Specific Identification of *Biomphalaria tenagophila* and *Biomphalaria occidentalis* Populations by the Low Stringency Polymerase Chain Reaction

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Although Biomphalaria occidentalis *and* B. tenagophila *are indistinguishable on the basis of shell morphology and the majority of their genital organs, only the latter is susceptible to infection with* Schistosoma mansoni. *Thus, the identification of these species is fundamental to epidemiological studies of schistosomiasis. Here we describe a simple and rapid method for differentiating* B. tenagophila *from* B. occidentalis *based on low stringency polymerase chain reaction and using a pair of primers specific for the amplification of the 18S rRNA gene. Analysis of the low stringency product profiles of populations of these snails from different geographical regions confirmed this approach as being applicable to the identification* of B. tenagophila *and* B. occidentalis *in cases where classical morphology is inconclusive.*

Key words*:* polymerase chain reaction - low stringency - *Biomphalaria tenagophila* - *Biomphalaria occidentalis* - schistosomiasis

Paraense (1981), described *Biomphalaria occidentalis*, a species which could not be differentiated from *B. tenagophila* by shell characteristics and by the morphology of most genital organs. The same author showed that in the laboratory, the two species are separated by absolute reproductive isolation. The differentiation of the two species can be accomplished only by careful dissection of the male and female genitalia and the demonstration of the presence of a vaginal pouch in *B. tenagophila* and its absence in *B. occidentalis* (Paraense 1981). *B. occidentalis* has never been successfully infected by the trematode *Schistosoma mansoni* (Paraense & Côrrea 1982, Coimbra & Engel 1982), and the identification of these species is important for the epidemiology and control of schistosomiasis mansoni. Bailey et al. (1986) used electrophoresis of the hemolymphs of these snails in agarose gel in order to differentiate them. Mascara and Morgante (1995) proposed isoenzyme patterns that might contribute to the identification of these molluscs. An alternative, and indeed very powerful, method to study the genetics of molluscs is the polymerase chain reaction (PCR). A modification of this technique is the random amplification of polymorphic DNA (RAPD), by which complex and informative genomic fingerprints can be readily generated without prior sequence determination (Williams et al. 1990, Welsh & McClelland 1990). This technique has already been used in the study of molluscs for the identification of *Bulinus* species (Langand et al. 1993) and in analysis of genetic variability of *B. glabrata* populations (Vidigal et al. 1994). A related methodology, described by Dias-Neto et al. (1993), was named low stringency PCR (LS-PCR). This method has been used for sex determination in *S. mansoni*, for the identification of *Leptospira* serovars (Caballero et al. 1994) and more recently in identification of *B. glabrata* and *B. tenagophila* (Vidigal et al. 1996). LS-PCR utilizes specific primers under LS conditions, in contrast to AP-PCR (arbitrarily primed PCR) where the choice of primer is arbitrary, although the conditions of the reaction remain the same. The result of complex LS-PCR amplification is a specific fragment defined by the primer used, together with a complex set of other fragments (known as LS products or LSPs) derived from LS interactions of the primers with other sequences in the target genome. Here we have used

Work partially supported by Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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Received 6 May 1996
Accepted 16 August 1996

LS-PCR with specimens of *B. tenagophila* and *B. occidentalis* from different geographical regions of Brazil showing that distinct profiles are consistently produced which distinguish these two planorbid species.

MATERIALS AND METHODS

Snail populations - The studies were undertaken using seven populations of *B. tenagophila* and six of *B. occidentalis* from different geographical regions of Brazil (Fig. 1).

Fig. 1: map of the regions in Brazil showing the origin of the snail isolates (see Table).

These snails were maintained in the Departamento de Malacologia, Instituto Oswaldo Cruz, Rio de Janeiro, except the specimens of *B. occidentalis* from Dracena, Assis, Presidente Epitácio and Palmital, State of São Paulo, which were maintained in the Departamento de Malacologia, Superintendência de Controle de Endemias. The snails from Campo Grande and Ladário, State of Mato Grosso do Sul and Vespasiano, State of Minas Gerais, were collected directly from the field. The dates of collections, descriptions of the sites and origin of the specimens are shown in the Table.

All the snails used in the study were reared and maintained at room temperature under identical conditions in aquaria with running water and sterilized earth, sand, and calcium carbonate. The snails were exposed to artificial diurnal lighting for 10 hr and fed with lettuce. In all cases, the snails were identified by means of comparative morphology based on the reproductive organs and shells (Paraense 1975). The snails were examined in order to determine whether they released any type of cercaria and none were found to be infected by *S. mansoni* or other trematodes.

Preparation of DNA - Total DNA was extracted from the foot of the snails basically as described for *B. glabrata* by Vidigal et al. (1994). Briefly, the foot of each snail was mechanically disrupted in 50mM Tris HCl ph 8.0, 100mM NaCl, 50mM EDTA, 0.5% SDS and incubated with 50 mg/ml proteinase K overnight at 37°C. Following phenol/chloroform extraction and ethanol precipitation, DNA was resuspended in 10 mM Tris-HCl, 1 mM EDTA pH 8.0 and the DNA concentration estimated by comparison with known quantitative standards in 1.5% ethidium bromide stained agar-

TABLE

Dates of collection of the snails used

Species (*Biomphalaria*)	Origin	Site	Date of collection
B. tenagophila	Paracambi	Stream	May, 1990
B. tenagophila	Imbé	N.A.	N.A.
B. tenagophila	Araçatuba	N.A.	May, 1981
B. tenagophila	Formosa	N.A.	August, 1981
B. tenagophila	Vespasiano	Stream	May, 1994
B. tenagophila	Joinvile	N.A.	N.A.
B. tenagophila	Vila Velha	N.A.	January, 1983
B. occidentalis	Campo Grande	N.A.	September, 1995
B. occidentalis	Ladário	N.A.	September, 1995
B. occidentalis	Assis	Stream	October, 1993
B. occidentalis	Dracena	Stream	January, 1994
B. occidentalis	Palmital	Stream	June, 1993
B. occidentalis	Presidente Epitácio	Stream	July, 1989

N.A. = not available

ose gels. A one nanogram template DNA was utilized for each reaction of the PCR.

DNA amplification by LS-PCR - The protocol used was that previously applied for the study of sex differentiation in *S. mansoni* (Dias-Neto et al. 1993). DNA samples from each individual were amplified using 1 ng of template DNA. Each reaction was undertaken in a final volume of 10µl containing 0.8 units of Taq DNA polymerase (Cenbiot, RS, Brazil), 200 µM each dNTP, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8.5, together with 3.2 pmoles of the primers ET1 (5'-GTCCAGACACTACGGGAAT-3') and NS1 (5'-TAGTCATATGCTTGTCTCAG-3'). The primers used are based on the sequence of the 18S rRNA gene from the bivalve mollusc *Placopecten* available in GenBank (access number X53899). The primers correspond to a conserved region of the gene and show a high degree of homology with human (access number X03205), *S. mansoni* (access number X53467), fungi (*Neurospora crassa*, access number X04971) and other 18S rRNA genes. The reaction mix was overlaid with 20 µl of mineral oil and, following an initial denaturation at 95°C for 5 min, was subjected to two cycles through the following temperature profile: 30°C for 2 min for annealing, 72°C for 1 min for extension and 95°C for 30 sec for denaturation followed by 33 cycles where the annealing step was altered to 40°C. In the final cycle, the extension step lasted 5 min. For analysis of the snail amplification products, 3µl of the final reaction mix was applied to the gel. Electrophoresis was undertaken using 4% polyacrylamide gels. Following the separation, the gels were fixed with 10% ethanol/0.5% acetic acid for 5 min and DNA bands revealed by staining with 0.2% silver nitrate for 10 min and reduction with 0.75 M NaOH/0.1 M formaldehyde for 5 min as previously described (Santos et al. 1993, Sanguinetti et al. 1994).

RESULTS

We analyzed seven populations of *B. tenagophila* and six populations of *B. occidentalis* from different geographical regions of Brazil (Fig. 1). Fig. 2 shows a comparison of the LSP profiles produced from two individuals of *B. tenagophila*. Fig. 3 shows two individuals of *B. occidentalis*, collected from the different localities using the primer pair NS1 and ET1 and one nanogram template DNA for each amplification. The LSP profiles were polymorphic for both species. However,

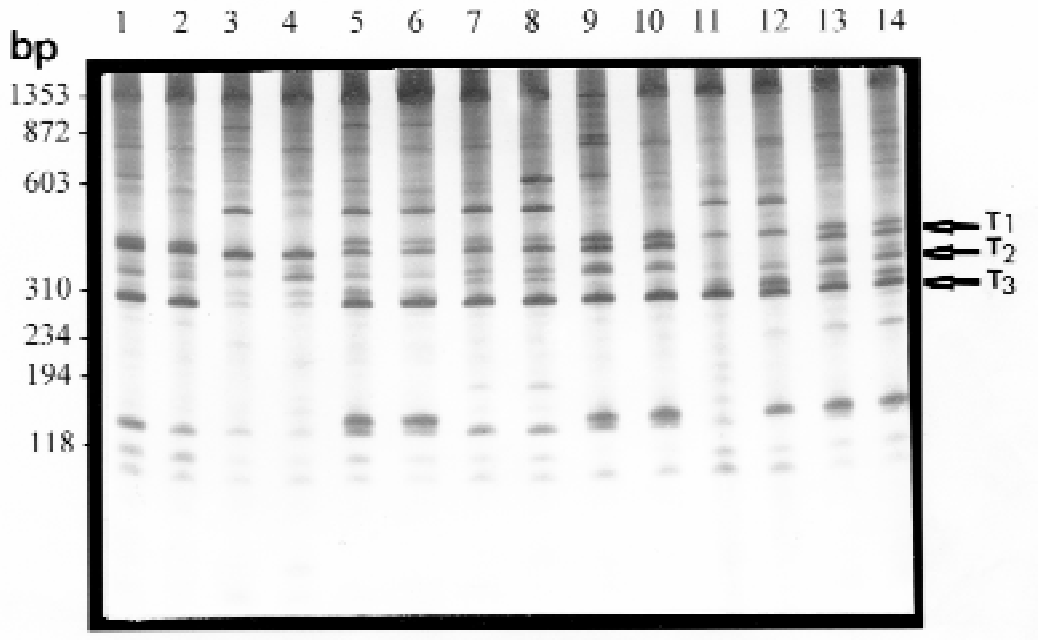

Fig. 2: comparison of LSP profiles of *Biomphalaria tenagophila* of different geographical regions obtained with the primer pair NS1-ET1 and 1 ng of DNA template. Lanes 1 and 2 are representative of *B. tenagophila* specimens from Formosa, GO. Lanes 3 and 4: *B. tenagophila* specimens from Imbé, RS. Lanes 5 and 6: *B. tenagophila* specimens from Joinvile, SC. Lanes 7 and 8: *B. tenagophila* specimens from Araçatuba, SP. Lanes 9 and 10: *B. tenagophila* specimens from Vitória, ES. Lanes 11 and 12: *B. tenagophila* specimens from Paracambi, RJ. Lanes 13 and 14: *B. tenagophila* specimens from Vespasiano, MG. The LS-PCR amplification products were visualized in a 4% polyacrylamide gel stained with silver.

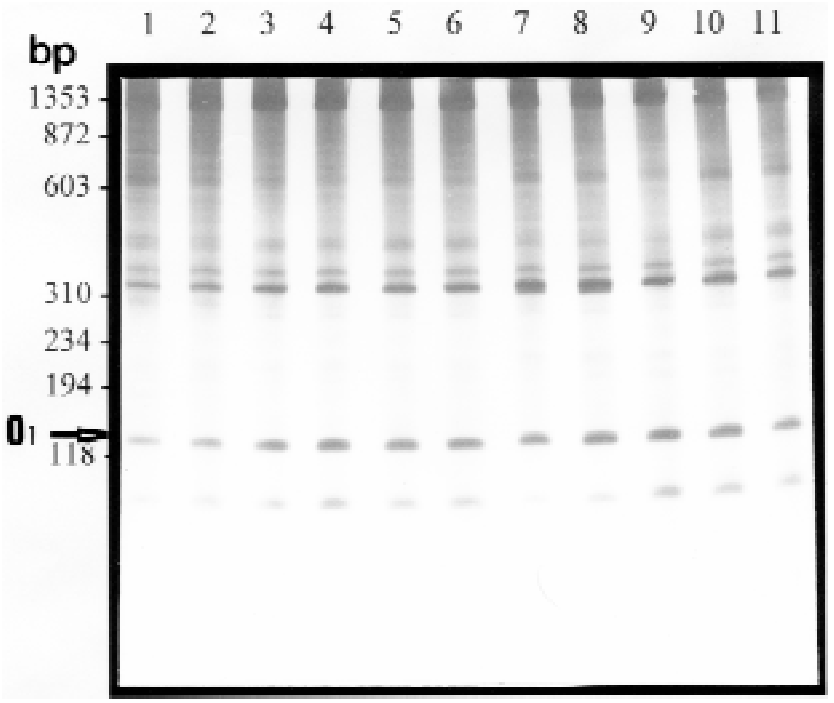

Fig. 3: comparison of LSP profiles of *Biomphalaria occidentalis* obtained with the primer pair NS1-ET1 and 1 ng of DNA template. Lanes 1 and 2: *B. occidentalis* specimens from Campo Grande, MS. Lanes 3 and 4: *B. occidentalis* specimens from Ladário, MS. Lanes 5 and 6: *B. occidentalis* specimens from Assis, SP. Lanes 7 and 8: *B. occidentalis* specimens from Dracena, SP. Lanes 9 and 10: *B. occidentalis* specimens from Palmital, SP. Lane 11: *B. occidentalis* specimen from Presidente Epitácio, SP. The LS-PCR amplification products were visualized in a 4% polyacrylamide gel stained with silver.

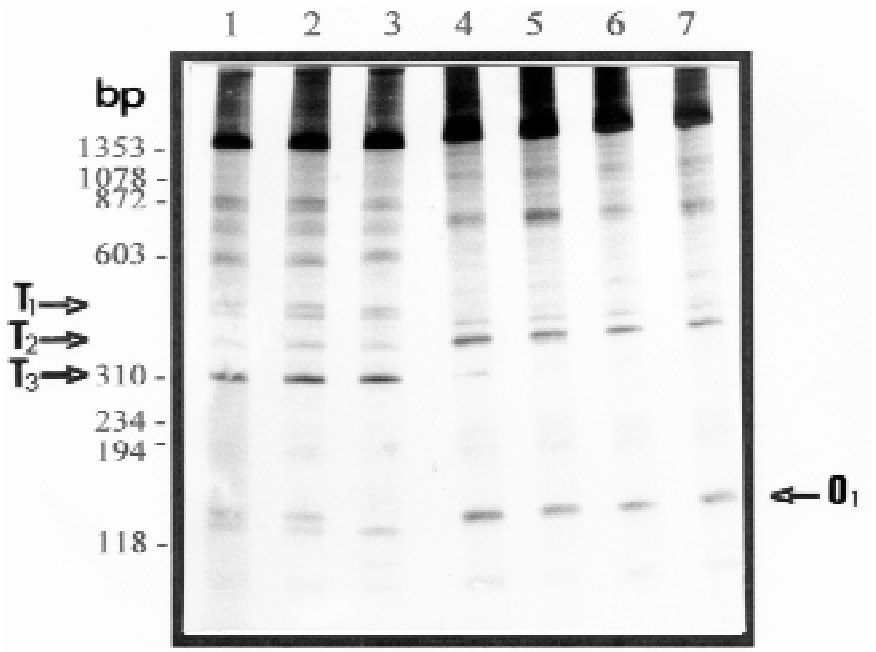

Fig. 4: comparison of LSP of profiles of *Biomphalaria occidentalis* and *B. tenagophila* obtained with the primer pair NS1-ET1 and 1 ng of DNA template. Lane 1 to 3: *B. tenagophila* specimens from Vespasiano; MG, Vitória; ES and Joinvile; SC, respectively. Lanes 4 to 7: *B. occidentalis* specimens from Campo Grande; MS, Ladário; MS; Assis; SP and Dracena; SP, respectively. The LS-PCR amplification products were visualized in a 4% polyacrylamide gel stained with silver.

a number of bands were consistently amplified from all the specimens of each species. The *B. tenagophila* specimens were characterized by the presence of three LSP: of 500 bp (T1), 400 bp (T2) and 310 bp (T3). The *B. occidentalis* specimens were characterized by the presence of one LSP of 130 bp (O1). Fig. 4 shows a reduced number of specimens of each species run on the same gel to demonstrate their different LSPs profiles: *B. tenagophila* continued presenting the three characteristics bands (T1, T2 and T3) and the *B. occidentalis* one characteristic band (O1).

DISCUSSION

The identification of *B. tenagophila* and *B. occidentalis* is difficult because these species are very similar and cannot be differentiated by shell characteristics or by the morphology of most of the genital organs (Paraense 1981). Thus, alternative approaches are required.

Bailey et al. (1986), used hemolymph analyzed by agarose gel electrophoresis in an attempt to separate these species but this approach demonstrated that *B. glabrata* and *B. straminea* could not be separated. Mascara and Morgante (1995) demonstrated that isoenzyme analysis may contribute to the identification of these two species of snails. These authors observed that the value of these techniques is questionable for taxonomic studies because quantitative and qualitative variations are associated with the age and size of individual snails.

The use of PCR provides a new method for the study of molluscs at the genomic level and has been used with success in the study of the genetic variability of *Bulinus* and *B. glabrata* populations (Langand et al. 1993, Vidigal et al. 1994), and in two species of marine gastropods: *Littorina saxalitis* and *L. arcana* (Crossland et al. 1993).

In the present study, LS-PCR was used as an approach to the identification of *B. tenagophila* and *B. occidentalis*. Analysis of the results with specimens obtained from widely separated localities probed with a pair of primers specific for the rRNA gene (NS1-ET1) indeed shows that these two species can be readily distinguished by this technique. It is noteworthy that in both cases, although there are polymorphisms, the LSP profiles are relatively consistent, facilitating identification and suggesting a reduced level of genetic variation in these species as compared with *B. glabrata* (Vidigal et al. 1994). The profiles of the two species, while allowing identification, are generally quite similar. This is consistent with the close phylogenetic relationship of the two species and, at a practical level, demonstrates that the test should be employed using standards and coelectrophoresis on the same gel to avoid errors.

ACKNOWLEDGMENTS

To Dr Wladimir Lobato Paraense and Dr Ligia Corrêa from the Departamento de Malacologia, Instituto Oswaldo Cruz, for providing the snails. To Cristiana Brito, Rosane Sturzeneker, Emmanuel Dias Neto and Patrícia Pinto from the Centro de Pesquisas René Rachou, by their invaluable advice and discussions, and Dr Álvaro Romanha for the facilities provided in the development of the project.

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