S* and 31 *ITS2* aligned sequences were collapsed into unique *COI*, *16S* and *ITS2* haplotypes using DNAsp v.5 (Librado & Rozas 2009). The *COI*, *16S* and *ITS2* sequences were submitted to GenBank.

The relationship between the haplotypes was investigated using algorithms implemented in TCS 2.1 (Clement et al. 2000), which clusters sequences by haplotype, calculates the frequency of these haplotypes in each location and estimates the relationships between them. Analysis of molecular variance (AMOVA) was used to estimate the level of variation amongst and within the northern and southern groups. Genetic structure indices were calculated in Arlequin 3.5.1.2 (Excoffier & Lischer 2010). The statistical significance of the AMOVA was calculated after 1,023 permutations of the data to simulate the null hypothesis. Appropriate nucleotide substitution models were selected with MrModeltest 2.3 (Nylander 2004).

The intraspecific phylogeny of *B. tenagophila* was inferred using a Bayesian Markov-Chain Monte Carlo (MCMC) analysis in MrBayes 3.1 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003). Four simultaneous independent searches were run for 1.5×10^6 generations, with trees saved every 100 generations and with the first 1,500 sampled trees of each search being discarded as "burn-in".

RESULTS

COI, *16S* AND *ITS2* DIVERSITY

The final alignment dataset for the 38 sequences of *COI*, 60 sequences of *16S* and 31 sequences of *ITS2* displayed a length of 545, 309 and 379 nucleotides, respectively. Collapsing of the sequences into haplotypes resulted in nine unique haplotypes for *COI*, seven haplotypes for *16S* and 11 unique sequences for *ITS2* (Table 2).

As a general pattern, it was observed that in the southern region, few sequences (1–5) collapsed into more numerous and also, especially for mitochondrial genes, less divergent haplotypes. In contrast, a large number of sequences collapsed into few haplotypes in the northern region. One third of the *COI* and *16S* haplotypes were shared between the northern and southern regions and only one haplotype was shared between the regions for *ITS2* (Table 3).

Table 2. Comparison of *COI*, *16SrRNA* and *ITS-2* nucleotide diversity calculated in DNAsp5: N – number of sequences, h – number of haplotypes, Hd – haplotype diversity, Π – nucleotide diversity, k – average number of nucleotide differences, Fu's Fs (FU 1997: equation 1) and Tajima's D statistics (TAJIMA 1989: equation 38)

	Region	N	h	Hd	Π	k	Fu's Fs	D
COI	Northern	21	3	0.624±0.068	0.01201	6.543	2.20736	2.90471
	Southern	17	8	0.875±0.053	0.00880	4.794	−0.84404	−0.57907
	Overall	38	9	0.826±0.096	0.01232	6.717	1.36642	1.60995
16S	Northern	34	2	0.487±0.044	0.00318	0.973	1.29872	1.95432
	Southern	26	7	0.683±0.086	0.00489	1.495	−0.23305	−0.90144
	Overall	60	7	0.664±0.039	0.00515	1.575	−0.23305	−0.21597
ITS2	Northern	17	4	0.419±0.141	0.00116	0.456	−1.39391	−1.37718
	Southern	14	8	0.868±0.076	0.00898	3.538	−0.35833	−0.24799
	Overall	31	11	0.665±0.096	0.00555	2.185	−1.60338	−1.24805

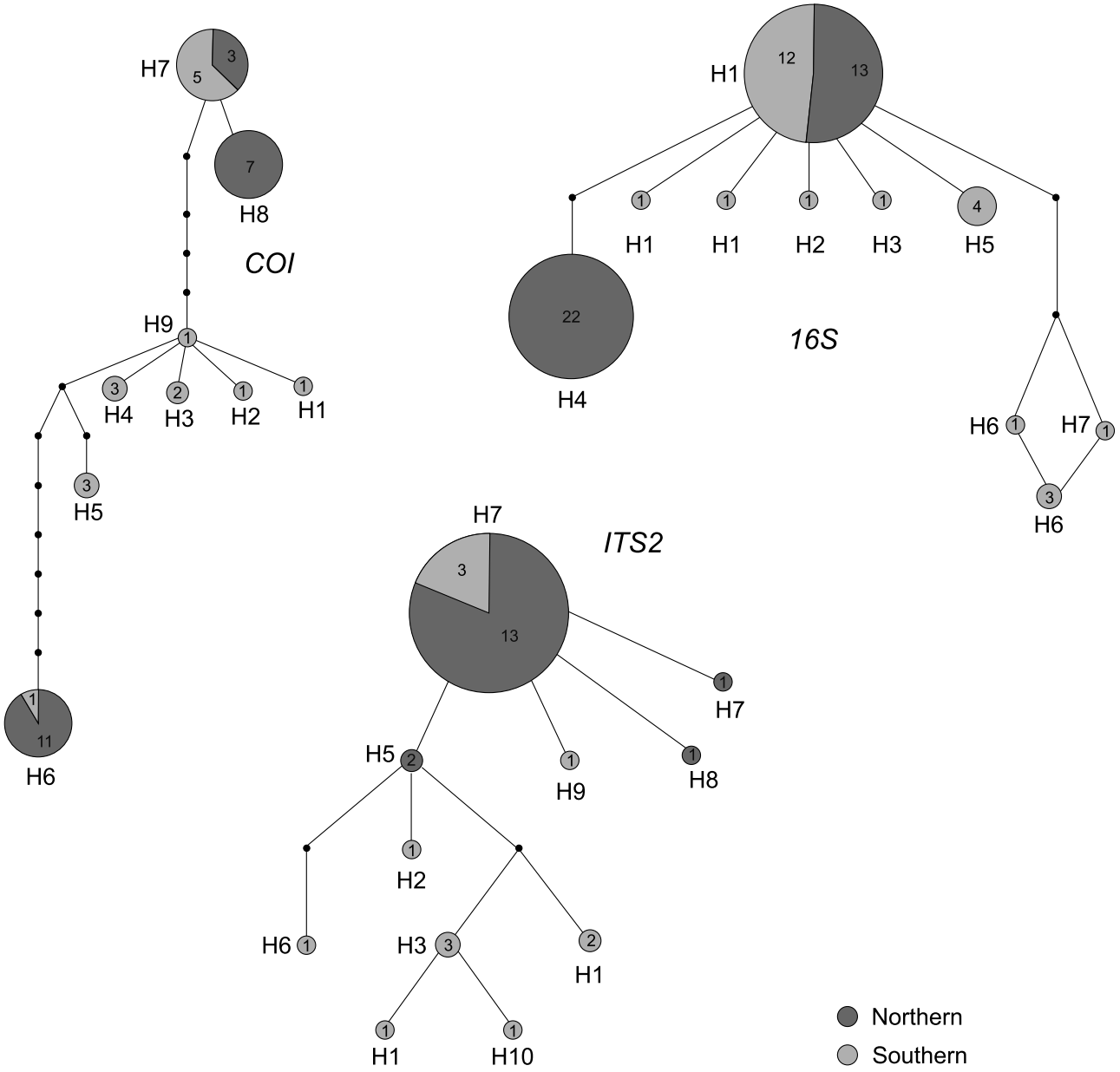


Fig. 4. TCS – haplotype networks for *Biomphalaria tenagophila* based on mitochondrial *COI*, *16S* and nuclear *ITS*2 sequences. Northern and southern sequences are represented in different shades of grey. Haplotype frequency is shown by the size of each haplotype

Table 3. Number of *COI*, *16S* and *ITS2* sequences collapsed in haplotypes for *Biomphalaria tenagophila* sampled in the northern and southern region

	Haplotypes	1	2	3	4	5	6	7	8	9	10
COI	Northern	0	0	0	0	0	11	3	7	0	–
	Southern	1	1	2	3	3	1	5	0	1	–
16S	Northern	13	0	0	21	0	0	0	–	–	–
	Southern	14	1	1	1	4	4	1	–	–	–
ITS2	Northern	0	0	0	0	2	0	13	1	1	0
	Southern	2	1	3	1	0	1	5	0	0	1

The diagram with sequences connected according to the number of mutations constructed in a parsimony network using TCS is shown in Fig. 4. As a result, the networks show higher frequencies of minor and unique haplotypes in the southern region than in the northern region.

PHYLOGENETIC ANALYSIS

The best-fit model of evolution estimated in MrModeltest with AIC parameters for the 38 *COI*, 60 *16S* and 31 *ITS2* aligned sequences used to conduct a Bayesian search for the best tree was K80, K2 and JC, respectively (Fig. 5).

ANALYSIS OF THE POPULATION GENETIC STRUCTURE

The results of AMOVA show that the highest values for genetic diversity (>80%) occur within regions and a smaller percentage (13–25%) was found in both the northern and southern regions (Table 4). The estimates of genetic differentiation between the populations (Φ) indicate that there is no genetic isolation between the northern and southern populations. AMOVA calculated for *COI*, *16S* genes and *ITS2* showed that the percentage of genetic variation within regions (75–80%) is three to four times as high as it is amongst snails collected in hydrologically disconnected regions (20–24%).

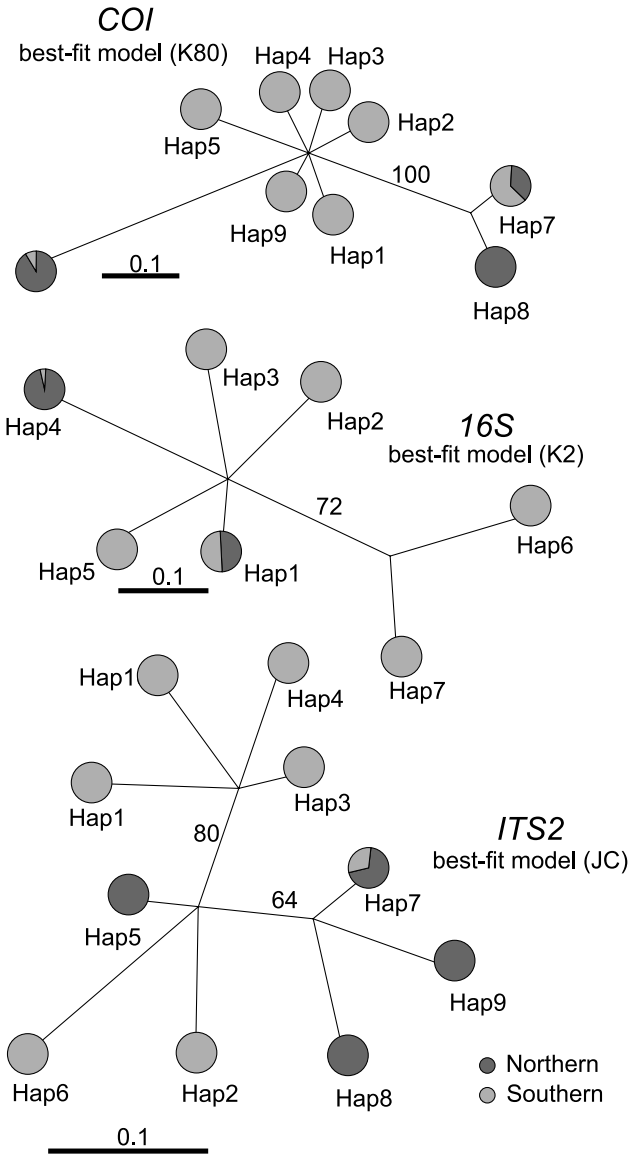


Fig. 5. Unrooted trees inferred under Bayesian approach with *COI*, *16SrRNA* and *ITS2* haplotypes of *Biomphalaria tenagophila*. Bayesian posterior probabilities given above each branch. (Average standard deviations of split frequencies for *COI*, *16S* and *ITS2* were 0.003889, 0.001944 and 0.002682, respectively). The shades of grey match the shades of grey of haplotypes in Fig. 4

Table 4. Analysis of Molecular Variance (AMOVA) amongst and within *COI*, *16S* and *ITS2* sequences in samples from the northern and southern regions: d.f. – degrees of freedom, Φ – fixation index

	AMOVA	d.f	Sum of squares	Variance components	% variation	Φ	p-value
COI	Amongst regions	1	2.051	0.08961 Va	19.59	0.19593	0.00000
	Within regions	36	13.238	0.36772 Vb	80.41		
	Total	37	124.263	3.81945			
16S	Amongst regions	1	3.032	0.09321 Va	24.60	0.24602	0.00000
	Within regions	58	16.568	0.28565 Vb	75.40		
	Total	59	19.600	0.37886			
ITS2	Amongst regions	1	1.050	0.04852 Va	13.71	0.13714	0.01466
	Within regions	29	8.853	0.30527 Vb	86.29		
	Total	30	9.903	0.35379			

DISCUSSION AND CONCLUSIONS

The pattern of molecular variation for *B. tenagophila* is widely different in snails collected within the southern and northern coastal freshwater systems, for both mitochondrial and nuclear markers. It can be concluded that the probability of collecting two genetically different snails seems not to be directly related to the geographical distance between the populations; instead, there is a greater chance of collecting two genetically different snails in the same hydrological ecosystem than in disconnected and distant freshwater systems.

This is in line with the earlier results obtained with mitochondrial and microsatellite markers in *Biomphalaria* and *Bulinus* collected in the Neotropics and Africa, which show that genetic variation in snails is in general partitioned into small populations within localities even across geographiclly connected water bodies (Sire et al. 2001, Mavárez et al. 2002, Charbonnel et al. 2002, Thiele et al. 2013, Standley et al. 2014, Zein-Eddine et al. 2017).

Samples collected in the southern region provided the largest set of mitochondrial haplotypes, the higher polymorphisms values on both mitochondrial and nuclear markers and the higher number of singletons in individuals collected across the study area. In the northern region a large number of sequences collapsed into one to three homogeneous haplotypes spread over the entire area. In the light of these results, two significant questions arise: why are some haplotypes unique to the southern region and do not spread over the entire study area, and why in the northern region a clonal pattern of sequences is observed?

In theory newly invasive populations are thought to have a reduced genetic variation compared to their source populations because of the genetic founder effect associated with the initially small population size during colonisation (Handley et al. 2011). The reduced genetic variation amongst specimens collected in the northern region could be the result of a more recent colonisation process.

B. tenagophila from the southern regions might originate from an independent source population, different from the endemic populations restricted to the southernmost inland freshwater systems. The hypothesis is supported by the fact that the southern region is a part of a hot spot corridor for biodiversity inside the Atlantic forest, holding a highly endemic fauna (Carnaval et al. 2014). Plens (2009) and Souza et al. (2005) found in shell mounds archaeological sites a significant diversity of shells of freshwater bivalves and gastropods which demonstrate, in theory, the ancient high diversity of the malacofauna in the Ribeira do Iguape region. In general terms, our study supports the hypothesis of Vaz (1989). According to this author, the Ribeira do Iguape basin was in the Paleocene the main dispersal route of *B. tenagophila* from inland to the southern coastal region.

The Bayesian analysis yielded trees with almost identical topologies in *COI*, *16S* and *ITS2* with phylogenetic relationships of some haplotypes well solved and some with weak support, especially haplotypes with sequences from the southern region. In general, the Bayesian analysis of the *ITS2* sequences corroborates the finding of differentiated *B. tenagophila* sequences as previously observed in the middle Paranapanema (Tuan & Santos 2007).

Understanding the population's genetic structure and the mechanisms of spread of *B. tenagophila* might fill some gaps in the knowledge of schistosome transmission. When the genetic and parasitological data were compared, Palasio (2013) observed that cercarial shedding of *B. tenagophila* from the northern region was significantly higher (60–80%) than in *B. tenagophila* from the southern region. Hence, it could conceivably be hypothesised that schistosomiasis transmission could be largely affected by the natural history of the snails.

ACKNOWLEDGEMENTS

The authors would like to thank Dr Luiz Augusto Magalhães, Unicamp, for his constructive comments, Dr Marisa Cristina de Almeida Guimarães, SUCEN, for helping with the collection of snails in Itariri, Ricardo Soares Lopes de Souza for helping in editing the figures and Marina Tuan Machado for review. The work was partly supported by the Superintendência de Controle de Endemias, FAPESP (ref. no. 07/03458-7) and partly by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes) which provided Raquel G. S. Palasio with a master's fellowship.

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Received: June 26th, 2018
Revised: October 25th, 2018
Accepted: October 27th, 2018
Published on-line: December 4th, 2018