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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.

1. INTRODUCTION

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

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

4

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 ℃ 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' TAAACTTCAGGGTGACCAAAAAATCA 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 ºC for 15 minutes;

261   followed by 35 cycles of denaturation at 95 ºC for 30 seconds, annealing at 50 ºC for

262   one minute, extension at 72 ºC for one minute; and a final elongation at 60 ºC 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

14

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 lumper 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   model 1 − 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

18

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

22

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* (Jousseaume 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 Bargues 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**

701 **Pilar Alda:** Conceptualization, Methodology, Investigation, Formal analysis, Writing -

702 Original Draft, Reviewing and Editing **Manon Lounnas:** Investigation, Writing -

703 Reviewing and Editing **Antonio A. Vázquez:** Investigation, Writing - Reviewing and

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714

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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

734   the content of this article.

735

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