

Article

Assembly of the *Delphinium densiflorum* Chloroplast Genome and Comparative Genomics Within *Delphinium*

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Abstract

Background/Objectives: Chloroplast genomes are essential for understanding the systematics and adaptive evolution of alpine plants, yet genomic data for high-altitude *Delphinium* species remain scarce. *Delphinium densiflorum*, a medicinal plant endemic to the Qinghai-Tibet Plateau, exhibits notable high-altitude adaptations, but its plastome features and evolutionary position are still unclear. This study aims to assemble and characterize its complete chloroplast genome and clarify its phylogenetic placement within *Delphinium*. **Methods:** Using Illumina NovaSeq data, we de novo assembled the *D. densiflorum* plastome, annotated it with CPGAVAS2, and compared it with 12 published Ranunculaceae plastomes. We analyzed IR-boundary dynamics, genome-wide sequence variation, and codon-usage bias and constructed a maximum-likelihood phylogeny based on 69 shared protein-coding genes. **Results:** The plastome is 154,161 bp (GC 38.24%) with a canonical quadripartite structure, encoding 131 genes (87 CDS, 8 rRNA, 37 tRNA). An IR expansion into the SSC region yields the shortest SSC reported among the compared *Delphinium* species and produces unique structural variants. Photosynthetic genes are extremely conserved (nucleotide diversity $P_i \leq 0.01$), whereas several loci (e.g., *ycf1* and *psaC*) are highly divergent ($P_i \geq 0.05$). Codon usage shows a strong bias toward AU-ending triplets. Phylogenetically, *D. densiflorum* forms a 100%-bootstrap clade with other high-altitude congeners, supporting the non-monophyly of *Delphinium*. **Conclusions:** This study delineates the plastome architecture and putative adaptive signatures of *D. densiflorum*, identifies robust candidate loci for DNA barcoding, and provides molecular evidence for taxonomic revision and conservation strategies in *Delphinium*.

Keywords: *Delphinium densiflorum*; chloroplast genome; comparative genomics



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1. Introduction

Delphinium densiflorum is a perennial herb endemic to the Qinghai-Tibet Plateau and adjacent highlands, where it occurs in valley thickets, river terraces, or alluvial fans at elevations of 3300–4500 m. The entire plant is used in traditional Tibetan and folk medicine as an antipyretic and detoxifying agent; decoctions are applied externally to treat dermatitis, furuncles, tinea, and psoriasis or taken orally to counteract Aconitum poisoning [1].

The genus *Delphinium* L. (Ranunculaceae) comprises approximately 365 species distributed throughout the north-temperate alpine and sub-alpine belts, with three primary diversity centres: (i) the European Alps-Carpathians, (ii) the Rocky Mountains-Mexican Plateau, and (iii) the Himalaya-Hengduan Mountains [2,3]. China harbours the richest assemblage, with 232 species ($\approx 64\%$ of global diversity) recorded to date [4].

For centuries, the tubers and roots of *Delphinium* have been employed in traditional materia medica across continents. In Anatolian folk medicine, they are prescribed against epilepsy, rabies, and tetanus [5]; in the UK and France, the dried and powdered tubers of *D. staphisagria* and *D. peregrinum* are used as an “insecticidal powder”; in military camps it is dusted onto clothing to kill body lice, and the same formula is also applied externally for scabies and cutaneous parasitic infections [3]; in Iran they are administered for splenic disorders, jaundice, and oedema [6], whereas in China preparations are utilised to relieve toothache, rheumatic pain, oedema, and cutaneous affections such as scabies and tinea [7].

Orogenic uplift of the Qinghai-Tibet Plateau since the Late Miocene, coupled with intensified monsoon dynamics and Quaternary climatic oscillations, has triggered rapid radiation, recurrent hybridisation, and extensive polyploidisation within *Delphinium*, producing widespread morphological convergence and cytotype mosaics that confound both classical taxonomy and molecular phylogenetic reconstruction [8,9]. Concomitantly, the chloroplast (cp) genomes of high-alpine lineages have experienced unique molecular trajectories—ranging from gene loss and pseudogenisation to IR boundary shifts and accelerated sequence divergence—which have been hypothesized to reflect potential adaptation to hypobaric hypoxia, high UV-B flux, and thermal instability [10,11], although neutral processes such as genetic drift and mutational bias may additionally contribute. Despite these insights, the phylogenetic placement of *Delphinium* within Ranunculaceae remains contentious, with morphological circumscriptions often contradicting molecular topologies [8,12].

Chloroplasts retain a semi-autonomous genetic system that integrates environmental cues with organellar gene expression [13,14]. The circular cp genome of photosynthetic angiosperms (115–165 kb) is present in high copy number and exhibits strong structural conservatism, making it an ideal locus for comparative evolutionary studies [15]. Elucidating the complete cp genome of *D. densiflorum* will therefore (i) clarify the deep-level relationships between *Delphinium* and other ranunculaceous genera—especially *Ranunculus*, with which it shares superficial morphological similarity—and (ii) provide a robust reference for future DNA-barcode design, germplasm conservation, and marker-assisted breeding programmes within the family.

Here, we used Illumina NovaSeq paired-end sequencing to generate a high-quality, fully annotated chloroplast genome of *D. densiflorum*. Through comparative genomic and phylogenomic analyses that incorporate all publicly available ranunculaceous cp genomes, we aim to (i) characterise structural innovations unique to the high-alpine *Delphinium* lineage, (ii) test the monophyly of traditional infrageneric classifications, and (iii) refine the temporal and biogeographic framework of Ranunculaceae diversification.

2. Materials and Methods

2.1. Sample Collection and DNA Extraction

D. densiflorum samples were collected from Qunjia Forest Farm, Huangzhong District, Xining City, Qinghai Province, China (101°41' E, 36°16' N). Healthy fresh leaves were harvested, silica-dried, and transported back to the laboratory for subsequent use. Species identification was verified by Associate Professor Jiuli Wang from Qinghai Minzu University.

Genomic DNA was extracted using the Plant Genomic DNA Kit (Cat. No. DP305; Tiangen Biotech (Beijing) Co., Ltd., Beijing, China) strictly following the manufacturer's

protocols. The extracted DNA samples were then sent to Nanjing GSH Biotechnology Co., Ltd. (Nanjing, China) for high-throughput sequencing.

2.2. Sequencing and Data Quality Control

Following qualification of the genomic DNA samples, the DNA was fragmented by ultrasonication. The fragmented DNA was then subjected to purification, end repair, 3'-dA tailing, and sequencing adapter ligation. Fragment size selection was performed via agarose gel electrophoresis, followed by PCR amplification to construct the sequencing library. The constructed library underwent quality inspection; qualified libraries were sequenced on the Illumina NovaSeq 6000 platform with a paired-end (PE) read length of 150 bp (PE150) [16].

Raw sequencing data were filtered using fastp software (version 0.20.0, <https://github.com/OpenGene/fastp>, accessed on 13 January 2026): potential sequencing adapters and primer sequences were trimmed from the reads; reads with an average quality score < Q5 were discarded to avoid interference with subsequent analysis accuracy; reads containing more than 5 Ns (undetermined bases) were removed. Strict quality control was implemented to reduce uncertainties in the sequencing results [17].

2.3. Genome Assembly

The chloroplast genome of *D. densiflorum* was assembled using SPAdes software (v3.10.1, <https://github.com/ablab/spades>, accessed on 13 January 2026) with k-mer values set to 55, 87, and 121, yielding a preliminary chloroplast genome scaffold. Gap filling was performed on the assembled sequence using Gapfiller v2.1.1 (<https://sourceforge.net/projects/gapfiller/>, accessed on 13 January 2026) [18]. Based on the typical quadripartite structure of chloroplast genomes, the corrected pseudo-genome was subjected to coordinate rearrangement to generate a complete circular chloroplast genome sequence [19]. Finally, the final chloroplast genome sequence of *D. densiflorum* was obtained after manual inspection and refinement.

To assess the quality and completeness of the assembled chloroplast genome, the filtered clean reads were mapped back to the final assembly using BWA (0.7.19) (Burrows-Wheeler Aligner) [20] with default parameters. The mapping results were processed using SAMtools (v2.1) [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 completeness and reliability of the assembly.

2.4. Genome Annotation

The assembled complete chloroplast genome sequence was annotated using CP-GAVAS2 (v2.1.0), a specialized tool for chloroplast genome annotation. Following manual inspection and correction, the chloroplast genome map was generated using Chloroplot (<https://irscope.shinyapps.io/Chloroplot/>, accessed on 13 January 2026), an organellar genome visualization tool. Finally, the annotated chloroplast genome was submitted to the GenBank database of the National Center for Biotechnology Information (NCBI) with the accession number OM022261.1.

2.5. Comparative Genomic Analysis

D. densiflorum belongs to the genus *Delphinium*. For comparative chloroplast genomic analysis, we selected 12 representative species with publicly available complete chloroplast genomes, including 11 *Delphinium* species (i.e., *Delphinium anthriscifolium*, *D. brunonianum* [22], *D. caeruleum*, *D. candelabrum* var. *monanthum*, *D. ceratophorum*, *D. nudatum* [23], *D. grandiflorum* [24], *D. maackianum*, *D. tangkulaense*, and *D. yunnan-*

nense) and one Ranunculaceae outgroup species (*Ranunculus tanguticus*). Combined with the chloroplast genome of *D. densiflorum* obtained in this study, a total of 12 complete chloroplast genomes were included in the comparative analysis.

2.5.1. IR Boundary Analysis

Previous studies have shown that the chloroplast genomes of *Delphinium* species typically possess a circular quadripartite structure, with four junction sites between the inverted repeat (IR) regions and single-copy (SC) regions: JSA (SSC-IRa), JLA (IRa-LSC), JLB (LSC-IRb), and JSB (IRb-SSC) [7]. IRscope (<https://irscope.shinyapps.io/irapp/>, accessed on 13 January 2026) was used to analyze the boundary positions between IR and SC regions of *D. densiflorum* and 11 other Ranunculaceae species, thereby determining the expansion and contraction events of the IR regions [25].

2.5.2. Global Sequence Variation Analysis

The CPJSDraw visualization tool (<http://cloud.genepioneer.com:9929>, accessed on 13 January 2026) [26] was employed to compare and analyze the boundary information of the four regions (LSC, SSC, IRa, IRb) in the chloroplast genomes of 12 Ranunculaceae species, allowing observation of the contraction and expansion dynamics of the IR regions. Using the chloroplast genome of *D. densiflorum* as the reference, homologous sequence alignment was performed via the online mVISTA tool (<https://genome.lbl.gov/vista/index.shtml>, accessed on 13 January 2026) [27] under the Shuffle-LAGAN (global alignment) mode.

2.5.3. Nucleotide Diversity Analysis

Nucleotide diversity (P_i) was calculated for protein-coding sequences (CDSs) and intergenic spacers (IGSs) using DnaSP v6.0 [28]. Complete chloroplast genome sequences of 12 species were aligned with MAFFT v7.0 [29] using the G-INS-i strategy. For CDS analysis, start and stop codons were excluded, and overlapping regions between adjacent genes were trimmed to avoid redundancy. For IGS analysis, intergenic regions between protein-coding and tRNA genes were extracted and concatenated. P_i values were computed using a sliding window approach (window size: 600 bp; step size: 200 bp) implemented in DnaSP to visualize the distribution of genetic diversity across the genome. Hypervariable hotspots were operationally defined as regions with $P_i \geq 0.06$ for IGSs and $P_i \geq 0.02$ for CDSs, whereas conserved loci were defined as $P_i \leq 0.01$. These thresholds were selected based on the overall distribution of nucleotide diversity in the dataset.

2.6. Phylogenetic Tree Construction

In this study, *R. tanguticus* (GenBank ID: OR625580.1) was selected as the outgroup. Shared protein-coding sequences (CDSs) were extracted from the chloroplast genomes of *D. densiflorum* and its related taxa (Table 1). After sequence integration and alignment using MAFFT, the best-fit nucleotide substitution model was determined using ModelTest-NG (v0.1.7), which selected the GTR + I + G model as optimal based on the Akaike Information Criterion (AIC). A phylogenetic tree was constructed via MEGA X software (v10.0) based on the maximum likelihood (ML) method [30]. The main parameters were set as follows: Model = GTR + I + G, bootstrap = 1000 replicates. Bootstrap support values $\geq 70\%$ were considered as strong support.

Table 1. Chloroplast genomes used for phylogenetic tree construction.

No	Family	Subfamily	Species	Genbank ID
1	Ranunculaceae	Ranunculus	Ranunculus tanguticus	OR625580.1
2	Ranunculaceae	Delphinioideae	Delphinium densiflorum	OM022261.1
3	Ranunculaceae	Delphinioideae	Delphinium anthriscifolium	MK253461.1
4	Ranunculaceae	Delphinioideae	Delphinium brunonianum	MT457851.1
5	Ranunculaceae	Delphinioideae	Delphinium caeruleum	PP234506.1
6	Ranunculaceae	Delphinioideae	Delphinium candelabrum var. monanthum	MW246165.1
7	Ranunculaceae	Delphinioideae	Delphinium ceratophorum	MK253460.1
8	Ranunculaceae	Delphinioideae	Delphinium denudatum	PV364607.1
9	Ranunculaceae	Delphinioideae	Delphinium grandiflorum	MN556604.1
10	Ranunculaceae	Delphinioideae	Delphinium maackianum	MN648402.1
11	Ranunculaceae	Delphinioideae	Delphinium tangkulaense	PP234555.1
12	Ranunculaceae	Delphinioideae	Delphinium yunnanense	MW246156.1

3. Results

3.1. Chloroplast Genome Characteristics

Approximately 5.01 Gb of paired-end sequencing data was generated in this study, providing an estimated coverage depth of $\sim 507\times$ (based on genome size of 154,161 bp) and 100% breadth, ensuring robust assembly quality. Read mapping validation showed that 99.7% of the chloroplast genome was covered by ≥ 10 reads and 96.2% by ≥ 50 reads, with an average mapping quality score of Q58, confirming high assembly accuracy and completeness. From these data, a circular chloroplast genome of *D. densiflorum* (Figure 1) was assembled, with a total length of 154,161 bp and a GC content of 38.24%. It exhibits a typical quadripartite structure consisting of a large single-copy (LSC) region of 84,881 bp, a small single-copy (SSC) region of 16,164 bp, and two identical inverted repeat (IR) regions (IRa and IRb) each of 26,558 bp.

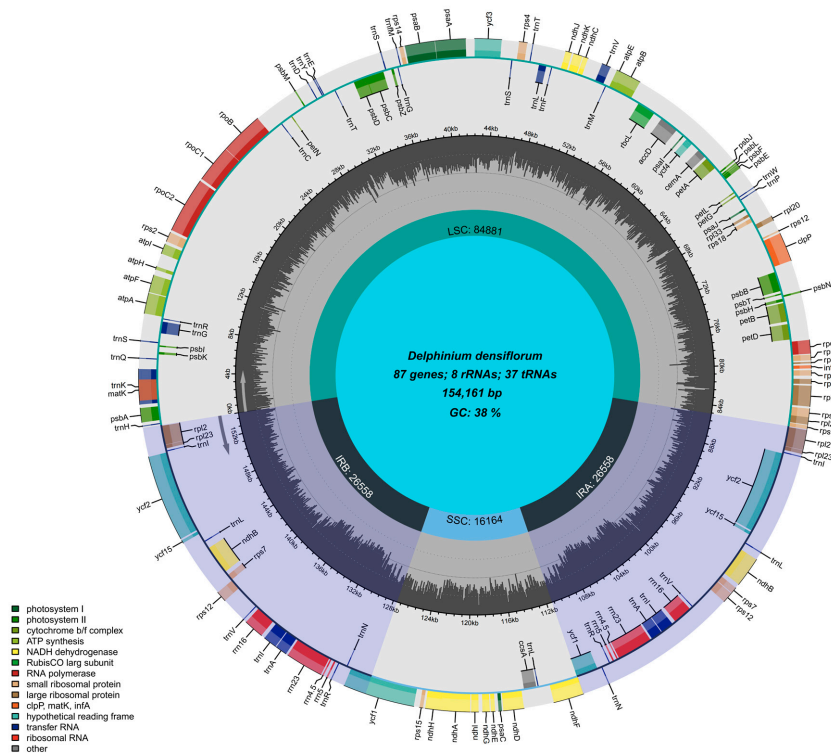


Figure 1. Chloroplast Genome Map of *D. densiflorum*. Genes drawn outside the circle are transcribed in the clockwise direction, whereas those inside are transcribed counter-clockwise. Different colored

boxes denote distinct functional gene categories. The inner gray ring represents GC content, with darker peaks indicating regions of higher GC content. LSC, large single-copy region (green); SSC, small single-copy region (light blue); IRa and IRb, inverted repeat regions (light purple). Genome size: 154,161 bp; average GC content: 38%.

The chloroplast genome of *D. densiflorum* contains 131 genes, which can be functionally classified into categories such as photosynthesis and self-replication (Table 2). Among these, there are 87 protein-coding sequences (CDSs), 8 ribosomal RNA (rRNA) genes, and 37 transfer RNA (tRNA) genes, with no pseudogenes detected.

Table 2. Gene composition of the *D. densiflorum* chloroplast genome.

Function	Gene Grouping	Gene Name
Photosynthesis-related genes	photosystem I	<i>psaA, psaB, psaC, psaI, psaJ</i>
	photosystem II	<i>psbA, psbB, psbC, psbD, psbE, psbF, psbH, psbI, psbJ, psbK, psbL, psbM, psbN, psbT, psbZ</i>
	cytochrome b/f complex	<i>petA, petB*, petD*, petG, petL, petN</i>
	ATP synthesis	<i>atpA, atpB, atpE, atpF*, atpH, atpI</i>
	NADH dehydrogenase	<i>ndhA*, ndhB*(2), ndhC, ndhD*, ndhE, ndhF, ndhG, ndhH, ndhI, ndhJ*, ndhK</i>
	RubisCO large subunit	<i>rbcL</i>
Self-replication	RNA polymerase	<i>rpoA, rpoB, rpoC1*, rpoC2*</i>
	Large ribosomal protein	<i>rpl2**(2), rpl14, rpl16*, rpl20, rpl22, rpl23, rpl33, rpl36</i>
	Small ribosomal protein	<i>rps2, rps3*, rps4, rps7(2), rps8, rps11, rps12**(2), rps14, rps15, rps16*, rps18, rps19</i>
	Ribosomal RNAs	<i>rrn16(2), rrn23(2), rrn4.5(2), rrn5(2)</i>
	Transfer RNAs	<i>trnA-UGC*(2), trnC-GCA, trnD-GUC, trnE-UUC, trnF-GAA, trnG-GCC, trnG-UCC, trnH-GUG, trnI-CAU*(2), trnI-GAU, trnK-UUU*, trnL-CAA(2), trnL-UAA*, trnL-UAG*, trnM-CAU, trnN-GUU(2), trnP-UGG, trnQ-UUG, trnR-ACG(2), trnR-UCU, trnS-GCU, trnS-GGA, trnS-UGA, trnT-GGU, trnT-UGU, trnV-GAC(2), trnV-UAC, trnW-CCA, trnY-GUA*, trnfM-CAU</i>
Other genes	Caseinolytic protease P	<i>clpP*</i>
	Maturase	<i>matK</i>
	Translation initiation factor	<i>infA</i>
	Chloroplast envelope membrane protein A	<i>cemA</i>
	AcetylCoA carboxylase β subunit	<i>accD</i>
	Ctype cytochrome synthesis factor A	<i>ccsA</i>
Hypothetical reading frame	hypothetical reading frame	<i>#ycf1, ycf2(2), ycf3*, ycf4**, ycf15(2)</i>

Notes: Gene*: Gene with one intron; Gene**: Gene with two introns; # Gene: Pseudo gene; Gene (2): Number of copies of multicopy genes.

3.2. IR Boundary Analysis Among *Delphinium* Species

Except for *D. densiflorum*, the JLB boundary (the boundary between the LSC and IRb regions) of all other species is located within the *rpl2* gene, approximately 1484 bp from the start site of this gene (Figure 2). The JLA boundary (the boundary between the IRa and LSC regions) of all species is situated on the same side as the *trnH* gene, with an interval of approximately 75 bp from the *trnH* gene. The JSB boundary (the boundary between the IRb and SSC regions) is near the right end of the *ndhF* gene in most species, at a distance of 68–72 bp, whereas this boundary has shifted in *D. densiflorum* due to IR region expansion.

The JSA boundary (the boundary between the SSC and IRa regions) is located within the *ycf1* gene; *D. densiflorum* exhibits a fragmented duplication of the *ycf1* gene at this boundary, with sequences duplicated at positions 3639 bp and 1677 bp, and a 27 bp short sequence immediately following the second duplication.

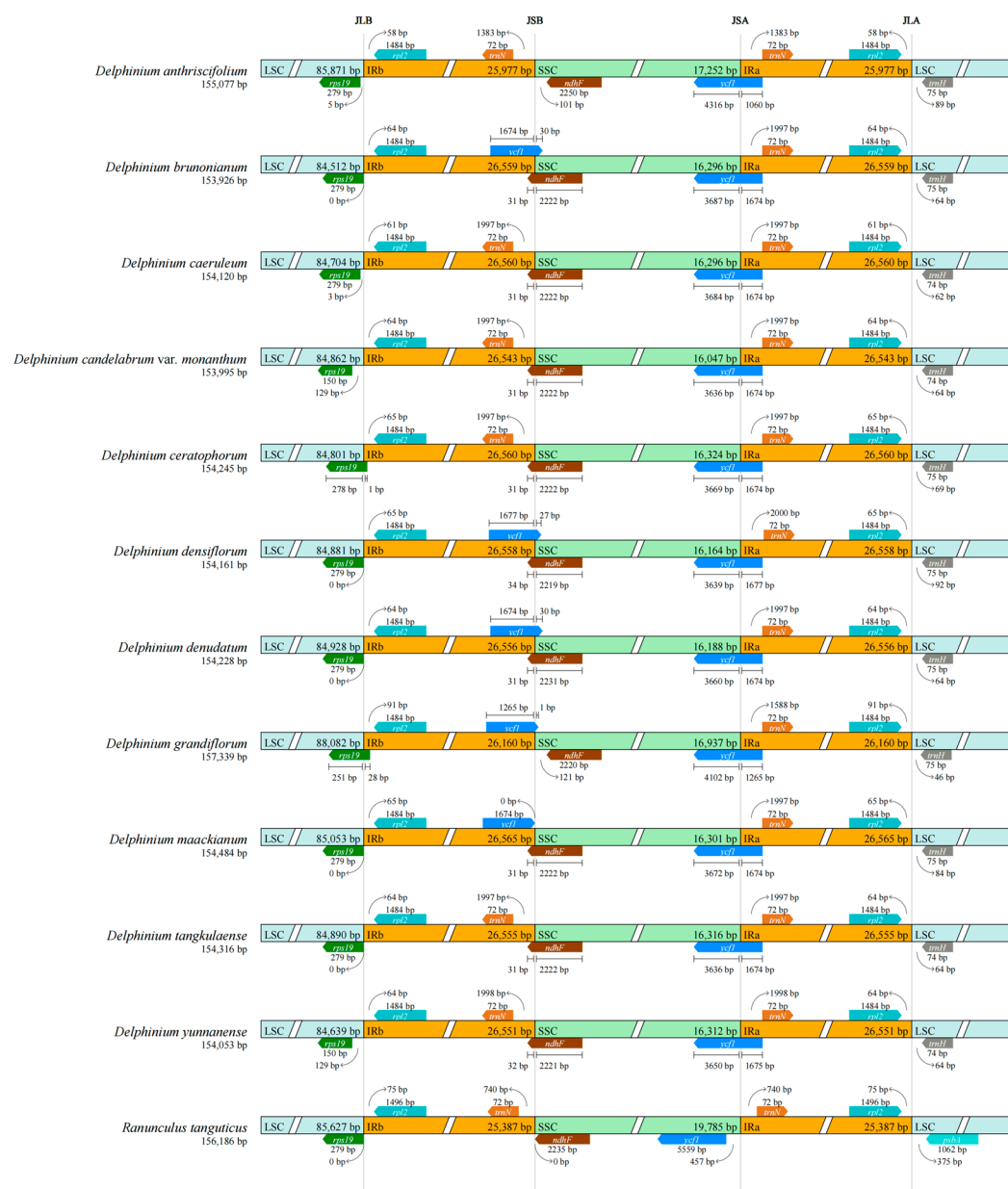


Figure 2. Comparative analysis of the four junction sites (JLB, JSB, JSA, and JLA) between the inverted repeat (IRa, IRb) and single-copy (LSC, SSC) regions in twelve *Delphinium* and one *Ranunculus* chloroplast genomes. Genes adjacent to the junctions are shown, with numbers indicating the distance (in base pairs) from each junction to the start or end of the neighboring gene. Different colored boxes denote distinct regions.

The length of the IR region in the chloroplast genomes of the 12 species ranges from 25,977 bp (*D. anthriscifolium*) to 26,565 bp (*Delphinium denudatum*). Among them, the IR region of *D. densiflorum* is 26,558 bp in length, and its SSC region is 16,164 bp, the shortest among the tested *Delphinium* species—further confirming that its IR boundary has undergone significant expansion toward the SSC region.

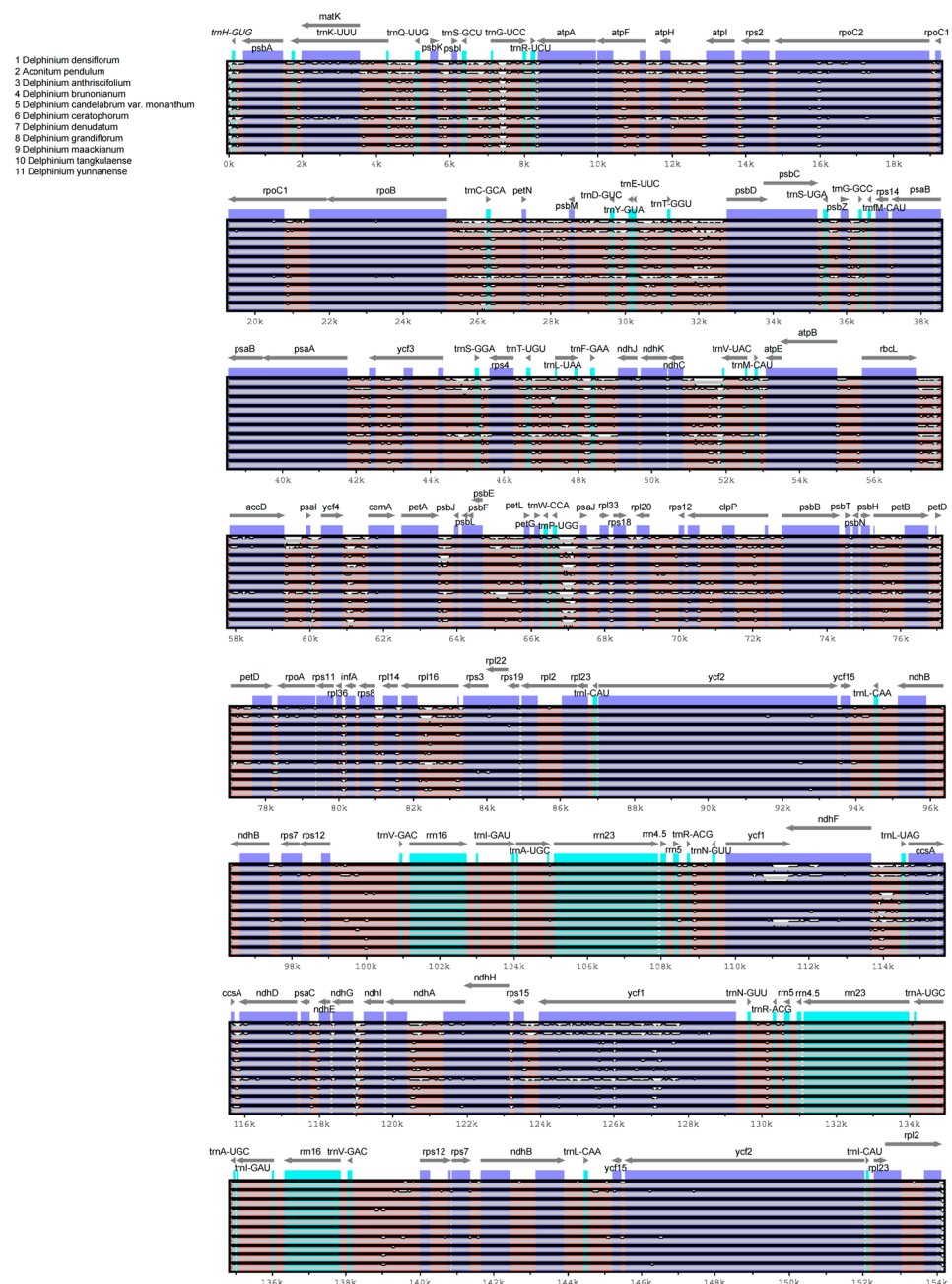


Figure 4. Sequence alignment of chloroplast genomes from *D. densiflorum* and related species using mVISTA. The x-axis represents the position in the reference genome (*D. densiflorum*). Purple bars indicate exons; gray arrows show gene orientation and transcription direction. Pink/red areas denote regions of high sequence conservation ($\geq 70\%$ identity), while light blue areas indicate non-coding or less conserved regions. Black vertical lines represent SNPs; green vertical lines indicate indels.

To assess the robustness of hypervariable hotspot identification, we performed sensitivity analyses by varying sliding-window parameters and taxon sampling. Using window sizes of 400, 600, and 800 bp (with proportional step sizes of 133, 200, and 267 bp), the six hypervariable CDS (*ycf1*, *psaC*, *rps11*, *rpl22*, *rps18*, and *rps16*) and eight IGS (*rps18-rpl20*, *trnD-GUC-trnY-GUA*, *ccsA-ndhD*, *ndhG-ndhI*, *psaI-rpl33*, *petA-psbJ*, *rps15-ycf1*, and *trnE-UUC-trnT-GGU*) regions remained consistent across all parameter combinations. Similarly, jackknife resampling (iteratively excluding one of the 12 species) recovered the same hotspot regions in $>90\%$ of iterations, indicating that the results are robust to single-taxon exclusion.

4.2. IR-Boundary Dynamics and Phylogenetic Signal Within the Genus

Comparative mapping of IR/SC junctions across 12 chloroplast genomes reveals pronounced interspecific shifts within *Delphinium*, yet the movement adheres to a “micro-slide” model: the JLA (IRa/LSC) and JLB (LSC/IRb) boundaries remain effectively stationary, whereas JSB (IRb/SSC) and JSA (SSC/IRa) undergo concerted displacement in *D. densiflorum* and its high-elevation allies. This synchronous repositioning constitutes a molecular synapomorphy that recovers the “plateau clade” with 100% bootstrap support [7,37]. While this boundary congruence provides a useful diagnostic trait for distinguishing high-altitude species, it does not necessarily imply adaptive significance; the shared structural pattern may equally reflect shared ancestry or common mutational tendencies at the IR-SSC junctions in this lineage.

4.3. Hypervariable Hotspots and DNA-Barcoding Potential

Sliding-window analysis identified four divergence hotspots ($P_i \geq 0.05$) within the intergenic spacers *trnK-rbcL*, *psbZ-psaB* and *rpl32-ycf1*, whereas core photosynthetic loci (*psbA*, *rbcL*, *atpB*, etc.) remained extremely conserved ($P_i \leq 0.01$), exemplifying a “functionally conserved, sequence-diverged” genomic landscape [37,38]. The single-copy gene *ycf1* exhibited the highest overall variability ($P_i = 0.1987$) and is increasingly advocated as a universal “super-barcode” because of its length polymorphism and strong phylogenetic resolving power [39]. We detected two tandem repeats within *ycf1* (3.6 kb and 1.7 kb) that can be targeted by specific primers for rapid molecular authentication of *D. densiflorum* against its look-alike congeners [39,40].

4.4. Codon-Usage Bias: A Potential Correlation with High-Altitude Environment

Relative synonymous codon usage (RSCU) analysis revealed that 87.5% of preferred codons in *D. densiflorum* are AU-ended. 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 [33,41]. The biased gene conversion and spontaneous deamination of cytosine to thymine in the single-stranded DNA state during replication contribute to the universal AT mutation bias in chloroplast genomes [42]. The plastid tRNA pool is similarly enriched for A/U anticodons, which may reinforce codon preference through translational selection efficiency [43], although the strength of this selection in plastomes is generally considered weak relative to prokaryotes [41].

In addition to these structural and mutational constraints, the observed AU bias has been hypothesized to confer physiological advantages in cold environments. Under the ‘cold-adaptation hypothesis; A/T-rich sequences may reduce DNA/RNA secondary structure stability, potentially facilitating transcription and translation at low temperatures [43,44]. Consistent with this hypothesis, *D. densiflorum* shows AT enrichment comparable to other Himalayan-Hengduan alpine species [10,23]. However, the low-elevation congener *D. grandiflorum* (distributed at <2000 m) exhibits similarly high AU bias (86.3% AU-ending codons) [24], indicating that this pattern is likely phylogenetically conserved across *Delphinium* rather than exclusively driven by altitude-dependent selection. Furthermore, direct experimental evidence linking codon usage to thermal performance is lacking, and the correlation may equally reflect shared ancestry or genetic drift in isolated alpine populations [45].

Distinguishing between neutral and adaptive explanations for codon usage bias requires heterologous expression experiments or comparative transcriptomics across temperature gradients [43]. We therefore interpret the AU-rich profile of *D. densiflorum* as a candidate pattern for functional validation rather than established evidence of adaptation.

4.5. Phylogenetic Placement and Evidence for the Non-Monophyly of *Delphinium*

The maximum-likelihood tree reconstructed from 69 shared CDS loci provisionally groups *D. densiflorum* with *D. caeruleum* and *D. denudatum* in a clade receiving 100% bootstrap support, nested within the “Himalaya-Hengduan rapid-radiation lineage.” In contrast, the morphologically similar, low-elevation *D. grandiflorum* is placed at the base of the tree. 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—whereby *D. grandiflorum* is distant from high-elevation congeners despite similar floral morphology—is consistent with either (i) convergent evolution of floral traits or (ii) maternal capture of divergent plastid lineages via hybridization [8,12]. 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 [8].

5. Conclusions

Based on the research background and hypotheses, this study is the first to decode the chloroplast genome of *D. densiflorum*, which not only conforms to the conservative characteristics of the genus *Delphinium* but also exhibits unique variations adaptive to high altitudes [23]. With a length of 154,161 bp, a GC content of 38.24%, and 131 annotated genes (87 CDSs, 8 rRNAs, and 37 tRNAs), its composition is highly consistent with previously reported *Delphinium* species, confirming the structural stability of chloroplast genomes in this genus. Notably, the significant expansion of the IR region (26,558 bp in length) toward the SSC region results in the shortest SSC region (16,164 bp) among the tested *Delphinium* species, accompanied by structural variations at the IR boundaries, specifically partial duplication of *ycf1* and truncation of *rps19* [38]. These findings document the structural differentiation of the chloroplast genome in *D. densiflorum*, consistent with patterns observed in other high-altitude lineages. While we discuss potential correlations with environmental pressures, causal relationships between IR expansion and high-altitude adaptation remain hypothetical and require further functional investigation.

Sequence variation analysis reveals a pattern of “conserved photosynthetic core genes (e.g., *psbA*, *rbcL*, with $P_i \leq 0.01$) and divergent auxiliary genes (e.g., *ycf1*, *psaC*, with $P_i \geq 0.05$)”, while the strong preference for A/U-ending codons (87.50% of high-frequency codons end with A/U) further illustrates the balanced strategy of *D. densiflorum* in maintaining basic life activities and adapting to the low-temperature high-altitude environment [23,44].

Phylogenetic analysis based on maternally inherited chloroplast CDSs provisionally clarifies the evolutionary position of *D. densiflorum* within the context of plastid evolution. This species clusters with high-altitude species such as *D. caeruleum* and *D. denudatum* into a clade with 100% bootstrap support. 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. Future studies incorporating ITSs and low-copy nuclear genes are essential to distinguish between true phylogenetic divergence and maternal lineage sorting or cytoplasmic capture events [8,12]. Consistent with previous molecular systematic studies, this result reveals potential misjudgments in traditional morphological classification due to over-reliance on convergent floral traits and confirms the driving role of the Qinghai-Tibet Plateau uplift and climate fluctuations in the narrow endemic specialization and rapid differentiation of species. Additionally, highly

variable genes such as *ycf1* and *rps11*, as well as variable hotspots including *trnK-rbcL* and *rpl32-ycf1*, can be developed into efficient DNA barcodes, providing practical technical support for the accurate identification of *Delphinium* species and the protection and rational utilization of medicinal resources.

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References

1. Sun, J.-Y.; Li, T.-C. A New Norditerpenoid Alkaloid from *Delphinium densiflorum*. *J. Chem. Res.* **2009**, *2009*, 306–307. [CrossRef]
2. Delpeuch, P.; Jabbour, F.; Damerval, C.; Schönenberger, J.; Pamperl, S.; Rome, M.; Nadot, S. A Flat Petal as Ancestral State for Ranunculaceae. *Front. Plant Sci.* **2022**, *13*, 961906. [CrossRef]
3. Yin, T.; Cai, L.; Ding, Z. An Overview of the Chemical Constituents from the Genus *Delphinium* Reported in the Last Four Decades. *RSC Adv.* **2020**, *10*, 13669–13686. [CrossRef] [PubMed]
4. Huang, S.; Wang, J.-Z.; Guo, Q.-J.; Xiao, Y.; Feng, Y.-M.; Chen, L.; Xie, J.; Zhou, X.-L. Diterpenoid Alkaloids from Two Species of *Delphinium*. *J. Asian Nat. Prod. Res.* **2023**, *25*, 718–730. [CrossRef] [PubMed]
5. Raza, M.; Shaheen, F.; Choudhary, M.I.; Sombati, S.; Rafiq, A.; Suria, A.; Rahman, A.-; DeLorenzo, R.J. Anticonvulsant Activities of Ethanolic Extract and Aqueous Fraction Isolated from *Delphinium Denudatum*. *J. Ethnopharmacol.* **2001**, *78*, 73–78. [CrossRef] [PubMed]
6. Kiumarsi, A.; Parvinzadeh Gashti, M.; Salehi, P.; Dayeni, M. Extraction of Dyes from *Delphinium zalil* Flowers and Dyeing Silk Yarns. *J. Text. Inst.* **2017**, *108*, 66–70. [CrossRef]
7. Song, C.; Zhu, J.; Li, H. Complete Chloroplast Genomes of Eight *Delphinium* Taxa (Ranunculaceae) Endemic to Xinjiang, China: Insights into Genome Structure, Comparative Analysis, and Phylogenetic Relationships. *BMC Plant Biol.* **2024**, *24*, 600. [CrossRef]
8. Jabbour, F.; Renner, S.S. A Phylogeny of Delphinieae (Ranunculaceae) Shows That Aconitum Is Nested within Delphinium and That Late Miocene Transitions to Long Life Cycles in the Himalayas and Southwest China Coincide with Bursts in Diversification. *Mol. Phylogenet. Evol.* **2012**, *62*, 928–942. [CrossRef]
9. Wang, W.; Liu, Y.; Yu, S.-X.; Gao, T.-G.; Chen, Z.-D. *Gymnaconitum*, a New Genus of Ranunculaceae Endemic to the Qinghai-Tibetan Plateau. *TAXON* **2013**, *62*, 713–722. [CrossRef]
10. Chen, Z.; Yu, X.; Yang, Y.; Wei, P.; Zhang, W.; Li, X.; Liu, C.; Zhao, S.; Li, X.; Liu, X. Comparative Analysis of Chloroplast Genomes within *Saxifraga* (Saxifragaceae) Takes Insights into Their Genomic Evolution and Adaption to the High-Elevation Environment. *Genes* **2022**, *13*, 1673. [CrossRef]
11. Scobeyeva, V.A.; Artyushin, I.V.; Krinitsina, A.A.; Nikitin, P.A.; Antipin, M.I.; Kuptsov, S.V.; Belenikin, M.S.; Omelchenko, D.O.; Logacheva, M.D.; Konorov, E.A.; et al. Gene Loss, Pseudogenization in Plastomes of Genus *Allium* (Amaryllidaceae), and Putative Selection for Adaptation to Environmental Conditions. *Front. Genet.* **2021**, *12*, 674783. [CrossRef]
12. Jabbour, F.; Renner, S.S. *Consolida* and *Aconitella* Are an Annual Clade of *Delphinium* (Ranunculaceae) That Diversified in the Mediterranean Basin and the Irano-Turanian Region. *TAXON* **2011**, *60*, 1029–1040. [CrossRef]
13. Zhang, Y.; Zhang, A.; Li, X.; Lu, C. The Role of Chloroplast Gene Expression in Plant Responses to Environmental Stress. *Int. J. Mol. Sci.* **2020**, *21*, 6082. [CrossRef] [PubMed]
14. Lv, R.; Li, Z.; Li, M.; Dogra, V.; Lv, S.; Liu, R.; Lee, K.P.; Kim, C. Uncoupled Expression of Nuclear and Plastid Photosynthesis-Associated Genes Contributes to Cell Death in a Lesion Mimic Mutant. *Plant Cell* **2019**, *31*, 210–230. [CrossRef] [PubMed]

15. Mehmetoğlu, E.; Kaymaz, Y.; Ateş, D.; Kahraman, A.; Tanyolaç, M.B. The Complete Chloroplast Genome of *Cicer Reticulatum* and Comparative Analysis against Relative *Cicer* Species. *Sci. Rep.* **2023**, *13*, 17871. [\[CrossRef\]](#)
16. Bolger, A.M.; Lohse, M.; Usadel, B. Trimmomatic: A Flexible Trimmer for Illumina Sequence Data. *Bioinformatics* **2014**, *30*, 2114–2120. [\[CrossRef\]](#)
17. Chen, S.; Zhou, Y.; Chen, Y.; Gu, J. Fastp: An Ultra-Fast All-in-One FASTQ Preprocessor. *Bioinformatics* **2018**, *34*, i884–i890. [\[CrossRef\]](#)
18. Bankevich, A.; Nurk, S.; Antipov, D.; Gurevich, A.A.; Dvorkin, M.; Kulikov, A.S.; Lesin, V.M.; Nikolenko, S.I.; Pham, S.; Pribelski, A.D.; et al. SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing. *J. Comput. Biol.* **2012**, *19*, 455–477. [\[CrossRef\]](#)
19. Tillich, M.; Lehwar, P.; Pellizzer, T.; Ulbricht-Jones, E.S.; Fischer, A.; Bock, R.; Greiner, S. GeSeq—Versatile and Accurate Annotation of Organelle Genomes. *Nucleic Acids Res.* **2017**, *45*, W6–W11. [\[CrossRef\]](#)
20. Li, H.; Durbin, R. Fast and Accurate Short Read Alignment with Burrows-Wheeler Transform. *Bioinformatics* **2009**, *25*, 1754–1760. [\[CrossRef\]](#)
21. Li, H.; Handsaker, B.; Wysoker, A.; Fennell, T.; Ruan, J.; Homer, N.; Marth, G.; Abecasis, G.; Durbin, R. 1000 Genome Project Data Processing Subgroup The Sequence Alignment/Map Format and SAMtools. *Bioinformatics* **2009**, *25*, 2078–2079. [\[CrossRef\]](#) [\[PubMed\]](#)
22. Li, Q.; Guo, X.; Yuan, F.; Nima, C.; Dongzhi, D.; Duoje Li, X. Characterization of the Complete Chloroplast Genome of the Musk Larkspur *Delphinium brunonianum* (Ranunculales: Ranunculaceae). *Mitochondrial DNA Part B* **2020**, *5*, 2394–2396. [\[CrossRef\]](#) [\[PubMed\]](#)
23. Li, H.; Jia, J.; Abdullah Sammad, A.; Shah, S.A.; Huang, Y.; Cui, Y.; Heidari, P.; Tian, X. Chloroplast Genomes Characterization of *Aconitum violaceum*, *Caltha palustris*, and *Delphinium denudatum* (Ranunculaceae). *Ecol. Evol.* **2025**, *15*, e72276. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Duan, H.; Zhang, Q.; Wang, C.; Li, F.; Tian, F.; Lu, Y.; Hu, Y.; Yang, H.; Cui, G. Analysis of Codon Usage Patterns of the Chloroplast Genome in *Delphinium grandiflorum* L. Reveals a Preference for AT-Ending Codons as a Result of Major Selection Constraints. *PeerJ* **2021**, *9*, e10787. [\[CrossRef\]](#)
25. Amiryousefi, A.; Hyvönen, J.; Poczei, P. IRscope: An Online Program to Visualize the Junction Sites of Chloroplast Genomes. *Bioinformatics* **2018**, *34*, 3030–3031. [\[CrossRef\]](#)
26. Li, H.; Guo, Q.; Xu, L.; Gao, H.; Liu, L.; Zhou, X. CPJSDraw: Analysis and Visualization of Junction Sites of Chloroplast Genomes. *PeerJ* **2023**, *11*, e15326. [\[CrossRef\]](#)
27. Frazer, K.A.; Pachter, L.; Poliakov, A.; Rubin, E.M.; Dubchak, I. VISTA: Computational Tools for Comparative Genomics. *Nucleic Acids Res.* **2004**, *32*, W273–W279. [\[CrossRef\]](#)
28. Rozas, J.; Ferrer-Mata, A.; Sánchez-DelBarrio, J.C.; Guirao-Rico, S.; Librado, P.; Ramos-Onsins, S.E.; Sánchez-Gracia, A. DnaSP 6: DNA Sequence Polymorphism Analysis of Large Data Sets. *Mol. Biol. Evol.* **2017**, *34*, 3299–3302. [\[CrossRef\]](#)
29. Katoh, K.; Standley, D.M. MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability. *Mol. Biol. Evol.* **2013**, *30*, 772–780. [\[CrossRef\]](#)
30. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549. [\[CrossRef\]](#)
31. Luo, D.; Yue, J.; Sun, W.; Xu, B.; Li, Z.; Comes, H.P.; Sun, H. Evolutionary History of the Subnival Flora of the Himalaya-Hengduan Mountains: First Insights from Comparative Phylogeography of Four Perennial Herbs. *J. Biogeogr.* **2016**, *43*, 31–43. [\[CrossRef\]](#)
32. Xiang, K.-L.; Aytaç, Z.; Liu, Y.; Espinosa, F.; Jabbour, F.; Byng, J.W.; Zhang, C.-F.; Erst, A.S.; Wang, W. Recircumscription of *Delphinium* Subg. *Delphinium* (Ranunculaceae) and Implications for Its Biogeography. *TAXON* **2017**, *66*, 554–566. [\[CrossRef\]](#)
33. Wicke, S.; Schneeweiss, G.M.; dePamphilis, C.W.; Müller, K.F.; Quandt, D. The Evolution of the Plastid Chromosome in Land Plants: Gene Content, Gene Order, Gene Function. *Plant Mol. Biol.* **2011**, *76*, 273–297. [\[CrossRef\]](#) [\[PubMed\]](#)
34. Leng, Z.; Pang, Z.; He, Z.; Gao, Q. Comparative Analysis of Chloroplast Genomes of 19 *Saxifraga* Species, Mostly from the European Alps. *Int. J. Mol. Sci.* **2025**, *26*, 6015. [\[CrossRef\]](#)
35. Zhao, S.; Gao, X.; Yu, X.; Yuan, T.; Zhang, G.; Liu, C.; Li, X.; Wei, P.; Li, X.; Liu, X. Comparative Analysis of Chloroplast Genome of *Meconopsis* (Papaveraceae) Provides Insights into Their Genomic Evolution and Adaptation to High Elevation. *Int. J. Mol. Sci.* **2024**, *25*, 2193. [\[CrossRef\]](#)
36. Tian, Z.; Wang, J.; Zeng, Z.; Chen, J.; Zhang, T.; La, Q. Chromosome-Level Genome Assembly of the Tibetan Medicinal Plant *Duyiwei* (*Phlomis rotata*). *Sci. Data* **2025**, *12*, 1576. [\[CrossRef\]](#)
37. Xin, Y.; Yu, W.-B.; Eiadthong, W.; Cao, Z.; Li, Q.; Yang, Z.; Zhao, W.; Xin, P. Comparative Analyses of 18 Complete Chloroplast Genomes from Eleven *Mangifera* Species (Anacardiaceae): Sequence Characteristics and Phylogenomics. *Horticulturae* **2023**, *9*, 86. [\[CrossRef\]](#)
38. Daniell, H.; Lin, C.-S.; Yu, M.; Chang, W.-J. Chloroplast Genomes: Diversity, Evolution, and Applications in Genetic Engineering. *Genome Biol.* **2016**, *17*, 134. [\[CrossRef\]](#)

39. Dong, W.; Xu, C.; Li, C.; Sun, J.; Zuo, Y.; Shi, S.; Cheng, T.; Guo, J.; Zhou, S. Ycf1, the Most Promising Plastid DNA Barcode of Land Plants. *Sci. Rep.* **2015**, *5*, 8348. [[CrossRef](#)]
40. Li, H.; Xiao, W.; Tong, T.; Li, Y.; Zhang, M.; Lin, X.; Zou, X.; Wu, Q.; Guo, X. The Specific DNA Barcodes Based on Chloroplast Genes for Species Identification of Orchidaceae Plants. *Sci. Rep.* **2021**, *11*, 1424. [[CrossRef](#)]
41. Parvathy, S.T.; Udayasuriyan, V.; Bhadana, V. Codon Usage Bias. *Mol. Biol. Rep.* **2022**, *49*, 539–565. [[CrossRef](#)]
42. Hershberg, R.; Petrov, D.A. Evidence That Mutation Is Universally Biased towards AT in Bacteria. *PLoS Genet.* **2010**, *6*, e1001115. [[CrossRef](#)]
43. Yang, C.; Wang, B.; Chen, J.; Sun, Q. Codon Usage Bias Analysis of the Chloroplast Genomes in Medicinal Plants of Ampelopsis Michaux. *S. Afr. J. Bot.* **2025**, *181*, 512–522. [[CrossRef](#)]
44. Anandan, J.; Shanmugam, R.; Alharbi, N.S.; Thiruvengadam, M. Green-Synthesized Silver Nanoparticles from Centella Asiatica and Ayapana Triplinervis: A Novel Approach to Treating Wound Infections and Reducing Antimicrobial Resistance. *S. Afr. J. Bot.* **2025**, *177*, 617–629. [[CrossRef](#)]
45. Plotkin, J.B.; Kudla, G. Synonymous but Not the Same: The Causes and Consequences of Codon Bias. *Nat. Rev. Genet.* **2011**, *12*, 32–42. [[CrossRef](#)]

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