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Assembly and Comparative Analyses of the Chloroplast Genomes of the Threatened Plant *Rosa anemoniflora*

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Abstract: Due to insufficient molecular biology and genetic research on *Rosa anemoniflora*, this endangered plant has not yet received effective protection. Therefore, the complete chloroplast genome sequence of *R. anemoniflora*, along with comparative analysis of the chloroplast genomes of related species, is necessary and crucial for reconstructing phylogenetic relationships and developing genetic markers to conserve these species. A series of analyses, including genome structure, GC content, gene number, selection pressure, and nucleotide diversity, were performed by comparing the chloroplast genomes of *R. anemoniflora* and its relatives. The results indicate that the chloroplast genomes of *R. anemoniflora* and its close relatives are highly conserved in all genome characteristics, and all protein-coding genes in *R. anemoniflora* have not experienced significant positive or negative selection pressures. Comparative analysis revealed several variation hotspots, such as the *atpH-atpI* region, which can serve as a DNA barcode for distinguishing *R. anemoniflora* from its close relatives. Finally, the results confirmed that *R. anemoniflora* belongs to *Rosa* section *Synstylae* and that *R. anemoniflora* and its close relatives likely originated from the Fujian and Taiwan regions of China and diverged approximately 3.24 million years ago. This study provides crucial information for future biodiversity conservation and genetic resource management.

Keywords: *Rosa anemoniflora*; genetic research; chloroplast genome; phylogenetic relationships; genetic markers; biodiversity conservation

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1. Introduction

Rosa anemoniflora Fortune ex Lindl. belongs to the genus *Rosa*, which is important within the Rosaceae family. This genus includes numerous species and serves as a source of many horticultural and medicinal plants. *R. anemoniflora* is a climbing shrub with a small stature. Its young branches are purple-brown and cylindrical. The plant has three small leaflets and light pink flowers, making it highly ornamental and an important plant germplasm resource. Currently, *R. anemoniflora* is found only in Nanping city, Fujian Province, China, and is primarily distributed on slopes, wastelands, roadsides, and riverbanks at altitudes ranging from 400 to 1000 m [1]. Its population is currently distributed in a patchy manner, with some small populations in a state of severe isolation. Field surveys have indicated a substantially insufficient population size, classifying it as a typical extremely small population, well below the globally recognized minimum viable population standard (approximately 10,000 individuals) for shrubby plants. Consequently, in 2020, *R. anemoniflora* was listed on the China Biodiversity Red List (https://www.mee.gov.cn/xxgk2018/xxgk/xxgk01/202305/W020230522536560832337.pdf, accessed on 16 May 2024) and designated as a second-level protected plant on the Chinese National Key Protected Wild Plants List.

R. anemoniflora's exserted and columnated styles provide strong evidence for its inclusion in the *Synstylae* section [2]. However, morphological characteristics have a limited ability to establish species boundaries due to small variations induced by environmental or genetic factors. Consequently, distinguishing *R. anemoniflora* from its close relatives based on external features is challenging, complicating the accurate identification of conservation targets. Genetic characterization studies on *R. anemoniflora* can clarify its phylogenetic position and evolutionary history and aid in the development of high-resolution molecular markers. To our knowledge, such studies have not been conducted previously, and filling this knowledge gap was the primary motivation for this study.

Studying the chloroplast genomes of this highly endangered species provides a solid foundation for its genetic characterization. Chloroplast genome sequences are widely employed in plant molecular phylogenetics, population genetics, and conservation genetics due to their slower evolution rate than nuclear genomes, maternal inheritance, and lower recombination rate [3,4]. In addition, chloroplast genomes are important for determining plant stress responses [5]. For instance, the upregulation of *psbA* significantly enhances the repair of photosystem II under strong light stress [6], *petL* is markedly upregulated during cold acclimation, potentially playing a role in a plant's response to low-temperature stress [7], and the expression of the chloroplast ribosomal protein-encoding gene *RPS1* is crucial for the response to heat stress [8]. Therefore, whole chloroplast genome sequences can provide a wealth of genetic information and are useful molecular markers for efficient conservation and management strategies [9–11].

In this study, the first complete chloroplast genome of the endangered species *R. anemoniflora* was assembled and compared with that of its close relatives. Our main goals were (1) to characterize and compare the chloroplast genomes of *Rosa* sect. *Synstylae* plants; (2) to identify hotspots of variation in the chloroplast genomes of *Rosa* sect. *Synstylae* plants and develop high-resolution molecular markers based on these hotspots; and (3) to clarify the taxonomic status, evolutionary history, and ancestral distribution of *R. anemoniflora*. Our study results will provide important scientific insights for the comprehensive conservation of *R. anemoniflora*.

2. Materials and Methods

2.1. Collection and Preservation of Experimental Material

The *R. anemoniflora* research material was collected from the Mangdangshan National Nature Reserve in Fujian (34°10′12″ N, 116°30′41″ E). The collection and experiments of plant materials complied with the relevant guidelines and regulations of the Mangdangshan National Nature Reserve. Species identification was performed by Guosheng He, a professor at Fujian Forestry Vocational and Technical College, and specimens were deposited in the Herbarium, Institute of Botany, Chinese Academy of Sciences (PE 01651707). Leaf samples were dried with silica gel and stored in the laboratory. The silica-dried leaf samples of *R. anemoniflora* were subsequently sent to Genepioneer Biotechnologies (Nanjing, China) for sequencing. Total genomic DNA was extracted using the Plant Genomic DNA Kit (TIANGEN, Beijing, China). DNA purity was assessed using a 1.0% agarose gel, and qualified DNA samples were used to construct libraries and perform paired-end sequencing (PE) with a read length of 150 bp on the Illumina NovaSeq platform. Sequencing was conducted by Nanjing Jessyn Bio Science & Technology Co., Ltd. (Nanjing, China).

2.2. Chloroplast Genome Assembly and Annotation

Fastp v0.20.0 [12] (https://github.com/OpenGene/fastp, accessed on 16 May 2024) software was used for initial data filtering. Subsequently, Bowtie2 v2.2.4 [13] (http://bowtie-bio.sourceforge.net/Bowtie2/index.shtml, accessed on 16 May 2024) was used to align the sequences to the chloroplast genome database. The resulting sequences were considered the study chloroplast genome sequences. SPAdes [14] generated the seed sequence, subjected to Kmer iterative extension and classified as a contig if an overlap group was formed. This sequence was designated a pseudogenomic sequence. Bowtie2 was used

to align the sequenced sequence to the pseudogenomic sequence, ensuring that the reads coverage of the assembly results was normal. SSPACE v2.0 (https://www.baseclear.com/, accessed on 16 May 2024) was used to connect contigs into scaffolds, and Gapfiller v2.1.1 (https://sourceforge.net/projects/gapfiller/, accessed on 16 May 2024) was used to fill in the scaffold sequences. In the case of gaps, primers were designed for PCR sequencing until a complete pseudogenomic sequence was obtained. Alignment to the pseudogenome verified the final assembly accuracy. Finally, using the large single-copy region as the starting point and referring to the orientation of the small single-copy region in *Rosa lucidissima* H. Lév. (MK782979.1.gbk), the pseudogenome was rearranged to construct a circular chloroplast genome sequence. For annotation, the CDSs were compared with those of the closely related species *R. lucidissima* on NCBI using BLAST [15]. The rRNA sequences of the *R. lucidissima* (MK782979) were extracted and aligned using MAFFT [16] (v, --auto), and then the hidden Markov model was constructed using the hmmbuild tool from the HMMER [17] package. Finally, nhmmer was used to search. Aragorn [18] was used to predict tRNAs, and OGDRAW (https://chlorobox.mpimp-golm.mpg.de/OGDraw.html, accessed on 16 May 2024) was used to generate circular plastome maps.

2.3. Chloroplast Genome Comparative Analysis

Seven species belonging to *Rosa* sect. *Synstylae* and sect. *Chinenses* were selected for analysis. *R. anemoniflora* and *R. lucidissima* are endangered plants, while *Rosa brunonii* Lindl., *Rosa maximowicziana* Regel, *Rosa multiflora* Thunb., *Rosa chinensis* Jacq. and *Rosa odorata* (Andr.) Sweet are non-endangered plants that served as controls (Table S1). The Geneious Prime 2024.0 [19] software was used to calculate the lengths of the different boundaries (SSC, LSC, and IR regions), the number and types of genes, GC content, and other information of the chloroplast genomes of seven species based on their GenBank annotation files. Mauve 2.4.1 [20] (https://darlinglab.org/, accessed on 16 May 2024) was used for homologous and collinear comparative analysis of the entire chloroplast genome sequences of other *Rosa* species (default parameters). IRscope was used to show the IR/SC boundaries of the tested chloroplasts. PAML 4.9j [21] was used for selection pressure analysis, with *R. anemoniflora* and *R. lucidissima* set as foreground branches. Under the branch model, if the dn/ds value is greater than 1 and the M0 model can be rejected, this indicates significant positive selection pressure on the foreground branch. If the dn/ds value of the foreground branch is less than the background value and the M0 model can be rejected, this indicates significant relaxed selection pressure on the foreground branch.

2.4. Molecular Marker Discovery

The chloroplast sequences of *R. anemoniflora* and 10 closely related species belonging to *Rosa* sect. *Synstylae* were selected for analysis (Table S1). MAFFT [16] was used for the alignment of the 11 chloroplast genome sequences, and DnaSP 6 [22] was used for sliding window analysis to calculate nucleotide diversity (Pi) values of the chloroplast genome sequences. The step size was set at 200 bp, and the window length was set at 600 bp. Regions with Pi values greater than 0.005 were designated single nucleotide polymorphism (SNP) hotspots. Gene typing analyses were used to determine whether SNPs in the hotspot regions could effectively differentiate the 11 tested *Rosa* species. MISA [23] (parameters: 1-8, 2-5, 3-3, 4-3, 5-3, and 6-3) was used to detect simple sequence repeats (SSRs) in the chloroplast sequence of *R. anemoniflora*. The chloroplast sequences of *R. anemoniflora* were aligned with those of 11 closely related species, and potential polymorphic sites were identified by comparing SSR sites with insertion-deletion sites.

2.5. Phylogenetic Analysis

Seventeen *Rosa* species were selected, representing *Synstylae*, *Chinenses*, *Caninae*, *Microphyllae*, and *Bracteatae*. *Rubus pedunculosus* and *Dasiphora glabra* were chosen as outgroups. The chloroplast genome data of the 19 plants were obtained from the GenBank database (Table S1). MAFFT [21] was used to align the complete chloroplast genome sequences.

Determined K3Pu+F+I as the best-fit model using ModelFinder [24]. RAxML-NG [25] was used to construct a phylogenetic tree via the maximum likelihood (ML) method by comparing the chloroplast genome sequences of different species with 1000 bootstrap replicates.

2.6. Molecular Dating Analysis

The alignment files of the chloroplast sequences were converted into nexus format using PhyloSuite v1.2.1 [26]. The optimal GTR+I+R model was obtained with the Model Finder plugin of PhyloSuite v1.2.3 software. Site model parameters were set with BEAUti in BEAST v1.10.4 [27] software. The molecular clock model was set by selecting the relaxed molecular clock model Relaxed clock log Normal with default parameters. The Yule tree prior was selected as the tree prior model. The separation of the genus *Rosa* and the outgroup at 50.0 Ma (with a 97.5% confidence interval between 51.0 and 49.0 Ma) and the crown age of Core *Synstylae* estimated to be 24.2 Ma (95% HPD: 15.4–33.7 Ma) were used as the calibration points [28]. The MCMC chain length set to 25,000,000 generations, with a sampling frequency of 1000 Tracer v1.7 [29], was used to construct the tracer distribution plot and determine the effective sample size (ESS). If the ESS value was >200, it was determined that the running parameters had converged. TreeAnnotator was used to discard 10% of the trees as burn-in and retain median height values. After the run, trees with time nodes were viewed in FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/, accessed on 16 May 2024).

2.7. Biogeographical Analysis

Those widely distributed *Rosa* species (*Rosa multiflora* and *Rosa chinensis*) whose central distribution could not be identified were not included in the analysis. The tested *Rosa* plants were divided into seven biogeographical regions according to the ancestral area reconstruction (AAR): (A) Northeast China, (B) Northwest China, (C) Southwest China, (D) Central China, (E) Taiwan Province and Fujian Province, (F) South China, and (G) outside China. RASP v4.2 [30] was used to analyze six models of ancestral geographical range estimation: dispersal–extinction cladogenesis (DEC), dispersal–extinction cladogenesis with jump (DEC+J), dispersal–vicariance analysis (DIVALIKE), dispersal–vicariance analysis with jump (DIVALIKE+J), BI for discrete areas (BAYAREALIKE), and BI for discrete areas with jump (BAYAPEALIKE+J). All parameters were set to default values.

3. Results

3.1. General Features and Comparative Analyses of Rosa anemoniflora Chloroplast Genomes

The chloroplast genome of *R. anemoniflora* exhibits the typical quadripartite structure found in typical land plant chloroplasts, with a total length of 156,578 bp. These included inverted repeat regions (IRA and IRB, 26,058 bp), a large single-copy region (LSC, 85,722 bp), and a small single-copy region (SSC, 18,740 bp) (Figure 1). The GC content of the chloroplast genome of *R. anemoniflora* was 37.23%. However, the GC content varied significantly among the three regions (LSC, SSC, and IR), with the highest GC content occurring in the IR region (42.73%), followed by that occurring in the LSC region (35.18%), and the lowest occurring in the SSC region (31.34%). The chloroplast genome of *R. anemoniflora* contained a total of 130 genes, including 8 rRNA genes, 37 tRNA genes, and 85 protein-coding genes, with the ycf1 gene identified as a pseudogene. The length of the chloroplast genomes among the seven species was approximately 156,543.571 bp $\pm$ 66.51 bp, the gene count was 131 $\pm$ 2.082, and the GC content was approximately 37.243% $\pm$ 0.008%. Overall, the chloroplast genome structure, total GC content, GC content in different regions, and gene number of *R. anemoniflora* were highly similar to those of the other tested species (Table 1).

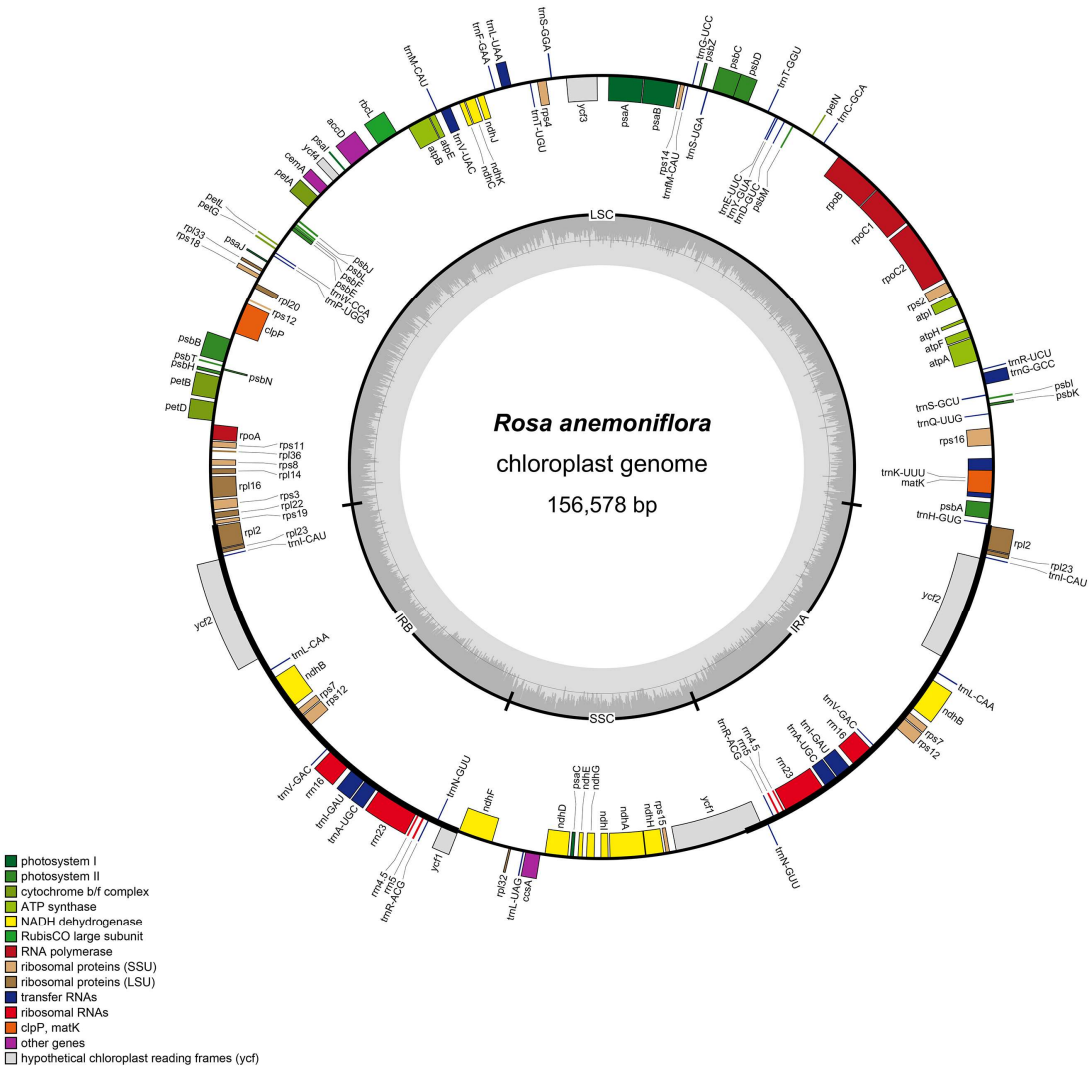


Figure 1. Gene map of the chloroplast genome of *Rosa anemoniflora*.

Table 1. Characteristics of selected chloroplast genomes.

Genome Features	*Rosa anemoniflora*	*Rosa brunonii*	*Rosa maximowicziana*	*Rosa multiflora*	*Rosa lucidissima*	*Rosa chinensis*	*Rosa odorata*
Genome size (bp)	156,578	156,544	156,405	156,519	156,588	156,591	156,580
LSC size (bp)	85,722	85,705	85,529	85,643	85,713	85,737	85,704
SSC size (bp)	18,740	18,743	18,760	18,760	18,779	18,766	18,780
IR size (bp)	26,058	26,048	26,058	26,058	26,048	26,044	26,048
Total GC content	37.23	37.24	37.25	37.24	37.25	37.24	37.25
GC content in LSC	35.18	35.2	31.32	35.2	35.2	35.2	35.21
GC content in SSC	31.34	31.3	35.21	31.32	31.35	31.32	31.35
GC content in IR	42.73	42.73	42.73	42.73	42.73	42.71	42.73
Number of genes	130	130	134	134	129	130	130
Protein-coding genes	84	85	88	88	83	85	85
tRNA genes	8	8	8	8	8	8	8
rRNA genes	37	37	37	37	37	37	37
Pseudo genes	1	0	1	1	1	0	0

3.2. Comparative Analysis of Gene Number and Selection Pressure

Compared with *R. brunonii*, *R. chinensis*, and *R. odorata*, *R. anemoniflora* lost *infA*, and *R. lucidissima* lost both *infA* and *rps19*. In contrast, *R. maximowicziana* and *R. multiflora* had more genes than *R. brunonii*, *R. chinensis*, and *R. odorata*, mainly due to the presence of two *orf42s* and one *orf18*. Interestingly, *ycf1* was pseudogenized in both *R. anemoniflora* and *R. lucidissima*, but their chloroplast genomes contained another normal copy of the *ycf1* gene. In *R. maximowicziana* and *R. multiflora*, *infA* was found to be pseudogenized. Among the seven species tested, a total of 82 core genes were found. The branch model test showed that none of these genes rejected the M0 model. This indicates that the selection pressure on all genes in *R. anemoniflora* and *R. lucidissima* is consistent with the other five non-endangered species (Table S2).

3.3. Comparative Analysis of Chloroplast Genome Structure

To assess structural differences in the genome sequences, Mauve 2.4.1 software was used to analyze the collinearity of the chloroplast genomes of *R. anemoniflora* and its close relatives (Figure 2). The chloroplast genome of *R. anemoniflora* showed complete collinearity with those of the other six *Rosa* species, with no large indels, inversions, or rearrangements. A comparative analysis of the IR-LSC and IR-SSC boundaries of the seven *Rosa* species was also conducted (Figure 3). The results indicated that the coding genes at the LSC/IR boundaries of the chloroplast genomes were highly conserved, with *rps19* and *rpl2* at both LSC/IRB boundaries and *rpl2* and *trnH* at both IRa/LSC boundaries. Based on the distribution of genes and boundary positions, there were no major expansions or contractions in the LSC or IR regions of the chloroplast genomes among these seven species, demonstrating a high degree of conservation.

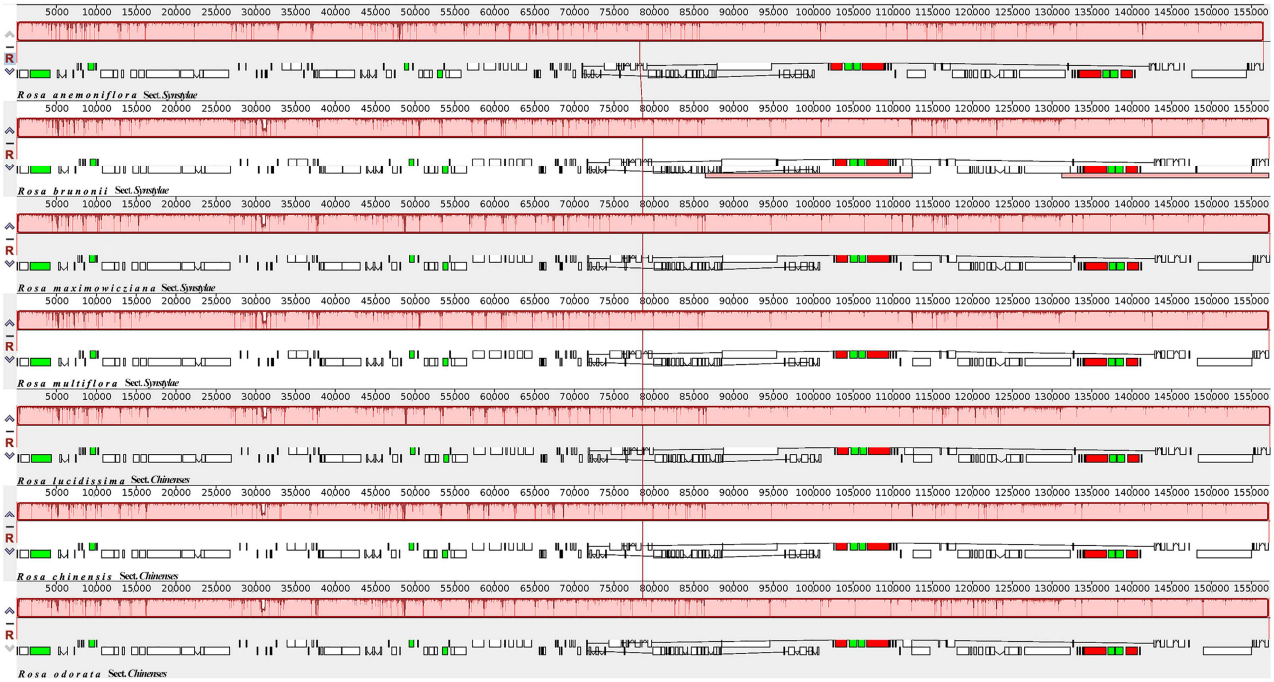


Figure 2. Mauve alignment of the seven *Rosa* chloroplast genomes revealing no rearrangements. Homologous regions between different chloroplast genomes are represented by the same color blocks.

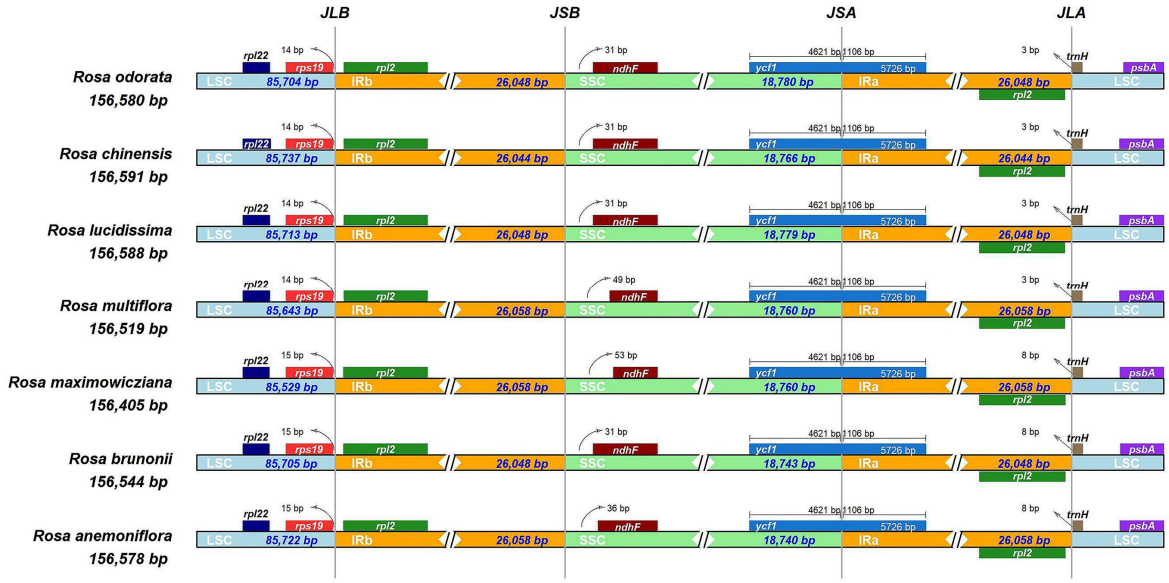


Figure 3. Comparison of genome boundaries in chloroplasts from seven tested *Rosa* species.

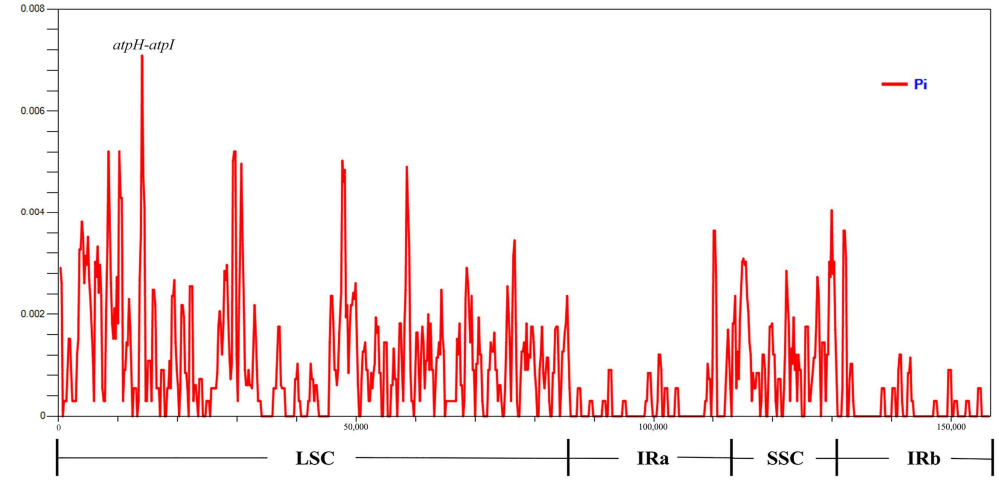
3.4. Highly Divergent Regions and Molecular Marker Development among Rosa Section Synstylae Plants

The results of sliding window analysis using DnaSP6 indicated that all seven mutation hotspots are located in the LSC region, suggesting that nucleotide variations in the chloroplast genome of sect. *Synstylae* of *Rosa* primarily occur in the LSC region (Figure 4A). In descending order of pi values, these seven mutation hotspots were found in five regions (*atpH-atpI*, *trnS_GCU-trnG_GCC*, *trnR_UCU-atpA*, *petN-psbM*, and *trnT_UGU-trnL_UAA*). The extraction of 10 SNP loci in the *atpH-atpI* region for gene typing revealed that these SNP loci could effectively distinguish the 11 tested species in sect. *Synstylae* (Figure 4B). According to the screening criteria, a total of 248 SSR loci were identified in the chloroplast genome of *R. anemoniflora*. Among them, there were 155 mononucleotide repeats, 11 dinucleotide repeats, 68 trinucleotide repeats, 11 tetranucleotide repeats, 1 pentanucleotide repeat, and 2 hexanucleotide repeats (Table S3). Additionally, 18 types of base repeat motifs were identified (Table S3), with A/T repeats being the most abundant (145). Based on sequence alignment, 48 SSR loci exhibiting interspecies polymorphisms were ultimately screened in the chloroplast genome of *R. anemoniflora*.

3.5. Phylogenetic Position, Species Divergence Time, and Ancestral Distribution Reconstruction of Rosa anemoniflora

Using chloroplast genome data from the tested plants, phylogenetic trees were constructed via the ML method. The bootstrap values of each node in the phylogenetic tree were generally high, indicating a reliable topology. In addition to the two outgroups, sect. *Microphyllae* and sect. *Bracteatae* were located at the base of the evolutionary tree. Although *Synstylae* and sect. *Chinensis* did not form separate branches, the sub-branches where *R. anemoniflora* is located were all from sect. *Synstylae*, providing sufficient evidence that *R. anemoniflora* belongs to sect. *Synstylae*. According to the evolutionary tree, *R. anemoniflora* was most closely related to *R. taiwanensis* and *R. pricei* (Figure 5). Subsequently, based on the chloroplast genome sequence dataset, the divergence time of the tested plants was estimated. The results indicated that *R. anemoniflora* and its closest relative, *R. taiwanensis*, diverged approximately 3.24 million years ago (Figure 5). In the reconstruction of ancestral geographical distributions, DEC was identified as the optimal model among the six models used to study the biogeographic evolution of the tested *Rosa* species. The results of biogeographic reconstruction showed that the common ancestors of *R. anemoniflora*, *R. taiwanensis*, and *R. pricei* likely originated from the Fujian and Taiwan regions of China, with a probability of 91.56% (Figure 6).

A



B

	13,706	13,821	13,904	13,905	14,125	14,158	14,159	14,166	14,277	14,441
Rosa anemoniflora	T	G	T	G	A	T	T	T	C	A
Rosa brunonii	T	G	T	G	G	T	T	T	C	A
Rosa helenae	T	A	T	T	G	C	T	T	C	A
Rosa lasiosepala	C	A	T	T	G	C	C	T	C	A
Rosa lichiangensis	T	A	T	T	G	T	T	T	A	A
Rosa longicuspis	T	A	T	T	G	C	C	T	C	A
Rosa maximowicziana	T	G	G	G	A	T	T	T	C	A
Rosa multiflora	T	G	G	G	A	T	T	T	C	A
Rosa pricei	T	G	T	G	A	T	T	T	C	A
Rosa soulieana	T	A	T	T	G	T	T	T	A	A
Rosa taiwanensis	T	G	T	G	A	T	T	T	C	A

Figure 4. Highly divergent regions and their snp loci. (**A**) Sliding window analysis of the entire chloroplast genome of 11 species from *Rosa* section *Synstylae* (window length: 600 bp; step size: 200 bp). *X*-axis: position of the midpoint of a window; *Y*-axis: nucleotide diversity of each window. (**B**) SNP loci of the *atpH-atpI* region based on 11 species from *Rosa* section *Synstylae*. T: Thymine; G: Guanine; A: Adenine; C: Cytosine, with the same bases represented by the same color blocks.

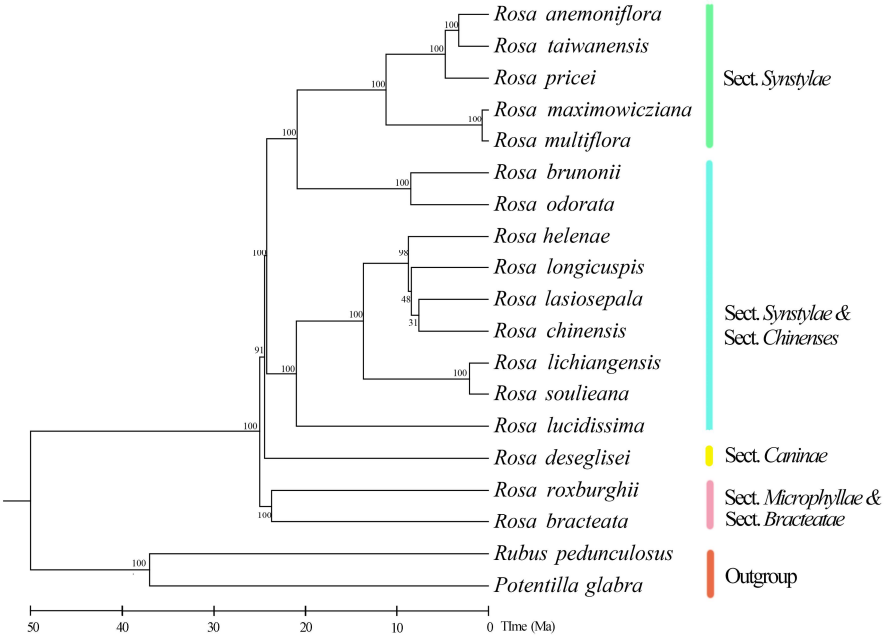


Figure 5. Maximum likelihood (ML) phylogenetic tree based on 19 plant chloroplast genomes.

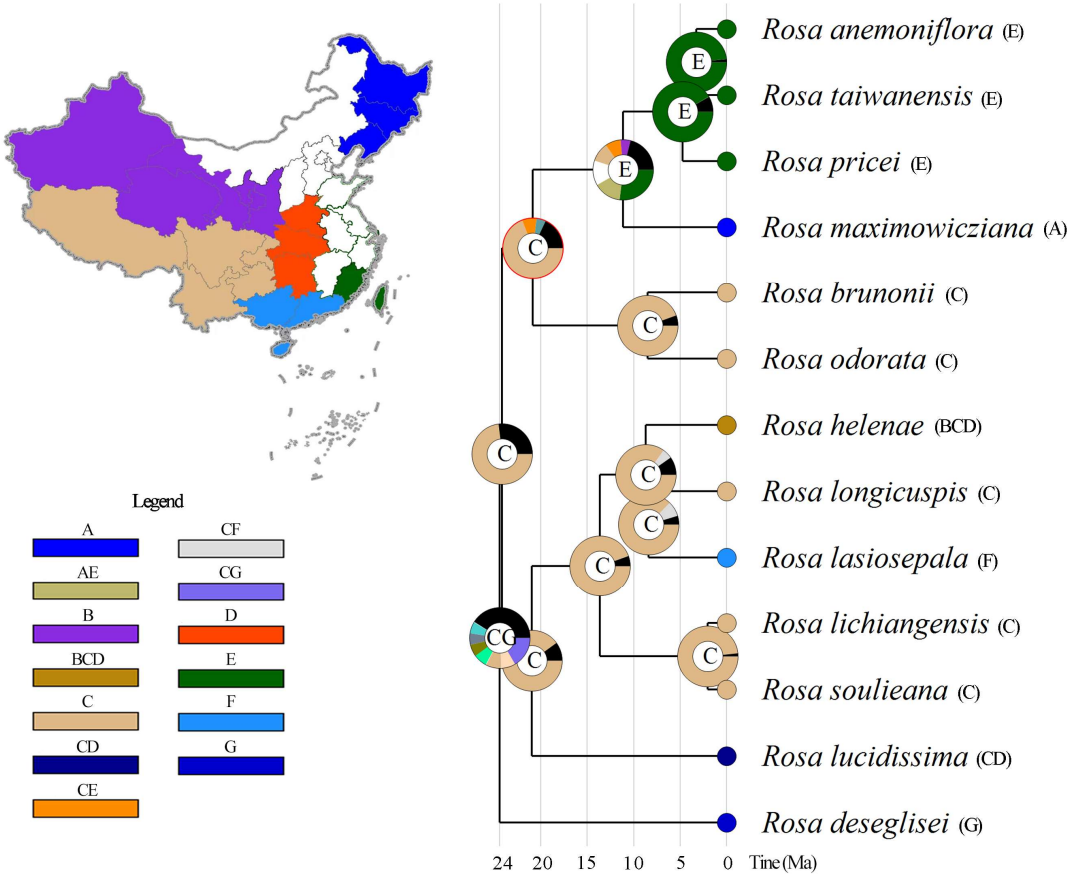


Figure 6. Results of ancestral distribution reconstruction.

4. Discussion

In this study, the chloroplast genome of *R. anemoniflora* was sequenced and assembled, and this information was applied for comparative analysis with that of six other related species in sect. *Synstylae* and sect. *Chinenses*. The size of the genome; length of the IR, LSC, and SSC regions; and GC number and content revealed high similarity among the genomes. The length of the seven chloroplast genomes was 156,544 bp, the number of genes was approximately 130, and the GC content was approximately 37.24%. These data closely resemble reports from other sections of the *Rosa* genus [31–33], suggesting that *Rosa* species share low diversity. The contraction and expansion of IR regions are the main driving forces of chloroplast genome structure variation in some taxa [34]. However, the IR/SC boundaries are conserved among our seven *Rosa* species. The reverse orientation of the SSC region has also been reported in a wide variety of plant species [35]. However, the SSC region was not inverted in the seven tested *Rosa* species. These results strongly suggest that the chloroplasts of *R. anemoniflora* and related species are highly conserved.

Endangered plants often exhibit weaker environmental adaptability, manifested in their excessive dependence on specific environments, while lacking sufficient interspecific competitiveness in other environments and leading to an inability to adapt to alternative conditions, which is commonly referred to as an "evolutionary dead-end" [36]. Many epiphytic orchids restricted to tropical regions serve as typical examples [37]. Positive selection pressure contributes to plants specializing in adapting to specific [38]. Conversely, relaxed selection pressure may lead to functional degradation of genes [39]. Considering the crucial role of chloroplast genes in photosynthesis and in the response to environmental stress, specialization or functional degradation of these genes can impact a plant's interspecific competitiveness. However, in our study, the chloroplast genes of *R. anemoniflora* did not experience positive or relaxed selection pressure, leading to the inference that the endan-

gered status of *R. anemoniflora* might not be attributed to differential selection pressures on chloroplast genes. Nevertheless, the chloroplast genome contains fewer genes, and their functions are relatively singular. Further comprehensive studies of nuclear genome sequences are necessary to fully understand whether *R. anemoniflora* specializes in a specific environment, consequently weakening its adaptability to other environments.

Accurately identifying conservation targets is the primary prerequisite and foundation for biodiversity protection. The diversity of *Rosa* species is vast and remarkable, encompassing approximately 200 species that thrive in various temperate and warm regions of the Northern Hemisphere [40]. This diversity is not only reflected in their wide range of morphological traits, such as flower color, size, and fragrance, but also in their complex genetic makeup resulting from frequent hybridizations and polyploidization events [41]. However, hybridization frequently occurs, leading to morphological similarities among species. This convergence in phenotypic characteristics complicates species identification [42,43]. Under such circumstances, highly variable regions in the chloroplast genome could be potential molecular markers for species identification. In this study, through the analysis of the chloroplast genome sequences of 11 species within *Synstylae*, we successfully identified seven variable hotspot regions. These regions are located in noncoding regions, where noncoding sequences appear to show higher variability than coding regions, providing more critical informative sites [44]. The *ndhF* and *ndhF-rpl32* regions have been widely employed as highly divergent regions within *Rosa* [45,46]. We reported the identification of a rarely reported variable hotspot region, namely, the *atpH-atpI* intergenic region, where SNP sites effectively differentiate the 11 species within sect. *Synstylae*. This region can serve as a barcode in systematic studies of *Rosa* sect. *Synstylae*, providing powerful tools for accurate species identification.

After the implementation of conservation measures, it is crucial to continuously monitor and evaluate the population dynamics of conservation targets by assessing genetic indicators [47] for the targeted adjustment of conservation strategies. Due to their maternal inheritance and high levels of within-population polymorphism, chloroplast-specific SSRs have been widely employed in conservation genetic research [48]. Similar to the chloroplasts reported for *Rosa* species [31,46], A/T-type SSRs had the highest proportion in the chloroplasts of *R. anemoniflora*. This may be attributed to the greater content of AT bases than GC bases in the chloroplast genome of *R. anemoniflora*, as the nucleotide composition of the genome can influence the dominant SSR types. This study identified highly conserved SSR loci at the interspecific level and plans to select appropriate SSR markers from the remaining 48 loci for conservation genetic analysis of the *R. anemoniflora* population.

The frequent hybridization between *Rosa* sect. *Chinensis* and Sect. *Synstylae*, defined by traditional taxonomic methods, makes the taxonomic status of *R. anemoniflora* difficult to ascertain based on morphological characteristics. Therefore, there is an urgent need for phylogenetic analysis. Our results support those of previous research, confirming that sect. *Chinensis* and sect. *Synstylae* are not monophyletic [28]. Based on the topology and nodal support of the phylogenetic tree, we strongly confirmed that *R. anemoniflora* indeed belongs to sect. *Synstylae*. Remarkably, *R. anemoniflora* and its two close relatives exhibit distinct geographical distribution characteristics and are restricted to the Fujian and Taiwan regions of China [49]. Molecular dating analysis revealed that *R. anemoniflora* and *R. taiwanensis* diverged approximately 1.82 million years ago. The Taiwan Strait between Taiwan and the Asian mainland formed approximately 10,000 years ago during the Holocene [50]. Since their divergence time predates the formation of the strait, this finding suggested that the difference between *R. anemoniflora* and *R. taiwanensis* was not solely attributable to geographic isolation caused by the Taiwan Strait. In addition to clarifying taxonomic status and evolutionary history, phylogenetics provides a novel approach for assessing biodiversity, evaluating conservation priorities, and quantifying species evolutionary history [51]. Studies indicate that if a species faces the threat of extinction, its close relatives also have an extinction risk higher than the average level [52]. In this context, rose species exclusively distributed in the Taiwan region, such as *R. pricei*

and *R. taiwanensis*, may also face the risk of endangerment, warranting further in-depth attention and research.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f15060940/s1, Table S1: The species used for this study; Table S2: Branch model test of selective pressures on genes; Table S3: Number of classified repeat types.

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