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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*

Table 2
Microsatellite primers used in the multiplex PCR detection of *Galba cubensis* (Lounnas et al., 2017b), *Galba schirazensis* (Lounnas et al., 2017a), and *Galba truncatula* (Trouvé et al., 2000). Also provided are the size (in base pairs) of the PCR product (alleles) and primer concentration. The size of the microsatellite locus for *G. truncatula* was retrieved from Chapuis et al. (2007). (f): forward; (r) reverse.

Name species (Primer)	Sequence (5′–3′)	Size (bp)	Primer concentration (mM)
Galba cubensis (Lc34)	GTCACTACTGCTTGTCTCAGC (f) AAAAGACTTTAACCCTTACCACCC (r)	179–200	2
Galba schirazensis (Ls23)	AARGACCCAGTGGGGAAG (f) TGGGGAAGGTTCAATTGTTT (r)	227–232	8
Galba truncatula (Lt37)	GTCCAGTCTTTGTATGTC (f) GTTAAGTACCCAACTTCTTC (r)	111–129	10

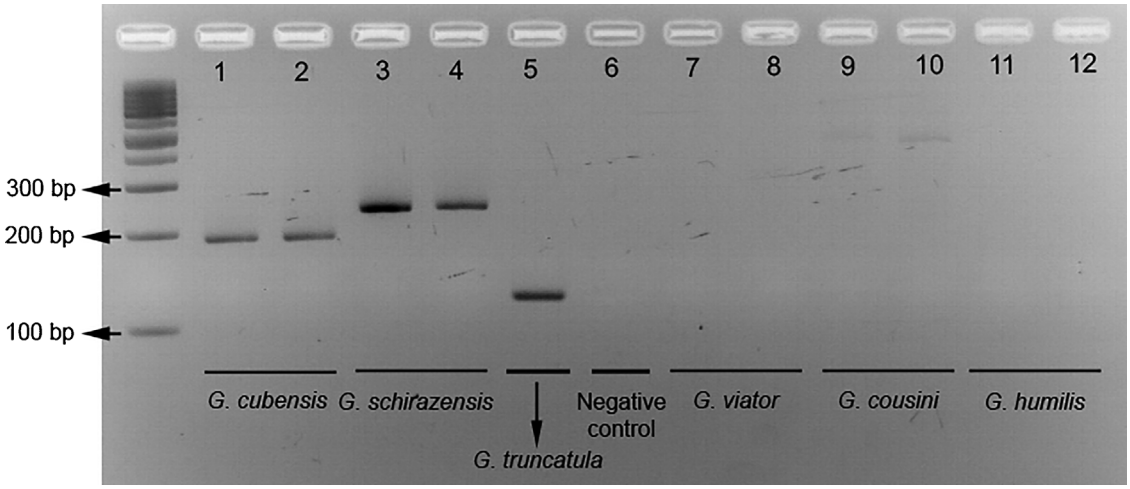

Fig. 1. Agarose gel electrophoresis of multiplex PCR products of individuals of the six *Galba* species. These individuals were previously identified based on ITS1, ITS2, CO1, and 18S sequences by Correa et al. (2011). See countries, sites, and GenBank accession numbers of CO1 sequences in Table 1.

(Table 2) in both single-species (Fig. 1) and pooled samples.

The newly-developed multiplex PCR method was validated using DNA extracts of 49 additional *Galba* previously identified by Correa et al. (2011) and Lounnas et al. (2017a,b) based on DNA sequences: 23 *G. cubensis*, 22 *G. schirazensis*, and 5 *G. truncatula* (Table 3). These individuals were removed from sites geographically distant from the sites in which the initial 11 individuals used to develop the method were sampled. DNA-extraction protocols of the individuals here studied are described in Correa et al. (2011) and Lounnas et al. (2017a).

3. Results

The multiplex PCR we designed successfully differentiated individuals of *G. cubensis*, *G. schirazensis*, and *G. truncatula* previously identified by sequencing DNA markers (Correa et al., 2011; Lounnas et al., 2017a,b). Each snail species was identified by the distinctive size of an amplified band produced in a multiplex PCR format, with each amplicon derived from a species specific microsatellite template. DNA bands of each species were definitively separated in a 5% agarose gel (Fig. S1). The loci chosen for the multiplex PCR have low to intermediate polymorphism, so as a consequence there were no overlapping bands among the species on agarose gels (Table 2; Fig. S1). The smallest difference in the size of amplified alleles was observed between some individuals of *G. cubensis* from Venezuela and Guadeloupe and individuals of *G. schirazensis*. Even at this size difference (27 bp according to Lounnas et al., 2017a,b), both species could be accurately identified. We did not find pairs of distinct DNA bands (putatively allelic) at any locus for any individual tested.

4. Discussion

The multiplex PCR we designed successfully identifies three cryptic *Galba* species that are intermediate hosts of *Fasciola hepatica*: *G. cubensis*, *G. schirazensis*, and *G. truncatula*. Until the present study, the only method by which cryptic *Galba* populations could be identified was by amplifying and sequencing mitochondrial or nuclear genetic markers. This technique is time-consuming because sequencing must usually be outsourced in countries where fasciolosis is widespread and laboratories doing DNA sequencing services are few. The multiplex PCR approach developed in this study is: (i) accurate, because it was validated by the results obtained by mitochondrial or nuclear markers; (ii) inexpensive compared to amplifying and sequencing molecular markers; (iii) simple, because a single PCR is sufficient to amplify the species-specific alleles; (iv) rapid, because the results are available in less than a day; and (v) reproducible and adaptable to a large set of individuals (because it is fast and cheap), including adults or juveniles. This method is, however, insufficiently sensitive to distinguish heterozygotes when the alleles differ by less than 15 bp (Fig. S1). Thus, we do not recommend it to estimate heterozygosity. Researchers wishing to both identify *Galba* populations and estimate their heterozygosity may wish to supplement the method proposed here with methods that allow distinguishing alleles differing by a single nucleotide, as in Meunier et al. (2004) and Lounnas et al. (2017a,b).

This multiplex PCR approach would be useful as a first approach for the study of *Galba* populations in unexplored and poorly-known regions of the world. *Galba* populations from the south of Brazil have been studied on the basis of morphology only, for example, and species could have been misidentified (Medeiros et al., 2014). Similarly, in North America authors recognize as many as 22 "*Fossaria*" species on the basis of shell and radula morphology (Burch, 1982; Johnson et al., 2013), most of which are indistinguishable from cryptic *Galba* species worldwide. Hubendick (1951) considered almost all of the nominal North American species to be synonyms of *G. humilis*, but resolution awaits molecular analysis, and comprehensive sampling across a broad

Table 3
Galba individuals (n = 49) used to test and validate the multiplex PCR. *Galba cubensis* has been identified based on sequence data by Lounnas et al. (2017b), *G. schirazensis* by Lounnas et al. (2017a), and *G. truncatula* from Peru by Lounnas et al. (2017b) and the others by Correa et al. (2011). GenBank accession numbers from Lounnas et al. (2017b) are for ITS2 sequences (except for KT461809 which is a CO1 sequence) and the ones from Correa et al. (2011) and Lounnas et al. (2017a) are for CO1 sequences. ND: no data.

Name species	Country	Site	Coordinates	Number of individuals	GenBank accession number
Galba cubensis	Cuba	Contramaestre	20°30′08”N 76°26′40”W	2	KU870347, KU870348
		Trinidad (Río El Junco)	21°43′57”N 79°33′23”W	2	KU870343, KU870344
	Ecuador	Las Dos Puertas	01°56′01”S 79°34′38”W	2	KT461809, KT461817
		Yaguachi	2°5′34”S 79°42′30”W	1	KT461814
	Guadeloupe	Pinadière	16°19′28”N 61°21′54”W	2	KU870355, KU870356
	Peru	Moquegua	17°19′24”S 70°59′29”W	2	KU870349, KU870350
		Ocoña	16°25′17”S 73°06′56”W	2	KU870351, KU870352
		Río Lurín	12°06′07”S 76°47′17”W	2	KU870353, KU870354
	Puerto Rico	Canal Salinas	ND	2	KU870345, KU870346
	Venezuela	Fincas 4M Tucacas	10°46′N 68°24′W	2	KT781217, KT781218
		Hato Río de Agua	10°34′48”N 62°59′22”W	1	KT781205
		La Linda	10°05′24”N 67°47′25”W	1	KT781202
		San Mateo	10°12′41”N 67°24′52”W	2	KT781214, KT781215
Galba schirazensis	Colombia	Finca Jocum Bucaramanga	07°06′25”N 73°04′60”W	2	KY198253, KY198254
	Ecuador	Hacienda Cienaga	00°46′18”S 78°37′10”W	1	KT781301
		Huagrahuma	02°47′32”S 79°16′31”W	2	KT781302, KT781304
		Manto de la Novia	01°24′03”S 78°17′49”W	2	KT781305, KT781315
		Nono	00°03′25”S 78°34′15”W	2	KY198255, KY198256
	La Reunion Island	Ravine du Gol	21°14′26”S 55°25′07”E	3	KY198257, KY198258, KY198259
	Peru	La Joya de Arequipa	16°28′56”S 71°49′07”W	2	KY198250, KY198260
	USA	Louisiana Bedico	30°26′11”N 90°15′01”W	1	KT781332
	Venezuela	La Trampa	08°33′31”N 71°27′13”W	2	KY198251, KY198252
		Los Nevados	08°27′41”N 71°04′28”W	1	KT781320
		Sabana Alto	08°36′11”N 71°27′45”W	3	KT781322, KT781323, KT781324
Galba truncatula	Morocco	ND	ND	1	JN614387
	Peru	Moquegua	17°19′24”S 70°59′29”W	1	KU870357
	Venezuela	El Sapo	08°52′04”N 70°48′29”W	2	JN614382, JN614381
		Paso El Cóndor	08°50′14”N 70°49′49”W	1	JN614383

geographic scale. In Africa, Europe, and Asia, *G. truncatula* has been the name used by many authors for decades (see references in Seddon et al., 2015). However, most studies identified this species based on shell morphology, and might rather be dealing with the cryptic, invasive, and widely distributed species *G. schirazensis*. We have found *Galba* populations of mixed species composition in some localities, with individuals within one species being indistinguishable ecologically or morphologically from the other (authors' unpublished data). The multiplex PCR developed here would identify these cryptic species, avoiding misidentifications.

Large-scale studies based on accurate species identification are essential to deepen our understanding of not only the snail, but also the epidemiology of fasciolosis. Population genetics studies would build profitably on accurate species identifications such as provided by multiplex PCR, and could eventually help to reveal the geographic origins, routes of invasion, and the ecological factors that make some *Galba* species better invaders than others. The method proposed here could also be helpful to monitor snail populations once range shifts and expansions have occurred, helping to develop management strategies with respect to the emergence or re-emergence of fasciolosis. We are not yet able to design a multiplex PCR to include the remaining two cryptic species (*G. humilis* and *G. viator*) because specific primers amplifying microsatellites have yet not been described. However, future efforts will be directed toward extending this technique to identify *G. humilis* and *G. viator*, as well as *G. cubensis*, *G. schirazensis*, and *G. truncatula*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vetpar.2018.01.006.

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