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Assessment of relationships between Iranian *Fritillaria* (*Liliaceae*) Species Using Chloroplast *trn*h-*psb*a Sequences and Morphological Characters

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Abstract
The genus *Fritillaria* comprises of 165 taxa of medicinal, ornamental and horticultural importance. Evolutionary relationships in this genus is an interesting research area, attracting many researchers. In this study, phylogenetic relationships among 18 native to endemic species in Iran belonging to four subgenera *Petilium*, *Theresia*, *Rhinopetalum* and *Fritillaria*, are assessed using chloroplast *trn*H-*psb*A IGS sequences. Fifteen variable morphological characters are studied, and used in constructing a numerical classification. Results of molecular data showed that subgenus *Fritillaria* in Iran was a polyphyletic group. Members of the section *Olostyleae* appeared as paraphyletic. Species non-monophyly was revisited for *Fritillaria crassifolia*. Both morphological and molecular data show that *Fritillaria zagrica* and *Fritillaria pinardii* were closely related taxa, although they may be retain as separate species based on some morphological differences. Multivariate analysis of morphological data arranged the species in consistent groups as with the phylogenetic tree based on sequence data. Results of this study revealed feasibility of the *trn*H-*psb*A sequences for contribution in phylogenetic reconstruction in the genus *Fritillaria*.

Key words: *Fritillaria*; Iran; Morphology; Phylogenetic; *trn*H-*psb*A

Introduction

Fritillaria L. (*Liliaceae* Juss. 1789) comprises of more than 165 taxa (about 100 species) which are distributed in Northern hemisphere. Most of the species in this genus belong to the main subgenus, *Fritillaria* (Rix *et al.*, 2001). The Mediterranean region is the center of genetic diversity of *Fritillaria* species, with most of the taxa described from Turkey (Rix, 1984; Ozhatay, 2000). Taxonomy of this genus is reviewed by several authors (Baker, 1874; Boissier, 1882; Bentham and Hooker, 1883; Turrill and Sealy, 1980), and the current classification which is proposed by Rix *et al.* (2001) is supported at sub generic level by the phylogenetic studies (Ronsted *et al.*, 2005; Day *et al.*, 2014), although, relationships among species remain, however, not resolved especially in the largest subgenus, *Fritillaria*.

The center of diversity of the genus *Fritillaria* may also be found in Iran (Rix, 1997), where groups from central Asia, the Mediterranean, and the Caucasus meet. Most of the taxa in the main subgen. *Fritillaria* in Iran (such as *F. olivieri* Backer, *F. kotschyana* Herb. ssp. *kotschyana*, *F. reuteri* Boiss., *F. atrolineata* Bakhshi-Khaniki, *F. chlororhabdota* Bakhshi-Khaniki, *F. chlorantha* Hausskn. & Bornm., *F. zagrica* Stapf, and the recently described *F. avromanica* Advay, Teksen & Maroofi) are diploid endemics with 2n = 24 (Bakhshi-Khaniki and Persson, 1997; Bakhshi Khaniki, 1997a,b; Bakhshi-Khaniki, 2002b,a,2005; Jafari *et al.*, 2014; Advay *et al.*, 2015). Circumscription of some species in Iran was uncertain, being debated or revised by various authors. For example, *F. zagrica* proposed to be decreased as the synonym for *F. pinardii* Boiss. (Celebi *et al.*, 2008; Teksen *et al.*, 2010) and *F. crassifolia* ssp. *poluninii* is raised to specific level, *F. poluninii* (Rix) Bakhshi-Khaniki and Persson.

Recent molecular phylogenetic studies using nuclear and plastid sequences have provided evidence for both polyphyly and species non monophyly in the main subgenus, *Fritillaria*.

ITS sequences are useful in phylogenetic studies which has vastly been used in many studies, and in

combination with cpDNA and/or mtDNA sequences. In an outstanding study by Zarrei *et al.* (2009), 393 new sequences of *Gagea* and *Lloydia* species were analyzed. Results of the four types of analyses confirmed close relationships of *Gagea* and *Lloydia*. Six *Lloydia* spp. and all *Gagea* accessions formed highly supported clades (BP 100%). Incongruence between results of uni-parentally inherited plastid sequences and bi-parentally inherited ITS sequences was evident for inter-specific relationships, which was potentially due to ancient hybridization and/or paralogy of ITS sequences (Zarrei *et al.*, 2009). Inconsistent sequence datasets for *Fritillaria* (Day *et al.*, 2014), necessitated more studies using different sources of data to be conducted. Aldrich *et al.* (1988) was first who showed prevalence of indels in *trn*H-*psb*A IGS sequences between closely related species. This region was then showed to be of value to systematics (Sang et al., 1997) as the variability of these sequences were higher than that of *mat*K or *trn*L-*trn*F. Several investigators then started using this region to study closely related genera and species (Azuma et al., 1999; Fukuda et al., 2003; Miller et al., 2003). This region is most useful at the specific level, but has also been used in an intraspecific investigation (Holdregger and Abbott, 2003). At higher levels, *trn*H-*psb*A has proven to be largely unalignable (Shaw *et al.*, 2005).

In the current study, chloroplast *trn*H-*psb*A IG of 18 species in Iran, from all four subgenera (*Petilium*, *Theresia*, *Rhinopetalum*, and *Fritillaria*) are sequenced, and used as a new source of data for this genus, in constructing a phylogenetic tree. Quantitative morphological characters are also observed in several specimens of all the sequenced species, to construct an ordination, in order to compare the results driven from the two different data sources.

Materials and Methods

Plant material

Samples of the genus *Fritillaria* were collected from different regions along Alborz and Zagros mountains of Iran (Table 1). Specimens were identified (Townsend, 1985; Wendelbo, 1990; Rix, 1997; Ozhatay, 2000) and vouchers preserved in the Herbarium of Faculty of Science at the University of Shahrekord.

DNA extraction, PCR amplification, and sequencing reaction

Genomic DNA was extracted from the dry frozen leaves of 22 *Fritillaria* samples following the CTAB DNA isolation protocol (Doyle and Doyle, 1987). The *trn*H^{GUG}-*psb*A region (Shaw and Small, 2005) was amplified at a final volume of 30 µl using 0.3 unit of *Taq* DNA Polymerase (Fermentase Life Sciences), 1X supplied *Taq*-buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 0.1 mM of each primer pair. After 1 min at 94 ºC, thirty-five cycles were performed with 20 s at 94 ºC, 30 s at 57 ºC and 60 s at 72 ºC, and a final extension step of 7 min at 72 ºC. PCR products were subjected to gel electrophoresis and were cleaned up using a PCR clean-up kit (Promega, USA). Purified PCR products were directly sequenced on an automated DNA sequencer (ABI/Prism 377, Applied Biosystems). Chromatograms were edited using MEGA ver 6.0 software and nucleotide sequences saved with FASTA format (Tamura *et al.*, 2011). The newly generated sequences were submitted to the GenBank (Table 1).

Phylogenetic analysis

Lilium ledebourii sequences (accession number EU939299.1) were retrieved from the GenBank and chosen as an out-group in the phylogenetic analysis. Maximum likelihood fits for 24 different nucleotide substitution models were assessed using MEGA 6.0 software package to achieve the best model for phylogenetic analysis (Tamura *et al.*, 2013). The phylogenetic analysis was performed in MEGA 6.0 using the Minimum Evolution (Rzhetsky and Nei, 1992), Maximum Likelihood (ML) and Neighbor Joining (NJ) methods with 1000 bootstrap replications (Felsenstein, 1985).

Morphology

Three to five samples of each species were measured for 15 quantitative morphological characters (Table 2). Measurements were entered in a formatted matrix and used for multivariate

analysis in NTSYS-pc ver. 2.11 software (Rohlf, 2000). Ordination analysis (PCO) was performed using Euclidean distances. The first four principal axes were extracted from the double centered distance matrix, and a three dimensional ordination diagram was generated using the first three axes.

Results

Phylogenetic relationships

Aligned matrix of *trn*H-*psb*A IGS sequences for 23 taxa (Table 1), contained 471 positions. *F. gibbosa* Boiss. and *F. ariana* (Loz.-Lozinsk. & Vved.) Rix showed the shortest amplicon length (309 bp) for the *trn*H-*psb*A region, while the longest amplicon (352 bp) was achieved for *F. assyriaca* Baker. The average length of sequences in 22 studied *Fritillaria* specimens (18 species, Table 1) was 321 bp. The GC content of the *trn*H-*psb*A IGS region ranged from 44.3% to 47.4% with an average of 46%. The number of parsimony informative sites in *trn*H-*psb*A IGS region was 3.82% for the studied taxa, markedly higher than that of *trn*L-*trn*F region (Turktas *et al.*, 2012). Phylogenetic relationships between studied taxa was inferred using analysis of the matrix by the ME method which resulted in an optimal tree with the sum of branch length= 0.083 (Fig. 1). Bootstrap values >70% with ×1000 replicates, are presented on the tree. Analysis of data matrix using Neighbor Joining and Maximum Likelihood methods resulted in similar topologies as ME, with just very minor differences in bootstrap supports (trees are not shown). The phylogenetic tree (Fig. 1) consisted of three clades with high bootstrap supports (clade A: 97%, clade B: 100% and clade C: 86%). Most of the species of the *Fritillaria* subgenus *Fritillaria* were fall in Clade A. This clade did not encompass all the members (Rix, 1997) of the subgenus *Fritillaria*. Members of subgenus *Rhinopetalum* (*F. gibbosa* and *F. ariana*) were basally attached to the clade A. Clade B only consisted of taxa in Caucasian group of sect. *Olostyleae*, and this clade similarly did not encompass all the taxa in Caucasian group. Clade C consisted of subgenera *Petilium* and *Theresia*. *F. straussii*, a member of group Crassifolia in sect. *Fritillaria* put at the base of clades B+C, separated from other members of the group Crassifolia.

Table 1. Plant material. Specimens are preserved at the Herbarium of Shahrekord University (SKU). All the specimens are sequenced in this study (except for *L. ledebourii*).

No.	Species	GPS Coordinates (Lat., long.) of Collection Site	Alt (m)	Voucher	GenBank accession
1	*F. ariana*	35.88059, 61.06716	1360	sku-1359	KU159159
2	*F. assyriaca*	35.28396, 47.11823	1865	sku1236	KU159168
3	*F. atrolineata*	37.29324, 45.16880	2030	sku-1244	KU159173
4	*F. avromanica*	35.21637, 46.28287	1800	sku-1251	KU159169
5	*F. caucasica*	38.38909, 46.87418	2015	sku-1287	KU159171
6	*F. chlorantha*	36.00272, 45.93134	2400	sku-1229	KU159172
7	*F. crassifolia* ssp. *kurdica*	38.40015, 46.86223	1685	sku-1190	KU159167
8	*F. crassifolia* ssp. *kurdica*	37.29658, 45.16564	2070	sku-1184	KU159166
9	*F. crassifolia* ssp. *kurdica*	35.29222, 46.20336	2148	sku-0151	KU159165
10	*F. gibbosa*	35.30826, 46.96277	1930	sku-0186	KU159158
11	*F. imperialis* var. *imperialis*	35.08423, 46.39893	2534	sku-1266	KU159156
12	*F. imperialis* var. *imperialis*	35.31708, 46.24309	2140	sku-1268	KU159155
13	*F. olivieri*	35.59461, 46.95255	2118	sku-0096	KU159162
14	*F. persica*	37.29658, 45.16564	2070	sku-1281	KU159160
15	*F. persica*	35.37421, 46.14301	1900	sku-1278	KU159161

16	*F. pinardii*	37.28335, 45.17288	2175	sku-1275	KU159176
17	*F. poluninii*	35.20228, 46.27422	2550	sku-0205	KU159175
18	*F. raddeana*	37.43834, 56.67463	1340	sku-1272	KU159157
19	*F. reuteri*	32.47199, 50.51035	2521	sku-0026	KU159163
20	*F. straussii*	35.21766, 46.29432	1718	sku-0136	KU159164
21	*F. uva-vulpis*	35.21588, 46.29469	1730	sku-0139	KU159170
22	*F. zagrica*	35.2824, 47.11922	1842	sku-1162	KU159174
23	*L. ledebourii*	-	-	Kew, 23346	EU939299.1

Morphology

Morphological data matrix consisted of 19 OTUs and 15 quantitative characters (Table 2). In the resultant ordination diagram (Fig. 2), subgenera *Petilium* and *Theresia* were clustered together in a group in which members of *Petilium* (*F. imperialis* L. and *F. raddeana* Regel.) are distinguished from *Theresia* (*F. persica* L.) along the third axis. Members of subgen. *Fritillaria* were split into three groups concordant with the infra-sectional classification of this subgenus. Groups Kotschyana and Crassifolia were adjacent clusters; implying for the sect. *Fritillaria.* Members of subgen. *Rhinopetalum* were situated near the members of subgenus *Fritillaria* sect. *Olostyleae* (=group Caucasica), but separated along the third axis. *F. zagrica*, *F. caucasica* Adam and *F. pinardii* are closely related in the Caucasian group, just slightly separated along the second axis. The split of subgenus *Fritillaria* into two sections, and the position of *Olostyleae* closer to *Rhinopetalum*, far from sect. *Fritillaria* (Fig. 2) based on morphological characters was similar to paraphyly of subgenus *Fritillaria* based on molecular data (Ronsted *et al.*, 2005; Day *et al.*, 2014). In overall, achieved groups in PCO diagram were concordant with subgenera and sections in Rix *et al.* (2001).

Discussion

Phylogenetic relationships in *Fritillaria*

The strict consensus tree in this study resolved the species into three major clades (Fig. 1). *F. reuteri* was reported as closely related with *F. zagrica* (Khourang *et al.*, 2014), or with *F. olivieri* (Ronsted *et al.*, 2005; Day *et al.*, 2014). In our study, *F. reuteri* was placed near *F. olivieri* in clade A. Close relationships of *F. persica* with members of subgen. *Petilium* was in common with previous studies by various authors (Ronsted *et al.*, 2005; Celebi *et al.*, 2008; Turktas *et al.*, 2012; Khourang *et al.*, 2014), and also consistent with morphological assessments in this study. The split of subgenus *Fritillaria* into two sections and three groups based on morphological characters is not supported by our phylogenetic tree (Fig. 1). *F. zagrica*, *F. pinardii*, and *F. chlorantha* (members of Caucasian group in sect. *Olostyleae*) are grouped together in clade B with high bootstrap support. Other species of the Caucasian group (*F. caucasica*, *F. atrolineata*, *F. assyriaca*, and *F. uva-vulpis* Rix), are not included in this clade. Group Kotschyana is represented in this study by *F. olivieri*, which fall in clade A, adjacent to *F. reuteri*, and is in agreement with the results of previous studies. Subgenus *Fritillaria* splits into two clades in our study. Day *et al.* (2014) showed that subgen. *Fritillaria* splits into two clades. One clade consisted of Chinese and Central Asian species (subgenus *Fritillaria* B), which was not closely related to the large predominantly European, Middle Eastern and North African Clade (subgenus *Fritillaria* A). This split was mainly based on geographical origin of the species. The split in Iranian species of subgenus *Fritillaria* was however different, as they were all collected from various regions of Iran.

Table 2. Morphological characters in *Fritillaria* species, averages of measurements and standard deviations. A: Stem length (cm), B: Bulb diameter (cm), C: Lower leaf length (cm), D: Lower leaf width (cm), E: Length of filament (cm), F: Length of anther (cm), G: Length of style (cm), H: Length of ovary (cm), I: Length of tepal (cm), J: Width of tepal (cm), K: Nectary length (cm), L: Distance of nectary from base of tepal (cm), M: Style branch length (cm), N: Number of flowers, O: Number of leaves.

	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**
F. imperialis	80±25.6	5±1.3	15±3.7	6±1.5	2.5±0.2	1±0.06	3±0.3	1.5±0.29	5±1.1
F. raddeana	70±22.2	4.5±1.2	14±3.5	5±1.3	1.7±0.1	0.8±0.06	2.7±0.3	1.3±0.24	4.2±0.92
F. persica	75±24.1	3±0.7	15±3.7	3±0.7	0.95±0.1	0.5±0.04	1±0.1	1±0.19	2.2±0.48
F. straussii	35±11.3	2±0.5	13±3.2	2.5±0.6	1±0.1	1±0.07	1±0.1	1±0.16	2.8±0.62
F. crassifolia	20±6.5	2±0.5	6±1.5	1.5±0.3	0.8±0.08	0.9±0.06	0.8±0.09	1.46±0.27	2±0.44
F. poluninii	20±6.2	1±0.2	10±2.5	1.5±0.3	0.7±0.07	0.9±0.06	0.8±0.08	0.52±0.09	1.6±0.35
F. gibbosa	30±9.6	1.5±0.3	7±1.7	1.3±0.3	0.8±0.08	0.2±0.01	0.7±0.08	0.3±0.05	1.4±0.31
F. Ariana	30±9.7	1.5±0.3	8±2	1.2±0.3	0.7±0.07	0.2±0.01	0.6±0.07	0.3±0.05	1.8±0.30
F. olivieri	50±16	2±0.5	14±3.2	1.2±0.3	0.9±0.09	0.7±0.05	1±0.1	0.8±0.15	3.5±0.71
F. kotschyana	45±14.4	2±0.5	10±2.4	2±0.5	0.9±0.08	0.7±0.05	0.9±0.1	0.8±0.15	4.8±1.02
F. assyriaca	22±7.1	2±0.5	7±1.8	0.8±0.2	0.8±0.08	0.6±0.05	0.8±0.09	0.8±0.15	2±0.41
F. zagrica	20±3.2	2±0.4	8±1.8	2±0.5	0.8±0.08	0.4±0.03	0.8±0.09	0.7±0.13	1.8±0.35
F. chlorantha	21±6.7	2±0.5	8±2	2.2±0.5	0.8±0.08	0.7±0.05	0.8±0.09	2±0.3	2.4±0.51
F. atrolineata	23±7.3	1±0.2	9±2.2	1.5±0.3	0.7±0.07	0.6±0.03	0.7±0.08	0.8±0.15	2±0.42
F. caucasica	26±8.3	2±0.5	8±2.1	1.5±0.3	1±0.08	0.6±0.04	1.4±0.08	0.9±0.17	2.1±0.47
F. pinardii	21±6.7	1±0.2	7±1.7	1±0.2	0.7±0.07	0.8±0.06	0.6±0.07	0.6±0.09	1.9±0.40
F. uva-vulpis	25±8	1±0.2	8±2	1.5±0.3	0.8±0.08	0.7±0.06	0.8±0.09	0.9±0.17	2.3±0.51
F. avromanica	20±6.1	2±0.5	10±2.4	4±1.1	0.6±0.06	0.56±0.04	0.7±0.08	0.5±0.09	2.2±0.42
F. reuteri	30±9.2	2±0.5	13±3.2	1±0.2	0.6±0.06	0.9±0.06	1.2±0.1	0.7±0.13	1.8±0.33
	J	**K**	**L**	**M**	**N**	**O**			
F. imperialis	1.8±0.3	0.58±0.13	0±0.00	0.3±0.05	8±5	26±9			
F. raddeana	1.7±0.3	0.35±0.08	0±0.00	0.27±0.04	7±6	25±10			
F. persica	0.8±0.18	0.3±0.06	0±0.00	0±0.00	20±12	23±12			
F. straussii	1.1±0.19	1.5±0.32	0.4±0.05	0.7±0.05	2±0	7±3			
F. crassifolia	0.8±0.16	1±0.23	0.5±0.05	0.4±0.03	2±1	5±2			
F. poluninii	0.65±0.13	0.7±0.16	0.2±0.03	0.5±0.03	2±1	8±2			
F. gibbosa	0.7±0.14	0.4±0.09	0±0.00	0±0.00	7±3	8±2			
F. Ariana	1±0.20	0.4±0.09	0±0.00	0±0.00	6±2	8±2			
F. olivieri	1.2±0.20	0.5±0.11	0.4±0.04	0.4±0.04	2±1	6±3			
F. kotschyana	1.5±0.25	0.5±0.11	04±0.04	0.3±0.04	2±1	6±3			
F. assyriaca	0.5±0.10	0.4±0.09	0.1±0.02	0±0.00	2±1	7±3			
F. zagrica	0.6±0.12	0.3±0.06	0.1±0.02	0±0.00	1±0	6±2			
F. chlorantha	0.6±0.12	0.3±0.06	0.1±0.02	0.1±0.01	1±0	6±2			
F. atrolineata	0.6±0.12	0.5±0.11	0.1±0.02	0±0.00	1±0	5±2			
F. caucasica	1±0.21	0.6±0.13	0±0.00	0±0.00	1±0	4±1			
F. pinardii	0.7±0.14	0.45±0.10	0.1±0.02	0.08±0.01	1±0	4±1			
F. uva-vulpis	0.7±0.14	0.45±0.10	0.1±0.02	0±0.00	1±0	5±2			
F. avromanica	0.5±0.10	0.38±0.08	0.1±0.02	0.1±0.01	2±1	6±2			
F. reuteri	0.7±0.13	1±0.21	0.5±0.04	0.3±0.04	2±1	7±2			

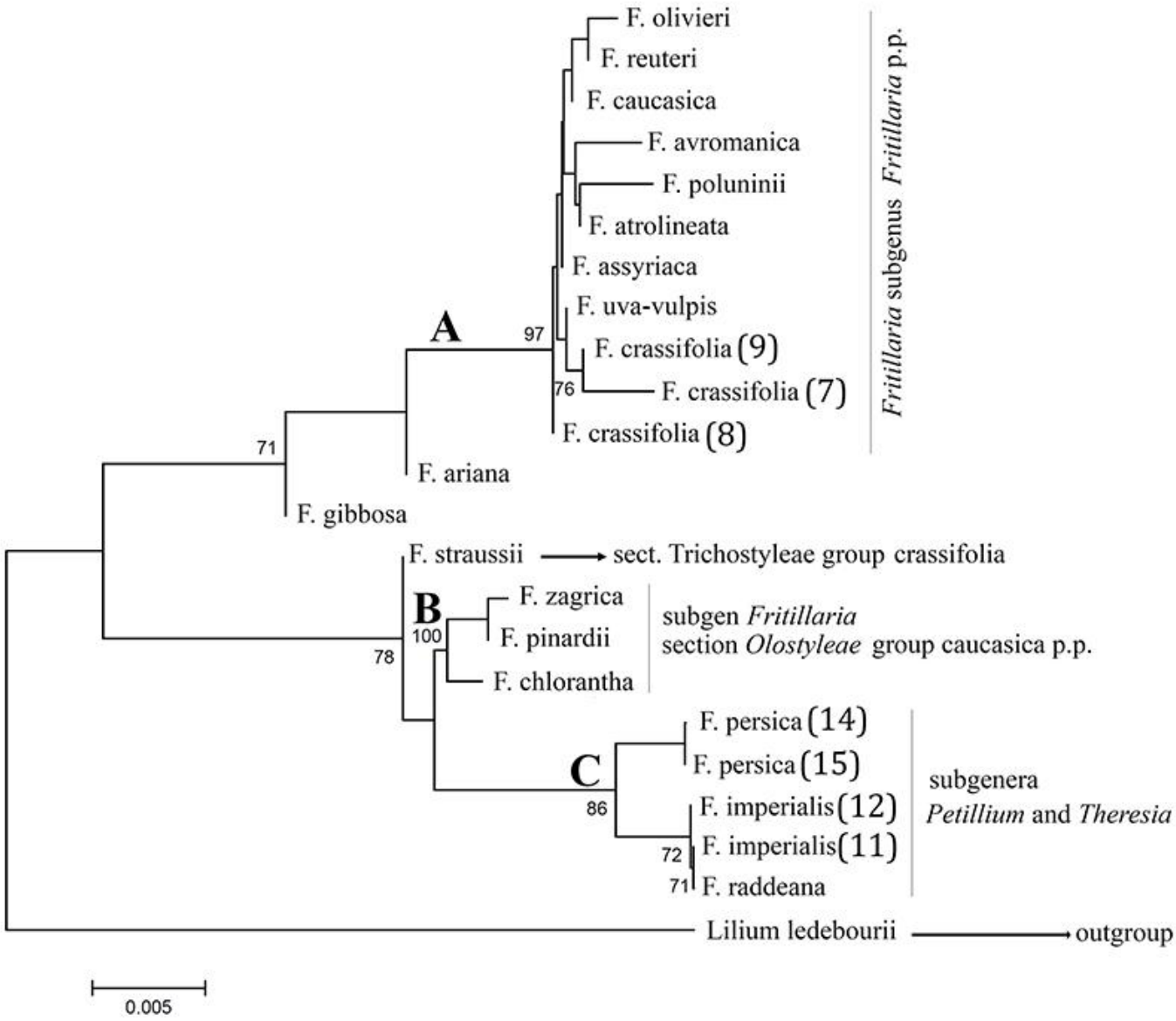

Fig. 1. Minimum Evolution (ME) tree of *Fritillaria* species with *Lilium ledebourii* as outgroup. Tree was inferred from analysis of *trn*H-*psb*A sequences. Bootstrap values >70 from 1000 replicates are shown at the nodes.

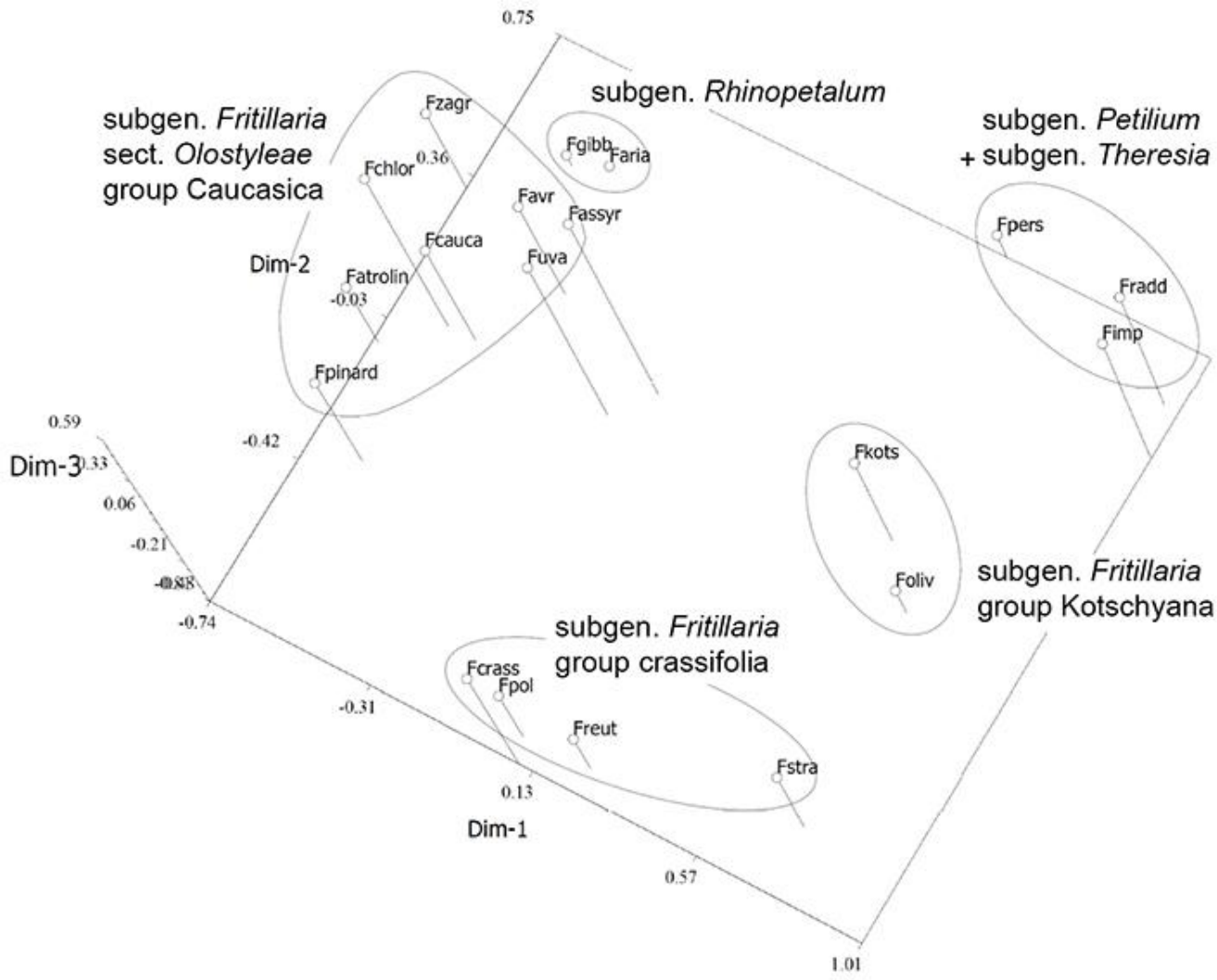

Fig. 2. PCO diagram for analysis of morphological characters. Abbreviations: Fzagr: *F. zagrica*, Fchlor: *F. chlorantha*, Favr: *F. avromanica*, Fassyr: *F. assyriaca*, Fuva: *F. uva-vulpis*, Fcauca: *F. caucasica*, Fatrolin: *F. atrolineata*, Fpinard: *F. pinardii*, Fgibb: *F. gibbosa*, Faria: *F. ariana*, Fpers: *F. persica*, Fradd: *F. raddeana*, Fimp: *F. imperialis*, Fkots: *F. kotschyana*, Foliv: *F. olivieri*, Fcrass: *F. crassifolia* subspecies *kurdica*, Fpol: *F. poluninii*, Freut: *F. reuteri*, Fstra: *F. straussii*.

F. zagrica was treated as the synonym for *F. pinardii* in the revision of the genus in the Mediterranean region (Teksen and Aytac, 2011). In our study, *F. zagrica*, *F. pinardii*, and *F. chlorantha* were grouped together in the clade B with 100% bootstrap, supportive for the close relationships of these taxa, and consistent with previous studies based on morphological characters (Ozhatay, 2000), and also with molecular phylogenetic analysis using combined plastid datasets (Day *et al.*, 2014). However, we may still retain them as separate species, as they show clear morphological differences (Table 2, Fig. 3). In molecular phylogenetic analyses of Day *et al.* (2014), *F. poluninii* and *F. crassifolia* distantly fall in one clade. *F. poluninii* Bakhshi-Khaniki (1998) was first described by Rix (1975) as a subspecies of *F. crassifolia* Boiss. and Huet. (1859). Similarly in our phylogenetic tree, *F. poluninii* and *F. crassifolia* distantly fall in one clade (Fig. 1).

Polyphyly of subgenus Fritillaria

Our results were inconsistent with one phylogenetic study of eight *Fritillaria* species in Iran, in which subgen. *Fritillaria* was resolved as monophyletic (Khourang *et al.*, 2014). The phylogenetic tree in this study provided clear evidence for the polyphyly of subgen. *Fritillaria*, which is in common with other recent reports (Ronsted *et al.*, 2005; Day *et al.*, 2014), supportive for the monophyly of all subgenera (Rix *et al.*, 2001), except for subgen. *Fritillaria*.

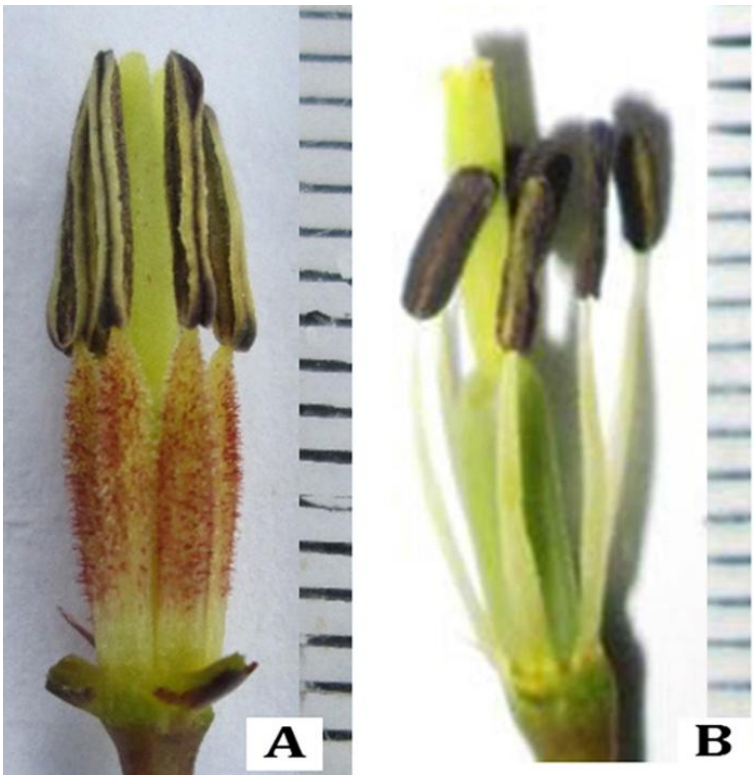

Fig. 3. Morphological differences in *Fritillaria pinardii* (A), and *Fritillaria zagrica* (B). These closely related taxa show clear morphological differences. Filament is smooth and thin (0.6 mm), and anther is short (3.7 mm) in *F. zagrica*, while, filament is 1.2 mm in width and the anther length is 6.2 mm in *F. pinardii*.

Species non-monophyly in F. crassifolia

Our data provided evidence for species non monophyly in *F. crassifolia*, a species denoted by Rix (1997) as a highly variable taxon in Iran. One specimen of *F. crassifolia* (no. 8, Table 1) collected from Urmia, fall at the base of clade A, separated from two other specimens (no. 7, 9, Table 1). Similarly, Day *et al.* (2014) reported that taxa with multiple individuals in their study (such as *F. crassifolia* and *F. assyriaca*), showed evidence of species non-monophyly. Khourang *et al.* (2014) showed on the other hand that all seven specimens of *F. crassifolia* from Iran, fell into one monophyletic clade. Species non monophyly in subgenus *Fritillaria* was also reported for *F. crassifolia* by Ronsted *et al.* (2005). Different processes such as hybridization, incomplete lineage sorting and also uncertainty in circumscription of taxa could result in polyphyly (Funk and Omland, 2003). It is not clearly known if which of these may have contributed to the species non-monophyly in *Fritillaria*.

Morphological assessment

Fritillaria species showed considerable morphological variability between species (Table 2). In the resultant diagram of PCO analysis, members of subgenus *Petilium* (*F. imperialis* and *F. raddeana*) grouped together and *F. persica* (subgenus *Theresia*) fell adjacent to them. *Theresia* is monotypic and is usually classified separated from *Petilium*, however, they are close groups based on several morphological characteristics, most notably number of flowers and leaves and the plant height. Members of sect. *Fritillaria* are separated in PCO plot into two adjacent groups, implying for the two groups Kotschyana and Crassifolia in this section. Our results were generally consistent with the classification of the genus proposed by Rix *et al.* (2001). Some authors, however, noted that morphological characters in the genus *Fritillaria* could be misleading. Ryan (2014), for example, showed that style division was highly labile and with little phylogenetic signal in subgenus *Liliorhiza*. Teksen and Aytac (2011) claimed that dark anthers of *F. zagrica* were not diagnostic; the character was labile, they wrote, when young plant got old. Accordingly, *F. zagrica* was, considered as a synonym of *F. pinardii*. Morphological observations in our study showed that these taxa may be retained as separate species, because although the color of anthers in both taxa were dark, anthers and filaments showed clearly different characters. Filament is smooth and thin (0.6 mm), and anther is short (3.7 mm) in *F. zagrica*, while, filament is 1.2 mm in width and the anther is 6.2 mm in length (Fig. 3). The two taxa were easily distinguishable species based on quantitative morphological characters (Table 2) and were separated in our study, along the second axis in the PCO diagram. Close relationship of *F. avromanica* with *F. assyriaca* (Advay *et al.*, 2015), is supported by quantitative characters in our study (Fig. 2), although, this was not supported by sequence data, as *F. avromanica* was most closely related to *F. poluninii* in the phylogenetic tree.

It is concluded that the phylogeny of *Fritillaria* species in the subgenus *Fritillaria* remains unresolved. This study examined a new source of data for the phylogeny of the genus *Fritillaria*. It should be noted that, taxonomic circumscription of morphologically variable taxa are to be revised, and molecular genetic variation studies are to be conducted on well sampled populations, before the phylogeny of *Fritillaria* is claimed as resolved. The evidence for species non-monophyly in species of subgen. *Fritillaria* reported by this study and by Day *et al.* (2014), is an important clue for the further studies.

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