

Genetic Diversity Evaluation of Medicinal *Papaver nudicaule* Based on Complete Chloroplast Genomes

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Abstract

Papaver nudicaule has long been valued as both a traditional medicinal herb and an ornamental plant owing to its colorful flowers. Comprehensive intraspecific analyses based on chloroplast genome can provide novel genetic resources to reveal the genetic variation and diversity of this species from a maternal inheritance perspective. In this study, we sequenced and assembled the complete chloroplast genomes of 12 genotypes of *P. nudicaule* collected from distinct geographical locations. The complete chloroplast genomes of these accessions ranged from 153,683 to 153,905 base pairs (bp) and exhibited a typical quadripartite structure, with a total of 113 annotated functional genes. Comparative analyses of 12 *P. nudicaule* chloroplast genomes revealed 26 polymorphic simple sequence repeats (SSRs), 236 polymorphic single nucleotide polymorphisms (SNPs), 75 insertion-deletion mutations (indels), and six inversions. Phylogenetic analysis indicated that the 12 genotypes clustered into two distinct clades. By characterizing SNPs, indels, inversions and SSRs through chloroplast genome comparison, our results provide a case study for determining intraspecific chloroplast genome variation patterns and serve as valuable genetic resources for the genetic improvement and conservation of this species, which has both ornamental and medicinal value.

Introduction

Papaver nudicaule (Iceland poppy) is a short-lived perennial herb within the tribe Papavereae of the Papaveraceae family. This species naturally distributes across subpolar zones of Europe, Asia and North America, alongside mountainous areas in Central Asia. Admired for its richly colored blooms, *P. nudicaule* has long been utilized in traditional medicine and horticultural landscaping¹. The plant biosynthesizes an array of alkaloids, such as amrensinine, reframidine, nudicauline and nudicaulonol. Previous pharmacological research has confirmed that nudicauline acts as an effective non-addictive analgesic². Alkaloid metabolites derived from *P. nudicaule* exhibit multiple valuable bioactivities, including narcotic analgesia, antibacterial activity, muscle relaxation, antitussive and anti-tumor effects^{3,4}. Wild germplasm resources of *P. nudicaule* are gradually depleted as a result of uncontrolled overharvesting and habitat destruction, which calls for strengthened conservation strategies. Given its dual medicinal and ornamental value, existing studies on *P. nudicaule* mainly center on alkaloid separation and quantification^{5,6}, transcriptome analysis⁷, transcription factor regulation⁸, and floral pigment metabolism^{9,10}. Nevertheless, research concerning its intraspecific genetic diversity is still inadequate, and the genetic differentiation among different *P. nudicaule* populations has not been systematically characterized to date.

Chloroplasts are essential plastids in plant cells that function as the core organelles for photosynthesis and serve as pivotal sites for the biosynthesis of multiple key metabolites, including pigments, starch, and fatty acids^{11,12}. Possessing independent genetic systems, the chloroplast genomes of higher plants typically range from 120 to 180 kb in size¹³. Compared with nuclear genomes, chloroplast genomes exhibit lower mutation rates and lack genetic recombination, rendering them ideal molecular resources for evolutionary research. In particular, chloroplast genome sequencing has become a prevalent and reliable tool for reconstructing plant phylogenetic relationships at systematic taxonomic levels^{14–16}. Chloroplast genomes generally follow a uniparental inheritance pattern: angiosperms exhibit predominant maternal inheritance, while most gymnosperms display paternal inheritance^{12,17}. Such uniparental genetic characteristics contribute to distinct geographical genetic structure, making chloroplast molecular markers widely applicable in plant phylogeographic studies. Structurally, chloroplast genomes are circular and feature a typical quadripartite architecture, consisting of a large single-copy (LSC) region, a small single-copy (SSC) region, and a pair of inverted repeat (IR) regions^{18–21}. Benefiting from the rapid

advancement and cost reduction of next-generation sequencing technology, complete chloroplast genomes of numerous plant species have been sequenced and annotated across multiple germplasm accessions. At the intraspecific level, chloroplast genomes harbor abundant genetic variations, including single nucleotide polymorphisms (SNPs), simple sequence repeats (SSRs), and insertion-deletion mutations (indels). These polymorphic loci have been extensively utilized to evaluate population differentiation, genetic diversity, gene flow patterns, and biogeographical structures of plant species^{22–25}. Clarifying the intraspecific genetic diversity of target species can further provide a theoretical basis for parental germplasm selection and molecular breeding²⁶.

In this study, we performed whole-genome sequencing, assembly, and annotation of 12 complete chloroplast genomes of *P. nudicaule* collected from distinct wild geographical populations. We systematically characterized the structural features of these chloroplast genomes and identified intraspecific sequence polymorphisms, including genetic variations and diversity among different accessions. Furthermore, we evaluated the population genetic diversity, clarified the phylogenetic relationships, and revealed the population genetic structure of *P. nudicaule*. This study represents the first comprehensive investigation of intraspecific genetic variation and diversity of *P. nudicaule* based on full chloroplast genomic resources. The results not only provide valuable insights into chloroplast genome mutation patterns at the intraspecific level but also offer reliable genetic germplasm resources for future molecular breeding and conservation utilization of this medicinally and horticulturally valuable species.

Materials and Methods

Sample Collection, DNA Extraction, and Sequencing

The samples of *P. nudicaule* were collected in twelve areas and represented the geographical distribution of this species. Sample information is provided in Fig. 1. The collection permits were secured from the National Forestry and Grassland Administration. Formal species identification of all plant samples was performed by Dr. Wenpan Dong. Fresh leaves were collected for silica gel conservation.. Voucher specimens of each collected species were deposited at Chinese Academy of Quality and Inspection Testing Herbarium (Voucher No. ENC851320, ENC851322, ENC851324, ENC861956-62, ENC861968 and ENC861969). Total genomic DNA was extracted from the leaves of each sample using a modified CTAB DNA extraction method of Li et al.²⁷. The quality and concentration of the extracted total DNA were determined using 1% (w/v) agarose gel electrophoresis and NanoDrop 2000 spectrophotometry (Wilmington, DE, USA). Each DNA with a total concentration > 1µg was used for Illumina sequencing. Using a genome skimming approach, the chloroplast genomes of *P. nudicaule* were subjected to sequencing. Total DNA was sheared to 350 bp fragments using an ultrasonicator, and 350 bp insert libraries were constructed for Illumina sequencing following the instructions of the manufacturer (Illumina, San Diego, CA, United States). Genomic libraries were paired-end sequenced (150 bp) on the Illumina HiSeq X-Ten platform (Yuebo biotechnology Co., Ltd., Beijing,China).

Chloroplast Genome Assembly and Annotation

Raw reads were trimmed using Trimmomatic v0.36, and the resulting clean data were used for assembly and subsequent analysis²⁸. Then the clean reads were assembled into contigs by using SPAdes v3.6.1²⁹ with K-mer length of 95. The resulting contigs were selected for chloroplast genome encoding contigs on BLAST searches with the complete *P. nudicaule* chloroplast genome as the reference. The selected contigs were secondarily

assembled using Sequencher 5.4.5 (Gene Codes, Ann Arbor, MI). The gaps between the contigs were filled using the clean reads that were mapped to the contigs using Geneious v8.1³⁰. The assembled chloroplast genomes were depicted using OGDRAW v1.3.1³¹. The information of annotated chloroplast genomes was uploaded to the NCBI database (Genbank accession number ON209180-191).

Repeat Sequences Analysis

The chloroplast simple sequence repeats were identified using GMATA³² software. The minimum numbers of repeats for mononucleotide, dinucleotides, trinucleotides, tetranucleotides, pentanucleotide, and hexanucleotides were 10, 5, 4, 3, 3, and 3, respectively. The HBLH genotype represents the species of *P. nudicaule* to count the number of repeats and SSRs.

Intraspecific Variations of *Papaver nudicaule*

Single nucleotide polymorphisms (SNP) and indels were identified to reveal intraspecific variations in the chloroplast genome sequences of *P. nudicaule*. The sequences of *P. nudicaule* chloroplast genomes were aligned using MAFFT v7³³, then alignments were inspected and adjusted manually using Se-Al 2.0³⁴. MEGA X³⁵ software was used to calculate SNPs and DnaSP 6³⁶ software was used to identify indels. The distribution, positions and orientation of SNPs and indels were determined with the chloroplast genome of the HBLC genotype serving as the reference sequence..

Phylogenetic Analysis

To reconstruct and confirm the inferred phylogenetic relationships of *P. nudicaule* genotypes, the chloroplast genomes were analyzed. These complete chloroplast genome sequences were aligned using MAFFT v7³³ with default parameters. The trimAl was used to detect the unreliable alignment regions with parameter automated³⁷. The concatenated data were analyzed using the maximum likelihood (ML) methodologies. The best-fit model for ML analysis was selected using the ModelFinder v.1.6³⁸ under the Bayesian information criterion. The ML tree was performed using RAXML-NG³⁹ with 500 rapid bootstrap replicates. The Markov chain Monte Carlo (MCMC) algorithm was run for $2 \times 10,000,000$ generations, with trees sampled every 1000 generations. Stationarity was deemed achieved once the average standard deviation of split frequencies dropped below 0.001 following a 25% burn-in.

Results

Chloroplast Genomes Organization

The chloroplast genomes of *P. nudicaule* ranged in length from 153,683 bp (SXPL) to 153,905 bp (NMAL) and displayed a typical circular quadripartite structure, consisting of a pair of inverted repeat (IR) regions (25,692 – 25,730 bp) separated by a large single copy (LSC) region (83,472 – 83,618 bp) and a small single copy (SSC) region (18,810 – 18,869 bp) (Fig. 2 and Table 1). The 12 complete chloroplast genomes generated were deposited in GenBank with accession numbers ON209180 to ON209191 (Table 1). The overall GC content of the chloroplast genome was 38.9% (Table 1).

We annotated 113 unique functional genes in the *P. nudicaule* chloroplast genome, which consisted of 79 protein-coding genes, 30 transfer RNA genes, and 4 ribosomal RNA genes (Table 2). A total of 61 protein-coding and 22 tRNA genes were located in the LSC region, 12 protein-coding and 1 tRNA gene in the SSC region, and 8 protein-coding, 7 tRNA, and all 4 rRNA genes in the IR region. The genes could be divided into four categories. We identified 44 genes associated with photosynthesis genes. 25 genes were associated with self-replication functions, including those encoding large and small ribosomal protein subunits as well as DNA-dependent RNA polymerases. 34 genes were RNA genes including 4 rRNA and 30 tRNA. The remaining genes were classified as other genes and unknown genes.

A total of 18 genes contained introns. Sixteen genes (*atpF*, *ndhA*, *ndhB*, *petB*, *petD*, *rpl2*, *rpl16*, *rpoC1*, *rps12*, *rps16*, *trnA-UGC*, *trnG-UCC*, *trnI-GAU*, *trnK-UUU*, *trnL-CAA*, *trnV-UAC*) each harbored a single intron, whereas *clpP* and *ycf3* possessed two introns apiece. Four intron-containing genes (*ndhB*, *rpl2*, *trnA-UGC*, *trnI-GAU*) were located within the two inverted repeat (IR) regions; twelve genes (*atpF*, *clpP*, *petB*, *petD*, *rpl16*, *rpoC1*, *rps16*, *trnG-UCC*, *trnL-UAA*, *trnK-UUU*, *trnV-UAC*, *ycf3*) resided in the large single-copy (LSC) region, and only *ndhA* was distributed in the small single-copy (SSC) region. Notably, *rps12* is a trans-spliced gene: its first exon lies in the LSC, while the remaining two exons are positioned in the IR regions. (Fig. 2).

We performed a comparative analysis of the IR boundary regions between the two *Papaver* species (Fig. 3), and the results showed that the borders of the *Papaver* chloroplast genome were very similar. In *Papaver* species, the boundary was located within the *rps19* gene at the LSC/IRb junction. The IRa/SSC border extended into *ycf1*.

Table 1
Sampling information and chloroplast genome sequences information of *P. nudicaule*.

ID	Location	LSC	IR	SSC	Total length (bp)	%GC	GenBank accession number
HBLH	Longhua, Hebei,China	83565	25712	18817	153806	38.9%	ON209191
QHDT	Datong, Qinghai, Chian	83618	25693	18836	153840	38.9%	ON209190
HBZL	Zhulu, Hebei, China	83553	25711	18813	153788	38.9%	ON209189
NMTM	Tumotezuoqi, Inner Mongolia, China	83546	25712	18842	153812	38.9%	ON209188
NMXB	Xinbaerhuzuoqi, Neimnenggu, China	83561	25714	18838	153827	38.9%	ON209187
NMYK	Yskesu, Inner Mongolia, China	83558	25730	18843	153861	38.9%	ON209186
NMXW	Xiwuzhumuqinqi, Inner Mongolia, China	83548	25711	18843	153813	38.9%	ON209185
NMXL	Xilinguolemengzhenglanqi, Inner Mongolia, China	83551	25712	18844	153819	38.9%	ON209184
NMAL	Alukeerqinqi,Inner Mongolia, China	83576	25730	18869	153905	38.9%	ON209183
SXPL	Pingli, Shanxi, China	83472	25692	18827	153683	38.9%	ON209182
NMCB	Chenbaerhuqi, Inner Mongolia, China	83530	25693	18810	153726	38.9%	ON209181
JLTM	Tumen, Jinlin, China	83557	25712	18841	153822	38.9%	ON209180

Table 2
List of genes present in the *P. nudicaule* chloroplast genome.

Category for genes	Group of gene	Name of gene
Photosynthesis related genes	Rubisco	<i>rbcL</i>
	Photosystem II	<i>psaA, psaB, psaC, psal, psaJ</i>
	Photosystem II	<i>psbA, psbB, psbC, psbD, psbE, psbF, psbH, psbl, psbJ, psbK, psbL, psbM, psbN, psbT, psbZ</i>
	ATP synthase	<i>atpA, atpB, atpE, atpF, atpH, atpI</i>
	Cytochrome b/f complex	<i>petA, petB, petD, petG, petL, petN</i>
	NADPH dehydrogenase	<i>ndhA, ndhB, ndhC, ndhD, ndhE, ndhF, ndhG, ndhH, ndhI, ndhJ, ndhK</i>
Self-replication	DNA dependent RNA polymerase	<i>rpoA, rpoB, rpoC1, rpoC2</i>
	Small subunit of ribosomal proteins	<i>rps2, rps3, rps4, rps7, rps8, rps11, rps12, rps14, rps15, rps16, rps18, rps19</i>
	Large subunit of ribosomal proteins	<i>rpl2, rpl14, rpl16, rpl20, rpl22, rpl23, rpl32, rpl33, rpl36</i>
RNA genes	ribosomal RNA	<i>rrn4.5, rrn5, rrn16, rrn23</i>
	transfer RNA	<i>trnA-UGC, trnC-GCA, trnD-GUC, trnE-UUC, trnF-GAA, trnM-CAU, trnG-GCC, trnG-UCC, trnH-GUG, trnI-CAU, trnI-GAU, trnK-UUU, trnL-CAA, trnL-UAA, trnL-UAG, trnM-CAU, trnN-GUU, trnP-UGG, trnQ-UUG, trnR-ACG, trnR-UCU, trnS-GCU, trnS-GGA, trnS-UGA, trnT-GGU, trnT-UGU, trnV-GAC, trnV-UAC, trnW-CCA, trnY-GUA</i>
Other genes	Maturase	<i>matK</i>
	Envelope membrane protein	<i>cemA</i>
	Protease	<i>clpP</i>
	Subunit of acetyl-coA-carboxylase	<i>accD</i>
	Translation initiation factor	<i>infA</i>
	C-type cytochrome synthesis gene	<i>ccsA</i>
Genes of unknown function	Conserved open reading frames	<i>ycf1, ycf2, ycf3, ycf4</i>

Repeat Sequences Analyses

A total of 26 SSRs were identified in the *P. nudicaule* chloroplast genome using MISA software (Table S1 and Fig. 4). These SSRs included 16 mononucleotide, 3 dinucleotide, 2 trinucleotide, 4 tetranucleotide, and 1 pentanucleotide SSRs. Mononucleotide repeats were the most abundant repeats, accounting for 61.5% of the total repeats, while tetranucleotide repeats accounted for 15.4%, and other SSRs (dinucleotide, trinucleotide, and pentanucleotide SSRs) accounted for 23.1%. Additionally, there were no hexanucleotide repeats in the *P. nudicaule* plastomes (Fig. 4A). Most of these SSRs were located in the LSC region (57.7%), followed by the SSC region (26.9%), and the IR region (15.4%) (Fig. 4B). Non-coding regions (spacers) contained the majority of SSRs (61.5%), protein-coding (exon) contained the remaining SSRs (38.5%) (Fig. 4C). The SSRs had a significant bias toward A/T bases, with all the mononucleotide repeats being A/T, and dinucleotide, trinucleotide, tetranucleotide, and pentanucleotide SSRs were especially rich in A/T (Fig. 4D).

Inversion Analysis

Six small inversions were detected in the *P. nudicaule* chloroplast genome, with lengths ranging from 2 to 21 bp (Table 3). All of the small inversions were associated with a pair of inverted repeats directly flanking the inversion, forming a stem-loop structure. Stem length ranged from 7 to 34 bp. No correlation was found between the length of the inversions and the inverted repeats. Four inversions occurred in the LSC region and two in the SSC region. All inversions were located in the intergenic spacers.

Table 3
The small inversion in the *P. nudicaule* chloroplast genome.

Region	Location	Length	
		loop	stem
LSC	<i>trnH-psbA</i>	8	24
LSC	<i>petA-psbJ</i>	11	34
LSC	<i>ycf1-ndhF</i>	3	7
LSC	<i>ycf1-ndhF</i>	21	12
SSC	<i>rpl32-trnL</i>	2	20
SSC	<i>ccsA-ndhD</i>	9	23

Numbers and Mutation Patterns of Single Nucleotide Polymorphisms

Alignments of the chloroplast genomes of the 12 *P. nudicaule* samples revealed a total length of 154,130 bp across the genomes, along with 236 polymorphic sites, 174 singleton variable sites, and 62 parsimony informative sites (Table 4 and Table S2). The SNPs included 128 transition (Ts) and 108 transversion (Tv) sites. The most frequent SNP mutation types were C to T and G to A; G to C or C to G mutations were less frequent (Fig. 5). There were 70 SNPs in LSC regions, 1 in IR regions, and 36 in SSC regions (Table S2). Sixty-one sequence regions, including 38 spacer regions, 17 coding regions, and 6 intron regions, harbored SNPs. The *ycf1* gene had the highest number of SNPs (13), followed by the *rpoC2* (5) and *ndhF-rpl32* (5).

The IR region exhibited the lowest divergence according to nucleotide diversity and number of haplotypes; overall nucleotide diversity was 0.00035. Based on complete chloroplast genome sequences, each sample had a unique haplotype, and the IR region contained only eight haplotypes.

Table 4
Sampling information and chloroplast genome sequences information of *P. nudicaule*.

Location	length	Polymorphic sites	Singleton variable sites	Parsimony informative sites	Nucleotide diversity	Number of Haplotypes	Haplotypes diversity
LSC	83740	157	122	35	0.0004	12	1
IR	25734	9	6	3	0.00009	8	0.924
SSC	18922	61	40	21	0.00081	12	1
complete chloroplast genome	154130	236	174	62	0.00035	12	1

Numbers and Length of Indels

We identified 75 indels in the 12 *P. nudicaule* chloroplast genomes (Fig. 6 and Table S3). The majority of indels were located in non-coding regions. A total of 42 sequence regions harbored indels, *ndhF-rpl32* had the most indels (7), followed by *trnT-psbD*, *trnF-ndhJ*, *ycf1* and *ndhC-trnV* (4). The length of indels ranged from 1 to 141 bp, and indels of 1, 2, 3 and 5 bp were the most common. The largest indel (141 bp) and the second largest indel (80bp) were found in the *trnF-ndhJ* spacer. Most of these indels were located in the LSC region (62.7%), followed by the SSC region (24.0%), and the IR region (13.3%).

Phylogenetic Relationships

Phylogenetic relationships among *P. nudicaule* genotypes were inferred using the chloroplast genomes; the phylogenetic tree is presented in Fig. 7. The ML tree supports grouping of the 12 genotypes into two branches with 100% bootstrap support. The NMXL and NMXW samples from Cluster A formed a clade that was the first diverged group. The genotypes NMXB and JLTM, HBZL and HBLH, NMXL and NMXW, and NMTM and NMYK, formed sister groups. The cluster A included nine genotypes, including six genotypes from Inner Mongolia autonomous region (NMXB, NMAL, NMTM, NMYK, NMXW and NMXL). The NMCB genotype was the earliest diverging lineage in the cluster B. The genotypes QHDT and SXPL formed sister groups.

Discussion

This study provides the first assessment of intraspecific diversity of the phylogeography of *P. nudicaule* in China using the complete annotated chloroplast genomes. In the current study, we sequenced complete chloroplast genome sequences for 12 samples of *P. nudicaule* from 12 different geographical distributions using second generation high throughput sequencing technology. We obtained complete chloroplast genomes through assembly and annotation. The results showed the chloroplast genomes were a typical quadripartite structure, including a single LSC region, a single SSC region and a pair of IR regions, which had been reported in many plant species^{40–43}. The size of the genome ranged from 153,683 (SXPL) to 153,905bp (NMAL), which was longer than the genome of *P. setigerum* (152,862bp)⁴⁴, *P. rhoeas* (152,905bp) and *P. orientale* (152,799bp)⁴⁵. This length was

within the size range of the chloroplast genomes of most angiosperms⁴⁶. Angiosperm chloroplast genomes usually contain 110–130 genes, with up to 80 protein-coding genes, four rRNA genes, and approximately 30 tRNA genes⁴⁷. In the current study, the *P. nudicaule* chloroplast genomes had 113 unique genes consisting of 79 protein-coding genes, 30 transfer RNA genes, and 4 ribosomal RNA genes. The chloroplast genome sequences of *P. nudicaule* were highly conserved in terms of size, gene count, GC content and gene structure; however, the full chloroplast genome sequencing of *P. nudicaule* unveiled unexplored genetic variation, including SNPs, SSRs, and indels, which are important for population genetics studies.

In fact, a significant amount of variation was detected within the *P. nudicaule* chloroplast genome. 236 SNPs were detected and a mutation bias in the SNP patterns was observed. Compared to other published species, *P. nudicaule* had a medium level of intraspecific variation, such as in *Forsythia suspensa*⁴⁸, *Prunus mume*⁴⁹, and soybean⁵⁰. CT to GA occurred more frequently; AT to TA and GC to CG occurred significantly less frequently (Fig. 5). In general, noncoding regions tend to contain more SNPs due to less natural selection. The most variable locus was *ycf1*, a gene of unknown function. It showed higher divergence at the species level. The region located in the IR region is conservative and two regions located in the SSC region are extremely variable, and thus they are suitable for phylogenetic studies or DNA barcoding at low taxonomic levels. Furthermore, two mutation hotspots (*ycf1a* and *ycf1b*) in the *ycf1* gene were identified by interspecific analysis^{51, 52}. More informative nucleotide variations were found in the *ndhF-rpl32* and *rpoC2* in this study.

SSRs, known as microsatellites, which consist of tandemly repeated motifs of 6 bp or less, are significant repetitive elements of the entire genome. They are repetitive units with different numbers of repeats and are located in homologous regions and play important roles in many applications, including species identification, population genetics, and phylogenetic studies^{53–56}. Chloroplast genome SSRs (cpSSRs) are usually distributed in intergenic regions⁵⁷. In the SSR analysis, twenty-six polymorphic SSRs were found across the *P. nudicaule* chloroplast genome, which were used to explore genetic divergence and gene flow among distinct populations and phylogeographical studies. The LSC region contained the majority of these SSR loci, followed by the SSC and IR regions. Mononucleotide repeats were the most prevalent and added to the A/T richness. These results are consistent with most reported angiosperms^{58–61}. Analysis of the SSRs identified in the chloroplast genome of *P. nudicaule* indicated that the chloroplast genome is highly conserved. The analysis revealed that chloroplast genes exhibited high sequence conservation, while intergenic regions displayed greater variability.

Indels are frequent mutations found in the genome of chloroplasts⁶². The significance of indels as a source of genetic variety has been demonstrated by earlier research, and it also shows that indels are derived from and accelerate nucleotide substitution mutations^{63–65}. The phylogenetic homoplasy of correctly coded indels and repeats is exceedingly low, and the identical indels and repeats do not occur accidentally across different species. In the *P. nudicaule* chloroplast genome, we identified 75 indel events, which is less than SNPs. The region within the non-coding region (*ndhF-rpl32*) containing the highest number of indels was variable region in the chloroplast genome, as previously indicated⁶⁶. Resolution is significantly increased by adding indel information compared with basic substitution-based matrices of chloroplast DNA sequences⁶².

During the evolution of the chloroplast genome, inversions frequently occur as a result of repeated structures caused by back or parallel mutations^{67, 68}. Inversions must be identified during alignment, but the gaps caused by inversions should be treated with caution, particularly above species levels. In brief, the structural mutations

frequently comprise both phylogenetic signals and noises. Only phylogenetically meaningful structural mutation information is taken into consideration when nucleotide data alone cannot clarify evolutionary relationships^{69, 70}.

The recent development of next-generation sequencing has provided large amounts of data, improving phylogenetic resolution. Complete chloroplast genomes have been used to estimate population differentiation, genetic diversity, gene flow, and biogeographical structure. Maternally transmitted chloroplast DNA shows restricted seed dispersal as a result of short-distance dispersal and inefficient seed migration pathways, which causes population differentiation. Analysis of the chloroplast genome sequences of *P. nudicaule* revealed significant genetic divergences. The phylogenetic position of 12 *P. nudicaule* genotypes was inferred by analyzing the complete chloroplast genome. The 12 genotypes were separated into two clades generally consistent with the geographical distribution (Fig. 7). The genotypes of NMXL and NMXW were found to be the earliest diverging lineage of the cluster A. The cluster A included nine genotypes and separated into five branches. Two genotypes from Hebei province (HBLH and HBZL) had closer relationships, confidently assigned to one clade. The genotypes of NMYK and NMTM could be confidently assigned to one clade. The four genotypes (NMXL, NMXW, NMYK and NMTM) derive from Inner Mongolia autonomous region. The results are consistent with the geographical distribution. The NMCB genotype was the earliest diverging lineage in the cluster B. The genotypes QHDT and SXPL formed a sister group. Our study provides valuable information on these 12 genotypes of *P. nudicaule* and will facilitate future phylogenetic, conservation biology studies as well as other studies of *P. nudicaule*.

Declarations

Conflicts of Interest

The authors declare no conflicts of interest.

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Author Contribution

Conceptualization, Y.S. and M.L.; methodology, writing-original draft, J.X.; Formal analysis, H.C and K.C.; Validation, M.Z. and Y.P.; investigation, X.J.; resources, C.L. and Y.N.; writing—review and editing, J.X., Y.S. and M.L. All authors have read and agreed to the published version of the manuscript.

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Data Availability

Annotated genome sequences are deposited in GenBank (<https://www.ncbi.nlm.nih.gov>) under Accession Numbers ON209180-91.

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Figures

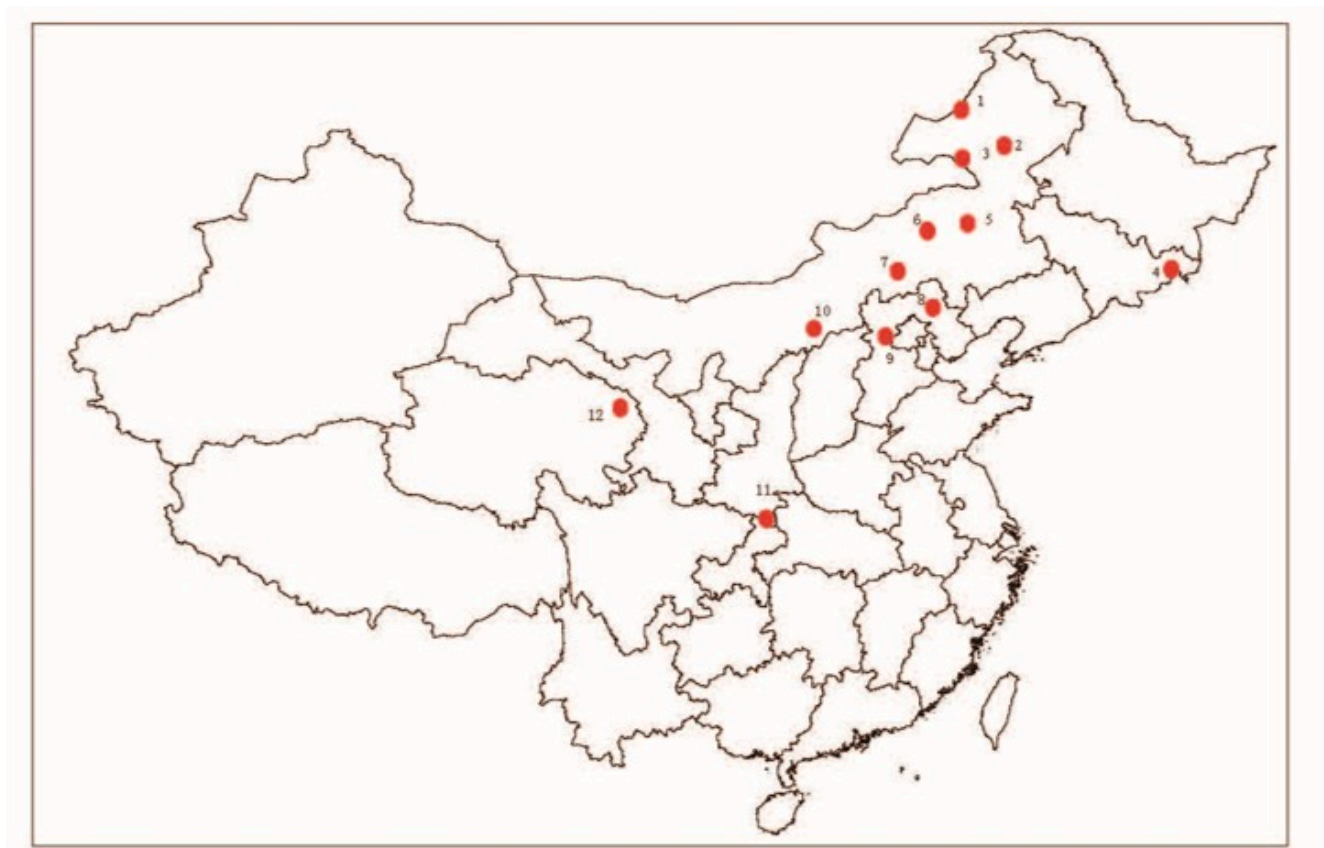


Figure 1

Sampling locations of the *P. nudicaule* individuals investigated. 1.NMCB, 2.NMYK, 3.NMxB, 4.JLTM, 5.NMAL, 6.NMXW, 7.NMXL, 8.HBLH, 9.HBZL, 10.NMTM, 11.SXPL, 12.QHDT

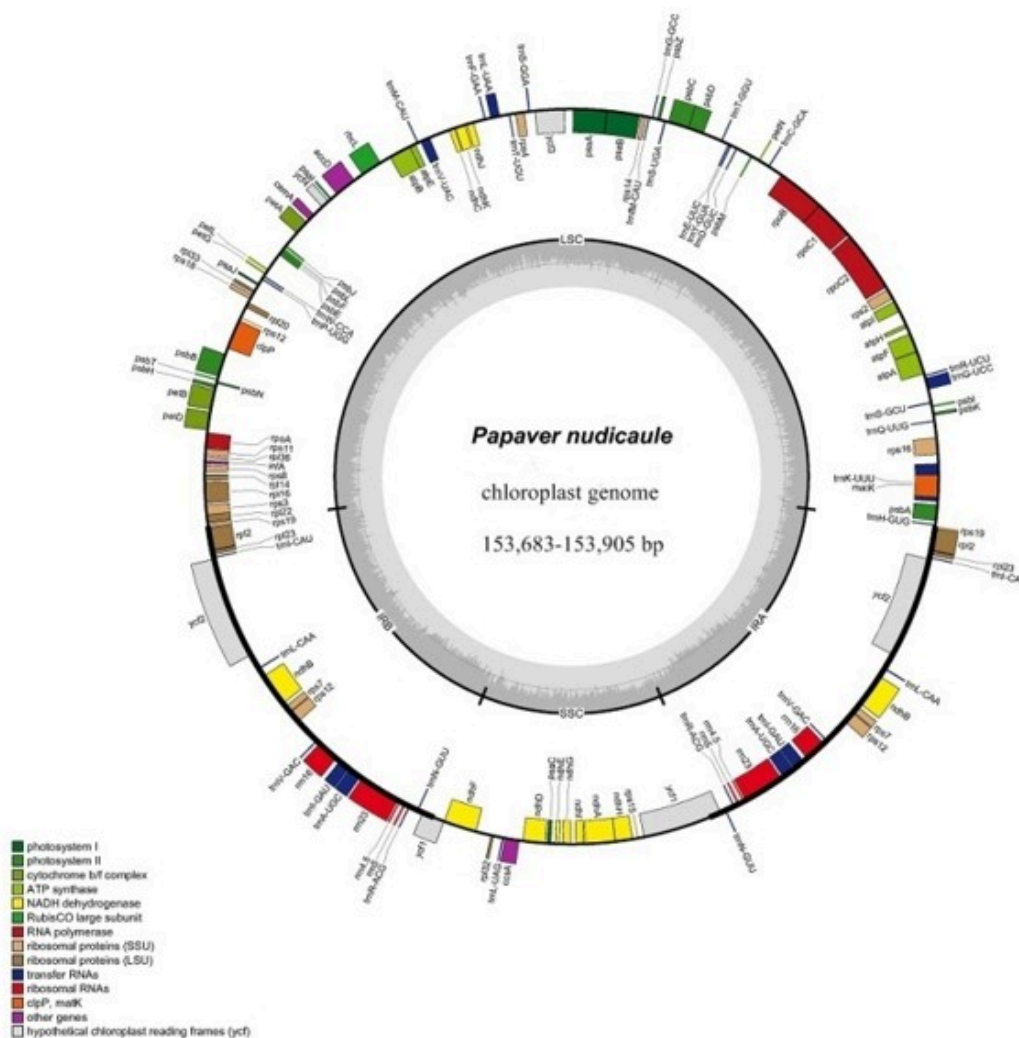


Figure 2

Gene map of the chloroplast genome of *P. nudicaule*. Genes outside the ring are transcribed in a counterclockwise direction, whereas those inside the ring are transcribed in a clockwise direction. The GC content of the chloroplast genome is shown in the dark gray region, and the AT content is shown in the light gray region.

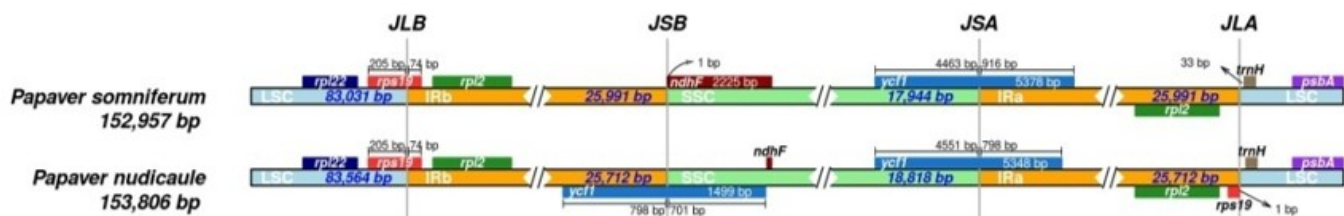


Figure 3

Comparison of the border positions of the LSC, SSC, and IR regions among the chloroplast genomes of two species of *Papaver*.

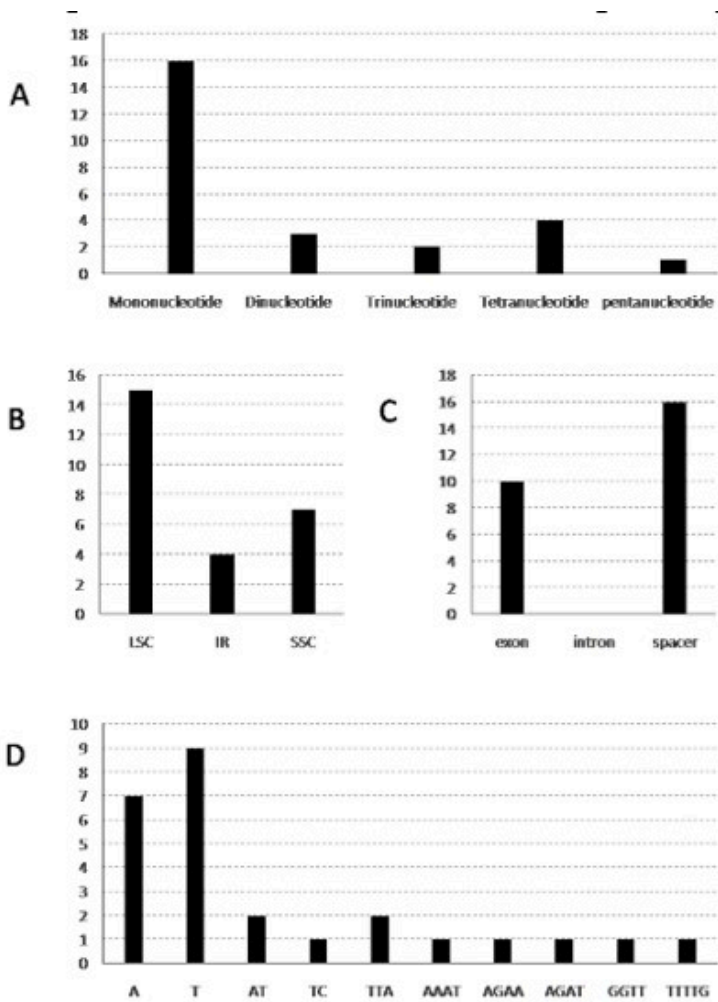


Figure 4

Type and distribution of simple sequence repeats (SSRs) in *P. nudicaule*. (A) Number of different SSR type detected by MISA. (B) Number of SSRs in the LSC, SSC, and IR regions. (C) Number of SSRs in spaces, exons, and introns. (D) Frequencies of identified SSR motifs in the different repeat classes.

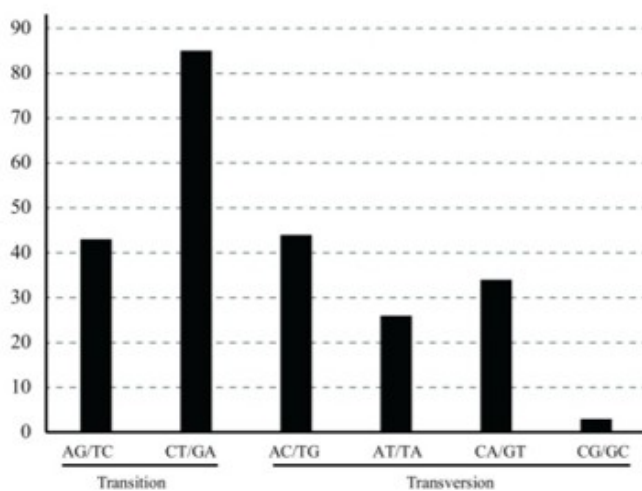


Figure 5

Patterns of SNPs among the *P. nudicaule* samples. Nucleotide substitutions were divided into six types as indicated by the six non-strand-specific base-substitution types (i.e., numbers of G to A and C to T sites for each respective set of associated mutation types).

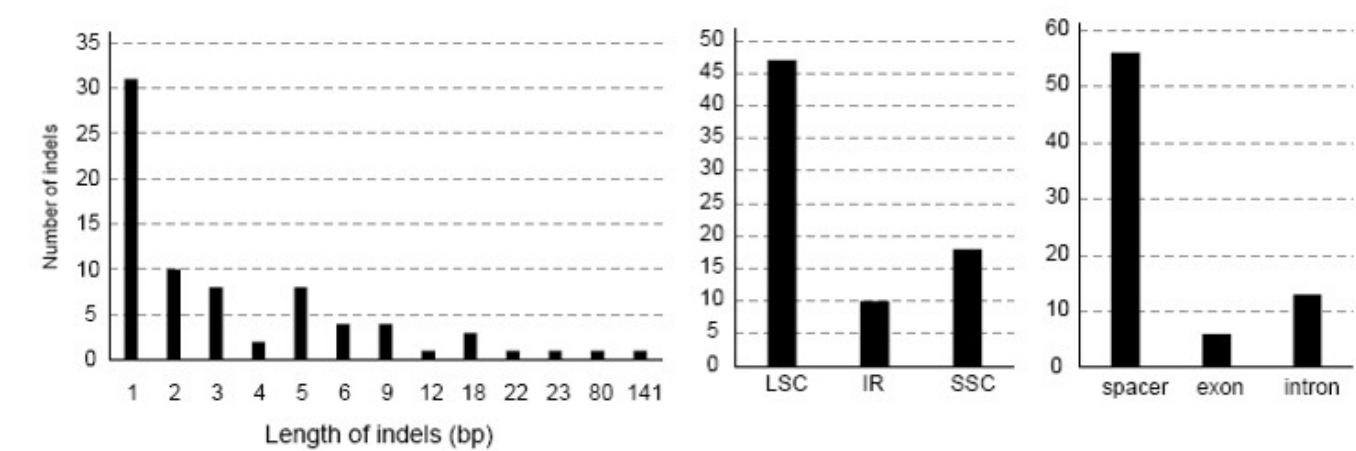


Figure 6

Numbers and patterns of indels among the *P. nudicaule* chloroplast genomes. (A) Numbers and sizes of indels. (B) Numbers of indels in the LSC, SSC, and IR regions. (C) Numbers of SSRs in spacers, exons, and introns.

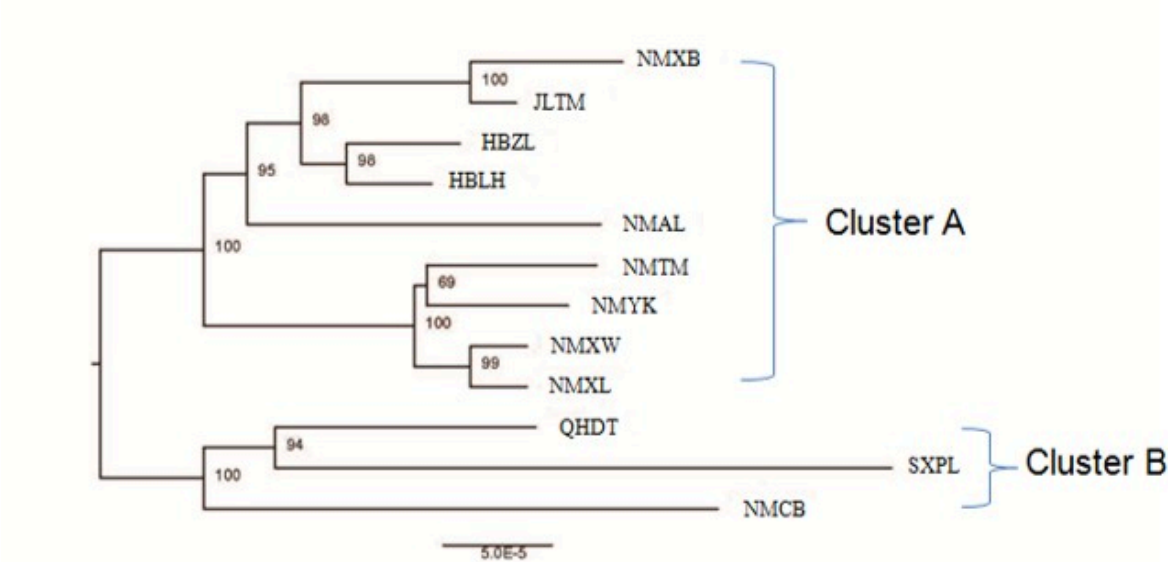


Figure 7

Phylogenetic relationships among *P. nudicaule* samples. ML topology is shown with the ML bootstrap support value at each node. ML values < 50 were not present.

Supplementary Files

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