Population structure and genetic diversity in the invasive freshwater snail *Galba schirazensis* (Lymnaeidae)

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Abstract

We studied the population genetic structure of the freshwater snail *Galba schirazensis* (Küster, 1862), a potential vector of infectious diseases such as fascioliasis. *Galba schirazensis* has now a worldwide distribution but a poorly known origin because this species has been distinguished only recently from the morphologically similar and cosmopolitan *Galba truncatula*. We developed specific microsatellite markers and sequenced a mitochondrial gene (cytochrome oxidase subunit I) to study individuals of *G. schirazensis* from the Old World and New World. We found very low genetic diversity within populations, no heterozygotes, and marked population structure—a pattern observed in other highly selfing lymnaeid species with recently enlarged distributions as a result of biological invasions. The total lack of observed heterozygosity in the few populations of *G. schirazensis* that displayed some allelic diversity suggests high selfing rates. We also found that the center of diversity, and by extension the origin area of this species, should be found in the New World, while Old World populations should rather result from a recent introduction of a genetically uniform population. The microsatellite markers developed here will help to clarify the history of expansion of *G. schirazensis* and might help understanding its role as a potential vector of infectious diseases.

Keywords: lymnaeids; *Lymnaea*; *Galba schirazensis*; *Galba truncatula*; vector; microsatellites; selfing.

1. Introduction

During the last century, human activities and climatic change have considerably accelerated the spread of species across their natural barriers and modified their areas of distribution (Kolar and Lodge 2001), threatening local biodiversity (Davis 2009). To better understand these threats, it is important to more fully characterize the species involved and the expansion pathways.

Freshwater systems are particularly sensitive to bioinvasion risks (Beisel and Lévêque 2010).

The Mollusca includes a large number of species invasive in freshwater habitats (Nunes et al. 2015). We here focus on the family Lymnaeidae in which several species have shown widespread long-distance colonization (Jabbour-Zahab et al. 1997; Meunier et al. 2001; Kopp et al. 2012; Lounnas et al. 2017). Long-distance dispersal can occur as a result of human activities, such as aquarium trade (Duggan 2010). Lymnaeids display marked resistance to desiccation which increases their survival probability (Chapuis and Ferdy 2012). If released in conducive new environments, even a single individual can found a population because lymnaeids are capable of self-fertilization, which also happens to be the main reproductive mode in some species (*e.g.*, Meunier et al. 2004b; Escobar et al. 2011; Lounnas et al. 2016). Such colonization events may also spread food- and water-borne trematodes carried by lymnaeids (Meunier et al. 2001; Mas-Coma et al. 2005; Correa et al. 2010), either because dispersing snails are themselves infected or because their presence in a new location facilitates subsequent transmission that originates from nearby infected vertebrates. Importantly, the worldwide expansion of the liver fluke *Fasciola hepatica*, a parasite infecting livestock and humans, has been facilitated by the dispersal of lymnaeids (Hurtrez-Boussès et al. 2001; Mas-Coma et al. 2005).

Despite their relevance to human and animal health, lymnaeids have often been misidentified especially the *Galba* species, a phylogenetically distinct group of small-shelled lymnaeids (Correa et al. 2010; 2011; Lounnas et al. 2016). These species,, exhibit high phenotypic

plasticity in shell shape and display extremely similar anatomical traits that make accurate species identification difficult in the absence of molecular data (Samadi et al. 2000; Correa et al. 2011). For instance, *Galba schirazensis* (Küster, 1862) first described by Bargues et al (2011), has only recently been distinguished from *Galba truncatula* in Europe and Asia and from *G. truncatula*, *Galba cubensis*, and *Galba viator* in the Americas (Correa et al. 2010; 2011; Bargues et al. 2011). However, *G. schirazensis* is now described in many regions of the world, probably favored by its wide habitat range and its capacity to survive outside water for extended time periods (Bargues et al. 2011). Recent molecular-based studies reported *G. schirazensis* in Iran, Egypt, Reunion Island, Spain, Dominican Republic, Mexico, Colombia, Venezuela, Ecuador, and Peru (Bargues et al. 2011; Correa et al. 2010; 2011; reported as *Lymnaea* sp. and *Galba* sp. by the latter authors), but went of course unnoticed because the species was recently described. However, only highly conserved genetic markers have been used so far in species discrimination (Bargues et al. 2011; Correa et al. 2010, 2011). The population structure and genetic diversity of the invasive lymnaeid *G. schirazensis* remains therefore largely unknown.

We developed microsatellite markers in *G. schirazensis* and use them to genotype 242 individuals from 18 localities in Peru, Ecuador, Colombia, Venezuela, USA, Spain, and Reunion Island. We characterized the population structure and genetic diversity, and discuss our result in light of the breeding system and recent history of expansion of this species. We also assessed the specificity of these markers by testing amplification in the morphologically similar and closely related *Galba* species, i.e. *G. truncatula*, *G. cubensis*, and *G. viator*. We also sequenced a mitochondrial gene (cytochrome oxidase subunit I; CO1) to further elucidate the phylogeographic relationships among these populations and the populations studied by Bargues et al. (2011) and Correa et al. (2011).

2. Materials and methods

Development of specific microsatellite markers

Snail DNA was extracted from four pooled individuals identified by Correa et al. (2011) as *Galba* sp to reach a total of 2–3 µg of total DNA. These individuals were collected in Río Negro, Antioquia, Colombia (N 06°07'21" W 75°26'57"). DNA was extracted from foot tissue using the DNeasy Blood and Tissue Kit (Qiagen) and individuals were identified to species using the nuclear genes 18S (GenBank accession numbers: JN614335, JN614339, JN614340, JN614342), ITS-1 (HQ283253, JN614429, JN614430, JN614432), ITS-2 (HQ283263, JN614455, JN614456, JN614458), and the mtDNA gene CO1 (JN614370, JN614371, JN614373, JN614374). All these sequences showed 99–100% similarity with the sequences of *G. schirazensis* reported by Bargues et al. (2011).

Microsatellite loci were isolated from two enriched libraries (TC10 and TG10) following protocols described in Dubois et al. (2005) and using biotin-labeled microsatellite oligoprobes and streptavidin-coated magnetic beads. The enriched molecules were cloned into the pGEMt vector used to transform XL1-Blue Supercompetent Cells. Recombinant clones were screened with TC10, TG10, and AGE1 (AAACAGCTATGACCATGATTAC) or AGE2 (TTGTAACGACGCGCCAGTG) oligonucleotides using a modified PCR method (Waldbieser 1995). We screened 228 clones, 163 of which gave a positive signal and were sequenced using an ABI Prism 3100 sequencer (Applied Biosystems). Ninety seven sequences included a repeated motif and flanking regions allowing determination of PCR primers that were designed using Primer3 (Rozen and Skaletsky 2000). We retained the 25 loci that showed the largest uninterrupted stretches of repeated motifs and selected the 22 ones that amplified successfully (Table 1). We tested these 22 microsatellite loci in six individuals from five localities from the Americas: Finca Jocum Bucaramanga (Colombia), Huagrahuma (Ecuador), Manto de la Novia (Ecuador), Louisiana Bedico (USA; 2 individuals), and Bodoque (Venezuela; Table 2, Fig. 1).

We finally selected the 13 loci that were polymorphic in these six individuals for further population genetic analyses.

To test the specificity of microsatellite markers, we also amplified these loci in individuals of three species closely related to *G. schirazensis*: *G. truncatula*, *G. cubensis*, and *G. viator*. We used two individuals of each species that were identified using the molecular markers 18S, ITS-1, ITS-2, and CO1 (GenBank accession numbers in Correa et al. 2011).

Population genetic analyses using microsatellite markers

We analyzed 238 individuals of *G. schirazensis* from 16 localities from Colombia, Ecuador, Peru, USA, and Venezuela, as well as individuals from the Old World, one from Spain and three from Reunion Island (Table 2, Fig. 1). The Spanish specimen was collected from within the geographic range of *G. truncatula* and the population from Reunion Island was first ascribed to the introduced species *G. truncatula* (Griffiths and Florens 2006). The Spanish specimen was identified by Correa et al. (2011) as *G. schirazensis* using 18S, ITS-1, ITS-2 and COI (reported as *Galba sp* by the authors, GenBank accession numbers : JN614341, JN614436, JN614454, JN614377). We clearly identified the three individuals from La Reunion as *G. schirazensis* by analyzing the CO1 gene (GenBank accession numbers in Table 2) which presented 99-100 % of similarity with the four sequences of *G. schirazensis* reported by Bargues et al. (2011) and only 90% with the sequence of *G. truncatula* from Spain reported by the same authors (AM494011) - see also this study showing that all the highly specific microsatellite of *G. schirazensis* successfully amplified in these four individuals-. Despite the small sample sizes, we included these individuals in our analyses because they constitute the first mention of *G. schirazensis* in these two areas. All samples were collected from small areas ($< 2\text{m}^2$) to prevent a Wahlund

effect (Meunier et al. 2004a). Snails were killed in 70 °C water and immediately stored in 70% ethanol.

For each specimen, we removed the distal part of the foot, which was twice compressed between paper towels to remove excess ethanol. DNA extractions were then 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 hours followed by gentle vortexing and a further incubation at 95 °C for 10 min. The mixture was gently vortexed and centrifuged at 10,000 g for 10 sec. The supernatant (100 µl) was collected, diluted 1: 10 in deionized water, and stored at -20 °C.

We amplified microsatellite loci in an Eppendorf Thermal Cycler, in a total volume of 10 µl containing 5 µl of Taq PCR Master Mix Kit (Qiagen), 1 µl of the primer mix, the forward being labeled with a fluorochrome (see Table 1 for the labels of each locus), and 1 µl of DNA. PCR conditions were as follow: 15 min activation at 95 °C, 35 cycles including 30 sec of initial denaturation at 94 °C, 90 sec of annealing at 55 °C, and 60 sec of extension at 72 °C, followed by 30 min of final extension at 60 °C.. For genotyping, we pooled 3 µl of diluted (1: 100) PCR products with 15 µl of Hi-Di Formamide and 0.15 µl of GeneScan-500 LIZ Size Standard and analyzed it on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). We performed multiplexed locus amplification by grouping loci as mentioned in Table 1. Multiplexing loci amplification was possible by using PCR products characterized by different sizes loci (no overlapping zones) and labelled with different fluorochromes. Allele sizes were read using GeneMapper® v.4.0 software (Applied Biosystems). Raw fragment sizes and corresponding peaks were manually checked one by one to discard error in this alleles calling step. Given the marked homozygosity, we evaluated our capacity to detect heterozygotes: we amplified loci combining DNA from two individuals exhibiting different genotypes. We both tested situations

in which alleles exhibit a small size difference (one base pair) or a large one (40 base pair). In all situations we were able to detect heterozygotes.

We used the individual genotypes of the 238 American individuals to estimate allelic richness, observed and expected heterozygosities, the F -statistic F_{IS} , and the selfing rate (s) estimated as $s = 2 F_{IS} / (1 + F_{IS})$ (Hartl and Clark 1997). The global and pairwise differentiations among populations were estimated using both F_{ST} and R_{ST} . We could not use software that detects null alleles because limnaeids are hermaphroditic. Standard Bonferroni corrections were applied in the case of multiple tests (Rice 1989). We analyzed data using Genodive (version 2.0b23; Meirmans and Van Tienderen, 2004) and FSTAT (version 2.9.3.2; Goudet, 1995) software. Individuals from Spain and Reunion Island were not included in the population genetic analysis because the small sample sizes ($N=1$ and $N=3$ respectively) do not allow proper estimation of population genetics parameters. However, we compared their genotypes to those of American individuals. Multilocus genotypes were clustered using a discriminant analysis of principal components (DAPC) using the adegenet package of R (Jombart et al. 2010). One of the main advantages of this analysis is that it does not rely on a particular population genetic model and is thus free of assumptions about Hardy-Weinberg or linkage equilibrium. Six principal components were retained based on the α -score. This score gives the number of principal components optimized in order to capture the best discrimination without overfitting.

Amplification, sequencing of COI and phylogeographic analysis

We amplified and sequenced the COI gene in 21 snails from 11 populations (Fig. 3). Because of amplification failure due to DNA conservation issues, we did not sequence it in individuals from Andaracas (Ecuador), Bodoque, Zea el Amparo, La Azulita, Bailadores, and San Eusebio (Venezuela). The individual from El Rocío (Spain) was previously sequenced by Correa et al

(2011) and we retrieved the sequence from Genbank (JN614377). The PCR primers were LCO1490 (5'-GGTCAACAACCTCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al. 1994). We used 2.5 µl of DNA, 12.5 µl of Taq PCR Master Mix Kit (Qiagen), 2.5 µl of each primer (2 µM), and 5 µl of distilled water. PCR conditions were as follow: 15 min activation at 95 °C, 35 cycles including 30 sec of initial denaturation at 95 °C, 1 minute of annealing at 50 °C, and 10 min of extension at 72 °C, followed by 10 min of final extension at 72 °C. Unidirectional DNA sequencing was performed by Eurofins MWG Operon (Germany) using the cycle sequencing technology (dideoxy chain termination / cycle sequencing) on ABI 3730XL sequencing machines (Applied Biosystems). Sequencing quality was assessed based on a quality score Q per base (values provided by Eurofins Genomics).

In addition, we downloaded from GenBank 12 CO1 sequences of *G. schirazensis* from Iran, Reunion Island, Peru, Ecuador, Colombia, Venezuela, and Mexico (JF272607–JF272610, Bargues et al. 2011; JN614370–JN614378, Correa et al. 2011), as well as one CO1 sequence of *G. truncatula* from France (JN614386, Correa et al. 2011), of *G. viator* from Argentina (JN872451, Standley et al. 2013) and of *G. cubensis* from Uruguay (JN614396, Correa et al. 2011).

These sequences were used for phylogeographic reconstruction, first aligning sequences using webPRANK (Löytynoja and Goldman 2010). The alignment was manually inspected using the electrophoregram of each sequence viewed on 4Peaks (Griekspoor and Groothuis 2005) and the quality report to discard Single Nucleotide Polymorphisms (SNPs) for which we had sequencing incertitudes ($Q < 20$). Then, we performed tree reconstruction using both Maximum Likelihood (ML) and Bayesian Inference (BI). ML analyses were conducted using the best-fitting model of sequence evolution. Model selection was based on Bayesian information criterion (BIC) using jModelTest (Posada 2008). We ran 5000

bootstrap replications to test node robustness. BI analyses were performed with MrBayes v3.2 (Ronquist and Huelsenbeck 2003). The tree space was explored using Markov Chain Monte Carlo (MCMC) analyses with random starting trees and four simultaneous, sequentially-heated independent chains sampled every 500 trees during five million generations. Suboptimal trees were discarded once the “burn-in” phase was identified and a majority-rule consensus tree, with posterior probability support of nodes, was constructed with the remaining trees. The tree was visualized using FigTree v1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>). Both trees were edited with Adobe Illustrator.

The number of haplotypes, the number of polymorphic sites, and the nucleotide diversity were calculated using DnaSp V5 (Librado and Rozas 2009). Genetic distances were estimated based on the number of base-pair differences per site (p-distance) among haplotypes using Mega7 (Kumar et al. 2016). A minimum-spanning network was constructed using the software SplitsTree4 (Huson and Bryant 2006) to represent the distances between haplotypes

3. Results

Microsatellite markers

The sequences and primers of the 22 microsatellite loci that we identified in *G. schirazensis* are available in GenBank (accession numbers in Table 1). No amplification was observed in the closely related species *G. truncatula*, *G. cubensis*, and *G. viator*, suggesting that these 22 loci are specific to *G. schirazensis*.

Approximate position of Table 1.

We characterized the variation at the 13 polymorphic loci in all American individuals. The number of alleles per locus was 2.846 ± 0.274 SD, the observed heterozygosity was 0 at all loci, and the mean expected heterozygosity was 0.007 ± 0.005 SD (Table 2). At most loci and populations, the lack of diversity prevented us from estimating F_{IS} (Table 2). Among the four loci and populations that showed allelic diversity at some loci (Table S1), the observed heterozygosity was always zero ($F_{IS} = 1$, $s = 1$; Table 2).

Approximate position of Table 2.

We found high genetic differentiation among populations (global $F_{ST} = 0.979$; $P < 0.001$; global $R_{ST} = 0.979$; $P < 0.001$). All population pairs coming from different countries (except for Venezuela and Colombia) showed significant F_{ST} after Bonferroni adjustment ($P = 0.0004$; Table S2). In contrast, populations from the same country or from adjacent countries usually showed no significant differentiation (Ecuador: $F_{ST} < 0.019$, NS; Venezuela and Colombia: $F_{ST} < 0.235$, NS). An exception is the Venezuelan population from La Trampa that differs significantly from most other Venezuelan and Colombian populations with F_{ST} between 0.3 and 0.45 (Table S2).

Nine multilocus genotypes were found (Fig. 1). Some loci (1–8) could not be genotyped in some individuals ($N = 65$). Thus, we did not ascribe these individuals with incomplete genotypes to any multilocus genotype. However, the loci that could be genotyped in these individuals were exactly the same as in the individuals where all the loci were genotyped (Table S1). To analyze the genetic structure of populations using DAPC, we used all the individuals except for 12 individual genotypes that had more than three loci that did not amplify (Table S1). Four clusters were detected by DAPC which grouped together individuals from (1) Colombia, Venezuela,

Spain, and Reunion Island, (2) Peru, (3) USA, and (4) Ecuador (Fig. 2; Table S1). Each cluster consisted of a single main multilocus genotype with small within-cluster variation (Table S1). Differences between the main genotypes and variants did not exceed one repeat (two base pairs), and was of one base pair in some cases. The exception is Spanish genotype that differed from the main type at two loci (Table S1). The main genotype from each cluster differed from those of other clusters at five loci or more (Table S1). The largest difference was observed between clusters from Ecuador and USA (all 13 loci fixed for different alleles).

Approximate position of Figure 1.

Approximate position of Figure 2.

COI sequences

We obtained 21 COI sequences of *G. schirazensis* (Table 2). The analysis of these sequences and sequences of *G. schirazensis* retrieved from GenBank revealed five haplotypes (number of sequences: 37, nucleotide diversity: 0.005 ± 0.001 SD and haplotype diversity: 0.499 ± 0.083 SD) determined by 10 polymorphic sites without indels (Table S3). The COI sequences diverged from 0.2% (USA and Mexico; USA and Ecuador) to 1.6% (USA and Peru – from Bargues et al. 2011) (Table S4).

The best model describing the evolution of the COI sequences was HKY+I. The Maximum Likelihood tree showed that these sequences pooled in three clades: (i) Ecuador (cluster 4 in DAPC), (ii) the single sequence from USA (cluster 3) and (iii) a worldwide clade including Peru (cluster 2), Venezuela, Colombia, Reunion Island and Spain (cluster 1). Genbank sequences from Iran, Venezuela, Colombia, and Reunion Island fell in the worldwide clade, those from Ecuador in the Ecuador clade and those from Mexico close to the USA sequence (Fig. 3). The Bayesian tree resulted in the same grouping (Fig. S1). However, the USA sequence grouped with the Ecuador clade in the ML tree while it grouped with the worldwide clade in the Bayesian tree.

In both trees these nodes were not well supported by posterior probabilities and maximum likelihood bootstrap (52-72%).

The topologies of the Maximum Likelihood tree and the Bayesian trees were consistent with the results from DAPC, grouping those individuals from the same cluster in the same clade (Figs. 2,3 and S1). These analysis showed congruence between nuclear and mitochondrial genetic structure. Each mitochondrial haplotype included one to five multilocus microsatellite genotypes, and no multilocus microsatellite genotype was found associated with different mitochondrial haplotypes. However, the distance between DAPC clusters was in some cases lower than the phylogenetic distances. For instance, clusters 1 (worldwide) and 2 (Peru) were genetically distant from each other in the DAPC but they gathered in the same worldwide clade (Figs. 2 and 3). Also, the clusters 3 (USA) and 4 (Ecuador) were genetically distant from each other in the DAPC (Fig. 2) but they were genetically closed in the tree and the CO1 sequences diverged a little from each other (0.2%, Table S3 and S4; Fig. 3 and 4). In the minimum-spanning network, North American haplotypes (3 and 4) appeared to be intermediates between worldwide and Ecuadorian haplotypes (1 and 5, Fig. 4). This inconsistency probably reflects the the fact that microsatellite loci are evolving much faster than mtDNA sequences.

Approximate position of Figure 3.

Approximate position of Figure 4.

4. Discussion

We developed 13 polymorphic genetic markers to study the population structure and genetic diversity of *G. schirazensis*, an invasive freshwater snail inhabiting Asia, Europe, and the New World. These microsatellite loci appear to be specific to *G. schirazensis* because we did not find

any amplification in the morphologically similar and most closely related species *G. truncatula*, *G. cubensis*, and *G. viator*.

Analyzing New World populations, we observed very low genetic diversity within populations, a total lack of heterozygotes, and marked population structure. The lack of observed heterozygosity in those few populations that showed allelic diversity suggests that *G. schirazensis* mainly reproduces by self-fertilization. This is consistent with the fact that under laboratory conditions, individuals isolated from birth are able to self fertilize (Bargues et al. 2011). Our population genetic evidence for self-fertilization in natural population only relies on five locus-by-population combinations, all involving relatively rare alleles segregated in the populations (Tables 2, S1). In the absence of self-fertilization, rare alleles are expected to be mainly in the heterozygous state, so it would be very improbable for them to appear only as homozygotes in these five cases. Therefore, a high selfing rate is the most likely explanation for the lack of heterozygosity. In addition, related lymnaeid species exhibiting selfing rates in the 80–100% range, for example *G. cubensis* (Lounnas et al. 2016), *G. truncatula* (Trouvé et al. 2000; Meunier et al. 2001; 2004a), *Omphiscola glabra* (Hurtrez-Boussès et al. 2005), and *Pseudosuccinea columella* (Nicot et al. 2008) which also show very low or no within-population allelic diversity, high F_{IS} , and large F_{ST} . In contrast, most populations of mainly outcrossing lymnaeids such as *Lymnaea stagnalis* (Puurtilinen et al. 2007, Kopp et al. 2012, Besnard et al. 2013) and *Radix* sp. (Pfenninger et al. 2011) have high polymorphism, low F_{IS} , and moderate F_{ST} . Therefore, *G. schirazensis* shares the common characteristics of highly selfing lymnaeid species studied so far. It also shares a similar ecology with *G. cubensis*, *G. truncatula*, and *O. glabra*. All of them live mainly in temporary habitats that undergo frequent flooding and droughts that create strong bottlenecks (Meunier et al. 2004b). In this context, self-fertilization allows a single individual to colonize vacant habitats and to produce a new population (Meunier et al. 2004a). Finally, the recently analyzed transcriptomes of both *G. truncatula* and *G.*

schirazensis bear molecular signatures consistent with ancient self-fertilization, dating back to their common ancestor (Burgarella et al. 2015).

It is very difficult to know *a priori* where *G. schirazensis* was present before 2010, hence whether each particular population is native or invasive. *Galba schirazensis* has probably been misidentified in previous reports of small lymnaeid species from all over the world, either as *G. truncatula* (everywhere) or as *G. cubensis* and *G. viator* (New World; Correa et al. 2010; 2011; Bargues et al. 2011) as they all have very similar shell morphology and internal anatomy. Thus, it is impossible to trace invasion routes using reports from the literature, except for places known from historical records to be devoid of native small-shelled lymnaeids, such as Reunion Island where the *G. schirazensis* populations necessarily result from introduction(s). The geographic origin of *G. schirazensis* remains unknown. Bargues et al. (2011) assumed that this species is native to the Middle East as it was first described in Iran (Küster 1862). From this postulate the authors hypothesize two spreading waves, first an Old World spread (from Iran into Africa, Europe and Asia) and second a Trans-Atlantic spread into the Americas through the livestock transported by the Spanish conquerors during the early colonization period. However, as is typical of species descriptions of that time, only a brief description of the shell of *G. schirazensis* was provided, which could in practice fit with that of many small-shelled lymnaeids, including the very widespread *G. truncatula*. Therefore, the snail lineage studied in the present paper, re-described on the basis of molecular sequences as *G. schirazensis* by Bargues et al. (2011), as *Lymnaea* sp. by Correa et al. (2010), and as *Galba* sp. by Correa et al. (2011), may not necessarily be (i) the same snail as that named *G. schirazensis* by Küster (1862) and (ii) native from the Middle East. Its current occurrence in Iran (Bargues et al. 2011) could well result from an introduction.

389 In this confused taxonomical situation, it is important to clearly list the available evidence on the
390 possible geographical origin of *G. schirazensis*. Any mention of this species can only be
391 ascertained based on molecular evidence in order to avoid misidentification. This eliminates all
392 mentions prior to 2010, including the first description by Küster (1862). As a consequence, we
393 must rely only on genetic diversity and phylogeny to identify likely origin areas. Bargues et al.
394 (2011; Table 1) showed that all the individuals they sampled in the Old World (Iran, Egypt, and
395 Spain) have exactly the same haplotypes at the nuclear genes 18S, ITS-2, ITS-1 and the
396 mitochondrial genes 16S and CO1. However, they observed two to four different haplotypes for
397 each gene in American populations (Dominican Republic, Mexico, Peru, Ecuador, and
398 Venezuela), including the Old World haplotype. Our microsatellite study corroborates this
399 pattern, as each of the four different regions sampled in the New World forms well-defined and
400 differentiated clusters and the few individuals from two very distant places in the Old World
401 (Reunion Island and Spain), including one from a known recent introduction (Reunion Island),
402 are genetically related to only one of these clusters (Fig. 1). The phylogeographic tree is
403 consistent with this pattern, since American CO1 sequences are variable while the sequences
404 from the Old World showed no variation and belong all to the large worldwide clade (Fig. 3 and
405 S2). All this suggests that the center of diversity, and by extension the origin area of this species,
406 should be found in the New World, while Old World populations should rather result from a
407 recent introduction of a genetically uniform population. In other snail species with the ability to
408 self-fertilize, genetic uniformity over large distances and many populations is indeed the
409 signature of recent introductions, for example in *Lymnaea stagnalis* introduced in New Zealand
410 (Kopp et al. 2012), *G. truncatula* introduced on the Bolivian Altiplano (Meunier et al. 2001) or
411 *P. columella* introduced in many parts of the world (Lounnas et al. 2017). Finally, an American
412 origin for *G. schirazensis* is also corroborated by the phylogeny of Correa et al. (2010), who
413 concluded that the whole *Galba* clade likely has an American origin, as most of its species are

native there (*G. cousini*, *G. viator*, *G. neotropica*, *G. cubensis*, *G. humilis*). The hypothesis of a post-Columbian introduction of *G. schirazensis* from Europe to the New World (Bargues et al. 2011), is not consistent with our findings: it would have been very unlikely to accumulate fixed differences at 13 distinct loci (as we observed between USA and Ecuador) in such a short time, while at the same time Venezuelan, Colombian, and Old World populations would remain nearly identical and devoid of diversity over huge geographical distances.

Although our data together along with the report of Bargues et al. (2011) seem sufficient to reject the hypothesis of a single recent introduction of *G. schirazensis* in the New World, more extensive sampling is needed (i) to test whether all the populations from the Old World have a single recent introduced origin, an hypothesis so far consistent with the results of conservative (Bargues et al. 2011, Correa et al. 2011, this study) and polymorphic (this study) markers and (ii) to more fully understand the genetic variation observed among South and North America isolates. The application of the genetic markers developed here to a more extensive sample that covers a worldwide range will therefore help to clarify the geographic origin and invasion routes of *G. schirazensis*.

Combining markers with contrasted mutation rates we observed discordances in genetic structuration between phylogenetic and DAPC analyses. Populations from Peru and populations distributed worldwide appeared to be significantly differentiated at a microsatellite level while they grouped in the same clade at a mitochondrial level (haplotype 1). Microsatellite loci have high mutation rates (between 10^{-2} and 10^{-4} per generation; Jarne and Lagoda 1996; Ellegren 2002) and tens of generations can generate mutations at least at one locus while several thousands of years are needed to generate one base substitution at the CO1 gene (around 1.35. 10^{-8} /site/year according Wilke et al. 2009). This result suggests that Peruvian population and

populations that belong to the worldwide genotype probably diverged recently. Similarly, we found that populations from Ecuador and USA were strongly differentiated at the nuclear level while they were genetically closed at the mitochondrial level (0.2% of divergence) suggesting a recent divergence event. Moreover sequences from USA and Mexico branched differentially between the Bayesian tree and the maximum likelihood tree with moderate posterior probabilities and maximum likelihood bootstrap. North American haplotypes appeared to be intermediate between haplotype 1 (worldwide) and haplotype 5 (Ecuador) that were strongly differentiated at both mitochondrial and microsatellite markers (1.2 %, Fig. 4). Finally, we cannot exclude the possibility of mtDNA introgression often responsible of mito-nuclear discordance (Toewls and Brelsford 2012). This lack of clear structuration of North and South American populations at a mitochondrial level shows that our sample size is too limited, and more genetic markers would have been required to understand the fine-scale population structure and bridging gaps in our understanding of both ancient population splits and more recent expansion events of *G. schirazensis*.

Understanding the population structure and the genetic diversity of *G. schirazensis* can also help elucidating its role in spreading infectious diseases. Caron (2015) reported high prevalences of *F. hepatica* in *G. schirazensis* sampled in Ecuador. However, Bargues et al. (2011) who experimentally infected snails from one Egyptian and one Spanish localities observed that the parasite did not complete its life cycle. Dreyfuss et al. (2014) in experimental infections on successive generations of Colombian *G. schirazensis* detected prevalences ranging from 0 to 15.3% but free cercaria were only observed in two of the 400 studied snails. It is worth mentioning that, in the two studies cited above, only allopatric combinations have been tested and no experiment has been conducted so far with sympatric parasites. It therefore seems to be wise to consider *G. schirazensis* as a putative, perhaps occasional, intermediate host of *F.*

hepatica. However, our capacity to identify which parasites are transmitted by *G. schirazensis* has hitherto been hindered by the morphological similarity among *Galba* species (Correa et al. 2011). We need to identify by molecular means morphologically cryptic *Galba* species and to identify the role of *G. schirazensis* as an intermediate host of food- and water-borne trematodes such as *F. hepatica*.

In conclusion, our analysis provides strong support to the ideas that this species is predominantly self-fertilizing, and has an American origin with recent colonization of the Old World by a genetically uniform strain related to populations from Venezuela and Colombia—a hypothesis that awaits confirmation by more extensive sampling.

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Table 1. Microsatellite loci in *Galba schirazensis*. Description of loci and genetic variation in six individuals from five localities (polymorphic loci in bold characters). Annealing temperature is 55 °C for all loci. Na, number of alleles detected; RAS, range of allele size (in base pairs).

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Locus	Primer sequences (5'→3')	Label dye	Multiplex set	GenBank accession number	Repeat motif	Na	RAS
Gsch_1	F: TTTTGGGTCAACATTAGGTTAGG R: GAACATTTGACACAGTAGCGTTG	PET	A	KT324711	(TC) ₁₃	3	213–249
Gsch_2	F: ACGTGCACACTTCTCCCTCT R: GCCTTGGTGCAGTTTTGTATT	PET		KT324709	(CT) ₂₃	1	185
Gsch_3	F: TGTAGGCAAAGGCACAAAAA R: AGGGTGTAAGGGCTGAATTG	PET	B	KT324702	(CT) ₁₃	4	152–178
Gsch_4	F: TTCATTGTCTGCTCCTGCTG R: CGCCACTGGTCGATAGACTT	VIC	B	KT324708	(TC) ₂₀ (CT) ₁₃	2	157–160
Gsch_5	F: GGTCGTTTAGCAGCCTAGCA R: CGACCGTTCAAACGTTACTG	NED	A	KT324710	(GT) ₁₀ (AG) ₁₃	2	179–185
Gsch_7	F: AAACGACCGTTTCTAAGGTGA R: CCCTGAACCAACAGAGCATT	VIC		KT324707	(AG) ₂₃	1	244
Gsch_9	F: GGCGGAAACGAAGAGAGTAA R: TACGTGCACACACTCAACCA	NED	B	KT324705	(TC) ₁₁	4	207–227
Gsch_10	F: AGGAGAGTCCCATTGAGCTG R: CGAAACTGTTGAAGGCATTG	PET		KT324706	(GT) ₁₁	1	218
Gsch_11	F: AACACATTTCCACCCACACA R: CTTTCTTTCGCGTGGGGTAT	FAM	B	KT324712	(CT) ₁₈	2	216–235
Gsch_12	F: CTGGGGCTAACCCAAGAATC R: TTTGGGGTTGAGGGCTTTAT	FAM		KT324713	(TG) ₁₀	1	151
Gsch_13	F: TCACGTTCTCGTGGTTCTCA R: GTCGATGGGGCTATGTGTCT	VIC	C	KT324714	(CA) ₁₀	5	166–173
Gsch_14	F: CGATGGCGCCAACTTATTTA R: TCATATCGCTACGGACATTCA	VIC	A	KT324715	(CA) ₁₀	2	201–222
Gsch_15	F: TGAGACGGGCAAGATTTCTC	NED		KT324716	(CT) ₁₅	1	154

	R: AGGGTTCGATTCCCATCTCT						
Gsch_17	F: CTTCATCCACGCAGCAAGTA R: GGGGGCCGATATTAATTTTT	PET		KT324717	(AC) ₁₃	1	205
Gsch_18	F: GACGGACCGTGAATTAGAGC R: ATTGTTGCGGCGGATAACTA	FAM		KT324718	(AG) ₁₁	1	210
Gsch_19	F: CGGTATTAGGGTGTCTGTGC R: CAGGGGGAACCATAAAGTTG	FAM		KT324719	(CA) ₁₂	1	171
Gsch_20	F: CAGTGAGACAGAGCCACGAA R: ATTGCCGACACGTTTGTGTT	FAM		KT324720	(AG) ₃₁	1	192
Gsch_21	F: CGCTAGACCTTTCTTTCTGTCC R: TTACAAGCTCTTGGAACGA	NED	C	KT324721	(TC) ₁₄	3	239–279
Gsch_22	F: TGTGTGTGTTTGTGGAGAGAGA R: GTGTTACGCATGGTGAACCT	PET	C	KT324722	(AG) ₁₁	3	249–322
Gsch_23	F: AATGACCCAGTGGGGAAG R: TGGGGAAGTTCAATTGTTT	NED	C	KT324723	(AC) ₁₆	2	227–232
Gsch_24	F: GCGTGCGTGTATGTGAAAGA R: GGGGCTCTTCAAGTGTGTGT	PET	A	KT324704	(AG) ₁₀	3	167–171
Gsch_25	F: AGCCAGACAAAGGGGGATAG R: GGGCAGGTTCACTACTCTGTTT	VIC	C	KT324703	(GA) ₁₉	2	188–190

Table 2. Populations of *Galba schirazensis* and their genetic variability. Genetic parameters were not calculated in the samples from Spain and La Réunion Island because of too small sample size. N, sample size; Na, mean number of alleles; He, expected heterozygosity; F_{IS} , inbreeding coefficient and P -value; NA, not available. Bonferroni corrections were applied ($P = 0.0125$). Observed heterozygosity was null in all populations.

Country	Location	Acronym	Coordinates	Sampling year	GenBank accession number	N	Na	He	F_{IS}	Estimated selfing rate
Peru	La Joya de Arequipa	LJ	16°28'56" S 71°49'07" W	2012	KY198250, KY198260	14	1.000	0.000	-	-
Ecuador	Huagrahuma	HU	02°47'32" S 79°16'31" W	2012	KT781302, KT781304	13	1.000	0.000	-	-
	Manto de la Novia	MN	01°24'03" S 78°17'49" W	2011	KT781305, KT781315	15	1.000	0.000	-	-
	Hacienda Cienaga	HC	00°46'18" S 78°37'10" W	2011	KT781301	12	1.000	0.000	-	-
	Andaracas	AN	00°26'10" S 78°32'22" W	2014	-	14	1.077	0.013	1 (<0.0001)	1
	Nono	NO	00°03'25" S 78°34'15" W	2014	KY198255, KY198256	16	1.000	0.000	-	-
Colombia	Finca Jocum Bucaramanga	FJ	07°06'25" N 73°04'60" W	2012	KY198253, KY198254	14	1.000	0.000	-	-
Venezuela	Bodoque	BO	08°16'15" N 71°48'51" W	2005	-	18	1.077	0.033	1 (<0.0001)	1
	Los Nevados	LN	08°27'41" N 71°04'28" W	2013	KT781320	18	1.000	0.000	-	-
	Zea el Amparo	ZA	08°21'40" N 71°46'01" W	2005	-	9	1.000	0.000	-	-
	La Azulita	LA	08°44'06" N 71°26'49" W	2005	-	11	1.000	0.000	-	-
	Bailadores	BA	08°14'05" N 71°50'26" W	2005	-	18	1.000	0.000	-	-
	San Eusebio	SE	08°38'39" N 71°23'42" W	2005	-	18	1.000	0.000	-	-
	Sabana Alto	SA	08°36'11" N 71°27'45" W	2013	KT781322, KT781323, KT781324	7	1.000	0.000	-	-

	La Trampa	LT	08°33'31" N 71°27'13" W	2013	KY198251, KY198252	20	1.154	0.048	¹ (<0.0001)	1
USA	Louisiana Bedico	LB	30°26'11" N 90°15'01" W	NA	KT781332	21	1.077	0.015	¹ (<0.0001)	1
Spain	El Rocío	ER	37°08'02" N 06°28'18" W	2010	JN614377	1	-	-	-	-
La Reunion Island (France)	Ravine du Gol	RG	21°14'26" S 55°25'07" E	2009	KY198257, KY198258, KY198259	3	-	-	-	-

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Figure legends

Figure 1. Geographic location of multilocus microsatellite genotypes of *Galba schirazensis*.

Each multilocus genotype is figured by a color and a letter. Each pie chart represents a population. See Table 2 for population acronyms.

Figure 2. Genetic population structure of *Galba schirazensis*. Scatter plot of 230 individuals on the first two DAPC axes (first axis: 61% of total variance; second axis: 22%). Individuals group in four clusters.

Figure 3. Maximum-likelihood phylogenetic tree of *Galba schirazensis* based on mtDNA CO1 sequences. Bootstrap values are indicated at each node. Sequences are given their GenBank accession numbers. Colored sequences are those obtained in this study, the others having been retrieved from GenBank. *Galba viator* JN872451 sequence (Standley et al. 2013) was used as outgroup. For each sequence obtained here, the color code refers to the cluster number derived from DAPC.

Figure 4. Minimum-spanning network of COI haplotypes from *Galba schirazensis*. Colors and haplotypes are those referred in the table S4. Each circle represents a haplotype, its size being proportional to the frequency of occurrence of a certain haplotype. Numbers of haplotype occurrences are indicated in each circle.

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