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

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Identifying vectors of fasciolosis: a new molecular tool to simultaneously distinguish three cryptic *Galba* species

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Abstract

We developed a molecular tool that will allow us to simultaneously identify cryptic freshwater snail species (genus *Galba*) involved in fasciolosis transmission, a worldwide infectious disease among humans and livestock. We designed a multiplex PCR that uses specific primers to amplify microsatellites and differentiate three *Galba* species (*G. cubensis*, *G. schirazensis*, and *G. truncatula*) based on the size of species-specific alleles at microsatellite loci. We tested and validated the accuracy of this new molecular tool by comparing the multiplex PCR results with species identification based on sequences at mitochondrial and nuclear markers. This new method is accurate, inexpensive, simple, rapid, and adaptable to large sets of individuals. It will also be helpful to monitor the invasion of *Galba* species and therefore to develop managing strategies of species involved in the emergence or re-emergence of fasciolosis.

Introduction

Understanding and preventing infectious diseases requires a good knowledge of vectors 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 Lymnaeidae family (Correa et al., 2010; Hurtrez-Boussès et al., 2001), serve as intermediate hosts of *Fasciola*, especially the *Galba* genus. For example, *Galba truncatula* and *Galba cubensis* are well-known vectors of the disease (Bargues et al., 2007; Jabbour-Zahab et al., 1997). Whether *Galba schirazensis* could also be a vector remains controversial. A first study suggested that *G. schirazensis* does not transmit the parasite (Bargues et al., 2011), but more recent ones showed that it does (Caron et al., 2017; Dreyfuss et al., 2015).

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

Absence of reliable morphological traits has, however, 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 marked 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 demonstrate high selfing rates (Chapuis et al., 2007; Lounnas et al., 2017a, 2017b; Meunier et al., 2004). 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, we suggest a rapid and inexpensive molecular approach to identify cryptic *Galba* species based on multiplex PCR. Such an approach has already been successfully used to identify a variety of cryptic parasites and their vectors, for example *Anopheles* mosquitoes, tapeworms, and protozoans diluted within feces or blood (Bohórquez et al., 2015; Kengne et al., 2001; Sumbria et al., 2015). Among lymnaeid species, real-time PCR based on species-specific melting temperatures can be used to identify *G. truncatula*, *G. viator*, *Pseudosuccinea columella*, and *Lymnaea diaphana* (Duffy et al., 2009). Here, we focus on three widely distributed cryptic *Galba* species, *G. cubensis*, *G. schirazensis*, and *G. truncatula*. We describe a multiplex PCR method using specific primers to amplify microsatellite loci, distinguishing species based on allele size. We were unable to design a multiplex PCR distinguishing the other two cryptic species (*G. humilis* and *G. viator*), because specific primers amplifying microsatellites have yet not been described. We test the accuracy of this new molecular method by comparing its results with species identification based on sequences at mitochondrial and nuclear loci.

Material and methods

We based our multiplex PCR method on species-specific primers amplifying microsatellite loci already described for the three targeted cryptic species: *G. truncatula* (Trouvé et al., 2000), *G. cubensis* (Lounnas et al., 2017b), and *G. schirazensis* (Lounnas et al., 2017a). We designed 11 primer mixes, 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. We also tested a range of concentrations for each primer: 2, 6, 8, and 10 mM.

We initially developed our primer mixes and concentrations in one negative control and 11 known controls: 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 single species and pooled DNA from multiple species.

We amplified DNA 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. We also tested different temperature of annealing in the PCR amplification: 50, 52, and 54ºC. Finally, we retained the PCR program that resulted in the sharpest bands in agarose gels. It has an initial denaturation step at 95 ºC for 15 minutes; followed by 35 cycles of denaturation at 94 ºC for 30 seconds, annealing at 52 ºC for 90 seconds, and extension at 72 ºC for one minute; the final extension was at 60 ºC for

30 minutes. The amplification products were electrophoretically resolved after 2 hours at 100V in 5% agarose gels and stained with EZ-Vision (Amresco).

By observing the agarose gel, we evaluated each of the 11 primer mixes 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, we developed a primer mix and concentration that allowed an accurate identification of *G. cubensis*, *G. schirazensis*, and *G. truncatula* (Table 2) in both single-species (Fig. 1) and pooled samples.

We validated our newly-developed multiplex PCR method using DNA extracts of 49 additional *Galba* previously identified by Correa et al. (2011) and Lounnas et al. (2017a, 2017b) 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, 2017b).

Results

The multiplex PCR that we designed successfully differentiated the individuals of *G. cubensis*, *G. schirazensis*, and *G. truncatula* previously identified by sequencing DNA markers (Correa et al., 2011; Lounnas et al., 2017a, 2017b; Table 1). We identified the species through the size of amplified alleles: DNA bands of each species were accurately separated in the 5% agarose gel (Fig. S1). The loci used in the multiplex PCR have low to intermediate polymorphism preventing 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 the individuals of *G. schirazensis*. Even at this size (27 bp according to Lounnas et al., 2017a, 2017b), both species could be accurately identified. We did not find pairs of distinct DNA bands (putatively allelic) at any locus for any individual tested.

Discussion

We designed a multiplex PCR that successfully identifies three cryptic *Galba* species that are vectors of fasciolosis: *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 fascioliasis is widespread and genetics laboratories 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. Our 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 the works of Meunier et al. (2004) and Lounnas et al. (2017a, 2017b).

We expect the multiplex PCR approach proposed here to prove 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 sampling at a comprehensive geographic scale. In Africa, Europe, and Asia, *G. truncatula* has been cited by many authors for decades (see references in Seddon et al., 2015). However, most studies identified this species based on shell morphology, and might indeed be dealing with the cryptic, invasive, and widely distributed species *G. schirazensis*. We have found in some localities *Galba* populations of mixed species indistinguishable ecologically as well as morphologically (authors' unpublished data). The multiplex PCR developed here would permit to easily distinguish these cryptic species and to avoid 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-genetic studies might reveal their geographic origins, their routes of invasion, and the ecological factors that make some *Galba* species better invaders than others. The method proposed here could be also helpful to monitor populations once invaded and to develop management strategies with regard to the emergence or re-emergence of fasciolosis. Future efforts will be directed toward extending our techniques to identify *G. humilis* and *G. viator*, as well as *G. cubensis*, *G. schirazensis*, and *G. truncatula*.

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Tables

Table 1. *Galba* individuals (n = 11) used to design the multiplex PCR. These individuals have been analyzed and identified by Correa et al. (2011) based on nuclear (ITS1 and ITS2) and mitochondrial (CO1 and 18S) sequences. The GenBank accession numbers provided here are those of 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

350 **Table 2.** Microsatellite primers used in the multiplex PCR-detection of *Galba cubensis*
351 (Lounnas et al., 2017b), *Galba schirazensis* (Lounnas et al., 2017a), and *Galba*
352 *truncatula* (Trouvé et al., 2000). We provide the GenBank accession number of
353 primers, the size (in base pairs) of the PCR product (alleles) and the primer
354 concentration. The size of the locus of *G. truncatula* was retrieved from Chapuis et al.
355 (2007). (f): forward; (r) reverse.

356

Name species (Primer)	Sequence (5' – 3')	GenBank accession number	Size (bp)	Primer concentration (mM)
Galba cubensis (Lc34)	GTCACTACTGCTTGTCTCAGC (f) AAAAGACTTTAACCCTTACCACCC (r)	AF226985	179–200	2
Galba schirazensis (Ls23)	AARGACCCAGTGGGGAAG (f) TGGGGAAGGTTCAATTGTTT (r)	KT285822	227–232	8
Galba truncatula (Lt37)	GTCCAGTCTTTGTATGTC (f) GTTAAGTACCCAACTTCTTC (r)	KT324723	111–129	10

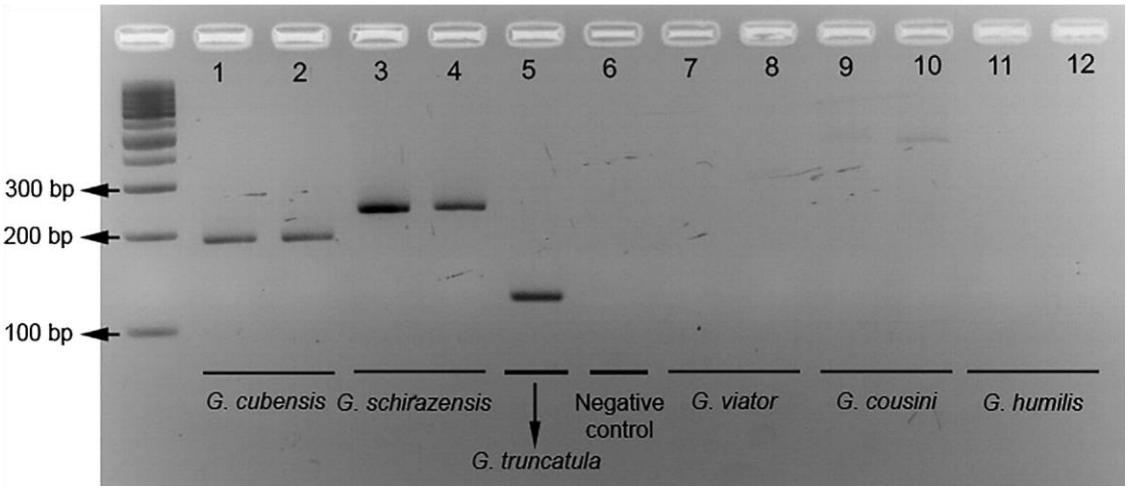
357 **Table 3.** *Galba* individuals (n = 49) used to test and validate the multiplex PCR. *Galba*
358 *cubensis* has been molecularly identified by Lounnas et al. (2017b), *G. schirazensis* by
359 Lounnas et al. (2017a), and *G. truncatula* from Peru by Lounnas et al. (2017b) and the
360 others by Correa et al. (2011). The GenBank accession numbers from Lounnas et al.
361 (2017b) are those ITS2 sequences (except for KT461809 that is, in fact, a CO1
362 sequence) and the ones from Correa et al. (2011) and Lounnas et al. (2017a) those of
363 CO1 sequences. ND: no data.
364

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

Figures captions

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



Supplementary Material

Table

Table S1. Microsatellite primer mixes tested in the 11 individuals of *Galba* species. Each mix contained a different combination of primers that were chosen such that the PCR products from the three species differ in size (range given in base pairs into parentheses). Primers have been already described by Trouvé et al. (2000) and Lounnas et al. (2017a, 2017b). The primer mix showed in row 10 is the one that allowed an accurate identification of *Galba cubensis*, *Galba schirazensis*, and *Galba truncatula*.

Mix	*Galba cubensis*	*Galba schirazensis*	*Galba truncatula*
1	Lc13 (191–200)	Ls23 (227–232)	Lt21 (104–116)
2	Lc19 (191–200)	Ls21 (239–279)	Lt21 (104–116)
3	Lc19 (191–200)	Ls22 (249–322)	Lt21 (104–116)
4	Lc19 (191–200)	Ls23 (227–232)	Lt21 (104–116)
5	Lc34 (179–200)	Ls07 (244)	Lt21 (104–116)
6	Lc34 (179–200)	Ls11 (216–235)	Lt21 (104–116)
7	Lc34 (179–200)	Ls21 (239–279)	Lt21 (104–116)
8	Lc34 (179–200)	Ls22 (249–322)	Lt21 (104–116)
9	Lc34 (179–200)	Ls23 (227–232)	Lt21 (104–116)
10	Lc34 (179–200)	Ls23 (227–232)	Lt37 (111–129)
11	Lc44 (179–200)	Ls23 (227–232)	Lt37 (111–129)

Figure legends

Figure S1. Diagram of an agarose gel electrophoresis showing the expected size of PCR products (white arrows) of *Galba* species identified by the multiplex PCR and the minimum range of separation between species (green arrows). The size of the loci was retrieved from Chapuis et al. (2007) and Lounnas et al. (2017a, 2017b).