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Evaluation of the genetic relationship between *Fritillaria* species from Turkey's flora using fluorescent-based AFLP

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Abstract: In this study, the genetic relationships among 12 *Fritillaria* taxa collected from different regions of Turkey were investigated using the amplified fragment length polymorphism (AFLP) technique. Seven primer pair combinations were used for the analysis. The neighbor-joining and principal coordinate analysis methods were implemented to identify the taxonomy of the species. The analyses generated congruent results with each other and with the previous studies. The neighbor-joining analysis clustered the species into 3 groups, while the principal coordinates analysis did not support separation of the third group. The subgenera *Fritillaria* and *Petilium* diverged from each other with strong support in all analyses. The neighbor-joining tree further divided the members of subgenus *Fritillaria* into 2 subgroups. Statistical analysis of the data set using NTSYS 2.1 showed a very good fit between the data matrix and the cophenetic matrix for the AFLP data with an r-value of 0.91. Generating a high polymorphic band profile, this analysis presented the feasibility of using AFLP for a genetic relationship analysis of *Fritillaria*. This primary report on AFLP analysis of *Fritillaria* species could facilitate achieving a deeper understanding of genetic relationships between the species.

Key words: AFLP, *Fritillaria*, neighbor joining, PCoA, genetic relationships

1. Introduction

Genus *Fritillaria* L. is a large genus of monocots with approximately 165 taxa grouped into 6 subgenera, 130 species, 17 subspecies, and 9 varieties (1,2). The bulbs of various *Fritillaria* species were traditionally used as an important antitussive, expectorant, and antihypertensive drug in Turkish, Chinese, Japanese, Pakistani and southeastern Asian folk medicines (3–6). In particular, *F. imperialis* and *F. persica* are important ornamental plants due to having large, attractive flowers.

The genus *Fritillaria* is cultivated in an area extending from Turkey to Pakistan (7). It is the fourth largest genus of geophytes in Turkey. Turkey is home to 41 taxa of *Fritillaria* with a 36.53% endemism rate and can be considered the center of diversity for the genus (8–13). The genus is phylogenetically close to the genus *Lilium*.

Fritillaria was one of the first horticultural crops in Europe, described by Linnaeus in 1753 (14). Since this first description, various classifications have been described (15–18). The last revision of the genus was done by Rix (19), who revised genus *Fritillaria* into 8 subgenera: *Davidii*, *Liliorhiza*, *Japonica*, *Fritillaria*, *Rhinopetalum*, *Petilium*, and monotypic *Theresia* and *Korolkowia*. According to Fay and Chase (20), *Fritillaria* spp. comprise 2 groups: North American and Eurasian species.

A karyology study of *Fritillaria* was done by Khaniki. This study used 3 Old World *Fritillaria* species belonging to the subgenera *Petilium* and *Theresia* (21).

The morphological and physiological characterization of *Fritillaria* has been studied by various researchers. However, a molecular systematic evaluation of the genus has not been thoroughly done. In recent times, molecular markers have been used by researchers to determine genetic relationships and to show the population structure of natural plant populations (22,23). The most extensive molecular systematic study was done by Rønsted et al. (24). *Fritillaria* spp. were analyzed using 3 plastid and nuclear DNA regions. Very recently, the genetic relationships among 10 *Fritillaria* spp. were analyzed using chloroplast *trn*L-*trn*F sequences (25).

The amplified fragment length polymorphism (AFLP) technique is based on polymerase chain reaction (PCR) amplification of a fraction of restriction fragments generated by the digestion of genomic DNA, and it is an informative molecular technique for determining genetic relationships. It is a highly sensitive method for the

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detection of diversity among and within species. It is also widely used for genetic relationship studies, particularly among closely related species (26–34).

In this study, 12 *Fritillaria* taxa were collected from several regions of Turkey and their genetic relationships were assessed using a fluorescent-based AFLP technique. The results were compared with previous studies. To our knowledge, this is the first study on the molecular taxonomy of the genus *Fritillaria* based on AFLP data, and the results provide useful information on the genetic structure of the genus.

2. Materials and methods

2.1. Sample collection

Twelve *Fritillaria* taxa consisting of 7 species were collected from their natural habitats in Turkey (Table 1). The voucher specimens were deposited in the collection garden of the Erzincan Horticultural Central Research Institute (EHRI). The molecular genetic relationships among *F. imperialis* (3 forms), *F. crassifolia* subsp. *kurdica*, *F. aurea* (2 forms), *F. michailovskyi* (3 forms), *F. latifolia*, *F. minuta*, and *F. caucasica* were evaluated (Table 1).

2.2. DNA isolation

Genomic DNA from leaves was isolated using the CTAB method as previously described (35). Briefly, 100 mg of frozen young leaves ground in liquid nitrogen was mixed with 750 µL of preheated CTAB solution including 10 mg of PVP; the mixture was incubated at 60 °C for 20 min. Next, 750 µL of a chloroform–octanol mixture was added to the samples and the tubes were vigorously shaken. After centrifugation at 13,000 rpm for 15 min, the supernatant was transferred to a new tube. Following the addition of 50% (w/w) 5 M NaCl and 200% (w/w) absolute ethanol, the samples were incubated at 4 °C for 20 min. The tubes were centrifuged at 13,000 rpm for 15 min, and then the DNA pellets were washed with 75% ethanol and resuspended in nuclease-free water. The quantity of the DNA samples was determined using a NanoDrop ND-1000 spectrophotometer, and their qualities were evaluated by electrophoresis on 0.8% agarose gel according to the method of Sambrook et al. (36).

2.3. AFLP analysis

AFLP fingerprints were obtained using fluorescently labeled primers of the AFLP Amplification Core Mix Module Analysis System (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Briefly, an enzyme master mix was prepared to perform the restriction-ligation reactions for each DNA sample, using 10X T4 DNA ligase buffer, 0.5 M NaCl, 1 mg/mL BSA, 1 U *Mse*I, 25 U *EcoR*I, and 2 U T4 DNA ligase. The restriction-ligation reactions created the template for the adaptors and then the adaptor pairs were ligated to the prepared template DNA. DNA digestion was carried out using *EcoR*I and *Mse*I (BioLabs, Beverly, MA, USA); 0.5 µg of genomic DNA was added to a reaction mixture containing 10X T4 DNA ligase buffer, which includes ATP, 1 mg/mL BSA, 5 pmol/µL *EcoR*I adaptor, 50 pmol/µL *Mse*I adaptor, and 1 µL enzyme master mix with water added to a final volume of 10 µL. After 2 h of incubation at 37 °C, 189 µL of $TE_{0.1}$ buffer was added to each restriction-ligation reaction.

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

Taxa	Specimen vouchers	Place of collection
Liliaceae *Fritillaria aurea* Schott Oesterr. Bot. Wochenbl. 4: 137. 1854.	M.Aslay F44043 (EHRI)	Malatya
Liliaceae *Fritillaria aurea* Schott Oesterr. Bot. Wochenbl. 4: 137. 1854.	M.Aslay F44043_8 (EHRI)	Malatya
Liliaceae *Fritillaria caucasica* Adams Beitr. Naturk. (Weber & Mohr) i. 51.	M.Aslay F75024 (EHRI)	Kars
Liliaceae *Fritillaria crassifolia* Boiss. & A.Huet subsp. *kurdica* (Boiss. & Noë) Rix Kew Bull. 29(4): 638 (1974 publ. 1975).	M.Aslay F65035 (EHRI)	Van
Liliaceae *Fritillaria imperialis* L. Sp. Pl. 1: 303. 1753 [1 May 1753].	M.Aslay F23001 (EHRI)	Elazığ
Liliaceae *Fritillaria imperialis* L. Sp. Pl. 1: 303. 1753 [1 May 1753].	M.Aslay F30013 (EHRI)	Hakkari
Liliaceae *Fritillaria imperialis* L. Sp. Pl. 1: 303. 1753 [1 May 1753].	M.Aslay F30013_6 (EHRI)	Hakkari
Liliaceae *Fritillaria latifolia* Willd. Sp. Pl., ed. 4 [Willdenow] 2(1): 92. 1799 [Mar 1799].	M.Aslay F75023 (EHRI)	Kars
Liliaceae *Fritillaria michailovskyi* Fomin Monit. Jard. Bot. Tiflis i. 18.	M.Aslay F36025 (EHRI)	Kars
Liliaceae *Fritillaria michailovskyi* Fomin Monit. Jard. Bot. Tiflis i. 18.	M.Aslay F04029 (EHRI)	Ağrı
Liliaceae *Fritillaria michailovskyi* Fomin Monit. Jard. Bot. Tiflis i. 18.	M.Aslay F25075 (EHRI)	Erzurum
Liliaceae *Fritillaria minuta* Boiss. & Noë Diagn. Pl. Orient. ser. 2, 4: 104. 1859. ser. 2, 4: 104 1859	M.Aslay F65032 (EHRI)	Van

Next, 4 µL of the 20-fold diluted mixture was amplified in the presence of 15 µL of the AFLP Core Mix (Applied Biosystems) and 1 µL AFLP preselective primer pairs with the following program: 2 min at 72 °C; 30 cycles of 30 s at 94 °C, 30 s at 56 °C, and 2 min at 72 °C; and 10 min at 60 °C. Following visualization of the preamplified products on 1.5% agarose gel, the products were again diluted 20-fold with $TE_{0.1}$ buffer. After that, selective amplifications were carried out with 7 fluorescently labeled primer pair combinations (*Eco*RI AGG*D4- *Mse*I CAA (B2), *Eco*RI AGG*D4- *Mse*I CTT (B3), *Eco*RI AGG*D4- *Mse*I CGA (B6), *Eco*RI AGG*D4- *Mse*I CGT (B7), *Eco*RI AGG*D4- *Mse*I CAT (B8), *Eco*RI AGG*D4- *Mse*I CAC (B9), and *Eco*RI AGG*D4- *Mse*I CCA (B10)). The same PCR mixture, except for the primers (*Mse*I [Primer–Cxx] at 5 µm and 1.0 µL *Eco*RI [Dye–primer–Axx] at 1 µM), was used as the preamplification reaction. The amplification was achieved with the following program setting: 1 cycle at 94 °C for 2 min; 13 cycles of 30 s at 94 °C, 30 s ramping from 65 to 56 °C (0.7 °C per cycle), and 1 min at 72 °C; 24 cycles of 30 s at 94 °C, 30 s at 56 °C, and 1 min at 72 °C; and 1 cycle at 60 °C for 30 min. A volume of 2.5 µL of amplification product was added to 17.5 µL of sample loading solution mixed with the DNA (size standard 400) and overlaid with mineral oil. The fluorescently labeled final PCR products were analyzed using the Genome Lab GeXP Genetic Analysis System (Beckman Coulter, Brea, CA, USA).

2.4. Statistical analysis

Polymorphic bands were scored automatically by the GeXP system as present (1) or absent (0). All fragments were given equal weights. In order to reduce scoring problems resulting from excess primer, peaks, and decreased signals of fragments longer than 500 bp, fragments in the range of 54 to 462 bp length were counted.

AFLP scores were prepared as a binary matrix, and the data were converted to a distance matrix using PhylTools (37). Jaccard's index was employed as a measure of similarity. The genetic tree generation, done using the neighbor-joining method of Saitou and Nei (38), was carried out using Phylip v.3.69 (39). A bootstrap analysis with 1000 replicates (40) was implemented to assess the accuracy of the tree. To determine the differences based on genetic similarity among the data set, a principal coordinates analysis (PCoA) was performed using NTSYS-pc v.2.1 (41). Samples were plotted as points in a 3-dimensional graph.

3. Results

3.1. Evaluation of AFLP primer combination

The obtained AFLP profiles showed high polymorphic patterns. Our analysis yielded an average of 87 fragments per primer pair, for a total of 606 fragments, all of which were polymorphic. The AFLP analysis with 7 primer combinations was carried out to assess the 12 taxa. Primer B7 generated the highest number of bands (131), whereas primer B8 produced the lowest number of bands (68).

3.2. AFLP divergence

The AFLP analysis arranged the 12 *Fritillaria* spp. into 3 clades (Figure 1). Clade 1 was composed of 3 *F. imperialis* accessions (EHRI 323001, EHRI 30013, and EHRI 30013_6). The second clade consisted of *F. latifolia*, *F. caucasica*, *F. aurea* (EHRI 44043 and EHRI 44043_8), *F. minuta*, and *F. michailovskyi* F25075 (EHRI), while *F. crassifolia* subsp. *kurdica* and *F. michailovskyi* (EHRI F36025 and EHRI F04029) formed the third clade.

Based on the AFLP data, similarity values were used for the determination of a cophenetic correlation coefficient (r-value) between the data matrix and the distance matrix, and it was found to be 0.91, suggesting a very good fit.

PCoA was used to investigate genetic variations between samples, as well. Eigenvectors are plotted in Figure 2. The first axis accounted for 22.24% of the variation in the whole dataset, in comparison with 12.69% for the second axis, and the first 2 axes accounted for 34.94% of the variation in the dataset (Table 2). The PCoA identified 2 major groups. Group 1 included *F. imperialis* F30013, *F. imperialis* F23001, and F. *imperialis* F30013 while Group 2 consisted of *F. crassifolia* subsp. *kurdica* F65035, *F. aurea* F44043, *F. michailovskyi* F36025, *F. michailovskyi* F04029, *F. aurea* F44043_8, *F. michailovskyi* F25075, *F. latifolia* F75023, *F. minuta* F65032, and *F. caucasica* F75024.

An inconsistency between the PCoA and the neighbor-joining (NJ) tree results was observed, as discussed below. The PCoA comprised 2 groups (Figure 2) while the samples in the NJ tree were clustered into 3 major groups.

4. Discussion

Having extensive variation provides the genus *Fritillaria* an agronomic and economic importance. However, there is a lack of information about its molecular systematics. In this study, 12 *Fritillaria* taxa were collected from their natural habitats and their genetic relationships were evaluated using an AFLP technique. We are aware that some *Fritillaria* species are missing from this study. However, the analyzed species are distributed in diverse regions of Turkey, and we aimed to represent the overall systematic status of *Fritillaria* species. Since the NJ and PCoA methods have been used widely in molecular systematic studies (42–44), we based our analyses on these methods.

The AFLP analysis showed that the 12 *Fritillaria* spp. clearly divided into 3 main clades. *F. imperialis* is the oldest ornamental plant, and different forms of this species were determined (45). In this study, we used 3 forms of the species. As a member of the subgenus *Petilium* (13),

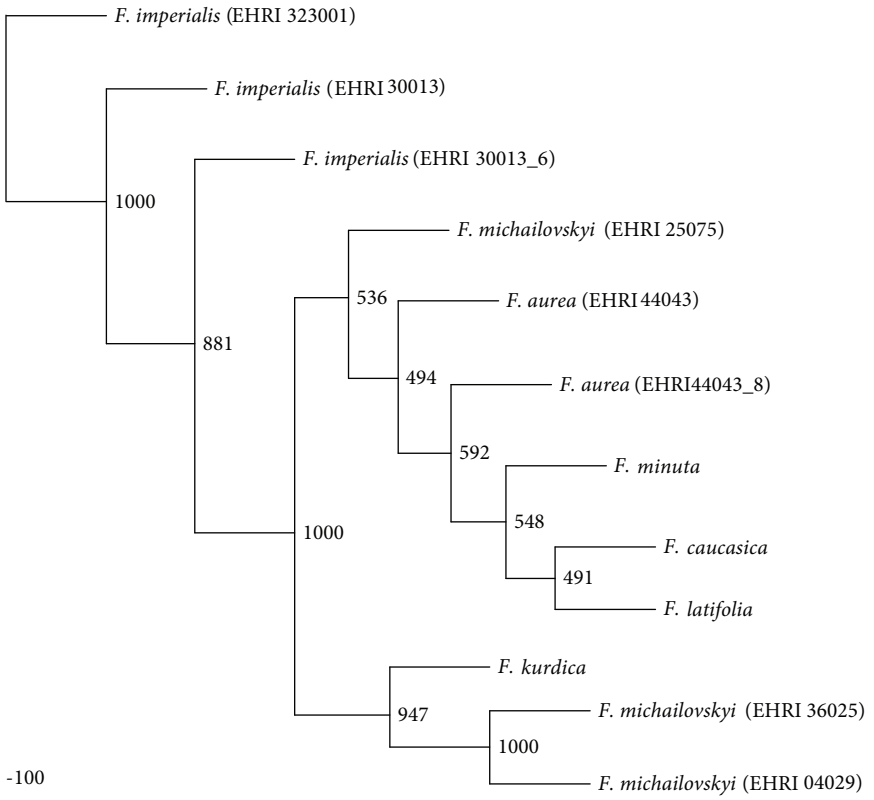

Figure 1. Neighbor-joining tree based on AFLP data. Bootstrap numbers are indicated near the branches.

3 of the *F. imperialis* specimens clearly diverged from the others, which belong to different subgenera, and grouped together forming the first clade of the NJ tree. A similar divergence was also observed from the chloroplast trnL-trnF sequence analysis (25). PCoA, which was used to evaluate genetic similarity, also supported the divergence of the 3 *F. imperialis*. As a result, all these analyses indicate an evident divergence of this subgenus from the others.

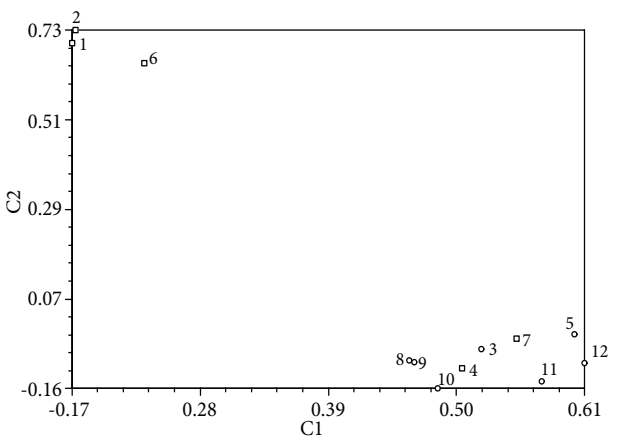

Figure 2. Plot of the first 2 axes from a principal coordinates analysis. Axis 1 (C1) accounts for 22.25% of the variation in the dataset and axis 2 (C2) accounts for 12.69%. The numbers correspond to individuals as indicated: 1) *F. imperialis* F30013, 2) *F. imperialis* F23001, 3) *F. crassifolia* subsp. *kurdica* F65035, 4) *F. aurea* F44043, 5) *F. michailovskyi* F36025, 6) F. *imperialis* F30013, 7) *F. michailovskyi* F04029, 8) *F. aurea* F44043_8, 9) *F. michailovskyi* F25075, 10) *F. latifolia* F75023, 11) *F. minuta* F65032, 12) *F. caucasica* F75024.

The second clade was composed of *F. latifolia*, *F. caucasica*, *F. aurea* (EHRI 44043 and EHRI 44043_8), *F. minuta*, and *F. michailovskyi* F25075 (EHRI). These species are known to be members of the subgenus *Fritillaria*. It is the largest subgenus within the genus. Within the subgenus, *F. caucasica* and *F. minuta* belong to section *Olostylea*, while the others are placed in section *Fritillaria* based on their style (19). The genetic tree based on the AFLP data grouped these 6 species together. However, our analysis did not support the section classification of Rix (19). Similarly, Rønsted et al. (24) could not find a corresponding section diversification. As discussed previously by Turrill and Sealy (18), these data show that style characteristics are not suitable for subclassification of these species. Even sequence-based analyses could not resolve subdivision of the subgenus *Fritillaria* with high support (24,25). As discussed by Després et al. (46), since polymorphic alleles in different lineages are sorted randomly, AFLP distributed among the whole genome will possibly be more informative than noncoding plastid

Table 2. Eigenvalues and percentages of variance of the first 2 factors.

Factor	Eigenvalue	Total variance (%)	Cumulative variance (%)
1	2.67	22.25	22.25
2	1.52	12.69	34.94

sequences. Accordingly, we found that AFLP analyses resulted in a better resolution compared to sequence data (25). However, subclassification of the subgenus *Fritillaria* was only solved to some extent in both analyses.

F. crassifolia subsp. *kurdica* and *F. michailovskyi* (EHRI F36025 and EHRI F04029) formed the third clade. The only discrepancy between the NJ tree and PCoA was observed in the grouping of subgenus *Fritillaria*. It was subdivided into 2 groups by the NJ tree, while PCoA did not support this finding. Similarly, a higher resolution of cluster analysis than that achieved with PCoA was reported previously (47). These 2 species were still grouped close to each other in analyses of ITS, plastid *rpl*16, and matK sequences (24). Interestingly, 2 of the *F. michailovskyi* specimens were clustered together, while *F. michailovskyi* (EHRI F25075) was placed separately from the other 2 with high support. A similar case was found with a chloroplast analysis of the species (25). However, in that study, *F. michailovskyi* (EHRI F04029) was placed distantly from the other 2 specimens, and it was discussed that the specimen might belong to a different subspecies. However, divergence of *F. michailovskyi* (EHRI F04029) had low support in the chloroplast analysis, while *F. michailovskyi* (EHRI F25075) diverged from the other 2 with very high support (100% bootstrapping) in the AFLP analysis. Additionally, *F. michailovskyi* (EHRI F04029) and *F. michailovskyi* (EHRI F36025) were separated from each other with 100% bootstrapping. It is suggested that these 3 individuals are evidently different forms of *F. michailovskyi*, but this needs to be confirmed with further morphological examinations. *F. crassifolia* subsp. *kurdica* and *F. minuta* could not be distinguished in a chloroplast analysis (25). In contrast, AFLP successfully separated these 2 species from each other. Rix (19) classified *F. crassifolia* subsp. *kurdica* in section *Fritillaria* of subgenus *Fritillaria*, whereas we did not observe a similar taxonomy. As discussed above, AFLP analysis did not support this subdivision in subgenus *Fritillaria* for a second time, indicating an inefficiency of style based on classification.

The only study on the molecular phylogenetic position of *F. latifolia* within genus *Fritillaria* was obtained from noncoding chloroplast sequence data (25). They showed that *F. latifolia* was clustered together with members of subgenus *Fritillaria*. Supporting that study, the NJ tree revealed the same topology with strong support.

To our knowledge, this is the first molecular systematic study on *Fritillaria* spp. based on AFLP analysis. The *matK* gene, *rpl16* plastid gene, *trnK*, and nuclear ribosomal ITS sequences of various members of genus *Fritillaria* were analyzed by Rønsted et al. (24). Moreover, Sucher et al. (47) patented 5S rDNA region sequences of 4 *Fritillaria* spp. The AFLP data revealed that the 12 *Fritillaria* taxa grouped into 3 clades. A previous report on the same *Fritillaria* cultivars showed their high potential for use in the ornamental plant sector (25). In that study, their genetic relationships were evaluated using the chloroplast *trn*L-*trn*F region. The AFLP analysis generated congruent results with the sequence data, but with a better resolution. These studies indicated that the use of molecular data is quite appropriate to assess genetic relationships of *Fritillaria* spp., and, furthermore, that using the AFLP technique improves the resolution of genetic trees in comparison to sequencing of certain regions.

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