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ARTICLE

Comparative Analysis of the Complete Chloroplast Genome Sequences of Four Origin Plants of Lonicerae Flos (*Lonicera*; Caprifoliaceae)

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ABSTRACT

Lonicerae Flos (LF) derived from the dried flower buds or opening flowers of four *Lonicera* plants (*Lonicera macranthoides*, *L. hypoglauca*, *L. confusa*, and *L. fulvotnetosa*), is a popular traditional Chinese medicine. Because the four origin plants are very similar in morphology, it is difficult to control the quality of LF in actual production. Over the past decade, many reports have pointed out the differences among them, including the botanical characteristics and active ingredients. However, there is still a lack of rapid methods that can be applied to the identification of the four origins. In this study, comparative analysis of the four chloroplast genomes was performed, and they showed low diversity (Pi = 0.00267), three variation hotspots regions (*rbcL-accD, rps12-ndhF* and *rps12-trnN-trnG*) were identified as potentially molecular marker of highly informative. Meanwhile, the most obvious difference in SSR comparative analysis is reverse and complement repeats were only identified in *L. confusa* and *L. hypoglauca*, respectively. Lastly, the phylogenetic tree showed that *L. confusa* is more closely related to *L. fulvotnetosa*, while *L. macranthoides* is closer to *L. hypoglauca*. This study systematically revealed the differences among the four chloroplast genomes, and it provides valuable genetic information for identifying the origin of LF.

KEYWORDS

Lonicerae Flos; *Lonicera*; chloroplast genome; origin plants; comparative genome

1 Introduction

Lonicerae Flos (LF, Shanyinhua in Chinese) is one of the most commonly used traditional Chinese medicines [1], which has been officially listed in Chinese Pharmacopeia (Edition 2020) and described as "Used for carbuncle boils, throat arthralgia, erysipelas, toxic blood dysentery, wind-heat cold, febrile fever". LF is the dried flower buds or opening flowers of four plants (*Lonicera macranthoides*, *L. hypoglauca*, *L. confusa*, and *L. fulvotnetosa*) [2], and they are widely cultivated in Southern China. Inflorescences and bracts are the main differences among the four origin plants in terms of botanical traits [3]. At present, *L. macranthoides* is the most cultivated one in the market, and *L. confusa* was the least cultivated and used [3,4]. Because they are so similar, how to distinguish the differences among them is

one of the difficulties of current research [5,6]. In order to solve this problem, studies on genetic diversity and relationships might be a good choice.

The use of plant DNA analysis to identify plant species, genotypes, and relationships has gradually replaced earlier techniques based on other biochemical markers [7]. With the rapid development of DNA analysis technology, it is becoming more accurate, conventional and low-cost, and is commonly used in plant research [8,9]. At present, phylogenetic analysis of plants is mainly based on the structure and changes of chloroplast genome and nuclear genome. However, it is difficult to screen low copy genes in plants due to the complexity of nuclear genomes. Chloroplast (cp) genome is of great significance in revealing the origin and evolution of species, genetic diversity, genetic relationship and biodiversity because of their small molecular weight (115 to 165 kb), non-recombination, highly conserved, and uniparental inheritance characteristics [10–13]. It has been widely accepted as a powerful tool for distinguishing the difference among related similar species [14,15]. With the accumulation of cp genome of *Lonicera*, comparative analysis of the complete cp genome of four origin plants of LF is helpful for deepening and expanding our systematic understanding of it.

In most previous report, studies focused on inferring the phylogeny of *Lonicera* or Caprifoliaceae based on complete chloroplast genome [16–19]. In this study, we report three sequenced complete cp genomes from three different origin plants of LF (*L. macranthoides*, *L. confusa*, and *L. fulvotnetosa*), respectively, and genomic comparative analyses with the other published cp genome (*L. hypoglauca*) [20]. The comparative analysis focuses on features, structure, nucleotide diversity, simple sequence repeats (SSRs) and phylogenetic analysis. The aims of our study are: (1) to comprehensive understanding the complete cp genome features from four origin plants of LF, (2) to the systematic analysis of similarities and differences from the four origin plants, (3) to infer the phylogenetic relationship among the four and between the four and Lonicerae Japonicae Flos (LJF, Jinyinhua in Chinese, *L. japonica*,), and (4) to provide genetic resources for developing chloroplast markers to identify LF species and future research on *Lonicera*.

2 Methods

2.1 Plant Material and Genome Sequencing

Fresh leaves samples were collected from three *Lonicera* species (*Lonicera macranthoides*, *L. confusa*, and *L. fulvotnetosa*; Table 1). Voucher specimens were deposited at the Hunan Academy of Forestry. A Genomic DNA extraction and high-throughput sequencing were performed with an Illumina NovaSeq6000 by Suzhou GENEWIZ Biotech. Co., Ltd. (Suzhou, China).

Table 1: Sample information

Species	Locality	Voucher No.	GenBank accession
L. macranthoides	Xiangxi, Hunan, China	Lm200523LS	MW493344
L. confusa	Guangzhou, Guangdong, China	Lc200523GZ	MW795591
L. fulvotnetosa	Duyun, Guizhou, China	Lf200523DY	MW795592

2.2 Assembly and Annotation of Chloroplast Genome

NGS QC Tool Kit software package was used for data quality detection and filtering to remove low quality sequences, joint sequences and sequences containing uncertain bases to obtain high quality sequences (clean reads). The clean reads then assembled using Velvet 1.2.10 [21], SSPACE v3.0 [22] and GapFiller v2.1.2 [23] with the cp genome of *L. japonica* (GenBank: KJ170923) as the reference [24].

The assembled sequence was annotated using the Plann [25], transfer RNAs (tRNAs) were detected in the genome using the program tRNAscan-SE [26] with default parameter settings and rRNA were identified by using RNAmmer [27]. All gene annotations were verified by Geneious 11.1.4 software [28] and the circular genome maps were drawn with OGDRAW (Organellar Genome DRAW) [29]. Finally, three *Lonicera* species annotated chloroplast genomes were submitted to GenBank (Table 2).

Table 2: Characteristics comparison of four origin plants of Lonicerae Flos chloroplast genomes

Characteristics	*L. macranthoides*	*L. confusa*	*L. fulvotnetosa*	*L. hypoglauca*
Accession number	MW493344	MW795591	MW795592	NC_054350
Reference	This study	This study	This study	Gu et al. [20]
Total size (bp)	155,515	155,157	155,126	154,581
LSC size (bp)	89,303	88,942	88,910	88,379
SSC size (bp)	18,656	18,661	18,662	18,646
IR size (bp)	23,778	23,777	23,777	23,778
Total number of genes	129	123	123	121 (129*)
Number of PCGs	83	76	76	80 (83*)
Number of tRNAs	38	39	39	33 (38*)
Number of rRNAs	8	8	8	8
GC content (%)	38.54	38.59	38.58	38.53
GC content of LSC (%)	36.99	37.06	37.06	36.95
GC content of SSC (%)	33.49	33.44	33.44	33.46
GC content of IR (%)	43.45	43.46	43.45	43.45

Note: *The number of the genes after additional verification based on this study method. PCGs: protein-coding genes.

2.3 Comparative Analysis of Chloroplast Genomes

This study, except for the *L. macranthoides*, *L. confusa* and *L. fulvotnetosa* complete chloroplast genomes sequenced here, *L. hypoglauca* chloroplast genome (NC_054350) [20] were used for comparative genomic analysis by mVISTA with LAGAN alignment program [30]. The major variations of gene contents or features in four *Lonicera* species chloroplast genome were manually identified with Geneious [28]. For accurate comparisons, gene annotations of NC_054350 was checked again with Plann, RNAmmer, and tRNAscan-SE [26]. DNA polymorphisms analysis including highly variable sites and nucleotide diversity (Pi) was performed using DnaSP (DNA Sequence Polymorphism) v6 [31], which the window length was set to 800 bp and the step size was set to 200 bp. The four chloroplast genome sequences were aligned by using Geneious.

2.4 Analysis of Simple Sequence Repeats

The repetitive simple sequence repeat (SSR) sequences in four chloroplast genomes of *Lonicera* were identified by MISA software (https://webblast.ipk-gatersleben.de/misa/) [32]. By using REPuter software [33] with a minimum repeat size of 20 bp, four types of repeat sequences (forward, reverse, complement and palindrome) were determined. The minimum repeat number thresholds for mononucleotide, dinucleotide, trinucleotide, tetranucleotide, pentanucleotide and hexanucleotide repeats were set to 10, 5, 4, 3, 3 and 3, respectively.

2.5 Phylogenetic Analysis

In the phylogenetic reconstruction of four LF species, 17 *Lonicera* species chloroplast genomes were used to conduct the phylogenetic tree. Except for the three LF species (*L. macranthoides*, *L. confusa* and *L. fulvotnetosa*) chloroplast genomes sequenced here, the other fourteen genomes were obtained from the NCBI (https://www.ncbi.nlm.nih.gov) included *L. hypoglauca* (NC_054350), *L. japonica* (KJ170923.1), *L. maximowiczii* (MN986996.1), *L. insularis* (MH028739.1), *L. sachalinensis* (MH028742.1), *L. maackii* (MH028741.1), *L. nervosa* (MK176510.1), *L. ferdinandi* (MK176512.1), *L. vesicaria* (MH028743.1), *L. hispida* (MK176511.1), *L. stephanocarpa* (MG738668.1), *L. praeflorens* (MH028740.1), *L. tragophylla* (MG738667.1), and *L. calcarata* (MN524650.1). MAFFT 7.487 (https://mafft.cbrc.jp/alignment/software/windows.html) [34] were used for multi-sequence alignment. Finally, by using IQTREE 1.6.12 software (http://www.iqtree.org) [35] with maximum likelihood method (GTR + I + G) and Figtree 1.4.4 software (http://tree.bio.ed.ac.uk/software/figtree/), the phylogenetic tree was built and edited.

3 Results and Discussion

3.1 Features of Chloroplast Genome

The chloroplast genome sizes of three *Lonicera* species (*L. macranthoides*, *L. confusa* and *L. fulvotnetosa*) were 155,515, 155,157 and 155,126 bp with 1198X, 1580X and 2154X depth, respectively. Each complete chloroplast genome sequences of three LF species had a typical quadripartite structure (Fig. 1), in which a large single-copy region (LSC) and a small single-copy region (SSC) were separated by two inverted repeats (IRs). *L. macranthoides*, *L. confusa* and *L. fulvotnetosa* chloroplast genome consisting of 89,303, 88,942 and 88,910 bp LSC region, 18,656, 18,661 and 18,662 bp SSC region and 23,778, 23,777 and 23,777 bp IRs regions (Table 2), respectively. 110 genes (75 PCGs, 31 tRNAs, 4 rRNAs), 106 genes (72 PCGs, 30 tRNAs, 4 rRNAs) and 111 genes (76 PCGs, 31 tRNAs, 4 rRNAs) were encoded by the three chloroplast genomes, respectively. In addition, The GC contents were identical in the three chloroplast genomes (Fig. 1; Table 2).

Comparison of chloroplast genomes from four origin plants of LF, *L. macranthoides* chloroplast genome had the largest genome size, however *L. hypoglauca* had the smallest genome size. Interestingly enough, "*L. macranthoides* and *L. hypoglauca*", "*L. confusa* and *L. fulvotnetosa*" shared the completely same number of genes. This is largely due to the simple and relatively conserved structure of chloroplast genome and the difference in chloroplast genome size may be caused by different homologous gene length for plants of the same genus.

3.2 Comparative Analyses of Lonicerae Flos Species

There were no large differences among the four origin plants of LF as a whole. Meanwhile, the chloroplast genomes of *L. confusa* and *L. fulvotnetosa* showing the least differences. It is worth noting that, when compared to the *L. macranthoides*, *L. confusa, L. fulvotnetosa* and *L. hypoglauca* have a large gap between *rbcL* and *accD* genes with five gaps located in conserved non-coding sequences (CNS) and two gaps located in *accD* (Fig. 2). In addition, *L. confusa* and *L. fulvotnetosa* have more variable sites compared to *L. macranthoides* and *L. hypoglauca*, *L. hypoglauca* has more variable sites than *L. macranthoides*.

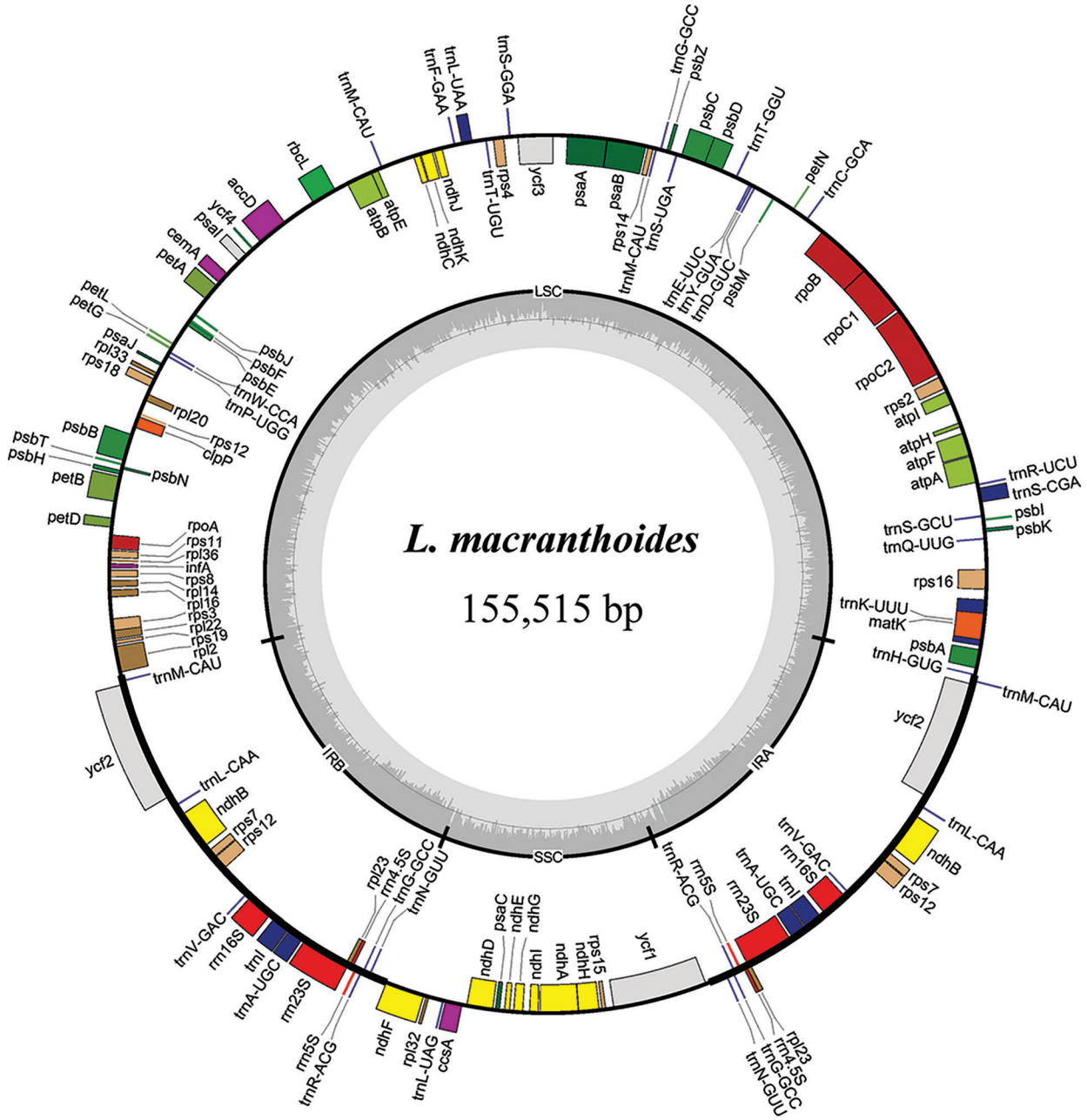
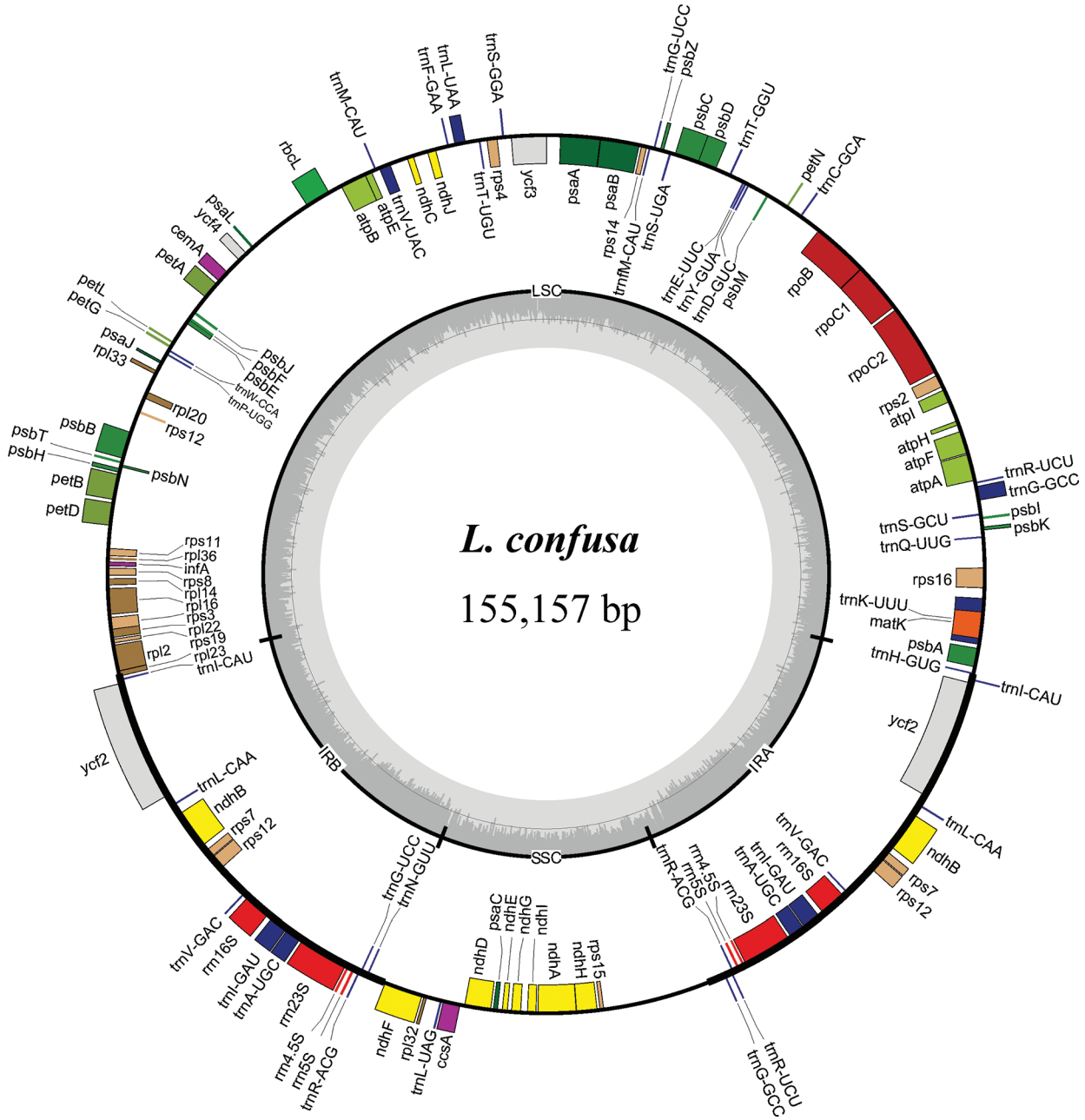


Figure 1: (Continued)



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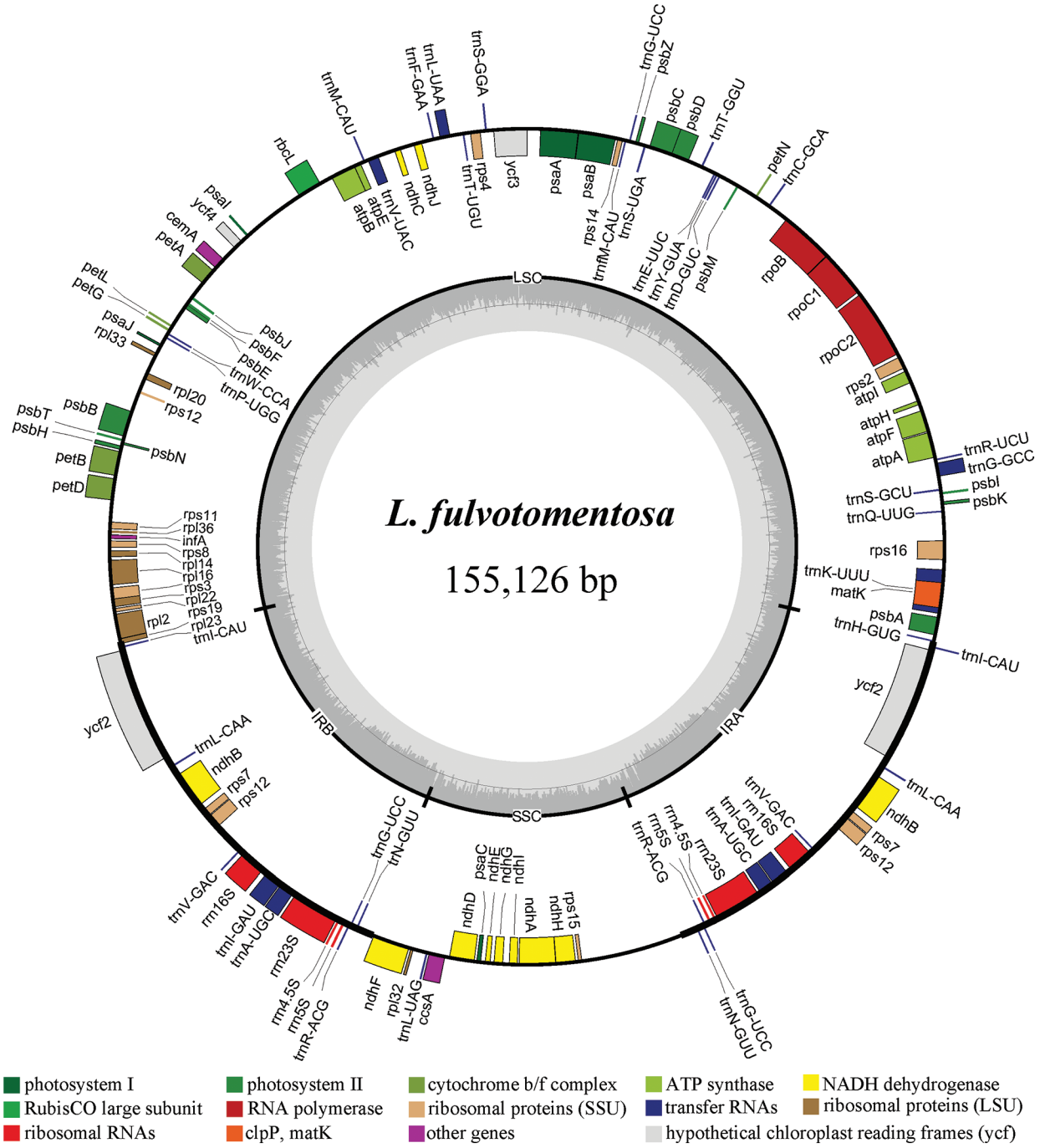

Figure 1: Chloroplast gene maps of *L. macranthoides*, *L. confusa* and *L. fulvotnetosa*. Different functional genes were shown in different colors. Transcribed clockwise or counter-clockwise were shown inside or outside the circle. LSC, large single-copy region; SSC, small single-copy region; IR, inverted repeat

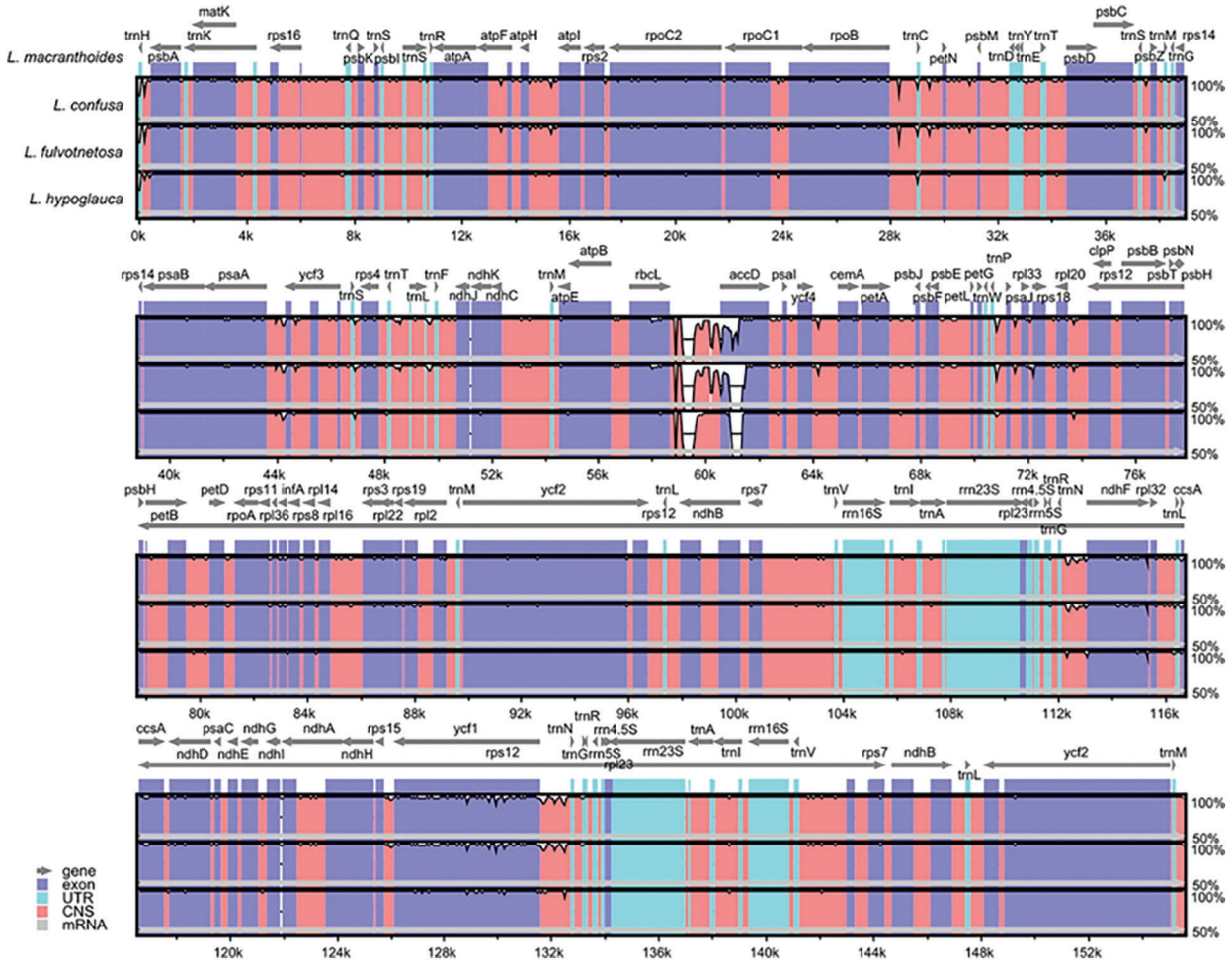

Figure 2: Comparisons of four Lonicerae Flos species chloroplast genomes. *L. macranthoides* chloroplast genome was used as reference sequence, the x-axis represents the aligned sequence of base and the y-axis represents the pairwise percent identity (50%–100%). Gray arrows, purple bars, sky blue, red bars and gray bars represent gene, exon, UTR, CNS and mRNA, respectively

By using DnaSP software, nucleotide diversity (Pi) was calculated to estimate the genetic distance among four LF species chloroplast genomes. The Pi value for four LF chloroplast genomes included (*L. macranthoides*, *L. confusa*, *L. fulvotnetosa* and *L. hypoglauca*) was 0.00267. By comparing the chloroplast genomes of four LF species, several variation hotspots were found (Fig. 3). There are three hotspots showed higher Pi values than other regions ($Pi > 0.02$), among these variation hotspots, *rbcL-accD* region showed the highest Pi (0.06875), followed by two regions (*rps12-ndhF* and *rps12-trnN-trnG*).

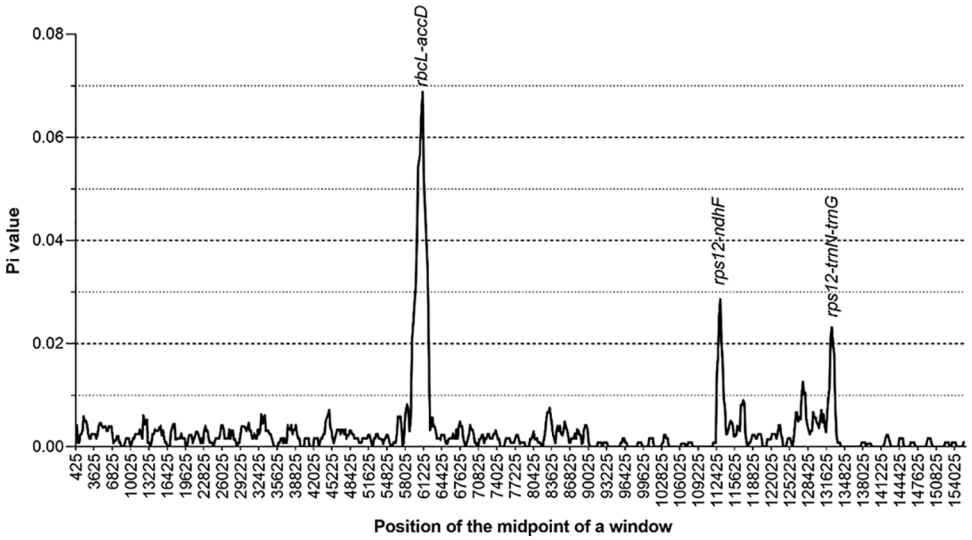

Figure 3: Nucleotide diversity of Lonicerae Flos chloroplast genomes. The x-axis represents the aligned sequence of base and the y-axis represents the Pi value. This graph shows each variation hotspot for Lonicerae Flos chloroplast genomes

3.3 Simple Sequence Repeats

There were some differences among these four chloroplast genomes. By using MISA software, 51, 54, 54 and 51 microsatellites were identified in *L. macranthoides, L. confusa, L. fulvotnetosa and L. hypoglauca*, respectively. For the 51 microsatellites identified from *L. macranthoides*, 32 mono-nucleotide, 7 di-nucleotide, 4 tri-nucleotide, 8 tetra-nucleotide repeats were identified. No penta-nucleotide or hexa-nucleotide was found (Fig. 4a). Among these microsatellites, 6, 13, and 32 microsatellites were located in the intron, exon and intergenic regions (Fig. 4b). Of the 54 microsatellites identified from the *L. confusa* and *L. fulvotnetosa*, 36 mono-nucleotide, 4 di-nucleotide, 2 tri-nucleotide, 8 tetra-nucleotide and 4 hexa-nucleotide repeats were identified. No penta-nucleotide was found (Fig. 4a). Among these microsatellites, 4, 7, and 43 microsatellites were located in the intron, exon and intergenic regions (Fig. 4b). Of the 51 microsatellites identified from the *L. hypoglauca*, 35 mono-nucleotide, 5 di-nucleotide, 3 tri-nucleotide, 7 tetra-nucleotide and 1 hexa-nucleotide repeats were identified. No penta-nucleotide was found (Fig. 4a). Among these microsatellites, 3, 10, and 38 microsatellites were located in the intron, exon and intergenic regions (Fig. 4b). Furthermore, there were forward (76.0%, 68.0%, 70.0% and 56.0%), palindromic (24.0%, 30.0%, 30.0% and 42.0%), reverse (0.0%, 2.0%, 0.0% and 0.0%) and complement (0.0%, 0.0%, 0.0% and 2.0%) in *L. macranthoides, L. confusa, L. fulvotnetosa* and *L. hypoglauca*, respectively (Fig. 4c). Reverse repeats and complement repeats were only identified in *L. confusa* and *L. hypoglauca* (Fig. 4c), respectively.

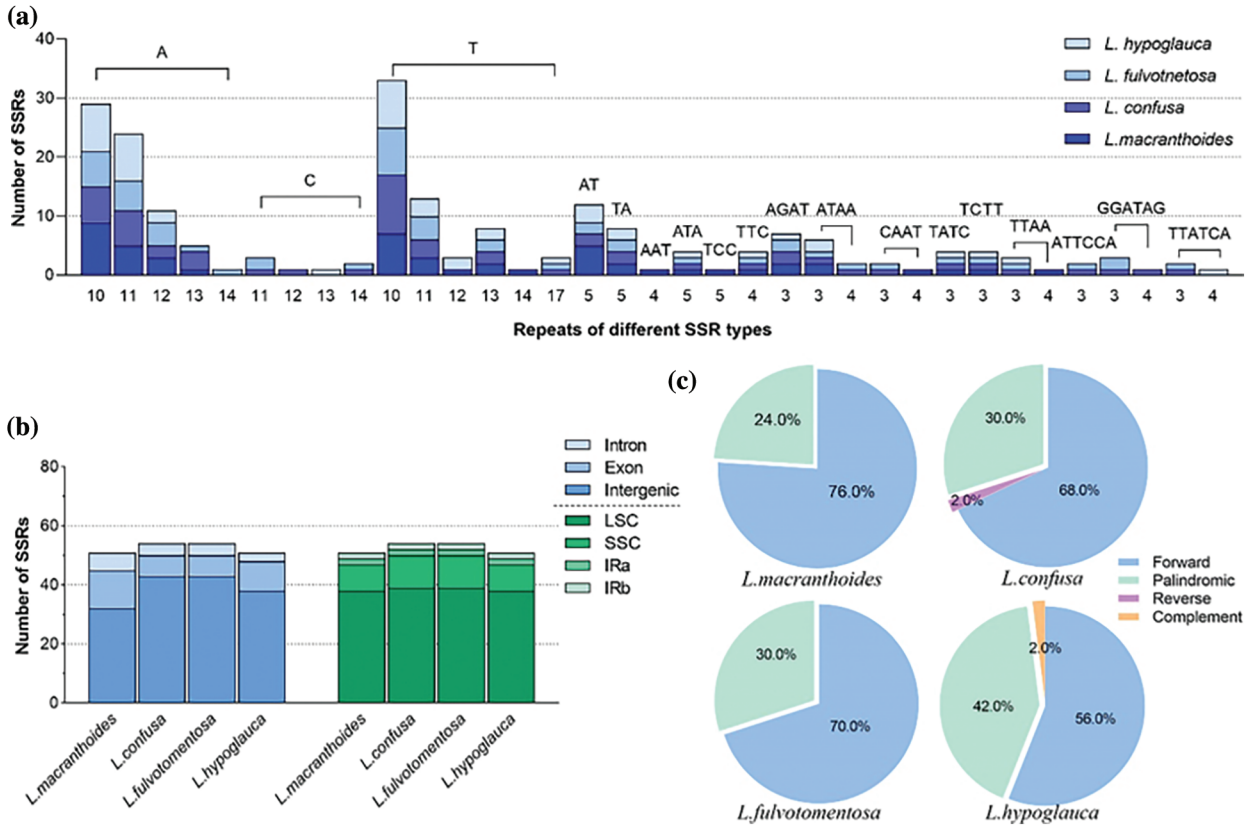

Figure 4: Statistics of Lonicerae Flos chloroplast genomes. (a) The number of repeat and repeated sequences; (b) Distribution of SSRs in the different regions; (c) The proportion of different SSR types

3.4 Phylogenetic Analysis

In this study, the phylogeny of *Lonicera* was reconstructed using four complete chloroplast genomes of LF species (*L. macranthoides, L. confusa, L. fulvotnetosa* and *L. hypoglauca*) and thirteen other species from the *Lonicera* genus. According to the phylogenetic tree (Fig. 5), two main clades can be identified, one of these includes four *Lonaria* species (96% bootstrap) and the remaining species, including the four species of interest, are located in the other clade (100% bootstrap). All four species of this study are included in the same clade (100% bootstrap) which is divided in two, with *L. confusa*, *L. fulvotomentosa* and *L. japonica* together in one clade, while *L. macranthoide* and *L. hypoglauca* along with *L. maximowiczii* are grouped in the other. In other words, the *L. confusa* is closer to *L. fulvotnetosa* and *L. macranthoides* is closer to *L. hypoglauca*. Compared to other species of *Lonicera* genus, the four origin plants of LF to be more closely related.

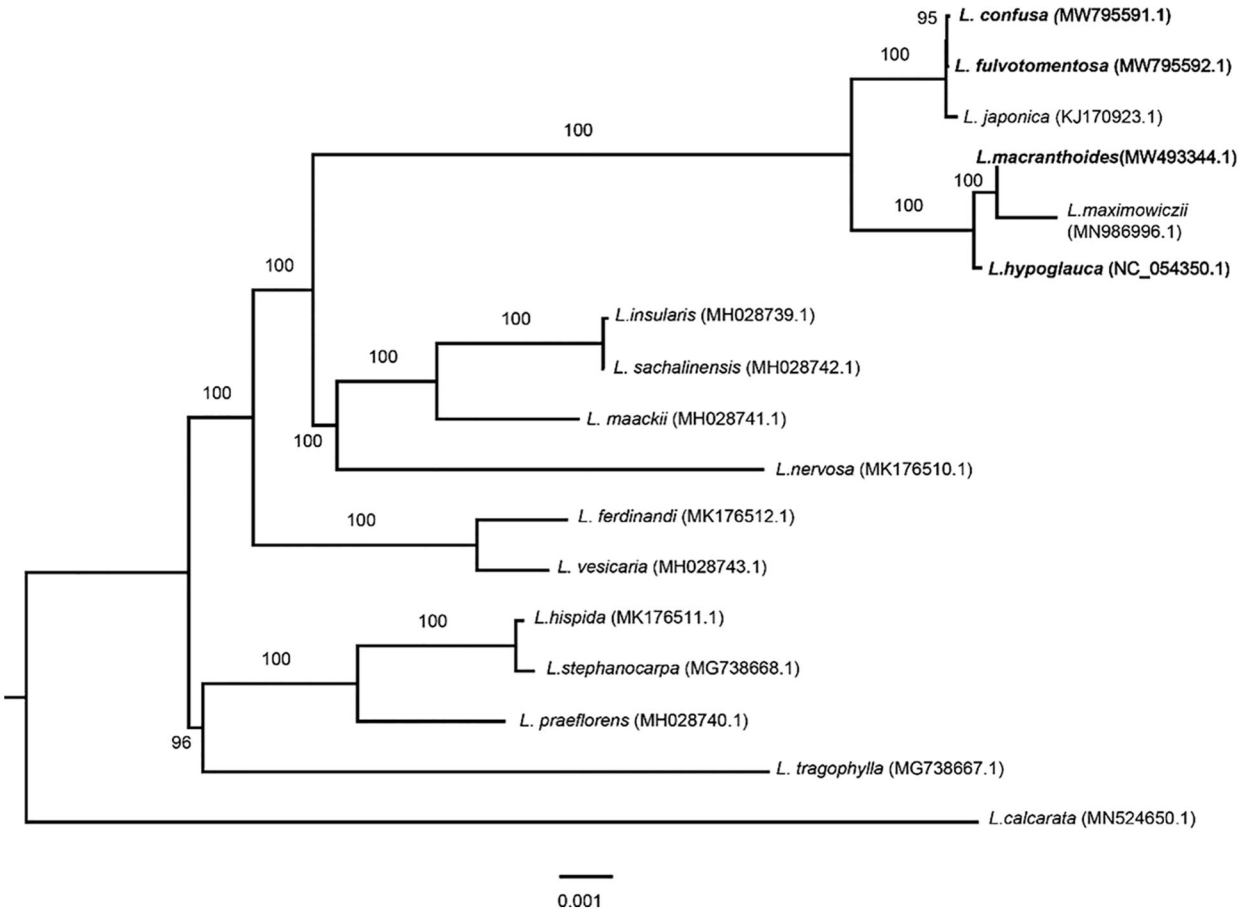

Figure 5: Phylogenetic analysis of seventeen *Lonicera* chloroplast genomes. The phylogenetic tree was built and edited by using IQTREE with maximum likelihood method and Figtree software. A total of 17 chloroplast sequences of *Lonicera* including *L. macranthoides* (MW493344.1)*, L. confusa* (MW795591.1)*, L. fulvotnetosa* (MW795592.1), *L. hypoglauca* (NC_054350), *L. japonica* (KJ170923.1), *L. maximowiczii* (MN986996.1), *L. insularis* (MH028739.1), *L. sachalinensis* (MH028742.1), *L. maackii* (MH028741.1), *L. nervosa* (MK176510.1), *L. ferdinandi* (MK176512.1), *L. vesicaria* (MH028743.1), *L. hispida* (MK176511.1), *L. stephanocarpa* (MG738668.1), *L. praeflorens* (MH028740.1), *L. tragophylla* (MG738667.1), and *L. calcarata* (MN524650.1)

4 Conclusions

Although chloroplast genomes of four origin plants of LF (*L. macranthoides, L. confusa, L. fulvotnetosa* and *L. hypoglauca*) have been reported [17,36,37], comparative analysis of the four chloroplast genomes for the first time in this study. In addition, the results of chloroplast genome assembly and annotation were different due to different plant varieties, sequencing platforms and assembly methods, the results of this study will be complementary. There is small difference in the feature of chloroplast genome among the four *Lonicera* plants, such as size of LSC, SSC and IR, the number of PCGs, tRNAs and rRNAs, GC content, etc. Within four origin plants of LF, the four chloroplast genomes showed low diversity (Pi = 0.00267), meanwhile, there are three variation hotspots regions which including *rbcL-accD*, *rps12-ndhF* and *rps12-trnN-trnG* were found. To find more differences, SSR analysis was performed. The results showed two obvious differences among these four chloroplast genomes. One is the percentage of microsatellites were located in the intron, exon and intergenic regions (Fig. 4b), and the other is the

proportion of different SSR types. Reverse, complement repeats were only identified in *L. confusa* and *L. hypoglauca* (Fig. 4c), respectively. Additionally, the chloroplast genome sequences of 17 *Lonicera* species were constructed the genetic phylogenetic analysis based on maximum likelihood method. According to the phylogenetic tree, four origin plants of LF (*L. macranthoides, L. confusa, L. fulvotnetosa* and *L. hypoglauca*), *L. japonica* and *L. maximowiczii* have a closer relationship.

The differences among the four origin plants of LF were evident in the genetic structure and repetitive sequences. Although *L. confusa* and *L. fulvotnetosa*, *L. macranthoides* and *L. hypoglauca* have the same number of microsatellites and coding regions without rearrangement, *L. confusa, L. fulvotnetosa* and *L. hypoglauca* have a large gap between *rbcL* and *accD* genes with seven small gaps compared to the *L. macranthoides*. Then by analyzing the SSR type, reverse and complement repeats were only found in *L. confusa* and *L. hypoglauca*, respectively. In order to better control the quality of traditional Chinese medicine, we need to identify the origin plants of Chinese medicinal materials quickly and accurately. Unquestionably, these differences were found in this study will benefit of the development of molecular markers to identify the origin of LF. Meanwhile, these results will provide more genetic information for molecular assisted breeding of LF.

Traditional Chinese medicine Lonicerae Japonicae Flos (LJF, Jinyinhua in Chinese) is dried flower buds or the flower with opening of *L. japonica*. Because LJF and LF both have the same pharmacologic effects and extremely similar appearances, there are easily confused, abuse and other phenomena [38–40]. As can be seen from Fig. 5, LF chloroplast genomes were classified into two branches, *L. japonica* was clustered into a branch with *L. confusa* and *L. fulvotnetosa*, however, *L. macranthoides* and *L. hypoglauca* were clustered into a branch with *L. maximowiczii*. The phylogenetic analysis showed that *L. japonica* have more closer relationship with *L. confusa* and *L. fulvotnetosa* than *L. macranthoides* and *L. hypoglauca*. Most current studies showed that it is difficult to point out their similarities or differences in-depth [41–43]. Therefore, studies on genetic diversity, relationships, bioactive compounds and modern pharmacological effects should be highlighted.

Author Contributions: Sisi Liu and Yongxin Li conceived and designed the experiments. Sisi Liu and Lisi Zhou performed the experiment and analyzed the data. Zhongquan Qiao provided technical guidance. Sisi Liu drafted the manuscript. Jiaoli Huang, Huijie Zeng and Gang Zhang revised the manuscript. All authors reviewed the manuscript.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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