Identification of evolutionary relationships and DNA markers in the medicinally important genus *Fritillaria* based on chloroplast genomics

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Abstract: Genus *Fritillaria* has attracted attention because of its medicinal and ornamental values. At least three reasons, including the accurate discrimination between various *Fritillaria* species, protection and sustainable development of rare *Fritillaria* resources as well as understanding of relationship of some perplexing species, have prompted phylogenetic analyses and development of molecular markers for *Fritillaria* species. Here we generated complete chloroplast (CP) genomes for *F. unibracteata*, *F. przewalskii*, *F. delavayi* and *F. sinica* through Illumina sequencing, followed by *de novo* assembly. The lengths of the genomes ranged from 151,076 in *F. unibracteata*) to 152,043 in *F. przewalskii*. Those CP genomes displayed a typical quadripartite structure, all including a pair of inverted repeats (26,078 to 26,355 bp) separated by the large single-copy (81,383 to 81,804 bp) and small single-copy (17,537 to 17,569 bp) regions. *Fritillaria*, *przewalskii*, *F. delavayi* and *F. sinica* equivalently encodes 133 unique genes consisting of 38 transfer RNA genes, 8 ribosomal RNA genes and 87 protein coding genes, whereas *F. unibracteata* contained 132 unique genes due to absence of the *rps16* gene. Subsequently, comparative analysis of the complete CP genomes revealed that *ycf1*, *trnL*, *trnF*, *ndhD*, *trnN-trnR*, *trnE-trnT*, *trnN*, *psbM-trnD*, *atpI* and *rps19* to be useful as molecular markers in taxonomic studies due to their inter-species variation. Based on the comprehensive CP genome data collected from 53 species in *Fritillaria* and *Lilium* genera, a phylogenomic study was carried out with three *Cardiocrinum* species and five *Amana* species as outgroups. *Fritillaria* genus and *Lilium* genus showed the closest relationship with a high support value, and the interspecific resolution within subgenus *Fritillaria* were much better than those of the phylogenetic trees in terms of the separate regions. The geographical distribution pattern of the 11 medicinal species appeared to map on the phylogenetic relationship based on CP genomes. Furthermore, phylogenetic analysis based on CP genome was a promising method to select potential novel medicinal resources to substitute current medicinal species that are on the verge of extinction. More importantly, the species-specific molecular identification for *F. taipaiensis*, *F. unibracteata* and *F. cirrhosa*, were preliminarily developed, respectively.

Abbreviations: CP, chloroplast; IR, inverted repeat; ITS, internal transcribed spacer; LSC, large single copy; SSC, small single copy; SSR, simple sequence repeats

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Introduction

The genus *Fritillaria* (*Liliaceae*), consisting of 140 known species, is widely distributed in Europe, Asia and North America (*Huang et al*., *2018*; *Rix et al*., *2001*). Based on the Flora of China, twenty-two species are distributed throughout most provinces in China, among which are four diversity hotspots (Xinjiang plain, East China plain, Hengduan Mountains and Northeast plain). *Fritillaria* species have attracted much attention because they are used in traditional Chinese medicine and sometimes as ornamental plants. Chinese Pharmacopeia (2020) reports dried bulbs from 11 species used in traditional Chinese medicine, which were divided into five main concoctions, including Chuan-Bei-mu (bulbs of the complex group of *F. cirrhosa*), Yi-Bei-mu (bulbs of *F. palilidiflora* and *F. walujewii*), Zhe-Bei-mu (bulbs of *F. thunbergii*), Ping-Bei-mu (bulbs of *F. ussuriensis*) and Hubei-Bei-mu (bulbs of *F. hupehensis*). Although the bulb of each original species has its own unique efficacy and bioactive compounds and should be used separately for given purposes in traditional prescription, various *Fritillaria* species were still used indiscriminately in clinical prescription due to their similar morphology and names. Especially, the morphological traits of the group that includes *F. cirrhosa*, including *F. cirrhosa*, *F. unibracteata*, *F. przewalskii*, *F. delavayi*, *F. taipaiensis* and *F. wabuensis*, were extremely complicated because of several highly variable characters including stem length, petal color, capsule winged or not, leaf curling; and scale number (*Luo et al*., *1996*). Therefore, it was vital to carry out taxonomic identification of various *Fritillaria* species.

At present, DNA-based classification is applied in angiosperm phylogeny because of their reliability (*Yang et al*., *2016*). Accurate identification (eg. using DNA markers) has been necessary to discriminate between the *Fritillaria* species and its adulterants. Secondly, since the bulbs of some *Fritillaria* species showed great economic value in Asian countries (*Yeum et al*., *2007*) and have long been used in traditional Chinese medicine, and due to this, wild *Fritillaria* populations decreased sharply because of long-term excessive harvesting. To date, four species of Chuan-Bei-mu and eight species in Xinjiang plain have been classified as rare resources based on the list of rare endangered higher plants of China (*Li et al*., *2018*). DNA markers have been helpful in understanding accurately genetic diversity and structure of *Fritillaria* population, and thus provided scientific approach for conservation requirement. Thirdly, a better understanding of the relationships within the genus could be of great significance for the medicinal use of *Fritillaria*. Some of the phylogenetically closest species might be analyzed for their potential medicinal values, and be used as substitutes to relieve survival pressure of species that are currently rare. Finally, the phylogenetic positions of some of the medicinal species of *Fritillaria*, such as *F. pallidiflora*, *F. wabuensis* and *F. davidii*, remain elusive. *Fritillaria pallidiflora* was always considered a member of subgenus *Fritillaria* by *Rix* (*2001*), whereas *Rønsted et al* (*2005*) linked it to subgenus *Petillium* based on the molecular and

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morphological analyses. *F. wabuensis* was discovered and nominated as a new species in *Fritillaria* (*Tang & Yue*, *1983*), but later it was classified as variant of *F. crassicaulis* (*Luo et al*., *1996*) and *F. unibracteata* (*Liu et al*., *2009*), respectively. Therefore, well resolved molecular phylogenies of *Fritillaria*, especially the medicinal species, were necessary.

Currently, the genus *Fritillaria* is divided into eight subgenera, including, *Liliorhiza* (including species mainly in North America), *Japonica* (including species mainly in Japan), *Fritillaria* (the biggest subgenus), *Rhinopetalum*, *Petilium*, and the monotypic *Davidii* (including only *F. davidii*), *Theresia* (only *F. persica*) and *Korolkowia* (only *F. sewerezowi*), by *Rix* (*2001*). At present, despite the frequent usage of nuclear DNA internal transcribed spacer (ITS) and several plastid genome regions (*trnL-trnF*, *matK*, *rbcL* and *rpl16*) in the classification of this genus, previous studies have found that these markers merely provided weak phylogenetic signal. In detail, *Rønsted et al* (*2005*), who primarily established the current understanding of evolutionary relationships within *Fritillaria*, investigated the phylogenetic position of 37 *Fritillaria* species using *matK*, *rpl16* intron and ITS. Consequently, *Fritillaria* was shown to be of two clades, in which one clade mainly included species from the North American subgenus *Liliorhiza* and the other clade comprised of species from the seven remaining subgenera. In common with the result of *Rønsted et al* (*2005*), *Khourang et al* (*2014*) revealed that the subgenus *Fritillaria* was sister to subgenus *Rhinopetalum* on the basis of the phylogenetic tree of nine Iranian species using the ITS and *trnL-trnF* regions. However, *Day et al* (*2014*) supported that the largest subgenus (subgenus *Fritillaria*) appeared to be polyphyletic and formed two clades with *matK* and *rbcL* sequences, in which one clade comprised taxa occurring mainly in Europe, the Middle East, Japan and North Africa, and the other clade comprised taxa distributing in China and Central Asia. In our previous research, various *Fritillaria* species from China were classified as North-China group and South-China group based on nrITS2 sequences, but 57.1 % species were not effectively resolved (*Zheng et al*., *2019*). Recently, *Li et al* (*2014*) presented high-quality chloroplast genome using single molecule real-time sequencing, and suggested that *rps19* gene varied greatest among various species. However, the noncoding regions showed higher variability and were potentially effective molecular markers. Therefore, it was proposed that genomics based on the entire chloroplast genome sequences might help identify molecular markers with higher resolution (*Xue et al*., *2019*).

The chloroplast (CP) genome has been extensively used for understanding phylogenetic relationships and discovering more effective molecular markers, some of which, such as *trnH-psbA*, *matK* and *rpl16*, have been used as universal plant DNA barcodes (*Bansala et al*., *2018*; Vinnersten & *Bremer*, *2001*). To date, the availability of 23 *Fritillaria* CP genomes in GenBank that can be used to enhance our understanding of the phylogenetic relationships and to identify molecular markers. Although previous reports (*Bi et al*., *2018*; *Chen et al*., *2019*; *Chen et al*., *2020*; *Huang et al*., *2020*; *Park et al*., *2017*) performed comparative analyses with *Fritillaria* CP genomes available on GenBank,

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species-specific identification has not been reported and the phylogenetic place of some ambiguous species remained elusive. At the initial stage of our study, the CP genomes of three important medicinal species (*F. unibracteata*, *F. przewalskii* and *F. delavayi*) and *F. sinica* have not been reported. The increasing CP genomes might not only provide a better phylogenetic analysis of this genus, but also promisingly develop species-specific identification method. Therefore, the CP genomes of these *Fritillaria* species were obtained using the Illumina platform in the present study. The objectives of this study included (1) analyzing the global structural patterns of the four CP genomes and comparing them with the available 23 CP genomes of *Fritillaria*; (2) assessing the phylogenetic relationships of the 11 medicinal species used in traditional Chinese medicine, by which to understand the phylogenetic position of some ambiguous species and find potential medicinal plants; and (3) obtaining candidate DNA markers (repeat sequences, SSRs, divergent regions and indels), and preliminarily developing species-specific identification of medicinal *Fritillaria* species in Chinese medicine market. To the best of our knowledge, we are the first to develop species-specific molecular identification of *Fritillaria* species.

Materials and methods

Plant material

The fresh leaves of *F. unibracteata*, *F. przewalskii*, *F. delavayi* and *F. sinica* were collected from the Huzhu County (36°50′15″N, 101°57′06″E), Xining city, Qinghai Province, respectively. The Huzhu County is located in north Hengduan Mountains and east of the Qinghai-Tibetan Plateau. All samples were immediately frozen in liquid nitrogen and stored at -80 °C until DNA extraction.

Chloroplast genome sequencing and assembly

Total genomic DNA was isolated from 100 mg of fresh leaves using a modified CTAB method. The DNA concentration (>50 ng μL^{-1}) was measured using a NanoDrop spectrophotometer. The isolated DNA was fragmented into small pieces using sonication. After end reparation and A-tailing, the short DNA fragments were ligated with the Illumina paired-end adaptors. Based on gel electrophoresis, the suitable fragments were purified and selected as templates for next-step PCR amplification, so as to create the final DNA library. The quality and quantity of the DNA library were measured using the Agilent 2100 Bioanalyzer. Finally, the library was sequenced from both the 5′ and 3′ ends using Illumina NovaSeq6000 PE150 Sequencing platform (Illumina, CA, USA). By use of NGSQCToolkit v2.3.3, the raw reads were filtered to remove the linker sequence and low-quality reads defined as having more than 10% bases with Q-value <20, and thus high-quality clean reads were obtained. The clean reads were then assembled using SPAdes (*Bankevich et al., 2012*) 3.10.1 (http://cab.spbu.ru/software/spades/) software with CP genome of *F. cirrhosa* as reference (NCBI accession number NC_024728.1). Finally, LSC/IR and SSC/IR junctions were further verified by

Sanger sequencing.

Genome annotation and sequence alignment

In order to predict putative gene function, the CDS, rRNA and tRNA genes were aligned using blast v2.2.25 (https://blast.ncbi.nlm.nih.gov/Blast.cgi), HMMER v3.1b2 (http://www.hmmer.org/) and aragorn v1.2.38 (http://130.235.244.92/ARAGORN/), respectively, with *E*-value of 10^{-5}. The OGDRAW (https://chlorobox.mpimp-golm.mpg.de/OGDraw.html) helped to make the chloroplast genome maps of *F. unibracteata*, *F. przewalskii*, *F. delavayi*, and *F. sinica*.

The vmatch v2.3.0 (http: //www.vmatch.de/) could identify their scattered repetitive sequences (*Askitis & Sinha, 2010*). MISA v1.0 (MIcroSAtellite identification tool, http://pgrc.ipk-gatersleben.de/misa/misa.html) helped to analyze CPSSR. The mafft v7.310 was used to perform and indel identification. (*Katoh & Standley, 2013*). After using the mafft to align the chloroplast genome sequences, BioEdit software was used to adjust the sequences manually (*Gupta et al., 2014*). DanSP v6.0 was used to perform sliding window analysis (step size =200 bp and window length=600 bp) for nucleotide variability (Pi) in the whole chloroplast genome (*Rozas et al., 2017*).

Phylogenetic analysis

The phylogenetic analysis was firstly performed based on *matK*, *psbA-trnH* and *rpl16*, respectively, by use of Neighbor Joining and Maximum Likelihood methods. Then, the chloroplast genomes in the phylogenetic analysis included the 27 species of *Fritillaria*, 26 species of *Lilium*, 3 species of *Cardiocrinum* and 5 species of *Amana*. The chloroplast genome evolutionary tree was constructed by BLAST2OGMSA script (https://github.com/fenghen360/BLAST2OGMSA) (*Bi, 2018*) and MEGA-X software (*Kumar et al., 2018*). Firstly, multi-sequence alignment was conducted using BLAST tool of NCBI (*Johnson et al., 2008*) and then, the initial alignment result was extracted by BLAST2OGMSA script to obtain homologous blocks. It was reported that BLAST2OGMSA relied on ProgressiveMauve, a kind of anchored alignment algorithm, to determine where locally collinear blocks (LCBs) represented the landmarks among organelle genomes (such as chloroplast and mitochondrial genomes). The co-exist LCBs among all organelle genomes were extracted and prepared for the further phylogenetic tree construction. In this study, the conserved CDS genes, functional non-coding regions, and rRNA genes as well were combined by BLAST2OGMSA. Finally, the alignment data from BLAST2OGMSA was imported into MEGA-X software to construct the phylogenetic tree using the Neighbor-Joining method and the Maximum likelihood method respectively.

Species-specific identification of *Fritillaria* species

After careful alignment on the CP genomes, three candidate indel markers for species-specific

test for *F. taipaiensis*, *F. unibracteata* and *F. cirrhosa* were verified preliminarily, respectively, using 11 species documented in the Chinese Pharmacopeia 2020 and 3 other species that were also frequently used in Chinese medicine market. Firstly, a unique indel with 137 bp deletion within intergenic space region, *accD-psaI*, of *F. taipaiensis* CP genome, made it a suitable target for developing species-specific test for *F. taipaiensis*. The test for *F. taipaiensis* was performed by routine PCR using 5'-GCG AAC GAG TAT TTA GTT CAT C -3' as former primer and 5'-AGG GTT CTT TCA CTC CTT TCT -3' as reverse primer. The routine PCR were performed under the following conditions (Table S1). All samples were performed in triplicate.

Similarly, two indels with 47 bp insertion and 6 bp deletion were also found within *trnG-GCC-trnR-UCU* and intron of *atpF* of *F. unibracteata* and *F. cirrhosa* CP genome respectively, were thus selected for development of real time PCR based marker. The species-specific tests for *F. unibracteata* and *F. cirrhosa* were performed by Taqman MGB real time PCR, respectively. The former primer (5'-GCT ACC CGC TTA ATA CAT AC-3'), reverse primer (5'-CCG GAA CAG ATC GAA CAG -3') and the probe (5'-FAM-CCA TTG TCT AAT GGA AAA GA-MGB-3') were used for identification of *F. unibracteata*. The former primer (5'-GCT ACC CGC TTA ATA CAT AC-3'), reverse primer (5'-CCG GAA CAG ATC GAA CAG -3') and the probe (5'-FAM-CCA TTG TCT AAT GGA AAA GA-MGB-3') were used for species-specific identification of *F. cirrhosa*. The TaqMan MGB real-time PCR was performed using 2×T5 Fast qPCR Mix (Qingke, China) with LightCycler 96 real-time fluorescence PCR instrument system (Roche) under the following conditions (Table S1). All samples were performed in triplicate.

Results

Genome sequencing, assembly, and genome features

Based on a stringent quality control, a total of 23,755,399 to 26,831,529 paired-end reads were obtained, generating 7,126,619,700 to 8,049,458,700 clean bases data, from the four *Fritillaria* species. The resultant clean paired-end reads were then employed to assemble the chloroplast genome using CP genome of *F. cirrhosa* as the reference. Totally, 471,385 to 652,632 mapping reads yielded average coverage of 934X to 1292X for each species, generating four full-length CP genomes that ranged from 151,076 in *F. unibracteata* to 152,043 in *F. przewalskii*. The CP genome contained identical structure including two IR regions (26,078 to 26,355 bp each), which were separated by one LSC region (81,383 to 81,804 bp) and one SSC region (17,537 to 17,569 bp) (Fig 1 and Table S2).

A total of 133 genes were annotated, including 87 protein-coding genes (PCGs), 38 tRNA and 8 rRNA genes. The global gene order and content were identical in the four species, except *F. unibracteata* that was lack of *rps16* gene. 21 genes were duplicated in the CP genome, including 8 tRNA genes, 4 rRNA genes and 9 PCGs. There were 13 genes containing intron, among which *clpP* and *ycf3* had two introns, whereas the other 13 genes had one intron. 8, 1, 4 and 2 introns were located

in the LSC, SSC, IRa and IRb region, respectively (Table 1 and Table S3). Table S3 listed the 15 intron-containing genes in the chloroplast genome of *F. unibracteata*, and those of *F. przewalskii*, *F. delavayi*, and *F. sinica* were included in Table S4, S5 and S6, respectively.

Four *Fritillaria* species had high sequence similarity (>90% identity). IR regions showed a lower level of sequence divergence than LSC and SSC regions. Contraction and expansion of IR regions, especially the boundary region, are important aspects of chloroplast genomes, which are the main reason of length variation in these genomes (*Abdullah et al*., *2020*). These 12 species have the same gene content and array in IR region, which is expanded in *rps19* and *ycf1* genes. The *rps19* gene in the 12 *Fritillaria* species crossed the LSC/IRb boundary and showed the same length of 279 bp which was similar to that of *Lilium superbum*, except that *F. cirrhosa* had *rps19* gene of 285 bp. In the LSC region, the length of *rps19* genes ranged from 250 to 268 bp, whereas that of *rps19* genes in the IRb region varied from 11 to 35 bp. Besides, the *rps19* genes lost their protein-coding function due to incomplete gene duplication. The similar event was also observed in the *ycf1* genes at the IRb/SSC border. The *ycf1* genes were largely located in the IRb and extended 16 to 32 bp into the SSC region, whereas the *ycf1* gene in *F. taipaiensis* was fully located in the IRb region, 58 bp from the IRb/SSC boundary. In the SSC/IRa boundary of 12 species of *Fritillaria* subgenus, *ycf1* was a key gene and almost equally distributed (Fig 2). *Ycf1* has a SSC region of 4320 bp in *F. unibracteata* and *F. przewalskii*, but 4314 bp in *F. delavayi*, *F. sinica*, *F. cirrhosa* and *F. taipaiensis*, and also has an IRa region of 1230 bp in all species. By comparing the LSC/IRb, SSC/IRa and IRa/LSC regions, it was found that there were obvious differences in IRb/SSC regions between the 12 *Fritillaria* species. The *ycf1* genes in *F. taipaiensis* and *F. cirrhosa* did not cross the IRb/SSC boundary, whereas those in other *Fritillaria* species extended 16 to 32 bp into the SSC region, which had a 16 to 32 bp overlap with *ndhF* gene.

Table 1

Fig. 1

Fig. 2

Repeat sequence, simple sequence repeats (SSRs) and divergent regions

The length of the repeat sequence distributed mainly from 15 to 20 bp and rarely from 21 to 38

bp among four *Fritillaria* species (Table S7). The repeating sequences were divided into forward repeating and palindrome sequences (including reverse and complementary sequences). The number of repeating sequences from 15 to 20 bp of *F. unibracteata* and *F. przewalskii* were more than 487, while those of *F. delavayi* and *F. sinica* were less than 350, respectively (Fig S1). The number of repeat sequences in *F. przewalskii*, *F. unibracteata*, *F. sinica* and *F. delavayi* were 1,200, 976, 656 and 425, respectively. Although repeat sequences from 21 to 38 bp were rare, several promising molecular markers were found. For instance, *F. unibracteata* had three forward repeating in repeat sequence at length of 23, 30 and 47 bp, respectively. *F. delavayi* also contained a palindrome in repeat sequence at length of 54 bp, and *F. przewalskii* contains two forward repeating sequences and a palindrome in repeat sequence at length of 23 bp.

We also found 77, 76, 75 and 72SSRs of at least 10 bp in *F. przewalskii*, *F. sinica*, *F. unibracteata* and *F. delavayi,* respectively (Table S7, Fig 3). These SSRs were mainly located in the LSC region, followed by 50 SSRs in IR region, and a few SSRs in the SSC region. The single- and three-nucleotide SSRs were the majority detected in these *Fritillaria* species, the double-and four-nucleotide SSRs were the minority detected and a few were five-nucleotide. Single- together with three-nucleotide repeats in *F. unibracteata*, *F. przewalskii*, *F. delavayi* and *F. sinica* accounted for 81.33%, 83.12%, 79.17%, and 81.58% of SSRs, respectively. The single-nucleotide SSR with eight to nine repeated units were the most abundant and accounted for 53.91% (Fig 3). The high variation of SSRs might provide abundant information for molecular marker studies and plant breeding.

Fig. 3

Using slide window analysis, 18 regions were eventually extracted to calculate the nucleotide variability with Pi value ranging from 0.0104 (*rpl12*) to 0.0159 (*ycf1*). 10 most divergent regions were identified and thus might be utilized as potential molecular markers for future phylogenetic analysis and species identification in genus *Fritillaria*. These regions included *ycf1*, *trnL*, *trnF*, *ndhD*, *trnN-trnR*, *trnE-trnT*, *trnN*, *psbM-trnD*, *atpI* and *rps19* (Fig S2). Due to its highest divergence, *ycf1* was used to construct phylogenetic tree in the following section.

Phylogenetic tree on the basis of CP genome

Prior to the phylogenetic analysis based on CP genomes, we attempted to construct phylogenetic trees based on three common DNA barcodes from CP genomes, including *matK*, *psbA-trnH* and *rpl16*. Also, the *ycf1* was used to construct phylogenetic tree. As a result, *matK*, *psbA-trnH* and *rpl16* obtained weakly supported trees, whereas phylogenetic tree based on *ycf1* was moderately supported as more than 50% and 60% branches got bootstrap of more than 90 BP using Neighbor joining (NJ)

(Fig S3) and Maximum Likelihood (ML) (Fig S4), respectively.

In addition, in comparison with four partial regions, the whole CP genomes obtained highly reliable phylogenetic tree. The CP genome matrix included the 27 species of *Fritillaria* genus and 26 of *Lilium* genus, with 3 of *Cardiocrinum* genus and 5 of *Amana* genus as outgroups. One average, (152,099) bp of the CP genome were aligned. The result of ML tree was similar to that of NJ tree (Fig S5). In the ML tree (Fig 4), the ingroup corresponding to *Fritillaria* and *Lilium* was strongly supported (100 BP), and sister to *Cardiocrinum*. In this analysis, *Lilium* was monophyletic (100 BP) and was sister to *Fritillaria* genus. Furthermore, *Lilium* was nested with *Fritillaria* with moderate bootstrap support (75 BP) than that (53 BP) of result of *Day et al*. *Fritillaria*, as the largest subgenus, was paraphyletic and majority of which fall in one strong supported Eurasain clade (A) except *F. maximowiczii* (subgenus *Liliorhiza*). Within the clade A, *F. davidii* appeared as successive sister taxa to the remaining Eurasian species (100 BP), which split into two well-supported clades. Clade A1 grouped with the monotypic subgenus *Rhinopetalum* (*F. karelinii*) as sister to two species from subgenus *Fritillaria* (*F. ussuriensia* and *F. meleagroides*), which occurred in North region of China. The sister clade (A2) was composed of the remaining 22 species that could be classified into two subclades (100 BP). Subclade B1 contained subgenus *Theresia* (*F. persica*) and subgenus *Petilium* (*F. eduardii*), which occur in the Middle East and Central Asia, while subclade B2 comprising subgenus *Fritillaria* includes 15 species from South China and five species (*F. tortifolia*, *F. verticillata*, *F. yuminensis*, *F. pallidiflora*, and *F. walujewii*) from Xinjiang plain (Fig 5). The 11 most valuable species used in traditional Chinese medicine were not included in monophyletic group, as *F. ussuriensis* was separated from the other 11 species. As a whole, the phylogenetic tree based on CP genome was highly supported, in which 91% (53 out of 58) branches obtained bootstrap values of more than 90 BP.

Fig. 4

Fig. 5

Preliminary species-specific test for *F. taipaiensis*, *F. unibracteata* and *F. cirrhosa*

As shown in Fig S6A, after routine PCR and electrophoresis, *F. taipaiensis* revealed a unique DNA band at length of 302 bp with limit of detection at 0.239 ng/μL (Lane 4), whereas the other *Fritillaria* species showed DNA band at length of 439 bp. As shown in Fig S6B, only *F. unibraceata* showed positive result and other *Fritillaria* species showed negative results, confirming an analysis specificity of 100%. Furthermore, the reactivity was detected at the limit of 0.1543ng/μL. Similarly, *F.*

cirrhosa showed unique positive result in Fig S6C with the limit of detection of 0.0145ng/μL.

Discussion

The overall structure of CP genome

With the development of *De novo* (Illumina) sequencing technology, the CP genome assembly has become cost-affordable and easier compared with previous Sanger method. Moreover, *De novo* sequencing technology has been widely used in transcriptome assembly in order to identify the biosynthetic and regulatory genes in traditional Chinese medicine, such as *Ligusticum chuanxiong* (*Song et al*., *2015*) and *Cassia obtusifolia* (*Deng et al*., *2018*). In this research, four new CP genomes of *Fritillaria* were obtained using *De novo* sequencing technology. The size in this research (from 151,076 to 152,043 bp) was in accordance with those of reported CP genomes, such as *F. ussuriensis* (151,524 bp), *F. taipaiensis* (151,693 bp), *F.cirrhosa* (151,991 bp), and etc. The four CP genomes contained similar genome structure, gene content and gene order that were typical of land plants. Compared with other three species, the number of *tRNA* and *rRNA* genes were identical, but the number of protein coding genes ranged from 77 to 78 due to the absence of *rps16* gene in *F. unibracteata*. The absence of *rps16* gene has also been observed in *Brassicaceae*, *Fabaceae* and *Populus* species (*Jin et al*., *2019*). The functional loss of *rps16* gene from the CP genome could be compensated by the mitochondrial and (or) nuclear-encoded *rps16* that could target chloroplast as well as mitochondria (*Ueda et al*., *2008*).

The highly conserved genomic structure and gene order as well as no rearrangement of the *Fritillaria* CP genomes has been observed. The 26 kb of IRs in the *Fritillaria* species was within the size range of most angiosperm CP genome (20 to 30 kb). The IR/LSC boundaries in the *Fritillaria* and *Lilium* (*Lilium superbum*) CP genomes expanded into the *rps19* gene, which might be a characteristic CP genome structure of *Fritillaria* genus and its relative genus. Similar expansion was also observed in other taxa from family *Liliaceae*, including *Lilium* (*Kim & Lee*., *2004*), *Fritillaria* (*Li et al*., *2014*), and *Cardiocrinum* (*Liu et al., 2018*). *Li et al* (*2017*) reported that the common location of IR/LSC junctions in *rps19* seemed to be an ancestral symplesiomorphy of *Liliaceae*. Here, the similar feature was also found that the whole *rps19* gene was contained inside the IR in *Smilax china*, *Oncidium gower* and *Allium chinense*, while in *Hordeum vulgare*, *rps19* did not extend into the IR (Fig 2). The similar IRb/LSC boundaries among *Fritillaria*, *Lilium* and *Cardiocrinum* implicated that these genera were closely related, which was coincided with the phylogenetic result based on CP genomes (Fig 4).

A careful comparison between repeat sequence and SSR regions revealed the important differences between various *Fritillaria* species leading to establish specific markers for molecular identification. In this study, a large number of repeat sequences, mainly ranging from 15 to 20 bp, were detected in the chloroplast genomes of four *Fritillaria* species, consistent with the results of

studies on the chloroplast genomes of *Cannabaceae* (*Zhang et al*., *2018*). SSRs have been used for the study of population genetics because of their high variability (*Asaf et al*., *2016*). In this study, the high ratio of SSRs in LSC region was also observed in *F. sichuanica* (*Chen et al*., *2019*). In the CP genomes of *F. unibracteata*, *F. przewalskii*, *F. delavayi* and *F. sinica*, the content of A/T repeats was far greater than that of G/C repeats, similar to the results of *Xue et al* (*2019*) and other studies (*Melotto-Passarin et al*., *2011*). Although several variable CP DNA markers, for instance *matK*, *rpl16*, *atpB* and *rbcL*, have been used in phylogenetic studies of *Fritillaria*, they showed small divergence (Pi value of 0.00717, 0.00571, 0.00391 and 0.00505, respectively) among 12 *Fritillaria* species. Based on the result of sliding window analysis, the 10 most divergent regions were identified with Pi value ranging from 0.0116 to 0.0159. These divergent regions included *ycf1*, *trnL*, *trnF*, *ndhD*, *trnN*, *atpI* and *rps19* in the coding region, and *trnN-trnR*, *trnE-trnT and psbM-trnD* in intergenic region. In respect to three common DNA barcodes, *ycf1* got more reliable phylogenetic tree and thus confirmed that divergent region was potential molecular markers for future phylogenetic analysis. The highly variable *trnE-trnT* and gene *ycf1* have also been found by *Li et al* (*2018*), and gene *ycf1* has been proposed as the most promising plastid DNA barcode of land plants (*Dong et al*., *2015*).

The phylogenetic analysis of medicinal genus *Fritillaria*

As compared to separate regions, the whole CP genome showed higher resolution than the former since no less than 91% branches with bootstrap value of 90 BP in Fig. 4. This result was consistent with the previous report (*Ronsted et al*., *2005*) that increasing additional gene regions would help to improve the resolution. As suggested by *Kress et al* (*2005*) and *Ng et al* (*2017*), the whole CP genome was promising to act as super DNA barcode to resolve various *Fritillaria* species efficiently. Furthermore, our findings indicated that *Fritillaria* and *Lilium* were evidently sisters, the closest relative being *Cardiocrinum* in a monophyletic genus (100 BP), similar with the result of *Chen et al* (*2019*). Such phylogenetic tree based on whole CP genome in this study showed similar topology with previous study (*Rønsted et al*., *2005*), but with higher resolution. Specially, genus *Fritillaria* was indicated as paraphyletic with higher bootstrap (100 BP) compared to 54 BP and 53 BP in the findings of *Rønsted et al* (*2005*) and *Day et al* (*2014*), respectively.

The subgenus *Fritillaria* also appeared to be a paraphyletic group, similar to the results of *Day et al* (*2014*). One important medicinal *Fritillaria* specie, *F. ussuriensis*, clustered with *F. meleagroides* and formed sister clade to *F. karelinii* of subgenus *Rhinopetalum*, similar to the result of *Huang et al* (*2020*), *Khourang et al* (*2014*) and *Li et al* (*2018*). *F. ussuriensis* and *F. meleagroides* were frequently considered as members of the large subgenus *Fritillaria* (*Rix 2001*). However, the two species do have some similarities with *F. karalinii* as both of them have small mastoid on filament, which was different from other species in Xinjiang plain with no mastoid. Similar conflict between molecules and morphology was also observed in other taxa (*Anand et al*., *2016*). Meanwhile, such mastoid on

filament was proposed to be a potential primitive feature, and our results partly supported this hypothesis because *F. karelinii* and *F. meleagroides* diverged early from other medicinal species from Xinjiang, such as *F. pallidiflora* and *F. walujewii*. Subgenus *Theresia* (*F. persica*) and *Petilium* (*F. eduardii*) had close relationship and formed monophyletic subclade B1, which was similar to the result of *Day et al* (*2014*) and *Li et al* (*2018*).

As shown in Fig 4 and Fig 5, 5 species from Xinjiang plain were included in a strongly supported subclade C1 (Fig 4), which was sister to subclade C2 containing the other 15 species from outside Xinjiang plain. This signified that the Xingjiang species had a close genetic relationship. All the four species that were distributed in east China plain, including *F. monantha*, *F. anhuiensis*, *F. thunbergii* and *F. hupehensis,* were included in a supported subclade (100 BP). The rest 11 species, including the complex group of *F. cirrhosa*, in another subclade were distributed in Hengduan Mountains (100 BP). The 11 important medicinal *Fritillaria* species were widely distributed in four hotspots, Xinjiang plain, northeastern China plain, east China plain and Hengduan Mountains. The former two regions constituted hotspots in North China, while the latter two regions constituted hotspots in South China. Interestingly, the eight species in the upper location of the clade originated from Xinjiang plain and northeastern China plain (*F. ussuriensis*), whereas the 12 species in the lower location distributed in East China and Hengduan Mountains region. Consequently, the geographical distribution pattern of the 11 medicinal species appeared to map on the phylogenetic tree, especially by plastid data (*Rønsted et al*., *2005*). The similar result was also reported by *Li et al* (*2018*), and thus the investigation on the correlation between distribution pattern and phylogenetic relationship was needed in the future.

Early in 1987, *F. unibracteata*, *F. cirrhosa*, *F. prezewalskii* and *F. delavayi* were recorded as national third-class endangered medicinal plants of China (*Konchar et al*., *2011*). That the most important medicinal species showed close relationship to widely cultivated members of subgenus *Fritillaria*, raised the possibility that the rare species were replaced by those widely cultivated species. Recent analyses have demonstrated that *F. crassicaulis*, showing closest relationship with *F. cirrhosa*, has been widely used as the substitution of *F. cirrhosa* by people of Naxi nationality and Tibetan since Ming/Qing Dynasty (*Tang & Xue*, *1992*). These findings highlighted those phylogenetic trees based on CP genomes were promising method to select potential novel medicinal species. In the future, those showing close relationship to the important species in traditional Chinese medicine , such as *F. sichuanica*, *F. dajinensis*, *F. yuzhongensis*, *F. sinica* and *F. crassicaulis*, might be investigated whether these bulbs contain the same bioactive compounds found in the complex of *F. cirrhosa*.

The phylogenetic placement and species-specific identification of some *Fritillaria* species

The non-monophyletic trait of subgenus *Fritillaria* indicated the incongruence in classification among some species, similar as the reports by *Rønsted et al* (*2005*) and *Day et al* (*2014*). Although *F. ussurinensis* was regarded as a member of subgenus *Fritillaria*, that its splitting from other members

of subgenus *Fritillaria* has also been observed by *Chen et al* (*2019*) and *Huang et al* (*2020*). There were several reports of natural interspecific hybrids (e.g. *F. ussurinensis* (*Ruan et al*., *2004*) and *F. eduardii* (*Wietsma et al*., *2011*)), which might promote the molecular phylogenetic non-monphyly (*Funk & Omland*, *2003*). Sencondly, *F. davidii* had rice-shaped bulbils, resembling the morphological character of subgenus *Liliorhiza*, and used to be grouped in subgenus *Liliorhiza*. But based on our results, it was distantly related to genus *Liliorhiza* and was thus placed in subgenus *Davidii* as described by *Rix* (*2001*). It was suggested that rice-shaped bulbils have independently evolved in *F. davidii* and subgenus *Liliorhiza* due to geographic separation, followed by a loss in some species in Eurasian clade during evolution (*Rønsted et al*., *2005*). Thirdly, *Rønsted et al* (*2005*) found that *F. pallidiflora* was resolved solely within the *Korolkowia*/*Petilium*/*Theresia* clade by combined plasmid *rpl16* and *matK* sequences. Our new results demonstrated that *F. pallidiflora* was clustered within subgenus *Fritillaria* and more closely related to *Petilium/Theresia*. The conflict in *F. pallidiflora* was likely to be solved by using whole CP genome instead of separate regions. In addition, based on the CP genome, *F. unibraceata* was sister to *F. wabuensis* with divergence of 0.003, which was more than that between *F. sichuanica* and *F. dajinensis* (0.002). If *F. sichuanica* and *F. dajinensis* were given at rank of specie, based on the CP genome it was preferable to follow *Tang & Yue* (*1983*) and rank *F. wabuiensis* as specie instead of rank of variant. However, this result was merely based on CP genome, the accurate placement of *F. wabuensis* kept further evaluation by nuclear genome comparison although it was extremely difficult to obtain.

Considering the incongruence between morphology and molecular phylogeny, the species-specific identification of *Fritillaria* species was highlighted. In this study, the repeated sequence, SSR, divergent region and indel analyses were performed, respectively, to identify candidate species-specific markers. As a result, three indels for *F. taipaiensis*, *F. unibracteata* and *F. przewalskii* were selected, respectively, due to uniqueness, fragment length and convenient design of primers. Further routine and real-time PCR obtained characteristic amplification using 14 *Fritillaria* species, including 11 most important medicinal species, as verifying materials. In previous researches, the indel-based methods have also been applied for plant identification with high reliability and convenience (*Hong et al*., *2017*; *Kim et al*., *2018*). Taking into account the limited individual samples we collected in this study, the results from our approach are very promising. In the future, the developing method is required to be investigated on multiple individuals.

Conclusion

The four CP genome of species from subgenus *Fritillaria* provided support for taxonomic clarification, phylogenetic relationship and development of DNA markers. The phylogenetic tree based on the whole CP genome was reliable since 91% branches obtained bootstrap of more than 90 BP, the result of which supported for the monophyly of genus *Lilium*, *Amana* and *Cardiocrinum*,

except that the largest genus *Fritillaria* was paraphyletic. The 11 members of subgenus *Fritillaria* that were used in traditional Chinese medicine were split into two clusters since *F. ussuriensis* was clustered with *F. meleagroides* and *F. karelinii*. In addition, the phylogenetic tree appeared to reflect a geographic distribution pattern of *Fritillaria* subgenus, and also highlighted the importance of CP genome in the evolutional analysis. The most important medicinal species, especially *F. cirrhosa* complex, were found to be close to species that were in widespread cultivation for medicinal and ornamental purposes. Excitingly, those closely related species from subgenus *Fritillaria* might be promising alternatives to balance the improving market and rare resources. Finally, the preliminary test among medicinal species in Chinese medicine market suggested the development of species-specific identification on the basis of CP genome was a promising approach, and in the future in depth investigation on multiple individuals is needed.

Availability of data and materials

The chloroplast genomes generated during the current study were deposited in NCBI with accession number of MW849272 (*F. unibraceata*), MW849274 (*F. przewalskii*), MW849275 (*F. delavayi*) and MW849273 (*F. sinica*), respectively. All the raw Illumina data of *F. unibracteata*, *F. przewalskii*, *F. delavayi* and *F. sinica* have been deposited in the Sequence Read Archive (SRA) of the NCBI under accession numbers of SRR14454932, SRR14455034, SRR14454929 and SRR14455331, respectively.

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