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Real-time PCR strategy for rapid discrimination among main lymnaeid species from Argentina

T. Duffy[a,*], F. Kleiman[b], S. Pietrokovsky[b], L. Issia[b], A.G. Schijman[a], C. Wisnivesky-Colli[b]

[a] *Laboratorio de Biología Molecular de la Enfermedad de Chagas, Instituto de Ingeniería Genética y Biología Molecular (INGEBI-CONICET), Vuelta de Obligado 2490, 1427 Buenos Aires, Argentina*

[b] *Unidad de Ecología de Reservorios y Vectores de Parásitos, Departamento de Ecología, Genética y Evolución, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina*

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ABSTRACT

Snails of the Family Lymnaeidae act as an intermediate hosts of *Fasciola hepatica* worldwide. The taxonomy of lymnaeid species is relevant for epidemiological studies and molecular strategies are increasingly used for that purpose. This work presents the first report of a real-time PCR approach used to identify the most important lymnaeid species in the Southern Cone of South America. Species discrimination is based on the sequence polymorphism located within the helix E10-1 of the variable region V2 of the 18S rRNA genes, which yields amplicons with clearly different melting temperatures. This procedure minimises the risk of carry-over contamination because it does not require post-PCR manipulations, and the whole protocol can be completed in less than 4 h with a single snail foot as starting material. This method was successfully carried out in a blind study that included a panel of 20 *Galba truncatula*, 5 *Lymnaea viatrix*, 5 *Lymnaea diaphana* and 5 *Pseudosuccinea columella* specimens from different endemic areas for fasciolosis. This molecular approach constitutes a key laboratory tool complementing ecological studies that ultimately will promote more efficient control strategies.

1. Introduction

Fasciolosis, produced by *Fasciola hepatica* is an important disease of livestock (Boray, 1967; Dargie, 1987). Recently, its medical interest has increased due to the re-emergence of human cases throughout the world (Oviedo et al., 1995; Mas-Coma et al., 1999).

The life cycle of *F. hepatica* includes snails of the Family Lymnaeidae, which act as an intermediate hosts (Malek, 1985). These freshwater snails are distributed worldwide, especially in tropical and subtropical regions of America, Europe, Asia, Africa and Oceania (Hubendick, 1951). The epidemiology of the disease varies with the geographical distribution and the lymnaeid species that are involved in the transmission (Mas-Coma, 2005). Thus, taxonomical studies are relevant for planning effective control strategies.

In the Southern Cone of South America, the most frequently cited lymnaeids are *Lymnaea viatrix*, which is the most widely distributed species (Paraense, 1982; Kleiman et al., 2004; Rubel et al., 2005; Cucher et al., 2006), and *Pseudosuccinea columella* in the North-Eastern area (Paraense, 1982; Prepelitchi et al., 2003). Other species reported are *Lymnaea diaphana* in Patagonia and the European native *Galba truncatula*. The latter has been found in the Bolivian Altiplano (Mas-Coma et al., 1999), Chile (Yahia, 1997) and Peru (Esteban et al., 2002). All the above mentioned species are competent intermediate hosts for *F. hepatica* (Malek, 1985; Graczyk and Fried, 1999; Prepelitchi et al., 2003). The similarity in the morphology of their shell and internal organs makes species identification difficult (Hubendick, 1951; Ueta, 1977; Paraense, 1982, 1983; Oviedo et al., 1995; Bargues et al., 1997; Bargues and Mas Coma, 1997; Samadi et al., 2000). In the Northern Bolivian Altiplano region, snails assigned to *L. viatrix* and *Lymnaea cubensis* on morphological grounds (Ueno et al., 1975; Paraense, 1982), were suggested by Oviedo et al. (1995) as being two extreme morphs of *G. truncatula*. This fact was subsequently confirmed by isoenzymatic (Jabbour-Zahab et al., 1997) and molecular studies (Bargues et al., 1997; Bargues and Mas Coma, 1997). In addition, specimens of *G. truncatula* from the Northern Bolivian Altiplano and Europe were found to belong to a same species based on identical 18S rDNA sequences (Bargues and Mas Coma, 1997).

In the last decades, molecular techniques have proven to be a useful tool to differentiate species and to solve evolutionary relationships among taxa. This is particularly the case for sequence analysis of the 18S rDNA gene of lymnaeids. Within the 18S rDNA

* Corresponding author. Tel.: +54 11 4783 2871x21; fax: +54 11 4786 8578.
E-mail address: duffy@dna.uba.ar (T. Duffy).

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molecule, the majority of polymorphic nucleotides in lymnaeid species reside in a small region included in the helix E10-1 of the variable region V2, enabling population and species group distinction (Bargues and Mas Coma, 1997; Stothard et al., 2000). Accordingly, the aims of the present study were to characterise the above mentioned region and to design a real-time PCR strategy for the direct, rapid and accurate differentiation of the four main lymnaeid species of Argentina, namely *P. columella*, *L. viatrix*, *G. truncatula* and *L. diaphana*.

2. Materials and methods

2.1. Snail material

We collected specimens from the following species: (1) *L. viatrix* var. ventricosa from (a) Province of Córdoba, around 31°25′S 64°48′W, March 1999 and (b) Province of Chubut, Locality of Cholila, 42°32′S 71°34′W, November 1999; (2) *L. diaphana* from Province of Chubut, Locality of Sarmiento, 45°35′S 69°05′W, April 2006; (3) *P. columella* from Province of Corrientes, Locality of Berón de Astrada, 27°33′S 57°32′W, December 2000; (4) *G. truncatula* from Province of Mendoza, 35°57′S 69°24′W, August 2006.

These species have been previously identified on the basis of their morphology according to Paraense (1984). All the specimens were examined for trematode infection and the feet of those proven to be uninfected were individually preserved for molecular studies.

2.2. DNA extraction and rDNA amplification for nucleotide sequencing

Individual snail feet were processed for DNA extraction, according to the procedure recommended by QIAGEN (QIAamp Tissue Kit Handbook, Qiagen, USA).

In order to determine the sequences of the helix E10-1 of the V2 variable region from the specimens of *L. viatrix*, *P. columella*, *G. truncatula* and *L. diaphana* collected in Argentina, PCR reactions were performed using 1 μM of primers LymFw (5′-TCCTACTTGGATAACTGTGGCA-3′) and LymRv (5′-TTACAAACATGGTAGGCATATC-3′) designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), 3 mM $MgCl_2$, 250 μM of each dNTP, 0.5 U of Platinum Taq polymerase, (Invitrogen, Life Technologies, USA) and 5 μL of sample DNA in a 50 μL reaction volume. After 5 min of preincubation at 95 °C, PCR amplification was carried out for 40 cycles (94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s).

PCR products were purified with QIAquick PCR Purification Kit (Qiagen, USA) and sequenced. Direct cycle sequencing was performed using a MEGABACE 500 automatic sequencer, (Amersham-Pharmacia, USA). The partial sequences of the 18S rDNA of *L. viatrix*, *P. columella*, *G. truncatula* and *L. diaphana* were deposited in the GenBank database under the following accession numbers: AY057089, EU241866, EU728668 and EU241865, respectively.

2.3. Sequence alignment and phylogenetic analyses

The sequences of the 18S rDNA genes of *L. viatrix*, *P. columella*, *G. truncatula* and *L. diaphana* were aligned together with those of six species available at the Genbank-EMBL, namely *Lymnaea auricularia* (GenBank accession no. Z73980), *Lymnaea peregra* (GenBank accession no. Z73981), *Lymnaea stagnalis* (GenBank accession no. Z73984), *Lymnaea palustris* (GenBank accession no. Z73983), *Lymnaea glabra* (GenBank accession no. Z73982) and *Lymnaea cubensis* (GenBank accession no. Z83831). Sequence alignment was conducted using MEGA version 4 (Tamura et al., 2007).

2.4. Identification of lymnaeid species by real-time PCR

In order to develop a real-time PCR strategy for differential identification of Argentinean lymnaeids, primers flanking the E10-1 helix of the V2 variable region were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The 20-μL reaction tube contained 1 μM of primers 213 (5′-ATTAGTTCAAAACCAATCGCCG-3′) and 297 (5′-CAAAGTTATCCAGAGTCACCAATGG-3′), 3 mM $MgCl_2$, 250 μM of each dNTP, 0.5 U of Platinum Taq polymerase, SYBR Green (both from Invitrogen, Life Technologies, USA) at a final concentration of 0.5× and 2 μL of sample DNA (25 ng/μL).

The PCR conditions consisted of an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 94 °C for 10 s, 55 °C for 10 s and 72 °C for 10 s, and a final step of 2 min at 72 °C. Amplification was immediately followed by a melt program with an initial denaturation of 5 s at 95 °C and then a stepwise temperature increase of 0.1 °C/s from 72 to 90 °C. An MJR-Opticon II device (Promega, USA) was used.

3. Results

The sequence of the helix E10-1 of the variable region V2 of the 18S rDNA is polymorphic in the four analysed species (Fig. 1). However, the fact that the amplicons from these species have similar lengths makes them indistinguishable by means of conventional agarose gel electrophoresis after a standard PCR procedure. Therefore, a real-time amplification strategy with subsequent analysis of the melting temperatures was investigated. In this case, the amplicons showed easily distinguishable melting temperatures in the real-time apparatus (Fig. 2 and Table 1). This method was sensitive enough to identify the four lymnaeid species from DNA extracted from individual feet. The results were reproduced in triplicates of feet specimens used as reference samples (Table 1). Specific melting temperature peaks were obtained when a panel of feet specimens from 20 *G. truncatula*, 5 *L. viatrix*, 5 *L. diaphana* and 5 *P. columella* snails was tested in a blind study (data not shown).

4. Discussion

The development of molecular laboratory tools for rapid and accurate identification of lymnaeid species is essential for the

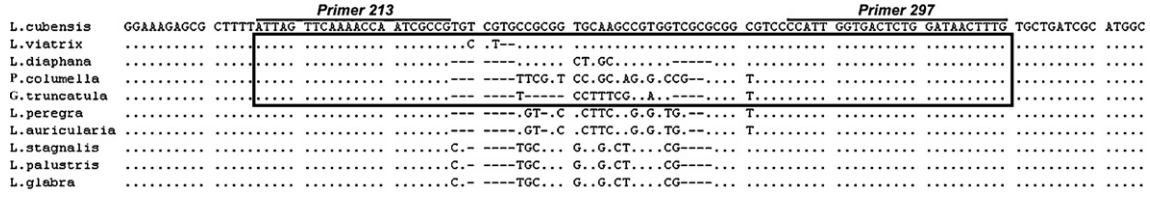

Fig. 1. Alignment of the E10-1 helix of the V2 variable region of the 18S rRNA gene for real-time PCR-based identification of lymnaeids. The amplicon sequence is boxed.

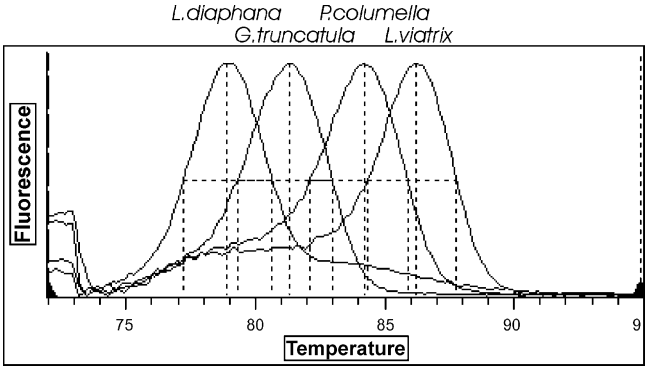

Fig. 2. Typical melting temperature peaks (°C) obtained for lymnaeid species identification by real-time PCR.

epidemiological studies. Carvalho et al. (2004) applied 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), using 12 restriction enzymes to identify *P. columella*, *L. viatrix* and *L. diaphana* from localities of Brazil, Argentina and Uruguay. This analysis revealed characteristic patterns for *P. columella* and *L. diaphana* that were concordant with classical morphological features, but *L. viatrix* populations rendered from 1 to 6 different profiles, making identification of this species cumbersome.

Real-time PCR strategies have proven to be more reliable, rapid, sensitive and specific than conventional typing methods for distinguishing morphologically similar variants of a wide spectrum of taxa, including bacterial pathogens (Jensen et al., 2005), parasitic protozoans (Mangold et al., 2005; Freitas et al., 2005), insect species (Harper et al., 2005) and fish eggs and larvae (Watanabe et al., 2004; Itoi et al., 2005).

This is the first report of a real-time PCR strategy based on the polymorphism within the E10-1 helix of the variable region V2 of the 18S rDNA sequence for lymnaeid identification. We were able to correctly identify specimens of a same species collected from geographically distant populations, as demonstrated when specimens of *L. viatrix* collected from Córdoba and Chubut, separated by approximately 1200 km, were typed. Although we did not find evidence of intra-specific variation, we believe that this method is capable of identifying cryptic species or hybrids due to the sensitivity of real-time melting temperature analysis to detect SNPs and to the variability in the E10-1 helix in lymnaeid species. Stothard et al. (2000) were able to characterise two different populations of *Lymnaea natalensis* and their respective hybrids using PCR–RFLP targeted to the helix E10-1. In fact, they found that the SNPs produced variations in the thermal stability of the E10-1 helix and this was correlated with the altitude at which the specimens were collected. This example clearly shows the power of resolution of the 18S rDNA-based typing methods in lymnaeid species.

In addition, this methodology is economically accessible (each assay costs approximately U$S 8.00) and shows the following advantages over conventional typing techniques: (1) the risk of carry-over contamination is minimised because the procedure does not require post-PCR manipulations such as gel electrophoresis, RFLP or sequencing; (2) the whole protocol, from DNA purification to species identification, can be completed in less than 4 h; (3) the result can be obtained from a single foot specimen; (4) the species-specific melting temperature peaks are clearly distinguishable.

Field data indicate that *L. viatrix* has an overlapping distribution with *G. truncatula* in Northern Patagonia, and with *L. diaphana* in Central Patagonia. In this context, this molecular approach constitutes a key laboratory tool complementing ecological studies that ultimately will promote more efficient control strategies.

Table 1
Mean melting temperatures (MT) and standard deviations (S.D.) obtained from real-time PCR experiments of triplicates from individual feet of each tested lymnaeid species.

Species	MT (°C)	Mean MT peak ± S.D. (°C)
Lymnaea diaphana	79.5 78.9 79.8	79.4 ± 0.46
Galba truncatula	81.5 81.3 81.6	81.5 ± 0.15
Pseudosuccinea columella	84.2 84.4 84.6	84.4 ± 0.2
Lymnaea viatrix	86.2 86.1 86.0	86.1 ± 0.1

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