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Research paper

A new multiplex PCR assay to distinguish among three cryptic *Galba* species, intermediate hosts of *Fasciola hepatica*

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ABSTRACT

A molecular tool described here allows in one step for specific discrimination among three cryptic freshwater snail species (genus *Galba*) involved in fasciolosis transmission, a worldwide infectious disease of humans and livestock. The multiplex PCR approach taken targets for each species a distinctive, known microsatellite locus which is amplified using specific primers designed to generate an amplicon of a distinctive size that can be readily separated from the amplicons of the other two species on an agarose gel. In this way, the three *Galba* species (*G. cubensis*, *G. schirazensis*, and *G. truncatula*) can be differentiated from one another, including even if DNA from all three were present in the same reaction. The accuracy of this new molecular tool was tested and validated by comparing multiplex PCR results with species identification based on sequences at mitochondrial and nuclear markers. This new method is accurate, inexpensive, simple, rapid, and can be adapted to handle large sample sizes. It will be helpful for monitoring invasion of *Galba* species and for developing strategies to limit the snail species involved in the emergence or re-emergence of fasciolosis.

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Table 1
Galba individuals (n = 11) used to design the multiplex PCR. These individuals had previously been identified by Correa et al. (2011) based on nuclear (ITS1 and ITS2) and mitochondrial (CO1 and 18S) sequences. GenBank accession numbers provided here are for the CO1 sequences. Some coordinates were corrected in order to match the specific locality: Frias (Argentina) and Owego (New York, USA).

Species	Country	Site	Coordinates	Number of individuals	Genbank accession number
Galba cubensis	USA	Charleston County (South Carolina)	32°45′59”N 79°49′35”W	2	JN614395, JN614394
Galba schirazensis	Colombia	Matasano (Antioquia)	06°25′58”N 75°22′28”W	1	JN614372
	Venezuela	La Trampa	08°33′29”N 71°27′15”W	1	JN614378
Galba truncatula	France	Limousin region	45°47′05”N 01°11′36”E	1	JN614386
Galba viator	Argentina	Frias	40°14′10”S 64°10′09”W	2	JN614397, JN614398
Galba cousini	Venezuela	Mucubají	08°47′54”N 70°49′33”W	2	JN614389, JN614388
Galba humilis	USA	Owego (New York)	42°06′01”N 76°15′04”W	2	FN182197, FN182198

1. Introduction

Understanding and preventing infectious diseases requires a thorough and accurate knowledge of hosts involved in parasite dynamics. Fasciolosis is a cosmopolitan disease causing significant economic losses in domestic livestock (Rim et al., 1994). Human cases of fasciolosis have been increasing throughout the world particularly in some regions of South America, suggesting the possibility of re-emergence in this area (Esteban et al., 1999; Mas-Coma et al., 2001). The parasites causing this disease are liver flukes (*Fasciola* spp.). Freshwater mollusks, mainly belonging to the family Lymnaeidae (Correa et al., 2010; Hurtrez-Boussès et al., 2001), serve as intermediate hosts of *Fasciola*, especially lymnaeids in the genus *Galba*. For example, *Galba truncatula* and *Galba cubensis* are well-known intermediate hosts of the disease (Bargues et al., 2007; Jabbour-Zahab et al., 1997). Whether *Galba schirazensis* is also an intermediate host remains controversial. One study suggested that *G. schirazensis* is not a host (Bargues et al., 2011), whereas more recent investigations suggested that it is (Caron et al., 2017; Dreyfuss et al., 2015).

Galba are small-shelled freshwater snails that mostly originating in the Americas and, subsequently invaded Europe, Africa, and Asia (Bargues et al., 2011; Correa et al., 2011, 2010; Lounnas et al., 2017a; Meunier et al., 2001). Their ability to survive drought and to reproduce by self-fertilization allow them to disperse over long distances and establish new populations from single individuals (Meunier et al., 2004, 2001). This high invasiveness has probably facilitated the worldwide expansion of fasciolosis.

Absence of reliable morphological traits has led to confusion regarding specific identities of *Galba* populations worldwide. Six species are considered valid: *Galba cousini*, *Galba cubensis*, *Galba schirazensis*, *Galba truncatula*, *Galba humilis*, and *Galba viator* (Correa et al., 2011). Of these six species only adults of *G. cousini* display distinct differences in shell morphology and internal anatomy (Paraense, 1995). The other five species cannot be accurately distinguished because their shell morphology exhibits greater intraspecific than interspecific variability, and their anatomy is homogeneous (Correa et al., 2011; Pointier, 2015; Samadi et al., 2000). Nor can they be distinguished with controlled crosses in the laboratory, as is possible in populations of the genus *Physa* (Dillon et al., 2011), because *Galba* populations typically exhibit high selfing rates (Lounnas et al., 2017a,b). Thus, except for *G. cousini* adults with their distinct morphology, individuals of *Galba* have in recent years been identified using DNA sequencing technology (Correa et al., 2011).

Amplifying and sequencing diagnostic loci can be time-consuming and expensive when sample sizes are large. Here, a rapid and inexpensive molecular approach based on multiplex PCR is provided to identify cryptic *Galba* species. Multiplex PCR has already been used successfully to identify a variety of cryptic parasites and their hosts, for example *Anopheles* mosquitoes, tapeworms, and protozoans diluted within feces or blood (Bohórquez et al., 2015; Kengne et al., 2001; Sumbria et al., 2015). With respect to lymnaeid species, real-time PCR based on species-specific melting temperatures can be used to differentiate among *G. truncatula*, *G. viator*, *Pseudosuccinea columella*, and *Lymnaea diaphana* (Duffy et al., 2009). The multiplex PCR method used in the present study focuses on three widely distributed cryptic *Galba* species, *G. cubensis*, *G. schirazensis*, and *G. truncatula*. Based on known microsatellite loci specific for each species, specific primers were used for each to enable amplification of a product that could be distinguishing in a species specific way from the other amplified products based on size. The accuracy of this new molecular method was tested by comparing its results with species identification based on mitochondrial and nuclear gene sequences.

2. Material and methods

The multiplex PCR method was based on species-specific primers amplifying microsatellite loci already described for each of the three targeted cryptic species: *G. truncatula* (Trouvé et al., 2000), *G. cubensis* (Lounnas et al., 2017b), and *G. schirazensis* (Lounnas et al., 2017a). Eleven primer mixes were designed, each including one species-specific primer pair for each of the three species (Table S1). Each mix contained a different combination of primers chosen such that the PCR products from the three species differed in size. A range of concentrations for each primer was also tested: 2, 6, 8, and 10 mM.

The primer mixes and concentrations were initially developed in one negative control and 11 known standards: two *G. cubensis*, two *G. schirazensis*, one *G. truncatula*, two *G. cousini*, two *G. humilis*, and two *G. viator*, all identified by Correa et al. (2011) on the basis of ITS2, ITS1, CO1, and 18S sequences (Table 1). Even though *G. cousini* is easily distinguishable using shell morphology and reproductive anatomy (Paraense, 1995), it was included in the development phase to evaluate the specificity of the multiplex PCR. Each candidate multiplex was tested using samples containing DNA from a single species and pooled DNA from multiple species.

DNA was amplified in a total volume of 10 µl containing 5 µl of Taq PCR Master Mix Kit (Qiagen), 1 µl of the primer mix, and 50–100 ng of DNA in an Eppendorf Thermal Cycler. Different annealing temperatures in the PCR amplification were also tested: 50, 52, and 54 °C. Finally, the PCR program retained was the one that consistently and accurately generated amplicons of distinct size for each species. The final PCR amplification protocol has an initial denaturation step at 95 °C for 15 min; followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 90 s, and extension at 72 °C for one min; the final extension was at 60 °C for 30 min. The amplification products were electrophoretically resolved after 2 h at 100 V in 5% agarose gels and stained with EZ-Vision (Amresco).

Observing the resultant agarose gel, each of the 11 primer mixes was then evaluated by three criteria: (1) each primer pair was required to amplify a single locus in the targeted species, (2) primers were not expected to yield PCR amplification in *G. cousini*, *G. humilis*, and *G. viator*, and (3) the PCR products from *G. cubensis*, *G. schirazensis*, and *G. truncatula* were required to differ noticeably in size. Ultimately, the primer mix and concentration retained was the one that allowed an accurate identification of *G. cubensis*, *G. schirazensis*, and *G. truncatula*