Comparative Chloroplast Genomes of Four Lycoris Species (Amaryllidaceae) Provides New Insight into Interspecific Relationship and Phylogeny

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Abstract: The genus Lycoris (Amaryllidaceae) consists of about 20 species, which is endemic to East Asia. Although the Lycoris species is of great horticultural and medical importance, challenges in accurate species identification persist due to frequent natural hybridization and large-scale intraspecific variation. In this study, we sequenced chloroplast genomes of four Lycoris species and retrieved seven published chloroplast (cp) genome sequences in this genus for comparative genomic and phylogenetic analyses. All these chloroplast genomes possess the typical quadripartite structure with conserved genome arrangement and gene content, yet their lengths varied due to expansion/contraction of the IR/SC boundaries. Phylogenetic relationships within Lycoris were resolved with high resolution using complete cp genome sequences. These results could not only offer a genome-scale platform for identification and utilization of Lycoris but also provide a phylogenomic framework for future studies in this genus.

Keywords: Lycoris; Amaryllidaceae; chloroplast genome; interspecific comparison; phylogenetic analysis
1. Introduction

The genus *Lycoris* Herb. is a group of perennial bulbous plants with high ornamental and medicinal values that belongs to the family Amaryllidaceae [1,2]. More than 110 Amaryllidaceae alkaloids were identified in *Lycoris*, which have the function of antitumor, antibacterial, cytotoxic, and cholinesterase inhibition activities [3]. Species of *Lycoris* are widely cultivated as ornamental plants for their large and beautiful flowers [4].

The genus contains about 20 species, mainly distributed in China (15 species recorded) and Japan, and a few in Myanmar and North Korea. It has been demonstrated that frequent interspecific hybridization and intraspecific morphological variation commonly happen in *Lycoris* [5], resulting in the difficulty to make a clear standard for germplasm identification at the morphological level. Moreover, new *Lycoris* species are being reported, such as, *L. hunanensis* which was published as a new species from Yuanling County in China, which showed some difference from *L. straminea* [6]. *Lycoris × hubeiensis* K. Liu was identified as a natural hybrid of putative parents *L. radiata* and *L. aurea* [7]. In the Tsinling Mountains in China, *L. tsinlingensis* was found and published as a new species, but it is largely similar to *L. chinensis* [8]. In fact, there are different opinions on whether these subtle differences could be used as a criterion for determining a new species, so a feasible evaluation standard was suggested to clarify whether it is a new species or a variant. It requires some specific sequences for germplasm identification and a clear interspecific relationship in *Lycoris*.

To explore the interspecific relationships and clarify the hypothesis of hybrid origin in *Lycoris*, molecular markers of RAPD (random amplified polymorphic DNA) [9], nuclear ITS (internal transcribed spacer) sequences [10], inter-simple sequence repeat (ISSR) [11], and SCoT (start codon targeted) [12] have been used. RAPD analysis was consistent with the classification based on chromosome karyotype, which divided 13 *Lycoris* species into two groups. Nuclear ITS sequences of 15 *Lycoris* species suggested the three infrageneric clades and the hybrid origin of *L. straminea*, *L. caldwellii*, and *L. albiflora*. However, the extensive sequence variation has existed in many plant genomes, the complex and unpredictable evolutionary behavior of ITS sequence reduced the utility for phylogenetic analysis [13]. Thus, more methods were developed, inter-simple sequence repeat (ISSR) analyses of 20 species and varieties indicated a high level of genetic variation among species in *Lycoris*, and four major groups clustered by UPGMA analysis presented a consistence with morphological and karyotype observations. SCoT markers of 14 *Lycoris* species were tested and clustered into four groups, in which *L. squamigera*, *L. incarnata*, and all hybrids with the characteristic of multi-colored flowers were gathered together, suggesting the possibility of the hybrid origin of these two species. Although several strategies were developed for the analysis of interspecific relationships in the genus *Lycoris*, each method offered limited resolution within closely related species, resulting in that they did not get unanimous conclusion. More effective molecular markers are needed to be developed for germplasm identification, conservation, utilization, and breeding of the *Lycoris* species.

Plastid genes are regularly utilized in biotechnology or phylogeny, but with the limitation of DNA sequencing costs, investigators always chose a dense taxon sampling, which had a small number of informative loci for molecular phylogenetic analysis in *Lycoris*. For example, the cpDNA *trnL-F* sequence of 15 *Lycoris* species was selected to construct a phylogeny tree, which contained three infrageneric clades and was basically consistent with the classification of morphology except for *L. longituba*, *L. aurea*, and *L. straminea*. Phylogenetic reconstruction was obtained using plastid markers (*trnS-trnF* and *trnC-ycf6*), which clustered *Lycoris* spp. into three clades and differed from that derived using ITS sequences [14]. Considering the rapid radiations and conservative genome evolution, limited sequence variation could be detected, particularly at low taxonomic levels. More sequence information and species were often desirable to increase phylogenetic resolution. Actually, complete chloroplast genome sequences were more highly discriminating and efficient as plant DNA barcodes. The development of next-generation DNA sequencing has brought the benefits of large numbers of genome data collection and allowed the rapid obtaining of complete organellar genomes. Whole plastome sequencing has been an efficient
option to increase the phylogenetic resolution for the phylogenetic analyses, especially at lower taxonomic levels [15,16]. In angiosperms, the cp genome is highly conserved in terms of structure, content, and order of genes [17]. They usually have a circular structure, where two large, inverted repeat (IR) regions were separated by a large single-copy (LSC) region and a small single-copy (SSC) region [18]. The cp genome sequences contain many noncoding and variation regions, which has provided an essential molecular source for interspecific phylogenetic and phylogeographic studies [19]. It has been successfully used in many families and genus, for example, Dracunculus (Araceae), Cardiocrinum and Amana (Liliaceae) [20,21], Artemisia (Asteraceae) [22], and Withania somnifera (Solanaceae) [23].

Since the first Lycoris complete cp genome of L. squamigera was published in 2018 [24], there are six Lycoris species that have been published, and a phylogenetic tree based on the complete cp genome sequences was constructed [25]. There is no doubt that more complete chloroplast genome sequences will provide more information and insights for phylogenetic relationship reconstruction. In this study, we sequenced complete cp genomes of four Lycoris species. Based on previous studies, we systematically analyzed the similarities and differences of global structural patterns, variations of genes, simple sequence repeats (SSRs), and inverted repeats. Then the phylogenetic relationship was constructed based on the complete chloroplast genome sequences of 11 species Lycoris. The comparative analysis has demonstrated the effectiveness and applicability of chloroplast genome sequences for Lycoris phylogeny and remarked on the potential applications for species identification, development of DNA barcoding, and future phylogenetic studies of the genus and family.

2. Materials and Methods

2.1. Plant Sample Collection, DNA Extraction and Sequencing

The bulbs of L. incarnata, L. shaanxiensis, L. straminea, and L. houdyshelii were planted in Nanjing Botanical Garden, Mem. Sun Yat-sen (E118_83, N32_06), Nanjing, China. The specimen of L. incarnata (No. SYS00024942) was stored at the herbarium of Sun Yat-Sen University, the specimen of L. straminea (No. 00110652) and L. houdyshelii (No. 00110525) were stored at the herbarium of the Institute of Botany, Chinese Academy of Sciences. The L. shaanxiensis was collected from Shanxi Province and identified in 2018, but there was no specimen record in the herbarium in China. Fresh leaves were collected, quick freezeed in liquid nitrogen, then stored at −80°C until use. Genomic DNA was extracted using the Plant Genomic DNA Kit (Huayueyang, Beijing, China). DNA integrity was examined by electrophoresis in 1% (w/v) agarose gel, and concentration was measured using a NanoDrop spectrophotometer 2000 (Thermo Scientific; Waltham, MA, USA), then accurate quantifications were completed by Qubit 2.0. High-quality DNA libraries were constructed and sequenced at Novogene Bioinformatics Technology Co., Ltd. (https://www.novogene.com/, accessed on March 2011 Tianjin, China). The strategy of Nova-PE150 was selected for high-throughput sequencing, with an insert size of 350 bp.

2.2. Complete Cp Genome Assembly, Annotation and Structure Analysis

The complete cp genomes were assembled using the organelle assembler NOVOPlasty (Version 3.3) [26] with the parameters of genome range (148,500–168,500) and k-mer (39). The complete cp genome sequence of L. radiata (GenBank accession no. MN158120) was set as a reference [27]. Assembled genome sequences were manually corrected by BLASTn comparison and circularized. GC content was calculated by Geneious software (version R11, http://www.geneious.com accessed on 3 October 2017). Correct cp genome sequences were input on web server CPGAVAS2 (http://www.herbalgenomics.org/cpgavas2, accessed on 14 October 2020.) for the cp genome annotation and visualization with the default parameters. Microsatellite sequences were identified with MISA [28], which set the unit_size/min_repeats as 1/10, 2/6, 3/5, 4/5, 5/5, and 6/5. The maximum length of a sequence between two SSRs was set as 100. MEGA [29] was performed for calculating the relative synonymous codon usage (RSCU) values. Seven previously reported Lycoris chloroplast genome sequences, i.e., L. squamigera (MH118290), L. radiata (MN158120), L. sprengeri...
(MN158986), L. longituba (MN096601), L. chinensis (MT700549), L. anhuiensis (MT700550), and L. aurea (NC_046752), were downloaded from the National Center of Biotechnology Information (NCBI) database. The obtained cp genome sequences in the present study were deposited in the NCBI, with the GenBank accession numbers of MW477439 (L. incarnata), MW477440 (L. shaanxiensis), MW477441 (L. straminea), and MW477442 (L. houdyshelii).

2.3. Interspecific Comparison of Chloroplast Genomes

To explore the divergence regions in Lycoris, the program IRscope (https://irscope.shinyapps.io/irapp/, accessed on 20 June 2021) was used to visualize the divergence on the boundaries of the junction sites of the 11 chloroplast genome sequences in Lycoris [30]. The mVISTA program (http://genome.lbl.gov/vista/index.shtml, accessed on 23 June 2021) [31] was used to align and compare the complete cp genomes of Lycoris with the default parameters. Each annotation of the Lycoris species was selected as a reference, and Shuffle-LAGAN mode was visualized in an mVISTA plot.

2.4. Phylogenetic Analyses

A total of 11 complete cp genome sequences of Lycoris were used for phylogenetic analysis, including seven reported species and four new species in this study, Narcissus poet-icus (MH706763) was selected as outgroup taxa. The reported sequences were downloaded from the NCBI database. Both the complete plastid sequences and 79 common PCGs (output by Geneious) were used for the ML tree construction. The nucleotide sequences were aligned using the MAFFT plugin [32,33] in Geneious with default settings. All gaps are treated as missing data. The complete alignment was used to reconstruct a maximum likelihood tree using PHYML [34] with 1000 bootstrap replicates. The GTR+G+I model suggested by jModelTest 2.1.4 [35] was used for each dataset.

3. Results and Discussion

3.1. General Features of the Cp Genomes of Lycoris

In the present study, we obtained four cp genomes of Lycoris by next-generation sequencing and de novo assembly, which were L. incarnata, L. shaanxiensis, L. straminea, and L. houdyshelii. A total of 7.2, 6.7, 5.5, and 4.8 million reads were obtained, and the average organelle coverage reached 7291×, 6761×, 5531× and 4581×, respectively (Table 1). The complete chloroplast genomes typically range from 120 to 170 kilobase pairs (kb) in length [17]. In Lycoris, the length of most species was around 158 kb [25]. In the present study, the full length of four species was 158,405, 158,498, 158,490, and 158,490 bp, respectively, with the same GC content of 37.8% (Table 1). The visualized circular map showed the typical angiosperm cp genome structure in Lycoris, which consists of one large single-copy (86,464–86,593 bp) and one small single-copy (18,352–18,499 bp) region, separated by a pair of inverted repeat (IR) (26,730–26,765) regions (Table 1 and Figure 1).

Table 1. Summary information of four chloroplast genomes of the Lycoris species.

| Genome Features        | L. incarnata | L. shaanxiensis | L. straminea | L. houdyshelii |
|------------------------|--------------|-----------------|--------------|----------------|
| Average organelle coverage | 7291×      | 6761×           | 5531×        | 4581×          |
| Genome size (bp)       | 158,405      | 158,498         | 158,490      | 158,490        |
| LSC size (bp)          | 86,593       | 86,469          | 86,473       | 86,464         |
| SSC size (bp)          | 18,352       | 18,499          | 18,487       | 18,496         |
| IR size (bp)           | 26,730       | 26,765          | 26,765       | 26,765         |
| GC content (%)         | 37.8         | 37.8            | 37.8         | 37.8           |
| No. of genes           | 113          | 113             | 113          | 113            |
| No. of PCGs            | 79           | 79              | 79           | 79             |
| No. of rRNAs          | 30           | 30              | 30           | 30             |
| No. of tRNAs          | 4            | 4               | 4            | 4              |
| Duplicated genes       | 20           | 20              | 20           | 20             |
Figure 1. The plastome features of the *Lycoris* species. The map contains four rings, from the center going outward, the first circle means the forward and reverse repeats connected with red and green arcs, respectively. The second circle shows the tandem repeats marked with short bars. The third circle shows the microsatellites. The fourth circle shows the gene structure on the plastome. The genes were colored based on the functional categories, which were shown in the center of the map.

Compared with reported *Lycoris* species [25], the complete cp genomes size ranged from 158,335 (L. radiata) to 158,687 bp (L. sprengeri). Here, the genome size of these three species is between the longest and shortest; the length of LSC and SSC regions made a greater contribution to the full size. On the contrary, the IR regions were relatively conservative. In *Lycoris* cp genomes, a total of 113 genes were annotated, including 79 protein-coding genes (PCGs), 30 tRNAs, and 4 rRNAs (Table 1 and Figure 1). Compared with our previous reports, we found that the number of genes was highly conservative in *Lycoris* cp genomes. They were divided into four categories; contained genes for photosynthesis, self-replication, other genes, and function unknown (Table 2). There were 20 genes
that were duplicated more than once. Four rRNAs were duplicated, which is consistent with other \textit{Lycoris} species \cite{25} and most plants, such as \textit{Allium} \cite{36} and \textit{Amomum} \cite{37}.

\textbf{Table 2.} Gene composition of four \textit{Lycoris} chloroplast genomes.

| Category of Genes | Group of Genes | Name of Genes |
|-------------------|----------------|---------------|
| Genes for photosynthesis | Subunits of photosystem I | psaB, psaA, psaI, psaJ, ycf4 |
| | Subunits of photosystem II | psbA, psbK, psbI, psbM, psbD, psbC, psbZ, psbL, psbF, psbE, psbB, psbT, psbN, psbH, ycf3 |
| | Subunits of NADH-dehydrogenase | ndhJ, ndhK, ndhC, ndhB \(^a\) (×2), ndhF, ndhD, ndhE, ndhG, ndhI, ndhA \(^a\), ndhH |
| | Subunits of cytochrome b/f complex | petN, petA, petL, petG, petB \(^a\), petD \(^a\), atpA, atpE \(^a\), atpH, atpI, atpE, atpB |
| | Subunits of ATP synthase | rbcL |
| | | |
| | Large subunit of ribosome | rpl33, rpl20, rpl36, rpl14, rpl16 \(^a\), rpl22, rpl2 \(^a\) (×2), rpl23 (×2), rpl32 |
| | DNA dependent RNA polymerase | rpoC2, rpoC1 \(^a\), rpoB, rpoA |
| | Small subunit of ribosome | rps16 \(^a\), rps2, rps14, rps4, rps12 \(^b\) (×2), rps11, rps8, rps3, rps19 (×2), rps7 (×2), rps15 |
| | Ribosomal RNAs | rnr16 (×2), rnr23 (×2), rnr4.5 (×2), rnr5 (×2) |
| | | trnK-UGC, trnQ-UGU, trnS-GCU, trnG-GCC \(^a\), trnR-UCU, trnC-GCA, trnD-GUC, trnY-GUA, trnE-UUC, trnT-GGU, trnS-UGA, trnG-GCC, trnMCAU, trnS-GGA, trnT-UGC, trnL-UAA \(^a\), trnF-GAA, trnV-UCAC \(^a\), trnM-CAU, trnW-CCA, trnP-UGC, trnH-GUG (×2), trn-lCAU (×2), trnL-CAU (×2), trnV-GAC (×2), trn-lGAU \(^a\) (×2), trnA-UUG \(^a\) (×2), trnR-ACG (×2)trnN-GULU (×2), trnL-UAG |
| | Transfer RNAs | |
| | Subunit of Acetyl-CoA-carboxylase | accD |
| | c-type cytochrome synthesis gene | ccsA |
| | Envelop membrane protein | cemA |
| | Protease | clpP |
| | Translational initiation factor | infA |
| | Maturase | matK |
| | Component of TIC complex | ycf1 (×2) |
| Other genes | Conserved open reading frames | ycf2 (×2) |

\(^a\) means the genes containing a single intron; \(^b\) indicates the genes containing two introns; (×2) indicates the genes duplicated in the IR regions.

Group II (G2) introns are self-splicing RNAs and mobile elements, which could provide rich characters for comparative analysis and phylogeny construction at both infrageneric and intrafamilial levels \cite{38–40}. For example, the matK open reading frame (ORF) has been used as a marker for plant evolutionary studies. Interestingly, trnK-UUU contains a group II intron (trnKII), which encodes the \textit{matK} ORF, which attracts interest because it represents an unusual form of a group II intron \cite{41}. In four \textit{Lycoris} species, there were 18 splitting genes in \textit{L. incarnata} and 17 in the other three species. There is one more \textit{ndhF} located in IR and SSC regions in \textit{L. incarnata}, which happened in \textit{L. radiata} and \textit{L. sprengeri} \cite{25}. Most of the splitting genes contain one intron and two exons, except for \textit{ycf3} and \textit{clpP}; they contained two introns and three exons (Table S1).

3.2. \textit{CpSSRs and Repeat Structures}

Chloroplast simple sequence repeats (cpSSRs) are microsatellites, showing typically mononucleotide tandem repeats. They commonly showed intraspecific variation in repeat numbers when they were located in the noncoding regions of the chloroplast genome \cite{42}. Some works have proved the potential applications of variations in the noncoding regions of the chloroplast genome for phylogenetic analysis at the level of genus and species \cite{43,44}. In the cp genomes of \textit{L. incarnata}, \textit{L. shaanxiensis}, \textit{L. straminea}, and \textit{L. houdyshelli}, there
were 51, 44, 45, and 45 SSRs, respectively. The same as other reported Lycoris species [25], type of mononucleotide (A/T) was the most variable, which was 48, 42, 44, and 44 in four species (Figure 2 and Table S2). One mononucleotide (C/G) was detected in only L. incarnata and L. houdyshelii. One dinucleotide (AT/AT) was commonly detected in Lycoris species except for the L. incarnata, which contains two SSRs of AT/AT. A previous study also showed one dinucleotide (AT/AT) in seven Lycoris species [25], suggesting the conservation of dinucleotide SSRs among species. Trinucleotide (AAT/ATT) was only detected in L. incarnata and L. shaanxiensis. The trinucleotide (ATT) was only detected in L. incarnata and L. shaanxiensis, which was existed in other four species (Figure 2), L. aurea, L. radiata, L. sprengeri, and L. squamigera [25], accounting for half of the reported Lycoris species. No tetranucleotide repetition was detected in all reported Lycoris species.

Figure 2. Statistics of simple sequence repeats of cp genomes of four Lycoris species. (A) Numbers of different repeat types; (B) Numbers of identified each SSRs motifs.

3.3. Statistics of Codon Usage

Codon usage analysis is beneficial for studies of evolution and new gene mining, which varies among different species [45]. Here, the complete cp genome sequences of four Lycoris were analyzed to investigate the amino acid frequency, the number of codon usage, the bias of codon usage, and relative synonymous codon usage (RSCU) (Table S3). Although the total number of codons was ranging from 48,207 to 49,641 in four species, showing a tiny change, the types of codons and amino acids were the same. A total of 64 codons were deduced, which were encoding 21 amino acids. Met and Trp were encoded by one codon usage, while others were encoding by multiple synonymous codons, ranging from two to six (Figure 3). The three highest frequency (AGA, GCT, and TTA) and four lowest frequency (AGC, GGC, GAC, and CTG) codons were observed in four species. It was defined as preferred codon usage when the RSCU value was >1.00 and vice versa. Except for methionine and tryptophan, there were 32 preferred and 30 non-preferred codon usages in L. incarnata, L. shaanxiensis, and L. houdyshelii, which was the same with the reported five Lycoris species, suggesting the main pattern of this codon usage in Lycoris [25]. In L. straminea, 31 preferred and 31 non-preferred codon usages were identified, which is different from any reported Lycoris species. The result will help us to understand the related patterns in Lycoris species and improve the research on codon usage in plant biology.
3.4. Inverted Repeats Contraction, Expansion, and Interspecific Comparison

The typical circular structure of the chloroplast genome consists of regions of IR, LSC, and SSC, which makes four boundaries (IRb/LSC, IRb/SSC, IRa/SSC, and IRa/LSC). The contraction or expansion of the IR regions commonly leads to the length variation of the chloroplast genomes among different plant species [20,46,47]. In the present study, we compared the IR/SC borders and the adjacent genes among the eleven Lycoris species, including the previously reported seven species [25,48,49] and four newly sequenced species. It showed the well-conserved genomic structure, but it also exhibited divergence at the IR/SC boundary regions among eleven Lycoris chloroplast genomes (Figure 4). In the most monocot plastid genome structure, IR regions expand into rps19 [47]; there was no obvious expansion at the IRb/LSC boundary in Lycoris, except for the L. radiata and L. incarnata, their IRb regions expanded by 37 bp toward the rps19 gene. In all Lycoris species, the IRa/SSC border extended into the ycf1 genes with 925–982 bp. In addition, the ndhF gene overlapped with the IRa/SSC border by 50 bp in seven species, including L. chinensis, L. anhuiensis, L. longituba, L. squamigera, L. shaanxiensis, L. straminea, and L. houdyshelii (Figure 4).

To rapidly identify the conserved sequences in long alignments, global interspecific comparisons were performed using software mVISTA [31,50]. A total of 11 Lycoris cp genome sequences (same as IR analysis) were selected for comparative analysis. Most of the sequence variations were found in the LSC and SSC regions, which being largely consistent with our previous studies [25], in which the IR regions showed the high sequence conservation of the 11 species (Figure 5). A lot of evidence indicated that ndhF has great power in discrimination at the low taxonomic level [51]. In Lycoris, ycf1 and ndhF presented the most divergence in all species, suggesting the potential molecular markers for phylogenetic analysis and species identification. Although the length and boundary distribution characteristics of ndhF suggested that the L. radiata was a putative female parent of L. incarnata, the comparative analysis suggested the closer relationship of seven related species that have similar IR boundaries features (Figures 4 and 5).
Figure 4. Comparison of border pattern of large single-copy regions (LSC), small single-copy regions (SSC), and an inverted repeat (IR) among 11 *Lycoris* chloroplast genomes.
Figure 5. Sequence identity plot using mVISTA based on the complete cp sequences of 11 Lycoris species with L. sprengeri as a reference. A 70% cut-off identity was used for the plots, and the Y-scale represents the percent identity from 50% to 100%.

3.5. Phylogenetic Analysis

To explore the interspecific relationship and phylogeny reconstruction, a total of 11 Lycoris species with complete chloroplast genome sequences were selected for the construction of the maximum likelihood (ML) tree and Narcissus poeticus was chosen as the outgroup taxa. Both the complete plastid genome sequences and 79 common protein-coding genes were used for phylogenetic analysis, and the phylogenetic trees based on these two datasets showed the same topology (Figure 6).

Here, 11 Lycoris species were clustered into three main groups, and L. sprengeri is basal for the other species in Lycoris; however, previous cpDNA sequences analysis showed that the L. radiata had a basal position within Lycoris [10]. It involves the discussion of the origin of hybrid species of Lycoris. Some studies have suggested that the four species of L. incarnata, L. shaanxiensis, L. straminea, and L. houdyshelii were hybrid origin species [52], plastid DNA sequences and SCoT analysis showed that natural hybrids L. incarnata and L. squamigera were located in same clade [12,14]. Here, two species, L. incarnata and L. shaanxiensis, with similar morphological characteristics, are not clustered together. L. incarnata was clustered with L. radiata, suggesting that the L. radiata may be the female donor of the L. incarnata. L. shaanxiensis showed the closest relationship to L. squamigera, which was also considered as a hybrid origin species [14], suggesting the same ancestor of these two species, and L. radiata may be their donor according to the phylogenetic analysis by complete cp genome sequences. L. straminea and L. houdyshelii showed the most similar morphological and ecological characteristics except for the flower color. L. straminea was a species with multiple ecological properties, which always exhibits a color change from light yellow to medium yellow degrees, but the flower of L. houdyshelii is white. If only based on morphological features, L. houdyshelii could be considered as a variant; however, evidence has shown that they have a totally different chromosome number and karyotype [12,53]. RAPD analysis also indicated that they were clustered into two groups [9]. Evaluation of different methods, including morphology, karyotypes, plastid sequences, and molecular
marker, produced both overlap and conflict on the interspecific relationship and phylogeny because of the variant resolutions and parameters.

Figure 6. Phylogenetic analysis of the 11 Lycoris species by maximum likelihood (ML) analyses, Narcissus poeticus was the outgroup taxa. (A) The topology was constructed by the complete cp genome sequences. (B) The tree was constructed using 85 common protein-coding genes.

Actually, the complete plastid sequence has been proved as an ideal method for phylogenetic relationship reconstruction. In the genus of Lycoris, some specific plastid gene sequences [14,54] and rDNA internal transcribed spacer (ITS) sequences [55,56] and have been developed before more complete cp genome sequences were available. More complete cp genome sequences provided adequate information and foundation for the clarification of inter-specific relationships and phylogenetic analysis. In the present study, we provided four cp genome sequences of Lycoris; they not only had similar morphological features but were also considered as the natural hybrid species. The phylogenic analysis supported the same group between L. straminea and L. houdyshelii, but L. houdyshelii showed a closer relationship with L. anhuiensis, L. chinensis, and L. longituba than L. straminea, the closest relationship of these five species also suggesting that L. straminea and L. houdyshelii may be derived from one of the three species.
4. Conclusions

In this study, we provided the complete cp genome sequences of *L. incarnata*, *L. shaanxiensis*, *L. straminea*, and *L. houdyshelii* and performed the interspecific comparison and phylogenetic analysis using whole cp genome sequences of 11 *Lycoris* species. The results not only showed the sequence conservation of genome size, gene number, and order but also distinguish the difference between IR-SC boundary regions. The interspecific comparison analysis supported the branch of phylogenetic analysis, where the species on the same sub-branch had the same border patterns, suggesting the high resolution and reliability of phylogenetic reconstruction by the complete cp genome sequences in *Lycoris*. Phylogeny analysis suggested that the *L. radiata* may be the female donor of the *L. incarnata*, *L. shaanxiensis*, and *L. squamigera*. *L. straminea* and *L. houdyshelii* may be derived from *L. anhuiensis*, *L. chinensis*, or *L. longituba*. The results will help to make the intraspecific relationship and evolution clear and benefit the identification, protection, and utilization of *Lycoris* germplasm resources.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/biology10080715/s1, Table S1: Splitting genes with introns and exons in the four *Lycoris* chloroplast genomes, Table S2: Chloroplast simple sequence repeats (cpSSRs) of four *Lycoris* species, Table S3: Relative synonymous codon usage (RSCU) in the four *Lycoris* chloroplast genomes.

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