Polymerase chain reaction and restriction fragment length polymorphism analysis of the ITS2 region for differatiation of Brazilian *Biomphalaria* intermediate hosts of the *Schistosoma mansoni*

Reação em cadeia da polimerase e polimorfismo de tamanho de fragmento de restrição da região do ITS2 para a diferenciação dos moluscos brasileiros do gênero *Biomphalaria* hospedeiros intermediários do *Schistosoma mansoni*

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ABSTRACT

We sequenced the internal transcribed spacer 2 of the ribosomal DNA (ITS2-DNAr) from the three Schistosoma mansoni *intermediate hosts in Brazil:* Biomphalaria glabrata, Biomphalaria tenagophila *and* Biomphalaria straminea. *Analysis of a restriction map from those sequences allowed us to select putative restriction enzymes able to identify the snail species under study. Four restriction enzymes were used and HpaII provided simple species-specific profiles easily visualized in polyacrylamide gels. The use of ITS2 is advantageous as it provides a small fragment of 460 bp which may be easily amplified by PCR. In the current work, we showed that the amplification of ITS2-DNAr together with HpaII enzyme restriction is an auxiliary molecular tool for the morphological identification of such snails as well as for taxonomic and phylogenetic studies of neotropical planorbids.*

Key-words: Biomphalaria glabrata. Biomphalaria tenagophila. Biomphalaria straminea. Polymerase chain reaction. Internal transcribed spacer 2.

RESUMO

O sequenciamento da região espaçadora transcrita interna 2 do DNA ribossomal (ITS2-DNAr) das espécies brasileiras gênero Biomphalaria *(*B. glabrata, B. tenagophila *and* B. straminea*) hospedeiras intermediárias do* Schistosoma mansoni *no Brasil, permitiu a análise dos sítios de restrição presentes nestas seqüências. A análise do mapa de restrição obtido dessas seqüências nos permitiu selecionar enzimas mais promissoras que gerassem perfis de restrição capazes de identificar essas espécies. Foram testadas 4 enzimas e a enzima HpaII foi selecionada por produzir perfis espécie específicos de fácil visualização em gel de poliacrilamida. A utilização da região ITS2 tem como vantagens a obtenção de um fragmento pequeno de 460bp, o qual pode ser facilmente amplificado por PCR. Neste trabalho, nos demonstramos que a amplificação da região ITS2-DNAr e a restrição deste com a enzima HpaII é uma ferramenta molecular auxiliar a identificação morfológica desses moluscos, bem como para estudos taxonômicos e filogenéticos de planorbídeos neotropicais.*

Palavras-chaves: Biomphalaria glabrata. Biomphalaria tenagophila. Biomphalaria straminea. *Reação em cadeia da polimerase. Região espaçadora transcrita interna 2.*

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Among the 10 Brazilian snail species and one subspecies of the genus *Biomphalaria* described up to the present, *B. glabrata*[17], *B. tenagophila*[8], *B. straminea*[5], *B. tenagophila guaibensis*[13] *B. peregrina*[8], *B. kuhniana*[3], *B. schrammi*[4], *B. amazonica*[9], *B. oligoza*[10], *B. intermedia*[16] and *B. occidentalis*[12] only the first three species have been found naturally infected by *Schistosoma mansoni*[14].

The correct identification of *Biomphalaria* snails is complicated due to the high intra-specific variation in anatomical and morphological characters or great similarity among some species[9 11 15]. The availability of methodologies based on molecular analysis has enabled the access to more consistent information on *Biomphalaria* populational structure among Planorbidae. Molecular taxonomy has been able to solve several problems considered insoluble so far by traditional morphology.

The polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) analysis of the internal transcribed spacer (ITS) region of the rDNA (1300pb approximately) and a part of COI region of mitochondrial DNA (mit-COI – 780bp approximately) have been used for identification of several *Biomphalaria* species from Brazil and some regions of South America[1 2 18 19 21 22]. Restriction enzymes were randomly selected due to the lack of available sequences from *Biomphalaria* ITS region. But for part of the mit-COI region, analysis of a restriction map available in a data base allowed us to select particular enzymes to be tested and used in PCR-RFLP. In the present work, we report the use of this methodology for specific identification of field populations of *B. glabrata, B. straminea* and *B. tenagophila* from different Brazilian localities using restriction profiles provided by the ITS2 region. The choice of such region was due to two specific reasons: 1) on account of the size of the generated fragment for *Biomphalaria*, after PCR amplification (approximately 460bp). This is a considerably small product when compared with the size of ITS-rDNA and the COI region from this genus, enabling an easier amplification with no need of a high quality DNA; 2) this region proved to be appropriate as it had been sequenced and analyzed in phylogenetic studies of Brazilian *Biomphalaria* species[20].

Ten specimens of each population were killed and fixed. Before fixing the specimens, a fragment of their foot was removed for subsequent DNA extraction. Fixed specimens were identified by means of comparative morphology of the reproductive organs and shells[11 12 13 15]. Total DNA was extracted from the foot of each snail using the Wizard Genomic DNA Purification Kit (Promega)[21]. The ITS2 region was amplified using the primers ITS2F (5'-CGTCCGTCTGAGGGTCGGTTTGC-3')[20] and ETTS1 (5'-TGCTTAAGTTCAGCGGGT-3')[7] anchored in the conserved extremities of the 5.8S and 28S ribosomal genes, respectively. The PCR amplification conditions were the same used by Vidigal et al[19], except for the annealing temperature, which was 60°C.

The PCR amplification of *Biomphalaria* ITS2 region, from four specimens of each species under study, resulted in a product of approximately 460bp. Theses products were digested using the following restriction enzymes: *Taq*I and *Mbo*I (Invitrogen, Life Science), *Rsa*I and *Hpa*II (Promega Co, USA). These enzymes were selected based on the restriction map analysis using the program Webcutter version 2.0, (www.firstmarket.com/cutter/cut2.html) of the ITS2 sequences available in the Genbank[20]. – The access numbers used in our study were: *B. glabrata,* AF198659, AF198660, AF198661, AF198662; *B. tenagophila* AF198654, AF198655, AF198656; *B. straminea* AF198668, AF198669, AF198670, AF198671, AF198672.

Afterwards, the fragments were visualized in 6% silver-stained polyacrylamide gels. Digestion and RFLP analysis were performed[18] and the gels photographed with a Mavica digital camera (Sony).

The profiles obtained with *TaqI* and *Rsa*I did not allow us to distinguish between the three species due to the high similarity among RFLP profiles (data not shown). The most promising profiles were those produced by *MboI* and *Hap*II, and the best result was obtained with *Hap*II (Figure 1), which provided a simple profile of four fragments for *B. glabrata*, (200, 130, 90 and 70bp), *B. tenagophila* (200, 120, 90 and 60bp) and two fragments for *B. straminea* (300 and 180bp). Although *B. glabrata* and *B. tenagophila* share the fragments of 200 and 90bp (Figure 1), they could be separated by other two non-shared fragments: Bg1 (130bp) and Bg2 (70bp) for *B. glabrata*; Bt1 (120bp) and Bt2 (70bp) for *B. tenagophila*. The restriction profile for *B. straminea* comprised: Bs1 (300bp) and Bs2 (180bp) (Figure 1). Reproducibility of the generated profiles with *Hap*II was supported by the use of specimens originated from different localities in Brazil (Figure 1).

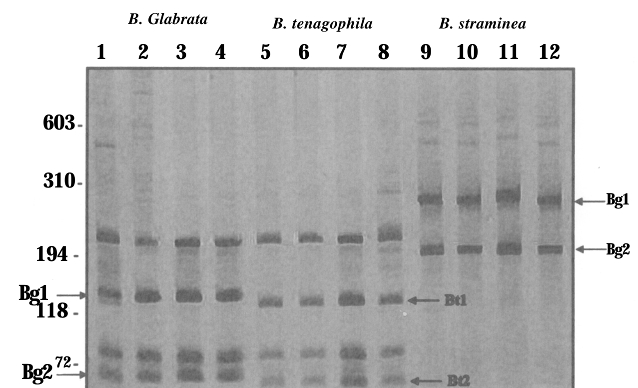

Figure 1 - Silver-stained 6% polyacrylamide gel showing the restriction profiles obtained with enzyme Hpa*II of the ITS2 region of DNA extracted from* Biomphalaria *species intermediate hosts of the* S. mansoni *in the Brazil. Lane 1:* B. glabrata *from Touros (State of Rio Grande do Norte); Lane 2:* B. glabrata *from Cururupu (State of Maranhão); Lane 3:* B. glabrata *from Jacobina (State of Bahia); Lane 4:* B. glabrata *from Sabará (State of Minas Gerais); Lane 5:* B. tenagophila *from Vespasiano (State of Minas Gerais); Lane 6:* B. tenagophila *from Formosa (State of Goiás); Lane 7:* B. tenagophila *from Florianópolis (State of Santa Catarina); Lane 8:* B. tenagophila *from Imbé (State of Rio Grande do Sul); Lane 9:* B. straminea *from Picos (State of Piauí); Lane 10:* B. straminea *from Jacobina (State of Bahia); Lane 11:* B. straminea *from Juiz de Fora (State of Minas Gerais); Lane 12:* B. straminea *from Guaíra (State of Paraná). The arrows and abbreviations indicate species specific fragments.* B. glabrata*: Bg1- 130bp and Bg2- 70 bp;* B. tenagophila*, Bt1- 120bp and Bt2- 70bp and* B. straminea*, Bs1 300bp and Bs2 180bp. Molecular size markers are shown on the left of each gel.*

These results demonstrated that PCR-RFLP of the ITS2 region, using *Hpa*II restriction enzyme, is an important tool to distinguish among *B. glabrata*, *B. straminea* and *B. tenagophila* species. Such data is in accordance with those produced for ITS and COI regions, through the same technique[19 22] and it also corroborates classical morphological taxonomy.

In general success of the amplification using degraded DNA is difficult to achieve and severely restricted in target size (degraded DNA results in amplification of relatively small fragments)[6]. Regarding this aspect, we believe that such methodology may be used in studies, in which degraded DNA is recovered from improperly conserved material (low molecular weight < 500bp). Thus, the fragment of 460bp correspondent to the ITS2 region may be more easily amplified by PCR than a region of approximately 1300bp (approximate size of ITS-rDNA from Planorbidae), due to the need for a more conserved or high quality DNA.

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