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Research article

Analysis of chloroplast genomes of ten central Asian *Fritillaria* species and their phylogenetic relationships

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Fritillaria is a taxonomically complex genus of Liliaceae that contains many important ornamental and medicinal species. In this study, we sequenced the chloroplast genomes of ten *Fritillaria* species distributed in central Asia and compared them to previously sequenced *Fritillaria* cp genomes. Comparison of 36 *Fritillaria* species' cp genomes identified as useful potential molecular markers due to their high level of divergence three coding regions and 11 intergenic spacers. Effective single sequence repeats (SSRs) for population genetic studies of *Fritillaria* were also identified. Phylogenetic analysis showed that two recently described species, *F. baisunensis* and *F. rugillosa* belong to *F.* subg. *Rhinopetalum*, and that the belonging of *F. ussuriensis* and *F. meleagroides* in *F.* subg. *Fritillaria* should be revised. These two species and *F. camschatsencese* currently assigned to *F.* subg. *Liliorhiza*, should be members of the same taxonomic unit. *Fritillaria* subg. *Liliorhiza*, although a small group, is more complicated than it was previously thought, and apparently comprises species too distant to be in one subgenus. *Fritillaria maximowischii*, currently assigned to *F.* subg. *Liliorhiza* should be treated as a new subgenus.

Keywords: central Asia, *Fritillaria*, chloroplast genome, comparative analysis, phylogeny, protein-coding genes

Introduction

The genus *Fritillaria* (Liliaceae) comprises approximately 158 species (POWO 2023) distributed across a vast range of temperate regions in the Northern Hemisphere, covering areas from North America through Europe, the Mediterranean, and central Asia, extending to China and Japan (Rønsted et al. 2005). Being one of the largest genera

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in Liliaceae, *Fritillaria* has considerable horticultural, medicinal, and ecological importance (Rix 2001, Day et al. 2014). The species of the genus are morphologically diverse reflecting their broad geographic distribution and long evolutionary history (Rønsted et al. 2005). *Fritillaria* flowers are usually nodding and solitary, but some species produce umbels or many-flowered racemes. The campanulate perianth often has contrasting light and dark patterns. Typical bulbs have fleshy scales and a thin tunic, though some resemble *Lilium* with naked bulbs and numerous bulblets (Rønsted et al. 2005). Nectary morphology is taxonomically important, with most species having linear, lanceolate or ovate nectaries while species from the subgenus *Rhinopetalum* have deeply depressed hairy nectaries (Khaniki and Persson 1997).

The taxonomic history of *Fritillaria* spans over 150 years. The traditional morphology-based taxonomy begins with the establishment of the genus *Fritillaria* by Linnaeus in 1753. Since then, the classifications were based primarily on such characters as bulb structure, flower morphology and leaf characteristics. In 1874, Baker divided *Fritillaria* into 10 subgenera based on features such as the bulb type, style shape, nectary form and capsule valves. Over the next century, various botanists built their revisions of the *Fritillaria* subgenera and sections upon Baker's classification (Boissier and Engelmann 1882, Bentham and Hooker 1883, Komarov 1935, Turrill and Sealy 1980). The latest morphology-based taxonomy by Rix (2001) recognizes eight subgenera (*Davidii, Liliorhiza, Japonica, Fritillaria, Rhinopetalum, Petilium, Theresia* and *Korolkowia*) defined by traits like bulb morphology and flower patterning. Bulbs of *Fritillaria* only possess 1–3 farinaceous scales and generally have no bulbils. However, subgenus *Liliorhiza* (including *F. maximowiczii*) and subgenus *Davidii* (*F. davidii*) both have bulbils. (Chen and Helen 2000)

The molecular era of *Fritillaria* taxonomy began in the mid-2000s with phylogenetic analyses utilizing a limited number of DNA regions, such as nuclear ITS and plastid *matK, rbcL* and *rpl16* (Rønsted et al. 2005, Day et al. 2014). These initial analyses yielded some conflicting results regarding subgeneric relationships. More recently, with the availability of next-generation sequencing approaches, the usage of complete chloroplast genomes allowed to resolve better the infrageneric phylogenetic relationships and to provide more robust taxonomic classifications of this complex genus (Chen et al. 2019, 2022, Zhang et al. 2021). Molecular data have also shed light on the biogeographic history of *Fritillaria*, suggesting its origin to be associated with uplift of the Qinghai-Tibetan Plateau in the early Miocene (17∼26 million years ago) followed by multiple migration events across Eurasia over the past 10–20 million years (Huang et al. 2018, Li et al. 2022). Unfortunately, while the phylogenetic position of East Asian *Fritillaria* species is well documented (Huang et al. 2018, Li et al. 2018, Chen et al. 2019, 2022), the molecular-based phylogeny of the central Asian species is poorly known. In order to fill this gap and support ongoing projects like 'Tree of Life: Monocots of Uzbekistan' (PFI-5), 'Taxonomic Revision of Polymorphic Plant Families of the Flora of Uzbekistan' (Sennikov et al. 2016), and 'Grid Mapping of the Flora of Uzbekistan' (), we gathered, sequenced, assembled, and analyzed the ITS of nine and the plastomes of ten central Asian *Fritillaria* species. Our study addressed the following questions: 1) what are the highly divergent regions of *Fritillaria* plastomes that can be useful for studying genus phylogeny? And 2) what are the phylogenetic relationships of central Asian *Fritillaria* species ?

Material and methods

Plant material

A total of 19 specimens (Supporting information), collected from ten species representing five subgenera of the genus *Fritillaria* (Fig. 1) were sampled from their wild habitats in Uzbekistan. The collected specimens were deposited at TASH; the voucher information is presented in the Supporting information. Fresh leaves of the collected specimens were dried with silica-gel. Besides the newly obtained chloroplast genomes of these 19 specimens, 35 cpDNA genomes and 53 ITS sequences were downloaded from GeneBank (Supporting information) and used for resolving genus phylogeny. Plastid genomes of *F. karelinii* and *F. eduardii* were resequenced, even though they were reported earlier in Li et al. (2018).

Sequencing, assembly, and annotation

Total genomic DNA of each sample was isolated from dried leaves using DP305 Plant Genomic DNA kits (Tiangen, Beijing, China) according to the manufacturer's instructions. The NEBNext UltraTM DNA Library Prep Kit for Illumina (NEB, USA, Catalog: E7370L) was utilized to generate the sequencing library, with index codes added to each sample as recommended by the manufacturer. Sonication was used to fragment the genomic DNA sample to a size of 350 bp. The fragmented DNA was then end-polished, A-tailed, and ligated with full-length adapters for Illumina sequencing, followed by further PCR amplification. The PCR products were purified using the AMPure XP system (Beverly, USA). Library quality was assessed on the Agilent 5400 system (Agilent, USA) and quantified by QPCR (1.5 nM). Then the libraries were pooled and sequenced on Illumina platforms using the PE150 strategy at Novogene Bioinformatics Technology Co. (Beijing, China), based on the effective library concentration and data amount required. The resulting clean reads were assembled using the GetOrganelle pipeline (Jin et al. 2020). Gene annotation was performed in Geneious ver. 10.0.2, with *Fritillaria cirrhosa* (KY646167) set as the reference. Start and stop codons and intron/exon boundaries for protein-coding genes were manually checked (Kearse et al. 2012). The raw sequencing data have been submitted to the Sequence Read Archive (SRA) database under the project number PRJNA1127285.

Simple sequence repeats (SSRs)

The MicroSatellite (MISA) web tool (Beier et al. 2017) was utilized to identify the simple sequence repeats (SSRs) in the chloroplast genome. The search parameters were configured

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Figure 1. The species of *Fritillaria* whose genome was recently extracted. (A) *F. bucharica,* (B) *F. baisunensis,* (C) *F. stenanthera,* photo by Gaziev. (D) *F. rugillosa,* (E) *F. karelinii,* photo by Beshko. (F) *F. gibbosa,* (G) *F. olgae,* (H) *F. ferganensis,* (I) *F. sewerzowi.* and (J) *F. eduardii*.

to detect mono-, di-, tri-, tetra-, penta-, and hexa-nucleotide motifs that were perfect and had a minimum of 10, 5, 4, 3, 3 and 3 repeats, respectively. To identify the repeats in the cp genomes, the REPuter program (Kurtz et al. 2001) was employed, which identified forward, reverse, palindrome and complement sequences. The repeat identification settings were set as follows: 1) hamming distance of 3; 2) minimal repeat size of 30 bp; and 3) maximum computed repeats of 90 bp.

Comparative analysis of chloroplast genomes

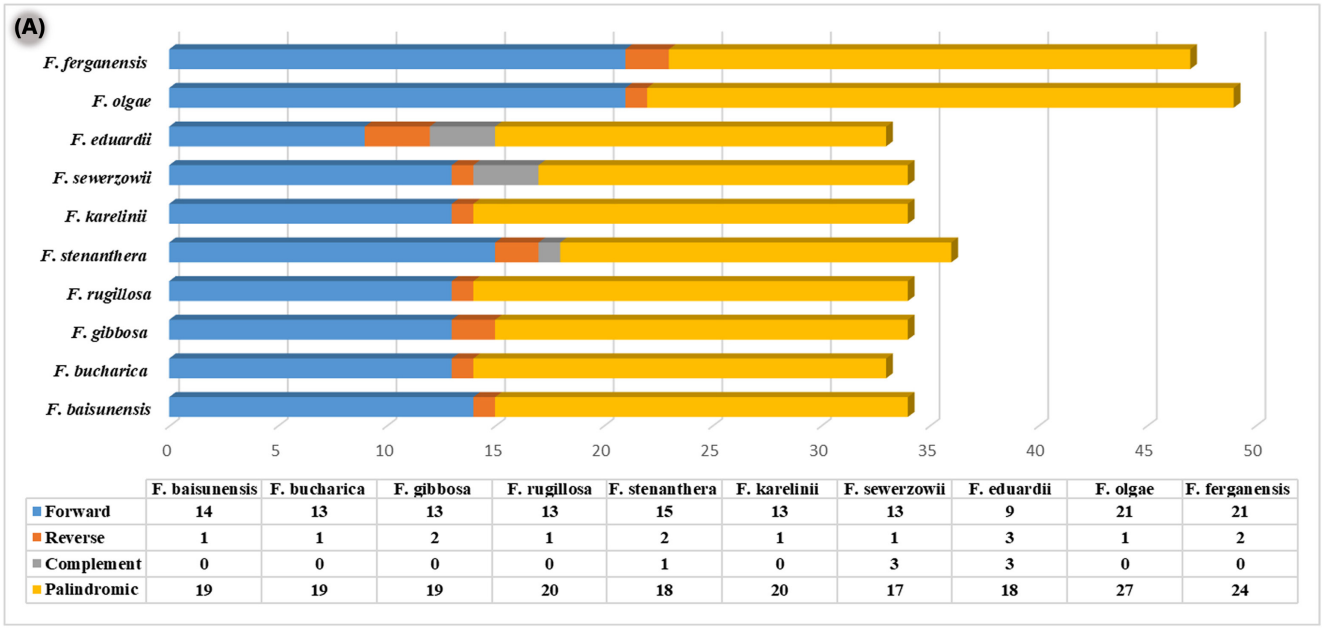
The chloroplast genome was visualized using OGDRAW ver. 1.1 (Lohse et al. 2007), and nucleotide variability (Xu et al.) was calculated using DnaSP ver. 6.12.03 software (Rozas et al. 2018). We calculated Pi for the whole chloroplast genome and protein-coding genes separately, using a window length of 800 bp and a step size of 200 bp. Additionally, we compared pairwise chloroplast genomic alignment among 10 species by mVISTA in Shuffle-LAGAN mode (Frazer et al. 2004), with *F. cirrhosa* (KY646167) serving as the reference.

Phylogenetic analysis

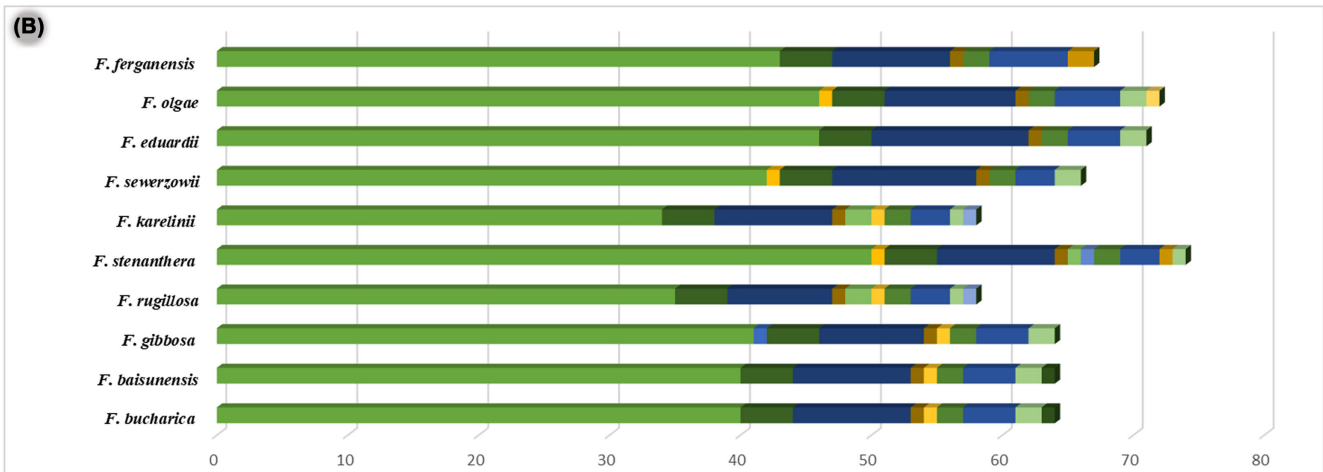
Phylogenetic tree reconstruction was performed using protein-coding sequences (CDS) of 54 plastomes representing 36 species, of which 35 were obtained from GenBank (Supporting information). In addition, 53 nuclear ITS sequences, of which 37 were obtained from GenBank (Supporting information) were used for phylogeny reconstruction. The sequences were aligned using MAFFT software (Katoh and Standley 2013). Three species from the genus *Lilium* served as the outgroup species.

The phylogenetic relationships of 37 *Fritillaria* species and the outgroups were determined through the use of maximum likelihood (ML), maximum parsimony (MP) and Bayesian inference (BI) methods. The ML tree was constructed using IQ-TREE ver. 2.1.2 software (Minh et al. 2021) with 1000

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	F. baisunensis	F. bucharica	F. gibbosa	F. rugillosa	F. stenanthera	F. karelinii	F. sewerzowii	F. eduardii	F. olgae	F. ferganensis
Forward	14	13	13	13	15	13	13	9	21	21
Reverse	1	1	2	1	2	1	1	3	1	2
Complement	0	0	0	0	1	0	3	3	0	0
Palindromic	19	19	19	20	18	20	17	18	27	24



	F. bucharica	F. baisunensis	F. gibbosa	F. rugillosa	F. stenanthera	F. karelinii	F. sewerzowii	F. eduardii	F. olgae	F. ferganensis
A/T	40	40	41	35	50	34	42	46	46	43
C/G	0	0	1	0	0	0	0	0	0	0
AC/GT	0	0	0	0	1	0	1	0	1	0
AG/CT	4	4	4	4	4	4	4	4	4	4
AT/AT	9	9	8	8	9	9	11	12	10	9
AAG/CTT	1	1	1	1	1	1	1	1	1	1
AAT/ATT	0	0	0	2	1	2	0	0	0	0
AGG/CCT	0	0	0	0	1	0	0	0	0	0
AAAC/GTTT	1	1	1	1	0	1	0	0	0	0
AAAG/CTTT	2	2	2	2	2	2	2	2	2	2
AAAT/ATTT	4	4	4	3	3	3	3	4	5	6
AAGT/ACTT	0	0	0	0	1	0	0	0	0	2
AATT/AATT	2	2	2	1	1	1	2	2	2	0
AAATG/ATTTC	0	0	0	1	0	1	0	0	0	0
AAAATT/AATTTT	0	0	0	0	0	0	0	0	1	0
AAAATG/ATTTTC	1	1	0	0	0	0	0	0	0	0

Figure 2. Analysis of simple repetitive sequences in ten *Fritillaria* cp genomes. (A) Number of four types of repeats, (B) total numbers of different SSR motifs.

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bootstrap replicates to evaluate clade support (Nguyen et al. 2015). The best model for the chloroplast genome was determined to be the GTR+G model based on the Akaike information criterion (AIC) using jModelTest2 on XSEDE (www.phylo.org). For MP analysis, PAUP* 4.0a169 (Swofford 2002 #72) was utilized with heuristic search, TBR branch-swapping, 1000 bootstrap replicates, a random addition sequence with 10 replicates, and a maximum of 1000 trees saved per round. Bayesian inference (BI) was performed using the MrBayes ver. 3.2 software (Ronquist et al. 2012). The models of partitions and jModelTest (the same set used in the likelihood analyses) for BI analyses were parameterized independently. Two independent runs of 10 million generations were performed using the BI analyses. Trees were sampled every 1000 generations, with the first 25% trees of each run discarded as the burn-in. The phylogenetic trees for nrDNA (ITS) data using MP, ML and BI methods were constructed as described above.

Results

Chloroplast genome features of Fritillaria species

The size of the newly sequenced plastid genomes of *F. gibbosa, F. bucharica*, *F. rugillosa, F. stenanthera, F. sewerzowi, F. ferganensis, F. baisunensis, F. eduardii*, *F. karelinii* and *F. olgae* varied from 152 206 (*F. stenanthera*) to 151 725 (*F. ferganensis*). The cp genomes presented a typical quadripartite structure including one LSC region (82 007–81 540 bp), one SSC region (17 559–17 280 bp), and a pair of IR regions (26 402–26 330 each), respectively. The GC (guanine+cytosine) contents of the plastid genomes of these study species were nearly similar, varying from 37.00% (*F. eduardii* and *F. ferganensis*) to 36.93% (*F. stenanthera*). A total of 114 distinct genes were annotated, including 78 PCGs, 30 tRNA genes, and four rRNA genes. Eighteen genes were duplicated in the cp genome, including eight tRNAs (*trnA-UGC*, *trnI-CAU*, *trnI-GAU, trnL-CAA, trnNGUU, trnR-ACG, trnV-GAC, trnH-GUG*), four rRNAs (*rrn16, rrn23, rrn4.5, rrn5*), and six PCGs (*ndh*B, *rpl*2, *rpl*23, *rps*12, *ycf*2, *rps7*). There were 18 intron-containing genes, among which two genes (*ycf*3 and *clp*P) had two introns each, while the other 16 had one intron, including 10 PCGs (*atp*F, *rpo*C1, *rpl*2, *ndh*B, *ndh*A, *pet*B, *pet*D, *rpl*16, *rps*16, *rps*12) and five tRNA genes (*trn*A-UGC, *trn*I-GAU, *trn*K-UUU, *trn*L-UAA, *trn*V-UAC). The mean p-distance among the seven species was 0.006408, ranging from 0.000119 to 0.01. The distance between *F. olgae* – *F. stenanthera* and the other five species was larger than that between the other five species, indicating that *F. olgae* and *F. stenanthera* were most divergent while *F. karelinii* and *F. rugilosa* (p-distance = 0.000119) were least divergent.

SSR analysis and repeat sequences

We identified a total of 310 repeats, including 147 palindromic, 142 forward, 14 reverse and 7 complement, in the ten newly sequenced *Fritillaria* plastomes using REPuter (Fig. 2A). *Fritillaria stenanthera* possessed the largest total number of repeats (36) followed by *F. olgae* and *F. ferganensis* (35 each), while *F. baisunensis* contained the fewest. With MISA, a total of 848 SSRs were identified across the 10 newly sequenced *Fritillaria* plastomes. Each plastome (or species) was found to contain a number of SSRs ranging from 58 (*F. karelinii*) to 74 (*F. stenanthera*) (Fig. 2B) and six types of SSRs were detected. Mono- (A/T), di- (mostly AG/AT), tri- (AAG), tetra- (AAAG/AAAT/AATT) were present in each plastome. Mono-nucleotide (C/G) SSRs were present only in *F. gibbosa*, di-nucleotide (AC) in only *F. stenanthera.* Tri-nucleotide (AAT) SSRs were present only in *F. stenanthera, F. rugillosa* and *F. karelinii* while AGG type SSRs were present only in *F. stenanthera*. Tetra-nucleotide (AAAC) SSRs were present in 5 plastomes (*F. gibbose, F. bucharica, F. rugillosa, F. baisunensis* and *F. karelinii*) while AAGT type SSRs were present only in the *F. stenanthera* plastome. Penta-nucleotide (AAATG) was found rarely in only two species (*F. rugillosa* and *F. karelinii*). Two hexa-nucleotide AAAATG/AAAATT SSRs each were found in only one species, *F. baisunensis* and *F. olgae*, respectively (Fig. 2B).

Genome-wide comparative analyses

The sliding window analysis identified the following most divergent among *Fritillaria* species regions: *mat*K – *rps*16, *trn*E-UUC – *trn*T-GGU, *rpo*B – *trn*C-GCA, *psb*M – *trn*D-GUC, *trn*T-UGU – *trn*F-GAA, *rbc*L – *acc*D, *acc*D – *psa*I, *ycf*4 – *cem*A, *pet*A – *psb*J, *psb*H – *pet*B, *ndh*F – *rpl*32 and *ycf*1 (Table 1). These regions are potentially useful molecular

Table 1. Twelve regions of highly variable sequences of *Fritillaria*.

No.	Highly variable marker	Length	Variable sites	Parsimony informative sites	Nucleotide diversity
1	*mat*K – *rps*16	4327	131	63	0.01007
2	*trn*E-UUC – *trn*T-GGU	852	44	18	0.018
3	*rpo*B – *trn*C-GCA	3970	88	44	0.0074
4	*psb*M – *trn*D-GUC	1233	38	22	0.01234
5	*trn*T-UGU – *trn*F-GAA	1823	59	32	0.01151
6	*rbc*L – *acc*D	3669	71	34	0.0066
7	*acc*D – *psa*I	2441	57	27	0.00796
8	*ycf*4 – *cem*A	2004	57	34	0.01101
9	*pet*A – *psb*J	1923	56	37	0.0107
10	*psb*H – *pet*B	1839	47	28	0.00942
11	*ndh*F – *rpl*32	2817	56	26	0.00614
12	*ycf*1	5580	202	117	0.01319

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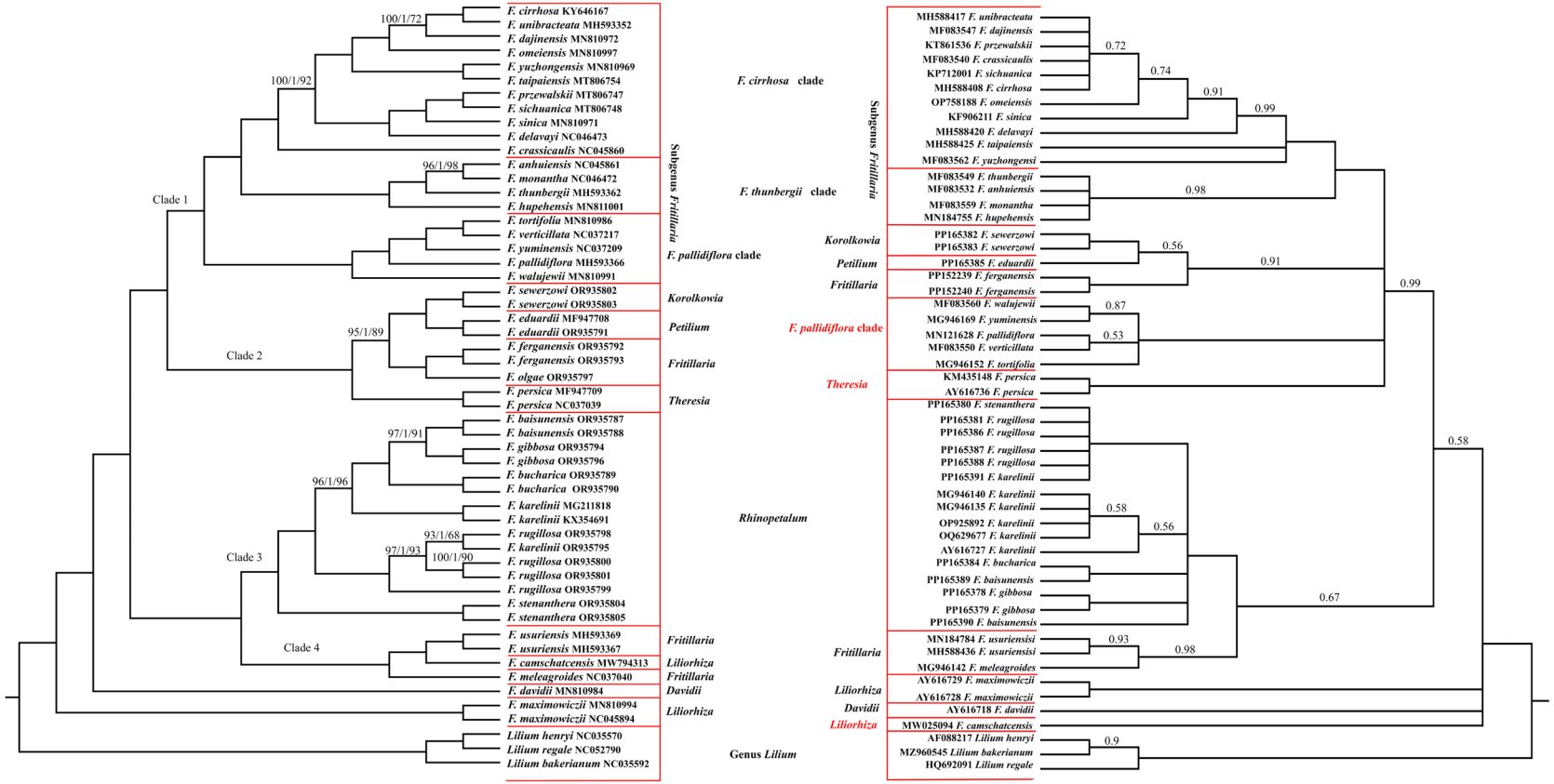


Figure 3. Phylogenetic relationship of *Fritillaria* species inferred using CDS (left) and ITS (right) sequences data. ML/BI/MPvalues are shown near corresponding nodes (only values below 100/100/1 are shown.

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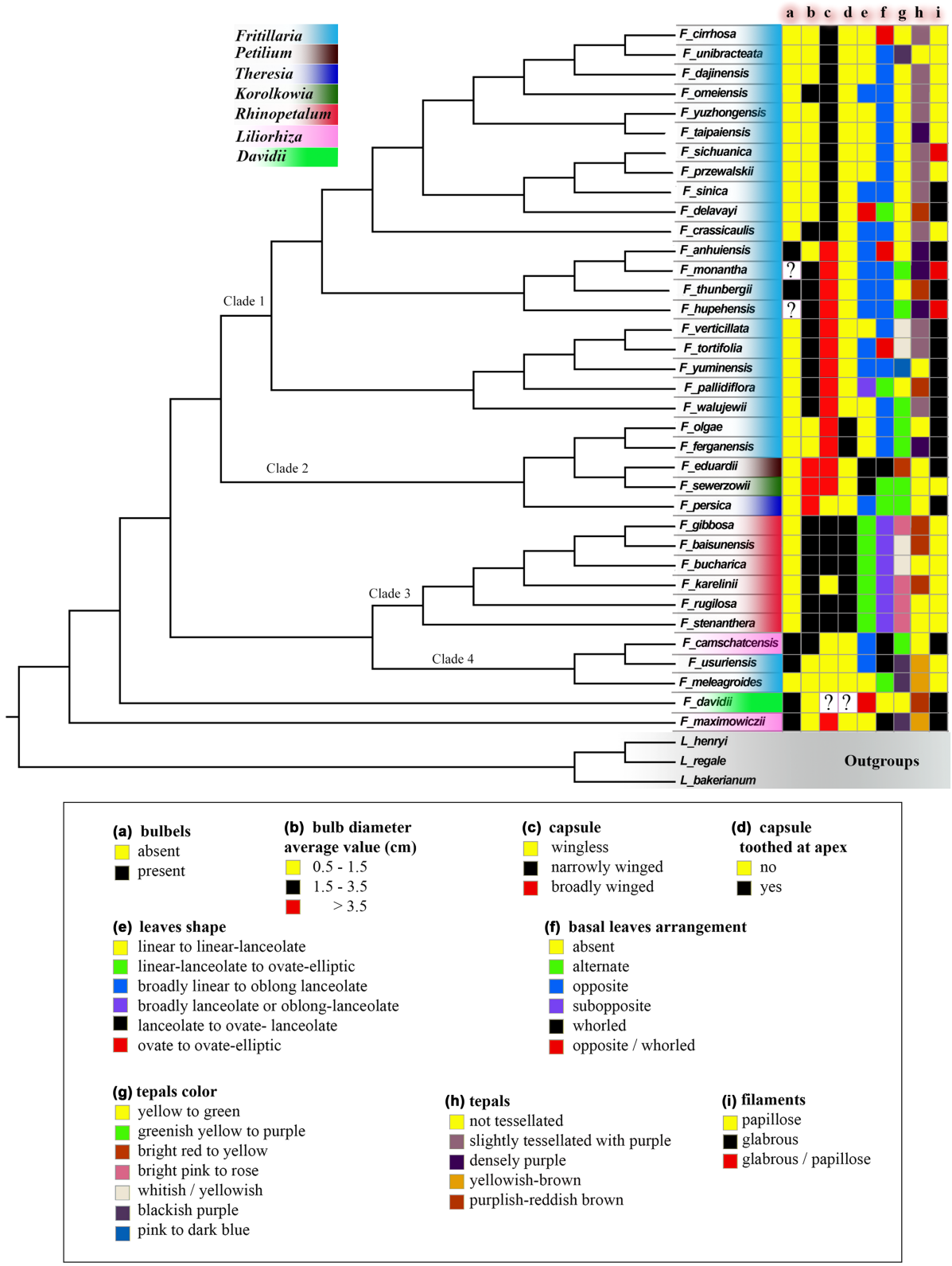


Figure 4. Comparison of morphological characteristics and the plastome phylogeny (? indicates no information).

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markers for conducting population genetics and phylogenetic studies.

Phylogenetic analysis

The aligned plastome and ITS datasets were 53 706 bp and 506 bp in length, respectively. The plastome matrix included 61 CDSs. For plastomes, the topologies of the ML, BI, and MP trees did not differ. For ITS trees, the ML, BI, and MP trees differed in their topology; the BI tree most similar to the CDS tree and with strongest clade support values is described below and used for discussion of the results.

In the plastome tree, *F. maximowiczii* had the basal position. This species and *F. davidii* were well separated from the other species organized into four distinct clades (BS = 100, PP = 1.00 for all clades) (Fig. 3). Clade 1 is composed of species predominantly distributed in China and is subdivided into three subclades. The *Fritillaria cirrhosa* subclade species is distributed in QTP-HHM (Qinghai Tibetan Plateau – Himalaya-Hengduan Mountains); the *Fritillaria anhuiensis* subclade species occur in Eastern Asia; the *Fritillaria tortifolia* subclade is sister to these two subclades, and this subclade is mostly distributed in the Xinjiang province of China. Clade 2 comprise species from the subgenera *Fritillaria, Petilium, Korolkowia* and *Theresia* distributed in central Asia and the Irano-Turanian region. Clade 3 comprise species from the subgenus *Rhinopetalum* of predominantly central Asian distribution. Clade 4 comprise *F. usuriensis, F. meleagroides* and *F. camschatcensis*.

In the ITS tree (Fig. 3), due to small size and relatively low variability of ITS sequences, many clades were unresolved concealing a general similarity of CDS and ITS trees. In particular, in both trees *F. eduardii, F. sewerzowi* and *F. ferganensis* clustered together, as well as *F. usuriensis* and *F. meleagroides*. In both trees, *F. maximowiczii* and *F. davidii* formed two separate basal clades. Incongruencies between the plastome and ITS trees were in positions of *F. camschatcensis* and *F. persica*.

Morphological analysis

In our study, we surveyed species in the Flora of China (FOC), Flora of the USSR, and Flora Iranica focusing on the morphological characters of *Fritillaria*. We compared these characters with the results of our plastome tree, as illustrated in Fig. 4. Our research focused on the role of bulb, capsule, leaves, and filament structure in the evolution of *Fritillaria*. Within clade 4, three species *F. usuriensis* and *F. meleagroides* from the *Fritillaria* subgenus, and *F. camschatcensis* from the *Liliorhiza* subgenus share several distinctive features. Despite belonging to different subgenera, these three species exhibit a number of common characteristics that set them apart from other *Fritillaria* species within the same clade. First, unlike other *Fritillaria* species that possess winged capsules, these three species have wingless capsules. Second, *F. usuriensis* and *F. camschatcensis* possess bulbils, similar to those found in subgenus *Liliorhiza*. Interestingly, in both *F. usuriensis* and *F. camschatcensis*, the basal leaves are arranged in a whorled pattern around the stem, further distinguishing them from other *Fritillaria* species. These shared features suggest a potential closer evolutionary relationship between these three species within clade 4.

Discussion

Chloroplast genome characteristics

The analyzed plastomes of ten *Fritillaria* species varied in size from 151 725 to 152 206 bp which is in line with the size of previously sequenced *Fritillaria* cp genomes (Li et al. 2018, Huang et al. 2020, Lu et al. 2021) and falls within the typical range of cp genome sizes for angiosperms. The gene content and gene order were the same in all species, containing 78 PCGs, 30 tRNA genes, and four rRNA genes, and the *inf*A but *ycf*15 genes were not annotated due to the region absence. All the analyzed cp genomes had *clp*P, which was absent in the *F. hupehensis* cp genome (Li et al. 2018).

We found from 58 to 74 SSRs in the chloroplast genomes of the ten *Fritillaria* species, mostly in noncoding regions, which is similar to findings of Huang et al. (2020), but much less than what was found by Park et al. (2017).

Earlier, Zhang et al. (2021) identified in 11 *Fritillaria* species *ycf1, trnL, trnF, ndhD, trnN-trnR, trnE-trnT, trnN, psbM-trnD*, *atpI* and *rps19* as the most variable regions. Wu et al. (2021) identified in 26 plastomes representing 10 *Fritillaria* species rps19 as the most variable protein-coding gene, and rpl22-rps19 and rpl16-rps3 as the most variable spacer regions. Huang et al. (2020) identified six hypervariable intergenic spacer regions (*rps16-trnQ, rbcL-accD, accD-psaI, psaJ-rpl33, petD-rpoA*, and *rpl32-trnL*) in the plastomes of 26 *Fritillaria* species. The results of our study of 36 *Fritillaria* plastomes confirm high variability of *rbcL-accD* and *accD-psaI* but not of the other regions reported by Huang et al. (2020) as hypervariable. In general, we see no consistency in the plastome regions reported as hypervariable from one *Fritillaria* study to another.

Phylogenetic relationships and taxonomic implications

In a phylogenetic study of *Fritillaria*, that by Huang et al. (2018), in the phylogenetic tree derived using three chloroplast markers, within the Old World clade of *Fritillaria*, all six subgenera delimited by Rix (2001) were strongly supported as monophyletic except *F.* subg. *Fritillaria*, and the relationships between subgenera were resolved. The polyphiletic *F.* subg. *Fritillaria* formed two well supported clades, one comprising species from the Mediterranean Basin and Europe, and another one with species mainly from China grouped into three monophyletic subclades. In all phylogenetic studies utilizing full plastome data (Huang et al. 2020, Zhang et al. 2021, Chen et al. 2022, Li et al. 2022) sampling was much more superficial than in Huang et al. (2018), and in all these studies samples from the Mediterranean Basin and Europe were absent. However, in Chen et al. (2022), *F.* subg. *Fritillaria* in China were divided into four clades, three identical with those in Huang et al. (2018), and a new one with

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F. meleagroides and *F. ussuriensis* not sampled by Huang et al. (2018). In Huang et al. (2018), as well as Li et al. (2018) and Zhang et al. (2021), *F. karelinii* from *F.* subg. *Rhinopetalum* was also a member of this clade. In contrast to these results, our CDS tree clearly shows that *F. karelinii* has a firm position within a clade comprising members of *F.* subg. *Rhinopetalum* only and having central Asian and Turano-Iranian distribution. Another important finding of our study is a close relationship of *F. camschatcensis* from *F.* subg. *Liliorhiza* with *F. meleagroides* and *F. ussuriensis*, and a sister position of their clade to the *F.* subg. *Rhinopetalum* clade.

Our study is the most representative analysis of *Fritillaria* phylogeny using complete chloroplast genomic data (37 species). Similar previous studies utilized 21 species (Chen et al. 2022), 26 species (Huang et al. 2020, Li et al. 2022) and 27 species (Zhang et al. 2021). More comprehensive sampling, especially species from central Asia, allowed new insights into genus phylogeny. From the plastome-based phylogeny, it is evident that *F.* subg. *Liliorhiza*, although a small group, is more complicated that it was previously thought, and apparently comprises species too distant to be in one subgenus. *Fritillaria ussuriensis* and *F. meleagroides*, although morphologically similar to members of *F.* subg. *Fritillaria*, have a different evolutionary history and their similarity is a result of convergence. *Fritillaria Camschatsencese*, previously assigned to *F.* subg. *Liliorhiza*, should be a member of the same taxonomic unit as the above two species. *Fritillaria maximowiczii* should be treated as a new subgenus.

It is important to stress that our CDS phylogenetic tree, like analogous plastome-based trees of Huang et al. (2020), Zhang et al. (2021), Chen et al. (2022), Li et al. (2022) has very strong support, much stronger than trees obtained with the three cp markers, ITS or their sequences combined (Rønsted et al. 2005, Day et al. 2014, Huang et al. 2018). In comparison with the ITS phylogeny, the plastome data are more consistent with the species distributions and members of the same clade usually share the same regional distribution. These two features of the plastome sequence data, i.e. strong clade support and good agreement with clade geographic distribution, appear to indicate that plastome data better reflect group evolutionary history than ITS and the revisions of the current taxonomy (which is based or morphology alone) should utilize plastome sequence data.

However, in comparison with the ITS-based phylogenetic trees, both previously published and done by us, the disagreements are not strong. ITS-based trees tend to be a bit more consistent with Rix's (2001) morphological classification of the genus. However, both tree types reveal problems in the current genus taxonomy. These problems mostly are in the phylogenetic position of some members of *F.* subg. *Fritillaria* which was found in all previous molecular studies to be polyphiletic (Rønsted et al. 2005, Day et al. 2014, Li et al. 2018, Huang et al. 2018, Zhang et al. 2021). The most problematic are *F. olgae* and *F. ferganensis*. The four central Asian species, *F. eduardii, F. sewerzowi, F. olgae, and F. ferganensis*, are members of the same clade in both the ITS and plastome trees, despite belonging to different subgenera sensu Rix (2001). In Rix' classification, *F. olgae* and *F. ferganensis* belong to *F.* subg. *Fritillaria*, *F. sewerzowi* to *F.* subg. *Korolkowii,* and *F. eduardii* to *F.* subg. *Petilium*. The latter two subgenera exhibit monophily within this clade, and it would be logical to consider *F. ferganensis* and *F. olgae* as members of a new subgenus. Alternatively, *F. ferganensis*, *F. olgae*, *F. sewerzowii*, *F. eduardii*, and such species as *F. chitralensis* and *F. raddiana* should all be considered as representatives of the same subgenus. Earlier, Rønsted et al. (2005) proposed to combine *F.* subg. *Petilium* with *F. sewerzowi*, *F. pallidiflora* and *F. persica* due to such similarities as large bulbs formed by several thick, fleshy scales and a strong stout stem. This recommendation cannot be approved since the ITS and plastome trees for *F. pallidiflora* do not support it.

In our study, the focus was on central Asian species insufficiently covered by the to-date published phylogenetic studies. In Rønsted et al. (2005), only *F. gibbosa* and *F. karelinii* represented *F.* subg. *Rhinopetalum*; in Huang et al. (2018), *F. gibbosa, F. bucharica* and *F. stenanthera* were included andin Day et al. (2014) *F. gibbosa, F. bucharica, F. karelinii* and *F. stenanthera*. Two recently described species, *F. baisunensis* and *F. rugillosa* (Rukšāns 2019, Naumenko and Zubov 2021) have been confirmed by both plastome and ITS data to belong to *F.* subg. *Rhinopetalum*. Because *F. karelini* differs from the other members of *F.* subg. *Rhinopetalum* in having wingless capsule and highly speckled perianth, and clustered together with *F. ussuriensis* and *F. meleagroides* in the phylogenetic trees of Zhang et al. (2021) and Chen et al. (2022), we newly sequenced *F. karelini* sampled in central Asia. The new plastome sequence turned out to be more similar to *F. rugilosa* than to the sequences of *F. karelini* samples from China that we downloaded from NCBI. This may indicate the existance of a '*Rhinopetalum* complex', a group of recent diversification and rapid evolution, characterized by extensive morphological variation (Supporting information) and probably hybridization, making this group taxonomy problematic.

Fritillaria ferganensis was synonymized with *F. walujewii* by Li et al. (2018). However, they were located in different clades in both the plastome and ITS trees which agrees well with the known morphological differences of the two species. *Fritillaria ferganensis* differs from *F. walujewii* by moderately angular perigonium, pinkish-violet tepals on the outer and inner sides, and capsule valves marked by prominently elevated ridges, culminating in acute hooks (Sennikov and Lazkov 2013).

Conclusions

Our reconstruction of the phylogeny of *Fritillaria* based on nrDNA ITS and plastid DNA sequences revealed that the existing morphology-based taxonomy requires considerable revision. For future studies of *Fritillaria* phylogeny we recommend, considering both level of variability and sequence length, usage of such highly variable genes and intergenic regions as *trnE-UUC – trnT-GGU*, *psbM – trnD-GUC*, *trnT-UGU – trnF-GAA*, *ycf4 – cemA, petA – psbJ*, *psbH – petB*.

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Data availability statement

Data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.1c59zw452 (Karimov et al. 2024).

Supporting information

The Supporting information associated with this article is available with the online version.

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