Comparison of the abilities of universal, super, and specific DNA barcodes to discriminate among the original species of Fritillariae cirrhosae bulbus and its adulterants

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Abstract

Fritillariae cirrhosae bulbus is a famous type of traditional Chinese medicine used for cough relief and eliminating phlegm. The medicine originates from dried bulbs of five species and one variety of Fritillaria. Recently, immature bulbs from other congeneric species, such as F. ussuriensis, have been sold as adulterants of Fritillariae cirrhosae bulbus in medicine markets owing to the high price and limited availability of the genuine medicine. However, it is difficult to accurately identify the bulbs from different original species of Fritillariae cirrhosae bulbus and its adulterants based on traditional methods, although such medicines have different prices and treatment efficacies. The present study adopted DNA barcoding to identify these different species and compared the discriminatory power of super, universal, and specific barcodes in Fritillaria. The results revealed that the super-barcode had strong discriminatory power (87.5%). Among universal barcodes, matK provided the best species resolution (87.5%), followed by ITS (62.5%), rbcL (62.5%), and trnH-psbA (25%). The combination of these four universal barcodes provided the highest discriminatory power (87.5%), which was equivalent to that of the super-barcode. Two plastid genes, ycf1 and psbM-psbD, had much better discriminatory power (both 87.5%) than did other plastid barcodes, and were suggested as potential specific barcodes for identifying Fritillaria species. Phylogenetic analyses indicated that F. cirrhosa was not a “good” species that was composed of multiple lineages, which might have affected the evaluation of the discriminatory ability. This study revealed that the complete plastid genome, as super barcode, was an efficient and reliable tool for identifying the original species of Fritillariae cirrhosae bulbus and its adulterants.
Introduction

Fritillaria L. is one of the most important genera in the family Liliaceae, and it includes approximately 140 species of perennial herbaceous plants worldwide [1,2]. Almost all known species in this genus grow in temperate regions of the Northern Hemisphere [3]. There are 24 species in China, most of which have medicinal value and are used for cough relief and eliminating phlegm, such as *F. cirrhosa* D. Don, *F. ussuriensis* Maxim., *F. walujewii* Regel, *F. thunbergii* Miq., and so on [4–6]. According to the Chinese Pharmacopoeia (2015), there are five traditional Chinese medicines that originate from the dried bulbs of *Fritillaria* species. Among them, Fritillarias cirrhosae bulbs (also known as “Chuanbeimu” in traditional Chinese medicine) has been regarded the best throughout Chinese history. The original species used to make this type of medicine include five species and one variety, namely *F. cirrhosa*, *F. przewalskii* Maxim., *F. unibracteata* Hsiao et K.C. Hsia, *F. delavayi* Franch., *F. taipaiensis* P.Y. Li, and *F. unibracteata var. wabuensis* (S.Y. Tang et S.C. Yue) Z.D. Liu., S. Wang et S.C. Chen [7].

Recently, the market price of Fritillariae cirrhosae bulbs has been increasing sharply due to its limited output from wild habitats, and thus immature bulbs of other *Fritillaria* species (e.g., *F. ussuriensis*, *F. thunbergii*, *F. pallidiflora* Schrenk, etc.) have been sold in medicinal markets imitating the original Fritillariae cirrhosae bulbs. Genuine Fritillariae cirrhosae bulbs originating from bulbs with different botanical origins are difficult to identify based on the morphological characteristics and traditional methods. The bulbs from each original species obviously differ in price and might also differ in their medicinal treatment efficacy, which could seriously affect their health benefits to consumers [8–11]. Thus, it is extremely important to be able to accurately discriminate between Fritillariae cirrhosae bulbs and its adulterants, as well as among the different original species used to make these medicines.

DNA barcoding is a technology used for species identification that employs a short and standardized DNA region [12–15]. It has been proven to be an effective tool for rapid and accurate species discrimination [16–19]. Based on a large number of studies, three plastid loci (*trnH-psbA*, *matK*, and *rbcL*) and one ribosomal DNA spacer region (the internal transcribed spacer or ITS), or their combinations, were proposed as universal barcodes for plants that could discriminate most plant species [20–22]. However, for taxonomically complicated groups, such as *Fritillaria*, the use of these universal barcodes and their combinations has achieved lower success rates in species discrimination, except *matK* (this barcode had low success rate of PCR and Sanger sequencing in *Fritillaria*), due to insufficient sequence variation [23, 24].

The complete plastid genome was suggested as a super-barcode for identification of plant species, especially for taxonomically difficult taxa such as the genera *Citrus*, *Oncidium*, and *Gossypium* [25–27]. This genome has been also used successfully to explore phylogenetic relationships among plant taxa [28–30]. Typically, the plastid genome in angiosperms is circular, ranges in size from 72 to 217 kb, and has a quadripartite structure composed of a large single copy region (LSC), small single copy region (SSC), and a pair of inverted repeats (IRs) [31–34]. In contrast with the nuclear and mitochondrial genomes, the plastid genome is largely conserved among taxa in terms of its gene content, organization, and structure. Moreover, plastid genomes are maternally inherited and therefore appropriate for use in genetic engineering due to lack of cross-recombination [35,36]. These characteristics of the plastid genome render it suitable for species discrimination within complex plant taxa [37,38]. The results of recent studies also supported the strong discriminatory power of this super-barcode when used in species identification [39,40]. For the genus *Fritillaria*, complete plastid genomes were previously employed to evaluate phylogenetic relationships among some species and produced results that were supported by high bootstrap values [41–43]. However, those previous studies...
generally sequenced only one individual per species, and thus they could not effectively compare the intra- and inter-specific genetic distances in *Fritillaria* species. This leads to the question of whether species of *Fritillaria*, especially the original plants used to make *Fritillariae cirrhosae bulbus* and its adulterants, could be correctly identified at the species level by analyses of the complete plastid genome.

In the present study, we used the complete plastid genome sequence as a super-barcode to: identify the botanical origins of *Fritillariae cirrhosae bulbus* and its adulterants; compare the discriminatory power of the super-barcode with that of universal DNA barcodes and their combinations; and scan the highly variable gene regions as potential specific barcodes for species identification of *Fritillariae cirrhosae bulbus*. The present study provided abundant information for further development of super-barcodes and broadened the horizon over which other rapid and accurate molecular techniques for species identification in *Fritillaria* can be explored.

**Materials and methods**

**Material sampling**

A total of 32 individuals of *Fritillaria* representing the five original species of *Fritillariae cirrhosae bulbus* and three species of its adulterants, as well as an individual of *F. anhuiensis* S.C. Chen & S.F. Yin as an outgroup, were used in tree-building analysis in this study (Fig 1, S1 Table). Among these species, individuals of the five original species that produce the genuine medicine were collected from wild habitats in Southwest China, while those of the adulterants were collected from cultivated populations in provinces of Zhejiang, Jilin, and Xinjiang, China. None of the species from the genus *Fritillaria* are listed as the national protected plants in China (Information System of Chinese Rare and Endangered Plants, http://www.iplant.cn/rep/) and therefore their collection is allowed for scientific research. Fresh leaves were sampled from healthy, mature individuals in the field or cultivation bases and then dried by using silica gel. Meanwhile, 3–5 individuals with flowers were dug up and preserved as voucher specimens. Subsequently, geographic information (latitude, longitude, altitude, etc.) for the sampling locations was obtained by a Global Positioning System receiver (GPS; Garmin, Olathe, KS, USA). All voucher specimens of *Fritillaria* were identified and then deposited at the Herbarium of Medicinal Plants and Crude Drugs of the College of Pharmacy and Chemistry, Dali University, Dali, China (S1 Table).

**DNA extraction, sequencing, and assembly.** Total genomic DNA was extracted from about 100 mg of dried leaf material using a modified CTAB method [44,45]. The DNA content was checked by electrophoresis on 1.2% agarose gels, and its concentration was determined using a SmartSpec™ Plus Spectrophotometer (Bio-Rad, Hercules, CA, USA). DNA extracts were then fragmented for the construction of 300 bp short-insert paired-end (PE) libraries and sequenced on an Illumina HiSeq 2000/2500 sequencer at the Beijing Genomics Institute (BGI, Shenzhen, China).

The raw data were filtered using Trimmomatic v.0.32 [46] with default settings. Paired-end reads in the clean data were then filtered and assembled into contigs using GetOrganelle.py [47] with *Fritillaria cirrhosa* (accession number: KF769143) as reference [48], calling the bowtie2 v., Blastn v., and SPAdes v.3.10 [49]. The de novo assembly graphs were visualized and edited using Bandage Linux dynamic v.8.0 [50], and then a whole or nearly whole circular plastid genome (plastome) was generated.

**Annotation and sequence submission**

The plastomes were annotated by aligning them to the complete plastid genome sequence available in NCBI using MAFFT [48,51] with default parameters, which was coupled with manual adjustment using Geneious v.11.1.4 [52]. Circular genome visualization was generated.
with OGDRAW v.1.2 [53]. Furthermore, the ITS sequences were sequenced using Illumina sequencing technology and extracted from the raw data. The annotated plastid genomes of the nine *Fritillaria* species and their ITS sequences were submitted to the NCBI under the accession numbers MH588404-MH588436 for ITS sequences and those listed in Table 1 for the complete plastid genomes.

**Variable site analysis**

After using MAFFT v.7.129 to align the plastid genome sequences, BioEdit software was used to adjust the alignment manually [51,54]. A sliding window analysis was conducted to determine the nucleotide variability (Pi) in the whole plastid genome using DnaSP v.6.11 [55]. The step size was set to 200 bp, with a 600 bp window length. Moreover, the DnaSP software was used to identify and quantify the insertions/deletions (indels), mutations, and nucleotide variability (Pi) in all aligned datasets. The p-distances, GC content, variable sites, and parsimony informative sites in the genomes were identified and analyzed by the software MEGA v.7.0.26 [56].
Species discrimination of universal, super, and specific barcodes

To evaluate the success rates of species discrimination with each barcode, we used a tree-building method to analyze 14 datasets for each of the single regions examined and their combinations. All single regions, including universal DNA barcodes and highly variable loci, were extracted from the complete plastid genome sequences, except the ITS/ITS2 region. These datasets were aligned with MAFFT [51] and used to build neighbor-joining trees (NJ) based on p-distances in the software MEGA [56]. The plastome of *F. anhuiensis* (accession number: MH593363) was used as the outgroup in these tree-building analyses. Species were regarded as being successfully discriminated if all the individuals of a given species formed a monophyletic group [57].

Table 1. Summary of complete plastid genomes obtained for the five original species of *Fritillariae cirrhosae* bulbus and three species of its adulterants, as well as the outgroup (*F. anhuiensis*).

| Species Code Total length GC% Number of | Species | Code | Total length | Large single copy (LSC, bp) | Small single copy (SSC, bp) | Inverted repeat (IR, bp) | Accession number |
|------------------|---------|------|-------------|-----------------------------|-----------------------------|--------------------------|------------------|
| 151,546 37.0% 115 | BM 1–1  | 151,546 | 81,402 | 17,542 | 26,301 | 115 MH593342 |
| 151,546 37.0% 115 | BM 1–2  | 151,546 | 81,402 | 17,542 | 26,301 | 115 MH593344 |
| 151,539 37.0% 115 | BM 2–1  | 151,998 | 81,755 | 17,545 | 26,349 | 115 MH244906 |
| 151,647 37.0% 115 | BM 2–2  | 151,605 | 81,467 | 17,534 | 26,302 | 115 MH593344 |
| 151,794 36.9% 115 | BM 3–1  | 152,035 | 81,794 | 17,541 | 26,350 | 115 MH593345 |
| 151,794 36.9% 115 | BM 3–2  | 152,035 | 81,794 | 17,541 | 26,350 | 115 MH593346 |
| 151,983 36.9% 115 | BM 6–1  | 151,983 | 81,744 | 17,539 | 26,350 | 115 MH244908 |
| 152,054 36.9% 115 | BM 6–2  | 152,054 | 81,816 | 17,538 | 26,350 | 115 MH593347 |
| 152,054 36.9% 115 | BM 7–1  | 151,955 | 81,715 | 17,540 | 26,350 | 115 MH593348 |
| 152,054 36.9% 115 | BM 7–2  | 151,960 | 81,722 | 17,538 | 26,350 | 115 MH593349 |
| 152,057 37.0% 115 | BM 8–1  | 151,058 | 81,339 | 17,539 | 26,090 | 115 MH244909 |
| 152,057 37.0% 115 | BM 8–2  | 151,057 | 81,338 | 17,539 | 26,090 | 115 MH593350 |
| 152,057 37.0% 115 | BM 9–1  | 151,012 | 81,295 | 17,537 | 26,090 | 115 MH593351 |
| 152,057 37.0% 115 | BM 9–2  | 151,078 | 81,398 | 17,538 | 26,071 | 115 MH593352 |
| 151,853 37.0% 115 | BM 10–1 | 151,853 | 81,602 | 17,513 | 26,369 | 115 MH593353 |
| 151,853 37.0% 115 | BM 10–2 | 151,854 | 81,603 | 17,513 | 26,369 | 115 MH593354 |
| 151,854 37.0% 115 | BM 10–3 | 151,854 | 81,603 | 17,513 | 26,369 | 115 MH593355 |
| 151,707 37.0% 115 | BM 11–1 | 151,707 | 81,451 | 17,552 | 26,352 | 115 MH244910 |
| 151,518 37.0% 115 | BM 11–2 | 151,518 | 81,268 | 17,546 | 26,352 | 115 MH593356 |
| 151,741 37.0% 115 | BM 12–1 | 151,741 | 81,478 | 17,561 | 26,351 | 115 MH593357 |
| 151,741 37.0% 115 | BM 12–2 | 151,741 | 81,478 | 17,561 | 26,351 | 115 MH593358 |
| 151,741 37.0% 115 | BM 12–3 | 151,741 | 81,478 | 17,561 | 26,351 | 115 MH593359 |
| 152,160 37.0% 115 | BM 16–1 | 152,160 | 81,895 | 17,565 | 26,350 | 115 MH244914 |
| 152,160 37.0% 115 | BM 16–2 | 152,160 | 81,895 | 17,565 | 26,350 | 115 MH593360 |
| 152,160 37.0% 115 | BM 16–3 | 152,160 | 81,895 | 17,565 | 26,350 | 115 MH593361 |
| 152,160 37.0% 115 | BM 17–1 | 152,160 | 81,895 | 17,565 | 26,350 | 115 MH593362 |
| 152,160 37.0% 115 | BM 17–2 | 152,160 | 81,895 | 17,565 | 26,350 | 115 MH593363 |
| 152,073 37.0% 115 | BM 23–1 | 152,073 | 81,779 | 17,514 | 26,390 | 115 MH593364 |
| 152,067 37.0% 115 | BM 23–2 | 152,067 | 81,763 | 17,528 | 26,388 | 115 MH593365 |
| 152,073 37.0% 115 | BM 23–3 | 152,073 | 81,780 | 17,513 | 26,390 | 115 MH593366 |
| 151,571 36.9% 115 | BM 26–1 | 151,571 | 81,773 | 17,126 | 26,336 | 115 MH593367 |
| 151,523 37.0% 115 | BM 26–2 | 151,523 | 81,741 | 17,122 | 26,330 | 115 MH593368 |
| 151,552 37.0% 115 | BM 26–3 | 151,552 | 81,764 | 17,124 | 26,332 | 115 MH593369 |
| 152,119 37.0% 115 | BM 20–2 | 152,119 | 81,817 | 17,560 | 26,371 | 115 MH593363 |

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Results

Plastid genome organization of *Fritillaria*

The 32 complete plastid genomes, consisting of a circular double-stranded DNA, ranged from 151,518 bp in *F. taipanensis* (accession number: MH593356) to 152,073 bp in *F. pallidiflora* (accession number: MH593366). The genomes possessed a typical quadripartite structure, which comprised a pair of IRs (26,090–26,390 bp), LSC (81,295–82,085 bp), and SSC (17,122–17,565 bp) regions (Table 1, S1 Fig). Overall GC content of the complete plastid genomes was 36.9%–37.0% (Table 1). Moreover, a total of 115 genes were found, namely 78 protein coding genes, 30 tRNA genes, and 4 rRNA genes, as well as 3 pseudogenes (Table 2, S1 Fig). The protein coding genes present in the plastid genome of *Fritillaria* included 9 genes for large ribosomal proteins, 12 genes for small ribosomal proteins, 5 genes for photosystem I, 15 genes for photosystem II, and 6 genes for ATP synthase (Table 2, S1 Fig).

### Highly variable regions in plastid genome

Seven highly variable regions from the plastid genomes, namely three intergenic regions (*psbM-psdD, rps4-trnL-UAA, and ndhF-trnL-UAG*), three gene regions (*matK, ndhD, and ycf1*), and one intron region (*petB-intron*), were selected as potential specific barcodes for use in species identification in *Fritillaria* (Fig 2). Among these regions, *ycf1* was the longest, followed by *psbM-psbD,* 

| Category for gene | Group of genes | Name of genes |
| ------------------|----------------|--------------|
| **Self-replication** | Large subunit of ribosome | rpl2\(^a\), rpl14\(^\circ\), rpl16\(^\circ\), rpl20, rpl22, rpl23\(^\circ\), rpl32, rpl33, rpl36 |
| | Small subunit of ribosome | rps2, rps3, rps4, rps7\(^\circ\), rps8, rps11, rps12\(^\circ\), rps14, rps15, rps16\(^\circ\), rps18, rps19 |
| | DNA dependent RNA polymerase | rpoA, rpoB, rpoC1\(^\circ\), rpoC2 |
| | rRNA gene | rrn4.5\(^\circ\), rrn5\(^\circ\), rrn16\(^\circ\), rrn23\(^\circ\) |
| | tRNA gene | trnK-UUU\(^\circ\), trnL-GAU\(^\circ\), trnA-UGC\(^\circ\), trnV-UAC\(^\circ\), trnL-UAA\(^\circ\), trnS-UAG, trnS-GCU, trnS-GGA, trnY-GUA, trnC-GCA, trnL-CA\(^{\circ}\), trnL-UAG, trnH-GUG\(^\circ\), trnD-GUC, trnM-CAU, trnW-CCA, trnP-UGG, trnI-CA\(^{\circ}\), trnR-AGG\(^\circ\), trnE-UUC, trnT-UGU, trnF-GAA, trnQ-UUG, trnR-UCU, trnT-GGU, trnM-CAU, trnV-GAC\(^\circ\), trnN-GUU\(^\circ\), trnV-GUC\(^\circ\), trnG-GCC |
| Gene for photosynthesis | Subunits of photosystem I | psaA, psaB, psaC, psaI, psaL |
| | Subunits of photosystem II | psbA, psbB, psbC, psbD, psbE, psbF, psbH, psbL, psbl, psbK, psbL, psbM, psbN, psbT, psbZ |
| | Subunits of NADH-dehydrogenase | ndhA\(^\circ\), ndhB\(^\circ\), ndhC, ndhD, ndhE, ndhF, ndhG, ndhH, ndhI, ndhK |
| | Subunits of cytochrome b/f complex | petA, petB\(^\circ\), petD\(^\circ\), petG, petL, petN |
| | Subunit for ATP synthase | atpA, atpB, atpE, atpF\(^\circ\), atpH, atpL |
| | Large subunit of rubisco | rbcL |
| Other genes | Translational initiation factor | infA |
| | Maturase | matK |
| | Protease | clpP\(^\circ\) |
| | Envelope membrane protein | cemA |
| | Subunit of Acetyl-carboxylase | accD |
| | C-type cytochrome synthesis gene | ccsA |
| | Open reading frames (ORF, ycf) | ycf1, ycf2\(^\circ\), ycf3\(^\circ\), ycf4, ycf15\(^\circ\), ycf68\(^\circ\) |

The lowercase letter a in superscript after gene names indicates genes located in IR regions. Asterisks indicate intron-containing genes.

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while petB-intron region was the shortest (Table 3). Furthermore, all highly divergent fragments were found in the LSC and SSC regions, whereas none were present in the IR regions.

**Variability of the barcodes and their combinations**

Among the regions and their combinations examined, the complete plastid genome clearly had the highest number of variable sites, parsimony-informative sites, and mutation sites (Table 3). Among the universal DNA barcodes, the ITS2 region was the most mutation-rich (Ps: 0.03304), followed by the ITS (0.02560), trnH-psbA (0.01288), and matK (0.00537), whereas the rbcL region (0.00295) was highly conserved (Table 3). Furthermore, among the highly variable regions, ndhF-trnL-UAG showed the highest variability (0.00994), and it was followed by psbM-psdD (0.00907), rps4-trnL-UAA (0.00892), ycf1 (0.00836), petB-intron (0.00703), and ndhD (0.00502).

**DNA barcoding gap assessment**

The inter- and intraspecific distances were calculated for each of the 14 datasets (Table 3). In these datasets, the ITS2 region exhibited the highest inter- and intraspecific distances (0.0326 and 0.0094, respectively), followed by the ITS (0.0235 and 0.0044), trnH-psbA (0.0147 and 0.0010) and ndhF-trnL-UAG (0.0112 and 0.0008), whereas these distances were the lowest for the rbcL (0.0033 and 0.0005). Meanwhile, the inter- and intraspecific distances of the complete plastid genome showed relatively low values (0.0037 and 0.0005, respectively) compared with those calculated for other datasets. Furthermore, the barcoding gap between inter- and intraspecific distances based on the p-distance model revealed that matK had the highest interspecific gap (divergence), but overlap between inter- and intraspecific distances existed for almost all single regions and their combinations, except for matK (Fig 3, S2 Fig).

**Discriminatory powers of all regions and their combinations**

We calculated the species discrimination ability of each region and their combinations based on 14 datasets using tree-building methods (Fig 4). The super-barcode, comprised of complete
plastid genomes, showed the highest power for species identification (87.5%), with strongest bootstrap values (Fig 5), except for F. cirrhosa, which is probably due to its polyphylectic nature. Among the universal DNA barcodes tested here, matK had the highest discriminatory power (87.5%), same as that of the super-barcode (Fig 4), followed by rbcL and ITS (both 62.5%). In contrast, trnH-psbA and ITS2, with relatively short DNA sequences (253 bp and 239 bp, respectively) and heavy overlaps between inter- and intraspecific distances, had relatively low success rates in distinguishing Fritillaria species (25% and 37.5%, respectively). Moreover, the combinations of the four barcodes (HKLI) or of the three plastid DNA regions (HKL) also showed the highest power for species discrimination (both 87.5%). Among the highly variable loci, psbM-psbD and ycf1 had the highest species discrimination rates (both 87.5%), followed by rps4-trnL-UAA (75%), ndhF-trnL-UAG ndhD, and petB-intron (both 50%) (Fig 4).

According to the NJ trees, each of the DNA regions psbM-psbD, ycf1, and matK alone could be used to efficiently identify the original plants of Fritillaria cirrhosae bulbus and its adulterants. In contrast, the ITS, ndhD, ndhF-trnL-UAG, and rps4-trnL-UAA regions identified only the adulterant species from the genuine ones in the medicine. Moreover, the ITS2 could discriminate the adulterant from the genuine medicine, but could not effectively identify any of

| Table 3. Analysis of the variability in different fragments and combination of fragments. |
|----------------------------------|----------------|----------------|----------------|----------------|----------------|----------------|
| No. sites | No. variable sites | No. parsimony information sites | No. mutations | No. InDels | Intraspecific distance | Interspecific distance | Nucleotide diversity (Pi) |
| Genome | 154,404 | 2,667 | 2,436 | 2,449 | 4,273 | 0–0.0021 | 0.0005 | 0.0007–0.0078 | 0.0037 | 0.00337 |
| HKLI | 3,890 | 180 | 132 | 170 | 44 | 0–0.0052 | 0.0012 | 0.0008–0.0191 | 0.0082 | 0.00784 |
| HKL | 3,255 | 76 | 74 | 71 | 19 | 0–0.0034 | 0.0006 | 0.0003–0.0117 | 0.0051 | 0.00466 |
| ITS2 | 239 | 52 | 22 | 55 | 9 | 0–0.0807 | 0.0094 | 0–0.1211 | 0.0326 | 0.03034 |
| ITS | 635 | 104 | 58 | 99 | 25 | 0–0.0338 | 0.0044 | 0–0.0709 | 0.0235 | 0.02560 |
| trnH-psbA | 253 | 17 | 17 | 16 | 19 | 0–0.0043 | 0.0010 | 0–0.0598 | 0.0147 | 0.01288 |
| matK | 1539 | 37 | 37 | 37 | 0 | 0–0.0039 | 0.0007 | 0.0006–0.0123 | 0.0059 | 0.00537 |
| rbcL | 1464 | 21 | 19 | 21 | 0 | 0–0.0027 | 0.0005 | 0–0.0075 | 0.0033 | 0.00295 |
| ndhF-trnL-UAG | 1,381 | 60 | 57 | 52 | 77 | 0–0.0052 | 0.0008 | 0–0.0321 | 0.0112 | 0.00994 |
| psbM-psbD | 3608 | 163 | 150 | 130 | 582 | 0–0.0058 | 0.0014 | 0–0.0202 | 0.0101 | 0.00907 |
| rps4-trnL-UAA | 1,323 | 56 | 53 | 49 | 138 | 0–0.0061 | 0.0016 | 0–0.0238 | 0.0098 | 0.00892 |
| ycf1 | 5,565 | 214 | 203 | 223 | 63 | 0–0.0044 | 0.0010 | 0–0.0166 | 0.0092 | 0.00836 |
| petB-intron | 855 | 30 | 29 | 28 | 17 | 0–0.0050 | 0.0012 | 0–0.0200 | 0.0078 | 0.00703 |
| ndhD | 1,509 | 39 | 36 | 41 | 6 | 0–0.0020 | 0.0006 | 0–0.0160 | 0.0056 | 0.00502 |

HKL and HKLI represent a combination of three fragments of the plastid genome and a combination of four fragments of universal barcodes, respectively (H: trnH-psbA; K: matK; L: rbcL; I: ITS)
Fig 3. Histograms of the frequencies (y-axes) of pairwise inter- and intraspecific divergences calculated based on the p-distances (x-axes) of each single regions and their combinations. (H: trnH-psbA; K: matK; L: rbcL; I: ITS).

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the original species of Fritillariae cirrhosae bulbus. Finally, the $\text{trnH-psbA}$, $\text{rbcL}$, and $\text{petB-intron}$ regions could neither discriminate the adulterant medicine from the genuine nor their original species.

**Definition of *Fritillaria cirrhosa***

In the NJ trees inferred from the whole plastid genomes, other DNA regions, or combinations, the six individuals of *F. cirrhosa* collected from three locations were clustered into two distinct clades: one was placed close to *F. unibracteata* and the other was close to *F. przewalskii* with strong support values (Fig 5).

**Discussion**

DNA barcoding has been demonstrated to be an efficient tool for identifying traditional medicines as well as their original species [23,58–60]. In the present study, this tool was used to discriminate among the five original species of Fritillariae cirrhosae bulbus and three of its adulterants.

**Performance of universal DNA barcodes**

In plants, the term universal DNA barcodes, which are identified based on Sanger sequencing, generally refers to $\text{rbcL}$ and $\text{matK}$, to be supplemented with $\text{trnH-psbA}$ and ITS/ITS2 [21,22]. Among the four universal DNA barcodes, $\text{matK}$ had the highest species discriminatory power and successfully distinguished all the original species of Fritillariae cirrhosae bulbus, except *F. cirrhosa*. In contrast, ITS2 and $\text{trnH-psbA}$ showed the lowest species resolution and failed to correctly identify the original species of Fritillariae cirrhosae bulbus, although these two barcodes were previously suggested as core barcode for species identification in traditional Chinese medicines [22,61]. ITS/ITS2 correctly discriminated the genuine medicine from the adulterants (Fig 5, S3 Fig). This result was nearly consistent with that of our previous study [24]. In fact, ITS/ITS2 were rich in variable sites and nucleotide diversity (Pi) compared with the studied plastid DNA regions (Table 3), but the obvious overlap between inter- and
intraspecific distances in such regions among and within *Fritillaria* species might impact their discriminating ability (Fig 3).

It should be noted that the *matK* sequences used for analysis were obtained from the complete plastid genome, not by Sanger sequencing. If the obstacles to Sanger sequencing of *matK*
are resolved, \textit{matK} will become the ideal candidate barcode for identifying the original species of \textit{Fritillariae cirrhosae} bulbus, despite its lower nucleotide diversity (Pi) when compared with that of other regions. The best performance of this barcode might be attributed to the existence of a clear barcoding gap (Table 3, Fig 3). The \textit{matK} gene has been confirmed to perform poorly in discriminating species of \textit{Fritillaria} and other genera such as \textit{Primula} [62] and \textit{Garcinia} [63] because of its low success rates in PCR amplification and Sanger sequencing [24]. However, next-generation sequencing (NGS) technology could resolve the aforementioned deficiency of the Sanger method, as it was revealed in the present study that this marker has satisfactory discriminatory power. Therefore, we propose that NGS technology should be adopted for DNA sequencing of \textit{matK} to overcome the disadvantages of the Sanger sequencing method for such fragments.

Furthermore, combined barcodes generally perform better in species discrimination than single barcodes [23]. In the present study, combinations of the four barcodes (HKLI) or three plastid regions (HKL) also showed strong species discrimination abilities (87.5%) (Fig 5, S3 Fig). In short, multi-locus combination could raise discrimination ability for \textit{Fritillaria} species and improve the reliability.

\section*{Super-barcode–a crucial candidate DNA barcode in \textit{Fritillaria}}

Because of the low power for species discrimination of universal DNA barcodes, new methods are necessary to discriminate closely related species [23,37]. Complete plastid genomes are extremely rich in genetic variations and have been shown to be powerful tools for resolving the phylogenetic relationships of complex groups [30,42,43,64,65]. Their use can greatly improve the resolution at lower taxonomic levels in plant phylogeny, phylegography, and population genetics; therefore, they were also proposed as a type of super DNA barcode that is likely to resolve the defects of the universal DNA barcodes [23,66]. In this study, plastid genomes of \textit{Fritillaria} species, with lengths from 151,518 to 152,073 bp, provided abundant informative sites for species identification. As a result, this super-barcode identified almost all of the original species of \textit{Fritillariae cirrhosae} bulbus and its adulterants with high bootstrap values, except for \textit{F. cirrhosa} because of its possible polyphyletic nature (Fig 5). Herein, the complete plastid sequences in the present study were obtained from dried leaf materials using NGS methods. Nevertheless, the use of this super-barcode might face extreme challenges if one needed to extract DNA from specific materials, such as kiln-dried specimens or medicines. However, recent procedures have been developed that can use total DNA as a template for genome skimming to assemble plastid genome, which not only solves the problem of extracting plastid DNA from dried or even degraded materials but also simplifies the whole process [23,67,68]. As sequencing technology and bioinformatics continue to improve rapidly, super DNA barcode will become more popular and may eventually replace Sanger-based DNA barcoding. Thus, super DNA barcodes could be adopted as useful complements to universal DNA barcodes, especially in identifying closely related species.

\section*{Specific DNA barcode–a trade-off between universal and super-barcode}

The present study revealed that, when adopting the NGS method, the universal DNA barcodes were limited in their ability to discriminate species, except \textit{matK} region, which showed high success rate for identifying species in \textit{Fritillaria} (87.5%). Although the super-barcode exhibited high discriminatory power and sufficient reliability in this study, its use might be limited due to complications in data analyses and expensive sequencing costs. Therefore, it is of great importance to search for specific barcodes from highly variable regions that can be used as a trade-off between universal and super DNA barcodes.
Highly variable regions in the plastome could help to effectively resolve phylogenetic relationships and identify species within complicated groups [30,69]. In the present study, six highly variable regions were extracted from the complete plastid genomes. Among these regions, ycf1 had the same high species discriminating ability (87.5%) as the combination of four markers (HKLI); similarly, psdM-psbD showed the strikingly high discriminatory power (87.5%) (Fig 4, Fig 5). In fact, ycf1 had been successfully used to reconstruct the phylogenies of the family Orchidaceae [70] and the genus Astragalus [71], and it showed an extremely strong power to resolve interspecific relationships.

Therefore, we conclude that ycf1 and psdM-psbD can be used as potential specific barcodes for Fritillaria. However, the overly long DNA sequences of these regions result in some difficulties during PCR amplification and DNA Sanger sequencing [72]. Therefore, designing suitable primers to amplify shorter sections of those regions (about 1000 bp) with more variation might be another approach to be used in Sanger sequencing.

Delimitation of F. cirrhosa and its effect on species discrimination abilities of DNA barcodes

In the current study, the NJ trees generated using plastid genomes, universal barcodes, specific barcodes, and their combinations failed to resolve the samples of F. cirrhosa from different locations into one clade (Fig 5, S3 Fig). In the tree based on genome sequences, six individuals were divided into two different clades, one (BM1-1, BM1-2, and BM 2–2) was sister to F. unibracteata, but the other (BM2-1, BM 3–1, and BM 3–2) was placed close to F. przewalskii.

These results indicated that F. cirrhosa is not a “good” species but contains multiple different lineages, which was also supported by the previous analysis of population genetics data based on amplified fragment length polymorphism (AFLP) markers (unpublished data). It is well known that F. cirrhosa has extremely complex variations in its morphology, especially its floral characteristics. According to the field survey, individuals from Lijiang (ZDQ15019) possess yellow-green tepals, but they are dark purple in individuals from Shangri-La (ZDQ13053). The complex morphology of this species unavoidably causes confusion in its taxonomy. Delimitation, as well as phylogeny, of F. cirrhosa and its closely related species is still controversial [8] and requires further study based on more samples and better markers. Thus, if the polyphyletic condition of this species is not considered, we could conclude that all of the examined fragments, including the whole plastid genomes, matK, ycf1, and psbM-psbD, as well as the combination HKLI, were able to discriminate all the original species of Fritillariae cirrhosae bulbus and its adulterants with a success rate of 100%.

Conclusion

In the present study, 32 individuals from eight species, representing five species of the original plants of Fritillariae cirrhosae bulbus and three of its adulterants, were employed to compare the species discriminatory powers of universal, super, and specific DNA barcodes. The results revealed that the whole plastid genome used as a super-barcode exhibited a powerful ability to identify Fritillaria species, with high reliability. Among the universal barcodes, only matK could discriminate almost all the original species when NGS methods are employed. It should be noted that ITS2 separated genuine Fritillariae cirrhosae bulbus from its adulterants, but it could not correctly identify the original species. Among the highly variable regions examined, ycf1 and psbM-psbD are considered the primary potential specific barcodes for Fritillaria species, but their successful sequencing using the Sanger method will depend on developing primers that will amplify the barcodes in sections. Moreover, NJ analysis based on complete plastid genomes, as well as other regions, revealed that F. cirrhosa was polyphyletic and the variations
in its morphology requires further research at the population level. As the costs of NGS con-
tinue to decrease and data analysis methods are simplified, the use of super-barcodes might
become the primary method for species discrimination in plants. Overall, the results in this
study help to recognize species discrimination ability of super, universal, and specific barcodes
in complex groups, and provide new knowledge to accurately identify the original plants of
Fritillariae cirrhosae bulbus and its adulterants.

Supporting information

S1 Fig. Gene map of Fritillaria plastid genomes. Genes shown on the outside of the circle are
transcribed clockwise, and genes shown on the inside of the circle are transcribed counter-
clockwise. Genes belonging to different functional groups are color-coded. The darker gray
color in the inner circle corresponds to the GC content, and the lighter gray color corresponds
to the AT content. (PDF)

S2 Fig. Histograms of the frequencies (y-axes) of pairwise intra- and interspecific diver-
gences based on the p-distances (x-axes) calculated for single regions and their combina-
tions that were not listed in Fig 3. (H: trnH-psbA; K: matK; L: rbcL; I: ITS) (PDF)

S3 Fig. Neighbor-joining (NJ) tree generated for 33 individuals, including 32 from the
original species of Fritillariae cirrhosae bulbus and its adulterants, based on analyses of
single regions and their combinations that were not listed in the Fig 5. (H: trnH-psbA; K:
matK; L: rbcL; I: ITS) (PDF)

S1 Table. Collection information for the eight original species and one outgroup in Fritil-
laria in this study. (DOCX)

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