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3

4 **Systematics and Geographical Distribution of *Galba* Species, a Group of Cryptic**
5 **and Worldwide Freshwater Snails**

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54

55 **ABSTRACT**

56 Cryptic species can present a significant challenge to the application of systematic and
57 biogeographic principles, especially if they are invasive or transmit parasites or
58 pathogens. Detecting cryptic species requires a pluralistic approach in which molecular
59 markers facilitate the detection of coherent taxonomic units that can then be analyzed
60 using various traits (e.g., internal morphology) and crosses. In asexual or self-fertilizing
61 species, the latter criteria are of limited use. We studied a group of cryptic freshwater
62 snails (genus *Galba*) from the family Lymnaeidae that have invaded almost all
63 continents, reproducing mainly by self-fertilization and transmitting liver flukes to
64 humans and livestock. We aim to clarify the systematics, distribution, and phylogeny of
65 these species with an integrative approach that includes morphology, molecular
66 markers, wide-scale sampling across America, and data retrieved from GenBank (to
67 include Old World samples). Our phylogenetic analysis suggests that the genus *Galba*
68 originated ca. 22 Myr ago and today comprises six species or species complexes. Four
69 of them show an elongated-shell cryptic phenotype and exhibit wide variation in their
70 genetic diversity, geographic distribution, and invasiveness. The remaining two species
71 have more geographically restricted distributions and exhibit a globose-shell cryptic
72 phenotype, most likely phylogenetically derived from the elongated one. We emphasize
73 that no *Galba* species should be identified without molecular markers. We also discuss
74 several hypotheses that can explain the origin of cryptic species in *Galba*, such as
75 convergence and morphological stasis.

76 **KEY WORDS:** phylogenetics, America, Lymnaeidae, vector snails, biological invasions,
77 self-fertilization.

78 **1. INTRODUCTION**

79 Cryptic species are groups of populations in which the intrapopulation phenotypic
80 variance exceeds interspecific phenotypic variance (see review in Bickford et al. 2007;
81 Fišer et al. 2018; Struck et al. 2018). They have been described in all taxonomic
82 kingdoms, although cryptic species seem to be more often reported in animals (Struck et
83 al. 2018). Their frequency might reach 10-20% of all animal species (Janzen et al.
84 2017), an estimate that should be considered quite approximate given our lack of
85 knowledge on the species totals. They also seem to be homogeneously distributed
86 among taxa and biogeographical regions (Pfenninger and Schwenk 2007). Four
87 hypotheses have been proposed for the origin of cryptic species (Bickford et al. 2007;
88 Fišer et al. 2018; Struck et al. 2018): (1) recent divergence, when distinguishing traits
89 have not as yet accumulated (e.g. cave fish, Niemiller et al. 2012); (2) parallelism,
90 where independent phenotypic traits evolve in different taxa from a similar and shared
91 ancestral trait (Struck et al. 2018); (3) convergence, where more distantly related
92 species evolve from dissimilar ancestors (e.g. sea stars, Zulliger and Lessios, 2010), and
93 (4) morphological stasis, where species remain similar over long periods of time (e.g.,
94 Gomez et al. 2004; Struck et al. 2018).

95 Although interesting as models for study of the speciation process (Coyne and
96 Orr 2004; De Queiroz 2007), cryptic species are problematic from at least two human
97 perspectives: biological invasion and disease transmission. Cryptic species may exhibit
98 wide differences in invasive ability and impact on invaded ecosystems and communities
99 (Fang et al. 2014). An accurate identification at the species level is required in such
100 situations to track invasions and mitigate any harmful consequences (Kolar and Lodge
101 2001; Dunn and Hatcher 2015; Jarić et al. 2019). Cryptic species may also exhibit

102 differences in disease transmission. This is the case in the *Anopheles gambiae* complex
103 which includes the most important vectors of malaria in Africa (Stevenson and Norris
104 2016). Some members of the complex are broadly zoophilic, while others feed more
105 strictly on humans. Accurate species identification is required for effective mosquito
106 control.

107 Snails, especially freshwater ones, are an interesting group for addressing
108 biogeographic issues in cryptic species. Although taxonomists have increasingly used
109 molecular markers over the last decades to identify snail species (Dayrat et al. 2011),
110 morphological characters, especially shell shape and sculpture, remain widely relied
111 upon—often resulting in large numbers of synonymous species (Qian et al. 2012). The
112 shells of some mollusk populations show significant phenotypic plasticity, however, in
113 reaction to temperature, pollution, or predation (Bourdeau et al. 2015). Others stay
114 stable for millions of years (e.g., Weigand et al. 2011; Weiss et al. 2018). This has
115 resulted both in the proliferation of species names and descriptions (e.g., Taylor, 2003),
116 most of which are invalid (Jarne et al. 2010; Dillon et al. 2011), as well as the
117 misidentification of valid species, yielding errors in the assessment of species invasion
118 ability and distributional range (e.g., Pfenninger et al. 2006; Rama Rao et al. 2018). For
119 example, an invasive Asian clam of the genus *Sinanodonta* has been overlooked in
120 Russia because it is morphologically indistinguishable from another invasive Asian
121 clam (Bespalaya et al. 2018). Such problems call for an integrated approach to
122 gastropod systematics and biogeography in which phenotypic traits are studied together
123 with appropriate molecular tools (Dayrat 2005).

124 Here we focus on small-bodied basommatophoran pulmonate snails of the genus
125 *Galba* (Hygrophila, Lymnaeidae), common inhabitants of unstable freshwater habitats

126 worldwide. Baker (1911) recognized 30 species and subspecies in the subgenera *Galba*
127 (*s.s.*) and *Simpsonia* in North America, on the basis of minor shell morphological
128 variation, where Hubendick (1951) suggested that as few as four biological species
129 might be valid: *humilis*, *truncatula*, *cubensis*, and *bulimoides*. The more recent work of
130 Burch (1982) proposed 22 North American species in the genus *Fossaria*, which we
131 here consider a junior synonym of *Galba*. In South America, Hubendick (1951)
132 recognized only two species of small-bodied, amphibious lymnaeids (*viator* and
133 *cousini*), but more recent work based on single-gene approaches and on fewer than 10
134 sequences per species distinguishes seven species (*viator*, *schirazensis*, *cousini*,
135 *neotropica*, *meridensis*, *truncatula*, and *cubensis*; Bargues et al. 2007, 2011b, 2011a;
136 Correa et al. 2010, 2011; Lounnas et al. 2017, 2018). Population genetic studies
137 suggest, however, that *neotropica* and *cubensis* could be synonymies. Uncertainty
138 prevails in *Galba* systematics mainly because most of the nominal *Galba* species share
139 a similar shell morphology and internal anatomy, as well as plasticity in shell, anatomy,
140 and life-history traits (Samadi et al. 2000; Correa et al. 2011). The exceptions are *Galba*
141 *cousini* (Paraense 1995) and *Galba meridensis* (Bargues et al. 2011b), which are
142 morphologically different from other *Galba* species. However, they are both
143 morphologically similar (shell and reproductive anatomy) and *G. meridensis* has been
144 described from a single locality (Bargues et al. 2011b). We therefore have two groups
145 of cryptic species, which are often misidentified within groups (Correa et al. 2010;
146 Bargues et al. 2011a). Overall, this uncertainty calls for a wider study that sheds light on
147 the phylogenetic relationships among *Galba* species.

148 A further challenge is that crossing cannot be used to distinguish species (Coyne
149 and Orr 2004), as has been done in other freshwater snails (e.g., *Physa* species, Dillon et

150 al. 2011), since *Galba* populations primarily reproduce by self-fertilization (Meunier et
151 al. 2004; Bargues et al. 2011a; Lounnas et al. 2017, 2018). Self-fertilization poses two
152 problems here: one practical and one theoretical. On the practical side, an evaluation of
153 whether phylogenetic groups might demonstrate reproductive isolation among each
154 other would be prohibitively labor-intensive, many generations being required to
155 demonstrate unsuccessful crosses at some rather-arbitrarily set confidence. The
156 theoretical problem is that if one applies the biological species concept to a 100% self-
157 fertilizing taxon, each individual would immediately produce a line of descent
158 reproductively isolated from all others, and thus deserve a species name (Coyne & Orr
159 2004). However, strict selfing has never been reported in any biological group,
160 including Lymnaeid snails (Cutter 2019). Population genetic studies, when variation is
161 sufficient to test it, report low outcrossing rates in some lymnaeid populations, but
162 generally not lower than 5-10% (Escobar et al. 2011, Lounnas et al., 2017). Rates as
163 low as 1%, yielding recombination every 100 generations, are probably sufficient, over
164 evolutionary time, to maintain genetic cohesion within a set of interbreeding
165 populations, reproductively isolated from other similar sets.

166 Moreover, at least two species, *G. schirazensis* and *G. truncatula*, have been
167 shown to be extremely efficient anthropogenic invaders, muddling our knowledge of
168 species distribution. Populations of *G. truncatula*, probably from Eurasia, have invaded
169 South America, especially the Bolivian Altiplano (Meunier et al. 2004). This is of
170 special concern since *Galba* populations are the main vectors of the liver fluke *Fasciola*
171 *hepatica* which causes fasciolosis in both livestock and humans (Mas-Coma et al. 2005)
172 and transmission efficiency and invasion ability differ among species (Vázquez et al.
173 2018).

174 Worldwide, the geographic distribution of *Galba* species is poorly known. The
175 dubious character of records based on morphological identification leaves us with a
176 small sample of molecular studies (Correa et al. 2010, 2011; Bargues et al. 2011b,
177 2011a, 2012; Lounnas et al. 2017, 2018) upon which to base a very large-scale pattern.
178 Our objectives here are to characterize the geographic distribution of *Galba* species at
179 continental scale, based on an extensive sampling over America, to reconstruct the
180 genus phylogeny to delimit species, and to explore the origin of cryptic species. We aim
181 to delineate species—the real scientific challenge of integrative taxonomy (Dayrat
182 2005)—noting that in practice *Galba* species are very difficult to delineate due to the
183 presence of cryptic species, wide geographical distribution, and mating system.
184 Previous studies reconstructing *Galba* phylogeny have used single genes and analyzed
185 fewer than 10 sequences per species, failing to account for the wide geographic
186 distribution of this genus (Correa et al. 2010, 2011; Bargues et al. 2011c, 2011a;
187 Standley et al. 2013). Here we employ morphological and molecular markers
188 (microsatellite loci and DNA sequences from two genes) to study more than 1,700
189 individual *Galba* from 161 sites. Our data set was augmented with a complete sample of
190 all the *Galba* DNA sequences available in GenBank and multiple phylogenetic analyses
191 conducted. We used both gene trees and multispecies coalescent models to shed light on
192 the phylogenetic relationships and on the origin of cryptic species in the genus *Galba*.

193

194 **2. MATERIALS AND METHODS**

195 2.1 Snail Sampling and Species Identification

196 We conducted simple searches for *Galba* populations in suitable habitats throughout the
197 New World over a span of two decades, 1998–2017. Overall, *Galba* populations were

198 detected in 161 sites and 1,722 individuals were sampled from nine countries:
199 Argentina, Canada, Colombia, Cuba, Ecuador, France (Guadeloupe and Martinique),
200 Peru, Venezuela, and USA (Table S1). *Galba* populations have previously been
201 reported from some of these sites in Venezuela and Ecuador by the authors (Pointier
202 2015; Orlando Narváez et al. 2017). In most cases, we discovered our *Galba*
203 populations in unstable habitats subject to frequent flooding and droughts. The sampled
204 habitats were characterized as brook, irrigation canal, ditch, oxbow lake, pond, marsh,
205 lake, tank, rice field, and river. Individual snails were often collected above the water
206 line, consistent with their amphibious habit. Some sites were visited up to five times.
207 Geographic coordinates were recorded for most sites. After collection, individuals were
208 placed in water at 70 °C for 30–45 s. This procedure allows fixation of individuals
209 without contraction of soft parts and facilitates a proper study of snail internal anatomy.
210 The body was carefully withdrawn from the shell using forceps and both body and shell
211 stored in 70% ethanol until morphological and DNA analyses (Pointier et al. 2004).

212 Species were characterized using a three-step procedure involving both
213 morphological and molecular markers (Fig. 1). Step 1 was an analysis of shell
214 morphology and reproductive anatomy. In step 2, we used a molecular tool that enables
215 us to distinguish *G. cubensis*, *G. schirazensis*, and *G. truncatula* (Alda et al. 2018). In
216 step 3, we sequenced mitochondrial and nuclear genes in individuals for which no PCR
217 amplification product was obtained in step 2. DNA from some of those individuals
218 identified in steps 1 and 2 were also sequenced in order to reconstruct the phylogeny of
219 *Galba*. Note that this three-step approach is less expensive than an approach based on
220 simply sequencing the same genes in all individuals.

221 2.1.1 Step 1: morphology of the shell and of internal organs

222 We photographed the shell of three to five adult snails from each site and dissected their
223 body under a stereoscopic microscope. We drew the anatomy of the penial complex,
224 prostate, and renal tube using a camera lucida attachment (Pointier et al. 2004). We did
225 not record any morphological measurements or perform any quantitative tests because
226 previous studies (Samadi et al. 2000, Correa et al. 2011) have shown that cryptic *Galba*
227 species cannot be delimited by such means. Our observations were qualitative only.

228 2.1.2 Step 2: multiplex PCR of microsatellite loci

229 We applied the multiplex PCR test designed by Alda et al. (2018) to all the 1,420
230 individuals that were not distinguishable based on shell and reproductive anatomy. This
231 method is based on species-specific primers amplifying three microsatellite loci (one
232 each per targeted species) and producing band sizes that are specific to these species
233 (179–200 pb in *G. cubensis*, 227–232 pb in *G. schirazensis* and 111–129 pb in *G.*
234 *truncatula*). DNA was extracted using a Chelex protocol following Estoup and Martin
235 (1996) as adapted for 96-well plates. Methods for DNA amplification and
236 electrophoretic resolution followed Alda et al. (2018).

237 2.1.3 Step 3: identification by sequencing

238 We amplified the internal transcribed spacer 2 (ITS2) and the cytochrome oxidase
239 subunit 1 (COI) genes in 35 individuals sampled from 15 sites in Argentina, Canada and
240 the USA (1 to 5 per population) where at least some individuals did not demonstrate an
241 amplification product in step 2, using the method of Lounnas et al. (2017, 2018). We
242 also amplified ITS2 and COI in 112 individuals that did return an amplification product
243 in step 2, including one individual identified as *G. cousini/meridensis*. To supplement
244 these results, we also amplified the internal transcribed spacer 1 (ITS1) and the ARN
245 ribosomal 16S in two individuals of *G. cubensis* from Bosque del Apache (USA) and in

246 one individual of *G. cousini/meridensis* from Ecuador (Table S1). With this approach,
247 we obtained at least one sequence from each hypothetical species represented by the
248 four genes and used them to delimit species. The total number of individuals amplified
249 was 151.

250 We used the primers NEWS2 (forward) 5' TGTGTCGATGAAGAACGCAG 3'
251 and ITS2-RIXO (reverse) 5' TTCTATGCTTAAATTCAGGGG 3' to amplify ITS2;
252 Lim1657 (forward) 5' CTGCCCTTTGTACACACCG 3' and ITS1-RIXO 5'
253 TGGCTGCGTTCTTCATCG 3' to amplify ITS1 (Almeyda-Artigas et al. 2000);
254 LCOI490 (forward) 5' GGTCAACAAATCATAAAGATATTGG 3' and HCO2198
255 (reverse) 5' TAAACTTCAGGGTGACCAAAAATCA 3' to amplify COI (Folmer et
256 al. 1994) and forward 5' CGCCTGTTTATCAAAAACAT 3' and reverse 5'
257 CCGGTCTGAACTCAGATCACGT 3' to amplify 16S (Remigio and Blair 1997). In all
258 cases, PCR amplification was performed in a total volume of 25 µl containing 12.5 µl of
259 Taq PCR Master Mix (Qiagen), 2.5 µl of each primer (10 mM) and 2 µl of DNA in an
260 Eppendorf Thermal Cycler with an initial denaturation step at 95 °C for 15 minutes;
261 followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 50 °C for
262 one minute, extension at 72 °C for one minute; and a final elongation at 60 °C for 30
263 minutes. The presence and size of amplification products were electrophoretically
264 confirmed in 1% agarose gels stained with EZ-Vision. DNA sequencing was performed
265 by Eurofins Genomics (Ebersberg, Germany) using PCR-amplified products as
266 templates. All sequences were uploaded to GenBank (Table S1) and assigned to a
267 species using the phylogenetic reconstruction.

268

269 2.2 Type Localities

270 Because of the longstanding confusion and uncertainty regarding the systematics of the
271 genus *Galba* worldwide, it is especially important to establish standard populations,
272 against which unknown populations can be compared. Type localities were specified by
273 the authors of all the more recently-described species, such as *neotropica* (Bargues et al.
274 2007) and *meridensis* (Bargues et al. 2011b), and others have been established by
275 subsequent use, for example *schirazensis* (Bargues et al. 2011a). But in his original
276 description of *Limnaeus viator*, D’Orbigny (1835) simply stated “Patagonia.” Pfeiffer
277 (1839) gave no locality data for his *Limnaeus cubensis* at all, beyond “Cuba”. Thus, we
278 selected a more precise spot within these wide-ranged type localities for *viator* and
279 *cubensis*. Type localities for all eight of the widely-recognized species in the genus
280 *Galba* are listed in Table 1. COI sequences from samples of all the populations
281 inhabiting these localities have been previously uploaded to GenBank, and most have
282 ITS1, ITS2, or 16S sequences available as well.

283

284 2.3 Retrieving Data on *Galba* spp. Distribution from Published Work

285 We searched the literature and GenBank for sequence data at four genes (COI, ITS1,
286 ITS2, and 16S) apparently attributable to lymnaeids of the genus *Galba*. Coordinates
287 were provided for most sites by the authors. When coordinates were not provided, we
288 inferred them from the locality data using GoogleEarth. We found 132 New World sites
289 in which *Galba* species have been molecularly characterized (Table S2), and 45 sites in
290 the Old World (Table S3, Fig. S1). The specific nomina attributed to these sequences by
291 their depositors in GenBank were 157 *truncatula*, 152 *schirazensis*, 70 *neotropica*, 57
292 *cubensis*, 44 *viator*, 20 *cousini*, 9 *humilis*, 6 *meridensis*, and 2 others. This corresponds
293 to 166 COI, 163 ITS2, 118 ITS1, and 70 16S sequences.

294

295 2.4 Phylogenetic and Ancestral Reconstruction Study

296 Phylogenetic analyses were conducted on the ITS2 and COI sequences obtained in this
297 study, together with ITS2, COI, ITS1, and 16S sequences retrieved from GenBank. This
298 is the first phylogenetic study conducted in *Galba* species that use such a large dataset
299 including 796 sequences: 90 for 16S, 251 for COI, 122 for ITS1, and 333 for ITS2.

300 Previous studies were based on single genes and smaller sample sizes. For instance, the
301 description of *G. meridensis* is based from a single locality (Bargues et al. 2011b).

302 Some GenBank accession numbers appear more than once in Tables S2–S3 because
303 individuals with identical sequences have been registered under the same GenBank
304 accession number.

305 Alignment was performed individually for each gene using MAFFT (Katoh and
306 Standley 2013). Ambiguously aligned sites were excluded using GBLOCKS with
307 default settings for a less stringent selection (Castresana 2000). The number of positions
308 in the final sequences was 412 for 16S (83% of the original 493 positions), 609 for COI
309 (86% of 707), 435 for ITS1 (48% of 888), and 333 for ITS2 (23% of 1,429). We
310 examined levels of saturation for each gene and for the first and second *versus* third
311 codon positions of COI using DAMBE (Xia 2017). We did not find evidence of
312 saturation in the four genes analyzed, including all codon positions of COI. We
313 partitioned codon positions and unlinked substitution models in phylogenetic analyses.

314 We used Bayesian inference in Beast2 (Bouckaert et al. 2014) for five reasons:
315 (1) to assign sequences to species; (2) to validate, and to mend if necessary, species
316 identity for sequences retrieved from GenBank; (3) to delimit species; (4) to reconstruct
317 the phylogeny and estimate time divergence; and (5) to assess the ancestral phenotypic

318 state of *Galba* species. Because we did not have identical sets of individuals (or
319 populations) across genes it was necessary to build four unlinked gene trees to address
320 questions (1) and (2). The best-fitting models of sequence evolution for each gene were
321 selected using bModelTest (Bouckaert and Drummond 2017). We estimated a model for
322 each COI partition (1st, 2nd, and 3rd position). The best model describing the evolution of
323 16S was HKY+G+I, 123424+G+I for COI (1st codon), 121321+G+I for COI (2nd
324 codon), TN93+G+I for COI (3rd codon), 123424+G+I for ITS1, and 121323 for ITS2.
325 We linked trees for the three COI codon partitions. The analyses were run using four
326 gamma categories and a proportion of 0.5 invariant sites. Uncorrelated relaxed-clock
327 models were chosen for all loci. The relative clock mean priors were all lognormal ($M =$
328 $0, S = 1$). We used a birth-death model as priors with lognormal birth and death rates.
329 These gene trees allowed us to evaluate species names and validate or mend species
330 identity for all sequences, whether obtained in this study or retrieved from GenBank.
331 All the MCMC were run for 200,000,000 generations storing every 20,000 generations.
332 The MCMC output was visualized using Tracer (Rambaut et al. 2018) and tree samples
333 summarized by TreeAnnotator (utility program distributed with the Beast package)
334 using a 10% burn-in. The species tree was visualized and edited in FigTree and GIMP
335 (<https://www.gimp.org>). The names of the eight widely-recognized species in the genus
336 *Galba* were ultimately assigned by reference to their type localities. We also built
337 haplotype networks for each gene using popART (Leigh and Bryant 2015) and
338 compared them with gene trees.

339 To address our question (3), species delimitation, we built ten multispecies
340 coalescent tree models in StarBeast2 (Ogilvie et al. 2017) differing in species
341 assignments, some models splitting species to as many as nine while others lumping to

342 as few as five (Fig. S2). We assigned the species identity obtained from tasks 1 and 2 to
343 each of the 796 sequences (Table S1–S3) and tested each scenario in turn. Species
344 assignments were inferred from the literature and our own observations. *Galba*
345 *truncatula*, *G. schirazensis*, and *G. humilis* were considered as different species in every
346 scenario. However, *G. cousini* and *G. meridensis* were considered as a single species in
347 half of the models and as two species in the other half since literature considers them as
348 separate species (Bargues et al. 2011b) but we suspect they could be the same species
349 since they both share the same distinct morphology and restricted distribution. *Galba*
350 *cubensis*, *G. viator*, *G. neotropica*, and *Galba* sp. “Bosque del Apache” were considered
351 as one, two, three, or four species depending the model since some studies considered
352 them different species (Bargues et al. 2007) but previous genetic population studies
353 (Lounnas et al. 2017) and the gene trees here constructed (task 1) suggest they could be
354 synonymies. We also created an eleventh model in which we separated the populations
355 of *G. viator* from Argentina and from Chile to test whether splitter models showed
356 higher support than lumpers models regardless of their biological meaning. In
357 StarBeast2, not all the individuals but all the hypothetical species must be represented
358 by at least one sequence of all genes. Thus why, we accordingly removed the sequences
359 from Ethiopia because this hypothetical species was represented by two genes.

360 In all our multispecies coalescent tree models, we used the same site models as
361 for reconstructing the gene trees. We assigned to the mitochondrial loci a gene ploidy of
362 0.5 and to nuclear loci a gene ploidy of 2.0 (diploid). We used constant population sizes
363 (fixed to 1) and uncorrelated relaxed-clock models for all loci. For nuclear loci, we used
364 the molecular clock rate reported by Coleman and Vacquier (2002) for ITS in bivalves
365 (0.00255 per Myr). For mitochondrial loci, we retained the molecular clock rate

366 estimated by Wilke et al. (2009) for COI in invertebrates (0.0157 per Myr). We used the
367 birth-death model as tree prior with lognormal birth and death rates. We ran each model
368 using Multi-Threaded Nested Sampling analysis with 10 particles, 8 threads, a chain
369 length of 100,000,000 and sub-chain length of 5,000. Then, we compared the trees by
370 computing Bayes factor (BF), a model selection tool that is simple and well-suited for
371 comparing species-delimitation models (Leaché et al. 2014).

372 BF is the difference between the marginal likelihoods of two models: $BF =$
373 $\text{model 1} - \text{model 2}$. If BF is larger than 1, model 1 is favored, and otherwise model 2 is
374 favored. When BF is between 20 and 150 the support is strong and when the BF is
375 above 150 the support is overwhelming (Leaché et al. 2014). We used Nested Sampling
376 implemented in the NS package to calculate the marginal likelihoods necessary to
377 obtain BF and also an estimate of the variance of the marginal likelihoods (Russel et al.
378 2019). This approach has proven to be an excellent tool for delimiting species (Leaché
379 et al. 2014; Matos-Maravi et al. 2018), assuming that tree reconstruction is of good
380 quality and that the best scenario to explain species delimitations is within the scenarios
381 tested (Leaché et al. 2014).

382 We also applied two other species-delimitation methods: Automatic Barcode
383 Gap Detection (ABGD; Puillandre et al. 2012) and Species Tree and Classification
384 Estimation in Beast2 (STACEY, Jones 2017). ABGD was run for each gene using the
385 default settings (<https://bioinfo.mnhn.fr/abi/public/abgd/>). ABGD does not rely on tree
386 shapes but on divergence and it requires an appropriate prior value of maximal
387 intraspecific divergence. This prior defines the threshold between intra- and
388 interspecific pairwise distances and is iterated from minimum to maximum through ten
389 steps (Puillandre et al. 2012). If this value is underestimated, the result is an oversplit

390 classification, and if overestimated, the classification is overlumped. Given the wide
391 genetic diversity observed within some species (Lounnas et al. 2017), we chose the
392 partition that showed high prior on intraspecific divergence (the penultimate partition).

393 The STACEY delimitation method, as the Nested Sampling analysis, also relies
394 on a Multispecies Coalescent Model. However, STACEY does not require setting up
395 predefined scenarios in terms of species number and sample assignment. The method
396 distinguishes very shallow species divergences with a statistic called “collapseHeight,”
397 which we set to a small value (0.0001) following Jones (2017). Given the results
398 obtained with ABGD and Nested Sampling analyses, we ran three multispecies
399 coalescence models with different collapseWeight parameters: (1) a lumping model
400 with six *Galba* species with 1/X distribution (initial value: 0.975, between 0 and 1), (2)
401 a splitting model with nine species with 1/X distribution (initial value: 0.952 between 0
402 and 1), and (3) a no-prior-taxonomic model with a Beta distribution ($\alpha = 2$, $\beta = 2$). The
403 initial value of the lumping and splitting models were calculated following Matos-
404 Maravi et al. (2018). All other parameters were set as in the Nested Sampling analysis.
405 We ran the three models for 250,000,000 generations with storing every 25,000
406 generations. The MCMC output was visualized using Tracer (Rambaut et al. 2018). For
407 STACEY, we used only the ITS2 and COI sequences because these both genes were
408 better represented in the dataset than ITS1 and 16S. Since the hypothetical number of
409 species in STACEY ranges from one to the number of individuals, each of our 113 snail
410 populations was considered as a minimal cluster.

411 To address our question (4), regarding species topology and divergence time, we
412 reran the multispecies tree model that showed the highest Bayes Factor in StarBeast2.
413 We used the same parameters as in the Nested Sampling analysis, but we ran the

414 MCMC for a longer time (250,000,000 generations stored every 25,000 generations).
415 The MCMC output was visualized using Tracer (Rambaut et al. 2018) and tree samples
416 summarized by TreeAnnotator (utility program distributed with the Beast package)
417 using a 10% burn-in. The species tree was visualized and edited in FigTree, GIMP, and
418 DensiTree (Bouckaert and Heled 2014). Some analyses were run in CIPRES Science
419 Gateway (Miller et al. 2012).

420 Finally, to address our question (5), the ancestral phenotypic state, we applied
421 Bayesian Binary MCMC (BBM, Ronquist and Huelsenbeck 2003), statistical dispersal-
422 vicariance analysis (S-DIVA; Yu et al. 2010), and Statistical dispersal-extinction-
423 cladogenesis (S-DEC; Ree and Smith 2008) in the software Reconstruct Ancestral State
424 in Phylogenies (RASP, Yu et al. 2015). We used the splitting model (scenario A) under
425 default settings. We added two phenotypic (shell) states: globose for *G. cousini* and *G.*
426 *meridensis* and elongated for all other species.

427

428 **3. RESULTS**

429 3.1 Morphology

430 Most individuals (N = 1,420 from 133 sites) were not distinguishable based on shell and
431 reproductive anatomy (Fig. S3). The exception was a single group comprising all
432 individuals from *G. cousini* and *G. meridensis* (N = 302). These tended to demonstrate
433 more globose shells with shorter spires, adult sizes in excess of 10 mm standard shell
434 length. *Galba cousini* and *G. meridensis* also differed from the other species in their
435 internal anatomy—a ureter with two distinct flexures, a wider and more ovate prostate,
436 a larger penial complex, and a penis sheath approximately the same length as the
437 preputium (Fig. S3). We did not find any anatomical differences between *G. cousini* and

438 *G. meridensis*, however, comparing individuals from Ecuador, Colombia, and
439 Venezuela (Fig. 2).

440

441 3.2 Multiplex PCR of Microsatellite Loci

442 DNA from the 1,420 American individuals with similar phenotypes was amplified using
443 the multiplex PCR procedure (step 2 from Fig. 1). We identified 541 individuals of *G.*
444 *cubensis*, 330 of *G. schirazensis*, and 349 of *G. truncatula* (Table S1; Fig. 2). No
445 amplification was observed in 200 individuals sampled in one site from Argentina and
446 14 sites from Canada and USA (Table S1).

447

448 3.3 Identification by Sequencing and Phylogenetic Analysis

449 Phylogenetic analysis of COI sequences returned six clusters (Fig. S4). Clusters I–V
450 each contained a single type population: *truncatula* (I), *schirazensis* (II), *humilis* (III),
451 *cousini* (IV), and *meridensis* (V). Cluster VI contained the type populations of *cubensis*,
452 *neotropica*, and *viator*. The posterior probabilities (PP) of all clusters were 1.0, except
453 for cluster III (*humilis*, PP = 0.95).

454 Analysis of 16S, ITS1, and ITS2 sequences confirmed the COI results in almost
455 all respects, although sequences were missing for at least one or two type populations in
456 each tree (Figs. S5–S7). However, we detected discrepancies between the mitochondrial
457 and nuclear gene trees. A striking example are sequences from Bosque del Apache
458 (USA; cluster VI) that exhibited very long branches in the mitochondrial gene trees
459 (Figs. S4–S5) but clustered tightly with the *cubensis* type population and similar
460 populations in the nuclear gene trees (Figs. S6–S7). The mitochondrial sequences from
461 Ethiopia (one per gene) also formed a long branch, although nuclear sequences were not

462 reported in GenBank for that population. Within cluster VI, we found that the *cubensis*
463 and *neotropica* type populations were located in separate subclades for the nuclear
464 genes but clustered together in the mitochondrial trees (Figs. S4–S7).

465 Gene trees (Figs. S4–S7) and haplotype networks (Figs. S8–S11) showed that
466 genetic diversity varied among the six clusters and four genes. Cluster II (*schirazensis*)
467 showed reduced variation, while cluster VI (*viator* / *cubensis* / *neotropica* / Bosque del
468 Apache sample) was larger and more diverse. Mitochondrial genes seemed more
469 diverse than nuclear genes. This observation may be biased, however, by a structural
470 correlation in our data between genes sequenced and regions sampled.

471 Most of the sequences uploaded to GenBank identified as one of the eight
472 species of *Galba* were accurately clustered into the six clades containing their type
473 populations. However, there were some exceptions. Eight sequences of COI uploaded
474 as *G. truncatula* from France appeared in cluster II with the *G. schirazensis* type
475 population (Table S3; Fig. S4). We reidentified these sequences as belonging to *G.*
476 *schirazensis*. The COI sequence from Ethiopia, uploaded as *G. truncatula*, clustered at
477 the base of *truncatula* clade I with low posterior probability (Fig. S4). The other
478 Ethiopian sequence (16S), also identified as *G. truncatula*, did not cluster with any of
479 the *Galba* clades (Fig. S5).

480 The topology of all gene trees showed that clusters III (*humilis*), IV (*cousini*),
481 and V (*meridensis*) grouped together. But inconsistent results among genes were
482 obtained for the remainder of the other identified clusters (Figs. S5–S8). The multilocus
483 multispecies tree returned three major groups: cluster I (*truncatula*) together with II
484 (*schirazensis*); cluster III (*humilis*) together with IV (*cousini*) and V (*meridensis*); and
485 the cluster VI group (*viator* / *cubensis* / *neotropica* / Bosque del Apache sample; Fig.

486 4). The multilocus multispecies tree visualized in DensiTree (Fig. 3) confirmed that
487 most tree topologies united the clusters into the three major groups outlined above,
488 although some topologies placed clusters differently reflecting the incongruence found
489 among the gene trees.

490

491 3.4 Species Delimitation

492 Figure 3 illustrates the results obtained using the three species-delimitation methods.
493 The Multi-Threaded Nested Sampling analysis (Fig. S12) suggested that scenario A
494 (nine species) is the best fit to the available data, demonstrating the largest maximum
495 likelihood estimate (Table S4). BF analysis preferred scenario A over scenario D
496 (current taxonomy) or scenario K, separating populations of *G. viator* from Argentina
497 and from Chile.

498 ABGD results varied depending on the gene analyzed (Fig. 3). Nine species
499 (scenario A) were suggested by ITS1, while six species only were returned by our
500 analysis of ITS2 and 16S, with *G. cubensis*, *G. viator*, *G. neotropica*, and *Galba* sp.
501 “Bosque del Apache” lumped. ABGD analysis of the COI gene indicated that *G. viator*
502 and *Galba* sp. “Bosque del Apache” are separate species, but that *G. cubensis* and *G.*
503 *neotropica* should be lumped together. The ITS2 and COI analyses also suggested that
504 some species (*G. cousini* and *G. truncatula*) might be represented by more than one
505 taxon.

506 The species-delimitation analysis implemented in STACEY suggested that six of
507 the nine clusters of scenario A might include more than one taxon. The exceptions were
508 *G. viator*, *G. meridensis*, and *Galba* sp. “Bosque del Apache”, the last two species

509 including only one population. Our STACEY results converged towards similar
510 MCMCs regardless of which prior was used for the collapseWeight parameter (Fig. 3).

511

512 3.5 Time of Divergence and State Reconstruction

513 The estimated divergence time from the most recent common ancestor of the *Galba*
514 group was 22.6 Mya [95% HPD interval: 14.6–33; Figs. 3–4]. Diversification within the
515 species complex *G. cousini* and *G. meridensis* and the one formed by *G. cubensis*, *G.*
516 *viator*, *G. neotropica*, and *Galba* sp. “Bosque del Apache” seems to have occurred 5
517 Mya, or less. The three analyses of phenotypic state reconstruction (S-DEC, S-DIVA,
518 and BBM) suggested that the most recent common ancestor of all *Galba* species
519 displayed the elongated-shell phenotype (Fig. 4). Thus, the globose-shell phenotype of
520 *G. cousini* and *G. meridensis* should be considered as derived from the elongated one
521 (Fig. 4).

522

523 4. DISCUSSION

524 4.1 *Galba* Comprises Six Species or Species Complexes

525 Here we report the largest study published to date of *Galba* systematics and distribution,
526 based on extensive sampling at a very large geographical scale and integration of
527 phenotypic and molecular approaches across all DNA sequences available in GenBank
528 for four genes (Dayrat 2005). The widespread occurrence of self-fertilization in these
529 populations essentially voids the biological species concept (Coyne and Orr 2004), and
530 the absence of any reliable morphological distinction obviates the typological one.
531 Thus, we are left with a phylogenetic approach, which suggests the existence of six
532 clusters, perhaps corresponding to as many as nine species, or as few as six. These

533 findings reinforce several previously-published works that have involved fewer genes
534 and smaller sample sizes (Correa et al. 2010, 2011; Bargues et al. 2011b, 2011a;
535 Standley et al. 2013; Lounnas et al. 2017).

536 We suggest that five of our six clusters are best understood as one species each.
537 The oldest taxonomic names for these are *Galba truncatula* (Müller 1774) for cluster I,
538 *Galba schirazensis* (Küster 1862) for cluster II, and *Galba humilis* (Say 1822) for
539 cluster III, *Galba cousini* (Jousseume 1887) for cluster IV, and *Galba meridensis*
540 (Bargues, Artigas, Khoubbane & Mas-Coma 2011) for cluster V. However, considering
541 *Galba cousini* and *G. meridensis* as two different species is essentially based on the
542 molecular divergence (estimated at 4.7 Mya; they always clustered together in our
543 phylogenetic reconstructions), and should be considered with caution. The two species
544 indeed inhabit northern regions of South America, and are not distinguishable based on
545 shell and reproductive anatomy characters, despite the claims of Bargues et al. (2011b)
546 given the known within-species variation for these characters. Moreover, *G. meridensis*
547 has been sampled in a single locality, and more extensive sampling is clearly required to
548 ascertain species status.

549 The sixth cluster includes the nomina *Galba viator* (d'Orbigny 1835), *Galba*
550 *cubensis* (Pfeiffer 1839), *Galba neotropica* (Bargues, Artigas, Mera y Sierra, Pointier &
551 Mas-Coma, 2007), and a population from southern USA (Bosque del Apache).
552 However, any distinction between the sets of populations within this cluster depends on
553 both the genes and phylogenetic methodology employed (Fig. 3). Most species
554 delimitation methods suggest that this cluster comprises four species but others suggest
555 two or even one species (Fig. 3). Discrepancies could be due to the genes analyzed.
556 Note, for instance, that *G. cubensis* is not monophyletic when analyzing the COI and

557 ITS2 trees and haplotype networks which would be affecting the species delineation
558 process. The distance separating *G. cubensis* and *G. neotropica* is limited (1 Mya).
559 Moreover, microsatellite markers defined in *G. cubensis* amplified effectively in
560 individuals of *G. neotropica* (Lounnas et al. 2017), suggesting a very short genetic
561 distance. Additional sampling, especially in the North and South of the distribution
562 range (Argentina and USA) and the analysis of other genes would help resolve the
563 status of this cluster. On the whole, a cautious position would be to suggest that cluster
564 VI corresponds to a species complex or a species with wide diversity, as has been found
565 in other freshwater snails from the clade Hygrophila (e.g., Ebbs et al. 2018 in *Physa*;
566 Mavárez et al. 2002 in *Biomphalaria*; Pfenninger et al. 2006 in *Radix*). We also note
567 that if we ultimately recognize a single species; its name should be *viator*, and not
568 *cubensis* or *neotropica*, based on prior description.

569 The *Galba* species tree that we constructed based on a multispecies coalescent
570 model returned three groups: one group uniting *G. truncatula* and *G. schirazensis*,
571 another uniting *G. humilis*, *G. cousini*, and *G. meridensis* and the last with *G. cubensis*,
572 *G. viator*, *G. neotropica*, and *Galba* sp. “Bosque del Apache”. This result partially
573 agrees with some gene trees published in previous work (Correa et al. 2010, 2011;
574 Barges et al. 2011a). However, previous trees were based on single genes and smaller
575 sample sizes. Our phylogenetic analysis also revealed some distinctive branches,
576 including the mtDNA sequences from Ethiopia (Dayrat et al. 2011) and the samples
577 from Bosque del Apache. These results may suggest undetected species, accelerated
578 molecular evolution or mitochondrial introgression with non-sampled populations or
579 species (Fourdrilis et al. 2016; Pinceel et al. 2005; Thomaz et al. 1996). More extensive

580 sampling and further phylogenetic analysis are required before reaching any
581 conclusions.

582 In North America, Burch (1982) recognized 22 species of small, mud-dwelling
583 lymnaeids, which he grouped into the genus *Fossaria* with two subgenera, *Fossaria*
584 (*s.s.*) with 11 species and *Bakerilymnaea* with 11 (see Table S5 for species names).
585 Johnson et al. (2013) transferred these species to the genus *Galba*, but otherwise
586 retained the Burch system. Included in the present analysis were topotypic samples of
587 *obrussa* from Philadelphia and *parva* from Cincinnati, both of which we here show to
588 be indistinguishable from topotypic *humilis*, collected at Owego, New York. Remigio
589 (2002) contributed to Genbank a 16S sequence from a Canadian population he
590 identified as *Fossaria obrussa*, also grouping with cluster III (*humilis*). We suggest that
591 *obrussa*, *parva*, and the seven other uniquely North American specific nomina listed
592 above ascribed by Burch (1982) to the subgenus *Fossaria* are junior synonyms of *G.*
593 *humilis*, setting aside American populations of *G. truncatula* as distinct. In addition to
594 his *obrussa* sequence, Remigio (2002) contributed a 16S sequence from Oklahoma to
595 Genbank which he labelled “*Fossaria bulimoides*”. This sequence grouped with cluster
596 VI in our analysis. We suggest that all 12 specific nomina ascribed by Burch (1982) to
597 the *Fossaria* subgenus *Bakerilymnaea* (Table S5) including *bulimoides* (Lea 1841), are
598 junior synonyms of the species or species complex of cluster VI (*viator* / *cubensis* /
599 *neotropica* / Bosque del Apache sample).

600 Two of the three species-delimitation models here used (Nested Sampling
601 analysis and STACEY) are based on Multispecies Coalescence, assuming gene flow
602 within species though not between species (Sukumaran & Knowles 2017). Thus, the use
603 of these models would be inappropriate in a fully selfing species group. As mentioned

604 above, the selfing rate in *Galba* species and populations is high (often ca. 0.9), but
605 outcrossing does occur (Chapuis et al. 2007; Lounnas et al. 2017, 2018). Further
606 research is needed to explore how selfing may affect speciation. *Galba* species would
607 be excellent models to investigate such questions, since selfing rates vary so greatly
608 among species and populations (Chapuis et al. 2007; Lounnas et al. 2017, 2018).

609

610 4.2 A Set of Cryptic Species

611 Our study has confirmed the previous reports of Samadi et al. (2000) and Correa et al.
612 (2011) using both classical approach and geometrical morphometry that most species of
613 *Galba* cannot be distinguished on the basis of shell morphology or internal anatomy.
614 Trait variability within species seems to be greater than variance among species, likely
615 attributable to phenotypic plasticity (Correa et al. 2011). Reproductive and growth traits
616 in *G. truncatula* have been shown to vary according to habitat characteristics at small
617 geographical scales (Chapuis et al. 2007), suggesting both that life-history traits are
618 phenotypically plastic, and that to rely on such traits for specific identification is not
619 advisable.

620 Our study also confirms that *G. cousini* and *G. meridensis* differ strikingly in
621 adult size, shell shape, and anatomy from all other *Galba* species. Our molecular results
622 suggest that their shell phenotype evolved from the phenotype exhibited by all other
623 *Galba* species. Interestingly, *G. cousini* and *G. meridensis* are the largest species within
624 the genus *Galba*, occurring in a specialized habitat and displaying a complex
625 reproductive anatomy that resembles the anatomy of known outcrossing species rather
626 than the simplified ones observed in selfing species (see Jarne et al. 2010; Escobar et al.
627 2011).

628 Among the freshwater pulmonates, cryptic species have previously been
629 documented in *Ancylus* (Weiss et al. 2018) and *Radix* (Pfenninger et al. 2006). Our
630 methods here were strictly qualitative, as was the case for *Ancylus* and *Radix*, because
631 previous studies have shown that the dimensions of internal organs depend on
632 physiological state and mating system and that shell shape and size depend on the
633 environment (Samadi et al. 2000; Correa et al. 2011; see Bourdeau et al 2015 for review
634 in gastropods). Hence species cannot be distinguished by means of such measurements.
635 We were therefore not able to include quantitative traits to differentiate species in our
636 reconstruction of the ancestral phenotypic state. Future, more comprehensive
637 phylogenetic approaches should combine discrete anatomical traits, environmental
638 variables, and mating systems to elucidate the origin and maintenance of *Galba* species.
639 Such an approach might provide insights into how evolutionary biologists should
640 describe and evaluate morphological and environmental diversity in cryptic species
641 complexes.

642 Four hypotheses have been offered to explain the occurrence of cryptic species:
643 recent divergence, parallelism, convergence, and stasis (Bickford et al. 2007; Fišer et al.
644 2018; Struck et al. 2018). The recent divergence hypothesis seems unlikely in this case.
645 *Galba* has no closely related groups; its closest relatives are probably the stagnicoline
646 lymnaeids of North America and Eurasia, which demonstrate a very distinctive
647 morphology (Aksenova et al. 2018). Our analyses suggest that the several species of
648 *Galba* are separated by more than 20 Myr (Burgarella et al. 2015). And indeed, the
649 morphological divergence demonstrated by *G. cousini* and *G. meridensis* suggests that
650 time has not been a significant constraint. The parallelism hypothesis also seems
651 unlikely given that, based on our phylogenetic reconstruction, the cryptic morphology is

652 ancestral for *Galba*, and the only other morphology that has evolved in the group, as
653 demonstrated by *G. cousini* and *G. meridensis*, is derived. Nor does the topology of
654 lymnaeid phylogeny fit the convergence hypothesis (Correa et al. 2010). So, by default,
655 morphological stasis is left as the most likely hypothesis to explain the presence of
656 cryptic species in the genus *Galba*, as has been proposed in other gastropod groups
657 (e.g., Gomez et al. 2004; Struck et al. 2018).

658 The challenge of identifying cryptic *Galba* species is aggravated by their wide
659 and poorly-known geographical distributions, recently scrambled by biological
660 invasion. For example, *G. schirazensis* and *G. truncatula* have broadly expanded their
661 distribution over recent decades (Brown 1994; Bargues et al. 2001, 2011a; Vinarski and
662 Kantor 2016; Lounnas et al. 2018). We have documented up to three *Galba* species
663 occurring in some South American sites (Table S1). Their identification is not possible
664 without molecular tools.

665 The specific identity of *Galba* populations is important because they are
666 involved in the transmission of fasciolosis caused by the liver fluke *F. hepatica*. Some
667 studies have shown that lymnaeid species demonstrate different patterns of
668 susceptibility, host-parasite compatibility and immunological resistance to *F. hepatica*
669 (Gutiérrez et al. 2003; Vázquez et al. 2014; Dreyfuss et al. 2015). Although all species
670 can be infected under laboratory conditions (Vázquez et al. 2018), field transmission
671 depends on ecological and sociological conditions. Cattle or wildlife do not occupy the
672 same grazing habitats as infecting snails in many parts of the world (Sabourin et al.
673 2018). Ecological studies should be performed to evaluate whether the several cryptic
674 *Galba* species differ with regard to habitat preference, since our current knowledge is
675 essentially limited to *G. truncatula* (Chapuis et al. 2007).

676

677 4.3 Conclusions and Future Directions

678 Lymnaeid populations of the genus *Galba* are of interest for addressing a variety of
679 questions, including wide-scale biogeography, biological invasions, evolution of mating
680 systems, and host-parasite interactions. Our work is a first attempt to clarify the
681 phylogeny, systematics, and biogeographical distribution of this interesting group in the
682 New World. We have constructed a variety of gene trees using classical approaches, as
683 well as a species tree based on a multispecies coalescent model that reconciles gene
684 trees and provides a much better estimation accuracy for species tree topology than, for
685 instance, concatenation (Heled and Drummond 2010). The inferred phylogenetic
686 relationships among species varied, depending on the genes analyzed and techniques
687 employed. Incomplete lineage sorting or introgressive hybridization of specific genes
688 may indeed lead to such a result (Felsenstein 2004). Future studies could investigate
689 which evolutionary processes (gene duplication, horizontal gene transfer, incomplete
690 lineage sorting, hybridization) gave rise to the incongruence we have observed in gene
691 and species trees. Although our study was conducted at an extremely large geographic
692 scale, especially in America, *Galba* populations occur on almost all continents. Much
693 more extensive sampling and molecular analysis will be required to get a worldwide
694 picture of the phylogeny and distribution of the genus.

695

696 **DATA AND CODE ACCESSIBILITY**

697 Xml files for phylogenetic analyses are available from the Zenodo repository
698 (<https://zenodo.org/record/3473937#.XZiPcC0ryTd>).

699

700 **CREDIT AUTHOR STATEMENT**

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731

732 **CONFLICT OF INTEREST DISCLOSURE**

733 The authors of this preprint declare that they have no financial conflict of interest with
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735

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