Molecular Phylogenetics and Evolution 157 (2021) 107035

Contents lists available at ScienceDirect



Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Systematics and geographical distribution of Galba species, a group of

cryptic and worldwide freshwater snails

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ARTICLE INFO

ABSTRACT

Keywords: Phylogenetics America Lymnaeidae Vector snails **Biological** invasions Self-fertilization

Cryptic species can present a significant challenge to the application of systematic and biogeographic principles, especially if they are invasive or transmit parasites or pathogens. Detecting cryptic species requires a pluralistic approach in which molecular markers facilitate the detection of coherent taxonomic units that can then be analyzed using various traits (e.g., internal morphology) and crosses. In asexual or self-fertilizing species, the latter criteria are of limited use. We studied a group of cryptic freshwater snails (genus Galba) from the family Lymnaeidae that have invaded almost all continents, reproducing mainly by self-fertilization and transmitting liver flukes to humans and livestock. We aim to clarify the systematics, distribution, and phylogeny of these species with an integrative approach that includes morphology, molecular markers, wide-scale sampling across America, and data retrieved from GenBank (to include Old World samples). Our phylogenetic analysis suggests that the genus Galba originated ca. 22 Myr ago and today comprises six species or species complexes. Four of

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Received 26 March 2020; Received in revised form 18 September 2020; Accepted 30 November 2020

Available online 4 December 2020

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https://doi.org/10.1016/j.ympev.2020.107035

them show an elongated-shell cryptic phenotype and exhibit wide variation in their genetic diversity, geographic distribution, and invasiveness. The remaining two species have more geographically restricted distributions and exhibit a globose-shell cryptic phenotype, most likely phylogenetically derived from the elongated one. We emphasize that no *Galba* species should be identified without molecular markers. We also discuss several hypotheses that can explain the origin of cryptic species in *Galba*, such as convergence and morphological stasis.

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

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

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

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

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

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

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

Step 1: Morphological study

2. Materials and methods

2.1. Snail sampling and species identification

We conducted simple searches for Galba populations in suitable habitats throughout the New World over a span of two decades, 1998-2017. Overall, Galba populations were detected in 161 sites and 1,722 individuals were sampled from nine countries: Argentina, Canada, Colombia, Cuba, Ecuador, France (Guadeloupe and Martinique), Peru, Venezuela, and USA (Table S1). Galba populations have previously been reported from some of these sites in Venezuela and Ecuador by the authors (Pointier, 2015; Orlando Narváez et al., 2017). In most cases, we discovered our Galba populations in unstable habitats subject to frequent flooding and droughts. The sampled habitats were characterized as brook, irrigation canal, ditch, oxbow lake, pond, marsh, lake, tank, rice field, and river. Individual snails were often collected above the water line, consistent with their amphibious habit. Some sites were visited up to five times. Geographic coordinates were recorded for most sites. After collection, individuals were placed in water at 70 °C for 30-45 s. This procedure allows fixation of individuals without contraction of soft parts and facilitates a proper study of snail internal anatomy. The body was carefully withdrawn from the shell using forceps and both body and shell stored in 70% ethanol until morphological and



Fig. 1. The three-step procedure followed to identify the 1,722 individuals of Galba species. The number of individuals identified at each step is indicated in the left and the species identified are indicated on the right. In Step 1, we photographed the shell and dissected three to five adult snails from each of the 166 sites. Fragments of the ITS2 and COI genes were sequenced in 146 in dividuals: Galba cousini/meridensis (1), Galba cubensis (41), Galba schirazensis (41), Galba viator (1).

DNA analyses (Pointier et al., 2004).

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

2.1.1. Step 1: Morphology of the shell and of internal organs

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

2.1.2. Step 2: Multiplex PCR of microsatellite loci

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

2.1.3. Step 3: Identification by sequencing

We amplified the internal transcribed spacer 2 (ITS2) and the cytochrome oxidase subunit 1 (COI) genes in 35 individuals sampled from 15 sites in Argentina, Canada and the USA (1 to 5 per population) where at least some individuals did not demonstrate an amplification product in step 2, using the method of Lounnas et al. (2017, 2018). We also amplified ITS2 and COI in 112 individuals that did return an amplification product in step 2, including one individual identified as *G. cousini/meridensis.* To supplement these results, we also amplified the internal transcribed spacer 1 (ITS1) and the ARN ribosomal 16S in two individuals of *G. cubensis* from Bosque del Apache (USA) and in one individual of *G. cousini/meridensis* from Ecuador (Table S1). With this approach, we obtained at least one sequence from each hypothetical species represented by the four genes and used them to delimit species. The total number of individuals amplified was 151.

We used the primers NEWS2 (forward) 5' TGTGTCGATGAA-GAACGCAG 3' and ITS2-RIXO (reverse) 5' TTCTATGCTTAAATT-CAGGGG 3' to amplify ITS2; Lim1657 (forward) 5 CTGCCCTTTGTACACACCG 3 ITS1-RIXO 5 and TGGCTGCGTTCTTCATCG 3' to amplify ITS1 (Almeyda-Artigas et al., 2000); LCOI490 (forward) 5' GGTCAACAAATCATAAAGATATTGG 3' and HCO2198 (reverse) 5' TAAACTTCAGGGTGACCAAAAAATCA 3' to amplify COI (Folmer et al., 1994) and forward 5' CGCCTGTTTAT-CAAAAACAT 3' and reverse 5' CCGGTCTGAACTCAGATCACGT 3' to amplify 16S (Remigio and Blair, 1997). In all cases, PCR amplification was performed in a total volume of 25 µl containing 12.5 µl of Taq PCR Master Mix (Qiagen), 2.5 µl of each primer (10 mM) and 2 µl of DNA in an Eppendorf Thermal Cycler with an initial denaturation step at 95 $^\circ$ C for 15 min; followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for one minute, extension at 72 °C for one minute; and a final elongation at 60 °C for 30 min. The presence and size of amplification products were electrophoretically confirmed in 1% agarose gels stained with EZ-Vision. DNA sequencing was performed by Eurofins Genomics (Ebersberg, Germany) using PCR-amplified products as templates. All sequences were uploaded to GenBank (Table S1) and assigned to a species using the phylogenetic reconstruction.

2.2. Type localities

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

2.3. Retrieving data on Galba spp. distribution from published work

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

2.4. Phylogenetic and ancestral reconstruction study

Phylogenetic analyses were conducted on the ITS2 and COI sequences obtained in this study, together with ITS2, COI, ITS1, and 16S sequences retrieved from GenBank. This is the first phylogenetic study conducted in *Galba* species that use such a large dataset including 796 sequences: 90 for 16S, 251 for COI, 122 for ITS1, and 333 for ITS2. Previous studies were based on single genes and smaller sample sizes. For instance, the description of *G. meridensis* is based from a single locality (Bargues et al., 2011b). Some GenBank accession numbers appear more than once in Tables S2–S3 because individuals with identical sequences have been registered under the same GenBank accession number.

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

We used Bayesian inference in Beast2 (Bouckaert et al., 2014) for five reasons: (1) to assign sequences to species; (2) to validate, and to mend if necessary, species identity for sequences retrieved from GenBank; (3) to

Table 1

Type localities of *Galba* species and sequences of individuals recovered in those localities. References mentioned are the ones in which sequences were provided and not in which species were described, except for articles describing *Galba meridensis* and *Galba neotropica* were the authors also provided sequences (Bargues et al., 2007, 2011b). The type localities for *Galba cubensis* and *Galba viator* were here restricted because original description of the species did not provide a locality, authors simply stated "Cuba" and "Patagonia", respectively (D'Orbigny, 1835; Pfeiffer, 1839).

Species	Country	Locality	Coordinates	ITS1	ITS2	165	COI	Reference
Galba cousini	Ecuador	Chanchu-Yacu	00°18′55′'S 78°34′02′'W	FN598157	FN598153	-	FN598161	Bargues et al. (2011b)
Galba cubensis	Cuba	Vaqueria 21	23°01′N 82°32′W	AM412226	AM412223	-	AM494009	Bargues et al. (2007)
Galba humilis	USA	Owego, New York	42°06'01''N	FN182193,	FN182191,	-	FN182197,	Correa et al.
			76°15′40′'W	FN182194	FN182192		FN182198	(2011)
Galba meridensis	Venezuela	Laguna Mucubají (Mérida)	08°47′52″N 70°49′32″W	FN598159	FN598154	-	FN598164	Bargues et al. (2011b)
Galba neotropica	Peru	Lima, Rio Rimac	12°02'S 76°56'–77°08'W	AM412228	AM412225	-	AM494008	Bargues et al. (2007)
Galba schirazensis	Iran	Taleb Abad river, Bandar Anzali, Gilan province	37°27′46″N 49°37′07″E	JF272603	JF272601	JF272605	JF272607	Bargues et al. (2011a)
Galba truncatula	Germany	Thuringia, Erfurt- Bindersleben	ND	-	-	-	EU818799	Albrecht et al. (2008)
Galba viator	Argentina	Frias	40°14′ S 64°10′ W	JN614428	HQ283265, JN614465	-	JN614397, JN614398	Correa et al. (2011)

delimit species; (4) to reconstruct the phylogeny and estimate time divergence; and (5) to assess the ancestral phenotypic state of Galba species. Because we did not have identical sets of individuals (or populations) across genes it was necessary to build four unlinked gene trees to address questions (1) and (2). The best-fitting models of sequence evolution for each gene were selected using bModelTest (Bouckaert and Drummond, 2017). We estimated a model for each COI partition (1st, 2nd, and 3rd position). The best model describing the evolution of 16S was HKY + G + I, 123424 + G + I for COI (1st codon), 121321 + G + Ifor COI (2nd codon), TN93 + G + I for COI (3rd codon), 123424 + G + I for ITS1, and 121,323 for ITS2. We linked trees for the three COI codon partitions. The analyses were run using four gamma categories and a proportion of 0.5 invariant sites. Uncorrelated relaxed-clock models were chosen for all loci. The relative clock mean priors were all lognormal (M = 0, S = 1). We used a birth-death model as priors with lognormal birth and death rates. These gene trees allowed us to evaluate species names and validate or mend species identity for all sequences, whether obtained in this study or retrieved from GenBank. All the MCMC were run for 200,000,000 generations storing every 20,000 generations. The MCMC output was visualized using Tracer (Rambaut et al., 2018) and tree samples summarized by TreeAnnotator (utility program distributed with the Beast package) using a 10% burn-in. The species tree was visualized and edited in FigTree and GIMP (https://www.gimp.org). The names of the eight widely-recognized species in the genus Galba were ultimately assigned by reference to their type localities. We also built haplotype networks for each gene using popART (Leigh and Bryant, 2015) and compared them with gene trees.

To address our question (3), species delimitation, we built ten multispecies coalescent tree models in StarBeast2 (Ogilvie et al., 2017) differing in species assignments, some models splitting species to as many as nine while others lumping to as few as five (Fig. S2). We assigned the species identity obtained from tasks 1 and 2 to each of the 796 sequences (Tables S1-S3) and tested each scenario in turn. Species assignments were inferred from the literature and our own observations. Galba truncatula, G. schirazensis, and G. humilis were considered as different species in every scenario. However, G. cousini and G. meridensis were considered as a single species in half of the models and as two species in the other half since literature considers them as separate species (Bargues et al., 2011b) but we suspect they could be the same species since they both share the same distinct morphology and restricted distribution. Galba cubensis, G. viator, G. neotropica, and Galba sp. "Bosque del Apache" were considered as one, two, three, or four species depending the model since some studies considered them

different species (Bargues et al., 2007) but previous genetic population studies (Lounnas et al., 2017) and the gene trees here constructed (task 1) suggest they could be synonymies. We also created an eleventh model in which we separated the populations of *G. viator* from Argentina and from Chile to test whether splitter models showed higher support than lumper models regardless of their biological meaning. In StarBeast2, not all the individuals but all the hypothetical species must be represented by at least one sequence of all genes. Thus why, we accordingly removed the sequences from Ethiopia because this hypothetical species was represented by two genes.

In all our multispecies coalescent tree models, we used the same site models as for reconstructing the gene trees. We assigned to the mitochondrial loci a gene ploidy of 0.5 and to nuclear loci a gene ploidy of 2.0 (diploid). We used constant population sizes (fixed to 1) and uncorrelated relaxed-clock models for all loci. For nuclear loci, we used the molecular clock rate reported by Coleman and Vacquier (2002) for ITS in bivalves (0.00255 per Myr). For mitochondrial loci, we retained the molecular clock rate estimated by Wilke et al. (2009) for COI in invertebrates (0.0157 per Myr). We used the birth-death model as tree prior with lognormal birth and death rates. We ran each model using Multi-Threaded Nested Sampling analysis with 10 particles, 8 threads, a chain length of 100,000,000 and sub-chain length of 5,000. Then, we compared the trees by computing Bayes factor (BF), a model selection tool that is simple and well-suited for comparing species-delimitation models (Leaché et al., 2014).

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

We also applied two other species-delimitation methods: Automatic Barcode Gap Detection (ABGD; Puillandre et al., 2012) and Species Tree and Classification Estimation in Beast2 (STACEY, Jones, 2017). ABGD was run for each gene using the default settings (https://bioinfo.mnhn. fr/abi/public/abgd/). ABGD does not rely on tree shapes but on divergence and it requires an appropriate prior value of maximal intraspecific divergence. This prior defines the threshold between intra- and

interspecific pairwise distances and is iterated from minimum to maximum through ten steps (Puillandre et al., 2012). If this value is underestimated, the result is an oversplit classification, and if overestimated, the classification is overlumped. Given the wide genetic diversity observed within some species (Lounnas et al., 2017), we chose the partition that showed high prior on intraspecific divergence (the penultimate partition).

The STACEY delimitation method, as the Nested Sampling analysis, also relies on a Multispecies Coalescent Model. However, STACEY does not require setting up predefined scenarios in terms of species number and sample assignment. The method distinguishes very shallow species divergences with a statistic called "collapseHeight," which we set to a small value (0.0001) following Jones (2017). Given the results obtained with ABGD and Nested Sampling analyses, we ran three multispecies coalescence models with different collapseWeight parameters: (1) a lumping model with six *Galba* species with 1/X distribution (initial value: 0.975, between 0 and 1), (2) a splitting model with nine species

with 1/X distribution (initial value: 0.952 between 0 and 1), and (3) a no-prior-taxonomic model with a Beta distribution ($\alpha = 2$, $\beta = 2$). The initial value of the lumping and splitting models were calculated following Matos-Maravi et al. (2019). All other parameters were set as in the Nested Sampling analysis. We ran the three models for 250,000,000 generations with storing every 25,000 generations. The MCMC output was visualized using Tracer (Rambaut et al., 2018). For STACEY, we used only the ITS2 and COI sequences because these both genes were better represented in the dataset than ITS1 and 16S. Since the hypothetical number of species in STACEY ranges from one to the number of individuals, each of our 113 snail populations was considered as a minimal cluster.

To address our question (4), regarding species topology and divergence time, we reran the multispecies tree model that showed the highest Bayes Factor in StarBeast2. We used the same parameters as in the Nested Sampling analysis, but we ran the MCMC for a longer time (250,000,000 generations stored every 25,000 generations). The MCMC



Fig. 2. Geographic distribution of Galba species in America, based on molecular identification. Coordinates are given in Tables S1–S2. The sites Perdriel (Argentina), Batallas and Tambillo (Bolivia), Ontario (Canada), San Rafael (Mexico), Canal Salinas (Puerto Rico) are not represented since coordinates are missing in the original publications.

output was visualized using Tracer (Rambaut et al., 2018) and tree samples summarized by TreeAnnotator (utility program distributed with the Beast package) using a 10% burn-in. The species tree was visualized and edited in FigTree, GIMP, and DensiTree (Bouckaert and Heled, 2014). Some analyses were run in CIPRES Science Gateway (Miller et al., 2012).

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

3. Results

3.1. Morphology

Most individuals (N = 1,420 from 133 sites) were not distinguishable based on shell and reproductive anatomy (Fig. S3). The exception was a single group comprising all individuals from *G. cousini* and *G. meridensis* (N = 302). These tended to demonstrate more globose shells with shorter spires, adult sizes in excess of 10 mm standard shell length. *Galba cousini* and *G. meridensis* also differed from the other species in their internal anatomy—a ureter with two distinct flexures, a wider and more ovate prostate, a larger penial complex, and a penis sheath approximately the same length as the preputium (Fig. S3). We did not find any anatomical differences between *G. cousini* and *G. meridensis*, however, comparing individuals from Ecuador, Colombia, and Venezuela (Fig. 2).

3.2. Multiplex PCR of microsatellite loci

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

3.3. Identification by sequencing and phylogenetic analysis

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

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

Gene trees (Figs. S4–S7) and haplotype networks (Figs. S8–S11) showed that genetic diversity varied among the six clusters and four genes. Cluster II (*schirazensis*) showed reduced variation, while cluster VI (*viator / cubensis / neotropica /* Bosque del Apache sample) was larger

and more diverse. Mitochondrial genes seemed more diverse than nuclear genes. This observation may be biased, however, by a structural correlation in our data between genes sequenced and regions sampled.

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

The topology of all gene trees showed that clusters III (*humilis*), IV (*cousini*), and V (*meridensis*) grouped together. But inconsistent results among genes were obtained for the remainder of the other identified clusters (Figs. S5–S8). The multilocus multispecies tree returned three major groups: cluster I (*truncatula*) together with II (*schirazensis*); cluster III (*humilis*) together with IV (*cousini*) and V (*meridensis*); and the cluster VI group (*viator / cubensis / neotropica /* Bosque del Apache sample; Fig. 4). The multilocus multispecies tree visualized in DensiTree (Fig. 3) confirmed that most tree topologies united the clusters into the three major groups outlined above, although some topologies placed clusters differently reflecting the incongruence found among the gene trees.

3.4. Species delimitation

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

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

The species-delimitation analysis implemented in STACEY suggested that six of the nine clusters of scenario A might include more than one taxon. The exceptions were *G. viator*, *G. meridensis*, and *Galba* sp. "Bosque del Apache", the last two species including only one population. Our STACEY results converged towards similar MCMCs regardless of which prior was used for the collapseWeight parameter (Fig. 3).

3.5. Time of divergence and state reconstruction

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



Fig. 3. Time-calibrated phylogenetic hypotheses of the model that best approximate species status in Galba and species-delimitation methods. The trees showed are the most probable topologies based on the multispecies tree model that showed the highest Bayes Factor in StarBeast2 (visualized in Densitree), as a function of time (in Mya). Greater topological agreement is visualized by a higher density of trees (on the left), whereas uncertainty in the height and distribution of nodes are represented by increased transparency. The most common topologies are shown in blue, the second most common topologies in red and the third in green. The six clusters built with individual gene trees are indicated on the extreme right. The number of species recovered varied with eight approaches (ABGD with each gene, StarBeast with all genes, STACEY with a variable number of species included as prior). For each approach, the colored bars represent different species. Bars were striped when the groups included more than one species. Clusters as appeared in text are shown in roman numbers. Scale bar represents branch length expressed as number of substitutions per site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

4.1. Galba comprises six species or species complexes

Here we report the largest study published to date of *Galba* systematics and distribution, based on extensive sampling at a very large geographical scale and integration of phenotypic and molecular approaches across all DNA sequences available in GenBank for four genes (Dayrat, 2005). The widespread occurrence of self-fertilization in these populations essentially voids the biological species concept (Coyne and Orr, 2004), and the absence of any reliable morphological distinction obviates the typological one. Thus, we are left with a phylogenetic approach, which suggests the existence of six clusters, perhaps corresponding to as many as nine species, or as few as six. These findings reinforce several previously-published works that have involved fewer genes and smaller sample sizes (Correa et al., 2010, 2011; Bargues et al., 2011b, 2011a; Standley et al., 2013; Lounnas et al., 2017).

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

The sixth cluster includes the nomina Galba viator (d'Orbigny, 1835), Galba cubensis (Pfeiffer, 1839), Galba neotropica (Bargues, Artigas, Mera y Sierra, Pointier & Mas-Coma, 2007), and a population from southern USA (Bosque del Apache). However, any distinction between the sets of populations within this cluster depends on both the genes and phylogenetic methodology employed (Fig. 3). Most species delimitation methods suggest that this cluster comprises four species but others suggest two or even one species (Fig. 3). Discrepancies could be due to the genes analyzed. Note, for instance, that G. cubensis is not monophyletic when analyzing the COI and ITS2 trees and haplotype networks which would be affecting the species delineation process. The distance separating G. cubensis and G. neotropica is limited (1 Mya). Moreover, microsatellite markers defined in G. cubensis amplified effectively in individuals of G. neotropica (Lounnas et al., 2017), suggesting a very short genetic distance. Additional sampling, especially in the North and South of the distribution range (Argentina and USA) and the analysis of other genes would help resolve the status of this cluster. On the whole, a cautious position would be to suggest that cluster VI corresponds to a species complex or a species with wide diversity, as has been found in other freshwater snails from the clade Hygrophila (e.g., Ebbs et al., 2018 in Physa; Mavárez et al., 2002 in Biomphalaria; Pfenninger et al., 2006 in Radix). We also note that if we ultimately recognize a single species; its



Molecular Phylogenetics and Evolution 157 (2021) 107035

Fig. 4. Most common topology of the species tree and phenotype of the most recent common ancestor of Galba inferred in StarBeast2. The probability of a cryptic phenotype in the most recent common ancestor of Galba species is 100% according to S -DEC and BBM and 78% according to S-DIVA. Clusters as appeared in text are shown in roman numbers. Node values indicate posterior probability and blue bars indicate 95% credibility intervals. The mating system is indicated on the right. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

name should be *viator*, and not *cubensis* or *neotropica*, based on prior description.

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

In North America, Burch (1982) recognized 22 species of small, muddwelling lymnaeids, which he grouped into the genus Fossaria with two subgenera, Fossaria (s.s.) with 11 species and Bakerilymnaea with 11 (see Table S5 for species names). Johnson et al. (2013) transferred these species to the genus Galba, but otherwise retained the Burch system. Included in the present analysis were topotypic samples of obrussa from Philadelphia and parva from Cincinnati, both of which we here show to be indistinguishable from topotypic humilis, collected at Owego, New York. Remigio (2002) contributed to Genbank a 16S sequence from a Canadian population he identified as Fossaria obrussa, also grouping with cluster III (humilis). We suggest that obrussa, parva, and the seven other uniquely North American specific nomina listed above ascribed by Burch (1982) to the subgenus Fossaria are junior synonyms of G. humilis, setting aside American populations of G. truncatula as distinct. In addition to his obrussa sequence, Remigio (2002) contributed a 16S sequence from Oklahoma to Genbank which he labelled "Fossaria bulimoides". This sequence grouped with cluster VI in our analysis. We suggest that all 12 specific nomina ascribed by Burch (1982) to the Fossaria subgenus Bakerilymnaea (Table S5) including bulimoides (Lea 1841), are junior

synonyms of the species or species complex of cluster VI (*viator / cubensis / neotropica /* Bosque del Apache sample).

Two of the three species-delimitation models here used (Nested Sampling analysis and STACEY) are based on Multispecies Coalescence, assuming gene flow within species though not between species (Sukumaran & Knowles, 2017). Thus, the use of these models would be inappropriate in a fully selfing species group. As mentioned above, the selfing rate in *Galba* species and populations is high (often ca. 0.9), but outcrossing does occur (Chapuis et al., 2007; Lounnas et al., 2017, 2018). Further research is needed to explore how selfing may affect speciation. *Galba* species would be excellent models to investigate such questions, since selfing rates vary so greatly among species and populations (Chapuis et al., 2007; Lounnas et al., 2017, 2018).

4.2. A set of cryptic species

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

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

Among the freshwater pulmonates, cryptic species have previously

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

Four hypotheses have been offered to explain the occurrence of cryptic species: recent divergence, parallelism, convergence, and stasis (Bickford et al., 2007; Fišer et al., 2018; Struck et al., 2018). The recent divergence hypothesis seems unlikely in this case. Galba has no closely related groups; its closest relatives are probably the stagnicoline lymnaeids of North America and Eurasia, which demonstrate a very distinctive morphology (Aksenova et al., 2018). Our analyses suggest that the several species of Galba are separated by more than 20 Myr (Burgarella et al., 2015). And indeed, the morphological divergence demonstrated by G. cousini and G. meridensis suggests that time has not been a significant constraint. The parallelism hypothesis also seems unlikely given that, based on our phylogenetic reconstruction, the cryptic morphology is ancestral for Galba, and the only other morphology that has evolved in the group, as demonstrated by G. cousini and G. meridensis, is derived. Nor does the topology of lymnaeid phylogeny fit the convergence hypothesis (Correa et al., 2010). So, by default, morphological stasis is left as the most likely hypothesis to explain the presence of cryptic species in the genus Galba, as has been proposed in other gastropod groups (e.g., Gomez et al., 2004; Struck et al., 2018).

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

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

4.3. Conclusions and future directions

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

Data and code accessibility

Xml files for phylogenetic analyses are available from the Zenodo repository (https://zenodo.org/record/3473937#.XZiPcCOryTd).

CRediT authorship contribution statement

Pilar Alda: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft. Manon Lounnas: Investigation, Writing - review & editing. Antonio A. Vázquez: Investigation, Writing - review & editing. Rolando Ayaqui: Resources. Manuel Calvopiña: Resources. Maritza Celi-Erazo: Resources. Mobert T. Dillon: Resources, Writing - review & editing. Luisa Carolina González Ramírez: Resources. Eric S. Loker: Resources. Jenny Muzzio-Aroca: Resources. Alberto Orlando Nárvaez: Resources. Oscar Noya: Resources, Writing - review & editing. Andrés Esteban Pereira: Resources. Luiggi Martini Robles: Resources. Richar Rodríguez-Hidalgo: Resources. Nelson Uribe: Resources. Patrice David: Resources, Writing - review & editing. Philippe Jarne: Conceptualization, Resources, Writing - review & editing. Jean-Pierre Pointier: Resources, Writing - review & editing. Sylvie Hurtrez-Boussès: Conceptualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We would like to express our gratitude to Nicolás Bonel for useful comments on earlier drafts of the manuscript and Harry G. Lee for advice and assistance on the taxonomy. We thank Jimena Guerrero, Björn Stelbrink and Thomas Wilke for suggestions on phylogenetic analyses and Graham R. Jones, Patricio Maturana Russel and Remco R. Bouckaert for assistance in running STACEY and Multi-Threaded Nested Sampling. We thank the reviewers Pável Matos-Maraví and Christelle Fraïsse and the recommender from PCI in Evolutionary Biology Fabien Condamine for their thoughtful comments and suggestions. Fellowships granted by Erasmus Mundus PRECIOSA and Méditerranée Infection supported research stays of PA at the Institute de Recherche pour le Développement, MIVEGEC (Montpellier, France). AV was supported by a grant from IRD (BEST) and ML by a doctoral fellowship from University of Montpellier and a post-doctoral grant from Labex CeMeb. This study was financially supported by IRD, CNRS, ECOS-SUD (A16B02) and Malacological Society of London. Version 3 of this preprint has been peer-reviewed and recommended by Peer Community In Evolutionary Biology (https://doi.org/10.24072/pci.evolbiol.100089).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2020.107035.

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Molecular Phylogenetics and Evolution 157 (2021) 107035

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