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Peer Review File

Standing genetic variation and introgression shape the cryptic radiation of *Aquilegia* in the mountains of Southwest China

Corresponding Author: Dr Zhi-Qiang Zhang

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors investigate the evolution of cryptic diversity in Aquilegia species from the biodiversity hotspot in SW China using whole-genome sequence data based on extensive intraspecific and intrapopulation field sampling. The manuscript addresses a highly relevant research topic, is well-focused, cleanly written, has a clear structure, well-formulated research questions, and a thorough discussion. I appreciate the extensive data processing with the use of a wide range of approaches and algorithms leading to well synthesized and interpreted results. The manuscript provides novel insights into the plant evolution, which I believe are of interest to a broad audience.
I have only a few minor comments that could improve the manuscript.
1) In several places, the results section contains parts dealing with discussion or interpretation of the data that go quite far beyond the results presented; they should be moved to the Discussion (lines 173-180, 188-190, 199-203, 330-333)
2) I do not like that the ASTRAL tree is shown in the main figure (Fig. 1d); given the large admixture detected by STRUCTURE and the low quartet score of the nodes, the hierarchical and bifurcating tree representation may not adequately describe the patterns of variation. A network would be more appropriate here (e.g. as in Fig. S4).
3) The authors have uncovered as many as 11 lineages within the three traditionally recognized species (morphospecies) and describe in detail the evolutionary processes that probably produced them. So the cryptic diversity is enormous, but somewhat disappointing to me is that the authors do not suggest what needs to be done to formally recognize this diversity. I do not want them to come to definitive taxonomic conclusions here, but a huge amount of data has been collected and analyzed (not just genomic, but also geographic, ecological, and morphological), so I would at least expect them to offer a suggestion or opinion to taxonomists for the future on whether species delimitation should be revised, whether new species should be described, etc., so that the diversity discovered is actually recognized. It is briefly mentioned (lines 199-203) that more evidence is needed, but I believe that the present study has provided a really detailed insight that is sufficient at least to propose some taxonomic solutions. Species are still the fundamental units of biodiversity, and without their formal description, biodiversity cannot be properly assessed and protected.

Reviewer #2

(Remarks to the Author)
The manuscript reported the mechanisms behind cryptic radiation in a group of Aquilegia species found in Southwest China. By analyzing the whole genomes of 158 individuals, the authors identified multiple paraphyletic lineages within each morphological species. They discovered that introgression (the transfer of genetic material between different species) played a significant role in the divergence of these lineages. They also found that standing genetic variation, which was present before the lineages formed, contributed to morphological similarities between different species. Additionally, introgression from non-sister lineages accelerated the genetic divergence of these species. These findings highlight the importance of both standing variation and introgression in the evolution of cryptic diversity in plants.
I found the overall manuscript was well-written and intriguing. I probably have one main consideration provided here for discussion.
The authors present three morphologically defined species of Aquilegia from Southwest China. However, the phylogenetic analysis reveals paraphyletic lineages, raising concerns about the accuracy of species delimitation. The authors are

suggested to strengthen the validity of their species delimitation and provide a more robust understanding of the evolutionary history of Aquilegia in Southwest China.
First, the current manuscript lacks specific morphological data to support the proposed species distinctions. Clear descriptions of morphological differences, particularly in floral and vegetative traits, are essential for a comprehensive understanding of species boundaries. It is crucial to distinguish between natural variation and potential artifacts, such as faded colors in specimens (here like the yellow or purple colors on the petal), when assessing morphological differences.
Second, the study should explicitly clarify the origin of the plant material, confirming that it is derived from natural populations and not horticultural cultivars or landraces. Considering the potential for artificial hybridization in cultivated Aquilegia plants, it is important to exclude such material to avoid misleading interpretations.
Third, to further evaluate the genetic and evolutionary relationships between the proposed species, conducting hybridization experiments would be valuable. If the species are reproductively compatible, it could indicate that they may represent intraspecific variation rather than distinct species, especially for those sympatric populations mixed with different floral types.
Some minor comments:
L59, between 'reconnected' populations.
L88, with or after the split of …
L98, common name of columbines would be appreciated to be mentioned with Latin name.
L106, please clarify the meaning of 'distribution', ecological or geographical.
L119, range 'shifts' instead of 'movements'
L161, a kind of confused with the classification of two datasets here
L292, in the analysis of Fst and Dxy, the authors used a sliding window size of 100kb. My understanding was that a window size of 10-50kb is more suitable. Smaller windows can capture more variation, and help to identify regions of the genome that have experienced recent selection or other evolutionary forces. The authors of the manuscript chose a larger window size (100kb) compared to the more common 10-50kb, which is likely due to the large number of SNPs in their dataset (10 million). It would be valuable to clarify the window size used here.
L1073, repeated species name
L1095, what is the meaning of "morphologically fixed loci"?

Reviewer #3

(Remarks to the Author)
I partially read this manuscript but stopped when I encountered what I perceive as a potentially highly problematic issue with the sequencing data. I have some comments about the clarity of writing and framing of the questions in the introduction. I will begin with my concerns about the handling of the sequencing data, and then post my comments about the introduction.

In particular, I have a strong concern over the haplotype phasing and genotype imputation step. The authors follow basic and accepted pipelines for library prep, read filtering, mapping, etc. However, at no point do the authors appear to filter sites for missing data (e.g., filtering out sites exceeding some threshold of individuals with missing data). Mean depth filters are applied (lines 542-543), which is good, but there are still probably many sites in the genome that are supported by only a few samples with data. This is highly problematic because the data are then phased and imputed with BEAGLE (line 544). This means there are an unknown number of sites (not reported) with data for potentially many missing individuals being imputed based on the inferred LD and genotype calls from potentially a small number of individuals. These are of course then used downstream for all analyses. Given the lack of a reference panel for proper genotype imputation and a geographically disparate set of input samples, each potentially with their own population-specific patterns of LD and allele frequencies, etc. I would highly advise not phasing and imputing genotypes with these data (at least not, for example, without more stringent missing data filters and some assessment of imputation error/ some way to assess confidence in imputed genotypes). Many of the analyses performed here are possible without phased, imputed genotypes, but we currently have no way of knowing how strongly that data processing step affects these results.

Other issues with the Introduction:
• Lines 53-69. The paragraph here discusses standing variation, adaptive introgression, and rapid genetic divergence, but it doesn't really connect these ideas and it is not clear why they are being discussed together.
• Lines 55-58. Balancing selection is not the only process responsible for standing variation.
• Lines 70-84. The discussion about hemiplasy is confusing. As it reads currently, the text implies hemiplasy is in some way related to the independent evolution of similar morphologies. This is a mischaracterization. Hemiplasy is the apparent convergent evolution of traits caused by incomplete lineage sorting (the traits have the same molecular basis – the problem is simply gene tree-species tree discordance).

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors addressed my previous concerns satisfactorily, both in their replies in the rebuttal letter and in the manuscript. I have no further comments.

Reviewer #2

(Remarks to the Author)
The authors have provided a well-revised manuscript, and my primary concerns have been adequately addressed. However, I remain somewhat uncertain about the assertion of reproductive incompatibility between the proposed species lineages. While I appreciate the inclusion of a brief summary in the revised manuscript (L428-432), which suggests that prezygotic barriers, such as differences in pollinators, may contribute to reproductive isolation, this hypothesis would benefit from additional experimental or ecological evidence to strengthen its validity.

In their response materials, the authors mentioned some controlled intra- and interspecific crossing results. Incorporating these findings into the manuscript would provide valuable support for their claims and enhance the overall robustness of the study. I recommend including this data in the next revision to further substantiate the discussion on reproductive isolation.

We thank the Editors and the three reviewers for constructive and helpful comments on our manuscript. We have followed their suggestions in our revision, and we believe this has generally improved our presentation. Below, we provide point-by-point responses to the queries raised (**our responses are in bold**).

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The authors investigate the evolution of cryptic diversity in Aquilegia species from the biodiversity hotspot in SW China using whole-genome sequence data based on extensive intraspecific and intrapopulation field sampling. The manuscript addresses a highly relevant research topic, is well-focused, cleanly written, has a clear structure, well-formulated research questions, and a thorough discussion. I appreciate the extensive data processing with the use of a wide range of approaches and algorithms leading to well synthesized and interpreted results. The manuscript provides novel insights into the plant evolution, which I believe are of interest to a broad audience.

I have only a few minor comments that could improve the manuscript.

Thank you for your recognition and thoughtful comments. We have revised our manuscript according to your comments and suggestions, which are very helpful in improving our paper. Below, we provide our point-to-point responses and clarify the changes that have been made in the manuscript.

In several places, the results section contains parts dealing with discussion or interpretation of the data that go quite far beyond the results presented; they should be moved to the Discussion (lines 173-180, 188-190, 199-203, 330-333)

We appreciate your suggestion and have revised the manuscript accordingly by relocating these sections from the Results section to the Discussion section (lines 381-391, L457-L461).

I do not like that the ASTRAL tree is shown in the main figure (Fig. 1d); given the large admixture detected by STRUCTURE and the low quartet score of the nodes, the hierarchical and bifurcating tree representation may not adequately describe the patterns of variation. A network would be more appropriate here (e.g. as in Fig. S4).

We have updated the figures based on your valuable suggestion. Specifically, Figure S4 has been incorporated into the main figure (Figure 1), and Figure 1d has been moved to the supplementary figures (Fig. S4).

The authors have uncovered as many as 11 lineages within the three traditionally recognized species (morphospecies) and describe in detail the evolutionary processes that probably produced them. So the cryptic diversity is enormous, but somewhat disappointing to me is that the authors do not suggest what needs to be done to formally recognize this diversity. I do not want them to come to definitive taxonomic conclusions here, but a huge amount of data has been collected and analyzed (not just genomic, but also geographic, ecological, and morphological), so I would at least expect them to offer a suggestion or opinion to taxonomists for the future on whether species delimitation should be revised, whether new species should be described, etc., so that the diversity discovered is actually recognized. It is briefly mentioned (lines 199-203) that more evidence is needed, but I believe that the present study has provided a really detailed insight that is sufficient at least to propose some taxonomic solutions. Species are still the fundamental units of biodiversity, and without their formal description, biodiversity cannot be properly assessed and protected.

Thank you very much for the insightful comment. We fully agree with your perspective on the importance of species classification as the fundamental unit of biodiversity. We also appreciate your helpful suggestion to propose a taxonomic solution or offer a suggestion/opinion based on analyzed geographic, ecological, or morphological data.

Following your suggestions, we have revised the manuscript by expanding on

potential avenues for species delimitation and classification. Specifically, we have conducted additional analyses of floral color measurements among different cryptic lineages within each morphologically defined species. The results suggested that floral color is a reliable trait for classifying some cryptic lineages (Fig. S9-S12 and Table S2). For example, the E2 lineage exhibits distinct floral color characteristics that differentiate it from other lineages within the morphological species *Aquilegia ecalcarata*. Similarly, measurable differences were observed among lineages within morphospecies *A. kansuensis* and *A. rockii*. Additionally, differences in geography and ecology among lineages were also observed. In the revised manuscript, we have included these findings and provided suggestions for future taxonomic work (lines 199-225, 438-462. Fig. S10-12). In the Discussion section, we emphasize the importance of integrating quantitative traits, such as floral color, with genomics to achieve robust species delimitation (lines 457-462). We hope these additions will help guide future efforts in recognizing and formally describing the cryptic diversity uncovered in our study.

Reviewer #2 (Remarks to the Author):

The manuscript reported the mechanisms behind cryptic radiation in a group of Aquilegia species found in Southwest China. By analyzing the whole genomes of 158 individuals, the authors identified multiple paraphyletic lineages within each morphological species. They discovered that introgression (the transfer of genetic material between different species) played a significant role in the divergence of these lineages. They also found that standing genetic variation, which was present before the lineages formed, contributed to morphological similarities between different species. Additionally, introgression from non-sister lineages accelerated the genetic divergence of these species. These findings highlight the importance of both standing variation and introgression in the evolution of cryptic diversity in plants. I found the

overall manuscript was well-written and intriguing. I probably have one main consideration provided here for discussion.

Thank you very much for your summary and recognition. We have revised our manuscript according to your constructive comments and suggestions. Please see the detailed responses to each comment below.

The authors present three morphologically defined species of Aquilegia from Southwest China. However, the phylogenetic analysis reveals paraphyletic lineages, raising concerns about the accuracy of species delimitation. The authors are suggested to strengthen the validity of their species delimitation and provide a more robust understanding of the evolutionary history of Aquilegia in Southwest China.

First, the current manuscript lacks specific morphological data to support the proposed species distinctions. Clear descriptions of morphological differences, particularly in floral and vegetative traits, are essential for a comprehensive understanding of species boundaries. It is crucial to distinguish between natural variation and potential artifacts, such as faded colors in specimens (here like the yellow or purple colors on the petal), when assessing morphological differences.

We fully agree with you on the importance of incorporating specific morphological data to strengthen the validity of species delimitation and to provide a clearer understanding of species boundaries. We appreciate your helpful suggestion and have conducted floral color measurements among cryptic lineages. Our results indicated that distinct floral color patterns were identified among some lineages within each morphospecies (Fig. S9-S12 and Table S2), which may reflect underlying genetic differentiation. This finding suggests that floral color could act as a reliable trait for classifying certain cryptic lineages. In addition, a previous study (Liu et al. 2018, https://doi.org/10.11646/phytotaxa.348.4.5) also found morphological differences in floral traits and fruit traits between K1 lineages and other cryptic lineages

(Lines 442-446). Although we have not yet come to definitive taxonomic conclusions, we did find some morphological differences between cryptic lineages. Based on these findings, we have proposed a taxonomic framework here (Lines 438-462), which lays a solid foundation for our future study of species delimitation among these cryptic lineages.

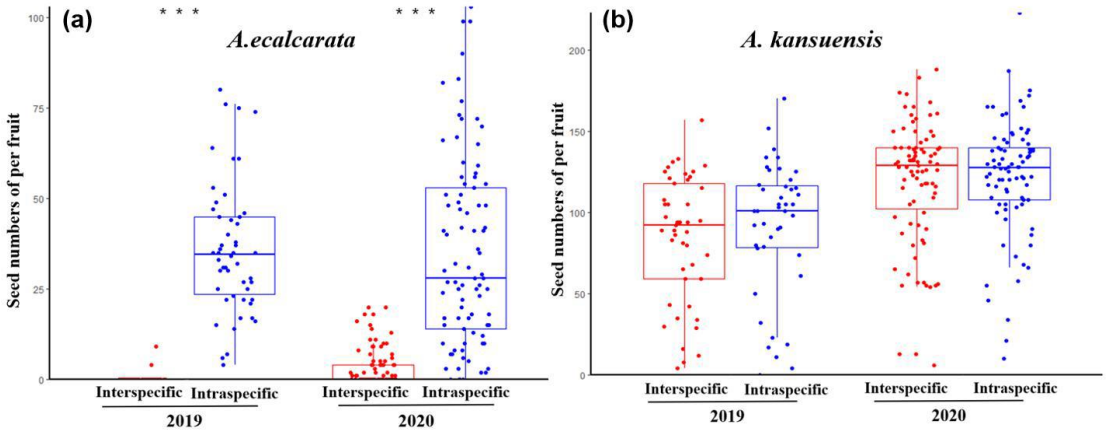
Second, the study should explicitly clarify the origin of the plant material, confirming that it is derived from natural populations and not horticultural cultivars or landraces. Considering the potential for artificial hybridization in cultivated Aquilegia plants, it is important to exclude such material to avoid misleading interpretations.

Thank you for pointing out the unclear statement about plant material. We collected all the plant material in the natural populations, which are located in remote places far away from potential horticultural cultivars. In addition, as far as we know, cultivated *Aquilegia* plants are not popular in cities in China, especially in western China. We have confirmed that all the plant materials were collected from natural populations (lines 585-586). After sequencing, we checked for individuals with unusually long branches and outliers in T-SNE and phylogenetic analyses, which could indicate artificial hybridization. Fortunately, no such individuals were identified in our dataset (Figure 1c, 1d, and S4-S6).

Third, to further evaluate the genetic and evolutionary relationships between the proposed species, conducting hybridization experiments would be valuable. If the species are reproductively compatible, it could indicate that they may represent intraspecific variation rather than distinct species, especially for those sympatric populations mixed with different floral types.

We appreciate your insightful comment regarding reproductively compatible. We agree that hybridization experiments are valuable for assessing reproductive compatibility and distinguishing intraspecific variation from distinct species, especially for those sympatric populations mixed with different floral types.

Aquilegia species are well known for their interspecies compatibility (reviewed in Kramer, E. M. 2009, _Annual Review of Plant Biology_ 60, 261-277.). However, an unpublished study of our lab indicated strong reproduction barriers between _Aquilegia_ species in sympatric populations. First, we observed a pattern of unilateral postzygotic incompatibility, in which pollen from K1 fails to fertilizes ovules of E1 (as the following Figure shown). Additionally, other reproductive ecological experiments indicate that gene flow from E1 to K1 was reduced by several prezygotic reproductive isolations. We have included a summary of these findings in the discussion section of the manuscript (Lines 428-432). These findings also provide evidence that the lineages identified in this study are more likely to represent interspecific variation rather than intraspecific variation.



Comparison of seed number of inter- and intra- specific pollinations between _A. ecalcarata_ (E1) and _A. kansuensis_ (K1) from a sympatric population in Qinghai province. (a) Comparison of the number of seed sets in interspecific cross-pollination of _A. ecalcarata_ as maternal parent, fig.(b) Comparison of the number of seed sets in interspecific cross-pollination of _A. kansuensis_ as maternal parent.

Some minor comments:

L59, between ‘reconnected’ populations.

We have revised it as suggested.

L88, with or after the split of …

We agree that "after" is more appropriate here and have revised the text accordingly.

L98, common name of columbines would be appreciated to be mentioned with Latin name.

We have added the common name of columbines alongside the Latin name at Line 90 in the revised manuscript.

L106, please clarify the meaning of ‘distribution’, ecological or geographical.

We have clarified the text to specify that the term "distribution" refers to geographical distribution (Line 111).

L119, range ‘shifts’ instead of ‘movements’

Done (Lines 125).

L161, a kind of confused with the classification of two datasets here

Thank you for pointing this out. We have clarified this by rephrasing the text to: “More detailed structure analysis of two datasets—5,875 SNPs from populations in the northern to eastern Sichuan Basin (Fig. 1f and Fig. S2) and 10,105 SNPs from populations in western Sichuan and Xizang (Fig. 1g and Fig. S3)” (Lines 167–168).

L292, in the analysis of Fst and Dxy, the authors used a sliding window size of 100kb. My understanding was that a window size of 10-50kb is more suitable. Smaller windows can capture more variation, and help to identify regions of the genome that

have experienced recent selection or other evolutionary forces. The authors of the manuscript chose a larger window size (100kb) compared to the more common 10-50kb, which is likely due to the large number of SNPs in their dataset (10 million). It would be valuable to clarify the window size used here.

We thank you for pointing this out. We agree that smaller window sizes, such as 10–50 kb, are better suited for detecting recent selection signals. However, smaller windows may not effectively capture link selection associated with gene density. For instance, the average gene density of the *Aquilegia kansuensis* genome used in this study is approximately 9.37 genes per 100 kb. Reducing the window size to 50 kb or 10 kb would lower the average number of genes per window to 4.7 and 0.9, respectively, disproportionately increasing the weight of marginal genes and potentially skewing the analysis. Additionally, while the full dataset of invariant sites contains 10 million SNPs, the dataset of variant sites is reduced to 2.7 million. Accurate detection of introgression signals requires at least 100 variant sites per window. Using a window size of 10 kb would result in nearly half of the windows being excluded due to insufficient variant sites, whereas a 100-kb window size reduces this exclusion rate to 5%. This makes the 100-kb window size more appropriate for observing genome-wide variation patterns while ensuring robust detection of introgression signals. And we have clarified these reasons in the manuscript (L314–L318).

L1073, repeated species name

The repeated species name has been corrected to "*A. ecalcarata*" (Lines 1168).

L1095, what is the meaning of "morphologically fixed loci"?

We have revised the term to "fixed loci" for clarity in the revised manuscript (Lines 1187).

Reviewer #3 (Remarks to the Author):

I partially read this manuscript but stopped when I encountered what I perceive as a potentially highly problematic issue with the sequencing data. I have some comments about the clarity of writing and framing of the questions in the introduction. I will begin with my concerns about the handling of the sequencing data, and then post my comments about the introduction.

We thank you very much for your thoughtful and constructive comments. Below, we provide point-by-point responses to each specific point, as well as the changes that have been made in the manuscript.

In particular, I have a strong concern over the haplotype phasing and genotype imputation step. The authors follow basic and accepted pipelines for library prep, read filtering, mapping, etc. However, at no point do the authors appear to filter sites for missing data (e.g., filtering out sites exceeding some threshold of individuals with missing data). Mean depth filters are applied (lines 542-543), which is good, but there are still probably many sites in the genome that are supported by only a few samples with data. This is highly problematic because the data are then phased and imputed with BEAGLE (line 544). This means there are an unknown number of sites (not reported) with data for potentially many missing individuals being imputed based on the inferred LD and genotype calls from potentially a small number of individuals. These are of course then used downstream for all analyses. Given the lack of a reference panel for proper genotype imputation and a geographically disparate set of input samples, each potentially with their own population-specific patterns of LD and allele frequencies, etc. I would highly advise not phasing and imputing genotypes with these data (at least not, for example, without more stringent missing data filters and some assessment of imputation error/ some way to assess confidence in imputed genotypes). Many of the analyses performed here are possible without phased, imputed genotypes, but we currently have no way of knowing how strongly that data processing step affects these results.

We sincerely thank you for the insightful comments regarding our haplotype phasing and genotype imputation procedures. In the previous version of our manuscript, we applied a missing data threshold of 0.9 but omitted this detail from the manuscript. We have now explicitly included this information in the revised version (Lines 605-606).

In our initial analysis, the BEAGLE phasing step was applied in the workflows related to the "ABBABABAwindows.py" and "popgenWindows.py" scripts. We acknowledge your concern that unknown sites could potentially be introduced by BEAGLE. In the revised manuscript, we re-ran all analyses involving BEAGLE, this time excluding the phasing step to evaluate its impact. The updated results (Fig. 3, Fig. 4, and Fig. S15-S22; Table S6; Lines 356-378) revealed only minor differences compared to our previous results. The main conclusions remain unchanged but are now more robust. We have revised methods and results accordingly.

Other issues with the Introduction:

Lines 53-69. The paragraph here discusses standing variation, adaptive introgression, and rapid genetic divergence, but it doesn't really connect these ideas and it is not clear why they are being discussed together.

We appreciate your comment and have revised this part and emphasized that standing variation and adaptive introgression can be two main genetic sources underlying the rapid genetic divergence of cryptic diversity (Lines 54-74). We hope these changes contribute to the clarity of introduction.

Lines 55-58. Balancing selection is not the only process responsible for standing variation.

We agree that balancing selection is not the only mechanism responsible for maintaining standing genetic variation. Accordingly, we have revised the

sentence as follows: "In contrast, standing variation might be maintained through long-term balancing selection within populations before speciation, providing an immediately accessible pool of genetic material that can facilitate rapid genetic divergence" (Lines 58-61). Thanks for your comment.

Lines 70-84. The discussion about hemiplasy is confusing. As it reads currently, the text implies hemiplasy is in some way related to the independent evolution of similar morphologies. This is a mischaracterization. Hemiplasy is the apparent convergent evolution of traits caused by incomplete lineage sorting (the traits have the same molecular basis – the problem is simply gene tree-species tree discordance).

Your insightful comment regarding hemiplasy is greatly appreciated. Our intended meaning in the manuscript aligns with your perspective. In the revised manuscript, we now have defined parallel evolution as occurring when different species independently evolve genotypes that produce similar phenotypes, following Stern (2013; *Nature Reviews Genetics* 14, 751-764). We use the term collateral evolution to describe scenarios where different lineages share the same genetic variations and evolve similar morphologies, encompassing both incomplete lineage sorting (ILS) and introgression. Additionally, we have replaced references to hemiplasy with ILS. These revisions have been made to ensure clarity and accuracy (Lines 77-85).

Below are point-by-point responses to the queries raised; queries are in regular font, and our responses are in bold.

Reviewer #1 (Remarks to the Author):

The authors addressed my previous concerns satisfactorily, both in their replies in the rebuttal letter and in the manuscript. I have no further comments.
We thank you for your insightful comments which have undoubtedly improved our work.

Reviewer #2 (Remarks to the Author):

The authors have provided a well-revised manuscript, and my primary concerns have been adequately addressed. However, I remain somewhat uncertain about the assertion of reproductive incompatibility between the proposed species lineages. While I appreciate the inclusion of a brief summary in the revised manuscript (L428-432), which suggests that prezygotic barriers, such as differences in pollinators, may contribute to reproductive isolation, this hypothesis would benefit from additional experimental or ecological evidence to strengthen its validity. In their response materials, the authors mentioned some controlled intra- and interspecific crossing results. Incorporating these findings into the manuscript would provide valuable support for their claims and enhance the overall robustness of the study. I recommend including this data in the next revision to further substantiate the discussion on reproductive isolation.

We sincerely appreciate your insightful comments. To address your concern, we have deposited the full dataset from our controlled intra- and interspecific crossing experiments in a preprint available on BioRxiv (Lines 440 and 1038-1040). We believe that providing these results further substantiates our claims and enhances the overall robustness of the study.