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CELLULAR AND MOLECULAR BIOLOGY

Phylogeny, morphology, and haplotypic distribution of *Biomphalaria straminea* populations from the five geographic regions of Brazil

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Abstract: *Biomphalaria straminea* is one of the three snails that act as the intermediate hosts of *Schistosoma mansoni* and is responsible for maintaining high rates of schistosomiasis in some areas of northeastern Brazil. The principal morphological characteristic of *B. straminea* is the marked vaginal wrinkling, although it is also present in congeneric species, a group known as the *B. straminea* complex. Based on the morphological and molecular analyses, we investigated the intraspecific variation of *B. straminea*. The specimens were obtained from 10 sites in Brazil, and the shells were analyzed, as well as 16 morphological structures of the reproductive system. The COI and ITS2 sequences were used for phylogenetic analysis, genetic divergence, and haplotype network (COI). We observed a large intraspecific variation in the morphological structures examined. The genetic divergence also demonstrated significant intraspecific variability in *B. straminea*: 0–3% in ITS2 sequences, and 0–6% in COI sequences. Analysis of the distribution of COI haplotypes recovered 16 haplotypes and haplotype diversity of 0.9088. These results indicate phenotypic variability that is not constrained to a locality or strictly controlled genetically by *B. straminea*, which may have driven a misidentified of these species.

Key words: *Biomphalaria* spp., haplotype network, COI, ITS2, phylogeny, Schistosomiasis.

INTRODUCTION

Schistosomiasis is a neglected parasitological disease that still remains a public health problem in Brazil, according to the most recent national prevalence survey (Katz 2018). Three species of snail of the genus *Biomphalaria* are responsible for the natural transmission of the parasite *Schistosoma mansoni* Sambon 1907 in Brazil. *Biomphalaria straminea* (Dunker 1848) is the most widespread species, occurring in 1325 municipalities, followed by *Biomphalaria glabrata* (Say 1818), which is found in 806 municipalities, and *Biomphalaria tenagophila* (d'Orbigny 1835), distributed in 603 municipalities (Scholte et al. 2012). The distribution of the species of the genus *Biomphalaria*, in particular *B. straminea*, in Brazil, contributes significantly to the progressive expansion of *S. mansoni*, including into areas previously free of the disease (Carvalho et al. 1988). Despite presenting the lowest rates of *S. mansoni* infection, *B. straminea* is responsible for maintaining high human infection rates in some areas of northeastern Brazil (Jansen 1946, Pellon & Teixeira 1950, Favre et al. 2002). It is important to note that *B. straminea* is the

species best adapted to the extremes of climate and ecological conditions found in Brazil and is especially tolerant of variation in physical-chemical factors (Paraense 1986). The potential of *B. straminea* as an invasive species due to its capacity to adapt to different biotic and abiotic conditions was pointed out by Yipp (1990) and Habib et al. (2018).

The principal morphological characteristic that differentiates *B. straminea* from its congeners is the presence of undulations in the dorsal wall of the vagina, a condition known as vaginal wrinkling. However, this wrinkling is also found in *Biomphalaria* species that do not transmit *S. mansoni*, which may have driven misidentification of these species. Given the morphological similarities observed among *B. straminea*, *Biomphalaria kuhniana* (Clessin 1833), and *Biomphalaria intermedia* Paraense & Deslandes 1962, Paraense (1988) proposed that they should be included in a group known as the "*B. straminea* complex". This author confirmed the complete reproductive isolation between specimens of *B. straminea* from Tangará, in the Brazilian state of Rio Grande do Norte, and *B. kuhniana* from the region of Tucuruí, on the Tocantins River, in Pará state, using albinism as a genetic marker. The study also described the diagnostic anatomical characteristics of these species, which according to the author, present different degrees of vaginal wrinkling, which is poorly developed in *B. kuhniana*, intermediate in *B. intermedia*, and conspicuous in *B. straminea*. The number of prostatic diverticula also varies among the species, with 4–9 being observed in *B. kuhniana*, 7–15 in *B. intermedia*, and 9–18 in *B. straminea,* and the distal segment of the spermiduct usually straight or slightly wavy in *B. kuhniana*, more or less curly in *B. straminea*. (Paraense & Deslandes 1955, 1962, Paraense 1988). The *B. straminea* complex was confirmed by Caldeira et al. (1998), who used a molecular analysis of the Restriction Fragment Length Polymorphism (RFLP) to identify three distinct groups, one of which contained *B. straminea* and *B. kuhniana*. The profile identified for one of the enzymes analyzed was the same in both *B. straminea* and *B. intermedia*. Dejong et al. (2001) and other sequenced based studies have also confirmed the *B. straminea* complex, a good confirmation of the work of Paraense (1988) and Caldeira et al. (1998).

In addition to this intra and interspecific variation, the morphological identification of the species is hampered by the small size of the mollusks, their maturity, and the lack of professional taxonomists with experience in the identification of the mollusks of the family Planorbidae, as well as the variation provoked by the distension of the organs during the fixation of the specimens (Paraense 1975, Caldeira et al. 2009). Given these problems, molecular tools have become increasingly important for the identification of taxa and have been widely employed in phylogenic studies of the genus *Biomphalaria*. Many studies have used the DNA barcode, that is, subunit I of the Cytochrome C oxidase (COI) gene (Campbell et al. 2000, Habib et al. 2018, Ohlweiler et al. 2020, Palasio et al. 2017, 2019, Tuan et al. 2012) and the Internal Transcriptional Spacers (ITS1 and ITS2) of the ribosomal DNA (Dejong et al. 2001, Caldeira et al. 1998, Campbell et al. 2000, Mavárez et al. 2002, Pepe et al. 2009, Spatz et al. 1999, Tuan & dos Santos 2007, Vidigal et al. 1998, 2000a, b, 2004) in both intra- and inter-specific analyses. These studies have shown that these molecular markers are effective for the differentiation of *Biomphalaria* species, as well as providing important tools for the diagnosis of cryptic species.

The correct identification of the species that act as the vectors of clinically-relevant parasites is vital for the discrimination of vectors from

non-vectors, and the implementation of the most effective measures for the monitoring and control of parasitic diseases such the schistosomiasis. Given this, the present study verified the potential intraspecific variation in specimens of *B. straminea* based on a combination of molecular and morphological analysis and, for the first time, with samples of many sites from all five geographic regions of Brazil, to provide a more solid baseline for future comparative studies about the *B. straminea* complex.

MATERIALS AND METHODS

Collection and processing of the samples

The present study was based on the analysis of 10 sites of *B. straminea*, which were sampled through the collection of specimens from the live colonies maintained in the molluscarium of the National Reference Laboratory for Schistosomiasis/Malacology at the Oswaldo Cruz Institute (LRNEM-IOC) in Rio de Janeiro, Brazil. The activities of the LRNEM-IOC are carried out following the Standard Operating Procedure (SOP), which has well-established protocols, to minimize errors and cross-contamination. The source populations for the captive colonies are from eight different Brazilian states (Fig. 1), representing the country's five geographical regions (North, Northeast, Midwest, Southeast, and South). The MidWest region is represented by two municipalities in the state of Goiás (Minaçu and Uruaçu), and the North region by two municipalities in the state of Pará

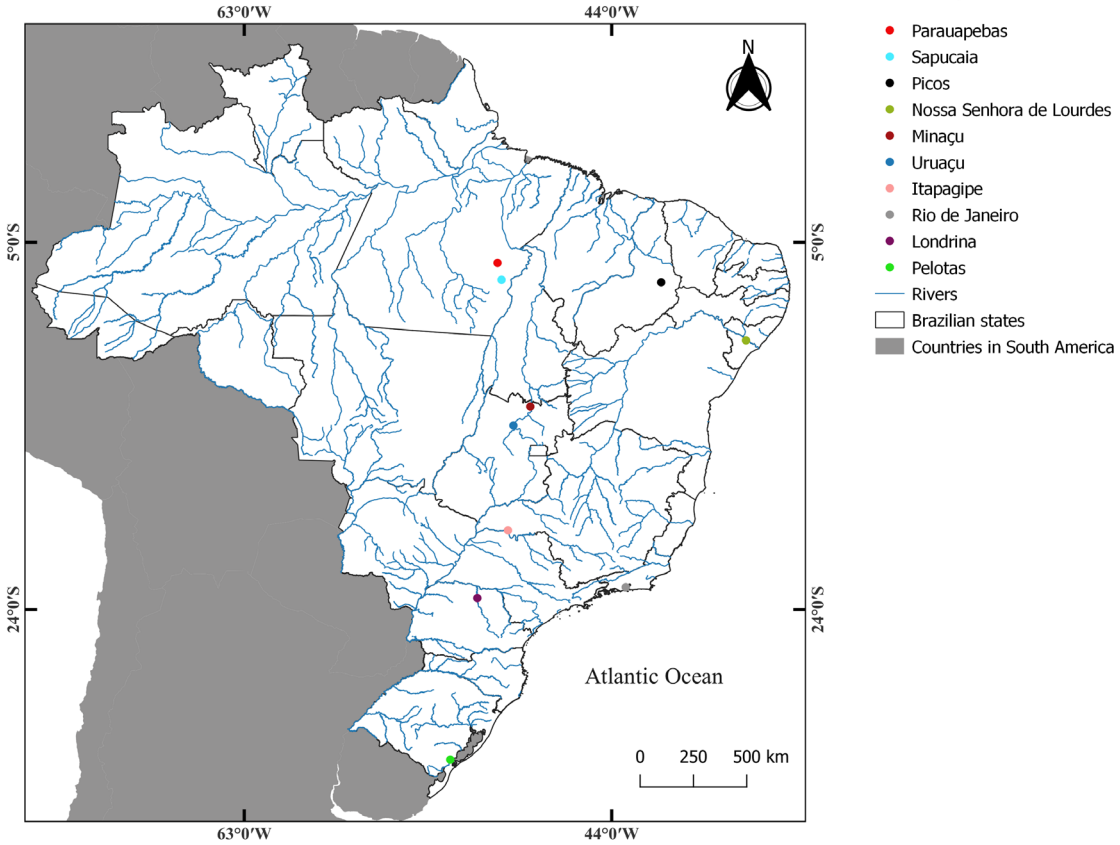

Figure 1. Map of Brazil showing the origins of the 10 *Biomphalaria straminea* sites sampled for the live colonies maintained in the Laboratório de Referência Nacional para Esquistossomose-Malacologia do Instituto Oswaldo Cruz (LRNEM-IOC) in Rio de Janeiro, Brazil.

(Parauapebas and Sapucaia). The populations from the Northeast region were from the municipalities of Picos in Piauí state and Nossa Senhora de Lourdes in Sergipe, while those from the South region were from Londrina in Paraná and Pelotas in the Rio Grande do Sul state. In the Southeast region, the sites were from Itapagipe in Minas Gerais state and Rio de Janeiro, in Rio de Janeiro state. The specimens were collected years ago (from five to more than 40 years), without receiving specimens from the field throughout time. They were identified as *B. straminea* mainly by Dr. Wladimir Lobato Paraense, who was specialist in the group, except the more recently collected specimens. All the specimens obtained were adults, with the largest available specimens being selected preferentially, to minimize the potential influence of morphological variation related to sexual maturity or very young individuals. The specimens are deposited at the Oswaldo Cruz Institute Mollusk Collection (CMIOC 13907 – 13917).

Five *B. straminea* specimens were obtained from each site, with a total sample of 50 individuals. The live animals were submerged in a solution of anesthetic (1% Hypnol) until their tissue had relaxed completely, which takes approximately six hours, and was confirmed when there was no reaction to prodding with tweezers. The specimens were then immersed in water at 70 °C for 40 seconds and subsequently in water at ambient temperature to cool them down. The soft part of each specimen was then extracted from the shell by the foot using fine-pointed tweezers and immersed in water. A tissue fragment was then extracted from the mantle/foot of each specimen and stored in individual Eppendorf tubes, which were frozen to -20°C for molecular analyses. The bodies were fixed in Raillet-Henry solution for the morphological analyses (Brasil 2008) and the shells were washed and dried at ambient temperature.

Morphological and conchological analyzes

Forty-five specimens were dissected under a stereomicroscope, and the principal structures of the reproductive system (both female and male) were drawn using a camera lucida and measured manually from these drawings using a ruler. A total of 16 morphological characters were analyzed in each specimen (Table I, Fig. 2), and eight indices were derived from these parameters (Table II).

For the conchological analysis, the shells were photographed under a Leica M205C stereomicroscope attached to a DMC2900 digital camera, and their length and width were measured with a caliper. The size of the shell of each specimen was analyzed, and the mean and standard deviation were calculated for each site.

DNA Extraction, amplification by PCR, and sequencing

Approximately 3 mm of foot tissue was removed from 89 snails and gDNA was extracted using the Qiagen DNA Easy Blood and Tissue kit following the manufacturer's protocol. The Polymerase Chain Reaction (PCR) was performed to amplify part of the subunit I of the mitochondrial Cytochrome Oxidase (COI, ~710 bp) gene using the forward LCO1490 (5' -GGTCAACAAATCATAAAGATATTGG - 3') and reverse HCO2198 (5' - TAAACTTCAGGGTGACCAAAAAATCA - 3') primers of Folmer et al. (1994). In addition, the second region of the Internal Transcriptional Spacer (ITS2, ~460 bp) of ribossomal DNA was amplified by PCR using the forward primer ITS2 (5' -CGTCCGTCTGAGGGTCGGTTTGC - 3') of Vidigal et al. (2000b) and the reverse primer, ETTS1 (5' - TGCTTAAGTTCAGCGGGT-3') of Kane & Rollinson (1994). The PCR was run in a 25 µL mix of the reagents containing a final concentration of 1X

Table I. Morphological parameters of the male and female reproductive systems in the *Biomphalaria straminea* specimens obtained from the municipalities and states representing the different regions of Brazil.

Character*[1]	Mean ± standard deviation (in mm) recorded in the population from:								
	Pelotas (RS)	Sapucaia (PA)	Londrina (PA)	Minaçu (GO)*[2]	Uruaçu (GO)	Itapagipe (MG)	Picos (PI)	Rio de Janeiro (RJ)	Parauapebas (PA)
1	2.39 ± 0.51	1.58 ± 0.19	2.48 ± 0.32	1.89 ± 0.37	1.90 ± 0.57	2.05 ± 0.38	2.40 ± 0.50	2.45 ± 0.41	2.44 ± 0.26
2	0.38 ± 0.19	0.28 ± 0.11	0.35 ± 0.05	0.31 ± 0.05	0.35 ± 0.08	0.41 ± 0.05	0.32 ± 0.05	0.34 ± 0.07	0.29 ± 0.07
3	0.69 ± 0.34	0.40 ± 0.11	0.57 ± 0.14	0.46 ± 0.19	0.50 ± 0.13	0.53 ± 0.21	0.66 ± 0.16	0.52 ± 0.06	0.76 ± 0.51
4	3.52 ± 1.00	2.15 ± 0.44	3.40 ± 0.39	2.93 ± 0.44	2.70 ± 0.49	2.70 ± 0.46	3.16 ± 0.55	3.22 ± 0.42	3.33 ± 0.44
5	0.19 ± 0.07	0.16 ± 0.03	0.17 ± 0.04	0.14 ± 0.02	0.19 ± 0.05	0.22 ± 0.04	0.23 ± 0.06	0.20 ± 0.06	0.16 ± 0.03
6	0.21 ± 0.11	0.10 ± 0.02	0.17 ± 0.02	0.13 ± 0.03	0.17 ± 0.03	0.18 ± 0.03	0.16 ± 0.03	0.17 ± 0.05	0.19 ± 0.04
7	11 ± 0	9 ± 1	11 ± 2	8 ± 1	10 ± 3	9 ± 2	12 ± 1	12 ± 1	12 ± 1
8	0.44 ± 0.09	0.35 ± 0.06	0.41 ± 0.11	0.33 ± 0.10	0.43 ± 0.08	0.61 ± 0.15	0.37 ± 0.10	0.36 ± 0.10	0.47 ± 0.13
9	5 ± 2	4 ± 3	5 ± 1	6 ± 4	6 ± 4	10 ± 4	5 ± 2	5 ± 2	6 ± 3
10	2.14 ± 0.26	0.93 ± 0.15	2.03 ± 0.49	0.87 ± 0.32	1.34 ± 0.47	1.19 ± 0.82	1.07 ± 0.20	1.35 ± 0.20	1.51 ± 0.30
11	0.83 ± 0.12	0.40 ± 0.11	0.67 ± 0.17	0.53 ± 0.13	0.52 ± 0.18	0.50 ± 0.34	0.78 ± 0.20	0.70 ± 0.16	0.52 ± 0.11
12	0.85 ± 0.15	0.65 ± 0.07	1.06 ± 0.06	0.64 ± 0.06	0.75 ± 0.22	0.76 ± 0.13	0.98 ± 0.18	0.95 ± 0.24	0.71 ± 0.15
13	1.25 ± 0.36	0.72 ± 0.12	1.24 ± 0.17	0.69 ± 0.15	0.86 ± 0.19	1.40 ± 0.39	1.08 ± 0.24	0.93 ± 0.25	1.01 ± 0.13
14	0.42 ± 0.18	0.36 ± 0.15	0.54 ± 0.04	0.41 ± 0.11	0.39 ± 0.06	0.61 ± 0.13	0.59 ± 0.26	0.44 ± 0.12	0.42 ± 0.20
15	0.69 ± 0.39	0.76 ± 0.19	0.86 ± 0.18	0.72 ± 0.22	1.07 ± 0.17	0.97 ± 0.33	0.87 ± 0.13	0.76 ± 0.19	0.76 ± 0.21
16	3 ± 2	4 ± 3	4 ± 0	4 ± 1	6 ± 2	5 ± 2	2 ± 1	3 ± 2	5 ± 2

***Characters[1]: 1: length of the prepuce; 2: width of the tip of the prepuce; 3: width of the base of the prepuce; 4: length of the penis sheath; 5: width of the penis sheath; 6: maximum width of the vas deferens; 7: number of prostatic diverticula; 8: length of the basal diverticulum; 9: number of branches of the basal diverticulum; 10: distance between the first and last diverticulum; 11: distance between the female opening and the insertion of the spermatheca; 12: length of the spermathecal duct; 13: length of the spermatheca; 14: width of the spermatheca; 15: extension of the area of vaginal wrinkling; 16: number of folds in the vaginal wrinkling. n=5, except Minaçu/GO n=6*[2].**

Ludwig 10 x buffer, 0.2 µM of dNTP, 2.5 µM Ludwig $mgCl_{2,}$ 0.2 µM of each primer, 1 µM of Taq Ludwig Hot Start, 2 µL of the DNA extracted from each sample, and a total reaction volume of 50 µL. The PCR cycle for the ITS2 was: 5 minutes at 95°C, 32 cycles of 45 seconds at 95°C, 1 minute at 60°C, 2 minutes at 72°C, 5 minutes at 72°C, and 4 minutes at 20°C (adapted from Vidigal et al. 1998). For the COI gene, the cycle was: 5 minutes at 95°C, 30 cycles of 1 minute at 95°C, 1 minute at 52°C, 1 minute at 72°C, 5 minutes at 72°C, and 4 minutes at 20°C (adapted from Hayes et al. 2009).

The PCR products were verified via gel electrophoresis and purified using the GFX PCR DNA and Gel Band Purification kit from GE Healthcare following the manufacturer's instructions. The cycle sequencing of the samples was performed in both directions with the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and analyzed via capillary electrophoresis on the ABI 3730XL (Applied Biosystem) at the sequencing facility at Fiocruz Technological Platform Network. The sequences of both markers were edited manually in the Seqman software v. 7 (DNAStar, INC) for errors and ambiguities.

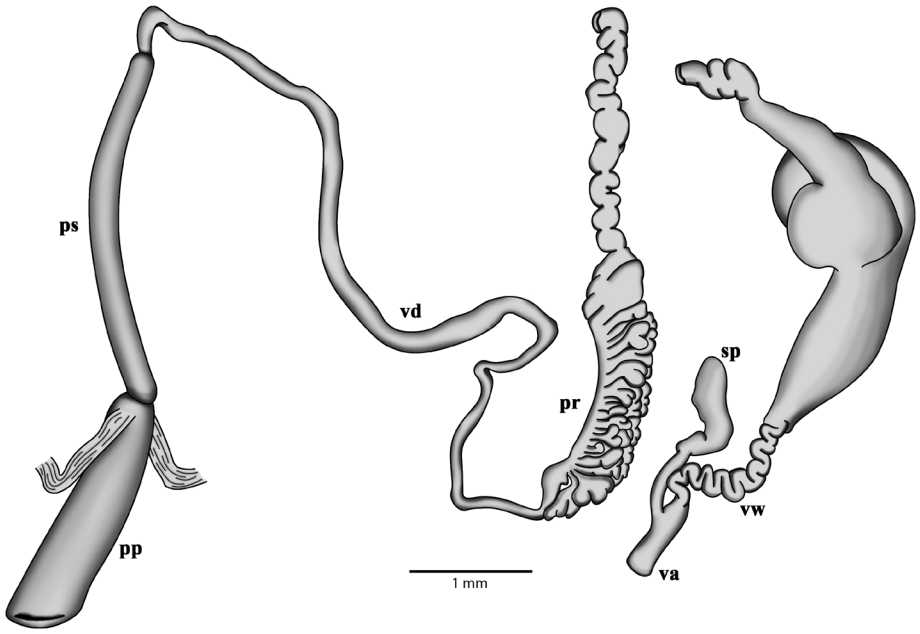

Figure 2. Diagram of the reproductive system of *Biomphalaria straminea* showing the principal structures analyzed. Abbreviations: **pp**: prepuce; **ps**: penis sheath; **vd**: vas deferens; **pr**: prostate; **sp**: spermatheca; **vw**: vaginal wrinkling; **va**: vagina.

Table II. Mean ratios between the morphological parameters recorded in each *Biomphalaria straminea* site (municipality).

	Mean ratio recorded in the population from:								
Ratio*[1]	**Pelotas**	**Sapucaia**	**Londrina**	**Minaçu*[2]**	**Uruaçu**	**Itapagipe**	**Picos**	**Rio de Janeiro**	**Parauapebas**
1	1.48	1.37	1.37	1.55	1.42	1.32	1.32	1.31	1.36
2	1.84	1.42	1.65	1.48	1.41	1.28	2.06	1.53	2.62
3	0.19	0.11	0.19	0.11	0.14	0.13	0.09	0.12	0.13
4	3	2.01	2.28	1.68	2.23	2.29	1.83	2.11	2.4
5	1.48	1.11	1.17	1.08	1.15	1.84	1.1	0.98	1.42
6	0.23	0.19	0.23	0.18	0.17	0.19	0.44	0.25	0.15
7	0.32	0.81	0.42	0.83	0.8	0.81	0.81	0.56	0.5
8	0.9	1.56	1.02	1.08	1.14	1.17	1.44	1.18	0.84

***[1]Morphometric ratios: 1: length of the penis sheath *vs.* length of the prepuce; 2: width of the prepuce at the base *vs.* the tip; 3: length of the insertion of the prostatic diverticula *vs.* the number of diverticula; 4: length *vs.* width of the spermatheca; 5: length of the spermatheca *vs.* length of the spermathecal duct; 6: length of the vaginal wrinkling *vs.* the number of folds; 7: length of the vaginal wrinkling *vs.* the length of the prostatic diverticula; 8: maximum width of the vas deferens *vs.* maximum width of the penis sheath. n=5, except Minaçu/GO n=6*[2].**

Alignment, phylogenetic analysis, genetic divergence, and haplotype network

The COI sequences were aligned in the online TranslatorX server (Abascal et al. 2010) using the algorithm for the alignment of multiple sequences ClustalW (Thompson et al. 1994). The ITS2 sequences were aligned using the online MAFFT algorithm, version 7 (Katoh et al. 2019), with the interactive E-INS-i refinement selected, and all other parameters on default. The nucleotide substitution models were selected using the Bayesian Information Criterion (BIC), run in jModelTest version 2.1.10 (Darriba et al. 2012).

Consensus sequences of COI and ITS2 were concatenated for the Bayesian Inference (BI) in

MrBayes 3.2.7 (Ronquist et al. 2012), including seventy-six sequences (COI: ON704917-54; ITS2: ON707165-202) previously identified as *B. straminea* as part of this work in addition to consensus sequences of *Bimphalaria* and *Helisoma* species obtained from GenBank. One species of *Helisoma* was selected as the outgroup of *Biomphalaria* species, according to the studies of Hubendick & Rees (1955), Bandoni et al. (1995), Sullivan et al. (1995), and Dejong et al. (2001), who referred both genera are closely-related planorbids.

The run of BI was configured to four simultaneous chains, in two independent runs with different random trees, consisting of four Markov chains, that were sampled every 100 generations over 10,000,000 generations. The branch support values were determined by the Bayesian Posterior Probabilities (BPPs) of the trees sampled following the removal manually of the first 10% of the trees as burn-in. The trees were visualized in FigTree 1.4.4 (Rambaut 2012) and edited in Adobe Photoshop 2021 Portable. Effective Sample Size (ESS) was estimated in Tracer v1.7 (Rambaut et al. 2018).

The intra- and inter-specific genetic divergence of COI and ITS2 were calculated in the Mega X software (Kumar et al. 2018) based on the Kimura 2-parameter model (Kimura 1980). The DnaSP software v. 6.12.03 (Rozas et al. 2017) was used to calculate the number of haplotypes (*h*) and produce the haplotype network. In this analysis we combinate 59 COI sequences of *B. straminea* (*N* = 50 from this work: ON704917-ON704954; ON714049-ON714060 and *N* = 9 available on GenBank: KF926184-91 and KF926195) from 14 sites in different regions of Brazil.

RESULTS

Morphological and conchological analyzes

The morphology of the reproductive system was analyzed in the five specimens obtained from all the study sites except that from Nossa Senhora de Lourdes. Unfortunately, it was not possible to measure the morphological structures in these specimens, although we observed through the qualitative examination that the vaginal wrinkling of one of them was very discreet and had a swollen appearance, which was quite distinct from the other individuals analyzed (Fig. 3). In addition, one of the specimens from Sapucaia, which was only analyzed molecularly, had only three prostatic diverticula (Fig. 4).

All the 16 parameters analyzed varied among individuals, not only from different sites but also from the same one (Table I), except from Pelotas, where all five specimens had 11 prostatic diverticula. Considerable variation was also observed in the degree of vaginal wrinkling, which is the principal diagnostic feature of *B. straminea*, including within the same site, as found in the specimens from Sapucaia, in which the undulations varied from discreet to pronounced (Fig. 5). The specimens from Londrina presented the least variation in the degree of vaginal wrinkling, with four of the specimens having four folds in the dorsal wall of the vagina (Fig. 6 a, c, d and e) and one specimen with three folds (Fig. 6b).

Another important characteristic that varied considerably was the number of prostatic diverticula, as observed in the specimens from Uruaçu, with 7–14 diverticula being recorded in different individuals (Fig. 7). This variation was also observed among the different sites and in individuals of approximately the same size.

Variation was also found in the proportional indices, such as the proportion between the length of the spermatheca and the spermathecal duct, and between the width of the penis sheath

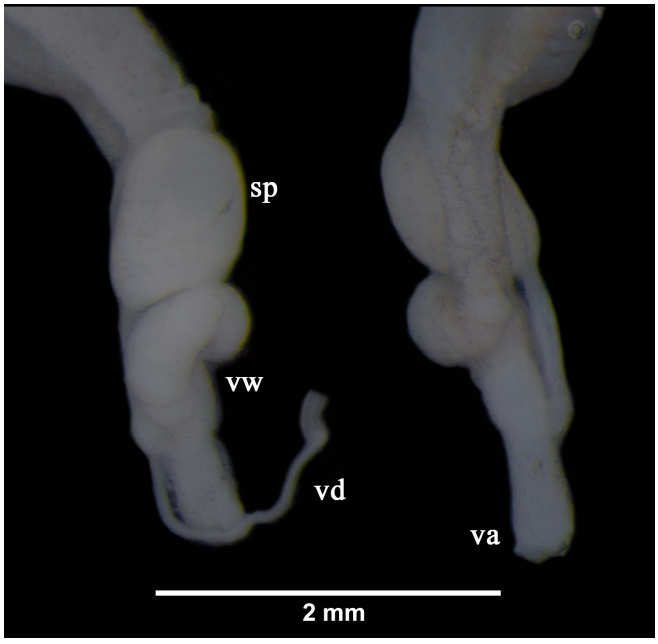

Figure 3. Photograph of the vaginal wrinkling of *Biomphalaria. straminea* specimen 7 from Nossa Senhora de Lourdes (Photograph by Eduardo Cinilha). Abbreviations: **sp**: spermatheca; **vw**: vaginal wrinkling; **vd**: vas deferens; **va**: vagina.

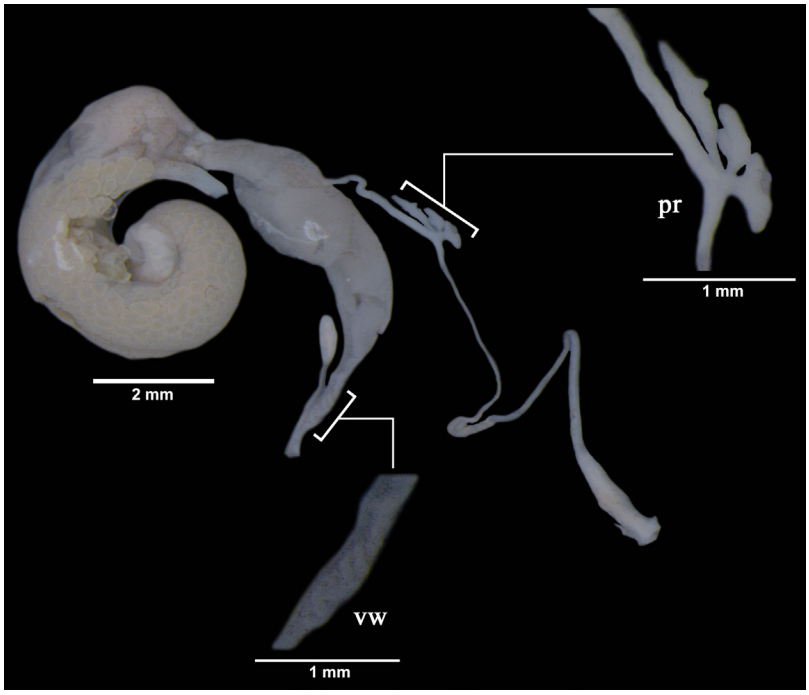

Figure 4. Photograph of the complete reproductive system of *Biomphalaria straminea* specimen 8 from Sapucaia, showing the three prostatic diverticula, as well as the vaginal wrinkling typical of the species (Photograph by Eduardo Cinilha). Abbreviations: **pr**: prostate; **vw**: vaginal wrinkling.

and the vas deferens. The spermathecal duct was shorter than the spermatheca in almost all the sites, except for that of Rio de Janeiro, and in some individuals from Minaçu, Uruaçu, Sapucaia, and Londrina. The width of the penis sheath was greater than that of the median portion of the vas deferens in most of the sites, except for Pelotas and Parauapebas. However, the ratio between the length of the penis sheath and the prepuce did not vary significantly, wire the sheath being invariably longer than the prepuce. The base of the prepuce was also invariably wider than its tip.

Considerable variation was also observed in both conchological characters measures (Table III). The highest mean values were recorded in

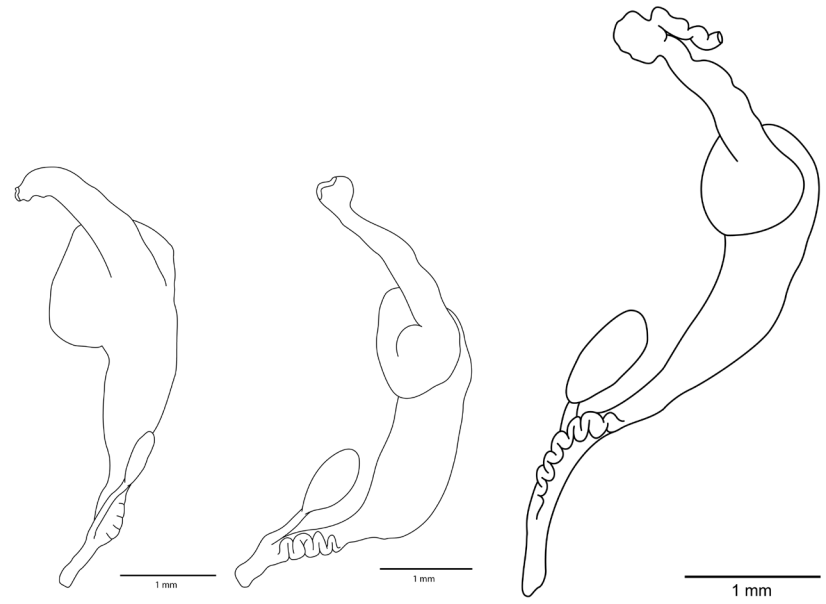

Figure 5. Diagram drawn by the authors, showing the intraspecific variation in the degree of vaginal wrinkling observed in the *Biomphalaria straminea* specimens from Sapucaia, in the Brazilian state of Pará (specimens 1, 2, and 5, from left to right).

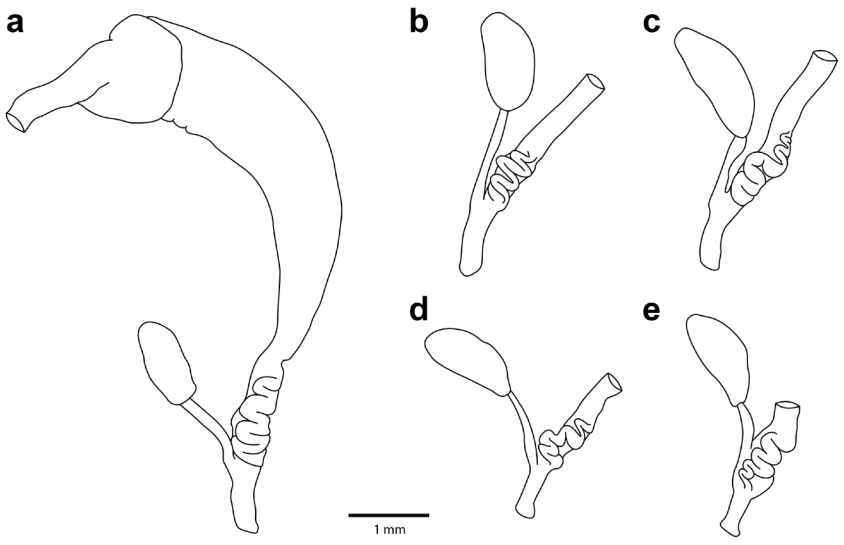

Figure 6. Diagram showing the relative lack of intraspecific variation in the degree of vaginal wrinkling observed in the *Biomphalaria straminea* specimens from Londrina, in the Brazilian state of Paraná (**a, b, c, d,** and **e** are specimens 9, 3, 2, 5, and 6, respectively).

specimens from Pelotas (Fig. 8a), for both shell length (7.82 mm) and width (3.23 mm), while the lowest means (length = 4.79 mm; width = 2.11 mm) were recorded in specimens from Sapucaia (Fig. 8b).

Phylogenetic analysis, genetic divergence, and haplotype network

The total length of the sequences was 1315 bps (COI = 1–655; ITS2 = 656–1315), including missing data for both genes and gaps from ITS2. The nucleotide substitution models were HKY+I+G (I: 0.58, G: 2.329) for COI and GTR+G (G: 0.502) for ITS2. The trees sampled by the Markov chains following the elimination of the burn-in had a mean score of lnL= -6567.2382. The values of Effective Sample Size (ESS) were all above 776 effectively independent samples for all the parameters, which indicates that the sample was robust (Lanfear et al. 2016).

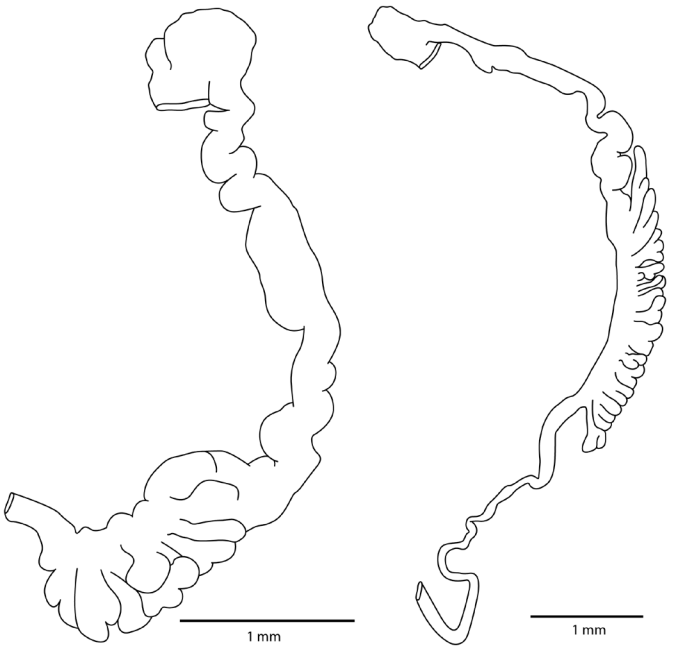

Figure 7. Diagram showing the intraspecific variation in the number of prostatic diverticula in the *Biomphalaria straminea* specimens from Uruaçu, in the Brazilian state of Goiás: specimen 5 (right, with 7 diverticula) and specimen 2 (left, with 14 diverticula).

Table III. Mean length and width of the *Biomphalaria straminea* shells collected from the different sites (municipalities) analyzed, n=5, except Minaçu/GO n=6*.

	Mean ± standard deviation (in mm) recorded in the population from:								
Character	**Pelotas**	**Sapucaia**	**Londrina**	**Minaçu***	**Uruaçu**	**Itapagipe**	**Picos**	**Rio de Janeiro**	**Parauapebas**
Length	7.82 ± 1.08	4.79 ± 0.43	7.60 ± 0.70	5.72 ± 1.13	6.15 ± 0.91	6.96 ± 0.37	7.61 ± 0.61	6.39 ± 0.53	6.19 ± 0.89
Width	3.23 ± 0.34	2.11 ± 0.20	2.77 ± 0.16	2.47 ± 0.30	2.54 ± 0.32	2.93 ± 0.06	2.81 ± 0.21	2.25 ± 0.17	2.37 ± 0.26

Phylogenetic analysis recovered a clade composed of specimens from the state of Pará (municipalities of Parauapebas and Sapucaia), including one with only three prostatic diverticula. The other specimens of *B. straminea* were in another clade, as a sister group of this clade, in addition to *B. kuhniana,* while the *B. intermedia* sequence was in a separate clade, sister group to the one grouping both clades. All these groups were supported strongly (BPP = 0.95–1.00; Fig. 9). The specimen from Nossa Senhora de Lourdes with the swollen dorsal wall of the vagina was grouped with *B. straminea* from other sites (Londrina, Parauapebas, and Rio de Janeiro), in which we observed typical vaginal wrinkling, in the analysis of the mitochondrial gene (data not shown).

The analysis based on the DNA barcode demonstrated an intraspecific genetic distance of 0–6%. The interspecific genetic distances between *B. straminea* and *B. kuhniana* ranged from 0% to 4%, while the distances between *B. straminea* and *B. intermedia* were 5–7%. The largest intraspecific distance observed in *B. straminea* in the nuclear marker was similar to the maximum distance found between *B. kuhniana* and *B. straminea* (3%), while the distance between *B. straminea* and *B. intermedia* was 6–11%. The intraspecific genetic distance is shown in Table IV.

To evaluate the haplotype distribution, we previously removed the missing data and the final length included 585 sites (number of polymorphic (segregating) sites, *S*: 428; the

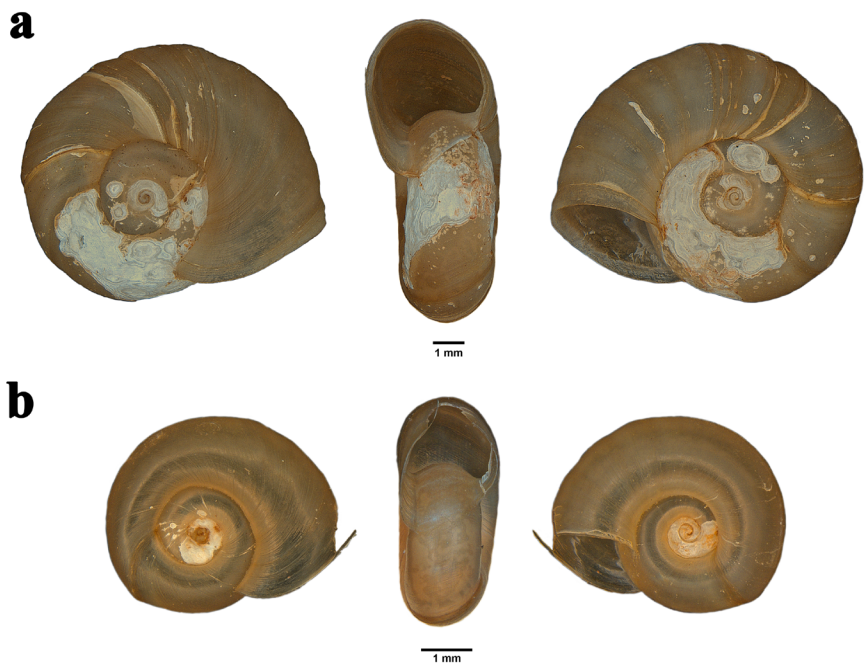

Figure 8. Shells of *Biomphalaria straminea* from (a) Pelotas (specimen 1), and (b) Sapucaia (specimen 3).

total number of mutations, *Eta*: 462; the total number of singleton mutations, *Eta(s)*: 9. Sixteen haplotypes (h = 16) were identified with a haplotype diversity of h_d = 0.9088, nucleotide diversity π = 0.0885, and mean number of nucleotide differences k = 51.809. The number of haplotypes in each site ranged from one to three, with Picos/PI, Londrina/PR, and Pelotas/RS not showing haplotypic diversity, while most sites analyzed (Nossa Senhora de Lourdes/SE, Rio de Janeiro/RJ, Sapucaia/PA, Parauapebas/PA, and Itapagipe/MG) and two sites from the state of Goiás (Uruaçu and Minaçu) presented two and three haplotypes, respectively (Fig. 10). The specimens from Parauapebas/PA, Rio de Janeiro/RJ, and Pelotas/RS shared a haplotype with specimens from Itariri/SP and Aparecida/SP. This haplogroup was recorded in the largest number of individuals and sites. Specimens from the state of São Paulo also share haplotypes with individuals from Rio de Janeiro/RJ and the whole of the specimens from Londrina/PR, Parauapebas/PA, and Sapucaia/PA. The specimens from Uruaçu and Minaçu shared only one haplotype even though both municipalities are relatively close in the state of Goiás. In contrast, two haplotypes of these sites are shared with the specimens from Itapagipe/MG.

DISCUSSION

The species of the *B. straminea* complex have been misidentified due to the phenotypic plasticity (Paraense 1988, Vidigal et al. 2000b) and have caused taxonomic confusion in this group composed of species considered refractory to infection by *S. mansoni* and *B. straminea*, which has epidemiological importance. Through our integrative analyzes, we found large variability in morphological and molecular characters of the different specimens of *B. straminea.*

These results are consistent with the pattern found in previous studies, such as that of Paraense (1957), who highlighted the presence of planorbid subpopulations with divergent characteristics, even within the same geographic region. The considerable variation found in

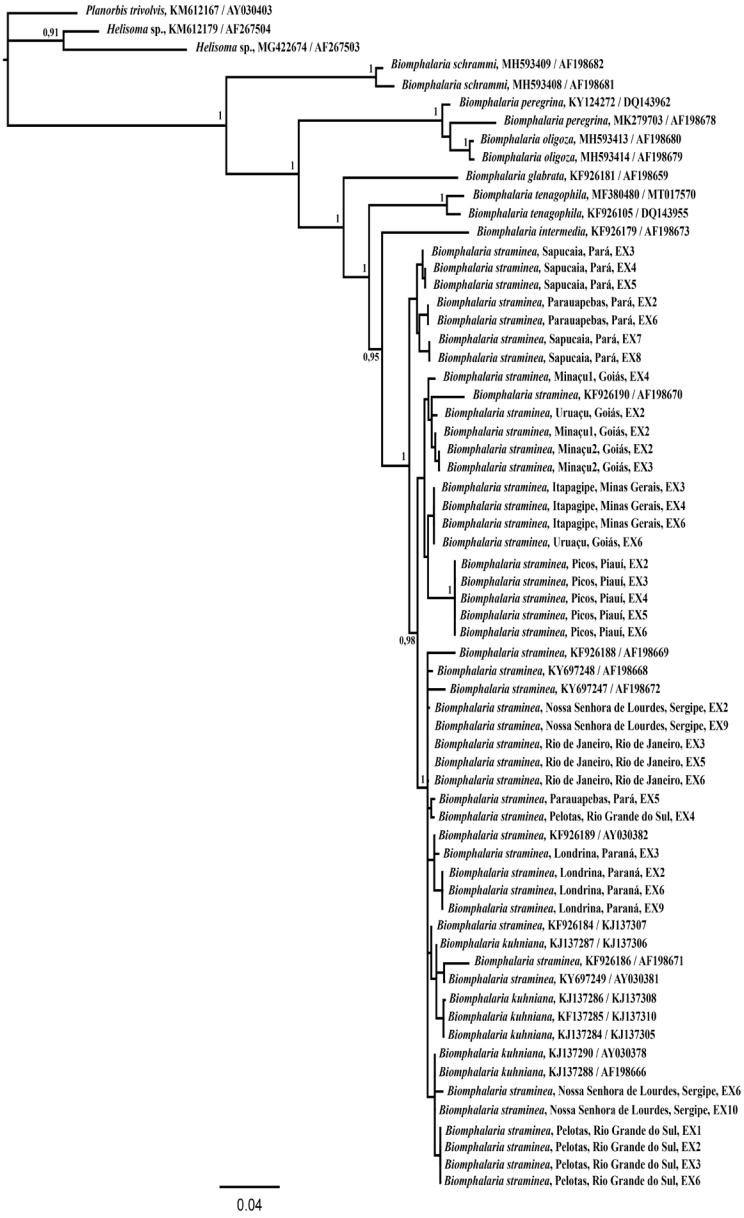

Figure 9. Phylogenetic tree generated by the analysis of the concatenated sequences (COI + ITS2) of the *Biomphalaria straminea* and outgroup included in the present study using Bayesian Inference. The node support values are Bayesian posterior probabilities (BPPs).

the principal diagnostic characteristic of *B. straminea* hampers the reliable identification of the species. As no conspicuous vaginal wrinkling–which is typical of *B. straminea*–was observed in some individuals, while one of the specimens presented swelling in this region, there is some potential overlap with the other species of the *B. straminea* complex, therefore, these morphological variations and similarities may lead to erroneous or inconclusive identifications. However, some authors have reported a lower variation in morphological characteristics in specimens of *B. straminea*. Palasio et al. (2017) observed that both *B. straminea* and *B. intermedia* had undulations in the dorsal wall of the vagina, albeit with considerable variation between the species, being undulated in *B. straminea* and swollen in *B. intermedia*. Habib et al. (2018) analyzed *B. straminea* specimens introduced into southern

Table IV. ***Biomphalaria straminea*** **intraspecific genetic distances obtained using the Kimura 2 Parameter model (Kimura 1980) in MEGAX software (Kumar et al. 2018). The genetic distance of the ITS2 sequences is above the diagonal and the COI below.**

Localities	Sapucaia	Itapagipe	Uruaçu	Pelotas	Londrina	N. Sra. de Lourdes	Parauapebas	Minaçu	Rio de Janeiro	Picos
Sapucaia	—	0.00–0.02	0.00–0.03	0.00–0.02	0.01–0.03	0.00–0.01	0.01–0.03	0.01–0.03	0.00–0.02	0.00–0.02
Itapagipe	0.02–0.04	—	0.00–0.01	0.00–0.02	0.00–0.01	0.00	0.01	0.01	0.00–0.01	0.00–0.01
Uruaçu	0.02–0.03	0.00–0.01	—	0.00–0.03	0.00–0.01	0.00–0.01	0.01–0.02	0.01–0.02	0.00–0.01	0.00–0.01
Pelotas	0.03	0.02–0.03	0.02	—	0.01–0.03	0.00–0.02	0.01–0.03	0.00–0.02	0.00–0.02	0.01–0.02
Londrina	0.03–0.04	0.02–0.04	0.02–0.04	0.01–0.02	—	0.01	0.01–0.02	0.00–0.02	0.00–0.02	0.01–0.02
N. Sra. de Lourdes	0.02–0.04	0.02–0.03	0.02–0.03	0.00–0.01	0.01–0.02	—	0.01–0.02	0.00–0.01	0.00–0.01	0.00–0.01
Parauapebas	0.00–0.03	0.02–0.03	0.02–0.03	0.00–0.03	0.01–0.04	0.00–0.04	—	0.01–0.02	0.01–0.02	0.01
Minaçu	0.02–0.03	0.00–0.02	0.00–0.02	0.02	0.02–0.03	0.02–0.03	0.02–0.03	—	0.01–0.02	0.01
Rio de Janeiro	0.03	0.01–0.03	0.01–0.02	0.00–0.03	0.01–0.04	0.00–0.03	0.00–0.03	0.01–0.03	—	0.00–0.01
Picos	0.04–0.05	0.3	0.3	0.04–0.05	0.04–0.06	0.04–0.05	0.04–0.05	03	0.03–0.05	—

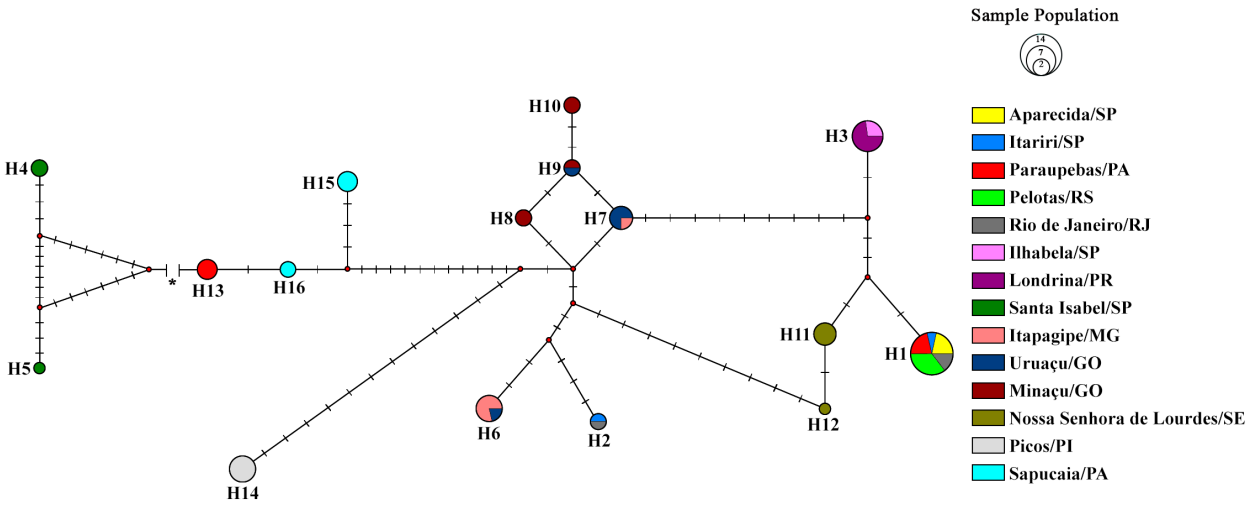

Figure 10. Haplotype network of the ***Biomphalaria straminea*** **sites. Each site is represented by a different color, as shown in the legend. Each circle represents a haplotype and its size is proportional to the number of individuals that share that haplotype. The red diamonds represent haplotypes that were not sampled or extinct. The gap marked with an asterisk (*) represents a total of 405 mutations.**

China in addition to specimens from Brazil and observed that the principal characteristics of the reproductive system were consistent with those described in previous studies.

The variation observed in the number of prostatic diverticula was lower than that found by Paraense (1975) in individuals from the state of Minas Gerais, which had between 5 and 20 diverticula, while Palasio et al. (2017) mentioned 20 diverticula and Habib et al. (2018) 10–20. The morphometric ratios were consistent with those reported by Palasio et al. (2017), such as the ratio between the width of the base and the tip of the prepuce, in which the free extremity of the prepuce was invariably larger, and that between the length of the penis sheath and of the prepuce, with the sheath being longer in all the sites. These findings contrast with those of Paraense (1975), however, in which the length of the penis sheath varied from slightly shorter

to much longer than the prepuce. The variation observed in the penis sheath–vas deferens width ratio (0.84–1.56) was also smaller than that recorded by Paraense & Deslandes (1955), i.e., 3.17±0.47mm.

The mean shell measurements observed in the nine sites analyzed were considerably lower than that reported by Paraense (1975), i.e., mean length of 16.5±6 mm and were more similar to the value recorded for *B. kuhniana* (7.5 mm) than for *B. straminea* (11.0–16.5 mm) by Paranse (1988). Even though some *Biomphalaria* species were first described based on the diagnosis of conchological characters, these parameters do not necessarily provide reliable criteria for identification, given their vulnerability to the influence of a range of variables, including environmental factors (Paraense 1961). In his study of the systematics of Brazilian planorbids, Paraense (1975) emphasized that the morphological characters used to describe each species are subject to considerable variation. One example is the lateral surface of the shell, which may be more or less concave or even concave on one side and convex on the other. The authors conclude that "...these features, which reflect the individual variation found in some populations, may become dominant traits in other populations, reflecting fluctuations in the relative frequency of the genes that determine these traits. This variation is responsible for the large number of synonyms found in the conchological literature" (Paraense 1975).

Our phylogenetic analysis corroborated the morphology and previous results which recovered *B. straminea* and *B. kuhniana* as a monophyletic group (Vidigal et al. 2000b, Dejong et al. 2001), sister group of *B. intermedia*. These results may indicate that *B. straminea* is more closely related to *B. kuhniana* than to *B. intermedia*, or could be due to the lack of sequences of this last species in Genbank, or even due to hybridization processes, which would produce inconsistent results between the nuclear and mitochondrial genes since the last is inherited from the mother (Dejong et al. 2001). Hybridization processes have already been reported in *Biomphalaria* species, such as between *Biomphalaria cousini*, Paraense 1966 and *Biomphalaria amazonica*, Paraense 1966 (Teodoro et al. 2011), but it needs to be further studied, mainly among the species of the *B. straminea* complex.

The branch length in the phylogenetic analysis of the ITS2 sequences indicates that they accumulate more differences than the COI sequences, which was already expected due to the internal transcribed spacer regions (ITS1 and ITS2) being highly variable and used in phylogenetic analyzes between related taxa that have diverged in the last fifty million years (Carvalho et al. 2008, Zhang et al. 2018), while coding genes are more conserved regions due to protein translation. Also, some of the longest branches of *B. straminea* are sequences from GenBank, which may be due to the sequences being older and less accurate.

The largest intraspecific distance in the mitochondrial gene of *B. straminea* (0–6%) was the same as the interspecific distances found between some specimens of *B. tenagophila* and *Biomphalaria occidentalis*, Paraense 1981, and between *B. intermedia* and *B. kuhniana*. It is important to note, however, that these pairs of species are members of the *B. tenagophila* and *B. straminea* complexes, respectively. While the greatest interspecific distance between *B. straminea* and *B. kuhniana* from GenBank was lower (4%). In the ribosomal gene, the largest intraspecific genetic distances observed in *B. straminea* were the same as the largest interspecific distance between *B. straminea* and *B. kuhniana* from GenBank (3%). Although Habib et al. (2018) contested the identification of the COI

sequences deposited by Attwood et al. (2015) for *B. kuhniana* (KJ137284–87), which they attributed to the possible misidentification of the samples, our findings showed that other sequences of *B. kuhniana* (AY030378-80; AY030382; AF198666) from different studies and countries (Dejong et al. 2001, Vidigal et al. 2000b) were identical to those of *B. straminea*.

Previous studies showed lower indices of intraspecific diversity than those recorded in the present study, Palasio et al. (2017) recorded a genetic diversity of 3% for *B. straminea* from São Paulo and found no overlap between the intraspecific and interspecific divergences. By contrast, Palasio et al. (2019) analyzed 18 COI sequences of *B. straminea* from São Paulo and recorded much less genetic variation (0–1%), which was much lower than that recorded in other studies. Even so, it was the highest intraspecific variation recorded in this study, in addition to *B. glabrata*, in which the same variation was observed (0-1%).

The analysis of the distribution of the haplotypes indicated gene flow between *B. straminea* specimens that have been maintained in the lab for 5-40 years and that come from different hydrographic basins or other water bodies, as observed in Uruaçu and Itapagipe, Rio de Janeiro and Itariri, and Londrina and Ilhabela (Fig. 11b, c and d). This may occur due to the passive transfer of these planorbids through human activities, fluvial transport, or other organisms. It is known that the egg mass and even the adult individuals are highly resistant to desiccation, and may be transported in the plumage of water birds (Woodruff & Mulvey 1997, Bilton et al. 2001, Figuerola & Green 2002), while human activities such as fish farming or the creation of artificial habitats on agricultural land may contribute to the dispersal of the mollusks (Mavárez et al. 2002). Similar processes may have contributed to the structuring of the first haplotype, which was shared by sites from the Brazilian North, South, and Southeast regions. In the specific case of the haplotype found in the two sites from the state of Goiás, the Cana Brava and Serra da Mesa hydroelectric reservoirs are located in the municipalities of Minaçu and Uruaçu, respectively (Fig. 11a), and these bodies of water are linked physically, which implies the potential for gene flow between the specimens.

Our results demonstrated the presence of a larger number of haplotypes (h = 16), haplotypic diversity (h_d = 0.9088), nucleotide diversity (π = 0.0885), and the mean number of nucleotide differences (k = 51.809) than those mentioned in previous studies. Palasio et al. (2017), for example, used the DNA barcode to identify the *Biomphalaria* species and analyzed different sites of *B. straminea* from São Paulo, in addition to a specimen of *B. intermedia*. These authors found six haplotypes, and the haplotype and nucleotide diversity, while the mean number of differences in the nucleotides recorded in the eleven specimens of *B. straminea* was hd = 0.836; π = 0.01199; k = 6.582, respectively. Habib et al. (2018) also recorded low levels of haplotype diversity based on the COI and 16S markers, with four and three distinct haplotypes, respectively, in sites from Brazil and China, with nucleotide diversity (π) of 0.004488 and 0.005587 for both genes. The high haplotype diversity observed in *B. straminea* may be due to the number of sequences analyzed in the present study and reinforces the importance of analyzing different sites from distinct regions (or the whole of the species' geographic range), as recommended by Paraense (1957).

These results reinforce the importance of integrative studies in taxonomy, in particular for the more reliable diagnosis of closely-related and cryptic species, such as those of the *B. straminea* complex. This approach provides essential tools for the understanding

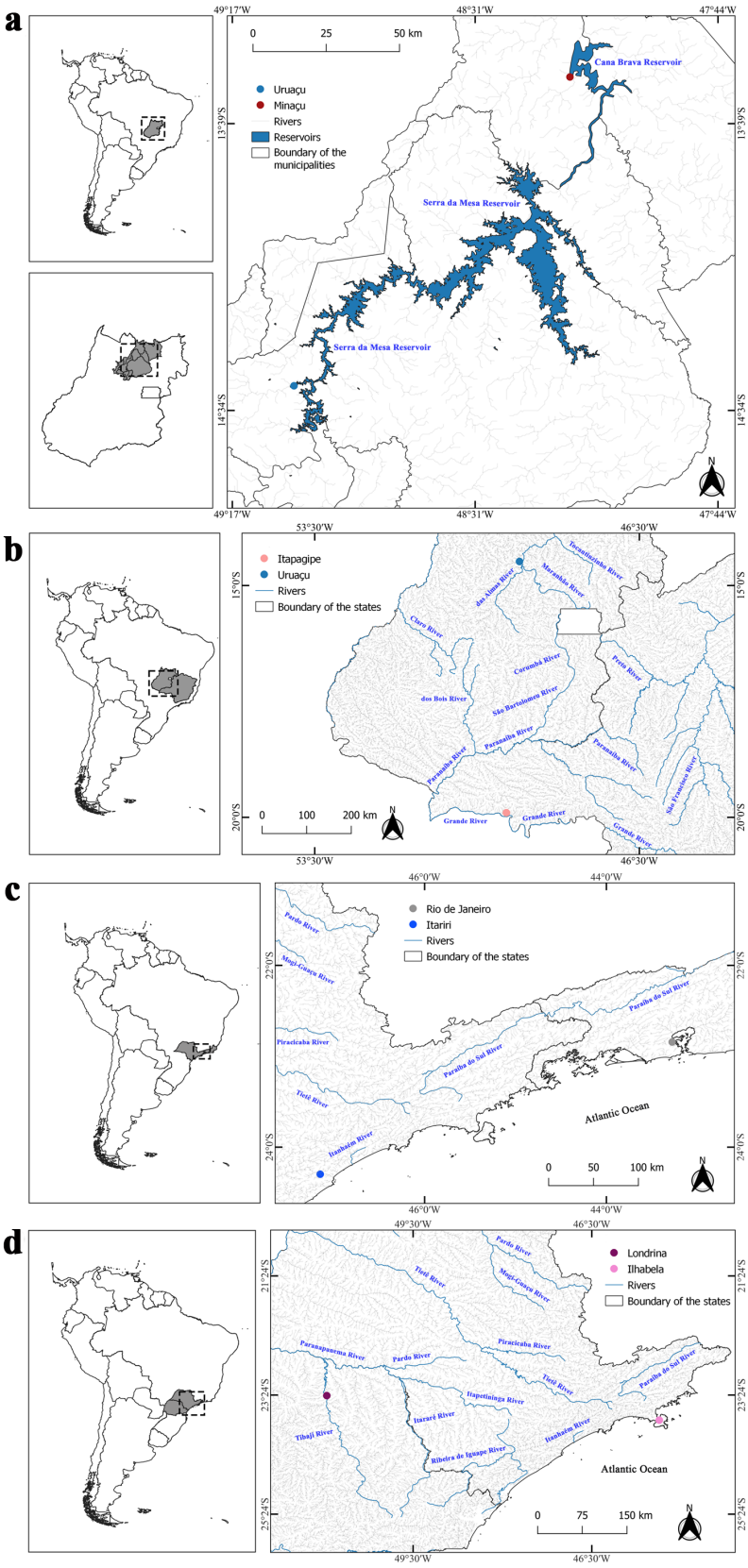

Figure 11. Locations of *Biomphalaria straminea* samples from different municipalities that shared haplotypes **(a)** Municipalities of Minaçu and Uruaçu in the state of Goiás, and the local reservoirs. **(b)** Municipalities of Uruaçu, in the Brazilian state of Goiás, and Itapagipe, in Minas Gerais, and their principal rivers. **(c)** City of Rio de Janeiro, and the municipality of Itariri, and the principal rivers of the Brazilian states of Rio de Janeiro and São Paulo. **(d)** Municipalities of Londrina, in the Brazilian state of Paraná, and Ilhabela, in São Paulo, and their principal rivers.

of the intra- and inter-specific evolutionary relationships of the planorbids, as well as the delimitation of species. The data are consistent with the findings of Palasio et al. (2019), which show that the genetic divergence and the similarities in the morphological characters of the *Biomphalaria* species indicate that these phenotypic characters, used traditionally to delimit species, are not adequate for the comprehensive description of the diversity of the group.

The considerable morphological and molecular variability found in *B. straminea*, and its similarities with *B. kuhniana*, emphasize the need for further studies with larger samples, especially of *B. kuhniana*, which should include specimens from the type locality, to better understand their evolutionary history, in particular, their taxonomic validity and possible processes of hybridization. Our findings represent an important contribution to the understanding of the morphological and molecular variation of the specimens of *B. straminea* that occur in Brazil, as well as expanding the genetic database of this species, which will enhance future comparative analyses of the other species of the *B. straminea* complex.

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RTN developed the project, performing the morphological, conchological, and molecular analyses of the samples, as well as data analyses and manuscript writing, in addition to image editing. SRG, MAF, and SCT were responsible for supervising the development of the project and reviewing the manuscript writing. KPB performed the phylogenetic analyzes, haplotype network and also assisted in writing and editing images. APS carried out the molecular analyzes of several samples. CRM contributed to map production and other issues. All authors discussed the results and reviewed the final manuscript.