The Use of the Polymerase Chain Reaction and Restriction Fragment Length Polymorphism Technique Associated with the Classical Morphology for Characterization of *Lymnaea columella*, *L. viatrix*, and *L. diaphana* (Mollusca: Lymnaeidae)

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The specific identification of Lymnaeid snails is based on a comparison of morphological characters of the shell, radula, renal and reproductive organs. However, the identification is complicated by dissection process, intra and interspecific similarity and variability of morphological characters. In the present study, polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) techniques targeted to the first and second internal transcribed spacers (ITS1 and ITS2) rDNA and to the mitochondrial 16S ribosomal gene (16S rDNAmt) were used to differentiate the species Lymnaea columella, L. viatrix, *and* L. diaphana *from some localities of Brazil, Argentina, and Uruguay as well as to verify whether the molecular results corroborates the classical morphological method. PCR-RFLP analysis of the ITS1, ITS2, and 16S using 12 restriction enzymes revealed characteristic patterns for* L. columella *and* L. diaphana *which were concordant with the classical morphology. On the other hand, for* L. viatrix *populations a number of 1 to 6 profiles were generated while morphology provided the species pattern results.*

Key words: *Lymnaea columella - Lymnaea viatrix - Lymnaea diaphana* - polymerase chain reaction and restriction fragment length polymorphism - first and second internal transcribed spacers of the RNA ribosomal gene - subunit 16S of mitochondrial RNA ribosomal gene

The genus *Lymnaea* Lamarck, 1799 (Basommatophora, Lymnaeidae) includes some freshwater snails that act as intermediate hosts of the liver-fluke *Fasciola hepatica* Linnaeus, 1758, a digenean that causes fascioliasis in animals and humans (Malek 1985, Hopkins 1992, Müller et al. 1998, Esteban et al. 1998).

Paraense (1976, 1982a,b, 1983, 1984, 1995), using the classical morphology, has reported for the South American region the following species: *L. viatrix* Orbigny, 1835, *L. columella* Say, 1817, *L. diaphana* King, 1830, *L. cousini* Jousseaume, 1887, *L. rupestris* Paraense, 1982b and *L. plicata* Scott, 1953. However, only *L. viatrix, L. columella* and *L. diaphana* have been found with natural infection by *F. hepatica* (Malek 1985).

The specific identification of these Lymnaeid snails is based on comparison of morphological characters of the shell, radula, renal and reproductive organs (Hubendick 1951, Paraense 1976, 1982a,b, 1983, 1984, 1995). The correct identification of these molluscs is important for the detection of populations in endemic areas. However, such identification is complicated by dissection processes, intra and interspecific similarity and variability of morphological characters (Hubendick 1951, Ueta 1977, Paraense 1982a, 1983, Samadi et al. 2000). Indeed, Paraense (1994) named *L. peregrina* Clessin, 1882 as a synonym of *L. columella*. Ueno et al. (1975) and Paraense (1982a) have reported for the Northern Bolivian Altiplano region the species *L. viatrix* and *L. cubensis* Peiffer, 1839. Oviedo et al. (1995), however, suggested that these two species correspond to two extreme morphs of the European species *L. truncatula*; data confirmed by isoenzymatic (Jabbour-Zahab et al. 1997) and molecular based studies (Bargues et al. 1997, Bargues & Mas-Coma 1997).

Due to those difficulties involving the snail species identification, molecular-based tools have been used in order to support parasitological research with a wide range of applicability. One of techniques used for such purpose is polymerase chain reaction-restriction fragmente length polymorphism (PCR-RFLP), which is based on the amplification of any DNA region for the subsequent step of digestion with restriction enzymes. Thus, by using primers that anchor conserved regions there will be amplification of a target region under study.

By using PCR-RFLP targeted to the first and second internal transcribed spacers (ITS1and ITS2) regions (nuclear DNA) and to the partial subunit 16S rDNA gene (mitochondrial DNA) the present study is aimed at differentiating the species *L. viatrix*, *L. columella*, and *L.*

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diaphana, obtained from some localities of South America, as well as verifying whether such results corroborate the classical morphological identification.

MATERIALS AND METHODS

Snail populations - Localities and numbers of specimens of each species used are shown in the Table. All the molluscs were obtained from the field, except for *L. viatrix* specimens from Rio Grande do Sul, which were obtained from the Laboratório de Helmintologia, Universidade Federal de Pelotas (Rio Grande do Sul, Brazil). The snails were identified by means of comparative morphology based on the shell, radula, renal region, and reproductive organs accordingly to Hubendick (1951) and Paraense (1976, 1982a b, 1983, 1984, 1995). A reference snail for each DNA isolate has been preserved at the malacological collection of the Laboratório de Helmintoses Intestinais, Centro de Pesquisas René Rachou-Fiocruz.

TABLE

Origin and number of samples of the *Lymnaea* species used in the study

Species of *Lymnaea*	Number of samples	Locality
L. columella	5	Acre (Brazil)
	5	Jaboticatubas/MG (Brazil)
	5	Santa Catarina (Brazil)
L. viatrix	3	Rio Grande do Sul (Brazil) [a]
	3	Buenos Aires (Argentina)
	6	Uruguay
	3	Chubut (Argentina)
	4	Rio Acima/MG (Brazil)
	4	Belo Horizonte/MG (Brazil)
L. diaphana	3	Santa Cruz (Argentina)

a: laboratory populations

DNA extraction - Total DNA was extracted from the feet of snails using the phenol/chloroform method as described in Vidigal et al. (1994).

rDNA-ITS1 amplification - The ITS1 region was amplified using the primers ETTS2 (5'-TAACAAGG TTTCCGTAGGTGAA-3') (Kane & Rollinson 1994) and ITS1r (5'ACGAGCGAGTGATCCACCGC 3') (Vidigal et al. 2004), anchored, respectively, in the conserved ends of 18S and 5.8S ribosomal genes. PCR amplification was undertaken in a final volume of 10 µl consisting of: 1-10 ng of DNA, buffer (1.5 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl, pH 8.5), 200 µM each dNTP, 0.5 U of *Taq* DNA polymerase, 5.0 pmol of each primer. PCR reactions were performed according to Vidigal et al. (2000a) except for the annealing temperature that was set to 60°C.

rDNA-ITS2 amplification - The ITS2 region was amplified using the primers ITS2f (5' CGTCCGTCTGA GGGTCGGTTTGC 3') (Vidigal et al. 2000b) and e ETTS1 (5' TGCTTAAGTTCAGCGGGT 3') (Kane & Rollinson 1994), anchored, respectively, in the conserved ends of 5.8S and 28S ribosomal genes. PCR amplification was undertaken in a final volume of 10 µl consisting of: 1-10 ng of DNA, buffer (1.5 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl, pH 8.5), 200 µM each dNTP, 0.5 U of *Taq* DNA polymerase, 1.0 pmol of each primer. PCR reactions were performed according to Vidigal et al. (2000a) except for the annealing temperature that was set to 60°C.

16S rDNAmt amplification - The 16S region was amplified using the primers 5'CGCCTGTTTATCAAA AACAT 3' and 5'CCGGTCTGAACTCAGATCACGT 3', described by Palumbi (1996). The PCR amplification was undertaken in a volume of 10 µl consisting of: 1-10 ng of DNA, buffer (1.5 µM MgCl2, 50 mM KCl, 10 mM Tris-HCl, pH 8.5), 200 mM each dNTP, 0.5 U of *Taq* DNA polymerase, 1.0 pmol of each primer. PCR reactions were performed according to Remigio and Blair (1997a).

A negative control (no template DNA) was included in all experiments. Three microliters of the amplification products were visualized on 6% silver stained polyacrylamide gels.

RFLP analysis - To evaluate possible enzymes that might yield informative RFLP profiles, five restriction enzymes (*Alu*I, *Msp*I, *Hpa*II, *Rsa*I and *Dde*I) for the ITS1 region, five enzymes (*Hae*III, *Hha*I, *Hpa*II, *Mbo*II and *Mnl*I) for the ITS2 region and 10 enzymes (*Alu*I, *Ava*I, *Dde*I, *Hae*III, *Hpa*I, *Hpa*II, *Mbo*I, *Mnl*I, *Mva*I and *Rsa*I) for the 16S region were randomly chosen. The digestion was performed according to Vidigal et al. (2000a). The results were recorded with the digital camera Mavica (Sony).

RESULTS

Morphological identification of snail populations - After a comparison of the morphological characters, the molluscs from Acre (Brazil), Jaboticatubas (Minas Gerais, Brazil), and Santa Catarina (Brazil) were identified as being *L. columella.* The populations from Rio Grande do Sul (Brazil), Belo Horizonte (Minas Gerais, Brazil), Rio Acima (Minas Gerais, Brazil), Uruguay, Chubut (Argentina) and Buenos Aires (Argentina) were identified as *L. viatrix*. The population from Santa Cruz (Argentina) was identified as *L. diaphana*.

RFLP analysis - PCR amplification of the ITS1 region generated a fragment of approximately 600 bp for all species under study (data not shown). This region presented restriction sites for all the enzymes and revealed characteristic specific patterns for *L. columella* and *L. diaphana*. However, the populations identified as *L. viatrix* were not grouped by none of the restrictions enzymes that generate polymorphic profiles: one for Rio Acima (Minas Gerais, Brazil) population, one for the populations from Rio Grande do Sul (Brazil) and Buenos Aires (Argentina), one for the populations from Uruguay and Chubut (Argentina) and another for the population from Belo Horizonte (Minas Gerais, Brazil). After enzyme digestion all samples exhibited reproducible profiles and only some specimens of each species are shown here. Fig. 1 shows the profile generated by the *Dde*I enzyme, exhibiting the most accurate profile for differentiating those species.

PCR amplification of the ITS2 region generated fragments ranging from approximately 500 bp (*L. columela* and *L. viatrix*) to 550 pb (*L. diaphana*) (data not shown). Except for *Hha*I, all the restriction enzymes tested cut the fragments. Results from ITS2 region showed to be similar to those from ITS1, allowing differentiation between *L.*

columella and *L. diaphana*. On the other hand, populations of *L. viatrix* showed distinct profiles for each population under study. *Hpa*II enzyme exhibited the most accurate profile for differentiating these species (Fig. 2).

DNA amplification of the 16S region generated fragments of approximately 460 bp for *L. columella*, 520 bp for *L. viatrix*, and 580 bp for *L. diaphana* (data not shown). Out of the ten restriction enzymes used, only *Rsa*I, *Dde*I and *Mnl*I showed restriction sites for all the populations. Out of these, only the enzyme *Dde*I (Fig. 3) produced invariant species-specific profiles, which allowed us to separate the species *L. columella*, *L. viatrix*, and *L. diaphana*. The *Rsa*I enzyme has also differentiated *L. columella* and *L. diaphana*; and for the *L. viatrix* populations, only that from Rio Acima showed distinct profiles (data not shown). The enzyme *Mnl*I produced similar results compared to the others used for ITS1 region (data not shown).

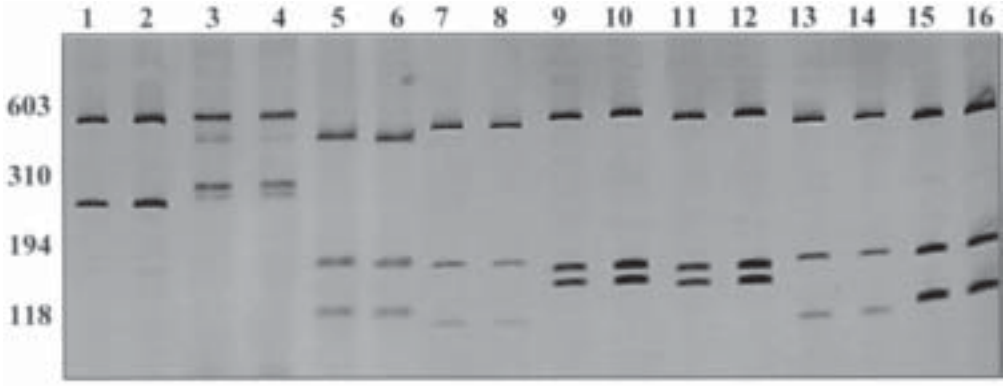

Fig. 1: silver-stained 6% polyacrylamide gel showing restriction fragment length polymorphism profiles obtained after digestion of the rDNA first internal transcribed spacer with *Dde*I enzyme. Lanes - 1: *Lymnaea columella* from Acre, Brazil (BR); 2: *L. columella* from Jaboticatubas, Minas Gerais, BR; 3, 4: *L. diaphana* from Santa Cruz, Argentina (AR); 5, 6: *L. viatrix* from Rio Acima, Minas Gerais, BR; 7, 8: *L. viatrix* from Rio Grande do Sul, BR; 9, 10: *L. viatrix* from Uruguay; 11, 12: *L. viatrix* from Chubut, AR; 13, 14: *L. viatrix* from Buenos Aires, AR; 15, 16: *L. viatrix* from Belo Horizonte, Minas Gerais, BR. Molecular size markers are shown on the left side of the figure. The molecular marker used was ∅ x 174.

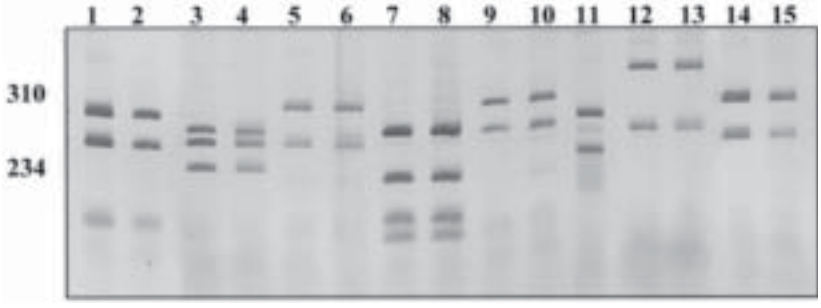

Fig. 2: silver-stained 6% polyacrylamide gel showing restriction fragment length polymorphism profiles obtained after digestion of the rDNA second internal transcibed spacer with *Hpa*II enzyme. Lanes - 1: *Lymnaea columella* from Acre, Brazil (BR); 2: *L. columella* from Jaboticatubas, Minas Gerais, BR; 3, 4: *L. diaphana* from Santa Cruz, Argentina (AR); 5, 6: *L. viatrix* from Rio Grande do Sul, BR; 7, 8: *L. viatrix* from Buenos Aires, AR; 9, 10: *L. viatrix* from Uruguay; 11: *L. viatrix* from Chubut, AR; 12, 13: *L. viatrix* from Belo Horizonte, Minas Gerais, BR; 14, 15: *L. viatrix* from Rio Acima, Minas Gerais, BR. Molecular size markers are shown on the left side of the figure. The molecular marker used was ∅ x 174.

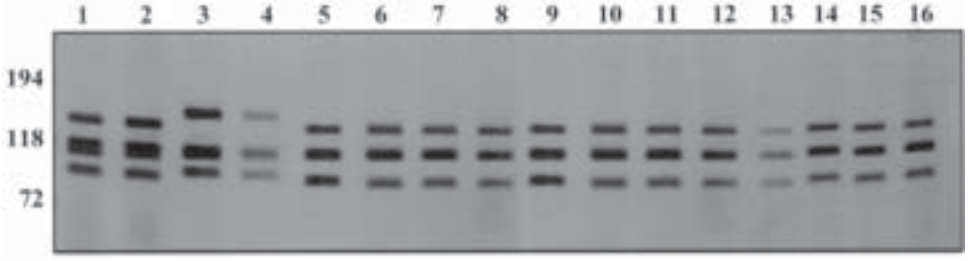

Fig. 3: silver-stained 6% polyacrylamide gel showing the restriction fragment length polymorphism profiles obtained by digesting of the partial subunit 16S rDNA mitochondrial using the *Dde*I enzyme. Lanes - 1: *Lymnaea columella* from Acre, Brazil (BR); 2: *L. columella* from Jaboticatubas, Minas Gerais, BR; 3, 4: *L. diaphana* from Santa Cruz, Argentina (AR); 5, 6: *L. viatrix* from Rio Acima, Minas Gerais, BR; 7, 8: *L. viatrix* from Rio Grande do Sul, BR; 9, 10: *L. viatrix* from Uruguay; 11, 12: *L. viatrix* from Chubut, AR; 13, 14: *L. viatrix* from Buenos Aires, AR; 15, 16: *L. viatrix* from Minas Gerais, Belo Horizonte, BR. Molecular size markers are shown on the left side of the figure. The molecular marker used was ∅ x 174.

DISCUSSION

In the present study, we used the PCR-RFLP technique targeted to different DNA regions (ITS, ITS2, 16S) and compared the molecular results obtained with the traditional morphology identification. Because there is a paucity of information about the taxonomy of the Lymnaeid group, especially those from South America, we decided to use molecular markers of different evolution rate in order to obtain more reliable molecular results. Indeed, Anderson (2001) has shown how molecular data from a single genetic marker can lead to ambiguous conclusions, emphasizing the importance of utilizing more than one marker in studies on parasite species.

From analyses of ITS1 and ITS2 region, we have observed that all the enzymes used for both regions produced profiles for *L. columella* and *L. diaphana* that were identical among the species and distinguishable from the others. Our molecular results are in accordance with the classical morphological identification. Nevertheless, those regions exhibited a high level of polymorphism for *L. viatrix* populations. By using the 16S region of rDNAmt, only *Dde*I, out of the ten restriction enzymes under study, was able to cluster all *L. viatrix* populations. Such polymorphism may be mirroring an intra-specific variation as already remarked on other organisms by using the same rDNA regions (Hope & McManus 1994, Stothard & Rollinson 1997, Bargues et al. 2001).

However, morphological and anatomical studies on *L. viatrix* carried out by Paraense (1976) and Hubendick (1951) have raised the issue whether all the specimens included in the same taxa really belong to that species. Moreover, Durand et al. (2002) analyzed the genetic variability of three *L. viatrix* populations (from Lima/Peru, Tacna/Peru, and Bahia Blanca/Argentina) based on isoenzymatic studies and observed the occurrence of several distinct genetic entities in those samples, suggesting that the South American taxa include at least three taxonomic units.

The PCR-RFLP technique has been successfully used for specific identification of several organisms. This molecular biology methodology is simple, robust, and rapid and has proved to be an important additional tool in studies on morphological identification of molluscs (Vidigal et al. 2000a, Caldeira et al. 2000, Carvalho et al. 2001, Stothard et al. 2001); arthropods (Clark et al. 2001); helminthes (Mayta et al. 2000, Caldeira et al. 2003) and protozoan (Kong et al. 2002). Recently, sequence analyses of the large subunit (16S) of the mitochondrial ribosomal DNA have provided a distinction among several Lymnaeid species and data for phylogenetic relationships studies (Remigio & Blair 1997a, Remigio 2002). Remigio and Blair (1997b), using the ITS1 and ITS2 of the nuclear ribosomal DNA for the three members of the subgenus *Stagnicola* s. str, demonstrated that they were similar and such data could be taken as evidence that the snails are conspecific or are sister species. Furthermore, these authors have shown the most useful markers to differentiate two very closely related subgenera (*Stagnicola* and *Hinkleyia*). Bargues et al. (2001) sequenced the ITS2 region from 66 populations of 13 European species and one North American species and obtained phylogenetic trees which could resolve supraspecific, specific and population Lymnaeid relationships, distinguishing many genotypes in a total of 11 species (including two sub-species) distributed in four different genera (one comprising two subgenera). Klossa-Kilia et al. (2002), using PCR-RFLP technique directed to 16S rDNAmt, obtained species specific restriction patterns that enabled clear discrimination among five fish roe Mugilidae species.

The results showed here suggest that further morphological and phylogenetic studies on *L. viatrix* are necessary, in order to better understand the actual taxonomical status of that species and association with other populations. On the other hand, results provided for the species *L. columella* and *L. diaphana* by using PCR-RFLP are in agreement with classical taxonomy, emphasizing the usefulness of such molecular technique for molluscs differentiation**.**

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