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Pattern of Genetic Divergence of Mitochondrial DNA Sequences in *Biomphalaria tenagophila* Complex Species Based on Barcode and Morphological Analysis

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1. Introduction

Neotropical region has a high diversity of *Biomphalaria* (Pulmonata: Basommatophora: Planorbidae) fauna with at least twenty-six of the world's estimated thirty- seven species recorded (Carvalho,2008). Nearly one –third of these species occur in freshwater ecosystems related to the main drainage river basins in Brazil (Estrada et al.,2006; Teodoro,2010),where *Biomphalaria glabrata* (Say,1818),*Biomphalaria tenagophila (*Orbigny,1835) and *Biomphalaria straminea (*Dunker,1848) have serious impact on human health for these species host *Schistosoma mansoni* Sambon 1907 (Figure 1).

Fig. 1. Map (according Palasio, R.G.S., 2011) showing the worldwide distribution of the 37 *Biomphalaria* species, the 26 neotropical *Biomphalaria* species and the 11 species and 1 subspecies naturally described in Brazil (•) (Brasil,Ministério da Saúde 2007; Carvalho et al., 2008; Teodoro et al.,2010). An asterisk indicates *Schistosoma mansoni* host species (Palasio, RGS, 2011).

B. tenagophila is the nominal member of a species complex of freshwater snails distributed widespread in the neotropical region (Figure 2). *B. tenagophila* is the main host of *S. mansoni* in the southeastern Brazil. (Paraense & Corrêa,1978).As currently defined,the *B. tenagophila* complex includes a sub-species *B. tenagophila guaibensis* (Paraense,1984) and a species *B. occidentalis* (Paraense,1981).

Fig. 2. Geographic distribution of *Biomphalaria tenagophila* complex in the southeastern Brazil. (R.G.S. Palasio & R. Tuan based on illustrations in Lima et al.,1993; Paraense 1984, 2001; Carvalho & Caldeira,2004).

The morphological characters of the genital organs employed by the malacologist WL Paraense permits all of us to correctly identify the taxonomic units of the *tenagophila* complex. But,in fact,identifying *Biomphalaria* species solely on morphological characters leads to a less precise identification of these taxonomical groups,which are found to be difficult to diagnose in a malacological work routine.

The following examples may illustrate how morphological or technical variations can lead to erroneous species identification with serious epidemiological consequences.

Example 1: Within the species of planorbid described in Brazil,a *B. glabrata* is the only species to feature pigmented renal ridge (Paraense and Deslandes 1959). This character is known to be the only to set the difference between *B. glabrata* (the most important host species of *S. mansoni* in the Americas) and *B. tenagophila*,responsible for the transmission of schistosomiasis in the southeastern region of Brazil. Pepe et al. (2009) reports that a pigmentation-like may appear associated to the renal ridge in *B. t. guaibensis,*as a result from an intraspecies variation. Such diagnosis would trigger an unnecessary alert once *B.t.guaibensis* is not an intermediary host of *S. mansoni.*

Variation in the pattern of the pigmentation of the renal ridge may also reflect the influences of the freshwater substrate types leading us to an erroneous identification of all *Biomphalaria* species.

Here we exemplified the two major aggravating factors in a morphological identification system: morph variation within the species and variation caused by interaction of the organism with the environment.

Example 2: According to Paraense (1984) an essential condition for a clear distinction of *B.tenagophila* from the subspecies *B. t. guaibensis "is that the animal body is preserved for dissection in a well relaxed condition"*,that is,for the identification of the subspecies,more rigorous laboratorial methods of looseness need to be used instead of the methods utilized in the current identification of mollusks. If the *B.t. guaibensis* subspecies were dispersed,it would be difficult to have a morphological differential diagnosis of these subspecies.

The intrinsic difficulties to the system of morphological identification of organisms limit the description of taxonomic discreet units,although they lead to the development of auxiliary molecular techniques to overcome these difficulties (Estrada et al.,2006; Carvalho,2008).

Species of planorbid snails belonging to the genus *Biomphalaria* have received increasing attention in molecular systematic and phylogeographic studies in recent years (Vidigal et al. 2000,2004; Carvalho et al. 2001; DeJong et al. 2001,2003; Mavárez et al. 2002a,b; Morgan et al. 2002; Pointier et al. 2005; Tuan and Santos,2007). The development of genetic markers and the databases generated in such studies can be further used for multiple purposes,of both theoretical and applied interest: species identification,analysis of population genetic structure and speciation patterns,inference of common/independent origins of susceptibility to *Schistosoma*,detection of natural hybridization,tracking of geographical expansions and biological invasions,evaluation of genetic differentiation between phenotypic variants and/or geographic isolates,amongst others. Genetic markers used in *Biomphalaria* studies include mitochondrial DNA (Spatz et al. 1999; DeJong et al. 2001,2003; Mavárez et al. 2002b; Pointier et al. 2005). The latter is especially useful for species identification.

The dispersal and colonizing ability of *B. tenagophila* highlights the need to monitor the spread of this species within and outside the neotropical region. The identification of *B. tenagophila* in Kinshasa,Africa (Pointier,2005) and Romania (Majoros et al. 2008) certainly caused great concern to public health authorities.

For *Biomphlalaria* species two important characteristics play an important role to successfully establish snail populations in a environment outside the native range of the species: self-fertilization and desiccation. *B.tenagophila* species are simultaneous hermaphrodites snails with the possibility of both self and cross-fertilization (Paraense,1955; Tuan & Simões,1989; Guimarães,2003). Such a complex mode of reproduction might probably increase colonization since new populations can arise from just one snail by self-fertilization.

On the other hand reproduction alone is not enough to establish a species because snails still must be able to survive conditions outside their native range. One important aspect to *B. tenagophila* survival it is the ability to withstand desiccation in response to water shortage (Tuan & Simões,1989; Ohlweiler & Kawano,2002) that greatly increases the possibility of recolonization after a population crash or colonization of new environments. These must be a major source of genetic diversity amongst the same species creating patchily distributed populations that can be easily distinguished by molecular markers.

In this study,we obtained mitochondrial COI and 16SrRNA sequences from *B. tenagophila*,*B. t. guaibensis*,and *B. occidentalis* specimens collected in the São Paulo state,and Rio Grande do Sul state,Brazil to estimate the divergence of each species amongst the three members of the *tenagophila* complex and evaluate the potential of COI and 16S rRNA mitochondrial genes for identification of closely related species.

2. Materials and methods

2.1 Molecular analysis

B. tenagophila and *B. occidentallis* snails were collected along the banks of different streams of Paranapanema,Ribeira do Iguape,Paraíba do Sul,Tietê,and Litoral Basins at São Paulo,Brazil. *B.t. guaibensis* specimens were collected at Rio Grande do Sul,Brazil (Table 1). Two samples from Argentina were also analyzed. A total of 66 specimens were used in this study. Prior to molecular analysis snails were identified through morphological analysis,according to Deslandes (1959) and Paraense (1975,1981,and 1984).

Total genomic DNA was extracted from foot tissue of individual snails using DNeasy Tissue Kits (Qiagen®) and was preserved as DNA vouchers specimens in the DNA voucher collection of the Laboratory of Molecular Biology and Biochemical at SUCEN. Amplification of COI gene was attempted using LCO-1490 and HCO-2198 (Folmer et al.,1994). Amplifications (in 50µl of total volume) consisted of 10-100 ng of DNA,0.2 mM of each dNTP,0.10 µM of each primer and 1 U of *Taq* DNA polymerase (BioLabs) with the supplied buffer. The following PCR temperature profile was used: a initial 3 min step at 95ºC for denaturation,25 iterations of 1 min at 95ºC,1 min at 47ºC and 1 min 30 sec at 72ºC,and a final extension step for 7 min at 72ºC. The amplification profile of 16SrRNA fragment included an initial denaturation step at 94°C for 2 min. and 35 cycles of 30 sec at 94°C,30 sec at 54°C and 1 minute at 72°C,and a final step at 72°C for 5 minutes. Amplification of 16S gene was attempted using a Palumbi forward and reverse primer (Palumbi, 1996).

PCR products were checked by agarose gel electrophoresis,cleaned using QIAquick PCR Purification Kits (Qiagen®). Cycle-sequencing of PCR products were carried out using terminal primers given above and the BigDye Terminator v3.1 kit of Applied Biosystems (ABI),purified using DyeEx-Kit (Qiagen®) according to the modified protocol and sequenced on an ABI 310 automated sequencer.

Species/Locality	Code	Geographical coordinates
B. tenagophila		
Ourinhos (SP),Brazil	*Btt_Our1 -6*	22°58′02″S 49°52″25″ W
Ipauçu (SP),Brazil	*Btt_Juq1-8*	23°02′54″S 49°34′41″W
Taubaté (SP),Brazil	*Btt_Tau1-3*	23°01'35"S 45°33'21"W
Pindamonhangaba (SP),Brazil	*Btt_Pin1-2*	22°53'30''S 45°28'09''W
Caraguatatuba (SP),Brazil	*Btt_Car1-7*	23°37'31"S 45°24'44"W
São Sebastião (SP),Brazil	*Btt_SSe1*	23°47′08″S 45°33′22″W
Campinas (SP),Brazil	*Btt_Cam1-4*	23°02'13''S 47°06'21''W
Sorocaba (SP),Brazil	*Btt_Sor1-5*	23°29'56"S 47°27'30"W
Registro (SP),Brazil	*Btt_Reg1-3*	24°29'20''S 47°51'06″W
Juquiá (SP),Brazil	*Btt_Juq1-8*	24°19'33″S 47°38'22''W
Araraquara (SP),Brazil	*Btt_Ara1*	23°01'35"S 45°33'21"W
Rio da Prata,Argentina	*Btt_Arg1-2*	NA
B. t. guaibensis		
Porto Alegre (RS),Brazil	*Btg_POA1-4*	29°59′52. 3′′S 51°15′38.6′′W
B. occidentalis		
Ourinhos (SP),Brazil	*Boc_Our 1-5*	22°58′02″S 49°52′25″W
Martinópolis (SP),Brazil	*Boc_Mar1-2*	22°14′04″S 51°09′36″W
Presidente Prudente (SP),Brazil	*Boc_PPr1*	22°10′12″S 51°22′34″W
Candido Mota (SP),Brazil	*Boc_CMo1*	22°44'52"S 50°23'08"W

NA,Not Available.

Table 1. Collecting data,code and geographical identification of the specimen used in molecular study.

Sequences were corrected using Chromas (Technelysium Pty Ltd.),aligned in ClustalX 1.8 (Thompson et al. 1997). The alignments of COI datasets comprised sequences with no gaps or indels. The alignments were subsequently adjusted by eye using BioEdit 7.0 (Hall, 1999). Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 5 (Tamura,Peterson,Stecher,Nei,Kumar 2011). Sequence divergence between species was estimated with Kimura 2 parameter model (K2P) (Kimura, 1980). The Neighbor-Joining (Saitou and Nei 1987) trees were obtained using K2P model and the support for the nodes was calculated using 1000 bootstrap replicates (Felsenstein, 1985). Nucleotide diversity (pi,Nei,1987,equations 10.5 or 10.6 and 10.7); and nucleotide divergence between sequence groups (D_{xy},Nei,1987,equation 10.20 using Jukes and Cantor correction) were calculated using DnaSP v.5 (Librado and Rozas,2009). F*st* was estimated following Hudson et al. 1992.

2.2 Morphology analysis

The morphological analysis was based on 10 dissected specimens *B. tenagophila* from Arambaré,Rio Grande do Sul (lot DPE 9011); 13 dissected specimens *B. t. guaibensis* from Porto Alegre,Rio Grande do Sul (lot DPE 9008,Figure 3) and 31 dissected specimens *B. occidentalis* from Tremembé,São Paulo (lot DPE 9028). Only large and adult specimens were used for dissection and anatomic study.

Fig. 3. A natural freshwater habitat at Porto Alegre where *B. tenagophila guaibensis* were collected. 29° 59′ 52 3′′S, 51° 15′ 38. 6′ W.

Snails were identified according to Paraense (1975). Soft parts were preserved under fixation as vouchers. The identification of the mollusks was done under the stereomicroscopy (Leica MZ 95) and was photographed with the image Program IMS 50. We took into consideration the morphology of the shell and soft parts,especially the reproductive system.
Three radulae and three jaws of the *B. occidentalis,B. tenagophila* and *B. t. guaibensis* were extracted and examined under a Scanning Electron Microscope LEO 440 at the Museum of the Zoology,São Paulo University (MZUSP).

3. Results

3.1 Molecular analysis

The evolutionary relationships of *tenagophila* complex were inferred using Neighbor-Joining method for COI and 16SrRNA mitochondrial genes (Figure 4). The evolutionary distances depicted as branch lengths were computed using the Kimura 2p method using 550 nucleotides positions for the COI dataset and 314 nucleotide positions for the 16S rRNA dataset. The *tenagophila* complex sequences were retrieved in two well distinct and bootstrap supported branches: one with *B. occidentalis* clustering with *B. t. guaibensis*,and another distinct branch with the nominal *B.tenagophila* sequences.
For each component that belongs to *tenagophila* complex we obtained intraspecific identical sequences that support morphological taxonomy.
Two distinct groups of *B. tenagophila* were recovered in each analysis (COI and rRNA16S).
The estimates of genetic divergence (Table2) under Kimura 2p model over the COI and 16S between sequences pairs of the taxa analyzed show that *B. t. guaibensis* are more closely related with *B. occidentallis* than with *B. tenagophila*.

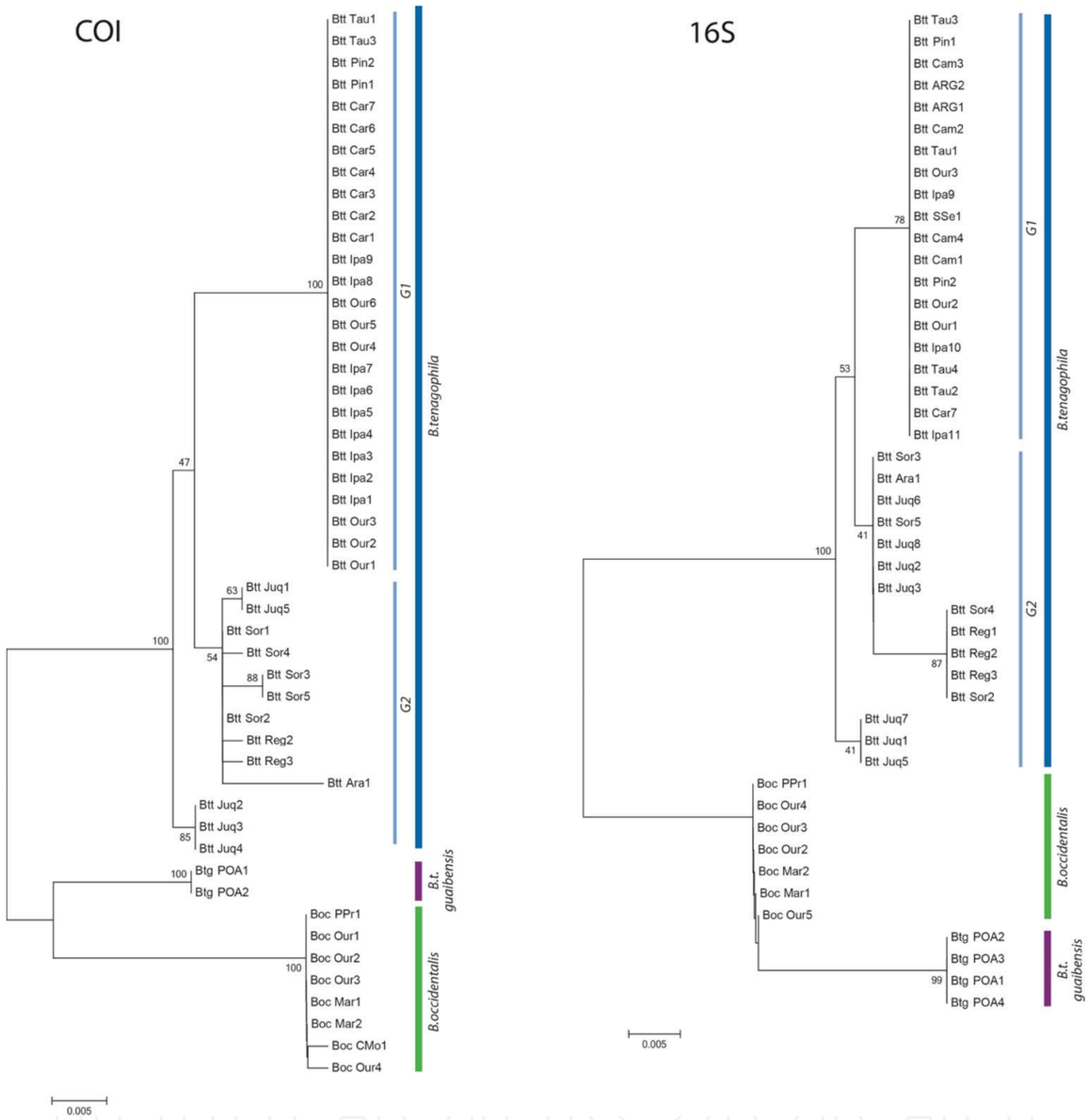

Fig. 4. Optimal trees inferred for a 550 fragment COI mtDNA(A),sum of branch length= 0.09775397,and for a 314 nucleotides 16SrRNA,sum of branch length= 0.07712277. Bootstrap values (1000 replicates) are shown next to the branches.

Species 1	Species 2	COI	16S
B.tenagophila	*B.t.guaibensis*	0.0436	0.0668
B.tenagophila	*B.occidentalis*	0.0546	0.0478
B.t.guaibensis	*B.occidentalis*	0.0362	0.0178

Table 2. Mean Pairwise distance values calculated under Kimura´s 2 –parameter model (K2P) for 49 COI sequences and 46 16SrRNA sequences.

The Figure 5 shows that no overlap exists between intraspecific and interspecific distribution distance calculated under Kimura 2P. Indeed,the distributions of the COI and 16S genetic distances are very similar (Figure 5).

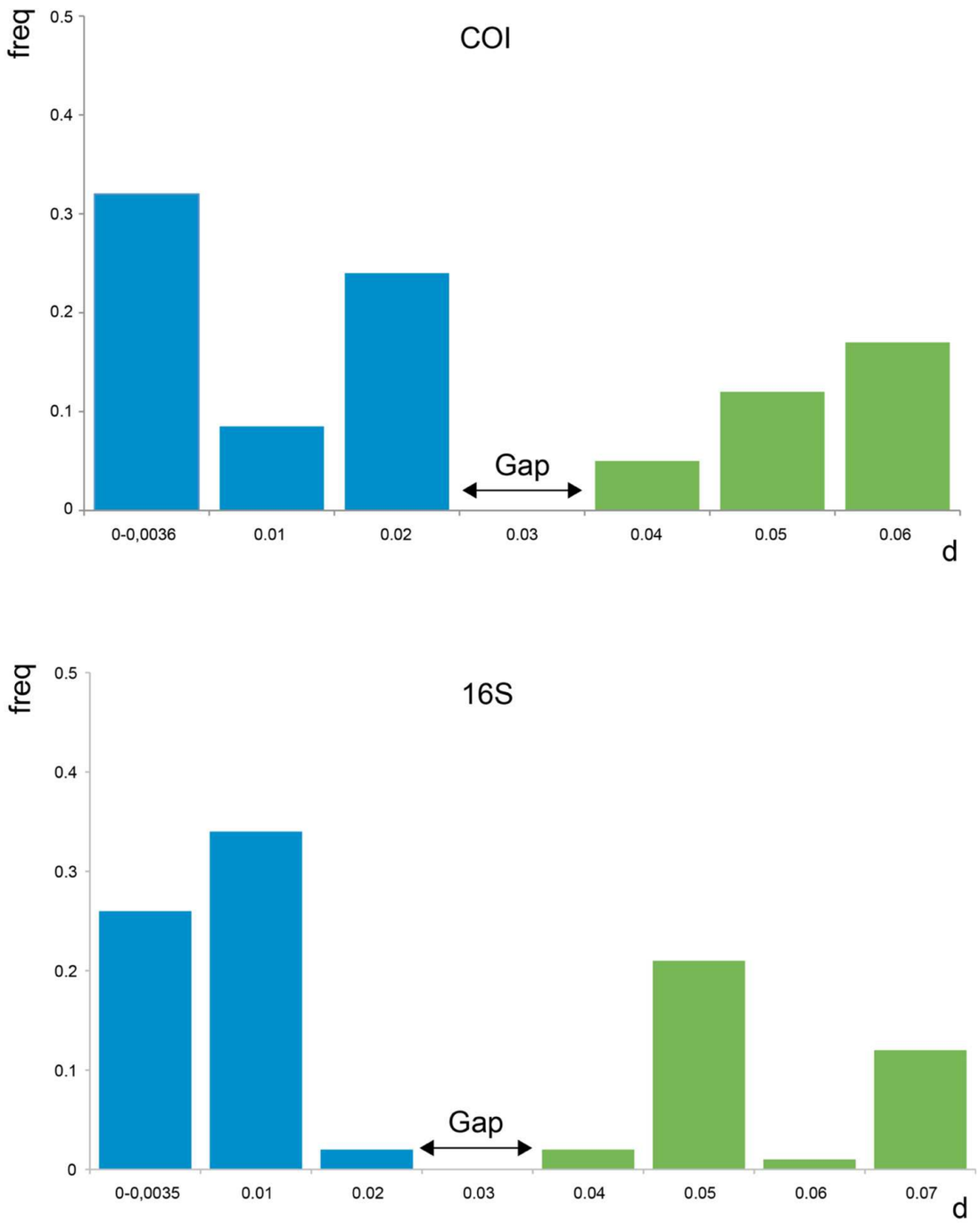

Fig. 5. Distribution of intraspecific variation genetic distance (in blue bars),and interspecific distance (in green bars) estimated by Kimura´s 2 –parameter model (K2P) for all the species studied

In order to examine the ability of the barcoding methodology to identify the three *Biomphalaria* taxa we plotted the intra and interspecific divergence of COI and 16S using a threshold of 3% minimum interspecific genetic variation and 3% maximum intraspecific genetic variation (Figure 6).
According the 3% threshold value for within and between species variation four quadrants are defined. Species on quadrant I (dark grey) are well-defined species. Groups on quadrant II (white colored) are composite species. Species on quadrant III (medium grey) are recent divergent species,and species on quadrant IV (light grey) are misidentified species.

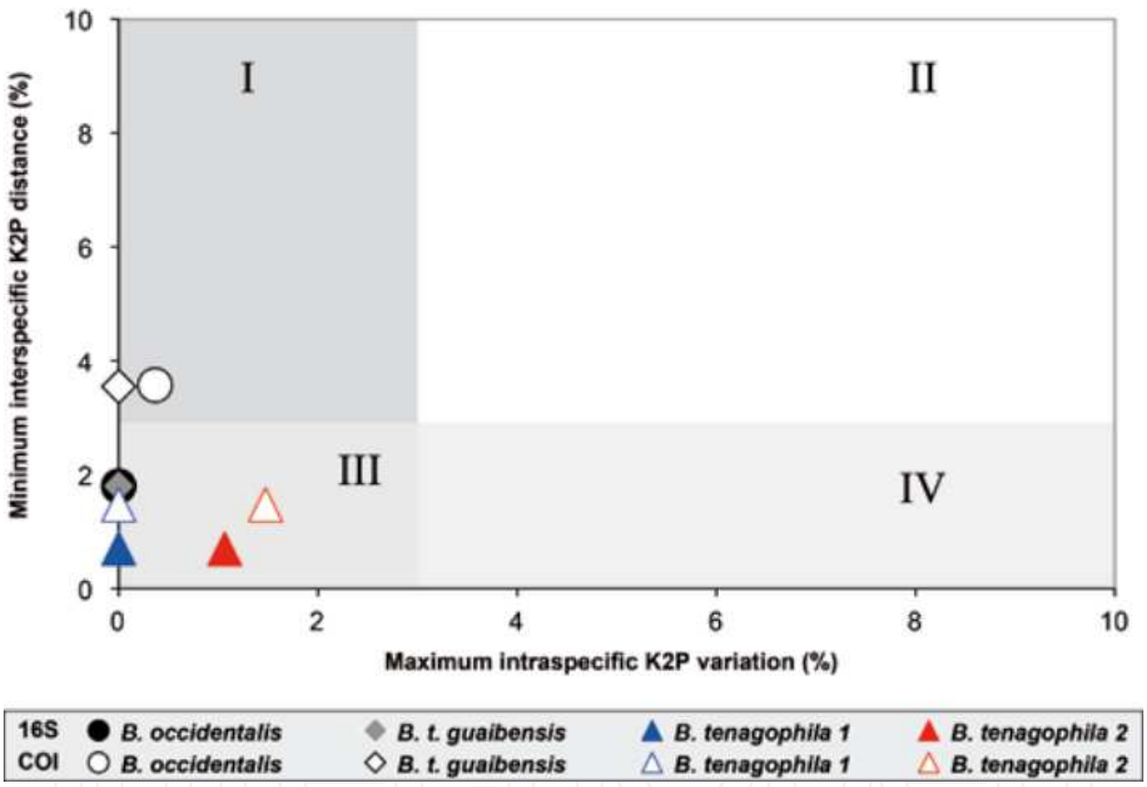

Fig. 6. Minimum Interspecific distances versus Maximum intraspecific distances of COI and 16S sequences for *B. tenagophila*,*B. occidentalis* and *B. t. guaibensis* (according Ballard et al.,2009).

3.2 Comparative analysis of shell,reproductive system and radula

B. tenagophila,*B.t. guaibensis and B. occidentalis* had no significant differences among the number of whorls. The species had typically six to eight whorls. Otherwise,the shell aperture would be shorter and narrower in *B.t guaibensis* than in the other two species analyzed (Figure 7).

Fig. 7. Shell. A: *B. t. guaibensis Biomphalaria tenagophila,* C: *B. occidentalis*

Biomphalaria snails can be distinguished based on differences in the shape and size of their male and female internal organs as described in Figure 8.

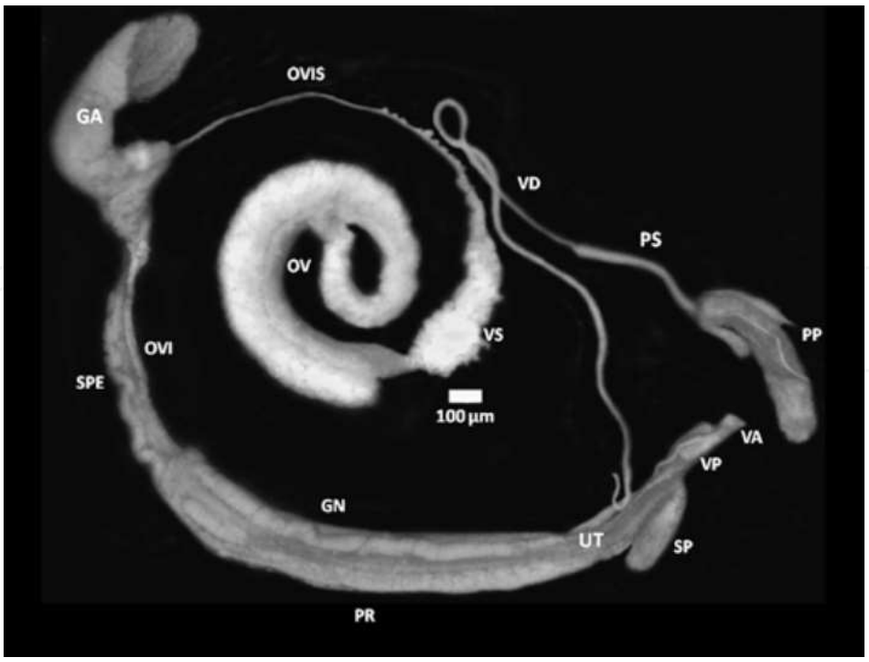

Fig. 8. Reproductive System from *Biomphalaria tenagophila*. GA: albumen gland,GN: nidamental gland,OV: ovotestis,OVI: oviduct,OVIS: ovispermiduct,PP: prepuce,PR: prostate,PS: penial sheath,SP: spermatheca,SPE: spermiduct,UT: uterus,VA: vagina,VD: vas deferens,VP: vaginal pouch, VS: seminal vesicle.

The hermaphrodite part did not present differences between *B.tenagophila,B.t. guaibensis* and *B. occidentalis*.

The differences between the species were mainly found in the male and female systems. The anatomical characters that clearly groups *B.tenagophila* and *B. t. guaibensis* in a same taxon is a well developed vaginal pouch located in the vaginal wall of this species,and also the shape and size of the prepuce and the volume of prostata (Table 5,Figure 9).

	Biomphalaria tenagophila	*Biomphalaria tenagophila guaibensis*	*Biomphalaria occidentalis*
Vagina	short	Long and slender	Long and slender
Vaginal pouch	Well developed	Well developed	absent
penis	thick	Long and slender	Long and slender
prepuce	Wider at the free end of the prepuce	Wider at the free end of the prepuce	Same diameter along the prepuce
Penis sheath	Shorter and slenderer than the prepuce	Shorter and slenderer than the prepuce	Shorter and slenderer than the prepuce
Próstata	Robust with a great number of diverticula	Robust with a great number of diverticula	Refined with a small number of diverticula
spermatheca	large	small	small

Table 5. Differences among *B. tenagophila* species complex based on reproductive parameters

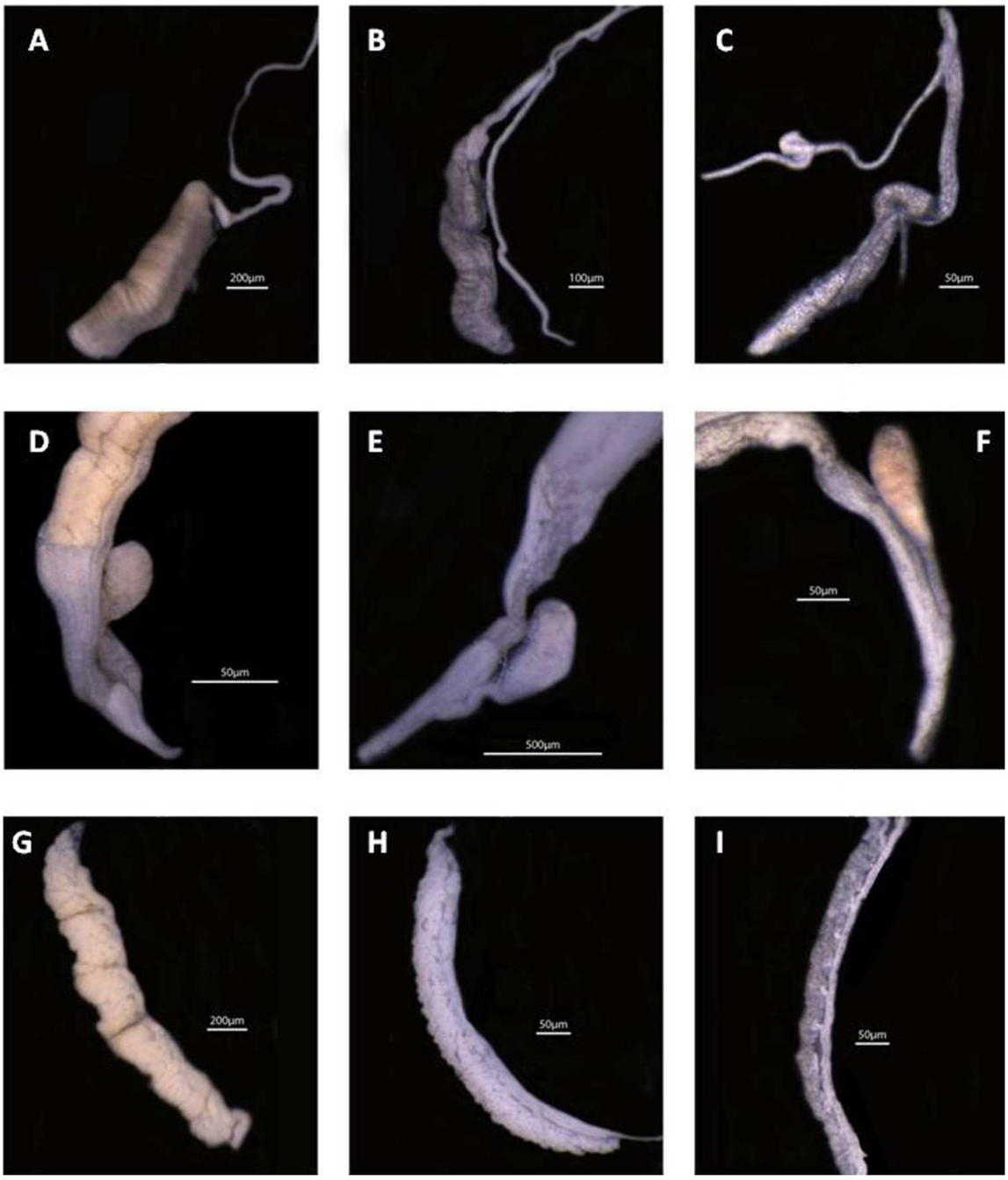

Fig. 9. Detail of reproductive system. A,D and G: *B. tenagophila*,B,E and H: *B. t. guaibensis*,C,F and I: *B. occidentalis*. A,B and C: Penis; D,E and F: Vaginal complex; G,H and I: Prostate

The radulae of *B tenagophila,B. t. guaibensis and B. occidentalis* consisted of a fused jaw (Figure 10) to which the teeth are attached.

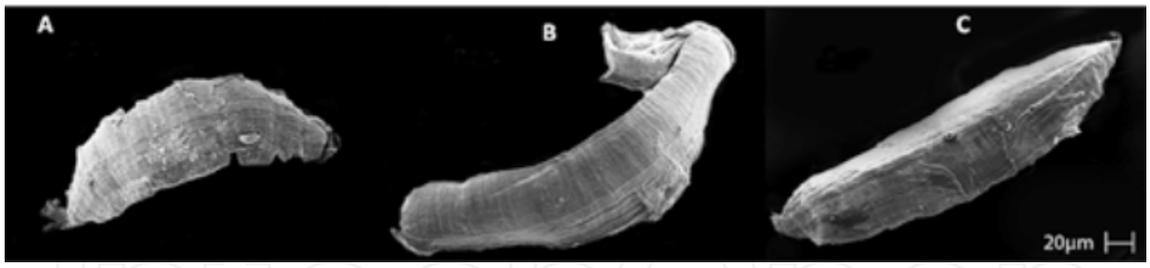

Fig. 10. Mandíbula. A.: *B. tenagophila,B.: B.t. guaibensis,C: B. occidentalis.*

In all the species analyzed there was only one central tooth with two cusps and several lateral teeth at each side of the central tooth. In *B.t. guaibensis* the central tooth showed variations that affected the inner and outer faces of the cuspids (Figure 11).

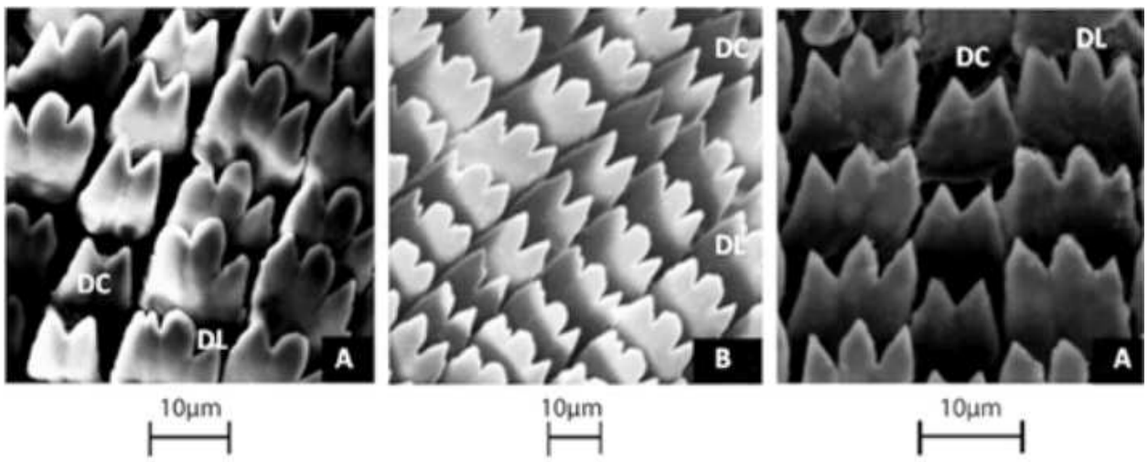

Fig. 11. Radula. A: *B. tenagophila*,B: *B.t. guaibensis*,C: *B. occidentalis*. DC: central tooth,DL: lateral teeth.

In all the three species the lateral were characterized with three cuspids. Small subcusps appear on lateral teeth near the margins given a serrated appearance for the radulae. (Figure 12)

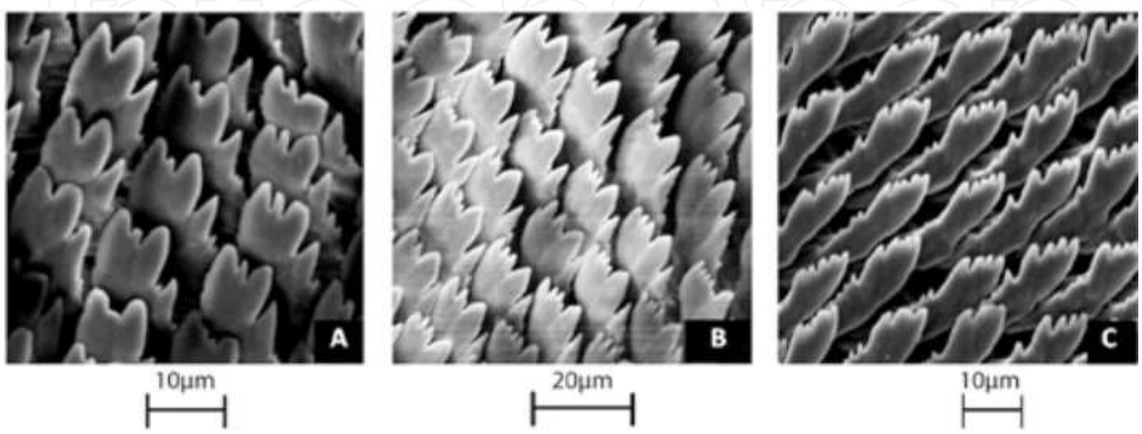

Fig. 12. Marginal teeth of the radula. A: *B. tenagophila,*B: *B.t. guaibensis*,C: *B. occidentalis*.

4. Discussion

Phylogenetic analysis using short sequences of the mitochondrial COI and 16SrRNA genes resulted in trees with high support values morphologycally pairs of species *B. tenagophila* and the subspecies *B. t. guaibensis,*and the *B. occidentalis* and *B. t guaibensis,*assigned as *tenagophila* species complex (Figure 4).

The molecular evidence thus strongly suggests that *B. occidentalis* and *B.t. guaibensis* are part of a clade of closely related species with a minimal genetical divergence between them (Table 2).

The result obtained here is in perfect agreement with our observations with respect to the resemblance between *guaibensis* and *occidentalis* morphology,both thin and elongated species when compared to *tenagophila* species. For taxonomically purposes,the vaginal pouch places *B. tenagophila* and *B.t. guaibensis* closer to each other (Paraense,1984). The vaginal pouch represents a character easy to score unambiguously (1 for presence 0 for absence) but instead of inferring a close relationship between *B.tenagophila* and *B.t guaibensis,*the vaginal pouch could also be considered a case of homoplasy and might not have great confidence and definition for such a complex taxonomic groups as in other gastropod groups (Collin,2003). To sustain such hypothesis,further additional sequences from other sites are clearly required.

Our sequence data (COI,16S) revealed significant intraspecific variation for *B. tenagophila*. Two major branches were resolved by phylogenetic analysis,one of which -G1- with very low internal variation (pi=0.0000 for COI and 16S) and a second branch -G2- with a significant degree of internal genetic differentiation amongst sequences (pi=0.00587 for COI and pi= 0.00995 for 16S).

The extremely low levels of genetic variation in G1 branch exists amongst populations across a broad geographic range area,including Ourinhos,Pindamonhangaba and Taubate. All these sites presented were in the past active areas of transmission of schistosomiasis (Piza,1972; Silva,1985),subjected to intensive use of moluscicide measure that resulted in cycles of extinction followed by recolonization of *B. tenagophila* (Tuan,1996).

Whereas G1 population occurs in historical unstable sites,G2 population inhabits freshwater sites at São Paulo (Registro,Juquia) with fragments of the Brazilian Atlantic Rain Forest,one of the most important hot- spots of genetic diversity in the world (Carnaval et al. 2009). The significant differentiation between G1 and G2 *B. tenagophila* populations (Fst COI= 0.82; Fst 16S=0.77) and the retention of even a small genetic diversity in G2 populations could be due to historical signatures since Atlantic coastal regions played an important role as a climatic refugee during the last Pleistocene (Pfenninger and Posada,2009). A taxonomist working with a series of snails of this species collected over a broad geographical range certainly would not recognize any significant morphological variation. This shows the lack of resolution of morphology and that DNA barcoding data can direct us to what we need to look further into: biodiversity.

According to Moritz and Cicero (2004) "accurate diagnosis" by a short COI DNA sequence,"depends on low intraspecific variation compared with that between species" So we can conclude that COI and 16S may be used for detection of species in *B. tenagophila* complex species (Figure 5). Furthermore,mtDNA lineages found in B. *tenagophila* may help explain heterogeneous patterns of schistosomiasis transmission.

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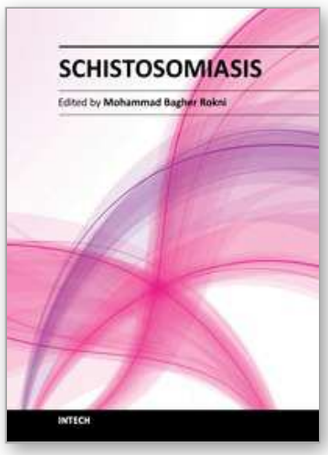

Schistosomiasis

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In the wake of the invitation by InTech, this book was written by a number of prominent researchers in the field. It is set to present a compendium of all necessary and up-to-date data to all who are interested. Schistosomiasis or blood fluke disease, also known as Bilharziasis, is a parasitic disease caused by helminths from a genus of trematodes entitled Schistosoma. It is a snail-borne trematode infection. The disease is among the Neglected Tropical Diseases, catalogued by the Global Plan to combat Neglected Tropical Diseases, 2008-2015 and is considered by the World Health Organization (WHO) to be the second most socioeconomically devastating parasitic disease, next to malaria. WHO demonstrates that schistosomiasis affects at least 200 million people worldwide, more than 700 million people live in endemic areas, and more than 200.000 deaths are reported annually. It leads to the loss of about 4.5 million disability-adjusted life years (DALYs).

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