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Distribution of genetic diversity of neotropical *Biomphalaria* (Preston 1910) (Basommatophora: Planorbidae) intermediate hosts for schistosomiasis in Southeast Brazil

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Introduction: *Biomphalaria glabrata*, *B. tenagophila*, and *B. straminea* occurrence are crucial for estimating the risk of infectious human schistosomiasis in the neotropics. How different geographic sample strategies influence snail genetic diversity estimations were here investigated for three *Schistosoma mansoni* hosts.

Methods: Mitochondrial gene sequences were employed for Cytochrome C Oxidase I (COI), ribosomal RNA (rRNA) 16S, and a dataset with concatenated gene sequences (COI+16S), resulting in an improved scientific hypothesis regarding the geographical distribution of snail species. This study compared the sequences of snails from the Middle Paranapanema (MP) hydrographic basin in a geographically restricted area (inner group) to snails widely distributed across a broad geographical range in São Paulo (outer group), Brazil from 1999 to 2017. DNA sequence polymorphisms and haplotype diversity were estimated using DNAsp software. Haplotype network trees were constructed using a network program. The geographical distribution of the haplotypes was mapped using QGIS. Haplotype variation and distribution were tested for population structure using analysis of molecular variance (AMOVA).

Results and discussion: The genetic diversity of *B. glabrata*, sampled from disconnected but geographically close freshwater collections, was partitioned into two sequence groups. The haplotype network showed that the diversity of *B. straminea* was more spatially partitioned than in *B. tenagophila*, which exhibited two population groups. The haplotype distribution pattern for *B. tenagophila*

showed many unique and exclusive haplotypes for all three loci. AMOVA showed that genetic diversity could be high in species inhabiting small geographical areas, and a large river is not a local geographical barrier for snail migration. This study found that the survey dimensions and snail samplings influenced the genetic diversity results obtained by mitochondrial DNA molecular markers.

KEYWORDS

schistosomiasis, *Biomphalaria*, mtDNA, haplotype diversity, spatial analysis, molecular diversity, sampling strategy

Introduction

Biomphalaria glabrata (Say, 1818) (Basommatophora: Planorbidae), *B. straminea* (Dunker, 1848), and *B. tenagophila* (Orbigny, 1835) freshwater snails act as intermediate hosts for *Schistosoma mansoni* Sambon 1907 (Strigeatida: Schistosomatidae), being the presence of these snails a key factor in the environmental management of these species that aims to establish snail control programs to prevent, control, and eliminate human schistosomiasis (1). Therefore, these snails are typically sampled from freshwater bodies on two geographic scales: a local scale to investigate hotspots for ongoing disease transmission and a regional scale to monitor the likelihood of future disease transmission. On both scales, numerous factors influence the occurrence and distribution range patterns of *Biomphalaria* (Preston, 1910) freshwater snails. Factors include the size and connectivity of the freshwater bodies (2), climatic and microclimate conditions (3, 4), large or small geographical areas (5), and land use for agriculture or urbanization. These factors act synergistically in colonizing freshwater habitats by *Biomphalaria* species, including non-native geographical areas.

Although animals have a low ability to move, *Biomphalaria* snails can disperse between different freshwater bodies with local, regional, or intercontinental ranges (6–8). According to genetic evidence, 14–33% of sampled snails in freshwater bodies in China have been identified as migrants from locations approximately 50 km away (7). The *B. straminea* invasion in Hong Kong in the 1970s and its subsequent spread to South China are good examples of the high potential for invasion of the host intermediate snail, increasing the risk of human schistosomiasis (8).

A significant change was recently observed in the geographic area occupied by *B. tenagophila* and *B. straminea*, as seen by studying previous surveys on freshwater collections located in the hydrographic basin of the Middle Paranapanema (MP) (9–12). *Biomphalaria tenagophila* suffered a marked reduction in previously occupied habitats. At the same time, *B. straminea* spread its geographical ranger across the MP region (13). In contrast, *B. glabrata* persisted in the same region as historically recorded.

Due to population growth, changes in land use, severe droughts, and other anthropogenic landscape changes, Southeast Brazil, particularly São Paulo (14), has experienced a plethora of disturbances that, in many cases, have led to local freshwater fragmentation and eutrophication (15). As a result, *B. tenagophila* and *B. straminea*, which often occur in São Paulo freshwater reservoirs, can now be found in contaminated and eutrophicated freshwater habitats, frequently close to urban areas (16, 17). The survival of the *Biomphalaria* species is boosted by a combination of their capacity to survive desiccation physiologically and self-fertilization (18–20).

The genetic diversity partitioning of different classes of molecular markers in *S. mansoni* snail hosts for different populations is potentially related to the mode of reproduction (especially inbreeding), population bottlenecks, and drift (21–28). Different populations include snails within the same or different water bodies (21, 29), different habitats (22–24), different regional scales (25, 26), and different countries (27, 28).

Investigating the genetic diversity of *Biomphalaria* in freshwater bodies in São Paulo led to some complex issues. An example includes whether the genetic variability characterized for the intermediate host species for *S. mansoni* at a few sites depicts the diversity inherent to these species or whether it is only a subset of the whole population diversity (13).

We conducted a genetic analysis of the three hosts of *S. mansoni* using two mitochondrial genes (Cytochrome C Oxidase I - COI and 16S ribosomal RNA - rRNA) and a dataset with concatenated gene sequences (COI+16S), owing to the robust results acquired using a couple of mitochondrial genes (30). In addition, we compared mtDNA sequences acquired from one watershed with a dataset of snail sequences sampled from eight water resource management units (UGRHI) in São Paulo's hydrographic regions (31). This comparison was made to explore factors that affect genetic variation at local and broad geographic scales. Finally, we deepen our analysis to investigate whether a large river potentially acts as a geographical barrier to genetic diversity among populations of the same species.

Materials and methods

Study area and approaches for the obtention of DNA sequences and genetic analyses

This study used 306 COI, 181 rRNA 16S, and 144 COI+16S mitochondrial DNA sequences with a length of 546 bp, 344 bp and 894 bp nucleotides. These sequences were obtained from snails sampled from freshwater bodies in nine hydrographic basins in São

Paulo (SP), Brazil, from 1999 to 2017. The nine hydrographic basins include: 1-"*Alto Tietê*" (AT), 2-Sorocaba-Middle Tietê (MT) and 3-Piracicaba-Capivari-Jundiaí (MT), 4-"*Jacaré-Tietê*" (JT), 5-"*Paraíba do Sul*" (PS), 6-"*Ribeira do Iguape*" valley (VR), 7-Northern Coast (LN), 8-"*Pontal do Paranapanema*" (PP), and 9- Middle Paranapanema (MP) (Figure 1; Table 1). In addition, the Sorocaba-Middle Tietê, and Piracicaba-Capivari-Jundiaí watershed data were combined due to a low number of sequences. All the freshwater bodies where the snails were surveyed are an extensive array of shallow and lentic freshwater bodies surrounded by urban and rural areas.

The methods for collecting snails, taxonomical identification based on the anatomical characters of reproductive systems, percent identity for each DNA Barcode sequences in NCBI reference library, and statistical tests DNA Barcode are described by Palasio et al. (13). Geographical coordinates of each collection site were provided by a Garmin eTrex GPS device (Garmin, Olathe, USA). All snails were identified simultaneously by morphology and DNA barcoding. In the case that DNA extraction was unsuccessful the morphology was used for identification (Table 1).

Amplification and sequencing of the mitochondrial genes used Folmer-LCO HCO primers (39) to amplify the mitochondrial COI region of ±600 bp, and the 16S ar/16S br primers (40) to amplify ±500 bp before aligned. Palasio et al. (32) and Tuan et al. (33) described the PCR reaction components and conditions. Alignments were performed using MAFFT version 7 (41) https://mafft.cbrc.jp/alignment/server/) under the Q-INS-I parameter. The final aligned sequences were edited and corrected using BioEdit version 7.2.5 (42, 43).

The COI and 16S sequences were concatenated using the SeaView 4 program (44). The 16S sequences obtained from snails sampled from MP were submitted to NCBI GenBank® (45) www.ncbi.nlm.nih.gov/genbank/) with accession numbers MK583964–MK584156. We also used COI and 16S sequences deposited in GenBank from Zanna (34), Tuan et al. (33), Palasio et al. (13, 26, 32), and Ohlweiler et al. (35) (Table 1).

The sequences were separated into the inner (MP watershed only) and outer group (AT+ MT+JT+PS+VR+LN+PP). Together, the inner and outer regions cover the most developed region in South America, with great changes in land use, including multiple hydroelectric power plants (46).

The inner group was marked by an intensive and extensive local sampling of *B. glabrata*, *B. straminea*, and *B. tenagophila*, along all

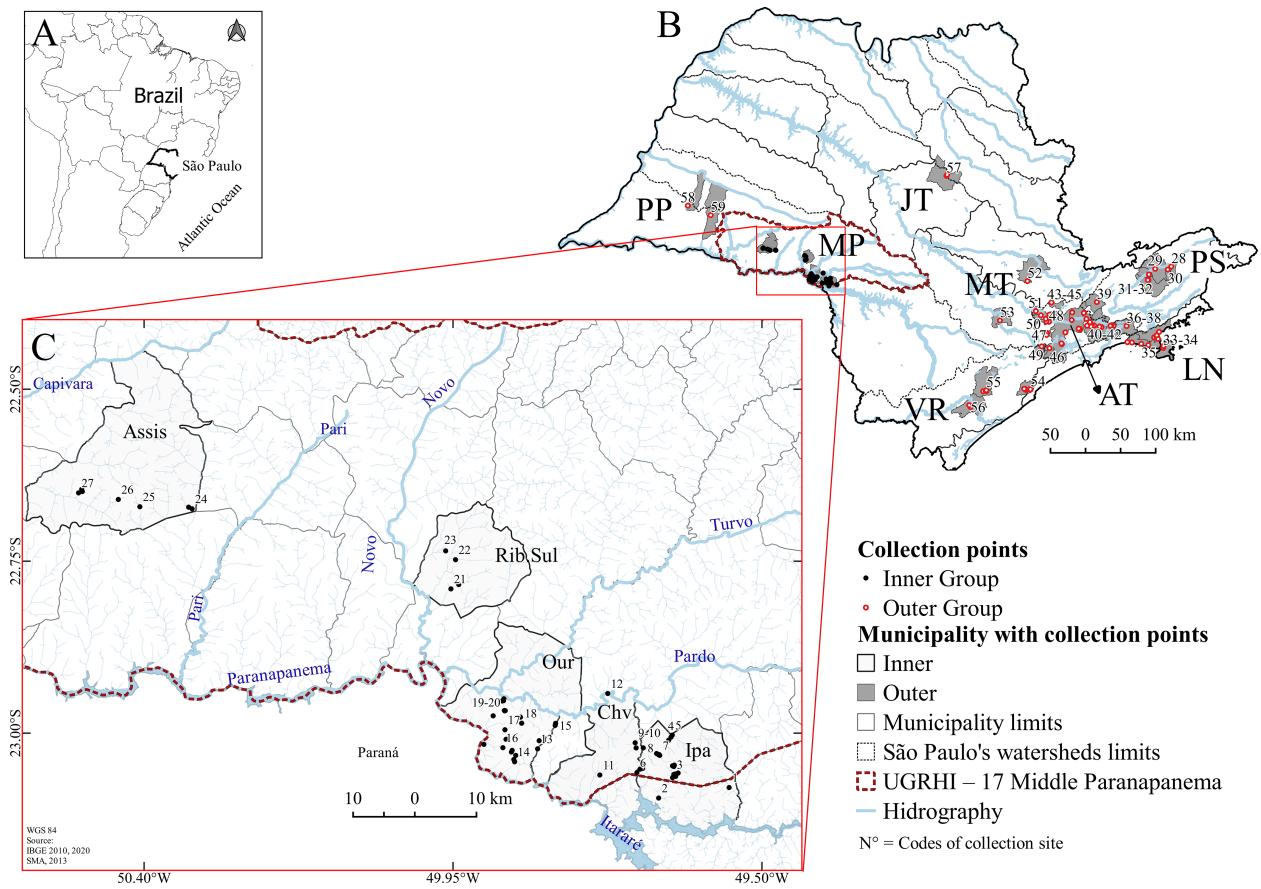

FIGURE 1
Collection points of *Biomphalaria glabrata, B. tenagophila*, and *B. straminea*. **(A)** Maps of South America, Brazil, the state of São Paulo; **(B)** Map showing the distribution of collection points in freshwater bodies in nine hydrographic basins in São Paulo; **(C)** Map of the Middle Paranapanema (MP) region, São Paulo, Brazil, from 1999 to 2018. **(B)** Hydrographic Basins: Outer Group – "Alto Tietê" (AT), Sorocaba-Middle Tietê and Piracicaba-Capivari-Jundiaí (MT), "Jacaré-Tietê" (JT), "Paraíba do Sul" (PS), "Ribeira do Iguape" valley (VR), Northern Coast (LN), "Pontal do Paranapanema" (PP) and Inner Group -Middle Paranapanema (MP); **(C)** Municipalities of: Assis (Assis), Ribeirão do Sul (Rib. Sul), Ourinhos (Our), Chavantes (Chv) and Ipaussu (Ipa), Water Resource Management Unit (UGRHI); Codes of the numbers of the water collection sites in Table 1. Source: 13, 26, 31, 34–38.

TABLE 1 GenBank accession numbers of nucleotide sequences for mitochondrial DNA COI and 16S rRNA and their respective geographic coordinates and references from snail species identified in the nine watersheds of the study, São Paulo, Brazil, from 1999 to 2018.

	watershed		M	C	Acession Number Genbank		Latitude (°)	Longitude (°)	Collection date
				D	COI	rRNA16S			
B. glabrata	Inner	MP	Ourinhos	13	MK395896-98[a]	MK584039	-23.022833	-49.826733	2016
					*	*	-23.010972	-49.824500	2015
				14	MK395903[a]	MK584040	-23.041650	-49.860233	2016
					MK395907-908[a]	MK584043-44	-23.027467	-49.864867	
				16	KX354434, 36[b], MK395933-34[a]	MK584059-60	-23.008944	-49.872750	2015-2016
				17	KF926178[b]		-22.995111	-49.874333	2012
				19	KX354433[b], MK395961-66[a]	MK584079-80	-22.967600	-49.874683	2015-2016
					*	*	-22.967117	-49.875167	2015-2017
					MK395969, 72-74[a], KX354437-38[b]	MK584084-86	-22.952833	-49.876333	2015-2016
					KX354435[b], MK395976[a]	MK584087	-22.950050	-49.875850	
					KF926110-111[d]	KF892019-20[e]	SI	SI	2008
					KF926182[b]		-22.967361	-49.873861	2012
					KF926181, 183[b]		-22.967361	-49.874194	
			Assis	26	MK396043-44[a]	MK584142	-22.660240	-50.437510	2017
	Outer	PS	Tremenbé	32		KF892017[e]	-22.958330	-45.549440	1999
B. straminea	Inner	MP	Ipaussu	1	*	*	-23.079100	-49.547817	2015-2017
				2	MK395804-05[a]	MK583971-72	-23.094333	-49.650417	2015
			Chavantes	11	MK395887[a]	MK584033	-23.060900	-49.736117	
				12	*	*	-22.942400	-49.724483	2016
			Ourinhos	14	*	*	-23.038333	-49.861400	
					MK395909[a]	MK584045	-23.025063	-49.863847	
				17	MK395938[a]	MK584063	-22.995111	-49.874333	2015-2016
				20	MK395986[a]	MK584099	-22.953222	-49.878306	2015-2017
				–		KF892025[e]	SI	SI	2002
			Ribeirão do Sul	21	MK396017-19[a]	MK584117-18	-22.790300	-49.953033	2015-2016
					MK396022-23[a]	MK584122	-22.783950	-49.941600	
				22	MK396031-32[a]	MK584134-35	-22.747833	-49.946317	2016-2017
				23	MK396033[a]	MK584138	-22.735033	-49.960667	2016
			Assis	24	*	*	-22.673883	-50.330050	2018
					*	*	-22.671483	-50.335083	
				27	MK396057[a]	MK584149	-22.650717	-50.495433	2017
					MK396058[a]		-22.648800	-50.489983	
					MK396059-60[a]	MK584150	-22.645400	-50.490810	
	Outer	PS	Aparecida	28	KF926184, 86[b]		-22.864444	-45.262778	2012
		LN	Ilha Bela	34	KF926187, 91[b]	KF840621-622[f]	-23.821528	-45.367056	2010-2012

(Continued)

TABLE 1 Continued

	watershed		M	C	Acession Number Genbank		Latitude (°)	Longitude (°)	Collection date
				D	COI	rRNA16S			
		AT	Santa Isabel	39	KF926189-190, 195[b]		-23.283389	-46.216417	2012
		JT	Araraquara	57	KF926112[e]	KF892031[e]	-21.778667	-48.134944	2008
		PP	Presidente Prudente	58	KF926113-118[d]	KF892026-30[e]	-22.146083	-51.457833	2008
			Martinopolis	59		KF892032[e]	-22.258056	-51.165556	2012
		VR	Itariri	54	KF926185[b]	KF840642[f]	-24.303278	-47.067806	
					KF926188[b]	KF840643[f]	-24.298222	-47.148611	
			Registro	56		KF840639-641[f]	-24.510278	-47.835528	2007
B. tenagophila	Inner	MP	Ipaussu	2	MK395806-09[a]	MK583973-76	-23.059556	-49.626778	2015
					MK395810-14[a]	MK583977-81	-23.058889	-49.623722	
					MK395815[a]	MK583983	-23.058028	-49.622028	
				3	MK395817-20[a]	MK583984-86	-23.064750	-49.630250	2015-2016
					MK395822-23[a]	MK583988	-23.060083	-49.628333	
					MK395824-28[a]	MK583989-90	-23.047528	-49.630250	
					MK395829-32[a]	MK583991-93	-23.046556	-49.627528	
					KF926119-128[c]	KF891996-97[c]	-23.048556	-49.628306	2003-2004
					KF926129[c]	KF891995[c]	-23.062785	-49.625581	2008
				4	MK395842[a]	MK584003	-23.010217	-49.635800	2015-2017
					MK395837-38[a]	MK583998	-23.005733	-49.632400	
				5	*	*	-23.002883	-49.630583	2015
				6	MK395848[a]	MK584007	-23.057333	-49.682083	
					MK395850[a]	MK584009	-23.052283	-49.677467	
				7	MK395851-53[a]	MK584010	-23.032350	-49.648867	2015-2017
					MK395864[a]	MK584019	-23.029617	-49.653433	
					MK395858-63[a]	MK584013-17	-23.031400	-49.650567	2015
					MK395855-57[a]	MK584011-12	-23.031267	-49.649600	
				8	MK395866[a]	MK584020	-23.021400	-49.672833	
			Chavantes	9	MK395877-82[a]	MK584026-30	-23.021483	-49.683233	2015-2017
				10	MK395883-84[a]	MK584031	-23.014033	-49.684667	
			Ourinhos	14	MK395906[a]	MK584042	-23.032400	-49.858617	2016
				15	MK395917[a]	MK584051	-22.988750	-49.801167	2016-2017
					MK395920[a]	MK584053	-22.985800	-49.800517	2016
				16	MK395928[a]	MK584058	-23.021167	-49.877278	2015-2016
					*	*	-23.016306	-49.905000	2015
				18	*	*	-22.976766	-49.851745	
					MK395949, 51[a]	MK584073	-22.985556	-49.849972	
					KF926192[b]				2012
				19	KF926193[b]		-22.974861	-49.891500	2012

(Continued)

TABLE 1 Continued

	watershed		M	C	Acession Number Genbank		Latitude (°)	Longitude (°)	Collection date
				D	COI	rRNA16S			
				20	KF926194[b]		-22.953222	-49.878306	2013
				–	KF926130-35[c]	KF891981-84[c]	SI	SI	2003-2004
			Assis	25	*	*	-22.670850	-50.406117	2017
	Outer	AT	Salesopolis	36	MH593493[g]		-23.564444	-45.835556	2016
			Biritiba Mirim	37	MH593396, 400,401[g]		-23.558333	-46.031389	2015
					MH593398, 399[g]		-23.557222	-46.038056	
					MH593397[g]		-23.565833	-46.040278	
					KF926204[b]		-23.561944	-46.001667	2012
					KF926203, 205[b]	KF891987[e]	-23.562222	-46.043056	
			Mogi das Cruzes	38	KF926202[b]	KF892001[e]	-23.576389	-46.156667	
					MH593420-22[g]		-23.571389	-46.182222	2016
					MH593402-04[g]		-23.556944	-46.251389	2015
			Suzano	40	MH593418[g]		-23.552222	-46.280833	2016
					MH593415,416[g]		-23.570833	-46.290833	
					MH593439-43[g]		-23.518611	-46.305000	
			Poa	41	MH593505[g]		-23.559722	-46.342222	
			Itaquaquecetuba	42	MH593444-448[g]		-23.480000	-46.350278	
			Guarulhos	43	MH593452-454[g]		-23.408333	-46.377778	
					MH593449-451[g]		-23.410833	-46.383889	
					MH593479-80[g]		-23.404722	-46.532222	
					MH593477-78[g]		-23.401111	-46.532222	
					MH593455-57[g]		-23.493333	-46.538611	
			São Paulo	44	MH593462-63[g]		-23.602500	-46.429167	
					MH593464-65[g]		-23.601389	-46.429722	
					MH593469-73[g]		-23.593889	-46.449722	
					MH593474-76[g]		-23.592500	-46.449722	
					MH593485-87[g]		-23.639167	-46.619167	
					MH593483-84[g]		-23.638889	-46.620556	
					MH593481-82[g]		-23.776667	-46.663611	
					MH593488-92[g]		-23.768056	-46.670000	
			Franco da Rocha	45	MH593496-502[g]		-23.287778	-46.798333	
B. tenagophila			Embu Guaçu	46	MH593494-495,03-04[g]		-23.823889	-46.823333	
			Embu das Artes	47	KF926197[b]	KF892000[e]	-23.647361	-46.853139	2012
					KF926198[b]		-23.669028	-46.861583	2013
			Barueri	48	MH593434-38[g]		-23.514722	-46.830000	2016
					MH593423-27[g]		-23.510556	-46.864167	
					MH593395,430-433[g]		-23.480278	-46.871389	

(Continued)

TABLE 1 Continued

	watershed		M	C	Acession Number Genbank		Latitude (°)	Longitude (°)	Collection date
				D	COI	rRNA16S			
			São Lorenço da Serra	49	KF926201[b]		-23.803056	-46.924167	2013
			Santana de Parnaiba	50	MH593466-68[g]		-23.430833	-46.823889	2016
					MH593458-61[g]		-23.438056	-46.933611	
			Pirapora do Bom Jesus	51	MH593407[g]		-23.390000	-46.998889	2015
		JT	Araraquara	57	KF926199-200[b]		-21.791750	-48.144750	2013
					KF926154[c]	KF891988[c]	-21.790278	-48.142472	2008
		LN	Caraguátatuba	33	KF926136-142[c],222[b]	KF840598, 03, 06,08,11,16[f]	-23.697083	-45.441889	2010-2012
					KF926219, 220[b]	KF840588-89, 93, 96[f]	-23.696222	-45.482750	2012
					KF926216[b]	KF840592, 595[f]	-23.693000	-45.449472	
						KF840586, 613[f]	-23.690083	-45.444750	2010
					KF926214[b]	KF840591, 594[f]	-23.678389	-45.455139	2012
					KF926215[b], MF380482[f]	KF840590, 597, 605[f]	-23.673917	-45.448417	2010-2012
					KF926217, 221[b]	KF840587,01, 12,14[f]	-23.634500	-45.420750	2012
					KF926106[b]	KF840610[f]	-23.634236	-45.420639	2010
					KF926105[b]	KF840615[f]	-23.633222	-45.419833	
					KF926218[b]	KF840619-620[f]	-23.632139	-45.419106	
			Ilha Bela	34	KF926212-213[b]	KF840599-600[f]	-23.799000	-45.362222	2012
			Sao Sebastiao	35	MF380476[f]	KF840609[f]	-23.787667	-45.556889	2010
					MF380475[f]	KF840604[f]	-23.772278	-45.647361	
					MF380479, 81[f]	KF840607, 618[f]	-23.757028	-45.766872	
					MF380478[f]	KF840617[f]	-23.753389	-45.825500	
					MF380477[f]	KF840602[f]	-23.716194	-45.431583	
		MT	Campinas	52		KF891989-93[c]	-23.036944	-47.105833	SI
			Sorocaba	53	KF881852-56[c]	KF891971-75[c]	-23.498889	-47.458333	2000
		PS	Aparecida	28	KF926196[b]		-22.864444	-45.262778	2012
			Pindamonhangaba	29	KF926144, 146[c]	KF891985-86[c]	-22.891750	-45.469361	2008
B. tenagophila			Roseira	30		KF891994[c]	-22.900552	-45.309000	2003
			Taubate	31	KF926103-04,43,45[c]	KF891976-79[c]	-23.024694	-45.563750	2008
			Tremenbé	32	KF881849-851[d]	KF891966-70, 80[e]	-22.958330	-45.549440	1999
		VR	Itariri	54	KF926206[b]	KF840628, 635[f]	-24.311083	-47.125389	2010
					KF926207[b], MF380480[f]	KF840644, 46-47[f]	-24.307306	-47.066361	
					KF926208-209[b]	KF840627, 645[f]	-24.303278	-47.067806	2012-2013
					KF926210[b]	KF840636[f]	-24.303750	-47.075472	2012

(Continued)

TABLE 1 Continued

	watershed		M	C	Acession Number Genbank		Latitude (°)	Longitude (°)	Collection date
				D	COI	rRNA16S			
					KF926211[b]	KF840638[f]	-24.298611	-47.135222	
			Juquiá	55	KT225580[b]	MF380486[f]	-24.327639	-47.673611	2013
					KT225577-79[b]	MF380483-85,87-88[f]	-24.315306	-47.632944	
					KF926147, 49-51[c]	KF840630, 33-34[f]	-24.326000	-47.631528	2008
						KF840623[f]	-24.322139	-47.639694	
					KF926148[c]	KF840629, 31-32[f]	-24.315750	-47.634194	
			Registro	56	MF380474[f]	KF840637[f]	-24.510278	-47.835528	2007
					KF926152-53[c]	KF840624-26[f]	-24.488917	-47.851694	2007-2008

Hydrographic Basins: Inner Group -Middle Paranapanema (MP); Outer Group – "Alto Tietê" (AT), Sorocaba-Middle Tietê and Piracicaba-Capivari-Jundiaí (MT), "Jacaré-Tietê" (JT), "Paraíba do Sul" (PS), "Ribeira do Iguape" valley (VR), Northern Coast (LN), "Pontal do Paranapanema" (PP). CD, Codes of water collections shown in Figure 1, M, Municipality, *only morphological identification, according Palasio et al. (13); Reference: [a]Palasio et al. (13); [b](32), [c] Tuan et al. (33), [d]Zanna (34), [e]Tuan et al. unpublished data, [f]Palasio et al. (26), [g]Ohlweiler et al. (35), Unmarked are sequences of this study.

the freshwater bodies located within the middle stretch of the Paranapanema river (MP, drainage area of ±16.749 Km^2) (47). This basin is situated between -50.57325, -23.14839, -49.52913, and -22.44481 in São Paulo, Brazil. This area of MP is bathed mainly by the Paranapanema and Pardo rivers and their tributaries, the Turvo, Pari, Novo, and Capivara rivers (Figure 1) (47). The Paranapanema River flows westward from the headwaters in São Paulo into the Paraná River, defining the natural border between Brazil, Paraguay, and Argentina (48). These samples were for a scientific research program developed from 2015 to 2018 (13) and studies previously from 2002 to 2015 (32–34) (Table 1).

The outer group includes COI and 16S mtDNA sequences from studies previously published on snails distributed in freshwater bodies over eight different river basins: PS, AT, MT (two watersheds), JT, LN, VR, and PP, from 1999 to 2016 (26, 32–35) (Figure 1; Table 1). The outer drainage area encompasses <89.416 km^2 (31) of suitable habitat areas *for B. tenagophila*, *B. straminea*, and *B. glabrata* (only one site).

The inner group counts *B. glabrata* (n = 33, 15, and 14 for COI, 16S, and COI+16S sequences, respectively), *B. straminea* (N = 18, 15, and 14), and *B. tenagophila* (N = 78, 51, and 50). The outer group contains *B. glabrata* (n = 1 for the 16S sequence), *B. straminea* (n = 16, 14, and 9 for COI, 16S, and COI+16S sequences, respectively), and *B. tenagophila* (n = 161, 85, and 57) (Table 2).

DNA sequence polymorphism and haplotype diversity of 129 COI, 81 16S mitochondrial, and 78 COI+16S sequences were obtained for *B. glabrata, B. tenagophila*, and *B. straminea* of the inner group. Further, this was also conducted for the 177 COI, 100 16S, and 66 COI+16S sequences obtained for the three species sampled assigned to the outer group. Next, the results were analyzed in DNAsp version 6.12 (49) to provide estimated values of gene diversity related to mitochondrial haplotypes (h, Hd), nucleotide diversity (π), and the average number of nucleotide differences between sequence pairs (k).

Intraspecific haplotype networks and spatial distribution of haplotypes

The distribution of the COI, 16S, and COI+16S sequences into haplotypes for each species was inferred using DNAsp version 6.12 (49). The resulting nexus data files were used to infer haplotype network trees based on the median-joining algorithm (50) for COI, 16S, and COI+16S through Network program version 5.0.1.1 (Fluxus Technology Ltd, http://www.fluxus-engineering.com).

The mitochondrial haplotypes of COI+16S rRNA genes obtained from DNAsp (49) for the inner group sequences were associated with the geographic reference data (corresponding latitudes/longitudes) of the freshwater collections. In addition, the mitochondrial haplotypes for sequences for the outer group were associated with the centroid of watersheds. These associations were conducted to produce graph-type diagram maps from their attributes using the QGIS program version 3.16 (51). The cartographic materials were obtained from the Brazilian Institute of Geography and Statistics (IBGE), the Integrated System for Water Resources Management (SigRH), and the Secretary of Environment of the State of São Paulo (SMA) (31, 36–38).

AMOVA

The gene-population structuring hypothesis was tested on COI, 16S, and COI+16S sequence clusters of species with differentiated haplotypes through analysis of molecular variance (AMOVA) using Arlequin v.3.5.2.2 software (52). This analysis was run following 1023 permutations on the data to simulate the null hypothesis and was estimated using standard F-statistics. In addition, population structure hypotheses were tested for the species *B. glabrata*, *B. straminea*, and *B. tenagophila* at two geographic scales. This first geographic scale is in the

TABLE 2 Haplotype genetic diversity indices for *Biomphalaria glabrata, B. straminea*, and *B. tenagophila* calculated for the inner (Middle Paranapanema - MP) and outer (eight watersheds in São Paulo, Brazil) group in DNAsp, from 1999 to 2017.

	species	Cluster	N	h	Hd	Π	K
COI	***B. glabrata***	**Inner**	33	4	0.536	0.001	0.841
		Outer	–	–	–	–	–
	B. straminea	**Inner**	18	2	0.471	0.010	5.647
		Outer	16	5	0.700	0.010	5.550
	B. tenagophila	**Inner**	78	2	0.026	0.000	0.282
		Outer	161	12	0.616	0.011	6.167
16S	***B. glabrata***	**Inner**	15	3	0.257	0.002	0.533
		Outer	1	nc	nc	nc	nc
	B. straminea	**Inner**	15	3	0.676	0.011	3.257
		Outer	14	6	0.736	0.013	3.978
	B. tenagophila	**Inner**	51	2	0.113	0.000	0.113
		Outer	85	9	0.666	0.005	1.570
COI+16S	***B. glabrata***	**Inner**	14	4	0.670	0.002	1.681
		Outer	–	–	–	–	–
	B. straminea	**Inner**	14	3	0.670	0.010	8.374
		Outer	9	5	0.833	0.010	8.778
	B. tenagophila	**Inner**	50	2	0.115	0.000	0.115
		Outer	57	16	0.819	0.010	8.363

"-", No information. N, number of sequences; h, number of haplotypes; Hd, haplotype diversity; π, Nucleotide diversity; k, average number of nucleotide differences between sequence pairs.

inner group (MP region) from 2015 to 2017 with genetic differences between mitochondrial haplotypes of *B. straminea* obtained from snails collected from the right and left banks of the Pardo River. In addition, *B. glabrata* snails were obtained in disconnected and geographically close freshwater streams. The second geographic scale is in all the São Paulo regions with genetic differences between of *B. straminea*, and *B. tenagophila* from inner and outer group. The genetic differentiation among groups was considered low when the fixation index (Fst) < 0.05, moderate when Fst = 0.05–0.15, high when Fst = 0.15–0.25, and very high when Fst > 0.25 (53).

Results

Genetics and haplotype distribution analysis

We analyzed a length of 546 bp of the COI gene for 306 *Biomphalaria* snails (*B. glabrata* =33, *B. straminea*= 34, *B. tenagophila*=239), a 344 bp in length of 16S rRNA gene for 181 snails (*B. glabrata*= 16, *B. straminea*= 29 and *B. tenagophila*= 139), a dataset of 894 bp for concatenated COI+ 16S sequences for 181 snails (*B. glabrata*= 14, *B. straminea*= 23 and *B. tenagophila*= 107), sampled in freshwater bodies in São Paulo state, Brazil (Figure 1; Table 1). The number of sequences used for *B. tenagophila* is 7-10 higher than those obtained for *B. glabrata* and *B. straminea*, being this difference the result of the large distribution and abundance of *B. tenagophila* in the freshwater bodies in São Paulo.

A summary of the parameters for h, Hd, π, and K for the haplotype sequences used for statistics is shown in Table 2. The values were estimated for haplotype-based sequences according to whether they belonged to the inner or outer groups. For the nucleotide diversity (π) of sequences, the values were generally low, between 0 and 0.02, showing no differences in the patterns among all the genes and species analyzed. The patterns of genetic diversity based on the haplotype diversity (Hd) and the average number of nucleotide differences (K) seem to be a more realistic metric to indicate the potential population divergence for the three loci here analyzed.

The analysis of the genetic diversity (Table 2) for *B. glabrata* is limited to the inner group, an area of São Paulo that is considered a hot-spot for the presence of the species. Because only one sequence was available for *B. glabrata* from the inner group of sequences, the number of haplotypes (h=3-4) for *B. glabrata* reflects only the potential diversity of the entire population sampled in the whole study area. Nevertheless, the genetic diversity of *B. glabrata* was partitioned in two different streams (Christoni and Sobra) into the inner group of sequences: a substantial amount of this variability was found in the Christoni stream which seems isolated from Sobra stream population (Figures 2, 3).

The haplotype diversity (hd) calculated for *B. straminea* show quantitative differences between inner and outer groups, with a high

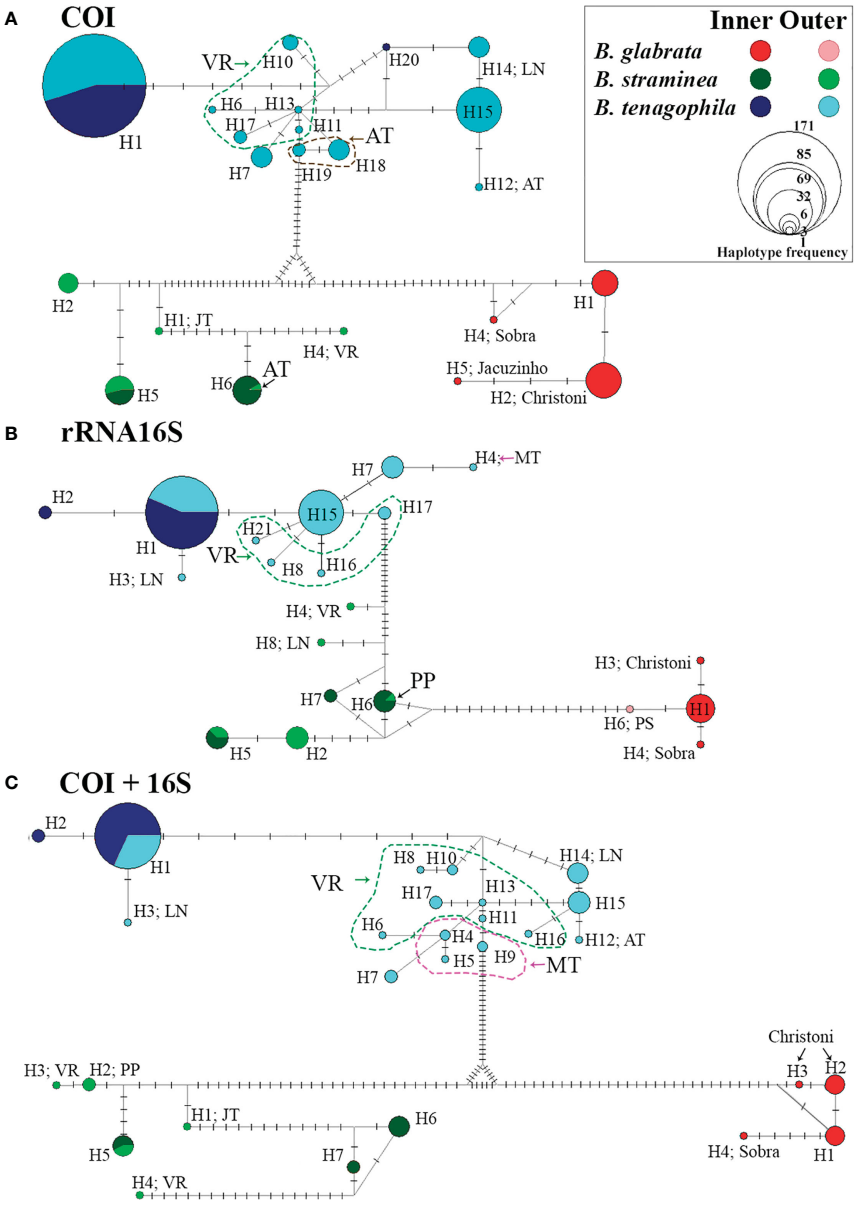

FIGURE 2
Haplotype network between the sequences: COI **(A)**, 16S **(B)**, and COI+16S **(C)** concatenated from the intermediate host species in the Middle Paranapanema (MP) region (inner) and the other eight watersheds (outer), São Paulo, Brazil, from 1999 to 2017. "Alto Tietê" (AT), Sorocaba- Middle Tietê and Piracicaba-Capivari-Jundiaí (MT), "Jacaré-Tietê" (JT), "Paraíba do Sul" (PS), "Ribeira do Iguape" valley (VR), Northern Coast (LN), "Pontal do Paranapanema" (PP).

chance of obtaining samples from distinct haplotypes in the outer group. Notably, a wide distribution of *B. straminea* haplotypes from 5 to 7 hydrographic basins across the area was observed in the study. Another interesting result can be seen in the haplotype network which shows that for *B. straminea*, the diversity was more spatially partitioned than that for *B. tenagophila*, which exhibited two different population groups (Figures 2, 4).

For *B. tenagophila* a remarkable loss of genetic diversity was observed in the inner group, as indicated by hd and K (Table 2). In general, the number of singletons was highest in the snails sampled from the outer group, a large area, encompassing water bodies across São Paulo. The geographical distribution of genetic variation for *B. tenagophila* was also widespread across all the hydrographic basins. The highest genetic diversity within *B. tenagophila* from the outer group was linked to the highest number of unique haplotypes, especially in the VR hydrographic basin (Figures 2, 5).

AMOVA

For all three mtDNA markers, the estimated genetic variation for *B. straminea* (72-82%) and *B. tenagophila* (70-81%) was higher within each group than among the groups. This difference shows that, for both species, no genetic structure was observed. Gene flow

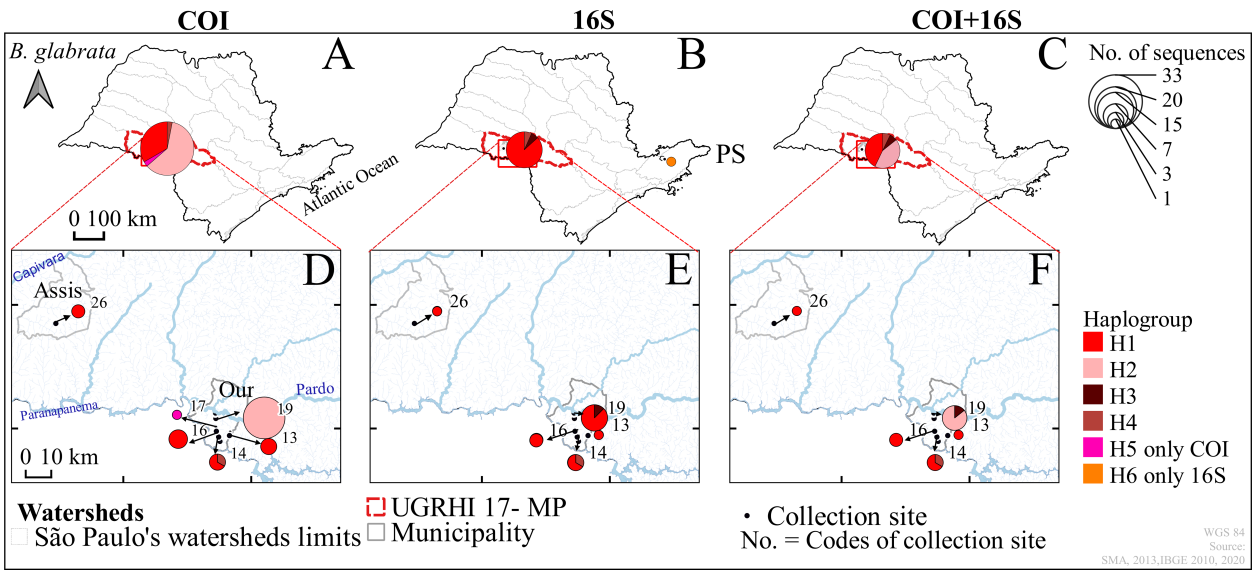

FIGURE 3
Haplotype distribution maps of the genes of *Biomphalaria glabrata*: COI, 16S, and COI+16S rRNA concatenated in the outer group **(A–C)** and the inner group **(D–F)**, São Paulo, Brazil, from 1999 to 2017. Hydrographic Basins: Outer Group – "Paraíba do Sul" (PS) and Inner Group -Middle Paranapanema (MP); Municipalities of: Assis (Assis) and Ourinhos (Our); * Codes of the numbers of the water collection sites in Table 1. Code - Name water Collection: 13-Lageadinho, 14-Sobra, 16-Jacu, 17-Jacuzinho, 19-Christoni, 26-Fortuninha.

occurs between *B. straminea* snails (Fst=0.28) sampled from inner group (MP) despite a large geographical barrier, such as the Pardo River. The larger genetic variation among *B. glabrata* snails (72-93%) sampled from disconnected streams indicates a higher likelihood for the genetic variation observed in small and local populations rather than in different geographical regions. The values for Fst (0.67-0.92) also suggest a considerable level of differentiation among *B. glabrata* sampled in different freshwater in a local geographical scale (Table 3).

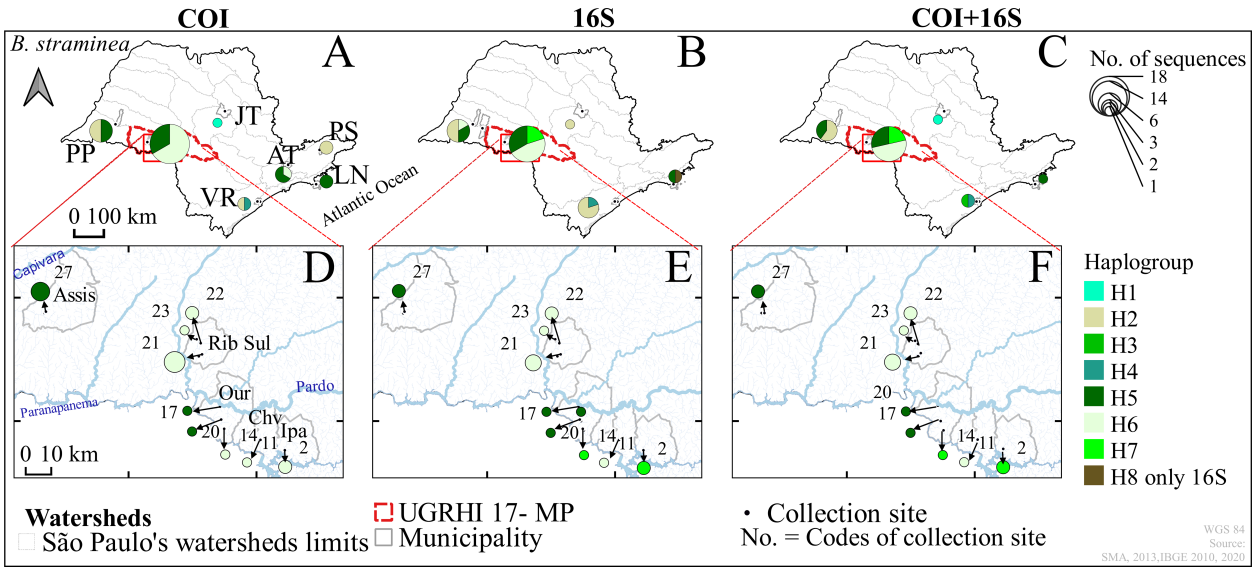

FIGURE 4
Haplotype distribution maps of the genes of *Biomphalaria straminea*: COI, 16S, and COI+16S rRNA concatenated in the outer group **(A–C)** and the inner group **(D–F)**, São Paulo, Brazil, from 2002 to 2017. Hydrographic Basins: Outer Group – "Alto Tietê" (AT), "Jacaré-Tietê" (JT), "Paraíba do Sul" (PS), "Ribeira do Iguape" valley (VR), Northern Coast (LN), "Pontal do Paranapanema" (PP) and Inner Group -Middle Paranapanema (MP); Municipalities of: Assis (Assis), Ribeirão do Sul (Rib. Sul), Ourinhos (Our), Chavantes (Chv) and Ipaussu (Ipa); * Codes of the numbers of the water collection sites in Table 1. Code - Name water Collection: 2-Boa Vista, 11-Colossinho, 14-Sobra, 17-Jacuzinho, 20-Agua da Veada, 21-Ribeirão do Pinto, 22-Matão, 23-Lagoa, 27-Baixadão.

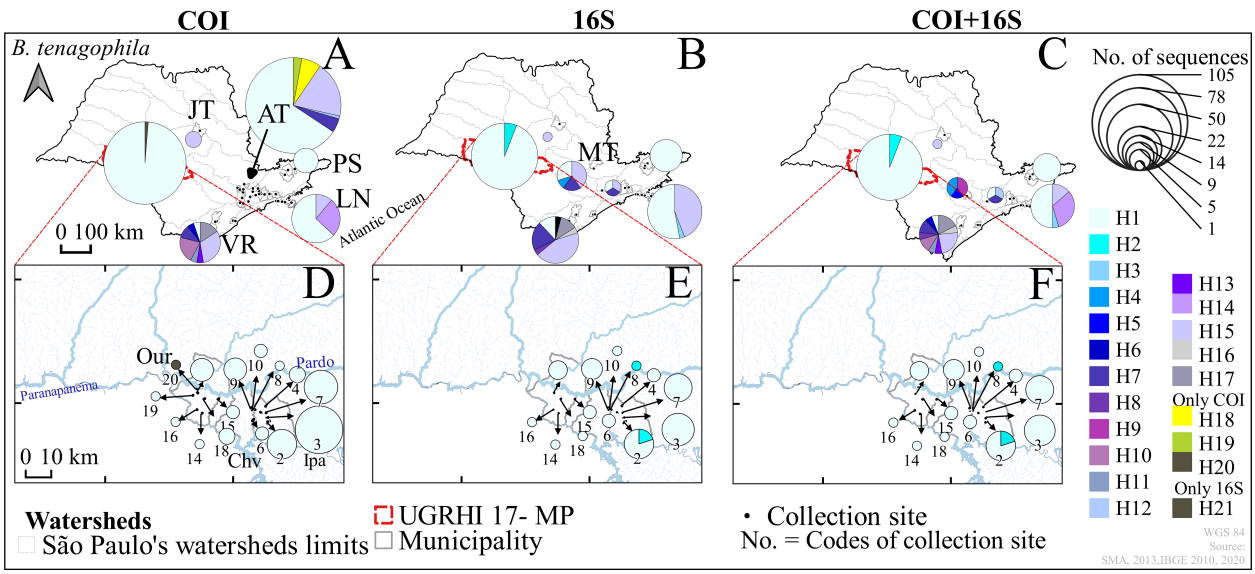

FIGURE 5
Haplotype distribution maps of the genes of *Biomphalaria tenagophila* COI, 16S, and COI+16S rRNA concatenated in the outer group **(A–C)** and the inner group **(D–F)**, São Paulo, Brazil, from 1999 to 2017. Hydrographic Basins: Outer Group – "Alto Tietê" (AT), Sorocaba-Middle Tietê and Piracicaba-Capivari-Jundiaí (MT), "Jacaré-Tietê" (JT), "Paraíba do Sul" (PS), "Ribeira do Iguape" valley (VR), Northern Coast (LN) and Inner Group -Middle Paranapanema (MP); Municipalities of: Ourinhos (Our), Chavantes (Chv) and Ipaussu (Ipa); * Codes of the numbers of the water collection sites in Table 1. Code - Name water Collection: 2-Boa Vista, 3-São Luiz, 4-Mombuquinha, 6-Barranco Vermelho, 7-Ribeirão Grande, 8-Boa Vista (Divisa), 9-Santo Antonio, 10-Piranhas, 14-Sobra, 15-Barreirinha, 16-Jacu, 18-Furninhas, 19-Chumbiadinha, 20-Agua da Veada.

Discussion

Intraspecific genetic diversity has been little explored as an important metric in understanding the potential for snail expansion leading to the risk of spreading schistosomiasis. An important aspect of the current study is the extent to which the geographical scales of snail sampling influence the assessment of genetic diversity. The results of this study show that the genetic diversity varies within all three species at different spatial collection scales. This finding allows for speculation regarding the contemporary and recent past processes that have driven the population differences.

In Brazil, the main range of *B. glabrata* is found in Minas Gerais and Bahia freshwater bodies. However, focal sites, which are separated from the main range, can be found in Southeast Brazil, in bodies of water in São Paulo, Paraná, and Rio Grande do Sul (54). Any analysis of the species carried out in Southeast Brazil is, therefore, limited by the fact that this species only occurs in a very limited number of freshwater bodies, which are hydrologically disconnected from the main breeding sites of the species. The geographical range of *B. glabrata* in the MP river basin is a good example of this pattern; only four in Ourinhos and one in Assis municipality out of more than 100 streams are inhabited by this species (13).

The distribution of *B. glabrata* in fragmented sites across Southeast Brazil limits any analysis of the species due to snail numbers and limited geographic range. Therefore, further analysis must be based on sequences of the COI and 16S genes deposited in GenBank. Nevertheless, GenBank has a few sequences from specimens collected in Brazil, outside São Paulo. These include 16S (N = 26) or COI (N = 8) sequences (N° of GenBank 16S: JQ886405, JQ886408, AF449597–AF449599, AY030208, AY198084–AY198089, AY198091–AY198092, AY198095–AY198104, AY204640, and AY737280 and COI: AF199091– AF199092, AF199094– AF199096, and KF926107– KF926109). The low number of sequences is impressive, given the epidemiological relevance of this species for schistosomiasis transmission in Brazil and Africa. Furthermore, the genetic diversity indices calculated for *B. glabrata* sequences of the inner group for 16S (pi = 0.01, h = 13, Hd = 0.952, and k = 6.867) and COI (pi = 0.02, H = 4, Hd = 0.867, and k = 13.733) genes were about 10 times lower than in DNAsp from the GenBank database, which includes sequences from animals collected in Brazil. This result means that the MP population represents only a fraction of the potential whole genetic diversity of the species.

It is unknown why the species has not spread to adjacent sites even though the inner group was sampled from a high-risk area for flooding, overflow, and inundation (55). In consequence, if floods and inundations occur regularly such factors should not be seen as significant drivers for spreading the species. The limited genetic variability of *B. glabrata* in the inner group could explain the containment of the species to these few streams. It would be worth considering the adaptability of snails to specific ecological and environmental factors as a possible explanation for their containment in the MP region. The low genetic diversity associated with mitochondrial genes would also hypothetically be an important factor, since, snails can alter their oxidative systems to decrease their plasticity tolerance under stressors and environmental changes (56, 57).

Biomphalaria tenagophila sequences showed identical mitochondrial genotypes in the inner group. Compared to the outer group, the lower genetic diversity found in *B. tenagophila* in the inner group suggests that the genetic variation differences might result from differences in inbreeding rates. Also, the difference

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TABLE 3 Analysis of Molecular variance (AMOVA) of the frequency of the 16S and COI haplotypes separated and concatenated (COI+16S) among and within the groups of *Biomphalaria glabrata, B. tenagophila*, and *B. straminea*.

Source		DF			SQ			Variance components			% of variation		
		COI	16S	COI +16S	COI	16S	COI +16S	COI	16S	COI +16S	COI	16S	COI +16S
a)All Groups Inner /Outer													
B. straminea	Among groups	1	1	1	2.221	1.981	1.223	0.11401 Va	0.11372 Va	0.07816 Va**	28	25	18
	within each group	32	27	21	9.250	9.019	7.690	0.28906 Vb	0.33404 Vb	0.36621 Vb**	72	75	82
B. tenagophila	Among groups	1	1	1	5.503	6.565	4.755	0.05035 Va**	0.09937 Va	0.08465 Va	19	30	26
	within each group	237	134	105	50.292	30.788	25.750	0.21220 Vb**	0.22976 Vb	0.24524 Vb	81	70	74
b) Pardo River as a geographical barrier													
B. straminea	Among righ and left bank	1	1	1	0.000*	1.024	1.024	-0.03125 Va*	0.10880 Va	0.10880 Va	-14*	28	28
	within righ/leftt bank	16	12	12	4.000*	3.333	3.333	0.25000 Vb*	0.27778 Vb	0.27778 Vb	114*	72	72
c) Different streams													
B. glabrata	Among streams	1	1	1	6.157	2.044	2.505	0.45906 Va	0.29422 Va	0.36565 Va	93	67	72
	within each stream	25	11	11	0.917	1.571	1.571	0.03667 Vb	0.14286 Vb	0.14286 Vb	7	33	28

All p-valor <0,04; exception *no significative difference for p=0.411.
All Fst, Fixation Index very high (Fst> 0,25), exception ** high (Fst= 0,15-0,25) (Wright, 1978; 52)
DF, degree of freedom; SQ, Sum of squares.
The population structure hypotheses at two geographic scales in São Paulo, Brazil.

between self-fertilization could, in theory, protect clonal lineages from the negative impacts of population bottlenecks that affect this species due to environmental droughts.

We suggest, for *B. tenagophila* the possibility of two independent genetic lineages: a clonal lineage, which would result from the selection of animals with high potential for self-fecundation, and a lineage of animals in which self-fecundation is used more parsimoniously. The clonal lineage would have the advantage of adaptability to new environments and being able to be dispersed on a large scale, which relies on a comparison between *B. tenagophila* samples collected throughout the distribution area of the species.

In *B. tenagophila*, it may be possible to find significant genetic differentiation among animals, even in close and similar freshwater environments in the outer group, as exemplified by the haplotype in the VR basin. This variation mostly occurs because of the high frequency of unique haplotypes, a pattern that may result from genetic drift.

In *B. straminea*, the genetic variation in both groups was similar, possibly reflecting a more recent spatial colonization potentialized by the high ability of the species to colonize new habitats (13, 58). The expansion of the heat shock protein family in the genome of *B. straminea* (59) can improve the adaptability of the species to environmental stressors, causing *B. straminea* to spread to new environments across local and continental geographic scales.

The values for genetic variations and the gene flow between the sampled snails on opposite banks of the Pardo River show that the river does not represent a geographical barrier to the spread of the species. The colonization of new environments can be achieved through natural connections between collections of water by the passive displacement of snails through floods and overflows (60, 61) and by dispersal over a very large distance by the resident or migratory waterbirds (62).

In conclusion, this study presents the first genetic analysis of all three Neotropical schistosomiasis hosts based on the distribution patterns of different mitochondrial DNA sequence loci. The results show that the geographical scale of *B. glabrata* and *B. tenagophila* sampling may lead to discrepancies in the assessment of genetic diversity in both species. Additional studies must be conducted to achieve new insights into the biological differences between both species, which may be linked to adaptive constraints for their spreading. The same argument does not seem to apply to *B. straminea* because of their high ability to inhabit diverse habitats across a wide range of geographical scales.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article.

Ethics statement

This study has the approval by the School of Public Health Committee for Ethics in Research (COEP) of USP through the Plataforma Brasil system (Ministry of Health, National Health Council) under reference number 53559816.0.0000.5421.

Author contributions

RP: The manuscript was part of the Doctoral thesis of RP. The author contributed with field and molecular data collection, analysis and interpretation of molecular data, geospatial mapping and manuscript preparation adding intellectual content. FC-N: Contribution to Geospatial Data, Analysis and interpretation, manuscript preparation, adding intellectual content. RT: Concept, design and coordination of the research project funded by Health Secretariat of São Paulo State. Molecular and morphological data analysis and interpretation. Manuscript preparation. Critical revision, adding intellectual content. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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