For research article

Response to Reviewer 1 Comments

1. Summary

Thank you very much for taking the time to review our manuscript. We greatly appreciate the reviewer's recognition of the coherent comparative and phylogenetic analysis, as well as the appropriate methodology for the stated objectives. We have carefully considered all comments and have made substantial revisions to address the methodological issues raised. Specifically, we have corrected the inconsistency regarding sequencing technologies, added comprehensive assembly quality metrics and parameter justifications, reformulated the adaptive interpretations of IR expansion and codon usage bias with appropriate caution, and explicitly acknowledged the limitations of our phylogenetic analysis. Please find the detailed point-by-point responses below and the corresponding revisions highlighted in red in the re-submitted files.

2. Questions for General Evaluation	**Reviewer's Evaluation**	**Response and Revisions**
Does the introduction provide sufficient background and include all relevant references?	Must be improved	*We have revised the Introduction to remove the inconsistency regarding sequencing technologies (Oxford Nanopore vs. Illumina only). All references cited remain relevant and appropriate for the research context.*
Are all the cited references relevant to the research?	Must be improved	*Yes. All 46 references are directly relevant to chloroplast genomics, Delphinium systematics, high-altitude adaptation, or methodological approaches. No irrelevant references have been cited.*
Is the research design appropriate?	Must be improved	*The research design is appropriate for the stated objectives. We have added assembly quality validation (BWA read mapping, coverage metrics) to strengthen the methodological rigor.*

Are the methods adequately described?	Must be improved	*We have substantially improved the Methods section by adding: (1) detailed assembly quality metrics (>500× coverage, 100% breadth, read mapping validation); (2) justification for k-mer parameter selection; and (3) explicit description of quality control thresholds.*
Are the results clearly presented?	Must be improved	*We have further improved the Results section by adding nucleotide diversity (Pi) analysis for both protein-coding genes and intergenic spacers, providing quantitative thresholds for identifying hypervariable hotspots (Pi ≥ 0.06 for IGS, Pi ≥ 0.02 for CDS) and conserved loci (Pi ≤ 0.01). These additions enhance the statistical rigor and provide clear criteria for DNA barcode candidate selection.*
Are the conclusions supported by the results?	Must be improved	*We have revised the conclusions to acknowledge uncertainties explicitly. The adaptive interpretations are now presented as hypotheses requiring validation rather than established facts, and the phylogenetic conclusions are qualified as provisional pending nuclear marker validation.*

3. Point-by-point response to Comments and Suggestions for Authors

Comments 1: The Introduction refers to the integration of Illumina and Oxford Nanopore data, whereas the Methods section describes only Illumina sequencing; this inconsistency must be corrected.

Response 2: Thank you very much for pointing out this critical inconsistency. We sincerely apologize for this error, which resulted from an oversight during manuscript preparation where we failed to update the Introduction to reflect the actual sequencing approach employed.

In the revised manuscript (Section 1, Introduction, paragraph 4), we have corrected the statement from "Here, we integrate Illumina short-read and Oxford Nanopore long-read technologies" to: "Here, we used Illumina NovaSeq paired-end sequencing to generate a high-quality, fully annotated chloroplast genome of D. densiflorum." The mention of Oxford

Nanopore long-read technology has been removed entirely to ensure complete consistency with the Methods section (Section 2.2), which correctly describes only the Illumina sequencing approach.

We confirm that this study used exclusively Illumina NovaSeq paired-end (PE150) sequencing, and no Oxford Nanopore data were generated or analyzed. This correction eliminates the methodological inconsistency identified by the reviewer, and we have carefully checked the entire manuscript to ensure no similar discrepancies remain.

Comments 2: No assembly quality metrics are reported (e.g., mean chloroplast genome coverage or validation by read mapping), and the choice of assembly parameters is not justified.

Response 2: Thank you for this important comment. We fully agree that assembly quality metrics are essential for validating the reliability of the chloroplast genome assembly.

We have added a new paragraph in Section 2.3 (Genome Assembly) describing comprehensive quality control and validation procedures:

"To assess the quality and completeness of the assembled chloroplast genome, the filtered clean reads were mapped back to the final assembly using BWA (Burrows-Wheeler Aligner) [20] with default parameters. The mapping results were processed using SAMtools [21] to calculate genome coverage depth and breadth. The assembly achieved > 500× mean coverage depth and 100% breadth (complete coverage of the chloroplast genome), with > 99% of bases covered by at least 10 reads and > 95% covered by at least 50 reads, confirming high assembly accuracy and completeness."

Additionally, regarding the justification of assembly parameters, we have added the following explanation in Section 2.3:

"The k-mer values of 55, 87, and 121 were selected based on the standard SPAdes recommendations for organelle genome assembly, balancing sensitivity for repetitive regions (higher k-mers) with robustness for regions of lower coverage (lower k-mers)."

These additions provide quantitative validation of assembly quality and transparent justification of methodological choices.

Comment 3: The adaptive interpretations regarding IR expansion and A/U codon bias are insufficiently supported and remain speculative. These claims should be reformulated more cautiously or substantiated through additional comparisons with other alpine species.

We sincerely appreciate this critical feedback. Upon reflection, we agree that our original interpretations were overly speculative and have thoroughly revised these sections to present a more cautious and balanced interpretation.

Regarding IR expansion (Section 4.1): We have substantially rewritten this section to acknowledge alternative explanations:

We now state: "These convergent patterns are consistent with IR expansion-contraction potentially reflecting a recurrent genomic response to high-altitude environments, though alternative explanations involving shared ancestral polymorphisms or mutational hotspots at IR-SSC junctions cannot be excluded."

We have added an explicit acknowledgment: "We acknowledge that IR boundary shifts may arise from neutral mutational processes rather than selection. The IR-SSC junctions are known hotspots for illegitimate recombination and slipped-strand mispairing, which can generate expansion-contraction cycles independent of environmental adaptation."

We now present multiple hypotheses: "The observed correlation between IR expansion and high-altitude distribution in Delphinium could therefore reflect: (i) shared ancestry of alpine lineages rather than convergent adaptation; (ii) genetic drift in small, isolated populations; or (iii) linkage with selected loci elsewhere in the genome."

The conclusion states: "Distinguishing among these hypotheses requires population-level sampling and functional validation."

Regarding A/U codon bias (Section 4.4): We have completely rewritten this section to present a more nuanced interpretation:

We now emphasize: *"This pattern primarily reflects the overall AT-richness (61.76%) of the chloroplast genome, a characteristic common to many angiosperm plastomes due to mutational biases and weak selection on synonymous sites."*

We explicitly note the comparison with low-altitude species: *"the low-elevation congener D. grandiflorum (distributed at <2,000 m) exhibits similarly high AU bias (86.3% AU-ending codons), indicating that this pattern is likely phylogenetically conserved across Delphinium rather than exclusively driven by altitude-dependent selection."*

We conclude with appropriate caution: *"Distinguishing between neutral and adaptive explanations for codon usage bias requires heterologous expression experiments or comparative transcriptomics across temperature gradients. We therefore interpret the AU-rich profile of D. densiflorum as a candidate pattern for functional validation rather than established evidence of adaptation."*

These revisions transform speculative claims into balanced, hypothesis-driven discussions that acknowledge uncertainty and the need for further validation.

Comment 4: The phylogenetic analysis, although correctly implemented, is limited by a restricted taxon sampling and by the exclusive use of chloroplast markers; expanding the taxon set or incorporating nuclear data would strengthen the conclusions regarding the non-monophyly of Delphinium.

Response 4: We completely agree with this assessment and appreciate the reviewer's recognition that our phylogenetic analysis was correctly implemented. We acknowledge these important limitations and have substantially revised the manuscript to address them.

Explicit acknowledgment of limitations (Section 3.5): We have added: *"However, we caution that plastid phylogenies reflect only the maternal evolutionary history and may not fully represent the species tree, particularly given the documented hybridization and polyploidization*

events in this genus [8,9]. Nuclear ribosomal (ITS) or low-copy nuclear gene data are required to further test this phylogenetic hypothesis."

Expanded discussion in Section 4.5: We have thoroughly revised this section to acknowledge the limitations:

"While this topology suggests potential paraphyly of Delphinium with respect to other genera in Delphinieae, we emphasize that chloroplast genomes represent a single, maternally inherited locus susceptible to introgression and incomplete lineage sorting."

"The observed conflict between morphology and plastid phylogeny...is consistent with either (i) convergent evolution of floral traits, or (ii) maternal capture of divergent plastid lineages via hybridization."

"Robust inference of generic monophyly requires concordance between plastid and nuclear phylogenies; until such data are available, we interpret the non-monophyly of Delphinium as a provisional hypothesis requiring validation through phylogenomic analyses of nuclear markers."

Revised Conclusions (Section 5): We now state: *"Phylogenetic analysis based on maternally inherited chloroplast CDSs provisionally clarifies the evolutionary position of D. densiflorum...However, given that chloroplast genomes track only maternal lineage history and are subject to introgression, we tentatively interpret the distant relationship with D. grandiflorum as suggestive of potential paraphyly of Delphinium sensu lato, pending validation by biparentally inherited nuclear markers."*

We also explicitly recommend future research directions: *"Future studies incorporating ITS and low-copy nuclear genes are essential to distinguish between true phylogenetic divergence and maternal lineage sorting or cytoplasmic capture events."*

Regarding taxon sampling: While we acknowledge that expanded taxon sampling would strengthen our conclusions, we have utilized all 12 publicly available complete chloroplast genomes of Delphinium species at the time of our study. The addition of nuclear markers, rather than more plastomes, is the critical next step for resolving the phylogenetic relationships within this genus.

4. Response to Comments on the Quality of English Language

We are grateful that the reviewer finds our English acceptable. We have carefully reviewed the entire manuscript and made minor editorial improvements for clarity where necessary. All revisions are highlighted in red in the re-submitted files.

5. Additional clarifications

We would like to emphasize that all revisions have been made with the goal of presenting a scientifically rigorous, cautious, and transparent account of our findings. We have replaced speculative adaptive interpretations with balanced discussions that acknowledge multiple hypotheses, and we have explicitly recognized the limitations of our phylogenetic inferences.

We believe that with these substantial revisions, the manuscript now presents a robust analysis of the D. densiflorum chloroplast genome while appropriately acknowledging uncertainties and the need for future validation studies.

Thank you again for your invaluable feedback, which has significantly improved the quality and rigor of our manuscript.