Article

The Complete Chloroplast Genomes of Two Lancea Species with Comparative Analysis

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Abstract: The genus Lancea is native to the Qinghai-Tibetan Plateau and consists of two species, Lancea tibetica Hook. f. et Thoms. and Lancea hirsuta Bonati. Here, we report the complete sequences of the chloroplast genomes of L. tibetica and L. hirsuta, which were 153,665 and 154,045 bp in length, respectively, and each included a pair of inverted repeated regions (25,624 and 25,838 bp in length, respectively) that were separated by a large single copy region (84,401 and 84,588 bp in length, respectively) and a smaller single copy region (18,016 and 17,781 bp in length, respectively). A total of 106 genes in L. tibetica and 105 in L. hirsuta comprised 79 protein-coding genes, and 4 ribosomal RNA (rRNA) genes, as well as 23 and 22 transfer RNA (tRNA) genes in L. tibetica and L. hirsuta, respectively. The gene order, content, and orientation of the two Lancea chloroplast genomes exhibited high similarity. A large number of informative repetitive sequences, including SSRs, were observed in both genomes. Comparisons of the genomes with those of three other Lamiales species revealed 12 highly divergent regions in the intergenic spacers and in the matK, rpoA, rps19, ndhF, ccsA, ndhD, and ycf1 coding regions. A phylogenomic analysis suggested that Lancea forms a monophyletic group that is closely related to the clade composed of the families Phrymaceae, Paulowniaceae, and Rehmanniaceae.

Keywords: Mazaceae; Laminales; organellar genome; phylogenetic analysis

1. Introduction

Chloroplasts, which originated from ancient endosymbiotic cyanobacteria, are specialized photosynthetic organelles for photosynthesis and carbon fixation as well as fatty acid synthesis, amino acid synthesis, and the immune response in plants [1]. Chloroplasts also possess their own genomes and genetic systems. Most chloroplast genomes range from 120 to 160 kb in length and have a typical quadripartite structure with two copies of inverted repeats (IRs) separating the large single copy (LSC) region and the small single copy (SSC) region [2]. Recently, lots of fragments from chloroplast genomes such as rbcL and matK have been widely used in plant systematics research due to its maternal inheritance and highly conserved structures [3]. With the reduction of sequencing cost, the complete chloroplast genome sequences are becoming an increasingly used and effective tool in the study of plant phylogenetic classification, molecular identification and genetic diversity [4]. The comparative analysis of chloroplast genomes is especially useful for inferring new and important insights to resolve many enigmatic phylogenetic relationships for their relatively stable genome structure, gene content, and gene order.

Lancea is a small genus consisting of two species, Lancea tibetica Hook. f. et Thoms. and Lancea hirsuta Bonati, both of which are native to the Qinghai-Tibetan Plateau. The main morphological
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differences between L. tibetica and L. hirsuta are their respective absence and presence of coarse
multicellular hairs on their stems and leaves [5]. L. tibetica is found along streams in grasslands and
sparse forests at an approximate altitude of 2000–4500 m above sea level in Gansu, Qinghai, Sichuan,
and the Tibet region of China as well as Bhutan, India, and Nepal. However L. hirsuta is exclusively
demic to northwest Sichuan, northwest Yunnan, southeast Qinghai and the Tibet region of China [6].
L. tibetica also is an important traditional Tibetan medicine that has been used in the treatment of
leukemia, intestinal angina, heart disease, and coughs [7–9].

The systematic position of Lancea has been debated for years. Wettstein (1891) placed Mazus,
Dodartia, and Lancea in the tribe Gratioleae, subtribe Mimulinae based on their morphological traits [10].
However, recent molecular studies have indicated that the Scrophulariaceae are not monophyletic [11].
Lancea was then transferred to Phrymaceae according to the phylogenetic analysis of chloroplast trnL/F
and nuclear ribosomal ITS and ETS sequence data [12]. However, no support was found for the
monophyly of Phrymaceae. The two subfamilies of Phrymaceae-Phrymoideae and Mazoideae do not
form a monophyletic clade in any of the trees in different studies [13]. Consequently, the Angiosperm
Phylogeny Group IV (APG IV) classification system separated Lancea, Mazus, and Dodartia from
Phrymaceae, placing them into a new family named Mazaceae, which is the outgroup to Paulowniaceae
and Orobancheaceae [14]. Although chloroplast and/or nuclear DNA data provide some information
about the taxonomy of Lancea, different phylogenetic relationships were inferred when phylogenies
were constructed using different sequence fragments, thus requiring confirmation by the use of
comprehensive genomic data [11–13,15–17]. Until now, comparative genomics approaches to the study
of genetic diversity and phylogenetics in Mazaceae has been limited.

In the present study, we report the complete chloroplast genomes of L. tibetica and L. hirsuta, which
were derived using next-generation sequencing. Genomic information about L. tibetica and L. hirsuta is
fundamental to supporting current conservation efforts especially phylogenetic analysis of these rare
species. Our aim was to compare the full chloroplast genomes of these two species, which will serve
as valuable genomic resources.

2. Materials and Methods

2.1. DNA Extraction and Sequencing

L. tibetica was sampled from a single plant collected in Qumalai (96°34′38.8″ E, 33°58′03.1″ N,
Qinghai, China), while a single sample of L. hirsuta was collected from Zaduo (95°00′16″ E, 32°51′51″ N,
Qinghai, China). The DNA of the two species was isolated from fresh leaves via the modified CTAB
method [18]. The DNA content was measured using a NanoDrop spectrophotometer (Thermo Scientific,
Carlsbad, CA, USA). Each DNA sample was randomly fragmented to construct paired-end libraries
according to the Illumina preparation manual (San Diego, CA, USA). We sequenced the complete
chloroplast genomes using the Illumina MiSeq platform at Novogene Biotech Co. (Beijing, China).

2.2. Chloroplast Genome Assembly and Annotation

Genomic sequences were screened and assembled with SOAPdenovo [19]. To test the assemble
accuracy around IR-LSC/SSC junctions, four primers listed in Table S1 were used to amplify the
junctions of IRs and the LSC/SSC. These PCR products were analyzed via Sanger sequencing using the
primers mentioned above. Annotation was performed with CpGAVAS (http://www.herbalgenomics.
org/cpgavas) [20] coupled with manual adjustment of start/stop codons and intron/exon borders
after BLAST searches. The gene homologies were confirmed by comparing them with NCBI’s
non-redundant (Nr) protein database, Clusters of orthologous groups for eukaryotic complete genomes
(KOG), KEGG (http://www.kegg.jp/), GO (http://www.geneontology.org), PFAM (http://xfam.org),
SWISS-PROT (http://web.expasy.org/docs/swissprotguideline.html), and TREMBL (http://www.
bioinfo.pte.hu/more/TrEMBL.htm) databases. TRNAscan-SE 1.21 were introduced to confirm the
transfer RNAs (tRNAs). The circular maps of the two *Lancea* chloroplast genomes were drawn with OrganellarGenomeDRAW (OGDRAW; http://ogdraw.mpimp-golm.mpg.de/index.shtml) [21]. The annotated genomic sequences have been submitted to GenBank under accession numbers MF593117 and MG551489 for *L. tibetica* and *L. hirsuta*, respectively.

2.3. Repeat Structure Analysis

Dispersed and palindromic repeats were identified by the REPuter program (http://bibiserv2.cebitec.uni-bielefeld.de/reputer) [22]. The minimal size was set to 30 bp with >90% identity (Hamming distance equal to 3) between the two repeats. MSDB 2.4 (https://code.google.com/archive/p/msdb/downloads) [23] was used to detect simple sequence repeats (SSRs) with minimal repeat numbers of 10, 5, 4, 3, 3, and 3 for mono-, di-, tri-, tetra-, penta-, and hexa-nucleotides, respectively.

2.4. Genome Comparison

The mVISTA program (http://genome.lbl.gov/vista/mvista/about.shtml) [24] was employed in Shuffle-LAGAN mode to determine differences in the chloroplast genomes of *L. tibetica* and *L. hirsuta* with those of *Rehmannia chingii* (KX426347), *Paulownia coreana* (KP718622), and *Erythranthe lutea* (KU705476), all of which were obtained from GenBank. The nucleotide variability (average pairwise divergence) between the *L. tibetica* and *L. hirsuta* chloroplast genomes was calculated using DnaSP v5.10 (http://www.ub.edu/dnasp/DnaSP_OS.html) [25] with a sliding window analysis. Window length was set to 400 bp, and the step size was 200 bp.

2.5. Phylogenetic Analysis

Phylogenetic analysis was performed among *L. tibetica*, *L. hirsuta*, and 21 outgroup Lamiales species (Table S2) on sequence alignments in two ways; one on the complete chloroplast genome sequences, and the other on 75 protein-coding genes. *Lactuca sativa* (Asteraceae) was used as the outgroup. First, sequences were aligned using MAFFT v7.0 (http://mafft.cbrc.jp/alignment/server/) [26]. Then, jModelTest2 implemented on XSEDE (2.1.6) at the CIPRES Science Gateway (http://www.phylo.org/) was used to select the best model for maximum likelihood (ML) and standard Bayesian inference (BI) analysis. ML analysis was implemented using RAxML-HPC2 on XSEDE (8.2.10) based on the GTR + G + I nucleotide substitution model as recommended by jModelTest2 with 1000 replications. Similarly, BI analysis was constructed by MrBayes on XSEDE (3.2.6) based on the GTR + G + I nucleotide substitution model. Two independent Markov chain Monte Carlo (MCMC) chains were run for 10,000,000 generations and sampled every 1000 generations with the first 25% of calculated trees was discarded as burn-in. All the generated trees were modified by Interactive Tree Of Life (iTOL, http://itol.embl.de/) [27].

3. Results

3.1. Characteristics of the Chloroplast Genomes

The *L. tibetica* and *L. hirsuta* chloroplast genomes were 153,655 bp and 154,045 bp in length, respectively. The genomes were like those of most angiosperms with a typical quadripartite structure consisting of a pair of inverted repeats (IRs) of 25,624 bp in *L. tibetica* and 25,838 bp in *L. hirsuta*, a large single copy (LSC) region of 84,401 bp in *L. tibetica* and 84,588 bp in *L. hirsuta*, and a small single copy (SSC) region of 18,016 bp in *L. tibetica* and 17,781 bp in *L. hirsuta* (Figure 1, Table 1). The GC content of the genomes were both 37.9%, but the IR regions had higher GC contents (43.3% and 43.2% in *L. tibetica* and *L. hirsuta*, respectively) than that of the LSC regions (35.9% and 35.8% in *L. tibetica* and *L. hirsuta*, respectively) and SSC regions (30% and 32% in *L. tibetica* and *L. hirsuta*, respectively).
Figure 1. Gene map of the two Lancea chloroplast genomes. Genes belonging to different functional groups are color-coded. Genes drawn inside the circle are transcribed clockwise, while outside are counterclockwise. Nucleotide position 1 was indicated by the red arrow and the sequence was in counterclockwise. Gene trnT-UGU was not found in L. hirsuta.

Table 1. The basic chloroplast genome characteristics of Lancea tibetica and L. hirsuta.

| Characteristics                      | L. tibetica | L. hirsuta |
|--------------------------------------|-------------|------------|
| Total cpDNA size (bp)                | 153,664     | 154,045    |
| Length of large single copy (LSC)    | 84,401      | 84,254     |
| Length of inverted repeat (IR) region| 25,624      | 25,838     |
| Length of small single copy (SSC)    | 18,016      | 17,781     |
| Total GC content (%)                 | 37.9        | 37.9       |
| LSC                                  | 35.9        | 35.8       |
| IR                                   | 43.3        | 43.2       |
| SSC                                  | 30.0        | 32.0       |
| Total number of genes                | 106         | 105        |
| Protein-coding genes                 | 79          | 79         |
| tRNAs genes                          | 4           | 4          |
| tRNAs genes                          | 23          | 22         |

The gene content, order, and orientation were similar across the two Lancea genomes. There were 106 predicted genes in L. tibetica including 79 protein-coding genes, 23 tRNA genes, and 4 rRNA genes, while the 105 genes predicted in L. hirsuta consisted of 79 protein-coding genes, 22 tRNA genes, and
4 rRNA genes (Table 2). Among the protein-coding genes, 63 were located in the LSC region, 11 were in the SSC region, and 6 genes (ndhB, rpl2, rpl23, rps7 and ycf2) were duplicated in the IR regions. There were 13 intron-containing protein-coding genes, two of which (ycf3 and clpP) contained two introns. As in most other land plants, the rps12 gene was a trans-spliced gene, with its 5′ end located in the LSC region and its duplicated 3′ ends in the IR regions. The ndhD gene contained the alternative ACG start codon, while rps19 started with GTG, which are common features of most homologous genes in the chloroplast genomes of other plants [28–32]. Approximately 54.8% of Lancea chloroplast genomes consisted of protein-coding genes (84,254 bp in L. tibetica and 84,474 bp in L. hirsuta), 1.4% of tRNAs (2198 bp L. tibetica and 2131 bp in L. hirsuta), and 6.1% of rRNAs (9396 bp in both species). As such, the non-coding regions consisting of introns, pseudogenes, and intergenic spacers accounted for 37.7% of the both genomes.

**Table 2.** Genes present in *Lancea tibetica* and *L. hirsuta* chloroplast genomes.

| Category | Name |
|----------|------|
| Rubisco  | rbcL |
| Photosystem I | psaA, B, C, I, J |
| Photosystem II | psbA, B, C, D, E, F, H, I, J, K, L, M, N, T, Z |
| ATP synthase | atpA, B, E, * F, H, I |
| Cytochrome b/f complex | petA, * B, * D, G, I, N |
| Cytochrome c synthesis | ccsA |
| NADPH dehydrogenase | * ndhA, *-* B, C, D, E, F, G, H, I, J, K |
| Transcription | rpsA, B, * Cl, C2 |
| Small subunit ribosomal proteins | rps2, 3, 4, * 7, 8, 11, ** 12, 14, 15, * 16, 18, 19, |
| Large subunit ribosomal proteins | rpl2, 14, * 16, 20, 22, * 23, 32, 33, 36 |
| Translation initiation factor | infA |
| Ribosomal RNA | * trn4.5, * 5, * 16, * 23 |
| RNA processing | matK |
| Carbon metabolism | ccmA |
| Fatty acid synthesis | accD |
| Proteolysis | ** clpP |
| Unknown function protein-coding gene | ycf1, 2, ** 3, 4 |
| Transfer RNA | trnC-GCA, trnD-GUC, trnE-UUC, trnF-GAA, trnG-GCC, trnH-GUG, |
| | * trnI-CAU, * trnL-CAA, trnL-UAG, * trnM-CAT, * trnN-GUU, |
| | trnP-UCC, trnQ-UUG, * trnR-AAC, trnR-UU, trnS-GCU, trnS-GGA, |
| | trnS-UGA, trnT-GGU, * trnT-UGU, * trnV-GCA, trnW-CCA, trnY-GUA |

* gene with one intron, ** gene with two introns, * gene with two copies, b gene not found in *L. hirsuta*.

### 3.2. Repeat and SSR Analysis

Total of 22 forward repeats and 24 palindromic repeats were found in the *L. tibetica* genome, while there were 20 forward repeats and 28 palindromic repeats in *L. hirsuta* (Figure 2A, Tables S3 and S4). The majority of repeats ranged in size from 30 to 44 bp, and the longest palindromic repeat (410 bp) was found in *L. hirsuta*. The repeats were mostly distributed in the intergenic spacers (IGS) and intron sequences, but eight repeats were also found in the coding sequences (CDSs) of psaB, ycf3, rps19, ndhB, ndhA and ycf2.

Simple sequence repeats (SSRs) are another important type of repeated sequence in genomes that are particularly useful molecular markers in genetic diversity research. A total of 50 perfect microsatellites, 574 bp in length, were detected in *L. tibetica* chloroplast genome, and there were 37, 3, 2, 4, 1, and 3 mono-, di-, tri-, tetra-, penta- and hexa-nucleotides repeats, respectively (Table S5). In *L. hirsuta*, the 46 SSRs with totally 496 bp in length included 37, 2, 2, 4, and 1 mono-, di-, tri-, tetra-, and penta-nucleotide repeats, respectively (Table S6). No hexa-nucleotide repeats were found in *L. hirsuta*. Most SSRs were located in non-coding regions, especially in the LSC region. AT content comprised 86% and 87% of SSRs in *L. tibetica* and *L. hirsuta*, respectively.
was the last gene, with 1607 bp between the spacer and the ends of the IRa regions. Overall, contraction of the SSC/IRa junctions in all five chloroplast genomes were crossed by repeated sequences. Similar results have been observed in previous studies [29,32].

3.3. IR Contraction and Expansion

Contraction and expansion at the borders of IR regions have been commonly reported in chloroplast genomes, which may explain the apparent size differences between chloroplast genomes [17]. Accordingly, the inferred assembly was checked to confirm contraction and expansion. Although the IR region of the five chloroplast genomes was highly conserved, structure variation was still found in the IR/SC boundary regions. As shown in Figure 3, the rps19-rp12 gene was located in the junctions of the LSC/IRb regions. The rps19 gene crossed the LSC/IRb region with 3–52 bp located in the IRb region. The ycf1-ndhF gene was located at the junctions of the IRb/SSC regions, though the trnN-ndhF sequence in E. lutea was missing the ycf1 gene in the IRb region. The ycf1 genes of R. chingii, L. tibetica, and L. hirsuta spanned the IRb and SSC regions, with 3–130 bp in the SSC region. The ndhF gene in P. coreana extended into the LSC region and overlapped with the ycf1 gene by 42 bp, while in E. lutea it extended 36 bp into the LSC region. The SSC/IRa junctions in all five chloroplast genomes were crossed by ycf1, with 751–1084 bp in the IRa region. Like the IRb/SSC boundary regions, the LSC/IRa regions were also variable. The rpl2-trnH genes of R. chingii, L. tibetica, and L. hirsuta were located at the junctions of IRa/LSC regions with 0, 114, and 106 bp, respectively, separating the spacer from the ends of the IRa regions. However, in P. coreana, the rps19 pseudogene was at one end of the IRa region. In E. lutea, the rpl2 gene was missing, and rpl23 was the last gene, with 1607 bp between the spacer and the ends of the IRa regions. Overall, contraction and expansion of the IR regions was detected across the five chloroplast genomes.

3.4. Sequence Divergence and Divergence Hotspot

To characterize genome divergence, we performed multiple sequence alignments between the five chloroplast genomes using the program mVISTA, with R. chingii being used as a reference (Figure 4). The comparison demonstrated that the coding regions are more conserved than the non-coding regions. In particular, the IR regions were less divergent than the LSC and SSC regions. The most highly divergent regions among the five chloroplast genomes were found among the intergenic spacers, including trnH-psbA, matK-rps16, rps16-psbK petN-psbM, psbZ-rps14, psaA-ycf3, rps4-ndhJ, ndhC-atpE, petA-psbJ, and ycf4-cemA in LSC as well as rpl32-ccsA and ndhG-ndhI in SSC. More divergence of coding regions was found in the matK, rpoA, rps19, ndhF, ccsA, ndhD, and ycf1 sequences. Similar results have been observed in previous studies [29,32].

Figure 2. Repeated sequences in Lancea chloroplast genomes. (A) Number of three repeat types within chloroplast genomes; (B) SSR type distribution within Lancea chloroplast genome.
Figure 3. Comparison of the borders of large single-copy (LSC), small single-copy (SSC), and inverted repeat (IR) regions among the chloroplast genomes of five species.

Figure 4. Comparison of five chloroplast genomes using the mVISTA alignment program with *Rehmannia chingii* as a reference. The x-axis represents the coordinates in the chloroplast genome. The y-axis indicates the average percent identity of sequence similarity in the aligned regions, ranging between 50% and 100%. Genome regions are color coded as protein coding, rRNA coding, tRNA coding or conserved noncoding sequences (CNS).
Nucleotide variability (pairwise divergence) was calculated to show divergence at the sequence level between the two *Lancea* chloroplast genomes. Between *L. tibetica* and *L. hirsuta*, the pairwise divergence values ranged from 0 to 0.09, with a mean of 0.00221. As shown in Figure 5, the IR regions were more conserved than the LSC and SSC regions. The most divergent region, *rps4-ndhJ*, showed a pairwise divergence value of 0.09 in the LSC region. The *petB* gene in the LSC region showed the highest degree of nucleotide variability, with a pairwise divergence of 0.0467. The low divergence values between the *L. tibetica* and *L. hirsuta* chloroplast genomes illustrated high similarity between the two species.

![Figure 5](image_url)

**Figure 5.** Sliding window analysis of nucleotide variability (pairwise divergence) between *Lancea tibetica* and *L. hirsuta*.

3.5. Phylogenomic Analysis

*Lancea* was traditionally placed in Scrophulariaceae. Recent studies have reported its phylogenetic relationship among other genera in the Lamiales based on chloroplast and/or nuclear ribosomal sequence data. However, the position of *Lancea* was still unclear, and thus required confirmation with additional data. In our present studies, the ML and BI analysis of the complete chloroplast genomes and 75 protein-coding genes showed that the two *Lancea* species were clustered into one monophyletic group (Figure 6). Alignment of the complete chloroplast genome sequences gave an obvious conflict between ML phylogenetic trees and BI phylogenetic trees, which may be caused by rapidly evolving and potentially poorly aligned sites [4,33,34]. On the other hand, alignment of 75 protein-coding genes strongly supported the *Lancea* genus as sister to a clade formed by Phrymaceae, Paulowniaceae, and Rehmanniaceae, rather than the Scrophulariaceae clade both in ML and BI phylogenetic trees. The relationships supported by our analysis are basically consistent with APG IV [14]. As there is a lack of published chloroplast genomes from the Mazus, Dodartia, and Phrymaceae taxa, the phylogenetic placement of Mazaceae and Phrymaceae remains uncertain.
Figure 6. Phylogenetic trees of 24 species based on complete chloroplast genomes and 75 protein-coding genes. (A) Maximum likelihood (ML) phylogenetic tree constructed with complete chloroplast genomes; (B) Bayesian inference (BI) phylogenetic tree constructed with complete chloroplast genomes; (C) ML phylogenetic tree constructed with 75 protein-coding genes; (D) BI phylogenetic tree constructed with 75 protein-coding genes. The *Lancea* species are shown in red.

4. Discussion

Using next-generation sequencing data, two complete *Lancea* chloroplast genomes were assembled, annotated, and analyzed. In the future, we plan to analyze chloroplast genomes of the genera *Mazus* and *Dodartia*, which were placed in Mazaceae in APG IV [14], in order to elucidate the phylogenetic relationships between *Lancea* and those species. Hence, the comprehensive data presented in this study not only characterizes the entire *Lancea* chloroplast genomes and enables the inference of their phylogenetic relationships, but also offers a valuable resource for future studies.

Supplementary Materials: Supplementary materials are available online. Table S1: List of all pairs of primers used for assembly validation; Table S2: The list of accession numbers of the chloroplast genome sequences used in the phylogenetic analysis; Table S3: Long repeat sequences in the *Lancea tibetica* chloroplast genome; Table S4: Long repeat sequences in the *Lancea hirsuta* chloroplast genome; Table S5: Distribution of SSRs in the *Lancea tibetica* chloroplast genome; Table S6: Distribution of SSRs in the *Lancea hirsuta* chloroplast genome.

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**Sample Availability:** Sequence data of two *Lancea* species are available from the authors.

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