Complete chloroplast genome sequences of *Lilium*: insights into evolutionary dynamics and phylogenetic analyses

Yun-peng Du\(^1\), Yu Bi\(^1,2\), Feng-ping Yang\(^1\), Ming-fang Zhang\(^1\), Xu-qing Chen\(^1\), Jing Xue\(^1\) & Xiu-hai Zhang\(^1\)

*Lilium* is a large genus that includes approximately 110 species distributed throughout cold and temperate regions of the Northern Hemisphere. The species-level phylogeny of *Lilium* remains unclear; previous studies have found universal markers but insufficient phylogenetic signals. In this study, we present the use of complete chloroplast genomes to explore the phylogeny of this genus. We sequenced nine *Lilium* chloroplast genomes and retrieved seven published chloroplast genomes for comparative and phylogenetic analyses. The genomes ranged from 151,655 bp to 153,235 bp in length and had a typical quadripartite structure with a conserved genome arrangement and moderate divergence. A comparison of sixteen *Lilium* chloroplast genomes revealed ten mutation hotspots. Single nucleotide polymorphisms (SNPs) for any two *Lilium* chloroplast genomes ranged from 8 to 1,178 and provided robust data for phylogeny. Except for some of the shortest internodes, phylogenetic relationships of the *Lilium* species inferred from the chloroplast genome obtained high support, indicating that chloroplast genome data will be useful to help resolve the deeper branches of phylogeny.

The genus *Lilium*, in the family Liliaceae, is economically and phylogenetically important and includes approximately 110 species distributed throughout the cold and temperate regions of the Northern Hemisphere, especially East Asia and North America\(^4-5\), with eastern Asia and the Himalayas established as the center of diversity for this species. De Jong\(^4\) and Patterson & Givnish\(^4\) have described southwestern China and the Himalayas as the point of origin for the genus *Lilium*. Many *Lilium* species, ornamental cultivars and hybrids (such as Oriental hybrid, LA-hybrid, OT-hybrid, Asiatic hybrid, LO-hybrid, Longiflorum, and Aurelian & Trumpet), are cultivated for their esthetic value. In addition, both the flowers and bulbs are regularly consumed as both food and medicine in many parts of the world, particularly in Asia\(^5\). Presently, the “medicine food homology” values of *Lilium* plants have received considerable attention with respect to their great commercial prospects.

Nevertheless, many natural distribution areas of the wild lily are being adversely affected by both natural and human forces\(^6-7\), and a growing number of *Lilium* species are on the verge of extinction (the IUCN Red List of Threatened Species (http://www.iucnredlist.org)). Thus, programs to protect and preserve lily resources (especially rare lily species) are urgently needed. Species endemic to China—*L. paradoxum* Stearn, *L. medogense* S. Y. Liang, *L. pinifolium* L. J. Peng, *L. saccatum* S. Y. Liang, *L. huidongense* J. M. Xu, *L. matangense* J. M. Xu, *L. stewartianum* I. B. Balfour et W. W. Smith, *L. habaense* F. T. Wang et Tang, *L. jinfushanense* L. J. Peng et B. N. Wang, *L. xanthellum* F. T. Wang et Tang and *L. fargesii* Franch.—have been put on the China Species Red List\(^8\).

*Lilium*, which is taxonomically and phylogenetically regarded as an important clade of the core Liliales, appears to have evolved in the Himalayas approximately 12 million years ago, despite the lack of fossil records\(^6,9\). Currently, the major phylogenetic clades of *Lilium* have been basically clear, and the updated system classifies the genus into seven sections primarily based on morphological taxonomy and molecular phylogenetic methods\(^10-15\). In the past two decades, the nuclear rDNA internal transcribed spacer (ITS)\(^10,12,16-17\) and several plastid...
and add seven species from GenBank 34–40. We then test the feasibility of phylogeny reconstruction using the chloroplast genome as a universal barcode and the existence of variable characters among species through NGS sequencing and assembly.

### Results

#### Genome sequencing and assembly.

Using the Illumina HiSeq 4000 system, nine *Lilium* taxa were sequenced to produce 3,719,304–11,167,835 paired-end raw reads (150 bp in average read length). *Lilium* cp genomes were de novo assembled using SPAdes 3.6.1. After these paired-end reads were screened through alignment with the chloroplast genome using Geneious V9, 47,505 to 988,478 cp genome reads were extracted with 46 X to 971 X coverage (Table 1). The four junction regions in each genome were validated by PCR-based sequencing.

#### Complete chloroplast genomes of *Lilium* species.

The nucleotide sequences of the 16 *Lilium* cp genomes range from 151,655 bp (*L. bakerianum*) to 153,235 bp (*L. fargesii*; Fig. 1, Table 2). The Chloroplast genomes assembled in single circular, double-stranded DNA sequences, displaying a typical quadripartite structure, consisting of a pair of IRs (26,394–26,990 bp) separated by the LSC (81,224–82,480 bp) and SSC (17,038–17,620 bp) regions. The overall GC content is 36.9–37.1%, indicating nearly identical levels among the 16 complete *Lilium* cp genomes. The *Lilium* cp genome contains 113 genes, including 79 protein coding genes, 30 tRNA genes, and 4 rRNA genes (Fig. 1, Table S1). All four rRNA genes are duplicated in the IR region. Fifteen distinct genes contain one intron, two of which contain two introns (*clpP* and *ycf3*). The *rps12* gene is a trans-spliced gene with the 5′ end located in the LSC region and the duplicated 3′ end in the IR region, as has been reported previously in other plants.

#### Simple Sequence Repeats (SSR) analysis of the *Lilium* cp genome.

We used MISA to detect the SSR sites of all 16 chloroplast genomes. The number of SSRs in chloroplast genomes differed among the sixteen *Lilium* species, as shown in Table 2. The number of SSRs varied from 53 to 78. The most abundant were mononucleotide repeats, which accounted for approximately 56.38% of the total SSRs, followed by dinucleotides and tetranucleotides (Table S2). Hexanucleotides are very rare across the cp genomes.

### Table 1. Summary of the sequencing data for nine *Lilium* species.

| Species         | Raw data no. | Mapped read no. | Mapped to reference genome (%) | cp genome coverage (X) |
|-----------------|--------------|-----------------|--------------------------------|------------------------|
| *L. fargesii*   | 3719304      | 47505           | 0.64%                          | 46.50                  |
| *L. brownii*    | 801417       | 988478          | 6.17%                          | 971.15                 |
| *L. lancifolium*| 5702538      | 174796          | 1.53%                          | 171.85                 |
| *L. nepalense*  | 6346201      | 248794          | 1.96%                          | 245.03                 |
| *L. leucanthum* | 5962131      | 131349          | 1.10%                          | 128.83                 |
| *L. davidii*    | 7409181      | 369289          | 2.49%                          | 362.86                 |
| *L. duchartrei* | 11167835     | 185719          | 0.83%                          | 182.93                 |
| *L. bakerianum* | 6897215      | 74085           | 0.54%                          | 73.28                  |
| *L. henryi*     | 9331411      | 322449          | 1.73%                          | 315.88                 |

Summary of the sequencing data for nine *Lilium* species.
Figure 1. Gene map of the 16 Lilium chloroplast genome. The genes inside and outside of the circle are transcribed in the clockwise and counterclockwise directions, respectively. Genes belonging to different functional groups are shown in different colors. The thick lines indicate the extent of the inverted repeats (IRa and IRb) that separate the genomes into small single copy (SSC) and large single copy (LSC) regions.

Table 2. Summary of complete chloroplast genomes of Lilium species.
marker and influence the ingroup topology in our research (Figs 4 and 5, Fig. S1). Smilax china species or added sect. Leucolirion Fritillaria had moderate to high support, except for the IR dataset, which received poor support. In addition, constructed based on five datasets (Figs 4 and 5, Fig. S1). The topologies based on the three methods of analysis and four outgroups as well as Lilium species were used to perform phylogenetic analysis. Using MP, ML and MrBayes analyses, phylogenetic trees were

Table 3. Variable site analyses in Lilium chloroplast genomes.

| Region  | Number of sites | Number of variable sites | Number of parsimony-informative sites | Nucleotide Diversity |
|---------|-----------------|--------------------------|---------------------------------------|----------------------|
| LSC     | 84,935          | 2281                     | 1066                                  | 0.00635              |
| SSC     | 17,935          | 665                      | 311                                   | 0.00839              |
| IR      | 26,663          | 113                      | 42                                    | 0.00093              |
| Complete cp genome | 156,551 | 3182 | 1449 | 0.00463 |

Figure 2. Frequency of simple sequence repeats (SSRs) in the sixteen Lilium chloroplast genomes.

In Lilium, all mononucleotides (100%) are composed of A/T, and a similar majority of dinucleotides (70.31%) are composed of A/T (Fig. 2). Our findings are comparable to previously reported findings that chloroplast genome SSRs are composed of polyadenine (polyA) or polythymine (polyT) repeats and rarely contained tandem guanine (G) or cytosine (C) repeats. Most of those SSRs are located in the LSC and SSC regions. In general, the SSRs of the sixteen Lilium species represent abundant variation and can be used in combination with nuclear SSRs developed in the genus for conservation or reintroduction, species biodiversity assessments and phylogenetic studies of Lilium in native or introduced areas.

Genome sequence divergence among Lilium species. We compared nucleotide diversity in the total, LSC, SSC, and IR regions of the cp genomes. The alignment revealed high sequence similarity across the Lilium cp genomes, suggesting that they are highly conserved. In total, 3,182 variable sites (2.03%), including 1,449 parsimony-informative sites in the total cp genomes were found (0.93%; Table 3). Among these regions, IR regions exhibit the least nucleotide diversity (0.00093) and SSC higher divergence (0.00839).

The p-distance and number of nucleotide substitutions were used to estimate divergence among the sixteen Lilium species. The p-distance among Lilium species ranges from 0.0001 to 0.0074, and the number of nucleotide substitutions was found to be 8 to 1,178 (Table S3). L. hansonii and L. tsingtauense show the greatest sequence divergence. L. sp. (from GenBank) exhibits only 8 nucleotide substitutions (L. tsingtauense), with the second lowest divergence being 63 nucleotide substitutions (L. hansonii).

Divergence of hotspot regions. Genome-wide comparative analyses among the sixteen Lilium species expected non-coding and SC regions to exhibit higher divergence levels than those of coding and IR regions, respectively (Fig. 3). Furthermore, to calculate the sequence divergence value within 800 bp was calculated (Fig. 3). In the Lilium cp genome, these values vary from 0 to 0.02247. We identified 10 hotspot regions for genome divergence that could be utilized as potential markers to reconstruct the phylogeny and plant identification in this genus: trnS-trnG, trnE-trnT-psbD, trnF-ndhJ, psbE-petL, trnP-psaJ-rpl33, psbB-psbH, petD-rpoA, ndhF-rpl32-trnL, ycf1a, and ycf1b. Seven of these (trnS-trnG, trnE-trnT-psbD, trnF-ndhJ, psbE-petL, trnP-psaJ-rpl33, psbB-psbH, and petD-rpoA) are located in the LSC, and three (ndhF-rpl32-trnL, ycf1a, and ycf1b) in the SSC region. Only two markers (ycf1a and ycf1b) are in coding regions. Among these, the coding marker ycf1b shows the highest variability (Fig. 3, Table 4).

Phylogenetic analysis. In the present study, five datasets (whole complete cp genome sequences, LSC, SSC, IR and ten combined variable regions) from cp genomes of sixteen Lilium and four outgroups as well as Smilax china were used to perform phylogenetic analysis. Using MP, ML and MrBayes analyses, phylogenetic trees were constructed based on five datasets (Figs 4 and 5, Fig. S1). The topologies based on the three methods of analysis were highly concordant in each dataset, as well as with the results of Rønsted et al.41 and the phylogenetic trees had moderate to high support, except for the IR dataset, which received poor support. In addition, Fritillaria species or added Smilax china were used as the outgroup. The results showed that different outgroups could not influence the ingroup topology in our research (Figs 4 and 5, Fig. S1).

The sixteen Lilium species were grouped into two branches (Fig. 4). All the datasets indicated that two sect. Leucomirion 6b species, L. brownii and L. longiflorum, form a monophyletic group and then cluster with three sect. Martagon species, L. sp. (from GenBank), L. tsingtauense and L. hansonii as well as species of L. cernuum, L.
Lancifolium and L. davidii var. willmottiae, which belong to sect. Sinomartagon 6a. In the other branch, L. superbum belongs to sect. Pseudolirium is distributed in North America; the other seven Lilium species are native to Hengduan Mountains and the Himalayas and form another monophyletic clade.

**Discussion**

**Chloroplast genome evolution in Lilium.** In this study, nine new chloroplast genome sequences of *Lilium* were sequenced using the Illumina HiSeq platform. The complete cp genomes range from 151,655 to 153,235 bp, which is within the range of cp genomes from other angiosperms 42. The cp genomes of *Lilium* are highly conserved, with identical gene content and gene order and genomic structure comprising four parts. Such a low GC content has also been found in other angiosperm chloroplast genomes 43.

Through a comparative analysis of *Lilium* cp genome sequences, we rapidly developed molecular markers such as single nucleotide polymorphism (SNPs), and SSRs a type of 1–7 nucleotide unit tandem repeat sequence frequently observed in cp genomes, have been shown to have significant potential applications. SSRs are These markers are widely used in population genetics and breeding program studies 44, 45 because of their high polymorphism even within species, due to slipped-strand mispairing on a single DNA strand during DNA replication 46.

In this study, 1,043 SSRs were identified in sixteen *Lilium* cp genomes. The most abundant are mononucleotide repeats, accounting for more than 56.38% of the total SSRs, followed by the di-, tri-, tetra-, and pentanucleotides. These new resources will be potentially useful for population studies in the *Lilium* genus, possibly in combination with other informative nuclear genome SSRs.

The nucleotide substitution rate is a central question in molecular evolution 47. Based on the number and distribution of SNP and proportions of variability, the sequence divergence of the IR region is lower than that in LSC and SSC regions, also occurring in many previously reported plants 48, 49. All pairwise sequence comparisons in our study reveal that DNA sequences evolve at different rates in different species. This result has also been found in other taxa 49.

Because *Lilium* contains more than 100 species, its DNA barcoding and taxonomy are difficult to assess. The *rbcL, matK, trnH-psbA*, and ITS genes have been widely used to investigate taxonomy and DNA barcoding at the interspecific level (China Plant BOL Group 2014). In DNA barcoding or molecular phylogenetic studies of *Lilium*, these markers had extremely low discriminatory power 11–13. The indel and SNP mutation events in the genome were not random but clustered as “hotspots.” Such mutational dynamics created the highly variable regions in the

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**Table 4.** Ten regions of highly variable sequences of *Lilium.*

| High variable marker | Length | Variable sites | Parsimony informative sites | Nucleotide diversity |
|----------------------|--------|----------------|----------------------------|---------------------|
| trnS-trnG            | 855    | 55             | 26                         | 0.01514             |
| trnE-trnT-psbD       | 881    | 49             | 21                         | 0.01364             |
| trnF-ndh1            | 628    | 35             | 18                         | 0.01678             |
| psbE-petL            | 755    | 33             | 20                         | 0.0146              |
| trnP-psa1-rpl33      | 665    | 36             | 20                         | 0.01726             |
| psbB-psbH            | 1049   | 49             | 27                         | 0.01287             |
| petD-rpoA            | 633    | 35             | 18                         | 0.0145              |
| ndhF-rpl32-trnL      | 1525   | 107            | 50                         | 0.01581             |
| ycf1a                | 1124   | 70             | 36                         | 0.0156              |
| ycf1b                | 710    | 52             | 23                         | 0.01833             |
| Combine              | 8825   | 521            | 259                        | 0.01533             |

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![Figure 3. Sliding window analysis of the entire chloroplast genome of 16 Lilium species (window length: 600bp; step size: 200bp). X-axis: position of the midpoint of a window; Y-axis: nucleotide diversity of each window.](chart.png)
Figure 4. Phylogenetic relationships of the 16 *Lilium* species inferred from maximum parsimony (MP), maximum likelihood (ML) and Bayesian (BI) analyses of different data partitions. (A) Whole chloroplast genome. (B) LSC region. (C) IR region. (D) SSC region. Numbers above nodes are support values with MP bootstrap values on the left, ML bootstrap values in the middle, and Bayesian posterior probabilities (PP) values on the right.

Figure 5. Phylogeny of the 16 *Lilium* species constructed using 10 regions of highly variable sequences. *Fritillaria* was used as the outgroup. Numbers above nodes are support values with MP bootstrap values on the left, ML bootstrap values in the middle, and Bayesian posterior probabilities (PP) values on the right.
Total genomic DNA was extracted using a plant genome extraction kit (Tiangen, Beijing, China). Specimens were deposited in the herbarium of the Institute of Botany, Chinese Academy of Sciences (PE) qualitatively assessed and assembled using SPAdes 3.6.1. The gaps were filled by PCR amplification and Sanger paired-end library in accordance with the Illumina HiSeq 4000 standard protocol. The paired-end reads were repeat units each for tetra-, penta-, and hexanucleotide SSRs.

Two rarely reported highly variable regions, petD-rpoA and trnE-trnT-psbD, present in the Lilium cp genome were Two rarely reported highly variable regions, petD-rpoA and trnE-trnT-psbD, present in the Lilium cp genome were Two rarely reported highly variable regions, petD-rpoA and trnE-trnT-psbD, present in the Lilium cp genome were Two rarely reported highly variable regions, petD-rpoA and trnE-trnT-psbD, present in the Lilium cp genome were

| Species       | Section          | Voucher       | Locality     |
|---------------|------------------|---------------|--------------|
| L. fargesii   | Lophophorum      | BOP040593     | Shaanxi      |
| L. brownii    | Leucolirion 6b   | BOP040602     | Hubei        |
| L. lancifolium| Sinomartagon     | BOP040607     | Hubei        |
| L. nepalense  | Lophophorum      | BOP040618     | Yunnan       |
| L. leucanthum | Leucolirion 6a   | BOP040622     | Chongqing    |
| L. davidii    | Sinomartagon     | BOP040624     | Yunnan       |
| L. duchartrei | Sinomartagon     | BOP040925     | Sichuan      |
| L. bakerianum | Leucolirion 6a   | BOP040932     | Yunnan       |
| L. henryi     | Leucolirion 6a   | BOP040933     | Hubei        |

Table 5. Sampled species and their voucher specimens used in this study.

Inferring the phylogeny with chloroplast phylogenomics in Lilium. Phylogenetic analyses based on complete plastid genome sequences have provided valuable insights into relationships among and within plant genera. Early studies have been conducted to position uncertain families in angiosperms, such as Amborellaceae51, Nymphaeaceae52, and Nelumbonaceae53. With the recent advent of NGS technology, chloroplast genomes have been widely used for phylogenetic studies54–56. Two rarely reported highly variable regions, petD-rpoA and trnE-trnT-psbD, present in the Lilium cp genome were identified in the present study.

Methods

Plant material and DNA extraction. Fresh leaves of nine Lilium species were sampled (Table 5). Specimens were deposited in the herbarium of the Institute of Botany, Chinese Academy of Sciences (PE) (Table 5). Total genomic DNA was extracted using a plant genome extraction kit (Tiangen, Beijing, China). Subsequently, DNA concentration was measured using a NanoDrop spectrophotometer 2000 (Thermo Fisher Scientific, America).

Genome sequencing, assembly and annotation. DNA was sheared to construct a 400 bp (insert size) paired-end library in accordance with the Illumina HiSeq 4000 standard protocol. The paired-end reads were qualitatively assessed and assembled using SPAdes 3.6.1. The gaps were filled by PCR amplification and Sanger sequencing. Sanger sequence reads were proofread and assembled with Sequencher 4.10 (http://www.genecodes.com).

All genes encoding proteins, transfer RNAs (tRNAs), and ribosomal RNAs (rRNAs) were annotated on the Lilium cp genomes. This study used the cp genome data to infer the phylogenetic relationships in Lilium, providing genome-scale support. The cp genome is expected to be useful in resolving the deeper branches of the phylogeny as more whole-genome sequences become available in Lilium.

Microsatellite analysis. Perl script MISA was used to detect microsatellites (mono-, di-, tri-, tetra-, penta-, and hexanucleotide repeats) with the following thresholds (unit size, min repeats): ten repeat units for mononucleotide SSRs, five repeat units for dinucleotide SSRs, four repeat units for trinucleotide SSRs, and three repeat units each for tetra-, penta-, and hexanucleotide SSRs.
Molecular marker identification and sequence divergence analysis. The sequences were first aligned using MAFFT v7\(^ {62} \) and then manually adjusted using BioEdit software. Subsequently, a sliding window analysis was conducted to evaluate the nucleotide variability (Pi) of the cp genome using DnaSP version 5.1 software\(^ {63} \). The step size was set to 200 base pairs, and the window length was set to 600 base pairs.

Variable and parsimony-informative base sites across the complete cp genomes and the large single copy (LSC), small single copy (SSC), and inverted repeat (IR) regions of the six cp genomes were calculated using MEGA 6.0 software\(^ {64} \). The p-distance among Lilium cp genomes was calculated to evaluate the divergence of Lilium species using MEGA software.

Phylogenetic analysis. Phylogenetic trees were constructed by maximum parsimony (MP), maximum likeliness (ML) and Bayesian analysis (BI) methods using the entire cp genome, LSC, SSC, IR regions and combining ten variable regions. The lengths of all alignment matrices of these datasets are shown in Table 3. In all phylogenetic analyses, Fritillaria or Smilax china were used as the outgroup (Figs 4 and 5, Fig. S1). MP analyses were conducted using PAUP v4b10\(^ {60} \) with heuristic searches with the ‘MulTrees’ option followed by tree bisection–reconnection (TBR) branch swapping. Branch support was assessed with 1,000 random addition replicates. All characters were unordered and were accorded equal weight, with gaps being treated as missing data. The best-fit substitution models were selected by running ModelTest 3.7\(^ {66} \) under the Akaike information criterion (AIC). ML analyses were performed using RAxML-HPC2 BlackBox v8.1.24 at the CIPRES Science Gateway website\(^ {77,78} \). For ML analyses, the best-fit models, general time reversible (GTR) + G, were used in all analyses, as suggested with 1,000 bootstrap replicates. BI was performed with MrBayes 3.2\(^ {69} \). Two independent Markov chain Monte Carlo (MCMC) chains were run, each with three heated and one cold chain for 50 million generations. Each chain started with a random tree, default priors and sampling trees every 1,000 generations, with the first 25% discarded as burn-in. Stationarity was considered reached when the average standard deviation of split frequencies remained below 0.01.

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Author Contributions
Y.P.D and X.H.Z. conceived the experiments, Y.P.D., M.F.Z. and X.Q.C. collected the samples, Y.P.D. and J.X. conducted the experiments, Y.P.D., Y.B. and F.P.Y. analyzed the results, Y.P.D. and Y.B. wrote the manuscript.
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