

Complete chloroplast genome sequence and characteristic analysis of *Paeonia suffruticosa* from the lower reaches of the Yangtze River basin

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Abstract

Paeonia suffruticosa cv. 'Yu Luo Chun' is an important representative of the peony groups from the Jiangnan region of China. However, little is known about the genetic basis of chloroplast genome phylogeny and the adaptive evolution of this group. In this study, high-throughput sequencing technology was used to sequence the complete chloroplast genome of leaves, and sequence assembly, annotation and feature analysis were performed by bioinformatics analysis methods. The results showed that the chloroplast genome of *P. suffruticosa* cv. 'Yu Luo Chun' exhibited a typical tetrad structure with a total length of 152,596 bp, including a large single-copy (LSC) region, a small single-copy (SSC) region, and two inverted repeats (IRs) whose lengths were 84,272 bp, 17,044 bp and 25,640 bp, respectively. A total of 126 genes were annotated, including 85 protein-coding genes, 37 tRNA genes and 4 rRNA genes. Bioinformatics analysis showed that a total of 71 simple sequence repeats (SSRs) were searched in the chloroplast genome, of which the numbers of mononucleotide, dinucleotide, trinucleotide and tetranucleotide repeat motifs were 47, 12, 7 and 5, respectively, and no pentanucleotide or hexanucleotide repeat motifs were found. The chloroplast genome prefers to end in A/T, among which leucine (Leu) is the most frequently used, while cysteine (Cys) is the least frequently used. Highly differentiated regions, such as *rpoC1*, *petB*, *rsp16*, *rps19*, *clpP*, *ccsA*, *ycf1*, *ndhF-trnL*, *ndhD-psaC* and *trnV-rps12*, were identified as DNA barcodes and potential genetic markers for interspecies relationships. We compared the gene selection pressure and identified 5 genes, *ndhF*, *petB*, *petD*, *rpoA* and *ycf2* that were positively selected, and these genes have important contributions to the adaptive evolution. The chloroplast genome sequences of 18 species, including *P. suffruticosa* cv. 'Yu Luo Chun', were clustered by the nearest neighbour combination method and the maximum likelihood method. Both methods gave the same result and indicated that this cultivar has a relatively close kinship with *P. qiui* and *P. rockii*. The results provide important references for species identification, genetic diversity analysis and systematic taxonomic research between *P. suffruticosa* cv. 'Yu Luo Chun' and other *Paeonia* species.

Keywords: chloroplast genome; comparative genomics; *P. suffruticosa*; phylogenetic analysis

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Introduction

Chloroplasts are essential organelles for photosynthesis in green plants and algae. Compared with the nuclear genome, the chloroplast genome is smaller and has uniparental inheritance. Its gene contents, gene sequence and base composition are highly conserved, and the frequency of nucleotide substitution is low. Therefore, the chloroplast genome has become an important marker for the study of plant phylogenetic evolution (Daniell *et al.*, 2016; Dobrogojski *et al.*, 2020). With the rapid development of large-scale sequencing technology in recent years, high-throughput next-generation sequencing technology using the Illumina platform has come to provide a high-throughput, low-cost, and efficient method for chloroplast and mitochondrial genome assembly. In recent years, an increasing number of plant chloroplast genomes have been assembled, and a large number of chloroplast genome sequences have been widely used in phylogenetic research (Sun *et al.*, 2018; Xiong *et al.*, 2020; Luo *et al.*, 2021; Mehmetoglu *et al.*, 2022).

The tree peony (*Paeonia suffruticosa* Andrews), known as the 'king of flowers' in China, is a famous traditional flower with both ornamental and medicinal value that is widely used in the country. In terms of plant taxonomy, it belongs to the genus *Paeonia* of the family Paeoniaceae. There are 34 species of the genus *Paeonia* in the world (Zhao *et al.*, 2016; Hong, 2021; Zhou *et al.*, 2021). The genus *Paeonia* is divided into three groups, namely, sect. *Moutan*, sect. *Paeonia* and sect. *Onaepia* (Stern, 1946, 1973). There are 7 species and 2 subspecies from sect. *Moutan* in China and 9 wild species from sect. *Paeonia* (Hong, 2021).

Although peony has been cultivated for a long time and the number of horticultural varieties is large, research on the classification and origin of peony is relatively incipient (Yang *et al.*, 2020; Zhou *et al.*, 2021). Nine species of the genus *Paeonia* are wild species which have been listed as rare in China (Hong, 2021). Tree peony varieties from China can be divided into 4 cultivated species groups according to the original wild species and cultivation area, namely, the peony groups in the Central region, the peony groups in the Jiangnan region (i.e., Jiangnan peony), the peony groups in the Southwest region, and the peony groups in the Northwest region of China (Wang, 1997; Li *et al.*, 2011). Among them, the peony groups in the Jiangnan region have a long history of cultivation, and it is an excellent group that is relatively resistant to humidity and heat (Hu, 2018). The concept of a breeding group is more convenient for use in practical production and has application value, but it is easy to confuse the genetic background in scientific research. In addition, after long-term artificial introduction and selection, most of the peony groups in the Jiangnan region are formed through repeated hybridization between varieties. Most of the cultivated hybrids have been classified as peonies. It is difficult to judge the origin and kinship of peonies only from the external phenotype. In addition, unclear genetic relationships, shared genetic backgrounds, and large and complex genomes ultimately limit the opportunities for Jiangnan peony breeding (Wang, 2009). Jiangnan peony groups have excellent characteristics of damp and heat resistance, and is an admirable material for peony breeding and resistance research. The origin and classification of Jiangnan peony groups have always been a research hotspot. Previous studies revealed that the groups of Jiangnan peony mainly originated from the central Plains, and was also influenced by the peony groups in the southwest and northwest (Zhang *et al.*, 2020; Hong, 2021). The origin and classification of the Jiangnan peony cultivation group are mainly focused on *Paeonia ostii*, believing that the *Paeonia ostii* plays an indirect role as one of the main original species of the Jiangnan peony groups. However, molecular evidence has been missing for the role of southwest and northwest peony groups in the formation of peony groups in Jiangnan.

Paeonia ostii is one of the nine native species of cultivated peony, the cultivated peony should be a hybrid, so the Latin name of peony in the latest "Flora of China" is modified to *Paeonia* × *suffruticosa* Andrews (<https://www.iplant.cn/info/Paeonia%20%C3%97%20suffruticosa>, accessed on 13 November 2024). Due to the climatic characteristics of the south of China and the biological characteristics of peony that is not resistant to heat, peony varieties are scarce in the south of China, and the flower pattern is single, and the genetic diversity is relatively narrow (Li, 2005; Wang, 2009; Li *et al.*, 2011; Zhu *et al.*, 2018). If the northwest peony or

southwest peony groups can be used, a new strategy can be provided for the creation of new varieties of Jiangnan peony by crossing. Therefore, it is necessary to analyse the other varieties in the Jiangnan peony groups.

Because Jiangnan peony plants grow in different ecological environments, they easily hybridize between distinct species, resulting in a complex genetic background. Thus, it is difficult to determine the exact biological characteristics of Jiangnan peony using traditional morphological classification methods. Therefore, it is necessary to explore more effective methods to analyse the formation and origin of Jiangnan peony varieties (Guo *et al.*, 2018; Guo *et al.*, 2020; Wu *et al.*, 2021). *P. suffruticosa* cv. 'Yu Luo Chun' is a traditional variety and one of the main ornamental varieties of the Jiangnan groups (Li *et al.*, 2011; Hu and Han, 2018). In recent years, different scholars have studied the chloroplast genome of *Paeonia*, but most of them focused on peony species from the Central, Northwest, and Southwest peony groups and on wild species in China (Guo *et al.*, 2020; Wu *et al.*, 2020, 2021). However, studies on the chloroplast genome of the peony groups in Jiangnan region only focus on *P. ostii*, and other Jiangnan peony varieties have not been reported (Guo *et al.*, 2018; Sun *et al.*, 2018). In this study, high-throughput sequencing technology was used to sequence and assemble the whole chloroplast genome of *P. suffruticosa* cv. 'Yu Luo Chun', which was annotated and characterized by bioinformatics analysis and compared with the additional seven reported wild species of *Paeonia*. Chloroplast genome sequences were subjected to comparative genomics and phylogenetic analysis. The purpose is to provide a theoretical reference for the phylogenetic evolution, cultivation origin and molecular marker development of *P. suffruticosa* cv. 'Yu Luo Chun' relative to other species of the genus *Paeonia*.

Materials and Methods

Plant material and DNA extraction

The 5-year-old *P. suffruticosa* cv. 'Yu Luo Chun' was used as the experimental material. The leaves of *P. suffruticosa* cv. 'Yu Luo Chun' for testing were collected from the Peony Germplasm Resource Garden of Zhejiang Institute of Landscape Plants and Flowers (30°06'N, 120°22'E), Linpu Town, Hangzhou City, Zhejiang Province, China, and the specimens were stored in Zhejiang Institute of Landscape Plants and Flowers (specimen number: 20210608001). The fresh leaves of the samples were collected in June 2021, then frozen in liquid nitrogen, and placed in a -80 °C refrigerator for later use.

The total genomic DNA of the leaves of *P. suffruticosa* cv. 'Yu Luo Chun' was extracted using a modified cetyltrimethylammonium bromide (CTAB) method (Porebski *et al.*, 1997). The purity and concentration of the DNA were determined by 1.0% agarose gel electrophoresis. DNA samples with higher concentration and better quality were selected and sent to BIOZERON (Shanghai, China) Co., Ltd. for sequencing using the Illumina NovaSeq 6000 platforms with a read length of 2 × 150 bp.

DNA sequencing, genome assembly and annotation

The chloroplast genome sequence of *Paeonia ostii* 'Feng Dan' (GenBank accession number: MG585274) was downloaded from GenBank as a reference. To ensure more accurate subsequent assembly, the original Illumina sequencing data were quality clipped, using Trimmomatic v0.39 Software (Bolger *et al.*, 2014). Chloroplast genome assembly was performed using NOVOPlasty v4.2 Software (github.com/ndierckx/NOVOPlasty) (Dierckxsens *et al.*, 2016). Sequences with sufficiently high coverage depth and long assembly length were selected as candidate sequences, and the chloroplast scaffolds were confirmed by aligning the NT library. The sequences were then connected based on overlap. The starting position and direction of the chloroplast assembly sequence were determined according to the reference genome, and the possible partition structure (LSC/IR/SSC) of the chloroplast was determined to obtain the final chloroplast genome sequence. Prediction of genes encoding proteins, tRNAs, and rRNAs in the

chloroplast genome was performed using GeSeq software (chlorobox.mpimp-golm.mpg.de/geseq.html/) (Tillich *et al.*, 2017). Finally, OGDRAW software (chlorobox.mpimp-golm.mpg.de/OGDraw.html) was used to display the sample genome in a circular map (Greiner *et al.*, 2019). The annotated sequence was submitted online to NCBI with BankIt, resulting in obtaining sequence accession number OK662586.

Genome structure and comparative analysis

MISA software (webblast.ipk-gatersleben.de/misa/) was used to perform microsatellite (SSR) scanning analysis (Beier *et al.*, 2017) on the chloroplast genome sequence of *P. suffruticosa* cv. 'Yu Luo Chun', and the parameters were set to mononucleotide, dinucleotide repeat numbers of, trinucleotide, tetranucleotide, pentanucleotide and hexanucleotide are 10, 5, 4, 3, 3 and 3, respectively. Using CodonW1.4.2 Software (<http://mobyli.pasteur.fr/cgi-bin/portal.py?form=codonw>), RSCU (Relative synonymous codon usage) of chloroplast genes was used for statistical analysis and for codon bias analysis (Sharp and Li, 1987).

Long repeats using the REPuter online program (bibiserv.cebitec.unibielefeld.de/reputer) (Kurtz *et al.*, 2001) at Hamming distance of 3, maximum computed repeats of 50 and minimal repeat size of 30 conditions. Chloroplast genome collinearity analysis of different species was performed using Mauve Software (Darling *et al.*, 2004).

The chloroplast genomes of 8 species of *P. ostii*, *P. qiui*, *P. jishanensis*, *P. rockii*, *P. decomposita*, *P. delavayi*, *P. ludlowii*, *P. suffruticosa* cv. 'Yu Lou Chun', *Paeonia* were compared using the mVISTA program (Frazer *et al.*, 2004), in Shuffle-LAGAN mode, use *P. ostii* as a reference. IRscope (irscope.shinyapps.io/irapp/) was used to compare the characteristics of IR border regions in chloroplast genomes of different species (Ali *et al.*, 2018).

The extracted 75 consensus protein-coding genes of *P. ostii*, *P. qiui*, *P. jishanensis*, *P. rockii*, *P. decomposita*, *P. delavayi*, *P. ludlowii*, *P. suffruticosa* cv Yu Lou Chun were compared by MAFFT7.037 software (Katoh and Standley, 2013). Nonsynonymous (Ka) and synonymous (Ks) rates were calculated using the DNAsp v5.10.01 (Librado and Rozas 2009), and finally the Ka/Ks graph was statistically drawn by Excel Software.

Phylogenetic analysis

The chloroplast genome sequences of 16 species of *Paeonia*, including *P. ostii* (MG585274), *P. qiui* (MK701992), *P. jishanensis* (MT210545), *P. rockii* (NC_037772), *P. decomposita* (MG571273), *P. delavayi* (NC_035718), *P. ludlowii* (NC_035623), *P. suffruticosa* cv Yu Lou Chun (OK662586), *P. mairei* (MN061945), *P. lactiflora* (MN868412), *P. anomala* (MT210549), *P. obovata* (MH191383), *P. intermedia* (MT210547), *P. emodi* (MT210548), *P. brownii* (MH191385), *P. veitchii* (KT894821), and *Vitis mustangensis* (NC_036009) and *Bergenia scopulosa* (NC_036061) as outgroups, were downloaded from NCBI. Multiple sequence alignments using MAFFT7.037 Software (Katoh and Standley, 2013) were performed, and the results were manually checked and adjusted. Phylogenetic analysis was performed, and the Maximum Likelihood (ML) analysis was performed with analysis was conducted with RAxML version.8.1.24 (Stamatakis, 2014), and Bayesian inference (BI) analysis was performed in MrBayes version 3.2.6 (Ronquist *et al.*, 2012).

Table 1. Summary of *P. suffruticosa* cv. 'Yu Luo Chun' chloroplast genome features

Region	Length (bp)	T/U (%)	C (%)	A (%)	G (%)	AT (%)	GC (%)
Genome	152596	31.15	19.53	30.48	18.84	61.63	38.37
LSC	84272	32.32	18.79	31.02	17.87	63.34	36.66
IRb	25640	28.44	20.8	28.49	22.27	56.93	43.07
SSC	17044	33.47	17.1	33.89	15.54	67.36	32.64
IRa	25640	28.49	22.27	28.43	20.8	56.93	43.07
Protein coding genes	78579	31.11	18.02	30.41	20.46	61.52	38.48
First position	26193	23.49	18.95	30.56	27.00	54.05	45.95
Second position	26193	32.41	20.49	29.24	17.85	61.66	38.34
Third position	26193	37.42	14.61	31.43	16.54	68.85	31.15
tRNA	2791	24.61	24.08	22.07	29.24	46.69	53.31
rRNA	9054	18.69	23.75	25.89	31.68	44.58	55.42

Eighteen genes in the chloroplast genome of *P. suffruticosa* cv. 'Yu Luo Chun' contain introns (Table S1). Among them, 10 protein-coding genes and 6 tRNA genes contained one intron, and 2 protein-coding genes (*clpP* and *ycf3*) contained two introns. Among them, the *trnK-UUU* gene had the longest intron, 2,447 bp, and the *trnL-UAA* gene had the shortest intron, 523 bp (Table S1). These genes can be divided into four categories according to their functions: self-replication-related genes; photosynthesis-related genes; other protein genes; and unknown function genes.

Among the chloroplast genomes of the eight species of the genus *Paeonia*, that of *P. ostii* was the smallest, with a length of 152,153 bp, and that of *P. delavayi* was the largest, with a length of 154,405 bp, and the was difference of 2,252 bp (Table S2). Among the four regions of the chloroplast genome, the LSC region was the longest, with a length of 82,993-85,373 bp; it was the smallest in *P. delavayi* and the largest in *P. ostii*, with a difference between the two of 2,380 bp. The length of the SSC region was between 16,983-17,054 bp; it was the smallest in *P. ludlowii* and the largest in *P. ostii*, with a difference between the two of 71 bp. The length of the IR region was 24,863-27,181 bp; it was the smallest in *P. ostii* and the largest in *P. delavayi*, with a difference between the two of 2,318 bp. The variation in the length of the LSC region and of the IR region was greater than that of the SSC region. The variation in genome length is mainly caused by the difference in length of the LSC and IR regions.

The total number of chloroplast genes in the eight *Paeonia* species varied from 124 to 141 (Table S2). *P. ostii* had the least genes and *P. ludlowii* had the most. *P. ostii* had the fewest protein-coding genes, with 85, and *P. ludlowii* had the most, with 92. The number of rRNAs genes was the most conserved, with 8. In terms of GC content, the minimum GC content was 38.32%, found in *P. ostii*, and the maximum GC content was 38.44%, found in *P. ludlowii*.

Codon usage analysis

Eighty-five coding DNA sequences (CDSs) were obtained from the chloroplast genome of *P. suffruticosa* cv. 'Yu Luo Chun'. To ensure the accuracy of the results, the length of the sequence was set to less than 300 bp, repeated gene sequences were eliminated, and the remaining 52 CDSs were used for codon bias analysis. There were 30 codons with relative synonymous codon usage (RSCU) values greater than 1.00, of which 28 ended in T/A and 2 ended in G/C (Table S3). This indicates that the chloroplast genome codons of *P. suffruticosa* cv. 'Yu Luo Chun' prefer to end in A or T but do not prefer to end in G or C. The number of codons encoding leucine (Leu) was the largest, 2,052; isoleucine (Ile) and glycine (Gly) codons ranked second and third, respectively, 1,677 and 1,454; and the number of cysteine (Cys) codons was the lowest, 217.

Analysis of SSR and repeat sequences

Seventy-one SSR loci were detected in the chloroplast genome of *P. suffruticosa* cv. ‘Yu Luo Chun’, of which 47, 12, 7 and 5 were mononucleotide, dinucleotide, trinucleotide and tetranucleotide repeats, respectively. Pentanucleotide and hexanucleotide repeat motifs were not detected (Figure 2). The most frequently occurring single nucleotide sequences were composed of A/T and C/G, of which 44 were composed of A/T, accounting for approximately 62.3% of all SSRs, and 3 were composed of C/G. There were a total of 12 dinucleotide sequences consisting of AG/CT and AT/AT, which accounted for approximately 16.90% of all SSRs. There were 4 tetranucleotide sequences, AAAC/GTTT, AAAG/CTTT, AAAT/ATTT and AGAT/ATCT, accounting for approximately 7.04% of the total SSRs. Regarding the distribution of SSRs by segment, 6.67% were located in the IR segment, 78.33% were located in the LSC segment, and 15.00% were located in the SSC segment. These findings indicate the heterogeneity of SSR distribution in the chloroplast genome of *P. suffruticosa* cv. ‘Yu Luo Chun’.

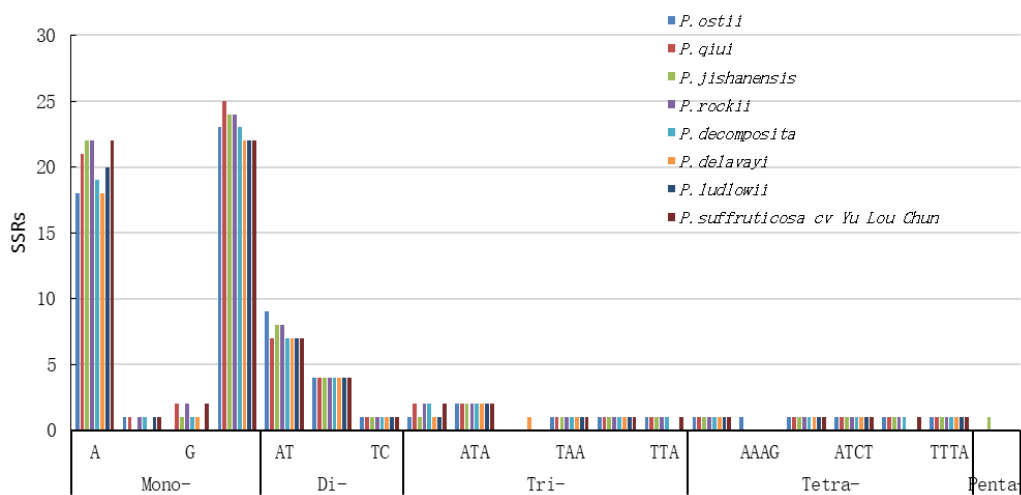


Figure 2. Comparison of SSR repeats in the chloroplast genome of eight *Paeonia* species

The SSRs of the chloroplast genomes of the eight *Paeonia* species were compared and analysed. None of the peonies had a pentanucleotide repeat motif, except for *P. jishanensis*. In the eight chloroplast genomes, the number of single nucleotide repeats ranged from 41 (*P. delavayi*) to 49 (*P. qiui*), and dinucleotide, trinucleotide, and tetranucleotide repeats were also found. The number of repeating motifs was 12-14, 5-7, and 4-6, respectively. Therefore, the main type of SSR in peony is a single nucleotide, and the vast majority of single-nucleotide SSRs are A/T-type SSRs.

The repeat sequences in the chloroplast genome were analysed by REPuter, and 43 repeats were found in *P. suffruticosa* cv. ‘Yu Luo Chun’, including 22 palindromic repeats and 21 forward repeats (Figure 3), but no reverse repeats or complementary repeats were found. A comparative analysis of the chloroplast genome repeats of eight *Paeonia* species found that *P. qiui*, *P. rockii*, *P. delavayi*, and *P. ludlowii* contained three types of repeats (palindromic repeats, forward repeats and reverse repeats); the chloroplast genomes of the other four species contained only palindromic repeats and forward repeats. Except for *P. jishanensis*, which had equal numbers of palindromic and forward repeats, the proportion of palindromic repeats was the highest in the other seven species.

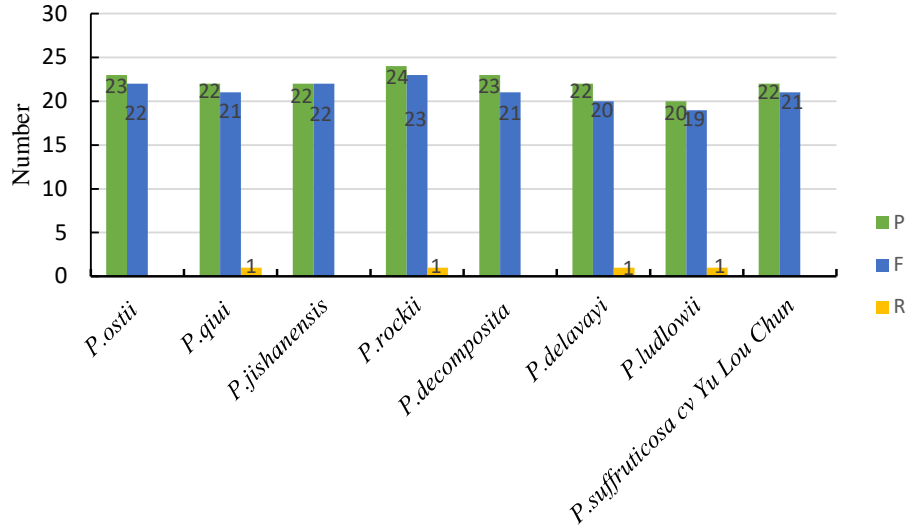


Figure 3. Comparison of long repeat sequences in the chloroplast genome of eight *Paeonia* species

Comparison of chloroplast genomes among Paeonia species

Collinearity analysis showed that no large-scale gene rearrangements were found in the chloroplast DNA sequences of the eight *Paeonia* species (Figure 4), indicating that the evolutionary models of these chloroplast genomes were highly conserved. To verify the possibility of chloroplast genome differentiation, we used mVISTA to compare the *Paeonia* chloroplast genome with the annotation of *P. ostii* as a reference (Figure 5). The results showed that *P. suffruticosa* cv. ‘Yu Luo Chun’ had high sequence homology with *Paeonia*. Intergenic regions *rpoC1*, *petB*, *rsp16*, *rps19*, *clpP*, and *ccsA* and the *ycf1* gene were highly conserved, while *psbA-matK*, *trnQ-psbK*, *trnS-trnR*, *rpoC1-rpoB*, *pspM-trnD*, *pspA-ycf3*, *atpB-rbcL*, *petA-psbL*, *rpl20-rps12*, *petD-rpoA*, *rps19-rpl2*, *rps7-rps12*, *ndhF-trnL*, *ndhD-psaC* and *trnV-rps12* were highly divergent and thus may be good candidate sequences for *Paeonia* species identification.

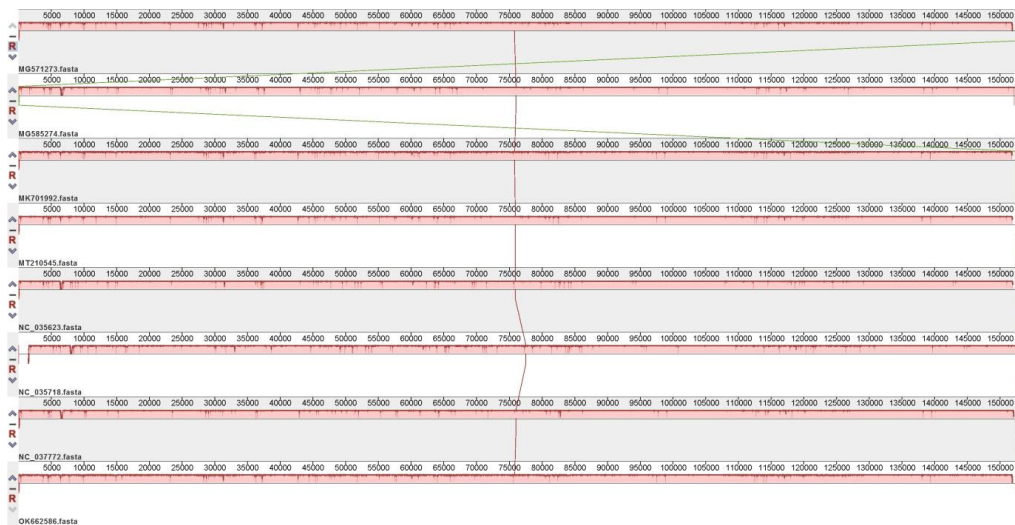


Figure 4. Mauve alignment in the chloroplast Genome of eight *Paeonia* species. *P. ostii* (MG585274), *P. qiui* (MK701992), *P. jishanensis* (MT210545), *P. rockii* (NC_037772), *P. decomposita* (MG571273), *P. delavayi* (NC_035718), *P. ludlowii* (NC_035623), *P. suffruticosa* cv ‘Yu Luo Chun’(OK662586)

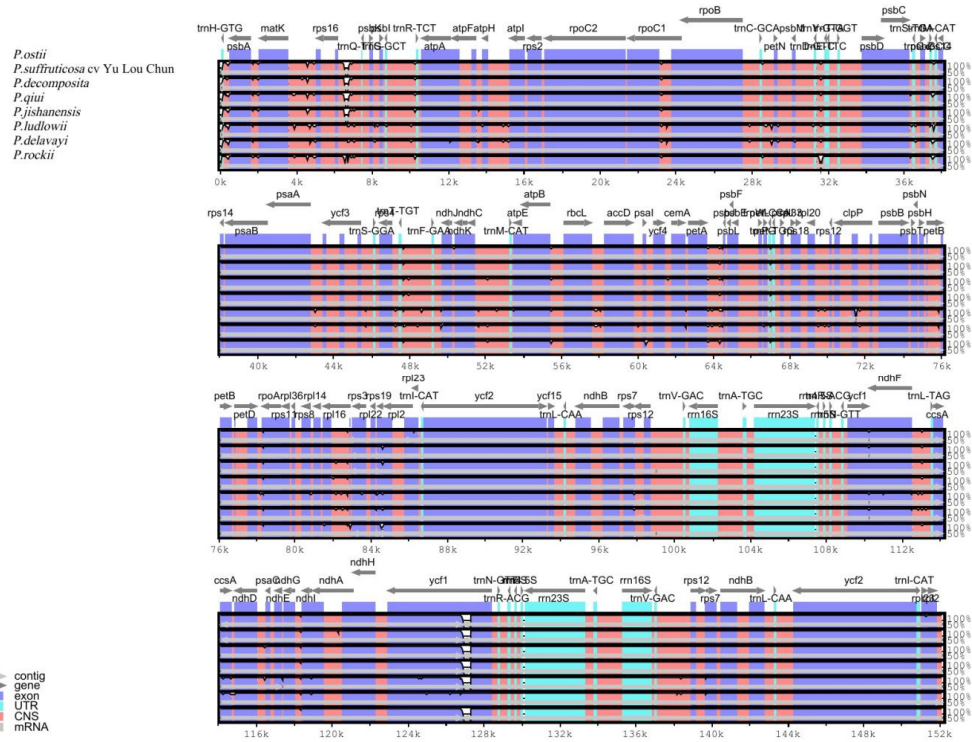


Figure 5. Comparison of chloroplast genomes of eight *Paeonia* species using mVISTA

IR contraction and expansion

To explore the potential expansion and contraction of IRs, the distribution of IR and single-copy border region genes in the chloroplast genomes of eight species of the genus *paeonia* was compared (Figure 6). As shown, genes distributed at the LSC/IR and SSC/IR boundaries included rps19, rpl2, ndhF, ycf1 and trnH. Among them, the LSC/IRb of *P. suffruticosa* cv. ‘Yu Luo Chun’, *P. ludlowii*, *P. delavayi*, *P. jishanensis* and *P. qiui* are in the rps19 gene, while the LSC/IRb of *P. rockii* and *P. ostii* are in the rpl22 coding region. The IRb/SSC of the eight species of the genus *paeonia* are all in the ndhF coding region, and the IRb/SSC of *P. suffruticosa* cv. ‘Yu Luo Chun’, *P. rockii* and *P. ostii* are also in the hypothetical chloroplast open reading frame 1 (ycf1) coding region. The SSC/IRa boundaries of the chloroplast genomes of the eight *Paeonia* species are all ycf1 coding regions, and the length of the ycf1 coding region differed. The length of the IRa region of the eight species of the genus *paeonia* was 1,078 bp. The SSC regions of *P. suffruticosa* cv. ‘Yu Luo Chun’, *P. decomposita*, *P. rockii*, *P. jishanensis*, and *P. qiui* were all 4,346 bp in length, while the SSC regions of *P. ludlowii*, *P. delavayi* and *P. ostii* were 4340, 4355 and 4,352 bp in length, respectively. The IRa/LSC boundaries of the eight species of the genus *paeonia* were diverse. The IRa/LSC boundaries of both *P. delavayi* and *P. rockii* lack trnH, and that of *P. rockii* was in the rpl2 coding region. The trnH gene was found in *P. suffruticosa* cv. ‘Yu Luo Chun’, *P. ludlowii*, *P. decomposita*, *P. jishanensis* and in both *P. qiui* and *P. ostii*, it was found in the LSC region at 3, 0, 4, 3, 4, and 79 bp away from the IRa/LSC boundary, respectively.

Inverted Repeats

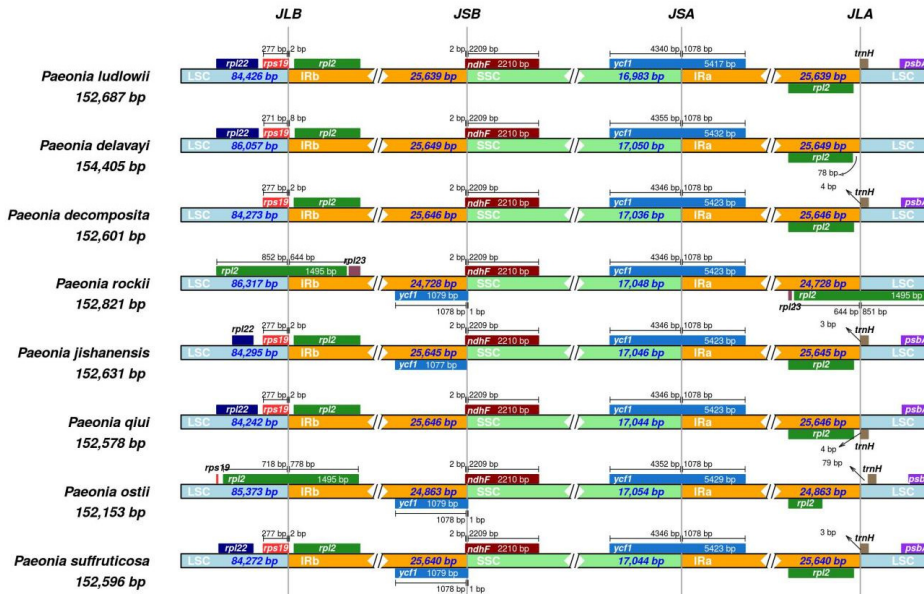


Figure 6. Comparison of LSC, SSC, and IR regions among eight *Paeonia* species

Adaptive evaluation analysis

The non-synonymous/synonymous substitution rate (Ka/Ks) ratio in the chloroplast genomes of the protein-coding genes of eight species of the genus *Paeonia* were calculated. The results showed that most genes had Ka/Ks ratios below 1 compared with those of other *Paeonia* species (Figure 7), and most genes were selected for purification. The Ka/Ks ratios of *ndhF*, *petB*, *petD*, *rpoA* and *ycf2* were usually greater than 1, indicating that these genes undergo positive selection and play a key role in the adaptation of *Paeonia* species.

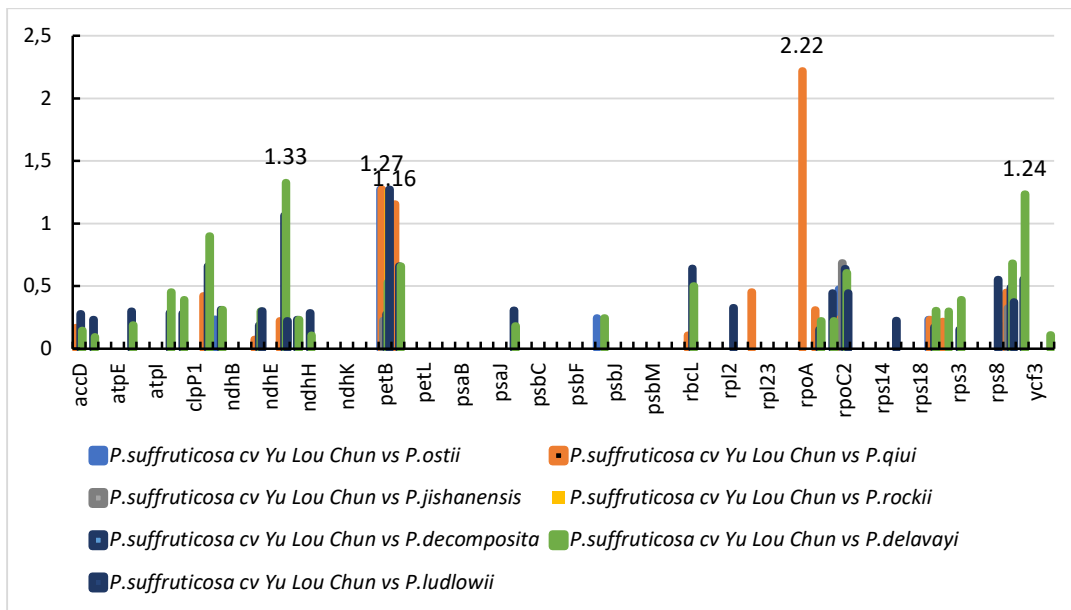
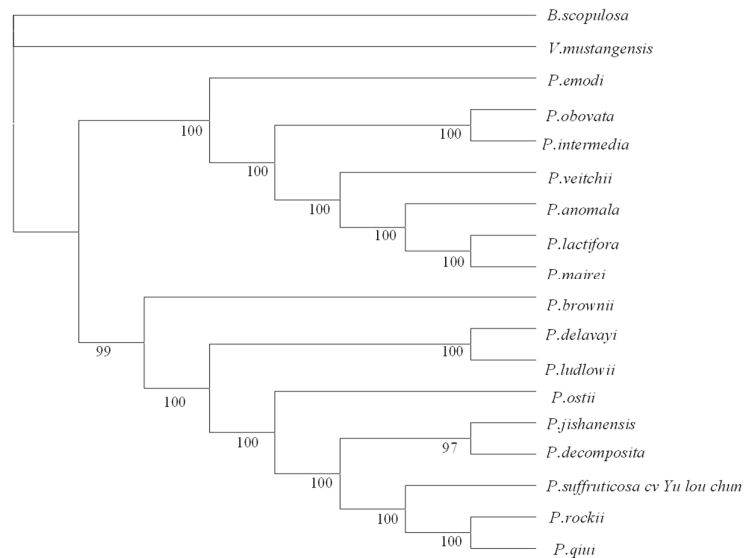


Figure 7. Ka/Ks ratio values of among the chloroplast genomes of eight *Paeonia* species

Phylogenetic analysis

We selected the chloroplast genomes of 18 species to build a phylogenetic tree. Taking *Vitis mustangensis* and *Bergenia scopulosa* as outgroups and including 16 species of *Paeonia*, phylogenetic maximum likelihood (ML) and Bayesian inference (BI) trees were constructed (Figure 8). The results show that the topological structures of the ML and BI trees were consistent, and only a few branches had slightly different support rates. The two outgroups *V. mustangensis* and *B. scopulosa* were first separated. *V. mustangensis* belongs to Vitaceae, *B. scopulosa* belongs to Saxifragaceae. Because they belong to different families and are distantly related, the phylogenetic trees can separate them well. The peony species were divided into two large clusters, namely, sect. *Paeonia* and sect. *Moutan*. Belonging to sect. *Paeonia*, *P. ostii*, *P. qiui*, *P. jishanensis*, *P. rockii*, *P. decomposita*, *P. delavayi*, *P. ludlowii*, *P. suffruticosa* cv. ‘Yu Luo Chun’ and *P. brownii* clustered into one large clade. *P. mairei*, *P. lactiflora*, *P. anomala*, *P. obovata*, *P. intermedia*, *P. emodi*, and *P. veitchii* all belong to sect. *Moutan* and gather into another large branch. Among them, *P. suffruticosa* cv. ‘Yu Luo Chun’, *P. qiui* and *P. rockii* clustered into a small clade, indicating that they are closely related.



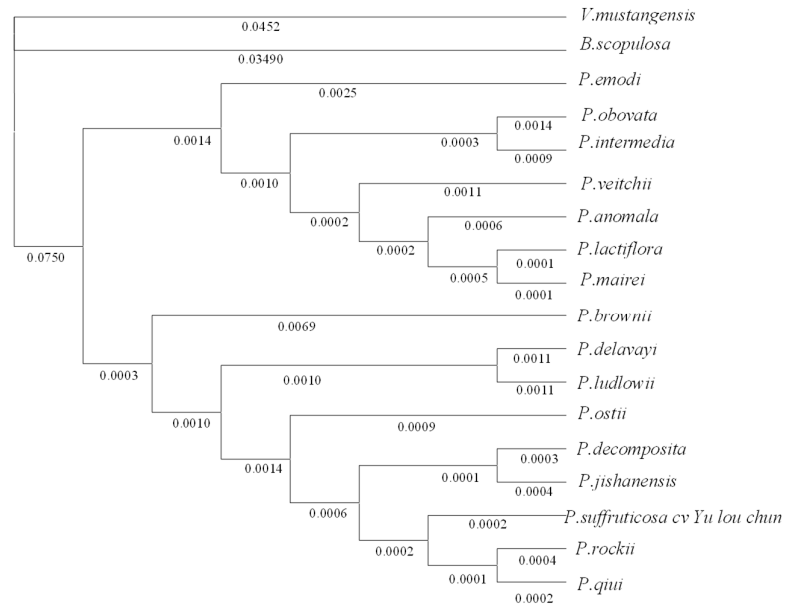


Figure 8. Phylogenetic trees constructed using (A) Maximum Likelihood (ML) and (B) Bayesian Inference (BI) methods, based on the chloroplast genomes of 16 *Paeonia* species, with *Vitis mustangensis* and *Bergeia scopulosa* as the outgroups

Discussion

Chloroplast genome features of P. suffruticosa cv. 'Yu Luo Chun'

In recent years, with the rapid development of high-throughput sequencing technology, the cost of genome sequencing has been greatly reduced, and the acquisition of chloroplast genomes has become more convenient. The chloroplast genome of *Paeonia* has also been revealed (Guo *et al.*, 2018; Sun *et al.*, 2018; Guo *et al.*, 2020; Wu *et al.*, 2020, 2021). In this study, the lengths of the whole chloroplast genome and LSC, SSC and IR regions of the eight *Paeonia* species were 82,993-85,373 bp, 16,983-17,054 bp and 24,863-27,181 bp, respectively. The difference in GC content was low. Overall, the chloroplast genomes of *Paeonia* species showed high similarity in gene composition, GC content and other parameters. This indicates that the chloroplast genome of *Paeonia* is highly evolutionarily conserved, and the variation in the length of the LSC and IR regions is greater than that of the SSC region. The variation in the genome length is mainly caused by the difference in the length of the LSC and IR regions. A similar phenomenon was also noted in the chloroplast genome of *Prunus* species. The GC content in the LSC and SSC regions of the *Prunus* chloroplast genome was significantly lower than that in the IR region. The foremost reason is that the eight rRNA genes with high GC content were all distributed in the IR region (Xue *et al.*, 2019).

The high GC content in the IR regions may be because these regions contain rRNAs with low A/T content in the chloroplast genome of *Paeonia* (Wu *et al.*, 2020). However, the overall GC content of all *Paeonia* regions (LSC and SSC) was higher than that of IR regions. Relatively high GC contents in IR regions are frequently characterized by rRNA and tRNA genes (He *et al.*, 2016, Shen *et al.*, 2017). In general, large IRs play an important role in maintaining the stability of the plastid genome (Wu *et al.*, 2011). The loss of a large number of IRs resulted in few differences in genome structure and gene content in the plastid genome (Yi *et al.*, 2013). There are no large IR regions in intact plastids of conifer species (Zeb *et al.*, 2022). Therefore, the GC content of different plant material sources in the LSC, SSC and IR regions was different.

This study also found that the chloroplast genome of *P. suffruticosa* cv. 'Yu Luo Chun' contained 131 functional genes, including 86 protein-coding genes, 37 tRNA genes and 8 rRNA genes. By comparing the number of genes in the chloroplast genomes of *P. suffruticosa* cv. 'Yu Luo Chun' with that of seven other species of the genus *Paeonia*, it was found that the total number of genes in *P. suffruticosa* cv. 'Yu Luo Chun' was intermediate among them (124-141), which also shows that the *Paeonia* chloroplast genome is highly conserved. This study supplemented the Jiangnan peony groups chloroplast genome that was missing from *Paeonia* and provides a data basis for further revealing the phylogenetic relationship and genetic diversity of *Paeonia*.

Codon usage and SSR analysis

There were certain differences in the base composition of the different chloroplast genomes (Chakraborty *et al.*, 2020; Wang *et al.*, 2022). The chloroplast genome of *P. suffruticosa* cv. 'Yu Luo Chun' tends to use codons containing A/T, similar to the findings for other Paeoniaceae species (Wu *et al.*, 2021). By comparing and analysing the codon preferences of the chloroplast genomes of *P. suffruticosa* cv. 'Yu Luo Chun' and seven other *Paeonia* species, it was found that the chloroplast genomes of the eight species of *Paeonia* were all biased to use codons containing A/T. This result is common in the chloroplast genomes of higher plants (Gichira *et al.*, 2017). Codon preference is closely related to the long-term evolutionary history of organisms (Iriarte *et al.*, 2021; Wu *et al.*, 2021). Therefore, studying the codon usage of *P. suffruticosa* cv. 'Yu Luo Chun' plays a major role in exploring the evolutionary pattern of *Paeonia* species. Related research provides very useful information for the transgenic strategy for the *P. suffruticosa* cv. 'Yu Luo Chun' chloroplast. For example, before the genetic transformation of the chloroplast genome, the codons of the target gene can be optimized to make it more efficiently expressed in the chloroplast of *P. suffruticosa* cv. 'Yu Luo Chun'.

SSRs in chloroplast genomes are highly variable at the interspecific and even intraspecific levels and are therefore commonly used as genetic markers in evolutionary studies (Park *et al.*, 2018). In the current study, among the eight species of *Paeonia*, mononucleotide and dinucleotide repeats were the most common, similar to findings reported for other *Paeonia* species (Guo *et al.*, 2018; Wu *et al.*, 2020; Wu *et al.*, 2021). In addition, the distribution of SSRs in different regions varied greatly, and SSRs were mainly found in the LSC region, followed by the SSC region. The SSRs were rich in A/T, and the polymeric A/T type was much larger than the polymeric G/C type, indicating the heterogeneity of SSR distribution in the chloroplast genomes of eight species of *Paeonia*. This is consistent with the results of other *Paeonia* species (Guo *et al.*, 2018; Sun *et al.*, 2018). Therefore, it is speculated that these SSRs with high A/T content may be one reason for the low content of CG in the chloroplast genome and for the deviation in base content in each region. The discovery of SSR loci has a certain significance for the identification of *Paeonia* species, population-level polymorphism detection and phylogenetic analysis.

Comparative analysis of genome structure

The expansion and contraction of the IR region of the chloroplast genome is a shared evolutionary phenomenon (Guo *et al.*, 2021). In the current study, colinear analysis showed that no large-scale gene rearrangements were found in the chloroplast DNA sequences of the eight species of *Paeonia*, indicating that the evolutionary models of these chloroplast genomes were highly conserved. This result was the same as that reported in a previous study (Wu *et al.*, 2020; Wu *et al.*, 2021). *Paeoniaceae* in the Campanian of the late Cretaceous was differentiated from Saxifragales, while in the late Oligocene or early Miocene, it was differentiated into woody and herbaceous clades. *Paeoniaceae* may have experienced 5 mutation events and 21 migrations in this historical event (Zhou *et al.*, 2021). However, the chloroplast genomes of the studied *Paeoniaceae* species are relatively conserved, with no rearrangement of gene organization.

Using mVISTA to compare the chloroplast genomes of the genus *Paeonia*, it was found that the chloroplast genome sequences of *P. suffruticosa* cv. 'Yu Luo Chun' and seven other species of *Paeonia* are in the *psbA-matK*, *trnQ-psbK*, *trnS-trnR*, *rpoC1-rpoB*, *pspM-trnD*, *pspA-ycf3*, *atpB-rbcL*, *petA-psbL*, *rpl20-rps12*, *petD-rpoA*, *rps19-rpl2*, *rps7-rps12*, *ndhF-trnL*, *ndhD-psaC* and *trnV-rps12* intergenic regions and in the *rpoC1*, *petB*, *rsp16*, and *rps19* genes. There are marked differences in the gene regions of *clpP*, *ccsA* and *ycf1*, and these regions can provide a reference for the screening of specific DNA barcodes for the identification of *Paeonia* species. Some studies identified genes with greater variation, including *trnK*, *trnR*, *psbZ*, *ycf3*, *rps3* and *rps19*, and believed that the variation in intergenic regions was significantly greater than that in gene regions (Wu *et al.*, 2020). Ten highly variable regions, *psbA*, *rps16*, *rps14*, *rbcL-accD*, *petA-psbJ*, *rps18*, *rpl22*, *ndhF*, *ccsA*, and *ycf1*, were identified in the chloroplast genomes of *P. ostii* 'Fengdan' and *P. lactiflora* 'Da fugui' (Sun *et al.*, 2018). There are differences in identification, which are related to the different materials used.

A series of cp fragments have been found as plant barcodes in *Paeonia* (Wu *et al.*, 2021). Changes in the position of the IR/SC boundary in the chloroplast genome between different species are relatively common; that is, some genes enter the IR region or SC region, and these changes are usually different in species of the same genus. In the current study, although the chloroplast genomes of the eight species of *Paeonia* were relatively conserved in structure and size, there were still certain changes in the position of IR boundaries among different species. Genes distributed at the LSC/IR and SSC/IR boundaries of the eight *Paeonia* species included *rps19*, *rpl2*, *ndhF*, *ycf1* and *trnH*. IRb/SSC were all in the *ndhF* gene, and the IRb/SSC of *P. suffruticosa* cv. 'Yu Luo Chun', *P. rockii* and *P. ostii* were also in the hypothetical chloroplast open reading frame 1 (*ycf1*) gene. The SSC/IRa boundaries were all in *ycf1* genes. The position of the IRa/LSC boundary still changes to some extent. Both the IRa/LSCs of *P. delavayi* and *P. rockii* lack *trnH*, and the IRa/LSCs of *P. rockii* are within the *rpl2* gene. Given that our findings are consistent with those of other plants (Guo *et al.*, 2018; Guo *et al.*, 2020; Wu *et al.*, 2020), the LSC/IRb boundary change is the main reason for the expansion and contraction of the IR region of the *Paeonia* chloroplast genome. These regions can provide a reference for the screening of specific DNA barcodes for the identification of *Paeonia* species.

Selective pressure in the evolution of P. suffruticosa cv. 'Yu Luo Chun'

The Ka/Ks ratio has been used to assess gene differentiation rates. A Ka/Ks ratio < 1 represents purifying selection, while a Ka/Ks ratio > 1 represents positive selection (Zheng *et al.*, 2019; Tian *et al.*, 2021). Synonymous nucleotide substitutions occur more frequently than non-synonymous nucleotide substitutions in most protein-coding genes (Mehmood *et al.*, 2020; Huang *et al.*, 2021). In this study, the Ka/Ks ratio was < 1 in most of the genes, indicating that most of the genes of the eight *Paeonia* species were purified by selection. However, the Ka/Ks ratios of *ndhF*, *petB*, *petD*, *rpoA* and *ycf2* were generally > 1, indicating that these genes undergo positive selection, which may help them adapt to the living environment. In the selective pressure analysis, we isolated two types of photosynthesis genomes: subunits of NADH dehydrogenase (*ndhF*), subunits of the cytochrome b/f complex (*petB*), and genes of unknown function (*ycf2*). Positively selected genes play an important role in the variation of *Paeonia* species under different environmental conditions.

Phylogenetic inferences

Phylogenetic analysis showed that the topological structures of the ML and BI trees were consistent, and only a few branches had slightly different support rates. *P. suffruticosa* cv. 'Yu Luo Chun' and *P. qiui* and *P. rockii* belong to the same clade and are closely related to each other. This discovery identifies the closest relationship among these species. This branching pattern was consistent with that of the phylogenetic tree constructed by using chloroplast markers. It coincides with the three groups divided by Stern and Hong (Stern, 1946, 1973; Hong, 2021), and the phylogenetic relationship between different species within each branch has also been well resolved. The phylogenetic tree constructed based on the *Paeonia* chloroplast whole genome

sequence is an effective way to solve the phylogenetic relationships among *Paeonia* species. This study can provide basic data for future research on the phylogenetic relationships of *P. lactiflora*. The chloroplast genome has been put forwards as a super barcode for species identification (Guo *et al.*, 2020; Yang *et al.*, 2020). In this study, the peony species had a very high support value in the phylogenetic tree based on the complete chloroplast genome. The complete chloroplast genome can provide adequate informative loci, which can help to elucidate the relationship of closely related species.

One of the outstanding features of peonies is homoploid speciation by reticulate evolution due to hybridization and polyploidization (Zhou *et al.*, 2021). The phylogenetic position of Jiangnan peony groups in the family *Paeoniaceae* has been hotly debated. The Jiangnan peony groups is divided according to the different peony cultivation sites and original species, so the source is more complicated (Wang, 1979; Wang, 1997; Hu and Han, 2018). The Jiangnan peony groups developed in the Jiangnan region of China is mainly cultivated for medicinal purposes, and there are few ornamental varieties. It is mainly cultivated in Anhui, Zhejiang, Jiangsu and other provinces. The Jiangnan peony groups are an excellent group that is relatively resistant to humidity and heat. The traditional Jiangnan peony groups adapt to the high temperature and humidity in the Jiangnan region. However, the existing varieties of Jiangnan peony were deficient, some excellent varieties were facing loss, and genetic diversity was reduced, which may further lead to the decline in growth vigour and stress resistance (Wang, 2009). In the past, the view was that *P. ostii* 'Fengdan' was considered to be the native species of the Jiangnan peony groups and played a major role in its origin (Wang, 1997; Zhang *et al.*, 2020; Hong, 2021). *P. suffruticosa* cv. 'Yu Luo Chun' is a traditional variety in the south of the Yangtze River and one of the main ornamental varieties in the Yangtze River Basin (Li *et al.*, 2011). In this study, the phylogenetic tree established by using the chloroplast genome of *Paeonia* showed that *P. suffruticosa* cv. 'Yu Luo Chun' was closely related to *P. qiui* and *P. rockii*. *P. suffruticosa* cv. 'Yu Luo Chun' was phylogenetically close to *P. qiui* and *P. rockii* rather than to *P. ostii*. This may be due to the wide variety of *Paeonia*, the complex genetic background, the easy interspecies hybridization within the genus, and the common intermediate and transitional types. Nine wild species of peony have made important contributions to the origin of cultivated varieties through hybridization. They originated the Jiangnan peony groups through crosses between multiple wild species (Wang, 2009; Hong, 2021). This result supports the idea of the origin of "multiple places and diversity" of cultivated peony in China.

P. qiui and *P. rockii* were important chloroplast providers to Jiangnan peony, indicating that they are involved in the breeding of Jiangnan peony groups. At the same time, the introduction and domestication of peonies in northern and southern China are also important reasons. We speculate that the southwest species group and the Jiangnan peony groups group have similar origins. Through this study, it is confirmed that some species of Jiangnan peony may have been directly cultivated and domesticated from the southwest variety for a long time, and we propose strategies and suggestions for the breeding and utilization of resources of Jiangnan peony. To cultivate breeding targets with heat resistance, shallow root systems and good ornamental value, excellent heat and humidity resources, such as *P. qiui* and *P. rockii*, can be used to breed new varieties suitable for the Jiangnan region through hybridization and other methods.

The peony groups in the north were moved south and cultivated since ancient times. As early as the Southern and Northern Dynasties of China (approximately 422 AD), Xie Lingyun said that "there are many peonies in the bamboo in the water between Yongjia and Wenzhou, Zhejiang" (Li, 2005). The Jiangnan peonies started being transplanted in the Tang Dynasty and their development peaked in the Song Dynasty. After the Qing Dynasty, the cultivation of peony in Shanghai increased considerably (Hu *et al.*, 2018). Currently, peony is mostly distributed in Shanghai, Anhui, Jiangsu and Zhejiang Provinces and cities (Li *et al.*, 2011). This demonstrates that the Jiangnan peony groups have an intricate relationship with other wild peony species. The results of this study showed that in the formation process of *P. suffruticosa* cv. 'Yu Luo Chun', *P. qiui* and *P. rockii* made a certain contribution to the formation of the Jiangnan peony groups. The classification of Jiangnan

peony groups is challenging. The use of the chloroplast genome can provide a reference for classifying Jiangnan peony groups; however, the number of published chloroplast genomes of this group is still very limited. Therefore, more chloroplast genomes of Jiangnan peony varieties are needed to better solve the phylogeny of Jiangnan peony groups.

Conclusions

In the current study, the chloroplast genome of *P. suffruticosa* cv. 'Yu Luo Chun' was assembled and annotated, and the chloroplast genome structure, gene composition and SSR distribution were compared with those of seven others wild *Paeonia* species. Seven fragment regions with high differences, including *rsp16*, *rps19*, *clpP*, *ccsA*, and *ycf1*, can be used as the basis for the selection of molecular barcodes in the chloroplast genome of *Paeonia*. five regions (*ndhF*, *petB*, *petD*, *rpoA* and *ycf2*) were screened by Ka/Ks analysis with positively selected genes, and the taxonomic position of *P. suffruticosa* cv. 'Yu Luo Chun' was clarified. *P. suffruticosa* cv. 'Yu Luo Chun' is closely related to *P. qiui* and *P. rockii*. Strategies and suggestions for the breeding and utilization of Jiangnan peony resources are put forwards. This study provides a reference for the taxonomic protection of *P. suffruticosa* cv. 'Yu Luo Chun' and for the species identification, interspecific evolution and phylogeny of *Paeonia*.

Authors' Contributions

Conceptualization: JQZ; Data curation and Writing-Original draft: JQZ; Data curation: HCL and JQZ; Software analysis, Visualization: XW; Writing-review and editing: JQZ and KYZ. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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