Round #1

Decision

by Fabien Condamine, 2019-07-19 16:00
Manuscript: https://doi.org/10.1101/647867

Decision for Ms. Alda et al. (https://www.biorxiv.org/content/10.1101/647867v1**): Revision needed**

Dear authors,

Thank you for soliciting the Peer Community in Evolutionary Biology to assess your study.

We have received the feedback of three reviewers for your preprint study. You will see that the three referees are positive about the paper but they also bring up very interesting and useful comments as well as suggestions that I am sure will improve the study. Overall, I agree with the reviewers that the study has many merits and that the findings are interesting. I also think the approach proposed here is original and may be useful for further studies in taxonomy and systematics of difficult groups, especially for invertebrate clades. That being said, the study should be more rooted in the concept of integrative taxonomy (e.g. Dayrat 2005 – Biol. J. Linn. Soc.). In addition, the study suffers from some methodological and conceptual issues. I think the main issues concern the phylogeny and dating analyses, but because these results are the cornerstone for the interpretation of species delimitation, the corresponding results may be inconclusive as it stands. The referees also felt that the manuscript suffers from a lack of clarity in several parts of the text, especially in the Methods and the interpretations (Results and Discussion).

Response 1: We would like to thank the recommender and the reviewers for their thoughtful comments that help us to greatly improve our manuscript. We tried to address all the methodological and conceptual issues raised by the reviewers. We (i) included more samples, (ii) added methods to statically delimit species and to date the species tree, and (iii) restructure the discussion section. Although the results are similar to those described in the previous version of the manuscript, the current conclusion is less subjective and more evidence-based. We also hope to have fixed all the text parts that were unclear in the previous version.

We think that the previous version of the manuscript was already rooted in the concept of integrative taxonomy since we tried to delimit species from multiple and complementary perspectives: phylogeography, comparative morphology, and ecology. However, we were not clear enough in this respect and we added a sentence in the Introduction section to mention this concept (Lines 171-172, clean version): “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 its crypsis, wide geographical distribution and mating system."

To summarize, I have identified six major points raised by the reviewers that you would need to carefully address. This includes the following:

(1) Running phylogenetic analyses removing the third position of mitochondrial coding genes from the alignment;

Response 2: We used two mitochondrial genes in this study: the non-coding protein 16S and the coding protein COI genes. In the previous version of the manuscript (Lines 380-382), we made a mistake in saying that the third position of the COI gene was highly saturated. We wrongly interpreted the results obtained in DAMBE (Xia 2017). We found that the index of substitution saturation (Iss) was significantly lower than the critical Iss value (Iss.c) when considering 32 OTUs (the maximum number of operational taxonomic units calculated in DAMBE) and a symmetrical topology (which is most probably the case of the history of this gene in *Galba*):

Positions 1 & 2: Iss = 0.253, Iss.cSym = 0.692, T = 12.747, df = 202, p = 0.0000

Position 3: Iss = 0.567, Iss.cSym = 0.690, T = 2.949, df = 202, p = 0.0036

So, the third position of COI was little saturated. Moreover, it was highly informative. We reran the phylogenetic analyses removing the third position of COI and the obtained tree was inaccurate (sequences of not-related individuals were grouped in the same cluster). Including the third position lead us to a better phylogenetic reconstruction than not including it. This is because excluding the third position left us with few substitutions to work on, but also because substitutions at this position might better conform to the neutral theory of molecular evolution than those at the other two positions (Salemi et al. 2009).

Thus, we conserved the third position of the COI gene and we replaced "We found that the third codon position of COI was highly saturated." by "We did not find evidence of saturation in the four genes analyzed, including all codon positions of COI." (Lines 305-306, clean version).

(2) Running a second dating analysis using relaxed clocks and compare the results obtained with a strict clock;

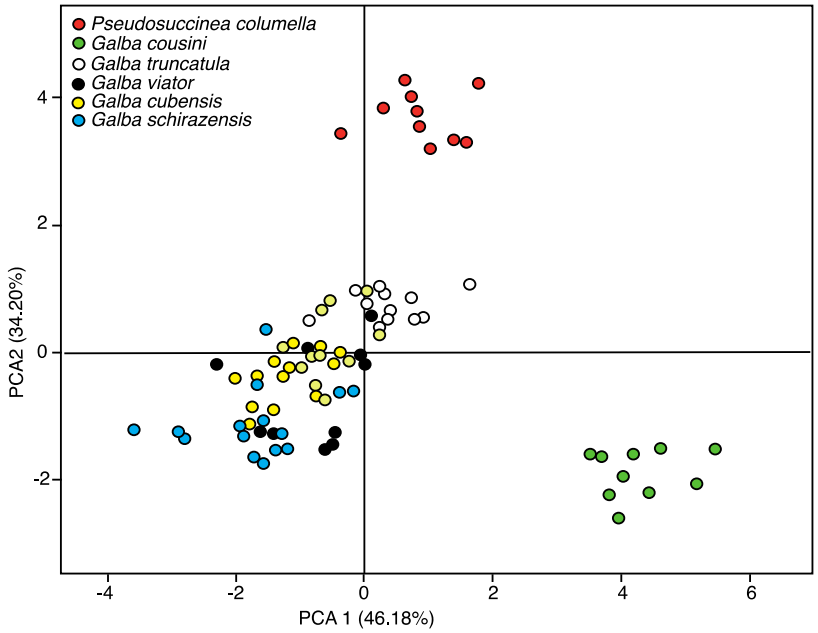
Response 3: Done. We reanalyzed data using relaxed clocks and compared the results obtained with a strict clock. Results were similar. The way of clustering species (or miniclusters) was the same under relaxed and strict clocks. However, topologies, posterior probabilities, and branch lengths changed a bit. For instance, the 16S tree had a different topology in the relaxed and strict clocks but the one with the relaxed clock was similar to the topology of the COI tree. We decided to keep the trees with relaxed clocks since it would be the ones that better reflect the history of the gene trees given the long evolutionary history of the group. Note that the gene trees were only used to assign names to species (or miniclusters, like *Galba* sp. from Bosque del Apache that seemed different from the other populations of *G. cubensis*) to then test and delimit species by using a multispecies coalescent model that reconcile gene trees.

Accordingly, we modified figures S5-S8 and replaced the following phrase in Lines 320-322 (clean version): "We used a strict clock and a birth-death model as priors with lognormal birth and death rates." by "Uncorrelated relaxed-clock models were chosen for all loci. The relative clock mean priors were all lognormal (M = 0, S = 1).". We also replaced the following phrase in Lines 342-343 (clean version) "... we used strict clock models for both partitions." by "We used constant population sizes (fixed to 1) and uncorrelated relaxed-clock models for all loci".

(3) Performing a multivariate analysis on some quantitative trait(s) and calculating a distance between clusters on this morphometric space;

Response 4: Unfortunately, it is not possible to quantitatively differentiated cryptic *Galba* species. As we mention in our manuscript (Lines 588-592, clean version), "Our methods here were strictly qualitative, as was the case for *Ancylus* and *Radix*, because previous studies (Samadi et al. 2000; Correa et al. 2011) have shown that the dimensions of internal organs depend on physiological state and hence that species cannot be distinguished by means of such measurements.".

Thus, we found useless to perform a quantitative test. Our research group performed such an analysis a few years and showed that cryptic *Galba* species have a very similar shell and internal anatomy (Correa et al. (2011). In this study, shell and internal anatomy were quantified in 81 individuals from the seven taxa considered in our study, clearly showing that shell or internal anatomy do not permit to determine the taxonomic status of individuals sampled in natural populations of *G. cubensis*, *G. neotropica* (considered as a synonym of *G. cubensis* in our manuscript), *G. viator*, *G. schirazensis* (*Galba* sp. in the original article) and *G. truncatula*. See the figure below extracted from Correa et al. (2011).



We included the following sentence in the Material and Methods section (Lines 217-220, clean version) and add a figure in Supplementary Materials (Fig. S2) to clarify why we think that this analysis would be useless: "We did not record any morphological

measurements or perform any quantitative tests because Correa et al. (2011) have shown that cryptic *Galba* species cannot be delimited by such means. Our observations were qualitative only (Fig S2).".

(4) Strengthening the analyses of ancestral state reconstructions, perhaps with the use of other models (e.g. maximum-likelihood models like Dispersal-Extinction-Cladogenesis; Ree & Smith 2008 – Syst. Biol.) and by including the uncertainty around the node estimates;

Response 5: We reran the analyses of ancestral state reconstructions applying three models to address this point: Bayesian Binary MCMC (BBM, Ronquist and Huelsenbeck 2003), statistical dispersal-vicariance analysis (S-DIVA; Yu et al. 2010), and Lagrange dispersal-extinction-cladogenesis (DEC; (Ree and Smith 2008). All points to the cryptic phenotype as the ancestral state.

We replaced the following phrases "We added a discrete trait partition with the phenotypic state (one for all cryptic species vs. another one for *G. cousini*) to infer the ancestral phenotypic state of *Galba*." by "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 Lagrange dispersal-extinction-cladogenesis (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 states: one for all cryptic species and another one for *G. cousini*/*meridensis*." in the Material and Methods section (Lines 393-399, clean version).

We also replaced the following phrases "The phenotypic state reconstruction showed that the most recent common ancestor of *Galba* species had a cryptic phenotype. Thus, the non-cryptic phenotype of *G. cousini* would be a derived state from the cryptic phenotype (Fig. 3)." by "The three phenotypic state reconstruction analyses (S-DEC, S-DIVA and BBM) suggested that the most recent common ancestor of *Galba* species displayed the cryptic phenotype (Fig. 4). Thus, the phenotype of *G. cousini* and *G. meridensis* should be considered as derived from the cryptic phenotype." in the Results section (Lines 492-496, clean version).

(5) Clarifying the rationale used for distinguishing cryptic species and the evolutionary scenarios tested;

Response 6: Done. In this new version of the manuscript, we added analyses to try to distinguish cryptic species and to clarify the evolutionary scenarios tested.

First, we statically delimit cryptic species by applying three methods: (1) running ten multispecies coalescent tree models that differ in species assignments and comparing them with by computing Bayes factor, (2) inferring species hypotheses based on automatized identification of barcode gaps between inter- and intraspecific pairwise distances in sequence data sets (ABGD), and (3) testing multispecies coalescent clusters using STACEY.

We added the following paragraphs in the Material and Methods section (Lines 331-381, clean version) to explain the methodology applied:

“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. We assigned the species identity obtained from tasks 1 and 2 to each individual and tested each scenario in turn. 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. Since all the hypothetical species generated in these tree models must be represented by all genes, we removed the sequences from Ethiopia in this analysis.

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 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).

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/). The prior on intraspecific divergence defines the threshold between intra- and interspecific pairwise distances and is iterated from minimum to maximum through ten steps (Puillandre et al. 2012). 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).
For the STACEY delimitation method, 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. The method distinguishes very shallow species divergences with a statistic called “collapseHeight,” which we set to a small value (0.0001) following Jones et al. (2015). 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 five

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. (2018). 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."

We also added the following paragraphs in the Results and Discussion sections:

Lines 456-476, clean version: "Figure 3 illustrates the results obtained using the three species-delimitation methods. The Multi-Threaded Nested Sampling analysis (Fig. S13) suggested that scenario A (nine species) is the best fit to the available data, demonstrating the largest maximum likelihood estimate (Fig. 3; see Table S4 for Likelihoods). BF analysis preferred scenario A over scenario D (current taxonomy) or scenario K, separating populations of *G. viator* from Argentina and from Chile. This suggests that further splitting the phylogeny of *Galba* (here to consider that *G.* meridensis and *G.* cousini are separate species) is not required.

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).".

Note that StarBeast2 can be used only when all hypothetical species are represented by all genes. We therefore amplified ITS1 and 16S in two individuals of *G. cubensis* from Bosque del Apache and one individual of *G. cousini*/*meridensis* from Ecuador for the current version of the manuscript. We accordingly added some sentences in the Material and Methods section:

Lines 238-243, clean version: "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.".

Lines 245-252, clean version: “We used the primers ... Lim1657 (forward) 5’ CTGCCCTTTGTACACACCG 3’ and ITS1-RIXO 5’ TGGCTGCGTTCTTCATCG 3’ to amplify ITS1 (Almeyda-Artigas et al. 2000); ... and forward 5’ CGCCTGTTTATCAAAAACAT 3’ and reverse 5’ CCGGTCTGAACTCAGATCACGT 3’ to amplify 16S (Remigio and Blair 1997).”.

Lines 294-296, clean version: “Ultimately, we included 796 sequences in our analyses: 90 for 16S, 251 for COI, 122 for ITS1 and 333 for ITS2.”.

Second, we time-calibrated the species tree. This analysis enabled us to withdraw the scenario of “recent divergence”, getting an overall divergence of 22.6 Mya. It is therefore very unlikely that crypticity among species is due to recent divergence.

We added the following paragraphs in the Material and Methods section:

Lines 343-346 (clean version): “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).”.

Lines 382-385 (clean version): “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).”

We also added the following sentences in the Result section (Lines 489-492, clean version): “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 *G. cousini*/*meridensis* species complex and the *G. cubensis*/*viator* species complex seems to have occurred 5 Mya ago, or less.”

(6) Taking into account all the comments that aim at improving the text, the hypotheses, and the figures.

Response 7: All recommender’s and reviewers’ comments were taken into account and we think that this new version of the manuscript is clearer and more straightforward than the previous version.

Based on the referees’ comments and my reading, I believe the manuscript will benefit from a revision and a second round of reviews. If you chose to resubmit a revised paper, please make a point-by-point reply to the comments (like for a traditional journal). For the moment, I do not recommend the study in PCi Evol. Biol. but if the revision is thorough (satisfies the reviewers) and the results still support the conclusions, I will be supportive for the paper as being recommended.

Dr. Fabien Condamine, recommender for PCi Evol. Biol.

Reviews

Reviewed by Pavel Matos, 2019-06-17 10:37

In this study, Pilar Alda et al aimed to delimit species in the freshwater snail genus Galba. In the process, they aimed to clarify the systematics and the evolution of morphological resemblance among species. They developed a 3-step approach where morphology, microsatellites, and DNA sequences of 4 loci informed species identities. By using recent phylogenetic methods including the multispecies coalescent and ancestral state inference, the study suggested the existence of at least 5 Galba species. However the inferred gene and species trees seem to support disparate phylogenetic relationships among species. If this gene tree discordance was caused by incomplete lineage sorting, the use of the multispecies coalescent model is largely justified. But other causes, such as hybridization and inter-species gene flow, have not been adequately treated by the methods in the manuscript and should be acknowledged in the text. Finally, the authors suggested that the most recent common ancestor of Galba was likely "cryptic", and they supported a hypothesis of morphological stasis through time, perhaps driven by stabilizing selection associated to environmental conditions.

Response 8: We acknowledge that *Galba* is a group that could have undergone historical hybridization—recall though that most of these species are highly selfing, limiting genetic exchanges. Gene flow between species could be one of the reasons of the observed incongruence between gene and species tree (Figs. S5-S8 vs. 4). We have already expressed this idea in the previous version of the manuscript (Lines 655-661, clean version): "The inferred phylogenetic relationships among species varied, depending on the genes analyzed and techniques employed. Such a discordance is not unusual and has been reported in many different studies (e.g., Kutschera et al., 2014; Stewart et al., 2014; Suh et al., 2015), including in mollusks (Krug et al. 2013; Sales et al. 2013). Incomplete lineage sorting or introgressive hybridization of specific genes may indeed lead to such a result (Felsenstein 2004).".

We agreed, however, with the reviewer that hybridization has not been tested in the manuscript. In a future work, we would like to study which evolutionary processes gave rise to the incongruence observed in gene and species trees. It would especially be interesting to test these processes in the two species complexes of *Galba* (*G. cubensis*/*viator* and *G. cousini*/*meridensis*) and to try to disentangle if they are two single species with a wide genetic diversity or, in fact, different species. However, since these species have a large geographic range and low inter-species gene flow, we think that more extensive sampling than the one used here and further population genetic studies are needed to adequately analyze these evolutionary processes. We added sentences in the Discussion section to include this future perspective:

> Lines 661-663 (clean version): "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."
>
> Lines 692-694 (clean version): " ... (ii) evaluate whether the *G. cubensis*/*viator* and *G. cousini*/*meridensis* groups constitute species complexes or species with wide diversity; ... ". We expect to tackle this goal in a near future.

This study used a comprehensive taxon sampling, multiple Lines of evidence, and state-of-the-art methods. The research questions are relevant to other fields in evolutionary biology and thus the manuscript can be of general interest. I believe that there are still few issues that need to be clarified or strengthened in order to improve the manuscript.

INTRODUCTION

Line 98-110:

These Lines need rewording. The first sentence of the paragraph refers to at least two issues that are problematic with cryptic species. But the following sentences discuss the second issue on biological invasions and a new third issue on disease transmission. Where is the first issue?

Response 9: Thank for this remark. The Lines were reworded. We now mention two issues that are problematic with cryptic species: biological invasions and disease transmission (Line 105, clean version).

MATERIAL AND METHODS

Line 193:

I suggest moving Fig S2 into the main article because it nicely explains your 3-step approach. The figure can be improved by adding the total number of individuals analyzed in each step.

Response 10: Done. Figure S2 (now Figure 1) was moved to the main text. The total number of individuals analyzed in each step was added. We also accordingly changed (incremented) figure numbers throughout the manuscript.

Line 209:

How many microsatellite loci have you targeted in the 1,722 individuals?

Response 11: Three microsatellite loci (one per species) were applied to all the 1,420 individuals that were cryptic species. We apologize for this mistake because in Lines 208 (previous version) it reads “1,722 individuals” but it should have said “1,420 individuals”. So, we corrected the number of individuals and we specified the number of microsatellite loci that are used in the multiplex PCR.

> We replaced the phrase “We applied the multiplex PCR designed by Alda et al. (2018) to all 1,722 individuals. It is based on species-specific primers amplifying microsatellite loci targeting three species ...” by “We applied the multiplex PCR 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 per each targeted species) ...” (Lines 228-231, clean version).

Line 281:

The third codon position of COI was highly saturated. It will be helpful to run the phylogenetic analyses removing such nucleotides from the alignment, and to compare it with the results presented in the manuscript.

Response 12: Thanks for this remark. Please, see Response 2.

Line 287:
The mitochondrial gene trees are in fact not independent. These loci are linked and thus share the same phylogenetic history.

Response 13: Right, the mitochondrial gene trees are not independent. However, in order to link trees across partitions (genes) you must have identical sets of taxa, which is not the case in our study, since most of the individuals were not sequenced at the four genes sequenced. In this study, we obtained the mitochondrial gene COI for 80 individuals and the 16S gene for 3 individuals. Moreover, in most of the sequences retrieved from GenBank, you cannot know which sequences belong to the same individual. This explain why we cannot link trees.

However, in this new version, we clarified this point, replacing "To address points 1 and 2, we built four independent gene trees" by "To address points 1 and 2, we built four gene trees. We unlink all gene trees because we could not link trees across genes since we did not have the identical sets of individuals (nor populations) for the four genes." (Lines 316-318, clean version).

Line 294:
Given the apparent long evolutionary history of the group, ~ 20Myr, the use of strict clock might not be adequate. It will be helpful to run a second comparative analysis using relaxed clocks.

Response 14: Please, see Response 3.

Line 300:
It is unclear how the gene trees allowed the identification or validation of species. Are you using any testable, quantitative criterion? It will also be informative to present in Fig. 2 the posterior probabilities on nodes.

Response 15: Thanks for this comment, highlighting that species delineation was not explained appropriately. We added methods, analyses, and figures that will help the reader to understand our methodology and that will delimitate species more robustly. In this new version of the manuscript, the gene trees and the haplotype networks were used only to assign sequences to hypothetical species. Because of the longstanding confusion and uncertainty regarding the systematics of the genus *Galba* worldwide, we established standard populations (the ones from species type localities), against which unknown populations can be compared. These hypothetical species (or mini-clusters) identified in the gene trees (and also illustrated in the haplotype networks) were then tested using three species-delimitation methods. We accordingly made several changes regarding this issue.

First, we added haplotype network analyses and clarified how species names were assigned to each sequence.

> Lines 330-336, clean version: "Species name of the eight widely-recognized species in the genus *Galba* was assessed by considering that all individuals clustering with the individual from the type locality belong to the same species. Since gene trees can be erroneously inferred due to incomplete sampling, incomplete lineage sorting or introgression between lineages, we also built

haplotype networks for each gene using popART (Leigh and Bryant 2015) and compared them with gene trees.".

Lines 444-445 (clean version): "Gene trees (Figs. S5–S8) and haplotype networks (Figs. S9–S12) showed that genetic diversity varied among the six clusters and four genes."

Second, we applied three species-delimitation methods to these hypothetical species identified in the gene trees and also illustrated in the haplotype networks. Please see Response 6 for details.

We completely modified Figure 3 (Figure 2 in the previous version of the manuscript). Thus, the suggestion about including posterior probabilities on nodes is no longer applicable. However, we included them in Figure 4 where we showed the most common topology of species tree.

Line 304:
It is unclear how the molecular dataset was used in StarBeast2. Have you used all individuals and assigned each individual a species identity?

Response 16: Yes, we used all individuals and assigned a species identity to each individual. We clarified this issue by adding the following sentence in Lines 339-340 (clean version): "We assigned species identity obtained in points 1 and 2 to each of the individuals ..."

Furthermore, StarBEAST2 simultaneously estimates gene and species trees, so it is not clear why you used BEAST2 before to infer gene trees? You could just show the gene trees estimated in StarBEAST2.

Response 17: Right. StarBEAST2 simultaneously estimates gene and species trees. However, to use StarBEAST2 we had to assign sequences to species and to validate or mend identities for sequences retrieved from GenBank. Thus, we preferred to used BEAST2 to infer gene trees and then used StarBEAST2 to infer the species tree using the results obtained from gene trees. We verified that the gene trees estimated with BEAST2 (Figs. S5-S8) show similar results to the ones estimated with StarBEAST2.

RESULTS
Line 327:
When reading this line, it is unclear the overall rationale for distinguishing cryptic species in this manuscript. Before this line, you acknowledged that morphological similarities among species occur in the genus Galba. But when it comes to G. cousini, you dismissed this rationale and assumed that the morphological similarity between individuals from Venezuela (known as a separate species, G. meridensis) and Ecuador/Colombia are because they are a single species, G. cousini.

Response 18: Right. In the previous version of the manuscript, we were assuming from the beginning that there was only one species (*Galba cousini*). In this new version, we start with two species, *Galba cousini* and *Galba meridensis* (because they have been described as such), and then we conclude that *G. cousini*/*meridensis* constitute a species complex or a single species with wide diversity. We accordingly modify the manuscript:

Lines 144-148 (clean version): We replaced “All these nominal species share a similar shell morphology and internal anatomy, except *Galba cousini* (Paraense 1995) plenty of phenotypic plasticity on shell, anatomy and life-history traits (Samadi et al. 2000; Correa et al. 2011) ...” by “All these nominal species share a similar shell morphology and internal anatomy, except *Galba cousini* (Paraense 1995) and *Galba meridensis* (Bargues et al. 2011b) that have a different—but very similar between them—shell morphology and internal anatomy. *Galba* species show plenty of phenotypic plasticity on shell, anatomy and life-history traits (Samadi et al. 2000; Correa et al. 2011) ...”

Lines 409-418 (clean version): We replaced “*Galba cousini* was the only species that could be identified at species level based on shell morphology of adults (Fig. S4) with their larger (> 10 mm) and more globose shells with shorter spires than other species. Shell length in the other species was less than 10 mm (Fig. S4). *Galba cousini* and the other species also differed with regard to their internal anatomy (i.e., ureter with two distinct flexures, ovate prostate wider than in the other species, bigger penial complex, and penis sheath about the same length than the preputium; see Fig. S4). We did not find any difference in shell morphology and internal anatomy when comparing individuals of *G. cousini* from Ecuador and Colombia on one side and from Venezuela (referred to as *G. meridensis*, Bargues et al. (2011) on the other side. Based on these differences in morphology, 302 individuals out of 1,722 were attributed to *G. cousini* and all were sampled in Venezuela, Colombia and Ecuador (Fig. 1).” by “Most individuals (N = 1,420 from 133 sites) were not distinguishable based on shell and reproductive anatomy (Fig. S4). A single group stands out including all individuals from *G. cousini* and *G. meridensis* (N = 302) and showing larger (> 10 mm vs. < 10 mm in the other species) and more globose shells with shorter spires than other species. These two species also differed from the other species in their internal anatomy (*i.e.*, ureter with two distinct flexures, ovate prostate wider than in the other species, bigger penial complex, and penis sheath about the same length than the preputium; Fig. S4). However, we did not find any difference when comparing individuals of these two species from Ecuador, Colombia and Venezuela, even if two species names have been ascribed to these individuals.”

Line 335:
It is unclear how you identified specimens using microsatellite loci. How many loci were used?

Response 19: See Response 10.

Line 341:
What is the criterion to identify clusters using COI sequences? Is it genetic distance? But in Fig. 2 the genetic divergence between clusters II and IV seem to be lower than the intra-cluster divergences of clade V (cubensis/viator). In addition, posterior probabilities for the crown nodes of clusters II and IV seem to be low, 0.9 and 0.7, respectively.

Response 20: Right. We acknowledge that this criterion is too subjective and that some clusters are better supported for some species/genes than others. See Responses 6 and 15, which explain how we deal with this issue.

DISCUSSION

Line 412:

You suggest that the specific status of G. viator depends on the gene consider, and take a cautious but ambiguous position to consider it part of a species complex or part of a single species with wide diversity. But I encourage you to take advantage of StarBeast2. You could estimate marginal likelihoods of two StarBeast2 runs, one assuming G. viator as a separate species from G. cubensis and another assuming G. viator and G. cubensis are the same species. Then, you could compare the marginal likelihoods by computing Bayes factors, and take a clearer position on this matter.
Alternatively, you could use your multi-locus dataset and multispecies coalescent methods that estimate species limits, such as BP&P or STACEY. This alternative approach will be a stronger criterion for delimiting species compared to the current approach consisting of identifying clusters.

Response 21: Thanks a lot for this suggestion which we took into account. See Response 15 for details.

Line 521:

You ruled out the "recent-divergence hypothesis" to explain the morphological resemblance among species because a previous study suggested that the genus had a 20-Ma origin. But you have not estimated divergence times among extant species in this study. An old crown age of Galba does not rule out very young species divergences.

Response 22: This is correct. In this new version of the manuscript, we include time calibrated trees which set the ground for ruling out the recent-divergence hypothesis. See Response 6 for more details.

Line 524:

You ruled out the "parallelism and convergence hypotheses" based on your ancestral inference of states "cryptic" or "non-cryptic". But as currently defined, these two qualitative states do not properly inform your hypotheses on parallelism and convergence. You would instead need to estimate ancestral states of measurable traits to really rule out such hypotheses. Or at the very least, consider and discuss this issue in the light of the Galba fossil record (https://paleobiodb.org/classic/basicTaxonInfo?taxon_no=307489). Were the extinct species also cryptic?

Response 23: The two qualitative states refer to two morphological types, well-defined and studied in previous articles (Paraense 1995; Samadi et al. 2000; Correa et al. 2011), as already stated in text. These qualitative states in fact refer to a suite of traits (shell and reproductive anatomy) showing more variation within than among species, and all separate the two types (see Response 4). We therefore think that we can infer the ancestral type. It remains of course possible that each lineage experienced morphological divergence in the course of its history, but we have no data to sustain this claim.

With regard to fossils, we did not consider the fossil record to reconstruct the phylogeny of *Galba* because we could not ascertain that the fossils that have been ascribed as *Galba* species actually belong to the *Galba* genus. In fact, although the fossil record of marine and terrestrial species is often of high quality for systematic purposes (see e.g. (Bouchet and Strong 2010), this is far less true with freshwater Pulmonates. The family Lymnaeidae to which *Galba* belongs is not an exception. The fossil record is extremely poor. Moreover, the fossils ascribed to *Galba* lack valuable taxonomic characters (e.g., protoconch, sculptures) and resemble those of other species that belong to other Lymnaeids. For instance, the *Galba*-like fossils reported from North America by Baker (1911), more than 100 Mya old, may be ancestor of the current *Galba* or belong to other Lymnaeid genders.

However, we now consider this issue in the manuscript:

> Lines 688-705 (clean version): "The integration of accurate fossil records would likely provide more accurate insights into macroevolutionary dynamics of *Galba* than molecular phylogenies alone. The fossil record has proved to be very helpful in determining the phylogeny of groups containing hard body parts like plants, mammals and mollusks (Magallón and Sanderson 2005; Agnarsson et al. 2011; Bolotov et al. 2016). However, in this study, we decided not to consider fossil record to reconstruct the phylogeny of *Galba* because we could not ascertain that the fossils that have been ascribed as *Galba* species are, in fact, species of that genus. Unlike marine snails that have a diverse fossil record with many valuable taxonomic characters (sculptures, ornamentation, coloration, protoconch; Bouchet and Strong 2010), fossil freshwater pulmonate snails are scarce and lack useful taxonomic characters. The fossils ascribed as *Galba* resemble those of other species that belong to other genders of Lymnaeids. For instance, Baker (1911) reported *Galba*-like fossils in North America, but these fossils could also belong to other genera of Lymnaeids or an ancestor of current Lymnaeids. Moreover, the soft parts of the mollusk—where are the most reliable diagnostic characters within *Galba*—are usually not preserved. For these two reasons (shells that resemble the ones of other species and soft parts not preserved), we cannot rely in *Galba* fossil records to reconstruct the phylogeny or estimate time divergence."

Line 529:

You seem to accept your "morphological stasis hypothesis" by default (literally written in the text). But again, you could discuss this in the light of the fossil record. Could we see this morphological stasis over millions of years?
In this respect, you also consider strong stabilizing selection related to environmental conditions as an agent for morphological stasis. However, given that you recorded habitat associations for sampled individuals (Line 183: "The sampled habitats were characterized …"), then why not associate this meta-data to back up your hypothesis of selection driven by environmental conditions? Are the cryptic species significantly associated with particular habitats compared to the non-cryptic species?
In Line 533 you seem to relate cryptic species to a variety of freshwater habitats, but nothing is mentioned about how you concluded this and if there is another type of associations for the non-cryptic species based on your sampling notes.

Response 24: See response 23 for the issue on fossils. With regard to habitat: *Galba* species indeed occupy a variety of habitats, as shown by our 30 years of sampling this genus, reflected in the variety of water bodies in which we found it (see Table S1). Our point is in fact that *Galba* species occupy habitats that are not occupied by other freshwater snails, especially transiently inundated habitats drying out during the dry season(s). In other words, they are more amphibious than other freshwater snails, which might allow them to decrease interspecific competition (Burgarella et al. 2015). Such habitats might impose strong stabilizing selection and morphological stasis on the shell. It is less clear why the reproductive anatomy remained so similar, but it might simply be the most simple anatomy that can be afforded by a preferentially selfing species (Jarne et al. 2010). On the whole, we suspect that further ecological studies might be useful to evaluate whether species segregate along ecological gradients—this would require detailed, long-term studies. Another research path would be to look for adaptive molecular divergence, and possibly to link it to environmental variables. We clarified this aspect in text (Lines 610-627, clean version).

Pável Matos-Maraví

Reviewed by Niklas Wahlberg, 2019-06-26 16:26

The authors report a very detailed study of the genetics of a group of cryptic species of snails in the genus Galba, mainly from the New World. The genus does have a worldwide distribution, and the taxonomic situation is not clear anywhere. This study is a first detailed step to clearing the taxonomy of the snails. I found the study to be well done and an excellent contribution to Galba taxonomy and systematics. The next step is highlighted by the authors, and that is to expand the specimen sampling to the rest of the world, preferably at the same detailed level. I have no suggestions for improvement, I enjoyed reading the manuscript, despite not being an expert in molluscs.

Response 25: Many thanks for these words.

Reviewed by Christelle Fraïsse, 2019-06-11 16:17

By means of a large-scale sampling and intense literature review (clearly presented in the Supplementary Tables), Alda et al. provide an excellent overview on the systematics and geographic distribution of cryptic freshwater snails of the genus Galba. This primarily descriptive and organism-centred work meets its objectives, and I think it will be valuable for future biogeographic research on the genus.

My main concern regards the assessment of the different scenarios to explain crypticity in Galba, which I believe is the most interesting part of the paper from an evolutionary perspective. These are presented from the introduction (L87 – 97) and on Figure S1: (i) recent divergence, (ii) parallelism, (iii) convergence and (iv) morphological stasis.

First, the authors only provide verbal arguments in the discussion to disentangle between the four scenarios (L516 – 537), while their Figure S1 suggests that a more quantitative test could be performed. For example, the level of disparity between clades could be measured by performing a multivariate analysis on some quantitative trait (e.g. shell morphology), and then by calculating a distance between clusters on this morphometric space (e.g. by using the coordinates of the clusters on the first

component). This will give a multivariate measure of disparity that then can be compared between different species pair. More generally, I really think that the authors should go beyond a qualitative description of morphology (L507-510) if they want to discuss scenarios on the evolution of crypticity. Given that they have photographs of the shells, it would be possible to perform a morphometric analysis using R packages such as Geomorph (Adams & Otarola-Castillo 2013:) and Morpho (Schlager 2015: http://cran.r-project.org/package=Morpho" \t "_blank".

Response 26: this is a point already made by another reviewer and the recommender. See Responses 4 and 23.

Second, the authors do not explain how the species pairs should be chosen in the phylogeny to compare their level of disparity (e.g. they take A1/A2 and A1/A3 for scenario A). Should the pairs being compared ("similar" vs "similar" ; "similar" vs "different") have similar level of molecular divergence?

Response 27: No, they don't (see Figure 4). All species, except *G. cousini*/*meridensis* exhibit the same morphology (see Response 18), irrespective of molecular distance. What we did was to delimit species, to infer the ancestral state, and to discuss the four hypotheses. See Response 5.

Third, the ancestral reconstruction trait analysis, as it stands, is not convincing enough. The root state posterior probabilities shown in Figure 3 (60% vs 40%) indicate rather weak evidence that "crypticity" is the ancestral state. Could you please add 95% confidence intervals around the ancestral values to assess the uncertainty around these estimates? And would it be possible to impose an informative prior on nodes based on information from the fossil record? Moreover, instead of using a dichotomic trait ("cryptic" vs "non-cryptic"), the authors may gain some power by using the multivariate scores of the morphometric analysis suggested above to reconstruct ancestral states. Finally, by using the scores reconstructed at the different nodes in the past, it may be possible to actually plot the level of disparity as a function of divergence time, as illustrated on your Figure S1.

Response 28: Thanks for these remarks, echoing points made above. They were addressed in details in Responses 5, 23 and 24.

Minor concerns are listed below.

→ Discrepancies between genes trees and species tree. I totally agree with the authors that conflicting genealogical histories is a major issue for phylogenetic reconstruction, whether it is due to sampling errors, incomplete lineage sorting or introgression between lineages. For this same reason, I think it would be more appropriate to represent the genealogical relationships between alleles of the four genes as genetic networks instead of gene trees (Figure S5 – S8).

Response 29: We do agree, but note first that we used gene trees to assign sequences to species, not to represent the genealogical relationships, as mentioned in Response 15. We now report haplotype networks, built with popART (Figs. S9-S12). Gene trees and networks lead to several scenarios of 'species hypotheses' that were tested as explained in Response 6. We also applied two other species-delimitation methods: ABGD and

STACEY. We replaced the unrooted gene trees by the best tree obtained with this approach (Fig. 3 in both versions). See Response 15 for details.

→ Evolution of crypticity.

- L503: "some habitats possibly favouring the emergence of cryptic species (like caves, Katz et al., 2018)." Briefly explain why please.

Response 30: Done. We modified the following phrase in Lines 90-94 (clean version). We replaced "... although it remains possible that some habitats such as caves and subterranean habitats fostered cryptic speciation (Katz et al. 2018)." by "...although it has been suggested that caves and subterranean habitats may promote cryptic speciation. Such isolated, dark, low-energy habitats promote diversification but constrain morphological differentiation since very specialized adaptations are needed to survive in them (Katz et al. 2018).".

- L531: "Selfing in Galba might have led to limited genetic variation favouring stasis". It is not entirely clear to me why this would be the case. Is there any evidence from the literature that this has been observed (e.g. in plants by contrasting outcrossers and selfers)?

Response 31: We clarified our point here by adding the following sentences in the Discussion section (Lines 621-627, clean version): "The stasis in reproductive anatomy we have documented in populations of *Galba* may simply reflect a shared adaption to self-fertilization in unpredictable habitat patches (Jarne et al. 2010). Once established, self-fertilization promotes the rapid erosion of genetic variation, as inbred populations become progressively unable to generate new genetic combinations through recombination (Noël et al. 2017). Thus, selfing could reinforce morphological stasis that might have evolved for other reasons.".

→ Figures and typos.

- Figure 3: Please, add numbers (I to V) after names to be consistent with Figure 2.

Response 32: Done. We added the cluster numbers (in this new version, from I to VI) in Figures S5-S12 to be consistent with the text and with Figures 3 and 4.

- Figure S1. Maybe remove one species (five instead of six) to be consistent with your biological system. Please, explain in the legend what the colours correspond to (green vs blue).

Response 33: Figure S1 was extracted from Struck et al. (2018). These authors illustrated the four evolutionary processes that can lead to cryptic species by using six hypothetical species, explaining the number of species in Figure S1. In the new version of the manuscript, we modified the figure and legend from Struck et al. (2018) to better fit our manuscript. Thus, there is no green and blue colors in the new figure.

- Figure S2. The numbers in the legend do not match: "111 individuals of Galba cubensis (41), Galba schirazensis (41) and Galba truncatula (29)". Please, specify how many individuals were used for Galba humilis and Galba viator in the third step.

Response 34: Done. We added the numbers of individuals analyzed at each step (Figure 1 in the new version) and a clearer, more informative legend is now proposed.

Lines 1104-1111, clean version: "**Figure 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 individuals: *Galba cousini*/*meridensis* (1), *Galba cubensis* (41), *Galba schirazensis* (41), *Galba truncatula* (30), *Galba humilis* (34) and *Galba viator* (1)."

- Figure S4. Please, add a scale for the photographs and the drawings.

Response 35: Right, thanks. Done.

- Figure S5 – S8: Please, specify the unit of the scale. Also, I cannot see any highlighting in yellow.

Response 36: We modified these figures since we reran the analyses. We now specify the scale unit and we replaced yellow by mustard to make it clearer.

- L100 and L105: I cannot see where the first issue is described in "with regard to at least two issues. The [second] issue is biological invasions" and "[third] issue arises when species".

Response 37: Done. See Response 9.

Other comments:

Dear Pilar,

In addition to the decision of Fabien Condamine about your MS#208, we would like that you take these points into account in your clean version.

As indicated in the 'How does it work?' section and in the code of conduct, please make sure that:

-Data are available to readers, either in the text or through an open data repository such as Zenodo (free), Dryad or some other institutional repository. Data must be reusable, thus metadata or accompanying text must carefully describe the data.

Response 38: Right. All the data was already available to readers in the text and as Appendices in the previous version of the manuscript.

-Details on quantitative analyses (e.g., data treatment and statistical scripts in R, bioinformatic pipeline scripts, etc.) and details concerning simulations (scripts, codes) are available to readers in the text, as appendices, or through an open data repository, such as Zenodo, Dryad or some other institutional repository. The scripts or codes must be carefully described so that they can be reused.

Response 39: we uploaded the script for the phylogenetic analysis (html) in the data repository Zenodo and we added the following phrase in Lines 717-719 (clean version): "Data and code accessibility:
Xml files for phylogenetic analyses are available from the Zenodo repository (https://zenodo.org/record/3473937#.XZiPcC0ryTd).".

-Details on experimental procedures are available to readers in the text or as appendices.

Response 40: Yes, they are.

-Authors have no financial conflict of interest relating to the article. The article must contain a "Conflict of interest disclosure" paragraph before the reference section containing this sentence: "The authors of this preprint declare that they have no financial conflict of interest with the content of this article." If appropriate, this disclosure may be completed by a sentence indicating that some of the authors are PCI recommenders: "XXX is one of the PCI XXX recommenders."

Response 41: Done. We added the conflict of interest disclosure with the following phrases "The authors of this preprint declare that they have no financial conflict of interest with the content of this article. Philippe Jarne is one of the PCI Evolutionary Biology recommender." (Lines 737-740, clean version).

Best
Thomas for PCI Evol Biol

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