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Molecular characterization of phylogeneticrelationships in *Fritillaria* species inferred from chloroplast trnL-trnF sequences

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Abstract: The genus *Fritillaria* embraces about 165 taxa in the family *Liliaceae*. In this study, the molecular phylogenetic relationships between 10 *Fritillaria* taxa were presented. *Fritillaria* spp. were collected from their natural habitats, and specimens with different morphological features were obtained via selective breeding. Specimens with the desired characters were presented as promising candidate cultivars for ornamental use. Phylogenetic analysis was based on DNA sequences of the chloroplast trnL-trnF region. The phylogeny was constructed using the neighbor joining, maximum parsimony, maximum likelihood, and Bayesian inference methods. The data showed that the examined *Fritillaria* spp. were evidently diverged into 2 *Fritillaria* subgenera. Members of the subgenus *Fritillaria* formed one clade while the other clade contained the subgenera *Theresia* and *Petilium*. Separation within the latter clade was strongly supported with bootstrap values, whereas resolution within the subgenus *Fritillaria* remained ambiguous. This analysis revealed the feasibility of the chloroplast trnL-trnF region DNA sequence for phylogeny of the *Fritillaria* species. Our study is the first phylogenetic analysis in *Fritillaria* spp. based on the trnL-trnF region. Fingerprint data of these cultivars would be a valuable source for their identification and for the generation of new cultivars in future.

Key words: *Fritillaria*, trnL, trnF, intergenic spacer, chloroplast, phylogeny, ornamental plants

Introduction

The genus *Fritillaria* is represented by approximately 165 taxa compiled of about 100 species, 17 subspecies, and 9 varieties. Members are distributed mainly in temporal regions of the northern hemisphere from the Middle East, through Europe, Central Asia, and to North America. Of the taxa, 41 are natural habitants of Turkey, of which 36.53% are endemic (1-5). Having a high number of species and endemism ratio, Turkey might be the center of genetic diversity of the genus (6). Members of the genus have great agronomic and economic importance. Aside from being one of the most desired ornamental plants, the bulbs of many fritillaries produce several alkaloids, and some have been used in traditional Chinese medicine for a long time (7,8).

After the first report of the genus *Fritillaria* by Linnaeus (9), numerous further classifications have been described (10-14). According to the last revision by Rix (14), the genus was subdivided into 8 subgenera, 2 sections, and 165 taxa. It was reported that *Fritillaria* spp. evidently divided in 2 groups:

North American and Eurasian species (15). Rønsted et al. (16) analyzed 3 plastid and nuclear DNA regions of *Fritillaria* spp., and their results supported the classification by Rix (14). There are supplementary categorizations of *Fritillaria* spp. based on numerous palynological characters, which have taxonomic importance for the genus (17-20). Some studies have attempted to classify the genus with karyological analysis (21). Although several publications are available about the morphological and physiological characterization of *Fritillaria*, molecular phylogenetic evaluation of the genus is not well studied.

Chloroplast markers are widely used for phylogenetic assessments (22-25). The low evolutionary rate of chloroplast DNA is the biggest drawback for the investigation of intraspecific relationships among sample sets. On the other hand, noncoding DNA sequences of the chloroplast genome evolve rapidly, and present a valuable source for phylogenetic studies (26,27). Since these noncoding DNA regions are mutational hot spots, the tRNA-Leu (trnL) intron, and the intergenic spacer (IGS) between the trnL 3' exon and tRNA-Phe (trnF) gene regions are quite suitable for the phylogenetic analysis of intraspecific variations (28). Accordingly, consensus primers were described for these noncoding regions (27).

Floriculture is an important industry. Worldwide, an estimated 223,105 ha are used for this aim, and the income from the sector is over 50 billion dollars (29). Inevitably, many studies have emerged to develop new ornamental plants via selective and hybridization breeding and genetic engineering. Members of the genus *Fritillaria* exhibit a broad range of variation in their morphological features and physiological responses to the environment. As a result of this diversity, they are one of the most widely used ornamental geophytes.

In this study, we address the determination of the phylogenetic relations among *Fritillaria* spp. For this purpose we evaluated the chloroplast trnL intron, and the trnL-trnF IGS regions. The phylogeny was constructed using the neighbor joining, maximum parsimony, maximum likelihood, and Bayesian inference methods. The results were comparable with the previous phylogenetic studies of the genus *Fritillaria*. In addition to molecular data, the morphological properties of the *Fritillaria* spp. were also evaluated. These molecular and morphological data would be a valuable source for the identification and generation of new *Fritillaria* varieties for ornamental purposes.

Materials and methods

Plant material

Samples of the genus *Fritillaria* were collected from different regions of Turkey. They were conserved in the collection garden of the Erzincan Horticultural Central Research Institute (EHRI). The plants were regularly checked for their specific morphological characters (plant height and width; flowering and vegetation time; size, number, color, and odor of tepals; and number, color, and size of the leaf). Specimens with the desired features were propagated by chipping the bulbs into 8 pieces. A total of 10 *Fritillaria* taxa consisting of 7 species were selected based on their morphological characters for further analyses. Of the 10, *F. michailovskyi* was represented by 3 specimens, while *F. imperialis* was represented by 2 specimens, and the others were represented by 1 specimen. The voucher specimens were deposited in the EHRI, Turkey. Sample information is presented in Table 1.

DNA extraction, PCR amplification, and sequencing reaction

Genomic DNA was extracted from the frozen leaves of 10 *Fritillaria* samples, as described previously (30). The intron of the chloroplast trnL (UAA) gene and partial trnL gene and the trnL-trnF IGS regions were amplified with primer combinations of c + d, and e + f, respectively (27). Polymerase chain reaction (PCR) amplifications were performed in a Bio-Rad cycler (Bio-Rad Laboratories, Inc., CA, USA) with an initial 2 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 45 s at 56 °C, and a final 1.5 min at 72 °C. Reactions were carried out in a total volume of 20 µL, containing 2.5 mM $MgCl_2$, 0.2 M of each dNTP, 1X PCR buffer (Fermentas, USA), 5 pmol of forward and reverse primers, 1.0 U of Taq DNA Polymerase, and 100 ng of DNA template. PCR products were subjected to gel electrophoresis and cleaned up using a PCR clean-up kit (Promega, USA). Purified PCR products

were directly sequenced from 2 directions, using the corresponding primers. Sequencing reactions were carried out using a DTCS cycle sequencing kit (Beckman Coulter, USA). DNA sequencing was performed using the GenomeLab™ GeXP Genetic Analysis System (Beckman Coulter).

Phylogenetic reconstruction

Sequences were edited using the SeqMan software of a DNAstar program package (DNAstar, USA). Edited sequences were subjected to Basic Local Alignment Search Tool searches (31) for preliminary analysis. The newly generated sequences were submitted to GenBank (Table 1). Multiple sequence alignment was carried out using the ClustalW program (32). The *F. persica* (NCBI accession number EU912327) sequence was the only available trnL-trnF region sequence for the genus *Fritillaria* (9 January, 2012), and was used to determine the boundaries of this region. Rønsted et al. (16) showed that *Fritillaria* and *Lilium* are sister taxa; thus *Lilium davidii* (NCBI accession number EU597205) was chosen as an outgroup in the analyses.

Phylogenetic analyses of the data matrix were performed based on the neighbor joining (NJ), maximum parsimony (MP), maximum likelihood (ML), and Bayesian Markov chain Monte Carlo methods. NJ analyses were conducted by calculating Kimura's 2-parameter distance model of the MEGA 4 software. MP trees were sought in the heuristic search of MEGA 4 with an initial 10,000 trees (33). The data set was analyzed using the ML method as well as the Tamura-Nei model of MEGA 4. Bootstrap values were calculated from 100 replicates (34), and bootstrap coefficiencies were indicated above each branch. The Bayesian inference (BI) method was conducted using MrBayes v.3.1.2 software (35). BI analysis was performed for 1,000,000 generations, and the trees were sampled every 10 generations, applying 2 runs with 4 heated chains (temperature parameter value of 0.2). The general time reversible model of molecular evolution with gamma-distributed rate variation across the sites was set. The halfcompat consensus tree was generated from the last 75,001 of the 100,001 trees sampled. The trees were viewed with TreeView v.32 (Lidor Systems, Radovis, Macedonia) (36).

Results

Morphological characters

After collection of the *Fritillaria* spp. from different regions of Turkey, they were cultivated in the EHRI. During this cultivation, a certain number of specimens exhibited different morphological features (Figure 1). Features of the *Fritillaria* cultivars are given in Table 2. *F. imperialis* F30013 forms flowers with 2 layers,

Table 1. *Fritillaria* species used in this study, with their voucher information, place of collection, and GenBank accession numbers.

Taxa	Specimen vouchers	Place of collection	GenBank accession number
F. aurea	M. Aslay F44043 (EHRI)	Malatya	JQ289553
F. caucasica	M. Aslay F75024 (EHRI)	Kars	JQ289557
F. imperialis	M. Aslay F23001 (EHRI)	Elazığ	JQ327135
F. imperialis	M. Aslay F30013 (EHRI)	Hakkari	JQ289551
F. crassifolia subsp. kurdica	M. Aslay F65035 (EHRI)	Van	JQ289552
F. latifolia	M. Aslay F75023 (EHRI)	Kars	JQ289555
F. michailovskyi	M. Aslay F36025 (EHRI)	Kars	JQ289554
F. michailovskyi	M. Aslay F04029 (EHRI)	Ağrı	JQ327136
F. michailovskyi	M. Aslay F25075 (EHRI)	Erzurum	JQ327137
F. minuta	M. Aslay F65032 (EHRI)	Van	JQ289556

Figure 1. Images of the taxa analyzed: a) *F. aurea* F44043, b) *F. caucasica* F75024, c) *F. crassifolia* subsp. *kurdica* F65035, d) *F. latifolia* F75023, e) F. *imperialis* F23001, f) *F. imperialis* F30013, g) *F. michailovskyi* F25075, h) *F. michailovskyi* F36025, i) *F. michailovskyi* F04029, and j) *F. minuta* F65032.

Table 2. Morphological features of 10 *Fritillaria* cultivars.

Species	**Morphological characters**
F. aurea M.Aslay F44043 (EHRI)	Narrow bell-shaped, red mottled on yellow tepals
F. caucasica M.Aslay F75024 (EHRI)	Tall length, deep purple tepals
F. imperialis M.Aslay F23001 (EHRI)	The tallest and the widest bell-shaped flowers within the species
F. imperialis M.Aslay F30013 (EHRI)	Two layered placement of red tepals
F. crassifolia subsp. *kurdica* M.Aslay F65035 (EHRI)	Multiflower and multileaf, tessellated
F. latifolia M.Aslay F75023 (EHRI)	The biggest flower size within the species
F. michailovskyi M.Aslay F36025 (EHRI)	Yellow striped, flat-end tepals
F. michailovskyi M.Aslay F04029 (EHRI)	Multiflower
F. michailovskyi M.Aslay F25075 (EHRI)	Claret striped on yellow tepals
F. minuta M.Aslay F65032 (EHRI)	Wide and multileaf

whereas this species normally has flowers with a single layer. The flower number of *F. crassifolia* subsp. *kurdica* and *F. michailovskyi* typically varies from 1 to 3 per plant, while *F. crassifolia* subsp. *kurdica* F65035 and *F. michailovskyi* F04029 produced several flowers (around 8 and 13 flowers, respectively). The flower number of *F. imperialis* normally changes from 3 to 10, while *F. imperialis* F30013 yielded 14 flowers. *F. crassifolia* subsp. *kurdica* normally has around 5-7 leaves, but *F. crassifolia* subsp. *kurdica* F65035 produced about 23 leaves. The typical flower size of *F. latifolia* is smaller than that of *F. latifolia* F75023 (approximately 3.5 cm). *F. caucasica* F75024 is also taller than a typical *F. caucasica*. Further analyses were performed on these selected specimens.

Amplification, sequencing, sequence characteristics

Due to a sequencing problem in the total trnL-trnF region, 2 primer combinations were used separately. c + d primers amplified the intron of the trnL, while e + f primers amplified the partial trnL gene and trnL-trnF IGS regions. PCR amplification of the trnL-trnF region was successfully obtained for all of the samples (Figure 2). The samples had PCR product of about 250 bp and 600 bp with the c + d and e + f primer sets, respectively. The amplicon sizes were similar among the samples for both primer pairs, whereas *F. imperialis* M. Aslay F23001 (EHRI) showed a clear difference in amplicon length for the e + f primer set (Figure 2). The PCR products were directly sequenced from both directions after gel purification.

Sequence alignment and divergence

Sequences of the trnL and trnL-trnF IGS regions of 11 *Fritillaria* taxa and 1 *Lilium* sp. were aligned to determine phylogenetic assessment. The *F. persica* and *L. davidii* sequences were downloaded from the NCBI databank. Since the sequence of *F. persica* was smaller than the rest, it was excluded only in this analysis. The total length of this region among the 10 *Fritillaria* samples ranged from 770 to 810 bp. Among the samples, the trnL region showed higher length divergence than the IGS region. In detail, the trnL sequence length ranged from 676 to 717 bp, while the IGS length varied from 93 to 94 bp. The average G + C content of the trnL and IGS regions was 32.3% and 43.8%, respectively. The trnL and IGS regions had 719 and 95 alignment characters, respectively. The trnL region showed 94.94% identity, whereas the IGS region had slightly lower conservation, with 93.68% identity. Within the whole region, 9 parsimony informative sites (1.11%) were found.

Phylogenetic relationships among Fritillaria species

In the databank, for the genus *Fritillaria*, the trnL-trnF sequence of *F. persica* is present (37) and was used for our phylogeny estimation. *L. davidii* was applied as an outgroup. The sequence of *F. persica* was 93 bp shorter (44 bp from the 5' end and 49 bp from the 3' end) than the newly generated sequences. Analyses were performed both excluding and including the missing regions, and the same phylogenetic trees were obtained (data not shown). Here, results obtained from whole region analysis

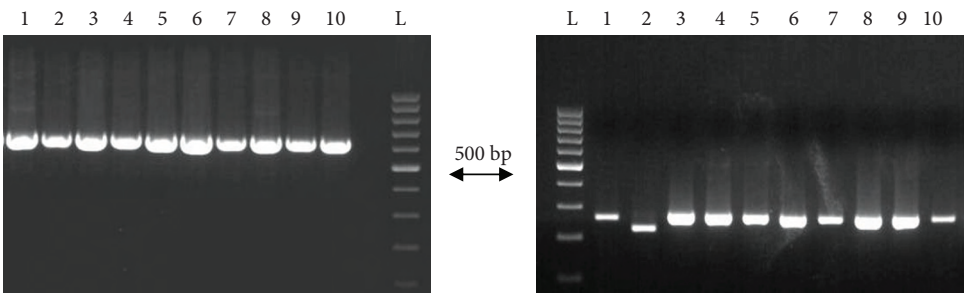

Figure 2. Amplified trnL (left), part of the trnL and trnL-trnF IGS regions (right) of 10 *Fritillaria* taxa. Order of the samples are: 1) F. *imperialis* F23001, 2) *F. imperialis* F30013, 3) *F. crassifolia* subsp. *kurdica* F65035, 4) *F. aurea* F44043, 5) *F. michailovskyi* F36025, 6) *F. michailovskyi* F04029, 7) *F. michailovskyi* F25075, 8) *F. latifolia* F75023, 9) *F. minuta F65032*, 10) *F. caucasica* F75024, and L) DNA ladder.

are presented. Four phylogenetic methods were carried out to investigate the relationships between 11 *Fritillaria* taxa, including 8 species. The majority-rule consensus trees resulting from NJ analyses of the trnL-trnF region were highly consistent with the MP, ML, and Bayesian trees (Figure 3). Analyses showed that *Fritillaria* spp. divided into 2 strongly supported clades. As members of the subgenus *Fritillaria*, *F. aurea*, *F. caucasica*, *F. crassifolia* subsp. *kurdica*, *F. latifolia*, *F. minuta*, and *F. michailovskyi* formed 1 clade. However, resolution within the clade was low. *F. caucasica* and *F. aurea* could not be distinguished by NJ analysis. The other 3 analyses generated weak resolution between these 2 species as well. The same case was observed between *F. kurdica* and *F. minuta*. The other clade contained *F. imperialis* subg. *Petilium* and *F. persica*. subg. *Theresia*.

Two of the *F. imperialis* specimens grouped together. Although all 3 *F. michailovskyi* specimens fell into the same clade, the *F. michailovskyi* M. Aslay F04029 (EHRI) specimen diverged from a different node.

Discussion

Due to high interest in ornamental plants, generating new varieties has increasing importance. Species of the genus *Fritillaria* are extensively used as ornamental plants, in addition to utilization for medical purposes. In the concept of this study, *Fritillaria* spp. were collected from their natural habitats, and propagated in a collection garden. During propagation, a certain number of specimens generated new forms, and they were selected for further analyses. Since plant florogenesis is under the control of genetic and environmental factors, development of new forms is widely seen among certain plants (38-40). Although the specimens in this study were cultivated under optimum conditions, the plants still had to acclimate to certain differences between their original ecology and the collection houses. In our study, plant responses to new ecological conditions might also be the reason for formation of the specimens with new morphological features. Many variations have been observed in natural populations of certain *Fritillaria* spp. as well (41). Due to its broad variation, the genus has great agronomic and economic importance. However, there is little information about its molecular phylogeny in the literature thus far. In this study, we elucidated the chloroplast trnL-trnF DNA region of 11 *Fritillaria* taxa.

Due to their reproducibility, DNA sequencing-based strategies are widely used for identification of cultivars and phylogenetic reconstruction studies (42). Therefore, we performed sequencing from both forward and reverse directions, and they successfully verified each other. The newly generated sequences were compared with relevant sequences in the databank to elucidate their borders. This fact has been explained with catalytic properties and secondary structure formation of this region (27).

Our phylogeny analysis revealed that the 11 *Fritillaria* spp. clearly divided into 2 main clades. The first clade contains species of the subgenus *Fritillaria*, the largest subgenus of the genus. However, bootstrap values are not highly supportive of the classification within this clade, and so the resolution level is not so reliable within the subgenus *Fritillaria*. Particularly, separation between *F. caucasica* and *F. aurea*, and also *F. kurdica* and *F. minuta* remained unsolved. Similarly, Rønsted et al. (16) was also unable to detect a good separation in this clade. This indicates that variations in the analyzed regions are not sufficient to resolve phylogenetic relationships within the subgenus. Analyzing different DNA regions might solve this uncertainty.

Rønsted et al. (16) proposed that a combination of different matrices improves the resolution and bootstrap values of the groups. In our analysis, the subgenera *Petilium* and monotypic *Theresia* were grouped together and composed the second clade. In addition to molecular data, similar karyotype and pollen structures of *Petilium* and *Theresia* also support the fact that these 2 subgenera are closely related (20,43). In our study, the subgenus *Petilium* was composed of 2 *F. imperialis* (crown imperial) specimens. The amplicon size of *F. imperialis* M. Aslay F23001 (EHRI) was clearly shorter than the rest, yet 2 specimens of *F. imperialis* were grouped together. On the other hand, all of the *F. michailovskyi* (Michael's flower) specimens fell into the subgenus *Fritillaria* clade, but *F. michailovskyi* M. Aslay F04029 (EHRI) was placed separately from the other 2. Although this divergence was moderately supported by bootstrap

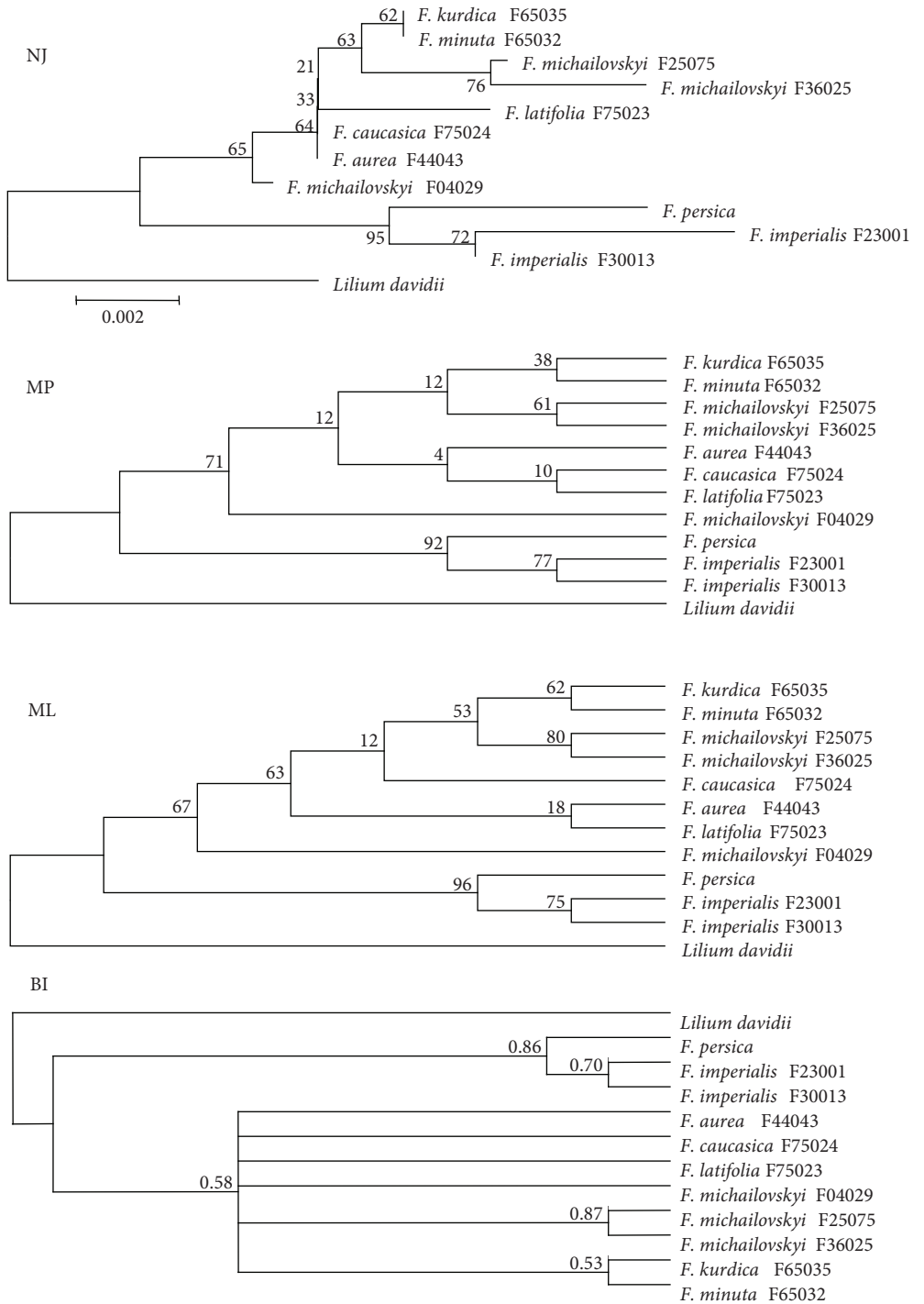

Figure 3. Majority-rule consensus trees of the *Fritillaria* species, obtained using the NJ, MP, ML, and BI methods. Bootstrap coefficiencies are shown above each branch.

numbers, it should be noted that this specimen differed from the other 2 in its morphological features, and it might belong to a different subspecies, and this might be the reason for this divergence.

The molecular phylogeny of many *Fritillaria* spp. has been analyzed by Rønsted et al. (16). However, *F. latifolia* was not included in that study. The only molecular taxonomy study on *F. latifolia* was based on the nuclear DNA sequence of its internal transcribed spacer (ITS) region (44). However, it was used as an outgroup in that publication, and its phylogenetic relationship with the genus *Fritillaria* was not evaluated. Our results demonstrated that *F. latifolia*, a member of the subgenus *Fritillaria*, was grouped together with the other members of the corresponding subgenus.

Rønsted et al. (16) analyzed comprehensively the *matK* gene, *rpl16* plastid gene, *trnK*, and nuclear ribosomal ITS sequences of various members of the genus *Fritillaria*. In addition, the 5S rDNA region sequences of 4 *Fritillaria* spp. were patented by Sucher et al. (45). To our knowledge, this is the first study on the phylogeny of *Fritillaria* spp. based on the trnL-trnF region. In summary, sequence analysis of the noncoding chloroplast trnL-trnF region demonstrated that the 11 *Fritillaria* taxa evidently grouped into 2 clades. While divergence of the subgenus *Fritillaria* from the subgenera *Petilium* and *Theresia* is strongly supported, resolution within the subgenus *Fritillaria* is not clear. Further studies, including examination of nuclear DNA regions or analysis with different marker systems, e.g., SSR, AFLP, could increase the resolution of *Fritillaria* spp. phylogeny. In the content of this study, the 10 *Fritillaria* cultivars showed high potential for their usage in the ornamental plant sector. Their cultivation is still progressing to generate cultivars with stable phenotypes. Ultimately, the most desirable cultivars will be systematically classified using appropriate keys as well. In view of the fact that molecular markers are acceptable to patent new varieties, these fingerprint data will be used for registering new cultivars in the ornamental plant sector. Moreover, one of the most popular methods for generating new varieties is hybridization. Hence, information about phylogenetic relationships among these species would be useful for future hybridization studies.

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