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Isolation, characterization and population-genetic analysis of microsatellite loci in the freshwater snail *Galba cubensis* (Lymnaeidae)

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ABSTRACT

The freshwater snail *Galba cubensis* (Pfeiffer, 1839) has a large distribution in the Americas. Despite being an intermediate host of *Fasciola hepatica*—the trematode causing fasciolosis in livestock and humans—its population genetics have never been studied. We isolated and characterized 15 microsatellite loci in *G. cubensis* to evaluate its genetic diversity, population-genetic structure and mating system. We tested the microsatellite loci in 359 individuals from 13 populations of *G. cubensis* from Cuba, Guadeloupe, Martinique, Puerto Rico, Venezuela, Colombia and Ecuador. We also tested cross-amplification in three closely related species: *G. truncatula*, *G. viator* and *G. neotropica*. We found that *G. cubensis* has a similar population structure to other selfing lymnaeids that live in temporary habitats: low genetic diversity, large departure from Hardy-Weinberg equilibrium, marked population structure and high selfing rate. We found that seven and six loci amplified in *G. truncatula* and *G. viator*, respectively, and that all 15 loci amplified in *G. neotropica*. This last finding suggests a close relatedness between *G. cubensis* and *G. neotropica*, probably being conspecific and synonymous. This new set of microsatellite markers will be a useful tool to study the genetic diversity of this snail species across a large geographical range and, consequently, to understand the emergence and re-emergence of fasciolosis in the Americas.

INTRODUCTION

Lymnaeids (Basommatophora) act as an intermediate host of a great diversity of trematodes (Plathyhelminthes) (Dawes, 1968). One of the most striking cases is that of the liver fluke *Fasciola hepatica*, a cosmopolitan parasite causing severe veterinary and public health problems (Cotruvo *et al.*, 2004), which can be transmitted by half of the lymnaeid species (Correa *et al.*, 2010). Understanding the population genetics of intermediate hosts can help to elucidate parasite transmission dynamics and to develop appropriate control methods (Standley *et al.*, 2014). For this purpose, the population genetics of a number of lymnaeid species that act as intermediate hosts of *F. hepatica* has been studied, such as *Galba truncatula* (O.F. Müller, 1774) (Trouvé *et al.*, 2000, 2003; Meunier *et al.*, 2001, 2004a, b), *Omphiscola glabra* (Hurtrez-Boussès *et al.*, 2005) and *Pseudosuccinea columella* (Nicot *et al.*, 2008). These studies have found that these hermaphroditic lymnaeid species reproduce mainly by self-fertilization and, hence, show little genetic variability within populations. These self-fertilizing populations should be more vulnerable to infection by *F. hepatica*, because genetically homogenous host populations are more vulnerable to infection than genetically diverse populations (King & Lively, 2012). Although *Galba cubensis* (Pfeiffer, 1839) transmits fasciolosis (Rojas *et al.*, 2010; Vázquez *et al.*, 2014; Pointier *et al.*, 2015) and has been recorded in many countries in the Americas (Burch, 1982; Malek, 1985; Correa *et al.*, 2011), its population genetics have not been studied so far.

The aim of this study is to provide a first characterization of the population genetics of *G. cubensis*. Therefore, we isolated and characterized microsatellite loci in *G. cubensis* and used them to study genetic diversity and population structure in 13 populations from Cuba to Ecuador. In addition, we tested cross-amplification in three closely related species that are morphologically indistinguishable from *G. cubensis* (Correa *et al.*, 2010, 2011): *G. truncatula*, *G. viator* (d'Orbigny, 1835) and *G. neotropica* (Bargues *et al.*, 2007).

MATERIAL AND METHODS

Isolation and characterization of microsatellite loci

We selected nine adult snails of *Galba cubensis* from three sites (three snails per site): Grande Ravine in Guadeloupe (16°23'10.3″N, 61°28'40.2″W), Alquizar in Cuba (22°43'52.3″N, 82°39'33.5″W) and La Habana in Cuba (23°1'12.7″N, 82°21'15.8″W). We chose these sites because *G. cubensis* was originally described from Cuba (Pfeiffer, 1839) and is easily distinguishable from the other lymnaeid that inhabits Cuba and Guadeloupe, *Pseudosuccinea columella* (Pointier, 2008; Vázquez *et al.*, 2014). We pooled the samples and extracted DNA from the distal part of the foot using the DNeasy Blood and Tissue Kit (Qiagen).

Microsatellite libraries were performed and analysed by the Geno Sat method (GenoScreen) using very high speed sequencing (GS-FLX®, Roche Diagnostic) and following protocols described by Malausa *et al.* (2011). We fragmented the DNA and enriched it with eight oligoprobes (TG, TC, AAC, AAG, AGG, ACG, ACAT, ACTC). The enriched DNA was then amplified by PCR with high-fidelity Taq polymerase. The amplification products served to construct GS-FLX libraries that were sequenced according to the GS-FLX protocol. We obtained 148,434 contigs, representing 39 Mb of sequence data. We identified 9,317 sequences containing microsatellite motifs. Among them, 308 presented suitable flanking regions for primer design. We identified 48 loci for preliminary PCR screening and of these we selected 15 that showed polymorphism and correct amplification in gel electrophoresis (Table 1).

Diversity at microsatellite loci

We used the 15 microsatellite loci to assess genetic diversity of 359 individuals from 13 populations of *G. cubensis* from Cuba (3 populations), Guadeloupe (2), Martinique (1), Puerto Rico (1), Venezuela (3), Colombia (1) and Ecuador (2; Table 2). We collected snails from small areas (<2 m^2) in order to prevent Wahlund effects and stored them in ethanol 80% until molecular analyses.

We took *c.* 2 mm^3 of snail tissue from the distal part of the foot and used it for DNA isolation. We pressed the tissue twice between paper towels in order to remove ethanol. Extractions were performed using 200 µl of 5% Chelex® (Chelex Bio Rad diluted in a Tris-EDTA buffer) solution incorporating 5 µl of proteinase K (Sigma) at a concentration of 20 mg/ml. This suspension was heated at 56 °C for 6 h, then gently vortexed and finally incubated at 95 °C for 10 min. The mixture was gently vortexed and centrifuged at 10,000×*g* for 10 s. The supernatant (100 µl) was taken, diluted 1:10 in deionized water and stored at −20 °C.

Since morphology of lymnaeids is insufficient for the accurate identification of species (Correa *et al.*, 2011), we identified *G. cubensis* by sequencing the gene internal transcribed spacer 2 (ITS-2) (Bargues *et al.*, 2007; Correa *et al.*, 2011). First, we amplified ITS-2 from one or two individuals taken at random from each sampling site, according to protocols described by Correa *et al.* (2011) with primers Forward News (5'-TGTGTCGATGAAGAACGCAG-3') and Reverse Rixo 2 (5'-TTCTATGCTTAAATTCAGGGG-3'). We then blasted the sequences obtained against sequences of *G. cubensis* available in GenBank: AM412223, JF514088 and JN614461

Table 1. Characteristics of microsatellite loci isolated from *Galba cubensis*.

Locus	GenBank acc. no.	Primer sequence (5'–3')	Dye label used for F	Repeat motif
Gcu_1	KT285812	F: TATATGGGAAAAGCAGGCCC	ATTO 550	AC
		R: AAGAGCATTAAGACTTGCTTAGACTAT		
Gcu_5	KT285813	F: GCATTTCATCTTCTGAACTCCC	ATTO 565	TG
		R: CAACAACCACACTCACTTCAA		
Gcu_13	KT285814	F: GTCTGTCACACAGTTTCATATTCTCA	ATTO 565	CA
		R: CAACGAATATTGAAATGGCCT		
Gcu_19	KT285815	F: ACAGAAAGTCAGTGAGAAAAAGACTG	ATTO 550	AG
		R: TCAGTTTTGACCTGCACCCT		
Gcu_21	KT285816	F: CGGACATTTTCTATGTGCTCG	ATTO 565	AAC
		R: CACAGCAATTCACTAATTTAAGACA		
Gcu_27	KT285819	F: TTTTCCCTTTGGAACCATGT	FAM	TC
		R: AGATATGACGTCAGTGTGACAGA		
Gcu_30	KT285820	F: AGAGGCAAGGGAGAAAGGAG	ATTO 550	AGTG
		R: CTCAATCACTCTCAAACTCACTCA		
Gcu_31	KT285821	F: CTTGTGGGCTCAGTGTTTGA	HEX	TG
		R: GGCTGCAAGATGTGTTGAGA		
Gcu_34	KT285822	F: AAAAGACTTTAACCCTTACCACCC	HEX	AC
		R: GTCACTACTGCTTGTCTCAGCA		
Gcu_35	KU865177	F: GTCAGTGTGACAGATACCACGAA	FAM	AG
		R: CCAAGAGCTTTGCTACAGACA		
Gcu_37	KT285823	F: GATTCGAGCTGAGGCAGAG	HEX	GA
		R: TTGTGCTATGTCTCTAAAAGGTAGTGA		
Gcu_39	KT285824	F: GTTTGTCTTGTATTTACCTGATTCTTT	ATTO 550	TG
		R: ACCTTCAGGTGTATTGATTGAAA		
Gcu_40	KT285825	F: AGGATACGGGCGATTTGAA	ATTO 565	AC
		R: TTATCACAAGTCATCATGAATCAA		
Gcu_41	KT285826	F: ACCGACCTATAAACAAACAGAAGA	HEX	AG
		R: TGGGCAAATTCCCATATTACA		
Gcu_44	KT285827	F: CAACTTTGGGACATGTTGGA	FAM	AC
		R: TGGACCTTTGTTGTTATCTTGG		

Annealing temperature for the 15 microsatellite loci was 55 °C.

Abbreviations: F, forward primer; R, reverse primer.

Table 2. Populations of *Galba* species tested for microsatellite loci of *Galba cubensis*.

Species	Country	Site	Coordinates	Date	Sample size	GenBank acc. no.
G. cubensis	Cuba	Bahía Honda	22°54'9.4″N 83°10'15.9″W	26 February 2003	23	ND
		Contramaestre	20°30'08″N 76°26'40″W	19 February 2003	17	KU870347; KU870348
		Trinidad (Río El Junco)	21°43'57″N 79°33'23″W	15 May 2002	32	KU870343; KU870344
	Guadeloupe (France)	Fond Barboteaux	16°10'04″N 61°39'11″W	14 December 2003	27	ND
		Pinadière	16°19'27.9″N 61°21'53.7″W	4 February 2014	21	KU870355; KU870356
	Martinique (France)	Ravine Mansarde au Robert	14°40'29″N 60°56'26″W	14 April 2011	43	ND
	Puerto Rico	Canal Salinas	ND	1 October 1998	9	KU870345; KU870346
	Venezuela	Hato Río de Agua	10°34'48.2″N 62°59'21.6″W	21 November 2006	10	KT781205
		Mesa de Esnujaque	9°2'18.9″N 70°42'58.1″W	19 October 2005	31	ND
		San Mateo	10°12'41.4″N 67°24'51.8″W	26 October 2010	10	KT781214; KT781215
	Colombia	El Progreso	06°12'01″N 75°35'06″W	7 September 2009	48	ND
	Ecuador	Las Dos Puertas	1°56'1.1″S 79°34'38.1″W	16 October 2014	44	KT461809; KT461817
		Yaguachi	2°5'34.4″S 79°42'29.8″W	15 October 2014	44	KT461814
G. truncatula	Peru	Moquegua	17°19'24.3″S 70°59'29″W	15 October 2012	3	KU870357
G. viator	Argentina	Frias	40°44'07″S 66°37'27″W	1 February 2004	3	KT781170
G. neotropica	Peru	Moquegua	17°19'24.3″S 70°59'29″W	15 October 2012	42	KU870349; KU870350
		Ocoña	16°25'16.8″S 73°6'55.8″W	13 October 2012	12	KU870351; KU870352
		Río Lurín	12°6'7.1″S 76°47'17.3″W	7 October 2012	51	KU870353; KU870354
	Venezuela	La Linda	10°5'24.1″N 67°47'25.4″W	18 November 2006	9	KT781202
		Fincas 4M Tucacas	10°46'N 68°24'W	19 September 2001	10	KT781217; KT781218

Some individuals were identified by sequencing ITS-2; GenBank accession numbers are provided.
ND, no data.

(Bargues *et al.*, 2007, 2011; Correa *et al.*, 2011). All the sequences showed 99–100% homology. Finally, we uploaded the sequences obtained to GenBank (see Table 2 for accession numbers). Because of insufficient DNA extracted, we were unable to sequence ITS-2 for individuals from some sites: El Progreso (Colombia), Bahía Honda (Cuba), Fond Barboteaux (Guadeloupe), Ravine Mansarde au Robert (Martinique) and Mesa de Esnujaque (Venezuela). However, the individuals from Cuba, Martinique and Guadeloupe were easily distinguishable from the other lymnaeid that inhabits these regions (Pointier, 2008; Vázquez *et al.*, 2014). The individuals from Colombia and Venezuela (Mesa de Esnujaque) were identified based on the morphology of the adult reproductive system (Pointier *et al.*, 2015). We confirmed the species identification in these populations by the correct amplification of all the microsatellite loci of *G. cubensis*.

Amplification of microsatellite loci was performed in 10 µl final volumes including 2 µl buffer 5× (Promega), 1 µl $MgCl_2$ 25 mM (Promega), 0.5 µl dNTPs 2.5 mM (Life Technologies), 0.2 µl of each primer 10 mM and 0.2 µl of Taq DNA polymerase (Promega). PCR conditions were as follows: 10 min of activation at 95 °C, 35 cycles including 30 s of initial denaturation at 95 °C, 30 s of annealing at 55 °C and 60 s of extension at 72 °C, followed by 10 min of final extension at 72 °C. For genotyping, we pooled 3 µl of diluted (1:100) PCR products with 15 µl of Hi-Di Formamide and 0.2 µl of GeneScan-500 LIZ Size Standard and analysed it on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). We performed multiplexing loci amplification for those PCR products characterized by different sizes and labelled with different fluorochromes. Allele sizes were estimated using GeneMapper® v. 4.0 software (Applied Biosystems).

Cross-species amplification of microsatellite loci

We tested amplification and analysed allelic polymorphism of the 15 isolated microsatellite loci of *G. cubensis* in three closely related

species that are morphologically indistinguishable from it (Correa *et al.*, 2010, 2011): *G. truncatula* from Moquegua (Peru), *G. viator* from Frias (Argentina) and *G. neotropica* from Río Lurín (Peru, the type locality from which the species was first described by Bargues *et al.*, 2007, 2011; Table 2). We tested the microsatellite loci in three individuals from each species. Since all amplifications were successful in the three individuals of *G. neotropica*, we analysed 121 additional individuals from three localities in Peru (including 48 additional individuals from Río Lurín) and two localities from Venezuela (Table 2).

Table 3. Allelic diversity in *Galba* species using microsatellite loci of *G. cubensis*.

Locus	Number of alleles			
	G. cubensis *N* = 271	*G. neotropica* *N* = 124	*G. truncatula* *N* = 3	*G. viator* *N* = 3
Gcu_1	1 (232)	2 (232; 282)	1 (221)	2 (221; 223)
Gcu_5	2 (291; 293)	2 (291; 293)	2 (257; 291)	NA
Gcu_13	2 (191; 193)	3 (193–200)	1 (190)	NA
Gcu_19	4 (142–150)	4 (144–172)	NA	NA
Gcu_21	6 (102–144)	6 (100–128)	NA	NA
Gcu_27	3 (125–135)	4 (125–145)	NA	NA
Gcu_30	9 (135–173)	4 (144–165)	NA	NA
Gcu_31	2 (131; 133)	2 (130; 133)	NA	1 (124)
Gcu_34	5 (183–197)	5 (179–200)	1 (181)	NA
Gcu_35	9 (108–125)	4 (121–127)	1 (113)	3 (104–125)
Gcu_37	4 (237–243)	3 (237–254)	1 (214)	3 (229–241)
Gcu_39	3 (118–124)	4 (114–122)	1 (104)	2 (106; 110)
Gcu_40	6 (124–142)	5 (124–182)	NA	NA
Gcu_41	7 (108–120)	4 (114–122)	NA	NA
Gcu_44	2 (188; 190)	4 (180–192)	NA	3 (175–182)

Allele size in basepairs is indicated in parentheses, with the range when more than two alleles were found.

Abbreviations: *N*, sample size; NA, no amplification.

Again, we identified one or two individuals from each species and locality by sequencing ITS-2 as described above and blasting the sequences against those already available in GenBank for *G. truncatula* (AJ296271; Bargues *et al.*, 2001), *G. viator* (AM412224; Bargues *et al.*, 2007) and *G. neotropica* (AM412225; Bargues *et al.*, 2007). For each species, the sequences showed 99–100% homology. The sequences were uploaded to GenBank (see Table 2 for accession numbers).

Population genetics of *G. cubensis* and *G. neotropica*

We used individual genotypes to estimate current parameters of population genetics in *G. cubensis* and *G. neotropica*: number of alleles, observed and expected heterozygosities (H_O and H_E), the inbreeding coefficient *F*-statistic F_{IS}, and selfing rate (s) as $s = 2F_{IS}/(1 + F_{IS})$ (Hartl & Clark, 1997). We also used the *F*-statistic F_{ST} to estimate differentiation between populations. Values are given as mean ± 1SD. We analysed data using FSTAT v. 2.9.3.2 (Goudet, 2001). Standard Bonferroni corrections were applied in the case of multiple tests.

RESULTS

We isolated and characterized 15 polymorphic microsatellite loci in *Galba cubensis*. In the 359 individuals, 14 showed polymorphism whereas locus Gcu_1 harboured a single allele (Table 3). The average number of alleles per locus was 4.33 ± 2.58 and ranged from 1 to 10 (Table 3). We obtained amplification at seven loci for samples of *G. truncatula*; one was polymorphic and none was in the allele size range of *G. cubensis*. We also obtained amplification at six loci for samples of *G. viator*; five were polymorphic and two were in the allele size range of *G. cubensis* (Table 3). Notably, we obtained amplification for the 15 loci for the 124 individuals of *G. neotropica*—all being polymorphic (including the locus Gcu_1, the one monomorphic in the 359 individuals of *G. cubensis*). We also found that all these loci shared one or three alleles with *G. cubensis* (Table 3).

Most populations of *G. cubensis* and *G. neotropica* showed low allelic diversity and very low H_O and H_E (mean $H_O = 0.001 \pm 0.001$; mean $H_E = 0.066 \pm 0.008$; Table 4). The highest allelic diversity

Table 4. Population-genetic parameters of *Galba cubensis* and *G. neotropica*.

Species	Country	Site	*N*	Mean number of alleles	H_O	H_E	F_{IS} (*P*-value)	*s*
G. cubensis	Cuba	Bahía Honda	23	1.07	0	0.013	1 (0.003)	1
		Contramaestre	17	1.73	0.008	0.117	**0.931 (<0.001)**	0.964
		Trinidad (Río El Junco)	32	2.6	0.005	0.455	**0.990 (<0.001)**	0.995
	Guadeloupe (France)	Fond Barboteaux	27	1.07	0.002	0.002	0 (1)	0
		Pinadière	21	1	0	0	–	–
	Martinique (France)	Ravine Mansarde au Robert	43	1	0	0	–	–
	Puerto Rico	Canal Salinas	9	1	0	0	–	–
	Venezuela	Hato Río de Agua	10	1.27	0	0.086	**1 (<0.001)**	1
		Mesa de Esnujaque	31	1.13	0	0.026	**1 (<0.001)**	1
		San Mateo	10	1.27	0	0.081	**1 (<0.001)**	1
	Colombia	El Progreso	48	1	0	0	–	–
	Ecuador	Las Dos Puertas	44	1	0	0	–	–
		Yaguachi	44	1	0	0	–	–
G. neotropica	Peru	Moquegua	42	1.07	0	0.003	1 (0.013)	1
		Ocoña	12	1.27	0	0.101	**1 (<0.001)**	1
		Río Lurín	51	1.6	0.007	0.035	**0.813 (<0.001)**	0.897
	Venezuela	La Linda	9	1.07	0	0.017	1 (0.067)	1
		Fincas 4M Tucacas	10	1.85	0	0.317	**1 (<0.001)**	1

In bold are the populations that significantly deviate from Hardy-Weinberg equilibrium following Bonferroni's adjustment ($P = 0.00033$).

Abbreviations: *N*, sample size; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient; *s*, estimated selfing rate.

(2.6) was observed in a population from Cuba (Trinidad, Río El Junco; Table 4). Most populations deviated from Hardy-Weinberg equilibrium (Table 4). The mean F_{IS} was high (0.894 ± 0.287; Table 4) and, consequently, the *s* was also high (0.888 ± 0.333; Table 4). The lack of diversity prevented inference of population structure for the populations of *G. cubensis* from Pinadière (Guadeloupe), Ravine Mansarde au Robert (Martinique), Canal Salinas (Puerto Rico), El Progreso (Colombia), Las Dos Puertas (Ecuador) and Yaguachi (Ecuador; Table 4).

We observed a significant difference among populations (total $F_{ST} = 0.882$; $P < 0.001$) after Bonferroni's adjustment ($P = 0.0003$). All the pairs of populations showed significant F_{ST} (Supplementary Material Table S1) except for Ravine Mansarde au Robert (Martinique) and Fond Barboteaux (Guadeloupe; $F_{ST} = 0.009$, $P = 0.387$), Yaguachi and Las Dos Puertas (Ecuador; $F_{ST} = 0$, $P = 1$), and Moquegua and Río Lurín (Peru; $F_{ST} = 0.067$, $P = 0.006$).

DISCUSSION

We developed 15 polymorphic microsatellite loci in *Galba cubensis*. The cross-amplification tests showed that seven of the 15 loci amplified in *G. truncatula*—with different allele sizes than in *G. cubensis*—and that six of the 15 amplified in *G. viator*—sharing some allele sizes with *G. cubensis*. These findings are consistent with previous results that showed that *G. truncatula*, *G. cubensis* and *G. viator* belong to a monophyletic group of small lymnaeids (Correa *et al.*, 2010, 2011). We found that the five polymorphic loci that amplified in *G. viator* (Table 3) could be used to assess genetic variability in this species.

Because the 15 loci designed for *G. cubensis* cross-amplified in *G. neotropica* and the allele size ranges overlap with those observed in *G. cubensis* (Table 3), we suggest that—although we cannot exclude homoplasy—both species are closely related. Phylogenetic analyses have shown that these species are two closely related but distinct clades (Bargues *et al.*, 2007; Correa *et al.*, 2010, 2011). Based on the similarity between *G. cubensis* and *G. neotropica* in conserved markers (ITS-1, ITS-2, 18S, COI; Correa *et al.* 2010, 2011), as well as in highly polymorphic markers (microsatellite loci; this study), we suggest these two taxa could in fact be conspecific, rather than being considered as distinct entities.

The population-genetic analysis of the polymorphic microsatellite loci in 18 populations of *G. cubensis* and *G. neotropica* from the Caribbean and northern South America showed that these species have low genetic diversity, large departures from Hardy-Weinberg equilibrium, high estimated selfing rates and marked population structure.

Our results are in line with those observed in other studies on lymnaeid snails such as *G. truncatula* (Trouvé *et al.*, 2000; Meunier *et al.*, 2001, 2004a, b; Hurtrez-Boussès *et al.*, 2010), *Omphiscola glabra* (Hurtrez-Boussès *et al.*, 2005) and *Pseudosuccinea columella* (Nicot *et al.*, 2008). The low H_O found in populations of these hermaphroditic snails is most probably due to high selfing rates. Indeed, Meunier *et al.* (2004b) have experimentally demonstrated that selfing prevails in *G. truncatula*. Moreover, these species live in temporary habitats that experience frequent flooding and droughts, affecting their survival and creating strong bottlenecks (Meunier *et al.*, 2004a). A single individual can recolonize or colonize new habitats since selfing is the major reproductive mode (Meunier *et al.*, 2004b), promoting strong genetic drift that leads to low genetic variability.

Our results revealed high levels of total differentiation among populations. The lack of differentiation between Ravine Mansarde au Robert (Martinique) and Fond Barboteaux (Guadeloupe), Yaguachi and Las Dos Puertas (Ecuador) and Moquegua and Río Lurín (Peru) might be explained by recent events of introduction from a single source. Indeed, these pairs of populations are relatively close to each other (200, 25 and 800 km, respectively). Recent introductions could also explain the total lack of diversity in El Progreso (Colombia), Pinadière (Guadeloupe), Ravine Mansarde au Robert (Martinique), Canal Salinas (Puerto Rico), and Yaguachi and Las Dos Puertas (Ecuador), as has previously also been shown for populations of *G. truncatula* in the Bolivian Altiplano (Meunier *et al.*, 2001). Freshwater snails can travel for long distances attached to large mammals or migratory waterbirds (Van Leeuwen *et al.*, 2013). Also, the aquarium trade has been responsible for the establishment of freshwater snail species in areas where they are not native (Duggan, 2010). Such colonization events must be carefully considered, since the spread of *G. cubensis* would favour the expansion of fasciolosis. Conversely, the highest allelic diversity was observed in a population from Cuba, suggesting an ancient presence of *G. cubensis* in this island. This result agrees with the fact that *G. cubensis* was first described from Cuba (Pfeiffer, 1839).

In conclusion, we found that *Galba cubensis*—like other selfing lymnaeid species—has a much larger proportion of genetic variation among, rather than within, populations. Our results support the close relatedness between *G. cubensis* and *G. neotropica*, implying that they could be conspecific.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Molluscan Studies* online.

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