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1 *Research paper - Veterinary Parasitology*

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

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46 **Key words:** Lymnaeidae, *Lymnaea*, *Fossaria*, multiplex PCR, microsatellites,
47 infectious disease.

48

49 **Abstract**

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

61 **Introduction**

62 Understanding and preventing infectious diseases requires a good knowledge of
63 vectors involved in parasite dynamics. Fasciolosis is a cosmopolitan disease causing
64 significant economic losses in domestic livestock (Rim et al., 1994). Human cases of
65 fasciolosis have been increasing throughout the world particularly in some regions of
66 South America, suggesting the possibility of re-emergence in this area (Esteban et al.,
67 1999; Mas-Coma et al., 2001). The parasites causing this disease are liver flukes
68 (*Fasciola* spp.). Freshwater mollusks, mainly belonging to the Lymnaeidae family
69 (Correa et al., 2010; Hurtrez-Boussès et al., 2001), serve as intermediate hosts of
70 *Fasciola*, especially the *Galba* genus. For example, *Galba truncatula* and *Galba*
71 *cubensis* are well-known vectors of the disease (Bargues et al., 2007; Jabbour-Zahab
72 et al., 1997). Whether *Galba schirazensis* could also be a vector remains controversial.
73 A first study suggested that *G. schirazensis* does not transmit the parasite (Bargues et
74 al., 2011), but more recent ones showed that it does (Caron et al., 2017; Dreyfuss et
75 al., 2015).

76
77 Populations of *Galba* are small-shelled freshwater snails mostly originating in the
78 Americas, subsequently invading Europe, Africa, and Asia (Bargues et al., 2011;
79 Correa et al., 2011, 2010; Lounnas et al., 2017a; Meunier et al., 2001). Their abilities to
80 survive drought and to reproduce by self-fertilization allow them to disperse over long
81 distances and establish new populations from single individuals (Bargues et al., 2011;
82 Lounnas et al., 2017a, 2017b, Meunier et al., 2004, 2001). This high invasiveness has
83 probably facilitated the worldwide expansion of fasciolosis.

84
85 Absence of reliable morphological traits has, however, led to confusion regarding
86 specific identities of *Galba* populations worldwide. Six species are considered valid:
87 *Galba cousini*, *Galba cubensis*, *Galba schirazensis*, *Galba truncatula*, *Galba humilis*,

88 and *Galba viator* (Correa et al., 2011). Of these six species only adults of *G. cousini*
89 display marked differences in shell morphology and internal anatomy (Paraense,
90 1995). The other five species cannot be accurately distinguished because their shell
91 morphology exhibits greater intraspecific than interspecific variability, and their
92 anatomy is homogeneous (Correa et al., 2011; Pointier, 2015; Samadi et al., 2000).
93 Nor can they be distinguished with controlled crosses in the laboratory, as is possible
94 in populations of the genus *Physa* (Dillon et al., 2011), because *Galba* populations
95 typically demonstrate high selfing rates (Chapuis et al., 2007; Lounnas et al., 2017a,
96 2017b; Meunier et al., 2004). Thus, except for *G. cousini* adults with their distinct
97 morphology, individuals of *Galba* have in recent years been identified using DNA
98 sequencing technology (Correa et al., 2011).

99

100 Amplifying and sequencing diagnostic loci can be time-consuming and expensive when
101 sample sizes are large. Here, we suggest a rapid and inexpensive molecular approach
102 to identify cryptic *Galba* species based on multiplex PCR. Such an approach has
103 already been successfully used to identify a variety of cryptic parasites and their
104 vectors, for example *Anopheles* mosquitoes, tapeworms, and protozoans diluted within
105 feces or blood (Bohórquez et al., 2015; Kengne et al., 2001; Sumbria et al., 2015).
106 Among lymnaeid species, real-time PCR based on species-specific melting
107 temperatures can be used to identify *G. truncatula*, *G. viator*, *Pseudosuccinea*
108 *columella*, and *Lymnaea diaphana* (Duffy et al., 2009). Here, we focus on three widely
109 distributed cryptic *Galba* species, *G. cubensis*, *G. schirazensis*, and *G. truncatula*. We
110 describe a multiplex PCR method using specific primers to amplify microsatellite loci,
111 distinguishing species based on allele size. We were unable to design a multiplex PCR
112 distinguishing the other two cryptic species (*G. humilis* and *G. viator*), because specific
113 primers amplifying microsatellites have yet not been described. We test the accuracy of
114 this new molecular method by comparing its results with species identification based
115 on sequences at mitochondrial and nuclear loci.

116

117 **Material and methods**

118 We based our multiplex PCR method on species-specific primers amplifying
119 microsatellite loci already described for the three targeted cryptic species: *G. truncatula*
120 (Trouvé et al., 2000), *G. cubensis* (Lounnas et al., 2017b), and *G. schirazensis*
121 (Lounnas et al., 2017a). We designed 11 primer mixes, each including one species-
122 specific primer pair for each of the three species (Table S1). Each mix contained a
123 different combination of primers chosen such that the PCR products from the three
124 species differed in size. We also tested a range of concentrations for each primer: 2, 6,
125 8, and 10 mM.

126

127 We initially developed our primer mixes and concentrations in one negative control and
128 11 known controls: two *G. cubensis*, two *G. schirazensis*, one *G. truncatula*, two *G.*
129 *cousini*, two *G. humilis*, and two *G. viator*, all identified by Correa et al. (2011) on the
130 basis of ITS2, ITS1, CO1, and 18S sequences (Table 1). Even though *G. cousini* is
131 easily distinguishable using shell morphology and reproductive anatomy (Paraense,
132 1995), it was included in the development phase to evaluate the specificity of the
133 multiplex PCR. Each candidate multiplex was tested using samples containing DNA
134 from single species and pooled DNA from multiple species.

135

136 We amplified DNA in a total volume of 10 µl containing 5 µl of Taq PCR Master Mix Kit
137 (Qiagen), 1 µl of the primer mix, and 50–100 ng of DNA in an Eppendorf Thermal
138 Cycler. We also tested different temperature of annealing in the PCR amplification: 50,
139 52, and 54°C. Finally, we retained the PCR program that resulted in the sharpest
140 bands in agarose gels. It has an initial denaturation step at 95 °C for 15 minutes;
141 followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 52 °C for
142 90 seconds, and extension at 72 °C for one minute; the final extension was at 60 °C for

143 30 minutes. The amplification products were electrophoretically resolved after 2 hours
144 at 100V in 5% agarose gels and stained with EZ-Vision (Amresco).

145

146 By observing the agarose gel, we evaluated each of the 11 primer mixes by three
147 criteria: (1) each primer pair was required to amplify a single locus in the targeted
148 species, (2) primers were not expected to yield PCR amplification in *G. cousini*, *G.*
149 *humilis*, and *G. viator*, and (3) the PCR products from *G. cubensis*, *G. schirazensis*,
150 and *G. truncatula* were required to differ noticeably in size. Ultimately, we developed a
151 primer mix and concentration that allowed an accurate identification of *G. cubensis*, *G.*
152 *schirazensis*, and *G. truncatula* (Table 2) in both single-species (Fig. 1) and pooled
153 samples.

154

155 We validated our newly-developed multiplex PCR method using DNA extracts of 49
156 additional *Galba* previously identified by Correa et al. (2011) and Lounnas et al.
157 (2017a, 2017b) based on DNA sequences: 23 *G. cubensis*, 22 *G. schirazensis*, and 5
158 *G. truncatula* (Table 3). These individuals were removed from sites geographically
159 distant from the sites in which the initial 11 individuals used to develop the method
160 were sampled. DNA-extraction protocols of the individuals here studied are described
161 in Correa et al. (2011) and Lounnas et al. (2017a, 2017b).

162

163 **Results**

164 The multiplex PCR that we designed successfully differentiated the individuals of *G.*
165 *cubensis*, *G. schirazensis*, and *G. truncatula* previously identified by sequencing DNA
166 markers (Correa et al., 2011; Lounnas et al., 2017a, 2017b; Table 1). We identified the
167 species through the size of amplified alleles: DNA bands of each species were
168 accurately separated in the 5% agarose gel (Fig. S1). The loci used in the multiplex
169 PCR have low to intermediate polymorphism preventing overlapping bands among the

170 species on agarose gels (Table 2; Fig. S1). The smallest difference in the size of
171 amplified alleles was observed between some individuals of *G. cubensis* from
172 Venezuela and Guadeloupe and the individuals of *G. schirazensis*. Even at this size
173 (27 bp according to Lounnas et al., 2017a, 2017b), both species could be accurately
174 identified. We did not find pairs of distinct DNA bands (putatively allelic) at any locus for
175 any individual tested.

176

177 **Discussion**

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

196

197 We expect the multiplex PCR approach proposed here to prove useful as a first
198 approach for the study of *Galba* populations in unexplored and poorly-known regions of
199 the world. *Galba* populations from the south of Brazil have been studied on the basis of
200 morphology only, for example, and species could have been misidentified (Medeiros et
201 al., 2014). Similarly, in North America authors recognize as many as 22 “*Fossaria*”
202 species on the basis of shell and radula morphology (Burch, 1982; Johnson et al.,
203 2013), most of which are indistinguishable from cryptic *Galba* species worldwide.
204 Hubendick (1951) considered almost all of the nominal North American species to be
205 synonyms of *G. humilis*, but resolution awaits molecular analysis, and sampling at a
206 comprehensive geographic scale. In Africa, Europe, and Asia, *G. truncatula* has been
207 cited by many authors for decades (see references in Seddon et al., 2015). However,
208 most studies identified this species based on shell morphology, and might indeed be
209 dealing with the cryptic, invasive, and widely distributed species *G. schirazensis*. We
210 have found in some localities *Galba* populations of mixed species indistinguishable
211 ecologically as well as morphologically (authors’ unpublished data). The multiplex PCR
212 developed here would permit to easily distinguish these cryptic species and to avoid
213 misidentifications.

214

215 Large-scale studies based on accurate species identification are essential to deepen
216 our understanding of not only the snail, but also the epidemiology of fasciolosis.
217 Population-genetic studies might reveal their geographic origins, their routes of
218 invasion, and the ecological factors that make some *Galba* species better invaders
219 than others. The method proposed here could be also helpful to monitor populations
220 once invaded and to develop management strategies with regard to the emergence or
221 re-emergence of fasciolosis. Future efforts will be directed toward extending our
222 techniques to identify *G. humilis* and *G. viator*, as well as *G. cubensis*, *G. schirazensis*,
223 and *G. truncatula*.

224

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340

341 **Tables**

342 **Table 1.** *Galba* individuals (n = 11) used to design the multiplex PCR. These
 343 individuals have been analyzed and identified by Correa et al. (2011) based on nuclear
 344 (ITS1 and ITS2) and mitochondrial (CO1 and 18S) sequences. The GenBank
 345 accession numbers provided here are those of the CO1 sequences. Some coordinates
 346 were corrected in order to match the specific locality: Frias (Argentina) and Owego
 347 (New York, USA).

348

Species	Country	Site	Coordinates	Number of individuals	Genbank accession number
<i>Galba cubensis</i>	USA	Charleston County (South Carolina)	32°45'59"N 79°49'35"W	2	JN614395, JN614394
<i>Galba schirazensis</i>	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
<i>Galba truncatula</i>	France	Limousin region	45°47'05"N 01°11'36"E	1	JN614386
<i>Galba viator</i>	Argentina	Frias	40°14'10"S 64°10'09"W	2	JN614397, JN614398
<i>Galba cousini</i>	Venezuela	Mucubají	08°47'54"N 70°49'33"W	2	JN614389, JN614388
<i>Galba humilis</i>	USA	Owego (New York)	42°06'01"N 76°15'04"W	2	FN182197, FN182198

349

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)
<i>Galba cubensis</i> (Lc34)	GTCACTACTGCTTGTCTCAGC (f) AAAAGACTTTAACCCCTTACCACC C (r)	AF226985	179–200	2
<i>Galba schirazensis</i> (Ls23)	AARGACCCAGTGGGGAAG (f) TGGGGAAGGTTCAATTGTTT (r)	KT285822	227–232	8
<i>Galba truncatula</i> (Lt37)	GTCCAGTCTTTGTATGTC (f) GTTAAGTACCCAACCTTCTTC (r)	KT324723	111–129	10

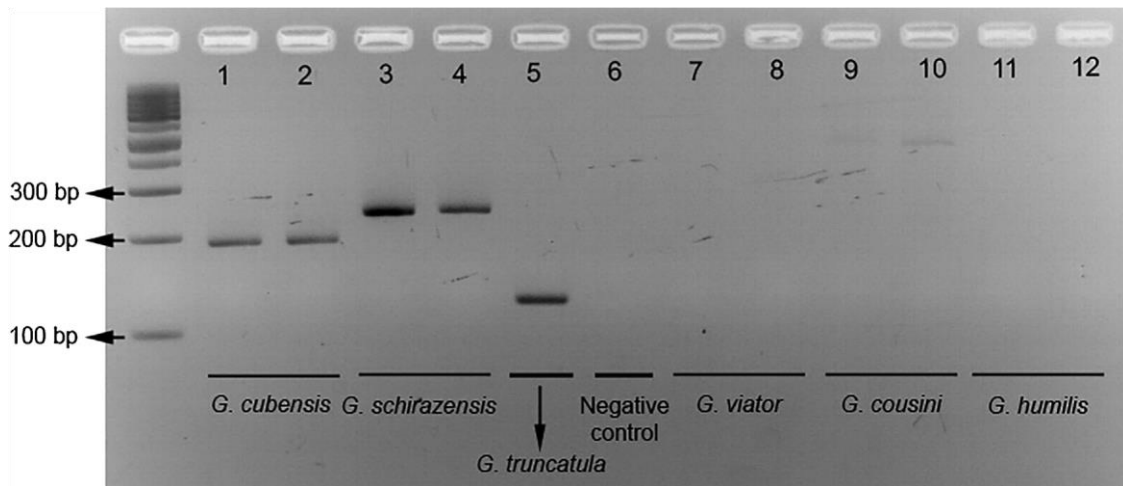
357 **Table 3.** *Galba* individuals (n = 49) used to test and validate the multiplex PCR. *Galba*
358 *cubensis* has been molecularly identified by Lounnas et al. (2017b), *G. schirazensis* by
359 Lounnas et al. (2017a), and *G. truncatula* from Peru by Lounnas et al. (2017b) and the
360 others by Correa 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
<i>Galba cubensis</i>	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
<i>Galba schirazensis</i>	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
<i>Galba truncatula</i>	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

365 **Figures captions**

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



370

371

372 **Supplementary Material**

373 **Table**

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

Mix	<i>Galba cubensis</i>	<i>Galba schirazensis</i>	<i>Galba truncatula</i>
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)

381

382 **Figure legends**

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