June 10, 2024

Dear recommender, Prof Felix Sperling,

My coauthors and I would like to thank you and the reviewers for your constructive comments and for inviting us to resubmit a revised version of our manuscript. We have found the comments of all three reviewers extremely helpful and have addressed all of them. We include point-by-point responses at the end of this letter with our replies and corresponding line numbers for the revision. We believe that our manuscript is much clearer and hope is now ready for publication in PCI Zoology.

Revision round #1

Decision for round #1 : *Revision needed*

This manuscript has recieved three very positive reviews. All of the reviews raise some editorial points, and two of them request more detailed information and discussion on the lab methods. I agree that it would help to flesh out the methods at least a bit more. Please revise your manuscript accordingly to take advantage of these supportive reviews, responding to (or refuting) each of the points raised.

*by **Felix Sperling**, 17 May 2024 06:40*

Manuscript: **https://doi.org/10.1101/2024.03.21.586057**

version: 1

Review by Michael Caterino, 22 Apr 2024 09:47

Title and abstract
Does the title clearly reflect the content of the article? [**X**] Yes, [ ] No (please explain), [ ] I don't know
Does the abstract present the main findings of the study? [**X**] Yes, [ ] No (please explain), [ ] I don’t know

Introduction
Are the research questions/hypotheses/predictions clearly presented? [**X**] Yes, [ ] No (please explain), [ ] I don’t know
Does the introduction build on relevant research in the field? [**X**] Yes, [ ] No (please explain), [ ] I don’t know

Materials and methods
Are the methods and analyses sufficiently detailed to allow replication by other researchers? [**X – with some commentary on exclusion criteria**] Yes, [ ] No (please explain), [ ] I don’t know
Are the methods and statistical analyses appropriate and well described? [**X**] Yes, [ ] No (please explain), [ ] I don’t know

Results

In the case of negative results, is there a statistical power analysis (or an adequate Bayesian analysis or equivalence testing)? [ ] Yes, [ ] No (please explain), [ ] I don't know, [**X**] N/A.
Are the results described and interpreted correctly? [**X**] Yes, [ ] No (please explain), [ ] I don't know

Discussion

Have the authors appropriately emphasized the strengths and limitations of their study/theory/methods/argument? [**X**] Yes, [ ] No (please explain), [ ] I don't know
Are the conclusions adequately supported by the results (without overstating the implications of the findings)? [**X**] Yes, [ ] No (please explain), [ ] I don't know

This is an impressive phylogenomic study of a complex lineage of ground beetles, while also serving as a powerful demonstration of the applicability of museomic techniques to such questions. The amount of data acquired from historic specimens was surprising to me (not having explored this area nearly as much as I would like), but the trees support that the vast majority of it is reliable and informative.
With regard to the criteria for excluding certain samples, these aren't too clear early on.

We want to thank Dr Caterino for his insightful comments on our article. In this study, we decided to present in the most honest way how a modern museomics study is conducted including failure attempts which happen to be numerous when working with highly degraded DNA. We detailed the entire filtering process and indicated the samples that were initially included but subsequently failed to be sequenced or resulted in too few loci. There are therefore several levels of sample filtering. Firstly, after the extractions, we excluded samples (38) with concentrations too low to be detected as explained L200-201. Then, after the capture and sequencing process, we also excluded from the final dataset samples (12) with too much missing data, which had a direct impact on the reliability of their phylogenetic placement (rogue taxa), as explained L242-243.

Apparently there was nothing detected for the samples initially excluded, rather than simply very low concentrations. (As an aside, it would be interesting to see if any markers could be amplified from those extracts. Given the higher than expected success rates overall, it seems not out of the question that below detectable quantities may still be amplifiable.) Exclusion of some lower concentration samples that produced some data might merit a little more discussion.

Based on our experience with these approaches (Toussaint et al. 2021 GBE; Gauthier et al. 2023 Syst Entomol), including samples with undetectable DNA concentrations is usually a waste of time and money, as we are almost certain that they would have yielded nothing in the end. Even some samples with detectable concentrations initially included had to be excluded at the end because they did not contain enough loci. These aspects are discussed in L464-471.

12 samples with <150 loci were left out. But it would be interesting to see if these could be placed on data sets limited to a smaller number of shared loci. I'm just interested to see what information can be squeezed out of these old specimens (thinking 100YO types and such) when necessary.

We have initially tested phylogenies including these samples as you mention to ensure that some information could or not be used for downstream estimations. Unfortunately these samples behaved as rogue taxa, with hazardous placements and were subsequently removed by standard data matrix thresholds of taxonomic/genomic completeness. You can see that in some datasets there are fewer taxa. These “bordeline” taxa are the ones that have limited genomic information but still could be included in some analyses. The rest were simply not considered in the study. We are aware that this level of transparency is rarely offered in museomics studies where authors generally simply present the final taxonomic sampling after discarding some samples. We believe it is more honest and more interesting for potential researchers interested in such approaches to have a more realistic view of the problems encountered when using degraded DNA.

Otherwise, there is a wealth, perhaps an overabundance of discussion of the fine points of Carabus relationships. Only the hardcore fans will find this useful, while the general readers interested more in museomics will begin to wonder whether to continue reading. In many other groups this might be problematic. I suppose in a paper dealing with such a popular genus, the committed readership will be somewhat higher than for many other taxa.

This is certainly a subjective topic and even though we may disagree on what is necessary or not to include in such discussion we believe that most included points are not found elsewhere in the literature. The genus *Carabus* is an interesting example of a publicly well-known group for which very little has been published in the literature except in field guides and non peer-reviewed booklets. We believe that an extensive discussion on the biogeography and systematics of the genus should be of interest for readers interested in beetle evolution as a whole.

I made a number of other minor corrections and comments in the pdf. But in general this is a very readable and clean manuscript, especially for a pre-print. The figures are attractive, clear, and sufficient to convey the results.

Download the review

Review by Julian Dupuis, 08 May 2024 18:06

Here, the authors use HyRAD-X on a combination of museum and contemporary specimens to generate a large phylogenomic dataset to assess diversification of a genus of ground beetles. This approach allowed them to generate quite a bit of data from a broad assemblage of diverse specimens, which they then used for a whole bunch of phylogenetic and population genetic analyses. From these, they focused discission on both taxonomic considerations within this genus as well as diversification/speciation dynamics. I am no carabid expert, so can’t judge that aspect of the study, however from a very superficial review of pertinent Carabus literature, the authors have done a fine job in addressing outstanding taxonomic questions at both the subgeneric and more microevolutionary levels.
Overall, the study is well-written, most analyses are conducted and presented appropriately, and the system-specific discussion is well-presented and as approachable as such system-specific discussions can be for non-experts in that group. From a phylogenomic perspective, bioinformatic pipelines (including code, input files, etc.),

analyses, and interpretations are solid. My only bigger-picture critique of the study is that I think more could be done to explore why some samples work and some don't work in this museomics context. As the authors point out, a lot of this is unknowable (specimen collection and preservation conditions), but the authors document that a FA was used on all raw DNA extracts, so I wonder if there are any trends with regard to fragment size distributions that might inform more interpretation of the results presented in Figure 1. Might turn out to just be too variable to make any conclusions there, but I'd say worth a bit of added discussion to the first paragraph of that section of the Discussion. As someone who is exploring these methods in depth, that kind of methodologically important information is quite valuable, even if it's somewhat conjecture or the author's best guess. Specific comments provided below. I think this study will be a valuable addition to both 1) ground beetle researchers and 2) systematists exploring this new field of museomics. With museomics being such a young field, studies such as these have a great opportunity to add extremely valuable context to the discipline moving forward.

First we would like to thank Dr. Dupuis for his insightful comments. Indeed, the FA results were also used to decide whether or not to include the samples. We found that the fragment size distribution for these historical samples was normal, with a mean between 50 bp and 100 bp. Unfortunately, for the samples that were excluded and had an undetectable DNA concentration at the qubit, the FA profiles were invariably flat.

Lines 79-81. Grammar a bit clunky—would rephrase.

Rephrased.

Line 92. Not sure what is meant by "inferences made in fine"?

Corrected.

Line 116. Should the abbreviated form of the subgenus name be used here, as it is used down in the results/discussion? i.e., "C. (Chaetocearabus)"? Formatting of subgenera goes back and forth throughout the manuscript it seems. Personally, I like the abbreviated form, since they're all Carabus subgenera.

As is the practice, the genus name has been given in full in the first occurance (L103-105), then in abbreviated form in the rest of the text. This has been corrected this way for all subgenera and species.

Lines 119-120. Reword to "where hybrids are found, for example, at the Katara Pass…"

Corrected.

Lines 193-194. Although I think I generally understand what is being said here, I would suggest rephrasing this sentence. "Processed" could be used to mean several diderent things in this context.

Rephrased : "Multiple specimens of the same taxa and geographic populations were initially selected to anticipate the risk of failure linked to hDNA degradation that can result in specimens being excluded."

Line 205-208. Thank you for this transparency! I completely agree. I would rephrase slightly as “Early sampling erosion and discarded samples are commonly not…”.

Corrected.

Section at line 216. Was the same RNAseq-based probe kit used as in Toussaint et al. 2021? It is unclear from this section here, and that seems to be a pivotal step in library prep for this method.

A clarifying sentence has been added: “The HyRAD protocol was applied as in (Toussaint et al. 2021) allowing to generate the same probe set and therefore a backward compatibility with the data of this previous study.”

Line 247-249. Commas should be used in the # loci here. Also maybe refer to Table 1 here, so it shows up near this part of the methods?

Corrected.

Line 288. Should be “SNPs”

Corrected.

Line 331. Although barplots and likelihood values are provided in the supplement, I’d also like to see the deltaK values (especially since that method was used to determine K, as in the methods) and would like to see the likelihood values plotted so they’re more interpretable to a reader. Ultimately, following some of the best practices for STRUCTURE (10.1111/mec.14187), seeing the various statistics developed by Puechmaille might help in determining the best value of K, but given the relatively low (and uneven) sample sizes, I don’t expect that they’d be terribly informative beyond what is presented here. But, as I note below, providing some more details about those analyses/interpretations would be a good addition here.

A plot representing the deltaK has been integrated to the Supplementary Figure 6. A more detailed description of the results has now been integrated into the Results.

Line 348. Rephrase to “enabled us to…”

Corrected.

Lines 350-351. What elution volume was used for these extractions? Without that, the concentrations are rather meaningless. Alternatively, are 260/280 or 260/230 ratios available to contextualize the quality of DNA? Expanding a bit more broadly, I wonder if any of those considerations could help explain the large spread in data return across the various extracts. Or some other consideration from the FA runs that were done on raw extracts? I am not the most familiar with HyRAD/HyRAD-X literature, but trying to figure out what the best practices are with these museomic extraction techniques seems to be the biggest sticking point that may be keeping the broader community from advancing these

methods to the next level. Adding a bit more detail/discussion to that logistical topic might be a nice balance to the very detailed taxonomic/biogeographic discussion.

Line 352. I would also argue the same for fresh samples (concentrations not being homogenous)—it looks like almost a bimodal distribution among fresh samples, which might be worth noting in the text (or at least the large spread).

The elution volume is now indicated in the Material and Methods. We've never been really satisfied with the reliability of Nanodrop results in the past, so we didn't use it for this study and don't have the 260/280 or 260/230 ratios. The factors that influence the quantity and quality of DNA in historical samples is a complex issue that would merit a dedicated study with more factors. Based on the literature, a paragraph dedicated to these questions is included in the discussion, L578-589.

Figure 2. Some mismatch between parts of this figure—I assume the result of bad pdf conversion or something like that. Also, as a color-blind person, I have trouble diderentiating the grey and red asterisks (the boxes are a bit easier), FYI. Also also, great illustration and pictures—cheers Conrad!

Yes, it was a problem converting to pdf. This has been corrected, as well as the colour in Figure 2.

Line 434. What value of K was determined to be best, and what did deltaK say? From figure 3, it seems like K=8 was supported, but looking at the supplemental barplots, K=7 shows virtually the same overall pattern, and it's really only fine-scale diderences that are showing up relative to K=6. More detail about the interpretation of these results would be warranted.

A plot representing the deltaK has been integrated to the Supplementary Figure 6. A more detailed description of the results has now been integrated into the Results.

Line 445. "i.e." not needed.

Corrected.

Line 668. Personally, I think one or all of these networks should be included in the maintext. The connection here to hybridization is nice, but from a purely species delimitation point of view, the diderences in the diderent mt genes is interesting. Or does a combined 3-gene network show an overall composite of the findings of the three genes individually? That might simplify a main-text figure to one thing, which I think would be a nice addition.

One of the focuses of the study is to show the effectiveness of the capture approach in recovering a large number of nuclear loci that are particularly informative for resolving phylogenies. The recovery of mitochondrial sequences is more of a by-product of the approach, which is certainly interesting, but is not the core of the study. We therefore prefer to leave these figures as supplementary.

Line 709. BioProject not currently available on NCBI—can't tell if just still embargoed or not.

Raw data is on the NCBI but under embargo yes, the data will be released once the paper is accepted.

Figure 3. Symbols are provided to contextualize specimens, as in fig 2, but aren't actually included in the figure (unless they also were subject to some pdf creation/conversion errors).

Symbols were mainly used to indicate the colors for the barplot; they have been replaced.

Note, I'd answer yes to virtually all of the questions presented in the PCI Zool reviewer's list, and think I've adequately addressed my concerns above.

Review by anonymous reviewer 1, 29 Apr 2024 18:45

Lines 105-108 explicitly mention which are the two species

Corrected.

Lines 108-109 structure references and parentheses appropriately

Corrected.

Linea 324 indicate the beta value

Indicated.

Figure 1 homogenize colors in pinned (< 30-year-old)

Corrected.

Does the title clearly reflect the content of the article? [X] Yes, [ ] No (please explain), [ ] I don't know
Does the abstract present the main findings of the study? [X] Yes, [ ] No (please explain), [ ] I don't know

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